Towards establishing a reverse genetics system to recover infectious African horse sickness virus

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DECLARATION

I declare that the dissertation, which I hereby submit for the degree M.Sc (Microbiology) at the University of Pretoria, is my own work and had not previously been submitted by me for a degree at this or any other tertiary institution.

Signed: …….. Date: ……..

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SUMMARY

Towards establishing a reverse genetics system to recover infectious African horse sickness virus

by

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African horse sickness virus (AHSV), a member of the *Orbivirus* genus within the *Reoviridae* family, is an arthropod-borne virus that is capable of causing severe disease in horses. Although progress has been made regarding structure-function analyses of individual AHSV proteins, studies into AHSV biology would be greatly enhanced if a reverse genetic system was available whereby individual genome segments could be genetically manipulated. Consequently, the aim of this study was essentially to develop a reverse genetic system for AHSV that would allow recovery of infectious from recombinant sources and/or allow for the targeted introduction of cDNA-derived genome segments into the viral genome.

Towards establishing a reverse genetic system for AHSV, it was first determined whether *in vitro*-transcribed AHSV ssRNA is infectious. The results indicated that infectious virus could be recovered following transfection of permissive cells with purified AHSV-4 core-derived ssRNA. These results therefore suggested that infectious AHSV may be recovered from recombinant sources, provided that the AHSV ssRNA bear authentic 5'- and 3'-terminal sequences and are capped at their 5' end. Subsequently, two DNA-based and a synthetic mRNA-based reverse genetic approach was evaluated for their ability to recover AHSV-4. The use of an entirely plasmid DNA-based reverse genetic system, in which full-length cDNA copies of the AHSV-4 genome segments are flanked by an upstream T7 RNA polymerase promoter and by a downstream hepatitis delta virus (HDV) ribozyme sequence, failed to recover AHSV-4 in BSR-T7 mammalian cells. Likewise, transfection of the mammalian cells with the T7 transcription cassettes of each cloned AHSV-4 cDNA genome segment did not

result in the recovery of infectious AHSV-4. Similar results were obtained when a mixture of *in vitro*-synthesised and -capped AHSV-4 T7 transcripts, using the T7 transcription cassettes as templates in these reactions, were transfected into BSR cells. The inability to recover infectious AHSV-4 from these recombinant sources may have been due to different technical complexities, including inefficient capping of transcripts that may lead to the activation of antiviral responses and difficulties associated with transfection of cells with a full complement of the ten DNA constructs or ssRNA transcripts. Consequently, it was next investigated whether recombinant AHSV could be generated by targeted replacement of a single genome segment with a cDNA-derived genome segment. Transfection of BSR cell monolayers with a mixture of *in vitro*-synthesised and -capped AHSV-4 segment 10 T7 transcripts and AHSV-3 core-derived ssRNA yielded reassortant plaques, of which the identity was confirmed by a serogroup discriminating polymerase chain reaction assay and nucleotide sequencing of the genome segment 10 amplicon.

The recovery of recombinant AHSV containing a plasmid cDNA-derived genome segment not only represents a valuable milestone toward the development of a reverse genetic system for AHSV, but also is a powerful tool for studies aimed at understanding AHSV biology. This reverse genetic approach is potentially applicable to all genome segments and has the potential to be used as a tool for future investigations into the functions of viral proteins in replicating AHSV, as well as the elucidation of genetic factors involved in viral pathogenesis and virulence.

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LIST OF ABBREVIATIONS

Acc.	accession
AHS	African horse sickness
AHSV	African horse sickness virus
ARCA	anti-reverse cap analogue
ATCC	American type culture collection
BHK	Baby hamster kidney
BLAST	Basic Local Alignment Search Tool
bp	base pair
BTV	Bluetongue virus
°C	degrees Celsius
C	carboxy
ca.	approximately
CAT	chloramphenicol acetyltranferase
cDNA	complementary DNA
cm^2	cubic centimeter
CO_2	carbon dioxide
CPE	cytopathic effect
cryo-EM	cryo-electron microscopy
DEPC	diethyl pyrocarbonate
dH_2O	deionized water
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside-5'-triphosphate
ds	double stranded
DTT	dithiothreitol
e.g.	*exempli gratia* (for example)
EBSS	Earle's Balanced Salt Solution
EDTA	ethylenediaminetetra-acetic acid
eGFP	enhanced green fluorescent protein
EMEM	Eagle's Minimal Essential Medium
et al.	*et alia* (and others)
FBS	foetal bovine serum
Fig.	figure
h	hour

HDV	hepatitis delta virus
i.e.	*id est* (that is)
kb	kilobase pairs
kDa	kilodalton
KOAc	potassium acetate
kV	kilovolt
LB	Luria-Bertani
M	molar
MAb	monoclonal antibody
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MOI	multiplicity of infection
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
N	amino
NaOAc	sodium acetate
NEAA	non-essential amino acids
ng	nanogram
nm	nanometer
N-MAb	neutralising monoclonal antibody
No.	number
nt	nucleotides
OD	optical density
OIE	Office International des Epizooties
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
pmol	picomole
PSB	protein solvent buffer
RdRp	RNA-dependant RNA polymerase
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease

RNP	ribonucleoprotein complex
s	second
SDS	sodium dodecyl sulphate
shRNA	short hairpin RNA
siRNA	small interfering RNA
ss	single stranded
TE	Tris-EDTA
TEM	transmission electron microscopy
TEMED	N',N',N',N'-tetramethylethylenediamine
Tris	Tris-hydroxymethyl-aminomethane
ts	temperature-sensitive
U	units
UV	ultraviolet
V	volts
v.	version
v/v	volume per volume
VIB	viral inclusion body
vRNA	viral RNA
w/v	weight per volume
× *g*	centrifugal force
µg	microgram
µl	microlitre
µm	micrometre

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CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

The discovery of "filterable disease agents", later to become known as viruses, by Dimitri Ivanovsky over a century ago, gave rise to the ever-expanding field of biology known as virology (Ruiz-Saenz and Rodas, 2010). Although tobacco plants were the hosts of the first described filterable agent, it was soon discovered that viruses exist wherever life is found (Suttle, 2005). It is likely that all organisms are susceptible to viral infection, which in most cases is asymptomatic. Diseases caused as the result of viral infections have, however, been the topic of much investigation due to their potentially devastating effects on human health and agriculture. Amongst these is African horse sickness (AHS), of which African horse sickness virus (AHSV) is the causative agent. AHS is one of the most lethal diseases of horses and is characterised by clinical signs that develop as a consequence of damage to the circulatory and respiratory systems, thus giving rise to serious effusion and small haemorrhages in various organs and tissues.

AHS was first described in Yemen in 1327, but is almost certainly African in origin and has caused numerous outbreaks in South Africa since Dutch occupation in the 18th century (Coetzer and Guthrie, 2004). It was not until 1900 that Sir John McFadyean used blood from infected animals to show that the causative agent of AHS was filterable, and therefore a virus (Erasmus, 2009). This constituted one of the first animal diseases for which a virus had been implicated as the aetiological agent. Subsequent studies aimed at characterising the structure and morphology of AHSV (Breese *et al.*, 1969; Oellermann *et al.*, 1970; Calisher and Mertens, 1998) led to its classification as a member of the *Orbivirus* genus within the family *Reoviridae*. Orbiviruses are characterised as non-enveloped, multi-layered virus particles that harbour a segmented double-stranded (ds) RNA genome, and are capable of infecting a broad range of vertebrate and insect hosts (Gorman, 1992; Calisher and Mertens, 1998). Although progress has since been made regarding structure-function relationships of some AHSV proteins (Uitenweerde *et al.*, 1995; Maree and Huismans, 1997; van Niekerk *et al.*, 2001; de Waal and Huismans, 2005; Stassen *et al.*, 2011), many areas of AHSV biology remain unclear. The roles of individual viral proteins in AHS disease and pathogenesis, as well as questions relating to how the virus and host proteins together orchestrate a successful infectious cycle remain largely unelucidated. Reverse genetics may represent a potentially powerful approach whereby these types of questions can be answered.

Contemporary molecular virology makes use of reverse genetic techniques to study defined mutations within the hereditary material of viruses and the effects that these mutations confer on viral replication and pathogenesis (Palese *et al.*, 1996; Walpita and Flick, 2005; Roy, 2005). However, due to the inherent nature of their segmented dsRNA genomes, orbiviruses have remained refractory to these technologies. Recently, the prospect of a reverse genetic system for AHSV has been raised as a consequence of reverse genetic techniques having been developed for other segmented dsRNA viruses, including rotavirus (Komoto *et al.*, 2006; Troupin *et al.*, 2010; Trask *et al.*, 2010), reovirus (Kobayashi *et al.*, 2007, 2010) and bluetongue virus (Boyce *et al.*, 2008).

This review will summarise the current literature concerning AHSV and will highlight the role of individual proteins in the infectious cycle of the virus. This will be followed by a brief discussion of RNA interference and its development as a tool for heterologous gene silencing in mammalian cells. The review will be concluded by a discussion of reverse genetic approaches and systems, some of which may be exploited for the future study of AHSV.

1.2 AFRICAN HORSE SICKNESS (AHS)

AHS is a highly infectious disease of equines with high mortality rates in horses (Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004). Although zebras have long been considered the natural vertebrate host and reservoir of AHSV (Erasmus *et al.*, 1978; Lord *et al.*, 1997; Barnard, 1998), antibodies to AHSV have been detected in camels, cattle, sheep, buffalo (Coetzer and Guthrie, 2004), dogs (van Rensburg *et al.*, 1981), donkeys (Fassi-Fihri *et al.*, 1998) and mules (el Hasnaoui *et al.*, 1998). A single incident of AHSV infection in humans by neurotropic vaccine strains of the virus (serotypes 1 and 6) has been reported (Swanepoel *et al.*, 1992; van der Meyden *et al.*, 1992).

AHS was thought to be a disease confined to sub-Saharan Africa, except for occasional outbreaks in northern Africa and the Arabian Peninsula (Mellor and Hamblin, 2004). In the late 1980s, however, repeated outbreaks in Spain and Morocco dispelled a long-held belief that AHSV could not survive European winter conditions (Mellor and Hamblin, 2004; MacLachlan and Guthrie, 2010). The occurrence of BTV in Europe is associated with an increasing geographical distribution of its vector insect, which has been linked to climate change (Purse *et al.*, 2005).

AHS is not contagious and the virus is transmitted primarily through the bites of adult female midges, belonging to the genus *Culicoides* (Wetzel *et al.*, 1970; Mellor, 1993). The major vector of AHSV in Africa is *C. imicola*, which has a distribution range throughout Africa, south-east Asia and southern Europe (Mellor and Hamblin, 2004). A second species of midge, *C. bolitinos*, has also been implicated in the transmission of AHSV (Venter *et al.*, 2000). *C. bolitinos* exhibits a different geographic range to *C. imicola*, preferring cooler highland areas where the latter is rare.

Upon infection, as result of blood feeding by an infected midge, AHSV is transported to the regional lymph nodes of the animal, where initial virus replication takes place. The virus is then disseminated throughout the body via the circulatory system, resulting in primary viraemia. Subsequent virus replication in target organs and pulmonary microvascular endothelial cells gives rise to secondary viraemia (Coetzer and Guthrie, 2004). Four distinct clinical syndromes have been described in horses with AHS. Each of these is associated with a specific pathogenesis and mortality, ranging between 95% (pulmonary form) and 0% (fever form) (Erasmus, 1973; Brown and Dardiri, 1990; Coetzer and Guthrie, 2004). The fever form of the disease develops after an incubation period of 5-14 days upon which the affected horses develop mild to moderate fever, scleral infection and mild depression, followed by complete recovery. The cardiac form has an incubation period of 7-14 days and results in mild fever, pericardial effusion, and oedema of subcutaneous and intramuscular tissue of the head, neck and chest. Mortality rates exceeding 50% have been reported for horses affected with this form of the disease. The most severe form of the disease is the pulmonary form, which results in mortality rates often exceeding 95%. This form of the disease develops rapidly (within 4-5 days of infection) and is characterised by high fever, pulmonary oedema and plural effusion. The most common form of AHS, however, is a mixed pulmonary and cardiac form, which exhibits symptoms of both forms of the disease and a mortality rate approaching 70%. All forms of the disease can occur in any one outbreak, but in susceptible horse populations the mixed (cardio-pulmonary) and pulmonary forms tend to predominate so that mortality rates in these animals are generally high (Coetzer and Guthrie, 2004).

Due to the severity of AHS disease and its consequent importance with regard to the maintenance and transport of animals within the equestrian industry, AHS is listed as a notifiable disease by the Office International des Epizooties (OIE). Notifiable diseases are defined as transmissible and have the potential for very serious and rapid spread, with

particularly severe socio-economic or public health consequences, and are of major importance in the international trade of animals and animal products (OIE, 2011). In southern Africa, AHS is controlled by vaccination using polyvalent, live attenuated vaccines that are administered twice in the first and second year of susceptible animals, and annually thereafter (Erasmus, 1976; MacLachlan *et al.*, 2007). These vaccines are, however, not without risks and drawbacks. These include incomplete protection, weak immunogenicity of some vaccine strains and possible reversion to virulence (Mellor and Hamblin, 2004).

1.3 AFRICAN HORSE SICKNESS VIRUS (AHSV)

1.3.1 Taxonomic classification

AHSV is a member of the genus *Orbivirus* in the family *Reoviridae* (Calisher and Mertens, 1998). *Reoviridae* can be distinguished from other virus families by their segmented dsRNA genomes, non-enveloped icosahedral virus particles, as well as their ability to infect a wide range of hosts. Members of the *Reoviridae* family have been isolated from a wide variety of terrestrial and non-terrestrial vertebrates, invertebrates and plants (Gorman, 1992). The orbiviruses can be distinguished from other members of the *Reoviridae* in that they replicate in both insects and vertebrates (Calisher and Mertens, 1998), show greater susceptibility to lipid solvents and detergents, and virus infectivity is lost in mildly acidic conditions (Gorman and Taylor, 1985). Moreover, orbiviruses are morphologically similar; the most characteristic feature being large, ring-shaped capsomeres on the inner shell of the virus particle (Borden *et al.*, 1971). Within the genus, viruses are divided into 21 distinct serogroups based on cross-reactivity in complement fixation tests, and serotypes within a serogroup are recognised by specific serum-neutralisation tests (Hazrati and Ozawa, 1968; Howell, 1962). To date, nine different AHSV serotypes (1 to 9) have been distinguished serologically (Howell, 1962).

1.3.2 Virion structure

AHSV, like bluetongue virus (BTV), is structurally distinct from other members of the *Reoviridae* family. Virions, which measure 88 nm in diameter, are composed of seven discrete structural proteins (VP1 - VP7) that are arranged into an inner and outer capsid (Roy, 1996). The outer capsid is composed of the two major structural proteins VP2 and VP5, while the inner capsid is composed of the two major proteins VP7 and VP3 that enclose three minor structural proteins (VP1, VP4 and VP6) and the viral genome (Roy, 1996) (Fig. 1.1).

The structure of the AHSV particle is comparable to that of BTV, of which the structure of single- and double-shelled virus particles has been determined by cryo-electron microscopy (cryo-EM) and by X-ray crystallography (Hewat *et al.*, 1992; Grimes *et al.*, 1998; Zhang *et al.*, 2010). BTV subcore particles are composed of 120 copies of VP3 (T=2), arranged as an icosahedron. VP3 is essentially triangular in shape and can occupy two positions and two distinct conformations, designated A and B (Grimes *et al.*, 1998). VP3A and VP3B both form pentamers, which interact with each other to form a decamer. Twelve such decamers form the inner shell of the core particle through interaction via a dimerization domain (Grimes *et al.*, 1998; Kar *et al.*, 2004). The interaction between five VP3A molecules at the five-fold axes leads to the formation of a small central pore. The VP3 shell is stabilised by the outer layer of the core particle that comprises of 780 copies of VP7, arranged as 260 trimers (Grimes *et al.*, 1998). The VP7 molecules are layered onto the VP3 scaffold in 13 distinct orientations, resulting from a mismatched symmetry with VP3. Due to this, individual VP7 trimers are capable of occupying five different positions on the surface of the core particle, assigned with increasing distance from the five-fold axis as P, Q, R, S (two-fold) and T, which forms the three-fold axis. The pores formed at the five-fold axes of the subcore, through which newly synthesised viral mRNA presumably passes, are completely blocked by the VP7 T trimer in non-transcriptionally active cores (Grimes *et al.*, 1998).

The VP7 trimers also act as attachment sites for the two outer capsid proteins VP2 and VP5 (Hewat *et al.*, 1992). Globular VP5 proteins (360 copies) occupy the channels formed by the hexameric rings of VP7 trimers, making contact with the core proteins on the external edges of these channels, thus effectively plugging the channels with their basal tails. VP2 (180 copies) trimers are bound by interactions with the surface of the core particle and project beyond VP5, forming 60 triskelion structures (Nason *et al.*, 2004; Zhang *et al.*, 2010). The outermost feature on the viral surface is a propeller-shaped spike, the top of which is angled upward, perpendicular to the viral surface.

The close association of the core transcriptase complexes, formed by VP1, VP4 and VP6 with viral genomic dsRNA, has hampered studies into the organisation of these proteins within the core particle (Grimes *et al.*, 1998). Studies using reconstructions of recombinant core-like particles have shown that VP1 and VP4 associate with the five-fold axes through attachment to the underside of VP3 (Nason *et al.*, 2004). Although not confirmed, it is thought that VP6 is also located at the inner five-fold axes of the core particle, in close association with VP1

and VP4 (Roy, 2008). BTV genomic dsRNA is not clearly visible using cryo-EM or X-ray crystallographic techniques and little is known regarding the properties of concentrated dsRNA solutions in general (Grimes *et al.*, 1998; Gouet *et al.*, 1999). It has, however, been suggested that the genome could be arranged in a highly organised manner as four concentric layers, made up of multiple strands, within the core particle (Gouet *et al.*, 1999).

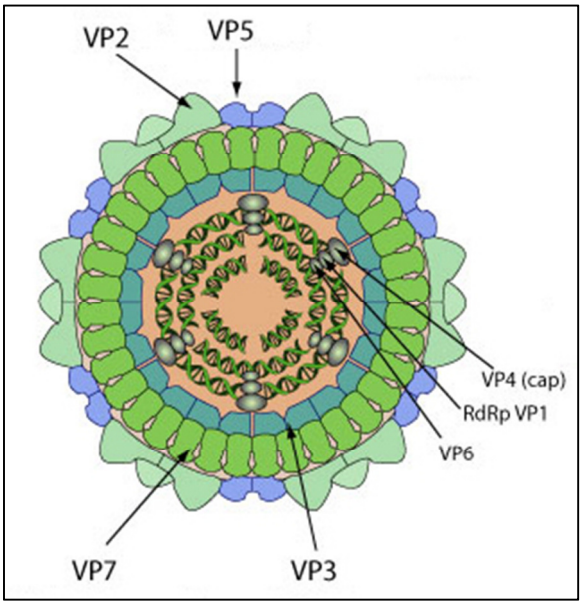


Fig. 1.1: Structure of the *Orbivirus* virion. Genomic dsRNA resides within the inner capsid and is associated with transcriptase complexes, which are composed of the RNA-dependent RNA polymerase (VP1), helicase (VP6) and capping enzyme (VP4). The transcriptase complexes are, in turn, located on the five-fold axes of the inner capsid formed by VP3 and VP7. An outer capsid, composed of VP2 and VP5, surrounds the inner capsid. Adapted from the ExPASy Bioinformatics Resource Portal (Gasteiger *et al.*, 2003).

1.3.3 The AHSV genome

The genome of AHSV is comprised of 10 linear dsRNA segments of varying sizes, which are grouped into large (L1-L3), medium (M4-M6) and small (S7-S10), according to their electrophoretic mobility through a polyacrylamide gel (Oellerman, 1970; Bremer, 1976). Each genome segment is monocistronic, except for S10, which encodes the two related nonstructural proteins NS3 and NS3A (van Staden *et al.*, 1991; Grubman and Lewis, 1992). The total viral genome is *ca.* 19.2 kilobase pairs (kb) in length. The 5' noncoding region of the genome segments ranges in size between 12 and 35 base pairs (bp), while the 3' noncoding regions are 29 to 100 bp in length (Roy *et al.*, 1994). In contrast to BTV, the

terminal hexanucleotide sequences of AHSV are not conserved throughout all of the segments (Rao *et al.,* 1983; Roy *et al.*, 1994). The 5'- and 3'-terminal sequences of each genome segment, however, display partial inverted complementarity. This feature is thought to play a role in the sorting and assembly of genome segments during viral replication (Anzola *et al.*, 1987; Cowley *et al.*, 1992).

1.3.4 AHSV proteins

In addition to the seven structural proteins (VP1-VP7), four nonstructural proteins (NS1, NS2, NS3 and NS3A) are also encoded by the viral genome. It should be noted that a fourth nonstructural protein, designated NS4, was recently identified for BTV (Belhouchet *et al.*, 2011), but not much is known regarding its function. The 10 orbivirus genome segments, together with their encoded proteins and likely functions, are summarised in Table 1.1.

1.3.4.1 Core proteins

The major core proteins, VP7 and VP3, form the outer layer of the viral core particle, and assemble spontaneously into core-like particles when co-expressed by recombinant baculoviruses (Maree *et al.*, 1998). Of the two proteins, VP7 is the most abundant protein in the core particle and self-assembles into trimers (Basak *et al.*, 1992), which forms the outermost shell of the core. In both AHSV and BTV, VP7 has been demonstrated to be a serogroup-specific antigen (Huismans and Erasmus, 1981; Chuma *et al.*, 1992). The top domain of AHSV and BTV VP7 both contain a surface-exposed RGD tripeptide motif, which in the case of BTV, has been shown to be responsible for the attachment of the core to *Culicoides* cells (Tan *et al.*, 2001). However, in contrast to BTV, AHSV VP7 forms flat hexagonal crystals in the cytoplasm of virus-infected (Burroughs *et al.*, 1994) and recombinant baculovirus-infected cells (Chuma *et al.*, 1992; Maree and Paweska, 2005). The functional significance of the VP7 crystalline structures is not yet known, but is thought to represent a by-product, rather than an essential component of AHSV replication (Burroughs *et al.*, 1994). The VP3 protein plays a major role in the structural integrity of the virus core and forms the protein scaffold on which the VP7 capsomeres are assembled (Stuart *et al.*, 1998; Kar *et al.*, 2004). The BTV VP3 protein contains group-specific antigenic determinants (Inumaru *et al.*, 1987) and is capable of binding single-stranded (ss) RNA (Loudon and Roy, 1992). The functional significance of this interaction is yet to be determined.

Table 1.1: Genome segments and protein products of a representative AHSV isolate (adapted from Mertens, 2004)

Segment	Length (bp)	Protein encoded	Amino acids	Size (Da)	Copies/ particle	Location	Function
L1	3965	VP1	1305	150292	10 / 12	Interior of core	RNA-dependant RNA polymerase
L2	3203	VP2	1051	122043	180	Outer capsid	Serotype-specific antigen, adsorption, neutralization, structural protein involved in determination of virulence
L3	2792	VP3	905	103269	120	Inner capsid	Structural protein, forms scaffold for VP7 trimers, controls size and organization of capsid structure
M4	1978	VP4	642	75826	20 / 24	Interior of core	Capping enzyme with guanyltransferase activity
M5	1748	NS1	548	63377	0	Nonstructural, forms tubules	Unknown, possible virulence determinant and role in viral egress
M6	1566	VP5	504	56900	360	Outer capsid	Structural protein, serotype determinant, role in membrane penetration early in infection
S7	1167	VP7	349	37916	780	Inner capsid	Group-specific structural protein, possibly involved in insect host cell attachment
S8	1166	NS2	365	41193	0	Nonstructural, forms cytoplasmic inclusion bodies	Forms viral inclusion bodies (VIBs) where progeny virus assembly takes place, binds viral ssRNA
S9	1169	VP6	369	38464	60 / 72	Interior of core	Helicase, binds ssRNA and dsRNA
S10	756	NS3	217	23659	0	Nonstructural, cell membrane-associated	Involved, along with cellular proteins, in virus release, potential virulence determinant
		NS3A	206	22481	0		

The three minor core proteins VP1, VP4 and VP6 form part of the transcriptase complex, and are solely responsible for the synthesis of capped and methylated transcripts of each dsRNA segment during the infectious cycle (Mertens and Diprose, 2004). The VP1 protein is an RNA-dependant RNA polymerase (RdRp) and exhibits a detectable RNA-elongation activity in the presence of single-stranded poly(U) template and a poly(A) primer (Roy *et al.*, 1988; Urakawa *et al.*, 1989). More recently, it was reported that soluble recombinant VP1 exhibited processive replicase activity, synthesising complete complementary RNA strands of *in vitro*-synthesised BTV ssRNA templates (Boyce *et al.*, 2004). Crystallographic modelling of VP1 revealed the structure and organisation of the protein to be typical of previously described RdRps, containing the conventional thumb, palm and finger domains (Wehrfritz *et al.*, 2007). The resolved polymerase domain (PD), N-terminal domain (NTD) and C-terminal domain (CTD) were shown to be analogous to the reovirus polymerase enzyme (Tao *et al.*, 2002). Additionally, mutagenesis studies of the catalytic GDD motif, located in the PD, concluded that this motif was essential to protein function (Wehrfritz *et al.*, 2007). Although the PD alone is insufficient for catalytic activity *in vitro*, function can be restored by providing purified NTD and CTD fragments, suggesting they play a role in polymerase activation (Wehrfritz *et al.*, 2007).

The 5' ends of the viral mRNA are believed to be capped and methylated during transcription, thus resulting in the stabilisation of the viral mRNA synthesised during infection (Roy, 1992). The VP4 protein of BTV possesses nucleotide phosphohydrolase (NTPase), guanylyltransferase (GTPase) and transmethylase type 1 and type 2 activities, which are consistent with the requirements for formation of Cap 1 structures (Le Blois *et al.*, 1992; Ramadevi *et al.*, 1998; Martinez-Costas *et al.*, 1998). Resolution of the atomic structure of VP4 revealed that it is an elongated molecule, in which the respective catalytic motifs are arranged sequentially along the molecule in discrete domains (Sutton *et al.*, 2007).

The third minor core protein, VP6, is a highly basic protein with a high affinity for both ss- and dsRNA (Hayama and Li, 1984; Roy *et al.*, 1990; de Waal and Huismans, 2005). Purified BTV VP6, upon incubation with dsRNA in the presence of ATP and Mg^{2+}, is capable of efficiently unwinding dsRNA templates with either blunt or overhanging (both 5' and 3') ends (Stauber *et al.*, 1997). Based on the above properties, it is likely that VP6 may function as a helicase during the transcription of viral mRNA, either to unwind dsRNA prior to transcription or to separate the parental and newly synthesised RNAs following transcription.

Based on its RNA-binding ability, VP6 may also be involved in the encapsidation of viral RNA. VP6 shows structural homology to other helicase proteins, exhibiting a hexameric form capable of forming a stable ring-shaped structure in the presence of ss- and dsRNA (Kar and Roy, 2003).

1.3.4.2 Outer capsid proteins

The VP2 protein, one of the two outer capsid proteins, is the most variable of the viral proteins (Potgieter *et al.*, 2003; Maan *et al.*, 2008), and is the major serotype-specific antigen (Huismans and Erasmus, 1981; Maan *et al.*, 2007) and viral haemagglutinin (Cowley and Gorman, 1987). AHSV and BTV VP2 elicit neutralising antibodies (Martinez-Torrecuadrada *et al.*, 2001) that can confer protection against subsequent challenge with the homologous virus serotype (Huismans *et al.*, 1987b; Martinez-Torrecuadrada *et al.*, 1994). Moreover, VP2 is involved in attachment of the virus to cells and has been reported to bind to sialic acid moieties of cellular receptors prior to internalisation of the virus particle (Hassan and Roy, 1999; Zhang *et al.*, 2010). In addition to its role in attachment, VP2 is also emerging as a key player in the control of BTV assembly and egress from infected cells. The protein interacts with vimentin and this interaction contributes to virus egress (Bhattacharya *et al.*, 2007; Celma and Roy, 2009). It has also been reported that the extracellular treatment of mammalian cells with a combination of VP2 and VP5 is sufficient to trigger apoptosis (Mortola *et al*., 2004).

Compared to VP2, the second outer capsid protein VP5 is more conserved (Gould and Pritchard, 1988; Oldfield *et al.*, 1991). In contrast to BTV VP5, which may play a supportive role to VP2 in enhancing the immune response (Marshall and Roy, 1990; Roy *et al.*, 1992), the AHSV VP5 protein is able to induce neutralising antibodies, albeit at lower titres than those induced by VP2 (Martinez-Torrecuadrada *et al.*, 1999). Recent studies on the biological activity of VP5 have shown that the protein permeabilises host cell membranes (Hassan *et al.*, 2001; Stassen *et al.*, 2011) and that BTV VP5 has the ability to induce cell-cell fusion when expressed on the cell surface (Forzan *et al.*, 2004). Both these activities are mediated by two N-terminal amphipathic α-helices and are believed to play a major role in destabilising the membrane of the endocytosed vesicle, thus allowing release of the viral core into the cytoplasm (Forzan *et al.*, 2007; Zhang *et al.*, 2010). BTV VP5 also interacts with membrane lipid rafts via a WHAL motif, and is likely to play an important role in docking VP5 with

plasma membranes for assembly and egress via membrane fusion (Bhattacharya and Roy, 2008).

1.3.4.3 Nonstructural proteins

The nonstructural viral proteins, NS1, NS2, NS3 and NS3A, are found in the cytoplasm of infected cells and are considered to play important roles in the replication, assembly and egress of orbiviruses (Eaton *et al.*, 1988; Hyatt *et al.*, 1993; van Staden *et al.*, 1998; Owens *et al.*, 2004). In AHSV-infected cells, NS1 and NS2 are synthesised abundantly and are responsible for the formation of two virus-specific structures, namely tubules and viral inclusion bodies (VIBs), respectively (Lecatsas, 1968). In contrast, NS3 and NS3A are barely detectable in virus-infected cells (French *et al.*, 1989; Wu *et al.*, 1992; van Staden *et al.*, 1995).

Tubules, which are a distinctive feature of orbivirus infection, are composed entirely from NS1 protein (Huismans and Els, 1979). The AHSV NS1 protein is particularly rich in cysteine residues and has several hydrophilic and hydrophobic regions spaced throughout the molecule, indicating that it is likely to have a highly ordered structure (Maree and Huismans, 1997). Site-directed mutagenesis, conducted on BTV NS1, revealed that cysteine residues at positions 240 and 337, as well as intact amino (N)- and carboxy (C)-termini are essential for tubule formation (Monastyrskaya *et al.*, 1994). The tubular structures are formed by helically coiled ribbons of NS1 dimers, but the biophysical character of the tubules differ between BTV and AHSV. In contrast to BTV tubules that have a diameter of 52 nm and lengths up to 1 µm (Hewat *et al.*, 1992), the AHSV tubules have a diameter of 23 nm and lengths up to 4 µm (Maree and Huismans, 1997). Several roles for NS1 have been proposed; however, its function in orbivirus infection still remains unclear. It was initially suggested that NS1 is involved in the trafficking of virus particles from the VIBs to the cell membrane prior to virus release (Eaton *et al.*, 1990) or, alternatively, that tubules inhibit the formation of core particles before the incorporation of minor proteins and genome segments (Eaton *et al.*, 1990; Hewat *et al.*, 1992). More recently, it has been proposed that NS1 may be a major determinant of BTV pathogenesis in the vertebrate host since it augments virus-cell association that ultimately leads to lysis of the infected cells (Owens *et al.*, 2004).

The NS2 protein is the predominant component of VIBs and expression of NS2, in the absence of other viral proteins in both insect and mammalian cells, results in the formation of inclusion bodies that are indistinguishable from VIBs found in virus-infected cells (Thomas *et al.*, 1990; Uitenweerde *et al.*, 1995). The VIBs have been shown to contain ssRNA, dsRNA, NS1, as well as complete and incomplete virus particles (Eaton *et al.*, 1988, 1990; Brookes *et al.*, 1993). These observations have led to the recognition of VIBs as the sites in which virus assembly occurs, leading to them being termed "virus factories". The NS2 protein has a strong affinity for ssRNA (Huismans *et al.*, 1987a; Theron and Nel, 1997; Lymperopoulos *et al.*, 2003), suggesting that it may have a role in the recruitment and packaging of viral ssRNA prior to encapsidation. NS2 is the only virus-specific protein that is phosphorylated in infected cells (Huismans *et al.*, 1987a; Theron *et al.*, 1994). Although the significance of NS2 phosphorylation is only partially understood, it has been reported that phosphorylation of NS2 downregulates its ssRNA binding activity (Theron *et al.*, 1994). In BTV, phosphorylation of NS2 is required for VIB formation and dephosphorylation of the protein is proposed to allow disassembly of the VIBs with subsequent release of the assembled viral cores (Modrof *et al.*, 2005). Interestingly, BTV NS2 displays phosphohydrolase (NTPase) activity, and can bind and hydrolyse both ATP and GTP to their corresponding nucleotide monophosphates (Horscroft and Roy, 2000; Taraporewala *et al.*, 2001). It has been suggested that the NTPase activity may play a role in providing energy for the assortment, movement, packaging or condensation of bound ssRNA (Horscroft and Roy, 2000).

The AHSV NS3 and NS3A proteins are encoded by RNA genome segment 10 from alternate in-phase translation initiation codons, and differ only with respect to 10 amino acid residues present at the N-terminal end of NS3 (van Staden and Huismans, 1991). The BTV NS3, in contrast to that of AHSV, has been shown to be glycosylated (Wu *et al.*, 1992), which may serve to protect it from degradation (Bansal *et al.*, 1998). The NS3/NS3A proteins are the only virus-encoded membrane-associated proteins (Wu *et al.*, 1992; van Niekerk *et al.*, 2001) and are localised to the sites of virus release in infected cells (Hyatt *et al.*, 1993; Stoltz *et al.*, 1996). NS3 has also been shown to interact specifically with VP2 in newly formed BTV virions (Beaton *et al.*, 2002), an activity which has been mapped to highly charged lysine residues on the C-terminal of the protein (Celma and Roy, 2009). These findings therefore suggest a role for NS3 during the final stages of viral morphogenesis by facilitating the release of progeny virus from infected cells.

1.4 ORBIVIRUS REPLICATION, ASSEMBLY AND RELEASE

Orbivirus replication can be divided into four major events: (i) adsorption and penetration, (ii) uncoating and formation of replicative complexes, (iii) formation of virus tubules and inclusion bodies within which core assembly takes place, and (iv) transport of progeny virions to the cell membrane for release. Due to the vast amount of information available for BTV, it will be used as a model to describe orbivirus replication and morphogenesis, which is illustrated in Fig. 1.2.

In mammalian cells, the binding and internalisation of BTV is mediated by the outer capsid VP2 protein (Huismans and van Dijk, 1990; Hassan and Roy, 1999). Although the cellular receptors to which BTV binds have not yet been identified, it has been reported that the VP2 protein attaches to sialoglycoproteins of mammalian cells prior to internalisation (Hassan and Roy, 1999; Zhang *et al.*, 2010). The virus enters the cell through AP2-dependent clathrin-mediated endocytosis and is incorporated into early endosomes (Forzan *et al.*, 2007). It has recently been speculated that the low pH within the endosome causes VP5 to undergo a conformational change, allowing the protein to have extensive hydrophobic contact with the endosomal membrane (Hassan *et al.*, 2001; Zhang *et al.*, 2010). This potentially causes VP5 to penetrate the endosome, in the process detaching itself and VP2 from the virus particle, thereby resulting in the release of an uncoated core into the cell cytoplasm (Zhang *et al.*, 2010). The replication of BTV is initiated by the synthesis and extrusion of capped and methylated mRNA from transcriptionally active cores within the cytoplasm. The mRNA transcripts function to encode proteins, and are also used as templates for production of negative-strands to form dsRNA genome segments encapsidated in the progeny virions (Mertens and Diprose, 2004). The mechanism whereby viral mRNAs are selected and encapsidated prior to replication is, however, unknown.

Soon after the initiation of transcription of BTV mRNAs, granular matrix structures accumulate near core particles (Hyatt *et al.*, 1987). These VIBs increase both in size and number as viral infection progresses (Eaton *et al.*, 1990). Newly synthesised viral transcripts, the four subcore proteins (VP1, VP3, VP4 and VP6), as well as assembled cores and subcores have been identified in the VIBs and therefore appear to be the sites of orbivirus replication and early viral assembly (Hyatt and Eaton, 1988). The assembly of the BTV VP3 subcore has been reported (Kar *et al.*, 2004). VP3 was reported to have three distinct domains, *i.e.* an

apical, a carapace and a dimerization domain. The dimerization domain mediates the formation of VP3 dimers, which pack together in decamers to form the icosahedral structure of the VP3 layer. Although it has been reported that BTV VP3 can bind to RNA (Loudon and Roy, 1992), it was more recently reported that BTV RNAs failed to associate with VP3 decamers (Kar *et al.*, 2004). Consequently, it was suggested that assembly of the BTV core may begin with a complex formed by minor core proteins and the VP3 decamers, and that these assembly intermediates recruit the viral RNA prior to completion of VP3 subcore assembly (Kar *et al.*, 2004). Co-expression of the BTV structural proteins with NS2 has indicated that VP7 requires co-expression of VP3 to be recruited to the VIBs and that neither of the outer capsid proteins VP5 and VP2 have an affinity for the VIBs (Modrof *et al.*, 2005; Kar *et al.*, 2007). Therefore, it would appear that progeny core particles are first produced inside VIBs, then moved to the periphery of the VIBs where they are coated by the outer capsid proteins VP5 and VP2 (Kar *et al.*, 2007). The nascent virions are subsequently released from the VIBs, possibly through the dephosphorylation of NS2 (Modrof *et al.*, 2005). In an alternative model, it was recently reported that VP5 of BTV associates with lipid rafts in the plasma membrane and that the core particles are transported to these sites for the final assembly of the outer capsid proteins (Bhattacharya and Roy, 2008). In addition to VIBs, NS1-rich tubules form part of the "insoluble" phase in the cell and become a characteristic structure of cells in the early stages of infection (Huismans and Els, 1979; Eaton *et al.*, 1988).

Investigations regarding virus release from mammalian cells have demonstrated a strong correlation between the presence of NS3 and NS3A, and virus release (Hyatt *et al.*, 1989; Stoltz *et al.*, 1996). The virions may leave infected cells in one of two ways. Early after infection, when the host cell metabolism is not completely inhibited and the integrity of the host plasma membrane is maintained, progeny virions have been observed to bud through the plasma membrane surface and acquire a transient envelope (Gould and Hyatt, 1994). Alternatively, during the latter stages of infection, when the integrity of the plasma membrane is not maintained, non-enveloped virions can be released by extrusion through the locally disrupted plasma membrane surface (Hyatt *et al.*, 1989; Han and Harty, 2004). More recently, the NS3 protein of BTV has been shown to interact with the cellular proteins p11 and Tsg101, and these interactions were furthermore shown to assist in the egress of virus particles from infected cells in a non-lytic manner (Beaton *et al.*, 2002; Wirblich *et al.*, 2006; Celma and Roy, 2009).

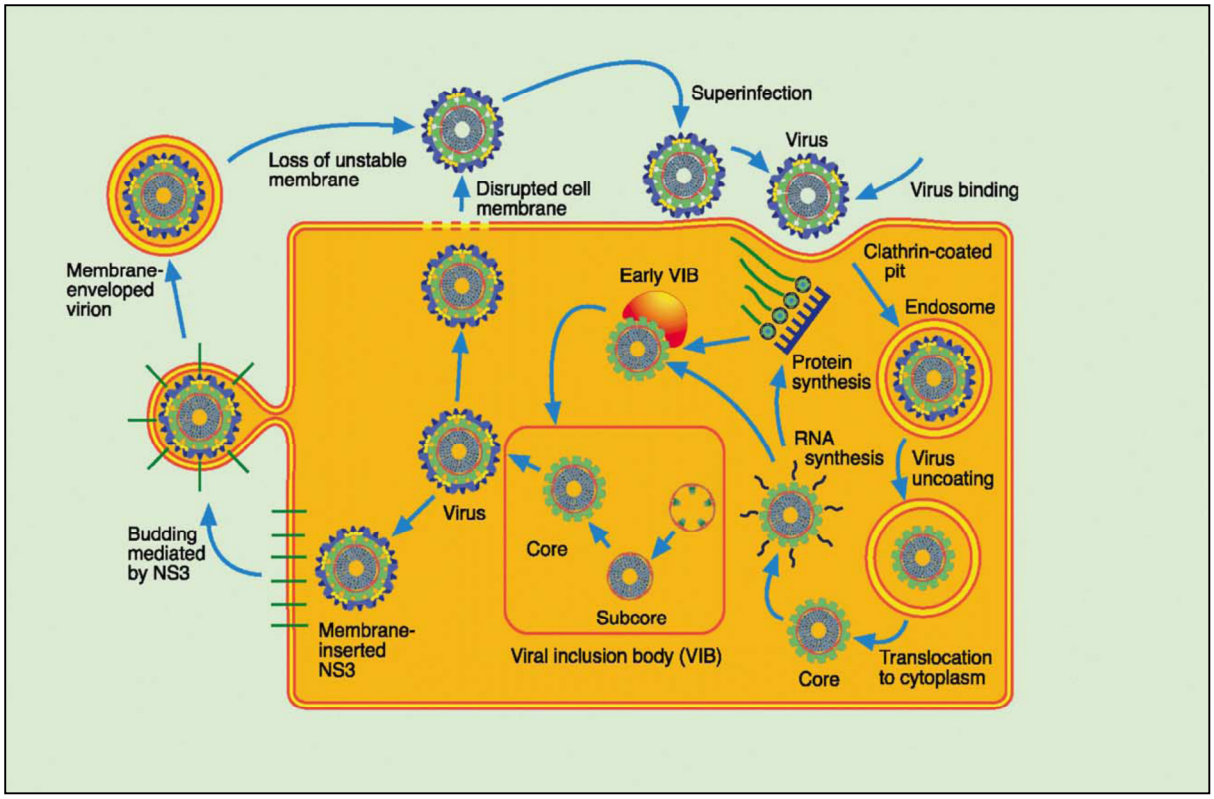


Fig. 1.2: Schematic diagram representing the replication cycle of BTV (Mertens, 2004). BTV enters the host cell by clathrin-mediated endocytosis and is incorporated into early endosomes where the outer capsid is shed, releasing the transcriptionally active core particle into the cytoplasm. BTV has 10 genome segments, which are transcribed within the core particle and extruded into the cytoplasm, where they form the templates for viral protein synthesis and genomic replication. BTV transcripts, along with BTV structural proteins are sequestered by the viral nonstructural protein NS2, which forms viral inclusion bodies where progeny virions are assembled. Progeny BTV leave the host cell by NS3-mediated budding or by lysis through a disrupted cell membrane.

1.5 RNA INTERFERENCE (RNAi)

RNA interference (RNAi) is an evolutionarily conserved gene silencing mechanism in metazoan eukaryotic cells, in which sequence-specific down-regulation of a target gene is caused by its cognate dsRNA. RNAi was first described in the nematode *Caenorhabditis elegans* (Fire *et al.*, 1998), but has subsequently been shown to be homologous to post-translational gene silencing (PTGS) in plants (Napoli *et al.*, 1990) and quelling in fungi (Cogoni *et al.*, 1996). The RNAi mechanism and its associated processes are thought to have evolved as a protective response by the host to invasion of the genome by mobile genetic elements such as transposons and viruses, in addition to maintaining normal growth and development (Agrawal *et al.*, 2003). As a result of its universal applicability, high specificity

and simplicity, RNAi has become a useful tool for the analysis of gene functions in diverse groups of organisms, including viruses (Mohr *et al.*, 2010). Recently, RNAi has also been exploited as a selection method to drive single-gene reverse genetics for rotavirus (Trask *et al.*, 2010) and thus warrants further discussion.

1.5.1 The mechanism and machinery of RNAi

Biochemical and genetic analyses have provided a mechanistic understanding of RNAi-mediated gene-silencing (Meister and Tuschl, 2004; Tomari and Zamore, 2005; Jinek and Doudna, 2009) (Fig. 1.3). In the first step, referred to as the RNAi initiating step, long dsRNA is typically cleaved into discrete 21-nucleotide (nt) RNA fragments, termed small interfering RNA (siRNA), by the RNase III-like enzyme Dicer (Bernstein *et al.*, 2001). Dicers are 200-kDa multidomain proteins and have a single dsRNA processing centre that contains two RNA cleavage sites (Provost *et al.*, 2002). Cleavage of the dsRNA at two nearby phosphodiester bonds on opposite RNA strands thus results in siRNA duplexes that are typically 21-nt in length, contain 5' phosphate and 3' hydroxyl groups and 2-nt overhangs at the 3'-termini (Zhang *et al.*, 2004). In the second step, referred to as the effector step of RNAi, the siRNAs are assembled into an RNA-induced silencing complex (RISC), which subsequently guides the sequence-specific recognition of the target mRNA (Hong *et al.*, 2008; Hutvagner and Simard, 2008). Every RISC contains a member of the Argonaute protein family, which is characterised by the presence of a PAZ domain and a PIWI domain (Cerutti *et al.*, 2000; Carmell *et al.*, 2002). It is thought that interaction between the PAZ domain and the 2-nt 3' overhangs of the siRNA duplexes allows for the transfer of one of the siRNA strands into RISC (Lingel and Izaurralde, 2004). This effectively creates a guide-strand that permits the RISC complex to bind to a complementary target mRNA, which forms a duplex with the antisense siRNA strand (Martinez e*t al.*, 2002). Based on the similarity of the PIWI domain with RNase H (Song *et al.*, 2004), it has been proposed that the Argonaute proteins act as the catalytic subunit, termed Slicer, that is responsible for hydrolysis of the target mRNA (Liu *et al.*, 2004; Parker *et al.*, 2005; Ma *et al.*, 2005). Cleavage of the target mRNA occurs at a single site in the centre of the siRNA:mRNA duplex region, 10 nt from the 5' end of the siRNA (Martinez and Tuschl, 2004).

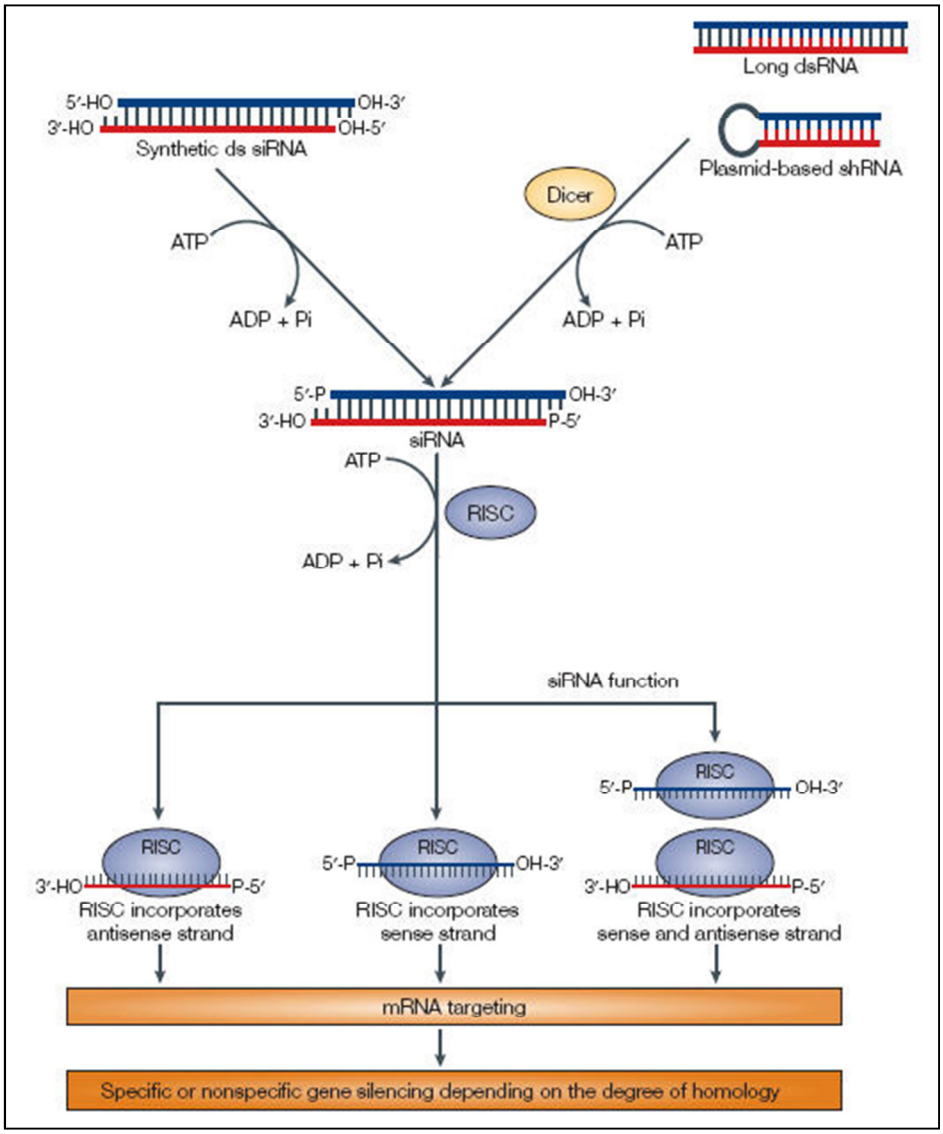


Fig. 1.3: RNAi-mediated gene silencing (Mittal, 2004). The processing of long dsRNA or plasmid-synthesised short hairpin RNA (shRNA) by Dicer leads to the formation of siRNAs, which consist of 21- to 23-nt RNA duplexes with symmetric 2-nt 3' overhangs and 5' phosphate groups. Exogenously provided synthetic siRNAs are converted into active functional siRNAs by an endogenous kinase that provides 5' phosphate groups in the presence of ATP. siRNAs associate with cellular proteins to form an RNA-induced silencing complex (RISC), which contains a helicase that unwinds the duplex siRNA in an ATP-dependant reaction. In an ideal situation, the antisense strand guides the RISC to the target mRNA for endonucleolytic cleavage. In theory, each of the siRNA strands can be incorporated into RISC and direct RNAi. The antisense strand of a siRNA can direct the cleavage of a corresponding sense RNA target, whereas the sense strand of a siRNA can direct the cleavage of an antisense target.

1.5.2 RNAi-mediated silencing in mammalian cells

Despite its utility in diverse organisms, it was initially difficult to detect potent and specific RNAi by dsRNA longer than 30 bp in commonly used mammalian cell culture systems (Agrawal *et al.*, 2003). This was largely due to the fact that introduction of the dsRNA into the cytoplasm of mammalian cells triggered an interferon response, which results in a systemic, nonspecific shut-down of protein synthesis (Stark *et al.*, 1998). These sequence-nonspecific effects can, however, be overcome by introducing synthetic 21- to 23-nt siRNA duplexes, rather than long dsRNA, into mammalian cells (Elbashir *et al.*, 2001). The siRNA duplexes avoid provoking an interferon response by virtue of their small size and are incorporated directly into the RNAi pathway by mimicking the products of the Dicer enzyme, which catalyse the initiation step of RNAi (see Section 1.5.1). In addition to the use of chemically synthesised siRNAs, various vector-based RNAi approaches, including plasmid DNA vectors (Brummelkamp *et al.*, 2002; Cheng and Chang, 2007; Wu *et al.*, 2009) and viral vectors such as adenoviruses, retroviruses and lentiviruses (Wadhwa *et al.*, 2004; Manjunath *et al.*, 2009), have been reported that allow for the production of short hairpin RNAs (shRNAs) that can be converted by Dicer into functional siRNAs (Fig. 1.3).

1.5.3 Application of RNAi to viruses with a segmented dsRNA genome

Since the discovery of RNAi activity in mammalian cell cultures (Caplan *et al.*, 2001; Elbashir *et al.*, 2001), numerous publications have subsequently described the use of RNAi to inhibit viruses from diverse virus families (Tan and Yin, 2004; Ketzinel-Gilad *et al.*, 2006; Csorba *et al.*, 2009). There have, however, been a limited number of studies exploring the potential of RNAi approaches to members of the *Reoviridae* family, and these have focussed mostly on rotaviruses. RNAi has been used for cell culture-based studies into the roles of both structural (Dector *et al.*, 2002; Zambrano *et al.*, 2007; Ayala-Breton *et al.*, 2009) and nonstructural proteins (Campagna *et al.*, 2005; Lopez *et al.*, 2005; Montero *et al.*, 2006). RNAi has also been applied to reovirus proteins associated with the formation of viral inclusions (Kobayashi *et al.*, 2006; Carvalho *et al.*, 2007; Kobayashi *et al.*, 2009). In the case of orbiviruses, siRNAs have been used to silence expression of the VP7 gene of AHSV, without affecting the expression of other viral genes (Stassen *et al.*, 2007).

