

Expression of rotavirus proteins and virus-like particles in bacterial and insect cells

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“Life before Death.
Strength before Weakness.
Journey before Destination.”
— Brandon Sanderson, *The Way of Kings*

“And so, does the destination matter? Or is it the path we take? I declare that no accomplishment has substance nearly as great as the road used to achieve it. We are not creatures of destinations. It is the journey that shapes us. Our callused feet, our backs strong from carrying the weight of our travels, our eyes open with the fresh delight of experiences lived.”

“If the journey itself is indeed the most important piece, rather than the destination itself, then I traveled not to avoid duty—but to seek it.”

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Abstract

Rotavirus infection remains a leading cause of severe gastroenteritis affecting infants and young children. Currently, only live-attenuated (LA) vaccines are licensed and globally administered. These vaccines are not as effective in low- and middle-income countries such as those in Sub-Saharan Africa and Asia compared to high-income countries. This immunity gap could be bridged by using non-replicating subunits vaccines such as virus-like particles (VLPs).

In this study, proteins VP2, VP6, and VP7 from two rotavirus strains, the South African RVA/Human-wt/ZAF/GR10924/1999/G9P[6] strain and SA11 RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] strain, were expressed in bacteria and insect cells. We attempted to reduce the toxicity associated with VP7 expression in bacteria by removing the endoplasmic reticulum leader peptide (amino acids 1-50). Although the bacteria expressed a soluble protein, a deletion of 159 bp (amino acids 121-174) occurred in the middle of the VP7 open reading frame (ORF). VP2 and VP6 was expressed in bacteria but the proteins were not soluble.

The ORFs of SA11 VP2/6/4 and VP7 were insect-cell codon-optimised. The ORFs of VP2 and VP6 were cloned into the pFBdual donor plasmid for double-layered particle (DLP) production. The ORFs of SA11 VP4 and VP7 were cloned in separate pFBdual donor plasmids for flexible transcapsidation. The Bac-to-Bac expression system was used for the generation of recombinant baculoviruses. Overexpression of VP2 could be observed with Coomassie-stained SDS-PAGE and VP6 expression through western blot. VP2/6 expression was confirmed through a novel immunofluorescent monolayer assay (IFMA) protocol and SDS-PAGE. DLP assembly was confirmed with transmission electron microscopy (TEM). VP4 expression was confirmed through PAGE. However, no VP7 expression could be detected on PAGE. On TEM, no triple-layered particles (TLPs) were detected even with the addition of 20 mM calcium supplemented in the media and buffers.

Keywords: Rotavirus, virus-like particles, South Africa, GR10924 G9P[6], SA11, VP2/6/4 and VP7, codon optimisation, bacterial expression, baculovirus expression, immunofluorescent monolayer assay, double-layered particles, triple-layered particles.

Opsomming

Rotavirus infeksie bly 'n hooforsaak van ernstige gastro-enteritis wat babas en jong kinders aantast. Tans word slegs lewende geattenuëerde (LA) entstowwe gelisensieer en wêreldwyd toegedien. Hierdie entstowwe is nie so effektief in ontwikkelende lande soos dié in Sub-Sahara Afrika en Asië in vergelyking met meer ontwikkelde lande nie. Hierdie gaping in immuniteit kan oorbrug word deur die gebruik van nie-repliserende subeenheid-entstowwe soos virusagtige partikels (VAPs).

In hierdie studie, is proteïene VP2, VP6, VP4 en VP7 van twee rotavirusstamme, die Suid-Afrikaanse RVA/Human-wt/ZAF/GR10924/1999/G9P[6] stam en SA11 RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] stam uitgedruk in bakterieë en insekselle. 'n Poging is aangewend om die toksisiteit wat verband hou met VP7-uitdrukking in bakterieë te verminder deur die endoplasmiese retikulumleierpeptied (amino sure 1-50) te verwyder. Alhoewel die bakterieë 'n oplosbare proteïen uitgedruk het, het 'n uitsnyding van 159 bp (amino sure 121-174) in die middel van die VP7 oop leesraam (OLR) plaasgevind. VP2 en VP6 is uitgedruk in bakterieë, maar nie as oplosbare proteïene nie.

Die OLRe van SA11 VP2/6/4 en VP7 is insekselkodon ge-optimaliseer. Die OLRe van VP2 en VP6 is gekloneer in die pFBdual skenkerplasmied vir dubbellaag partikels (DLP) produksie. Die OLRe van SA11 VP4 en VP7 is in afsonderlike pFBdual skenkerplasmiede gekloon vir buigsame transkapsidasie. Die Bac-to-Bac uitdrukkingstelsel is gebruik om rekombinante baculovirusse te genereer. Ooruitdrukking van VP2 is waargeneem met Coomassie-gekleurde NDS-PAGE en VP6 uitdrukking deur westerlike klad. VP2/6-uitdrukking is bevestig deur middel van 'n nuwe immunofluoreserende monolaag-toets (IFMT)protokol en NDS-PAGE. DLP-samestelling is bevestig met transmissie-elektronmikroskopie (TEM). VP4-uitdrukking is bevestig deur middel van PAGE. Geen VP7-uitdrukking kon egter met PAGE opgespoor word nie. Met TEM is geen TLPs opgespoor nie, selfs met die byvoeging van 20 mM kalsium aangevul in die media en buffers.

Sleutelwoorde: Rotavirus, virusagtige partikels, Suid-Afrika, GR10924 G9P[6], SA11, VP2/6/4 en VP7, kodon-optimalisering, bakteriële uitdrukking, baculovirusuitdrukking, immunofluoreserende monolaag-toets, dubbellaag partikels, trippellaag partikels.

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Chapter 1 Literature Review

1.1 An overview of rotavirus

In 1958, Dr. Hubert Malherbe isolated 15 viruses from overtly healthy vervet monkeys (*Cercopithecus aethiops pygerythrus*) during the poliovirus vaccine era at the National Institute of Virology, Johannesburg, South Africa. One of these viruses was, designated as Simian Agent 11, i.e., SA11 (Malherbe & Harwin, 1963), and was later identified as rotavirus.

In 1973, Bishop and associates first identified human rotavirus while studying electron micrographs of epithelial cells of duodenal mucosa of children with acute non-bacterial gastroenteritis (Bishop *et al.*, 1973). The virus was initially thought to be orbivirus-like due to its morphology, size, and the occurrence of both enveloped and naked particles within cisternae. However, it was later correlated to work done in 1958 by Dr. Malherbe.

Symptoms of rotavirus (RV) infection include vomiting, severe diarrhoea, and abdominal pain. The significant factors causing deaths in these cases are dehydration and electrolyte imbalance associated with RV infection. RV is commonly spread through the faecal-oral route (Ansari *et al.*, 1991) and can be infectious for a long time outside the body, thus increasing the risk of infection in low- and middle-income countries. Acute gastroenteritis (AGE) was still the fourth leading cause of death in infants and children under five years in 2015 when rotavirus accounted for more than a third of these deaths (Troeger *et al.*, 2018). Vaccination provides the only reasonable means to reduce the associated morbidity, mortality, and economic impact.

Two commercially licensed, live-attenuated rotavirus vaccines have been developed and used since 2006, namely Rotarix™ and RotaTeq®. In 2018, two new live-attenuated vaccines, namely Rotasiil® and Rotavac® were implemented. Initial vaccine trials indicated efficient protection against RV-

associated diarrhoea, resulting in the WHO recommending these vaccines' implementation into global immunization programs in 2009 (WHO, 2009). Follow-up studies on vaccine impact indicated that the efficiency of these two vaccines varied between developed and developing countries, with the latter showing reduced efficacy, see Section 1.6 (Armah *et al.*, 2012; Madhi *et al.*, 2010; Zaman *et al.*, 2010).

One of the most efficient ways to bridge the gap in immunity in developing countries, that have already received commercially licensed RV vaccines, is by introducing regionally specific, non-replicating subunit vaccine boosters such as virus-like particles (VLPs). Recombinantly expressed rotavirus VLPs can elicit a humoral and cellular immune response while being non-infectious in animal models (Fernandez *et al.*, 1998). Chimeric RV-VLPs, displaying VP7 and VP4 from human strains, can be assembled onto a universal double-layered particle (DLP) backbone, providing the ideal platform for regionally or seasonally specific vaccine candidates (Jere *et al.*, 2014)

Rotavirus VLPs have been expressed in various expression systems throughout the years, each with advantages and drawbacks. At the North-West University, the primary means of VLP production were bacterial and insect cells expression systems (Jere, 2012; Naude, 2014). Bacterial expression systems have proven difficult to establish, with limited protein expression and infrequent repeatability of VLP production in the last few years. Using the baculovirus Bac-to-Bac expression system, insect cell expression had more success regarding protein expression and chimeric VLP assembly. See Section 1.7.4.

This MSc aimed to develop an SA11 VP2/VP6 DLP as a universal platform for presenting various regional rotavirus VP7 and VP4 antigens as future subunit booster vaccines.

1.2 Rotavirus classification

The Baltimore classification system places viruses into families, depending on the type of genome they have and the replication method (Baltimore, 1971) (Figure 1). According to this system, rotavirus belongs to Group III, comprised of dsRNA viruses that infect a wide range of hosts, including animals, plants, fungi, and bacteria. The most prominent member of this Group is the *Reoviridae* family, of which rotaviruses form one genus (ICTV, 2020).

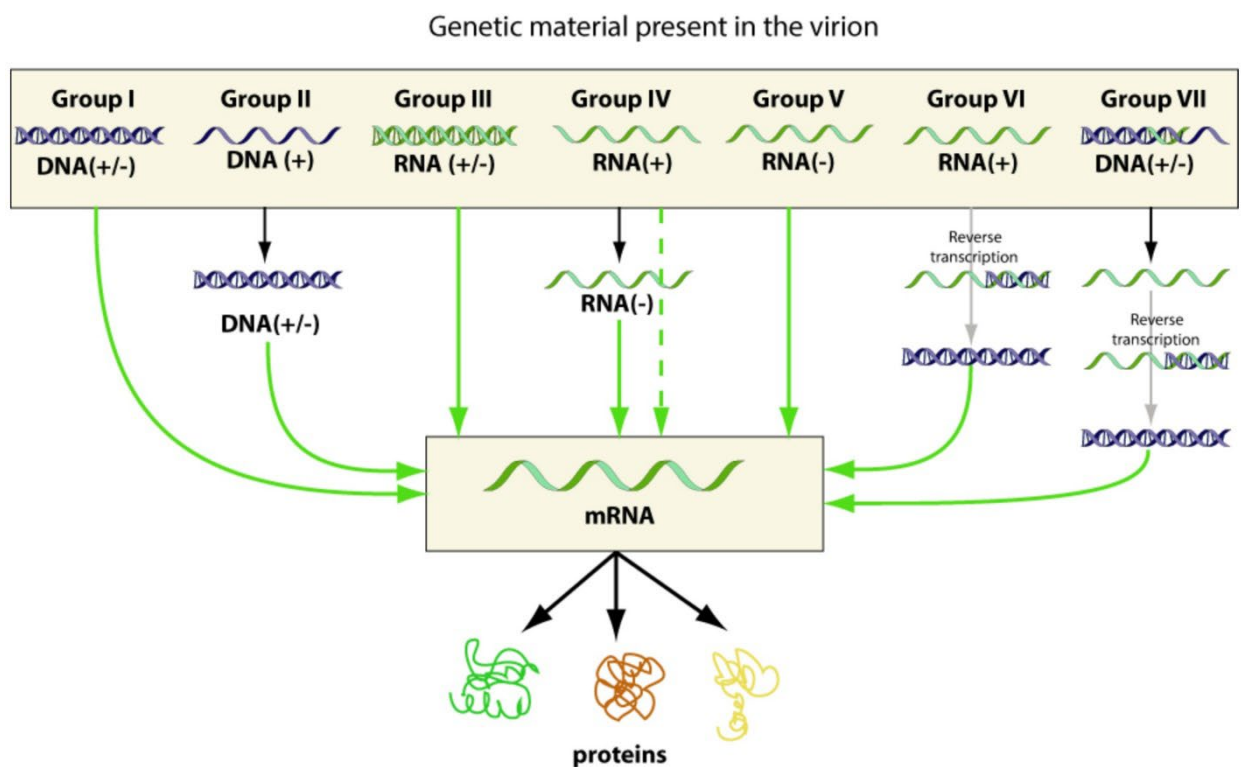


Figure 1: The Baltimore classification system categorises viruses into families according to their genome type as well as their method of replication. Images taken from ViralZone 20/11/2020 (public domain).

The *Reoviridae* family (Table 1) is divided into two subfamilies: *Sedoreovirinae*, entailing six genera, and *Spinoreovirinae* entailing nine genera. *Reo* is an acronym for the respiratory enteric orphan because the first family members were discovered in the respiratory and enteric tracts of humans and animals but did not cause any illness (Maclachlan & Dubovi, 2010). Viruses belonging to the *Reoviridae* family have multi-layered, non-enveloped, icosahedral capsids. The

genome of these viruses is composed of 10-12 segments of double-stranded RNA (dsRNA). The rotaviruses belong to the subfamily *Sedoreovirinae*, as the inner capsid of the virus is smooth (Attoui & Mertens, 2007).

Table 1: Classification of the *Reoviridae* family.
([https://viralzone.expasy.org/104?outline=all by species](https://viralzone.expasy.org/104?outline=all%20by%20species))

Sub-family	Genera	Number of species	Genome segments	Examples of selected types of species
<i>Sedoreovirinae</i>	<i>Cardoreovirus</i>	1	11	Eriocheir sinensis reovirus
	<i>Mimoreovirus</i>	1	11	Micromonas pusilla reovirus
	<i>Orbivirus</i>	22	10	Bluetongue virus
	<i>Phytoreovirus</i>	3	12	Wound tumor virus
	<i>Rotavirus</i>	8	11	Rotavirus A
	<i>Seadornavirus</i>	3	11	Banna virus
<i>Spinareovirinae</i>	<i>Aquareovirus</i>	7	11	Aquareovirus A (Chum Salmon reovirus CS)
	<i>Coltivirus</i>	2	12	Colorado tick fever virus (strain Florio)
	<i>Cypovirus</i>	16	10	Lymantria dispar cypovirus 1
	<i>Dinovernavirus</i>	1	9	Aedes pseudoscutellaris reovirus
	<i>Fijivirus</i>	9	10	Fiji disease virus
	<i>Idnoreovirus</i>	5	10 – 11	Idnoreovirus 1: Diadromus pulchellus
	<i>Mycoreovirus</i>	3	11 or 12	Mycoreovirus 1: Cryphonectria parasitica mycoreovirus-1
	<i>Orthoreovirus</i>	6	10	Mammalian orthoreovirus
	<i>Oryzavirus</i>	2	10	Rice ragged stunt virus

1.2.1 Serologic and genetic classification of rotavirus

Rotaviruses can be divided into nine distinct serological groups (A to D, F to J). Antibodies to viral protein 6 (VP6), one of the major structural proteins, differentiate the subgroups. Serogroups A, B, and C infect humans and animals, with Group A rotaviruses being the primary cause of RV-associated infections in

humans (Changotra & Vij, 2017). Groups D and F to J have only been found in animals to date.

The serogroups are further divided into serotypes based on neutralizing epitopes of VP4 (protease-sensitive outer capsid protein) and VP7 (glycoprotein), annotated as various G- and P-serotypes. Historically, serological approaches were used to differentiate between various rotavirus G- and P-types. However, recently classification has moved to a more sequenced-based approach. Both the immunogenic and PCR-based methods have been used to determine the G- and P-types. Although a connection between serotypes and genotypes is well established, only a short region of VP4 defines the specific P-type (Gorziglia *et al.*, 1990).

In 2008, a whole-genome classification system was developed based on the nucleotide sequence and appropriate sequence cut-off limits (Matthijssens *et al.*, 2008). The identification of RVA strains has become more informative by assigning a specific genotype to each of the 11 RVA genome segments. The VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genome segments of RV strains are described using the abbreviations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, respectively. The letter of the genotype name used in the abbreviation is derived from the encoded protein's main characteristic (Matthijssens *et al.*, 2011), as shown in Table 2.

Table 2: RVA genotype diversity adapted from Matthijssens showing the rotavirus genes along with the number of genotypes as in 2015.

Gene product	Number of genotypes	The primary function of the gene product
VP7	41G	G lycosylated
VP4	[57]P	P rotease sensitive
VP6	31I	Inner capsid
VP1	27R	R NA-dependent RNA polymerase
VP2	23C	C ore protein
VP3	23M	M ethyltransferase
NSP1	38A	Interferon A ntagonist
NSP2	27N	N TPase
NSP3	27T	T ranslation enhancer
NSP4	31E	E nterotoxin
NSP5	27H	P Hosphoprotein

This system, as well as the nomenclature, is maintained by the Rotavirus Classification Working Group. The guidelines they recommend for uniform nomenclature dictate that as much information as possible for each strain must be included, namely, the RV group / species of origin / country of identification / typical name / year of identification / G- and P-type (Matthijssens *et al.*, 2011). To give an example of how it is combined, simian agent 11 (SA11) selected for this project is described as RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] with the complete genotype of G3-P[2]-I2-R2-C5-M5-A5-N5-T5-E2-H5.

Table 3: Illustration of the classification and nomenclature to the structural and nonstructural protein-encoding genome segments for selected human, bovine, porcine, simian, and avian rotavirus strains (Matthijssens *et al.*, 2008). With permission from the publisher.

			VP[7]	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Wa	Hu	Wa-like	G1	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
KU	Hu	Wa-like	G1	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
Dhaka16-03	Hu	Wa-like	G1	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
D	Hu	Wa-like	G1	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
P(rice)	Hu	Wa-like	G3	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
ST3	Hu	Wa-like	G4	- P[6]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
IAL28	Hu	Wa-like	G5	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
WI61	Hu	Wa-like	G9	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
B3458	Hu	Wa-like	G9	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
RMC321	Hu	Wa-like	G9	- P[19]	- I5	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
Dhaka12-03	Hu	Wa-like	G12	- P[6]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
Matlab13-03	Hu	Wa-like	G12	- P[6]	- I1	- R1	- C1	- M1	- A1	- N1	- T2	- E1	- H1
B4633-03	Hu	Wa-like	G12	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
Dhaka25-02	Hu	Wa-like	G12	- P[8]	- I1	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
DS-1	Hu	DS-1-like	G2	- P[4]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
TB-Chen	Hu	DS-1-like	G2	- P[4]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
B1711	Hu	DS-1-like	G6	- P[6]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
DRC86	Hu	DS-1-like	G8	- P[6]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
DRC88	Hu	DS-1-like	G8	- P[8]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
69M	Hu	DS-1-like	G8	- P[10]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E2	- H2
L26 ^a	Hu	DS-1-like	G12	- P[4]	- I2	- R2	- C2	- M1/M2	- A2	- N1	- T2	- E2	- H1
N26-02	Hu	DS-1-like	G12	- P[6]	- I2	- R2	- C2	- M2	- A2	- N1	- T2	- E6	- H2
RV176-00	Hu	DS-1-like	G12	- P[6]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E6	- H2
RV161-00	Hu	DS-1-like	G12	- P[6]	- I2	- R2	- C2	- M2	- A2	- N2	- T2	- E1	- H2
Au-1	Hu	AU-1-like	G3	- P[9]	- I3	- R3	- C3	- M3	- A3	- N3	- T3	- E3	- H3
T152	Hu	AU-1-like	G12	- P[9]	- I3	- R3	- C3	- M3	- A12	- N3	- T3	- E3	- H6
NCDV-Lincoln	Bo		G6	- P[1]	- I2	- R2	- C2	- M2	- A3	- N2	- T6	- E2	- H3
BRV033	Bo		G6	- P[1]	- I2	- R2	- C2	- M2	- A3	- N2	- T6	- E2	- H3
RF	Bo		G6	- P[1]	- I2	- R2	- C2	- M2	- A3	- N2	- T6	- E2	- H3
Uk	Bo		G6	- P[5]	- I2	- R2	- C2	- M2	- A3	- N2	- T7	- E2	- H3
WC3	Bo		G6	- P[5]	- I2	- R2	- C2	- M2	- A3	- N2	- T6	- E2	- H3
KJ44	Bo		G5	- P[1]	- I1	- R1	- C1	- M2	- A1	- N1	- T1	- E1	- H1
KJ75	Bo		G5	- P[5]	- I1	- R1	- C1	- M2	- A1	- N1	- T1	- E1	- H1
A131	Po		G3	- P[7]	- I5	- R1	- C2	- M1	- A1	- N1	- T1	- E1	- H1
Gottfried	Po		G4	- P[6]	- I1	- R1	- C1	- M1	- A8	- N1	- T1	- E1	- H1
OSU	Po		G5	- P[7]	- I5	- R1	- C1	- M1	- A1	- N1	- T1	- E1	- H1
A253	Po		G11	- P[7]	- I5	- R1	- C2	- M1	- A1	- N1	- T1	- E1	- H1
YM	Po		G11	- P[7]	- I5	- R1	- C1	- M1	- A8	- N1	- T1	- E1	- H1
PO-13	Av		G18	- P[17]	- I4	- R4	- C4	- M4	- A4	- N4	- T4	- E4	- H4
SA11g4"O" (5N)	Si		G3	- P[1]	- I2	- R2	- C5	- M5	- A5	- N5	- T5	- E2	- H5
SA11-5S	Si		G3	- P[1]	- I2	- R2	- C5	- M5	- A5	- N5	- T5	- E2	- H5
SA11-30/19	Si		G3	- P[1]	- I2	- R2	- C5	- M5	- A5	- N5	- T5	- E2	- H5
SA11-30/1A	Si		G3	- P[1]	- I2	- R2	- C5	- M5	- A5	- N5	- T5	- E2	- H5
SA11-H96	Si		G3	- P[2]	- I2	- R2	- C5	- M5	- A5	- N5	- T5	- E2	- H5
SA11-Both	Si		G3	- P[2]	- I2	- R2	- C5	- M5	- A5	- N2	- T5	- E2	- H5

1.3 Rotavirus particles, genome structure, and proteins

The rotavirus group A genome has a total size of 18 522 base pairs (bp) and consists of 11 double-stranded RNA (dsRNA) segments, ranging from 667 - 3 302 bp (Estes & Cohen, 1989). The genome segments are monocistronic, except for genome segment 11, which encodes two proteins, NSP5 and NSP6, in two, out of frame, overlapping ORFs (Mitchell & Both, 1988). The eleven viral dsRNA genome segments have a distinctive migration profile in polyacrylamide gels (Figure 2).

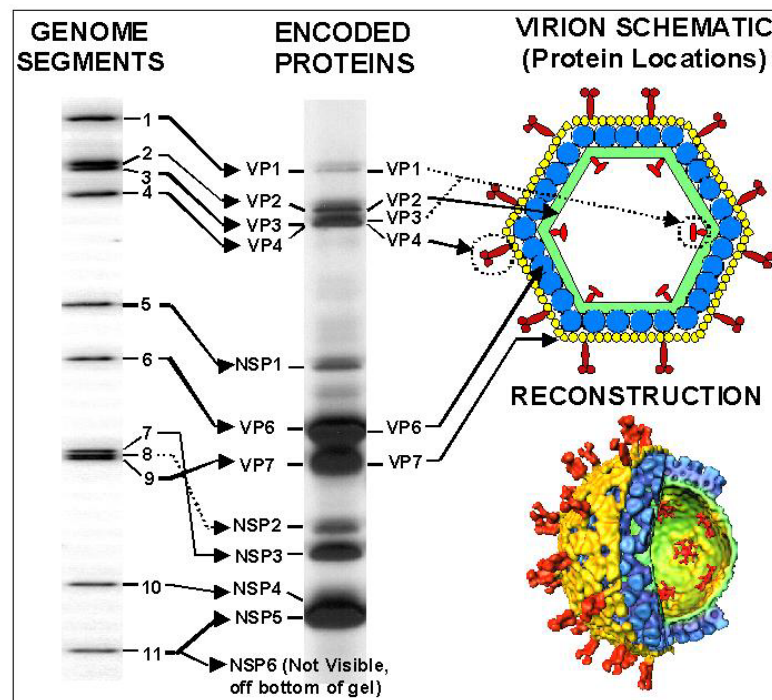


Figure 2: Rotavirus dsRNA genome segments resolved by SDS-PAGE along with the encoded protein products and the virion schematic. The image was taken from Reoviridae.org Date: 15/01/2020 (public domain). With permission from the publisher.

The genome segments' coding assignment and the main functions of the proteins encoded by each of the 11 genome segments are reasonably well understood and summarised in Table 4.

There are six structural (VP) and six non-structural proteins (NSP). The complete virion is composed of three concentric protein layers that enclose the genome. The six non-structural proteins take part in viral replication but are not incorporated in the mature virion (Greenberg & Estes, 2009).

Table 4: Rotavirus genome segments and protein products obtained from Virus Taxonomy, 2011

Genome segment	Size (bp)	Protein*	Protein size (kDa)	Protein copy number/particle	Location	Function
Seg1	3302	VP1 (Pol)	125.0	≤12	core	RNA-dependent RNA polymerase, minor core component, activated by VP2
Seg2	2690	VP2 (T1)	102.4	120	innermost capsid	Core shell protein, RNA binding, sub-group specificity antigen
Seg3	2591	VP3 (Cap)	98.1	≤12	core	Guanylyltransferase, methyltransferase, ssRNA binding, minor core component
Seg4	2362	VP4 VP5* VP8*	86.8 60.0 28.0	180	outer capsid	P-type neutralization antigen, viral attachment protein, homotrimer, spike, hemagglutinin, activated by trypsin cleavage (VP5*, membrane penetration; VP8*, carbohydrate binding)
Seg5	1611	NSP1	58.7	0	cytoplasm	Antagonist of interferon expression, putative viral E3 ubiquitin ligase, RNA binding, RING domain
Seg6	1356	VP6 (T13)	44.8	780	intermediate capsid	Group and sub-group specificity antigen, trimeric, major virion protein
Seg7	1104	NSP3	34.6	0	cytoplasm	Binds 3' end of viral mRNA and cellular eIF4G, promotes circularization of viral mRNAs, surrogate of PABP, inhibits host translation
Seg8	1059	NSP2 (ViP)	36.7	0	viroplasm	Octamer with NTPase, RTPase, and helix destabilizing activities, ssRNA binding, essential viroplasm component, interacts with NSP5
Seg9	1062	VP7 (1) VP7 (2) cleaved form	37.4 33.9	780	outer capsid	G-type neutralization antigen, virion surface glycoprotein, forms Ca ²⁺ -stabilized trimer
Seg10	751	NSP4	20.3	0	RER membrane	RER transmembrane glycoprotein, binds DLPs, essential for budding into ER and addition of outer capsid, age-dependent diarrhea-inducing enterotoxin, disrupts Ca ²⁺ homeostasis
Seg11	667	NSP5	21.7	0	viroplasm	Phosphorylated, O-linked glycosylation, serine-threonine rich, RNA binding, essential viroplasm component, interacts with NSP2
		NSP6	11.0	0	viroplasm	Product of second ORF, RNA binding, interacts with NSP5, non-essential viroplasm component

*Protein structure/function: Pol, RNA polymerase ; Cap, capping enzyme (guanylyltransferase and transmethylase); T1, inner virus structural protein with T = 1 symmetry with two molecules in the icosahedral asymmetric unit (interpreted in the orbiviruses as T = 2 symmetry); T13, inner virus structural protein with T = 13 symmetry; ViP, viral inclusion body or viroplasm matrix protein. Viruses from other species within the genus may have proteins with significant differences in sizes.

The fully infectious, non-enveloped rotavirus particle contains three concentric layers of proteins (Figure 3). The mature virion is also referred to as the triple-layered particle (TLP). When viewed under an electron microscope, the ~80nm mature TLP resembles a wheel, Latin *rota*, thus leading to the name rotavirus (Flewett *et al.*, 1974).

The RNA-dependent RNA polymerase (VP1) and the RNA capping enzyme (VP3) form the replication enzyme complex. These two proteins are located inside the single-layered core particles VP2. VP2 comprises about 90% of the core particle mass, and consists of 120 viral protein molecules arranged in 60 dimers. The VP2 layer is surrounded by 260 trimers of VP6, making up the double-layered particle. VP6 is the major protein in the rotavirus's final organisation by interacting with the outer layer proteins, VP7 and VP4, and the inner protein VP2. VP6 trimers are directly below the VP7 trimers, forming aqueous channels linking the outer surface with the inner core (Mathieu *et al.*, 2001).

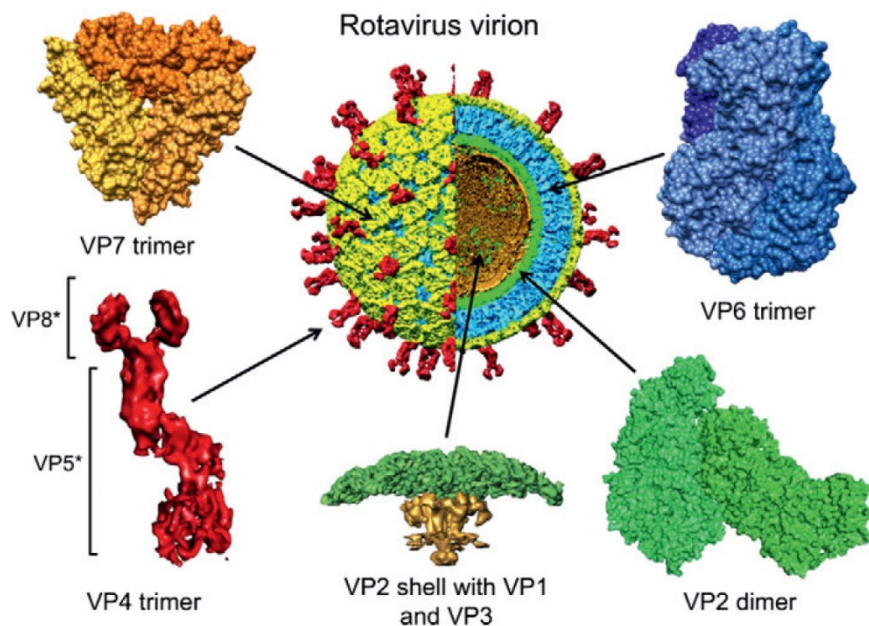


Figure 3: Rotavirus virion. Cryo-EM reconstructed image of a virion with a cut away view. This triple-layered particle shows the association and interaction of the structural proteins with one another. Image taken from Virus Taxonomy, 2012

The virion contains 132 of these aqueous channels, which are a distinct feature of the TLP. These channels are divided into three types based on their position, size, and function. Type I channels, of which there are twelve in total, are used to export the messenger RNA from the replication complex into the cytoplasm for

either translation or templates for replication. Another noteworthy channel is type II, which is closely associated with the VP4 spike, with 60 of these channels spread across the VLP surrounding the fivefold icosahedral axis (Estes & Greenberg, 2013).

The VP4 spike protein, consisting of 60 trimers, has two distinct domains, called the foot (VP5) and head (VP8) (Figure 4 A). The foot domain is anchored in VP6, and the head domain forms the top of the spike protruding out of the VP7 shell. The spike is held in place by both VP6 and VP7. The outer layer of the TLP comprises VP4 and VP7. 260 trimers of VP7 are grouped at the icosahedral and local three-fold axes of $T = 13$ icosahedral lattices. VP7 is a glycoprotein and a calcium-binding protein. Two Ca^{2+} ions are bound at each subunit interface in the trimer. The removal of these Ca^{2+} ions causes destabilization of the VP7 outer layer (Aoki *et al.*, 2009)(Figure 4 B).

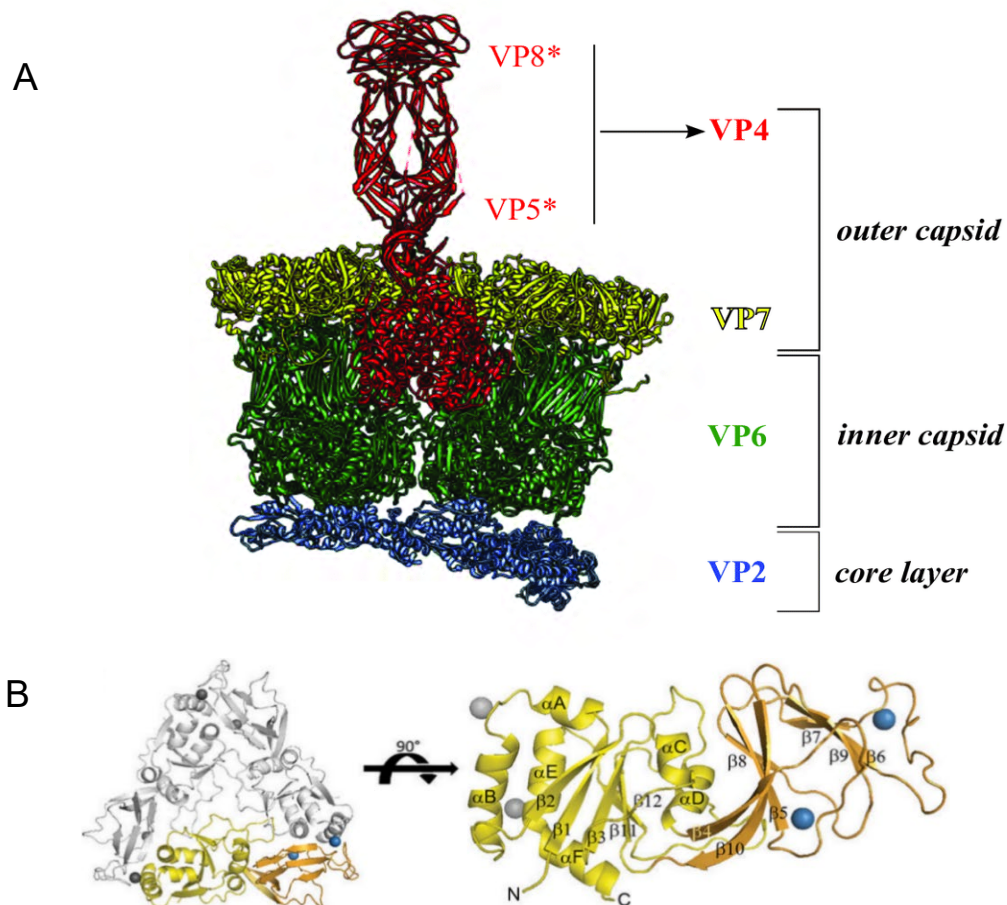


Figure 4: Interaction of rotavirus structural proteins. A: VP4 shown to be divided into VP8* and VP5*. The foot domain is shown to be anchored into VP6, while the head domain protrudes out of VP7 and forms the spike (Settembre *et al.*, 2011). **B:** Ribbon diagram of VP7 in trimer formation (Left). Close up view of one VP7 trimers with the Ca^{2+} ions in blue colour (Right) (Aoki *et al.*, 2009). With permission from the publishers.

1.4 Replication cycle of rotavirus

The RV replication cycle (Figure 5) can be summarised into the following steps: particle attachment, particle penetration, and un-coating, (+)sense mRNA synthesis, viroplasm formation, (+)ssRNA packaging, and (-)sense RNA replication, DLP formation and virion maturation and egress.

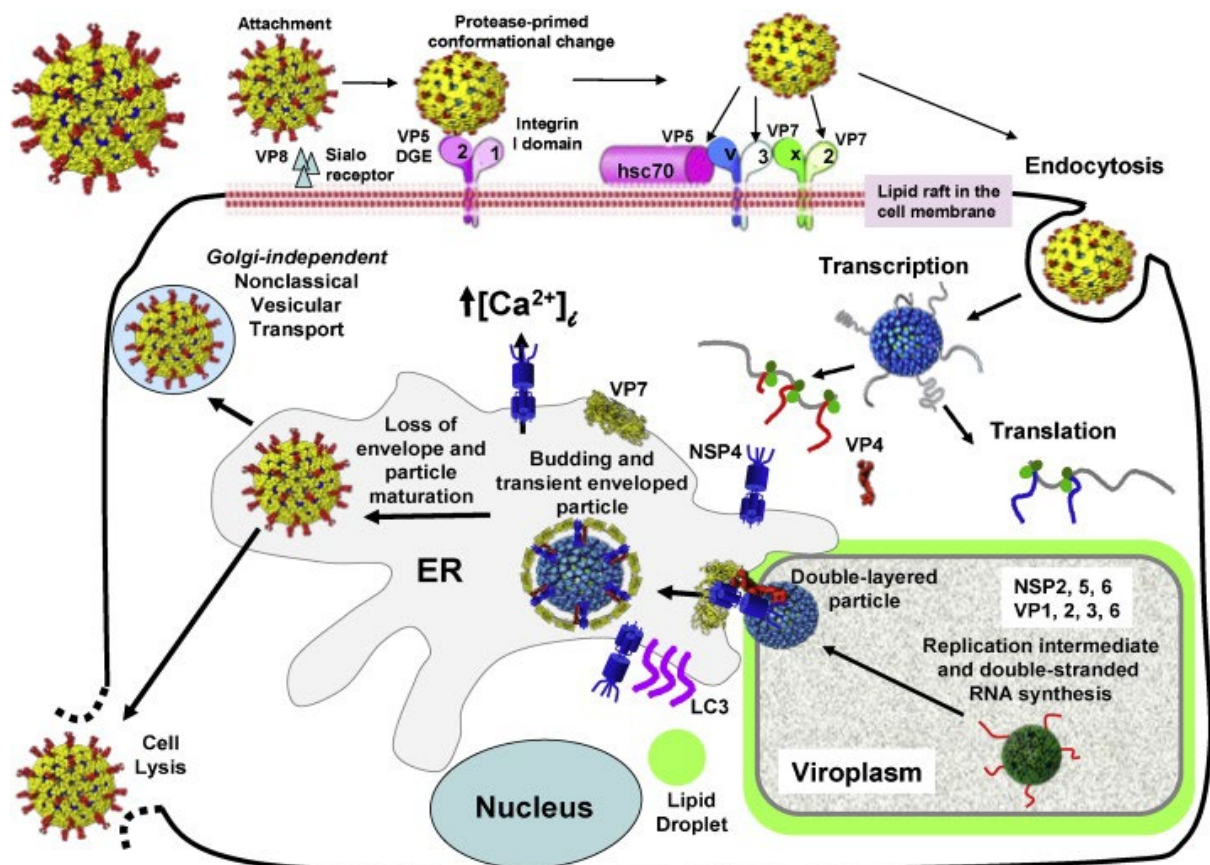


Figure 5: Schematic illustration of the rotavirus' replication cycle. VP4 is cleaved with trypsin and undergoes conformational changes to allow the particle to interact with cellular receptors. The virus is transported to the endosome through endocytosis. The low calcium concentration within the endosome triggers the disassociation of VP7 trimers and the DLP is released into the cytoplasm for viral mRNA transcription. Viral mRNAs are used to translate proteins and as a template for RNA genome replication as well as packaging into newly made DLPs. This assembly and replication happen in the viroplasm formed by NSP2 and NSP5. The newly formed DLP binds to NSP4, that acts as an intracellular receptor, and buds into the endoplasmic reticulum. VP4 and VP7, assemble to form the TLP, during which the transient enveloped is lost. The mature virus is released either through exocytosis or viral lysis. With permission from the publishers (Estes & Greenberg, 2013).

Newly assembled rotavirus virions are not infectious. The VP4 spike first needs to be proteolytically cleaved by a trypsin-like protease of the host gastrointestinal

tract into two subunits, VP5 and VP8. Rotavirus replication occurs in enterocytes in the small intestine, which are rich in proteases (Jayaram *et al.*, 2004). Most of the studies on the replication cycle of rotavirus used SA11 and Rhesus rotavirus (RRV). The exact cellular binding receptors vary between strains (Arias *et al.*, 2015). Strains like SA11 use the sialic acid receptors, and the strain G10P[11] binds to non-sialic acid-containing histo-blood group antigens via the Gal β 1-4GlcNac motif (Desselberger, 2014). To keep the host cell from going into apoptosis, the virus suspends the cell cycle in the S phase (Bagchi *et al.*, 2010).

The initial mechanism by which rotavirus attaches to the host cell is a multi-step process that involves the interaction/binding of the VP4 protein on the cell's surface receptors. Attachment is mediated on the virus through a shallow groove on the VP8 subunit. It is followed by a second slower binding step that includes the co-receptor binding of the VP5 subunit with various integrins. Cellular integrins include α 2 β 1, α 4 β 1, α 4 β 7, and hsc70 (Guerrero *et al.*, 2002; Ruiz *et al.*, 2009). Recent studies have identified a specific amino acid sequence on RRV VP4 that plays a crucial role in binding rotavirus to hsc70 (heat shock protein 70) (Mohanty *et al.*, 2017).

The virus is transported to the endosome through endocytosis. Studies have shown that viruses that initiate infection through the means of receptor-mediated endocytosis often rely on the help of acidification of endosomes (Ludert *et al.*, 1987). However, when the endocytosis inhibitors were introduced, rotavirus could still enter the cells (Wolf *et al.*, 2011). Rotavirus entry into cells has been monitored through the interactions of VP4 and liposomes, using co-entry toxins and fluorescent dyes (Liprandi *et al.*, 1997). These observations confirmed the hypothesis that rotavirus enters the cells through endocytosis after a direct interaction with receptors on the plasma membrane (Nandi *et al.*, 1992).

It is hypothesised that the outer capsid proteins are dissociated within an endocytic vesicle because of the low Ca²⁺ concentration found inside the vesicle (Abdelhakim *et al.*, 2014) (Figure 4). This leads to conformational changes in the capsid, capsid solubilisation, and vesicle lysis. Studies have been done to increase the intracellular Ca²⁺ concentration during the early stages of replication to prove this hypothesis. With the increase of Ca²⁺, the outer capsid could not

un-coat within the intracellular microenvironment (Ludert *et al.*, 1987), illustrating the importance of Ca^{2+} during viral replication as a target for therapeutics or culturing techniques.

With the un-coating of the outer capsid, the DLP is released into the cytoplasm for viral mRNA synthesis, referred to as (+)ssRNA transcription (Ruiz *et al.*, 1997). Of these transcripts, several remain in the cytoplasm for viral protein synthesis, whereas others are caught in the newly formed viroplasms. The viroplasm is a non-membrane-bound electron-dense structure, the site used for assortment, packaging, and dsRNA replication (Suzuki, 2019). NSP2 and NSP5 are the two major proteins that interact with each other to produce the viroplasm. NSP5 interacts with RNA as well. The viroplasm is where (+)ssRNA is incorporated into the replication intermediates and where DLP assembly occurs. Upon initial infection, multiple small viroplasms are formed inside the cells. However, since the number of viroplasms decreases with time and the remaining viroplasms enlarge, it is an indication that fusion, facilitated by cellular microtubular networks of the viroplasm, has occurred (Eichwald *et al.*, 2012).

