

MOLECULAR CHARACTERIZATION OF A PORCINE PICOBIRNAVIRUS RNA-
DEPENDENT RNA POLYMERASE.

by

Maanda Noaxe Phosiwa

A dissertation submitted in fulfilment of part of the requirements for the degree of Master of Science in Veterinary Tropical Diseases (Molecular Biology) at the University of Pretoria

Supervisor: Dr. A.C. Potgieter

University of Pretoria, Onderstepoort Campus

November 2008



DECLARATION

I declare that, this dissertation is the original work of the author and has not been submitted for any degree in any other institute, and that all sources used or quoted have been indicated and acknowledged by complete references

CANDIDATE: Maanda Noaxe Phosiwa

SIGNATURE:

DATE:



DEDICATION

To my parents, Mavis and Joseph Phosiwa, I wish you were still with us and be proud of my achievement and reap the reward of the hard work and discipline you installed in me. I love you, may peace be with your souls

To my daughter Lutendo and my family for their love, support and patience through out the course of my studies.

ACKNOWLEDGEMENTS

I would like to acknowledge the following people and organisation without whom, this work would not have been possible:

My Supervisor, Dr. A.C. Potgieter, for his timeless support, critique and encouragement through out the years

Friends and colleagues at OVI Biochemistry Department for accommodating me in their facilities

DST students in OVI for their support and encouragement during the course of the study

Agricultural Research Council (ARC), Department of Science and Technology (DST) and Department of Agriculture (DoA) for funding

Dr. Dennis Bamford, Peter Sarin, Antii and Riitta from the Centre of Excellence (COE) of the University of Helsinki, Finland, for inviting, accommodating and assisting me with some aspects of my study

And Dr. A.J. Musoke, for his support and faith in my abilities, his encouragement when times got harder and his fatherly support through out my time of study.

ABSTRACT

Picobirnavirus is an unclassified dsRNA virus, which is associated with viral gastroenteritis in humans and animals. Picobirnavirus dsRNA has been detected in many cases when diagnostic PAGE screening for rotavirus dsRNA is performed. During this routine diagnosis, picobirnavirus dsRNA has been detected in the faeces of patients with and without viral gastroenteritis. Despite the common occurrence of picobirnavirus infection in humans and animals, its direct involvement in causing gastroenteritis has not been established. No molecular studies have been done on picobirnavirus except sequencing and epidemiology studies. Like all RNA viruses, picobirnavirus encodes a RNA-dependent RNA polymerase. The picobirnavirus RNA-dependent RNA polymerase has only been identified on the basis of its amino acid sequence. The catalytic activity of the polymerase has not been studied to date. In this study, a porcine picobirnavirus was studied at a molecular level to establish the activity of the protein encoded by segment 2 of its genome. To determine the identity of this putative picobirnavirus RNA-dependent RNA polymerase, its open reading frame (ORF) was successfully amplified by PCR, cloned and sequenced. Subsequently the ORF was successfully sub-cloned into baculovirus and bacterial expression vectors. The protein encoded by picobirnavirus segment 2 was successfully expressed as a recombinant protein in a soluble form in both baculovirus and bacterial expression systems. In the baculovirus system, two recombinant baculoviruses were constructed. One recombinant baculovirus expressing a histidine tagged protein and another one expressing an untagged protein. In bacterial expression systems, a recombinant protein fused to a Glutathione-S-Transferase (GST) tag at the N terminal end was expressed. The GST tag allowed easy purification of the expressed GST fusion protein by affinity chromatography on immobilized glutathione. Subsequently the GST tag could be removed from the purified recombinant protein by proteolysis with thrombin. Both tagged and untagged putative picobirnavirus RNA-dependent RNA polymerase from the bacterial expression system were shown to have an affinity for heparin. This implies that the protein might have an affinity for nucleic acids. Picobirnavirus genome segment 2 ssRNA was generated from full length picobirnavirus genome segment 2 cDNA by *in vitro* transcription. The recombinant *E.coli* expressed

proteins (tagged and untagged) was tested for ssRNA binding and RNA replicase activity. No RNA binding or replicase activity was observed with either tagged or untagged recombinant protein. This study reports the first evidence other than conserved polymerase motifs, that the protein encoded by segment 2 of picobirnavirus is most likely a RNA-dependent RNA polymerase.

TABLE OF CONTENT

Title page.....	i
Declaration.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Abstract.....	v
Abbreviation.....	xii

CHAPTER I – INTRODUCTION AND LITERATURE REVIEW

Introduction.....	1
Literature review.....	5
Picobirnavirus.....	5
Picobirnavirus segment 2 (S2).....	7
Overview of viral RNA-dependent RNA polymerase proteins.....	7
1.2.3.1. RNA-dependent RNA polymerases structure.....	8
1.2.3.2. Active form of dsRNA virus RNA-dependent RNA polymerases.....	10
1.2.3.3. Active form of ssRNA virus RNA-dependent RNA polymerase.....	12
Rationale of the study.....	12

CHAPTER II – PCR AMPLIFICATION, SUB-CLONING AND SEQUENCING OF PICOBIRNAVIRUS SEGMENT 2 cDNA

2.1. Introduction.....	14
2.2. Materials and methods.....	15
2.2.1. PCR design.....	15

2.2.2. PCR amplification of picobirnavirus S2 ORF from cloned cDNA.....	16
2.2.3. Standard procedure used for agarose gel electrophoresis.....	17
2.2.4. Agarose gel electrophoresis.....	18
2.2.5. Gel extraction and purification of DNA.....	18
2.2.6. Cloning of PCR products.....	19
2.2.6.1. Preparation of pGEM-T vector.....	19
2.2.6.2. Ligation reaction.....	19
2.2.6.3. Preparation of competent <i>E.coli</i> cells.....	20
2.2.6.4. Transformation of competent <i>E.coli</i> Sure cells.....	21
2.2.6.5. Purification of recombinant plasmids.....	22
2.2.6.6. Restriction enzyme digestion of the recombinant plasmids.....	23
2.2.6.7. Sequencing.....	23
2.3. Results and discussion.....	25
2.3.1. PCR amplification of the picobirnavirus S2 ORF.....	25
2.3.2. Cloning of S2 ORF PCR products into pGEM-T vector.....	29
2.3.3. Sequencing of insert in the recombinant plasmids.....	31
2.4. Summary.....	35

CHAPTER III – EXPRESSION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE USING THE BACULOVIRUS EXPRESSION SYSTEM

3.1. Introduction.....	36
3.2. Materials and methods.....	39
3.2.1. Sub-cloning of picobirnavirus S2 ORF into pFastBac donor plasmids.....	39
3.2.1.1. Plasmids used in this chapter.....	39
3.2.1.2. Restriction enzyme digestion of the donor plasmids.....	39
3.2.1.3. Preparation of insert DNA(s).....	41
3.2.1.4. Ligation reaction.....	41

3.2.1.5. Transformation of competent <i>E.coli</i> Sure cells.....	41
3.2.1.6. Purification (miniprep), restriction digestion and sequencing of recombinant pFastbac plasmids.....	41
3.2.2. Generation of recombinant Bacmid.....	42
3.2.2.1. Transformation of AcBac Δ CC cells with pFastBac constructs.....	42
3.2.2.2. Purification of recombinant Bacmid.....	42
3.2.2.3. Analysis of the recombinant Bacmid by PCR.....	43
3.2.3. Transfection of Sf9 and pilot expression of the putative picobirnavirus RNA-dependent RNA polymerase using the Bac-to-Bac baculovirus expression system.....	44
3.2.3.1. Transfection of insect cells with recombinant bacmid DNA.....	44
3.2.3.2. Pilot expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase.....	45
3.2.3.3. Standard procedure used for SDS-PAGE.....	45
3.2.3.4. Cell lysis and SDS-PAGE analysis.....	47
3.3. Results and discussion.....	49
3.3.1. Preparation of donor plasmids and picobirnavirus segment 2 ORF for cloning.....	49
3.3.2. Sub-cloning of picobirnavirus segment 2 ORF into pFastBac donor plasmids.....	49
3.3.3. Sequencing of insert in the recombinant donor plasmid.....	52
3.3.4. Generation of recombinant bacmid(s).....	54
3.3.5. Transfection and pilot expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase in Bac-to-Bac baculovirus system.....	55
3.4. Summary.....	57

CHAPTER IV – EXPRESSION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE USING A BACTERIAL SYSTEM

4.1. Introduction.....	58
4.2. Materials and methods.....	59
4.2.1. Sub-cloning of picobirnavirus S2 ORF into the pGEX-4T vector for bacterial expression.....	59
4.2.2. Expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase in bacteria cells.....	59
4.2.2.1. Transformation of <i>E.coli</i> BL21 cells with recombinant pGEX-4T-PBV 2B.....	59
4.2.2.2. Expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase in recombinant <i>E.coli</i> BL21 cells.....	60
4.2.2.3. Determination of the yield and solubility of recombinant fusion protein expressed under different conditions.....	61
4.3. Results and discussion.....	62
4.3.1. Sub-cloning of picobirnavirus S2 ORF into bacterial expression vector pGEX-4T.....	62
4.3.2. Expression of the putative picobirnavirus RNA-dependent RNA polymerase in bacterial cells.....	65
4.3.3. Expression and solubility of the putative picobirnavirus RNA-dependent RNA polymerase expressed under different conditions.....	66
4.4. Summary.....	68

CHAPTER V – PURIFICATION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA PLOYMERASE – GST FUSION PROTEIN

5.1. Introduction.....	69
5.2. Materials and methods.....	70
5.2.1. Protein extraction from bacterial cells.....	70
5.2.2. Purification of GST fusion protein.....	70
5.2.3. GST tag removal procedure.....	71
5.3. Results and discussion.....	73
5.3.1. Purification of recombinant putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein.....	73
5.3.2. Removal of the GST tag from recombinant putative picobirnavirus RNA-dependent RNA polymerase.....	75
5.4. Summary.....	77

CHAPTER VI – MOLECULAR PROPERTIES OF PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE

6.1. Introduction.....	78
6.2. Materials and methods.....	70
6.2.1. <i>In vitro</i> generation of authentic picobirnavirus S2 RNA transcripts.....	80
6.2.1.1. Generation of full length picobirnavirus S2 transcription containing RNA polymerase promoters.....	80
6.2.1.2. <i>In vitro</i> RNA transcription.....	82



6.2.2. Mobility shift assay.....	83
6.2.3. Binding of soluble putative picobirnavirus RNA-dependent RNA polymerase to heparin.....	83
6.2.4. Heparin binding to thrombin treated GST fusion protein.....	84
6.2.5. RNA replication assay.....	85
6.3. Results and discussion.....	87
6.3.1. Generation of RNA synthesis template (picobirnavirus S2 cDNA) by PCR....	87
6.3.2. Generation of ssRNA template.....	88
6.3.3. Mobility shift assay.....	91
6.3.4. Affinity purification of soluble putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein using heparin.....	93
6.3.5. Heparin binding of thrombin treated protein (untagged putative picobirnavirus RNA-dependent RNA polymerase).....	95
6.3.6. RNA replicase assay.....	96
6.4. Summary.....	99
CHAPTER VII – CONCLUDING REMARKS.....	100
CHAPTER VIII – REFERENCE.....	103
CHAPTER IX – APPENDICES.....	110
Appendix 1: List of figures.....	110
Appendix 2 Protocol manuals.....	116

ABBREVIATIONS

3'	3' terminus
5'	3'terminus
°C	degree celsius
µl	micro-liter
µg/ml	microgram per millilitre
pmol/µl	picogram per microlitre
A	adenine
aa	amino acid
ATP	adenosine triphosphate
bp	base pair
BTV	bluetongue virus
C	cytosine
CaCl ₂	calcium chloride
cDNA	complementary DNA
CTP	cytidine triphosphate
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dsRNA	double-stranded RNA
<i>E.coli</i>	<i>escherichia coli</i>
EDTA	ethylenediamine tetra-acetate
EtBr	ethidium bromide

FBS	foetal Bovine Serum
G	guanine
g	grams
GC	guanine-cytosine
GDD	glycine-aspartate-aspartate
GST	glutathione-S-transferase
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
His	histidine
hrs	hours
ICTV	International Committee for the Taxonomy of Viruses
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilo-bases
KCl	potassium chloride
kDa	kilo Daltons
KH ₂ PO ₄	potassium phosphate
LB	Luria broth
M	molar
MgCl ₂	magnesium chloride
Mg(CH ₃ COO) ₂ ·H ₂ O	magnesium acetate
ml	millilitres
min	minute(s)

MnCl ₂	manganese chloride
mRNA	messenger RNA
NaCl	sodium chloride
Na ₂ EDTA	sodium ethyl diamine tetra-acetate
Na ₂ HPO ₄	sodium phosphate
ng	nanogram
NH ₄ CH ₃ COO	ammonium acetate
NCBI	National Centre for Biotechnology Information
nm	nanometres
NP40	Nonidet P-40 (octyl phenoxy polyethoxy ethanol)
nt	nucleotides
NTP	deoxyribo-nucleoside triphosphate
ORF	open reading frame
OD ₆₀₀	optical density at 600nm
OVI	Onderstepoort Veterinary Institute
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered solution
PBV	picobirnavirus
PCR	polymerase chain reaction
Pipes	piperazine-N, N'-bis[2-ethane-sulfonic acid]
pl	isometric point
pmol	picomole
pol	polymerase
<i>polh</i>	poyhedrin promoter
RdRp	RNA-dependent RNA polymerase
RHDV	rabbit hemorrhagic disease virus

RNA	ribonucleic acid
rpm	revolutions per minute
rNTP	ribo-nucleoside triphosphate
s	second(s)
SDS	sodium dodecyl sulphate
ssRNA	single stranded RNA
Sf 9	<i>spodeptera Frugiperda</i>
T	thymine
TAE	tris acetate EDTA
<i>Taq</i>	<i>thermus aquaticus</i>
TB	transformation buffer
TBE	tris borate EDTA
TC	transcription complex
Tm	melting temperature
TTP	thymine triphosphate
Tris	tris-(hydroxymethyl)-aminomethane
U	units
UV	ultraviolet
V	voltage
VP	viral protein
v/v	volume to volume ratio
X-gal	d-galactopyranoside
w/v	weight to volume ratio

CHAPTER I INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Double-stranded RNA viruses (dsRNA viruses) represent a large, diverse group of viruses that infects a large range of hosts. Their hosts range from the terrestrial and non-terrestrial vertebrates to arthropods, molluscs, plants, fungi, and prokaryotes. Several dsRNA viruses are pathogens and therefore of major medical, veterinary or agricultural importance. Many of these pathogens have an impact on the economy (Knipe *et al.*, 2001; Mertens, 2004). Among these are rotavirus (major cause of infant mortality), bluetongue virus that causes disease in ruminants, specifically sheep, African horsesickness virus that causes disease in equids, especially horses, birnaviruses that cause losses in the poultry and fishery industries, and cypoviruses that cause infection in arthropods and present a threat to silk industry (Mertens, 2004). Because of the economic importance of dsRNA viruses, it is wise to expand our knowledge about these viruses in order to develop ways in which they can be controlled.

Currently, dsRNA viruses are grouped into eight distinct families, recognized by the International Committee for the Taxonomy of Viruses (ICTV) (Knipe *et al.*, 2001). Those are the *Birnaviridae*, *Cystoviridae*, *Chrysovriidae*, *Hypoviridae*, *Partitiviridae*, *Reoviridae*, *Totiviridae*, and *Varicosavirus* (Table 1). Only two of these families of dsRNA viruses infect animal cells, namely those from the families *Reoviridae* and *Birnaviridae*. The rest of the families infect fungi (*Partitiviridae*, *Totiviridae* and *Hypoviridae*), protozoa (*Totiviridae*), plants (*Reoviridae*) or bacteria (*Cystoviridae*) (Knipe *et al.*, 2001). The dsRNA viruses are distinguished from one another by their genome organization, protein coding strategies, virion structure and host range (Knipe *et al.*, 2001).

Most dsRNA virions consist of one or two protein shells and a segmented dsRNA genome enclosed inside the protein shell(s) (Fig. 1). The viral particle sizes range from 30-360nm (Knipe *et al.*, 2001, Mertens, 2004). The dsRNA viruses share some common structural

features. For example, the outer capsid of reoviruses (family *Reoviridae*), the single capsid of birnaviruses (family *Birnaviridae*), and the capsid of *Cystoviruses* (family *Cystoviridae*) are arranged in a T=13 symmetry, a feature unique to these viruses (Knipe *et al.*, 2001). Also, the inner capsid of reoviruses, the single capsid of *Totiviruses* (family *Totiviridae*) and the inner capsid of *Cystoviruses* are arranged in a T=1 symmetry (Knipe *et al.*, 2001). Apart from that, the majority of the dsRNA viruses have an icosahedral structure, with the exception of the *Hypoviridae* which do not have a capsid, but a pleomorphic vesicle and *Varicsovirus* which have a rod shape (Table 1) (Mertens, 2004).

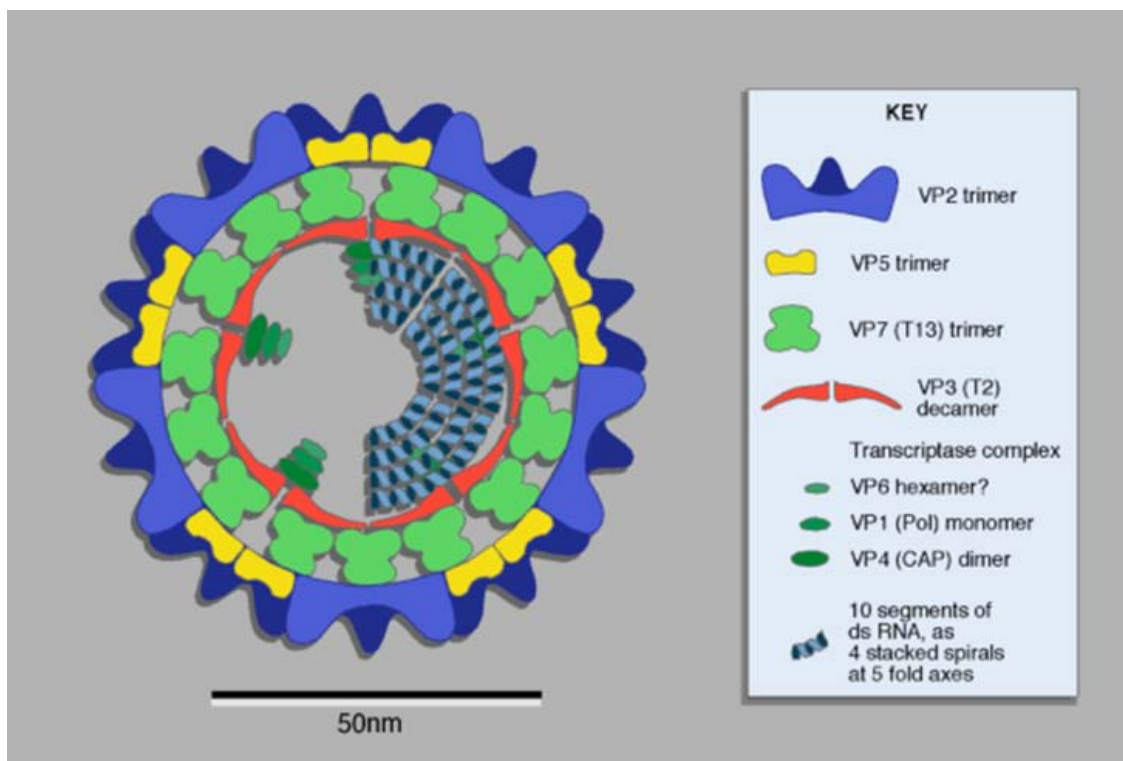


Figure 1. An illustration of the structure of a bluetongue virus particle. The picture illustrates the segmented dsRNA genome encapsidated by two protein layers. The polymerase complex at the 5 fold axis is also illustrated. Picture courtesy of P.P.C. Mertens (Mertens, 2004).

Table 1. The families of dsRNA viruses.

Family	Number of genome segments	Types of virus particle	Host
<i>Birnaviridae</i>	2	Icosahedral single shell	Fish, insect, birds, moluscus
<i>Cystoviridae</i>	3	Icosahedral nucleocapsid	bacteria
<i>Chrysoviridae</i>	3	Icosahedral protein capsid	Fungi
<i>Hypoviridae</i>	1	Pleomorphic vesicles	Fungi
<i>Partitiviridae</i>	2	Icosahedral protein capsid	Fungi, plants
<i>Reoviridae</i>	10, 11, or 12	Icosahedral 1/2/3 layered protein capsid)	Insect, plants, reptiles, birds, mammals, arachnids, fungi, arthropods, crustaceans
<i>Totiviridae</i>	1	Icosahedral	Fungi
<i>Varicosavirus</i>	2	Rod shaped	Plants

Adapted from Mertens, 2004.

Another common feature of the dsRNA viruses is that they contain a mRNA synthesizing enzyme (RNA-dependent RNA polymerase) which is contained in the capsid together with the viral genome. All dsRNA viruses have a segmented dsRNA genome, with the number of segments ranging from 2 in the family *Birnaviridae* up to 12 in some members of the family *Reoviridae* (Table 1). The dsRNA genome is contained safely inside the protein capsid (shell). The capsid of dsRNA viruses can be single, double or triple-layered (Mertens, 2004). Double or triple layered capsid increase the protection of the genome so that viral particles can survive for extended periods in the extracellular environment and

be protected from host defense mechanisms during infection. There are 135 dsRNA virus species that are recognized by the ICTV, with additional 52 viruses that have not yet been classified. The majority of these impressive numbers of virus species is classified within the family *Reoviridae*, with 74 classified and 30 unclassified viruses (Mertens, 2004).

Despite the diversity of dsRNA viruses, most share the same replication pathway or strategy (Mertens, 2004, Mertens and Diprose, 2004). For example, upon entry of the host cell, the virion is converted into transcriptionally active viral particles, which can synthesize positive ssRNA(s) from the genomic dsRNA (Mertens and Diprose, 2004). The ssRNA is transported out of the viral particle to the cytoplasm of the host cell where it is translated into viral protein using the host cell's protein synthesis machinery. The viral proteins and the ssRNA then assemble to form new transcriptionally active viral particles, which package the positive ssRNA from the old viral particles. The packaged particles will develop into mature viruses which are released by lysis of the host cell or budding (Mertens and Diprose, 2004). Therefore, both replication of the viral genome and its transcription depend on the transcriptionally active viral particle. Transcriptionally active particles must contain an active polymerase, which will transcribe and replicate the viral genome.

One of the unclassified dsRNA viruses namely picobirnavirus (PBV) is of interest in this study. Of particular interest is the protein encoded by genome segment 2, which has been proposed to be a RNA-dependent RNA polymerase.

1.2. LITERATURE REVIEW

1.2.1. Picobirnavirus

Picobirnaviruses (PBV) were first detected in faecal specimens from humans and rats in 1988 (Wakudu *et al.*, 2005). In recent years it has been detected in faecal samples of infants with gastroenteritis. Picobirnavirus dsRNA is usually detected in stool samples from humans and animals with gastroenteritis, when diagnostic PAGE (Polyacrylamide Gel Electrophoresis) is performed for the detection of rotavirus, a dsRNA virus which is a major cause of viral gastroenteritis (Rosen *et al.*, 2000, Wakudu *et al.*, 2005). The virus has also been detected in faecal samples from domestic animal and wild animals like pigs, guinea pigs, rats, hamsters, giant anteaters, foals, calves, and chickens (Gatti *et al.*, 1989, Ludert *et al.*, 1991, Pereira *et al.*, 1988, Wakudu *et al.*, 2005).

Like most dsRNA viruses, the picobirnavirus capsid has an icosahedral structure and the virus particles have an un-descriptive surface when viewed by electron microscopy (Ludert *et al.*, 1991, Rosen *et al.*, 2000, Wakudu *et al.*, 2005). Their sizes range from 35-41nm in diameter. The virions are non-enveloped with a buoyant density of 1.38-1.40g/ml in caesium chloride (Ludert *et al.*, 1991, Rosen *et al.*, 2000, Wakudu *et al.*, 2005). Since picobirnavirus is usually detected in animals and humans with gastroenteritis, it has been suggested that the virus might be an opportunistic pathogen or innocuous virus in the intestine. However, the direct involvement of the virus in gastroenteritis has not yet been established (Wakudu *et al.*, 2005). The genome of picobirnavirus consists of two dsRNA segments (Fig. 2), hence it was given the name picobirnavirus (pico meaning small and bi-rna-virus means bisegmented RNA virus). The sizes of the two segments varies from 2.3-2.6kb for the larger segment (segment 1) and 1.5-1.9kb for the smaller segment (segment 2). It has been suggested that segment 2 encodes the RNA-dependent RNA polymerase and that segment 1 may encode the capsid protein(s) (Rosen, *et al.*, 2000). Interestingly it has also been suggested that the genome size is too small to code for all the required proteins to create a complete viral particle. Further studies are therefore

required to investigate how many proteins segment 1 codes for and whether they are indeed the capsid proteins.

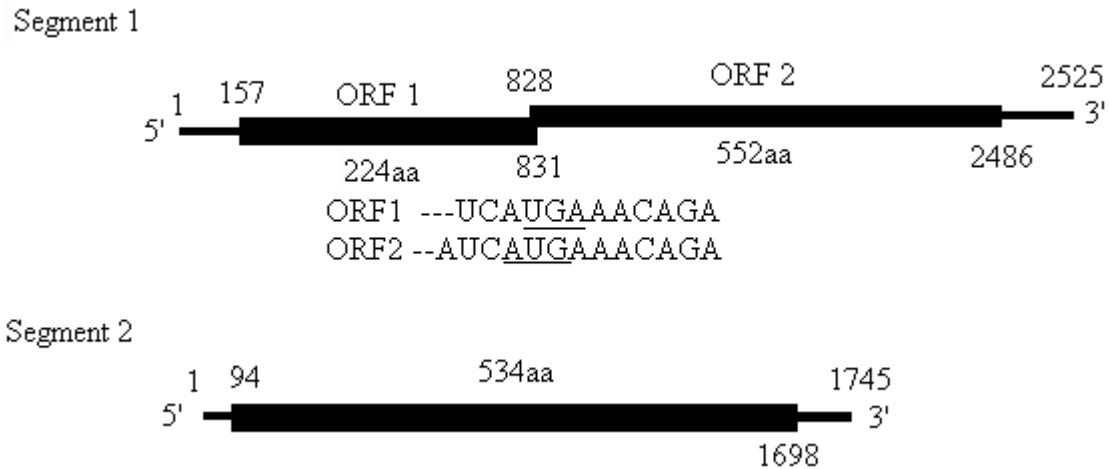


Figure 2. Genome organization of segment 1 and segment 2 of picobirnavirus. The numbers indicate the positioning of the nucleotide for the ORFs in each segment. Segment 1: Positions 1 and 2525 represent the start and end of full length segment 1 respectively. Positions 157 and 831 represent the start and end of segment 1 ORF1 respectively. Position 828 and 2486 represents the start and end of segment 1 ORF 2 respectively. The termination codon for ORF1 (UGA) and initiation codon for ORF2 (AUG) in segment 1 overlap. Segment 2: Position 1 and 1745 represent the start and end of the full length segment 2 respectively, and position 94 and 1698 represents the start and end of segment 2 ORF respectively. Adapted and modified from Wakuda *et al*, 2005.

There are two types of picobirnavirus species, referred to as typical and atypical picobirnavirus. The typical picobirnavirus has been detected in the animals as mentioned above. Atypical picobirnavirus have been isolated from oocysts of *Cryptosporidium parvum* and in human stool containing *Cryptosporidium parvum* (Wakudu *et al.*, 2005). In contrast to the typical picobirnavirus, the size of atypical picobirnavirus genome is 1.8kb for segment 1 compared to the typical picobirnavirus's 2.3-2.5kb, and segment 2 is 1.4kb compared to the typical picobirnavirus's 1.5-1.7kb (Wakudu *et al.*, 2005). Apart from the

difference in their genome size, segment 1 of the atypical picobirnavirus has an ORF (open reading frame) encoding a putative RNA-dependent RNA polymerase and segment 2 may encode the capsid protein, which is the opposite in typical picobirnavirus (Wakudu *et al.*, 2005). These features discriminate the viruses from one another. The porcine picobirnavirus that will be used in this study is a typical picobirnavirus.

1.2.2. Picobirnavirus segment 2 (S2)

In 2005, the genome sequence of both segments of a picobirnavirus from an infant with acute non-bacterial gastroenteritis was published (Wakudu *et al.*, 2005). In 2000, Rosen, *et al.*, reported that the deduced amino acid sequence of S2 has three conserved RNA-dependent RNA polymerase motifs, which are found in most RNA-dependent RNA polymerase proteins. All three motifs were observed between amino acid 259 and 363, with motif 1 observed between amino acid 259 and 269, motif 2 observed between amino acid 318 and 334, and motif 3, which contain the GDD sequence, is observed between amino acid 359 and 363. The protein consists of 534 amino acids with a theoretical molecular weight of 60kDa and a pI of 8.13 (Wakudu *et al.*, 2005). Apart from this basic molecular data for the picobirnavirus RNA-dependent RNA polymerase, no further studies have been conducted to prove its identity. The involvement of the polymerase in the life cycle of the virus has also not yet been established, i.e. its involvement in replication and/or transcription of the genome, and whether or not it needs accessory proteins like a helicase to assist with replication of the genome.

1.2.3. Overview of viral RNA-dependent RNA polymerase proteins

The RNA-dependent RNA polymerase (RdRp) is one of the essential proteins encoded by all dsRNA viruses. dsRNA viruses have only one copy of each genome segment and therefore only one copy of the segment encoding the RNA-dependent RNA polymerase (Bruenn, 1991, Bruenn, 2003, Hey *et al.*, 1986, Van Dijk *et al.*, 2004). The RNA-dependent RNA polymerases catalyze the transcription of genomic dsRNA into single-

stranded sense copies (mRNA) and then replicate the sense RNA to form genomic dsRNA. Therefore, it is responsible for transcription of positive sense RNA from genomic dsRNA and also for replication of viral genome during packaging. Both these functions make the RNA-dependent RNA polymerase an essential enzyme for the life cycle of RNA viruses (Bruenn, 1991, Butcher *et al.* 2001, Van Dijk *et al.*, 2004). This enzyme is also important since the host cells do not contain a RNA-dependent polymerase. Therefore, each RNA virus needs to have its own copy of RNA-dependent RNA polymerase. RNA polymerases are usually enclosed in a transcription complex, and a single virion can have one or more transcription complexes (Mertens *et al.*, 2004, Van Dijk *et al.*, 2004).

1.2.3.1. RNA-dependent RNA polymerase structure

The structures of several RNA-dependent RNA polymerases have been determined using X-ray crystallography, for example, the RNA-dependent RNA polymerase of Hepatitis C virus (Biswal *et al.*, 2005) and RNA-dependent RNA polymerase of Infectious Bursal Disease Virus (Pan *et al.*, 2007). In bluetongue virus, the structure of the transcription complex has been determined by X-ray crystallography of BTV cores (Mertens and DeRose, 2004). This transcription complex is composed of three minor structural proteins namely VP1, the polymerase, VP6, the helicase and VP4, the capping enzyme (Mertens, *et al.*, 2004). All the RNA-dependent RNA polymerases assume a 3-dimensional right hand conformation, with a palm, thumb and finger domain (Figures 3 and 4). There is no significant primary amino acid sequence conservation between the RNA-dependent RNA polymerase of RNA viruses, but, as mentioned before, there are several conserved motifs (Bruenn, 1991, Butcher *et al.* 2001, Maraver *et al.*, 2003, O'Reilly and Kao, 1998, Philipps *et al.*, 2005). The conserved motifs are found in the secondary structure of each polymerase in an ordered manner and all form part of the palm domain (O'Reilly and Kao, 1998). These motifs can fold onto one another to form a large domain of about 120-210 amino acids long (Fig. 5).

The four motifs have been named motif A, B, C, D, and E (Fig. 5). Motif A is situated in the N-terminal followed by motif B, with motif E in the C-terminal of the polymerase

(Maraver *et al.*, 2003, O'Reilly and Kao, 1998, Philipps *et al.*, 2005). Among the four motifs, motif C is the most conserved amongst the RNA-dependent RNA polymerases. It is embedded in the hydrophobic residues and characterized by a GDD (Gly-Asp-Asp) amino acid sequence. This has also been defined as the 'polymerase site' (Poch *et al.*, 1989, Van Dijk *et al.*, 2004). The two aspartate amino acids in motif C are involved in RNA synthesis. They coordinate with a magnesium ion during RNA synthesis and are also involved in sugar selection so that only ribonucleotides are incorporated instead of deoxyribonucleotides (O'Reilly and Kao, 1998).

