# Generation of a set of SA11 expression plasmids for the development of a T7-RNA polymerase-dependent rotavirus reverse genetic system

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“All we have to decide is what to do with the time that is given us.”

J.R.R. Tolkien, The Fellowship of the Ring

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# ABSTRACT

Rotavirus (RV) is a member of the family *Reoviridae* which contains a segmented double-stranded RNA genome comprised of 11 double-stranded RNA genome segments. Rotavirus is still the leading cause of severe diarrhoea worldwide in children less than five years of age and causes 215 000 deaths per year, most of which occur in Africa (Trask et al., 2016)

Reverse genetic systems have been developed for members of the *Reoviridae* family, which include bluetongue virus (Boyce et al.,2008) orthoreovirus (Kobayshi et al., 2007) African horsesickness virus (Matsuo et al., 2010) and epizootic haemorrhagic disease virus (Yang et al., 2015). Reverse genetics allows for the manipulation of the viral genomes at cDNA level and also for the generation of information regarding the replication, pathogenesis and biological characterisation of these viruses. Until 2017, no reverse genetic system for rotavirus had been developed. Several helper virus dependent reverse genetic systems for rotaviruses have been described). However, they all depend on the presence of a helper virus and require strong selection. A true rotavirus reverse genetic system, which is free of any selection and allows manipulation of any genome segment, will enhance the understanding of the rotavirus replication cycle and elucidation of detailed host-pathogen interaction.

This study was an attempt at developing a plasmid-based reverse genetic system for rotavirus with the use of cDNA expression plasmids based on the consensus SA11 sequence. The expression plasmids were constructed by cloning cDNA representing the consensus sequence of the 11 genome segment sequences of the rotavirus SA11 strain which were produced by PCR, into pSMART by means of FastCloning and In-Fusiong®HD cloning. The genome segments were flanked by a T7 promoter sequence on the 5’ end followed by a hepatitis delta virus (HDV) ribozyme sequence at the 3’ sequence to generate exact (+)ssRNA when transfected in mammalian cell cultures.

The SA11 consensus sequence expression plasmids were transfected to BHK-T7 and BSR-T7 cells. Lysates of BHK-T7 and BSR-T7 cells were used to infect MA104 cells to generate viable virus indicating viral rescue. Viral rescue was evaluated with the use of immunofluorescent staining. Despite the indication of viral translation in one attempt of transfection, no viable virus was recovered following infection of MA104 cells with BHK-T7 and BSR-T7 cell lysates. The development of a reverse genetic system was unsuccessful in this study. Thus, the constructed set of SA11 CS expression plasmids will be the basis for further development towards a more robust rotavirus reverse genetic system

Keywords: Rotavirus; reverse genetics; rotavirus SA11 strain; consensus sequence; FastCloning; In-Fusion HD cloning; transfection; virus rescue; Immunofluorescent staining

# OPSOMMING

Rotavirus (RV) is 'n lid van die *Reoviridae* familie wat 'n gesegmenteerde dubbelstring RNA-genoom bevat wat uit 11 dubbelstring RNA-genoom segmente bestaan. Rotavirus is steeds die hoof oorsaak van die voorkoms van ernstige diarree in kinders jonger as vyf jaar wêreldwyd, en veroorsaak 215 000 sterftes jaarliks waarvan meeste in Afrika voorkom (Trask et al., 2016)

Tru-genetika sisteme is al ontwikkel vir lede van die Reoviridae-familie, insluitend bloutongvirus (Boyce et al., 2008) orthoreovirus (Kobayshi et al., 2007) Afrika perdesiekte virus (Matsuo et al., 2010) en epizootiese hemorragiese siekte virus (Yang et al., 2015). Tru-genetika maak die manipulering van die virus genoom op cDNA-vlak moontlik en het ook gelei tot die bydrae van inligting rakende verskeie aspekte van virus replisering, patogenese en biologiese karakterisering van hierdie virusse. Tot en met 2017 was daar geen plasmied-gebaseerde tru-genetiese stelsel vir rotavirus nie. Verskeie helpervirus-afhanklike tru-genetiese sisteme is voorheen beskryf vir rotavirusse, maar die sisteme is almal afhanklik van die teenwoordigheid van 'n helpervirus en vereis ook sterk seleksie prosesse. 'n Ware rotavirus tru-genetiese stelsel, wat vry is van enige seleksie en die manipulasie van enige genoomsegment toelaat, sal die begrip van die rotavirus-repliseringsiklus en gedetailleerde gasheer-patogeen interaksie verbeter.

Hierdie studie het gepoog om 'n plasmied-gebaseerde tru-genetiese sisteem vir rotavirus te ontwikkel met die gebruik van cDNA-uitdrukkingsplasmiede. Die uitdrukkingsplasmied konstrukte is ontwikkel deur die klonering van cDNAs wat die konsensusvolgorde van die 11 genoomsegmente van die rotavirus SA11-stam verteenwoordig wat deur PKR geproduseer is en in pSMART te kloneer met behulp van FastCloning en In-Fusiong®HD klonering. Die genoomsegmente bevat 'n T7 promoter volgorde aan die 5'-terminus gevolg deur 'n hepatitis delta virus (HDV) ribosiem volgorde aan die 3 ' terminus om sodoende presiese (+)ssRNA te genereer tydens transfeksie in selle.

Die SA11 konsensusvolgorde uitdrukkingsplasmide is in BHK-T7 en BSR-T7 selle getransfekteer. Geliseerde BHK-T7 en BSR-T7 selle is gebruik om MA104 selle te infekteer om infektiewe virus te vermeerder. Virale redding is geëvalueer met die gebruik van immunokleuring. Ten spyte van die aanduiding van die teenwoordigheid van virus proteïene in een poging van transfeksie, is geen infektiewe virus herwin na infeksie van MA104-selle met BHK-T7 en BSR-T7 sel lisate. Dus sal die stel SA11 konsensusvolgorde uitdrukkings plasmiede wat tydens hierdie studie ontwikkel is gebruik word as die basis vir die ontwikkeling van ‘n meer kragtige rotavirus tru-genetika sisteem.

Sleutelwoorde: Rotavirus; tru-genetika; rotavirus SA11 stam; konsensusvolgorde; FastCloning; In-Fusion HD klonering; transfeksie; virus redding; Immunokleuring

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| | |
|---|---|
| AGE | agarose gel electrophoresis |
| AHSV | African horse-sickness virus |
| ATP | Adenosine triphosphate |
| Bp | Base pairs |
| BTV | Bluetongue virus |
| CPE | Cytopathic effect |
| DNA | Deoxyribonucleic acid |
| DLP | Double-layered particle |
| dsRNA | Double-stranded ribonucleic acid |
| EB | Elution buffer |
| EDTA | Ethylene-diamine-tetra-acetic acid |
| ER | Endoplasmic reticulum |
| FBS | Foetal bovine serum |
| FDA | Food and Drug Administration |
| HA | Haemagglutinin |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| MDA5 | melanoma differentiation associated gene 5 |
| Ml | Millilitre |
| NA | Neuraminidase |
| NTPase | Nucleoside triphosphatase |
| NSP | Non-structural protein |
| ORF | Open reading frame |
| GS | genome segment |
| HCl | hydrochloric acid xii |
| HDVR | Hepatitis delta virus ribozyme |
| HEPES | N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) |
| IFMA | immunofluorescent monolayer assay |
| KCl | potassium chloride |
| LAV | live attenuated vaccine |
| MgCl2 | magnesium chloride |
| ml | milliliter |
| mM | millimol |
| mRNA | messenger RNA |
| NAb | neutralising antibodies |

| | |
|---|---|
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| ng | nanogram |
| nm | nanometer |
| NS | non-structural viral proteins |
| PABP | Poly (A) binding proteins |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PKR | dsRNA-dependent kinase |
| RdRp | RNA-dependent RNA polymerase |
| RIG-I | retinoic inducible gene I |
| RG | reverse genetics |
| RNA | Ribonucleic acid |
| Rpm | Revolutions per minute |
| siRNA | Small interfering ribonucleic acid |
| S | segment (genome) |
| ss | single-stranded |
| SOC | Super Optimal broth with catabolite repression |
| ssRNA | Single-stranded ribonucleic acid |
| TAE | tris-acetate EDTA xiii |
| TBE | tris-borate EDTA |
| TC | tissue culture |
| TLP | Triple-layered particle |
| U | Unit |
| UTR | Untranslated terminal region |
| VP | Structural viral protein |
| $C^o$ | Degrees Celsius |
| µl | micro liter |

# CHAPTER 1: LITERATURE REVIEW

## 1.1 Introduction

From the early 1800s through to the 1900s gastroenteritis was referred to as typhoid or choleramorbus. During the late 1800s to 1900s researchers still believed that the cause for gastroenteritis was mainly due to bacteriological infections. The first report of an epidemic gastroenteritis illness caused by viral agent was published in 1929 by the American physician, John Zahorsky (Zahorsky, 1929) and termed the illness “hyperemesis hiemes” or winter vomiting disease. Years later calves were inoculated with faecal filtrate from infected new-borns to induce the same state of diarrhoea, but it was not possible to adapt the causative agent to cultured cells (Light and Hodes 1943).

In 1968 faecal samples were collected from students and teachers showing symptoms of acute diarrhoea at an elementary school in Norwalk. The pathogen responsible for the Norwalk outbreak could only be discovered in 1972 when Kapikian and co-workers found viral particles in the faecal matter of a volunteer infected with a purified stool sample isolated from a Norwalk outbreak patient. Electron microscopy identified particles measuring between 27-32 nm, and the virus was named the Norwalk virus (Kapikian et al., 1972).

The first rotavirus (RV) to be described was the simian agent 11 (SA11). This virus was isolated from a Cercopithecus monkey by Dr Hubert Malherbe at the National Institute of Virology located in Johannesburg, South Africa (Malherbe and Strickland-Cholmley 1967). In 1973 Ruth Bishop and her colleagues found the first link with RV and diarrhoea when they identified a viral agent in the duodenal mucosa of infants with severe gastroenteritis which very much looked like SA11 (Bishop et al., 1973). In the year that followed, Thomas Hendry Flewett observed that rotavirus particles resembled a wheel when seen through an electron microscope. The name rotavirus was then proposed (Flewett et al., 1974). The name was officially recognised by the international committee on taxonomy in 1979.

Serotypes for RV were first defined in the 1980s (Birch et al., 1988, Coulsen et al., 1987). Viruses are classified into serotypes defined by reactivity in neutralization assays against the outer capsid neutralization antigens VP4 and VP7. With such assays, 27 VP7 (or G [for glycoprotein]) serotypes have been identified and strains of animal and human origin may fall within the same G serotype. For G types, serotypes and genotypes are synonymous, For P types, there are many more P genotypes than reference sera determining P serotypes. Rotaviruses are then classified by a binary system in which distinct types of VP4 and VP7 are recognised.

A major breakthrough came the following year when rotavirus WA isolated from an infant stool sample was adapted to replicate in cultured cells (Wyatt et al., 1980). This was followed by a range of rotavirus strains being successfully adapted to cell cultures which made it much easier to study rotavirus replication and develop vaccine strategies.

Rotavirus is one of several viruses which is known to cause a self-limited gastroenteritis. Acute gastroenteritis is one of the most common diseases amongst humans worldwide. Every year an estimate of 1 billion diarrhoea cases are reported of which 2.4 – 5 million are fatal (Trask et al., 2016). Approximately 250 000 of these deaths can still be attributed to RV, most occur in children aged under 5 years. The epidemiology of these infections is complex and related to winter peaks in temperature climates. In tropical and subtropical regions infections occur throughout the year.

The detailed understanding of molecular mechanisms underlying RV replication and pathogenesis has been hampered by the lack of RV reverse genetic (RG) systems. The first RG system was the recovery of a λ-phage and SV40 hybrid which was rescued from monkey kidney cells (Goff and Berg, 1976). For RNA viruses, the first reverse genetics system for positive-sense RNA viruses was that for the poliomyelitis virus (Racaniello and Baltimore, 1981a). Negative-sense RNA viruses have been less compliant with genetic manipulation. The best illustration of the power of RG is that of the influenza virus RG system. In this system, ribonucleo-proteins and cDNA were transfected into cultured cells together with a helper influenza A virus infection (Luytjes et al., 1989). The function of the helper virus was to incorporate cDNA genome segments in order to create a recombinant virus. The reverse genetics system for influenza viruses used today has undergone multiple improvements and primarily makes use of recombinant cDNA plasmids (Neumann et al., 2012, Neumann et al., 1999). Originally, the influenza virus reverse genetics approach employed the transfection of 12 plasmids for the recovery of viable virus. Eventually the system was reduced to 8 plasmids and finally only 5 plasmids were needed to rescue infectious influenza virus (Hoffmann and Webster, 2000, Neumann et al., 2005). The influenza virus reverse genetics systems were cardinal in the development of influenza vaccines (Subbarao and Katz, 2004). The current reverse genetic system for influenza is the result of many developments and improvements (Neumann and Kawaoka, 2001, Neumann et al., 1999, Pleschka et al., 1996) and as a result, viable influenza virus can now be recovered from the transfection of 5 expression plasmids with this robust system the influenza vaccine is updated yearly. The mechanisms for the development of reverse genetic systems along with research and findings on RG systems for dsRNA viruses will be discussed in detail in Sections 1.9 – 1.11

## 1.2 Rotavirus classification

Rotaviruses comprise the genus *Rotavirus* within the family *Reoviridae*, one of 15 genera of *Reoviridae* family subdivided into sub-families which share characteristic morphological and biochemical properties (Mertens et al., 2004). The genus Rotavirus contains 7 distinct groups with cross-reacting antigens and are classified serologically by a scheme that allows for the presence of multiple groups (serogroups) and multiple serotypes within each group. The group specificity is predominately determined by the serological reactivity and genetic variability of VP6 (Hoshino and Kapikian, 2000). Group A, B and C rotaviruses are found in both humans and animals, with group A being the cause of most disease outbreaks (Matthijnssens, Ciarlet et al. 2011). Rotaviruses in groups D, E, F and G have been found only in animals to date (Matthijnssens et al., 2012). Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups (Yolken et al., 1988). Serotypes within group A are defined by the reactivity of neutralising antibodies of glycoprotein VP7(serotype G) and the protease cleaved protein VP4(Serotype P) (Birch et al., 1988). Over the years 27 VP7 (or G) and 37 VP4 (P) serotypes have been identified with over 70 combinations of P and G serotypes (Theuns et al., 2015).

## 1.3 Rotavirus structure, genome organisation and coding assignments

### 1.3.1 Virion structure

Rotaviruses are comprised of 11 double-stranded (ds)RNA genome segments encapsidated in a triple-layered capsid. Each genome segment encodes for one protein except genome segment 11 which encodes for 2 viral proteins in some strains (Section 1.2.3). The three layers are the inner core, which is made up of VP2; the middle/intermediate layer comprised of VP6 and together the inner core and middle layer make up DLPs; the outer layer is made of VP4 and VP6 (Figure: 1-1)

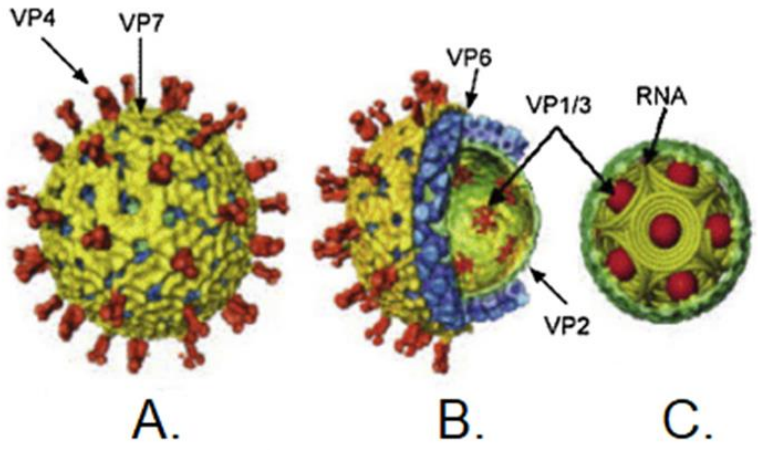


**Figure 1.1 Reconstruction of the rotavirus virion structure Jayaram et al. (2004).** (A) A triple-layered particle with VP4 spike protein (orange) and VP7 (yellow) forming the outermost layer. (B) Cutaway view of the rotavirus TLP showing the inner VP6 (blue) and VP2 (green) layers and the transcriptional enzymes (in red) anchored to the inside of the VP2 layer at the fivefold axes. (C) Schematic depiction of genome organization in rotavirus. The genome segments are represented as inverted conical spirals surrounding the transcription enzymes (shown as red balls) inside the VP2 layer in green

### 1.3.2 Genome structure, (+) ssRNAs, coding assignments and function

The 11 dsRNA genome segments are contained within the core capsid made of VP2. Virus particles contain their own RNA-dependent RNA polymerase to transcribe the individual RNA segments into mRNA. The packaging of these RNA segments into the rotavirus capsid, however, requires intimate protein-RNA interaction (Kapahnke et al., 1986). The structural proteins VP1, VP2, and VP3 may be responsible for the packaging, however the specific protein directly responsible for packaging remains unknown (Estrozi et al., 2013). The first known genome sequence was that of the rotavirus SA11 strain. The genome of RVs is highly ordered within the particle. Each positive sense RNA [(+) ssRNA] genome segment starts with a 5′ guanidine (illustrated in Figure 1.2) followed by a set of conserved sequences forming part of the noncoding 5’ region. The 5’ noncoding region is followed by an open reading frame (ORF) followed by another noncoding sequence which contains a subset of conserved terminal 3’ sequence ending with two 3’ terminal cytidines (Trask et al., 2010). There is variation between the 5’- and 3’ terminal end sequences among RV in different groups with an example thereof listed in Table1.1

**Table 1.1 Sequence variations in the 5' and 3' terminus of different RV strains**

| Group | Strain | 5’-terminal sequence | 3’-terminal sequence |
|---|---|---|---|
| A | SA11 | 5’-GGC$(A/U)_7$- | -A/GCC-3’ |
| B | IDIR | 5’-GGC/U- | -ACCC-3’ |
| C | Bristol | 5’-GGCC$(A/U)_7$- | -GGCU-3’ |
| D | HS-58 | 5’-GG$(U)_5(A)_7$- | -GACC-3’ |
| H | ADR-N | 5-GGCACU- | -ACCCC-3’ |

The ORF of each genome segment codes for a specific protein. Messenger RNAs (mRNAs), mostly end with the consensus sequence 5′UGUGACC3′ (Lu et al., 2008). This consensus sequence contains essential signals for genome replication and gene expression which will be discussed in more detail in Section 1.5. Translation is enhanced by the last four nucleotides of the mRNA (Chizhikov and Patton, 2000) The lengths of the 3′ and 5′ noncoding sequences vary for different genome segments, but the noncoding sequences of homologous strains are highly conserved. The dsRNA segments are base paired end to end, and the (+) strands contain a 5′ cap sequence m7GpppG(m)GC (Gouet et al., 1999).

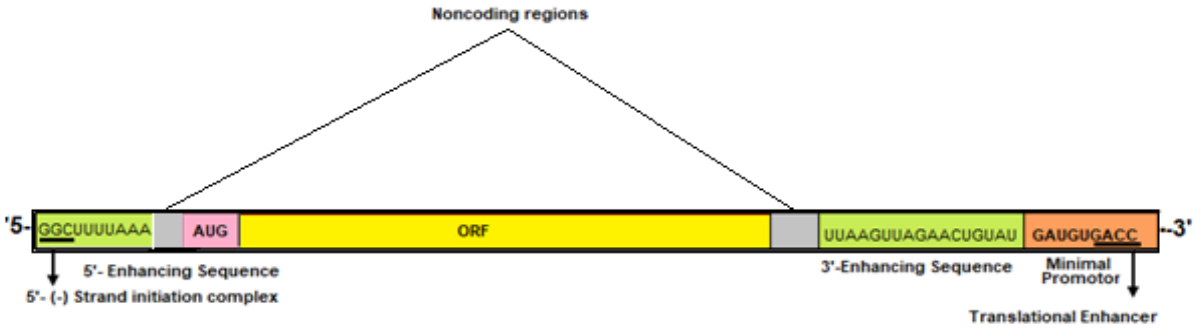


**Figure 1.2 RV genome segment structure.** All 11 RV genes lack a polyadenylation signal, are A+U rich and have conserved consensus sequence at the 5' and 3' end. SA11 genome segments range between 667bp and 3302 bp with segment 11 being the smallest and segment 1 the largest genome segment**.**

All 11 mRNAs must be distinguished from one another for packaging. Therefore, mRNAs contain a unique *cis*-acting signal due to mRNAs being replicated by the same polymerase which recognises the sequenced in a base-specific manner. In most cases, the genome of group A viruses is therefore composed out of four size groups of dsRNA segments, genome segment 1 to 4, which have a high molecular weight. Followed by Segment 5 and Segment 6, which have lower molecular weight, then a triplet of segments (segment 7 to segment 9) and then two smaller segments (segment 10 and segment 11) (Mathieu et al., 2001).

Viruses within each serogroup are capable of genetic reassortment, but reassortment does not occur among viruses in different groups. The segmented nature of the genome allows reassortment of genome segments during mixed infections, which is the major distinguishing feature of RV genetics. Reassortment has been a powerful tool for mapping mutations and other determinants of biological phenotypes to specific genome segments. However, more detailed genetic analysis of RVs is currently limited by the inability to perform reverse genetics.

### 1.3.3 Virus proteins, coding assignments and the viral replication cycle

As mentioned in Section 1.3.1 rotavirus has 11 dsRNA genome segments each encoding a specific protein, except for genome segment 11 which encodes for two viral proteins. Coding assignments were first determined for the type species SA11. The genome segments code for 6 structural proteins, which are found in virus particles, and six non-structural proteins found in the infected cells. Each protein has a specific role and properties (Table 1.2).

**Table 1.2 RV proteins and function**

| Genome segment | Protein product | Function |
|---|---|---|
| **1** | VP1 | RNA-dependent RNA polymerase, ss-RNA binding, forms complex with VP3, |
| **2** | VP2 | RNA binding, required for replicase activity of VP1 |
| **3** | VP3 | Guanylyltransferase, methyltransferase, ss-RNA binding, forms complex with VP1 |
| **4** | VP4 | Hemagglutinin, cell attachment, neutralisation antigen, protease enhanced infectivity, virulence, putative fusion region |
| **5** | NSP1 | Basic, zinc finger, RNA binding, virulence in mice, interact with and degraded IRF-3, non-essential for some strains |
| **6** | VP6 | Hydrophobic, trimer, subgroup antigen, protection (intracellular neutralization), required for transcription. |
| **7** | NSP3 | Acidic dimer binds 3' end of viral mRNAs, competes with cellular PABp for interactions with eIF-4G1, inhibits host |
| **8** | NSP2 | Basic, RNA binding, oligomer, NTPase, helicase forms viroplasms with NSP5 |
| **9** | VP7 | RER integral membrane glycoprotein, calcium-dependent trimer, neutralization antigen |
| **10** | NSP4 | RER transmembrane glycoprotein, intracellular receptor for DLP, role in morphogenesis, interacts with viroplasm, |
| **11** | NSP5 | Basic phosphoprotein, RNA binding, protein kinase, forms viroplasm with NSP2 interacts with VP2 and NSP6 |

*Table was compiled from Fields Virology 2016, Estes and Greenberg

VP1, VP2 and VP3 are encoded by genome segments 1, 2 and 3 respectively. These structural proteins form the enzymatic machinery, along with structural protein VP6, are used in the synthesis of capped mRNA during RV replication (Jayaram et al., 2004). VP1 is the RNA-dependent RNA polymerase (Valenzuela et al., 1991). VP3 is a guanylyl and methyltransferase protein (Chen et al., 1999). VP2 plays a crucial role in the genomic organisation of the viral core and exhibits the ability to assemble independently to form the core structure also known as the SLP.

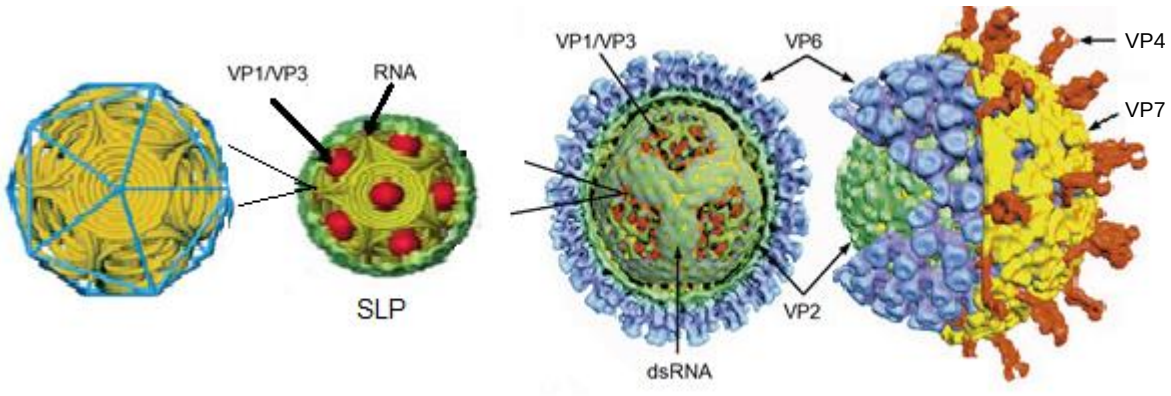


**Figure 1.3. Schematic illustration of core particle within the TLP.** Figure adapted from Pesavento et al., 2001 and Jayaram et al., 2004

The structural assembly of the viral proteins during replication is determined by VP2 (Zeng et al., 1998). The structural integrity of the virus particle is maintained by VP6, encoded by genome segment 6, by ensuring the organisation of the transcriptional complex (Estes and Cohen 1989). The outer capsid of the virion is comprised of the glycoprotein VP7, and hemagglutinin proteins VP4. VP 7 is encoded by genome segment 9 and may modulate the cell attachment and cell entry functions of VP4 (encoded by genome segment 4) (Mèndez et al., 1996). VP7 also plays a part in the rotavirus entry and assembly steps and is highly immunogenic (Aoki et al., 2009). VP4 is mainly involved in cell penetration, virulence, neutralisation, hemagglutination, host range specificity and the attachment to sialic-acid containing cellular receptor (Shaw et al., 1996).

Before RV can infect a cell VP4 must be converted to VP5* and VP8*. The cleavage of VP4 functions to increase viral infectivity. Rotavirus non-structural proteins (NSPs) coordinate various stages of genome replication and viral assembly. NSP3 is encoded by genome segment 7 and is proposed to facilitate the translation of the rotaviral mRNA transcripts and to suppress host protein synthesis through antagonism of the poly A binding protein (PABP) (Chung and McCrae 2011). Rotaviruses rely on the host translation machinery to produce the viral proteins encoded by their genome. During viral infection, NSP3 interacts with host immune responses increasing the translation of viral transcripts (Groft and Burnley 2002). NSP2 is encoded by genome segment 8 and plays a critical role in the formation of the viroplasm as well as genome encapsidation and genome replication (Fabretti et al., 1999). During viroplasm formation, NSP2 interacts with NSP5 as well as structural proteins VP2 and VP1. NSP5 competes with ssRNA for binding to NSP2 (Jiang et al., 2006) and therefore suggests that one of the functions of NSP5 is to regulate NSP2-RNA interactions during genome replication. The ssRNA-binding and helix-destabilising activities of NSP2 are required for relaxing mRNA templates in preparation for dsRNA synthesis (Taraporewala and Patton 2001). However, the roles for the observed NTPase, RTPase, and NDP kinase activity of NSP2 during RV replication remain unclear.

Despite various properties that are attributed to NSP5 from *in vivo* and *in vitro* studies, the only role firmly established for this protein is as a binding partner of NSP2 in the formation of viroplasms. In addition to NSP2, NSP5 has been shown to interact with other rotavirus proteins such as VP1, VP2 and NSP6 (Torres-Vega et al., 2000), and also with single-stranded and double-stranded RNA in a sequence-independent manner (Vende et al., 2002). Studies also reveal NSP5 is involved in many processes such as the dynamics and regulation of viroplasms and as an adapter to integrate the various functional properties of NSP2 with other rotavirus proteins during viral genome replication/encapsidation (Contin et al., 2010).

NSP4, encoded by gene segment 10, is a multifunctional protein and many of the NSP4 functions have been mapped to distinct domains of the protein. NSP4 is synthesised as an ER transmembrane glycoprotein and consists of three hydrophobic domains (H1–H3). The H3 domain is highly amphipathic and was recently shown to disrupt cellular calcium homeostasis by the viroporin-mediated release of ER calcium stores (Hyser et al., 2010). A secreted form of NSP4, which contains the integrin I domain binding site, is involved in diarrhoea induction through interaction with cellular plasma membrane integrin I domains. Other NSP4 activities include disruption of plasma membrane integrity (Newton et al., 1977), inhibition of sodium absorption by epithelial sodium channels (ENaC) and sodium glucose transporter 1 (SGLT1) (Ousingsawat et al.,2011) and remodeling of the cellular microtubule and actin networks (Yang and McCrae 2012).

### 1.4 Replication Cycle

The RV replication cycle (Figure1.3) starts with virus attachment which is mediated by structural haemagglutinin protein VP4 and glycoprotein VP7 followed by penetration and uncoating of the virus capsid. After adsorption and partial uncoating, rotaviruses produce (+)ssRNA transcripts in the cytoplasm which are either translated into viral proteins or packaged and transformed to dsRNA. Viral propagation takes place in the viroplasms formed by the non-structural proteins NSP2 and NSP5. Subviral particles are assembled, and genome replication takes place. As NSP2 and NSP5 are essential for viroplasm formation, viral RNA replication cannot occur when the function of either of these non-structural proteins are blocked. Viral particles mature from double-layered particles to triple-layered infectious virions in the cytoplasm after the release from viroplasms. The infectious virions are then released by means of cell lysis. Before RV can infect a cell VP4 must be converted to VP5* and VP8*. The cleavage of VP4 functions to increase viral infectivity. Rotavirus non-structural proteins (NSPs) coordinate various stages of genome replication and viral assembly. NSP3 is encoded by genome segment 7 and is proposed to facilitate the translation of the rotaviral mRNA transcripts and to suppress host protein synthesis through antagonism of the poly A binding protein (PABP) (Chung and McCrae 2011). Rotaviruses rely on the host translation machinery to produce the viral proteins encoded by their genome. During viral infection, NSP3 interacts with host immune responses increasing the translation of

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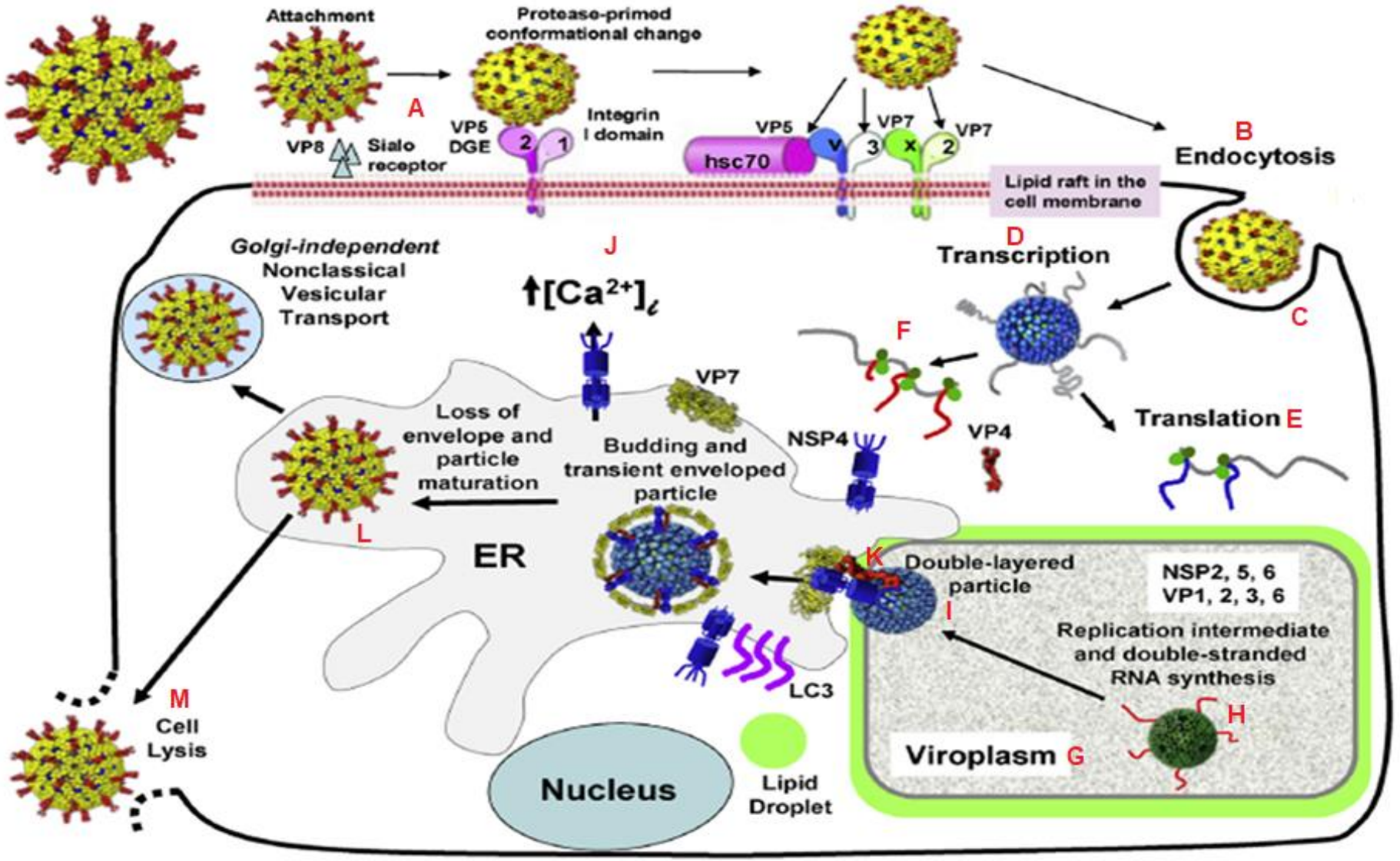


**Figure 1.4 Overwiev of RV replication cycle**

Rotavirus attaches to the cell by cleavage of VP4 in the presence of trypsin producing by VP5* and VP8*(A). The virus enters the cell through endocytosis (B) and the double-layered particle is released into cytoplasm of the cell (C). Transcription of capped (+) ssRNAs is initiated (D). (+) ssRNA then functions either as mRNAs for viral replication (E) or as template for synthesis of dsRNA during genome replication (F). NSP2 and NSP5 interact to form the main structure of the viroplasm (G). dsRNA is synthesised by VP1 within the inner VP2 core(H) VP6 assembles onto VP2 to form the DLP(I). NSP4 Increases the intracellular $Ca^{2+}$ levels(J) and recruits VP4 and the DLP to the ER where the NSP4-VP4-DLP complex buds into the ER(K). VP7 attaches to the DLP to form the mature TLP(L) which is then released from the cell through either exocytosis or cell lysis(M). Modified from Hu et al., 2010 and Estes and Greenberg 2013

### 1.4.1 Virus particle attachment

RV particle attachment is an intricate process (López and Arias,2004) and is depicted in Figure 1.4 (A). The rotavirus-triple layered particle first reacts with the cellular receptor via the VP4 spikes containing sialic acid (SA) (Dormitzer et al., 2002). Attachment is mediated by a subunit of VP4, VP8*, which interacts with sialic acids on cellular glycans. (Dormitzer et al., 2002) Several cellular surface molecules react with integrin ligand motifs. VP7 of VP5* and act as co-receptors post attachment (Gutiérrez et al., 2010).

### 1.4.2 Virus particle penetration and uncoating

Upon contact with the cellular receptors, the VP4 spikes of rotavirus triple-layered particles undergo structural changes resulting in the removal of the outermost layer. Trypsin cleavage product VP5*, which is normally hidden under trypsin cleavage product VP8*, takes the form of a 'post-penetration umbrella' structure, depicted in Figure 1.5 (Settembre et al.,2011). Full infectivity of the triple-layered particles is achieved through the treatment of rotavirus particles with trypsin (Estes et al., 1981). The mechanism of cell penetration of rotavirus particles, following binding, remains unclear. In some RV strains, cell membrane entry occurs in the presence of GTPase and cholesterol (Sánchez-San Martín et al., 2004). RV entry also requires the 'endosomal sorting complex for transport' (ESCRT) (Silva-Ayala et al., 2013).