In addition to the above studies aimed at probing gene function, RNAi has also been used as a means to develop both a genetic complementation system and a reverse genetic platform for rotavirus. A complementation system was devised for the analysis of the rotavirus NSP2 protein, which serves a key function in the formation of viroplasms in infected cells (Taraporewala *et al.*, 2006). In this system, MA104 cells were infected with a mutant SA11 rotavirus encoding a temperature-sensitive NSP2 protein that showed a 100-fold decrease in viroplasm formation at non-permissive temperatures. Additionally, siRNA-mediated degradation of these NSP2 transcripts further abrogated the formation of viroplasms, resulting in NSP2 expression being undetectable in cells which had been transfected with siRNA prior to viral infection. This provided the basis for the complementation of NSP2 activity by the *in vivo* transcription and expression of an RNAi-resistant NSP2 cDNA clone. This approach allowed for investigations into the effect of targeted mutations on NSP2 activity and the subsequent effects of the mutations on rotavirus replication.

Although the above method resulted in the knockdown of viral NSP2 to negligible levels, siRNA was only delivered to 80-90% of infected cells and therefore resulted in background levels of temperature-sensitive NSP2 being produced. Subsequently, the genetic complementation system was refined with the aim of recovering recombinant rotavirus containing a cDNA-derived wild-type NSP2 genome segment (Trask *et al.*, 2010) (Fig. 1.4). For this purpose, a stable cell line capable of expressing temperature-sensitive NSP2-specific shRNAs was constructed that ensures that all cells in the monolayer are capable of degrading the temperature-sensitive NSP2 transcripts. When the mutant SA11 rotavirus encoding a temperature-sensitive NSP2 protein was used to infect the engineered cell line at non-permissive temperatures, plasmid-derived wild-type NSP2 transcripts capable of RNAi escape were efficiently incorporated into virus genomes. By making use of growth at an elevated temperature and RNAi (via the introduction of silent mutations into the region targeted by the siRNA) as selection mechanisms, NSP2 recombinant virus was readily recovered with negligible temperature-sensitive helper virus contamination. This reverse genetic method was used successfully to generate a panel of viruses with chimeric NSP2 genes (Trask *et al.*, 2010).

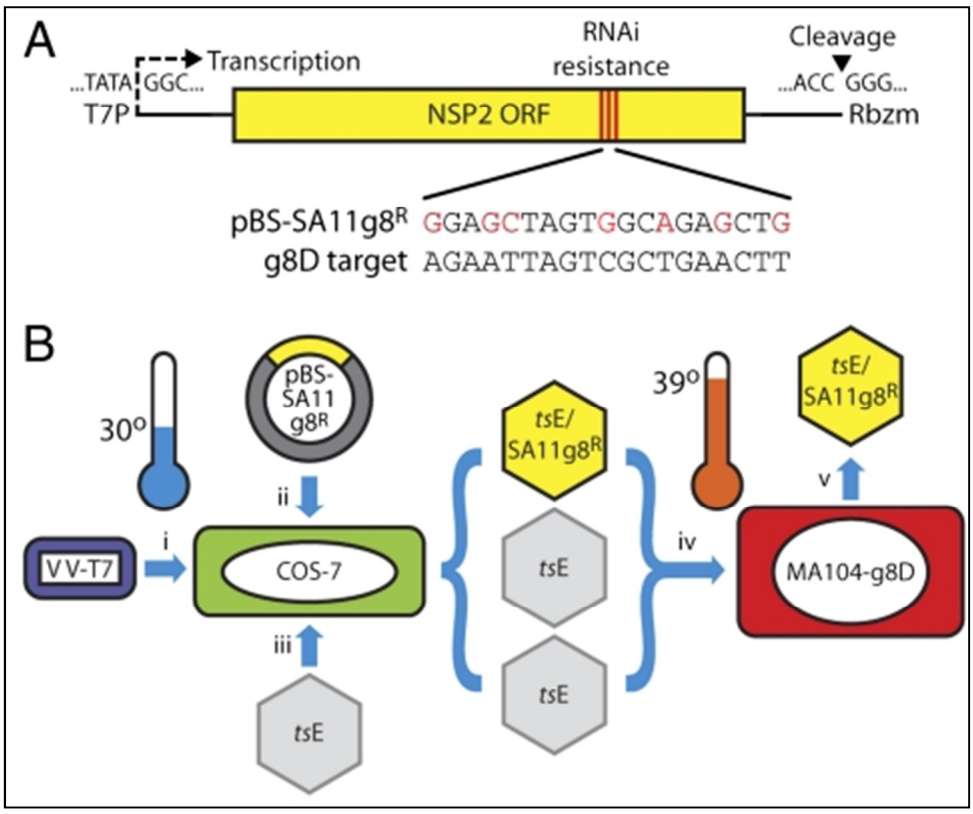


Fig. 1.4: Generation and recovery of NSP2 gene recombinant rotavirus using dual selection (Trask *et al.*, 2010). **(A)** Diagram of NSP2 gene cDNA. A T7 RNA polymerase promoter (T7P) initiates transcription of SA11 NSP2 gene mRNA with a native 5'-terminal sequence. The g8D RNAi target site is modified so that T7 transcripts escape RNAi-mediated cleavage. Autocatalytic cleavage of a 3' HDV ribozyme sequence equips the transcripts with a native 3'-terminal sequence. **(B)** Schematic representation of the reverse genetic method. COS-7 cells are infected with vaccinia virus expressing T7 RNA polymerase (VV-T7) *(i)* and transfected with the recombinant NSP2 gene plasmid (pBS-SA11g8^R) *(ii)* before infection with the *t*emperature-*s*ensitive helper virus (*ts*E) at 30°C *(iii)*. Progeny viruses from COS-7 cell lysates, which contain *ts*E helper viruses and recombinant *ts*E/SA11g8^R viruses, are passaged at 39°C in the *ts*E NSP2-specific shRNA-expressing cell line MA104-g8D *(iv)*, leading to the selection and isolation of recombinant *ts*E/SA11g8^R viruses *(v)*.

1.6 REVERSE GENETIC ENGINEERING OF VIRAL GENOMES

Prior to the development of reverse genetic systems for animal RNA viruses, forward genetics has typically been used. Forward genetics refers to the Darwinian method whereby viral mutants are firstly selected and only then characterised using further genetic studies (Kobayashi *et al.*, 2007). In contrast, reverse genetic systems allow for genetic engineering of specific viral genes, followed by investigations regarding the effect of the engineered mutation. Therefore, there is no longer a need to select mutants in order to study gene function, but rather the gene of interest can be specifically targeted and its function revealed

through subsequent investigations. Reverse genetic systems based on the use of cDNA clones have been established for DNA viruses (Goff and Berg, 1976), RNA viruses with positive-strand genomes, *e.g.* bacteriophage Qβ (Taniguchi *et al.*, 1978) and poliovirus (Racaniello and Baltimore, 1981), as well as RNA viruses with negative-strand RNA genomes, *e.g.* measles virus (Radecke *et al.*, 1995) and influenza A virus (Bridgen and Elliot, 1996). However, AHSV and many other members of the *Reoviridae*, which possess segmented dsRNA genomes, have remained stubbornly refractory to the development of methods for the rescue of infectious virus from cloned nucleic acid (reverse genetics). Indeed, it is only recently that selected members of the *Reoviridae* have been made amenable to reverse genetic manipulation.

1.6.1 Reverse genetic manipulation of RNA viruses with segmented genomes

Influenza virus has a segmented, negative-strand RNA genome and has long been used as the prototype virus for the development of reverse genetic systems for viruses containing segmented RNA genomes (Bridgen and Elliot, 1996; Hoffmann *et al.*, 2000; Neumann *et al.*, 2005). As a result, many of the techniques devised for influenza viruses have applications for members of the *Reoviridae* family. The development of methods capable of manipulating the genomic segments of influenza viruses has allowed the construction of recombinant viruses expressing a variety of foreign genes (Krammer *et al.*, 2010) and gene-silencing constructs (Varble *et al.*, 2010), as well as the elucidation of viral gene function (Vreede and Fodor, 2010) and vaccine production (O'Neill and Donis, 2009).

Negative-strand RNA viruses differ from positive-strand RNA viruses in that their genomic RNA is not infectious and they must therefore deliver their own RNA-dependant RNA polymerase into the infected cell to initiate viral mRNA synthesis. Initially, genetically engineered influenza virus was obtained through the *in vitro* reconstitution of biologically active ribonucleoprotein complexes (RNPs) and their subsequent transfection into helper virus-infected cells (Luytjes *et al.*, 1989; Enami *et al.*, 1990). The helper virus provides the viral proteins needed for the transcription of the synthetic RNP complex, which is comprised of synthetic RNA and purified nucleoprotein and polymerase proteins. Inclusion of a synthetic segment into the genome of the helper virus by reassortment was then selected for by host range, temperature sensitivity or neutralising antibodies.

Subsequent refinements to the system involved the intracellular reconstitution of RNP complexes, the components of which were expressed from plasmids (Pleschka *et al.*, 1996). This circumvented the need to purify viral proteins and the complicated process of transfecting RNPs. The system comprised of a plasmid encoding the synthetic gene driven by a human DNA-dependant RNA polymerase I (Pol I) promoter and terminated by a hepatitis delta virus (HDV) ribozyme, leading to transcripts with native 5' and 3' influenza viral sequences. Additionally, the Pol I-driven plasmid was co-transfected into human 293 cells with plasmids expressing the influenza virus polymerase subunits PB1, PB2 and PA, and the influenza virus nucleoprotein (NP) under the control of human DNA-dependant RNA polymerase II (Pol II). This system, however, still relied on the use of helper virus and therefore a powerful selection system, such as neutralising monoclonal antibodies (N-MAbs), were required for elimination of helper virus.

Contemporary reverse genetic systems for influenza viruses do not require helper virus, but only cDNA clones of all the viral genomic segments. Transfection of eight plasmids driven by a Pol I promoter, each encoding a viral RNA (vRNA) genomic segment, and four plasmids expressing the functional components of RNP complexes, under the control of a Pol II promoter, resulted in the rescue of infectious virus (Fodor *et al.*, 1999; Neumann *et al.*, 1999). Subsequently, a bidirectional reverse genetic system was developed for influenza viruses (Hoffmann *et al.*, 2000) (Fig. 1.5). In this system, cDNA encoding negative-strand vRNA is inserted between a Pol I promoter and terminator sequence, while the positive-strand of the same cDNA clone is inserted between a Pol II promoter and polyadenylation site. This reduces the number of plasmids needed to eight, as mRNA and vRNA for each segment are produced from the same cDNA clone. Recent improvements to the unidirectional system have involved the cloning of up to eight Pol I transcription cassettes (one for each genome segment) into a single plasmid, allowing virus to be rescued from cell lines that are difficult to transfect (Neumann *et al.*, 2005).

Due to the limited number of mammalian cell lines that recognise human Pol I and Pol II promoters, a unidirectional T7 RNA polymerase-based reverse genetic system has been described for recovery of influenza in avian cell lines (de Wit *et al.*, 2007). During influenza virus infection, vRNA and mRNA synthesis takes place in the nucleus of infected cells. Consequently, T7 polymerase with a nuclear-localisation signal was provided from a eukaryotic expression vector to enable nuclear transcription of influenza cDNA.

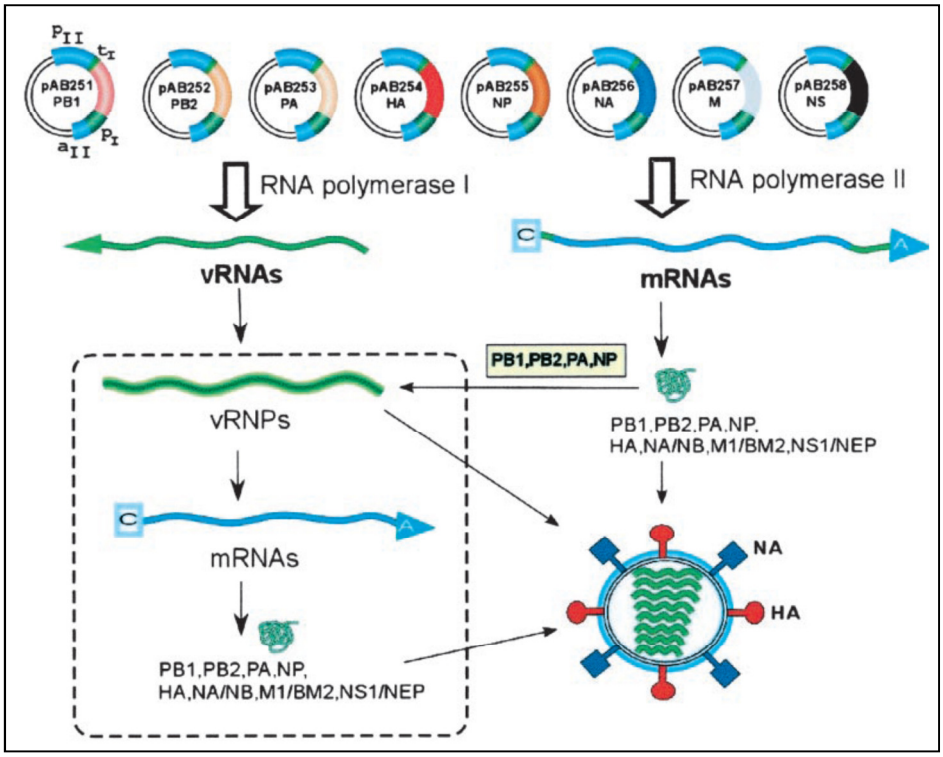


Fig. 1.5: A schematic representation of an eight-plasmid bidirectional reverse genetic system for influenza virus recovery (Hoffmann *et al.*, 2000). The system constitutes the transfection of eight recombinant plasmids, harbouring cDNA representing each of the eight gene segments of influenza virus. In each plasmid, gene segment cDNA is flanked by Pol I (P_I) promoter/terminator (t_I) sequences. The RNA Pol I transcription cassette is flanked by Pol II (P_{II}) promoter/polyadenylation (a_I) sequences. Following transfection of the plasmids into COS-7 cells, cellular Pol I synthesises virus-like negative-strand vRNAs and cellular Pol II synthesises capped mRNAs encoding the viral proteins. After the translation of RNP protein constituents (PA, PB1, PB2 and NP), the eight negative-strand vRNAs are transcribed and replicated. Ultimately, RNPs and structural proteins produced from cellular transcription or viral amplification (dashed square) are assembled into progeny virions.

1.6.2 Rotavirus reverse genetics

A reverse genetic system was developed for rotavirus (Komoto *et al.*, 2006), based on procedures originally described for influenza viruses (Luytjes *et al.*, 1989; Enami *et al.*, 1990; Enami and Palese, 1991). This system relies on the *in vivo* transcription of a cDNA gene fragment to yield a positive-strand transcript that corresponds to the native viral mRNA, a helper virus that provides the RNA-dependent RNA-polymerase required for recognition and replication of the viral mRNA, and a strong selection mechanism to recover transfectant virus containing the cDNA-derived gene.

In establishing the helper virus-driven reverse genetic system, Komoto *et al.* (2006) constructed a recombinant plasmid that harboured a full-length cDNA copy of the VP4 gene segment of the simian rotavirus strain SA11. The cDNA fragment was flanked by an upstream T7 RNA polymerase promoter and by a downstream HDV ribozyme sequence, followed by a T7 RNA polymerase terminator. Thus, transcription of the cloned cDNA fragment, followed by autocatalytic cleavage, yields a transcript with 5' and 3' ends that correspond to the ends of authentic SA11 VP4 mRNA. The recombinant plasmid DNA was transfected into COS-7 cells that had been infected with a recombinant vaccinia virus expressing T7 RNA polymerase, after which the transfected cells were infected with the human rotavirus strain KU as the helper virus. To select for a transfectant virus that contains the SA11 cDNA-derived VP4 segment in the background of KU helper viruses, the culture supernatant was passaged in MA104 cells in the presence of N-MAbs against the VP4 protein of the KU helper virus. This selection strategy therefore results in elimination of KU viruses, without affecting the replication of viruses with VP4 of the SA11 strain. Although the efficiency of this approach was exceptionally low (one transfectant virus per three transfections on 60-mm plates), viruses containing copies of the SA11 VP4 segment were nevertheless recovered. This low efficiency was attributed to the strict control under which genomic segment packaging occurs in *Reoviridae*, in addition to the limited localisation of the synthesised mRNA transcript to the viroplasm where virus replication and assembly occurs (Silvestri *et al.*, 2004). In addition to the rescued transfectant virus with the authentic SA11 VP4 gene, infectious transfectant viruses containing site-specific mutations in the VP4 gene were also rescued using this approach, indicating that it is possible to manipulate the rotavirus genome at the DNA level (Komoto *et al.*, 2006). It should be noted, however, that this system appears to be restricted to segments encoding antigenically distinct viral surface proteins (like VP4), because the selection of transfectant viruses requires the use of specific and potent neutralising antibodies to eliminate the helper virus.

Recently, two different approaches have been published whereby recombinant rotaviruses can be produced that contain a cDNA-derived genome segment encoding for modified nonstructural proteins. Both approaches rely on the same mechanism of viral genome segment introduction to that described by Komoto *et al.* (2006). As discussed previously (Section 1.5.3), the first of the approaches relies on the use of a dual selection mechanism, *i.e.* growth temperature and RNAi, to recover recombinant rotaviruses in which replacements of the NSP2 gene have been engineered (Trask *et al.,* 2010). An alternative approach is based on

the observation that in group A rotaviruses an RNA segment is sometimes replaced by a rearranged RNA segment, which is derived from its counterpart by partial sequence duplication (Desselberger, 1996). Based on the preferential packaging of rearranged RNA segments, Troupin *et al.* (2010) reported a reverse genetic system for rotavirus. Using this system, wild-type or *in vitro*-engineered forms of rearranged segment 7 from a human rotavirus, encoding the NSP3 protein, derived from cloned cDNAs and transcribed in the cytoplasm of COS-7 cells with the help of T7 RNA polymerase, were shown to replace the wild-type segment of a bovine helper virus strain. The recombinant rotaviruses were recovered by propagating the viral progeny in MA104 cells, with no need for additional selective pressure. However, extensive passaging at a high multiplicity of infection (MOI) was required to recover recombinant rotavirus. The usefulness of this approach has been questioned, as the requirement for an elongated recombinant gene results in a genetically abnormal and potentially unstable rotavirus (Trask *et al.*, 2010). Moreover, the system might not be applicable to helper viruses that grow poorly in cell culture or to genome segments for which rearrangements have not yet been identified in viable rotaviruses (Troupin *et al.*, 2010).

1.6.3 Orthoreovirus reverse genetics

Orthoreovirus was the first member of the *Reoviridae* family to be made amenable to reverse genetics, when a method detailing the replacement of the s2 genome segment, encoding the σ2 protein, with a chimeric s2-CAT reporter gene was described (Roner and Joklik, 2001). The method represents a modification of an infectious RNA system previously established by Roner *et al.* (1990). The basic features of the infectious reovirus RNA system include transfection of L929 mouse fibroblast cells with core-derived viral mRNA and viral dsRNA, as well as with a rabbit reticulocyte lysate in which ssRNA or melted dsRNA had been translated, followed by infection of the transfected cells with a helper virus of a different serotype. To allow for the incorporation of the s2-CAT gene into the reovirus genome, the basic system was modified as follows. The wild-type s2 RNA was removed from the mixture of 10 ssRNA species by RNase H degradation, following hybridisation of the s2 RNA with a complementary DNA oligonucleotide. The set of nine ssRNAs was then supplemented with an *in vitro*-synthesised s2-CAT transcript and the mixture was transfected into L929 cells, engineered to constitutively express the wild-type σ2 protein, together with a rabbit reticulocyte lysate in which the ssRNA mixture had been translated. Using this approach, *ca.*

50% of the recovered viruses displayed CAT activity. Although this method has shown some success for the study of individual genome segments such as s2 (Roner and Joklik, 2001; Roner *et al.*, 2004), m1 (Roner and Steele, 2007) and l1 (Roner and Steele, 2007), its use is limited due to its highly complicated nature.

More recently, a helper virus-independent reverse genetic system, based entirely on the use of recombinant plasmid DNA constructs harbouring cloned cDNA copies of each genome segment, has been developed for mammalian orthoreovirus (Kobayashi *et al.*, 2007). The principle behind this reverse genetic system is based on transcription of full-length viral mRNAs from plasmids encoding each of the 10 viral gene segments (Fig. 1.6). The viral mRNAs are transcribed from T7 RNA polymerase promoters directly upstream of the cloned fragments and equipped with native 3' ends through the autocatalytic cleavage by an HDV ribozyme sequence, directly downstream of the cloned segments. This transcription strategy consequently yields 10 mRNA species, each of which possesses the native reovirus terminal sequences. The 10 recombinant plasmids were subsequently transfected into murine fibroblast cells, which had been infected with a recombinant vaccinia virus expressing T7 RNA polymerase, thereby resulting in cytoplasmic transcription. This reverse genetic approach generated high titres (10^4-10^6 pfu/ml) of progeny virus from cell culture supernatants. The above system has already been used successfully to study several aspects of reovirus infection, including the role of outer capsid proteins σ1 and σ3 during attachment to the JAM-A host cell receptor (Kobayashi *et al.*, 2007; Kirchner *et al.*, 2008) and the roles of μ2 and μ3 in reovirus cell tropism (Ooms *et al.*, 2010).

The above reverse genetic system was recently refined (Kobayashi *et al.*, 2010). One of the limitations of the previous reverse genetic system was that transfection of the 10 plasmid DNAs was inefficient. This was subsequently overcome by constructing recombinant plasmids that contained two or four transcriptional cassettes, thereby effectively reducing the number of plasmids from 10 to four. Moreover, virus recovery was conducted in BHK-21 cell monolayers that had been engineered to express T7 RNA polymerase, negating the requirement for a recombinant vaccinia virus. Compared to the previous system, this four-plasmid system was reported to result in increased virus recovery (at least 10-fold higher) by three mechanisms. Firstly, there are fewer plasmids that need to be transfected into each cell, and thus there is a higher probability that a full set of viral RNA will accumulate in a cell for replication initiation. Secondly, by decreasing the number of plasmids the probability that a

cell contains a sufficient number of intact transcripts is higher. Thirdly, the constriction of transcription and translation activities to the same micro-environment would greatly facilitate protein-protein and protein-RNA interactions required for virus recovery (Kobayashi *et al.*, 2010).

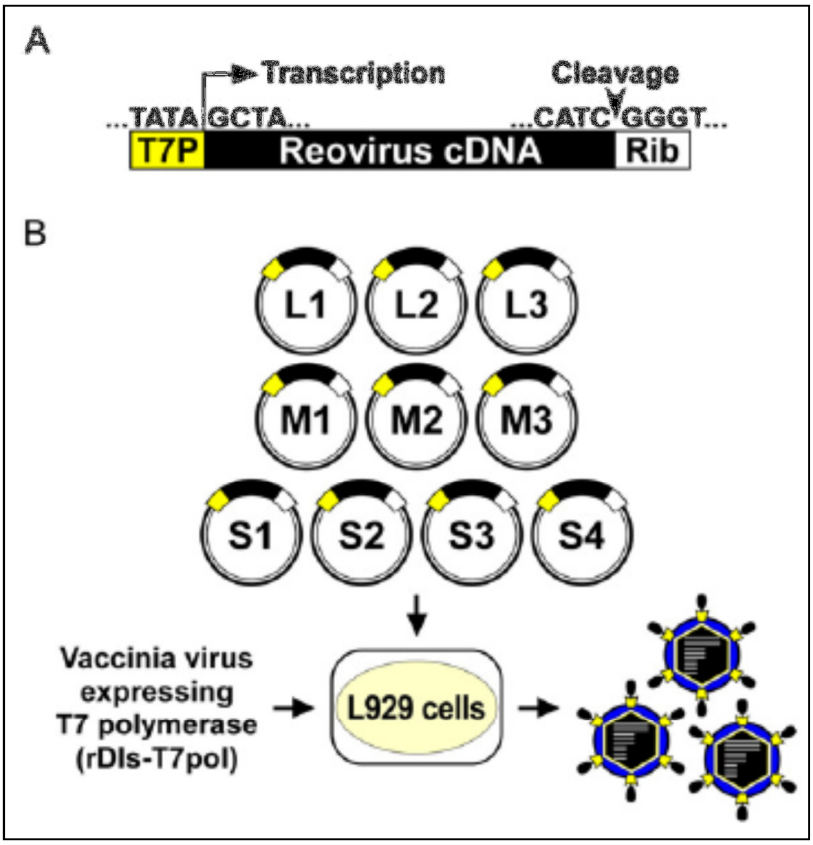


Fig. 1.6: Schematic representation of the helper virus-independent plasmid-based reverse genetic system for the recovery of orthoreovirus (Kobayashi *et al.*, 2007). **(A)** In each recombinant plasmid, cytoplasmic transcription of reovirus gene segment cDNA is driven by an upstream T7 RNA polymerase promoter (T7P), producing gene segment transcripts with a native 5'-terminal sequence. The T7 transcripts are equipped with native 3'-terminal sequences through the autocatalytic cleavage of an HDV ribozyme (Rib) sequence. **(B)** To generate reovirus, ten recombinant plasmids, each harbouring a reovirus gene segment transcription cassette, are transfected into L929 cells that have been infected with recombinant vaccinia virus expressing T7 RNA polymerase. Cytoplasmic transcription of the recombinant plasmid inserts yields positive-strand transcripts capable of acting as a template for reovirus protein synthesis and genome replication, resulting in the recovery of infectious reovirus.

1.6.4 Bluetongue virus reverse genetics

A major problem that hampered the development of a reverse genetic system for orbiviruses was the pervading belief that *in vitro*-transcribed viral RNA is not infectious when transfected into permissive mammalian cells. Nevertheless, Boyce and Roy (2007) reported that BTV mRNA, synthesised *in vitro* using purified core particles, were infectious and indeed led to the recovery of viable progeny virus after being transfected into permissive BSR cells. This discovery was soon followed by a report in which an RNA-based reverse genetic system for BTV was described (Boyce *et al.*, 2008).

The ensuing reverse genetic system makes use of T7 cDNA clones to produce synthetic mimics of BTV mRNA segments *in vitro*. The transcription cassette in each cDNA clone contains a T7 RNA polymerase promoter and a unique restriction enzyme site, with the BTV genome segment located between these elements (Fig. 1.7). The BTV genome segment in each clone was positioned relative to the other two sequence elements such that the T7 transcript derived from plasmid DNA linearised with the restriction enzymes was predicted to have exactly the same sequence as the mRNA strand of the corresponding BTV genome segment. The run-off T7 transcripts obtained by *in vitro* transcription reactions, using the linearised plasmid cDNA clones, were capped on the 5'-terminal with a commercially available cap analogue. The transcripts therefore resembled the native BTV mRNAs that are generated during infection. The T7 transcripts were subsequently transfected into BSR cell monolayers and led to the recovery of high titres of viable progeny virus (1-3 × 10^7 pfu/ml) that were shown to be morphologically indistinguishable from wild-type BTV (Boyce *et al.*, 2008). In addition, it was also shown that it is possible to introduce targeted mutations into selected genome segments. The introduction of a silent mutation in the cDNA clone of segment 8, encoding NS2, resulted in an altered restriction enzyme digestion profile in progeny virus, confirming that it is possible to engineer viable mutations into BTV segments using this reverse genetic system (Boyce *et al.*, 2008). The developed reverse genetic system has subsequently been used to confirm the interaction of NS3 with the outer capsid protein VP2 and the cellular Tsg101 protein (Celma and Roy, 2009), as well as to confirm the essential role played by VP6 during BTV replication (Matsuo and Roy, 2009).

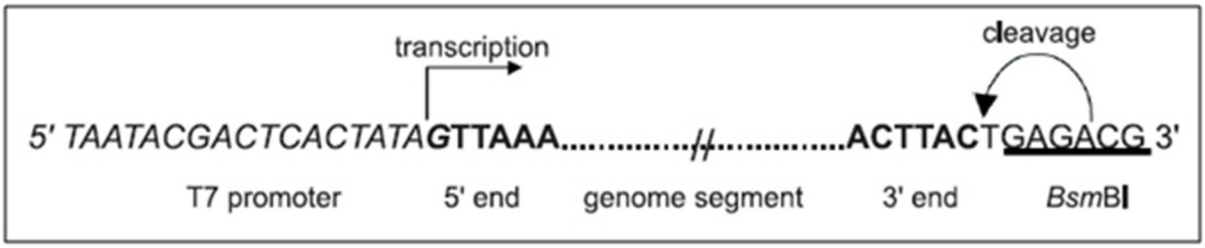


Fig. 1.7: A reverse genetic system for the recovery of BTV using T7 transcripts produced from BTV genome segment cDNA clones (Boyce *et al.*, 2008). The system uses 10 recombinant plasmids, each containing a BTV genome segment cDNA, which is flanked by a T7 RNA polymerase promoter and restriction endonuclease cut site. *In vitro* transcription of each linearised recombinant plasmid yields BTV gene segment transcripts with native 5'- and 3'-terminal sequences. BSR cells are then transfected with the T7 transcripts, which act as templates for BTV protein production and genome synthesis, resulting in the recovery of infectious BTV.

This system has been refined through the inclusion of a double transfection step, which was shown to significantly increase virus recovery (Matsuo and Roy, 2009). For this purpose, BSR cells were first transfected with T7 transcripts of BTV genome segments encoding proteins believed to be associated with primary replication events, namely VP1 (L1), VP4 (M1), VP6 (S3), VP3 (L3), NS1 (M2) and NS2 (S2). At 18 h post-transfection, the cell monolayers were transfected for a second time with T7 transcripts corresponding to all 10 genome segments. It was reported that, compared to a single-transfection rescue experiment, the inclusion of an initial transfection with six primary replication transcripts led to a *ca.* 50-fold increase in the number of rescue events (one to three plaques following a single transfection of all ten segments compared to 68 to 80 plaques using the double-transfection procedure) (Matsuo and Roy, 2009).

1.7 AIMS OF THE STUDY

From the review of the literature, it is evident that many aspects regarding AHSV replication, morphogenesis and egress, as well as the role of individual proteins in these processes still need to be elucidated. Studies regarding structure-function relationships of different AHSV genes and encoded gene products have benefited greatly from gene cloning, genetic engineering and protein expression technologies, whereas RNAi technologies have facilitated investigations regarding virus gene function in the context of virus-infected cells. However, it can be envisaged that studies on AHSV would benefit significantly from the availability of a

reverse genetic system that would allow for genetic manipulation of the virus. Indeed, the successful establishment of reverse genetic systems for most positive- and negative-strand RNA viruses, which allows for manipulation of viral genes at the cDNA level followed by rescue of infectious virus, have provided a better understanding, amongst others, of RNA virus gene function and replication (Walpita and Flick, 2005; Pasternak *et al.*, 2006). In the case of *Reoviridae*, the development of reverse genetic systems has been hampered by the nature of the genome, which is comprised of 10 to 12 dsRNA segments that are densely packed within the viral particle and transcribed and replicated within a subviral structure (Chandran and Nibert, 2003; Mertens and Diprose, 2004; Patton *et al.*, 2004). In recent years, the introduction of plasmid-derived sequences into the dsRNA genomes of various members of the *Reoviridae* has become possible, notably for orthoreovirus (Roner and Joklik, 2001) and, to a limited extent, for rotaviruses (Komoto *et al.*, 2006; Troupin *et al.*, 2010; Trask *et al.*, 2010). Recently, helper virus-independent reverse genetic systems have been established for mammalian orthoreovirus and BTV, an orbivirus related to AHSV. The strategies are based on the transfection of a complete set of plasmid cDNAs or plasmid cDNA-derived mRNAs that allow for the rescue of recombinant infectious virus (Kobayashi *et al.*, 2007, 2010; Boyce *et al.*, 2008). This therefore suggests that the development and establishment of a reverse genetic system for AHSV is tractable. Consequently, the aims of this study where the following:

- To determine whether AHSV core-derived ssRNA is infectious when transfected into permissive mammalian cell cultures.

- To evaluate different reverse genetic approaches, based on transfection of plasmid cDNA clones, T7 transcription cassettes and T7 transcripts, for their ability to rescue infectious AHSV in mammalian cell culture.

- To develop a methodology whereby single-gene replacements can be introduced into the AHSV genome.

CHAPTER TWO

RECOVERY OF INFECTIOUS AFRICAN HORSE SICKNESS VIRUS FROM CORE-DERIVED TRANSCRIPTS

2.1 INTRODUCTION

African horse sickness virus (AHSV), a member of the genus *Orbivirus* within the *Reoviridae* family, is structurally related to bluetongue virus (BTV). Like BTV, the prototype orbivirus, AHSV virion particles consist of two concentric layers of proteins that are organised into capsids, *i.e.* an outer capsid of two proteins (VP2 and VP5), and an inner capsid or core. The core is composed of two major proteins, VP3 and VP7, which enclose the three minor proteins VP1, VP4 and VP6, in addition to the viral genome of 10 double-stranded (ds) RNA segments (Verwoerd and Huismans, 1972; Bremer *et al.*, 1990; Roy *et al.*, 1994).

During the early stage of orbivirus replication, shortly after cell entry, the outer capsid proteins are removed and a transcriptionally active core particle is released into the host cell cytoplasm (Hassan *et al.*, 2001; Forzan *et al.*, 2007). The released uncoated core particle is a highly stable structure, which provides a robust compartment for the viral genome that remains within the core throughout the infection cycle. Since the dsRNA genome of the virus is therefore never freely distributed in the cytosol, this replication strategy minimises the induction of the host interferon response (Roy, 2005). Thus, the core is the endpoint of virus disassembly within which the 10 dsRNA genome segments can repeatedly be transcribed by the core-associated enzymes, resulting in the extrusion of the newly synthesised capped and methylated mRNA (Mertens and Diprose, 2004). The extruded transcripts, in turn, function as templates for the synthesis of viral structural and nonstructural proteins, and also act as templates for the synthesis of genomic RNA following their encapsidation in progeny viral cores (Patton and Spencer, 2000; Mertens and Diprose, 2004). *In vitro* BTV mRNA synthesis can be activated by proteolytic degradation of the outer capsid of the virus (van Dijk and Huismans, 1980, 1988). The structural integrity of the core particle appears to be essential for maintaining an efficient transcriptional activity which, like other members of the *Reoviridae* family, occurs by a highly conserved process that requires a series of enzyme activities encoded by VP1 (RNA-dependant RNA polymerase), VP4 (capping-enzyme) and VP6 (helicase) (Boyce *et al.*, 2004; Ramadevi *et al.*, 1998; Stauber *et al.*, 1997).

Despite being able to synthesise BTV transcripts from purified core particles *in vitro*, it has long been believed that the viral transcripts are non-infectious. Indeed, this belief appeared to be reaffirmed when Roner and Joklik (2001) reported that ssRNA of orthoreovirus was not infectious in L929 cells. Recently, it was reported that it is possible to recover infectious

BTV wholly from ssRNA synthesised *in vitro* using BTV core particles (Boyce and Roy, 2007). This discovery was soon followed by a report describing the establishment of a reverse genetic system for BTV, based entirely on the use of synthetic T7-derived ssRNA transcripts (Boyce *et al.*, 2008). Towards the long-term goal of establishing a reverse genetic system for AHSV, the aim of this part of the study was to determine whether AHSV ssRNA could likewise lead to the recovery of infectious virus. Moreover, the efficiency by which infectious AHSV could be rescued in different mammalian cell lines was also assessed.

2.2 MATERIALS AND METHODS

2.2.1 Cell cultures and virus

Baby hamster kidney-21 (BHK-21; ATCC CL-10), Vero (ATCC CL-81) and BSR (a clone of BHK-21) cells were propagated and maintained as monolayers in 75 cm^2 tissue culture flasks. The cells were cultured in Eagle's Minimal Essential Medium (EMEM) with Earle's Balanced Salt Solution (EBSS) and L-glutamine (Hyclone), supplemented with 5% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA) and antibiotics (10 000 U/ml of penicillin, 10 000 µg/ml of streptomycin and 25 µg/ml of amphotericin B) (Hyclone). The flasks were incubated at 37°C in a humidified incubator with a constant supply of 5% CO_2. AHSV-4 (isolate HS32/62; 10S-10BHK-3LP-5Vero-1BHK) was provided by Dr. A. C. Potgieter (Deltamune, Pretoria). AHSV-4 was propagated in confluent BSR monolayers using a low-passage stock virus as inoculum. For infections, BSR monolayers were rinsed with serum-free EMEM medium and then infected with AHSV-4 at a multiplicity of infection (MOI) of 1 pfu/cell. Virus infection was performed at room temperature for 1 h, followed by incubation of the cell monolayers in complete EMEM medium for 4 days. The titres of virus stocks were determined on BSR cells according to the method described by Oellermann (1970).

2.2.2 Purification of AHSV-4 cores

BSR cell monolayers (1.5×10^7 cells/150 cm^2 flask; 36 flasks in total) were infected with AHSV-4 at a MOI of 0.08 pfu/cell and incubated at 37°C in a CO_2 incubator. At 72 h post-infection, the cells were harvested from the surface of the tissue culture flasks, collected by centrifugation at 3 000 × *g* at 4°C for 15 min and washed twice in chilled 1 × phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.2H_2O$, 1.4 mM

KH_2PO_4; pH 7.4). AHSV-4 cores were then purified using a modification of the method described by Boyce and Roy (2007). The AHSV-4 infected cells were lysed by incubation on ice for 30 min in 7 ml chilled 100 mM Tris-HCl (pH 8.8), 50 mM NaCl, 10 mM EDTA, 0.5% (v/v) NP-40. To ensure lysis, the cells were passed 10 times through a 28G hypodermic needle fitted to a 20-ml syringe. Nuclei were removed by centrifugation at 1000 × *g* at 4°C for 15 min. To remove the outer capsid proteins of virions, α-chymotrypsin (Sigma-Aldrich) was added to the cytoplasmic extract to a final concentration of 60 µg/ml, and incubated at 37°C for 1 h. Following incubation, *N*-lauroyl sarcosine (Merck) was added to the cytoplasmic extract to a final concentration of 0.2% (w/v) and the sample was incubated at 25°C for 1 h. Thereafter, 4 ml of the cytoplasmic extract was loaded onto a 1 ml sucrose cushion (40% [w/v] sucrose prepared in 600 mM $MgCl_2$, 20 mM Tris-HCl [pH 8.0]) and subjected to ultracentrifugation at 141 000 × *g* at 20°C for 2 h in a Beckman Coulter Optima™ L-100 XP ultracentrifuge using an SW 55Ti swing-bucket rotor. The pellet, consisting of partially purified AHSV-4 core particles, was suspended in 80 µl of 20 mM Tris-HCl (pH 8.0) and stored at 4°C.

2.2.3 Characterisation of AHSV-4 cores

2.2.3.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

An aliquot of the AHSV-4 core sample (12 µl) was mixed with an equal volume of 2 × protein solvent buffer (PSB; 125 mM Tris [pH 6.8], 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol, 0.06% [w/v] bromophenol blue). The samples were heated for 5 min in boiling water prior to SDS-PAGE. Proteins in partially purified cores were subsequently resolved by electrophoresis in a discontinuous gel system, as described by Laemmli (1970). A 5% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide separating gel was used, of which the acrylamide:bisacrylamide ratio was 30:0.8. The low porosity separating gel (0.375 mM Tris-HCl [pH 8.8], 0.1% [w/v] SDS) and high porosity stacking gel (0.125 M Tris-HCl [pH 6.8], 0.1% [w/v] SDS) were each polymerised by addition of 0.08% (w/v) ammonium persulphate and 10 µl of TEMED. Electrophoresis was performed in a Hoefer miniVE™ electrophoresis unit at 100 V for 3 h in 1 × TGS buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS; pH 8.3). Following electrophoresis, gels were stained for 20 min with 0.125% Coomassie brilliant blue (prepared in 50% methanol and 10% acetic acid) and then destained in a solution containing 25% methanol, 10% acetic acid. The sizes of the

resolved proteins were estimated by comparison to reference molecular mass proteins (Spectra™ Multicolor Broadrange Protein Ladder; Fermentas).

2.2.3.2 Transmission electron microscopy (TEM)

Aliquots of the partially purified AHSV-4 cores were absorbed onto 400-mesh Formvar- and carbon-coated copper grids for 90 s, rinsed with dH_2O, and then negatively stained with 2% (w/v) uranyl acetate. The grids were examined under a JEOL 2100F transmission electron microscope at 100 kV.

2.2.4 Preparation of AHSV-4 core-derived ssRNA

2.2.4.1 Synthesis of AHSV-4 ssRNA *in vitro*

AHSV-4 ssRNA was synthesised *in vitro*, using reaction conditions similar to those described by Boyce and Roy (2007), in reaction volumes of either 50 or 100 µl. Partially purified AHSV-4 cores (75 µg/ml) were incubated at 30°C for 6 h in AHSV core transcription buffer (100 mM Tris-HCl [pH 8.0], 4 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 500 µM *S*-adenosylmethionine, 6 mM DTT, 9 mM $MgCl_2$, 5 U/µl of Protector RNase inhibitor [Roche Diagnostics]).

2.2.4.2 Purification of AHSV ssRNA

Infectious AHSV-4 core particles were removed from the *in vitro* transcription reactions by centrifugation at 20 000 × *g* at 4°C for 30 min in an Eppendorf 5804R benchtop centrifuge. The supernatants were collected and centrifuged for a second time, as above, to ensure removal of residual core particles. The ssRNA was precipitated from the supernatants by addition of 8 M LiCl (Merck), to a final concentration of 2 M, followed by incubation at 4°C for 16 h. The samples were subsequently centrifuged at 17 000 × *g* at 4°C for 30 min and the pellets were suspended in 100 µl of DEPC-treated dH_2O. To ensure complete removal of contaminating proteins and dsRNA, the AHSV-4 ssRNA was purified with the Nucleospin® RNA Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. Briefly, 600 µl of lysis-binding buffer was added to the ssRNA samples and the resulting mixture passed through an RNA-binding column by centrifugation at 8 000 × *g* for 30 s. The columns, containing bound AHSV-4 ssRNA, were washed twice with membrane desalting buffer (supplied in the kit) and the ssRNA was eluted from the column with 60 µl of RNase-

free dH_2O. An aliquot of the purified AHSV ssRNA was mixed with an equal volume of denaturing RNA loading buffer (Fermentas) and analysed by electrophoresis on a 1% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5) (Sambrook *et al.*, 1989). The concentration and purity of the ssRNA was determined on a Nanodrop® ND-1000 spectrophotometer (Thermo Fischer Scientific). The samples were stored at -70°C until required.

2.2.5 Extraction of dsRNA from infected BSR cells

Total RNA was extracted from virus-infected cells using the Aurum™ Total RNA extraction kit (BioRad) according to the manufacturer's instructions. Briefly, the culture medium was removed and the cells were rinsed once with 1 × PBS. The cell monolayers were suspended in 350 µl of lysis buffer and an equal volume of 70% ethanol was added. The cells were lysed by vigorous pipeting before being centrifuged through a RNA-binding column at 20 000 × *g* for 30 s. The column was then rinsed with wash solution and treated for 15 min at room temperature with RNase-free DNase I to remove contaminating genomic DNA. The RNA was eluted from the column in 30 µl of the supplied elution buffer. To precipitate ssRNA, 8 M LiCl was added to a final concentration of 2 M and the samples were incubated at 4°C for 16 h. Following centrifugation at 17 000 × *g* at 4°C for 30 min, the supernatant was collected and the dsRNA was precipitated from the supernatant by addition of 0.1 volume of 3 M NaOAc and 2 volumes of absolute ethanol. The samples were centrifuged at 17 000 × *g* at 4°C for 30 min and the pellets were washed twice with 70% ethanol before being suspended in 20 µl of DEPC-treated dH_2O. The dsRNA was then analysed by electrophoresis on a 1% (w/v) agarose gel.

2.2.6 Transfection of BSR cells with purified AHSV ssRNA, dsRNA or AHSV cores

BSR cells were seeded 4-6 h preceding transfection in 24-well tissue culture plates at a confluency of 80% in antibiotic-free EMEM medium, which had been supplemented with 5% (v/v) FBS. Monolayers were then transfected with AHSV-4 ssRNA using Lipofectamine™ 2000 reagent (Invitrogen) according to the double-transfection protocol, as described by Matsuo and Roy (2009). Briefly, 800 ng of purified AHSV-4 ssRNA and 2 µl of Lipofectamine™ 2000 reagent were each diluted in 50 µl of serum- and antibiotic-free EMEM medium, incubated at room temperature for 5 min and then mixed to allow the formation of RNA-lipofectamine complexes. Following incubation at room temperature for 20 min, the

solutions were added drop-wise to the BSR cell monolayers and the tissue culture plates were incubated at 37°C in a CO_2 incubator. A second transfection, identical to the first, was performed on the monolayers 16 h subsequent to the first, followed by incubation at 37°C for 72 h.

As controls, BSR cell monolayers were also transfected, as above, with 800 ng of AHSV-4 dsRNA and 100 ng of AHSV-4 cores, as well as with 800 ng of AHSV-4 ssRNA, 800 ng of dsRNA and 100 ng of AHSV-4 cores digested with either Proteinase K or RNase A. Digestions were performed at 37°C for 1 h using 100 µg/ml Proteinase K or 50 µg/ml RNase A. The Proteinase K-digested samples were heated to 65°C for 15 min in order to denature the enzyme.

2.2.7 Plaque assays

At 72 h post-second transfection, the BSR cells were harvested in the culture medium, transferred to Eppendorf tubes and subjected to three successive freeze-thaw cycles to lyse the cells. Ten-fold dilutions of cell lysates were subsequently used to infect confluent BSR cell monolayers in 6-well tissue culture plates. Following incubation at 37°C for 1 h, the inoculums were aspirated and the cells were overlaid with a 0.5% (w/v) agarose solution (Seakem LE agarose; prepared in complete EMEM medium). The tissue culture plates were subsequently incubated at 37°C for 96 h in a CO_2 incubator. To visualise plaques, the cell monolayers were flooded with 500 µl of Neutral Red (0.05% [w/v] in 1 × PBS) and incubated at 37°C for a further 48 h, after which the Neutral Red solution was aspirated. Individual plaques were selected randomly, and each plaque was plucked as an agarose plug with a pipette and placed in an Eppendorf tube containing 100 µl of complete EMEM medium. Virus was eluted from the agarose plugs by incubation at 37°C for 24 h. Individual wells of a 6-well tissue culture plate, each containing a confluent BSR monolayer, were inoculated with a separate plaque pickup (100 µl) in a final volume of 1 ml of complete EMEM medium. Following incubation at 37°C for 4 days the cell monolayers were harvested and dsRNA was extracted, as described previously.

2.2.8 Immunoblot analysis

BSR cells, seeded at 80% confluency in 6-well tissue culture plates, were transfected with 2 µg of AHSV-4 ssRNA, as described above, except that 4 µl of Lipofectamine™ 2000 reagent,

diluted in 250 µl of serum- and antibiotic-free EMEM medium, was used. NS2 protein expression was analysed at 12-h intervals, over a time course of 72 h, using a guinea pig polyclonal antiserum raised against AHSV-9 NS2 (Uitenweerde *et al.*, 1995). Whole-cell lysates, prepared at each time interval, were resolved by SDS-PAGE and immunoblot analysis was performed by transferring the separated proteins on an unstained SDS-polyacrylamide gel to a Hybond™-C membrane (Amersham Biosciences). The nitrocellulose membrane, cut to the same size as the gel, was equilibrated in transfer buffer (25 mM Tris, 186 mM glycine) for 20 min prior to gel transfer. The proteins were electroblotted onto the membrane for 90 min at 28 V with a Mighty Small™ Transphor electroblotting apparatus (Hoefer). After transfer, the membrane was washed once in 1 × PBS for 5 min and nonspecific binding sites were blocked by incubating the membrane overnight in blocking solution (1% [w/v] fat-free milk powder in 1 × PBS). The membrane was then incubated at room temperature for 2 h with the anti-NS2 antibody (diluted 1:100 in 1 × PBS). Following incubation, the unbound primary antibodies were removed by washing the membrane three times for 5 min each in wash buffer (0.05% [v/v] Tween-20 in 1 × PBS). The secondary antibody, Protein A conjugated to horseradish peroxidase (Sigma-Aldrich) and diluted 1:1000 in 1 × PBS, was added to the membrane and then incubated at room temperature for 1 h. The membrane was washed three times for 5 min each in wash buffer and once for 5 min in 1 × PBS. To detect the NS2 protein, the membrane was immersed in freshly prepared enzyme substrate solution (60 mg 4-chloro-1-naphtol in 20 ml methanol and 60 µl hydrogen peroxide in 100 ml of 1 × PBS, mixed just before use). Once the bands became visible, the membrane was rinsed with dH_2O and air-dried.

2.2.9 Evaluation of different mammalian cell lines for recovery of infectious AHSV-4

BHK-21, BSR and Vero cells were seeded at 80% confluency in 24-well tissue culture plates and transfected twice with 800 ng of AHSV-4 ssRNA, as described above (Section 2.2.6). At 72 h post-second transfection, the cell monolayers were harvested, subjected to three freeze-thaw cycles and the viral titre determined using each of the respective cell-lines, *i.e.* BHK-21 cell lysates were titrated on BHK-21 cell monolayers, BSR cell lysates were titrated on BSR cell monolayers, and Vero cell lysates were titrated on Vero cell monolayers. For comparative purposes, each of the respective cell lines was infected with AHSV-4 at a MOI of 1 pfu/cell. The cell monolayers were harvested at 72 h post-infection and processed in an identical manner to the cell monolayers transfected with AHSV-4 ssRNA. Three independent

experiments, with two replicates each, were performed for both ssRNA-transfected and AHSV-4 infected cell lines.

2.3 RESULTS

2.3.1 Purification of AHSV-4 cores and *in vitro* synthesis of AHSV-4 ssRNA

It has been reported previously that conversion of BTV particles to core particles, either through centrifugation of purified virions on CsCl gradients at pH 7 (Verwoerd, 1970) or through the enzymatic removal of the outer capsid proteins with chymotrypsin (van Dijk and Huismans, 1980), yields cores that are transcriptionally active *in vitro*. More recently, it was shown that BTV core-derived ssRNA is infectious when transfected into permissive cells, leading to the recovery of infectious virus (Boyce and Roy, 2007). Whether the same holds true for AHSV was not known at the inception of this study.

Towards investigating the possibility that AHSV ssRNA is infectious, it was necessary to obtain AHSV cores that could then be used for the synthesis of full-length capped ssRNAs. For this purpose, AHSV-4 cores were purified from virus-infected BSR cells at 72 h post-infection, as described in Materials and Methods (Section 2.2.2). The protein composition of the core particles was analysed by SDS-PAGE. Analysis of the Coomassie-stained SDS-polyacrylamide gel indicated the presence of some contaminating cellular proteins present in the sample. Nevertheless, proteins of which the molecular mass corresponded to that of VP1 (150 kDa), VP3 (103 kDa) and VP4 (75 kDa) could be identified readily. The core proteins VP6 (38.4 kDa) and VP7 (37.9 kDa) co-migrated as a single band in the SDS-polyacrylamide gel (Fig. 2.1A). To confirm that the outer capsid proteins had been removed by α-chymotrypsin treatment, the AHSV-4 core particles were examined by negative-staining transmission electron microscopy. The results indicated the presence of large quantities of spherical particles, measuring *ca.* 65 nm in diameter (Fig. 2.1B). These particles displayed the characteristic capsomeres of orbivirus particles and were smaller in size than intact virion particles (*ca.* 80 nm in diameter) (Borden *et al.*, 1971; Kantor and Welch, 1976). Together, these results therefore indicated that AHSV-4 core particles, lacking the outer capsid proteins VP2 and VP5, were isolated successfully.

