The Expression of Shuni Virus Nucleocapsid Protein in *Nicotiana benthamiana* for Use as a Diagnostic Reagent

By

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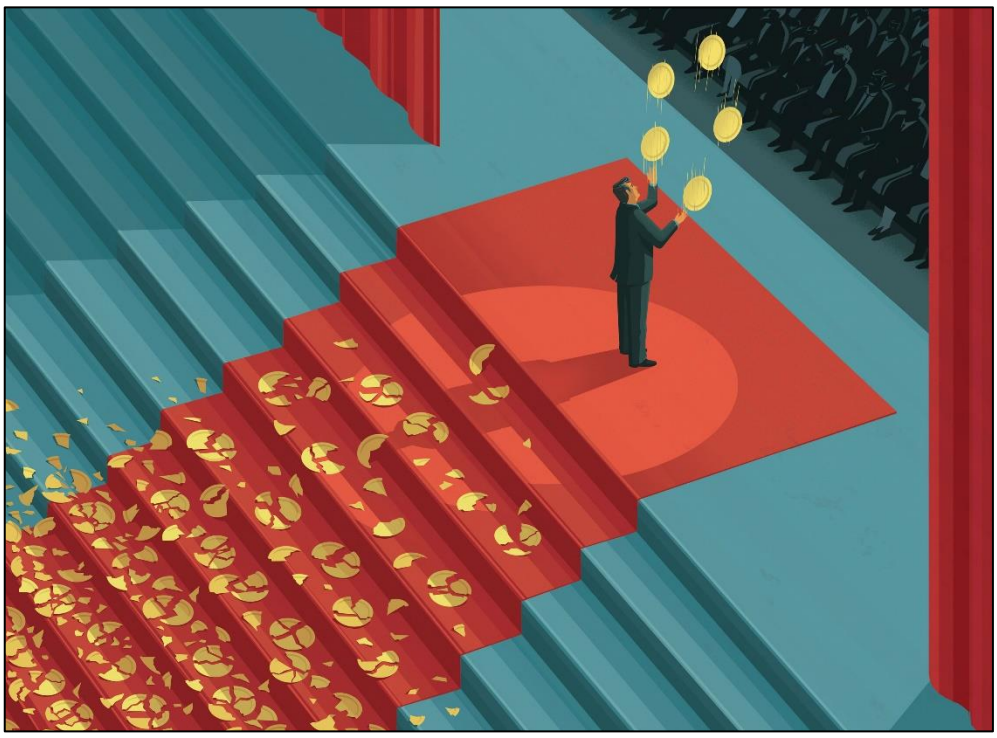

- “Key to Success” (Schmitz, 2017)

Declaration

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Abstract

Many devastating zoonotic viruses such as West Nile and Rift Valley fever viruses are endemic to South Africa, affecting livestock and ultimately, through their arthropod vectors, also infecting humans. One such zoonotic virus that is of interest is Shuni virus (SHUV). SHUV belongs to the viral genus *Orthobunyavirus*, family *Peribunyaviridae*., and order *Bunyavirales.* Discovered in arthropods and humans in Nigeria, it was soon identified as a possible cause for cases of neurological disease in horses within South Africa. Studies have shown South African veterinarians who had come into contact with such cases tested positive for antibodies against the virus. Therefore, SHUV is being further investigated as a potential cause of neurological disease within humans and there is a need to develop appropriate quick and effective diagnostic reagents to allow for surveillance of the virus.

The main focus for this study was the development of diagnostic reagents centred around the nucleocapsid (N) protein of the SHUV. The N proteins of closely related members of the order *Bunyavirales* have shown to be highly abundant in infection and cause an immune response in the infected hosts thus making it the ideal target.

Using available SHUV genome sequences and data, the N protein gene was designed and synthesised to be expressed in both *Escherichia coli* and plant expression systems.

The expression of the N protein in *E. coli*, followed by subsequent washing with BugBuster, led to a final mass of 5.1 mg of the SHUV N protein from a 1000 ml culture. This led to a SHUV N yield of 5.1 µg/ml of culture and was measured to make up 69.5% of the total soluble protein. The immunisation of rabbits with this recombinantly expressed SHUV N allowed for the development of polyclonal antibodies which were successfully used in immunoblot studies to detect plant produced SHUV N protein.

Plants are an effective and possibly cheaper alternative production system to bacterial, mammalian, or insect cell cultures and thus the N protein was transiently expressed in *N. benthamiana* plants using *Agrobacterium tumefaciens*-mediated infiltration.

The recombinant protein produced underwent purification using nickel affinity chromatography. This led to yields of 2.248 mg of SHUV N protein from 35 plants which gave a yield of 9.9 mg/kg of raw plant material.

This purified plant produced N protein acted as an antigen for diagnostic assays such as ELISA, which was used to screen known SHUV infected sera. This led to mixed results due to the limited sera samples available. However, as a proof of concept, it has shown great potential and thus opens the door to a possible inexpensive dual-use assay for use in the diagnoses of both animal and human SHUV infection.

Abbreviations

A	Absorption
AEC	Animal Research Ethics Committee
ACP	Acepromazine
AHS	African Horse Sickness
APTG	p-amino-phenyl-β-D-thio-galactosidase
BCIP	4-chloro-3-indoxyl-phosphate
BeYDV	Bean Yellow Dwarf Virus
BRU	Biopharming Research Unit
BSA	Bovine Serum Albumin
BSL	Biosafety Level
CaMV	Cauliflower Mosaic Virus
CCHFV	Crimean Congo Haemorrhagic Fever Virus
dpi	Days Post Infiltration
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FMDV	Foot-and-mouth disease virus
g	Grams
GFP	Green-fluorescent protein
His	Histidine
HT	Hyper-Translatable
IPTG	Isopropyl b-D-1-thiagalactopyranoside
kb	Kilobase
kDa	Kilo-Dalton
LB	Luria-Bertani
LIR	Long Intergenic Region
M	Molar
MES	2-morpholinoethanesulfonic acid
MDV	Middleburg Virus

mg	Milligram
min	Mins
ml	Millilitres
MW	Molecular Weight Marker
N	Nucleocapsid
N/A	Not Applicable
nm	Nanometres
ng	Nanograms
OD	Optical Density
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
RVFV	Rift Valley Fever Virus
RT-PCR	RNA using reverse- transcription PCR
sec	Seconds
SHUV	Shuni Virus
SIR	Short Intergenic Region
siRNA	Small Interfering RNAs
TBS	Tris-Buffered Saline
TbsV	tomato bushy stunt virus
TSP	Total Soluble Protein
UCT	University of Cape Town
UV	Ultraviolet
VLPs	Virus-Like particles
WT	Wild-Type
X-gal	5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside
ZRU	Zoonosis Research Unit
µg	Micrograms
µl	Microliter

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Chapter 1: Literature Review

1.1 Introduction

Many devastating zoonotic viruses such as West Nile (WNV) and Rift Valley fever (RVFV) viruses are endemic to South Africa, affecting livestock and ultimately, through their arthropod vectors, infecting humans. One such zoonotic virus that is of interest is Shuni virus (SHUV). This belongs to the viral genus *Orthobunyavirus*, family *Peribunyaviridae,* within the newly created order *Bunyavirales* (Adams et al., 2017).

Originally discovered in a cow in Sokoto, Nigeria (Causey et al., 1972, Kemp et al., 1973), it has been found in small ruminants in Israel (Golender et al., 2015) along with arthropods, cattle, goats and even equine hosts within Southern Africa (Coetzer and Erasmus, 1994). SHUV was identified as a possible cause for several cases of neurological disease and possible encephalitis affecting a variety horse cases within South Africa (van Eeden et al., 2012). Further studies have shown South African veterinarians who have had exposure to ruminants, equine and arthropods associated with such livestock, to test positive for antibodies against SHUV, highlighting the possible zoonotic aspect of the virus (van Eeden et al., 2014b).

Many cases of viral encephalitis and neurological disease in animals and humans remain undiagnosed throughout South Africa (van Eeden et al., 2012). Current methods of SHUV detection require the use of immunologic assays such as viral neutralization assays, or screening through the use of nucleic acid based assays such as PCR (van Eeden et al., 2014a). However, both conventional methods of immunogenic and nucleic acid-based diagnostics suffer from downsides that limit them in terms of cost, turnaround time and specificity.

The development of a fast, easy and cost-effective diagnostic tool using transient plant expression systems will give the ability to understand the prevalence and effect of SHUV in a South African context and thus shed light on various unknowns around this highly understudied virus. This will ultimately allow for the investigation of SHUV as a potential cause of neurological disease within animals and humans.

1.2 Shuni Virus

1.2.1 Virus Classification

The SHUV is an enveloped negative sense, single stranded RNA Group V virus that belongs to newly created virus order of *Bunyavirales* (van Eeden et al., 2014a). Originally SHUV was classified in the family *Bunyaviridae*; however, in 2017 the International Committee of Taxonomy of Viruses (ICTV) shifted it from the taxonomic rank of family to the order of *Bunyavirales* (Adams et al., 2017). The order *Bunyavirales* includes many zoonotic viruses of great concern to public health such as Crimean Congo haemorrhagic fever virus (CCHFV) and Rift Valley fever virus.

SHUV now falls under the newly created taxonomic family of *Peribunyaviridae* in the genus *Orthobunyavirus*. Within this genus there are 18 serogroups and 48 species of virus, of which Bunyamwera virus is the type species virus. SHUV belongs to the largest serogroup within the genus, the Simbu serogroup (van Eeden et al., 2014a). Species are normally distinguished at this level using cross neutralization and cross-haemagglutinin-inhibition assays (Saeed et al., 2001).

1.2.2 Animal Infection and Geographical Distribution

Surprisingly little is known about the role of SHUV, not only in a South African context, but globally. The very first identification and isolation of SHUV originated from an apparently asymptomatic cow in an abattoir during an arborvirus study in Sokoto, Nigeria, in 1966 (Causey et al., 1972). The SHUV is named after Shuni region and specifically Shuni market in Sokoto, Nigeria (Causey et al., 1972). The original strain (An10107) was isolated as described (Causey et al., 1972), where infant mice were intracerebrally inoculated with various infectious samples that were taken from the aforementioned cow and were probed for using general Simbu antibodies (Causey et al., 1972) Viral neutralization assays were performed that allowed for type identification of the virus.

This was performed as part of an arbovirus identification study that was being conducted in the region over the course of 1964-1969. This study not only revealed the prototype SHUV from the initial isolation and identification but subsequently revealed two more cases of cattle and a single sheep in the region to be infected with the newly discovered virus (Causey et al., 1972).

The study went on further to identify a variety of dairy and trade cattle in the region that were also shown to have neutralising antibodies to SHUV (Causey et al., 1972, Kemp et al., 1973). The study performed by Causey and by Kemp et al. revealed that the blood of 25 out of the 28 domestic trade livestock tested at the local Shuni Market tested positive for neutralizing antibodies (Causey et al., 1972; Kemp et al., 1973). Following this, blood samples were taken from 36 dairy cows in the region, and the cows were tested at yearly intervals after the initial blood test. All were shown to contain neutralizing antibodies to the virus that did not diminish, and were thus exposed to SHUV over the course of their lifetime (Causey et al., 1972).

An entomological study between 1967 – 1970 in Ibadan, Nigeria, sampled a variety of midges of *Culicoides* genus to gain insight into the possible route of transmission for a variety of identified zoonotic viruses in the region. The study found *Culicoides* spp. midges localized to the University of Ibadan's cattle housing to contain SHUV. While this did not confirm the role of an arthropod vector, it did hint at the possible route of transmission (Lee, 1979).

Between 1970 and 1979, SHUV was identified not only within sub-Saharan Africa but within the borders of South Africa. Several cases of asymptomatic cattle and a single goat in the province of Kwa-Zulu Natal were screened and shown to test positive for the presence of the virus (Mclntosh et al., 1972). As further insight into the role that arthropod vectors may play in the life cycle or transmission of the virus, *Culex theileri* mosquitos caught close to Johannesburg were shown to contain the virus (Coetzer and Erasmus, 1994).

In 1977, two horses originating from South Africa and Zimbabwe were shown to be suffering from severe neurological and nervous diseases. Initial investigation suspected rabies disease due to the nature of the symptoms, however, further testing allowed for the isolation of SHUV from the brains of the deceased horses (Coetzer, 1998). While these cases were unfortunately not further investigated, it differed from the asymptomatic nature of the virus identified in earlier cattle cases.

In January 2009 a single yearling crossbreed horse in Vaalwater Limpopo, South Africa was found in a paddock displaying progressively worsening ataxic signs. The yearling displayed signs of severe neurological disease such as muscle spasms and tremors before it was euthanized when recumbent. Post-mortem investigation into the causative agent of the disease revealed the horse to be infected with SHUV (van Eeden et al., 2012).

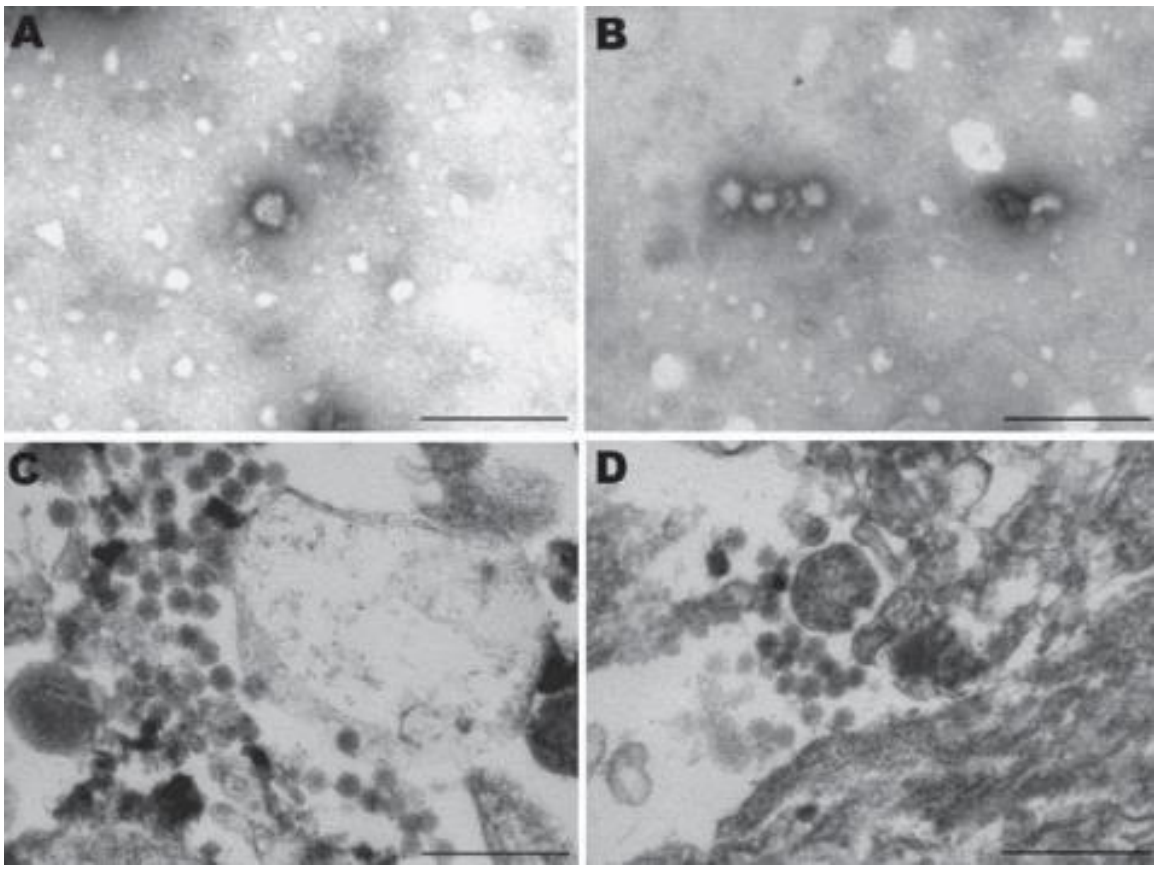


Figure 1.1: Electron micrographs taken of Vevo cells infected with virus from infected horse case SAE 18/09 displaying bunyavirus sized particles present.
Particles are seen in the 80-100 nm range. All scale bars are 250nm. This image was duplicated with permission (van Eeden et al., 2012).

Between 2009 and December 2010, SHUV infection of horses has been identified in a handful of cases across South Africa. The identification of seven horses displaying similar severe neurological symptoms between this period followed by another two horses in 2011, prompted investigations into the role of SHUV as a possible cause for undiagnosed cases of neurological diseases in horses across South Africa (van Eeden et al., 2012).

More recently however, between 2014-2015, SHUV was identified in northern Israel in the villages of Yokneam and Sde Ya'akov located in the northern Izre'el Valley (Golender et al., 2015). Various samples were collected from 15 affected small ruminants in the northern valley and additional surrounding regions. These ruminants displayed severe foetus malformation that led to abortion. A total of 23 of the 27 samples taken, tested positive for SHUV RNA through reverse transcription polymerase chain reaction (RT-PCR) screening (Golender et al., 2015). The authors conducting this study inoculated homogenate material from these various malformed foetuses into test mice intracerebrally and PCR screening performed on these mice confirmed the presence of SHUV cerebral infection which was suitable for further cell line propagation. Furthermore, SHUV was isolated from the foetal brain of a malformed lamb which, as the authors describe, is a highly unusual finding as Simbu viruses are usually only isolatable from exposed specimens or vectors during the initial 4 days of viremia. This suggests that the affected foetus may be a reservoir for the virus and that replication in the foetal nervous system is a possibility (Golender et al., 2015).

Overall, the geographical distribution of SHUV is incredibly circumscribed and available literature on its prevalence is limited. It is unknown whether, from its original discovery in Nigeria, the virus spread to the countries described and shown in Figure 1.2, or whether the virus had existed in these countries beforehand, but its occurrence had only recently been discovered. This also indicates that the virus may be prevalent in other African countries but is yet to be discovered due to the lack of available studies, resources, and available diagnostic reagents.

Figure 1.2: A map displaying various locations of SHUV incidence.
As can be seen, various cases of SHUV infection have been documented in South Africa, Zimbabwe, Israel, and the original discovery in Nigeria.

1.2.3 Human Infection

During the 1960s a virus isolation study was being performed across Nigeria as part of a surveillance program on zoonotic and arbovirus activity. This involved the routine examination of blood specimens from feverish children with unexplained causes at the outpatient clinic at the University College Hospital in Ibadan, Nigeria (Moore et al., 1975). In 1966, the first human case of SHUV infection was identified and isolated from a 1.5 year old febrile child admitted at the General Outpatients Clinic at the University Collage Hospital (Moore et al., 1975). Further patient data was unfortunately not made available; however, this was the first identified case of human SHUV infection.

Since this individual case, no further human cases of SHUV infection have been identified over the last couple of decades until a recent study performed by van Eeden et al. (2014), who invited South African veterinarians who have regular exposure to possible arthropod vectors due to outdoor work and those who work closely with bovine, small ruminants and equines, to donate blood for study. Screening of the 123 samples found five South African veterinarians testing positive for SHUV neutralizing antibodies (van Eeden et al., 2014b).

While it is not known whether arthropod vectors were involved in the transmission of the virus to human hosts or whether this was due to direct exposure to infected animals, this does highlight the zoonotic potential of the virus. While these numbers are low in terms of incidence, screening for the virus in humans has been limited due to the low profile of SHUV. However, recent investigation into febrile symptoms and neurological disease in overlooked patients in Gauteng hospitals has led to the identification of the several cases of the unrelated West Nile virus infection (Zaayman, 2012). This has spurred the idea that many unsolved or overlooked cases of neurological disease in humans may be due to undiagnosed SHUV infection due to the limited epidemiological knowledge and the correlation of equine cases with neurological disease. Thus, the clinical significance of SHUV infection should be investigated.

1.2.4 Clinical Symptoms

As described earlier, the clinical symptoms and manifestations of SHUV infection seem to differ between the species of infected hosts, which may play a large role in the low profile of the virus and lack of research into the virus' epidemiology. The virus is highly understudied; thus, being able to describe the disease progression through classified clinical stages cannot currently be done.

The original SHUV strain recorded was isolated from an asymptomatic cow in an abattoir in Sokoto Nigeria in 1966 (Causey et al., 1972). This was due to the arbovirus study being performed in the region and not due to symptomatic or clinical investigation. As mentioned in section 1.2.2, the general trend in recorded bovine cases such as trade and dairy cattle in Nigeria (Causey et al., 1972) and cattle in Kwa-Zulu Natal, South Africa (Mclntosh et al., 1972), has led to the consensus that SHUV infection presents with a lack of noticeable symptoms in bovines. This is not true of non-bovine ruminants as shown earlier in a study by Golender et al., where identified cases of SHUV infection in flocks of goats and sheep in northern Israel were associated with foetus malformations and eventual abortion (Golender et al., 2015). The ability to cause such symptoms is a general trend seen amongst most known *orthobunyaviruses*. Furthermore, these foetuses showed the potential to act as a viral reservoir should they be born alive (Golender et al., 2015).

Further study of these infected cases by Golender et. al. led to the intracerebral inoculation of mice with the SHUV infected sheep tissue. These mice displayed neurological nervousness symptoms associated with early stage neurological disease during the course of the experiment (Golender et al., 2015).

The deformities of small ruminant foetuses in Israel as described by Goldender et al. was not the first case of malformation (Golender et al., 2015). Using South African local SHUV isolates from both cattle and *Culicoides* Spp., the University of Pretoria began a study during the 1970s centred around the ability of the virus to impact the teratogenic potential of chicken embryos and its capability to induce congenital malformations (Mendes, 1984).

Figure 1.3: Deformities in 18-day old chicken embryos infected with SHUV.
Panel A shows the control embryo sacrificed at 18 days for comparison while panel B shows four different chicken embryos at 18 days after being infected with SHUV at day 4. Malformation of the embryos is seen. Image taken with permission from (Mendes, 1984) .

In this study, various chicken embryos were inoculated at 4 days post lay with SHUV and harvested at 18 days post lay. Mortality was the principal outcome seen; however, deformities and malformation of the chicken embryos were observed as shown in Figure 1.3 (Mendes, 1984). This shows that while cattle cases have been generally shown to be asymptomatic (Causey et al., 1972), foetal malformations can be associated with SHUV exposure as is evident from the field study of small ruminants in Israel (Golender et al., 2015) and laboratory study of chicken embryos performed at the University of Pretoria (Mendes, 1984).

This study had the unfortunate drawback that the chicken embryo model lacked a placenta, which plays an important role in virus progression in mammalian models. In natural infections of mammals, the virus must infect and circulate around the pregnant host before having to cross the placenta in order to allow infection of the foetus and induce malformations. While in the chicken embryo model, should the host be infected, the virus would be in direct contact with the embryo during egg formation therefore completely bypassing the passage through the placenta (Mendes, 1984).

Equine cases have presented very differently to both small ruminants and bovines. Horses are known to be highly sensitive to arbovirus infection and thus are a good indicator of difficult-to-detect viruses in a region. Looking at the recorded equine cases of SHUV infection within South Africa, seven of the nine different horses identified with SHUV infection between 2009 and 2011 were shown to suffer from severe neurological symptoms that range from ataxia and tremors to complete quadriplegia, encephalitis and eventual death. The remaining cases were shown to suffer from severe febrile disease that included fever, anorexia and leukopenia (van Eeden et al., 2012).

There have been isolated identified cases of wildlife suffering from SHUV infection. There were four unrelated cases of a crocodile, rhino, warthog and buffalo in South Africa reported to be suffering from the sudden onset of paralysis In most cases this led to the eventual death of the animals which were then screened for arboviruses to reveal SHUV infection (Venter, 2010). While there is little further information on these cases, it showcases the ability of SHUV to infect a wide range of hosts with largely different symptoms presenting, however, due to the inability to screen or diagnose wildlife due to either lack of tools, practicality or need, it hints that the epidemiological spread of SHUV may be broader than initially seen.

As mentioned previously, the first known human case of SHUV infection was diagnosed in a child in Ibadan, Nigeria in 1966. The child was treated at the University College Hospital and shown to display febrile symptoms. Unfortunately, no further patient data was made available (Moore et al., 1975). South African veterinarians who tested positive for neutralising antibodies against SHUV seem to be asymptomatic at the time of sampling with no clear history of disease linked to possible previous infection (van Eeden et al., 2014b).

It is clear from the literature that SHUV infection is highly understudied as the limited number of animal and human cases display a wide spectrum of clinical symptoms ranging from cases of severe neurological diseases to being generally asymptomatic. This highlights the need to map and diagnose SHUV infection within South Africa to understand the prevalence and effect it has on populations and to fully characterize the epidemiology of the virus.

1.2.5 Virus Vectors and Transmission

Members of the order *Bunyavirales* are known to often be transmitted by arthropod vectors such as ticks, mites, midges and mosquitos and are therefore classified under the term arboviruses (Beaty and Bishop, 1988). The exception is members of the family *Hantaviridae* which do not have insect vectors into their lifecycle (Soldan and Gonzalez-Scarano, 2005). Particularly, studies show that Simbu viruses are transmitted by mosquitos of the *Culex* genus and midges of the *Culicoides* genus (Coetzer et al., 2004). SHUV is suspected to follow similar trends in terms of arthropod transmission.

As discussed previously, there have been a handful of cases of SHUV detection and isolation from arthropods. After the initial discovery of the virus in cattle in Ibadan, Nigeria, the virus was twice discovered in *Culicoides spp.* midges during field work by Lee et al., when sampling midges around cattle housing between 1967 and 1970 (Lee, 1979). In South Africa, two batches of *Culex theileri* mosquitos caught near Johannesburg during the 1970s were shown to harbour SHUV (McIntosh et al., 1972). It is possible that while arthropod vectors may be the main route of transmission, exposure to infected tissue may also contribute to transmission of the virus (van Eeden et al., 2010). These examples illustrate not only the possible mechanism by which SHUV infection may spread but once again highlight the lack of epidemiological knowledge and literature there is on the transmission of the virus.

1.2.6 Virus Structure

Although not yet fully characterised, the SHUV genome is consistent with those of orthobunyaviruses and consists of three single stranded negative sense RNA segments, namely the small (S), medium (M) and large (L) RNA segments. These are 850bp, 4351bp, and 6910bp in length, respectively and named appropriately after their respective base pair sizes. (van Eeden et al., 2014a). Each one of these segments uses a different reading frames to produce a set number of both structural and non-structural proteins involved in the structure and life cycle of the virus (Schmaljohn, 1996).

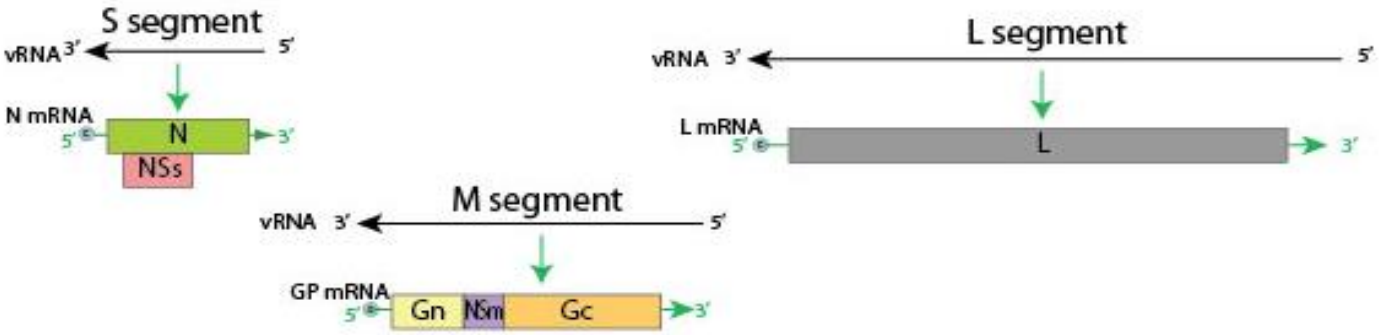


Figure 1.4: The genomic structure of SHUV.
The single stranded, negative sense RNA genome is made up of 3 segments, namely the small (S), medium (M), and large (L) segments. Image taken from ViralZone:www.expasy.org/viralzone *SIB Swiss Institute of Bioinformatics*.

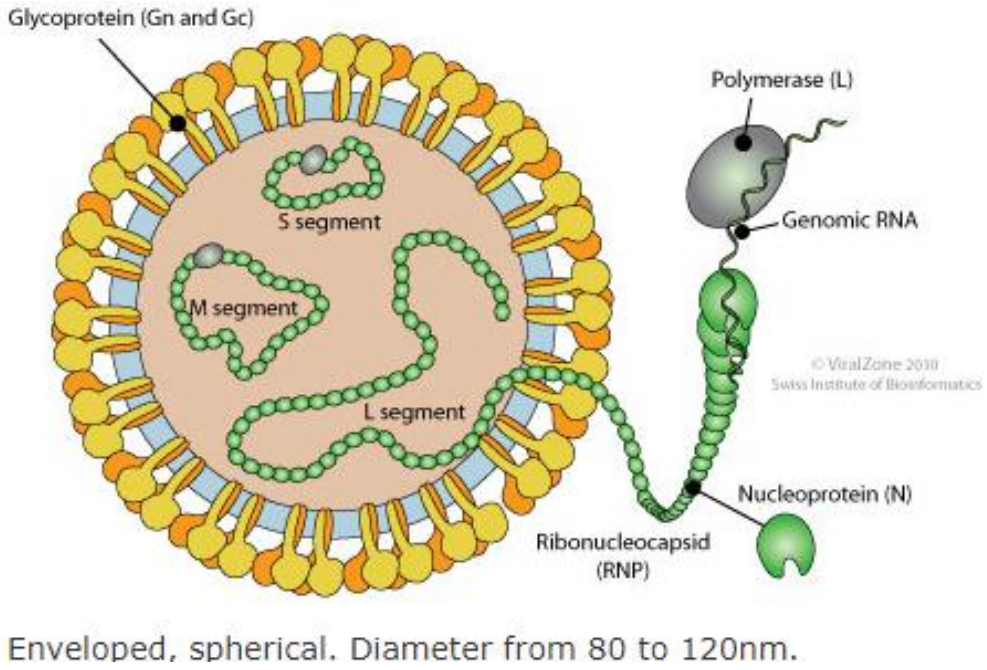


Figure 1.5: The structure of an enveloped, typical bunyavirus virion.
The typical virion is approximately 80-120 nm in size. The Gn, Gc, and N proteins are seen localized to their respective parts of the virion. Image taken from ViralZone: www.expasy.org/viralzone *SIB Swiss Institute of Bioinformatics*.

The L segment is known to be responsible for the production of the RNA-dependent RNA polymerase involved replication and transcription of the viral genome of all orthobunyaviruses (Alain, 2006). The M segment codes for the precursor to the viral envelope glycoproteins Gn and Gc (also referred to as G1 and G2) which form the outer structure of the virus while also coding for non-structural protein (NSm) through the use of different frames of translation (Elliott, 1990). The Gn and Gc proteins are likely transcribed as a single protein before being post-translationally modified and cleaved into two separate glycoproteins(Elliott, 1990, van Eeden et al., 2014a). These play an important role in formation of the viral envelope and thus participate in viral entry and cell-type recognition (Atkinson, 2016). Finally, the S segment codes for the nucleocapsid (N) protein and another non-structural protein (NSs) (Elliott, 1990, van Eeden et al., 2014a) .

1.3 Diagnostics

1.3.1 Nucleocapsid (N) Protein

The N protein is known to be the most abundant viral protein produced by members of the *Bunyavirales* order. It is the N protein that forms a ribonucleocapsid with the viral RNA, essential for the packaging of the viral genome. The RNA segments are all encapsulated with several copies of the viral N protein in order to form the ribonucleo-complex referred to as the ribonucleoproteins (RNPs) (Ariza et al., 2013). It is the formation of these RNPs that is required for the packing of each segment during the assembly of the virus particle. The association of the RNPs is crucial for gene expression through the use of the viral polymerase (Overby, 2007)

Due to the abundance and immunogenicity of the N protein in bunyaviruses, it has been used as a marker of current and previous infection for bunyaviruses such as Rift Valley Fever virus RVFV and CCHFV (Mbewana 2017, Atkinson et al., 2016) In CCHFV, the protein has been shown to generate abundant specific antibodies, thus making it an ideal target for infection diagnosis (Marriott et al.,

1994). There is no literature on the abundance of N protein involved in SHUV infection or its immunogenicity; however, extrapolating from related bunyaviruses CCHFV and RVFV suggests that the same should be true.

Prediction of potential viral outbreaks is key to managing the spread of bunyaviruses – such as, for example, CCHFV. These outbreaks often affect developing countries which do not have the facilities, funding, or required expertise to diagnose infection and manage the spread of disease. Previous work performed by the Biopharming Research Unit (BRU) between 2016 and 2018 allowed for the development of two different cost-effective indirect enzyme linked immunosorbent assays (ELISA) as a diagnostic tool. This was done using recombinantly plant expressed CCHFV N protein and RVFV N protein. These diagnostic tool allowed for the successful screening of infected sera for antibodies against the wildtype virus in humans and ruminants respectively (Atkinson et al., 2016, Mbewana et al., 2018).

1.3.2 Current Assays

Current methods of SHUV detection in animals and humans are both immunogenic and nucleic acid-based methods. The most prominent of these is viral neutralisation assay, which requires the culturing of live virus. While this method has the advantage of being highly specific, it suffers from long turnaround times due to the requirement of correct facilities such as labs at a minimum biosafety level of three (BSL3), based on the pathogenicity of the cultured virus. This comes with its own set of hurdles such as the need for correctly trained personnel as well as being a resource-heavy and expensive assay (van Eeden et al., 2014a, van Eeden et al., 2012).

The second method of screening is RT-PCR and quantitative PCR (qPCR) to amplify viral RNA from samples taken from the specimen. These PCR based methods have shown promise for detecting viral infection during the three or four days of viremia stages. As an example, during the Golender et al. study in which ruminants were assessed for SHUV infection, diagnostic RT-PCR was performed using Simbu specific primers (Golender et al., 2015). However, once the initial stage of viraemia has lapsed during SHUV infection, this method may be unsuitable to diagnose infection based on blood samples alone. This fact, coupled with lengthy turnaround time, primer design, the cross-amplification of closely related bunyaviruses via RT-PCR , and the assay not consistently being able to provide data on lifetime exposure, suggest that immunogenic diagnostic assays may be the preferred method of diagnosis (van Eeden et al., 2014a, Kuno et al., 1996).

1.4 Plant Based Protein Expression

1.4.1 Plant Expression

The use of plants as production systems for various biological molecules is a complex but blossoming field that has been around for over 30 years. Plants as vehicles of expression are used for the production of recombinant proteins and are seen as safe and possible cost-effective alternatives to production compared to the use of bacterial, mammalian, or insect cell cultures (Rybicki, 2014). The advantages of such systems are that plants are not susceptible to human pathogens which allows them to be used safely in the production of human and animal vaccines and, unlike bacterial and other culture systems, they do not have to be grown in sterile environments (Rybicki, 2010). Plants are able to post-translationally modify the expressed recombinant proteins thus allowing morphologically intact and antigenically applicable mammalian proteins to be produced (Streatfield, 2007). In addition, plant production systems are

able to produce the required protein or antigen in large and, if need be, agricultural scale with low associated production costs (Fischer et al., 2004).