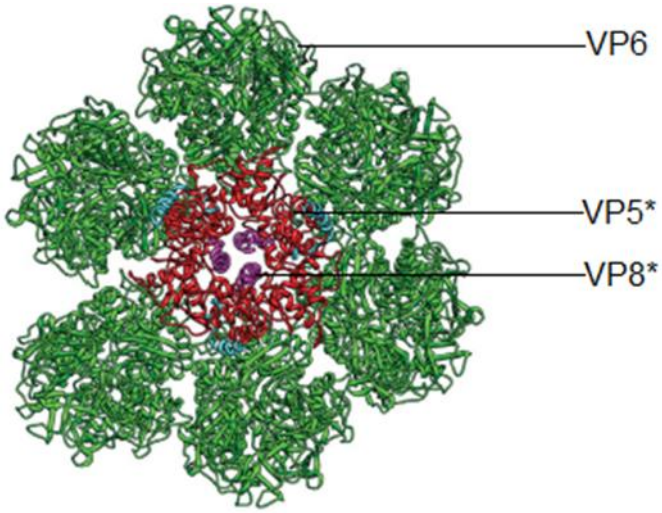


**Figure 1.5. Post-penetration umbrella configuration of the double-layered particle.** The VP5 segment forms the coiled coil in the 'post-entry' conformation (red) which is normally hidden under VP8 (blue) and can be visualised after the removal of the outer capsid. Figure adapted from Settembre et al., 2011

### 1.4.3 RV (+) ssRNA synthesis

As discussed in Section 1.3.3 RV particles possess their own transcription machinery, consisting of VP1 and VP3 localised at the inner surface of the VP2 (Jayaram et al., 2004). The transcription compounds form complexes with specific dedicated viral RNA genome segments (Perizet al.,

2013). Capped, non-polyadenylated, (+)ssRNA transcripts are produced in the cytoplasm by double-layered rotavirus particles from the negative strand of the genomic RNA. The (+) ssRNAs are then released from the double-layered particles. Early in the replication cycle, the transcripts serve as templates for translation of virus-encoded proteins. However, later in the replication cycle transcripts serve as templates for genome replication resulting in the dsRNA genomes (Silvestri et al., 2004). 12 different genome segments are produced from (+)ssRNAs by the *Reoviridae* family. RV, however, produces 11 genome segments. RV double-layered particles become transcriptionally active with sufficient ATP as an energy source and precursors and produce copious amounts of (+)ssRNAs (Lu et al.,2008). Secondary transcription is indicated when newly synthesised, transcriptionally active, double-layered particles produce (+)ssRNAs exponentially (Ayala-Breton et al., 2009). Genome segment specific (+)ssRNAs are forced out of the double-layered particles into the cytoplasm and then translated into encoded proteins as described in Section 1.3.3

### 1.4.4 Viroplasm formation and function

Non-structural proteins NSP2 and NSP5 are essential for the formation of viroplasms. RV proteins and RNAs interact specifically in the viroplasms. When NSP2 and NSP5 are not present in sufficient amounts, the result is the production of viroplasm-like structures (Fabbretti et al., 1999). Viroplasm formation can also be prevented by the blocking of NSP2 and NSP5 or the use of NSP2-or NSP5-mutants (Campagna et al., 2005). NSP2 in the cytoplasm forms complexes with VP1, VP2 and tubulin to form the viroplasm tubulin component (Criglar et al., 2014). These complexes, through acetylation, also induce microtubule depolymerisation and stabilisation (Eichwald et al.,2012). Functional proteasomes and components of the autophagic pathway are essential for viroplasm formation and RV replication (Arnoldi et al., 2014). Grooves in the NSP2 octamer are binding sites for which NSP5 and ssRNAs compete, as mentioned in Section 1.3.3, thus NSP2 is thought to regulate the balance between rotavirus RNA translation and RNA replication (Jianget al., 2006).

### 1.4.5 RNA packaging, minus-strand RNA synthesis and DLP formation

The specific molecular details of core particle formation and RNA replication are not well understood, and it is unclear how the packaging of the correct set of 11 (+)ssRNAs into individual particles is controlled. The primary replication complexes (VP1, VP3 and the (+)ssRNA) presumably interact with a VP2 decamer (Berois et al., 2003) After the formation of the core particles, they are promptly transcapsidated by VP6 within the viroplasm (Figure 1.6), resulting in the formation of the double-layered particles (Desselberger et al., 2013). The 11 different (+) ssRNAs are then maintained and interact with viral core proteins for packaging and replication of

the dsRNAs.. This interaction leads to the formation of core particles. The formation of the VP2 complex is essential for the activity of the VP1 RNA dependent RNA polymerase.

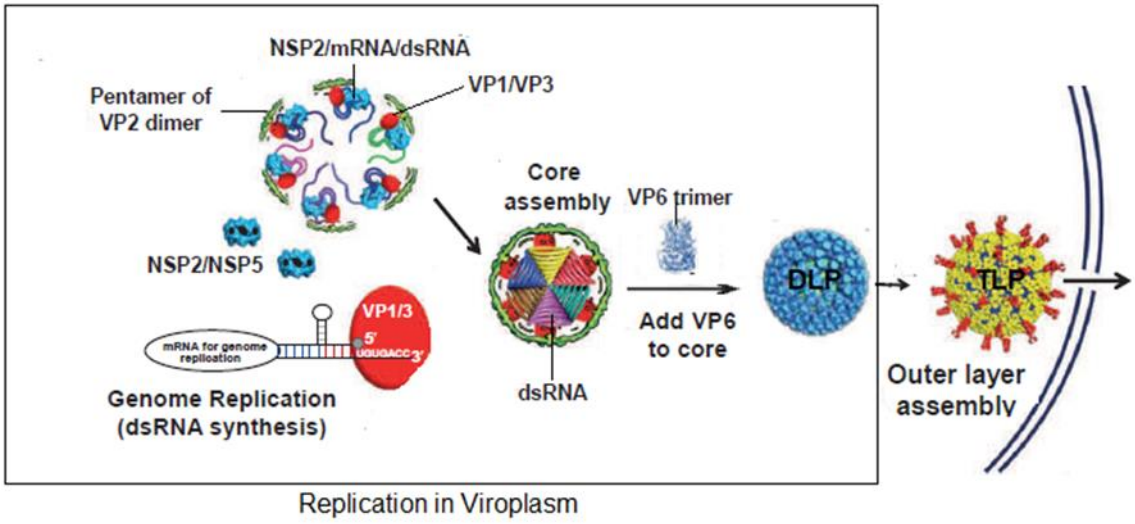


**Figure 1.6. Replication of dsRNA within the viroplasm.** (+) RNAs that are thought to form panhandle structures that are translated into viral proteins and replicated into genomic double-stranded RNA (dsRNA) in viroplasms. dsRNA is packed within the core whereafter the VP6 trimers are added to the core particle allowing the formation of the double-layered particle. Figure adapted from Fields Virology 2016

### 1.4.6 Virus particle (virion) maturation and release

The double-layered RV particles reach maturation when leaving the viroplasm by budding through the endoplasmic reticulum (ER) as depicted in Figure 1.4K. During this process NSP4 interacts with VP6 and serves as an intracellular receptor (Taylor et al., 1996). Rotavirus particles acquire the outer layer consisting of VP4 and VP6 (Estes and Greenberg, 2013). The interaction of the double-layered particle with VP4 followed by the interaction with VP7 is essential for full infectivity of the triple-layered particle (Trask and Dormitzer, 2006). However, virus particle maturation can be affected by the blockage of NSP4 expression by siRNA which leads to maturation defects and the inhibition of RNA replication (Silvestri et al., 2005). The matured triple-layered particles are released from cells either by lysis [Figure 1.5(M)] or by a budding process that does not kill the cell immediately (Gardet et al., 2006).

## 1.5 Immune response to rotavirus infectivity

The virus enters the cells within the body, and viral replication occurs. The precise mechanisms of RV infections remain to be understood. However, some studies have reported on the different immune responses that occur in hosts during RV infection. These responses include the innate immune response (Figure 1.7) and acquired immunity namely the humoral immune response and cellular immune response.

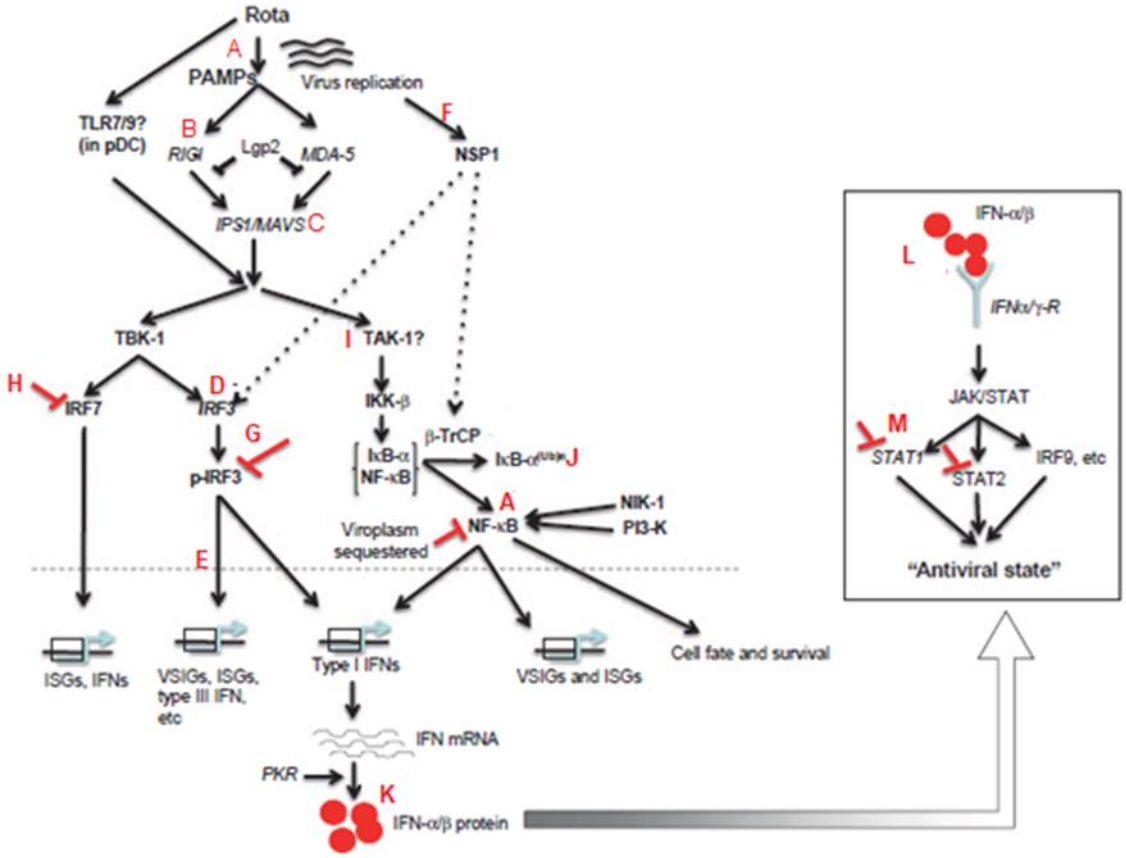


**Figure 1.7 RV interactions with host innate immune response**. The innate immune response is triggered upon viral entry and leads to the activation of various molecular pathways and pathogen recognition receptors. Figure adapted from Fields Virology 2016. Upon viral entry pathogen-associated molecular pathways (PAMPs) (A) are generated activating cytosolic pathogen recognition receptors (PRRs) RIG-I and MDA-5 (B), which in turn leads to mitochondrial-associated adaptor protein IPS-1/MAVS–dependent activation (C) of transcription factor IRF3 by the kinase TBK-1 (D). Early in the replication cycle transcription of ISGs is induced by IRF 3 (E). NSP1 is expressed as a result of viral replication (F) and leads to the degradation of IRF3. Degradation of IRF 3 is made possible by the TBK-1 dependent phosphorylation of the IRF3 carboxyl-terminus (G). Other interferon regulating factors including IRF5 and IRF7 are also be proteasomally degraded by NSP1 (H) (Estes and Greenberg 2016). Nuclear factor-kB(NF-kB) activated by distinct signalling pathway (I) is also required for interferon response in RV infected cells following the proteasomal degradation of its inhibitory partner, IkB-a (J). The double-stranded RNA (dsRNA)-dependent protein kinase PKR mediates IFN secretion by an unknown mechanism (K). RV triggers IFN secretion by a process that is likely a result of viral genomic dsRNA-mediated TLR7/9 signalling (steps 1 and 2). The result of IFN secretion from RV-infected cells is the establishment of an antiviral state in bystander cells mediated by signalling through the transcription factors STAT1, STAT2, and IRF9 (L). A second viral strategy exists to counter this phase of the IFN response by sequestration of STAT1 and STAT2 (M), although the viral factors involved are not known. Other interferons including type II and III IFNs may further restrict virus replication and dissemination in the host and may exert different effects depending on the tissue, strain, and stage of pathogenesis (Estes and Greenberg 2016).

While investigating the innate immune response to RV transcripts at the NWU, Dr L. Mlera found that RIG-I sensed RV transcripts in transfected cells. However, no MDA5 response was observed (Mlera, 2012). Dr Mlera also found that RV transcripts induce elevated levels of cellular mRNA encoding the cytokines IFN-1β, IFN-λ1, CXCL10 and TNF-α while other cytokines namely IFN-α, IL-10, IL-12 p40 and the kinase RIP1 revealed little to no induction. Inhibiting the RNA-dependent

protein kinase R (PKR) reduced the induction of cytokines IFN-1β, IFN-λ1, CXCL10 and TNF-α, but the expression levels were not abrogated.

During his studies on the effect of rotavirus transcripts on specific interferon pathways in the presence of various RV Wa and SA11 proteins at the NWU, Dr J.F. Wentzel found that rotavirus transcripts induced elevated levels of the expression of the cytokines IFN- α1, IFN 1β, IFN-λ1 and CXCL10 (Wentzel, 2014). He also found that the expression of VP3, VP7 and NSP5/6 was more likely to stimulate interferon responses. In contrast to this finding, viral proteins VP1, VP2, VP4 and NSP1 actively suppressed the expression of specific cytokines. This resulted in the suppression of interferon responses stimulated by RV transcripts. Dr Wentzel also found that cells transfected with the plasmids encoding NSP1, NSP2 or a combination of NSP2 and NSP5 significantly reduced the expression of specific cytokines induced by RV transcripts. From these studies, Dr Wentzel concluded that in addition to the NSP1 degradation of IRF, there are other possible viral innate suppression mechanisms.

Their insights regarding the role of the innate immune response after transfection of rotavirus into cells should aid the development of a true rotavirus reverse genetics system. Transfection of RV transcripts revealed the induction of interferon of type I and type III IFN stimulated by transfection of RV transcripts, which had not been described yet. Since the induction of IFN induces apoptosis the basis of cell death was directly linked to the transfection of RV transcripts. The induction of cytokines by RV transcripts resulted in an antiviral state in transcript infected cells. It was also determined that the antiviral state could be the critical event preventing RV recovery

### 1.6 Rotavirus disease burden and vaccines

While diarrhoeal diseases remain one of the leading infectious diseases with high rates of mortality in children under the age of 5 (Trask et al., 2015), rotavirus infection is classified as the major pathogen associated with severe dehydrating gastroenteritis in children (Kotloff et al., 2013) with the greatest disease burdens in south-east Asia and sub-Saharan Africa (Parashar et al., 2013). There are at least 215,000 (range 197,000–233,000) occurring RV deaths globally, 56% of these deaths are reported in sub-Saharan Africa and 22% in India alone (Tate et al., 2016). A study published in September reported that rotavirus vaccines had been introduced to 33 African countries by 2016 and estimated that the introduction of these vaccines would lead to a reduction in rotavirus hospitalizations and deaths in these 33 countries which would, in turn, lead to the introduction of rotavirus vaccines across the continent (Shah et al., 2017).

The primary aim of a RV vaccine has been to prevent severe rotavirus gastroenteritis during the first 2 to 3 years of life, the period when RV disease is most severe and takes its greatest toll. These observations suggest that the effectiveness of a rotavirus vaccine largely depends on its

ability to stimulate transport of antibodies into the gut lumen or to stimulate local production of antibodies. Most efforts to date have focused on live attenuated vaccines that are administered orally.

There are currently two main licenced vaccines i.e., RotaTeq® and Rotarix®. Besides these two commercial and WHO-prequalified vaccines, three additional live attenuated orally administered rotavirus vaccines have obtained national licensure in the country of manufacture (Kirkwood et al., 2017). RotaVac is an Indian vaccine (Bhandri et al., 2014) which was included in the Universal Immunization Programme (UIP) of India in 2016. The Lanzhou Lamb Rotavirus (LLR-85) was vaccine developed by the Lanzhou Institute of Biological Products, China and Rotavin-M1, a live attenuated human RV vaccine developed in Vietnam (Huong et al., 2009)

### 1.6.1 RotaTeq®

The RotaTeq vaccine is manufactured by Merck and was licensed by the FDA in 2006. RotaTeq is a genetically engineered vaccine made of live, attenuated human-bovine reassortant rotaviruses expressing human rotavirus VP7 from serotypes G1, G2, G3 or G4 and VP4 (P[8]). Other genotypes, including G9P[8], G12P[8], and G2P[4], have predominated for a year or two in specific locations, but overall G1P[8] has remained the predominant genotype in countries using RotaTeq.

### 1.6.2 Rotarix®

Rotarix is a genetically engineered vaccine made of live attenuated human rotavirus G1P[8] strain (Parashar 2016) and is administered at 2 and 4 months of age. In the African clinical trial of Rotarix conducted in Malawi and South Africa (O'Ryan et al., 2015), great diversity of circulating rotavirus strains was observed, with the G1P[8] vaccine-type strains accounting for 57% of strains detected in South Africa and only 13% of strains in Malawi. Nevertheless, the vaccine demonstrated good efficacy against a range of G types (G1, G12 and G8) and circulating P types (P[8], P[4] and P[6]) Both vaccines are effective and accumulated evidence show reductions in RV related hospitalizations in countries where vaccines have been introduced.

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## 1.7 Simian agent 11

The simian agent 11 (SA11; RVA/Simian-tc/ZAF/SA11/1958/G3P[2]) was isolated at the National Institute of Virology, Johannesburg, South Africa in 1958 by Dr Hubert Malherbe. This prototype strain of the SA11 group was isolated from a rectal swab taken from an overtly healthy vervet monkey (Cercopithecus aethiops pygerythrus) (Malherbe and Strickland-Cholmley, 1967). SA 11

is one of 15 South African *Cercopithecus* monkey viruses (SA 1-15) (Malherbe and Harwin, 1957; Malherbe et al., 1963). A number of these viruses belong to broader groups such as entero-, adeno-, reo- and herpes-viruses.

Using electron microscopy, ultra-thin sections of duodenal mucosa from children with acute gastroenteritis was examined by Ruth Bishop and Ian Holmes in May 1973 in Australia (Bishop et al., 1973). In their study Bishop and Holmes identified an abundance of rotavirus particles in the cytoplasm of mature epithelial cells lining duodenal villi and faeces of the children admitted to the Royal Children's Hospital, Melbourne. At that time, the virus particles were identified to be reovirus-like/orbivirus-like and only after the virus was linked to previous descriptions in the literature provided by Dr Malherbe the virus could be identified as rotavirus.

The SA11 strain was chosen for this study due to its ability to propagate very well in cell culture, and it has not been reported to cause disease in humans or animals. Therefore, SA11 is an ideal model to investigate rotavirus growth, virulence, genome replication, rotavirus proteins encoded by genome segments and their function (Estes, 2001).

### 1.8 Viral reverse genetics

Reverse genetics (RG) techniques are designed to investigate the phenotypical traits that are conferred by a defined genomic sequence and variations thereof. One of the most definitive ways in which to study the roles of specific sequences in viral genomes is to modify them and to generate infectious virus, that is, to 'rescue' the virus, with these modified sequences. Viral RGs involves the generation of infectious virus particles in cell culture from cDNA clones or 'infectious' (+) ssRNA transcripts. Using reverse genetics, the viral genome can be manipulated with recombinant DNA techniques to introduce directed mutations or generate chimeric viruses by exchanging coding regions. The ability to engineer recombinant mutant viruses makes it possible to study the biology of the virus and also to generate rationally designed vaccines (Ebihara et al., 2005).

Before the advent of recombinant DNA and sequencing technologies, classical genetic analysis, namely random isolation and characterisation of virus mutants, was one of the few effective methods for identifying, mapping, and characterising virus genes, and the only method for obtaining virus mutants.

As mentioned in Section 1.1, the first RG system for RNA viruses was the development of the positive-sense RNA poliomyelitis virus (Racaniello and Baltimore, 1981a). The poliomyelitis RG system was developed by cloning a cDNA copy of whole genomic RNA into a pBR322 plasmid and transfecting the recombinant plasmid into mammalian cell cultures which resulted in the recovery of infectious poliovirus (Racaniello and Baltimore, 1981a). The system was later

optimised by the use of SP6 polymerase to generate poliovirus transcripts from cDNA templates (Kaplan et al., 1985) which after transfection into HeLa 3 cells also resulted in the recovery of infectious poliovirus. The development of a RG system for the poliomyelitis virus was performed with ease due to the positive-sense nature of the genomic RNA of the virus. Development of RG systems for negative-sense RNA viruses is much more complicated.

To date, RG systems are in place for a variety mammalian RNA and DNA viruses, which include influenza A viruses, bornaviruses, flaviviruses, picornaviruses and paramyxoviruses (Neumann et al., 1999, Yun et al., 2003 Racaniello and Baltimore, 1981, Perez et al., 2003). These RG systems enabled the gathering of information regarding the natural characterisation, replication and pathogenesis of these viruses.

The best illustration of the power and potential of RG systems is that of the influenza virus. The influenza RG system was based on the transfection of ribonucleoproteins and cDNA into cultured cells with in the presence of a helper influenza A virus to incorporate cDNA genome segments allowing the formation of recombinant virus (Luytjes et al., 1989). Over the years the influenza RG system has been improved continuously resulting in the use of 5 cDNA plasmids (Neumann et al., 2012). The 5-cDNA plasmid transfection process was reduced from the original 12 cDNA plasmid recovery procedure (Neumann et al., 1999). With the use of the influenza virus RG systems as basis, many other RG systems were developed.

### 1.9 Reverse genetic systems for mammalian dsRNA viruses

Rescuing dsRNA viruses is more complex because many of these viruses (such as rotavirus) have multiple genome segments which means that cells must be transfected with constructs for each of the genome segments as well as for the replication proteins. When developing RG systems two main strategies are followed: plasmid-based RG and transcript-based. Plasmid-based RGs rely on the construction of recombinant expression plasmids containing a cDNA copy of the entire viral genome placed under the control of an upstream promoter sequence (cytomegalovirus IE or T7 polymerase promoters). Plasmids are then transfected into mammalian cultured cells which support the replication of the specific virus. Viable viruses are then rescued from the cell cultures. Plasmid-based RG systems have been developed for many animal RNA viruses. Transcript-based RG systems rely on the transfection of transcriptively active (+) ssRNA transcripts generated from genome segment templates for the generation of infectious virus particles.

Over the years many attempts at developing methods to engineer segmented, double-stranded RNA from the *Reoviridae* family have been made resulting in the development of RG systems for mammalian orthoreovirus (MRV) (Kobayashi et al., 2007), bluetongue (BTV) virus (Boyce et al.,

2008), African horsesickness virus (AHSV) (Matsuo et al., 2010). and epizootic haemorrhagic disease virus (EHDV) (Yang et al., 2015).

### 1.9.1 Mammalian orthoreovirus

A plasmid-based RG system for mammalian orthoreovirus was described in 2007 (Kobayashi et al., 2007). Initially, cDNA copies of all 10 viral genome segments were placed in individual plasmids under the control of a T7 promoter and transfected into L929 cells (Figure 1.8). This system was improved by introducing multiple genome segments into a single plasmid. This improvement resulted in only four plasmids to be transfected (Kobayashi et al., 2010). Viable orthoreoviruses could be rescued after 48 hours. In the RG system depicted in figure 1.8, the complete set of reovirus genome segments were individually fused at their native 5'- termini to a T7 polymerase promoter and cloned into separate plasmids. The constructs also included a hepatitis delta virus (HDV) ribozyme (Rib) at the 3'-end which enabled the generation of (+)ssRNAs containing exact 3'-end sequences. To enable the recovery of reovirus, L929 cells were infected with a recombinant vaccinia virus (rDIs-T7pol) which provided the T7 polymerase for transcription and capping of reovirus transcripts. This was followed by transfecting the 10 plasmids and recovery of reovirus after 5 days incubation of cell cultures.

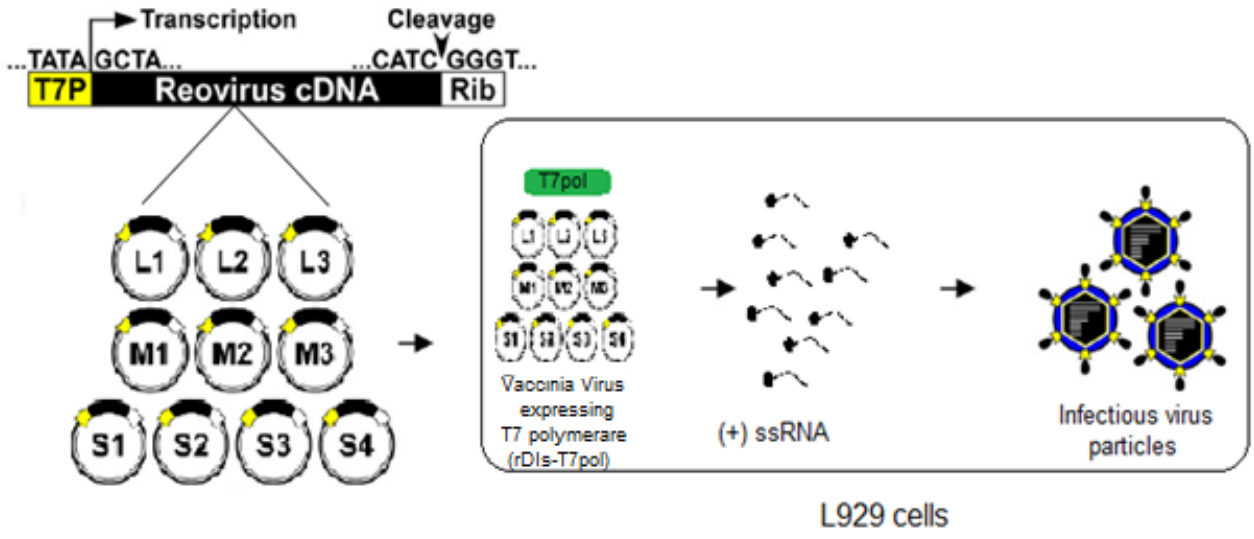



**Figure 1.8 Schematic representation of the reverse genetic system of mammalian orthoreovirus** Prototype reovirus genome segment cDNA in the plasmid is also illustrated. cDNA plasmids are transfected into mammalian cultured cells to generate infectious virus particles. Adapted from Kobayashi et al., 2008

### 1.9.2 Bluetongue virus

Similar to the RG system for MRV the fundamental principle of BTV RGs relies on the transcriptionally active subviral particle delivered during virus entry initiating viral replication by

extruding (+)ssRNAs into the host cell cytosol. BTV can be recovered using the complete set of 10 pure viral mRNAs transcripts obtained *in vitro* either from transcriptionally active viral cores or using T7-polymerase transcription of viral cDNA (Figure 1.9) (Taniguchi and Komoto, 2012). Infectivity of BTV RNA was first demonstrated by recovery of infectious virus following transfection of highly purified *in vitro* transcribed (+)ssRNAs derived from isolated BTV subviral particles (Matsua and Roy 2007). In this system, the addition of dsRNA did not affect the efficiency of BTV recovery. In the following year, Boyce and co-workers (2008) reported the recovery of reassortants containing genome segments from BTV serotype 1 (BTV-1) and BTV serotype 9 (BTV-9). This was achieved by the transfection of core-derived BTV-1 and BTV-9 ssRNA into BSR cells, a clone of BHK-21 cells. Furthermore, synthetic transcripts, of the entire genome, derived from *in vitro* transcription of cDNA templates with T7 polymerase were used to recover viable BTV (Boyce et al., 2008). It was possible to create reassortants using T7 polymerase derived transcripts

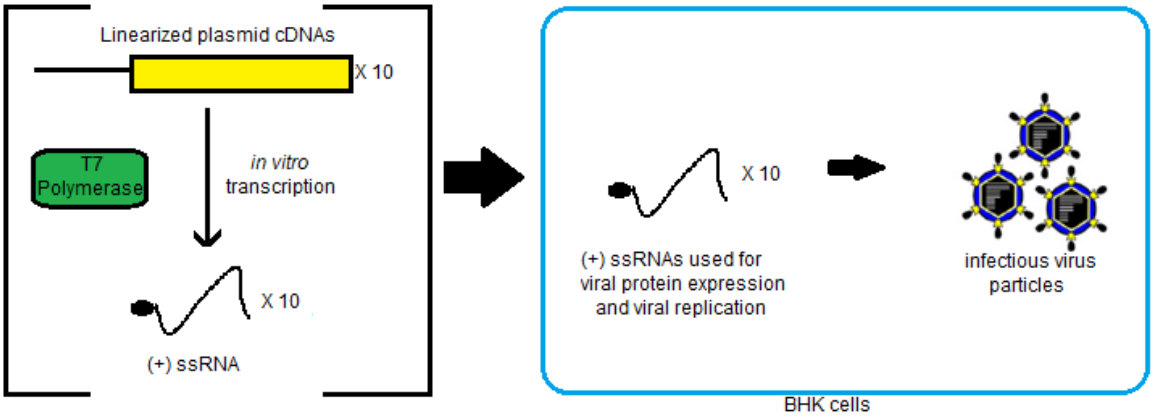



**Figure 1.9 Reverse genetic system used for the rescue of BTV.** mRNA transcripts, under the control of a T7 promoter sequence, are transfected into mammalian cultured cells to generate infectious virus particles. Figure adapted from Trask et al., 2012

Transfection of a complement of (+)ssRNAs containing just a single uncapped segment effectively prevents recombinant BTV recovery (Matsuo and Roy 2007). Why capped RNAs are required is not understood, although the presence of a cap most likely increases (+)ssRNA stability and enhances translation from viral (+)ssRNAs in transfected cells. An important technical advance for BTV reverse genetics came in the use of a ‘‘double-transfection’’ strategy, in which cells are transfected twice (separated by several hours) with BTV (+)ssRNAs; this approach significantly improved the efficiency of recombinant virus recovery (Ratinier et al., 2011).

Thus, (+)ssRNA capping and enhanced virus recovery by double-transfection highlight the importance of viral protein translation in BTV reverse genetics and indicate that it may be the rate-limiting step in the formation of infectious, recombinant virions.

### 1.9.3 African horsesickness virus (AHSV)

Similar to the recovery of BTV from core-derived (+)ssRNA, African horsesickness virus (AHSV) was recently rescued from the transfection of core-derived transcripts (Matsuo et al., 2010). It was also possible to recover reassortants by transfecting (+)ssRNA from two different AHSV serotypes. AHSV recovery was more efficient following two (+)ssRNA transfections that were 18 hours apart, indicating that AHSV genome replication occurred in two phases (Matsuo et al., 2010). The second transfection is thought to provide transcripts that are replicated and packaged as genomic dsRNA. In 2016 novel improvements were made that increased the flexibility of AHSV (Conradie et al., 2016) and reduced plasmids required for virus rescue to 5 plasmids instead of 10 which lead to an increase of virus recovery efficiency (Conradie et al., 2016). Conradie and co-workers improved the basic AHSV-4 RG system by including a T7 RNA polymerase expression cassette onto the genetic backbone of the reduced 5 expression plasmids containing the 10 cDNA clones representing the AHS viral genome (Conradie et al., 2016).

### 1.9.4 Epizootic haemorrhagic disease Virus (EHDV)

The development of a RG system for EHDV followed similar approaches used for BTV and AHSV. Construction of 10 T7 plasmid clones (Seg1 – Seg10) used to produce EHDV RNA transcripts for the RG system was carried out as described for BTV (Boyce et al., 2008). To facilitate the expression of viral protein expression plasmids were also constructed. Transcripts were transfected into mammalian BHK-21 cells following a double transfection with expression plasmids (Kaname et al., 2013). Virus rescue was observed 48 hours post plasmid transfection.

## 1.10 Rotavirus reverse genetics

At the start of this study in 2015, no true RG system for RV had been developed. However, there were three selection dependent reverse genetics systems that had been developed for RVs that depend on helper viruses with strong selection conditions. These systems permit the manipulation of only one of the eleven genome segments. Komoto and colleagues designed the system to manipulate genome segment 4 (VP4) (Komoto et al., 2006). In this system they utilised plasmids which contained the entire genome segment 4 sequence of rotavirus SA11 and placed the plasmid under the control of a T7 promoter. After 20 hours of transfection with the genome segment 4 plasmid, cells were infected with a KU strain helper virus. The helper virus was suppressed after 24 hours of infection allowing the recombinant virus containing VP4 to be rescued in the presence of neutralising antibody directed against the VP4 of the helper virus. The

system was later modified by Troupin and co-workers to enable the rescue and rearrangement of NSP3 from an *in vitro* modified cDNA plasmid expressing a rearranged genome segment 7(Troupin et al., 2010). In this case, the use of a helper bovine RF strain was employed. However, with no selection conditions being applied the bovine helper virus was only removed after multiple serial passages (Kobayashi et al. 2010, Troupin et al., 2010). Further modification of these systems led to an innovative approach by Trask and co-workers who took advantage of a temperature sensitive (*ts*) RV mutant as a helper virus (Trask et al., 2010). A cDNA plasmid containing genome segment 8 (NSP2) under the control of a T7 promoter sequence was transfected into cultured COS-7 cells infected with rDIs-T7pol(VV-T7), as depicted in Figure 1.8, followed by the transfection of the *ts* mutant RV(*ts*E*)* propagated at 30ºC. The incubation temperature was raised to 39°C after a specific period of incubation in order to select for the recombinant viruses which were then passaged to MA104-g8D cells allowing the rapid isolation of the *ts*E/SA11g8R virus (Trask et al., 2010).

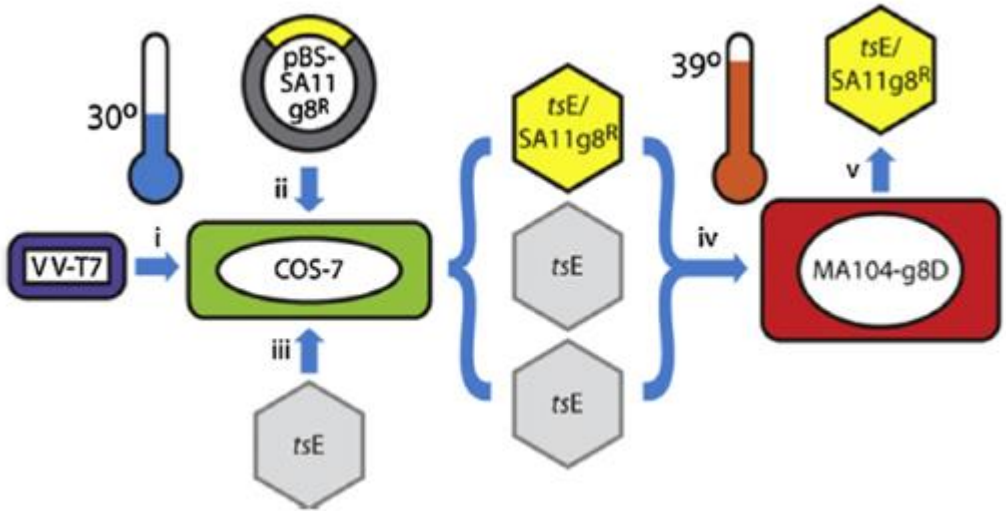



**Figure 1.10 Schematic illustration of the reverse genetics method of genome segment 8 using temperature sensitive RV strain as helper virus.** The Rescue of genome segment 8 is made possible by increasing the incubation temperature making the passage of selected recombinant viruses into MA104 cells possible. Figure adapted from Trask et al., 2010a

The cDNA plasmid was constructed with genome segment 8 having an authentic 5' end and an engineered RNAi target site to prevent the targeting of recombinant mRNAs. The authentic 3' end is generated by HDV ribozyme cleavage. Engineering partial gene duplications and heterologous cDNA sequences into the 3' region of NSP2 have been made possible by using this technique (Navarro et al., 2013). In an attempt to rescue viable RVs Richards and co-workers followed the protocol described by Boyce and co-workers, who rescued infectious bluetongue virus (Boyce et al., 2008) by co-transfecting 10 full-length (+)ssRNA transcripts as described in Section 1.10.2. However, Richards and co-workers were not able to rescue viable RV from the 11 full-length (+)ssRNA transcripts because the ssRNAs were not infectious and unable to be translated in cultured cells (Richards et al.,2013). Work performed to generate plasmid-based and transcript-

based RV RG systems made the incorporation of *in vivo* biotinylated VP6 into infectious RV particles possible (De Lorenzo et al., 2012).

In February 2017, a breakthrough in the RV RG world was made by Yuta Kanai and her research team at the Research Institute for Microbial Diseases of the Osaka University, Japan. Kanai et al reported the development of helper virus-free RV RG system, depicted in Figure 1.11 (Kanai et al., 2017). Kanai and co-workers constructed 11 plasmids which contained the full-length genome segments (1-11) of the SA11 L2 strain individually. The SA11 genome segments were flanked by a T7 RNA polymerase (T7Pol) promoter at the 5' end followed by the antigenomic HDV ribozyme at the 3' end. Plasmids were transfected into BHK cells expressing T7 polymerase thereby allowing exact full-length viral (+) ssRNA transcripts to be synthesised. As the transfection of cDNA plasmids did not work in the past as for BTV, RV replication was increased by the expression of a reovirus fusion-associated small transmembrane (FAST) protein. It had been found in a previous study that FAST proteins exponentially increased the yield of heterologous mammalian orthoreovirus (Ciechonska and Duncan, 2014). Plasmids expressing a vaccinia virus-capping enzyme were also constructed and transfected as it was found to increase translatability of reovirus (+) ssRNAs (Kobayashi et al., 2007). By expressing both FAST proteins and capping enzymes the efficiency of rescue of mammalian orthoreovirus was significantly improved and therefore applied to the development of a RV RG system.