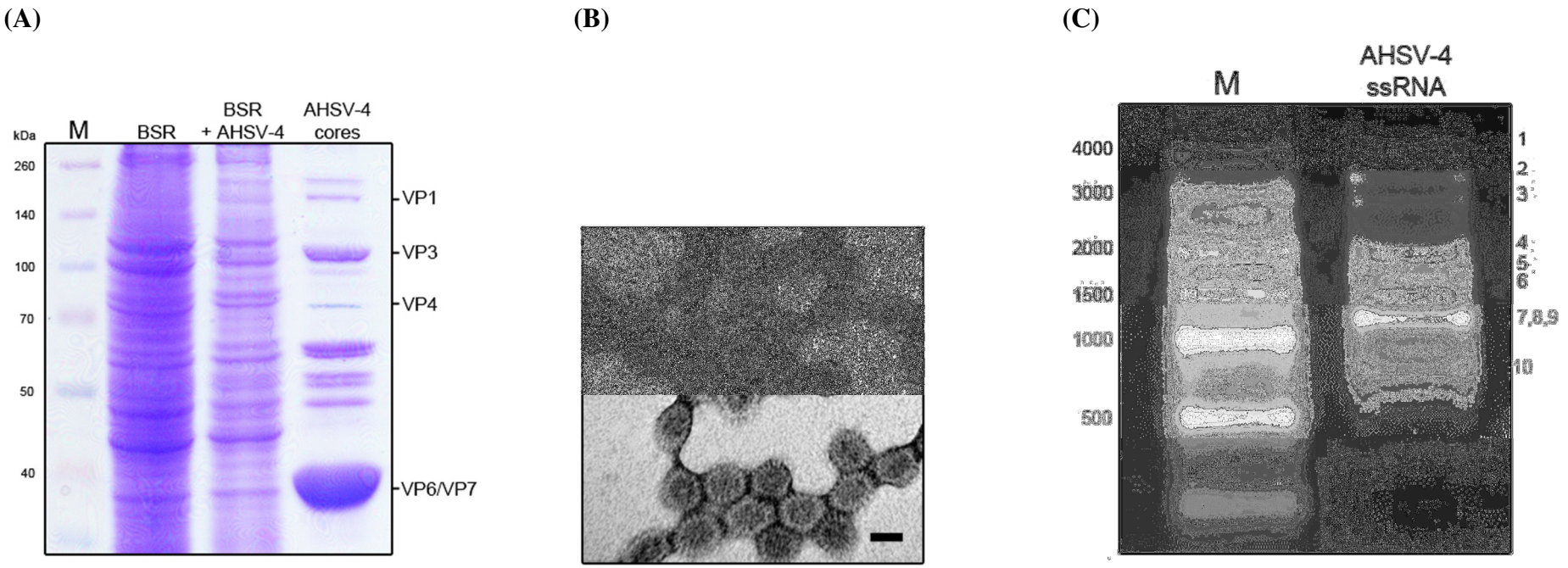


Fig. 2.1: Purification of AHSV-4 core particles from infected BSR cells and their use in *in vitro* transcription reactions. (**A**) SDS-PAGE of purified core particles, showing representative proteins. (**B**) TEM micrograph of purified core particles, scale bar = 60 nm. (**C**) AHSV-4 core-derived ssRNA generated following *in vitro* transcription of purified core particles.

Having confirmed the presence of the core proteins in intact particle structures, the AHSV-4 cores were subsequently used for *in vitro* ssRNA synthesis. Following *in vitro* transcription, the core particles were removed from the reaction mixtures by centrifugation and the ssRNA was selectively precipitated with LiCl and then purified using a commercial kit to ensure separation of the ssRNA from residual core particles. The purified AHSV-4 core-derived transcripts were analysed by agarose gel electrophoresis. The results indicated that all 10 viral transcripts were synthesised and purified, with no obvious evidence of premature termination or degradation of the transcripts (Fig. 2.1C). These results therefore indicated not only that AHSV-4 cores are transcriptionally active, but also suggested that these *in vitro*-synthesised transcripts should have the same coding potential as AHSV-4 ssRNA produced within virus-infected cells.

2.3.2 Expression of NS2 in BSR cells transfected with purified AHSV-4 ssRNA

During the infectious cycle of orbiviruses, the uncoated core particle serves as the source of mRNA that is translated and replicated in infected cells (Mertens and Diprose, 2004). To determine whether AHSV-4 core-derived ssRNA could synthesise viral proteins *in vivo*, BSR cells were transfected with AHSV-4 ssRNA and harvested at different time intervals, with the presence or absence of cytopathic effect (CPE) recorded concurrently. As a marker whereby the expression of viral proteins could be assessed over time the nonstructural protein NS2 was used. The NS2 protein was specifically chosen for this purpose since it is synthesised in large amounts throughout the replication cycle and accumulates in inclusion bodies, which are the sites in which virus replication and morphogenesis occurs (Hyatt and Eaton, 1988; Uitenweerde *et al.*, 1995; Kar *et al.*, 2007). Consequently, whole-cell lysates were prepared at each time interval, resolved by SDS-PAGE and subjected to immunoblot analysis using an anti-NS2 polyclonal antibody. Expression of NS2 was detected from 60 h post-transfection onward, which correlates with the appearance of CPE indistinguishable from that caused by viral infection (Fig. 2.2). The results thus indicate that *in vitro*-synthesised AHSV-4 ssRNA can serve as templates for virus protein synthesis *in vivo*.

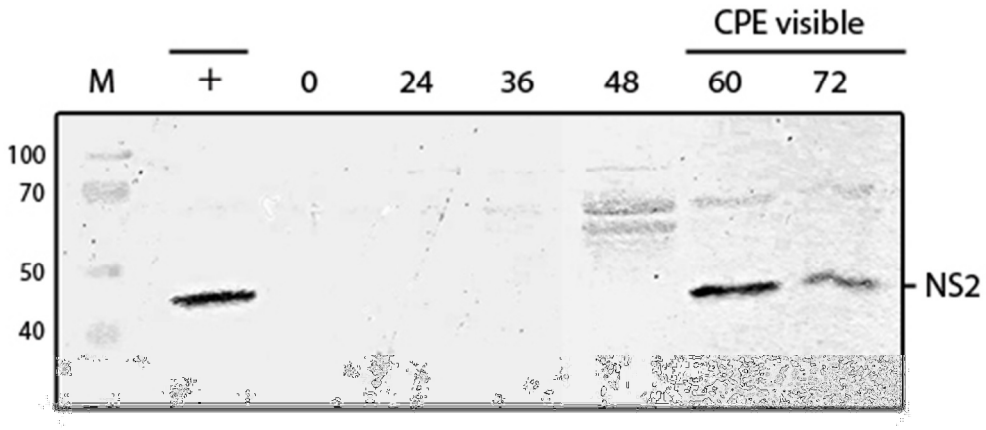


Fig. 2.2: Viral NS2 protein expression in BSR cells transfected with AHSV-4 ssRNA. BSR monolayers were transfected twice with AHSV-4 core-derived ssRNA and expression of the NS2 protein was determined at 12-h intervals, over a time course of 72 h, by immunoblotting. CPE was observed by light microscopy at 60 h post-second transfection. BSR cells infected with AHSV-4 served as a positive control.

2.3.3 Recovery of infectious virus from BSR cells transfected with purified AHSV-4 ssRNA

To determine whether infectious AHSV-4 could be generated from AHSV-4 ssRNA, BSR cells were transfected with different quantities of the ssRNA (800 ng - 2 µg), as it was expected that the recovery of virus would occur at lower frequency than viral protein expression in transfected cells. At 72 h post-second transfection, a CPE indistinguishable from that caused by AHSV-4 infection was seen in the transfected cell monolayers and, upon plaque assays, clear defined plaques were observed (Fig. 2.3). These results therefore indicated that infectious virus can be rescued from AHSV-4 ssRNA transcripts upon transfection into cells.

To confirm virus recovery and to determine whether the viral ssRNA had been replicated to produce new genomic dsRNA, viruses from individual plaques were amplified by picking plaques randomly and infecting BSR cells. At 120 h post-infection, dsRNA was extracted and analysed on agarose gels. The migration patterns of dsRNA segments extracted from AHSV-4 plaques derived from transfection were identical to that of the parental AHSV-4 derived from infection (Fig. 2.3). These results demonstrated that infectious AHSV-4 was present in each plaque and furthermore confirmed that AHSV-4 was recovered from the core-derived transcripts.

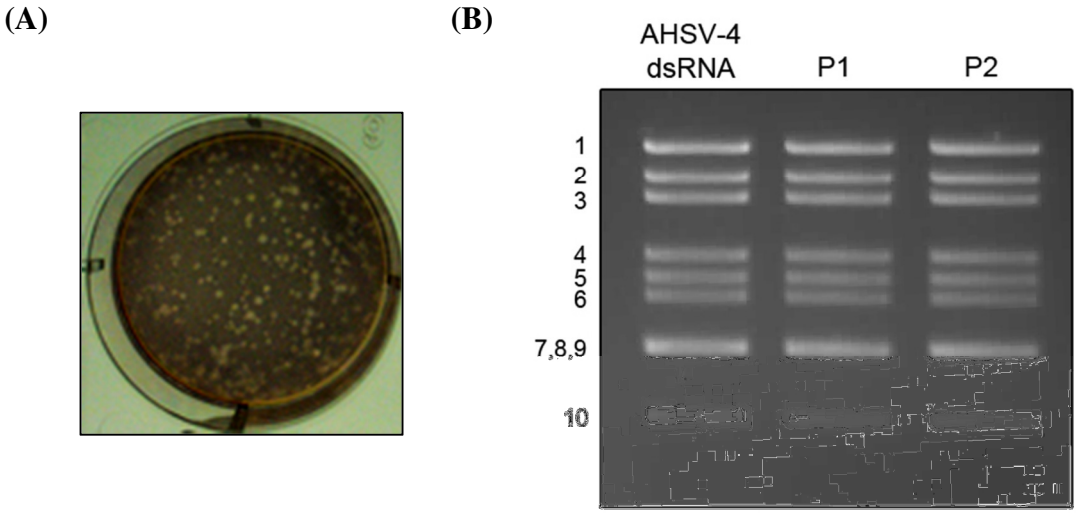


Fig. 2.3: Recovery of infectious AHSV-4 following transfection of BSR cells with AHSV-4 ssRNA. **(A)** Results of a plaque assay performed on the transfection lysate of BSR cells, which had been transfected twice with 800 ng of AHSV-4 ssRNA. **(B)** Agarose gel electrophoresis of AHSV-4 dsRNA compared to dsRNA extracted from BSR cells inoculated with plaques (P1 and P2) picked following plaque assay.

To characterise the infectious material in the AHSV-4 ssRNA preparation, its sensitivity to RNase A and Proteinase K was compared with that of AHSV-4 cores and dsRNA. In the case of cores, the dsRNA genome is protected by the VP3 and VP7 layers of the core particles. In these assays, 800 ng of each the AHSV-4 ssRNA and dsRNA, as well as 100 ng of AHSV-4 cores was digested with RNase A or Proteinase K prior to transfection of the BSR cells. The transfected BSR cell monolayers were incubated for 72 h and then plaque assayed. The infectivity of the AHSV-4 ssRNA was reduced to zero by the RNase A treatment, whereas the infectivity of the AHSV-4 cores was unaffected. In contrast, Proteinase K treatment of core particles completely inhibited their ability to cause infection, whereas the infectivity of AHSV-4 ssRNA was unaffected. As expected, the AHSV-4 dsRNA, which is resistant to the action of RNase A and Proteinase K under physiological conditions, was not infectious (Fig. 2.4). These results thus eliminated the possibility that some cores had survived the purification of the ssRNA, demonstrating that the unprotected ssRNA was the source of the infectivity.

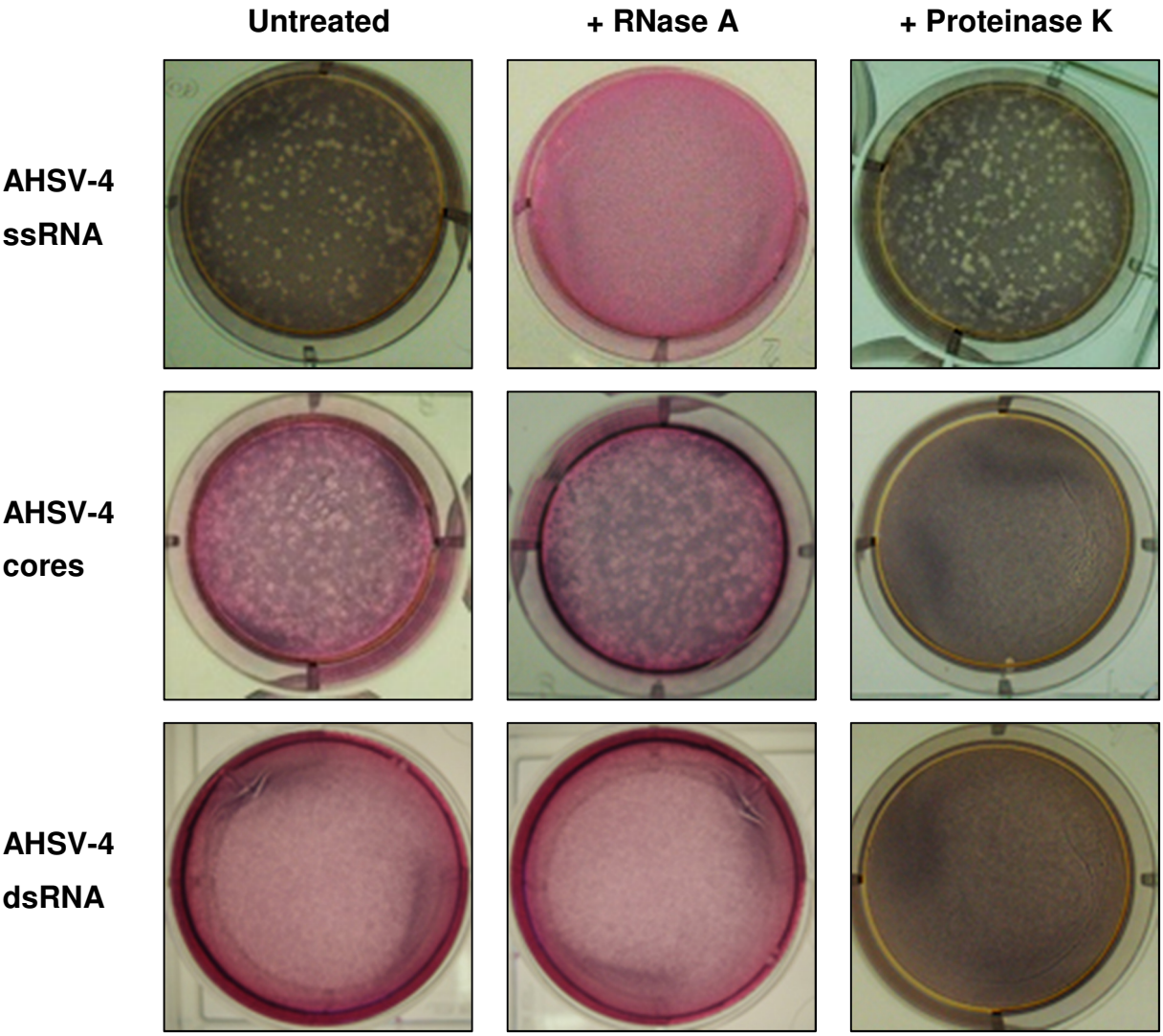


Fig. 2.4: Recovery of AHSV-4 from BSR cells transfected with AHSV-4 ssRNA. Treatment of AHSV-4 ssRNA with RNase A prior to transfection precludes viral recovery, but does not affect the ability of AHSV-4 cores to cause infection. Treatment of AHSV-4 ssRNA with Proteinase K prior to transfection does not affect virus recovery; however, AHSV-4 cores treated with Proteinase K do not produce virus infection. Transfection of BSR cells with AHSV-4 genomic dsRNA does not result in virus replication.

2.3.4 Evaluation of different mammalian cells for the efficient recovery of infectious AHSV-4

Different mammalian cell cultures, including BSR (Chapter 2, this study), BHK-21 (Burroughs *et al.*, 1994; Paweska *et al.*, 2003) and Vero (Stoltz *et al.*, 1996; Meiring *et al.*, 2009), have been used for the propagation and study of AHSV. It was therefore of interest to determine whether these cell cultures are equally proficient at allowing recovery of infectious AHSV-4 from transfected ssRNA transcripts. To investigate, monolayers of each of these cell

cultures were either infected with AHSV-4 at a MOI of 1 pfu/cell or transfected twice with an equal amount (800 ng) of AHSV-4 ssRNA. Following incubation for 72 h, the cells were harvested, lysed by successive freeze-thaw cycles and then titrated on cells from which the lysate originated.

The results, presented in Fig. 2.5, indicated that the BSR, BHK-21 and Vero cells were permissive for AHSV-4 replication and no significant differences in the virus titres could be observed (*ca.* 10^6 pfu/ml). As expected, the titres of infectious AHSV-4 rescued in the respective cell lines were lower compared to the corresponding titre of virus-infected cells. However, despite numerous attempts, infectious AHSV-4 could not be rescued in Vero cells transfected with the AHSV-4 ssRNA. In contrast, infectious AHSV-4 could be rescued in BSR and BHK-21 cells, but the titre of infectious virus was *ca.* 100-fold less in BHK-21 cells as compared to that in BSR cells (2.5×10^3 pfu/ml and 2.19×10^5 pfu/ml, respectively). This data therefore indicates that the mammalian cell line used for virus recovery may have a significant influence on the results.

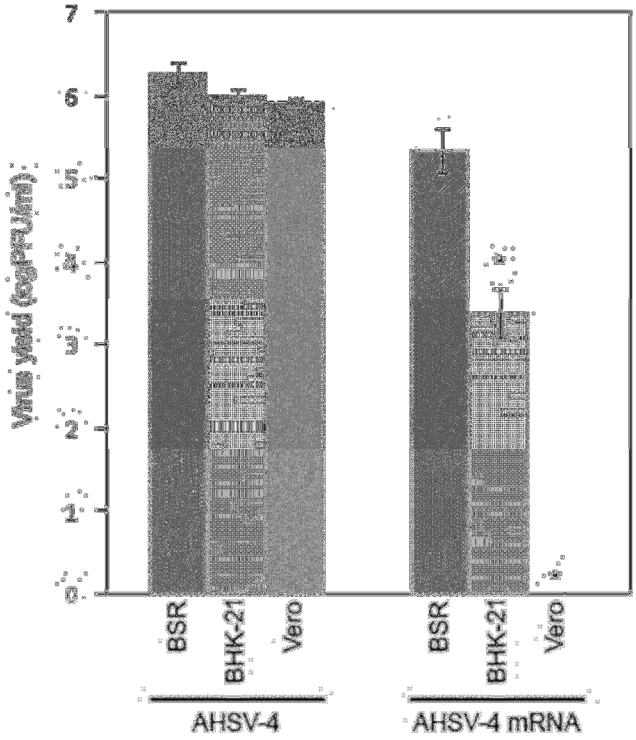


Fig. 2.5: Effect of cell line on AHSV-4 recovery following transfection with AHSV-4 core-derived ssRNA. BSR, BHK-21 and Vero cells were transfected twice with 800 ng of AHSV-4 core-derived ssRNA, incubated for 72 h and then subjected to plaque assays. AHSV-4 was included as a positive control. Bars represent the averages and standard deviations from three independent experiments. *, $P<0.05$ as determined by students t-test in comparison with BSR.

2.4 DISCUSSION

In the *Reoviridae* family, core particles synthesise and release capped viral ssRNA into the cytoplasm of infected cells. The extruded transcripts act both as mRNAs for viral protein synthesis and as templates for the synthesis of new dsRNA segments (Chandran and Nibert, 2003; Mertens and Diprose, 2004; Patton *et al.*, 2004). Other than the production of viral ssRNA transcripts, the core particle itself has no known role in infection. It therefore follows that the delivery of viral ssRNA to the cytoplasm of a cell by a route other than extrusion from an infecting particle should lead to the production of infectious virus. Consequently, in this part of the study, it was investigated whether AHSV-4 transcripts synthesised *in vitro* are infectious when introduced into BSR cells by transfection.

Based on reports indicating that BTV core particles are transcriptionally active *in vitro* (Verwoerd and Huismans, 1972; van Dijk and Huismans, 1980, 1988), the first step was therefore to determine whether purified AHSV core particles could likewise serve as a source for *in vitro*-synthesised viral ssRNA. For this purpose, a procedure was optimised allowing for the large-scale preparation of AHSV-4 cores that is considerably less time-consuming as compared to previously described methods for the purification of BTV core particles (van Dijk and Huismans, 1980; Mertens *et al.*, 1987; Boyce and Roy, 2007). By making use of the purified AHSV-4 core particles in *in vitro* transcription reactions, it was demonstrated that the core particles are capable of synthesising ssRNA transcripts of which the sizes correspond to that of the full-length viral mRNAs (Fig. 2.1C). The core-derived ssRNA was also shown to act as mRNA for the synthesis of viral proteins, as was evidenced by the expression of NS2 in the transfected BSR cell monolayers (Fig. 2.2). Several lines of evidence indicated that infectious virus could be recovered following transfection of BSR cells with the AHSV-4 ssRNA. Not only could CPE be observed in the transfected monolayers, but plaques were visualised following titration of the transfection cell lysates, and the presence of CPE in BSR cells incubated with the plaque isolates was observed. Moreover, viral genomic dsRNA was extracted from amplified plaque isolates, demonstrating that replication of the viral genome had indeed occurred (Fig. 2.3).

Although the above results provide strong evidence for the production of infectious AHSV-4 in BSR cells transfected with core-derived ssRNA, the possibility nevertheless existed that the infectivity of the viral ssRNA may have been due to the presence of trace amounts of

contaminating core particles. This is not a trivial matter, as it has been reported for both BTV (Mertens *et al.*, 1987) and AHSV (Burroughs *et al.*, 1994) that core particles are infectious in BHK-21 cells. In this study, great care was taken to remove core particles from the ssRNA transcripts used for transfection of BSR cells. This was achieved firstly through centrifugation and secondly by denaturation of residual core-particles with guanidium thiocyante included in the lysis buffer used during column purification of the ssRNA. In addition, Proteinase K treatment of core particles prior to transfection into BSR cell monolayers resulted in no virus being recovered, whereas Proteinase K treatment of purified AHSV-4 core-derived ssRNA prior to transfection did not inhibit the recovery of infectious AHSV-4. In contrast, the infectivity derived from cores was shown to be resistant to treatment with RNase A, whereas RNase A treatment of the AHSV-4 ssRNA resulted in no virus being recovered. The results therefore provide convincing proof that the infectivity present in purified AHSV-4 ssRNA is dependent on the integrity of the unprotected viral ssRNA and is not derived from core particles surviving the purification methods used. Furthermore, the transfection of AHSV-4 genomic dsRNA did not result in the recovery of virus. The lack of infectivity of AHSV dsRNA is not surprising as there is no known mechanism for dsRNA to be used as a template for the synthesis of viral proteins.

It is interesting to note that expression of the NS2 protein, which is known to be expressed at high levels throughout the infectious cycle (Huismans, 1979; Eaton *et al.*, 1990), could only be detected from 60 h onward in BSR cells transfected with the AHSV-4 ssRNA. In addition, CPE was only detected in the transfected BSR cells at 60 h post-transfection. These results suggest that the rescue of infectious virus occurs at a low frequency. This is in agreement with results reported regarding the recovery of BTV from core-derived ssRNA. It was estimated that the rescue of infectious virus occurs in *ca.* 1 in 10 000 transfected cells (Boyce and Roy, 2007). Various possibilities may account for the observed low efficiency in recovering infectious AHSV-4 from BSR cells transfected with the core-derived ssRNA. It is possible that the infectivity of ssRNA is reduced through degradation of the ssRNA by RNases present in the cell cytoplasm. This would not only limit the extent of viral protein synthesis, but would also reduce the probability of packaging a full complement of 10 intact mRNA segments. A second possibility is that productive infection is initiated only when a certain critical amount of viral ssRNA is introduced into the cell. If rescue is to occur, a transfected cell must receive copies of each of the 10 ssRNA transcripts. Moreover, enough copies of each transcript must be present in order to allow for viral proteins to be synthesised,

as well as to allow for packaging and subsequent replication of each transcript. Once progeny cores have been assembled, the transcripts produced by the progeny cores will lead to amplification of gene expression and so to further core particle and virion production (Matsuo and Roy, 2009). It therefore follows that if cells are not transfected with each of the 10 viral ssRNA or are transfected with too few copies of each ssRNA transcript, then there would be no rescue of virus. Another possibility may be related to the processes of genome packaging and replication, which occurs within the viral inclusion bodies of orbivirus-infected cells. The core particle has been shown to be localised in the viral inclusion bodies (Eaton *et al.*, 1988, 1990), thereby supplying viral ssRNA for packaging and replication at the site of assembly of progeny cores (Kar *et al.*, 2004; Modrof *et al.*, 2005; Kar *et al.*, 2007). Since the introduction of viral ssRNA into cells by transfection omits the presence of a core particle and therefore would not mimic this situation, a reduction in the recovery of virus is to be expected.

Although reverse genetic systems have been reported for various members of the *Reoviridae* family, it is striking that the rescue of infectious virus is limited to the use of a single mammalian cell line only. For example, recovery of infectious BTV from either viral ssRNA or *in vitro*-synthesised T7 transcripts has been reported in BSR cells only (Boyce and Roy, 2007; Boyce *et al.*, 2008; Matsuo and Roy, 2009; Van Gennip *et al.*, 2010; Celma and Roy, 2009, 2011), whereas different reverse genetic systems for rotavirus are all reliant on recovery of recombinant rotavirus in MA104 cells (Komoto *et al.*, 2006; Trask *et al.*, 2010; Troupin *et al.*, 2010). In the case of orthoreovirus, recovery of recombinant virus has been performed almost exclusively in murine L929 fibroblast (L) cells (Roner *et al.*, 1990; Roner and Joklik, 2001; Kobayashi *et al.*, 2007; 2010) and, more recently, in BHK-T7 cells (Kobayashi *et al.*, 2010). This observation prompted an investigation into the efficiency whereby infectious AHSV-4 could be recovered from core-derived ssRNA in mammalian cell lines that are typically used for the isolation, propogation and/or study of AHSV. In this study, BSR, BHK-21 and Vero cells were evaluated. Although these mammalian cell lines are permissive for AHSV replication, it is interesting to note that only BSR cells and BHK-21 cells, albeit to a lesser extent, were capable of supporting virus recovery. In contrast, infectious AHSV-4 could not be recovered following transfection of Vero cells with the ssRNA. It is unlikely that the observed differences in virus recovery are due to differences in transfection efficiency since, in three independent experiments, the same results were obtained. In addition, this laboratory has successfully obtained high transfection efficiencies

(in excess of 80%) for plasmid DNAs and siRNAs in Vero cells using Lipofectamine™ 2000 reagent (unpublished results). Considering that the AHSV-4 isolate used in this investigation had been adapted for growth in BSR cells, it therefore follows that recovery of infectious virus is the most efficient in these cells. Although the origin of BSR cells is not clear, it is frequently referred to as a "clone" or "derivative" of BHK-21 cells (Sato *et al.*, 1976; Boyce and Roy, 2007; Celma and Roy, 2011). Thus, it may therefore not be surprising that infectious AHSV-4 was recovered from transfected BHK-21 cells. Notably, Vero cells have been reported to undergo spontaneous gene deletions that have resulted in the lack of interferon genes (Desmyter *et al.*, 1968; Mosca and Pitha, 1986; Ge *et al.*, 2003; Matskevich *et al.*, 2009). Whether this phenomenon may be related to the inability to recover infectious AHSV-4 from transfected Vero cells requires further investigation. Whatever the reasons for the observed differences in virus recovery, the results nevertheless point to the importance of evaluating different mammalian cell lines for their suitability with regards to the development and establishment of reverse genetic systems.

In conclusion, the results obtained in this part of the study demonstrate that recovery of infectious AHSV-4 is possible in BSR cells transfected with core-derived ssRNA transcripts. Moreover, the results also indicate that beyond the synthesis of viral transcripts in the cytoplasm of infected cells, there is no essential role for the core particle. The discovery that AHSV ssRNA is infectious represents a significant step towards the development of a reverse genetic system for AHSV. The details of these investigations are provided in the following Chapters.

CHAPTER THREE

EVALUATION OF DIFFERENT REVERSE GENETIC APPROACHES FOR RECOVERY OF AFRICAN HORSE SICKNESS VIRUS

3.1 INTRODUCTION

One of the major technological advances in virology has been the development of reverse genetic systems for all major groups of DNA- and RNA-containing viruses (Goff and Berg, 1976; Racaniello and Baltimore, 1981; Radecke *et al.*, 1995; Bridgen and Elliot, 1996). Despite variations in their molecular design and methodology, they all share a common feature, which is the availability of cloned cDNA encoding viral genomes that can be manipulated and subsequently used to isolate viruses containing engineered changes in their genomic nucleic acids. The use of such reverse genetic systems has led to the accumulation of a significant amount of new knowledge regarding the replication, biological properties and pathogenesis of several important viruses (Walpita and Flick, 2005; Pasternak *et al.*, 2006; Kiraly and Kostolansky, 2009).

RNA virus rescue systems typically entail intracellular transcription of full-length genomic or antigenomic RNAs from plasmids transfected into permissive cells, frequently in conjunction with transient expression of essential components of the viral replicase (Racaniello and Baltimore, 1981; Fodor *et al.*, 1999; Neumann *et al.*, 1999). Furthermore, translational competence of positive-strand RNA genomes has enabled rescue of infectious virus from cells following introduction of *in vitro*-generated genomic RNAs free of viral proteins (van der Werf *et al.*, 1986). In contrast, the development of reverse genetic systems for double-stranded (ds) RNA viruses of the family *Reoviridae* has been slow due to the technical complexities involved in manipulating a segmented genome containing 10 or more segments. This critical technological gap has been the single most important limitation to studies of this family of viruses.

In recent years the development of reverse genetic systems has been reported for several genera in the *Reoviridae* family. The first reverse genetic system was a helper virus-dependant system for mammalian orthoreovirus (Roner and Joklik, 2001). This approach combined reovirus infection of permissive cells and transfection with viral dsRNA, viral mRNA, a T7 transcript and *in vitro*-translated viral mRNA. Another helper virus approach has allowed the replacement of a rotavirus outer capsid protein with the corresponding protein of a different serogroup (Komoto *et al.*, 2006). The expression of the introduced genome segment was driven within the cell cytoplasm by a recombinant T7 vaccinia virus system, and selective pressure against the equivalent helper virus protein was provided by the

use of monoclonal antibody selection. These approaches have been used successfully to define packaging signals in reovirus RNAs (Roner and Steele, 2007) and to isolate rotaviruses containing engineered mutations in the VP4 viral attachment protein (Komoto *et al.*, 2006). However, neither of the reovirus or rotavirus reverse genetic systems in their current configurations permits selective introduction and recovery of designed mutations in each individual viral genome segment. More recently, mammalian orthoreovirus has been recovered using a helper virus-independent plasmid DNA-based system similar to the T7-driven systems first used for negative-strand RNA viruses (Kobayashi *et al.*, 2007). In this case, expression of all 10 genome segments is driven by either a recombinant T7 vaccinia virus system or BHK cells engineered to express the T7 RNA polymerase (Kobayashi *et al.*, 2010). This was soon followed by a report detailing the establishment of a reverse genetic system for bluetongue virus (BTV), a member of the *Orbivirus* genus, based on the use of *in vitro*-synthesised T7 transcripts with a cap analogue at the 5' end that functionally substitutes for transcripts synthesised by core particles (Boyce *et al.*, 2008). The respective reverse genetic systems have since been applied successfully to the study of different reovirus structural proteins (Kobayashi *et al.*, 2007; Kirchner *et al.*, 2008; Ooms *et al.*, 2010) and BTV nonstructural proteins (Celma and Roy, 2009, 2011) during the course of viral infection.

Comparative analysis of the reverse genetic systems that have been developed for members of the *Reoviridae* family indicate that they have some features in common that are most likely responsible for their success (Boyce *et al.*, 2008). Firstly, the genome segments derived from cDNA clones are provided as message sense transcripts in the transfected cells. Secondly, the cDNA-derived transcripts have the same 5' and 3' end sequences as the corresponding viral transcript. Finally, the cDNA-derived transcripts, like those of authentic viral transcripts, are capped, either *in vitro* by a cap analogue or intracellularly by the cross-capping activity associated with recombinant T7 vaccinia viruses. It therefore follows that provided these criteria are met, it may be possible to establish a reverse genetic system for AHSV. Consequently, the primary aim of this part of the study was to evaluate different reverse genetic approaches for their ability to rescue infectious AHSV in mammalian cell culture. These approaches comprised of the use of an entirely plasmid-based reverse genetic system, the use of linear T7 transcription cassettes of each viral genome segment, and the use of *in vitro*-synthesised and -capped T7 transcripts.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

Escherichia coli JM109 strains were cultured routinely in LB broth (1% [w/v] tryptone, 1% [w/v] NaCl, 0.5% [w/v] yeast extract; pH 7.4) (Sambrook *et al.*, 1989) at 37°C with shaking at 200 rpm, and maintained at 4°C on LB agar (LB broth containing 1.2% [w/v] bacteriological agar) or at -70°C as glycerol cultures. For plasmid DNA maintenance in *E. coli*, the medium was supplemented with 50 µg/ml kanamycin (Melford). The plasmids used in this study comprised of recombinant plasmid pKM-eGFP, which harbours an eGFP-encoding gene flanked by AHSV-4 hexanucleotide sequences, as well as recombinant pKM plasmids that contain cloned chemically synthesized (synthetic) copies of each of the 10 AHSV-4 genome segments. The latter plasmids are referred to individually as pKM-S1 through pKM-S10 or collectively as pKM(AHSV-4). All plasmid constructs were obtained as a kind gift from Dr. A. C. Potgieter (Deltamune, Pretoria).

3.2.2 Cell cultures

BSR cells were propagated and maintained as monolayers in 75 cm^2 tissue culture flasks in Eagle's Minimal Essential Medium (EMEM) with Earle's Balanced Salt Solution (EBSS) and L-glutamine (Hyclone), supplemented with 5% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA) and antibiotics (10 000 U/ml of penicillin, 10 000 µg/ml of streptomycin and 25 µg/ml of amphotericin B) (Hyclone). The flasks were incubated at 37°C in a humidified incubator with a constant supply of 5% CO_2. BHK-T7 and BSR-T7 cells, both of which have been engineered to express phage T7 RNA polymerase from their genome, were obtained from Dr. A. C. Potgieter (Deltamune, Pretoria). These cells were maintained as described above for BSR cells, except that 600 µg/ml Hygromycin (Roche Diagnostics) or Geneticin (Sigma-Aldrich) was added to the BHK-T7 and BSR-T7 cells, respectively, after every 10 cell divisions.

3.2.3 Large-scale preparation of recombinant pKM(AHSV-4) plasmids

3.2.3.1 Preparation of competent cells

Competent *E. coli* JM109 cells were prepared and transformed according to the method of Sambrook *et al.* (1989). A single colony of a freshly streaked *E. coli* JM109 culture was inoculated into 20 ml of LB broth and incubated overnight at 37°C with shaking. An aliquot

(1 ml) of the overnight culture was then used to inoculate 100 ml of pre-warmed (37°C) LB broth and the culture was incubated at 37°C with shaking until an OD_{600} of 0.55 was reached. Cells from 30 ml of the culture were harvested by centrifugation at 8000 × *g* at 4°C for 8 min. The supernatant was discarded and the pellet suspended in 10 ml of an ice-cold solution comprising 80 mM $CaCl_2$ and 50 mM $MgCl_2$. Following incubation on ice for 10 min, the cells were centrifuged, as above, and then suspended in 5 ml of ice-cold 100 mM $CaCl_2$. Glycerol (1.5 ml) was added and aliquots of 200 µl were pipetted into 1.5-ml Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -70°C until needed.

3.2.3.2 Transformation of competent cells

Competent cells (200 µl) were allowed to thaw on ice and mixed with 1 µl (*ca.* 100 ng) of recombinant plasmid DNA. Following incubation on ice for 30 min, the tubes were heat-shocked at 42°C for 90 s. The tubes were then immediately chilled on ice for 2 min, after which 800 µl of LB broth was added and the tubes were incubated at 37°C for 1 h with shaking. The transformation mixtures were plated in volumes of 100 µl onto LB agar supplemented with 50 µg/ml kanamycin. The agar plates were incubated overnight at 37°C. A positive control (100 ng of non-recombinant pCMV-Script® plasmid) and a negative control (competent cells only) were also included to determine the competency of the *E. coli* JM109 cells and to test for contamination, respectively.

3.2.3.3 Plasmid DNA extraction and quantification

Plasmid DNA was extracted and purified with the Qiagen Plasmid Midi kit according to the manufacturer's instructions. A single bacterial colony was inoculated into 250 ml of selective LB medium and incubated overnight at 37°C with shaking. The bacterial cells from 50 ml of the culture were harvested by centrifugation at 6000 × *g* at 4°C for 15 min and the bacterial pellet was suspended in 4 ml of resuspension buffer P1 (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 µg/ml RNase A), followed by the addition of 4 ml of lysis buffer P2 (200 mM NaOH, 1% [w/v] SDS). Following incubation at room temperature for 5 min, 4 ml of chilled neutralization buffer P3 (3M KOAc; pH 5.5) was added and the suspension was incubated on ice for 15 min. The lysate was cleared by centrifugation at 20 000 × *g* at 4°C for 30 min and the supernatant was added to a DNA-binding column. The DNA-binding column was washed twice with 10 ml of buffer QC (1 M NaCl, 50 mM MOPS [pH 7.0], 15% [v/v] isopropanol), and the DNA was eluted in 5 ml of buffer QF (1.25 M NaCl, 50 mM Tris-HCl [pH 8.5], 15%

[v/v] isopropanol). Plasmid DNA was precipitated by addition of 0.7 volumes isopropanol and recovered by centrifugation at 20 000 × *g* at 4°C for 30 min. The DNA pellet was washed twice with 70% ethanol, air-dried and suspended in 100 µl of dH_2O. The plasmid DNA concentration and purity was determined with a NanoDrop® ND-1000 spectrophotometer (Thermo Fischer Scientific).

3.2.4 Characterisation of recombinant pKM(AHSV-4) plasmids

3.2.4.1 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). A horizontal 1% (w/v) agarose gel was cast and electrophoresed at 90 V in 1 × TAE buffer (40 mM Tris-HCl, 20 mM NaOAc, 1 mM EDTA; pH 8.5). To allow visualisation of the DNA when viewed on a UV transilluminator, the gel was supplemented with 0.5 µg/ml ethidium bromide. Where applicable, the size of the DNA fragments was determined according to their migration pattern in an agarose gel compared to that of a DNA molecular weight marker (O'Generuler™ 1kb DNA ladder; Fermentas).

3.2.4.2 Nucleotide sequencing and sequence analysis

The nucleotide sequence of cloned insert DNA in the pKM plasmids was determined using the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions. In addition to the AHSV-RGR and AHSV-RGF sequencing primers, which respectively anneal to upstream and downstream sequences flanking the insert DNA cloned into pKM(AHSV-4) plasmids, gene-specific internal primers were also used in the sequencing reactions (Table 3.1). Each sequencing reaction mixture (10 µl) contained 100-200 ng of purified plasmid DNA, 2 µl of BigDye™ Termination Mix, 2 µl of 5 × Sequencing buffer and 3.2 pmol of the sequencing primer. Cycle sequencing reactions were performed in a Perkin-Elmer GeneAmp® 2700 thermal cycler with 25 of the following cycles: denaturation at 94°C for 10 s, primer annealing at 50°C for 15 s, and elongation at 60°C for 4 min. The extension products were precipitated by addition of 2 µl of 3 M NaOAc (pH 4.6), 60 µl of absolute ethanol and 11 µl of dH_2O. The tubes were incubated for 30 min on ice, centrifuged at 20 000 × *g* for 30 min and the pellet was washed twice with 70% ethanol. The extension products were resolved on an ABI PRISM® Model 377 automated sequencer (Applied Biosystems). The nucleotide

sequences were assembled and analysed with the BioEdit v.7.0.4.1 software package (Hall, 1999). Pair-wise alignments were performed with ClustalW, included in the software package.

Table 3.1: Primers used in sequencing reactions

Primer*	Sequence
NS1 633R	5'-GCCCTTCAATCAGATCCTTT-3'
NS1 555F	5'-ATGTTGATGGATTGATTCCG-3'
NS1 1256F	5'-AAGGAAGCGATTACTGTAACTG-3'
NS2 611R	5'-ACATCATTTCTTCCTCCTCCA-3'
NS2 501F	5'-TTAAAGATGAAGGAGAACAGGG-3'
NS3 524R	5'-TTATCGGAAATCTTACCCGC-3'
NS3 239F	5'-CTATGGCGGAAGCATTGC-3'
VP1 681R	5'-TCTTCACGCATTCGGTACCAC-3'
VP1 500F	5'-CTCTTGGGTTATTGGTGGC-3'
VP1 1214F	5'-GTGATAACTATGTTCAGCGC-3'
VP1 1926F	5'-AGAGTTTGGATATGGAGAGG-3'
VP1 2636F	5'-TGAAGTCATCGATTATCGGG-3'
VP1 3348F	5'-AGGGAATAGAGAAAGTCCG-3'
VP2 632R	5'-TGGTAGATAATGTCGGTTCCG-3'
VP2 550F	5'-GAGATGCGGTTAATGAAAGG-3'
VP2 1251F	5'-GATGGAGTAAACGTCTTGAC-3'
VP2 1961F	5'-ACAAGTTGAAGTAACGAAGG-3'
VP2 2663F	5'-TTTCAAC TCACAGCTTTCG-3'
VP3 657R	5'-CCGCTCTAAACACATCCACA-3'
VP3 500F	5'-GCAGAGATCGCAGATCCA-3'
VP3 1171F	5'-AATGTTTACTTACGGSCCGS-3'
VP3 1800F	5'-CCCGTTCAGATTTACTTGAG-3'
VP3 2411F	5'-GACGAGATACTGACGGCA-3'
VP4 693R	5'-CCGGAGCAATTGGATCTATGC-3'
VP4 584F	5'-GTGTATTATGTCGGATGTGG-3'
VP4 1316F	5'-TCGAACAGGCAGAATTTCAC-3'
VP5 623R	5'-TCAACGCGTCAAATTTCTCT-3'
VP5 401F	5'-TAGCGATTCGAAAGATTGTG-3'
VP5 1028F	5'-ATCATGTTAGGATCCCAGG-3'
VP6 623R	5'-CCCATCAACCTTTGTCTTCTC-3'
VP6 467F	5'-GGTGAGGTACCATCTGG-3'
VP7 623R	5'-AAGTGACGCACCTTGAGGA-3'
VP7 429F	5'-CTGGCAGATATTACGTACCG-3'
AHSV-RGR#	5'-CCCAATACGCAAGGAAACAG-3'
AHSV-RGF#	5'-CGAATTGAAGGAAGGCCGTC-3'

* For each primer, the numbers following the relevant nonstructural (NS) or structural (VP) gene designation denote the nucleotide position at which the primer anneals to the target gene. F - forward primer; R - reverse primer

\# Anneal to the pKM plasmid backbone and flank the insertion site of each respective synthetic genome segment

3.2.5 Transient expression of eGFP in BHK-T7 and BSR-T7 cells

3.2.5.1 Transfection of cell monolayers with the pKM-eGFP reporter plasmid

Prior to transfection, BHK-T7 and BSR-T7 cells were removed from confluent monolayers by adding Trypsin/Versene (Highveld Biological) to the cells. The cells were then seeded into 24-well tissue culture plates and allowed to reach confluencies between 50 and 90% within 12 h of incubation at 37°C in a CO_2 incubator. The cell monolayers were subsequently transfected with pKM-eGFP using the TransIT®-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Briefly, purified plasmid DNA (1.5 µg) and 3 µl of TransIT®-LT1 reagent per 1 µg of DNA (*i.e.* 4.5 µl) was diluted in 50 µl of EMEM medium lacking serum and antibiotics. Following incubation for 20 min at room temperature, the cell monolayers were overlayed with the transfection mixture while gently rocking the plate back and forth. Subsequently, 500 µl of EMEM medium, supplemented with 5% FBS, was added to each well and the tissue culture plates were incubated at 37°C for 24 h in a CO_2 incubator. Mock-transfected cells were included as a control.

3.2.5.2 Fluorescence microscopy

To observe expression of eGFP, monolayers of transfected BHK-T7 and BSR-T7 cells were examined at 24 h post-transfection under a Zeiss Axiovert 200 fluorescent microscope fitted with the no. 10 Zeiss filter set (excitation at 450-490 nm; emission at 515-565 nm). The images of different microscope fields were captured with a Nikon DXM1200 digital camera.

3.2.6 Amplification of pKM(AHSV-4) T7 transcription cassettes

3.2.6.1 Primers

The primers used in PCR reactions to amplify T7 transcription cassettes contained in the respective pKM(AHSV-4) plasmid DNAs are indicated in Table 3.2. The primers were obtained from Dr. A. C. Potgieter (Deltamune, Pretoria) and included a single forward primer, corresponding to the plasmid backbone upstream of the AHSV-4 genome segment inserts, and genome segment-specific reverse primers.

Table 3.2: Primers used to amplify T7 transcription cassettes from pKM(AHSV-4)

Primer	Sequence
T7-orbi F-1	5'-AGGAAGGCCGTCAAG-3'
VP1R	5'-GTAAGTGTTTTGAGCTG-3'
VP2R	5'- GTATGTGTATTCACATGG-3'
VP3R	5'- GTAAGTGTAATTCTGCCC-3'
VP4R	5'-GTAAGGTTATTAGGATTCCC-3'
VP5R	5'-GTATGTGTTTTCTCCGC-3'
VP6R	5'-GTAAGTTTTAAGTTGCCTG-3'
VP7R	5'-GTAAGTGTATTCGGTATTG-3'
NS1R	5'-GTAAGTTTGTGAACCAGG-3'
NS2R	5'-GTATGTTGAAATCCGC-3'
NS3R	5'-GTATGTTGAAATCCGC-3'
eGFPR	5'-GTAGGAAGAGCTTATCTAGATCC-3'

3.2.6.2 Polymerase chain reaction (PCR)

The PCR reaction mixtures (50 µl) contained 0.5 ng of the relevant pKM(AHSV-4) plasmid DNA as template, 10 pmol of each the T7-orbi F-1 forward and appropriate genome segment-specific reverse primers (Table 3.2), 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% [v/w] TritonX-100), 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP) and 1 U of *Ex Taq* Proofreading DNA polymerase (Takara Bio, Inc.). The tubes were placed in a Perkin-Elmer GeneAmp® 2700 thermal cycler. Following initial denaturation at 94°C for 3 min, the reactions were subjected to 30 cycles of denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s and elongation at 72°C for 3 min. After the last cycle, a final elongation step was performed at 72°C for 10 min to complete synthesis of all DNA strands. For control purposes, an identical reaction mixture lacking template DNA was included. The PCR reaction mixtures were electrophoresed on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

3.2.6.3 Purification of amplicons from agarose gels

The PCR-amplified T7 transcription cassettes were purified from agarose gels with a Zymoclean Gel DNA Recovery™ kit (Zymo Research Corporation) according to the manufacturer's instructions. Briefly, the DNA fragment was excised from the agarose gel, mixed with 3 volumes of Agarose Dissolving Buffer™ and incubated at 55°C for 15 min. The dissolved agarose solution was centrifuged through a Zymo-Spin I™ DNA-binding column at 10 000 × *g* for 30 s. The column was then washed twice with the supplied wash buffer and

the DNA was eluted in 10 µl of dH_2O. The concentration of each DNA fragment was determined using a NanoDrop® ND-1000 spectrophotometer (Thermo Fischer Scientific).

3.2.7 Transfection of BSR-T7 cell monolayers with pKM(AHSV-4) or PCR-amplified T7 transcription cassettes

BSR-T7 cells were seeded in 24-well tissue culture plates to reach 80% confluency within 6-8 h of incubation at 37°C in a CO_2 incubator. The cell monolayers were subsequently transfected with the recombinant pKM(AHSV-4) plasmids or with the PCR-amplified AHSV-4 T7 transcription cassettes in equimolar amounts (Table 3.3), in accordance with the approach described by Kobayashi *et al.* (2007). The same transfection protocol as described above was followed (Section 3.2.5.1), except that all 10 pKM(AHSV-4) plasmids or amplicons were mixed in a single tube prior to its addition to the diluted TransIT®-LT1 transfection reagent (3 µl per 1 µg of DNA). Following incubation of the tissue culture plates at 37°C for 18 h in a CO_2 incubator, the cell monolayers were transfected for a second time with the same protocol used for the first round of transfections. The tissue culture plates were then incubated for 72 h at 37°C in a CO_2 incubator. Mock-transfected cells and cells infected with AHSV-4 were included in the assays.

Table 3.3: Recombinant plasmids containing synthetic AHSV-4 genome segments

Plasmid	AHSV-4 genome segment	No. of base pairs (bp)	Protein encoded	Plasmid equimolar mass (ng)*	Transcription cassette equimolar mass (ng)+	Transcript equimolar mass (ng)#
pKM-S1	1	3965	VP1	200	250	160
pKM-S2	2	3229	VP2	175	205	131
pKM-S3	3	2793	VP3	161	176	113
pKM-S4	4	1978	VP4	135	125	80
pKM-S5	5	1748	NS1	128	111	71
pKM-S6	6	1566	VP5	122	100	64
pKM-S7	7	1167	VP7	109	75	48
pKM-S8	8	1165	NS2	109	75	48
pKM-S9	9	1160	VP6	109	73	47
pKM-S10	10	757	NS3/NS3A	96	48	31

* 1.344 µg total
\+ 1.238 µg total
\# 793 ng total

3.2.8 Transfection of BSR cells with AHSV-4 T7 transcripts

3.2.8.1 *In vitro* synthesis of AHSV-4 T7 transcripts

The gel-purified T7 transcription cassettes (Section 3.2.6) were used as templates to synthesise T7 transcripts *in vitro* with a 5' cap analogue. For this purpose, the MessageMax™ T7 ARCA-capped Message Transcription kit (Epicentre Biotechnologies) was used according to the manufacturer's instructions. Each reaction mixture contained 1 µg of template, 2 µl of 10 × transcription buffer, 8 µl of ARCA/dNTP premix, 2 µl of 100 mM DTT, 0.5 µl of RNase Inhibitor, 2 µl of MessageMax™ T7 enzyme solution and RNase-free dH_2O to a final volume of 20 µl. In these transcription reactions the ratio of anti-reverse cap analogue (ARCA) to rGTP was 4:1. The reaction mixtures were subsequently incubated at 37°C for 2 h. Following incubation, the DNA template was removed by addition of 1 µl of RNase-free DNase I and incubation at 37°C for 15 min. The ssRNA transcripts were purified with the Nucleospin® RNA Clean-up kit (Macherey-Nagel) and eluted in 60 µl of RNase-free dH_2O. An aliquot of each purified T7 transcript was analysed by electrophoresis on a 1% (w/v) agarose gel, using denaturing loading buffer (Fermentas), and their concentration and purity was determined with a Nanodrop® ND-1000 spectrophotometer (Thermo Fischer Scientific).

3.2.8.2 Transfection of BSR cell monolayers with AHSV-4 T7 transcripts

BSR cells were seeded 4-6 h preceding transfection in 24-well tissue culture plates at a confluency 80% in antibiotic-free EMEM medium, which had been supplemented with 5% (v/v) FBS. Prior to transfection the BSR cell monolayers were washed twice with serum- and antibiotic-free EMEM, followed by the addition of antibiotic-free complete EMEM to the wells. Monolayers were then transfected with *in vitro*-synthesised AHSV-4 T7 transcripts using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. Briefly, equimolar amounts of AHSV-4 T7 transcripts (Table 3.3) and 2 µl of Lipofectamine™ 2000 reagent were each diluted in 50 µl serum- and antibiotic-free EMEM medium, incubated at room temperature for 5 min and then mixed. Following incubation at room temperature for 20 min, the solutions were added drop-wise to the BSR cell monolayers and the tissue culture plates were incubated at 37°C in a CO_2 incubator. A second transfection, identical to the first, was performed on the monolayers 16 h subsequent to the first. The cell-culture plates were incubated at 37°C in a CO_2 incubator for 72 h. Mock-transfected and AHSV-4 infected monolayers were included as controls.

3.2.9 Plaque assays

Plaque assays were used to determine whether infectious AHSV-4 could be recovered following transfection of BSR-T7 cell monolayers with the 10 recombinant pKM(AHSV-4) plasmid DNAs or PCR amplified T7 transcription cassettes, and following transfection of BSR cell monolayers with the 10 *in vitro*-synthesised T7 transcripts. The plaque assays were performed on BSR cell monolayers according to the method described by Oellermann (1970). The transfected cells were harvested in the culture medium, transferred to Eppendorf tubes and subjected to three successive freeze-thaw cycles to lyse the cells. Undiluted cell lysates were then used to infect confluent BSR cell monolayers in 6-well tissue culture plates. Following incubation at 37°C for 1 h, the inoculums were aspirated and the cells were overlaid with a 0.5% (w/v) agarose solution (Seakem LE agarose; prepared in complete EMEM medium). The tissue culture plates were subsequently incubated at 37°C for 96 h in a CO_2 incubator. To visualise plaques, the cell monolayers were flooded with 500 µl of Neutral Red (0.05% [w/v] in 1 × PBS) and incubated at 37°C for a further 48 h, after which the Neutral Red solution was aspirated. The tissue culture plates were photographed using a Sony DSC-V3 digital camera.

