HOMOLOGOUS RECOMBINATION-MEDIATED INSERTION OF ANTI-HBV CRISPR/CAS9-ENCODING SEQUENCES INTO THE HELPER-DEPENDENT ADENOVIRAL VECTOR GENOME

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DECLARATION

I, Tasneem Farhad, declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

01/07/2024

(Signature of candidate) Date

PRESENTATIONS AND PUBLICATIONS

- **T. Farhad, P. Arbuthnot, M. B. Maepa.** Homologous recombination-mediated insertion of transgenes into the helper-dependent adenoviral vector genome. Molecular Bioscience Research Thrust (MBRT) Research Day 2022, University of the Witwatersrand. 3rd Prize poster presentation
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ABSTRACT

Hepatitis B is caused by the hepatitis B virus (HBV), which maintains a stable DNA genome in infected hepatocytes that prolongs infection. Current treatments serve only to reduce the effects of the infection, but do not remove the viral genome from infected cells. As such, gene therapy approaches have been explored to target and eradicate the HBV genome from infected cells. Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) has been shown to be effective against HBV, but it is difficult to efficiently deliver these sequences to targeted cells. Viral vectors like adenoviruses are often used as delivery vehicles for gene therapy. Helper-dependent adenoviral vectors (HDAdVs) are widely used, and though they are well suited to target hepatocytes, they have a large genome that is difficult to insert transgenes into. The aim of this study was to design a system to efficiently insert transgenes into the HDAdV genome through homologous recombination with an anti-HBV CRISPR/Cas9-expressing shuttle plasmid.

An anti-HBV CRISPR/Cas9 sequence and a nanoLuciferase (nLuc) reporter gene was inserted into a previously constructed shuttle plasmid, that was designed to include two regions that are homologous to regions within the HDAdV genome. Each transgene-expressing shuttle was used with a plasmid expressing the HDAdV genome to co-transform *recA* expressing *Escherichia coli* (*E. coli*) cells, wherein homologous recombination between the two regions of homology was induced. However, restriction analysis of the resultant recombinants indicated significant instability that hindered their characterisation. A low copy *ori* and a 400 bp fragment of the adenovirus genome associated with increased vector stability was inserted into the shuttle and recombination between this nLuc-expressing shuttle and the HDAdV genome resulted in stable recombinants. These, however, did not include the transgene. This indicated that despite the improved stability of the recombinants, unintended recombination events still occurred that excluded the transgene from the final product. As such, further work must be done to insert the transgene elsewhere in the shuttle to ensure that recombination events include the transgene in the final recombinant. The implications of this work are that one the biggest drawbacks of HDAdV application can be improved to streamline the process and potentially expand on the future use of HDAdVs in gene therapy.

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TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
AdV	adenoviral vector
ADP	adenovirus death protein
ALT	alanine aminotransferase
apri-miRNA	artificial pri-miRNA
ASO	antisense oligonucleotide
CAR	coxsackie adenovirus receptor
cccDNA	covalently closed circular DNA
Chi	crossover hotspot instigator
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats/ CRISPR associated protein 9
crRNA	CRISPR RNA
dCas9	dead Cas9
dsDNA	double-stranded DNA
DMEM	Dulbecco's modified Eagles medium
dUTPase	dUTP pyrophophatase
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FBS	foetal bovine serum
gRNA	guide RNA

HBcAg	hepatitis B virus core antigen
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HBx	hepatitis B virus X protein
HDAdV	helper-dependent adenoviral vector
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HPRT	human hypoxanthine-guanine phosphoriboslytransferase
HPV	human papillomavirus
HSV-1	herpes simplex virus 1
HV	helper virus
ITR	inverted terminal repeat
KanR	kanamycin resistance gene
LB	Luria Bertani
LHB	large HBV surface antigen
LNP	lipid nanoparticle
MAR	matrix attachment region
MHB	medium HBV surface antigen
MHC	major histocompatibility complex
miRNA	microRNA
MSP-1	merozoite surface protein 1
nLuc	NanoLuc® luciferase reporter gene

OD	optical density
ORF	open reading frame
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PEG	polyethylene glycol
pgRNA	pregenomic RNA
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
MAR	matrix attachment region
MHC	major histocompatibility complex
RID	receptor internalisation and degradation protein
RNAi	RNA interference
saCas9	*Staphylococcus aureus* Cas9
SEM	standard error of the mean
sgRNA	single guide RNA
SHB	small HBV surface antigen
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOC	Super Optimal broth with Catabolite repression
spCas9	*Streptococcus pyogenes* Cas9
ssDNA	single-stranded DNA
TAE	Tris-Acetate-EDTA
TALEN	transcription activator-like effector nuclease

LIST OF ABBREVIATIONS

tracrRNA	trans-activating CRISPR RNA
VA	virus-associated
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZFN	zinc-finger nuclease

CHAPTER 1

1. INTRODUCTION

1.1 Hepatitis B

In 2019, approximately 1.5 million people were infected with hepatitis B virus (HBV) worldwide, leading to approximately 820 000 deaths that year (World Health Organization, 2021). HBV is a hepatotropic virus that causes both acute and chronic hepatitis B which may lead to cirrhosis and liver cancer. HBV infection is prevalent in the Western Pacific region and in Africa, where the disease is spread through perinatal transmission or exposure to infected blood or bodily fluids (World Health Organization, 2017). HBV is a member of the *Hepadnaviridae* family and is a non-cytopathic DNA virus with a 3.2 kb relaxed circular genome encapsulated by an outer envelope with embedded surface proteins and an inner nucleocapsid core (Figure 1.1A). The genome contains four partially overlapping open reading frames (ORFs) named *P* (*polymerase*), *S* (*surface*), *C* (*core*), and *X* (*HBx*). The *P* ORF encodes for the HBV polymerase enzyme (Pol) and the *S* ORF encodes for the three HBV surface antigens (HBsAg): namely, small (SHBs), medium (MHBs), and large (LHBs) surface antigens. The *C* ORF encodes the core (HBcAg) and the e (HBeAg) antigens, while the *X* ORF encodes the HBx protein (Figure 1.1B) (Seto et al., 2018; Yuen et al., 2018). Upon infection of hepatocytes with HBV through binding to the sodium taurocholate cotransporting polypeptide (NTCP) receptor (Yan et al., 2012), the relaxed circular viral genome (rcDNA) is converted to covalently closed circular DNA (cccDNA) by the host cells' DNA repair machinery. This cccDNA remains in the nucleus of infected cells and serves as the template for viral DNA replication while contributing to the longevity of HBV infection (Fanning et al., 2019).

In an infected individual, the main viral antigens found in the blood are HBeAg and HBsAg. There are various phases of HBV infection that are characterised by serum levels of HBV DNA, HBsAg, HBeAg, and alanine aminotransferase (ALT). The initial phase following infection is referred to as the HBeAg-positive chronic infection, where there is a high level of serum HBV DNA and HBsAg, but little to no liver damage and normal levels of serum ALT (Seto et al., 2018). The second phase is the HBeAg-positive chronic hepatitis phase, which has decreasing levels of serum

HBV DNA and HBsAg with increased HBeAg seroconversion, but more liver damage in the form of inflammation and fibrosis, and higher levels of serum ALT (Dusheiko et al., 2023). The third phase is the HBeAg-negative chronic infection phase, which has normal levels of serum ALT, low serum HBsAg, and low to undetectable levels of HBV DNA. This phase then progresses to the fourth phase, the HBeAg-negative chronic hepatitis phase, where there are fluctuating levels of serum HBV DNA and ALT (Dusheiko et al., 2023; Seto et al., 2018).

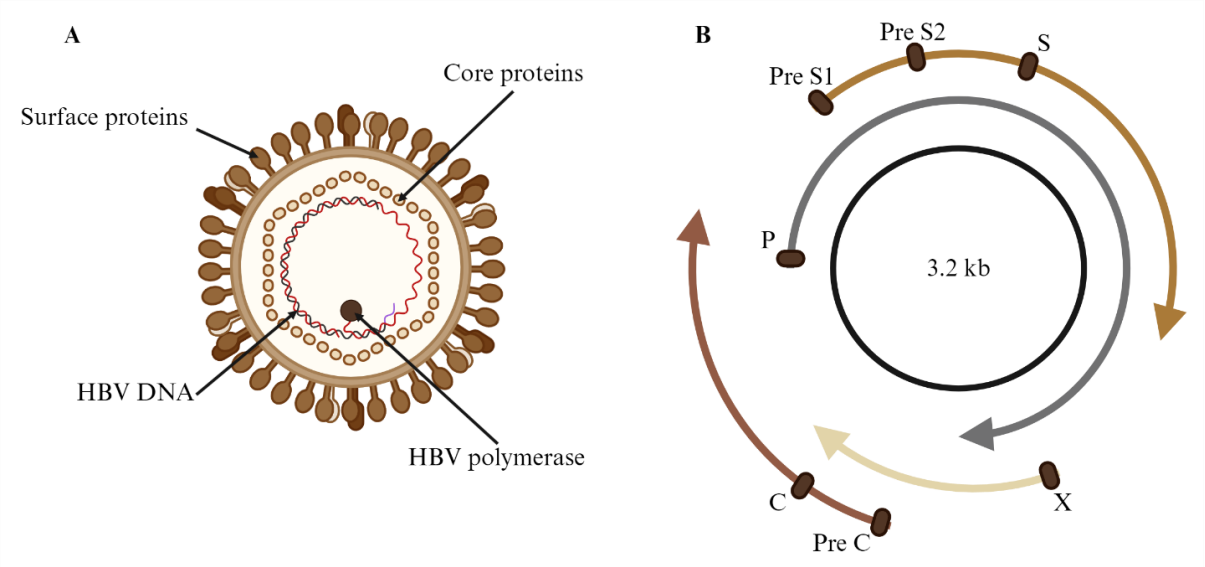


Figure 1.1: Hepatitis B virus particle and genome.

*Schematic representation of (**A**) the HBV particle and (**B**) the HBV genome with the four ORFs indicated by arrows and the start codons for the different encoded proteins indicated by oval shapes (Created with Biorender.com).*

The primary means of preventing HBV-related disease is by vaccination. The current vaccine regimen against HBV uses a three-dose vaccine series, the first of which is administered to infants soon after birth, followed by two or three booster shots a few weeks later (World Health Organization, 2017; Yuen et al., 2018). This vaccine is made of recombinant HBsAg that is generally produced in a yeast cell line (Kao, 2015). The recombinant HBsAg vaccine is known to successfully induce a protective effect similar to the first HBV vaccine produced, a plasma-derived HBsAg vaccine that was limited in its use due to the cost of production (Kao, 2015; Van Den Ende et al., 2017). Although this is an effective vaccine strategy against the disease, the recombinant HBsAg protein vaccine is not as effective in individuals older than 40 years and those that are

immunocompromised (Tian et al., 2021; Van Den Ende et al., 2017). Approximately 10% of adults vaccinated are unable to mount an effective immune response to HBV, which has been associated with a variety of factors including age, BMI, concomitant diseases, and lifestyle habits such as smoking and alcohol consumption (David et al., 2015; Yang et al., 2016; Yuen et al., 2018). In these cases, it has been found that there is a reduced level of T cell activation or a lack of recognition of the HBsAg by T cells (Desombere et al., 2000; Yuen et al., 2018). Considering these limitations, HBV infection remains a major health concern in many parts of the world, and the effects of chronic HBV infections must be lessened more effectively.

1.1.1 Anti-HBV treatment

The ideal endpoint for HBV treatment would be a so-called complete cure; the total removal of HBV cccDNA, silencing of any integrated viral genes, and a lack of any disease symptoms (Dusheiko et al., 2023; Seto et al., 2018). This, however, is currently difficult to achieve as it is difficult to target cccDNA. Therefore, a more achievable endpoint of treatment is referred to as a functional cure of HBV; a complete clearance of HBsAg in the blood, no detectable HBV DNA at the end of treatment, no disease-associated immune response, and no further spread of the disease (Dusheiko et al., 2023; Revill et al., 2019; Seto et al., 2018). This can be achieved using oral nucleoside or nucleotide analogues that inhibit viral DNA replication, including entecavir and tenofovir as first-line agents, along with other antivirals such as lamivudine, adefovir, and telbivudine (Seto et al., 2018; Yuen et al., 2018). The use of interferon alfa and pegylated interferon alfa has also been shown to be an effective first-line treatment for chronic HBV infections. This immunomodulatory approach uses weekly subcutaneous injections to induce adaptive immunity against HBV as well as using the inherent antiviral activity of interferon alfa to elicit an innate immune response against HBV (Fanning et al., 2019; Yuen et al., 2018). These HBV treatments serve to mitigate symptoms of the disease by suppressing HBV replication and preventing long-term liver damage, but do not clear the infection. Failure of the current treatments to target and eliminate the persistent cccDNA results in relapse following treatment (Fanning et al., 2019).

As such, various novel approaches are being investigated to target various points in the HBV replication cycle (Figure 1.2). HBV entry inhibitors such as bulevirtide (Volz et al., 2013), ezetimibe (Lucifora et al., 2013), and cyclosporin derivatives (Shimura et al., 2017; Watashi et al., 2014) are examples of agents that aim to prevent the entry of HBV into hepatocytes and therefore

halt disease progression (Fanning et al., 2019). Small molecules that act as capsid assembly modulators that disrupt capsid formation and encapsidation of genomic HBV DNA, and inhibit the release of HBV particles from infected cells (Cole, 2016; Nijampatnam and Liotta, 2019) have been shown to transiently reduce serum HBV DNA, but do not affect the expression of HBsAg in infected cells (Dusheiko et al., 2023). Nucleic acid polymers such as REP 2139 and REP 2165 have also been investigated as anti-HBV agents where they prevent the trafficking of subviral HBV particles (Real et al., 2017). Immunomodulatory approaches have also been used in attempts to boost HBV-specific cell mediated immune responses in conjunction with other forms of therapy (Bertoletti and Le Bert, 2018; Wu and Ning, 2017). These therapies do not eliminate HBV cccDNA, commonly requires life-long administration, and it is likely that a combination of agents may be necessary for complete clearance of HBV in infected cells. Gene editing is therefore an attractive approach in designing therapeutics with long-term activity and curative effects against HBV.

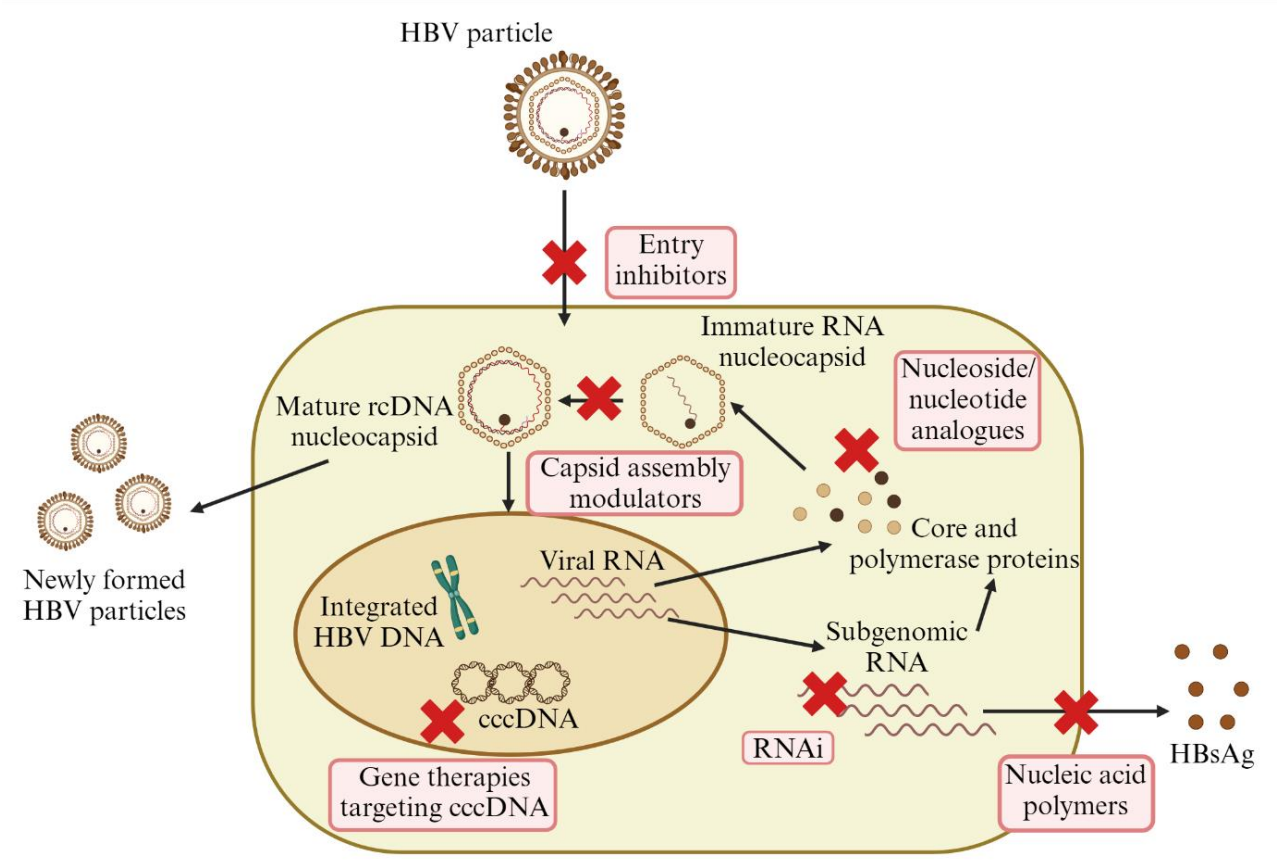


Figure 11.2: The HBV replication cycle and the mechanisms of HBV therapies.
HBV particles bind to NTCP receptors of hepatocytes via the HBsAg and enter the cell through receptor-mediated endocytosis. The envelope is removed and the nucleocapsid releases the HBV genome into the nucleus. Host DNA repair mechanisms convert the rcDNA to cccDNA from which HBV mRNA and pregenomic RNA (pgRNA), which binds to HBV polymerase, is transcribed. The viral mRNA encodes capsid proteins that package the pgRNA, which is reverse-transcribed and assembled into mature nucleocapsid particles that are released from the infected cell. Indicated in red crosses are the various points in the HBV lifecycle where different therapies act to treat HBV infection (Created with Biorender.com).

1.2 Gene therapy tools against HBV

Gene therapy involves the use of nucleic acids to treat disease (Verma and Weitzman, 2005). One of the most basic examples of gene therapy uses RNA interference (RNAi) to silence gene expression. In the RNAi pathway, small RNA molecules like microRNA (miRNA) regulate gene expression. miRNA encoding genes are first transcribed to produce primary miRNA (pri-miRNA) that are processed into precursor miRNA (pre-miRNA). This is then exported out of the cell nucleus and into the cytoplasm where it is further processed into mature miRNA duplexes. A single strand of these duplexes is then used as a guide that targets a complementary RNA molecule to degrade it or inhibit its translation. This pathway has been manipulated using synthetic short interfering RNAs (siRNAs) that can target specific sequences to inhibit translation from disease-associated RNAs (Agrawal et al., 2003). Short hairpin RNA (shRNA) and artificial pri-miRNA (apri-miRNA) have also been used to mimic pre-miRNA and pri-miRNA to target specific RNA sequences respectively (Li et al., 2019; Rao et al., 2009; Torrecilla et al., 2016; F. van den Berg et al., 2020; F. T. van den Berg et al., 2020).

There have been several promising RNAi-based gene therapies that have been developed against HBV infections. These include the siRNAs ARC-520 (NCT02065336) (Wooddell et al., 2017; Yuen et al., 2020), ARB-001467 (NCT02621096), and ALN-HBV (NCT02826018) that have reached Phase 2 clinical trials. shRNAs and apri-miRNA expression cassettes have also been used to target key overlaps in the HBV genome and subsequently silence HBV gene expression (Carmona et al., 2006; Crowther et al., 2008; Ely et al., 2009, 2008; Maepa et al., 2017; Mowa et al., 2014, 2012). However, this approach still does not target cccDNA directly and might have the

same limitations as the current therapies. Gene editing offers an attractive option for the treatment of a chronic HBV infection by targeting both cccDNA and integrated HBV DNA in host cells.

A versatile gene editing tool discovered in 1985 is the zinc finger nuclease (ZFN) (Klug, 2010). ZFNs use two domains to cleave target DNA; paired zinc finger protein domains of three to six zinc fingers that bind to between 9 and 18 bp of DNA, and paired nuclease domains from the restriction enzyme *Fok* I which are used to induce double-stranded breaks in DNA that is targeted by the paired zinc finger domains (Carlson et al., 2012; Negi et al., 2023). Since their discovery, ZFNs have been widely studied as a means of correcting disease-causing DNA sequences including in a phase 1 clinical trial in the treatment of haemophilia (NCT02695160), and two phase 1 safety trials using a T-cell product where the *CCR5* gene that encodes the HIV receptor on T cells was disrupted using ZFNs (NCT01044654 and NCT00842634) (Klug, 2010; Schacker and Seimetz, 2019; Urnov et al., 2010). A few studies have developed ZFNs against HBV and demonstrated cccDNA targeting and reduction in HBV replication markers (Cradick et al., 2010; Weber et al., 2014; Zhao et al., 2013). However, ZFNs have become less widely used compared to other gene editors due to the complex assembly requirements to produce them (Bloom et al., 2018).

Another gene editing tool is the transcription activator-like effector nuclease (TALEN). TALENs also use two binding domains from a TALE protein to target 15 to 20 bp of target DNA, which is then cleaved by heterodimeric *Fok* I nuclease domains (Wu and Zhang, 2023). It has been widely studied to eliminate genetic causes of sickle cell disease (Zarghamian et al., 2023), cancer (Bonini et al., 2023), and human papillomavirus (HPV) infection (Hu et al., 2015), to name a few, with clinical trials to investigate their efficacy against HPV-related malignant neoplasm and acute myeloid leukaemia (Schacker and Seimetz, 2019). TALENs have been used to target the *S* and *C* ORFs (Bloom et al., 2013), and the *P* and *C* ORFs (Chen et al., 2014) in the HBV genome and demonstrated significant reduction in viral replication both *in vitro* and *in vivo.*

Despite the above methods of gene editing having proven their usefulness, one of the most popular gene editing tools in recent years is clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) system. ZFNs and TALENs have been associated with lower efficiency, greater off-target effects, and higher costs of production than CRISPR/Cas9-based gene editing (Khalil, 2020; Wu and Zhang, 2023).

1.2.1 CRISPR/Cas9 system

The CRISPR/Cas system is a prokaryotic adaptive defence mechanism used to protect against invading pathogens like phages and plasmids (Makarova et al., 2006). Upon initial infection by a pathogen, foreign DNA is inserted into a so-called CRISPR array in the host bacterial genome (Barrangou et al., 2007; Garneau et al., 2010). This CRISPR sequence refers to a series of repeated sequences of DNA that are separated by stretches of foreign DNA called spacers. These unique spacer sequences act as a record of previously invading pathogens that can be transcribed by the bacteria to produce CRISPR RNA (crRNA). This can be used to guide a Cas protein to cleave the complementary sequence present in a newly invading pathogen (Garneau et al., 2010) (Figure 1.3). The Cas9 protein uses a small RNA molecule transcribed from the sequence upstream of the CRISPR array and *Cas9* gene, called trans-activating CRISPR RNA (tracrRNA), as a scaffold to link to the crRNA (Deltcheva et al., 2011; Wiedenheft et al., 2012). Generally, when taken together, crRNA and tracrRNA are referred to as the guide RNA (gRNA), or single guide RNA (sgRNA) (Doudna and Charpentier, 2014; Jinek et al., 2012). A critical component of the CRISPR/Cas system is the protospacer adjacent motif (PAM). This is a sequence within the viral genome that is used by the Cas protein to identify and bind to the target DNA sequence (Jinek et al., 2012) and subsequently cleave the DNA adjacent to the PAM (Jiang and Doudna, 2017). Cas proteins from different bacterial species have different PAM sequences, which often occur with different frequencies within the target genome (Adli, 2018). Another important element is the seed region, which is the DNA sequence just beyond the PAM where complete complementarity is necessary for targeted cleavage of DNA (Semenova et al., 2011).

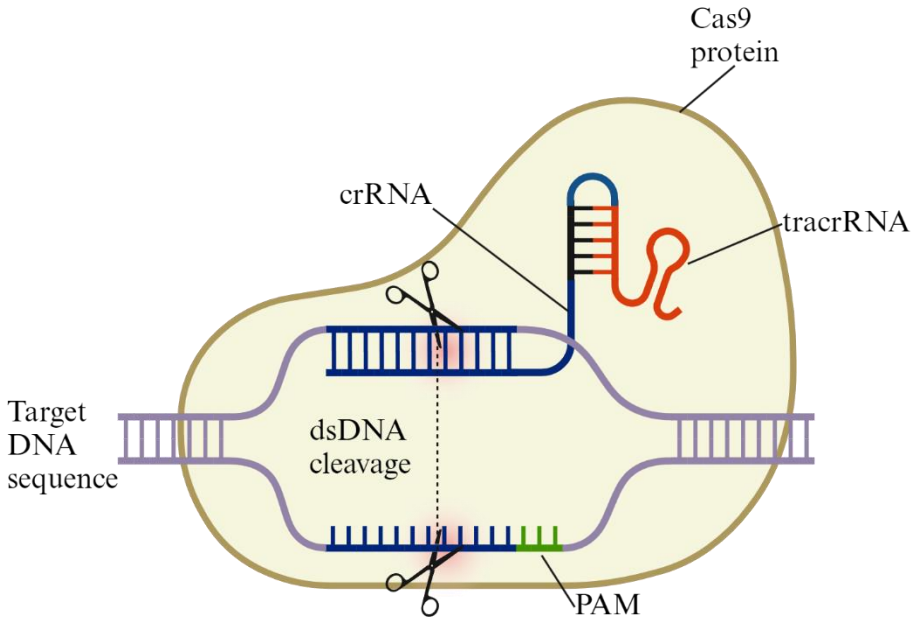


Figure 1.3: Cas9-mediated dsDNA cleavage of a target sequence.

Schematic diagram of dsDNA cleavage by a Cas9 protein guided by the crRNA and tracrRNA, with the location of the PAM relative to the cleavage site (Created with Biorender.com).

1.2.2 CRISPR/Cas9 as a tool against viral infections

The CRISPR/Cas DNA targeting system has been adapted for therapeutic use where sgRNA is artificially synthesized and exogenously supplied to target the DNA sequence of any organism. Not only can the Cas9 protein be used to cleave and inactivate target DNA, but variants of the Cas protein have also been engineered for a variety of purposes. A dead Cas9 (dCas9) protein can be used for chromatin imaging of target DNA (Adli, 2018), while a nickase Cas9 can be used to induce single-strand nicks in a DNA sequence to inactivate a gene (Jinek et al., 2012). In the prevention of HIV, one approach targeted the CCR5 receptor that HIV binds to with CRISPR/Cas9, where disruption of this receptor in induced pluripotent stem cells rendered the cells resistant to HIV infection (Ye et al., 2014). To treat a latent HIV infection, Ebina *et al*. used CRISPR/Cas9 to target and inactivate integrated HIV DNA in infected cells (Ebina et al., 2013), which was also used in animal models of HIV (Kaminski et al., 2016). Various herpesviruses such as the Epstein-Barr virus (EBV) and herpes simplex virus-1 (HSV-1) have also been successfully

targeted with CRISPR/Cas9 in cell culture (Roehm et al., 2016; van Diemen et al., 2016; Wang and Quake, 2014). HPV, which is linked to cervical and other anogenital cancers, has been targeted by CRISPR/Cas9 systems to inhibit the virus-driven cancer progression both *in vitro* and *in vivo* (Zhen et al., 2014).

Based on the success of these pre-clinical studies, there have been numerous clinical trials initiated using CRISPR/Cas9 systems to treat viral infections. CRISPR/Cas9 system targeting HIV-1 pro-viral DNA, EBT-101, is currently being investigated to assess the long-term efficacy in HIV-1 infected patients currently receiving antiretroviral therapy (NCT05144386) (Zhang et al., 2023). Another study aims to disrupt HPV DNA in cervical cancer using CRISPR/Cas9 to target HPV E6 and E7 genes, whose expression is linked to cancer progression (NCT03057912) (de Buhr and Lebbink, 2018; Hu et al., 2014). Another trial is examining the effect of the novel gene editor BD111 which targets HSV-1 in patients with refractory herpetic viral keratitis that leads to blindness using CRISPR/Cas9 mRNA (NCT04560790) (Luthra et al., 2021). CRISPR/Cas9 systems have also been widely studied as a means of targeting the HBV genome and have since been shown to be effective both *in vitro* and *in vivo* in models of HBV infection. Lin *et al.* first demonstrated the efficacy of a CRISPR/Cas9 system that successfully reduced the production of HBV surface antigen and core protein using gRNAs that targeted a range of HBV ORFs including *X* and *P* both in single gRNA systems and in multiplexed systems (Lin et al., 2014). Since then, Dong *et al.* investigated different sgRNAs that targeted conserved regions of overlaps in the ORFs within the HBV genome both *in vitro* and *in vivo* using a mouse model of HBV (Dong et al., 2015). This was expanded upon by Ramanan *et al.*, where they found that targeting conserved regions of the HBV genome had a significant effect on the expression of HBV cccDNA both *in vitro* and *in vivo* (Ramanan et al., 2015). A system using dual gRNA CRISPR/Cas9 similarly showed significant suppression of HBsAg and HBeAg production (Wang et al., 2015), while another study used a CRISPR/Cas9 nickase system to induce ssDNA breaks in targets within the *S* and *X* ORFs of the HBV genome (Karimova et al., 2015). The opportunity to use multiplexed CRISPR/Cas9 systems with a series of sgRNAs targeting different regions of the HBV genome makes CRISPR/Cas9 systems a more attractive choice than other gene editors, as it allows a single expression cassette to target more than one region of the genome at the same time. A multiplexed CRISPR/Cas9 system targeting multiple regions in the HBV genome was shown to have a greater anti-HBV effect than that of a TALEN array that also targeted multiple points in the HBV genome

(Schiwon et al., 2018). Further studies tested different gRNA sequences to target different genotypes of HBV (Liu et al., 2015) and in transgenic mouse models of HBV (Zhu et al., 2016).

This study uses an anti-HBV CRISPR/Cas9 system designed by Scott *et al*. In the study *Staphylococcus aureus* Cas9 (saCas9) and sgRNAs were delivered to an HBV-infected HepG2-derived cell line expressing the human NTCP receptor to assess HBV replication suppression. Here, 10 sgRNAs that target the overlap of the S and P ORFs of the HBV genome were identified. Of the 10 guide sequences identified, one in particular, 5' - CAAGAAUCCUCACAAUACCG – 3', was found to be particularly effective in targeting HBV and reducing serum levels of HBsAg *in vitro* (Scott et al., 2017). These, among many other studies, demonstrate the efficacy of CRISPR/Cas9 systems as a means of totally eradicating HBV from infected cells (Kennedy et al., 2015; Seeger and Sohn, 2016, 2014). However, several challenges need to be overcome before the goal of HBV eradication can be realised. Among these challenges, the delivery of anti-HBV gene editor sequences is the most daunting.

1.2.3 Delivery of anti-HBV gene therapeutics

Along with considering the various gene editing tools that can be used to treat HBV and other viral infections, there must be careful consideration given to the mechanism of delivery of these tools, with different viral vector- and non-viral vector-based methods of delivery having unique benefits and shortfalls that guide their choice as a vector. Lipids, lipid-like nanoparticles, and ribonucleoparticles are examples of non-viral vectors that have been used to deliver an increasingly wide variety of therapeutics against infectious diseases, including anti-HBV gene therapies to both infected cells *in vitro* (Martinez et al., 2022; Suzuki et al., 2021) and animal models of HBV infection (Jiang et al., 2017). Lipid nanoparticles (LNPs) are comparatively safer as they are not highly immunogenic (Cullis and Hope, 2017). They also allow for much safer repeated dosing of the same formulations (Kenjo et al., 2021). LNPs can also be targeted to specific cell types (Akinc et al., 2010; Shi et al., 2023; Yin et al., 2017), and they can allow for transient expression of transgenes that minimize the chances of off-target effects (Kowalski et al., 2019). However, these vectors often suffer from low transfection efficiency compared to viral vectors and their transient effects are not suited for the treatment of chronic HBV infection (Martinez et al., 2022; Shin et al., 2020).

1.2.4 Viral vectors used in anti-viral gene therapy

Viral vectors have been widely studied for a variety of uses including as vaccine vectors and anti-viral therapies. They are generally more efficient than non-viral vectors both in their delivery and the duration of gene expression which makes them more attractive for application in gene therapy where prolonged gene expression is often a requirement. Several viruses are commonly used as vectors, the most common of which are lentiviruses, adeno-associated viral vectors (AAVs), and adenoviral vectors (AdVs) (Ramamoorth and Narvekar, 2015).

Lentiviruses are a kind of single-stranded RNA retrovirus that includes HIV (Merten et al., 2016). Lentivirus genomes include *gag*, *pol*, and *env* genes, among others, with two flanking ITRs to produce functional viruses. One of the first lentivirus-based vector systems is based on HIV-1 (Bulcha et al., 2021). These vectors have an 8 kb carrying capacity for transgenes (Mátrai et al., 2010), and can be targeted to specific cell types through pseudotyping (Sakuma et al., 2012). However, they readily integrate into the host genome which contributes to host genome instability and constitutive expression that leads to off-target cleavage (Martinez et al., 2022; Mátrai et al., 2010). Along with the expected immune response induced by the lentivirus itself, studies have shown that cellular proteins from the packaging cells used to produce the lentivirus are often incorporated into the virion during the packaging process to which immune responses can be induced (Annoni et al., 2013; Wheeler et al., 2007). Despite these shortfalls, lentiviruses have been used in a variety of applications, including in clinical trials for the treatment of HIV and in animal models of diseases such as β-thalassemia, haemophilia, and liver diseases, among others (Escors and Breckpot, 2010). Lentiviruses have been used in vaccines against influenza (Gallinaro et al., 2018), SARS-CoV-2 (Ku et al., 2021), and HIV (Norton et al., 2019). A lentiviral-based vaccine has recently been shown to induce HBV-specific T-cell responses to chronic HBV infections in both naïve mice and in a mouse model of chronic HBV infection (Zhang et al., 2024). In the treatment of HBV infections, lentiviruses have been used to deliver shRNA that targets pre-genomic HBV RNA to inhibit HBV replication (Deng et al., 2009). They have also been used for the delivery of anti-HBV CRISPR/Cas9 sequences both *in vitro* (Kennedy et al., 2015; Ramanan et al., 2015) and *in vivo*, (Kayesh et al., 2020), as well as CRISPR/Cas-mediated base editors that target and inactivate HBV cccDNA (Yang et al., 2020).

AAVs are dependoparvoviruses that lack the essential genes necessary for their replication and expression, and therefore require an Adenovirus (Ad) to provide these genes *in trans*. AAVs are single-stranded DNA viruses with a 4.7 kb genome containing four ORFs: *rep, cap, membrane-associated accessory protein (MAAP),* and *assembly-activating protein (AAP)* (Bulcha et al., 2021). AAVs are used as vectors by removing all of their coding sequences, leaving only their ITR sequences. The use of AAVs in the treatment of infectious diseases has been widely investigated, with two Phase I clinical trials involving HIV currently underway (NCT01937455 and NCT03374202), vaccines against SARS-CoV-2 (Zabaleta et al., 2021) and a mouse model of Ebola having been tested (Van Lieshout et al., 2018), and even AAVs encoding antibodies against simian immunodeficiency virus (SIV) (Martinez-Navio et al., 2020). In the treatment of HBV, they have been used to deliver various anti-HBV CRISPR/Cas9 (Scott et al., 2017) and ZFN (Weber et al., 2014) sequences to infected cells *in vitro* and *in vivo*, more efficiently than lentiviruses (Kayesh et al., 2020). There are currently three gene therapies using AAVs that have been approved in the United States, and another in Europe, to treat haemophilia and diseases associated with the eye and muscles (Kohn et al., 2023). However, AAVs are relatively small, with a carrying capacity of approximately 5 kb, which limits their use in most applications (Wu et al., 2010). They also tend to integrate into the host genome and cause continuous expression of transgenes that causes off-target effects (Miller et al., 2005; Samulski and Muzyczka, 2014; Xiao et al., 1996).

1.3 Adenoviruses

Adenoviruses are members of the *Adenoviridae* family, which is divided into five genera, including the *mastadenoviridae,* which is the genus human adenoviruses fall under. These are again divided into seven species named A to G, which include close to 70 different serotypes that can infect humans, and generally cause opportunistic and persistent asymptomatic infections (Watanabe et al., 2021). One of the most common and widely studied species of Ad that infects humans is the species C serotype 5 adenovirus (Ad5). This Ad infects the upper respiratory system and binds to host cells via the coxsackie adenovirus receptor (CAR) (Bergelson et al., 1997), which is expressed on various cell types including myoblasts, epithelial cells, and hepatocytes (Freimuth et al., 2008; Tatsis and Ertl, 2004). Ads are non-enveloped, double-stranded DNA viruses with an icosahedral structure between 70 and 90 nm in size, comprised of the outer capsid proteins and

inner core proteins (McConnell and Imperiale, 2004) (Figure 1.4). The capsid is made up of three major proteins, hexons, pentons, and fibre proteins, along with the minor structural hexon-associated proteins IIIa, VI, VIII, and IX (Tatsis and Ertl, 2004; Zhang and Zhou, 2016). There are 240 hexon proteins present on the Ad capsid that form the facets of the icosahedral, and 12 penton proteins that form the vertices (Liu and Seol, 2020). The penton base anchors the fibre proteins that have a knob protein at their C terminus which acts as the primary receptor of the Ad (McConnell and Imperiale, 2004). The core proteins V, VII, and X are associated with the viral DNA and serve various functions including interactions with the nuclei of host cells, viral assembly, DNA binding and initiation of replication, and viral chromosome condensation, respectively (Zhang and Zhou, 2016).

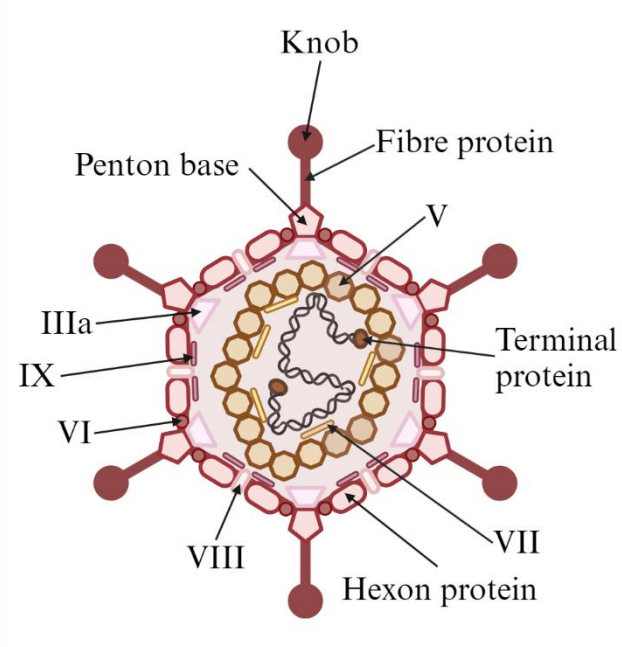


Figure 1.4: Adenovirus particle structure.

The icosahedral Ad virion is comprised of outer capsid structural proteins and inner core proteins that encapsulate the viral genome. The capsid proteins include the major hexon, penton, and fibre proteins, with minor hexon-associated proteins IIIa, VI, VIII, and IX. The DNA-associated inner core proteins include the terminal proteins, V, and VII proteins. (Created with Biorender.com).

Ad virions bind via the knob protein of the fibre to CARs, and an RGD motif on the penton base interacts with α_v integrins to facilitate viral internalisation through endocytosis (Ricobaraza et al., 2020; Rosewell et al., 2011). The acidic environment of the endosome allows for the escape of

virions into the cytoplasm, where the virions are trafficked to the nucleus. Here, they interact with the nuclear pore complex where the capsid is disassembled, allowing for viral DNA replication and transcription of the Ad genome in the host nucleus (McConnell and Imperiale, 2004). The Ad genome ranges from 26 to 45 kb in size and consists of two groups of genes that are differentially expressed either before or after viral DNA replication and are therefore referred to as the early and late region units respectively (Zhang and Zhou, 2016) (Figure 1.5). The early units, E1 to E4, encode various proteins necessary for the replication of the viral genome, while the late units, L1 to L5 encode the proteins that form the viral capsid (McConnell and Imperiale, 2004; Watanabe et al., 2021). Following viral DNA replication and transcription to produce the necessary viral proteins, the Ad is assembled, and the viral genome is packaged within the assembled viral capsid. Host cell lysis is induced by Ad proteins which release the newly produced Ad virions for further infection (McConnell and Imperiale, 2004).

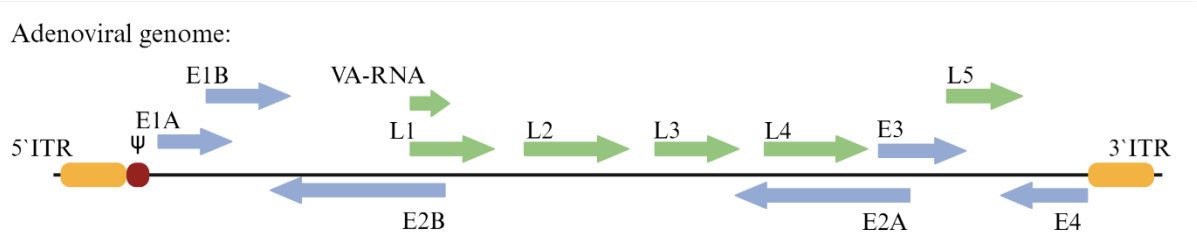


Figure 1.5: The adenovirus genome.

Schematic representation of the 26 to 45 kb adenovirus genome. It is comprised of the early units E1 to E4, indicated in blue, and late units L1 to L5, indicated in green. Also shown are the two inverted terminal repeats (ITRs) and the packaging signal (ψ) next to the left ITR.