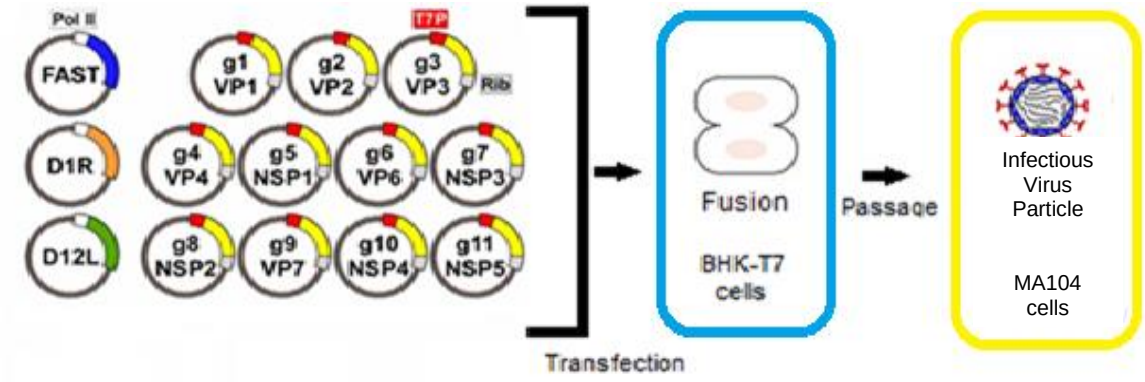



**Figure 1.11 Plasmid-based reverse genetic system for RV.** Expression plasmids are transfected into mammalian culture cells along with plasmids expressing FAST proteins and a vaccinia virus capping enzyme to generate infectious virus particles. Figure adapted from Kanai et al., 2012

Transfection of 11 cDNA plasmids together with plasmids expressing FAST proteins and vaccinia capping enzyme lead to the rescue of viable RV following passage of trypsin-activated lysates in

MA104 cells. Restriction recognition sites were engineered into the cDNAs to confirm the genuine rescue of RV SA11.

With the development of the RV RG system many unanswered questions may now be addressed including:

- The identification of the packaging signals of the RV RNA,
- The different receptor specificities of different RV strains
- Details of the interaction of viroplasms with lipid droplets
- The mechanisms controlling assortment and reassortment of 11 RNA segments in replication complexes (i.e. RNA/RNA interactions)
- The mechanisms controlling RV RNA packaging
- The formation and loss of temporary enveloped RV particles in the ER during the viral maturation process
- The potential of intestinal organoid cell cultures for the study of RV replication
- The factors (mutations) determining RV pathogenicity and virulence
- The molecular basis for host range and organ/cell specific viralreplication
- The significance of the different arms of the immune response inestablishing protection against RV disease
- The full identification of the correlates of protection against RV disease
- The factors determining RV spread
- The reasons for decreased efficacy of RV vaccines in reserve-strapped settings and areas of low socioeconomic conditions
- The further evolution of human RVs under the condition of universal mass vaccination against RV disease
- The development of alternative (non-live attenuated) RV vaccine candidates and the development of RV antivirals (Desselberger, 2014).

## 1.11 Problem identification, Aims and Objectives

### 1.11.1 Problem identification

At the start of this study in 2015, no true helper virus selection free reverse genetic system for rotavirus was available. Developing a RG system at the NWU will enable us to study the certain biological aspects of RV. Many elements of rotavirus biology and replication are not yet known such as the mechanisms for packaging, replication of the dsRNA genome and the cause(s) of pathogenicity. With a RV RG system, many of these issues can be addressed as RGs enable us to genetically manipulate viruses to create a rationally designed virus. This modification helps to better understand viral gene function and interaction with host cells. Reverse genetics is also a

powerful technique used in prevention and control of human viral diseases by means of vaccine development such as the influenza vaccine. No vaccines for African strains of rotavirus have yet been developed the development of RG system will enable us to do so.

### 1.11.2 Long-term aim

The long-term aim of this study is to develop a plasmid-based rotavirus reverse genetic system for advancement of research and the development of local rotavirus strain based vaccines.

### 1.11.3 Project-specific aims

The primary aim of this project was to construct a set of SA11 consensus sequence plasmids which produce exact (+)ssRNA copies of all 11 genome segments individually as a basis for a plasmid-only RV RG system. Apart from the rotavirus genome segments the components of the expression plasmid for plasmid-only reverse genetic system will be a T7 promoter region, HDV ribozyme region and a T7 termination region**.** The specific aims were:

- To correct a design flaw in 4 existing multigenome expression plasmids by removing three additional guanines at the 5' terminus of the genome segments.
- To design primers to amplify the 11 rotavirus SA11 genome segments from 4 multigenome expression plasmids
- To clone all 11 genome cassettes into pSMART using FastCloning and/or In-Fusion® HD cloning
- To sequence plasmid constructs to ensure that no mutations have been introduced
- To transfect expression plasmids into BHK-T7 and BSR- T7 cells

# CHAPTER 2: CONSTRUCTION OF A SET OF SA 11 EXPRESSION PLASMIDS

## 2.1 Introduction

As mentioned in Chapter 1 reverse genetics (RG) enables the manipulation of selected viral genomes at cDNA level allowing the generation of rationally designed viruses. Several projects towards the development of a RV RG system have been conducted by researchers at the North-West University (NWU).

Dr L. Mlera performed preparatory investigations for the development of a transcript-based RV RG system. He determined the whole genome consensus nucleotide sequence of the prototype rotavirus DS-1 and SA11 strains (Mlera et al., 2011). He also evaluated the effect of transfected RV transcripts obtained from *in vitro* transcription and studied the cellular innate immune response (Mlera, 2013).

Dr J.F. Wentzel investigated the importance of co-expressed RV proteins in the development of a selection-free RG system (Wentzel 2014). He optimised the transfection conditions for the expression of RV SA11 proteins from RNA transcripts and cDNA plasmids. He also evaluated the effect of RV transcripts on specific interferon pathways in the presence of various expressed rotavirus proteins. He found that apoptotic cell death occurred within 24 hours in cells only when transfected with *in vitro* derived RV transcripts and that cell death was reduced by the co-expression of NSP2 and NSP5 plasmids (Wentzel 2014)

The work performed by Dr Mlera and Dr Wentzel produced important information for future attempts at the development of a RV RG system at the NWU as both studies combined aspects of both a plasmid-based and transcript-based approach. Plasmid-based RG systems consist of cDNAs representing each of the 11 RV dsRNA genome segments. These segments are introduced into plasmids at sites flanked by a T7 promoter and the hepatitis delta virus (HDV) ribozyme. These recombinant plasmids are typically then transfected into mammalian cell cultures which support the replication of RV and viable viruses are then rescued from cultured cells. Comprehensive plasmid-based RG system for the prototype of the *Reoviridae* family, orthoreovirus was described in 2007 (Kobayashi et al., 2007).

For this M.Sc. project, a set of SA11 plasmids were developed to contain the cDNA corresponding with the individual 11 RV dsRNA genome segments of the SA11 strain producing exact (+)ssRNA copies. Plasmids were designed to have individual genome segments flanked by a T7 promoter at the 5' terminus and a HDV ribozyme at the 3' terminus followed by a T7 terminator. Multigenome expression plasmids Alpha, Beta, Gamma and Delta designed and constructed by Dr Wentzel were used as templates for the construction of the individual SA11 plasmids.

However, the genome sequences of the four multigenome expression plasmids contained a design flaw (due to a design issue) at the 5' terminus of each genome segment resulting in three extra guanines which had to be corrected. Figure 2.1 illustrates the design of the genome segment insert as well as the genome segment composition of the four multigenome expression plasmids Alpha, Beta, Gamma and Delta.

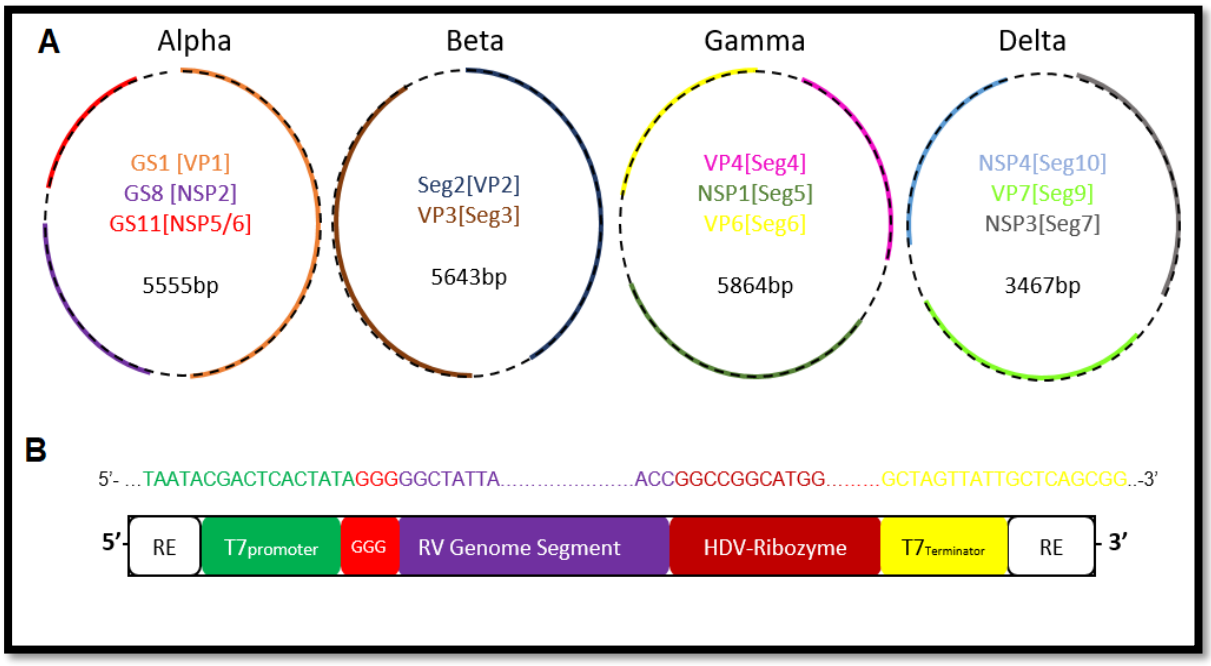



**Figure 2.1 Design of the multigenome expression plasmids.** (A) A schematic depiction of the four multigenome expression plasmids designed by Dr JF Wentzel for rotavirus reverse genetics used as template for this study. (B) Each genome segment was cloned into a pSMART LC vector flanked by the T7 promoter and the HDV ribozyme followed by the T7 terminator. The three extra Gs design flaw is indicated in red.

My M.Sc. project started in January 2015 when no selection-free RV RG had been developed. In February 2017 Kanai and co-workers published an article on their development of an entirely plasmid-based reverse genetic system for RV (Kanai et al., 2017). The system was successfully tested in September 2017 by Prof. A.C. Potgieter at Deltamune. The approach for the RV RG system described in my M.Sc. turned out to be fundamentally similar to the Japanese RV RG except for the additional fusion and capping enzymes (Kanai et al., 2017).

At the NWU our work towards a RV RG system uses the consensus sequence (CS) of the SA11 genome and pSMART-T7 vector. The SA11 CS used in this study differs slightly from the SA11-L2 strain sequence used by Kanai et al (2017), namely in genome segment 4 (VP4) (4%), genome segment 5 (NSP1) (3%) and genome segment 8 (NSP2) (79.79%). The construction of plasmids also differed slightly as the pSMART-T7 backbone vector we used (constructed from a pSMART HC plasmid with an AHSV S2(VP2) insert provided by Prof. A.C. Potgieter) was approximately 1050bp smaller resulting in a lower plasmid load and more plasmid per microgram being

transfected. The genome segments were also flanked with a different HDV ribozyme variant at the 3' terminus.

## 2.2 Materials and Methods

### 2.2.1 Primer design

For this study, 24 different primers were designed. In order to amplify the genome segments of interest, 11 primer pairs were designed to create 11 individual SA11 genome segment amplicons. Primers were designed to amplify inserts from the previously constructed SA11 multigenome expression plasmids Alpha, Beta, Gamma and Delta (Figure 2.1). The primers were designed to correct the design flaw of the three extra 5' guanines mentioned in the introduction. Each of these primers was also designed to have a 16+ nucleotide overhang homologous to that of a pSMART-T7 vector (Table 2.1). The pSMART HC vector plasmid used in this study contained a genome segment encoding African horsesickness virus VP2, constructed and provided by Prof. A.C. Potgieter, Deltamune. Primers for the vector plasmid were designed to amplify only the pSMART plasmid sequence and the T7 promoter sequence, HDV ribozyme and T7 terminator region (Figure 2.3)

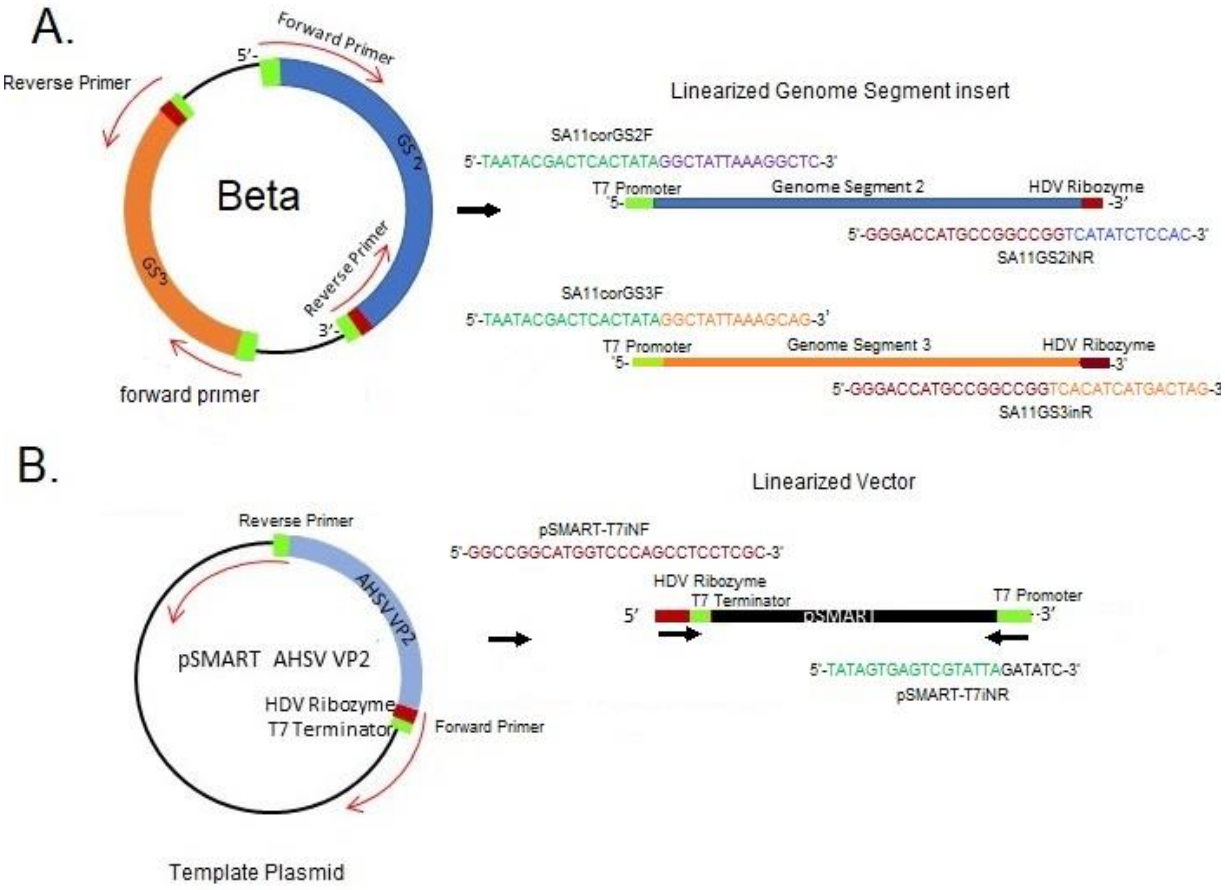



**Figure 2.2 Primer designs for the amplification of the RV genome segments and pSMART-T7 backbone** (A) Amplification of genome segments 2 and 3 from the multigenome expression plasmid Beta. (B) Preparation of pSMART-T7 backbone from the pSMART- AHSV-VP2.

Primers (Table 2.1) were designed using CLC Bio freeware. Primer designs were checked for predicted primer dimer formation using Snapgene software (www.snapgene.com). All primers were purchased from TIBMolBiol (Berlin, Germany). Stock solutions of the primers were prepared according to the manufacturer's instructions by dissolving lypophilised DNA in $dH_2O$ (Ion Torrent nuclease-free $H_2O$, ThermoFisher) to a final concentration of 100 µM.

**Table 2.1 Primers for the PCR amplification of SA11 genome segments and pSMART-T7 vector**

| Primer name | Sequence 5' – 3' | Length (bases) |
|---|---|---|
| SA11corGS1F | 5'-TAATACGACTCACTATAGGCTATTAAAGCTG-3' | 31 |
| SA11corGS2F | 5'-TAATACGACTCACTATAGGCTATTAAAGGCTC-3' | 32 |
| SA11corGS3F | 5'-TAATACGACTCACTATAGGCTATTAAAGCAG-3' | 30 |
| SA11corGS4F | 5'-TAATACGACTCACTATAGGCTATAAAATGGC-3' | 31 |
| SA11corGS5F | 5'-TAATACGACTCACTATAGGCTTTTTTTTTGAAAAG-3' | 34 |
| SA11corGS6F | 5'-TAATACGACTCACTATAGGCTTTTAAACGAAG-3' | 32 |
| SA11corGS7F | 5'-TAATACGACTCACTATAGGCATTTAATGC-3' | 29 |
| SA11corGS8F | 5'-TAATACGACTCACTATAGGCTTTTAAAGCG-3' | 30 |
| SA11corGS9F | 5'-TAATACGACTCACTATAGGCTTTAAAAAGAG-3' | 31 |
| SA11corGS10F | 5'-TAATACGACTCACTATAGGCTTTTAAAAGTTC-3' | 32 |
| SA11corGS11F | 5'-TAATACGACTCACTATAGCTTTTAAAGCGC-3' | 30 |
| pSMART-T7inF | 5'-GGCCGGCATGGTCCCAGCCTCCTCGC-3' | 26 |
| SA11GS1iNR | 5'-GGGACCATGCCGGCCGGTCACATCTAAGCG-3' | 30 |
| SA11GS2iNR | 5'-GGGACCATGCCGGCCGGTCATATCTCCAC-3' | 29 |
| SA11GS3iNR | 5'-GGGACCATGCCGGCCGGTCACATCATGACTAG-3' | 32 |
| SA11GS4iNR | 5'-GGGACCATGCCGGCCGGTCACATCCTCTAG-3' | 30 |
| SA11GS5iNR | 5'-GGGACCATGCCGGCCGGTCACATCCTCTAG-3' | 31 |
| SA11GS6iNR | 5'-GGGACCATGCCGGCCGGTCACATCCTCTCAC-3' | 31 |
| SA11GS7iNR | 5'-GGGACCATGCCGGCCGGCCACATAACGCCC-3' | 30 |
| SA11GS8iNR | 5'-GGGACCATGCCGGCCGGTCACATAAGCGC-3' | 29 |
| SA11GS9iNR | 5'-GGGACCATGCCGGCCGGTCACATCATACAATTC-3' | 33 |
| SA11GS10iNR | 5'-GGGACCATGCCGGCCGGTCACATTAAGACC-3' | 30 |
| SA11GS11iNR | 5'-GGGACCATGCCGGCCGGTCACAAAACGGGAG-3' | 30 |
| pSMART-T7inR | 5'-TATAGTGAGTCGTATTAGATATC-3' | 23 |

* T7 promoter sequence – green. Genome segment sequence – purple. HDV ribozyme sequence – red. pSMART sequence – blue

### 2.2.2 Transformation of *E. Cloni* ® 5-Alpha chemically competent cells

The transformation of the expression plasmids Alpha, Beta, Gamma and Delta, obtained from Dr J.F. Wentzel, was carried out using *E.cloni* 5-alpha chemically competent cells. The transformation procedures were carried out in accordance with the manufacturer's protocols. Before transformation nutrient agar plates containing 100 µg/ml ampicillin were prepared. In order

for the plasmid DNA added to be less than 5% of the total volume, 4 µl of DNA was added to an aliquot of 50 µl of *E.cloni*® 5-Alpha cells. The transformation mixture was then incubated on ice for 30 min. Incubation on ice was followed by heat shock carried out in a 42°C water bath for 45 seconds. Following heat shock, the transformation mixture was placed on ice for an incubation period of two min. After incubation 950 µl of pre-warmed recovery medium from the supplies was added to the transformation mixture which was then placed in a shaking incubator and incubated for one hour at 37°C and 250 rpm. A total of a 100 µl of the transformation mixture was spread out on pre-warmed agar plates containing 100 µg/ml ampicillin. Agar plates were then incubated overnight allowing colonies to grow at 37°C.

### 2.2.3 Extraction of plasmid DNA

#### 2.2.3.1 Miniplasmid preparation

The QIAprep Spin Miniprep kit (Qiagen, Whitehead Scientific) is based on alkaline lysis of bacteria cells which is followed by the absorption of DNA onto a silica membrane in the presence of high salt concentration. The miniprep procedure enables the rapid small-scale isolation of plasmid DNA from bacteria. The miniprep kit essentially follows a simple bind-wash-elute procedure and allows for the purification of plasmid DNA using centrifugation. The minipreparation procedure was carried out according to the miniprep protocol. Aliquots of 5 ml LB culture media, containing 100 µg/ml ampicillin, were inoculated with a single colony of the 4 transformed plasmids (Alpha, Beta, Gamma and Delta) picked from the agar plates which had been incubated for no longer than 18 hours. These aliquots were then incubated overnight with vigorous shaking (300 rpm) at 37°C. The total volume of overnight culture was then centrifuged at 11000 xg for 6 minutes to collect the bacterial cells. The supernatants were discarded and the pelleted cells were then resuspended in 250 µl of buffer P1 (kept at 4°C) containing RNaseA and LyseBlue reagent (1:1000). For cell lysis to occur 250 µl of Buffer P2 was added followed by the sample being inverted 6 times resulting in the solution turning blue. The cell lysis was followed by adding 350 µl of the neutralisation buffer N3 and inverting the mixtures 6 times resulting in the solutions turning colourless. The samples were then centrifuged at 13 000 xg in an Eppendorf 2J20 bench centrifuge for 10 min. After centrifugation, the supernatants were applied to the QIAprep spin columns by pipetting and then centrifuged for 60 seconds 13 000 xg. Spin columns were then washed with 750 µl of the wash buffer PE and 60 seconds of centrifugation at 13 000 xg. The wash buffer was then discarded and spin columns were centrifuged for 60 seconds at 13000 xg to remove residual wash buffer. QIAprep spin columns were then transferred to a 1.5 ml LoBind microcentrifuge tube. DNA was eluted with 50 µl of nuclease-free $H_2O$ (Ion torrent nuclease-free $H_2O$, ThermoFisher) added to the centre of the spin column. Samples were then left to stand for

one minute followed by one-minute centrifugation Plasmid DNA was then quantified, and the purity was determined using a NanoDrop® ND-1000 spectrophotometer. Quantifications were carried out according to the manufacturer's instructions. The purity of the samples was determined by the $OD_{260/280}$ nm. Pure DNA samples generally have a ratio of 1.8. Ratios lower than that of 1.8 indicates the presence of contaminants.

#### 2.2.3.2 Endotoxin-free plasmid DNA extraction and purification

Endotoxins, also known as lipopolysaccharides or LPS, are cell membrane components of Gram-negative bacteria (bacterial cells used in transformation protocols) possessing a hydrophobic, hydrophilic, and charged region. These unique features allow LPS to have various interactions with other molecules. When it comes to the transfection of DNA into mammalian cells increased levels of endotoxins reduce transfection efficiencies dramatically. As the plasmids containing the various genome segments of RV SA11 were used for transfection into mammalian cultured cells, all possible endotoxins had to be removed.

The EndoFree® Plasmid Maxi kit (Qiagen, Whitehead Scientific) is a purification procedure also based on alkaline lysis. During lysis, bacterial endotoxins are released and they are removed by various wash steps. Single bacterial colonies with plasmids containing the various SA11 genome segments were inoculated into 100 ml LB culture medium containing 100 µg/ml ampicillin and incubated with vigorous shaking (300rpm) at 37ºC overnight in a New-Brunswick shaking incubator. The cultures were then poured into 50 ml Falcon tubes, ThermoFisher Scientific, and centrifuged at 6000 xg at 4ºC for 15 min. The bacteria were then resuspended in 5 ml buffer P1, containing RNase A and LyseBlue reagent in a 1:1000 ratio, and then mixed to a final volume of 10 ml. Following resuspension 10 ml of lysis buffer P2 was added and mixed by inverting the tube 6 times and then incubated for 5 minutes at room temperature. The cell suspension turned blue due to the presence of the LyseBlue reagent. During incubation, the QIAfilter cartridges were prepared by screwing the caps onto the outlet nozzles and placing the cartridges into 50 ml Falcon tubes. Buffer P3 (chilled) was added to the lysate at a volume of 10 ml and mixed by inverting the sample 6 times. The lysate mixture was transferred to the QAIfilter cartridge and incubated for 10 minutes. After incubation, the caps were removed from the outlet nozzles and the plungers were inserted. The cell lysate was then filtered into a 50ml Falcon tube. Following filtration 2.5 ul Buffer ER was added to the filtered lysate and incubated on ice for 30 minutes. During incubation, the QAIGEN-tip 500 column was equilibrated with 10 ml buffer QBT allowing the column to empty by gravity flow. The filtered lysate was transferred to the QAIGEN-tip column and allowed to enter the resin by gravity flow. The flow-through was then discarded and QAIGEN-tip column was then washed with 60 ml Buffer QC (Qiagen, Whitehead Scientific). The QAI-tip was then placed in a 50 ml endotoxin free tube, and DNA was eluted in 15 ml buffer QN. DNA was then precipitated

by adding 10.5 ml isopropanol and centrifuged for 30 minutes at 15 000g. Following centrifugation, the supernatant was discarded carefully as not to remove the DNA pellet. The DNA pellet was washed with 5 ml endotoxin-free ethanol (70%) at RT and centrifuged for 10 minutes at 15 000g. The DNA pellet was allowed to air-dry for 5 – 10 minutes whereafter the DNA was redissolved in 100 ul TE buffer. The DNA product was then evaluated with the use of AGE (Section 2.2.5) and the yield and purity was determined with a Nanodrop® ND-1000 spectrophotometer.

### 2.2.4 PCR amplification

The pSMART-T7 vector was amplified from the AHSV2 plasmid provided by Prof. A.C. Potgieter (as mentioned in Section 2.1) and the SA11 genome segment inserts were all PCR amplified using the plasmid DNA Alpha, Beta, Gamma and Delta as template using appropriate primers described in section Section 2.1 using Phusion High-Fidelity DNA polymerase (Thermo Scientific). Phusion DNA polymerase has a unique structure with a novel *Pyrococcus*-like enzyme which is fused to a processivity-enhancing DNA binding domain. This unique structure increases speed and fidelity. Phusion High-Fidelity DNA polymerase is a thermostable polymerase and possesses 5'-3' polymerase activity with 3'-5' exonuclease activity and generates PCR products with blunt-ends. The PCR reactions were carried out in accordance with guidelines set out in the Phusion High-Fidelity DNA polymerase protocol of Thermo Scientific. The DNA concentration of the pSMART backbone and multigenome expression plasmids were above 100 ng/µl. Working solutions were prepared by diluting the pSMART and multigenome expression plasmid DNA to a final concentration of 10 ng/µl using nuclease-free $H_2O$. The PCR reactions were performed in a total volume of 25 µl and run on T100™ Thermal Cycler (Bio-Rad). The 25 µl reactions contained 5 µl 5X Phusion HF buffer, 200 µM 10 mM dNTPs, 10 pmol forward primer, 10 pmol reverse primer, 10 ng template DNA and 0.02 U/µl Phusion DNA polymerase. Unless otherwise indicated the PCR parameters were 95ºC for 5 min denaturation. (95ºC 30 sec, annealing temperatures as indicated in results between 50ºC and 70ºC 30 sec, extension at 72ºC 3 min) x 18 cycles, final extension at 72ºC 10 min. Samples were stored at 4ºC at the end of cycling.

### 2.2.5 Agarose gel electrophoresis

Amplicons were analysed using agarose gel electrophoresis (AGE). With the use of AGE nucleic acids can be separated based on their respective DNA/RNA bp sizes. This sieving matrix is formed as the heated agarose mixture cools down and the agarose polymers associate non-covalently allowing the gelatine structure to form pores through which DNA molecules migrate. Negatively charged DNA molecules are separated in AGE with the use of an electric field flowing

in a negative to positive direction. Due to the negatively charged nature of DNA, the DNA fragment will migrate to the positively charged anode when the current is applied. The rate of migration of a DNA molecule through a gel is, however, determined not only by the size of the molecule but also by the electrophoresis buffer and agarose concentration. The denser the agarose gel, the slower the migration of the DNA molecule. The conformation of the DNA molecule may also influence migration speed of DNA as well as the voltage of the electrical current applied and the presence of ethidium bromide. Supercoiled plasmid DNA, because of its compact conformation, moves through the gel fastest, followed by a linear DNA fragment of the same size, with the open circular form travelling the slowest. Agarose gels and accompanying buffers were made according to standard molecular procedures (Russel & Sambrook 2001). Tris-Acetate-EDTA (TAE) buffer and 1% TAE agarose gels were used in this study. Although TAE buffers have lower ionic strength and lower buffering capacity than TBE, TAE provides the best resolution for larger DNA fragments. Therefore, TAE buffers and gels were selected for this study. The dimensions of the gels used were 10 cm x 5 cm x 0.7 cm. The agarose powder was dissolved by boiling the mixture. The gel mixture was then cooled to approximately 60°C and ethidium bromide (EtBr) was added to 0.5 µg/ul. EtBr is the most commonly used DNA stain. EtBr releases energy in the form of light when exposed to UV light and also works by intercalating with the DNA molecule which not only makes it possible to visualise the DNA bands under UV light (302 nm) but also serves as an estimation of the amount of DNA based on the intensity of the DNA bands. Gels were cast at approximately 45–55°C and allowed to set at room temperature for 40 min. Gels were then placed in the electrophoresis tank containing TAE. The samples were mixed with 2 µl of loading dye (6X mass ruler DNA loading dye). The loading dye adds density to the DNA samples allowing the samples to sink into the well and also allows for the estimation of the DNA travel distance because loading dyes move at standard rates. 5 µl DNA standard (GeneRuler™ 1kb DNA Ladder) was used as a marker. It contains a mixture of pre-determined DNA fragment sizes for size comparison of the loaded DNA samples. The gel electrophoresis was performed in a BioRad system where the gel was then subjected to a constant voltage of 95 V for 1 hour.

### 2.2.6 Gel purification of PCR amplicons

Agarose gels are able to set at 30ºC and melt at 65ºC. Temperate range is far below the melting temperature of DNA and therefore make the recovery of DNA from agarose gels possible. The gel purifications in this study were carried out using the MiniElute®Gel extraction kit (Qiagen, Whitehead Scientific). All experimental procedures were carried out according to the manufacturer's protocol. Only samples used in InFusion®HD cloning reactions were gel purified as this is not a requirement for the FastCloning protocol. 20-25 µl of PCR product, mixed with 5 ul loading dye, was loaded onto a 1% TAE agarose gel and run at 90 V for 1 hour. The gel was

then observed on a dark reader. The amplicons were excised from the gel with a sterile sharp scalpel blade. The DNA segments were then placed into pre-weighed 2 ml RNase, DNase free microcentrifuge tubes. In order to determine the weight of the DNA the weight of the microcentrifuge tubes was measured again. QC buffer was added at a volume three times more than the weight of the agarose gel. The sample was then incubated in a heating block for 10 minutes at 50ºC until the gel slices were dissolved. Isopropanol was added at a volume equal to the gel weight, the sample was mixed by inverting. The sample was then transferred to separate MiniElute columns (stored at 4ºC) to bind DNA. The MiniElute columns were placed in collection tubes and then centrifuged for one min at 11 000 xg. The flow-through was then discarded and 500 µl of buffer QC was added and samples were centrifuged for 30 seconds at 11 000 xg. The membranes were then washed with 750 µl Buffer PE and centrifuged for 30 seconds at 11 000 xg. The DNA was eluted in buffer EB to a final volume of 20 µl.

### 2.2.7 Cloning reactions

RNA viruses such as rotavirus have multiple genome segments. To rescue viable virus cells must be transfected with constructs containing each genome segment expressing exact (+)ssRNA copies of the 11 genome segments individually to ensure correct packaging and replication. Plasmid constructs were generated using both FastCloning and In-Fusion®HD cloning. Both cloning methods are based on seamless cloning allowing the sequence-independent insertion of a DNA fragment into a selected plasmid vector with no extra base pairs added between the joined fragments.

#### 2.2.7.1 FastCloning

The FastCloning method was first published in 2011 by Li and co-workers (Li et al., 2011). FastCloning is a purification free, ligation-independent *in vivo* assembly cloning system based on *DpnI* digestion and the use of a High-Fidelity polymerase eliminating the need for enzymatic assembly. *DpnI* only cuts methylated DNA as *DpnI* requires methylation at the recognition sites for efficient cleavage of DNA to occur. PCR generates products with no methylation, *DpnI* digests only the methylated plasmid DNA template leaving the PCR product intact and decreasing the background for negative colonies. FastCloning does not require gel purification of the inserts and vector

FastCloning reactions were set-up in a total volume of 25 µl, according to the guidance of Dr C.P.S Badenhorst, in 200 µl PCR tubes. The restriction enzyme *DpnI* 10 U/µl (New England Biolabs, Inqaba Biotec) was added to both the vector and inserts separately after amplification and mixed thoroughly by pipetting. The insert mix was then added to the vector mix, vortexed to assure the sample mixture has covered the entire area of PCR tubes so that the entire template is digested during incubation at 37ºC for 2 hours. After the 2-hour incubation, the vector insert

mix was further incubated for 20 minutes at 80ºC for deactivation of the *DpnI* activity. The sample was then allowed to cool down before transformation into chemically competent Zymo Mix and Go® Dh5α cells. The FastCloning reactions for both amplified vector and inserts contained 19 µl PCR product and 1ul DpnI (10 U/µl). All incubation steps were carried out in a T100™ Thermal Cycler (Bio-Rad).

#### 2.2.7.2 InFusion® HD cloning

In-Fusion® HD cloning is a commercialised cloning method of the FastCloning procedure. In-Fusion is a directional and ligation-independent cloning method. This method allows for fast, single-tube cloning with high cloning accuracy and little to no background thereby increasing the chances of obtaining correct clones at every cloning reaction.

The InFusion® HD cloning reactions were set-up according to the manufacturer's (Clontech, Takara) specifications. For higher cloning efficiency 50-200 ng of gel-purified pSMART-T7 vector amplicon and SA11 genome segments inserts were mixed at 1:2 molar ratio. The reactions contained 100-200 ng template DNA, 2ul of HD enzyme premix and nuclease-free $H_2O$ to a final volume of 10 µl in a single PCR tube. The reaction mixtures were then incubated for 15 minutes at 50ºC. Following incubation, mixtures were put on ice and then transformed into competent DH5α cells. The transformation procedure for Zymo Mix & Go DH5α cells varies from the procedures described in Section 2.2.2. To keep the DNA to competent cell ratio less than 5%, 5 µl of cloning mixture was added to 30 µl of DH5α cells. Cells were then incubated on ice for five minutes after that 300 µl of pre-warmed Zymo™Broth was added to the cell mixture for cell recovery. A total volume of 100 µl was then spread out on LB agar plates pre-warmed to 37ºC containing 100µg/ml ampicillin. Spread out plates were then incubated overnight at 37ºC.

### 2.2.8 Restriction enzyme digestion

Restriction digestion reactions were performed as follows. The plasmid DNA was digested with restriction enzymes (*BamHI, EcoRV, PstI, NcoI* and *HindIII*) that recognize 6 consecutive corresponding nucleotides. Restriction enzymes for each construct were selected using CLC Bio software based on whether the enzymes had recognition sites in the inserts or vector. Constructs were linearised to determine the lengths of each construct. If the length of the various constructs corresponded with that of the theoretical sizes another digestion reaction was performed with enzymes with two or more recognition sites. Reactions were set-up in a total volume of 30 ul containing 5 ul of plasmid DNA (about 100 ng), 3ul of 10X NEB buffer and 2 ul of the 10 U/ul restriction enzyme. The reaction mixtures were then incubated for six hours at 37ºC in a BioRad T100™ Thermal cycler. Digested products were then analysed with AGE as described in Section 2.2.5.

### 2.2.9 DNA sequencing

The sequences of the SA11 constructs were verified with both Sanger and Next Generation Sequencing (NGS). Automated Sanger sequencing was performed at the Central DNA Sequencing facility (University of Stellenbosch) and was performed prior to NGS to check the 5' and 3' regions of the genome segments. NGS was performed at the NWU with the S5 instrument from Ion Torrent (ThermoFisher) Sequencing was performed with the Ion 540™-Chef Kit. The kit included all reagents and materials to prepare the chip with the Ion Chef™ system and then sequence the loaded 540 chip. The Ion Chef™System provides automated, high-throughput template preparation and chip loading for use with the Ion S5™ system. After a planned run with specific sequencing parameters was created in the Torrent suite, library samples were diluted, according to manufacturer's specifications, and reagents were allowed time to thaw. Following dilution, the sequencing chip was loaded into the Ion Chef along with the consumables, reagents and library. After completion of the chip preparation in the Ion Chef™ the chip was loaded into the Ion S5™ instrument and sequencing data was generated in the sequencing run. Sequencing was also performed at the National Institute for Communicable Diseases (NICD) with the Illumina MiSeq instrument. Sequence analyses was performed using the CLC Genomic workbench software.