3.3 RESULTS

3.3.1 Characterisation of recombinant pKM(AHSV-4) and pKM-eGFP plasmid DNAs

With the aim of establishing a plasmid cDNA-based reverse genetic system for AHSV, similar to that described for orthoreovirus (Kobayashi *et al.*, 2007), recombinant pKM plasmids containing synthetic cDNA copies of each of the 10 AHSV-4 genome segments were obtained from Dr. A. C. Potgieter. Each of the cloned virus genome segments had been constructed so that they are flanked by an upstream T7 RNA polymerase promoter and by a downstream antigenomic hepatitis delta virus (HDV) ribozyme sequence. Thus, upon transfection of the cDNA clones into cells expressing T7 RNA polymerase, the synthesised transcripts are expected to correspond to viral mRNAs containing the native 5' end, while autocatalytic cleavage by the HDV ribozyme is expected to generate the native 3' end. Theoretically, this transcription strategy therefore has the potential to yield AHSV-4 ssRNA that are capable of serving as templates for viral protein synthesis and genomic dsRNA synthesis.

Prior to evaluating these recombinant plasmids for their ability to recover infectious AHSV-4, it was first necessary to determine whether the supplied plasmids indeed contained cloned cDNA copies of the respective AHSV-4 genome segments. To investigate, the plasmid DNA was analysed by agarose gel electrophoresis. Differences were observed in the migration of the respective plasmid DNAs through the agarose gel, indicating that each plasmid DNA harboured an insert DNA of a distinct size (Fig. 3.1A). To confirm that this was indeed the case, PCR was performed by making use of primers AHSV-RGF and AHSV-RGR (Table 3.1), together with each of the respective recombinant pKM(AHSV-4) plasmids as template DNA. Following agarose gel electrophoresis of the reaction mixtures, amplicons corresponding in size to the expected size of the individual AHSV-4 genome segments were observed. No amplification products were observed in a negative control reaction in which template DNA was omitted (Fig. 3.1B).

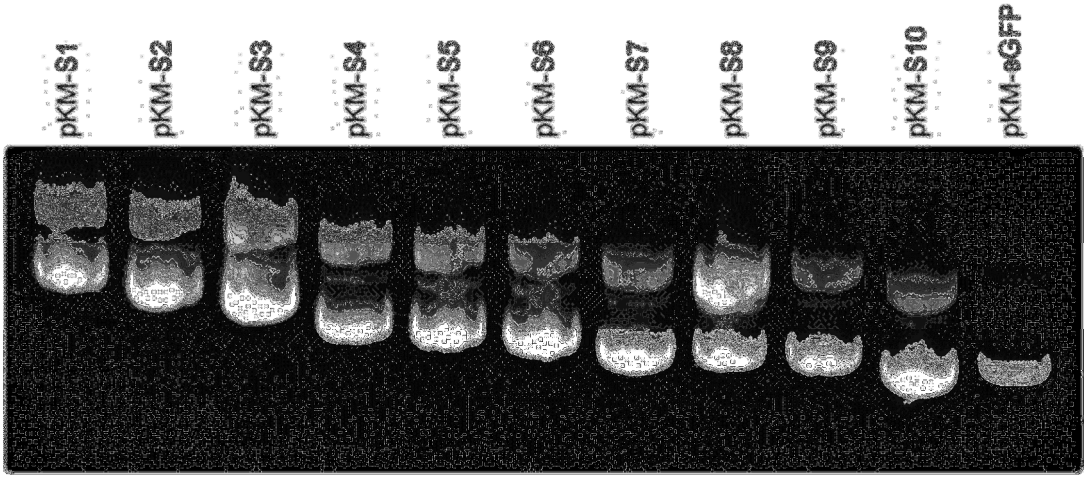
Based on the above results it was concluded that the pKM(AHSV-4) plasmid DNAs contained differently sized insert DNAs of which the sizes were in agreement with those of individual AHSV-4 genome segments. Consequently, the plasmid DNAs were transformed into competent *E. coli* cells and large-scale plasmid extractions were performed in order to obtain sufficient amounts of highly purified pKM(AHSV-4) plasmid DNAs for further use.

3.3.2 Evaluation of BHK-T7 and BSR-T7 mammalian cells for use in AHSV-4 recovery

As indicated above, each of the full-length AHSV-4 genome segments had been constructed to be under the transcriptional control of the T7 RNA polymerase promoter. To allow for synthesis of viral transcripts, the transfected cells can be infected with a T7 RNA polymerase-expressing vaccinia virus strain (Kobayashi *et al.*, 2007) or, alternatively, mammalian cell lines that have been engineered to stably express T7 RNA polymerase can be used (Kobayashi *et al.*, 2010). The use of the latter has been reported to simplify the virus rescue protocol, as well as being easier to maintain and propagate than the recombinant vaccinia virus strain (Kobayashi *et al.*, 2010).

In this study, two mammalian cell lines, designated BHK-T7 (Ito *et al.*, 2003) and BSR-T7 (Buchholz *et al.*, 1999), which have both been engineered to stably express T7 RNA polymerase under control of a cytomegalovirus promoter, were selected for use. The two cell lines were evaluated for their ability to express the T7 RNA polymerase enzyme and they were also compared with regards to their ability to take up plasmid DNA. For this purpose,

(A)



(B)

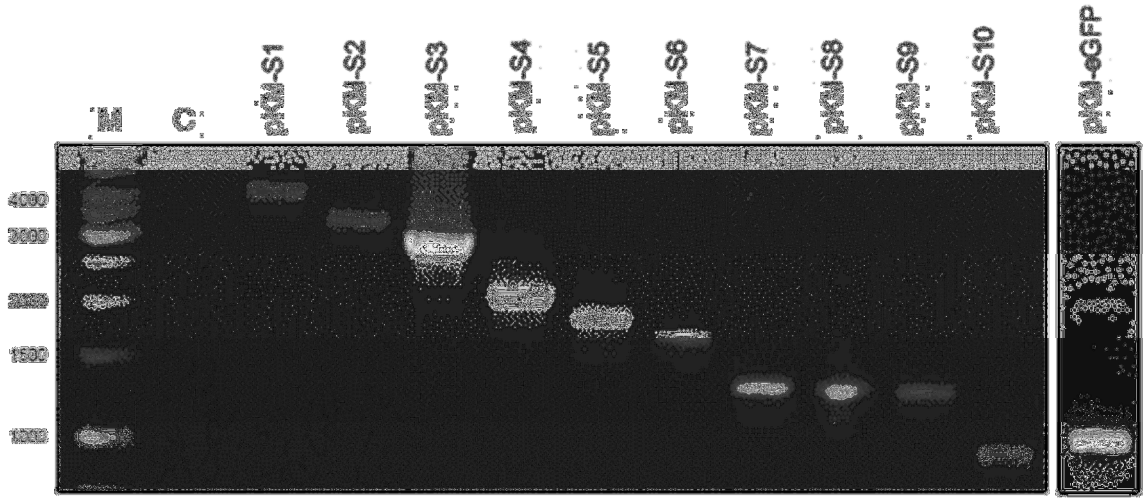


Fig. 3.1: Characterization of recombinant pKM(AHSV-4) and pKM-eGFP plasmid DNAs. **(A)** Agarose gel electrophoretic analysis of the recombinant plasmids. **(B)** DNA products obtained by PCR amplification using the individual pKM(AHSV-4) and pKM-eGFP plasmid DNA as template, and primers AHSV-RGF and AHSV-RGR. The sizes of the DNA molecular weight marker (M), O'GeneRuler 1 kb DNA Ladder (Fermentas), are indicated to the left of the figure. As a negative control, a PCR reaction mixture lacking template DNA was included in the assay (C).

the reporter plasmid pKM-eGFP was used. The eGFP transcription cassette contained in this plasmid comprises of an upstream T7 RNA polymerase promoter, followed by an eGFP reporter gene and a downstream HDV ribozyme sequence.

To evaluate and compare the BHK-T7 and BSR-T7 cell lines, cell monolayers at different confluencies (50-90%) were transfected with the pKM-eGFP reporter plasmid. The transfected cell monolayers were examined at 24 h post-transfection by fluorescence microscopy. Three experimental repeats were conducted and representative results are presented in Fig. 3.2. The results indicated that although cells in both the BHK-T7 and BSR-T7 monolayers expressed the eGFP protein, many more cells in the BSR-T7 cell monolayers expressed the eGFP protein across all cell confluencies investigated. The reason for the observed differences between the BHK-T7 and BSR-T7 cell lines is unclear, but it may reflect differences in the level of the expressed T7 RNA polymerase in the respective cell lines, an increased toxicity of the transfection reagent towards the BHK-T7 cells or a difference in efficiency whereby the cells can be transfected. These aspects were not further investigated in this study. Nevertheless, based on the above results, indicating that the BSR-T7 cells expressed T7 RNA polymerase and could be transfected efficiently, it was decided to continue with this cell line for use in all subsequent experiments.

Since it is reasonable to expect that there may be a dose-dependent inhibitory effect of the plasmid DNA concentration on the transfection reagent, it therefore follows that using higher concentrations of the plasmid DNA would not necessarily result in efficient transfection. This could pose a problem, as the strategy envisaged for recovery of infectious AHSV-4 would require that the cells be transfected with 10 different plasmid DNAs; the final concentration of which would be higher than the 1 µg of plasmid DNA used in the above experiments. Therefore, the BSR-T7 cells were transfected at a confluency of 80% with different concentrations of the pKM-eGFP reporter plasmid (0.8-2 µg). In these experiments, cell monolayers at 80% confluency were used since it was reasoned that if the transfection reagent is cytotoxic to the cells, then there would still be enough viable cells available that could be transfected, as opposed to the number of viable cells in monolayers of lower confluency. The results that were obtained (Fig. 3.3) indicated an increase in the number of fluorescent cells with an increase in the concentration of plasmid DNA transfected into the cells, and furthermore indicated that up to 2 µg of plasmid DNA can be used to transfect the cells efficiently.

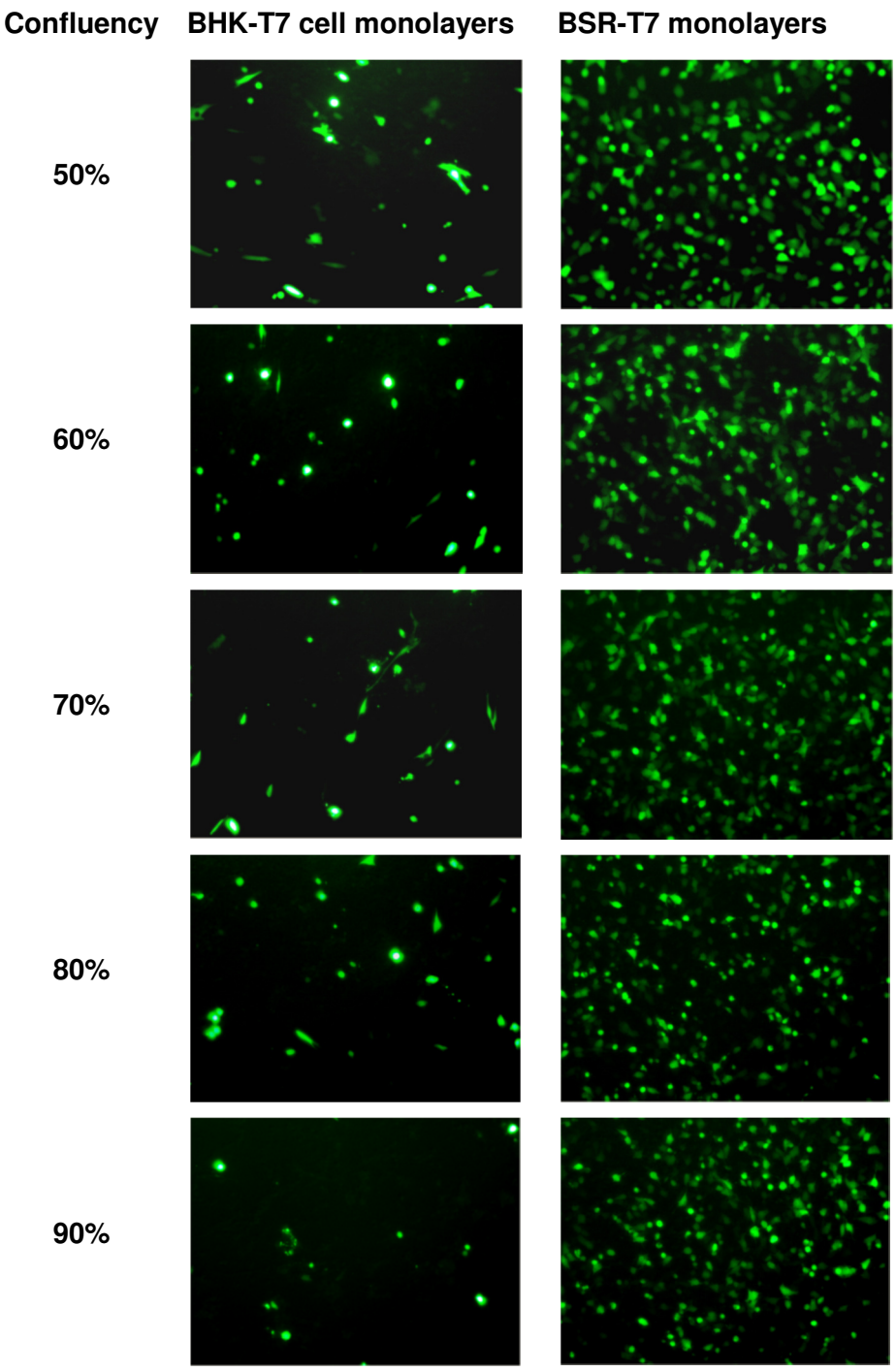


Fig. 3.2: Fluorescence microscopy of eGFP gene expression in BHK-T7 and BSR-T7 cells. Cell monolayers, at the indicated confluencies, were transfected with 1 µg of pKM-eGFP and analysed for eGFP protein expression at 24 h post-transfection with a Zeiss Axiovert fluorescence microscope. Magnification, 10×.

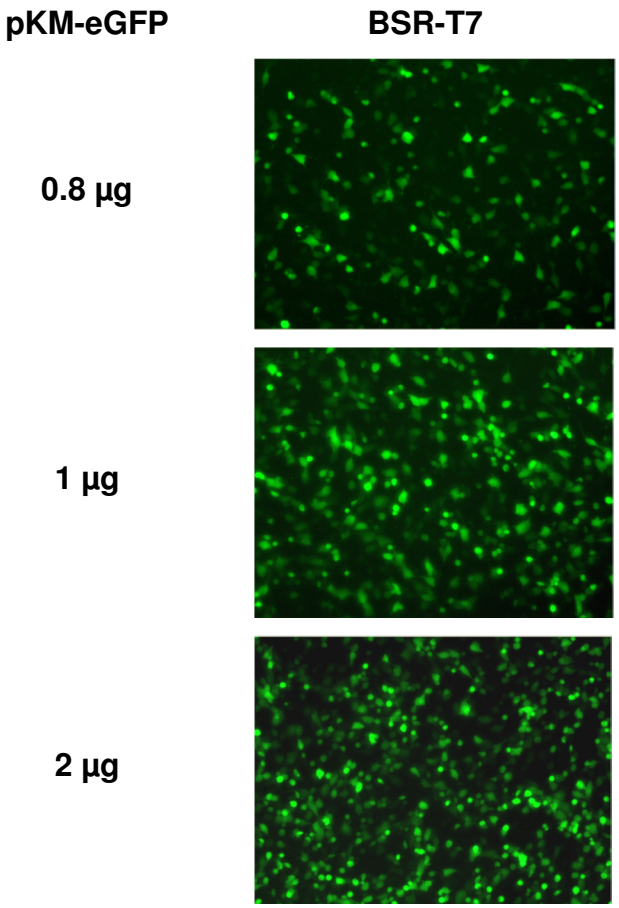


Fig. 3.3: Fluorescence micrographs of eGFP gene expression in BSR-T7 cells transfected with increasing amounts of the pKM-eGFP reporter plasmid. Cell monolayers at 80% confluency were transfected with the indicated concentrations of pKM-eGFP and analysed for eGFP protein expression at 24 h post-transfection with a Zeiss Axiovert fluorescence microscope. Magnification, 10 ×.

3.2.3 Recovery of infectious AHSV-4 from plasmid cDNA clones

To determine whether infectious AHSV-4 could be recovered from the 10 recombinant pKM(AHSV-4) cDNA clones, BSR-T7 cells were transfected with equimolar amounts of each plasmid DNA (Kobayashi *et al.*, 2007). Furthermore, to increase the chances of recovering infectious virus, the BSR-T7 cells were transfected with a mixture of all 10 cDNA clones, followed by a second transfection with the same amount at 18 h after the first transfection. This double-transfection procedure was shown previously to allow for the efficient recovery of infectious AHSV-4 from core-derived ssRNA (Chapter 2) and was also reported to be more efficient than a single transfection for recovery of infectious BTV (Matsuo and Roy, 2009). Following incubation of the transfected BSR-T7 cells for 72 h, the cells were harvested and plaque assays of the lysed cells performed. No plaques could be

visualised, indicating that infectious AHSV-4 could not be recovered using this approach. In addition to repeating these experiments numerous times, attempts were also made to optimise the experimental protocol by varying the amounts of transfected plasmid DNA and transfection reagent used. Moreover, since the viral titre in transfection lysates might have been below the limit of detection at 72 h post-second transfection, the lysates were also used to infect BSR cells and incubated for varying periods of time prior to performing plaque assays. However, none of these modifications resulted in the recovery of AHSV-4. A summary of the results obtained is presented in Fig. 3.4.

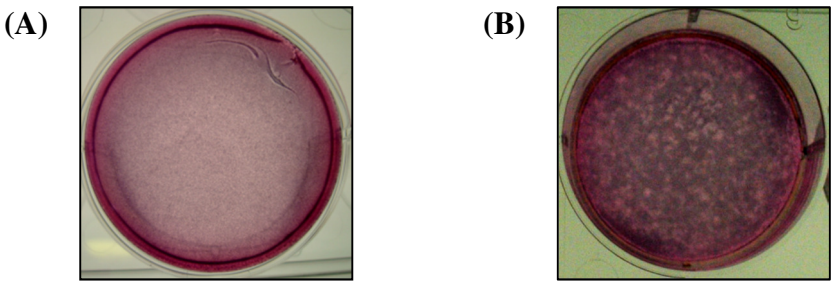


Fig. 3.4: Results of cell transfection experiments with purified pKM(AHSV-4). **(A)** Plaque assay of an undiluted BSR-T7 transfection lysate, of which the cells were transfected twice with equimolar amounts of the 10 recombinant pKM(AHSV-4) plasmid DNAs and then incubated for 72 h. **(B)** Plaque assay of BSR cells that had been infected with AHSV-4 at a MOI of 1 pfu/ml.

3.3.4 Sequence analysis of pKM(AHSV-4) plasmid inserts

In addition to several technical complexities that may have prevented the recovery of infectious AHSV-4 from the plasmid cDNA clones (refer to Discussion), it is also possible that mutations within the cloned AHSV-4 genome segments may lead to the synthesis of proteins with impaired enzyme activity (*e.g.* VP1, VP4 and VP6) and/or lead to the disruption of protein-protein or protein-RNA interactions required during virus replication and morphogenesis. In addition, despite having shown that the recombinant pKM(AHSV-4) plasmids contain insert DNA corresponding in size to the genome segments of AHSV-4 (Fig. 3.1B), the integrity of the cloned insert DNA had not been verified. Therefore, the nucleotide sequence of the insert DNA in each of the 10 recombinant pKM(AHSV-4) plasmid DNAs was determined by automated sequencing. A combination of vector- and genome segment-specific primers was used to generate overlapping sequences that were then assembled in the BioEdit software programme to yield the full-length sequence for each viral cDNA segment.

The nucleotide sequences of the individual AHSV-4 genome segments are provided in the Appendix to this dissertation.

The nucleotide sequence of the entire AHSV-4 genome, as contained in the respective recombinant pKM(AHSV-4) plasmids, was found to comprise 19.528 kilobase pairs (kb), and ranged in size from 3.965 kb for the VP1-encoding gene to 0.757 kb for the NS3/NS3A-encoding gene (Fig. 3.5). Each of the viral genome segments contained conserved 5' (5'-GTT(A/T)A(T/A)……) and 3' (……(A/C)C(T/A)TAC-3') terminal hexanucleotides. The nucleotide sequences that were determined during the course of this investigation were subsequently compared to those used for the chemical synthesis of each AHSV-4 genome segment, and no differences were observed between the respective sequences. The results therefore excluded the possibility that deleterious mutations were introduced into the synthetic AHSV-4 genome segments during chemical synthesis of the individual DNA segments. It should also be noted that the insert DNA of the recombinant pKM(AHSV-4) plasmid DNAs was likewise sequenced, following their amplification in *E. coli*. No differences were observed in the sequences compared to that determined directly for the synthetic viral cDNA genome segments.

Although analysis of the sequence data indicated that each of the cloned AHSV-4 genome segments in the recombinant pKM(AHSV-4) plasmids was flanked by an upstream T7 RNA polymerase promoter and a downstream HDV ribozyme sequence, a second vector-borne T7 RNA polymerase promoter was also identified. The second T7 RNA polymerase promoter is located 45 bp upstream of the promoter initially designed to drive transcription of the AHSV-4 cDNA segments (Fig. 3.5). The sequence of the vector-borne T7 RNA polymerase promoter differed from that of the promoter directly adjacent to the AHSV-4 genome segments at the final two nucleotide positions (GG as opposed to TT). As such, the sequence of the T7 RNA polymerase promoter on the vector backbone is identical to the consensus T7 RNA polymerase promoter sequence (Dunn and Studier, 1983; Richardson, 1983). It is therefore conceivable that this strong upstream T7 RNA polymerase promoter may lead to promoter occlusion, thereby interfering with the transcription from the downstream T7 RNA polymerase promoter. Based on the sequence data, it was concluded that the inability to recover infectious AHSV-4 from the transfected plasmid cDNA clones can, in part, be ascribed to the synthesis of AHSV-4 transcripts that may not correspond to the authentic viral transcripts synthesised in infected cells.

Genome segment	Size (kb)	T7 RNA polymerase promoter (vector)	T7 RNA polymerase promoter (gene segments)	Hexanucleotides flanking segment	HDV ribozyme sequence
		TAATACGACTCACTATAGGG	TAATACGACTCACTATAGTT		GGGTCGG…//…AGAGCTC
1	3.965			GTTAATTTGAG…//…AAACAACTTAC	
2	3.229			GTTAAATTCAC…//…AATACACATAC	
3	2.793			GTTTATTTTCT…//…ATTACACTTAC	
4	1.978			GTTAATTTAGG…//…AATAACCTTAC	
5	1.748			GTTAAAGAACC…//…CACAAACTTAC	
6	1.566			GTTAATTTTTC…//…AAAACACATAC	
7	1.167			GTTAAAATTCG…//…AATACACTTAC	
8	1.165			GTTAAAAATCC…//…TTTCAACATAC	
9	1.160			GTTAAATAAGT…//…TTAAAACTTAC	
10	0.757			GTTAAAATTAT…//…TAACAACTTAC	

Fig. 3.5: Diagram indicating the organization of the synthetic AHSV-4 genome segments, and the presence of a second upstream vector-borne T7 RNA polymerase promoter. The two T7 RNA polymerase promoters and the HDV ribozyme sequence are present in each of the ten pKM(AHSV-4) plasmid constructs. The nucleotide sequence indicated in black represents the first and last nucleotides of the respective AHSV-4 genome segments, while the terminal hexanucleotides are indicated in blue. Black arrows indicate the transcription initiation sites on the T7 RNA polymerase promoters and the autocatalytic cleavage site for the HDV ribozyme. Orange arrows denote primer annealing positions for PCR amplification of the transcription cassettes, excluding the second vector-borne T7 RNA polymerase promoter.

3.3.5 Recovery of infectious AHSV-4 from T7 transcription cassettes

Due to the presence of two T7 RNA polymerase promoters upstream of the cloned AHSV-4 cDNA genome segments, it is plausible that transcription may yield a mixture of AHSV-4 transcripts of which the 5' ends may or may not correspond to the 5' ends of authentic viral mRNA. This therefore necessitated that the experimental approach be modified so as to prevent transcription from being initiated from the vector-borne T7 RNA polymerase promoter, thereby ensuring that the transcripts will contain 5' ends that are identical to that of viral mRNA. Consequently, a PCR-based strategy was devised aimed at selective amplification of the T7 transcription cassette contained in each of the recombinant pKM(AHSV-4) cDNA clones. For this purpose, a primer was designed to anneal upstream of the T7 RNA polymerase promoter immediately adjacent to the cloned AHSV-4 genome segment, and downstream primers were designed to anneal to the 3'-terminus of each individual AHSV-4 genome segment (Fig. 3.5; Table 3.2). Care was taken with the design of the PCR primers to ensure that transcription of the PCR-amplified T7 transcription cassettes would yield ssRNA with authentic AHSV-4 terminal sequences.

It was demonstrated previously that transfection of both BSR-T7 and BHK-T7 cells with the reporter plasmid pKM-eGFP resulted in successful expression of the eGFP protein (Figs. 3.2 and 3.3). However, by taking the sequence data into account, these results do not allow for a definitive conclusion to be drawn as to whether both of the T7 RNA polymerase promoters present in recombinant pKM plasmids are transcriptionally active. Therefore, it was important to determine whether the T7 RNA polymerase promoter present in PCR-amplified T7 transcription cassettes is capable of driving transcription. To investigate, the T7 transcription cassette was PCR-amplified from the reporter plasmid pKM-eGFP with appropriately designed primers, purified from an agarose gel and then transfected into BSR-T7 cells. Examination of the cell monolayers at 24 h post-transfection under a fluorescent microscope indicated the presence of fluorescing cells. Compared to eGFP expression in BSR-T7 cells transfected with the pKM-eGFP reporter plasmid, BSR-T7 cell monolayers transfected with only the eGFP transcription cassette showed less fluorescing cells and a reduced overall level of fluorescence. However, this result nevertheless demonstrated that the T7 RNA polymerase promoter present in the T7 transcription cassette is indeed capable of driving transcription.

Subsequently, the T7 transcription cassettes contained in each of the 10 recombinant pKM(AHSV-4) cDNA clones was obtained through PCR amplification using a strategy similar to that described above. An aliquot of each reaction mixture was analysed by agarose gel electrophoresis and an amplicon of the expected size was obtained from each cDNA clone. An equimolar mixture of the gel-purified T7 transcription cassettes were transfected twice into BSR-T7 cells at 18-h intervals and plaque assays were performed at 72 h post-second transfection. Despite several attempts and further optimisation of the system, no plaques were visualised and thus indicated that infectious AHSV-4 could not be recovered with this approach. A summary of the results is presented in Fig. 3.6.

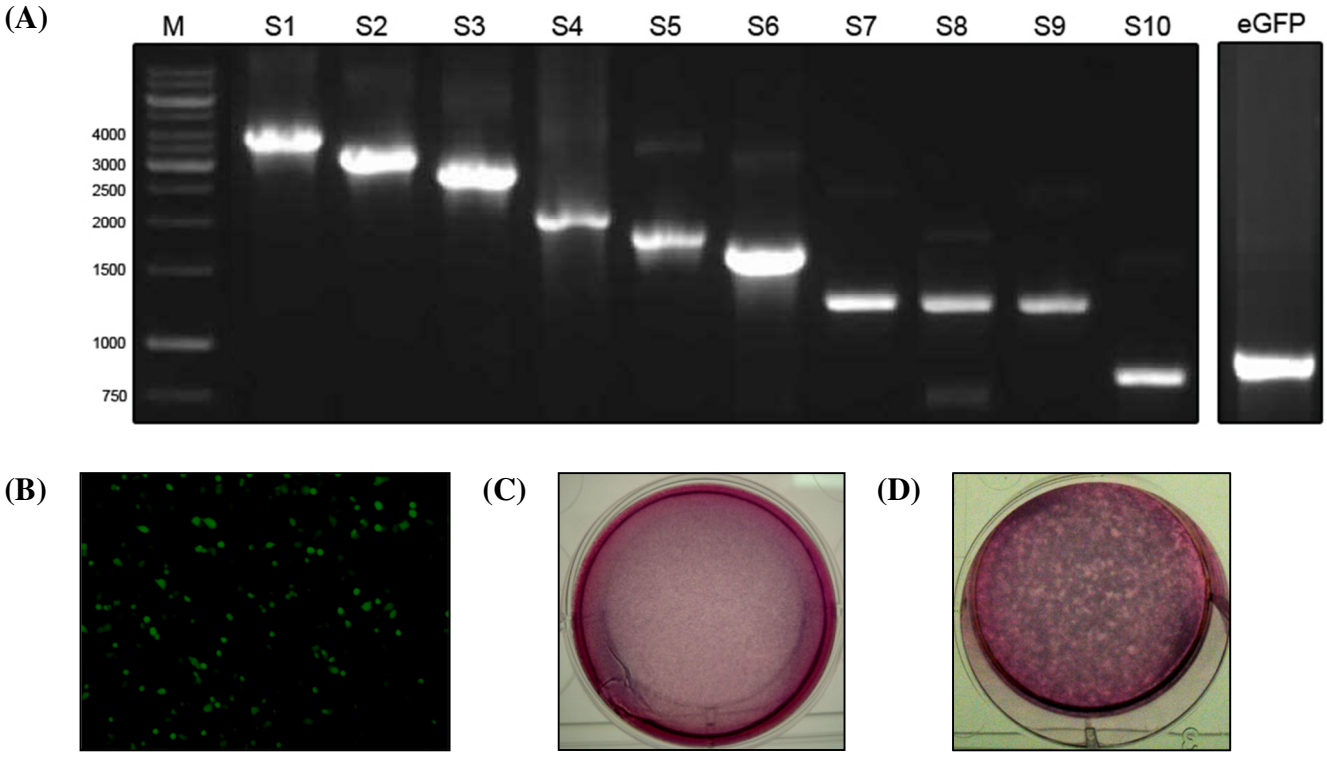


Fig. 3.6: Results of cell transfection experiments with PCR-amplified T7 transcription cassettes from the 10 recombinant pKM(AHSV-4) plasmid DNAs. **(A)** Agarose gel electrophoretic analysis of the PCR-amplified T7 transcription cassettes from the pKM(AHSV-4) and pKM-eGFP plasmid DNA. The sizes of the DNA molecular weight marker, O'GeneRuler 1 kb DNA Ladder (Fermentas), are indicated to the left of the figure. **(B)** Fluorescence micrograph of eGFP gene expression in BSR-T7 cells transfected with 800 ng of the eGFP T7 transcription cassette. The transfected cells were analysed at 24 h post-transfection with a Zeiss Axiovert fluorescence microscope. Magnification, 10 ×. **(C)** Plaque assay of an undiluted BSR-T7 transfection lysate, of which the cells were transfected twice with equimolar amounts of the 10 PCR-amplified AHSV-4 T7 transcription cassettes and then incubated for 72 h. **(D)** Plaque assay of BSR cells that had been infected with AHSV-4 at a MOI of 1 pfu/ml.

3.3.6 Recovery of infectious AHSV-4 from *in vitro*-synthesised T7 transcripts

The results obtained above indicated that it was not possible to recover infectious AHSV-4 from the two DNA-based approaches evaluated in this study. A possible explanation may relate to the absence of a cap structure on the 5' end of transcripts produced from the AHSV-4 cDNA clones and T7 transcription cassettes, which would decrease the stability of the transcripts. Consequently, an alternative approach, based on the use of *in vitro*-synthesised and -capped T7 transcripts, was evaluated. This approach is similar to that described by Boyce *et al.* (2008), in which the transfection of all 10 BTV segments transcribed *in vitro* from linearised plasmid cDNA clones was shown to lead to the recovery of infectious virus.

To investigate, the PCR-amplified T7 transcription cassettes of each AHSV-4 genome segment were used as templates to synthesise T7 transcripts *in vitro* by making use of the MessageMax™ T7 ARCA-capped Message Transcription kit. The reagents included in the kit have been optimised to produce ssRNA with an anti-reverse cap analogue (ARCA) on the 5' end. In contrast to ARCA, conventional cap analogues are asymmetrical and up to 50% of the capped ssRNA produced can have the cap in the reverse orientation, which results in sub-optimal intracellular and *in vitro* translation of the transcripts (Pasquinelli *et al.*, 1995). Since the ARCA molecule included in the kit cannot be incorporated in the reverse orientation, all of the caps in the ssRNA produced are in the correct orientation, thus increasing translational efficiency of the ssRNA (Grudzein *et al.*, 2004). Theoretically, the AHSV-4 T7 transcripts synthesised in the presence of cap analogue can therefore be considered to be functionally equivalent to both core-derived ssRNA and authentic viral transcripts.

Following *in vitro* transcription in the presence of ARCA, aliquots of the reaction mixtures were analysed on an agarose gel. The results indicated the synthesis of 10 transcripts of which the sizes were in agreement with those of AHSV-4 core-derived ssRNA. The synthesised full-length and capped T7 transcripts were purified and subsequently transfected in equimolar amounts into BSR cells using the double-transfection protocol described earlier. At 72 h post-second transfection, plaque assays were performed on the transfected BSR cell lysates. This approach, however, did not lead to the recovery of infectious AHSV-4, as was evidenced by the absence of plaques on BSR cells. Subsequent optimisation of the system with regards to the amount of T7 transcripts transfected or by performing plaque assays following serial amplification of the virus also failed to allow for the recovery of infectious AHSV-4. A summary of the results is presented in Fig. 3.7.

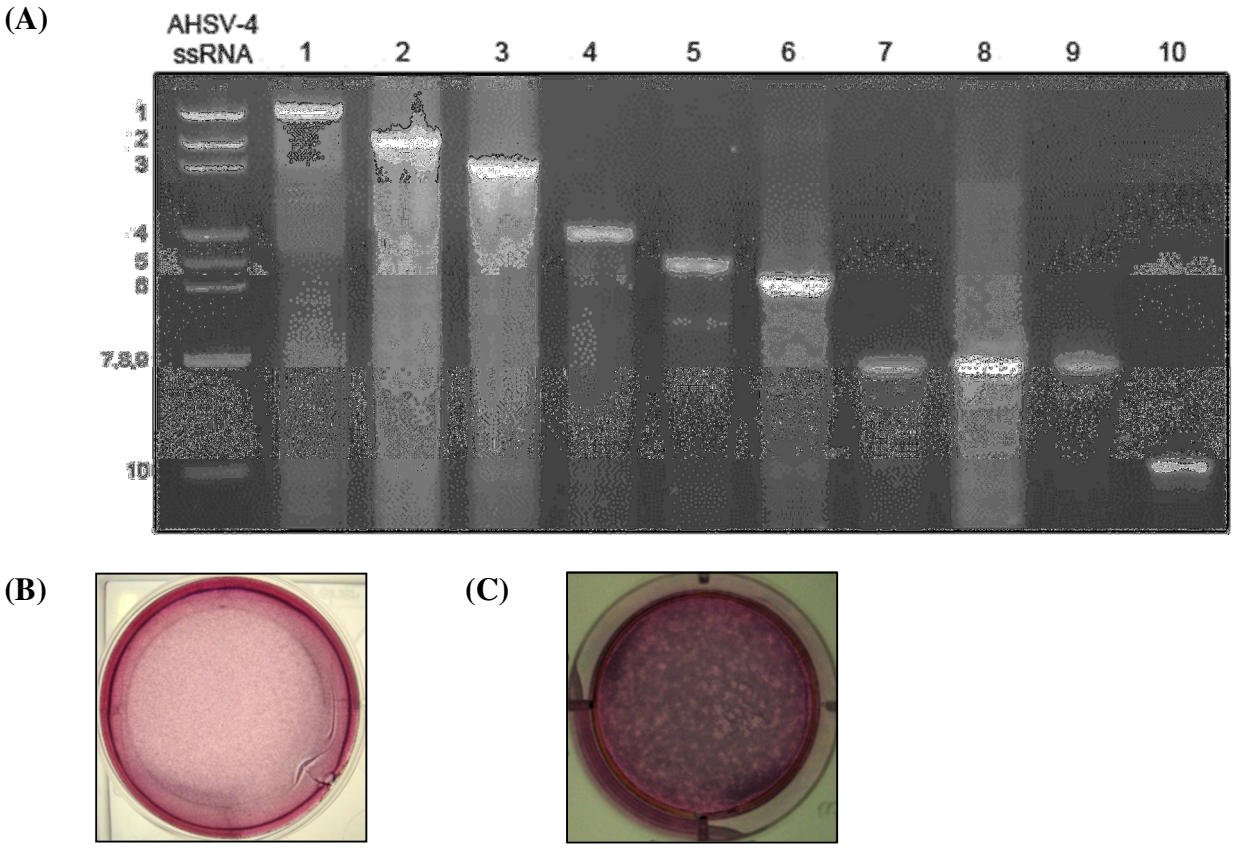


Fig. 3.7: Results of cell transfection experiments with *in vitro*-synthesised and -capped AHSV-4 T7 transcripts. **(A)** Agarose gel electrophoretic analysis of the 10 synthetic RNA products that were produced by *in vitro* transcription using the AHSV-4 T7 transcription cassettes as template. AHSV-4 mRNA derived from core particles is shown for comparative purposes. **(B)** Plaque assay of an undiluted BSR transfection lysate, of which the cells were transfected twice with equimolar amounts of the 10 *in vitro* synthesised- and-capped AHSV-4 T7 transcripts and then incubated for 72 h. **(C)** Plaque assay of BSR cells that had been infected with AHSV-4 at a MOI of 1 pfu/ml.

3.4 DISCUSSION

In the previous Chapter it was shown that AHSV-4 core-derived transcripts are infectious when transfected into permissive BSR cells, thus opening the door to the development of a reverse genetic system for AHSV. Indeed, recent advances for orthoreovirus (Kobayashi *et al.*, 2007) and BTV (Boyce *et al.*, 2008) have demonstrated that it is possible to recover these viruses entirely from recombinant sources. Consequently, towards establishing a reverse genetic system for AHSV, the ability to recover infectious AHSV from plasmid cDNA clones and synthetic mRNA transcripts were evaluated in this part of the study.

In the first approach, it was investigated whether AHSV-4 could be recovered from plasmid cDNA clones that had been designed to yield transcripts corresponding to authentic viral mRNA. During the course of these investigations, experiments were performed to characterise the plasmid cDNA clones and to select the most appropriate T7 RNA polymerase-expressing mammalian cell line for use with the recombinant pKM(AHSV-4) constructs. Unfortunately, no infectious virus could be rescued following transfection of BSR-T7 cells with the respective plasmid cDNA clones. Towards identifying factors that may account for the lack of AHSV-4 recovery, the nucleotide sequence of the insert DNA in each of the 10 recombinant pKM(AHSV-4) plasmid DNAs was determined. Although no differences were observed between the sequences determined in this study and those used for the synthesis of the synthetic AHSV-4 genome segments, a vector-borne T7 RNA polymerase promoter was identified that is situated upstream of the promoter included during the design of the synthetic AHSV-4 genome segments (Fig. 3.5).

Albeit unexpected, the presence of this second upstream T7 RNA polymerase promoter may provide a plausible explanation for the lack of infectious AHSV-4 recovery from the pKM(AHSV-4) plasmid cDNA clones. In both the orthoreovirus and BTV reverse genetic systems, great emphasis is placed on generating RNA transcripts with 5'- and 3'-terminal sequences that are identical to those of the viral mRNA (Kobayashi *et al.*, 2007; Boyce *et al.*, 2008). Although it can be expected that self-cleavage of the HDV ribozyme would yield authentic 3'-terminal sequences in the transcripts generated from the recombinant pKM(AHSV-4) plasmid DNAs, the same may not be true for the 5' ends of the transcripts. Due to the presence of a second upstream T7 RNA polymerase promoter, it is possible that transcription may have been initiated from this promoter. Since these transcripts would include vector sequences, it therefore follows that they would not bear any resemblance to the 5' ends of viral mRNA transcripts. The transcripts would therefore not be packaged into progeny virion particles and would thus not result in the generation of viable virus. However, it is also likely that transcription may have originated from both the T7 RNA polymerase promoters. In this case, a mixed population of transcripts would be generated, *i.e.* those with and those without the authentic 5' end of viral transcripts. Considering that the ratios of these transcripts are not known, it is possible that transcripts containing authentic 5' ends may have been present in lesser amounts compared to those that were transcribed from the vector-borne T7 RNA polymerase promoter. In this regard, it is interesting to note that the initiation region of T7 RNA polymerase promoters is highly conserved, particularly at nucleotides +1 to +3

(GGG, where +1 is the first transcribed nucleotide) (Imburgio *et al.*, 2000). In the case of the T7 RNA polymerase promoter immediately upstream of the synthetic AHSV-4 genome segments, the GGG sequence was mutated to GTT in order to produce transcripts with authentic AHSV 5'-terminal sequences. However, these base substitutions may diminish the affinity of the T7 RNA polymerase for the promoter region and reduce transcription efficiency. Indeed, site-directed mutagenesis studies of the T7 RNA polymerase promoter initiation sequence indicated that mutations resulting in the incorporation of uracil in any of the first six positions on the resulting RNA transcript dramatically reduced T7 RNA polymerase-driven transcription efficiency (Imburgio *et al.*, 2000).

In addition to the above, note should be taken that this approach may also be hampered by technical difficulties. Since it is not possible to ensure that all 10 pKM(AHSV-4) plasmid clones will be taken up by a given cell, it may be that the cells were not transfected with a full complement of the recombinant plasmids. In this regard, it is interesting to note that Kobayashi *et al.* (2007) reported that productive virus infection was established in only a small fraction of the cells that were transfected with plasmids containing the 10 orthoreovirus genome segments (*ca.* 1 in 10^5 - 10^6 cells that gave rise to between 10 and 100 viable virus particles). Notably, subsequent refinement of the plasmid DNA-based orthoreovirus reverse genetic system, by reducing the number of transfected plasmids to four, resulted in a 10- to 100- fold increase in virus recovery (Kobayashi *et al.*, 2010), furthermore indicating, as had been noted with influenza virus (Neumann *et al.*, 2005), that the number of plasmids directly influences virus recovery.

To overcome complications that may be associated with the presence of the conserved T7 RNA polymerase promoter present in the pKM vector backbone, the experimental strategy was modified. In this second approach, the T7 transcription cassette present in the respective pKM(AHSV-4) plasmids was obtained by PCR amplification and used in subsequent cell transfection experiments. Not only did this strategy result in the exclusion of the vector-borne T7 RNA polymerase promoter, but it was also anticipated that the lower molecular weight of the amplicons, as compared to that of the plasmid cDNA clones, would result in a higher transfection efficiency and thus a higher probability of a single cell containing a full complement of viral T7 transcription cassettes. However, transfection of BSR-T7 cell monolayers with the T7 transcription cassettes did not result in recovery of infectious AHSV-4. During the course of these investigations, the T7 RNA polymerase promoter immediately

flanking the viral genome segment was shown to be transcriptionally active, as was evidenced by fluorescing cells after transfection of BSR-T7 cells with the PCR-amplified T7 transcription cassette from the reporter plasmid pKM-eGFP. These results also indicated that the intracellularly synthesised transcripts can indeed serve as templates for translation. Interestingly, although not quantified, less fluorescent cells were observed and the fluorescence intensity appeared to be diminished, as compared to BSR-T7 cells transfected with the pKM-eGFP reporter plasmid. It is therefore tempting to suggest that this observed difference may be related to a difference in the strength of the respective T7 RNA polymerase promoters. This would, however, need to be confirmed by additional studies in which reporter plasmids, containing the individual T7 RNA polymerase promoters, are compared with regards to eGFP expression. Alternatively, these results may reflect on the stability of the transfected linear dsDNA in the intracellular environment. It has been reported previously that linear dsDNA products, as apposed to covalently closed circular plasmid DNA, are more sensitive to cytoplasmic degradation (Hsu and Uludag, 2008).

In addition to the strict requirement for authentic 5'- and 3'-terminal sequences to enable packaging and subsequent replication of the orbivirus mRNA, another factor that may have directly influenced recovery of infectious AHSV-4 concerns capping of the T7 transcripts derived from both the plasmid cDNA clones and the PCR-amplified T7 transcription cassettes. The absence of a cap structure at the 5' end of the cDNA-derived transcripts can greatly diminish their stability and the level of translation from these transcripts (Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977; Shatkin, 1985). For example, studies regarding transcription of a plasmid-based CAT reporter gene in cells that stably express T7 RNA polymerase have shown that the reporter gene transcripts are uncapped and therefore do not serve as efficacious templates for protein synthesis (Elroy-Stein and Moss, 1990). However, in the same study, CAT expression was increased 500-fold following infection of the transfected cells with vaccinia virus, which has an associated capping enzyme.

To investigate whether inefficient capping of the DNA-derived constructs may account for the lack of AHSV-4 recovery, a third experimental approach was subsequently adopted. For this purpose, transcripts were produced from PCR-amplified T7 transcription cassettes present in each of the pKM(AHSV-4) cDNA clones, using a commercial *in vitro* transcription and capping kit. The capped synthetic AHSV-4 RNA transcripts produced by this manner can thus be considered to be functional equivalents of AHSV core-derived transcripts. However,

infectious AHSV-4 could not be recovered following transfection of BSR cells with these *in vitro*-synthesised and -capped AHSV-4 transcripts. It should be noted that although the anti-reverse cap analogue that is incorporated into the 5' end of the transcripts can purportedly increase the proportion of transcripts that are capped in the correct orientation to 80% (Jemielity *et al.*, 2003), some variability in the efficiency of capping has been noted. Boyce *et al.* (2008) reported that the efficiency whereby infectious BTV could be recovered from synthetic RNA transcripts, prepared in a similar manner to that indicated above for AHSV, varied greatly (15-80%) and the variation in this efficiency was suggested to reflect variation in the quality or degree of capping of different T7 transcript preparations. Moreover, the recovery of BTV from T7 transcripts was reported to lead to the recovery of 100-fold fewer plaques than when an equivalent quantity of core-derived viral transcripts is used. Additionally, the uncapped transcripts have a 5' triphosphate moiety that is known to be a pathogen-associated molecular pattern recognised by RIG-I that may lead to the induction of antiviral responses (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006; Cuietal, 2008) and can thus influence the recovery of virus adversely. It is therefore tempting to conclude that variable capping of the AHSV-4 transcripts, together with the induction of antiviral responses by the uncapped transcripts, may have resulted in the inability to recover infectious AHSV-4.

The pKM(AHSV-4) DNA inserts were chemically synthesised based on a consensus sequence constructed from ultra-deep pyrosequencing data for an AHSV-4(HS32/62)isolate that had been passaged through suckling mouse brain, as well as BHK-21 and Vero cell monolayers. In addition, the BSR cell culture-adapted AHSV-4(HS32/62) isolate that was used for the successful recovery of AHSV-4 from core-derived mRNA displayed the same consensus sequence (Dr. A. C. Potgieter, personal communication). However, due to their error-prone RNA-dependant RNA polymerases, RNA viruses exist as a population of closely-related genetic variants (or quasispecies), which makes it impossible to predict the outcome of an infection with an isolated clone (Lauring and Andino, 2010). Thus, an RNA virus genome consensus sequence is by its very nature an average of multiple quasispecies produced during normal viral infection, and does not take into account the possibility of select sub-populations being responsible for viral replication under specific conditions. No data is currently available regarding predominating orbivirus sub-populations in cell culture; however, selection of distinct viral sub-populations has been reported upon passage between different vertebrate and invertebrate hosts for BTV (Bonneau *et al.*, 2001). A consensus sequence inclusive of an AHSV isolate that has been passaged through suckling mouse brain

may therefore obscure the predominance of a cell culture-specific viral sub-population, which is not selected for during *in vivo* neuronal replication. AHSV core-derived mRNA by comparison is likely to contain sub-populations of each segment that may be more suited to the recovery of virus from transcripts, and are therefore selected in combinations that ensure efficient virus recovery.

In conclusion, to achieve infectious AHSV recovery, gene expression must be sufficient to allow the assembly of progeny core particles, which themselves are transcriptionally active and therefore will lead to an amplification of gene expression. It therefore follows that a high level of gene expression is needed to assemble these incomplete virions. The results that were obtained during the course of this part of study suggest that levels of intracellular viral gene expression were likely insufficient to enable the assembly of progeny core particles. The low expression levels may be as a consequence of using a weak T7 RNA polymerase promoter to drive transcription of the AHSV-4 genome segments and especially the presence of uncapped or inefficiently capped cDNA-derived AHSV-4 transcripts. The latter would not only result in transcript instability, but also in inefficient protein synthesis and the induction of antiviral responses. The aim of a helper virus-independent reverse genetic system for AHSV is one that therefore requires ongoing pursuit. However, it is a pursuit that is entirely reasonable to achieve and will offer the ability to manipulate and study individual or multiple viral segments in the context of AHSV infection.

CHAPTER FOUR

GENERATION OF DIRECTED SEGMENT-SPECIFIC REASSORTANTS OF AFRICAN HORSE SICKNESS VIRUS

4.1 INTRODUCTION

The genus *Orbivirus* of the family *Reoviridae* comprises a number of important animal pathogens, such as African horse sickness virus (AHSV) and bluetongue virus (BTV). In contrast to BTV, which represents the best-studied orbivirus (Roy, 2008), many aspects regarding AHSV replication, morphogenesis and release, as well as the role of individual viral proteins in these processes still need to be elucidated. A combination of mutagenesis and re-expression of AHSV proteins in heterologous hosts has allowed progress to be made in relation to the structure-function relationships among some of the AHSV proteins, including structural proteins such as VP5 (Stassen *et al.*, 2011), VP6 (de Waal and Huismans, 2005) and VP7 (Burroughs *et al.*, 1994), as well as nonstructural proteins such as NS1 (Maree and Huismans, 1997), NS2 (Uitenweerde *et al.*, 1995) and NS3 (van Staden *et al.*, 1995; van Niekerk *et al.*, 2001). Despite these advances, definitive roles of many of these proteins in AHSV replication remain unresolved.

During the last decade, RNA interference (RNAi) has become a powerful research tool whereby loss-of-function phenotypes can be generated, thus allowing virus gene function to be determined within the context of an infected host cell. Amongst orbiviruses, this approach has been used to study the roles of BTV VP2 (Forzan *et al.*, 2007) and NS3 (Wirblich *et al.*, 2006) in virus entry and egress, respectively, as well as the role of AHSV VP7 in stable capsid assembly (Stassen *et al.*, 2007). Despite the success and usefulness of RNAi-based gene silencing approaches, several shortcomings to this technology have been identified, including the transient nature of gene silencing in the case of small interfering RNAs (siRNAs) (Elabshir *et al.*, 2002; Rao *et al.*, 2009), induction of apoptosis in the case of using vector-based RNAi approaches (Ghodgaonkar *et al.*, 2009; Kadan-Kulangara *et al.*, 2010), and the inability to study mutant versions of selected genes in the absence of complete gene silencing (Venter *et al.*, 2005). Many of these hurdles can be overcome if a reverse genetic system was available for AHSV that would allow for genetic manipulation of the virus. Indeed, the introduction of defined mutations into the genomes of many viruses has enabled rational approaches to the molecular dissection of viral gene products (Palese *et al.*, 1996; Walpita and Flick, 2005; Roy, 2005).

Recently, major advances were made regarding the development of reverse genetic systems for some *Reoviridae*. For mammalian orthoreoviruses and BTV, reverse genetic systems

based on the transfection of plasmid cDNA (Kobayashi *et al.*, 2007, 2010) or cDNA-derived T7 transcripts (Boyce *et al.*, 2008), corresponding to a complete set of the 10 genome segments have been established, allowing the rescue of infectious viral progeny. Results obtained during the course of this investigation indicated that AHSV remained recalcitrant to similar reverse genetic approaches (Chapter 3), although it is possible to recover infectious AHSV from viral mRNAs synthesised from core particles *in vitro* (Chapter 2). This therefore necessitated that alternative reverse genetic approaches for AHSV be investigated. A potentially useful approach relies on the construction of directed or "synthetic" reassortant viruses. In the case of BTV, it has been shown that *in vitro*-synthesised T7 transcripts, derived from cDNA clones, can be introduced into the genome of BTV using either a mixture of T7 transcripts and core-derived mRNAs (Boyce *et al.*, 2008) or through the transfection of a T7 transcript into virus-infected cells (van Gennip *et al.*, 2010). The targeted replacement of genome segment 7 or 10, as well as the simultaneous replacement of genome segments 2 and 5 was demonstrated in these studies. Although these approaches obviate the need for a full set of 10 cDNA clones, screening is required to identify the desired reassortant virus and the efficiency of these methods is variable. For example, it has been reported that the percentage of rescued reassortant virus varies between 3-5% (van Gennip *et al.*, 2010) and 15-80% (Boyce *et al.*, 2008). Notably, Trask *et al.* (2010) reported a methodology that combines characterised rotavirus temperature-sensitive mutants and validated siRNA targets to isolate a virus containing a single recombinant gene. In this helper virus-independent single-gene replacement strategy, a mutant SA11 rotavirus encoding a temperature-sensitive defect in the NSP2 protein was coupled with RNAi-mediated degradation of NSP2 mRNA to isolate virus containing a single recombinant gene that evades both selection mechanisms. The method appears to be efficient, since after two rounds of selective passage the virus population was found to be almost 100% recombinant (Trask *et al.*, 2010).