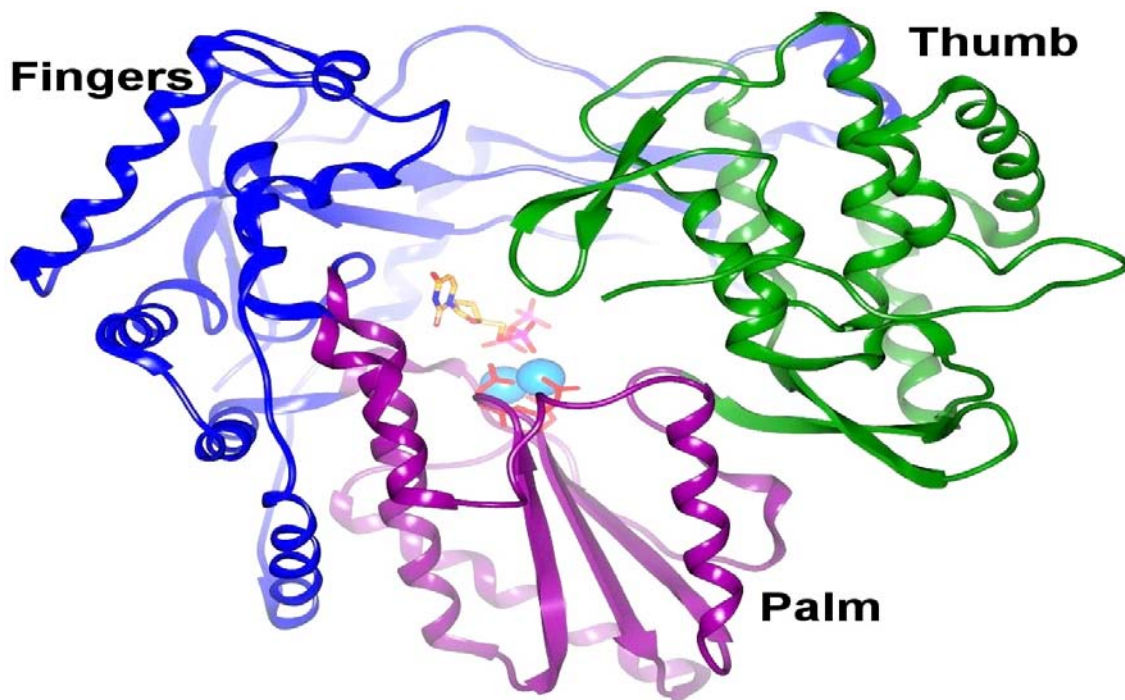


Figure 3. The three-dimensional structure of the phi6 RNA-dependent RNA polymerase showing the three domains. Blue, purple and green colours corresponding to finger, palm and thumb respectively (Makeyev and Grimes, 2004).

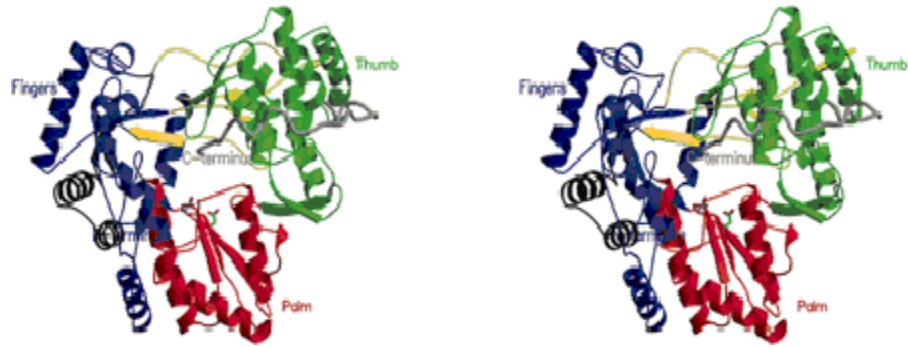


Figure 4. The three dimensional structure of Hepatitis C Virus RNA-dependent RNA polymerase showing the three domains. Blue, red and green colours correspond to the finger, palm and thumb domains, respectively. The yellow colour represents the loop domain (Biswal *et al.*, 2005).

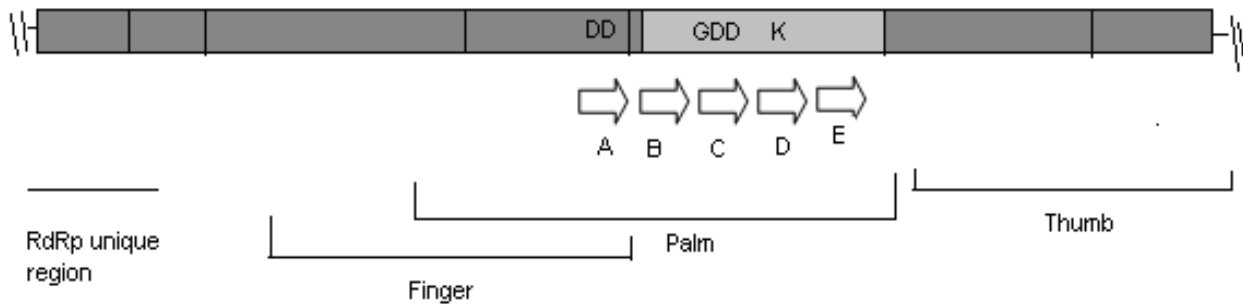


Figure 5. Conserved motif in the palm domain of RNA-dependent RNA polymerase from a HCV virus. Adapted and modified from O'Reilly and Kao, 1998.

1.2.3.2. Active forms of dsRNA virus RNA-dependent RNA polymerases

RNA-dependent RNA polymerases exist in two different active forms depending on the virus. The polymerase can either be fully active only when it is in complex with other viral structural proteins, or when it is not associated with other viral proteins, for example when expressed as recombinant subunits (Bernstein and Hruska, 1981). In dsRNA viruses, RNA-dependent RNA polymerases are mostly found in association with other capsid (structural) proteins to form a complex, which is referred to as polymerase complex.

For instance, in rotavirus (dsRNA virus, family *Reoviridae*), the RNA-dependent RNA polymerase (VP1) forms part of the capsid proteins (VP2) (Bernstein and Hruska, 1981, Gorziglia and Espraza, 1981, Patton *et al.*, 1997). In fact, rotavirus VP1 is only active in complex with other viral proteins (VP2 and VP6). Despite this, rotavirus VP1 can bind to nucleotides and ssRNA when it is isolated from the complex.

In reovirus (the first dsRNA virus to be characterized, family *Reoviridae*), the RNA polymerase ($\lambda 3$) is situated in the interior of the viral core particle in a complex with other viral proteins (Gillies *et al.*, 1971, Starnes and Joklik, 1993, Tao *et al.*, 2002). The polymerase complex is formed when $\lambda 3$ is associated with $\mu 2$, the enzyme responsible for removal of 5'- γ -phosphate prior to capping and $\lambda 2$, the capping enzyme (Starnes and Joklik, 1993, Tao *et al.*, 2002). Both these viral proteins assist the polymerase activity by capping the nascent strands. The capped transcript then serves as mRNA for protein translation and for packaging in the new viral particles prior to its replication to form the dsRNA genome. Protein $\lambda 3$ is not active in isolation, therefore, for complete polymerase activity, $\lambda 3$ needs to be associated with other reovirus core proteins.

In bluetongue virus (BTV, family *Reoviridae*), the RNA-dependent RNA polymerase (VP1) forms a transcription complex with two other structural viral proteins. BTV VP1 is the subunit responsible for the polymerase activity. BTV VP4 is responsible for capping the newly synthesized strands and BTV VP6 is responsible for unwinding dsRNA in order for VP1 to bind to ssRNA prior to transcription (Mertens and Diprose, 2004). Therefore, the complex assists the VP1 subunit by unzipping the dsRNA and allows VP1 to bind to the open strand and synthesise the mRNA. Boyce *et al.*, (2004) reported that purified recombinant BTV VP1 was able to synthesise dsRNA from a viral positive strand template in a template-independent manner. This was the first polymerase from the *Reoviridae* family to show replication activity in the absence of other viral proteins. It was demonstrated that BTV polymerase activity is template-independent by *in vitro* synthesis of dsRNA from ssRNA templates of BTV S10 (822nt, smallest BTV genome segment), L1 (3,954nt, Largest BTV genome segment), L2 (2,926nt) and M4 (2,011nt, medium size

segment) (Boyce *et al.*, 2004). The BTV VP1 is able to synthesise these segments in the absence of primers, a process referred to as *de novo* synthesis.

In contrast to most RNA-dependent RNA polymerases of the family *Reoviridae*, the RNA-dependent RNA polymerase of $\Phi 6$ (dsRNA bacteriophage with three genome segments), is completely active in isolation. It can synthesise mRNA from dsRNA and dsRNA from single-stranded mRNA without being associated with other viral proteins (Juuti and Bamford, 1997, Makeyev and Bamford, 2000a, Makeyev and Grimes, 2004). In addition, it was found that the polymerase could efficiently replicate a broad range of both phage-specific and heterologous ssRNA templates *in vitro*. In the virus particle, the RNA-dependent RNA polymerase subunit of $\Phi 6$ is also found in a polymerase complex. Within this complex there are three other viral proteins, which either assist in structural support (P1) or in RNA packaging and particle stability (P4 and P7) (Gottlieb *et al.*, 1990, Juuti and Bamford, 1997, Makeyev and Bamford, 2000b, Makeyev and Grimes, 2004). In this complex only the P2 polymerase subunit is responsible for genome replication and transcription (Makeyev and Grimes, 2004). During dsRNA transcription, it seems as if the polymerase subunit requires the whole complex to be present since it cannot initiate dsRNA transcription, but with the whole complex assembled, the P2 polymerase is able to initiate transcription. $\Phi 6$ P2 needs other protein(s) to unwind the dsRNA genome (Makeyev and Grimes, 2004). After initiation, the polymerase subunit does not require any further assistance by the proteins in the complex. It extends the nascent strand to full-length ssRNA without assistance from other proteins in the polymerase complex (Makeyev and Grimes, 2004).

1.2.3.3. Active forms of ssRNA virus RNA-dependent RNA polymerase

In contrast to some of the RNA-dependent RNA polymerases of dsRNA viruses, the ssRNA virus RNA-dependent RNA polymerases are mainly active in isolation. For example the RNA-dependent RNA polymerase of *Calicivirus*, a virus with a sense ssRNA

genome and in particular rabbit haemorrhagic disease virus, is active in isolation. It can replicate the viral genome independent of other viral proteins (Ng *et al.*, 2002). This may be because a helicase enzyme is not required to unwind the segments as with the genome of dsRNA viruses. The rabbit hemorrhagic disease virus RNA polymerase has also been successfully expressed as a recombinant protein in bacteria and purified (Lopez-vazquez *et al.*, 1998, Makeyev and Bamford, 2000a, Ng *et al.*, 2002). Its ability to replicate the ssRNA template with divalent ions has been demonstrated (Ng *et al.*, 2002, Van Dijk *et al.*, 2004). The same system is employed by the poliovirus polymerase (P2), where the polymerase has been shown to replicate ssRNA template independent of other viral proteins (Makeyev and Bamford, 2000a, Morrow *et al.*, 1987, Neufeld *et al.*, 1991a, Neufeld *et al.*, 1991b). Apart from the examples above, many RNA-dependent RNA polymerases of different ssRNA and dsRNA viruses have been studied. These studies provide important information on the replication, packaging and transcription of ssRNA and dsRNA viral genomes during virus replication (Morrow *et al.*, 1987, Neufeld *et al.*, 1991a, Neufeld *et al.*, 1991b).

1.3. Rationale of this study

Picobirnavirus genome segment 2 was of a particular interest in this project and more specifically the segment 2 of a porcine picobirnavirus field strain from Bari, Italy. In a collaboration between the University of Bari (Italy) and the ARC-OVI (South Africa), the segments of this virus was amplified, cloned and sequenced at the Biochemistry section at ARC-OVI using the methods described by Potgieter *et al.*, 2002. When the porcine picobirnavirus segment 2 sequence is translated into amino acid sequence and compared to other sequences in the GenBank database (NCBI-National Centre for Biotechnology Information), the results show that the sequence encodes a protein belonging to the RNA-dependent RNA polymerase family of single-stranded RNA viruses, with closest relationship to the RNA-dependent RNA polymerase of rabbit haemorrhagic disease virus (Potgieter A.C. unpublished data). The questions which arise from the sequence comparison are: does picobirnavirus segment 2 encode a RNA-dependent RNA polymerase? If so, is it translated into an active form without needing post-translational

modification (activation by proteolytic cleavage), or is it active in isolation? Since the translated protein is so small (about half the molecular weight of bluetongue VP1, 150kDa) does it form dimers or trimers? Does it have helicase activity or does this activity reside in the capsid of the virus? How does this dsRNA virus unwind the dsRNA during transcription? No other dsRNA virus with such a small RNA-dependent RNA polymerase has been studied. Knowledge of the above-mentioned questions about picobirnavirus RNA-dependent RNA polymerase activity might provide more information on the replication, transcription and genome packaging of this understudied virus.

The aims of the project were:

- To clone segment 2 of a porcine picobirnavirus and express the protein it encodes using recombinant DNA techniques
- To assess if the recombinant protein encoded by segment 2 has any of the properties of a RNA-dependent RNA polymerase.

CHAPTER II

PCR AMPLIFICATION, SUB-CLONING AND SEQUENCING OF PICOBIRNAVIRUS SEGMENT 2 cDNA

2.1. INTRODUCTION

Picobirnaviruses have a double-stranded RNA genome with two genome segments. The full length sequences of both segments of a human isolate have been successfully sequenced and published (Wakudu *et al.*, 2005). The genome of a porcine strain of picobirnavirus, supplied by Dr Vito Martella from University of Bari, Italy, has recently been amplified using the sequence-independent method described by Potgieter *et al.*, 2002. Several copies of the large and small segments of this isolate were cloned in pGEM-T vector and sequenced completely (Potgieter, unpublished data).

This chapter describes the PCR amplification and cloning of the open reading frame (ORF) of the porcine picobirnavirus segment 2 (S2) with the intention to sub-clone the ORF into expression vectors used for recombinant protein expression in insect and bacterial cells.

2.2. MATERIALS AND METHODS

2.2.1. Primer design

Prior to PCR, the full length picobirnavirus genome segment 2 cDNA sequence (Fig. 7, page 26) was analyzed, using DNAMAN (Lynnon Corporation). Three sequence-specific primers for PCR were designed that included the restriction enzyme sites for *Bgl* II, *Nco* I and *Xho* I. The recognition sequences of these restriction enzymes are not present in segment 2 of the picobirnavirus used in this study. The following PCR primers were designed:

- **Forward primers**

Primer 1 : *Nco* I forward primer
 Sequence : 5'-p-ATTTGCCATGGCTAAAAATAATGC-3'
 Restriction enzyme site : *Nco* I
 Length : 24 bases
 GC content : 33.3%
 Tm : 62.3°C

Primer 2 : *Bgl* II forward primer
 Sequence : 5'-p-TGGAAGATCTATGCCTAAAAATAATGC-3'
 Restriction enzyme site : *Bgl* II
 Length : 27 bases
 GC content : 33.3%
 Tm : 61.9°C

- **Reverse primer**

Primer 3 : *Xho* I reverse primer
 Sequence : 5'-p-TCCCTCGAGTCACTTTGACTTC-3'
 Restriction enzyme site : *Xho* I
 Length : 20 bases
 GC content : 50%
 Tm : 60.8°C

2.2.2. PCR amplification of picobirnavirus S2 ORF from cloned cDNA

The ORF of picobirnavirus S2, was amplified by PCR with the primers described above as follows: Three dilutions of each of the three original pGEM-T cDNA clones of picobirnavirus segment 2 (from the field strain of the porcine picobirnavirus) were made in ultra pure water namely: 1:10 (~300ng/μl), 1:100 (~30ng/μl) and 1:1000 (~3ng/μl). For the purpose of this study the original pGEM-T cDNA clones were named clone 1, clone 2 and clone 3 respectively. For PCR, 1μl of each dilution was used as template. Two PCR reactions were performed in 25μl reaction volumes containing the following: 1 × PCR buffer (Roche, catalogue number: 1699121 - 10X: 100mM Tris-HCl, 500mM KCl, pH 8.3 and 25mM MgCl₂), 0.2mM dNTP (dATP, dCTP, dGTP and dTTP each) (Roche, catalogue number: 1581295), 0.5U of *Taq* polymerase (Roche, catalogue number: 1146165), 50pmol of each primer, and Ultra-pure water to 25μl. The PCR reaction was carried out in the Eppendorf MasterCycler[®] gradient thermocycler. The reaction components were mixed as prescribed by the manufacturer (*Taq* polymerase instruction manual, Roche).

The program for the PCR reaction performed with primer 1 and 3 and primer 2 and 3 respectively contained the following steps:

Steps	Conditions	Number of Cycles
1.	120 seconds at 95°C – Denaturation	x1
2.	30 seconds at 35°C – Primer hybridisation	x1
3.	90 seconds at 72°C – Elongation	x1
4.	30 seconds at 94°C – Denaturing	} x15
5.	30 seconds at 60°C – Primer hybridisation	
6.	90 seconds at 72°C – Elongation	
7.	180 seconds at 72°C – Final Elongation	x1

2.2.3. Standard procedure used for agarose gel electrophoresis (Sambrook *et al.*, 1989)

The following buffers and solutions were used in all agarose gel electrophoresis (AGE) throughout the study unless otherwise stated:

Solutions/buffers	Stock	Final concentration
6x Loading dye		
Bromophenol blue	0.25%	0.063%
Xylene Cyanol FF	0.25%	0.063%
Glycerol	30%	7.5%

For each agarose electrophoresis, a loading sample with a ratio of sample to loading dye of 3:1 was prepared before loading into the gel

10x TBE buffer

Tris	890mM	44.5mM
EDTA	20mM	1mM
Boric acid	890mM	44.5mM

50x TAE buffer

Tris	2M	40mM
Glacial acetic acid	5.7% (v/v)	0.15% (v/v)
EDTA	50mM	1mM

1% agarose gel

1% (w/v) agarose added to 0.5 x TBE buffer or 1 x TAE buffer.

The agarose was dissolved by boiling in a microwave until the agarose was completely melted.

The gels were rapidly cooled in crushed ice and Ethidium bromide was added to a final concentration of 0.5µg/ml where indicated.

2.2.4. Agarose gel electrophoresis

The PCR products (3 μ l) were mixed with 1 μ l of 6 \times loading dye and subjected to electrophoresis in stained 1% TBE-agarose in 0.5 \times TBE buffer for 1 hour at 100V.. After electrophoresis, the EtBr-stained DNA could be visualized by trans-illumination with long wavelength ultraviolet (UV-160A). Ethidium bromide intercalates with the DNA. Upon UV exposure, ethidium bromide fluoresces with an orange colour. Images of the gel were captured using an AutochemiTM UVP BioImaging System for recording and analysis purposes. The fragment sizes of the PCR products were estimated by comparison to DNA size marker XVII from Roche (catalogue number: 1855646).

2.2.5. Gel extraction and purification of DNA

For quantitative purification of PCR products, the remaining PCR products from all the reactions were loaded on an unstained 1% TAE-agarose gel and subjected to electrophoresis in 1 \times TAE buffer at 80V for 2 hour. The gels were prepared as described in 2.2.3, but TAE buffer was used instead of TBE buffer. The gel was stained with SYBR Gold nucleic acid gel stain (Invitrogen, catalogue number: S-11494) for 1 hour. After staining, the SYBR gold stained bands could be visualized by trans-illumination with blue light (Dark Reader, DR-45M, Clare chemical research). Blue light and SYBR gold was used instead of UV light in order to avoid the DNA damage which is caused by UV light, hence preserving the DNA integrity for down-stream applications. UV light causes structural damage to the DNA by causing a cross-link between cytosine and thymine bases, thereby creating a pyrimidine dimer. This DNA damage usually results in introduction of mutation during DNA replication. The band corresponding to the expected PCR product size was excised using a sterile surgical blade. The DNA was extracted and purified from the agarose using a MinElute gel extraction kit (Qiagen, catalogue number: 28604). In principle, the excised gel is dissolved by adding high salt concentration solution buffer QG (Qiagen) to the gel and incubation at 50 $^{\circ}$ C for 10 minutes. The high salt solution also assists in DNA binding to the QIAquick membrane (silica). Nucleic acid in the solution is bound to the silica column (QIAquick membrane) and washed of all impurities (such as primers, dye, unincorporated nucleotides and salts) by adding an

ethanol containing buffer, buffer PE (Qiagen) and centrifugation at $16100 \times g$ for 1 minute. The bound nucleic acid/DNA is eluted with a low salt and basic buffer solution, pH 8.5, buffer EB (Qiagen). The eluted DNA is stored at -20°C or used immediately. The gel purified PCR products ($3\mu\text{l}$) were mixed with $1\mu\text{l}$ of $6\times$ loading dye and subjected to electrophoresis in stained TBE-agarose gel as described before (2.2.3).

2.2.6. Cloning of PCR products

2.2.6.1. Preparation of pGEM-T vector

The purified PCR products were cloned into pGEM-T vector (Promega, catalogue number: A3600). Prior to cloning, pGEM-T vector was treated with shrimp alkaline phosphatase (Roche, MB grade, catalogue number: 1758250) in order to remove the phosphate groups at the terminal ends and prevent re-ligation of the plasmid ends. The dephosphorylation reaction was performed in a $10\mu\text{l}$ reaction volume containing the following: $1\times$ dephosphorylation buffer (Roche, 10X: 0.5M Tris-HCl, 50mM MgCl_2 , pH8.5), 50-100ng pGEM-T vector, 1U Alkaline Phosphatase (Roche), and Ultra pure water to $10\mu\text{l}$. The reaction mixture was incubated at 37°C for an hour, and then incubated at 65°C for 5 minutes in order to inactivate the enzyme.

2.2.6.2. Ligation reaction

The purified PCR product was ligated to the dephosphorylated pGEM-T using the T4 DNA ligation kit from Roche (Fig 9, page 29). The method used for cloning is referred to as T/A cloning. The pGEM-T vector is linearized and contains a single thymidine base at both 3' ends. The PCR product (2.2.1) was generated using an enzyme (*Taq* polymerase) which has terminal transferase activity by which a single adenosine base is added to the at the 3' ends. Ligation of the vector and PCR product will result in the A overhang on the PCR product and T overhang on the vector forming a T-A pair, hence the method is called T/A cloning.

Ligation reactions were performed in 20µl reaction volumes containing the following: 10µl of the dephosphorylated pGEM-T vector, 4µl purified PCR product, 1× T4 DNA ligation buffer (Roche), and 0.5U T4 DNA ligase (Roche, catalogue number: 1635379). Ligation reactions were incubated at 4°C for 16hours (overnight). Ligation reactions were used to transform competent *E.coli* cells (2.2.6.4).

2.2.6.3. Preparation of competent *E.coli* cells

Prior to transformation, competent *E.coli* cells were prepared using the method of Inoue *et al*, (1990), with minor modifications. This method was used since it produces competent cells with a transformation efficiency of at least 1×10^8 colony forming units/µg plasmid and is one of the most simple and effective methods to date (Inoue *et al.*, 1990). Furthermore, the reagents required for this procedure are cheap and readily available in most Molecular Biology laboratories. Stock *E.coli* Sure[®] cells (Stratagene, catalogue number: 200294, genotype: *endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC e14- Δ(mcrCB-hsdSMR-mrr)171 F'[proAB⁺ lacI^q lacZΔM15 Tn10]*) stored in liquid nitrogen were thawed at room temperature and streaked on LB-agar plate supplemented with (40µg/ml) tetracycline and incubated overnight at 37°C. Sterile LB and sterile LB-agar plates were prepared as follows:

Sterile Luria-Bertani (LB)	Final concentration
Yeast extract	0.5% (w/v)
Tryptone	1% (w/v)
NaCl	1% (w/v)

The media was sterilized by autoclaving at 127°C and 1.5kg/cm² for 20 minutes

Sterile LB-agar plates	Final concentration
Yeast extract	0.5% (w/v)
Tryptone	1% (w/v)
NaCl	1% (w/v)
Agar	3% (w/v)

The media was sterilized by autoclaving at 127°C and 1.5kg/cm² for 20 minutes. Where appropriate, antibiotics were added to the cooled sterile LB-agar. LB-agar plates were prepared by pouring 20ml of the cooled agar into 75cm³ sterile Petri dishes (Promex).

From the streaked plates, eight colonies were picked using a sterile plastic loop and inoculated into 1ml sterile Luria-Broth (LB) and grown for one and a half hour at 37°C. Fresh sterile LB (125ml) was inoculated with 0.5ml of the culture and incubated at 18°C until the culture reached an OD₆₀₀ of 0.5 (approximately 16 hours). The culture was transferred into three 50ml centrifuge bottles (40ml per tube), cooled on ice for 30 minutes, and centrifuged at 2500 × g for 10 minutes at 4°C. Each pellet was resuspended in 26ml ice-cold Transformation buffer and incubated on ice for 10 minutes.

Transformation buffer was prepared as follows:

Transformation buffer	Final concentration
Pipes (piperazine-N, N'- bis[2-ethane-sulfonic acid])	10mM
MnCl ₂	55mM
CaCl ₂	15mM
KCl	250mM

All salts except MnCl_2 were added and the pH was adjusted to 6.7 with 1M KOH. The MnCl_2 was then added. The buffer was filter-sterilized with 0.2 μm filters.

The cells were centrifuged again at 2500 \times g for 10 minutes. Finally, the pellets were resuspended in 6.6ml transformation buffer in each tube and DMSO was added to a final concentration of 7%. The resulting cell suspension was incubated on ice for 10 minutes and 550 μl aliquots were dispensed into 2ml tissue-culture tubes (Nunc) and immediately chilled by immersion in liquid nitrogen. Competent cells were stored in liquid nitrogen for up to 2 years.

2.2.6.4. Transformation of competent *E.coli* Sure cells

Competent *E.coli* Sure cells were transformed with the ligation reaction (2.2.6.2) as follows: the competent cells were thawed at room temperature and place on ice. 200 μl of the cells were added into a micro-centrifuge tube containing each of the ligation reactions and incubated on ice for 20 minutes. The cells were heat-shocked at 42 $^\circ\text{C}$ for 30s and transferred to ice for 2 minutes. The sudden change in temperature causes pores in the cell membrane to open allowing plasmid DNA to enter the cells. When the cells are transferred to a lower temperature, the pores close trapping the plasmid inside the cells. Fresh sterile LB (800 μl) was added to the transformation reaction and transferred to a 15ml polypropylene tube. The cells were incubated at 37 $^\circ\text{C}$ for 1h in a shaking incubator (300rpm-350rpm) during which the cell walls are repaired. After incubation, 100 μl of the cell suspensions were spread on sterile LB-agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin, 100 $\mu\text{g}/\text{ml}$ IPTG and 40 $\mu\text{g}/\text{ml}$ X-gal. The sterile LB-agar plates were prepared as in 2.2.6.3. The plates were incubated overnight (16hrs) at 37 $^\circ\text{C}$.

2.2.6.5. Purification of recombinant plasmids

Blue/white colony selection was used to select recombinant colonies. In principle, successful cloning of an insert into pGEM-T vector results in interruption of the lac Z

gene. The lacZ gene encodes the β -galactosidase enzyme, which metabolises galactose into lactose and glucose. β -galactosidase can also convert the colourless X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) into galactose and 5-bromo-4-chloro-3-hydroxyindole which is oxidized into an insoluble blue coloured product (5,5'-dibromo-4,4'-dichloro-indigo). Therefore, successful interruption of the lacZ gene with a cloned segment results in colonies which can not convert X-gal into the insoluble coloured product. After transformation, cells containing the recombinant plasmid are deficient of the β -galactosidase gene and are unable to convert the X-gal into the insoluble blue coloured product.

Single white colonies were picked from the plates (2.2.6.4) and inoculated into 5ml fresh LB media supplemented with 100 μ g/ml ampicillin and grown overnight at 37°C with shaking (300-350rpm). Recombinant plasmids were extracted from 2ml of the overnight cultures using a QIAprep Spin miniprep kit (Qiagen, catalogue number: 27104). The cells were pelleted by centrifugation at 16100 \times g for 1 minute. The cells were lysed by alkaline methods (Sambrook *et al.* 1989) as follows: The cell pellet was re-suspended in Tris buffer containing RNase, (Buffer P1, Qiagen). The lysate was treated with buffer solution P2 (Qiagen), which contains NaOH and SDS. The SDS weakens the proteins in the cell membrane and assists in the release of plasmid DNA from the cells and NaOH denatures any genomic DNA that is released from the cells. The genomic DNA and cellular proteins were precipitated by adding buffer solution N3 (Qiagen) which contains potassium acetate. In combination, the buffers used results in a supernatant with high salt concentration which contains the plasmid DNA. The high salt concentration assists the binding of the plasmid DNA to the silica membrane (Qiagen). The genomic DNA and cell debris were removed by centrifugation at 16100 \times g for 10 minutes. The supernatants were loaded to the silica membrane. The bound DNA was washed of all the impurities (such as cellular protein, metabolites and RNA) by adding buffer solution PB and PE sequentially (both from Qiagen) and centrifugation at 16100 \times g for 1 minutes. The bound plasmid DNA was eluted with low salt, basic buffer solution, buffer EB (Qiagen) which

contains 10mM Tris, pH 8.5. The eluted plasmid DNA was stored at -20°C or used immediately.

2.2.6.6. Restriction enzyme digestion of the recombinant plasmids

Restriction enzyme digestions were carried out in 10ul volumes. Double digestions were performed in the same buffer for 3 hours or overnight (16hours). The restriction digestion reactions were performed in 10µl reaction volumes containing the following: 1µl × 10 reaction buffer (Roche), 0.5U of each restriction enzyme (Roche), 100ng plasmid DNA and Ultra-pure water. After digestion, 5µl of the reaction mixture was subjected to electrophoresis in TBE-agarose gel as described in 2.2.4. The following restriction enzymes were used in these experiments: *Bgl* II (Roche, catalogue number: 1047604), *Xho* I (Roche, catalogue number: 703770) and *Nco* I. (Roche, catalogue number: 835315).

2.2.6.7. Sequencing

The pGEM-T vector plasmids contain the binding sites for pUC/M13 forward and reverse primers. Therefore, the recombinant plasmids with the correct insert sizes were sequenced using the M13 primers (forward primer: 5'-TGTAACGACGGCCAGT-3' and reverse primer: 5'-GTTTTCCAGTCACGAC-3') as well as sequence specific primers (designed by Dr. A.C. Potgieter, unpublished data). Sequencing was performed by the ARC-OVI Molecular Biology Sequencing Laboratory (Onderstepoort, South Africa) using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit, version 2.0 (Applied Biosystems, Foster city, CA, USA). Sequences were analyzed on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystem), according to the manufacturer's specifications. Approximately 300-500ng of each of the recombinant plasmids were sequenced using 2-4pmol of each of the primers.

2.3. RESULTS AND DISCUSSION

2.3.1. PCR amplification of the picobirnavirus S2 ORF

The sequence of cloned picobirnavirus genome segment 2 cDNA was analyzed, using DNAMAN (Fig. 7). The following features were identified during full length picobirnavirus segment 2 sequence analyses:

- The complete segment is 1730bp in length
- The 5' and 3' non-coding regions are 44bp and 51bp in length respectively,
- The segment has a GC content of 46.4 %,
- The segment contains a single open reading frame (ORF) of 1602bp,
- The ORF encodes a polypeptide of 534 amino acids, with a theoretical molecular weight of 60kDA,
- The encoded polypeptide has a theoretical pI of 8.13.

Restriction enzyme recognition sites for restriction enzymes, *Bgl* II, *Nco* I and *Xho* I, are not present in the sequence of picobirnavirus S2 cDNA. Therefore, these sites were incorporated in primers designed for PCR to allow sub-cloning of PCR products into expression vectors. Using the sequence information, sequence specific PCR primers were designed to remove the non-coding regions (NCR) of picobirnavirus S2 and incorporate restriction enzyme recognition sites at the terminal ends of the PCR amplicons. Three primers were designed, one common reverse primer and two forward primers. On the reverse primer an *Xho* I restriction site was incorporated and on the forward primers, *Bgl* II and *Xho* I restriction sites were incorporated respectively. The PCR products were expected to have the restriction enzyme recognition sites as illustrated in Figure 6. The binding sites for the primers on full length picobirnavirus segment 2 cDNA are shown in Figure 7.

PCR was performed using the dilutions of the original porcine picobirnavirus S2 cDNA clones as template. A PCR product of approximately 1600bp in size was expected. Figure

8 shows the gel purified PCR products and it can be seen that an amplicon of approximately 1600bp in size was generated effectively using both primer sets from all of the clones. From a template dilution of 1:1000 (3ng/μl) of the original cDNA clone, a large amount of PCR product was generated from all the original cDNA clones (Figure 8, A and B, lanes 4, 7, and 10). The results correlate with the expected fragment size of picobirnavirus S2 ORF (1602bp). Therefore an amplicon of the correct size was generated by PCR. For reference purposes, PCR product generated by primer 1 and 3 will be referred to as PBV 2N and products generated by primer 2 and 3 will be referred to as PBV 2B.