### 2.2.10 Preparation of glycerol stocks of transformed bacterial cells

Overnight LB broth cultures with correct plasmid constructs of SA11 genome segments were stored in aliquots of 1 ml containing 15% glycerol. These stocks are stored at -80ºC for further use.

## 2.3 Results

### 2.3.1 Cloning strategy for the construction of the SA11 CS expression plasmids

In order to generate 11 plasmids containing individual SA11 genome segments, flanked by a T7 promoter and HDV ribozyme, the individual genome segments had to be synthesised and cloned into a backbone containing both the T7 promoter region as well as the HDV ribozyme region. The experimental approach taken to generate these plasmid constructs was using seamless cloning. One method was to use a published method, Fastcloning (Li et al., 2011) as implemented in the laboratory of Dr C.P.S. Badenhorst at the University of Greifswald, Germany. The other was a commercial kit, In-Fusion®HD cloning (Clonetech, Takara). Seamless cloning is a sequence-independent, simplified and efficient PCR-based cloning technique. This technique allows one to insert any DNA fragment into the desired vector at any desired cloning site. With FastCloning inserts and vectors are amplified separately using a high-fidelity DNA polymerase (Li et al., 2011). After amplification inserts and vectors are digested with *DpnI* and the transformed into chemically

competent cells. For the insert to be cloned into the vector, amplified inserts must have a 16+ base pair overhang corresponding to that of the vector. In-Fusion®HD (Clonetech, Separations) cloning, a cloning technique similar to FastCloning needs a 15 bp overhang and relies on the 3'-5' exonuclease activity of poxvirus DNA polymerase (Hamilton et al., 2007). Both FastCloning and In-Fusion®HD cloning techniques are ligation independent. Figure 2.3 illustrates the steps used in both FastCloning and In-Fusion HD cloning which was discussed in detail in Section 2.2.8.

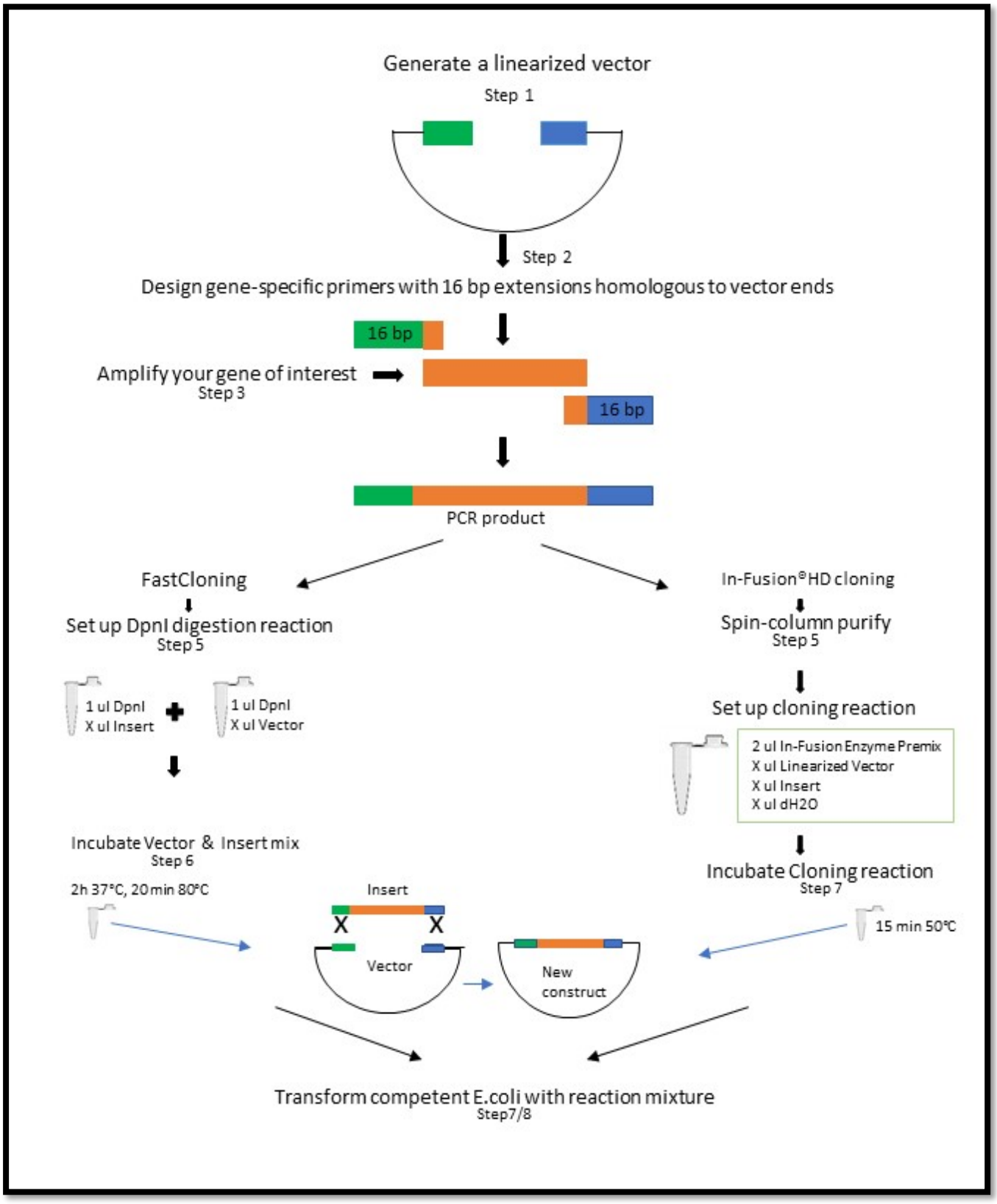



**Figure 2.3 Schematic representation of the In-Fusion®HD and FastCloning seamless cloning procedure.**

### 2.3.2 PCR amplification of SA 11 genome segment inserts and pSMART-T7 vector

#### 2.3.2.1 Temperature gradient for Alpha, Beta, Gamma, and Delta multigenome expression plasmids and pSMART-T7 backbone

For PCR reactions to be carried out the optimal annealing temperatures for the primers of the genome segments within the multigenome expression plasmids had to be determined. The selection of annealing temperature is a critical component for the optimisation of the PCR reaction. Optimal annealing temperature increases primer binding and reduces the generation of non-specific secondary amplicons. By performing a gradient PCR, the optimal annealing temperature could be empirically determined. The PCR temperature gradient was performed in a BioRad RT100 thermal cycler with a gradient function allowing the evaluation of 8 different annealing temperatures in a single run. Phusion®High Fidelity DNA polymerase was used in conjunction with the forward and reverse primers (Table 2.1) along with the template DNA from the four multigenome expression plasmids and the pSMART vector plasmid to generate linearised amplicons. Using the gradient function of the thermal cycler a gradient from 50ºC – 70ºC was set. The PCR parameters were denaturation at 95ºC for 5 min, extension (95ºC 30 sec, 50ºC-70ºC 30 sec, 72ºC 3 min) x 18 cycles, and elongation at 72ºC 10 min and 4ºC infinite. Linearized amplicons were evaluated with AGE (Section 2.2.5). Amplicon sizes were determined by using the GeneRuler™ 1kb DNA Ladder as a size marker reference. The GeneRuler™ ladder is comprised of a mixture of DNA fragments between 250 – 10 000 bp making it suitable for the evaluation of both the SA11 genome segment amplicons and pSMART-T7 amplicon. Genome segments within each multigenome expression plasmid were selected randomly as calculated annealing temperatures revealed little variation within the different genome segments except Alpha containing both genome segment 1 and genome segment 11 (varying 2000bp in size). Genome segment 1 was therefore selected. Therefore only 4 temperature gradients were performed instead of 11 temperature gradient PCR reactions. Primers with high melting point values can also be used in PCR reactions with a wide range of annealing temperature. The linearized pSMART-T7 vector amplicon size was expected to be 2024 bp, the expected sizes of the linearized genome segments from the multigenome expression plasmids were 3334 bp (Alpha Seg1), 2687 bp (Beta Seg2), 1350bp (Gamma Seg6) and 1057bp (Delta Seg9).

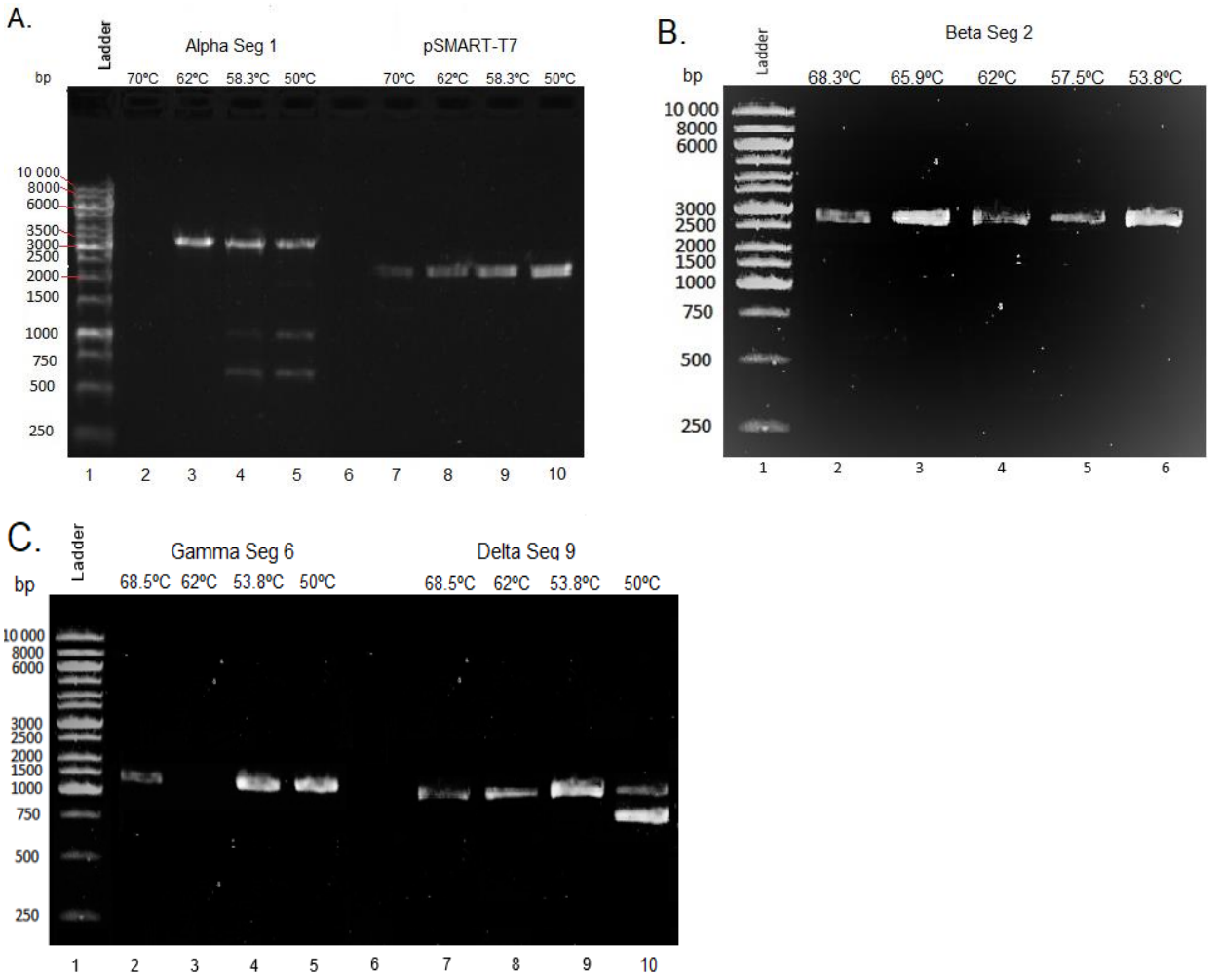



**Figure 2.4 Agarose gel analysis of amplicons generated from temperature gradient PCRs** (A) Amplicons generated from the Alpha expression plasmid (Lanes 2 to 5) and the pSMART-T7 vector amplicons generated from the AHSV2 plasmid (Lanes 7 to 10). Lane 6 was left empty. (B) Amplicons generated from the Beta expression plasmid (Lanes 2 to 6) (C) amplicons generated from the expression plasmids Gamma (Lanes 2 to 5) and Delta (Lanes 7 to 10). Lane 6 was left empty. Lane 1 contained the 1 Kb GeneRuler Ladder used as size reference.

The results depicted in Figure 2.4 A indicates the optimal annealing temperatures for Alpha and pSMART-T7. The optimal annealing temperature should produce a higher yield of amplicon and no non-specific amplicons. At 70ºC (Figure 2.4 A, Lane 2) there was no amplification for Alpha Seg 1 which could mean that an experimental pipetting error occurred or that the primers did not anneal and therefore amplification did not occur. At 62ºC (Figure 2.4 A, Lane 3) a high-density Alpha Seg 1 amplicon band with no non-specific bands could be visualised at approximately 3334 bp. At both 58.3ºC (Figure 2.4 A, Lane 4) and 50ºC (Figure 2.4 A, Lane 5) non-specific amplicons were observed at approximately a 1000bp and 500bp. The optimal annealing temperature for Alpha Seg 1 was then determined to be at 62ºC (Figure 2.4 A, Lane 3) due to a high-density amplicon product with no non-specific amplicons and a size of approximately 3334 bp which corresponds with the expected size of Alpha Seg 1. Al four annealing temperatures for pSMART-T7 produced a specific amplicon. However, the yields of the amplicons varied. At 70ºC (Figure

2.4 A, Lane 7) and 62ºC (Figure 2.4 A, Lane 8) a much lower amplicon yield was observed than at 58.3ºC (Figure 2.4 A, Lane 9) and 50ºC (Figure 2.4 A, Lane 10) with 70ºC having the lowest amplicon product yield. The highest amplicon product yield with an approximate size of 2024 bp was observed at 50ºC thereby determining the optimal annealing temperature for the pSMART-T7 vector to be 50ºC.

The optimal annealing temperature for multigenome expression plasmid Beta Seg 2 was determined using the AGE results indicated in Figure 2.4 B. Specific amplification was observed at all annealing temperatures. At 65.9ºC (Figure 2.4 B, Lane 3) and 53.8ºC (Figure 2.4 B, Lane 6) amplicon products with the highest yields were observed at almost double the amount than amplicons visualised at 68.3ºC (Figure 2.4 B, Lane 2), 62ºC (Figure 2.4 B, Lane 4) and 57.5ºC (Figure 2.4 B, Lane5). All amplicons were also approximately 1350 bp in size confirming the amplification of Seg 2. The optimal annealing temperature for Beta Seg 2 was then determined to be both 68.3ºC and 53.8ºC. As it is generally ideal to have annealing temperatures near the extension temperature (72ºC indicated in Section 2.2.4) 68.3ºC was used as the optimal annealing temperature.

The optimal annealing temperature for multigenome expression plasmid Gamma Seg 6 and Delta Seg 9 was determined using the AGE results indicated in Figure 2.4 C. At 68.5ºC (Figure 2.4 C, Lane 2) a low yield amplicon was observed. At 62ºC (Figure 2.4 C, Lane 3) no amplification was observed, this was unexpected, and the cause was attributed to experimental error. At both 53.8ºC (Figure 2.4 C, Lane 4) and 50ºC (Figure 2.4 C, Lane 5) amplicons with a high DNA yield were observed. All amplicons were approximately 1350 bp. The optimal annealing temperature for Gamma Seg 6 was then determined to be 53.8ºC or 50ºC. Delta Seg 9 revealed specific amplicons at 68.5ºC (Figure 2.4 C, Lane 7) 62ºC (Figure 2.4 C, Lane 8) and 53.8ºC (Figure 2.4 C, Lane 9), with amplification at 53.8ºC resulting in the amplicon with the highest DNA yield. At 50ºC non-specific amplicons resulted from PCR amplification. The optimal annealing temperature for Delta Seg 9 was thus determined to be 53.8ºC due to a higher amplicon yield and the amplicon size being approximately 1053 bp in length

With the optimal annealing temperatures of selected genome segments within the multigenome expression plasmids and the pSMART-T7 vector determined, the PCR amplification of the SA11 genome segments for seamless cloning could be performed.

#### 2.3.2.2 PCR amplification of the full set of SA 11 genome segments

To generate the full set of genome segment amplicons for seamless cloning, PCR reactions were carried out with the optimal annealing temperature of the genome segments within the

multigenome expression plasmids as determined in the temperature gradient PCR experiment. Phusion®High Fidelity DNA polymerase was again used in conjunction with the forward and reverse primers (Table 2.1) along with the template DNA from the four multigenome expression plasmids. Unless stated otherwise the PCR parameters were denaturation at 95ºC for 5 min, extension (95ºC 30 sec, [62ºC (Alpha), 65.9ºC (Beta) and 53.8ºC (Gamma and Delta), 30 sec], 72ºC 3 min) x 18 cycles, elongation at 72ºC for 10 min and samples were stored at 4ºC after cycling . Linearized amplicons (Figure 2.5) were visualised with AGE

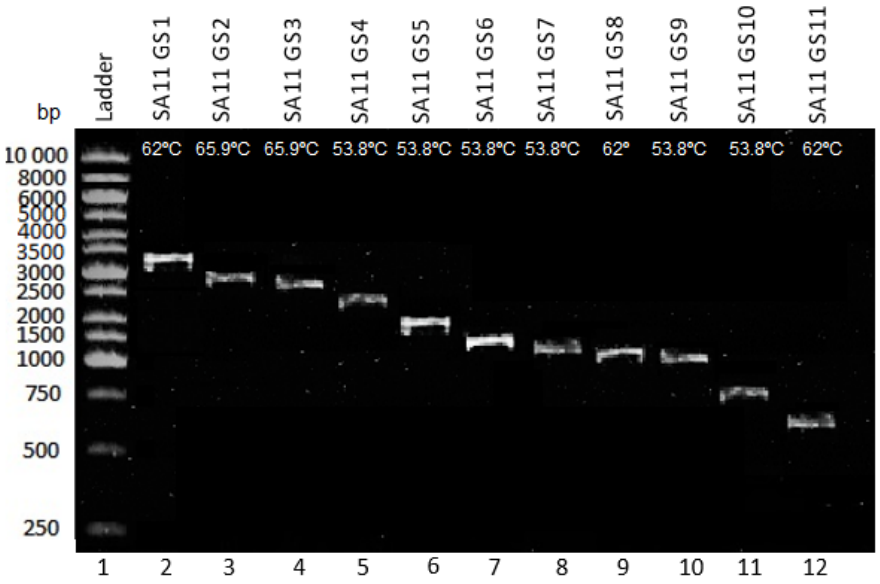



**Figure 2.5 Agarose gel analysis of SA11 genome segment insert amplicons generated from multigenome expression plasmids**. Lane 1 contained the GeneRuler™ 1kb DNA ladder. Lanes 2 – 12 contained SA 11 gene insert amplicons.

All 11 SA11 genome segments were amplified. All amplicons of the genome segments had the expected sizes: 3334 bp (GS1), 2687 bp (GS2), 2585 bp (GS3), 2356 bp (GS4), 1608 bp (GS5), 1350 bp (GS6), 1099 bp (GS7), 1053 bp (GS8), 1057 bp (GS9), 745 bp (GS10), 660 bp (GS11). It was thus concluded that the correct genome segments had been amplified. Inserts could then further be processed for plasmid construction with the use of FastCloning and In-Fusion®HD cloning.

### 2.3.3 Preparation of a set of expression plasmids for each of the rotavirus SA11 CS genome segments using seamless cloning.

Cloning reactions were performed using both FastCloning and In-Fusion® HD cloning. FastCloning reactions were carried out for the construction of the SA11 CS T-7 expression plasmids containing SA11 genome segments 1, 2, 3, 5, 6, and 10 as described in Section 2.2.7.1. In-Fusion reactions were carried out for the construction of the SA11 CS T-7 expression plasmids

containing SA11 genome segments 4, 7, 8, 9 and 11. FastCloning and In-Fusion products were transformed into chemically competent Zymo Mix and Go® DH5α cells. 50 ul and 100 ul transformed cells were spread out on agar plates for the FastCloning and InFusion transformations respectively. 20 Colonies out of approximately 80 – 100 (FastCloning) and 200+ (In-Fusion) colonies were picked from each agar plate and screened by DNA extraction with the use of the QAIprep® Spin Miniprep kit. Master plates containing the selected colonies were prepared and grown overnight at 37ºC in an incubator. After master plate colonies were incubated, the agar plates were sealed with parafilm and stored at 4ºC. Master plates are prepared to have access to correct screened colonies for further molecular applications such as the preparation of glycerol stocks or endotoxin-free plasmid purification. After DNA extraction DNA samples from selected colonies were analysed with AGE as described in Section 2.2.5. DNA samples resembling the expected sizes of the 11 SA11 constructs were then further analysed with the use of restriction enzyme digestion.

### 2.3.4 Restriction enzyme digestion for verification of SA11 constructs

To further screen for correct SA11 plasmids constructs restriction enzyme digestion was performed restriction enzymes were first used to linearise the constructs to determine whether the sizes correlated with that of the expected construct sizes. When the sizes of selected DNA samples corresponded with that of the expected sizes (bp) DNA samples were digested again in a new restriction reaction containing restriction enzymes having 2 or more recognition sites within the particular plasmid. Table 2.2 indicates selected restriction enzymes corresponding to the sequence of the SA11 plasmid constructs for both linearizations of the plasmids and digestion of plasmids into 2 or more segments. The expected sizes of the plasmids after digestion are also listed.

**Table 2.2 Restriction enzymes used for screening of the SA11 plasmid constructs**

| Construct | 1st Digestion (Linearization) | Expected length (bp) | 2nd Digestion | Expected Length (bp) |
|---|---|---|---|---|
| pSMART-T7 Seg 1 | BamHI | 3334 | EcoRV | 2792;1799;1050 |
| pSMART-T7 Seg 2 | BamHI | 2687 | EcoRV | 2066; 1799; 634 & 212 |
| pSMART-T7 Seg 3 | HindIII | 2585 | BamHI | 2907 & 1710 |
| pSMART-T7 Seg 4 | PstI | 2356 | EcoRV | 1799; 1785 & 796 |
| pSMART-T7 Seg 5 | BamHI | 1608 | EcoRV | 1799; 1013 & 820 |
| pSMART-T7 Seg 6 | BamHI | 1350 | EcoRV | 1799 & 1575 |
| pSMART-T7 Seg 7 | PstI | 1099 | EcoRV | 1799; 911 & 413 |
| pSMART-T7 Seg 8 | BamHI | 1053 | EcoRV | 1799 & 911 |
| pSMART-T7 Seg 9 | NcoI | 1057 | BamHI | 2208 & 970 |
| pSMART-T7 Seg 10 | PstI | 745 | EcoRV | 1799 & 970 |
| pSMART-T7 Seg 11 | BamHI | 660 | EcoRV | 1799 & 885 |

Restriction enzymes *BamHI, NcoI, Pst*I and *HindIII* were selected for the linearization of the indicated plasmids (Table 2.2) since there is only one recognition site in the plasmids.

The restriction enzymes *BamHI* (pSMART-T7 Seg 3 and 9) and *EcoRV* (pSMART-T7 Seg 1, 2, 4, 5, 6, 7, 8, 10 and 11) were used for the second digestion. Both restriction enzymes had two or more recognition sites within the indicated plasmids (Table 2.2). The restriction enzyme digestion reactions resulted in plasmids being cut at 2 or more sites within the plasmids leading to 2 or more plasmid DNA fragments, as listed in Table 2.2. Restriction digestion fragments were analysed using AGE as described in Section 2.1.5 and depicted in Figure 2.5.

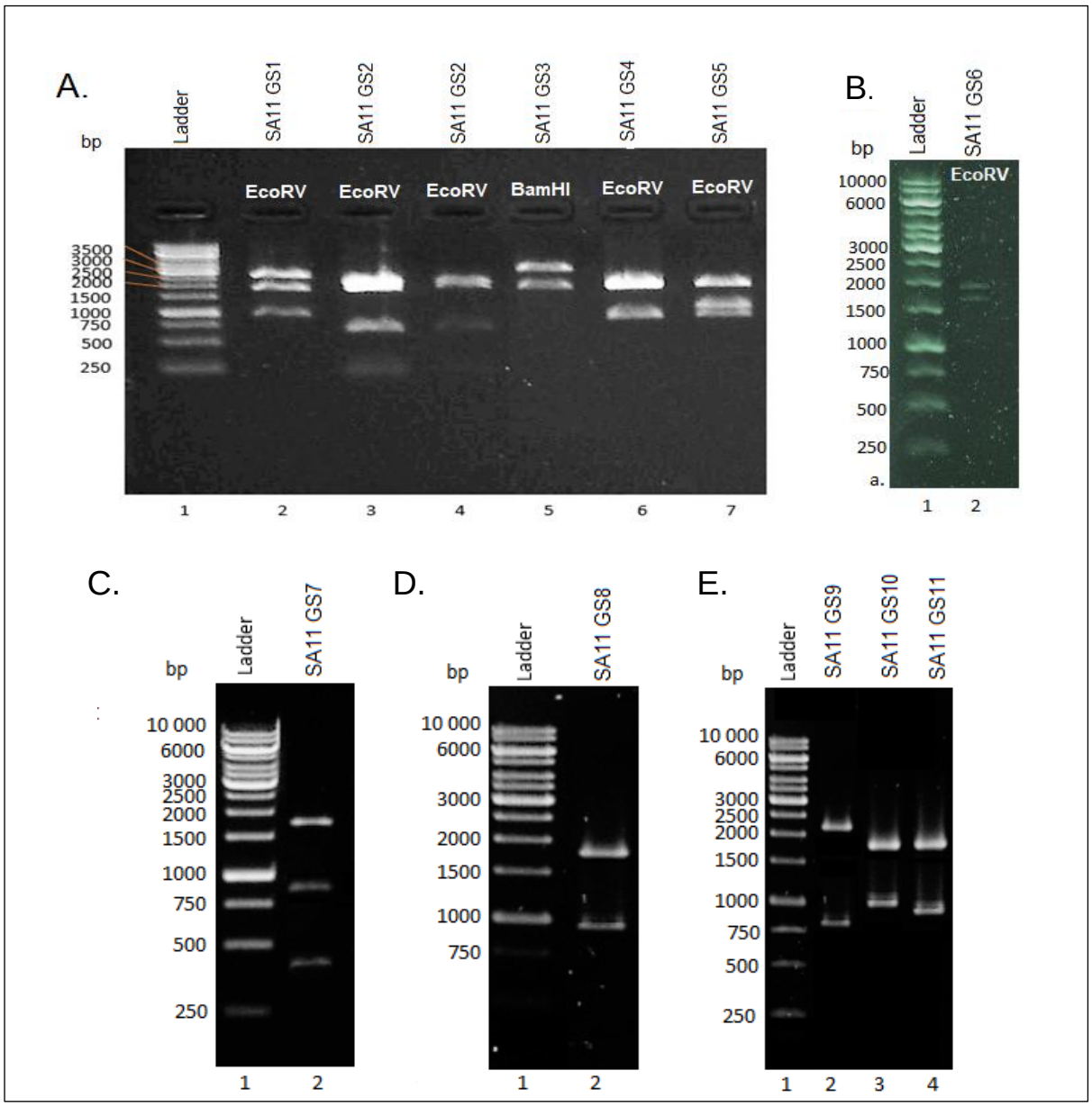



**Figure 2.6 Agarose gel analysis of the restriction patterns of the SA11 plasmid constructs** (A) pSMART-T7 Seg 1 to 5 (B) pSMART-T7 Seg6 (C) pSMART-T7 Seg7. (D) pSMART-T7 Seg8. (E) pSMART-T7 Seg 9 to 11. Lane 1 contained the GeneRuler™ 1kb DNA ladder (Thermo Fisher, Thermo Scientific. Plasmid constructs were digested with indicated restriction plasmids (Table 2.2)

The restriction digestion results of newly generated SA11 CS expression plasmids corresponded to the expected sizes for all 11 SA11 plasmids as listed in Table 2.2. Thus, it was concluded that a full set of SA11 expression plasmids containing the individual CS genome segments were constructed. The SA11 plasmids were then plasmid purified using an endotoxin-free plasmid purification kit using the colonies from the master plates. Purified plasmid samples were then prepared for next generation sequencing.

### 2.3.5 DNA sequencing

After confirmation of constructs, samples were prepared to be sequenced as described in Section 2.2.11. Sequence analysis was performed with CLC Genomic workbench. Constructs were screened for unwanted mutations using the concensus sequence (CS) SA11 plasmid construct sequences as a reference.

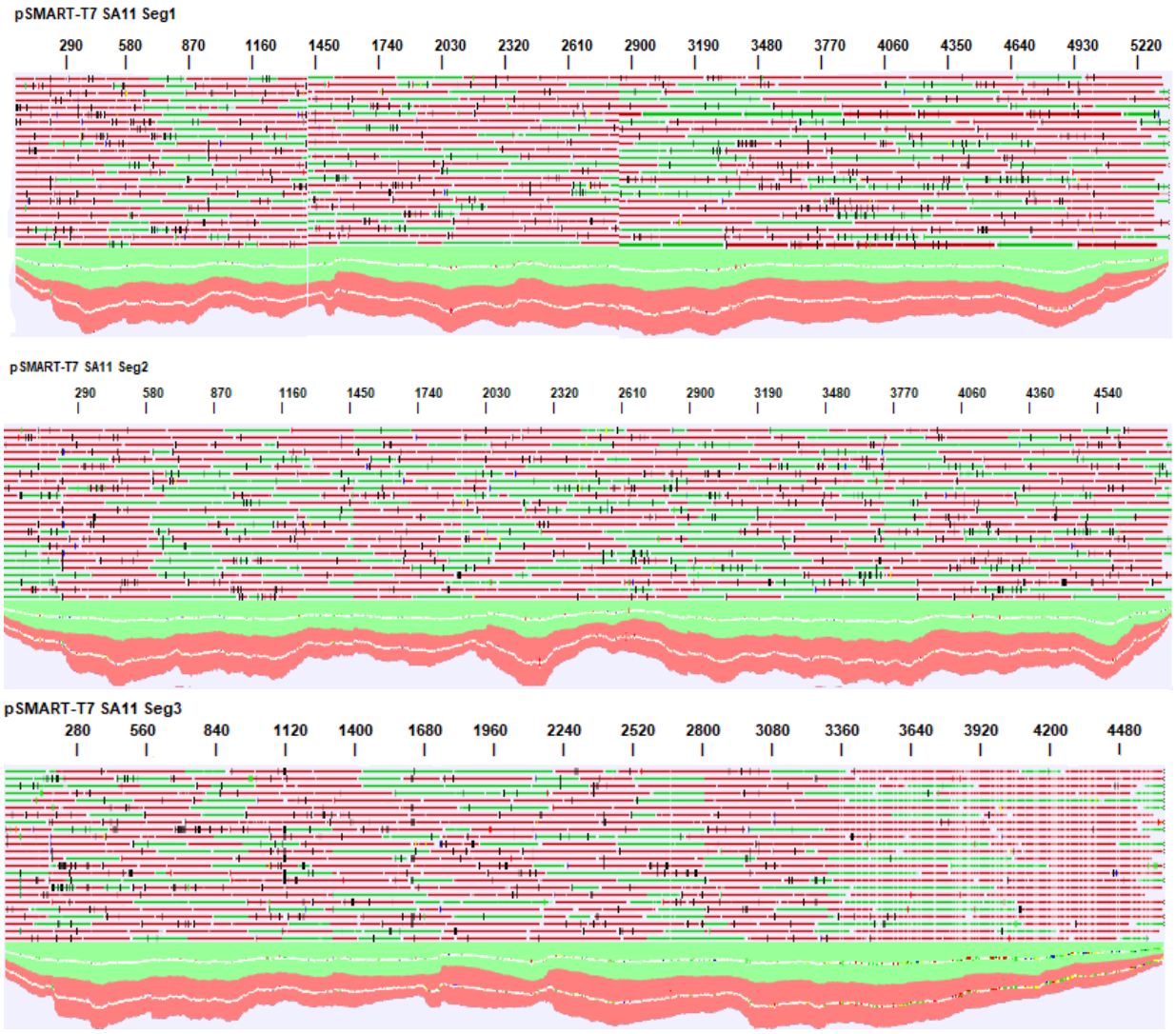



**Figure 2.7 Representation of the coverage pattern from mapping sequence reads of the pSMART-T7 SA11 CS expression plasmids 1 to 3** Representation of the coverage pattern of the pSMART-T7 plasmids containing CS genome segment 1 to 3 sequences. The green pattern below the reads indicate the coverage pattern of the forward reads, whereas the reverse read coverage can be observed from the red pattern. Sequenced reads were mapped against plasmid construct reference sequences

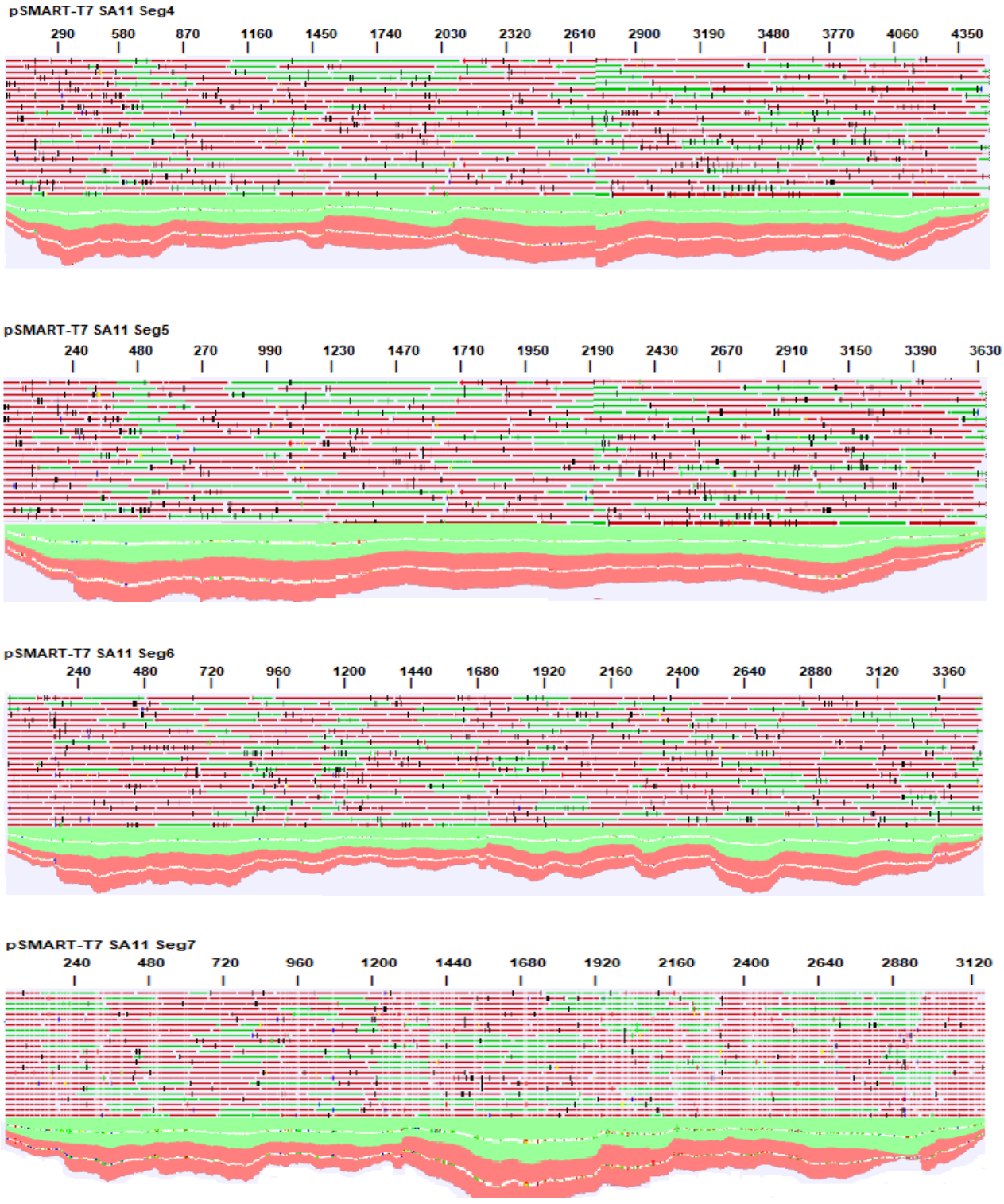



**Figure 2.8 Representation of the coverage pattern from mapping sequence reads of the pSMART-T7 SA11 CS expression plasmids 4 to 7.** Representation of the coverage pattern of the pSMART-T7 plasmids containing CS genome segment 4 to 7 sequences. The green pattern below the reads indicate the coverage pattern of the forward reads, whereas the reverse read coverage can be observed from the red pattern. Sequenced reads were mapped against CS plasmid construct reference sequences.