1.4.2 Transgenic Expression

The initial proof of concept around the transgenic expression of proteins in plants for vaccine application was seen in 1989 with the successful expression of mouse hybridoma-derived monoclonal antibody (mAb) in *Nicotiana tabacum* (Hiatt et al., 1989). Following this the original expression of virus like particles (VLPs) using Hepatitis B surface antigens in 1992 (Mason et al., 1992). Originally, the driving idea behind plant expression was to allow for the expression of recombinant proteins for use as vaccines to be transgenically targeted within edible crops such as fruits, tubers, edible leafy plants and even seeds to allow for easier route of inoculation; however, this idea has been largely unrealised. Small successes such as the expression of HPV-16 L1 protein in transgenic tomatoes as part of a prophylactic and therapeutic human papillomavirus (HPV) vaccine showed success in stimulating both T-cell and antibody responses, but unfortunately lacked an efficient yield to make it worth it (Paz de la Rosa, 2009). Another avenue of expression and delivery was through edible leafy crops. A study in 1999 showed that mice having undergone oral immunisation with transgenic alfalfa leaves expressing recombinant foot and mouth disease virus (FMDV) VP1 protein were successfully challenged with live FMDV (Wigdorovitz, 1999). This shows that the concept of orally fed vaccines can, in principal, be feasible; however the concept suffers major flaws. Yields of recombinantly expressed proteins are often too low in crops to offer effective immunisation when ingested. Following this, the yields of expressed proteins in these crops is not only inconsistent at best but also difficult to measure to ensure reliability of delivery. This makes the effective oral dosing of required quantities unmanageable and often unrealistic (Rybicki, 2010).

The idea that plant produced vaccines should be edible has been largely dismissed in recent times; however, plant expression systems as a concept has not. Amongst the many advantages of plant expression listed previously, plant material is easily processed and homogenised to allow extraction of the expressed protein which, in the case of a vaccine candidate, can then be administrated as an injectable (Rybicki, 2009). Various plant models have been used for non-edible plant expression of recombinant proteins, from *Arabidopsis thaliana* and alfalfa through to a variety of *Nicotiana* spp.

As shown above, transgenic expression of recombinant proteins and vaccines has shown to be incredibly promising, with many more successful and recent examples having not been mentioned, however, transgenic expression is not without its disadvantages (Rybicki, 2010). Experimental evidence has shown the yield of recombinant expressed proteins from most transgenic lines, not only in edible crops as mentioned above, to be relatively low compared to the high costs used to generate such plant lines (Wroblewski T., 2005). In order to make yields more efficient, high costs associated with increase plant numbers, better quality purifications and concentration processes would be seen. This is over and above the already lengthy time required to develop the correct transgenic line (Gleba, 2007, Rybicki, 2009).

1.4.3 Transient Expression

In more recent times there has been an increasing shift to the use of transient expression systems as opposed to initially developed transgenic expression systems. There are many factors that influenced this move, but most important were the speed of expression and higher yields seen (Fischer, 1999, Rybicki, 2010). Transient expression systems offer the ability to generate large amounts of the required recombinant protein within a couple days after the required molecular

cloning and transformation steps were completed, compared to the many months required for transgenic expression (Fischer, 1999). Transient expression serves the required purpose of quick and bountiful expression for low associated cost, overcoming the main hurdle seen with transgenic expression (Wroblewski T., 2005).

Agrobacterium tumefaciens-mediated gene transfer has been used to create stable transformed plants for a number of years. The bacterium has the ability to transfer the bacterial T-DNA and virulence gene elements (referred to as the T-complex) into the host plant cells. A gene of interest is inserted in between 25bp direct repeats that form the left and right borders of the single-stranded T-DNA. This is directly transferred into the host plant cell nucleus and may integrate into the plant chromosome (Zupan et al., 2000). However, while a small fraction of the T-DNA may integrate, a large number of T-DNA copies present in the nucleus do not and are therefore present only transiently in the plant host. These are transcribed regardless of their transient nature, and can thus lead to much higher levels of expression of the target protein as there are no nuclear location effects (Kapila et al., 1997, Maclean et al., 2007).

Transient expression has had many successful cases since its inception, such as the high levels of expression seen of FMDV VP1 by agroinfiltration of Alfalfa leaves reported by Habibi (2014). Here the authors were able to produce the recombinant subunit vaccine on a large-scale without any major disadvantages. Another case was the production of FMDV VP1, VP4 and T-cell epitopes of 2C and 3D via transient expression in *Nicotiana benthamiana* that successfully provided immunity and protection in guinea-pigs in challenge experiments (Andrianova, 2011). Currently, the industry is poised to take plant expressed pharmacetuicals to very large scales of production. This is seen with research into the development of industrial scale levels of production of pharmecetuicals such as monoclonal antibodies within plants (Buyel et al., 2017).

1.4.4 Plant Expression Vectors

The high yields associated with transient agroinfiltration-mediated expression are partly due to the use of viral vector constructs, and the replacement of the tumour inducing plasmid (Ti plasmid) in *A. tumefaciens* with recombinant plasmids derived from a swathe of plant viruses that can be both self-replicating and not (Gleba, 2007, Gelvin, 2003). An example of replicating vectors would be the creation of the 'launch vector' by Musiychuk et al. derived from a tobacco mosaic virus (TMV)-based construct which has shown success in the expression of HPV E7 and H5N1 influenza proteins (Musiychuk, 2007), both of which were shown to be protective in viral challenge experiments in mice and ferrets respectively (Massa, 2007, Mett, 2008). Non-replicating vectors of interest to this study are the pEAQ *HT* and pTRAc HT plant expression vectors, discussed in section 3.1.

Self-replicating viral vectors have shown huge promise in terms of increasing the provided yield of the chosen recombinant protein. This is achieved through the self-replication of the viral vector within the targeted cells which leads to increased translation and protein upregulation (Regnard et al., 2010). The inclusion of replication elements such as the Rep/RepA cassette found in the Bean yellow dwarf virus (BeYDVm) based ssDNA-derived viral vector, pRIC (Regnard et al., 2010), allows for rolling circle self-replication of the vector itself within the host cell. This amplification leads to high copy numbers of the gene of interest through the production of circular DNA transcription templates referred to as replicons. The presence of these replicons leads to the advantage that continuous transcription leads to much high levels of protein translation in comparison to basic viral vectors where transcription is often limited and short lived (Hanley-Bowdoin et al., 2013).

Gene elements and signal sequences included on the viral vector allow for the targeting of the recombinant protein translation to specific organelles within the host cell or allow for combined translation and post-translation modification to occur in multiple organelles. The potato derived *rbcS1* gene, referred to as a chloroplast-transit peptide sequence, allows for chloroplast targeted expression of the gene of interest (Maclean et al., 2007) while the well-known (SEKDEL) sequence allows for the endoplasmic reticulum (ER) retention for further folding of the protein and post-translation modification such as *O*-glycosylation (Stornaiuolo et al., 2003, Maclean et al., 2007).

These developments in transient plant expression are not without an appropriate response from plants, however, which have developed their own plant defences mechanisms to combat the infiltration and transfection of virus based vectors. An example of which would be the ability of plants to prevent viral RNA replication and translation through RNA silencing with siRNA molecules. Nevertheless, plant viruses such as the tomato bushy stunt virus (TbSV), have developed silencing suppressors such as the p19 silencing suppressor which act as a countermeasure to ensure that viral RNA is able to be translated and expressed. This is achieved by preventing the accumulation of Argonaute-1 and by preventing the binding of the viral RNAs to the host plant's RNA-induced silencing complex (Csorba and Burgyán, 2016, Van Zyl et al., 2016, Gunter, 2017).

Overall, plants have been hugely successful in producing many antibody and virus-derived proteins for use as vaccine candidates, reagents and antigens in diagnostic ELISA tests (Rybicki, 2014). As of 2018, the BRU has had numerous successes using agroinfiltration in conjunction with *N. benthamiana* to produce various recombinant VLPs such as those used for the African horse sickness virus (AHSV) vaccine candidate (Dennis et al., 2018), along with many diagnostic antigens such as CCHFV N protein (Atkinson et al., 2016) and RVFV N protein (Mbewana 2017). These diagnostic antigens have been used specifically in indirect ELISAs to test for the presence of antibodies to wildtype virus in infected sera of human patients (CCHFV) and sheep specimens (RVFV), both of which have been highly successful.

1.5 Conclusion

Many cases of viral encephalitis and neurological disease in both animals and humans remains undiagnosed throughout South Africa, and largely, the world. Symptomatically, the virus has presented very differently in the variety of hosts it has been identified in infecting.

In bovines it has shown to present generally asymptomatically and thus was only identified due to arbovirus studies being performed in select regions (Causey et al., 1972). In the case of small ruminants in northern Israel, severe foetus malformation was seen that led to abortion with the possibility of aborted foetuses acting as a reservoir for the virus (Golender et al., 2015). Similar experiments conducted by the University of Pretoria showed chicken embryos to become malformed when exposed to the virus during development (Mendes, 1984). Infection of horses with SHUV was characterised by neurological disease that often led to the eventual death or euthanasia of the animal while in humans it was shown to present with febrile symptoms with regards to the initial case or asymptomatically in the case of the veterinarians (van Eeden et al., 2012, van Eeden et al., 2014b). This variety and range of symptoms highlights the lack of general knowledge around the virus and the need to further pursue studies to fill such gaps in literature.

The development of a reliable, cost effective and accurate diagnostic tool using plant expression systems would give the ability to understand the prevalence and effect of SHUV in a South African context. This is to be achieved through the recombinant expression of SHUV N proteins in *N. benthamiana* for use as a diagnostic antigen in indirect ELISA screening of sera. The recombinant expression of SHUV N protein has not be documented before in the literature.

The One Health approach is highly applicable in this regard. The inherent idea is that the health of the environment and animals, be it livestock or otherwise, are intrinsically connected to human wellbeing. The ability to monitor diseases that spread through animal populations and the environment will give sufficient benefits in managing potential human health threats.

Through the diagnosing of symptomatic animals or those thought to be infected with SHUV, there is an increase in survival and animal wellbeing thus lowering the socio-economic impact of severe cattle, horse and general ruminant losses due to viral disease. The ability to provide early diagnosis of viral infection would lead to better animal management and thus keep the spread of infection to a minimum. The monitoring of animal health can lead to the ability to predict the spread of disease as animal populations can serve as early warning markers for problematic zoonotic diseases.

This diagnostic tool would give the ability to map SHUV distribution amongst animal populations which would give insight into the mechanisms, prevalence and clinical signs of the virus.

This falls in line with the One Health concept by approaching potential health hazards in a collaborative and trans-disciplinary approach, where the development of diagnostic reagents allows for prediction of the spread of a potential threat. Understanding whether a disease is connected to underlying conditions seen in populations will allow multidisciplinary management of the disease.

1.6 Project Aims and Objectives

Current methods of detection used for the identification of SHUV infection are currently limited to the culturing of live virus which requires substantial facilities and expertise; and PCR methods, which have issues with turnaround time, specificity, and diagnosis outside of viraemia stages. It is established that immunogenic assays are favoured in the diagnosis of viral infections such as SHUV.

The overarching aim of this project was to develop a diagnostic reagent from the SHUV nucleocapsid (N) protein: to date there have been no published examples of the expression of SHUV N protein in any expression system. This project aim was to be achieved through two main objectives.

The first objective of this study was to express the full length codon-optimised SHUV N protein using an *E coli* expression system. The SHUV N gene would be constructed through a consensus sequence taken from seven different isolates in which the full sequence of the N gene was known. To allow the detection of the expressed protein, a histidine affinity tag (6xHis) would be attached to the N-terminus of the target protein. Once expressed and evaluated on a small and then large-scale, rabbits were to be immunised using *Escherichia coli* cell extract containing the overexpressed SHUV N protein. This would allow the rabbits to generate antibodies against the SHUV N protein, and to have a positive control for downstream studies.

The second objective of this project was to express and purify the SHUV N protein using a plant expression system; specifically, by transient expression in *N. benthamiana*. The viability of plant expression of the target protein would be assessed by choice of expression vector, the inclusion of a 6xHis affinity tag, and time of expression. Once optimised, a large-scale purification would be developed.

Finally, the third and main objective of this project was to attempt to assess the reactivity of sera from known SHUV-infected horses. This was to be achieved through the use of the purified plant expressed SHUV N protein (the diagnostic reagent) as an antigen in indirect ELISAs (the diagnostic assay) to detect antibodies in the sera resulting from wildtype virus infection. This has previously shown to be successful for related viruses of the *Bunyavirales* order such as CCHFV (Atkinson et al., 2016) and RVFV (Mbewana 2017), where the N protein of each respective virus was transiently expressed in plants and used as an antigen in diagnostic ELISAs.

The development of this diagnostic tool would allow for the quick, reliable and cost-effective diagnosis of animal sera for the presence of antibodies against the wildtype virus, which can be a marker of current or previous infection. This diagnostic tool will give the ability to map SHUV distribution amongst animal and even human populations in South Africa, which would give valuable insight into the prevalence and clinical signs of the virus.

Chapter 2: Development of a Specific Antiserum against Shuni Virus N Protein

2.1 Introduction

In terms of recombinant protein expression systems, *Escherichia coli* cell cultures are the laboratory standard due to a variety of factors such as the low cost of culture, being easily culturable and having been significantly studied and characterised (Chen, 2012). The choice of *E.coli* strain can impact factors such as post-translational modifications of the target protein or amount of recombinant expression before growth is affected (Makino et al., 2011). *E. coli* expression vectors such as pProEX, allow for lactose operon-derived promoter elements to control the expression of the gene of interest. These can be easily induced by the addition of the lactose analogue, isopropyl β-D-thiogalactopyranoside (IPTG) (Terpe, 2006).

The nucleocapsid (N) proteins of bunyaviruses have been shown to be highly abundant and immunogenic in the infected host (Ariza et al., 2013). This is therefore an excellent target for the development of a plant-expressed diagnostic reagent for the relatively unknown and undocumented bunyavirus, SHUV.

The downstream expression of recombinant SHUV N protein will be performed in the chosen plant expression system, *N. benthamiana*. However, the ability to detect this recombinantly plant expressed protein is dependent on the assay involved and the availability and specificity of the detecting reagents. Usual methods used to assess plant-expressed recombinant proteins are through antibody related assays such as western blot on the crude plant extract, and enzyme-linked immunosorbent assay (ELISA), both of which are dependent on the availability and specificity of antibodies. In order to achieve antibody detection of the protein of interest, antibodies specifically raised against the target protein are needed. This chapter contains the process of development of polyclonal rabbit antibodies specific to the SHUV N protein.

In order to get around having to use polyclonal antibodies specific to the protein of interest, multiple expression systems make use of expression vectors that allow for the tagging of the recombinant protein with a variety of affinity tags. Affinity tags are able to serve a duel purpose; these are not only available for detection through antibodies but may also help with downstream purification of the target protein through the ability to bind to specific resins.

An example of such a dual purpose affinity tag would be the intein-chitin binding domain tag (intein-CBD tag), which contains a self-cleaving intein element attached to a chitin binding domain for protein purification purposes (Kimple et al., 2013). Commercial antibodies raised against the CBD are available for probing purposes in immunogenic assays such as western blot and ELISA which will allow for detection of the recombinantly tag protein. The CBD element is also available for binding to chitin resin for protein purification; however, the chitin resin suffers from nonspecific binding that may affect the purity of the eluted sample. Chitin affinity chromatography is also known to not be compatible with denaturing reagents such as urea (Kimple et al., 2013, Chong et al., 1997)

Another example of an applicable affinity tag would be the popular β-galactosidase (β-gal) or LacZ tag. While the protein tag is inherently large (1024 aa), it has been shown to increase expression of

the fusion protein in *E. coli* expression systems (Sambrook and Fritsch, 1989). The LacZ fusion proteins are able to be purified through substrate affinity chromatography on immobilized p-amino-phenyl-β-D-thio-galactosidase (APTG) and eluted through a change in pH with high pH borate buffer(Kimple et al., 2013). However, compared to other affinity tags, detection can be achieved through a colorimetric enzymatic assay with its substrate 5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside (X-gal) and does not require antibodies specific to it for visualization in western blots and ELISAs. The unfortunate downside is that, due to the large size of the LacZ tag, it can alter the folding and functionality of the attached recombinant protein. In addition to that is has a tendency to form multimers with itself (Kimple et al., 2013, Sambrook and Fritsch, 1989).

One of the more commonly used affinity tags in recombinant protein expression is the polyhistidine tag. This comprises of six histidine residues (6xHis) on either the amine or carboxyl terminus (N or C terminus) of the chosen protein. Commercial antibodies raised against the 6xHis tag epitope can be used as a probing reagent in immunogenic assays in a similar fashion to the affinity tags described above. Not only does the 6xHis tag benefit the recombinant protein in detection, but histidine residues are able to form coordination bonds with various immobilized transition metal ions such as Zn^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, and most commonly Ni^{2+}. The use of immobilised metal ions can be incorporated into protein purification through the use of the ions in the stationary matrix as part of column chromatography (Kimple et al., 2013).

The 6xHis affinity tag has benefits such as allowing for the easy probing of the tagged protein without protein-specific antibodies, and it allows one antibody to be used in a variety of recombinant protein studies (Kimple et al., 2013). However, 6xHis probing comes with disadvantages and drawbacks.

Antibodies raised against the 6xHis residue protein tag have a lower specificity than antibodies raised against an entire target protein. This manifests in a high level of background during antibody assays such as western blots and ELISAs. The non-specific binding of the 6xHis antibody may lead to misrepresentation of whether or not the tagged recombinant protein is present in the sample, and thus gives rise to false positives. This is notorious due to the high number of histidine residues in insect and mammalian protein expression systems. The probing antibody may bind to artefacts, background proteins or constituently expressed proteins from the chosen expression system (Kimple et al., 2013). Detection can be limited should the 6xHis tag be folded within the structure of the tagged recombinant protein or cleaved by a protease. The polyhistidine tag has been reported to sometimes influence the folding of the target protein, which poses problems in structure specific proteins (Bornhorst and Falke, 2000).

Should resources be available and the study benefit from protein specific antibodies, the described issues can be solved by the development of polyclonal antibodies specific against the target protein.

This can be achieved through the use of animal systems to generate large quantities of polyclonal antibodies against the recombinant protein of choice. This is done by the injection/vaccination of sufficient quantities of the recombinant protein or required epitopes into the test animal in order to elicit an immune response (Kascsak et al., 1987). Should the protein be highly immunogenic and be delivered in a sufficient quantity over the course of the trial, the test animal will produce a spectrum of antibodies, such as IgMs and IgGs, against the target protein (Kascsak et al., 1987).

Collection and treatment of the animal blood will yield large quantities of polyclonal antibodies in the serum that can be used as probing reagents in the antibody-based assays mentioned before such as western blots and ELISAs, given the correct secondary antibodies are used.

2.2 Materials and Methods

2.2.1 Codon Optimisation

The SHUV N gene coding sequence was obtained through the alignment of the SHUV N gene from isolate sequences shown in Table 1 below. This was aligned using the bioinformatics software CLC Bio (CLC Bio, Denmark).

Table 1: Various isolates and their respective Genebank No. used in the alignment and construction of the consensus sequence of the SHUV N gene.

Isolate	Genbank No.	Location	Publication Source
Ib An 10107	HE800143	Nigeria	(Goller et al., 2012)
SAE1809	KC510272	South Africa	(van Eeden et al., 2014a)
Shuni/2504/2/14	KP900878	Israel	(Golender et al., 2015)
Shuni/274/14	KP900867	Israel	
SHUV/ISR-274/14	KT946779	Israel	
Shuni/263/14	KP900860	Israel	
Shuni/2417/1/14	KP900872	Israel	
An10107	AF362405	Nigeria	(Saeed et al., 2001)

The isolates described in Table 1 were used to construct the consensus sequence This consensus sequence, referred to here as the SHUV N gene, was submitted to GenScript Inc. (USA) for codon optimisation for expression in *N. benthamiana*: this was done using the OptimumGene™ by GenScript Inc (USA). The optimisation parameters used were:

- GC Content adjustment
- Codon usage
- Removal of unwanted restriction enzyme sites
- Repeat sequences
- Cis-acting elements.

2.2.2 Gene Synthesis

The consensus SHUV N gene was synthesised by GenScript Inc (USA) as a single gene consisting of 723bp and was cloned into the GenScript standard pUC57 vector using the blunt end restriction enzyme *EcoR*I. This led to the creation of the pUC57 SHUV N construct by GenScript Inc. This construct contained the required ampicillin resistance gene native to the pUC57 cloning vector to be used for downstream selection purposes.

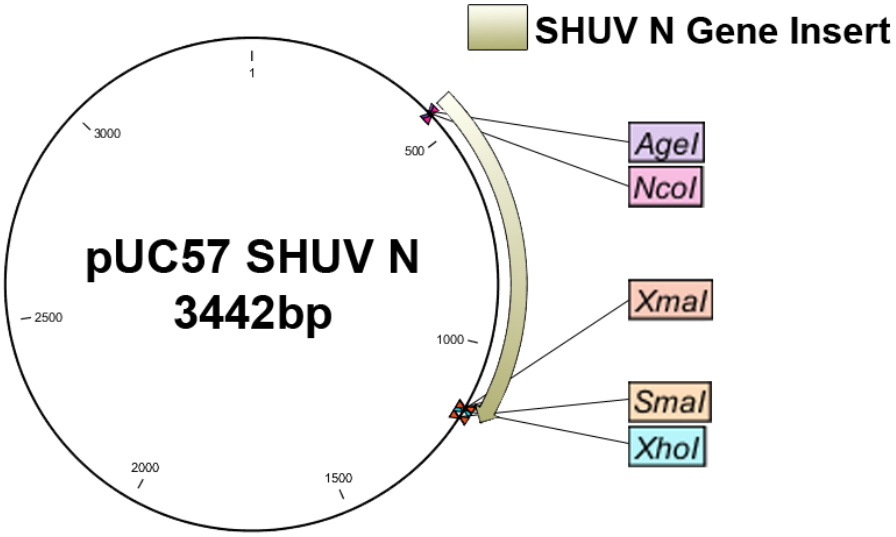


Figure 2.1: The synthesized SHUV N Gene cloned into a standard pUC57 vector.
The SHUV N gene insert is shown in pUC57 plasmid, along with the variety of restriction enzyme digest sites used for sub-cloning of the gene.

2.2.3 Expression Vector pProEX HTb

The *E. coli* expression vector pProEX HTb (Invitrogen, Thermo Fisher Scientific Inc , USA) was used for the general expression of the SHUV N gene in *E. coli* for the downstream development of a polyclonal antibodies raised against the expressed target protein. The pProEX HTb expression vector contains an ampicillin resistance gene for downstream selection purposes.

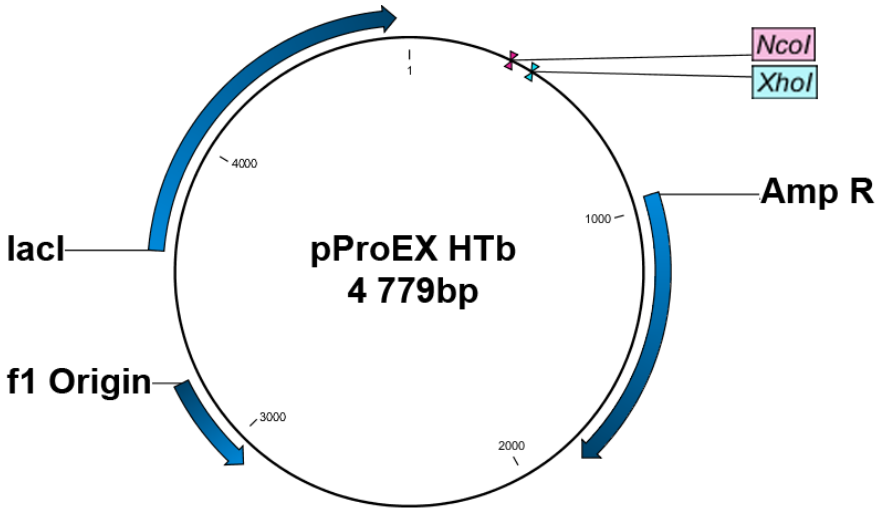


Figure 2.2: The vector map for the *E. coli* expression vector, pProEX HTb.
The vector map describes the location of the multiple cloning site before the cloning of the SHUV N gene. The location of restriction enzyme sites are shown along with the location of the origin of replication (ori), promoter sites and necessary genes for selective expression in *E. coli*. (Invitrogen, Thermo Fisher Scientific Inc , USA)

Table 2: The cloning vector and *E. coli* expression construct used in this study along with the corresponding working concentration of antibiotic used in the selective media.

Plasmid	Antibiotic Working Concentration
pUC57 SHUV N	Ampicillin at 100 ug/ml
pProEX HTb	
pProEX HTb SHUV N	

2.2.4 Cloning Strategy

The SHUV N gene was synthesised as described in section 2.2.2 using the restriction enzyme sites *NcoI* and *XhoI* on both the 5' and 3' termini to allow for direct sub-cloning into both the *E. coli* expression vector described here, and the plant expression vectors described in the following chapter.

A 15 µl sample of suspended DH5α *E. coli* (E. cloni® 10G cells, Lucigen) were transformed with 1 µl of pUC57 SHUV N construct DNA. This suspension was incubated on ice for 30 min before being heat shocked at 37°C for 1 min. 400 µl of Luria-Bertani lysogeny broth (LB) (Lennox, 1955) was added and the cell suspension was incubated on at 37°C for 1 hour before being spread plated on selective LB agar media supplemented to a working concentration of 100 µg/ml with ampicillin.

Single colonies were picked and grown up in 10 ml LB liquid with the correct antibiotic. A number of 1 ml glycerol stocks were created from these consisting of grown culture in LB liquid media mixed in a 1:1 ratio with 50% glycerol, these were stored at -80°C for future work. This was similarly and concurrently done with DH5α *E. coli* that contained the empty pProEX HTb expression vector. Once the cultures were grown, cells were pelleted at 15 000 x g for both the respective plasmids and DNA was obtained through the use of the QIAGEN DNA Miniprep Kit (Qaigen, USA) as per the manufacturer's instructions. All DNA was quantified through the NanoDrop™ 2000c spectrophotometer (Thermo Fischer Scientific, USA).

A 6xHis tag on the N-terminus of the expressed protein was required and thus pProEX HTb was selected as the *E. coli* expression vector of choice. Both pProEX HTb (the vector) and pUC57 SHUV N (the donor) underwent double restriction enzyme (RE) digest using *NcoI* and *Xho*I. The cut vector and donor were electrophoresed through a 1% agarose gel alongside GeneRuler™ 1kb DNA Ladder and the required bands of both the vector back bone and SHUV N insert were excised from the gel and purified using the-QAIGEN Gel Extraction Kit (Qaigen, USA) as per the manufacturer's instructions. The excised pProEX HTb backbone and excised SHUV N insert were then ligated at 4°C overnight using T4 Ligase (Roche, Basel, Switzerland) as per the manufacturer's instructions

The ligation mixture was transformed into chemically competent E.Cloni® 10G cells and plated on selective LB agar media supplemented to a working concentration of 100 µg/ml with ampicillin. All single colonies were screened using colony PCR with the primers described in Table 3.

All PCRs were performed in a BioRad Biocycler automated temperature cycler. These were carried out in 20 µL reactions with 0.5 Units (U) of KAPA Taq polymerase (Merck & Co, Kenilworth, USA),1x buffer, 200 µM dNTPs, 2 mM MgCl2, and 10 µM of each primer. Each colony was sampled and introduced into the PCR mixture. PCR cycles followed the procedure of denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing for 30 s at the temperature given in Table 3, and extension at 72 °C for 30 s. These cycles were followed by a final elongation step at 72 °C for 5 min

Once PCR was complete, selected colonies were grown up, DNA extracted and underwent double restriction enzyme digestion with restriction enzymes *NcoI* and *Xho*I to confirm the inclusion of the insert within the vector backbone.

Table 3: PCR primers used for screening colonies in this study. The sequence, length and T_m of the primers are shown.

Primer Name	Nucleotide Sequence (5' – 3')	Length (bp)	T_m
pProEX HT f	TTC TTC TTC TTG CTG ATT GG	20	56°C
pProEX HT r	CAC AGA AAA CCG CTC ACC	18	56°C

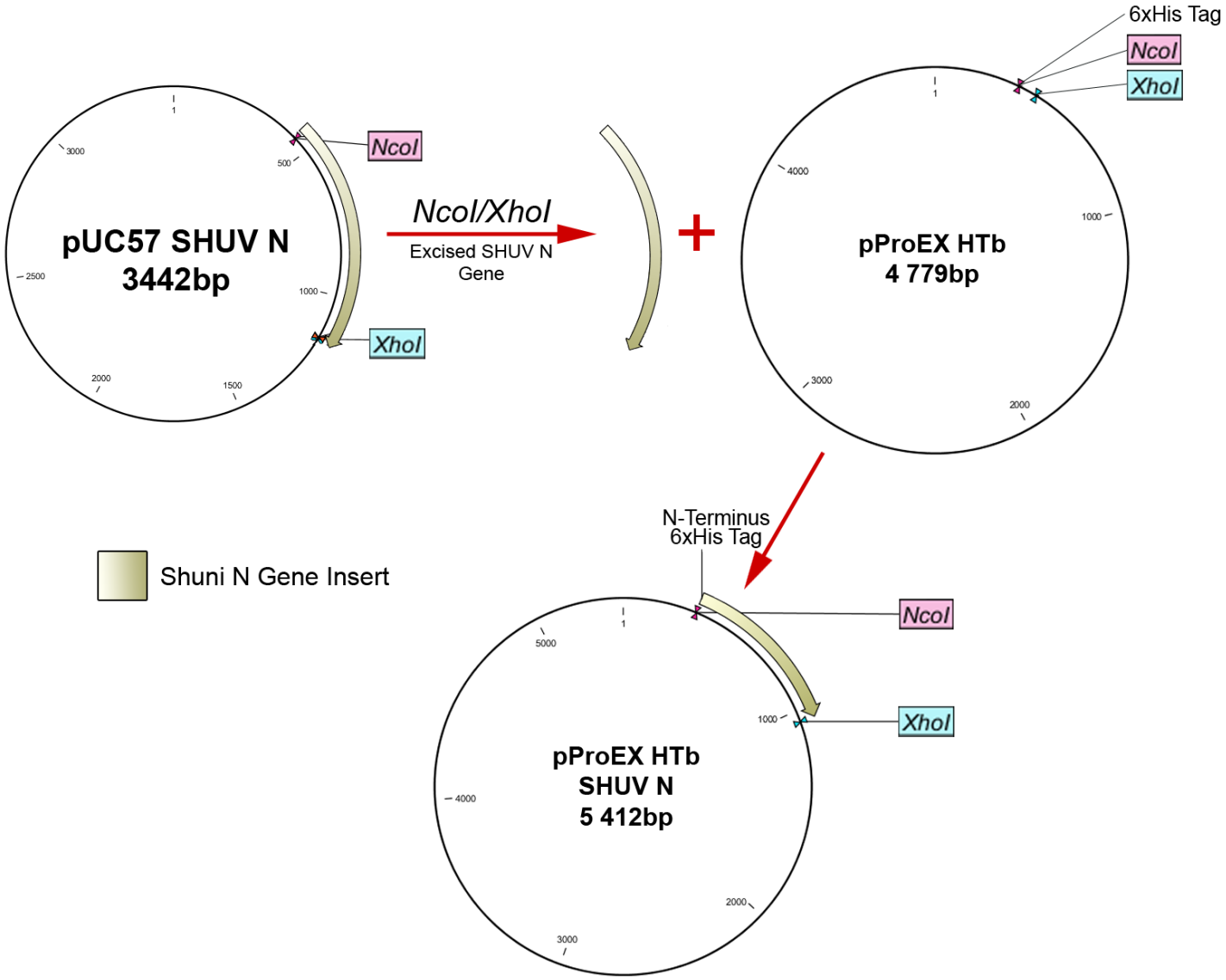


Figure 2.3: Flow diagram describing the direct subcloning of the SHUV N gene.
The diagram shows the step wise process of exercising the synthesized SHUV N gene from the cloning vector pUC57 SHUV N using REs *Nco*I and *Xho*I and subcloning into the *E. coli* expression vector pProEX HTb.

2.2.5 Expression in *E. coli* (Small and Large-scale)

Small Scale

Expression studies were first performed on a small-scale before being up scaled to larger volumes. Both the pProEX HTb SHUV N construct and the empty vector pProEX HTb (negative control) were each inoculated from 1 ml 50% glycerol stocks into a separate 10 ml of LB supplemented with ampicillin at 100 ug/ml working concentration. Each culture was left to grow overnight shaking at 37°C.

The overnight cultures were used to inoculate 10ml of fresh LB media containing appropriate antibiotics. The optical density (OD) at 600nm was monitored until an OD_{600nm} of 0.5 was achieved for both cultures. Once reached, each culture was inoculated with commercial isopropyl β-D-1-thiogalactopyranoside (IPTG) (Merck & Co, Kenilworth, USA) to a recommended working concentration of 0.5 mM to induce expression. The IPTG concentration was not varied or optimised. For each culture, 1 ml samples were taken each hour and pelleted. The supernatant and pellet were both assessed for expression.

Large-Scale

Using the conditions determined in the small-scale experiment, large-scale expression was performed in the following way. Both the pProEX HTb SHUV N expression construct and empty pProEX HTb were each inoculated from 1 ml 50% glycerol stocks into separate 10 ml LB media with ampicillin at a working concentration of 100 µg/ml. This was grown overnight shaking at 37°C before 10 ml of each culture was used to inoculate 50ml LB. Following a similar procedure, the entire 50 ml culture was grown overnight and used to inoculate separate 500 ml LB media. Each volume of media was supplemented with the correct antibiotic.

Once grown to the required OD_{600nm} of 0.5, each culture was inoculated with IPTG to a concentration of 0.5mM to induce expression. At a time point of 3 hours post induction, the cultures were centrifuged at 22 000 x g and the pelleted collected.