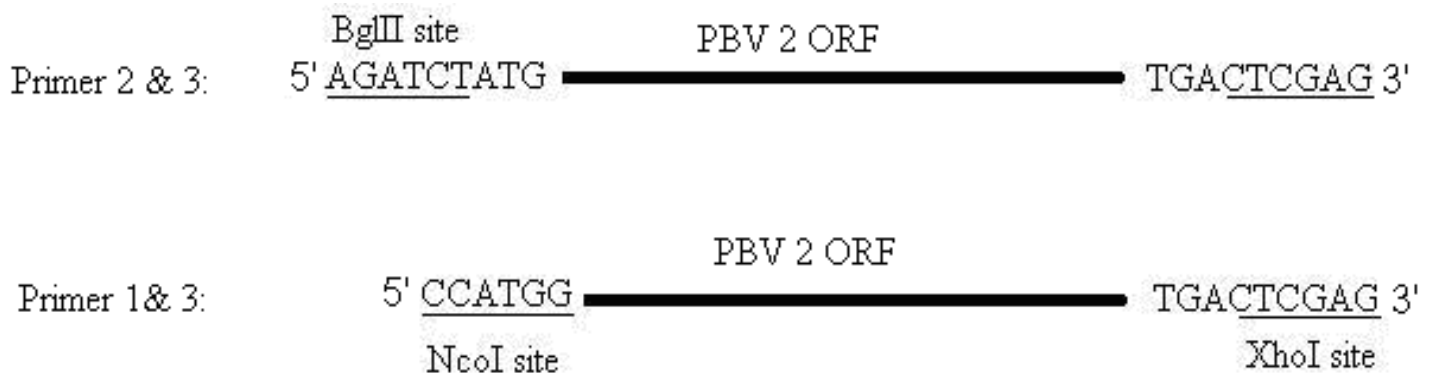


Figure 6. Graphic representation of the PCR products expected to be generated using the primers as indicated. Picobirnavirus genome segment 2 ORF in the PCR product is represented by the solid line and the restriction enzyme sites in the PCR products are underlined.

Figure 7 is on another format, print separately NB

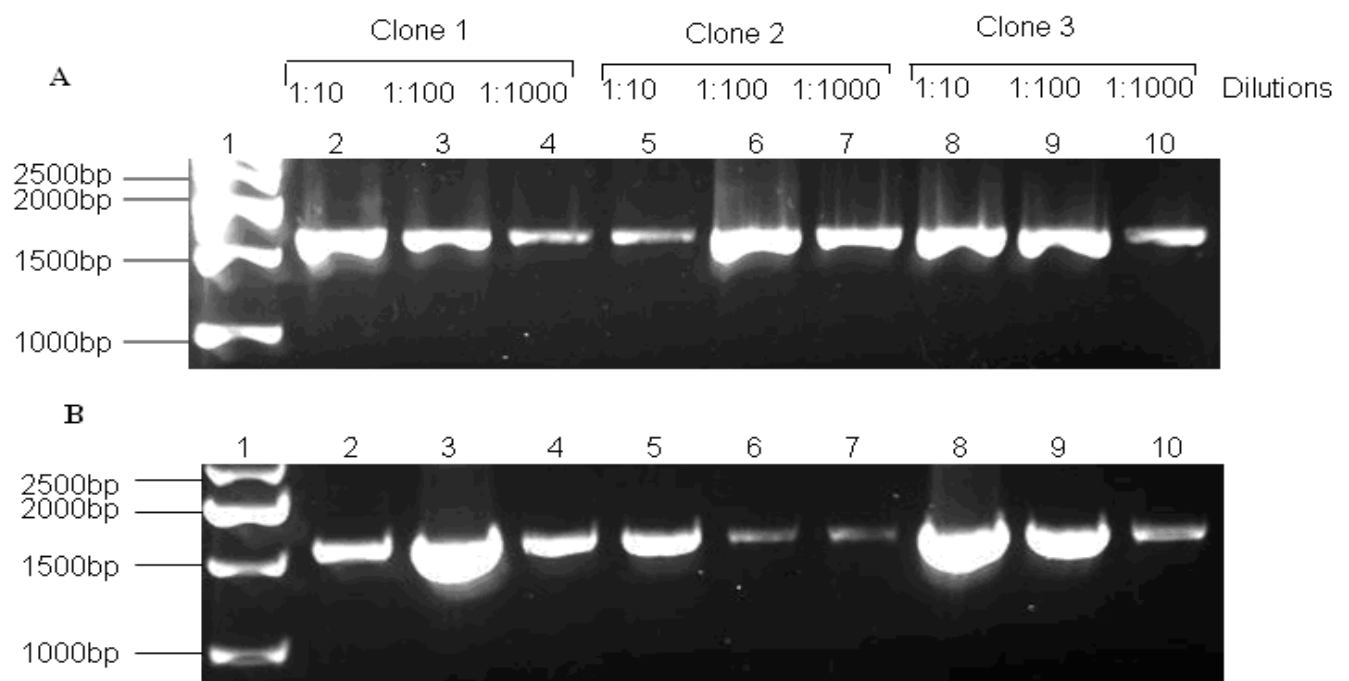


Figure 8. Agarose gel (1%) electrophoresis analysis of purified PCR products of picobirnavirus segment 2 ORF using sequence specific primers. A: PCR products generated by primers 1 and 3, **B:** PCR products generated by using primers 2 and 3. In both Figures **A** and **B**: lane 1: DNA size marker XVII (Roche), lanes 2-4: PCR amplicon generated from clone 1 in decreasing template concentration, lanes 5-7: PCR amplicon generated from clone 2 in decreasing template concentration and lanes 8-10: PCR amplicon generated from clone 3 in decreasing template concentration.

2.3.2. Cloning of the S2 ORF PCR product in the pGEM-T vector

Several white colonies were obtained after cloning. Single colonies from each plate were picked and inoculated into fresh LB for recombinant plasmid DNA extraction (2.2.6.5). To confirm that cloning was successful, the recombinant plasmids were extracted from bacterial cells (2.2.6.5) and digested with restriction enzymes to confirm the presence of the insert in the recombinant plasmid (2.2.6.6). Digestion was performed using restriction enzymes for which the recognition sites were incorporated into the terminal ends of the PCR product (Fig. 6). Digestion of the plasmid DNA with these enzymes were expected to result in the removal of the insert from the recombinant plasmid, generating an empty plasmid with a size of 3000bp and the insert with an expected size of 1600bp. In Figure 10, lane 1, it can be seen that digestion of pGEM-T-PBV 2B with *Bgl* II and *Xho* I resulted in generation of two DNA fragments with sizes corresponding to that of the insert size (1600bp) and empty plasmid size (3000bp). In Figure 10, lane 3, it can be seen that digestion of pGEM-T-PBV 2N with *Nco* I and *Xho* I also resulted in formation of two DNA fragments with sizes corresponding to that of the empty plasmid (3000bp) and the insert (1600bp). These results show that PCR products with the correct size (1600bp) and restriction enzyme sites were cloned successfully into the pGEM-T vector.

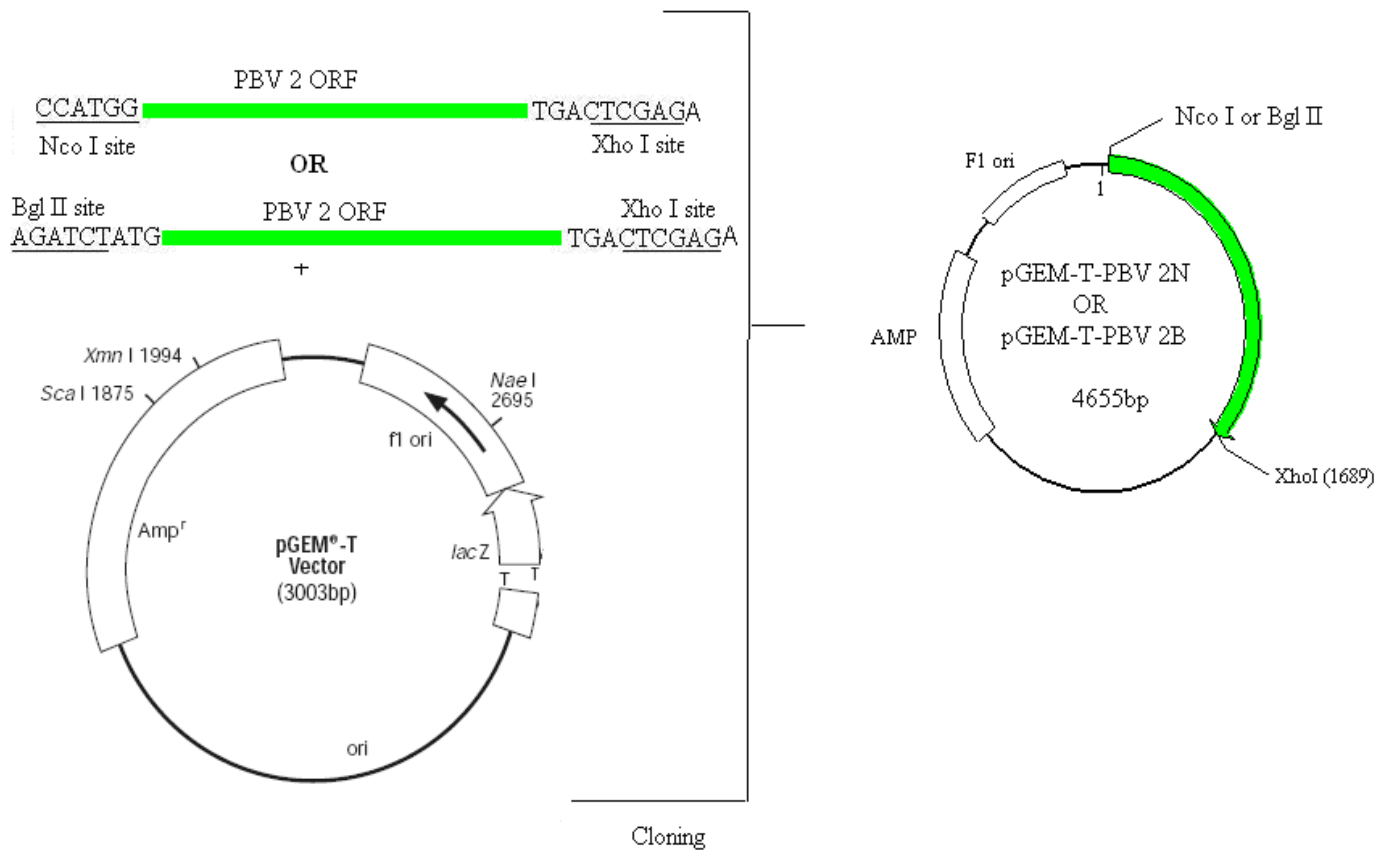


Figure 9. Graphic representation of the cloning of the picobirnavirus segment 2 ORF (PCR products) into dephosphorylated pGEM-T vector. Ligation of purified PCR product, PBV 2N and PBV 2B, into dephosphorylated pGEM-T vector resulted in the formation of recombinant plasmids pGEM-T-PBV 2N and pGEM-T-PBV 2B respectively.

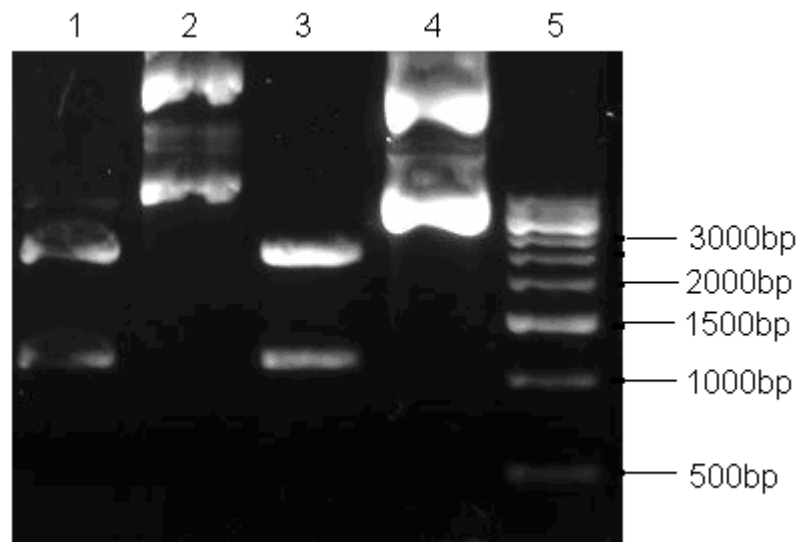


Figure 10. Agarose gel (1%) electrophoresis analysis of restriction digestion of the recombinant pGEM-T plasmids. Lane 1: pGEM-T-PBV 2B digested with *Bgl* II and *Xho* I, lane 2: Un-digested pGEM-T-PBV 2B. Lane 3: pGEM-T-PBV 2N digested with *Nco* I and *Xho* I, lane 4: Un-digested pGEM-T-PBV 2N and lane 5: DNA size marker XVII (Roche).

2.3.3. Sequencing of the inserts in the recombinant plasmids

To determine if the generated recombinant pGEM-T clones contained the picobirnavirus segment 2 ORF, the recombinant pGEM-T vectors were sequenced with M13 primers and internal sequence specific primers. The following sequence-specific primers were used for primer walking:

ss_1F: 5'-CAT CAC AGT TCG TGC C-3',
 ss_2F: 5'- CTC AAT GTC TTC CAT GCG-3',
 ss_1R: 5'-ACC AAG CGA AGT CTA CC-3',
 ss_2R: 5'- GAT GCC AAA GCT GTA GC -3'.

Sequence results received from the OVI Molecular Biology Sequencing Laboratory were analyzed by DNAMAN (Lynnon Corporation). The sequences obtained using the pUC/M13 forward and reverse primers were edited in the following manner:

- The sequences obtained using the forward primers were directly aligned with the porcine picobirnavirus segment 2 consensus cDNA sequence.
- The sequences obtained using the reverse primers were converted to the reverse complementary sequence, before alignment with the porcine picobirnavirus segment 2 consensus cDNA sequence. The sequence was converted because during sequencing, the reverse primers generates the anti-sense strand sequence, and the consensus sequence used for alignment is the sense strand of picobirnavirus S2 cDNA sequence.
- A comparative alignment was performed using both the forward and reverse sequences
- The consensus sequence of the cloned segments were assembled from overlapping forward and reverse sequences

Figure 11 shows that the inserts in the recombinant plasmids are that of picobirnavirus genome segment 2 ORF cDNA. The internal sequences of both PCR products were identical with few silent mutations found on the sequence of both inserts in the recombinant plasmid (Figure 11, in the highlighted letters, red). These mutations were not of great concern as they did not alter the composition/sequence of the encoded amino acid.

Fig. 11 on another format, print separately

2.4. SUMMARY

Using the complete sequence of picobirnavirus S2 cDNA provided by Dr. A.C. Potgieter, sequence-specific PCR primers were designed to amplify the ORF of this segment and to incorporate restriction enzyme recognition sites into its terminal ends. Using these primers and cDNA of porcine picobirnavirus S2 as template, amplicons of the correct size were generated by PCR (Fig. 8). Subsequently PCR products of the correct size and with the correct restriction enzyme sites were successfully cloned into plasmid pGEM-T (Fig. 10). Sequencing of the cloned inserts confirmed that the clones contained the ORF of porcine picobirnavirus S2 (Fig. 11). Sequencing also showed that the restriction enzyme recognition sites were successfully incorporated into both terminal ends of each of the insert at the expected positions. The sequence obtained was consistent with the sequence provided by Dr. A.C Potgieter with some silent mutations. The sub-cloning of the picobirnavirus ORF into baculovirus expression vectors and the recombinant expression of its encoded polypeptide in insect cells will be described in Chapter III.

CHAPTER III

EXPRESSION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE USING THE BACULOVIRUS EXPRESSION SYSTEM

3.1. INTRODUCTION

The baculovirus expression system is a recombinant protein expression system which utilizes recombinant baculoviruses for the over-expression of proteins of interest in insect cells. In the normal life cycle of the baculovirus *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV), which infects the fall armyworm *Spodoptera frugiperda*, large amounts of proteins, which are non-essential for viral replication in cell culture, are synthesized in the late stage of the virus replication cycle (O'Reilly *et al.*, 1994, Poly, 2004). Polyhedrin is one of these proteins and it is expressed under control of a strong promoter called the polyhedrin promoter (*polh*). In most commercial baculovirus expression systems this promoter is utilized to express the gene of interest. This is achieved by removal of the polyhedrin gene from the baculovirus genome and replacing it with the gene of interest downstream of the polyhedrin promoter (O'Reilly *et al.*, 1994, Philipps *et al.*, 2005, Poly, 2004). This results in the protein encoded by the gene of interest being expressed at a high level in cells infected by the recombinant virus. The produced recombinant proteins are post-translationally modified by the insect cell translational machinery (O'Reilly *et al.*, 1994, Poly, 2004). This assists the protein to fold to its natural three-dimensional conformation. The baculovirus expression system has been successfully used to express viral proteins of many dsRNA viruses. This includes the RNA-dependent RNA polymerases of several viruses including rotavirus, bluetongue virus, reovirus and bovine viral diarrhoea virus (Boyce *et al.*, 2004, Neufeld *et al.*, 1991a, Sankar and Porror, 1991, Zhong *et al.*, 1998).

In this chapter, expression of recombinant putative picobirnavirus RNA-dependent polymerase using the Bac-to-Bac baculovirus system and Sf9 (*Spodoptera frugiperda*) cells is described. The Bac-to-Bac system comprises of donor plasmids, a baculovirus

shuttle vector (baculovirus genome in bacteria) and a helper plasmid which contain a gene for transposase (Fig. 12) (Luckow and Summers, 1989). The donor plasmids are used to clone the gene of interest, which results in formation of a recombinant donor plasmid with the gene of interest under control of the *polh* promoter. The expression cassette is flanked by transposon sites (Tn7), also called mini Tn7 elements. The recombinant donor plasmid is used to transform the *E.coli* cells containing both the Baculovirus genome (bacmid) and the helper plasmid. The bacmid contains the kanamycin resistance gene and *lacZ α* gene, which are used for the selection of recombinants by blue-white screening in the presence of kanamycin, X-gal and IPTG. The *lacZ* gene contains the attachment site for bacterial transposition Tn7. This site is recognized by a transposase which binds to it during transposition (Luckow and Summers, 1989). The recombinant bacmid is generated by the transposition of the mini Tn7 element in the donor plasmid (or recombinant donor plasmid) to the mini Tn7 into the bacmid, thereby generating a recombinant bacmid containing the gene of interest under control of the polyhedrin promoter. Recombinant baculoviruses expressing the gene of interest are generated by transfecting insect cells with the recombinant bacmid DNA.

In this chapter, two baculovirus donor plasmids were used to sub-clone picobirnavirus S2 ORF, namely pFastbac1 and pFastbac-HT. Both donor plasmids have the strong polyhedrin promoter for high level expression of protein of interest. pFastBac-HT allows the fusion of a N-terminal hexa-histidine tag to the recombinant protein for easy purification using nickel chelate chromatography. The plasmid, pFastBac-HT, also contains a TEV protease cleavage site for the removal of the histidine tag after purification. pFastBac1 does not have any tag, therefore its use results in production of a wild-type protein. The expression of two forms of recombinant picobirnavirus S2 protein, wild-type and histidine tagged, using the Bac-to-Bac baculovirus system is described. This was done because the tagged protein is easy to purify and is preferred over the untagged protein. However, the fusion protein may be insoluble after expression and the wild-type (un-tagged) protein without the tag may be soluble.

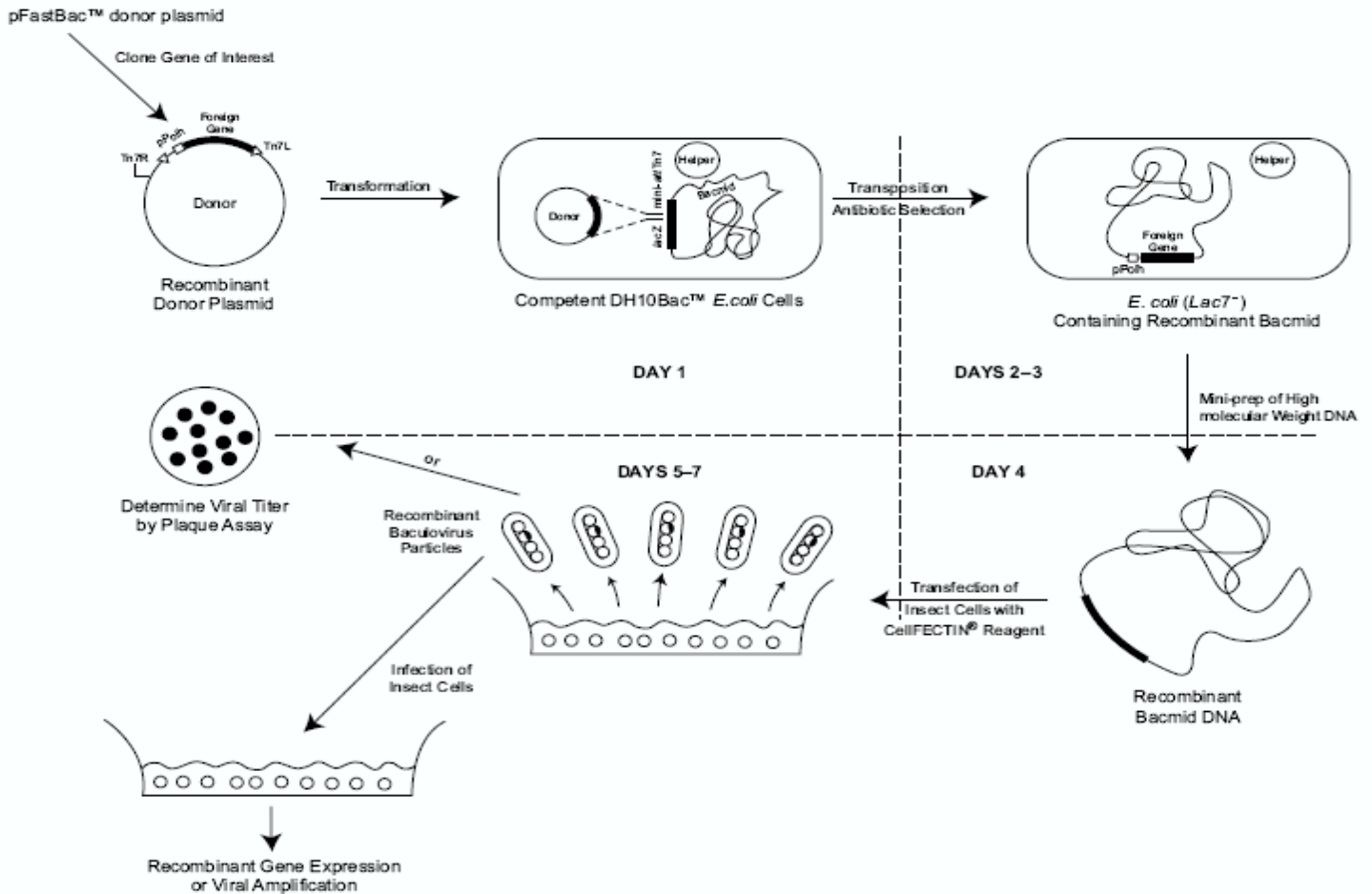


Figure 12. Graphical representation of the generation of recombinant baculovirus and gene expression with the Bac-to-Bac expression system. Taken from Bac-to-Bac product manual (Invitrogen).

3.2. MATERIALS AND METHODS

3.2.1. Sub-cloning of the picobirnavirus S2 ORF into pFastBac donor plasmids

3.2.1.1. Plasmids used in this chapter

Two baculovirus donor plasmids were used to sub-clone picobirnavirus S2 ORF, namely pFastbac1 and pFastbac-HT (Invitrogen, catalogue numbers 10360-014 and 10584-027 respectively). The plasmid maps for pFastBac1 and pFastBac-HT A, are shown in Figures 13 and 14 respectively. pFastbac1 was used to sub-clone picobirnavirus segment 2 ORF (PBV 2B, section 2.3.1) and pFastbac-HT was used to sub-clone picobirnavirus segment 2 ORF (PBV 2N, section 2.31).

3.2.1.2. Restriction enzyme digestion of the donor plasmids

The pFastbac donor plasmids were subjected to double restriction enzyme digestion and gel extraction as described sections 2.2.6.6 and 2.2.4, respectively. The following restriction enzymes were used in these experiments: *Bam* HI, *Xho* I and *Nco* I. pFastBac1 plasmid was digested with *Bam* HI and *Xho* I whereas pFastBac-HT plasmid was digested with *Nco* I and *Xho* I. After gel purification, 50µl of each digested donor plasmid was recovered, and 3µl of each plasmid was subjected to electrophoresis and visualized as described in section 2.2.4.

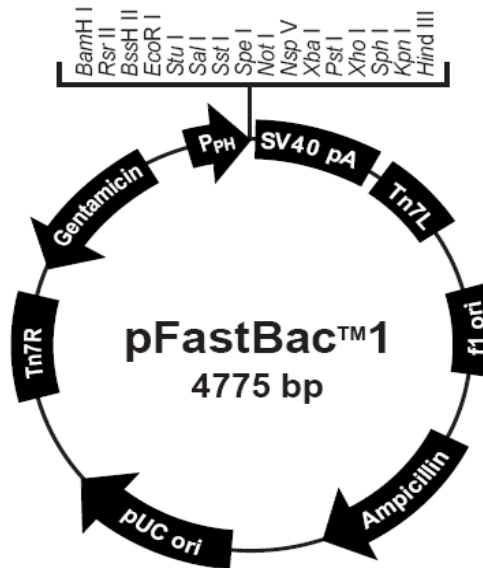


Figure 13. Plasmid map of the baculovirus donor plasmid, pFastbac1.

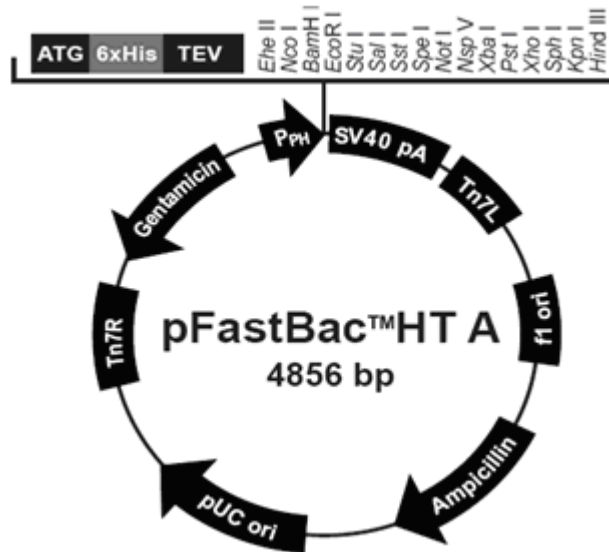


Figure 14. Plasmid map of the baculovirus donor plasmid, pFastbac-HT A.

3.2.1.3. Preparation of insert DNA(s)

Picobirnavirus segment 2 ORF inserts (PBV 2N and PBV 2B) were prepared by double digestion of recombinant pGEM-T vectors (pGEM-T-PBV 2N and pGEM-T-PBV 2B) as described in section 2.2.6.6, with subsequent purification by gel extraction as described in section 2.2.4. pGEM-T-PBV 2N plasmid was digested with *Nco* I and *Xho* I whereas pGEM-T-PBV 2B plasmid was digested with *Bgl* II and *Xho* I.

3.2.1.4. Ligation reaction

Ligation of purified picobirnavirus segment 2 ORF inserts into digested and purified donor plasmids was performed as described during T/A cloning (2.2.6.2). However, in this experiment ligation reactions were incubated for 5min at room temperature and not overnight at 4°C as described in section 2.2.6.2.

3.2.1.5. Transformation of competent *E.coli* Sure cells

Competent cells were transformed with ligation reactions (3.2.1.4) using the methods described in section 2.2.6.4. Undigested donor plasmid, pFastbac1, was used as transformation control. The following antibiotics were used for selective growth on LB plates: 100µg/ml gentamycin and 100µg/ml ampicillin.

3.2.1.6. Purification (miniprep), restriction digestion and sequencing of recombinant pFastbac plasmids

Recombinant donor plasmids were propagated and purified as described before (2.2.6.5). The purified recombinant donor plasmids were digested with two restriction enzymes as described in section 2.2.6.6. The following restriction enzymes from Roche were used in these experiments: *Xho* I, *Bgl* II and *Eco* RI (Roche, catalogue number: 1175084). Recombinant donor plasmids were sequenced using a pFastBac sequence-specific

primer that binds to the polyhedrin promoter namely **pFB-PF**: 5'- TCC GGA TTA TTC ATA CCG TC -3'. Sequencing was performed and sequences analyzed as described in section 2.2.6.7. Approximately 300-500ng of each of the recombinant plasmids were sequenced using 2-4pmol of each of the primers. Stock recombinant plasmids were stored at -20°C and recombinant *E.coli* Sure cells containing the recombinant plasmid were stored at -70°C in 25% glycerol.

3.2.2. Generation of recombinant bacmid

3.2.2.1. Transformation of AcBac Δ CC cells with recombinant pFastBac constructs

To generate recombinant bacmid(s), *E.coli* cells containing the complete baculovirus genome with transposon sites, were transformed with the recombinant pFastbac plasmid(s) according to the instruction of the manufacturers with minor modifications as indicated (Bac-to-Bac Baculovirus expression system, Invitrogen). The pFastBac recombinant plasmids and controls were used for transformation of competent cells containing the *chitinase* and V-cathepsin deficient bacmid (AcBac Δ CC) genome instead of DH10BAC cells. The use of *chitinase/v-cath* deficient bacmid genome protects recombinant proteins from degradation by these proteases (Kaba *et al.*, 2004). These cells, made competent as before (section 2.2.6.3) were provided by Dr. A.C. Potgieter. The cells were transformed with the recombinant pFastBac plasmids (pFastBac1-PBV 2B and pFastBac-HT-PBV 2N) and control plasmid (pFastbac1 without insert) respectively. For each transformation, competent AcBac Δ CC cells were transformed as described in section 2.2.6.4 with minor modifications as indicated. 10ng of each recombinant donor plasmid and 1ng of intact pFastBac1 plasmid were used to transform competent cells. After transformation, 100 μ l of each transformation reaction mixture was plated on LB plates containing 50 μ g/ml kanamycin, 7 μ g/ml gentamicin, 10 μ g/ml tetracycline, 100 μ g/ml X-gal and 40 μ g/ml IPTG. The plates were incubated for 48hrs at 37°C. Two white colonies were picked from each plate and streaked on LB plates containing the same antibiotics as above and incubated overnight at 37°C.

3.2.2.2. Purification of recombinant bacmid

From the plates described above, individual colonies were inoculated into 5ml LB medium supplemented with antibiotics (50µg/ml kanamycin, 7µg/ml gentamicin and 10µg/ml tetracycline) and incubated overnight at 37°C with shaking at 300-350rpm. Recombinant bacmid was extracted from the overnight cultures using the protocol described in Bac-to-Bac product manual, page 51 and NucleoBond® buffers S1, S2 and S3 (Macherey-Nagel). In principle, cells were lysed by the alkaline/SDS procedure (Sambrook *et al*, 1989). The overnight cultures were centrifuged at 16100 × g for 1min and the cell pellets were re-suspended in NucleoBond buffer S1. This buffer contains Tris buffer and RNase A, which degrade all the RNA from the cells. The cells were lysed by the addition of Nucleobond buffer S2, which contains SDS, EDTA and sodium hydroxide. Buffer S2 denatures the proteins in the cell wall and assists in the release of bacmid DNA from the cells and denatures the genomic DNA. The genomic DNA and cellular proteins were precipitated by addition of Nucleobond buffer S3, which contain acidic (pH 5.5) potassium acetate. The buffers used also neutralize the lysate. The lysate was incubated on ice for 5-10min. The genomic DNA and cell debris were removed by centrifugation at 16100 × g for 10min while the bacmid DNA remains in solution. The bacmid DNA was precipitated from the supernatant by the addition of 800µl of ice-cold isopropanol. The solution was subjected to centrifugation at 16100 × g to pellet the bacmid DNA. The pellet was washed with 70% ethanol and air dried. Finally, the DNA was dissolved in 50µl TE-buffer.

3.2.2.3. Analysis of the recombinant bacmid by PCR

The purified recombinant bacmid(s) were analyzed by PCR to verify the transposition of picobirnavirus segment 2 ORF expression cassette into the bacmid. PCR was performed using the M13 forward and reverse primers (M13 forward primer: 5'-GTTTCCCAGTCACGAC-3', M13 Reverse primer 5'-CAGGAAACAGCTATGAC-3', Invitrogen). M13 primers were used because the bacmid contains the M13 priming site on

the both sides of the mini-*attTn7* (Bac-to-Bac expression system protocol manual). The PCR reactions were performed in 25µl reaction volumes containing the following: 1× PCR buffer (TaKaRa), 0.5U of Ex *Taq* polymerase (TaKaRa), 0.2mM dNTP (dATP, dCTP, dGTP and dTTP each), 50pmol of each primer, 1µl of purified recombinant bacmid and Ultra-pure water to 25µl. The PCR reactions were carried out in the Eppendorf MasterCycler^R gradient thermocycler. The program for the PCR reaction performed with the M13 primer contained the following steps:

Steps	Conditions	Number of cycle
1	120 seconds at 95°C – Initial denaturation	x1
2	30 seconds at 94°C – Denaturation	} x 24
3	30 seconds at 56°C – Primer hybridisation	
4	180 seconds at 72°C – Elongation	
6	180 seconds at 72°C – Final elongation	x1

PCR products (3µl) were mixed with 1µl of loading dye and subjected to electrophoresis in stained 1% TBE-agarose gel in 0.5× TBE as described in section 2.2.4. Recombinant bacmid(s) that yielded PCR products of the expected size were used immediately to transfect insect cells.