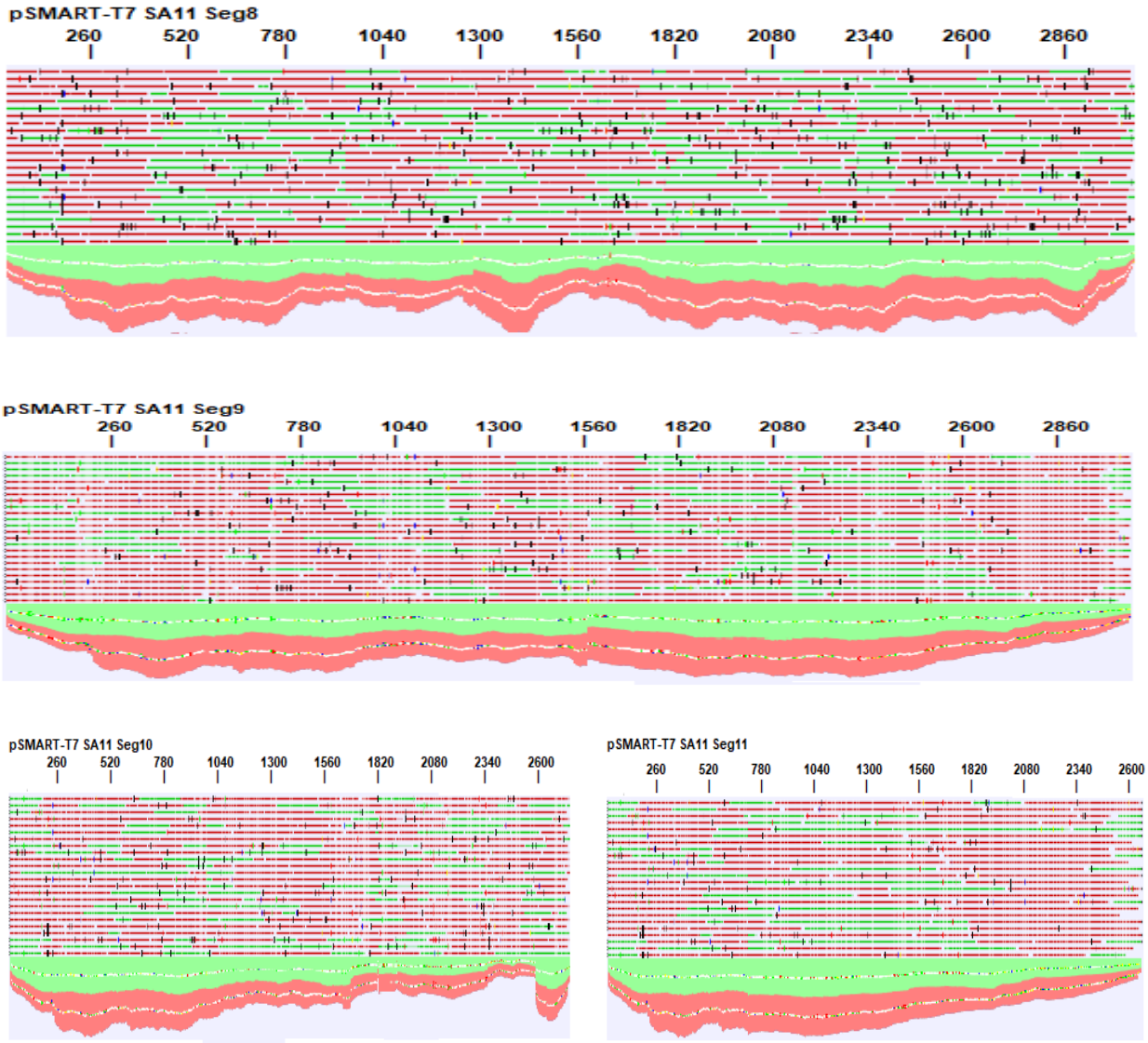



**Figure 2.9 Representation of the coverage pattern from mapping sequence reads of the pSMART-T7 SA11 CS expression plasmids 8 to 11.** Representation of the coverage pattern of the pSMART-T7 plasmids containing CS genome segments 8 to 11 sequences. The green pattern below the reads indicate the coverage pattern of the forward reads, whereas the reverse read coverage can be observed from the red pattern. Sequenced reads were mapped against CS plasmid construct reference sequences.

The mapping and coverage patterns achieved reveal that the constructed pSMART-T7 SA11 CS plasmids matches up to the theoretical plasmid construct reference sequences. The CS reference plasmid constructs were constructed using DNAman software (Lynnon, Biosoft), by inserting the SA11 CS sequences into the pSMART-T7 backbone lacking the AHSV genome segment 2 sequence. Each SA11 genome segment therefore replaced the AHSV VP2 sequence to form the 11 reference sequences used for mapping of the constructed SA11 plasmids. These results confirmed that the 11 constructed SA11 CS plasmids indeed contained each genome segment flanked with a T7 promoter, HDV ribozyme and T7 terminator sequence. In addition the 5' and 3' termini were correct and had no possible mutations.

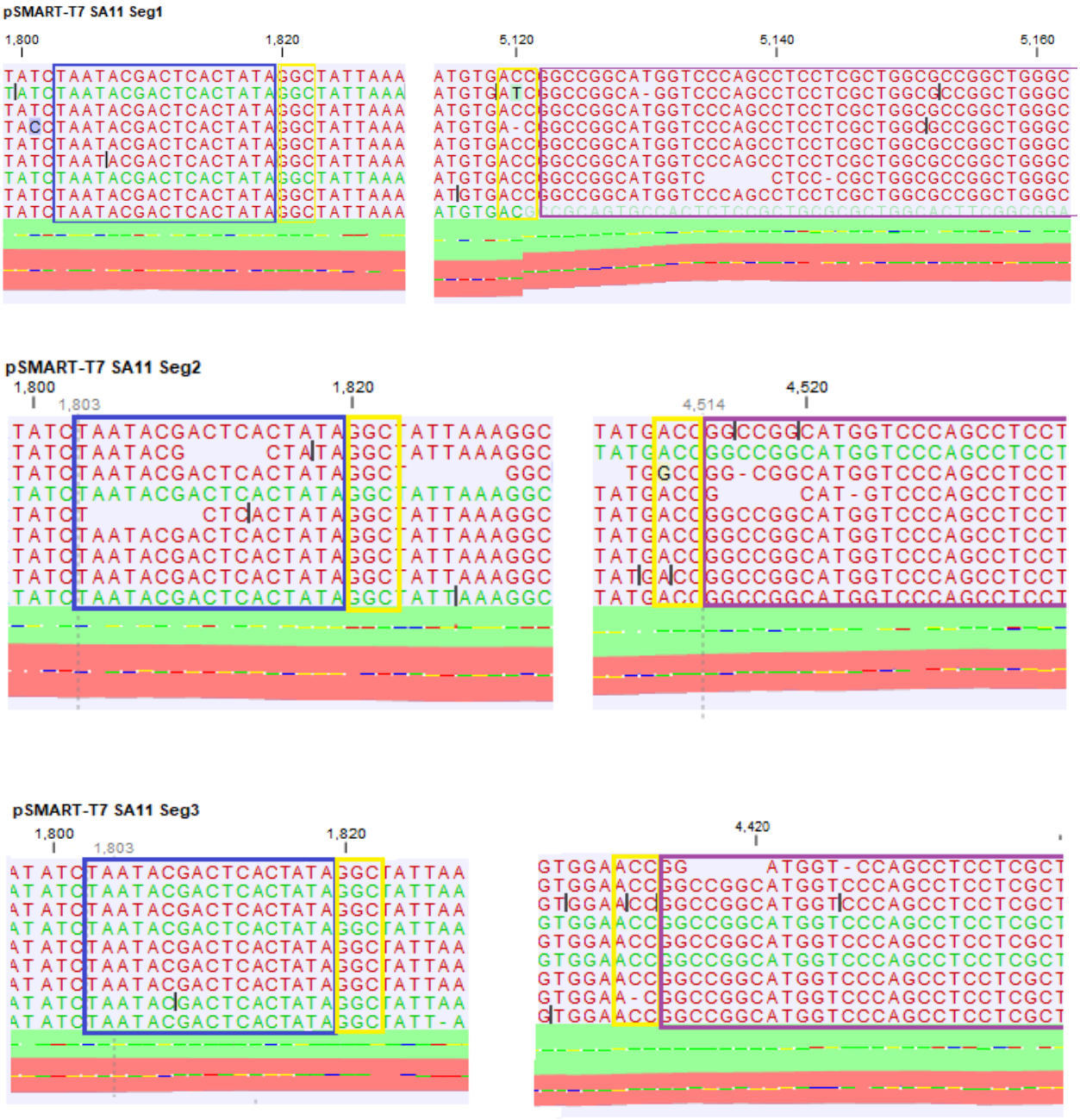



**Figure 2.10 NGS data analysis of the 5' and 3' termini of the SA11 CS plasmid constructs 1 to 3.** NGS data analysis of the 5’ and 3’ termini of pSMART-T7 SA11 Seg 1 to 3. T7 promoter sequences are highlighted in the blue boxes, 5’ and 3’ sequences are highlighted in yellow boxes and the HDV ribozyme sequences are highlighted in purple

S

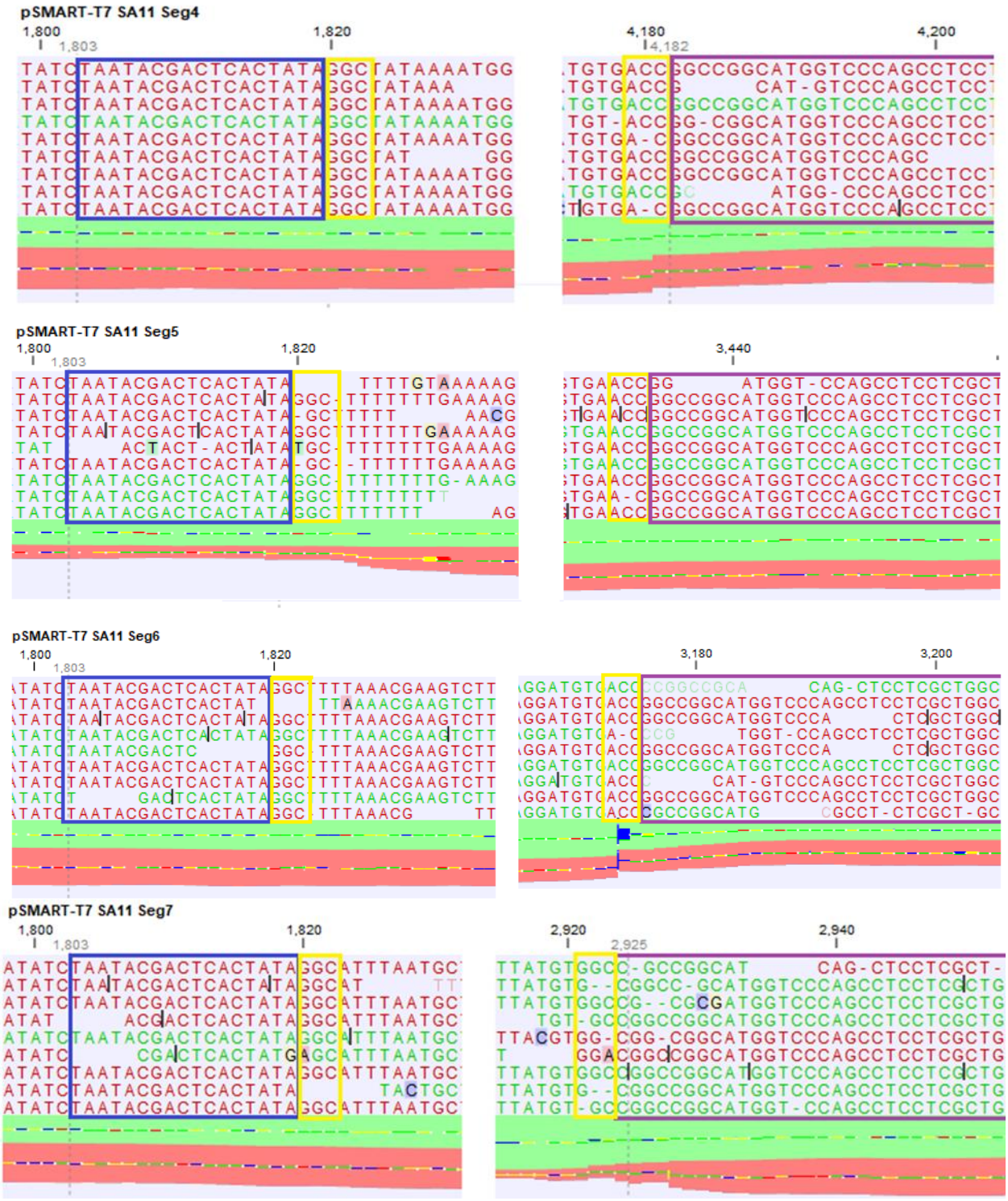



**Figure 2.11 NGS data analysis of the 5' and 3' termini of the SA11 CS plasmid constructs 4 to 7**. NGS data analysis of the 5’ and 3’ termini of pSMART-T7 SA11 Seg 4 to 7. T7 promoter sequences are highlighted in the blue boxes, 5’ and 3’ sequences are highlighted in yellow boxes and the HDV ribozyme sequences are highlighted in purple

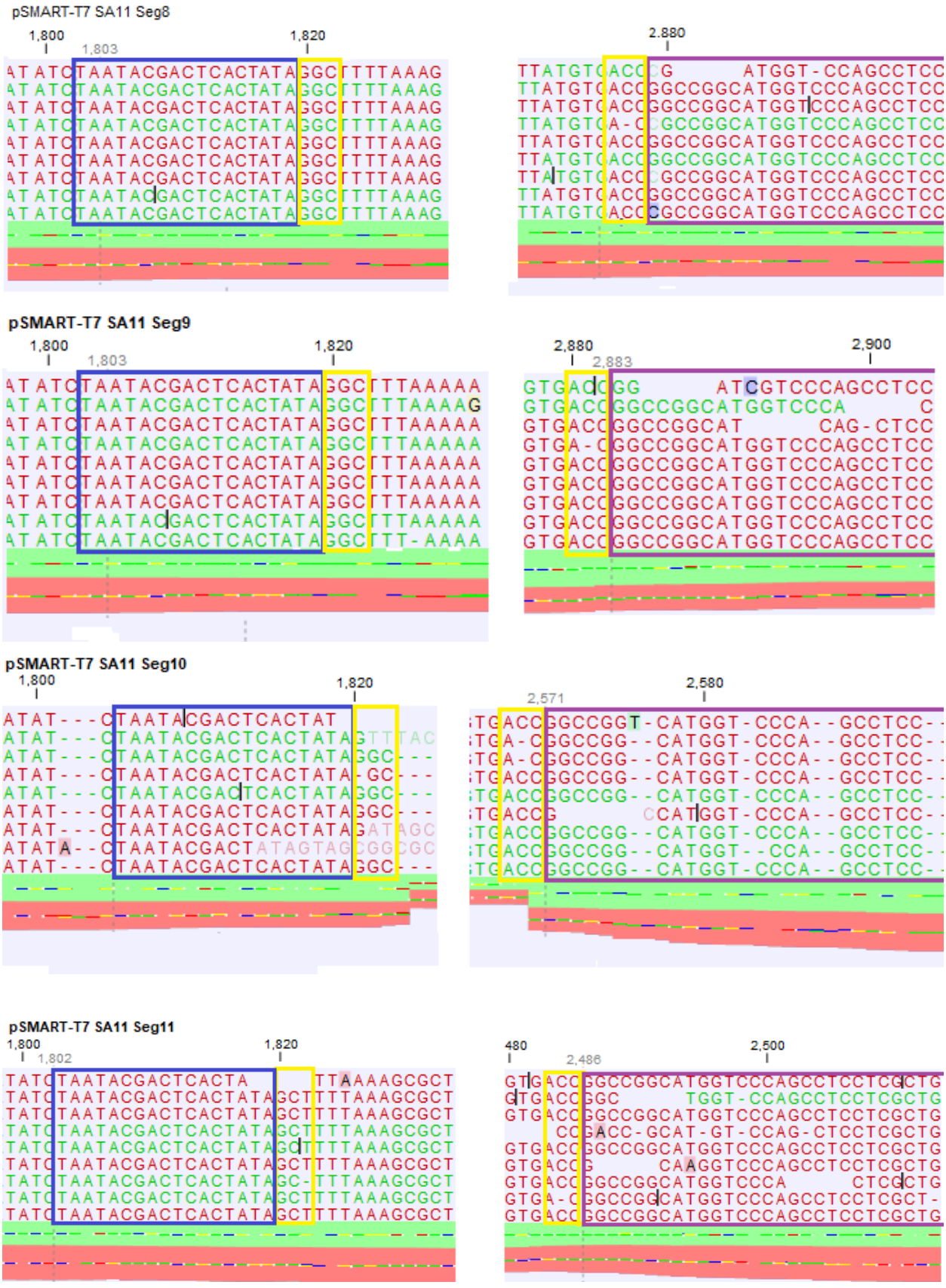



**Figure 2.12 NGS data analysis of the 5' and 3' termini of the SA11 CS plasmid constructs 8 to 11**. NGS data analysis of the 5’ and 3’ termini of pSMART-T7 SA11 Seg 8 to 11. T7 promoter sequences are highlighted in the blue boxes, 5’ and 3’ sequences are highlighted in yellow boxes and the HDV ribozyme sequences are highlighted in purple

With the 5’ and 3’ termini (Figure2.7) indicating that no mutations were introduced thereby remaining conserved, the generation of exact (+)ssRNA is possible, making the constructed SA11 CS plasmids suitable for transfection experiments.The next chapter will focus on the transfection of the SA11 CS expression plasmids in cultured mammalian cells.

## 2.4 Summary

Primers were designed for the PCR amplification of all 11 SA11 genome segments and linearization of the pSMART-T7 backbone vector. Each genome segment was cloned into the pSMART-T7 vector with seamless FastCloning and In-FusionHD cloning methods in order to generate the 11 SA11 expression plasmids. Expression plasmids therefore contained cDNA corresponding to the consensus SA11 sequence. The Genome segments were flanked by a T7 polymerase promoter at the 5’ position followed by a HDV ribozyme and T7 terminator sequence at the 3’ end. Plasmids were analyzed by restriction enzyme digestion and AGE. After restriction enzyme digestion was performed correct plasmid constructs were sequenced by both automated Sanger sequencing at the Central DNA Sequencing facility (University of Stellenbosch) and next generation sequencing performed at the NWU using S5 instrument, as well as the National Institute for Communal Diseases (NICD) with the Illumina MiSeq instrument. Sequence data was compared to the reference sequence data with CLC Genomics workbench. All sequences were characterised with no additional base pairs introduced during cloning and the design flaw of the three extra guanines was corrected. Following to the construction of the correct SA11 expression plasmids subsequent experiments could be performed. All 11 plasmids were purified with endotoxin-free plasmid purification kit for transfection into mammalian cell culture which will be described in Chapter 3.

# CHAPTER 3: TRANSFECTION OF THE CONSTRUCTED SA11 CS TRANSCRIPTION PLASMIDS AND EVALUATION OF TRANSFECTION

## 3.1 Introduction

As mentioned in Chapter 1, one of the most definitive ways with which to study the roles of specific sequences in viral genomes is to modify them and to generate infectious virus, that is, to 'rescue' the virus, with these modified sequences. Therefore, the 11 SA11 CS expression plasmids corresponding the 11 genome segments were constructed as described in Chapter 2. Rescuing dsRNA viruses is more complex especially those with multiple genome segments. To rescue virus, mammalian cultured cells are transfected with the 11 SA11 CS expression plasmids containing the 11 genome segments.

cDNA plasmids are transfected into mammalian cultures expressing the T7 RNA polymerase. As mentioned in Chapter 1, RG systems rely on site-specific initiation of transcription by T7 RNA polymerase to generate the authentic 5' end of recombinant RNA segments. Transcription is halted by the T7 polymerase terminator sequence thereby producing exact (+)ssRNA. The authentic 3' end of each segment is generated by the HDV ribozyme and. The transcribed (+)ssRNA then serves as a template for viral expression and translation resulting in the formation of recombinant infectious virus particles.

The RG system developed for BTV demonstrated that BTV utilises a primary and secondary (two-stage) replication cycle (Boyce et al., 2008). Therefore, an increase in viral recovery efficiency is reached when transcripts needed for the assembly of the replication complex is transfected first, followed by the transfection of the 10 genome segments. Formation of empty virions is prevented by the lack of VP2, VP5 and NS3 during the initial transfection (Ratinier et al., 2011). Establishment of a BTV reverse genetic system lead to the development of a reverse genetic system for AHSV which was first reported by Matsuo and Roy in 2009 (Matsuo and Roy, 2009). The reverse genetic system for AHSV also utilises a two-stage replication cycle where the primary replication is achieved by transfection of AHSV core proteins (VP1, VP3, VP4, VP6 and VP7) and non-structural proteins (NS1 and NS2) (Matsuo et al., 2010). Secondary replication is achieved by the transfection of the individual 10 genome segments capped (+)ssRNAs.

Kanai and co-workers introduced two important modifications during the development of a RG system for RV. These modifications were to increase nonfusogenic RV replication that in turn leads to enhanced recombinant virus recovery. They speculated that FAST proteins (Fusogenic orthoreovirus proteins), due to their ability to promote viral replication and pathogenesis, could accelerate the replication of Rotavirus (Kanai et al., 20017). To increase translation Kanai and

co-workers added plasmids that express the Vaccinia Virus (VV) capping enzyme to the transfection reaction since transcripts synthesised from T7 polymerase rescue plasmids are not capped and thus poorly translated. The VV capping enzyme consists of two subunits D1R and D12L. The co-expression of plasmids encoding the FAST protein and the VV capping enzyme subunits increased the yield of transcription and improved the RV rescue efficiency (Kanai et al., 2017)

The experimental approach in this study was to transfect the 11 SA11 CS plasmid constructs into BHK-T7 and BSR-T7 cells along with polymerase II promotor (Pol II)-driven expression plasmids encoding FAST and the D1R and D12L capping enzymes. Co-transfection of SA11 CS plasmids with codon-optimised (CO) expression plasmids was also performed in an attempt to produce a rotavirus replication scaffold. The CO expression plasmids were designed by GenScript for the experimental purposes of Dr J.F. Wentzel during his PhD study (Wentzel 2014).

Seven codon-optimised (OptimumGene™, GenScript) reading frames, encoding for the replication complex (VP1, VP2, VP3 and VP6), viroplasm-like structure (NSP2and NSP5) and innate immune response suppression protein (NSP1) were designed for the expression in MA104 cells and placed under the control of a CMV promoter. The individual SA11 codon optimised gene segments were cloned into a phCMVDream expression plasmid and flanked by *BsmBI* restriction sites (Wentzel 2014). Only six of the original eight constructed plasmids were used in this study.

## 3.2 Materials and method

### 3.2.1 Plasmids

The 11 SA11 CS expression plasmids used in this study were synthesised as described in Chapter 2 and are listed in Table 3.1. Details regarding the CO optimised expression plasmids and plasmids expressing FAST proteins and VV capping enzymes are listed in Table 3.2 and Table 3.3, respectively.

**Table 3.1 Expression plasmids used in the attempt to rescue RV SA11**

| Name of plasmid | Genome segment | Virus | Plasmid | 5'end flanking sequence | 3' end flanking sequence |
|---|---|---|---|---|---|
| pSMART-T7 SA11 Seg1 | 1 | VP1 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11Seg2 | 2 | VP2 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg3 | 3 | VP3 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg4 | 4 | VP4 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg5 | 5 | NSP1 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg6 | 6 | VP6 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11Seg7 | 7 | NSP3 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg8 | 8 | NSP2 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg9 | 9 | VP7 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg10 | 10 | NSP4 | pSMART | T7 promotor[a] | HDV ribozyme[b] |
| pSMART-T7 SA11 Seg11 | 11 | NSP5/6 | pSMART | T7 promotor[a] | HDV ribozyme[b] |

[a]T7 promotor sequence: TAATACGACTCACTATA

[b]HDV ribozyme sequence: GGCCGGCATGGTCCCAGCCTCCTCGCTGGCGCCGGCTGGGCAACATT CCGTAGGGGA

**Table 3.2 Codon-optimised expression plasmids constructed by GenScript**

| Name of plasmids | Genome segment | Virus | Plasmid | 5' and 3' flanking sequence | Promotor |
|---|---|---|---|---|---|
| VP1 CO | 1 | VP1 | phCMVDream | BsmBI | CMV |
| VP2 CO | 2 | VP2 | phCMVDream | BsmBI | CMV |
| VP3 CO | 3 | VP3 | phCMVDream | BsmBI | CMV |
| VP6 CO | 6 | VP6 | phCMVDream | BsmBI | CMV |
| NSP1 CO | 5 | NSP1 | phCMVDream | BsmBI | CMV |
| NSP2 CO | 8 | NSP2 | phCMVDream | BsmBI | CMV |

**Table 3.3 Plasmids expressing FAST proteins and VV capping enzymes**

| Name of plasmids | Expressing | Plasmid | Cloning site | Promotor |
|---|---|---|---|---|
| pCAG-FAST | Fusogenic orthoreovirus proteins | phCMVDream | NcoI | Pol II |
| pCAG-D1R | VV capping enzyme subunit D1R | phCMVDream | NcoI | Pol II |
| pCAG-D12L | VV capping enzyme subunit D12L | phCMVDream | NcoI | Pol II |

### 3.2.2 Mammalian cell lines used for transfection

The mammalian cell lines, BSR-T7 and BHK-T7 (provided by Deltamune (Pty) Ltd) were chosen for this study as they stably express T7 RNA polymerase. Epithelial monkey kidney (MA104) cells were also selected as RV SA11 propagates well in MA104 cells. The BHK-T7 cells were developed by integrating the T7 RNA polymerase gene into the chromosome of a stable baby hamster kidney (BHK-21) cell. Therefore allowing constitutively cytoplasmic T7 RNA polymerase expression (Zheng et al., 2009). BSR-T7 cells are referred to as a clone of BHK-21 cells, although the origin of these cells is not clear (Buchholz et al., 1999). Both BHK-T7 and BSR-T7 cells were grown in 25 $cm^2$ tissue culture flasks as monolayers. Both cell lines were cultured routinely in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAX™ which contained 5% foetal bovine serum (FBS) and 1X Anti-Anti Gibco (antibiotic/antimycotic) mixture (penicillin 10 000 U/ml, 10 000 µg/ml streptomycin, 25 µg/ml fungizone). The tissue culture flasks were incubated in a humidified tissue culture (TC) incubator (ESCO) at 37ºC with a constant supply of 5% CO2.

### 3.2.3 Maintenance of T7 expression in BHK-T7 and BST-T7 cells

In order to maintain the expression of the T7 polymerase, BHK-T7 cells were supplemented with 5 %Hygromycin (50mg/ml) after every tenth passage. BSR-T7 cells were supplemented with 10% Geneticin (150 mg/ml). Cultured cells were allowed to reach a confluency of 80 – 100 % where after the supernatant was removed followed by rinsing the monolayer twice with tissue culture PBS. The cell monolayer was then rinsed with 0.05% trypsin-EDTA, most of the trypsin -EDTA was then removed leaving only a minimal (enough to cover monolayer cells) volume trypsin-EDTA. The cultured cells were then incubated for a 2-minute period in a humidified incubator at

37ºC. Detachment of the cells is facilitated by the incubation at 37ºC. Following incubation, the detached cells were resuspended in an appropriate volume of DMEM plus GlutaMAX™ containing 5 % FBS and then transferred to new tissue culture flasks (25 $cm^2$). Cells were split at 1:6 ratio and maintained in 25 $cm^2$ flasks.

### 3.2.4 Equimolar transfection of SA11 CS expression plasmids into BHK-T7 cells

BHK-T7 cells were grown to a confluency of 80% in a 25 $cm^2$ flask. Once confluency was reached the medium of the BHK-T7 was removed and changed to OPTI-MEM®I Reduced serum medium (Gibco, Life Technologies). BHK-T7 were co-transfected with a total of 10 µg DNA of transfection plasmids (Table 3-1) in equimolar amounts using 2ul of TranslT-LT1 transfection reagent per µg plasmid DNA used. 0.8 µg of each capping enzyme sub-unit plasmid D1R and D12L was added per 2.2 µg of transfection mix and 0.015 µg of the FAST phusion plasmid was added at a concentration of 0.015 µg per 2.2 µg DNA.

**Table 3.4 Calculated amounts of the SA11 CS transfected plasmids in BHK-T7 cells per 25 $cm^2$**

| SA 11 CS expression plasmids | Amount |
|---|---|
| pSMART-T7 SA11 Seg1 | 1308.27 ng |
| pSMART-T7 SA11Seg2 | 1158.74 ng |
| pSMART-T7 SA11 Seg3 | 1133.37 ng |
| pSMART-T7 SA11 Seg4 | 1077.12 ng |
| pSMART-T7 SA11 Seg5 | 893.20 ng |
| pSMART-T7 SA11 Seg6 | 829.3 ng |
| pSMART-T7 SA11Seg7 | 768.1 ng |
| pSMART-T7 SA11 Seg8 | 733.1 ng |
| pSMART-T7 SA11 Seg9 | 757.7 ng |
| pSMART-T7 SA11 Seg10 | 681.0 ng |
| pSMART-T7 SA11 Seg11 | 660.1 ng |
| **Total** | **10 000 ng** |

**Table 3.5 Amounts of FAST and capping enzyme expression plasmids added to BSR-T7 transfection mix in 25$cm^2$ flask**

| FAST and Capping Enzyme Plasmids | Amount |
|---|---|
| pCAG-FAST | 41.0 ng |
| pCAG-D12L | 2200.0 ng |
| pCAG-D1R | 2200.0 ng |
| **Total** | **4441.0 ng** |

Approximately 24 hours after the transfection, BHK-T7 cells were seeded with MA104 cells. Both BHK-T7 and MA104 cells were trypsinated and incubated at 37ºC allowing cells to detach from the cell culture flasks. Both BHK-T7 cells and MA104 cells were then resuspended in an appropriate amount of DMEM containing 5% FBS. Resuspended cells were then added together

in a volume ratio of 1:1 and 2:1 (BHK-T7: MA104) and transferred to 25cm$^2$ culture flasks. The transfected BHK-T7 and MA104 cells were co-cultured for approximately 24 hours whereafter the culture medium was replaced with medium containing 0.5 µg/ml porcine trypsin (Sigma) and incubated for 48 hours. After incubation cells were lysed by freeze/thaw at -20ºC for 30 min three times. Lysates containing possible rescued rotavirus were then activated by adding 1µg/ml porcine trypsin to the lysate followed by a 30 min incubation period at 37ºC. The activated lysate was added to a monolayer of naive MA104 cells and incubated in DMEM containing 1µg/ml porcine trypsin and non-essential amino acids for one hour. Following adsorption, the MA104 cells were then washed three times with PBS and cultured for three days in medium containing non-essential amino acids and 0.5µg/ml porcine trypsin. Monolayers were monitored for typical cytopathic effect (CPE) as an indication of the presence of rescued virus. pGFP plasmid was used as a positive control for transfection and expression.

### 3.2.5 Transfection of BSR-T7 cells

BSR-T7 cells were grown in 25cm$^2$ flasks to a confluency of 80% whereafter the BSR-T7 monolayer was transfected with 7 µg SA11 CS expression plasmid and 3µreplication complex and viroplasm CO expression plasmids (Table 3.6) in equimolar amounts, using 2.5 µl Lipofectamine™2000 (Invitrogen, Life Technologies) per ug transfected DNA in OPTI-MEM®I Reduced serum medium (Gibco, Life Technologies). The BSR-T7 cells were also transfected with 2.2 µg capping enzyme expression plasmids D1R and D12L as well as 0.041 µg of the pCAG-FAST (Table 3.7) expression plasmid (Kanai et al., 2017). The plasmid mixture and Lipofectamine™2000 was added to 250µl OPTI-MEM®I Reduced serum medium separately and incubated at room temperature for 5 min. Following the 5 min incubation period the plasmid mixture was added to the Lipofectamine™2000 in OPTI-MEM®I Reduced serum medium and incubated for 20 min at room temperature. The transfection mixture was added in a dropwise fashion to the BSR-T7 monolayer and incubated at 37°C for 24 h.

**Table 3.6 Calculated equimolar amounts of the SA11 transfected plasmids in BSR-T7 cells per 25 cm$^2$**

| Transfection plasmid | Amount |
|---|---|
| pSMART-T7 SA11 Seg1 | 915,8 ng |
| pSMART-T7 SA11Seg2 | 811,1 ng |
| pSMART-T7 SA11 Seg3 | 793,4 ng |
| pSMART-T7 SA11 Seg4 | 754.0 ng |

| | |
|---|---|
| pSMART-T7 SA11 Seg5 | 625,2 ng |
| pSMART-T7 SA11 Seg6 | 580,5 ng |
| pSMART-T7 SA11Seg7 | 537,6 ng |
| pSMART-T7 SA11 Seg8 | 513,2 ng |
| pSMART-T7 SA11 Seg9 | 530,4 ng |
| pSMART-T7 SA11 Seg10 | 476,7 ng |
| pSMART-T7 SA11 Seg11 | 462,1 ng |
| **Total:** | **7 000 ng** |
| **Expression plasmid** | **Amount** |
| VP1 CO | 1182,8 ng |
| VP2 CO | 543,6 ng |
| VP3 CO | 516,7 ng |
| VP6 CO | 249,1 ng |
| NSP1 CO | 307,5 ng |
| NSP2 CO | 200,2 ng |
| **Total** | **3000 ug** |
| **Total** | **10 000 ug** |

**Table 3.7 Amounts of FAST and capping enzyme expression plasmids added to BSR-T7 transfection mix in 25cm$^2$ flask**

| FAST and Capping Enzyme Plasmids | Amount |
|---|---|
| pCAG-FAST | 41.0 ng |
| pCAG-D12L | 2200.0 ng |
| pCAG-D1R | 2200.0 ng |
| **Total** | **4441.0 ng** |

Approximately 24 hours after transfection, BSR-T7 cells were seeded with MA104 cells. Both BSR-T7 and MA104 cells were trypsinated and incubated at 37ºC allowing cells to detach from the cell culture flasks. Both BSR-T7 cells and MA104 cells were then resuspended in an appropriate amount of DMEM containing 5% FBS. Resuspended cells were then added together in a 1:1 volume ratio and transferred to 25cm$^2$ culture flasks. The transfected BSR-T7 and MA104 cells were co-cultured for approximately 24 hours and the culture medium was replaced with medium containing 0.5 µg/ml porcine trypsin (#T5266,Sigma-Aldrich (Merk)) and incubated for 48 hours. After incubation cells were lysed by three freeze/thaw cycles at -20ºC for 30 min. The lysates containing possible rescued rotavirus were then activated by adding 1µg/ml porcine trypsin to the lysed cells followed by a 30 min incubation period at 37ºC. Activated lysates were added to a fresh monolayer MA104 cells and incubated in DMEM containing 1µg/ml porcine trypsin and non-essential amino acids for 1 hour. Following adsorption, the MA104 cells were washed three times with PBS and cultured for three days in medium containing non-essential amino acids and 0.5µg/ml porcine trypsin. Monolayers were monitored for typical cytopathic effect

(CPE) as an indication of the presence of any rescued rotavirus. pGFP was used as a positive control for transfection and virus expression.

### 3.2.6 Equimicrogram transfection of SA11 CS expression plasmids in BHK-T7 Cells

BHK-T7 cells were grown and seeded as discribed in Section 3.2.5). BHK-T7 sells were then transfected with 2.5 µg of each of the SA11 CS expression plasmids (Table 3.8), together with 2.2 µg capping enzyme expression plasmids (D1R and D12L) and 0.041 µg of the FAST protein expression plasmid (Kanai et al., 2017). Transfection was carried out as described in Section 3.2.5

**Table 3.8 Calculated amounts of SA11 expression plasmids in equal microgram amount per 25 cm$^2$**

| SA11 CS Expression plasmids | Amount |
|---|---|
| pSMART-T7 SA11 Seg1 | 2.5 µg |
| pSMART-T7 SA11Seg2 | 2.5 µg |
| pSMART-T7 SA11 Seg3 | 2.5 µg |
| pSMART-T7 SA11 Seg4 | 2.5 µg |
| pSMART-T7 SA11 Seg5 | 2.5 µg |
| pSMART-T7 SA11 Seg6 | 2.5 µg |
| pSMART-T7 SA11Seg7 | 2.5 µg |
| pSMART-T7 SA11 Seg8 | 2.5 µg |
| pSMART-T7 SA11 Seg9 | 2.5 µg |
| pSMART-T7 SA11 Seg10 | 2.5 µg |
| pT7-NSP5SA11 (Kanai et al., 2017) | 2.5 µg |
| **Total:** | **27.5 ng** |

**Table 3.9 Amounts of FAST and capping enzyme expression plasmids transfected into BHK-T7 cells**

| FAST and Capping Enzyme Plasmids | Amount |
|---|---|
| pCAG-FAST | 41.0 ng |
| pCAG-D12L | 2200.0 ng |
| pCAG-D1R | 2200.0 ng |
| **Total** | **4441.0 ng** |

Approximately 24 hours after the transfection, BHK-T7 cells were co-cultured with MA104 cells as described as before, but cells were mixed at a 2:1 ratio. The transfected BHK-T7 and MA104 cells were co-cultured as described in section 3.2.5. Monolayers were monitored for typical cytopathic effect (CPE).

