The Generation of Double Transgenic Cell Lines for the Production and Regulated Expression of the Nipah Virus Nucleocapsid Protein:

A Technical Approach that will Facilitate Further Investigations into how the Over-expression of the Nipah Virus Nucleocapsid Protein Blocks Viral Replication

Bonnie Solylo

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Medical Microbiology
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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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MASTER OF SCIENCE

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Abbreviations

Ψ^+	retroviral packaging signal
°C	degrees celcius
µ	micro
A	amperage
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
BME	beta-mercaptoethanol
BSA	bovine serum albumin
BSL4	Biosafety Level 4
C	C protein
C-terminus	carboxy terminus
$CaCl_2$	calcium chloride
CDC	Center for Disease Control and Prevention
cm	centimeters
CMV	Cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
CRFK	Crandell-Rees feline kidney
dd	double distilled
DMEM	Dulbecco's Modified Eagles medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotides (dATP, dCTP, dGTP and dTTP)
dox	doxycycline
EDTA	ethylenediaminne tetraacetic acid
Env	envelope gene (retroviral envelope)
EtBr	ethidium bromide
F	fusion protein
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
g	grams
G	glycoprotein
G418	neomycin
Gag	group antigen gene (retroviral core structural proteins)
GFP	green fluorescent protein
GLB	gel loading buffer
HA	hemagglutinin
HCl	hydrogen chloride
HEK	human embryonic kidney
HEPA	high efficiency particulate air
HeV	Hendra virus

HRP	horse radish peroxidase
IDV	integrated density value
IFA	immunofluorescence assay
IgG	immunoglobulin G
in. Hg.	inches of mercury
IPTG	isopropyl-beta-D-thiogalactopyranoside
JE	Japanese encephalitis
kb	kilobases
kDa	kiloDalton
l	liters
L	large protein (polymerase)
LB	Luria-Bertani
log	logarithm
m	meters; milli
M	matrix protein; molar
$MgCl_2$	magesium chloride
min	minutes
MLV	Moloney Murine Leukemia virus
MOI	multiplicity of infection
M_r	molecular mass
n	nano
N	nucleoprotein
N-terminus	amino terminus
NaCl	sodium chloride
Neo_r	neomycin resistance
NiV	Nipah virus
NP40	nonidet-P40
OD	optical density
oligo	oligonucleotide
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	potential hydrogen
P_{minCMV}	minimal immediate early promoter of cytomegalovirus
pol	polymerase gene (retroviral reverse transcriptase and integrase)
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
rtetR	reverse tetracycline repressor
RT-PCR	reverse trascriptase polymerase chain reaction
rtTA	retroviral regulatory factor
SDS	sodium dodecyl sulfate
TAE	tris acetic acid + ethylenediaminetetra acetic acid

$TCID_{50}$	50% tissue culture infective dose
TE	Tris-Ethylenediamine Tetraacetic Acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
tet	tetracycline
TRE	tetracycline response element
Tris	trishydroxymethylaminomethane
TSS	transformation and storage solution
U	units
UV	ultaviolet
V	V protein; volts
VP16	virion phosphoprotein 16 (Herpes Simplex)
VSV	Vesicular Stomatitis virus
w/v	weight to volume
x g	x gravity (relative centrifugal force)
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

ABSTRACT

Nipah virus (NiV) is a Biosafety Level 4 paramyxovirus that causes fatal encephalitis in up to 70% of reported human cases. In view of the fact that NiV recently emerged in 1998 and studies to unveil the pathogenesis of this virus are scarce, it is not surprising that there are no licensed vaccines or treatments currently available. However, recent preliminary transfection studies have shown that NiV replication and the production of *de novo* progeny virus is suppressed more than 10,000 fold in the presence of over-expressed viral nucleocapsid (N) protein.

To facilitate further investigations and reveal the mechanisms behind how increased levels of the NiV N protein blocks viral replication, double transgenic cell lines were generated where one transgene regulates the expression of the NiV N protein located on a second transgene. The creation of these stable cell lines that express recombinant NiV gene products is a novel technical approach for studying NiV biology.

This study demonstrates that when using the RevTet-On system to generate stable cell lines, it is best to select and screen for homologous populations to achieve optimal expression of the recombinant NiV N protein, rather than polyclonal populations where expression among cells were few and far between.

This study also demonstrates that when using the RevTet-On system to generate double transgenic cell lines, the "stably" integrated NiV N transgene was silenced or lost after several passages, suggesting that this type of transgenic technology does not give indefinite long-term expression of the recombinant viral

protein of interest. This system must therefore be used with caution, especially when utilized to identify the mechanisms behind N's ability, when over-expressed, to block viral replication.

CHAPTER 1: Introduction

I.I: Nipah Virus

I.I.I: Taxonomy. Originally considered a Morbillivirus, Nipah virus, along with the closely related Hendra virus, have recently been classified under the newly generated genus *Henipavirus* within the order *Mononegavirales*, family *Paramyxoviridae*, and subfamily *Paramyxovirinae* (96, 81). Even though NiV and HeV are genetically and biologically similar to other paramyxoviruses, there are several features that distinguish them from other members of the subfamily. Genetically, they contain unique genus-specific 3' leader and 5' trailer sequences, variations in the conserved catalytic site of the transcriptase protein, a 15% longer genome, a novel P/V/C gene and larger phosphoproteins than other members of the subfamily *Paramyxovirinae* (139, 57, 141, 56, 140). Biologically, they are different in the sense that they have a broad host range both *in vivo* and *in vitro*, are only zoonotic and highly pathogenic, and demonstrate cross-reactivity only with each other and not with any of the other paramyxoviruses (20, 27).

I.I.II: Epidemiology. Nipah virus (NiV), is a newly emergent paramyxovirus that was first discovered and identified in 1999 as the causal agent of an outbreak of encephalitis in peninsular Malaysia (20, 21). The name "Nipah" was adopted from the location where it was detected in Malaysia, the village of Sungai Nipah. Before precise identification of the virus in 1999, Malaysian health authorities mistook the

etiologic agent with Japanese encephalitis (JE) which is endemic to the region and clinically similar to NiV infections in humans. Nonetheless, epidemiological factors of the outbreak did not correspond with JE and further investigations of the outbreak, headed by a team from CDC in Atlanta, identified the culprit to be a new "Hendra-like" virus known as Nipah virus (37).

Investigations of the Malaysian outbreak in Perak, Negeri Sembilan, and Selangor from September 1998 to May 1999 revealed that 105 people, out of the 256 infected with Nipah virus, died (20, 21, 29, 135). Consequential of importing infected swine into Singapore from Malaysia, one of 11 abattoir workers infected with Nipah virus, died (20, 21, 106). Containment of the outbreak was accomplished by culling over one million swine (21, 27). Since the Malaysian outbreak, several more recognized outbreaks of Nipah virus, reaching mortality rates of up to 75%, were reported in 2001, 2003, 2004 and 2005 in Bangladesh (22, 65, 2, 1). Most recently, in neighbouring parts of India, an outbreak of Nipah virus is ongoing (71).

Based on seroprevalence data and virus isolations, the primary natural reservoir of Nipah virus are Old World fruit bats including *Pteropus vampyrus* (Malayan flying fox), *Pteropus hypomelanus* (Island flying fox), and *Pteropus giganteus* (Indian flying fox) (156, 102, 30, 121). Moreover, geographical locations of Nipah virus outbreaks correlate with *Pteropus* species habitats. Nipah virus has also been shown to infect humans and pigs and be seropositive in dogs, cats, horses, and goats (27, 63,

88, 82). Experimental hosts of Nipah virus include golden hamsters and felines (94, 148).

During the Malaysian outbreak, 93% of infected individuals had occupational exposure to NiV infected swine, signifying the role of swine intermediates during human transmission (105, 24, 27, 45, 132). The transmission of NiV from fruit bats to swine to humans is thought to result from the increasing overlap between bat habitats and pig farms. For example many Malaysian piggeries have nectar-bearing trees where infected bats can transmit the virus through partially eaten fruit, urine and faeces to swine (28, 36). Once established among swine herds the virus would have ample time to adapt to human abattoir hosts. Alternatively, epidemiological studies of the Bangladesh outbreaks indicate the absence of a swine intermediate during human transmissions indicating direct transmission from the natural fruit bat host (65). Humans are likely contracting the virus directly from fruit bats due to the increasing movement of humans into bat territory such as pig farms and the increasing levels of bats habituating areas of agriculture and human dwellings. Fruit bats are known to drink from the same clay pots humans use to collect date palm juice at night (1). Furthermore, investigations of the 2004 outbreak in Faridpur district in Bangladesh indicate for the first time the possibility of human-to-human transmission of the virus by close contact with infected individuals (3, 2).

I.I.III: Pathogenesis. Pathologically, NiV infections present mainly as a systemic, multi-organ vasculitis, particularity in the CNS and lung, caused by endothelial damage including syncytial giant cell formation, thrombosis and necrosis. (149, 82). Another histopathological feature characteristic of Nipah virus infections are the presence of viral inclusions in the brain (149). It has been shown that the increase in frequency of these pathological features is concomitant with the rise of viral antigen in the CNS (149). Furthermore, viral loads are predominantly high in endothelial cells and neurons and are most likely the reason why the vasculitis observed in the CNS is the most severe (149).

I.I.IV: Clinical Manifestations. Symptomatic Nipah virus infections of humans include highly fatal acute febrile encephalitis and to a lesser extent, 25% of cases, in addition to the neurological distress, exhibit acute respiratory illness (29, 45, 150). Incubation periods can range between a very fast onset of two weeks, which the majority of patients experience, to two months (150). Infected swine display signs of neurological illness, severe respiratory illness characterized by a harsh non-productive cough and low mortality rates (20, 89).

I.I.V: Treatment and Safety. Besides intensive supportive care, there are currently no effective treatments, drug therapies or vaccines available to prevent or cure Nipah virus infections. However, preliminary studies have shown that the antiviral drug Ribavirin may reduce mortality among patients with encephalitis caused by Nipah

virus (26, 146). Further investigation into the efficacy of Ribavirin to cure NiV infections is necessary.

As a result of the lack of effective treatment and vaccine, and high fatality rates, Nipah virus must be handled under Biosafety Level 4 (BSL4) containment. In addition to the latter, the availability, and ease of production and dissemination are reasons why NiV may be considered for use during a bioterrorism attack. Thus, NiV has been listed as a critical Category C biological agent by The Centers for Disease Control and Prevention (19).

Conducting experiments with NiV is restricted due to the constraints of having a Level 4 lab. Nipah virus is capable of growing to high titres in a range of cultured tissues from diverse species (13). Following infection, cells will fuse, forming giant multinucleated cells known as syncytia, a pathogenic hallmark of the paramyxovirus CPE (46).

I.II: Molecular Characteristics of Nipah Virus

I.II.I: Viral genome. Nipah virus has a helical, non-segmented, single stranded RNA genome of negative polarity. Complete sequencing of the entire genome (18,246 - 18, 252 nucleotides) has revealed a closely related genetic arrangement to members of the genera *Respirovirus* and *Morbillivirus* (25, 55). The genome consists of six transcriptional units that each contain their own conserved

untranslated transcriptional gene start and stop control sequences (reviewed in 139 and 75). These six transcripts, that are separated by intergenic regions, encode for six major structural proteins, the nucleocapsid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and polymerase (L) (figure 1). Like all the other genes, the gene encoding for the P protein transcribes a single mRNA, however due to the multiple ORFs within the gene, three proteins are encoded for rather than just one, the phosphoprotein, the V protein and the C protein (57). Located at the genomic termini are two highly conserved extracistronic regions known as the 3' leader and the 5' trailer (56). It is believed that the leader sequence contains the promoter for transcription of positive-sense RNA while the trailer complement sequence contains the promoter responsible for the synthesis of the negative-strand RNA during viral replication (53). These terminal sequences may also contain regions specific for initiating encapsidation (10).

I.II.II: Virus Structure. Even though filamentous forms of NiV can be observed, the virus is generally spherical in shape ranging in size from 40 to 600 nm in diameter (66). Nipah virus has a structure that is typically found within the family *Paramyxoviridae* and is reviewed by Lamb and Parks (75). Briefly, Nipah virus contains an envelope which is acquired from the plasma membrane of the host cell and is embedded with integral glycoproteins F and G. Beneath the lipid membrane lays a shell of matrix proteins which associates with the membrane and is important in virion architecture.

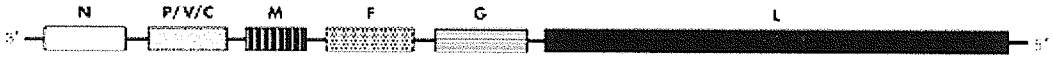


Figure 1: Nipah virus genetic map. The gene abbreviations and proteins they encode for are as follows: N – nucleoprotein, P/V/C – phosphoprotein/V protein/C protein, M – matrix protein, F – fusion protein, G – glycoprotein, L – large protein (RNA polymerase)

This figure can be found in the 5th Ed. of Fields virology by Lamb and Parks (75).

Contained within the envelope are all the components imperative for initiating intracellular virus replication including an N encapsidated RNA genome and structural proteins P and L.

I.II.III: Nucleocapsid Protein. The NiV N gene of 2242 nucleotides contains a 1599 nt ORF that encodes for the N protein (139, 57). This protein has a calculated molecular mass (M_r) of approximately 58 kDa (139). The N-terminal domain of the N protein is highly conserved while the C-terminal domain is not (57).

Like in all paramyxoviruses, the N gene of NiV is the first to be transcribed and is therefore the most abundant protein in the purified virion. The N protein is an RNA-binding protein that encapsidates the RNA genome to form the helical nucleocapsid template, the only biologically active form of these viral RNAs. Encapsidation is also thought to protect the viral genome from nuclease digestion, aid in the alignment of distal RNA segments to generate a functional 3' promoter, and provide interaction sites for assembly of progeny nucleocapsids into budding virions (75). Assembly of the helical nucleocapsids first involves associating free N subunits with either the viral RNA anti-genome or genome followed by the association of the P-L protein complex. It has also been noted that N proteins, in the absence of other viral components, have the ability to self-assemble into herringbone nucleocapsid-like structures indicating that N-N protein interactions are required to drive nucleocapsid assembly (133). In addition to encapsidation, the N protein also forms part of the replicase complex necessary for viral replication (113, 7, 10, 50, 107).

I.II.IV: Virus Life Cycle. Nipah virus replication is thought to be similar to that of other paramyxoviruses and is reviewed by Lamb and Parks, 2007 (75). The NiV life cycle begins with the cytoplasmic entry into mammalian host cells. Recognition of the virus to the host cell is accomplished by having the viral G surface protein bind cell surface ligands ephrin-B2, or alternative receptor ephrin-B3 (11, 99, 100). Ephrin-B2 is expressed on many cell types including endothelial cells, neurons, and smooth muscle cells, while ephrin-B3 is found less extensively on certain neuronal subsets and lymphoid cells (78). The high prevalence of these receptors among different species helps to explain the broad host range of Nipah virus (99). Subsequently, viral penetration is accomplished by the F protein fusing the virion envelope to the host cell membrane. Cytoplasmic entry is followed by the release of the M protein and uncoating of the nucleocapsid core permitting the release of the negative sense RNA genome.

Following viral entry and uncoating, all six genes found on the NiV genome are transcribed into six separate capped and polyadenylated transcripts for further translation into their respective proteins. This is believed to be facilitated by a transcriptase complex comprised of P-L and various cellular factors (34, 41, 42, 113). A gradient of transcripts is generated since L's ability to re-initiate transcription at each of the gene junctions decreases as it gets further downstream from the 3' end. Therefore, the structural protein expressed at the highest levels, because of its positioning at the utmost 3' end of the genome, is the nucleocapsid (N) protein. In addition, it is believed that a replicase complex composed of N-P-L and possible

cellular factors are responsible for mediating production of anti-genomic and genomic viral RNA for replication (113, 7, 10, 50, 107). The replication of NiV is known to comply with the "rule of six" which stipulates that the RNA polymerase efficiently replicates viral genomes whose lengths are in multiples of 6 nucleotides (53). This can be made possible if the template for trascription and replication are nucleotides in which each nucleoprotein subunit associates with six nucleotides of genomic RNA.

The newly generated viral genomes and proteins come together followed by the release of progeny virions from the plasma membrane by the budding process.

I.III: Transgenic Technology

I.III.I: Transgenic techniques. Transgenic technology, where genetic elements are introduced into cultured mammalian cells, has long been explored in the biomedical and pharmaceutical research fields (116). Transgenics can be achieved using various transfection methods including Calcium Phosphate co-precipitation, electroporation, heat shock, magnetofection, particle bombardment, cationic lipids and polymers, or direct transfer using a gene gun (127, 6, 40). These methods can all introduce recombinant DNA transiently into adherent cells. While transient transfections are advantageous for rapid analysis of genes, they may give inconsistent results since the gene introduced can be lost from the cell at any time (116). Stable transfections on the other hand, where the gene of interest is integrated into the target cell's chromosome, provide a means of gene expression that is more long-term,

reproducible and well-defined. Major applications for stable transfections include the analysis of gene function and regulation, large scale protein production, drug discovery, and gene therapy (116).

I.III.II: Mechanism of Stable Integration. Stable expression is achieved by first introducing the gene of interest into the target cell nucleus followed by integration into the chromosomal DNA. The two factors influencing stable expression are the transfection method used and the vector containing the gene of interest.

a) Transfection methods. The transfection technique used is influenced by the cell lines targeted for stable chromosomal integration. For instance, transfection and viral delivery mechanisms are the only two methods that allow efficient delivery of DNA into reputably hard-to-transfect suspension cells and primary cell lines. Viral systems are capable of integrating foreign DNA molecules into the host chromosome using viral integration mechanisms (52) while transfection introduces DNA molecules into the host cell chromosome using the cell's machinery, perhaps using DNA repair and recombination enzymes (51).

b) Vectors. Regulation of transgene expression and the selection conditions for stably expressing cells are both influenced by the type of vector used for stable integration. The promoter cloned upstream of the expression vector and the integration site influence the level and time of expression of the gene of interest (151). For instance, CMV and Simian Virus 40 promoters are well characterized and

permit high constitutive expression while inducible promoter systems are also available and allow for more regulated gene expression (80). Selection of the stably transfected cells is accomplished by the co-expression of antibiotic resistant genes such as neomycin phosphotransferase which confers resistance to neomycin (G418) (151, 116). Either bulk heterologous populations of resistant cells can be collected where the transgene is integrated at various positions throughout the target cell genome or clonal homologous populations can be picked which have a single integration event.

I.III.III: Viral Systems and Transgenic Cell Lines. Currently, retroviral vectors are the most commonly used method for gene delivery and stable integration into eukaryotic cells (4, 33, 62, 83, 93). The oncoretrovirus, Moloney murine leukemia virus (MLV) and its ability to stably integrate genetic material into the actively dividing host cell chromosome is what the majority of the retroviral systems are based on (32, 86). The system works by first packaging the retroviral vector, carrying the gene of interest, into retroviral particles. Following infection of the target cell with the retrovirus, the vector along with the gene of interest is permanently integrated into the host chromosome. In the end, transgenic cell lines are generated which stably express the gene of interest.

More specifically, retroviral particles are packaged by co-expressing MLV-based retroviral vectors with structural MLV genes *gag* (core structural proteins), *pol* (reverse transcriptase and integrase), and *env* (coat glycoproteins). These structural

genes can be provided in *trans* by either transient transfection or packaging cell lines which constitutively express the genes (84, 85, 131). The viral *Env* gene determines the cellular host range of the packaged virus through the recognition of specific cellular receptors. To achieve a virus that is capable of infecting a broader host range of mammalian cells, modifications can be made where *Env* is replaced with vesicular stomatitis virus glycoprotein (VSV-G) (17, 76, 155). Additionally, the VSV-G protein more stably associates with virions than retroviral envelope proteins allowing a higher-titer of packaged retrovirus to be generated (155). In the end, infectious retroviral particles that consist of the retroviral vector carrying a gene of interest and the retroviral structural gene products are generated. However, the retroviral structural genes themselves are absent from the generated virus, thus preventing replication. Also, to obtain a high titer of the virus, retroviral packaging signals such as Ψ^+ are included on the retroviral vector. The overall packaging process is depicted in figure 2.

These newly generated high-titer replication-incompetent viruses are then harvested and used to infect cultured mammalian cells. Cell division during infection is essential to allow the pre-integration complex to migrate to the nucleus and gain access to the host cell genome (87).

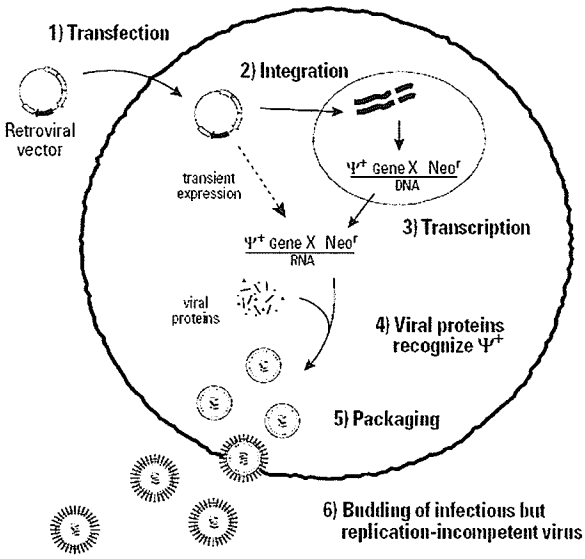


Figure 2: Packaging of retroviral particles. These viruses are packaged by transfecting the retroviral vector, containing the gene of interest, antibiotic selection marker (ie. Neo_r) and packaging signal (Ψ^+), into a cell expressing the genes necessary for particle formation (ie. *gag*, *pol* and *env*). The retroviral vector is either transiently expressed or stably integrated into the host cell. The packaging signal on the retroviral vector is then recognized by the viral proteins then packaged and released as infectious, replication-incompetent retroviral particles.

This figure can be found in the 2004 RevTet System User Manual provided by Clontech (31)

Stable integration of the retroviral vector can be selected for by the antibiotic selection markers that are included on the retroviral vector. In the end, a new transgenic cell line expressing the gene of interest is created.

Stable cell lines can either be monoclonal or polyclonal. Monoclonal or homologous cells are populations of stable cells that have a common integration site since they originate from one transduced cell. Alternatively, polyclonal or heterologous cells are populations of stable cells where the integration events in each cell can be in a different location of the chromosome.

