

Adeno-associated viral vectors expressing anti-HBV TALENs from an mTTR promoter

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Declaration

I, Ridhwaanah Jacobs (student number: 722944) 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.

.....

.....day of, 2019

Declaration

Dedication

To my loved ones

Conference Presentations

1. **Jacobs.R, Maepa.B and Arbuthnot.P.** Adeno-associated viral vectors expressing anti-HBV TALENs from an MTTR promoter. Molecular Bioscience Research Thrust (MBRT) research day 2017. University of the Witwatersrand, November 2017.
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Abstract

Hepatitis B virus (HBV) infection is a major public health concern accounting for 2 billion infections world-wide. HBV infection results in acute and chronic liver disease as well as Hepatocellular carcinoma. The HBV genome contains four overlapping open reading frames (ORFs) S, C, P and X. The S ORF encodes for the HBsAg and is one of the first viral markers to be detected in the serum of an infected individual. Upon infection with HBV, the viral genome is transported to the nucleus, where it is repaired to covalently closed circular DNA (cccDNA). The cccDNA serves as a template for the transcription of viral RNA and translation of viral proteins. Several drugs have been developed to target HBV, but they are not effective in eliminating the cccDNA, which results in relapse following treatment. This has paved the way for designer nucleases such as Transcription activator-like effector nucleases (TALENs) to be explored in targeting cccDNA. TALENs induce double stranded breaks (DSBs) within the viral DNA. The DSBs are repaired by error prone Non-homologous end joining (NHEJ), which results in insertions and deletions. In a study conducted by the Antiviral Gene Therapy Research Unit (AGTRU), anti-HBV TALENs were designed to target the ORFs of the HBV genome. A higher percentage of targeted mutagenesis was observed when using TALENs targeting the S ORF. To improve the effectiveness and efficiency of the anti-HBV TALENs, these TALENs were expressed from a liver-specific murine transthyretin promoter (mTTR) in this study. A major obstacle of the translation of this therapeutic to the clinic is the lack of a safe and efficient delivery vehicle. Adeno-associated viral vectors (AAVs) are a promising gene delivery vehicle as they are non-pathogenic, have high transduction efficiency and can transduce a variety of cell types. Thus far, no study has investigated the ability of AAVs to deliver anti-HBV TALENs. In this work, AAV genome bearing plasmids were successfully engineered to express anti-HBV TALENs from an mTTR promoter. An in-depth characterization of these plasmids demonstrated that the anti-HBV TALENs achieved over 80 % gene silencing and 5.89 % detectable targeted mutagenesis without any toxic effects. These plasmids were then used to produce viral vectors containing anti-HBV TALEN encoding sequences. The viral vectors were able to achieve 55 % gene silencing at day 2 post transduction. No toxicity to the viral vectors used was detected. This is the first study to identify AAVs as a delivery vehicle for anti-HBV TALENs and will contribute significantly to the progression of these nucleases into the clinic.

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List of Abbreviations

1. Ads -Adenovirus
2. AAV- Adeno associated virus
3. AGTRU- Antiviral Gene Therapy Research Unit
4. Ab- Antibody
5. cccDNA- covalently closed circular DNA
6. CRISPR- clustered regularly-interspaced short palindromic repeats
7. Cas 9- CRISPR-associated protein 9
8. crRNA- CRISPR-RNA
9. CMV- cytomegalovirus promoter
10. CNS- Central Nervous System
11. dsDNA- double stranded DNA breaks
12. ds- double strand
13. ER- Endoplasmic Reticulum
14. FDA- U.S Food and Drug Administration
15. GFP- Green Fluorescent Protein
16. HE- Homing Endonucleases
17. HBV- Hepatitis B virus
18. HCC- Hepatocellular carcinoma
19. HBsAg- Hepatitis B Surface antigen
20. HBeAg – Hepatitis B e antigen
21. HBcAg- Hepatitis B core antigen
22. HSPG- heparan sulfate proteoglycan
23. HR- Homologous recombination
24. hFIX- Human Factor IX
25. IFN- α - interferon- α LHBs- large HBV surface protein
26. MHBs- middle HBV surface protein
27. mRNA- messenger RNA
28. mTTR- liver-specific murine transthyretin promoter
29. NTCP- sodium taurocholate co-transporting polypeptide
30. NHEJ- Non-homologous end-joining repair pathway
31. NPC- Nuclear Pore Complex
32. ORF- Open Reading Frame
33. pgRNA- pregenomic RNA

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- 34. pri-miR- primary microRNA
- 35. rcDNA- relaxed circular DNA genome
- 36. RVDs- Repeat Variable Diresidues
- 37. rAAVs- recombinant Adeno-associated viral vectors
- 38. RBS- rep binding site
- 39. Rpm- revolutions per minute
- 40. SHBs- small HBV surface protein
- 41. SA- South Africa
- 42. ss- single stranded
- 43. ssAAVs- single stranded AAVs
- 44. scAAVs- self-complimentary AAVs
- 45. sgRNA- single-guide RNA
- 46. TALEs- transcription activator-like-effectors
- 47. TALENs- Transcription activator-like effector nucleases
- 48. tracrRNA- trans-activating crRNA
- 49. TRS- terminal resolution site
- 50. WHO- World Health Organization
- 51. ZFs- Zinc Fingers
- 52. ZFNs- Zinc Finger Nucleases

Chapter 1

Introduction

Hepatitis B virus (HBV) infection is a global health problem with 2 billion people being infected and viral hepatitis accounting for up to 1.34 million deaths per year (WHO, 2017). It is hyper-endemic in Africa and Eastern Asia, with an estimated 2.5 million individuals being infected in South Africa [(SA) (Burnett et al., 2012)]. It is the 10th leading cause of death, despite the availability of an effective vaccine. HBV infection is associated with complications of the liver, including cirrhosis and hepatocellular carcinoma (HCC). There are approximately 240 million chronic carriers worldwide and more than 620 000 deaths occur each year as a result of cirrhosis or HCC (Gerlich, 2013). Without an effective and accelerated response to this serious public health problem, the number of people living with HBV infection is set to remain high for the next 30-40 years (WHO, 2017).

HBV infection results in both acute and chronic liver disease. Acute infection is usually asymptomatic. However, certain individuals that are acutely infected may have symptoms such as, jaundice of the skin and eyes, extreme fatigue, nausea, vomiting and abdominal pain. Chronic infection is life threatening, resulting in cirrhosis and HCC. Individuals that are chronically infected may be asymptomatic carriers for the first few years of infection (Kramvis and Kew, 2007). Various forms of transmission of the virus exist, which can occur perinatally from an infected mother to child, horizontally from one person to another, sexually or socially, which includes sharing of infected needles or male to male transmission [reviewed in (Aspinall et al., 2011)].

The prevalence of HBV infection is largely associated with different geographical regions that are characterized into high, intermediate and low areas of endemicity [reviewed in (Aspinall et al., 2011)]. Approximately half of the world's population is said to live in HBV endemic areas, where seroprevalence of hepatitis B surface antigen (HBsAg) is about 8 % (Inan and Tabak, 2015). In low prevalent areas such as Western Countries, infection is

mainly due to sexual contact and needle sharing among drug users. In high prevalent areas such as SA and Eastern Asia, the source of infection is mainly due to horizontal transmission among children around the ages of 2-7 years and perinatally in Eastern Asia (Grimm et al., 2011). Eight HBV genotypes (A-H) have been identified with distinct geographical locations. Genotypes A and D are most commonly found in regions including the USA and Europe, whereas genotypes B and C are endemic to areas such as China and Asia [Reviewed in (Aspinall et al., 2011)]. In a study conducted to investigate the HBV variants from SA, the isolated variants from this country clustered among the HBV A1 strain (Forbi et al., 2013).

1.1. Hepatitis B Virology

HBV belongs to the family of Hepadnaviruses. Other viruses in this family include woodchuck hepatitis virus and ground squirrel hepatitis virus (Morikawa et al., 2016). Key features of this family of viruses include; a partially double stranded DNA genome consisting of a complete negative strand of DNA linked to an RNA dependent polymerase and an incomplete positive strand of DNA (Schädler and Hildt, 2009). The HB virion, also known as the Dane particle, is spherically shaped, encapsulated by a lipid bilayer and is approximately 42 nm in diameter (Liang 2010). It consists of a ~3 kb partially double stranded relaxed circular DNA genome (rcDNA) within a nucleocapsid (core) that is surrounded by a lipid bilayer (Joung and Sander, 2013, Block et al., 2007). The nucleocapsid is 27 nm in diameter and is assembled from 240 viral capsid proteins and contains a single copy of rcDNA and DNA polymerase that is covalently linked to the 5' end of the negative strand of DNA (Wang and Seeger, 1992). The viral genome contains four overlapping open reading frames (ORF), which are; Surface (S), Core (Core), Polymerase (P) and X [Figure 1.1(Hollinger and Liang, 2001)]. The S ORF encodes for the surface antigen [(HBsAg) (Liang, 2009)]. The 3 envelope proteins forming the HBsAg are: large HBV surface protein (LHBs), middle HBV surface protein (MHBs) and small HBV surface protein [(SHBs) (Schädler and Hildt, 2009)]. The LHBs comprises of the PreS1, PreS2 and surface domain. The MHBs consists of the PreS2 and surface domains, whereas the SHBs consist of the surface domain only (Ganem and Prince, 2004). The PreS1 domain of the LHBs appears to be essential in the initial attachment of the HB virion to the receptors present on the hepatocyte and plays an important role in initiating infection [reviewed in (Block et al., 2007)]. The MHBs does not play a role in virion formation, secretion or infectivity but does however induce humoral immune responses during the early stages of infection (Chisari and Ferrari, 1995). The importance of the SHBs is unclear but it is suspected to play an important role in immune evasion [reviewed in (Block

et al., 2007)]. The C ORF is divided into either the pre-core or the core regions. The pre-core gene encodes for the non-structural protein HBeAg (HBV E antigen), whereas the core gene encodes for the structural HBcAg (HBV core antigen). The P ORF encodes for the polymerase region, which is divided up into 3 domains with distinct functions. These domains respectively are: the terminal protein domain, the reverse transcriptase domain and the ribonuclease H domain. The X ORF of the HBV genome encodes for the HBxAg, which is a regulatory protein (Liang, 2009).