Based on the above, the primary aim of this part of the study was to investigate the targeted replacement of an AHSV genome segment with a T7 transcript derived from a cDNA clone as a method whereby cloned sequences can be introduced into the AHSV genome. In addition, the use of RNAi-mediated degradation of targeted mRNA as a means to reduce the virus background was also investigated. For these studies, the segment 10 gene, encoding the nonstructural protein NS3/NS3A, was selected for targeted replacement.

4.2 MATERIALS AND METHODS

4.2.1 Cells and viruses

BSR cells were propagated and maintained as monolayers in 75 cm^2 tissue culture flasks in Eagle's Minimal Essential Medium (EMEM) with Earle's Balanced Salt Solution (EBSS) and L-glutamine (Lonza, BioWhittaker®), supplemented with 5% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NEAA) and antibiotics (10 000 U/ml of penicillin, 10 000 µg/ml of streptomycin and 25 µg/ml of amphotericin B) (Hyclone). The flasks were incubated at 37°C in a humidified incubator with a constant supply of 5% CO_2. AHSV-4 and AHSV-3, kindly provided by Mr. F. Wege (Department of Genetics, University of Pretoria), was propagated in confluent BSR cell monolayers using low-passage stock viruses as inoculum.

4.2.2 Purification of AHSV-3 core particles and synthesis of *in vitro* transcripts

BSR cell monolayers were infected with AHSV-3 at a multiplicity of infection (MOI) of 0.08 pfu/cell and incubated at 37°C in a CO_2 incubator for 72 h. Transcriptionally active core particles were purified using the same methods as described previously for the purification of AHSV-4 core particles (Chapter 2, Section 2.2.2). Essentially, the virus-infected cells were lysed and nuclei removed by centrifugation. The outer capsid proteins of virions present in the recovered supernatant was removed by incubation with α-chymotrypsin, after which the core particles were purified by ultracentrifugation through a 40% (w/v) sucrose cushion. The pellet was suspended in 80 µl of 20 mM Tris-HCl (pH 8.0) and stored at 4°C. AHSV-3 core-derived transcripts were synthesised and purified using methodology similar to that used for AHSV-4 (Chapter 2, Section 2.2.4). Briefly, the AHSV-3 core particles (75 µg/ml) were incubated at 30°C for 6 h in AHSV core transcription buffer (100 mM Tris-HCl [pH 8.0], 4 mM ATP, 2 mM GTP, 2 mM CTP, 2 mM UTP, 500 µM *S*-adenosylmethionine, 6 mM DTT, 9 mM $MgCl_2$, 5 U/µl of Protector RNase inhibitor [Roche Diagnostics]). The AHSV-3 core-derived ssRNAs were then precipitated with LiCl and purified with a Nucleospin® RNA Clean-up kit (Macherey-Nagel). An aliquot of the purified ssRNA was analysed by electrophoresis on a 1% (w/v) agarose gel, and the purity and concentration of the viral ssRNA was determined with a Nanodrop® ND-1000 spectrophotometer (Thermo Fischer Scientific).

4.2.3 *In vitro* synthesis of AHSV-4 segment 10 transcripts

Recombinant plasmid pKM-S10, containing a synthetic cDNA copy of the AHSV-4 segment 10 gene under the transcriptional control of a T7 promoter, has been described (Chapter 3, Section 3.2.1). For synthesis of T7 transcripts corresponding to AHSV-4 gene segment 10, the T7 transcription cassette was PCR-amplified from plasmid pKM-S10 with primers T7-orbi F1 (5'-AGGAAGGCCGTCAAG-3') and NS3R (5'-GTATGTTGAAATCCGC-3'), and the reaction conditions described previously (Chapter 3, Section 3.2.6). The amplicon was purified from an agarose gel with the Zymoclean Gel DNA Recovery™ kit (Zymoclean Research Corporation) and subsequently used as template for *in vitro* transcription reactions. Transcripts with a 5' cap analogue were generated with the MessageMAX™ T7 ARCA-capped Message Transcription kit (Epicentre Biotechnologies), using a 4:1 ratio of anti-reverse cap analogue to rGTP, according to the manufacturer's instructions. Following removal of the DNA template with RNase-free DNase I, the transcripts were purified with the Nucleospin® RNA Clean-up kit (Macherey-Nagel). The concentration of the *in vitro*-synthesised and -capped S10 transcripts was determined with a Nanodrop® ND-1000 spectrophotometer (Thermo Fischer Scientific).

4.2.4 Co-transfection of AHSV-3 mRNA and synthetic AHSV-4 segment 10 transcripts into BSR cells

BSR cells were seeded in 24-well tissue culture plates to reach 80% confluency within 6 h of incubation at 37°C in a CO_2 incubator. The BSR cells were then transfected with a mixture of AHSV-3 core-derived ssRNA and the T7 transcript of AHSV-4 segment 10 using Lipofectamine™ 2000 reagent (Invitrogen), according to the manufacturer's instructions. Briefly, 800 ng of AHSV-3 core-derived ssRNA and 800 ng of the AHSV-4 segment 10 T7 transcript was added together and the mixture was then diluted in 50 µl of EMEM medium (without serum and antibiotics). Likewise, 2 µl of Lipofectamine™ 2000 reagent was diluted in 50 µl of serum- and antibiotic-free EMEM medium. Following incubation at room temperature for 5 min, the two solutions were mixed and incubated at room temperature for 20 min to allow the formation of RNA-lipofectamine complexes. The cell monolayers were prepared for transfection by rinsing twice with 500 µl of EMEM medium (without serum and antibiotics), followed by addition of 500 µl of antibiotic-free EMEM medium containing 5% (v/v) FBS. The BSR cell monolayers were subsequently overlaid with the RNA-lipofectamine complexes and the tissue culture plates were incubated at 37°C for 16 h in a

CO_2 incubator. Following incubation, the cell monolayers were transfected for a second time, as described above, and incubated for a further 72 h in the CO_2 incubator. The cell monolayers were then processed for plaque assays as described below.

4.2.5 Enhancing AHSV single-gene reassortment using an siRNA-mediated selection mechanism

4.2.5.1 Small interfering RNA (siRNA)

Previously, siRNAs targeting three distinct regions on the AHSV-3 segment 10 mRNA have been designed and evaluated for their ability to silence NS3 gene expression. Quantitative real-time PCR analyses indicated that the siRNA, designated siNS3-266, was capable of reducing the level of AHSV-3 mRNA transcripts in virus-infected mammalian cells in excess of 90% (Barnes, 2011). Consequently, siNS3-266 was selected for use as a selection mechanism to drive single-gene reassortment in AHSV. The siRNA has the following sequence: Sense strand, 5'-GAACCGAUACGUCAAAUAAtt-3'; Antisense strand, 5'-UUAUUUUGACGUAUCGGUUCtg-3'.

4.2.5.2 Co-transfection of siNS3-266 and synthetic AHSV-4 segment 10 mRNA into BSR cells prior to infection with AHSV-3

BSR cells were seeded in 24-well tissue culture plates to reach 80% confluency within 6 h of incubation at 37°C in a CO_2 incubator. The BSR cell monolayers were prepared for transfection and then transfected with a mixture of siNS3-266 (20 pmol) and the T7 transcript of AHSV-4 segment 10 (800 ng) using Lipofectamine™ 2000 reagent (Invitrogen), as described above. The tissue culture plates were incubated at 37°C for 12 h in a CO_2 incubator. Following incubation, a second transfection, identical to the first, was performed on the BSR cell monolayers, which were subsequently incubated for a further 12 h. The transfected BSR cell monolayers were then infected with AHSV-3 at a MOI of 0.1 pfu/cell. After 1 h of infection, the virus inoculum was removed and 500 µl of complete EMEM medium was added to the BSR monolayers. At 24 h post-infection, the cell monolayers were processed for plaque assays.

4.2.6 Plaque assays and amplification of picked plaques

Transfected BSR cells were harvested in the culture medium, transferred to Eppendorf tubes and subjected to three successive freeze-thaw cycles to lyse the cells. Ten-fold dilutions of

the cell lysates were subsequently used to infect confluent BSR cell monolayers in 6-well tissue culture plates. Following incubation at 37°C for 1 h, the inoculums were aspirated and the cells were overlaid with 0.5% (w/v) agarose solution (Seakem LE agarose; prepared in complete EMEM medium). The tissue culture plates were subsequently incubated at 37°C for 96 h in a CO_2 incubator. To visualise the plaques, the cell monolayers were flooded with 500 µl of Neutral Red (0.05% [w/v] 1 × in PBS) and the tissue culture plates were incubated at 37°C for a further 48 h, after which the Neutral Red solution was removed. Individual plaques were selected randomly, and each plaque was plucked as an agarose plug with a pipette and placed in an Eppendorf tube containing 100 µl of complete EMEM medium. Virus was eluted from the agarose plugs by incubation at 37°C for 24 h. Individual wells of a 6-well tissue culture plate, each containing a confluent BSR monolayer, were inoculated with a separate plaque pickup (100 µl) in a final volume of 1 ml of complete EMEM medium. Following incubation at 37°C for 4 days the cell monolayers were harvested and screened for the presence of reassortant virus as described below.

4.2.7 Screening of transfection-derived AHSV plaques for reassortants containing the introduced genome segment

4.2.7.1 Primers

Primers for amplification of the segment 10 gene of the respective AHSV serotypes were designed based on the nucleotide sequence of the AHSV-4 NS3 gene (as determined in Chapter 3) and of the AHSV-3 NS3 gene (GenBank Acc. No. DQ868782). The primers were designed with PerlPrimer v.1.1.18 (Marshall, 2004) and each of the primers was subjected to a BLAST*n* analysis to verify target sequence specificity. The primers, indicated in Table 4.1, were obtained from Inqaba Biotechnical Industries.

Table 4.1: Primers for screening AHSV segment 10 cDNA

Primer*	Sequence
A3S10-186F	5'-GATACTTAACCAAGCCATGTC-3'
A3S10-700R	5'-GTTTGATCCACCCAACACTG-3'
A4S10-239F	5'-CTATGGCGGAAGCATTGC-3'
A4S10-742R	5'-GTATGTTGTTATCCCACTCC-3'

*A3/A4S10 denotes the virus serotype and segment which primers target; the numbers which follow denote the nucleotide position at which the primer anneals to the target gene. F - forward primer; R - reverse primer

4.2.7.2 Extraction of dsRNA from recovered AHSV

Total RNA was extracted from BSR cell monolayers infected with plaque-picked AHSV isolates using the Aurum™ Total RNA Extraction kit (BioRad) according to the manufacturer's instructions. The dsRNA was then selectively purified as follows. To precipitate ssRNA, 8 M LiCl was added to a final concentration of 2 M and the samples were incubated at 4°C for 12 h. Following centrifugation at 17000 × *g* at 4°C for 25 min, the supernatant was collected and the dsRNA was precipitated from the supernatant by addition of 0.1 volume of NaOAc and 2 volumes of absolute ethanol. The samples were centrifuged at 17 000 × *g* at 4°C for 30 min and the pellets were washed twice with 70% ethanol before being suspended in 20 µl of DEPC-treated dH_2O. The dsRNA was then analysed by electrophoresis on a 1% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris-HCl, 20mM NaOAc, 1 mM EDTA; pH 8.5) (Sambrook *et al.*, 1989).

4.2.7.3 cDNA synthesis

The extracted dsRNA was reverse transcribed with the RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions. Prior to reverse transcription, each dsRNA preparation was incubated with 1 µl of RNase-free DNase I to ensure removal of contaminating DNA. The enzyme was subsequently inactivated by incubation at 65°C for 10 min. For reverse transcription, 1 µg of the dsRNA sample was mixed with 1 µl of random hexamer primers and nuclease-free water was added to a final volume of 12 µl. Following incubation at 65°C for 5 min, 4 µl of 5 × Reaction buffer, 1 µl of RiboLock RNase inhibitor (20 U/µl), 2 µl of a 10 mM dNTP mixture and 1 µl of RevertAid™ H Minus M-MuLV reverse transcriptase (200 U/µl) was added to the reaction. The reaction mixture was incubated at 42°C for 1 h, after which the enzyme was inactivated by heating to 70°C for 5 min. As controls, reaction mixtures lacking either reverse transcriptase enzyme or dsRNA template were included.

4.2.7.4 Polymerase chain reaction (PCR)

To identify AHSV reassortants, PCR amplification was performed with primer pairs specific for either the AHSV-3 or AHSV-4 segment 10 gene (Table 4.1). Each of the PCR reaction mixtures (20 µl) contained 1 µl of a 10-fold dilution of the cDNA as template, 10 pmol of each the forward and reverse primers, 1 × PCR buffer (75 mM Tris-HCl [pH 8.8], 16 mM $(NH_4)_2SO_2$, 0.1% [v/v] Tween-20), 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1 U of

SUPERTHERM *Taq* DNA polymerase (Southern Cross Biotechnology). The PCR was performed in a Perkin-Elmer GeneAmp® 2700 thermal cycler. The DNA was initially denatured at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s and elongation at 72°C for 1 min. The last cycle was followed by an elongation step at 72°C for 3 min to complete synthesis of all DNA strands. A negative control was also included in which the DNA template had been omitted. Aliquots of the reaction mixtures were analysed by electrophoresis on a 1% (w/v) agarose gel in the presence of an appropriate DNA molecular weight marker.

4.2.7.5 Nucleotide sequencing and analysis

The nucleotide sequence of amplicons derived from putative reassortant viruses was determined using the ABI-PRISM® BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Perkin-Elmer Applied Biosystems) on an ABI-PRISM® Model 377 DNA sequencer. The sequencing reactions were performed, as described previously (Chapter 3, Section 3.2.4.2), by making use of NS3 239F (5'-CTATGGCGGAAGCATTGC-3') as the sequencing primer. Nucleotide sequences were analysed with the BioEdit v.7.0.4.1 software package (Hall, 1999) and multiple sequence alignments were performed with ClustalW included in the software package.

4.3 RESULTS

4.3.1 Purification of core particles, synthesis of core-derived transcripts and recovery of AHSV-3

AHSV-3 core particles were purified from virus-infected BSR cells at 72 h post-infection according to the procedure used previously to successfully purify AHSV-4 cores (Chapter 2). The protein composition of the core particles was verified by SDS-PAGE analysis and core particles were detected by negative-staining transmission electron microscopy. The results respectively indicated the presence of all five of the core proteins (VP1, VP3, VP4, VP6 and VP7) in the absence of both of the outer capsid proteins VP2 and VP5 (Fig. 4.1A), and core particles of *ca.* 65 nm in diameter were detected by electron microscopy (Fig. 4.1B). The purified AHSV-3 core particles were subsequently used for *in vitro* ssRNA synthesis. The core-derived transcripts were purified and analysed by agarose gel electrophoresis, the result of which confirmed the presence of all 10 viral transcripts (Fig. 4.1C).

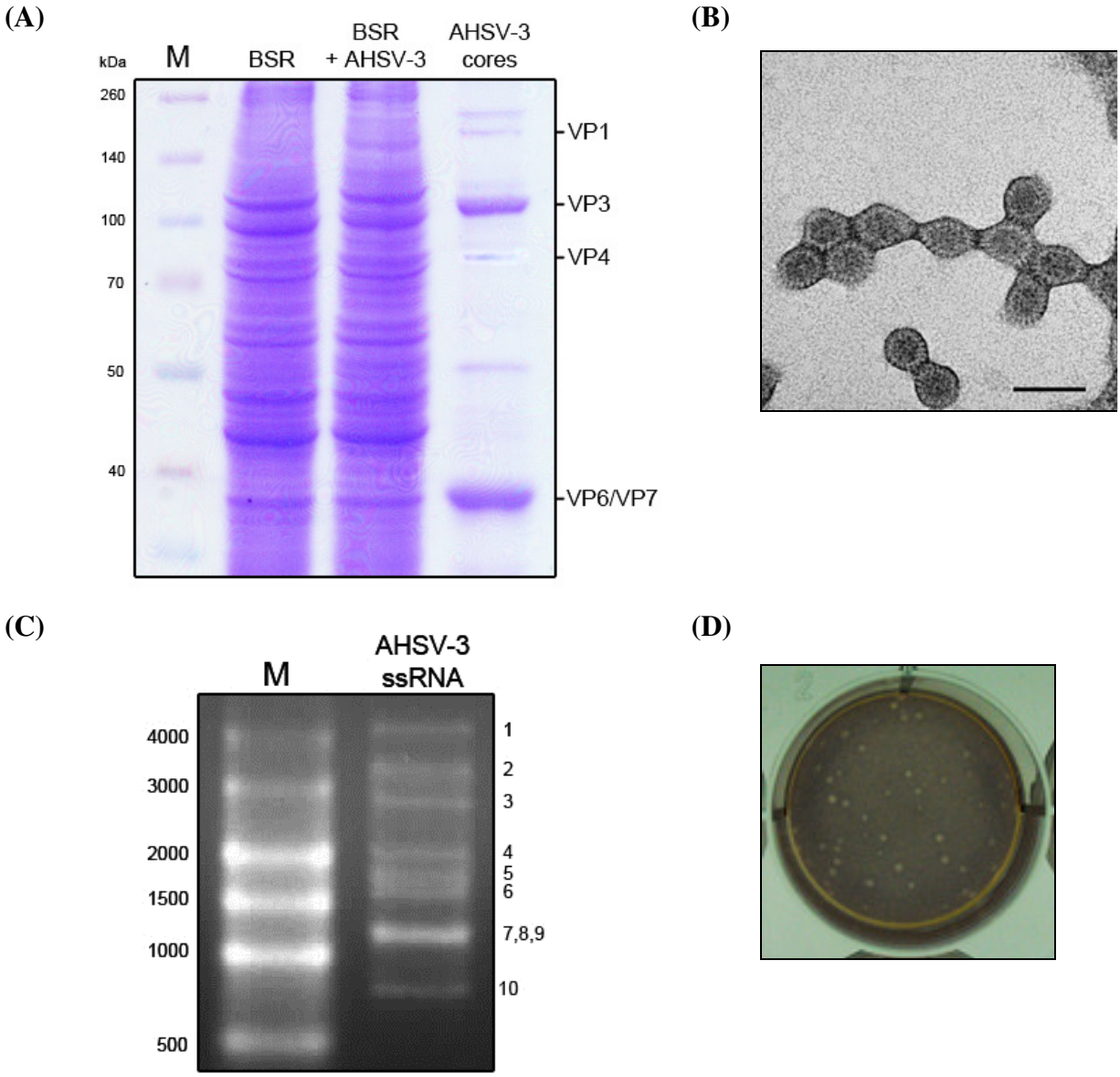


Fig. 4.1: Purification of AHSV-3 core particles, *in vitro* transcription of AHSV-3 core-derived transcripts and recovery of AHSV-3. AHSV-3 core particles were purified from BSR cells, using the same method as described for AHSV-4 in Chapter 2. **(A)** SDS-PAGE of purified AHSV-3 core particles with representative proteins shown. **(B)** TEM micrograph of purified core particles, scale bar = 80 nm**. (C)** AHSV-3 core-derived ssRNA generated following *in vitro* transcription of purified core particles. **(D)** Results of plaque assay performed on the transfection lysate of BSR cells, which had been transfected twice with 800 ng of the *in vitro*-synthesised AHSV-3 ssRNA.

To determine whether AHSV-3 could be recovered from the core-derived ssRNA, BSR cells were transfected twice with the AHSV-3 core transcripts at 16-h intervals. Plaque assays were performed on the transfection lysate at 72 h post-second transfection, and clear well-defined plaques were observed (Fig. 4.1D). Individual plaques were subsequently picked and the virus was amplified by infecting BSR cells. Following incubation for 96 h, dsRNA was extracted and analysed on an agarose gel. The migration patterns of the extracted dsRNA segments were identical to that of the parental AHSV-3 serotype (results not shown). Cumulatively, these results therefore confirmed that AHSV-3 can be recovered from its corresponding core transcripts upon transfection into permissive cells.

4.3.2 Introduction of an AHSV-4 segment derived from a cDNA clone into the AHSV-3 genome

Reassortment between genome segments of two different serotypes of AHSV occurs naturally during co-infection (Potgieter *et al.*, 2009; Meiring *et al.*, 2009), indicating that some degree of variation in genome segments does not abrogate packaging or replication. With the aim of developing a reverse genetic system for AHSV, the targeted replacement of a genome segment with a T7 transcript from a cDNA clone was investigated. For this purpose, the introduction of the AHSV-4 segment 10 T7 transcript into the genome of AHSV-3 was selected.

The AHSV-4 segment 10 T7 transcript was produced from a T7 transcription cassette, which had been PCR-amplified from recombinant plasmid pKM-S10 using appropriate primers. The amplified transcription cassette has a T7 RNA polymerase promoter to generate the authentic 5' end sequence, whereas the downstream primer was designed to ensure that the authentic 3' end sequence is present. Following *in vitro* synthesis of the segment 10 T7 transcript in the presence of a 5' cap analogue, the full-length T7 transcript was purified (Fig. 4.2A). The AHSV-4 segment 10 T7 transcript was subsequently mixed with AHSV-3 transcripts produced from core particle transcription reactions and then transfected into BSR cells by making use of the double-transfection protocol described above. A plaque assay was performed at 72 h post-second transfection. Typically, *ca.* 15 plaques were obtained after a 10^{-3} dilution of the transfected cell lysate.

The recovery of viruses containing the cDNA-derived AHSV-4 segment 10 was screened for by first amplifying the virus from randomly selected plaques. The dsRNA was purified from each amplified plaque, cDNA was synthesised and the origin of the dsRNA genome segments

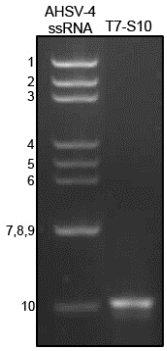
was then determined by PCR using primers specific for the S10 segment of AHSV-4 and AHSV-3 in order to discriminate between reassortants and wild-type AHSV. The products were resolved by electrophoresis in agarose gels and the identity of amplicons obtained by PCR amplification using the AHSV-4 segment 10-specific primers was furthermore confirmed by nucleotide sequencing. The sequence of this segment 10 gene was 100% identical to that of the pKM-S10 cDNA. Representative results are provided in Fig. 4.2. The frequency at which reassortant progeny was recovered was 8%. This data serves to illustrate the successful introduction of a cDNA-derived AHSV-4 segment into the genome of viable AHSV-3.

4.3.3 Alternative approach for single-gene replacement in AHSV

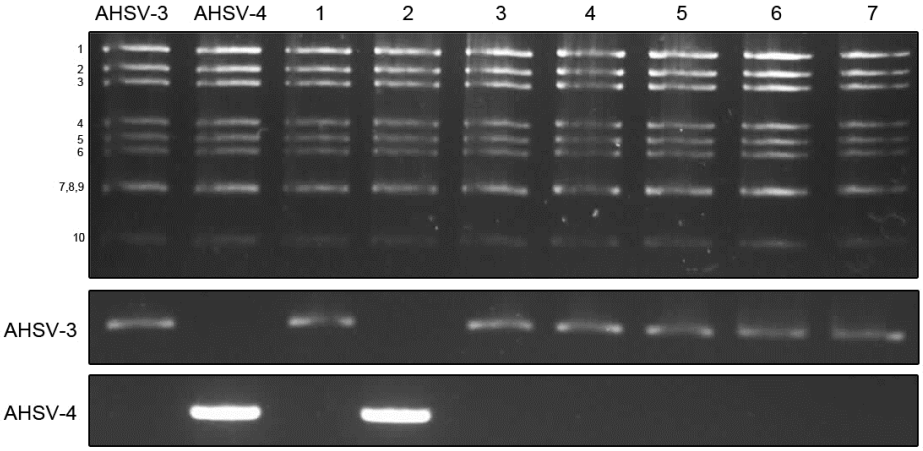
Although the above represents a useful reverse genetic system, disadvantages associated with the approach are the requirement for purified core particles and the inefficient recovery of progeny virus. To overcome these impediments, an alternative method for targeted single-gene replacement was investigated. This system is based on the uptake of an *in vitro*-synthesised viral RNA in an ongoing viral infection, thus obviating the need for core-derived ssRNA transcripts. Moreover, siRNA transfection was incorporated into the methodology since it represents a quick and technically simple method to diminish the transcription of a targeted parental virus gene. With regards to the latter, three different siRNAs were evaluated previously for their ability to silence expression of the AHSV-3 NS3 gene using both a NS3 expression reporter plasmid and an *in vitro* model of infection. Results were obtained that showed that a synthetic siRNA, siNS3-266, silenced AHSV-3 mRNA and protein expression effectively (Barnes, 2011). Therefore, this AHSV-3 NS3-directed siRNA was selected for further use in this study.

To evaluate the feasibility of such a system, BSR cell monolayers were transfected twice at 12-h intervals with a mixture of siNS3-266 and the *in vitro*-synthesised T7 transcript of the S10 segment of AHSV-4. The transfected cells were subsequently infected with AHSV-3 at a MOI of 0.1 pfu/cell, and at 24 h post-infection, the cells were harvested and subjected to plaque assays. To verify the presence of recombinant virus in the samples, plaque isolates were amplified and screened for the presence of the newly introduced S10 genome segment as described above. In this case, none of the virus screened contained the S10 genome segment originating from AHSV-4 (Fig. 4.3). This result therefore suggests that this approach is inefficient to drive single-gene reverse genetics for AHSV.

(A)



(B)



(C)

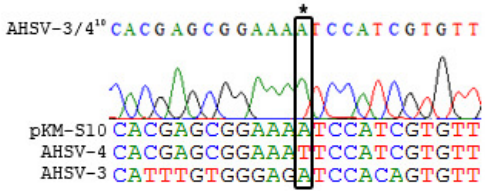


Fig. 4.2: Generation of an AHSV-3/4[10] reassortant virus using AHSV-3 core-derived ssRNA and a plasmid cDNA-derived AHSV-4 segment 10 T7 transcript. **(A)** AHSV-3 transcripts were derived from core-particles, whereas a T7 transcript of the AHSV-4 genome segment 10 (T7-S10) was prepared using a commercially available *in vitro* RNA synthesis and capping kit. **(B)** A mixture of the AHSV-3 core-derived ssRNA and *in vitro*-synthesised AHSV-4 segment 10 T7 transcript was transfected twice into BSR cells. At 72 h post-second transfection, a plaque assay was performed and individual plaques were picked, amplified and the viral dsRNA extracted. Subsequently, cDNA was synthesised and the origin of the viral segment 10 was determined by serotype-specific (AHSV-3 or -4) PCR amplification. **(C)** To confirm the identity of the segment 10 genome segment amplified by AHSV-4 specific primers from putative reassortant virus (Isolate 2) the nucleotide sequence of the amplicon was determined. The sequence was identical to that of the pKM-S10 nucleotide sequence, but differed from the nucleotide sequence of the AHSV-3 S10 gene segment. The nucleotide difference (indicated by asterisk) between the pKM-S10 sequence and that of AHSV-4 S10 is due to the pKM-S10 insert having been synthesised from an AHSV-4 consensus sequence (see Chapter 3).

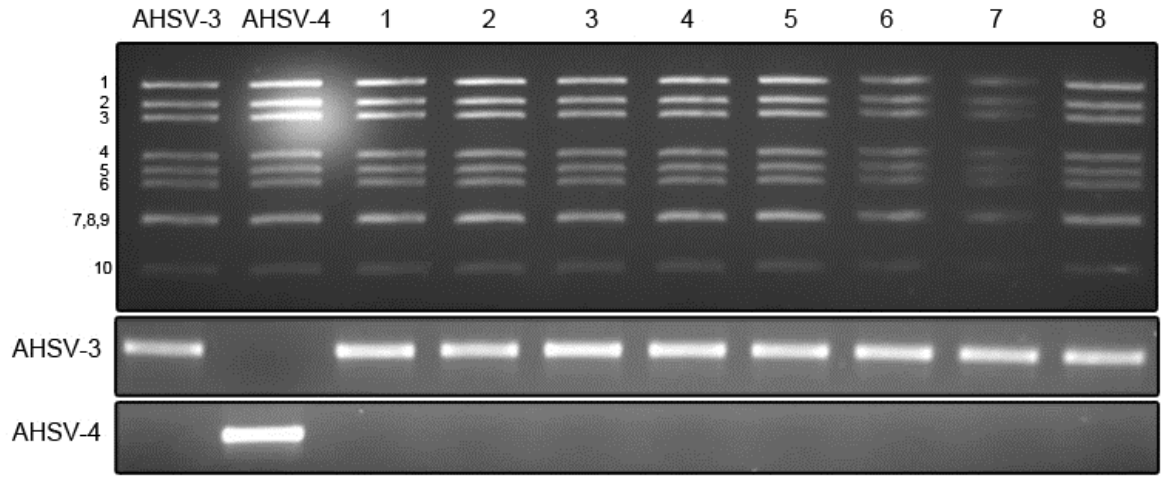


Fig. 4.3: Screening for the production of AHSV-3/4[10] reassortant virus by an siRNA-mediated selection mechanism. BSR cells were transfected twice with a mixture of the AHSV-4 segment 10 T7 transcript and siNS3-266 before being infected with AHSV-3. At 24 post-infection, plaque assays were performed and individual plaques were picked, amplified and the viral dsRNA extracted. Subsequently, cDNA was synthesised and the origin of the viral segment 10 was determined by serotype-specific (AHSV-3 or -4) PCR amplification. No reassortant virus, containing AHSV-4 genome segment 10, was isolated.

4.4 DISCUSSION

One of the greatest hurdles that have hampered research into AHSV is the inability to manipulate the 10-segmented dsRNA genome through reverse genetics. Recent advances for orthoreovirus (Kobayashi *et al.*, 2007, 2010) and BTV (Boyce *et al.*, 2008) have demonstrated that it is possible to recover infectious virus entirely from recombinant sources. In contrast, results obtained during the course of this investigation have indicated that AHSV is recalcitrant to similar methods (Chapter 3). Consequently, in this part of the study, an alternative reverse genetic method was investigated for its ability to generate recombinant AHSV.

In the case of BTV, it has been reported that recombinant virus can be generated by mixing core-derived ssRNA and a plasmid cDNA-derived T7 transcript (Boyce *et al.*, 2008). Thus, the targeted introduction of an AHSV-4 segment 10 T7 transcript into the genome of AHSV-3 was investigated in order to determine whether the facile introduction of cDNA-derived transcripts into AHSV is likewise possible. Transfection of BSR cell monolayers with a mixture of the AHSV-4 T7 transcript and AHSV-3 core-derived ssRNA yielded reassortant

plaques, of which the identity was confirmed by a serogroup discriminating PCR assay and nucleotide sequencing of the genome segment 10 amplicon. These results indicate that genome segment 10 of AHSV-4 is therefore functionally compatible with a background of AHSV-3 genome segments, both at the levels of RNA packaging and replication, and NS3 function. Importantly, these results furthermore demonstrate that this single-gene replacement system represents a feasible reverse genetic approach for AHSV.

Despite the success of this approach, recombinant AHSV was generated at a relatively low frequency (8%). This low efficiency may reflect variation in the degree of capping of the T7 transcript preparations since only a portion of T7 transcripts generated in the presence of cap analogue have the cap analogue incorporated at the 5' end (Jemielity *et al.*, 2003). In addition to being poorly translated, the uncapped transcripts may be defective during RNA packaging and replication, or transcription in the next round of infection. Moreover, the induction of antiviral responses to the 5' triphosphate of the uncapped T7 transcripts via RIG-1 may also influence the recovery of virus (Cui *et al.*, 2008; Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). Although not investigated in this study, it can be envisaged that the described method can be improved to increase the percentage of recombinant AHSV. This could be achieved by increasing the amount of *in vitro*-synthesised T7 transcript in the transfected cells, either through repeated transfection or through intracellular RNA synthesis by making use of BSR-T7 cells and the recombinant plasmid pKM-S10, which contains a cDNA copy of AHSV-4 genome segment 10 flanked by an upstream T7 RNA polymerase promoter and a downstream ribozyme sequence.

Alternative methods whereby targeted single-gene replacements can be made have been described for BTV and rotavirus. In the case of BTV, van Gennip *et al.* (2010) reported a method that is based on the uptake of one *in vitro*-synthesised viral transcript in an ongoing infection. An obvious advantage of this method is that it obviates the need to isolate and purify core particles as a source of viral mRNA, but it was reported that a high percentage of parental virus do not reassort with the transfected *in vitro*-synthesised T7 transcript. However, Trask *et al.* (2010) described an efficient method for the generation of recombinant rotavirus in which RNAi-mediated degradation of segment-specific transcripts was used to decrease the parental virus background. Consequently, these two approaches were combined in order to develop a simplified, cheap and more efficient single-gene reverse genetic system for AHSV. To investigate, BSR cell monolayers were transfected with a mixture of *in vitro*-

synthesised AHSV-4 segment 10 T7 transcript and a siRNA directed against the corresponding gene of AHSV-3, followed by infection of the transfected cells with AHSV-3. In contrast to the method discussed above, this approach did not result in the recovery of recombinant AHSV.

The failure to recover recombinant AHSV may have been due to several factors. It should be kept in mind that siRNA-mediated gene silencing is transient and it is therefore inevitable that the RNAi effect is abrogated over time. It is also unlikely that all of the cells were transfected with siRNA and it is thus plausible that the untransfected cells might provide a reservoir for unrestricted parent virus growth. Under these experimental conditions and by taking into account that the frequency of generating recombinant virus is low, it therefore follows that the recombinant will be a minor component of the virus population (relative to AHSV-3). Initial reports have indicated that a single mismatch between the antisense strand of the siRNA and the target mRNA can abolish siRNA activity (Tuschl *et al.*, 1999; Elbashir *et al.*, 2001). More recent reports, however, have indicated that, depending on the position of the mismatch, siRNA activity could be affected to different extents (Ge *et al.*, 2010). Single mutations within the centre of a siRNA duplex appear to be more discriminating than mutations located at the 5' end or 3' end, and in some cases there is enough activity left to mediate significant gene silencing (Amarzguioui *et al.*, 2003). Considering that the siRNA used in this study displays a single mismatch between the NS3 gene sequences of AHSV-3 and AHSV-4 and that the mismatch is present 6 nt from the 5' end of the antisense strand, it is possible that the transfected AHSV-4 segment 10 T7 transcript may also have been targeted for degradation by the RNAi machinery, thereby diminishing the prospect of its incorporation into progeny virions. Notably, results obtained with rotavirus have indicated that siRNAs directed to a given transcript impaired the synthesis of the corresponding protein, but not of the corresponding RNA genome segment (Silvestri *et al.*, 2004). It was proposed that viral mRNAs establish two functionally different and physically separated pools. One pool is outside of the viroplasms (the intracellular site of virus morphogenesis and replication), which undergoes translation and are susceptible to siRNA-mediated degradation, whereas the second pool is located inside viroplasms, which undergoes replication and are inaccessible to the RNAi machinery. The formation of virus inclusion bodies (VIBs) during AHSV replication may similarly present a challenge for the RNAi machinery. In contrast to the cytoplasmic AHSV-4 segment 10 T7 transcripts, the AHSV-3 core particle is localised to VIBs where it supplies viral mRNA for packaging and replication in nascent virions. Since

the viral mRNA is protected from RNAi-mediated degradation in the VIBs, it follows that the parent virus will dominate the virus population.

In conclusion, the results obtained in this part of the study indicate that using a mixture of authentic viral transcripts and T7 transcripts is representative of a feasible reverse genetic system for AHSV. Since the recovery of recombinant virus containing one plasmid cDNA-derived genome segment requires the construction of a single clone or PCR product, this targeted single-gene replacement system is potentially applicable to any genome segment. The utility of the system can be expanded through the uptake of mutated genome segments, thereby opening many new possibilities for investigating AHSV protein function. As such, it not only represents a valuable milestone toward the development of a reverse genetic system for AHSV, but also a powerful tool for studies aimed at understanding AHSV biology.

CHAPTER FIVE

CONCLUDING REMARKS

Although much progress has been made regarding the structure-function relationship of different African horse sickness virus (AHSV) genes and encoded gene products (Uitenweerde *et al.*, 1995; Maree and Huismans, 1997; van Niekerk *et al.*, 2001; de Waal and Huismans, 2005; Stassen *et al.*, 2011), knowledge of the molecular biology of AHSV and the role of each protein in the infectious cycle is still lacking. Research into AHSV could benefit greatly from a reverse genetic system that would allow for genetic manipulation of the individual genome segments. Strategies to rescue infectious viruses from cloned cDNA or *in vitro*-synthesised T7 transcripts have only recently been reported for some members of the *Reoviridae* family, including orthoreovirus (Kobayashi *et al.*, 2007, 2010) and bluetongue virus (BTV) (Boyce *et al.*, 2008). Consequently, the aims of this study were essentially to determine whether AHSV core-derived ssRNA is infectious when transfected into permissive cells, and to develop a reverse genetic system for AHSV. In this conclusion, the information that has evolved during the course of this study will be summarised briefly and some suggestions regarding future research will be made.

In this study, AHSV-4 core-derived ssRNA was shown to be infectious, as evidenced by the recovery of infectious virus upon transfection of the ssRNA into permissive BSR mammalian cells (Chapter 2). The methodology developed and optimised during the course of this study was shown to be reproducible, since infectious AHSV-3 could also be recovered using an identical approach (Chapter 4). Interestingly, AHSV-4 could only be recovered in BSR and BHK-21 cells, but not in Vero cells. The former is not unexpected, since BSR cells are considered to be a clone of BHK-21 cells and the AHSV-4 isolate used in this study had previously been adapted for growth in BSR cell monolayers. At present there is no clear explanation for the lack of AHSV-4 recovery in Vero cells, albeit that these cells are permissive for AHSV replication. Importantly, the results obtained in this part of the study suggested that infectious AHSV may be recovered from recombinant sources, provided that the AHSV ssRNA bear authentic 5'- and 3'-terminal sequences and are capped at their 5' end.

Subsequently, different DNA-based and synthetic mRNA-based reverse genetic approaches for the recovery of infectious AHSV-4 were evaluated (Chapter 3). An entirely plasmid-based reverse genetic system, in which chemically synthesised full-length cDNA copies of the AHSV-4 genome segments are flanked by an upstream T7 RNA polymerase promoter and by a downstream hepatitis delta virus (HDV) ribozyme sequence, was initially used to

transfect BSR-T7 monolayers. However, this approach did not allow for the recovery of infectious AHSV-4. Sequence analysis of the recombinant plasmid DNA indicated the presence of a second vector-borne T7 RNA polymerase promoter that is located upstream of the promoter flanking the AHSV-4 genome segments, and has a sequence identical to the consensus sequence of T7 RNA polymerase promoters (Richardson, 1983). It is therefore likely that transcription is preferentially initiated from this promoter, thereby resulting in the synthesis of aberrant transcripts with an extended 5'-terminus that are incapable of serving as templates for packaging and subsequent replication. To overcome this impediment, T7 transcription cassettes of each cloned AHSV-4 cDNA genome segment were PCR amplified, thus excluding the vector-borne T7 RNA polymerase promoter. However, transfection of the T7 transcription cassettes into BSR-T7 cells did not result in virus recovery. In addition to technical challenges, *e.g.* the inability to transfect a single cell with all 10 plasmid cDNA clones or T7 transcription cassettes and the greater susceptibility of linear dsDNA to intracellular nuclease degradation (Hsu and Uludag, 2008), it is also possible that the *in vivo*-transcribed AHSV-4 transcripts are not efficiently capped. Since T7 RNA polymerase does not possess capping activity, capping of the transcripts is dependent on the synthesis of VP4, which, in the case of BTV, has been reported to posses nucleotide phosphohydrolase (NTPase), guanylyltransferase (GTPase) and transmethylase type 1 and type 2 activities (Ramadevi *et al.*, 1998; Martinez-Costas *et al.*, 1998). Notably, transcripts produced in the absence of a 5'-terminal capping mechanism have been reported to be prone to degradation and display reduced translation efficiency (Furuichi *et al.*, 1977; Shimotohno *et al.*, 1977). Consequently, it is likely that the viral proteins were not synthesised at levels high enough to allow for the formation of nascent core particles, thus leading to an absence of capped AHSV-4 transcripts. To address the possibility of inefficient capping of the AHSV-4 transcripts, the PCR-amplified T7 transcription cassettes were subsequently used as templates for the production of *in vitro*-synthesised and -capped T7 transcripts using a commercially available kit. In contrast to the above DNA-based reverse genetic approaches, the synthetic AHSV-4 ssRNA reverse genetic method represents a more robust method that allows for qualitative and quantitative evaluation of the *in vitro*-synthesised AHSV-4 genome segment transcripts prior to cell transfection. However, it was still not possible to recover infectious AHSV-4 using this approach. In addition to differences in the capping efficiency between AHSV-4 core-derived ssRNA and the *in vitro*-synthesised and -capped AHSV-4 T7 transcripts, the only other difference between them concern their sequence. The DNA templates for the latter had been chemically synthesised based on a consensus sequence,

following ultra-deep pyrosequencing of the parental virus isolate that had been passaged in suckling mouse brain, as well as BHK-21 and Vero cell monolayers (Dr. A. C. Potgieter, personal communication). It is thus tempting to speculate that the derived virus population following transfection of the cells may not be the most genetically fit population adapted for growth in the cultured mammalian cells. In this regard, it is interesting to note that different viral sub-populations have been shown to exist upon passage of BTV between different vertebrate and invertebrate hosts (Bonneau *et al.*, 2001).

Although there is great merit in developing a plasmid cDNA-based reverse genetic system for AHSV, the current system and approach may need further refinement. The currently available pKM(AHSV-4) constructs would need to be modified in order to delete or inactivate the vector-borne T7 RNA polymerase promoter. Moreover, plasmids need to be constructed that harbour multiple AHSV-4 genome segments, thereby increasing the likelihood that transfected cells will contain the full complement of 10 AHSV-4 transcripts. To ensure efficient intracellular capping of the newly transcribed AHSV-4 transcripts, BSR-T7 cells may be infected with a virus that displays cross-capping activity or, alternatively, BSR cells may be used that have been infected with a recombinant T7 vaccinia virus or fowl pox-T7 virus, which do display cross-capping activity (Elroy-Stein and Moss, 1990; Boot *et al.*, 2001; Kobayashi *et al.*, 2007). The virus-infected cells can then be transfected with the modified AHSV-4 plasmid cDNA clones.

The use of reassortant viruses to study AHSV NS3 protein function was recently reported (Meiring *et al.*, 2009). In these studies, Vero cells were co-infected with different AHSV serotypes, but it was found that the NS3 gene was exchanged with mostly other gene segments. Although this represents a meaningful approach towards the study of AHSV protein function, these types of studies could benefit greatly from targeted gene replacements. In this study, a method based on the use of an *in vitro*-synthesised T7 transcript of one AHSV serotype and core-derived ssRNA from a second AHSV serotype was demonstrated to yield recombinant AHSV (Chapter 4). These findings extend the earlier discovery that AHSV mRNA is infectious when transfected into permissive cells and demonstrates that an *in vitro*-synthesised T7 transcript with a cap analogue can functionally substitute for a transcript synthesised by core particles. Although the method in its current form is not very efficient, it is nevertheless easy to perform, and mass screening of reassortant candidates can be performed depending on the targeted gene and available tools, such as discriminating

monoclonal antibodies (MAbs) or, as used in this investigation, discriminating PCR assays. This targeted single-gene replacement system may be of particular value for research focussing on one genome segment, since there is no need to construct a full set of 10 cDNA clones. Indeed, during the course of this study an almost identical system was published detailing the successful introduction of plasmid DNA-derived T7 transcripts from AHSV-4 genome segments 4 and 5 into the genome of AHSV-6 (Matsuo and Roy, 2011). Despite the success of this method, it should be noted that a recombinant virus is generated. In contrast, recovery of infectious virus entirely from a complete set of 10 plasmid cDNAs or plasmid cDNA-derived transcripts would allow the generation of AHSV mutants with a consistent genetic background. This approach would be especially useful in the recovery of mutants that are expected to have a slow replication phenotype, as the screening of plaques for the desired mutant amongst parental plaques is not required. Therefore, there is still great merit in developing and establishing a plasmid cDNA-based reverse genetic system for AHSV, and should continue to be investigated as a matter of urgency.

An alternative method, based on the uptake of an *in vitro*-synthesised T7 transcript in an ongoing viral infection coupled with RNA interference (RNAi)-mediated degradation of the corresponding parent virus transcript, proved unsuccessful as a means to recover recombinant AHSV (Chapter 4). Various factors that may have limited the usefulness of this approach had been identified, including the use of small interfering RNA (siRNA) that may not have been able to discriminate between the exogenous and endogenous virus transcripts, as well as unrestricted parent virus replication in untransfected cells. Although there may still be merit in pursuing this strategy, the methodology would need to be modified. These modifications could include the use of an appropriate plasmid cDNA clone, rather than an *in vitro*-synthesised T7 transcript, to allow for constitutive RNA synthesis in transfected BSR-T7 cells. This would allow plasmid-derived transcript synthesis to coincide with maximum RNAi-knockdown efficiency, thereby increasing the likelihood of genome segment reassortment. A further improvement might be the use of a short hairpin RNA (shRNA) that is targeted to a unique sequence of the parent virus transcript only or, alternatively, a number of silent mutations can be introduced into the cDNA clone to render it resistant to RNAi-mediated degradation. Also, a stable shRNA-expressing BSR cell line could be constructed to ensure that the entire cell population expressed the discriminating shRNA, thus overcoming the inherent variability encountered in cell transfection experiments. However, the time and

effort required to construct shRNA-expressing cell lines for each viral genome segment and to engineer RNAi-resistant cDNA clones may outweigh its potential benefits.

In summary, this study has shown that a reverse genetic system for AHSV is tractable. The targeted single-gene replacement system described herein can be expanded to also allow for the uptake of mutated genome segments. The ability to introduce specific mutations into the genes of AHSV will undoubtedly further understanding of the functions of the viral proteins in replicating virus and allow the corroboration of functions already assigned in previous studies. Moreover, studies regarding the *cis*-acting RNA sequences required for replication, packaging and expression of the individual genome segments can be investigated in greater detail, while it may also be possible to identify determinants of pathogenicity of AHSV.