3.2.3. Transfection of *Sf9* cells and pilot expression of putative picobirnavirus RNA-dependent RNA polymerase using the Bac-to-Bac baculovirus expression system

3.2.3.1. Transfection of insect cells with recombinant bacmid DNA

Insect cells (*Sf9* cells) were grown in Grace's medium (Lonza) containing 10% (v/v) FBS, 1% (v/v) L-arginine, 1% (v/v) Antibiotics mixture (Pen/Strep/Fungizone mixture - Whitehead Scientific) and 1% (v/v) Pluronic at 27°C with slow shaking (100rpm). When

the cell count reached about 1.4×10^6 cells/ml (counted using a haemocytometer), cultures were diluted to approximately 0.4×10^6 cells/ml with Grace's medium containing 10% (v/v) FBS, 1% (v/v) L-arginine and 1% (v/v) antibiotics mixture. 3ml of the diluted cells were seeded into single wells of a 6-well plate and allowed 30 minutes for the cells to attach to the plastic surface. A master mix containing transfection reagents for transfection of multiple samples was prepared as follows: 3ml of Grace's medium and 75 μ l Fugene (Roche) was mixed in 5ml polystyrene tubes. Polystyrene tubes were used instead of normal Eppendorf tubes because Fugene binds to the normal Eppendorf tubes. 200 μ l from the master mix was mixed with 12 μ l of the recombinant bacmid DNA and incubated for 30min at room temperature. 190 μ l of this mixture (Transfection reagent and recombinant bacmid) was added to each well. The plates were swirled gently to disperse the transfection mix evenly. The 6-well plate was incubated at 27°C for 72 hours inside a partially closed container with a damp tissue/cloth to maintain the moisture in the plates. After 72 hours, the medium was removed from the cells in each well and placed in sterile 2ml Eppendorf tube. The cells were removed by centrifugation at 2440 \times g for 2min. The supernatant containing the recombinant baculovirus virus (transfection supernatant) was collected and placed on ice or stored at 4°C. The supernatant was used to infect fresh insect cells for pilot expression of picobirnavirus segment 2 protein.

3.2.3.2. Pilot expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase

Fresh Sf9 cells in log phase were seeded in 6-well plates as described in section 3.2.3.1. Transfection supernatants containing recombinant baculoviruses (300 μ l) were diluted to 1ml with fresh Grace's medium. The medium from seeded cells was removed and the diluted transfection supernatant was added to the cells. The plate was placed on a rocker at 12 oscillations per minute for one hour after which 2ml of fresh medium was added. The plates were incubated at 27°C for 72 hours inside a partially closed container with a damp tissue/cloth for moisture. After 72 hours incubation, the infected cells were carefully

resuspended in the medium inside the wells. The re-suspended cells were dispensed into 2ml micro-centrifuge tubes and centrifuged at 500 × g for 5min at 4°C. The supernatant was collected and stored as passage 1 viral stock at 4°C and -70°C. The cell pellet was collected and prepared for SDS-PAGE analysis.

3.2.3.3. Standard procedure used for SDS-PAGE (Ausubel *et al.* 1994-2004)

The following buffer solutions were used for SDS-PAGE electrophoresis throughout the whole study unless otherwise indicated:

2× SDS sample buffer	Stock	Final concentration
Tris-HCl, pH 6.8	125mM	62.5mM
Glycerol	20% (v/v)	10% (v/v)
SDS	4% (w/v)	2% (w/v)
β-mercapto-ethanol	2% (w/v)	1% (w/v)
Bromophenol blue	0.01% (w/v)	0.005% (w/v)
5 × Tris-Glycine buffer		
Tris base	125mM	25mM
Glycine	7.2% (w/v)	1.2% (w/v)
SDS	0.5% (w/v)	0.1% (w/v)
Separating gel (10%)	amount (ml)	for SDS-PAGE

30% acrylamide/0.8% bisacrylamide	5
4x Tris.Cl/SDS, pH 8.8	3.75
H ₂ O	6.25
10% (w/v) ammonium persulfate	0.05
TEMED	0.01

Stacking gel

30% acrylamide/0.8% bisacrylamide	0.65
4x Tris.Cl/SDS, pH 6.8	1.25
H ₂ O	3.05
10% (w/v) ammonium persulfate	0.025
TEMED	0.005

Fairbanks A stain

Coomasie brilliant blue	0.05% (w/v)
Isopropanol	25% (v/v)
Acetic acid	10% (v/v)

Fairbanks D (De-stain)

Acetic acid	10% (v/v)
-------------	-----------

PBS pH 7.4

KCl	2.7mM
CaCl ₂ .2H ₂ O	0.9mM

KH ₂ PO ₄	1.5mM
MgCl ₂ .6H ₂ O	0.5mM
NaCl	137mM
NaHPO ₄	9.6mM

3.2.3.4. Cell lysis and SDS-PAGE analysis

The cell pellet from 3.2.3.1 was washed gently with 1ml of PBS by gentle re-suspension and centrifugation at 500 × g for 2min at 4°C. After washing, 200µl of lysis buffer (PBS containing 0.5% NP40 and protease inhibitor from Roche) was added to the pellet and mixed gently. The mixture was incubated at room temperature on a rocker at 12 oscillations per min for 1 hour. 50µl of this mixture was transferred to a fresh tube and treated as the total protein fraction. The remaining 150µl was centrifuged at 16100 × g for 10 min and the supernatant was collected (soluble protein fraction).

To confirm that expression of the recombinant picobirnavirus segment 2 protein was successful, both collected mixtures (30µl) were mixed with 2XSDS sample buffer at a ratio of 1: 1 and boiled in a water bath (95°C) for 3min and then subjected to 10% SDS-PAGE for 1½ hours in 1× Tris-Glycine buffer. The polyacrylamide gel was stained using Fairbanks A stain for 30min and de-stained using Fairbanks D for 2 -16 hours. Prior to staining and de-staining, the gel (in the staining or de-staining solution) was boiled in a microwave oven for 30-90 seconds to increase the speed of staining and de-staining. After de-staining, the stained protein bands could be visualized after illumination of the gel with white light. Gel images were captured using an Autochemi™ UVP BioImaging System. The molecular weights of protein bands were estimated by comparison to protein molecular weight standards (BioRad).

3.3. RESULTS AND DISCUSSION

3.3.1. Preparation of donor plasmids and picobirnavirus segment 2 ORF for cloning

Picobirnavirus segment 2 (S2) open reading frame (ORF) was cloned into pFastBac donor plasmids. Digestion of pFastBac1 plasmid with *Bam* HI and *Xho* I was expected to generate nucleotide overhangs compatible to those of picobirnavirus segment 2, PBV 2B digested with *Bgl* II and *Xho* I. Similarly, digestion of pFastBac-HT plasmid with *Nco* I and *Xho* I was expected to generate nucleotide overhangs compatible to picobirnavirus segment 2 ORF, PBV 2N digested with the same enzymes. Digested pFastBac donor plasmids and inserts were successfully recovered from the gel extraction procedure (Fig. 14, lane 2: pFastBac-HT and lane 3: pFastBac1)

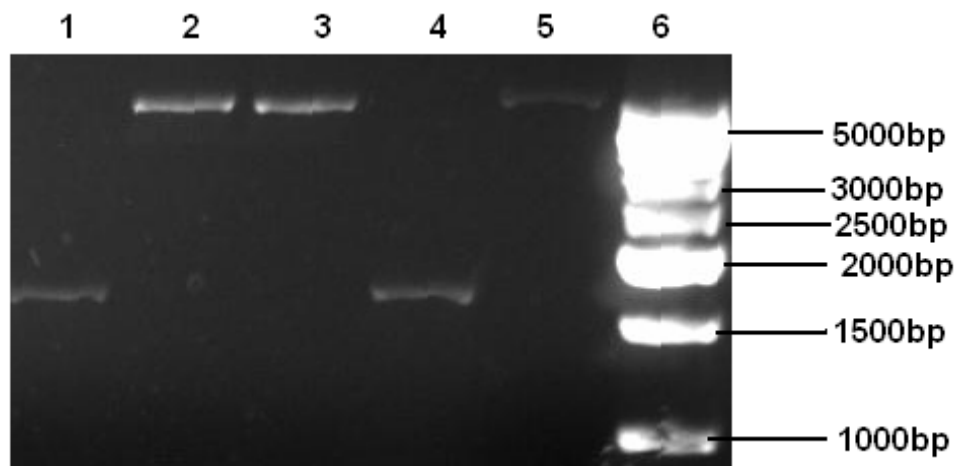


Figure 15. Agarose gel (1%) electrophoresis analysis of gel purified pFastbac plasmids and inserts after restriction digestion and purification. Lane 1: PBV 2N, lane 2: pFastBac-HT, lane 3: pFastBac1, lane 4: PBV 2B, lane 5: pFastBac1 and lane 6: Roche DNA size marker XVII.

3.3.2. Sub-cloning of picobirnavirus segment 2 ORF into pFastBac donor plasmids

After ligation and transformation several colonies were obtained. Single colonies were picked and inoculated into fresh LB for recombinant plasmid DNA extraction (3.2.1.6). As expected the control ligation, where no insert was included in the ligation reaction, did not yield any colonies. To confirm that sub-cloning was successful, the recombinant plasmids were extracted from bacterial cells and digested with restriction enzymes (3.2.1.6). Digestion of pFastBac-HT-PBV 2N with *Eco* RI and *Xho* I was expected to result in two fragments with sizes of 853bp and 5528bp. The *Eco* RI recognition site in the multiple cloning site of the donor plasmid was removed during cloning. Therefore, in the recombinant plasmid, *Eco* RI only recognises the site in the insert and *Xho* I recognizes the site in the 3' end of the inserts. Figure 18, lane 4, shows that digestion of recombinant pFastBac-HT-PBV 2N with *Eco* RI and *Xho* I resulted in generation of two DNA fragments with the expected sizes.

Digestion of pFastBac1-PBV 2B with *Bgl* II and *Xho* I was expected to result in the generation of DNA fragments with sizes of 470bp, 2704bp and 3202bp. This is due to the fact that PBV 2B was ligated to the *Bam* HI and *Xho* I site in the donor plasmid and ligation of *Bgl* II and *Bam* HI overhangs results in the formation of site which is not recognized by either *Bgl* II or *Bam* HI. Hence, the removal of PBV 2B from the recombinant plasmid will not be observed. However, *Bgl* II recognition sites are present in the plasmid, which will result in fragmentation of the plasmid after digestion as observed (Fig. 18, lane 4). Figure 18, lane 2, shows that digestion of recombinant pFastBac-1-PBV 2B with *Bgl* II and *Xho* I resulted in generation of three DNA fragments with the expected sizes.

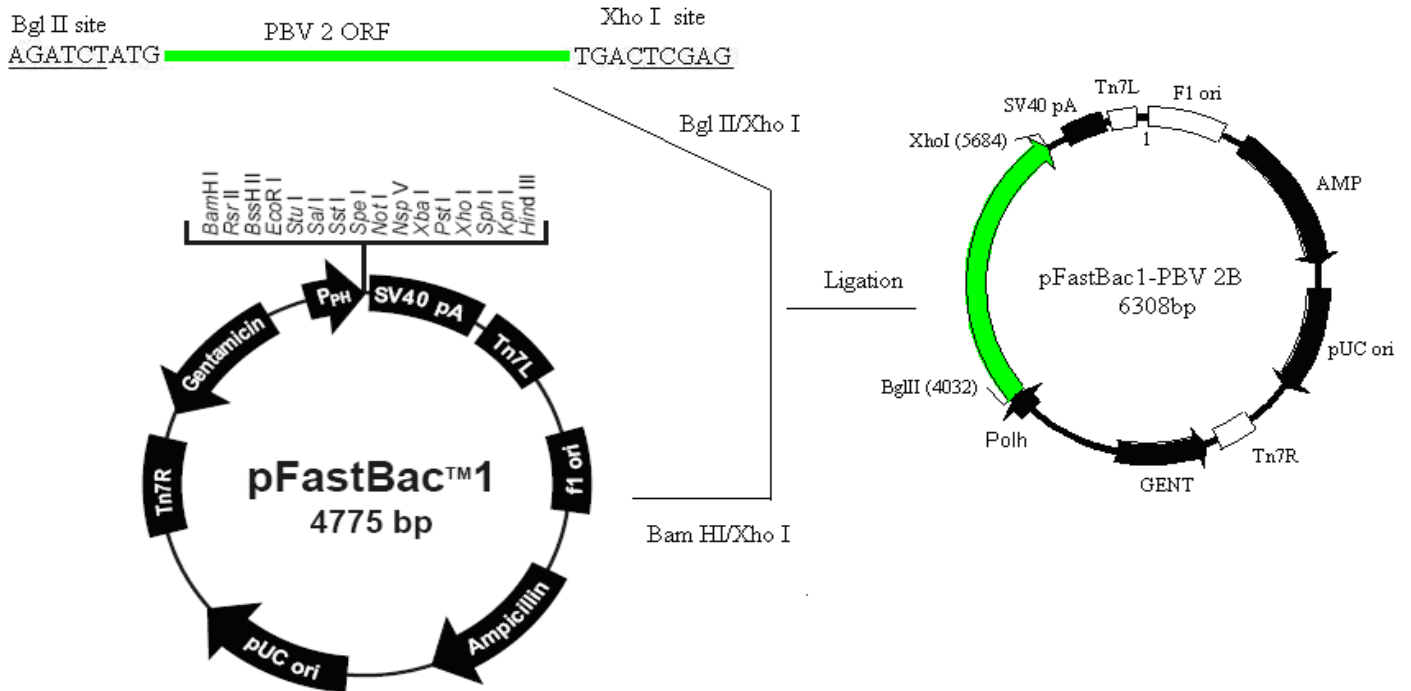


Figure 16. Graphic representation of cloning of picobirnavirus segment 2 cDNA into digested pFastbac1 vector. Ligation of purified PBV 2B from recombinant pGEMt-PBV 2B, into digested pFastbac1 vector resulting into formation of a recombinant pFastBac1-PBV 2B.

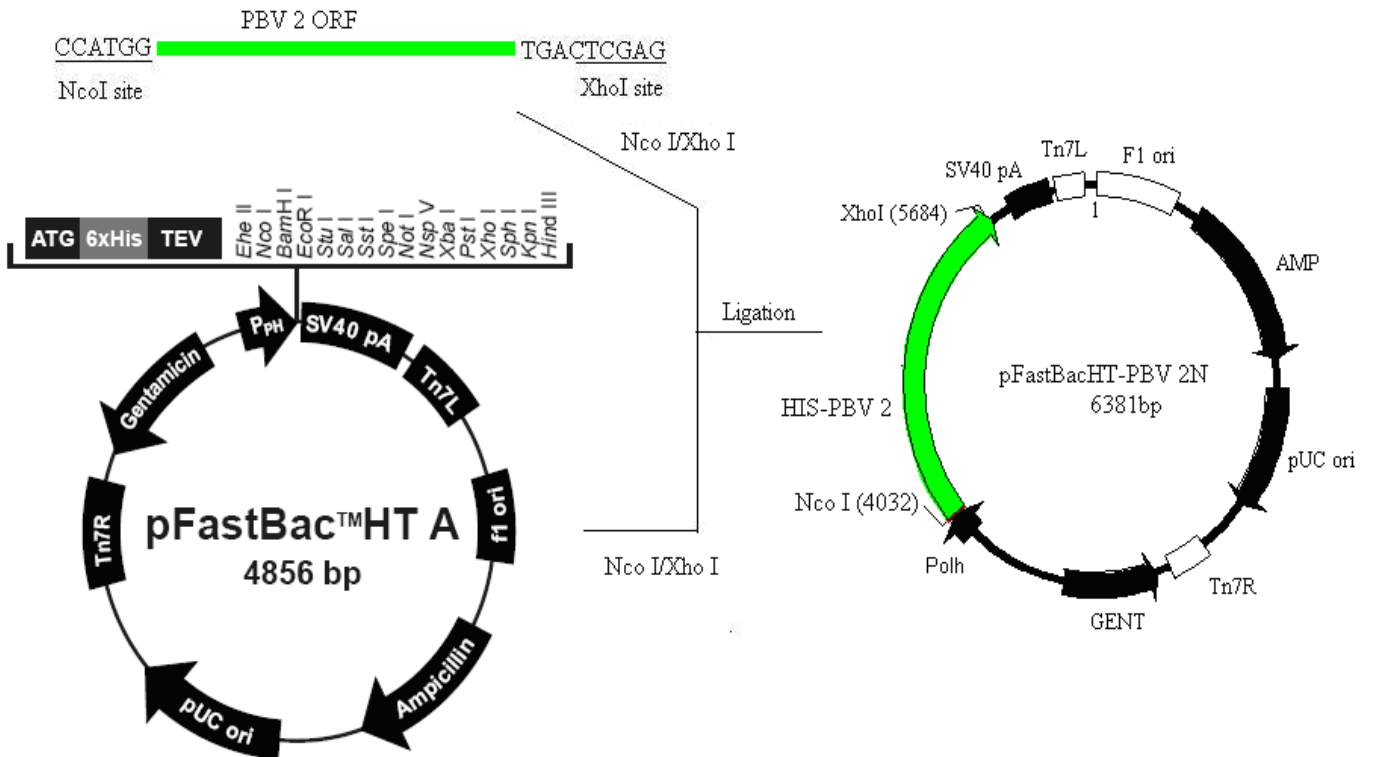


Figure 17. Graphic representation of cloning of picobirnavirus segment 2 cDNA into digested pFastbac-HT vector. Ligation of purified PBV 2N from recombinant pGEMt-PBV 2B, into digested pFastbac1 vector resulting into formation of a recombinant pFastBac1-PBV 2N.

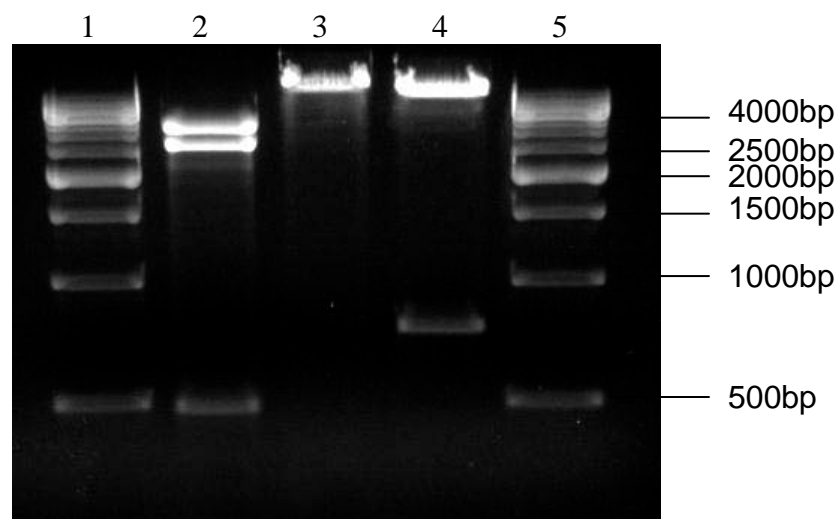


Figure 18. Agarose gel (1%) electrophoresis analysis of restriction digestion of recombinant pFastBac constructs. Lane 1: DNA size marker XVII (Roche), lane 2: *Bgl* II and *Xho* I digested

pFastBac1-PBV 2B, lane 3: *Xho* I digested pFastBac1-PBV 2B, lane 4: *Eco* RI and *Xho* I digested pFastBac-HT-PBV 2N and lane 5: DNA size marker XVII (Roche).

3.3.3. Sequencing of the insert in the recombinant donor plasmids

To confirm that the generated recombinant donor plasmids contained the correct insert, the recombinant donor plasmids were sequenced using a pFastBac sequence specific primer. In this experiment, only the 5' end of the insert was sequenced and compared to the original sequence from Chapter II. Sequencing was performed to confirm that the insert was that of picobirnavirus S2 ORF cDNA. Sequencing outputs from OVI Molecular Biology Sequencing Laboratory were analyzed by DNAMAN as described in Chapter II (2.3.3). Sequencing results show that sub-cloning of picobirnavirus S2 ORF(s) into the baculovirus transfer vectors was successful and the insert in recombinant plasmid pFastBac-HT-PBV 2N was in the correct reading frame (Fig. 19.) Recombinant donor plasmids containing picobirnavirus S2 were stored at -20°C and used for downstream analysis.

FIGURE 19, PRINT SEPARATE, ON DIFFERENT FORMAT

3.3.4. Generation of recombinant bacmid(s)

Recombinant bacmids were generated by transforming *E.coli* cells containing bacmid DNA (competent Acc cells) resulting in transposition of the expression cassette from the recombinant pFastBac donor plasmids to the bacmid DNA as described in section 3.1 and 3.2.2.1. Blue and white colony selection was used to select the recombinant bacmid containing cells. Several white colonies were obtained from the plates containing transformants. Individual white colonies were picked, propagated and used for recombinant bacmid purification and analysis as described in section 3.2.2.2 and 3.2.2.3, respectively.

To confirm transposition of the expression cassette from the donor plasmids into the bacmid DNA, the purified recombinant bacmid DNA was subjected to PCR using M13 forward and reverse primers as described (3.2.2.3). A PCR product of 2300bp or 2430bp in size was expected from the recombinant bacmid DNA transposed with empty pFastbac1 or pFastBac-HT donor plasmid, respectively. A PCR product of 2300bp or 2430bp plus the size of the insert (in this experiment, picobirnavirus S2 ORF, 1602bp) was expected from the recombinant bacmid transposed with recombinant donor plasmid. In Figure 19, lane 1, an amplicon of 3900bp (2300bp plus 1602bp) generated using the M13 primers is shown. Interestingly, in lane 2, the recombinant bacmid was also transposed with recombinant pFastBac1-PBV 2B but yielded two PCR products. One of the PCR products was of the expected size, 3900bp, as in lane 1. The larger PCR product (5500bp), in lane 2, suggests that two inserts were transposed into the bacmid DNA because the PCR product is 5500bp (expected 2300bp plus 2×1605bp).

In Figure 19, lanes 3 and 4, the recombinant bacmid DNA was transposed with recombinant pFastBac-HT-PBV 2N and the figure shows that the expected amplicon of 4035bp (2430bp plus 1602bp) was generated. The results in Figure 20 correlate with the expected DNA fragment sizes and therefore, recombinant bacmid DNA bearing both picobirnavirus S2 ORF, PBV 2B and PBV 2N, was successfully generated.

For reference purposes, the generated recombinant bacmid DNA transposed with pFastBac1-PBV 2B was referred to as Bac-PBV 2B-1 (Fig. 20, lane 1) and Bac-PBV 2B-2 (Fig. 20, lane 2). The recombinant bacmid DNA transposed with pFastBac-HT-PBV 2N as Bac-HT-PBV 2N-1 (Fig. 19, lane 3) and Bac-HT-PBV 2N-2 (Fig. 20, lane 4). Recombinant bacmid of which the PCR results are represented in lane 1 to 4 were used to generate recombinant baculoviruses.

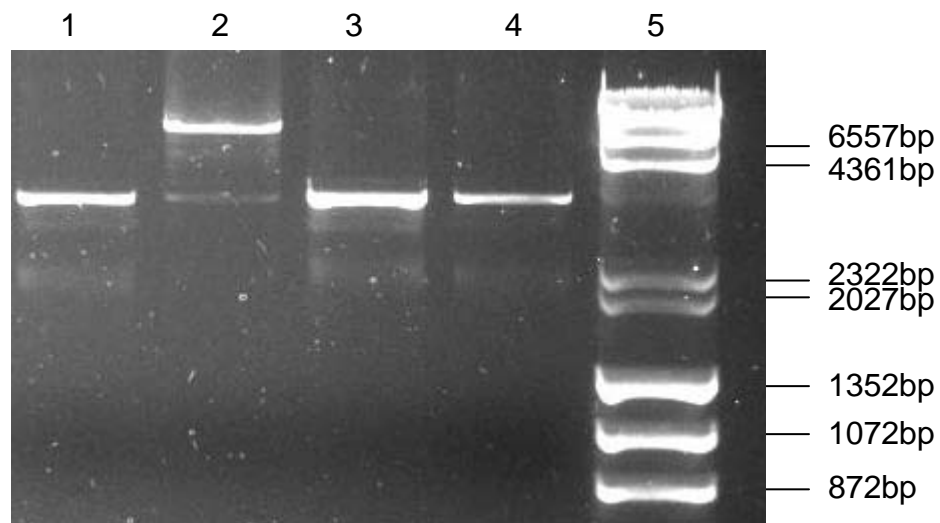
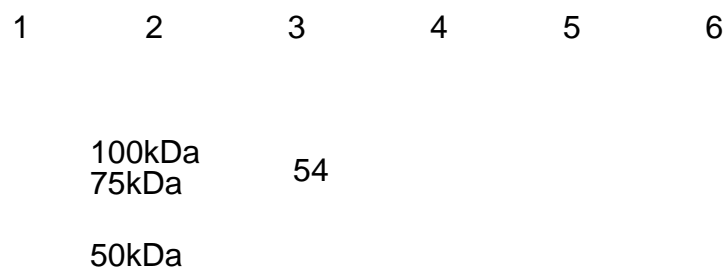


Figure 20. Agarose gel (1%) electrophoresis analysis of PCR amplicons generated from the recombinant bacmid DNA using M13 primers. Lanes 1 and 2: PCR products generated from recombinant bacmid containing PBV 2B (Bac-PBV 2B), lanes 3 and 4: PCR products generated from recombinant bacmid containing PBV 2N (Bac-HT-PBV 2N), lane 5: DNA size marker (λ DNA *Hind III* digest and ϕ X174 DNA *Hae III* digest (Finnzymes)).

3.3.5. Transfection and pilot expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase in the Bac-to-Bac baculovirus system

Recombinant baculoviruses were generated by transfecting fresh insect (*Sf9*) cells with the recombinant bacmid DNA described in section 3.2.3.1. To confirm if the resulting baculoviruses expressed the putative picobirnavirus RNA-dependent RNA polymerase, fresh insect cells were infected with the recombinant baculoviruses (transfection supernatant) as described in section 3.2.3.2. The infected cells were collected, lysed and analyzed by SDS-PAGE as described in section 3.2.3.4. A control was included in the expression experiment in the form of a mock control (no virus was added to the cells). Figure 21, lane 1, shows the total protein fraction from mock infection experiment compared to the infection experiments (lane 3-6). A histidine fusion protein of 61kDa (0.82kDa histidine tag plus 60.9kDa picobirnavirus 2 protein) in molecular weight was expected from the Bac-HT-PBV 2N-1 and Bac-HT-PBV 2N-2 derived recombinant baculoviruses. Figure 21, lanes 3 and 4, shows that a recombinant protein with a molecular weight of approximately 60kDa was observed from the Bac-HT-PBV 2N-1 and Bac-HT-PBV 2N-2 derived recombinant baculoviruses.

A recombinant protein with a molecular weight of 60kDa was also expected from the Bac-PBV 2B-1 and Bac-PBV 2B-2 derived recombinant baculoviruses. Figure 21, lane 5, show that no expression of a recombinant PBV protein size was produced. This is possibly be due to the fact that the recombinant bacmid DNA used to transfect the insect cells had a double insert (Fig. 20, lane 2). Figure 21, lane 6 shows that a protein with a molecular weight of approximately 50kDa was produced. The reduced size of the expressed protein is possibly due to the fact that some proteins contain more negative charges because of its amino acid composition and therefore does not always migrate according to its theoretical molecular weight.



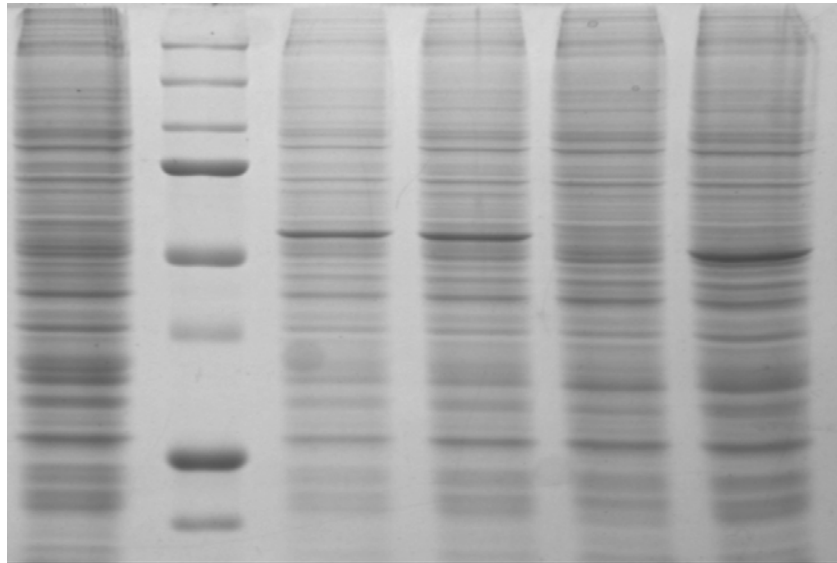


Figure 21. SDS-PAGE analysis of total fraction of protein expressed in SF9 cells infected with recombinant baculovirus (Bac-HT-PBV 2N-1, Bac-HT-PBV 2N-2, Bac-PBV 2B-1 and Bac-PBV 2B-2 respectively). Lane 1: uninfected insect cells, lane 2: Protein molecular weight standards (BioRad, catalogue number: 161-0374), lane 2: Bac-HT-PBV 2N-1 recombinant protein, lane 3: Bac-HT-PBV 2N recombinant protein, lane 4: Bac-PBV 2B-2 recombinant protein and lane 5: Bac-PBV 2B-1 recombinant protein.

3.4. SUMMARY

Picobirnavirus segment 2 (S2) open reading frames PBV 2N and PBV 2B from Chapter II were successfully sub-cloned into two pFastBac donor plasmids, pFastBac-HT and pFastBac1. The recombinant donor plasmids were sequenced with a pFastBac specific primer. It was found that the insert (picobirnavirus S2 ORF) was that of segment 2 of picobirnavirus. Using the recombinant donor plasmids, recombinant bacmids were generated, namely Bac-PBV 2B-1, Bac-PBV 2B-2, Bac-HT-PBV 2N-1 and Bac-HT-PBV 2N-2 (Figure 20). Recombinant bacmid DNA was used to transfect insect cells thereby creating recombinant baculoviruses. Proteins with more or less the expected molecular weights were expressed in *Sf9* cells upon infection. The histidine tagged protein had a molecular weight of approximately 60kDa and the wild-type (untagged) protein had a molecular weight of approx 50kDa (Figure 21). However, the expression levels of the putative picobirnavirus RNA-dependent RNA polymerase was very low. In addition

baculovirus expression is time consuming and expensive. Therefore, the baculovirus expression system described here was reserved as a back-up system in case expression of the protein in bacterial cells was not successful. Chapter IV will describe attempts to express the putative picobirnavirus RNA-dependent RNA polymerase in a bacterial system.

CHAPTER IV

EXPRESSION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE USING A BACTERIAL EXPRESSION SYSTEM

4.1. INTRODUCTION

Bacterial expression systems have been used to produce a large number of recombinant eukaryotic proteins for biochemical analysis (Sahdev *et al.*, 2008). Bacterial protein expression has the advantages that it is easy to culture bacterial cells, bacterial cells grow faster than eukaryotic cells and expression is easily controlled. In bacterial expression systems, the expression plasmids harbour antibiotic resistance genes for selective growth and recombinant protein expression is under the control of a strong promoter, for example the *lac*^R promoter or the T7 phage RNA polymerase promoter (Sahdev *et al.*, 2008). Expression is usually controlled by the *lac* operator and induction with IPTG. Purification of the recombinant proteins of interest has been made much simpler by the use of fusion tags and the availability of commercial kits to purify the resulting fusion proteins.

Bacterial expression systems have been used successfully to produce the RNA-dependent RNA polymerase of a picornavirus, rabbit haemorrhagic disease virus (Lopez-vazquez *et al.*, 2001) as well as the RNA-dependent RNA polymerase of the dsRNA bacteriophages Phi6 and Phi12 (Makeyev and Grimes, 2004). This chapter describes the bacterial expression of the putative RNA-dependent RNA polymerase of a porcine picobirnavirus, using expression plasmid pGEX-4T and *E.coli* BL21 cells. pGEX-4T allows

expression of a GST protein (tag) fused to the recombinant protein of interest. The expression plasmid also has an ampicillin resistance gene for selective growth.

4.2. MATERIALS AND METHODS

4.2.1. Sub-cloning of picobirnavirus S2 ORF into the pGEX-4T vector for bacterial expression

The bacterial expression plasmid, pGEX-4T, was used to sub-clone picobirnavirus S2 ORF, PBV 2B (section 2.3.1). To sub-clone the DNA, 100-150ng pGEX-4T plasmid was digested with restriction enzymes, *Bam* HI and *Xho* I, as described in section 2.3.2. Picobirnavirus S2 ORF (PBV 2B) was removed from recombinant plasmid pGEM-T-PBV 2B by digestion with with *Bgl* II and *Xho* I, separated from the plasmid by agarose gel electrophoresis and gel purified as described in section 2.2.6.5.

Ligation of purified picobirnavirus S2 ORF DNA into the digested pGEX-4T was performed as described in section 3.2.1.4. The ligation reactions were used to transform competent *E.coli* Sure cells as described in section 2.2.6.4. Single colonies from the plates were inoculated into 5ml LB medium supplemented with 100µg/ml ampicillin, for selective growth, and grown overnight at 37°C with shaking. Recombinant plasmids were extracted from 2ml of the overnight culture as described in section 2.2.6.5. The purified recombinant plasmids were analyzed by restriction enzyme digestion and agarose gel electrophoresis. The clones which were shown to contain the correct insert were selected and used for expression of the putative picobirnavirus RNA-dependent RNA polymerase in bacterial cells. Stock recombinant plasmids and recombinant *E.coli* Sure cells were stored as described in section 2.2.6.5.