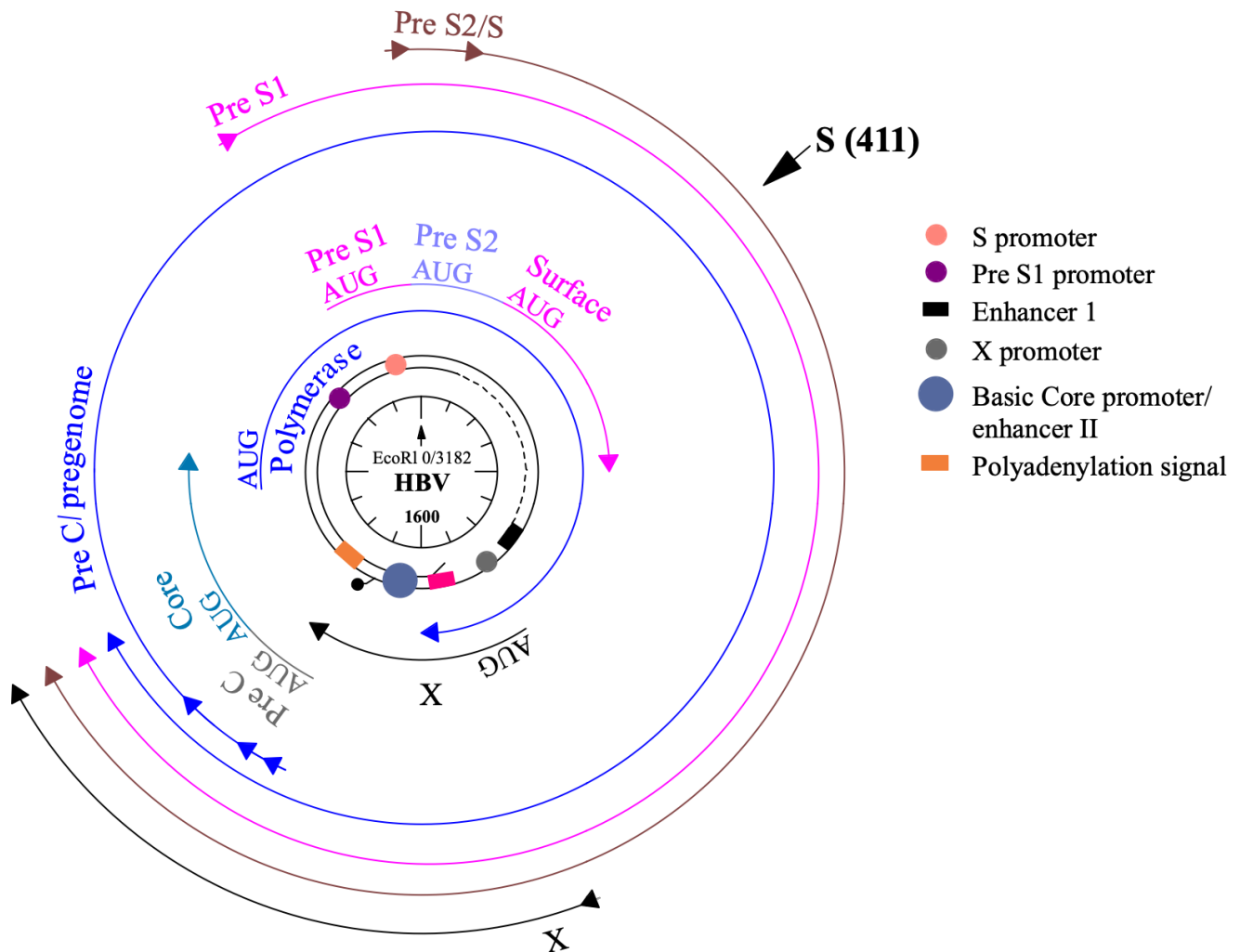


Figure 1.1 Schematic representation of the HBV genome. The center of the circular genome map illustrates the partially double-stranded relaxed circular DNA (rcDNA), which contains the positive and negative strand of DNA. The *EcoR* I sites are used to calculate the nucleotide co-ordinates in a clockwise orientation. The approximate location of the promoter, enhancer and cis-regulatory elements are indicated in the centre as circles and squares. The four overlapping open reading frames (ORFs) of the HBV genome, core, polymerase, surface and X are shown as arrows surrounding the centre. The four outer arrows surrounding the HBV genome represent the viral transcripts. The region

of the HBV genome targeted by the Surface anti-HBV TALENs is indicated by an axial arrow. [Figure adapted from (Bloom et al., 2013)].

1.2 Replication of Hepatitis B virus

HBV infection of hepatocytes is thought to follow a multistep process in a newly infected individual (Figure 1.2). The virus travels inside the host via the bloodstream towards the target organ, the liver. The initial attachment of the virus is facilitated by the preS1 region of the LHBs to the heparan sulfate proteoglycan (HSPG) present on the surface of the hepatocyte (Glebe and Urban, 2007). As a secondary step of entry, the HB virions transfer from the HSPG to the sodium taurocholate co-transporting polypeptide NTCP, which has been identified as an HBV entry receptor (Yan et al., 2012). Following binding to the specific receptors, the virions enter the hepatocyte through host factors induced endocytosis. Development of HBV infection is dependent on the expression of Rab 5 and Rab 7, which are host factors. Once in the cytoplasm, the nucleocapsid containing the rcDNA is transported to the nucleus using the nuclear pore complex [(NPC) (Ezzikouri et al., 2014)]. This transportation is facilitated by the nucleocapsids interaction with microtubules in the cytoplasm. Evidence suggests that the disassembly of the capsid and release of the rcDNA into the nucleus is facilitated by the nuclear basket of the NPC (Block et al., 2007). Once in the nucleus, the incomplete positive strand of DNA is completed using the viral DNA polymerase attached to the 5' end of the negative strand of DNA and removal of the RNA primer from the positive strand of rcDNA. The positive and negative strand of DNA bind covalently to form covalently closed circular DNA (cccDNA). cccDNA also known as a minichromosome, contains histone and nonhistone-like proteins and serve as a template for the transcription of viral mRNAs (Urban et al., 2010). cccDNA is the key molecule in the establishment of HBV infection as it is capable of persisting in an infected hepatocyte for its lifetime. It is relatively stable and durable, which is reflected by the observation of viral rebound after the cessation of antiviral therapy. Viral rebound is found to be associated with surviving cccDNA and remains the ultimate target for the elimination of HBV. cccDNA serves as a template for host RNA polymerase II to transcribe cccDNA into 4 groups of viral messenger RNA (mRNAs) that vary in length. These are 3.5 kb pre-core mRNA and pregenomic RNA (pgRNA), 2.4 kb mRNA for LHBs protein, 2.1 kb mRNA for MHBs and SHBS proteins, and a 0.7 kb mRNA for the X protein [Figure 1.1]. Pre-core mRNA is translated into pre-Core protein, which is proteolytically cleaved into E antigen. HBeAg is not a component of viral and sub viral particles but its detection in circulation is associated

with high levels of viral replication (Magnius and Espmark, 1972). The pgRNA that is transcribed plays a major role in HBV replication as it serves as a reverse transcription and translation template (Schädler and Hildt, 2009). The pgRNA is a greater than normal length transcript containing direct repeat 1 (DR1), the Epsilon signal and poly A tail (Schädler and Hildt, 2009). Reverse transcription is initiated by the binding of host polymerase II to the 5' Epsilon site present on the pgRNA to initiate nucleocapsid assembly and initiate DNA negative strand synthesis [reviewed in (Block et al., 2007)]. The Epsilon-polymerase interaction primes positive DNA strand synthesis and following a multistep reverse transcription leads to rcDNA formation. Once the rcDNA is formed, the nucleocapsids mature and can either complete their viral replication cycle by interacting with envelope proteins and be secreted as infectious virions or they can re-deliver their rcDNA to the nucleus where a cccDNA pool can be established (Bruss, 2007, Zhang et al., 2003). The nucleocapsids bud into the endoplasmic reticulum (ER), where they acquire an envelope embedded with all 3 surface proteins (Seeger and Mason, 2015). All 3 of the envelope proteins have been detected on the ER (Morikawa et al., 2016). The HB virion is said to be secreted from the cell by exocytosis (Tuttleman et al., 1986, Wu et al., 1990).

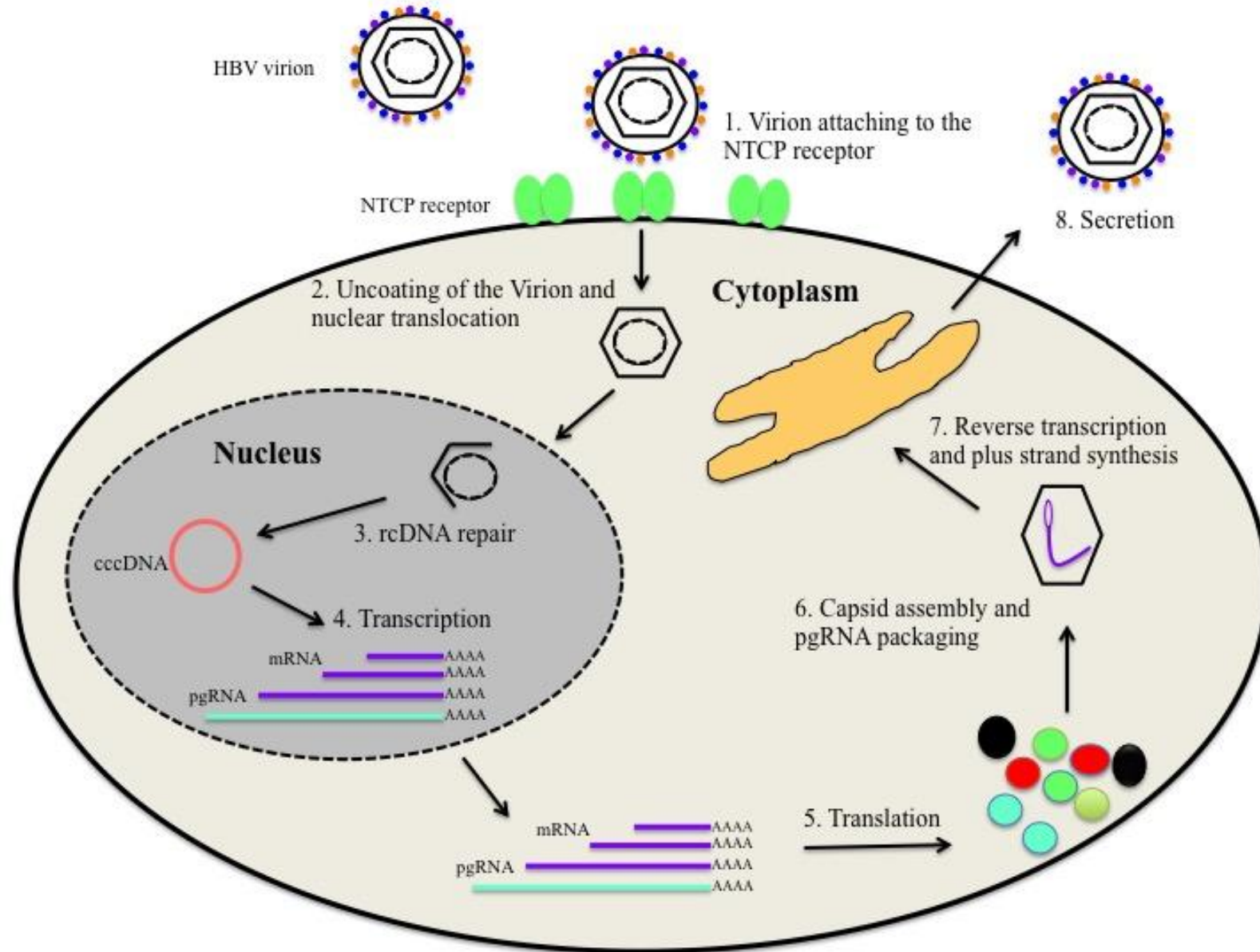


Figure 1.2: Schematic representation of the HBV replication cycle: 1) The attachment of the HB virion to the sodium taurocholate co-transporting polypeptide (NTCP) and other receptors facilitate viral entry. 2) Uncoating of the virus and nuclear translocation of the virus occurs, with the relaxed circular DNA (rcDNA) entering the nucleus. 3) Within the nucleus, the rcDNA is repaired and released to form covalently closed circular DNA (cccDNA). 4) The cccDNA serves as a template for the transcription of pre-genomic RNA (pgRNA) and subgenomic RNA, which are transported to the cytoplasm for translation. 5) The pgRNA is translated into polymerase, HBV e antigen, HBX, surface and core proteins. 6) Once translated, the polymerase binds to pgRNA and initiates encapsidation. The pgRNA is reverse transcribed to the negative strand of DNA, which serves as a template for positive DNA strand synthesis. This produces a mature nucleocapsid that contains rcDNA. 7) The nucleocapsid binds to the endoplasmic reticulum, where it receives its envelope embedded with surface proteins. 8). The HBV virion is secreted from the hepatocyte via exocytosis. [Figure adapted from (Maepa et al., 2015)].