The major proteins transcribed by the Ad genome are described in Table 1.1. Of the early units, the E1A unit, located just next to the left ITR and the packaging signal, is the first gene transcribed and allows for the transcription of other viral genes (McConnell and Imperiale, 2004). The proteins transcribed by this unit, including the E1A control gene expression, enable viral DNA replication, assist in immune evasion, disrupt cell cycle control in infected cells, and inhibit host cell apoptosis (McConnell and Imperiale, 2004; Russell, 2009; Watanabe et al., 2021). The E1B region has various anti-apoptotic functions, inhibits host protein synthesis, and selectively stabilises viral RNA (Tatsis and Ertl, 2004). The E2 unit facilitates viral replication by encoding a polymerase

and various DNA-binding proteins such as the single-stranded DNA-binding protein and terminal protein precursor pTP (De Jong et al., 2003; McConnell and Imperiale, 2004). The E3 unit is not essential for viral replication, though it encodes immunomodulatory proteins that contribute to the escape of viral particles from the host immune system and protects Ad-infected cells from cytotoxic T cells and receptor-mediated apoptosis (Lichtenstein et al., 2004). The E3 region has a total of nine ORFs from which seven proteins are transcribed; E3-12.5K, whose function is currently unknown, E3-6.7K, E3-gp19K, the adenovirus death protein (ADP), the receptor internalization and degradation protein α (RIDα), RIDβ, and E3-14.7K. E3-gp19K prevents the translocation of the major histocompatibility complex (MHC) class I protein to the cell surface and prevents the binding of viral antigens to the MHC class I molecule (McConnell and Imperiale, 2004). E3-14.7K contributes to the inhibition of apoptosis of Ad-infected cells, while RIDα and RIDβ remove chemokine receptors from the surface of infected cells to prevent apoptosis. The ADP is expressed later in infections and allows for the release of newly formed Ad virions from an infected cell (Lichtenstein et al., 2004).

The E4 unit has several open reading frames (ORFs) which encode proteins that affect viral DNA replication and transcription, nuclear export of viral RNA, and various host cell functions (Weitzman, 2005). The first ORF, E4orf1 is transcribed to produce mRNA that tends to accumulate in host cells after infection and is suggested to be involved in the lytic cycle of Ads. The E4orf1 sequence was shown to resemble dUTP pyrophosphatase (dUTPase) enzymes, but lack any functional activity relating to this, and it was suggested that this region was derived from ancestral Ad dUTPase (Täuber and Dobner, 2001; Weiss et al., 1997). Little is known about the function of E4orf2, while E4orf3 was the first gene product of the E4 region to be identified. It encodes an 11 kDa protein that is functionally redundant with the product of E4orf6 and is associated with the nuclear matrix. This protein allows for the reorganisation of nuclear structures that are involved in genomic stability, DNA repair, and transcriptional control, among other functions, and it has been suggested that E4orf3 facilitates the initiation of viral replication. Both E4orf3 and E4orf6 have been shown to prevent the formation of concatemers upon viral infection of host cells (Evans and Hearing, 2003). E4orf4 encodes a protein of 114 amino acids that, while not essential for the growth and replication of the Ad, was found to play a role in the regulation of viral DNA replication by controlling protein phosphorylation. It is also suggested that it controls the splicing of RNAs expressed later in the viral replication cycle and has been shown to induce apoptosis in

host cells to allow for the further spread of viral particles (Weitzman, 2005). E4orf6 is one of the most studied E4 gene products. It is a 34 kDa protein that is associated with viral DNA replication, RNA processing and transport of viral mRNA, the inhibition of host cell protein synthesis, as is involved in the prevention of concatemerisation of the viral genome like E4orf3(Huang and Hearing, 1989). The fusion protein of E4orf6 and E4orf7 uses 58 amino acids from the N terminus of E4orf6 and the 92 amino acids encoded by E4orf7 to produce a protein that controls transcription and can be used to replace the function of the E1A unit (McConnell and Imperiale, 2004; Täuber and Dobner, 2001; Weitzman, 2005).

Also encoded by the Ad genome are two virus-associated (VA) RNAs which form double-stranded hairpin loops that are needed for the translation of Ad genes, while the *IX* gene encodes a minor component of the Ad capsid that contributes to the stability of the viral particle (Tatsis and Ertl, 2004). As previously mentioned, the late units encode most of the major and minor viral capsid proteins including the minor capsid and core proteins, with the hexon protein encoded by the L3 unit, the penton base encoded by L2, while the fibre protein is encoded by L5 (Tatsis and Ertl, 2004).

Table 1.1: List of proteins encoded by the Ad genome.

Protein	**Function/s**	**Transcription Unit**
E1A	Viral DNA replication, immune evasion, cell cycle control, and inhibiting host cell apoptosis	E1A
E1B 19 K	Anti-apoptotic functions, inhibits host protein synthesis, and stabilises viral RNA	E1B
E1B 55 K		
DNA binding protein	Participates in viral DNA replication	E2A
Terminal protein precursor pTP	Core protein	E2B
E3-gp19K	Preventing translocation of MHC class I to the cell surface and prevents the binding of viral antigens to the MHC class I molecules	E3
E3-12.5K	Unknown function	
ADP	Allows release of Ad virions from an infected cell	
RIDα and RIDβ	Removes chemokine receptors from infected cell surface and prevents apoptosis	
E3-14.7K	Inhibition of apoptosis of Ad infected cells	
E4orf1	Involved in Ad lytic cycle	E4
E4orf2	Unknown function	
E4orf3	Prevents the formation of concatemers upon viral infection	
E4orf4	Regulation of viral DNA replication	
E4orf6	Prevents the formation of concatemers upon viral infection	
E4orf6/7	Controls viral transcription	

pIIIa	Minor structural protein	L1
Penton	Major capsid protein	L2
IIIa	Minor structural protein	
V	Core protein	
VII	Core protein	
X	Core protein	
VI	Minor structural protein	L3
Hexon	Major capsid protein	
100K	Minor structural protein	L4
33K	Minor structural protein	
22K	Minor structural protein	
VIII	Minor structural protein	
Fibre	Major capsid protein	L5

1.3.1 Adenoviral vectors

Adenoviruses are used as vectors by modifying the wildtype virus through the deletion of various viral genes to produce three generations of adenoviral vectors (AdVs) (Figure 1.6). These AdVs are particularly useful as vectors due to their large carrying capacity (Breyer et al., 2001), good safety profile (Douglas, 2007), ability to infect a variety of replicating and quiescent cell types (Tatsis and Ertl, 2004), their long-term gene expression (Douglas, 2007), and their non-integrative nature which contributes to their safety and stability (Watanabe et al., 2021). AdVs also have a high transduction efficiency and a subsequently lower dose requirement that contributes to their low toxicity. These vectors are easy and cheap to produce, especially at a large scale, and therefore are much more appealing in regions of low income like Africa and the western Pacific (Farhad et al., 2022). The inherent hepatotropism of Ads makes AdVs an ideal candidate for liver-directed therapies.

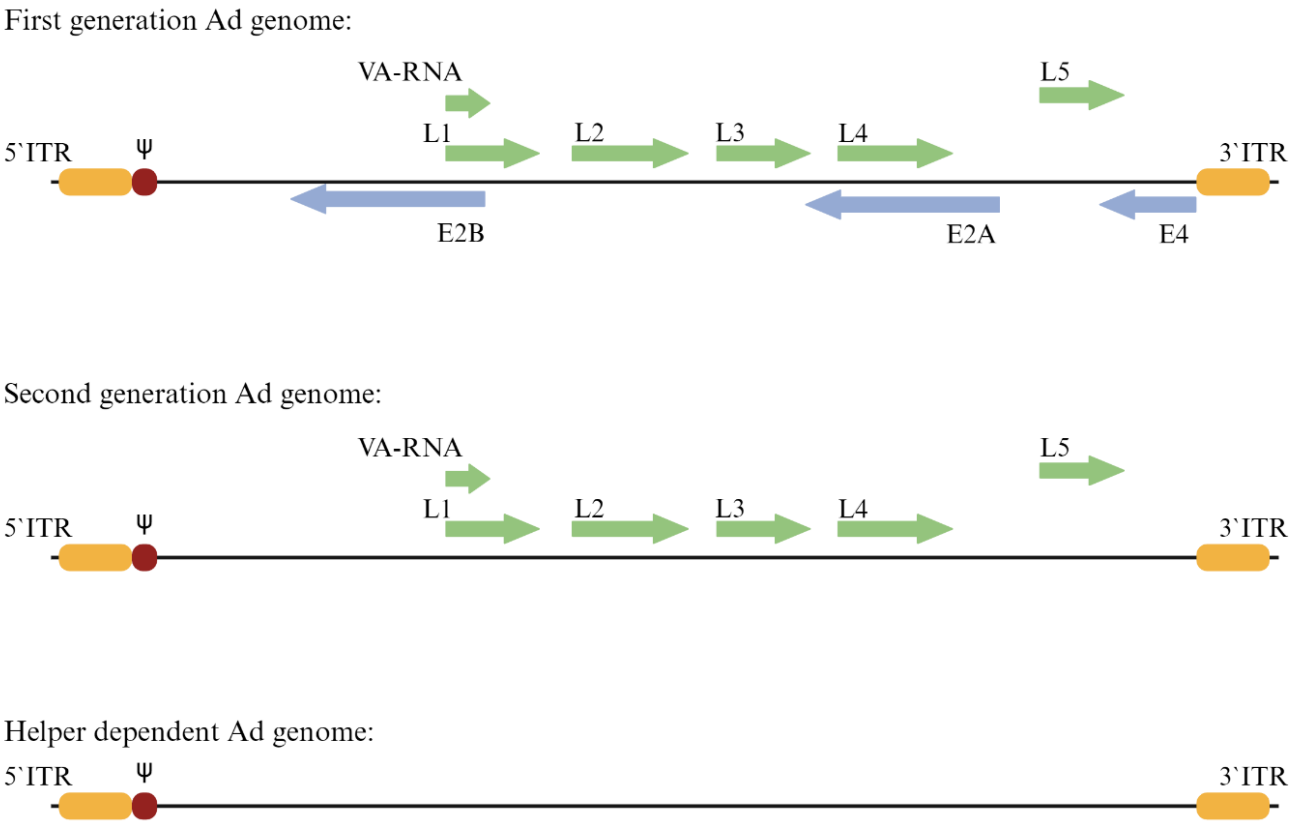


Figure 1.6: Genomes of the three generations of adenoviral vectors.

*The genomes of (**A**) a first-generation AdV with the E1 and E3 regions deleted, a (**B**) second-generation AdV with all the early units deleted, and a (**C**) third generation AdV with all early and late units deleted, leaving only the non-coding ITRs and packaging signal. (Created with Biorender.com).*

First-generation AdVs are constructed by deleting the E1 region, rendering the vector replication-deficient, which prevents unwanted amplification of the viral vector in a host (Figure 1.6A) (Ghosh-Choudhury et al., 1986). The non-essential E3 region may also be deleted to increase the carrying capacity of these vectors to approximately 8 kb (Saito et al., 1985). First-generation AdVs have been used against various infections such as human immunodeficiency virus (HIV), rabies virus, Ebola virus, hepatitis B virus (HBV), and measles virus, to name a few (Tatsis and Ertl, 2004). They are commonly used in vaccine development due to the strong immune response they elicit (Tatsis and Ertl, 2004). These vectors are known to facilitate strong B cell, and T cell responses against the transgene they express, which facilitates good vaccine efficacy (Lasaro and

Ertl, 2009; Wold and Toth, 2013). Hence, there have been numerous first-generation AdV vaccine clinical trials such as the notable STEP trial, which used three first-generation AdVs expressing various HIV proteins (Buchbinder et al., 2008; Priddy et al., 2008), and another vaccine trial against HIV, referred to as HVTN, which used an AdV as a booster for an initial DNA vaccine expressing HIV proteins (Hammer et al., 2013; Wold and Toth, 2013). Since then, there have been numerous clinical trials established using first-generation AdVs as vaccines against diseases such as COVID-19 (Folegatti et al., 2020; Sadoff et al., 2022; Voysey et al., 2021; Zeng et al., 2022), Ebola (Anywaine et al., 2019), influenza (Gao et al., 2006), malaria (Sedegah et al., 2011), and hepatitis B (Chinnakannan et al., 2020; Martin et al., 2015), among others.

These studies demonstrate the efficacy of first-generation AdVs as vectors, with an inarguable benefit in their use. However, first-generation AdVs are not favoured as vectors for gene therapy due to the strong immune response seen against both the virus itself and the transgene it may express. Additionally, first-generation AdVs do not allow for long-term expression of transgenes, and these vectors have been linked to strong immune responses against the vector itself and toxicity in transduced tissues, which makes them less useful as vectors of gene therapy (Yang et al., 1994a).

Second-generation AdVs are like first-generation vectors, but have also the E2 and/or the E4 regions deleted to further increase the carrying capacity of the vectors to approximately 14 kb and reduce the potential cytotoxic effects associated with the expression of viral proteins (Figure 1.6B) (McConnell and Imperiale, 2004; H. Zhang et al., 2023). These vectors have not been as widely used as other AdVs, with variable results seen in animal models of diseases such as cystic fibrosis (Engelhardt et al., 1994a; Yang et al., 1994b), atherosclerosis (Van Craeyveld et al., 2011) and liver-directed therapy (Engelhardt et al., 1994b). There is also conflicting data regarding their lower toxicity and improved duration of transgene expression compared to first-generation AdVs (Rosewell et al., 2011; H. Zhang et al., 2023), and production of these second-generation AdVs is less efficient than that of first-generation AdVs, making them a less appealing choice of vector.

Third generation AdVs, also known as gutless, high capacity, or helper-dependent adenoviral vectors (HDAdVs), have all viral genes deleted, leaving only the ITR sequences and the cis-acting packaging signal (Ψ) (McConnell and Imperiale, 2004) (Figure 1.6C). These vectors use a helper virus (HV) to provide the necessary viral genes *in trans* to produce a functional viral particle (Palmer and Ng, 2005), unlike earlier generation AdVs which can be produced in cell lines that

express the necessary viral proteins such as HEK 293T cells (Breyer et al., 2001). HDAdVs serve as useful vectors of gene therapy because of their weaker immune stimulation, long-term transgene expression, and large carrying capacity of close to 36 kb, which is significantly larger than other viral vectors (Brunetti-Pierri and Ng, 2011; McConnell and Imperiale, 2004). HDAdVs use a so-called stuffer sequence to maintain the size of the vector for successful encapsidation (Alba et al., 2005). These non-coding sequences are necessary for the stability of the resultant vector and viral DNA in the host cells (Parks et al., 1999). A common stuffer sequence used in HDAds is a DNA fragment from intron 1 of the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene which includes a matrix attachment region (MAR) that is important for the binding of the Ad genome to the nuclear matrix of host cells for stability *in vivo* (Parks et al., 1999; Sandig et al., 2000). It was assumed that using DNA with a human origin as a stuffer would provide better results, particularly if repeat sequences were avoided and integration into the host genome was avoided through rearranging the stuffer sequence. It was shown that using different human stuffer sequences affected the expression levels from the vectors, which was attributed to the interaction of the vector with cellular matrix proteins, or with the level of DNA condensation and methylation (Sandig et al., 2000).

There have been numerous applications of HDAdVs as antiviral therapies. HDAdVs have been shown to perform better than first-generation AdVs that express the same antigen, demonstrated in studies that compared the expression levels of HBsAg (Kron et al., 2011) and in the expression of the malaria protein merozoite surface protein 1 (MSP-1) (Zong et al., 2011), where the HDAdVs were shown to have higher levels of transgene expression. HDAdV-based vaccines were demonstrated to elicit greater transgene-specific cytotoxic T-cell responses and antibody production against the transgenes, to offer a greater protective effect than first-generation AdVs (Brunetti-Pierri and Ng, 2017; Weaver et al., 2009). There has been work to produce HDAdVs that express cytokines against HBV (Crettaz et al., 2009) and the hepatitis C virus (HCV) (Aurisicchio et al., 2005) which resulted in appreciable antiviral effects. In the treatment of hepatitis B, HDAdVs have successfully been used to deliver RNAi activator sequences that target overlaps in the HBV genome to infected cells (Crowther et al., 2014; Mowa et al., 2014, 2012). As a potential treatment against human papillomaviruses (HPV), HDAds have been used to deliver HPV-specific clustered regularly interspaced short palindromic repeats CRISPR/Cas9 sequences that selectively targeted and induced apoptosis in HPV-infected cells (Ehrke-Schulz et al., 2020)

Although induction of an immune response to the viral capsid is still a challenge with HDAdVs there are approaches that can be used to minimise the immune response induced by these vectors. These include immune suppression prior to administration, pegylation of vectors with polyethylene glycol (PEG) and capsid modification to evade immune responses to capsid proteins (Crowther et al., 2008; Fontanellas et al., 2010; Prill et al., 2011; Weaver and Barry, 2008; Weklak et al., 2021).

1.3.2 Producing recombinant adenoviral vectors

The basic steps in AdV production involve vector genome engineering to insert transgenes, production of AdVs by transfecting the recombinant genomes into a producer cell line carrying the E1 unit, with the HEK 293 cells being the most commonly used, followed by vector amplification and purification. These steps are common across the three generations, except that production and amplification of HDAdVs require the use of a helper virus (HV) to supply all the essential viral genes *in trans*. This HV is later separated from HDAdV during vector purification. Using an HV with a non-functional packaging signal and with a different genome size to the HDAdV prevents its propagation during vector production and facilitates its separation by size during purification (Kochanek et al., 1996; Palmer and Ng, 2005; Ricobaraza et al., 2020). The most commonly used HV has its packaging signal flanked by *lox p* sites for cre-mediated excision of the packaging signal in cre expressing producer cell line. This renders the HV genome unpackageable and mediates selective replication of the HDAdVs only (Parks et al., 1996). A similar approach uses the yeast derived FLP/frt recombination system in the same way, where frt sites flank the HV packaging signal to prevent its encapsidation while maintaining its gene expression (Ng et al., 2001; Umana et al., 2001). An alternative method to avoid HV contamination is to instead provide the necessary genes for Ad replication and packaging in a hybrid baculovirus, which contains the Ad packaging signal that can be excised with cre-recombinase rendering it unable to be packaged into infectious viral particles (Cheshenko et al., 2001), or to use a herpes simplex virus (HSV) amplicon-adenovirus hybrid to deliver the necessary Ad genes, after which it can be easily be separated from the HDAdV being produced (Kubo et al., 2003). Other methods of HDAdV production that are HV free have also been described where a helper plasmid is used rather than a virus (Liu and Seol, 2020).

Although the large genome of AdVs allows for its increased carrying capacity, this genome size makes it difficult to insert transgenes into the genome as traditional ligation methods are extremely inefficient when using larger DNA fragments (Chartier et al., 1996; He et al., 1998). Hence several methods for insertion of transgenes in to the vector genome have been developed.

1.3.3 Methods of transgene insertion into the vector genome

Several methods have been developed to improve the efficiency of transgene insertion into the vector genome (Figure 1.7). One such method uses a smaller shuttle plasmid. This shuttle plasmid is made to contain a transgene of interest and a DNA sequence identical to the left-most end of the Ad genome and used with the Ad genome to co-transfect human embryonic kidney 293 (HEK 293) cells, or another cell line that expresses the *E1* gene *in trans* (Chinnadurai et al., 1979; Stratford-Perricaudet et al., 1992), such as 911 (Fallaux et al., 1996), or PER.C6 (Fallaux et al., 1998) cells. Here, recombination occurs between the region of homology between the shuttle and the Ad genome to replace the E1 region with the transgene to be expressed, while rendering the recombinant Ad replication deficient (Danthinne and Imperiale, 2000). This method is inefficient, with low levels of recombination occurring, even after a time-consuming and labour-intensive procedure (Danthinne and Imperiale, 2000; H. Zhang et al., 2023). Another method uses a shuttle plasmid containing a transgene of interest and another plasmid containing most of the Ad genome, where a Cre/*loxP* system is used to induce a more efficient recombination between the plasmids in HEK 293 cells (Aoki et al., 1999; H. Zhang et al., 2023).

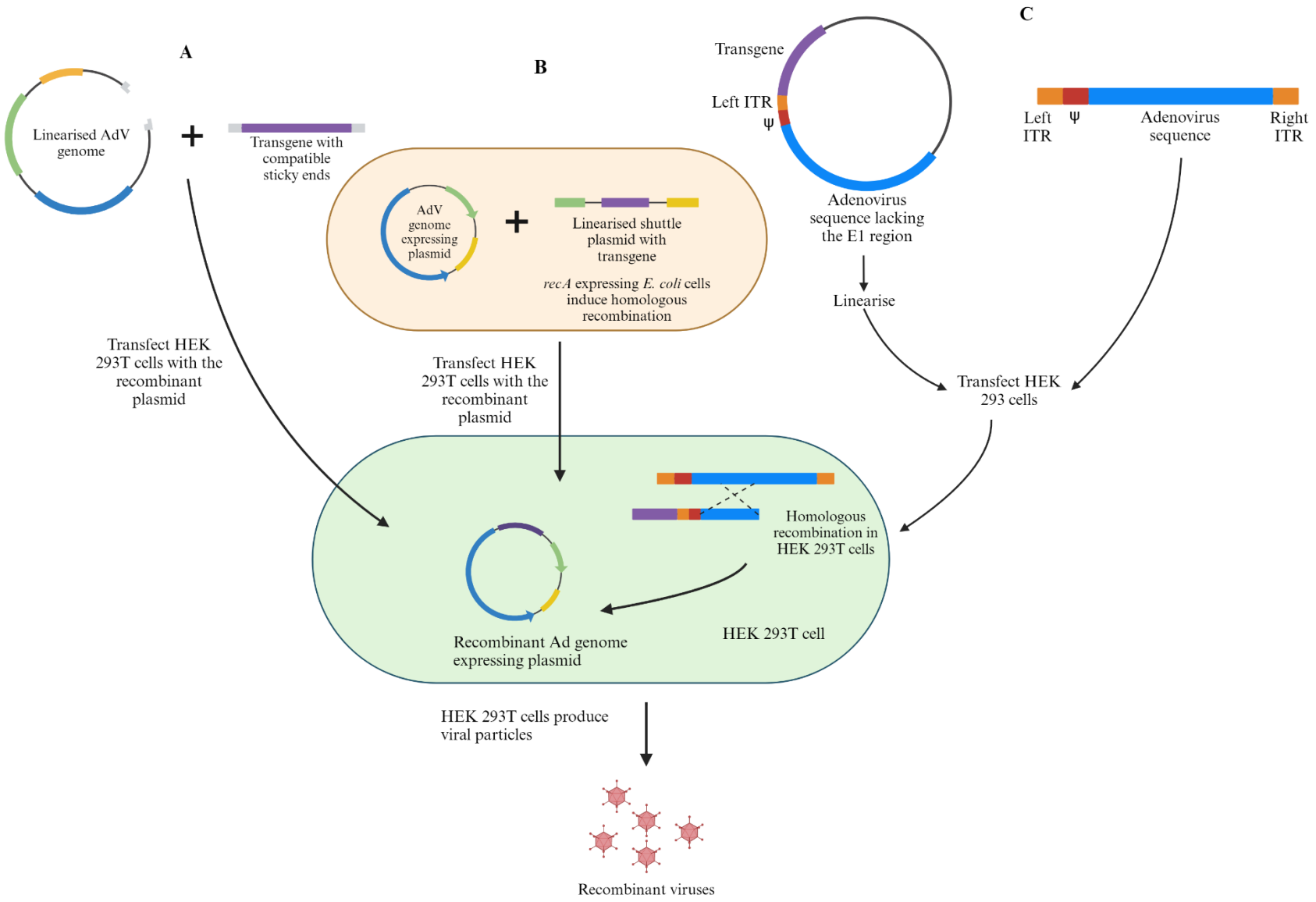

A
Linearised AdV genome
Transgene with compatible sticky ends
B
AdV genome expressing plasmid
Linearised shuttle plasmid with transgene
recA expressing E. coli cells induce homologous recombination
C
Transgene
Left ITR
ψ
Adenovirus sequence lacking the E1 region
Linearise
Left ITR
ψ
Adenovirus sequence
Right ITR
Transfect HEK 293T cells with the recombinant plasmid
Transfect HEK 293T cells with the recombinant plasmid
Transfect HEK 293 cells
Homologous recombination in HEK 293T cells
Recombinant Ad genome expressing plasmid
HEK 293T cell
HEK 293T cells produce viral particles
Recombinant viruses

Figure 1.7: Common methods of producing recombinant AdVs.

*Schematic representation of three common methods of producing recombinant AdVs. The first (**A**) involves the direct cloning of a transgene into the AdV genome, followed by transfection of HEK 293T cells to produce the recombinant viruses. The second (**B**) uses a shuttle plasmid with two regions of homology with the AdV genome. These plasmids are used to co-transform recA expressing E. coli where homologous recombination occurs to insert the transgene into the AdV genome. This recombinant plasmid is then used to transfect HEK 293T cells to produce the recombinant viruses. The third method (**C**) uses a transgene-expressing shuttle plasmid with a DNA sequence identical to the left-most end of the Ad genome, along with the Ad genome itself to co-transfect HEK 293T cells. Recombination between the region of homology between the shuttle and the Ad genome replaces the E1 region with the transgene. (Created with Biorender.com).*

An alternative to transfecting HEK 293 cells to facilitate recombination is the widely used AdEasy system, which is designed to insert transgenes into a first-generation AdV using a shuttle plasmid in *recA* expressing in *Escherichia coli* cells (He et al., 1998). This shuttle plasmid is significantly smaller than the plasmid containing the AdV, and as such, it is significantly easier to insert transgenes into this plasmid. The shuttle plasmid is designed to include two regions that are identical to two regions within the AdV plasmid, and when the linearised shuttle plasmid is used with the plasmid containing the AdV to co-transform *recA* expressing *E. coli* cells. The bacterial DNA repair machinery induces homologous recombination between the two identical DNA sequences using the RecBCD pathway, and in doing so, inserts the transgene in the shuttle plasmid into the AdV genome. The resultant recombinant AdV can then be used to produce viral particles expressing the transgene (He et al., 1998) In the AdEasy system, homologous recombination occurs when RecA recombinase proteins bind to single stranded DNA (ssDNA) that is formed when double-stranded DNA (dsDNA) breaks, forming a nucleoprotein filament (Val et al., 2019). This filament is then able to identify a region of homology in dsDNA, at which point there is a strand exchange between the two to form heteroduplex DNA. The dsDNA is used as a template by a DNA polymerase to synthesize a DNA strand complementary to the template, which is then ligated to the original single stranded DNA to repair the initial double stranded break (del Val et al., 2019; Radding, 1981). A critical element of this process is an eight-nucleotide sequence (5`-GCTGGTGG-3`) referred to as the crossover hotspot instigator (*chi*) (Lam et al., 1974; Smith et al., 1984). The RecBCD complex works to unwind dsDNA or degrade a stand of the DNA until it

encounters this sequence, upon which it cleaves the DNA and allows RecA proteins to be loaded onto the DNA to form the nucleoprotein filament (Michel and Leach, 2012).

These methods of transgene insertion are well defined and available for production of first generation AdVs. Different stuffer sequences are generally used in HDAdV genomes, hence systems like these must be designed for specific genome stuffer sequences used. Hence insertion of transgenes into the HDAdV genomes commonly still involves direct cloning of transgenes into the HDAdV genome bearing plasmids. However, the shuttle plasmid can be adapted for use with HDAdVs genomes. Toietta *et al.*, has demonstrated the feasibility of this by designing a specific shuttle plasmid for the HDAdV plasmid used, in their case pΔ28 carrying an ampicillin resistance marker (Toietta et al., 2002). Here, two homology arms were included in the shuttle plasmid, corresponding to the HPRT stuffer sequence with the right ITR and the C346 stuffer sequence with the left ITR in pΔ28. This shuttle plasmid also contained a kanamycin resistance gene along with the transgene to be expressed, β-galactosidase. Their study successfully demonstrated the effectiveness of a specifically designed shuttle plasmid that can be used to insert a transgene into the HDAd genome through homologous recombination (Figure 1.8). They were able to generate recombinant adenoviruses by essentially replacing the bacterial sequence in pΔ28 containing an ampicillin resistance marker with the shuttle plasmid sequence containing a kanamycin resistance gene and the transgene.

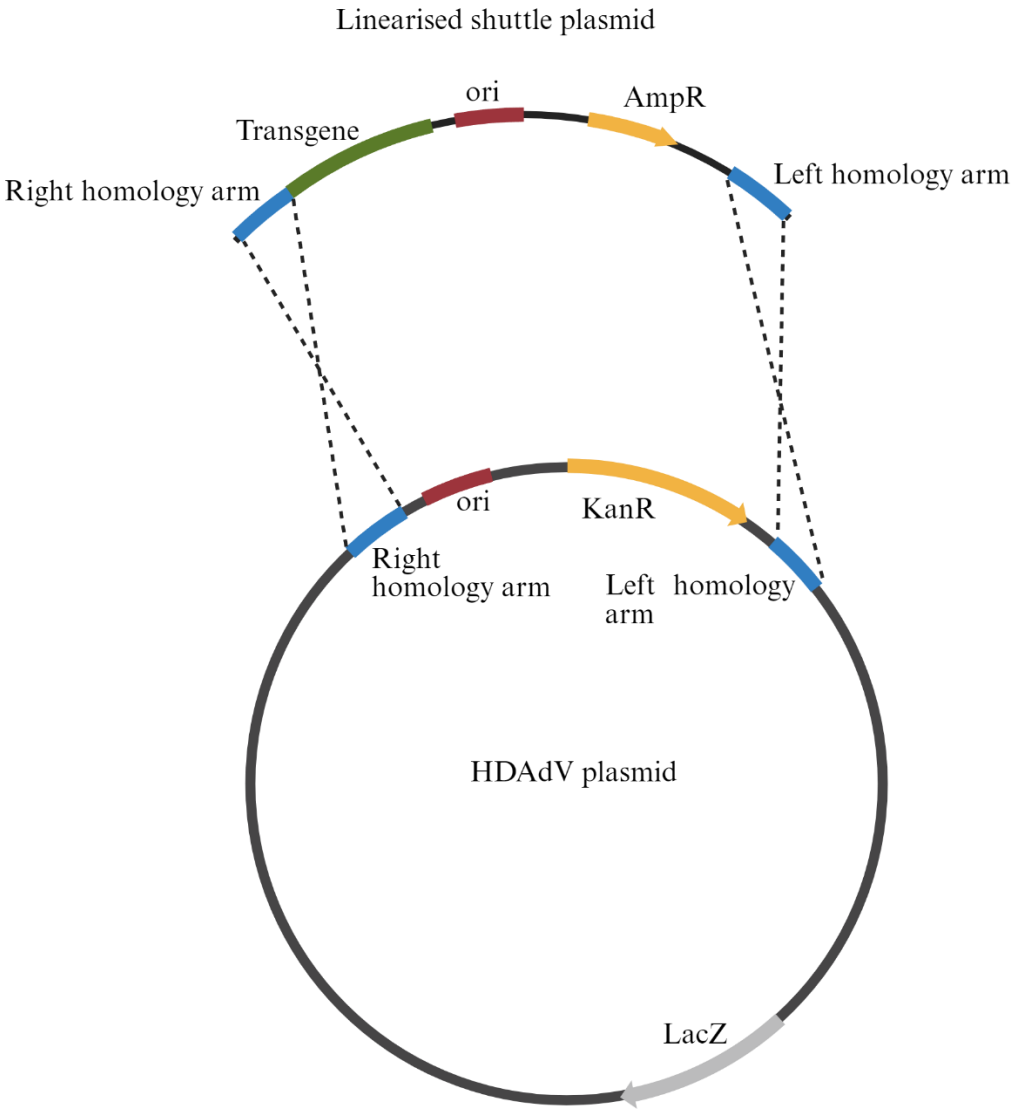


Figure 1.8: Recombination between a transgene expressing shuttle plasmid and the HDAdV genome expressing plasmid.

Once a transgene has been inserted into a shuttle, the plasmid is linearized and used to co-transform recA*-expressing cells with the HDAdV genome-bearing plasmid. The homology regions are indicated, and crosses indicate where homologous recombination occurs. (Created with Biorender.com).*

The HDAdV genome-containing plasmids used in gene therapy may contain different stuffer sequences, hence there is a need to have uniquely designed shuttle plasmids to use with each

plasmid. In addition, the system described by Toietta *et al.* is not accessible for general use. As such, to apply this system to the HDAdV genome containing plasmids available, a specific shuttle must be designed and characterised. A shuttle plasmid (pGEMShuttle) was constructed in my honours project, but its functionality was not tested.

1.4 Aim and objectives.

This study aimed to produce an efficient system to insert transgenes into the HDAd genome using a shuttle plasmid. The following objectives were set:

1. To insert the nanoLuciferase (nLuc) reporter gene and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) encoding sequences targeting the hepatitis B virus (HBV) genome into the shuttle plasmid (pGEMShuttle) using standard DNA ligation.
2. To assess the level of transgene expression from pGEMShuttle in tissue culture using a luciferase assay and an ELISA to assess HBsAg knockdown.
3. To insert the transgenes into the HDAd genome-bearing plasmid by homologous recombination.
4. To assess the functionality of the resultant recombinant plasmids for production of infectious HDAdVs.

CHAPTER 2

2. METHODS AND MATERIALS

2.1 Bacterial culturing

DH5α, DH10β (NEB, Massachusetts, USA), and BJ 5183 (Agilent, California, USA) strains of *Escherichia coli* (*E. coli*) were cultured in Luria Bertani (LB) liquid medium (Appendix 5.1.2) or grown on LB agar plates (Appendix 5.1.1) at 37°C or 30°C overnight, with liquid cultures shaken at 150 rpm. Bacteria expressing an ampicillin resistance marker were cultured in the presence of 100 µg/mL ampicillin (Appendix 5.1.6), and bacteria expressing a kanamycin resistance marker were grown in the presence of 50 µg/mL of kanamycin (Appendix 5.1.7). To assess for *lacZ* expression in blue-white screening, 40 µL of 20 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Appendix 5.1.5) diluted in 500 µL of LB was spread evenly on agar plates containing the appropriate antibiotic before plating.

2.1.1 Preparation of chemically competent *E. coli* cells

A pre-culture was prepared by inoculating a single colony or 20 µL of a freezer stock of *E. coli* DH10B or *E. coli* DH5α cells in 10 mL of LB and incubated overnight at 37°C while shaking at 150 rpm. This pre-culture was diluted 100-fold in 150 mL of LB and incubated at 37°C while shaking at 150 rpm until the optical density (OD) measured at 600 nm had an absorbance reading ranging between 0.4 to 0.6. The culture was divided into three 50 mL tubes and centrifuged at 4000 × g for 25 minutes at 4°C. The resultant bacterial pellets were then resuspended in 10 mL of transformation buffer (Appendix 5.1.8) and combined into a single 50 mL tube, and the final volume was adjusted to 35 mL with transformation buffer. The cells were incubated on ice for 30 minutes, and then centrifuged at 2 500 × g for 5 minutes at 4°C. The bacterial pellet was then resuspended in 1.5 mL of transformation buffer. Aliquots were made of 100 µL of cells, which were stored at -80°C.

2.1.2 Heat shock transformation of chemically competent *E. coli* DH10B and DH5α cells

DNA was added to 50 µL of chemically competent cells. This mixture was then incubated on ice for 20 minutes, followed by heat shock at 42°C for 90 seconds. The mixture was then immediately

incubated on ice for 5 minutes and rescued with up to 500 µL of SOC outgrowth media (NEB, Massachusetts, USA). The mixture was incubated at 37°C for one hour, and was then plated on agar plates with the appropriate antibiotic, and was incubated overnight at 37°C.

2.1.3 Electroporation of electrocompetent *E. coli* cells

Electrocompetent DH5α, DH10β (NEB, Massachusetts, USA), and BJ 5183 (Agilent, California, USA) *E. coli* cells were allowed to thaw on ice while tubes and cuvettes are stored on ice to allow them to cool. Rescue media was warmed to 37°C in an incubator. An appropriate amount of DNA, (no more than 10% of the volume of cells) was added to 50 µL of electrocompetent cells, and the mixture then added to the bottom of a chilled 2 mm electroporation cuvette. The cells were then electroporated using the Bio-Rad Gene Pulsar Xcell Electroporation system (Bio-Rad, California, United States) with a voltage of 2 000 V, a capacitance of 25 µF, and a resistance of 200 Ω. Immediately after electroporation, the cells were rescued in 1 mL of pre-warmed rescue media and transferred to a sterile 2 mL tube. Cells were incubated at 37°C for 60 mins while shaking at 250 rpm, then plated on agar plates with the appropriate antibiotic present, and incubated overnight at 37°C. For co-transformation of electrocompetent of *E. coli* BJ 5183 cells (Agilent, California, USA) a total of 100 ng of Ad genome bearing plasmid pD28E4LacZ or pD24.7 in no more than 1 µL of water and 1000 ng of linearised shuttle plasmid DNA in no more than 4 µL of water were combined before electroporation as above. Various volumes of cells, typically 100 µL, 300 µL, and 600 µL, were then plated on agar plates with the appropriate antibiotic present, and incubated for 1 to 2 days at 37°C.

2.2 DNA isolation and purification

2.2.1 Plasmids used in this study.

Plasmids used and constructed in this study are described in Table 2.1.

Table 2.1: List of plasmids used.

Plasmid Name	Description	Reference
pGEMShuttle	The initial shuttle plasmid derived from pGEM-T® Easy containing two regions of homology with the HDAdV genome.	Previous Honours study
pD28E4LacZ	An HDAdV genome bearing plasmid expressing a *lacZ* reporter gene.	Donated by Phillip Ng
pD27.4	An HDAdV genome bearing plasmid lacking a reporter gene.	Donated by Phillip Ng

pAAV-nLuc	A plasmid carrying the *nLuc* reporter gene.	AGTRU, Donated by Njabulo Mnyandu (Unpublished))
pTZ-SaU6-sgRNA-8	pTZ derived plasmid containing the sgRNA sequenc.	(Scott et al., 2017)
pAAV-CMV-SaCas9-Bgh-U6sgRNA	Plasmid containing the saCas9 and a non-HBV targeting sgRNA sequence	(Scott et al., 2017)
pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8	A plasmid containing the SaCas9 and HBV-specific sgRNA sequence.	This study
pGEM-T® Easy	A commercial TA cloning vector.	Promega, Wisconsin, USA
pCH-9/3091	A replication competent HBV plasmid with a sequence greater than the length of the HBV genome.	(Nassal et al., 1990)
pGEM-T EasynLuc	A pGEM-T® Easy derived plasmid containing the *nLuc* reporter gene.	This study
pGEM-T EasyCRISPR/Cas9	A pGEM-T® Easy derived plasmid containing the anti-HBV CRISPR/Cas9 sequence.	This study
pGEMShuttlenLuc	pGEMShuttle carrying the *nLuc* reporter gene	This study
pGEMShuttleCRISPR/Cas9	pGEMShuttle carrying the anti-HBV CRISPR/Cas9 sequence.	This study
pUC57-KanR-Ad ITR-E4	pUC57 derived plasmid constructed by Genscript Biotech (New Jersey, USA) containing the Ad ITR and a 400 bp fragment of the Ad E4 ORF1 sequence.	This study
pGEM-T Easy-pUC57 PCR	A pGEM-T® Easy derived plasmid containing the Ad ITR and a 400 bp fragment of the Ad E4 ORF1 sequence.	This study
pShuttle-CMV	The shuttle plasmid used in the AdEasy system to produce first generation Ads.	(He et al., 1998)
pGEMShuttleLCori	The modified pGEMShuttle containing the mutation that makes it a low copy number plasmid.	This study
pGEMShuttleLCoriE4	The modified pGEMShuttleLCori containing the Ad ITR and a 400 bp fragment of the Ad E4 ORF1 sequence.	This study
pGEMShuttleLCoriE4nLuc	The modified shuttle plasmid pGEMShuttleLCoriE4 carrying the *nLuc* reporter gene.	This study

2.2.2 Alkaline lysis plasmid extraction (mini prep)

A single colony was inoculated into 10 mL of LB containing the appropriate antibiotic and incubated overnight at 37°C while shaking at 250 rpm. The cultures were then centrifuged for 5 minutes at 4 000 × g at room temperature. The pellets were then resuspended in 180 µL of Buffer P1, the resuspension buffer (Appendix 5.2.1). The cells were then lysed with 160 µL of Buffer P2, the lysis buffer (Appendix 5.2.2), and the mixture inverted before incubating at room temperature for 5 minutes. Following this, 120 µL of Buffer P3, the neutralisation buffer (Appendix 5.2.3), was

added and mixed by inverting before incubating on ice for 5 minutes. The mixture was then centrifuged at 12 000 × g for 15 minutes at 4°C. The supernatant was then transferred to a new tube, and the plasmid DNA was precipitated from the solution by adding 600 µL of cold isopropanol. The mixture was inverted and incubated at room temperature for 2 minutes, and was then centrifuged at 12 000 × g for 20 minutes at 4°C. The resultant pellet was then washed with 150 µL of cold 70% ethanol and centrifuged for 5 minutes at 4°C. The supernatant was then removed, and the pellet was allowed to air dry for approximately 10 minutes. The DNA was then dissolved in 50 µL of distilled water.

2.2.3 Large scale plasmid extraction (maxi prep)

Larger quantities of purified plasmid DNA samples were extracted using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). Briefly, a single colony was inoculated in 300 mL of LB with the appropriate antibiotic and was incubated overnight at 37°C while shaking at 250 rpm. The culture was then centrifuged at 6 000 × g for 15 minutes at 4°C. The pellet was then resuspended in 10 mL of resuspension buffer, and the bacteria lysed using 10 mL of lysis buffer, with the mixture inverted for thorough mixing. The mixture was then incubated at room temperature for 5 minutes, after which 10 mL of cold neutralisation buffer was added and the mixture inverted, before incubating on ice for 20 minutes. The mixture was then centrifuged at 4 000 × g for 45 minutes at 4°C, during which time, the columns were prepared by first filling with distilled water and allowing it to flow through the column. Once empty, 10 mL of equilibration buffer (Appendix 5.2.6) was added and allowed to flow through the column. Once the sample had been centrifuged, it was filtered by adding it to a syringe filled with fish tank wool and loaded into the column. The column was washed twice with 30 mL of wash buffer (Appendix 5.2.4), and the DNA eluted into 10.5 mL of cold isopropanol by adding 15 mL of elution buffer (Appendix 5.2.5). The sample was then centrifuged at 6 000 × g for 60 minutes at 4°C. The supernatant was then discarded, and the pellet washed with 5 mL of cold 70% ethanol, and centrifuged for 20 minutes at 4 000 × g at 4°C. The supernatant was discarded, and the pellet was allowed to air dry for approximately 10 minutes. The DNA pellet was then dissolved in 300 µL of distilled water. The DNA concentration was determined through NanoDrop spectrophotometry using a NanoPhotometer® (Implen, California, USA)

2.2.4 Agarose gel electrophoresis

An agarose gel of the appropriate concentration was made by adding agarose powder to 1 × TAE running buffer (1 g of agarose powder to 100 mL of TAE running buffer for a 1% agarose gel) and was briefly mixed by swirling before heating in the microwave for approximately 2 minutes. The volume of the solution was adjusted back to 100 mL after microwaving due to the loss of volume through evaporation. The solution was allowed to cool slightly before ethidium bromide was added (10 µL of 10 mg/mL ethidium bromide) to allow for the visualisation of DNA in the agarose gel under UV light. The gel was poured into a casting tray and a comb added, and the gel was allowed to solidify. Purple (6×) gel loading dye, (NEB, Massachusetts, USA) was added to DNA samples to a 1× final concentration, and the samples were then loaded into the wells in the gel along with a Quick-Load® Purple 1 kb Plus or Quick-Load ® 1 kb Extend DNA Ladder (NEB, Massachusetts, USA). The samples were run at 120 V after which the DNA was visualised under UV light using the Omega Fluor™ Gel Documentation system (Aplegen, Inc, California, USA).