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APPENDIX

1. ClustalW ALIGNMENTS OF AHSV-4(HS32/62) CONSENSUS SEQUENCE, pKM(AHSV-4) INSERTS AND *E. coli*-AMPLIFIED pKM(AHSV-4) INSERTS

Key:

AHSV-4 VP1: AHSV-4(32/62) VP1 consensus sequence used to synthesise insert cDNA

pKM-S1: pKM-S1 insert sequence

pKM-S1 Amp: pKM-S1 insert sequence subsequent to plasmid amplification in *E. coli*

1.1 AHSV-4(32/62) VP1

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60
AHSV-4 VP1       GTTAATTTGA GCGATGGTCA TCACCGTGCA AGGTGCAGAT CTAGTCAGGA GGGCTTTAAA
pKM-S1           GTTAATTTGA GCGATGGTCA TCACCGTGCA AGGTGCAGAT CTAGTCAGGA GGGCTTTAAA
pKM-S1 Amp       GTTAATTTGA GCGATGGTCA TCACCGTGCA AGGTGCAGAT CTAGTCAGGA GGGCTTTAAA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          70         80         90        100        110        120
AHSV-4 VP1       TCGATTATTT AAATATGGGA GGATAGATGG AACTAAAATG TATTATGAGT ATTATAGATA
pKM-S1           TCGATTATTT AAATATGGGA GGATAGATGG AACTAAAATG TATTATGAGT ATTATAGATA
pKM-S1 Amp       TCGATTATTT AAATATGGGA GGATAGATGG AACTAAAATG TATTATGAGT ATTATAGATA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 VP1       TTCAAGTAAA ATGAGGGAGA CTAGGAGGAA GAAAGGAACG AAATATAAAA CGGATGATGA
pKM-S1           TTCAAGTAAA ATGAGGGAGA CTAGGAGGAA GAAAGGAACG AAATATAAAA CGGATGATGA
pKM-S1 Amp       TTCAAGTAAA ATGAGGGAGA CTAGGAGGAA GAAAGGAACG AAATATAAAA CGGATGATGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 VP1       GTTTTTAGAG CGCGAGAGGG ATGCGGGCAG GTTGAAGCTT TACGATTTAC AAGTGATACG
pKM-S1           GTTTTTAGAG CGCGAGAGGG ATGCGGGCAG GTTGAAGCTT TACGATTTAC AAGTGATACG
pKM-S1 Amp       GTTTTTAGAG CGCGAGAGGG ATGCGGGCAG GTTGAAGCTT TACGATTTAC AAGTGATACG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 VP1       AGAGGCATCC TGGGAAGATT TGCTGTACGA GAACGTTCAT ACTGCAGAGT TAGATATCTA
pKM-S1           AGAGGCATCC TGGGAAGATT TGCTGTACGA GAACGTTCAT ACTGCAGAGT TAGATATCTA
pKM-S1 Amp       AGAGGCATCC TGGGAAGATT TGCTGTACGA GAACGTTCAT ACTGCAGAGT TAGATATCTA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 VP1       CGTTAGATCA ATCTTGAAGT TAGAGGATTT AGAGCCGGAA GAGGAATTTT TGCGAAATTA
pKM-S1           CGTTAGATCA ATCTTGAAGT TAGAGGATTT AGAGCCGGAA GAGGAATTTT TGCGAAATTA
pKM-S1 Amp       CGTTAGATCA ATCTTGAAGT TAGAGGATTT AGAGCCGGAA GAGGAATTTT TGCGAAATTA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 VP1       TGCGGTTTGT GATGGTGTGC ATCCGCTGAA AGATTTCGTT GAGATGAGGG CAAAAAACGA
pKM-S1           TGCGGTTTGT GATGGTGTGC ATCCGCTGAA AGATTTCGTT GAGATGAGGG CAAAAAACGA
pKM-S1 Amp       TGCGGTTTGT GATGGTGTGC ATCCGCTGAA AGATTTCGTT GAGATGAGGG CAAAAAACGA
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         430        440        450        460        470        480
AHSV-4 VP1       AATGCAGATT TTTGGTGATA TGCCTATTAA AGCTTGGATC TCAGTATTAA TGGAAATTTC
pKM-S1           AATGCAGATT TTTGGTGATA TGCCTATTAA AGCTTGGATC TCAGTATTAA TGGAAATTTC
pKM-S1 Amp       AATGCAGATT TTTGGTGATA TGCCTATTAA AGCTTGGATC TCAGTATTAA TGGAAATTTC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         490        500        510        520        530        540
AHSV-4 VP1       GCGTGAAACA AAGCATAAAC CTCTTGGGTT ATTGGTGGCA TCGGATTTTG TTGGAAGATT
pKM-S1           GCGTGAAACA AAGCATAAAC CTCTTGGGTT ATTGGTGGCA TCGGATTTTG TTGGAAGATT
pKM-S1 Amp       GCGTGAAACA AAGCATAAAC CTCTTGGGTT ATTGGTGGCA TCGGATTTTG TTGGAAGATT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         550        560        570        580        590        600
AHSV-4 VP1       CGGTTCGCCT TTTGAGCAGA ATTTCAGAGA TTTGTCACAG ATCAACGAAT ATGGTTATTG
pKM-S1           CGGTTCGCCT TTTGAGCAGA ATTTCAGAGA TTTGTCACAG ATCAACGAAT ATGGTTATTG
pKM-S1 Amp       CGGTTCGCCT TTTGAGCAGA ATTTCAGAGA TTTGTCACAG ATCAACGAAT ATGGTTATTG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650        660
AHSV-4 VP1       TTACTCAAGT CCATTATTAT TTGAGATGTG CGTGACTGAA TCAATTTTGG AGTTTAATAT
pKM-S1           TTACTCAAGT CCATTATTAT TTGAGATGTG CGTGACTGAA TCAATTTTGG AGTTTAATAT
pKM-S1 Amp       TTACTCAAGT CCATTATTAT TTGAGATGTG CGTGACTGAA TCAATTTTGG AGTTTAATAT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         670        680        690        700        710        720
AHSV-4 VP1       GTGGTACCGA ATGCGTGAAG AGAGAATACA ATCACTGAAA TTTGGATTGG AAACAATTGA
pKM-S1           GTGGTACCGA ATGCGTGAAG AGAGAATACA ATCACTGAAA TTTGGATTGG AAACAATTGA
pKM-S1 Amp       GTGGTACCGA ATGCGTGAAG AGAGAATACA ATCACTGAAA TTTGGATTGG AAACAATTGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         730        740        750        760        770        780
AHSV-4 VP1       CCCTTTCAAA CTGATACAAG AATTCTTTGA AATATGTTTG CCACATCCTA AAAAGATAAA
pKM-S1           CCCTTTCAAA CTGATACAAG AATTCTTTGA AATATGTTTG CCACATCCTA AAAAGATAAA
pKM-S1 Amp       CCCTTTCAAA CTGATACAAG AATTCTTTGA AATATGTTTG CCACATCCTA AAAAGATAAA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         790        800        810        820        830        840
AHSV-4 VP1       CAACACGTTG CGTTCACCGT ATTCGTGGTT CGTTAAAAAT TGGGGGATCG GATGTCCGAG
pKM-S1           CAACACGTTG CGTTCACCGT ATTCGTGGTT CGTTAAAAAT TGGGGGATCG GATGTCCGAG
pKM-S1 Amp       CAACACGTTG CGTTCACCGT ATTCGTGGTT CGTTAAAAAT TGGGGGATCG GATGTCCGAG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         850        860        870        880        890        900
AHSV-4 VP1       AGTGAAAGTT CTAACATCTA TTGGAGGTGA GGATCGAAAC TCAAAGGAGA TTTTTTATAC
pKM-S1           AGTGAAAGTT CTAACATCTA TTGGAGGTGA GGATCGAAAC TCAAAGGAGA TTTTTTATAC
pKM-S1 Amp       AGTGAAAGTT CTAACATCTA TTGGAGGTGA GGATCGAAAC TCAAAGGAGA TTTTTTATAC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950        960
AHSV-4 VP1       TGGTTATCAT GAGACAGAAA ATCTATACTC AGAGATTGTC TTGAAATCAA AGTTTTATAG
pKM-S1           TGGTTATCAT GAGACAGAAA ATCTATACTC AGAGATTGTC TTGAAATCAA AGTTTTATAG
pKM-S1 Amp       TGGTTATCAT GAGACAGAAA ATCTATACTC AGAGATTGTC TTGAAATCAA AGTTTTATAG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         970        980        990        1000       1010       1020
AHSV-4 VP1       AGAGAGTCTA AAGCAAAATA TGACAAAAAC GGAAGAAGCA ATTAGCTATT CGCAAAAGCT
pKM-S1           AGAGAGTCTA AAGCAAAATA TGACAAAAAC GGAAGAAGCA ATTAGCTATT CGCAAAAGCT
pKM-S1 Amp       AGAGAGTCTA AAGCAAAATA TGACAAAAAC GGAAGAAGCA ATTAGCTATT CGCAAAAGCT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1030       1040       1050       1060       1070       1080
AHSV-4 VP1      TGGCAATCAC GGGAGAACAA TGCCTATTTT CCTGAAAATG TTAAAAGCGG TATATACAAC
pKM-S1          TGGCAATCAC GGGAGAACAA TGCCTATTTT CCTGAAAATG TTAAAAGCGG TATATACAAC
pKM-S1 Amp      TGGCAATCAC GGGAGAACAA TGCCTATTTT CCTGAAAATG TTAAAAGCGG TATATACAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1090       1100       1110       1120       1130       1140
AHSV-4 VP1      GGAGTTTGAT CCAACAAAAA TAAGTCACGT TATACTAGCG TCATTATGCT TAAGCATACA
pKM-S1          GGAGTTTGAT CCAACAAAAA TAAGTCACGT TATACTAGCG TCATTATGCT TAAGCATACA
pKM-S1 Amp      GGAGTTTGAT CCAACAAAAA TAAGTCACGT TATACTAGCG TCATTATGCT TAAGCATACA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1150       1160       1170       1180       1190       1200
AHSV-4 VP1      AACGATAACG GGGTACGGGA GGGCGTGGGT AGTTAACAAA TCTAGCGATT TGGAAGCGCA
pKM-S1          AACGATAACG GGGTACGGGA GGGCGTGGGT AGTTAACAAA TCTAGCGATT TGGAAGCGCA
pKM-S1 Amp      AACGATAACG GGGTACGGGA GGGCGTGGGT AGTTAACAAA TCTAGCGATT TGGAAGCGCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1210       1220       1230       1240       1250       1260
AHSV-4 VP1      GATGAAACCA AGTAGTGATA ACTATGTTCA GCGCGTATGT GACTATACAA AGAATAACTT
pKM-S1          GATGAAACCA AGTAGTGATA ACTATGTTCA GCGCGTATGT GACTATACAA AGAATAACTT
pKM-S1 Amp      GATGAAACCA AGTAGTGATA ACTATGTTCA GCGCGTATGT GACTATACAA AGAATAACTT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1270       1280       1290       1300       1310       1320
AHSV-4 VP1      TATAAAAGCC TATGAGGAAG CGAGACGAGG GGGTGAAGAG ATCGTGATGC CTGAAGATAT
pKM-S1          TATAAAAGCC TATGAGGAAG CGAGACGAGG GGGTGAAGAG ATCGTGATGC CTGAAGATAT
pKM-S1 Amp      TATAAAAGCC TATGAGGAAG CGAGACGAGG GGGTGAAGAG ATCGTGATGC CTGAAGATAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1330       1340       1350       1360       1370       1380
AHSV-4 VP1      GTATACATCG ATATTACGTC TTGCTAAAAA CACAAGCTCA GGCTTTTCAA CTAGTATTGA
pKM-S1          GTATACATCG ATATTACGTC TTGCTAAAAA CACAAGCTCA GGCTTTTCAA CTAGTATTGA
pKM-S1 Amp      GTATACATCG ATATTACGTC TTGCTAAAAA CACAAGCTCA GGCTTTTCAA CTAGTATTGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1390       1400       1410       1420       1430       1440
AHSV-4 VP1      TGTTTTTAAG CGGTATGGTC CCAACGCGAA AGGTGGACGT GGAGAAAAGA TCCAAATAAC
pKM-S1          TGTTTTTAAG CGGTATGGTC CCAACGCGAA AGGTGGACGT GGAGAAAAGA TCCAAATAAC
pKM-S1 Amp      TGTTTTTAAG CGGTATGGTC CCAACGCGAA AGGTGGACGT GGAGAAAAGA TCCAAATAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1450       1460       1470       1480       1490       1500
AHSV-4 VP1      TTCGCGGATT AAAGCGTTAG TGATTTTCAC AAAAGGCCAC GAAATATTTA CGCCAAAAAA
pKM-S1          TTCGCGGATT AAAGCGTTAG TGATTTTCAC AAAAGGCCAC GAAATATTTA CGCCAAAAAA
pKM-S1 Amp      TTCGCGGATT AAAGCGTTAG TGATTTTCAC AAAAGGCCAC GAAATATTTA CGCCAAAAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550       1560
AHSV-4 VP1      TCTAGCTTTG AAGTATAACA CTACCGAATT TTTCCAAACG AAAGGTTCAA GAGATGTCCC
pKM-S1          TCTAGCTTTG AAGTATAACA CTACCGAATT TTTCCAAACG AAAGGTTCAA GAGATGTCCC
pKM-S1 Amp      TCTAGCTTTG AAGTATAACA CTACCGAATT TTTCCAAACG AAAGGTTCAA GAGATGTCCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1570       1580       1590       1600       1610       1620
AHSV-4 VP1      GATCAAATCT ACGAGAATAG TGTACTCTAT CAATCTTTCA ATTTTAGTAC CGCAGCTTAT
pKM-S1          GATCAAATCT ACGAGAATAG TGTACTCTAT CAATCTTTCA ATTTTAGTAC CGCAGCTTAT
pKM-S1 Amp      GATCAAATCT ACGAGAATAG TGTACTCTAT CAATCTTTCA ATTTTAGTAC CGCAGCTTAT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1630       1640       1650       1660       1670       1680
AHSV-4 VP1      AGTAACGTTA CCCTTGAATG AGTATTTCGC GAGAGCAGGA GGGAGTACTT TGCCAGAAAC
pKM-S1          AGTAACGTTA CCCTTGAATG AGTATTTCGC GAGAGCAGGA GGGAGTACTT TGCCAGAAAC
pKM-S1 Amp      AGTAACGTTA CCCTTGAATG AGTATTTCGC GAGAGCAGGA GGGAGTACTT TGCCAGAAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1690       1700       1710       1720       1730       1740
AHSV-4 VP1      ACAACGTATG GGCGGGAAAA TTATCGTTGG AGATTTGGAG GCTACTGGAT CACGTGTGAT
pKM-S1          ACAACGTATG GGCGGGAAAA TTATCGTTGG AGATTTGGAG GCTACTGGAT CACGTGTGAT
pKM-S1 Amp      ACAACGTATG GGCGGGAAAA TTATCGTTGG AGATTTGGAG GCTACTGGAT CACGTGTGAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1750       1760       1770       1780       1790       1800
AHSV-4 VP1      GGACGCTGCA GACACATTCA GGAATTCTTC GGATCCACTC AATCTAACCA TTGCGATCGA
pKM-S1          GGACGCTGCA GACACATTCA GGAATTCTTC GGATCCACTC AATCTAACCA TTGCGATCGA
pKM-S1 Amp      GGACGCTGCA GACACATTCA GGAATTCTTC GGATCCACTC AATCTAACCA TTGCGATCGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1810       1820       1830       1840       1850       1860
AHSV-4 VP1      TTACAGTGAA TTTGATACAC ATTTGACGCC TTATAACTTC AGAAATGGGA TGCTAGATGG
pKM-S1          TTACAGTGAA TTTGATACAC ATTTGACGCC TTATAACTTC AGAAATGGGA TGCTAGATGG
pKM-S1 Amp      TTACAGTGAA TTTGATACAC ATTTGACGCC TTATAACTTC AGAAATGGGA TGCTAGATGG

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                       1870       1880       1890       1900       1910       1920
AHSV-4 VP1      CATACGGGAG GCAATGCGTA GATATCAACA TTTGAGATAT GAGGGATACA CATTAGATGA
pKM-S1          CATACGGGAG GCAATGCGTA GATATCAACA TTTGAGATAT GAGGGATACA CATTAGATGA
pKM-S1 Amp      CATACGGGAG GCAATGCGTA GATATCAACA TTTGAGATAT GAGGGATACA CATTAGATGA

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                       1930       1940       1950       1960       1970       1980
AHSV-4 VP1      ATTAATAGAG TTTGGATATG GAGAGGGTCG CGTGCTGAAC ACATTATGGA ATGGAAAAAG
pKM-S1          ATTAATAGAG TTTGGATATG GAGAGGGTCG CGTGCTGAAC ACATTATGGA ATGGAAAAAG
pKM-S1 Amp      ATTAATAGAG TTTGGATATG GAGAGGGTCG CGTGCTGAAC ACATTATGGA ATGGAAAAAG

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                       1990       2000       2010       2020       2030       2040
AHSV-4 VP1      GCGAGTATTC AAAGTGGCAT TTGAGGATTA TGTTATGTTA AGTGATGAAG ATAAAGTTCA
pKM-S1          GCGAGTATTC AAAGTGGCAT TTGAGGATTA TGTTATGTTA AGTGATGAAG ATAAAGTTCA
pKM-S1 Amp      GCGAGTATTC AAAGTGGCAT TTGAGGATTA TGTTATGTTA AGTGATGAAG ATAAAGTTCA

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                       2050       2060       2070       2080       2090       2100
AHSV-4 VP1      GGGAGTTTTT AAACCACCTA TTGGAGTGAA GCCAGTAAAG AACATTAAAA TTTGTGAAGA
pKM-S1          GGGAGTTTTT AAACCACCTA TTGGAGTGAA GCCAGTAAAG AACATTAAAA TTTGTGAAGA
pKM-S1 Amp      GGGAGTTTTT AAACCACCTA TTGGAGTGAA GCCAGTAAAG AACATTAAAA TTTGTGAAGA

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                       2110       2120       2130       2140       2150       2160
AHSV-4 VP1      ACTGGAGAAA AAGGCAGACG GCCGTGATTT GATTTTGGTT TCACCTACGG ATGGAAGCGA
pKM-S1          ACTGGAGAAA AAGGCAGACG GCCGTGATTT GATTTTGGTT TCACCTACGG ATGGAAGCGA
pKM-S1 Amp      ACTGGAGAAA AAGGCAGACG GCCGTGATTT GATTTTGGTT TCACCTACGG ATGGAAGCGA

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                       2170       2180       2190       2200       2210       2220
AHSV-4 VP1      TCTAGCGTTG ATCAATACCC ATTTATCCGG AGAAAATTCC ACGTTGATCG CAAATAGCTT
pKM-S1          TCTAGCGTTG ATCAATACCC ATTTATCCGG AGAAAATTCC ACGTTGATCG CAAATAGCTT
pKM-S1 Amp      TCTAGCGTTG ATCAATACCC ATTTATCCGG AGAAAATTCC ACGTTGATCG CAAATAGCTT
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                       2230       2240       2250       2260       2270       2280
AHSV-4 VP1      ACATAACCTG GCGATTGGGA CTGTTATTCG TGAAGAAGTC AAGCGTATTT TTGGTGATGA
pKM-S1          ACATAACCTG GCGATTGGGA CTGTTATTCG TGAAGAAGTC AAGCGTATTT TTGGTGATGA
pKM-S1 Amp      ACATAACCTG GCGATTGGGA CTGTTATTCG TGAAGAAGTC AAGCGTATTT TTGGTGATGA

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                       2290       2300       2310       2320       2330       2340
AHSV-4 VP1      TATTTCATTT AAGTCAGAGC AGTACGTCGG TGATGACACT TTATTCTATA CTGAATTGAG
pKM-S1          TATTTCATTT AAGTCAGAGC AGTACGTCGG TGATGACACT TTATTCTATA CTGAATTGAG
pKM-S1 Amp      TATTTCATTT AAGTCAGAGC AGTACGTCGG TGATGACACT TTATTCTATA CTGAATTGAG

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                       2350       2360       2370       2380       2390       2400
AHSV-4 VP1      GACGCGATCA GTCGAGCGGT TTGATTCAAT TGTCGATACG ATTTTTGAAG TTATTAAGAA
pKM-S1          GACGCGATCA GTCGAGCGGT TTGATTCAAT TGTCGATACG ATTTTTGAAG TTATTAAGAA
pKM-S1 Amp      GACGCGATCA GTCGAGCGGT TTGATTCAAT TGTCGATACG ATTTTTGAAG TTATTAAGAA

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                       2410       2420       2430       2440       2450       2460
AHSV-4 VP1      GAGTGGTCAC GAAGCCTCAA TGTCAAAAAC TCTCATAGCT CCCTTTTCAG TCGAAAAAAC
pKM-S1          GAGTGGTCAC GAAGCCTCAA TGTCAAAAAC TCTCATAGCT CCCTTTTCAG TCGAAAAAAC
pKM-S1 Amp      GAGTGGTCAC GAAGCCTCAA TGTCAAAAAC TCTCATAGCT CCCTTTTCAG TCGAAAAAAC

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                       2470       2480       2490       2500       2510       2520
AHSV-4 VP1      ACAAACGCAC GCCAAGCAGG GAATATATGT CCCTCAGGAT CGGATGATGC TAGTTTCGTC
pKM-S1          ACAAACGCAC GCCAAGCAGG GAATATATGT CCCTCAGGAT CGGATGATGC TAGTTTCGTC
pKM-S1 Amp      ACAAACGCAC GCCAAGCAGG GAATATATGT CCCTCAGGAT CGGATGATGC TAGTTTCGTC

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                       2530       2540       2550       2560       2570       2580
AHSV-4 VP1      TGAACGAAGA AAGGATATTG AGGATGTTGC AGGGTACCTG AGATCCCAAG TTCAAACATT
pKM-S1          TGAACGAAGA AAGGATATTG AGGATGTTGC AGGGTACCTG AGATCCCAAG TTCAAACATT
pKM-S1 Amp      TGAACGAAGA AAGGATATTG AGGATGTTGC AGGGTACCTG AGATCCCAAG TTCAAACATT

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                       2590       2600       2610       2620       2630       2640
AHSV-4 VP1      GACTACGAAA ATTAGTCGTG GTTTCTCGCA TGAATTGGCG CAGCTCATTT TTATGATGAA
pKM-S1          GACTACGAAA ATTAGTCGTG GTTTCTCGCA TGAATTGGCG CAGCTCATTT TTATGATGAA
pKM-S1 Amp      GACTACGAAA ATTAGTCGTG GTTTCTCGCA TGAATTGGCG CAGCTCATTT TTATGATGAA

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                       2650       2660       2670       2680       2690       2700
AHSV-4 VP1      GTCATCGATT ATCGGGCATA GGAAGCTGAA GAGAACGATT AAGGATGGAG GGTACAGAGA
pKM-S1          GTCATCGATT ATCGGGCATA GGAAGCTGAA GAGAACGATT AAGGATGGAG GGTACAGAGA
pKM-S1 Amp      GTCATCGATT ATCGGGCATA GGAAGCTGAA GAGAACGATT AAGGATGGAG GGTACAGAGA

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                       2710       2720       2730       2740       2750       2760
AHSV-4 VP1      TAGGAAATAC GATGATGACA AGGAGGACGG CTTCACTTTG ATAATGTTGC GAGATCCACT
pKM-S1          TAGGAAATAC GATGATGACA AGGAGGACGG CTTCACTTTG ATAATGTTGC GAGATCCACT
pKM-S1 Amp      TAGGAAATAC GATGATGACA AGGAGGACGG CTTCACTTTG ATAATGTTGC GAGATCCACT

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                       2770       2780       2790       2800       2810       2820
AHSV-4 VP1      TATTGCTTTT TATCCTGTTG AATGGAATGG TTTTGGGGCG CACCCGGCGG CGATGAATAT
pKM-S1          TATTGCTTTT TATCCTGTTG AATGGAATGG TTTTGGGGCG CACCCGGCGG CGATGAATAT
pKM-S1 Amp      TATTGCTTTT TATCCTGTTG AATGGAATGG TTTTGGGGCG CACCCGGCGG CGATGAATAT
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                       2830       2840       2850       2860       2870       2880
AHSV-4 VP1      AATAATGACT GAAGATATGT TTGTGGATTC AGTGATGAGA GGGGAGTGTA GGGCGTGGAT
pKM-S1          AATAATGACT GAAGATATGT TTGTGGATTC AGTGATGAGA GGGGAGTGTA GGGCGTGGAT
pKM-S1 Amp      AATAATGACT GAAGATATGT TTGTGGATTC AGTGATGAGA GGGGAGTGTA GGGCGTGGAT

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                       2890       2900       2910       2920       2930       2940
AHSV-4 VP1      GGAACCTTTA GTTAAGTTGA TTGATCAATC TCCGCCCCTG TGGAATGAAA CGAGCGCGGA
pKM-S1          GGAACCTTTA GTTAAGTTGA TTGATCAATC TCCGCCCCTG TGGAATGAAA CGAGCGCGGA
pKM-S1 Amp      GGAACCTTTA GTTAAGTTGA TTGATCAATC TCCGCCCCTG TGGAATGAAA CGAGCGCGGA

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                       2950       2960       2970       2980       2990       3000
AHSV-4 VP1      TAAAAGGATG ATTGGTACGG ACAGTACGAT GTCTTTTTTC TCTAGAATGG CGAGGCCAGC
pKM-S1          TAAAAGGATG ATTGGTACGG ACAGTACGAT GTCTTTTTTC TCTAGAATGG CGAGGCCAGC
pKM-S1 Amp      TAAAAGGATG ATTGGTACGG ACAGTACGAT GTCTTTTTTC TCTAGAATGG CGAGGCCAGC

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                       3010       3020       3030       3040       3050       3060
AHSV-4 VP1      GGTTCGAACA GTATTAACGA ATACTGAGGT TGGAGATGCC GTGAGAAGTT TGCCTCTGGG
pKM-S1          GGTTCGAACA GTATTAACGA ATACTGAGGT TGGAGATGCC GTGAGAAGTT TGCCTCTGGG
pKM-S1 Amp      GGTTCGAACA GTATTAACGA ATACTGAGGT TGGAGATGCC GTGAGAAGTT TGCCTCTGGG

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                       3070       3080       3090       3100       3110       3120
AHSV-4 VP1      AGACTTCTCT CCTTTCAATA TCTCTAAGAC TATGATGCAT TCGGCGCTGT TAAAGGAAAA
pKM-S1          AGACTTCTCT CCTTTCAATA TCTCTAAGAC TATGATGCAT TCGGCGCTGT TAAAGGAAAA
pKM-S1 Amp      AGACTTCTCT CCTTTCAATA TCTCTAAGAC TATGATGCAT TCGGCGCTGT TAAAGGAAAA

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                       3130       3140       3150       3160       3170       3180
AHSV-4 VP1      GAACGCCCGT TCATTACTCA CTCCGGCGTA TGAGATGGAA TATCAAAAAG AGTTGCAAGG
pKM-S1          GAACGCCCGT TCATTACTCA CTCCGGCGTA TGAGATGGAA TATCAAAAAG AGTTGCAAGG
pKM-S1 Amp      GAACGCCCGT TCATTACTCA CTCCGGCGTA TGAGATGGAA TATCAAAAAG AGTTGCAAGG

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                       3190       3200       3210       3220       3230       3240
AHSV-4 VP1      TTGGAGACCA AGACAGAAGA GGTTTTTAGT GACATCTAAC GAAATGGAAA TCACCACGAA
pKM-S1          TTGGAGACCA AGACAGAAGA GGTTTTTAGT GACATCTAAC GAAATGGAAA TCACCACGAA
pKM-S1 Amp      TTGGAGACCA AGACAGAAGA GGTTTTTAGT GACATCTAAC GAAATGGAAA TCACCACGAA

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                       3250       3260       3270       3280       3290       3300
AHSV-4 VP1      TTACATGAAA ATGTTTAACG TCAGTAAAAT CCCACTGCAC GGTTTGGCGT TGAAGTTTTT
pKM-S1          TTACATGAAA ATGTTTAACG TCAGTAAAAT CCCACTGCAC GGTTTGGCGT TGAAGTTTTT
pKM-S1 Amp      TTACATGAAA ATGTTTAACG TCAGTAAAAT CCCACTGCAC GGTTTGGCGT TGAAGTTTTT

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                       3310       3320       3330       3340       3350       3360
AHSV-4 VP1      CCCAGATGTT AACTTGTCGA AGGAATTTTT CTTACAGAAG AGCGTTTTAG GGAATAGAGA
pKM-S1          CCCAGATGTT AACTTGTCGA AGGAATTTTT CTTACAGAAG AGCGTTTTAG GGAATAGAGA
pKM-S1 Amp      CCCAGATGTT AACTTGTCGA AGGAATTTTT CTTACAGAAG AGCGTTTTAG GGAATAGAGA

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                       3370       3380       3390       3400       3410       3420
AHSV-4 VP1      AAGTCCGAGA GCGCGAATGT CATATGTTGA TAGAATTGAC TCAATACTAC GCGGCGATGT
pKM-S1          AAGTCCGAGA GCGCGAATGT CATATGTTGA TAGAATTGAC TCAATACTAC GCGGCGATGT
pKM-S1 Amp      AAGTCCGAGA GCGCGAATGT CATATGTTGA TAGAATTGAC TCAATACTAC GCGGCGATGT
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                        3430       3440       3450       3460       3470       3480
AHSV-4 VP1      TGTAATGCGA GGGTTCATTA CTGCGAATAC TATCATTAAC ATATTGGAGA AATTGGGACA
pKM-S1          TGTAATGCGA GGGTTCATTA CTGCGAATAC TATCATTAAC ATATTGGAGA AATTGGGACA
pKM-S1 Amp      TGTAATGCGA GGGTTCATTA CTGCGAATAC TATCATTAAC ATATTGGAGA AATTGGGACA

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                        3490       3500       3510       3520       3530       3540
AHSV-4 VP1      CACGCACTCG GCGAGCGATT TGACTACGTT ATTCGAAATA ATGAACTTAT CATCAAGCGT
pKM-S1          CACGCACTCG GCGAGCGATT TGACTACGTT ATTCGAAATA ATGAACTTAT CATCAAGCGT
pKM-S1 Amp      CACGCACTCG GCGAGCGATT TGACTACGTT ATTCGAAATA ATGAACTTAT CATCAAGCGT

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                        3550       3560       3570       3580       3590       3600
AHSV-4 VP1      CGCTCAGCGG CTATCAGAGT ATATCACGAC CGAACGCGTT CGCTTCGATG CGATGAAACT
pKM-S1          CGCTCAGCGG CTATCAGAGT ATATCACGAC CGAACGCGTT CGCTTCGATG CGATGAAACT
pKM-S1 Amp      CGCTCAGCGG CTATCAGAGT ATATCACGAC CGAACGCGTT CGCTTCGATG CGATGAAACT

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                        3610       3620       3630       3640       3650       3660
AHSV-4 VP1      ATCAAAGAGG GGCATATGTG GTGACGAATT CTCAATGTCA TTGGATGTTT GCACACAAAC
pKM-S1          ATCAAAGAGG GGCATATGTG GTGACGAATT CTCAATGTCA TTGGATGTTT GCACACAAAC
pKM-S1 Amp      ATCAAAGAGG GGCATATGTG GTGACGAATT CTCAATGTCA TTGGATGTTT GCACACAAAC

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                        3670       3680       3690       3700       3710       3720
AHSV-4 VP1      GATGGTGGAT AAATATATAC GTGCGCCGAC ACAGTTTACT AAAACGGAGC TCGACGCTGT
pKM-S1          GATGGTGGAT AAATATATAC GTGCGCCGAC ACAGTTTACT AAAACGGAGC TCGACGCTGT
pKM-S1 Amp      GATGGTGGAT AAATATATAC GTGCGCCGAC ACAGTTTACT AAAACGGAGC TCGACGCTGT

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                        3730       3740       3750       3760       3770       3780
AHSV-4 VP1      AAATTTGTAT GTTGCCCAAC ATATTATGTT AGATGCGGCG ACGGGGTTAA CGCCATCACG
pKM-S1          AAATTTGTAT GTTGCCCAAC ATATTATGTT AGATGCGGCG ACGGGGTTAA CGCCATCACG
pKM-S1 Amp      AAATTTGTAT GTTGCCCAAC ATATTATGTT AGATGCGGCG ACGGGGTTAA CGCCATCACG

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                        3790       3800       3810       3820       3830       3840
AHSV-4 VP1      ATATGATATT AATGTATCGG GAGACGAAAG GGTACGATTT AAGCAGCGCG TTGCCCGATT
pKM-S1          ATATGATATT AATGTATCGG GAGACGAAAG GGTACGATTT AAGCAGCGCG TTGCCCGATT
pKM-S1 Amp      ATATGATATT AATGTATCGG GAGACGAAAG GGTACGATTT AAGCAGCGCG TTGCCCGATT

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                        3850       3860       3870       3880       3890       3900
AHSV-4 VP1      TAACACACAT TTACCCAAGA TGCGGATGGT CAAAAGATTG ATCGAAACGG AGAGGTTGTC
pKM-S1          TAACACACAT TTACCCAAGA TGCGGATGGT CAAAAGATTG ATCGAAACGG AGAGGTTGTC
pKM-S1 Amp      TAACACACAT TTACCCAAGA TGCGGATGGT CAAAAGATTG ATCGAAACGG AGAGGTTGTC

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                        3910       3920       3930       3940       3950       3960
AHSV-4 VP1      CGCGAGGCTG GTTCAGAACC AGTTTGTCTG ATTAGAACTA GCACCCCACA GCTCAAAACA
pKM-S1          CGCGAGGCTG GTTCAGAACC AGTTTGTCTG ATTAGAACTA GCACCCCACA GCTCAAAACA
pKM-S1 Amp      CGCGAGGCTG GTTCAGAACC AGTTTGTCTG ATTAGAACTA GCACCCCACA GCTCAAAACA

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AHSV-4 VP1      CTTAC
pKM-S1          CTTAC
pKM-S1 Amp      CTTAC
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1.2 AHSV-4(32/62) VP2

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                         10         20         30         40         50         60
AHSV-4 VP2      GTTAAATTCA CTATGGCGTC CGAGTTTGGA ATATTGATGA CAAATGAAAA ATTTGACCCA
pKM-S2          GTTAAATTCA CTATGGCGTC CGAGTTTGGA ATATTGATGA CAAATGAAAA ATTTGACCCA
pKM-S2 Amp      GTTAAATTCA CTATGGCGTC CGAGTTTGGA ATATTGATGA CAAATGAAAA ATTTGACCCA

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                         70         80         90        100        110        120
AHSV-4 VP2      AGCTTAGAGA AAACCATTTG CGATGTTATA GTTACGAAGA AGGGAAGAGT GAAGCATAAA
pKM-S2          AGCTTAGAGA AAACCATTTG CGATGTTATA GTTACGAAGA AGGGAAGAGT GAAGCATAAA
pKM-S2 Amp      AGCTTAGAGA AAACCATTTG CGATGTTATA GTTACGAAGA AGGGAAGAGT GAAGCATAAA

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                        130        140        150        160        170        180
AHSV-4 VP2      GAGGTGGATG GCGTATGTGG ATACGAGTGG GATGAAACGA ATCACCGATT CGGATTGTGT
pKM-S2          GAGGTGGATG GCGTATGTGG ATACGAGTGG GATGAAACGA ATCACCGATT CGGATTGTGT
pKM-S2 Amp      GAGGTGGATG GCGTATGTGG ATACGAGTGG GATGAAACGA ATCACCGATT CGGATTGTGT

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                        190        200        210        220        230        240
AHSV-4 VP2      GAGGTGGAAC ACGACATGTC TATATCGGAA TTTATGTACA ATGAGATCAG ATGTGAGGGG
pKM-S2          GAGGTGGAAC ACGACATGTC TATATCGGAA TTTATGTACA ATGAGATCAG ATGTGAGGGG
pKM-S2 Amp      GAGGTGGAAC ACGACATGTC TATATCGGAA TTTATGTACA ATGAGATCAG ATGTGAGGGG

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                        250        260        270        280        290        300
AHSV-4 VP2      GCATATCCAA TTTTTCCGCG TTATATAATT GATACGTTAA AATACGAGAA ATTTATTGAT
pKM-S2          GCATATCCAA TTTTTCCGCG TTATATAATT GATACGTTAA AATACGAGAA ATTTATTGAT
pKM-S2 Amp      GCATATCCAA TTTTTCCGCG TTATATAATT GATACGTTAA AATACGAGAA ATTTATTGAT

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                        310        320        330        340        350        360
AHSV-4 VP2      AGGAATGACC ATCAAATTAG AGTGGATAGA GATGATAACG AAATGAGGAA AATATTGATA
pKM-S2          AGGAATGACC ATCAAATTAG AGTGGATAGA GATGATAACG AAATGAGGAA AATATTGATA
pKM-S2 Amp      AGGAATGACC ATCAAATTAG AGTGGATAGA GATGATAACG AAATGAGGAA AATATTGATA

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                        370        380        390        400        410        420
AHSV-4 VP2      CAGCCGTATG CAGGTGAGAT GTACTTTTCG CCGGAATGTT ATCCGAGCGT TTTTCTTCGG
pKM-S2          CAGCCGTATG CAGGTGAGAT GTACTTTTCG CCGGAATGTT ATCCGAGCGT TTTTCTTCGG
pKM-S2 Amp      CAGCCGTATG CAGGTGAGAT GTACTTTTCG CCGGAATGTT ATCCGAGCGT TTTTCTTCGG

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                        430        440        450        460        470        480
AHSV-4 VP2      AGGGAAGCGC GAAGTCAAAA GCTTGATCGG ATTCGGAATT ATATTGGAAA GAGAGTCGAA
pKM-S2          AGGGAAGCGC GAAGTCAAAA GCTTGATCGG ATTCGGAATT ATATTGGAAA GAGAGTCGAA
pKM-S2 Amp      AGGGAAGCGC GAAGTCAAAA GCTTGATCGG ATTCGGAATT ATATTGGAAA GAGAGTCGAA

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                        490        500        510        520        530        540
AHSV-4 VP2      TTTTATGAAG AGGAGAGTAA GAGAAAAGCA ATCCTTGATC AGAATAAGAT GTCTAAGGTT
pKM-S2          TTTTATGAAG AGGAGAGTAA GAGAAAAGCA ATCCTTGATC AGAATAAGAT GTCTAAGGTT
pKM-S2 Amp      TTTTATGAAG AGGAGAGTAA GAGAAAAGCA ATCCTTGATC AGAATAAGAT GTCTAAGGTT

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                        550        560        570        580        590        600
AHSV-4 VP2      GAACAATGGA GAGATGCGGT TAATGAAAGG ATTGTGAGTA TCGAACCAAA GCGAGGTGAG
pKM-S2          GAACAATGGA GAGATGCGGT TAATGAAAGG ATTGTGAGTA TCGAACCAAA GCGAGGTGAG
pKM-S2 Amp      GAACAATGGA GAGATGCGGT TAATGAAAGG ATTGTGAGTA TCGAACCAAA GCGAGGTGAG
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                         610        620        630        640        650        660
AHSV-4 VP2       TGCTATGATC ACGGAACCGA CATTATCTAC CAATTCATAA AAAAGCTGAG ATTTGGAATG
pKM-S2           TGCTATGATC ACGGAACCGA CATTATCTAC CAATTCATAA AAAAGCTGAG ATTTGGAATG
pKM-S2 Amp       TGCTATGATC ACGGAACCGA CATTATCTAC CAATTCATAA AAAAGCTGAG ATTTGGAATG

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                         670        680        690        700        710        720
AHSV-4 VP2       ATGTACCCAC ACTATTATGT TTTGCATAGT GATTACTGTA TTGTACCAAA TAAGGGGGGA
pKM-S2           ATGTACCCAC ACTATTATGT TTTGCATAGT GATTACTGTA TTGTACCAAA TAAGGGGGGA
pKM-S2 Amp       ATGTACCCAC ACTATTATGT TTTGCATAGT GATTACTGTA TTGTACCAAA TAAGGGGGGA

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                         730        740        750        760        770        780
AHSV-4 VP2       ACTAGTATTG GATCATGGCA TATAAGAAAA CGTACTGAGG GTGATGCGAA AGCTTCTGCT
pKM-S2           ACTAGTATTG GATCATGGCA TATAAGAAAA CGTACTGAGG GTGATGCGAA AGCTTCTGCT
pKM-S2 Amp       ACTAGTATTG GATCATGGCA TATAAGAAAA CGTACTGAGG GTGATGCGAA AGCTTCTGCT

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                         790        800        810        820        830        840
AHSV-4 VP2       ATGTATTCTG GAAAAGGTCC ACTGAATGAC TTACGAGTTA AAATTGAGCG GGATGATTTA
pKM-S2           ATGTATTCTG GAAAAGGTCC ACTGAATGAC TTACGAGTTA AAATTGAGCG GGATGATTTA
pKM-S2 Amp       ATGTATTCTG GAAAAGGTCC ACTGAATGAC TTACGAGTTA AAATTGAGCG GGATGATTTA

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                         850        860        870        880        890        900
AHSV-4 VP2       TCTCGAGAGA CAATTATTCA GATCATTGAG TACGGTAAGA AATTTAATTC ATCAGCAGGT
pKM-S2           TCTCGAGAGA CAATTATTCA GATCATTGAG TACGGTAAGA AATTTAATTC ATCAGCAGGT
pKM-S2 Amp       TCTCGAGAGA CAATTATTCA GATCATTGAG TACGGTAAGA AATTTAATTC ATCAGCAGGT

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                         910        920        930        940        950        960
AHSV-4 VP2       GATAAGCAGG GGAACATTTC AATTGAAAAA TTGGTAGAGT ATTGTGATTT TTTGACAACA
pKM-S2           GATAAGCAGG GGAACATTTC AATTGAAAAA TTGGTAGAGT ATTGTGATTT TTTGACAACA
pKM-S2 Amp       GATAAGCAGG GGAACATTTC AATTGAAAAA TTGGTAGAGT ATTGTGATTT TTTGACAACA

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                         970        980        990        1000       1010       1020
AHSV-4 VP2       TTCGTTCATG CGAAGAAGAA AGAAGAGGGT GAGGATGATA CTGCTCGACA GGAGATAAGA
pKM-S2           TTCGTTCATG CGAAGAAGAA AGAAGAGGGT GAGGATGATA CTGCTCGACA GGAGATAAGA
pKM-S2 Amp       TTCGTTCATG CGAAGAAGAA AGAAGAGGGT GAGGATGATA CTGCTCGACA GGAGATAAGA

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                         1030       1040       1050       1060       1070       1080
AHSV-4 VP2       AAAGCATGGG TTAAGCGGAT GCCTTATATG GATTTCTCAA AACCGATGAA AATCACGCGT
pKM-S2           AAAGCATGGG TTAAGCGGAT GCCTTATATG GATTTCTCAA AACCGATGAA AATCACGCGT
pKM-S2 Amp       AAAGCATGGG TTAAGCGGAT GCCTTATATG GATTTCTCAA AACCGATGAA AATCACGCGT

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                         1090       1100       1110       1120       1130       1140
AHSV-4 VP2       GGATTCAACA GAAATATGCT TTTCTTTGCG GCGCTCGATT CATTCAGAAA GAGGAACGGT
pKM-S2           GGATTCAACA GAAATATGCT TTTCTTTGCG GCGCTCGATT CATTCAGAAA GAGGAACGGT
pKM-S2 Amp       GGATTCAACA GAAATATGCT TTTCTTTGCG GCGCTCGATT CATTCAGAAA GAGGAACGGT

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                         1150       1160       1170       1180       1190       1200
AHSV-4 VP2       GTAGATGTTG ATCCGAATAA GGGTAAGTGG AAAGAACATA TAAAGGAGGT AACCGAAAAA
pKM-S2           GTAGATGTTG ATCCGAATAA GGGTAAGTGG AAAGAACATA TAAAGGAGGT AACCGAAAAA
pKM-S2 Amp       GTAGATGTTG ATCCGAATAA GGGTAAGTGG AAAGAACATA TAAAGGAGGT AACCGAAAAA
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                         1210       1220       1230       1240       1250       1260
AHSV-4 VP2       TTGAAGAAAG CGCAAACCGA AAATGGAGGA CAACCATGCC AAGTGTCGAT CGATGGAGTA
pKM-S2           TTGAAGAAAG CGCAAACCGA AAATGGAGGA CAACCATGCC AAGTGTCGAT CGATGGAGTA
pKM-S2 Amp       TTGAAGAAAG CGCAAACCGA AAATGGAGGA CAACCATGCC AAGTGTCGAT CGATGGAGTA

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                         1270       1280       1290       1300       1310       1320
AHSV-4 VP2       AACGTCTTGA CTAACGTAGA TTACGGTACG GTTAATCATT GGATAGATTG GGTAACAGAT
pKM-S2           AACGTCTTGA CTAACGTAGA TTACGGTACG GTTAATCATT GGATAGATTG GGTAACAGAT
pKM-S2 Amp       AACGTCTTGA CTAACGTAGA TTACGGTACG GTTAATCATT GGATAGATTG GGTAACAGAT

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                         1330       1340       1350       1360       1370       1380
AHSV-4 VP2       ATAATTATGG TTGTACAAAC TAAACGTTTG GTGAAAGAGT ATGCATTTAA AAAACTAAAG
pKM-S2           ATAATTATGG TTGTACAAAC TAAACGTTTG GTGAAAGAGT ATGCATTTAA AAAACTAAAG
pKM-S2 Amp       ATAATTATGG TTGTACAAAC TAAACGTTTG GTGAAAGAGT ATGCATTTAA AAAACTAAAG

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                         1390       1400       1410       1420       1430       1440
AHSV-4 VP2       AGCGAAAACT TACTTGCTGG AATGAATAGT TTAGTTGGGG TATTAAGATG TTATATGTAT
pKM-S2           AGCGAAAACT TACTTGCTGG AATGAATAGT TTAGTTGGGG TATTAAGATG TTATATGTAT
pKM-S2 Amp       AGCGAAAACT TACTTGCTGG AATGAATAGT TTAGTTGGGG TATTAAGATG TTATATGTAT

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                         1450       1460       1470       1480       1490       1500
AHSV-4 VP2       TGCTTAGCTT TAGCGATCTA TGATTTTTAT GAAGGGACTA TTGATGGTTT TAAGAAAGGC
pKM-S2           TGCTTAGCTT TAGCGATCTA TGATTTTTAT GAAGGGACTA TTGATGGTTT TAAGAAAGGC
pKM-S2 Amp       TGCTTAGCTT TAGCGATCTA TGATTTTTAT GAAGGGACTA TTGATGGTTT TAAGAAAGGC

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                         1510       1520       1530       1540       1550       1560
AHSV-4 VP2       TCGAATGCTT CCGCTATCAT TGAAACTGTC GCGCAGATGT TTCCGGACTT TCGCAGAGAA
pKM-S2           TCGAATGCTT CCGCTATCAT TGAAACTGTC GCGCAGATGT TTCCGGACTT TCGCAGAGAA
pKM-S2 Amp       TCGAATGCTT CCGCTATCAT TGAAACTGTC GCGCAGATGT TTCCGGACTT TCGCAGAGAA

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                         1570       1580       1590       1600       1610       1620
AHSV-4 VP2       CTTGTCGAAA AATTCGGTAT AGATTTAAGG ATGAAGGAAA TCACGCGTGA GTTGTTTGTT
pKM-S2           CTTGTCGAAA AATTCGGTAT AGATTTAAGG ATGAAGGAAA TCACGCGTGA GTTGTTTGTT
pKM-S2 Amp       CTTGTCGAAA AATTCGGTAT AGATTTAAGG ATGAAGGAAA TCACGCGTGA GTTGTTTGTT

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                         1630       1640       1650       1660       1670       1680
AHSV-4 VP2       GGTAAGAGCA TGACGTCAAA ATTTATGGAG GAAGGTGAAT ATGGATATAA GTTCGCCTAT
pKM-S2           GGTAAGAGCA TGACGTCAAA ATTTATGGAG GAAGGTGAAT ATGGATATAA GTTCGCCTAT
pKM-S2 Amp       GGTAAGAGCA TGACGTCAAA ATTTATGGAG GAAGGTGAAT ATGGATATAA GTTCGCCTAT

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                         1690       1700       1710       1720       1730       1740
AHSV-4 VP2       GGATGGCGTA GGGATGGCTT CGCGGTGATG GAAGATTACG GAGAAATTTT GACAGAAAAA
pKM-S2           GGATGGCGTA GGGATGGCTT CGCGGTGATG GAAGATTACG GAGAAATTTT GACAGAAAAA
pKM-S2 Amp       GGATGGCGTA GGGATGGCTT CGCGGTGATG GAAGATTACG GAGAAATTTT GACAGAAAAA

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                         1750       1760       1770       1780       1790       1800
AHSV-4 VP2       GTGGAGGACC TATATAAGGG TGTACTTTTA GGACGAAAGT GGGAGGATGA GGTTGATGAT
pKM-S2           GTGGAGGACC TATATAAGGG TGTACTTTTA GGACGAAAGT GGGAGGATGA GGTTGATGAT
pKM-S2 Amp       GTGGAGGACC TATATAAGGG TGTACTTTTA GGACGAAAGT GGGAGGATGA GGTTGATGAT
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                       1810       1820       1830       1840       1850       1860
AHSV-4 VP2      CCAGAGAGTT ATTTTTATGA TGATCTTTAT ACTAATGAGC CCCACAGAGT GTTTCTAAGC
pKM-S2          CCAGAGAGTT ATTTTTATGA TGATCTTTAT ACTAATGAGC CCCACAGAGT GTTTCTAAGC
pKM-S2 Amp      CCAGAGAGTT ATTTTTATGA TGATCTTTAT ACTAATGAGC CCCACAGAGT GTTTCTAAGC

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                       1870       1880       1890       1900       1910       1920
AHSV-4 VP2      GCAGGAAAGG ATGTGGATAA TAATATCACG CTTCGATCGA TTTCGCAGGC GGAAACCACG
pKM-S2          GCAGGAAAGG ATGTGGATAA TAATATCACG CTTCGATCGA TTTCGCAGGC GGAAACCACG
pKM-S2 Amp      GCAGGAAAGG ATGTGGATAA TAATATCACG CTTCGATCGA TTTCGCAGGC GGAAACCACG

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                       1930       1940       1950       1960       1970       1980
AHSV-4 VP2      TATCTATCGA AACGTTTCGT ATCATATTGG TATAGAATAT CACAAGTTGA AGTAACGAAG
pKM-S2          TATCTATCGA AACGTTTCGT ATCATATTGG TATAGAATAT CACAAGTTGA AGTAACGAAG
pKM-S2 Amp      TATCTATCGA AACGTTTCGT ATCATATTGG TATAGAATAT CACAAGTTGA AGTAACGAAG

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                       1990       2000       2010       2020       2030       2040
AHSV-4 VP2      GCGCGTAATG AAGTTCTGGA CATGAATGAG AAACAGAAGC CGTATTTTGA ATTTGAATAT
pKM-S2          GCGCGTAATG AAGTTCTGGA CATGAATGAG AAACAGAAGC CGTATTTTGA ATTTGAATAT
pKM-S2 Amp      GCGCGTAATG AAGTTCTGGA CATGAATGAG AAACAGAAGC CGTATTTTGA ATTTGAATAT

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                       2050       2060       2070       2080       2090       2100
AHSV-4 VP2      GATGATTTCA AACCCTGTTC AATTGGAGAG TTGGGGATCC ATGCATCCAC ATATATATAT
pKM-S2          GATGATTTCA AACCCTGTTC AATTGGAGAG TTGGGGATCC ATGCATCCAC ATATATATAT
pKM-S2 Amp      GATGATTTCA AACCCTGTTC AATTGGAGAG TTGGGGATCC ATGCATCCAC ATATATATAT

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                       2110       2120       2130       2140       2150       2160
AHSV-4 VP2      CAGAACCTAC TGGTCGGACG TAATAGAGGT GAGGAAATAC TTGATTCGAA AGAGCTCGTC
pKM-S2          CAGAACCTAC TGGTCGGACG TAATAGAGGT GAGGAAATAC TTGATTCGAA AGAGCTCGTC
pKM-S2 Amp      CAGAACCTAC TGGTCGGACG TAATAGAGGT GAGGAAATAC TTGATTCGAA AGAGCTCGTC

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                       2170       2180       2190       2200       2210       2220
AHSV-4 VP2      TGGATGGATA TGTCACTTTT AAATTTTGGA GCGGTCAGAT CTCACGATAG GTGCTGGATC
pKM-S2          TGGATGGATA TGTCACTTTT AAATTTTGGA GCGGTCAGAT CTCACGATAG GTGCTGGATC
pKM-S2 Amp      TGGATGGATA TGTCACTTTT AAATTTTGGA GCGGTCAGAT CTCACGATAG GTGCTGGATC

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                       2230       2240       2250       2260       2270       2280
AHSV-4 VP2      TCCTCAAGCG TCGCGATTGA GGTGAATTTA CGTCATGCAC TAATAGTTAG GATTTTTTCA
pKM-S2          TCCTCAAGCG TCGCGATTGA GGTGAATTTA CGTCATGCAC TAATAGTTAG GATTTTTTCA
pKM-S2 Amp      TCCTCAAGCG TCGCGATTGA GGTGAATTTA CGTCATGCAC TAATAGTTAG GATTTTTTCA

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                       2290       2300       2310       2320       2330       2340
AHSV-4 VP2      CGCTTTGACA TGATGTCGGA AAGAGAAACG TTTTCAACCA TTTTAGAAAA AGTCATGGAG
pKM-S2          CGCTTTGACA TGATGTCGGA AAGAGAAACG TTTTCAACCA TTTTAGAAAA AGTCATGGAG
pKM-S2 Amp      CGCTTTGACA TGATGTCGGA AAGAGAAACG TTTTCAACCA TTTTAGAAAA AGTCATGGAG

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                       2350       2360       2370       2380       2390       2400
AHSV-4 VP2      GATGTGAAAG AGTTGAGATT TTTCCCGACA TATCGTCATT ATTATTTGGA AACTCTCCAA
pKM-S2          GATGTGAAAG AGTTGAGATT TTTCCCGACA TATCGTCATT ATTATTTGGA AACTCTCCAA
pKM-S2 Amp      GATGTGAAAG AGTTGAGATT TTTCCCGACA TATCGTCATT ATTATTTGGA AACTCTCCAA
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                       2410       2420       2430       2440       2450       2460
AHSV-4 VP2      CGTGTCTTTA ACGATGAGAG ACGCTTAGAA GTTGATGACT TTTATATGAG GTTATATGAT
pKM-S2          CGTGTCTTTA ACGATGAGAG ACGCTTAGAA GTTGATGACT TTTATATGAG GTTATATGAT
pKM-S2 Amp      CGTGTCTTTA ACGATGAGAG ACGCTTAGAA GTTGATGACT TTTATATGAG GTTATATGAT

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                       2470       2480       2490       2500       2510       2520
AHSV-4 VP2      GTGCAGACAA GGGAGCAGGC ACTAAATACT TTCACGGATT TTCACAGGTG TGTTGAGTCG
pKM-S2          GTGCAGACAA GGGAGCAGGC ACTAAATACT TTCACGGATT TTCACAGGTG TGTTGAGTCG
pKM-S2 Amp      GTGCAGACAA GGGAGCAGGC ACTAAATACT TTCACGGATT TTCACAGGTG TGTTGAGTCG

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                       2530       2540       2550       2560       2570       2580
AHSV-4 VP2      GAACTGCTCT TACCGACACT TAAACTTAAC TTTCTGCTGT GGATTGTTTT TGAAATGGAA
pKM-S2          GAACTGCTCT TACCGACACT TAAACTTAAC TTTCTGCTGT GGATTGTTTT TGAAATGGAA
pKM-S2 Amp      GAACTGCTCT TACCGACACT TAAACTTAAC TTTCTGCTGT GGATTGTTTT TGAAATGGAA

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                       2590       2600       2610       2620       2630       2640
AHSV-4 VP2      AATGTTGAAG TGAACGCGGC GTACAAGCGT CATCCGCTTT TAATCTCAAC TGCCAAAGGG
pKM-S2          AATGTTGAAG TGAACGCGGC GTACAAGCGT CATCCGCTTT TAATCTCAAC TGCCAAAGGG
pKM-S2 Amp      AATGTTGAAG TGAACGCGGC GTACAAGCGT CATCCGCTTT TAATCTCAAC TGCCAAAGGG

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                       2650       2660       2670       2680       2690       2700
AHSV-4 VP2      TTAAGGGTTA TCGGCGTTGA TATTTTCAAC TCACAGCTTT CGATATCAAT GAGCGGATGG
pKM-S2          TTAAGGGTTA TCGGCGTTGA TATTTTCAAC TCACAGCTTT CGATATCAAT GAGCGGATGG
pKM-S2 Amp      TTAAGGGTTA TCGGCGTTGA TATTTTCAAC TCACAGCTTT CGATATCAAT GAGCGGATGG

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                       2710       2720       2730       2740       2750       2760
AHSV-4 VP2      ATTCCGTATG TCGAACGGAT GTGCGCGGAG AGTAAAGTTC AAACAAAATT GACGGCTGAT
pKM-S2          ATTCCGTATG TCGAACGGAT GTGCGCGGAG AGTAAAGTTC AAACAAAATT GACGGCTGAT
pKM-S2 Amp      ATTCCGTATG TCGAACGGAT GTGCGCGGAG AGTAAAGTTC AAACAAAATT GACGGCTGAT

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                       2770       2780       2790       2800       2810       2820
AHSV-4 VP2      GAGCTGAAAT TGAAGAGGTG GTTCATCTCA TATTATACGA CGTTGAAATT GGACCGCAGA
pKM-S2          GAGCTGAAAT TGAAGAGGTG GTTCATCTCA TATTATACGA CGTTGAAATT GGACCGCAGA
pKM-S2 Amp      GAGCTGAAAT TGAAGAGGTG GTTCATCTCA TATTATACGA CGTTGAAATT GGACCGCAGA

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                       2830       2840       2850       2860       2870       2880
AHSV-4 VP2      GCGGAGCCAC GTATGAGTTT CAAATTTGAG GGGTTGAGTA CATGGATCGG TTCGAACTGC
pKM-S2          GCGGAGCCAC GTATGAGTTT CAAATTTGAG GGGTTGAGTA CATGGATCGG TTCGAACTGC
pKM-S2 Amp      GCGGAGCCAC GTATGAGTTT CAAATTTGAG GGGTTGAGTA CATGGATCGG TTCGAACTGC

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                       2890       2900       2910       2920       2930       2940
AHSV-4 VP2      GGAGGTGTTA GGGATTACGT AATACAGATG CTTCCTACCA GAAAACCTAA ACCGGGAGCT
pKM-S2          GGAGGTGTTA GGGATTACGT AATACAGATG CTTCCTACCA GAAAACCTAA ACCGGGAGCT
pKM-S2 Amp      GGAGGTGTTA GGGATTACGT AATACAGATG CTTCCTACCA GAAAACCTAA ACCGGGAGCT