2.2.6 Protein Extraction and Purification

Each pelleted culture underwent lysis and protein extraction with BugBuster® 10x Protein Extraction Kit (Merck & Co, Kenilworth, USA) which was performed by following the inclusion body cell extraction protocol as per the manufacturer's instructions. The pelleted culture was gently resuspended in BugBuster before 25 units/ml of Benzonase (Merck & Co, Kenilworth, USA) was added in order to facilitate lysis and DNA degradation. This was incubated while shaking at room temperature for 20 mins. Following this, the suspension was centrifuged at 16 000 x g at 4°C for 20 mins. Supernatant was removed and used to asses protein solubility. Once again, the pellet was resuspended in BugBuster before being supplemented with 200 µg/ml lysozyme (Merck & Co, Kenilworth, USA) and incubated at room temperature for 10 mins. Once complete, six volumes of 1:10 BugBuster was added to the suspension before undergoing centrifugation at 16 000 x g at 4°C for 15 mins. This is referred to as Wash 1 (W1). The supernatant was removed and assessed as before before the pellet was washed twice more (W2 and W3) with 1:10 diluted BugBuster and pelleted. The resulting pellet was washed four times with one volume of endotoxin free 1xPBS before being pelleted and resuspended in endotoxin free 1xPBS. This was stored at -20°C until

animal trials began. The solubility and expression of the SHUV N protein was analysed at every step of the protocol via SDS-PAGE and western blot.

Endotoxin levels of the resuspended pellet sample were assessed using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript Biotechnologies, Piscataway, NJ, USA) following the manufacturer's instructions. Contaminant bacteria was tested for by plating samples on LB agar plate with no antibiotics. This was incubated overnight at 37°C and assessed for growth.

2.2.7 Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For both the pProEX HTb SHUV N and pProEX HTb empty cultures, the resulting washed cell lysate was aliquoted out and denatured with 5x Sample Application Buffer (SAB) consisting of 25% (v/v) glycerol, 0.5M DTT, 5% (w/v) SDS, 0:001% (w/v) bromothymol blue, at 95°C for 10 mins. Following this, denatured samples underwent fractionation by SDS-PAGE through a 15% polyacrylamide gel at 120V for 90 mins using the electrophoresis apparatus Biorad Mini-PROTEAN® Tetra Cell Systems (Bio-Rad Laboratories, Hercules, USA). The Color Prestained Protein Standard Broad Range 11-245 kDa marker (New England Biolabs, Ipswich, USA) was used in all subsequent SDS-PAGE gels.

The resulting polyacrylamide gel was incubated in Coomassie brilliant blue stain consisting of 0.1%(m/v) Brilliant Blue R-250, 50% (v/v) methanol and 10% (v/v) glacial acetic acid, at 37°C for 1 hour before being incubated in de-staining solution made up of 45% (v/v) methanol and 10% (v/v) glacial acetic acid, at room temperature (roughly 25°C) overnight with constant shaking.

2.2.8 Western Blot

After SDS-PAGE was performed, the resulting polyacrylamide gel underwent semi-dry electrophoresis transfer onto nitrocellulose membrane (Amersham™ Protran™ Premium 0.45 µm NC) using the Bio-Rad Trans-Blot Semi-dry transfer blotter (Bio-Rad Laboratories, Hercules, USA) at 15 V for 90 mins.

The nitrocellulose membrane was then blocked at room temperature for 30 mins with 5% w/v non-fat milk (Pick N' Pay, South Africa) in 1xPBS, pH 7.4, as a blocking buffer. Once complete, this was poured off and probed with blocking buffer containing the primary antibody, mouse anti-6xHis (Thermo Fisher Scientific, Waltham, USA) at a 1:2000 dilution and left shaking at 4°C overnight.

Following this, the membrane was washed repeatedly with 1xPBS containing 0.05% Tween 20 and probed using the secondary antibody, goat anti-mouse alkaline phosphatase (AP) conjugated antibody (Merck & Co, Kenilworth, USA) at a 1:10 000 dilution. This was washed repeatedly with 1xPBS and developed using 4-chloro-3-indoxyl-phosphate (BCIP/NBT) (SeraCare Life Sciences Inc., Milford, USA) solution left at room temperature for 60 mins if not stated otherwise.

A similar procedure was performed for the visualisation of SHUV N protein through the use of the raised rabbit sera as probing reagent. This rabbit sera contained the polyclonal antibodies raised against the *E. coli* expressed SHUV N protein. This rabbit sera was made up in the specified dilutions starting at 1:2000 before being serially diluted to 1:20 000 in blocking buffer. This was used to probe nitrocellulose membranes as a primary antibody. This was left shaking at 4°C overnight, after which the membrane was washed repeatedly with 1xPBS containing 0.05% Tween 20 and probed using the secondary antibody, goat anti-rabbit AP conjugated antibody (Merck & Co, Kenilworth, USA) at a 1:10 000 dilution.

2.2.9 Quantification of Protein

Quantification of the expressed protein was achieved through the use of SDS-PAGE and Coomassie Brilliant Blue staining as described above in section 2.2.7. The sample was run at varying dilutions alongside a known BSA standard series. Once thoroughly stained with Coomassie Brilliant Blue, the polyacrylamide gel was scanned and assessed using the computer quantification program GeneTools (Syngene, India) through the method of gel densitometry. A standard curve was created using the BSA standard of known concentrations and used to evaluate the concentrations of each sample.

2.2.10 Immunization of Rabbits for Polyclonal Antibody Production

Two female New Zealand White rabbits were obtained from the University of Cape Town Research Animal Facility (UCT RAF, South Africa) at approximately 3 months of age. The animals were kept in standard biosafety level 1 facilities within large animal pens at the Research Animal Unit with the Faculty of Health Science Faculty at UCT.

This study ran over the course of 5 weeks and was assessed and approved by the Animal Research Ethics Committee at UCT (AEC No. 017-010).

At the start of the trial, a 5 ml pre-bleed was collected from each rabbit before vaccinations began. Each rabbit was immunized at day 0 with follow up booster injections at day 14, day 21 and day 28. All immunizations were performed via subcutaneous injection mixed in a 1:1 ratio with Incomplete Freund's Adjuvant (Merck & Co, Kenilworth, USA). Each dose contained 500 to 600 µg of protein to a total volume of 1ml per injection per rabbit injected. This was injected into two different sites on the rabbits back.

Each booster injection was accompanied with 5ml bleeds to assess antibody titres against the injected protein. Each bleed was taken from the central ear artery after intravenous administration of acepromazine (ACP) to dilate the artery and calm the rabbits.

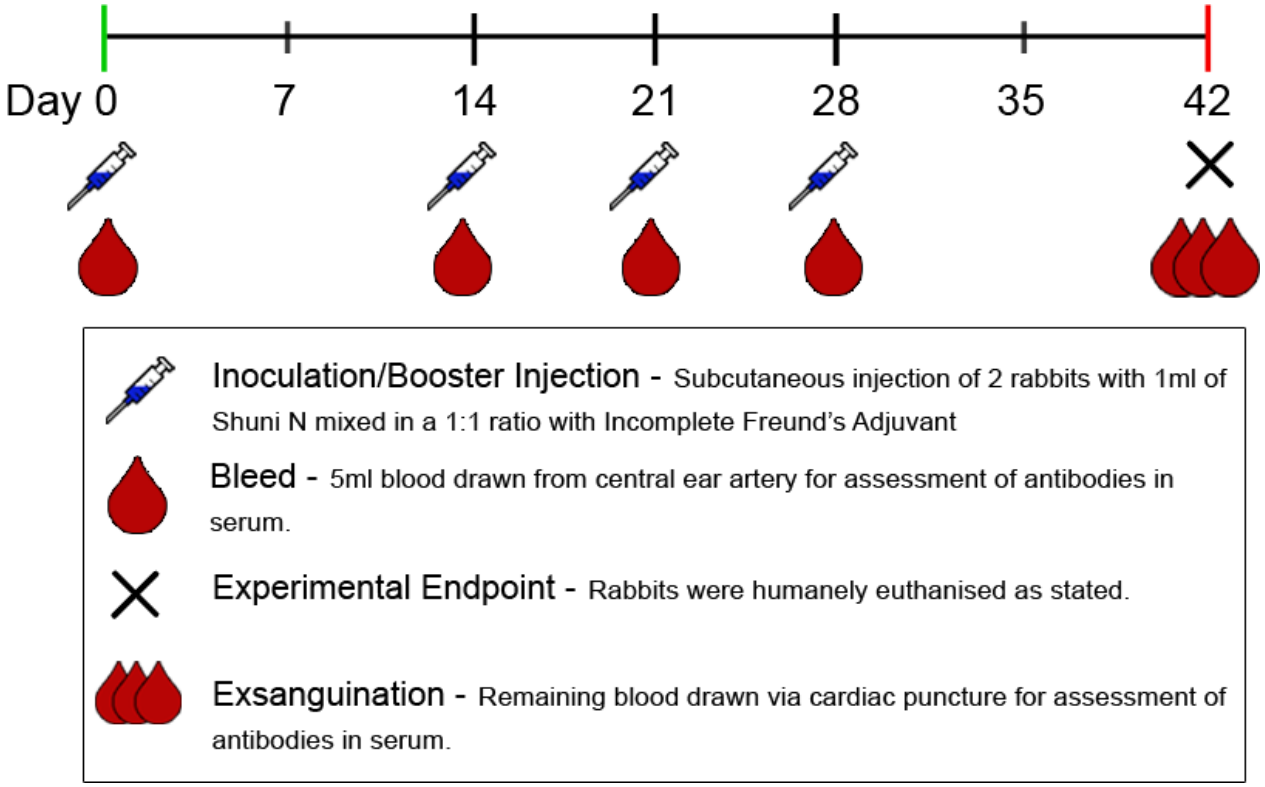


Figure 2.4: Experimental timeline for the inoculation of two rabbits.
The experimental timeline stating the inoculation of two rabbits with *E. coli* produced SHUV N protein at set timepoints, along with subsequent bleeds. The experimental endpoint and exsanguination is seen.

The bleeds from day 28 were assessed for the sufficient titres of antibodies raised against our target protein via western blot, whereafter it was decided that day 42 would be the experimental endpoint. The rabbits were once again intravenously injected with Acepromazine (ACP) followed by a mixture of ketamine and xylazine of no more than 6 mg/kg and 0.7 mg/kg of body weight respectively. The duration of the anaesthesia was 45-60 mins. The animals were then exsanguinated via cardiac puncture over a period of 10-15 mins. The death of these rabbits was then confirmed by intra-cardiac injection of potassium chloride (KCl).

All blood collected was in 15ml centrifuge tubes containing Percoll (GE Health Care, USA) and centrifuged at roughly 1300 x g for 10 mins in order to separate the samples into plasma and cells. This plasma (serum) was aliquoted out and stored at 4°C for short term use or -20°C for long term storage.

2.3 Results

2.3.1 Designing the SHUV Nucleocapsid (N) Gene

Consensus Sequence

A consensus sequence for the SHUV N gene was compiled through the alignment of complete nucleocapsid (N) gene sequences from various SHUV isolates across many locations to give a complete consensus sequence. The table of compiled N gene sequences can be seen in Table 1.

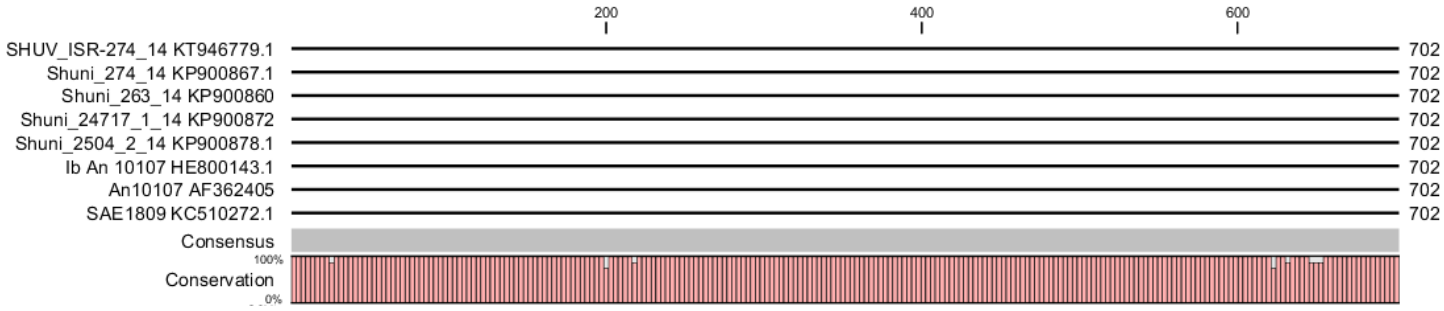


Figure 2.5: Sequence alignment of the various SHUV N gene sequences
The open reading frame of eight SHUV N gene sequences are aligned to create the consensus SHUV N gene sequence to be used during this study. The conservation of the consensus sequence is shown in pink.

Optimisation

Codon and GC content optimisation for optimal expression in *N. benthamiana* were performed on the consensus SHUV N gene sequence. As shown in Figure 2.6, the Codon Adaptation Index (CAI) was improved from a score of 0.73 to a score of 0.92 while the Frequency of Optimal Codons (FOP) in Figure 2.7 was improved upon so that 72% of all codons fell within the highest usage frequency for *N. benthamiana*.

The GC content of the sequence was brought down to 39.84% from 46.50% before optimisation, closer to the GC content of *N. benthamiana* itself: see Figure 2.8.

Internal RE sites that would have possibly interfered with subsequent subcloning of the gene were removed, while external RE sites were added to aid in subcloning of the gene into various expression vectors downstream in the correct orientation. Various cis-acting elements were removed in the optimized sequence, namely a single PolyA (TATAAA) site and two Destabilizing (ATTTA) sites.

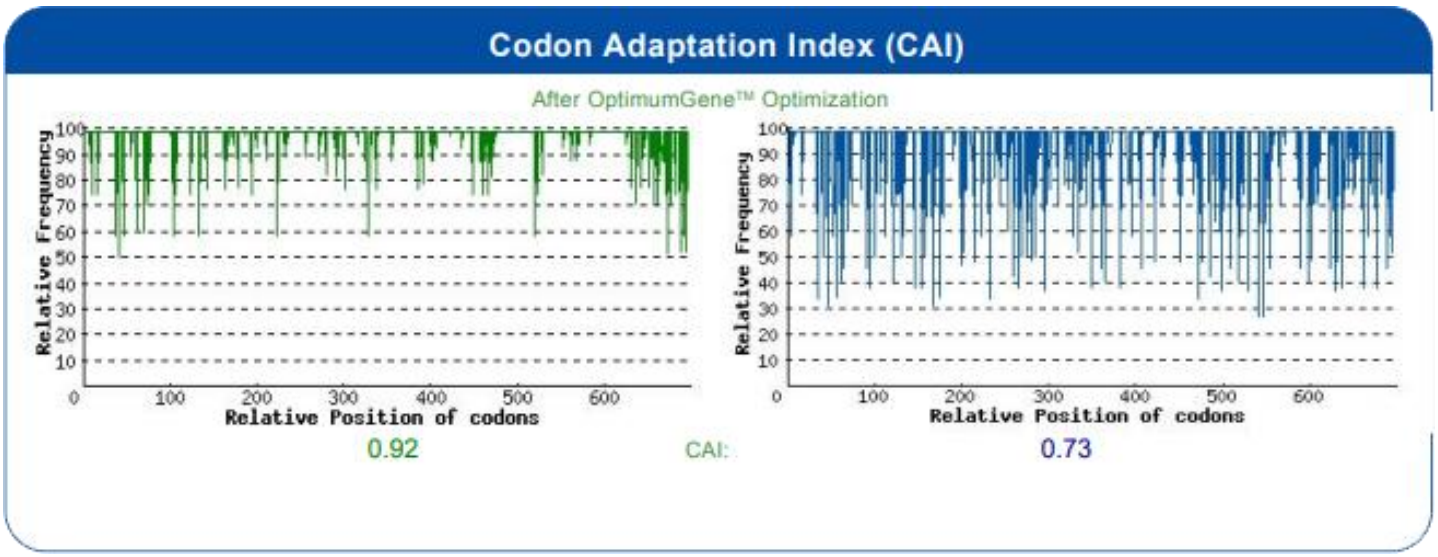


Figure 2.6: The results of codon and GC optimisation on the consensus SHUV N gene sequence for *N. benthamiana* expression.

Codon and GC content optimisation of the SHUV N in preparation for *N. benthamiana* expression. The distribution of codon usage frequency along the length of the gene sequence is shown in the top figure. A Codon Adaption Index (CAI) of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

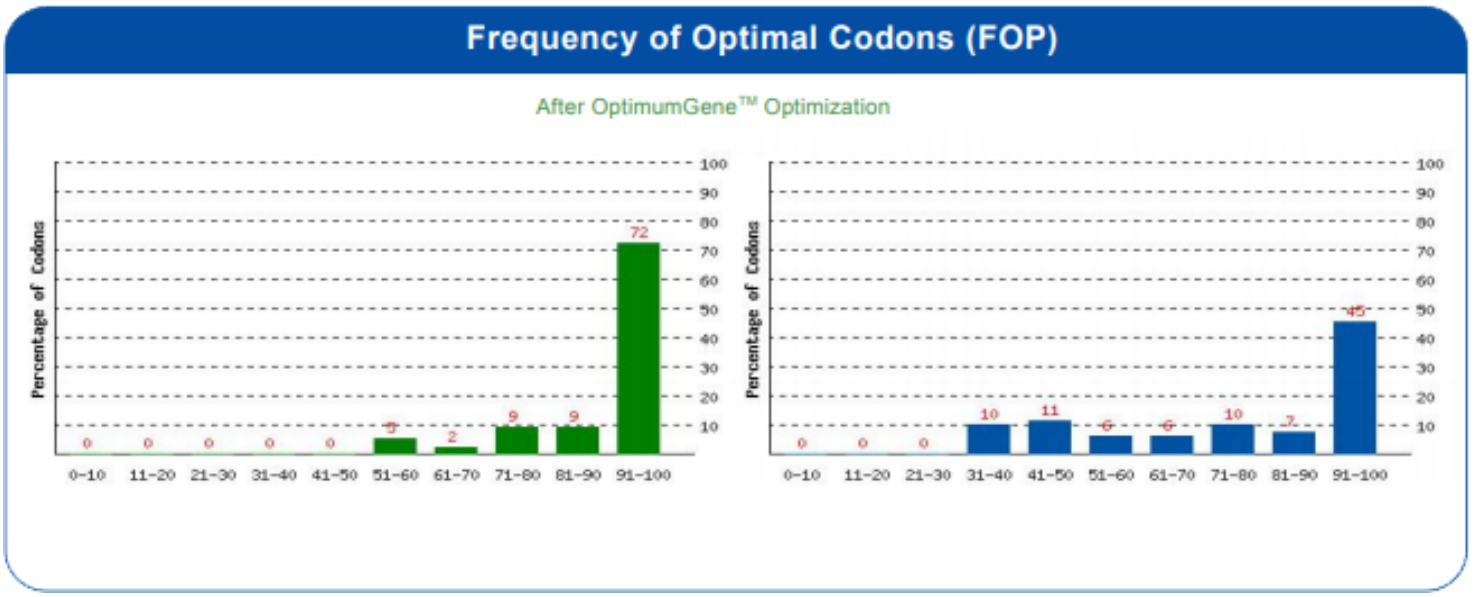


Figure 2.7: The percentage distribution of codons in computed codon quality groups.

The value of 100 is set for the codon with the highest usage frequency for a given amino acid in the desired expression organism.

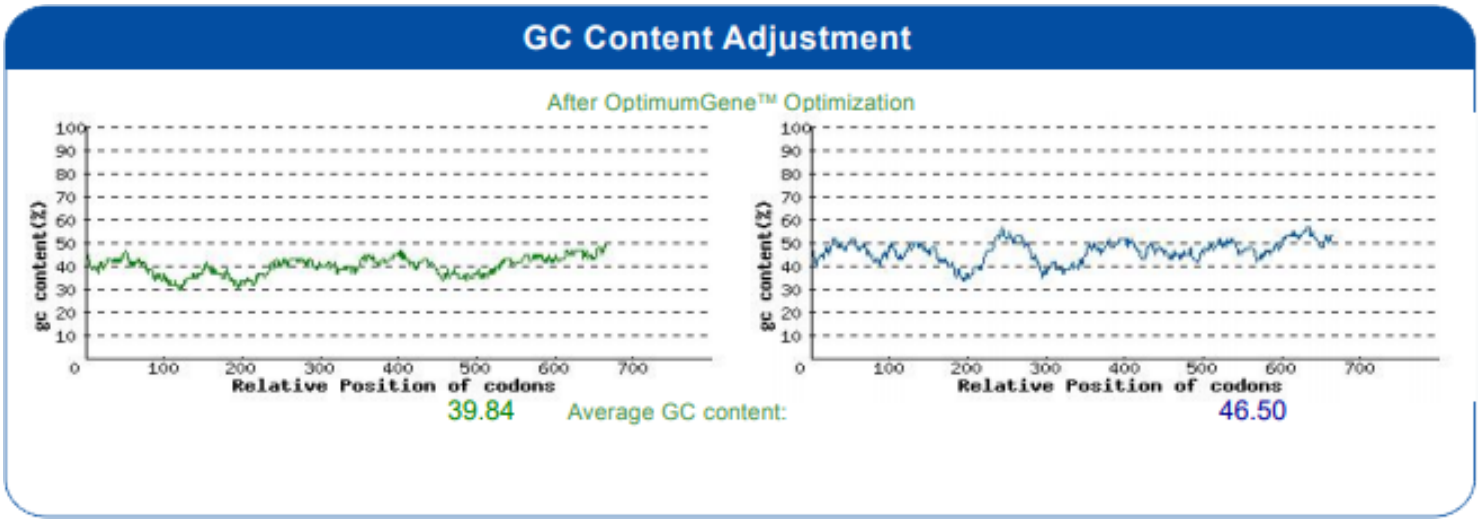


Figure 2.8: GC content adjustment level for the optimised sequence.
The ideal percentage range of GC content lies between 30% and 70%. Peaks of GC content in a 60 base pair window have been removed.

2.3.2 Cloning

Creation of pProEX SHUV (N) Expression Construct

The SHUV N gene was directly subcloned into the pProEX HTb using RE sites *NcoI* and *XhoI*. The resulting construct contained the 723bp gene of interest preceeded by a N-terminus 6xHis tag for downstream detection and possible purification of the SHUV N protein. Following this, the pProEX HTb SHUV N expression construct was successfully transformed into *E. coli* (DH5α *E. coli*) as described in section 2.2.4. Colony PCR was performed with vector primers flanking the multiple cloning site in order to select successful clones as shown in Figure 2.9. This can be seen by the amplification of an 854bp band. This correlates with the predicted size *in silico* for the inserted SHUV N gene within the multiple cloning site (MCS) of the vector.

Further confirmation of the inserted gene was performed through RE digest with *Nco*I and *XhoI* to give the expected band of 723bp (Figure 2.10). All subsequent *E. coli* expression studies were performed using the pProEX HTb SHUV N expression clone.

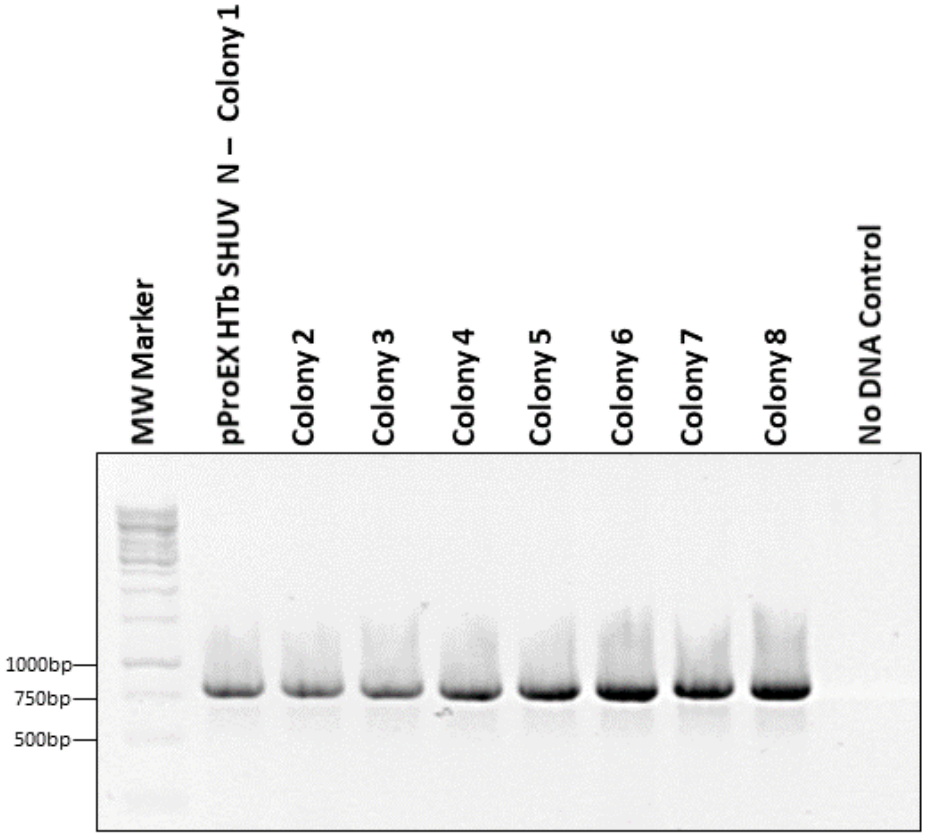


Figure 2.9: Colony PCR of *E. coli* transformants.
Each colony underwent PCR with pProEX specific primers to reveal the inclusion of a gene of expected size of 854bp across the MCS of the expression vector.

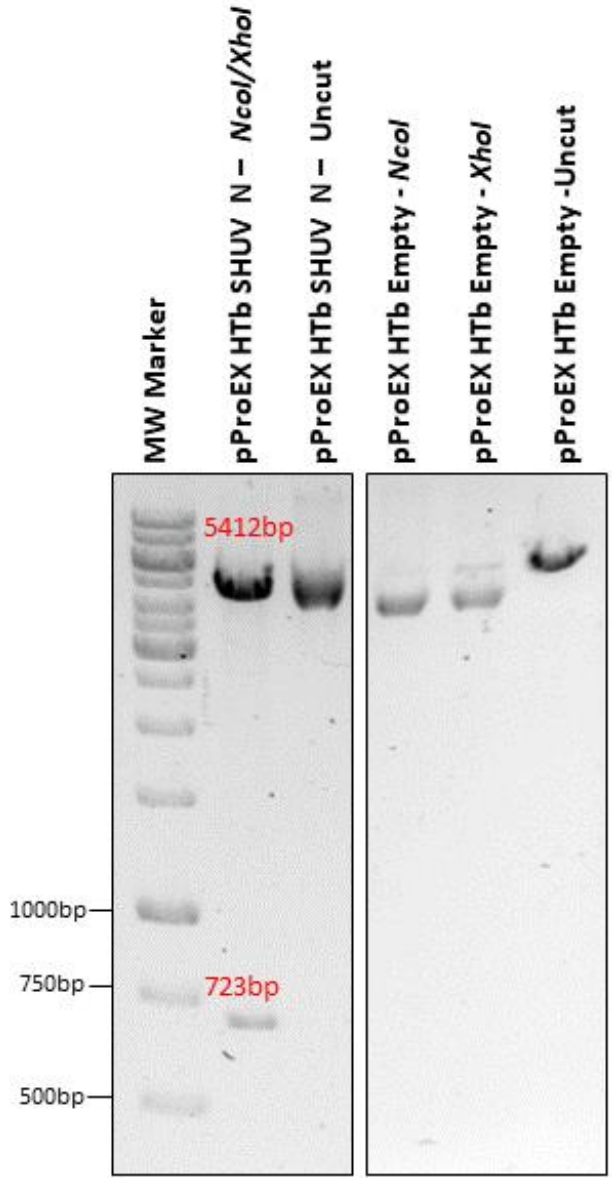


Figure 2.10: Restriction enzyme digest of pProEX HTb SHUV N DNA.
Restriction enzyme digest was performed using *NcoI* and *XhoI* restriction enzymes to target sites either side of the inserted SHUV N gene. The 723bp band is the expected size of the cut insert while the 5412bp band is the expected size of the digested vector backbone.

2.3.3 Protein Expression

Small-scale Induction

Once *E. coli* was successfully transformed with the pProEX HTb SHUV N expression construct, small-scale expression studies were performed to check for expression of the target protein and optimise times post induction for the highest yield expression. This was performed on the expression clone of interest, pProEX HTb SHUV N, as well as our negative expression control, pProEX HTb Empty, a pProEX HTb clone without the subcloned SHUV N gene insert.

Time trial expression studies began once the starter cultures reached an OD_{600nm} of 0.5. The culture was inoculated with IPTG to induce protein expression at concentration of 0.5 mM as described in section 2.2.6 and sampled every hour post induction, lysed and pelleted. Both the pelleted culture and supernatant was assessed qualitatively for protein expression through western blotting using anti-6xHis tag primary antibody as described in section 2.2.5 to 2.2.8 in order to assess the solubility of the expressed SHUV N protein.

The supernatant of the pelleted cultures was assessed for the presence of protein expression and did not show the presence of any of the expected bands, in either the sample or the negative control. This leads to the understanding that the *E. coli* produced SHUV N protein may in fact be insoluble in nature as it is found in the insoluble pellet and not the aqueous supernatant after lysis.

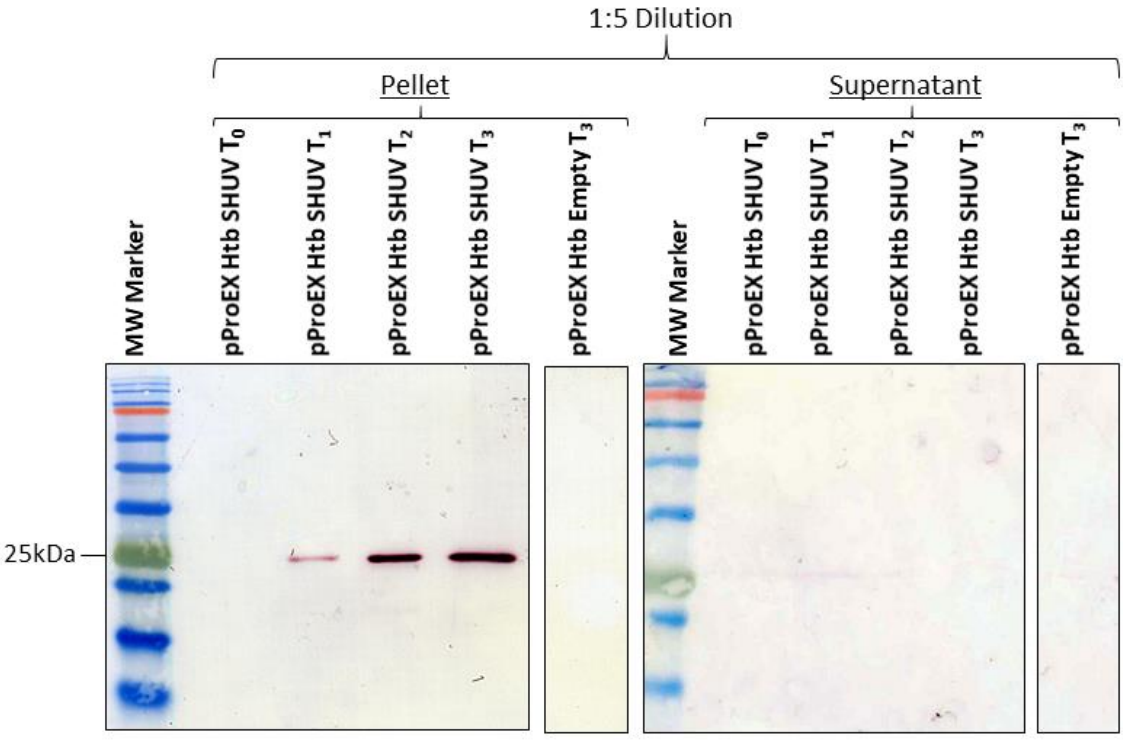


Figure 2.11: Western blot of pelleted *E. coli* pProEX HTb SHUV N extract and its supernatant after small-scale expression.
Western blot comparing pelleted *E. coli* cultures containing the pProEX HTb SHUV N expression construct and its supernatant versus the pProEX HTb empty vector culture. Expression was induced at T_0 and measured for a subsequent three hours afterwards. Each pelleted culture was **diluted 1:5** and probed using Mouse Anti-6xHis 1:2000. The expected monomer is seen at 25kDa.

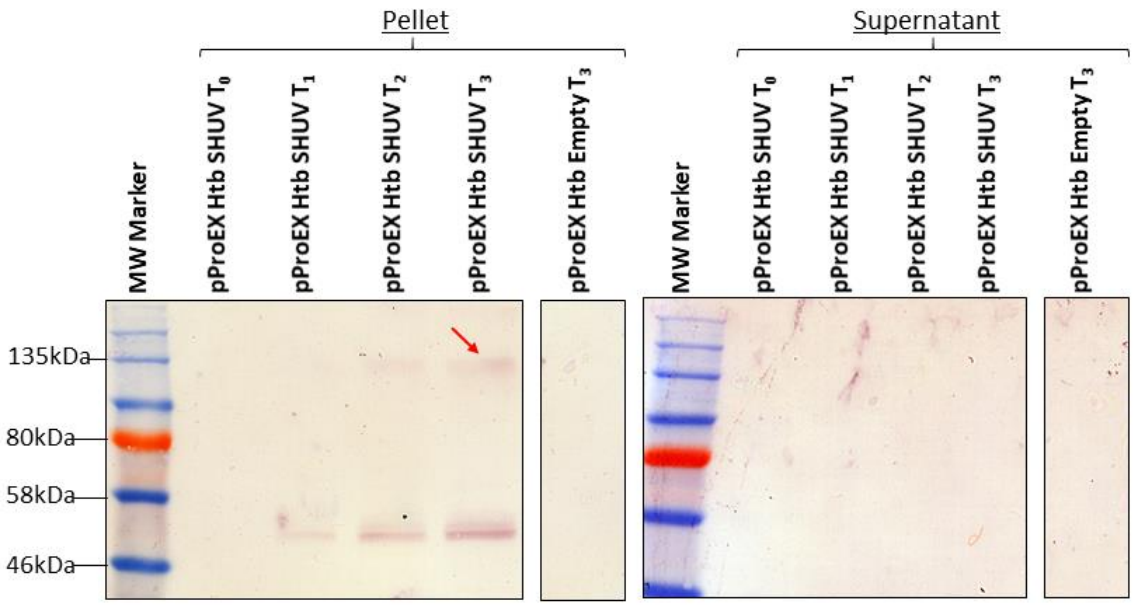


Figure 2.12 Western blot of pelleted *E. coli* pProEX HTb SHUV N culture and its supernatant after small-scale expression.
Western blot comparing pelleted *E. coli* cultures containing the pProEX HTb SHUV N expression construct and its supernatant versus the pProEX HTb empty vector culture equivalent. **Undiluted** crude pellet shows the formation of possible dimers and multimers. Probed using Mouse Anti-6xHis 1:2000.