Viroplasm maturation is dependent on NSP4 as well. NSP4 plays a role in the distribution of several viral proteins. NSP4 can be found in three pools inside an infected cell. The first pool is distributed in the cytoplasm. These NSP4 molecules act as a viroporin that releases Ca^{2+} from intracellular stores (Hyser *et al.*, 2010). It is theorized that the increase of Ca^{2+} in the cytoplasm acts as a trigger for NSP5 to form the viroplasm (Berkova *et al.*, 2006). These NSP4 molecules also bind to VP6 at a later stage during the infection, thus inhibiting transcription, this allows RNA replication to proceed and virus assembly to be completed (Silvestri *et al.*, 2004). The second NSP4 minor pool is found in the ER-Golgi intermediate compartment. These molecules can be recycled back into the ER, or they are secreted out of the cell during the early stage of infection and act as a viral enterotoxin by binding to receptors of uninfected cells (Ball *et al.*, 1996), as discussed in Section 1.5.

NSP4 also plays a significant function in the maturation of the viral proteins by acting as an intracellular receptor for viral morphogenesis on the ER membrane. NSP4 binds to the DLPs assembled in the viroplasm and mediates the budding

of DLPs into the lumen of the ER (Taylor *et al.*, 1996). The maturation of the virus is a calcium-dependent process. When viruses are grown in calcium-depleted environments, the cells produce almost entirely DLPs, and no fully mature virions are formed (Estes & Greenberg, 2013). Because VP7 forms calcium-dependent trimers, calcium is needed to assemble VP7 onto the DLPs as VP7 trimers clamp onto the VP6 trimers (Figure 4) (Chen *et al.*, 2009). This interaction between VP7 and VP6 implies that VP4 needs to be attached first as the shell forms while VP7 partly covers the base of the VP4 spike, holding VP4 in place (Figure 4).

When the TLP is formed, the fully infectious virus is ready for release. Studies have shown that host cell lysis occurs in non-polarised cells resulting in the cell's death (Altenburg *et al.*, 1980). However, polarised epithelial cells brush border membranes are destabilised by some budding method in which the cell is not lysed immediately (Gardet *et al.*, 2006).

1.5 Pathogenesis and immune response to rotavirus infection

1.5.1 Pathogenesis of rotavirus infection

As discussed previously, NSP4 is present in three pools. Two pools of which were discussed in section 1.4, reside in the cell. The third pool is a secreted form of NSP4, containing an integrin I domain binding site, which binds to cellular plasma membrane integrin-I receptors (Hu *et al.*, 2012). Through these interactions, a phospholipase is activated, leading to an increase of Ca^{2+} into the cytoplasm from the intracellular stores (Figure 6). The 2 to 4-fold increase of Ca^{2+} in the cell increases the plasma membrane's cation permeability (Hyser *et al.*, 2010). Additionally, NSP4 has also been found to disrupt plasma membrane integrity and inhibiting the sodium absorption of epithelial sodium channels and sodium-glucose transporter (Blanchard *et al.*, 2007; Hu *et al.*, 2012). These all lead to the impairment of the epithelium cells to absorb and digest nutrients. The destruction of these cells leads to the loss of nutrients and water, resulting in

osmotic diarrhoea (Changotra & Vij, 2017; Osborne *et al.*, 1991; Pesavento *et al.*, 2006; Ramig, 2004).

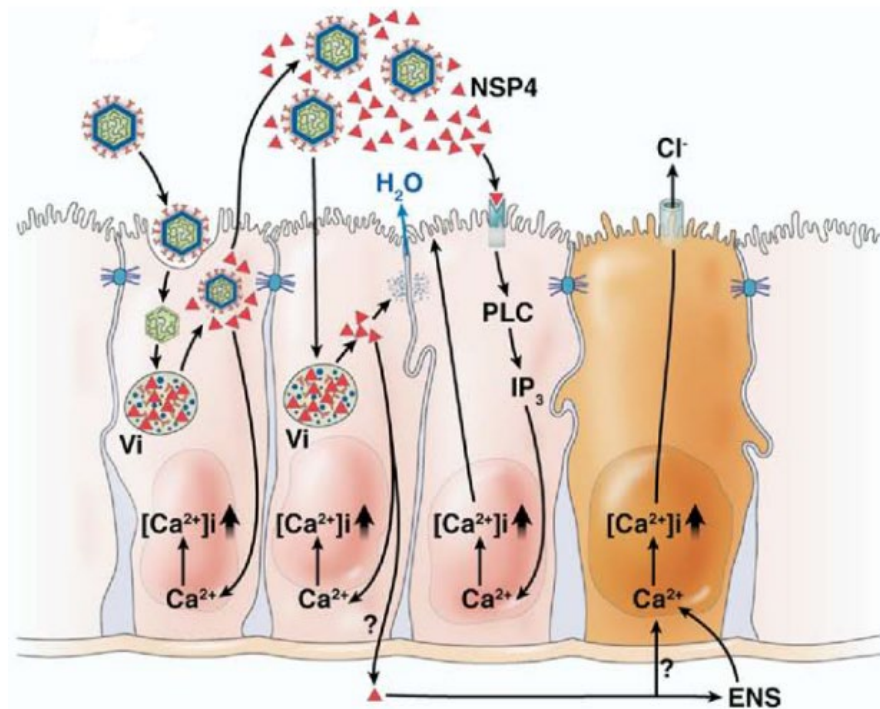


Figure 6: Mechanisms by which rotavirus causes diarrhoea. Rotavirus replication occurs with the release of the virion. NSP4 is also released by the non-secretory pathway. Intercellular NSP4 release Ca^{2+} from the intercellular ER stores. NSP4 from previously infected cells binds to receptors on uninfected cells leading to a signalling cascade through phosphatase C. This also releases Ca^{2+} from the intracellular stores and increases the concentration of Ca^{2+} in the cells, thus disrupting the cells from absorbing nutrients. NSP4 can also stimulate the enteric nervous system (ENS) resulting in the secretion of Cl^- . This cascade on the ENS stimulation elicits diarrhoea (Greenberg & Estes, 2009). With permission from the publishers.

NSP3 can shut off host protein synthesis and, together with NSP1, can spread the virus extra-intestinally (Greenberg & Estes, 2009). NSP1 also suppresses innate immune responses by degrading the interferon regulatory factor 3 (IRF3). The IRF3 is needed to mediate the interferon responses to rotavirus infections (Sen *et al.*, 2011).

1.5.2 Immune response to rotavirus infection

Currently, the precise correlates of protection against RV-associated diarrhoea are not well understood. Animal models have shown adaptive immunity through neutralising and non-neutralising antibodies. However, it is uncertain how this extends to humans (Desselberger & Huppertz, 2011). Factors that play a role in this include but are not limited to obtaining specimens from patients at the correct time and dietary factors that need to be investigated. Studies have shown that vitamin A deficiency can impair serum antibody and cell-mediated immune response to rotavirus (Ahmed *et al.*, 1991).

The first two months of an infant's life are relatively devoid of rotavirus infection (Pratibha *et al.*, 2007). This corresponds with the transferal of maternal antibodies through breast milk or the placenta. IgA neutralising antibodies found in breast milk might protect the infant in the first few months of life. However, this also reduces the efficacy of the vaccines administered, as discussed in Section 1.6 (Chan *et al.*, 2011). The IgA in breast milk coats the infant's mucosal surfaces to cover the microvilli, thus giving active protection against infection. Breast milk also contains a glycoprotein lactadherin. This protein prevents rotavirus infection by binding to the virus, thus making lactadherin a non-antibody glycol-conjugate that inhibits specific pathogens' activity (Newburg *et al.*, 1998). This passive immunity (IgG) is taken over by active immunity as IgA, from breast milk, cannot enter the infant's circulation.

In the gut, the first line of defence is from the epithelial cells of the villi from the small intestines. These cells release specific cytokines, chemokines, and interferons. This further elicits antigen-specific immunity by activating B- and T-cells (Borsa *et al.*, 1979; Changotra & Vij, 2017). The B-cells are responsible for long-term protection, which helps prevent re-infection. T-cells mediate short-term reduced susceptibility to re-infection. Both these cells clear the primary infection. However, T-cells do this more efficiently than B-cells (McNeal *et al.*, 1997).

1.6 Vaccine related research and development

Two live-attenuated, oral rotavirus vaccines were introduced in 2006 (WHO, 2009). RotaTeq™ (Merck & Co., USA) and Rotarix® (GlaxoSmithKline Biologicals, Belgium). With the success these two vaccines have shown in several high and middle-income countries' immunization programs, the WHO recommended these be implemented into immunization programs in all countries in 2009 (WHO, 2009).

It is more than ten years since rotavirus vaccines have been introduced in many countries' national immunisation programs. These vaccines have had a significant impact on diarrhoea morbidity and mortality rates. Rotavirus mortality decreased by 22% in Brazil and around 41% in Mexico (Burnett *et al.*, 2018). South Africa has decreased all-cause diarrhoea hospitalization in children under the age of five by approximately 50% during the first two years of vaccination (Page *et al.*, 2018).

Two newly licensed vaccines, ROTAVAC® (Bharat Biotech, India) and ROTASIIL® (Serum Institute of India PVT, India), have been prequalified by the WHO for global use in 2018 and are currently in use in India and Palestine (Burke *et al.*, 2019) (Table 5).

Table 5: Licensed rotavirus vaccines. Adapted from Burnett (Burnett *et al.*, 2018). With permission from publishers.

Name	Manufacturer	Composition
RotaTeq™	Merck and Co	Live-attenuated pentavalent bovine-human reassortant composed of human G1, G2, G3, G4, and P[8] strains.
RotaRix®	GlaxoSmithKline Biologicals	Live-attenuated monovalent human G1P[8] strain
ROTAVAC®	Bharat Biotech, Hyderabad, India	Live-attenuated neonatal rotavirus strain, G9P[11]
ROTAIIL®	Serum Institute of India	Live-attenuated bovine-human reassortant rotavirus, G1, G2, G3, G4, G9

As of the end of 2018, 92 countries worldwide introduced rotavirus vaccines into their immunization program, of which 46 are Gavi-eligible. However, 57% of all children, ~70 million, still lack access to rotavirus vaccines (Rota Council, <https://preventrotavirus.org/rotavirus-disease/global-burden> Date accessed: 7 July 2019)

Pre-vaccine data indicated that more than 66% of rotavirus infections globally were attributed to G1P[8], and ~20% were due to G2P[4], G3P[8], and G4P[8] strains. As a result, the majority of vaccination efforts were focused on these strains. However, these strains only accounted for 23% of RV infections in Africa and 34% in Asia (Burnett *et al.*, 2018). The vaccines have proven to be quite effective (>85%) in high-income countries. However, they are less effective (40% to 70%) in low to middle-income countries (Changotra & Vij, 2017). Many factors could play a role in this in-effectiveness, including but not limited to RV strain diversity, sanitation, malnutrition, HIV, TB, malaria, infant microbiome, and maternal antibody levels (Burke *et al.*, 2019; Chan *et al.*, 2011).

Post-vaccination data revealed that countries using Rotarix® (G1P[8]) had an increase in the prevalence of G3 strains with a G2 increase in countries using RotaTeq™ (G1, G2, G3, G4 and P[8]) (Kirkwood *et al.*, 2011). This could indicate that vaccine-induced selection pressure occurred, leading to the emergence of rare/novel rotavirus or genetically divergent strains, posing a threat to the long-term vaccination efficacy.

In randomised control trials in South Africa, waning immunity after one year of age was observed (Madhi *et al.*, 2012). This raises a concern that waning immunity may leave the vaccinated children vulnerable for reinfection later in life.

The LA RV vaccines consist of a 2-dose regimen, with a 3-dose regimen considered to address the aforementioned coverage. Alternatively, a booster dose for rotavirus has been proposed to address this problem. Simulation studies indicated that roughly 3-16% mortality could have been averted if a booster had been administered (Burnett *et al.*, 2017). All this data shows that a booster such as a non-replicating virus-like particle (VLP) is needed. As rotavirus strains continuously evolve, VLPs would provide better serotype coverage as needed geographically, as discussed in Section 1.7.

1.7 Rotavirus virus-like particles (VLPs) and expression systems used for generating recombinant proteins

VLPs mainly comprise multi-subunit structural proteins that retain self-assembly without containing any of the viral genetic material. Thus, VLPs mimic the overall structure of the native virus but cannot replicate, eliminating the risk of reassortment. With the particle assembly, conformational epitopes are displayed, allowing the VLP to stimulate both the humoral and cellular pathways of the immune system (Changotra & Vij, 2017). VLPs are also being considered for subcutaneous administration. This could overcome the neutralizing antibodies present in breast milk and other barriers associated with reducing efficacy hurdles experienced with oral vaccines (Burke *et al.*, 2019).

Over the years, numerous expression systems have been used to generate recombinant rotavirus proteins and VLPs. These systems include plant, yeast, bacteria, and insect cells (Figure 7). Each of these systems has its advantages and drawbacks discussed briefly.

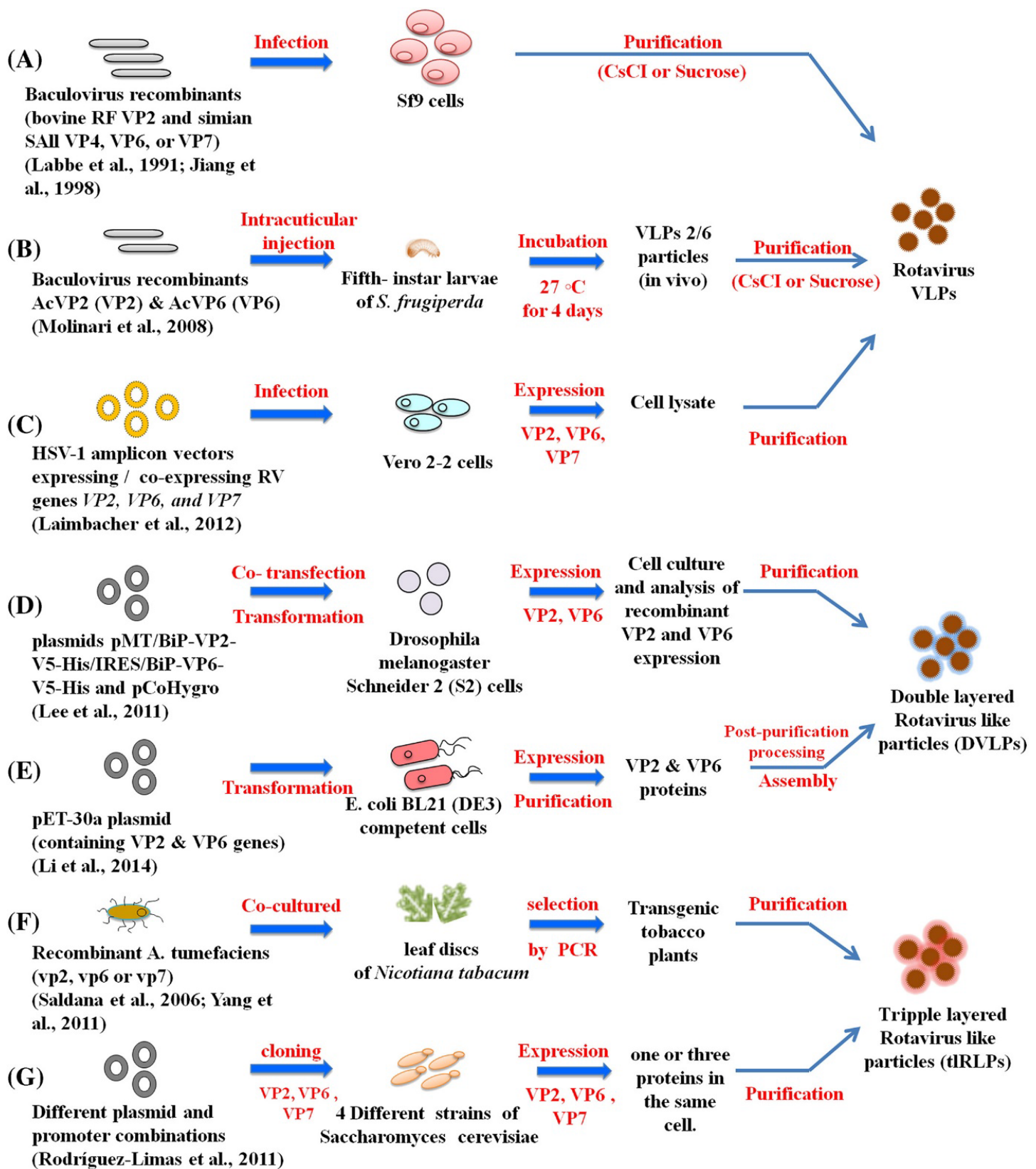


Figure 7: Schematic representation of various strategies used in the production of rotavirus-like particles (Changotra., 2017). With permission from the publishers.

1.7.1 Expression in plant cells

Plants have demonstrated outstanding potential to produce recombinant proteins. Plant expression is rapid, highly scalable, cost-effective, easy to handle, and harbours no mammalian pathogens. The downstream processing of foreign proteins, such as glycosylation, phosphorylation, and folding, is similar to mammalian cells. Several expression methods exist, such as transient transformation using plant viral vectors or the plastid genomes (membrane-bound organelles found in plant cells) (Kushnir *et al.*, 2012).

Rotavirus VLPs has been successfully expressed in transgenic tomato and tobacco plants (Figure 7) (Pera *et al.*, 2015; Saldana *et al.*, 2006; Yang *et al.*, 2011). The expressed VP2 and VP6 produced in the tomato fruit have molecular weights similar to those found in native rotaviruses (Figure 8). However, only a small fraction of VP2 and VP6 self-assembled into VLPs. Nevertheless, detectable levels of anti-rotavirus antibodies in serum were obtained when mice were immunized with the VLPs (Saldana *et al.*, 2006). Similar results were obtained from a study done with VP2/VP6 and VP2/VP6/VP7 VLPs produced in tobacco plants (Yang *et al.*, 2011).

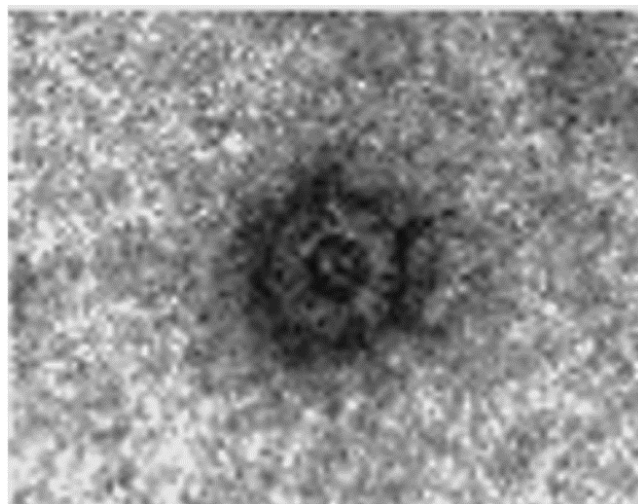


Figure 8: Rotavirus-like (DLP) particles consisting of VP2 and VP6 from transgenic tomatoes. (Saldaña, 2006) With Permission from the publisher.

1.7.2 Expression in yeast cells

Recombinant protein expression in yeast is well-established. Yeast cells can proliferate and are easier to work and less expensive than mammalian and insect cell work. The drawbacks of this system are the difference in glycosylation with regards to mammalian cells. Yeast cells also contain a rigid cell wall making the transfection of cells and recovering non-secreted proteins difficult (Reyes-Ruiz & Barrera-Saldana, 2006).

Multi-layered rotavirus-like particles of VP2/VP6/VP7 were produced for the first time in yeast by Rodriguez-Limas and co-workers in 2011 (Rodriguez-Limas *et al.*, 2011) (Figure 9). Mice were subjected to yeast extracts containing rotavirus proteins and VLPs. Protection against rotavirus infection was observed, but no increase in rotavirus-specific antibodies. The authors theorised that cellular responses were responsible for protection instead of a humoral response (Rodriguez-Limas *et al.*, 2014).

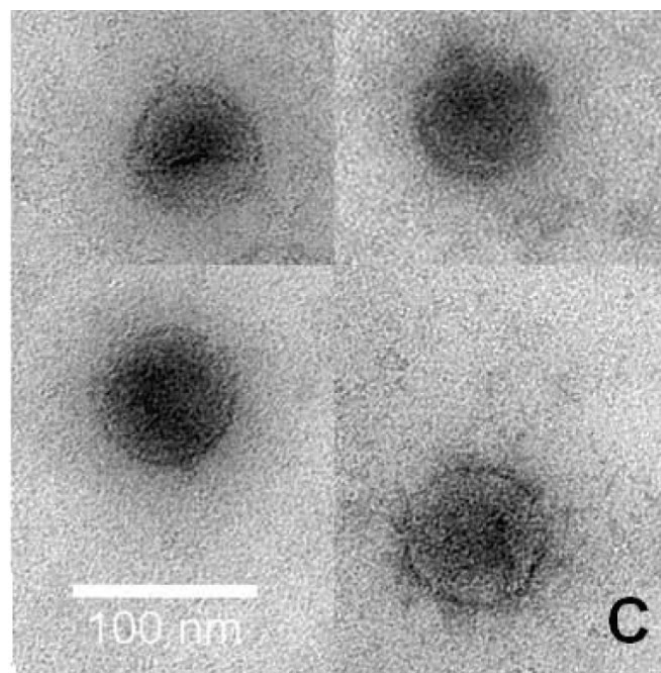


Figure 9: Transmission electron micrographs of VP2/VP6/VP7 produced in yeast cells (Rodriguez-Limas, 2011). Open access article.

1.7.3 Expression in bacteria

Bacterial expression systems are the most widely used expression systems to produce recombinant proteins. Bacterial expression systems have the advantage of low cost, rapid growth, high expression level and are more amenable to up-scaling strategies than other expression systems. Prokaryotes do not contain any mammalian-like post-translational modification systems, affecting the recombinant protein's solubility, structure, stability, and immunogenicity (Yin *et al.*, 2007). Thus, bacteria are not usually preferred for VLP production.

Single- and double-layered rotavirus particles were produced in *Escherichia coli* BL21 (DE3) (Li *et al.*, 2014). cDNA was prepared from wild-type rotavirus dsRNA and cloned in expression plasmids. VP2 and VP6 recombinant proteins were expressed and isolated from the bacteria. Assembly of VLPs was done *in vitro* by incubating the viral proteins in a phosphate buffer (Figure 10). This resulted in VLPs with better thermal stability and antigenicity (Li *et al.*, 2014).

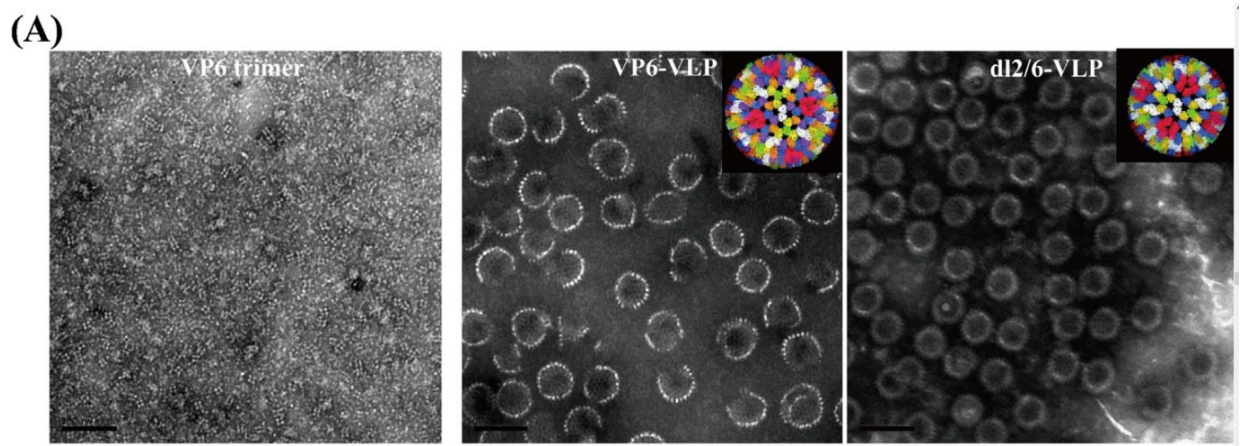


Figure 10: Transmission electron micrographs of VP6 trimers, VP6 particles, and VP2/VP6 DLPs produced in bacteria (Li *et al.*, 2014). Permission from the publisher.

Bacterial expression of codon optimised RVA/Human-wt/ZAF/GR10924/1999/G9P[6] VP2 and VP6 was done at the North-West University by L.A. Naude in 2014. Both genes were cloned into a pETDuet-1 vector. VP6 was only present in the total fraction, meaning it is not water-soluble. Soluble VP6 was obtained by transforming the plasmid into an Origami cell line

containing the pGro7 (groES-groEL) chaperones, which aided in folding the expressed proteins. However, VP2 had no detectable expression even with the changes in an expression vector and with the addition of chaperones. Soluble VP2 was expressed when using pColdTF. This vector contains a trigger factor, a prokaryotic ribosome-associated chaperone, which increases protein folding during cold shock. The use of cold shock (or heat shock) proteins provides a unique expression system that could aid in the expression of challenging proteins, as illustrated by Naude's work.

1.7.4 Expression in insect cells

Baculovirus-insect cell expression is the industry standard for the large-scale production of rotavirus VLPs. Insect cells possess eukaryotic type post-translation modifications such as glycosylation, resembling that of mammalian cells. These cells can accommodate the high-level accumulation of foreign proteins, making overexpression more viable and enabling co-expression. Insect cells lack mammalian pathogens, reducing the risk of off-target reactions and increasing the product's general safety (Roy & Noad, 2009). A drawback of this system includes the contamination of target baculovirus particles with co-produced particles not carrying the target proteins, thus reducing the system efficacy.

SF9 and High Five are two of the insect host cell lines suitable for expressing recombinant proteins. SF9 cells are the traditional cell lines used with the baculovirus system. These cells are derived from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda*. This cell line is readily used for transfection, plaque purification, generating the titre of stocks, and expression of recombinant proteins.

High Five cells originated from the ovarian cells of the cabbage looper, *Trichoplusia ni*. They have a shorter doubling time than the SF9 cells, however, they form irregular monolayers making it difficult to identify plaques. One of the main advantages of using these cells is their ability to express high levels of

recombinant proteins. Studies have indicated that they have 5-10-fold higher secreted expression for selected proteins than the SF9 cells (Davis *et al.*, 1992).

The Bac-to-Bac baculovirus expression system (Invitrogen) efficiently utilises a viral system for expressing heterologous genes in cultured insect cells. This method uses a site-specific transposition of an expression cassette that contains the genes of interest into a baculovirus shuttle vector, called the bacmid, which is propagated in *E. coli*. The bacmid is then transfected into insect cells to produce recombinant baculovirus particles (Invitrogen, 2015).

Complex self-assembly of multi-layered VLPs in insect cells has been efficiently achieved for rotavirus (Charpilienne *et al.*, 2002; Jere *et al.*, 2014; Shoja *et al.*, 2013; Zeng *et al.*, 1996). In the late 1980s, Mary Estes and colleagues used the baculovirus expression system to obtain basic information on protein processing and viral morphogenesis (Crawford *et al.*, 1994) (Figure 11). Understanding what influences rotaviruses' genetic and antigenic variability and the gastrointestinal tract infection outcome has also been extensively studied (Bertolotti-Ciarlet *et al.*, 2003).

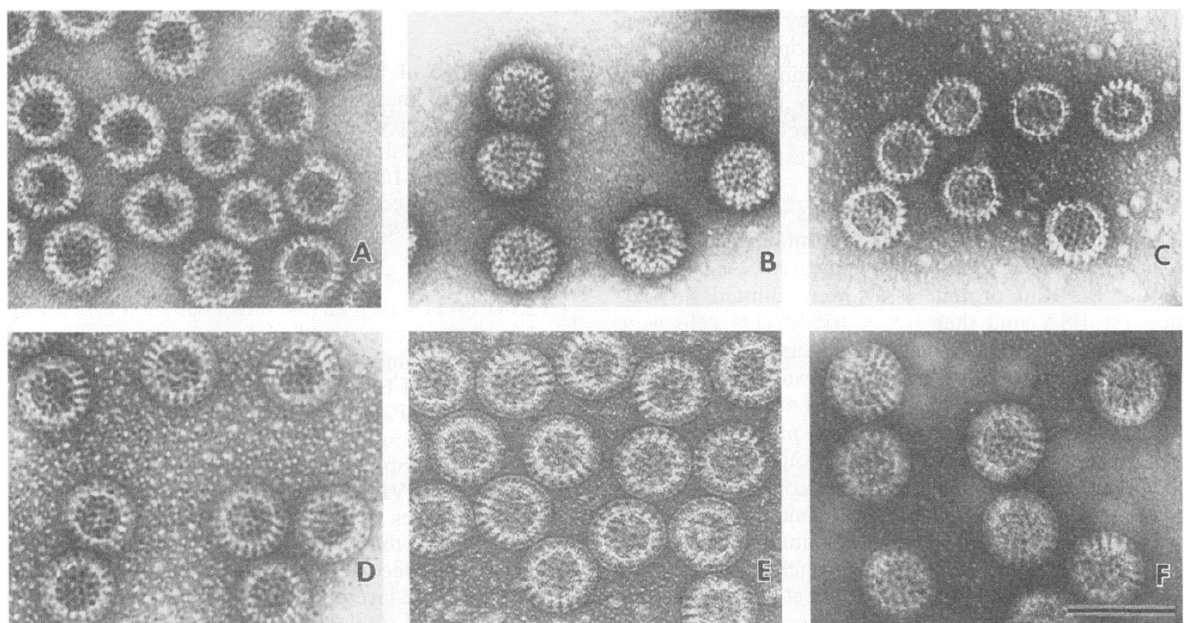


Figure 11: Electron micrographs of different formulations of SA11 VLPs produced by coexpression of baculovirus recombinants. (A) VP2/VP6 particles expressed in insect cells; (B) natural reoccurring DLPs of SA11 rotavirus; (C) Rotavirus-like particle consisting of VP2/VP6/VP4; (D) Rotavirus-like particle consisting of VP2/VP6/VP7; (E) VP2/VP6/VP4/VP7 TLP particle expressed in insect cells; (F) natural reoccurring TLPs of SA11 (Crawford *et al.*, 1994). With permission from the publisher.

In our laboratory, Dr Jere produced chimeric VLPs in insect cells directly by using the consensus insect cell codon optimised sequences of African rotavirus field strains (genotypes G2, G8, G12, P[4], P[6], P[8]) obtained from faeces (Jere *et al.*, 2014). These strains were assembled onto a DS1-like VP2/VP6 DLP (Figure 12). However, further optimisation is needed to improve the assembly efficiency of both homologous and chimeric triple-layered RV-VLPs, as less than 30% of recovered VLPs partially contained VP7. Personal communication with Sue Crawford revealed that the insect cell culture media used in the 1990s contained enough Ca^{2+} for VP7 to remain intact on the VLP. However, the media's formulation changed over the years and must now be supplemented with an additional 20mM Ca^{2+} .

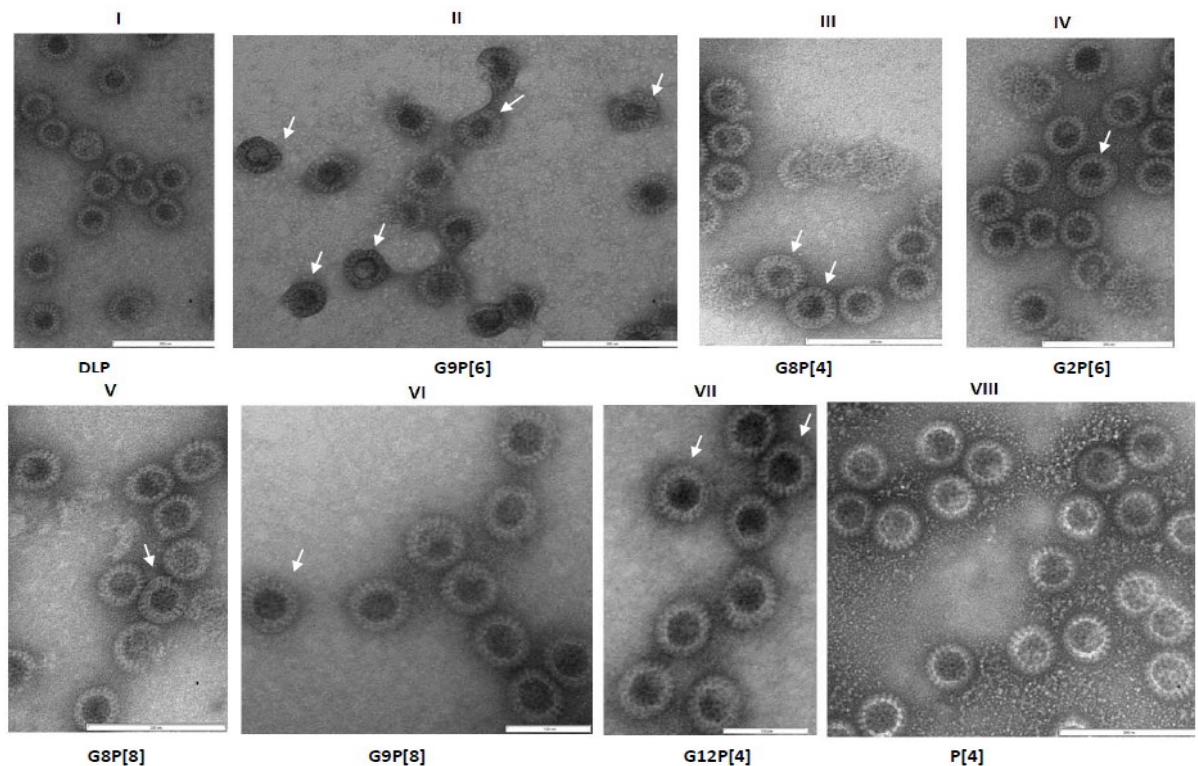


Figure 12: Electron micrographs of rotavirus-like particles produced in High Five® and SF9 cells; (I) VP2/VP6 DLPs (II, III, IV, V, VI, VII, VIII) VP2/VP6/VP4/VP7 TLPs. Chimeric particles were produced using different African strain VP4/VP7 on a DS1-like backbone (Jere *et al.*, 2014). Open access article.

1.8 Motivation and aims of the project

Despite the success commercially licenced rotavirus vaccines have shown, there is still a need for cheaper, rational designed and regionally-specific rotavirus VLPs vaccine candidates to combat rotavirus associated morbidity and mortality in developing countries such as South Africa and India. This project will focus on the expression and auto-assembly of rotavirus proteins using bacterial and insect cell systems.

1.8.1 Main objectives of the project

Bacterial expression of RV VP2/6/4/7

- To rationally design and construct bacterial codon optimised, consensus sequence-based, regional-specific expression vectors encoding GR10924 (VP2/VP6), SA11 (VP2/VP4/VP6/VP7) GR10924 (VP7-leader peptide).
- To evaluate the plasmid-based expression of rotavirus VLPs in various bacterial cell lines, such as Tuner, Origami, BL21, Origami B, with the addition of various chaperones.

Insect cell expression of RV VP2/6/4/7

- To rationally design and construct SF9 insect cell codon optimised, consensus sequence-based, regionally specific expression vectors encoding SA11 (VP2/VP4/VP6/VP7).
- To generate recombinant baculovirus vectors expressing target proteins using the Bac-to-Bac system.
 - To successfully transform the various donor plasmids for expression of SA11 (VP2/VP4/VP6/VP7) into $\Delta\Delta$ AccBac bacterial cells to generate recombinant bacmid DNA.
 - To transfect recombinant bacmid DNA into Sf9 cells to generate recombinant baculoviruses expressing VP2, VP6, VP4 and VP7.
- To evaluate assembly of VLPs in Sf9 cells in terms of yield and VLP integrity.

- To optimise the expression of single-, double- and triple-layered VLP assembly through various means such as supplementing the medium with Ca^{2+} , SF9 insect cell codon optimisation, and varying the multiplicity of infection (MOI).

Chapter 2

Bacterial expression of rotavirus proteins

2.1 Introduction

As discussed previously, bacterial expression systems remain one of the most common approaches for recombinant proteins as their benefits include ease of scalability, affordability, and simplicity to work with. The primary drawback of bacterial expression systems, however, is that they lack eukaryotic post-translational modifications. Considering these drawbacks, the advantages associated with bacterial expression still make it a viable option for generating recombinant VLPs. Various attempts were made during this project to establish a stable expression system for rotavirus proteins.

Students in our laboratories used various combinations of different bacterial strains and expression vectors to generate recombinant rotavirus proteins. The initial goal was the expression of GS2 (VP2) and GS6 (VP6) of the human strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] (G9-P[6]-I2-R2-C2-M2-A2-N2-T2-E2-H2 genotype), followed by auto-assembly into RV DLPs (Jere *et al.*, 2011). It was possible to express RV_GR10924 GS6 in pCold_GR10924_VP6 transformed into *E. coli* cells (personal communication, Dr R van der Sluis). In the pCold expression vector, the RV GS6 was under the control of a cold-shock protein-induced promoter and allowed expression of VP6 at low temperatures (15°C). This allowed proper folding of the protein and prevented intercellular aggregation of the VP6. Soluble RV_GR10924 VP2 expression was obtained in pColdTF as described in 1.7.3. In this project, the work previously done was reattempted with slight modifications to the technical approach. These modifications included different expression vectors, different induction methods, and more advanced bacterial cell lines.

Due to the glycosylation sites and toxic nature of RV VP7 (GS9), its expression is associated with a myriad of complications. Considering the lack of glycosylation machinery in bacterial cells, it was attempted to express RV_GR10924 GS9 (VP7) in various cell lines through the truncation of the N-

terminal leader peptide. This short N-terminal aids in transferring VP7 to the ER in eukaryotic cells during viral replication and new virion formation and maturation. Since bacteria do not have an ER, the 5' sequence encoding the signal peptide was removed from the expression vector to prevent intercellular accumulation of VP7 and reduce its toxicity (Figure 13). This hypothesis was further supported by the fact that the peptide is not needed for VLP formation and is excised from VP7 before assembly (during natural infection in eukaryotic cells) (Stirzaker *et al.*, 1990).

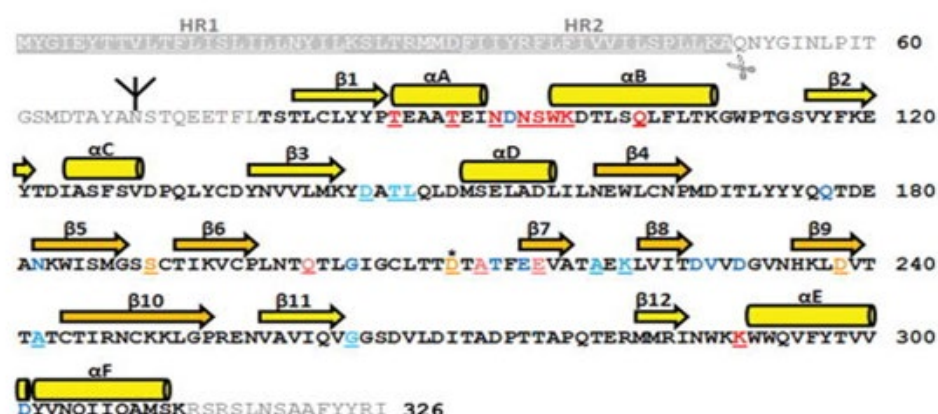


Figure 13: Amino acid sequence of rhesus rotavirus VP7. Alpha-helices and beta-strands are shown as cylinders and arrows, respectively. Coloured, underlined, are residues that contribute to Ca^{2+} ions ligation. Scissor symbol: position of signal peptidase cleavage. The branched symbol at Asn69: position of N-linked glycan (Aoki *et al.*, 2009). With permission from the publisher.

In 2017 a breakthrough was made in producing whole viruses using a plasmid-only reverse genetics system (Kanai *et al.*, 2017). This shifted our focus to the production of RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2] (G3-P[2]-I2-R2-C5-M5-A5-N5-T5-E2-H5 genotype) VP2/VP6 DLP particles. The RV_SA11 DLPs would be used for antibody production and serve as a baseline for investigating the structural restraints to RV reassortment capacity. This platform would also help identify genetic restraints to reassortment through indirect evaluation, i.e., if the assembly of VP4 and VP7 can be achieved on DLP *in vivo* but could not be done via plasmid-based reverse genetics, it would suggest genetic restriction instead of structural restrictions.

2.2 Materials and Methods

2.2.1 Design of plasmids for expression of rotavirus proteins in different bacterial cell lines

The ORFs for VP2/6/4 and VP7 were used in this study and were codon optimised for expression in *E. coli* and purchased from GeneArt, USA (Table 6). The figures for the plasmids can be found in the appendix. GR10924_VP2 was already used in a previous experiment and was subcloned into pET28a(+) with a kanamycin resistance marker for this current project. Plasmids received from GeneArt were delivered in lyophilized form. The vials were spun before opening to make sure that no product is lost. Nuclease-free water was used to dissolve the product to a final concentration of 100ng/μl. Plasmids were stored at -20°C for long-term storage as discussed in 2.2.8.

Table 6: Plasmids used in this experiment

Plasmid name	Vector	Gene encoding	Antibiotic resistance
pET28_VP2	pET28a(+)	GR10924_VP2	Kanamycin
pET100_VP6	pET100	GR10924_VP6	Ampicillin
pET151_VP7	pET151	GR10924_VP7	Ampicillin
pET100_SA11_VP2_BacO	pET100	SA11_VP2	Ampicillin
pET100_SA11_VP6_BacO	pET100	SA11_VP6	Ampicillin
pET100_SA11_VP4_BacO	pET100	SA11_VP4	Ampicillin

Table 7: Summary of bacterial cell lines used in this study

Cells	Strain	Genotype	Features
Cloning (Thermo Scientific)	DH5 α	F ⁻ ϕ 80lacZ Δ M15 Δ (lacZYA-argF)U169 recA1 endA1 hsdR17(rK ⁻ , mK ⁺) phoA supE44 λ ⁻ thi-1 gyrA96 relA1	Mutations introduced in these cells are used to disable recombinase proteins as well as inactivate homologous recombination.
	DH10 β	F ⁻ mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 endA1 araD139 Δ (ara-leu)7697 galU galK λ ⁻ rpsL(StrR) nupG	This cell line is adapted to eliminate the restriction of DNA methylation, making them ideal for generating cDNA or genomic libraries.
Expression (Novagen)	BL21(DE3)	F ⁻ ompT hsdSB (rB ⁻ , mB ⁻) gal dcm (DE3)	BL21 is a reputable cell line to use, as both <i>Lon</i> and <i>OmpT</i> proteases are removed for increased target protein stability.
	Origami(DE3)	Δ (ara-leu)7697 Δ lacX74 Δ phoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac ⁺ lacI q pro] (DE3) gor522::Tn10 trxB (KanR, StrR, TetR)4	Origami cells enhance disulphide bond formation due to the two mutations in the thioredoxin and glutathione reductase genes.
	Tuner(DE3)	F ⁻ ompT hsdSB(rB ⁻ mB ⁻) gal dcm lacY1 (DE3)	A mutant strain of BL21 with a deletion of <i>lacZY</i> . This allows (isopropyl β -D-1-thiogalactopyranoside) IPTG to enter the cells uniformly, allowing more control of protein expression. Low-level expression can enhance protein solubility.
	Origami B(DE3)	F ⁻ ompT hsdSB(rB ⁻ mB ⁻) gal dcm lacY1 aphC (DE3) gor522::Tn10 trxB (KanR, TetR)	These cells contain the same mutation as Origami and Tuner cells.