4.2.2. Expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase in bacterial cells

4.2.2.1. Transformation of *E.coli* BL21 cells with recombinant pGEX 4T-PBV 2B

E.coli BL21 cells (Invitrogen, Genotype: *F ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*) were made competent as described in section 2.2.6.3 and stored in liquid nitrogen. Competent *E.coli* BL21 cells were transformed with recombinant pGEX-4T-PBV 2B (~150ng) and empty pGEX-4T, separately, using the methods described in section 2.2.6.4

4.2.2.2. Expression of recombinant putative picobirnavirus RNA-dependent RNA polymerase protein in *E.coli* BL21 cells

Single colonies from the plates (4.2.2.1) were inoculated separately into 5ml LB supplemented with 100µg/ml ampicillin and incubated at 37°C overnight with shaking (300-350rpm). Overnight cultures were diluted 1:20 with LB supplemented with antibiotics, to a total volume of 5ml. The diluted cultures were grown to an optical density at 600nm (OD₆₀₀) of approximately 0.6 (log phase), in a shaking incubator (300-350rpm) at 37°C. The culture was placed on ice for 10 minutes, and induced with by adding IPTG (Roche, catalogue number: 1 411 446) to a final concentration of 1mM. The induced cultures were incubated for another 4 hours in a shaking incubator (225-250rpm) at 37°C. After incubation, proteins were extracted from the bacterial cells and prepared for SDS-PAGE. As a control BL21 cells transformed with empty expression plasmid was used (pGEX 4-T). The bacterial cells were harvested separately by centrifugation at 10 000 x g for 20 min in a pre-cooled centrifuge (4°C). The cell pellets were re-suspended in BugBuster protein extraction reagent (Novagen, catalogue number: 70584-3) at room temperature. Bugbuster reagent utilises non-ionic detergents to gently disrupt the bacterial cell walls, resulting in the release of recombinant protein. Benzonase (Novagen, catalogue number: 70746-6) and recombinant lysozyme (Novagen, catalogue number: 71110-4) were added to the cell suspension to a final concentration of 25U and 1kU per ml of Bugbuster respectively. Benzonase is a genetically engineered endonuclease from *Serratia marcescens*, which degrades all DNA and RNA in the solution. Lysozyme breaks the bonds between muramic acid polymers in the cell wall, thereby increasing the efficiency of cell lysis and protein extraction. The cell suspensions were incubated on

a rocker (The Belly Dancer[®], Stovall Life Science Inc) at room temperature for 30min. An aliquot of the total cell lysate was collected (30 μ l), and stored on ice as the total protein fraction. Cell debris and insoluble protein-inclusion bodies were removed from the lysate by centrifugation at 10 000 x g for 20 min at 4°C. An aliquot of the supernatant containing the soluble protein fractions were collected and stored on ice. An aliquot of the collected fractions were prepared for SDS-PAGE. 2xSDS sample buffer was added to the aliquot and the mixture was boiled at 95 for 3min. The rest of the lysates were stored at 4°C and -20°C (see section 3.2.3.1 for SDS-PAGE procedure).

4.2.2.3. Determination of the yield and solubility of recombinant fusion protein expressed under different conditions

Single colonies from the plates (4.2.2.1) were inoculated separately into 5ml LB supplemented with 100 μ g/ml ampicillin and incubated at 37°C overnight with shaking (300-350rpm). Overnight cultures were diluted 1:20 with LB supplemented with antibiotic, to different volumes including 5ml (\times 2), 10ml, 25ml and 50ml. The diluted cultures were grown to an OD₆₀₀ of approximately 0.6, in a shaking incubator (300-350rpm). One of the 5ml cultures was immediately induced by adding IPTG to a final concentration of 1mM and incubated for another 4 hours in a shaking incubator (300-350) at 37°C. The remaining cultures (5ml, 10ml, 25ml and 50ml) were placed on ice for 10min and induced by adding IPTG to a final concentration of 1mM. These cultures were induced overnight (16hours) in a shaking incubator (300-350rpm) at 18°C. After incubation, proteins were extracted from the bacterial cells and prepared for SDS-PAGE as described in section 4.2.2.2.

4.3. RESULTS AND DISCUSSION

4.3.1. Sub-cloning of picobirnavirus S2 ORF into bacterial expression vector pGEX4-T

Expression plasmid, pGEX-4T and picobirnavirus S2 ORF DNA was prepared by restriction digestion and subsequent gel purification as described in section 4.2.1. Digestion of pGEX-4T with *Bam* HI and *Xho* I was expected to generate a linearized plasmid with nucleotide overhangs compatible to the nucleotide overhangs on the picobirnavirus S2, PBV 2B, with an expected size of 4900bp. Figure 23, lane 1 and 2, shows that digested pGEX-4T plasmids was successfully recovered from gel extraction procedure. Figure 23, lane 3, show that picobirnavirus S2 ORF DNA was successfully separated from the recombinant pGEM-T vector and recovered from the gel as described in section 2.2.6.6.

The recombinant expression plasmid pGEX-4T-PBV 2B generated in section 4.2.1, was analyzed by restriction digestion (4.2.1). Digestion of recombinant pGEX-4T-PBV 2B with *Eco* RI and *Xho* I was expected to result in the removal of a DNA fragment from the recombinant plasmid with a size of approximately 758bp. The *Eco* RI recognition site is expected to be present only in the insert, hence a fragment of the insert will be removed. However, Figure 24, lane 1 shows that three fragments with sizes of approximately 750bp, 1700bp and 5000bp were generated. The results show that there is another *Eco* RI recognition site present in the recombinant plasmid. This results shows that there are two inserts in the plasmid. This is because the total size of all the fragments is estimated to be larger than 7000bp by combining the fragments obtained in Figure 24, lane 1. Hence there are two inserts in the plasmid. The same fragments were obtained when generation of recombinant pGEX-4T was repeated. The recombinant plasmid was not sequenced and it was decided to transform *E.coli* cells with this plasmid for expression of recombinant protein. Recombinant plasmids which were shown to contain the insert (picobirnavirus segment 2 DNA) was stored as before (2.2.5.5) and used for transformation of BL21 cells.

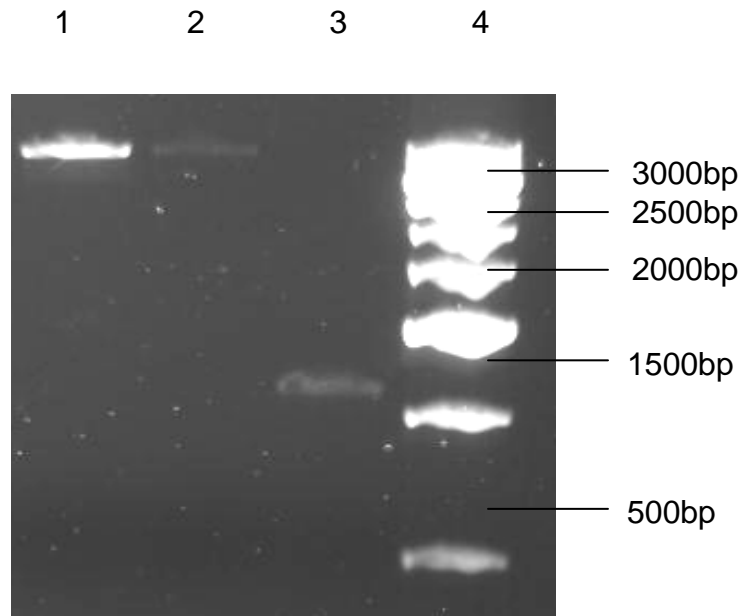


Figure 23. Agarose gel (1%) electrophoresis analysis of digested and gel purified pGEX-4T plasmid and picobirnavirus segment 2 ORF. Lane 1: digested and gel purified pGEX-4T plasmid after digestion with *Bam* HI and *Xho* I, lane 2: digested and gel purified pGEX-4T plasmid after digestion with *Bam* HI and *Xho* I, lane 3: gel purified picobirnavirus segment 2 ORF (PBV 2B) and lane 4: DNA size marker XVII (Roche).

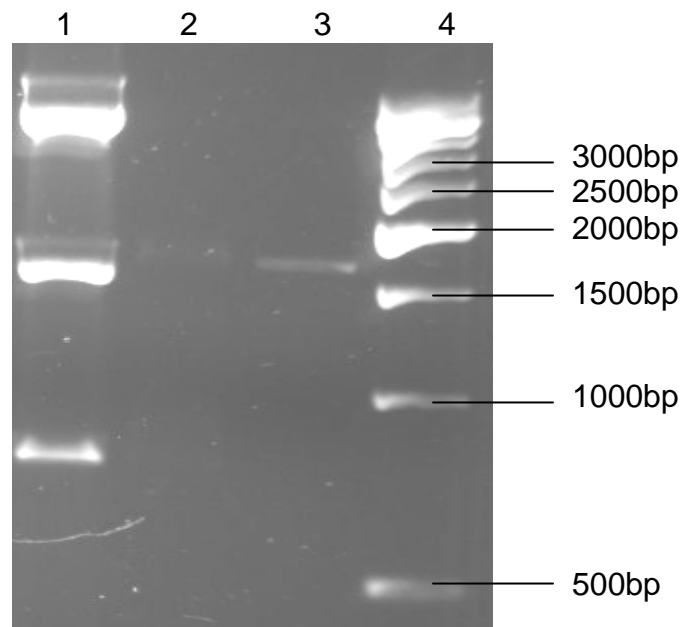


Figure 24. Agarose gel (1%) electrophoresis analysis of restriction digested recombinant pGEX 4T plasmids and gel purified insert. Lane 1: recombinant pGEX-4T-PBV 2B digested with *EcoR* I and *Xho* I, lane 2: recombinant pGEX-4T-PBV 2B digested with *EcoR* I and *Xho* I, lane 3: gel purified insert and lane 4: DNA size marker XVII (Roche).

lanes 2: purified picobirnavirus S2 ORF (PBV 2B) and 3: purified Picobirnavirus S2 ORF (PBV 2B) and lane 4: DNA size marker XVII (Roche).

4.3.2. Expression of putative picobirnavirus RNA-dependent RNA polymerase in bacterial cells

To confirm the recombinant expression of the putative picobirnavirus RNA-dependent RNA polymerase, proteins expressed from empty expression plasmid (pGEX4-T), was compared to the proteins expressed from the recombinant plasmid (pGEX 4-T-PBV-2B). BL21 cells containing either of the plasmids were propagated and protein expression was induced as described (4.2.2.2). The recombinant protein expressed from empty pGEX 4-T was expected to have a molecular weight of 26kDa, while the protein expressed from plasmid pGEX-4T-PBV-2B was expected to yield a protein with a molecular weight of approximately 85kDa (60kDa from the picobirnavirus S2 ORF plus 26kDa of the GST tag). Figure 25, lanes 2 and 3 shows that a recombinant protein of the expected molecular weights for GST tag was expressed. However, Figure 25, lanes 4 and 5, shows that expression from pGEX-4T-PBV-2B plasmid resulted in expression of a recombinant protein with the molecular weight of approximately 75kDa.

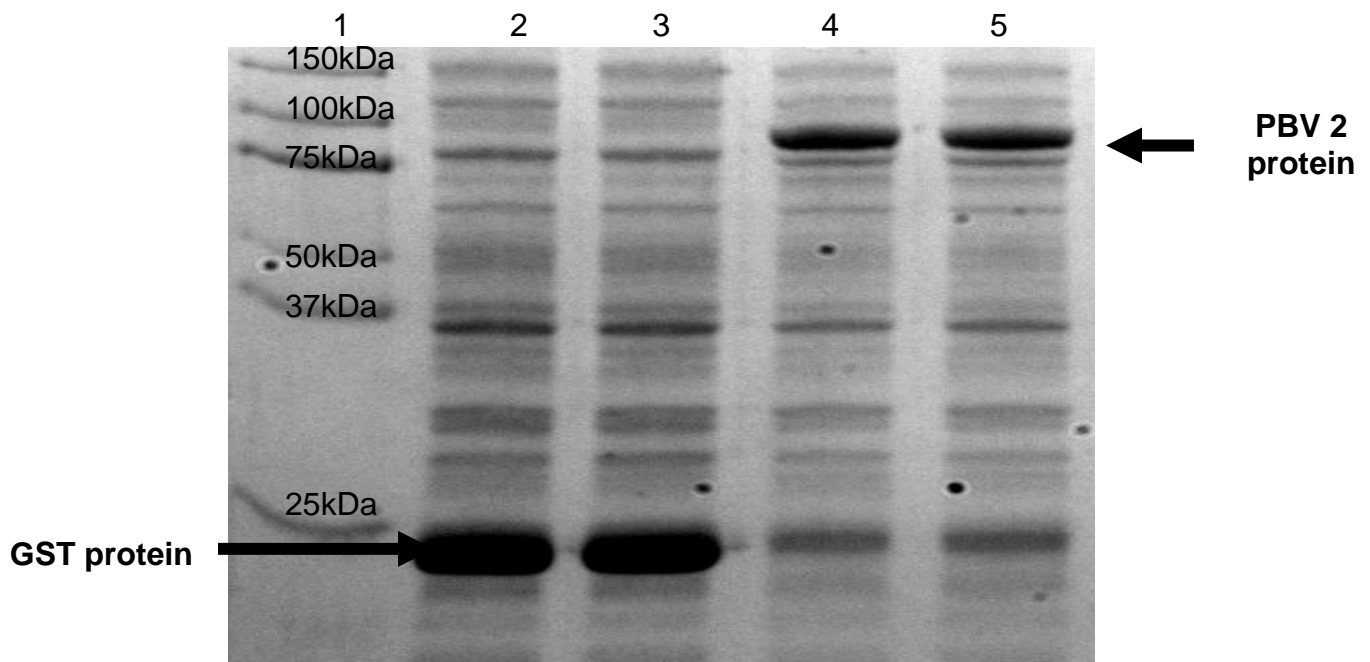


Figure 25. SDS-PAGE (10%) analysis of proteins expressed in recombinant bacterial cells containing an empty expression plasmid (pGEX-4T) and recombinant PBV S2 expression plasmid, respectively. Lane 1: Precision plus protein standards (BioRad), lane 2: Total protein fraction of recombinant cells containing an empty expression plasmid (pGEX-4T), lane 3: Soluble protein fraction of cells containing an empty expression plasmid (pGEX-4T), lane 4: Total protein fraction of recombinant cells containing recombinant expression plasmid (pGEX-4T-PBV 2B) and lane 5: soluble protein fraction of recombinant cells containing recombinant expression plasmid (pGEX-4T-PBV 2B).

4.3.3. Expression and solubility of putative picobirnavirus RNA-dependent RNA polymerase expressed under different conditions

Expression of the recombinant fusion protein was performed under different conditions (temperatures and expression culture volumes) with the aim to find the optimal conditions for expression of soluble recombinant fusion protein. From cultures induced in different volumes and incubated at different temperatures, the total protein fractions and soluble protein fractions were analyzed by SDS-PAGE as described (4.2.2.2). Induction was expected to result in expression of a recombinant protein with a molecular weight of 85kDa (60kDa from the picobirnavirus S2 ORF plus 26kDa of the GST tag). However, induction of 5ml culture grown at 37°C for 4 hours resulted in expression of a recombinant protein with the molecular weight of approximately 75kDa (Fig. 26, lane 2 (total fraction) and 3 (soluble fraction)). The recombinant proteins expressed in 5ml culture at 37°C were found to be highly insoluble (Fig. 26, lane 2 compared to lane 3). Therefore, expression of the recombinant protein at 37°C was not considered favourable. Figure 26, lane 4, shows that when the expression was conducted at 18°C in 5ml cultures, the expression yielded high amount of recombinant proteins when compared to expression at 37°C (Fig. 26, lane 4 compared to lane 2). In addition, Figure 26, lane 5 shows that more soluble recombinant protein was yielded by expression the protein at 18°C (Fig. 26, lane 5 compared to lane 3). To assess if the volume of the culture has an effect in recovery of soluble protein, culture volumes of 10ml, 25ml and 50ml were tested. Figure 26, lane 6 and 8, shows that when the volume of the expression culture was increased to 10 ml, the expression level remained the same (10ml, Fig. 26, lane 6) However further increase in culture volume resulted in decreased yields (25ml and 50ml, Fig. 26, lanes 8 and 10), which means that expression in a 5ml culture at 18°C was most favourable for this study.

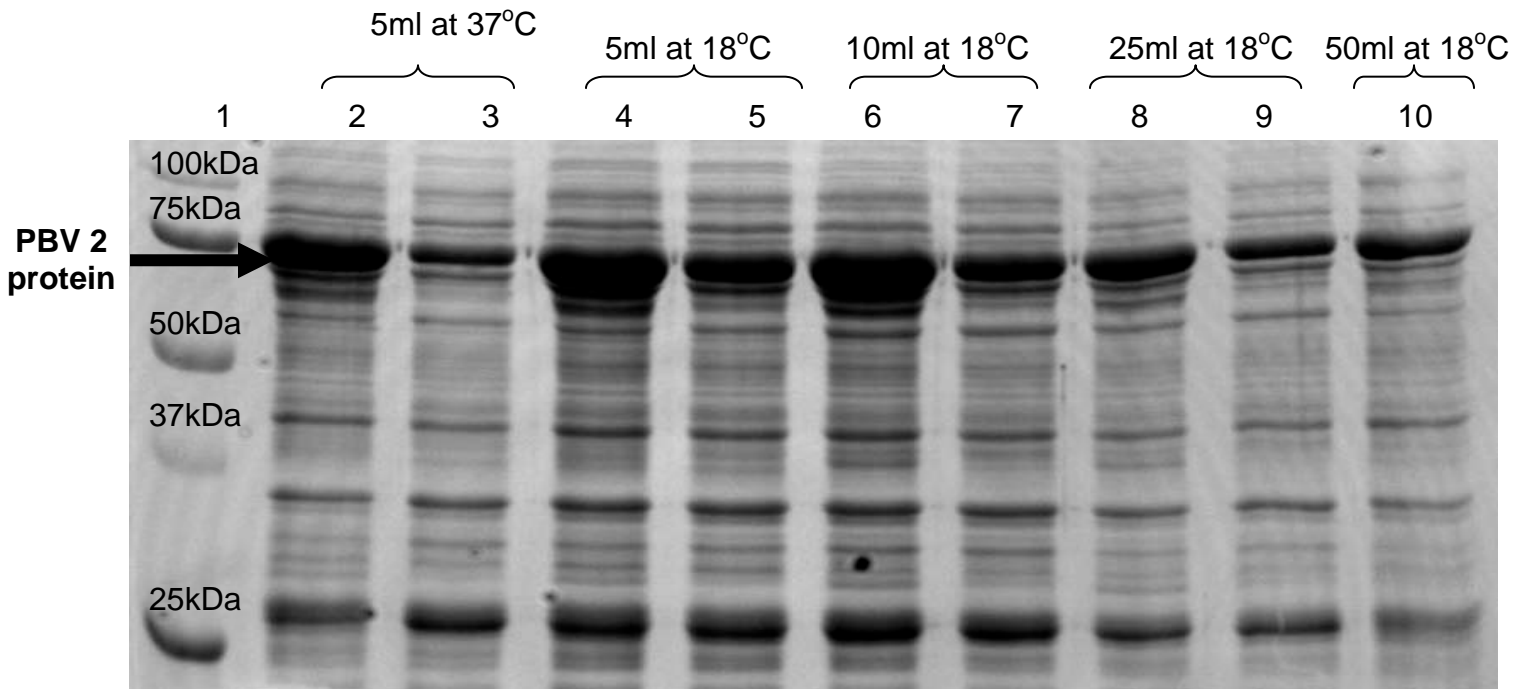


Figure 26. 10% SDS-PAGE analyses of the total fractions and soluble fractions of protein expressed in recombinant bacterial cells under different conditions. Lane 1: Precision plus protein standards (BioRad), lane 2: Total protein fraction at 37°C for 4hrs (5ml culture), lane 3: Soluble protein fraction expressed at 37°C for 4hrs (5ml culture), lane 4: Total protein fraction expressed at 18°C for 16hrs (5ml culture), lane 5: Soluble protein fraction expressed at 18°C for 16hrs (5ml culture), lane 6: Total protein fraction expressed at 18°C for 16hrs (10ml culture) lane 7: soluble protein fraction expressed at 18°C for 16hrs (10ml culture), lane 8: Total protein fraction expressed at 18°C for 16hrs (25ml culture), lane 9: Soluble protein fraction expressed at 18°C for 16hrs (25ml culture), lane 10: Soluble protein fraction expressed at 18°C for 16hrs (50ml culture).

4.4. SUMMARY

The cloned porcine picobirnavirus S2 ORF described in Chapter II was successfully sub-cloned into a bacterial expression plasmid pGEX-4T that allows expression of a GST protein fused to the recombinant protein of interest. Recombinant bacterial cells (*E.coli* BL21 cells) transformed with the recombinant expression plasmid (pGEX-4T-PBV 2B) successfully expressed a recombinant protein upon induction with IPTG (Fig. 25). However, the expressed recombinant protein was 10kDa less than the expected molecular weight (85kDa). This might be due to the fact that not all protein migrates to their expected molecular weight on the PAGE. Other factors, than molecular weight, influence the migration of proteins on PAGE, such as the overall charge of the protein. The recombinant GST fusion protein expressed using this system was found to be more soluble when expressed at 18°C than when expressed at 37°C (Fig. 26). In addition the optimal volume for expression was determined.

CHAPTER V

PURIFICATION OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE – GST FUSION PROTEIN

5.1. INTRODUCTION

The bacterial expression plasmid used to express the putative picobirnavirus RNA-dependent RNA polymerase, pGEX-4T, is an expression plasmid that allows fusion of a recombinant protein with a GST (Glutathione-S-Transferase) tag at the amino terminus (N terminus). The GST fusion protein can subsequently be purified by affinity chromatography using immobilized glutathione and eluted under mild, non-denaturing conditions using reduced glutathione (GST gene fusion system handbook, Edition AA - Amersham Biosciences). If needed the GST tag can be removed from the fusion protein using a site specific protease (Thrombin). This chapter describes the purification of the putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein expressed in Chapter IV, using affinity chromatography and subsequent removal of the GST tag with thrombin.

5.2. MATERIALS AND METHODS

5.2.1. Protein extraction from bacterial cells

Bacterial cultures containing plasmid (pGXE-4T-PBV 2B), were induced overnight as described in section 4.2.2.2. The bacterial cells were harvested and proteins were extracted as described in section 4.2.2.2. Aliquots of the extracts were prepared for SDS-PAGE as described in section 4.2.2.2.

5.2.2. Purification of GST tagged proteins

The following buffer solutions were used in GST fusion protein purification (Novagen, catalogue number: 70534-3):

10× GST bind/wash buffer (pH 7.3)	Stock	Final concentration
Na ₂ HPO ₄	43mM	4.3mM
KH ₂ PO ₄	4.7 mM	0.47mM
NaCl	1.37M	0.14mM
KCl	27mM	2.7mM

10× GST elution buffer (pH8.0)

Tris-HCl	500mM	50mM
Reduced glutathione	100mM	10mM

The expressed GST fusion protein (GST-PBV 2B) was purified from the soluble fraction of the cell lysate (5.2.1) using a method adapted from the GST BIND kit (Novagen, catalogue number: 70534-3) with minor modifications as indicated. The GST column was prepared by adding 3ml of the GST-BIND resin (Novagen, catalogue number: 70541-3) into a plastic column with a bottom stopper and allowed to settle at room temperature. The column was washed with 10ml distilled water. The column was equilibrated with 5ml 1× GST bind/wash buffer (Novagen). The protein extracts (5.2.1) collected as soluble fractions was loaded into the column, mixed gently with the resin by pipetting up and down and allowed to settle for 10min. An aliquot of the flow through fraction was allowed to drain into a collection tube and stored on ice. The column was washed with 10ml 1× GST bind/wash buffer and an aliquot of the flow through fraction was collected as the “wash fraction” and stored on ice. The GST fusion protein was eluted with 5ml 1× GST

elution buffer (Novagen), in a fraction of 1ml. The 1ml elution fractions were collected separately and stored on ice. Aliquots from these fractions were collected for SDS PAGE analysis. All fractions were prepared for and separated by SDS-PAGE as described in section 4.2.2.2.

5.2.3. GST tag removal procedure

The following buffer solution was used in the removal of the GST tag from purified fusion protein:

10x thrombin cleavage buffer (pH 8.4)	Stock	Final concentration
Tris-HCl	200mM	20mM
NaCl	1.5M	0.15mM
CaCl ₂	25mM	2.5mM

The recombinant picobirnavirus S2 (GST-PBV 2B) protein expressed contain a N-terminal GST tag. The tag can be removed by proteolysis using thrombin. The GST tag removal experiment was performed using the elution fraction from section 5.2.2 (Fig. 26, lanes 6 and 7). Prior to treating the GST fusion protein, the elution fraction from section 5.2.2 were combined and concentrated by the use of vivaspin columns (Sartorius, catalogue number: vs0601) as follows: PBS (2ml) was added to the vivaspin column and centrifuged in a pre-cooled centrifuge for 5 minutes at 16100 × g at 4°C. The elution fractions from Figure 26, lanes 6 and 7 were loaded into the vivaspin column and subjected to centrifugation at 16100 × g for 10 minutes at 4°C. Buffer exchange was achieved by adding PBS (5ml) was added to the same vivaspin column and the column was subjected to centrifugation at 16100 × g for 30 minutes at 4°C. After centrifugation, 500µl of protein solution was left in the column and this solution was collected and stored.

GST column was prepared as described before (section 5.2.2). The purified GST fusion protein solution (buffer changed) was loaded on the column and allowed to settle for 10min. The column was washed with 5ml 1× GST bind/wash buffer. The resin-protein complex was equilibrated by washing with 5ml 1× thrombin cleavage buffer (Novagen, catalogue number: 69232-3). The resin-protein complex was re-suspended into 2ml 1× thrombin cleavage buffer on the column, by pipetting up and down. Biotinylated thrombin (25U, Novagen, catalogue number: 69672-3) was added to the resin-protein complex and incubated for up to 2hrs at room temperature in an orbital shaker. After thrombin cleavage, Streptavidin agarose which binds the biotinylated thrombin (800µl) (Novagen, catalogue number: 69203-3) was added to the resin-protein-thrombin mixture, mixed by pipetting up and down and incubated for 10min at room temperature on the orbital shaker. The flow through was collected and stored on ice. To increase the amount of the protein recovered, 1.25ml of 1× thrombin cleavage buffer was added to the treated column, collected in the flow through and stored on ice. The collected fractions were prepared for and separated by SDS-PAGE as described in Chapter IV.

5.3. RESULTS AND DISCUSSION

5.3.1. Purification of recombinant putative picobirnavirus RNA-dependent RNA polymerase –GST fusion protein

The GST fusion protein was purified from the soluble fraction by affinity chromatography as described (5.2.2). Figure 27 shows that most of the recombinant protein was soluble. Proteins from the flow through showed that some of the fusion protein bound successfully to the GST column since there was less recombinant protein in the flow-through than in the total soluble fraction. However, approximately 40% of the expressed proteins did not bind to the column (Fig. 27, lane 4). This might be due to overloading of the column since the 1ml of resin has the capacity to bind 5-8mg of GST tagged protein. However, the amount of protein added on the column was not determined, but it is speculated that more than 10mg was added. The expressed protein bound effectively to the column because very little of the recombinant GST fusion protein was washed off during the washing step (Fig. 27, lane 5). Reduced glutathione successfully displaced the GST fusion proteins from the column by (Fig. 27, lane 4-9) since the elution fractions contained the purified recombinant fusion protein. In Figure 27, lane 6 and 7 contained contaminating protein with a lower molecular weight. The contaminating protein is the same molecular weight as the GST tag alone and therefore it is speculated that expressed tag was co-purified with the GST fusion protein. The eluted proteins could be stored at -20°C and 4°C for at least six months without precipitating out of the solution.

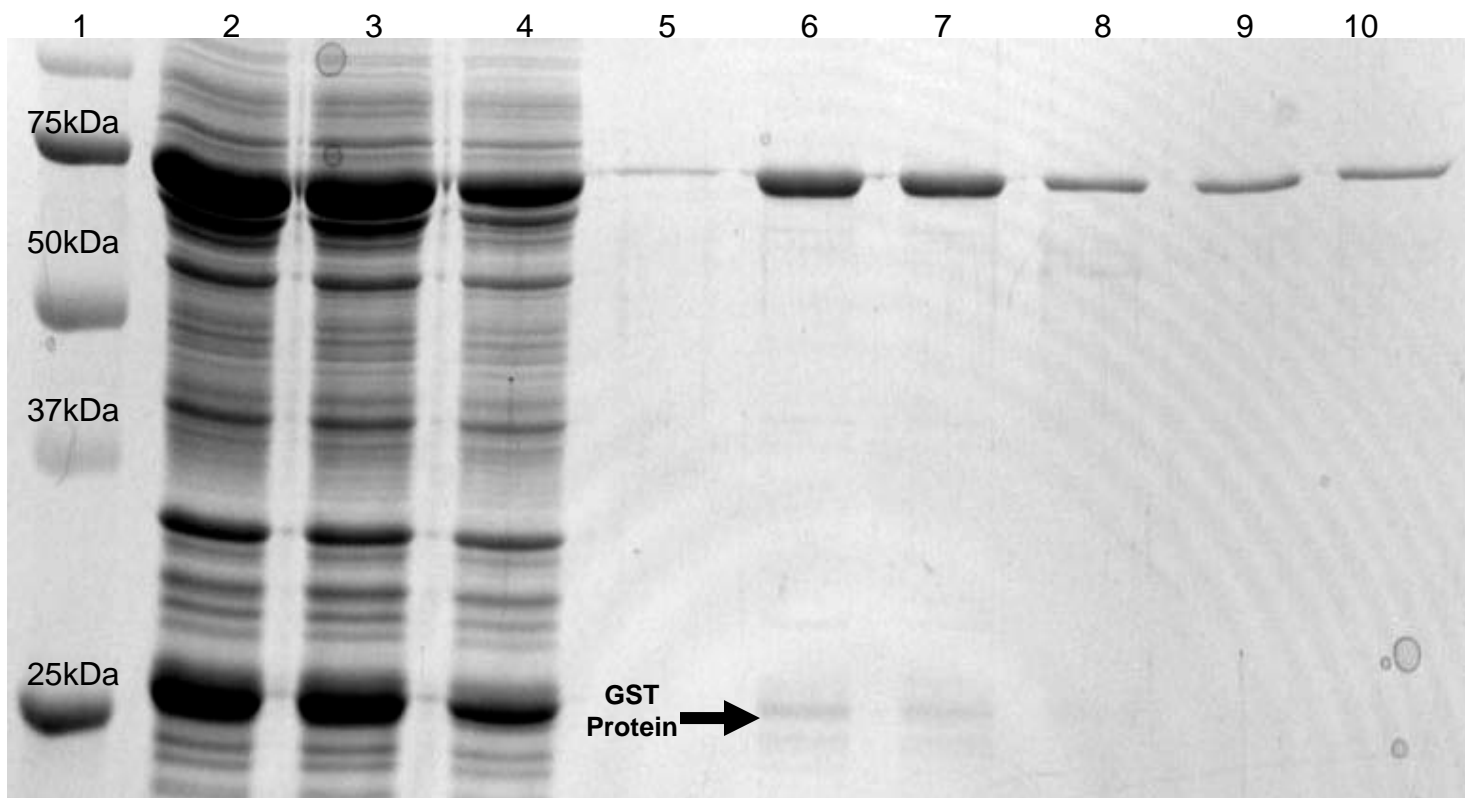


Figure 27. SDS-PAGE (10%) analysis of fractions from the purification of GST tagged picobirnavirus 2 protein. Lane 1: Precision plus protein standards (BioRad, catalogue number: 161-0374), lane 2: total protein fraction, lane 3: soluble protein fraction, lane 4: proteins collected from the column flow-through, fraction lane 5: proteins collected from the column wash fraction, lane 6-10: protein fractions eluted from the column by the addition of reduced glutathione.

5.3.2. Removal of the GST tag from recombinant putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein

The recombinant picobirnavirus S2 protein, expressed in Chapter IV, is fused to a N-terminal GST tag. This tag may interfere with the activity and function of the protein. The tag may either interfere with proper folding of the protein or it may block the active site of the protein and prevent proper functioning of the protein. Hence, an experiment was conducted to remove the GST tag from the expressed recombinant picobirnavirus S2 protein. This was also done so that the untagged and tagged proteins could be compared when binding assays were conducted (Chapter VI). The untagged protein would serve as the “native protein”. The tag can be removed by treatment with thrombin. There is a thrombin cleavage site between the GST tag and the recombinant protein in the fusion protein (Figures 22 and 28).

The GST tag was removed from the expressed recombinant protein (GST-PBV 2) by treating the recombinant protein with biotinylated thrombin and analysed by SDS-PAGE as described (5.2.2). The thrombin cleavage reaction was expected to result in the generation of a protein with a molecular weight of approximately 60kDa, i.e. the size of picobirnavirus S2 protein. Figure 29, lanes 4-6, shows that thrombin treatment resulted in the generation of protein with a molecular weight of approximately 60kDa. However, in this experiment the molecular weight of the expressed and purified recombinant protein was observed to be 80kDa in molecular size, which is 5kDa larger than observed during expression (Fig. 26). The band representing the GST tag will not be observed because the tag will remain bound to the GST column. Biotinylated thrombin binds to streptavidin agarose (used as described in 5.2.3), therefore streptavidin will hold the thrombin in the column and hence it will not be observed in the elution fractions. The untagged protein solutions were stored at 4°C and -20°C. The protein was found to be stable for at least two months.