As shown in Figure 2.11 and Figure 2.12, after induction, the pelleted cultures were probed and a protein band correlating with the expected size of the SHUV N protein, approximately 25kDa, was shown to be present in the pelleted pProEX HTb SHUV N clones. Expression is seen from 1 hour after induction and was greatest 3 hours after induction. This band was not present in our negative expression control. Undiluted pelleted samples shown in figure 2.12 showed large over-exposed bands at roughly 25kDa indicative of overloaded lanes and this was not seen in the supernatant.

Figure 2.11 shows the presence of this band when the samples were diluted 1:5 as to not cause overloading of the individual lanes. Undiluted samples in Figure 2.12 showed the presence of possible dimers and multimers at roughly 50kDa and 135kDa respectively in the pProEX HTb SHUV N cultures. These bands were not seen in the negative expression control. These bands were present 1 hour after induction and were greatest 3 hours after induction.

Optimal conditions for *E. coli* SHUV N expression were found to be pelleting and lysing of the culture 3 hours after induction with 0.5mM IPTG.

Large-scale Induction and Expression

E. coli expression of the SHUV N protein was upscaled to 500 ml for antigen production for downstream animal trials. pProEX HTb SHUV N cultures and pProEX HTb without insert as a negative expression control were grown and spun down 3 hours after induction. Figure 2.13 shows the supernatant after pelleting the culture after the various wash steps (W) during the protcol. The final product is pelleted and resuspended for animal inoculation. As can be seen in Figure 2.13, the overloaded pellet lane shows the abundance of SHUV N protein expression in the culture; thus, the bands seen in the various wash fractions are the expected minimal amounts of protein being lost during each resuspension and wash step.

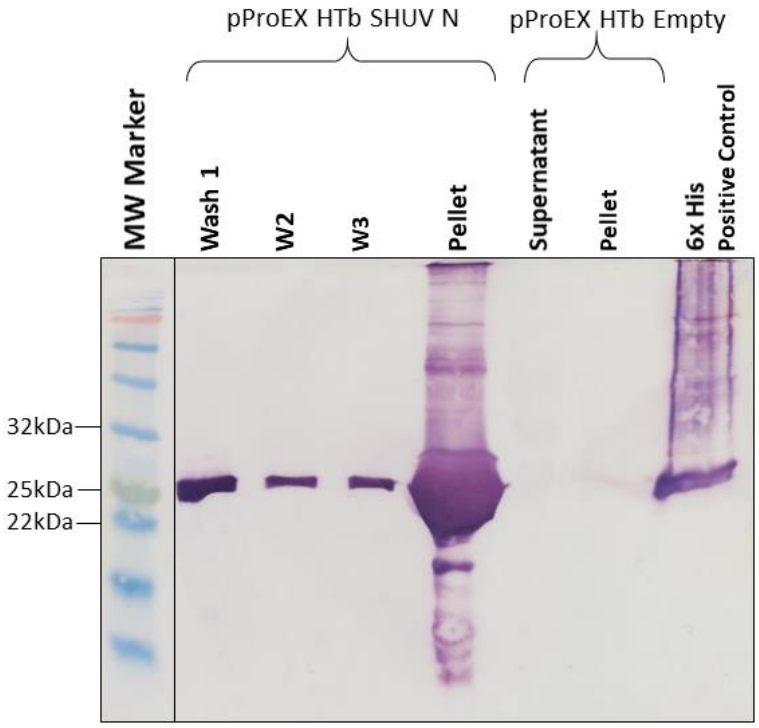


Figure 2.13 Western blot of induced *E. coli* pProEX HTb SHUV N culture after BugBuster washes. Supernatants of induced pelleted crude culture were taken during BugBuster washes (W). The final washed *E. coli* pProEX HTb SHUV N pellet is shown. Empty vector supernatant and washed pellet are negative controls. Positive control is unwashed *E. coli* extract of induced pProEX HTb SHUV N after 3 hours. Probed using Mouse Anti-6xHis 1:2000.

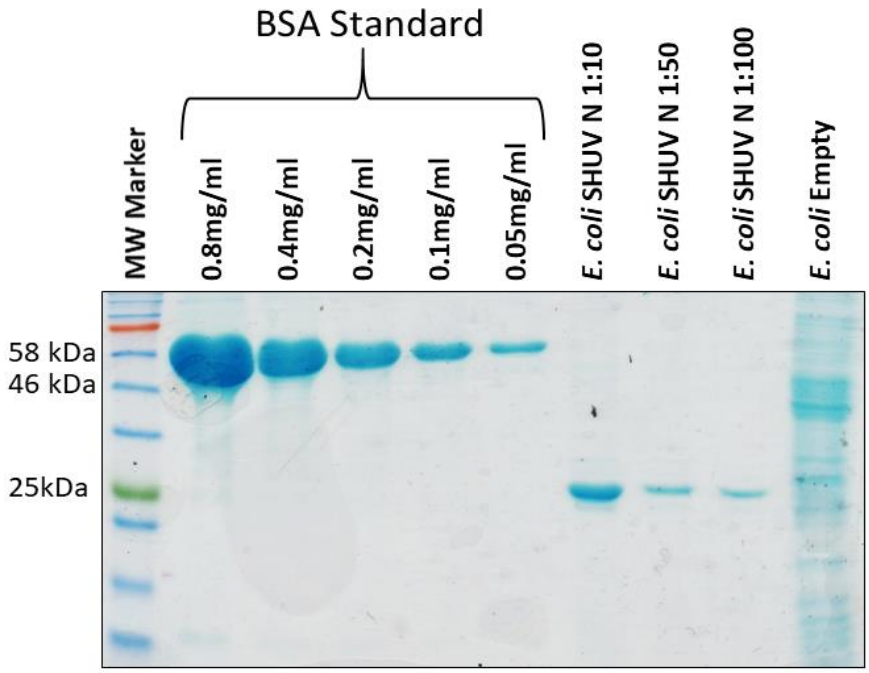
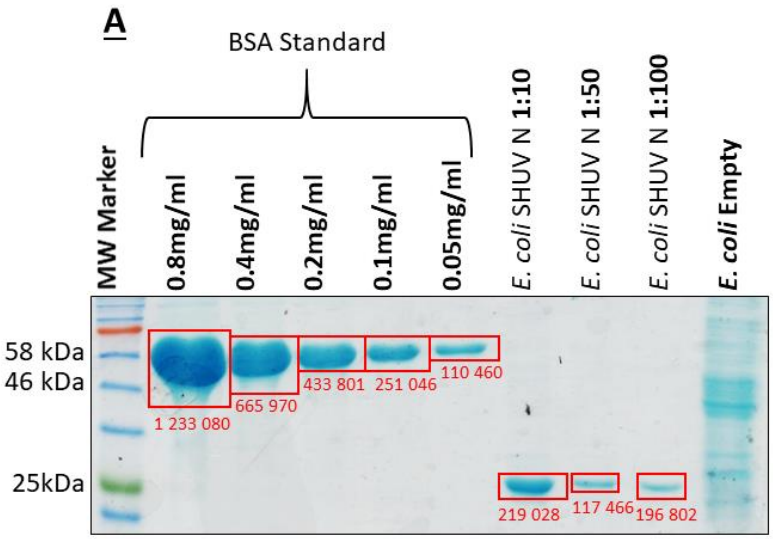


Figure 2.14: SDS-PAGE after Coomassie Brilliant Blue staining of BSA standard and washed *E. coli* pProEX HTb SHUV N culture.
BSA Standard compared to *E. coli* produced SHUV N with dilutions from 1:10 to 1:100 to be quantified through gel densitometry.



B

BSA Standard (mg/ul)	Signal
0,8	1233080
0,4	665970
0,2	433801
0,1	251046
0,05	110460
SHUV N **1:10**	219028
SHUV N **1:50**	117466
SHUV N **1:100**	196802 (Deemed an outlier)

Figure 2.15: Evaluation of Coomassie Brilliant Blue stained acrylamide gel through densitometry and subsequent results.

The quantification of *E. coli* expressed SHUV N protein evaluated through densitometry analysis of the appropriate band. This was determined through the use of a BSA protein standard loaded with equal volume. The concentration of each standard is indicated above the lane along with the densitometric signal being indicated under each of the highlighted bands (A). The determined signal for each standard and sample is indicated in the table (B). The SHUV N 1:100 signal was deemed an outlier.

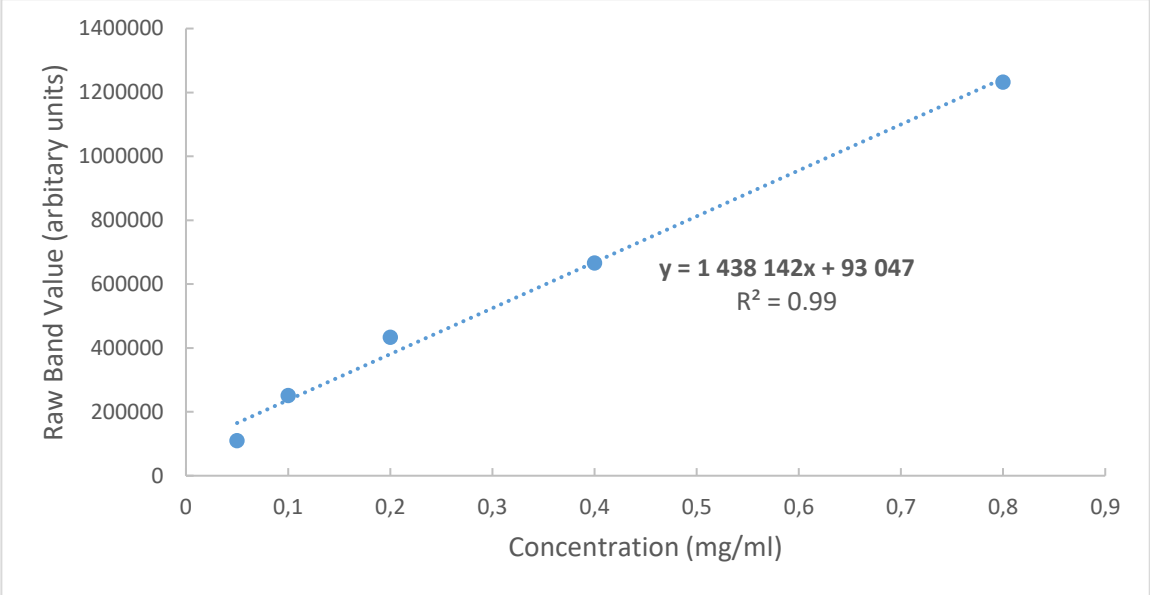


Figure 2.16: The standard curve created through the densitometric anaylsis of the BSA protein standard seen in Figure 2.15.

Densitometry analysis comparing the select BSA protein standard bands with their ascribed concentration. The R^2 value and equation shown were used for quantification of recombinant *E. coli* produced SHUV N protein.

While the final washed sample was not pure, the expressed SHUV N protein was present in much larger quantities than endogenously expressed *E. coli* proteins. The presence of dimers and multimers was seen in far smaller quantities after the BugBugster washes were performed. The final washed pellet was resuspended in 6ml of endotoxin free 1x PBS and led to a final concentration of 0.853mg/ml of the SHUV N protein. The concentration of the washed SHUV N protein extract was quantified and calculated from a linear standard curve as described in section 2.2.9, against known BSA dilutions, using gel densitometry. Further assessment documented that the SHUV N protein made up 69.5% of the total soluble protein (TSP) post wash. Thus 1 litre (2x 500ml) of induced *E. coli* pProEX HTb SHUV N culture, after BugBuster wash, yielded a final mass of 5.117 mg of SHUV N protein.

2.3.4 Polyclonal Antibody Generation

Further purification of the *E. coli* produced SHUV N protein sample did not take place due to the overwhelming quantities of the expressed protein in relation to the endogenously expressed *E. coli* proteins. This allowed the expressed SHUV N protein to probably be the most immunodominant protein in each inoculation. The protein sample was then prepared for antibody production through animal trials as described in section 2.2.10.

With each inoculation, 5ml of blood was collected to check for the presence and formation of polyclonal antibodies to the injected SHUV N protein. Two weeks after the initial inoculation, presence of anti-SHUV N antibodies was shown in the sera of both rabbits. This was performed via western blotting against *E. coli* produced SHUV N protein at first (Figure 2.17), before being tested against transiently expressed SHUV N protein in plant crude extract (Figure 2.18). This was repeated with each subsequent bleed. Looking at Figure 2.17, a large band of 25kDa can be seen across all dilutions which correlates with the expected size of the SHUV N protein. An anti-6xHis control was used to confirm the presence of the protein. A small non-specific band can be seen just below the 25kDa band in lanes probed with anti-SHUV N and anti-6xHis. It is assumed this is an endogenous *E. coli* protein binding antibody found in rabbit sera that have possibly been raised against it when inoculated with the same *E. coli* produced protein. In the case of the lane probed with anti-6xHis, it is assumed this is either non-specific binding or possible degradation of the SHUV N protein, hence the reduced size and the fact it was still recognised by the 6xHis antibody.

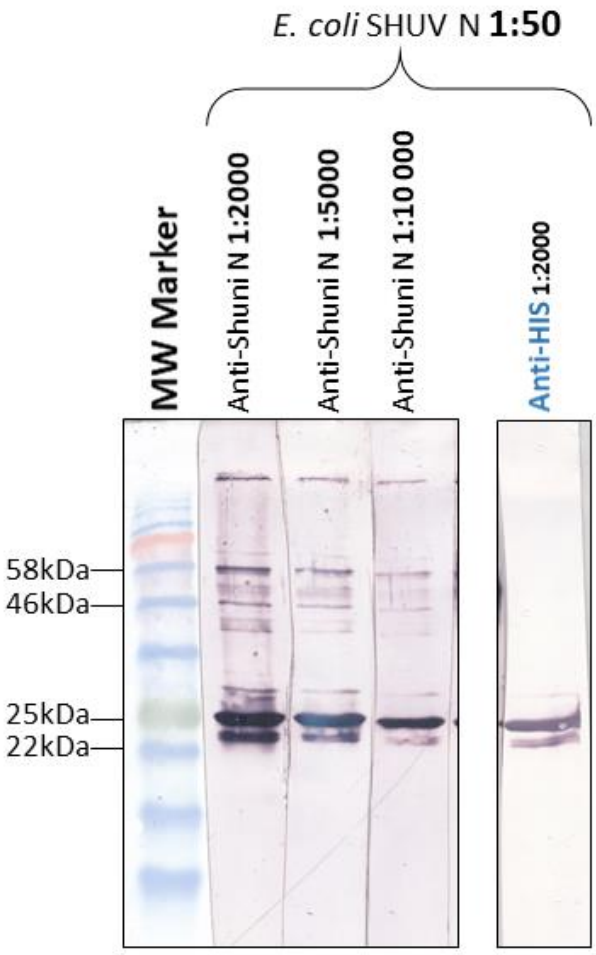


Figure 2.17: Western blot of *E. coli* produced SHUV N protein probed with dilutions of animal sera.
Western blot of *E. coli* produced SHUV N probed using the given dilutions of rabbit serum (Anti-Shuni N) containing antibodies raised against *E. coli* expressed SHUV N. All *E. coli* produced SHUV N was diluted **1:50**. Positive control is the probing of the *E. coli* produced SHUV N with Anti-6xHis at 1:2000.

All plant crude extracts used in the testing of this polyclonal antibody were transiently expressed SHUV N protein in *N. benthamiana,* which will be expanded upon in Chapter 3. Plants infiltrated with expression constructs pEAQ *HT* C-term SHUV N and empty pEAQ *HT* were used for +ve and -ve plant crude respectively.

Once the animal trial had reached the end point, each rabbit was exsanguinated, the blood collected, and sera obtained as described in section 2.2.10. A dilution series was created for the sera and tested against 6 µg TSP of transiently expressed SHUV N protein in crude plant extract labelled '+ve plant crude', and equivalent with empty vector infiltrated plant crude referred to as '-ve plant crude'.

As can be seen in Figure 2.18, bands of roughly 25kDa and 50kDa are detected in the +ve plant crude using the rabbit sera containing possible anti-SHUV N antibodies. This correlates with the expected size of our transiently plant expressed SHUV N protein, both the monomer and its dimer respectively. The monomer is seen in sera dilutions of 1:2000 – 1:20 000 while dimer is seen only in sera dilutions of 1:2000 – 1:10 000. The presence of each band becomes fainter with increased sera dilutions as expected.

With the -ve plant crude samples, a band of approximately 22kDa is visible in the 1:2000 – 1:5000 sera dilutions but is no longer visible in the higher dilutions. It is thought this band is indirect binding of the anti-SHUV N antibody to endogenously expressed plant protein.

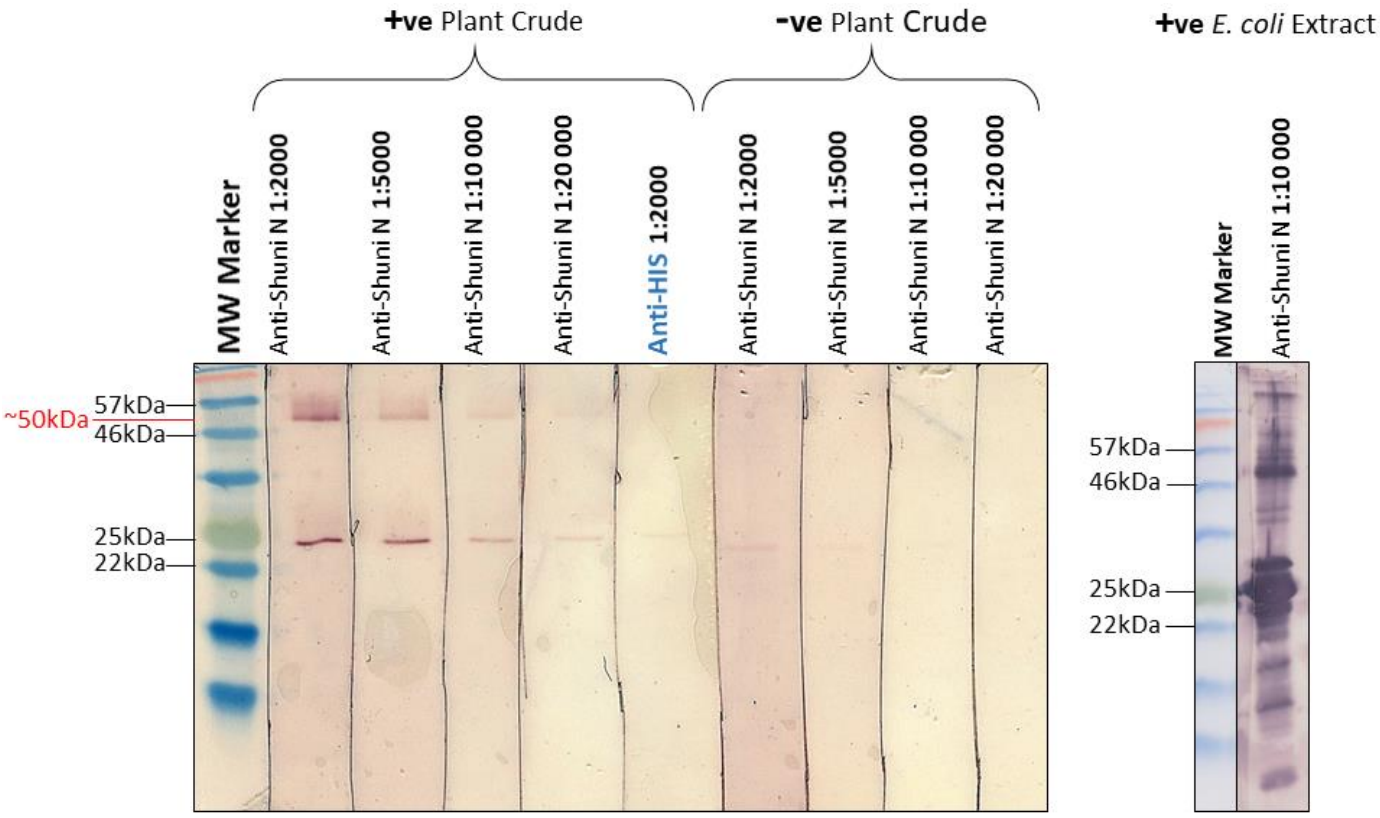


Figure 2.18 Western blot of plant produced SHUV N protein with dilutions of animal sera inoculated with SHUV N.
Western blot of plant crude extract, both positive (+ve) and negative (-ve) for the expression of SHUV N. This is probed using dilutions of rabbit serum containing antibodies raised against *E. coli* expressed SHUV N. The probing of *E. coli* extract expressing SHUV N is seen for comparison.

2.4 Discussion

The production of recombinant proteins in bacterial systems such as *E. coli* has been shown to be cost effective, easily manageable, and antigen applicable proteins can be made without the need for specialised eukaryote expression systems (Chen, 2012, Terpe, 2006).

In this study, the SHUV N gene sequence was constructed as a consensus sequence from seven different sequences from SHUV isolates and subsequently used for all downstream cloning and expression in both *E. coli* and plant expression systems. It was constructed from four separate sequences isolated from ruminants found in Israel (Golender et al., 2015), two sequences isolated from Nigeria (Goller et al., 2012, Saeed et al., 2001) and a single sequence obtained from a horse in South Africa (van Eeden et al., 2014a). This was due to the limited number of complete SHUV N gene sequences being made available at the time of synthesis. Once this was created, it was optimised specifically for downstream expression in *N. benthamiana* as described in chapter 3.

The use of the pProEX HTb expression vector allowed for the easy cloning and design of the expression construct. The pProEX HT expression system is renowned for its high efficiency translation a the ability to be easily inducible(Polayes and Hughes Jr, 1994). This was seen in this study with the high yields of around 0.853 mg/ml of SHUV N protein after extensive BugBuster washes. This in comparison to other *E. coli* expression studies showed the yield of the SHUV N protein was above average. *The E. coli* expression of polioviral proteins VP0, VP3, VP1 and 3AB gave a final yield of around 0.016-0.036 mg/ml for each expressed protein (Uma et al., 2016) while a study on the development the *E. coli* expression of porcine circovirus-2 (PCV-2) CP protein within the BRU led to yields of between 0.25 – 0.35 mg/mL (Gunter, 2017). While both studies did further purification through the use of a nickel affinity purification due to all proteins containing an affinity tag, the loss in yield due to this purification was not significant.

While improvements in this regard are not needed, it is possible that more extensive optimisation may lead to improved yields. The expression time trial ran over the course of three hours post induction with IPTG. It is possible a longer sampling period would lead to a larger yield. IPTG was used at a concentration of 0.5mM which is recommended by the commercial pProEX HT expression protocol (Invitrogen, Thermo Fisher Scientific Inc , USA). Varying concentrations of IPTG was not tested due to the recommended concentration being sufficient, however, future work could incorporate titrations of IPTG from 0.02 mM to 2.0 mM in order to asses changes to expression yield (Terpe, 2006).

It is possible that the strain of *E. coli* used may have an effect on the yield of the expressed protein. While this study chose the *E. coli* DH5α strain for protein expression, it has been shown that *E. coli* BL21 and K12 strains may be better candidates for recombinant protein expression due both strains and their derivatives being deficient in *lon* (Phillips et al., 1984) and *ompT* proteases (Terpe, 2006). It has also been suggested that the T7 RNA polymerase system, found in some Bl21 derivatives (Studier and Moffatt, 1986) which is under lac promoter derivate L8-UV5 lac, may increase yields when compared to the main *lac* promoter expression system (Terpe, 2006).

The use of BugBuster as described allowed for optimal cell lysis along with the removal of soluble cell debris and proteins to successfully extract the required amount of SHUV N protein. This came at the expense of slight SHUV N protein loss in the wash steps, however whether this was due to slight solubility of the protein or unrefined technique is debatable. Following BugbBuster washes,

it was seen that the pellet was difficult to resuspend due to aggregation. It may be the case that the SHUV N protein becomes insoluble once it begins to aggregate and that the small amount of unaggregated protein is washed off during said BugBuster washes, hence the bands seen in wash lanes in Figure 2.13. Needless to say, 1000ml of culture allowed for the production of 5.1174 mg of extracted and washed SHUV N protein. Thus, any loss of protein seen during the washes was negligible despite being so obvious in the western blot shown in Figure 2.13. While the washes performed helped reduce the amount of soluble cell debris and proteins, the final pellet cannot be considered pure despite a large portion of the pellet being expressed SHUV N protein. Evaluation of the final extract showed that the recombinantly expressed SHUV N protein made 69.5% of the TSP in the extract.

The large amounts of this expressed protein far outweighed any constitutively expressed *E. coli* protein left behind after the use of BugBuster. This allowed for the inoculation of rabbits with the confidence that the expressed SHUV N protein would be immunodominant over the lower concentration constituent *E. coli* proteins (Burns, 2009).

While purification through the use of a His Trap column or nickel affinity batch purification was an option, the abundance of the protein after washes indicated it was not necessary for the development of polyclonal antibodies. The insoluble nature of the *E. coli* expressed protein would have proven to be a challenge to purify due to the need for the protein to be soluble in the mobile phase. Without this, the binding of the insoluble protein to the nickel resin would have been difficult without first denaturing the protein and thus possibly losing the necessary epitopes required to act as an antigen. This was possibly the case when making PCV-2 CP in this lab, where the insoluble nature of the *E. coli* expressed PCV-2 CP protein led to poor binding to the nickel affinity gel and, while not disastrous, subsequent protein loss was seen (Gunter, 2017)

The sera obtained over the course of the animal trials were tested in by western blot. Blots showed a reasonable amount of binding against constituently expressed *E. coli* protein, as expected due to the inoculum having originated from *E. coli* cell cultures. Despite this leading to a large amount of background binding, specific binding is seen for both the monomer and dimer of the expressed SHUV N protein. This background is not seen when using the sera to probe the plant crude extract containing plant expressed SHUV N protein, which is highly relevant when using the sera as a probing reagent for plant expression or in ELISA based assays.

Looking back at the screening of plant produced SHUV N through western blot assays, the slight non-specific binding seen in -ve plant crude extracts could possibly be attributed to plant-specific antibodies in the rabbit sera. To improve on this, pre-absorption of the rabbit sera to un-infiltrated plant crude extract will help reduce the number of plant protein binding antibodies found in the sera without drastically reducing the antibodies specific to the SHUV N protein.

Currently, there are no SHUV antibodies available commercially, or any described in literature. Both the reagents created during this study, the *E. coli* expressed SHUV N and resulting antisera, show progress in the development of analytical assays and reagents used for the identification of recombinantly expressed N protein, and more importantly, general future SHUV studies. The importance of diagnostic antibodies cannot be highlighted enough and allows for immunogenicity based assays such as immunofluorescence, which has applications in assessing possible cell line virus studies, western blots , which require probing agents to assess the identity of expressed

proteins, and ELISA which can assess anything from cell lysate to fixed viral antigens and recombinant protein expression (Burns, 2009).

This can be applied to the current study where the generated antisera shows the potential to evaluate plant produced SHUV N without the need for an affinity tag on the recombinantly expressed protein, which in turn provides a number of benefits in its ability to act as an antigen. When using this plant produced SHUV N as an antigen in downstream ELISAs, the generated antisera will act as positive control when assessing possibly infected horse sera for the presence of antibodies against the wildtype SHUV N protein, thus bolstering the assay.

In conclusion, this study clearly demonstrated that rabbits were able to raise polyclonal antibodies to the *E. coli* produced SHUV N protein, as the protein was evidently highly immunogenic. Secondly, the anti-SHUV N antibodies were able to detect not only *E. coli* produced SHUV N protein but could be used to screen plant crude extract for the presence of plant-produced SHUV N with little to no background. This makes the sera containing anti-SHUV N antibodies a useful lab reagent for the detection and screening of plant crude extracts for the presence of expressed SHUV N protein. This serum can be used as a probing agent in western blotting techniques as shown here, or as a probing reagent in more quantitative assays such as ELISA.

Chapter 3: Development of the Diagnostic Reagent

3.1 Introduction

Predicting, monitoring and managing the spread of emerging arboviruses is essential for not only veterinary surveillance but, in the case of known zoonotic viruses, plays an important role in human disease surveillance.

In the case of emerging viral diseases where epidemiological and clinical data is limited, being able to map the spread of the responsible virus and document the clinical symptoms and manifestations of the disease is of the utmost importance. However, lack of reagents for the identification of the virus in suspected cases, or for seroepidemiological studies, hampers this work. Such is the case with the understudied SHUV. This can be rectified through the development of quick and effective diagnostic tools that do not require specialised equipment, expertise and facilities with assays such as viral culture, which is still a mainstay of viral diagnostics.

The nucleocapsid (N) proteins of members of the virus order *Bunyavirales* have been shown to be highly immunogenic in the infected host, due to it being one of the most abundant viral proteins in infected hosts (Ariza et al., 2013). The N protein has been shown to generate specific antibody responses in patients infected with the important tick-transmitted bunyavirus, CCHFV, thus making the N protein ideal target for serodiagnosis for SHUV and related viruses (Marriott et al., 1994). The choice of the N protein as opposed to a viral envelope glycoprotein came down to certain factors such as the ease of expression, the complexity of the protein(s), and the abundance of the protein. Therefore, the N protein is logically a good target for the development of a plant produced diagnostic reagent for SHUV, as it has been for related bunyaviruses (Atkinson et al., 2016). The recombinant expression of the SHUV N protein has not yet been reported in plants or, in fact, in any other expression platform. Thus, the expression of this viral protein in both *E. coli* and plant expression systems is inherently novel.

During the process of bunyaviral infection, antibodies would target a variety of viral proteins, and particularly the highly abundant N protein (Ariza et al., 2013). The ability to detect antibodies raised against the N protein of the virus in the serum of the infected host, allows for the diagnosis of current or previous infection and exposure to the virus.

In work reported in this chapter, the recombinant transient expression of the SHUV N protein in *N. benthamiana* was investigated. This involved the cloning of the SHUV N gene into a variety of virus vector-based expression constructs both with and without a 6xHis tag. The polyhistidine (6xHis) affinity tag is often used in recombinant protein expression thanks to its low immunogenicity, minimal effect on protein structure and function along with its small size (0.84kDa) (Atkinson et al., 2016, Chong et al., 1997, Young et al., 2012). Various virus-derived expression vectors were of interest to this study. These can be divided into the non-replicating virus vectors, specifically the pEAQ *HT* and pTRAc expression vectors, along with the self-replicating geminivirus-derived expression shuttle vector, pRIC.

The pEAQ *HT* vector was derived and constructed from essential elements from the Cowpea mosaic virus (CPMV) RNA-2 in combination with the pBINPLUS binary vector. It is hypertranslatable (*HT*) RNA elements allow for much higher levels of protein translation (Sainsbury et al., 2009). A total of 7kb of nonessential sequence was removed from the pBINPLUS backbone and T-DNA region to

make room for the 5'-untranslated region (UTR) and the 3'-UTR from the CPMV-HT to be housed within the binary vector pBINPLUS (Sainsbury et al., 2009). This included an expression cassette containing the Cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) terminator (van Engelen et al., 1995). This allowed for insertion of the target gene sequence, in between in the 5' and 3' UTR (Sainsbury et al., 2009).

The pTRAc vector is originally derived from the binary vector pPAM (Genbank AY027531). It was designed to incorporate a cauliflower mosaic virus (CaMV) 35S promoter along with transcriptional enhances, namely the CaMV 35S polyadenylation signal (pA35S) and chalcone synthase 5' untranslated region (CHS). Two copies of the expression cassette, the tobacco Rb7 scaffold attachment region (SAR) were included within the vector (Maclean et al., 2007). The pTRAc HT variant contains a 6xHis affinity tag sequence. This was created by T. Rademacher in the Fischer Lab, Fraunhofer IME, Aachen (Maclean et al., 2007)

Another expression vector of interest to this study is the autonomously replicating gene expression shuttle vector, pRIC. The pRIC vector was derived from the ssDNA geminivirus BeYDVm (Halley-Stott et al., 2007). During the virus' life cycle, viral DNA replication takes place after entering the host, leading to a double stranded DNA replicative intermediate to be formed in the host nucleus. This intermediate DNA is then replicated through rolling circle replication which uses the virus' Rep/RepA gene elements as well as plant host proteins to achieve viral DNA propagation (Hefferon and Dugdale, 2003). This feature was incorporated into the design of pRIC in order to increase recombinant expression yields through vector replication within the host (Regnard et al., 2010). In order for the vector to be release and re-ciruclarize after amplification and delivery into the host cell, the vector is bounded by two copies of the BeYDVm long intergenic region (LIR), includes a short intergenic region (SIR), rep gene and a viral promoter between the two LIR. This allows for DNA primer binding for complementary DNA strand synthesis. This vector contains a CaMV 35S promoter taken from pTRAc (Regnard et al., 2010).

In order to express this protein within these *N. benthamiana*, recombinant *A. tumefaciens* infiltration takes place to introduce the expression constructs of choice (Rybicki, 2010). The plant expression of the SHUV N protein is not only to be assessed through western blot probing of the plant crude with anti-6xHis antibodies but with the protein specific anti-SHUV N antibodies that were developed in Chapter 2.

In order to purify the target protein from the expressed plant crude, ammonium sulphate precipitation techniques were investigated, along with affinity tag purification. The use of this 6xHis tag for purification purposes is achieved using nickel affinity batch purification for small-scale studies and the use of an automated column chromatography system (ÄKTA Explorer, GE Healthcare) for large-scale processing.

Following the bulk expression and purification of the SHUV N protein in *N. benthamiana*, the protein was tested as a capture antigen, the diagnostic reagent, for use in indirect ELISA. This was used to assess the seropositivity of horse samples for wildtype SHUV infection, and specifically the N protein. Due to the limited availability of known positive sera, the optimisation of this protocol would be performed by screening sera from horses with no known exposure to SHUV.

The development of an effective indirect ELISA based diagnostic kit using purified recombinant plant expressed SHUV N protein as the diagnostic antigen was the overall goal of this project, as it

will allow for the quick and effective analysis of animals who have come into contact with the virus. This will in turn provide a wealth of data on the epidemiological spread of SHUV which can be further analysed to help characterise the virus in terms of associated symptoms, manifestations, as well as help give an idea of the mechanisms of infection.

3.2 Materials and Methods

3.2.1 Plant Expression Vectors

Various plant expression vectors were used in this study, specifically two polyhistidine 6xHis affinity tagged non-replicating plant expression vectors and one non-6xHis tagged replicating expression vector.

Non-replicating vectors such as pEAQ *HT*, based on CPMV RNA2 (Sainsbury et al., 2009), and the pTRAc HT vector were used in the study. The pTRAc HT vector is a 6xHis affinity tagged version of the initial pTRAc vector (Maclean et al. 2007) originating from the binary vector, pPAM (Genbank AY027531) with an included cauliflower mosaic virus (CaMV) 35S promotor. This 6xHis affinity tagged variant was created by the BRU.

The replicating geminivirus-derived expression vector without a 6xHis affinity tag, pRIC 4.0, is a derivative of the expression vector pRIC 3.0 (Regnard et al., 2010) but includes a TAV 2b silencing suppressor cassette. This was also used to assess whether the attachment of the 6xHis tag affects protein expression.