2.2.2 Preparation and transformation of chemical competent *Escherichia coli* cells

Tuner(DE3) and Origami(DE3) cells were purchased from Novagen and used in a previous study. Glycerol stocks were used to make the cells competent using a one-step procedure (Chung *et al.*, 1989) with slight modifications. The cells were streaked out on LB-agar (1% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar) plates and grown overnight at 37°C. A single colony of the overnight culture was used to inoculate 5 ml of LB and grown overnight with shaking at 225 rpm at 37°C. From this, 1.2 ml was used to inoculate a 50 ml culture shaking at 225 rpm at 37°C until an OD₆₀₀ of 0.3 - 0.4. Cells were collected by centrifugation at 6000 x *g* for 10 minutes at 4°C. The supernatant was removed, and cell pellet was re-suspended in 5ml ice-cold TSS (1.6% (w/v) tryptone, 1% (w/v) yeast extract, 1% (w/v) NaCl, 10% PEG 6000, 5% DMSO and 30 mM MgCl₂) and incubated for 20 minutes on ice.

For long-term storage of these competent cells, glycerol was added to a final concentration of 15%. The cells were aliquoted in 200 µl volumes in 1.5 ml polypropylene tubes, frozen in liquid N₂, and stored at -80°C.

To transform these cells, 100 ng of plasmid DNA was mixed with the 200 µl cells on ice for 30 minutes. 800 µl, pre-heated TSSG (TSS media with 20 mM glucose) was added and incubated for 1 hour at 37°C, shaking at 225 rpm. Cells were aliquoted and plated in a serial dilution on LB-agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

2.2.3 DNA transformation into commercial competent cells

Transformation of plasmids, upon arrival, was performed in a DH5α cloning cell line for propagation according to Thermo Scientific's protocol. In short, DH5α competent cells were thawed in an ice bath before 100 µl of cells were transferred to a 14 ml round-bottom tube. Approximately 1 µl (100 ng) of plasmid DNA was

added to the cells and incubated on ice for 30 minutes. Cells were transferred to a water bath and incubated for exactly 45 seconds at 42°C, after which they were returned to the ice bath for 2 minutes. Pre-warmed SOC medium (0.5% (w/v) yeast extract, 2% (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added up to a final volume of 1 ml. The transformation mixture was incubated by shaking at 225 rpm for 1 hour at 37°C. The cells were plated in a serial dilution on LB-agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

2.2.4 In-Fusion® HD cloning

In-fusion HD cloning is a method used to clone fragments of DNA into a vector. It requires that both the vector and inserts be linearised either through PCR or restriction enzyme digestion. Linearisation through PCR, as used in this experiment, used primers designed with overlapping complementary regions for both vector and DNA insert (Table 8). The PCR reaction mixtures were set up as follows: 10 µl GoTaq reaction buffer, 1 µl (10 mM) dNTPs, 1 µl (10 µM) of each appropriate primer, 500 ng DNA, 0.25 µl GoTaq DNA polymerase, and nuclease-free H₂O to a final volume of 50 µl.

The reaction was incubated in a thermocycler (T100 Thermal Cycler, Biorad, California, USA) at 95°C for initial denaturing for 2 minutes followed by 35 cycles of 95°C for 60 seconds, 60°C for 60 seconds and 72°C for 2 minutes, and a final elongation cycle at 72°C for 5 minutes. The samples were held at 4°C until further use.

After amplification, each amplicon was separated through agarose gel electrophoresis, and gel extraction was performed per the manufacturer's protocol, discussed in 2.2.5 (Qiagen, Cat. No. 27106). The In-Fusion reaction contained 200 ng of the DNA insert, 100 ng of vector DNA along with 2 µl of the 5x In-Fusion HD enzyme premix (Clontech, Takara. Cat. No. 638920). The total reaction volume was adjusted to 10 µl using nuclease-free water. The reaction

mixture was incubated for 15 minutes at 50°C using a thermocycler (T100 Thermal Cycler, Biorad, California, USA). After incubation, the mixture was placed on ice until the transformation step.

The In-Fusion premix contains a 3'-exonuclease that digests the amplicons resulting in "sticky ends" ssDNA overhangs that cause the vector and the DNA fragment to hybridise in a circular plasmid. However, the phosphate backbone is only repaired after transformation using the bacterial cells' own ligase. A volume of 5 µl of the In-Fusion reaction was used to transform chemically competent Stellar cells, provided in the In-Fusion HD kit, using heat shock as described in Section 2.2.3.

Table 8: Primers used for In-Fusion purchased from TIB Molbiol.

pET151_VP7 forward	5'- AGCAGAGATTACCTACACACGATAATAGAATGCTGCGC -3'
pET151_VP7 reverse	5'- GCAGAGATTACCTAAATCAGATCTGCCAGTTCGC -3'
pCOLD1 forward	5'- GGTAATCTCTGCTTAAAAGCACAGAATC-3'
pCOLD1 reverse	5'- CCTACCTTCGATATGATGATGATGATGATGATG-3'

2.2.5 Plasmid isolation

Plasmid isolation was performed using the Qiaprep Spin Miniprep kit (Qiagen, Cat. No. 27106), following the manufacturer's protocol. This protocol uses alkaline lysis conditions combined with the detergent SDS described in Birnboim and Doly (Birnboim & Doly, 1979).

Overnight cultures of 5 ml LB, with appropriate antibiotics, were prepared from a single colony. Cultures were pelleted at room temperature at 6000 x *g* for 5 minutes. After centrifugation, the supernatant was removed, and the pellet was left to air dry. The cell pellet was resuspended in 250 µl buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNase A, 1 µl/ml LyseBlue). LyseBlue is a visual identification method that turns blue, indicating that the mixture's pH turned alkaline. The EDTA is used to chelate divalent cations to prevent DNases from damaging the plasmid. The bacterial cells were lysed with the addition of 250 µl

chilled buffer P2 (200 mM NaOH, 1% SDS), after which the tubes were inverted 6 times. The SDS was used to solubilize the cell membrane by removing the phospholipids and protein components. The NaOH denatures cellular proteins and the genomic and plasmid DNA by disrupting the hydrogen bonds between the bases, resulting in double-stranded DNA becoming single-stranded DNA. However, the plasmid DNA is circular and remains topologically constrained, meaning the two strands, although denatured, remain together. This reaction step should not be left to proceed more than 5 minutes as this may result in degradation of the plasmid DNA too.

The lysate was neutralised by adding 350 μ l buffer N3 (4.2 M Gu-HCl, 0.9 M potassium acetate, pH 4.8). With the potassium acetate added, the pH is neutralised from the strong alkaline conditions, allowing the circular DNA to renature. This neutralization is indicated by the solution turning from blue to colourless. The renaturing of the plasmid DNA allows the plasmid to stay in solution. The high salt environment causes denatured proteins, sheared ssDNA, cellular debris, and SDS to precipitate. The solution was mixed gently and thoroughly to ensure complete precipitation. This solution was centrifuged at 17 000 $\times g$ for 10 minutes to separate the plasmid DNA solution from the insoluble genomic DNA-protein complex.

After centrifugation, 800 μ l of the high salinity solution containing the plasmids were transferred to a Qiaprep 2.0 spin column through pipetting. The spin-column and the supernatant were subjected to centrifugation at 17 000 $\times g$ for 1 minute. The column is packed with a silica membrane that binds DNA, while the rest of the contaminants such as endotoxins, residual cellular proteins, RNA, and EDTA pass through the column during centrifugation.

The spin-column membrane containing the plasmid DNA was washed with 750 μ l buffer PE (10 mM Tris-HCl pH 7.5, 80% ethanol). This wash step removes salts efficiently and is centrifuged again for 60 seconds. The flow-through was discarded, and the column was centrifuged again to remove any residual wash buffer. The spin-column was moved to a sterile 1.5 ml microcentrifuge tube, and 50 μ l of preheated (60°C) nuclease-free water was added. This was allowed to

incubate for 1 minute, after which it was centrifuged again for 1 minute to elute the DNA.

2.2.6 Spectrophotometric evaluation of nucleic acids

The purity of DNA was determined using a NanoDrop One spectrophotometer (Thermo Scientific, Waltham, MA, USA). Before use, the NanoDrop was cleaned using 2 µl of molecular grade water on each pedestal. The NanoDrop was blanked using the same buffer used for the elution of the DNA sample. Maximum absorbance of DNA is observed at OD 260 nm and RNA at OD 280 nm. To measure the concentration and purity of the DNA sample, the OD_{260/280 nm} values were measured. Pure DNA values are between 1.7 and 1.9. OD_{260/280 nm} values lower than 1.7 indicate protein contamination. Values higher than 1.9 indicate RNA contamination.

2.2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyse the plasmid DNA and restriction enzyme digestions of plasmids. The principle behind this technique is to separate nucleic acid fragments based on their size through a uniform electrical field. Nucleic acids are negatively charged and move from the cathode to the anode. The fragments will separate as they move through the porous matrix. Smaller fragments migrate faster than bigger fragments.

Standard 1% (w/v) agarose gels in 1 x TAE buffer (40 mM Tris-acetate pH8.1, 1 mM EDTA) were used for analysis and visualisation. The procedure as described in Sambrook & Russell (2001) was used to prepare these gels.

A GeneRuler™ 1kb DNA ladder mix (ThermoFisher Cat. No. SM0311) was used as a DNA molecular size marker. Nucleic acid samples were diluted 5:1 with the

6 x loading dye before the samples were added to the wells. The gels were electrophoresed at 70 V until DNA fragments in the samples were sufficiently separated. All gel electrophoresis was done using a Bio-Rad PowerPac system. Ethidium bromide (1 µg/ml) facilitated the visualisation of DNA on an ultraviolet light trans-illuminator. This step was performed in a ChemiDoc™ MP Imaging System from Bio-Rad laboratories.

2.2.8 Glycerol stocks for long term storage of bacterial samples

Bacterial cultures of interest were stored at -80°C using glycerol to a final concentration of 15%. Glycerol is used as it prevents ice-crystal formation at such low temperatures, keeping the cells structurally stable. Glycerol stocks were prepared from a 5 ml overnight culture of a single colony.

2.2.9 DNA sequence determination

Sanger sequencing was done to confirm that no mutations occurred during the transformation and propagation of the plasmids. After plasmid extraction, 15 µl of 100 µg/µL of plasmid DNA samples were sent to the Central Analytical DNA Sequencing Facility (CAF) (Stellenbosch University, South Africa). The standard T7 promoter (forward) and T7 terminator (reverse) primers supplied by the CAF sequencing unit were used (Table 9).

Sequencing results were obtained via email and viewed using the SnapGene™ chromatogram viewer. The sequences were aligned to the reference sequence using BioEdit software.

Table 9: Primers used for Sanger sequencing

Primer name	Sequence	Length	Tm
T7 forward	5' - TAA TAC GAC TCA CTA TAG GG - 3'	20 mer	48°C
T7 reverse	5' - GCT AGT TAT TGC TCA GCG G - 3'	19 mer	54°C

2.2.10 Expression of rotavirus proteins using a pET expression system

Protein expression for the pET plasmid sets was done using the pET System Novagen Manual protocol (TB055 10th Edition Rev.B 0403). An overnight culture containing the appropriate antibiotic was made from the expression vector glycerol stock. 2 ml of this overnight culture was used to inoculate 100 ml LB, with the appropriate antibiotics, in a 500 ml Erlenmeyer flask. This culture was incubated at 37°C with shaking at 225 rpm until the OD_{600nm} reached approximately 0.5. Before induction, the culture was split into two 50 ml cultures. One of the cultures was used as an un-induced control. The other culture was induced with 0.4 mM IPTG. Both were incubated with shaking at 37°C for 3 hours.

2.2.11 Cell lysis using BugBuster protein extraction reagent

The BugBuster (Novagen, Cat. No. 70584-4) reagent mixture contains non-ionic detergents capable of cell wall perforation to lyse bacterial cell walls to liberate the content of the cells. Benzonase® (Merck, Cat. No. 70746-10KUN) was added according to the manufacturer's protocol. This genetically engineered endonuclease degrades all forms of DNA and RNA.

After incubation, the flasks were placed on ice for 5 minutes to reduce the cells' metabolism, after which they were centrifuged at 10 000 x *g* for 10 minutes in pre-weighed tubes. The supernatant was removed, and the weight of the wet cell pellet was determined. BugBuster and benzonase were added according to the manufacturer's protocol, 5 ml BugBuster per gram of wet cell paste, and 1 µl

benzonase per ml of BugBuster reagent was used. The cells were entirely re-suspended by pipetting the mixture.

This cell mixture was incubated at room temperature on a slow-moving platform for 20 minutes. Then, 500 µl of the mixture was removed as the total fraction. The rest of the mixture was centrifuged at 16 000 x *g* for 20 minutes to remove all the insoluble cell debris. The supernatant was recovered and represented the soluble fraction.

2.2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The method of Laemmli (1970), as described in Sambrook and Russell (2001), was used. A 5% stacking gel and 12% resolving gel was used. The resolving gel was poured into an assembled 0.75 mm Bio-Rad Mini Protean gel casting apparatus (BIORAD, California, USA).

The protein samples were prepared by diluting the samples in a 3:1 ratio with the sample buffer. The sample buffer consists of 4 x Laemmli Sample Buffer (BIORAD Cat. No. 161-0747) and 10% β-mercaptoethanol (βME). The samples were heated to 95°C for 5 minutes to denature the proteins before loading them onto the gel.

TGS buffer (25 mM Tris, 250 mM Glycine, 0.1% (m/v) SDS, pH 8.3) was used in the tank to provide an electrical field. Samples were electrophoresed at 130 V until the bromophenol blue migrated to the bottom of the resolving gel.

The SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 stain (0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 40% H₂O, and 10% glacial acetic acid solution) for 4 hours on a slowly rocking platform at room temperature. The staining solution was discarded and the gel was rinsed with distilled water. The gels were destained with methanol: acetic acid solution (50% methanol, 40% H₂O, and 10% glacial acetic acid) on a slowly rocking platform for 4 hours, changing the destaining solution 3 to 4 times. The gels were visualised by white illumination using a BioRad ChemiDoc™ MP imaging system.

2.3 Results and Discussion

2.3.1 Expression of rotavirus VP7-ER in different bacterial cell lines

The GR10924_VP7 was purchased from GeneArt. Using primers listed in Table 8, infusion was used to sub-clone GR10924_VP7-ER (the VP7 construct lacking the leader peptide discussed in Section 2.1) (Data not shown) into the pColdI vector (Figure 43).

To confirm that rotavirus VP7 can be expressed in bacterial cells without the leader peptide, the pCOLDI_VP-ER plasmids were transformed into expression cell lines Tuner and Origami expressing the T7 polymerase. The experimental outline for protein expression is discussed in Section 2.2.10. Bugbuster was used to lyse the bacterial cell, and the expression of rotavirus VP7 was analysed on SDS-PAGE (Figure 14).

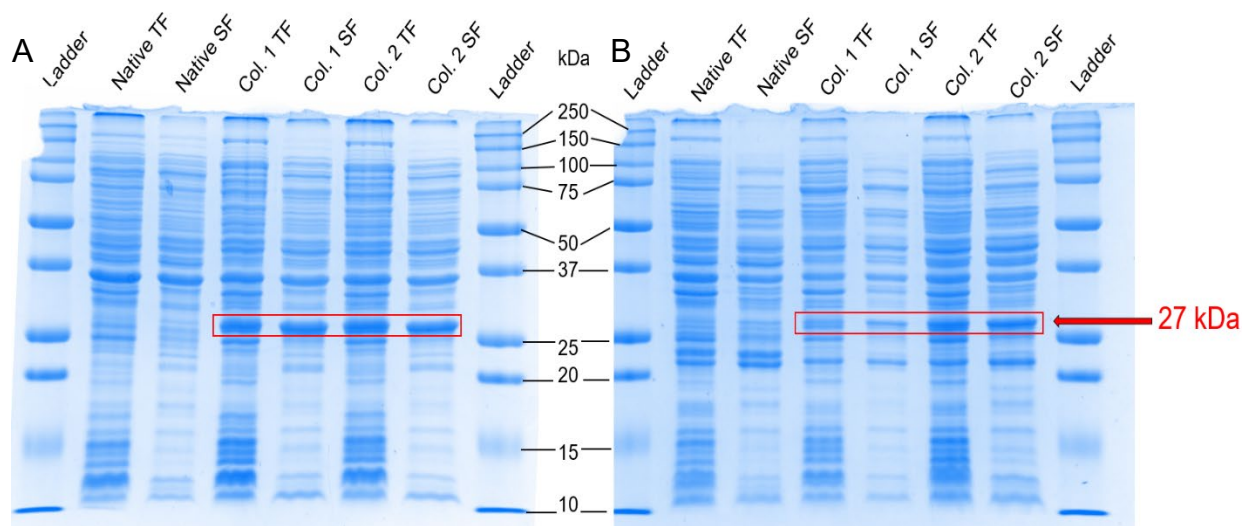


Figure 14: SDS-PAGE analysis of pCOLDI_GR10924_VP7. TF: Total fraction; SF: Soluble fraction. A: Tuner cell line expression. B: Origami cell line expression. Red boxes indicate the expressed protein. Col 1 and Col 2 represent the bacterial colony selected for use.

Expression of soluble, recombinant VP7 was successful. However, the expected 34 kDa VP7 (37 kDa – including the leader peptide) was not visible. Instead, a 27 kDa protein was expressed and observed in all samples. Sequence analysis of the pCOLDI_VP7-ER vector indicated that during the transformation, a 159 bp fragment of the ORF (bp 365-524) encoding for 53 amino acids (AA 121-174) was deleted (Figure 15 B). This deletion spans the variable regions VR6 and VR7 of VP7. This truncation of the proteins disrupted two disulphide bonds in the protein's tertiary structure (Aoki *et al.*, 2009) (Figure 15 B). Although the protein produced from this experiment was incomplete, its immunogenic epitopes were still intact and could be used in future work to establish an immunogenic response specific to RV_GR10924 VP7. See appendix for pCOLDI_VP7-ER sequencing data.

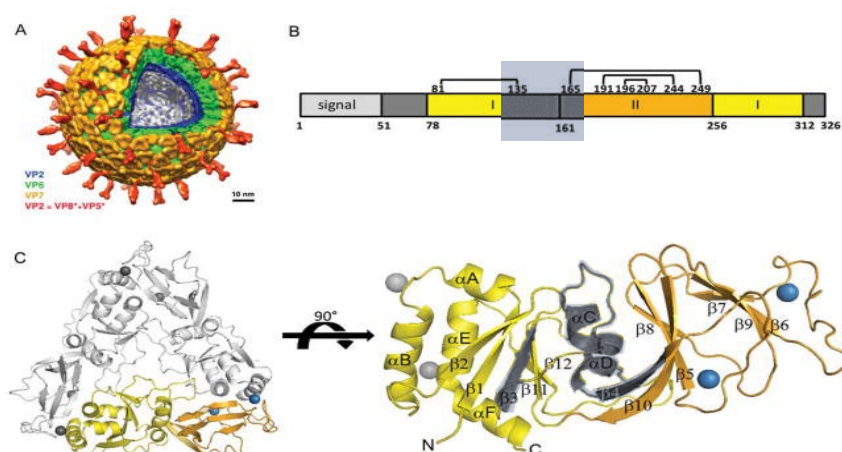


Figure 15: **A.** CryoEM structure of the complete virion 25 Å resolution. **B.** Schematic diagram of VP7 primary structure, including the signal sequence (residues 1-50, light gray). The two domains are in the same colours as in A; The pattern of the intrasubunit disulphide bonds with numbers corresponding to the positions of the cysteine residues. **C.** Ribbon diagram of the VP7 trimer (left), with one subunit and one pair of Ca^{2+} ions in colour. The Rossmann-fold domain (domain I) is in yellow; the β -barrel domain (domain II), in orange; the Ca^{2+} ions, in blue. A single subunit, inside view, is shown on the right, with secondary structure elements labelled (Aoki *et al.*, 2009). With permission from publishers.

2.3.2 Rotavirus VP2 and VP6 expression

For a previous experiment, the plasmid pET28_GR10924_VP2 was constructed and purchased from GeneArt™ (Mr JJ van Greunen). The GR10924_VP2 plasmid was used for co-expression with pET_GR10924_VP6, purchased from GeneArt™, and thus was put under a different antibiotic resistance control (kanamycin selection). (See appendix Figure 37, Figure 39).

Various expression conditions were utilised for the expression of RV VP2/VP6 DLPs in bacteria. These conditions included using different expression cell lines, different IPTG concentrations for induction of expression, various expression temperatures (using thermo-sensitive induction promoters), induction times, chaperones, and different expression media.

Expression cell lines listed in Table 7 illustrate the various features that have been exploited during this experiment. As described before, in Section 2.2.1, Tuner cells were used to slow the expression of recombinant proteins through the regulation of the IPTG concentration. The expression can be regulated from deficient levels to robust, fully induced levels commonly associated with pET hosts. The lower-level expression may enhance the solubility of toxic proteins or proteins with complicated secondary structures. IPTG concentration can be adjusted between 0.1 mM to 4 mM. Temperatures of 20°C, 30°C, and 37°C have been used to change the bacteria's metabolic rate, which aids in folding the desired proteins, further increasing their solubility and reducing their toxicity (Vera *et al.*, 2007).

The optimal induction time of the protein in bacterial cells is 4 hours, according to the manufacturer's manual for the pET vector. Overnight induction can lead to the depletion of the antibiotics used for the selection of recombinant cells (Novagen, 2003). This, in turn, might lead to increased representation of non-recombinant cells, thus lowering expression efficacy. With this drawback in mind, overnight expression was still attempted to increase overall protein yield. Lower temperature overnight expression was also attempted to overcome the depletion of resources previously described (Novagen, 2003).

Additionally, the folding of proteins was modulated through the incorporation of various chaperones. Although the protein's amino acid sequence contains information needed to adapt the protein's tertiary structure, some proteins are misfolded due to environmental stresses and the lack of eukaryotic co-factors. The chaperone set pGro7 (GroEL/GroES), induced with L-arabinose, was used to prevent aggregation of insoluble proteins and aid in forming soluble proteins (Figure 16).

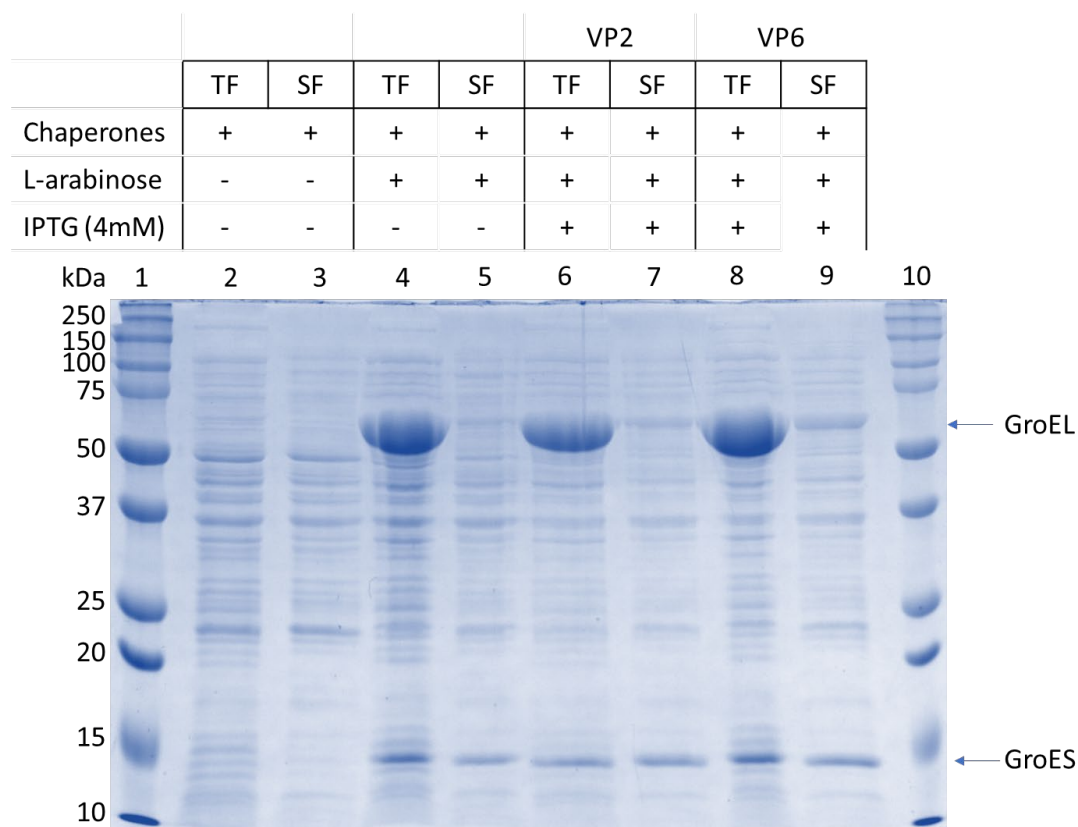


Figure 16: SDS-PAGE analysis of VP2 and VP6 in Origami cells with GroEL/GroES chaperones. TF: Total fraction, SF: Soluble fraction. GroEL molecular weight in its monomeric form is 60 kDa and is visible in lanes 4, 6, and 8. GroES molecular weight is 10 kDa but can be seen here at around 14 kDa in both the TF and SF. Neither VP2 nor VP6 expression can be observed. Lane 2 and 3 indicated no expression of the chaperones as expected in the uninduced control. Lane 4 and 5 show chaperone expression in the induced control. Lane 6 and 7 reveal the GR10924_VP2 with chaperones. No expression for VP2 at 102 kDa can be observed, even though overexpression for chaperones can be seen. Lane 8 and 9 have no expression for VP6 (48 kDa), but chaperones' expression can be observed.

Bacteria were grown in normal Luria Broth (LB) during propagation, transformation, and induction. However, Terrific Broth (1.2% (w/v) tryptone, 2.4%

(w/v) yeast extract, 4% glycerol, with an additional 0.17 M K_2PO_4 , 0.72 M K_2HPO_4 after autoclave) was also tried as this media increases the plasmid load in a cell. Terrific Broth contains more nutrients than LB broth and glycerol, thus increasing the yield of bacteria per volume. The addition of potassium phosphates in the media reduced cell death by preventing the media's pH from decreasing.

All the deviations from the standard protocol still resulted in mixed results. Neither Tuner nor Origami cells gave optimal expression. Since we made these cell lines competent using our in-house one-step methods, a mutation might have occurred, which resulted in these two cell lines not expressing as they should. No well defined expression of VP2 (102 kDa) and VP6 (48 kDa) was observed with the commercially competent cell lines Origami B and BL21 (Desselberger, 2014) (Figure 17 and Figure 18).

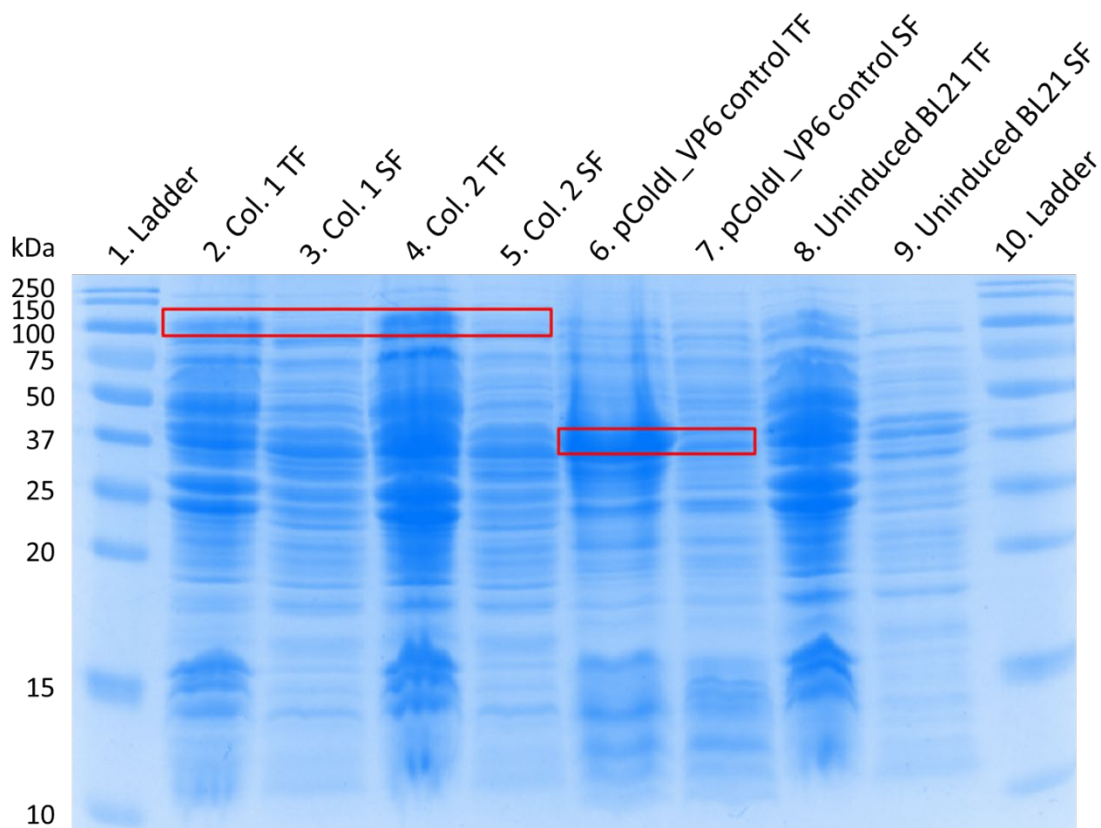


Figure 17: SDS-PAGE analysis of BL21_SA11_VP2. Two colonies were selected for VP2 expression. The highlighted region in lanes 2 to 5 indicate bands that cannot be observed in uninduced lanes 8 and 9. Lane 6 and 7 were used as a positive control for VP6 expression.

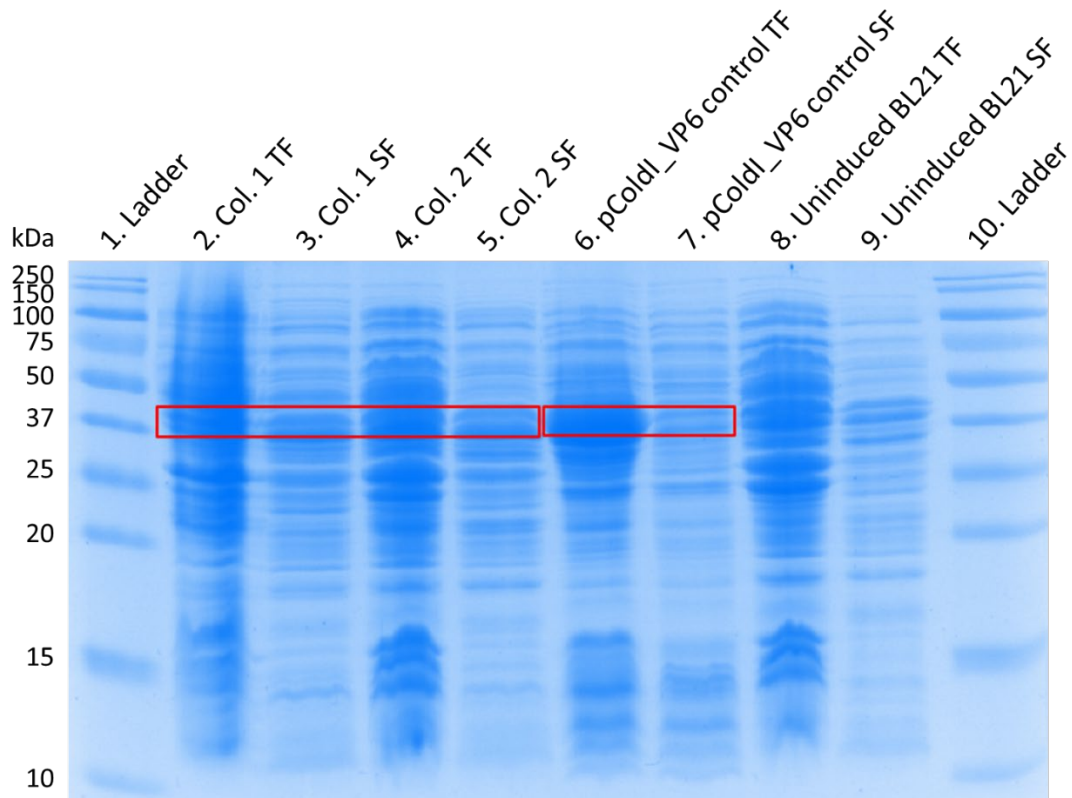


Figure 18: SDS-PAGE analysis of BL21_SA11_VP6. Two colonies were selected for VP6 expression. With the oversaturated protein concentration in each lane, no definite expression can be seen in the sample lanes nor the positive control lanes.

2.4 Summary

This study aimed to express bacterial codon optimised African GR10924 G9P[6] field strain VP2/6/7 and SA11 VP2/6/4. The ORF of GR10924 VP7 without the N-terminal endoplasmic reticulum signal peptide (amino acids 1-50) was sub-cloned into pCOLDI_GR10924_VP7. It was hypothesised that the toxicity of VP7 could be decreased by removing the leader peptide which is cleaved off before TLP formation. Expression of the truncated protein was obtained in Tuner and Origami cells (Figure 14). However, an additional truncation occurred. Instead of the desired 34 kDa protein, a truncated 27 kDa protein was expressed. Unfortunately, expression of this truncated protein could not be repeated.

No expression of GR10924 VP2/6 and SA11 VP2/6 could be confirmed with SDS-PAGE analysis despite the variety of expression conditions used, such as expression time, expression in bacteria that co-express chaperones (Figure 16), and different media in the experimental procedures. The expression time varied between 4h to 16h expression. Due to time constraints further, bacterial expression was halted and thus VP4 expression was not attempted.

Since our laboratory previously produced soluble VP6 fused to a trigger factor, it should be investigated again to see if it is possible to express VP2 and VP6 using this method. Recent studies in our laboratory aimed at expressing glycine-N-acyltransferase, GLYAT used the small ubiquitin modifying protein (SUMO) tag to express GLYAT in bacteria. SUMO is a yeast-derived N-terminal protein with chaperoning effects. Due to its hydrophobic inner core and hydrophilic surface, proteins are rapidly folded correctly (Butt *et al.*, 2005). The use of SUMO tag for the expression of rotavirus structural protein should also be investigated.

Chapter 3

Baculovirus expression of SA11 rotavirus-like particles

3.1 Introduction

Insect cells have the advantage of expressing high recombinant protein levels and are easy to scale up using suspension cultures. Since insect cells have many post-translational modification pathways like mammalian cells, proteins produced by the insect cells are mostly antigenically, immunogenically, and functionally similar to mammalian proteins.

The high recombinant protein production levels in insect cells have made it one of the most sought-after systems for protein production and assembly, such as VLPs. Dr. Jere previously used the baculovirus expression system in our laboratory to assemble VP4 and VP7 of African rotavirus strains on the same VP2/VP6 double-layered particle. This gave rise to the idea to make a universal backbone for the assembly of the most prominent rotavirus strains for each country. In our laboratory, SA11 VP2/VP6 was selected to be the backbone. As a first step, we aimed to generate SA11 VP2/VP6/VP4/VP7. For this, SA11 VP2/VP6 needs to be expressed in insect cells, and SA11 VP4 and VP7 must auto-assemble on double-layered particles.

The ORFs encoding SA11 VP2/VP6 were purchased in the same pFASTBACdual expression vector as these two proteins need to be expressed simultaneously to produce the double-layered particle. The ORFs of SA11 VP4 and VP7 were purchased in different expression vectors to make it easier to exchange the different strains for future experiments. Recombinant baculoviruses were produced using the Bac-to-Bac expression system. Following confirmation of rotavirus protein production on an SDS-PAGE, the sample will be ultracentrifuged to purify the particles before being sent for TEM for validation.

3.2 Materials and Methods

3.2.1 The principle of the Bac-to-Bac® expression system

The bac-to-Bac® expression system was developed at Monsanto and provides a efficient method to generate recombinant baculoviruses (Ciccarone *et al.*, 1998). The Bac-to-Bac expression system consists of three major components (Figure 19).

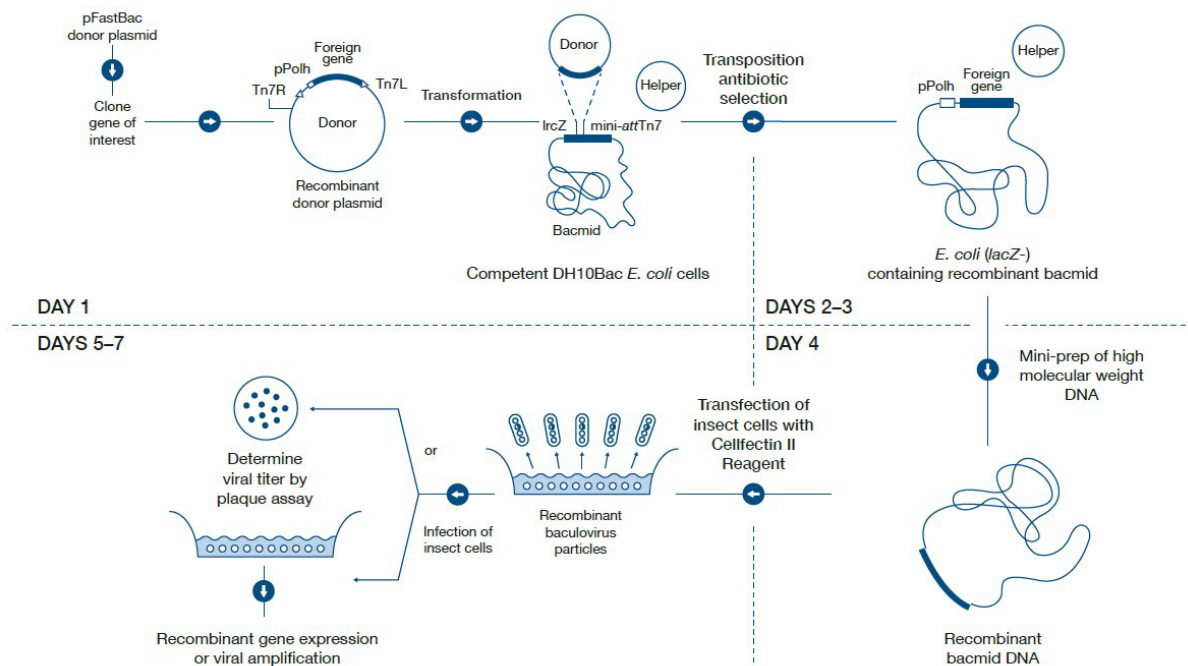


Figure 19: Schematic representation of the Bac-to-Bac® system. Flow diagram depicting the generation of a recombinant baculovirus. A gene of interest is cloned into the recombinant donor plasmid. The donor plasmid is transformed into competent DH10Bac cells containing the bacmid DNA. Transposition moves the expression cassette into the bacmid, making it recombinant. The recombinant bacmid DNA is extracted from the bacteria and transfected into SF9 cells. The recombinant virus is generated and selected through plaque assay. The image was taken from Thermofisher.com public domain (20/09/2020).

The first is the donor plasmid containing the gene(s) of interest under a baculovirus-specific promoter's control. The donor plasmid used for this MSc was pFASTBACdual (pFBd) containing two promoters and cloning sites, allowing the expression of two genes (Figure 20), one from the polyhedrin and another from

the p10 promoter. These two promoters are derived from the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). The donor plasmid contains two antibiotic resistance markers. One code for ampicillin is used during plasmid propagation, and the second is a gentamycin resistance marker (GmR) that will be transposed along with the gene of interest into the bacmid. The gene(s) of interest, promoters, and the GmR gene are collectively known as the expression cassette. Two mini-Tn7 elements flank the expression cassette. The mini-Tn7 is used to transfer the cassette into the bacmid.

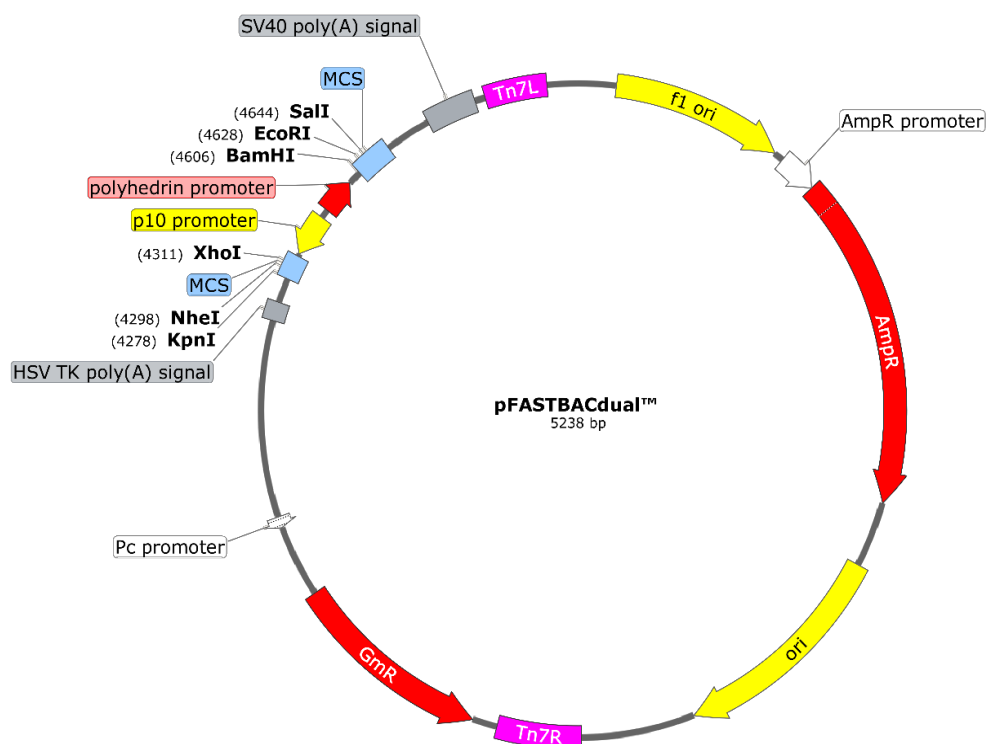


Figure 20: Map of the donor plasmid pFASTBACDual. pFBd consist of a p10 and polyhedrin promoter, ampicillin and gentamycin antibiotic resistance genes. The expression cassette is flanked by two mini-Tn7 elements.