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                       2950       2960       2970       2980       2990       3000
AHSV-4 VP2      TTGATGGTGG TATACGCGCG GGATTCGAGA ATCGAGTGGA TCGAAGCAGA GCTATCACAG
pKM-S2          TTGATGGTGG TATACGCGCG GGATTCGAGA ATCGAGTGGA TCGAAGCAGA GCTATCACAG
pKM-S2 Amp      TTGATGGTGG TATACGCGCG GGATTCGAGA ATCGAGTGGA TCGAAGCAGA GCTATCACAG
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                         3010       3020       3030       3040       3050       3060
AHSV-4 VP2       TGGCTGCAAA TGGAAGGTTC GCTTGGTTTG ATCCTCGTTC ATGATTCAGG TATAATAAAT
pKM-S2           TGGCTGCAAA TGGAAGGTTC GCTTGGTTTG ATCCTCGTTC ATGATTCAGG TATAATAAAT
pKM-S2 Amp       TGGCTGCAAA TGGAAGGTTC GCTTGGTTTG ATCCTCGTTC ATGATTCAGG TATAATAAAT

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                         3070       3080       3090       3100       3110       3120
AHSV-4 VP2       AAGAGCGTAT TGAGAGCGAG AACTCTGAAA ATTTACAATA GGGGTTCGAT GGATACTTTA
pKM-S2           AAGAGCGTAT TGAGAGCGAG AACTCTGAAA ATTTACAATA GGGGTTCGAT GGATACTTTA
pKM-S2 Amp       AAGAGCGTAT TGAGAGCGAG AACTCTGAAA ATTTACAATA GGGGTTCGAT GGATACTTTA

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                         3130       3140       3150       3160       3170       3180
AHSV-4 VP2       ATTCTAATTT CGAGTGGAGT TTACACTTTC GGAAATAAAT TCTTGTTGTC GAAGTTACTC
pKM-S2           ATTCTAATTT CGAGTGGAGT TTACACTTTC GGAAATAAAT TCTTGTTGTC GAAGTTACTC
pKM-S2 Amp       ATTCTAATTT CGAGTGGAGT TTACACTTTC GGAAATAAAT TCTTGTTGTC GAAGTTACTC

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                         3190       3200       3210       3220
AHSV-4 VP2       GCAAAAACGG AATAGCAACG TGACTGTTGC TCCATGTGAA TACACATAC
pKM-S2           GCAAAAACGG AATAGCAACG TGACTGTTGC TCCATGTGAA TACACATAC
pKM-S2 Amp       GCAAAAACGG AATAGCAACG TGACTGTTGC TCCATGTGAA TACACATAC
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1.3 AHSV-4(32/62) VP3

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                          10         20         30         40         50         60
AHSV-4 VP3       GTTTATTTTC TGCTGTGAAC TCCAGAATGC AAGGGAATGA GAGAATACAA GATAAAAATG
pKM-S3           GTTTATTTTC TGCTGTGAAC TCCAGAATGC AAGGGAATGA GAGAATACAA GATAAAAATG
pKM-S3 Amp       GTTTATTTTC TGCTGTGAAC TCCAGAATGC AAGGGAATGA GAGAATACAA GATAAAAATG

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                          70         80         90        100        110        120
AHSV-4 VP3       AGAAGGAGAA GGCGTATGCA CCATATCTTG ACGGAGCGAG CGTATCAACG GATAATGGGC
pKM-S3           AGAAGGAGAA GGCGTATGCA CCATATCTTG ACGGAGCGAG CGTATCAACG GATAATGGGC
pKM-S3 Amp       AGAAGGAGAA GGCGTATGCA CCATATCTTG ACGGAGCGAG CGTATCAACG GATAATGGGC

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                         130        140        150        160        170        180
AHSV-4 VP3       CCATTTTATC AGTGTTTGCC TTACAGGAAA TCATGCAAAA AATTAGGCAA AATCAGAGCG
pKM-S3           CCATTTTATC AGTGTTTGCC TTACAGGAAA TCATGCAAAA AATTAGGCAA AATCAGAGCG
pKM-S3 Amp       CCATTTTATC AGTGTTTGCC TTACAGGAAA TCATGCAAAA AATTAGGCAA AATCAGAGCG

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                         190        200        210        220        230        240
AHSV-4 VP3       ATATGGCTGC ACATGCTCCC GATGTTGATG GGGCGATACC AGAGGTAATG ACTATAATCA
pKM-S3           ATATGGCTGC ACATGCTCCC GATGTTGATG GGGCGATACC AGAGGTAATG ACTATAATCA
pKM-S3 Amp       ATATGGCTGC ACATGCTCCC GATGTTGATG GGGCGATACC AGAGGTAATG ACTATAATCA

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                         250        260        270        280        290        300
AHSV-4 VP3       GTGGAATTAA GGGATTGCTG GAAGAAAAGG ATTACAAAGT AATAAACGCG CCACCGAATT
pKM-S3           GTGGAATTAA GGGATTGCTG GAAGAAAAGG ATTACAAAGT AATAAACGCG CCACCGAATT
pKM-S3 Amp       GTGGAATTAA GGGATTGCTG GAAGAAAAGG ATTACAAAGT AATAAACGCG CCACCGAATT
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                         310        320        330        340        350        360
AHSV-4 VP3       CTTTTCGCAC GATCCCGATG CAGAGTACGG AGTATGTTTT GCAAGTGAAC ACGTTTTATG
pKM-S3           CTTTTCGCAC GATCCCGATG CAGAGTACGG AGTATGTTTT GCAAGTGAAC ACGTTTTATG
pKM-S3 Amp       CTTTTCGCAC GATCCCGATG CAGAGTACGG AGTATGTTTT GCAAGTGAAC ACGTTTTATG

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                         370        380        390        400        410        420
AHSV-4 VP3       AGAGGATGTC GGAAATTGGA GGGCCGGTGG ATGAAACGGA TCCAATTGGA TTTTATGCAT
pKM-S3           AGAGGATGTC GGAAATTGGA GGGCCGGTGG ATGAAACGGA TCCAATTGGA TTTTATGCAT
pKM-S3 Amp       AGAGGATGTC GGAAATTGGA GGGCCGGTGG ATGAAACGGA TCCAATTGGA TTTTATGCAT

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                         430        440        450        460        470        480
AHSV-4 VP3       TGATACTGGA GAAATTGAAA TTTTTGAAAT CCGAGGGGGC TTTCATCTTG CAGGGGATAG
pKM-S3           TGATACTGGA GAAATTGAAA TTTTTGAAAT CCGAGGGGGC TTTCATCTTG CAGGGGATAG
pKM-S3 Amp       TGATACTGGA GAAATTGAAA TTTTTGAAAT CCGAGGGGGC TTTCATCTTG CAGGGGATAG

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                         490        500        510        520        530        540
AHSV-4 VP3       CTACTAAAGA TTATAGGGGT GCAGAGATCG CAGATCCAGA GATAATCGGG GTTAGCTTCC
pKM-S3           CTACTAAAGA TTATAGGGGT GCAGAGATCG CAGATCCAGA GATAATCGGG GTTAGCTTCC
pKM-S3 Amp       CTACTAAAGA TTATAGGGGT GCAGAGATCG CAGATCCAGA GATAATCGGG GTTAGCTTCC

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                         550        560        570        580        590        600
AHSV-4 VP3       AAAATGCTTT GAGTCATTTG GCGGCAATAG ATCGTCAAAT CATACAGGAT ACTCTCAACG
pKM-S3           AAAATGCTTT GAGTCATTTG GCGGCAATAG ATCGTCAAAT CATACAGGAT ACTCTCAACG
pKM-S3 Amp       AAAATGCTTT GAGTCATTTG GCGGCAATAG ATCGTCAAAT CATACAGGAT ACTCTCAACG

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                         610        620        630        640        650        660
AHSV-4 VP3       GGATGATCAT TGAAAATGGA TTAGTGGCAG ATAGAAATGT GGATGTGTTT AGAGCGGCGA
pKM-S3           GGATGATCAT TGAAAATGGA TTAGTGGCAG ATAGAAATGT GGATGTGTTT AGAGCGGCGA
pKM-S3 Amp       GGATGATCAT TGAAAATGGA TTAGTGGCAG ATAGAAATGT GGATGTGTTT AGAGCGGCGA

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                         670        680        690        700        710        720
AHSV-4 VP3       TGTCGGACCC AATCTATCGG ATACACAATG TATTACAAGG ATATATTGAA GGTATACAAT
pKM-S3           TGTCGGACCC AATCTATCGG ATACACAATG TATTACAAGG ATATATTGAA GGTATACAAT
pKM-S3 Amp       TGTCGGACCC AATCTATCGG ATACACAATG TATTACAAGG ATATATTGAA GGTATACAAT

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                         730        740        750        760        770        780
AHSV-4 VP3       ATGGGGAGTT GCGTGAATCG GTCAATTGGT TGATGAGGCT TGGACTAAGG AAGAGAATCG
pKM-S3           ATGGGGAGTT GCGTGAATCG GTCAATTGGT TGATGAGGCT TGGACTAAGG AAGAGAATCG
pKM-S3 Amp       ATGGGGAGTT GCGTGAATCG GTCAATTGGT TGATGAGGCT TGGACTAAGG AAGAGAATCG

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                         790        800        810        820        830        840
AHSV-4 VP3       AGTTTGCCAA CGATTTCTTG ACTGATTTTA GACGCGCGGA CACTATTTGG ATAATATCAC
pKM-S3           AGTTTGCCAA CGATTTCTTG ACTGATTTTA GACGCGCGGA CACTATTTGG ATAATATCAC
pKM-S3 Amp       AGTTTGCCAA CGATTTCTTG ACTGATTTTA GACGCGCGGA CACTATTTGG ATAATATCAC

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                         850        860        870        880        890        900
AHSV-4 VP3       AAAGGTTACC GATTAATGCT AATGTAATTT GGAATGTTCC AAGATGCCAT ATAGCAAATC
pKM-S3           AAAGGTTACC GATTAATGCT AATGTAATTT GGAATGTTCC AAGATGCCAT ATAGCAAATC
pKM-S3 Amp       AAAGGTTACC GATTAATGCT AATGTAATTT GGAATGTTCC AAGATGCCAT ATAGCAAATC
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                         910        920        930        940        950        960
AHSV-4 VP3       TAATCACAAA TGTCGCGTTA TGCTTACCGA CTGGAGAGTA TTTGATGCCC AACCCAAGAA
pKM-S3           TAATCACAAA TGTCGCGTTA TGCTTACCGA CTGGAGAGTA TTTGATGCCC AACCCAAGAA
pKM-S3 Amp       TAATCACAAA TGTCGCGTTA TGCTTACCGA CTGGAGAGTA TTTGATGCCC AACCCAAGAA

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                         970        980        990       1000       1010       1020
AHSV-4 VP3       TTAATTCGAT CACAATCACA CAGCGTATCA CTCAGACTAA TCCATTTTCA ATAATTTCAG
pKM-S3           TTAATTCGAT CACAATCACA CAGCGTATCA CTCAGACTAA TCCATTTTCA ATAATTTCAG
pKM-S3 Amp       TTAATTCGAT CACAATCACA CAGCGTATCA CTCAGACTAA TCCATTTTCA ATAATTTCAG

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                        1030       1040       1050       1060       1070       1080
AHSV-4 VP3       GGTTAACCCC GACAGCGGTT CAAATGAATG ATGTTAGAAA AATATATCTT GCATTAATGT
pKM-S3           GGTTAACCCC GACAGCGGTT CAAATGAATG ATGTTAGAAA AATATATCTT GCATTAATGT
pKM-S3 Amp       GGTTAACCCC GACAGCGGTT CAAATGAATG ATGTTAGAAA AATATATCTT GCATTAATGT

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                        1090       1100       1110       1120       1130       1140
AHSV-4 VP3       TTCCCAACCA AATTATTCTG GATATAAAAC CAGACAGCTC GCATGCGGTA GATCCAGTTT
pKM-S3           TTCCCAACCA AATTATTCTG GATATAAAAC CAGACAGCTC GCATGCGGTA GATCCAGTTT
pKM-S3 Amp       TTCCCAACCA AATTATTCTG GATATAAAAC CAGACAGCTC GCATGCGGTA GATCCAGTTT

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                        1150       1160       1170       1180       1190       1200
AHSV-4 VP3       TGCGAATGGT TGCGGGAGTA TTGGGCCATG TAATGTTTAC TTACGGACCG ATCATGACAA
pKM-S3           TGCGAATGGT TGCGGGAGTA TTGGGCCATG TAATGTTTAC TTACGGACCG ATCATGACAA
pKM-S3 Amp       TGCGAATGGT TGCGGGAGTA TTGGGCCATG TAATGTTTAC TTACGGACCG ATCATGACAA

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                        1210       1220       1230       1240       1250       1260
AHSV-4 VP3       ACATAACTCC AACGATGGCT GAATTGCTTG ATGCTGCGCT GAGCGATTAT TTATTGTACA
pKM-S3           ACATAACTCC AACGATGGCT GAATTGCTTG ATGCTGCGCT GAGCGATTAT TTATTGTACA
pKM-S3 Amp       ACATAACTCC AACGATGGCT GAATTGCTTG ATGCTGCGCT GAGCGATTAT TTATTGTACA

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                        1270       1280       1290       1300       1310       1320
AHSV-4 VP3       TGTATAACAA TCGGATTCCT ATAAATTACG GTCCAACAGG TCAACCCCTA GATTTTCGCA
pKM-S3           TGTATAACAA TCGGATTCCT ATAAATTACG GTCCAACAGG TCAACCCCTA GATTTTCGCA
pKM-S3 Amp       TGTATAACAA TCGGATTCCT ATAAATTACG GTCCAACAGG TCAACCCCTA GATTTTCGCA

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                        1330       1340       1350       1360       1370       1380
AHSV-4 VP3       TTGGAGCAAG AAATCAGTAT GATTGTAACG CTTTCCGAGC GGACCCACAA ACGGGTCGAG
pKM-S3           TTGGAGCAAG AAATCAGTAT GATTGTAACG CTTTCCGAGC GGACCCACAA ACGGGTCGAG
pKM-S3 Amp       TTGGAGCAAG AAATCAGTAT GATTGTAACG CTTTCCGAGC GGACCCACAA ACGGGTCGAG

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                        1390       1400       1410       1420       1430       1440
AHSV-4 VP3       GTTACAACGG GTGGGGGGTG GTTGATGTTC AGAGAGTCCA GCCGAGTCCA TATGATCATG
pKM-S3           GTTACAACGG GTGGGGGGTG GTTGATGTTC AGAGAGTCCA GCCGAGTCCA TATGATCATG
pKM-S3 Amp       GTTACAACGG GTGGGGGGTG GTTGATGTTC AGAGAGTCCA GCCGAGTCCA TATGATCATG

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                        1450       1460       1470       1480       1490       1500
AHSV-4 VP3       TTCAACGTGT AATCCGTTAC TGTGACATTG ATTCAAGGGA GATCATAGAT CCAAGAACTT
pKM-S3           TTCAACGTGT AATCCGTTAC TGTGACATTG ATTCAAGGGA GATCATAGAT CCAAGAACTT
pKM-S3 Amp       TTCAACGTGT AATCCGTTAC TGTGACATTG ATTCAAGGGA GATCATAGAT CCAAGAACTT
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                       1510       1520       1530       1540       1550       1560
AHSV-4 VP3      ATGGGATGAA TATGACATAT CCAATATTTC GGGAAATGCT TAGAATGCTA GTTGCTGCGG
pKM-S3          ATGGGATGAA TATGACATAT CCAATATTTC GGGAAATGCT TAGAATGCTA GTTGCTGCGG
pKM-S3 Amp      ATGGGATGAA TATGACATAT CCAATATTTC GGGAAATGCT TAGAATGCTA GTTGCTGCGG

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                       1570       1580       1590       1600       1610       1620
AHSV-4 VP3      GAAAAGATCA AGAAGCAGCT TATTTGAGAC AGATGCTGCC TTTTCATATG ATTCGTTTCG
pKM-S3          GAAAAGATCA AGAAGCAGCT TATTTGAGAC AGATGCTGCC TTTTCATATG ATTCGTTTCG
pKM-S3 Amp      GAAAAGATCA AGAAGCAGCT TATTTGAGAC AGATGCTGCC TTTTCATATG ATTCGTTTCG

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                       1630       1640       1650       1660       1670       1680
AHSV-4 VP3      CGCGAATAAA TCAAATTATA AATGAAGATT TGCTCAGTGC TTTCTCTCTG CCAGATCAGA
pKM-S3          CGCGAATAAA TCAAATTATA AATGAAGATT TGCTCAGTGC TTTCTCTCTG CCAGATCAGA
pKM-S3 Amp      CGCGAATAAA TCAAATTATA AATGAAGATT TGCTCAGTGC TTTCTCTCTG CCAGATCAGA

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                       1690       1700       1710       1720       1730       1740
AHSV-4 VP3      ATTTCGATGT AGTTCTACAC AACTTGATAC AAGGTAATTT TGGAGAAACG GATCCGGTAG
pKM-S3          ATTTCGATGT AGTTCTACAC AACTTGATAC AAGGTAATTT TGGAGAAACG GATCCGGTAG
pKM-S3 Amp      ATTTCGATGT AGTTCTACAC AACTTGATAC AAGGTAATTT TGGAGAAACG GATCCGGTAG

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                       1750       1760       1770       1780       1790       1800
AHSV-4 VP3      TTTTGGAGGT GAGTTGGGCT TCGATCTGGT TCGCCTTCGT GAGGCGTTTC GAACCCATCG
pKM-S3          TTTTGGAGGT GAGTTGGGCT TCGATCTGGT TCGCCTTCGT GAGGCGTTTC GAACCCATCG
pKM-S3 Amp      TTTTGGAGGT GAGTTGGGCT TCGATCTGGT TCGCCTTCGT GAGGCGTTTC GAACCCATCG

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                       1810       1820       1830       1840       1850       1860
AHSV-4 VP3      CCCGTTCAGA TTTACTTGAG GCAGCTCCTC TGATTGAAGC TAGATATGCA GCTGAATTAT
pKM-S3          CCCGTTCAGA TTTACTTGAG GCAGCTCCTC TGATTGAAGC TAGATATGCA GCTGAATTAT
pKM-S3 Amp      CCCGTTCAGA TTTACTTGAG GCAGCTCCTC TGATTGAAGC TAGATATGCA GCTGAATTAT

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                       1870       1880       1890       1900       1910       1920
AHSV-4 VP3      CCACGATGCA GATGGATGTA CAGCAGCTGA GGATGATGCG AGCCAGAGTC CCGGACACTG
pKM-S3          CCACGATGCA GATGGATGTA CAGCAGCTGA GGATGATGCG AGCCAGAGTC CCGGACACTG
pKM-S3 Amp      CCACGATGCA GATGGATGTA CAGCAGCTGA GGATGATGCG AGCCAGAGTC CCGGACACTG

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                       1930       1940       1950       1960       1970       1980
AHSV-4 VP3      TGATTAATGC GACGCCAAGT CAATGCTGGA AAGCGGTCTT GAAGAATGCG CCAGAGCCAA
pKM-S3          TGATTAATGC GACGCCAAGT CAATGCTGGA AAGCGGTCTT GAAGAATGCG CCAGAGCCAA
pKM-S3 Amp      TGATTAATGC GACGCCAAGT CAATGCTGGA AAGCGGTCTT GAAGAATGCG CCAGAGCCAA

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                       1990       2000       2010       2020       2030       2040
AHSV-4 VP3      TTAAAAATTT AATGAACTTG TCTCATTCTT TCAGTTTTGT GAATGTACGA GATATTGTAA
pKM-S3          TTAAAAATTT AATGAACTTG TCTCATTCTT TCAGTTTTGT GAATGTACGA GATATTGTAA
pKM-S3 Amp      TTAAAAATTT AATGAACTTG TCTCATTCTT TCAGTTTTGT GAATGTACGA GATATTGTAA

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                       2050       2060       2070       2080       2090       2100
AHSV-4 VP3      GGTGGAGTCA ACAGAGGGAC ATTCAGGAGT CGTTAGCGTA TGTTTTGAAT AGGGAAGCTT
pKM-S3          GGTGGAGTCA ACAGAGGGAC ATTCAGGAGT CGTTAGCGTA TGTTTTGAAT AGGGAAGCTT
pKM-S3 Amp      GGTGGAGTCA ACAGAGGGAC ATTCAGGAGT CGTTAGCGTA TGTTTTGAAT AGGGAAGCTT
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                       2110       2120       2130       2140       2150       2160
AHSV-4 VP3      GGGCGATTGC TAATGACTTT GAAGACTTGA TGTTAGTGGA TCACGTGTAT ATACAGCGCA
pKM-S3          GGGCGATTGC TAATGACTTT GAAGACTTGA TGTTAGTGGA TCACGTGTAT ATACAGCGCA
pKM-S3 Amp      GGGCGATTGC TAATGACTTT GAAGACTTGA TGTTAGTGGA TCACGTGTAT ATACAGCGCA

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                       2170       2180       2190       2200       2210       2220
AHSV-4 VP3      CGATGTTACC TGAACCTAGG TTAGACGATA TCAATGAGTT CAGGCGGCAA GGATTCTTCC
pKM-S3          CGATGTTACC TGAACCTAGG TTAGACGATA TCAATGAGTT CAGGCGGCAA GGATTCTTCC
pKM-S3 Amp      CGATGTTACC TGAACCTAGG TTAGACGATA TCAATGAGTT CAGGCGGCAA GGATTCTTCC

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                       2230       2240       2250       2260       2270       2280
AHSV-4 VP3      ATACGAATAT GATCGATGGA GCGCCACCAA TTGGAGATGT TACTCACTAC ACCTATGCGA
pKM-S3          ATACGAATAT GATCGATGGA GCGCCACCAA TTGGAGATGT TACTCACTAC ACCTATGCGA
pKM-S3 Amp      ATACGAATAT GATCGATGGA GCGCCACCAA TTGGAGATGT TACTCACTAC ACCTATGCGA

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                       2290       2300       2310       2320       2330       2340
AHSV-4 VP3      TTGCAAATCT ACAGGCGAAT ATGGGACAGT TCAGAGCGGC GATCAGGCGT ACACTGGATG
pKM-S3          TTGCAAATCT ACAGGCGAAT ATGGGACAGT TCAGAGCGGC GATCAGGCGT ACACTGGATG
pKM-S3 Amp      TTGCAAATCT ACAGGCGAAT ATGGGACAGT TCAGAGCGGC GATCAGGCGT ACACTGGATG

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                       2350       2360       2370       2380       2390       2400
AHSV-4 VP3      ATAACGGTTG GATTCAGTTT GGTGGTATGT TACGGAATAT CAAGATTAAA TTTTTTGATT
pKM-S3          ATAACGGTTG GATTCAGTTT GGTGGTATGT TACGGAATAT CAAGATTAAA TTTTTTGATT
pKM-S3 Amp      ATAACGGTTG GATTCAGTTT GGTGGTATGT TACGGAATAT CAAGATTAAA TTTTTTGATT

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                       2410       2420       2430       2440       2450       2460
AHSV-4 VP3      CGCGGCCGCC CGACGAGATA CTGACGGCAA TGCCTTATGT GTACACGGAG GAGGAGAGGG
pKM-S3          CGCGGCCGCC CGACGAGATA CTGACGGCAA TGCCTTATGT GTACACGGAG GAGGAGAGGG
pKM-S3 Amp      CGCGGCCGCC CGACGAGATA CTGACGGCAA TGCCTTATGT GTACACGGAG GAGGAGAGGG

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                       2470       2480       2490       2500       2510       2520
AHSV-4 VP3      ATGGAGTGAG GATGGTCGCT TTTAAATACG CGACAACCGC GACTGCCTAT TTCTTATTGT
pKM-S3          ATGGAGTGAG GATGGTCGCT TTTAAATACG CGACAACCGC GACTGCCTAT TTCTTATTGT
pKM-S3 Amp      ATGGAGTGAG GATGGTCGCT TTTAAATACG CGACAACCGC GACTGCCTAT TTCTTATTGT

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                       2530       2540       2550       2560       2570       2580
AHSV-4 VP3      ATAACGTTGA GTATTCAAAT ACGCCAGATA CTTTGATCAC AGTGAACCCA ACATTTACGA
pKM-S3          ATAACGTTGA GTATTCAAAT ACGCCAGATA CTTTGATCAC AGTGAACCCA ACATTTACGA
pKM-S3 Amp      ATAACGTTGA GTATTCAAAT ACGCCAGATA CTTTGATCAC AGTGAACCCA ACATTTACGA

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                       2590       2600       2610       2620       2630       2640
AHSV-4 VP3      TGACGAAAAT TCATATGCGA AAGAAAATTG TTAGACGAGT TCGCGCTCCA GATGTGCTAT
pKM-S3          TGACGAAAAT TCATATGCGA AAGAAAATTG TTAGACGAGT TCGCGCTCCA GATGTGCTAT
pKM-S3 Amp      TGACGAAAAT TCATATGCGA AAGAAAATTG TTAGACGAGT TCGCGCTCCA GATGTGCTAT

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                       2650       2660       2670       2680       2690       2700
AHSV-4 VP3      CACAAGTTAA CAAACGTTTA GTCGCGTACA AAGGTAAGAT GCGCTTAATG GATGTAACGA
pKM-S3          CACAAGTTAA CAAACGTTTA GTCGCGTACA AAGGTAAGAT GCGCTTAATG GATGTAACGA
pKM-S3 Amp      CACAAGTTAA CAAACGTTTA GTCGCGTACA AAGGTAAGAT GCGCTTAATG GATGTAACGA
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                       2710       2720       2730       2740       2750       2760
AHSV-4 VP3      AATGCCTAAA GACTGGAGTT CAACTTGCGC GACCAACGAT TTAGCAGCCT GACCACCTGG
pKM-S3          AATGCCTAAA GACTGGAGTT CAACTTGCGC GACCAACGAT TTAGCAGCCT GACCACCTGG
pKM-S3 Amp      AATGCCTAAA GACTGGAGTT CAACTTGCGC GACCAACGAT TTAGCAGCCT GACCACCTGG

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                       2770       2780       2790
AHSV-4 VP3      TGAATCGTTC TAGCGGGCAG AATTACACTT AC
pKM-S3          TGAATCGTTC TAGCGGGCAG AATTACACTT AC
pKM-S3 Amp      TGAATCGTTC TAGCGGGCAG AATTACACTT AC
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1.4 AHSV-4(32/62) VP4

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                         10         20         30         40         50         60
AHSV-4 VP4      GTTAATTTAG GATGGAACCT TACGCGATAT TGTATGTTAC GCAGGAGATC GAATACCTAC
pKM-S4          GTTAATTTAG GATGGAACCT TACGCGATAT TGTATGTTAC GCAGGAGATC GAATACCTAC
pKM-S4 Amp      GTTAATTTAG GATGGAACCT TACGCGATAT TGTATGTTAC GCAGGAGATC GAATACCTAC

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                         70         80         90        100        110        120
AHSV-4 VP4      TCAAAGATAG TTTTCTTCCA AAGTGGGAAC TTGATGGGAT TAAAGATCTT AATACATTAT
pKM-S4          TCAAAGATAG TTTTCTTCCA AAGTGGGAAC TTGATGGGAT TAAAGATCTT AATACATTAT
pKM-S4 Amp      TCAAAGATAG TTTTCTTCCA AAGTGGGAAC TTGATGGGAT TAAAGATCTT AATACATTAT

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                       130        140        150        160        170        180
AHSV-4 VP4      GGTTGGAGAG GGGCAGAATG GCGTGTGACA CATACGCGGT TGGAAAAATT GAGCAATGGT
pKM-S4          GGTTGGAGAG GGGCAGAATG GCGTGTGACA CATACGCGGT TGGAAAAATT GAGCAATGGT
pKM-S4 Amp      GGTTGGAGAG GGGCAGAATG GCGTGTGACA CATACGCGGT TGGAAAAATT GAGCAATGGT

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                       190        200        210        220        230        240
AHSV-4 VP4      CGGTGCGGCA GCTACGCGCA CATAGATTTT TGTTTATAAG TACGAAAAGG AAGATCAGGC
pKM-S4          CGGTGCGGCA GCTACGCGCA CATAGATTTT TGTTTATAAG TACGAAAAGG AAGATCAGGC
pKM-S4 Amp      CGGTGCGGCA GCTACGCGCA CATAGATTTT TGTTTATAAG TACGAAAAGG AAGATCAGGC

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                       250        260        270        280        290        300
AHSV-4 VP4      TGAAGGATTG CACGATTTCG CCCGACATAT TCATTTTGAA AAAAGAATTA AAGGAGTATG
pKM-S4          TGAAGGATTG CACGATTTCG CCCGACATAT TCATTTTGAA AAAAGAATTA AAGGAGTATG
pKM-S4 Amp      TGAAGGATTG CACGATTTCG CCCGACATAT TCATTTTGAA AAAAGAATTA AAGGAGTATG

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                       310        320        330        340        350        360
AHSV-4 VP4      ATATGAAGAG ATTCGAAACG TTAATTGGCA GAAGAAGGGT AACGTTAAGG AAGAGTTTCG
pKM-S4          ATATGAAGAG ATTCGAAACG TTAATTGGCA GAAGAAGGGT AACGTTAAGG AAGAGTTTCG
pKM-S4 Amp      ATATGAAGAG ATTCGAAACG TTAATTGGCA GAAGAAGGGT AACGTTAAGG AAGAGTTTCG

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                       370        380        390        400        410        420
AHSV-4 VP4      GGAATATGTT AAGGGCTTAT GCCTTCCAAC ACGTGACTGT CCTACACGGA AGTGAGGCGG
pKM-S4          GGAATATGTT AAGGGCTTAT GCCTTCCAAC ACGTGACTGT CCTACACGGA AGTGAGGCGG
pKM-S4 Amp      GGAATATGTT AAGGGCTTAT GCCTTCCAAC ACGTGACTGT CCTACACGGA AGTGAGGCGG
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                         430        440        450        460        470        480
AHSV-4 VP4       AAACGTTGAG TTATGCGGAT CCGAAGAGAC ACGTCGTGAA GGGCCAGCCT AAAGCTGCTC
pKM-S4           AAACGTTGAG TTATGCGGAT CCGAAGAGAC ACGTCGTGAA GGGCCAGCCT AAAGCTGCTC
pKM-S4 Amp       AAACGTTGAG TTATGCGGAT CCGAAGAGAC ACGTCGTGAA GGGCCAGCCT AAAGCTGCTC

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                         490        500        510        520        530        540
AHSV-4 VP4       CAATGTATGA TCATCCAGAC AGATGGTGGC GAGACGTTGA TGATGGACCA ACCGATAAGA
pKM-S4           CAATGTATGA TCATCCAGAC AGATGGTGGC GAGACGTTGA TGATGGACCA ACCGATAAGA
pKM-S4 Amp       CAATGTATGA TCATCCAGAC AGATGGTGGC GAGACGTTGA TGATGGACCA ACCGATAAGA

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                         550        560        570        580        590        600
AHSV-4 VP4       AATTAGTTAG TATGCTTGAT TACATTATAT ATAGTGCGGA TGAAGTGTAT TATGTCGGAT
pKM-S4           AATTAGTTAG TATGCTTGAT TACATTATAT ATAGTGCGGA TGAAGTGTAT TATGTCGGAT
pKM-S4 Amp       AATTAGTTAG TATGCTTGAT TACATTATAT ATAGTGCGGA TGAAGTGTAT TATGTCGGAT

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                         610        620        630        640        650        660
AHSV-4 VP4       GTGGTGATTT AAAAACACTC GAACAATTCG CGTCTAGAGA TAGAAAACGG TTTGACAGAA
pKM-S4           GTGGTGATTT AAAAACACTC GAACAATTCG CGTCTAGAGA TAGAAAACGG TTTGACAGAA
pKM-S4 Amp       GTGGTGATTT AAAAACACTC GAACAATTCG CGTCTAGAGA TAGAAAACGG TTTGACAGAA

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                         670        680        690        700        710        720
AHSV-4 VP4       TCAAATGGAT ATGCATAGAT CCAATTGCTC CGGAAACATC GTACGCTAAT GTAAAGGTTG
pKM-S4           TCAAATGGAT ATGCATAGAT CCAATTGCTC CGGAAACATC GTACGCTAAT GTAAAGGTTG
pKM-S4 Amp       TCAAATGGAT ATGCATAGAT CCAATTGCTC CGGAAACATC GTACGCTAAT GTAAAGGTTG

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                         730        740        750        760        770        780
AHSV-4 VP4       TAAAAGAAAA AGTTGTGTCA GCGCGTGACC TGAAACATTA TTTGATGCGC GATGAGGTTG
pKM-S4           TAAAAGAAAA AGTTGTGTCA GCGCGTGACC TGAAACATTA TTTGATGCGC GATGAGGTTG
pKM-S4 Amp       TAAAAGAAAA AGTTGTGTCA GCGCGTGACC TGAAACATTA TTTGATGCGC GATGAGGTTG

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                         790        800        810        820        830        840
AHSV-4 VP4       AGCGATTATT AATTTGGGAT GTGAGTGCAG ATGGGCTGAA GGGGACAATT GAGTGGGAGA
pKM-S4           AGCGATTATT AATTTGGGAT GTGAGTGCAG ATGGGCTGAA GGGGACAATT GAGTGGGAGA
pKM-S4 Amp       AGCGATTATT AATTTGGGAT GTGAGTGCAG ATGGGCTGAA GGGGACAATT GAGTGGGAGA

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                         850        860        870        880        890        900
AHSV-4 VP4       AGCGGAGATT TAAGGAGGAT CGTAATGGCG AAAACATCGC GGAAGCGTTG TGCTCAGATT
pKM-S4           AGCGGAGATT TAAGGAGGAT CGTAATGGCG AAAACATCGC GGAAGCGTTG TGCTCAGATT
pKM-S4 Amp       AGCGGAGATT TAAGGAGGAT CGTAATGGCG AAAACATCGC GGAAGCGTTG TGCTCAGATT

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                         910        920        930        940        950        960
AHSV-4 VP4       TTGCTTTAGC TTTGATTAAA CATCGAATAC CAGAAGAGAG TGATGAATAT ATTTGCAGGA
pKM-S4           TTGCTTTAGC TTTGATTAAA CATCGAATAC CAGAAGAGAG TGATGAATAT ATTTGCAGGA
pKM-S4 Amp       TTGCTTTAGC TTTGATTAAA CATCGAATAC CAGAAGAGAG TGATGAATAT ATTTGCAGGA

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                         970        980        990        1000       1010       1020
AHSV-4 VP4       GCTCTTGGCT ACTACCACAG CCCGGGGCTC CAATAACGAT GTATGAGCTA CGAAATCTCA
pKM-S4           GCTCTTGGCT ACTACCACAG CCCGGGGCTC CAATAACGAT GTATGAGCTA CGAAATCTCA
pKM-S4 Amp       GCTCTTGGCT ACTACCACAG CCCGGGGCTC CAATAACGAT GTATGAGCTA CGAAATCTCA
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                       1030       1040       1050       1060       1070       1080
AHSV-4 VP4      TGCGTTTGGA CGGATATTCG CATGTTGAAA GGAAGCACAT ACCAAGAGCG CACGTCCGGA
pKM-S4          TGCGTTTGGA CGGATATTCG CATGTTGAAA GGAAGCACAT ACCAAGAGCG CACGTCCGGA
pKM-S4 Amp      TGCGTTTGGA CGGATATTCG CATGTTGAAA GGAAGCACAT ACCAAGAGCG CACGTCCGGA

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                       1090       1100       1110       1120       1130       1140
AHSV-4 VP4      AAATCGATGC GGAAGTTGCG AGAAGATTAG TTGAGGAGTA TCATGGAGAG GATGTTGGAC
pKM-S4          AAATCGATGC GGAAGTTGCG AGAAGATTAG TTGAGGAGTA TCATGGAGAG GATGTTGGAC
pKM-S4 Amp      AAATCGATGC GGAAGTTGCG AGAAGATTAG TTGAGGAGTA TCATGGAGAG GATGTTGGAC

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                       1150       1160       1170       1180       1190       1200
AHSV-4 VP4      GATTGTTGAA ACGATCTTTA TATGAAGATA TACATATTGA GCGCGCTGAT GGGTTGACGG
pKM-S4          GATTGTTGAA ACGATCTTTA TATGAAGATA TACATATTGA GCGCGCTGAT GGGTTGACGG
pKM-S4 Amp      GATTGTTGAA ACGATCTTTA TATGAAGATA TACATATTGA GCGCGCTGAT GGGTTGACGG

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                       1210       1220       1230       1240       1250       1260
AHSV-4 VP4      ATGGTGATGA GAGAACGAGA GCGGACCTCT TTTATTTGAC AAATATGCGG AACGCTGCGT
pKM-S4          ATGGTGATGA GAGAACGAGA GCGGACCTCT TTTATTTGAC AAATATGCGG AACGCTGCGT
pKM-S4 Amp      ATGGTGATGA GAGAACGAGA GCGGACCTCT TTTATTTGAC AAATATGCGG AACGCTGCGT

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                       1270       1280       1290       1300       1310       1320
AHSV-4 VP4      TTATGCATGA TGTATATCGA GTAGTTGAAA AAAGTTTTAT TTCTACCCTG TGGGTCTCGA
pKM-S4          TTATGCATGA TGTATATCGA GTAGTTGAAA AAAGTTTTAT TTCTACCCTG TGGGTCTCGA
pKM-S4 Amp      TTATGCATGA TGTATATCGA GTAGTTGAAA AAAGTTTTAT TTCTACCCTG TGGGTCTCGA

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                       1330       1340       1350       1360       1370       1380
AHSV-4 VP4      ACAGGCAGAA TTTCACATAT GATGATGTCC CGGTTAATAG GAATTTTATT ACATTACGCT
pKM-S4          ACAGGCAGAA TTTCACATAT GATGATGTCC CGGTTAATAG GAATTTTATT ACATTACGCT
pKM-S4 Amp      ACAGGCAGAA TTTCACATAT GATGATGTCC CGGTTAATAG GAATTTTATT ACATTACGCT

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                       1390       1400       1410       1420       1430       1440
AHSV-4 VP4      TTTCGAAAAA GAATCGACGA GTGCTCGATG GGAATGGAGC AATTCTGTTT CTGATGTGGC
pKM-S4          TTTCGAAAAA GAATCGACGA GTGCTCGATG GGAATGGAGC AATTCTGTTT CTGATGTGGC
pKM-S4 Amp      TTTCGAAAAA GAATCGACGA GTGCTCGATG GGAATGGAGC AATTCTGTTT CTGATGTGGC

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                       1450       1460       1470       1480       1490       1500
AHSV-4 VP4      AGCATCCGAA GGATTTTCCA AAAACTATGA ACTATGACCC CAGTTGGGCG GAGAATTATG
pKM-S4          AGCATCCGAA GGATTTTCCA AAAACTATGA ACTATGACCC CAGTTGGGCG GAGAATTATG
pKM-S4 Amp      AGCATCCGAA GGATTTTCCA AAAACTATGA ACTATGACCC CAGTTGGGCG GAGAATTATG

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                       1510       1520       1530       1540       1550       1560
AHSV-4 VP4      CTGTCATTTT CTATCATGCG TTAACGAGTC CGGTTCCGGA TCTTTCATTA TGCAGATTTA
pKM-S4          CTGTCATTTT CTATCATGCG TTAACGAGTC CGGTTCCGGA TCTTTCATTA TGCAGATTTA
pKM-S4 Amp      CTGTCATTTT CTATCATGCG TTAACGAGTC CGGTTCCGGA TCTTTCATTA TGCAGATTTA

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                       1570       1580       1590       1600       1610       1620
AHSV-4 VP4      TTGGATTAAG GTTGATGTCT TCAACTTTGA GGATAAATTC AGATCGTCCA CATCAGGTAA
pKM-S4          TTGGATTAAG GTTGATGTCT TCAACTTTGA GGATAAATTC AGATCGTCCA CATCAGGTAA
pKM-S4 Amp      TTGGATTAAG GTTGATGTCT TCAACTTTGA GGATAAATTC AGATCGTCCA CATCAGGTAA
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                       1630       1640       1650       1660       1670       1680
AHSV-4 VP4      CTGATATCTT GAAGAAATTG GGTTTGGACG TATCGGGTCA TCTATTTATT TGCTTAATGT
pKM-S4          CTGATATCTT GAAGAAATTG GGTTTGGACG TATCGGGTCA TCTATTTATT TGCTTAATGT
pKM-S4 Amp      CTGATATCTT GAAGAAATTG GGTTTGGACG TATCGGGTCA TCTATTTATT TGCTTAATGT

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                       1690       1700       1710       1720       1730       1740
AHSV-4 VP4      CGAATTCATA TGTTGCTGAT CTTGATTGGT GGTTTCGTAT GATCCTAGAG TGGTCAGTTA
pKM-S4          CGAATTCATA TGTTGCTGAT CTTGATTGGT GGTTTCGTAT GATCCTAGAG TGGTCAGTTA
pKM-S4 Amp      CGAATTCATA TGTTGCTGAT CTTGATTGGT GGTTTCGTAT GATCCTAGAG TGGTCAGTTA

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                       1750       1760       1770       1780       1790       1800
AHSV-4 VP4      AGGATAGAGA AGGGAAGCTG GCGGCGTTAA GTGAAGCGAA GGCAGAACTC ATTGAGTGGA
pKM-S4          AGGATAGAGA AGGGAAGCTG GCGGCGTTAA GTGAAGCGAA GGCAGAACTC ATTGAGTGGA
pKM-S4 Amp      AGGATAGAGA AGGGAAGCTG GCGGCGTTAA GTGAAGCGAA GGCAGAACTC ATTGAGTGGA

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                       1810       1820       1830       1840       1850       1860
AHSV-4 VP4      AGGATGAGAA AGCGGACGAG CCTTGGCACA TAAAGAATGA TTTGCTGGCG GCGTTGTTTG
pKM-S4          AGGATGAGAA AGCGGACGAG CCTTGGCACA TAAAGAATGA TTTGCTGGCG GCGTTGTTTG
pKM-S4 Amp      AGGATGAGAA AGCGGACGAG CCTTGGCACA TAAAGAATGA TTTGCTGGCG GCGTTGTTTG

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                       1870       1880       1890       1900       1910       1920
AHSV-4 VP4      AGTTTATGTA TTTTGCGAAA CATTTTGAGA TAAATGAGAG GTATGTCGAG TCCTGGATAC
pKM-S4          AGTTTATGTA TTTTGCGAAA CATTTTGAGA TAAATGAGAG GTATGTCGAG TCCTGGATAC
pKM-S4 Amp      AGTTTATGTA TTTTGCGAAA CATTTTGAGA TAAATGAGAG GTATGTCGAG TCCTGGATAC

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                       1930       1940       1950       1960       1970
AHSV-4 VP4      AATATCTGCG TAACGCTTAA AGGTGACACT TAGCGAGGGG GAATCCTAAT AACCTTAC
pKM-S4          AATATCTGCG TAACGCTTAA AGGTGACACT TAGCGAGGGG GAATCCTAAT AACCTTAC
pKM-S4 Amp      AATATCTGCG TAACGCTTAA AGGTGACACT TAGCGAGGGG GAATCCTAAT AACCTTAC
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1.5 AHSV-4(32/62) NS1

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                        10         20         30         40         50         60
AHSV-4 NS1      GTTAAAGAAC CTAGGCGGTT TGGCAACACA CAAACATGGA TAGATTCTTG ACTTATTTCC
pKM-S5          GTTAAAGAAC CTAGGCGGTT TGGCAACACA CAAACATGGA TAGATTCTTG ACTTATTTCC
pKM-S5 Amp      GTTAAAGAAC CTAGGCGGTT TGGCAACACA CAAACATGGA TAGATTCTTG ACTTATTTCC

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                        70         80         90        100        110        120
AHSV-4 NS1      AGGTACGAGG AGAGAGAGCA AACGCTGTTC GGCTGTTTGG AGAGATTTCC GAACAAATAG
pKM-S5          AGGTACGAGG AGAGAGAGCA AACGCTGTTC GGCTGTTTGG AGAGATTTCC GAACAAATAG
pKM-S5 Amp      AGGTACGAGG AGAGAGAGCA AACGCTGTTC GGCTGTTTGG AGAGATTTCC GAACAAATAG

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                       130        140        150        160        170        180
AHSV-4 NS1      ATTGCTCACA TCTCAAACGA GATTGCTTTG TAAATGGAAT ATGTGCAAGA CAACACTTTA
pKM-S5          ATTGCTCACA TCTCAAACGA GATTGCTTTG TAAATGGAAT ATGTGCAAGA CAACACTTTA
pKM-S5 Amp      ATTGCTCACA TCTCAAACGA GATTGCTTTG TAAATGGAAT ATGTGCAAGA CAACACTTTA
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                       190        200        210        220        230        240
AHSV-4 NS1      AAGAATGCTG TAATATTGCT ACAGATAATG GCTCACGCAC AAATGCAGAT AAATTAGTGG
pKM-S5          AAGAATGCTG TAATATTGCT ACAGATAATG GCTCACGCAC AAATGCAGAT AAATTAGTGG
pKM-S5 Amp      AAGAATGCTG TAATATTGCT ACAGATAATG GCTCACGCAC AAATGCAGAT AAATTAGTGG

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                       250        260        270        280        290        300
AHSV-4 NS1      CTTTAGCTTT GCGAGCACTT TTAGATAGAC AAACTATTTG GACTTGTGTC ATCAAAAATG
pKM-S5          CTTTAGCTTT GCGAGCACTT TTAGATAGAC AAACTATTTG GACTTGTGTC ATCAAAAATG
pKM-S5 Amp      CTTTAGCTTT GCGAGCACTT TTAGATAGAC AAACTATTTG GACTTGTGTC ATCAAAAATG

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                       310        320        330        340        350        360
AHSV-4 NS1      CGGATTACGT TAGTCAATAT GCTGATGAAC AGATGGAGGA AGAAGTTAAT AAGCTGTATG
pKM-S5          CGGATTACGT TAGTCAATAT GCTGATGAAC AGATGGAGGA AGAAGTTAAT AAGCTGTATG
pKM-S5 Amp      CGGATTACGT TAGTCAATAT GCTGATGAAC AGATGGAGGA AGAAGTTAAT AAGCTGTATG

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                       370        380        390        400        410        420
AHSV-4 NS1      ATGTCTATCT CCAGAGCGGG ACGAGAGAGG AATTTGAAGG ATTTAGACAG AGGAATAGAC
pKM-S5          ATGTCTATCT CCAGAGCGGG ACGAGAGAGG AATTTGAAGG ATTTAGACAG AGGAATAGAC
pKM-S5 Amp      ATGTCTATCT CCAGAGCGGG ACGAGAGAGG AATTTGAAGG ATTTAGACAG AGGAATAGAC

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                       430        440        450        460        470        480
AHSV-4 NS1      CAAGTAGAGT TGTGATGGAT GATAGCTGCT CAATGCTCTC ATATTTTTAC ATTCCAATGA
pKM-S5          CAAGTAGAGT TGTGATGGAT GATAGCTGCT CAATGCTCTC ATATTTTTAC ATTCCAATGA
pKM-S5 Amp      CAAGTAGAGT TGTGATGGAT GATAGCTGCT CAATGCTCTC ATATTTTTAC ATTCCAATGA

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                       490        500        510        520        530        540
AHSV-4 NS1      ATCAAGGGAA TCCAGCTCCA GTTGCGAAGC TTAGCCGATG GGGTCAATTT GGAATTTGTT
pKM-S5          ATCAAGGGAA TCCAGCTCCA GTTGCGAAGC TTAGCCGATG GGGTCAATTT GGAATTTGTT
pKM-S5 Amp      ATCAAGGGAA TCCAGCTCCA GTTGCGAAGC TTAGCCGATG GGGTCAATTT GGAATTTGTT

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                       550        560        570        580        590        600
AHSV-4 NS1      ACTATGATAG AACAAATGTT GATGGATTGA TTCCGTATGA TGAGATCGGT TTAGCTCAAG
pKM-S5          ACTATGATAG AACAAATGTT GATGGATTGA TTCCGTATGA TGAGATCGGT TTAGCTCAAG
pKM-S5 Amp      ACTATGATAG AACAAATGTT GATGGATTGA TTCCGTATGA TGAGATCGGT TTAGCTCAAG

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                       610        620        630        640        650        660
AHSV-4 NS1      CTATAGATGG CTTAAAGGAT CTGATTGAAG GGCGATTGCC CGTTTGCCCT TATACTGGAG
pKM-S5          CTATAGATGG CTTAAAGGAT CTGATTGAAG GGCGATTGCC CGTTTGCCCT TATACTGGAG
pKM-S5 Amp      CTATAGATGG CTTAAAGGAT CTGATTGAAG GGCGATTGCC CGTTTGCCCT TATACTGGAG

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                       670        680        690        700        710        720
AHSV-4 NS1      CGAATGGCAG GATTAATGCT GTTTTACATC TACCATTAGA GATGGAGGTG ATTATGGCGG
pKM-S5          CGAATGGCAG GATTAATGCT GTTTTACATC TACCATTAGA GATGGAGGTG ATTATGGCGG
pKM-S5 Amp      CGAATGGCAG GATTAATGCT GTTTTACATC TACCATTAGA GATGGAGGTG ATTATGGCGG

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                       730        740        750        760        770        780
AHSV-4 NS1      TGCAGGAAAA TGCAACACAA TTAATGCGTA GAGCGGCACA GGATTTCAAA TTCATCACAC
pKM-S5          TGCAGGAAAA TGCAACACAA TTAATGCGTA GAGCGGCACA GGATTTCAAA TTCATCACAC
pKM-S5 Amp      TGCAGGAAAA TGCAACACAA TTAATGCGTA GAGCGGCACA GGATTTCAAA TTCATCACAC
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                        790        800        810        820        830        840
AHSV-4 NS1      ACGCTGGATG GAGGCTATAT CCAAGATTGT TGCGACAACG GTTCGCGATC GAGGACGCTA
pKM-S5          ACGCTGGATG GAGGCTATAT CCAAGATTGT TGCGACAACG GTTCGCGATC GAGGACGCTA
pKM-S5 Amp      ACGCTGGATG GAGGCTATAT CCAAGATTGT TGCGACAACG GTTCGCGATC GAGGACGCTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        850        860        870        880        890        900
AHSV-4 NS1      CGGAAGGGGT GATTCATCAT GTGATGCTAG GCCATTTAAG ATATTATGAT GAAGATACAA
pKM-S5          CGGAAGGGGT GATTCATCAT GTGATGCTAG GCCATTTAAG ATATTATGAT GAAGATACAA
pKM-S5 Amp      CGGAAGGGGT GATTCATCAT GTGATGCTAG GCCATTTAAG ATATTATGAT GAAGATACAA

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                        910        920        930        940        950        960
AHSV-4 NS1      GTATCGTGAA GTATCGCTTC CTTAACGATG GATCTTTAGA TTGGAGGACT TGGACAATTC
pKM-S5          GTATCGTGAA GTATCGCTTC CTTAACGATG GATCTTTAGA TTGGAGGACT TGGACAATTC
pKM-S5 Amp      GTATCGTGAA GTATCGCTTC CTTAACGATG GATCTTTAGA TTGGAGGACT TGGACAATTC

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                        970        980        990       1000       1010       1020
AHSV-4 NS1      CTTTACATCT GATGCGGACA GCAAGGTTGG GACATCTGCA ACCGGAATCA ATTTTAGTCT
pKM-S5          CTTTACATCT GATGCGGACA GCAAGGTTGG GACATCTGCA ACCGGAATCA ATTTTAGTCT
pKM-S5 Amp      CTTTACATCT GATGCGGACA GCAAGGTTGG GACATCTGCA ACCGGAATCA ATTTTAGTCT

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                       1030       1040       1050       1060       1070       1080
AHSV-4 NS1      TTATGCATAA AAAGCTCACA TGTCAGGTAT GTTTTATGGT TGACCTCGCT CTGCTTGACA
pKM-S5          TTATGCATAA AAAGCTCACA TGTCAGGTAT GTTTTATGGT TGACCTCGCT CTGCTTGACA
pKM-S5 Amp      TTATGCATAA AAAGCTCACA TGTCAGGTAT GTTTTATGGT TGACCTCGCT CTGCTTGACA

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                       1090       1100       1110       1120       1130       1140
AHSV-4 NS1      CAATCCCAGT GGTTGATTCA AAAATTGCTG AACTAACAGG AGGCACAGAT GTATTTTATA
pKM-S5          CAATCCCAGT GGTTGATTCA AAAATTGCTG AACTAACAGG AGGCACAGAT GTATTTTATA
pKM-S5 Amp      CAATCCCAGT GGTTGATTCA AAAATTGCTG AACTAACAGG AGGCACAGAT GTATTTTATA

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                       1150       1160       1170       1180       1190       1200
AHSV-4 NS1      CACGTGCGTA TGTACATGCG GACAATCACA AAGTGCCAAA TGTCAGAGAT TTGATGATGA
pKM-S5          CACGTGCGTA TGTACATGCG GACAATCACA AAGTGCCAAA TGTCAGAGAT TTGATGATGA
pKM-S5 Amp      CACGTGCGTA TGTACATGCG GACAATCACA AAGTGCCAAA TGTCAGAGAT TTGATGATGA