Table 4: The plant expression constructs used in this study along with the working concentration of antibiotic used in the selective media

Plasmid	Antibiotic Working Concentration
pEAQ *HT*	Kanamycin at 50 ug/ml
pTRAc HT	Ampicillin at 100 ug/ml
pRIC 4.0 HT	Ampicillin at 100 ug/ml

3.2.2 Cloning Strategy

As described in section 2.2.1, the SHUV N gene was synthesised by GenScript Inc (USA) to include the *AgeI*, and *NcoI* sites on the 5' terminus along with *XmaI*, *SmaI,* and *XhoI* sites on the 3' termini to allow for sub cloning from the pUC57 vector that houses the SHUV N gene, into all plant expression vectors described in Table 4 and the *E. coli* expression vector described in section 2.2.3 in chapter 2.

Multiple 10ml LB broths were each inoculated separately with transformed DH5α *E. coli* containing pUC57 SHUV N construct and *E. coli* containing the plant expression vectors, pEAQ-*HT*, pTRAc HT and pRIC 4.0. These were grown up with the antibiotics in Table 4 and DNA isolation was performed as described in section 2.2.4.

Each separate plasmid vector and aliquot of pUC57 SHUV N plasmid underwent restriction enzyme digest with the specified enzymes in Table 5 with the methods described in section 2.2.4. This is described by the flow diagram in Figure 3.1 and Figure 3.2.

Table 5: The restriction enzyme digests used on both the plasmid vector backbone (Vector RE) and on pUC57 SHUV N plasmid (Insert RE) to allow for generation of each of the constructs through subcloning.

Construct Name	Vector RE	Insert RE
pEAQ *HT* N-term SHUV N	*XmaI/XhoI*	*AgeI/XhoI*
pEAQ *HT* C-term SHUV N	*AgeI/SmaI*	*AgeI/SmaI*
pEAQ *HT* SHUV N	*AgeI/XhoI*	*AgeI/XhoI*
pTRAc HT SHUV N	*NcoI/XhoI*	*NcoI/XhoI*
pRIC 4.0 SHUV N	*AflIII/XhoI*	*NcoI/XhoI*

Following RE digest, each construct was ligated, transformed and plated for colonies as described in section 2.2.4 (Sambrook and Fritsch, 1989).

All single colonies were screened using colony PCR as described in section 2.2.4 using vector specific primers as shown in Table 6. DNA was isolated as previously stated and RE digestion was performed to confirm the inclusion of the insert within the vector backbone as stated in Table 7. These RE are not entirely the same as those listed in Table 5 due to not all RE sites being regenerated after subcloning.

Table 6: Various primers used during this study. The sequence, length and T_m is shown.

Name	Nucleotide Sequence (5'-3')	Length (bp)	T_m	Notes
pEAQ *HT* f	TTC TTC TTC TTG CTG ATT GG	20	56°C	Used for all pEAQ *HT* constructs
pEAQ *HT* r	CAC AGA AAA CCG CTC ACC	18	56°C	
pTRA-f	CAT TTC ATT TGG AGA GGA CAC G	22	64°C	Used for both pTRAc HT and pRIC 4.0 constructs
pTRA-r	GAA CTA CTC ACA CAT TAT TCT GG	23	64°C	

Table 7: The restriction enzyme digests used to confirm the inclusion of the SHUV N gene within the various constructs.
The expected sizes of the confirmed insert is as shown. The sizes may differ to the original cloned insert as not all RE sites were regenerated and upstream sites were used in confirmation.

Construct Name	Confirmation RE	Expected Band Size
pEAQ *HT* N-term SHUV N	*AgeI/XhoI*	744
pEAQ *HT* C-term SHUV N	*AgeI/SmaI*	710
pEAQ *HT* SHUV N	*AgeI/XhoI*	717
pTRAc HT SHUV N	*NcoI/XhoI*	710
pRIC 4.0 SHUV N	*EcoRV/XhoI*	858

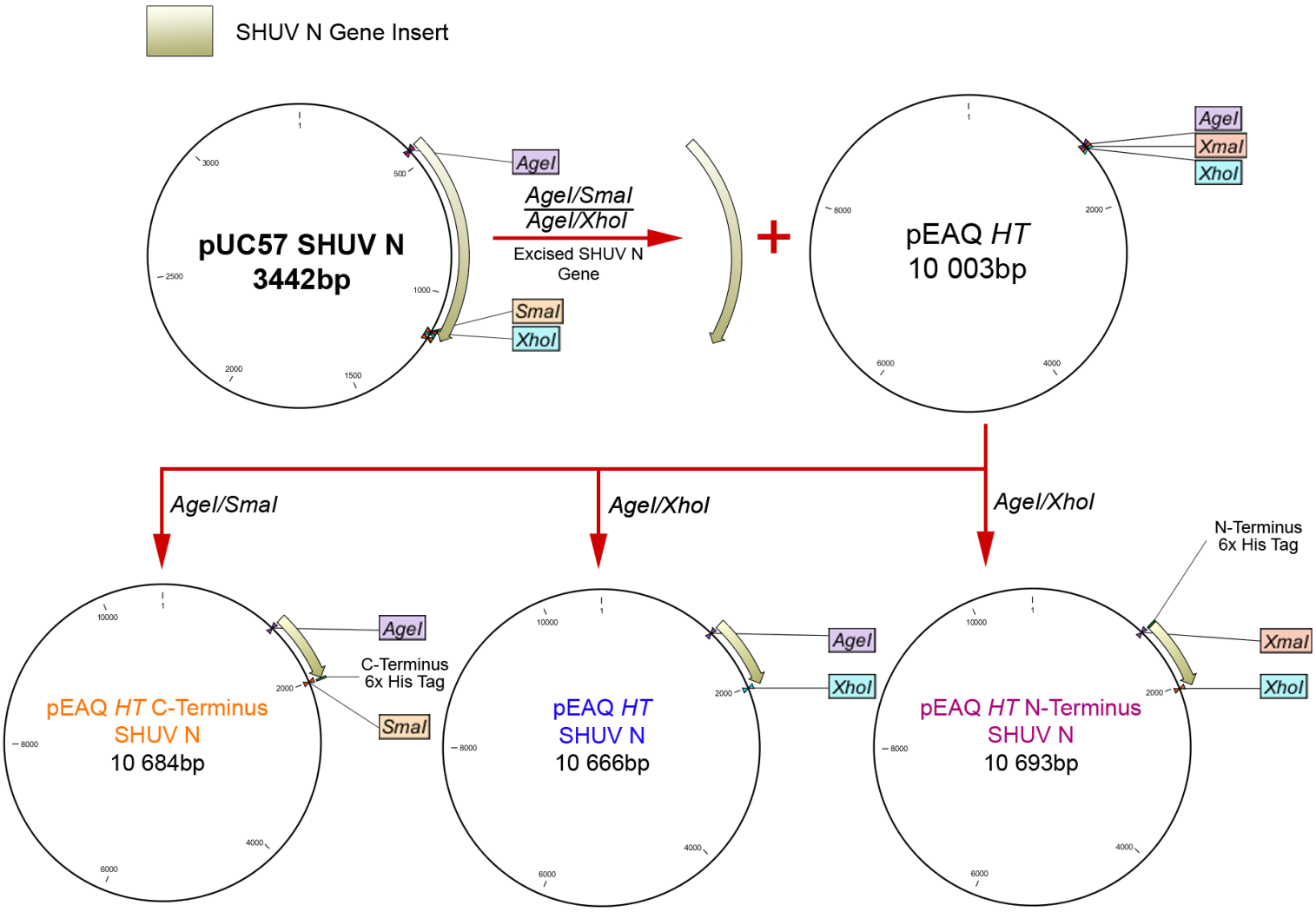


Figure 3.1: Direct subcloning strategy used for the creation of the various pEAQ *HT* SHUV N expression constructs.

The schematic shows the excision of the SHUV N gene from the synthesised pUC57 SHUV N construct and directly subcloned into pEAQ-*HT* using the appropriate restriction enzymes to allow for different configurations of the 6xHis tag to be expressed on the protein of interest.

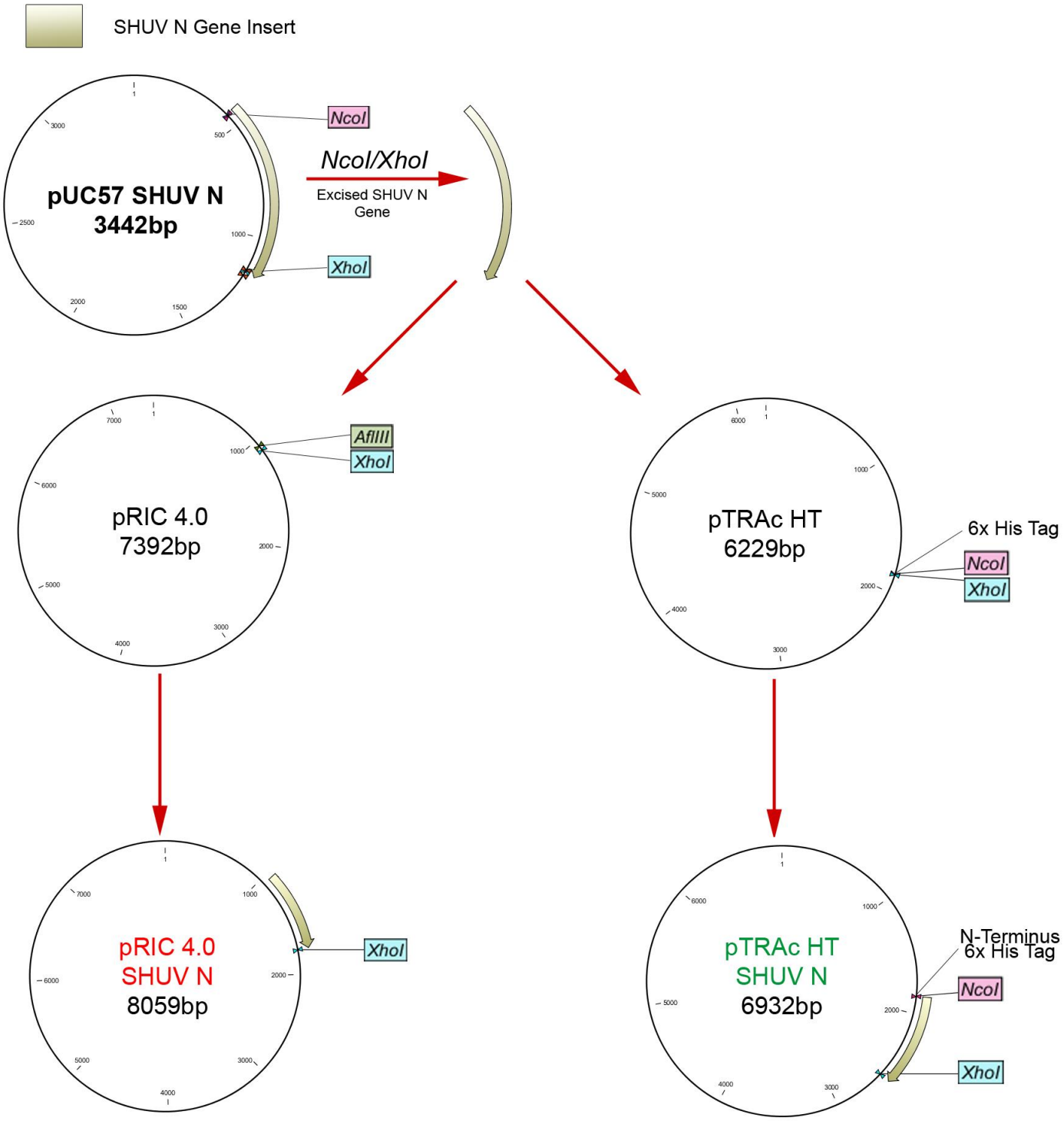


Figure 3.2: Direct subcloning strategy used for the creation of the pTRAc HT SHUV N and pRIC 4.0 SHUV N expression constructs.

The schematic shows the excision of the SHUV N gene from the synthesised pUC57 SHUV N construct and directly subcloned into pTRAc HT and pRIC 4.0 expression vectors. The pRIC 4.0 SHUV N construct does not contain a 6xHis tag to be expressed on the protein of interest.

3.2.3 *A. tumefaciens* Transformation

All *A. tumefaciens* transformations were done using electroporation (Maclean et al., 2007). Two strains of *A. tumefaciens* were used, namely *A. tumefaciens* GV3101::pMP90RK for transformation of pTRAc HT and pRIC 4.0 based expression constructs and *A. tumefaciens* LBA4044 for all pEAQ *HT* based constructs. Electrocompetent cells for both strains were prepared as described (Shen and Forde, 1989).

All constructs housed in *E. coli* listed in Table 5 were grown up and DNA isolated as described in section 2.2.4.

Electroporation of the *A. tumefaciens* competent cells was performed by mixing 100-200 ng (at 50 ng/µl) of plasmid DNA with 100 µl of competent cells from the specified strain (Table 8) in a 1mm electroporation cuvette. Each cuvette was electroporated with a Bio-Rad GenePulser (Bio-Rad Laboratories, USA) at 1.8 kV, 200 Ω, with a capacitance of 25 µF across the two electrodes (Maclean et al., 2007).

Once complete, the newly transformed *A. tumefaciens* cells were incubated in LB while shaking at 180rpm for 3 hours at 27°C for initial cell growth and recovery before being plated on selective LB agar media with the respective antibiotics seen in Table 8, specific to the backbone expression vector and *A. tumefaciens* strain. This was left to be incubated at 27°C for 2-3 days or until sizeable colonies were observed.

Single colonies were picked and underwent colony PCR following the procedure described in section 2.2.4 using the primers and T_m stated in Table 6 for each of the respective constructs. Any colonies showing the correct sized PCR product were grown in 10 ml LB broth with appropriate antibiotics over the course of 2 days, shaking at 180 rpm at 27°C. Each *A. tumefaciens* culture was pelleted and lysed to obtain DNA through the use of the QAIGEN DNA Miniprep Kit (Qaigen, USA) before transformed back into competent DH5α *E. coli* as described in section 2.2.4. This was performed due to the inability to obtain sufficient yields of DNA from *A. tumefaciens* cultures to allow for RE digest confirmation of the insert within the expression vector. The back transformed *E. coli* clones were grown up in 10 ml LB broth, lysed, before the DNA was extracted and underwent restriction enzyme digest to confirm the presence of the SHUV N expression constructs which originated in the *A. tumefaciens* clones.

Table 8 : Various expression vectors and the required strain of *A. tumefaciens* used for transformation.
The respective antibiotics and additives used for the growth of each plasmid and strain combination is shown.

***A. tumefaciens* Strain**	**Plasmid**	**Antibiotics Used**	**Notes**
GV3101::pMP90RK	pTRAc HT & pRIC 4.0	Carbenicillin 50 µg/ml	
		Kanamycin 30 µg/ml	
		Rifampicin 50 µg/ml	
LBA4044	pEAQ-*HT*	Kanamycin 30 µg/ml	2 mM $MgSO_4$ Added
		Rifampicin 50 µg/ml	

3.2.4 Small-scale Agroinfiltration of *N. benthamiana* for Transient Protein Expression Trials

Each *A. tumefaciens* SHUV N expression clone was grown up in 10 ml LB with appropriate antibiotics, shaking overnight at 180rpm at a temperature of 27°C. This was used to inoculate 50ml induction medium which consisted of LB broth supplemented with 10 mM MES, 1 mM Acetosyringone and 2 mM $MgCl_2$ at a pH of 5.6.

This was left to grow overnight shaking at 27°C. The culture was pelleted at 2000 x g for 10 mins at 4°C. The supernatant was discarded, and the pelleted cells were resuspended in infiltration media consisting of 10 mM Acetosyringone, 10 mM MES, 2 mM $MgCl_2$, made up in dH_2O at a pH of 5.6. This allowed for the respective cultures to be diluted by infiltration media to an OD_{600} of 0.25 and 0.5. Once diluted, each culture was left at room temperature for 2 hours to allow induction of the *vir* genes by the Acetosyringone, which will be responsible for integration of the target SHUV N expression construct into the *N. benthamiana* host after infiltration. Thus, each respective suspension was then loaded into a needless syringe and injected (infiltrated) into the abaxial airspaces of the ventral side of *N. benthamiana* leaves. Each plant was at an age of 6-8 weeks old at time of infiltration and a minimum of three newer leaves of substantial size were infiltrated.

Each *A. tumefaciens* infiltration culture underwent PCR using the appropriate primers stated in Table 6 for each of the specified constructs. This confirmed the presence of each construct in each culture prior to infiltration.

3.2.5 Small-scale Protein Extraction and Assessment

N. benthamiana plants were grown in a growth room at roughly 25°C and 45% relative humidity. Each room followed 16/8-hour artificial day/night cycle both before and after agroinfiltration with a light intensity of 60 – 80 µE/m2/s.

Each plant was at six weeks of age when infiltrated with an *A. tumerfaciens* clone at a culture OD_{600} of both 0.25 and 0.5. Each plant was harvested daily for total of 7 days post infiltration (dpi). A total of three leaf-disk clippings were taken each day's harvest with one clipping per infiltrated leaf. This was done using the cap of a 1.5ml Eppendorf microcentrifuge tube. A total of 3 plants were infiltrated for each construct at the specified OD_{600} to ensure biological repeats of the experiment in triplicate.

Once harvested, all leaf clippings were flash frozen and stored at -80°C until all time points had been harvested. Once collected, all leaf clippings were thawed and 200µl of extraction buffer consisting of 1xPBS was added. Two 60mm diameter ceramic beads (Merck & Co , Kenilworth, USA) were added to each 2ml microcentifuge tube containing the frozen leaf clippings in PBS. These were left to shake on a vortex mixer at the highest setting for approximately 20 min or until all clippings were sufficiently pulverized. This was performed at 4°C. This was repeated a total of three times as both a technical and biological repeat. A single repeat of 1xPBS + 1% Triton-100x extraction buffer was used for plant material infiltrated with pEAQ *HT* N-term SHUV N, pEAQ *HT* C-term SHUV N, and pTRAc HT SHUV N agrobacterium clones, in order to assess whether the addition of a strong detergent benefited the extraction process. Following this, each tube was centrifuged at 15 000 x *g* for 10 min to pellet insoluble material, the supernatant was then collected. This extract was prepared for SDS-PAGE with the addition of 5 x SDS sample application buffer as mentioned in section 2.2.7 and denatured at 95°C for 10 min. Once complete, all samples were frozen at -20°C until for further analysis.

Once SDS-PAGE was performed, plant expression of each constru ct was assessed through western blot following the protocol specified in section 2.2.8. This was performed on plant crude infiltrated with pEAQ *HT* N-term SHUV N, pEAQ *HT* C-term SHUV N, and pTRAc HT SHUV N constructs. These were initially probed using mouse anti-6xHis antibodies at a 1:2000 dilution followed by a secondary antibody of goat anti-mouse AP at a dilution of 1:10 000. Following this, the plant expression of all constructs listed in Table 5 and Table 6 was subsequently assessed using western blots and probed with rabbit anti-SHUV N antibodies at a 1:10 000 dilution. Secondary antibody of goat anti-rabbit AP was used at a 1:10 000 dilution.

3.2.6 Large-scale Agroinfiltration of *N. benthamiana*

Large-scale agroinfiltration began with the growing up of *A. tumerfaciens* pEAQ-*HT* C-term SHUV N in 10 ml LB media supplemented with specified antibiotics (Table 8) overnight. This pre-culture was used to inoculate 50 ml LB media (section 2.2.5), containing the correct antibiotics which was once again incubated overnight. Following this, the fully grown 50ml culture was used to inoculate 500ml of induction media (section 3.2.4) containing the correct antibiotics. This was left to incubate overnight. All described incubation steps were performed at 27°C on a shaking platform at 180rpm. This exact protocol was performed in parallel with the *A. tumerfaciens* pEAQ-*HT* empty clone, to act as a negative control.

Once grown, the OD_{600} of each 500ml culture was determined and diluted to a value of 0.5 with resuspension infiltration media (section 3.2.4) and Acetosyringone was added to a concentration of 200 µM. This was left for 2 hours to induce *vir* genes. Following this, 35x *N. benthamiana* plants of approximately 6 weeks of age, were vacuum agroinfiltrated by submerging the entire plants in the diluted culture and applying a vacuum of approximately -100KPa. The reapplication of atmospheric pressure caused the diluted culture to be pushed into the abaxial airspaces of the *N. benthamiana* leaves in a roughly uniform manner. These plants were then housed in a growth room at roughly 25°C in an environment 45% relative humidity with 16/8-hour artificial day/night cycle with light intensity of 60 – 80 µE/m2/s. This was done until harvest at 3 dpi.

3.2.7 Large-scale Protein Extraction

Roughly 225.3 g of infiltrated *N. benthamiana* leaves were harvested from 35x plants 3 dpi, they were stored and frozen at -80°C before being pulverized and weighed. 1xPBS was added in a ratio of 2ml/g of leaf material. This was homogenized with an Ultra-Turrax® homogeniser (IKA® Works Inc., NC, USA) until the required consistency was seen. Once complete, this was incubated at 4°C shaking for 1-2 hours. Following this all subsequent steps were performed at 4°C. The extract mixture was filtered through Miracloth™ (22-24 µm pore, Millipore, USA) before being centrifuged in a J-14 rotor within an Avanti J-30I centrifuge (Beckman Coulter, USA) at 15 000 x *g* for 20 min at a temperature of 4°C. The supernatant was collected and stored at 4°C for further analysis.

3.2.8 Ammonium Sulphate Precipitation

The previously prepared extract underwent $(NH_4)_2SO_4$ precipitation which was achieved by the slow addition of solid $(NH_4)_2SO_4$ to the filtered extract. This was done at 4°C while the mixture was constantly stirring. The initial precipitation experiment required a 0-70% increase in $(NH_4)_2SO_4$ saturation at 10% intervals. The required amount of $(NH_4)_2SO_4$ needed for each increase in 10% saturation was calculated using the following online tool at http://www.encorbio.com/protocols/AM-SO4.htm.

Each amount of solid $(NH_4)_2SO_4$ was weighed out and slowly added to the stirring solution to prevent protein crash out. The solution was centrifuged at 20 000 x *g* for 20min at 4°C to pellet the precipitated protein, which was stored at 4°C for later assessment. The supernatant was kept for further precipitation of a higher percentage. This was done for each 10% fraction increase. For each pelleted fraction, the pelleted precipitate was resuspended in 1xPBS with 500 mM NaCl. All fractions were analysed through SDS-PAGE and western blot as described in section 2.2.8. The resulting western blot was probed using anti-6xHis antibodies due to anti-SHUV N antibodies not yet being available at this point in the project timeline. Equal volume of precipitate was loaded into each lane.

For large-scale, the 40-60% fractions were used and taken forward for dialysis in order to remove $(NH_4)_2SO_4$ from the solution for downstream purification. Dialysis was performed using tubing with a molecular weight cut-off of 10 kDa (Thermo Fischer Scientific, USA) in 1x PBS (500 mM NaCl) . This was done stirring overnight at 4°C with a dialysate to protein solution ratio of 250:1. The dialysate was changed out once or more if needed. This precipitation procedure was also performed on plant material infiltrated with empty pEAQ *HT* vector as a negative control.

3.2.9 Small-scale Nickel Affinity Batch Purification

Small-scale nickel affinity batch purification was performed by aliquoting 100 µl of Ni affinity resin (Thermo Fischer Scientific, USA) into a 1.5ml microcentrifuge tube. This was centrifuged at 5000 x g for 30 seconds and the supernatant was removed. Following this, the resin was equilibrated using 200 µl wash buffer consisting of 50 mM HNa_2PO_4 and 0.3 M NaCl at a pH 8. Once complete, the tube was centrifuged at 5000 x g for 30 seconds and supernatant removed before 100 µl of the dialysed 40 – 60% $(NH_4)_2SO_4$ fraction was loaded onto the affinity resin and vortexed vigorously for 1 min to allow binding of any potential proteins. This was once again centrifuged at 5000 x g for 30 seconds and the supernatant removed and kept at 4°C for SDS-PAGE analysis. After which the resin was washed with 100µl wash buffer as described earlier and vortexed, in which the tube was centrifuged at 5000 x g for 30 seconds and the supernatant saved for analysis. This wash step was repeated four times. Following this, the target protein was eluted by the addition of 100 µl elution buffer consisting of 50 mM HNa_2PO_4, 0.3M NaCl and 250 mM Imidazole at a pH 8 into the nickel mixture. The tube was vortexed vigorously for 1 min, centrifuged at 5000 x g for 30seconds and the supernatant removed and stored for analysis, this elution was repeated 3 times in total. The entire process was repeated with plant material infiltrated with empty pEAQ *HT* vector as a negative control. This plant material had undergone the exact same process, treatment and precipitation as the main extract.

3.2.10 Stability Study

Following the small-scale nickel affinity batch purification of the dialysed 40 – 60% $(NH_4)_2SO_4$ fraction. All elution fractions were pooled and 40 µl was aliquoted out for each time point (0 Weeks, 6 weeks, and 12 weeks) and for each temperature within those time points (4°C, -20°C, and -80°C). This was done in duplicate for a total of 18 samples prepared. Each was placed at its required temperature and stored for the allocated time period. Samples stored for 0 weeks were briefly exposed to the storage temperature over 1 day. Once the allocated time period had elapsed, the sample was removed, placed at room temperature and 5x SAB (section 2.2.7) was added before the sample was denatured at 95°C for 10 mins. The denatured sample was stored at -20°C until all timepoints had elapsed and the required SDS-PAGE was performed.

3.2.11 Nickel Affinity Chromatography

The dialysed 40 – 60% $(NH_4)_2SO_4$ precipitated sample was filtered through 0.20 µm polyvinylidene fluoride (PVDF) syringe filter. Both the wash buffer (1xPBS with 500 mM NaCl) and elution buffer (1x PBS, 500 mM NaCl and 500 mM imidazole) were both filtered through Whatman Grade 3 filter paper of pore size 6 µm (GE Healthcare, UK) via suction filtration before being used on the AKTA Explorer 100 system.

Purification was performed using an automated column chromatography system (ÄKTA Explorer, GE Healthcare) fast protein liquid chromatography (FPLC) system (The ÄKTA Explorer 100) in combination with a 5 ml HisTrap HP pre-packed column (GE Healthcare, UK).

Nickel affinity chromatography was performed at a buffer flow rate of 5 ml/min column volume per min was used. The column was equilibrated with 10 column volumes of wash buffer (described above) which after the sample was loaded onto the column using a 100 ml SuperLoop. Once loaded, the sample was washed through with 20 column volumes of wash buffer before elution of the target protein began by the introduction of the elution buffer (described above) in a gradient from 0-100% over the course of 30 (5 ml) column volume fractions with a static plateau at 10% for 10 column volumes. The fractions were captured in single column volume (5 ml) fractions at varying absorbance readings and stored at 4°C for future work.

3.2.12 Protein Quantification

All eluted fractions were assessed via SDS-PAGE and Coomassie Brilliant Blue staining as described in section 2.2.7. Each fraction was mixed with 5x SAB and denatured at 95°C for 10min. The fraction samples were run alongside a known BSA (Merck & Co , Kenilworth, USA) standard series of known concentration prepared via serial dilution. Once thoroughly stained with Coomassie Brilliant Blue, the polyacrylamide gel was scanned, and bands of corrected size were assessed for concentration using the Genetools (Syngene, Synoptics Group, United Kingdom) through gel densitometry.

3.2.13 Serum Samples

Serum samples of horse subjects thought to have had exposure to SHUV infection were obtained from Prof Marietjie Venter of the Zoonosis Research Unit (ZRU) based at the University of Pretoria, Pretoria, (UP REF No.: H012-16). Each serum sample was chemically inactivated by the addition of 0.5% Tween 20 before being assessed for the presence of IgGs against SHUV by means of viral neutralization assay; unfortunately, antibody titres of each sera sample were not made available. Serum samples are listed in Table 9.

Further horse serum samples used for optimisation were obtained as part of an African horse sickness virus study in our laboratory: these animals had no known exposure to SHUV. These were sampled from the greater Western Cape area with ethics approval (FHS AEC REF No.: 017/006 and ECRA REF No.: 07/17). No screening for IgGs against SHUV had been performed on these samples prior to this study. Therefore, these samples cannot be confirmed to have had no prior exposure to SHUV; however, all subjects from which the sera was obtained were shown to be in good health with no indication of exposure to the virus. These serum samples are listed in Table 10.

Table 9: Various serum samples obtained from the ZRU for use in ELISA based serum screening for IgGs against SHUV.

Sample Number	Sample ID/Animal name	Date Sampled	Location	Main Syndrome	SHUV PCR Assay	SHUV IgG Assay
SAE28/09	Blue Wings	N/A	Mphumalanga	Fever	Negative	Positive
SAE29/10	9	N/A	KZN	Neurological	Negative	Positive
SAE66/11	Temp Lady Foal	24/03/2011	Northern Cape	Fever	N/A	Negative
SAE131/11	Jetmaster	01/05/2011	Western Cape	Neurological	Negative	Positive
SAE140/11	Tanzanite	09/06/2011	Gauteng	Neurological	Negative	Positive
ZRU84/12	Ravenswood	N/A	Gauteng	Neurological	Negative	Positive
ZRU490/17	Chestnut Champ	12/12/2017	North West	Fever and Neuro	Negative	N/A
ZRU484/17	Barak van der Kjerst	05/12/2017	Western Cape	Neuro and Respiratory	Negative	N/A
ZRU76/18	Belle of the Ball	16/03/2018	North West	Neurological	Positive	N/A

Table 10: Various serum samples obtained as part of the BRU AHSV study for use in the optimisation of the ELISA based serum screening for IgGs against SHUV.

Sample Number	Sample ID/Animal name	Date Sampled	Location
Horse 1	Lucky Lips	26th June 2018	Driftsands, Delft, Western Cape
Horse 2	Liberty		
Horse 8	Lily		

3.2.14 SHUV N Protein Antigenicity Assessment

In order to assess the antigenicity of the plant expressed recombinant SHUV N protein and thus its ability to act as an antigen in downstream ELISAs against SHUV positive sera, mouse ascites fluid, raised against SHUV infected cell extract (strain An10107) and mouse ascites raised against Middleburg infected cell extract were provided by the ZRU. This originated from the US Centers for Disease Control and Prevention, Fort Collins, Colorado. This was used in order to assess whether the SHUV N antigen would allow differentiation from antibodies raised against both related and unrelated live viruses and thus asses the specificity of the antigen.

A SDS-PAGE and western blot were performed with crude extract from plants infiltrated with pEAQ *HT* C-term SHUV N and Empty pEAQ *HT* alongside the same plant crude that had undergone $(NH_4)_2SO_4$ precipitation (section 3.2.8) and purified through nickel affinity batch purification (section 3.2.9). This was probed using a 1:400 dilution of the mouse ascites fluid (anti-SHUV) in blocking buffer.

3.2.15 Preparation of Mock Antigen

Mock antigen was obtained from *N. benthamiana* plant material, agroinfiltrated with empty pEAQ vector as described in section 3.2.6, that underwent a mirrored extraction and purification process as used for the SHUV N antigen (section 3.2.7 – 3.2.12). In order for the plant produced and purified SHUV N antigen and the mock antigen to be comparable in downstream ELISAs, equal volumes of both the antigen and mock antigen were used when dilutions were made in coating buffer (10 mM Tris at pH 8.5). This is due to the antigen containing possible background proteins that were not cleared during purification plus additional over-expressed SHUV N protein while the mock antigen containing only possible background proteins. Therefore, protein concentration and TSP is not an effective method of standardising antigen against mock antigen.

3.2.16 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

All indirect ELISAs were performed on Nunc 96-well PolySorp plates (Nalge Nunc International, USA). Each well was coated with 100 µl of coating buffer (10 mM Tris at pH 8.5) containing 100-200ng of nickel chromatography purified plant produced SHUV N protein as an antigen and equal volume mock antigen as specified in section 3.2.15. Coating was performed at 4°C overnight while shaking

Following this, each well was emptied and washed out with 200 µl wash buffer (1x Tris, pH 7.5, and 0.05% Tween 20) four times. The antigen was blocked with 200 µl milk-based blocking buffer (1x Tris, pH 7.5, and %5 (v/v) Non-fat milk) at 37°C for 1 hour, shaking. Once complete, wells were emptied and washed with wash buffer as shown earlier. 100 µl of horse serum, mouse serum or purified antibody was made up in blocking buffer at the specified dilutions described in each ELISA experiment. These were added to each well and incubated at 37°C for 1 hour, shaking. Following this, wells were emptied and washed with 200 µl wash buffer containing Tween 20 at a concentration of 0.05%. This wash step was performed four times.

Secondary antibody was made up in the appropriate blocking buffer at the specified dilution of 1:5000 for Anti-Equine AP or 1:10 000 for Anti-Mouse AP. 100µl of the required secondary antibody was loaded into each well and incubated at 37°C for 1 hour while shaking. Once complete, wells were emptied and washed with 200 µl of final wash buffer (1xTBS, pH 9), this was repeated four times.

The final step involved visualisation by pipetting 200µl of p-Nitrophenyl phosphate substrate (Merck & Co , Kenilworth, USA) into each well and allowing the reaction to proceed for 30 min before absorbance was measured at 405nm. The reaction was stopped by the addition of 100µl of 2M NaOH solution to each well.

Initial screening was performed on horse sera provided by the ZRU, shown in Table 9. Each sera was tested at 1:32, 1:64, and 1:128 dilutions in blocking buffering containing 1xTBS, 5% (v/v) non-fat milk, at pH 7.5. Of the samples listed in Table 9, samples SAE131/11, SAE29/10, ZRU 84/12, and ZRU 76/18 were treated as potential positive samples. Samples SAE131/11, SAE29/10, and ZRU 84/12 having undergone viral neutralisation assay within the ZRU to confirm the presence of IgG antibodies against the virus with the exception of ZRU 76/18, which had not yet been assessed for IgGs but was shown to test positive in the PCR screening of the serum.

For each ELISA performed, wells coated with 200 ng/well antigen and equal volume mock antigen were repeated in triplicate against the respective sera dilutions. The data were plotted with the average of each triplicate and standard deviation being shown. Rabbit anti-SHUV N was used as an antigen positive control. Secondary antibody and substrate only controls were shown rule out any contributions these factors would play in each A_{405} reading.

Following initial screening of horse sera, optimisation of the ELISA protocol took place in later experiments shown in section 3.3.8. During this optimisation of the protocol, many factors were changed. 200 µl BSA-based blocking buffer (1x TBS, pH 7.5, and %3 (w/v) BSA) was used in place of the milk-based blocking buffer. The number of subsequent wash steps following the application of the sera was increased from four repeats to six repeats and involved shaking steps of 30 seconds once wash buffer was applied to the wells in order to increase the efficiency of the wash.