Other common viral systems used for generating transgenic cell lines include lentiviral vectors and adenoviral vectors (reviewed in 157 and 35). While lentiviral systems use similar mechanisms of gene delivery compared to that used with the retroviral system, adenoviral systems do not. Both lentiviral and adenoviral systems are capable of transducing non-proliferating cells including neuronal cells, drug- or growth- arrested cells or even primary cells that have traditionally been extremely resistant to transduction. Furthermore, adenoviral vectors, unlike lentiviral and retroviral vectors, remain episomal and do not integrate into the host cell genome. This results in unstable transient transgene expression and is further limited by cellular and humoral immune responses against "leaky" expression of wild type adenovirus gene products in the transduced cells (153, 152).

I.III.IV: Retroviral-Mediated Tet-Regulation: BD RevTet-On™ System. The BD RevTet-On™ System combines the advantages of retrovirus-mediated gene delivery and stable integration with tetracycline-regulated control of gene expression (reviewed in 31). Two MLV derived plasmids are involved in this system—pRevTet-On, a regulatory plasmid and pRevTRE, a response plasmid (Appendix C). Briefly, expression of the gene of interest, which is cloned into the retroviral response plasmid, is controlled by *trans*-acting factors provided by the regulatory plasmid. The retroviral regulatory factor rtTA that is provided in *trans* consists of the reverse Tet repressor protein, rTetR, and VP16. The rTetR protein normally works to block transcription by binding tet operator sequences in the presence of tetracycline (tet) or the tetracycline derivative doxycycline (dox). Addition of VP16 converts the rTetR from a transcriptional repressor to a transcriptional activator. Therefore, in the presence of tet or dox, rtTA is capable of binding the tetracycline responsive-element (TRE) allowing subsequent transcription from the minimal immediate early promoter of cytomegalovirus (P_{minCMV}) (figure 3). The enhancer from the CMV promoter has been removed to ensure silencing of the gene of interest in the absence of a bound Tet-controlled transactivator. Ultimately, this allows for expression of the gene of interest in a dose-dependent manner.

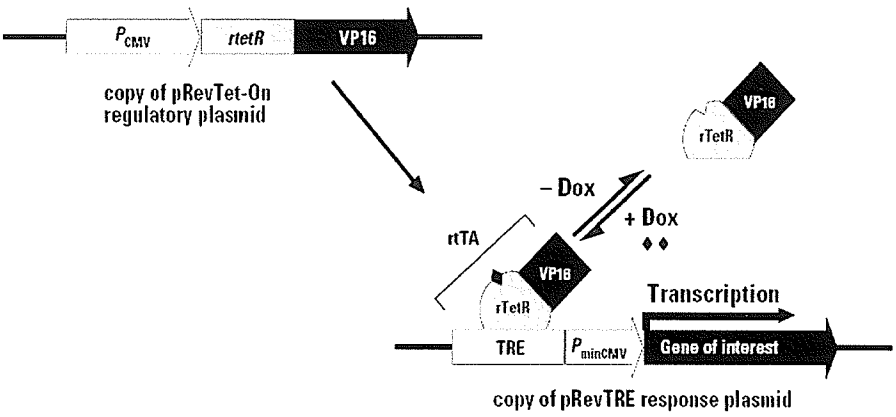


Figure 3: Mechanism of BD RevTet-On™ gene expression. The tetracycline-responsive element (TRE) is located upstream of the P_{minCMV}, which is silent in the absence of activation. In the presence of Tet or Dox, rtTA (which consists of rTetR and VP16) binds the TRE and activates transcription.

This figure can be found in the 2004 RevTet System User Manual provided by Clontech (31).

I.IV: Thesis Direction

I.IV.I: Recent Research. Recent transfection studies have shown that by providing increasing levels of the NiV N protein, alone or in the presence of the P protein, prior to NiV challenge, viral transcription, translation and replication all decrease. Specifically, the production of progeny virus was suppressed by 5 orders of magnitude in a dose-dependent manner (115).

I.IV.II: Hypothesis. It is possible that the relative availability of the N protein and a precise balance of viral components are imperative for encapsidation and formation of a functional replicase complex. Therefore, we hypothesized that the over-expression of NiV N protein binds vRNA, and/or viral gene products P and/or N in an aberrant manner during encapsidation and formation of the replicase complex, causing specific steps of the NiV replication cycle to be inhibited.

IV.III: Thesis Objective. The overall goal of this project is to create a genetic tool that can facilitate further investigation into how high levels of the NiV N protein blocks viral replication.

I.IV.IV: Experimental Approach. To achieve this goal stable cell lines were created using the RevTet-On^{TM} System that, upon induction, over-expressed either the full-length N protein or various truncations. Infection of these induced cell lines with NiV followed by various read-out systems to measure viral replication can be

done to help reveal the mechanisms and functional domains of the N protein involved in its ability to abrogate production of *de novo* progeny virus. More importantly, they can be used to see if an established NiV infection can be "cured" in the presence of high levels of the N protein.

CHAPTER II: Materials and Methods

II.I: Cells

II.I.I: Tissue Culture Maintenance. All cell lines were cultured in polystyrene Corning® tissue culture flasks of appropriate size in monolayers at 37°C in a 5% CO_2 incubator. Stock human embryonic kidney cells (HEK) 293T cells (ATCC, Manassas, VA) and HEK 293 cells (Stratagene, La Jolla, CA) were maintained in DMEM (Sigma, St. Louis, MO) supplemented with 10% heat-treated (56°C for 1 hour) FBS (Multicell, Wisent Inc., Quebec, CA). Transgenic cell line 293 Tet-On™ (Clontech, Mountain View, CA) were propagated in DMEM supplemented with heat-treated 10% Tet System Approved FBS (Clontech) and 400 μg/ml neomycin (Clontech). Double transgenics pRevTet-On (Clontech) and pRevTRE (Clontech) were grown in DMEM supplemented with heat-treated 10% Tet System Approved FBS, 400 μg/ml neomycin, and 200 μg/ml hygromycin (Clontech).

All cell lines were kept below a passage number of 60. To passage, cells were dislodged from the tissue culture flasks by first washing the cells with PBS then treating the cells for 15 minutes with 0.05% trypsin-EDTA (Invitrogen, Burlington, ON). The cells, now in suspension, were diluted and cultured under the appropriate growth conditions as described above. Passaging was done every 2-6 days in a HEPA filtered biological safety cabinet.

II.I.II: Tissue Culture Storage. For long-term storage, frozen cultures of the cell lines were made. To accomplish this, cells grown to confluency were brought into suspension using trypsin treatment. Diluted cell suspensions were counted using a hemocytometer then centrifuged at 500 x g for 5 minutes. Cells were resuspended in solution A (80% heat-treated FBS, 20% DMEM) then mixed with an equivalent amount of solution B (80% heat-treated FBS, 20% DMSO (Sigma)) to a final concentration of 5 x 10^6 cells/ml. After storing 1 ml aliquots of the prepared sample overnight at -80°C in a Cryo 1°C Freezing Container (Nalgene, Rochester, NY) containing 2-propanol (Fisher Scientific, Ottawa, ON) they were then stored in liquid nitrogen for indefinite lengths of time. Cells were brought out of liquid nitrogen by placing the frozen samples directly into a 37°C water bath. Once thawed, the samples were immediately diluted out with non-supplemented 37°C DMEM, and spun down at 500 x g for 5 minutes. Cells were then gently resuspended and maintained in a T25 using the appropriate growth media and temperatures required for propagation of that particular cell line.

II.I.III: Chemical-Competent Cells. Competent cells for transformations were either ordered through Invitrogen (OneShot® Top10 chemically-competent *E. coli* cells) or made in-house. To generate a large stock of chemically-competent cells in-house the transformation and storage solution method (TSS) was used. This was accomplished by growing up an overnight culture of commercial OneShot® Top10 chemically-competent *E. coli* cells in LB (Lennox) broth. A 0.5 ml subculture of this was grown in 50 ml LB broth at 37°C until an OD of 0.5-0.8 was reached. Following

a 20 minute incubation on ice, the cells were then centrifuged at 2500 x g for 10 minutes at 4°C. The pellet was resuspended in 5 ml TSS buffer (85% LB broth, 10% PEG 8000 (Sigma), 5% DMSO, and 50 mM $MgCl_2$ (fisher scientific)) then aliquoted and stored at -80°C.

II.II: Virus

II.II.I: Handling. Biosafety level 4 (BSL4) practices following the in-house Laboratory Centre for Disease Control Material Safety Data Sheet and the Health Canada Laboratory Biosafety Guidelines (http://www.hc-sc.gc.ca/pphb-dgspsp/publicat/lbg-ldmbl-96/index.html) were adopted for handling NiV.

II.II.II: NiV Amplification and Storage. To amplify NiV (provided by the Centers for Disease Control and Prevention, Atlanta, GA) and generate virus stocks, an 80% subconfluent monolayer of Crandell-Rees feline kidney (CRFK) cells (ATCC) were infected with NiV at an MOI of 1. After 48 of incubation at 37°C, the supernatant was harvested and the cell debris pelleted. End point titrations and CPE analysis along with reverse-transcriptase PCR (RT-PCR) assays were used to calculate the infectivity titer of the amplified virus. Nipah virus stocks of $5x10^6$ infectious units (IU)/ml were aliquoted and stored in liquid nitrogen.

II.III: Chromosomal and Plasmid DNA

II.III.I. Plasmid Acquisition. Plasmids were either generated in-house or obtained by a manufacturer. Plasmids pNiVN-IRES$_{cmv}$, pGFP$_{cmv}$, and pGag/Pol, pVSVG were generated in-house and provided by Charlene Ranadheera, National Microbiology Laboratory, Canada. The plasmid pNiVN-IRES$_{cmv}$ contains a CMV promoter which controls expression of the downstream NiV N gene. To reflect promoter activity an IRES was inserted between the promoter and NiV N gene. This plasmid was utilized in all studies requiring transient expression of the NiV N protein. The plasmid pNiVN-IRES$_{cmv}$ was also used to amplify and clone the NiV N ORF into pCR®-Blunt (Invitrogen). The resulting pCR-Blunt-NiVN constructs were used to subclone the NiV N ORF into the response plasmid pRevTRE. Plasmid pGFP$_{cmv}$ expresses the green fluorescent protein and is used as a transfection and IFA control. Plasmids pGag/Pol and pVSVG were used to transiently express the structural proteins necessary for packaging new retroviral particles. Plasmids pRevTRE and pRevTet-On from the retroviral gene expression system BD RevTet™ were provided by Clontech. Briefly, pRevTRE is the response plasmid where the NiV N gene will be inserted while pRevTet-On is the regulatory plasmid that, in the presence of doxycycline, will turn on and control expression of the NiV N gene.

II.III.II: Plasmid Transformation and Storage. Trasformations of plasmid DNA into bacterial cells were performed to allow for propagation of plasmid DNA. Following the manufacturer's instructions, plasmid DNA was either transformed into

OneShot® Top10 chemically-competent *E. coli* cells (Invitrogen), or with slight modification into the TSS-competent *E. coli* cells made in-house. To 50 µl One Shot® TOP10 cells, 0.5 µg of plasmid DNA was added, whereas if using the chemically-competent cells made in-house, 1.0 µg of plasmid DNA would be added to 100 µl competent cells. Following a 30 minute incubation on ice, the cells were heat shocked for 45 seconds at 42°C then placed on ice again for 2 minutes. To each reaction with One Shot® TOP10 cells, 250 µl of room temperature SOC medium (Invitrogen) was added, whereas when the homemade competent cells were used, 400µl of SOC was added (54). Each reaction was incubated at 37°C with shaking at 225 rpm for 1 hour. Each transformation was plated on selective LB plates coated with 40 µl of 20 mg/ml x-gal (dissolved in DMF, Invitrogen), 8 µl of 100 mg/ml IPTG (Invitrogen) and the appropriate selection agent. For example, plasmids pNiVN-IRES$_{cmv}$, pRevTRE, pRevTet-On, pGPF, pGag/Pol, and pVSVG were selected using 100 µg/ml ampicillin (Sigma) while pCR®-Blunt was selected using 50 µg/ml kanamycin (Sigma). The volume of each reaction plated also depended on the type of competent cells used. For instance when using the One Shot® TOP10 cells, 100 µl of each transformation reaction were plated, whereas when the competent cells made in-house were used, the entire 500 µl was plated. Plates were incubated overnight at 37°C. Using the appropriate selection agent (100 µl/ml ampicillin or 50 µg/ml kanamycin), transformants were grown overnight in LB broth. For long-term storage of transformed bacterial cells, glycerol stocks were made (75% glycerol (Fisher Scientific), 25% overnight culture of selected transformant) then stored at -80°C.

II.III.III: Plasmid Purification and Concentration. Depending on the desired yield, plasmid DNA extractions and purifications were performed according to the general guidelines provided with either the QIAprep® Spin Miniprep Kit (Qiagen, Mississauga, ON) or the QIAfilter™ Plasmid Maxi kit (Qiagen). DNA concentrations were determined using the Nano Drop® ND-1000 Spectrophotometer and respective Nano Drop software (NanoDrop Technologies, Inc., Wilmington, DE). If considered necessary, samples were concentrated using a DNA120 SpeedVac® (ThermoSavant, Holbrook, NY) for various lengths of time. Samples were stored at -20°C.

II.III.IV: Genomic DNA Isolation. Genomic DNA isolations were performed on the heterologous stable cell lines using DNAzol® Reagent (Invitrogen) according to the manufacturer's instructions. Once isolated and precipitated, the DNA was solubilized in 0.2-0.3 mls TE buffer. Concentrations were determined using the Nano Drop® ND-1000 Spectrophotometer and respective Nano Drop software (NanoDrop Technologies, Inc.).

II.IV: Amplification of the Nipah Virus N ORF and Truncated Variants

II.IV.I: PCR. All PCR amplification reactions were performed using *PfuTurbo*® (Stratagene), a high fidelity DNA polymerase with proofreading ability. Primer sets were designed in-house to amplify both the full-length 1599 bp ORF of the NiV N protein (Appendix B) and various N- and C-terminal truncations from pNiVN-

$IRES_{cmv}$. Included within the design of the forward and reverse primers were restriction digest sites *Bam*H1 and *Sph*1 respectively. These restriction sites were included to allow for further unidirectional subcloning of the insert into the expression vector pRevTRE. Also included in the design of the reverse primers was the sequence encoding a 9 amino acid HA epitope derived from Influenza virus to allow for protein expression (147). Since past transfection studies showing suppression of NiV replication involved over-expression of C-terminal HA-tagged NiV N protein, it was considered ideal to keep the studies consistent and also add HA epitopes to the C-terminus (115). The primer sequences, which were synthesized by DNA Core, National Microbiology Laboratory or Operon, Huntsville, AL, are listed in Table 1, while the primer sets used for each amplification reaction are listed in Table 2.

All the components of the master mix, provided by Stratagene, were gently combined before addition of the template DNA, except in the case of the negative control where the DNA template is absent (Table 3). Immediately after combining all components, each PCR reaction was subject to varying temperatures, time and cycling numbers in a Biometra® Thermoocycler (Table 4). PCR products were then purified using a QIAquick® PCR purification kit (Qiagen).

Table 1: Oligonucleotide sequences for the amplification of the full-length NiV N ORF and truncated variants.

Oligo Name	Cut sites and tags	Sequence (5' to 3') and base pair location on N ORF * Cut sites and tags are underlined
BS001 (forward)	*Bam*H1	ATT GGA TCC ATG AGT GAT ATC TTT GAA GAG 1
BS002 (reverse)	*Sph*1	AAT GCA TGC TCA CAC ATC AGC TCT GAC G 1599
BS003 (forward)	*Bam*H1	ATT GGA TCC ATG GCA CTT GAT GTG ATT AGA TCT C 163
BS004 (reverse)	*Sph*1 HA tag	AAT GCA TGC TCA AGC ATA ATC TGG AAC ATC ATA TGG ATA CAC ATC AGC TCT GAC GAA ATC 1596
BS005 (forward)	*Bam*H1	ATT GGA TCC ATG CTG GTC TCT GCA GTT ATC AC 481
BS006 (forward)	*Bam*H1	ATT GGA TCC ATG TTC GCA ACC ATC AGA TTC GG 802
BS007 (reverse)	*Sph*1 HA tag	AAT GCA TGC TCA AGC ATA ATC TGG AAC ATC ATA TGG ATA GGA TGT GCT CAC AGA ACT G 1401
BS008 (reverse)	*Sph*1 HA tag	AAT GCA TGC TCA AGC ATA ATC TGG AAC ATC ATA TGG ATA TGC TGA TTT TTG GCC TAG TC 1101
BS009 (reverse)	*Sph*1 HA tag	AAT GCA TGC TCA AGC ATA ATC TGG AAC ATC ATA TGG ATA AAT CCT GCC ATA CCA GTT TC 800
BS010 (reverse)	*Sph*1 HA tag	AAT GCA TGC TCA AGC ATA ATC TGG AAC ATC ATA TGG ATA CAC ATC AGC TCT GAC GAA ATC 1596

Table 2: Primer sets used for the amplification of the full-length NiV N ORF and truncated variants.

Amplicon	Primer Set
NiV N 1-1599 (NiV N ORF)	BS001, BS002
NiV N 1-1599-HA	BS001, BS010
NiV N 163-1599-HA	BS003, BS004
NiV N 481-1599-HA	BS005, BS004
NiV N 802-1599-HA	BS006, BS004
NiV N 1-1401-HA	BS001, BS007
NiV N 1-1101-HA	BS001, BS008
NiV N 1-801-HA	BS001, BS009
NiV N 163-1401-HA	BS003, BS007
NiV N 802-1101-HA	BS006, BS008

Table 3: General PCR Reaction composition

Component	Volume/Reaction
Master Mix	
10x PCR Buffer	5µl
$MgCl_2$ (25mM)	0-4µl*
dNTP mix (10mM of each)	2µl
Forward Primer (20µM)	1µl
Reverse Primer (20µM)	1µl
pfu DNA polymerase (100U)	1µl
Distilled Water	up to 50µl total volume
Template DNA	0.1 - 500 ng
Total Volume	50µl

* to obtain optimal yields of PCR product, various amounts of $MgCl_2$ were added

Table 4: General PCR Thermocycling Parameters

Cycle Step	Temperature (°C)	Time (min)	Number of Cycles
pfu DNA polymerase activation	95	10	1
Denaturation	95	2	40
Annealing	50-62	1	
Extension	68-72	1-2	
Final extension	72	7	1
Hold	4	-	-

II.IV.II: Agarose Gel Electrophoresis. Amplification of the desired NiV N ORF fragment was verified by loading the purified amplicons on a 1% agarose gel followed by electrophoresis. UltraPure™ Agarose (Invitrogen) was first dissolved in 1.0 x TAE pH 8.0 by bringing the solution to a boil, followed by constant stirring to cool the mixture to approximately 60°C. To allow for visualization of the DNA bands, 0.5 µl 1% EtBr (Fisher Scientific) was added per 50 ml gel solution. The solution was poured into a gel rack (0.5 inches in thickness) with comb and solidified. Five µl of each reaction were mixed with 6 x Gel Loading Buffer (30% glycerol (Fisher Scientific) and 0.25% Bromophenol Blue (Fisher Scientific)) then loaded into each well. For approximation of amplicon size the molecular weight marker 2-Log DNA Ladder™ (New England Biolabs, Appendix A) or Ready-Load™ 100 bp DNA Ladder (Invitrogen, Appendix A) was loaded and run alongside the samples. Electrophoresis was then carried out at 120V for ~40 minutes. For analysis, the gel was placed on a UV lightbox where the image was captured by either using a Gel Cam with Photo-documentation Hood (FisherBiotech) or AlphaImager® with its respective AlphaEase® FC software (Alpha Innotech).

II.V: Cloning

II.V.I: High Efficiency Cloning of PCR Amplicons

a) Ligation. The Zero Blunt® PCR Cloning Kit (Invitrogen) is designed to provide a high efficiency system for cloning blunt-ended PCR products. Following the general manufacturer's guidelines and using the materials provided with this kit, the PCR

amplicons of both the full-length and truncated versions of the NiV N ORF were ligated into the pCR®-Blunt vector (Table 5, Appendix C). For optimal ligation, reactions were incubated for 1 hour at 16°C.

b) Tansformation. The newly ligated construct was then transformed into either commercial OneShot® Top10 chemically-competent *E. coli* cells or TSS-Competent *E. coli* cells made in-house. Either 5 µl of the pCR®-Blunt ligation reaction were added to 50 µl of One Shot® TOP10 cells or 10 µl to 100 µl of the competent cells made in-house. Refer to section II.III.II for transformation protocol.

c) Restriction Digest Screening. Transformants were analyzed by restriction digests. Colonies grown from the transformation were picked and grown overnight at 37°C in 2 ml LB broth containing 50 µg/ml kanamycin. Plasmids were isolated using a QIAprep® Spin Miniprep Kit, and then subjected to an *Eco*R1 restriction digest to confirm the presence of the desired insert. Digestion reactions were set up as follows: 0.5 µl *Eco*R1 (20,000 U/ml, New England Biolabs, Pickering, ON), 2 µl 10 x NE Buffer 2 (New England Biolab), 2 µl isolated plasmid and sterile water up to a volume of 20 µl. Reactions were incubated for 3 hours at 37°C and then subjected to agarose gel electrophoresis for further analysis (Section II.IV.II). To allow for size comparison empty pCR®-Blunt vectors were run alongside all the samples.

d) Sequencing. Positive clones indicated by restriction digest were sent to DNA Core (National Microbiology Laboratory, Canada) for sequencing. Universal

sequencing primers M13 forward and M13 reverse have priming sites within pCR®-Blunt that flank the multiple cloning site where the fragment would be inserted (Table 6). Both M13 primers were synthesized and provided by DNA Core (National Microbiology Laboratory, Canada). Results were analyzed using Chromas 1.45 (Technelysium, Tewantin, Australia) and Clone Manager 6 (Sci Ed Central, Durham, NC) software. Positive clones that lack unwanted mutations were then chosen, purified and grown to high yields using QIAfilter™ Plasmid Maxi kit.