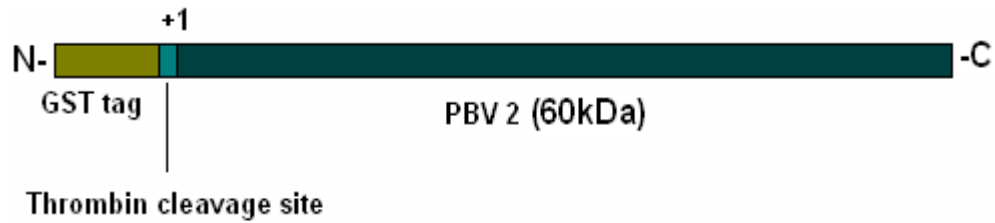


Figure 28. Graphic representation of the GST picobirnavirus S2 fusion protein. The number (+1) represents the start of picobirnavirus segment 2 protein. The letter N and C represents the terminal ends of the recombinant protein (GST-PBV 2), with N representing the amino terminal end and C representing the carboxyl terminal end.

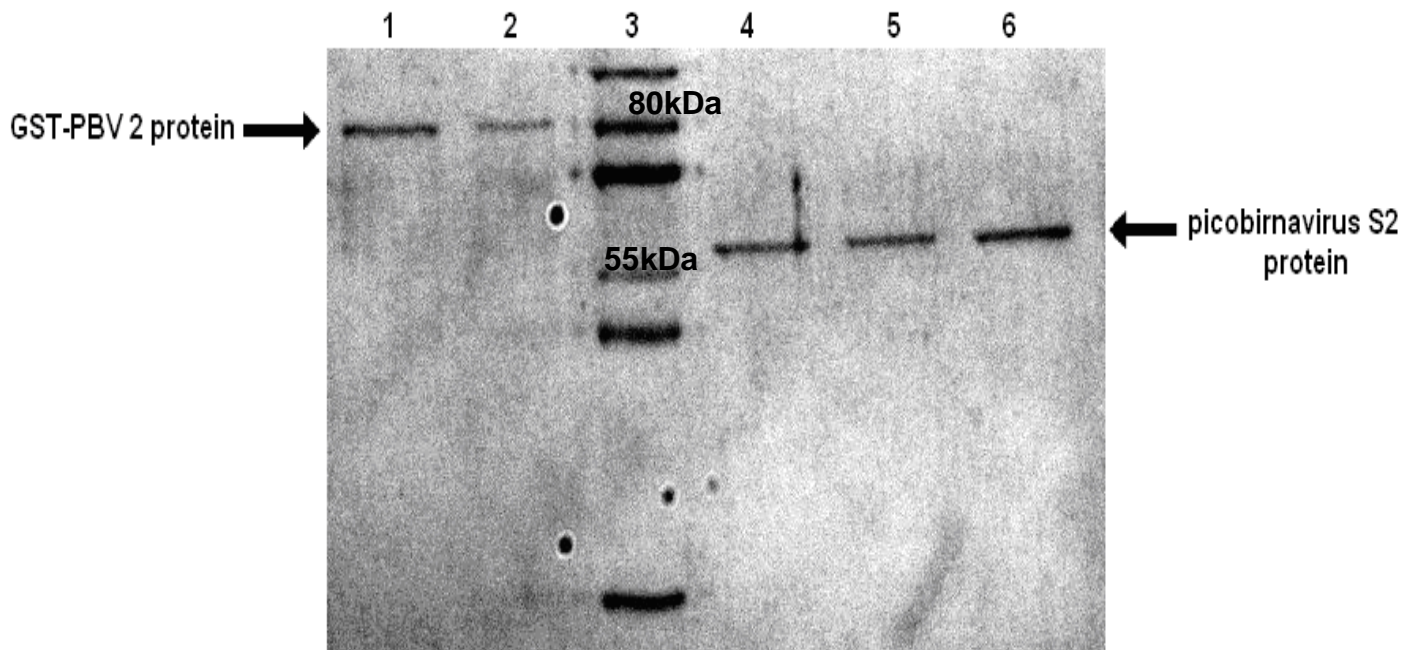


Figure 29. 10% SDS-PAGE analysis of protein fractions before and after GST tag removal from the tagged picobirnavirus segment 2 protein. Lanes 1 and 2: GST tagged picobirnavirus segment 2 protein (recombinant GST-PBV 2 elution fraction from Figure 26, lanes 6 and 7 respectively), lane 3: Protein molecular weight marker (Amersham, catalogue number: RPN800), lanes 4, 5 and 6: protein eluted from the column after thrombin treatment.

5.4. SUMMARY

The putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein expressed in Chapter IV could be purified using affinity chromatography with a commercial GST resin. The recovery of GST fusion protein was very efficient since the fusion protein bound to the column and almost all of the bound protein could be recovered in the first elution fractions (Fig. 27). The recombinant protein expressed and purified using this system was stable for at least six months and was used in Chapter V to try and characterize the expressed protein. The expressed fusion contained a GST tag which could be removed by treating the fusion protein with thrombin. It was shown that the GST tag in the expressed GST fusion protein could be removed by using thrombin (Fig. 29), but the resulting untagged protein was not stable because it precipitated out of the solution (became insoluble) within 36 hours. It is well documented that GST tag not only facilitates purification of the expressed GST fusion protein, but it also help solubilize the recombinant fusion protein. Therefore, if the GST tag is removed from the tag, the recombinant protein may become insoluble.

In the next chapter, experiments with the proteins purified in this chapter will be conducted to determine if the recombinant protein has any of the biochemical characteristics of a RNA-dependent RNA polymerase.

CHAPTER VI

MOLECULAR PROPERTIES OF THE PUTATIVE PICOBIRNAVIRUS RNA-DEPENDENT RNA POLYMERASE

6.1. INTRODUCTION

Picobirnavirus segment 2 (S2) contains the coding sequences of conserved RNA-dependent RNA polymerase motifs (Wakudu *et al.*, 2005, Rosen *et al.*, 2004). RNA-dependent RNA polymerases have the ability to transcribe and replicate RNA. Proteins with this ability also have an affinity for nucleic acids (Makeyev and Bamford, 2000b). The most common experiments that are conducted to prove the identity of a putative RNA-dependent RNA polymerase include RNA binding, heparin binding and RNA replication assays.

Some of the viral RNA-dependent RNA polymerases have been demonstrated to bind to RNA by mobility shift experiments. In mobility shift experiments, the putative RNA-dependent RNA polymerase protein is incubated with RNA which forms a complex. Complex formation is analyzed by agarose or polyacrylamide gel electrophoresis (PAGE) in which the mobility of the RNA complexed with protein (polymerase) will be retarded. For example Hepatitis C virus polymerase (NS5B) has been shown in this way to bind to RNA (Tomei *et al.*, 2000). In order to conduct the RNA binding and polymerase assays, a ssRNA template is required. In mobility shift experiments, the RNA used is from the same virus from which the polymerase protein is derived and the RNA is usually radioactively labelled. Thus authentic viral RNA, or a RNA probe synthesized using authentic virus RNA sequence is normally used. Most of the RNA-dependent RNA polymerases of dsRNA viruses have been shown to be specific to the 3' end sequence of their natural viral RNA. For example, the rotavirus RNA polymerase (VP1) is specific for the sense and anti-sense 3' end sequences in the non-coding regions. On the sense strand the consensus sequence, UGUGACC, is critical for dsRNA synthesis, and on the anti-sense the consensus sequence, A/U₇₋₈GCC, is critical for transcription (Patton, 2006). The RNA transcribed from bluetongue cores has been shown to be cap methylated (Mertens *et al.*,

2004). Recently, Boyce *et al.*, 2008 published the rescue of BTV from ssRNA transcripts made *in vitro*. Rescue only worked with ssRNA transcripts that were capped. In addition Patton *et al.*, 2006 showed the presence of a cap pocket in the RNA-dependent RNA polymerase of rotavirus, which suggests that replication is only sufficient in the presence of capped ssRNA. Given the above, it is essential that authentic virus ssRNA transcripts are used for mobility shift assays and RNA replication experiments.

Most proteins with affinity for DNA or RNA, including RNA-dependent RNA polymerases have been shown to have an affinity for heparin. Structurally, heparin is highly negatively charged and is composed of disaccharide units, which form a three-dimensional structure which is similar to that of a DNA or RNA. Therefore, binding of a protein to heparin is an indication that the protein may have an affinity for nucleic acids. This property has also been exploited to purify nucleic acid binding proteins. For example, during the purification process of recombinant Phi6 polymerase, heparin columns are used (Makeyev and Bamford, 2000b, Makeyev and Grimes, 2004). Mutant hepatitis C virus RNA-dependent RNA polymerase (NS5B) has also been shown to have affinity to heparin and its purification is facilitated by the use of heparin columns (Tomei *et al.*, 2000).

All the viral RNA-dependent RNA polymerases have been demonstrated to have RNA replicase activity. In RNA replicase assays, the putative RNA-dependent RNA polymerase protein is used to convert the ssRNA into dsRNA in the presence of authentic or synthetic RNA and commonly with radioactively labelled nucleotides. The replicase assay product is analyzed by agarose or polyacrylamide gel electrophoresis (PAGE). For example Phi6 polymerase have been shown to effectively replicate all phi6 specific positive-sense RNA substrate into dsRNA, However, phi6 polymerase has also been shown to replicate a broad range of heterologous ssRNA template (Makeyev and Bamford, 2000b). Other polymerases have been shown only to replicate short probes. For example, Hepatitis C virus RNA-dependent RNA polymerase (NS5B) has been shown to replicate a poly(rA)-poly(rU) and poly(rC)-poly(rG) templates (Tomei *et al.*, 2000)

In this chapter, the *in vitro* synthesis of positive sense ssRNA template with terminal ends identical to that of authentic picobirnavirus RNA will be described. Using these transcripts in combination with the recombinant putative picobirnavirus RNA-dependent RNA

polymerase expressed in Chapter IV, the following experiments were conducted to establish the identity of the protein:

- RNA binding (Mobility shift) experiments
- Heparin binding
- RNA replication assays

6.2. MATERIALS AND METHODS

5.2.1.1. *In vitro* generation of authentic picobirnavirus S2 RNA transcripts

6.2.1.1. Generation of full length picobirnavirus S2 transcription templates containing RNA polymerase promoters

To generate DNA template for the *in vitro* transcription of authentic picobirnavirus S2 ssRNA, three S2 sequence-specific primers were designed based on the complete sequence of porcine picobirnavirus S2 (section 2.3.1). The RNA polymerase promoter sequences for T7 and SP6 RNA polymerases were added to the forward primers. Primers were designed so that the RNA polymerase promoter sequences were immediately followed by the first nucleotide of the authentic picobirnavirus sequences. The downstream primer was designed so that the amplicon would end with the exact same sequence as the authentic picobirnavirus transcripts.

The following primers were designed:

▪ **Forward primers**

Primer 4

Sequence	: 5'- TAATACGACTCACTATAGTAAAATTTTG -3'
Promoter sequence	: T7 polymerase
Length	: 29bp
GC content	: 27.6%
T _m	: 57.1°C

Primer 5

Sequence	: 5'- ATTTAGGTGACACTATAGTAAAATTTTG -3'
Promoter sequence	: SP6 polymerase
Length	: 29
GC content	: 27.6%
Tm	: 58°C

- **Reverse primer**

Primer 6

Sequence	: 5'-GCAGTTGGAACCAAGTTTG-3'
Length	: 18
GC content	: 47.4%
Tm	: 55.0°C

The primers were synthesized by Invitrogen and they were used to generate full length picobirnavirus S2 cDNA containing RNA promoter sequences at the 5' end by PCR as indicated in Fig. 29. Clones 1 and 2, the original pGEM-T cDNA clones containing full length picobirnavirus S2 cDNA (section 2.2.2) were used as template for PCR. PCR amplification was performed using 1µl (300ng/µl) of each sample as template. Two PCR reactions (one from each template) were performed in 25µl reaction volumes containing the following: 1x PCR buffer (Roche, catalogue number: 1699121) (10X: 100mM Tris-HCl, pH 8.85, 250mM KCl, 50mM (NH₄)₂SO₄, and 20mM MgSO₄), 0.2mM dNTPs (dATP, dCTP, dGTP and dTTP each) (Roche, catalogue number: 1581295), 0.5U of *Pwo* polymerase (Roche, catalogue number: 1644947), 50pmol of each primer and Ultra-pure water up to 25µl. The PCR reactions were carried out in the Eppendorf MasterCycler^R gradient thermocycler. The program for the PCR reaction performed with primers 4 and 6, and primers 5 and 6 respectively contained the following steps:

Steps	Conditions	Number of Cycles
1.	120 seconds at 94°C	x1
2.	150 seconds at 50°C	x1
3.	90 seconds at 72°C	x1
4.	30 seconds at 94°C	} x15
5.	90 seconds at 63.8°C	
6.	90 seconds at 72°C	
7.	180 seconds at 72°C	x1

The PCR products from both reactions were subjected to electrophoresis in an unstained 1% TAE-agarose gel at 80V for 2 hours in 1x TAE buffer as described before in section 2.2.4. The gels were stained as before and the PCR amplicons were purified as described in section 2.2.5. The purified PCR products were used in the *in vitro* generation of uncapped picobirnavirus S2 ssRNA template (6.2.1.2).

6.2.1.2. *In vitro* RNA transcription

The purified PCR products were used in the transcription of unlabelled ssRNA using T7 and SP6 RNA polymerase (Epicentre, catalogue number: AS3107). For each transcription reaction, 5µl of the purified full length picobirnavirus segment 2 cDNA containing the T7 and SP6 promoter sequence (purified PCR product, 6.2.1.1) was used separately. Full length DNA fragments of picobirnavirus S2 generated from clones 1 and 2 (6.2.1.1) were used as *in vitro* transcription templates and they were referred to as template 1 and 2 respectively. The *in vitro* transcription reactions were performed in 50µl volumes containing the following: 1x transcription buffer (Epicentre, catalogue number: AS3107), 7.5mM rNTPs (ATP, CTP, GTP and UTP each) (Epicentre, catalogue number: AS3107) and nuclease free water up to 50µl. Reactions were initiated by adding 1x enzyme mix

(T7 and SP6 RNA polymerase solution, separately, containing an RNase inhibitor - Epicentre, catalogue number: AS3107). The reactions were incubated at 37°C for 2-3hrs.

An aliquot of the reaction (3µl) was subjected to electrophoresis in an ethidium bromide stained 1% agarose gel as described before (section 2.2.3). The gel apparatus (tank, gel tray and comb) was treated with 3.5 % (w/v) sodium hypochlorite (Regular Bleach, Pick 'n Pay) and rinsed with HPLC water (BDH) to remove RNases.

RNA products from the remaining reaction mixtures were purified by ammonium acetate precipitation according to the manufacturer's instructions (Epicentre, Ampliscribe T7, T3 and SP6 RNA High yield transcription kit protocol manual). Purification of RNA by ammonium acetate relies on the fact that ammonium acetate selectively precipitates RNA, leaving most of the DNA and unincorporated nucleotides in the supernatant after centrifugation. RNase-free ammonium acetate was added to a final concentration of 2.5M and the solutions were incubated on ice for 15 minutes. The precipitated RNA was pelleted by centrifugation at 10 000 x g for 15 minutes. The pellet was washed in 70% ethanol and re-suspended in 50µl of RNase-free water. An aliquot of the purified ssRNA products (3µl) were subjected to electrophoresis in an ethidium bromide stained 1% agarose gel at 80V for one hour in 1× TAE buffer as described before (section 2.2.4) with treatment of electrophoresis equipment as described above.

To determine the identity of the RNA (whether it was indeed RNA), the ssRNA products were treated with RNase A by adding buffer solution P1 from a Qiagen Spin miniprep kit, which contains RNase A (Qiagen, catalogue number: 27104). RNase reactions were performed in 6µl reaction volumes containing 3µl purified ssRNA and 3µl P1 buffer (QIAprep miniprep kit). The RNase reactions were incubated at room temperature for 30 minutes. The reaction mixtures were analyzed after separation on EtBr stained 1% TAE-agarose gels as described before (section 2.2.3). The rest of the ssRNA generated from both DNA templates were stored at -20 until further use. DNA size markers were included for comparison only and not for ssRNA size estimation.

6.2.2. Mobility shift assay

The ssRNA generated in 6.2.1.2 was used in combination with purified recombinant protein, from section 5.3.1, to assess whether the recombinant protein would bind to authentic picobirnavirus S2 ssRNA transcripts. Mobility shift assays were performed in 10µl reaction volumes containing the following: 50ng ssRNA (6.2.1.2), 5µl purified recombinant protein (from 5.3.1) and 4U protector RNase inhibitor (Roche). The reaction mixtures were incubated at 30°C for 30 minutes in a water bath. The reaction mixtures were analyzed after separation on a EtBr stained RNase free 1% TAE-agarose gel as described above (6.2.1.2).

6.2.3. Binding of soluble recombinant putative picobirnavirus RNA-dependent RNA polymerase protein to heparin

In this section, experiments were conducted to assess if the putative RNA-dependent RNA polymerase of picobirnavirus can be purified using a heparin column. The following buffer solutions were used in purification of the expressed putative picobirnavirus RNA-dependent RNA polymerase using a heparin column (method used from Makeyev and Grimes, 2004):

10x Heparin binding buffer (pH 8.4)	Stock	Final Concentration
Tris-HCl	200mM	20mM
EDTA	1mM	0.1mM
NaCl	100mM	10mM

The recombinant putative picobirnavirus RNA-dependent RNA polymerase was expressed and prepared for purification as described in section 5.2.1. The purification of

the expressed polymerase was conducted using the total soluble fraction of the cell lysate (section 5.2.1) and a heparin column as follows:

A heparin column was prepared by adding 3ml of immobilized heparin gel slurry (Separations, catalogue number: 20207) into a commercial plastic column with a bottom stopper. The slurry was allowed to settle at room temperature. The column was washed with 10ml of HPLC water (BDH) by allowing the water to drain through the column. The column was equilibrated by with 6ml of 1× Heparin binding buffer. The soluble protein extract (prepared as in section 5.2.1) was loaded on the column and allowed to settle for 10 minutes. The flow through was allowed to drain into a collection tube and stored on ice as the flow through fraction. The column was washed with 3ml heparin binding buffer (i.e. three times, 1ml per wash) and the first “wash fraction” was collected and stored on ice. The protein was eluted from the heparin column by adding heparin binding buffer containing NaCl. The elution was performed in fractions of 1ml per elution, with an increasing concentration of NaCl in increments of 200mM. The 1ml elution fractions were collected separately and stored on ice. Aliquots of the collected fractions were prepared for and separated by SDS-PAGE as described before (4.2.2.2).

6.2.4. Heparin binding of thrombin treated GST fusion protein

The purified recombinant putative picobirnavirus RNA-dependent RNA polymerase from which the GST tag was removed was used in a heparin binding assay. The recombinant protein without the GST tag was prepared as before (section 5.2.3). However, in this experiment GST resin and streptavidin agarose was not used to remove the GST tag and thrombin as before. The heparin binding assay for this protein was different to the procedure described above. The heparin agarose was prepared by adding heparin slurry (1ml) (Separations) into a 2ml micro-centrifuge tube and centrifugation at 16100 × g in a bench-top centrifuge at room temperature for 2 minutes. The heparin agarose was washed with 2ml HPLC water (BDH) three times. The agarose was washed by adding the HPLC water (BDH) into the micro-centrifuge tube, mixing by inversion, centrifugation at 16100 × g for 1 minute and decanting the supernatant. The heparin agarose was

equilibrated by washing with 4ml of 1× heparin binding buffer. The thrombin treated protein was added and the protein-heparin mixture incubated for 10 minutes at room temperature followed by centrifugation at 16100 × g for 1 minute. The supernatant was

collected as “flow through” and stored on ice. The slurry was washed with 1ml 1× heparin binding buffer by adding the buffer in the 2ml micro-centrifuge tube, mixing by inversion and centrifugation at 16100 × g for 1 minute. The supernatant was collected as the “wash fraction” and stored on ice. The bound protein was eluted from the heparin column with 1× heparin binding buffer in increasing concentrations of NaCl (200mM-800mM) in 200mM increments. The elution was performed in fractions of 1ml per elution. Aliquots of the collected fractions were prepared for and separated by SDS-PAGE as described before (section 4.2.3.1).

6.2.5. RNA replication assay

The ssRNA produced in section 6.2.1.2 and the protein purified in section 5.2.2 protein was used in the polymerase assays. The reaction buffer components for the polymerase assay using the putative picobirnavirus RNA-dependent RNA polymerase were formulated by comparing and mixing components from reaction buffers described for the rabbit haemorrhagic disease virus RNA-dependent RNA polymerase (Lopez-Vazquez *et al*, 1998) and the hepatitis C virus polymerase reaction buffers (Biswal *et al*, 2005). Phi6 polymerase reaction buffer (Makeyev and Bamford, 2000b) was used as a control buffer solution. The following buffers, prepared in RNase free water was used in the polymerase assays unless stated otherwise:

2× picobirnavirus reaction buffer	Stock	Final concentration
HEPES (pH 8.0)	100mM	50mM
DTT	8mM	4mM
DMSO	20% (w/v)	10% (w/v)
Mg(CH ₃ COO) ₂ ·H ₂ O	6mM	3mM

MnCl ₂	2mM	1mM
EDTA	0.2mM	0.1mM
Triton X-100	0.2 % (w/v)	0.1% (w/v)
PEG 3500	12% (w/v)	6% (w/v)

2x Phi6 reaction buffer (pH 7.8)

HEPES	100mM	50mM
NH ₄ CH ₃ COO	40mM	20mM
MgCl ₂	10mM	5mM
MnCl ₂	2mM	1mM
EDTA	0.2mM	0.1mM
Triton X-100	0.2% (w/v)	0.1% (w/v)
PEG 3500	12% (w/v)	6% (w/v)

The polymerase assays were performed in 15µl reactions containing the following: 2µl ssRNA (6.2.12), 2µl purified picobirnavirus S2 – GST protein (from 4.3.2.3) or 2µl purified untagged picobirnavirus S2 protein (6.2.1.5), 1x picobirnavirus 2 reaction buffer or 1x Phi 6 reaction buffer, 2.5mM rNTP (ATP, UTP, GTP and CTP each) (Ambion), 4U RNase inhibitor (Roche, catalogue number: 3335399001) and RNase-free water up to 15µl. As controls, the same reactions without the ribonucleotides were performed. The control reactions also served as the mobility shift assays similar to those described in 6.2.1.3. The reaction mixtures were incubated at 30°C for one hour in a water bath. The reaction temperature was chosen based on the temperature used for rabbit haemorrhagic disease virus polymerase assay. The temperature was also chosen because phi6 polymerase, used as the positive control, is active at 28°C but it has also been shown to be active at 30°C. Phi6 polymerase was a kind gift from Professor Dennis Bamford (Centre of Excellence, University of Helsinki, Finland). Aliquots of the reaction mixtures were

subjected to electrophoresis in a stained 1.2% agarose gel at 80V for 1hrs in 1x TAE buffer as described before (6.2.1.2).

6.3. RESULTS AND DISCUSSION

6.3.1. Generation of RNA synthesis template (picobirnavirus S2 cDNA) by PCR

Using full length picobirnavirus S2 cDNA sequence information, sequence specific PCR primers were designed using DNAMAN, to amplify the full length picobirnavirus S2 by PCR. The 5' primers were designed to incorporate promoter sites for T7 or SP6 RNA polymerase in the 5' end of the DNA and allow the start of transcription to be at the authentic 5' end of the picobirnavirus segment 2 DNA. The reverse primer was designed so that the PCR product would run for the full-length of segment 2 and ends with the authentic picobirnavirus segment 2 RNA 3' sequence. An enzyme (*Pwo* DNA polymerase) with proof-reading ability was chosen to minimize the chances of PCR produced mutations and to ensure that all the PCR products were blunt ended. Transcription from template with overhangs is often not efficient (Jorgensen *et al.*, 1991). PCR products with the expected size of approximately 1700bp were generated with both primer sets from both clones 1 and 2. The products were subsequently gel-purified and analyzed on an agarose gel. Figure 31 shows a picture of the purified products.

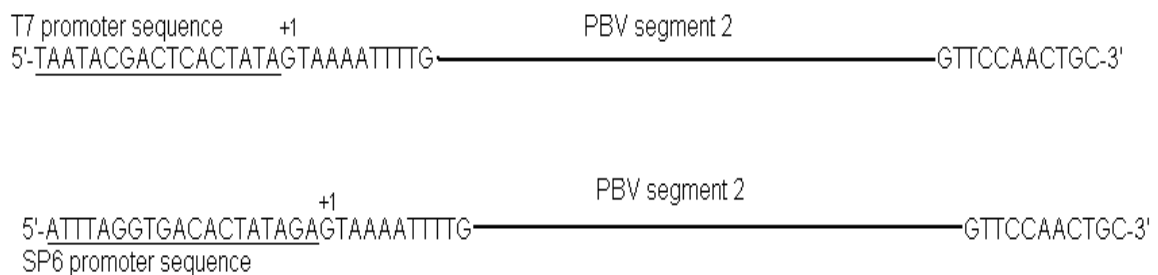


Figure 30. Graphic representation of the expected PCR products using promoter region incorporated primers. The location of the T7 and SP6 promoters sequences on the PCR products are indicated by the underlined bases. The first base-pair of picobirnavirus segment 2 is indicated by a +1.

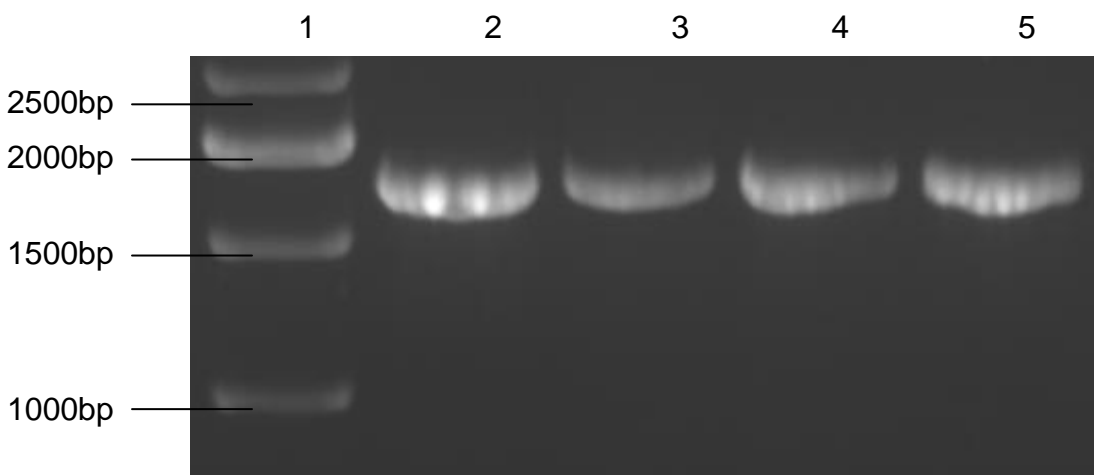


Figure 31. Agarose gel (1%) electrophoresis analysis of purified PCR products of the full length picobirnavirus S2 cDNA using sequence specific primers containing RNA polymerase promoter sites. Lane 1: DNA size marker XVII (Roche), lane 2: PCR products generated from clone 1 using primers 4 and 6, lane 3: PCR products generated from clone 2 using primers 4 and 6, lane 4: PCR products generated from clone 1 using primers 5 and 6, lane 5: PCR products generated from clone 2 using primers 5 and 6.

6.3.2. Generation of ssRNA template

The purified PCR products containing the T7 and SP6 RNA polymerase promoter sequences in the 5' end (Fig. 30) from both clone 1 and clone 2 templates were used for the generation of ssRNA. *In vitro* transcription was performed with the PCR products as template and T7 or SP6 RNA polymerase. A ssRNA product was only produced from templates 1 and 2 containing the T7 RNA polymerase site. Figure 32, lane 2 and 4, show that a product with a size of approximately 850bp in size (when compared to dsDNA) was generated. However, two non-specific RNA products with sizes of approximately 1500bp and 600bp were also generated. The use of templates 1 and 2 template containing SP6 RNA polymerase site did not produce and RNA products (results not shown). The yield of

ssRNA products from the Epicentre kits was very low, especially after precipitation with ammonium acetate.

Alternate production and purification methods were required to obtain high yield of ssRNA. A RiboMAX kit from Promega (catalogue number: P1300) was tested in combination with the Megaclear kit from Ambion (catalogue number: 1908) for purification. *In vitro* transcription performed using T7 RNA polymerase from Promega was performed according to manufacturer's instruction (Promega, RiboMAX Large Scale RNA Production System instruction manual). Purification of the RNA products with the Megaclear kit relies on the use of a filter cartridge which selectively binds the RNA. The bound RNA is then washed of impurities with a solution containing ethanol. The bound RNA is eluted in a low salt buffer. Using these kits, large amounts of ssRNA were recovered (Fig. 33, lanes 2-4). However, a larger band can be observed. This band represents the DNA templates since no DNase treatment was done, and the smearing below the RNA band is due to possible RNase contamination in the gel.

To confirm the identity of the transcription products, they were treated with RNase A. Figure 34 shows that the bands observed in Figure 33, lane 2-4, are RNA, since RNase treatment resulted in complete degradation of the RNA products (Fig. 34, lane 1-4). It was concluded that ssRNA was successfully generated using T7 RNA polymerase. However it could not be determined if the RNA was the correct size since ssRNA markers were not available for comparison. The ssRNA was stable when stored at -20°C for at least 12 months without any sign of degradation.

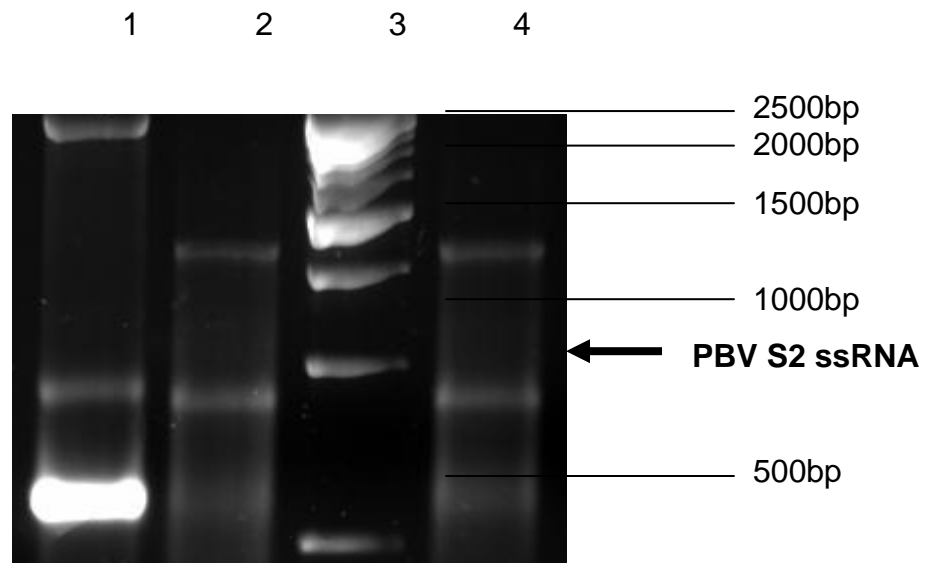


Figure 32. Agarose gel (1%) electrophoresis analysis of ssRNA products generated from the full length picobirnavirus S2 cDNA using T7 RNA polymerase (T7 ampliscribe kit, Epicentre). Lane 1: Control template supplied with the kit (Epicentre), lane 2: ssRNA generated from the clone 1 template containing T7 RNA polymerase site, lane: 3 DNA size marker XVII (Roche), lane 4: ssRNA from clone 2 template containing T7 RNA polymerase site.