The % (v/v) of Tween 20 used in the initial wash buffer was optimised from 0.05% to 0.15% to help reduce non-specific binding interactions. All sera (including positive control - rabbit made anti-SHUV N) was pre-absorbed against un-infiltrated plant crude extract as described in section 3.2.17, in order to reduce the number potential plant binding antibodies found in the sera.

3.2.17 Plant Control Antigen

In order to assess if binding was due to the fact that the antigen was produced in plants, an unrelated plant produced antigen from an ongoing BRU human papillomavirus (HPV) vaccine study was used as a plant produced antigen control. The HPV 16 L1 antigen was produced in *N. benthamiana* and extracted as described in section 3.2.6. This antigen does not contain a 6xHis affinity tag and was purified by iodixanol density gradient ultracentrifugation (Muzyczka, 1999) and not through the process of $(NH_4)_2SO_4$ precipitation and FPLC AKTA purification as described for the SHUV N antigen. This HPV 16 L1 antigen was diluted at 1:100 in BSA-based blocking buffer and used to coat wells as described above in section 3.2.16. The concentration of protein is not known.

3.2.18 Pre-absorption of Sera

Where specified, sera samples were pre-absorbed against uninfiltrated plant crude extract in order to lower the amount of plant protein specific antibodies found in the animal sera and thus lead to cleaner and more specific representations in antibody-based assays such as western blot and ELISA (Mbewana et. al, 2018).

This pre-absorption was performed by homogenising uninfiltrated plant crude extract in which 1xPBS was added in a ratio of 2 ml/g of leaf material. A 2 cm x 12 cm strip of nitrocellulose membrane (Amersham™ Protran™ Premium 0.45 µm NC) was cut out and incubated with the homogenised plant material mixture for a period of 3 hours at room temperature while shaking to allow for intrinsic plant proteins to bind to the membrane. The membrane was removed from the mixture and subsequently wash four times with 1xPBS.

Once done, the membrane was incubated in the required sera for both ELISA or western blot assays. This was incubated for a period of 3 hours at room temperature, shaking. The membrane was then removed, discarded, and the sera dilution was used in the further ELISAs and Western blots.

3.2.19 Indirect ELISA Data Analysis

As mentioned in section 3.2.16, probing of each antigen, mock antigen, and plant control antigen with each of the respective antibodies or sera was performed in triplicate as technical repeats on the same 96 well plate. This includes primary antibody only, secondary antibody only, and substrate only controls.

The mean of the absorbance at 405 nm across each of the three wells was taken and plotted for each of probing reagents and respective antigens. Standard deviation was calculated and plotted for each of the respective means.

3.3 Results

3.3.1 Cloning

Confirmation of Expression Vectors

Three vectors were chosen for plant expression of the SHUV N protein, namely the following: pEAQ-*HT*, pTRAc HT and pRIC 4.0. Each empty vector had its identify confirmed through restriction enzyme digest with *EcoRV* and the expected number of fragments of the correct size were observed for each vector (data not shown).

Creation of Plant Expression Constructs

A total of five expression constructs containing the SHUV N gene were planned using the three given vectors.

Three different constructs using variants of the pEAQ *HT* expression vector were planned, each version contained either no affinity tag gene, or the required gene sequence for a 6xHis affinity tag on either N or C terminus of the expressed protein. The SHUV N gene was successfully subcloned into the pEAQ *HT* vector using the restriction enzymes shown in Table 5 in section 3.2.2. This gave fragments of the size shown in Table 11. Following this, all expression constructs were successfully transformed into DH5α *E. coli* (E.Cloni® 10G cells). The inclusion of the SHUV N gene into the respective constructs was confirmed first through colony PCR (data not shown) and then subsequently through RE digestions shown in Figure 3.3 using the REs shown in Table 7 with the expected sizes as shown in the table. It must be noted that these expected sizes differ from those stated in Table 11 due to the fact that not all RE sites were regenerated after subcloning. This gave three pEAQ *HT* based expression constructs with a 6xHis affinity tag on the N terminus (N-Term), C terminus (C-Term), or without said tag.

Table 11: Various SHUV N plant expression constructs created. The size of the digested insert, vector and the final construct is shown. The inclusion and position of the 6xHis affinity tag in the construct is specified.

Construct	Insert Size (bp)	Vector Size (bp)	Construct Size (bp)	6xHis Affinity Tag
pEAQ *HT* N-term SHUV N	717	10 003	10 639	N-terminal 6xHis Tag
pEAQ *HT* C-term SHUV N	710		10 684	C-terminal 6xHis Tag
pEAQ *HT* SHUV N	717		10 666	N/A
pTRAc HT SHUV N	710	6229	6932	C-terminal 6xHis Tag
pRIC 4.0 SHUV N	710	7392	8059	N/A

Two remaining expression constructs were created using the pTRAc HT vector and the self-replicating vector. pRIC 4.0. The vector pTRAc HT allows for the expression of a 6xHis tag on the N terminus while pRIC 4.0 does not express a 6xHis affinity tag. The SHUV N gene was successfully subcloned into both the pTRAc HT and pRIC 4.0 vector with the REs specified in Table 5. As seen in Figure 3.4, the inclusion of the SHUV N gene within the respective vectors was confirmed through colony PCR at first, then RE digest using the RE specified in Table 7.

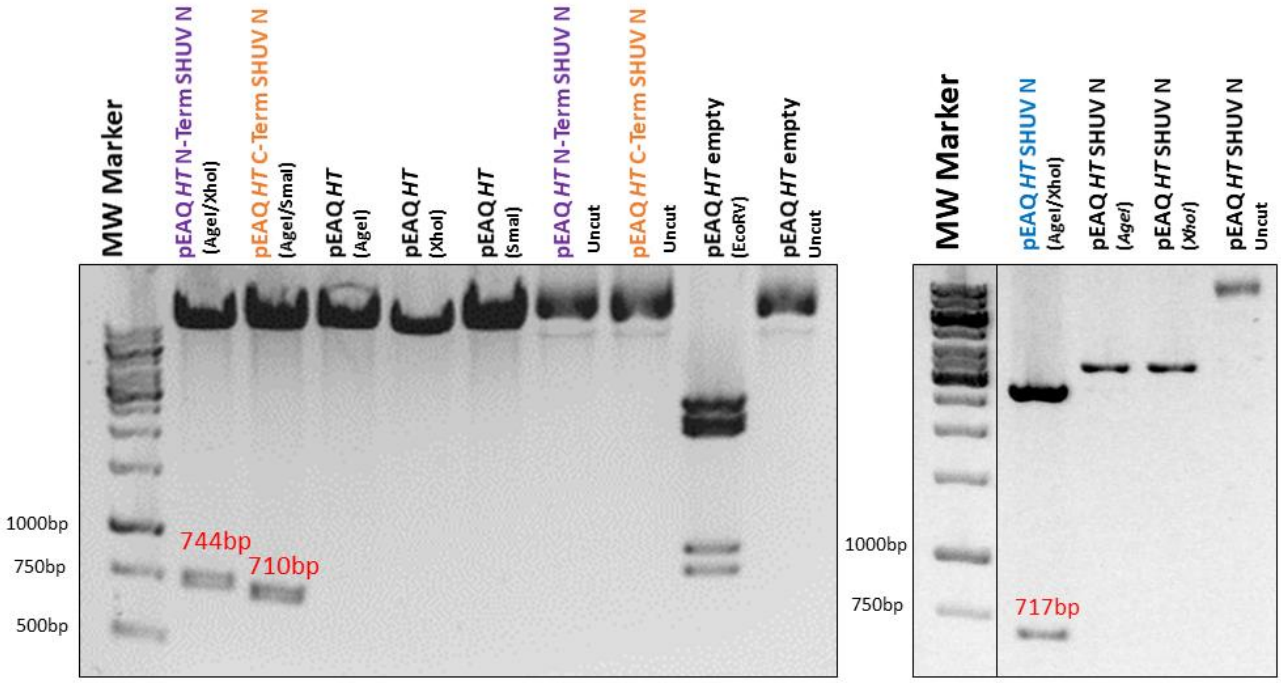


Figure 3.3: Restriction enzyme digest of the pEAQ *HT* N-term SHUV N, pEAQ *HT* C-term SHUV N and pEAQ *HT* SHUV N plant expression constructs.
1% agarose electrophoresis gel showing the excision of the SHUV N gene from the various pEAQ *HT* SHUV N constructs with the listed restriction enzymes.

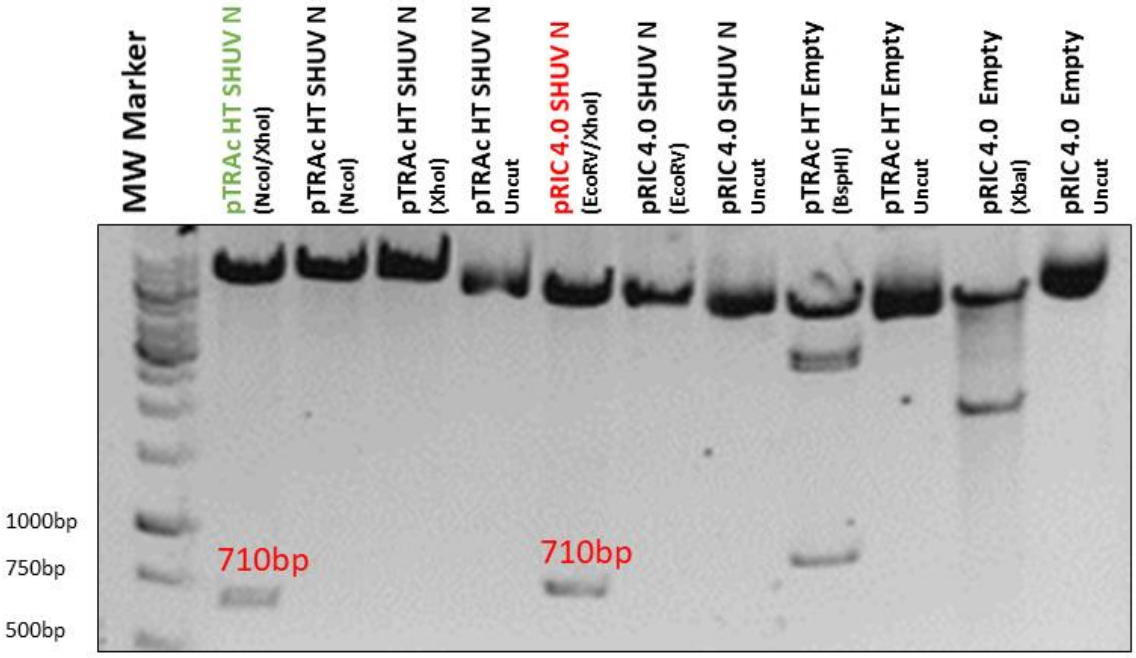


Figure 3.4: Restriction enzyme digest of pTRAc HT SHUV N and the pRIC 4.0 SHUV plant expression constructs.
1% agarose electrophoresis gel showing the excision of the SHUV N gene from the pTRAc HT SHUV N and pRIC 4.0 SHUV N constructs with the listed restriction enzymes.

Once all the expression vectors had been created, the specified *A. tumefaciens* strain was successfully transformed with the created constructed through electroporation as described in section 3.2.3. Colony PCR was performed once more with vector specific primers described in Table 6 over the multiple cloning site. This was able to select successfully transformed *A. tumefaciens* clones that contained the correct construct by giving the expected band sizes as seen in Table 12. As seen in Figure 3.5, the amplification of a band that correlates with the size of the inserted SHUV N gene into each construct shows the successful selection of *A. tumefaciens* clones.

To ascertain that the plasmid with the SHUV N gene was not lost, each agrobacterium culture was subjected to PCR to confirm the insert prior to infiltration.

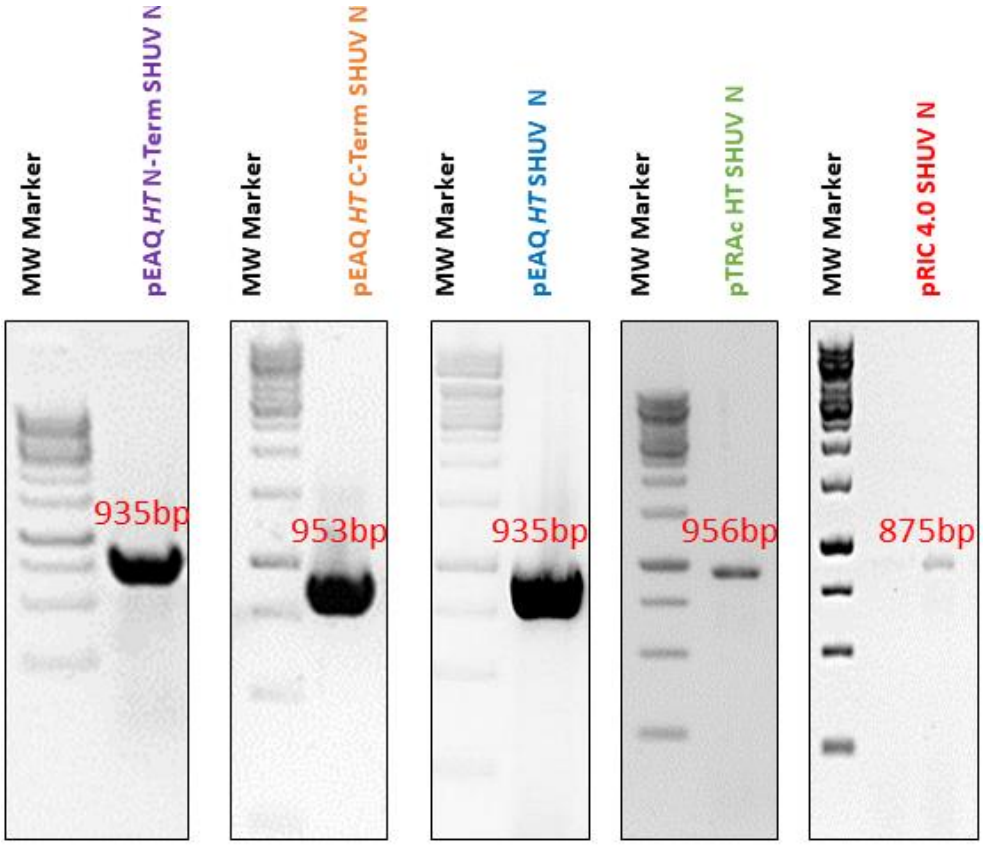


Figure 3.5: Colony PCR of *A. tumefaciens* transformants.
1% Agarose gel showing the amplification of the SHUV N gene insert in the various expression constructs. External vector specific primers were used to amplify over the multiple cloning site of each construct.

Table 12: Expected band sizes of the PCR amplified *E. coli* and *A. tumefaciens* colonies containing each of the following constructs.

Construct	PCR Product Size (bp)
pEAQ *HT* N-term SHUV N	935
pEAQ *HT* C-term SHUV N	953
pEAQ *HT* SHUV N	935
pTRAc HT SHUV N	956
pRIC 4.0 SHUV N	875

3.3.2 Small-Scale Infiltration and Protein Expression

Small-scale Infiltration

Plant based expression time trials were setup on a small-scale to compare the various expression constructs through *A. tumefaciens* mediated infiltration of *N. benthamiana,* this is described in section 3.2.4. For two different concentrations of *A. tumefaciens*, measured at OD_{600} to be 0.25 and 0.5, expression was assessed over 7 days post infiltration.

Initial small-scale expression experiments were performed only with constructs that contained the 6xHis affinity tag sequence: pEAQ *HT* N-term SHUV N, pEAQ *HT* C-term SHUV N, and pTRAc HT SHUV N and were detected through the use of anti-6xHis antibodies in Western blots.

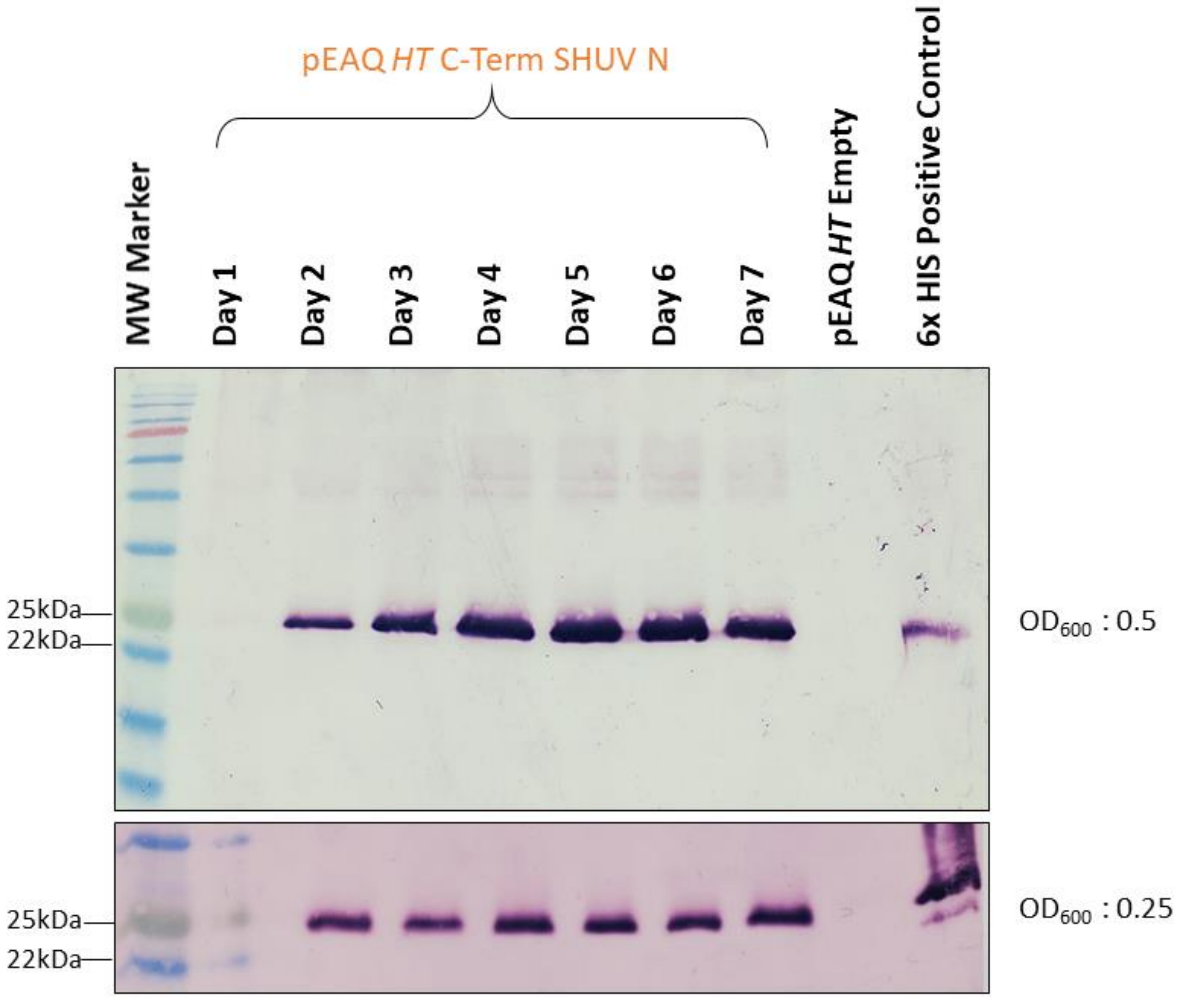


Figure 3.6: Western blot screening of pEAQ *HT* C-Term SHUV N construct at an *A. tumefaciens* OD_{600} of 0.5 (top) and 0.25 (bottom).
Western blot of two separate plant crude extracts which underwent agrobacterium mediated infiltration of the pEAQ *HT* C-Term SHUV N expression construct at two different concentrations of agrobacterium culture. Plant crude infiltrated with empty vector is negative control. Positive control is *E. coli* expressed SHUV N. Probed using mouse anti-6xHis antibodies at 1:2000 dilutions. 30 µg of TSP was loaded per lane.

Leaf crude extracts infiltrated with the expression constructs were qualitatively assessed through western blots. Looking at Figure 3.6, each lane was loaded with 30µg of total soluble protein (TSP) from the crude extract and probed. As can be seen, the expression construct pEAQ *HT* C-Term SHUV N showed a band of roughly 25kDa. This correlates with the expected band size of the expressed SHUV N protein, with possible dimer formation seen with a faint band at roughly 50kDa. Expression was seen starting at 2 dpi before peaking at 4 dpi and plateauing between 4 and 7 dpi. No expression was seen in plant crude extract infiltrated with pEAQ *HT* empty vector, the negative expression control. Comparison between plants infiltrated at an OD_{600} of 0.25 and 0.5 was performed and infiltrating at an *A. tumefaciens* OD_{600} of 0.5 expressed a subjectively higher amount of SHUV N protein when viewed via western blot. While the difference is not entirely contrasting, the logical conclusion is that the higher *A. tumefaciens* concentration possibly leads to a larger number of plant cells being transfected with the expression construct. These factors led to an *A. tumefaciens* concentration, measured at an OD_{600} of 0.5, to be used for all subsequent large-scale expression with this construct.

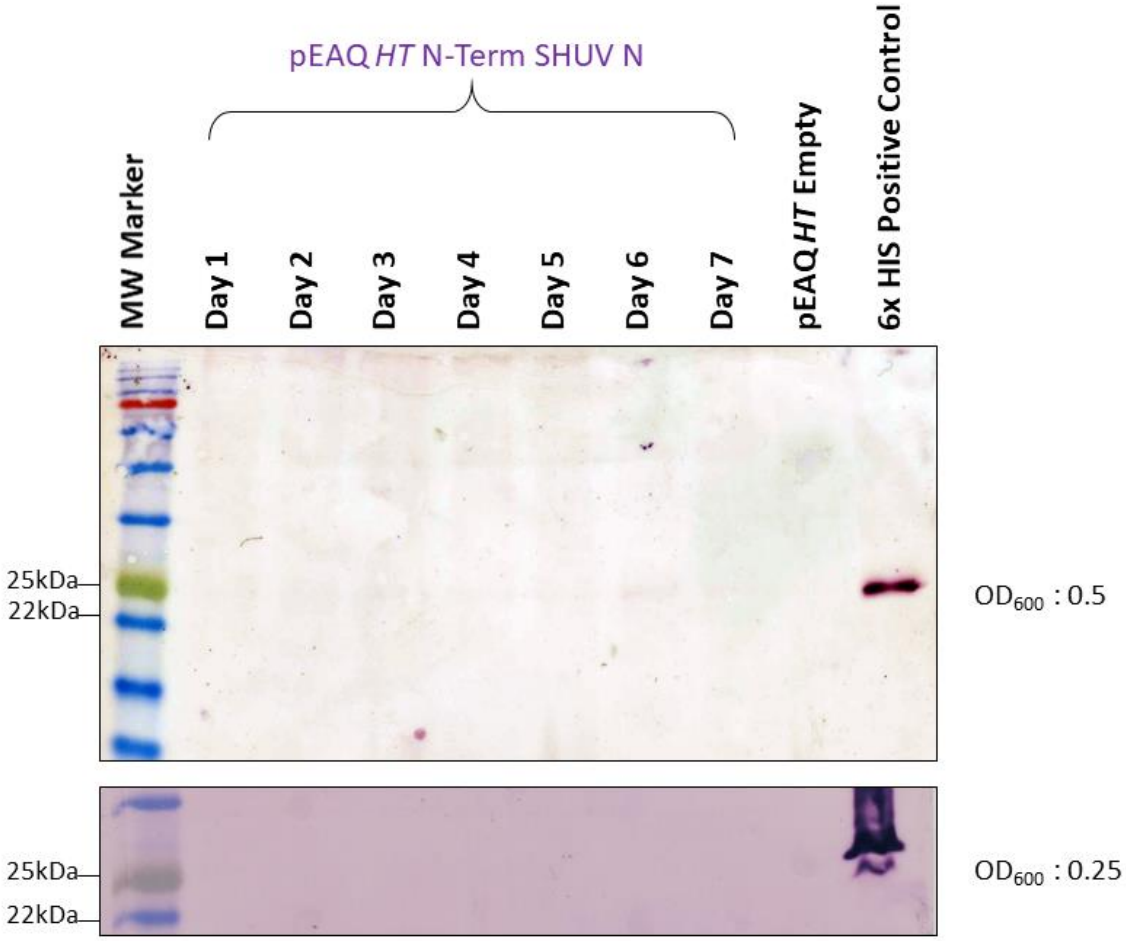


Figure 3.7: Western blot screening of pEAQ *HT* N-Term SHUV N construct with an *A. tumefaciens* OD of 0.5 (top) and 0.25 (bottom).
Western blot of two separate plant crude extracts which underwent agrobacterium mediated infiltration of the pEAQ *HT* N-Term SHUV N expression construct at two different concentrations of agrobacterium culture. Plant crude infiltrated with empty vector is negative control. Positive control is *E. coli* expressed SHUV N. Probed using mouse anti-6xHis antibodies at 1:2000 dilutions. 30 µg of TSP was loaded per lane.

Leaf crude extracts infiltrated with the expression construct pEAQ *HT* N-Term SHUV N showed little to no expression of the expected SHUV N protein as seen in Figure 3.7. Each lane was loaded with 30µg TSP from the crude extract. A faint band of roughly 25kDa can be seen in crude extract infiltrated at an OD_{600} of 0.5 between 6-7 dpi but this is barely discernible. This may be due to low levels of SHUV N expression. Three repeats of small-scale expression were performed with no change in detection or visible expression.

To determine if lack of detection of protein expression is due to extraction, 1% Triton-100X was added to the PBS extraction buffer, but no change and no expression was seen (data not shown). A change in extraction buffer conditions suggests that it isn't necessarily the extraction method that is responsible for the lack of detection but possibly the inability of the construct to express in *N. benthamiana*.

Finally, leaf crude extracts infiltrated with the expression construct pTRAc HT SHUV N were assessed for SHUV N expression. Each lane was loaded with 30 µg TSP from the crude extract. The western blots showed no detection of the expected SHUV N protein when assessed with anti-6xHis antibodies as the probing reagent. This was performed at an OD_{600} of both 0.25 and 0.5 with no difference between. Three repeats of small-scale expression were performed with no detection of expression (data not shown) The inclusion of 1% Triton-100X in 1x PBS extraction buffer showed no change and no expression was seen (data not shown).

The pEAQ *HT* N-Term SHUV N and pTRAc HT SHUV N constructs expresses a 6xHis affinity tag on the N terminus of the expressed protein. Thus, it is possible that due to the presence of the 6xHis tag on this terminus has led to trouble in the expression of the target protein in plants.

3.3.3 Screening of Constructs using Anti-Shuni N Specific Polyclonal Antibody

Plant expression was once again assessed per construct at *A. tumefaciens* infiltration culture OD_{600} of 0.25 and 0.5. This was visualised using the rabbit anti-SHUV N antibody described in chapter 2 and following the protocol described in section 3.2.5. For each construct, 6 µg of TSP from the crude plant extract was loaded into each lane for clear visualisation.

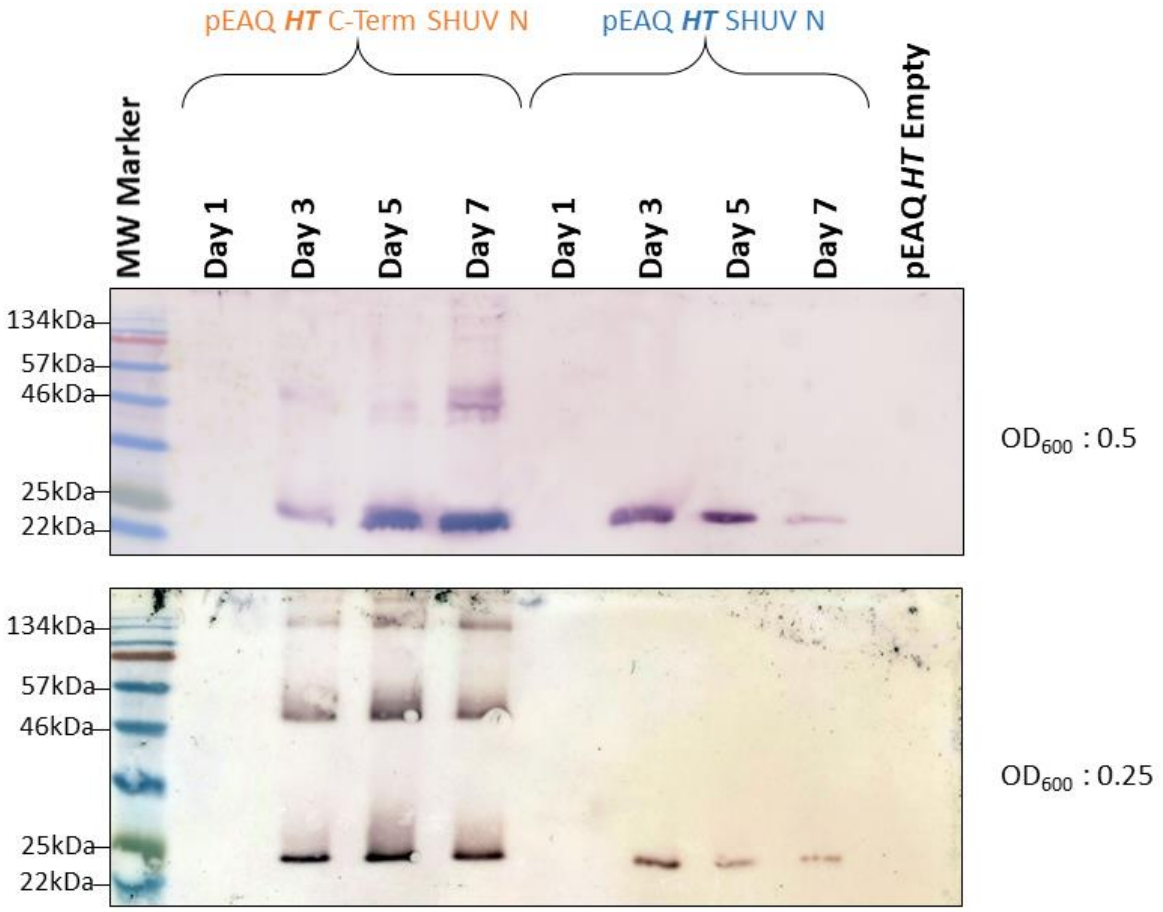


Figure 3.8: Western blot screening of pEAQ *HT* constructs at an *A. tumefaciens* OD_{600} of 0.5 (top) and 0.25 (bottom).
Western blot of plant crude extract having been infiltrated with *A. tumefaciens* containing pEAQ *HT* C-term SHUV N and pEAQ *HT* SHUV N expression constructs both at a culture OD_{600} of **0.5** and **0.25**. Plant crude infiltrated with empty vector is negative control. Probed using rabbit serum containing anti-SHUV N antibodies at 1:10 000 dilutions. 6µg of TSP was loaded per lane.

In Figure 3.8 plant crude infiltrated with pEAQ *HT* C-term SHUV N showed similar expression pattern when probed with anti-SHUV N as was seen when probed with anti-6xHis in Figure 3.6. Expression was not present at Day 1 but a band of roughly 25kDa was seen starting 3 dpi and increasing in intensity until 7 dpi. Had everyday been sampled, it is expected that expression would have been seen 2 dpi as seen previously in Figure 3.6. Qualitative assessment of western blots at the different ODs showed subjectively more expression at an OD_{600} of 0.5 when compared to an OD_{600} of 0.25. The ability to discern both the dimer and multimer of SHUV N was subjectively better at an OD of 0.25 when compared to an OD of 0.5. When comparing blots probed with anti-6xHis to that probed with anti-SHUV N, the ability to detect dimers and multimers of the SHUV N protein was far better with the polyclonal anti-SHUV N antibody at both ODs when compared to anti-6xHis.

When looking at the expression construct pEAQ *HT* SHUV N, it was assessed for plant expression solely through detection with anti-SHUV N due to the lack of 6xHis tag on the expressed protein and as such probing using anti-6xHis antibody would be ineffective. Looking to Figure 3.8, expression of a 25kDa band is seen, starting 3 dpi before subsequently becoming progressively fainter towards 7 dpi. Expression of the SHUV N protein was assessed to be greater at an OD_{600} of 0.5 as opposed to 0.25. No dimer or multimer formation was detected with this construct at either OD_{600} suggesting that either detection of such multimers is limited without the presence of a 6xHis affinity tag.

Plant expression of the SHUV N protein from pEAQ *HT* N-term SHUV N construct was assessed using anti-SHUV N antibodies and showed no possible product at either OD_{600}.This was performed without the inclusion of detergent in the extraction buffer. No dimer or multimer formation is detected with this construct at either OD_{600}. Data is not shown.

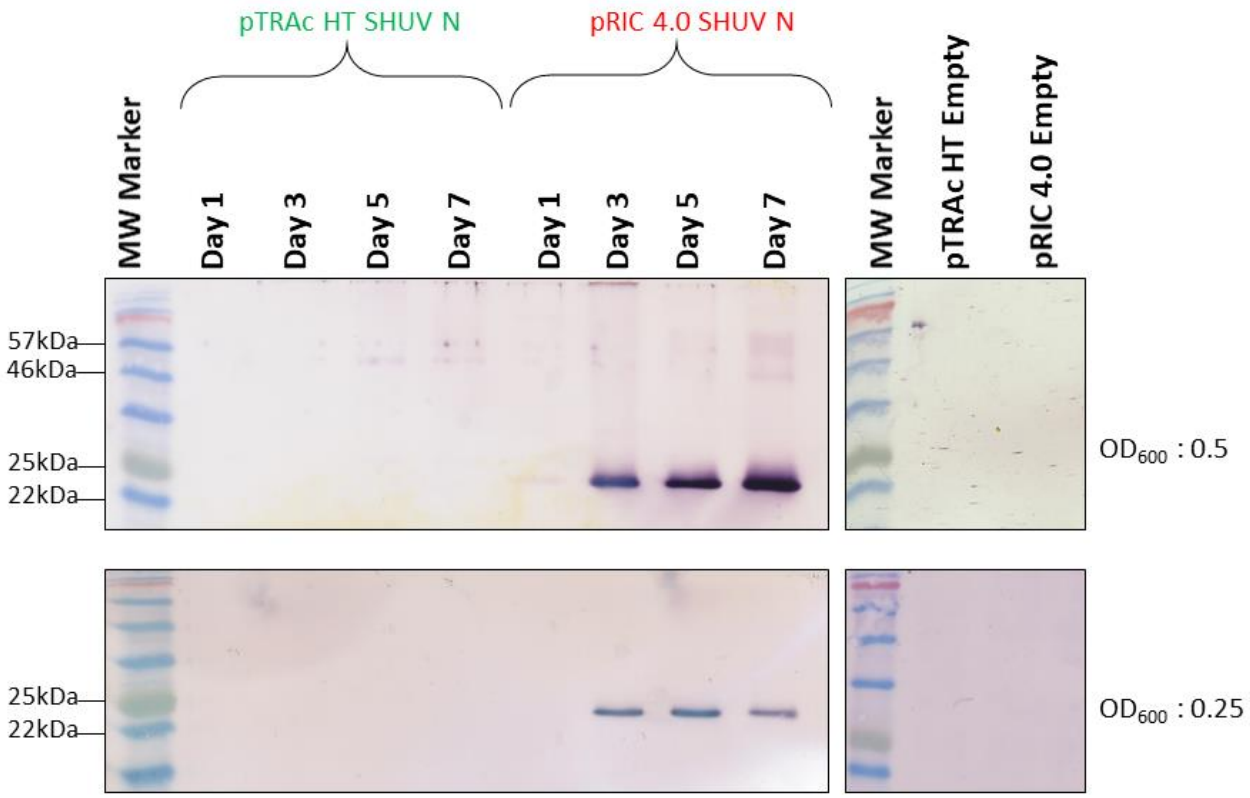


Figure 3.9: Western blot screening of pTRAc HT and pRIC 4.0 constructs at an *A. tumefaciens* OD_{600} of 0.5 (top) and 0.25 (bottom).
Western blot of plant crude extract having been infiltrated with *A. tumefaciens* containing pTRAc HT SHUV N and pRIC 4.0 SHUV N expression constructs both at a culture OD_{600} of **0.25** and **0.5**. Plant crude infiltrated with empty vector is negative control. Probed using rabbit serum containing anti-SHUV N antibodies at 1:10 000 dilutions. 6 µg of TSP was loaded per lane.