1.3. Current vaccine and therapies for infection of Hepatitis B virus

A safe and effective HBV vaccine has been available since the 1980s. The World Health Organization (WHO) in 1991 recommended that all countries should introduce a universal HBV vaccination, which contains inactivated HBsAg, into their expanded programme of immunization (Kane, 1995). It is estimated that with the introduction of the vaccine, more than 1.3 million deaths have been prevented (Spearman and Sonderup, 2014). SA was one of the first 10 countries in Africa to implement a universal HBV vaccine that is to be given in a 3-dose schedule. However, no birth dose is being administered despite the high risk of perinatal and early childhood transmission. This is one of the factors contributing to the high mortality associated with HBV, despite the availability of an effective vaccine. Other factors associated with a reduced response to vaccination include; immunosuppression, liver disease, renal failure, smoking, obesity, co-infection with HIV and an absence of catch-up vaccination programmes (Shouval et al., 1994, Spearman and Sonderup, 2014).

For the treatment of HBV infection, the U.S Food and Drug Administration (FDA) have approved two main therapeutic strategies. The first strategy consists of nucleoside/nucleotide reverse transcriptase inhibitors such as Lamivudine, Entecavir, Telbivudine, Adefovir, dipivoxil and Tenofovir disoproxil (Ye et al., 2015). The second strategy consists of immune modulators such as interferon- α [(IFN- α) (D'souza and Foster, 2004)]. IFN- α facilitates viral clearance by up regulating immune response and is only effective in a minority of people (Clark and Hu, 2015). The nucleoside/nucleotide reverse transcriptase inhibitors disrupt viral replication as they can target viral DNA during replication, reducing hepatic cccDNA but not eliminating it (Ye et al., 2015, Grimm et al., 2011). In people chronically infected with HBV, cccDNA is able to persist and contribute to relapse after treatment (Weber et al. 2014). The main drawbacks associated with these drugs are the indefinite duration of therapy, resistance to treatment and side effects (Grimm et al., 2011). Anti-viral therapies utilizing DNA based cleavage strategies that directly target the HBV viral replicating intermediate, cccDNA are being evaluated in the hopes of preventing HBV reactivation. The main goal of anti-viral therapy for chronically infected individuals would be the complete removal of cccDNA from infected hepatocytes. This is not possible with the currently available drugs and paves the way for the utilization of gene editing technologies.

1.4 Gene editing technologies

Genome engineering involves the modification of the genome at a precise location and is readily being explored for the treatment of a variety of diseases. Genome engineering has been successful in treating monogenic diseases such as SCID and Wiskott-Aldrich syndrome (Howe et al., 2008, Aiuti et al., 2013) and has recently gained momentum in treating latent and persistent viral infections. Genome editing using engineered nucleases was named “method of the year” for 2011 by Nature Methods (Nemudryi et al., 2014). Using its DNA-binding domain, this technology is able to bind to DNA and cleave it to form double-stranded DNA breaks [(dsDNA) (Joung and Sander, 2013)]. Currently available genome editing tools include; homing endonucleases (HEs), the clustered regularly-interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas) (CRISPR/Cas) system, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The rationale underlying use of engineered nucleases such as ZFNs (Cradick et al., 2010), TALENs (Bloom et al., 2013) and CRISPR-Cas 9 (Lin et al., 2014b) to target cccDNA is based on the fact that cleavage of cccDNA will activate either non-homologous end-joining repair pathway (NHEJ) or homologous recombination (HR). Error prone NHEJ is favoured and eventually leads to the formation of insertions and deletions in the cccDNA. These mutations result in the production of mutated RNA that will be translated into non-functional proteins (Joung and Sander, 2013). Engineered nucleases are therefore capable of disabling cccDNA permanently without resulting in toxicity and off-target effects [reviewed in (Ely et al., 2016)].

1.4.1 Designer nucleases

HEs also known as meganucleases are small proteins derived from bacteria, archaea and unicellular eukaryotes. They are rare-cutting enzymes encoded by introns and inteins. HEs can target long DNA sequences of around 14-40 bps and cause double stranded breaks, which differ to restriction enzymes that typically recognize sequences of around 4-8 bps (Chevalier and Stoddard, 2001, Belfort and Bonocora, 2014). Other characteristic features of HEs that differ from those of restriction endonucleases are the lack of sequence specificity, recognition properties, molecular associations and phylogenetic origins. HEs have been grouped into 5 different families based on a small conserved amino acid motif and cleave the target DNA in different ways (Chevalier et al., 2001). The amino acid motifs are; LAGLIDADG, GIY-YIG, HNH, His-Cys box and PD-(D/E)xxk. HEs work by causing insertions, deletions, mutations

and corrections in the target DNA in a highly controlled and site specific manner (Belfort and Bonocora, 2014, Belfort and Roberts, 1997). Major challenges associated with the utilization of HEs have been with re-directing HE cleavage specificity. If there is more than one DNA-binding domain of HEs within the same protein, the cleavage activity of HEs is lowered. (Schiffer et al., 2012).

The clustered regularly interspaced palindromic repeats (CRISPR) and its associated Cas 9 are commonly referred to as the bacterial adaptive immune system (Jinek et al., 2012). CRISPR-Cas 9 is used by the bacteria to protect themselves against foreign organisms. This system has been explored for treatment of vast number of diseases and viral infections. CRISPR consists of two components, the single-guide RNA (sgRNA) that forms a complex with the CRISPR associated Cas 9 protein to direct cleavage at the target site (Mali et al., 2013). The sgRNA is composed of CRISPR-RNA (crRNA) and trans-activating crRNA (tracrRNA). Short segments of foreign DNA known as 'spacers' are found within the CRISPR genome loci and are transcribed into crRNAs (Gaj et al., 2013), which direct the nucleolytic activity of Cas 9 protein (Hsu et al., 2014). The tracrRNAs have a 24-nucleotide sequence complementarity to the repeat regions of crRNA precursor transcripts (Deltcheva et al., 2011). Targeted recognition of the Cas 9 protein is dependent on a protospacer adjacent motif (PAM) sequence within the target sequence. Cas 9 derived from *Streptococcus pyogenes* (SpCas9) is the most widely used [reviewed in (Moyo et al., 2018)]. Cas 9 nucleases derived from other bacterial species are utilized and each system differs in the sgRNA properties and the PAM sequence required. A number of publications have utilized CRISPR-Cas 9 to target cccDNA of HBV (Jinek et al., 2012, Scott et al., 2017, Lin et al., 2014a) but the methods used to detect mutagenic events vary greatly and the specificity of these methods are low, making it difficult to compare their efficiencies [reviewed in (Bloom et al., 2018)].

Zinc Fingers (ZFs) are abundant multifunctional proteins that exist naturally as transcription factors. Zinc Finger nucleases (ZFNs) are composed of a DNA binding domain and a FokI nuclease domain (Kim et al., 1996). The Cys2-His2 zinc-fingers make up the DNA binding domain of ZFNs (Gaj et al., 2013). An individual zinc-finger consists of approximately 30 amino acids in a conserved $\beta\beta\alpha$ configuration and the α -helix of each zinc finger interacts with 3 base pairs within the major groove of DNA, with varying levels of specificity (Gaj et al., 2013). ZFNs typically consist of three to six ZFs (Pabo et al., 2001) that is capable of

binding to a DNA sequence of around 9-18 bp (Kim and Kim, 2014). Cleavage of target DNA is dependent on two ZFNs monomers to form an active nuclease. The requirement for dimerization increases the length of recognition sites thus increasing its specificity. The major disadvantage of ZFNs is the difficulty associated with their construction as DNA binding is dependent on surrounding ZFs.

There is a greater efficiency, specificity and less toxicity when using TALENs compared to ZFNs (Nemudryi et al., 2014, Sander et al., 2011, Tesson et al., 2011, Reyon et al., 2012, Hockemeyer et al., 2011, Bloom et al., 2013). Derivatives of DNA binding proteins utilizing TALENs have compared favourably to the above-mentioned technologies and pose an exciting potential to be used for antiviral gene therapy. Thus, the ease of design and high rates of cleavage activity make them suitable to be utilized as an anti-HBV therapeutic.

1.4.2 TALEN structure and design

TALENs' development arose from studying the transcription activator-like-effectors (TALEs) from a phytopathogenic bacteria of the genus *Xanthomonas*, which results in severe disease in many crop plants (Boch et al., 2009). TALEs encoded by proteobacteria are injected into host plant cells cytoplasm via a Type III secretion system and were found to result in the plant being more susceptible to pathogenic bacteria (Nemudryi et al., 2014). These TALE proteins contain a central domain that is able to bind to DNA, nuclear localization signal and an acidic transcriptional activation domain [Figure 1.3 (Boch et al., 2009)]. Each TALE protein is composed of 12-20 tandem repeats of around 33-35 amino acids (Chen et al., 2014). The repeat sequences are almost identical except for the amino acids at position 12 and 13, which are referred to as repeat variable diresidues [(RVDs), (Chen et al., 2014)]. These RVDs are responsible for single nucleotide recognition and site-specific DNA binding. Typically the RVDs comprise of NN, NI, NG and HD and bind to nucleotides G, A, T and C respectively (Boch et al., 2009). There is some degeneracy in the code, as in some cases more than one RVD may bind to the same base or one RVD may bind to different bases. Artificially designed TALENs consist of nuclease domains fused to TALEs to form TALENs, which are capable of sequence-specific cleavage. TALENs function in pairs and require a right and left TALEN monomer. The two-sequence specific TALENs bind to DNA and based on the design of the TALE monomers, a spacer region of between 12-19 bp is formed. The spacer region allows the endonuclease domain of the FokI to dimerize and create a ds-break in the DNA (Chen et al., 2014). ZFNs and TALENs are

similar as they both have a DNA binding domains attached to a Fok I endonuclease domain that recognize, binds and results in site specific cleavage of DNA (Nemudryi et al., 2014). The characteristic features of TALENs such as its specificity and ease of design enables it to be used as a molecular tool for cleavage of any desired DNA sequence. TALENs have been used for various genetic conditions including the correction of a mutation found in patients suffering from alpha-1-antitrypsin (AAT) deficiency. AAT deficiency is a genetic disorder that predisposes patients to cirrhosis and HCC (Ramalingam et al., 2014). TALENs were also used to correct a sickle cell disease (SCD) mutation in patient-derived human induced pluripotent stem cells (hiPSCs). The SCD mutation was corrected using HR repair pathway. SCD is caused by a single mutation in the human β -globulin gene and is the most common human genetic disease. The TALENs generated against SCD were highly specific and generated minimal off-target effects. The TALEN-mediated gene targeting left no residual ectopic sequences at the site of correction and the corrected hiPSCs retain full pluripotency and a normal karyotype. This study demonstrated that the utilization of TALENs for the treatment of genetic diseases such as SCD represents a significant advance toward hiPSC-based cell and gene therapies (Sun and Zhao, 2014).