II.V.II: Subcloning Nipah Virus N ORF and Truncated Variants into Expression Vector pRevTRE

a) Linearization and purification of Insert and Vector. Both the pCR®-Blunt clones, containing either the full-length or truncated versions of the NiV N ORF, and the inducible expression vector pRevTRE® (Appendix C) were linearized with *Bam*H1 and *Sph*1 to create compatible sticky ends. Each reaction consisted of 1 µl *Sph*1 (5 U/µl, New England Biolabs), 0.5 µl *Bam*H1 (10 U/µl, New England Biolabs), 2 µl 10 x *Bam*H1 Buffer (New England Biolabs), 2 µg plasmid DNA and sterile water up to a volume of 20 µl. Initially, the water, buffer, plasmid DNA and *Sph*1 enzyme were mixed together and incubated overnight at 37°C. The following day, *Bam*H1 was added to the mix and incubated again at 37°C for 3 hours. The digested insert and vector were gel purified using a QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

Table 5: General ligation reaction using pCR®-Blunt

Component	Volume
pCR®-Blunt (25ng)	1µl
Blunt PCR product	2µl
10x Ligation Buffer (with ATP)	1µl
Sterile water	5µl
T4 DNA Ligase (4 U/µl)	1µl
Total Volume	10µl

Table 6: Sequencing primers to confirm NiV N insert in pCR®-Blunt and pRevTRE

Cloning Vector	Oligo Name	Sequence (5' to 3') and base pair location on cloning vector
pCR®-Blunt	M13 forward	GTAAAACGACGGCCAG 442
	M13 reverse	CAGGAAACAGCTATGAC 204
pRevTRE	TRE forward	AACCGTCAGATCGCCTGGAG 3197
	TRE reverse	AATGGCGTTACTTAAGCTAG 3439

b) Ligation and Transformation. The following reactions were set up to ligate the insert, containing either the full-length or truncated versions of the NiV N ORF, to the inducible expression vector pRevTRE®; 1 µl T4 DNA ligase (500 U, Roche, Indianapolis, IN), 2 µl 10 x ligation buffer (Roche), 0.5 µl vector, sufficient insert to reach a 6:1 and 10:1 ratio of insert to vector, and sterile water up to a volume of 20 µl. Insert to vector ratios were determined by subjecting the purified linearized insert and vector to agarose gel electrophoresis. Band intensities were then compared and ratios were determined accordingly. Finally, all reactions were incubated at 16°C overnight. Transformations were performed as previously described in section II.III.II.

c) Restriction Digest Screening. To confirm the presence of positive pRevTRE®-NiV N clones, transformants were grown up overnight in selective LB broth containing 100 µg/ml ampicillin, purified using a QIAprep® Spin Miniprep Kit, then digested with *Bam*H1 to confirm the presence of the desired insert. Digestion reactions were set up as follows; 0.5 µl *Bam*H1 (10 U/µl, New England Biolabs), 2 µl 10 x *Bam*H1 Buffer (New England Biolabs), 2 µl plasmid DNA, and sterile water up to a volume of 20 µl. Reactions were incubated at 37°C for 3 hours then subjected to agarose gel electrophoresis for further analysis (Section II.IV.II). To allow for size comparison empty pRevTRE® vectors were run alongside all the samples.

d) Sequencing. Primers that have binding sites flanking the multiple cloning site within pRevTRE® were designed in-house then synthesized by DNA Core (National

Microbiology Laboratory, Canada) (Table 6). These primers, TRE forward and TRE reverse were sent along with the positive clones indicated by restriction digest to DNA Core for sequencing. Results were analyzed as previously described in Section II.V.I and positive clones with the least amount of unwanted mutations were chosen, purified and grown to large yields using QIAfilter™ Plasmid Maxi kit. Clones with unwanted mutations were fixed using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's general guidelines. All mutagenic oligonucleotide primers used were designed in-house and synthesized by DNA Core (sequences not shown).

II.VI: Protein Expression and Detection

II.VI.I: Transfections. Protein expression can be achieved by transiently transfecting plasmids and constructs into a eukaryotic cell. Transfections were carried out as follows in 6 well poly-D-lysine hydrobromide coated plates (Sigma) and adjusted accordingly for plates of smaller or larger size. Two separate reactions were set up per well, one where 4 μg plasmid DNA was added to 250 μl Opti-MEM® (Invitrogen), and the other where 5 μl Lipofectamine™ 2000 Reagent (Invitrogen) was added to 250μl OptiMEM. Following a 5 minute incubation at room temperature, the two solutions were gently mixed together and incubated again at room temperature for 15 minutes. This mixture was then added, taking the place of the growth media, to cells that were 70% confluent. Following a 24 hour incubation at 37°C, the mixture was removed and replaced with 2 mls DMEM

supplemented with 2% FBS. This was incubated for another 24 hours before harvesting. To ensure experimental validity, positive controls using $pGFP_{cmv}$ and mock controls that lacked the addition of DNA were always included.

II.VI.II: Protein size Prediction. Protein size predictions were based on nucleotide sequence and the amino acids encoded for using the online ExPASy Proteomics Server (39).

II.VI.III: Western Immunoblots. Cell samples (from a 6 well dish) were harvested for Western Blot by first scraping the cells off the plate and into the media. After spinning down the samples at 500 x g for 5 minutes, the pellets were mixed with 250 µl 2 x SDS gel loading buffer (100 ml stock of 4 x SDS GLB: 20 ml 1M Tris-HCL, 20 ml 20% SDS, 35 ml glycerol, 0.5 g bromophenol blue and 20 ml BME (Fisher Scientific)) and 10% BME. Samples were vortexed, spun down, then boiled at 99°C for 10 minutes.

Discontinuous SDS polyacrylamide mini gels were prepared between two glass plates 0.75 cm apart. The bottom gel was a 7% resolving gel (Per gel: 2.8 ml ddH_20, 0.9 ml 40% Acrylamide/Bis (Bio-Rad, Hercules, CA), 1.25 ml resolving buffer (1.5 M Tris pH 8.8), 0.05 ml 10% w/v SDS, 5 µl TEMED (Fisher Scientific), and 25 µl 10% APS (Sigma)) while the top gel was a 4% stacking gel (Per gel: 3.2 ml ddH_20, 0.5 ml 40% Acrylamide/Bis (Bio-Rad), 1.25 ml stacking buffer (0.5 M Tris pH 6.8), 0.05 ml 10% w/v SDS, 5 µl TEMED (Fisher Scientific), and 25 µl 10% APS

(Sigma)). Once the gels were poured and solidified, samples along with protein markers (MagicMark^{TM}XP (Ivitrogen, Appendix A) and 1 x See Blue®Plus2 (Invitrogen)) and positive controls (cells transfected with pNiVN-IRES$_{cmv}$) were loaded into each lane then subjected to electrophoresis. Gels were run in 1 x SDS gel running buffer (1 x Tris Glycine) at 100 V for approximately 60 minutes or until the bromophenol blue dye ran to the bottom of the plate.

Samples on the SDS polyacrylamide gel were transferred under semi-dry conditions to a Hybond^{TM}-P PVDF transfer membrane (Amersham Biosciences, Piscataway, NJ). For this, all components including filter pads (Bio-Rad), membrane (rinsed with methanol), and the SDS polyacrylamide gel were equilibrated for up to 20 minutes in transfer buffer (2.9 g Trizma-Base (Fisher Scientific), 1.47 g glycine, 0.2 g SDS, 100 ml methanol, ddH_20 up to 500 ml). A transfer "sandwich" was then assembled in which all components were stacked one on top of the other as follows: filter pad, gel, membrane, filter pad. Each gel was run on a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) at 60 mA for 90 minutes.

Membranes were blocked with blocking buffer (5% Difco^{TM} skim milk (BD Biosciences), 1% Tween® 20 (Fisher Scientific), PBS) overnight at 4°C. Membranes were washed twice (PBS/1% Tween) followed by a 1 hour antibody staining. Both washing and probing steps were performed at room temperature with rocking. To recognize NiV N recombinant proteins with an HA-tag, the primary antibody Rabbit anti-HA (Sigma) (1/500 dilution in blocking solution supplemented with 1/100

dilution of normal goat serum) was used. Following 3 more 5 minute washes of the membrane in PBS/Tween, the membrane was probed with a secondary HRP conjugated antibody, Goat anti-Rabbit-HRP (1/10000 dilution in blocking solution, Sigma). Full-length recombinant Nipah virus N proteins could also be detected in the absence of an HA-tag using the primary monoclonal antibody Guinea Pig 89 anti-NiV (1/1000 dilution in blocking solution supplemented with 1/100 dilution of normal rabbit serum) which was provided by Dr. Hana Weingartl (NCFAD, Canadian Food Inspection Agency, Winnipeg). The secondary antibody used in this case was an HRP-conjugated Rabbit anti-Guinea Pig antibody (1/10000 dilution in blocking solution, Sigma). After probing with the secondary antibodies, the membrane was washed 3 more times, 5 minutes each in PBS/Tween.

For detection of HRP, 2 ml of a 40:1 mixture of solution A and B (Amersham ECL Western Blotting Detection Reagents, Amersham Biosciences) was allowed to incubate over the entire surface of the membrane for 5 minutes at room temperature. After draining the excess reagent off the membrane, it was wrapped in SaranWrap and placed protein side up in an Autoradiography Cassette (FisherBiotech). The membrane was then exposed for 1 minute on Hyperfilm™ High Performance Chemiluminescence Film (Amersham Biosciences), and then developed using a Feline™14 X-Ray Film Processor (Fischer Industries Inc.). Membranes were sealed and stored at -20°C.

To strip antibodies off a membrane for re-probing, membranes were submerged and incubated with stripping buffer (62.5 mM Tris-HCl, 2% SDS) for 30 minutes at 50°C. Membranes were then washed twice for 10 minutes with PBS/Tween, blocked then probed as per usual.

II.VI.IV: Protein Band Quantification. To quantify recombinant NiV N proteins detected by Western Blot, membranes were stripped and re-probed (following the methods stated in section II.VI.III) with primary monoclonal antibody Mouse anti α-tubulin (Sigma) (1/1000 dilution in blocking solution supplemented with 1/100 dilution of normal goat serum) followed by a secondary staining with HRP-conjugated Goat anti-Mouse antibody (1/10000 dilution in blocking solution, Sigma). Developed x-ray films from the stained immunoblots were scanned and subjected to analysis using AlphaEaseFC^{TM} – Spot Denso (Alpha Innotech, San Leandro, CA) software. After adjusting for background, band intensities of both NiV N and α-tubulin were measured and quantified in IDV (integrated density values). The amount of cells loaded (representative of the α-tubulin loading control) were standardized and used to accurately quantify the level of NiV N expression. Standardizing was done by dividing the calculated NiV N IDV by the α-tubulin IDV.

II.VI.V: Dot Blots. To harvest samples (from a 6 well dish) for Dot Blots, cells were first scraped in 1ml fresh lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP40). Following rotation of the samples in an eppendorf tube for 1 hour at 4°C, they were spun down at 13 000 rpm for 10 minutes. Supernatants were collected and

loaded onto a dot blot apparatus containing a PBS activated Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences). Samples were then transferred to the membrane by vacuum filtration for 2 minutes at 15 in. Hg. Membranes were blocked then probed for recombinant NiV N proteins according to the Western Blot protocol in Section II.VI.III.

II.VI.VI: Immunofluorecense Assay (IFA). Cells in monolayers that were to be analyzed by IFA were fixed and permeabilized with 3.7% formaldehyde (Fisher Scientific) and 0.6% triton®-X-100 (Fisher Scientific) for 30 minutes at 37°C. Cells were rinsed with PBS then blocked with blocking buffer (1% BSA (Fisher Scientific) in PBS) for 30 minutes at 37°C. After blocking, 200 µl (for a 24 well plate) of primary antibody, diluted in PBS, was added for 1 hour at room temperature. To detect HA-tagged recombinant NiV N proteins, the primary antibody Rabbit anti-HA (Sigma) was used at a dilution of 1/200 along with a 1/100 dilution of normal goat serum. After removing the primary antibody, the cells were washed 3 times with PBS. The secondary antibody, Goat anti-Rabbit conjugated FITC (Sigma) was added (1/100 dilution in PBS) and incubated for 1 hour at room temperature in the dark. As a positive control HEK 293T cells transfected with pNiVN-IRES$_{cmv}$ were always included and stained for. To ensure non-specific binding of the secondary antibody these cells transfected with pNiVN-IRES$_{cmv}$ were also stained solely with the secondary antibody. Another negative control stained for were cells that lacked expression of NiV N. Fluorescence was detected using an Axiovert 200M Imaging

Universal Microscope and related Axiovision Release software (Zeiss, Maple Grove, MN).

II.VI.VII: Fluorescence-Activated Cell Sorting (FACS). To harvest samples for flow cytometry, cells were first dislodged from the plate/flask using Accumax™ (CHEMICON, Temecula, CA) treatment for 30 minutes at room temperature. The dislodged cells were then spun down at 1000 x g for 10 minutes. After discarding the supernatants, the pellets were washed in 1 x Dulbecco's PBS, which lacks chemical compounds $CaCl_2$ and $MgCl_2$, (Invitrogen) and spun down again at 1000 x g for 10 minutes. Supernatants were discarded and the remaining cells were fixed for a minimum of 30 minutes in 10% formalin.

The fixed cell suspension was diluted with 1 x Dulbecco's PBS until cells were visually distinguishable on a hemocytometer. Once counted, 100 000 cells were loaded into 5 ml polystyrene round-bottom tubes (BD Biosciences) and brought up to a volume of 4 mls with 1 x Dulbecco's PBS. To permeabilize the fixed cells, 0.5% triton®-X-100 (Fisher Scientific) was added to the cell suspension and incubated for 20 minutes at room temperature. Cells were pelleted by centrifugation at 1000 x g for 10 minutes then blocked with 10 µl human IgG (1 mg/ml, Sigma). For detection of HA-tagged recombinant NiV N proteins, 100 µl of the primary antibody Rabbit anti-HA (Sigma) (1/400 dilution in 1 x Dulbecco's PBS with 1/100 dilution of normal goat serum) was added for 1 hour at 4°C followed by two washing steps where cells were first rinsed with 1 x Dulbecco's PBS then centrifuged for 10

minutes at 1000 x g. Next, 100 µl of the secondary antibody Goat anti-Rabbit conjugated FITC (Sigma) (1/5000 dilution in 1 x Dulbecco's PBS) was added and incubated for another hour at 4°C. Following hybridization of the secondary antibody to the primary antibody, cells were washed two more times with 1 x Dulbecco's PBS. The stained cells were gently resuspended in 250 µl 1 x Dulbecco's PBS then run through the BD FACS Calibur™ and analyzed using related CellQuest™Pro (BD Biosciences) software. Data were collected for 10 000 cells/sample at an event rate of over 100 events per second. Negative controls, where fixed cells expressing the N protein were either stained solely with the secondary antibody or not stained at all, were gated upon for comparison purposes of all other samples. To ensure the validity of the staining, positive controls with cells expressing the N protein and stained with both the primary and secondary antibody were also included and compensated for to prevent overlapping emissions from multiple fluorophores. FACS experiments were repeated at least three times.

II.VII: Generation of Double Transgenic Cell Lines

II.VII.I: Retrovirus Production and Infection. HEK 293 cells (Stratagene) were seeded in 6 well poly-D-lysine hydrobromide coated plates (Sigma) and grown to 70% confluency. According to the transfection protocol in Section II.VI.I, Cells were co-transfected with pGag/Pol, pVSVG and the pRevTRE-NiV N construct of interest. Transfections were incubated for 48 hours in Opti-MEM® (GIBCO) at 37°C. Supernatants were collected and clarified using 0.45 µm PVDF 13 mm syringe

filters (Fisher Scientific). The purified retrovirus was then adsorbed for 1 hour at 37°C at 50 x g to single transgenic 293 Tet-On™ (Clontech) seeded in a 12 well poly-D-lysine hydrobromide coated plate (Sigma) grown to 80% confluency. The retrovirus was then removed and replaced with 1 ml DMEM supplemented with 2% FBS, followed by a 48 hour incubation at 37°C.

II.VII.II: Heterologous Stable Cell Line Selection. In order to represent transgene activity among a polyclonal population of stable cells, integration events between each cell must be at different locations within the chromosome. To accomplish this, infected cells from the 12 well plates (from section II.VI.I) were trypsinized, re-seeded into polystyrene coated 10 cm Primaria™ Tissue Culture Dishes (Falcon), and grown up in 10 mls selective DMEM (10% heat treated Tet System Approved FBS, 400 μg/ml neomycin, and 200 μg/ml hygromycin). Foci withstanding the selection pressure were grown up, trypsinized, and collected as one polyclonal population. The heterologous cell lines were scaled up and induced with 1 μg/ml doxycycline (Clontech). Doxycycline was chosen for induction rather than tetracycline since in the RevTet-On system, doxycycline has a 100-fold higher affinity for rtTA than tetracycline ((48)). This will allow for a greater activation of rtTA and a higher level of induction of the target gene. Expression of the recombinant NiV N proteins was detected via Western Blot and IFA while presence of the N transgene was confirmed via PCR. For the PCR reactions, genomic DNA from the stable cell lines was isolated according to the protocol in section II.III.IV.

Then using the primer sets listed in Table 7, PCR was performed as previously mentioned within section II.IV.I on each of the double transgenic cell lines.

II.VII.III: Homologous Stable Cell Line Selection. In order to represent transgene activity among a monoclonal population of stable cells, integration events between each cell must at the same location within the chromosome. For this, retroviral infected 293 Tet-On™ (Clontech) were re-seeded into polystyrene coated 10 cm Primaria™ Tissue Culture Dishes (Falcon) and grown up in 10 mls selective DMEM (10% heat treated Tet System Approved FBS, 400 μg/ml neomycin, and 200 μg/ml hygromycin). Once foci were visible, the media was removed and rinsed off with PBS. Foci were picked one by one by gently pipetting up and down with 5 μl trypsin. The picked foci were then seeded into 96 well poly-D-lysine hydrobromide coated plates (Sigma). Each clonal population of cells that withstood the strong selection pressure were scaled up and induced with 1 μg/ml doxycycline (Clontech) and analyzed for NiV N protein expression via high-throughput Dot Blots and Western Blots. An alternative method used to isolate monoclonal populations was to serially dilute out the few stable cells, among heterologous populations (from section II.VII.II), that were expressing the recombinant NiV N protein. One in 10 serial dilutions were done until single cells were isolated in the 96 well plates. The isolated cells were then analyzed for recombinant NiV N protein expression via high-throughput Dot Blots and Western Blots.

Table 7: Primer sets and sequences utilized for the detection of the NiV N transgene in double transgenic cell lines.

Primer Sets	Primer Sequence (5' to 3') and base pair location on N ORF	Sequences Amplified
N802fw N1594rv	TTCGCAACCATCAGATTCGG 802 CATCAGCTCTGACGAAATCA 1594	NiV N 1-1599 NiV N 1-1599-HA NiV N 163-1599-HA NiV N 481-1599-HA NiV N 802-1599-HA
N5fw N795rv	GTGATATCTTTGAAGAGGCG 5 TGCCATACCAGTTTCCTCGA 795	NiV N 1-1401-HA NiV N 1-1101-HA NiV N 1-801-HA
N802fw N1092rv	TTCGCAACCATCAGATTCGG 802 TTGGCCTAGTCTGAAATACA 1092	NiV N 802-1101-HA

II.VIII: Replication of NiV in Stable Cell Lines Over-Expressing Inducible NiV N and Truncated Variants

II.VIII.I. NiV Infection of Double Transgenic Cell Lines. Infections were done in 6 well plates with either non-induced or 48 hour doxycycline induced (1 μg/ml) double transgenic cell lines at 80% confluency. Five hundred μl of NiV CRFK stock were added to the cells at an MOI of 1 and adsorbed for 1 hour at 37°C. Negative controls which lacked the addition of NiV were also included. Virus was removed and replaced with 1 ml DMEM supplemented with 2% heat-inactivated Tet system approved FBS, and doxycycline (1 μg/ml). Infected cells were incubated overnight at 37°C in the dark then supernatants collected and stored at -80°C for $TCID_{50}$s, and cells harvested for Western Blot. To ensure inactivation of NiV, cell lysates collected for Western Blots were performed as per usual but boiled at 99°C for 15 minutes then transferred into new tubes before being brought out of BSL4 containment. The samples for FACS sat overnight in 10% formalin before transferred into fresh tubes and brought out of L4.

II.VIII.II: $TCID_{50}$. Supernatants from original infection were serially diluted in OptiMEM to 10^{-6}. In triplicate, 0.5 ml was added to HEK 293 cells at 80% confluency in 24 well plates. This was incubated for 1 hour at 37°C. After virus adsorption, supernatants were removed then replaced with DMEM supplemented with 2% heat-inactivated Tet system approved FBS. Cells were then incubated for several days with a close watch on CPE. $TCID_{50}$ were determined using the Reed and Muench method (119) and only performed once.