The second component is the *E. coli* host strain, DH10Bac, containing a baculovirus shuttle vector (bacmid). This bacmid is propagated as a bacterial artificial chromosome (BAC). It contains the low-copy number mini-F replicon, a kanamycin resistance marker, and a DNA segment that codes for the lacZ α

peptide. The N-terminal region of the lacZ α contains the attachment site for the mini-Tn7 element (mini-*att*Tn7). This attachment site does not disrupt the reading frame of the lacZ α peptide. However, if the transposition of the expression cassette has taken place, then the lacZ α peptide reading frame will have been disrupted.

The transposition of the expression cassette is facilitated by the third component, a helper plasmid. The helper plasmid confers resistance to tetracycline and encodes for the transposase protein, which is needed to insert the mini-Tn7 site into the mini-*att*Tn7 target site on the bacmid. Once the pFBd plasmid is transformed into DH10Bac cells, transposition occurs between these two elements. With a successful transposition, the expression of the lacZ α peptide is disrupted, which leads to the production of white colonies when grown in the presence of a chromogenic substrate such as X-gal and the inducer IPTG.

To address the issues with proteases, Kaba and co-workers modified the baculovirus vector by deleting specific genes, chitinase, and v-cathepsin (Kaba *et al.*, 2004). This modification improved the expression of recombinant secretory proteins. Chitinase is involved in host insect cell liquefaction which burdens the protein translocation machinery of the cell. V-cathepsin is a protease also involved in insect cell liquefaction and may cause proteolysis of the recombinant proteins. With this modification, the name of the bacmid changed from bMON14272 to AcBAC Δ CC.

3.2.2 Codon optimisation and construction of donor plasmids

pFASTBacdual (pFBd) vector constructs were designed and sent to GenScript for insect cell codon optimisation. This study used the rotavirus strain RVA/Simian-tc/ZAF/SA11-H96(N5)/1958/G3P[2]. SA11_VP2 and SA11_VP6 genome segment sequences were sent to GenScript for codon optimisation and insertion in the pFastBacDual vector. The ORF of SA11_VP2 was placed under the p10 promoter using *Xho* I and *Nhe* I restriction sites (RE sites), and

SA11_VP6 ORF was placed under the polyhedrin promoter using *EcoR I* and *Sal I* sites (Figure 21). These RE sites were added at the 5' and 3' ends of the ORF, ensuring the ORF is placed in the correct orientation in the multiple cloning site. In the RV virion, there are 120 copies of VP2 and 780 copies of VP6. The p10 promoter is activated a few hours earlier than the polyhedrin promoter (Roelvink *et al.*, 1992). However, expression under the p10 promoter generally remains lower than expression under the polyhedrin promoter.

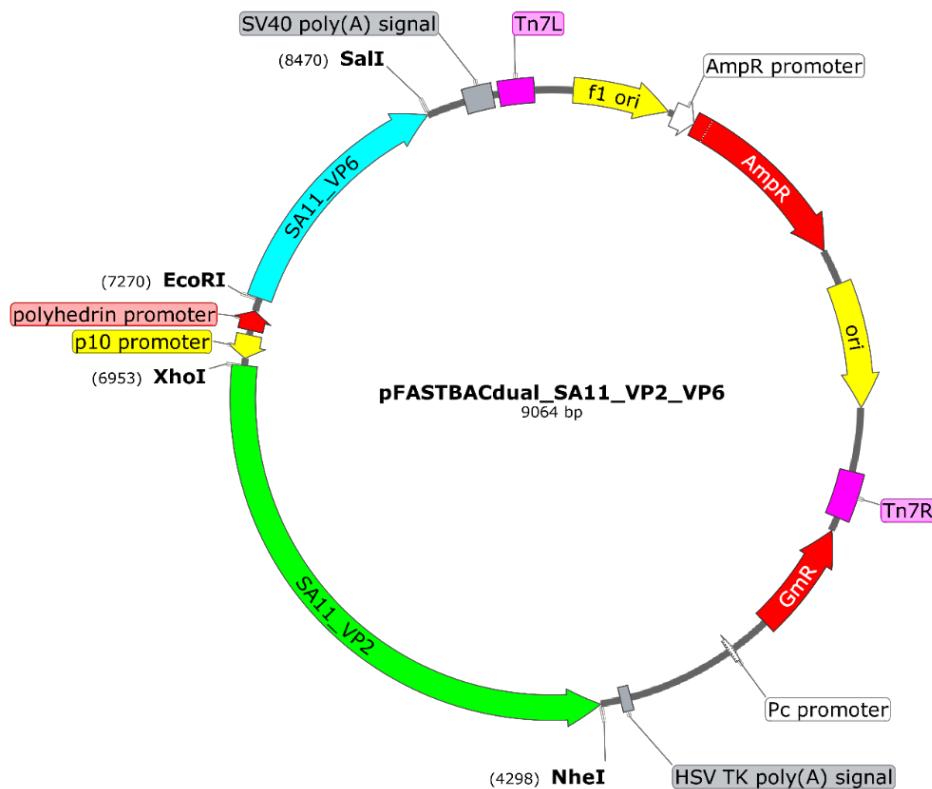


Figure 21: Map of pFASTBACdual_SA11_VP2_VP6. The ORF encoding SA11_VP2 (Green) under the p10 promoter using *Xho I* and *Nhe I* and SA11_VP6 (Cyan) under the polyhedrin promoter using *EcoR I* and *Sal I*. Plasmid maps constructed using SnapGene.

The ORFs encoding SA11_VP4 and SA11_VP7 were codon optimised and cloned in separate pFBdual plasmids to allow easy exchange of RV ORFs of different strains. The ORF of SA11_VP4 (180 copies) was also subcloned under the p10 promoter using *Kpn I* and *Xho I*, and the ORF of SA11_VP7 (780 copies) was placed under the polyhedrin promoter, using *BamH I* and *EcoR I* (Figure 22 and Figure 23).

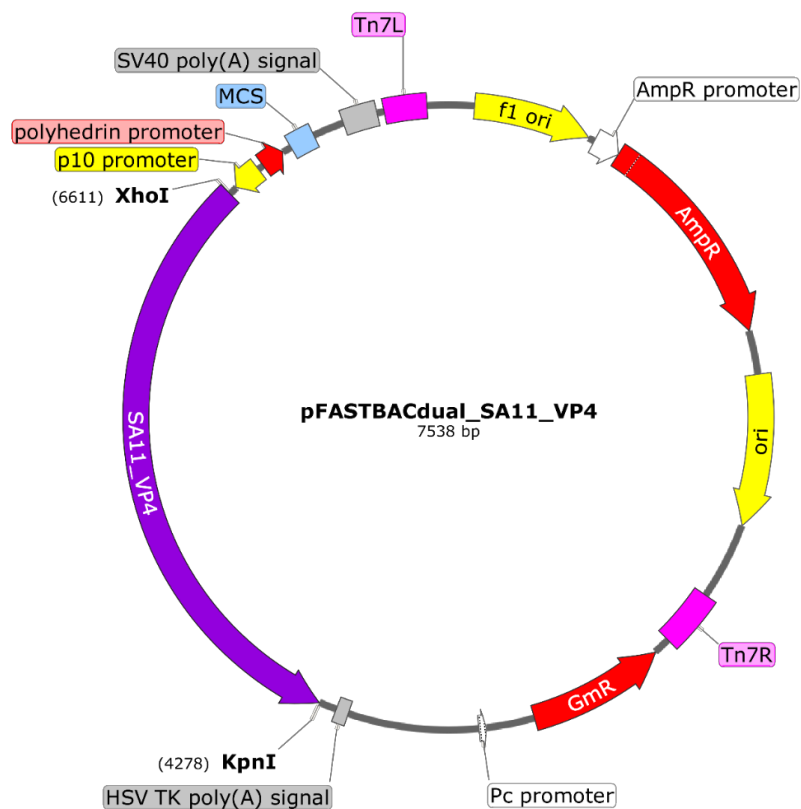


Figure 22: Map of pFASTBACdual_SA11_VP4. SA11_VP4 (Purple) under the p10 promoter using *Kpn I* and *Xho I* restriction enzyme sites. Plasmid maps constructed using SnapGene.

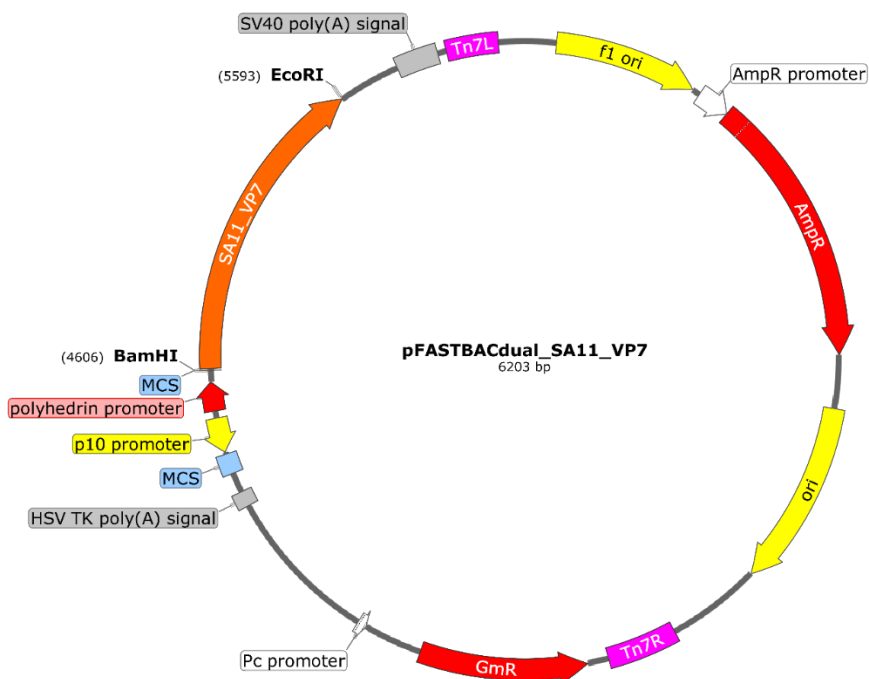


Figure 23: Map of pFASTBACdual_SA11_VP7. SA11_VP7 (orange) under the polyhedrin promoter using *BamH I* and *EcoR I*. Plasmid maps constructed using SnapGene.

3.2.3 Transformation, purification, and validation of donor plasmids

Plasmids were received as lipolysed samples from GenScript. The tubes containing the plasmids were first centrifuged to ensure that the plasmids were concentrated at the tubes' bottom. To get the final concentration of 100 ng/μl, 40 μl of nuclease-free water was added. The plasmids were dissolved in the water for 10 minutes before the tubes were centrifuged again, ensuring the plasmid solution is collected at the bottom of the tube.

For propagation, 1 μl of the pFBd plasmid was transformed into commercially competent DH5α cells using the heat-shock method described in Section 2.2.3. A serial dilution of the transformation mix was plated on LB agar plates containing 100 μg/ml carbenicillin overnight at 37°C. Six well-separated colonies were picked and inoculated in 5 ml of LB media with 100 μg/ml carbenicillin overnight at 37°C shaking at 225 rpm for aeration. The plasmid DNA from single colonies was isolated using the Qiagen miniprep kit by following the manufacturer's instructions as described in Section 2.2.5. Plasmids isolated from single colonies were sent for Sanger sequencing to ensure that they were correct before they were transformed into chemically competent AcBACΔCC cells (Section 2.2.2).

3.2.4 Transposition of pFBd expression cassettes into bacmid DNA

For the generation of recombinant bacmids, 100 ng pFBd DNA was transformed into chemically competent AcBACΔCC cells prepared using the DMSO method (Section 2.2.2). A serial dilution of the transformation mixture was plated on LB agar plates containing 100 μg/ml kanamycin, 14 μg/ml gentamicin, 50 μg/ml tetracycline, 4% IPTG, and 32 μg/ml X-gal. Plates were incubated for 24 to 48 hours at 37°C and then moved to 4°C for improving colour development.

To ensure that the colonies' phenotype is genuinely white, 10 white and 2 blue colonies were streaked out again on fresh LB agar plates containing the

appropriated antibiotics with IPTG and X-gal and incubated overnight at 37°C. Single colonies (white and blue) from these plates were picked up again and inoculated in 5 ml LB media with the correct antibiotics, at 37°C, 225 rpm overnight to reach the stationary phase. Of this overnight culture, 3 ml was used for bacmid extraction, and the rest was used to make glycerol stocks to a final concentration of 15% glycerol and stored at -80°C.

3.2.5 Purification of recombinant bacmid DNA

This protocol uses alkaline lysis conditions combined with the detergent SDS described in Birnboim and Doly with slight modifications (Birnboim & Doly, 1979). A 1.5 ml volume of an overnight culture was collected by centrifugation at 14 000 x g for 1 minute. This was repeated for a total of 3 ml of cells pelleted in a 1.5 ml conical micro-centrifuge tube. The supernatant was removed, and the cells were resuspended in 200 µl GTE buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris/HCl pH 8, 0.1 mg/ml RNase A). A volume of 400 µl 0.2 M NaOH/1% SDS solution was added to each sample, mixed gently, and incubated for 5 minutes at room temperature. To this, 300 µl chilled (4°C) KAc solution (60 ml 5 M potassium acetate pH 4.8-5.2, 28.5ml ddH₂O, and 11.5 ml glacial acetic acid) was slowly added to the solution and mixed gently. Samples were placed on ice for 10 minutes. The thick white precipitate of protein and genomic DNA formed and agglutinated into a ball. The suspension was centrifuged at 14 000 x g in a benchtop centrifuge for 10 minutes. After centrifugation, the supernatant was carefully removed from the tube, to avoid the white precipitate material, and transferred to a new microcentrifuge tube. This supernatant was centrifuged again to ensure that none of the white precipitate will be present in downstream reactions.

During the centrifugation, 800 µl of absolute isopropanol at room temperature was added to new 1.5 ml conical tubes. The supernatant was gently transferred to the tube containing the isopropanol. The tubes were mixed by inverting the tube several times and placed on ice for 10 minutes. The samples were

centrifuged for 15 minutes at 14 000 x *g* to precipitate the DNA. The supernatant was carefully removed, and the DNA pellets were washed with 500 µl 70% ethanol. The tubes were inverted several times to wash the pellet and centrifuged for 5 minutes at 14 000 x *g*. This wash step was repeated. The supernatant was carefully removed to ensure that the DNA pellet remains behind, after which the pellets were left to dry. The pellet was dissolved in 40 µl molecular grade water, heated to 60°C, by tapping the tube's side. The DNA concentration and its purity were determined with a NanoDrop spectrophotometer (Section 2.2.6). Further analysis was done with PCR.

3.2.6 PCR analysis of recombinant DNA

To confirm if the expression cassette was transposed to the bacmid, PCR was used. M13 amplification primers were used as they target sequences directly on either side of the mini-*att*Tn7 site within the bacmid (Figure 24 and Table 10). The Sanger sequencing primers were synthesised and HPLC purified by TIB Molbiol™ and are set out in Table 9. The PCR reaction mixtures were set up as follows: 10 µl 5x Q5 reaction buffer, 1 µl (10 mM) dNTPs, 2.5 µl (10 µM) of each appropriate primer, 100 ng bacmid DNA, 0.5 µl Q5 DNA polymerase, and nuclease-free H₂O to a final volume of 50 µl.

The reaction was incubated in a thermocycler (T100 Thermal Cycler, Biorad, California, USA) at 98°C for initial denaturing for 3 minutes followed by 30 cycles of 98°C for 45 seconds, 55°C for 45 seconds and 72°C for 5 minutes, and a final elongation cycle at 72°C for 10 minutes. The samples were held at 4°C until further use.

Table 10: Primers used for bacmid PCR and Sanger sequencing.

Bacmid PCR primers	Sequence	Length	T _m
M13 Forward (-40)	5' - GTTTTCCCAGTCACGAC - 3'	17 mer	51°C
M13 Reverse	5' - CAGGAAACAGCTATGAC - 3'	17 mer	46°C

Sanger sequencing primers			
pFBd_polH_for	5' - GGAGATAATTAATAATGATAACCATCTCGC - 3'	29 mer	56°C
pFBd_polH_rev	5' - GATTATGATCCTCTAGTACTTCTCGAC - 3'	27 mer	56°C
pFBd_pol10_for	5' - GGACCTTTAATTCAACCCAACAC - 3'	23 mer	56°C
pFBd_pol10_rev	5' - CAGTTAGCCTCCCCCATCTC - 3'	20 mer	59°C
SA11_VP4_for	5' - CCTGTGGGACAGTGGC - 3'	16 mer	56°C
SA11_VP4_rev	5' - CAGGGAGAAGTTGTATGATCCG - 3'	22 mer	57°C
SA11_VP2_for	5' - GACATGTTTCATCAGGGAGTCC - 3'	21 mer	56°C
SA11_VP2_rev	5' - GTTAGGGATCACGGTCAGC - 3'	19 mer	56°C

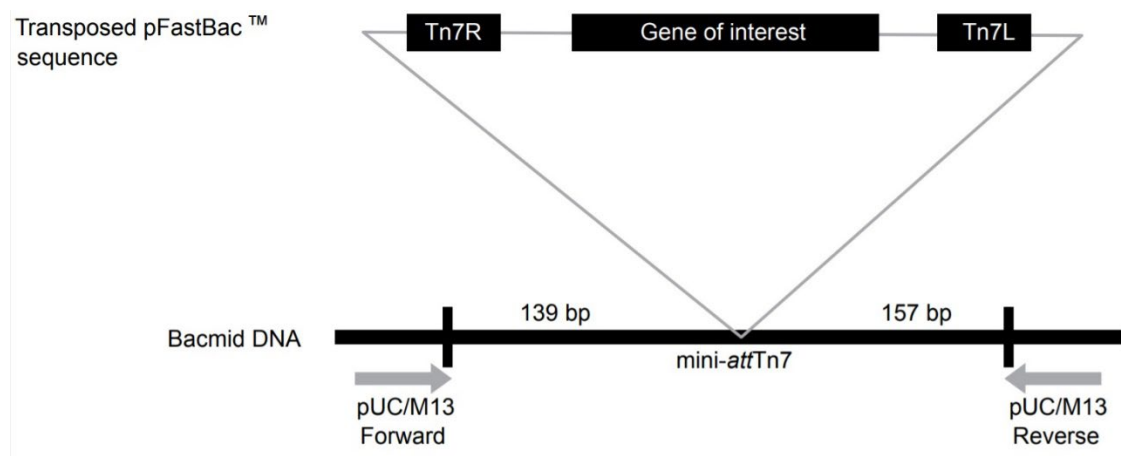


Figure 24: Schematic representation of the M13 forward and M13 reverse priming sites flanking the mini-*attTn7* site in bacmid DNA.

Table 11: The expected PCR amplicons with the M13 primers.

Sample	Size of PCR product
Bacmid alone	~ 300 bp
Bacmid + pFBd + gene of interest	~ 2562 bp + 300 bp + size of insert
Bacmid + pFBd + VP2/VP6	~ 6705 bp
Bacmid + pFBd + VP4/VP7	~ 6174 bp
Bacmid + pFBd + VP4	~ 5193 bp
Bacmid + pFBd + VP7	~ 3843 bp

3.2.7 Sanger sequencing of recombinant bacmids

Although PCR analysis helps determine whether the expression cassette has been successfully transposed, the bacmids were sent for sequencing to determine that no mutations occurred during transformation and transposition in the expression cassettes.

Primers were designed that were in the promoter and terminator regions of the pFASTBAC plasmids. However, since Sanger sequencing usually only reaches about 1000 bp, internal primers for the ORF of VP2 and VP4 were also designed (Figure 25). PCR was performed on extracted bacmids, and those with the correct sizes were sent for Sanger sequencing. These products were sent to the Central Analytical DNA Sequencing Facility (Stellenbosh University, South Africa), where they performed PCR clean-up before sequencing.

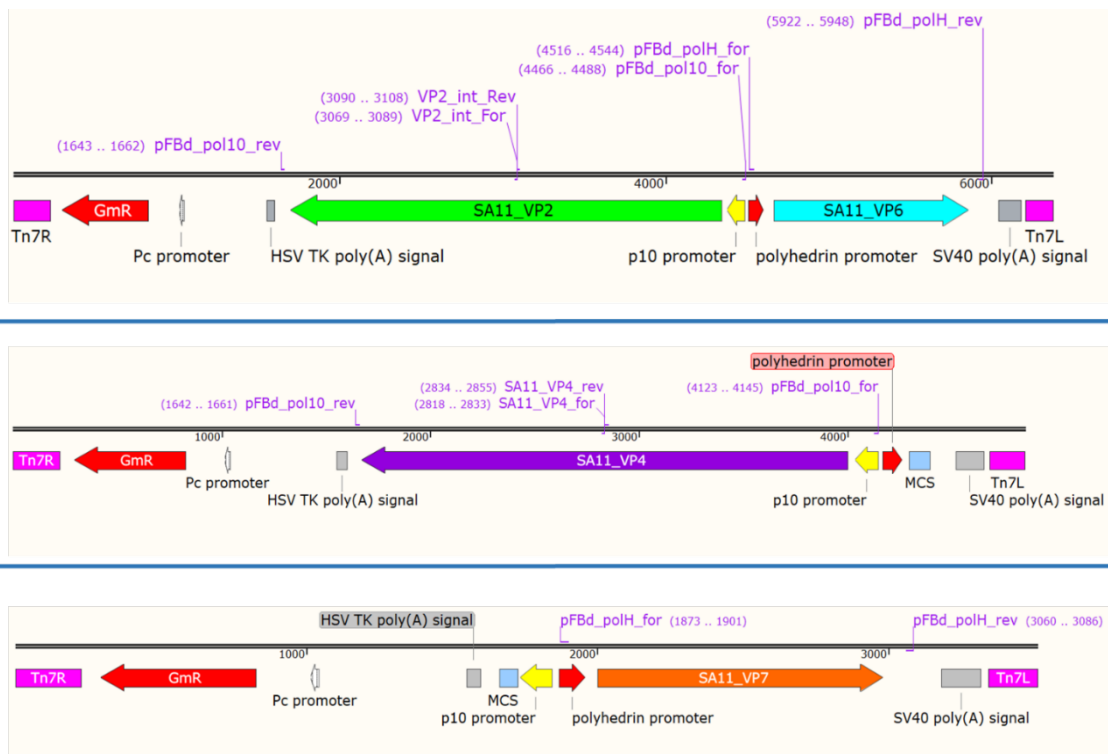


Figure 25: Expression cassette of each pFASTBAC plasmid. This image depicts the primer binding sites for each of the plasmids for sequencing. (Top) Expression cassette for pFASTBACdual_SA11_VP2_VP6. VP2 has 2 additional primers to increase the chance for complete sequence coverage for Sanger sequencing. (Middel) Expression cassette for pFASTBACdual_SA11_VP4. VP4 also contains two additional primers. (Bottom) Expression cassette for pFASTBACdual_SA11_VP7.

3.2.8 Propagation of SF9 insect cells

Cells were grown at 27°C in TC-100 media (Gibco, Thermo Scientific, MA, USA; (Cat. No. 13055025), 10% fetal bovine serum (FBS) (Gibco, Thermo Scientific, MA, USA) (Cat. No. 10493106), 1% antibiotic and antimycotic solution (penicillin, streptomycin, and fungizone) (Thermo Scientific, MA, USA; Cat. No. 15240062) and 0.1% pluronic F68 (Gibco, Thermo Scientific, MA, USA; Cat. No. 24040032) if cells were grown in suspension culture. Media supplemented with FBS, antibiotics, and antimycotic is referred to as complete growth media.

3.2.9 Transfecting insect cells

According to the manufacturer's protocol, the transfection reagent FuGENE® HD (Promega Corporation, Madison, WI, USA; Cat. No. PRE2311) was used. In short, one day before transfection, cells were seeded in a 6-well plate at 1×10^6 cell/ml. In a sterile 15 ml Falcon polystyrene round-bottom tube 96 µl, prewarmed to room temperature, media was added. 40 µl of extracted recombinant DNA was diluted in 60 µl opti-MEM (Gibco, Thermo Scientific, MA, USA; Cat. No. 31985062). A volume of 16 µl FuGENE® HD transfection reagent was added for a 4:1 FuGENE® HD transfection reagent: DNA ratio and mixed immediately. This transfection reagent/DNA complex was incubated for 15 minutes at room temperature, added dropwise onto the cells, and incubated in a humidified incubator at 27°C until signs of viral infection were visible for approximately 72 hours. The cells were detached from the bottom of the well, and the supernatant was collected from each well and centrifuged at $500 \times g$ for 5 minutes to pellet cells and debris. The clarified supernatant was transferred to a new tube as viral stocks and stored at 4°C, protected from light, until further use.

3.2.10 Amplifying recombinant baculoviruses and expression of recombinant proteins

SF9 cells were seeded at 1×10^6 cells/ml a day before infection in a 6-well plate. A volume of 300 μ l of the clarified transfection supernatant was diluted to 500 μ l with complete growth media. The seeded media was removed from the 6-well plates before infecting the cells with the 500 μ l. The 6-well plates containing the infected cells were incubated at room temperature on a gentle rocking platform for one hour. An additional 1.5 ml of complete growth media was added before the plates were incubated at 27°C in a humidified incubator until signs of viral infection were visible. The cells were detached from the bottom of the well, and the supernatant was collected from each well and centrifuged at 500 x g for 5 minutes to pellet cells and large debris. The supernatant was transferred to a new tube and kept as the P1 viral stocks.

The cells were lysed for one hour with 200 μ l of RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0,5% sodium deoxycholate, 0,1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH8)) and complete mini-EDTA free protease inhibitors (Thermo Scientific, MA, USA; Cat. No. A32953). A volume of 50 μ l of the lysed cells was taken as the total fraction before the lysates were subjected to centrifugation. Collection of the soluble fraction followed centrifugation at 16 000 x g for 5 minutes. Both protein fractions were analysed through SDS-PAGE (section 2.2.12).

3.2.11 Viral plaque assay

To determine the titre and to plaque purify the viral stocks, agarose overlay plaque assays were performed as described in the Invitrogen™ Bac-to-Bac® Baculovirus Expression System manual (Version D). A plaque is formed when an infected cell within the immobilised cell monolayer lyses, and viruses are released that spread to adjacent cells where the process is repeated.

The P1 stock of the recombinant baculoviruses was used to make an 8-log serial dilution by taking 100 µl of the clarified supernatant and adding it to 900 µl TC-100, FBS free, media. This was done sequentially until a dilution of 10^{-8} was achieved.

SF9 cells seeded at a density of 5×10^5 cells/ml were infected by 100 µl of each serial dilution in two 6-well plates. The cells were incubated with the virus on a rocking platform for 1 hour. Following the 1 hour incubation, the media containing the virus was removed and replaced with 2 ml of the plaquing medium [50% Grace's Insect medium (2X) (Thermo Scientific, MA, USA; Cat. No. 11667037) (supplemented with 20% FBS and 1% antimycotic-antimicrobial), 25% distilled water and 25% of melted 4% low melting point agarose gel (Sigma, St. Louis, Missouri, USA; Cat. No. A9045-25G)]. After allowing the plaquing media to set for 20 minutes at room temperature, the plates were moved to a 27°C humidified incubator for 7-10 days until plaques are visible and ready to count.

Plaques were visualised by staining the plates with Neutral Red. Neutral Red is a dye that needs to be actively transported into the cell, thus it will only stain live cells (Gonzalez-Hernandez *et al.*, 2013). Plates containing the plaques assay were stained with 0.5 ml Neutral Red (1 mg/ml) (Sigma, St. Louis, Missouri, USA; Cat. No. N4638-5G) and incubated at room temperature for 2 hours. After the two-hour incubation, the excess stain was removed with a pipette, and the clear spots (plaques) were counted.

3.2.12 Plaque purification

To generate viral stocks from a single infectious virus, plaque purification was used. Single plaques were carefully selected and picked up with a P1000 pipette. The agarose plug containing the virus was transferred to a 1.5 ml microcentrifuge tube containing 500 µl media. SF9 cells at a density of 5×10^5 cells/ml were infected by 100 µl of purified plaque solution and incubated at 27°C, in a humidified incubator until cytopathic effect (CPE) is complete.

The supernatant was removed and stored for further use. The remaining monolayer was used to perform an Immunofluorescent monolayer assay (IFMA) (Section 3.2.14) to see if the selected viruses contained the recombinant virus. The supernatant was passaged again on SF9 cells as described in Section 3.2.10 to amplify the viral stock.

3.2.13 Viral titre and multiplicity of infection (MOI) determination

The viral titre is defined by the number of plaque-forming units (PFU) per milliliter in a sample. To determine the viral titre, a plaque assay was performed as described in Section 3.2.11. To calculate the viral titer, the number of plaques is multiplied by the dilution factor.

$$\text{Titre } \left(\frac{\text{pfu}}{\text{well}} \right) = \text{number of plaques} \times \text{dilution factor} \times 1/(\text{mL of inoculum/well})$$

After the viral titre was determined, the MOI was calculated for optimal protein expression. The MOI represents the ratio of virus to cell. An MOI of 5 means 5 viruses per cell are present in an inoculum.

$$\text{Volume of virus} = \frac{(\text{desired MOI})(\text{number of cells})}{\text{titre of viral stock}}$$

3.2.14 Immunofluorescent monolayer assay (IFMA)

The basic principle of this technique is the same as an enzyme-linked immunosorbent assay (ELISA). However, this technique is performed on a cell culture monolayer. The cells and their proteins are fixed on the plate by adding

2 ml of ice-cold organic solvent solution (1:1 methanol: acetone) and stored for 1 hour at -20°C. The cells become permeable during this step which allows the primary antibody to attach to specific proteins. Before the primary antibody was placed on the monolayer, the cells were first treated with 2 ml blocking buffer (1x PBS, 0.05% Tween-20 and 1% (w/v) tryptone) and incubated for 1 hour at 37°C. The proteins in the blocking buffer bind to all the remaining surfaces of the plate. This improves the sensitivity of the assay by reducing background interference. Without the blocking buffer, the signal-to-noise ratio would be high.

After the 1 hour incubation period, the blocking buffer was removed and replaced with the 2 ml anti-SA11_RV DLP VP2/VP6 rabbit whole serum (provided by Prof. Potgieter, Deltamune, RSA, in association with Prof. H.G. O'Neill, University of the Free State, RSA) (1:1000, primary antibody: blocking buffer) for another hour at 37°C. After this step, the primary antibody was washed off with blocking buffer and replaced with 2 ml Alexa 488 goat anti-rabbit IgG (ThermoFisher, Cat. No. A-11008) (1:2000, secondary antibody: blocking buffer). After the secondary antibody attachment was complete, the surplus was washed off. The secondary antibody contains a fluorescent complex making visualisation of the monolayer possible under a fluorescent microscope (Nikon eclipse TE2000-S, Nikon Instruments Inc. Melville, NY, USA), containing a UV light source and a green filter.

3.2.15 Western blot analysis

Western blot analysis was performed to separate and verify the expression of recombinant proteins and determine their size. The proteins were size separated by SDS-PAGE. However, the gels were not stained with Coomassie blue. The proteins on the gels were electro-blotted onto a nitrocellulose membrane (Whatman® GmbH. Dassel. Germany) with Trans-Blot® Turbo™ transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol) using a Trans-Blot Turbo Transfer System at 25 V for 30 minutes.

The nitrocellulose membranes were submerged in blocking buffer (5 % non-fat skim milk [Nestle (Pty) Ltd, Randburg, South Africa] in TNT buffer [0.05 M Tris-HCl (pH7.4), 0.05% Tween20 and 0.2 M NaCl]) for 1 hour at room temperature. The blocking buffer was removed and replaced with the appropriate primary antibody (anti-SA11_RV DLP VP2/VP6 rabbit whole serum), diluted to 1:2000 in blocking buffer for 1 hour at room temperature or overnight at 4°C. The membranes were washed three times (1 x 10 minutes and 2 x 5-minutes) with TNT buffer to remove any residual primary antibody. A secondary antibody (HRP conjugated goat anti-rabbit) 1:10000 in blocking buffer was added and incubated for 1 hour at room temperature. The surplus secondary antibody was washed off with TNT buffer as described above.

To detect the horseradish peroxidase (HRP) conjugated secondary antibody, the membrane was submerged in AEC buffer (0.03% AEC, 3-amino-9-ethylcarbazole in 100% Dimethylformamide, 0.2% H₂O₂ in 0.05 M sodium acetate buffer pH5.2), until the reaction with the viral proteins was clear. The reaction was stopped by removing the AEC buffer and washing the membrane with H₂O.

3.2.16 Isolation of rotavirus virus-like particles for transmission electron microscopy (TEM)

For VLP purification, cells and media were harvested using the method described in 3.2.10. The supernatant obtained from the cells and media was centrifuged through a 40% sucrose cushion in Tris-calcium buffer (10 mM Tris-HCl, pH7.4, 20 mM CaCl₂) using a Sorvall W X Ultra Series centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) with the Surespin 630 rotor (36 ml) at 100,000 x *g* for 2 h at 4°C. The supernatant was removed, and the pellet containing the viral particles was resuspended in 400 µl Tris-calcium buffer.

To determine if viral particles were obtained a volume of 10 ml of sample was placed in a 36 ml ultracentrifuge tube. The tube was filled with sucrose by adding

the sucrose underneath the sample using a needle until 1-2 mm is left open on the top of the tube. The tubes were weighed to ensure that they were all the same weight before being subjected to ultracentrifugation. The sucrose was decanted, and the pellet was resuspended in 400 µl TC buffer. After confirmation, the rest of the samples were filled with Tris-calcium to ~5 ml and centrifuged again using a Surespin 630 rotor at 100,000 x *g* for 90 minutes at 4°C. This centrifugation step was to ensure that all the sucrose is removed for visualisation using a TEM. After the centrifugation, the pellet was resuspended again in 200 µl of Tris-calcium buffer.

The viral particles were verified by adding a drop of viral suspension on a 300-mesh carbon-covered grid. Excess fluid was drained from the grid with filter paper before staining with 1.5% ammonium molybdate (aqueous) pH 7 for 3 minutes. The surplus stain was removed with filter paper and allowed to dry for 5 minutes before being examined in a Tecnai G12 transmission electron microscope (FEI, Netherlands) at 120 kV. Micrographs were recorded with a Gatan bottom mount camera and Digital Micrograph software.

3.3 Results and Discussion

3.3.1 Generating three SA11 recombinant bacmids

Recombinant bacmids were generated by transforming 100ng of each SA11 pFBd donor plasmids (pFBd_VP2/VP6, pFBd_VP4, and pFBd_VP7) into the *E. coli* host strain, AcBACΔCC as described in Section 2.2.2. Due to the insertion into the LacZ gene within the bacmid, white colonies represent successful recombination (Section 3.2.1). However, this alone is not a full confirmation because it is possible that in some cases where the majority colonies are white, i.e., recombinant, a small sub-population may be non-recombinants (Grose *et al.*, 2021). These non-homogenous colonies start out white but gradually develop

into bulls' eyes (white colonies with a blue centre). Bulls' eyes were not used for bacmid isolation as they would yield mixed populations of unaltered and recombinant bacmids. Fully blue colonies containing unaltered bacmids were used as negative controls and were designated as wild-type baculoviruses. Colonies were selected for bacmid extraction as described in Section 3.2.5. The DNA concentration for the high molecular weight bacmid DNA was obtained using a NanoDrop One spectrophotometer described in Section 3.2.5. This concentration was not an absolute representation of bacmid yield, as the crude extraction method still leaves RNA fragments and salt contaminants. It was only used to ensure that bacmid extraction was successful before PCR validation was done.

pFASTBACdual SA11 VP2/VP6

PCR analysis was performed on 30 colonies containing pFBd_SA11_VP2/VP6 to verify that recombinant bacmids were obtained and to identify colonies with a mixture of recombinant and wild-type bacmids (bulls' eyes). The expected results for the PCR fragments are summarised in Table 11. Figure 26 shows the PCR results for the 30 VP2/VP6 colonies along with 2 wild-type colonies. Even though the 30 colonies appeared to be white during the second blue/white screening test, PCR results indicated that 14 colonies (number 2,3,4,5,6,13,21,23,25,26,27,28,28 and 30) contained a mixture of recombinant and wild type bacmids. The 14 mixed colonies yielded the expected 6705 bp amplicon and the 300 bp negative control amplicon (Section 3.2.6 Table 11). These could not be used further in the experiment as the wild-type baculovirus would out-compete the recombinant baculoviruses.

Seven colonies (number 7,9,10,11,12,15, and 16) appeared not to contain any wild-type bacmids and were the only ones used for further testing. Figure 26, Figure 27, and Figure 28 contained a non-specific band at around 1800 bp. Colony 31 was a blue colony selected, and colony 32 is from untransfected competent cells used as a negative control, thus the expected size of 300 bp was obtained for their amplicons.

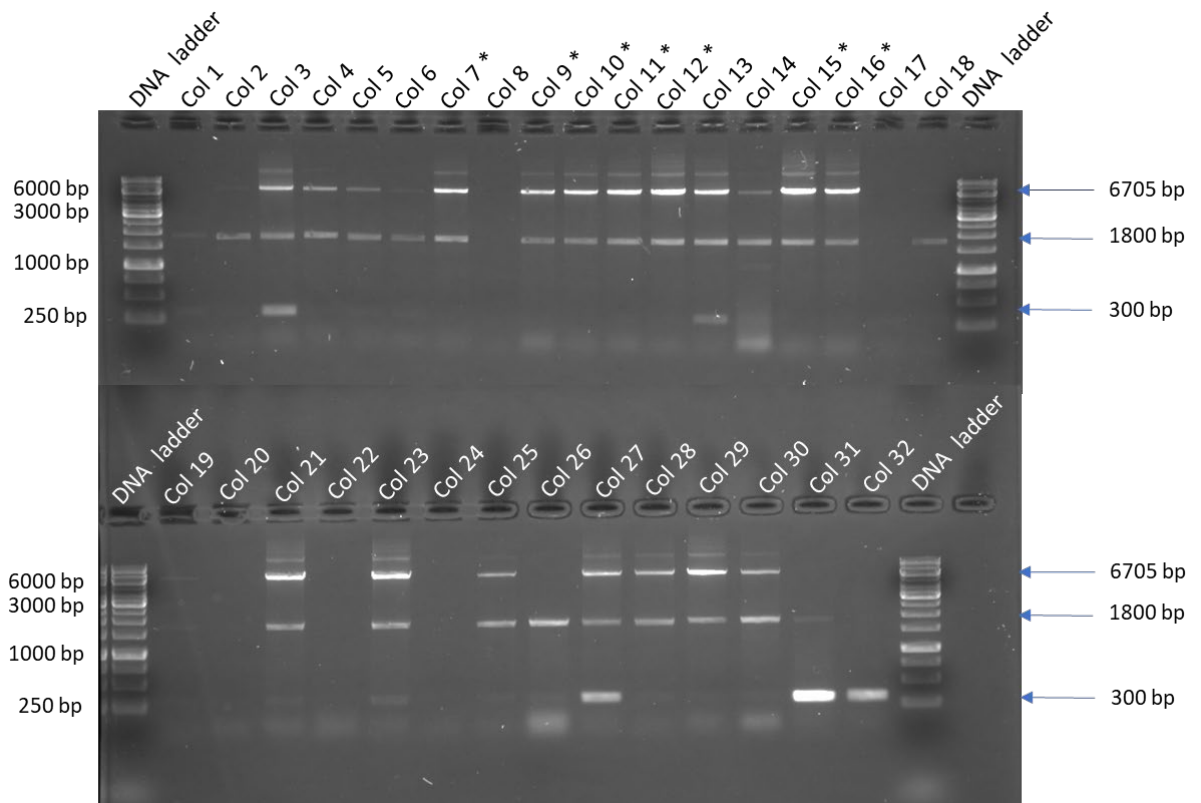


Figure 26: Agarose gel electrophoresis of recombinant SA 11 VP2/VP6 bacmid PCR amplicon to confirm transposition of expression cassette from donor pFBd plasmid into respective bacmid DNA. The first and last lane of each row contain an O'GeneRuler DNA Ladder Mix molecular marker. For confirmation, 30 white colonies and 2 blue colonies, as a negative control, were selected for PCR. Expected PCR amplicon size of recombinant SA11 VP2/VP6 bacmid is 6,705 bp and negative control is 300 bp. Colonies selected for further use are indicated with a star (*).

One of the drawbacks of the baculovirus system is the screening of recombinant bacmids for differentiation from wild-types. The Bac-to-Bac system has several checkpoints such as antibiotic selection, blue-white screening, PCR analysis of amplicons, and plaque purification. However, several blue colonies were still obtained under gentamycin selection, such as colony 31. The blue colonies indicate that the attTn7 site in the bacmid was not interrupted even though the expression cassette has been transposed for the selection marker to work. In 2021, another attTn7 site was discovered in the bacterial chromosome at the end of the vital glmS gene (Grose et al., 2021). This suggests that the expression cassette can also be transposed into the chromosomal DNA rather than into the bacmid, as shown with colony 31.

pFASTBACdual SA11 VP4

Twelve white colonies were selected to amplify recombinant VP4 bacmid, as can be seen in Figure 27. Colonies 1, 2, 3, 4, 5, 7, 9, 11, and 12 had no amplification of the recombinant bacmid. Only colony 6, 8, and 10 contained the correct size amplicon of the expected 5,193 bp.