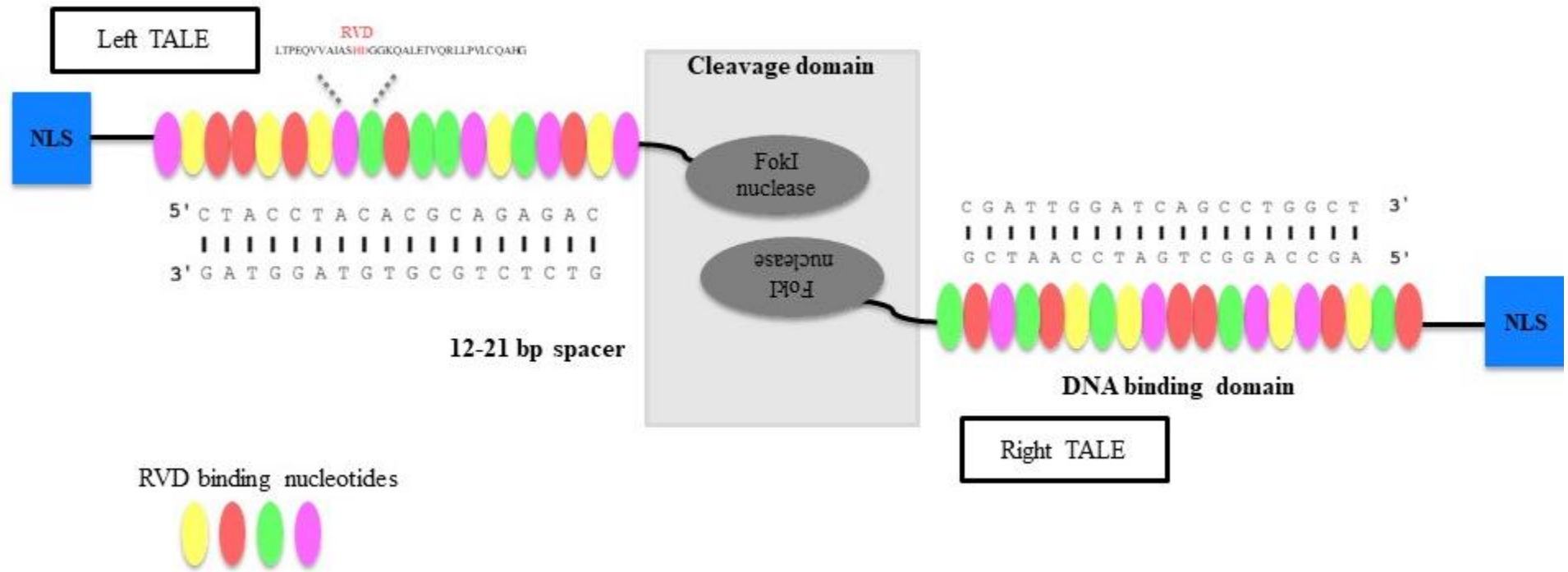


Figure 1.3: Schematic structure of TALENs. Representation of a TALEN, which is comprised of a Nuclear localization signal (NLS), a DNA binding domain and a FokI nuclease domain. The spacer region of around 12-21 bp allows for the dimerization of the FokI endonucleases so that DNA cleavage of the target sequence can occur. This requires a right and left monomer. The right and left TALEN monomers are comprised of 33-35 amino acids, which contain repeat variable diresidues (RVDs). The RVDs promote binding to the DNA. [Figure adapted from (Kim and Kim, 2014)].

1.4.3 Utilization of TALENs against HBV

The efficacy of anti- HBV TALENs was first demonstrated by Bloom et al, 2013. Four dimeric TALENs were designed to target the S, C, X and P ORF of the HBV genome (Bloom et al., 2013). The S and C TALENs were the most effective. A significant decrease in levels of HBsAg and around 35 % targeted mutagenesis was observed with the S TALENs *in vitro*. *In vivo*, greater than 90 % knockdown of HBsAg was achieved using the S TALENs and between 58-87 % targeted mutagenesis was observed. In a subsequent study conducted by Chen et al, TALENs were designed against conserved regions within the C and P ORF of the HBV genome. Targeted mutagenesis of 32 % in cccDNA molecules was observed when utilizing the anti-HBV TALENs against the core (Chen et al., 2014). Both studies utilized plasmids that expressed the anti-HBV TALENs from a cytomegalovirus promoter (CMV). In another study, linear donor DNA that included missense mutations was co-transfected with anti-viral trimeric primary microRNAs (pri-miRs) and a combination of anti-HBV TALENs that target the S and C ORF. This approach utilized the HR repair pathway to insert the anti-viral trimeric pri-miRs into the viral DNA. This resulted in the viral genome being permanently disrupted. Moreover, after HR, the HBV DNA should transcribe an antiviral sequence from its own rearranged viral genome. This study demonstrated the ability to combine gene editing and gene silencing in an effort to combat chronic HBV infection (Dreyer et al., 2016).

To ensure expression of the anti-HBV TALENs occurs primarily in the liver, the TALEN sequences were expressed from a liver-specific murine transthyretin promoter (mTTR) in this study. The mTTR promoter gene is regulated by two DNA regions that allow for hepatocyte-specific expression (Costa and Grayson, 1991). Promoters are composed of multiple binding sites for different transcription factors that cooperate to activate the RNA polymerase II complex. In the case of liver specific promoters such as mTTR, many of the transcription factors are found within the liver with a minority of them found in other tissues (Costa and Grayson, 1991). Utilization of the mTTR promoter to drive expression of the anti-HBV TALENs will ensure expression to occur only in the liver, reducing any off-target effects and cell toxicity. Mowa et al utilized a helper dependent (HD) adenoviral (Ad) vector that expressed anti-HBV Primary-microRNAs from the mTTR promoter. Expression of antiviral sequences from the mTTR promoter *in vivo* resulted in effective gene silencing without evidence of toxicity and the transgene expression was more sustained compared to the CMV-

promoter derived sequences (Mowa et al., 2014b).

Despite the availability of efficient anti-HBV TALENs, finding the safest and effective delivery system remains the major obstacle for progression of this nucleases in to the clinic.

1.5 Gene delivery

One of the main goals of gene therapy for the past 40 years has been to ‘correct’ genes at the molecular level, in particular monogenic diseases [Reviewed in (Naso et al., 2017)]. The identification of gene delivery vehicles that can safely deliver their therapeutic has lagged in this goal. Various non-viral systems exist for the delivery of a gene of interest to a target cell and can be classified into physical methods, electrical methods and chemical carrier methods. Physical methods such as micro-injection is highly inefficient for *in vivo* application [reviewed in (Ramamoorth and Narvekar, 2015)]. Concerns around microinjection surround transgene expression. Only around 60 % of microinjection derived mice show transgene expression and in animals demonstrating expression there are problems associated with low-level of expression or non-specific expression [reviewed in (Smith, 2004)]. Electroporation has been utilized to exert an electrical force on cells, which disrupts the membrane of cells, facilitating the intracellular delivery of therapeutic DNA. Electroporation is beneficial for the utilization on cancer cells and the delivery of plasmid DNA to the skin and muscle. Gene delivery is achieved by generating pores on a cell membrane through electric pulses. Advantages of this type of delivery are that it is efficient and can be repeated. However, tissue damage resulting in cell death is possible when the field strength and long pulses of voltage is applied to cells [reviewed in (Ramamoorth and Narvekar, 2015, Gascón et al., 2013)]. Chemical carriers include synthetic and polymer-based vectors. Synthetic based vectors such as cationic lipids have been among the more efficient gene delivery reagents. The structure of cationic lipids includes a positively charged hydrophilic head, a hydrophobic tail and a linker structure connecting the head and tail. The positively charged head binds with the negatively charged phosphate backbone in nucleic acids, to form a structure called lipoplexes (Felgner et al., 1987). Lipoplexes target mainly epithelial cells, endothelial cells, hepatocytes and muscle cells. They are very safe to use. However, decreased efficiency has been associated with the difference in surface charge, as it reduces the half-life of the lipoplexes. Lipoplexes are also cytotoxic to cells if the ratio of lipid:DNA exceed 3:1 [reviewed in (Ramamoorth and Narvekar, 2015, Gascón et al., 2013)]. Polymer based vectors such as cationic polymers interact with DNA to form nanosized

particles, known as polyplexes. Polymer based vector such as PEI are considered to be a gold standard for *in vivo* and *in vitro* gene transfer (Boussif et al., 1995). PEI creates a ‘protein sponge’ on cells, neutralizing the endosomal pH, which results in an influx of chloride ions. This ruptures the endosomal membrane, allowing the polyplexes to enter the cells. The usage of PEI for gene delivery is limited due to its low transfection efficiency, short duration of gene expression and elevated toxicity to cells (Chollet et al., 2002). One of the main drawbacks of non-viral vectors, is their poor efficiency of delivery, decreasing the expression of their transgenes and various questions surround their safety.

One of the earliest approaches investigated was the utilization of viruses and exploiting their natural ability to deliver nucleic acids into the host cell. (Ni et al., 2016). The viral vectors most commonly used are lentiviruses, Adenoviruses (Ads), Adeno-associated viruses (AAV) and herpes virus vectors (Samulski and Muzyczka, 2014). These viral vectors have been modified by deleting certain areas of their genomes to allow transgene insertion. Despite Ads having a natural hepatocyte tropism, which makes them attractive to use for anti-HBV gene therapy, they are highly immunogenic. They also result in short term anti-HBV effects and are not suitable to use for chronic HBV treatment (Raper et al., 2002). Herpesvirus vectors have the greatest potential for gene delivery to the nervous system, tumours and cancer cells (Nayerossadat et al., 2012). Even though lentiviral vectors result in a lifetime therapeutic effect, their integrative nature reduces their clinical safety. AAVs are promising tools for gene therapy as they are non-pathogenic, have high transduction efficiencies, and can transduce a variety of dividing and non-dividing cell types (Santiago-Ortiz et al., 2015a). This has prompted many researches to utilize them in human gene therapy trials.