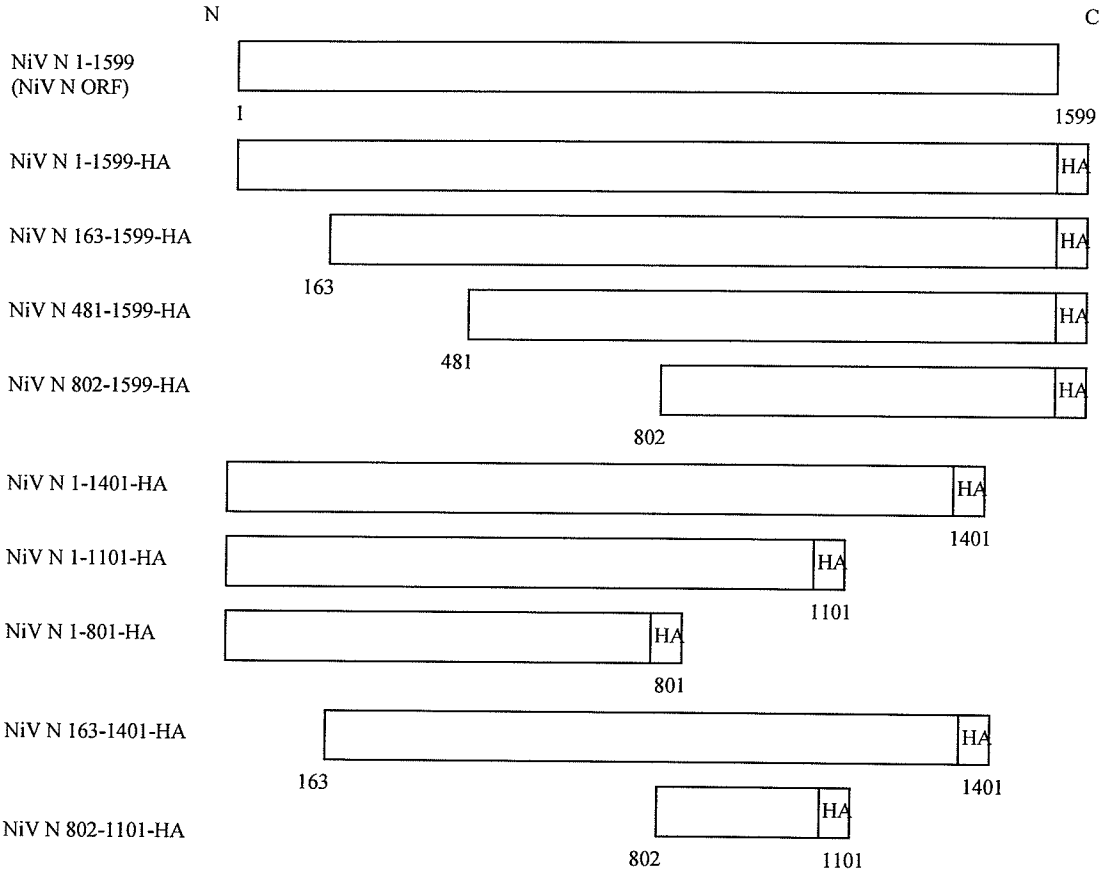
CHAPTER III: Results

III.I: Amplification of NiV N ORF and Truncated Variants

Using standard PCR methods, the 1599 bp NiV N ORF (Appendix B) and several N-terminal and C-terminal truncations were amplified from pNiVN-IRES$_{cmv}$ and HA-tagged for further detection purposes. Ten amplicons were generated in total: 1) NiV N 1-1599; 2) NiV N 1-1599-HA; 3) NiV N 163-1599-HA; 4) NiV N 481-1599-HA; 5) NiV N 802-1599-HA; 6) NiV N 1-1401-HA; 7) NiV N 1-1101-HA; 8) NiV N 1-801-HA; 9) NiV N 163-1401-HA; and 10) NiV N 802-1101-HA (Figure 4).

III.II: Cloning Full-Length and Truncated NiV N PCR Amplicons into pCR®-Blunt Vector

After ligating the various PCR amplicons to the pCR®-Blunt vector and transforming into competent cells, plasmids were isolated and subjected to an *Eco*R1 restriction digest. In the 3.5 kb pCR®-Blunt vector, *Eco*R1 cut sites flank the fusion joint where the blunt NiV N ORF amplicons are inserted (Appendix C). An *Eco*R1 cut site is also found in the 1599 bp NiV N ORF at base pair 856. Therefore with the presence of these cut sites, unique banding patterns on agarose gels can be used to differentiate between the empty vector and positive clones with the NiV N insert. For example, presence of the vector will be confirmed by a 3.5 kb band while the insert will be represented by two smaller bands of various sizes. To ensure the absence of unwanted point mutations, all clones showing the expected banding pattern were sequenced. Positive clones were selected for further experimentation.


N
C
NiV N 1-1599
(NiV N ORF)
1
1599
NiV N 1-1599-HA
HA
NiV N 163-1599-HA
HA
163
NiV N 481-1599-HA
HA
481
NiV N 802-1599-HA
HA
802
NiV N 1-1401-HA
HA
1401
NiV N 1-1101-HA
HA
1101
NiV N 1-801-HA
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1401
NiV N 802-1101-HA
HA
802
1101

a)

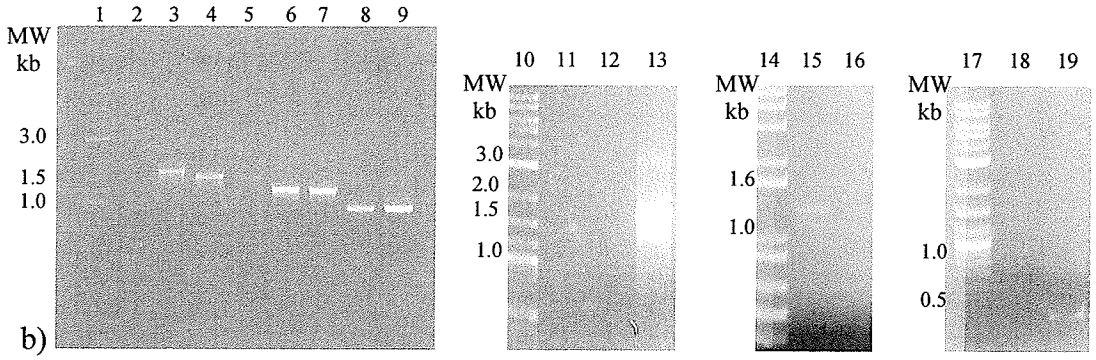


Figure 4: NiV N PCR amplicons. a) Schematic representation of HA-tagged full-length and truncated NiV N PCR amplicons. **b)** Agarose gel confirming PCR amplification of full-length NiV N ORF and N- and C-terminal truncations from pNiVN-IRES$_{cmv}$. PCR primers BS001 and BS002 were used to amplify the 1599 bp full-length NiV N ORF (lane 3) while primers BS001 and BS010 were used to amplify the full-length HA-tagged NiV N ORF (lane 13). Primers BS003 with BS004, BS005 with BS004, and BS006 with BS004 were used to amplify HA-tagged N-terminal truncations including NiVN163-1599 (lane 4), NiVN481-1599 (lane 6) and NiVN802-1599 (lane 8) respectively. Primers BS001 with BS007, BS001 with BS008 and BS001 with BS009 were used to amplify HA-tagged C-terminal truncations including NiVN1-1401 (lane 5), NiVN1-1101 (lane 7) and NiVN1-801 (lane 9) respectively. PCR amplicons with both N- and C-terminal truncations, NiVN163-1401-HA (lane 15) and NiVN802-1101-HA (lane 19), were generated using primers BS003 with BS007 and BS006 with BS008 respectively. Negative controls that lack the presence of pNiVN-IRES$_{cmv}$ DNA was included in lanes 2, 12, 16 and 18 while lane 11 is empty. For band size comparison 2-Log DNA Ladder^{TM} (New England Biolabs) was included in lanes 1, 10, and 17 while lane 14 consists of a 1 kb DNA ladder^{TM} (GIBCO).

Agarose gels showing *Eco*R1 restriction digest of pCR®-Blunt clones are shown in Figure 5 while a complete summary of the results are listed in Table 8.

III.III: Subcloning Full-Length and Truncated NiV N PCR Amplicons into pRevTRE Expression Vector

The full-length and truncated NiV N ORF located within the respective pCR®-Blunt-NiVN constructs were subcloned using restriction enzymes *Bam*H1 and *Sph*1 into the expression vector pRevTRE. To screen for positive clones restriction digests using *Bam*H1 were performed. In newly generated pRevTRE-NiV N clones there is only one *Bam*H1 cut site. This site is located at bp 3305 on pRevTRE and is the site of insert ligation. Therefore after a *Bam*H1 digestion of pRevTRE-NiV N, positive clones are detected by an upward shift of bands on an agarose gel compared to that of the empty 6.5 kb pRevTRE vector. In other words, the upward shift visualized on the agarose gel is representative of the NiV N insert. To ensure the absence of unwanted point mutations, all clones showing upwards shifts of their bands compared to that of the empty pRevTRE vector were sequenced. Positive clones were selected for further experimentation. Agarose gels showing *Bam*H1 restriction digest of pRevTRE clones are shown in Figure 6 while a complete summary of the results are listed in Table 9.

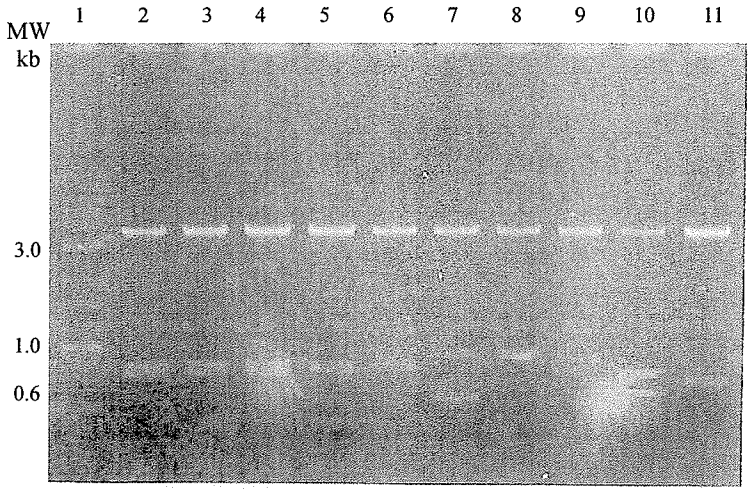


Figure 5: *Eco*R1 restriction digests to screen pCR®-Blunt clones carrying either the full-length or truncated versions of the NiV N ORF. Agarose gel lanes were loaded with the following digested pCR-Blunt-NiVN transformants: 1) pCR-Blunt-NiVN1-1599 in lane 2; 2) pCR-Blunt-NiVN1-1599-HA in lane 3; 3) pCR-Blunt-NiVN-163-1599-HA in lane 4; 4) pCR-Blunt-NiVN-481-1599-HA in lane 5; 5) pCR-Blunt-NiVN-802-1599-HA in lane 6; 6) pCR-Blunt-NiVN-1-1401-HA in lane 7; 7) pCR-Blunt-NiVN-1-1101-HA in lane 8; 8) pCR-Blunt-NiVN-1-801-HA in lane 9; 9) pCR-Blunt-NiVN-163-1401- HA in lane 10; and 10) pCR-Blunt-NiV-802-1101-HA in lane 11. For DNA band size comparison, 2-Log DNA Ladder™ was included in lane 1

Table 8: Expected outcome and Results of *Eco*R1 restriction digest to screen pCR®-Blunt clones carrying either the full-length or truncated versions of the NiV N ORF

pCR®-Blunt-clones	Bands expected after *Eco*R1 digest	# screened by restriction digest	Bands visualized after *Eco*R1 digest	Positive clone confirmed by sequencing
NiVN-1-1599	3.5 kb 856 bp 743 bp	4	3.5 kb 856 bp 743 bp	√
NiVN-1-1599-HA	3.5 kb 856 bp 770 bp	30	3.5 kb 856 bp 770 bp	√
NiVN-163-1599-HA	3.5 kb 693 bp 770 bp	4	3.5 kb 693 bp 770 bp	√
NiVN-481-1599-HA	3.5 kb 375 bp 770 bp	16	3.5 kb 375 bp – faint because of small size and bromophenol blue darkening of the gel at this level 770 bp	√
NiVN-802-1599-HA	3.5 kb 55 bp 770 bp	16	3.5 kb 55 bp band absent – too small to see on gel 770 bp	√
NiVN-1-1401-HA	3.5 kb 856 bp 572 bp	20	3.5 kb 856 bp 572 bp	√
NiVN-1-1101-HA	3.5 kb 856 bp 272 bp	12	3.5 kb 856 bp 272 bp band absent – cannot visualize because of bromophenol blue darkening of the gel at this level	√
NiVN-1-801-HA	3.5 kb 801 bp	4	3.5 kb 801 bp	√
NiVN-163-1401-HA	3.5 kb 693 bp 572 bp	25	3.5 kb 693 bp 572 bp	√
NiVN-802-1101-HA	3.5 kb 54 bp 272 bp	6	3.5 kb 54 bp band absent – too small to see on gel 272 bp band absent – cannot visualize because of bromophenol blue darkening of the gel at this level	√

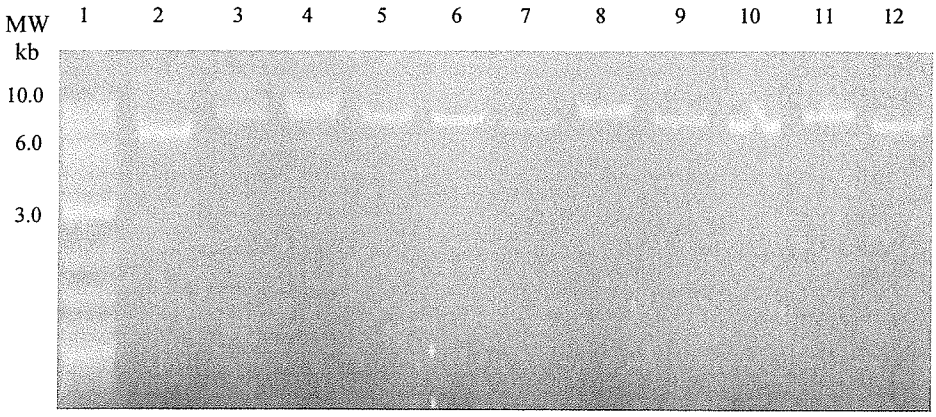


Figure 6: *Bam*H1 Restriction digests to screen pRevTRE® clones carrying either the full-length or truncated versions of the NiV N ORF. Agarose gel lanes were loaded with the following digested pRevTRE-NiVN transformants: 1) pRevTRE-NiVN1-1599 in lane 3; 2) pRevTRE-NiVN1-1599-HA in lane 4; 3) pRevTRE-NiVN163-1599-HA in lane 5; 4) pRevTRENiVN481-1599-HA in lane 6; 5) pRevTRE-NiVN802-1599-HA in lane 7; 6) pRevTRE-NiVN1-1401-HA in lane 8; 7) pRevTRE-NiVN1-1101-HA in lane 9; 8) pRevTRE-NiVN1-801-HA in lane 10; 9) pRevTRE-NiVN163-1401-HA in lane 11; and 10) pRevTRE-NiVN802-1101-HA in lane 12. To determine DNA band size 2-Log DNA Ladder™ was included in lane 1. To help detect presence of insert, which can be observed by a small upward shift of the DNA band, all samples were run alongside a *Bam*H1 digested empty pRevTRE vector. This control can be seen in lane 2.

Table 9: Results of *Bam*H1 restriction digest to screen pRevTRE® clones carrying either the full-length or truncated versions of the NiV N ORF

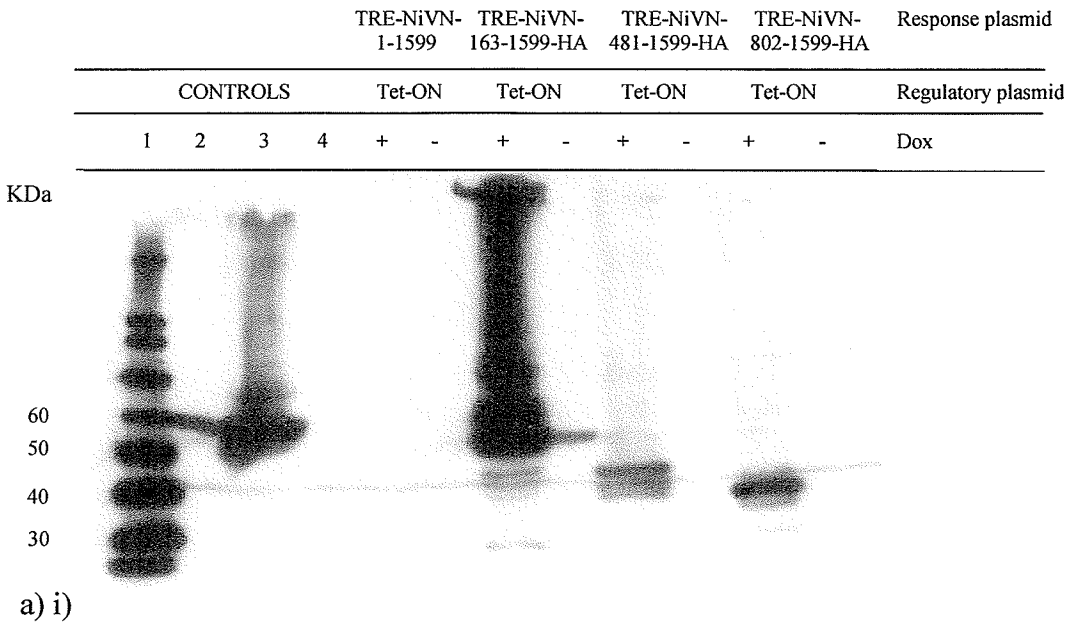
pRevTRE®-clones	Number screened by restriction digest	Upward band shift visualized after *Bam*H1 digest	Positive clone confirmed by sequencing
NiVN-1-1599	7	√	√
NiVN-1-1599-HA	20	√	√
NiVN-163-1599-HA	71	√	√
NiVN-481-1599-HA	14	√	√
NiVN-802-1599-HA	14	√ - very slight upward shift	√
NiVN-1-1401-HA	62	√	√
NiVN-1-1101-HA	16	√	√
NiVN-1-801-HA	38	√- very slight upward shift	√
NiVN-163-1401-HA	28	√	√
NiVN-802-1101-HA	10	X - small size of insert could not be detected by an upward shift of the band on the gel. Must rely on sequencing.	√

III.IV: Protein Expression from pRevTRE-NiV N Clones

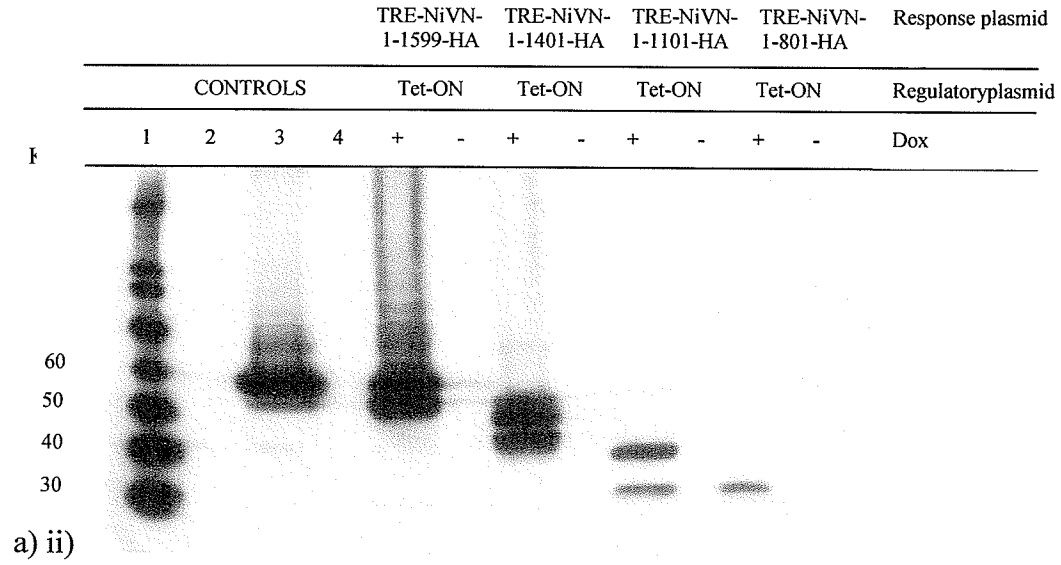
To ensure the RevTet^{TM} system is functional, protein expression from doxycycline induced 293T cells co-transfected with the regulatory plasmid pRevTet-ON and each of the pRevTRE-NiV N clones was detected via Western Blot. The predicted protein masses of the full-length and truncated NiV N proteins are shown in Table 10. Figure 7 confirms protein expression from all the pRevTRE-NiV N clones. For example, after doxycycline induction of the full-length N clones, pRevTRE-NiVN1-1599 and pRevTRE-NiVN1-1599-HA, strong protein expression can be observed just below the 60 kDa marker (Figure 7 b) and a)ii) respectively). Protein sizes correspond to the predicted protein mass shown in Table 10. Also, the banding pattern and size is the same as that seen with the control in lane 3, which has 293T cells transfected with pNiVN-IRES$_{cmv}$. Routinely, there are two bands observed, one dark band at the expected size and one of smaller size around 51 kDa (38). Even though this is a common phenomenon with NiV N expression, it is unknown whether the smaller band is due to multiple transcriptional or translational start-stop sites or post-translational cleavage. In support of the latter, there is *in vitro* evidence of proteolytic digestion of other paramyxovirus N proteins (59, 58, 91, 129, 133).