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                       1210       1220       1230       1240       1250       1260
AHSV-4 NS1      ATGAAGTCTT CAGGAAGATC GATGATCATT GGGTGATTCA GAAGTGTCAT ACAACGAAGG
pKM-S5          ATGAAGTCTT CAGGAAGATC GATGATCATT GGGTGATTCA GAAGTGTCAT ACAACGAAGG
pKM-S5 Amp      ATGAAGTCTT CAGGAAGATC GATGATCATT GGGTGATTCA GAAGTGTCAT ACAACGAAGG

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                       1270       1280       1290       1300       1310       1320
AHSV-4 NS1      AAGCGATTAC TGTAACTGCA ATTCAGATTC AGAGGTCGAT CAGAGGTGAT GGGCAGTGGG
pKM-S5          AAGCGATTAC TGTAACTGCA ATTCAGATTC AGAGGTCGAT CAGAGGTGAT GGGCAGTGGG
pKM-S5 Amp      AAGCGATTAC TGTAACTGCA ATTCAGATTC AGAGGTCGAT CAGAGGTGAT GGGCAGTGGG

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                       1330       1340       1350       1360       1370       1380
AHSV-4 NS1      ATACTCCGAT GTTTCACCAA TCAATGGCTC TGTTAACACG ATTGATTGTT TATTGGTTGA
pKM-S5          ATACTCCGAT GTTTCACCAA TCAATGGCTC TGTTAACACG ATTGATTGTT TATTGGTTGA
pKM-S5 Amp      ATACTCCGAT GTTTCACCAA TCAATGGCTC TGTTAACACG ATTGATTGTT TATTGGTTGA
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                       1390       1400       1410       1420       1430       1440
AHSV-4 NS1      CGGATGTGAC TGAGAGAAGT GCTATCTTTC GGCTGACTTG TTTCGCAATC TTCGGATGTA
pKM-S5          CGGATGTGAC TGAGAGAAGT GCTATCTTTC GGCTGACTTG TTTCGCAATC TTCGGATGTA
pKM-S5 Amp      CGGATGTGAC TGAGAGAAGT GCTATCTTTC GGCTGACTTG TTTCGCAATC TTCGGATGTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1450       1460       1470       1480       1490       1500
AHSV-4 NS1      AGCCAACGGC TCGAGGTAGA TATATTGATT GGGATGATCT TGGAACATTC ATGAAGAATG
pKM-S5          AGCCAACGGC TCGAGGTAGA TATATTGATT GGGATGATCT TGGAACATTC ATGAAGAATG
pKM-S5 Amp      AGCCAACGGC TCGAGGTAGA TATATTGATT GGGATGATCT TGGAACATTC ATGAAGAATG

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                       1510       1520       1530       1540       1550       1560
AHSV-4 NS1      TGTTGGATGG AAGAGATTTG ACTGTTTTGG AAGATGAAAC ATGTTTTATT TCGATGATGA
pKM-S5          TGTTGGATGG AAGAGATTTG ACTGTTTTGG AAGATGAAAC ATGTTTTATT TCGATGATGA
pKM-S5 Amp      TGTTGGATGG AAGAGATTTG ACTGTTTTGG AAGATGAAAC ATGTTTTATT TCGATGATGA

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                       1570       1580       1590       1600       1610       1620
AHSV-4 NS1      GGATGGCGAT GTTGCATGTG CAGAGATCCA AGGTAGTGTG CGCAACTGTG TTGGAGGCGC
pKM-S5          GGATGGCGAT GTTGCATGTG CAGAGATCCA AGGTAGTGTG CGCAACTGTG TTGGAGGCGC
pKM-S5 Amp      GGATGGCGAT GTTGCATGTG CAGAGATCCA AGGTAGTGTG CGCAACTGTG TTGGAGGCGC

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                       1630       1640       1650       1660       1670       1680
AHSV-4 NS1      CATTGGAAAT ACAACAGGTT GGCCAGATCG TTGAAGTACC CTTTGATTTC ATGCATAATT
pKM-S5          CATTGGAAAT ACAACAGGTT GGCCAGATCG TTGAAGTACC CTTTGATTTC ATGCATAATT
pKM-S5 Amp      CATTGGAAAT ACAACAGGTT GGCCAGATCG TTGAAGTACC CTTTGATTTC ATGCATAATT

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                       1690       1700       1710       1720       1730       1740
AHSV-4 NS1      AGATCAAATT TAAAAGTTTG TACCCGGGAA GTAGGTTAAG ATCATATCCC CCTGGTTCAC
pKM-S5          AGATCAAATT TAAAAGTTTG TACCCGGGAA GTAGGTTAAG ATCATATCCC CCTGGTTCAC
pKM-S5 Amp      AGATCAAATT TAAAAGTTTG TACCCGGGAA GTAGGTTAAG ATCATATCCC CCTGGTTCAC

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AHSV-4 NS1      AAACTTAC
pKM-S5          AAACTTAC
pKM-S5 Amp      AAACTTAC
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1.6 AHSV-4(32/62) VP5

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
AHSV-4 VP5      GTTAATTTTT CCAGAAGCCA TGGGAAAGTT CACATCTTTT TTGAAGCGCG CGGGCAATGC
pKM-S6          GTTAATTTTT CCAGAAGCCA TGGGAAAGTT CACATCTTTT TTGAAGCGCG CGGGCAATGC
pKM-S6 Amp      GTTAATTTTT CCAGAAGCCA TGGGAAAGTT CACATCTTTT TTGAAGCGCG CGGGCAATGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
AHSV-4 VP5      GACCAAGAGG GCGCTGACGT CGGATTCAGC AAAGAAGATG TATAAGTTGG CGGGGAAAAC
pKM-S6          GACCAAGAGG GCGCTGACGT CGGATTCAGC AAAGAAGATG TATAAGTTGG CGGGGAAAAC
pKM-S6 Amp      GACCAAGAGG GCGCTGACGT CGGATTCAGC AAAGAAGATG TATAAGTTGG CGGGGAAAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 VP5      GTTACAGAGA GTGGTAGAAA GTGAAGTTGG AAGTGCAGCG ATCGATGGCG TGATGCAGGG
pKM-S6          GTTACAGAGA GTGGTAGAAA GTGAAGTTGG AAGTGCAGCG ATCGATGGCG TGATGCAGGG
pKM-S6 Amp      GTTACAGAGA GTGGTAGAAA GTGAAGTTGG AAGTGCAGCG ATCGATGGCG TGATGCAGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 VP5      GGCGATACAA AGCATAATAC AAGGCGAAAA CCTTGGTGAT TCAATTAAGC AGGCGGTTAT
pKM-S6          GGCGATACAA AGCATAATAC AAGGCGAAAA CCTTGGTGAT TCAATTAAGC AGGCGGTTAT
pKM-S6 Amp      GGCGATACAA AGCATAATAC AAGGCGAAAA CCTTGGTGAT TCAATTAAGC AGGCGGTTAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 VP5      TTTAAATGTT GCGGGGACAT TGGAATCGGC GCCAGACCCG TTGAGCCCAG GGGAGCAGCT
pKM-S6          TTTAAATGTT GCGGGGACAT TGGAATCGGC GCCAGACCCG TTGAGCCCAG GGGAGCAGCT
pKM-S6 Amp      TTTAAATGTT GCGGGGACAT TGGAATCGGC GCCAGACCCG TTGAGCCCAG GGGAGCAGCT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 VP5      CCTTTACAAT AAGGTTTCTG AAATCGAGAA AATGGAAAAA GAGGATCGAG TGATTGAAAC
pKM-S6          CCTTTACAAT AAGGTTTCTG AAATCGAGAA AATGGAAAAA GAGGATCGAG TGATTGAAAC
pKM-S6 Amp      CCTTTACAAT AAGGTTTCTG AAATCGAGAA AATGGAAAAA GAGGATCGAG TGATTGAAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 VP5      ACACAATGCG AAAATAGAAG AAAAATTTGG TAAAGATTTA TTAGCGATTC GAAAGATTGT
pKM-S6          ACACAATGCG AAAATAGAAG AAAAATTTGG TAAAGATTTA TTAGCGATTC GAAAGATTGT
pKM-S6 Amp      ACACAATGCG AAAATAGAAG AAAAATTTGG TAAAGATTTA TTAGCGATTC GAAAGATTGT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480
AHSV-4 VP5      GAAAGGCGAG GTTGATGCAG AAAAGCTGGA AGGTAACGAA ATTAAGTACG TAGAAAAAGC
pKM-S6          GAAAGGCGAG GTTGATGCAG AAAAGCTGGA AGGTAACGAA ATTAAGTACG TAGAAAAAGC
pKM-S6 Amp      GAAAGGCGAG GTTGATGCAG AAAAGCTGGA AGGTAACGAA ATTAAGTACG TAGAAAAAGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        490        500        510        520        530        540
AHSV-4 VP5      GCTTAGCGGT TTGCTGGAGA TAGGGAAAGA TCAGTCAGAA CGCATTACAA AGCTATATCG
pKM-S6          GCTTAGCGGT TTGCTGGAGA TAGGGAAAGA TCAGTCAGAA CGCATTACAA AGCTATATCG
pKM-S6 Amp      GCTTAGCGGT TTGCTGGAGA TAGGGAAAGA TCAGTCAGAA CGCATTACAA AGCTATATCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        550        560        570        580        590        600
AHSV-4 VP5      CGCGTTACAA ACAGAGGAAG ATTTGCGGAC ACGAGATGAG ACTAGAATGA TAAACGAATA
pKM-S6          CGCGTTACAA ACAGAGGAAG ATTTGCGGAC ACGAGATGAG ACTAGAATGA TAAACGAATA
pKM-S6 Amp      CGCGTTACAA ACAGAGGAAG ATTTGCGGAC ACGAGATGAG ACTAGAATGA TAAACGAATA
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650        660
AHSV-4 VP5       TAGAGAGAAA TTTGACGCGT TGAAAGAAGC GATTGAAATC GAGCAGCAAG CGACACATGA
pKM-S6           TAGAGAGAAA TTTGACGCGT TGAAAGAAGC GATTGAAATC GAGCAGCAAG CGACACATGA
pKM-S6 Amp       TAGAGAGAAA TTTGACGCGT TGAAAGAAGC GATTGAAATC GAGCAGCAAG CGACACATGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         670        680        690        700        710        720
AHSV-4 VP5       TGAAGCGATT CAAGAGATGC TCGACTTAAG CGCGGAAGTA ATTGAGACTG CGTCGGAGGA
pKM-S6           TGAAGCGATT CAAGAGATGC TCGACTTAAG CGCGGAAGTA ATTGAGACTG CGTCGGAGGA
pKM-S6 Amp       TGAAGCGATT CAAGAGATGC TCGACTTAAG CGCGGAAGTA ATTGAGACTG CGTCGGAGGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         730        740        750        760        770        780
AHSV-4 VP5       GGTACCAATC TTCGGCGCTG GGGCGGCGAA CGTTATCGCC ACAACCCGCG CAATACAGGG
pKM-S6           GGTACCAATC TTCGGCGCTG GGGCGGCGAA CGTTATCGCC ACAACCCGCG CAATACAGGG
pKM-S6 Amp       GGTACCAATC TTCGGCGCTG GGGCGGCGAA CGTTATCGCC ACAACCCGCG CAATACAGGG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         790        800        810        820        830        840
AHSV-4 VP5       GGGGTTAAAA CTAAAGGAAA TTGTTGATAA GCTTACGGGC ATAGATTTGA GCCATTTGAA
pKM-S6           GGGGTTAAAA CTAAAGGAAA TTGTTGATAA GCTTACGGGC ATAGATTTGA GCCATTTGAA
pKM-S6 Amp       GGGGTTAAAA CTAAAGGAAA TTGTTGATAA GCTTACGGGC ATAGATTTGA GCCATTTGAA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         850        860        870        880        890        900
AHSV-4 VP5       GGTGGCCGAC ATTCATCCAC ACATCATTGA AAAGGCAATG CTACGTGATA CTGTAACGGA
pKM-S6           GGTGGCCGAC ATTCATCCAC ACATCATTGA AAAGGCAATG CTACGTGATA CTGTAACGGA
pKM-S6 Amp       GGTGGCCGAC ATTCATCCAC ACATCATTGA AAAGGCAATG CTACGTGATA CTGTAACGGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950        960
AHSV-4 VP5       CAAAGATTTG GCGATGGCAA TTAAGTCAAA AGTGGATGTA ATTGACGAGA TGAACGTAGA
pKM-S6           CAAAGATTTG GCGATGGCAA TTAAGTCAAA AGTGGATGTA ATTGACGAGA TGAACGTAGA
pKM-S6 Amp       CAAAGATTTG GCGATGGCAA TTAAGTCAAA AGTGGATGTA ATTGACGAGA TGAACGTAGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         970        980        990        1000       1010       1020
AHSV-4 VP5       AACGCAGCAC GTAATCGATG CCGTTCTACC GATAGTTAAA CAAGAATATG AGAAACATGA
pKM-S6           AACGCAGCAC GTAATCGATG CCGTTCTACC GATAGTTAAA CAAGAATATG AGAAACATGA
pKM-S6 Amp       AACGCAGCAC GTAATCGATG CCGTTCTACC GATAGTTAAA CAAGAATATG AGAAACATGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1030       1040       1050       1060       1070       1080
AHSV-4 VP5       TAACAAATAT CATGTTAGGA TCCCAGGTGC ATTGAAGATA CATTCAGAGC ACACGCCTAA
pKM-S6           TAACAAATAT CATGTTAGGA TCCCAGGTGC ATTGAAGATA CATTCAGAGC ACACGCCTAA
pKM-S6 Amp       TAACAAATAT CATGTTAGGA TCCCAGGTGC ATTGAAGATA CATTCAGAGC ACACGCCTAA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1090       1100       1110       1120       1130       1140
AHSV-4 VP5       GATACATATA TATACGACCC CATGGGATTC GGATAGCGTC TTCATGTGTA GAGCCATTGC
pKM-S6           GATACATATA TATACGACCC CATGGGATTC GGATAGCGTC TTCATGTGTA GAGCCATTGC
pKM-S6 Amp       GATACATATA TATACGACCC CATGGGATTC GGATAGCGTC TTCATGTGTA GAGCCATTGC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1150       1160       1170       1180       1190       1200
AHSV-4 VP5       ACCGCATCAT CAACAACGAA GCTTTTTCAT TGGATTTGAT CTAGAAATTG AATATGTCCA
pKM-S6           ACCGCATCAT CAACAACGAA GCTTTTTCAT TGGATTTGAT CTAGAAATTG AATATGTCCA
pKM-S6 Amp       ACCGCATCAT CAACAACGAA GCTTTTTCAT TGGATTTGAT CTAGAAATTG AATATGTCCA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1210       1220       1230       1240       1250       1260
AHSV-4 VP5      TTTTGAAGAT ACTTCAGTTG AGGGACATAT ATTACATGGA GGGGCAATAA CCGTTGAGGG
pKM-S6          TTTTGAAGAT ACTTCAGTTG AGGGACATAT ATTACATGGA GGGGCAATAA CCGTTGAGGG
pKM-S6 Amp      TTTTGAAGAT ACTTCAGTTG AGGGACATAT ATTACATGGA GGGGCAATAA CCGTTGAGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1270       1280       1290       1300       1310       1320
AHSV-4 VP5      TAGAGGATTT CGACAGGCGT ATACTGAGTT CATGAATGCA GCGTGGGGGA TGCCAACAAC
pKM-S6          TAGAGGATTT CGACAGGCGT ATACTGAGTT CATGAATGCA GCGTGGGGGA TGCCAACAAC
pKM-S6 Amp      TAGAGGATTT CGACAGGCGT ATACTGAGTT CATGAATGCA GCGTGGGGGA TGCCAACAAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1330       1340       1350       1360       1370       1380
AHSV-4 VP5      CCCAGAGCTC CATAAACGTA AGCTACAAAG GAGTATGGGA ACTCATCCGA TCTATATGGG
pKM-S6          CCCAGAGCTC CATAAACGTA AGCTACAAAG GAGTATGGGA ACTCATCCGA TCTATATGGG
pKM-S6 Amp      CCCAGAGCTC CATAAACGTA AGCTACAAAG GAGTATGGGA ACTCATCCGA TCTATATGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1390       1400       1410       1420       1430       1440
AHSV-4 VP5      ATCGATGGAT TACGCTATAA GCTACGAACA GCTGGTTTCT AACGCGATGA GATTAGTTTA
pKM-S6          ATCGATGGAT TACGCTATAA GCTACGAACA GCTGGTTTCT AACGCGATGA GATTAGTTTA
pKM-S6 Amp      ATCGATGGAT TACGCTATAA GCTACGAACA GCTGGTTTCT AACGCGATGA GATTAGTTTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1450       1460       1470       1480       1490       1500
AHSV-4 VP5      TGATTCCGAG TTACAAATGC ATTGTCTCCG TGGGCCTCTA AAATTTCAAC GCCGCACGCT
pKM-S6          TGATTCCGAG TTACAAATGC ATTGTCTCCG TGGGCCTCTA AAATTTCAAC GCCGCACGCT
pKM-S6 Amp      TGATTCCGAG TTACAAATGC ATTGTCTCCG TGGGCCTCTA AAATTTCAAC GCCGCACGCT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       1510       1520       1530       1540       1550       1560
AHSV-4 VP5      AATGAACGCG CTTCTATATG GTGTGAAAAT AGCTTGAAAG CCTCACGGCG CGGAGAAAAC
pKM-S6          AATGAACGCG CTTCTATATG GTGTGAAAAT AGCTTGAAAG CCTCACGGCG CGGAGAAAAC
pKM-S6 Amp      AATGAACGCG CTTCTATATG GTGTGAAAAT AGCTTGAAAG CCTCACGGCG CGGAGAAAAC

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AHSV-4 VP5      ACATAC
pKM-S6          ACATAC
pKM-S6 Amp      ACATAC
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1.7 AHSV-4(32/62) VP7

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
AHSV-4 VP7      GTTAAAATTC GGTTAGGATG GACGCGATAG CAGCAAGAGC CTTGTCCGTT GTACGGGCAT
pKM-S7          GTTAAAATTC GGTTAGGATG GACGCGATAG CAGCAAGAGC CTTGTCCGTT GTACGGGCAT
pKM-S7 Amp      GTTAAAATTC GGTTAGGATG GACGCGATAG CAGCAAGAGC CTTGTCCGTT GTACGGGCAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
AHSV-4 VP7      GCGTCACAGT GACAGATGCG AGAGTTAGCT TGGATCCAGG AGTGATGGAG ACGTTAGGGA
pKM-S7          GCGTCACAGT GACAGATGCG AGAGTTAGCT TGGATCCAGG AGTGATGGAG ACGTTAGGGA
pKM-S7 Amp      GCGTCACAGT GACAGATGCG AGAGTTAGCT TGGATCCAGG AGTGATGGAG ACGTTAGGGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 VP7      TTGCAATTAA TAGGTACAAT GGTTTAACGA ATCATTCGGT ATCGATGAGG CCACAAACCC
pKM-S7          TTGCAATTAA TAGGTACAAT GGTTTAACGA ATCATTCGGT ATCGATGAGG CCACAAACCC
pKM-S7 Amp      TTGCAATTAA TAGGTACAAT GGTTTAACGA ATCATTCGGT ATCGATGAGG CCACAAACCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 VP7      AAGCAGAACG AAATGAAATG TTTTTTATGT GTACTGATAT GGTTTTAGCG GCATTGAACG
pKM-S7          AAGCAGAACG AAATGAAATG TTTTTTATGT GTACTGATAT GGTTTTAGCG GCATTGAACG
pKM-S7 Amp      AAGCAGAACG AAATGAAATG TTTTTTATGT GTACTGATAT GGTTTTAGCG GCATTGAACG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 VP7      TCCAAATTGG GAATATTTCA CCAGATTATG ATCAGGCGTT GGCAACTGTG GGAGCTCTTG
pKM-S7          TCCAAATTGG GAATATTTCA CCAGATTATG ATCAGGCGTT GGCAACTGTG GGAGCTCTTG
pKM-S7 Amp      TCCAAATTGG GAATATTTCA CCAGATTATG ATCAGGCGTT GGCAACTGTG GGAGCTCTTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 VP7      CAACGACTGA AATTCCATAT AATGTTCAGG CCATGAATGA CATCGTTAGA ATAACGGGTC
pKM-S7          CAACGACTGA AATTCCATAT AATGTTCAGG CCATGAATGA CATCGTTAGA ATAACGGGTC
pKM-S7 Amp      CAACGACTGA AATTCCATAT AATGTTCAGG CCATGAATGA CATCGTTAGA ATAACGGGTC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 VP7      AAATGCAAAC ATTCGGACCA AGCAAAGTAC AGACGGGACC TTATGCAGGA GCAGTTGAGG
pKM-S7          AAATGCAAAC ATTCGGACCA AGCAAAGTAC AGACGGGACC TTATGCAGGA GCAGTTGAGG
pKM-S7 Amp      AAATGCAAAC ATTCGGACCA AGCAAAGTAC AGACGGGACC TTATGCAGGA GCAGTTGAGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480
AHSV-4 VP7      TGCAACAATC TGGCAGATAT TACGTACCGC AAGGTCGAAC ACGTGGTGGG TACATTAATT
pKM-S7          TGCAACAATC TGGCAGATAT TACGTACCGC AAGGTCGAAC ACGTGGTGGG TACATTAATT
pKM-S7 Amp      TGCAACAATC TGGCAGATAT TACGTACCGC AAGGTCGAAC ACGTGGTGGG TACATTAATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        490        500        510        520        530        540
AHSV-4 VP7      CAAATATTGC TGAAGTGTGT ATGGATGCAG GCGCTGCGGG ACAGGTCAAT GCGCTGCTAG
pKM-S7          CAAATATTGC TGAAGTGTGT ATGGATGCAG GCGCTGCGGG ACAGGTCAAT GCGCTGCTAG
pKM-S7 Amp      CAAATATTGC TGAAGTGTGT ATGGATGCAG GCGCTGCGGG ACAGGTCAAT GCGCTGCTAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        550        560        570        580        590        600
AHSV-4 VP7      CCCCAAGGAG GGGGGACGCA GTCATGATCT ATTTTGTTTG GAGACCATTG CGTATATTTT
pKM-S7          CCCCAAGGAG GGGGGACGCA GTCATGATCT ATTTTGTTTG GAGACCATTG CGTATATTTT
pKM-S7 Amp      CCCCAAGGAG GGGGGACGCA GTCATGATCT ATTTTGTTTG GAGACCATTG CGTATATTTT
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650        660
AHSV-4 VP7       GTGATCCTCA AGGTGCGTCA CTTGAAAGCG CTCCAGGGAC TTTTGTCACC GTTGATGGAG
pKM-S7           GTGATCCTCA AGGTGCGTCA CTTGAAAGCG CTCCAGGGAC TTTTGTCACC GTTGATGGAG
pKM-S7 Amp       GTGATCCTCA AGGTGCGTCA CTTGAAAGCG CTCCAGGGAC TTTTGTCACC GTTGATGGAG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         670        680        690        700        710        720
AHSV-4 VP7       TAAATGTTGC GGCTGGAGAT GTCGTCGCAT GGAATACCAT TGCACCAGTG AATGTTGGAA
pKM-S7           TAAATGTTGC GGCTGGAGAT GTCGTCGCAT GGAATACCAT TGCACCAGTG AATGTTGGAA
pKM-S7 Amp       TAAATGTTGC GGCTGGAGAT GTCGTCGCAT GGAATACCAT TGCACCAGTG AATGTTGGAA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         730        740        750        760        770        780
AHSV-4 VP7       ATCCTGGGGC ACGCAGATCA ATTTTACAGT TTGAAGTGTT ATGGTATACG TCTTTGGATA
pKM-S7           ATCCTGGGGC ACGCAGATCA ATTTTACAGT TTGAAGTGTT ATGGTATACG TCTTTGGATA
pKM-S7 Amp       ATCCTGGGGC ACGCAGATCA ATTTTACAGT TTGAAGTGTT ATGGTATACG TCTTTGGATA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         790        800        810        820        830        840
AHSV-4 VP7       GATCGCTAGA CACGGTTCCG GAATTGGCTC CAACGCTCAC AAGATGTTAT GCGTATGTTT
pKM-S7           GATCGCTAGA CACGGTTCCG GAATTGGCTC CAACGCTCAC AAGATGTTAT GCGTATGTTT
pKM-S7 Amp       GATCGCTAGA CACGGTTCCG GAATTGGCTC CAACGCTCAC AAGATGTTAT GCGTATGTTT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         850        860        870        880        890        900
AHSV-4 VP7       CTCCCACTTG GCACGCATTA CGCGCTGTCA TTTTTCAGCA GATGAATATG CAGCCTATTA
pKM-S7           CTCCCACTTG GCACGCATTA CGCGCTGTCA TTTTTCAGCA GATGAATATG CAGCCTATTA
pKM-S7 Amp       CTCCCACTTG GCACGCATTA CGCGCTGTCA TTTTTCAGCA GATGAATATG CAGCCTATTA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950        960
AHSV-4 VP7       ATCCGCCAAT TTTTCCACCG ACTGAAAGGA ATGAAATTGT TGCGTATCTA TTAGTAGCTT
pKM-S7           ATCCGCCAAT TTTTCCACCG ACTGAAAGGA ATGAAATTGT TGCGTATCTA TTAGTAGCTT
pKM-S7 Amp       ATCCGCCAAT TTTTCCACCG ACTGAAAGGA ATGAAATTGT TGCGTATCTA TTAGTAGCTT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         970        980        990        1000       1010       1020
AHSV-4 VP7       CTTTAGCTGA TGTGTATGCG GCTTTGAGGC CAGATTTCAG AATGAATGGT GTTGTTGCGC
pKM-S7           CTTTAGCTGA TGTGTATGCG GCTTTGAGGC CAGATTTCAG AATGAATGGT GTTGTTGCGC
pKM-S7 Amp       CTTTAGCTGA TGTGTATGCG GCTTTGAGGC CAGATTTCAG AATGAATGGT GTTGTTGCGC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1030       1040       1050       1060       1070       1080
AHSV-4 VP7       CAGTAGGCCA GATCAACAGA GCTCTTGTGC TAGCAGCCTA CCACTAGTGG CTGCGGTGTT
pKM-S7           CAGTAGGCCA GATCAACAGA GCTCTTGTGC TAGCAGCCTA CCACTAGTGG CTGCGGTGTT
pKM-S7 Amp       CAGTAGGCCA GATCAACAGA GCTCTTGTGC TAGCAGCCTA CCACTAGTGG CTGCGGTGTT

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1090       1100       1110       1120       1130       1140
AHSV-4 VP7       GCACGGTCAC CGCTTTCATT AGTGTCGCGT CGGTTCTTAT GCTGATAAAG TACGCATAAG
pKM-S7           GCACGGTCAC CGCTTTCATT AGTGTCGCGT CGGTTCTTAT GCTGATAAAG TACGCATAAG
pKM-S7 Amp       GCACGGTCAC CGCTTTCATT AGTGTCGCGT CGGTTCTTAT GCTGATAAAG TACGCATAAG

                 ....|....| ....|....| ....|..
                         1150       1160
AHSV-4 VP7       TAATACGTCA ATACCGAATA CACTTAC
pKM-S7           TAATACGTCA ATACCGAATA CACTTAC
pKM-S7 Amp       TAATACGTCA ATACCGAATA CACTTAC
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1.8 AHSV-4(32/62) NS2

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
AHSV-4 NS2      GTTAAAAATC CGTTCGTCAT CATGGCAGAG GTCAGAAAGC AACAACAATT CACGCGATCA
pKM-S8          GTTAAAAATC CGTTCGTCAT CATGGCAGAG GTCAGAAAGC AACAACAATT CACGCGATCA
pKM-S8 Amp      GTTAAAAATC CGTTCGTCAT CATGGCAGAG GTCAGAAAGC AACAACAATT CACGCGATCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
AHSV-4 NS2      GTTTGTGTTC TTGATTTAGG ACAAAAGACT TATTGCGGTA AAGTGGTTAG AGCAGTAAAT
pKM-S8          GTTTGTGTTC TTGATTTAGG ACAAAAGACT TATTGCGGTA AAGTGGTTAG AGCAGTAAAT
pKM-S8 Amp      GTTTGTGTTC TTGATTTAGG ACAAAAGACT TATTGCGGTA AAGTGGTTAG AGCAGTAAAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 NS2      GGAGTGTATT ATACCATTAA AATTGGGAGA ACTGTACAAT GTGGGGTTAC GCCAACCCCG
pKM-S8          GGAGTGTATT ATACCATTAA AATTGGGAGA ACTGTACAAT GTGGGGTTAC GCCAACCCCG
pKM-S8 Amp      GGAGTGTATT ATACCATTAA AATTGGGAGA ACTGTACAAT GTGGGGTTAC GCCAACCCCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 NS2      ATTCCAAAAA GTTATGTTTT GGAGATTCGT GAATGCGGAG CTTACCGTAT TCAAGATGGG
pKM-S8          ATTCCAAAAA GTTATGTTTT GGAGATTCGT GAATGCGGAG CTTACCGTAT TCAAGATGGG
pKM-S8 Amp      ATTCCAAAAA GTTATGTTTT GGAGATTCGT GAATGCGGAG CTTACCGTAT TCAAGATGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 NS2      ACGGATGTTT TAAGTTTAAT GATTACTGAA AGTGGGATTG AGGTAACGCA AAACCGATGG
pKM-S8          ACGGATGTTT TAAGTTTAAT GATTACTGAA AGTGGGATTG AGGTAACGCA AAACCGATGG
pKM-S8 Amp      ACGGATGTTT TAAGTTTAAT GATTACTGAA AGTGGGATTG AGGTAACGCA AAACCGATGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 NS2      GAGGAGTGGA GTTTTGAAGC GTTAACACCA GTACCGATGG CTGTGGCGGT GAATGTAGGG
pKM-S8          GAGGAGTGGA GTTTTGAAGC GTTAACACCA GTACCGATGG CTGTGGCGGT GAATGTAGGG
pKM-S8 Amp      GAGGAGTGGA GTTTTGAAGC GTTAACACCA GTACCGATGG CTGTGGCGGT GAATGTAGGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 NS2      AGAGGCTCGT TTGACACTGA GATTAAATAT GTGAGAGGAA GCGGTGCGGT TCCACCTTAT
pKM-S8          AGAGGCTCGT TTGACACTGA GATTAAATAT GTGAGAGGAA GCGGTGCGGT TCCACCTTAT
pKM-S8 Amp      AGAGGCTCGT TTGACACTGA GATTAAATAT GTGAGAGGAA GCGGTGCGGT TCCACCTTAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480
AHSV-4 NS2      ACGAAGAATG GAATGGATCG AAGAGCGATG CCTTCTTTAC CAGGAATAAC AACTTTGGAT
pKM-S8          ACGAAGAATG GAATGGATCG AAGAGCGATG CCTTCTTTAC CAGGAATAAC AACTTTGGAT
pKM-S8 Amp      ACGAAGAATG GAATGGATCG AAGAGCGATG CCTTCTTTAC CAGGAATAAC AACTTTGGAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        490        500        510        520        530        540
AHSV-4 NS2      GTTGGAGTTA GAGATTTGCG TTTAAAGATG AAGGAGAACA GGGAGGCAGA AAGGGAGAAG
pKM-S8          GTTGGAGTTA GAGATTTGCG TTTAAAGATG AAGGAGAACA GGGAGGCAGA AAGGGAGAAG
pKM-S8 Amp      GTTGGAGTTA GAGATTTGCG TTTAAAGATG AAGGAGAACA GGGAGGCAGA AAGGGAGAAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        550        560        570        580        590        600
AHSV-4 NS2      ATGGAACGAG CCCTAAGTGG TGGGCTCGAT ATGGGAAGCT GTAGAATGTA TGGAGGAGGA
pKM-S8          ATGGAACGAG CCCTAAGTGG TGGGCTCGAT ATGGGAAGCT GTAGAATGTA TGGAGGAGGA
pKM-S8 Amp      ATGGAACGAG CCCTAAGTGG TGGGCTCGAT ATGGGAAGCT GTAGAATGTA TGGAGGAGGA
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650        660
AHSV-4 NS2      AGAAATGATG TGCGTGAGAT CACCTTGGAT GAGGCCGGAC CATCACGTAC ACCAAGGAAA
pKM-S8          AGAAATGATG TGCGTGAGAT CACCTTGGAT GAGGCCGGAC CATCACGTAC ACCAAGGAAA
pKM-S8 Amp      AGAAATGATG TGCGTGAGAT CACCTTGGAT GAGGCCGGAC CATCACGTAC ACCAAGGAAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        670        680        690        700        710        720
AHSV-4 NS2      CTTTCTGTTC AGAGCAATGA AAGTCGTTCA GATGATGTGG CACGAAGACA TGCTGAGTTG
pKM-S8          CTTTCTGTTC AGAGCAATGA AAGTCGTTCA GATGATGTGG CACGAAGACA TGCTGAGTTG
pKM-S8 Amp      CTTTCTGTTC AGAGCAATGA AAGTCGTTCA GATGATGTGG CACGAAGACA TGCTGAGTTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        730        740        750        760        770        780
AHSV-4 NS2      GTGGAGATGG AGCGACTAAG AATGATGAAG AATGAACCAG TACGTACAGA GAGTATGTGG
pKM-S8          GTGGAGATGG AGCGACTAAG AATGATGAAG AATGAACCAG TACGTACAGA GAGTATGTGG
pKM-S8 Amp      GTGGAGATGG AGCGACTAAG AATGATGAAG AATGAACCAG TACGTACAGA GAGTATGTGG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        790        800        810        820        830        840
AHSV-4 NS2      TGTCAAAGTG ATAGTGATGA TCAATCTGAT GAGGATCACG AGGTTGGGAG TACAGAGCCA
pKM-S8          TGTCAAAGTG ATAGTGATGA TCAATCTGAT GAGGATCACG AGGTTGGGAG TACAGAGCCA
pKM-S8 Amp      TGTCAAAGTG ATAGTGATGA TCAATCTGAT GAGGATCACG AGGTTGGGAG TACAGAGCCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        850        860        870        880        890        900
AHSV-4 NS2      GAAAATTACA TTACTGAGGA GTATACACGT AGGCTGAACG AGGTAAAGAC GAAATATTCA
pKM-S8          GAAAATTACA TTACTGAGGA GTATACACGT AGGCTGAACG AGGTAAAGAC GAAATATTCA
pKM-S8 Amp      GAAAATTACA TTACTGAGGA GTATACACGT AGGCTGAACG AGGTAAAGAC GAAATATTCA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        910        920        930        940        950        960
AHSV-4 NS2      AAGGAATTAT CTTCATTGGC GATGAGAGTT CCAAAGAATG AGGGCAATTG TGGAAAACCG
pKM-S8          AAGGAATTAT CTTCATTGGC GATGAGAGTT CCAAAGAATG AGGGCAATTG TGGAAAACCG
pKM-S8 Amp      AAGGAATTAT CTTCATTGGC GATGAGAGTT CCAAAGAATG AGGGCAATTG TGGAAAACCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        970        980        990        1000       1010       1020
AHSV-4 NS2      ATTTTTTCTA AAAAGTGTAA ATGGGAGAAT GTTCCGATCT ACAATTACGA TGAAGCTAGC
pKM-S8          ATTTTTTCTA AAAAGTGTAA ATGGGAGAAT GTTCCGATCT ACAATTACGA TGAAGCTAGC
pKM-S8 Amp      ATTTTTTCTA AAAAGTGTAA ATGGGAGAAT GTTCCGATCT ACAATTACGA TGAAGCTAGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1030       1040       1050       1060       1070       1080
AHSV-4 NS2      GGAAACTATC GTTTTGTGTC AGTGGGAAGT GCTACACATT ACCACTGCTG TGCTAATGAC
pKM-S8          GGAAACTATC GTTTTGTGTC AGTGGGAAGT GCTACACATT ACCACTGCTG TGCTAATGAC
pKM-S8 Amp      GGAAACTATC GTTTTGTGTC AGTGGGAAGT GCTACACATT ACCACTGCTG TGCTAATGAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1090       1100       1110       1120       1130       1140
AHSV-4 NS2      TTGAGTTACA TGATTCTCCC AGCAGGGGGG AGCGGTTGAT CCTCCTGGGT GTAGCTGGCG
pKM-S8          TTGAGTTACA TGATTCTCCC AGCAGGGGGG AGCGGTTGAT CCTCCTGGGT GTAGCTGGCG
pKM-S8 Amp      TTGAGTTACA TGATTCTCCC AGCAGGGGGG AGCGGTTGAT CCTCCTGGGT GTAGCTGGCG

                ....|....| ....|....| ....|
                        1150       1160
AHSV-4 NS2      CATGTAACCG CGGATTTCAA CATAC
pKM-S8          CATGTAACCG CGGATTTCAA CATAC
pKM-S8 Amp      CATGTAACCG CGGATTTCAA CATAC
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1.9 AHSV-4 (32/62) VP6

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
AHSV-4 VP6      GTTAAATAAG TTGTCTCATG TCTTCGGCAT TACTCCTCGC ACCTGGCGAT CTGATGGTTA
pKM-S9          GTTAAATAAG TTGTCTCATG TCTTCGGCAT TACTCCTCGC ACCTGGCGAT CTGATGGTTA
pKM-S9 Amp      GTTAAATAAG TTGTCTCATG TCTTCGGCAT TACTCCTCGC ACCTGGCGAT CTGATGGTTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
AHSV-4 VP6      AAGCAAAGCG TGAGCTCGAG CAGCGCTCGA TTAGCTCATT GCTGCGCTCT ACGAGCGGAG
pKM-S9          AAGCAAAGCG TGAGCTCGAG CAGCGCTCGA TTAGCTCATT GCTGCGCTCT ACGAGCGGAG
pKM-S9 Amp      AAGCAAAGCG TGAGCTCGAG CAGCGCTCGA TTAGCTCATT GCTGCGCTCT ACGAGCGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 VP6      AACCGAAGGA AGAGAGGAAA GAAAAGCAGA ACAACCAAAA AGACGGGGAA AAGGAGGACA
pKM-S9          AACCGAAGGA AGAGAGGAAA GAAAAGCAGA ACAACCAAAA AGACGGGGAA AAGGAGGACA
pKM-S9 Amp      AACCGAAGGA AGAGAGGAAA GAAAAGCAGA ACAACCAAAA AGACGGGGAA AAGGAGGACA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 VP6      AGGCGGAAAA GGGTGAGGAA AAGATACCAA AAGATGGAGG ATTGGGATCA GCAAAATCAG
pKM-S9          AGGCGGAAAA GGGTGAGGAA AAGATACCAA AAGATGGAGG ATTGGGATCA GCAAAATCAG
pKM-S9 Amp      AGGCGGAAAA GGGTGAGGAA AAGATACCAA AAGATGGAGG ATTGGGATCA GCAAAATCAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 VP6      CAGAACCAGA ACCAGCGGAT GGATCAGGAG AATCAGCAAA ATCGACAGGA GGAGATGGAG
pKM-S9          CAGAACCAGA ACCAGCGGAT GGATCAGGAG AATCAGCAAA ATCGACAGGA GGAGATGGAG
pKM-S9 Amp      CAGAACCAGA ACCAGCGGAT GGATCAGGAG AATCAGCAAA ATCGACAGGA GGAGATGGAG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 VP6      GTGGAGGAGC AGGGCGCGGG GCTGGAGGGA GAGGAGTGGG CGGAGTGGCT GGAGGGGCTG
pKM-S9          GTGGAGGAGC AGGGCGCGGG GCTGGAGGGA GAGGAGTGGG CGGAGTGGCT GGAGGGGCTG
pKM-S9 Amp      GTGGAGGAGC AGGGCGCGGG GCTGGAGGGA GAGGAGTGGG CGGAGTGGCT GGAGGGGCTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 VP6      GACGACGTGG AGGATCACTT CGTGGGGGAC GGGATGCAGG ATTGGGCGAA TCTACGTCCG
pKM-S9          GACGACGTGG AGGATCACTT CGTGGGGGAC GGGATGCAGG ATTGGGCGAA TCTACGTCCG
pKM-S9 Amp      GACGACGTGG AGGATCACTT CGTGGGGGAC GGGATGCAGG ATTGGGCGAA TCTACGTCCG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480
AHSV-4 VP6      GAACAAATCA TATCACTAAT GATGATGCAA CACGCAATGC TGGTTCGGGT GAGGTACCAT
pKM-S9          GAACAAATCA TATCACTAAT GATGATGCAA CACGCAATGC TGGTTCGGGT GAGGTACCAT
pKM-S9 Amp      GAACAAATCA TATCACTAAT GATGATGCAA CACGCAATGC TGGTTCGGGT GAGGTACCAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        490        500        510        520        530        540
AHSV-4 VP6      CTGGTGGAGT CACTTCAGGA AGTTCTCAAG GCGGAGGAGG GGGCGCTACA ACGAGTGGTG
pKM-S9          CTGGTGGAGT CACTTCAGGA AGTTCTCAAG GCGGAGGAGG GGGCGCTACA ACGAGTGGTG
pKM-S9 Amp      CTGGTGGAGT CACTTCAGGA AGTTCTCAAG GCGGAGGAGG GGGCGCTACA ACGAGTGGTG

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        550        560        570        580        590        600
AHSV-4 VP6      AATCTGGCGG GCAACCACTG GATCGGAAGA GCGGCGCAAG CGGAGATACA AGATCTCCAA
pKM-S9          AATCTGGCGG GCAACCACTG GATCGGAAGA GCGGCGCAAG CGGAGATACA AGATCTCCAA
pKM-S9 Amp      AATCTGGCGG GCAACCACTG GATCGGAAGA GCGGCGCAAG CGGAGATACA AGATCTCCAA
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650        660
AHSV-4 VP6       GCGAGAAGAC AAAGGTTGAT GGGGGCGATC GACGGGATGG GGGATTAGCT ACTCAAGAGA
pKM-S9           GCGAGAAGAC AAAGGTTGAT GGGGGCGATC GACGGGATGG GGGATTAGCT ACTCAAGAGA
pKM-S9 Amp       GCGAGAAGAC AAAGGTTGAT GGGGGCGATC GACGGGATGG GGGATTAGCT ACTCAAGAGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         670        680        690        700        710        720
AHSV-4 VP6       TAGTCGATTA TGTCAGAAAG AAGGTTGGAG TTGAAGTTCC GATTTATCAG AAGGGAATGA
pKM-S9           TAGTCGATTA TGTCAGAAAG AAGGTTGGAG TTGAAGTTCC GATTTATCAG AAGGGAATGA
pKM-S9 Amp       TAGTCGATTA TGTCAGAAAG AAGGTTGGAG TTGAAGTTCC GATTTATCAG AAGGGAATGA

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         730        740        750        760        770        780
AHSV-4 VP6       GCAACTTATT TACTGTAGAT AGGGGCTTGC TTGAGCGGGG GGGTTTATCT AAAGATGATC
pKM-S9           GCAACTTATT TACTGTAGAT AGGGGCTTGC TTGAGCGGGG GGGTTTATCT AAAGATGATC
pKM-S9 Amp       GCAACTTATT TACTGTAGAT AGGGGCTTGC TTGAGCGGGG GGGTTTATCT AAAGATGATC

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         790        800        810        820        830        840
AHSV-4 VP6       TGCTACATCA ATCTGATATC GTAAAAGAGG CGAAGGCCAA TGATAAAAAA TTGAAAGTGG
pKM-S9           TGCTACATCA ATCTGATATC GTAAAAGAGG CGAAGGCCAA TGATAAAAAA TTGAAAGTGG
pKM-S9 Amp       TGCTACATCA ATCTGATATC GTAAAAGAGG CGAAGGCCAA TGATAAAAAA TTGAAAGTGG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         850        860        870        880        890        900
AHSV-4 VP6       TTCCACTCTC AACAGTCAAA AGAATTATTG CGGAATTTGG AGGGTCGGAA GAAGAAGATG
pKM-S9           TTCCACTCTC AACAGTCAAA AGAATTATTG CGGAATTTGG AGGGTCGGAA GAAGAAGATG
pKM-S9 Amp       TTCCACTCTC AACAGTCAAA AGAATTATTG CGGAATTTGG AGGGTCGGAA GAAGAAGATG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         910        920        930        940        950        960
AHSV-4 VP6       TAAAGGGAAT GCAGACACAA AGTTCTTCCA TAAGATACAT AAGCAATAGA ATGGAAGATG
pKM-S9           TAAAGGGAAT GCAGACACAA AGTTCTTCCA TAAGATACAT AAGCAATAGA ATGGAAGATG
pKM-S9 Amp       TAAAGGGAAT GCAGACACAA AGTTCTTCCA TAAGATACAT AAGCAATAGA ATGGAAGATG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         970        980        990        1000       1010       1020
AHSV-4 VP6       TTCCTAAAGC GAAGGCCATG TTCACGGCGC CGACTGGAGA TGAAGGGTGG AAGGAGGTCG
pKM-S9           TTCCTAAAGC GAAGGCCATG TTCACGGCGC CGACTGGAGA TGAAGGGTGG AAGGAGGTCG
pKM-S9 Amp       TTCCTAAAGC GAAGGCCATG TTCACGGCGC CGACTGGAGA TGAAGGGTGG AAGGAGGTCG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1030       1040       1050       1060       1070       1080
AHSV-4 VP6       CAAAAGCGGC GACGTTGAGG CCAAATATCA TGGCTTACGT TCATGAAGGG GAAGGGGATG
pKM-S9           CAAAAGCGGC GACGTTGAGG CCAAATATCA TGGCTTACGT TCATGAAGGG GAAGGGGATG
pKM-S9 Amp       CAAAAGCGGC GACGTTGAGG CCAAATATCA TGGCTTACGT TCATGAAGGG GAAGGGGATG

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         1090       1100       1110       1120       1130       1140
AHSV-4 VP6       GACTGAAGGA GCTTTTACAT TTAATAGATC ATATCTAGGT CCAGGGGTAA ACGGCAGCTT
pKM-S9           GACTGAAGGA GCTTTTACAT TTAATAGATC ATATCTAGGT CCAGGGGTAA ACGGCAGCTT
pKM-S9 Amp       GACTGAAGGA GCTTTTACAT TTAATAGATC ATATCTAGGT CCAGGGGTAA ACGGCAGCTT

                 ....|....| ....|....|
                         1150       1160
AHSV-4 VP6       GCAGGCAACT TAAAACTTAC
pKM-S9           GCAGGCAACT TAAAACTTAC
pKM-S9 Amp       GCAGGCAACT TAAAACTTAC
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1.10 AHSV-4(32/62) NS3

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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
AHSV-4 NS3      GTTAAAATTA TCCCTTGTCA TGAATCTAGC TACAATCGCC AAGAATTATA GCATGCATAA
pKM-S10         GTTAAAATTA TCCCTTGTCA TGAATCTAGC TACAATCGCC AAGAATTATA GCATGCATAA
pKM-S10 Amp     GTTAAAATTA TCCCTTGTCA TGAATCTAGC TACAATCGCC AAGAATTATA GCATGCATAA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
AHSV-4 NS3      TGGAGAGTCG GGGGCGATCG TCCCTTATGT GCCACCACCA TACAATTTCG CAAGTGCTCC
pKM-S10         TGGAGAGTCG GGGGCGATCG TCCCTTATGT GCCACCACCA TACAATTTCG CAAGTGCTCC
pKM-S10 Amp     TGGAGAGTCG GGGGCGATCG TCCCTTATGT GCCACCACCA TACAATTTCG CAAGTGCTCC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
AHSV-4 NS3      GACGTTTTCT CAGCGTACGA GTCAAATGGA GTCCGTGTCG CTTGGGATAC TTAACCAAGC
pKM-S10         GACGTTTTCT CAGCGTACGA GTCAAATGGA GTCCGTGTCG CTTGGGATAC TTAACCAAGC
pKM-S10 Amp     GACGTTTTCT CAGCGTACGA GTCAAATGGA GTCCGTGTCG CTTGGGATAC TTAACCAAGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        190        200        210        220        230        240
AHSV-4 NS3      CATGTCAAGT ACAACTGGTG CGAGTGGGGC GCTTAAAGAT GAAAAAGCAG CATTCGGTGC
pKM-S10         CATGTCAAGT ACAACTGGTG CGAGTGGGGC GCTTAAAGAT GAAAAAGCAG CATTCGGTGC
pKM-S10 Amp     CATGTCAAGT ACAACTGGTG CGAGTGGGGC GCTTAAAGAT GAAAAAGCAG CATTCGGTGC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        250        260        270        280        290        300
AHSV-4 NS3      TATGGCGGAA GCATTGCGTG ATCCAGAACC CATACGTCAA ATTAAAAAGC AGGTGGGTAT
pKM-S10         TATGGCGGAA GCATTGCGTG ATCCAGAACC CATACGTCAA ATTAAAAAGC AGGTGGGTAT
pKM-S10 Amp     TATGGCGGAA GCATTGCGTG ATCCAGAACC CATACGTCAA ATTAAAAAGC AGGTGGGTAT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350        360
AHSV-4 NS3      CAGAACTTTA AAGAACCTAA AGATGGAGTT AGCAACAATG CGTCGAAAGA AATCGGCATT
pKM-S10         CAGAACTTTA AAGAACCTAA AGATGGAGTT AGCAACAATG CGTCGAAAGA AATCGGCATT
pKM-S10 Amp     CAGAACTTTA AAGAACCTAA AGATGGAGTT AGCAACAATG CGTCGAAAGA AATCGGCATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        370        380        390        400        410        420
AHSV-4 NS3      AAAAATAATG ATCTTTATTA GTGGATGCGT AACGTTAGCT ACATCGATGG TTGGGGGATT
pKM-S10         AAAAATAATG ATCTTTATTA GTGGATGCGT AACGTTAGCT ACATCGATGG TTGGGGGATT
pKM-S10 Amp     AAAAATAATG ATCTTTATTA GTGGATGCGT AACGTTAGCT ACATCGATGG TTGGGGGATT

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        430        440        450        460        470        480
AHSV-4 NS3      GAGTATCGTT GACGACGAAA TATTAAGAGA TTATAAGAAC AACGATTGGT TAATGAAGAC
pKM-S10         GAGTATCGTT GACGACGAAA TATTAAGAGA TTATAAGAAC AACGATTGGT TAATGAAGAC
pKM-S10 Amp     GAGTATCGTT GACGACGAAA TATTAAGAGA TTATAAGAAC AACGATTGGT TAATGAAGAC

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        490        500        510        520        530        540
AHSV-4 NS3      TATACATGGG CTGAATTTGT TATGTACTAC AGTTTTGTTA GCGGCGGGTA AGATTTCCGA
pKM-S10         TATACATGGG CTGAATTTGT TATGTACTAC AGTTTTGTTA GCGGCGGGTA AGATTTCCGA
pKM-S10 Amp     TATACATGGG CTGAATTTGT TATGTACTAC AGTTTTGTTA GCGGCGGGTA AGATTTCCGA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        550        560        570        580        590        600
AHSV-4 NS3      TAAAATGCAA GAGGAGATTT CACGGACTAA ACGTGACATT GCGAAAAGAG AGTCTTACGT
pKM-S10         TAAAATGCAA GAGGAGATTT CACGGACTAA ACGTGACATT GCGAAAAGAG AGTCTTACGT
pKM-S10 Amp     TAAAATGCAA GAGGAGATTT CACGGACTAA ACGTGACATT GCGAAAAGAG AGTCTTACGT
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                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        610        620        630        640        650        660
AHSV-4 NS3      GTCAGCGGCG AGTATGTCGT GGAGTGGAGA TACTGAGATG TTATTACAGG GAATTAAGTA
pKM-S10         GTCAGCGGCG AGTATGTCGT GGAGTGGAGA TACTGAGATG TTATTACAGG GAATTAAGTA
pKM-S10 Amp     GTCAGCGGCG AGTATGTCGT GGAGTGGAGA TACTGAGATG TTATTACAGG GAATTAAGTA

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        670        680        690        700        710        720
AHSV-4 NS3      TGGCGAGAGC TAGTATGACC TCCACGAGCG GAAAATCCAT CGTGTTGGAT GGATGGAACG
pKM-S10         TGGCGAGAGC TAGTATGACC TCCACGAGCG GAAAATCCAT CGTGTTGGAT GGATGGAACG
pKM-S10 Amp     TGGCGAGAGC TAGTATGACC TCCACGAGCG GAAAATCCAT CGTGTTGGAT GGATGGAACG

                ....|....| ....|....| ....|....| ....|..
                        730        740        750
AHSV-4 NS3      CCTAGATCGT TTTCTAGGGA GTGGGATAAC AACTTAC
pKM-S10         CCTAGATCGT TTTCTAGGGA GTGGGATAAC AACTTAC
pKM-S10 Amp     CCTAGATCGT TTTCTAGGGA GTGGGATAAC AACTTAC
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