2.2.5 DNA extraction from an agarose gel

A scalpel was used to excise DNA fragments from an agarose gel for purification. The lid of a 2 mL tube was cut off, and a needle heated by the flame of a Bunsen burner was used to poke a hole through the bottom of a 1.5 mL tube. Fish tank wool was added to the bottom of the 1.5 mL tube, and the excised gel fragment was added to this. The 1.5 mL tube was added to the 2 mL tube, and this was centrifuged for approximately 10 minutes at 14 000 × g. The fish tank wool acts as a filter to separate the agarose gel from the DNA. Once sufficient fluid was collected in the 2 mL tube, a 0.75 × volume of phenol-chloroform was added to the solution. This was mixed thoroughly then centrifuged at 14 000 × g for 2 minutes. The upper aqueous phase was then removed and added to a new tube, to which an equal volume of chloroform was added. This was mixed thoroughly and centrifuged at 14 000 × g for 2 minutes. The upper aqueous phase was then removed and added to a new tube, and the previous chloroform extraction step was repeated at least once more to remove any residual phenol. Following this, 2.5 × the sample volume of 100% ethanol was added, and 0.1 × the sample volume of 3 M sodium acetate at pH 5.2 was added. This was then centrifuged for 10 minutes at 14 000 × g at 4°C. The supernatant was then removed, and the pellet washed with 150 µL of 70% ethanol, and centrifuged for 10 minutes at 14 000 × g at 4°C. The supernatant was

then discarded, and the DNA pellet allowed to air dry for approximately 10 minutes, after which it was then dissolved in 15 µL of distilled water.

2.2.6 Isopropanol precipitation

Room temperature isopropanol of 3 × the sample volume was added to the DNA. The sample was mixed and immediately centrifuged at 4℃ at 14 000 × g for 30 minutes. The supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol. This was then centrifuged for 15 minutes at 4°C at 14 000 × g. The supernatant was then discarded the pellet was washed repeatedly with 150 µL of 70% ethanol. The pellet was then allowed to air dry for 5 – 10 minutes before being resuspended in distilled water.

2.3 DNA modification

2.3.1 Restriction analysis of plasmid DNA

Restriction digests were performed as per the manufacturers' instructions (Thermo Fisher Scientific, Massachusetts, USA or NEB, Massachusetts, USA). In brief, DNA was added to a 1.5 mL tube, along with the appropriate volume of enzyme, reaction buffer, and distilled water to the required reaction volume. The reactions were mixed well and incubated at 37℃ for durations ranging from 1 hour to overnight.

For partial digestion, plasmid DNA was added to a reaction mixture with a reaction buffer in which the enzyme was only 50% active, and the reaction was incubated at 37℃ for durations ranging from 5 minutes to 20 minutes. The reactions were then immediately heat inactivated at 65°C for 20 minutes.

2.3.2 Plasmid DNA dephosphorylation

Linear fragments of plasmid DNA were dephosphorylated to prevent re-ligation of the ends of the DNA fragment. The phosphate groups on the ends of the DNA fragments were removed using 1 unit of FastAP (Thermo Fisher Scientific, Massachusetts, USA) per a maximum of 1 µg DNA. Following heat inactivation of restriction digest reaction at 65°C for 15 minutes, the FastAP was added directly and incubated at 37°C for 1 hr.

2.3.3 Polymerase chain reaction (PCR)

For TA cloning of PCR products into a TA cloning vector, Ampliqon Taq DNA Polymerase 2× Master Mix Red (Ampliqon, Odense, Denmark) was used as per the manufacturer's instructions. Each 20 µL reaction contained 10 ng of template DNA, 0.2 µM of both the forward and the reverse primers, and 2× Taq Master Mix to a final concentration of 1×. The primers used are listed in Table 2.2, and the cycling conditions are outlined in Table 2.3.

Table 2.2: Primers used for amplification in PCR.

Primer	Primer Sequence	Description	Source
NLUC FWD	5` - GGC GCG CCG CCA ACT CCA TCA CTA GG - 3`	Forward primer used to amplify the nanoLuciferase sequence, with an *Asc* I site engineered at the end, indicated in orange.	This study.
NLUC RV	5` - GGC GCG CCT CCC CCT GAA CCT GAA AC - 3`	Reverse primer used to amplify the nanoLuciferase sequence, with an *Asc* I site engineered at the end, indicated in orange	This study
PAAVF1	5` - GAT CGG CGC GCC CCT CTA GAC TCG AGG CGT - 3`	Forward primer used to amplify the anti-HBV CRISPR/Cas9 sequence, with an *Asc* I site engineered at the end, indicated in orange.	Donated by Betty Maepa (AGTRU)
PAAVR1	5` - GAT CGG CGC GCC GTT CCT GCG GCC GCA AA - 3`	Reverse primer used to amplify the anti-HBV CRISPR/Cas9 sequence, with an *Asc* I site engineered at the end, indicated in orange.	Donated by Betty Maepa (AGTRU)
ORI FRAG FWD	5` - CTC CAA GCT GGG CTG TGT GCA C - 3`	Forward primer used to amplify the ori fragment from pShuttle-CMV.	This study
ORI FRAG RV	5` - CTT TTT CCG AAG GTA ACT GGC TTC AGC AG - 3`	Reverse primer used to amplify the ori fragment from pShuttle-CMV.	This study

Table 2.3: PCR cycling conditions.

Step	**Temperature**	**Duration**	**Cycles**
Initial denaturation	95°C	3 mins	1
Denaturation	95°C	30 secs	×30
Annealing	55°C	30 secs	
Extension	72°C	2 mins	
Final Extention	72°C	2 mins	
Hold	4°C	∞	1

2.3.4 DNA ligation

Ligation reactions were performed using the pGEM®-T Easy Vector System (Promega, Wisconsin, USA), T4 DNA ligase (NEB, Massachusetts, United States), or the Fast-Link DNA Ligation Kit (Biosearch Technologies, Hoddesdon, United Kingdom) as per manufacturer's instructions. In brief, each reaction mixture contained 1 × ligation buffer, 1 µL of ligase, between 50 ng to 100 ng of backbone DNA, and various ratios of insert DNA, with ATP also included in the Fast-Link ligations. The reactions were incubated overnight at 4°C and were used to transform appropriate *E. coli* bacterial cells.

2.3.5 Construction of transgene expressing pGEMShuttle and homologous recombination to produce HDAdVs recombinant plasmids

The initial cloning strategy to produce transgene expressing HDAdVs is outlined in Figure 2.1. To insert transgenes into pGEMShuttle (Appendix 5.5), the anti-HBV CRISPR/Cas9 sequence was first prepared by digesting both the SaCas9-containing plasmid pAAV-CMV-SaCas9-Bgh-U6sgRNA (Appendix 5.8) and the sgRNA containing plasmid pTZ-SaU6-sgRNA-8 with *Kpn* I and *Not* I to excise the sgRNA sequence and provide compatible sticky ends in the SaCas9 vector to which it was ligated to. The resultant plasmid, pAAV-CMV-saCas9-Bgh-U6-sgRNA-8 (Appendix 5.9), was used as the template in a PCR with the forward and reverse primers PAAVF1 and PAAVR1 respectively, synthesised by Inqaba Biotech, Pretoria, South Africa, to amplify the anti-HBV CRISPR/Cas9 sequence and engineer *Asc* I restriction sites to the ends of the amplicon. The nLuc-containing plasmid, pAAV-nLuc (Appendix 5.7), was similarly used as the template in a PCR with the forward and reverse primers NLUC FWD and NLUC RV respectively, synthesised by Inqaba Biotech, Pretoria, South Africa, to add the same restriction sites to the ends of the nLuc transgene. Each of these amplicons were TA cloned into the pGEM®-T Easy cloning vector (Appendix 5.6, 5.10), and once the sequences were confirmed by Sanger sequencing (Inqaba Biotech, Pretoria, South Africa) (Appendix 5.4), each transgene was excised from the backbone through *Asc* I digestion. The shuttle plasmid pGEMShuttle was linearised by *Asc* I, and each transgene was ligated to the shuttle to produce two plasmids, pGEMShuttlenLuc and pGEMShuttleCRISPR/Cas9. Each of these transgene-containing shuttle plasmids were linearised by *Pac* I then used with the HDAdV genome bearing plasmid pD28E4LacZ (Appendix 5.12) to co-transform BJ 5183 *E. coli* cells, wherein the bacterial cells DNA repair mechanisms facilitated

homologous recombination between the two plasmids to insert the transgenes into the HDAdV genome to produce the two plasmids pD28nLuc and pD28CRISPR/Cas9.

2.3.6 Replacement of the high copy number ori with a low copy number ori in pGEMShuttle

To replace the high copy number ori present in pGEMShuttle with a low copy number ori, the ori fragment that controls the copy number of the plasmid was excised from pGEMShuttle through partial digestion with *Acu* I, followed by further digestion with *Bse* YI. Partial *Acu* I digestion was necessary because this restriction site cleaves pGEMShuttle at two points, within the ori and within the *KanR* gene. To remove the ori fragment, it was necessary to only linearise pGEMShuttle within the ori, then digest the resultant fragment with *Bse* YI to excise the ori fragment. The resultant DNA fragment was then used as the backbone into which the new ori fragment could be inserted. pShuttle-CMV (Appendix 5.13), a low copy number plasmid, was used as the template in a PCR with the forward and reverse primers ORI FRAG FWD and ORI FRAG RV respectively, synthesised by Inqaba Biotech, Pretoria, South Africa, to amplify the region of the ori that controls the copy number of the plasmid. These primers were designed to encompass the *Acu* I and *Bse* YI sites that flank the ori fragment of interest. The resultant amplicons were digested with *Acu* I and *Bse* YI to produce compatible sticky ends with the pGEMShuttle fragment generated earlier. The two fragments were ligated together, and the ori of the resultant plasmid, pGEMShuttleLCori, was sequenced (Inqaba Biotech, Pretoria, South Africa) to confirm successful replacement of the ori sequence.

2.3.7 Insertion of the E4 sequence in the pGEMShuttleLCori

To insert a fragment of the Ad E4 ORF1 sequence into pGEMShuttleLCori, an insert containing this region, a multiple cloning site, and the Ad ITR sequence was synthesised (Genscript, New Jersey, USA) and received in a *KanR* expressing pUC plasmid. This plasmid, and pGEMShuttleLCori, were digested with *Asc* I and *Spe* I to excise the Ad E4 ORF1 and ITR fragment and create compatible sticky ends that allowed the two fragments to be ligated to each other. The resultant plasmid, pGEMShuttleLCoriE4 (Appendix 5.14) was then used for downstream applications to assess the stability of recombinants formed from this plasmid.

METHODS AND MATERIALS

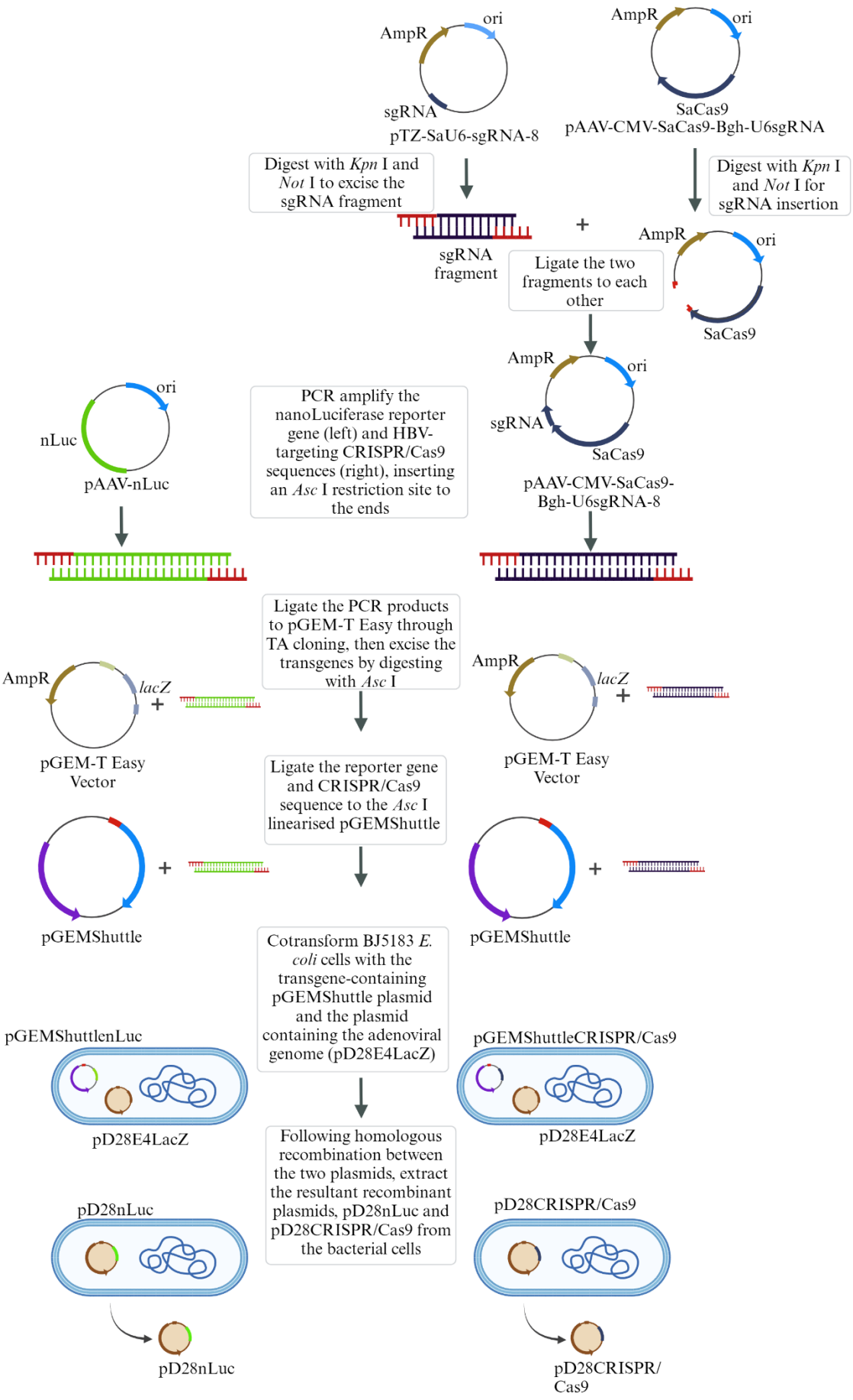


Figure 2.1: Cloning strategy to produce transgene expressing HDAdVs.
Schematic representation of the cloning strategy used to insert either an nLuc reporter gene or an anti-HBV CRISPR/Cas9 sequence into pD28E4LacZ using the shuttle plasmid pGEMShuttle. (Created with Biorender.com).

2.4 Tissue culturing

2.4.1 Mammalian cell culturing and maintenance

Human embryonic kidney derived HEK293T cells and human liver derived Huh7 cells were grown in optimal conditions in a humidified incubator at 37℃ and 5% CO_2. Cells were grown in Dulbecco's modified Eagles medium (DMEM) (Appendix 5.3.1). The medium was supplemented with 10% or 5% Foetal Bovine serum (FBS) (Sigma-Aldrich, Missouri, USA) (Appendix 5.3.3) and the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml) (Appendix 5.3.2). Cells were first grown from a liquid nitrogen freezer stock from which they were thawed by incubation at 37°C. The cells were then added to 9 mL of DMEM with 10% FBS and centrifuged for 3 minutes at 800 rpm. This pellet was then resuspended in 1 mL of DMEM with 10% FBS and added to a T25 cell culture flask with 4 mL of media. The cells were then incubated at 37℃ and 5% CO_2 until confluent. Cells were detached from the flasks by first removing the media and adding saline-EDTA solution (for HEK293T cells) or TrypLE Express (Gibco, Thermo Fisher Scientific, Massachusetts, USA) (Appendix 5.3.4) (for Huh 7 cells) to the cells and incubated for 3 minutes at 37℃ and 5% CO_2 after which the flask was lightly tapped to detach the cells. The TrypLE Express solution was inactivated with 3 mL of DMEM with 10% FBS. These suspended cells were then transferred to a T75 cell culture flask if necessary. The procedure for splitting cells is similar, but the resuspended cells are divided between the required flasks to a confluency and final volume of media specified by the flasks' supplier. The media in the flasks were replaced every 48 hours.

2.4.2 Transfection

Mammalian cells were transfected using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, Massachusetts, United States) according to manufacturer's instructions. Cells were seeded such that they were between 70-90% confluent on the day of transfection. Lipofectamine ™ 3000 Reagent amount equal to DNA amount was diluted in Opti-MEM to make up the

Lipofectamine™ mixture, while a DNA mixture was prepared with DNA diluted in Opti-MEM containing P3000™ Reagent. Along with the relevant DNA, eGFP encoding plasmid was also included to assess transfection efficiency through fluorescence microscopy using an EVOS Fluorescence Microscope (Invitrogen, Massachusetts, USA). Diluted DNA mixture was added to the diluted Lipofectamine™ 3000 Reagent to form DNA-lipid complex at a ratio of 1:1. This was allowed to incubate at room temperature for 15 minutes, after which the DNA-lipid complex was added to the cells and allowed to incubate at 37℃ and 5% CO_2.

2.4.3 Assessment of HBsAg knockdown using an ELISA

HEK 293T cells were seeded in a 24 well plate. Cells were transfected using Lipofectamine™ 3000 as per Section 2.4.2 with 100 ng pCH-9/309 (Appendix 5.15) and 200 ng total DNA of the various anti-HBV CRISPR/Cas9 sequence bearing plasmids, and transfections were performed in quadruplicate. Two days after transfection, media was collected and the concentration of HBsAg present was measured using the Monolisa HBsAg Ultra Kit (Bio-Rad, California, USA) as per the manufacturer's protocol. In brief, 100 µL of media was added to the provided 96 well plate, and 50 µL of conjugate solution was added to each well. The plate was sealed and incubated for 90 mins at 37°C. The plate was then washed five times using the ImmunoWash 1575 Microplate Washer (Bio-Rad, California, USA), and 100 µL of development solution was then added. The reactions were developed in the dark for 30 mins, after which 100 µL of stopping solution was added. The optical density of each sample was measured at 490 nm and 655 nm using the iMark™ Microplate Absorbance Reader (Bio-Rad, California, USA).

2.4.4 Luciferase assay

Huh 7 cells were seeded in a 24 well plate and transfected using Lipofectamine™ 3000 as per Section 2.4.2, with 500 ng of nLuc-expressing plasmid DNA, and transfections were performed in quadruplicate. Two days after transfection, nLuc activity was assess using the Nano-Glo® Luciferase Assay System (Promega, Wisconsin, USA) as per the manufacturer's instructions. In brief, an equal volume of spent media and Nano-Glo® Luciferase Assay Reagent, 100 µL each, was added to each well of a 96 well plate. After at least three minutes, the luminescence was measured using the GloMax Explorer Microplate Reader (Promega, Wisconsin, USA)

2.5 Statistical analysis

Data was expressed as the mean ± the standard error of the mean (SEM). Statistical significance was calculated using Microsoft Excel and was considered to be significant when a two-tailed, paired Student's T test indicated $P < 0.05$.

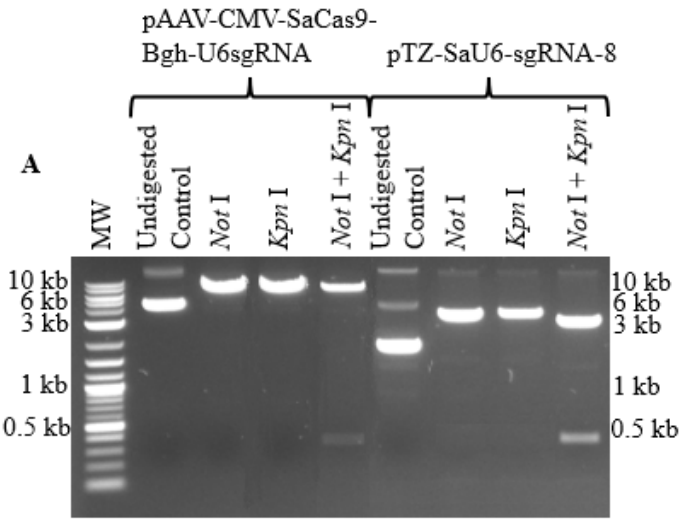
CHAPTER 3

3. RESULTS

3.1 Preparation of transgene donor plasmids

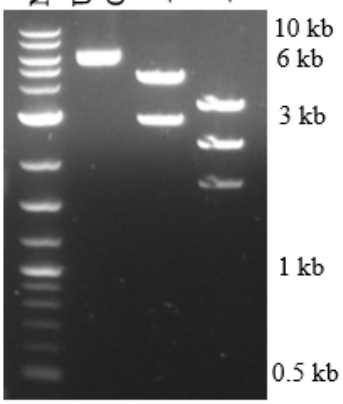
To assemble the anti-HBV CRISPR/Cas9 sequence, the previously constructed plasmids pAAV-CMV-SaCas9-Bgh-U6sgRNA (Appendix 5.8) carrying the saCa9 and non-HBV targeting sgRNA encoding sequences and pTZ-SaU6-sgRNA-8 carrying HBV-targeting sgRNA encoding sequence were used (Ran et al., 2015; Scott et al., 2017). The identity of the two plasmids was confirmed by restriction analysis using *Not* I and *Kpn* I. As expected *Not* I and *Kpn* I linearised both plasmids to give 7 445 bp and 3 240 bp fragments respectively. When used together, the two enzymes produced a 352 bp fragment and a 7 093 bp fragment from pAAV-CMV-SaCas9-Bgh-U6sgRNA or a 2 888 bp fragment from pTZ-SaU6-sgRNA-8 (Figure 3.1A). The 352 bp, *Not* I and *Kpn* I digested fragment from pTZ-SaU6-sgRNA-8 encoded the anti-HBV guide and was ligated to the similarly digested pAAV-CMV-SaCas9-Bgh-U6sgRNA to replace the non-HBV targeting sgRNA encoding sequence and produce pAAV-CMV-saCas9-Bgh-U6-sgRNA-8, a single plasmid containing both the saCas9 and the sgRNA8 encoding sequences. The ligation was of relatively high efficiency and low background owing to the dephosphorylation of the backbone. Following screening of the eight colonies picked (results not shown), the sequence integrity of the plasmid from one positive clone was further assessed by restriction analysis using the restriction enzymes *Not* I, *Xba* I, *Nde* I, and *Hind* III. The 7 452 bp plasmid was cut twice with *Not* I and *Xba* I to give two fragments of 2 881 bp and 4 571 bp, while it was cut thrice by *Hind* III and *Nde* I to give three fragments of 1 762 bp, 2 365 bp, and 3 325 bp, confirming the successful insertion of the anti-HBV sgRNA encoding sequence into the pAAV-CMV-SaCas9-Bgh-U6sgRNA plasmid (Figure 3.1B).

The nanoLuciferase (nLuc) encoding sequence was obtained from the previously constructed pAAV-nLuc plasmid (Appendix 5.7). Restriction analysis of pAAV-nLuc plasmid was performed using *Asc* I, *Apa* LI, and *Bam* HI. *Asc* I linearised the 5 308 bp plasmid, while *Apa* LI cleaved it twice to give two fragments of 1 246 bp and 4 062 bp, and *Bam* HI cleaved it twice to give two fragments of 1 618 bp and 3 690 bp, confirming the identity of the plasmid. (Figure 3.1C).

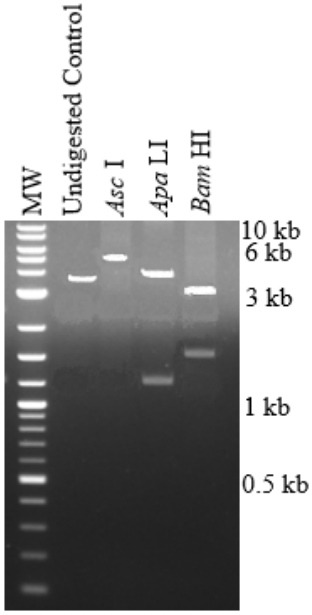


Fragment sizes of pAAV-CMV-SaCas9-Bgh-U6sgRNA after digestion:
Not I: 7 445 bp
Kpn I: 7 445 bp
Not I + *Kpn* I: 352 bp + 7 093 bp

Fragment sizes of pTZ-SaU6-sgRNA-8 after digestion:
Not I: 3 240 bp
Kpn I: 3 240 bp
Not I + *Kpn* I: 352 bp + 2 888 bp



Fragment sizes of pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8 after digestion:
Not I + *Xba* I: 2 881 bp + 4 571 bp
Hind III + *Nde* I: 1 762 bp + 2 365 bp + 3 325 bp
Same banding pattern expected with the digestion of the pAAV-CMV-SaCas9-Bgh-U6sgRNA backbone.



Fragment sizes of pAAV-nLuc after digestion:
Asc I: 5 308 bp
Apa LI: 1 246 bp + 4 062 bp
Bam HI: 1 618 bp + 3 690 bp

Figure 3.1: Restriction analysis of the transgene donor plasmids.

*Agarose gel showing the restriction analysis of (**A**) pAAV-CMV-SaCas9-Bgh-U6sgRNA and pTZ-SaU6-sgRNA-8 with Kpn I and Not I, (**B**) pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8 using the restriction enzymes Not I with Xba I and Hind III with Nde I and (**C**) pAAV-nLuc with the enzymes Asc I, Apa LI, and Bam HI. Fragment sizes were determined using the molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

An ELISA was performed to assess the functionality of the anti-HBV CRISPR/Cas9 sequences in pAAV-CMV-saCas9-Bgh-U6-sgRNA-8 as compared to when the sgRNA and the saCas9 are expressed from separate plasmids. HEK 293T cells were co-transfected with pCH-9/3091 (Nassal et al., 1990), a replication competent HBV plasmid and the experimental plasmid. The supernatant from the transfected cells was then harvested and used to assess HbsAg expression knockdown using the MONOLISA anti-HBs Kit (Bio-Rad, California, USA). The knockdown effect seen from the anti-HBV CRISPR/Cas9 sequences in a single plasmid and two separate plasmids were comparable to each other, both of which resulted in over 95% reduction in HbsAg levels relative to the HbsAg expression from cells transfected with a plasmid expressing saCas9 and a non-HBV targeting sgRNA. As expected, no HbsAg expression was detected in untransfected cells. This demonstrated that the combined anti-HBV CRISPR/Cas9 sequences were functional, and that there was no difference in function as compared to when the sgRNA sequence and Cas 9 sequence are in separate plasmids (Figure 3.2).

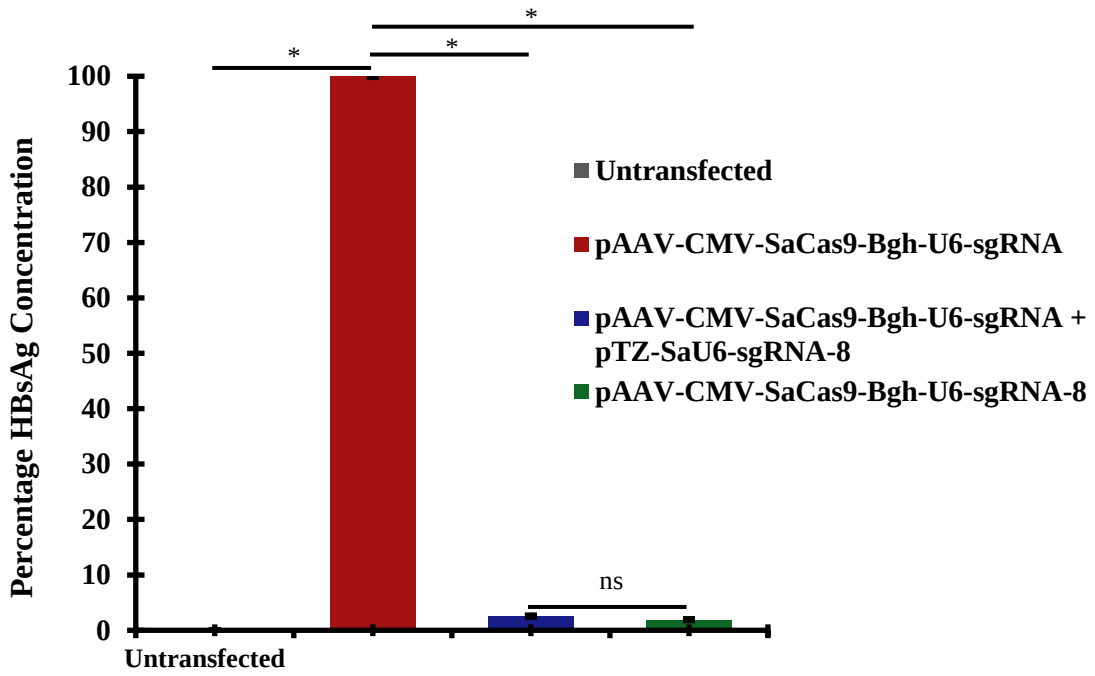
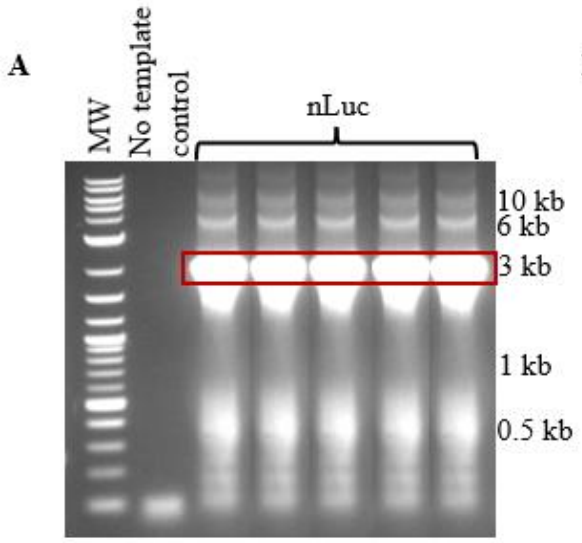


Figure 3.2: Effect of different anti-HBV CRISPR/Cas9 plasmids on HBsAg expression.
*HEK 293 T cells were co-transfected with HBV genome containing plasmid (pCH-9/3091) and anti-HBV CRISPR/Cas 9 plasmid/s. HBsAg expression was measured using an ELISA. Values represent normalised means and standard error of mean (SEM). A two tailed, paired, T test was used to compare data, where * indicates a significant difference of $P < 0.05$ and ns indicates no statistical significance.*

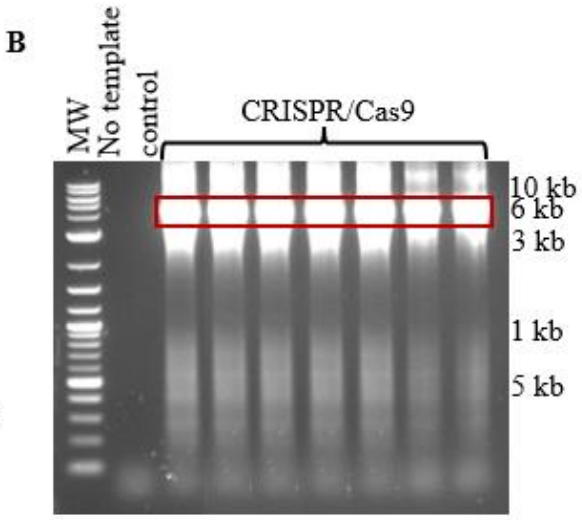
3.2 Engineering of restriction sites for transgene insertion into the shuttle plasmid

To amplify each of the transgenes using pAAV-nLuc and pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8 as templates, polymerase chain reactions (PCRs) were performed using primers designed to have *Asc* I restriction site. Following nLuc sequence amplification using pAAV-nLuc as a template, an expected 1983 bp PCR product was obtained (Figure 3.3A). PCR amplification using pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8 as a template to amplify the anti-HBV CRISPR/Cas9 sequence resulted in an expected 4601 bp product (Figure 3.3B). The PCRs resulted in significant non-specific amplification, however, subsequent gel extractions and cloning of these fragments were unaffected.

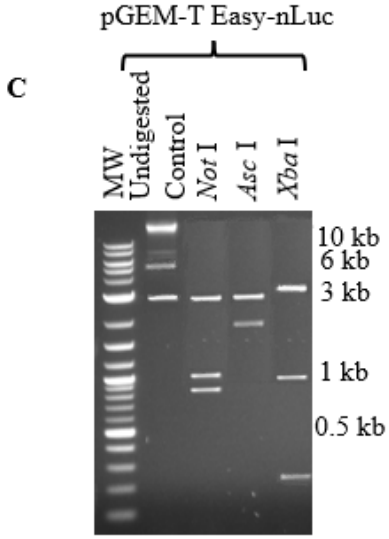
TA-cloning of the 1983 bp and 4 601 bp PCR products into the cloning vector pGEM®-T Easy (Promega, Wisconsin, USA) produced pGEM-T Easy-nLuc and pGEM-T Easy-CRISPR/Cas9 plasmids respectively (Appendix 5.10). Following screening (data not shown), further restriction analysis was performed to confirm sequence integrity of the plasmid from one positive clone. As expected, digestion of pGEM-T Easy-nLuc with *Not* I gave three fragments of 923 bp, 1 094 bp, and 2 981 bp while digestion with *Asc* I gave two fragments of 1 973 bp and 3 025 bp, and *Xba* I resulted in three fragments of 261 bp, 1 101 bp, and 3 636 bp (Figure 3.3C). Digestion of pGEM-T Easy-CRISPR/Cas9 with *Ava* I produced four fragments of 240 bp, 1 263 bp, 1 387 bp, and 4 726 bp with 1 263 bp and 1 387 bp fragments migrating together. Digestion with *Eco* RI resulted in three fragments of 631 bp, 2 997 bp, and 3 988 bp while *Nde* I produced three fragments of 235 bp, 3 254 bp, and 4 127 bp with 3 254 bp and 4 127 bp fragments migrating closer to each other. This confirms successful cloning of the anti-HBV sequence (Figure 3.3D). Sequencing confirmed sequence integrity of both inserts (Appendix 5.4).



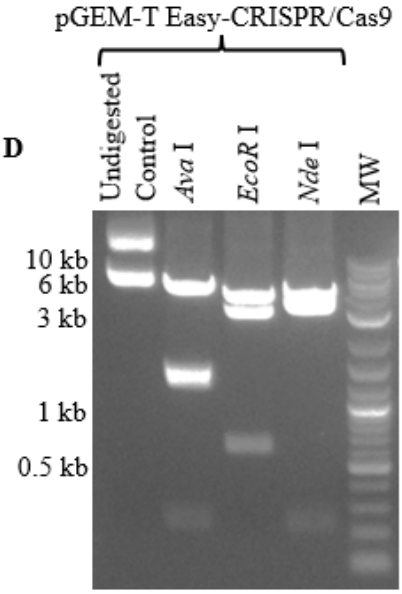
Expected fragment sizes after PCR:
1 983 bp



Expected fragment sizes after PCR:
4 601 bp



Fragment sizes of pGEM-T Easy-nLuc after digestion:
Not I: 923 bp + 1 094 bp + 2 981 bp
Asc I: 1 973 bp + 3 025 bp
Xba I: 261 bp + 1 101 bp + 3 636 bp
Expected fragment sizes of pGEM®-T Easy backbone after digestion:
Not I: 36 bp + 2 981 bp
Asc I: Undigested
Xba I: Undigested



Fragment sizes of pGEM-T Easy-CRISPR/Cas9 after digestion:
Ava I: 240 bp + 1 263 bp + 1 387 bp + 4 726 bp
Eco RI: 631 bp + 2 997 bp + 3 988 bp
Nde I: 235 bp + 3 254 bp + 4 127 bp
Expected fragment sizes of pGEM®-T Easy backbone after digestion:
Ava I: Undigested
Eco RI: 20 bp + 2 997 bp
Nde I: 3 017 bp

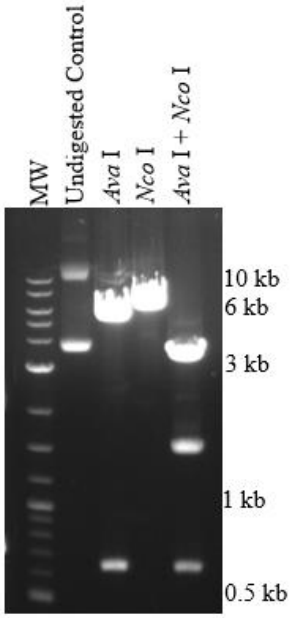
Figure 3.3: PCR amplification of the transgenes and the screening of their ligations to pGEM®-T Easy.

*Agarose gels showing the (**A**) PCR amplification of the nLuc transgene from pAAV-nLuc and (**B**) the PCR amplification of the anti-HBV CRISPR/Cas9 sequence from pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8, with the intended DNA fragments highlighted in red. Agarose gel showing the restriction analysis of (**C**) pGEM-T Easy-nLuc with Not I, Asc I, and Xba I, and (**D**) pGEM-T Easy-CRISPR/Cas9 with Ava I, Eco RI, and Nde I. Fragment sizes were determined using the molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

3.3 Insertion of transgenes into pGEMShuttle

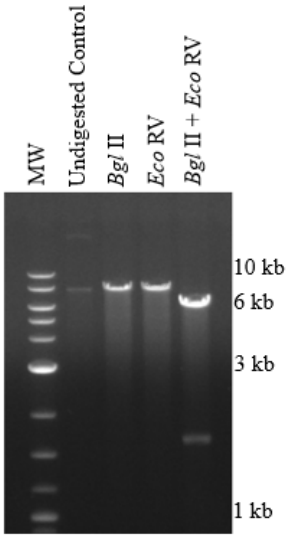
Previously constructed pGEMShuttle (not published) was first digested with *Ava* I and *Nco* I as single or double digest to verify its identity (Figure 3.4A). Digestion with *Ava* I produced two fragments of 687 bp and 5 150 bp, while digestion with *Nco* I linearised the plasmid to result in one fragment of 5837 bp. The double digest cleaved the plasmid three times to give fragments of 687 bp, 1 575 bp, and 3 575 bp. The nLuc and anti-HBV CRISPR/Cas9 sequences were then excised from their respective donor plasmids using restriction digestion with *Asc* I and ligated into the *Asc* I digested pGEMShuttle. Following screening (data not shown), one positive clone was confirmed by further restriction analysis. A single digestion of pGEMShuttlenLuc with *Bgl* II or *Eco* RV, linearized the plasmid to give a 7 785 bp band, and a double digest with *Bgl* II and *Eco* RV resulted in two fragments of 1 695 bp and 6 090 bp (Figure 3.4B). Restriction of pGEMShuttleCRISPR/Cas9 with *Kpn* I linearized the plasmid and resulted in a 10 428 bp band, whereas *Apa* LI cleaved the plasmid three times to give fragments of 1 246 bp, 4 290 bp, and 4 892 bp with 4 290 bp and 4 892 bp bands migrating closer to each other (Figure 3.4C). A double digest with both enzymes resulted in four fragments of 1 246 bp, 1 704 bp, 3 188 bp, and 4 290 bp. This confirms a successful insertion of transgenes into the pGEMShuttle.

A



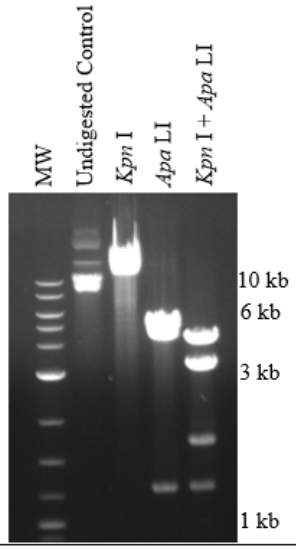
Fragment sizes of pGEMShuttle after digestion:
Ava I: 687 bp + 5 150 bp
Nco I: 5 837 bp
Ava I + *Nco* I: 687 bp + 1 575 bp + 3 575 bp

B



Fragment sizes of pGEMShuttle-nLuc after digestion:
Bgl II: 7 785 bp
Eco RV: 7 785 bp
Bgl II + *Eco* RV: 1 695 bp + 6 090 bp
Expected fragment sizes of pGEMShuttle backbone after digestion:
Bgl II: Undigested
Eco RV: 5 837 bp
Bgl II + *Eco* RV: 5 837 bp

C



Fragment sizes of pGEMShuttle-CRISPR/Cas9 after digestion:
Kpn I: 10 428 bp
Apa LI: 1 246 bp + 4 290 bp + 4 892 bp
Kpn I + *Apa* LI: 1 246 bp + 1 704 bp + 3 188 bp + 4 290 bp
Expected fragment sizes of pGEMShuttle backbone after digestion:
Kpn I: Undigested
Apa LI: 1 246 bp + 4 591 bp
Kpn I + *Apa* LI: 1 246 bp + 4 591 bp

Figure 3.4: Digests to verify the identity of pGEMShuttle and pGEMShuttle carrying transgenes.

*Agarose gel of (**A**) the empty pGEMShuttle plasmid digested with Ava I and Nco I, (**B**) pGEMShuttlenLuc digested with Bgl II and/or Eco RV, and (**C**) pGEMShuttleCRISPR/Cas9 digested with Kpn I and/or Apa LI. Fragment sizes were determined using the molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

To verify the functionality of the CRISPR/Cas9 sequence inserted, silencing of HBsAg expression was assessed by ELISA following co-transfection of HEK 293T cells with pCH-9/3091 and pGEMShuttleCRISPR/Cas9 (Figure 3.5). Transfection with pGEMShuttle-CRISPR/Cas9 resulted in ~ 85% knockdown of HBsAg expression, compared to close to 95% knockdown by pAAV-CMV-SaCas9-Bgh-U6-sgRNA-8. This demonstrated that pGEMShuttleCRISPR/Cas9 was able to effectively express the anti-HBV transgene and silence HBV gene expression. As expected, empty pGEMShuttle did not result in HBV gene expression knockdown and no HBsAg was detected from untransfected cells.