Table 10: Full-length and truncated NiV N protein mass predictions

NiV N Construct	Predicted Protein Mass (kDa)
pRevTRE-NiVN1-1599	58.0
pRevTRE-NiVN1-1599-HA	59.0
pRevTRE-NiVN163-1599-HA	53.3
pRevTRE-NiVN1-1401-HA	52.3
pRevTRE-NiVN163-1401-HA	47.6
pRevTRE-NiVN481-1599-HA	41.8
pRevTRE-NiVN1-1101-HA	41.9
pRevTRE-NiVN802-1599-HA	29.8
pRevTRE-NiVN1-801-HA	30.6
pRevTRE-NiVN802-1101-HA	12.5


TRE-NiVN-1-1599
TRE-NiVN-163-1599-HA
TRE-NiVN-481-1599-HA
TRE-NiVN-802-1599-HA
Response plasmid
CONTROLS
Tet-ON
Tet-ON
Tet-ON
Tet-ON
Regulatory plasmid
1 2 3 4 + - + - + - + -
Dox
KDa
60
50
40
30

a) i)


TRE-NiVN-1-1599-HA
TRE-NiVN-1-1401-HA
TRE-NiVN-1-1101-HA
TRE-NiVN-1-801-HA
Response plasmid
CONTROLS
Tet-ON
Tet-ON
Tet-ON
Tet-ON
Regulatoryplasmid
1 2 3 4 + - + - + - + -
Dox
60
50
40
30

a) ii)

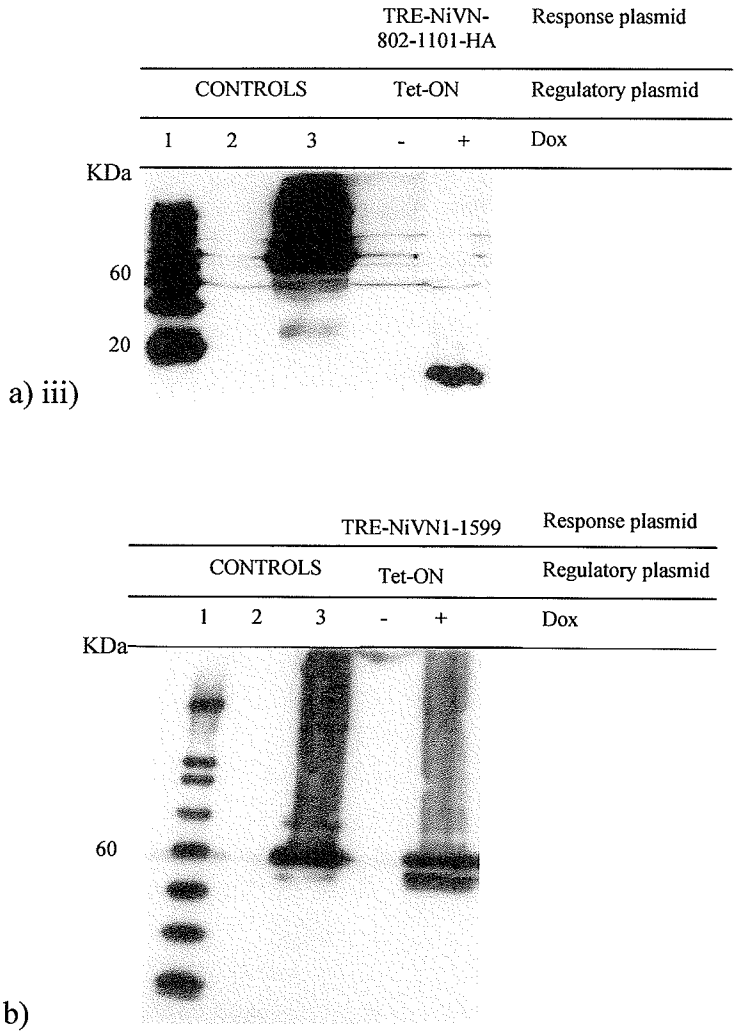


Figure 7: Expression of full-length NiV N protein and various truncations by transiently co-transfecting pRevTRE-NiVN clones with regulatory plasmid pRevTet-On. Co-transfections were done in HEK 293T cells and induced 48 hours with doxycycline. **a)** Western blot detection of recombinant NiV N proteins by HA-tag epitopes. This was done by probing primarily with a 1/500 dilution of Rabbit anti-HA followed by a secondary 1/10000 dilution of Goat anti-Rabbit conjugated HRP. **b)** Western blot detection of recombinant NiV N protein epitope. This was done by probing with a 1/1000 dilution of monoclonal Guinea Pig 89 anti-NiV followed by a secondary 1/10000 dilution of Rabbit anti-Guinea Pig conjugated HRP. Lane 3 on all blots in a) and b) was loaded with a positive control where HEK 293T cells were transfected with pNiVN-IRES$_{cmv}$. For a negative control, mock transfected cells were loaded in every lane 2. Other negative controls include HEK 293T cells transfected only with the regulatory plasmid pRevTet-ON. These are loaded in a) i) and ii) lane 4. MagicMark^{TM}XP is loaded in lane 1 on every blot.

The induced pRevTRE-NiV N clones expressing the N- and C-terminal truncations also display strong protein expression. Figure 7 a)i), ii), and iii) clearly indicates that as the N- and C-terminal truncations of NiV N protein increase, the protein size on the Western Blot is correspondingly smaller. The slight difference in band sizes compared to the predicted protein masses and the difference observed with the banding patterns is most likely dependent on the type of transcriptional or translational processing taking place. Expression is also strongly observed only after the addition of doxycycline. However, with pRevTRE-NiVN163-1599-HA there is slight expression of the N protein even without addition of doxycycline. This expression is much less than the induced and may either be attributed to protein gel leakage into the neighboring lane or a leaky RevTet^{TM} system.

Protein expression of the pRevTRE-NiVN163-1401-HA clone was not confirmed and was therefore, not used for further experiments. Protein expression from this clone was either too low to be detected by Western blot or completely absent. Detection may have been hampered as a result of the recombinant NiV N protein misfolding in a way that prevented access of the Western blot antibodies to the HA tag and NiV N epitopes.

III.V: Protein Expression of pRevTRE-NiV N Clones in Single Transgenic 293Tet-On cells

To confirm the functionality of the single transgenic 293Tet-On cell line ordered from Clontech, the response plasmid pRevTRE-NiVN 1-1599-HA, expressing a full-length recombinant NiV N protein, was transfected into them. Only in the presence of doxycycline is N protein expression observed (Figure 8).

III.VI: NiV N Protein Expression from Heterologous Double Transgenic Cell Lines

After retroviral infection and polyclonal selection of HEK 293 cells carrying both the pRevTet-On and pRevTRE-NiVN transgenes, protein expression was confirmed, in the presence and absence of doxycycline, via Western Blot. To ensure that negative results would not be a result of insufficient amounts of doxycycline or inadequate lengths of induction time, a dose response using doxycycline for 48 and 72 hours was performed.

As seen in Figure 9 a), d), e), f), g), h), and i), there was no NiV N protein expression from polyclonal double transgenic 293-Tet-On-TRE-NiVN; 1-1599, 1-1401-HA, 481-1599-HA, 1-1101-HA, 802-1599-HA, 1-801-HA and 802-1599-HA, respectively. Banding that is visualized, can be attributed to leakage from the positive control given that the bands are either the wrong size or present in the absence of doxycycline and absent with induction.

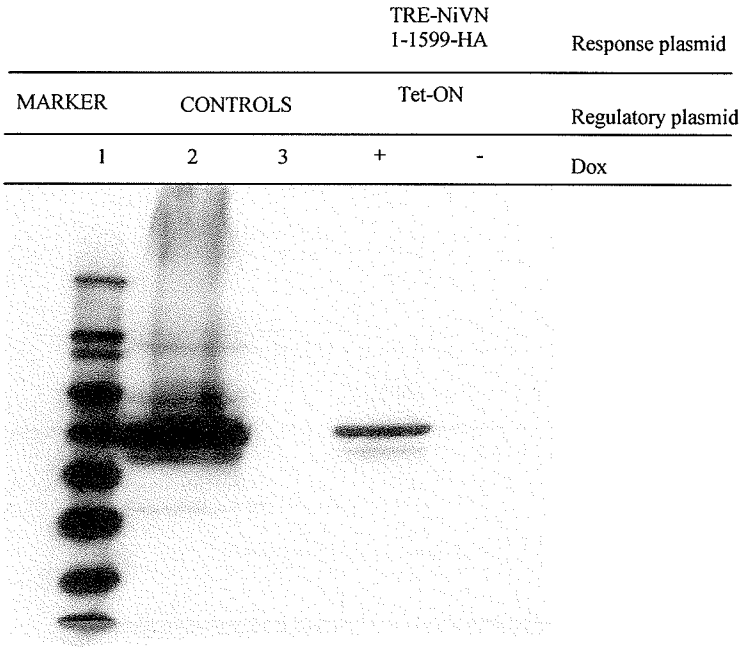
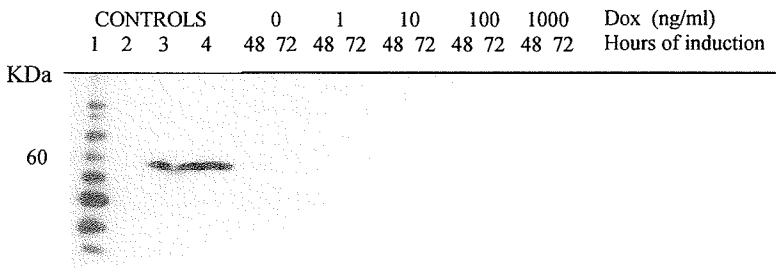
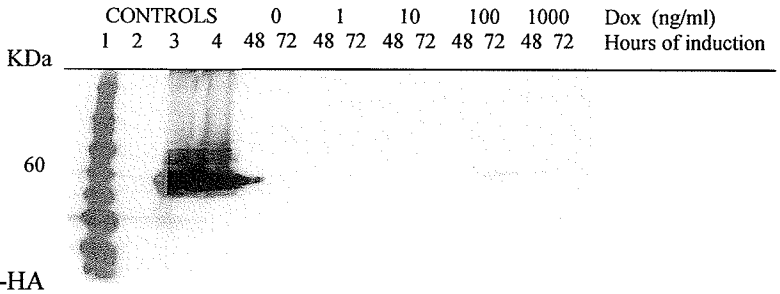


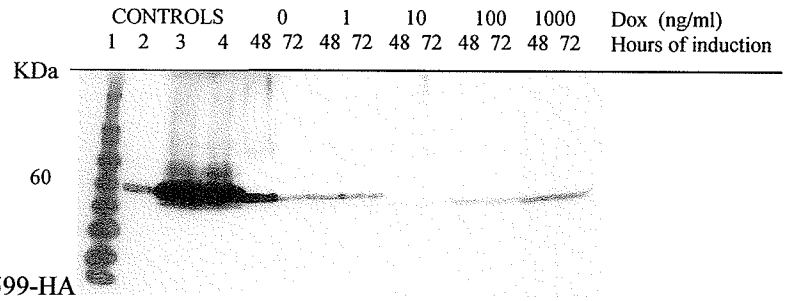
Figure 8: Doxycycline induction of single transgenic 293-Tet-ON cells regulates expression of transfected construct pRevTRE-NiVN1-1599-HA. Western blot analysis shows that the addition of doxycycline for 48 hours causes expression of the full-length NiVN-HA tagged protein. Blot was probed with a 1/500 dilution of Rabbit anti-HA followed by a secondary 1/10000 dilution of an HRP conjugated Goat anti-Rabbit antibody. Without doxycycline, there is no expression. Lane 3 are mock transfected 293-Tet-ON cells while lane 2 is a positive control where 293-Tet-ON cells are transfected with pNiVN-IRES$_{cmv}$. MagicMark^{TM}XP is loaded in lane 1.



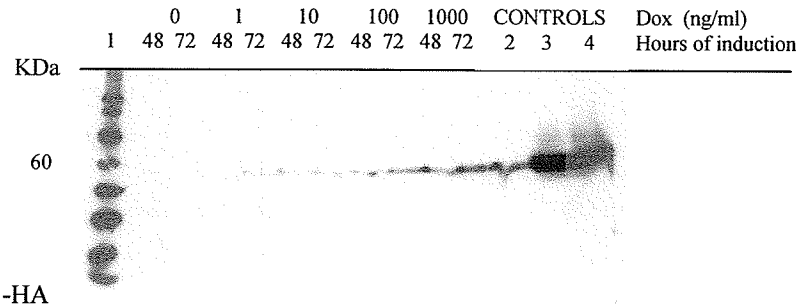
a) NiV N1-1599



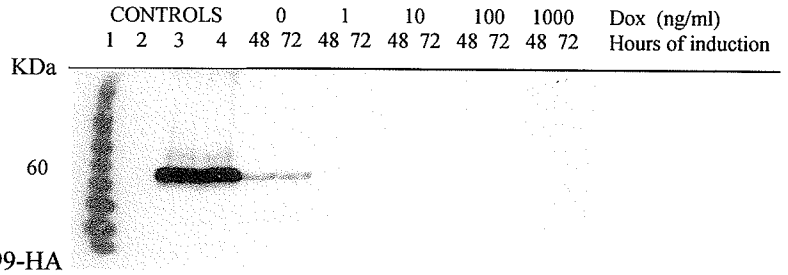
b) NiV N1-1599-HA



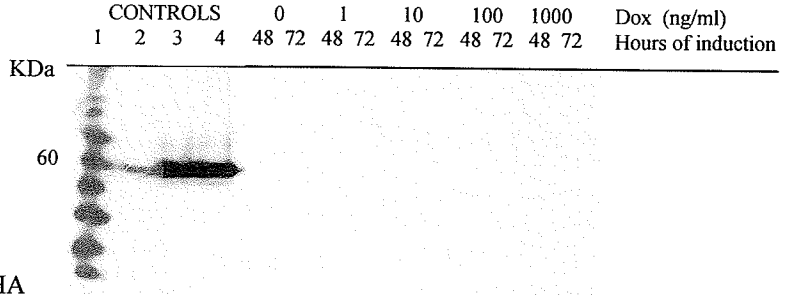
c) NiV N163-1599-HA



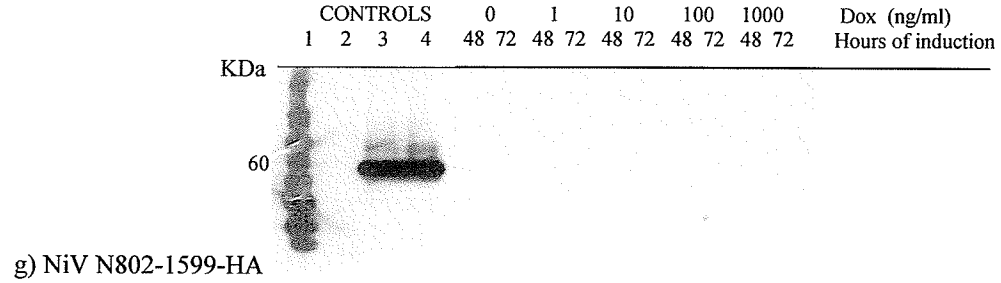
d) NiV N1-1401-HA



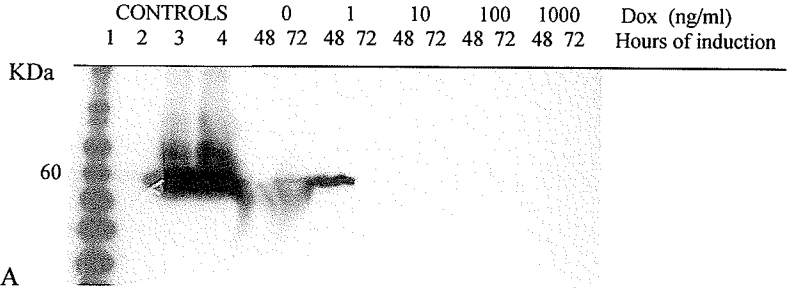
e) NiV N481-1599-HA



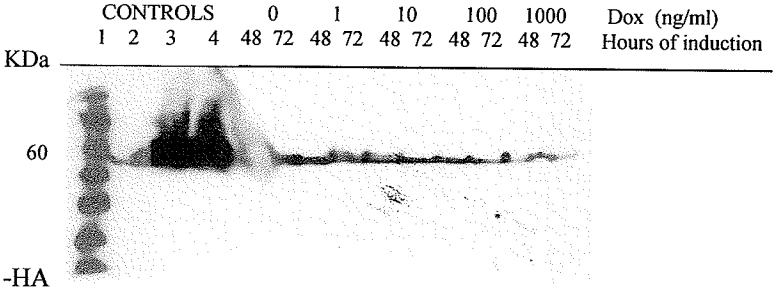
f) NiV N1-1101-HA



g) NiV N802-1599-HA



h) NiV N1-801-HA



i) NiV N802-1101-HA

Figure 9: Time course and dose response of doxycycline induced heterologous double transgenic 293-Tet-ON^{TM}-TRE-NiVN cells. Probing of recombinant NiV N proteins was done with a 1/500 dilution of Rabbit anti-HA followed by a secondary 1/10000 dilution of Goat anti-Rabbit conjugated HRP. Lanes 3 and 4 are positive controls with 293-Tet-ON cell lines tranfected with pNiVN-IRES$_{cmv}$. Lane 4 was induced 48 hours with doxycycline while lane 3 was not. Lane 2 represents mock transfected 293-Tet-ON cells. MagicMark^{TM}XP is loaded in lane 1.

Expression of the NiV N protein was observed with doxycycline induced 293-Tet-On-TRE-NiVN 1-1599-HA (figure 9 b)) and 293-Tet-On-TRE-NiVN 163-1599-HA (figure 9 c)). NiV N expression from 293-Tet-On-TRE-NiVN 1-1599-HA is very weak but seems to be the best after 72 hours of induction with 100 ng/ml doxycycline. Expression of recombinant NiV N is still present with 1000 ng/ml and seems to be expressed to the same level whether induced for 48 or 72 hours. NiV N expression from 293-Tet-On-TRE-NiVN 163-1599-HA is somewhat stronger than 293-Tet-On-TRE-NiVN 1-1599-HA but overall it is still weak compared to the control. Expression for this cell line was highest when 1000 ng/ml doxycycline was used to induce expression. The time of induction however did not make a difference in the level of expression. Therefore, experiments from this point forward were induced for 48 hours with 1000 ng/ml doxycycline.

To give a clearer picture of the percent of doxycycline induced 293-Tet-On-TRE-NiV N 1-1599-HA and 293-Tet-On-TRE-NiV N 163-1599-HA cells among the polyclonal population that are expressing the recombinant NiV N protein, and the level of expression, immunofluorescent assays were carried out. Level of expression would generally depend on the chromosomal location of the transgene and the number of insertion events. The IFA pictures shown in Figure 10, demonstrate expression of the NiV N protein. Only about 5-15% of the cells express varying levels of the NiV N protein, whereas about 60% of the positive control of 293T cells, transfected with pNiVN-IRES$_{cmv}$, strongly or weakly express the N protein.

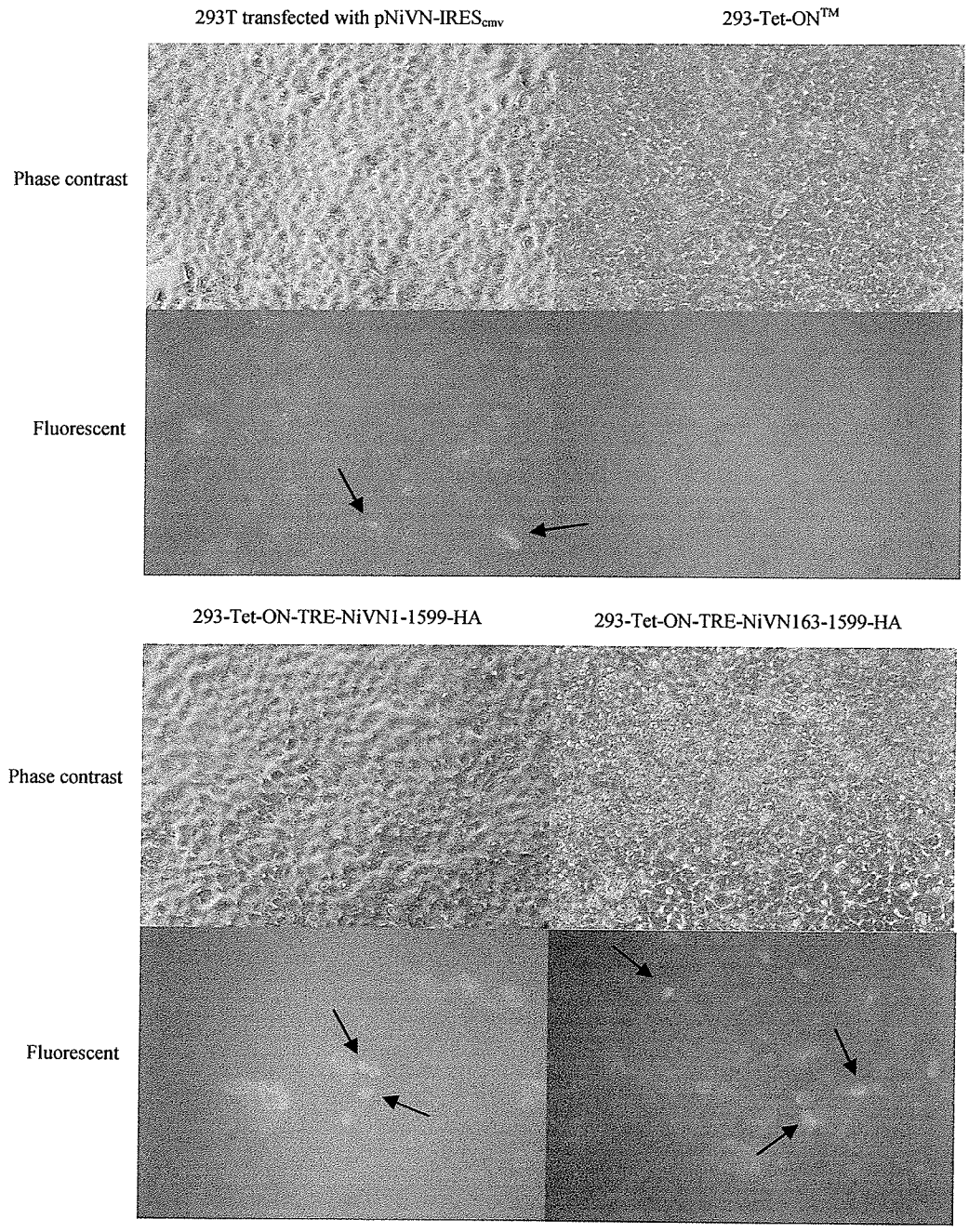


Figure 10: IFA showing expression from induced heterologous double transgenics 293-Tet-ON™-TRE-NiVN1-1599-HA and 293-Tet-ON™-TRE-NiVN163-1599-HA. After doxycycline induction for 48 hours, cells were stained primarily with a 1/200 dilution of Rabbit anti-HA followed by a secondary 1/100 dilution of Goat anti-Rabbit conjugated FITC. A select few of the positive cells are indicated with arrows.

III.VII: Confirmation of pRevTRE-NiVN Transgene in Heterogenous Stable cell lines

Due to the fact that very few of the heterologous cell lines selected for lacked expression of the recombinant NiV N protein, it was necessary to ensure the presence of the pRevTRE-NiV N transgene among the single transgenic 293-Tet-On chromosome. To accomplish this, PCR was performed on DNA isolated from each of the polyclonal double transgenic cell lines. Separate primers were designed to detect the N transgene with either N-terminal truncations, C-terminal truncations or both. The primer set used to detect the full-length and N-terminal truncations of the N transgene were designed to amplify base pairs 802-1594. The resulting 792 bp amplicon was detected in 293-Tet-ON-TRE-NiVN; 1-1599-HA, 163-1599-HA, 481-1599-HA and 802-1599-HA (Figure 11 a), lanes 7-10 respectively). The only cell line where the TRE-NiV N transgene was not detected was 293-Tet-ON-TRE-NiVN1-1599 (Figure 11 a), lane 6). The primer set used to detect the C-terminal truncations of the N transgene were designed to amplify base pairs 5-795. The resulting 790 bp amplicon was detected in 293-Tet-ON-TRE-NiVN; 1-1401-HA, 1-1101-HA, and 1-801-HA (Figure 11 b), lanes 16-18 respectively). Finally, the primer set used to detect the N transgene with both N- and C-terminal truncations were designed to amplify base pairs 802-1092. The resulting 290 bp amplicon was detected in 293-Tet-ON-TRE-NiVN802-1101-HA (Figure 11 b), lane 23).