### 3.2.7 Immunofluorescent monolayer assay (IFMA)

The immunofluorescent monolayer assay (IFMA) was used to detect the expression of viral proteins of RV SA11. Infected monolayers were fixed with an organic solvent methanol/acetone solution at a 1:1 ratio for 20 minutes at -20ºC. Organic solvent fixation, such as methanol/acetone, does not require a permeabilisation step as it removes lipids in the cell membrane, dehydrates the cell and precipitates the proteins on the cellular structure. Monolayers were blocked with a 1x PBS 0.05 % Tween-20 solution containing 1% tryptone and incubated for 30 min at 37ºC. The blocking solution was then removed, and the monolayer was incubated with a 1x PBS 0.05 % Tween solution containing 1% Tryptone and RV immune rabbit serum diluted at 1:2000 for 30 min at 37ºC. The antibody solution was removed. The cell monolayer was then washed three times with the 1x PBS 0.05% Tween-20 solution. The cell monolayer was incubated for 30 minutes at 37ºC with a PBS 0.05% Tween20 solution containing 1% Tryptone and 1:2000 goat anti-rabbit conjugate (Alexa Fluor™488 goat anti-rabbit IgG[H+L]) (Invitrogen, Thermo Fisher Scientific) provided by Prof. A.C. Potgieter, Deltamune. Fluorescent staining was visualised with an Eclipse TE2000-S microscope. MA104 cells were transfected with RV SA11 as positive control after all other cells were harvested to prevent contamination.

## 3.3 Results

To rescue infectious virus particles, plasmids containing cDNA corresponding to the RV genome segments were transfected into BSR-T7 and BHK-T7 cells.

### 3.3.1 Evaluation of RV rescue after transfection of equimolar amount of expression plasmids into BHK-T7 cells

A 25 $cm^2$ flask with confluent BHK-T7 cells grown in monolayer (Figure 3.1: A) was transfected with an equimolar mix of SA11 CS expression plasmids, plasmids expressing VV capping enzyme subunits (D1R and D12L) and FAST protein expression plasmid pCAG-FAST

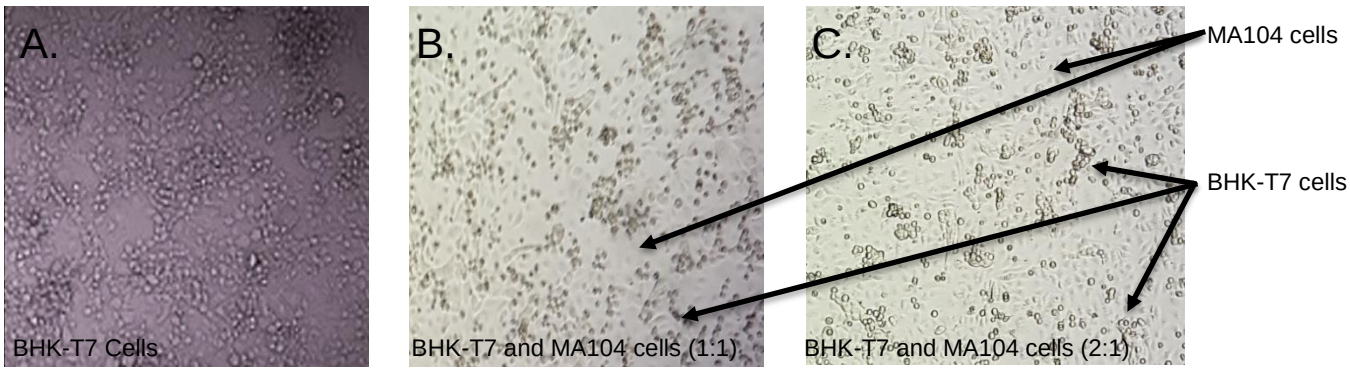



**Figure 3.1 "Image of cells transfected with RV SA11 CS plasmids** (A) BHK-T7 cells before transfection. (B) BHK-T7 sell co-seeded with MA104 cells at a 1:1 ratio. (C) BHK-T7 cells co-seeded with MA104 cells at a 2:1 ratio.

At 24 hours of transfection with the SA11 CS plasmids, transfected BHK-T7 cells were still attached to the culture flask and no cell death was observed (Figure 3.1: A). BHK-T7 cells were trypsinised and co-seeded with MA104 cells in 25 $cm^2$ flasks in a 1:1 (Figure 3.1: B ) and 2:1 (Figure 3.1: C) volume ratio and incubated at 37ºC for 24 hours. At 24 hours post incubation co-seeded MA104 cells and BHK-T7 cells were still attached to the culture flask (Figure 3.1 A and B).

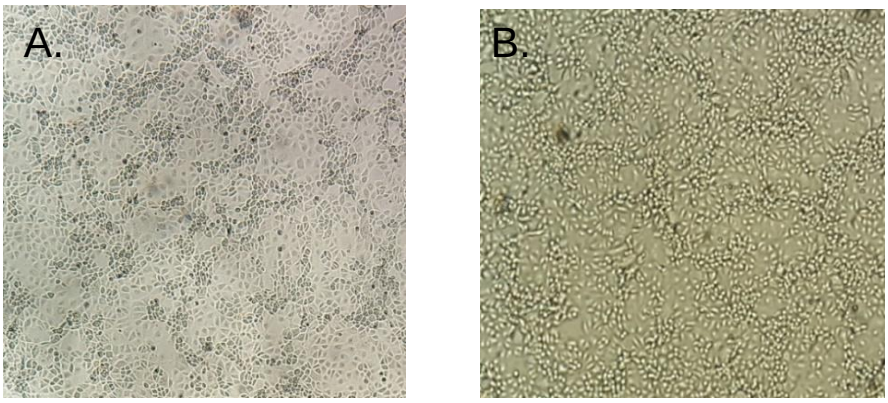



**Figure 3.2 MA104 cells after the 72-hour incubation with porcine trypsin activated BHK-T7/MA104 lysates** MA104 cells infected with trypsin activated lysates of BHK-T7 cells co-seeded with MA104 cells at a 1:1 (A) and 2:1 (B) ratio after 72 hours of incubation

After 24 hours of incubation co-seeded cells were activated with porcine trypsin and activated lysates containing possible rescued rotavirus were added to a naive monolayer of MA104 cells. After 3 days of incubation, no CPE was detected in either MA104 cells containing the 1:1 MA104: BHK-T7 lysates (Figure 3.2: A) and 2:1 MA104: BHK-T7 lysates and cells were still attached to the culture flask. Possible rotavirus translation of ss(+) RNA into viral proteins was then evaluated with IFMA. Both MA104 sells innoculated with the 1:1 MA104: BHK-T7 lysate (Figure 3.3 B) and 2:1 MA104: BHK-T7 lysate (Figure 3.3 C) revealed no indication of possible rescue of rotavirus when compared to the IFMA of the pGFP positive control (Figure 3.3 A). Rescue of rotavirus SA11 was thus unsuccessful.

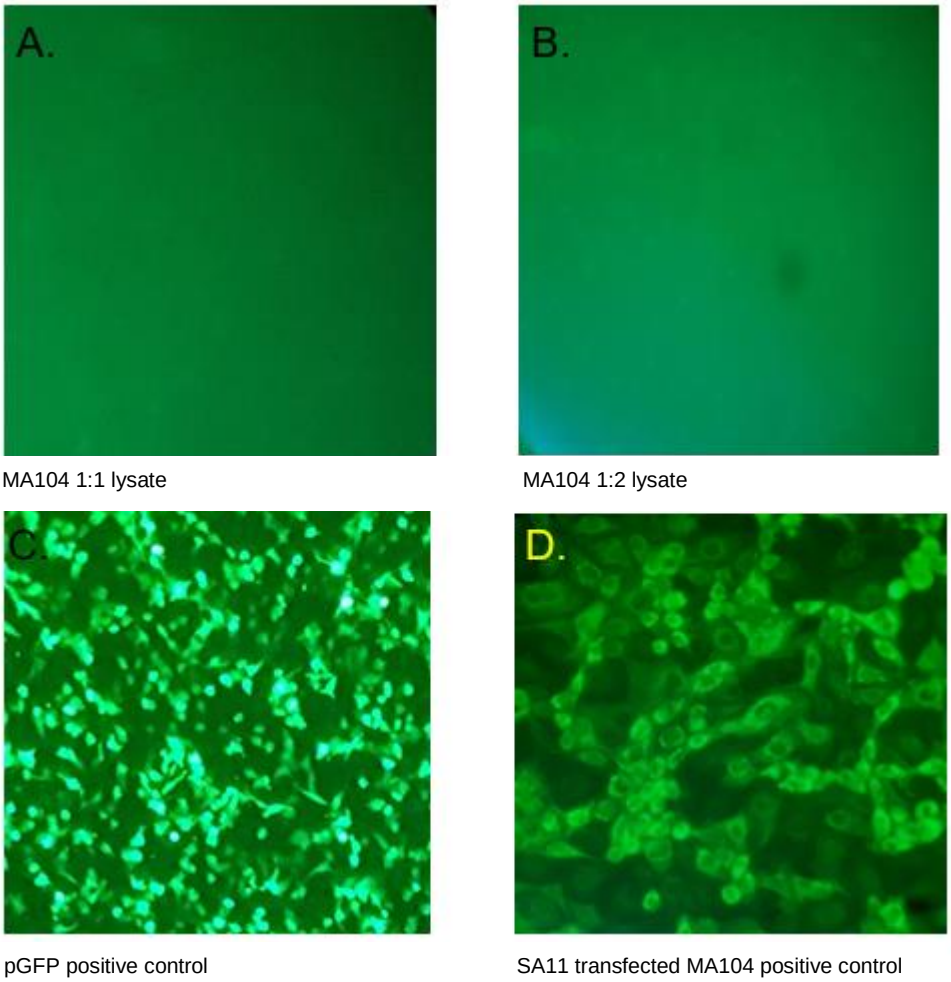
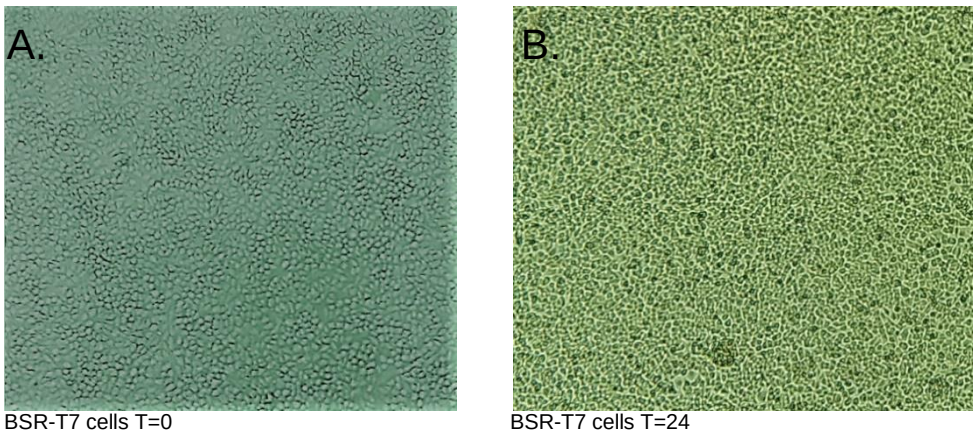



**Figure 3.3 IFMA results of SA11 CS expression plasmids transfected into BHK-T7 cells** Immunostaining results of MA104 cells infected with BHK-T7 lysates(A, B) using pGFP plasmid as positive control for transfection and expression (C). SA11 transfected MA104 cells used as positive control for viral protein expression(D).

### 3.3.2 Evaluation of RV rescue after transfection of equimolar SA11 CS expression plasmids and, CO VP 1,2,3,6, NSP 2 and 5 expression plasmids into of BSR-T7 cells

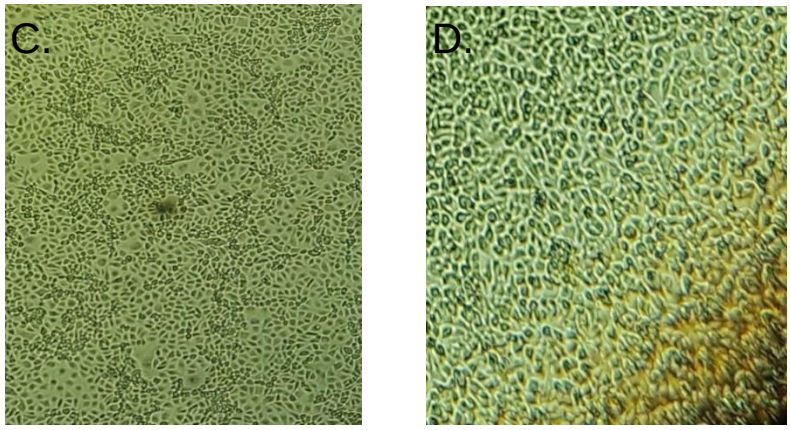
After failing to rescue RV from expressing SA11 CS plasmids in BHK-T7 cells, a BSR-T7 cell monolayer was transfected with SA 11 CS expression plasmids, CO replication complex and viroplasm forming plasmids, of each capping enzyme subunit (D1R and D12L) and pCAG-FAST expression plasmid using Lipofectamine™2000.




BSR-T7 cells T=0 BSR-T7 cells T=24

**Figure 3.4 Transfection of BSR-T7 cells with equimolar SA11 CS and CO expression plasmids** (A)BSR-T7 cells before transfection. (B) BSR-T7 transfected with SA11 CS and CO expression plasmids after 24 hours of incubation. 24 hours post transfection morphological differences in the cells were observed as a result of fused cells caused by the expression of the FAST protein.

At 24 hours post transfection, BSR-T7 cells were still attached to the culture flasks (Figure 3.4 B). With the use of LF2000 it is normal that cells take a knock after transfection as LF2000 can harm cells s and may appear toxic to cells but cells fully recover Cells were then trypsinized and co-seeded with MA104 in a 1:1 volume ration and co-cultured in serum-free medium supplemented with 0.5 ug/ml porcine trypsin.




MA104 and BSR-T7 cells (1:1) T= 24 MA104 with lysate T= 72

**Figure 3.5 Image of BSR-T7 cells after transfection with equimolar SA11 CS and CO expression plasmids** (A) BHK-T7 cells co-seeded with MA104 cells at a 1:1 ratio. (B) MA104 cells infected with a BHK-T7 MA104 lysate after 72 hours of incubation

After 24 hour incubation of co-seeded MA104 and BSR-T7 cells the monolayer remained attached to the culture flask with little to no cell death detected (Figure 3.5 A) . BSR-T7 and MA104 cells were lysed and lysates were added to a naive monolayer of MA104 cells. At 72 hours of incubation MA104 cells innoculated with the lysates showed no sign of potential CPE suggesting that rescue of rotavirus SA 11 using SA11 CS expression plasmids in conjunction with the CO expression plasmids was unsuccessful. This observation was verified by a lack of viral protein expression as observed using IFMA.

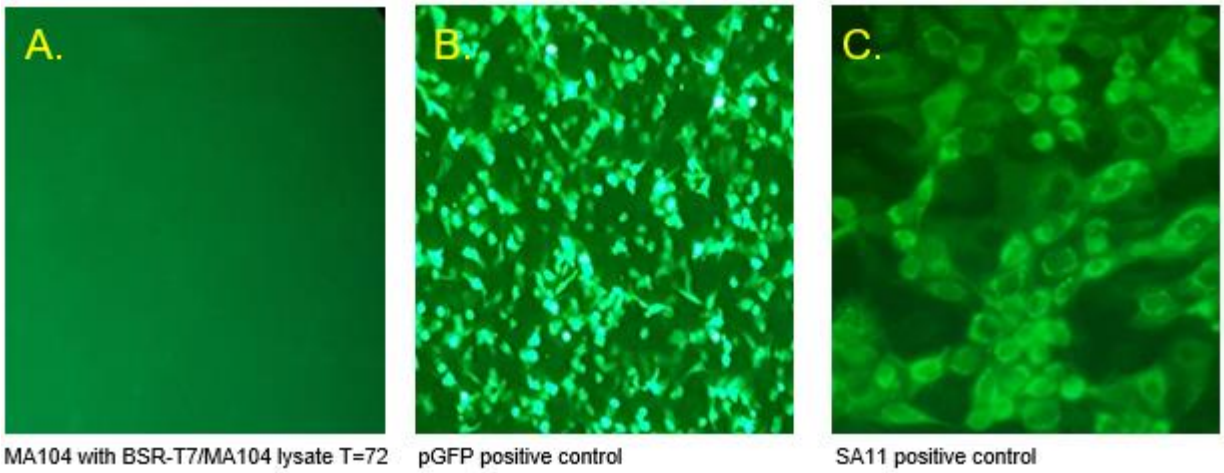



**Figure 3.6 IFMA image of BSR-T7 transfected with SA11 CS expression plasmids with CO expression plasmids.** (A) MA104 cells infected with BHK-T7 lysates. (B) pGFP transcription positive control. (C) SA11 positive control for viral protein expression.

No fluorescence could be visualised in the MA104 cells following immunofluorescent staining (Figure 3.6 B) when compared to the IFMA results of the pGFP positive control (Figure 3.6 A) this indicated that no viral proteins were expressed and the rescue of RV SA11 using the constructed SA 11 CS plasmids was unsuccessful.

Following the unsuccessful rescue of RV by the 2 transfection approaches, an investigation into the possible cause(s) was undertaken. Using the pGFP plasmid as a positive control for transfection and SA11 transfected MA104 cells as positive control for viral translation, contamination of reagents could be ruled out. Next generation sequence data of plasmids were then inspected again, and it was found that the plasmid construct containing genome segment 11 was constructed with a 5' sequence of 5-GCT following the T7 promoter sequence instead of the conserved 5' sequence 5-GGC. This difference in 5' sequence certainly resulted in the generation of an exact (+)ssRNA transcript with the wrong 5' terminus used for viral transcription, which may be the cause of unsuccessful rescue of RV. Plasmids constructs were constructed using consensus sequence of SA11 which was retrieved from GenBank at the time and using previous constructed cDNA plasmids as template. Although Dr. Mlera indicated the sequences correctly in his PhD study and paper highlighting the conserved 5'-GGC and 3'-ACC genome segments termini (Mlera et al., 2013) a mistake was made when uploading the sequences to GenBank. This resulted in the construction of an expression plasmid with an unknown design flaw in the 5' sequence of SA11 genome segment 11. The incorrect SA11 genome segment 11 sequence used can be seen in Figure 3.7.

>Seq11 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 11

GCTTTTAAAGCGCTACAGTGATGTCTCTCAGTATTGACGTGACGAGTCTTCCTTCTATTCCTT
CAACTATATATAAGAATGAATCGTCTTCAACAACGTCAACTCTTTCTGGAAAATCTATTGGTAG
GAGTGAACAGTACATTTCACCAGATGCAGAAGCATTCAATAAATACATGCTGTCGAAGTCTCC
AGAGGATATTGGACCATCTGATTCTGCTTCAAACGATCCACTCACCAGTTTTTCGATTAGATC
GAATGCAGTTAAGACAAATGCAGACGCTGGCGTGTCTATGGATTCATCAGCACAATCACGAC
CTTCAAGTAATGTCGGATGCGATCAAGTGGATTTCTCCTTAAATAAAGGCTTAAAAGTAAAAG
CTAATTTGGACTCATCAATATCAATATCTACGGATACTAAAAAGGAGAAATCAAAACAAAACCA
TAAAAGTAGGAAGCACTACCCAAGAATTGAAGCAGAGTCTGATTCAGATGATTATGTACTGGA
TGATTCAGATAGTGATGATGGTAAATGTAAGAACTGTAAATATAAGAAGAAATACTTCGCATTA
AGAATGAGAATGAAACAAGTCGCAATGCAATTGATTGAAGATTTGTAAGTCTGACCTGGGAAC
ACACTAGGGAGCTCCCCACTCCCGTTTTGTGACC

**Figure 3.7 Consensus sequence of SA11 Genome Segment 11** Incorrect 5' GCT sequence indicated in yellow

Taniguchi and Komoto also suggested that for plasmid-based reverse genetic systems, genome segment 11 could influence the efficiency of RV replication as the 11$^{th}$ genome segment is not present in other family members of *Reoviridae* which have RG systems in place (Tanighuchi and Komoto 2012). For proof of concept, another transfection reaction was performed substituting the SA11 genome segment 11 expression plasmid constructed in this study with the SA11 Genome segment 11 expression plasmid constructed by Kanai and co-workers (Kanai et al., 2017). A final transfection experiment was conducted with the assistance of Mr M.G.J. Huysers. The constructed SA11 genome segment 11 plasmid was replaced with the correct SA 11 genome segment 11 expression plasmid constructed by Kanai and co-workers used for the successful development of a plasmid-based RV RG system. Although the plasmid was constructed with the use of a different SA11 strain genome sequence, it would not influence the replication of RV SA11 as reassortment of RVs from different strains within the same group can occur. When evaluating the difference in NGS data between the SA11 genome segment 11 CS and the SA11-L2 genome segment 11 sequence Kanai et al. used the sequence was almost exact except for a nucleotide difference (T-C) at position 272. This nucleotide change is, however, synonymous and does not affect the amino acid sequence.

### 3.3.3 Evaluation of RV rescue after transfection of equimicgrogram amounts of SA11 CS expression plasmids into BHK-T7 cells

With the assistance of Mr M.G.J. Huyzers, another transfection was carried out. Equimicrogram amounts of each SA11 CS expression plasmids (Table 3.1) were transfected into confluent BHK-T7 cells grown in monolayer. The SA11 genome segment 11 plasmids was substituted with SA11

genome segment 11 plasmid constructed by Kanai et al which will be discussed in the summary of this chapter. Transfection was carried out using transfection reagent TransIT-LT1 as described in Section 3.2.3. 2.5µg of each capping plasmid (D1R and D12L) along with 49.5 ng of the plasmid expressing FAST proteins.

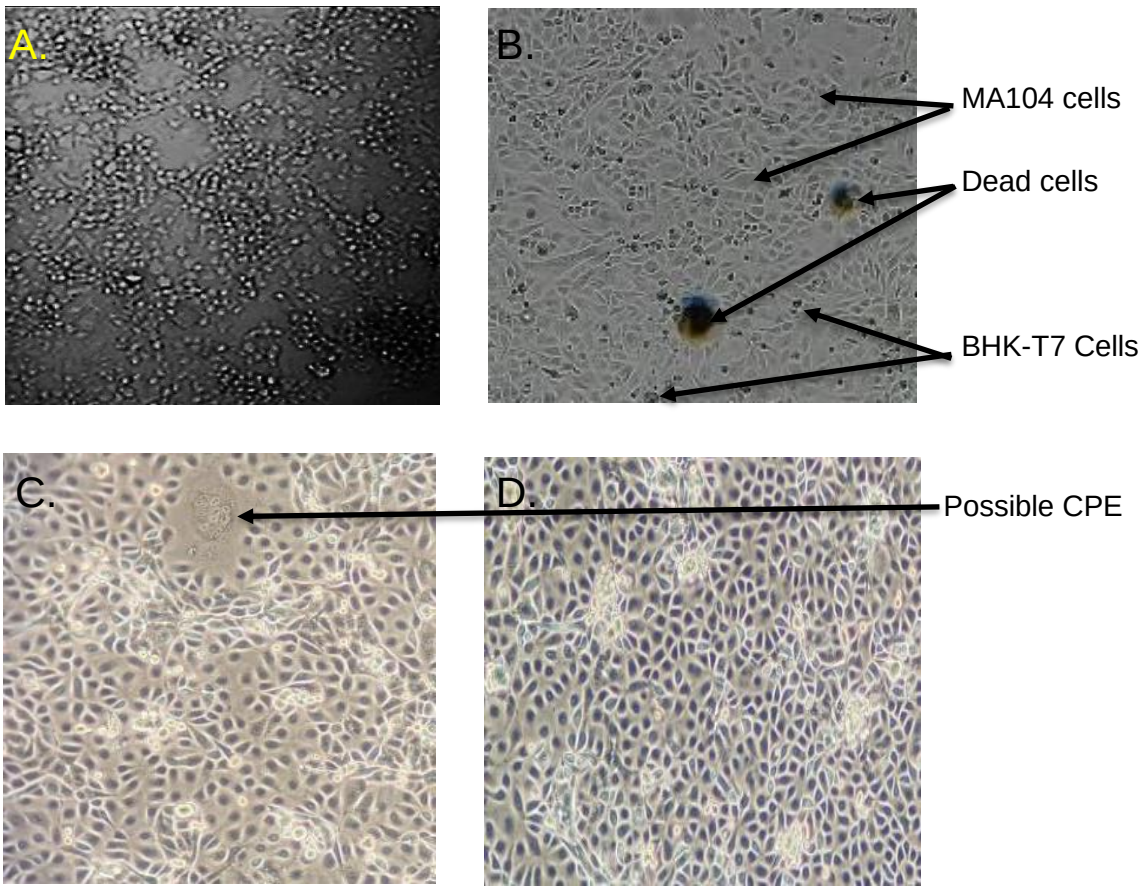



**Figure 3.8 Image of BHK-T7 cells fransfected with equal microgram amounts of SA11 CS expression plasmids in BHK-T7 cells** (A)BHK-T7 cells before transfection. (B) BHK-T7 cell co-seeded with MA104 cells at a 2:1 ratio. (C) MA104 cells infected with a (2:1) BHK-T7 MA104 lysate after 24 hours of incubation. (D) MA104 cells infected with a (2:1) BHK-T7 MA104 lysate after 24 hours of incubation

After 24 hours of transfection, BHK-T7 cells were still attached to the culture flask with little indication of CPE. BHK-T7 cells were then trypsinated and co-seeded with MA104 cells in a 2:1 volume ratio. At 24 hours post incubation of co-seeded MA104 and BHK-T7 cells were still attached to the culture flask although cell death was indicated (Figure 3.8 B). MA104 and BHK-T7 cells were then lysed, and lysates were added to a naive monolayer of MA104 cells. Following 24 hours of incubation with lysates, one focus of possible CPE was detected (Figure 3.8 C). At 72 hours post incubation (Figure 3.8 D) cells were still attached to the culture flask and possible CPE was observed. Possible rescue of rotavirus was then evaluated with IFMA immunostaining.

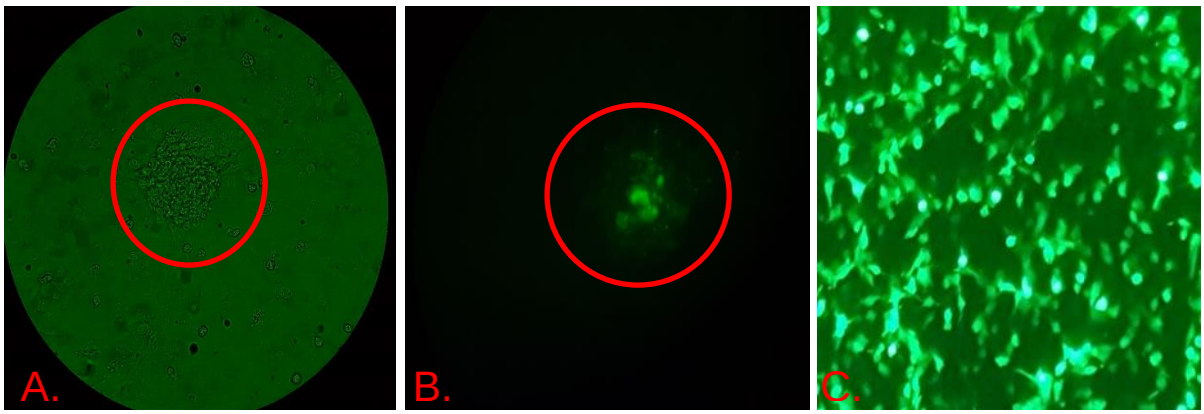



**Figure 3.9 IFMA result of transfection of equal microgram amounts of constructed SA11 expression plasmids** (A) Immunostained MA104 cells viewed without fluorescence indicating CPE. (B) Immunostained MA104 cells viewed under fluorescence. (C) pGFP positive control

When comparing the IFMA results of the MA104 cells, transfected with MA104/BHK-T7 lysates, to the positive GFP results(Figure 3.7 C) the IFMA indicated that viral protein was present (Figure 3.7 A and B) but the virus did not spread to uninfected cells. For virus to be efficiently rescued, the transfection procedure should be optimised.

## 3.4 Summary

In this study, three main transfection experiments were performed. Expression plasmids were transfected into BHK-T7 and BSR-T7 cells because both cell lines actively express T7 polymerase. MA104 cells were selected as rotavirus propagates well in this cell line.

In the first transfection experiment, an equimolar amount of SA11 CS expression plasmids were transfected into BHK-T7 cells along with capping enzyme expression plasmids and expression plasmid expressing FAST proteins. Rescue of rotavirus SA11 using equimolar SA11 CS expression plasmids in BHK-T7 was unsuccessful.

The second transfection was carried out in BSR-T7 cells, transfecting equimolar amounts of SA11 CS expression plasmids along with replication complex and viroplasm CO expression plasmids. CO expression plasmids were transfected in order to replicate the two-step transfection procedure used to rescue BTV and AHSV (Boyce et al., 2008, Matsuo et al., 2010). Rescue of rotavirus SA11 using SA11 CS expression plasmids in conjunction with CO expression plasmids, however, was unsuccessful.

The failure to recover RV from SA11 CS expression plasmids could be attributed to several factors in the replication cycle, transfected cells or construction of the expression plasmids. During transfection viroplasm formation and the synthesis of genomic dsRNA could not have occurred meaning that the virus could not replicate and increase within mammalian cultured cells. It was

only later discovered that failure to rescue RV from SA11 CS expression plasmids might be due to a design flaw in the 5’ terminus of the SA11 CS genome segment 11 plasmid.

A third transfection experiment was carried out in BHK-T7 cells with the assistance of Mr. M.G.J. Huyzers. Evaluation of transfection with immunofluorescent staining revealed that virus proteins might be present within cells (Figure 3.9 B) however, virus did not spread between cells. Virus would have to be isolated and sequenced in order to confirm that the virus or proteins indicated by IMFA are RV SA11. Further transfection experiment would need to be performed to optimise transfection procedures and also maybe increase virus replication. The design flaw in the genome segment 11 plasmid would also have to be corrected so that transfection with a full set of SA11 CS pSMART plasmids can be evaluated. These experiments could not be performed due to time restriction and must be done in future studies.

# CHAPTER 4: CONCLUDING REMARKS AND PROSPECTS

Over the years, numerous attempts toward the development of a rotavirus reverse genetic system were made, following the development of reverse genetic systems for other members of the *Reoviridae* family such as bluetongue virus (BTV) (Boyce et al., 2008), mammalian orthoreovirus (MRV) (Kobayashi et al., 2007), African horsesickness virus (AHSV) (Matsuo et al., 2010) and epizootic haemorrhagic disease virus (EHDV) (Yang et al., 2015). No reverse genetic system for rotavirus had been developed at the start of this study in 2015. Some success was however achieved with the rescue of viruses that were single GS reassortants with the assistance of helper viruses under strong selection conditions (Komoto et al., 2006, Trask et al., 2010, Troupin et al., 2010, Richards et al., 2013). The development of a plasmid-based reverse genetic system in 2017 resulted in excitement and anticipation that many of the outstanding issues of replication and correlates of protection can now be answered.

The overall aim of this study was to develop T7 polymerase-dependent plasmid-only reverse genetic system for rotavirus SA11, very similar to the plasmid-based reverse genetic system developed by Kanai and co-workers, using the SA11 consensus sequence. The project consisted of five specific aims:

**Correct the design flaw in the four multigenome expression plasmids.**
The three extra guanines at the 5' end of the genome segments within the four expression plasmids Alpha, Beta, Delta and Gamma was removed with PCR amplification due to the specific primer designs as listed in Chapter 2; Table 2.1. Primers were designed to amplify the 11 CS SA 11 genome segment sequences and lacked the three extra nucleotides (GGG) in the forward primer sequence so that genome segments would be amplified without the annealing of the three guanines. Genome segment lengths were determined using agarose gel electrophoresis of PCR amplified sequences to ensure that correct genome segments had been amplified.

**Design primers to PCR amplify the 11 rotavirus SA11 genome segments from these four expression plasmids**.
Primers were designed to amplify the genome segments from four multigenome expression plasmids. Primers were designed for all 11 genome segments in order to amplify single segment to have 11 SA11 consensus sequences of cDNA to be cloned into a selected backbone.

**Clone the 11 genome segments into pSMART vectors individually using FastCloning and In-Fusion® HD cloning**.

The 11 genome segments were each then cloned into pSMART vectors, containing a transcription initiation T7 promoter sequence as well as the HDV ribozyme sequence responsible for the production of exact 3' ends, provided by Prof A.C. Potgieter at Deltamune with the use of FastCloning and In-Fusion HD cloning. Vectors were linearised using PCR amplification and also checked with agarose gel electrophoresis. The FastCloning procedure produced a few difficulties at the start as it was a published method excluding essential steps for the optimisation of the procedure. Only after communication with Dr C.P.S. Badenhorst who was familiar with the method, genome segments were successfully cloned into pSMART vectors. Genome cloning products were incubated for two hours at 37ºC instead of the hour incubation as described by Li and co-workers (Li et al., 2011). This extension of incubation period also increased the number of correct colonies after transformation. FastCloning procedure was followed as it is a less expensive technique than In-Fusion HD cloning. In-Fusion cloning, however, allows for the construction of plasmids a lot quicker than FastCloning as cloning takes place with a 15-min incubation period followed by transformation. Nevertheless, both seamless cloning methods proved to be effective in the cloning of genome segments into vector plasmids resulting in the construction of the SA11 CS expression plasmid constructs. All 11 constructs were linearized with restriction enzymes BamHI (genome segments 1, 2, 5, 6, 8 and 11), Hind III (genome segment 3), PstI (genome segments 4, 7 and 10) and NcoI (genome segment 9) in order to determine the length of each constructed plasmid. Plasmids were then digested with restriction enzymes BamHI (genome segments 3 and 9) and EcoRV (genome segments 1, 2, 4, 5, 6, 7, 8, 10 and 11) cutting at specific sites resulting in 2 or more fragments of each digested expression plasmid in order to validate that correct plasmids had been constructed. Following evaluation of restriction enzyme digestion with agarose gel electrophoresis, expression plasmids were purified and sent for next generation sequencing.

**Sequence plasmid constructs to ensure no mutations had been introduced during the cloning process**

Nextgeneration sequencing was performed at NCID using the MiSeq (Illumina) sequencing platform. Next-generation sequencing was also performed at the NWU with the S5 instrument. Sequence data was analysed with use of reference sequences and proved that all constructed plasmids contained correct T7 promoter sequences and correct 5' and 3' virus genome sequences as well as the correct HDV ribozyme sequences. This revealed the successful construction of a set of SA11 T7 polymerase-dependent expression plasmids.

**Transfect expression plasmids into BHK-T7 and BSR- T7 cells**

To prove that expression plasmids could be used to generate viable rotavirus, plasmids were transfected into BHK-T7 and BSR-T7 cells along with plasmids expressing FAST proteins and a vaccinia capping enzyme to increase virus translation and replication (Kanai et al., 2017) The first equimolar transfection was attempted in BHK-T7 cells. SA11 CS expression plasmids were transfected with the TransIT-LT1 transfection reagent. However, no viable rotavirus was recovered following the attempt to infect MA104 cells with BHK-T7 lysates. The second transfection was carried out in BSR-T7 cells with Lipofectamine 2000 transfection reagent. Equimolar amounts of the SA 11 expression plasmids and codon optimised expression plasmids (Wentzel 2014) were transfected. The RIG-I signalling pathway is defectiveabsent in BSR cells and it was assumed that the viral rescue would be more efficient in BSR-T7 cells because the RIG-I mediated interferon signalling is bypassed thereby reducing the interferon response. A reduction in interferon response allows for sufficient viral translation. However, no viable virus could be recovered from this transfection procedure either.

A final transfection experiment was carried out in BHK-T7 cells. This time SA11 expression plasmids were transfected in equal microgram amounts. During the final transfection, the expression plasmid containing genome segment 11 was replaced with the genome segment 11 expression plasmid constructed by Kanai and co-workers (Kanai et al., 2017), due to a flaw in the 5' sequence of the SA 11 genome segment plasmid constructed in this study. The incorrect reference sequence was used to construct the SA11 genome segment 11 plasmids it was also used as a reference for analysis of NGS sequencing data. Infection of MA104 cells with BHK-T7 lysates indicated one focus area where virus might be present (Figure 3.9 B), however, the virus did not spread from cell to cell which could indicate that viral proteins were translated but viral replication and/ or spread did not occur. So, this could not be concluded as successful viral rescue.

Despite the inability to recover rotavirus during this study the work performed is still significant as the presence of viral proteins in the MA104 cells infected with transfected BHK-T7/MA104 lysates (Figure 3.9 A, B) could indicate that viral protein translation did occur. Further optimisation of the transfection protocol such as adjusted amounts of pCAG-FAST plasmids and capping enzyme subunits etc. may lead to the rescue of viable rotavirus. The flaw in the 5' sequence of the SA11 genome segment 11 expression will have to be corrected in order to evaluate the transfection of the full set of SA 11 pSMART expression plasmids. The development of the entirely plasmid-based reverese genetic system for RV in February 2017 also offers a method for comparison and will also assist in the optimisation of the expression plasmids constructed in this study that may

lead to the first rotavirus reverse genetic system in South Africa. Thus, the set of SA11 CS expression plasmids designed and constructed in this study will now also be used as the basis for further development towards a more robust rotavirus reverse genetic system

As mentioned, Kanai and co-workers (Kanai et al., 2017) constructed 11 plasmids which contained the full-length genome segments (1-11) of the SA11-L2 strain individually. The SA11 genome segments were flanked by a T7 RNA polymerase (T7Pol) promoter at the 5' end followed by the antigenomic HDV ribozyme at the 3' end. Plasmids were transfected into BHK cells expressing T7 polymerase thereby allowing exact full-length viral (+)ssRNA transcripts to be synthesized. As the transfection of cDNA plasmids did not work in the past as for BTV, RV replication was increased by the expression of a reovirus fusion-associated small transmembrane (FAST) protein. It had been found in a previous study that FAST proteins exponentially increased the yield of heterologous mammalian orthoreovirus rescue (Ciechonska and Duncan, 2014) apart from enhancing translation, cell fusion may also bring pools of transcripts into the same space hence avoiding missing certain players for virus assembly. Plasmids expressing a vaccinia virus-capping enzyme were also constructed and transfected as it was found to increase translatability of reovirus (+)ssRNAs (Kobayashi et al., 2007). The work performed by Kanai and co-workers represents a key achievement in RV research and will move the field forward in many laboratories as reverse genetics systems for many other RNA viruses have done.