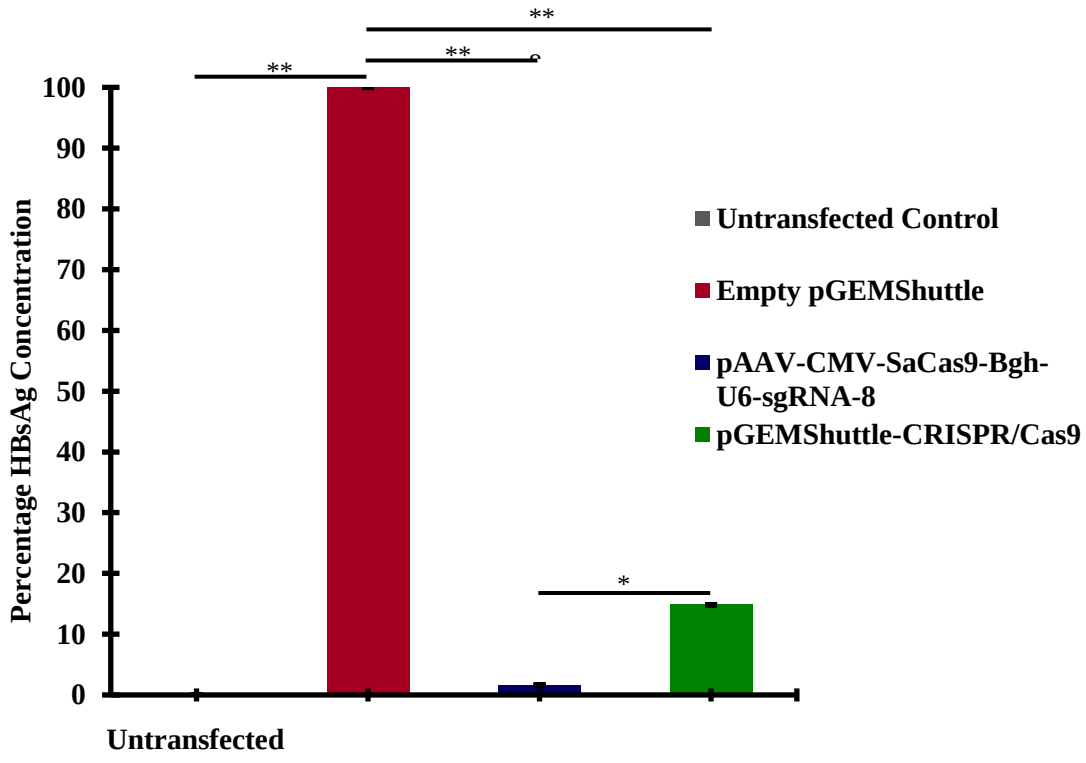


Figure 3.5: Assessment of HBV gene expression silencing by pGEMShuttleCRISPR/Cas9.

*An ELISA was performed to assess the ability of pGEMShuttle-CRISPR/Cas9 to knockdown HBsAg expression in HEK 293T cells transfected with pCH-9/3091. Values represent normalised means of the biological quadruplicate samples and standard error of mean (SEM). A two tailed, paired, T test was used to compare data, where * indicates a significant difference of P < 0.05, and ** indicates a significant difference of P < 0.001.*

A luciferase assay was performed to assess the level of gene expression from pGEMShuttlenLuc using the Nano-Glo® Luciferase Assay System (Promega, Wisconsin, USA) (Figure 3.6). Contrary to Huh 7 cells transfected with pGEMShuttle, cells transfected with either pAAV-nLuc or pGEMShuttle-nLuc showed significant levels of luciferase expression. As expected, untransfected cells did not show any luciferase expression.

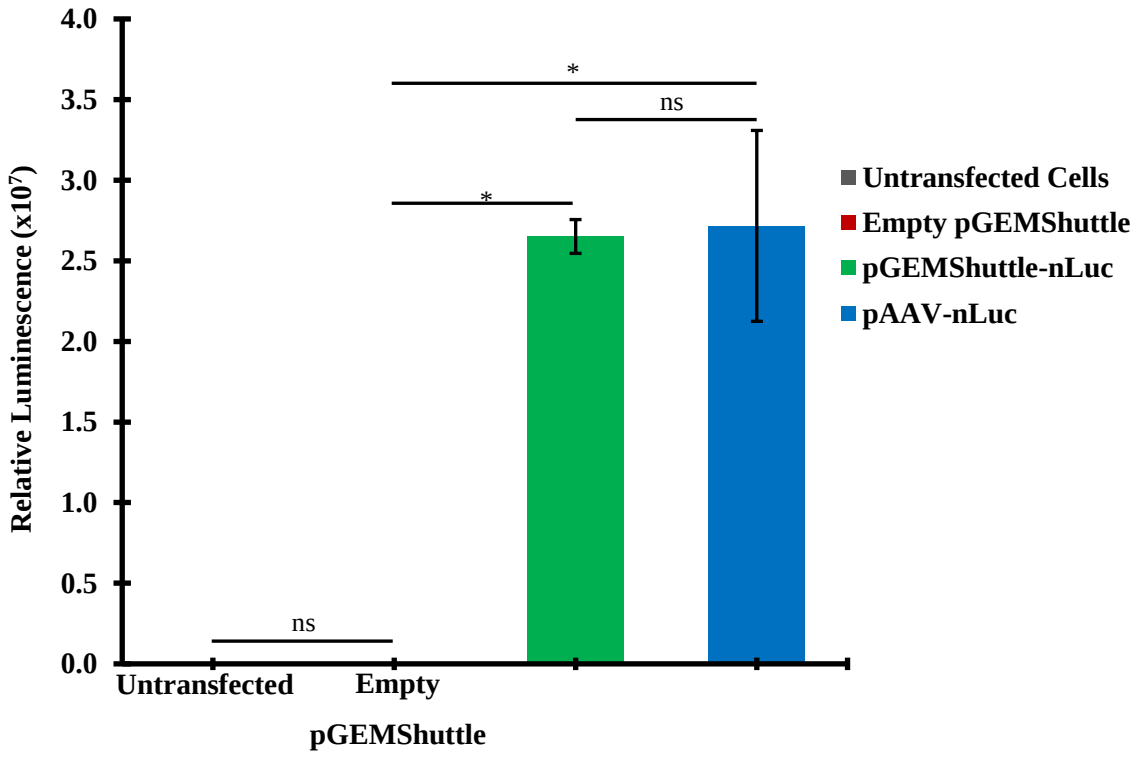


Figure 3.6: Luciferase assay to assess gene expression from pGEMShutlenLuc.

A luciferase assay was used to assess the levels of nLuc expression from pGEMShuttle-nLuc. The luminescence from samples transfected with pGEMShuttlenLuc was compared to that of samples from cells transfected with empty pGEMShuttle, or pAAV-nLuc. Values represent means of the

*biological triplicate samples and standard error of mean (SEM). A two tailed, paired T test was used to compare data, where * indicates a significant difference of P < 0.005 and ns indicates no significance.*

Based on the results obtained from each of these assays, the transgenes and shuttle plasmid were deemed functional, and could be inserted into the HDAdV genome through homologous recombination.

3.4 Insertion of transgenes into the adenoviral genome

To facilitate homologous recombination between pD28E4LacZ and pGEMShuttlenLuc, *recA* expressing *E. coli* BJ 5183 cells (Agilent, California, United States) were transformed with a mixture of pD28E4LacZ and *Pac* I linearized pGEMShuttle-nLuc via electroporation. Following plating, the resultant bacterial colonies were as expected, of two sizes: the larger colonies possibly represented bacteria containing undigested pGEMShuttlenLuc, while the smaller colonies represented bacteria potentially carrying pD28E4LacZnLuc recombinants (Figure 3.7). Five of the smaller colonies were picked, and the plasmid DNA was extracted after overnight culture in LB supplemented with ampicillin. These clones were run uncut on a 1% agarose gel to identify large-sized plasmid species running higher than the uncut pGEMShuttlenLuc plasmid. The potential recombinants all appear to run higher than the background pGEMShuttlenLuc it is compared to because these plasmids are expected to be over 25 kb larger than pGEMShuttlenLuc, which is the only other plasmid species that could be present in the selected bacterial colonies. However, identification of these larger plasmids may have been more accurate had the pGEMShuttlenLuc control been run on the same gel. By identifying these large plasmid species, potential recombinants between pD28E4LacZ and pGEMShuttle can be further screened (Figure 3.8A-B). To avoid further recombination events, DNA from these clones were used to transform a *recA* deficient *E. coli* DH10β cells, which were then plated on ampicillin-containing agar plates, and two colonies were picked from each plate to miniprep. Following restriction analysis, none of the plasmids extracted resulted in the expected banding pattern. Digestion of one such sample with *Bgl* II, *Eco* RV or *Xho* I resulted in a banding pattern indistinguishable from the undigested control sample, with this plasmid running significantly lower than the undigested clones extracted from the *E. coli* BJ 5183 cells as well as the undigested pGEMShuttlenLuc (Figure 3.8B-C). This result

was consistently seen in numerous plasmids extracted following repeated recombination reactions (not shown).

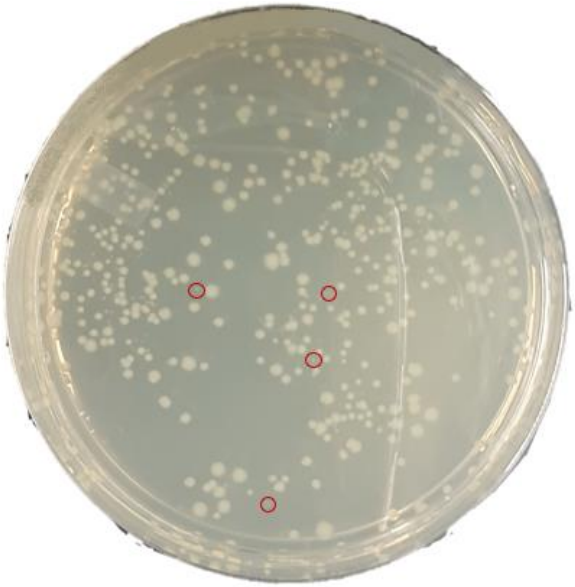
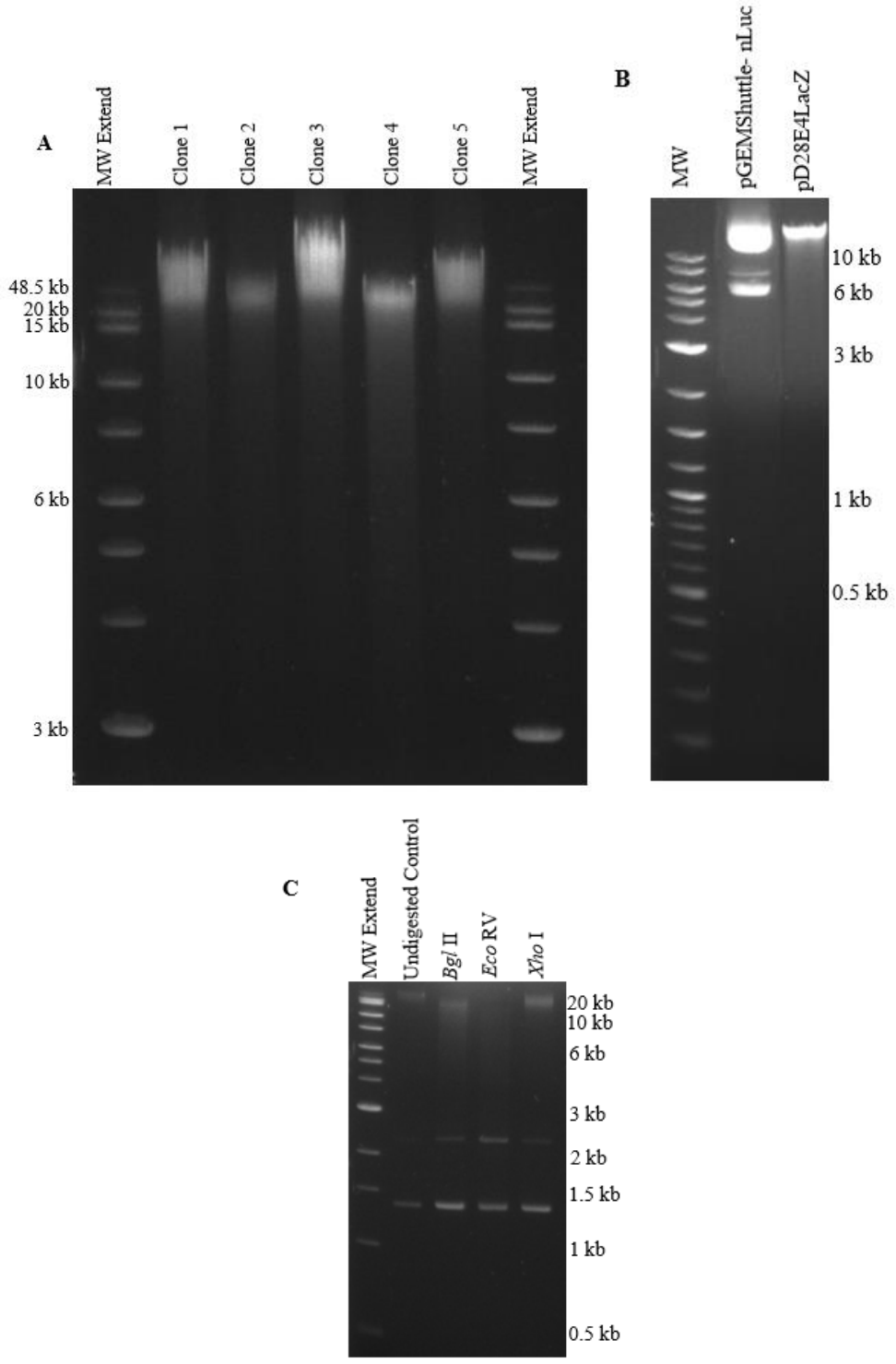


Figure 3.7: Agar plate containing BJ 5183 E. coli cells transformed with pD28E4LacZ and pGEMShuttlenLuc.

BJ 5183 E. coli cells were transformed through electroporation with pD28E4LacZ and linearised pGEMShuttlenLuc. The transformants were plated on ampicillin-containing agar plates. Red circles highlight some of the smaller colonies.



Approximate fragment sizes of pD28E4LacZ-nLuc recombinants expected after digestion:
Bgl II: 6 192 bp + 6 383 bp + 9 065 bp + 10 493 bp
Eco RV: 308 bp + 3 485 bp + 8 326 bp + 9 890 bp + 10 124 bp
Xho I: 3 308 bp + 3 894 bp + 7 315 bp + 17 616 bp

Figure 3.8: Screening of recombinants between pD28E4LacZ and pGEMShuttlenLuc.
*Agarose gels showing (**A**) five isolated recombinants between pD28E4LacZ and pGEMShuttlenLuc run undigested, (**B**) pGEMShuttlenLuc and pD28E4LacZ run undigested as a comparison, and (**C**) one of these recombinants digested with Bgl II, Eco RV, and Xho I. DNA fragment sizes were compared using molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder and (MW Extend) Quick-Load® 1 kb Extend DNA Ladder (NEB).*

Similar reactions were set up using pD28E4LacZ and *Pac* I linearised pGEMShuttleCRISPR/Cas9. BJ 5183 *E. coli* cells (Agilent, California, United States) were transformed with these plasmids and plated. The expected two sizes of bacterial colonies were seen, with the larger colonies representing possible background growth of pGEMShuttle-CRISPR/Cas9, and the smaller colonies representing potential recombinants of pD28E4LacZCRISPR/Cas9 (Figure 3.9). The smaller colonies were picked, and the plasmid DNA was extracted after overnight culture in LB supplemented with ampicillin. These clones were run uncut on a 1% agarose gel to identify large sized plasmid species running higher than the uncut pGEMShuttleCRISPR/Cas9 plasmid, and at a similar level as the uncut pD28E4LacZ. Despite the presence of faint bands indicating high molecular weight plasmid species, running at a similar level to that of uncut pD28E4LacZ, there are clearly much smaller bands visible running significantly lower than even pGEMShuttleCRISPR/Cas9. This suggests the presence of very small plasmids that strongly suggested that unintended recombination had occurred resulting in the resultant recombinants being much smaller than anticipated. This may be due to the host BJ 5183 *E. coli* cells the plasmids were extracted from, which may often cause recombination in plasmids due to its *recA* activity (Figure 3.10A). As earlier, DH10β *E. coli* cells were transformed with some these plasmids and screened. Five of these samples were digested with *Bgl* II, however, all the samples were identical to their undigested controls, demonstrating that none of these plasmids were digested with this enzyme (Figure 3.10B). Each sample also appeared to run at different levels, suggesting that each plasmid was of a different size. Similar results were seen in repeated experiments (not shown).

Generation of smaller plasmids that carried an ampicillin resistance maker following retransformation and propagation suggested that unwanted recombination events are occurring that affected the stability of the recombinants. To determine if the strain of cells being retransformed

was causing the recombination, the plasmid DNA from the supposed pD28E4LacZnLuc and pD28E4LacZCRISPR/Cas 9 recombinants were retransformed into another *recA* deficient *E. coli* XL 10-Gold cells, which yielded similar results (not shown).

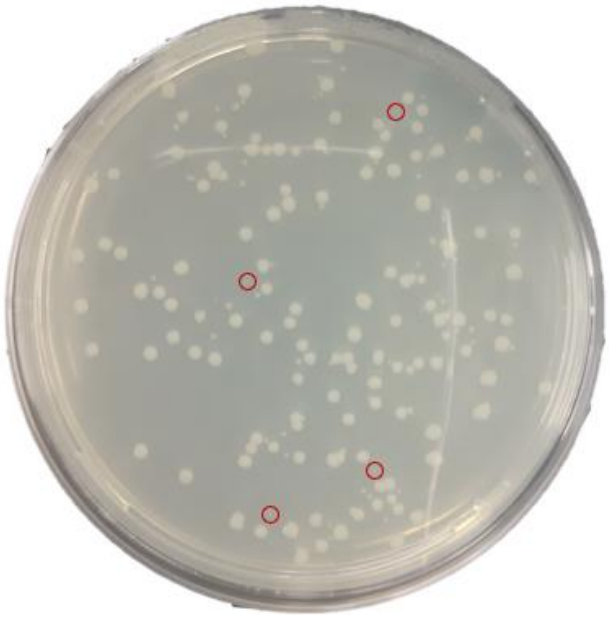
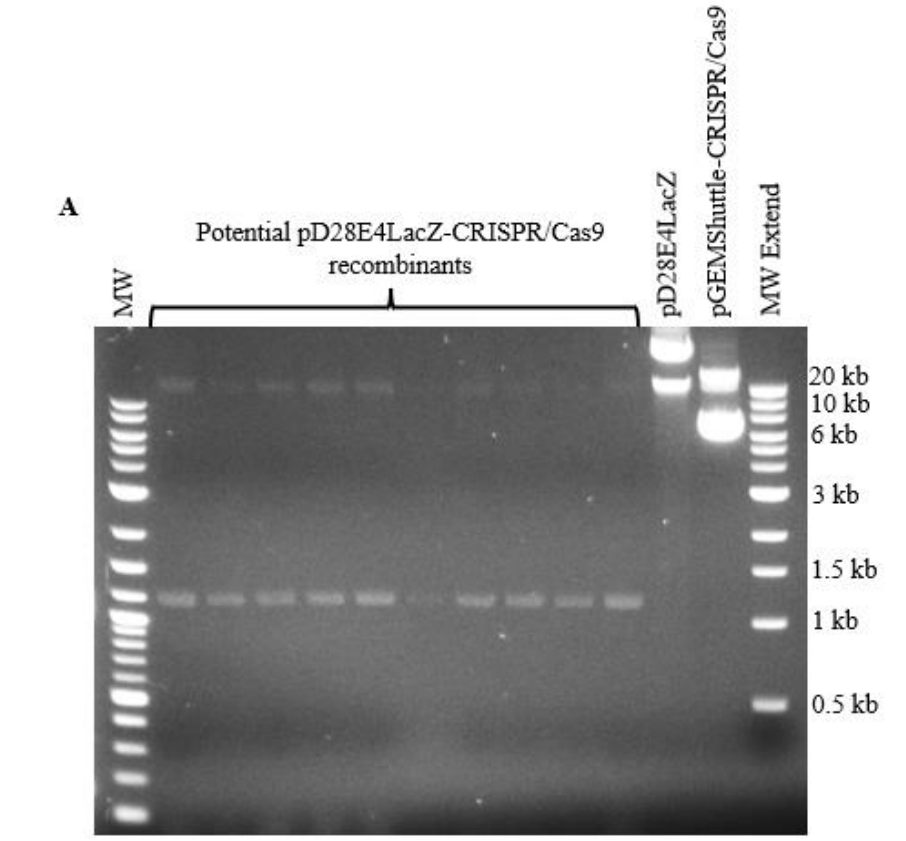
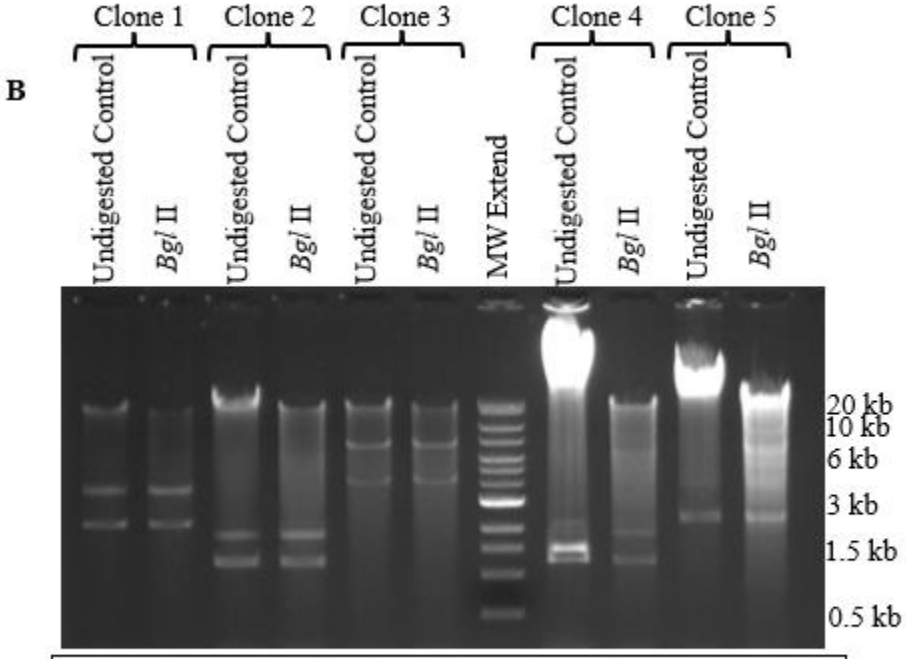


Figure 3.9: Agar plate with BJ 5183 cells transformed with pD28E4LacZ and pGEMShuttleCRISPR/Cas9.

BJ 5183 E. coli cells were transformed through electroporation with pD28E4LacZ and linearised pGEMShuttleCRISPR/Cas9. The transformants were plated on ampicillin-containing agar plates. Red circles indicate some of the smaller colonies.





Approximate fragment sizes of pD28E4LacZ-CRISPR recombinants expected after digestion:
Bgl II: 6 192 bp + 6 383 bp + 9 065 bp + 10 493 bp

Figure 3.10: Screening of recombinants between pD28E4LacZ and pGEMShuttleCRISPR/Cas9.

*Agarose gels showing (**A**) five isolated recombinants between pD28E4LacZ and pGEMShuttle-nLuc run undigested for analysis, with pD28E4LacZ and pGEMShuttle-CRISPR/Cas9 run undigested as a comparison, and (**B**) five of these recombinants digested with Bgl II. DNA fragment sizes were compared using molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder and (MW Extend) Quick-Load® 1 kb Extend DNA Ladder (NEB).*

3.5 Replacement of the high copy ori in pGEMShuttle

Considering the obvious instability of the recombinants previously obtained, a new strategy to produce stable recombinants was needed. Sequence alignment between pD28E4LacZ and pGEMShuttle showed that pD28E4LacZ contained an A-G point mutation within the ori sequence that caused the plasmid to have a low copy number. This mutation alters the efficiency of plasmid DNA replication thus lowering the copy number of the plasmid (Lin-Chao et al., 1992; Minton et al., 1988). It is possible that once homologous recombination occurred and the bacterial sequence in the HDAdV genome-bearing plasmid is replaced by the pGEMShuttle ori, the high copy number ori sequence present in pGEMShuttle cannot successfully replicate the larger plasmid of over 25 kb. Consequently, the ori sequence was replaced to produce a new shuttle, pGEMShuttleLCori (Figure 3.11)

pShuttle-CMV (Appendix 5.13), the shuttle plasmid used in the AdEasy system, was used as a template from which the low copy number ori sequence of 244 bp was obtained and used to replace the pGEMShuttle ori. The ori fragment in pGEMShuttle was removed by partial digestion with *Acu* I followed by *Bse* YI complete digestion. First, pGEMShuttle was partially digested with *Acu* I. As *Acu* I cuts the plasmid twice, the complete digestion results in two fragments of 1 012 bp and 4 825 bp. A partial digestion resulted in three bands of 1 012 bp, 4 825 bp and one larger 5 837 bp fragment representing a linearised plasmid cleaved at either one of the two *Acu* I restriction sites (Figure 3.12A). The 5 837 bp fragment, indicated in red, was extracted from the agarose gel, and this was then digested with *Bse* YI. As a comparison, the complete pGEMShuttle was also digested with *Bse* YI, which linearised the plasmid. The *Acu* I linearised pGEMShuttle was cut in one of two restriction sites, either within the ori, which is the intended site, or within the *AmpR* gene.

When these linearised fragments were then cut by *Bse* YI which cuts the plasmid once, the sizes of the resultant two fragments differed depending on where the *Acu* I cleaved the plasmid and were able to be separated on the gel. When *Acu* I cleaved within the *AmpR* gene, the resultant fragment sizes were 1 256 bp kb and 4 581 bp, and when *Acu* I cleaved at the intended site within the ori, the resultant fragments were 244 bp and 5 593 bp. The 5.6 kb fragment, indicated in red, represented the fragment of pGEMShuttle that had the intended 244 bp removed from the ori (Figure 3.12B). This fragment was then used as a backbone to insert the new ori sequence from pShuttle-CMV.

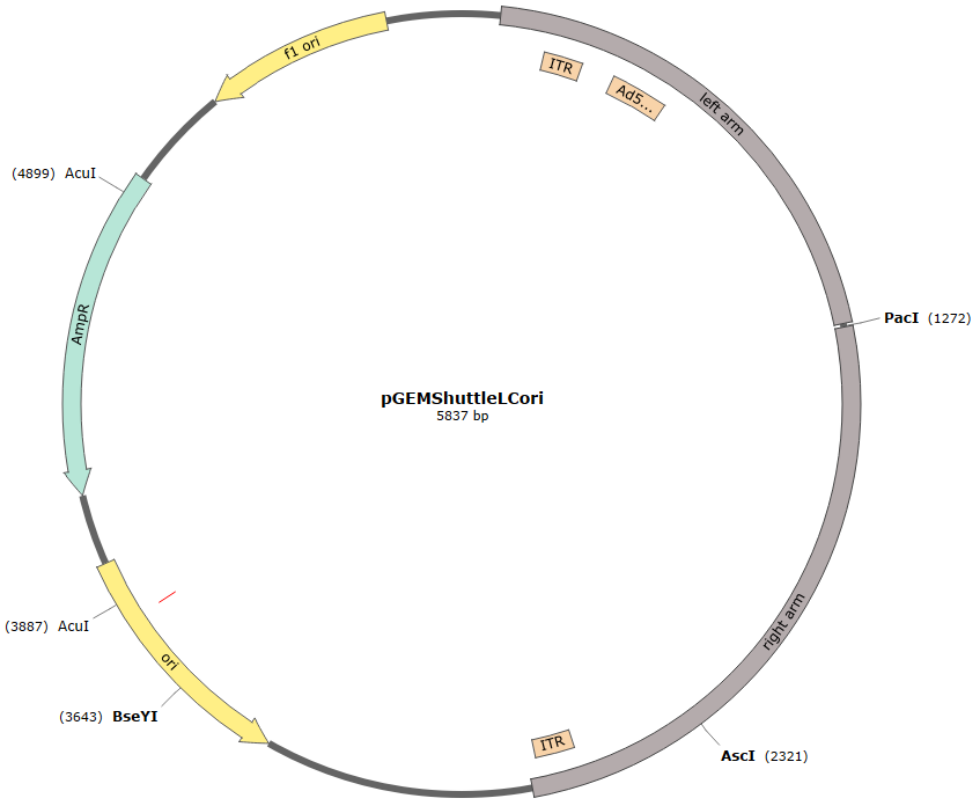
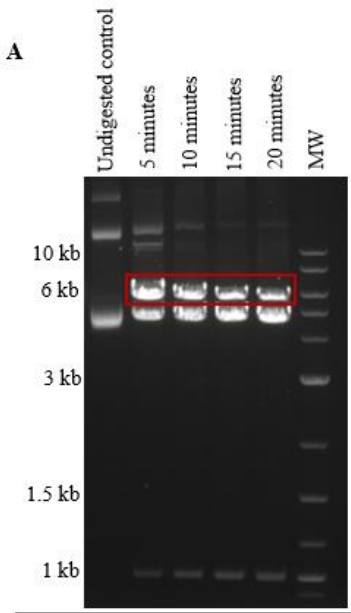


Figure 3.11: Plasmid map of pGEMShuttleLCori.

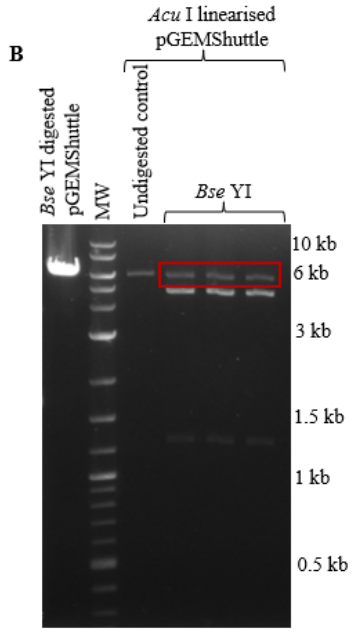
Plasmid map of the newly constructed pGEMShuttleLCori. The region of the ori containing the point mutation that controls the copy number of pGEMShuttle was excised through partial digestion with Acu I followed by digestion with Bse YI. The same region was PCR amplified from pShuttle-CMV and ligated to the Acu I and Bse YI backbone of pGEMShuttle, thereby replacing the region in pGEMShuttle with that of pShuttle-CMV. The resultant plasmid, pGEMShuttleLCori,

contained the A-G mutation that made it a low copy number plasmid. (Plasmid map obtained from Snapgene).

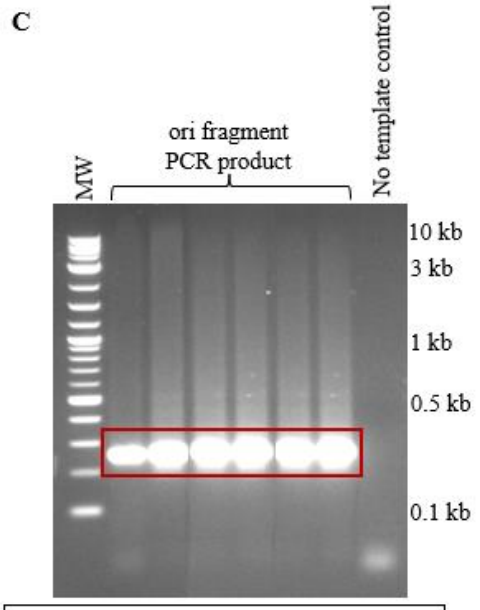
Although the 255 bp pShuttle-CMV ori fragment bearing the A-T mutation was flanked by *Acu* I and *Bse* YI sites, digestion of pShuttle-CMV with these enzymes produced fragments that could not be separated from the desired ori fragment. Hence, primer sequences that included the pShuttle-CMV *Bse* YI and *Acu* I sites were used to PCR amplify the ori fragment bearing the A-G point mutation, which produced an expected fragment of 255 bp (Figure 3.12C). The PCR product was extracted from the agarose gel and digested with *Acu* I and *Bse* YI, then ligated to the similarly digested pGEMShuttle to produce a plasmid, referred to henceforth as pGEMShuttleLCori in reference to the new low copy number ori present in the plasmid (Figure 3.11). Following screening of the clones (data not shown), the positive clones were further verified by restriction digestion with *Ban* I, which produced the expected bands of 711 bp, 1 097 bp, 1 283 bp, and 2 746 bp, similar to pGEMShuttle digestion (Figure 3.12D). Sequencing of the positive clones confirmed the presence of the A-G nucleotide difference between the original ori and the new ori (Figure 3.13).



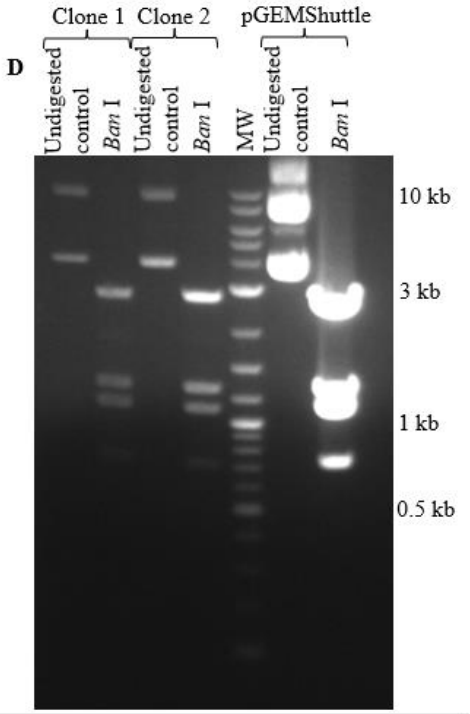
Fragment sizes of pGEMShuttle after *Acu* I digestion:
Complete digestion: 1 012 bp + 4 825 bp
Partial digestion: 1 012 bp + 4 825 bp + 5 837 bp



Fragment sizes after *Bse YI* digestion:
pGEMShuttle: 5 837 bp
Acu I linearised pGEMShuttle: 5 593 bp + 244 bp (not visible) OR 4 581 bp + 1 253 bp



Expected fragment sizes after PCR:
255 bp



Fragment sizes of pGEMShuttleLCori after *Ban* I digestion:
711 bp + 1 097 bp + 1 283 bp + 2 746 bp
Same banding pattern expected with digestion of pGEMShuttle

Figure 3.12: Replacement of the high copy number ori in pGEMShuttle.

*(**A**) Agarose gel showing the partial digestion of pGEMShuttle by Acu I. Each lane has indicated the length of time the plasmid was allowed to be digested. (**B**) Agarose gel showing the Bse YI of the Acu I linearised pGEMShuttle. (**C**) Agarose gel showing the PCR products following amplification of part of the ori sequence from pShuttle-CMV. (**D**) Agarose gel showing the Ban I digest to screen for positive clones of the low copy ori fragment to pGEMShuttle. DNA fragment sizes were determined using the molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

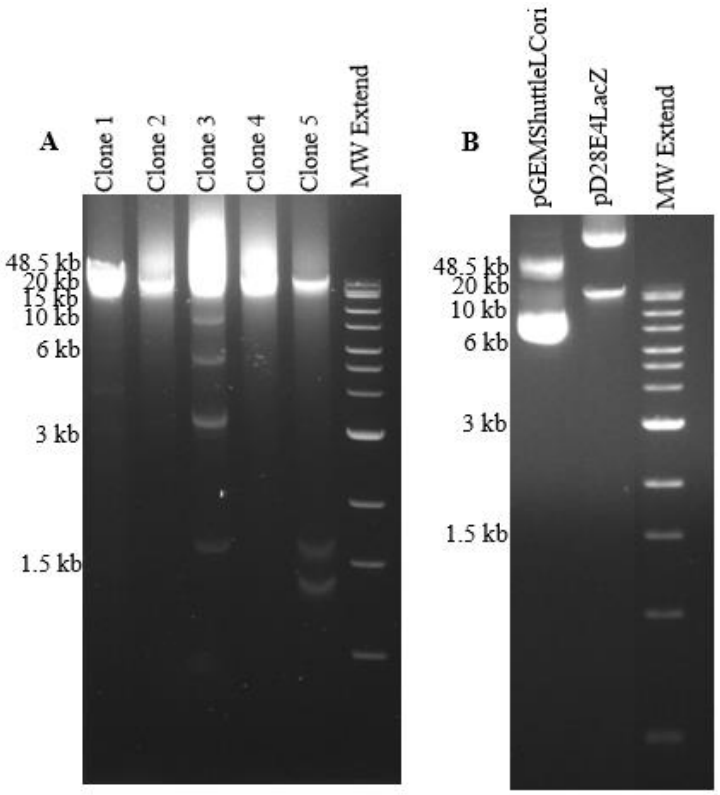
Figure 3.13: Sequence alignment of pGEMShuttleLCori to the reference sequence pGEMShuttle.

The upper line represents the sequence of the newly generated pGEMShuttleLCori, while the lower line represents the original pGEMShuttle sequence. The highlighted nucleotide represents the mutation within the ori sequence that affects the copy number of a plasmid, where there is an A in the high copy number pGEMShuttle, and a G in the low copy number pGEMShuttleLCori.

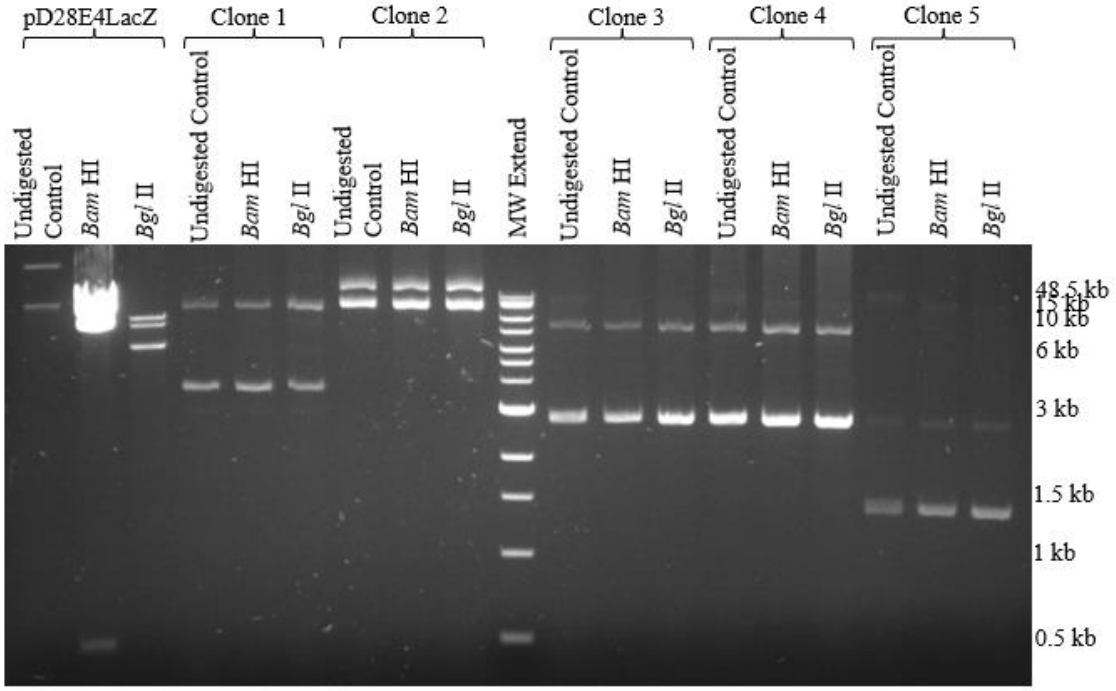
3.6 Homologous recombination between pGEMShuttleLCori and pD28E4LacZ

To assess the effect of using a low copy number shuttle plasmid on the stability of the resultant recombinants, homologous recombination between *Pac* I linearised pGEMShuttleLCori and pD28E4LacZ was performed before insertion of transgenes. A total of five potential recombinant clones were screened first by running the extracted uncut plasmid DNA on an agarose gel (Figure

3.14A-B). Three of the five clones showed high molecular weight DNA and two showed a mixture of low and high molecular weight DNA, which could have been a mixture of pGEMShuttleLCori and a recombinant clone. Unintended early recombination events, which is common in BJ 5183 can also result in the smaller bands observed in the mixed clones. This can be supported by the level at which the undigested pGEMShuttleLCori sample runs in comparison. All five clones were retransformed in *E. coli* DH5α cells and the isolated plasmids digested with *Bam* HI or *Bgl* II (Figure 3.14C). As expected pD28E4LacZ digestion with *Bam* HI produced four fragments of 456 bp, 8 412 bp, 11 106 bp, and 12 159 bp with the 1 106 bp, and 12 159 bp migrating together and *Bgl* II digestion resulted in four fragments of 6 192 bp, 6 383 bp, 9 065 bp, and 10 493 bp with 6 192 bp and 6 383 bp migrating together. However, digestion of the potential recombinants with *Bam* HI or *Bgl* II resulted in banding patterns similar to respective undigested controls and migration rate was different between the clones. Further purification of Clone 2, which showed to be of higher molecular weight by phenol-chloroform purification and redigestion still showed banding pattern similar to uncut plasmid (data not shown). These suggest again the instability of the recombinants produced using pGEMShuttleLCori.