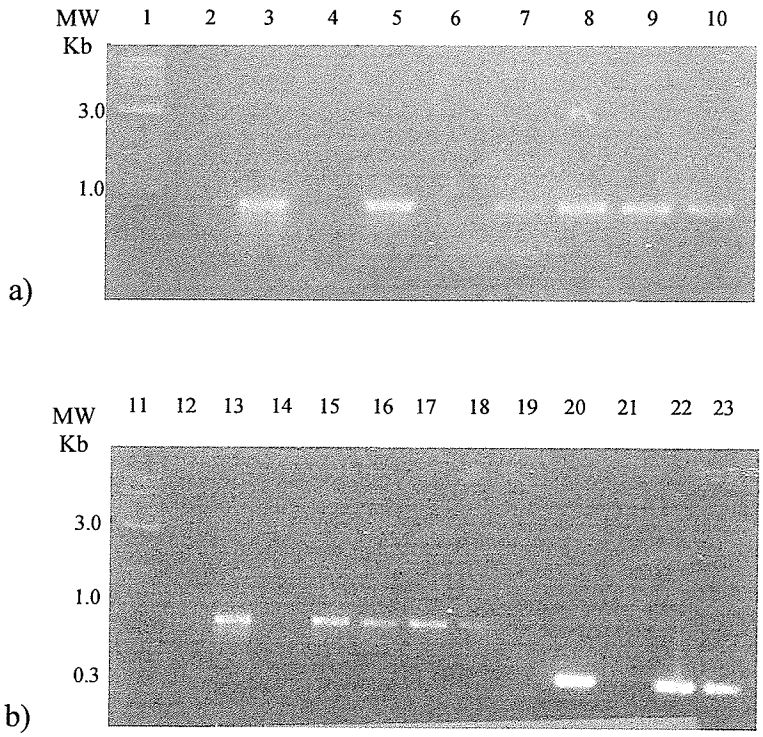
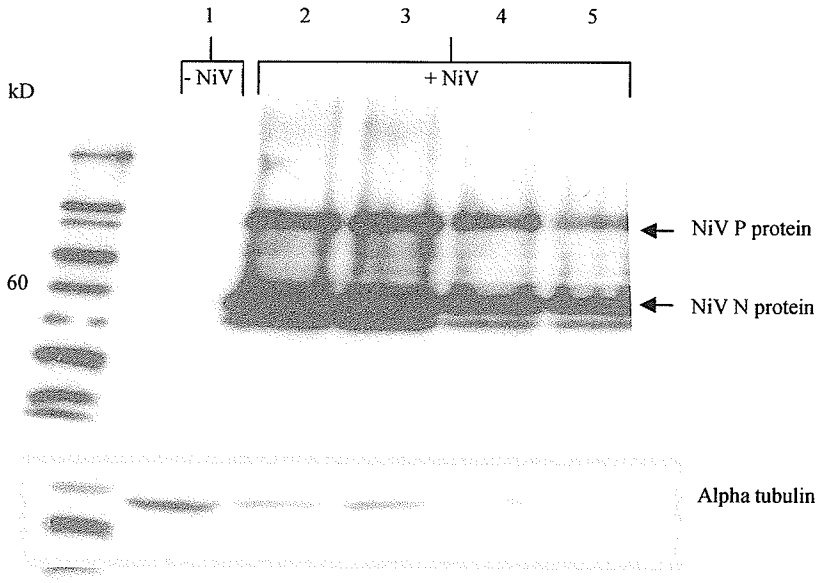


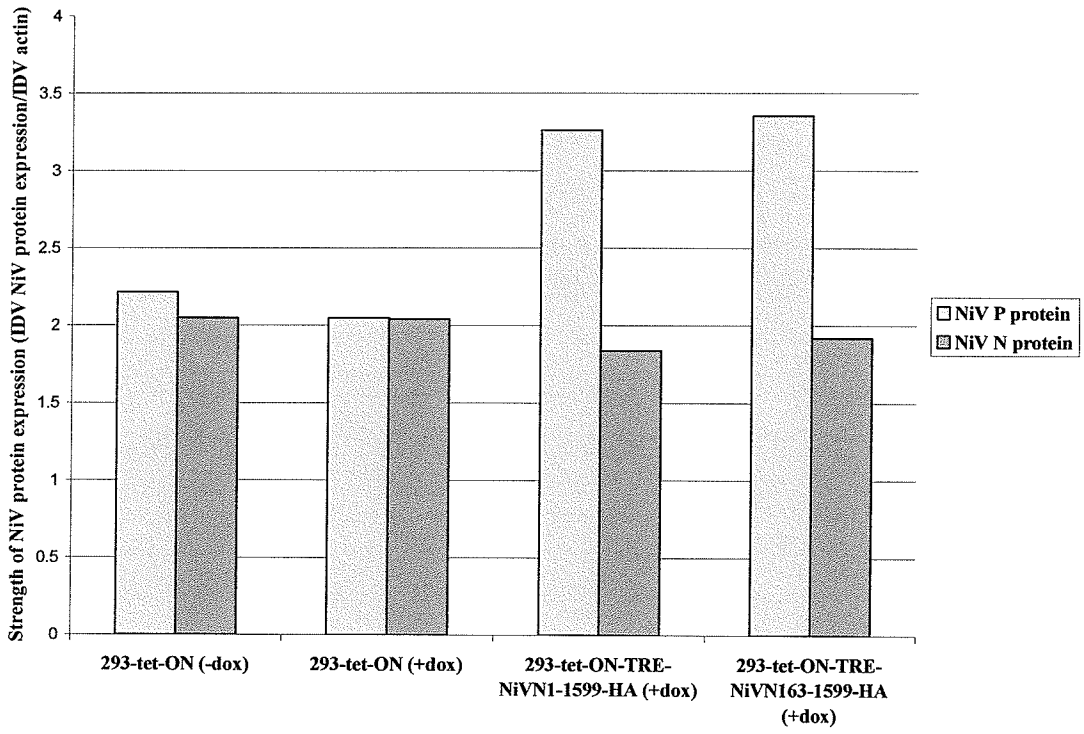
Figure 11: PCR amplification of NiV N transgene from DNA preparations of heterologous double transgenic 293-Tet-ON^{TM}-TRE-NiVN cells. DNA genomes of the heterologous stable cell lines were isolated, subjected to PCR then run on agarose gels. **a)** Amplification of the full-length and N-terminal truncated recombinant NiV N proteins. Primers N802 and N1594 were used in lanes 2-10 where lane 6 is 293-Tet-ON-TRE-NiVN1-1599, lane 7 is 293-Tet-ON-TRE-NiVN1-1599-HA, lane 8 is 293-Tet-ON-TRE-NiVN163-1599-HA, lane 9 is 293-Tet-ON-TRE-NiVN481-1599-HA and lane 10 is 293-Tet-ON-TRE-NiVN802-1599-HA. **b)** Amplification of both C- and N- and C-terminal truncated recombinant NiV N proteins. Primers N5 and N795 were used in lanes 12-18 where lane 16 is 293-Tet-ON-TRE-NiVN1-1401-HA, lane 17 is 293-Tet-ON-TRE-NiVN1-1101-HA, and lane 18 is 293-Tet-ON-TRE-NiVN1-801-HA. Primers N802 and N1092 were used in lanes 19-23 where lane 23 is 293-Tet-ON-TRE-NiVN802-1101-HA. Water controls were included in lanes 2, 12 and 19. Positive controls using pNiVN-IRES$_{cmv}$ were included in lanes 3, 13 and 20 while 293T cells transfected with pNiVN-IRES$_{cmv}$ were included in lanes 5, 15 and 22. Negative controls using non-transfected 293T cells were included in lanes 4, 14 and 21. Lanes 1 and 11 contain 2-Log DNA Ladder^{TM}.

III.VIII: Effects of NiV N Replication in Induced Heterologous Stable Cell Lines.

Induced heterologous cell lines 293-Tet-ON-TRE-NiVN1-1599-HA and 293-Tet-ON-TRE-NiVN163-1599-HA which, upon induction, were previously shown to carry the transgene and express low levels of the recombinant NiV N protein were challenged with NiV. Cell lysates were collected post infection and run on Western blot. At first glance NiV N and P protein production looks to have dropped relative to the controls (figure 12a), however when comparing to the loading control, alpha tubulin, there was no significant drop (figure 12b). Even though the stable cell lines challenged with NiV were expressing recombinant NiV N protein, expression of N was to the same level as the controls. This level of N protein expression may represent N protein from the challenging virus plus the recombinant N protein from the induced stable cell lines. If this is the case, then expression of the N protein from the virus itself decreased. On the other hand, the level of N protein expression observed may only represent N protein from the challenging virus. The low expression levels of the recombinant N protein from the stable cell lines may not have been detected. Unlike the N protein, expression of the NiV P protein increased.



a)



b)

Figure 12: NiV N and P protein quantification in induced heterologous stable cell lines 293-Tet-On-TRE-NiVN1-1599-HA and 293-Tet-On-TRE-NiVN163-1599-HA after being challenged with NiV. Heterologous stable cell lines were induced 48 hours prior to NiV challenge at an MOI of 1. **a)** Western blot analysis depicting strength of viral proteins N and P after induced heterologous stable cell lines were challenged with NiV. Lanes were loaded as follows: lane 1, unchallenged and uninduced stable cells; lane 2, NiV challenged induced 293-Tet-On cells; lane 3, NiV challenged non-induced 293-Tet-On cells; lane 4, NiV challenged induced 293-Tet-On-TRE-NiVN1-1599-HA cells; and lane 5, NiV challenged induced 293-Tet-On-TRE-NiVN163-1599-HA. Blots were probed initially using a 1/1000 dilution of Guinea pig anti-NiV followed by a 1/10000 dilution of HRP conjugated rabbit anti-guinea pig antibody. For quantification purposes, the blot was stripped then re-probed for alpha tubulin using 1/1000 dilution mouse anti-alpha tubulin followed by a 1/10000 dilution HRP conjugated goat anti-mouse antibody. Protein standard, MagicMark^{TM}XP was included in the utmost left hand lane on the blot. **b)** Bar graph depicting strength of NiV N and P protein expression from NiV challenged induced heterologous stable cell lines. Strength was determined by dividing IDVs, from NiV N and P expression by the IDV from the alpha tubulin controls. IDVs were determined from protein band strength in part a). Results were then graphed.

To investigate whether or not NiV replication in the induced heterologous 293-Tet-ON-TRE-NiVN1-1599-HA and 293-Tet-ON-TRE-NiVN163-1599-HA stable cell lines is affected, supernatants from the infection were collected and used to determine the $TCID_{50}$. Figure 13 shows that both cell lines, one expressing the full-length NiV N protein and the other a 10% N terminal truncated version of the protein, suppress viral replication by more than one log. These results are consistent with previous transfection studies where cells transiently over-expressing the NiV N protein suppress viral replication by 5 orders of magnitude (115). However, viral replication in this study was not suppressed to the same extent since there is very low expression of the recombinant NiV N protein in the heterologous stable cell lines. In our case, only 5-15% of the cells among the induced heterologous stable cell line population expressed the recombinant NiV N protein. This level of expression is much lower than expression levels reached with transfections which can reach efficiencies of approximately 60%.

Even though these results are suggestive that the NiV N protein, at increased levels, can suppress viral replication, it is questionable as to whether or not the level of protein expression obtained in these heterologous stable cell lines are adequate enough for use in further experimentation, which may address more complicated aspects such the mechanism and functional domains involved.

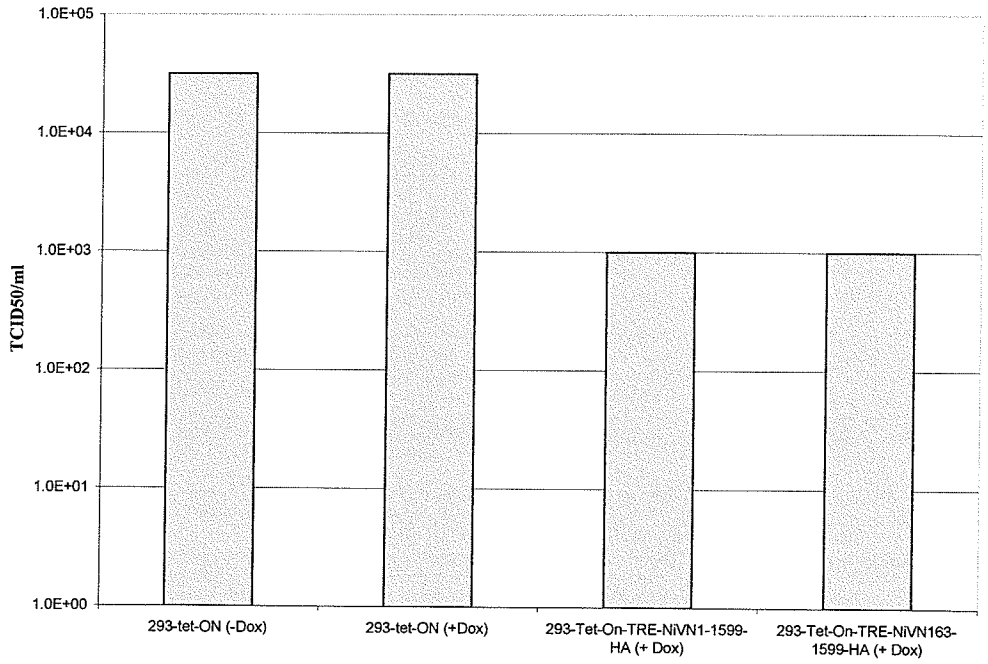


Figure 13: $TCID_{50}$ of induced heterologous stable cell lines challenged with NiV. Bar graph depicting NiV replication in HEK 293 cells. Virus was collected from supernatants of 48 hour induced heterologous stable cell lines 293-Tet-On-TRE-NiVN1-1599-HA and 293-Tet-On-TRE-NiVN163-1599-HA challenged with NiV. $TCID_{50}$s were determined according to degree of CPE using the Reed and Muench method. Experiment was not repeated.

Such low levels of N protein expression may result in an inaccurate and insufficient biological response after challenging with NiV. Without seeing a distinct cause and effect, definitive conclusions cannot be made. Therefore, to increase and mimic N protein expression levels used throughout previous experiments, where over-expression of the N protein blocks viral replication, homologous stable cell lines were explored.

III.IX: NiV N Protein Expression from Homologous Double Transgenic Cell Lines

In order to increase the number of cells expressing the recombinant NiV N protein, monoclonal cell lines were selected. Once a positive clone was identified via Dot blot analysis, it was amplified for use in further experiments. Expression of the recombinant NiV N protein expression from each clone was quantified by Western blot and FACs analysis.

a) Dot blot. Out of 175 homologously selected 293-Tet-On-TRE-NiVN-HA cell lines, containing the various NiV N transgenes, 32 induced clones showed strong or weak expression of the N protein (figure 14). The 32 stable cell lines with confirmed NiV N protein expression included 293-Tet-On-TRE-NiVN; 1-1599-HA, 143-1599-HA, 481-1599-HA, 1-1401-HA and 802-1401-HA.

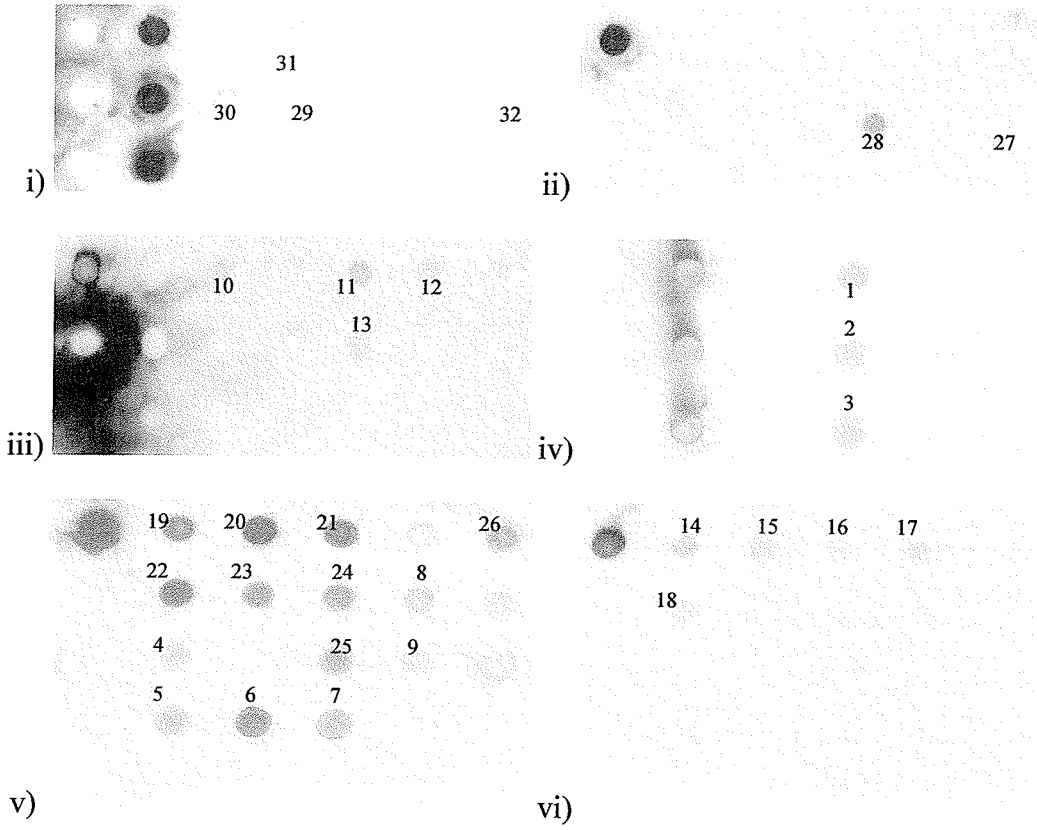
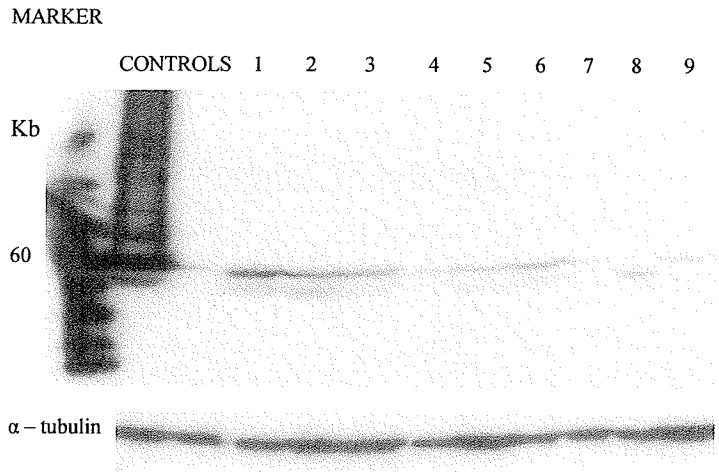
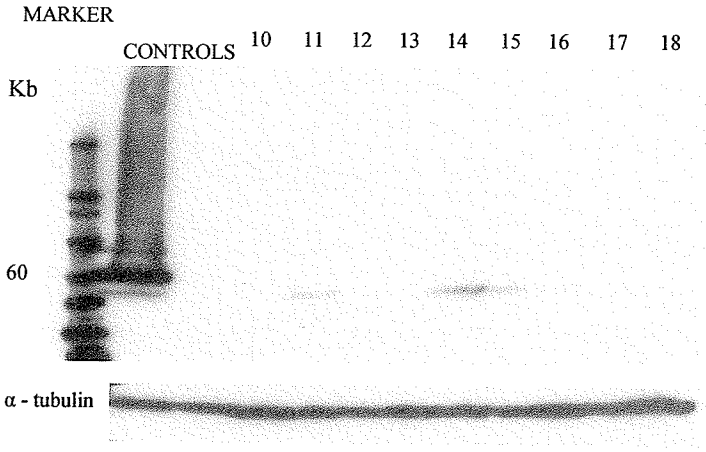


Figure 14: Dot blot analysis of NiV N protein expression from induced monoclonal 293-Tet-ON-TRE-NiVN cells. Cells were induced for 48 hours with doxycycline. Dot blots were probed with a 1/500 dilution of Rabbit anti-HA followed by a secondary 1/10000 dilution of Goat anti-Rabbit conjugated HRP. As positive controls, 293T cell lines tranfected with pNiVN-IRES$_{cmv}$. Mock transfected cells included as negative controls. Labels 1-32 are the clones picked to run on Western blot.

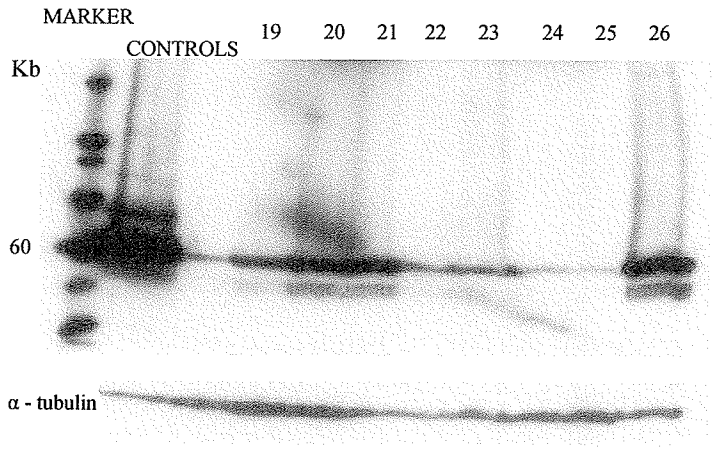
Western Blot analyses of each of the 32 induced clones were done in order to standardize the amount of cells loaded and accurately quantify the level of NiV N expression (figure 15). Results are depicted diagrammatically in figure 16. Clone number 26, 293-Tet-On-TRE-NiVN1-1599-HA, was selected for further experimentation since it showed the strongest expression of the recombinant NiV N protein out of all the stable cell lines containing the TRE-NiVN1-1599-HA transgene. Clone number 14, 293-Tet-On-TRE-NiVN163-1599-HA, was also selected for further experimentation since out of all the stable cell lines containing the TRE-NiVN163-1599-HA transgene, it showed the strongest expression. For comparison purposes in later experiments, another 293-Tet-On-TRE-NiVN1-1599-HA clone, with the same level of NiV N protein expression as clone 14 293-Tet-On-TRE-NiVN163-1599-HA was also chosen. For this, clone number 2 was chosen. All clones below the 0.4 mark on the y axis in figure 16 were not considered for further experimentation since NiV N protein expression could not be detected or was extremely low on the respective Western and Dot Blots (figures 14 and 15). For this reason, these clones, which have both transgenes selected for but lack expression of recombinant NiV N proteins, were considered ideal for use as a negative control for further experimentation. Specifically, clone 10 was chosen as a control.