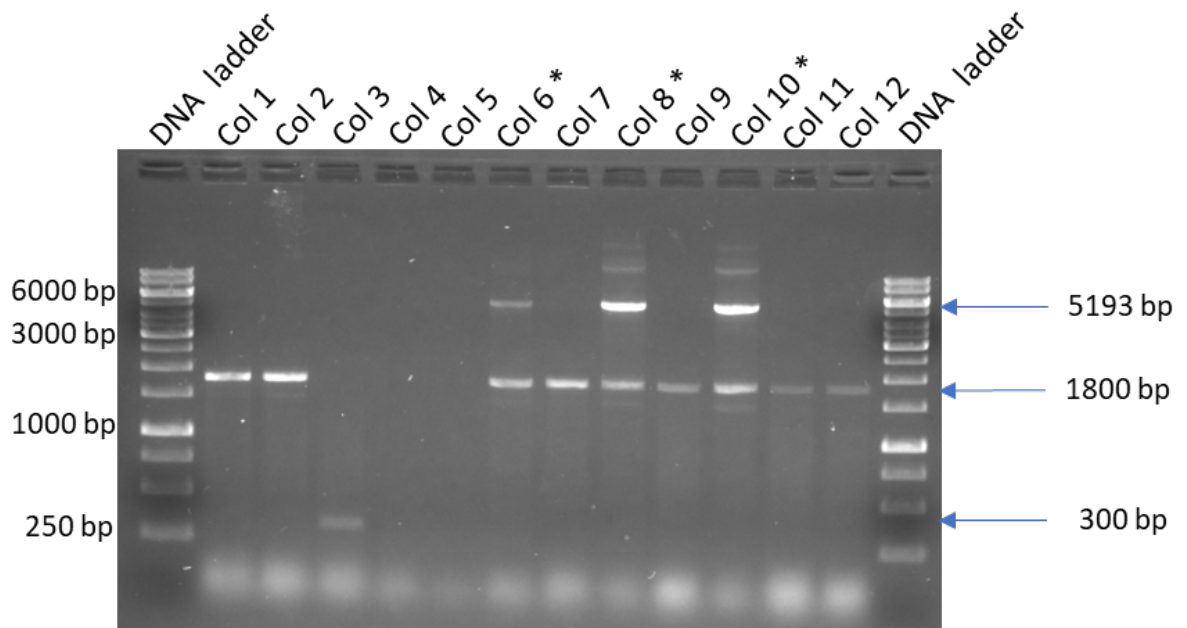


Figure 27: Agarose gel electrophoresis of recombinant SA11 VP4 bacmid PCR amplicon to confirm transposition of expression cassette from donor pFBd plasmid into respective bacmid DNA. The first and last lanes contain an O'GeneRuler DNA Ladder Mix molecular marker. The expected PCR amplicon for the recombinant SA11 VP4 is 5193 bp. Poor amplification can be observed as only 3 colonies contain the correct amplicon. Colonies 6, 8, and 10 contained the correct size amplicon mark with a star (*). Colony 3 was the only sample that contained wild-type bacmid, even though no other amplification can be seen. Primer dimerization is seen as well.

pFASTBACdual SA11 VP7

Six colonies (Colony numbers 1,2,3,4,5, and 12) contained no amplification of the recombinant cassette. However, colony 1, 5 and 12 contains the 1800 bp non-specific amplification as described above. Only colony 6, 7, 8, 9, 10, and 11 have the correct amplification of 3,843 bp for it to be recombinant VP7. No blue colony containing wild-type bacmid was used as the VP2/VP6 PCR results already contained wild-type bacmids used in all the experiments.

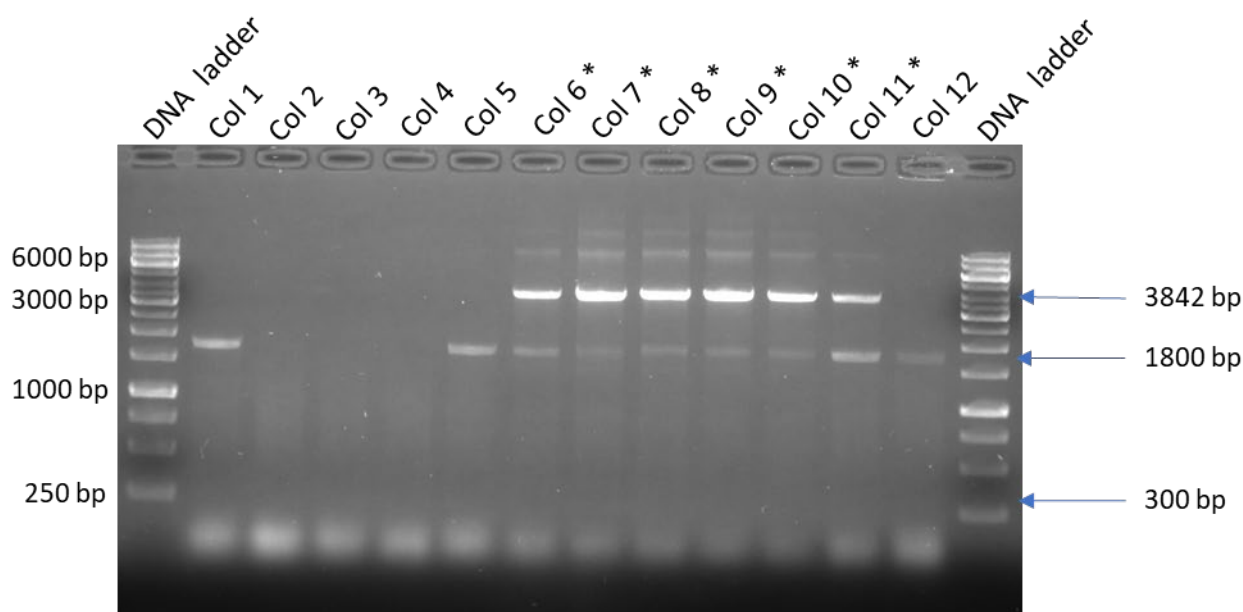


Figure 28: Agarose gel electrophoresis of recombinant SA11 VP7 bacmid PCR amplicon to confirm transposition of expression cassette from donor pFBd plasmid into respective bacmid DNA. The first and last lanes contain an O'GeneRuler DNA Ladder Mix molecular marker. The expected PCR product size for the recombinant VP7 bacmid is 3842 bp. Colonies 6 - 11 contained the correct amplicon size as expected.

3.3.2 Generation of recombinant baculoviruses expressing SA11 VP2/VP6

The purified bacmid DNA from colonies 7,9,10,11,12,15 and 16 (Figure 26) was transfected into SF9 cells to generate recombinant baculoviruses expressing rotavirus structural proteins VP2 and VP6 as described in Section 3.2.9. After complete CPE was observed, the viral stock was amplified as described in Section 3.2.10. The media containing the virus was removed from the 6 wells. An IFMA was performed on the monolayer to confirm that the baculovirus sample being amplified expressed VP2 and VP6 as described in Section 3.2.14.

Figure 29 shows normal uninfected SF9 cells under a brightfield microscope view (Figure 29 A) and the same field visualised for fluorescence (Figure 29 B). The baculovirus stock generated from colony 11 bacmid DNA is shown under brightfield (Figure 29 C) and fluorescence as well (Figure 29 D).

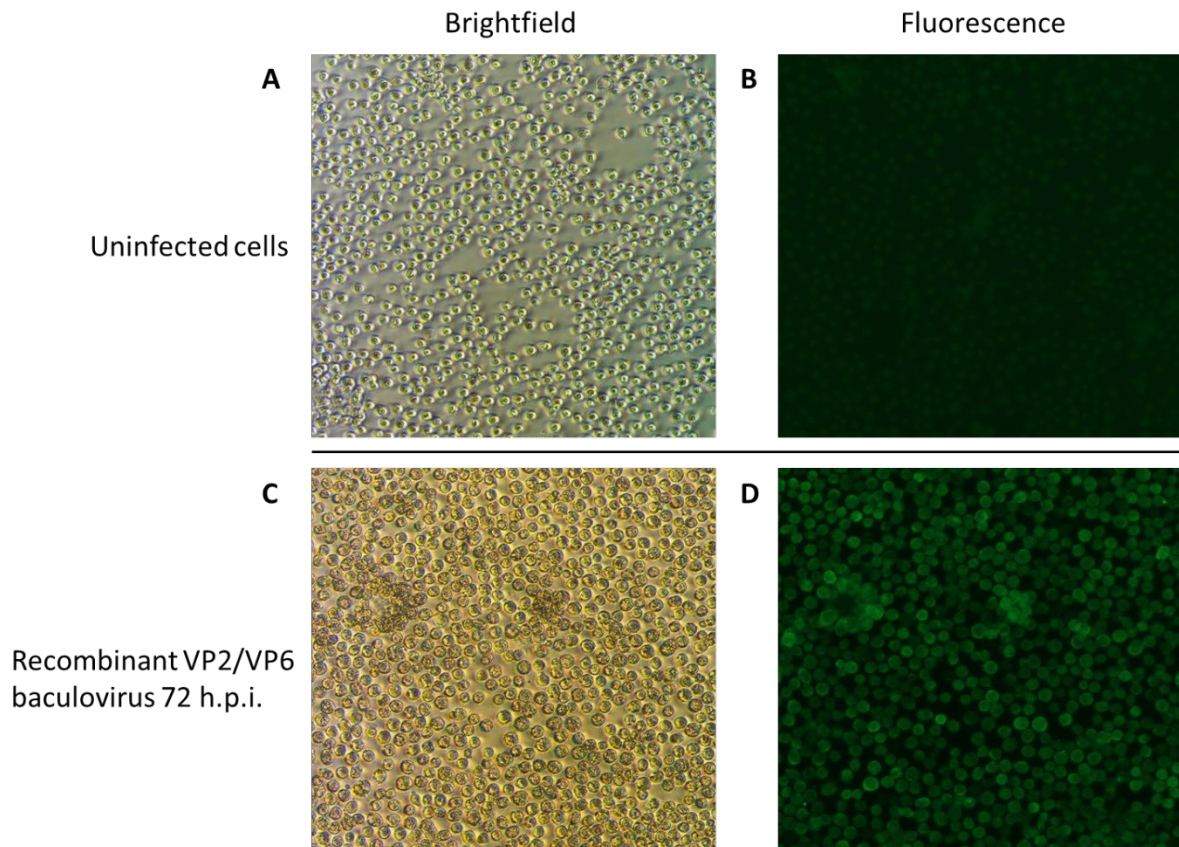


Figure 29: IFMA results for recombinant VP2/VP6. **A:** SF9 cells not infected with baculovirus in brightfield. **B:** Negative control SF9 cells viewed under fluorescence. **C:** Brightfield of SF9 cells infected with recombinant SA11 VP2/VP6. **D:** Infected Sf9 cells with recombinant VP2/VP6 viewed under fluorescence. Primary antibody: anti-SA11-RV DLP VP2/VP6 rabbit serum. Secondary antibody: Alexa 488 goat anti-rabbit. Low background fluorescences can be observed in the negative control. However, the positive response in recombinant SA11 VP2/VP6 clearly shows that the baculovirus expressed rotavirus SA11 VP2/VP6.

Figure 29 C shows clear signs of infection and plaques compared to Figure 29 A. The fluorescence of the IFMA indicated that colony 11 baculovirus expressed VP2 and VP6 in the majority of the cells. A plaque purification assay was done to ensure that the viral stock did not contain recombinant and wild-type baculovirus mixture. Ten plaques were picked as described in Section 3.2.10 and 3.2.11. After plaques were picked, the cells from the 6 well plates of the serial dilution were used for IFMA to determine if it is possible to see if the plaque that was picked contained recombinant VP2/VP6 (Figure 30).

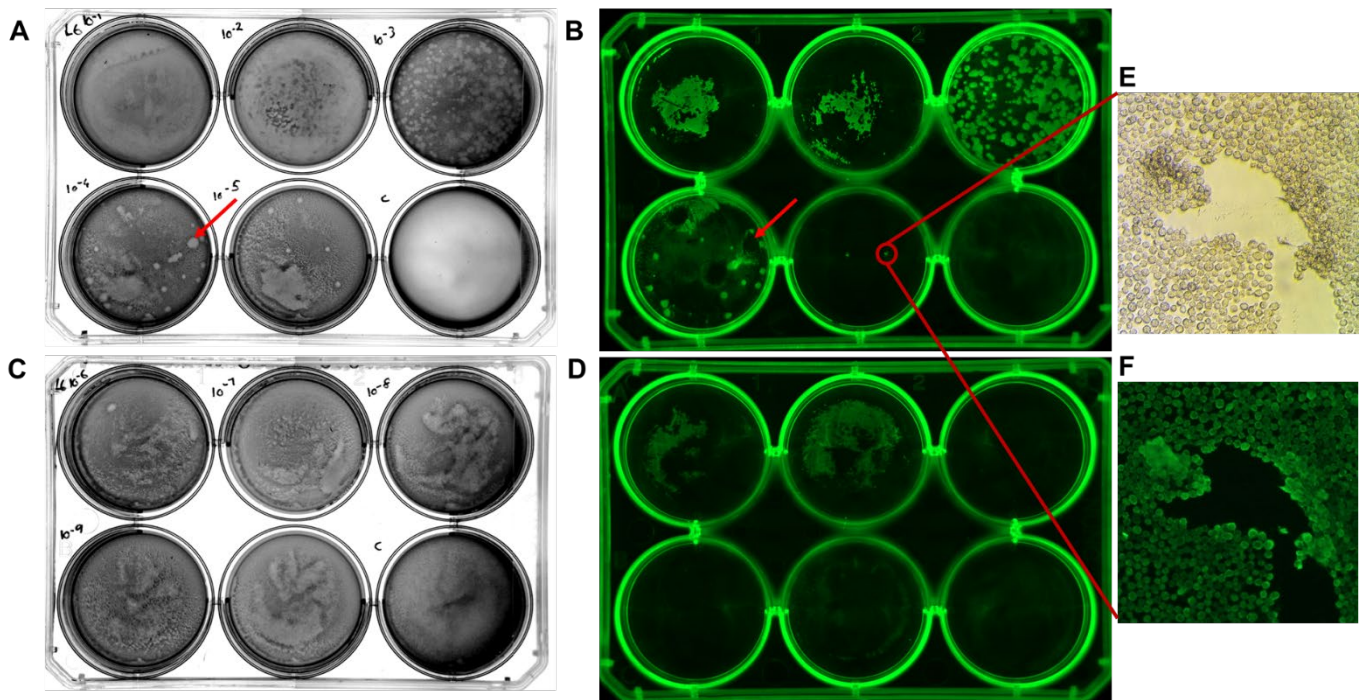


Figure 30: Plaque assay and IFMA of one colony containing recombinant VP2/VP6.
A and C: SF9 cells infected with a serial dilution of recombinant VP2/VP6. Plaques can be seen as white dots against the black/grey background of living cells. Individual plaques can be seen at 10^{-3} . B and D: IFMA of the 6 well plates after plaques were picked. Green zones are cells infected with recombinant VP2/VP6. E and F: Zoomed-in photo of a random plaque that was not selected. The red arrow indicates a plaque that was picked.

Plaques were observed from 10^{-3} dilution. However, single isolated plaques from 10^{-4} dilution were picked. One of the plaques that were picked is indicated with a red arrow, although the entire monolayer is infected, as shown in Figures B 10^{-4} dilution and Figure 30 F. Figure 30 E and F display a plaque that was not picked. The edges of the plaques fluoresce brighter as the viral yield is higher in these cells since the virus spreads through infection of neighbouring cells.

3.3.3 Baculovirus protein expression of SA11 VP2/VP6

The picked plaques were amplified as described in Section 3.2.12, and the lysate from the P2 stock cell pellet was run on SDS-PAGE (Figure 31). The total and

soluble fractions of baculovirus stock from colony 11 and colony 15 are depicted. VP2 expression was visible at 102 kDa and VP6 at 48 kDa. The expressed proteins were present in both the total and the soluble fractions and cannot be seen in lanes 8 and 9, representing the wild type. The wild-type total protein yield is lower than that of the recombinant viruses as the wild-type grows faster, resulting in quicker cell death.

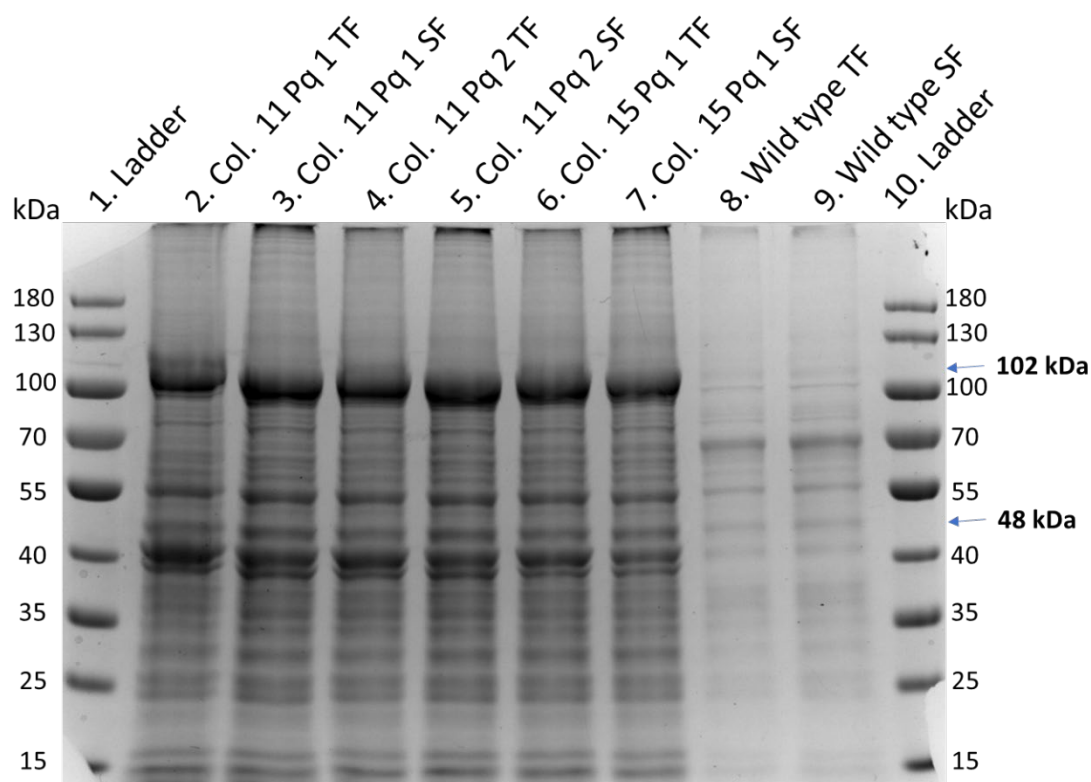


Figure 31: SDS PAGE analysis of recombinant VP2/VP6 amplified plaques after purification. A volume of 12 μ l sample was used in each lane. TF: Total fraction; SF: Soluble fraction; Lane 1: Protein molecular weight marker; Lane 2: Colony 11 plaque 1 total fraction; Lane 3: Colony 11 plaque 1 soluble fraction; Lane 4: Colony 11 plaque 2 total fraction; Lane 5: Colony 11 plaque 2 soluble fraction; Lane 6: Colony 15 plaque 1 total fraction; Lane 7: Colony 15 plaque 1 soluble fraction; Lane 8: Wild type baculovirus total fraction; Lane 9: Wild type baculovirus soluble fraction; Lane 10: Protein molecular weight marker.

Large-scale production of rotavirus VP2/VP6 was performed by making a 50 ml suspension culture of SF9 cells infected at a cell density of 1×10^6 cells/ml with baculovirus expressing rotavirus SA11 VP2/VP6. In parallel, a 50 ml suspension culture of SF9 cells was infected at a cell density of 1×10^6 cells/ml with

baculovirus expressing wild-type baculovirus as a control. Both infections were performed at an MOI of 5 as described in Section 3.2.10. The cells were harvested 5 days after infection via centrifugation. The media was removed, and the cell pellet was washed 1 x PBS after which the cells were lysed with the lysis buffer as described in Section 3.2.10. The recombinant samples and a negative control cell lysate were run on an SDS-PAGE and shown in Figure 32 A. From the 7 colonies transfected, only colonies 11 and 15 were chosen to be used further as they showed a higher titre by producing CPE more rapidly than all the other infections. Lanes 2 to 5 contain the soluble fraction of recombinant VP2/VP6. VP2 overexpression can be observed at around 102 kDa. Some expression can be observed for VP6 at 48 kDa. This expression is also seen on the western blot depicted in Figure 32 B. Lane 6 contains the soluble fraction of the wild-type baculovirus.

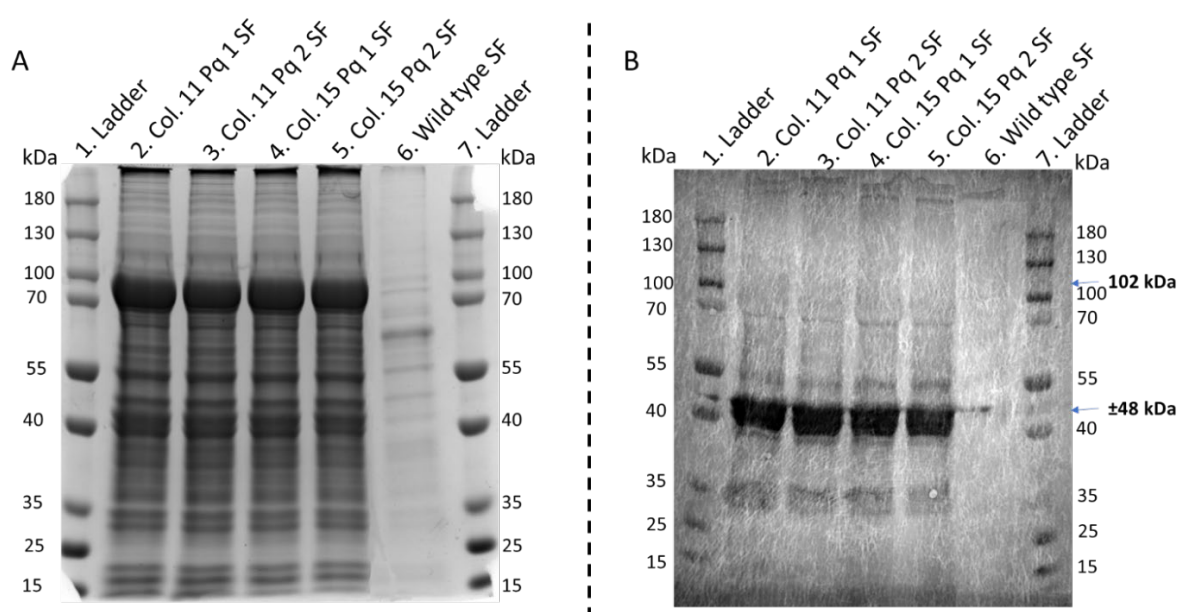


Figure 32: A: SDS-PAGE analysis of recombinant SA11 VP2/6 expressed in SF9 suspension cultures. Lanes contain 12 μ l of each sample infected with recombinant virus. Lane 1: Protein molecular weight marker; Lane 2: Colony 11 plaque 1 proteins; Lane 3: Colony 11 plaque 2 proteins; Lane 4: Colony 15 plaque 1 proteins; Lane 5: Colony 15 plaque 2 proteins; Lane 6: Wild type baculovirus obtained from a blue colony; Lane 7: Protein molecular weight marker. **B: Western blot analysis of recombinant VP2/6 expressed in SF9 suspension cultures.** A duplicate gel with the same samples was run along with the SDS page gel. Lane 1: Protein molecular weight marker; Lane 2 to 5: Soluble fraction of recombinant baculoviruses expressing VP2/VP6. VP6 overexpression can be seen at around ± 48 kDa. VP2 outlines can be observed in these lanes. Lane 6: negative control some VP6 of can be seen that have spilled over in the lane. Lane 7: Protein molecular weight marker.

Western blot analysis of the recombinant VP2/VP6 is shown in Figure 32 B. The primary antibody used in this experiment was anti-SA11_RV DLP VP2/VP6 rabbit whole serum. The secondary antibody was HRP conjugated goat anti-rabbit. VP6 is the group-specific antigen of rotavirus. Faint bands at the position of VP2 can be seen on the western blot suggesting that little to no VP2 antibody was present in the serum.

3.3.4 Verification of assembly of SA11 VP2/VP6 DLPs

The protein samples from the previous section were used for ultracentrifugation. DLPs were semi-purified using a 40% sucrose cushion described in Section 3.2.16. Protein isolation was visualised by SDS-PAGE (Figure 33).

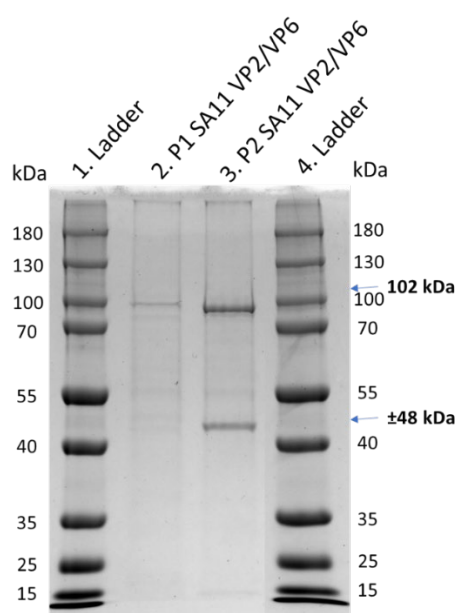


Figure 33: SDS PAGE of recombinant VP2/VP6 isolated with a 40% sucrose cushion. Lanes contain 10 μ l of sample for verification. Lane 1: Protein molecular weight marker; Lane 2: Plaque 1 SA11 VP2V/P6; Lane 3: Plaque 2 SA11 VP2/VP6 Lane 4: Protein molecular weight marker.

Residual sucrose was removed from the samples by diluting the sample in more TC buffer, as this will decrease the sucrose concentration in the sample. The samples were centrifuged again, and the protein pellet was resuspended in new

TC buffer. The recombinant VP2/VP6 DLPs were visualised using transmission electron microscopy (Figure 34). The sample contained a mixture of DLPs (A), partial assembly of VP6 on VP2 (B), and incomplete protein assembly of either VP2 or VP6 at C. Double-layered particles have a rough particle appearance because their periphery show projecting trimeric subunits (Estes & Greenberg, 2013).

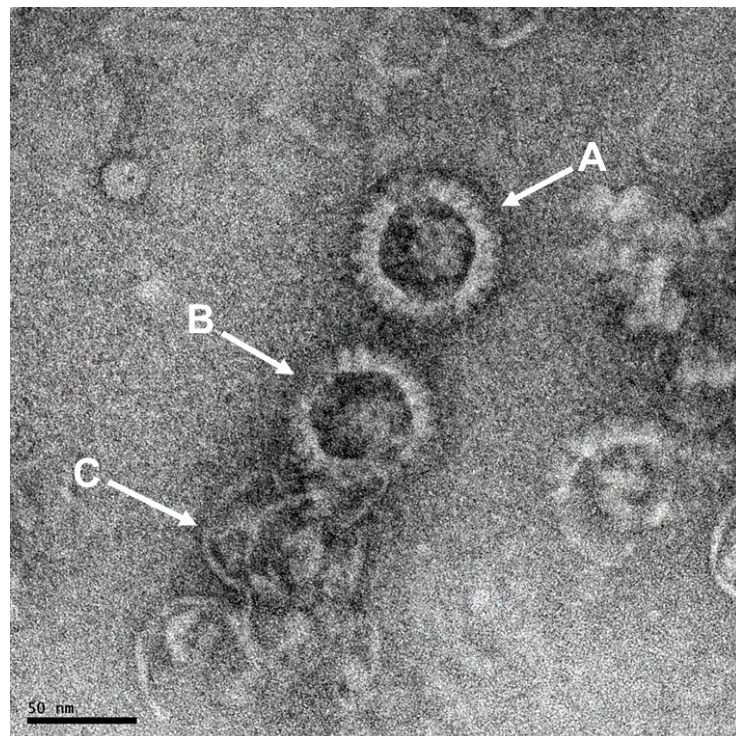


Figure 34: Rotavirus DLPs produced in insect cells. VP2/6 DLP produced from infected SF9 cells was approximately 71 nm. VP2 particle formation can be seen as small incomplete spheroids. Scale bar 50 nm.

3.3.5 Baculovirus expression of SA11 VP4 and SA11 VP7

The protocol followed for the expression of both SA11 VP4 and SA11 VP7 in a 6-well plate was the same as described in Section 3.3.2. Figure 35 depicts the proteins present in the total, and soluble fraction of three of the plaques picked for SA11 VP4 colony 8 before the titre was determined. A 75 kDa protein was

present in the total and soluble fraction of all the picked plaques implying that VP4 might be present. Lane 8 and 9 represent the wild-type.

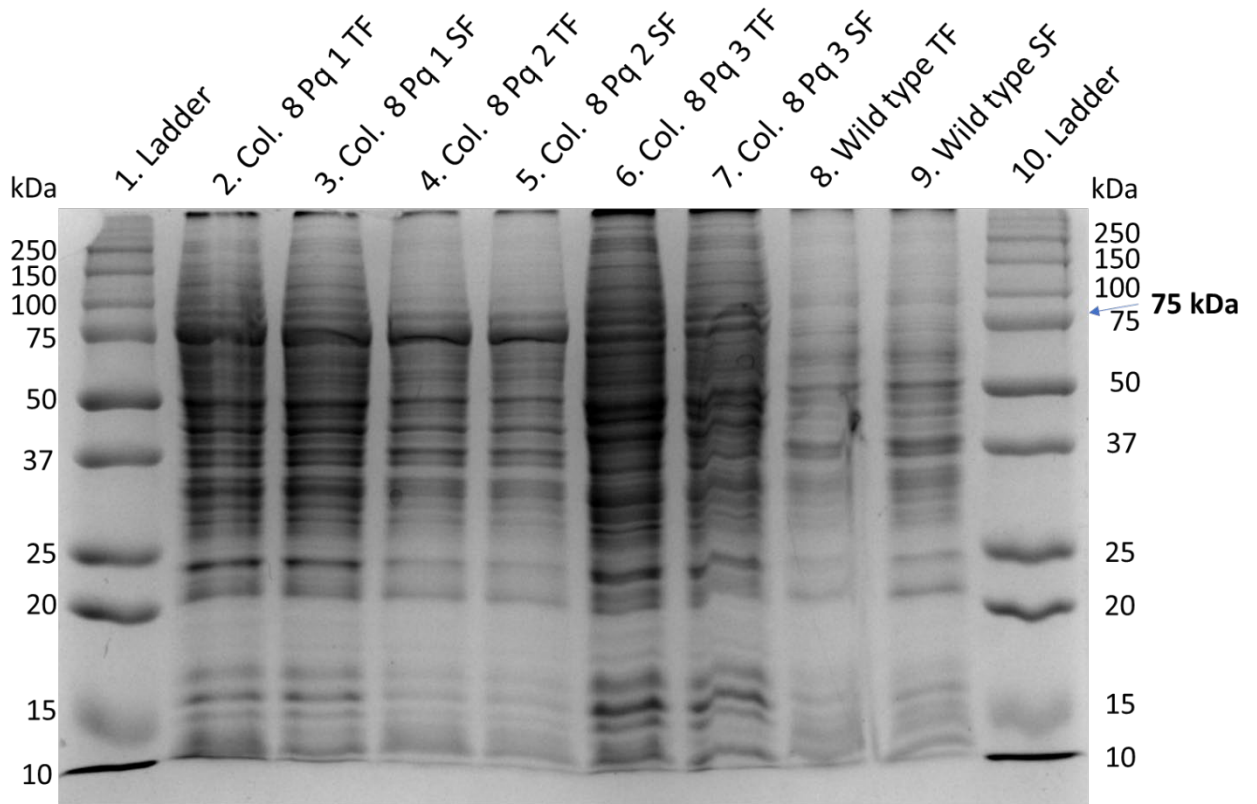


Figure 35: SDS-PAGE of recombinant VP4 amplified plaques after purification. A volume of 12 µl sample was used in each lane. TF: Total fraction; SF: Soluble fraction; Lane 1: Protein molecular weight marker; Lane 2: Colony 8 plaque 1 total fraction; Lane 3: Colony 8 plaque 1 soluble fraction; Lane 4: Colony 8 plaque 2 total fraction; Lane 5: Colony 8 plaque 2 soluble fraction; Lane 6: Colony 8 plaque 3 total fraction; Lane 7: Colony 8 plaque 3 soluble fraction; Lane 8: Wild type baculovirus total fraction; Lane 9: Wild type baculovirus soluble fraction; Lane 10: Protein molecular weight marker.

Figure 36 shows the total and soluble fraction of three plaques picked for SA11 VP7 colony 7. No expression of VP7 glycoprotein can be seen on the SDS-PAGE. This was expected as VP7 is difficult to observe on SDS-PAGE VP7 have been shown to be toxic in *E. coli* and eukaryote cell expression systems (Emslie *et al.*, 1995; McCrae & McCorquodale, 1987). VP7 expression was observed only by western blot analysis by Dr Jere and co-workers (Jere *et al.*, 2014). Glycoproteins are difficult to stain with Coomassie brilliant blue as the carbohydrate moiety of glycoproteins can interfere with the binding of Coomassie

brilliant blue (Osset *et al.*, 1989). Glycosylation affects proteins by giving them a negative charge. This interferes with the van der Waals forces used by the Coomassie brilliant blue to stain the proteins. VP7 expression should have been present at 37 kDa.

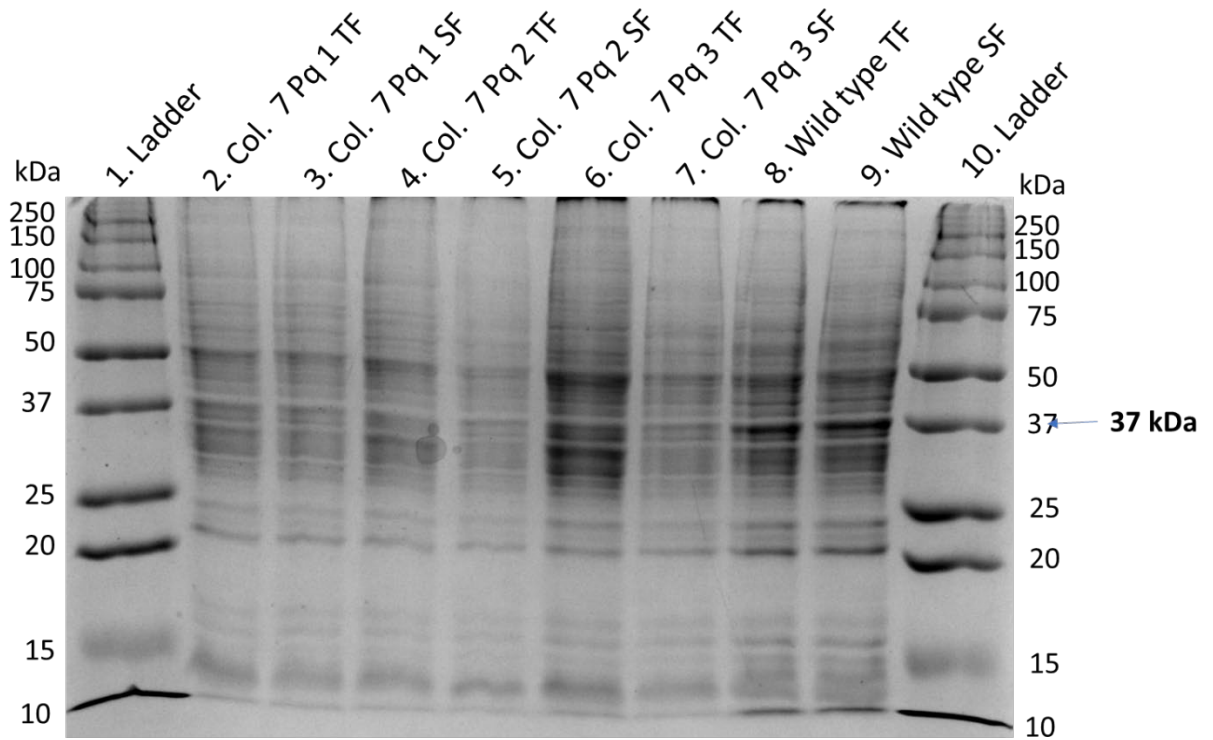


Figure 36: SDS-PAGE of recombinant VP7 amplified plaques after purification. A volume of 12 μ l sample was used in each lane. TF: Total fraction; SF: Soluble fraction; Lane 1: Protein molecular weight marker; Lane 2: Colony 7 plaque 1 total fraction; Lane 3: Colony 7 plaque 1 soluble fraction; Lane 4: Colony 7 plaque 2 total fraction; Lane 5: Colony 7 plaque 2 soluble fraction; Lane 6: Colony 7 plaque 3 total fraction; Lane 7: Colony 7 plaque 3 soluble fraction; Lane 8: Wild type baculovirus total fraction; Lane 9: Wild type baculovirus soluble fraction; Lane 10: Protein molecular weight marker.

3.4 Summary

The project's primary goal was to express SA11 TLPs as a base for structural compatibility studies and improve the assembly issues Dr. Jere experienced (Jere *et al.*, 2014). This overarching goal was subdivided into three sub-goals, the co-expression of the SA11 VP2/6 DLP (core) and the expression of the two

SA11 outer capsid proteins VP4 and VP7. Lastly, to improve the assembly of these proteins into TLPs by adding 20 mM calcium chloride in the media.

The expression and auto-assembly of the insect codon optimised SA11 VP2/6 DLP was confirmed through IFMA, SDS-PAGE, western blot, and TEM. The use of IFMA to identify plaques expressing VP2/VP6 was a novel approach which decreased the time interval from transfection to confirmation of protein expression.

With regards to SA11 VP4, soluble expression was confirmed on SDS-PAGE. Expression of SA11 VP7 was difficult to confirm due to its innate weak expression and resistance to Coomassie staining. The only plausible way to confirm VP7 expression is through the use of western blot. However, commercial antibodies were not available. Thus, VP7 expression can only be confirmed indirectly by observing TLPs on TEM.

During co-expression of SA11 VP2/VP6/VP4 and VP7 for the production of TLPs, no expression of any of the proteins was observed on SDS-PAGE even with the addition of 20 mM calcium chloride in the cell culture media and buffers used in purifying TLPs. TEM images from the TLP samples only showed DLPs (data not shown) even though an MOI of 5 was used for each viral stock used for co-infection. Further studies can be conducted to see if a variation of MOI or time of infection (TOI) can help increase the expression, and furthermore whether the passage number of the cells play a role in the level of protein expression.

Since we do not have any antibodies for VP4 and VP7, it was not possible to say for sure that the recombinant viral stock we have does not contain any wild-type virus since plaques picked only show low expression on SDS-PAGE for VP4.

Since I completed my experimental work, we became aware of mycoplasma contamination in our cell cultures. Mycoplasma contamination can alter the level of protein, RNA, and DNA synthesis of cells (Miller *et al.*, 2003). These complications need to be addressed in order to confirm if this contamination reduced the efficacy of assembly. The next step will be to use the pFASTBACquad (pFBq) as donor plasmid for the baculovirus system to express TLPs instead of the pFB and pFBd system.

Concluding remarks

Rotavirus is still one of the most common causes of gastric ailments such as vomiting and severe diarrhoea in children under the age of 5 and contributes to more than 215 000 deaths per year globally (Tate *et al.*, 2016). The efficacy of commercially live-attenuated licensed vaccines RotaTeq® and Rotarix™ range from 39% to 77% in developing countries of Africa and Asia (Madhi *et al.*, 2010; Zaman *et al.*, 2010), as described in Chapter 1 (Section 1.6). One of the factors that can influence the wide efficacy range is the different strains prevalent in these regions (Seheri *et al.*, 2014). It is generally anticipated that the gap in immunogenicity might be bridged by using regional-specific vaccine boosters.

Several alternatives to live-attenuated vaccines have been considered. Foremost among these is the production of VLPs. VLPs contain the same antigenic properties as the virus but cannot replicate because they do not contain any genetic material, making them a safer alternative to attenuated vaccines. Studies have shown that rotavirus VLPs (VP2/6/4/7) can function as an immune booster because they still invoke an immunogenic response (Azevedo *et al.*, 2010). Most of these studies have been done in the baculovirus expression system. However, this system is a much more expensive vaccine production platform than live-attenuated platforms. Therefore, if triple-layered particles can be produced in bacteria, the poorer countries can use a suitably subunit rotavirus vaccine with cheaper produced VLPs. Rotavirus protein expression was attempted internationally and in this laboratory. This MSc continues the work done previously by Dr Jere where he assembled different VP4 and VP7 African strains on a GR10924 VP2/VP6 DS-1 core. TEM images of the RV TLPs that he published shows that tRV-VLPs have partly smooth but mostly rough edges suggesting that assembly of VP7 and VP4 was not complete. Since the insect cell media currently on the market only contains 8 mM calcium, as appose to 20 mM they contained in the 1990s, I supplemented the media with an additional 20 mM calcium chloride.

The first experimental chapter (Chapter 2) focused on the expression of rotavirus bacterial codon optimised ORF of the structural proteins VP2, VP6, and VP7. Expression of VP7 was achieved by removing the N-terminal endoplasmic reticulum signal peptide (amino acids 1-50) from the sequence and moving the ORF from pET151_GR10924_VP7 to pCOLDI_GR10924_VP7. Although the expressed VP7 was soluble, a truncation of 159 bp of the ORF (bp 365-524) encoding 53 amino acids (amino acids 121-174) occurred. This truncation seems to have occurred following the transformation step of the In-fusion protocol when the VP7 ORF was moved from pET151_GR10924_VP7 to pCOLDI_GR10924_VP7, since the Sanger sequencing of pET151_GR10924_VP7 indicated that the VP7 ORF without ER sequence was correct. An explanation can be that the bacteria identified either the VP7 ORF or the protein produced (from leaky expression) as toxic and thus removed a portion of the sequence to reduce the toxicity.

Further studies are required to determine the impact this truncation has on the immunogenicity of the protein. Another approach can be to change the cell line from gram-negative to a gram-positive strain. SA11 VP7 without the ER leader peptide has been expressed previously in *Lactococcus lactis* NZ9800 cells by adding the Usp 45 signal peptide to excrete VP7 into the media (Perez *et al.*, 2005). Gram-positive bacteria have a different intracellular environment than gram-negative bacteria, such as *E. coli*, which occasionally improves the production of recombinant proteins (Saito *et al.*, 2019).