C



Expected fragment size of pD28E4LacZ after *Bam* HI and *Bgl* II digestion:
Bam HI: 456 bp + 8 412 bp + 11 106 bp + 12 159 bp
Bgl II: 6 192 bp + 6 383 bp + 9 065 bp + 10 493 bp
Recombinants were expected to show a similar banding pattern

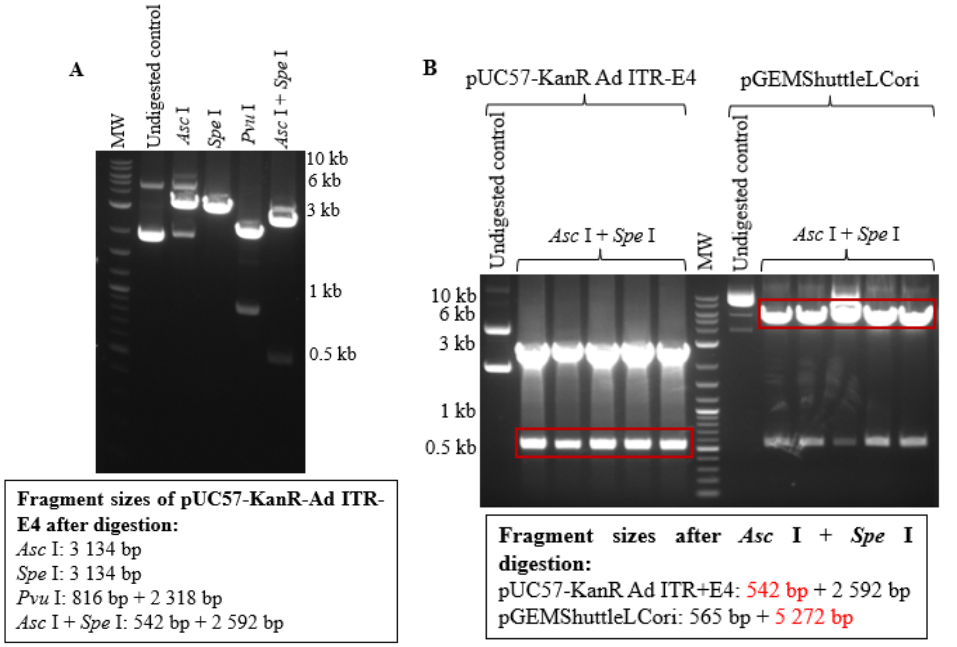
Figure 3.14: Screening of potential recombinants produced using the pGEMShuttleLCori.
*(**A**) Agarose gel of the five isolated clones of pD28E4LacZ-pGEMShuttleLCori run undigested compared to (**B**) undigested pGEMShuttleLCori and pD28E4LacZ. (**C**) Agarose gel showing the Bam HI and Bgl II digestion of potential positive recombinants of pD28E4LacZ and pGEMShuttleLCori after retransformation. DNA fragment sizes were compared using molecular weight ladder (MW Extend) Quick-Load® 1 kb Extend DNA Ladder (NEB).*

3.7 Insertion of the E4 fragment into pGEMShuttleLCori

To elucidate the cause of instability in the recombinants, the sequences outside of the bacterial sequence of pGEMShuttleLCori, was compared to that of the shuttle plasmid designed by Toietta *et al*. The difference was a 400 bp fragment of the non-coding E4 promoter region of the Ad genome, which they claim improves vector stability and infectivity (Toietta et al., 2002). This is supported by the fact that this fragment is also present in the pShuttle-CMV, which demonstrably produces stable recombinants (He et al., 1998; Luo et al., 2007).

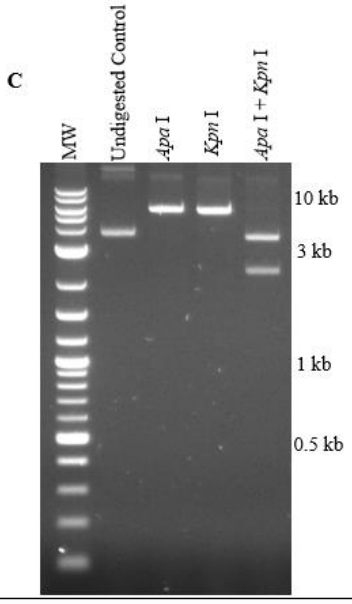
To determine if this fragment increases the stability of the recombinants, the 400 bp E4 fragment was then inserted in the pGEMShuttleLCori, directly before the right ITR. An approximately 0.6 kb fragment containing the Ad ITR as well as 400 bp of the Ad E4 ORF1 was synthesized by Genscript Biotech (New Jersey, USA) and received in a kanamycin resistance expressing pUC57 plasmid, referred to as pUC57-KanR-Ad ITR-E4. Also included in this fragment was a multiple cloning site that included the restriction sites *Spe* I, *Pme* I, and *Asc* I, among others, for downstream subcloning. The identity of this plasmid was confirmed by restriction digestion with *Asc* I, *Spe* I, and *Pvu* I, where *Asc* I or *Spe* I linearised the 3 134 bp plasmid, though partial digestion by *Asc* I resulted in faint bands above and below the expected fragment corresponding with the bands seen in the undigested control sample. *Pvu* I cleaved the plasmid twice to give two fragments of 816 bp and 2 318 bp, and double digestion with both *Asc* I and *Spe* I excised the 542 bp ITR and E4 fragment leaving the 2 592 bp backbone (Figure 3.15A). Both pGEMShuttleLCori and the pUC57 plasmid containing the Ad ITR and E4 fragment were digested with *Asc* I and *Spe* I to create compatible sticky ends for cloning the 542 bp insert into the 5 272 bp pGEMShuttleLCori backbone (Figure 3.15B), with care taken to not include partially digested backbone in the extracted fragment. The resultant plasmid, henceforth referred to as pGEMShuttleLCoriE4 was

verified by restriction digestion with *Apa* I and *Kpn* I. Each enzyme linearised the 5 814 bp plasmid, and together, resulted in two fragments of 2 318 bp and 3 496 bp, confirming the successful insertion of the E4 fragment into the pGEMShuttleLCori (Figure 3.15C).



Fragment sizes of pUC57-KanR-Ad ITR-E4 after digestion:
Asc I: 3 134 bp
Spe I: 3 134 bp
Pvu I: 816 bp + 2 318 bp
Asc I + *Spe* I: 542 bp + 2 592 bp

Fragment sizes after *Asc* I + *Spe* I digestion:
pUC57-KanR Ad ITR+E4: 542 bp + 2 592 bp
pGEMShuttleLCori: 565 bp + 5 272 bp



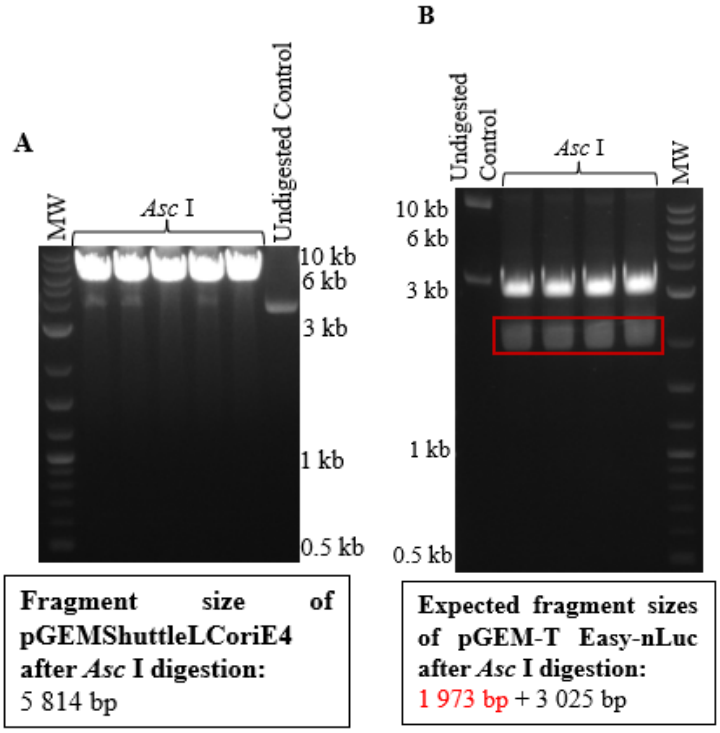
Fragment sizes of pGEMShuttleLCoriE4 after digestion:
Apa I: 5 814 bp
Kpn I: 5 814 bp
Apa I + *Kpn* I: 2 318 bp + 3 496 bp
Expected fragment sizes of pGEMShuttleLCori after digestion:
Apa I: 5 837 bp
Kpn I: Undigested
Apa I + *Kpn* I : 5 837 bp

Figure 3.15: Insertion of E4 fragment into pGEMShuttleLCori.

*(**A**) Agarose gel showing the screening of the synthesised plasmid pUC57-KanR Ad ITR+E4 with Asc I, Spe I, and Pvu I. (**B**) Agarose gel showing the Asc I and Spe I digestion of both pUC57-KanR Ad ITR+E4 and pGEMShuttleLCori, with the fragments gel extracted for ligation highlighted in red. (**C**) Agarose gel showing the Apa I and Kpn I screening of pGEMShuttleLCoriE4. DNA fragment sizes were compared using molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

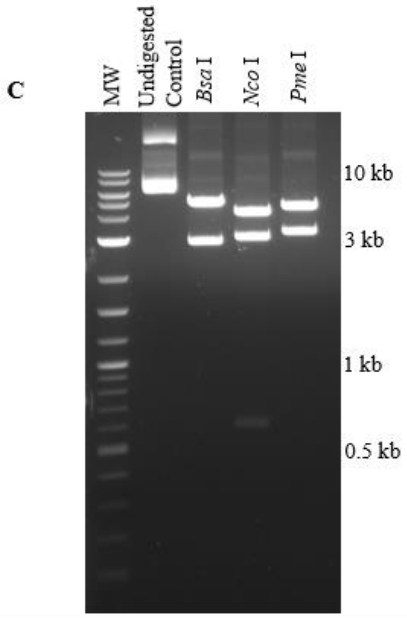
3.8 Insertion of nLuc into pGEMShuttleLCoriE4

To assess the potential for producing functional and stable recombinants between pGEMShuttleLCoriE4 and either pD24.7 or pD28E4LacZ, an nLuc transgene was inserted in the *Asc* I site within the shuttle plasmid. pD24.7 was used here because it is smaller than pD28E4LacZ and may have been able to produce smaller and more stable recombinants. pGEMShuttleLCoriE4 was linearised by *Asc* I, with fainter bands corresponding to the undigested band (Figure 3.16A), while pGEM-T Easy-nLuc was digested with *Asc* I and the 1 973 bp nLuc transgene was excised (Figure 3.16B). The two fragments were ligated to each other, and the resultant plasmid, pGEMShuttleLCoriE4nLuc, was verified by restriction digestion with *Bsa* I, *Nco* I, and *Pme* I (Figure 3.16C). Digestion with *Bsa* I gave two fragments of 2 828 bp and 4 959 bp, *Nco* I resulted in three fragments of 603 bp, 3 009 bp, and 4 175 bp, while digestion with *Pme* I produced two fragments of 3 168 bp and 4 619 bp, confirming the successful insertion of nLuc into the pGEMShuttleLCoriE4.



Fragment size of pGEMShuttleLCoriE4 after *Asc* I digestion:
5 814 bp

Expected fragment sizes of pGEM-T Easy-nLuc after *Asc* I digestion:
1 973 bp + 3 025 bp



Fragment sizes of pGEMShuttleLCoriE4-nLuc after digestion:
Bsa I: 2 828 bp + 4 959 bp
Nco I: 603 bp + 3 009 bp + 4 175 bp
Pme I: 3 168 bp + 4 619 bp
Expected fragment size of pGEMShuttleLCoriE4 after digestion:
Bsa I: 5 814 bp
Nco I: 5 814 bp
Pme I: 2 646 bp + 3 168 bp

Figure 3.16: Insertion of nLuc into pGEMShuttleLCoriE4.

*Agarose gels showing (**A**) the Asc I linearisation of pGEMShuttleLCoriE4, the (**B**) Asc I digestion of pGEM-T Easy-nLuc where the nLuc transgene was excised, and (**C**) the restriction analysis of pGEMShuttleLCoriE4-nLuc with Bsa I, Nco I, and Pme I. DNA fragment sizes were compared using molecular weight ladder (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

Before attempting to recombine pGEMShuttleLCoriE4 nLuc with pD24.7 or pD28E4LacZ, transgene expression from the shuttle was first assessed as before, with a luciferase assay using Huh 7 cells transfected with pGEMShuttleLCoriE4nLuc (Figure 3.17). Luciferase activity from this plasmid was compared to that of pAAV-nLuc and empty pGEMShuttleLCoriE4, where it was shown that luciferase expression in cells transfected with pGEMShuttleLCoriE4nLuc and pAAV-nLuc were comparable to each other and the slight difference seen was not statistically significant. As expected, cells transfected with empty pGEMShuttleLCoriE4 and untransfected cells did not produce significant levels of luminescence. This indicates that the newly constructed shuttle plasmid functions effectively to express the transgene inserted.

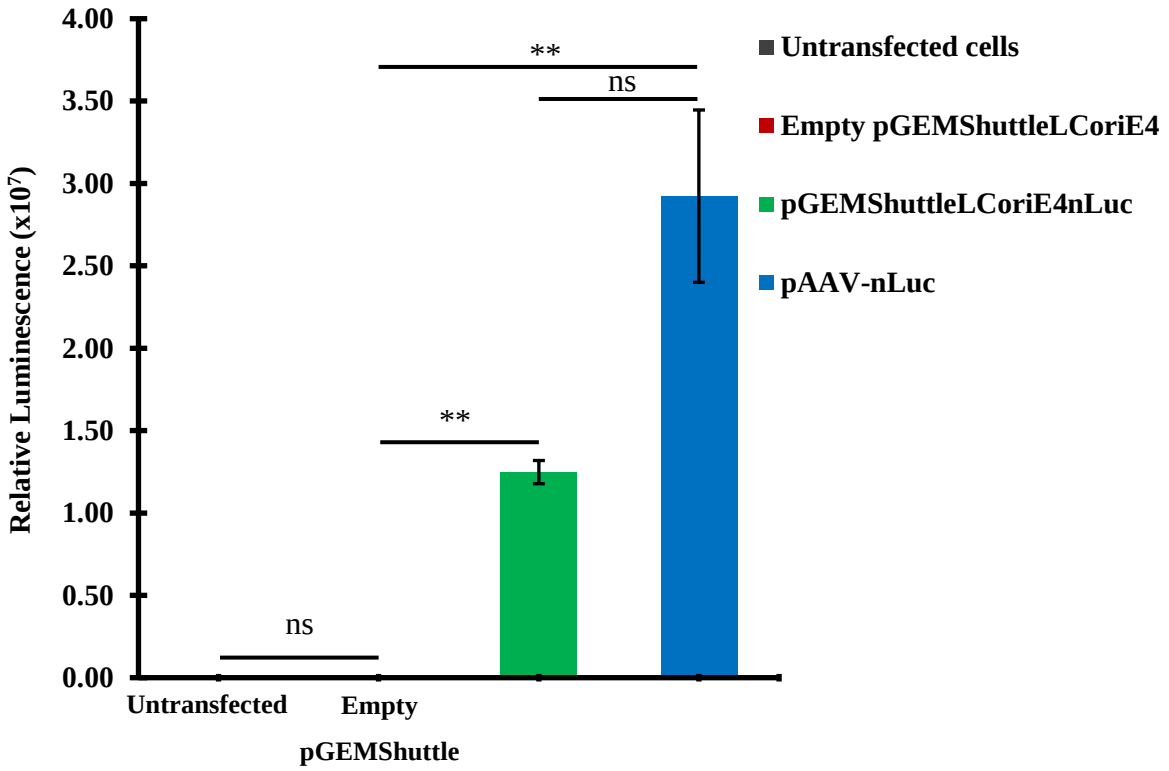


Figure 3.17: Luciferase expression from pGEMShuttleLCoriE4nLuc.

*A luciferase assay was used to assess the levels of nLuc expression from pGEMShuttleLCoriE4-nLuc. The luminescence from samples transfected with pGEMShuttleLCoriE4nLuc was compared to that of samples from cells transfected with empty pGEMShuttleLCoriE4 or pAAV-nLuc. Values represent means of the biological triplicate samples and standard error of mean (SEM). A two tailed, paired T test was used to compare data, where ** indicates a significant difference of P < 0.01 and ns indicates no significance.*

3.9 Homologous recombination between pGEMShuttleLCoriE4nLuc and pD28E4LacZ and pD24.7

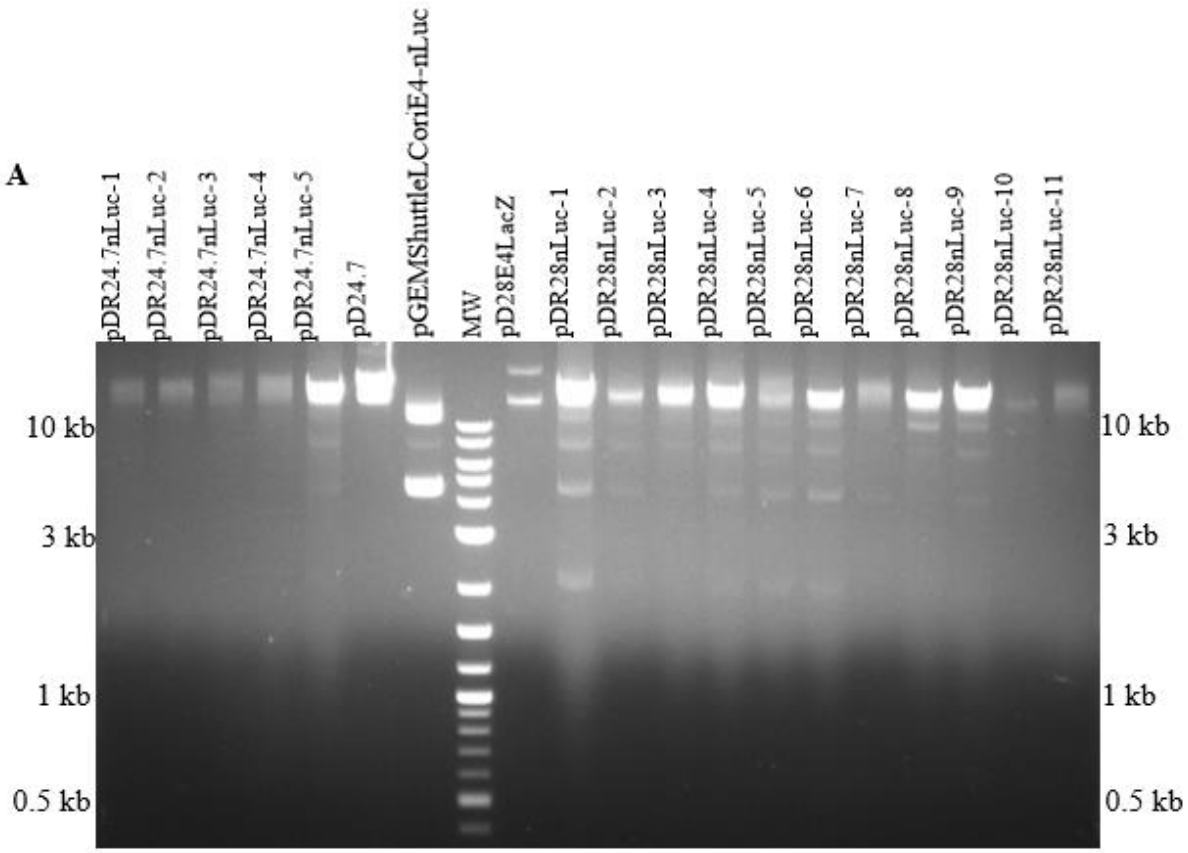
Using pGEMShuttleLCoriE4nLuc and either pD24.7 or pD28E4LacZ, homologous recombination was performed as before. The transformants were initially plated on ampicillin agar plates and inoculated in ampicillin-containing LB cultures. However, many of these colonies did not grow, or grew very slowly over a 48-hour period, which prompted the use of super optimal broth with catabolite repression (SOC) medium to boost growth of the recombinants by offering more optimal

metabolic conditions than ordinary LB medium. Plating both the BJ 5183 and DH5α *E. coli* transformants on SOC agar plates, and inoculating the smallest colonies present after 48 hours in SOC medium resulted in 5 potential recombinants of pD24.7 and pGEMShuttleLCoriE4-nLuc, and 11 recombinants of pD28E4LacZ and pGEMShuttleLCoriE4-nLuc, which were run undigested on an agarose gel for initial screening (Figure 3.18A).

Of the potential recombinants screened, most seemed to run higher than the bands seen in the pGEMShuttleLCoriE4-nLuc control and similar to the pD24.7 and pD28E4LacZ controls. Several samples also had smaller bands visible running at and below 10 kb, which is expected with plasmid DNA isolated from *E. coli* BJ 5183 cells. A few of the potential recombinants were selected for retransformation in DH5α *E. coli* cells, and of these, only four samples, pDR28nLuc-9-1, pDR28nLuc-10-1, pDR28nLuc-10-2, and pDR24.7nLuc-4-1 were digested with *Not* I. As expected pGEMShuttleLCoriE4-nLuc was cut three times by *Not* I to produce three fragments of 1 629 bp, 2 981 bp, and 3 177 bp with the latter two fragments running together. The top fainter bands may be a result of incomplete digestion. Both pD28E4LacZ and pD24.7 were not cleaved by Not I as expected (3.18B). However, the fragment sizes after digestion of the recombinants were not as expected. This fragment with a size discrepancy corresponds to the region of the plasmids that include the nLuc transgene. Regardless, these findings were promising, as it demonstrated that there was successful recombination due to the *Not* I sites that were present in the recombinants but not the original pD28E4LacZ or pD24.7 plasmids.

These recombinants were further screened with *Pme* I digestion, which was expected to cut the recombinants and the original plasmids twice, such that the bacterial sequences are removed from the larger plasmid for downstream viral production. However, while the anticipated banding pattern was present in the recombinants with a 3.2 kb fragment present in all four samples, there was also an unknown fragment of approximately 500 bp present in all four recombinants that could not be accounted for (Figure 3.19A). The recombinants were further screened through *Asc* I digestion, the restriction enzymes previously used to clone the transgene into the shuttle plasmid. It was expected that digestion with *Asc* I would result in the excision of the 1.9 kb transgene from the recombinants. However, none of the recombinants were digested by *Asc* I, despite pD28E4LacZ and pD24.7 being successfully linearised by the enzyme, as expected, and

pGEMShuttleLCoriE4 having the 1.9 kb transgene successfully excised from the plasmid (Figure 3.19B).



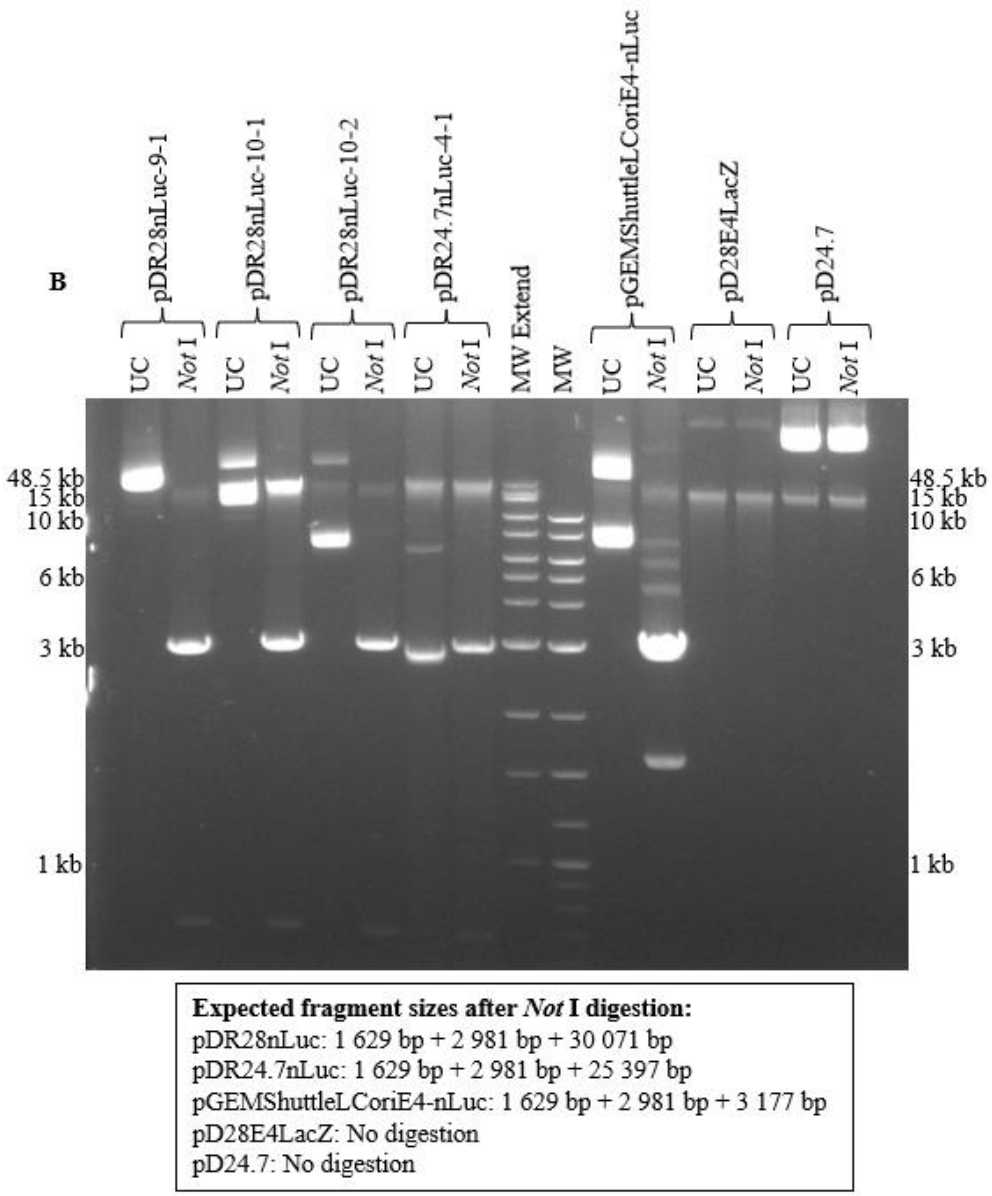
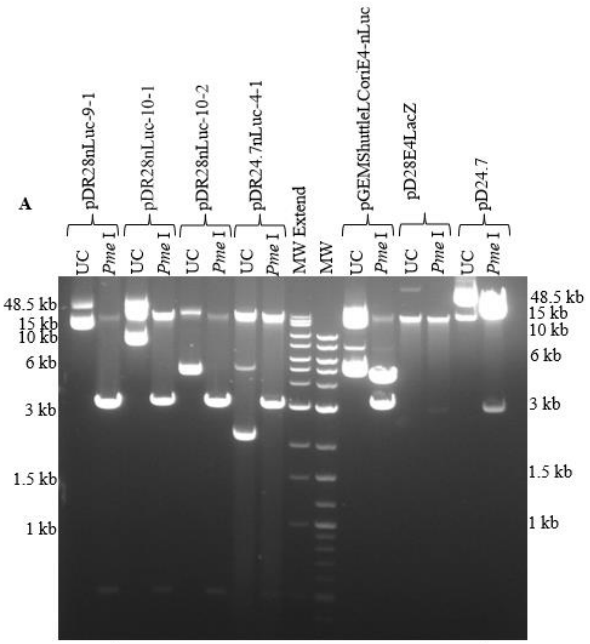
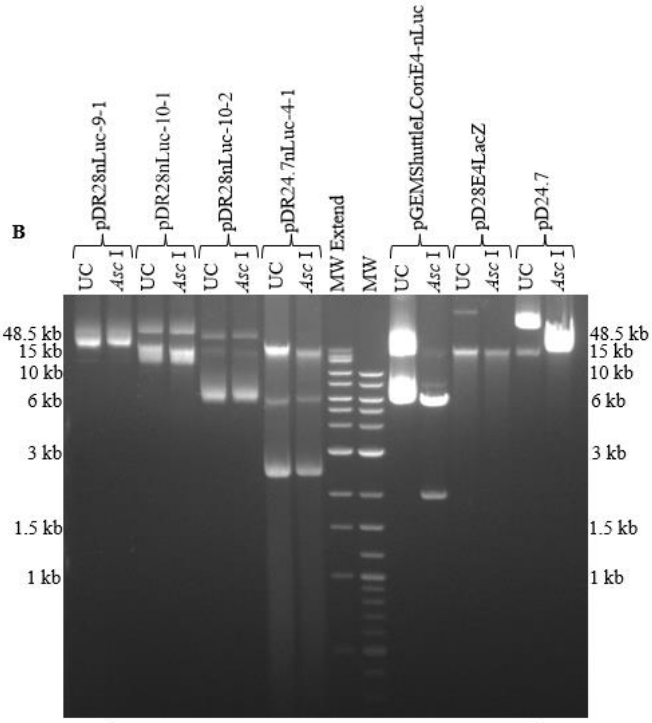


Figure 3.18: Initial screening of recombinants between pGEMShuttleLCoriE4nLuc and pD28E4LacZ or pD24.7.

Agarose gel showing (A) the potential recombinants between pGEMShuttleLCoriE4nLuc and either pD24.7 or pD28E4LacZ run undigested for analysis, and (B) the Not I digestion of potential recombinants between pGEMShuttleLCoriE4-nLuc and pD28E4LacZ or pD24.7. DNA fragment sizes were compared using molecular weight ladder (MW Extend) Quick-Load® 1 kb Extend DNA Ladder and (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).



Expected fragment sizes after *Pme* I digest:
pD28nLuc recombinant: 3 168 bp + 31 513 bp
pD24.7 recombinants: 3 168 bp + 26 839 bp
pGEMShuttleLCoriE4-nLuc: 3 168 bp + 4 619 bp
pD28E4LacZ: 2 949 bp + 29 184 bp
pD24.7: 2 949 bp + 24 510 bp



Expected fragment sizes after *Asc* I digest:
pD28nLuc recombinants: 1 973 bp + 32 708 bp
pD24.7 recombinant: 1 973 bp + 28 034 bp
pGEMShuttleLCoriE4-nLuc: 1 973 bp + 5 814 bp
pD28E4LacZ: 32 133 bp
pD24.7: 27 459 bp

Figure 3.19: Screening of recombinants between pGEMShuttleLCoriE4-nLuc and pD28E4LacZ or pD24.7.

*Agarose gels showing (**A**) the Pme I digestion of potential recombinants between pGEMShuttleLCoriE4 + nLuc and pD28E4LacZ or pD24.7, and (**B**) the Asc I digestion of potential recombinants between pGEMShuttleLCoriE4 + nLuc and pD28E4LacZ or pD24.7. DNA fragment sizes were compared using molecular weight ladder (MW Extend) Quick-Load® 1 kb Extend DNA Ladder and (MW) Quick-Load® 1 kb Plus DNA Ladder (NEB).*

It is obvious that recombination did in fact occur to produce these plasmids, as they were clearly larger than the shuttle plasmid when viewed on an agarose gel and were capable of replicating in the presence of ampicillin, which would not have been possible had recombination not occurred. However, the difficulties in screening these plasmids suggested that recombination events did not occur between the intended homology arms, resulting in a yet undeterminable bacterial sequence having been inserted into the HDAdV genomes, which was corroborated by the discrepancies seen in the *Not* I and *Asc* I digestion of the plasmids. To determine if the recombinants contained the transgene, a luciferase assay was performed using Huh 7 cells transfected with each of the recombinants, and pGEMShuttleLCoriE4nLuc as a positive control. While pGEMShuttleLCoriE4nLuc displayed significant luciferase activity, as expected, all four recombinants showed none, with luminescence comparable to that of both the empty pGEMShuttleLCori and the untransfected cells (Figure 3.20). This suggested that the recombination event that produced these recombinants excluded the nLuc transgene from the final product.

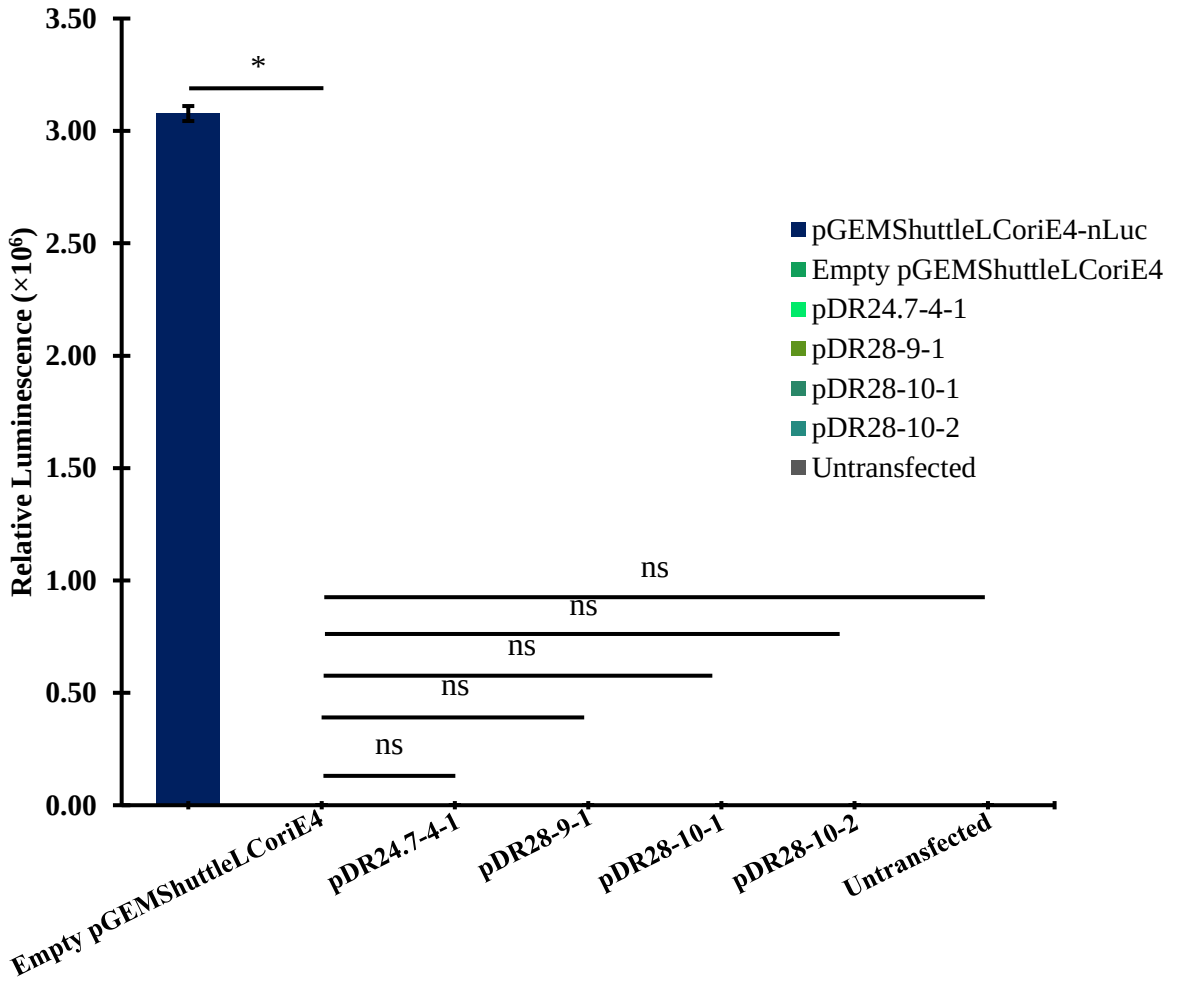


Figure 3.20: Luciferase expression from potential recombinants.

*A luciferase assay was used to assess the levels of nLuc expression from the various pD28nLuc and pD24.7nLuc recombinants. The luminescence from samples transfected with the recombinants was compared to that of samples from cells transfected with empty pGEMShuttleLCoriE4 and pGEMShuttleLCoriE4-nLuc. Values represent means of the biological triplicates and standard error of mean (SEM). A two tailed, paired T test was used to compare data, where * indicates a significant difference of P < 0.01 and ns indicates no significance.*

The four recombinants assessed here likely lack the intended transgenes due to the regions at which recombination occurred between the shuttle plasmid and the HDAdV plasmids. Again, because these plasmids were able to replicate both in liquid culture and solid agar in the presence of ampicillin and had the *Not* I sites, it suggests that recombination did in fact occur between the two plasmids to produce recombinants. However, the incorrect banding pattern after restriction

analysis and failure to express nLuc suggests that the recombination occurred such that the transgene was excluded from the recombinants. It is possible that this exclusion of the transgene from the recombinants is due to a small region of homology within the shuttle adjacent to the transgene that is a fragment of one of the homology arms present after the transgene was inserted into the shuttle. This fragment is approximately 103 bp, and its small size compared to the intended sites of homologous recombination, which is 1 046 bp and 1 174 bp had suggested that recombination there would be less favoured as compared to the larger regions of homology. To verify that the recombination occurred as suggested above to result in the four plasmids that lacked the nLuc transgene, Sanger sequencing of the transgene was performed (Inqaba Biotech, Pretoria, South Africa) to determine the exact sequence present in these recombinants. Primers were designed to flank the region in which the transgene was expected to be present within the recombinants, and the schematic below indicates where these sequences align with reference sequences constructed for each type of recombinant. In both the pD24.7 vector and the pD28E4LacZ vectors, the nLuc transgenes have clearly been excluded from the sequences indicating that the recombination event that generated these plasmids did not include the transgene in the final product. The presence of the E4 sequence from the shuttle plasmid confirms that recombination occurred at the 103 bp sequence (Figure 3.21).

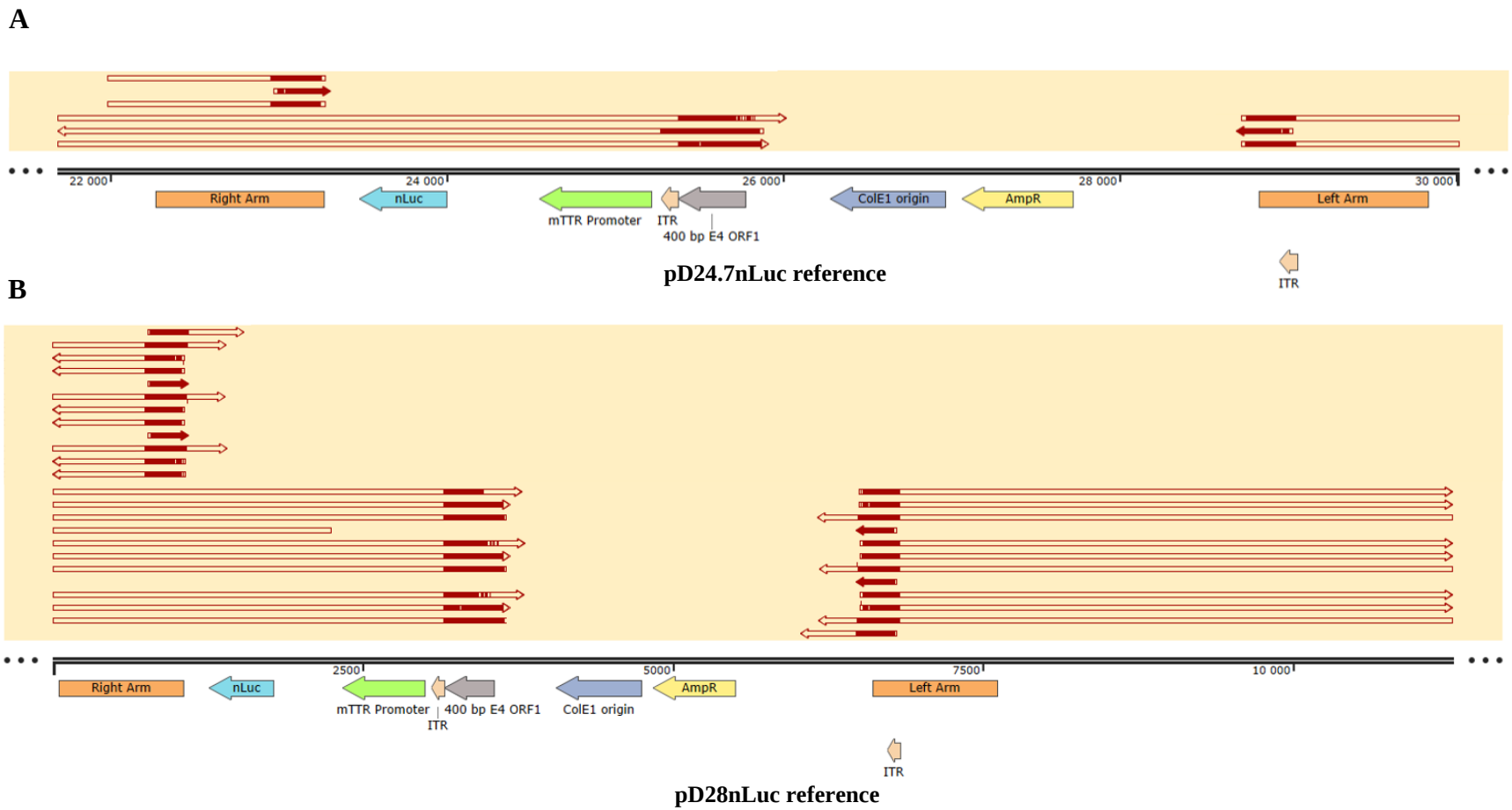


Figure 3.21: Alignment of the sequences of the recombinants.

*Schematic of the sequence alignment of (**A**) reads from pDR24.7-4-1 against a reference sequence of pD24.7nLuc and (**B**) reads from pDR28-9-1, pDR28-10-1, and pDR28-10-2 against a reference sequence of pD28nLuc. Sequence reads were generated through Sanger sequencing. (Images obtained from Snapgene).*

CHAPTER 4

4. DISCUSSION

Hepatitis B remains a global health concern despite the well-established vaccine regimen in place to prevent the infection. The lack of a complete cure for hepatitis B means that there is still significant work needed to develop treatment for chronic HBV infection. As the persistence of HBV cccDNA is the major contributor of the difficulties in curing this disease, gene editing offers an ideal solution to target and eradicate the presence of this viral DNA from infected cells. Of the gene editors currently available, CRISPR/Cas9 systems are arguably the best, as they are versatile, easy to use, and broadly applicable to a variety of diseases. Potent CRISPR/Cas 9 sequences against HBV have been designed and the potential of adenoviral vectors in the delivery of these sequences has been demonstrated (Kato et al., 2021; Schiwon et al., 2018). As a result of their natural tropism for the liver, Ads are better suited in the development of chronic HBV infection treatment. HDAdVs have a large carrying capacity that allows them to package and express a wide variety of transgenes, including anti-HBV CRISPR/Cas9 sequences. The challenge with these vectors, however, is the process of inserting transgenes into their larger genome efficiently. The purpose of this study was to design a system to insert anti-HBV CRISPR/Cas9 sequences into the HDAdV genome through homologous recombination with a shuttle plasmid, rather than directly ligating a transgene into the HDAdV genome, which is inefficient.

4.1 Successful construction of shuttle plasmids expressing anti-HBV CRISPR/Cas9 and the nLuc reporter

A shuttle plasmid, pGEMShuttle, was previously constructed but its functionality was not tested. To assess the functionality of this plasmid, the anti-HBV CRISPR/Cas9 or the nLuc sequences were first cloned in to produce pGEMShuttlenLuc or pGEMShuttleCRISPR/Cas9 (Figure 3.4B and Figure 3.4C). The anti-HBV activity of pGEMShuttleCRISPR/Cas9 was assessed by measuring the knockdown of HBsAg expression in HEK 293T cells after co-transfection with pCH-9/3091. HBsAg levels were reduced by approximately 85% in cells transfected with pGEMShuttleCRISPR/Cas9 as compared to those transfected with empty pGEMShuttle (Figure 3.5). When the same anti-HBV CRISPR/Cas9 sequences used in this study were expressed from

different plasmids, approximately 95% knockdown of HBsAg was observed (Scott et al., 2017). Scott *et al.*, performed a co-transfection experiment using a 7.4 kb plasmid expressing Cas9 and a 3.2 kb plasmid expressing sgRNA. The slightly lower level of knockdown seen in this study can be attributed to the increased size of pGEMShuttleCRISPR/Cas9, which is 10.4 kb, compared to the smaller plasmids used by Scott *et al.* It has been shown that an increased plasmid size results in both lower transfection efficiency (Yin et al., 2005) and lower transgene expression (Cheah et al., 1987), which may explain the lower level of knockdown seen from pGEMShuttleCRISPR/Cas9. In this study, both Cas9 and sgRNA are expressed from one plasmid hence the ratio of Cas9 to sgRNA encoding gene copies will be 1:1. Whereas Scott *et. al*. used equal amount of Cas9 and sgRNA expressing plasmids, there will be about 1:2 ratio of Cas9 to sgRNA encoding gene copies in the transfection and this may account for differences observed in this study.