Plant crude infiltrated with pTRAc HT SHUV N showed no detection of the target protein when probed with anti-6xHis antibodies. Here in Figure 3.9, plant crude infiltrated with pTRAc HT SHUV N was probed with anti-SHUV N antibody at both an *A. tumefaciens* culture OD_{600} of 0.25 and 0.5. As was the case with the previous probing, there was no visible detection of the target protein.

Finally, the plant expression construct pRIC 4.0 SHUV N was assessed for possible expression of the SHUV N protein as it could not be assessed previously due to the lack of a 6xHis tag within the construct. Plant crude extract infiltrated with pRIC 4.0 SHUV N at an OD_{600} of 0.25 and 0.5 was probed using anti-SHUV N antibody. As shown in Figure 3.9, a 25kDa band can been from 3 dpi for both and culture OD_{600} of 0.25 and 0.5. Intensity plateaus from 3 to 7 dpi at an OD_{600} of 0.25 but steadily increasing in intensity from day 3 to 7 at an OD_{600} of 0.5. It is seen that, in constructs lacking a 6xHis tag, multimer formation is decreased. This may suggest that multimer formation is due to association through the 6xHis tag. A 50kDa band representative of dimer formation was only seen 7 dpi in plants infiltrated at an *A. tumefaciens* culture OD_{600} of 0.5. The increased *A. tumefaciens* OD and general expression suggests that only at higher concentrations of the target protein does multimer formation become apparent without the possible help through association with the 6xHis affinity tag, however it may be possible that multimer formation is purely a factor of increased protein concentration

For all expression constructs, empty vectors were infiltrated and harvested 7 days post infiltration. These serve as negative expression controls. Plant crude extracts infiltrated with empty vectors such as pEAQ *HT* Empty, pTRAc HT Empty and pRIC 4.0. showed no bands after probing with anti-SHUV N at the same dilution and same amount of TSP loaded as used with positive samples.

3.3.4 Large-Scale Infiltration and Protein Expression

The expression construct pEAQ *HT* C-Term SHUV N was taken forward as the construct of choice. Large-scale infiltration was performed at an *A. tumefaciens* OD_{600} of 0.5 on 35 *N. benthamiana* plants and harvested 3 dpi. This decision was based on information obtained from small-scale protein expression studies performed with anti-6xHis antibodies rather than anti-SHUV N antibodies.

3.3.5 Purification of SHUV (N) Protein

Ammonium Sulphate Precipitation

Plant crude extract from 35x *N. benthamiana* plants that had been infiltrated with pEAQ *HT* C-term SHUV N underwent $(NH_4)_2SO_4$ precipitation. The extract was precipitated into seven different fractions, increasing in $(NH_4)_2SO_4$ saturation by 10% between each fraction. The resulting western blot is shown in Figure 3.10. The majority of SHUV N protein is seen to precipitate between the 40-60% fraction as shown by the presence of a 25kDa band, 50kDa and approximate 125kDa band that may represent the monomer, dimer and multimer respectively. The precipitate between 0 – 40% was discarded and the protein precipitated between 40-60% was kept for further downstream purification of the SHUV N protein.

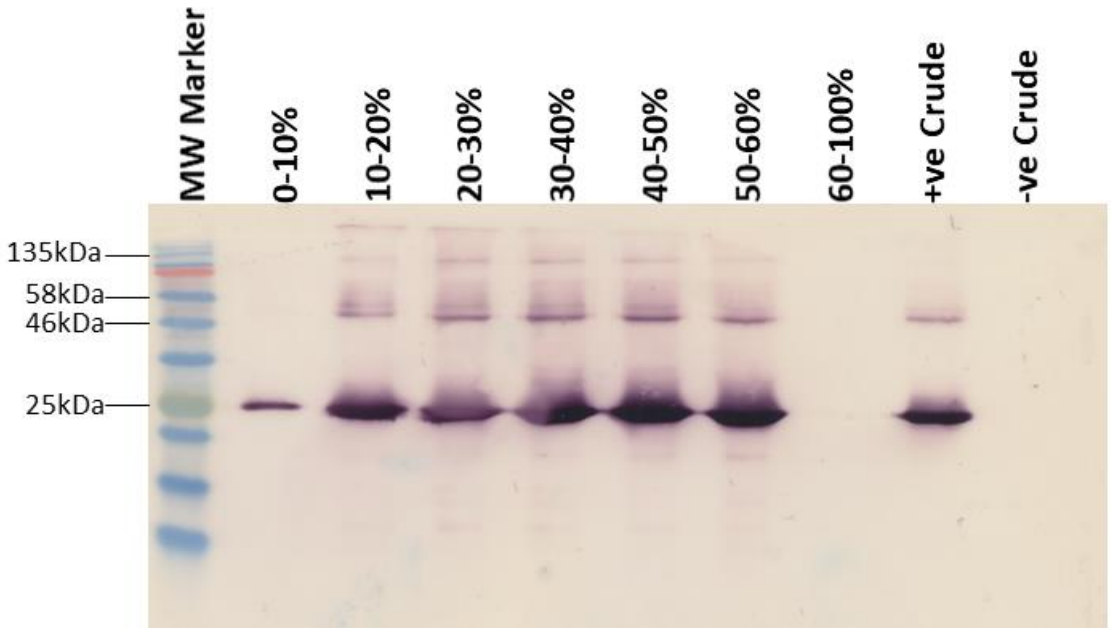


Figure 3.10: Western blot of various $(NH_4)_2SO_4$ precipitated fractions.
Western blot shows the presence of expected bands in the various percentage precipitated fractions. Equal volume was loaded in each lane. Plant crude infiltrated with pEAQ *HT* C-term SHUV N was used as a positive control while plant crude infiltrated with pEAQ *HT* Empty was used as a negative control. Probed using mouse anti-6xHis antibodies at 1:2000 dilution.

Small-Scale Nickel Affinity Batch Purification

Once the SHUV N protein had been precipitated in the 40-60% fraction, it was resuspended and dialysed in order to remove $(NH_4)_2SO_4$ from the solution. This sample then underwent small-scale nickel affinity batch purification to determine if the 6xHis tag was suitable for downstream affinity purification, small-scale nickel affinity batch purification was performed on the sample.

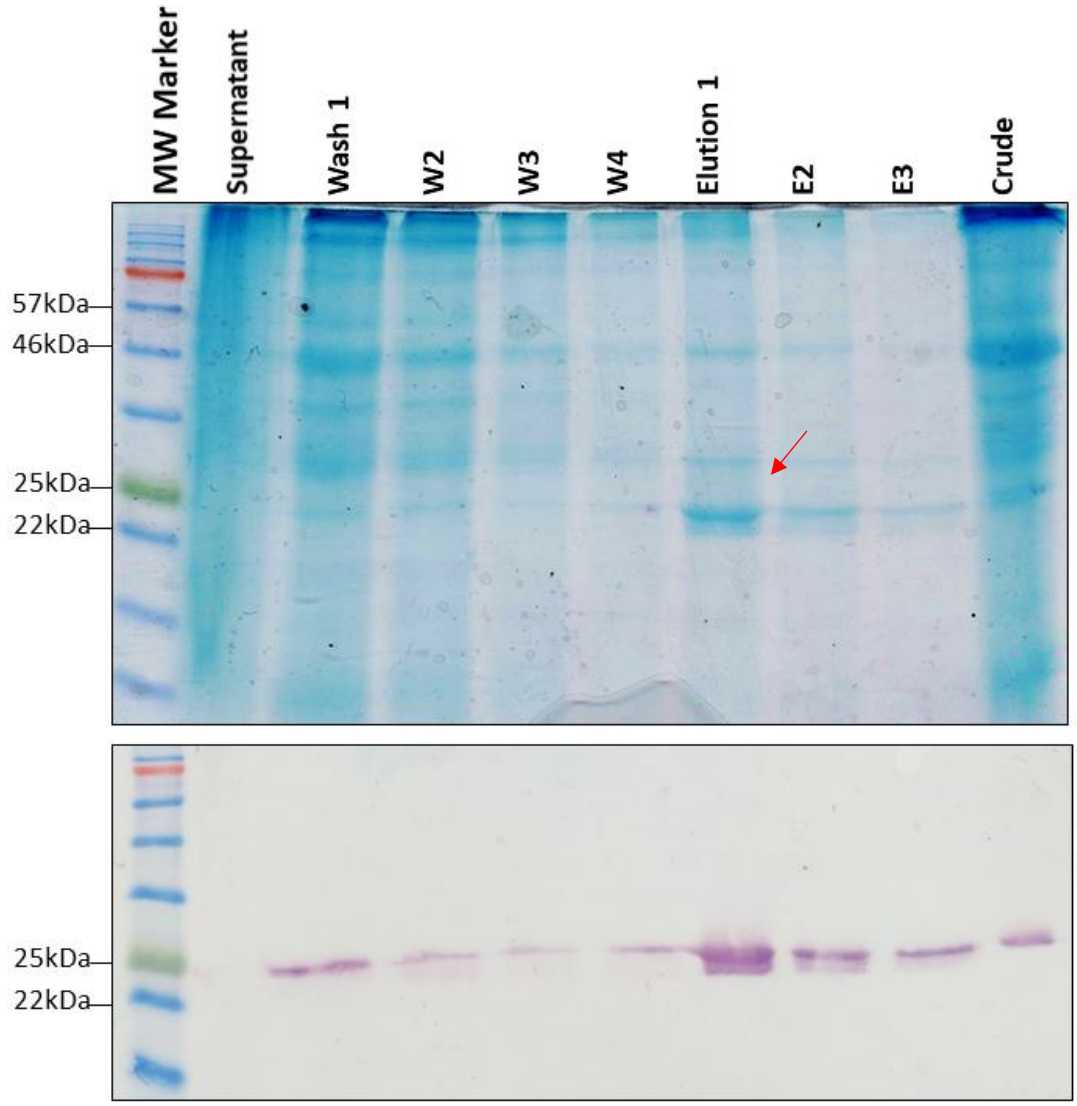


Figure 3.11: Coomassie Brilliant Blue stained gel (top) and western blot (bottom) showing the various fractions from small-scale nickel affinity batch purification.
The Coomassie stained electrophoresis gel shows the decreasing amounts of protein in the wash (W) fractions and the elution of a band of the expected size in elution (E) fractions 1 – 3 after small-scale nickel affinity batch purification. The western blot shows and confirms the presence of bands of expected size in wash fractions 1 – 4 and elution fractions 1 – 3. Probed using mouse anti-6xHis antibodies at 1:2000 dilution.

Looking at resulting Coomassie stained acrylamide gel in Figure 3.11, wash fractions 1 – 4 shows the elution of unbound protein in decreasing amounts while the western blot shows the elution of small amounts of unbound SHUV N protein in similarly decreasing amounts. It is quite possible the nickel resin was saturated with bound protein and thus unbound SHUV N was being washed off. Looking to the elution fractions 1- 3 in Figure 3.11, the presence of an intense 25kDa band and faint 50kDa band (thought to be the monomer and dimer respectively) in these fractions demonstrates the ability for the target protein to be able to bind to the nickel resin and be eluted off the resin with the natural competitor, imidazole. A non-specific band of around 30kDa is seen along with bands towards the top of the acrylamide gel. This may be indicative of non-specific binding of natural plant proteins to the nickel resin. The presence of the expected bands suggests that the 6xHis affinity tag is available for binding within the non-denatured SHUV N protein structure.

Nickel Affinity Chromatography

Once batch purification had shown the ability of the SHUV N protein to effectively bind to the nickel affinity resin, the next step for large-scale purification of the target protein was through the use of the FPLC system, the AKTA Explorer 100, in combination with a 5ml nickel HisTrap HP pre-packed column (GE Healthcare, USA).

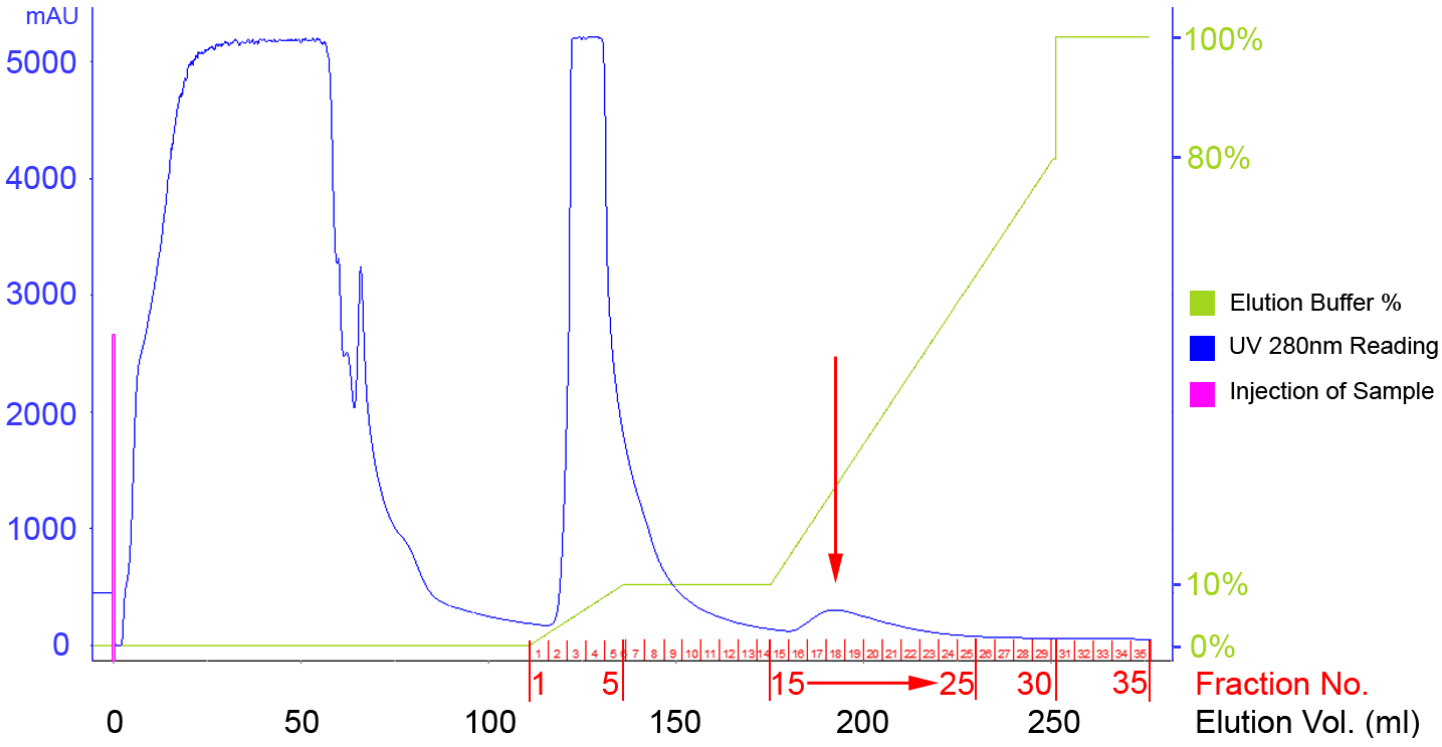


Figure 3.12: Elution profile of the AKTA 100 FPLC using the 5 ml nickel HisTrap HP pre-packed column as a stationary phase.
The increasing percentage of elution buffer that makes up the mobile phase is shown along with UV 280 nm reading of each eluted fraction. Arrow indicates the UV 280nm peak due to the elution of the SHUV N protein.

An elution profile was run to allow for the purification of the SHUV N sample obtain from the dialysed 40-60% fraction $(NH_4)_2SO_4$ fraction. The profile was run and each 5 ml fraction was collected and assessed through SDS-PAGE and stained via Coomassie. Looking at the elution profile in Figure 3.12, the elution buffer was introduced into the mobile phase to make up 0 – 10% of the total mobile phase over the course of the first 5 fractions. This then plateaued at 10% for fractions 5 to 15. This led to the elution of a large peak as seen from the UV 280 nm readings for fractions 2 – 10. This corresponded with change in colour of the physical liquid fractions from clear to a dark brown before returning to clear. This may correlate with the elution of unbound proteins and extract. The increase in the inclusion of the elution buffer in the mobile phase from a percentage of 10 – 80% over fractions 15-30 showed the presence of a second UV 280 nm peak between fractions 15 and 25 as indicated by the arrow. It is thought that this second 280 nm peak is due to the elution of the target protein and its multimers. The Coomassie stained acrylamide gel is shown Figure 3.13.

A 25kDa and a 50kDa band can be seen in fractions 18 – 25; this corresponds with our possible SHUV N monomer and dimer respectively. The concentration of each eluted fractions was determined by gel densitometry and was shown to sit between 26 µg/ml and 63 µg/ml. Each of the 8 fractions were pooled which resulted in the pooled sample concentration of 56.2 µg/ml. This was to be used as an antigen in downstream indirect ELISAs.

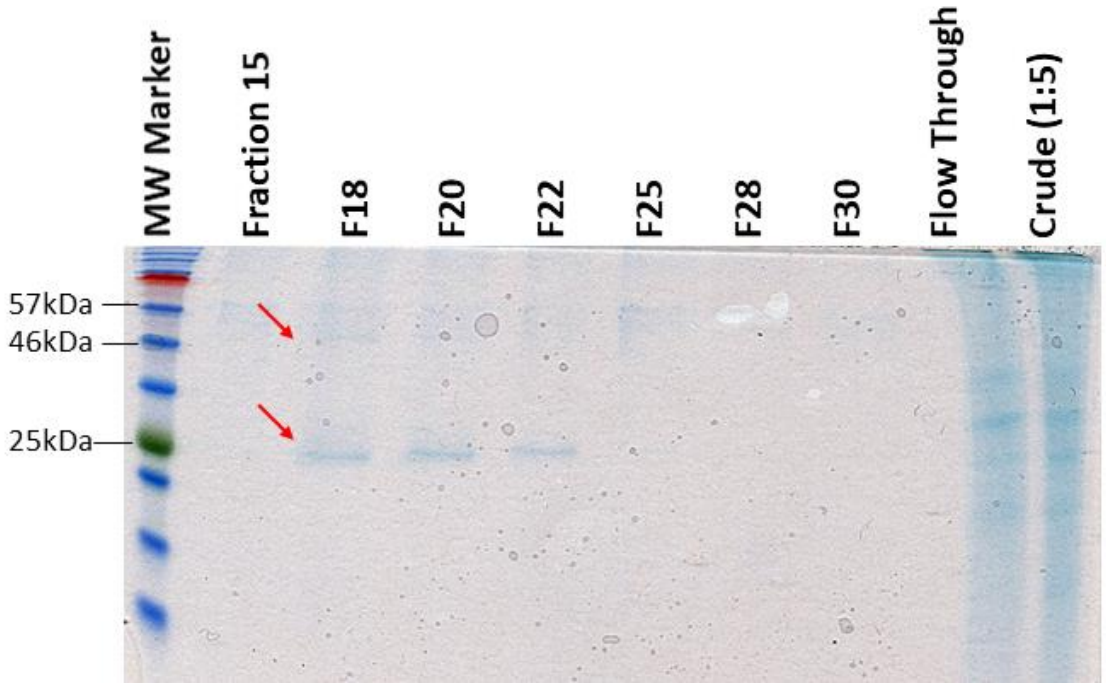


Figure 3.13: Coomassie Brilliant Blue stained gel showing the eluted fractions after affinity chromatography.
AKTA 100 FPLC purification was performed with a 5 ml nickel HisTrap HP pre-packed column and fractions shown. Fractions 18-25 show the presence of 25kDa and 50kDa bands that correspond with the expected size of the SHUV N monomer and dimer. Flow through and crude is shown.

Stability Study

In order to assess whether the eluted protein was stable over time, a degradation study was conducted as described in section 3.2.11. As can be seen in the Coomassie stained gel in Figure 3.14, the batch purified protein was assessed at three different temperatures and stored for 0, 6 or 12 weeks at each temperature. The 25kDa band seen was fairly consistent in its intensity across all temperatures and time points except for two specific points: these were 6 weeks at 4°C and 6 weeks at -20°C. Seeing as the following timepoints at 12 weeks were shown to be consistent with the those at 0 weeks, it is possible that the protein itself does not degrade at noticeable rate and that the faint bands seen at 4°C at 6 weeks and -20°C at 6 weeks were possibly due to pipetting error when preparing the sample for storage rather than degradation. At 4°C, dimer formation is only slightly seen after 12 weeks, while at -20°C it appeared at 0 and 12 weeks, once again justifying that the timepoint at 6 weeks was due to pipetting error. Dimer formation seen in all the -80℃ timepoints with little to no degradation shown. A nonspecific band of 30kDa is seen across all time and temperature points and is possibly due to nonspecific binding of a natural plant protein to the nickel resin during purification. The same band is seen in Figure 3.11 when conduction small-scale batch purification.

To confirm the identity of these bands, western blot was performed and probed using standard anti-6xHis antibody and not anti-SHUV N due to the antibody not being available at this point in the project timeline. As seen in the western blot in Figure 3.14, bands consistent with the expected SHUV N size were seen across all temperatures and timepoints. Once again two timepoints are slightly less intense than the remaining timepoints, 6 weeks at 4°C and 6 weeks at 20°C. Due to the reasons stated above, it is thought this lack of intensity is due to pipetting error when preparing the sample for storage rather than degradation as both the Coomassie and western blot show the same pattern. However, this experiment was only performed once, and more repeats would be needed to confirm this. Across all remaining timepoints, bands are consistent in intensity and no smaller bands are observed which suggests the protein does not degrade at a noticeable rate. Dimers are seen at 12 weeks at both 4°C and -20°C. While this has shown that the protein did not degrade noticeably by the selected timepoints, it does not allow insight into whether the protein would be biological stable or functional for the intended seroassay after said timepoints.

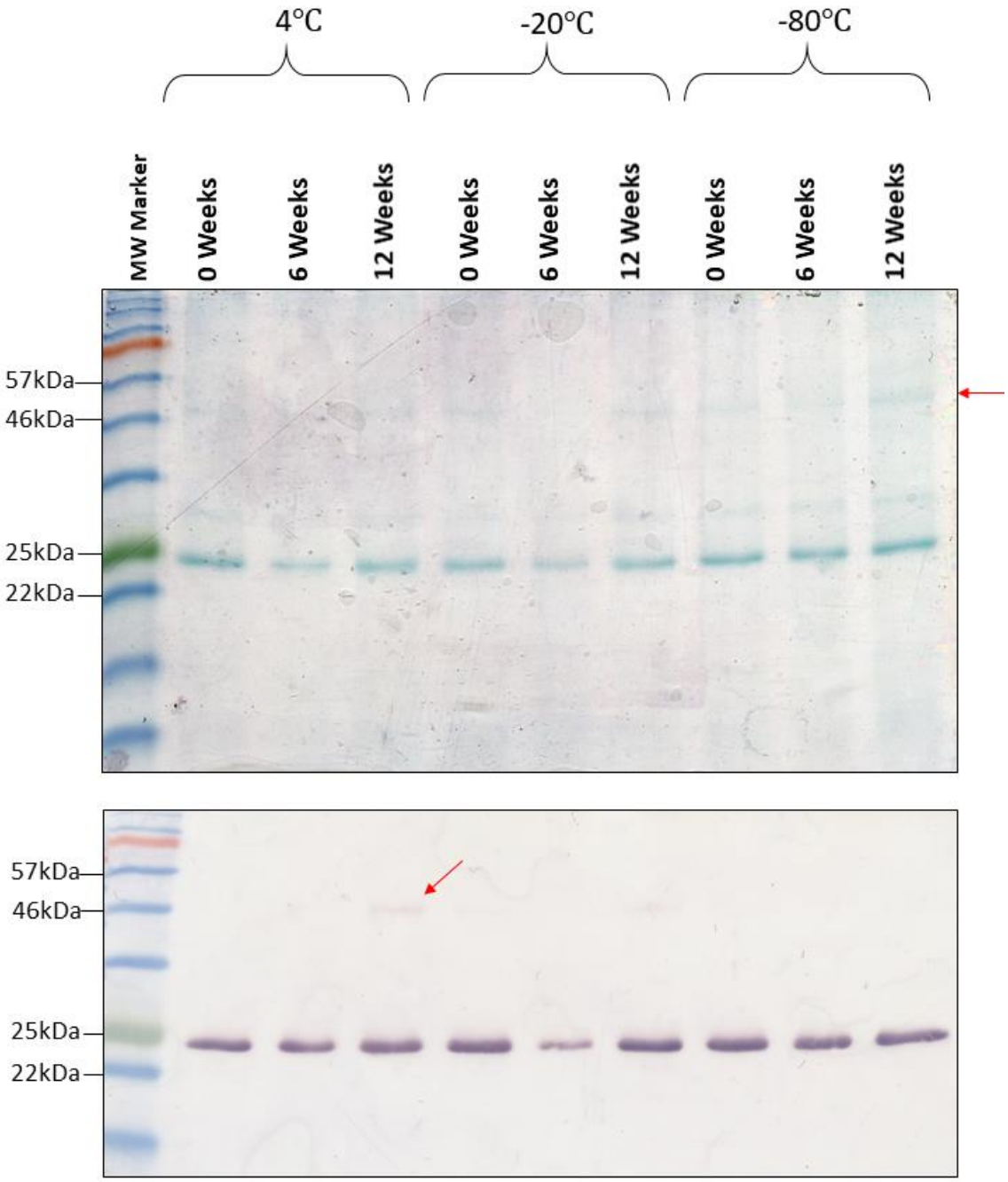


Figure 3.14: Coomassie Brilliant Blue stained gel (top) and western blot (bottom) showing the assessment of degradation of purified fractions stored at various temperatures for set times. The Coomassie stained electrophoresis gel shows expected bands from samples stored at for 4℃ , −20℃ and −80℃ at 0, 6 and 12 weeks for each temperature. A non-specific band of 30kDa is also seen. Western blot shows the presence of expected bands from samples stored at for 4℃, −20℃, and −80℃ at 0, 6, and 12 weeks for each temperature. Probed using mouse anti-6xHis antibodies at 1:2000 dilution. Arrows indicate the presence of dimers.

3.3.6 Testing of SHUV (N) Protein Antigenicity

Mouse ascites fluid raised against live SHUV was used to detect the plant produced SHUV N antigen in westerns and ELISAs to determine if this plant produced antigen is suitable for diagnostic assays.

Western Blot

Plant produced SHUV N protein was probed with mouse ascites fluid. Both the monomer and dimer can be seen in SHUV N infiltrated plant crude extract, the purified plant extract, but are absent in plant crude extract infiltrated with empty vector (Figure 3.15).

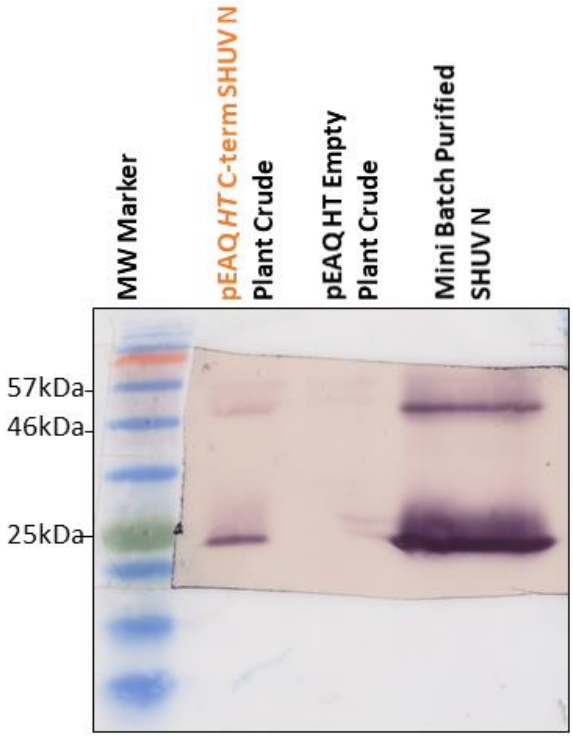


Figure 3.15: Western blot of plant produced and batched purified SHUV N being probed with mouse ascites fluid.
Western blot assessing the ability of mouse ascites fluid, raised against virus infected cell extract, to detect plant produced SHUV N in plant crude and after purification. Western blot shows expected bands for monomer and dimer in infiltrated plant crude and small-scale batch purified SHUV N. Plant crude infiltrated with pEAQ HT Empty was used as a negative control. Probed using mouse ascites fluid provided by the ZRU at 1:400 dilution.

Indirect ELISA

AKTA-purified plant-expressed SHUV N protein is subsequently referred to as 'antigen' while plant crude infiltrated with empty vector that underwent a mirrored purification process is referred to as 'mock antigen'.

As seen in Figure 3.16, an indirect ELISA was performed with SHUV N as an antigen against the specified mouse ascites fluid raised against SHUV infected cell extract (referred to as anti-SHUV). When probing with this ascites fluid, high A_{405} values were seen against the purified antigen, of 3.965 and 3.669 for dilutions of 1:3200 and 1:6400 respectively.

This was not seen against mock antigen where A_{405} values of 0.129 and 0.113 were seen and where A_{405} values of 0.106 and 0.107 were seen against coating buffer only controls for the respective dilutions. This suggest that specific binding of antibodies in the ascites fluid was seen with the plant produced SHUV N protein.

To prove the specificity of the antibodies, present in the SHUV raised ascites fluid, ascities fluid raised against the unrelated Middelburg virus was also tested on the SHUV N protein. This mouse ascites fluid raised against Middleburg virus infected cell extract (referred to as anti-MDV) gave low A_{405} values of 0.110 and 0.115 seen for 1:1600 and 1:3200 dilutions of the anti-MDV mouse ascites fluid. Looking at mock antigen and coating buffer controls, similarly low A_{405} values are seen with 0.110 and 0.111 seen for the respective dilutions against the mock antigen and values of 0.111 and 0.108 seen against coating buffer only controls. Overall this suggest that there was little to no binding taking place between anti-MDV antibodies in the ascites fluid and the plant produced SHUV N antigen. Unfortunately, no Middleburg virus antigens could be acquired for use as a positive control in this regard.

The binding of antibodies in SHUV-specific mouse ascites fluid to plant produced SHUV N antigen indicates that the plant produced antigen should be sufficient and able to bind the native antibodies that may be found in infected sera when performing downstream diagnostic ELISAs, which was the main objective of this project.

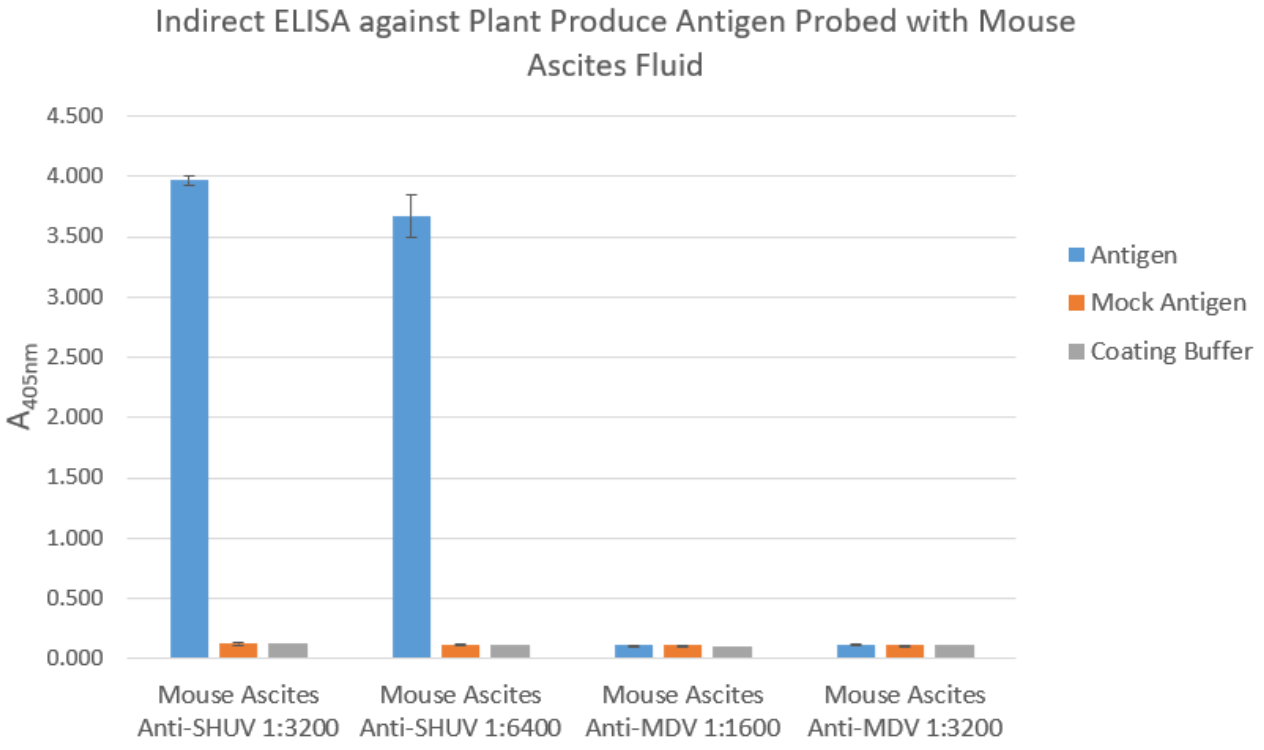


Figure 3.16: Indirect ELISA against plant produced antigen with ascites fluid.
Indirect ELISA was performed with plant produced SHUV N used as a capture antigen and probed with mouse ascites fluid raised against both SHUV Virus (Anti-SHUV) and Middleburg Virus (Anti-MDV). Mock antigen and wells containing only the coating buffer were used as neagtive control. This was performed at the given dilutions of the probing reagent.