Bacterial codon optimised ORFs of both GR10924 and SA11 genome segments 2 (VP2) and genome segment 6 (VP6) were purchased from GeneArt in pET100 (GR10924_VP6, SA11_VP2 and SA11_VP6) and pET28 (GR10924_VP2). The study aimed to evaluate the individual and co-expression of VP2 and VP6. However, no expression of these proteins was observed. Several modifications were investigated to try and improve the expression namely (i) Different bacterial cell lines: namely Tuner™ (adjustable levels of protein expression through regulation of IPTG concentration, along with increased resilience to toxic proteins) and Origami™ (enhances disulphide bond formation facilitating folding of proteins) (ii) IPTG concentration variation from 0.1 mM to 4 mM (iii) Induction

time variation from 4 hours to 16 hours (iv) The use of pGro7 chaperones to aid in the folding of proteins (v) Different types of media namely Luria Broth and Terrific Broth (more nutrient-rich than Luria broth because terrific broth contains glycerol which increases the bacteria yield per volume). Future studies can include moving the ORF to vectors such as pCOLD. Previously GR10924_VP6 was expressed soluble in pCOLDTF (Naude, 2014). Thus the SA11_VP2 and SA11_VP6 could be moved to pCOLD as well to increase solubility. Additionally, SUMO tag is a novel tag used in both prokaryotic and eukaryotic systems to aid in protein solubility, expression and folding. The exact mechanism this tag uses to aid in folding is not yet understood but it is hypothesised that it acts like chaperones. SUMO might increase protein yield by translocating the proteins from the cytosol to the nucleus thus decrease their susceptibility to proteolytic degradation (Butt *et al.*, 2005).

The expression of the structural rotavirus proteins in insect cells using the Bac-to-bac expression system was the focus of the second experimental chapter (Chapter 3). The ORF of the insect codon optimised genome segment 2 (VP2) and genome segment 6 (VP6) were purchased in the same vector, namely pFASTBACdual and was named pFBd_SA11_VP2/VP6. The ORF of genome segment 4 (VP4) and genome segment 9 (VP7) were each insect codon optimised and purchased in different vectors named pFBd_SA11_VP4 and pFBd_SA11_VP7, respectively. Each of the pFBd plasmids was transformed into *E. coli* host strain, AcBACΔCC as described in section 3.2.3. Recombinant colonies were selected through blue-white screening and PCR analysis as described in section 3.2.4 before the bacmids were transfected into SF9 cells (Section 3.2.9).

The primary purpose of the experiments described in Chapter 3 was to evaluate the expression and co-expression of SA11 VP2 and VP6 and their assembly into a double-layered particle (DLP) and to use different strains of VP4 and VP7 on the DLP. Co-expression of VP2 and VP6 was accomplished and confirmed with IFMA, SDS-PAGE and western blot. DLP assembly was confirmed using TEM (Section 3.3.3). Low VP4 expression was observed on SDS-PAGE (Section 3.3.5). VP7 expression could not be confirmed with SDS-PAGE (Section 3.3.5).

A combination of low expression and the difficulty of staining VP7 with Coomassie contributed to complications in confirming expression of VP7. Co-infection was attempted to produce triple-layered particles, but without any success, even with 20 mM calcium supplementation added to the media. The particles observed using TEM were DLPs and not TLPs.

The next step will be to use the pFBquad donor plasmid system to construct bacmids for TLPs expression instead of the pFB and pFBdual system (Jere, 2012). This will ensure that every infected cell will be expressing all four recombinant proteins. A study comparing bacmid construction using multiple monocistronic pFB donor plasmids, representing VP2, VP6 and VP7 vs a single tricistronic approach containing VP2, VP6 and VP7 found an increase of roughly 35% of purified rotavirus VLPs with the tricistronic infection (Vieira *et al.*, 2005).

Although the initial aim of bacterial expression could not be fully verified, a soluble, truncated VP7 was obtained which could have future applications. The insect cell expression and auto-assembly of RV TLPs could not be verified. However, DLPs were successfully expressed and assembled fully as confirmed through TEM. Due to the inherently complex nature of confirming the expression of VP4 and VP7, further work is required to optimise TLP auto-assembly. In addition to this, severe mycoplasma contamination in the insect cells used throughout the project seems to have hindered the full evaluation of TLP assembly. This will have to be re-evaluated following full decontamination. Finally, the pFBquad system should be evaluated with the addition of 20 mM calcium chloride for optimised TLP assembly. The work presented in this MSc provided adequate information for other studies and will aid in finalising other RV VLP assembly projects.

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Appendix A: List of common reagents used:

Product	Catalog / Batch number
Acetone	SAAR1022040LC
Acrylamide / Bis-acrylamide	A3574-100ML
AEC	A5154-50G
Agar	A20021-500.0
Agarose	MEL-A20096-100.0
Ammonium persulfate	215589-100G
CaCl ₂	C-2510
Carbenicillin	C0109
Coomassie Blue	1.12553.0025
Dimethylformaldehyde	BB103226L
DMSO	D8418-50ML
dNTPs	R1121
EDTA	03685
Ethanol	E7023-500ML
Gentamicin	G1914
Glacial Acetic acid	1.00063.2500
Glucose	G1400
Glycerol	G22020-015
Glycine	G0769
IPTG	34060
Isopropanol	1.09634.2500
K ₂ HPO ₄	PO573.1000
K ₂ PO ₄	A2121
Kac	P42000-1000.0
Kanamycin	K01120
KCl	P9541-1KG
L-arabinose	A3256-100G

Low melting agarose	A9045-25G
Methanol	230-4
MgCl ₂	M2450
MgSO ₄	M24300-500.0
NaCl	1.06404.0500
NaOH	S0523.1000
Nitrocellulose membrane	162-0094
Nuclease free water	W4502-1L
Nunc 6-well plate	140675
Nunc T75	156472
PBS	10010-15
PEG 6000	528877
Protease Inhibitor tablets EDTA-free	88666F
Q5 buffer	B90275
Q5 polymerase	M0491L
RNase A	1007885
SDS	B2008
Skim milk	21860179D1
Sodium deoxycholate	D6750-1000
Sucrose	S0389-500G
Tetracycline	T7660
Tris	B2005
Triton X-100	1.08603.1000
Tryptone	1.11931.1000
Tween 20	P1362
X-Gal	R0404
Yeast extract	1322.00
β-mercaptoethanol	M6250

Appendix B: List of plasmid maps and sequences:

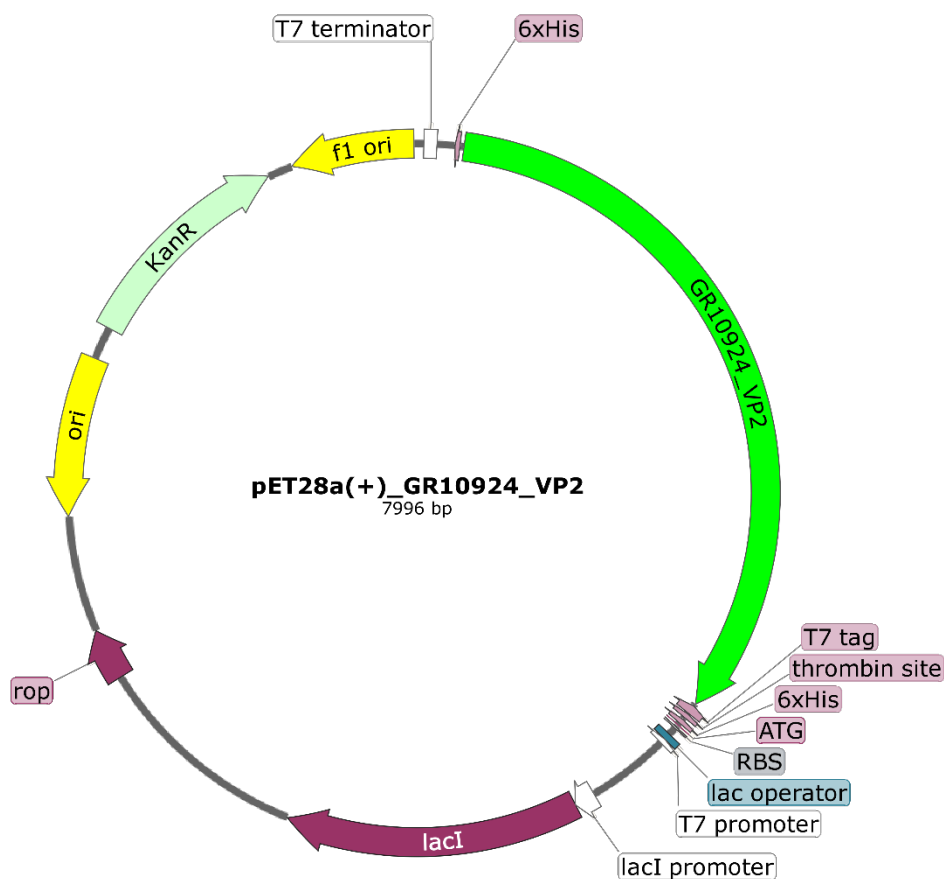


Figure 37: Map of pET28(+)_GR10924_VP2_BacO

	10	20	30	40	50
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	---GGTTGAG	TCGTTCCCTC	TAGAAATAAT	TTTGTTTAAC	TTTAAGAAGG
	60	70	80	90	100
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AGATATACCA	TGGCTTATAG	GAAAAGAGGA	GCTAGGAGAG	AAGCAAACTCT
	110	120	130	140	150
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AAACAACAAC	GATCGTATGC	AGGAGAAGAT	CGATGAAAAA	CAGGACAGCA
	160	170	180	190	200
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ACAAGATTCA	ACTGAGCGAC	AAAGTGCTGA	GCAAGAAAGA	GGAAATTGTT

	210	220	230	240	250
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ACCGATAGCC	ACGAGGAAGT	GAAGGTTACC	GACGAGCTGA	AGAAAAGCAC
	260	270	280	290	300
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CAAAGAGGAG	AGCAAACAGC	TGCTGGAAGT	GCTGAAGACC	AAAGAGGAAC
	310	320	330	340	350
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ACCAGAAAGA	GATCCAATAC	GAAATTCTGC	AAAAGACCAT	CCCGACCTTC
	360	370	380	390	400
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GAGCCGAAGG	AAACCATCCT	GCGTAAACTG	GAGGACATTC	AGCCGGAACT
	410	420	430	440	450
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGCGAAGAAA	CAAACCAAAC	TGTTCCGTAT	CTTTGAACCG	AAGCAGCTGC
	460	470	480	490	500
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CGATTTATCG	TGCGAACGGC	GAGCGTGAAC	TGCGTAACCG	TTGGTACTGG
	510	520	530	540	550
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AAACTGAAGA	AAGATACCCT	GCCGGACGGC	GATTACGACG	TGCGTGAGTA
	560	570	580	590	600
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTTTCTGAAC	CTGTACGACC	AGGTTCTGAC	CGAAATGCCG	GATTATCTGC
	610	620	630	640	650
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TGCTGAAGGA	CATGGCGGTT	GAGAACAAAA	ACAGCCGTGA	TGCGGGCAAG
	660	670	680	690	700
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GTGGTTGACA	GCGAAACCGC	GAGCATCTGC	GATGCGATTT	TTCAAGATGA
	710	720	730	740	750
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGAAACCGAG	GGTGC GG TGC	GTCGTTTTAT	CGCGGAAATG	CGTCAGCGTG

	760	770	780	790	800
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTCAAGCGGA	TCGTAACGTG	GTTAACTACC	CGAGCATCCT	GCACCCGATT
	810	820	830	840	850
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GACTATGCGT	TCAACGAGTA	CTTTCTGCAG	CACCAACTGG	TGGAACCGCT
	860	870	880	890	900
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GAACAACGAT	ATCATTTTCA	ACTATATCCC	GGAGCGTATT	CGTAACGATG
	910	920	930	940	950
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTAACTACAT	CCTGAACATG	GGACCGTAAC	CTGCCGAGCA	CCGCGCGTTA
	960	970	980	990	1000
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TATTCGTCCG	AACCTGCTGC	AGGATCGTCT	GAACCTGCAC	GACACTTTGA
	1010	1020	1030	1040	1050
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AGCCTGGTGG	GATACCATCA	CCACCAGCAC	TACATTCTGG	CGCGTAGCGT
	1060	1070	1080	1090	1100
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGATCGGACT	GAAAGAGCTG	GGTAGCACGA	GCGCAGATTC	GAAGATGGAG
	1110	1120	1130	1140	1150
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CAGAACCTGC	AACTGGAGGG	CCTGACATCG	AGCGAATCGA	TACTGACCGG
	1160	1170	1180	1190	1200
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CTATTTAACA	GTCGGTATTA	TAGCGCATGC	CTCTTTCGGG	CTTTGTTAGC
	1210	1220	1230	1240	1250
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AGCCGGATCT	CAGTGGTGGT	GGTGGTGGTG	CTCGAGTTAC	AGTTCGTTCA
	1260	1270	1280	1290	1300
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TAATACGCAT	GTTGTCAAAC	GCAACCGCGT	TGATCGGCTC	AACGGTGTCC
	1310	1320	1330	1340	1350
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GCGCTCACAA	ACGCCAGCAG	ATCGCTGTAC	ACGGTGAAGG	TCAGGTTGCT

	1360	1370	1380	1390	1400
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGTCAGCATG	TGCATGCTGT	TACGAAAGTC	GAATTGCTGC	GGCACCTGTT
	1410	1420	1430	1440	1450
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TATAAACCTT	GGTGGTGCTG	GTCGGAACCC	AATCGTAGTT	CGCCACCAGA
	1460	1470	1480	1490	1500
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TAGAAGTCGT	TGCTATCGCT	GTTAATTTTG	TACAGGATCG	GCTTCAGGGT
	1510	1520	1530	1540	1550
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GTCAACTTTA	CGCAGCTTCA	CGATCTGCGC	AAAAACGGTC	GCGTCCAGCT
	1560	1570	1580	1590	1600
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TCGCAATCAG	GCTGATAACG	CTGCTATCGG	TAATGAACGG	CAGCGCGCCA
	1610	1620	1630	1640	1650
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ACCAGCGCCA	CCGGCTGGTT	GTTTCAGCAGC	ATGTTGGTGA	TCTGCGCGTA
	1660	1670	1680	1690	1700
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ATCACCGCTA	CGCATCAGTT	CCTCCAGGTT	AATTTGCTGA	AAGCCCTCCA
	1710	1720	1730	1740	1750
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGTTACGCGC	GATGTTAACA	TAACCGTACA	TTTCGTCACG	CTCCAGGTGC
	1760	1770	1780	1790	1800
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	ATATCACGAT	ACGCAATGAT	CACGCCTTGC	GCAATTTTGT	CGCTCGCACG
	1810	1820	1830	1840	1850
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTTCGATCTGA	TCCATGTTCA	TCAGAATCAG	GTTGAAGATG	TCCAGACGAC
	1860	1870	1880	1890	1900
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GGATCTCCAC	CGGCAGCAGA	CGCAGACGAT	CACGCAGACG	GTACATTTGA

	1910	1920	1930	1940	1950
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TCGTCCGGAA	CACGGCTCAC	GTCAAAAATA	TACAGACGCT	TCAGGAAATC
	1960	1970	1980	1990	2000
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTCAACGATC	GCTTTCATTT	TCTTCTGGTA	CAGGTTTCTAGA	CGGTTTCGCCG
	2010	2020	2030	2040	2050
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CGGTAATGAT	CGCCACCGCG	TCGTTGATAC	GCTCGTTATA	GTTGCTGTGA
	2060	2070	2080	2090	2100
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AAGTTAACGT	TCACGTTATA	GTAGTGGAAC	AGGGTTTGCG	GGCTCGGAAT
	2110	2120	2130	2140	2150
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	AACGGTCGCG	TTACCGATCA	GCATGCACAG	GCTGGTCACG	CTGGTCAGCT
	2160	2170	2180	2190	2200
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	GCAGCTTTTC	GGTGGTCAGG	TTGGACGTGC	TGCATGTTCA	TGATGATGCA
	2210	2220	2230	2240	2250
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	CGCCATCAGG	TCTCATAGTT	GACGCCAGC	AGACGGATCA	GTCACCCAGG
	2260	2270	2280	2290	2300
GR10924_VP2_BacO_reference					
GR10924_VP2_BacO_sequenced	TTGGTCCCAG	ACGGTGGCTC	AGCAGCAGAA	TCCCGCCTTG	GCATCCTACG
	2310	2320	2330		
GR10924_VP2_BacO_reference				...	...
GR10924_VP2_BacO_sequenced	TTTGGTAATC	CAGTTCCGGG	C-----	---	---

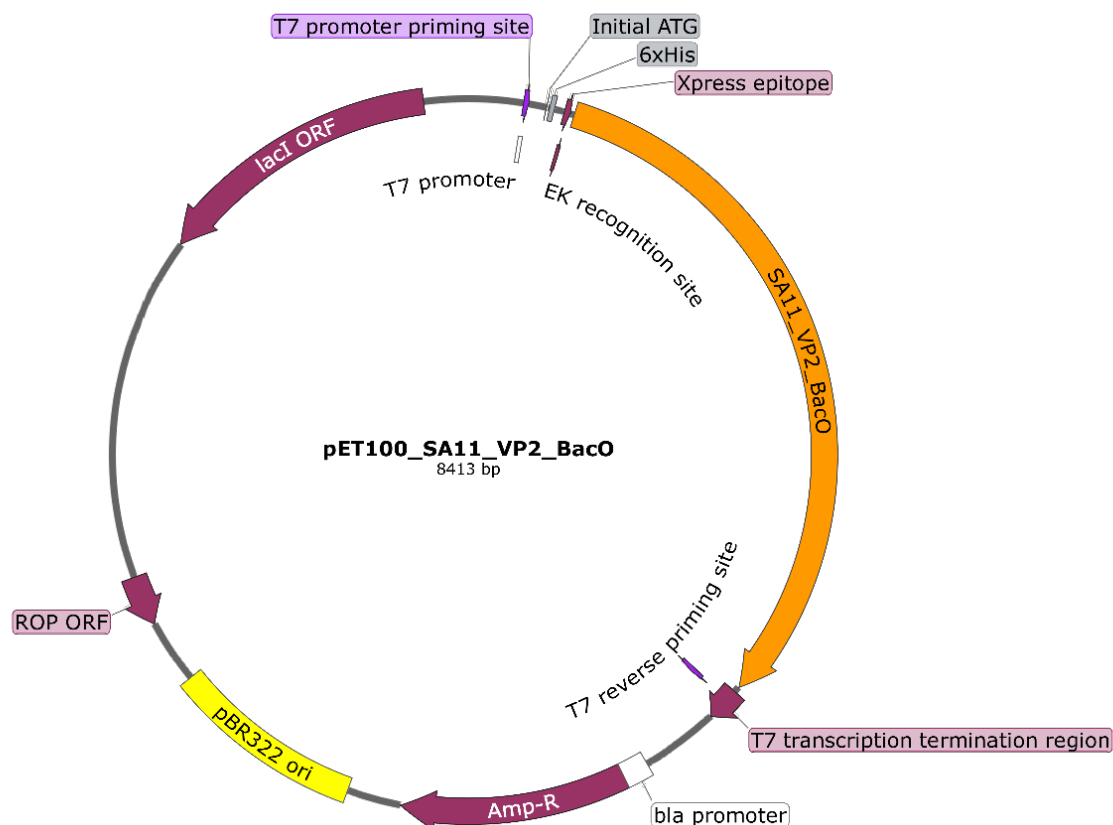


Figure 38: Map of pET100_SA11_VP2_BacO

	10	20	30	40	50
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	AYKWGGGACC	GGACAKTTCC	CTCYAGAAAT	AATTTTGTTT	AACTTTMAGA
	60	70	80	90	100
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	RGGAGATATA	CATATGCGGG	GTTCTCATCA	TCATCATCAT	CATGGTATGG
	110	120	130	140	150
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	CTAGCWTGAC	TGGYGGACAG	CAAATGGGTC	GGGATCTGTA	CGACGATGAC
	160	170	180	190	200
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GATAAGGATC	ATCCCTTCAC	CATGGCCTAT	CGTAAACGTG	GTGCACGTCG
	210	220	230	240	250
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	TGAAACCAAT	CTGAAACAGG	ATGAACGTAT	GCAAGAAAAA	GAGGACAGCA

	260	270	280	290	300
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AGAACATTAA	TAACGATAGC	CCGAAAAGCC	AGCTGAGCGA	AAAAGTTCCTG
SA11_VP2_BacO_sequenced	AGAACATTAA	TAACGATAGC	CCGAAAAGCC	AGCTGAGCGA	AAAAGTTCCTG
	310	320	330	340	350
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AGCAAAAAAG	AAGAGATCAT	CACCGATAAC	CAAGAAGAGG	TGAAAATCTC
SA11_VP2_BacO_sequenced	AGCAAAAAAG	AAGAGATCAT	CACCGATAAC	CAAGAAGAGG	TGAAAATCTC
	360	370	380	390	400
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CGACGAAGTG	AAAAAAAGCA	ACAAAGAGGA	ATCAAAACAG	CTGCTGGAAG
SA11_VP2_BacO_sequenced	CGACGAAGTG	AAAAAAAGCA	ACAAAGAGGA	ATCAAAACAG	CTGCTGGAAG
	410	420	430	440	450
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TGCTGAAAAC	CAAAGAAGAA	CATCAGAAAG	AAGTGCAGTA	CGAGATCCTG
SA11_VP2_BacO_sequenced	TGCTGAAAAC	CAAAGAAGAA	CATCAGAAAG	AAGTGCAGTA	CGAGATCCTG
	460	470	480	490	500
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CAGAAAACCA	TTCCGACCTT	TGAACCGAAA	GAAAGCATTG	TGAAAAAGCT
SA11_VP2_BacO_sequenced	CAGAAAACCA	TTCCGACCTT	TGAACCGAAA	GAAAGCATTG	TGAAAAAGCT
	510	520	530	540	550
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GGAAGATATC	AAACCGGAAC	AGGCACAAAA	ACAGACCAAA	CTGTTTCGCA
SA11_VP2_BacO_sequenced	GGAAGATATC	AAACCGGAAC	AGGCACAAAA	ACAGACCAAA	CTGTTTCGCA
	560	570	580	590	600
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TTTTTTGAGCC	GAAACAACCTG	CCGATTTATC	GTGCAAAATGG	TGAACGTGAA
SA11_VP2_BacO_sequenced	TTTTTTGAGCC	GAAACAACCTG	CCGATTTATC	GTGCAAAATGG	TGAACGTGAA
	610	620	630	640	650
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CTGCGTAATC	GTTGGTATTG	GAAACTGAAA	CGTGATACCC	TGCCGGATGG
SA11_VP2_BacO_sequenced	CTGCGTAATC	GTTGGTATTG	GAAACTGAAA	CGTGATACCC	TGCCGGATGG
	660	670	680	690	700
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TGATTATGAT	GTTTCGTGAAT	ATTTCCCTGAA	CCTGTATGAT	CAGGTTCTGA
SA11_VP2_BacO_sequenced	TGATTATGAT	GTTTCGTGAAT	ATTTCCCTGAA	CCTGTATGAT	CAGGTTCTGA
	710	720	730	740	750
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TGGAATGACC	GGATTATCTG	CTGCTGAAAG	ATATGGCAGT	GGAAAAACAA
SA11_VP2_BacO_sequenced	TGGAATGACC	GGATTATCTG	CTGCTGAAAG	ATATGGCAGT	GGAAAAACAA
	760	770	780	790	800
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AATAGCCGTG	ATGCAGGTAA	AGTTGTTGAT	AGCGAAACCG	CAGCAATTTG
SA11_VP2_BacO_sequenced	AATAGCCGTG	ATGCAGGTAA	AGTTGTTGAT	AGCGAAACCG	CAGCAATTTG
	810	820	830	840	850
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TGATGCAATT	TTTCAGGATG	AAGAAACCGA	AGGTGCAGTG	CGTCGTTTTA
SA11_VP2_BacO_sequenced	TGATGCAATT	TTTCAGGATG	AAGAAACCGA	AGGTGCAGTG	CGTCGTTTTA

	860	870	880	890	900
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TTGCAGAAAT	GCGTCAGCGT	GTT-CAGGCA	GATCGTAATG	TTGTTAATT-
SA11_VP2_BacO_sequenced	TTGCAGAAAT	GCGTCAGCGT	GTTTCAGGCA	GATCGTAATG	TTGTTAATTT
	910	920	930	940	950
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	ATCCGAGCAT	TCTGCATCCG	ATTGATCATG	CCTTTAACGA	ATACTTTCTG
SA11_VP2_BacO_sequenced	ATCCGAGCAT	TCTGCATCCG	ATTGATCATG	CCTTTAACGA	ATACTTTCTG
	960	970	980	990	1000
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CAGCATCAGC	TGGTTGAACC	GCTGAACAAT	GATATTATCT	TCAACTATAT
SA11_VP2_BacO_sequenced	CAGCATCAGC	TGGTTGAACC	GCTGAACAAT	GAWATTATCT	TCAACTATAT
	1010	1020	1030	1040	1050
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	TCCGGAACGC	ATTTCGAACG	ATGTGAAC TA	TATCCTGAAT	ATGGATCGTA
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1060	1070	1080	1090	1100
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	ATCTGCCGAG	CACCGCACGT	TATATTCTGC	CGAATCTGCT	GCAGGACCGT
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1110	1120	1130	1140	1150
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CTGAATCTGC	ATGATAATTT	TGAAAGCCTG	TGGGATACCA	TTACCACGAG
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1160	1170	1180	1190	1200
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CAATTATATC	CTGGCACGTA	GCGTTGTTCC	GGATCTGAAA	GAAC TGGTTA
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1210	1220	1230	1240	1250
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GCACCGAAGC	ACAGATCCAG	AAAATGAGCC	AGGATCTGCA	GCTGGAAGCA
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1260	1270	1280	1290	1300
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CTGACCATT C	AGAGCGAAAC	CCAGTTTCTG	ACCGGTATTA	ATAGCCAGGC
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1310	1320	1330	1340	1350
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AGCAAACGAT	TGCTTTAAAA	CCCTGATTGC	AGCAATGCTG	AGCCAGCGTA
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1360	1370	1380	1390	1400
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CCATGAGCCT	GGATTTTGTT	ACCACCAATT	ATATGAGCCT	GATTAGCGGT
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----
	1410	1420	1430	1440	1450
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	ATGTGGCTGC	TGACCGTTAT	TCCGAATGAC	ATGTTTATTC	GTGAAAGTCT
SA11_VP2_BacO_sequenced	-----	-----	-----	-----	-----

	1460	1470	1480	1490	1500
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GGTTGCATGT	CAGCTGGCCA	TTATTAACAC	CATTGTTTAT	CCTGCATTTG
	1510	1520	1530	1540	1550
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GCATGCAGCG	TATGCATTAT	CGTAATGGTG	ATCCGCAGAC	ACCGTTTCAG
	1560	1570	1580	1590	1600
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	ATCGCAGAAC	AGCAAATTCA	GAATTTTCAG	GTTGCAAACT	GGCTGCACCT
	1610	1620	1630	1640	1650
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	TGTGAATTAC	AATCAGTTTC	GTCAGGTGGT	GATTGATGGT	GTGCTGAATC
	1660	1670	1680	1690	1700
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	AGGTGCTGAA	TGATAACATT	CGCAATGGTC	ATGTTGTGAA	CCAACTGATG
	1710	1720	1730	1740	1750
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GAAGCCCTGA	TGCAGCTGAG	CCGTCAGCAG	TTTCCGACCA	TGCCGGTTGA
	1760	1770	1780	1790	1800
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	TTATAAACGT	AGCATTTCAG	GTGGTATCCT	GCTGCTGTCA	AATCGTCTGG
	1810	1820	1830	1840	1850
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GTCAGCTGGT	AGATCTGACC	CGTCTGCTGA	GCTATAACTA	TGAAACACTG
	1860	1870	1880	1890	1900
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	ATGGCATGCA	TCACCATGAA	TATGCAGCAT	GTTCA-GACC	CTGACCACCG
	1910	1920	1930	1940	1950
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	AAAACTGCA	GCTGACC-AG	CGTTACCTCA	CTGTGTATGC	TGATTGGTAA
	1960	1970	1980	1990	2000
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	TGCAACCGTG	ATTCCGAGTC	CGCAGACCTT	ATTT-CATTA	TTACAA-TGT
	2010	2020	2030	2040	2050
SA11_VP2_BacO_reference					
SA11_VP2_BacO_sequenced	GAATGTGAAC	TTCCACAGCA	ACTATAACGA	ACGCATTAAT	GATGCCGTTG

	2060	2070	2080	2090	2100
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CGATTATTAC	CGCAGCCAAT	CGCCTGAATC	TGTACCAGAA	AAAAA-TGAA
SA11_VP2_BacO_sequenced	CGATTATTWC	CGCAGCCAAT	CGCCTGAATC	TGTACCAGAA	AAAAAATGAA
	2110	2120	2130	2140	2150
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AAGCATCTGTG	GAAGATTTTC	TGAAACGCCT	GCAGATTTTT	GATGTTGCAC
SA11_VP2_BacO_sequenced	AAGCATCTGTG	GAAGATTTTC	TGAAACGCCT	GCAGATTTTT	GATGTTGCAC
	2160	2170	2180	2190	2200
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GTTGTCCGGA	TGATCAGATG	TATCGTCTGC	GTGATCGCCT	GCGTCTGCTG
SA11_VP2_BacO_sequenced	GTTGTCCGGA	TGATCAGATG	TATCGTCTGC	GTGATCGCCT	GCGTCTGCTG
	2210	2220	2230	2240	2250
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CCGGTGGAAA	TTCGTCGTCT	GGATATCTTT	AATCTGATCG	CAATGAACAT
SA11_VP2_BacO_sequenced	CCGGTGGAAA	TTCGTCGTCT	GGATATCTTT	AATCTGATCG	CAATGAACAT
	2260	2270	2280	2290	2300
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GGAACAAATT	GAACGTGCGA	GCGATAAAAT	TGCACAGGGT	GTTATTATTG
SA11_VP2_BacO_sequenced	GGAACAAATT	GAACGTGCGA	GCGATAAAAT	TGCACAGGGT	GTTATTATTG
	2310	2320	2330	2340	2350
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CCTATCGTGA	TATGCAGCTT	GAACGCGACG	AAATGTATGG	CTATGTTAAT
SA11_VP2_BacO_sequenced	CCTATCGTGA	TATGCAGCTT	GAACGCGACG	AAATGTATGG	CTATGTTAAT
	2360	2370	2380	2390	2400
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	ATTGCACGCA	ACCTGGATGG	TTTCCAGCAG	ATTAACCTGG	AAGAACTGAT
SA11_VP2_BacO_sequenced	ATTGCACGCA	ACCTGGATGG	TTTCCAGCAG	ATTAACCTGG	AAGAACTGAT
	2410	2420	2430	2440	2450
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GCGTAGTGGC	GATTATGCAC	AGATTACCAA	TATGCTGCTT	AATAATCAGC
SA11_VP2_BacO_sequenced	GCGTAGTGGC	GATTATGCAC	AGATTACCAA	TATGCTGCTT	AATAATCAGC
	2460	2470	2480	2490	2500
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	CGGTTGCACT	GGTTGGTGCA	CTGCCGTTTA	TTACCGATAG	CAGCGTTATT
SA11_VP2_BacO_sequenced	CGGTTGCACT	GGTTGGTGCA	CTGCCGTTTA	TTACCGATAG	CAGCGTTATT
	2510	2520	2530	2540	2550
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	AGCCTGATTG	CCAAACTGGA	TGCCACCGTT	TTTGCCCAGA	TTGTAAACT
SA11_VP2_BacO_sequenced	AGCCTGATTG	CCAAACTGGA	TGCCACCGTT	TTTGCCCAGA	TTGTAAACT
	2560	2570	2580	2590	2600
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GCGCAAAGTG	GATACCCTGA	AACCGATTCT	GTATAAAATC	AACAGCGATA
SA11_VP2_BacO_sequenced	GCGCAAAGTG	GATACCCTGA	AACCGATTCT	GTATAAAATC	AACAGCGATA
	2610	2620	2630	2640	2650
SA11_VP2_BacO_reference					
SA11_VP2_BacO_reference	GCAACGACTT	TTATCTGGTG	GCAAACATATG	ATTGGATTCC	GACCAGCACC
SA11_VP2_BacO_sequenced	GCAACGACTT	TTATCTGGTG	GCAAACATATG	ATTGGATTCC	GACCAGCACC

	2660	2670	2680	2690	2700
					
SA11_VP2_BacO_reference	ACCAAAGTGT	ATAAACAGGT	TCCGCAGCAG	TTTGATTTTC	GTGCCAGCAT
SA11_VP2_BacO_sequenced	ACCAAAGTGT	ATAAACAGGT	TCCGCAGCAG	TTTGATTTTC	GTGCCAGCAT
	2710	2720	2730	2740	2750
					
SA11_VP2_BacO_reference	GCATATGCTG	ACCAGTAATC	TGACCTTTAC	CGTTTATAGC	GATCTGCTGG
SA11_VP2_BacO_sequenced	GCATATGCTG	ACCAGTAATC	TGACCTTTAC	CGTTTATAGC	GATCTGCTGG
	2760	2770	2780	2790	2800
					
SA11_VP2_BacO_reference	CATTTGTTAG	CGCAGATACC	GTTGAACCGA	TTAATGCAGT	TGCCCTTTGAT
SA11_VP2_BacO_sequenced	CATTTGTTAG	CGCAGATACC	GTTGAACCGA	TTAATGCAGT	TGCCCTTTGAT
	2810	2820	2830	2840	2850
					
SA11_VP2_BacO_reference	AACATGCGTA	TCATGAACGA	ACTGTAAAAG	GGCGAGCTCA	ACGATCCGGC
SA11_VP2_BacO_sequenced	AACATGCGTA	TCATGAACGA	ACTGTAAAAG	GGCGAGCTCA	ACGATCCGGC
	2860	2870	2880		
					
SA11_VP2_BacO_reference	TGCTAACAAA	GCCCGAA---	-----	-----	
SA11_VP2_BacO_sequenced	TGCTAACAAA	GCCCGAAAAG	AARGSKKRWT	MTTKYKT	

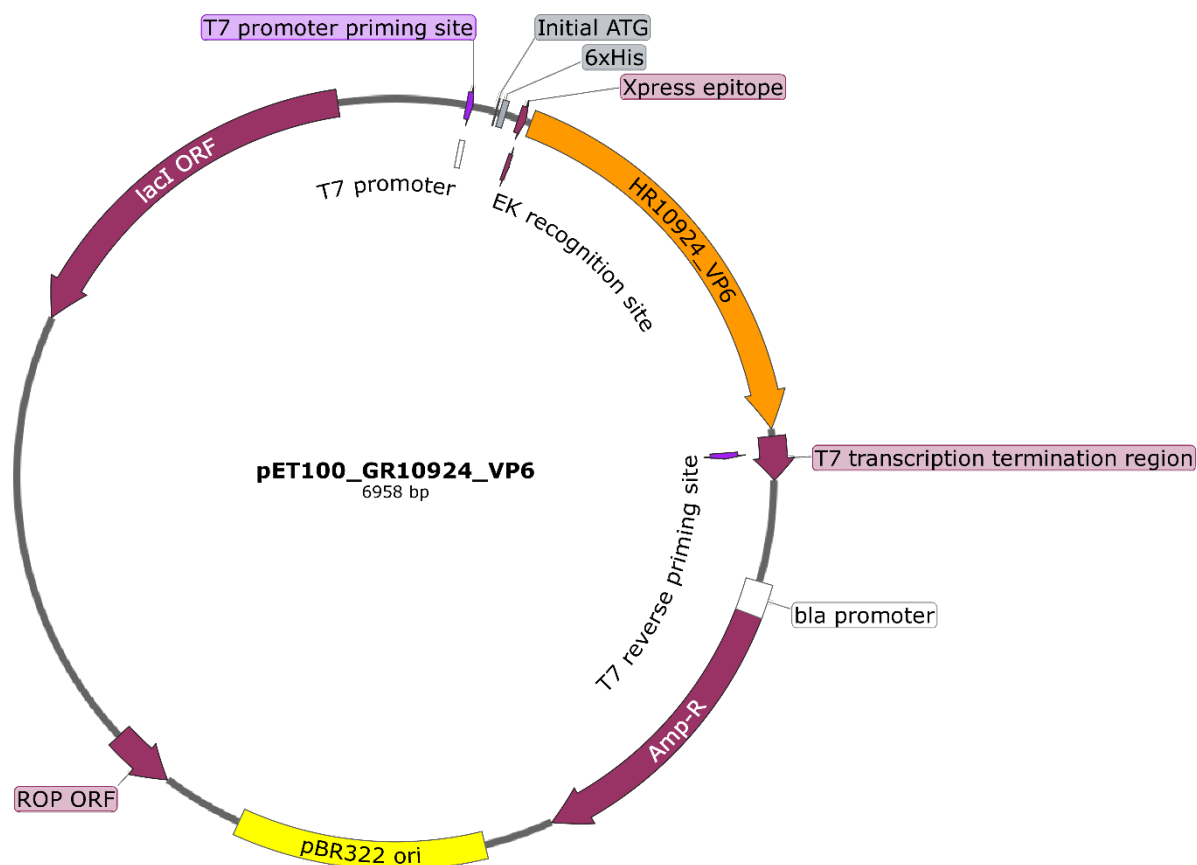


Figure 39: Map of pET100_GR10924_VP6_BacO

	10	20	30	40	50
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	-----GG	ATCTGTACGA	CGATGACGAT		
	60	70	80	90	100
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	AAGGATCATC	CCTTCACCAT	GGATGTACTA	TACTCATTGA	GTAAGACACT
	110	120	130	140	150
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	AAAGGATGCT	AGGGATAAGA	TAGTTGAGGG	CACCCGTGAT	AGCAACGTGA
	160	170	180	190	200
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	GCGATCTGAT	TCAGCAATTC	AACCAGATGA	TCATTACCAT	GAACGGCAAC
	210	220	230	240	250
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	GAATTTCAAA	CCGGTGGCAT	CGGTAACCTG	CCGATTTCGT	ACTGGAACCT

	260	270	280	290	300
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	CGACTTTGGT	CTGCTGGGCA	CCACCCTGCT	GAACCTGGAT	GCGAACTACG
	310	320	330	340	350
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	TTGAGACCGC	GCGTAACACC	ATCGACTATT	TCGTGGACTT	TGTTGATAAC
	360	370	380	390	400
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	GTGTGCATGG	ATGAGATGGT	GCGTGAAAGC	CAGCGTAACG	GCAATTGCGCC
	410	420	430	440	450
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	GCAAAGCGAC	AGCCTGCGTA	AGCTGAGCGG	TATCAAGTTC	AAGCGTATCA
	460	470	480	490	500
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	ACTTCGATAA	CAGCAGCGAG	TACATCGAAA	ACTGGAACCT	GCAGAACCGT
	510	520	530	540	550
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	CGTCAACGTA	CCGGTTTCAC	CTTTCACAAA	CCGAACATTT	TCCCGTATAG
	560	570	580	590	600
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	CGCGAGCTTT	ACCCTGAACC	GTAGCCAGCC	GGCGCACGAC	AACCTGATGG
	610	620	630	640	650
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	GTACCATGTG	GCTGAACGCG	GGCAGCGAAA	TCCAAGTTGC	GGGTTTTGAT
	660	670	680	690	700
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	TACAGCTGCG	CGATTAACGC	GCCGGCGAAC	ACCCAGCAAT	TCGAGCACAT
	710	720	730	740	750
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	CGTTCAGCTG	CGTCGTGTGC	TGACCACCGC	GACCATTACC	CTGCTGCCGG
	760	770	780	790	800
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	ATGCGGAACG	TTTCAGCTTT	CCGCGTGTTA	TCAACAGCGC	GGATGGCGCG
	810	820	830	840	850
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_sequenced	ACCACCTGGT	ACTTCAACCC	GGTGATTCTG	CGTCCGAACA	ACGTTGAGGT

	860	870	880	890	900
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	GGAATTTCTG	CTGAACGGCC	AGATCATTAA	CACCTATCAA	GCGCGTTTCG
GR10924_VP6_BacO_sequenced	GGAATTTCTG	CTGAACGGCC	AGATCATTAA	CMCCTATCAA	GCGCGTTTCG
	910	920	930	940	950
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	GTACCATCGT	GGCGCGTAAC	TTCGACACCA	TTCGTCTGAG	CTTTCAGCTG
GR10924_VP6_BacO_sequenced	GTACCATCGT	GGCGCGTAAC	TTCGACACCA	TTCGTCTGAG	CTTTCAGCTG
	960	970	980	990	1000
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	ATGCGTCCGC	CGAACATGAC	CCCGAGCGTT	GCGGCGCTGT	TCCCGAACGC
GR10924_VP6_BacO_sequenced	ATGCGTCCGC	CGAACATGAC	CCCGAGCGTT	GCGGCGCTGT	TCCCGAACGC
	1010	1020	1030	1040	1050
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	GCAACCGTTT	GAGCACCACG	CGACCGTGGG	TCTGACCCCTG	AAGATCGAGA
GR10924_VP6_BacO_sequenced	GCAACCGTTT	GAGCACCACG	CGACCGTGGG	TCTGACCCCTG	AAGATCGAGA
	1060	1070	1080	1090	1100
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	GCGCGGTTTG	CGAAAGCGTG	CTGGCGGATG	CGAGCGAGAC	CATGCTGGCG
GR10924_VP6_BacO_sequenced	GCGCGGTTTG	CGAAAGCGTG	CTGGCGGATG	CGAGCGAGAC	CATGCTGGCG
	1110	1120	1130	1140	1150
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	AACGTTACCA	GCGTGCCTCA	GGAATACGCG	ATCCCGGTTG	GTCCGGTGTT
GR10924_VP6_BacO_sequenced	AACGTTACCA	GCGTGCCTCA	GGAATACGCG	ATCCCGGTTG	GTCCGGTGTT
	1160	1170	1180	1190	1200
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	TCCGCCGGGC	ATGAACTGGA	CCGACCTGAT	TACCAACTAT	AGCCCGAGCC
GR10924_VP6_BacO_sequenced	TCCGCCGGGC	ATGAACTGGA	CCGACCTGAT	TACCAACTAT	AGCCCGAGCC
	1210	1220	1230	1240	1250
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	GTGAAGATAA	CCTGCAACGT	GTTTTACCG	TGGCGAGCAT	CCGTAGCATG
GR10924_VP6_BacO_sequenced	GTGAAGATAA	CCTGCAACGT	GTTTTACCG	TGGCGAGCAT	CCGTAGCATG
	1260	1270	1280	1290	1300
GR10924_VP6_BacO_reference					
GR10924_VP6_BacO_reference	CTGGTGAAT	AAAAGGGCGA	GCTCAACGAT	CCGGCTGCTA	ACAAAGCCCG
GR10924_VP6_BacO_sequenced	CTGGTGAAT	AAAAGGGCGA	GCTCAACGAT	CCGGCTGCTA	ACAAAGCCCG
	1310	1320			
GR10924_VP6_BacO_reference			.		
GR10924_VP6_BacO_reference	AA-----	-----	-		
GR10924_VP6_BacO_sequenced	AACYAAAGTA	TCGTTCGGCG	T		