Following transfection of Huh 7 cells with pGEMShuttlenLuc, significant expression of nLuc was seen, with a relative luminescence of approximately 2.5×10^7 RLU (Figure 3.6). Although statistically not significant, nLuc expression from pGEMShuttlenLuc, which is 7.8 kb was relatively lower as compared to the expression from pAAV-nLuc, which is 5.3 kb. This corroborates the results obtained with pGEMShuttleCRISPR/Cas9, that the bigger plasmid size may be contributing to low transfection efficiency which translates to lower transgene expression. The levels of nLuc expression seen here are comparable to that of other studies using the same reporter gene where luminescence ranging between 1.5×10^7 and 4×10^7 RLU was observed. These include published data by the Promega Corporation where they compared expression levels of nLuc to that of other luciferase reporters such as firefly luciferase and *Renilla* luciferase ("Nano-Glo Luciferase Assay System," 01/22), and by Matsuda *et al.*, where they used plasmids bearing the *nLuc* reporter gene to assess the effect of a frameshift mutation on transgene expression (Matsuda et al., 2018). Overall, the findings with both the CRISPR/Cas9 and nLuc demonstrate the ability of the shuttle plasmids to successfully express transgenes.

4.2 Homologous recombination between shuttle plasmids and HDAdV-genome expressing plasmids

Following co-transformation of BJ 5183 *E. coli* cells with the shuttle plasmids and the HDAdV genome-bearing plasmids, there were two sizes of bacterial colonies present on the agar plates

(Figures 3.7 and 3.9). The core principle of using a shuttle plasmid to insert transgenes into the HDAdV genome involves the presence of two homology arms within the shuttle that are identical to regions within the HDAdV genome-bearing plasmid. The resultant plasmid formed from homologous recombination between the two plasmids has significant differences compared to its parent plasmids. It is larger than both plasmids, and it bears a different antibiotic resistance gene to the original HDAdV plasmid; the recombinant has ampicillin resistance acquired from the shuttle plasmid while the original HDAdV genome plasmid has kanamycin resistance. By plating the transformed BJ 5183 *E. coli* cells on ampicillin-containing agar plates, the original HDAd plasmid was selected against and was unable to grow, leaving only ampicillin resistance-bearing plasmids able to survive. Of the two species of plasmids with ampicillin resistance, the smaller shuttle plasmids and the larger recombinants, there is a clear size difference between the colonies carrying each of these plasmids. The rate of plasmid replication has been shown to be linked and proportional to their size, where larger plasmids take longer to replicate than smaller plasmids. As such, the bacterial cells they transform are also affected, where cells transformed by larger plasmids grow slower than those transformed by smaller plasmids (Cheah et al., 1987). Hence, the larger, faster growing colonies most probably contained only shuttle plasmid and the smaller, slower growing plasmids most probably contained recombinants between the two plasmids (Figures 3.7 and 3.9). This is similar to what was seen by He *et al.* and Luo *et al.*, amongst others, in the production of both first generation AdVs (He et al., 1998; Luo et al., 2007), and Toietta *et al.* in the production of HDAdVs (Toietta et al., 2002). They successfully demonstrated that the smaller colonies present on the agar plates contained recombinants of the two plasmids, while the larger colonies did not.

This size difference between the recombinants and the shuttle plasmid was also used to differentiate the two species through gel electrophoresis, where larger plasmids migrate slower through the gel than smaller plasmids. The plasmids extracted from the smaller colonies on the agar plates were initially screened by running them undigested on an agarose gel, where the recombinants were expected to run higher than the shuttle plasmid due to their size difference (Figures 3.8A). This was generally the case, and there were clearly plasmids obtained that appeared to run at the expected level. However, recombinants produced with pGEMShuttle were proven to be unstable, where retransformation of the plasmids into stable cells that should have prevented further recombination as they were *recA* negative, resulted in significantly smaller plasmids

(Figure 3.8C and 3.10B). Attempts to screen these plasmids through restriction analysis were unsuccessful, likely due to the unintended recombination events having removed the known restriction sites from the plasmid. Restriction analysis of the plasmids obtained from the BJ 5183 *E. coli* cells was also unsuccessful, suggesting that further recombination events also occurred in the first transformation of BJ 5183 *E. coli* cells. These results were seen in multiple repeat experiments using both pGEMShuttlenLuc and pGEMShuttleCRISPR/Cas9.

4.3 Construction of a low copy number shuttle plasmid

Further sequence alignment of pGEMShuttle and the HDAdV genome bearing plasmids identified a single nucleotide difference between the two plasmids within their ori sequences. pGEMShuttle, which was derived from the cloning vector pGEM®-T Easy, is a high copy number plasmid that replicates at a rate of approximately 500 copies per cell at 37°C. pD28E4LacZ and pD24.7, on the other hand, are low copy number plasmids that replicate at a rate of approximately 20 copies per cell at 30°C. Most plasmid ori sequences are derived from ColE1, including pUC plasmids such as pGEM®-T Easy. pUC plasmids have been demonstrated as having a higher copy number than its parent plasmid. Plasmid DNA replication is initiated from the ori sequence upon synthesis of RNA II, an inactive RNA primer. This hybridises with template DNA and is cleaved at a specific site by RNaseH which leaves a 3` OH available for DNA synthesis by DNA polymerase I. Another RNA molecule, RNA I, functions as an inhibitor of plasmid DNA synthesis through interaction with RNA II to induce a change in the conformation of RNA II and prevents its binding to the template DNA to inhibit plasmid DNA synthesis. ColE1-derived plasmid replication is also controlled by the Rop/Rom protein, which enhances the binding of RNA I to RNA II to further inhibit DNA replication (Lin-Chao et al., 1992). Minton *et al.* identified a point mutation within the ori sequence of pUC plasmids that resulted in RNA I transcripts being three nucleotides shorter than the wildtype transcript. They suggested that this mutation affects the transcription initiation site of RNA I, which in turn affects its ability to inhibit plasmid DNA replication (Minton et al., 1988). The effect of this, in turn, means that plasmid DNA replication is enhanced, which leads to the increased copy number of pUC plasmids compared to ColE1-derived plasmids. It was also observed that temperature plays a role in affecting the copy number of plasmids, where high copy number plasmids that contained the point mutation showed increased DNA replication at 42°C

compared to the wildtype, low copy number plasmids, which had consistent DNA replication rates at temperatures between 30°C and 42°C (Lin-Chao et al., 1992).

To determine if lowering the copy number of pGEMShuttle would improve the stability of the resultant recombinants, the region of the ori containing the point mutation identified by Minton *et al.* was replaced with a fragment taken from another low copy number plasmid, pShuttle-CMV (Figure 3.12). The new plasmid, pGEMShuttleLCori, was able to grow and replicate as expected, confirming the functionality of the mutant ori. However, homologous recombination between it and pD28E4LacZ still did not result in stable recombinants, with none of the resultant recombinants screened able to be digested by restriction enzymes that did successfully digest pD28E4LacZ (Figure 3.14C). The continued instability of the recombinants suggested that despite successful alteration of the ori sequence in the shuttle, the copy number was likely not the only player in the instability of the recombinant plasmids.

4.4 Inclusion of the E4 sequence in the shuttle plasmid

Comparison between pGEMShuttleLCori and the shuttle plasmid produced by Toietta *et al.* to produce stable and functional recombinants revealed the presence of a non-coding 400 bp fragment from the E4 ORF1 of the Ad genome in the Toietta shuttle that was missing in the pGEMShuttleLCori (Toietta et al., 2002). They observed that the inclusion of this fragment improved the stability and infectivity of the vectors they produced, an observation that was first reported by Sandig *et al.*, where they reported that this non coding and non-essential element conferred some form of growth advantage to the HDAdVs through an undetermined mechanism (Sandig et al., 2000). However, it is not clear wither this sequence would confer stability of the plasmids carrying the HDAdV genome in bacterial cells. A majority of the recombinants obtained following homologous recombination with pGEMShuttleLCoriE4nLuc and either pD28E4LacZor pD24.7 appeared to be of high molecular weight even after retransformation in *recA* negative *E. coli* cells, which was not the case in previous attempts of recombination with shuttle plasmids that lacked this sequence (Figure 3.18A). Interestingly, these recombinants were more difficult to grow in ordinary LB media and on agar plates (Data not shown). Propagating these plasmids in SOC medium and plating transformants on SOC agar plates was more successful. It is well established that larger plasmids place a higher metabolic burden on their host cells, and this would be further exacerbated by a high copy number. Certainly, a combination of a lower copy number and the use

of SOC growth media resulted in better growth of potential recombinants by lowering their metabolic requirements for replication and increasing the concentration of nutrients present in their growth media.

Homologous recombination is one of the innate DNA repair mechanisms in both prokaryotic and eukaryotic cells to repair double stranded DNA breaks. In bacteria, this repair may occur through the RecBCD pathway. This uses an enzyme complex called RecBCD, which binds to the end of a double stranded break in DNA. The RecB and RecD subunits have helicase activity that unwinds the dsDNA until the complex reaches an eight-nucleotide sequence referred to as the Chi site. Recognition of this region causes the RecBCD complex to cleave the DNA strand and load multiple RecA proteins onto the newly formed ssDNA strand to form a nucleoprotein filament. This is then able to search for and bind to a homologous DNA sequence and insert itself into the DNA duplex it identifies, where the 3` overhang displaces a strand of the DNA duplex to create a D-loop. This D-loop is cleaved, and the resultant DNA strand anneals to the invading strand to form a Holliday junction. The 3` end of the invading strand then acts as a primer for DNA synthesis to form a replication fork that fills in the gap in the broken DNA to repair the break (del Val et al., 2019; Dillingham and Kowalczykowski, 2008; Kowalczykowski et al., 1994; Michel and Leach, 2012). The important element here is the Chi site, which has the sequence 5`-GCTGGTGG-3`. The name Chi comes from the phrase “crossover hotspot instigator” as these sequences were identified as being hotspots for recombination (Lam et al., 1974). It is clear that this sequence is vital for homologous recombination to occur, and the sequence is known for being fairly ubiquitous in the bacterial genome (Amundsen et al., 2016; Dixon and Kowalczykowski, 1991; Smith et al., 1984). Coincidentally, the first seven of these eight nucleotides, 5`-GCTGGTG-3`, are present within the E4 fragment that was included in both pGEMShuttleLCoriE4, and the shuttle produced by Toietta *et al*. It is possible that the presence of the majority of this sequence is what facilitated successful homologous recombination and improved stability of the recombinants, despite only seven of the eight nucleotides being present. However, this hypothesis will need to be further investigated.

Despite the successes in producing stable recombinants, recombination between pGEMShuttleLCoriE4nLuc and the HDAdV genome bearing plasmids did not result in transgene bearing plasmids. Assessing the luciferase activity in cells transfected with each of the

recombinants compared to that of cells transfected with pGEMShuttleLCoriE4nLuc indicated that there was no luciferase activity (Figure 3.20). Sequencing the region of these recombinants that was expected to include the nLuc transgene indicated that the transgene was excluded from the resultant plasmids (Figure 3.21). The shuttle plasmid is designed such that ideally, homologous recombination will occur between the two larger homology arms to replace the bacterial sequence in the HDAdV genome bearing plasmid with the shuttle plasmid (Figure 4.1). However, it is possible for recombination to also occur between one of the homology arms and the ori sequence. This is often the case, and has also been demonstrated by Luo *et al.*, where recombination between a homology arm and the ori sequence is just as likely (Luo et al., 2007). Hence the transgene must be placed such that should the ori participate in recombination the transgene is retained.

The shuttle plasmid used in this study was designed such that the transgenes are inserted into the *Asc* I site that is present within the right homology arm in the shuttle. In doing so, the homology arm is split in two homology sequences of 103 bp and 1046 bp flanking the transgene (Figure 4.1). If recombination occurs using the right homology arm and the 103 bp sequence, the result would be a recombinant with ampicillin resistance, but not the transgene, which is the case in all the recombinants seen here. It is surprising that this smaller fragment is preferred over the larger 1046 bp homology arm. Interestingly insertion of the E4 sequence placed the potential 7 bp Chi sequence closer to the 103 bp homology sequence which may make it the preferred region for recombination. Recombination using this small sequence will also result in smaller recombinants which might outcompete the larger recombinants during phenotypic expression. To remedy this, it may be necessary to move the point at which the transgene is inserted away from the homology arms, so that recombination at any site of the homology arms retains the transgene.

Sequence alignment of pGEMShuttle and both pD28E4LacZ and pD24.7 also identified a 300 bp region of homology bacterial sequence of both HDAdV genome bearing plasmids that is homologous to a region of the *AmpR* gene in pGEMShuttle. Interestingly there is a potential Chi sequence closer to this *AmpR* homology sequence (Figure 4.1). While this region was shown to be non-functional, as neither plasmid is able to grow on ampicillin-containing agar plates, it may contribute to unintended recombination. As a result of time constrains and the difficulty in the ligation of HDAdV plasmids, deletion of this region from the HDAdV plasmid was attempted but could not be completed (data not shown).

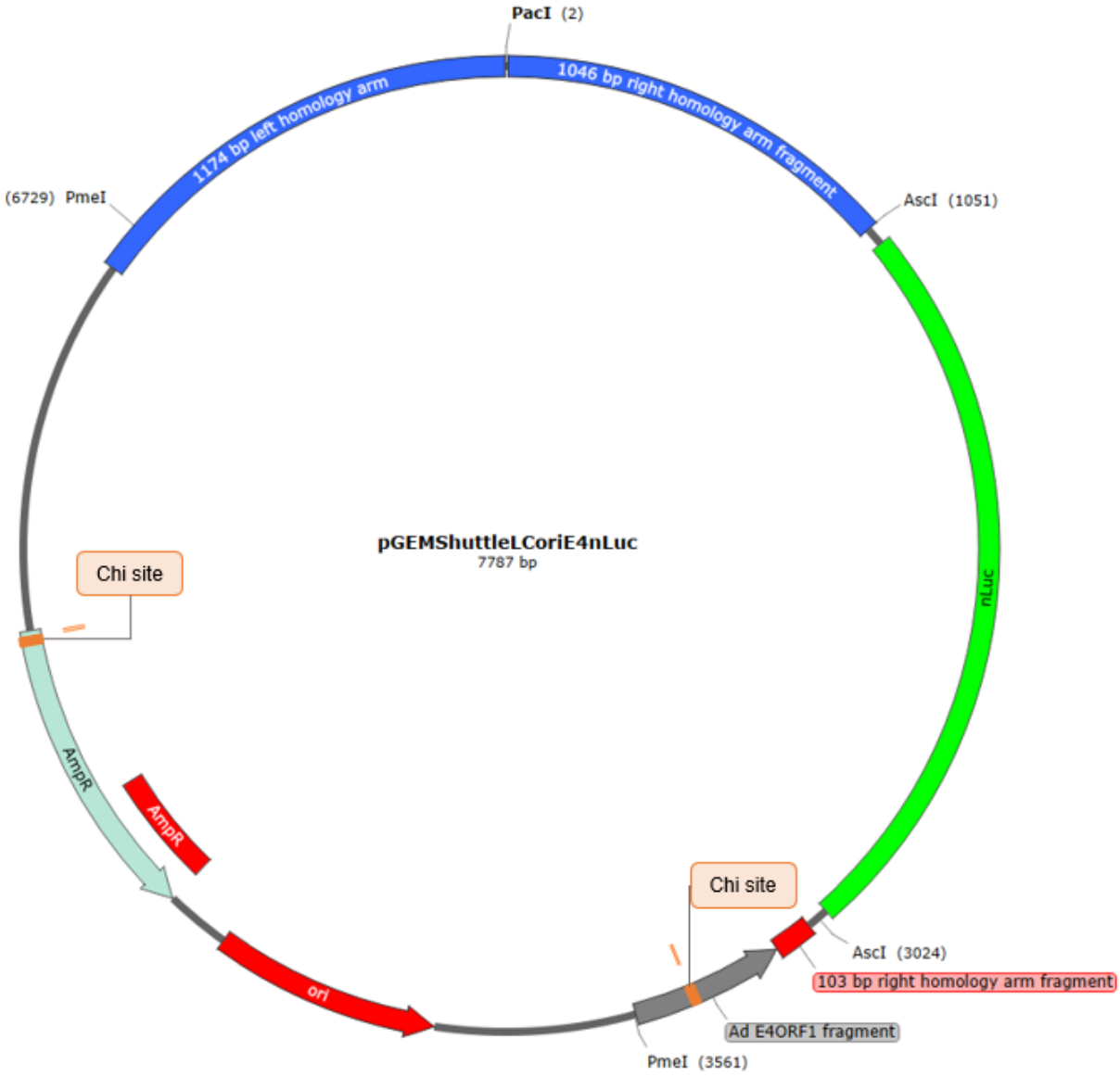


Figure 4.1: Plasmid map of pGEMShuttleLCoriE4nLuc with the various regions of homology with the HDAdV genome indicated.

Plasmid map of pGEMShuttleLCoriE4nLuc illustrating the left and right homology arms, in blue, where homologous recombination with the HDAdV was intended to occur. Indicated in red are the various other regions of homology with the HDAdV genome, including the ori, a fragment of the AmpR gene, and the small fragment of the right homology arm separated from the larger fragment when the transgene, in green, was inserted into the shuttle plasmid. Also indicated in orange are two Chi sites that are suggested to be vital for homologous recombination to occur.

4.5 Conclusions

The overall aim of this study, to develop a system to produce anti-HBV CRISPR/Cas9 expressing HDAdVs efficiently and easily, is yet to be met. Though significant improvements have been made to the shuttle plasmid constructed here, further work to produce stable and complete recombinants is necessary before the study can be considered complete. Up to this point, this study proposes using a system that is designed to simplify one of the most challenging aspects of HDAdV production, which is the insertion of transgenes into the vector. The system described here can readily be applied for insertion of a variety of transgenes, making this a versatile system that could be used for a variety of diseases beyond HBV.

4.6 Future work

To further reduce the chance of recombination occurring at unintended sites, the *AmpR* homology sequence in HDAd genome bearing plasmid will be deleted. To avoid recombination that excludes the transgene, the transgene insertion site will be moved away from the homology arms and such that homology between the arm and the ori retains the transgene. The refined shuttle plasmid will be used for homologous recombination with HDAd genome bearing plasmid. Once the correct recombinant plasmids are obtained, their ability to produce HDAdV particles will be assessed by transfecting HEK 293T cells. The stability, infectivity, effectiveness, and safety of the transgene-expressing viral particles will be assessed *in vitro* and in mice.

CHAPTER 5

5. APPENDIX

5.1 Bacterial culturing

5.1.1 Luria Bertani (LB) agar media

Ten grams of tryptone, 5 g yeast extract, 5 g NaCl, and 10 g agar was added to 1 L of distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2. The media was allowed to cool to approximately 55°C before the required antibiotic was added (100 µg/mL ampicillin or 50 µg/mL kanamycin). The media was then poured into bacterial petri dishes and allowed to set overnight.

5.1.2 Luria Bertani (LB) media

Ten grams of tryptone, 5 g yeast extract, and 5 g NaCl was added to 1 L of distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2 and allowed to cool.

5.1.3 Super optimal broth with catabolite repression (SOC) media

Ten grams of tryptone, 2.5 g yeast extract, 0.25 g NaCl, and 0.09 g KCl was added to 450 mL of distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2 and allowed to cool. Ten mM $MgCl_2$, 10 mM $MgSO_4 \cdot 7H_2O$, and 20 mM glucose was added to this, and the final volume adjusted to 500 mL.

5.1.4 Super optimal broth with catabolite repression (SOC) agar

Ten grams of tryptone, 5 g agar, 2.5 g yeast extract, 0.25 g NaCl, and 0.09 g KCl was added to 450 mL of distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2 and allowed to cool. Ten mM $MgCl_2$, 10 mM $MgSO_4 \cdot 7H_2O$, and 20 mM glucose was added to this, along with the required antibiotic (100 µg/mL ampicillin), and the final volume adjusted to 500 mL. The media was then poured into bacterial petri dishes and allowed to set overnight.

5.1.5 X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)

Two hundred milligrams of X-gal were dissolved in 10 mL of dimethyl formamide. The solution was stored at -20°C wrapped in foil due to light sensitivity.

5.1.6 Ampicillin (1000x stock)

Five grams of ampicillin was dissolved in a mixture 25 mL of distilled water and 25 mL of 100% ethanol. The solution was filter sterilized and stored at -20°C.

5.1.7 Kanamycin (50x stock)

Five hundred milligrams of kanamycin was dissolved in 10 mL of distilled water. The solution was filter sterilized and stored at -20°C in 500 µL aliquots wrapped in foil due to light sensitivity.

5.1.8 Bacterial transformation buffer

One hundred mM $CaCl^2$ (1.47 g), 10 mM PIPES-HCl (0.3 g), and 15% glycerol (15 mL) was added to 70 mL of distilled water. The solution was adjusted to pH 7 with NaOH, and the final volume adjusted to 100 mL with distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2 and stored at -20°C.

5.2 DNA isolation and purification

5.2.1 Resuspension buffer - Buffer P1

One litre of resuspension buffer was prepared by dissolving 6.06 g Tris base and 3.72 g of $Na_2EDTA.2H_2O$ in 800 mL of distilled water. The solution was adjusted to pH 8 using HCl, and the final volume adjusted to 1000 mL with distilled water. This was then autoclaved for 20 mins at 120° and 1 kg/cm^2 and allowed to cool before 100 mg of RNase A was added, and the buffer stored at 4°C.

5.2.2 Lysis buffer - Buffer P2

One litre of lysis buffer was prepared by adding 8 g of NaOH and 10 g of SDS to 500 mL of distilled water. The final volume was adjusted to 1000 mL, and the solution was autoclaved for 20 mins at 120° and 1 kg/cm^2 then stored at room temperature.

5.2.3 Neutralisation buffer - Buffer P3

One litre of neutralisation buffer was prepared by dissolving 294.5 g of potassium acetate in 500 mL of distilled water. The solution was adjusted to a pH of 5.5 with approximately 300 mL of acetic acid, and adjusted to a final volume of 1000 mL with distilled water before being autoclaved for 20 mins at 120° and 1 kg/cm^2 then stored at 4°C.

5.2.4 Wash buffer – Buffer QC

One litre of wash buffer was prepared by dissolving 58.44 g NaCl and 10.46 g MOPS in 800 mL of distilled water. The solution was adjusted to pH 7 with NaOH, and 150 mL of isopropanol was added, before the final volume was adjusted to 1000 mL with distilled water and stored at room temperature.

5.2.5 Elution buffer – Buffer QF

One litre of elution buffer was prepared by dissolving 73.05 g of NaCl and 6.06 g of Tris base in 800 mL of distilled water. The solution was then adjusted to a pH of 8.5 with HCl and was then autoclaved for 20 mins at 120° and 1 kg/cm^2. Approximately 150 mL of isopropanol was added to reach a final volume of 1000 mL, and the buffer was then stored at room temperature.

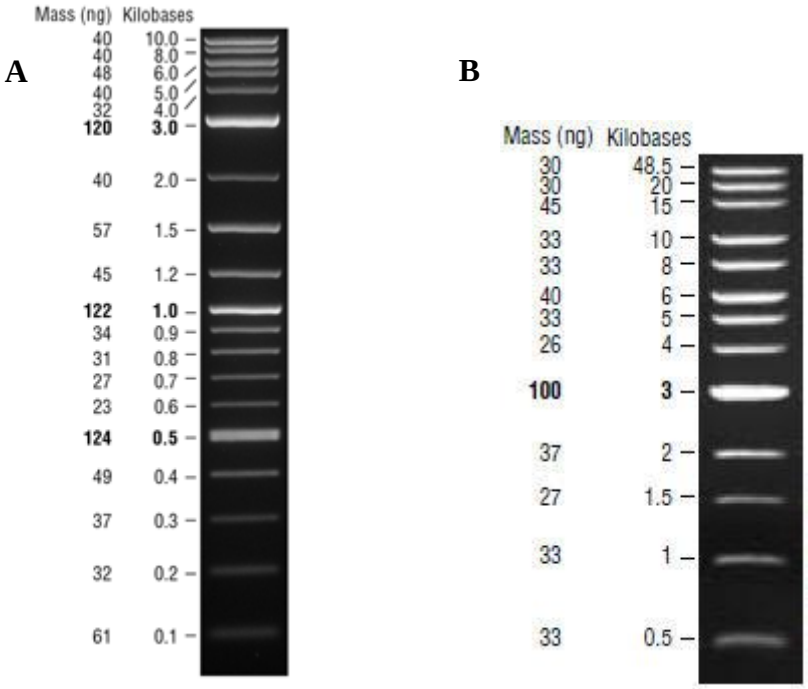
5.2.6 Equilibration buffer – Buffer QBT

One litre of equilibration buffer was prepared by dissolving 43.83 g of NaCl and 10.46 g of MOPS in 800 mL of distilled water. The solution was adjusted to pH 7 using NaOH, and 150 mL of isopropanol was added, followed by 15 mL of 10% Triton-X solution. The final volume was then adjusted to 1000 mL, and the buffer was then stored at room temperature.

5.2.7 Tris-acetate-EDTA (TAE) gel electrophoresis running buffer

A 50×TAE stock was prepared by dissolving 242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8) 800 mL of distilled water, and the final volume adjusted to 1000 mL with distilled water. To then make 1×TAE buffer, 225 ml of 50×TAE was added to 10 L of distilled water, and the volume then adjusted to 11.25 L.

5.2.8 Molecular weight markers



The molecular weight markers used were (**A**) NEB Quick-Load® 1 kb Plus DNA Ladder and (**B**) NEB Quick-Load® 1 kb Extend DNA Ladder

5.3 Tissue culturing

5.3.1 Dulbecco's modified Eagles medium (DMEM)

One litre of DMEM was prepared by dissolving 13.38 g of DMEM powder and 3.7 g $NaHCO_2$ in 1000 ml of Sabax water. The pH was adjusted to 6.7 with HCl and the medium then filter sterilised and stored at 4C.

5.3.2 Penicillin and Streptomycin 1000x

Stock solutions of 100 000 units/mL of penicillin and 100,000 µg/mL of streptomycin were prepared. Upon adding to culture media, 0.5 mL of the 1000× stock is added to 500 mL of media, resulting in working concentration of 100 units/mL of penicillin and 100 µg/mL of streptomycin.

5.3.3 Foetal Bovine serum (FBS)

FBS was heat inactivated and filter sterilised, then aliquoted into 50 mL working stocks and stored at -20°C.

5.3.4 TrypLE™ Express

TrypLE™ Express (Thermo Fisher Scientific, Massachusetts, USA) was diluted 2-fold with saline-EDTA solution, was filter sterilised, then stored at 4°C.

5.4 Sequence alignments

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            1536                                                                1617
nLuc TA C... ttatcgatcacgagactagcctcgatcgaggtcaattcacgcgagttaataattaccagcgcgggccaaataaataatccgc
Easy_D2_M... TTATCGATCACGAGACTAGCCTCGATCGAGGTCAATTCACGCGAGTTAATAATTACCAGCGCGGGCCAAATAAATAATCCGC
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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            1618                                                                1699
nLuc TA C... gaggggcaggtgacgtttgcccagcgcgcgctggtaattattaacctcgcgaatattgattcgaggccgcgattgccgcaat
Easy_D2_M... GAGGGGCAGGTGACGTTTGCCCAGCGCGCGCTGGTAATTATTAACCTCGCGAATATTGATTCGAGGCCGCGATTGCCGCAAT
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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            1700                                                                1781
nLuc TA C... cgcgaggggcaggtgacctttgcccagcgcgcgttcgccccgccccggacggtatcgataagcttaggagcttgggctgcag
Easy_D2_M... CGCGAGGGGCAGGTGACCTTTGCCCAGCGCGCGTTCGCCCCGCCCCGGACGGTATCGATAAGCTTAGGAGCTTGGGCTGCAG
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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            1782                                                                1863
nLuc TA C... gtcgagggcactgggaggatgttgagtaagatggaaaactactgatgacccttgcagagacagagtattaggacatgtttga
Easy_D2_M... GTCGAGGGCACTGGGAGGATGTTGAGTAAGATGGAAAACTACTGATGACCCTTGCAGAGACAGAGTATTAGGACATGTTTGA
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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            1864                                                                1945
nLuc TA C... acaggggccgggcgatcagcaggtagctctagaggatccccgtctgtctgcacatttcgtagagcgagtgttccgatactct
Easy_D2_M... ACAGGGGCCGGGCGATCAGCAGGTAGCTCTAGAGGATCCCCGTCTGTCTGCACATTTCGTAGAGCGAGTGTTCCGATACTCT
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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            1946                                                                2027
nLuc TA C... aatctccctaggcaaggttcatatttgtgtaggttacttattctccttttgttgactaagtcaataatcagaatcagcaggt
Easy_D2_M... AATCTCCCTAGGCAAGGTTCATATTTGTGTAGGTTACTTATTCTCCTTTTGTTGACTAAGTCAATAATCAGAATCAGCAGGT
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_M... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------
Easy_D2_F... ----------------------------------------------------------------------------------

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             2028                                                                2109
nLuc TA C... ttggagtcagcttggcagggatcagcagcctgggttggaaggaggggtataaaagccccttcaccaggagaagccgtcaca
Easy_D2_M... TTGGAGTCAGCTTGGCAGGGATCAGCAGCCTGGGTTGGAAGGAGGGGGTATAAAAGCCCCTTCACCAGGAGAAGCCGTCACA
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2110                                                                2191
nLuc TA C... cagatccacaagctcctgccaccatggtgagctagcgatcaatctagatgcattcgcgaggtaccgagctcgtttagtgaac
Easy_D2_M... CAGATCCACAAGCTCCTGCCACCATGGTGAGCTAGCGATCAATCTAGATGCATTCGCGAGGTACCGAGCTCGTTTAGTGAAC
Easy_D2_F... -------TARRCCTTCTTGCMCCATGGTGAGCTAGCGATCAATCTAGATGCATTCGCGAGGTACCGAGCTCGTTTAGTGAAC
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2192                                                                2273
nLuc TA C... cgtcagatcactagaagctttattgcggtagtttatcacagttaaattgctaacgcagtcagtgcttctgacacaacagtct
Easy_D2_M... CGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTGCTTCTGACACAACAGTCT
Easy_D2_F... CGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGTGCTTCTGACACAACAGTCT
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2274                                                                2355
nLuc TA C... cgaacttaagctgcagtgactctcttaaggtagccttgcagaagttggtcgtgaggcactgggcaggtaagtatcaaggtta
Easy_D2_M... CGAACTTAAGCTGCAGTGACTCTCTTAAGGTAGCCTTGCAGAAGTTGGTCGTGAGGCACTGGCAGGTAAGTATCAAGGTTAC
Easy_D2_F... CGAACTTAAGCTGCAGTGACTCTCTTAAGGTAGCCTTGCAGAAGTTGGTCGTGAGGCACTGGGCAGGTAAGTATCAAGGTTA
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2356                                                                2437
nLuc TA C... caagacaggtttaaggagaccaatagaaactgggcttgtcgagacagagaagactcttgcgtttctgataggcacctattgg
Easy_D2_M... AGACAGGTTTAAGAGACCAATARAAACTGGGCTTGTCGAGACAGAGAGACTCTTGCGTTTCTGATAGCACCTATGGTCTTAC
Easy_D2_F... CAAGACAGGTTTAAGGAGACCAATAGAAACTGGGCTTGTCGAGACAGAGAAGACTCTTGCGTTTCTGATAGGCACCTATTGG
Easy_D2_M... ---------------------------AGGTTACMGCAGGTAGGAACATAGAATGCTKCGACAGRAAGCTYTTGCGTTCGAT
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2438                                                                2519
nLuc TA C... tcttactgacatccactttgcctttctctccacaggtgtccactcccagttcaattacagctcttaaggctagagtacttaa
Easy_D2_M... TGAMATCCACTTTGCCTTTCTYCTCCMCAGTGTCCMCTCCCAGTCAATACAGCTCTAGCTAGAGTACTAATACSGACTCACT
Easy_D2_F... TCTTACTGACATCCACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGTTCAATTACAGCTCTTAAGGCTAGAGTACTTAA
Easy_D2_M... AGCMCCTATGTCTACTGACATCACTGCTTTCTCTCCACAGGTGTCMCTCCCAGTTCAATACAGCCTAAGGCTAGAGTACTAA
Easy_D2_F... ------------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------------

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             2520                                                                2601
nLuc TA C... tacgactcactataggctagcctcgaggtgtcgacccgggcGGCCGC-atgggcggtaggcgtgtacggtgggaggtctata
Easy_D2_M... ATAGGCTAGCTCGAGGGTGTCGACCCGGCGGCGCATTGGCGTTAGGCYGTTACGTGAGCCTATAAGCAGGCTCGATACGTGC
Easy_D2_F... TACGACTCACTATAGGCTAGCCTCGAGGTGTCGACCCGGGCGGCCGCAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
Easy_D2_M... TACGACYCACTATAGGCTAGCCTCGAGTGTCGACCCGGGCGGCCGCAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
Easy_D2_F... ------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------

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             2602                                                                2683
nLuc TA C... taagcagagctcgtttagtgaaccgtcagatcactagaagctttattgcggtagtttatcacagttaaattgctaacgcagt
Easy_D2_M... AACGCTCGATCCWCTAGAAGCT--------------------------------------------------------
Easy_D2_F... TAAGCAGGGCTCGTTTAGTGAACCGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGT
Easy_D2_M... TAAGCAGGGCTCGTTTAGTGAACCGTCAGATCACTAGAAGCTTTATTGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGT
Easy_D2_F... ------------------------------------------------------------------------------
Easy_D2_F... ------------------------------------------------------------------------------

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             2684                                                                2765
nLuc TA C... cagtgggcctcggcggccaagcttggcaatccggtactgttggtaaagccaccatggtcttcacactcgaagatttcgttgg
Easy_D2_M... ------------------------------------------------------------------------------
Easy_D2_F... CAGTGGGCCTCGGCGGCCAAGCTTGGCAATCCGGTATTGTTGGTAAAGCCACCATGGTCTTCACACTCGAAGATTTCGTTGG
Easy_D2_M... CAGTGGGCCTCGGCGGCCAAGCTTGGCAATCCGGTATTGTTGGTAAAGCCACCATGGTCTTCACACTCGAAGATTTCGTTGG
Easy_D2_F... ---------------------------------------------CMCMAAKGGGGTCCTTCCACTCGAAGATTTCGTTGG
Easy_D2_F... ------------------------------------------------------------------------------

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             2766                                                                2847
nLuc TA C... ggactggcgacagacagccggctacaacctggaccaagtccttgaacagggaggtgtgtccagtttgtttcagaatctcggg
Easy_D2_M... ------------------------------------------------------------------------------
Easy_D2_F... GGACTGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTTCAGAATCTCGGG
Easy_D2_M... GGACTGGCGACAGACAGCCGGCTACAACCTGGACCAAGTCCTTGAACAGGGAGGTGTGTCCAGTTTGTTTCAGAATCTCGGG
Easy_D2_F... GGACTGGCGACAGACAGCCGGCTACAACCTGGACCMAGTYCTTGAACAGGGAGGTGTGTCCAGTTTGTTTCAGAATCTCGGG
Easy_D2_F... ------------------------------------------------------------------------------

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             2848                                                                2929
nLuc TA C... gtgtccgtaactccgatccaaaggattgtcctgagcggtgaaaatgggctgaagatcgacatccatgtcatcatcccgtatg
Easy_D2_M... ------------------------------------------------------------------------------
Easy_D2_F... GTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATG
Easy_D2_M... GTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATG
Easy_D2_F... GTGTCCGTAACTCCGATCCAAAGGATTGTCCTGAGCGGTGAAAATGGGCTGAAGATCGACATCCATGTCATCATCCCGTATG
Easy_D2_F... ------------------------------------------------------------------------------

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             2930                                                                3011
nLuc TA C... aaggtctgagcggcgaccaaatgggccagatcgaaaaaattttaaggtggtgtaccctgtggatgatcatcactttaaggt
Easy_D2_M... ------------------------------------------------------------------------------
Easy_D2_F... AAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGT-GTGTACCCTGTGGATGATCATCACTTTAAGKG
Easy_D2_M... AAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGT
Easy_D2_F... AAGGTCTGAGCGGCGACCAAATGGGCCAGATCGAAAAAATTTTTAAGGTGGTGTACCCTGTGGATGATCATCACTTTAAGGT
Easy_D2_F... ------------------------------------------------------------------------------

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            3012                                                                        3093
nLuc TA C... gatcctgcactatggcacactggtaatcgacggggttacgccgaacatgatcgactatttcggacggccgtatgaaggcatc
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... ATCCTGCACTATGGCACACTGGTAATCGACGGGGTTACSCCGAACATGATCGACTATTTCGGACGGCGTATGAAGGCATCGC
Easy_D2_M... GATCCTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTATTTCGGACGGCCGTATGAAGGCATC
Easy_D2_F... GATCCTGCACTATGGCACACTGGTAATCGACGGGGTTACGCCGAACATGATCGACTATTTCGGACGGCCGTATGAAGGCATC
Easy_D2_F... ------------------------------------------------------------------------------------

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            3094                                                                        3175
nLuc TA C... gccgtgttcgacggcaaaaagatcactgtaacagggaccctgtggaacggcaacaaaattatcgacgagcgcctgatcaacc
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... GTGTTCGACGGCAAAAGATCMCTTGTAACAGGGACCTGTGGACGCACAAATTATCGACGAGCGCTGATCAGCCGACGGCTCC
Easy_D2_M... GCCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACC
Easy_D2_F... GCCGTGTTCGACGGCAAAAAGATCACTGTAACAGGGACCCTGTGGAACGGCAACAAAATTATCGACGAGCGCCTGATCAACC
Easy_D2_F... ------------------------------------------------------------------------------------

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            3176                                                                        3257
nLuc TA C... ccgacggctccctgctgttccgagtaaccatcaacggagtgaccggctggcggctgtgcgaacgcattctggcgtaattcta
Easy_D2_M... ------------------------------------------------------------------------------------
Easy_D2_F... CTGCTKTTCGAGTACATCACGATGACGCTGCGCTGGCSACGCATTCTGCGTATTCTAAGTCCGGCGCCGATCSATCAGCGAA
Easy_D2_M... CCGACGGCTCCCTGCTGTTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGTAATTCTA
Easy_D2_F... CCGACGGCTCCCTGCTGTTCCGAGTAACCATCAACGGAGTGACCGGCTGGCGGCTGTGCGAACGCATTCTGGCGTAATTCTA
Easy_D2_F... ------------------------------------------------------------------------------------

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            3258                3281
nLuc TA C... gagtcggggcggccggccgcttcg
Easy_D2_M... ------------------------
Easy_D2_F... CTGATAGATACCTGATGATTTGGA
Easy_D2_M... GAGTCGGGGCGGCCGGCCGCTTCG
Easy_D2_F... GAGTCGGGGCGGCCGGCCGCTTCG
Easy_D2_F... ------------------------

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Figure 5.1: Sequence alignment of the nLuc transgene.

Sequence alignment of the positive clone of the nLuc transgene against the reference sequence, generated using primers designed to flank the nLuc gene and the mTTR promoter sequences.