3.3.7 Screening of Infected Animal Sera with SHUV (N) Protein

In order to obtain the initial parameters for the diagnostic ELISA, varying amounts of antigen were tested against varying concentrations of rabbit made anti-SHUV N antibodies, developed as explained in chapter 2.

The outcome showed 100-200ng of antigen per well was required for a positive signal to be seen against the rabbit made anti-SHUV N antibody at a dilution of 1:10 000 (data not shown) and thus this amount of antigen should be sufficient to see a positive signal against possible positive test serum. Mock antigen and coating buffer control showed no reaction to the anti-SHUV N antibodies as expected.

As seen in Figure 3.17, indirect ELISAs were performed with serum samples listed in Table 9. The serum sample SAE 131/11 displayed very similar readings for both the antigen and mock antigen for each dilution, such as ODs of 0.600 and 0.300 for a 1:32 to 1:128 dilution of the serum. As show in Figure 3.18, the same trend is seen with the full 2-fold dilution series, with coating buffer control showing background values slightly lower than that of antigen and mock antigen of each dilutions. This was not observed for coating buffer controls in Figure 3.18 and thus high coating buffer values may be down to experimental error and technique variation while performing the assay.

Samples SAE28/09, SAE29/10, SAE140/11, and ZRU84/12 displayed this same characteristic trend seen in Figure 3.18 with inconsistent readings for the coating buffer control across these samples (data not shown).

These readings are indicative of the non-specific binding of native antibodies found in the sample horse sera. The specific binding of native IgGs in the sera to the SHUV N antigen is unable to be discerned through the high level of background and thus the ELISA protocol was further optimized in order to make this possible.

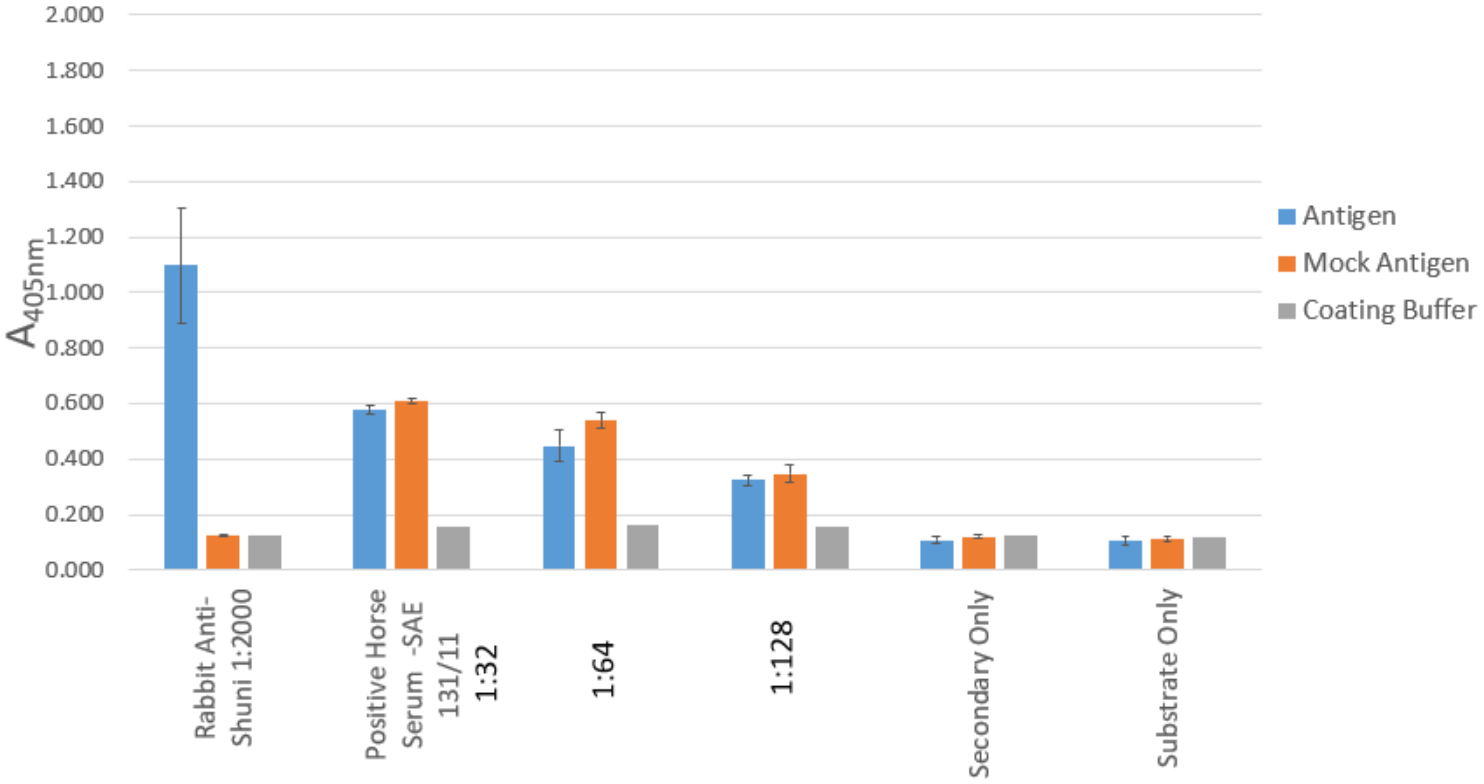


Figure 3.17: Indirect ELISA against plant produced antigen with horse serum at 1:32 – 1:128 dilution range.

Indirect ELISA using plant produced SHUV N as a capture antigen, being probed with SAE131/11 horse serum samples. This serum has previously shown to test positive for IgG's against SHUV. A_{405} readings for the probing with horse serum is done at varying dilutions of serum. Secondary antibody and substrate only controls are seen. Mock antigen and wells containing only the coating buffer were used as negative control.

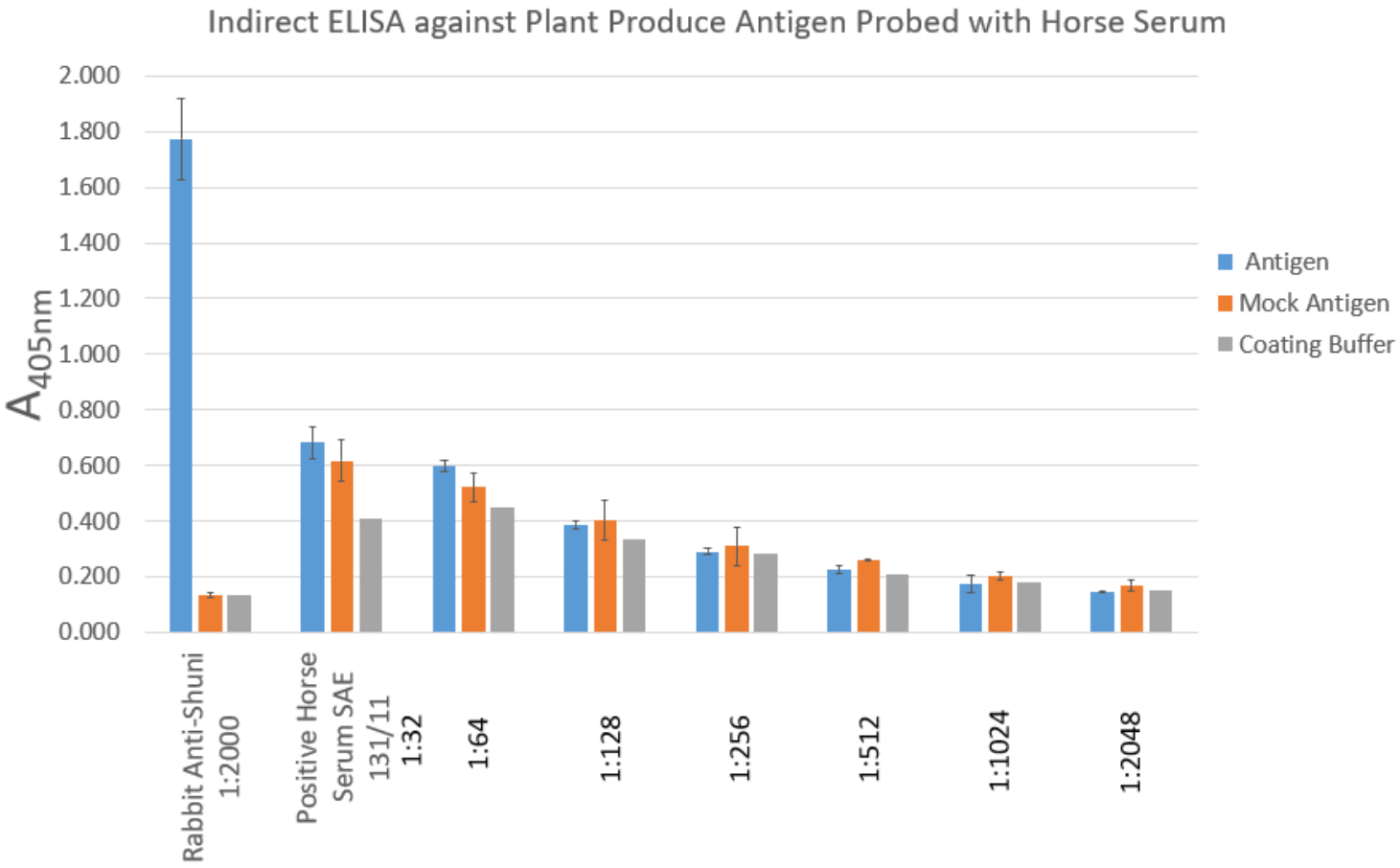


Figure 3.18: Indirect ELISA against plant produced antigen with horse serum at an extended dilution range.

Indirect ELISA using plant produced SHUV N as a capture antigen, being probed with SAE131/11 horse serum samples. This serum has previously shown to test positive for IgG's against SHUV. A_{405} readings for the probing with horse serum is down at an extended 2-fold dilution series of the serum. Positive serum control is the probing with Anti-Shuni N. Secondary antibody and substrate only controls are seen. Mock antigen and wells containing only the coating buffer were used as negative control.

3.3.8 Further Screening of Infected Sera with SHUV (N) Protein Post Optimisation

During this process of optimisation, horse sera was obtained from an AHSV study being performed by the BRU, shown in Table 10 as stated by the protocol in section 3.2.13. These horses had no known exposure to SHUV and samples were used in place of the limited "positive" sera obtained from the ZRU for the process of optimisation. The initial ELISAs performed against these new horse sera samples showed a similar trend of high background and non-specific binding to those seen in the "positive samples" obtained from the ZRU.

Once improvement of the protocol was complete, indirect ELISAs were performed against the various horse sera used during the optimisation, specifically sera samples from Lucky Lips, Liberty, and Lily. The serum sample SAE 131/11 provided by ZRU was used alongside as antibodies against wildtype SHUV had previously been detected in the sample using viral neutralization assay.

As can be seen in Figure 3.19, all horse sera showed reduced binding to mock antigen and coating buffer control, especially when compared to previous ELISAs shown in Figure 3.17 and Figure 3.18, however, non-specific binding was seen in wells containing plant produced SHUV N antigen. This was shown to be non-specific due to the binding seen against unrelated plant produced HPV 16 L1 antigen used to test the specificity of this binding.

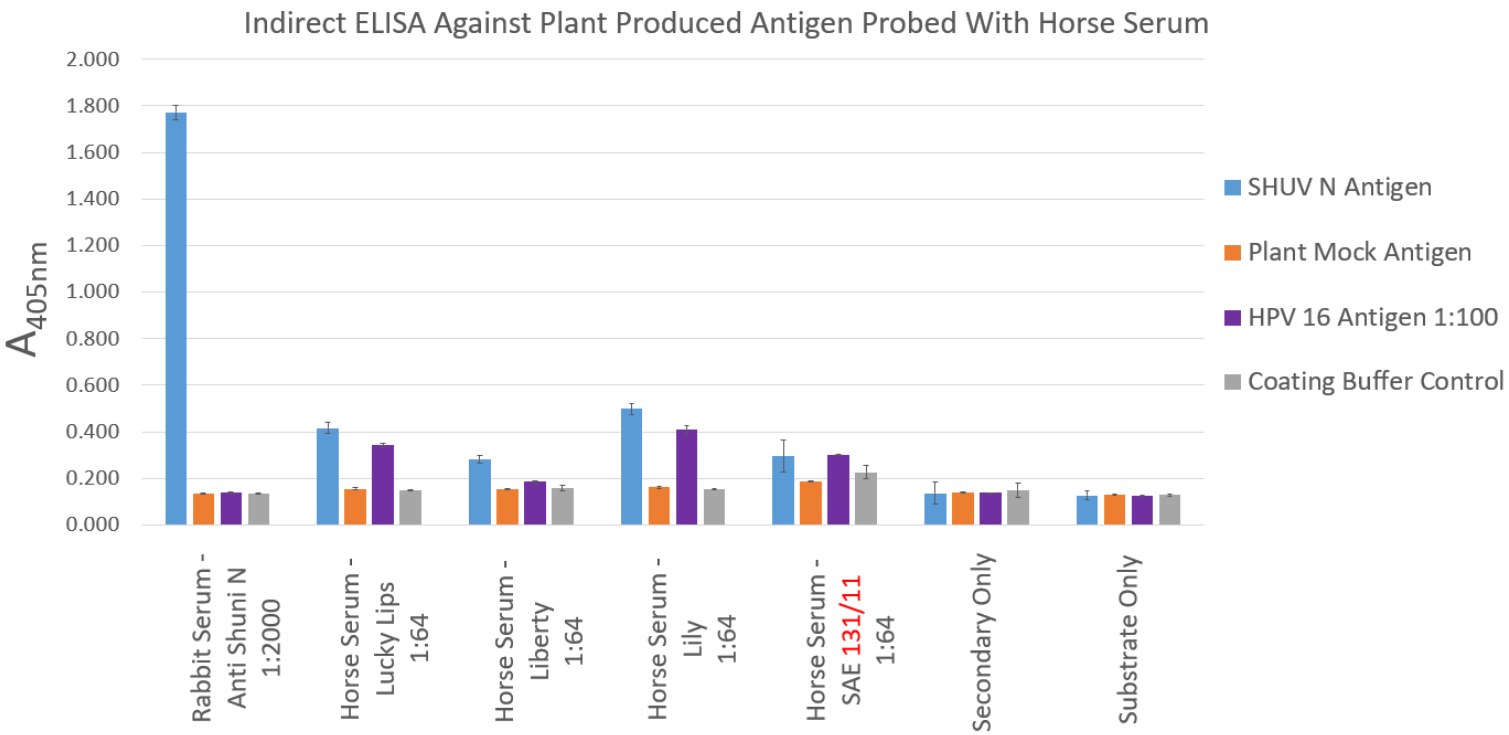


Figure 3.19: Indirect ELISA against plant produced antigen probed with horse serum, post optimization.

Indirect ELISA results showing four different horse sera against the specified antigens at a sera dilution of **1:64**. Sample SAE 131/11 is a known positive and has previously been tested to contain IgGs against SHUV infection. The remaining three have no known exposure to SHUV. Positive serum control is the probing with Anti-Shuni N. Secondary antibody and substrate only controls are seen. Plant control antigen is shown as HPV 16 Antigen at a 1:100 dilution. Mock antigen and wells containing only the coating buffer were used as negative control.

Across all sera, the A_{405} values for SHUV N antigen and HPV 16 antigen were roughly 0.100 in difference but significantly elevated from mock antigen and coating buffer control. The readings for mock antigen and coating buffer control showed comparable levels between the positive control, tested samples as well as secondary and substrate only controls. All sera had undergone pre-absorption against plant crude extract in order to reduce the number of plant binding antibodies in the sera.

3.4 Discussion

The ability to diagnose SHUV infection is limited and the literature on the virus itself, let alone diagnostics, is limited. Current methods of detection are limited to PCR assays that use Simbu group specific primers in order to monitor and evaluate sera samples (Golender et al., 2015, van Eeden et al., 2014a). Unfortunately, in-field monitoring of the virus through PCR is unfeasible due to the short-lived stage of viraemia for known bunyaviruses (Shepherd et al., 1989), the need for specialised equipment, expense, and the need for trained personal (Kuno et al., 1996). While immunologic assays are preferred, this is currently limited to viral neutralization assays which dangerously require the culturing of live virus in high biosafety facilities with specially trained personnel. Diagnostic indirect ELISA is a far simpler, more efficient and cost effect immunoassay method of diagnosing related bunyaviruses (Vanhomwegen et al., 2012). In the case of SHUV, the detection of IgG antibodies raised against SHUV infection and specifically the highly abundant N protein shown to be present in related bunyaviruses (Ariza et al., 2013).

Looking at the initial small-scale expression experiments, when probing with anti-6xHis, the pEAQ *HT* C-term SHUV N expression construct was shown to be expressing sufficient amounts of the SHUV N protein by 4dpi before plateauing until 7dpi. An *A. tumefaciens* OD_{600} of 0.5 was comparatively better as opposed to 0.25, however both showed a similar expression profile. This was not seen with either the pEAQ *HT* N-Term SHUV N or pTRAc HT SHUV N constructs, where no expression was observed for either.

Once the antisera against SHUV N was developed as described in Chapter 2, it allowed for reassessment of the previously stated constructs along with analysis of expression of the two constructs without a 6xHis affinity tag: pEAQ *HT* SHUV N and pRIC 4.0 SHUV N.

The pRIC 4.0 SHUV N construct showed sufficient expression 3dpi and continued and possibly increased expression into 7dpi. This was seen at both an agrobacterium culture OD_{600} of 0.25 and 0.5. Comparison between the CCHFV N and SHUV N protein expression through respective pRIC 3.0 HT and pRIC 4.0 expression vectors showed conflicting patterns. While CCHFV N peaked at 3dpi and began to gradually fall in expression in subsequent days, SHUV N did not (Atkinson, 2016).

The pEAQ *HT* SHUV N construct shows sufficient expression 3dpi but then progressively gets fainter towards 7dpi. This is seen when infiltrated at both a culture OD_{600} of 0.25 and 0.5. It may be possible that, without the 6xHis tag, the protein is not retained and is degraded *in planta.* However, the replicating construct, pRIC 4.0 SHUV N, while lacking a 6xHis tag, did not show this pattern. The expression profile seen for pEAQ *HT* SHUV N does not seem to be specific to the pEAQ *HT* vector used as the pEAQ *HT* C-term SHUV N construct does not suffer the same problem. Comparing this to the expression of the RVFV N protein (Mbewana et al., 2018) and CCHFV N protein (Atkinson, 2016), of which both the studies used the pEAQ *HT* vector, conflicting patterns of expression are seen. There was little discernible expression seen with the CCHFV pEAQ HT N protein expression construct while the RVFV pEAQ HT N protein construct showed an expression profile similar to that of pEAQ *HT* C-term SHUV N, with expression of the RVFV N protein peaking 4pi and plateauing (Atkinson, 2016, Mbewana 2017). Both were expressed in *N. benthamiana*.

When evaluated with the antisera to SHUV N from chapter 2, The pEAQ *HT* N-Term SHUV N continued to show no expression of SHUV N protein even after attempts had been made to amend extraction methods by increasing the amount of detergent in the extraction buffer. Similarly, the pTRAc HT SHUV N clone too showed no expression despite changes made to extraction buffer.

Both constructs showed no expression at either OD_{600} of agrobacterium thus it does not seem to be a factor of agrobacterium concentration. Had cleavage of the 6xHis tag taken place, the probing with anti-SHUV N antisera would have revealed cleaved or partial expression, should the epitopes been conserved. The same argument applies to the idea the 6xHis affinity tag may have been folded into the structure of the protein, thus 'hiding' the epitope from detection antibodies, or the idea that the 6xHis affinity tag was cloned out of frame. The ability to probe with anti-SHUV N antisera would reveal any expression and thus rules out both scenarios.

The fact that the infiltration cultures underwent PCR prior to infiltration and showed the correct product, rules out the idea that the expression construct was ejected from the bacterium. Thus it is assumed that the presence of the 6xHis tag targeted to the N terminus of the expressed protein has led to trouble in the plant expression of the SHUV N protein; specifically with the pEAQ *HT* N-Term SHUV N and pTRAc HT SHUV N constructs.

Looking at the literature around the effects of affinity tags, specifically 6xHis tags, have on the recombinantly expressed proteins show a changing opinion. Initially, 6xHis tags were used for not only their ability to act as an epitope while also allowing for downstream purification applications, but because they were inherently stable due to their small size (2.5kDa) and thus minimal effect on the structure and function of recombinant proteins (Graslund et al., 2008). However, this opinion is changing, as studies have shown that the effects that position and varying length of His tags do play a role in the expression and behaviour of a membrane protein (Mohanty and Wiener, 2004 , Booth et al., 2018). This being said, studies on the expression and structure of a zinc finger protein have showed that, while the choice of terminus (C or N) for 6xHis tag does have an effect on the structure and activity of the protein, expression levels are not noticeably affected (Zhao and Huang, 2016). This leads to conflicting ideas on whether expression levels may in fact be hindered or boosted by the position of such a tag. During the development of the plant expressed RVFV N protein, constructs that included a 6xHis tag on either the N or C terminus were shown to not express well, once again giving weight to the argument that affinity tags do in fact effect expression levels (Mbewana 2017).

The extensive multimerization of the SHUV N protein is seen in plant crude infiltrated with the pEAQ *HT* C-term SHUV N construct. Comparatively, the multimerization seen in blots probed with anti-6xHis is far fainter than blots probed with protein specific anti-SHUV N (Figure 3.6 and Figure 3.8 respectively). No multimerization is seen in plant crude infiltrated with the pEAQ *HT* SHUV N construct thus it may be possible that the expressed SHUV N proteins are multimerising via the 6xHis affinity tag on the C terminus of the protein. When viewing plant crude infiltrated with the pRIC 4.0 SHUV N construct, seen in Figure 3.9, no multimers are seen when infiltrated at an OD_{600} of 0.25 while a possible slight dimer band is seen in plant crude infiltrated at an OD_{600} of 0.5. This shows it is possible for multimerization to occur without the 6xHis affinity tag however it is thought the attachment of the affinity tag does sufficiently aid the formation of dimers and multimers. That being said, it may be possible that multimerization is purely a factor of protein concentration as the increased OD_{600} of the infiltration culture correlated with the increase

multimers. Should the data be needed, future work could entail the quantifying the crude expression for each of the respective expression constructs in order to assess if there is a correlation between the presence of the 6xHis tag and multimer formation.

Following this, the pEAQ HT C-term SHUV N construct was chosen to be the expression construct of choice due to the level of expression, time frame in which said expression peaks and the fact that the expression construct included a 6xHix on the C-terminus which aided in purification.

Having undergone both $(NH_4)_2SO_4$ precipitation and dialysis, the required fraction was initially tested with small-scale nickel affinity batch purification to assess the ability of the protein to bind to the nickel resin with sufficient affinity to allow for use of the FPLC AKTA Explorer 100 purification system. The presence of the expected bands suggests that the 6xHis affinity tag was available within the non-denatured SHUV N protein structure for binding and could bind with sufficient affinity for downstream purification. While the presence of the 6xHis tag on the protein had been confirmed earlier through western blotting with anti-6xHis antibodies, it was not assumed the affinity tag would be available on the non-denatured protein structure to allow for binding to the nickel affinity gel in its native conformation. Thus having tested the precipitated sample with the nickel affinity gel, there was confidence in the use of the FPLC AKTA Explorer 100 system and HisTrap Column setup for purification

Looking at the feasibility of this study for future production. The initial large-scale infiltration was conducted on 35x *N. benthamiana* plants. This led to a total biomass of 225.3g, specifically fresh leaf material harvested 4dpi. After undergoing extraction, precipitation and affinity purification, a total of 8 AKTA fractions, of 5ml each, were pooled (40ml) and quantified to give a SHUV N concentration of 56.2µg/ml and thus a total of 2.248mg of SHUV N protein from 35 plants. This gives a ratio of 9.9 mg/kg of SHUV N protein to raw plant material. These yields are far lower than the expression of RVFV N protein, which gave yields of 558 mg/kg of fresh leaf material (Mbewana 2017). However, the expression of CCHFV N protein gave much closer yields, having undergone a similar affinity based purification, of roughly 1.6mg/kg plant material (Atkinson, 2016) while the expression of unrelated proteins, such as the PCV-2 CP, gave a similar yield of 2mg/kg plant material (Gunter, 2017).

While the yield of this study may seem low in comparison to RVFV N protein (Mbewana 2017), the yield was shown to be sufficient for the purpose of diagnostic ELISAs. Considering the design and layout of a 96 well diagnostic ELISA, should each well require 100ng of purified plant produced SHUV N protein as an antigen. A single 96 well plate requires 9.6 µg of purified protein, which equates to a possible 234 plates that may be coated with antigen derived from expression in 35x *N. benthamiana* plants.

The comparison of expression systems in this study show various pros and cons for both in terms of the expression of the SHUV N protein. In terms of yield for the required biomass, the *E. coli* based expression of the target protein far outweighs the plant based with a yield of 5.1174 mg in direct comparison to a yield of 2.248 mg for each system respectively. While the *E. coli* system showed a far greater yield and is easily expressible, it suffers disadvantages previously shown in literature. Recombinant proteins produced in *E. coli* often suffer from being incorrectly folded, which is important in the design of diagnostic antigens and potential vaccines targeting humans and livestock. Secondly, *E. coli* produced proteins are often insoluble and aggregate, both of which was observed in this study (Larrick et al., 2001, Mbewana et al., 2018).

This leads to the inefficiency, sensitivity and overall utility for diagnostic ELISAs. Thus it is highly preferred to use a eukaryotic expression host , such as plants, that facilitate proper folding and post-translationally modification of the recombinant protein to allow for the correct antigenicity for diagnostic assays such as the one developed in this study (Warner, 2000).

Initial tests against positive sera showed severe problems such as a large amount of non-specific binding and thus a large background signals being seen in the assay across antigen, mock antigen, and coating buffer control. While access to test sera and volumes of sera provided by the ZRU (Table 9) was limited; every effort was made to ensure the assay could be improved upon and thus the ELISA protocol was optimised. Horse sera obtained as part of an AHSV study performed by the BRU (Table 10) was used to optimise the protocol due to the abundance of this serum. Following these results, improvements were made regarding various wash steps, volumes and buffer makeup.

In order to reduce the possibility of antibodies raised against plant protein, intrinsic to animal sera, from binding to our plant produce antigens; each serum was pre-absorbed against plant extract to reduce the titre of these antibodies. While these improvements to the ELISA protocol significantly reduced the amount of background signal observed to an acceptable level, assumed non-specific binding of antibodies in the sera was still seen as shown in Figure 3.19.

This was further explored, as seen in Figure 3.19 by testing antibodies against both the plant produced recombinant proteins, the SHUV N protein and unrelated plant produced HPV 16 L1 protein. Once again, non-specific binding was seen across all serum samples, both those listed in Table 9 and Table 10. Thus, this is thought to be intrinsic to the antibodies found in general in horse sera and not those because of SHUV infection. The fact that both unrelated proteins that were used as antigens were expressed within plants indicates that a more extensive pre-absorption treatment may help reduce this non-specific binding as it is quite possible that significant levels of plant binding antibodies remain behind in the sera after pre-absorption or possibly other non-specific antibodies that recognise epitopes on the coated antigens. Unfortunately, the concentration of the HPV 16 L1 sample used was not known and purified in different buffers with a different purification method. Thus, the comparison of these two plant produce proteins is limited but was designed to give insight into the possible traits that contribute to the extensive background signal seen.

Comparatively, plant produced CCHFV N protein was used as an ELISA antigen and screened against a panel of serum that was shown to test positive for CCHFV antibodies (Atkinson et al., 2016). This allowed for assessment of the protein's antigenicity. The results showed that the positive panel had ELISA readings that were 8 – 10-fold higher between the antigen and mock antigen. Comparing the positive to the negative panel showed that patients with CCHFV positive sera showed ELISA readings roughly 4 – 10 fold higher than the negative panel when tested against the plant produced CCHFV N protein antigen (Atkinson et al., 2016, Atkinson, 2016). This could be seen as a model for the expected signals should the future optimisation and troubleshooting of SHUV diagnostic ELISA work in reducing non-specific binding and thus reduce background signal.

Taking this diagnostic ELISA forward, various factors could be improved upon in order to fully optimise this assay. While changes to the wash steps could be improved upon, such as increased washes and increased Tween 20 concentration, previous optimization already made improvements in this area. Further increases in these areas may yield little improvement or enact negative change on readings by reducing the number of specific antibodies bound or the amount of antigen coating each of the wells.

In order to assess the antigenicity of the plant expressed SHUV N protein, the mouse ascites fluid, raised against live wildtype SHUV, was used to probe both crude extract and purified SHUV N (Figure 3.15). This showed the ability to bind to the SHUV N in both the crude plant extract and purified sample and thus showed promise in terms of specificity of the plant expressed protein to recognised by antibodies generated through native viral infection in the horse sera samples.

This showed similar results in the initial ELISA described in Figure 3.16. The specificity of the antigen against the mouse ascites fluid infers that native antibodies to SHUV infection found in infected horse sera should show similar binding, thus showing promise for the diagnostic ELISA.

This implies that when looking at the ELISA results in Figure 3.19 that the inability to discern specific antibody binding to the antigen in known infected horse sera samples may not be due to the antibodies not recognising the antigen but rather the masking effect of non-specific binding hiding the potential absorbance signal. While the results in Figure 3.16 demonstrate that the required epitopes should be present on the antigen, it is possible that incorrect folding of the protein or slight changes in the sequence may reduce the number of epitope sites on the protein thus limiting the signal seen in the ELISA results shown in Figure 3.19. It must also be said that while the mouse ascites fluid was raised against SHUV infected cell extract, this most likely led to an enormous antibody titre that is not comparable with antibody titres found in the natural sera of infected hosts. In which case may exaggerate the signal expected from a diagnostic ELISA. As antibody titre data was not available for the infected sera used, it is unable to be confirmed whether the antibody titre level was below the possible detection limit of the assay. Whether this in combination with the masking effect played by the non-specific binding, was responsible for the lack of credible signal seen in positive sera, is still to be determined. In order to help resolve this, non-specific binding to the antigen would need to be reduced drastically.

It is possible that improvements such as the use of enhancer solutions may improve the sensitivity of ELISA instead of conventional buffer, however this also created unwanted enhancement of background signals (Takai et al., 2009). Possibilities lie in expanding the current set of blocking buffers from a milk and BSA based blocking system, to commercial buffers such as Superblock, BlockAce and Immunoblock, which may help reduce background by reducing nonspecific binding (Takai et al., 2009, Craig et al., 1994).

The limited volumes and access to infected sera played a significant hindering role in the development of this diagnostic reagent. Had more sera been available, extensive optimisation to reduce non-specific binding would have been performed. A larger sample size of positive sera samples would have allowed for better assessment of specific binding to the antigens post ELISA optimisation and would have helped give insight into the cause of the non-specific binding observed.

The initial construction and alignment of the SHUV N gene reported in section 2.2.1. could be a factor in my results. Due to the unavailability of complete sequences from SHUV isolates, the majority of sequences used (4/8) were derived from SHUV infections in Northern Israel as specified in Table 1. While the Israel isolate sequences show minimal differences to those isolated from both Nigeria and South Africa, it was thought that the overall dominance of the Israeli isolates may have played a role the in the final consensus sequence being skewed towards the Israeli strains, and that this slight difference may have influenced protein structure - and thus the antibodies in sera from horses infected in South Africa might not recognise the recombinant protein. Looking at this, the South African isolate showed a 683/702 (97%) similarity when comparing the nucleotide sequence to the consensus sequence used. The Israeli' sequences showed between a 701/702 (99%) and 698/702 (99%) nucleotide similarity when they were similarly compared to the consensus sequence, thus reinforcing the idea that the consensus sequence is skewed towards Israeli strains.

However, the alignment of the eight sequences showed that while some slight nucleotide variation was seen, this had no effect on the translated amino acid sequence.

While the recombinant nucleocapsid protein was constructed with available sequences, it is possible that the South African wildtype SHUV that infected the horses whose sera were used in this study does not share similar epitopes on the nucleocapsid protein due to variation in its own gene sequences.

Once again this is difficult to prove, as there is no genomic data on the virus involved in the infected sera - and looking at the experimental ELISA data in section 3.3.8, the high level of non-specific background signal produced in the assay shown may be masking an effective binding on top of this.

Following this a western blot performed using the infected positive sera provided by ZRU to probe SHUV N plant crude, purified SHUV N and empty plant crude showed absolutely no binding and thus was not included in results. This may be due to antibody titres being below the detectable limit as both secondary and conjugate controls were shown to work (data not shown).

Chaper 4: Conclusion

To conclude, an overall hindering factor of this research project was the limited access to known positive sera. This mired the development of the diagnostic assay in many ways, such as optimization of the protocol and the ability to assess whether known positive samples were able to bind to the recombinant plant-expressed SHUV N protein. The lack of data on the sera such as antibody titres meant I could not rule out various factors that may have affected the outcome. Therefore, troubleshooting and optimisation could not be performed to any great extent. It is possible that antibody titres in the supposedly "known positive" sera were below the detectable limit due to the immune-response against SHUV nucleocapsid protein inherently being much lower than shown with the bunyavirus CCHFV (Marriott et al., 1994). If the intrinsic antibody response to the virus' N protein was lower than thought, the sensitivity of the diagnostic ELISA would need be increased to allow for the signal to be discernible amongst the non-specific binding seen earlier.

Notwithstanding this negative aspect, however, various objectives were achieved through this study. As Chapter 2 described, a specific anti-SHUV N polyclonal antibody was created through the successful *E. coli* expression of the SHUV N protein, and animal immunisation led to the raising of antibodies to said protein. The antibodies were used within this study to screen plant crude extracts, assess antigenicity and act as a positive control in diagnostic ELISAs. Seeing as currently there are no SHUV antibodies available commercially, and the literature does describe the development of any, the creation of this antiserum could be very useful for future SHUV studies. It can be used as a reagent in immunogenicity-based assays such as immunofluorescence for cell line assessment, future ELISAs and for its ability to assess recombinant expression of viral N protein. Thus, it could make a significant contribution to SHUV research and research materials.

Furthermore, this study described the successful expression of the SHUV N protein in *N. benthamiana* and the setup of a purification method based on nickel affinity-based chromatography. This led to the creation of purified, plant expressed, SHUV N antigen for use as a diagnostic reagent. While initial testing was hindered by restricted serum samples and high levels of background, antigenicity was assessed through the probing of mouse ascites fluid raised against live virus, which showed positive results. The diagnostic assay shows great promise and as new cases of SHUV infection are identified, assessed and the sera and data are made available, will allow for further optimisation of the diagnostic reagent and assay, help reduce background levels of non-specific binding and lead to the creation of a successful SHUV diagnostic kit.

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