The development of the rotavirus reverse genetic system will have many applications which include the identification of the packaging signals of the RV RNAs, the discovery of sequences of individual RNA segments determining the assortment process, and the exploration of the components of complex biological phenotypes, such as host range restriction, virulence, and attenuation. Rotaviruses prominently exposing highly cross-reactive epitopes (inducing cross-protective immunity) may lead to a universal rotavirus vaccine (Desselberger 2017). The reverse genetic system has already proven to be useful in viral replication studies as it was used to demonstrate that NSP6 is not essential for viral replication in cell cultures (Komoto et al 2017). Therefore, many unanswered questions can now be addressed with the implementation of the rotavirus reverse genetic system.

The future prospects of this study are however to first correct the design flaw in the 5' terminus of the SA 11 CS genome segment 11 construct. Another transfection can then be performed with a (correct) full set of SA11 CS expression plasmids in order to determine whether rotavirus can be rescued from the SA11 plasmids constructed at the NWU.

As seen in the past rotavirus reverse genetic system also leaves room for improvement for example the construction of a single expression plasmid containing all 11 genome segments or multiple genome segments on 4 expression plasmids, as the approach of Dr. J.F. Wentzel (Wentzel 2014), reducing the number of plasmids to be transfected. This may lead to an improved transfection efficiency as in the influenza reverse genetic system were expression plasmids where reduced from 12 plasmids to 5 expression plasmids (Neumann and Kawaoka, 2001).

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# ANNEXURES

## ROTAVIRUS A RVA/SIMIAN-TC/ZAF/SA11-CS/1958/G3P[2] SEGMENT 1-11

**>Seq1 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 1**

GGCTATTAAAGCTGTACAATGGGGAAGTACAATCTAATCTTGTCAGAATATCTATCATTTATATATAATTCACAATC
TGCAGTTCAAATTCCAATATATTACTCTTCCAACAGTGAATTAGAAAATAGATGTATTGAATTTCATTCCAAGTGTT
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CGGAGGAATATACGGACTCACTAATGGATCCAGCAATTTTAACTTCGCTATCATCAAATTTAAATGCAGTCATGTT
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GTAGCCTCAACGATAAATAAATATGGCGTACCAAGGCATAACGCAAAGTACAGATATGAATACGACGTAATGAAA
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TGATTTCAATTCACCGGACTTGGAGAAGCTGATAAGAATACCATTTAAGGGAAAAATACCAGCTGTTACATTCATA
TTACACTTATATGCAAAGCTAGAAGTTATAAACTACGCTATAAAAAATGGTTCATGGATAAGCCTATTTTGCAATTA
CCCTAAATCAGAAATGATAAAATTATGGAAGAAGATGTGGAACATCACGTCATTACGTTCGCCGTACACTAACGC
GAACTTCTTTCAAGATTAGAACGCTTAGATGTGACC

**>Seq2 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 2**

GGCTATTAAAGGCTCAATGGCGTATCGAAAACGTGGAGCGCGTCGTGAGACGAATCTAAAACAAGATGAACGAA
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CATGTAGTCAACCAATTAATGGAAGCTCTGATGCAATTATCTAGACAACAGTTTCCCACAATGCCAGTTGATTATA
AAAGATCTATACAGAGAGGAATTTTGCTGCTTTCTAACAGACTTGGTCAGCTTGTCGATTTAACAAGATTGTTATC
ATACAATTATGAGACATTAATGGCATGCATAACAATGAATATGCAGCATGTTCAAACATTAACAACTGAAAAATTG
CAATTAACATCAGTAACATCATTATGTATGCTAATTGGAAATGCTACGGTTATACCGAGTCCGCAAACATTGTTCC
ATTACTATAATGTGAATGTCAATTTTCATTCAAATTATAATGAAAGAATTAATGACGCAGTTGCAATTATAACTGCG
GCAAATAGATTAAATTTATATCAAAAGAAAATGAAATCAATAGTTGAGGACTTTCTGAAAAGATTACAGATATTTGA
TGTTGCGAGAGTACCAGATGACCAAATGTATAGATTGAGAGATAGATTAAGACTATTACCAGTTGAAATAAGAAG
ATTAGATATTTTTAATTTGATAGCAATGAATATGGAACAGATTGAACGTGCATCAGATAAAATTGCACAAGGAGTT
ATAATAGCATACCGAGATATGCAGTTAGAACGAGATGAGATGTATGGTTACGTCAATATTGCCAGAAACTTGGAC
GGATTTCAACAAATAAATCTTGAAGAATTGATGAGATCAGGAGATTATGCTCAAATTACTAACATGCTACTTAATAA
TCAACCAGTAGCTTTAGTTGGAGCGCTACCATTTATAACGGATTCATCAGTGATTTCGTTAATAGCTAAACTAGAT
GCAACCGTTTTTGCACAGATTGTCAAACTTAGAAAGGTCGACACGTTAAAACCCATCCTATATAAGATAAATTCAG
ATTCTAATGACTTTTATTTGGTGGCTAATTATGATTGGATTCCTACATCTACTACAAAAGTGTATAAACAAGTTCCA
CAACAATTTGATTTTAGAGCGTCAATGCATATGTTAACGTCTAACCTAACATTTACCGTATATTCAGATTTGCTTGC
GTTCGTTTCAGCTGATACTGTTGAACCAATTAATGCTGTTGCTTTTGATAATATGCGCATCATGAACGAACTGTAA
ACGCCAACCCCATTGTGGAGATATGACC

**>Seq3 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 3**

GGCTATTAAAGCAGTACCAGTAGTGTGTTTTACCTCTGATGGTGTAAACATGAAAGTACTAGCTTTAAGACACAGT
GTGGCTCAAGTGTATGCAGACACTCAAGTCTACGTTCATGATGATACAAAAGATAGTTATGAAAACGCTTTTTTAA
TCTCTAATCTTACGACCCATAATATTTTATACTTAAATTATAGCATTAAAACATTAGAAATATTAAATAAGTCAGGAA
TAGCTGCAATTGCTTTACAATCACTTGAAGAATTATTCACATTAATAAGGTGTAATTTCACTTATGATTATGAACTT
GATATAATATATTTACATGATTATTCATATTATACCAATAATGAAATTAGAACAGACCAACATTGGATAACAAAAAC
AAATATTGAAGAATATTTACTACCTGGATGGAAATTAACATATGTTGGTTATAATGGAAGTGAAACTAGAGGACAT
TATAACTTTTCATTTAAATGTCAAAACGCTGCAACAGATGATGATCTAATAATTGAATACATTTATTCAGAAGCGTT
GGACTTCCAAAATTTTATGTTAAAAAAGATAAAGGAAAGAATGACTACATCGTTGCCTATAGCTAGATTATCTAAC
AGAGTATTTAGGGATAAGTTATTCCCATCATTATTGAAAGAACATAAGAATGTAGTGAACGTTGGTCCGCGTAATG
AATCTATGTTTACATTTTTAAATTATCCAACTATAAAACAATTTTCAAATGGTGCGTATTTAGTAAAAGATACTATAA
AATTAAAACAAGAACGATGGTTAGGTAAAAGGATATCTCAGTTTGATATTGGTCAGTATAAAAATATGCTGAATGT
TCTTACAGCAATTTATTATTACTATAATTTATATAAAAGTAAACCAATTATATATATGATCGGATCTGCTCCATCTTA
TTGGATATATGACGTTAGGCATTATTCCGATTTTTTCTTTGAAACTTGGGATCCATTGGACACACCATATTCATCAA
TCCATCACAAAGAATTATTTTTTATAAATGATGTGAAGAAACTGAAGGATAACTCAATATTGTATATTGATATAAGA
ACCGATAGGGGCAATGCTGATTGGAAAAAATGGAGAAAGACAGTAGAAGAACAAACTATTAATAATTTGGACATA

GCTTATGAATATTTACGAACGGGTAAAGCGAAGGTGTGTTGTGTTAAGATGACAGCTATGGATTTGGAACTGCCA
ATTTCAGCTAAATTACTGCACCACCCAACTACGGAAATAAGATCAGAATTTTATTTATTACTAGATACTTGGGATTT
AACTAACATTAGGAGGTTCATTCCTAAAGGCGTGTTATATTCATTTATAAACAATATAATAACTGAAAATGTGTTTA
TTCAACAACCATTTAAAGTAAAAGTACTGAATGATAGTTATATTGTAGCGTTATATGCATTATCAAATGATTTTAATA
ATAGATCAGAAGTAATTAAATTAATTAATAATCAGAAACAATCTCTAATAACTGTTAGAATAAATAATACGTTTAAG
GATGAACCAAAAGTTGGGTTCAAAAATATCTATGATTGGACCTTTCTTCCAACCGACTTTGATACCAAAGAAGCTA
TAATTACTTCATACGACGGTTGTTTAGGACTCTTTGGTTTGTCTATATCGTTAGCATCAAAACCAACAGGGAATAA
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ACACGCACCAATAGAATTAATTTATGCATGTAGATCAGCAAAAGAATTTGCTACATTGCAGGATGACTTAACTGTA
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TATCGGAATACAATTTAGAAATATACCATATAAATATGATGTTAAAATACCGCATTTAACCTTCGGAGTATTACATA
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GACCAGTTATACATATATGTTATCAGATGGAATCTACGTAGCAAATGTTAGTGGAGTATTATCTACATACTTTAAAA
TCTATAACGTATTTTATAAAAATCAAATAACTTTTGGCCAATCCAGAATGTTTATTCCGCACATAACATTAAGCTTC
AATAACATGAGAACAGTAAGGATAGAGACTACTAAATTACAAATTAAATCCATTTATTTAAGAAAGATTAAGGGTG
ATACAGTGTTTGATATGGTTGAGTGAGCTAAAAACTTAACACACTAGTCATGATGTGACC

**>Seq4 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 4**

GGCTATAAAATGGCTTCGCTCATTTATAGACAATTGCTCACGAATTCTTATACAGTAGATTTATCCGATGAGATAC
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TAACTGGGGACCTGGAGAAATTAATGATTCTACGACAGTTGAACCATTGCTGGATGGGCCTTATCAACCAATGAC
ATTCAATCCACCAGTCGATTATTGGATGTTACTGGCTCCAACGACACCTGGCGTAATTGTTGAAGGTACAAATAA
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ACTGCTTTTTGTGACTTTTATATAATTCCTAGATCTGAAGAGTCTAAATGTACGGAATACATTAATAATGGATTACC
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CAATACACATATACTCGTGATGGTGAAGAAGTTACCGCACATACTACTTGTTCAGTGAATGGCGTTAATGACTTCA
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GGATTAGCGAATTCAGTTTCAACACTGACAGATTCTTTATCAGACGCAGCATCATCAATATCAAGAGGTTCATCTA
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CCTGACATTGTTACTGAAGCATCGGAAAAATTCATACCAAATAGGGCTTACCGCGTTATAAACAACGATGATGTG
TTTGAAGCGGAATTGATGGAAAATTTTTTGCTTATAAAGTGGATACATTTGAGGAAATACCATTTGATGTACAAAA
ATTCGCTGACTTAGTTACAGATTCTCCAGTAATATCCGCTATAATTGATTTTAAAACACTTAAAAATTTGAACGATA
ATTACGGCATTACTAAGCAACAAGCATTTAATCTTTTAAGATCTGACCCAAGAGTTTTACGTGAATTCATTAATCAG
GACAATCCTATAATTAGAAATAGAATTGAACAACTGATTATGCAATGCAGGTTGTGAGTAATTTCTAGAGGATGTG
ACC

**>seq5 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 5**

GGCTTTTTTTTGAAAAGTCTTGTGTTAGCCATGGCTACTTTTAAAGATGCATGCTTTCATTATCGTAGATTAACTGC
TTTAAATCGGAGATTATGCAACATTGGTGCAAATTCTATTTGgATGCCAGTTCCTGATGCGAAAATTAAGGGGTGG
TGTTTAGAATGTTGTCAAATAGCTGATTTAACCCATTGTTATGGTTGCTCATTGCCGCATGTTTGCAAATGGTGTG
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AGACAAATTACAGTGTATCATAGACTTGTACAATATAATATTTCCAATTAATGATAAAGTAATTAGAAAATTTGAAA
GAATGATAAAGCAAAGAGAATGTAGGAATCAATATAAAATTGAATGGTATAATCATTTGCTGCTCCCAATTACATT
AAATGCTGCTGCATTTAAGTTTGATGAAAATAATCTTTATTATGTTTTTGGGTTATATGAGAAATCAGTCAGTGATA
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GTCCAATCTACCAATAGAGTTGAGAAACCATTACGCAAAGAAATACTTCCAATTATCAAGACTGCCATCATCAAAA
CTAAAGCAAATTTACTTTTCAGATTTTACTAAAGAAACTGTGATTTTTAATACTTATACAAAAACGCCAGGAAGATC
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AATTAATTGCTGCAATGATGACGAGTAAGTATACTCGGTTCTATGCTCATGATAATAATTTTGGAAGGTTGAAAAT
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GTCACTATCTAATTATACAGTATTTAGCCATCACAAGACCGTCCAGACTAGAGTAGCGCCTAGCTGGCAAAATAC
TGTGAACC

**>Seq6 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 6**

GGCTTTTAAACGAAGTCTTCAACATGGATGTCCTATACTCTTTGTCAAAGACTCTTAAAGACGCTAGAGACAAAAT
TGTCGAAGGCACATTGTATTCTAACGTGAGTGATCTAATTCAACAATTTAATCAAATGATAATTACTATGAATGGAA
ATGAATTTCAAACTGGAGGAATCGGTAATTTGCCAATTAGAAACTGGAATTTTAATTTCGGGTTACTTGGAACAAC
TTTGCTGAACTTAGACGCTAATTATGTTGAAACGGCAAGAAATACAATTGATTATTTCGTGGATTTTGTAGACAAT
GTATGCATGGATGAGATGGTTAGAGAATCACAAAGGAACGGAATTGCACCTCAATCAGACTCGCTAAGAAAGCT
GTCAGCCATTAAATTCAAAAGAATAAATTTTGATAATTCGTCGGAATACATAGAAAACTGGAATTTGCAAAATAGA
AGACAGAGGACAGGTTTCACTTTTCATAAACCAAACATTTTTCCTTATTCAGCATCATTTACACTAAATAGATCACA
ACCCGCTCATGATAATTTGATGGGCACAATGTGGTTAAACGCAGGATCGGAAATTCAAGTCGCTGGATTTGACTA
CTCATGTGCTATTAACGCACCAGCCAATATACAACAATTTGAGCATATTGTGCCACTCCGAAGAGTGTTAACTACA
GCTACGATAACTCTTCTACCAGACGCGGAAAGGTTTAGTTTTCCAAGAGTGATCAATTCAGCTGACGGGGCAACT
ACATGGTTTTTCAACCCAGTGATTCTCAGGCCGAATAACGTTGAAGTGGAGTTTCTATTGAATGGACAGATAATAA

ACACTTATCAAGCAAGATTTGGAACTATCGTAGCTAGAAATTTTGATACTATTAGACTATCATTCCAGTTAATGAGA
CCACCAAACATGACACCAGCAGTAGCAGTACTATTCCCGAATGCACAGCCATTCGAACATCATGCAACAGTGGG
ATTGACACTTAGAATTGAGTCTGCAGTTTGTGAGTCTGTACTCGCCGATGCAAGTGAAACTCTATTAGCAAATGTA
ACATCCGTTAGGCAAGAGTACGCAATACCAGTTGGACCAGTCTTTCCACCAGGTATGAACTGGACTGATTTAATC
ACCAATTATTCACCGTCTAGGGAGGACAATTTGCAACGCGTATTTACAGTGGCTTCCATTAGAAGCATGCTCATT
AAATGAGGACCAAGCTAACAACTTGGTATCCAACTTTGGTGAGTATGTAGCTATATCAAGCTGTTTGAACTCTGTA
AGTAAGGATGCGTATACGCATTCGCTACACAGAGTAATCACTCAGATGGTATAGTGAGAGGATGTGACC

**>Seq7 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 7**

GGCATTTAATGCTTTTCAGTGGTTGATGCTCAAGATGGAGTCTACGCAACAGATGGCCGTCTCAATTATTAACTC
TTCTTTTGAAGCTGCAGTTGTAGCTGCAACCTCAGCTCTTGAGAATATGGGAATAGAATATGATTATCAGGATATA
TATTCTAGAGTAAAGAATAAATTTGATTTTGTGATGGACGATTCTGGTGTTAAAAATAATCTGATTGGTAAAGCAAT
AACTATTGATCAAGCTTTGAATAATAAATTTGGATCTGCTATAAGAAATAGAAACTGGCTTGCTGATACTTCTAGA
GCAGCTAAATTGGATGAGGATGTAAACAAACTAAGAATGATGTTATCATCAAAAGGAATTGATCAAAAAATGAGA
GTTTTAAACGCATGCTTCAGTGTAAAAAGAATACCTGGAAAATCATCATCTATTATTAAATGCACAAAATTGATGC
GTGATAAATTGGAACGTGGTGAAGTTGAAGTGGATGATTCATTTGTGGATGAAAAAATGGAAGTGGATACCATTG
ACTGGAAATCGCGCTATGAGCAATTGGAGCAAAGGTTTGAATCATTGAAATCCAGGGTAAATGAAAAATATAATA
ATTGGGTGTTGAAAGCAAGAAAAATGAATGAAAATATGCATTCTCTTCAAAATGTCATCTCTCAACAGCAAGCACA
TATAGCTGAGCTTCAAGTGTACAATAATAAACTAGAACGTGATTTGCAAAATAAAATTGGATCCCTTACTTCTTCG
ATTGAATGGTATTTAAGATCAATGGAATTAGACCCTGAAATAAAGGCAGACATTGAACAGCAAATTAACTCAATTG
ATGCGATAAATCCATTGCACGCTTTTGATGACTTAGAATCAGTAATACGTAATTTGATATCTGATTATGACAAATTA
TTCCTTATGTTCAAAGGATTAATACAGAGATGTAATTATCAATATTCATTTGGTTGCGAATAACCATTTTGATACAT
GTTGAACAATCAAATACAGTGTTAGTATGTTGTCATCTATGCATAACCCTCTATGAGCACAATAGTTAAAAGCTAA
CACTGTCAAAAACCTAAATGGCTATAGGGGCGTTATGTGGCC

**>Seq8 Rotavirus A RVA/Simian-tc/ZAF/SA11-H96 CS/1958/G3P[2] segment 8**

GGCTTTTAAAGCGTCTCAGTCGCCGTTTGAGCCTTGCGGTGTAGCCATGGCTGAGCTAGCTTGCTTTTGCTATC
CTCATTTGGAGAATGATAGCTATAAATTTATTCCTTTTAATAATTTAGCTATTAAAGCTATGCTGACAGCTAAAGTA
GACAAAAAGGACATGGATAAGTTTTATGATTCAATTATTTATGGAATAGCACCGCCTCCTCAATTTAAGAAACGGT
ATAATACTAATGATAATTCAAGAGGCATGAATTTTGAAACAATTATGTTTACTAAGGTGGCTATGTTGATATGTGAA
GCTCTAAATTCATTGAAAGTGACGCAAGCAAACGTCTCTAATGTATTATCACGAGTAGTATCAATAAGGCATTTAG
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ATTGCTATTGGACAGTCTAAAGAAATTGAAACTACAATAACTGCAGAAGGAGGAGAAATTGTATTTCAAAACGCTG
CCTTCACCATGTGGAAACTAACTTATTTAGAACATCAATTGATGCCAATTCTGGATCAGAATTTTATTGAATATAAA
GTTACATTGAACGAAGATAAACCAATTTCAGATGTTCATGTTAAAGAATTAGTCGCTGAACTTCGATGGCAATATA
ACAAGTTTGCTGTAATCACACATGGTAAGGGTCATTATAGAATTGTAAAGTATTCATCAGTTGCTAATCACGCTGA
CAGAGTATATGCAACTTTCAAGAGTAATGTTAAAACTGGAGTTAATAATGATTTTAACCTACTTGATCAAAGAATTA
TTTGGCAAAACTGGTATGCATTTACATCATCAATGAAACAGGGTAATACACTTGACGTGTGTAAAAGGTTGCTTTT
CCAAAAAATGAAACCAGAAAAAAATCCATTTAAAGGGCTGTCAACGGATAGAAAAATGGACGAAGTTTCTCAAGT
TGGCGTTTAATTCGCTATCAATTTGAGGATGATGATGGCTTAGCAAGAATAGAAAGCGCTTATGTGACC

**>Seq9 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 9**

GGCTTTAAAAAGAGAGAATTTCCGTTTGGCTAGCGGTTAGCTCCTTTTAATGTATGGTATTGAATATACCACAGTT
CTAACCTTTCTGATATCGATTATTCTACTAAATTACATACTTAAATCATTAACTAGAATAATGGACTTTATAATTTAT

AGATTTCTTTTTATAATTGTGATATTGTCACCATTTCTCAGAGCACAAAATTATGGTATTAATCTTCCAATCACAGG
CTCCATGGACAtTGCATACGCTAATTCAACGCAAGAAGAAACATTCCTCACTTCTACACTTTGCCTATATTATCCG
ACTGAGGCTGCGACTGAAATAAACGATAATTCATGGAAAGACACACTGTCACAACTATTTCTTACGAAAGGGTGG
CCAACTGGATCCGTATATTTTAAAGAATATACTAACATTGCATCGTTTTCTGTTGATCCGCAGTTGTATTGTGATTA
TAACGTAGTACTAATGAAATATGACGCGACGTTGCAATTGGATATGTCAGAACTTGCGGATCTAATATTAAACGAA
TGGTTGTGTAATCCAATGGATATTACTCTGTATTATTATCAGCAAACTGACGAAGCGAATAAATGGATATCAATGG
GCTCATCATGTACAATTAAAGTATGTCCACTTAATACACAAACTCTTGGAATTGGATGCTTGACAACTGATGCTAC
AACTTTTGAAGAAGTTGCGACAGCTGAAAAGTTGGTAATTACTGACGTGGTTGATGGCGTTAATCATAAGCTGGA
TGTCACAACAGCAACGTGTACTATTAGAAACTGTAAGAAATTGGGACCAAGAGAAAACGTAGCCGTTATACAAGT
TGGTGGTTCTGACATCCTCGATATAACTGCTGATCCAACTACTGCACCACAGACAGAACGGATGATGCGAATTAA
CTGGAAAAAATGGTGGCAAGTTTTTTATACTGTAGTAGACTATGTAGATCAGATAATACAAGTTATGTCCAAAAGA
TCAAGATCACTAAATTCAGCAGCATTTTATTACAGAGTGTAGGTATAACTTAGGTTAGAATTGTATGATGTGACC

**>Seq10 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 10**

GGCTTTTAAAAGTTCTGTTCCGAGAGAGCGCGTGCGGAAAGATGGAAAAGCTTACCGACCTCAATTATACATTGA
GTGTAATCACTCTAATGAACAATACATTGCACACAATACTTGAGGATCCAGGAATGGCGTATtTTCCTTATATAGC
ATCTGTCTTAACAGTTTTGTTTGCGCTACATAAAGCATCCATTCCAACAATGAAAATTGCATTGAAAACGTCAAAAT
GTTCATATAAAGTGGTGAAATATTGTATTGTAACAATTTTTAATACGTTGTTAAAATTGGCAGGTTATAAAGAGCAG
ATAACTACTAAAGATGAGATAGAAAAGCAAATGGACAGAGTAGTCAAAGAAATGAGACGCCAGCTAGAAATGATT
GACAAATTGACTACACGTGAAATTGAACAAGTAGAGTTGCTTAAACGCATTTACGATAAATTGACGGTGCAAACG
ACAGGCGAAATAGATATGACAAAAGAGATCAATCAAAAAAACGTGAGAACGCTAGAAGAATGGGAAAGTGGAAA
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GCGGAGTTCTTTACAGTAAGCACCATCGGACCTGATGGCTGACTGAGAAGCCACAGTCAGCCATATCGCGTGTG
GCTCAAGCCTTAATCCCGTTTAACCAATCCGGTCAGCACCGGACGTTAATGGAAGGAACGGTCTTAATGTGACC

**>Seq1 Rotavirus A RVA/Simian-tc/ZAF/SA11-CS/1958/G3P[2] segment 11**

GCTTTTAAAGCGCTACAGTGATGTCTCTCAGTATTGACGTGACGAGTCTTCCTTCTATTCCTTCAACTATATATAA
GAATGAATCGTCTTCAACAACGTCAACTCTTTCTGGAAAATCTATTGGTAGGAGTGAACAGTACATTTCACCAGAT
GCAGAAGCATTCAATAAATACATGCTGTCGAAGTCTCCAGAGGATATTGGACCATCTGATTCTGCTTCAAACGAT
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AGCACTACCCAAGAATTGAAGCAGAGTCTGATTCAGATGATTATGTACTGGATGATTCAGATAGTGATGATGGTA
AATGTAAGAACTGTAAATATAAGAAGAAATACTTCGCATTAAGAATGAGAATGAAACAAGTCGCAATGCAATTGAT
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# PSMART-T7 BACKBONE SEQUENCE


TsoI
XcmI
PvuI
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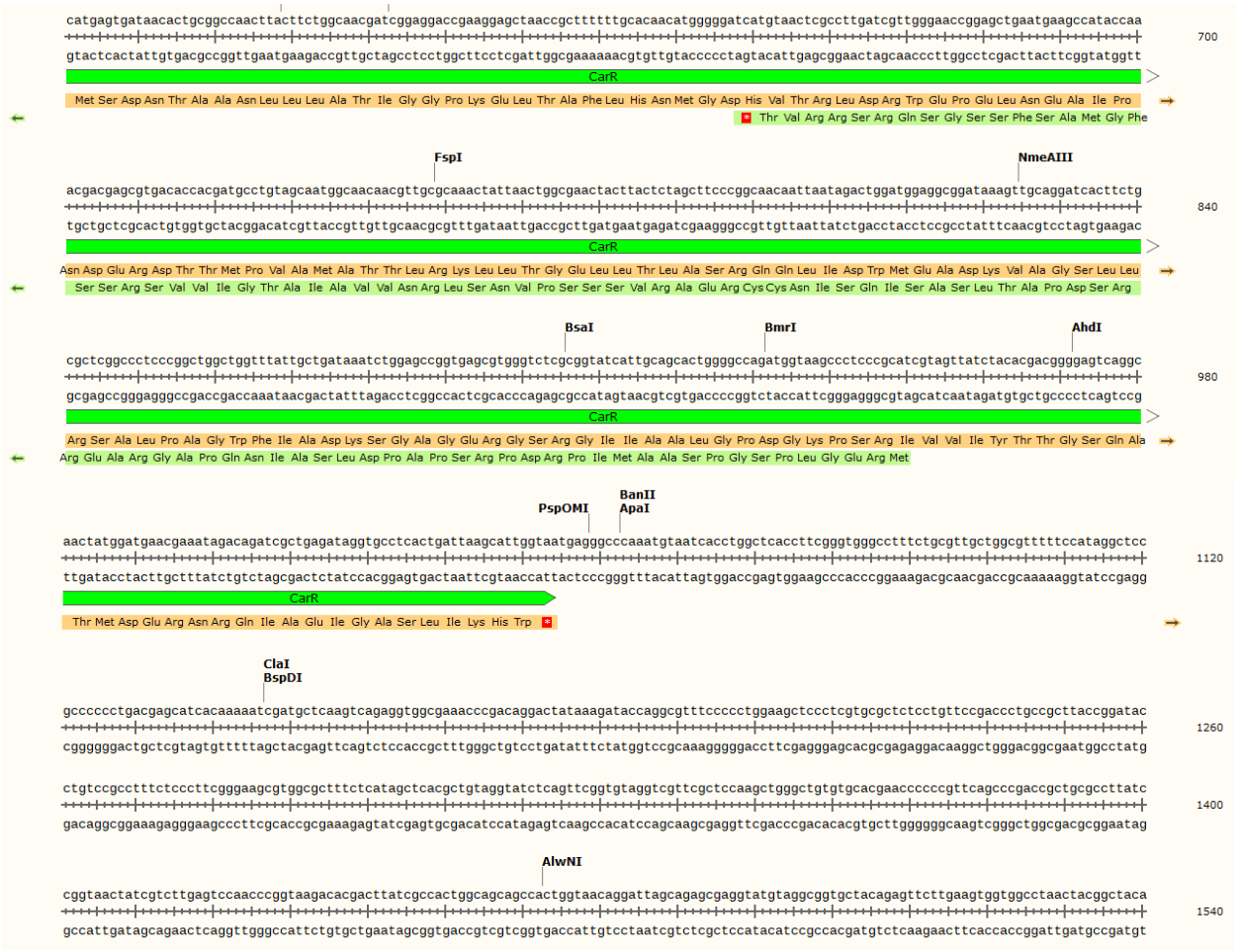
5'
Ribozyme mRNA
T7 terminator

# SEQUENCES OF THE SA11 CS EXPRESSION PLASMID DESIGNED AND CONSTRUCTED IN THIS STUDY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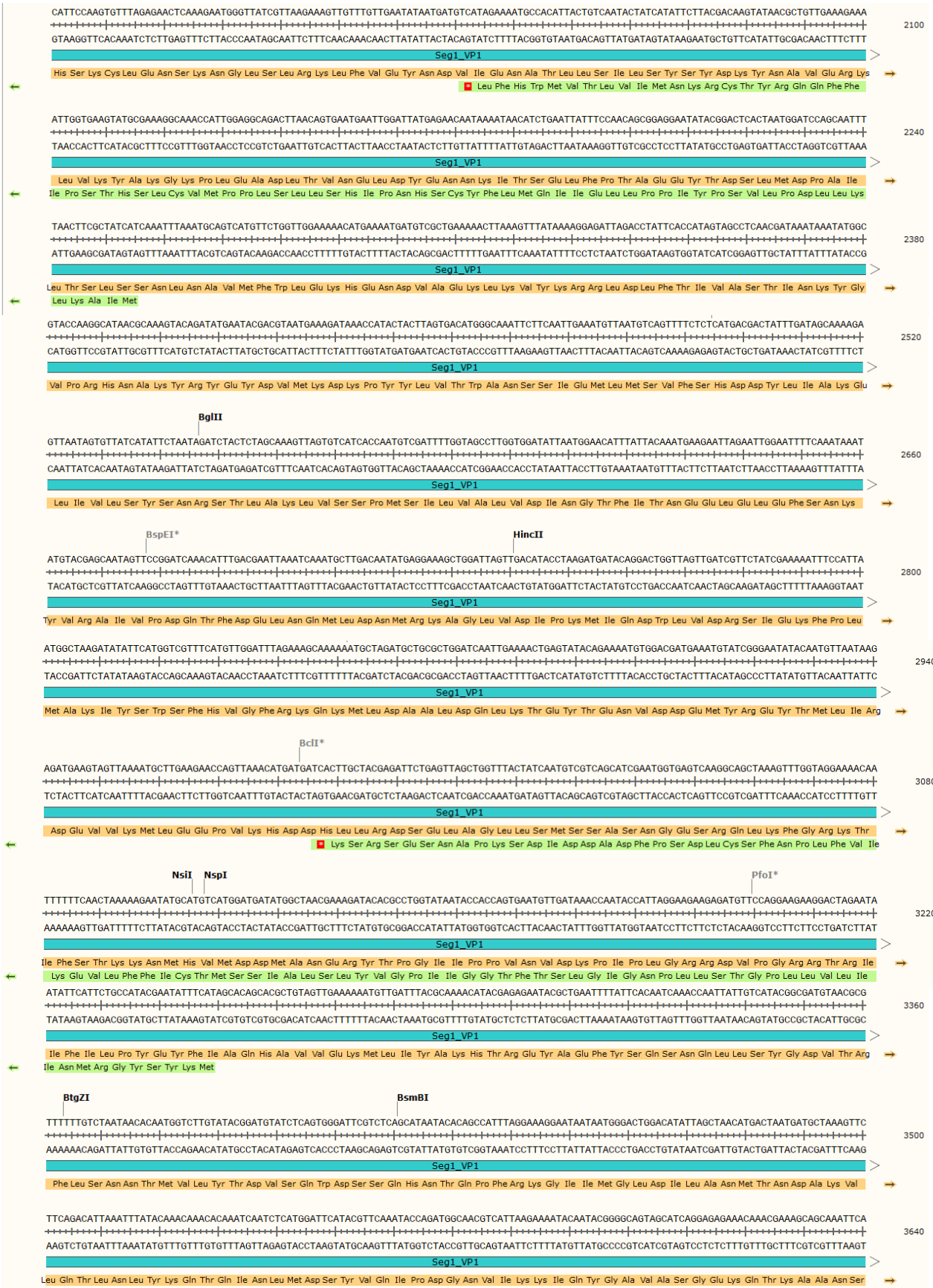
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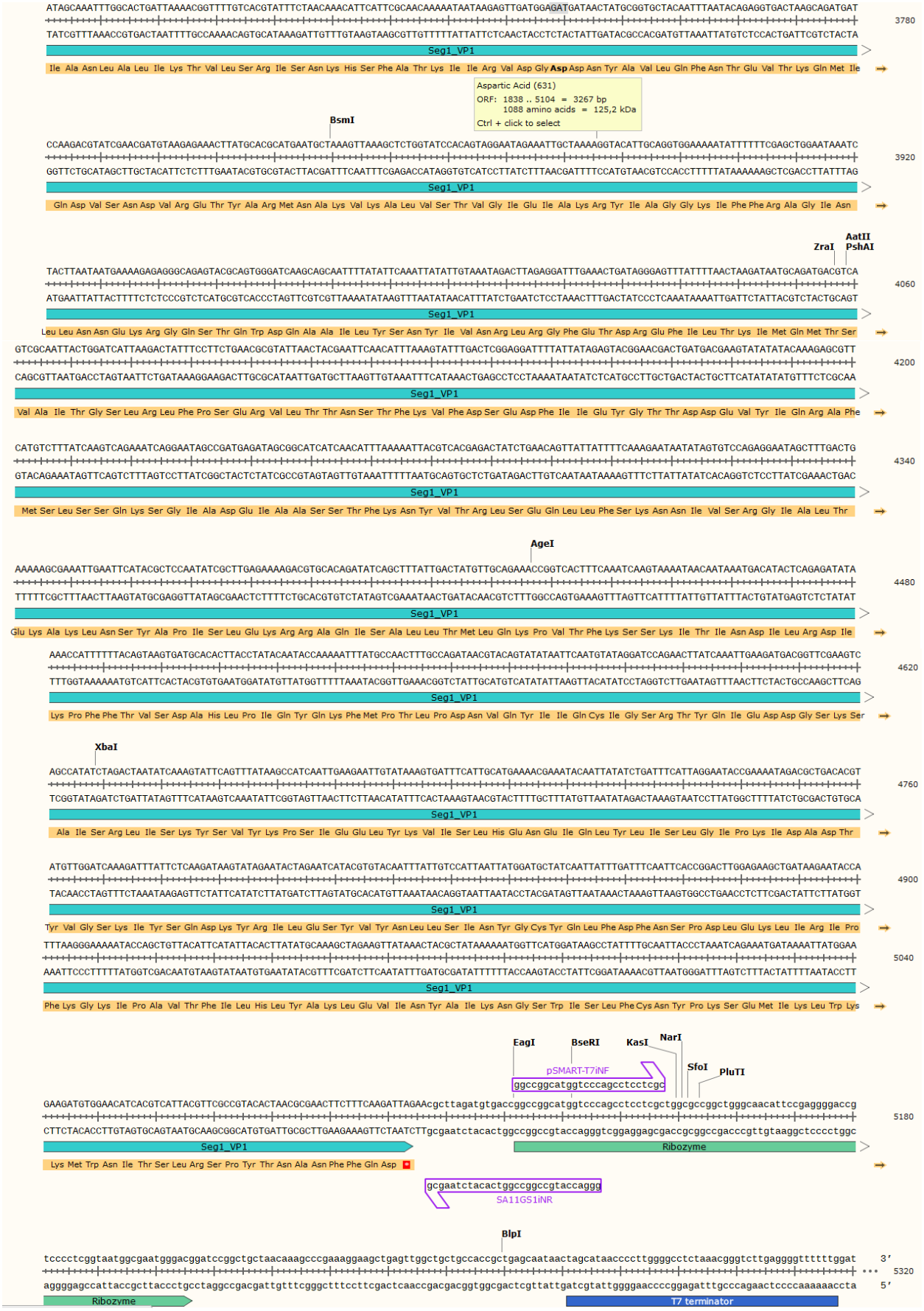
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Ribozyme
T7 terminator