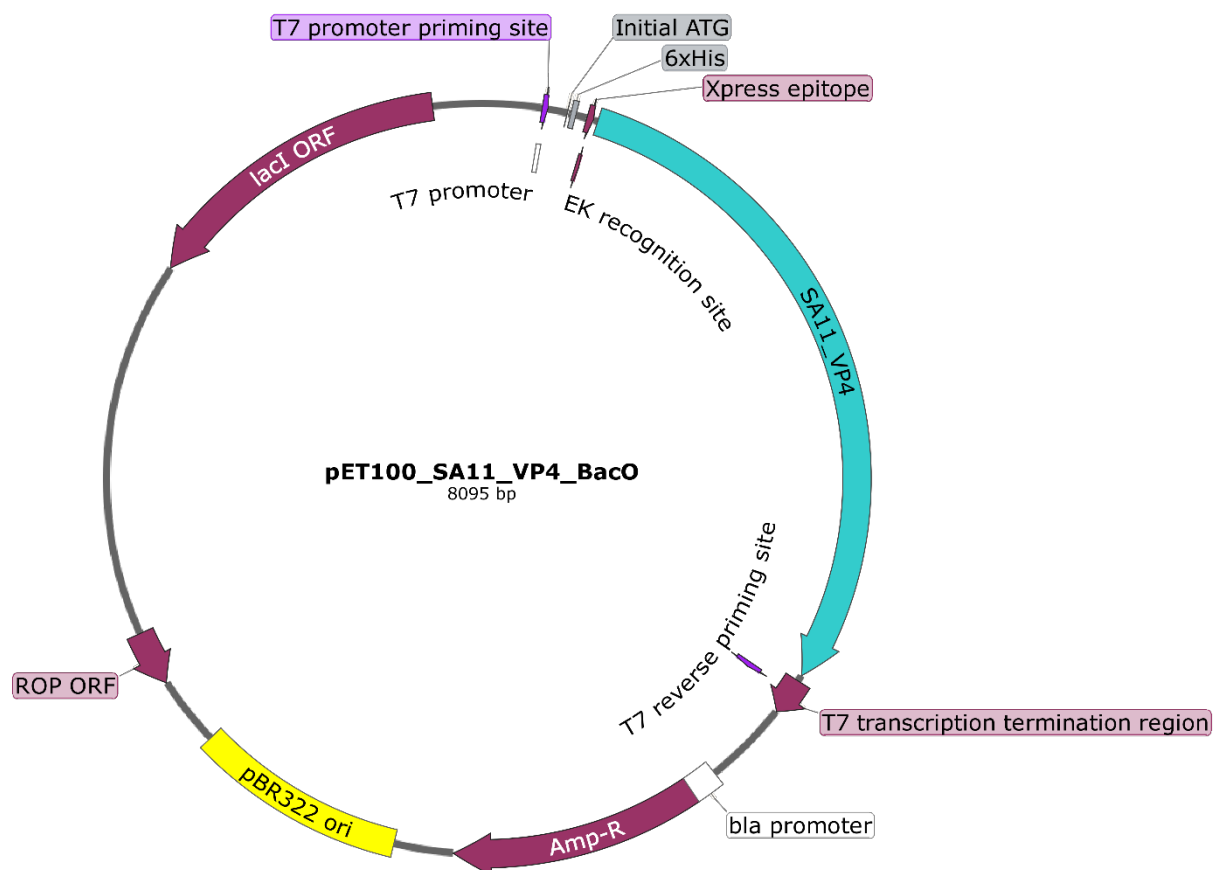


Figure 40: Map of pET100_SA11_VP4_BacO

	10	20	30	40	50
SA11_VP4_BacO_reference					
SA11_VP4_BacO_reference	-----ATG	GCCAGCCTGA	TTTATCGTCA		
SA11_VP4_BacO_sequenced	GATGACGATA	AGGATCATCC	CTTCACCATG	GCCAGCCTGA	TTTATCGTCA
	60	70	80	90	100
SA11_VP4_BacO_reference	GCTGCTGACC	AATAGCTATA	CCGTTGATCT	GAGTGATGAA	ATCCAAGAAA
SA11_VP4_BacO_sequenced	GCTGCTGACC	AATAGCTATA	CCGTTGATCT	GAGTGATGAA	ATCCAAGAAA
	110	120	130	140	150
SA11_VP4_BacO_reference	TTGGTAGCAC	CAAAGCCAG	AACGTTACCA	TTAATCCGGG	TCCGTTTGCA
SA11_VP4_BacO_sequenced	TTGGTAGCAC	CAAAGCCAG	AACGTTACCA	TTAATCCGGG	TCCGTTTGCA
	160	170	180	190	200
SA11_VP4_BacO_reference	CAGACCGGTT	ATGCACCGGT	TAATTGGGGT	CCGGGTGAAA	TTAATGATAG
SA11_VP4_BacO_sequenced	CAGACCGGTT	ATGCACCGGT	TAATTGGGGT	CCGGGTGAAA	TTAATGATAG
	210	220	230	240	250
SA11_VP4_BacO_reference	CACCACCGTT	GAACCGCTGC	TGGATGGTCC	GTATCAGCCG	ATGACCTTTA
SA11_VP4_BacO_sequenced	CACCACCGTT	GAACCGCTGC	TGGATGGTCC	GTATCAGCCG	ATGACCTTTA

	260	270	280	290	300
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ATCCGCCTGT	TGATTATTGG	ATGCTGCTGG	CACCGACCAC	ACCGGGTGTT
	310	320	330	340	350
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ATTGTTGAAG	GCACCAATAA	TACCGATCGT	TGGCTGGCAA	CCATTCTGAT
	360	370	380	390	400
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGAACCGAAT	GTTTCAGAGCG	AAAATCGTAC	CTATACCATC	TTTGGTATTC
	410	420	430	440	450
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	AAGAACAGCT	GACCGTTAGC	AATACCAGCC	AGGATCAGTG	GAAATTTATC
	460	470	480	490	500
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GATGTTGTTA	AAACCACCGC	CAATGGTTCA	ATTGGTCAGT	ATGGTAGCCT
	510	520	530	540	550
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GCTGAGCAGC	CCGAAACTGT	ATGCAGTTAT	GAAACATAAC	GAGAAACTGT
	560	570	580	590	600
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ACACCTATGA	AGGTCAGACC	CCGAATGCAC	GTACCGGTCA	TTATTCAACC
	610	620	630	640	650
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ACCAATTATG	ATAGCGTTAA	CATGACCGCC	TTTTGCGACT	TTTATATCAT
	660	670	680	690	700
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TCCGCGTAGC	GAAGAAAGCA	AATGCACCGA	ATATATCAAT	AATGGTCTGC
	710	720	730	740	750
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	CTCCGATTCA	GAATACCCGT	AATGTTGTTC	CGCTGAGCCT	GACCGCACGT
	760	770	780	790	800
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GATGTTATTTC	ATTATCGTGC	ACAGGCCAAT	GAAGATATCG	TTATTAGCAA
	810	820	830	840	850
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	AACCTCGCTG	TGGAAGAGAA	TGCAGTATAA	TCGCGATATT	ACCATCCGCT

	860	870	880	890	900
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TTAAATTTCGC	CAACACCATT	ATCAAAAGCG	GTGGTCTGGG	TTATAAAATGG
	910	920	930	940	950
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TCCGAAATTA	GCTTTAAACC	GGCAAACAT	CAGTATACCT	ATACGCGTGA
	960	970	980	990	1000
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGGTGAAGAA	GTTACCGCAC	ATACCACCTG	TAGCGTTAAT	GGTGTGAATG
	1010	1020	1030	1040	1050
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ATTTTTCCTT	TAATGGTGGT	AGTCTGCCGA	CCGATTTTGT	TGTTAGCAAA
	1060	1070	1080	1090	1100
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TTTGAGGTGA	TCAAAGAAAA	CAGCTACGTG	TATATCGATT	ATTGGGATGA
	1110	1120	1130	1140	1150
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TAGCCAGGCC	TTTCGTAATG	TTATGTATGT	TCGTAGCCTG	GCAGCAAATC
	1160	1170	1180	1190	1200
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGAATAGCGT	TATGTGTACC	GGTGGTAGCT	ATAACTTTAG	CCTGCCGGTT
	1210	1220	1230	1240	1250
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GGTCAGTGGC	CTGTTCTGAC	CGGTGGTGCA	GTTAGCCTGC	ATAGTGCCGG
	1260	1270	1280	1290	1300
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGTTACCCTG	AGCACCCAGT	TTACAGATTT	TGTGAGCCTG	AATAGCCTGC
	1310	1320	1330	1340	1350
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GTTTTTCGTTT	TCGTCTGGCA	GTTGAAGAAC	CGCATTTTAA	ACTGACCCGT
	1360	1370	1380	1390	1400
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ACACGTCTGG	ATCGTCTGTA	TGGCCTGCCT	GCAGCCGATC	CGAATAATGG
	1410	1420	1430	1440	1450
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TAAAGAATAT	TATGAAATTG	CCGGTCGCTT	TTCCCTGATT	AGCCTGGTTC

	1460	1470	1480	1490	1500
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	CGAGCAATGA	TGATTATCAG	ACACCGATTG	CAAATAGCGT	GACCGTTCGT
	1510	1520	1530	1540	1550
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	CAGGATCTGG	AACGTCAGCT	GGGTGAAGT	CGTGAAGAAT	TTAATGCACT
	1560	1570	1580	1590	1600
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GAGCCAAGAA	ATCGCAATGA	GCCAGCTGAT	TGATCTGGCA	CTGCTGCCGC
	1610	1620	1630	1640	1650
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGGATATGTT	TAGCATGTTT	AGTGGTATTA	AGAGCACCAT	CGATGCAGCA
	1660	1670	1680	1690	1700
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	AAAAGCATGG	CAACCAATGT	GATGAAAAAA	TTCAAAAAAA	GCGGTCTGGC
	1710	1720	1730	1740	1750
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	CAATAGCGTT	TCAACCCTGA	CCGATAGCCT	GTCAGATGCC	GCAAGCAGCA
	1760	1770	1780	1790	1800
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TTAGCCGTGG	TAGCAGCATT	CGTAGCATTG	GTAGCTCAGC	AAGCGCATGG
	1810	1820	1830	1840	1850
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	ACCGATGTTA	GTACCCAGAT	TACCGATATT	TCAAGCAGCG	TGAGCAGCGT
	1860	1870	1880	1890	1900
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TAGCACCAG	ACCTCAACCA	TTAGTCGTCG	TCTGCGTCTG	AAAGAGATGG
	1910	1920	1930	1940	1950
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	CGACCCAGAC	CGAAGGTATG	AATTTTGATG	ATATTAGCGC	AGCCGTGCTG
	1960	1970	1980	1990	2000
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	AAAACCAAAA	TTGATAAAAG	CACACAGATT	AGCCCGAACA	CCATTCCGGA
	2010	2020	2030	2040	2050
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TATTGTTACC	GAAGCAAGCG	AAAAATTTCAT	TCCGAATCGT	GCATATCGCG

	2060	2070	2080	2090	2100
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGATCAATAA	CGATGATGTT	TTTGAAGCAG	GCATCGACGG	TAAATTCTTT
	2110	2120	2130	2140	2150
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GCCTATAAAG	TGGATACCTT	TGAAGAGATC	CCGTTTCGATG	TTCAGAAATT
	2160	2170	2180	2190	2200
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TGCAGATCTG	GTTACCGATA	GTCCGGTTAT	TAGCGCGATT	ATTGATTTCA
	2210	2220	2230	2240	2250
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	AAACCCTGAA	GAACCTGAAC	GATAACTATG	GCATTACCAA	ACAGCAGGCA
	2260	2270	2280	2290	2300
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	TTTAATCTGC	TGCGTAGCGA	TCCGCGTGTT	CTGCGTGAAT	TTATCAATCA
	2310	2320	2330	2340	2350
SA11_VP4_BacO_reference					
SA11__VP4_BacO_sequenced	GGATAATCCG	ATTATTGCA	ACCGTATTGA	GCAGCTGATC	ATGCAGTGTG
	2360	2370	2380		
SA11_VP4_BacO_reference				..	
SA11__VP4_BacO_sequenced	GTCTGTAA--	GGTAWGTTGA	CAAGACCCCG	AC	

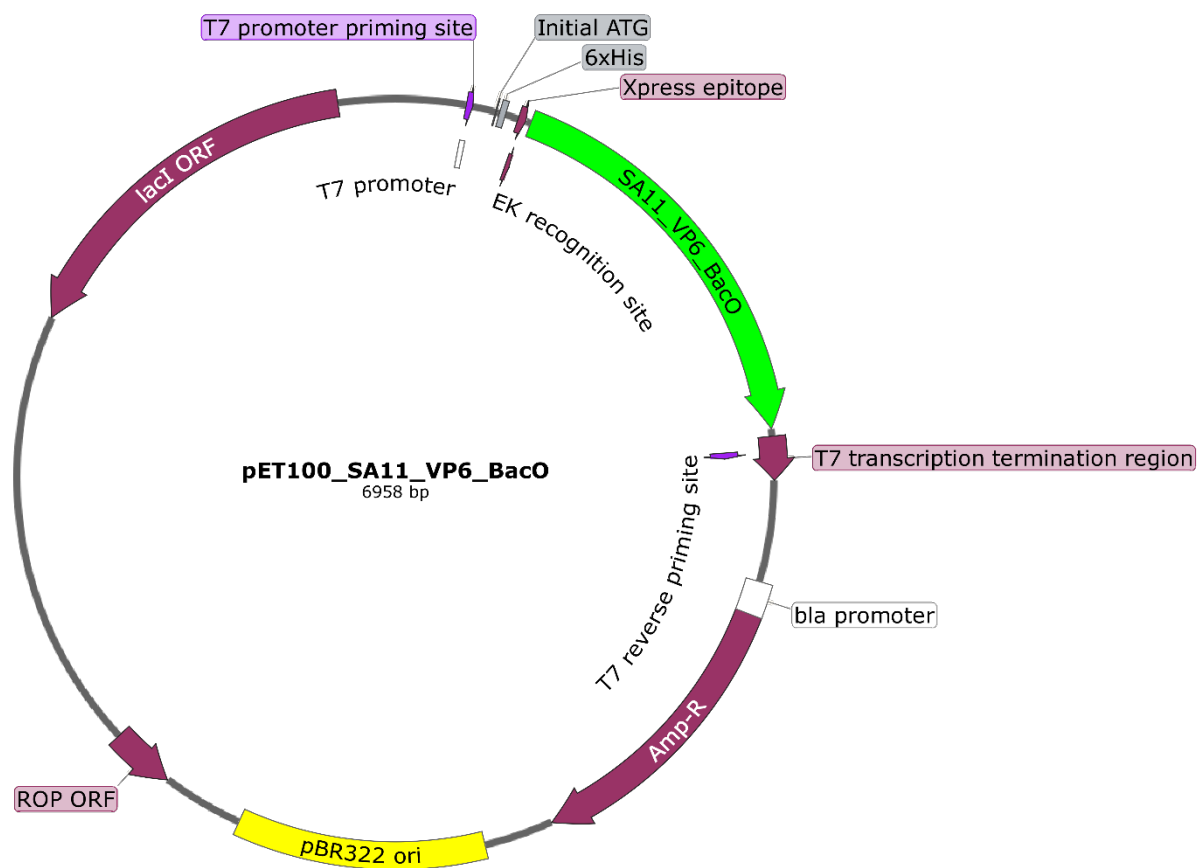


Figure 41: Map of pET100_SA11_VP6_BacO

	10	20	30	40	50
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	-----GGATC	TGTACGACGA	TGACGATAAG		
	60	70	80	90	100
SA11_VP6_BacO_reference	GATCATCCCT	TCACCATGGA	TGTTCTGTAT	AGCCTGAGCA	AAACCCTGAA
SA11_VP6_BacO_sequenced	GATCATCCCT	TCACCATGGA	TGTTCTGTAT	AGCCTGAGCA	AAACCCTGAA
	110	120	130	140	150
SA11_VP6_BacO_reference	AGATGCACGT	GATAAAATTG	TTGAAGGCAC	CCTGTATAGC	AATGTGAGCG
SA11_VP6_BacO_sequenced	AGATGCACGT	GATAAAATTG	TTGAAGGCAC	CCTGTATAGC	AATGTGAGCG
	160	170	180	190	200
SA11_VP6_BacO_reference	ATCTGATTCA	GCAGTTTAAC	CAGATGATTA	TTACCATGAA	CGGCAACGAA
SA11_VP6_BacO_sequenced	ATCTGATTCA	GCAGTTTAAC	CAGATGATTA	TTACCATGAA	CGGCAACGAA
	210	220	230	240	250
SA11_VP6_BacO_reference	TTTCAGACCG	GTGGTATTGG	TAATCTGCCG	ATTTCGTAATT	GGAACTTTAA
SA11_VP6_BacO_sequenced	TTTCAGACCG	GTGGTATTGG	TAATCTGCCG	ATTTCGTAATT	GGAACTTTAA

	260	270	280	290	300
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	CTTTGGTCTG	CTGGGTACAA	CCCTGCTGAA	CCTGGATGCC	AATTATGTTG
SA11_VP6_BacO_sequenced	CTTTGGTCTG	CTGGGTACAA	CCCTGCTGAA	CCTGGATGCC	AATTATGTTG
	310	320	330	340	350
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	AAACCGCACG	TAACACCATC	GATTATTTTG	TCGATTTTGT	GGACAACGTG
SA11_VP6_BacO_sequenced	AAACCGCACG	TAACACCATC	GATTATTTTG	TCGATTTTGT	GGACAACGTG
	360	370	380	390	400
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	TGCATGGATG	AAATGGTTCG	TGAAAGCCAG	CGTAATGGTA	TTGCACCGCA
SA11_VP6_BacO_sequenced	TGCATGGATG	AAATGGTTCG	TGAAAGCCAG	CGTAATGGTA	TTGCACCGCA
	410	420	430	440	450
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	GAGCGATAGC	CTGCGTAAAC	TGAGCGCCAT	TAAATTCAAA	CGCATCAACT
SA11_VP6_BacO_sequenced	GAGCGATAGC	CTGCGTAAAC	TGAGCGCCAT	TAAATTCAAA	CGCATCAACT
	460	470	480	490	500
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	TTGATAACAG	CAGCGAGTAT	ATTGAGAATT	GGAATCTGCA	GAATCGTCGT
SA11_VP6_BacO_sequenced	TTGATAACAG	CAGCGAGTAT	ATTGAGAATT	GGAATCTGCA	GAATCGTCGT
	510	520	530	540	550
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	CAGCGTACCG	GTTTTACCTT	TCATAAACCG	AACATTTTTC	CGTATAGCGC
SA11_VP6_BacO_sequenced	CAGCGTACCG	GTTTTACCTT	TCATAAACCG	AACATTTTTC	CGTATAGCGC
	560	570	580	590	600
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	AAGCTTTACC	CTGAATCGTA	GCCAGCCTGC	ACATGATAAT	CTGATGGGCA
SA11_VP6_BacO_sequenced	AAGCTTTACC	CTGAATCGTA	GCCAGCCTGC	ACATGATAAT	CTGATGGGCA
	610	620	630	640	650
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	CCATGTGGCT	GAATGCAGGT	AGCGAAATTC	AGGTTGCAGG	TTTTGATTAT
SA11_VP6_BacO_sequenced	CCATGTGGCT	GAATGCAGGT	AGCGAAATTC	AGGTTGCAGG	TTTTGATTAT
	660	670	680	690	700
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	AGCTGCGCAA	TTAATGCACC	GGCAAATATC	CAGCAGTTTG	AACATATTGT
SA11_VP6_BacO_sequenced	AGCTGCGCAA	TTAATGCACC	GGCAAATATC	CAGCAGTTTG	AACATATTGT
	710	720	730	740	750
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	TCCGCTGCGT	CGTGTTCTGA	CCACCGCAAC	CATTACACTG	CTGCCGGATG
SA11_VP6_BacO_sequenced	TCCGCTGCGT	CGTGTTCTGA	CCACCGCAAC	CATTACACTG	CTGCCGGATG
	760	770	780	790	800
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	CAGAACGTTT	TAGCTTTCCG	CGTGTTGATTA	ATAGCGCAGA	TGGTGC AACC
SA11_VP6_BacO_sequenced	CAGAACGTTT	TAGCTTTCCG	CGTGTTGATTA	ATAGCGCAGA	TGGTGC AACC
	810	820	830	840	850
SA11_VP6_BacO_reference					
SA11_VP6_BacO_reference	ACCTGGTTTT	TTAACCCGGT	TATTCTGCGT	CCGAATAATG	TGGAAGTTGA
SA11_VP6_BacO_sequenced	ACCTGGTTTT	TTAACCCGGT	TATTCTGCGT	CCGAATAATG	TGGAAGTTGA

	860	870	880	890	900
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	ATTTCTGCTG	AATGGCCAGA	TCATTAATAC	CTATCAGGCA	CGTTTTGGCA
	910	920	930	940	950
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	CCATTGTTGC	ACGTAATTTT	GATACCATTG	GCCTGAGTTT	TCAGCTGATG
	960	970	980	990	1000
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	CGTCCGCCTA	ATATGACACC	GGCAGTTGCA	GTTCTGTTTC	CGAATGCACA
	1010	1020	1030	1040	1050
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	GCCTTTTGAA	CATCATGCAA	CCGTTGGTCT	GACCCGTGCGT	ATTGAAAGCG
	1060	1070	1080	1090	1100
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	CAGTTTGTA	AAGCGTTCTG	GCAGATGCAA	GCGAAACACT	GCTGGCAAAAT
	1110	1120	1130	1140	1150
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	GTTACCAGCG	TTCGTCAAGA	ATATGCAATT	CCGGTTGGTC	CGGTTTTTCC
	1160	1170	1180	1190	1200
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	GCCTGGTATG	AATTGGACAG	ATCTGATTAC	AAATTATAGC	CCGAGCCGTG
	1210	1220	1230	1240	1250
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	AAGATAATCT	GCAGCGTGTT	TTTACCGTTG	CAAGCATTCG	TAGCATGCTG
	1260	1270	1280	1290	1300
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	ATCAAATAAA	AGGGCGAGCT	CAACGATCCG	GCTGCTAACA	AAGCCCGAA-
	1310	1320			
SA11_VP6_BacO_reference					
SA11_VP6_BacO_sequenced	GGAAARRSYK	ARTWTTTTYYY	G		

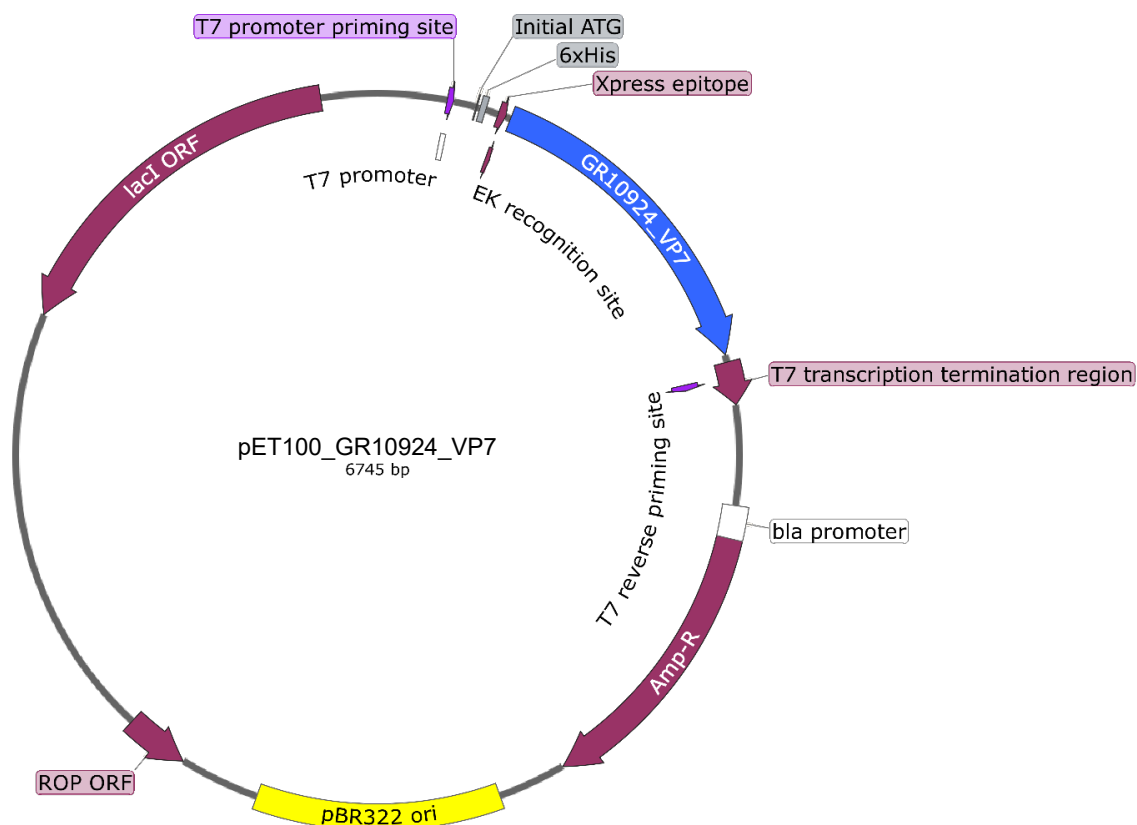


Figure 42: Map of pET151_GR10924_VP7_BacO

	10	20	30	40	50
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	ATCTGTACGA	CGATGACGAT	AAGGATCATC	CCTTCACCAT	GTATGGCATC
	60	70	80	90	100
GR10924_VP7_BacO_reference	GAATATACCA	CCATTCTGAC	CTTTCTGATT	AGCATTGTTC	TGCTGAAC TA
GR10924_VP7_BacO_sequenced	GAATATACCA	CCATTCTGAC	CTTTCTGATT	AGCATTGTTC	TGCTGAAC TA
	110	120	130	140	150
GR10924_VP7_BacO_reference	CATCCTGAAA	AGCCTGACCA	GCGCAATGGA	TTTTATCATT	TATCGTTTTC
GR10924_VP7_BacO_sequenced	CATCCTGAAA	AGCCTGACCA	GCGCAATGGA	TTTTATCATT	TATCGTTTTC
	160	170	180	190	200
GR10924_VP7_BacO_reference	TGCTGCTGAT	TGTTATTGCC	AGCCCGTTTG	TTAAAACCCA	GAATTATGGT
GR10924_VP7_BacO_sequenced	TGCTGCTGAT	TGTTATTGCC	AGCCCGTTTG	TTAAAACCCA	GAATTATGGT
	210	220	230	240	250
GR10924_VP7_BacO_reference	ATTAACCTGC	CGATTACCGG	TAGCATGGAT	ACCGCATATG	CAAA TAGCAG
GR10924_VP7_BacO_sequenced	ATTAACCTGC	CGATTACCGG	TAGCATGGAT	ACCGCATATG	CAAA TAGCAG

	260	270	280	290	300
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CCAGCAAGAA	ACATTTCTGA	CCAGCACCTT	GTGTCTGTAT	TACCCGACCG
	310	320	330	340	350
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	AAGCAAGCAC	CCAGATTGGT	GATACCGAAT	GGAAAGATAC	CCTGAGCCAG
	360	370	380	390	400
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CTGTTTCTGA	CAAAAGGTTG	GCCGACCGGT	AGCGTTTATT	TCAAAGAATA
	410	420	430	440	450
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	TACCGATATC	GCCAGCTTTA	GCATTGATCC	GCAGCTGTAT	TGTGATTATA
	460	470	480	490	500
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	ATGTGGTGCT	GATGAAATAC	GATAGCACCC	TGGAACCTGGA	TATGAGCGAA
	510	520	530	540	550
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CTGGCAGATC	TGATTCTGAA	TGAATGGCTG	TGTAATCCGA	TGGATATTAC
	560	570	580	590	600
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CCTGTATTAT	TACCAGCAGA	CCGATGAAGC	CAATAAATGG	ATTAGCATGG
	610	620	630	640	650
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	GTCAGAGCTG	TACCATTAAA	GTTTGTCGCG	TGAATACCCA	GACCCCTGGGT
	660	670	680	690	700
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	ATTGGTTGTA	TTACCACAAA	TACCGCAACC	TTTGAAGAAG	TTGCAACCAG
	710	720	730	740	750
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CGAAAACTG	GTTATTACCG	ATGTTGTTGA	TGGCGTGAAT	CATAAACTGG
	760	770	780	790	800
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	ATGTGACCAC	CAATACCTGC	ACCATTCTGT	ATTGTAAAAA	ACTGGGTCCG
	810	820	830	840	850
GR10924_VP7_BacO_reference					
GR10924_VP7_BacO_sequenced	CGTGAAAATG	TGGCAATTAT	TCAGGTGGT	GGTAGTGATG	TTCTGGATAT

	860	870	880	890	900
					
GR10924_VP7_BacO_reference	CACCGCAGAT	CCGACCACCG	CACCGCAGAC	AGAACGTATG	ATGCGTGTTA
GR10924_VP7_BacO_sequenced	CACCGCAGAT	CCGACCACCG	CACCGCAGAC	AGAACGTATG	ATGCGTGTTA
	910	920	930	940	950
					
GR10924_VP7_BacO_reference	ATTGGAAAAA	ATGGTGGCAG	GTCTTTTATA	CCGTGGTGGA	TTACATTAAT
GR10924_VP7_BacO_sequenced	ATTGGAAAAA	ATGGTGGCAG	GTCTTTTATA	CCGTGGTGGA	TTACATTAAT
	960	970	980	990	1000
					
GR10924_VP7_BacO_reference	CAGATCGTTC	AGGTTATGAG	CAAACGTAGC	CGTAGCCTGA	ATAGCGCAGC
GR10924_VP7_BacO_sequenced	CAGATCGTTC	AGGTTATGAG	CAAACGTAGC	CGTAGCCTGA	ATAGCGCAGC
	1010	1020	1030	1040	
					.
GR10924_VP7_BacO_reference	ATTCTATTAT	CGTGTGTAA-	-----	-----	-
GR10924_VP7_BacO_sequenced	ATTCTATTAT	CGTGTGTAAA	AGGGCGAGCT	CAACGATCCG	G

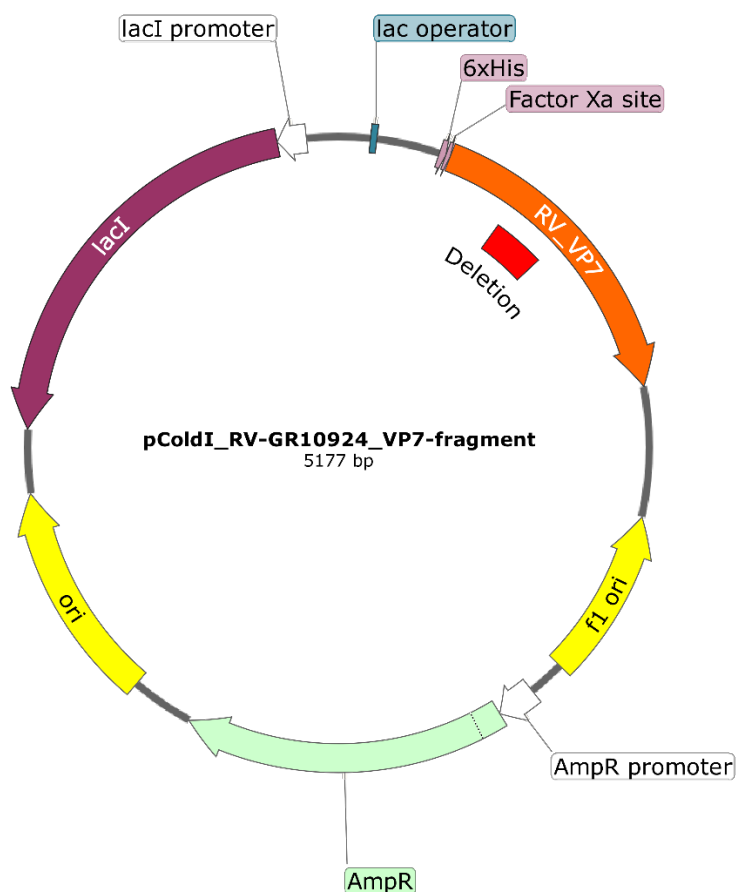


Figure 43: Map of pCOLDI_RV-GR10924_VP7-fragment

	10	20	30	40	50
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GGATCTGTAC	GACGATGACG	ATAAGGATCA	TCCCTTCACC	ATGTATGGCA
	60	70	80	90	100
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TCGAATATAC	CACCATTCTG	ACCTTTCTGA	TTAGCATTGT	TCTGCTGAAC
	110	120	130	140	150
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TACATCCTGA	AAAGCCTGAC	CAGCGCAATG	GATTTTATCA	TTTATCGTTT
	160	170	180	190	200
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TCTGCTGCTG	ATTGTTATTG	CCAGCCCGTT	TGTTAAAACC	CAGAAATTATG
	210	220	230	240	250
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GTATTAACTT	GCCGATTACC	GGTAGCATGG	ATACCGCATA	TGCAAAATAGC
	GTATTAACTT	GCCGATTACC	GGTAGCATGG	ATACCGCATA	TGCAAAATAGC

	260	270	280	290	300
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	AGCCAGCAAG	AAACATTTCT	GACCAGCACC	CTGTGTCTGT	ATTACCCGAC
	310	320	330	340	350
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	CGAAGCAAGC	ACCCAGATTG	GTGATACCGA	ATGGAAGAT	ACCCAGAGCC
	360	370	380	390	400
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	AGCTGTTTCT	GACAAAAGGT	TGGCCGACCG	GTAGCGTTTA	TTTCAAAGAA
	410	420	430	440	450
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TATACCGATA	TCGCCAGCTT	TAGCATTGAT	CCGCAGCTGT	ATTGTGATTA
	460	470	480	490	500
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TAATGTGGTG	CTGATGAAAT	ACGATAGCAC	CCTGGAAGTG	GATATGAGCG
	510	520	530	540	550
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	AACTGGCAGA	TCTGATTCTG	AATGAATGGC	TGTGTAATCC	GATGGATATT
	560	570	580	590	600
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	ACCCGTGATT	ATTACCAGCA	GACCGATGAA	GCCAATAAAT	GGATTAGCAT
	610	620	630	640	650
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GGGTTCAGAGC	TGTACCATTG	AAGTTTGTC	GCTGAATACC	CAGACCCCTGG
	660	670	680	690	700
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GTATTGGTTG	TATTACCACA	AATACCGCAA	CCTTTGAAGA	AGTTGCAACC
	710	720	730	740	750
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	AGCGAAAAAC	TGGTTATTAC	CGATGTTGTT	GATGGCGTGA	ATCATAAACT
	760	770	780	790	800
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GGATGTGACC	ACCAATACCT	GCACCATTCG	TAATTGTAAA	AAACTGGGTC
	810	820	830	840	850
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	CGCGTGAAAA	TGTGGCAATT	ATTCAAGGTT	GTGGTAGTGA	TGTTCTGGAT

	860	870	880	890	900
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	ATCACC	GACGAG	ATCCGACCAC	CGCACC	GACGAG
	910	920	930	940	950
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	TAATTG	GAAA	AAATGG	TGGC	AGGTCT
	960	970	980	990	1000
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	ATCAGAT	TCGT	TCAGGT	TATG	AGCAAAC
	1010	1020	1030	1040	1050
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	GCATTCT	TATT	ATCGTG	TGTA	AAAGGG
	1060	1070			
GR10924_VP7_BacO_reference					
pCOLDI_GR10924_VP7_sequenced	CAAAGCCC	GATA	ACAAAGC	CCGAA	

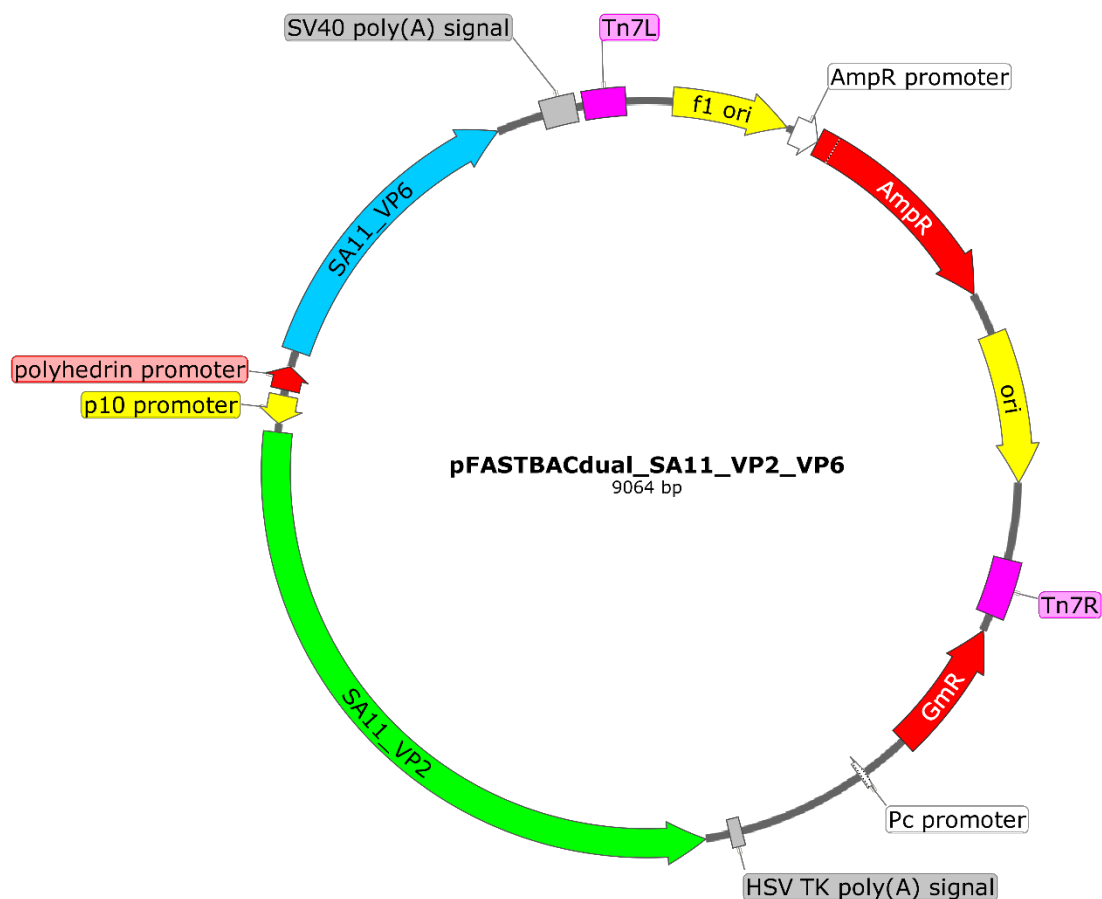


Figure 44: Map of pFASTBACdual_SA11_VP2_VP6

SA11 VP2 insect cell codon optimised

	10	20	30	40	50
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CAATCACTCG	ACGAAGACTT	GATCACCCGG	GATCTCGAGA	TGGCCTACAG
	60	70	80	90	100
SA11_VP2_InsO_reference	GAAGAGAGGT	GCTCGCCGTG	AGACCAACCT	GAAGCAGGAC	GAACGTATGC
SA11_VP2_InsO_sequenced	GAAGAGAGGT	GCTCGCCGTG	AGACCAACCT	GAAGCAGGAC	GAACGTATGC
	110	120	130	140	150
SA11_VP2_InsO_reference	AGGAGAAGGA	AGACAGCAAG	AACATCAACA	ACGACTCCCC	CAAGAGCCAG
SA11_VP2_InsO_sequenced	AGGAGAAGGA	AGACAGCAAG	AACATCAACA	ACGACTCCCC	CAAGAGCCAG
	160	170	180	190	200
SA11_VP2_InsO_reference	CTGTCTGAGA	AGGTGCTGAG	CAAGAAGGAG	GAAATCATCA	CCGACAACCA
SA11_VP2_InsO_sequenced	CTGTCTGAGA	AGGTGCTGAG	CAAGAAGGAG	GAAATCATCA	CCGACAACCA

	210	220	230	240	250
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	GGAGGAAGTC	AAGATCTCTG	ACGAGGTGAA	GAAGTCAAAC	AAGGAGGAAT
SA11_VP2_InsO_sequenced	GGAGGAAGTC	AAGATCTCTG	ACGAGGTGAA	GAAGTCAAAC	AAGGAGGAAT
	260	270	280	290	300
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	CCAAGCAGCT	GCTGGAAGTC	CTGAAGACCA	AGGAGGAACA	CCAGAAGGAG
SA11_VP2_InsO_sequenced	CCAAGCAGCT	GCTGGAAGTC	CTGAAGACCA	AGGAGGAACA	CCAGAAGGAG
	310	320	330	340	350
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	GTGCAGTACG	AAATCCTGCA	GAAGACCATC	CCTACTTTTCG	AGCCCAAGGA
SA11_VP2_InsO_sequenced	GTGCAGTACG	AAATCCTGCA	GAAGACCATC	CCTACTTTTCG	AGCCCAAGGA
	360	370	380	390	400
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	ATCTATCCTG	AAGAAGCTGG	AGGACATCAA	GCCAGAACAG	GCCAAGAAGC
SA11_VP2_InsO_sequenced	ATCTATCCTG	AAGAAGCTGG	AGGACATCAA	GCCAGAACAG	GCCAAGAAGC
	410	420	430	440	450
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	AGACCAAGCT	GTTCCGCATC	TTCGAGCCAA	AGCAGCTGCC	TATCTACCGC
SA11_VP2_InsO_sequenced	AGACCAAGCT	GTTCCGCATC	TTCGAGCCAA	AGCAGCTGCC	TATCTACCGC
	460	470	480	490	500
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	GCTAACGGCG	AGCGTGAACT	GCGCAACCGT	TGGTACTGGA	AGCTGAAGCG
SA11_VP2_InsO_sequenced	GCTAACGGCG	AGCGTGAACT	GCGCAACCGT	TGGTACTGGA	AGCTGAAGCG
	510	520	530	540	550
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	CGACACTCTG	CCCGACGGAG	ACTACGACGT	CCGTGAGTAC	TTCTTGAACC
SA11_VP2_InsO_sequenced	CGACACTCTG	CCCGACGGAG	ACTACGACGT	CCGTGAGTAC	TTCTTGAACC
	560	570	580	590	600
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	TGTACGACCA	GGTGCTGATG	GAAATGCCAG	ACTACCTGCT	GCTGAAGGAC
SA11_VP2_InsO_sequenced	TGTACGACCA	GGTGCTGATG	GAAATGCCAG	ACTACCTGCT	GCTGAAGGAC
	610	620	630	640	650
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	ATGGCCGTGG	AGAACAAGAA	CAGCAGAGAC	GCTGGCAAGG	TGGTGGACTC
SA11_VP2_InsO_sequenced	ATGGCCGTGG	AGAACAAGAA	CAGCAGAGAC	GCTGGCAAGG	TGGTGGACTC
	660	670	680	690	700
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	TGAAACCGCT	GCCATCTGCG	ACGCTATCTT	CCAGGACGAG	GAAACTGAGG
SA11_VP2_InsO_sequenced	TGAAACCGCT	GCCATCTGCG	ACGCTATCTT	CCAGGACGAG	GAAACTGAGG
	710	720	730	740	750
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	GCGCCGTCAG	GAGATTTCATC	GCTGAAATGA	GGCAGAGAGT	GCAGGCCGAC
SA11_VP2_InsO_sequenced	GCGCCGTCAG	GAGATTTCATC	GCTGAAATGA	GGCAGAGAGT	GCAGGCCGAC
	760	770	780	790	800
SA11_VP2_InsO_reference					
SA11_VP2_InsO_reference	CGCAACGTGG	TCAACTACCC	CAGCATCCTG	CACCCAATCG	ACCACGCTTT
SA11_VP2_InsO_sequenced	CGCAACGTGG	TCAACTACCC	CAGCATCCTG	CACCCAATCG	ACCACGCTTT