1.5.1 Adeno-associated virus

AAVs were discovered in 1965 as a contaminant of Ads isolates and belongs to the family paroviridae (Atchison et al., 1965). Twelve serotypes (AAV1-AAV12) have been identified and AAV-2 is the most extensively studied (Daya and Berns, 2008). AAV serotypes 2,3,5 and 6 were discovered from human cells, whereas AAV serotypes 1,4 and 7-11 have non-human primates origins (Gao et al., 2004, Gao et al., 2002). AAV is a small non-enveloped virus that has a single stranded linear DNA genome of approximately 5 kb in length (Berns and Parrish, 2007). The virion consists of an icosahedral capsid and is 25 nm in diameter (Samulski and Muzyczka, 2014). The AAV genome contains two inverted terminal repeats (ITRs) that flank two genes; *rep* and *cap* [Figure 1.4 (Gaj et al., 2013)]. The first 125

nucleotides of the ITRs contain palindromic sequences that form internal T-shaped hairpin structures and the other 20 bases remain unpaired, and are called the D sequence (Gaj et al., 2013, Daya and Berns, 2008). The Rep binding site (RBS) and the terminal resolution site (TRS) are found within the ITRs and play an important role in the replication and packaging of the AAV genome [Figure 1.5 (Ni et al., 1998)]. The *rep* gene encodes 4 proteins namely; Rep78, Rep68, Rep50 and Rep 42, which are required for viral genome replication (Gaj et al., 2013). The DNA encoding the Rep78/68 and Rep50/42 proteins are transcribed from two different promoters, the p5 and p19 respectively. The p5 promoter contains a TATA box, a RBS, a TRS, the Yin Yang 1 binding site and a downstream sequence that can form a hairpin structure (de Oliveira and Fraefel, 2010). The *cap* gene encodes 3 structural proteins namely VP1, VP2 and VP3 from a single promoter p40. VP3, the smallest of the structural proteins is important for full capsid assembly, VP1 is important for particle infectivity and VP2 is reported to be redundant structurally or functionally. The capsid proteins (VP1, VP2 and VP3) are present in a 1:1:10 molar ratio (Daya and Berns, 2008). The assembly activating protein (AAP) is translated from an alternative ORF with a non-canonical CTG start codon present within the *cap* gene. The AAP is involved in directing the capsid proteins to the nucleolus and also plays a role in the assembly of the viral capsids (Santiago-Ortiz et al., 2015b).

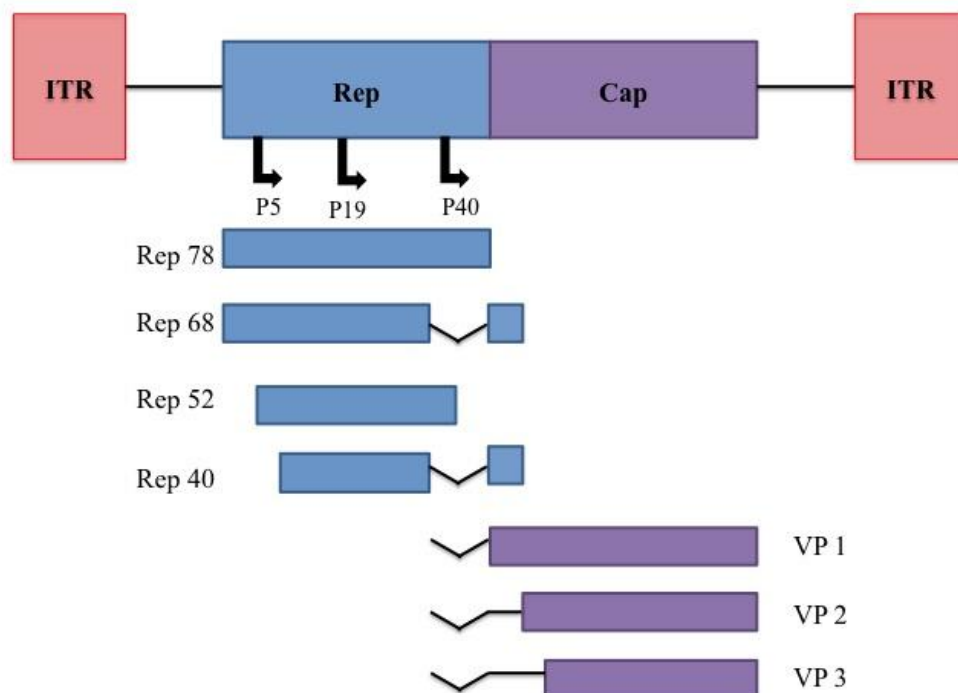


Figure 1.4: Schematic representation of wild type AAV-2. The inverted terminal repeats (ITRs) flank the rep and cap genes, with a poly A signal present downstream of the cap genes. The DNA

encoding the rep 78 and rep 68 proteins are transcribed from the P5 promoter, whereas the DNA encoding the rep 52 and rep 40 proteins are transcribed from the p19 promoter. The DNA encoding the 3 structural proteins (VP1, VP2 and VP3) are transcribed from the p40 promoter. [Figure adapted from (Samulski and Muzyczka, 2014)].

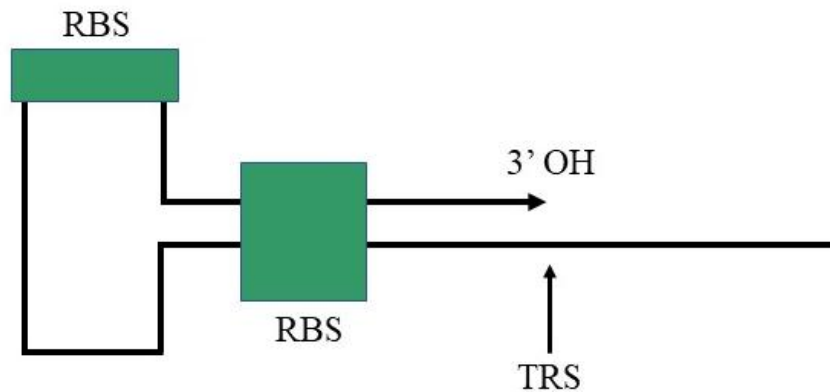


Figure 1.5: Schematic representation of the ITRs. The secondary structure formed by the inverted terminal repeats (ITRs). The first 125 nucleotides remain paired, whereas the remaining 20 bases remain unpaired. Depicted are the Rep binding site (RBS), the terminal resolution site (TRS) and 3'hydroxyl group. [Figure adapted from (de Oliveira and Fraefel, 2010).

1.5.2 AAV replication cycle

There are two forms of the AAV replication cycle, the lytic and lysogenic cycle. AAV-2 gains entry into cells by binding to the heparin sulfate proteoglycan receptor and co-receptor fibroblast growth factor receptor (Daya and Berns, 2008). An additional cellular molecule, avb5 has been identified, which plays an important role in AAV-2 binding and uptake (Qing et al., 1999). Following receptor binding, AAV is endocytosed via clathrin-coated vesicles (Weitzman and Linden, 2011). AAV is released from these vesicles and is uncoated in the nucleus. The exact mechanism of how AAV gains entry into the nucleus is unknown (Daya and Berns, 2008). In the absence of a helper virus, the lysogenic stage is initiated. During this stage there is limited expression of Rep78/68, AAV replication and viral gene expression is repressed and the AAV establishes latency by integrating into a 4 kb region present on chromosome 19 known as the AAVS1 (Srivastava et al., 1983). In the presence of a helper virus, the lytic cycle is initiated where the replication of AAV DNA is said to be based on a

rolling hairpin model. The ITRs are the origin of replication and serves as a primer for second strand synthesis. The 3' hydroxyl group allows for unidirectional synthesis to occur. Co-infection with Ad activates both the p5 and p19 promoter, which results in the transcription of the *rep* genes. Once the AAV genome has been copied, the Rep78/68 binds to the RBS present within the ITRs and creates site and strand specific nicks within the TRS. The nick created within the TRS regenerates a 3' hydroxyl end for the replication through the viral ITR. The Rep78/68 proteins play an important role in the replication process through their DNA binding, endonuclease and helicase activity. The endonuclease activity directed at the TRS is indirectly ATP dependent and rep helicase activity is required to allow the TRS to be single stranded, making it easily accessible to nicking activity. The regulatory activity of Rep seems to be involved in the maintenance of the ratio of the rep and cap proteins during infection. This allows a balance between AAV genome replication and packaging. The replication cycle of AAV generates a double stranded full length AAV genome or a single stranded full length AAV displacement product. It is speculated that the double stranded AAV genome serves as a template for further replication whereas the single stranded AAV genome is packaged (de Oliveira and Fraefel, 2010, Weitzman and Linden, 2011). If nicking at the TRS fails, elongation of the DNA occurs, generating linear double length double stranded molecules known as duplex dimers [(dd), (Gonçalves, 2005)]. The dd have either a head-to-head or tail-to-tail configuration, which are covalently connected to a single ITR [Figure 1.6 (Hong et al., 1994)].