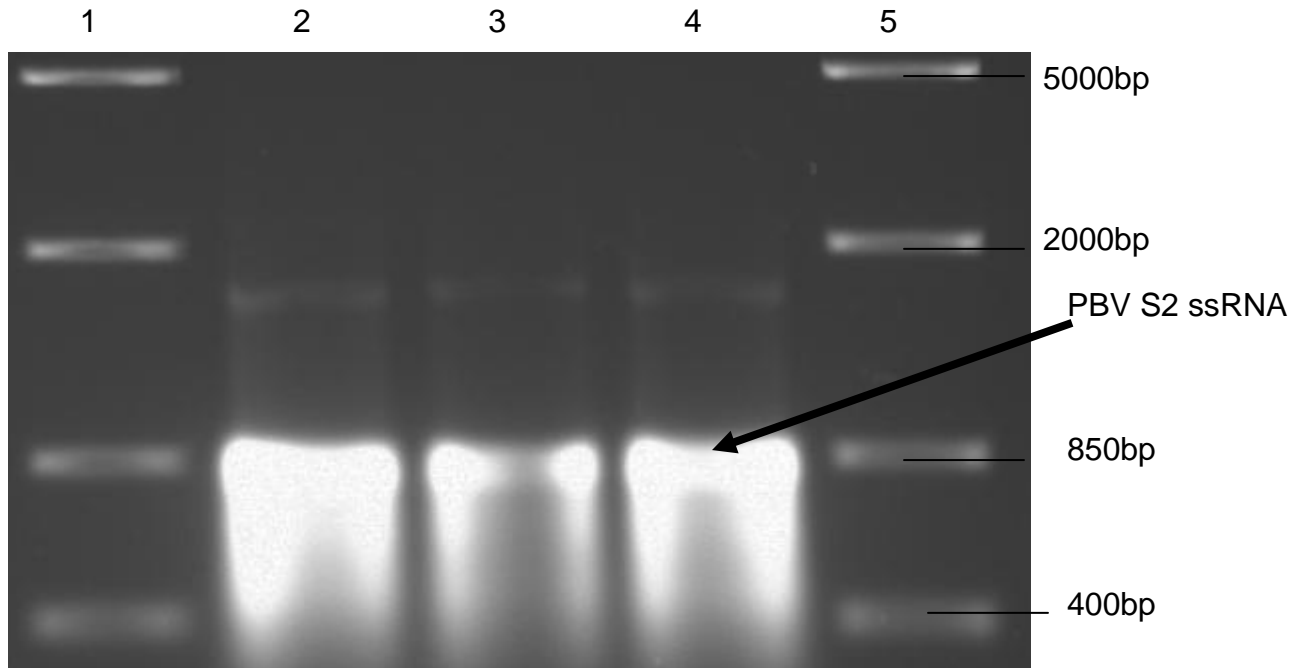


Figure 33. Agarose gel (1%) electrophoresis analysis of RNA products generated from the full length picobirnavirus S2 cDNA using T7 RNA polymerase from Promega (Ribomax kit.). Lanes 1 and 5: DNA size marker SM1113 (Fermentas), lane 2: ssRNA generated from template 1 containing the T7 RNA polymerase site, lane 3: ssRNA generated from template 2 containing the T7 RNA polymerase site and lane 4: ssRNA generated from template 1 containing T7 RNA polymerase promoter.

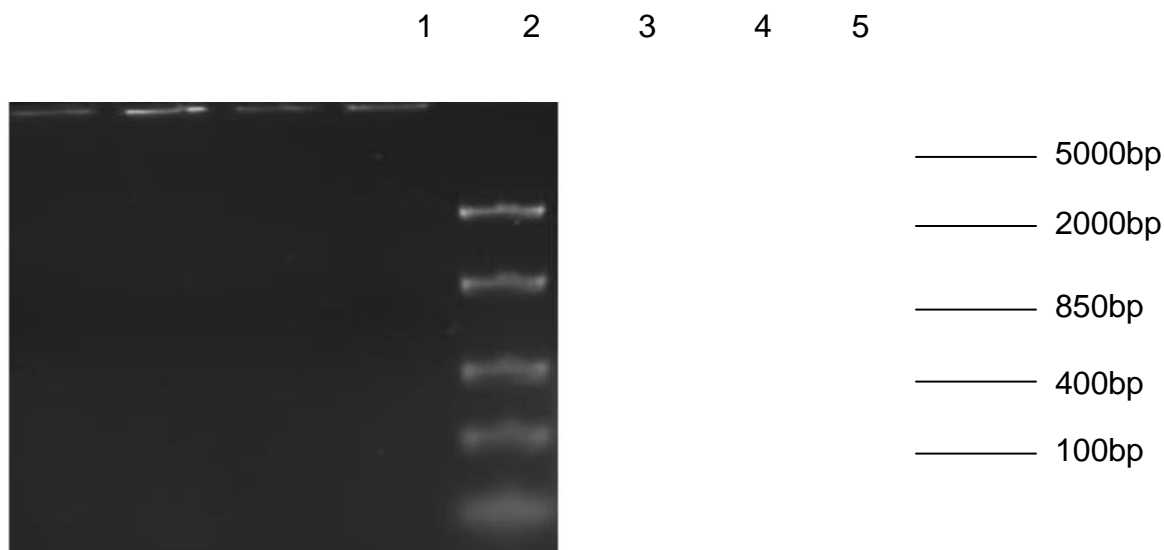


Figure 34. Agarose gel (1%) electrophoresis analysis of the RNA products after RNase treatment with RNase A. Lane 1: RNase treated Clone 1 ssRNA, lane 2: RNase treated Clone 2 ssRNA, lane 3: clone 1 ssRNA treated with 3 μ l of 1:3 Qiagen buffer P1, lane 4: clone 2 ssRNA treated with 3 μ l of 1:3 Qiagen buffer P1 and lane 5: DNA size marker (Fermentas, catalogue number: SM1113).

6.3.3. Mobility shift assay

The purified recombinant putative picobirnavirus RNA-dependent RNA polymerase fusion protein (GST-PBV 2) was analyzed for RNA binding affinity. RNA binding assay was performed by mixing the purified recombinant protein and the purified RNA template as described (6.3.2). The migration of the RNA template was expected to be reduced due to the increased size of the RNA/protein complex. Figure 35 shows that the protein had an effect on the migration of the ssRNA in the agarose gel, although not the expected effect. Figure 35A, lane 2 and 3, shows that the ssRNA migration on the gel was increased with a formation of two lower size bands (compare Fig. 35A, lane 1 to lanes 2 and 3). The increase in RNA migration was again observed when the binding assay was repeated (Fig. 35B, lanes 2-5). When the bands in Figure 35 are compared to the bands in Figure 34, where the RNA template was treated with RNase containing buffer, it can be

observed that with RNase treatment (Fig. 34), the RNA template is completely degraded. However, after incubation with the purified GST-PBV 2 protein (Fig. 35) some RNA template is still present. Therefore, it is possible that the increase in migration is not due to RNase digestion but a result of the putative polymerase binding to the RNA thereby changing its secondary structure as shown in Figure 36. It has been proposed that some RNA-dependent RNA polymerases bind to the ssRNA and causes it to loop back into the protein as shown in Figure 36 (Patton, 2006). The ssRNA folding into a secondary structure as shown would increase its mobility on an agarose gel and not retard it. However, since this proposed mechanism has not been proven or published in a peer-reviewed journal, the results here offer no proof of this but only offer a possible explanation for the increased mobility of the RNA on the agarose gel.

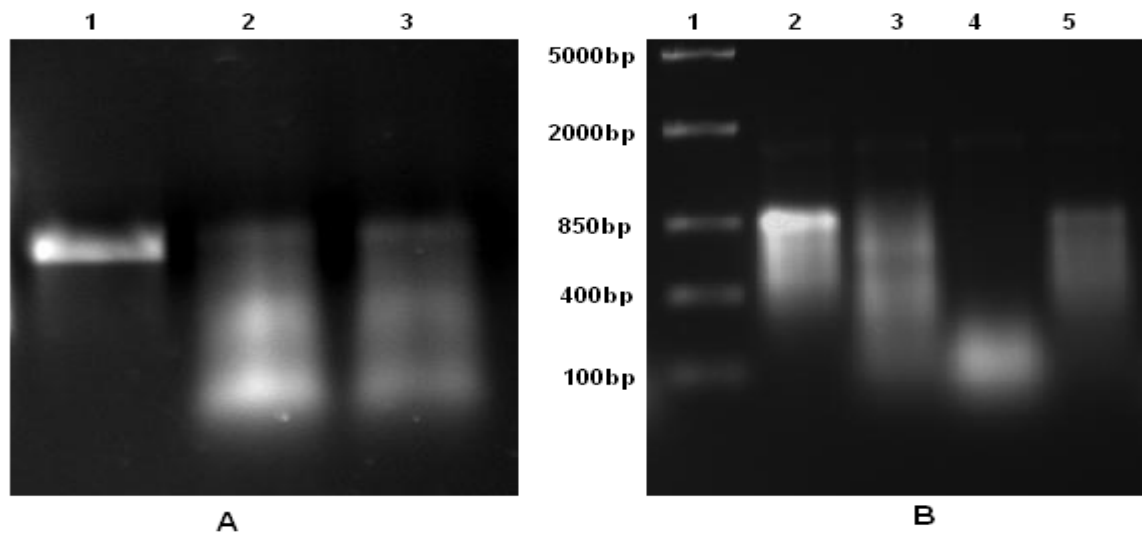


Figure 35. Agarose gel (1%) electrophoresis analysis of the putative picobirnavirus RNA-dependent RNA polymerase (GST-PBV 2 protein) complexed with ssRNA. A: lane 1: ssRNA from template 1, lane 2: ssRNA from template 1 combined with purified GST-PBV 2 protein lane 3: ssRNA from template 2 combined with purified GST-PBV 2 protein. **B:** lane 1: DNA size marker SM1113 (Fermentas), lane 2: template 1 ssRNA combined with purified GST-PBV 2 protein, lane 3: template 2 ssRNA combined with purified GST-PBV 2 protein, lane 4: template 2 ssRNA combined with GST-PBV 2 protein and lane 5: template 1 ssRNA combined with purified GST-PBV 2 protein.

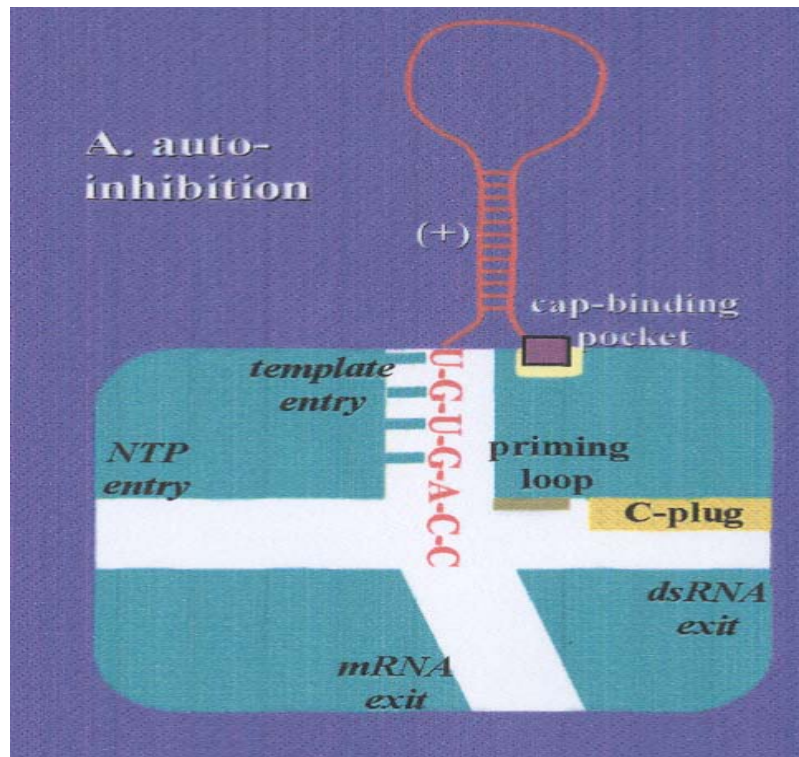


Figure 36. Graphic representation of rotavirus polymerase binding to RNA during replication. The capped 5' end of the RNA binds to the cap binding pocket and the 3' end enters to the active site. Taken from Patton J.T., 2006.

6.3.4. Affinity purification of soluble putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein using heparin

Heparin binds to most proteins with affinity for DNA/RNA such as DNA/RNA binding proteins, growth factors, coagulation protein and steroid receptors. The binding of heparin to these proteins is stable between pH 5 and pH 10. However, the binding is relatively weak. The binding is also not very specific since many proteins have been shown to bind heparin, not only those with affinity for nucleic acids. Heparin is commonly used during the purification of RNA-dependent RNA polymerases. An experiment was conducted to assess if the GST fusion protein would bind to heparin. Figure 37, lane 5 and 6 show that

the expressed GST fusion protein bound to the heparin column. However, most of the recombinant proteins were in the flow through showing that the binding efficiency was not very good (Fig. 37, lane 3). The binding was remarkably specific since only the recombinant protein was eluted from the column after purification. The protein could be eluted from the heparin column with binding buffer containing as little as 200mM NaCl, and all the proteins were eluted at a salt concentration of 400mM (NaCl). Elution of the bound protein with elution buffer containing salt concentration higher than 400mM NaCl (600mM and 800mM) did not yield any protein (results not shown). This showed that the binding to the column was very weak as the protein was eluted at a relatively low salt concentration as compared to Phi6 polymerase which is only eluted from heparin with elution buffer containing 400mM NaCl. Phi 6 polymerase is not eluted from heparin at a NaCl concentration of 200mM (Makeyev and Bamford, 2000b). The eluted protein was stored at -20°C and 4°C for further processing. After 24-36hrs of storage at both 4°C and -20°C, the protein in both the elution buffer with 200mM and 400mM NaCl, precipitated and could not be used for further processing. This is in contrast to the high stability of the protein after purification using GST columns. The difference in stability is probably due to the high salt concentration in the heparin elution buffer.

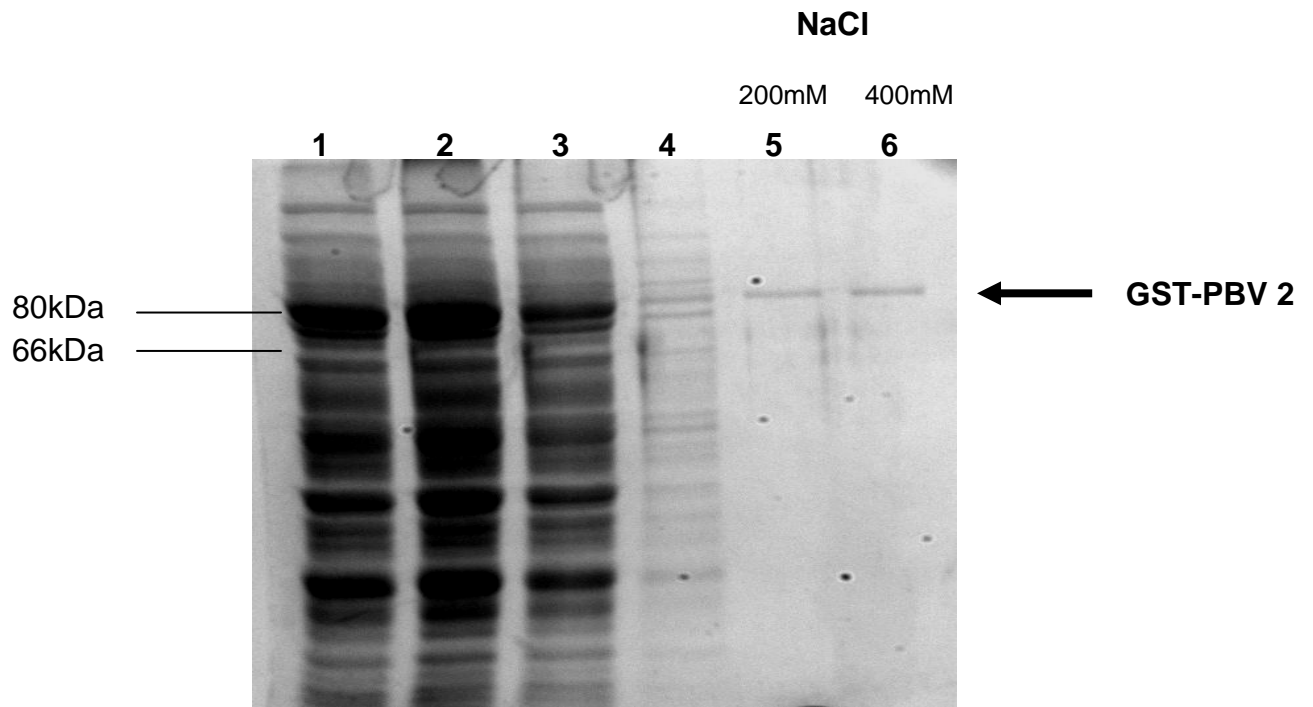


Figure 37. SDS-PAGE (10%) of fractions from the purification of GST tagged PBV2 protein with heparin. Lane 1: total protein fraction after 16hrs of induction, lane 2: soluble protein fraction after 16hrs of induction, lane 3: proteins from the column flow through fraction, lane 4: proteins from column wash fraction, lane 5: proteins from the fraction eluted with 200mM NaCl, and lane 6: proteins from the fraction eluted with 400mM NaCl.

6.3.5. Heparin binding of thrombin treated protein (untagged putative picobirnavirus RNA-dependent RNA polymerase)

The binding of recombinant putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein to heparin was shown to be relatively weak (6.3.4). An experiment was conducted to assess the binding affinity of untagged putative picobirnavirus RNA-

dependent RNA polymerase to heparin by combining the GST tag removal and heparin binding assays. The GST fusion protein was treated with thrombin as before but the GST tag and the thrombin was not removed as before. Thrombin cleavage was expected to result in the generation of proteins with molecular weights of approximately 60kDa and 26 kDa. Figure 38 show that the GST tag was again successfully cleaved from the fusion protein, with the resulting proteins having the expected molecular weight of 60kDa and 26kDa respectively (Fig. 38, lane 5).

The cleaved protein bound to the heparin column (Fig. 38 lanes 4, 5, and 6), however, some of the proteins were lost in the flow through (Fig. 38, lane 2). The cleaved protein bound effectively to the column since none of the proteins, with a molecular weight of 60kDa (untagged protein), were washed off the column in the wash step (Fig. 38, lane 3). Only the proteins with a lower molecular weight can be observed in the wash fraction. These proteins are thought to be the GST tag and thrombin although their identity was not confirmed. GST tag and thrombin are observed in this experiment because GST agarose and streptavidin were not included in this experiment (as compared to section 5.2.3). The proteins that were bound to heparin were eluted using buffer containing as little as 200mM NaCl as with the heparin assay with the complete fusion protein (Fig. 38, lane 4).

However, even after elution of the untagged protein with the buffer containing 600mM and 800mM NaCl, some of the 60kDa protein was still bound to the heparin (Fig. 38, lanes 6, and 7, respectively). At the higher salt concentration, the lower molecular weight proteins are no longer observed in the fraction, but only a faint band representing the 60kDa protein is observed (Fig 38, lanes 7 and 8). These results show that the untagged protein (putative picobirnavirus RNA-dependent RNA polymerase) bound more efficiently to the heparin than the GST-fusion protein. The purified untagged protein was stable when stored at -20°C for up to three months.

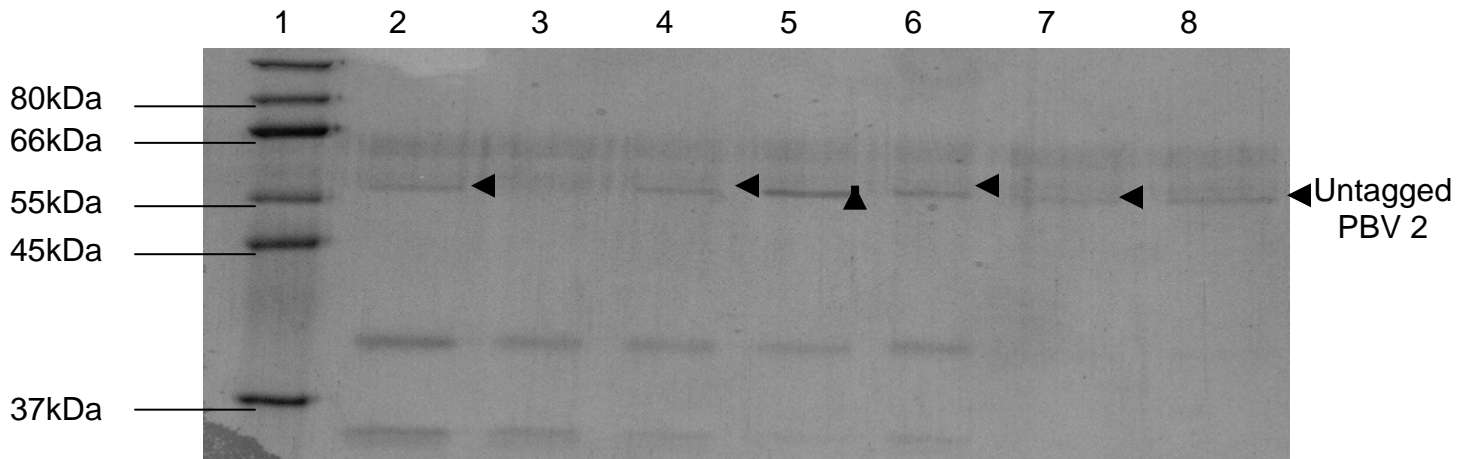


Figure 38. SDS-PAGE (10%) analysis of protein fractions from the heparin binding assay using untagged putative picobirnavirus RNA-dependent RNA polymerase. Lane 1: Protein molecular weight standards RPN8500 (Amersham), lane 2: Column flow through, lane 3: Column wash fraction with binding buffer, lane 4: elution fraction with binding buffer plus 200mM NaCl, lane 5: elution fraction with binding buffer plus 400mM NaCl, lane 6: Elution fraction with binding buffer plus 600mM NaCl, lanes 7 and 8: Elution fractions with 1x binding buffer plus 800mM NaCl.

6.3.6. RNA replication assay

Viral RNA-dependent RNA polymerases have the ability to transcribe and replicate RNA. However, most of them are only active when they are associated with other viral proteins in a complex referred to as the polymerase complex (Chapter I, section 1.2.3.2). A non-radioactive polymerase assay was performed to assess if the recombinant putative picobirnavirus RNA-dependent RNA polymerase can replicate the authentic picobirnavirus S2 ssRNA (6.3.2) *in vitro* without the presence of other viral proteins. The polymerase assay and ssRNA binding reactions were performed using either putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein, untagged putative picobirnavirus RNA-dependent RNA polymerase or phi6 polymerase (control) and the authentic picobirnavirus S2 ssRNA template from section 6.3.2. The reactions were analyzed by agarose gel electrophoresis as described (6.2.1.6). Once again the ssRNA binding reactions were expected to result in the reduction of ssRNA mobility on agarose

gel. The polymerase assay was expected to result in the generation of dsRNA which could be viewed as bands with a size of approximately 1700bp.

ssRNA binding reaction: Figure 39, lane 3 and 4 shows that both untagged putative picobirnavirus RNA-dependent RNA polymerase and phi6 polymerase, respectively, had no effect on the migration of the ssRNA on the agarose gel, when picobirnavirus segment 2 buffer was used (compare to the control reaction, Fig. 39, lane 5). However, the putative picobirnavirus RNA-dependent RNA polymerase – GST fusion protein exhibited RNase activity on the ssRNA (Fig. 39, lane 2). The RNase activity was not expected because RNase inhibitor was included in the ssRNA binding reactions. The same results were obtained when phi6 polymerase buffer was used for ssRNA binding reactions (Fig. 40, lanes 2, 3 and 4). The results show that the untagged putative picobirnavirus RNA-dependent RNA polymerase solutions did not contain RNase contamination observed with GST fusion protein solution.

RNA replication assay: The RNA replication assay was expected to result in the *de novo* synthesis of dsRNA with a size of 1700bp. However in Figure 39, lanes 8 and 9 such a product could not be observed whether untagged putative picobirnavirus RNA-dependent RNA polymerase or phi 6 polymerase was used (compare to control reaction, Fig. 39, lane 10). The GST fusion protein exhibited the same RNase activity observed during ssRNA binding reaction (Fig. 39, lane 7 compared to lane 2). The same results were observed when the buffer for phi6 polymerase was used (Fig. 40, lanes 7, 8 and 9). These results were surprising since Phi6 polymerase has been shown to replicate a wide range of RNA templates from different dsRNA viruses (Makeyev and Bamford, 2000b). In fact Phi6 polymerase is currently available commercially (Biolabs, catalogue number: MO255L or MO255S), and it is used for the *de novo* conversion of ssRNA into dsRNA. The sequence of the ssRNA template used in this study might have a unique 3' end sequence compared to the 3' end phi6 virus RNA template. Hence phi6 polymerase was unable to recognize, bind and replicate the RNA template. It is also possible that dsRNA was indeed generated, but only in very small quantities. However, since radioactively labelled nucleotides were not used, the reaction products could not be observed. It was

shown by Makeyev, *et al* 2000b, that no reaction products could be observed with Phi6 polymerase if unlabelled nucleotides were used in the reaction mixture. However, in a later publication, after conditions were optimized, it was clearly shown that the same phi6 polymerase used in this study could produce enough dsRNA from several different ssRNA templates to be viewed on normal EtBr stained agarose gels. Therefore, in this study, unlabeled nucleotides were used in the hope that enough dsRNA products would be generated to view on EtBR stained gels. This was however not the case.

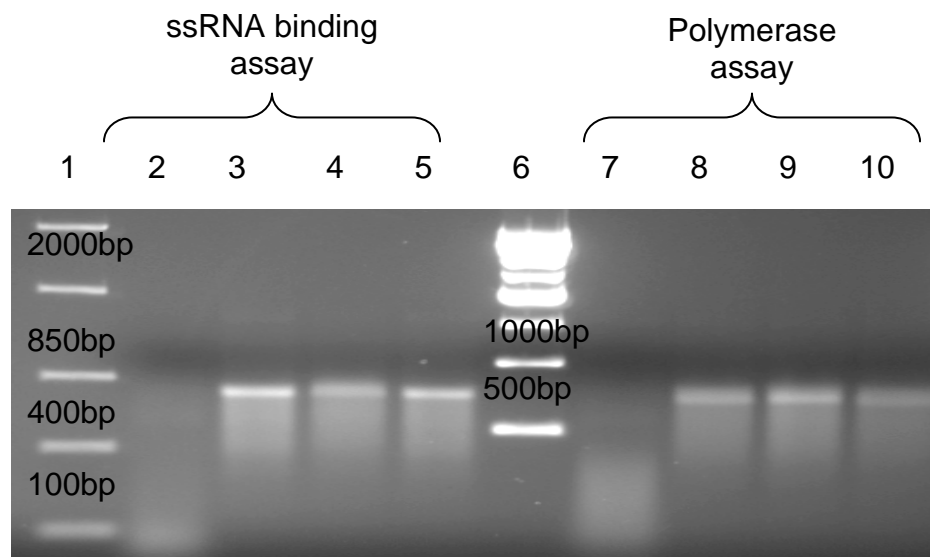


Figure 39. Agarose gel (1.2%) analysis of the ssRNA binding assay and polymerase assay products using picobirnavirus reaction buffers. Lane 1: DNA size marker (Fermentas, catalogue number: SM1113), lane 2: ssRNA/GST-PBV 2 protein complex, lane 3: ssRNA/untagged PBV2 protein complex, lane 4: ssRNA/Phi6 polymerase complex, lane 5: binding assay control, lane 6: DNA size marker XVII

(Roche), lane 7: GST-PBV 2 protein reaction product, lane 8: untagged PBV 2 protein reaction product, lane 9: Phi6 polymerase reaction product and lane 10: polymerase assay control.

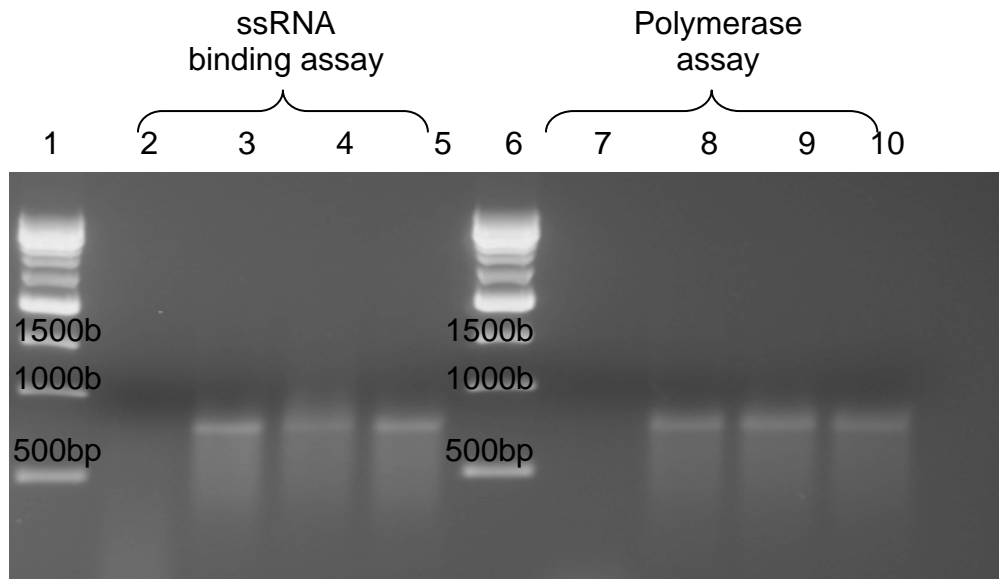


Figure 40. Agarose gel (1.2%) analysis of the ssRNA binding assay and polymerase assay products using phi6 polymerase buffer. Lane 1: DNA size marker XVII (Roche), lane 2: ssRNA/GST-PBV 2 protein complex, lane 3: ssRNA/untagged PBV2 protein complex, lane 4: ssRNA/Phi6 polymerase complex, lane 5: binding assay control, lane 6: DNA size marker XVII (Roche), lane 7: GST-PBV 2 protein reaction product, lane 8: untagged PBV 2 protein reaction product, lane 9: Phi6 polymerase reaction product and lane 10: polymerase assay control.

6.4. SUMMARY

Full length picobirnavirus S2 RNA transcription templates containing SP6 and T7 RNA polymerase promoters was successfully amplified from two original pGEM-T cDNA clones (Fig. 31). *In vitro* transcription was only successful when the template containing the T7 RNA polymerase promoter was used (Fig. 32). This may be due to the fact that the RNA promoter efficiency is influenced by the sequences immediately downstream of the promoter (Jorgensen *et al.*, 1991). When the ssRNA generated in the T7 polymerase reaction was treated with a solution containing RNase, it was completely degraded showing that it was RNA (Fig. 34). The ssRNA binding study showed that the GST tagged picobirnavirus S2 protein solution either contained traces of RNase or that the secondary

structure of the RNA may have been altered by the putative polymerase (Fig. 35). An attempt to remove the traces of RNase from the purified GST-PBV 2 protein solution by not using benzonase during protein extraction as prescribed by the BugBuster protein extraction protocol (section 5.2.2) was unsuccessful (results not shown).

The recombinant GST-tagged putative picobirnavirus polymerase protein was shown to have an affinity for heparin as it could be purified from cell extract with a heparin column (Fig. 37). Affinity of the putative picobirnavirus polymerase for heparin was confirmed when it was shown that untagged putative picobirnavirus polymerase also binds to heparin (Fig. 38). The affinity of putative picobirnavirus polymerase for heparin binding shows that they have an affinity for nucleic acids (Makeyev and Bamford, 2000). The untagged protein bound more efficiently to heparin compared to the GST tagged protein. Therefore, the GST tag on the GST fusion protein may interfere with the activity of the protein by blocking the heparin binding motifs. This result is significant since it shows that the GST tag may block or inhibit the amino acid regions in the putative polymerase that are responsible for heparin binding. The presence of the GST tag may therefore influence the enzymatic activity of the protein as well. In addition it may explain why the GST tagged protein did not bind ssRNA as expected.

No polymerase activity was observed with tagged or untagged putative picobirnavirus polymerase or with Phi6 polymerase (Figures 39 and 40). Since unlabelled nucleotides were used small amounts of product that may have been produced could not be observed. Labelled nucleotides, especially radioactively labelled nucleotides, need to be included to facilitate observation of reaction products from the polymerase reaction.

CHAPTER VI

CONCLUDING REMARKS

Viral gastroenteritis is common in humans and animals. The major cause of viral gastroenteritis in both humans and animals is rotavirus (family *Reoviridae*). Picobirnavirus, an unclassified dsRNA virus, has been detected in many cases when diagnostic PAGE screening for rotavirus dsRNA is performed. During this routine diagnosis, picobirnavirus dsRNA has been detected in the faeces of patients with and without viral gastroenteritis. The virus dsRNA is mostly detected in infants with HIV-AIDS (Gatti *et al*, 1989, Luderts *et al*, 1991, Perera *et al*, 1988,). In animals, picobirnavirus dsRNA is also detected whether animals are suffering from gastroenteritis or not. As in humans it sometimes occurs as a secondary infection and the presence of the virus is associated with diseases which result in weight loss (Wakuda *et al*, 2005). Despite the common occurrence of picobirnavirus infection in humans and animals, its direct involvement in causing gastroenteritis has not been established. No molecular studies have been done on picobirnavirus except sequencing and epidemiology studies. In order to understand how picobirnavirus replicates and how it is transmitted from host to host, thorough molecular studies need to be performed.

Studies on the RNA-dependent RNA polymerase enzymes of RNA viruses with single or double-stranded RNA genomes have revealed the requirement of this protein for virus replication and transcription. These studies also led, in most cases, to an understanding of how these viruses replicate, assemble and package their genomes. Identifying the genes and proteins responsible for the polymerase activity and understanding the polymerase activity of each viral RNA-dependent RNA polymerase is vital in understanding the life cycle of RNA viruses.

In this study, a porcine picobirnavirus was studied at a molecular level to establish the activity of the protein encoded by segment 2 of its genome. Picobirnavirus genome segment 2 sequence encodes a protein that contains conserved RNA-dependent RNA polymerase motifs (Rosen *et al*., 2000).

To determine the identity of this putative picobirnavirus RNA-dependent RNA polymerase, its open reading frame (ORF) was successfully amplified by PCR, cloned and sequenced. Subsequently the ORF was successfully sub-cloned into baculovirus and bacterial expression vectors. The protein encoded by picobirnavirus genome segment 2 was successfully expressed as a recombinant protein in both the baculovirus and bacterial expression systems. Using the Bac-to-Bac baculovirus expression system, two recombinant baculoviruses were constructed. One expressing a histidine tagged protein and another one expressing an untagged protein.