MARKER
CONTROLS 1 2 3 4 5 6 7 8 9
Kb
60
α – tubulin

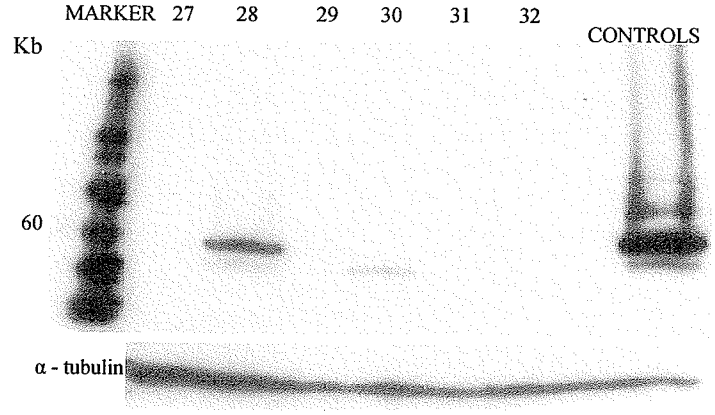
i)


MARKER
CONTROLS 10 11 12 13 14 15 16 17 18
Kb
60
α - tubulin

ii)



iii)



iv)

Figure 15: Western blot analysis of NiV N protein expression from induced monoclonal 293-Tet-ON-TRE-NiVN clones 1-32. Cells were induced for 48 hours with doxycycline. Western blots were probed with a 1/500 dilution of Rabbit anti-HA followed by a secondary 1/10000 dilution of Goat anti-Rabbit conjugated HRP. As a positive control, 293T cell lines tranfected with pNiVN-IRES$_{cmv}$ were also included either in the utmost left hand lane beside the protein marker or utmost right hand lane on the blot. Adjacent to the positive controls were mock transfected cells. Protein standard, MagicMark^{TM}XP was included in the utmost left hand lane on the blot. Membranes were stripped and re-probed for loading control alpha-tubulin (1/1000 dilution mouse anti-alpha tubulin followed by a 1/10000 dilution HRP conjugated goat anti-mouse antibody). This was done by staining with a 1/1000 dilution of primary antibody mouse anti α-tubulin followed by a 1/1000 dilution of secondary antibody HRP-conjugated Goat anti-Mouse antibody.

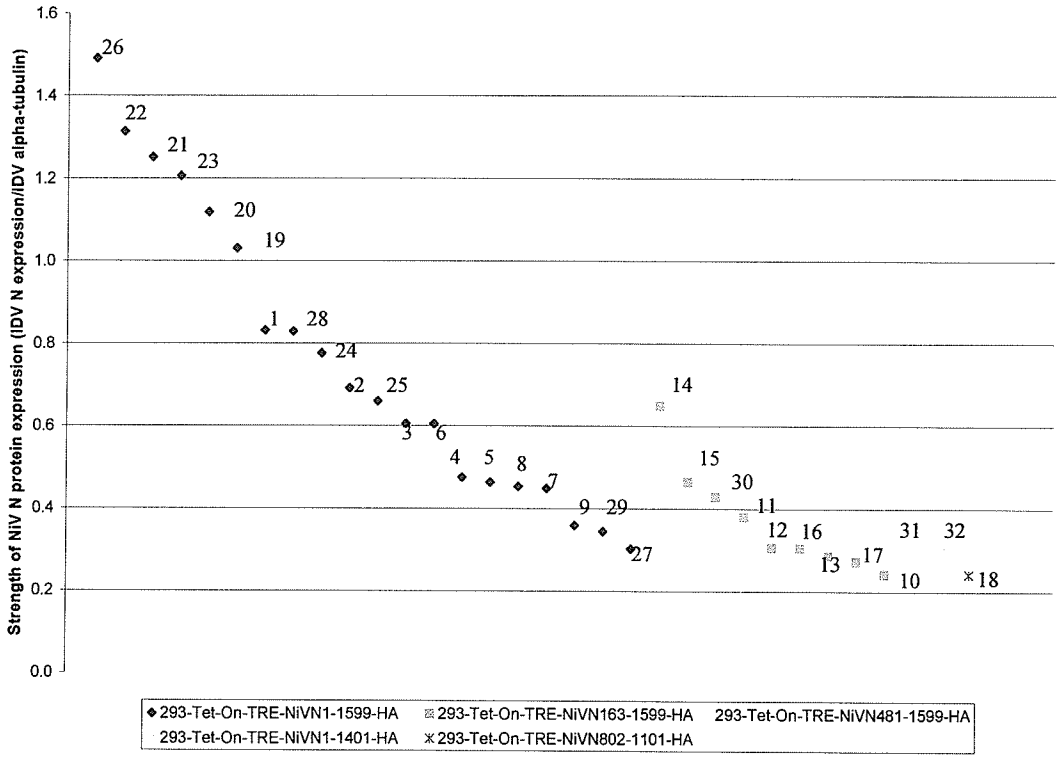
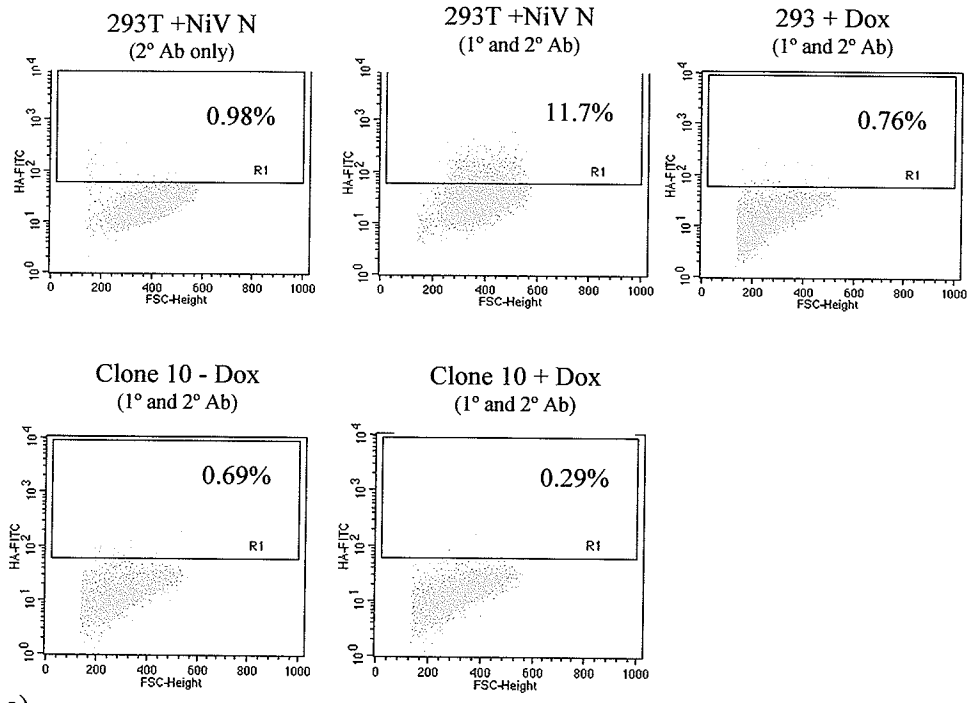


Figure 16: Scatter plot depicting strength of NiV N protein expression of induced monoclonal 293-Tet-ON-TRE-NiVN clones 1-32. Cells were induced for 48 hours with doxycycline. Strength was determined by quantification of protein band strength from figure c. IDVs from NiV N protein expression was divided by the IDV from the alpha tubulin controls then plotted.

b) FACS. Monoclonal populations of cells, in this case, are cells that all express the recombinant NiV N protein to the same degree. To ensure that this is the case, protein expression levels from clones 26, 2 and 14 were quantified on a per cell basis using FACS analysis. Clones 26, 2 and 14 were shown previously by Dot blot analysis to express the NiV N protein. However, after passaging these clones, inducing them with doxycycline, and subjecting them to flow cytometry, there was no detectable expression of the recombinant NiV N protein (Figure 17).

For a complete summary of the production of stable cell lines refer to Table 11.

Controls:


293T +NiV N
(2° Ab only)
0.98%
R1
293T +NiV N
(1° and 2° Ab)
11.7%
R1
293 + Dox
(1° and 2° Ab)
0.76%
R1
Clone 10 - Dox
(1° and 2° Ab)
0.69%
R1
Clone 10 + Dox
(1° and 2° Ab)
0.29%
R1
HA-FITC
FSC-Height

a)

b)

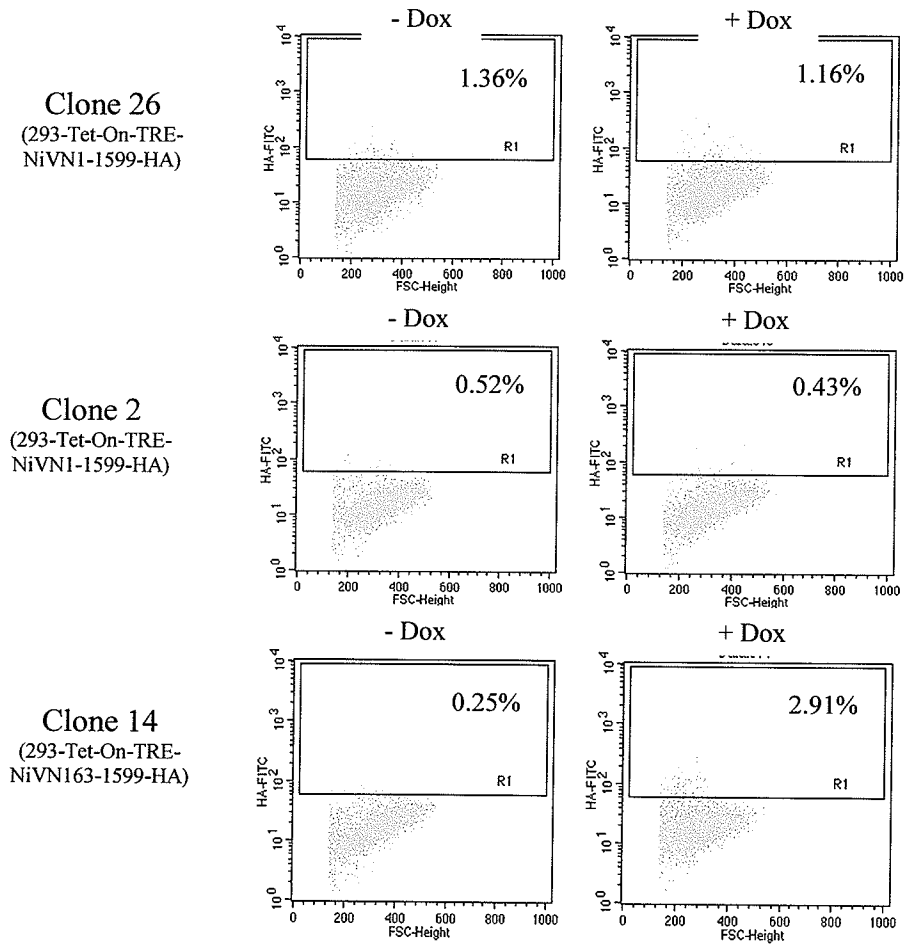


Figure 17: FACS analysis to detect presence of recombinant NiV N protein in induced 293-Tet-On-NivN monoclonal cell lines. In the presence or absence of induction agent doxycycline, cells were harvested, fixed and permeabilized. The primary antibody used was Rabbit anti-HA (1/400 dilution) while the secondary antibody was a FITC conjugated Goat anti-Rabbit (1/5000 dilution). Following staining, cells were run through the BD FACS Calibur™ and analyzed using related CellQuest™Pro software. Detected fluorescence is labeled on the y-axis of the graph. **a)** Controls. Control 293T cells transfected with pNiV-IRES$_{cmv}$ were stained with either the primary and secondary antibody or just the secondary on its own to confirm the absence of non-specific binding. Doxycycline induced control 293 cells that lacked the expression of HA-tagged recombinant NiV N protein were stained with both the primary and secondary antibody. Clone 10, another negative control, was stained with both the primary and secondary antibody both in the presence and absence of doxycycline. Negative control cells from this section were gated upon (R1) and used for comparison purposes to analyze all further samples. **b)** Monoclonal 293-Tet-On-NiV N samples. Clones 26, 2, and 14 were all stained with the primary and secondary antibody in the presence and absence of doxycycline. The percent of cells crossing gate R1 (labeled in the upper right hand corner of the graph) are indicative of the monoclonal cell lines that are expressing their respective recombinant NiV N proteins/variants.

Table 11: Summary table of stable cell lines

	Cloned into pRevTRE Expression Vector	Confirmed NiV N expression from pRevTRE expression vector (Transfection, Western Blot)	Confirmed NiV N transgene expression from Heterologous Stable Cell Line		Confirmed NiV N transgene expression from Homologous Stable Cell Line			
			Western Blot	IFA	Number of Clones Screened	Dot Blot	Western Blot	FACS
NiV N 1-1599	√	√			2			
NiV N 1-1599-HA	√	√	√	√	99	√	√	lost
NiV N 163-1599-HA	√	√	√	√	39	√	√	lost
NiV N 481-1599-HA	√	√			17	√		
NiV N 802-1599-HA	√	√						
NiV N 1-1401-HA	√	√			4	√		
NiV N 1-1101-HA	√	√			11			
NiV N 1-801-HA	√	√			3			
NiV N 163-1401-HA	√							
NiV N 802-1101-HA	√	√				√		

CHAPTER IV: DISCUSSION

IV.I: Understanding how the Over-Expression of the NiV N Protein Suppresses Viral Replication

NiV, a member of the family *Paramyxovirinae*, is a deadly biosafety level four agent. Previous transfection studies have shown that in the presence of over-expressed NiV N protein, specific steps of the viral replication cycle are suppressed (115). To study this phenomenon, stable cell lines using the RevTet-On system were generated, allowing expression of inducible recombinant NiV N proteins. Challenging stable cell lines that express different variants of the NiV N protein, with NiV at various time points (prior to and after induction) will give insight as to what the mechanisms are behind N's ability to suppress viral replication.

IV.II: Rationale for using Retroviral-Mediated, Tet-Regulated RevTet-On System for Generating Stable Cell Lines that Express Inducible Recombinant NiV N Proteins

Biological and medical technologies available for quantitative control of exogenous genes have recently been advanced with the generation of tetracycline-regulated gene expression. Briefly, the system consists of two plasmids, a regulatory plasmid and a response plasmid which contains the gene of interest. With the addition of induction agent tetracycline or the tetracycline derivative doxycycline, the regulatory factors from the regulatory plasmid can bind the response element on the response plasmid and promote transcription of the gene of interest.

Transfer of this unique inducible system into mammalian cells can be accomplished in a variety of ways including transient transfection or stable integration of the gene using transfections or viral gene delivery methods. Even though transient transfections are advantageous for rapid analysis of genes, the transfection efficiency is never consistent nor do they give reproducible results. Alternatively, plasmid DNA transfections used for generating stable cells can be tedious, time-consuming, and show false positives by inferring resistance without integration. This method also lacks stability where 1 to 10% of integrated vectors is not intact and show deletions that hamper future analysis (116).

Modifications to circumvent such limitations include using methods of viral gene delivery. The well characterized retroviral gene delivery systems have several advantages including (103): 1) efficient gene transfer in cell culture since retroviral vectors are produced at high titers; 2) stable integration into the host chromosome is a natural part of the retroviral life cycle and is therefore genetically heritable to all daughter cells; 3) vectors can easily be rendered replication-defective to prevent secondary infection by deletion of specific viral genes; and 4) are defective in viral gene expression therefore minimizing the development of an antiviral immune response.

An inducible gene expression system together with retroviral gene delivery technology creates a powerful tool for implementing stable cell lines where expression of a protein of interest can be regulated. One such system that offers both the advantages of retroviral-mediated and tet-regulated expression of a gene of

interest is the RevTet-On™ System. Unlike most inducible mammalian gene expression systems, where induction is nonspecific and expression levels cannot be precisely regulated, tetracycline control in RevTet systems exhibit tight on/off regulation, high induction levels, high absolute expression, rapid induction times and lack pleiotropic effects (31). Also, the levels of tetracycline or doxycycline necessary for the full range of gene expression are not cytotoxic nor do they effect cell proliferation (31). Furthermore, by having separate regulatory and response vectors, the ratio of the two may vary allowing a variety of expression levels to be attained. These significant advantages of the RevTet system provide a rationale for customizing this particular system to stably integrate and over-express both the full-length and truncated versions of the NiV N protein. Ultimately this system and technical approach can be implemented down the road to investigate the extraordinary phenomenon of the ability of the NiV N protein to block NiV replication.

Stable cell lines generated using the retroviral tet-regulated gene expression system can either be monoclonal or polyclonal. Polyclonal stable cell lines, where a population of cells has different integration sites, have several advantages over monoclonal cell lines. For instance, measuring a polyclonal group of events is less-time consuming and more representative of an entire population, reflecting the overall activity of the transgene rather than activity from just one entity. For these reasons polyclonal populations of cells were initially selected for to induce recombinant NiV N protein expression. However, this approach proved not to be very efficient since only few cells among the polyclonal population expressed the

recombinant NiV N protein. This makes it less likely to see biological effects of the induced NiV N protein. In an attempt to overcome this problem, monoclonal stable cell lines, where a population of cells have a common integration site, were generated. Working with monoclonal cell lines requires many clones to be picked, selected, screened and applied to obtain results that represent the transgene activity in not just one cell, but many. Although working with monoclonal cell lines is tedious, it has the potential of generating an entire population of cells strongly expressing the recombinant NiV N protein.

IV.III: Heterogenous Stable Cell Lines

The full-length NiV N ORF and various N- and C- terminal truncations were cloned into the retroviral response vector, pRevTRE. Subsequently, the single transgenic target cells 293-Tet-On, containing a chromosomal integration of the RevTet-On regulatory transgene, were infected with retrovirus particles packaged with the response pRevTRE-NiV N transgene. The entire population of cells was collected and stable heterogenous integration of the pRevTRE NiV N transgene was selected for with hygromycin. Following selection, several of the doubly-stable 293-Tet-On-TRE-NiVN clones were screened for: a) presence of the transgene using PCR; and b) NiV N expression using Western Blot. NiV N protein expression was confirmed in two of the polyclonal cell lines, one expressing the full-length N and the other a 10% N terminal truncation of the N protein: 293-Tet-On-TRE-NiVN 1-1599-HA and 293-Tet-On-TRE-NiVN 163-1599-HA respectively. Expression of the N protein among the two hygromycin resistant polyclonal cell lines was quite different. For example,

polyclonal cell line 293-Tet-On-TRE-NiVN 1-1599-HA displayed much lower expression of the N protein in the presence of doxycycline than did 293-Tet-On-TRE-NiVN 163-1599-HA.

There is also the question of what the signal to noise ratio is. For example, NiV N protein expression observed may result from many cells expressing low levels of the protein or a few cells expressing high levels of the protein. Therefore, it is imperative to quantify expression levels on a per cell basis via methods such as immunofluorescence or FACS. Immunofluorescene of both polyclonal cell lines showed strong expression in approximately 5 to 10% of the cells with the rest containing minimal to zero protein expression. Due to the higher levels of recombinant NiV N protein expression in 293-Tet-On-TRE-NiVN 163-1599-HA, compared to that seen with 293-Tet-On-TRE-NiVN 1-1599-HA, the dose dependency and time of induction with doxycycline could be more clearly observed. Furthermore, basal expression of the N protein in the absence of doxycycline is undetectable, indicating the tight control of the system. The NiV N transgene was transcribed only in the presence of doxycycline.

It can be speculated that variation of NiV N protein expression might result from the location of integration of the retrovirus into the host cell genome. Studies have shown that proper gene expression of genes requires a sophisticated set of proximal and long-distance regulatory elements (15, 23). It is possible that if integration of the pRevTRE-NiVN retrovirus integrates adjacent to a regulator element within the mammalian chromosome, transcriptional activity could be altered. Independent of distance and orientation, the class of regulatory elements known as the enhancers and

activators, which are found specifically in active human haematopoietic cells, can dramatically elevate transcriptional activity of homologous and heterologous promoters located in *cis* (73, 49, 44, 8, 90, 142).

Even complete silencing of the transgene is common depending on the integration site (95, 104). This would explain why expression of the transgene is absent in the majority of the heterogenous 293-Tet-On-TRE-NiVN 1-1599-HA cell lines.

Whether or not integration is a random event or not remains a controversial topic. Several integration studies within mammalian genomes found that the general integration process was not sequence specific and that a large number of sites in the host genome could serve as integration targets (14, 18, 136, 143). Conversely, other studies have suggested that specific regions of the chromosome are more favorable target sites than others. Such factors include transcription level (128, 130, 144) or DNase I hypersensitivity (47, 125, 126, 137). Other features that are considered hot spots for DNA integration into host cell chromosomes are methylated alternating CpG dinucleotides (74) or even sharp bends such as the most distorted sites within the nucleosome core (92, 109, 110, 111). Consistent with this idea, DNA binding proteins such as the *E. coli* integration host factor create sharp bends in the DNA and hence a more favorable integration site (12). Alternatively, some DNA binding proteins suppress integration near their binding sites such as bacterial transcriptional repressors (112).

Another reason why protein expression seems to be so low may be that the cells lacking an integrated transgene are out-growing the cells that are carrying the

transgene. This can occur by "insertional mutagenesis" causing detrimental effects to the target cell and hence hampering its growth rate (134). Either way, protein expression always tends to be lower in stable cell lines than during a transfection.

To avoid the negative effects that can arise from the location of integration of the transgene into the mammalian chromosome, several strategies including site-specific, homologous and transposon –mediated integration have been developed (74, 92). These strategies were not implemented in the current study and should be considered for use when troubleshooting the lack of NiV N protein expression in inducible stable cell lines.