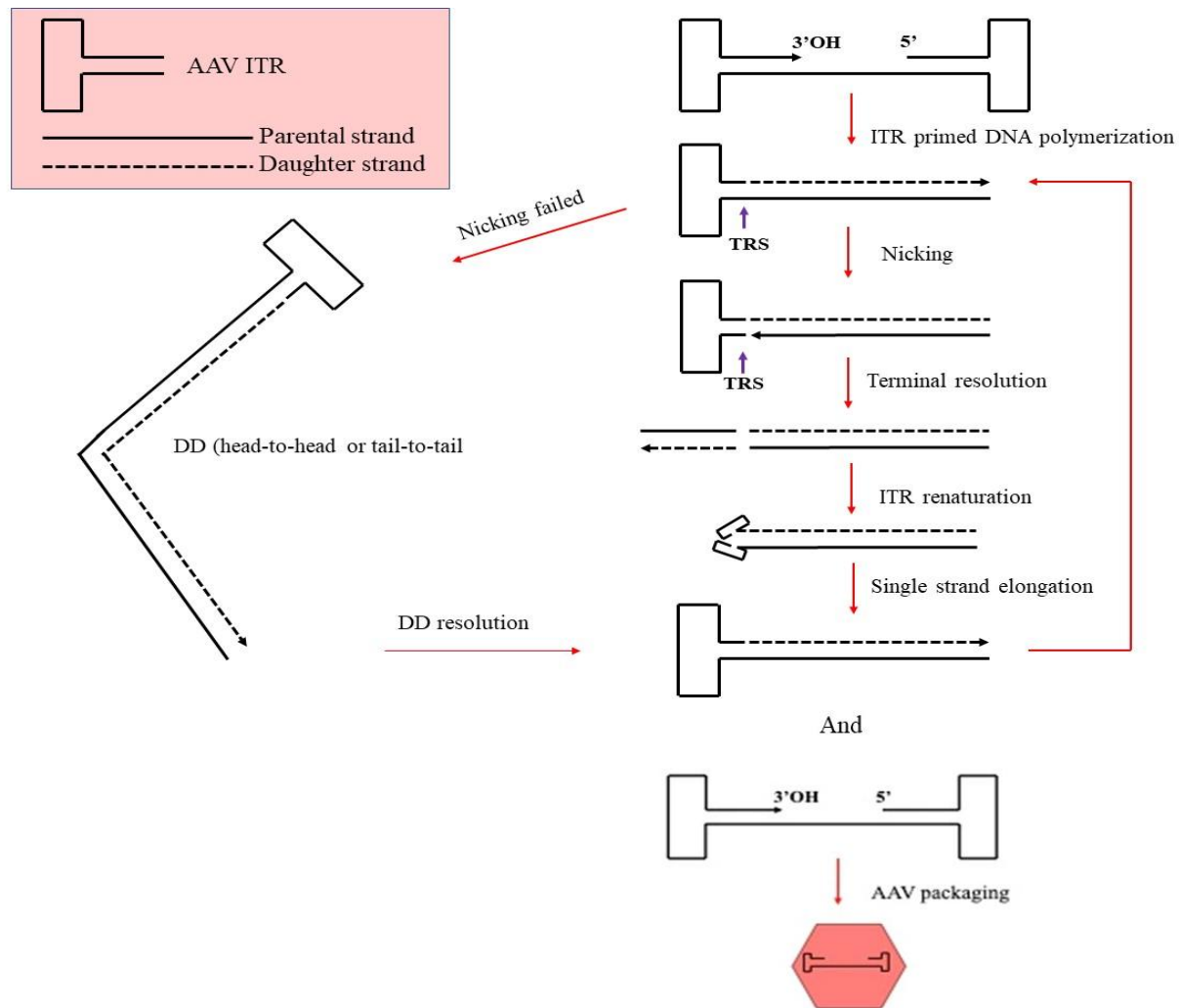


Figure 1.6: Schematic representation of AAV replication. AAV replication involves a self-priming single strand displacement mechanism that is initiated by DNA polymerase binding to the 3' hairpin primer. This leads to the formation of double strand DNA molecules that contain a single ITR. Nicking occurs at the terminal resolution site (TRS), which allows the newly generated 3'OH group to provide a substrate for DNA polymerase to copy the ITRs. The palindromic linear duplex termini can renature into terminal hairpins putting the 3'OH group in position for single strand displacement synthesis. The resulting double strand AAV genome can re-enter the replication cycle and the single strand AAV genome can be packaged. If nicking does not occur within the TRS, elongation proceeds throughout the covalently closed hairpin structures, generating duplex dimers (DD) with either a head-to-head or tail-to-tail configuration. The DD can be resolved into double strand AAV genomes, through the ITR sequences located at the axis of symmetry [Figure adapted from (Gonçalves, 2005)].

1.5.3 AAV as a gene delivery vector

AAV derived vectors (rAAVs) can be generated by eliminating the *rep* and *cap* genes and providing them in trans during vector production while retaining only the ITRs, which flank the transgene (Xiao et al., 1998). Particles generated in this manner can carry any type of transgene cassette that does not exceed the size of the genome of the wild-type virus, which is usually around 4.7 kb. (Knipe et al., 2013). rAAVs mainly exist in 2 types; self-complementary AAVs (scAAVs) and single stranded AAV (ssAAVs). The transgene expressions of scAAVs are quicker than ssAAVs, as ssAAVs genome require the conversion to ds DNA before transcription. A scAAV vector is designed in such a manner that a ss ITR folds back on itself to form a ds genome when entering infected cells. The transgene capacity of scAAVs is 2.35 kb, which is half the size of wild type AAVs. The transgene is packaged as a dimer, with the two ITRs on either side of the transgene and closed covalently by a hairpin at the terminal repeats (Raj et al., 2011). During vector production, the deleted *rep* and *cap* genes are supplied in a separate plasmid referred to as the AAV helper or packaging plasmid (Hermonat and Muzyczka, 1984). The production of rAAV also requires the addition of Ad genes *E1A*, *E1B*, *E2* and *E4* to provide additional helper functions (Daya and Berns, 2008).

Stable transgene expression has been demonstrated in mouse hepatocytes when using vectors based on AAV-2 encoding a variety of self and foreign transgenes that are capable of targeting either the lung, muscle, central nervous system (CNS), liver and retina (Xiao et al., 1998). Goa et al showed that AAV-2 and AAV-8 have greater transduction efficiencies to the liver compared to other serotypes (Gao et al., 2002). AAV-2 and AAV-8 consistently achieved a 10- to-100-fold improvement in gene transfer efficiency as compared to the other vector serotypes. Over the last two decades, numerous clinical studies were performed using AAVs to deliver therapeutic transgenes to the liver, muscle and CNS. These studies included hundreds of patients and resulted in safe record of AAV vectors for gene transfer in humans. One such success story is the identification of Alipogene tiparvovec (Glybera®). In November 2012, the European Commission approved the product Glybera® to be utilized as a form of treatment for patients with lipoprotein lipase (LPL) deficiency. Glybera® uses an AAV-1 vector to replace the gene responsible for the production of LPL. If LPL is deficient, it results in life-threatening pancreatitis (Bryant et al., 2013). rAAVs have also displayed efficacy and safety in numerous pre-clinical disease

models and clinical trials mainly for Duchenne muscular dystrophy, inherited blindness and Haemophilia B (Bowles et al., 2012, Stroes et al., 2008, Manno et al., 2003).

1.5.4 AAV vectors as a means of delivering anti-HBV gene therapy

AAV vectors have been used to successfully deliver anti-HBV ZFNs in cell culture. Three anti-HBV ZFNs were generated that target the HBV polymerase, core and X genes. The anti-HBV ZFNs were delivered using scAAVs vectors and their activity was tested in HepAD38 cells. The anti-HBV ZFNs were effective but cytotoxicity was noticed when using one of the anti-HBV ZFNs (Weber et al., 2014). In a study conducted by Scott et al, the Cas 9 protein derived from *Staphylococcus Aureus* and sgRNA were incorporated into ssAAV vector to assess functionality of this all-in-one vector (Scott et al, 2017). The sgRNA utilized was directed towards the S ORF of the HBV genome and resulted in efficient inactivation of HBV replication and mutation of cccDNA in cultured cells. Off-target analysis did not reveal any non-specific mutagenesis but the requirement of a PAM sequence and tracrRNA limits the utilization of CRISPR-Cas 9. Although knock down in HBsAg levels were noted, use of anti-HBV TALENs achieves better HBsAg knockdown. Although AAVs are currently the most promising gene therapy, their small transgene capacity and tissue specificity of the AAV serotypes are the major limiting factors for their utilization in the clinical settings (Katrekar et al., 2018). This study overcame the small transgene capacity of ssAAVs, by packaging the right and left anti-HBV TALENs into two vectors. Two vectors are thus required to express a complete anti-HBV TALEN dimer. This ensures a greater specificity and reduced the risk of off-target effects as cleavage cannot occur with a single TALEN monomer.

No studies have thus far tested the ability of AAV-2 to deliver anti-HBV TALENs that target the cccDNA of the HBV genome. This study will be the first to investigate the efficacy of viral vector AAV-2 to deliver anti-HBV TALENs *in vitro*.

Aim

To generate and characterize pairs of rAAV serotype 2 viral vector that express TALEN subunits from an mTTR promoter.

Study Objectives

- To construct AAV plasmids that express anti-HBV TALEN sequences from an mTTR promoter.
- To produce and propagate rAAVs that express anti-HBV TALEN sequences from an mTTR promoter.
- To determine *in vitro* expression of the anti-HBV TALENs in a mammalian cell line using immunocytochemical staining.
- To assess *in vitro* silencing of the HBV gene expression by anti-HBV TALENs in a mammalian cell line using an ELISA.
- To determine the cleavage of the HBV genome in HepG 2.2.15 cells by anti-HBV TALENs using a T7E1 assay.
- To measure toxicity of the anti-HBV TALENS in Huh 7 cells by an MTT assay.

Chapter 2

Methods and Materials

2.1 Bacterial culturing and manipulations

2.1.1 Culturing of *Escherichia coli* strains

XL-blue and DH5 α *E. coli* strains were grown on Luria Bertani Agar (LA) plates (Appendix 5.1.2) or Luria Bertani medium [LB, (Appendix 5.1.1)] at 37°C overnight. To grow strains that carried a plasmid with antibiotic resistance marker, medium was supplemented with 100 μ g/mL ampicillin (Appendix 5.1.3) or 50 μ g/mL kanamycin (Appendix 5.1.4). The liquid cultures were incubated at 37°C overnight at 150 revolutions per minute (rpm). All freezer stocks were frozen in 25 % glycerol at -80 °C.

2.1.2 Preparation and transformation of chemically competent *E. coli* cells

A single colony of XL-blue or DH5 α *E. coli* strain was picked and grown in 10 ml LB, incubated at 37°C with shaking overnight at 200-250 rpm. The pre-culture was diluted 100-fold in 100 ml of LB and incubated at 37°C with shaking at 200-250 rpm. The optical density (OD) was measured at a wavelength of 600 nm until an absorbance reading of between 0.4 – 0.6 was observed. The culture was transferred to two 50 ml Falcon tubes and centrifuged at 8000 rpm for 20 mins at 4°C. The supernatants were discarded, and the pellets were re-suspended in 10 ml of transformation buffer (Appendix 5.1.5), mixed in one tube and incubated on ice for 20 mins. The cells were centrifuged at 8000 rpm for 15 mins. The supernatant was discarded, and the pellet re-suspended in 1 ml of transformation buffer. Aliquots of 100 μ l were made and stored at -80°C.

Chemically competent XL-blue or DH5 α cells (100 μ l) were mixed with 10 μ l of pre-chilled DNA ligation mix or 10 ng of circular plasmid. The mixture was incubated on ice for 20 mins, thereafter, heat shocked at 42°C for 90 secs. The cells were then placed on ice for 5 mins and rescued with 500 μ l of pre-warmed LB medium. The mixture was then placed in the incubator at 37°C for 1 hour. The entire volume of mixture was added to pre-warmed LA plates supplemented with the correct antibiotic (Appendix 5.1.3-5.1.4). The plates were placed in the incubator at 37°C overnight to allow for bacterial growth.

2.2 DNA isolation, purification and analysis

2.2.1 Small scale plasmid DNA isolation by alkaline lysis

A single colony was inoculated into 1 ml of LB supplemented with either ampicillin (100µg/ml) or kanamycin (50µg/ml) and incubated overnight at 37°C at 150 rpm. The bacterial culture was transferred to a 1.5 ml Eppendorf and centrifuged at 13 000 rpm for 1 min at room temperature. The supernatant was removed, and the pellet was resuspended in 180 µl of buffer 1 (Appendix 5.2.1). Buffer 2 [160 µl (Appendix 5.2.2)] was added, the lysate was mixed by inverting gently and left at room temperature for 5 mins. Following incubation, 120 µl of buffer 3 (Appendix 5.2.3) was added. The lysate was mixed by inverting and placed on ice for 5 mins. The lysate was centrifuged at 13 000 rpm for 10 mins at 4°C. The resulting supernatant was transferred to a 1 ml Eppendorf. To allow for precipitation of plasmid DNA, 600 µl of 100 % isopropanol was added and incubated at room temperature for 2 mins. Centrifugation was performed at 13 000 rpm for 20 mins at 4°C. The supernatant was removed, and the pellet washed in 150 µl of 70% ethanol. Centrifugation was performed at 13 000 rpm for 2 mins at 4°C. The supernatant was removed, and the pellet was air dried. The pellet was resuspended in 50 µl of dH₂O. The concentration of plasmid DNA was determined using the NanoDrop spectrophotometer (Thermofisher Scientific, MA, USA).