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            1464                                                                1545
CRISPR-Ca... gacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccgcgttac
CRSIPR_D1... GACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTAC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            1546                                                                1627
CRISPR-Ca... ataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccata
CRSIPR_D1... ATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            1628                                                                1709
CRISPR-Ca... gtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgt
CRSIPR_D1... GTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            1710                                                                1791
CRISPR-Ca... atcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagtacatgaccttatg
CRSIPR_D1... ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            1792                                                                1873
CRISPR-Ca... ggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatggg
CRSIPR_D1... GGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            1874                                                                                1955
CRISPR-Ca... cgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatc
CRSIPR_D1... CGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATC
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------

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            1956                                                                                2037
CRISPR-Ca... aacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatat
CRSIPR_D1... AACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------

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            2038                                                                                2119
CRISPR-Ca... aagcagagctctctggctaactaccggtgccaccATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCA
CRSIPR_D1... AAGCAGAGCTCTCTGGCTAACTACCGGTGCCACCATGGCCCCAAAGAAGAAGCGGAAGGTCGGTATCCACGGAGTCCCAGCA
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------

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            2120                                                                                2201
CRISPR-Ca... GCCAAGCGGAACTACATCCTGGGCCTGGACATCGGCATCACCAGCGTGGGCTACGGCATCATCGACTACGAGACACGGGACG
CRSIPR_D1... GCCAAGCGGAACTACATCCTGGGCCTGGACATCGGCATCACCAGCGTGGGCTACGGYATCATCGACTACGAGACACGGGACG
CRSIPR_D1... ----------------------------------------------------------CTYSSAAACTWACGGRGACACGGGACG
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------

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            2202                                                                                2283
CRISPR-Ca... TGATCGATGCCGGCGTGCGGCTGTTCAAAGAGGCCAACGTGGAAAACAACGAGGGCAGGCGGAGCAAGAGAGGCGCCAGAAG
CRSIPR_D1... TGATCGATGCCGGCGTGCGGCTGTTCAAAGAGGCCAACGTGGAAAACAACGAGGGCARGCGGAGCAAGAGAGGCGCCAGAAG
CRSIPR_D1... TGATCGATGCCGGCGTGCGGCTGTTCAAAGAGGCCAACGYGGAAAACAACGAGGGCAGGCGGAGCAAGAGAGGCGCCAGAAG
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------
CRSIPR_D1... ----------------------------------------------------------------------------------

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             2284                                                                                2365
CRISPR-Ca... GCTGAAGCGGCGGAGGCGGCATAGAATCCAGAGAGTGAAGAAGCTGCTGTTCGACTACAACCTGCTGACCGACCACAGCGAG
CRSIPR_D1... GCTGAAGCGGCGGAGGCGGCATARAWTCCAGAGAGTGAAGAAGCTGCTGTTCGACTACAACCTGCTGACCGACCACAGCGGR
CRSIPR_D1... GCTGAAGCGGCGGAGGCGGCATAGAATCCAGAGAGTGAAGAAGCTGCTGTTCGACTACAACCTGCTGACCGACCACAGCGAG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             2366                                                                                2447
CRISPR-Ca... CTGAGCGGCATCAACCCCTACGAGGCCAGAGTGAAGGGCCTGAGCCAGAAGCTGAGCGAGGAAGAGTTCTCTGCCGCCCTGC
CRSIPR_D1... GCTGAGCGGCATCAAYCCCTACGAGGCAGRKKGAAGGGCCTKGAGCMAWAAGCTGACSAGGAARAGTTYTYYKGYCGCCTSK
CRSIPR_D1... CTGAGCGGCATCAACCCCTACGAGGCCAGAGTGAAGGGCCTGAGCCAGAAGCTGAGCGAGGAAGAGTTCTCTGCCGCCCTGC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             2448                                                                                2529
CRISPR-Ca... TGCACCTGGCCAAGAGAAGAGGCGTGCACAACGTGAACGAGGTGGAAGAGGACACCGGCAACGAGCTGTCCACCAgAGAGCA
CRSIPR_D1... CYKSMACCTGGCCAARARAARAGGCGTGCMCAWSCGTGAACGARGTGGAGAAGAMCACTGGCAACARGCTKGTCCACCAAAG
CRSIPR_D1... TGCACCTGGCCAAGAGAAGAGGCGTGCACAACGTGAACGAGGTGGAAGAGGACACCGGCAACGAGCTGTCCACCAAAGAGCA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             2530                                                                                2611
CRISPR-Ca... GATCAGCCGGAACAGCAAGGCCCTGGAAGAGAAATACGTGGCCGAACTGCAGCTGGAACGGCTGAAGAAAGACGGCGAAGTG
CRSIPR_D1... RACGRAWTCASCGGAACGCAGCTTGGAGAAAAWATCGTGGCAACTGTGCCARCKGGAACCGMCTGTGAGCA-----------
CRSIPR_D1... GATCAGCCGGAACAGCAAGGCCCTGGAAGAGAAATACGTGGCCGAACTGCAGCTGGAACGGCTGAAGAAAGACGGCGAAGTG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             2612                                                                                2693
CRISPR-Ca... CGGGGCAGCATCAACAGATTCAAGACCAGCGACTACGTGAAAGAAGCCAAACAGCTGCTGAAGGTGCAGAAGGCCTACCACC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... CGGGGCAGCATCAACAGATTCAAGACCAGCGACTACGTGAAAGAAGCCAAACAGCTGCTGAAGGTGCAGAAGGCCTACCACC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            2694                                                                                2775
CRISPR-Ca... AGCTGGACCAGAGCTTCATCGACACCTACATCGACCTGCTGGAAACCCGGCGGACCTACTATGAGGGACCTGGCGAGGGCAG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... AGCTGGACCAGAGCTTCATCGACACCTACATCGACCTGCTGGAAACCCGGCGGACCTACTATGAGGGACCTGGCGAGGGCAG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            2776                                                                                2857
CRISPR-Ca... CCCCTTCGGCTGGAAGGACATCAAAGAATGGTACGAGATGCTGATGGGCCACTGCACCTACTTCCCCGAGGAACTGCGGAGC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... CCCCTTCGGCTGGAAGGACATCAAAGAATGGTACGAGATGCTGATGGGCCACTGCACCTACTTCCCCGRGGAACTGCGGAGC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            2858                                                                                2939
CRISPR-Ca... GTGAAGTACGCCTACAACGCCGACCTGTACAACGCCCTGAACGACCTGAACAATCTCGTGATCACCAGGGACGAGAACGAGA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... GTGAAGTACGCCTACAACGCCGACCTGTACAACGCCCTGAACGACCTGAACAATCTCGTGATCACCAGGGACGAGAACGAGA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            2940                                                                                3021
CRISPR-Ca... AGCTGGAATATTACGAGAAGTTCCAGATCATCGAGAACGTG-TTCAAGCAGAAGAAGAAGCCCACCCTGAAGCAGATCGCCA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... AGCTGGAATATTACGAGAAGTTCCAGATCATCGAGAACGTGTTTCAAGCAGAAGAAGAAGCCCACCCTGAAGCAGATCGCCA
CRSIPR_D1... -----GRGRRRRRRKTYYMSGAWTCATCCGGRAGAACGTGTTCAAGCAGGAAGAAGAAGGCCCACCCTGAAGCAGATCGCCA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3022                                                                                3103
CRISPR-Ca... AAGAAATCCTCGTGAACGAAGAGGATATTAAGGGCTACAGAGTGACCAGCACCGGCAAGCCCGAGTTCACCAACCTGAAGGT
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... AAGAAATCCTCGTGAACG-AGAGGATATTAAGGGCTACAGAGTGACCAGCCACCGGCAGCCCGAGTTCMCCAACCTGAAGTG
CRSIPR_D1... AAGAAATCCTCKWGAACGAAGAGGATATTAAGGGCTACAGAGTGACCAGCACCGGCAAGCCCGAGTTCACCAACCTGAAGGT
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3104                                                                3185
CRISPR-Ca... GTACCACGACATCAAGGACATTACCGCCCGGGAAAGAGATTATTGAGAACGCCGAGCTGCTGGATCAGATTGCCAAGATCCTG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... TACCACGACATCAAGGACATTACCGCCCGGGAAAGARAATTATTGAGAACGCCGAGCTGCTGGATCAGATTGCAGATCTKGA
CRSIPR_D1... GTACCACGACATCAAGGACATTACCGCCCGGAAAGAGATTATTGAGAACGCCGAGCTGCTGGATCAGATTGCCAAGATCCTG
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3186                                                                3267
CRISPR-Ca... ACCATCTACCAGAGCAGCGAGGACATCCAGGAAGAACTGACCAATCTGAACTCCGAGCTGACCCAGGAAGAGATCGAGCAGA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... CCATYTACAGAGCAGCGAGGACATCCAGGAAGACTGAYCATTCTGACTCCGAGCTGGACCYCAGGAGRGATCSARCRRATYT
CRSIPR_D1... ACCATCTACCAGAGCAGCGAGGACATCCAGGAAGAACTGACCAATCTGAACTCCGAGCTGACCCAGGAAGAGATCGAGCAGA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3268                                                                3349
CRISPR-Ca... TCTCTAATCTGAAGGGCTATACCGGCACCCACAACCTGAGCCTGAAGGCCATCAACCTGATCCTGGACGAGCTGTGGCACAC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... YYTTATCTGAAGGGCTWTACCGACCCAACCTKRGCCTGAAGGCATCACTKGATTCTGGACGAGCTTGGACTCACGTATCGAT
CRSIPR_D1... TCTCTAATCTGAAGGGCTATACCGGCACCCACAACCTGAGCCTGAAGGCCATCAACCTGATCCTGGACGAGCTGTGGCACAC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3350                                                                3431
CRISPR-Ca... CAACGACAACCAGATCGCTATCTTCAACCGGCTGAAGCTGGTGCCCAAGAAGGTGGACCTGTCCCAGCAGAAAGAGATCCCC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... GSATCTCTAAACTGTAKGAGCWTGTGTGTACA----------------------------------------------------
CRSIPR_D1... CAACGACAACCAGATCGCTATCTTCAACCGGCTGAAGCTGGTGCCCAAGAAGGTGGACCTGTCCCAGCAGAAAGAGATCCCC
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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            3432                                                                3513
CRISPR-Ca... ACCACCCTGGTGGACGACTTCATCCTGAGCCCCGTCGTGAAGAGAAGCTTCATCCAGAGCATCAAAGTGATCAACGCCATCA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... ACCACCCTGGTGGACGACTTCATCCTGAGCCCCGTCGTGAAGAGAAGCTTCATCCAGAGCATCAAAGTGATCAACGCCATCA
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------
CRSIPR_D1... --------------------------------------------------------------------------------

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             3514                                                                                3595
CRISPR-Ca... TCAAGAAGTACGGCCTGCCCAACGACATCATTATCGAGCTGGCCCGCGAGAAGAACTCCAAGGACGCCCAGAAAATGATCAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TCAAGAAGTACGGCCTGCCCAACGACATCATTATCGAGCTGGCCCGCGAGAAGAACTCCAAGGACGCCCAGAAAATGATCAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             3596                                                                                3677
CRISPR-Ca... CGAGATGCAGAAGCGGAACCGGCAGACCAACGAGCGGATCGAGGAAATCATCCGGACCACCGGCAAAGAGAACGCCAAGTAC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... CGAGATGCAGAAGCGGAACCGGCAGACCAACGAGCGGATCGAGGAAATCATCCGGACCACCGGCAAAGAGAACGCCAAGTAC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             3678                                                                                3759
CRISPR-Ca... CTGATCGAGAAGATCAAGCTGCACGACATGCAGGAAGGCAAGTGCCTGTACAGCCTGGAAGCCATCCCTCTGGAAGATCTGC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... CTGATCGAGAAGATCAAGCTGCACGACATGCAGGAAGGCAAGTGCCTGTACAGCCTGGAAGCCATCCCTCTGGAAGATCTGC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             3760                                                                                3841
CRISPR-Ca... TGAACAACCCCTTCAACTATGAGGTGGACCACATCATCCCCAGAAGCGTGTCCTTCGACAACAGCTTCAACAACAAGGTGCT
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TGAACAACCCCTTCAACTATGAGGKGGACCACATCATCCCCAGAAGCGTGTCCTTCGACAACRGCTTCAACAACAAGGTGCT
CRSIPR_D1... ---GSCKTYMMMYWKKGRRGGGTGGGRCMACRTCRTCCSSAGAAGSGTGTCSTTCGACAACAGCTTCAACAACAAGGCTGTT
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             3842                                                                                3923
CRISPR-Ca... CGTGAAGCAGGAAG-AAAACAGCAAGAAGGGCAACCGGACCCCATTCCAGTACCTGAGCAGCAGCGACAGCAAGATCAGCTA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... CGTGAAGCAGGAAGAAAAACAGCAAGAGGCACCCGGACCCCATTCCAGTACCTGRGCAGCARCGACRGCAGATCWGCTACGA
CRSIPR_D1... CGWGAAGCAGGAAG-AAAACAGCAAGAAGGGCAACCGGACCCCATTCCAGTACCTGAGCAGCAGCGACAGCAAGATCWGCTA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            3924                                                                                4005
CRISPR-Ca... CGAAACCTTCAAGAAGCACATCCTGAATCTGGCCAAGGGCAAGGGCAGAATCAGCAAGACCAAGAAAGAGTATCTGCTGGAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... AWCCTTCAAGAGCACATCCTGGATTCTGGGCCAAGGGCAGGCARATTCAGCAGACAAGAAGRAGWTYTGCTGARGACGGGAM
CRSIPR_D1... CGAAACCTTCAAGAAGCACATCCTGAATCTGGCCAAGGGCAAGGGCAGAATCAGCAAGACCAAGAAAGAGTATCTGCTGGAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            4006                                                                                4087
CRISPR-Ca... GAACGGGACATCAACAGGTTCTCCGTGCAGAAAGACTTCATCAACCGGAACCTGGTGGATACCAGATACGCCACCAGAGGCC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TTCACAGGTYTCCGTGCRAAAGACTTCATCACGGACTGGTGATACCAGAAWCSCCACAAGGCTGATGACTGGYTGGCGGGTA
CRSIPR_D1... GAACGGGACATCAACAGGTTCTCCGTGCAGAAAGACTTCATCAACCGGAACCTGGTGGATACCAGATACGCCACCAGAGGCC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            4088                                                                                4169
CRISPR-Ca... TGATGAACCTGCTGCGGAGCTACTTCAGAGTGAACAACCTGGACGTGAAAGTGAAGTCCATCAATGGCGGCTTCACCAGCTT
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... YTCAAGTAMCTGACTAGTGASTCATCATGGCGTTCACCACRCTTCTYTCSGCCG--------------------------
CRSIPR_D1... TGATGAACCTGCTGCGGGGCTACTTCAGAGTGAACAACCTGGACGTGAAAGTGAAGTCCATCAATGGCGGCTTCACCAGCTT
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            4170                                                                                4251
CRISPR-Ca... TCTGCGGCGGAAGTGGAAGTTTAAGAAAGAGCGGAACAAGGGGTACAAGCACCACGCCGAGGACGCCCTGATCATTGCCAAC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TCTGCGGCGGAAGTGGAAGTTTAAGAAAGAGCGGAACAAGGGGTACAAGCACCACGCCGAGGACGCCCTGATCATTGCCAAC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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            4252                                                                                4333
CRISPR-Ca... GCCGATTTCATCTTCAAAGAGTGGAAGAAACTGGACAAGGCCAAAAAAGTGATGGAAAACCAGATGTTCGAGGAAAgGCAGG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... GCCGATTTCATCTTCAAAGAGTGGAAGAAACTGGACAAGGCC-AAAAAGTGATGGAAAACCAGATGTTCGAGGAAAAGCAGG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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APPENDIX

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               4334                                                                           4415
CRISPR-Ca...   CCGAGAGCATGCCCGAGATCGAAACCGAGCAGGAGTACAAAGAGATCTTCATCACCCCCCACCAGATCAAGCACATTAAGGA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   CCGAGAGCATGCCCGAGATCGAAACCGAGCAGGAGTACAAAGAGATCTTCATCACCCCCCACCAGATCAAGCACATTAAGGA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------

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               4416                                                                           4497
CRISPR-Ca...   CTTCAAGGACTACAAGTACAGCCACCGGGTGGACAAGAAGCCTAATAGAGAGCTGATTAACGACACCCTGTACTCCACCCGG
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   CTTCAAGGACTACAAGTACAGCCACCGGGTGGACAAGAAGCCTAATAGAGAGCTGATTAACGACACCCTGTACTCCACCCGG
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------

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               4498                                                                           4579
CRISPR-Ca...   AAGGACGACAAGGGCAACACCCTGATCGTGAACAATCTGAACGGCCTGTACGACAAGGACAATGACAAGCTGAAAAAGCTGA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   AAGGACGACAAGGGCAACACCCTGATCGTGAACAATCTGAACGGCCTGTACGACAAGGACAATGACAAGCTG-AAAAGCTGA
CRSIPR_D1...   -------------------------------------------------------------------AARRCCKTKGWAWCA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------

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               4580                                                                           4661
CRISPR-Ca...   TCAACAAGAGCCCCGAAAAGCTGCTGATGTACCACCACGACCCCCAGACCTACCAGAAACTGAAGCTGATTATGGAACAGTA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   TCAACAAGAGCCCCGAAAAGCTGCTGATGTACCACCACGA-CCCCAGACCTACCAG-AACTGAAGCTGATTATGGAACAGTA
CRSIPR_D1...   ACAAGGAAGCCCCGAAAAGGCTGCTGATGTACCACCACGACCCCCAGACCTACCAGAAACTGMAGCTGATTATGGAACAGTA
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------

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               4662                                                                           4743
CRISPR-Ca...   CGGCGACGAGAAGAATCCCCTGTACAAGTACTACGAGGAAACCGGGAACTACCTGACCAAGTACTCCAAAAAGGACAACGGC
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   CGGCGACGAGAAGAATCCCCTGTACAGTACTACGAGGAAACCGGACTACCTGACCAGTACTCCAAAAAGGACACGGCCCGTG
CRSIPR_D1...   CGGCGACGAGAAGAATCCCCTGTACAAGTACTACGAGGAAACCGGGAACTACCTGACCAAGTACTCCAAAAAGGACAACGGC
CRSIPR_D1...   ----------------------------------------------------------------------------------
CRSIPR_D1...   ----------------------------------------------------------------------------------

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             4744                                                                                4825
CRISPR-Ca... CCCGTGATCAAGAAGATTAAGTATTACGGCAACAAACTGAACGCCCATCTGGACATCACCGACGACTACCCCAACAGCAGAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ATCAGAAGATAAGTATACGGCAACAACTGAACGCCCATCTGGAMATCACGACGACTACCCACAGCAGAACAGGTCTGGAGCT
CRSIPR_D1... CCCGTGATCAAGAAGATTAAGTATTACGGCAACAAACTGAACGCCCATCTGGACATCACCGACGACTACCCCAACAGCAGAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------

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             4826                                                                                4907
CRISPR-Ca... ACAAGGTCGTGAAGCTGTCCCTGAAGCCCTACAGATTCGACGTGTACCTGGACAATGGCGTGTACAAGTTCGTGACCGTGAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... KCCTTGAAGCCTACRATCCACGGTACTGACAATGGWGTTACAGTYCKAACGGTGAGACYGATGCATCCAAAGAAGCTATCCG
CRSIPR_D1... ACAAGGTCGTGAAGCTGTCCCTGAAGCCCTACAGATTCGACGTGTACCTGGACAATGGCGTGTACAAGTTCGTGACCGTGAA
CRSIPR_D1... ------------------------------------------------------------------------------TKC
CRSIPR_D1... ------------------------------------------------------------------------------------

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             4908                                                                                4989
CRISPR-Ca... GAATCTGGATGTGATCAAAAAAGAAAACTACTACGAAGTGAATAGCAAGTGCTATGAGGAAGCTAAGAAGCTGAAGAAGATC
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ATGTAGCAGGCTWGAGGARYCTAAGAACCTGTGRACGAT------------------------------------------
CRSIPR_D1... GAATCTGGATGTGATCAAAAAAGAAAACTACTACGAAGTGGATAGCAAGTGCTATGAGGAAGCTAAGAAGCTGAAGAAGATC
CRSIPR_D1... RTTCCAAAAAAAGACATCTCTCGRAGTSGATWGGCMAGTGKCTTWAGGGAGGYTARRAGGTSGAAGAAGATTCAGACACAAGG
CRSIPR_D1... ------------------------------------------------------------------------------------

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             4990                                                                                5071
CRISPR-Ca... AGCAACCAGGCCGAGTTTATCGCCTCCTTCTACAACAACGATCTGATCAAGATCAACGGCGAGCTGTATAGAGTGATCGGCG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... AGCAACCAGGCCGAGTTTATCGCCTCCTTCTACAACAACGATCTGATCAAGATCAACGGCGAGCTGTATAGAGTGATCGGCG
CRSIPR_D1... CCSRAGTTWTWTCSTTCCCTTTCTCMAMCACGAATCTGAWTCAARAATCAACSGSGGMKCTKTWWAGRRKKAWTCCGCGKKA
CRSIPR_D1... ------------------------------------------------------------------------------------

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             5072                                                                                5153
CRISPR-Ca... TGAACAACGACCTGCTGAACCGGATCGAAGTGAACATGATCGACATCACCTACCGCGAGTACCTGGAAAACATGAACGACAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TGAACAACGACCTGCTGAACCGGATCGAAGTGAACATGATCGACATCACCTACCGCGAGTACCTGGAAAACATGAACGACAA
CRSIPR_D1... ACCACGACTKGYKAACCCGGATTCGAAGKGAACATGATCGACATCMCCTWCCGCCGAGKWCCTGGAAAACATGAACGACAAG
CRSIPR_D1... ------------------------------------------------------------------------------------

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             5154                                                                                5235
CRISPR-Ca... GAGGCCCCCCAGGATCATTAAGACAATCGCCTCCAAGACCCAGAGCATTAAGAAGTACAGCACAGACATTCTGGGCAACCTG
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... GAGGCCCCCCAGGATCATTAAGACAATCGCCTCCAAGACCCAGAGCATTAAGAAGTACAGCACAGACATTCTGGGCAACCTG
CRSIPR_D1... AGSCCCCCCCAGGATCATTAAGACAATCGCYTCCAGGCCCAGRAGCATTAAGAAGTACAGCMCAGACATTCTGGGCAACCTG
CRSIPR_D1... ------------------------------------------------------------------------------------

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             5236                                                                                5317
CRISPR-Ca... TATGAAGTGAAATCTAAGAAGCACCCTCAGATCATCAAAAAGGGCAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAA
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TATGAAGTGAAATCTAAGAAGCACCCTCAGATCGTCAAAAAGGGCAAAAGGCCGGCGGCCACGAAAAAGGCCGGCCAGGCAA
CRSIPR_D1... TATGAAGTGAAATCTAAGAAGCACCCTCAGATCGTCAAAAAGGSCAAAAGGCCGGCGGCCACGAAAAAGGCCGCCCAGGCAA
CRSIPR_D1... ------------------------------------------------------------------------------------

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             5318                                                                                5399
CRISPR-Ca... AAAAGAAAAAGggatcctacccatacgatgttccagattacgcttacccatacgatgttccagattacgcttacccatacga
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... AAAAGAAAAAGGGATCCTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTTACCCATACGA
CRSIPR_D1... AAAAGAAAAAGGGATCCTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTTACCCATACGA
CRSIPR_D1... ----------------------------------------ACCCTTACSGAWTGKTTCMAGATTACGCTTACCCATACGA

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             5400                                                                                5481
CRISPR-Ca... tgttccagattacgcttaagaattcctagagctcgctgatcagcctcgactgtgccttctagttgccagccatctgttgttt
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... TGTTCCAGATTACGCTTAAGAATTCCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTG-TT
CRSIPR_D1... TGTTCCAGATTACGCTTAAGAATTCCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT
CRSIPR_D1... TGTTCCAGATTACGCTTAAGAATTCCTAGAGCTCRMTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT

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             5482                                                                                5563
CRISPR-Ca... gcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgca
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... GCCCCTCCCCCGTGCCTTCCTTGACCTGGAAGKGGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCAT
CRSIPR_D1... GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCA
CRSIPR_D1... GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCA

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            5564                                                                                5645
CRISPR-Ca... ttgtctgagtaggtgtcattctattctgggggggtggggtggggcaggacagcaagggggaggattgggaagagaatagcagg
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... GTCTGAGTAGGKGTCATTCTATTCTGGGGGGTGGGKGGGGCAGACAGCAAGGGGGARGATTGGTAGRAGAATTRCAGCATGC
CRSIPR_D1... TTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAGAATAGCAGG
CRSIPR_D1... TTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGAGAATAGCAGG

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            5646                                                                                5727
CRISPR-Ca... catgctggggaggtacctgagggcctatttcccatgattccttcatatttgcatatacgatacaaggctgttagagagataa
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... CTGGGGAGGTACGAGGGCCTATTCCATGATCCTCATTTGCATACGATCAAGGCSTGTAGAGAGAATCTGCATTAATTGACGT
CRSIPR_D1... CATGCTGGGGAGGTACC-GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAA
CRSIPR_D1... CATGCTGGGGAGGTACC-GAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAA

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            5728                                                                                5809
CRISPR-Ca... ttggaattaatttgactgtaaacacaaagatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgc
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... GACCAGCATTAGTCCAAATCGTGGMCGTSAAGAGTACGTC------------------------------------------
CRSIPR_D1... TTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGC
CRSIPR_D1... TTGGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGC

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            5810                                                                                5891
CRISPR-Ca... agttttaaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttatatatc
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... ------------------------------------------------------------------------------------
CRSIPR_D1... AGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATGTATC
CRSIPR_D1... AGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATGTATC

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            5892
CRISPR-Ca... ttGTGG
CRSIPR_D1... ------
CRSIPR_D1... ------
CRSIPR_D1... ------
CRSIPR_D1... ------
CRSIPR_D1... ------
CRSIPR_D1... TTGTGG
CRSIPR_D1... TTGTGG

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```

Figure 5.2: Sequence alignment of the CRISPR/Cas9 transgene.

Sequence alignment of the positive clone of the anti-HBV CRISPR/Cas9 transgene against the reference sequence, generated using primers designed to flank the SaCas9 and sgRNA sequences.

```
            1262                                                                                1343
Recombina... ttacgccagaatgcgttcgcacagccgccagccggtcactccgttgatggttactcggaacagcagggagccgtcggggttg
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1344                                                                                1425
Recombina... atcaggcgctcgtcgataattttgttgccgttccacagggtccctgttacagtgatctttttgccgtcgaacacggcgatgc
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1426                                                                                1507
Recombina... cttcatacggccgtccgaaatagtcgatcatgttcggcgtaaccccgtcgattaccagtgtgccatagtgcaggatcacctt
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1508                                                                                1589
Recombina... aaagtgatgatcatccacagggtacaccaccttaaaaatttttcgatctggcccatttggtcgccgctcagaccttcatac
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1590                                                                1671
Recombina... gggatgatgacatggatgtcgatcttcagcccattttcaccgctcaggacaatcctttggatcggagttacggacaccccga
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1672                                                                1753
Recombina... gattctgaaacaaactggacacacctccctgttcaaggacttggtccaggttgtagccggctgtctgtcgccagtccccaac
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1754                                                                1835
Recombina... gaaatcttcgagtgtgaagaccatggtggctttaccaacagtaccggattgccaagcttggccgccgaggcccactgactgc
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1836                                                                1917
Recombina... gttagcaatttaactgtgataaactaccgcaataaagcttctagtgatctgacggttcactaaacgagctctgcttatatag
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            1918                                                                                1999
Recombina... acctcccaccgtacacgcctaccgcccatGCGGCCgcccgggtcgacacctcgaggctagcctatagtgagtcgtattaagt
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            2000                                                                                2081
Recombina... actctagccttaagagctgtaattgaactgggagtggacacctgtggagagaaaggcaaagtggatgtcagtaagaccaata
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

................................................................................................

            2082                                                                                2163
Recombina... ggtgcctatcagaaacgcaagagtcttctctgtctcgacaagcccagtttctattggtctccttaaacctgtcttgtaacct
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            2164                                                                                2245
Recombina... tgatacttacctgcccagtgcctcacgaccaacttctgcaaggctaccttaagagagtcactgcagcttaagttcgagactg
pD28-10-2... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------------------------------------------------
pD28-10-2... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------
pD28-10-1... ----------------------------------------------------------------------------------

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            2246                                                                                2327
Recombina... ttgtgtcagaagcactgactgcgttagcaatttaactgtgataaactaccgcaataaagcttctagtgatctgacggttcac
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2328                                                                                2409
Recombina... taaacgagctcggtacctcgcgaatgcatctagattgatcgctagctcaccatggtggcaggagcttgtggatctgtgtgac
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... ----------------------------------------TCCAGYRAAAACACCWATGCCATGATACGTCAAGCCWTTAGT
pD28-10-2... ------------------------------------------------TTCACAGAAAAACATCAKAGCYAATRTTATCGCCCA
pD28-10-1... ----------------------------------------------------CTCMTMGGATACASACTATGRCCATGAT
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2410                                                                                2491
Recombina... ggcttctcctggtgaaggggctttataccccctccttccaacccaggctgctgatccctgccaagctgactccaaacctgc
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... RACCATWGGAWCTCAGCTATGCATGCAACGCGTTGRAGCTCCTCCCATATGKTCGACCTGCAGGCGGCGCGGAATCACTAGT
pD28-10-2... AGCWTTTAGGKACAYTATGRAWACYCAGCCATKCATCACSCCGTTGGAGCTCCYCATATGKACGAATTGCRGKCGCCGCKAA
pD28-10-1... ACCCCAGCTATTTGKGACCTATGATATCYAAGCTAWGCACACGCGTGRRGCTCCCATAWGGTCGACCGCAGGCGCSCAATTC
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2492                                                                                2573
Recombina... tgattctgattattgacttagtcaacaaaaggagaataagtaacctacacaaatatgaaccttgcctagggagattagagta
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... GATTAAAACACMMCAWTAWCAACTGAAAMACCCTCCTGCCTAGGCAAAAWRGCATCCTCCCGCTCCAGAACAACATACAGCG
pD28-10-2... WTCACTARTKTTAAACACMMCAKTAACAACCTGAAMAACTCTCCTGCCTAGCMAAAATAGCWCCCYCTCGCTCCARAACAAC
pD28-10-1... ACTAGTKTTAAACMCACAATAACWCTGAAAAACCCYTCTKCCTAGGCAAAAATAGCACCCCTCCGCCTCCARAACAACAKAC
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2574                                                                                2655
Recombina... tcggaacactcgctctacgaaatgtgcagacagacggggatcctctagagctacctgctgatcgcccggcccctgttcaaac
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... CTGTCACAGCGGCAGCCATWACAGTCAGCCTWACCAGTAAAAAAGAAACCCTATTAAAAAAAACACACTCGACACGGCACCA
pD28-10-2... AAWCAGCGCYTCCACAGCGCAGCCATAACAGTCAGCCTTACCAGWAAAAAGAAAACCTATAAAAAAACACWCTCKACACKGC
pD28-10-1... AGCGCTTCACWGCGGCAGCCATAACAGTCAGCCTTACCAGTAAAAAGAAACCTATTAAAAAAACACCACTTCGACACGGCAC
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2656                                                                                2737
Recombina... atgtcctaatactctgtctctgcaagggtcatcagtagttttccatcttactcaacatcctcccagtgccctcgacctgcag
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... GTCCCAATCAGTCMCTGTGTAAWAACGRRCGCCAGTGAATTGTAATACGACTCACTAT------------------------
pD28-10-2... ACAGCCCMMTCATCCYGTGTWATACSRACGGCCAGTGAATTGTAATACGACTCACTAT------------------------
pD28-10-1... CAGCCCATCAGTCACTGTGTAWAACGGRCGGCCAGTGAATTGTAATACGACTCACTAT------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2738                                                                                2819
Recombina... cccaagctcctaagcttatcgataccgtccggggcggggcgaacgcgcgctgggcaaaggtcacctgcccctcgcgattgcg
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... ----------------------------AGGGCGAATTGGGCCCG----------ACGTCGCATGCTCCCGGCCGCCATG
pD28-10-2... ----------------------------AGGGCGAATTGGGCCCG----------ACGTCGCATGCTCCCGGCCGCCATG
pD28-10-1... ----------------------------AGGGCGAATTGGGCCCG----------ACGTCGCATGCTCCCGGCCGCCATG
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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            2820                                                                                2901
Recombina... gcaatcgcggcctcgaatc--aatattcgcgaggttaataattaccagcgcgcgctgggcaaacgtcacctgcccctcgcgg
pD28-10-2... ------------------------------------------------------------------------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-9-1_... GCGGCCGCGG----GAATTCGATTGCTAGCGAAAGCGAAAG----GAGCGGGCGCTAGG----------------------
pD28-10-2... GCGGCCGCGG----GAATTCGATTGCTAGCGAAAGCGAAAG----GAGCGGGCGCTAGG----------------------
pD28-10-1... GCGGCCGCGG----GAATTCGATTGCTAGCGAAAGCGAAAG----GAGCGGGCGCTAGG----------------------
pD28-9-1_... ------------------------------------------------------------------------------------
pD28-10-2... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------
pD28-10-1... ------------------------------------------------------------------------------------

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             2902                                                                                2983
Recombina... attatttatttggcccgcgctggtaatt------attaactcgcgtgaattgacctcgatcgaggctagtctcgtgatcgat
pD28-10-2... ---------------------------------------------------------------------------------
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-9-1_... ----------------GCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCG---------CCGCGCTTAAT
pD28-10-2... ----------------GCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCG---------CCGCGCTTAAT
pD28-10-1... ----------------GCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCG---------CCGCGCTTAAT
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-10-2... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------

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             2984                                                                                3065
Recombina... aaaattttgt-cgacagatctaggaacccctagtgatggagttggcGGCGCGCCGGTACCCTCGAGTCGCGAAGATCTcatc
pD28-10-2... ---------------------------------------------------------------------------------
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-9-1_... GCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGATCGAATTAATTCTTAATGAATTCGTTTAAA------------CATC
pD28-10-2... GCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGATCGAATTAATTCTTAATGAATTCGTTTAAA------------CATC
pD28-10-1... GCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGGATCGAATTAATTCTTAATGAATTCGTTTAAA------------CATC
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-10-2... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------

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             3066                                                                                3147
Recombina... atcaataatataccttattttggattgaagccaatatgataatgaggggggtggagtttgtgacgtggcgcggggcgtgggaa
pD28-10-2... ---------------------------------------------------------------------------------
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-9-1_... ATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAA
pD28-10-2... ATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAA
pD28-10-1... ATCAATAATATACCTTATTTTGGATTGAAGCCAATATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAA
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-10-2... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------

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             3148                                                                                3229
Recombina... cggggcgggtgacgtaggttttagggcggagtaacttgtatgtgttgggaattgtagttttcttaaaatgggaagtgacgta
pD28-10-2... ---------------------------------------------------------------------------------
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-9-1_... CGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAACTTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTA
pD28-10-2... CGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAACTTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTA
pD28-10-1... CGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAACTTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTA
pD28-9-1_... ---------------------------------------------------------------------------------
pD28-10-2... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------
pD28-10-1... ---------------------------------------------------------------------------------

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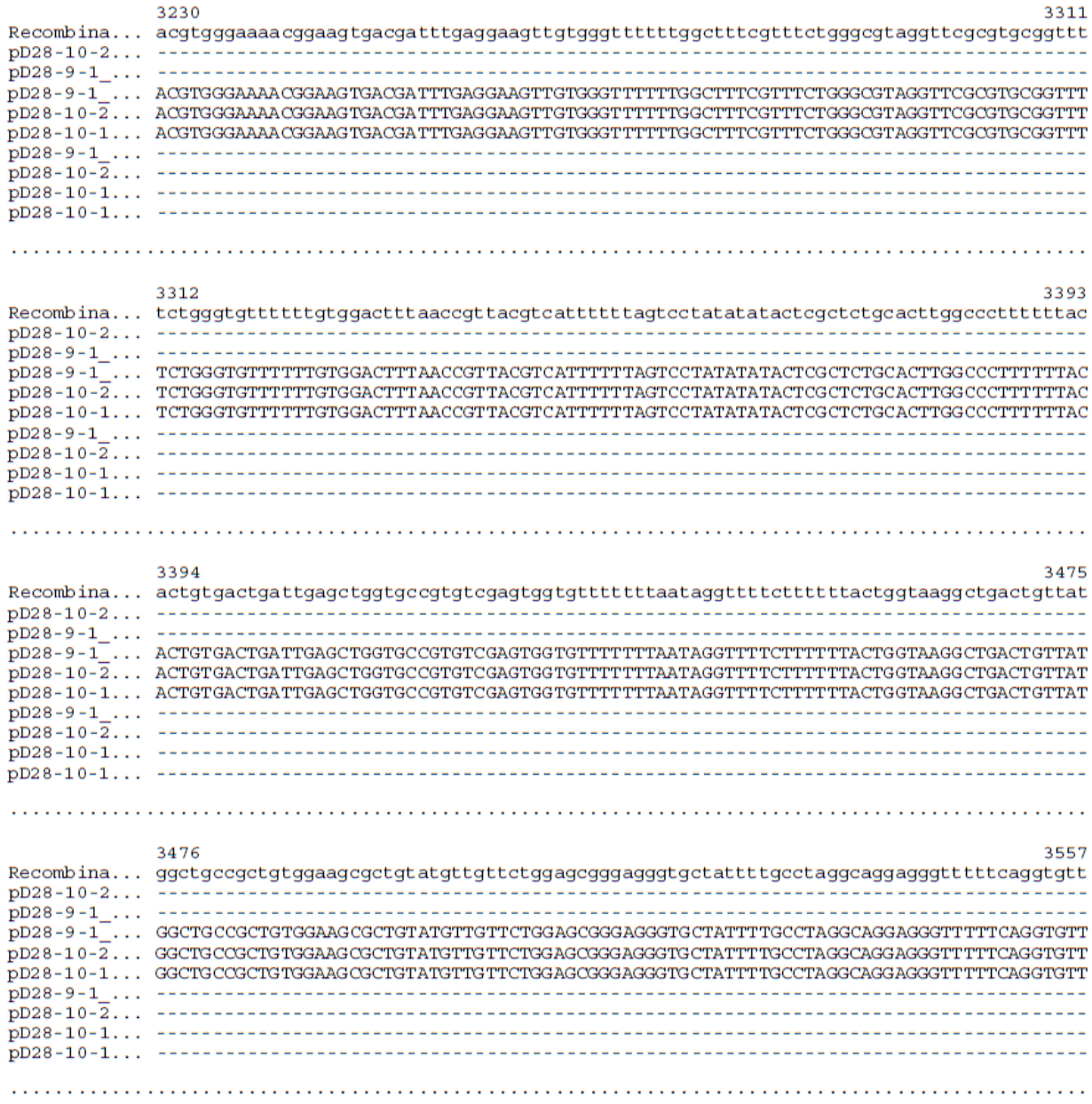

Figure 5.3: Sequence alignment of the recombinants with pD28E4LacZ.

Sequence alignments of the three recombinants between pD28E4LacZ and pGEMShuttleLCoriE4nLuc, demonstrating the exclusion of the nLuc sequences from the recombinants.

```
             23271                                                                23352
Recombina... GCGCCtccccctgaacctgaaacataaaatgaatgcaattgttgttgttaacttgtttattgcagcttataatggttacaaa
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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             23353                                                                23434
Recombina... taaagcaatagcatcacaaatttcacaaataaagcatttttttcactgcattctagttgtggtttgtccaaactcatcaatg
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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             23435                                                                23516
Recombina... tatcttatcatgtctgctcgaagcggccggccgccccgactctagaattacgccagaatgcgttcgcacagccgccagccgg
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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             23517                                                                23598
Recombina... tcactccgttgatggttactcggaacagcagggagccgtcggggttgatcaggcgctcgtcgataattttgttgccgttcca
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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             23599                                                                23680
Recombina... cagggtccctgttacagtgatctttttgccgtcgaacacggcgatgccttcatacggccgtccgaaatagtcgatcatgttc
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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             23681                                                                23762
Recombina... ggcgtaaccccgtcgattaccagtgtgccatagtgcaggatcaccttaaagtgatgatcatccacagggtacaccaccttaa
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            23763                                                                   23844
Recombina... aaatttttttcgatctggcccatttggtcgccgctcagaccttcatacgggatgatgacatggatgtcgatcttcagcccatt
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            23845                                                                   23926
Recombina... ttcaccgctcaggacaatcctttggatcggagttacggacaccccgagattctgaaacaaactggacacacctccctgttca
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            23927                                                                   24008
Recombina... aggacttggtccaggttgtagccggctgtctgtcgccagtccccaacgaaatcttcgagtgtgaagaccatggtggctttac
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            24009                                                                   24090
Recombina... caacagtaccggattgccaagcttggccgccgaggcccactgactgcgttagcaatttaactgtgataaactaccgcaataa
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            24091                                                                   24172
Recombina... agcttctagtgatctgacggttcactaaacgagctctgcttatatagacctcccaccgtacacgcctaccgcccatGCGGCC
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            24173                                                                   24254
Recombina... gcccgggtcgacacctcgaggctagcctatagtgagtcgtattaagtactctagccttaagagctgtaattgaactgggagt
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------
pD24.7-4-... ----------------------------------------------------------------------------------

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            24255                                                                       24336
Recombina... ggacacctgtggagagaaaggcaaagtggatgtcagtaagaccaataggtgcctatcagaaacgcaagagtcttctctgtct
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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            24337                                                                       24418
Recombina... cgacaagcccagtttctattggtctccttaaacctgtcttgtaaccttgatacttacctgcccagtgcctcacgaccaactt
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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            24419                                                                       24500
Recombina... ctgcaaggctaccttaagagagtcactgcagcttaagttcgagactgttgtgtcagaagcactgactgcgttagcaatttaa
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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            24501                                                                       24582
Recombina... ctgtgataaactaccgcaataaagcttctagtgatctgacggttcactaaacgagctcggtacctcgcgaatgcatctagat
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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            24583                                                                       24664
Recombina... tgatcgctagctcaccatggtggcaggagcttgtggatctgtgtgacggcttctcctggtgaaggggcttttatacccctc
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... ----------------------CAMRGAAAMCARYCATGACATGATACGCAGCTATAGKACCTATGAATCYCAGCTATGCAT
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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            24665                                                                       24746
Recombina... cttccaacccaggctgctgatccctgccaagctgactccaaacctgctgattctgattattgacttagtcaacaaaaggaga
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... ACACGCGTGGAGCTCTCCCAWATGGTCGAGCTKGCAGCGTCGCGAATTCACTAGTGTTAAACWCAMCATAAACACCTGAAAA
pD24.7-4-... --------------------------------------------------------------------------------
pD24.7-4-... --------------------------------------------------------------------------------

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APPENDIX

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            24747                                                                       24828
Recombina... ataagtaacctacacaaatatgaaccttgcctagggagattagagtatcggaacactcgctctacgaaatgtgcagacagac
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... CCCTCCTGCCTAGGCAAAAAWAGCACCTCCCGCTCARAACAACAWWACAGCGCTTCCACAGCGGCAGCCATAACAGTCAGCC
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            24829                                                                       24910
Recombina... ggggatcctctagagctacctgctgatcgcccggcccctgttcaaacatgtcctaatactctgtctctgcaagggtcatcag
pD24.7-4-... ------------------------------------------------------------CSKMMCTCTATAGG
pD24.7-4-... ------------------------------------------------------------AMMTTMMCTWATAGG
pD24.7-4-... TTACAGTAAAAAGAAAACCTATTAAAAAAACACACTCGACACGGCACCAGCTCAATCAGTCACWGTGTAWWGCGACGGCCAG
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            24911                                                                       24992
Recombina... tagttttccatcttactcaacatcctcccagtgccctcgacctgcagcccaagctcctaagcttatcgataccgtccggggc
pD24.7-4-... GGCGATTGRGCCCGACGTCGCATGCTCCCGGCCGCCATGGC----------GGCCGCGGGAATTCGATTGCTAGCGAAAGC
pD24.7-4-... GGCGATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGC----------GGCCGCGGGAATTCGATTGCTAGCGAAAGC
pD24.7-4-... TGAATTGTAATACGACTCACTAT----------------------------------------------------AGGGC
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            24993                                                                       25074
Recombina... ggggcgaacgcgcgctgggcaaaggtcacctgcccctcgcgattgcggcaatcgcggcctcgaatc--aatattcgcgaggt
pD24.7-4-... GAAAGGAGCGGGCGCTAG-------------GGCGCTGGCAAGTGTAGCGGT-----------------------------
pD24.7-4-... GAAAGGAGCGGGCGCTAG-------------GGCGCTGGCAAGTGTAGCGGT-----------------------------
pD24.7-4-... GAATTGGGCCCG----------ACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGG----GAATTCGATTGCTAGCGAAAG
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            25075                                                                       25156
Recombina... taataattaccagcgcgcgctgggcaaacgtcacctgcccctcgcggattatttatttggcccgcgctggtaatt------a
pD24.7-4-... ---------------CACGCTGCGCGTAACCACCACACCCGCCGCGCTTAAT----------GCGCCGCTA----------
pD24.7-4-... ---------------CACGCTGCGCGTAACCACCACACCCGCCGCGCTTAAT----------GCGCCGCTA----------
pD24.7-4-... CGAAAG----GAGCGGGCGCTAGG-----------------------------------------GCGCTGGCAAGTGTAGCGG
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            25157                                                                       25238
Recombina... ttaactcgcgtgaattgacctcgatcgaggctagtctcgtgatcgataaaattttgt-cgacagatctaggaacccctagtg
pD24.7-4-... -CAGGGCGCGTCCATTCGCC--------------ATTCAGGATCGAATTAATTCT------------------------TA
pD24.7-4-... -CAGGGCGCGTCCATTCGCC--------------ATTCAGGATCGAATTAATTCT------------------------TA
pD24.7-4-... TCACGCTGCGCGTAACCACCACACCCG---------CCGCGCTTAATGCGCCGCTACAGGGCGCGTCCATTCGCCATTCAGG
pD24.7-4-... ------------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------------

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            25239                                                                 25320
Recombina... atggagttggcGGCGCGCCGGTACCCTCGAGTCGCGAAGATCTcatcatcaataatataccttattttggattgaagccaat
pD24.7-4-... ATGAATTCGTTTAAA---------------------------CATCATCAATAATATACCTTATTTTGGATTGAAGCCAAT
pD24.7-4-... ATGAATTCGTTTAAA---------------------------CATCATCAATAATATACCTTATTTTGGATTGAAGCCAAT
pD24.7-4-... ATCGAATTAATTCTTAATGAATTCGTTTAAA------------CATCATCAATAATATACCTTATTTTGGATTGAAGCCAAT
pD24.7-4-... ------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------

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            25321                                                                 25402
Recombina... atgataatgaggggggtggagtttgtgacgtggcgcggggcgtgggaacggggcgggtgacgtaggttttagggcggagtaac
pD24.7-4-... ATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAAC
pD24.7-4-... ATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAAC
pD24.7-4-... ATGATAATGAGGGGGTGGAGTTTGTGACGTGGCGCGGGGCGTGGGAACGGGGCGGGTGACGTAGGTTTTAGGGCGGAGTAAC
pD24.7-4-... ------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------

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            25403                                                                 25484
Recombina... ttgtatgtgttgggaattgtagttttcttaaaatgggaagtgacgtaacgtgggaaaacggaagtgacgatttgaggaagtt
pD24.7-4-... TTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTAACGTGGGAAAACGGAAGTGACGATTTGAGGAAGTT
pD24.7-4-... TTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTAACGTGGGAAAACGGAAGTGACGATTTGAGGAAGTT
pD24.7-4-... TTGTATGTGTTGGGAATTGTAGTTTTCTTAAAATGGGAAGTGACGTAACGTGGGAAAACGGAAGTGACGATTTGAGGAAGTT
pD24.7-4-... ------------------------------------------------------------------------------
pD24.7-4-... ------------------------------------------------------------------------------

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            25485                                                                 25566
Recombina... gtgggttttttggctttcgtttctgggcgtaggttcgcgtgcggttttctgggtgttttttgtggactttaaccgttacgtc
pD24.7-4-... GTGGGTTTTTTTGGCTTTCGTTTCTGGGCGTAGGTTCGCGTGCGGTTTTCTGGGTGTTTTTTTGTGGACTTTAACCGTTACGTC
pD24.7-4-... GTGGGTTTTTTTGGCTTTCGTTTCTGGGCGTAGGYTCGCGTGCGGTTTTCTGGGTGTTTTTTTGTGGACTTTAACCGTTACGTC
pD24.7-4-... GTGGGTTTTTTTGGCTTTCGTTTCTGGGCGTAGGTTCGCGTGCGGTTTTCTGGGTGTTTTTTTGTGGACTTTAACCGTTACGTC
pD24.7-4-... ------------------------------------------------------------------------------A
pD24.7-4-... ------------------------------------------------------------------------------

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            25567                                                                 25648
Recombina... atttttttagtcctatatatactcgctctgcacttggccctttttttacactgtgactgattgagctggtgccgtgtcgagtgg
pD24.7-4-... ATTTTTTTAGTCCTATATATACTCGCTCTGCACTTGGCCCTTTTTTTACACTGTGACTGATTGAGCTGGTGCCGTGTCGAGTGG
pD24.7-4-... ATTTTTTTAGTCCTATATATACTCGCTCTGCACTTGGCCCTTTTTTTACACTGTGACTGATTGAGCTGGTGCCGTGTCGAGTGG
pD24.7-4-... ATTTTTTTAGTCCTATATATACTCGCTCTGCACTTGGCCCTTTTTTTACACTGTGACTGATTGAGCTGGTGCCGTGTCGAGTGG
pD24.7-4-... GGCCCATTCCGCGGGTCGTTTCCCAAGGGTTTTTAWWAACTGAGCCCAAGAGGGGGGCCTGAAGGGCTCCGCMCGAGTTTMA
pD24.7-4-... ------------------------------------------------------------------------------

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            25649                                                                 25730
Recombina... tgttttttaataggttttctttttttactggtaaggctgactgttatggctgccgctgtggaagcgctgtatgttgttctgg
pD24.7-4-... TGTTTTTTTTAATAGGTTTTCTTTTTTTACTGGTAAGGCTGACTGTTATGGCTGCCGCTGTGGAAGCGCTGTATGTTGTTCTGG
pD24.7-4-... TGTTTTTTTTAATAGGTTTTCTTTTTTTACTGGTAAGGCTGACTGTTATGGCTGCCGCTGTGGAAGCGCTGTATGTTGTTCTGG
pD24.7-4-... TGTTTTTTTTAATAGGTTTTCTTTTTTTACTGGTAAGGCTGACTGTTATGGCTGCCGCTGTGGAAGCGCTGTATGTTGTTCTGG
pD24.7-4-... ATCTCCATGSTGMCCCCCTAAGCTTTACTGGGTTGGTGGCTCTATGAAGARTTGGGTCCCTGATGTTGWGGGTATATCATCT
pD24.7-4-... ------------------------------------------------------------------------------

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Figure 5.4: Sequence alignments of the pD24.7 recombinant.