IV.IV: Homologous Stable Cell Lines

In attempt to optimize NiV N protein expression, and avoid complications that can arise due to the location of integration, monoclonal populations of cells rather than heterologous cells were selected for. Although it is possible to avoid various mechanisms that may counteract protein expression, there are many factors to consider when working with monoclonal populations. Not only is this process labor intensive, the monoclonal population being worked with may not be a true representative of the biological response. In order to have statistically relevant results, many clones need to be screened and tested. Furthermore, monoclonal selection must carefully be considered since the highest expression level may also not correlate with the most representative biological response.

Monoclonal populations of cells containing each of the full-length and truncated versions of the pRevTRE-NiV N transgene were picked and selected for with hygromycin. Thirty two of 175 tested 293-Tet-On-TRE-NiV N clones showed expression of the N protein via Dot Blot. Twenty of these were clones expressing the full-length NiV N protein: 293-Tet-On-TRE-NiV N 1-1599-HA , 9 were clones expressing N-terminal truncated NiV N protein: 293-Tet-On-TRE-NiV N 163-1599-HA, while the other three were 293-Tet-On-TRE-NiV N 481-1599-HA, 293-Tet-On-TRE-NiV N 1-1401-HA, and 293-Tet-On-TRE-NiV N 802-1101-HA. Protein quantification of each of the 32 clones by Western Blot indicated varying expression levels of the NiV N protein. This can be again attributed to the location of integration of the pRevTRE-NiVN retrovirus in the host cell chromosome. The best candidates with the highest NiV N protein expression were clones 26 and 14 expressing TRE-NiV N 1-1599-HA and TRE-NiV N 163-1599-HA respectively. These clones were picked, frozen down and stored for further analysis and experimentation. Clone 2, 293-Tet-On-TRE-NiV N 1-1599-HA, was also chosen since it displayed the same level of NiV N protein expression as clone 14. Several passages later, protein expression levels in these monoclonal cell lines were quantified on a per cell basis using flow cytometry. Unfortunately, after repeated attempts to induce and express the N protein from these three previously positive clones, expression remained undetectable.

Even though in principle, the traditional concept of stable cell lines are cells that indefinitely and at a constant level express a transgene, it is a common phenomenon that stable expression is silenced after passaging the cells for some time (60, 108,

118). Known as "position effect variegation", genes within a subpopulation of a culture can be inactivated by the spread of heterochromatin to positions adjacent to the euchromatin-associated transgene (124, 61, 138, 72). The genetic rearrangements of chromatin generates a strong repressive structure that is attributed to methods such as promoter methylation and histone deacetylation that occur by mechanisms either in the mammalian cell or the retrovirus itself (64, 77, 108, 118, 123, 98, 104, 70, 79, 97). Therefore, in some instances like this one, it may prove beneficial to use a mammalian promoter which would be recognized as a homotypic component. Also, as part of a cells defense mechanism against high copy numbers of viruses and transposons, multiple copies and integration of the transgene will be recognized and silenced (9, 43). All in all, the mechanisms of gene silencing are not well understood and require further research to fully understand each and every mechanism.

Silencing by methylation and histone deacetylation can be combated with the use of chromatin insulators such as β-globin cHS4 (108, 118, 117, 154, 122). Independent of the genome integration site, insulators have the capacity, when positioned on each side of the vector, to protect transgenes from chromatin position effects (118, 145, 16). Overall, insulators are good candidates to fight these negative effects that hamper transgene expression and should therefore be considered for use in future studies.

Another possible mechanism that may account for the loss of protein expression is deletions in the promoter. Cells frequently maintain the parts of the vector they need to survive and dispose of those they do not need, particularly if those parts place a notable burden on the cell from over-expressing a "useless" protein or one that is

slightly toxic. Toxicity of the N protein, however, can be disregarded in this case since cells used throughout this study were induced with doxycycline and passaged several times without any visual cytotoxic effects.

Finally, it is possible for the retroviral vector to never stably integrate into the host cell chromosome. Integration of the retroviral vector is dependent on cell division. Therefore, if the cell was dividing slowly at the time, the MLV retroviral vector, which has a half-life ranging between 5.5 to 7.5 hours, will lose its stability and not integrate into the host cell chromosome (5). Therefore, if the retroviral vector was maintained as an episome, chances of losing it over time are greater. However, this was not the reason why, in this experiment, recombinant NiV N protein expression in the stable cell lines was lost. If the retroviral vector was absent, antibiotic resistance would also be lost, which it was not.

IV.V: NiV Replication in Induced Stable Cell Lines Expressing Recombinant NiV N Protein

Induced heterologous stable cell lines 293-Tet-On-TRE-NiVN1-1599-HA and 293-Tet-On-TRE-NiVN163-1599-HA, which upon induction were shown to express very low levels of the recombinant NiV N protein, were challenged with NiV. Like previous experiments, replication of NiV was suppressed. It was also observed that NiV N protein expression was maintained at the same level as that of the control while P protein expression was increased. The different expression levels of the NiV N and P protein observed may be occurring at the translational level of virus replication. However, this result does not correlate with previous experiments where

cells transiently expressing high levels of NiV N protein had a 98% reduction of NiV P protein compared to cells that did not express any recombinant NiV N protein (115). This difference in observed protein expression may result from using different biological systems. There is also the possibility that the differences in expression levels of the NiV N protein had an effect on the biological response. Therefore, to ensure that there is sufficient expression of the recombinant N protein homologous stable cell lines could be used.

IV.VI: Implementing Stable Cell Lines to Better Understand how Increased Levels of the NiV N Protein Suppresses Viral Replication

Once optimized to express high levels of either the full-length or truncated variants of the NiV N protein the NiV N protein, these double transgenic cell lines will be beneficial tools that can be used to investigate the role over-expressed NiV N protein plays in blocking viral replication. For example, it will be interesting to see if results from previous transfection studies, which showed a drop in viral replication with the over-expression of the N protein, are consistent with infection studies performed with the inducible cell lines expressing high levels of the N protein. This question should be asked for the reason that the chromatin environment of an integrated transgene may give totally different results compared to episomal vectors. Studies have shown that the chromatin structure of circular test plasmids is remodeled in the sense that the nucleosomes are located at different positions throughout the DNA and may therefore affect the experimental results (120).

The regions on the N protein involved in its ability to block viral replication can be identified using these cell lines. To achieve such a goal the induced stable cell lines that are over-expressing the N protein variants can be infected with NiV followed by analysis of viral replication via various read out systems including Northern Blots to detect transcription, Western Blot and flow cytometry to detect translation and real-time PCR to detect replication of the full-length genome. What can be expected, according to previous data, is that over-expression of the full-length NiV N protein will block viral replication (115). If the same effect is observed with truncated versions of the N protein, then it can be concluded that the regions of the N protein remaining are important for abrogating production of progeny virus. On the other hand, if replication is not blocked with over-expression of the truncated N protein then the regions of N remaining are not important for blocking replication.

Once regions of the over-expressed N protein are implicated in blocking viral replication, correlations can be made to link the regions identified to known binding sites on the N protein. For example, binding domains for P and other N molecules have previously been identified (114). Even though the binding site for vRNA has not yet been elucidated, the cell lines expressing various truncations can be a useful tool for such a study. However, it may be the case that none of the already generated truncated constructs will give insight as to what domain on N is responsible for blocking production of progeny virus. This may result if the mechanism involved is more intricate and complex, involving multiple binding domains of both viral and cellular components. In this case stable cells expressing different deletions and truncations will need to be generated.

Another question to investigate is the degree of suppression observed with viral replication in the presence of over-expressed NiV N protein. In previous transfection studies, NiV replication is suppressed to more than five orders of magnitude in cells over-expressing the N protein (115). Transfection efficiencies of less than 100% used throughout that study do not correlate with the drop observed with viral replication (115). The massive drop observed in viral replication is only possible if close to every cell was over-expressing the NiV N protein, an unlikely occurrence with transfections. It may be that the NiV N protein on its own is secreted and taken up by neighbouring cells. To investigate this, flow cytometry on non-permeabilized doxycycline induced double transgenic cell lines can be performed to see if the N protein is secreted.

Furthermore, if previous studies are correct in that viral replication can be blocked with over-expression of the NiV N protein, it may also be possible to "cure" a Nipah virus infection. Nipah virus is capable of persistently infecting NiH3T3 cells. Therefore, by creating stable cells lines that can regulate N expression and persistently infecting them with NiV, N expression can be turned "on" in hopes of wiping out the existing viral infection.

Before any of these questions can be explored, it must be made certain that the monoclonal double transgenic cell lines encode stably the NiV N protein. Since passaging and culturing these cells for certain lengths of time is problematic, stocks when each was first generated will have to be revisited and tested for N expression. If viral expression is still absent then other means of gene delivery and vectors

should be explored. Also, only two of the double transgenics, at one time, were successful in expressing the N protein, the full-length N and one truncated 10% from the N terminal. The other truncations must also be expressed from the stable cell lines in order to narrow down the potential domains on N responsible for blocking viral replication. Furthermore, it is essential to optimize expression of the N protein among these stable cell lines. To accomplish this and find good expressers many more clones will have to be picked and upon induction screened for NiV N expression. Once good expressers are identified several homologous clones of each stable cell line expressing various truncations will have to be analyzed to ensure statistical relevance during further experimentation.

IV.VII: Research Outlook and Summary

Once the mechanism and functional domains of the N protein are identified in its ability to abrogate production of progeny virus, potential implications include molecular crystallization or even comparative molecular modelling of the identified domains. Generation of lead structures can then be used in further antiviral studies towards Nipah virus, a deadly paramyxovirus to which there are no current treatments or vaccines available. Due to the possibility of this having both a global impact on improving human health and agriculture (such as piggeries), it is imperative that studies like this be pursued.

In the end, 293-Tet-On-TRE-NiVN cell lines were successfully established using a retrovirus-mediated tet-regulated gene expression system, RevTet-On, for inducible,

doubly-stable *in vitro* expression of either the full-length or truncated versions of the NiV N gene. This is the first study to utilize the well characterized technology of retroviral gene delivery to generate stable cell lines and investigate the role of NiV proteins with prospects to identify domains on the N protein involved in its ability, when over-expressed, to abrogate production of *de novo* Nipah progeny virus. This system provides a means of controlling N expression in an on/off manner as well as quantitatively by administering doxycycline. Even though the cell lines were generated, NiV N protein expression was eventually lost after carrying them for some time (up to 30 times). This proves that the reproducibility of stably expressing cell lines is inconsistent and unpredictable over time.

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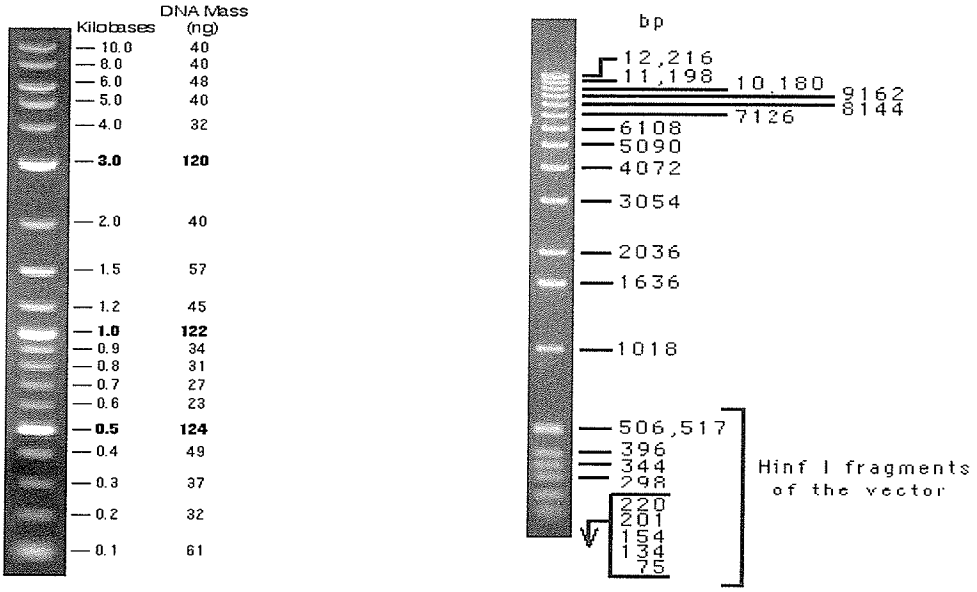
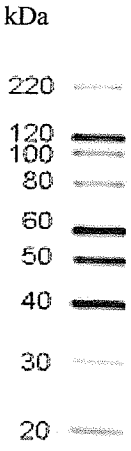
APPENDIX A: DNA and Protein Markers



a) b)

DNA ladders. a) New England Biolabs 2-Log DNA Ladder™ (101) **b)** Gibco 1 kb DNA ladder™ (69). Both gels are stained with EtBr and run on a 1% agarose gel.



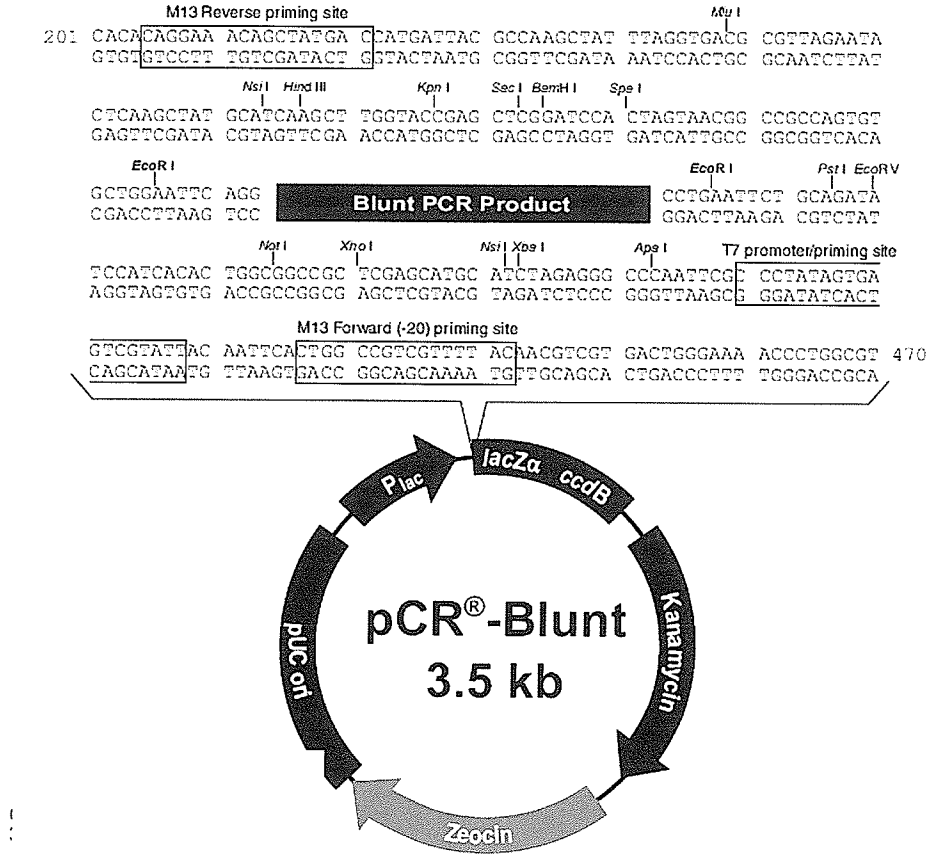
Magic Mark™ XP Protein Standard (68)

APPENDIX B: Sequence of Nipah Virus N ORF

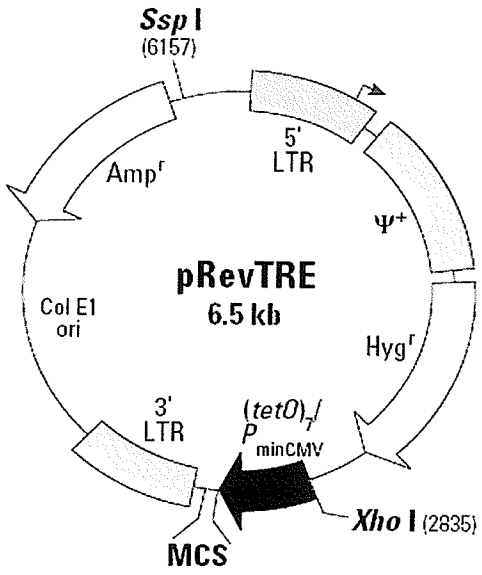
1	ATGAGTGATA	TCTTTGAAGA	GGCGGCTAGT	TTTAGGAGTT	ATCAATCTAA
51	GTTAGGGAGA	GATGGGAGGG	CTAGTGCAGC	AACTGCTACT	TTGACAACCA
101	AGATAAGGAT	ATTTGTACCA	GCTACTAATA	GTCCAGAGCT	CAGATGGGAA
151	CTAACATTGT	TTGCACTTGA	TGTGATTAGA	TCTCCGAGTG	CTGCCGAGTC
201	AATGAAAGTT	GGAGCTGCTT	TCACACTCAT	CTCTATGTAT	TCAGAGAGAC
251	CCGGGGCTCT	CATTAGAAGT	CTCCTCAATG	ACCCAGACAT	TGAAGCTGTA
301	ATAATAGATG	TTGGATCAAT	GGTCAACGGA	ATACCAGTAA	TGGAGAGGAG
351	AGGAGACAAG	GCTCAGGAGG	AGATGGAAGG	CTTGATGAGA	ATCCTCAAAA
401	CTGCTCGAGA	CAGCAGCAAG	GGAAAAACAC	CTTTTGTTGA	CAGCCGAGCT
451	TACGGCCTAC	GGATAACAGA	CATGAGCACC	CTGGTCTCTG	CAGTTATCAC
501	CATCGAGGCC	CAGATCTGGA	TACTGATCGC	TAAAGCAGTT	ACAGCTCCCG
551	ACACTGCCGA	GGAAAGTGAA	ACTAGAAGAT	GGGCTAAATA	CGTCCAACAA
601	AAGAGAGTCA	ATCCGTTCTT	TGCTCTAACT	CAGCAATGGC	TAACAGAAAT
651	GAGGAATCTG	CTCTCCCAGA	GTCTATCAGT	AAGGAAGTTC	ATGGTTGAGA
701	TCCTCATAGA	AGTCAAGAAA	GGAGGATCTG	CTAAAGGCAG	AGCAGTAGAA
751	ATAATCTCAG	ACATCGGAAA	CTATGTCGAG	GAAACTGGTA	TGGCAGGATT
801	CTTCGCAACC	ATCAGATTCG	GGTTGGAGAC	AAGGTATCCA	GCACTTGCAC
851	TCAACGAATT	CCAGAGTGAC	CTCAACACCA	TCAAAAGCTT	GATGCTACTC
901	TACAGAGAAA	TTGGCCCAAG	AGCCCCTTAT	ATGGTGCTTC	TTGAAGAATC
951	AATTCAGACT	AAATTTGCCC	CTGGAGGTTA	CCCATTATTG	TGGAGCTTTG
1001	CCATGGGTGT	GGCTACTACT	ATTGACAGGT	CTATGGGGGC	ATTGAATATC
1051	AATCGTGGTT	ATCTTGAGCC	TATGTATTTC	AGACTAGGCC	AAAAATCAGC
1101	ACGTCACCAT	GCTGGAGGAA	TTGATCAGAA	CATGGCAAAT	AGACTGGGAC
1151	TAAGTTCAGA	TCAAGTTGCA	GAACTCGCTG	CTGCAGTTCA	GGAAACATCA
1201	GCAGGAAGGC	AAGAGAGTAA	TGTTCAGGCT	AGAGAGGCAA	AATTTGCTGC
1251	AGGAGGTGTG	CTCATTGGAG	GCAGTGATCA	AGATATCGAT	GAAGGGGAAG
1301	AACCTATAGA	ACAGAGTGGC	AGACAGTCAG	TTACCTTCAA	AAGGGAGATG
1351	AGTATTTCAT	CCCTTGCTAA	CAGTGTGCCG	AGCAGTTCTG	TGAGCACATC
1401	CGGTGGGACC	AGATTGACTA	ATTCATTACT	AAACCTCAGA	TCAAGACTGG
1451	CTGCAAAAGC	AGCAAAAGAA	GCCGCCTCAT	CCAATGCAAC	AGATGATCCA
1501	GCAATCAGCA	ACAGAACTCA	AGGGGAATCA	GAGAAGAAGA	ATAATCAAGA
1551	CCTCAAACCT	GCTCAAAATG	ACCTTGATTT	CGTCAGAGCT	GATGTGTGA

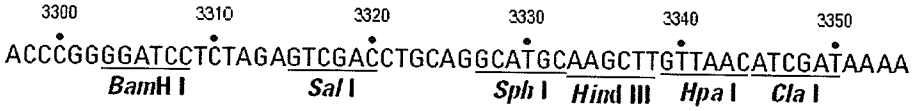
NiV N ORF. 1599 bp DNA (GenBank Accession number AF212302).

APPENDIX C: Cloning Vectors

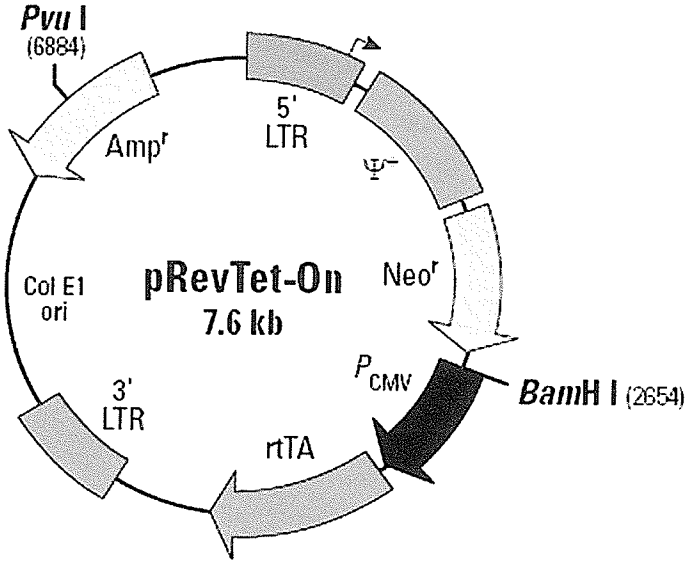


Map of pCR®-Blunt (67).





Map of pRevTRE (31).



Map of pRevTet-On (31).