2.2.2 Large scale plasmid DNA isolation

Plasmid DNA that was required in large quantities was isolated using the Qiagen Plasmid Maxi kit (Qiagen, MD, USA) according to the manufacturer's instructions. Briefly, a single colony was inoculated in 200-300 ml LB supplemented with either ampicillin or kanamycin and incubated overnight at 37°C at 150 rpm. The bacterial cells were harvested by centrifugation at 8000 rpm for 15 mins at 4°C. The supernatant was removed, and the bacterial pellet was resuspended in 10 ml resuspension buffer that contains RNase A (Thermofisher Scientific, MA, USA). To lyse cells, 10 ml lysis buffer was added. The lysate was mixed by gently inverting 4-6 times and incubating at room temperature for 5 mins. To neutralize the solution, 10 ml of neutralization buffer was added. The lysate was mixed by inverting 4-6 times and incubating on ice for 20 mins. Centrifugation was

performed at 12 000 rpm for 45 mins at 4°C. Qiagen columns were equilibrated by adding 10 ml of buffer QBT and allowing the column to empty by gravity flow. Filter paper was utilized to ensure clear lysate entered the column and flowed through the resin by gravity flow. The column was washed twice with 30 ml Buffer QC. Elution of plasmid DNA was performed by the addition of 15 ml Elution buffer. The eluted plasmid DNA was precipitated by the addition of 10.5 ml of isopropanol. The precipitated DNA was centrifuged at 12 000 rpm for 40 mins at 4°C. The supernatant was removed, and the pellet was washed in 70 % ethanol. The solution was centrifuged at 6000 rpm for 20 mins at 4°C. The supernatant was discarded, and the pellet was resuspended in 100-300 µl of dH₂O. The concentration of plasmid DNA was determined using the Nanodrop Spectrophotometer (ThermoFisher Scientific, MA, USA).

2.2.3 DNA analysis by agarose gel electrophoresis

A 1 % agarose gel was made by weighing out 1 g of powdered agarose and adding it to 100 ml of 1 × TAE gel electrophoresis running buffer (Appendix 5.2.7). The flask was swirled to mix contents and microwaved until the agarose was dissolved. This was allowed to cool until ~ 55°C. Once cooled, 5 µl of 10 mg/ml ethidium bromide was added. The gel was poured into an assembled casting tray and allowed to solidify at room temperature. The gel was placed into an electrophoresis tank that contained 1 × TAE gel electrophoresis running buffer. Tracking dye (Thermo Fisher Scientific, MA, USA) was added to each sample and loaded into the wells. A 1 kb O' generuler (Thermo Fisher Scientific, MA, USA) was loaded into the first well. A voltage of 120 V was applied for 1 hour or until the loading dye reached the bottom of the gel. The gel was viewed using a Syngene G:BOX Gel Documentation and Analysis system (Syngene, UK).

2.2.4 Purification of DNA following agarose gel electrophoresis

For purification of DNA fragments, the Qiagen minELute gel extraction kit was utilized as per the manufacturer's instructions (Qiagen, MD, USA). The DNA fragment of interest was excised from the agarose gel using a clean, sharp scalpel and placed into a 1.5 ml Eppendorf tube. The gel slice was weighed and buffer QG was added in a 3:1 ratio to gel weight. This was incubated at 50°C for 10 mins to allow the gel piece to dissolve.

Isopropanol was added to the sample in a 1:1 ratio to gel weight. The DNA was added to the minElute column and centrifuged at 13 000 rpm for 1 min at 4°C. The flow through was discarded and the column placed back into the collection tube. The addition of DNA to the column and discarding of flow through was repeated until the total volume of DNA was added to minElute column. Buffer QG (500 µl) was added to the spin column and centrifuged at 13 000 rpm for 1 min at 4°C. The flow through was discarded and 750 µl of buffer PE was added to the column. The column was centrifuged at 13 000 rpm for 1 min at 4°C. The flow through was discarded and the minElute column was centrifuged for an additional 1 min at 13 000 rpm. The minElute column was placed in a 1.5 ml eppendorf. The DNA was eluted by the addition of 50 µl dH₂O and centrifuged at 13 000 rpm for 1 min. The concentration of DNA was determined using the Nanodrop Spectrophotometer (Thermofisher Scientific, MA, USA).

2.3 Plasmid DNA manipulations

2.3.1 Restriction digests

Restriction digest reactions were carried out according to the manufacturer's instructions (Thermofisher Scientific, MA, USA). Reactions were carried out as follows: 1-2 µg of the appropriate plasmid DNA, 1-2 U of enzyme, 1 × appropriate reaction buffer and dH₂O was added to make the total volume to 20 µl. Restriction digests were incubated at 37°C for 2-3 hours.

2.3.2 Blunting of plasmid DNA using Klenow polymerase

To create blunt ended DNA, blunting reactions were carried out using Klenow enzyme according to the manufacturer's instructions (Thermofisher Scientific, MA, USA). Reactions were setup as follows: 0.1-4 µg of linearized plasmid DNA, 1 × reaction buffer, 0.5 µl of 2mM dNTP mix, 1-4 U klenow fragment and dH₂O was added to make the final volume to 20 µl. The reaction was incubated at 37°C for 10 mins and was stopped by heating at 75°C for 10 mins.

2.3.3 Dephosphorylation of plasmid DNA

To prevent vector re-circularization due to compatible ends, restriction enzyme digested DNA was treated with Antarctic phosphatase, according to the manufacturer's instructions (NewEngland Biolabs, MA, USA). The reaction was setup as follows: 1-5 U of antarctic phosphatase, 1-5 µg of linearized plasmid DNA, 1 × reaction buffer and appropriate volume of dH₂O to ensure that a final volume was 20 µl. The reaction was incubated at 37°C for 2 hours.

2.3.4 Polymerase chain reaction (PCR)

KAPA Taq 2 × ready mix PCR kit (KAPA Biosystems, CT, SA) was used for DNA amplification according to the manufacturer's instructions. The reactions were set up as follows: 1 ×KAPA Taq ready mix, 0.25 µM forward primer, 0.25 µM reverse primer, 25 ng of plasmid DNA and PCR grade water to a final volume of 25 µl. The primers utilized are listed in Table 1 and the PCR cycling conditions are indicated in Table 2.

Table 1: Primer sequences used for the amplification of the mTTR promoter, FokI poly A and firefly luciferase sequences

Primer name	Primer sequence	Description	Source/Reference
pCi-mTTR (F)	GATC CATATGAGATCT GT CGACAAAATTTATCGATC ACG	Forward primer with engineered <i>Nde I</i> and <i>Bgl II</i> restriction sites used for the amplification of mTTR sequence.	This study
pCi-mTTR (R)	GATC CTCGAG CTCGGTAC CTCGCGAATG	Reverse primer with engineered <i>Xho I</i> restriction site used for the amplification of mTTR sequence.	This study
pVAX-FokI poly A (F)	GATC CTCGAG CTATTGTG GCCCAGCTGAGC	Forward primer with engineered <i>Xho I</i> restriction site used for the amplification of the FokI-poly A sequence.	This study
pVAX-FokI poly A (R)	GATC GGTCTCCAATTGCC ATAGAGCCCACCGCATCC	Reverse primer with engineered <i>Bsa I</i> and <i>Mfe I</i> restriction sites used for the amplification of the FokI-poly A sequence.	This study
pCi-FluC (F)	GATC GACCTC CACAGTTA	Forward primer with engineered	This study

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	AATTGCTAACGCAGTCAGT GC	<i>Sac</i> I restriction site used for the amplification of firefly luciferase sequence.	
Pci-FluC (R)	GATC <i>GTTTAAAC</i> CCCTCA CTAAAGGGAAGCGGCCG	Reverse primer with engineered <i>Pme</i> I restriction site used for the amplification of firefly luciferase sequence.	This study

Key: Colour bases represent the respective engineered restriction sites.

Table 2: PCR cycling conditions.

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	3 mins	1
Denaturation	95°C	30 secs	×35
Annealing	55°C	30 secs	
Extension *	72°C	2 mins	
Final extension	72°C	2 mins	1
Hold	4°C	∞	1

* The extension time was adjusted for each PCR product according to the product size.

2.3.5 DNA ligation

DNA ligations were performed using the InsTAclone PCR cloning kit (Thermofisher Scientific, MA, USA) or 1-5 U T4 DNA ligase (Thermofisher Scientific, MA, USA) according to the manufacturer's instructions. Each reaction contained: 1 × ligation buffer, 1 µl T4 DNA ligase, 50-55 ng of plasmid DNA, insert DNA at either a 3:1 or 6:1 ratio to plasmid and nuclease free water to a final volume of 30 µl. The reaction was mixed briefly by tapping and centrifuged for 10 secs. The ligation was incubated at 16°C overnight. The ligation reactions were transformed into the competent DH5α *E.coli* cells (Section 2.1.1).

2.3.6 Construction of rAAV plasmids expressing anti-HBV TALENs from an mTTR promoter

Standard molecular cloning techniques described above were utilized for the construction of rAAVs that express anti-HBV TALENs from an mTTR promoter. To generate a plasmid that carries the FokI endonuclease domain and poly A sequence, a PCR based technique was utilized. The mTTR promoter was amplified from a previously constructed pCi-FluC plasmid (Table 3, Appendix 5.3.5) using primer pCi-mTTR (F) and pCi-mTTR (R) (Table 1). The resulting amplicon was ligated with a PCR cloning vector pTZ57R/T (Table 3, NewEngland Biolabs, MA, USA) to generate pTZ-mTTR. The FokI poly A sequence was amplified from a previously described plasmid, pVAX-CMV-TALEN [(Bloom et al., 2013), Appendix 5.3.1) using primers pVAX-FokI poly A (F) and pVAX-FokI poly A (R) (Table 1). The resulting amplicon was ligated with a PCR cloning vector pTZ57R/T to generate pTZ-FokI poly A

(Table 3). Positive clones were identified by restriction analysis and sent for sequencing to Inqaba Biotec (Inqaba Biotec, Pretoria, SA) to assess any PCR errors that may have occurred. The FokI poly A tail sequence containing fragment was digested with restriction enzymes *Xho* I and *Bsa* I, purified and ligated into pTZ-mTTR to generate pTZ-mTTR-FokI poly A (Figure 2.3.6.1, Table 3, Appendix 5.3.2).