	810	820	830	840	850
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CAACGAGTAC	TTCCTGCAGC	ACCAGCTGGT	CGAACCCCTG	AACAACGACA
	860	870	880	890	900
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	TCATCTTCAA	CTACATCCCA	GAGCGCATCC	GTAACGACGT	GAAC TACATC
	910	920	930	940	950
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CTGAACATGG	ACAGGAACCT	GCCTAGCACC	GCCAGGTACA	TCAGACCCAA
	960	970	980	990	1000
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CCTGCTGCAG	GACAGACTGA	ACCTGCACGA	CAACTTCGAA	TCTCTGTGGG
	1010	1020	1030	1040	1050
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	ACACTATCAC	CACTAGCAAC	TACATCCTGG	CTCGTTCTGT	GGTCCCTGAC
	1060	1070	1080	1090	1100
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CTGAAGGAGC	TGGTGTCAAC	CGAAGCCCAG	ATCCAGAAGA	TGTCCAGGA
	1110	1120	1130	1140	1150
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CCTGCAGCTG	GAGGCTCTGA	CTATCCAGTC	AGAAACCCAG	TTCTTGACTG
	1160	1170	1180	1190	1200
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GAATCAACTC	CCAGGCTGCC	AACGACTGCT	TCAAGACCCCT	GATCGCTGCC
	1210	1220	1230	1240	1250
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	ATGCTGAGCC	AGAGAACTAT	GTCTCTGGAC	TTCGTCACCA	CTAACTACAT
	1260	1270	1280	1290	1300
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	G TCACTGATC	TCCGGAATGT	GGCTGCTGAC	CGTGATCCCT	AACGACATGT
	1310	1320	1330	1340	1350
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	TCATCAGGGA	GTCCCTGGTG	GCTTGCCAGC	TGGCCATCAT	CAACACCATC
	1360	1370	1380	1390	1400
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GTGTACCCCG	CCTTCGGTAT	GCAGCGCATG	CACTACCGTA	ACGGCGACCC

	1410	1420	1430	1440	1450
					
SA11_VP2_InsO_reference	ACAGACTCCT	TTCCAGATCG	CCGAACAGCA	GATCCAGAAC	TTCCAGGTCG
SA11_VP2_InsO_sequenced	ACAGACTCCT	TTCCAGATCG	CCGAACAGCA	GATCCAGAAC	TTCCAGGTCG
	1460	1470	1480	1490	1500
					
SA11_VP2_InsO_reference	CTAACTGGCT	GCACTTCGTG	AACTACAACC	AGTTCCGCCA	GGTGGTCATC
SA11_VP2_InsO_sequenced	CTAACTGGCT	GCACTTCGTG	AACTACAACC	AGTTCCGCCA	GGTGGTCATC
	1510	1520	1530	1540	1550
					
SA11_VP2_InsO_reference	GACGGTGTGC	TGAACCAGGT	CCTGAACGAC	AACATCCGTA	ACGGCCACGT
SA11_VP2_InsO_sequenced	GACGGTGTGC	TGAACCAGGT	CCTGAACGAC	AACATCCGTA	ACGGCCACGT
	1560	1570	1580	1590	1600
					
SA11_VP2_InsO_reference	GGTCAACCAG	CTGATGGAGG	CTCTGATGCA	GCTGAGCAGG	CAGCAGTTCC
SA11_VP2_InsO_sequenced	GGTCAACCAG	CTGATGGAGG	CTCTGATGCA	GCTGAGCAGG	CAGCAGTTCC
	1610	1620	1630	1640	1650
					
SA11_VP2_InsO_reference	CCACCATGCC	AGTGGACTAC	AAGAGGAGCA	TCCAGAGAGG	AATCCTGCTG
SA11_VP2_InsO_sequenced	CCACCATGCC	AGTGGACTAC	AAGAGGAGCA	TCCAGAGAGG	AATCCTGCTG
	1660	1670	1680	1690	1700
					
SA11_VP2_InsO_reference	CTGTCTAACA	GGCTGGGTCA	GCTGGTGGAC	CTGACCAGAC	TGCTGTCTTA
SA11_VP2_InsO_sequenced	CTGTCTAACA	GGCTGGGTCA	GCTGGTGGAC	CTGACCAGAC	TGCTGTCTTA
	1710	1720	1730	1740	1750
					
SA11_VP2_InsO_reference	CAACTACGAG	ACCCTGATGG	CCTGCATCAC	TATGAACATG	CAGCACGTCC
SA11_VP2_InsO_sequenced	CAACTACGAG	ACCCTGATGG	CCTGCATCAC	TATGAACATG	CAGCACGTCC
	1760	1770	1780	1790	1800
					
SA11_VP2_InsO_reference	AGACTCTGAC	CACTGAAAAG	CTGCAGCTGA	CCAGCGTCAC	TTCTCTGTGC
SA11_VP2_InsO_sequenced	AGACTCTGAC	CACTGAAAAG	CTGCAGCTGA	CCAGCGTCAC	TTCTCTGTGC
	1810	1820	1830	1840	1850
					
SA11_VP2_InsO_reference	ATGCTGATCG	GCAACGCTAC	CGTGATCCCT	TCACCCAGAG	CTCTGTTCCA
SA11_VP2_InsO_sequenced	ATGCTGATCG	GCAACGCTAC	CGTGATCCCT	TCACCCAGAG	CTCTGTTCCA
	1860	1870	1880	1890	1900
					
SA11_VP2_InsO_reference	CTACTACAAC	GTGAACGTCA	ACTTCCACTC	CAACTACAAC	GAGAGGATCA
SA11_VP2_InsO_sequenced	CTACTACAAC	GTGAACGTCA	ACTTCCACTC	CAACTACAAC	GAGAGGATCA
	1910	1920	1930	1940	1950
					
SA11_VP2_InsO_reference	ACGACGCTGT	CGCCATCATC	ACTGCTGCCA	ACAGACTGAA	CCTGTACCAG
SA11_VP2_InsO_sequenced	ACGACGCTGT	CGCCATCATC	ACTGCTGCCA	ACAGACTGAA	CCTGTACCAG
	1960	1970	1980	1990	2000
					
SA11_VP2_InsO_reference	AAGAAGATGA	AGTCAATCGT	GGAAGACTTC	CTGAAGCGCC	TGCAGATCTT
SA11_VP2_InsO_sequenced	AAGAAGATGA	AGTCAATCGT	GGAAGACTTC	CTGAAGCGCC	TGCAGATCTT

	2010	2020	2030	2040	2050
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CGACGTGGCC	CGTGTCCCAG	ACGACCAGAT	GTACAGGCTG	AGAGACCGCC
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CGACGTGGCC	CGTGTCCCAG	ACGACCAGAT	GTACAGGCTG	AGAGACCGCC
	2060	2070	2080	2090	2100
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	TGCGTCTGCT	GCCTGTGGAG	ATCCGCCGTC	TGGACATCTT	CAACCTGATC
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	TGCGTCTGCT	GCCTGTGGAG	ATCCGCCGTC	TGGACATCTT	CAACCTGATC
	2110	2120	2130	2140	2150
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GCTATGAACA	TGGAGCAGAT	CGAACGTGCC	TCCGACAAGA	TCGCTCAGGG
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GCTATGAACA	TGGAGCAGAT	CGAACGTGCC	TCCGACAAGA	TCGCTCAGGG
	2160	2170	2180	2190	2200
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	AGTCATCATC	GCCTACAGGG	ACATGCAGCT	GGAGAGAGAC	GAAATGTACG
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	AGTCATCATC	GCCTACAGGG	ACATGCAGCT	GGAGAGAGAC	GAAATGTACG
	2210	2220	2230	2240	2250
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GCTACGTGAA	CATCGCTCGC	AACCTGGACG	GATTCCAGCA	GATCAACCTG
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GCTACGTGAA	CATCGCTCGC	AACCTGGACG	GATTCCAGCA	GATCAACCTG
	2260	2270	2280	2290	2300
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GAGGAACCTGA	TGCGTTCAGG	AGACTACGCT	CAGATCACCA	ACATGCTGCT
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GAGGAACCTGA	TGCGTTCAGG	AGACTACGCT	CAGATCACCA	ACATGCTGCT
	2310	2320	2330	2340	2350
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GAACAACCAA	CCAGTGGCTC	TGGTGGGTGC	TCTGCCTTTC	ATCACTGACT
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	GAACAACCAA	CCAGTGGCTC	TGGTGGGTGC	TCTGCCTTTC	ATCACTGACT
	2360	2370	2380	2390	2400
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CCAGCGTCAT	CTCCCTGATC	GCTAAGCTGG	ACGCCACCGT	GTTTCGCTCAG
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CCAGCGTCAT	CTCCCTGATC	GCTAAGCTGG	ACGCCACCGT	GTTTCGCTCAG
	2410	2420	2430	2440	2450
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	ATCGTCAAGC	TGAGGAAGGT	GGACACTCTG	AAGCCAATCC	TGTACAAGAT
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	ATCGTCAAGC	TGAGGAAGGT	GGACACTCTG	AAGCCAATCC	TGTACAAGAT
	2460	2470	2480	2490	2500
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CAACTCTGAC	TCAAACGACT	TCTACCTGGT	GGCCAACCTAC	GACTGGATCC
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CAACTCTGAC	TCAAACGACT	TCTACCTGGT	GGCCAACCTAC	GACTGGATCC
	2510	2520	2530	2540	2550
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CTACCTCCAC	CACTAAGGTG	TACAAGCAGG	TCCCCAGCA	GTTTCGACTTC
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CTACCTCCAC	CACTAAGGTG	TACAAGCAGG	TCCCCAGCA	GTTTCGACTTC
	2560	2570	2580	2590	2600
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CGTGCTTCAA	TGCACATGCT	GACCTCCAAC	CTGACCTTCA	CTGTCTACTC
SA11_VP2_InsO_reference					
SA11_VP2_InsO_sequenced	CGTGCTTCAA	TGCACATGCT	GACCTCCAAC	CTGACCTTCA	CTGTCTACTC

	2610	2620	2630	2640	2650
					
SA11_VP2_InsO_reference	AGACCTGCTG	GCCTTCGTCT	CCGCTGACAC	TGTGGAGCCT	ATCAACGCTG
SA11_VP2_InsO_sequenced	AGACCTGCTG	GCCTTCGTCT	CCGCTGACAC	TGTGGAGCCT	ATCAACGCTG
	2660	2670	2680	2690	2700
					
SA11_VP2_InsO_reference	TGGCCTTCGA	CAACATGAGG	ATCATGAACG	AACTGTAA--	-----
SA11_VP2_InsO_sequenced	TGGCCTTCGA	CAACATGAGG	ATCATGAACG	AACTGTAAAGC	TAGCAGCTGA
	2710				
					
SA11_VP2_InsO_reference	-----	-----			
SA11_VP2_InsO_sequenced	TGCWWGARMY	YGCCCC			

SA11 VP6 insect cell codon optimised:

	10	20	30	40	50
					
SA11_VP6_InsO_reference	-----	-----	-----	-----	-----
SA11_VP6_InsO_sequenced	TTATTCTATC	CGTCCCACCA	TCGGGCGCGG	ATCCCGGTCC	GAAGCGCGCG
	60	70	80	90	100
					
SA11_VP6_InsO_reference	-----ATGG	ACGTCCTGTA	CTCTCTGTCA	AAGACCCTGA	AGGACGCTCG
SA11_VP6_InsO_sequenced	GAATTCTATG	ACGTCCTGTA	CTCTCTGTCA	AAGACCCTGA	AGGACGCTCG
	110	120	130	140	150
					
SA11_VP6_InsO_reference	CGACAAGATC	GTCGAGGGTA	CTCTGTACTC	TAACGTGTCA	GACCTGATCC
SA11_VP6_InsO_sequenced	CGACAAGATC	GTCGAGGGTA	CTCTGTASTC	TAACGTGTCA	GACCTGATCC
	160	170	180	190	200
					
SA11_VP6_InsO_reference	AGCAGTTCAA	CCAGATGATC	ATCACCATGA	ACGGTAACGA	GTTCCAGACT
SA11_VP6_InsO_sequenced	AGCMRTTCAA	CCAGATGATC	ATCACCATGA	ACGGTAACGA	GTTCCAGACT
	210	220	230	240	250
					
SA11_VP6_InsO_reference	GGTGGCATCG	GCAACCTGCC	CATCAGGAAC	TGGAACCTCA	ACTTCGGACT
SA11_VP6_InsO_sequenced	GGTGGCATCG	GCAACCTGCC	CATCAGGAAC	TGGAACCTCA	ACTTCGGACT
	260	270	280	290	300
					
SA11_VP6_InsO_reference	GCTGGGTACC	ACTCTGCTGA	ACCTGGACGC	TAACTACGTC	GAGACCGCCA
SA11_VP6_InsO_sequenced	GCTGGGTACC	ACTCTGCTGA	ACCTGGACGC	TAACTACGTC	GAGACCGCCA
	310	320	330	340	350
					
SA11_VP6_InsO_reference	GAAACACTAT	CGACTACTTC	GTGGACTTCG	TGGACAACGT	GTGCATGGAC
SA11_VP6_InsO_sequenced	GAAACACTAT	CGACTACTTC	GTGGACTTCG	TGGACAACGT	GTGCATGGAC
	360	370	380	390	400
					
SA11_VP6_InsO_reference	GAGATGGTGC	GCGAATCTCA	GCGTAACGGA	ATCGCCCCAC	AGTCTGACAG
SA11_VP6_InsO_sequenced	GAGATGGTGC	GCGAATCTCA	GCGTAACGGA	ATCGCCCCAC	AGTCTGACAG

	410	420	430	440	450
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	CCTGCGCAAG	CTGTTCAGCTA	TCAAGTTCAA	GCGTATCAAC	TTTCGACAACT
	460	470	480	490	500
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	CCAGCGAGTA	CATCGAAAAC	TGGAACCTGC	AGAACC GCCG	TCAGCGCACC
	510	520	530	540	550
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	GGATTCACTT	TCCACAAGCC	TAACATCTTC	CCCTACTCTG	CCTCATTCAC
	560	570	580	590	600
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	CCTGAACCGT	TCCCAGCCTG	CTCACGACAA	CCTGATGGGT	ACTATGTGGC
	610	620	630	640	650
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	TGAACGCTGG	CTCCGAAATC	CAGGTGGCCG	GATTTCGACTA	CAGCTGCGCT
	660	670	680	690	700
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	ATCAACGCTC	CAGCCAACAT	CCAGCAGTTC	GAGCACATCG	TCCCTCTGAG
	710	720	730	740	750
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	GAGAGTGCTG	ACCAC TGCCA	CCATCACTCT	GCTGCCTGAC	GCTGAAAGGT
	760	770	780	790	800
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	TCTCCTTCCC	CAGAGTCATC	AACAGCGCTG	ACGGTGCCAC	CAC TTGGTTC
	810	820	830	840	850
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	TTCAACCCCG	TGATCCTGAG	GCCAAACAAC	GTGGAGGTCG	AGTT CCTGCT
	860	870	880	890	900
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	GAACGGACAG	ATCATCAACA	CCTACCAGGC	CAGGTTCCGGT	ACTATCGTCG
	910	920	930	940	950
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	CTCGCAACTT	CGACACCATC	CGTCTGAGCT	TCCAGCTGAT	GAGACCTCCC
	960	970	980	990	1000
SA11_VP6_InsO_reference					
SA11_VP6_InsO_sequenced	AACATGACTC	CTGCTGTGGC	CGTCCTGTTC	CCAAACGCCC	AGCCTTTCGA

	1010	1020	1030	1040	1050
					
SA11_VP6_InsO_reference	GCACCACGCT	ACCGTGGGCC	TGACTCTGCG	CATCGAGTCT	GCCGTCTGCG
SA11_VP6_InsO_sequenced	GCACCACGCT	ACCGTGGGCC	TGACTCTGCG	CATCGAGTCT	GCCGTCTGCG
	1060	1070	1080	1090	1100
					
SA11_VP6_InsO_reference	AATCAGTGCT	GGCTGACGCC	TCTGAGACCC	TGCTGGCCAA	CGTCACTTCA
SA11_VP6_InsO_sequenced	AATCAGTGCT	GGCTGACGCC	TCTGAGACCC	TGCTGGCCAW	CGTCACTTCA
	1110	1120	1130	1140	1150
					
SA11_VP6_InsO_reference	GTGCGTCAGG	AATACGCTAT	CCCCGTCGGC	CCAGTGTTC	CACCTGGAAT
SA11_VP6_InsO_sequenced	GTGCGTCAGG	AATACGCTAT	CCCCGTCGGC	CCAGTGTTC	CACCTGGAAT
	1160	1170	1180	1190	1200
					
SA11_VP6_InsO_reference	GAAC TGGACC	GACCTGATCA	CTAACTACTC	CCCCAGCAGG	GAAGACAACC
SA11_VP6_InsO_sequenced	GAAC TGGACC	GACCTGATCA	CTAACTACTC	CCCCAGCAGG	GAAGACAACC
	1210	1220	1230	1240	1250
					
SA11_VP6_InsO_reference	TGCAGAGAGT	CTTCACCGTG	GCTTCCATCC	GCAGCATGCT	GATCAAGTAA
SA11_VP6_InsO_sequenced	TGCAGAGAGT	CTTCACCGTG	GCTTCCATCC	GCAGCATGCT	GATCAAGTAA
	1260	1270	1280	1290	1300
					
SA11_VP6_InsO_reference	-----	-----	-----	-----	-----
SA11_VP6_InsO_sequenced	GTCGACGAGC	TCACTAGTCG	CGGCCGCTTT	CGAATCTAGA	GCC TGC AKCT
	1310				
					
SA11_VP6_InsO_reference	-----				
SA11_VP6_InsO_sequenced	CSACTGCCCT				

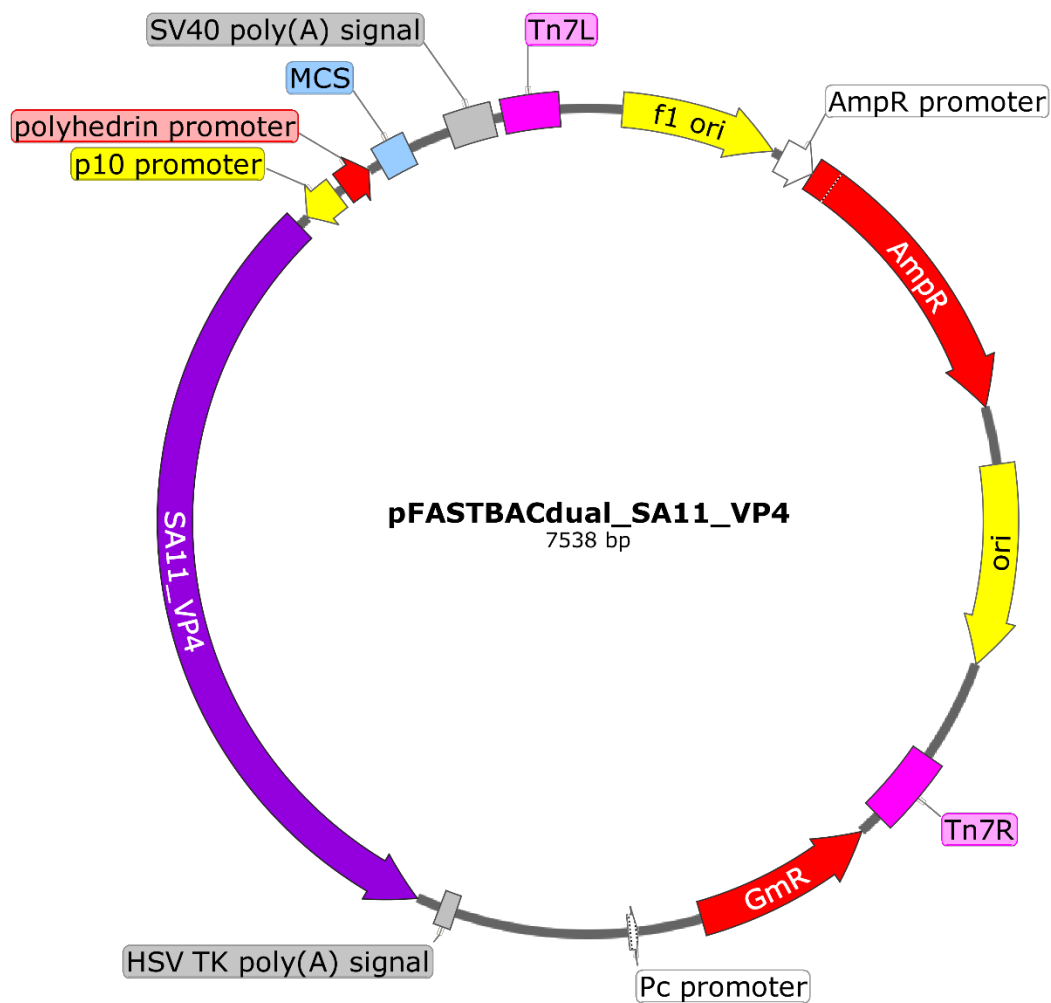


Figure 45: Map of pFASTBACdual_SA11_VP4

	10	20	30	40	50
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	TACAATCACT	CGACGAAGAC	TTGATCACCC	GGGATCTCGA	GATGGCATCC
	60	70	80	90	100
SA11_VP4_InsO_reference	CTCATCTACA	GACAGCTCTT	GACGAACAGT	TACACGGTTG	ACCTCTCCGA
SA11_VP4_InsO_sequenced	CTCATCTACA	GACAGCTCTT	GACGAACAGT	TACACGGTTG	ACCTCTCCGA
	110	120	130	140	150
SA11_VP4_InsO_reference	CGAAATCCAG	GAAATTGGCA	GCACAAAGTC	CCAGAACGTG	ACTATCAACC
SA11_VP4_InsO_sequenced	CGAAATCCAG	GAAATTGGCA	GCACAAAGTC	CCAGAACGTG	ACTATCAACC
	160	170	180	190	200
SA11_VP4_InsO_reference	CCGGACCATT	CGCTCAGACC	GGTTACGCC	CAGTGAAGTG	GGGACCTGGA
SA11_VP4_InsO_sequenced	CCGGACCATT	CGCTCAGACC	GGTTACGCC	CAGTGAAGTG	GGGACCTGGA

	210	220	230	240	250
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	GAGATCAACG	ACAGCACCAC	TGTCGAACCC	CTGCTGGACG	GTCCCTTACCA
SA11_VP4_InsO_sequenced	GAGATCAACG	ACAGCACCAC	TGTCGAACCC	CTGCTGGACG	GTCCCTTACCA
	260	270	280	290	300
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	GCCCATGACC	TTCAACCCTC	CCGTCTGACTA	CTGGATGCTG	CTGGCTCCAA
SA11_VP4_InsO_sequenced	GCCCATGACC	TTCAACCCTC	CCGTCTGACTA	CTGGATGCTG	CTGGCTCCAA
	310	320	330	340	350
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	CCACTCCTGG	TGTGATCGTC	GAGGGCACTA	ACAACACCGA	CCGTTGGCTG
SA11_VP4_InsO_sequenced	CCACTCCTGG	TGTGATCGTC	GAGGGCACTA	ACAACACCGA	CCGTTGGCTG
	360	370	380	390	400
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	GCCACCATCC	TGATCGAGCC	AAACGTGCAG	TCTGAAAACA	GGACCTACAC
SA11_VP4_InsO_sequenced	GCCACCATCC	TGATCGAGCC	AAACGTGCAG	TCTGAAAACA	GGACCTACAC
	410	420	430	440	450
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	TATCTTCGGC	ATCCAGGAAC	AGCTGACCGT	CAGCAACACT	TCTCAGGACC
SA11_VP4_InsO_sequenced	TATCTTCGGC	ATCCAGGAAC	AGCTGACCGT	CAGCAACACT	TCTCAGGACC
	460	470	480	490	500
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	AGTGGAAGTT	CATCGACGTG	GTCAAGACCA	CTGCTAACGG	CTCAATCGGA
SA11_VP4_InsO_sequenced	AGTGGAAGTT	CATCGACGTG	GTCAAGACCA	CTGCTAACGG	CTCAATCGGA
	510	520	530	540	550
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	CAGTACGGTT	CCCTGCTGTC	CAGCCCCAAG	CTGTACGCCG	TGATGAAGCA
SA11_VP4_InsO_sequenced	CAGTACGGTT	CCCTGCTGTC	CAGCCCCAAG	CTGTACGCCG	TGATGAAGCA
	560	570	580	590	600
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	CAACGAGAAG	CTGTACACCT	ACGAGGGTCA	GACTCCAAAC	GCTAGGACCG
SA11_VP4_InsO_sequenced	CAACGAGAAG	CTGTACACCT	ACGAGGGTCA	GACTCCAAAC	GCTAGGACCG
	610	620	630	640	650
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	GCCACTACAG	CACCACTAAC	TACGACTCTG	TCAACATGAC	TGCCTTCTGC
SA11_VP4_InsO_sequenced	GCCACTACAG	CACCACTAAC	TACGACTCTG	TCAACATGAC	TGCCTTCTGC
	660	670	680	690	700
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	GACTTCTACA	TCATCCCTAG	ATCAGAGGAA	TCCAAGTGCA	CCGAGTACAT
SA11_VP4_InsO_sequenced	GACTTCTACA	TCATCCCTAG	ATCAGAGGAA	TCCAAGTGCA	CCGAGTACAT
	710	720	730	740	750
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	CAACAACGGA	CTGCCACCTA	TCCAGAACAC	TCGCAACGTG	GTCCCCCTGT
SA11_VP4_InsO_sequenced	CAACAACGGA	CTGCCACCTA	TCCAGAACAC	TCGCAACGTG	GTCCCCCTGT
	760	770	780	790	800
SA11_VP4_InsO_reference					
SA11_VP4_InsO_reference	CCCTGACCGC	TCGTGACGTG	ATCCACTACA	GGGCTCAGGC	CAACGAGGAC
SA11_VP4_InsO_sequenced	CCCTGACCGC	TCGTGACGTG	ATCCACTACA	GGGCTCAGGC	CAACGAGGAC

	810	820	830	840	850
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	ATCGTCATCA	GCAAGACCTC	TCTGTGGAAG	GAAATGCAGT	ACAACAGAGA
	860	870	880	890	900
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CATCACTATC	CGCTTCAAGT	TCGCCAACAC	CATCATCAAG	TCTGGTGGCC
	910	920	930	940	950
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	TGGGTTACAA	GTGGTCAGAG	ATCTCCTTCA	AGCCAGCTAA	CTACCAGTAC
	960	970	980	990	1000
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	ACCTACACTC	GCGACGGCGA	GGAAGTGACT	GCCCACACCA	CTTGCTCAGT
	1010	1020	1030	1040	1050
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	GAACGGAGTC	AACGACTTCA	GCTTCAACGG	AGGTTCTCTG	CCTACCGACT
	1060	1070	1080	1090	1100
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	TCGTGGTGTC	CAAGTTCGAG	GTCATCAAGG	AAAACAGCTA	CGTCTACATC
	1110	1120	1130	1140	1150
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	GACTACTGGG	ACGACTCCCA	GGCTTTCAGA	AACGTGATGT	ACGTCCGCTC
	1160	1170	1180	1190	1200
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CCTGGCTGCC	AACCTGAACA	GCGTCATGTG	CACTGGCGGA	TCATACAACF
	1210	1220	1230	1240	1250
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	TCTCCCTGCC	TGTGGGACAG	TGGCCTGTCC	TGACCGGTGG	CGCTGTGAGC
	1260	1270	1280	1290	1300
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CTGCACTCTG	CTGGTGTAC	TCTGAGCACC	CAGTTCACTG	ACTTCGTGTC
	1310	1320	1330	1340	1350
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	ACTGAACFCC	CTGAGGTTCA	GATTCCGCTT	GGCCGTCGAG	GAACCACTCT
	1360	1370	1380	1390	1400
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	TCAAGCTGAC	CCGTACTAGG	CTGGACAGGC	TGTACGGCCT	GCCTGCTGCT

	1410	1420	1430	1440	1450
					
SA11_VP4_InsO_reference	GACCCAAACA	ACGGAAAGGA	GTA ~~CT~~ ACGAA	ATCGCTGGAA	GGTTCAGCCT
SA11_VP4_InsO_sequenced	GACCCAAACA	ACGGAAAGGA	GTA ~~CT~~ ACGAA	ATCGCTGGAA	GGTTCAGCCT
	1460	1470	1480	1490	1500
					
SA11_VP4_InsO_reference	GATCTCTCTG	GTGCCTTCTA	ACGACGACTA	CCAGACTCCC	ATCGCCAAC ~~T~~
SA11_VP4_InsO_sequenced	GATCTCTCTG	GTGCCTTCTA	ACGACGACTA	CCAGACTCCC	ATCGCCAAC ~~T~~
	1510	1520	1530	1540	1550
					
SA11_VP4_InsO_reference	CAGTGACCGT	CCGTCAGGAC	CTGGAGAGGC	AGCTGGGAGA	ACTGAGAGAG
SA11_VP4_InsO_sequenced	CAGTGACCGT	CCGTCAGGAC	CTGGAGAGGC	AGCTGGGAGA	ACTGAGAGAG
	1560	1570	1580	1590	1600
					
SA11_VP4_InsO_reference	GAATTC AACG	CTCTGTCACA	GGAGATCGCC	ATGTCC CAGC	TGATCGACCT
SA11_VP4_InsO_sequenced	GAATTC AACG	CTCTGTCACA	GGAGATCGCC	ATGTCC CAGC	TGATCGACCT
	1610	1620	1630	1640	1650
					
SA11_VP4_InsO_reference	GGCTCTGCTG	CCTCTGGACA	TGTTCTCAAT	GTTCTCCGGA	ATCAAGTCTA
SA11_VP4_InsO_sequenced	GGCTCTGCTG	CCTCTGGACA	TGTTCTCAAT	GTTCTCCGGA	ATCAAGTCTA
	1660	1670	1680	1690	1700
					
SA11_VP4_InsO_reference	CTATCGACGC	TGCCAAGTCA	ATGGCTACCA	ACGTGATGAA	GAAGTTC AAG
SA11_VP4_InsO_sequenced	CTATCGACGC	TGCCAAGTCA	ATGGCTACCA	ACGTGATGAA	GAAGTTC AAG
	1710	1720	1730	1740	1750
					
SA11_VP4_InsO_reference	AAGTCCGGTC	TGGCCAACAG	CGTCTCTACC	CTGACTGACT	CACTGTCCGA
SA11_VP4_InsO_sequenced	AAGTCCGGTC	TGGCCAACAG	CGTCTCTACC	CTGACTGACT	CACTGTCCGA
	1760	1770	1780	1790	1800
					
SA11_VP4_InsO_reference	CGCTGCCTCT	TCAATCAGCA	GAGGCTCCAG	CATCCGCAGC	ATCGGATCTT
SA11_VP4_InsO_sequenced	CGCTGCCTCT	TCAATCAGCA	GAGGCTCCAG	CATCCGCAGC	ATCGGATCTT
	1810	1820	1830	1840	1850
					
SA11_VP4_InsO_reference	CAGCTTCTGC	CTGGACCGAC	GTGTCAACCC	AGATCACTGA	CATCTCCAGC
SA11_VP4_InsO_sequenced	CAGCTTCTGC	CTGGACCGAC	GTGTCAACCC	AGATCACTGA	CATCTCCAGC
	1860	1870	1880	1890	1900
					
SA11_VP4_InsO_reference	TCTGTGTCAT	CCGTCTCCAC	CCAGACTAGC	ACCATCTCTC	GCCGTCTGCG
SA11_VP4_InsO_sequenced	TCTGTGTCAT	CCGTCTCCAC	CCAGACTAGC	ACCATCTCTC	GCCGTCTGCG
	1910	1920	1930	1940	1950
					
SA11_VP4_InsO_reference	TCTGAAGGAG	ATGGCTACTC	AGACCGAAGG	CATGAAC TTC	GACGACATCT
SA11_VP4_InsO_sequenced	TCTGAAGGAG	ATGGCTACTC	AGACCGAAGG	CATGAAC TTC	GACGACATCT
	1960	1970	1980	1990	2000
					
SA11_VP4_InsO_reference	CTGCTGCCGT	GCTGAAGACT	AAGATCGACA	AGAGCACCCA	GATCTCTCCT
SA11_VP4_InsO_sequenced	CTGCTGCCGT	GCTGAAGACT	AAGATCGACA	AGAGCACCCA	GATCTCTCCT

	2010	2020	2030	2040	2050
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	AACACTATCC	CCGACATCGT	CACCGAGGCT	TCAGAAAAGT	TCATCCCTAA
	2060	2070	2080	2090	2100
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CAGAGCCTAC	CGCGTGATCA	ACAACGACGA	CGTCTTCGAG	GCTGGCATCG
	2110	2120	2130	2140	2150
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	ACGGAAGTT	CTTCGCCTAC	AAGGTGGACA	CTTTCGAGGA	AATCCCATTC
	2160	2170	2180	2190	2200
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	GACGTCCAGA	AGTTCGCTGA	CCTGGTGACC	GACTCACCTG	TCATCTCCGC
	2210	2220	2230	2240	2250
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CATCATCGAC	TTCAAGACTC	TGAAGAACCT	GAACGACAAC	TACGGTATCA
	2260	2270	2280	2290	2300
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CCAAGCAGCA	GGCTTTCAAC	CTGCTGCGTA	GCGACCCCCG	TGTGCTGAGG
	2310	2320	2330	2340	2350
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	GAATTCATCA	ACCAGGACAA	CCCTATCATC	CGTAACCGTA	TTGAACAAC
	2360	2370	2380	2390	
SA11_VP4_InsO_reference					
SA11_VP4_InsO_sequenced	CATTATGCAG	TGCCGCCTCT	AA		

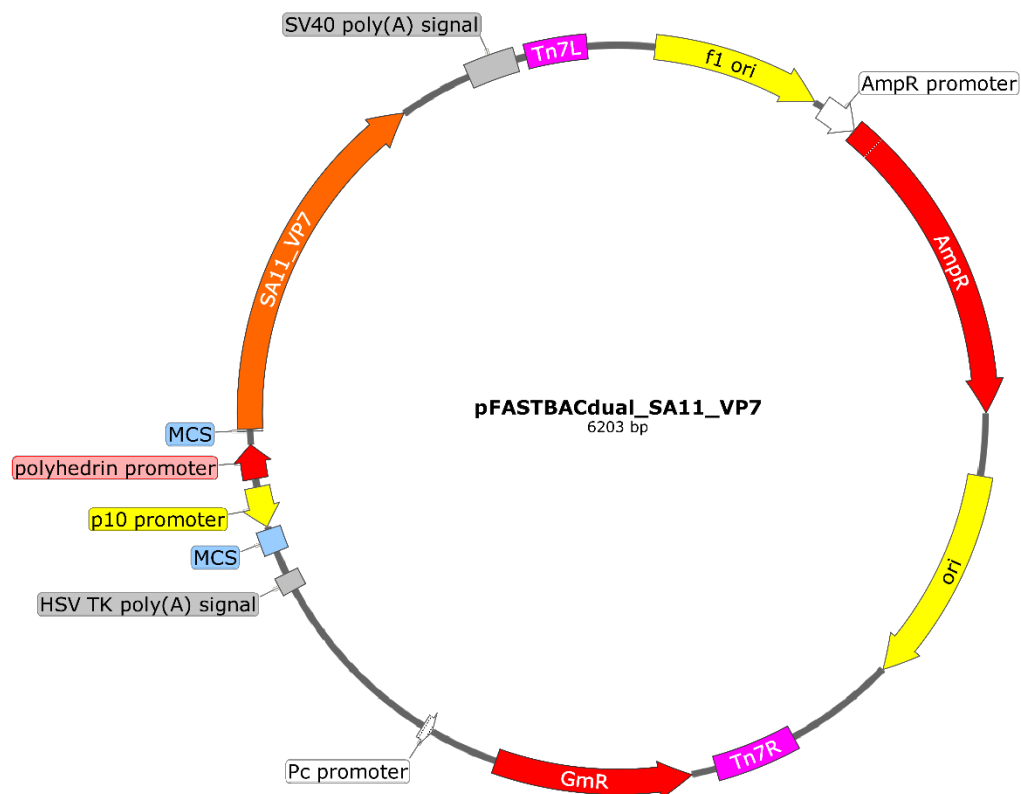


Figure 46: Map of pFASTBACdual_SA11_VP7

	10	20	30	40	50
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					
	60	70	80	90	100
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					
	110	120	130	140	150
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					
	160	170	180	190	200
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					
	210	220	230	240	250
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					
	260	270	280	290	300
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced					

	310	320	330	340	350
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	AACGACAAC	CCTGGAAGGA	CACCCGTGAGC	CAGCTGTTCC	TGACCAAGGG
	360	370	380	390	400
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	CTGGCCCACT	GGTTCCGTGT	ACTTCAAGGA	GTACACTAAC	ATCGCCTCTT
	410	420	430	440	450
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	TCTCAGTCGA	CCCACAGCTG	TACTGCGACT	ACAACGTGGT	CCTGATGAAG
	460	470	480	490	500
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	TACGACGCTA	CCCTGCAGCT	GGACATGTCC	GAGCTGGCCG	ACCTGATCCT
	510	520	530	540	550
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	GAACGAATGG	CTGTGCAACC	CTATGGACAT	CACCCTGTAC	TACTACCAGC
	560	570	580	590	600
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	AGACTGACGA	GGCTAACAAAG	TGGATCAGCA	TGGGCTCCAG	CTGCACATATC
	610	620	630	640	650
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	AAGGTGTGCC	CCCTGAACAC	CCAGACTCTG	GGAATCGGTT	GCCTGACCAC
	660	670	680	690	700
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	TGACGCCACC	ACTTTCGAGG	AAGTGGCTAC	CGCCGAAAAG	CTGGTTCATCA
	710	720	730	740	750
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	CTGACGTGGT	CGACGGAGTG	AACCACAAGC	TGGACGTCAC	CACCTGCTACC
	760	770	780	790	800
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	TGCACTATCA	GGAACGTCAA	GAAGCTGGGT	CCTAGAGAGA	ACGTGGCCGT
	810	820	830	840	850
SA11_VP7_InsO_reference					
SA11_VP7_InsO_sequenced	CATCCAGGTC	GGTGGCAGCG	ACATCCTGGA	CATCACCCTG	GACCCATACCA

	860	870	880	890	900
					
SA11_VP7_InsO_reference	CTGCTCCTCA	GACTGAACGC	ATGATGCGTA	TCAACTGGAA	GAAGTGGTGG
SA11_VP7_InsO_sequenced	CTGCTCCTCA	GACTGAACGC	ATGATGCGTA	TCAACTGGAA	GAAGTGGTGG
	910	920	930	940	950
					
SA11_VP7_InsO_reference	CAGGTGTTCT	ACACCGTGGT	CGACTACGTC	GACCAGATCA	TCCAAGTGAT
SA11_VP7_InsO_sequenced	CAGGTGTTCT	ACACCGTGGT	CGACTACGTC	GACCAGATCA	TCCAAGTGAT
	960	970	980	990	1000
					
SA11_VP7_InsO_reference	GTCAAAACGC	AGCAGGAGCC	TCAACTCAGC	AGCCTTCTAC	TACCGTGTCT
SA11_VP7_InsO_sequenced	GTCAAAACGC	AGCAGGAGCC	TCAACTCAGC	AGCCTTCTAC	TACCGTGTCT
	1010	1020			
					
SA11_VP7_InsO_reference	AA-----	-----	-----		
SA11_VP7_InsO_sequenced	AACYARCGAC	CYACCTTCTC	GCATT		

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Appendix D: Conference outputs

- MGJ Huyzers, **LJC Theart**, AA van Dijk. Bacterial expression of mature rotavirus protein 7. 11th African Rotavirus Symposium in Lilongwe, Malawi from the 28 – 30 of May 2017.
- **LJC Theart**, MGJ Huyzers, AA van Dijk. Bacterial expression of a truncated rotavirus protein 7 of a South African G9 strain. 7th European Rotavirus Biology Meeting (ERBM) in Cork, Ireland, June 18 – 21 2017.
- **LJC Theart**, AA van Dijk. Developing SA11 viral protein 2 and viral protein 6 double-layered particle as a universal backbone for chimeric particles. SASBMB – FASBMB conference, Potchefstroom 8 – 11 July 2018.
- MGJ Huyzers, AC Potgieter, **LJC Theart** and AA van Dijk. Local implementation and optimisation of entirely plasmid-based SA11 rotavirus reverse genetics. 13th International Double-Stranded RNA Virus Symposium, Houffalize, Belgium, 24 – 28 September 2018.
- **LJC Theart**, P Le Roux, AA van Dijk. Developing a SA11 DLP as a universal backbone for chimeric TLPs. 12th African Rotavirus Symposium, Johannesburg, 30 July – 1 August 2019.