Sequence alignments of the recombinant between pD24.7 and pGEMShuttleLCoriE4nLuc, demonstrating the exclusion of the nLuc sequences from the recombinant.

5.5 Plasmid maps

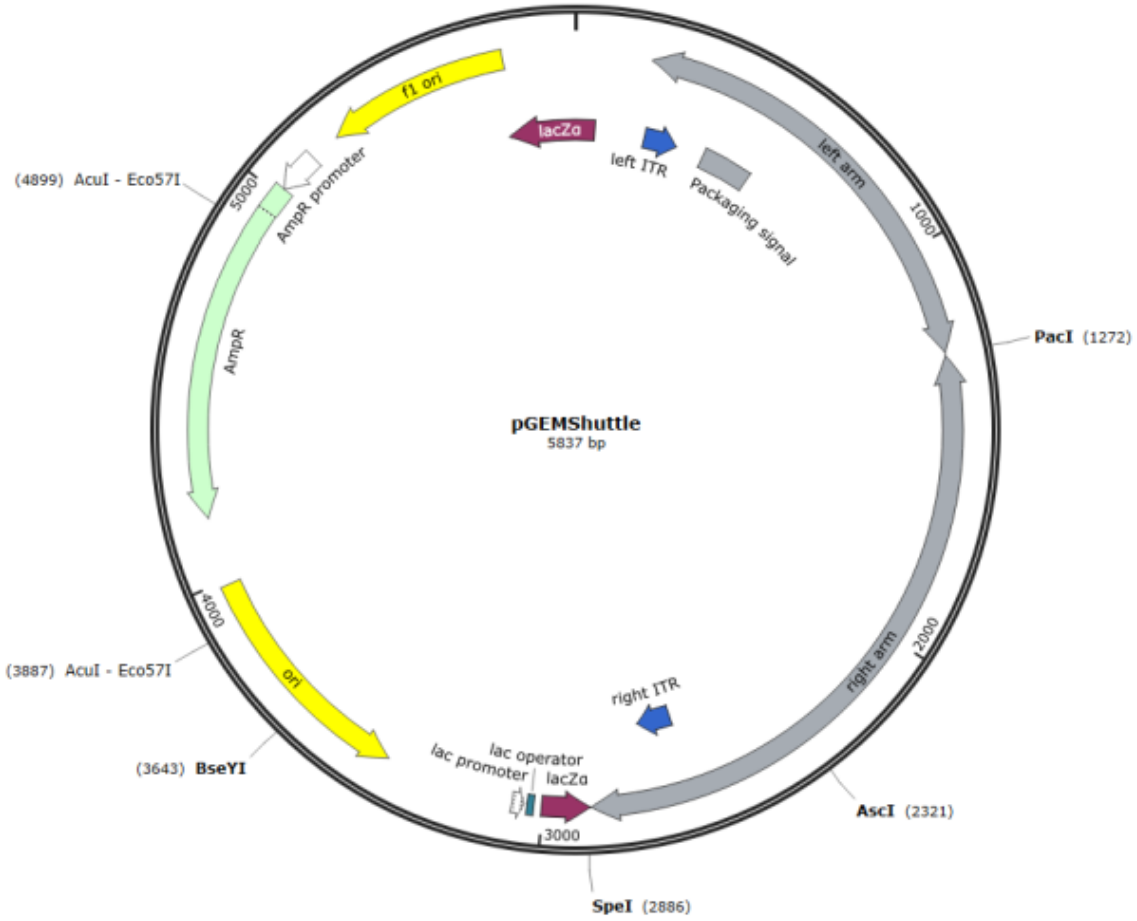

Figure 5.5: Plasmid map of the shuttle plasmid pGEMShuttle used in the production of transgene-expressing adenoviral recombinants.

A 5 837 bp plasmid derived from the cloning vector pGEM-T Easy with an ampicillin resistance selection marker into which a left and right homology arm were inserted to produce a shuttle plasmid into which transgenes can be inserted.

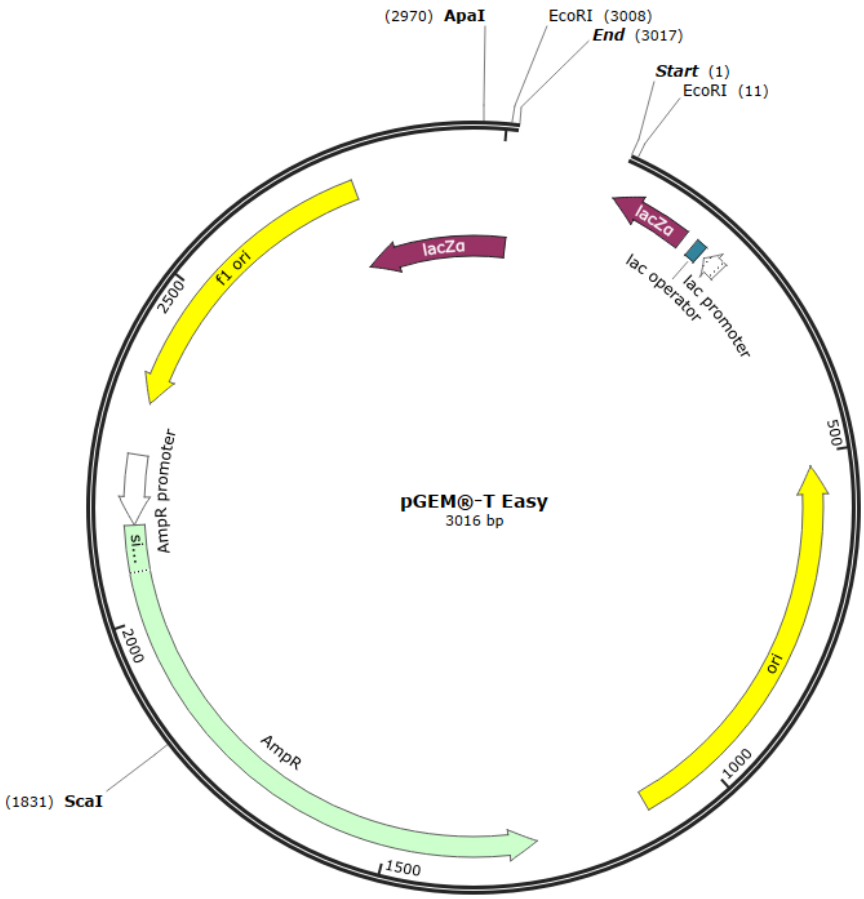

Figure 5.6: Plasmid map of the cloning vector pGEM®-T Easy used in the subcloning of transgenes and other inserts.

A linear 3 016 bp plasmid with an ampicillin resistance selection marker and a lacZ sequence for blue-white screening.

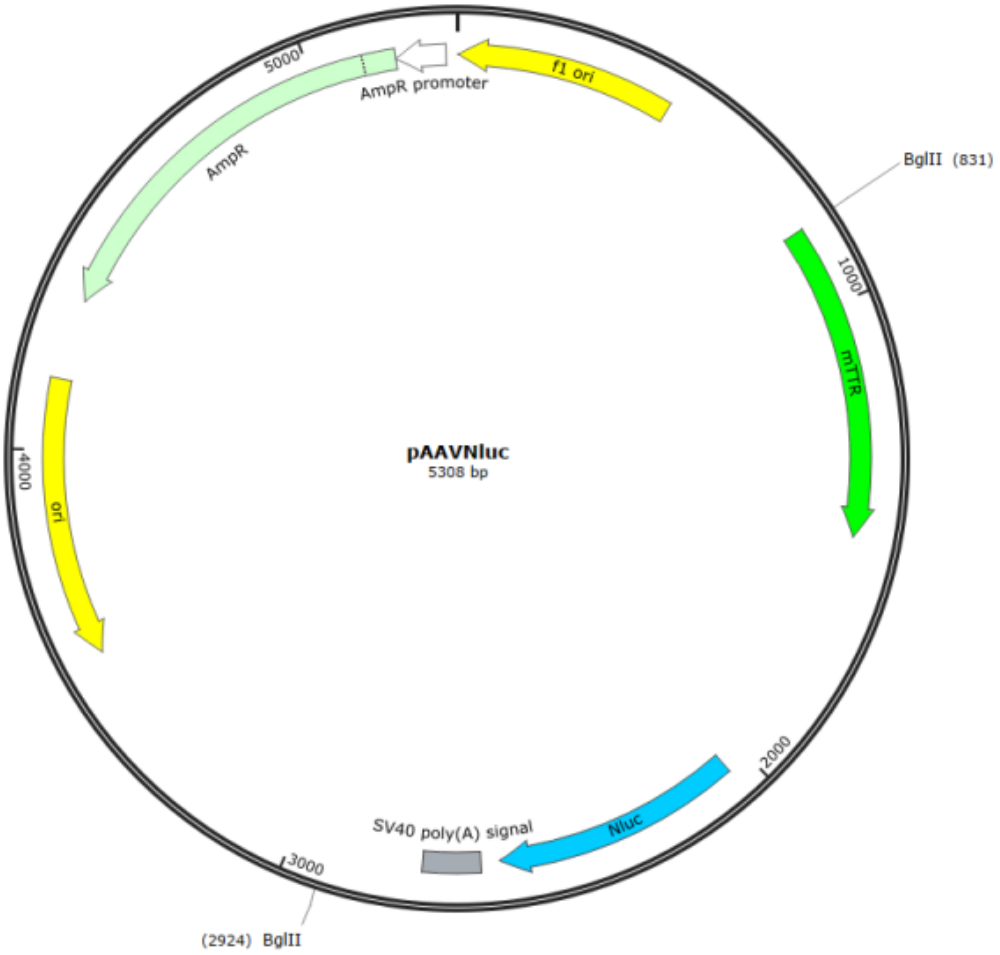

Figure 5.7: Plasmid map of the pAAV-nLuc plasmid used as a source of the transgene nLuc.

A 5 308 bp plasmid containing the nLuc sequence, from which the nLuc transgene was excised and used in further subcloning.

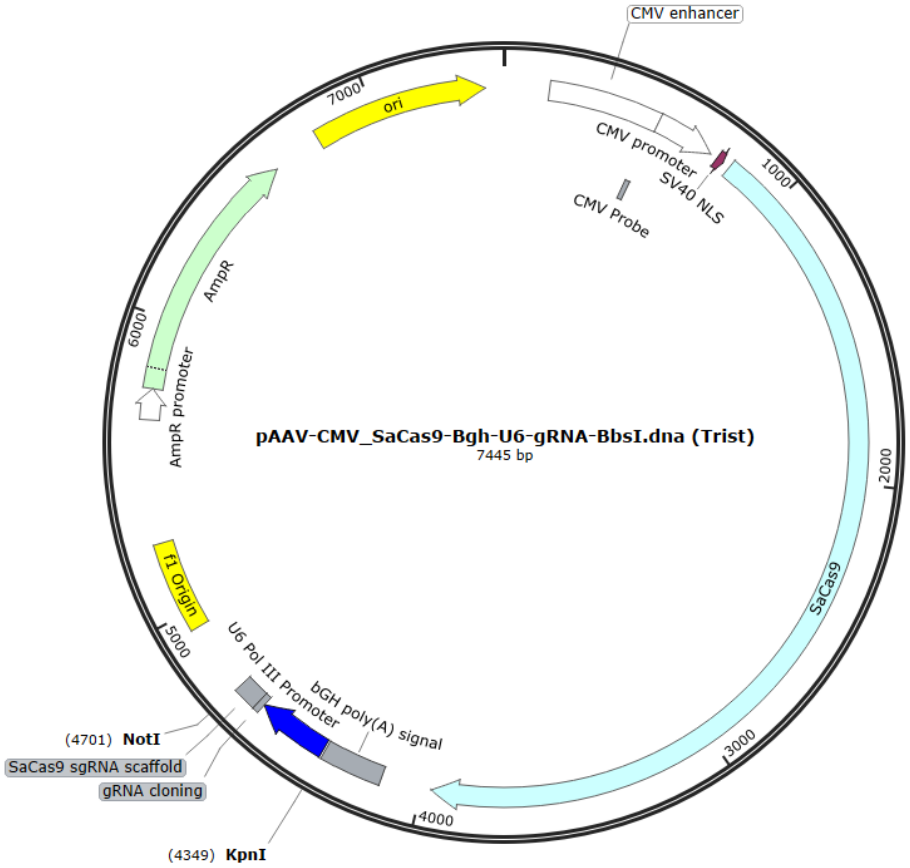

Figure 5.8: Plasmid map of the pAAV-CMV-saCas9-Bgh-U6-gRNA plasmid used as a source of the saCas9 sequence.

A 7 445 bp plasmid with an ampicillin resistance selection marker, containing an saCas9 sequence used to target HBV in infected cells when an appropriate sgRNA sequence is included.

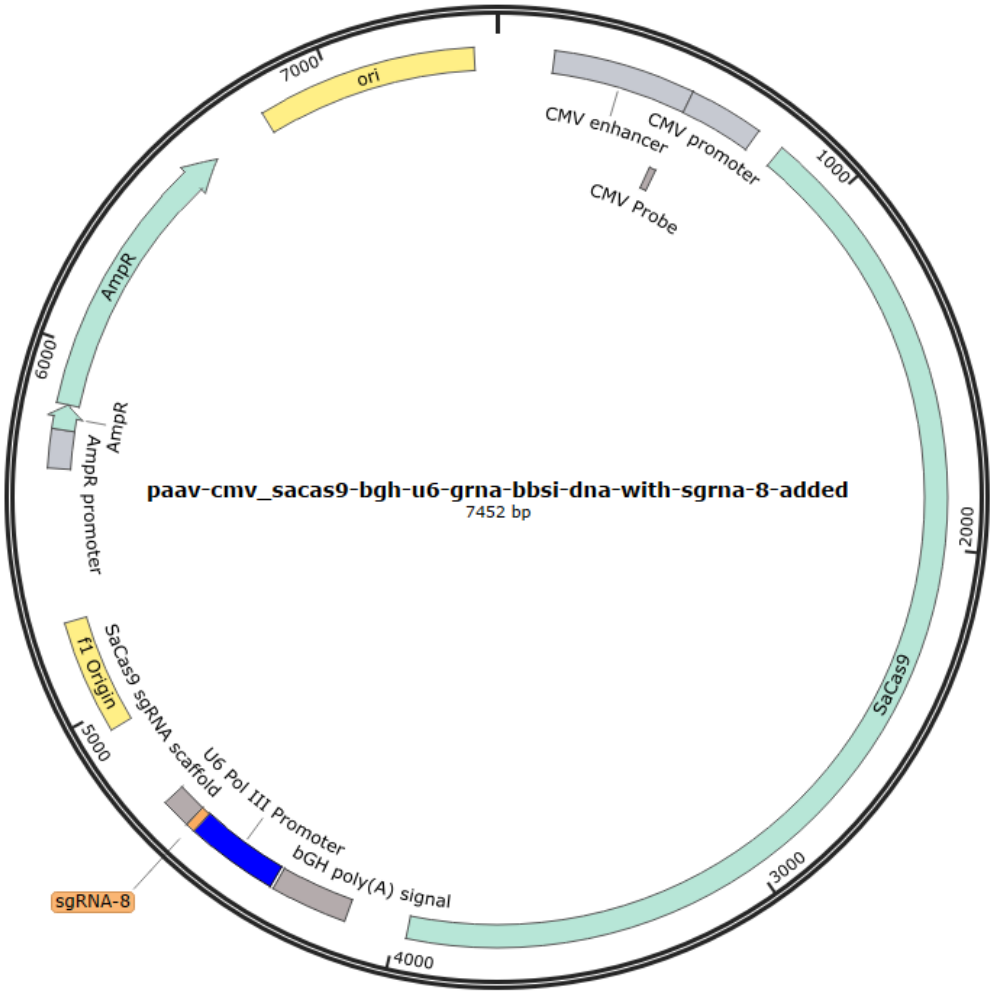

Figure 5.9: Plasmid map of the pAAV-CMV-saCas9-Bgh-U6-sgRNA8 plasmid used as a source of the anti-HBV CRISPR/Cas9 sequence.

A 7 452 bp plasmid with an ampicillin resistance marker, containing an saCas9 sequence that targets the polymerase of HBV using the sgRNA sequence added, referred to as sgRNA-8

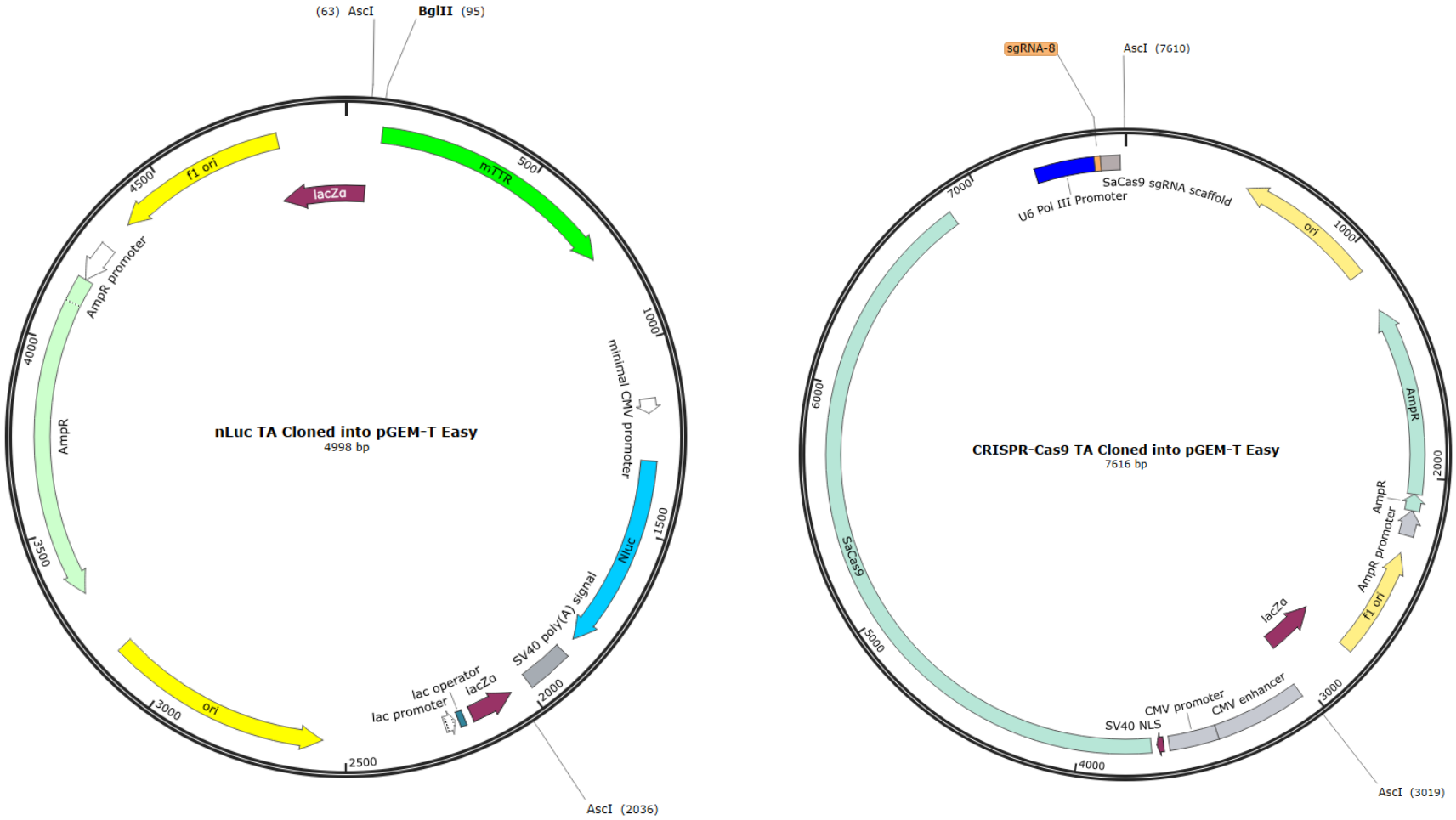

Figure 5.10: Plasmid maps of the nLuc and CRISPR/Cas9 transgenes cloned into pGEM®-T Easy.

The (A) 4 998 bp plasmid of the nLuc transgene TA cloned into pGEM®-T Easy, and (B) the 7 616 bp plasmid of the anti-HBV CRISPR/Cas9 sequence TA cloned into pGEM®-T Easy.

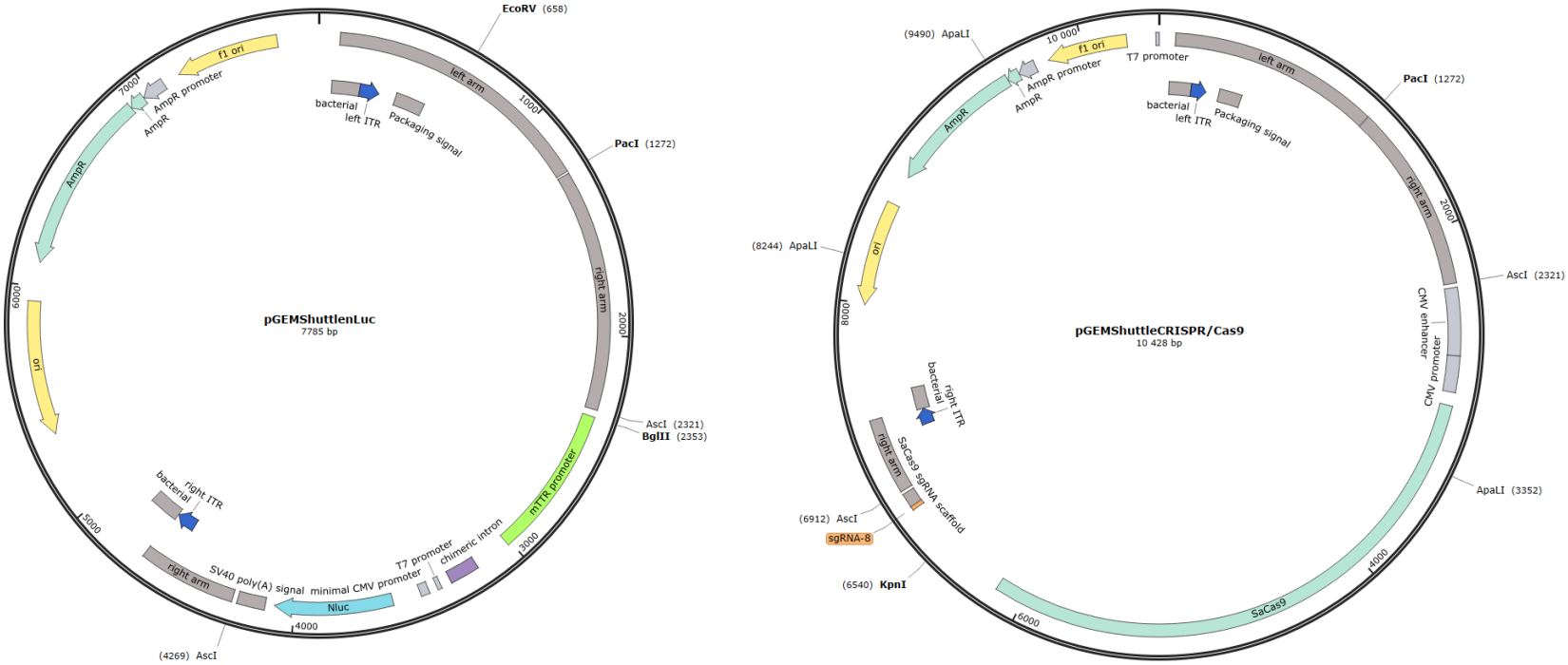

Figure 5.11: Plasmid maps of the nLuc and CRISPR/Cas9 transgenes cloned into pGEMShuttle.

The (A) 7 785 bp plasmid of the nLuc transgene ligated to pGEMShuttle, and (B) the 10 428 bp plasmid of the anti-HBV CRISPR/Cas9 sequence ligated to pGEMShuttle.

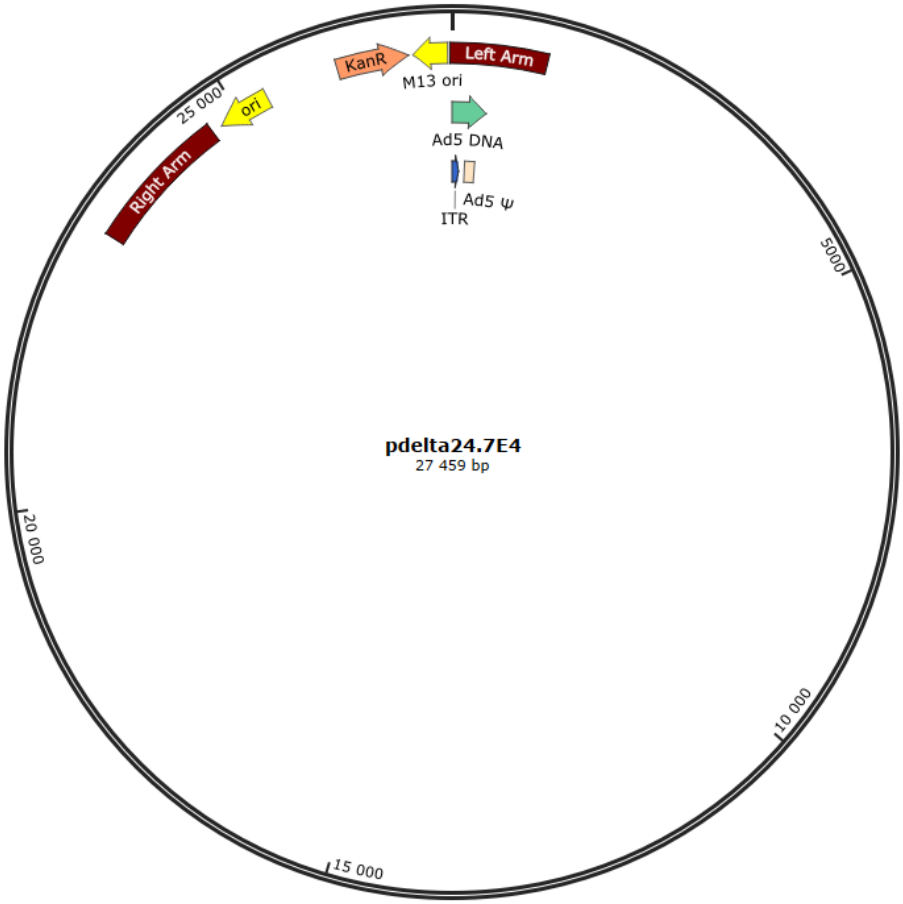

Figure 5.12: Plasmid map of the pD24.7 plasmid that contains the HDAdV genome.

A 27 459 bp plasmid with a kanamycin resistance selective marker used with the anti-HBV CRISPR/Cas9 transgene to produce anti-HBV HDAdVs.

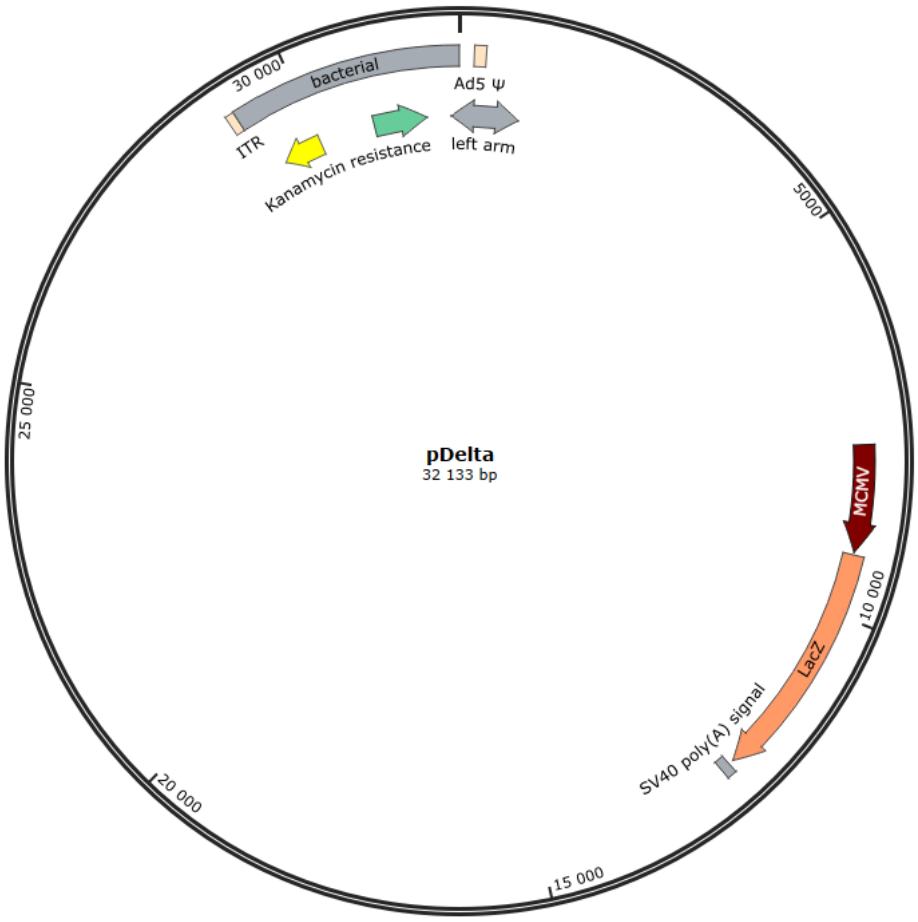

Figure 5.13: Plasmid map of the pD28E4LacZ plasmid that contains the HDAdV genome.

A 32 133 bp plasmid with a kanamycin resistance selection marker and a lacZ sequence used to produce HDAds.

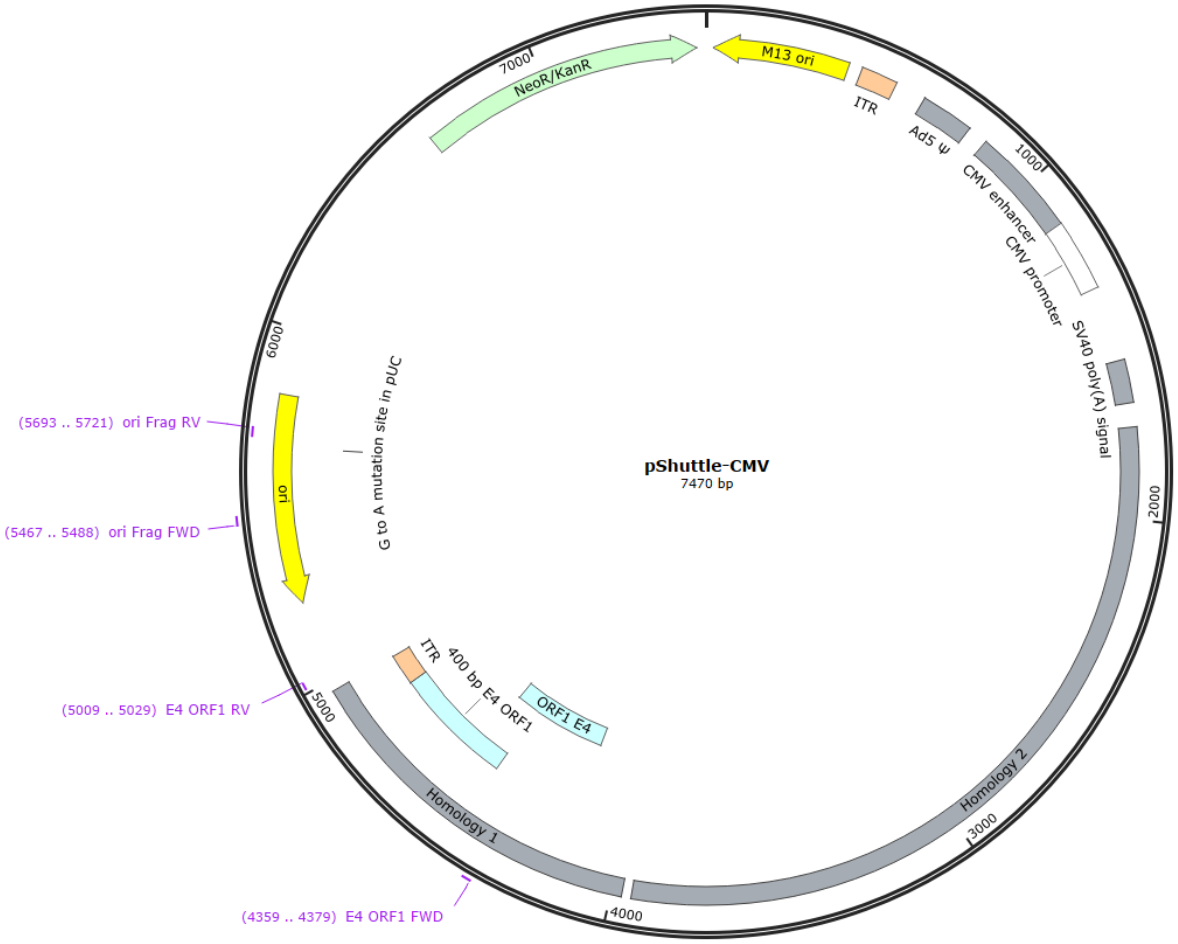

Figure 5.14: Plasmid map of pShuttle-CMV.

Plasmid map of the 7 470 bp shuttle plasmid used in the AdEasy system to insert transgenes into the first generation AdV genome. Also indicated are the two sets of primers designed to amplify fragments inserted into pGEMShuttle to modify it.

Figure 5.15: Plasmid map of pGEMShuttleLCoriE4.

Plasmid map of the modified shuttle plasmid pGEMShuttleLCoriE4 with the modified ori sequence rendering it a low copy number plasmid, and the 400 bp fragment of the Ad E4 ORF1 added to confer greater stability.

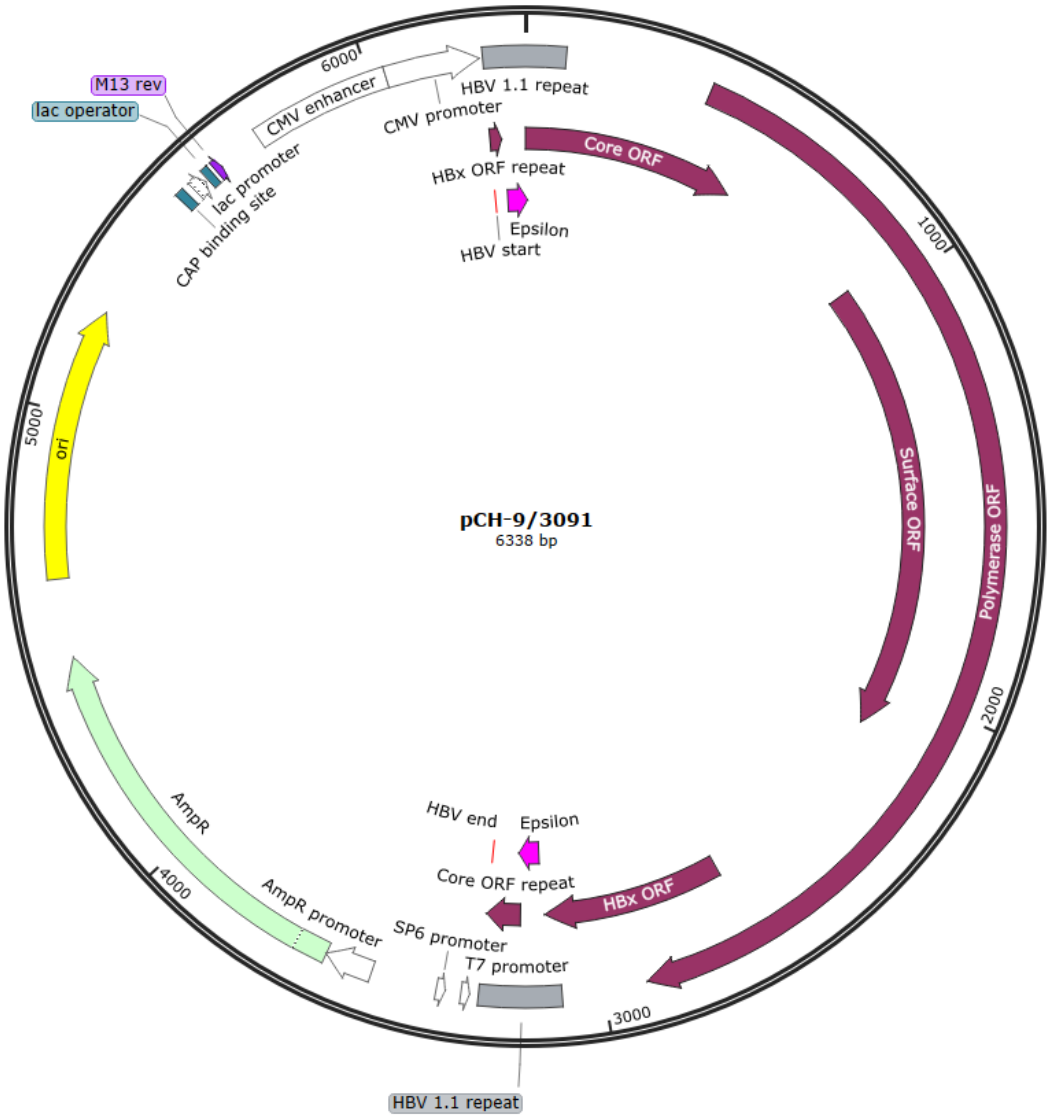

Figure 5.16: Plasmid map of pCH-9/3091.

A 6 338 bp plasmid containing the HBV genome for expression of HBV antigens.

CHAPTER 6

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