The recombinant protein expressed in bacteria (*E.coli*) was fused to a GST tag at the N terminal end. Therefore, the soluble recombinant protein expressed in bacterial cells could be purified. Subsequently the GST tag could be removed from the purified protein by cleavage with thrombin. Both tagged and untagged proteins were used in experiments to assess if the protein has any of the properties of a RNA-dependent RNA polymerase. In the first experiment, picobirnavirus segment 2 ssRNA was generated from full-length cDNA by *in vitro* transcription. The ssRNA in combination with the purified recombinant proteins (tagged and untagged) was used to test if the protein binds to authentic picobirnavirus RNA and if the protein had enzymatic (replicase) activity. Binding of the putative RNA-dependent RNA polymerase to RNA caused an unexpected increase in the mobility of the RNA on an agarose gel. Whether this was due to RNase contamination or folding of the RNA into a secondary structure known as the pan-handle structure is not known. No RNA replicase activity was observed with GST tagged or untagged picobirnavirus segment 2 protein. It is possible that dsRNA was produced, but since non-radioactive labelled nucleotides were used the dsRNA products could not be observed. Most of the polymerase assays conducted on the viral polymerases are conducted using radioactive labelled nucleotide, and without the label, the dsRNA products are rarely observed. However, it was observed that the GST tagged and untagged putative picobirnavirus polymerase from the bacterial expression system, both have an affinity for heparin. This implies that the protein might have an affinity for nucleic acids.

In this study two significant contributions were made:

- The optimal conditions for recombinant expression of the putative picobirnavirus RNA-dependent RNA polymerase protein in bacteria were determined.
- The binding of the recombinant putative RNA-dependent RNA polymerase to heparin provides further evidence that picobirnavirus genome segment 2 probably encodes an RNA-dependent RNA polymerase.

Failure to show replicase activity of the putative polymerase might be attributed to the following:

- There was RNase contamination of the GST tagged protein preparation
- The GST tag might have blocked the active polymerase sites
- The polymerase protein may not be active in isolation but needs to be associated with other virus proteins to function properly
- It is possible that dsRNA may have been produced during the replication assays but the products were not visible since radioactive labelled nucleotides were not used
- The ssRNA template used in the RNA binding and replicase assays was not cap methylated. Other studies indicate that ssRNA templates of viruses infecting mammalian cells are capped and that some of the enzymes contain a cap pocket that binds the RNA. This implies that ssRNA templates used in polymerase assays should be cap methylated

More studies are needed to confirm the identity of the protein encoded by picobirnavirus segment 2. The following is suggested:

- Purification of recombinant protein expressed in bacteria in the absence of RNases
- The use of cap methylated ssRNA as template for RNA binding and replication assays
- The use of radioactively labelled nucleotides in the polymerase assay to allow the visualization of dsRNA products
- The co-expression of the putative capsid and polymerase proteins to establish if polymerase activity is dependent on other viral proteins
- Determination of the optimal buffer conditions for replicase activity

CHAPTER VIII
REFERENCE

1. AUSUBEL, F.M., BRENT, R., KINGSTON, R.E., MOORE, D.D., SEIDMAN, J.G. and SMITH, J.A. 1994. *Current protocols in molecular biology*, Vol. 1 and 2. Brooklyn, New York John: Wiley and Sons, Inc.:1994-2004.
2. BENEVIDES, J.M., JUUTI, J.T., TURNA, R., BAMFORD, D.H. and THOMAS Jr., G.J. 2002. Characterization of subunit-specific interaction in dsRNA viruses: Raman difference spectroscopy of the $\Phi 6$ procapsid. *Biochemistry*, 41:11946-11953.
3. BERNSTEIN, J.M. and HRUSKA, J.F. 1981. Characterization of RNA polymerase of *Nebraska calf diarrhea virus* and *SA11 rotavirus*. *Journal of Virology*, 37(3):1071-1074.
4. BISWAL, B. K., CHERNEY, M. M., WANG, M., CHAN, L., YANNOPOULOS, C. G., BILIMORIA, D., NICOLAS, O., BERDARD, J. and JAMES, M. N. G. 2005. Crystal structure of RNA-dependent polymerase genotype 2a of Hepatitis C virus reveal two conformations and suggest mechanism of inhibition by non-nucleoside inhibition. *Journal of Biological Chemistry*, 280 (18):18202-18210.
5. BOYCE, M., CELMA, M. and ROY, P. 2008. Development of reverse genetics for bluetongue virus: recovery of infectious virus from synthetic RNA transcripts. *Journal of virology*, in press (accepted 18 June 2008).
6. BOYCE, M., WEHRFRITZ, J., NOAD, R. and ROY, P. 2004. Purified recombinant bluetongue virus VP1 exhibits RNA replicase activity. *Journal of Virology*, 78(8): 3994-4002.

7. BRUENN, J.A. 1991. Relationships among the positive strand and double-strand RNA viruses as viewed through their RNA-dependent RNA polymerases. *Nucleic Acid Research*, 19(2): 217-226.
8. BRUENN, J.A. 2003. A structural and primary sequence comparison of the viral RNA-dependent RNA polymerases: survey and summary. *Nucleic Acid Research*, 31(7):1821-1829.
9. BUTCHER, S.J., GRIMES, J.M., MAKEYEV, E.V., BAMFORD, D.H. and STUART, D.I. 2001. A mechanism for initiating RNA-dependent RNA polymerase. *Nature*, 410:235-240.
10. GATTI, M.S.V., PESTANA DE CASTRO, A.F. and FERRAZ, M.M. 1989. Viruses with bisegmented double-stranded RNA in pig faeces. *Research in Veterinary Science*, 47:397-398.
11. GILLIES, S., BULLIVANT, S. and BELLAMI, A.R. 1971. Viral polymerase; electron microscopy of reovirus reaction core. *Science*, 174:694-696.
12. GORZIGLIA, M. and ESPRAZA, J. 1981. Poly (A) polymerase in human rotavirus. *Journal of General Virology*, 53:357-362.
13. GOTTLIEB, P., STRASSMAN, J., QIAO, X., FRUCHT, A. and MINDICH, L. 1990. *In vitro* replication, packaging, and transcription of the segmented double-stranded RNA genome of bacteriophage $\Phi 6$: studies with procapsid assembled from plasmid-encoded proteins. *Journal of Bacteriology*, 172:5774-5784.
14. HEY, T.D., RICHARDS, O.C. and EHRENFELD, E. 1986. Synthesis of plus- and minus-strand from poliovirion RNA template *in vitro*. *Journal of Virology*, 58(3):790-796.

15. INOUE, H., NOJIMA, H. and OKAYAMA, H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene*, 96:23-28.
16. JORGENSEN, E.D., DURBIN, R.K., RISMAN, S.S. and MCALLISTER, W.T. 1991. Specific contacts between the bacteriophage T3, T7, and SP6 RNA polymerases and their promoters. *The journal of biological chemistry*, 266(1):645-651.
17. JUUTI, J.T. and BAMFORD, D.H. 1997. Protein P7 of phage phi6 RNA polymerase complex, acquiring of RNA packaging activity by *in vitro* assembly of purified protein onto deficient particles. *Journal of Molecular Biology*, 266:891-900.
18. KABA, S.A., SALCEDO, A.M., WAFULA, P.A., VLAK, J.M. and VAN OERS, A.M. 2004. Development of chitinase and v-cathepsin negative bacmid for improved integrity of secreted recombinant proteins. *Journal of Virological Methods*. 122:113-118.
19. KNIPE, D.M. and HOWLEY, P.M. 2001. *Fields: Virology*. Volume 1, 4th ed. Philadelphia, USA: Lippincott Williams and Wilkins: 105-118, 1680-1687.
20. LAHSER, F.C. and MALCOLM, B.A. 2004. A continuous non-radioactive assay for RNA-dependent RNA polymerase activity. *Analytical Biochemistry*, 325:247-254.
21. LOPEZ-VAZQUEZ, A., MARTIN-ALONSO, J.M. CASAIS, R., BOGA, J.A. and PARRA, F. 1998. Expression of enzymatically active *Rabbit hemorrhagic disease virus* RNA-dependent polymerase in *Escherichia coli*. *Journal of Virology*, 74(4):2999-3004
22. LOPEZ-VAZQUEZ, A., MARTIN-ALONSO, J.M. and PARRA, F. 2001. Characterisation of the RNA-dependent RNA polymerase from *Rabbit hemorrhagic virus* produced in *Escherichia coli*. *Archives of Virology*, 146:59-69.

23. LUCKOW, V.A. and SUMMERS, M.D. 1989. High level of nonfused foreign genes with *Autographa californica* nuclear polyhydrosis virus expression system. *Virology*, 170: 31-39.
24. LUDERT, J.E., HIDALGO, M. and LIPRANDI, F. 1991. Identification in porcine feacal of a novel virus with a bisegmented double-stranded RNA genome. *Archives of Virology*, 117:97-107.
25. MAKEYEV, E.V. and BAMFORD, D.H. 2000a. The polymerase subunit of a dsRNA virus plays a central role in the regulation of viral RNA metabolism. *The EMBO Journal*, 19(22):6275-6284.
26. MAKEYEV, E.V. and BAMFORD, D.H. 2000b. Replicase activity of the purified recombinant protein P2 of double-stranded bacteriophage ϕ 6. *The EMBO Journal*, 19(1):124-133.
27. MAKEYEV, E.V. and GRIMES, J.M. 2004. RNA-dependent RNA polymerase of dsRNA bacteriophages. *Virus Research*, 101(1):45-55.
28. MARAVER, A., CLEMENTE, R., RODRIGUEZ, J.F. and LOMBARDO. 2003. Identification and molecular characterization of the RNA polymerase-binding motif if infectious bursal disease virus inner capsid protein VP3. *Journal of Virology*, 77(4): 2459-2468.
29. MERTENS, P. 2004. The dsRNA viruses. *Virus Research*, 101:3-13.
30. MERTENS, P.P.C. and DIPROSE, J. 2004. The bluetongue virus core: a nano-scale transcription machine. *Virus Research*, 101:29-43.

31. MORROW, C.D., WARREN, B. and LENTZ, M.R. 1987. Expression of enzymatically active poliovirus RNA-dependent RNA polymerase in *Escherichia coli*. *Prot. Natl. Acad. Sci.*, 84:6050-6054.
32. NEUFELD, K.L., RICHARDS, O.C. and EHRENFELD, E. 1991a. Expression and characterisation of poliovirus proteins 3B^{VPg}, 3C^{pro}, and 3D^{pol} in recombinant baculovirus-infected *Spodoptera frugiperda* cells. *Virus Research*, 19:173-188.
33. NEUFELD, K.L., RICHARDS, O.C. and EHRENFELD, E. 1991b. Purification, characterization, and comparison of poliovirus RNA polymerase from native and recombinant sources. *Journal of Biological Chemistry*, 266(35):24212-24219.
34. NG, K.K.S., CHERNEY, M., LOPEZ-VAZGUEZ, A., MACHIN, A., MARTIN-ALONSO, J.M., PARRA, F. and JAMES, M.N.G. 2002. Crystal structure of active and inactive conformations of a *Calicivirus* RNA-dependent RNA polymerase. *Journal of Biological Chemistry*, 277(2):1381-1387.
35. O'REILLY, E.K. and KAO, C.C. 1998. Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structure and computer prediction of secondary structure. *Virology*, 252:287-303.
36. O'REILLY, D.R., MILLER, L.K. and LUCKOW, V.A. 1994. *Baculovirus expression vectors: a laboratory manual*. New York, Oxford: Oxford University Press
37. PAN, J., VAKHARIA, V. N. and TAO, Y. J. 2007. The structure of a birnavirus polymerase reveals a distinct active site topology. *PNAS*, 104(18): 7385-7390.
38. PATTON, J.T. 2006. Structure and function of the Rotavirus RNA polymerase. 9th dsRNA virus symposium. South Africa, Cape Town. 21-26 October 2006.

39. PATTON, J.T., JONES, M.T., KALBACH, A.N., HE, Y. and XIAOBO, J. 1997. Rotavirus RNA polymerase requires the core shell protein to synthesize the double-stranded RNA genome. *Journal of Virology*, 71(12):9618-9626.
40. PEREIRA, H.G., FLEWETT, T.H., CANDEIAS, J.A.N. and BARTH, O.M. 1988. A virus with a bisegmented double-stranded genome in rat (*Oryzomys nigripes*) intestine. *Journal of General Virology*, 69:2749-2754.
41. PHILIPPS, B., FORSTNER, M. and MAYR, L.M. 2005. A baculovirus expression vector system for simultaneous protein expression in insect and mammalian cells. *Biotechnology*, 21:708-711.
42. POCH, O., SAUVAGET, I., DELARUE, M. and TORDO, N. 1989. Identification of four conserved motifs among polymerase encoding elements. *The EMBO Journal*, 8(12):3867-3874.
43. POLY, R. 2004. Baculovirus solves a complex problem. *Nature Biotechnology*, 22(12):1527-1528.
44. PORANEN, M.M. and TUMA, R. 2004. Self-assembly of dsRNA bacteriophages. *Virus Research*, 101(1):93-100.
45. POTGIETER, A.C., STEELE, A.D, VAN DIJK, and A.A. 2002. Cloning of complete genome sets of six dsRNA viruses using an improved cloning method for large dsRNA gene. *Journal of General Virology*, 83(9):2215-2223.
46. ROSEN, B.I., FANG, Z., GLASS, R.I. and MONROE, S.S. 2000. Cloning of Human picobirnavirus segments and development of an RT-PCR detection assay. *Virology*, 277:316-329.

47. SAHDEV, S., KHATTAR, S.K and SAINI, K.S. 2008. Production of active eukaryotic proteins through bacterial expression systems: a review of the existing biotechnology strategies. *Molecular and cell biochemistry*, 307: 249-264.
48. SANKAR, S. and PORROR, A.G. 1991. Expression, purification, and properties of recombinant Encephalomyocarditis virus RNA-dependent RNA polymerase. *Journal of Virology*, 65(6): 2993-3000.
49. SAMBROOK, L., FRITSCH, E.F. and MANIATIS, T. 1989. Molecular cloning: A laboratory manual, 2nd edition. USA: Cold Spring harbour laboratory expression. ISBN: 0-87969-309-6
50. STARNES, M.C. and JOKLIK, W.K. 1993. Reovirus protein $\lambda 3$ is a poly(C) –dependent poly(C) polymerase. *Virology*, 193:356-366.
51. TAO, Y., FARSETTA, D.L., NIBERT, M.L. and HARRISON, S.C. 2002. RNA synthesis in a cage-structural studies of reovirus polymerase $\lambda 3$. *Cell*, 111:733-745.
52. TOMEI, T., VITALE, R.L., INCITTI, I., SERAFINI, S., ALTAMURA, S., VITELLI, A. and DE FRANCESCO, R. 2000. Biochemical characterisation of hepatitis C virus RNA-dependent RNA polymerase mutant lacking the C-terminal hydrophobic sequence. *Journal of General Virology*, 82: 759-767.
53. VAN DIJK, A.A., MAKEYEV, E.V. and BAMFORD, D.H. 2004. Initiation of viral RNA-dependent RNA polymerase. *Journal of General Virology*, 85:1077-1093.
54. WAKUDA, M., PONGSUWANNA, Y. and TANIGUCHI, K. 2005. Complete nucleotide sequence of two RNA segments of human picobirnavirus. *Journal of Virology Methods*, 126:165-169.

55. YANG, H., GOTTLIEB, P., WEI, H., BAMFORD, D.H. and MAKEYEV, E.V. 2003. Temperature requirement for initiation of RNA-dependent RNA polymerization. *Virology*, 314:706-715.
56. ZHONG, W., GUTSHALL, L.L. and DEL VECCHIO, A.M. 1998. Identification and characterization of an RNA-dependent RNA polymerase activity within the non-structural protein 5B region of bovine diarrhea virus. *Journal of Virology*, 72(11): 9365-9369.

CHAPTER IX

APPENDIX 1: LIST OF FIGURES

Figure 1. An illustration of the structure of a bluetongue virus particle. The picture illustrates the segmented dsRNA genome encapsidated by two protein layers. The polymerase complex at the 5 fold axis is also illustrated. Picture courtesy of P.P.C. Mertens (Mertens, 2004).

Figure 2. Genome organization of segment 1 and segment 2 of picobirnavirus. The numbers indicates the positioning of the nucleotide each segment. Segment 1: Positions 1 and 2525 represent the start and end of full length segment 1 respectively. Positions 157 and 831 represent the start and end of segment 1 ORF1 respectively. Position 828 and 2486 represents the start and end of segment 1 ORF 2 respectively. Segment 2: Position 1 and 1745 represent the start and end of the full length segment 2 respectively 94 and 1698 represents the start and end of segment 2 ORF respectively. The termination codon for ORF1 (UGA) and initiation codon for ORF2 (AUG) in segment 1 are overlapped. Adapted and modified from Wakuda et al, 2005.

Figure 3. The three-dimensional structure of the phi6 RNA-dependent RNA polymerase showing the three domains. Blue, purple and green colours corresponding to finger, palm and thumb respectively (Makeyev and Grimes, 2004).

Figure 4. The three dimensional structure of Hepatitis C Virus RNA-dependent RNA polymerase showing the three domains. Blue, red and green colours correspond to the finger, palm and thumb domains, respectively. The yellow colour represents the loop domain (Biswal *et al.*, 2005).

Figure 5. Conserved motif in the palm domain of RNA-dependent RNA polymerase from a HCV virus. Taken and modified from O'Reilly and Kao, 1998.

Figure 6. Graphic representation of expected PCR products using primers as indicated. Picobirnavirus genome segment 2 ORF in the PCR product is represented by the solid line and the restriction sites in the PCR products are underlined.

Figure 7. Positions of the PCR primers on the segment of porcine picobirnavirus genome segment 2 cDNA. The nucleotide sequences of the primers are indicated by the underlined bold face letters. The sequence of the full primer sequence is underlined and the restriction enzyme recognition site is represented by italic letters.

Figure 8. Agarose gel (1%) electrophoresis analysis of purified PCR products of picobirnavirus segment 2 ORF using sequence specific primers. **A:** PCR products generated by primer 1 and primer 3, **B:** PCR products generated by using primer 2 and primer 3. In both figures **A** and **B:** lane 1: DNA size marker XVII (Roche), lane 2-4: PCR amplicon generated from clone 1 in decreasing template concentration, lane 5-7: PCR amplicon generated from clone 2 in decreasing template concentration and lane 8-10: PCR amplicon generated from clone 3 in decreasing template concentration.

Figure 9. Graphic representation of the cloning of the picobirnavirus segment 2 ORF (PCR products) into dephosphorylated pGEM-T vector. Ligation of purified PCR product, PBV 2N and PBV 2B, into dephosphorylated pGEM-T vector resulted in the formation of recombinant plasmids pGEM-T-PBV 2N and pGEM-T-PBV 2B respectively.

Figure 10. Agarose gel (1%) electrophoresis analysis of restriction digestion of the recombinant pGEM-T plasmids. Lane 1: pGEM-T-PBV 2B digested with *Bgl* II and *Xho* I, lane 2: Un-digested pGEM-T-PBV 2B. Lane 3: pGEM-T-PBV 2N digested with *Nco* I and *Xho* I, lane 4: Un-digested pGEM-T-PBV 2N and lane 5: DNA size marker XVII (Roche).

Figure 11. Sequencing results of the cloned picobirnavirus segment 2 ORF in the recombinant pGEM-T vector (pGEMt-PBV 2B and pGEMt-PBV 2N). The sequence was compared to the full length picobirnavirus genome segment 2 (PBV 2) sequence, the consensus sequence provided by Dr. A.C. Potgieter. The insert is in upper case and the plasmid in lower case, the position of incorporated restriction site is underlined. The start and end of the ORF in represented by bold italic letters.

Figure 12. Graphical representation of the generation of recombinant baculovirus and gene expression with the Bac-to-Bac expression system. Taken from Bac-to-Bac product manual (Invitrogen).

Figure 13. Plasmid map of the baculovirus donor plasmid, pFastbac1.

Figure 14. Plasmid map of the baculovirus donor plasmid, pFastbac-HT.

Figure 15. Agarose gel (1%) electrophoresis analysis of gel purified pFastbac plasmids and inserts after restriction digestion and purification. Lane 1: PBV 2N, lane 2: pFastBac-HT, lane 3: pFastBac1, lane 4: PBV 2B, lane 5: pFastBac1 and lane 6: DNA size marker XVII.

Figure 16. Graphic representation of cloning of picobirnavirus segment 2 cDNA into digested pFastbac1 vector. Ligation of purified PBV 2B from recombinant pGEMt-PBV 2B, into digested pFastbac1 vector resulting into formation of a recombinant pFastBac1-PBV 2B.

Figure 17. Graphic representation of cloning of picobirnavirus segment 2 cDNA into digested pFastbac-HT vector. Ligation of purified PBV 2N from recombinant pGEMt-PBV 2B, into digested pFastbac1 vector resulting into formation of a recombinant pFastBac1-PBV 2N.

Figure 18. Agarose gel (1%) electrophoresis analysis of restriction digestion of recombinant pFastBac constructs. Lane 1: DNA size marker XVII (Roche), lane 2: *Bgl* II and *Xho* I digested pFastBac1-PBV 2B, lane 3: *Xho* I digested pFastBac1-PBV 2B, lane 4: *Eco* RI and *Xho* I digested pFastBac-HT-PBV 2N and lane 5: DNA size marker XVII (Roche).

Figure 19. Sequencing results of the cloned picobirnavirus segment 2 ORF in the recombinant pFastBac donor plasmids (pFastBac-HT-PBV 2B and pFastBac1-PBV 2N). The sequence was compared to the picobirnavirus segment 2 ORF, the sequence results from Chapter II (section 2.3.3, Figure 8). The insert is represented by upper case letters and the plasmid is represented by lower case letters. The dots (...) represent the area where no match was found. The start the ORF in represented by boldface italic capped letters and the primer sequence is underlined.

Figure 20. Agarose gel (1%) electrophoresis analysis of PCR amplicon generated from the recombinant bacmid DNA using M13 primers. Lanes 1 and 2: PCR products generated from recombinant bacmid containing PBV 2B (Bac-PBV 2B), lanes 3 and 4: PCR products generated from recombinant bacmid containing PBV 2N (Bac-HT-PBV 2N), lane 5: DNA size marker (λ DNA *Hind* III digest and \emptyset X174 DNA *Hae* III digest (Finnzymes).

Figure 21. SDS-PAGE analysis of total fraction of protein expressed in SF9 cells infected with recombinant baculovirus (Bac-HT-PBV 2N-1, Bac-HT-PBV 2N-2, Bac-PBV 2B-1 and Bac-PBV 2B-2 respectively). Lane 1: uninfected insect cells, lane 2: Protein molecular weight standards (BioRad, catalogue number: 161-0374), lane 2: Bac-HT-PBV 2N-1 recombinant protein, lane 3: Bac-HT-PBV 2N recombinant protein, lane 4: Bac-PBV 2B-2 recombinant protein and lane 5: Bac-PBV 2B-1 recombinant protein.

Figure 22. Graphic representation of the cloning of picobirnavirus segment 2 cDNA into digested pGEX-4T-1 vector. The ligation of purified PBV 2B from recombinant pGEMt-PBV 2B, into digested pGEX-4T-1 vector resulting in the formation of recombinant plasmid pGEX4T-PBV 2B, is illustrated. The position of the GST coding sequence at the 5' end of the insert (PBV 2B) is indicated

Figure 23. Agarose gel (1%) electrophoresis analysis of digested and gel purified pGEX-4T plasmid and picobirnavirus segment 2 ORF. Lane 1: digested and gel purified pGEX-4T plasmid after digestion with *Bam* HI and *Xho* I, lane 2: digested and gel purified pGEX-4T plasmid after digestion with *Bam* HI and *Xho* I, lane 3: gel purified picobirnavirus segment 2 ORF (PBV 2B) and lane 4: DNA size marker XVII (Roche)

Figure 24. Agarose gel (1%) electrophoresis analysis of restriction digested recombinant pGEX 4T plasmids and gel purified insert. Lane 1: recombinant pGEX 4T-PBV 2B digested with *EcoR* I and *Xho* I, lanes 2: purified picobirnavirus S2 ORF (PBV 2B) and 3: purified Picobirnavirus S2 ORF (PBV 2B) and lane 4: DNA size marker XVII (Roche).

Figure 25. SDS-PAGE (10%) analysis of proteins expressed in recombinant bacterial cells containing an empty expression plasmid (pGEX 4-T) and recombinant PBV S2 expression plasmid, respectively. Lane 1: Precision plus protein standards (BioRad), lane 2: Total protein fraction of recombinant cells containing an empty expression plasmid (pGEX-4T), lane 3: Soluble protein fraction of cells containing an empty expression plasmid (pGEX-4T), lane 4: Total protein fraction of recombinant cells containing recombinant expression plasmid (pGEX-4T-PBV 2B) and lane 5: soluble protein fraction of recombinant cells containing recombinant expression plasmid (pGEX-4T-PBV 2B).

Figure 26. SDS-PAGE (10%) analyses of the total fractions and soluble fractions of protein expressed in recombinant bacterial cells under different conditions. Lane 1: Precision plus protein standards (BioRad), lane 2: Total fraction proteins at 37°C for 4hrs (5ml culture), lane 2: Soluble fraction proteins expressed at 37°C for 4hrs (5ml culture), lane 4: Total fraction proteins expressed at 18°C for 16hrs (5ml culture), lane 5: Soluble fraction proteins expressed at 18°C for 16hrs (5ml culture), lane 6: Total fraction proteins expressed at 18°C for 16hrs (10ml culture) lane 7: soluble fraction proteins expressed at 18°C for 16hrs (10ml culture), lane 8: Total fraction proteins expressed at 18°C for 16hrs (25ml culture), lane 9: Soluble fraction proteins expressed at 18°C for 16hrs (25ml culture), lane 10: Soluble fraction proteins expressed at 18°C for 16hrs (50ml culture).

Figure 27. SDS-PAGE (10%) analysis of fractions from the purification of GST tagged picobirnavirus 2 protein. Lane 1: Precision plus protein standards (BioRad, catalogue number: 161-0374), lane 2: total protein fraction, lane 3: soluble protein fraction, lane 4: proteins collected from the column flow-through, fraction lane 5: proteins collected from the column wash fraction, lane 6-10: protein fractions eluted from the column by the addition of reduced glutathione.

Figure 28. Graphic representation of the GST picobirnavirus S2 fusion protein. The number (+1) represents the start of picobirnavirus segment 2 protein. The letter N and C represents the terminal ends of

the recombinant protein (GST-PBV 2), with N representing the amino terminal end and C representing the carboxyl terminal end.

Figure 29. 10% SDS-PAGE analysis of fractions from removal of GST tagged picobirnavirus segment 2 protein and the untagged picobirnavirus segment 2 protein after treatment with thrombin. Lanes 1 and 2: GST tagged picobirnavirus segment 2 protein (recombinant GST-PBV 2 elution fraction from Fig. 26, lanes 6 and 7 respectively), lane 3: Protein molecular weight marker (Amersham, catalogue number: RPN800), lanes 4, 5 and 6: protein eluted from the column after thrombin treatment.

Figure 30. Graphic representation of the expected PCR products using promoter region incorporated primers. The location of the T7 and SP6 promoters sequences on the PCR products are indicated by the underlined bases. The first base-pair of picobirnavirus segment 2 is indicated by a +1.

Figure 31. Agarose gel (1%) electrophoresis analysis of purified PCR products of the full length picobirnavirus S2 cDNA using sequence specific primers containing RNA polymerase promoter sites. Lane 1: DNA size marker XVII (Roche), lane 2: PCR products generated from clone 1 using primers 4 and 6, lane 3: PCR products generated from clone 2 using primers 4 and 6, lane 4: PCR products generated from clone 1 using primers 5 and 6, lane 5: PCR products generated from clone 2 using primers 5 and 6.

Figure 32. Agarose gel (1%) electrophoresis analysis of ssRNA products generated from the full length picobirnavirus S2 cDNA using T7 RNA polymerase (T7 ampliscribe kit, Epicentre). Lane 1: Control template supplied with the kit (Epicentre), lane 2: ssRNA generated from the clone 1 template containing T7 RNA polymerase site, lane: 3 DNA size marker XVII (Roche), lane 4: ssRNA from clone 2 template containing T7 RNA polymerase site.

Figure 33. Agarose gel (1%) electrophoresis analysis of RNA products generated from the full length picobirnavirus S2 cDNA using T7 RNA polymerase from Promega (Ribomax kit,). Lanes 1 and 5: DNA size marker SM1113 (Fermentas), lane 2: ssRNA generated from template 1 containing the T7 RNA polymerase site, lane 3: ssRNA generated from template 2 containing the T7 RNA polymerase site and lane 4: ssRNA generated from template 1 containing T7 RNA polymerase promoter.

Figure 34. Agarose gel (1%) electrophoresis analysis of the RNA products after RNase treatment with RNase A. Lane 1: RNase treated Clone 1 ssRNA, lane 2: RNase treated Clone 2 ssRNA, lane 3: clone 1 ssRNA treated with 3µl of 1:3 Qiagen buffer P1, lane 4: clone 2 ssRNA treated with 3µl of 1:3 Qiagen buffer P1 and lane 5: DNA size marker (Fermentas, catalogue number: SM1113).

Figure 35. Agarose gel (1%) electrophoresis analysis of the putative picobirnavirus RNA-dependent RNA polymerase (GST-PBV 2 protein) complexed with ssRNA. **A:** lane 1: ssRNA from template 1, lane 2: ssRNA from template 1 combined with purified GST-PBV 2 protein lane 3: ssRNA from template 2 combined with purified GST-PBV 2 protein. **B:** lane 1: DNA size marker SM1113 (Fermentas), lane 2: template 1 ssRNA combined with purified GST-PBV 2 protein, lane 3: template 2 ssRNA combined with purified GST-PBV 2 protein, lane 4: template 2 ssRNA combined with GST-PBV 2 protein and lane 5: template 1 ssRNA combined with purified GST-PBV 2 protein.

Figure 36. Graphic representation of rotavirus polymerase binding to RNA during replication. The capped 5' end of the RNA binds to the cap binding pocket and the 3' end enters to the active site. Adapted from Patton J.T., 2006.

Figure 37. SDS-PAGE (10%) of fractions from the purification of GST tagged PBV2 protein with heparin. Lane 1: total protein fraction after 16hrs of induction, lane 2: soluble protein fraction after 16hrs of induction, lane 3: proteins from the column flow through fraction, lane 4: proteins from column wash fraction, lane 5: proteins from the fraction eluted with 200mM NaCl, and lane 6: proteins from the fraction eluted with 400mM NaCl

Figure 38. SDS-PAGE (10%) analysis of protein fractions from the heparin binding assay using untagged putative picobirnavirus RNA-dependent RNA polymerase. Lane 1: Protein molecular weight standards RPN8500 (Amersham), lane 2: Column flow through, lane 3: Column wash fraction with binding buffer, lane 4: elution fraction with binding buffer plus 200mM NaCl, lane 5: elution fraction with binding buffer plus 400mM NaCl, lane 6: Elution fraction with binding buffer plus 600mM NaCl, lanes 7 and 8: Elution fractions with 1x binding buffer plus 800mM NaCl.

Figure 39. Agarose gel (1.2%) analysis of the ssRNA binding assay and polymerase assay products using picobirnavirus reaction buffers. Lane 1: DNA size marker (Fermentas, catalogue number: SM1113), lane 2: ssRNA/GST-PBV 2 protein complex, lane 3: ssRNA/untagged PBV2 protein complex, lane 4: ssRNA/Phi6 polymerase complex, lane 5: binding assay control, lane 6: DNA size marker XVII (Roche), lane 7: GST-PBV 2 protein reaction product, lane 8: untagged PBV 2 protein reaction product, lane 9: Phi6 polymerase reaction product and lane 10: polymerase assay control.

Figure 40. Agarose gel (1.2%) analysis of the ssRNA binding assay and polymerase assay products using phi6 polymerase buffer. Lane 1: DNA size marker XVII (Roche), lane 2: ssRNA/GST-PBV 2 protein

complex, lane 3: ssRNA/untagged PBV2 protein complex, lane 4: ssRNA/Phi6 polymerase complex, lane 5: binding assay control, lane 6: DNA size marker XVII (Roche), lane 7: GST-PBV 2 protein reaction product, lane 8: untagged PBV 2 protein reaction product, lane 9: Phi6 polymerase reaction product and lane 10: polymerase assay control.

CHAPTER X

APPENDIX 2: PROTOCOL MANUALS

1. Ampliscribe™ T7, T3 and SP6 high yield transcription kits. (EPICENTRE Biotechnologies)
2. Bac-to--bac
3. GST.Bind™ kit. 2002. (Novagen)
4. GST gene fusion system handbook. Edition AA. (Amersham Biosciences)
5. MEGAclear™: Purification for large scale transcription reactions. (Ambion)
6. pGEM®-T and pGEM®-T Easy vector system. Revised 11/98. (Promega)
7. GIAprep® Miniprep handbook: For purification of molecular biology grade DNA. June 2005. (QIAGEN)
8. QIAquick® Spin handbook: For QIAquick gel extraction kit. March 2006. (QIAGEN)
9. RiboMAX™ Large scale RNA production system-SP6 and T7. Revised 9/01 (Promega)
10. Rapid DNA ligation kit. September 2003. Version D. (Roche)
11. Thrombin kit. 1992-1998. (Novagen)