SwaI
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XmnI
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CarR
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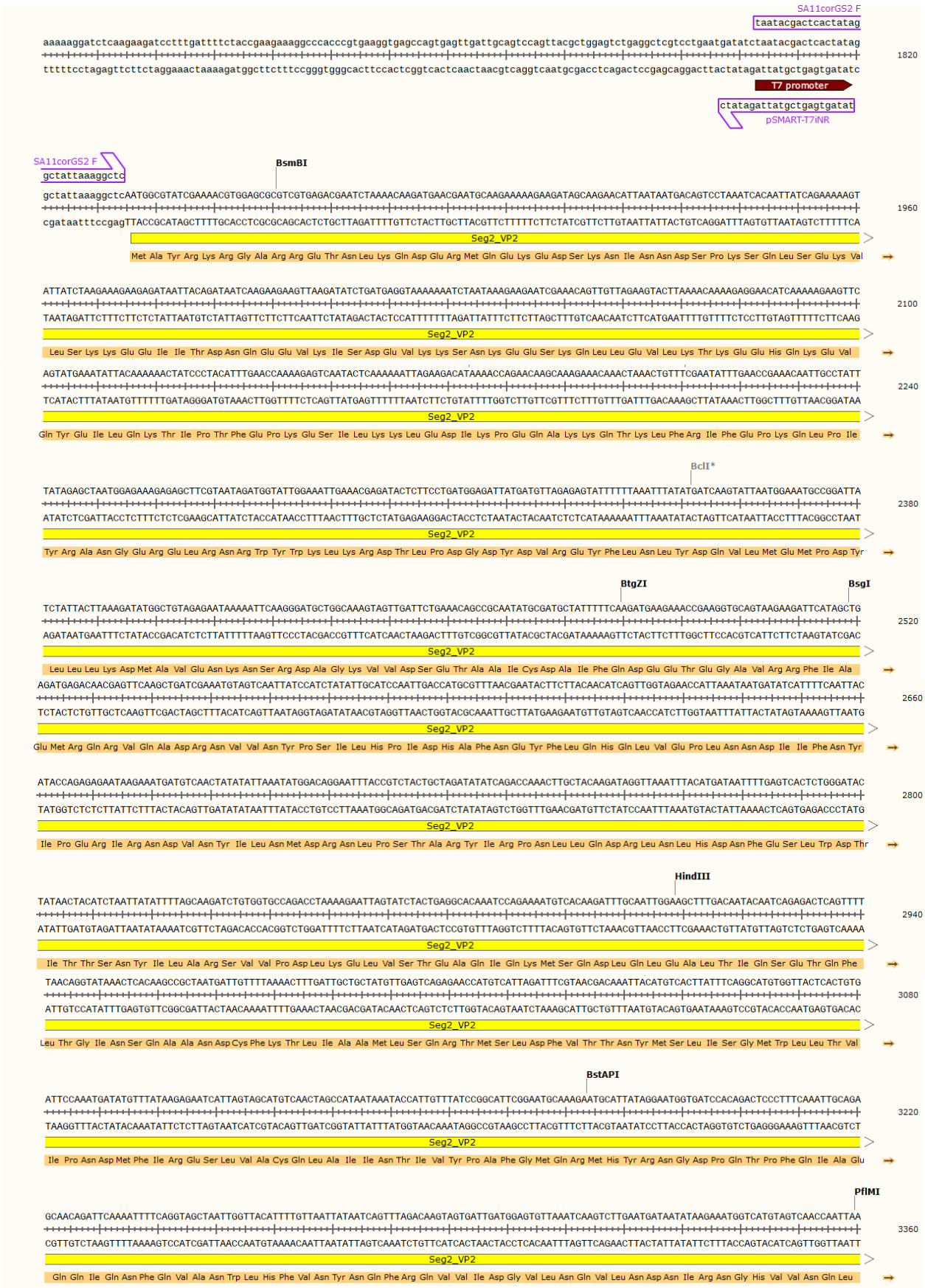
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Seg2_VP2
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BstXI
AfeI
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BlpI
StyI
T7 terminator


5'
3'
Bpu10I
XmnI
CarR
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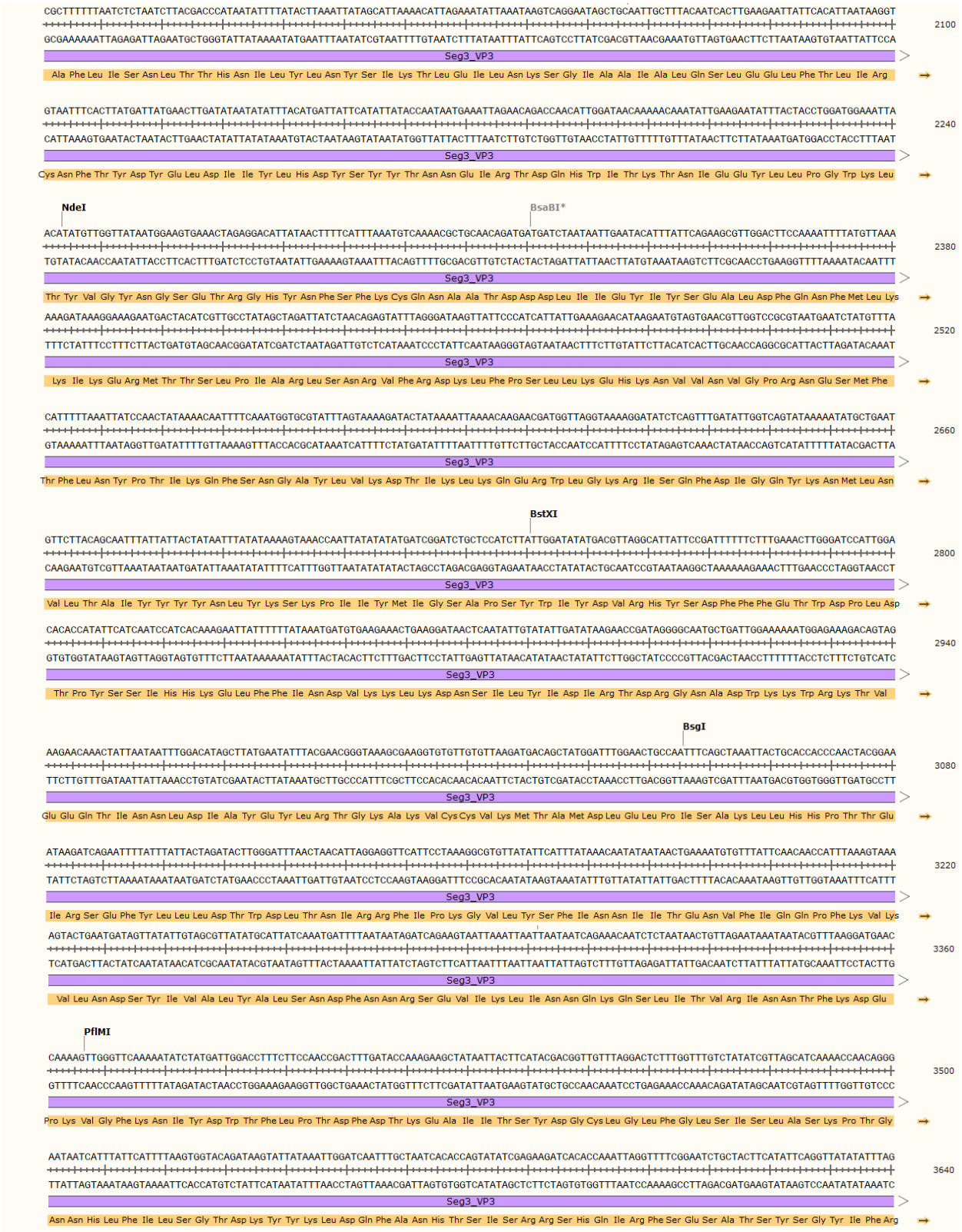
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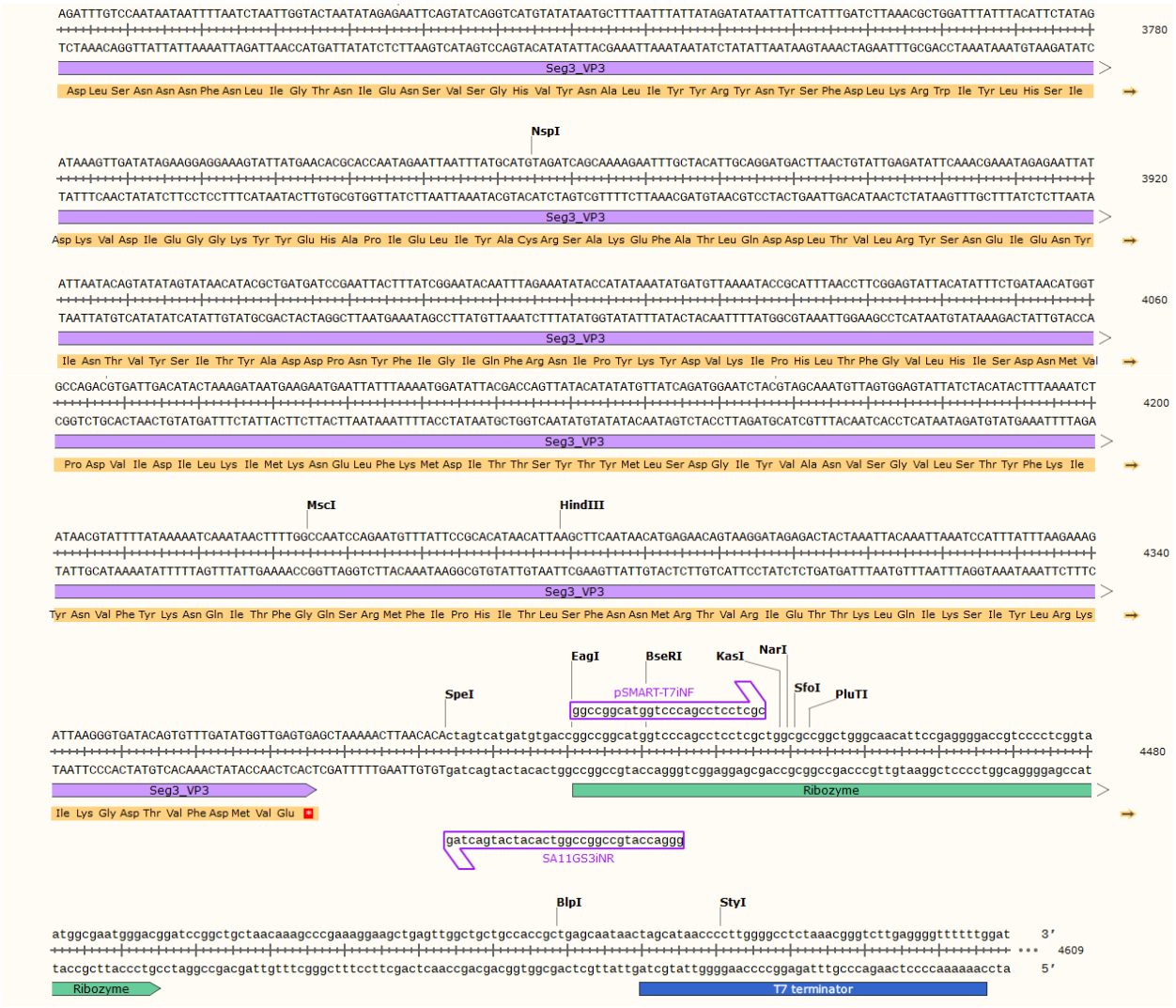
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BlpI
StyI
T7 terminator
Bpu10I
CarR


CarR
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BsaI
CarR
CarR
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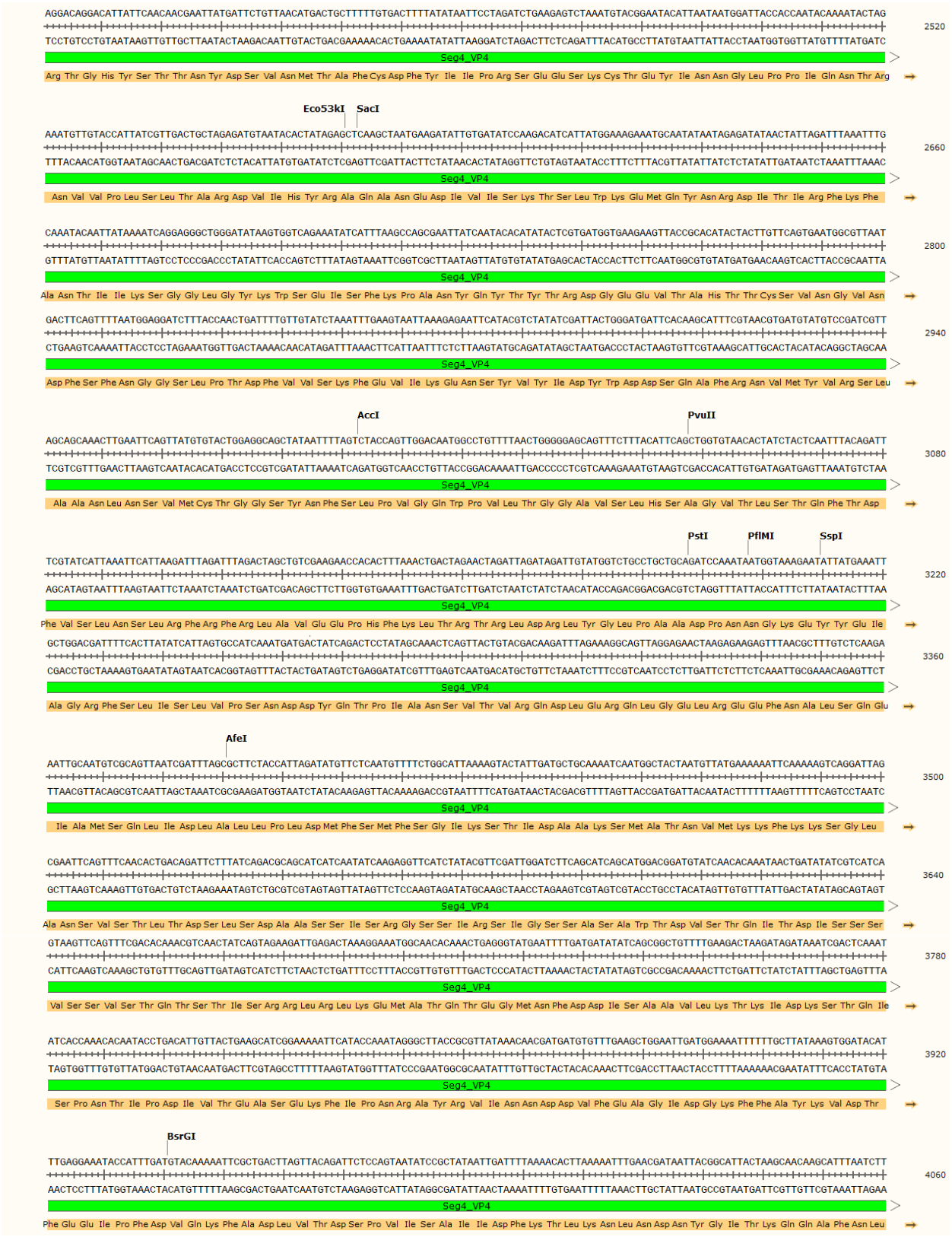
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T7 terminator
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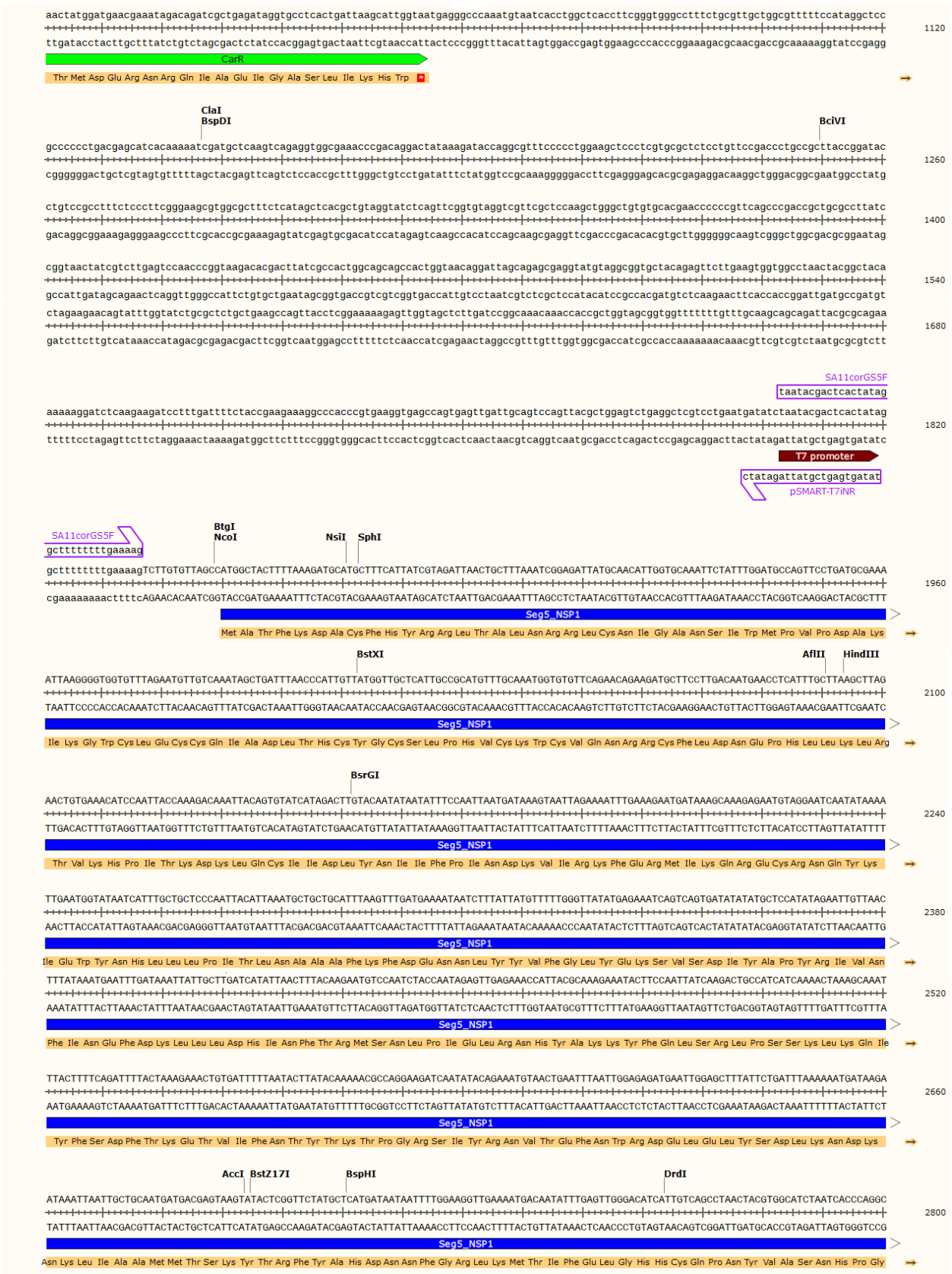
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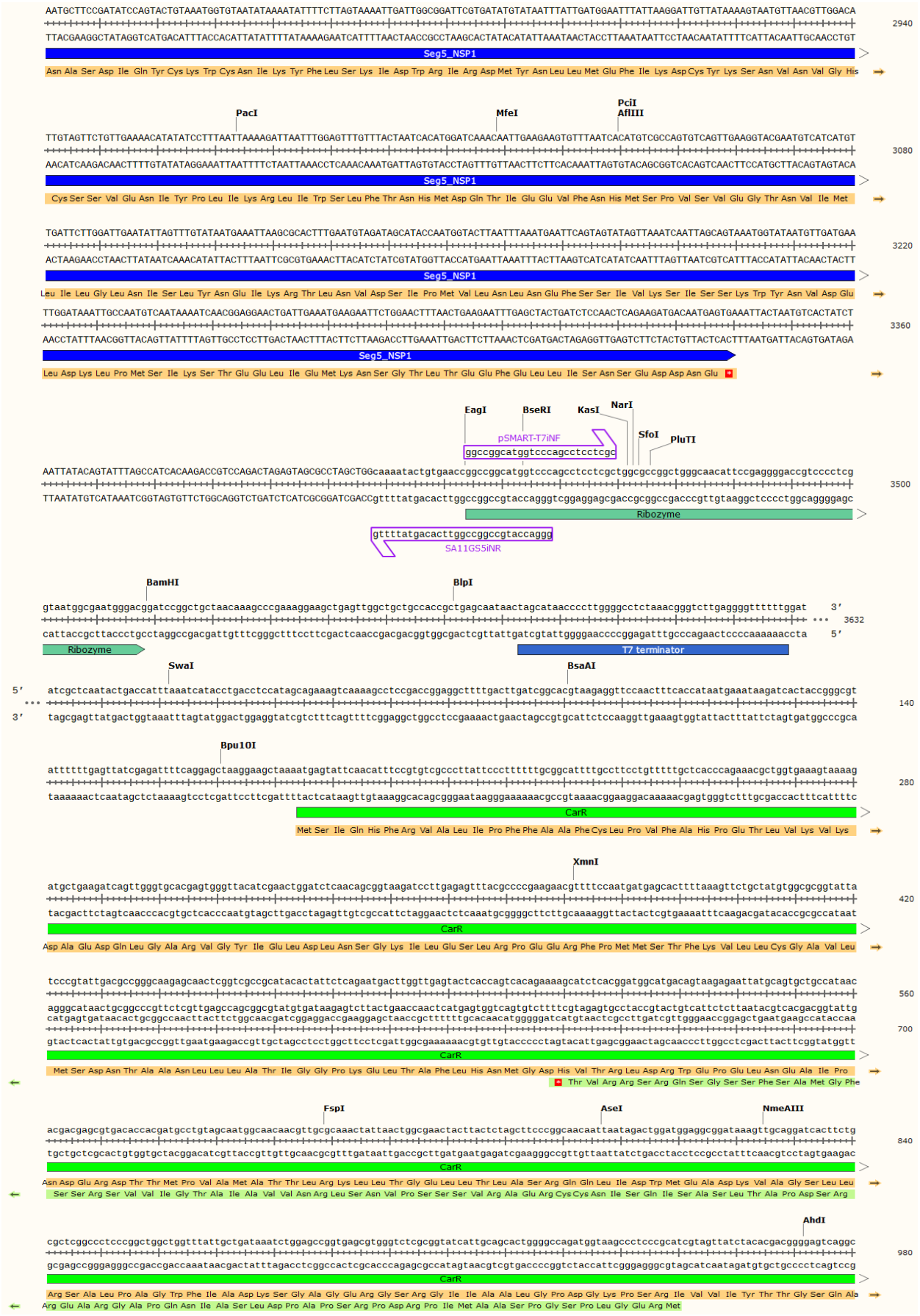
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CarR
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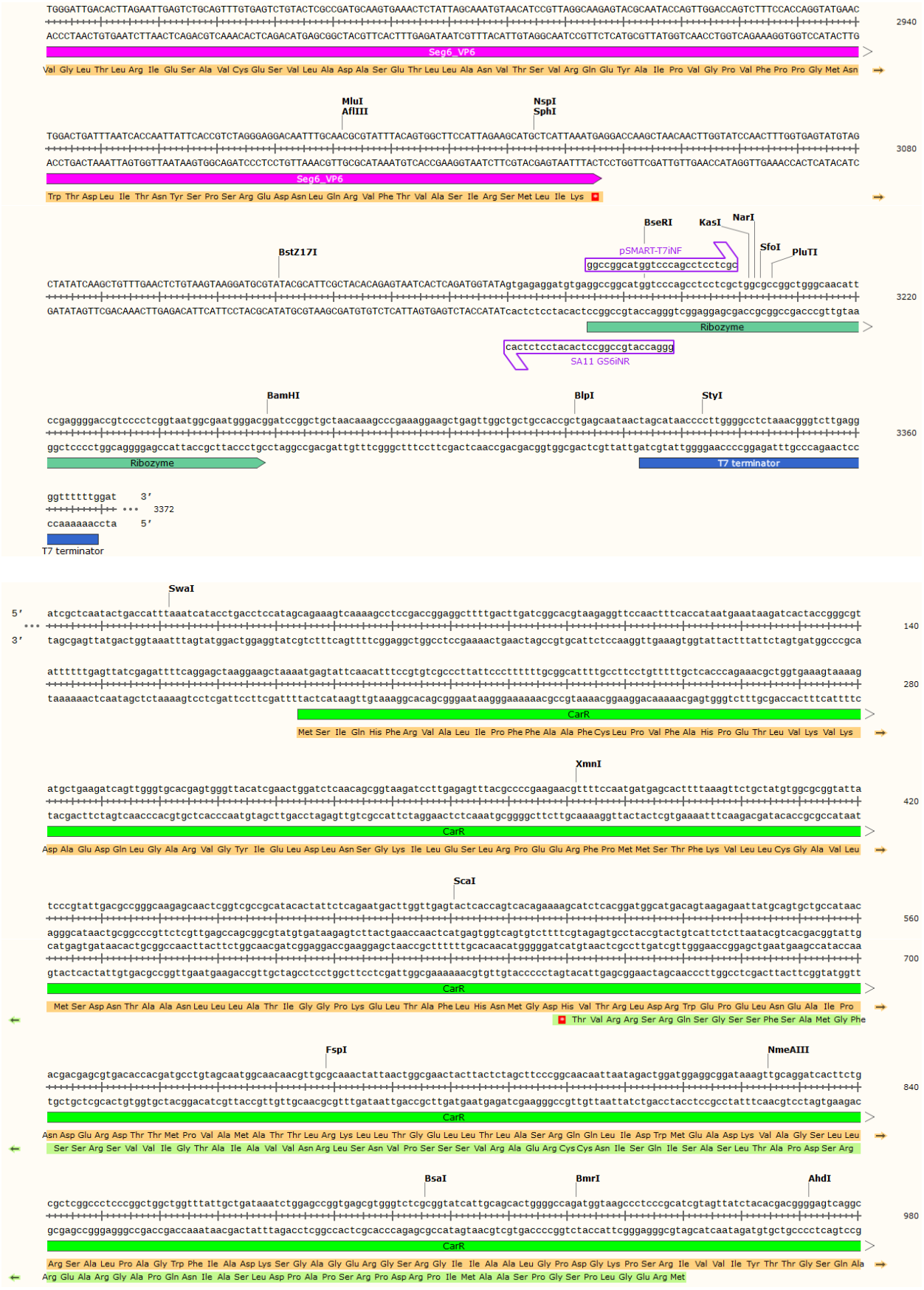
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CarR
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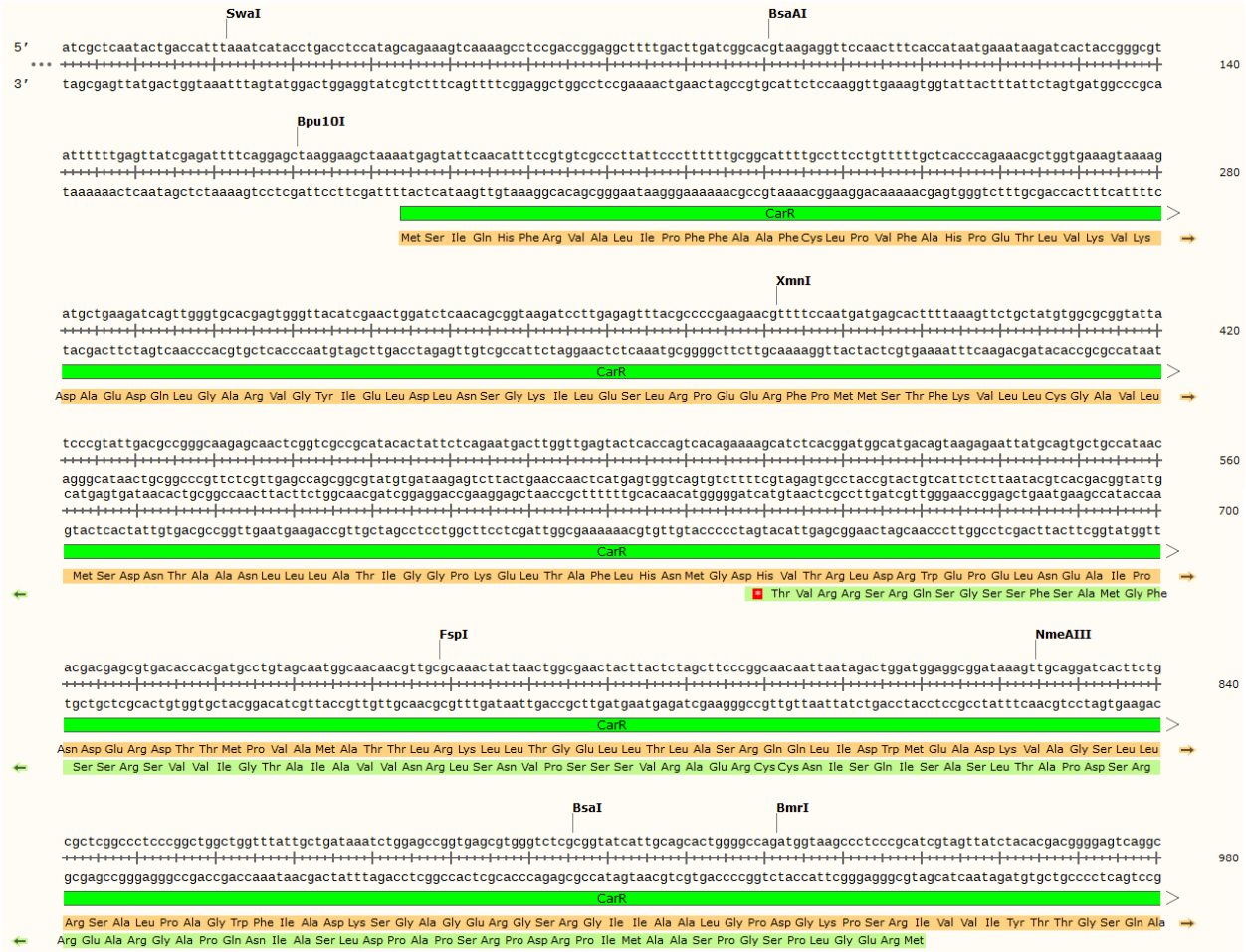
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CarR
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AlwNI
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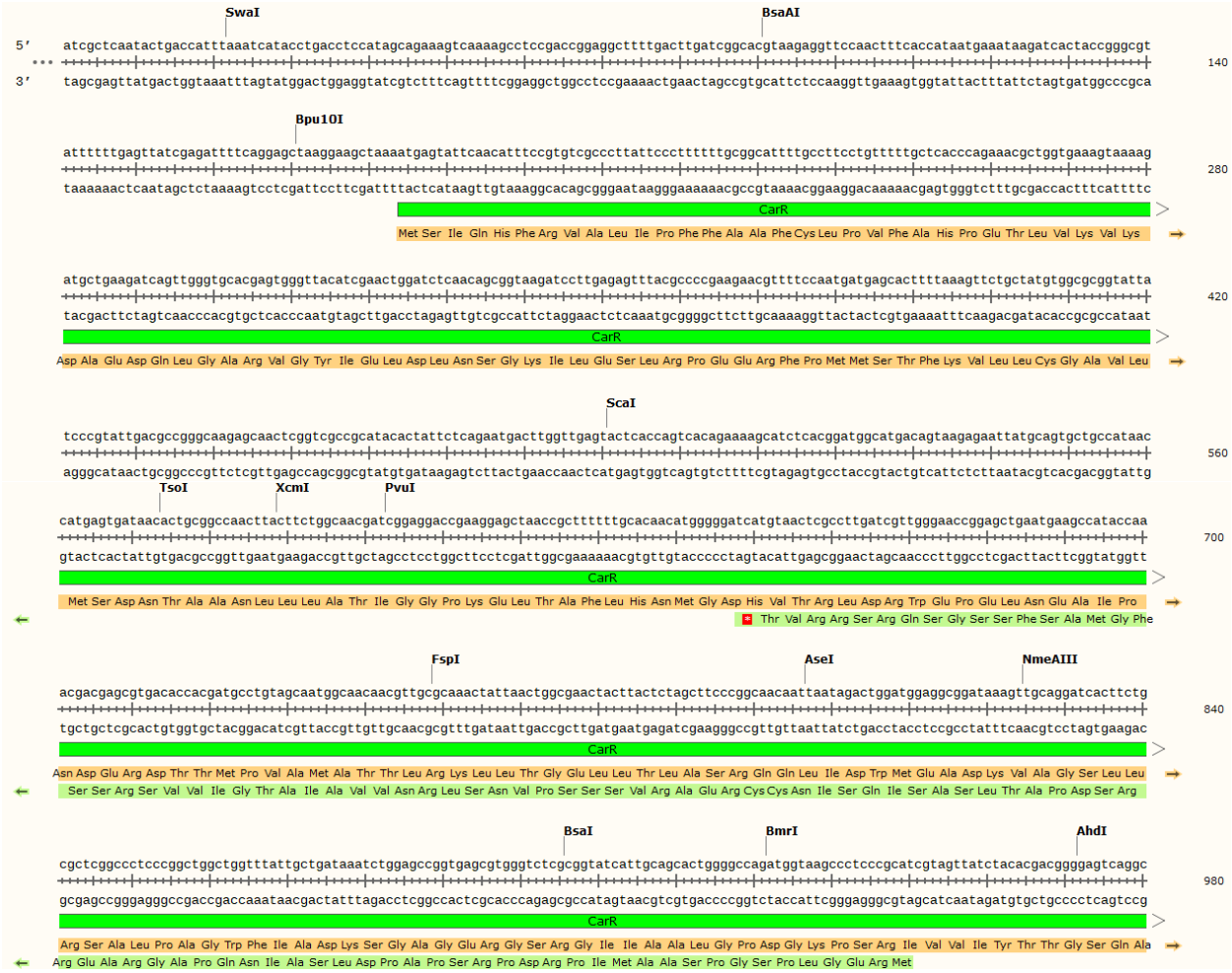
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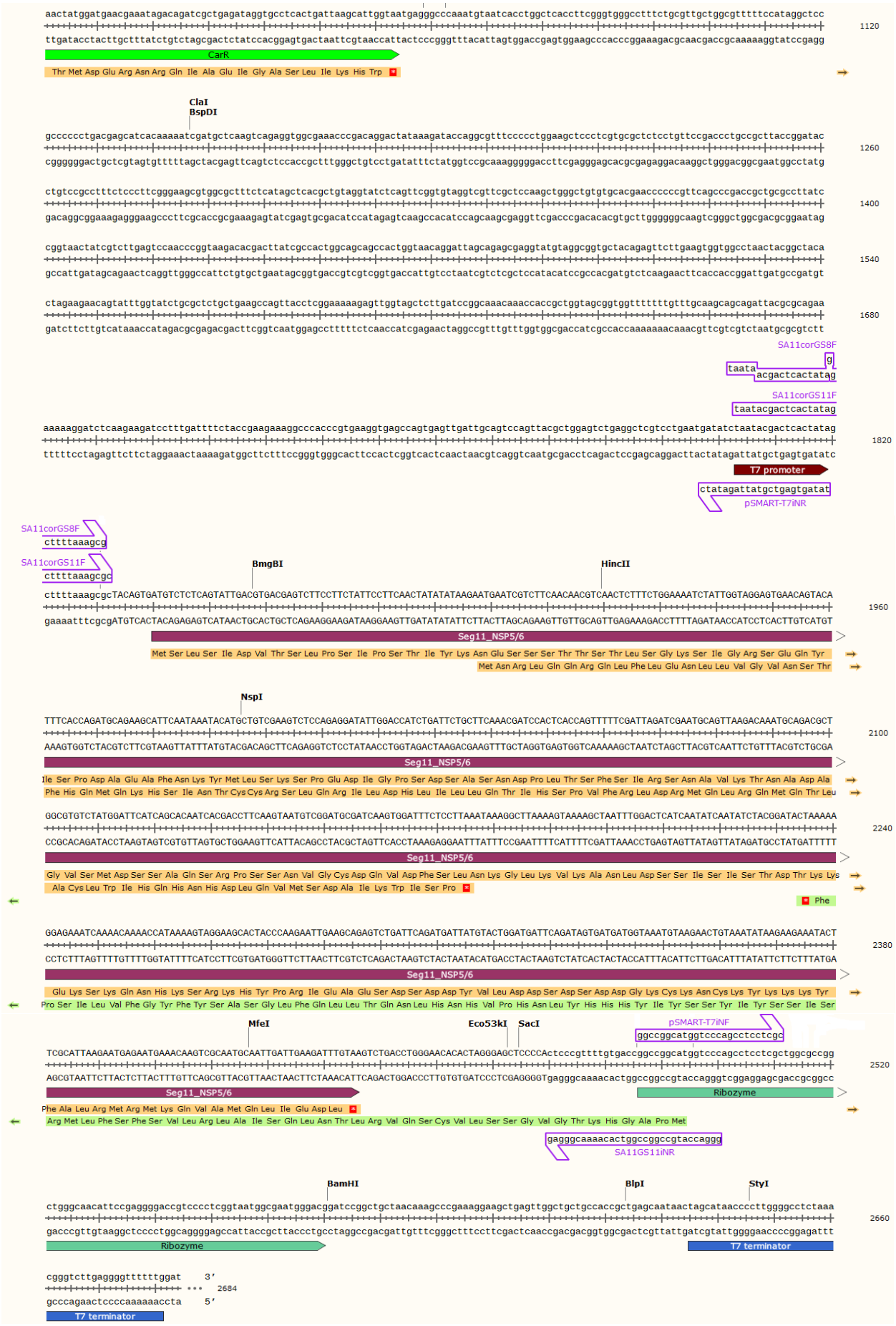
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