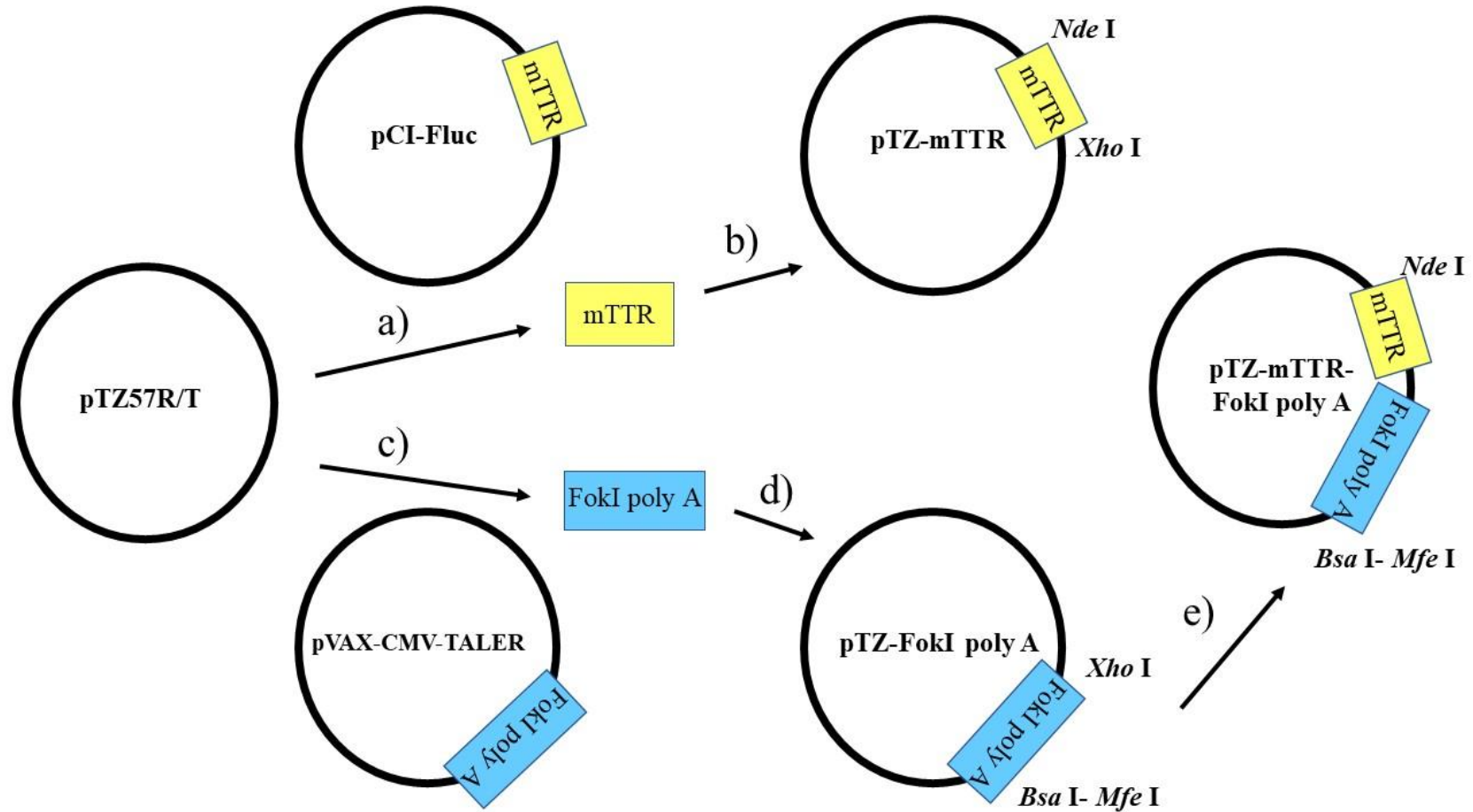


Figure 2.3.6.1: Schematic representation of the cloning strategy implemented for the generation of pTZ-mTTR- FokI poly A. a) The mTTR promoter was PCR amplified from pCI-Fluc. b) The mTTR promoter was cloned into a pTZ57R/T cloning vector to generate pTZ-mTTR. c) The FokI poly A sequence was PCR amplified from pVAX-CMV-TALENL. d) The FokI poly A sequence were cloned into a pTZ cloning vector to generate pTZ-FokI poly A. e) The FokI poly A sequence was sub-cloned from pTZ-FokI poly A into pTZ-mTTR to generate pTZ-mTTR-FokI poly A.

To insert the mTTR-FokI poly A sequence in to the AAV plasmid, pAAV-CAG-GFP [Table 3, (Mao et al., 2011)] was digested with *Mfe* I, blunted with Klenow and purified (Section 2.3.2). The purified fragment was digested with *Nde* I and purified. The pTZ-mTTR-FokI poly A tail plasmid was digested with *Nde* I and *Sma* I, mTTR-FokI poly A fragment was purified and ligated with the *Nde* I-blunt ended *Mfe* I present in the linear AAV plasmid. This generated pAAV-mTTR-FokI poly A (Appendix 5.3.3). This plasmid was used as a negative control for all experiments. The right or left anti-HBV TALEN sequences targeting the S ORF were digested from pVAX-CMV-TALEN(R) or pVAX-CMV-TALEN(L) (Bloom et al., 2013) using the *Sac* I and *Spe* I restriction sites (Appendix 5.3.4). The right and left TALE sequences were ligated in the *Sac* I and *Spe* I sites present in the pAAV-mTTR-FokI poly A plasmid. This generated pAAV-mTTR-TALE(R)-FokI poly A and pAAV-mTTR-TALE(L)-FokI poly A (Figure 2.3.6.2).

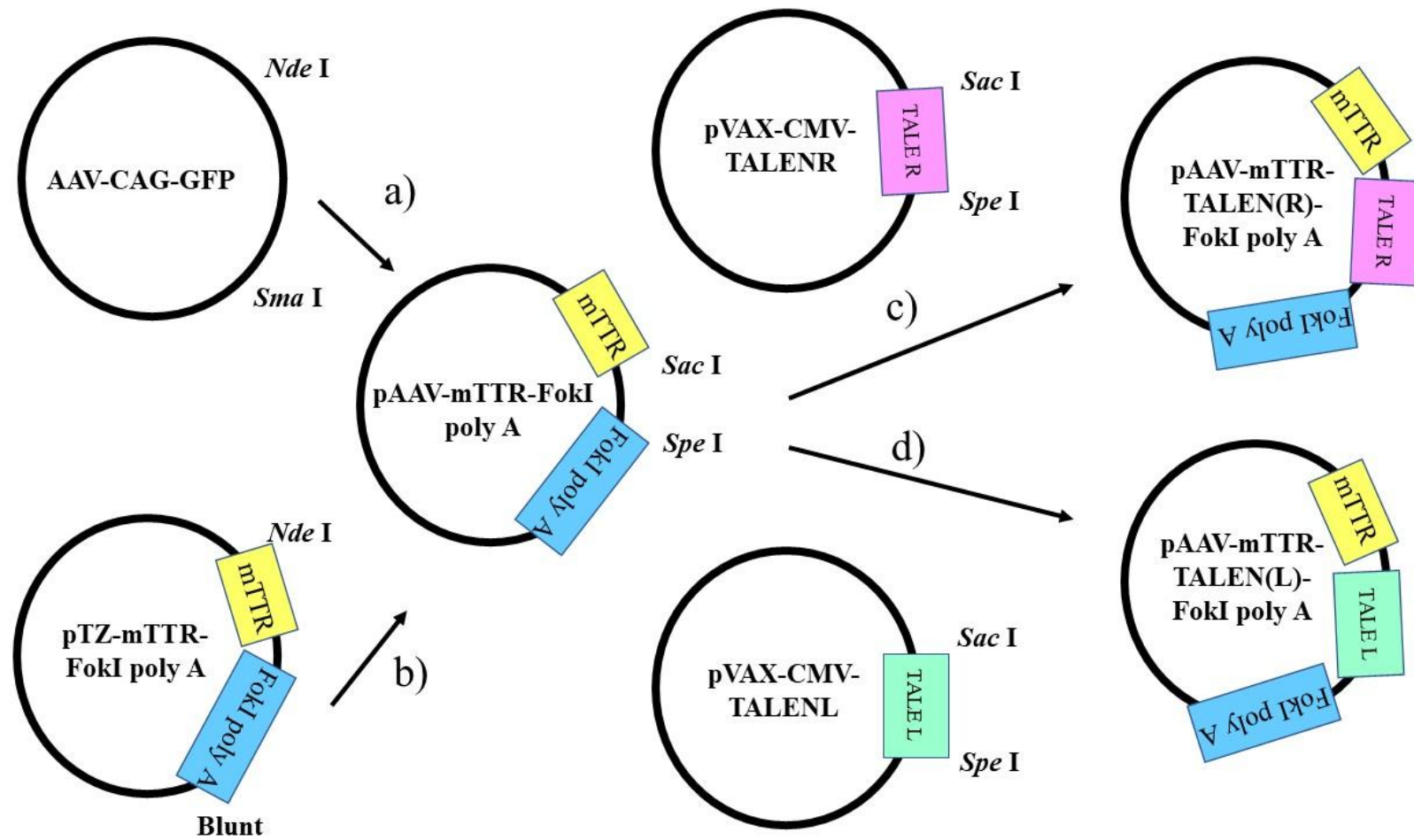


Figure 2.3.6.2: Schematic representation of the cloning strategy employed for the generation of rAAV plasmids expressing anti-HBV TALEN sequences. a) The AAV-CAG-GFP plasmid was digested with *Nde* I and *Sma* I to remove the CAG promoter and GFP sequence. b) The pTZ-mTTR-FokI poly A plasmid was enzymatically digested with *Nde* I and blunt ended *Mfe* I. This allowed for the insertion of the mTTR-FokI poly A sequence into pAAV, to generate pAAV-mTTR-FokI poly A. c and d) The right and left anti-HBV TALENs were digested with *Sac* I and *Spe* I and ligated with the same restriction sites present within pAAV-mTTR-FokI poly A. This generated pAAV-mTTR-TALEN(R)-FokI poly A and pAAV-mTTR-TALEN(L)-FokI poly A respectively.

2.3.7 Construction of rAAV plasmid expressing Firefly luciferase from an mTTR promoter

To enable easy assessment of transgene expression, a plasmid carrying a firefly luciferase (FluC) gene downstream of an mTTR promoter was constructed. The FluC gene was PCR amplified from the pCi-FluC (Appendix 5.3.5) plasmid using primer pCi-FluC (F) and pCi-FluC (R) (Table 1). The resulting amplicon was ligated with PCR cloning vector pTZ57R/T, to generate pTZ-FluC (Table 3). Positive clones, identified by restriction analysis, were sequenced by Inqaba Biotec to assess any PCR errors that may have occurred. The FluC sequence containing fragment was removed from pTZ-FluC using restriction enzymes *Sac* I and *Pme* I. The pAAV-mTTR-FokI poly A plasmid was digested with *Sac* I and *Pme* I, which removed the Fok I endonuclease domain retaining the poly A sequence. The FluC gene was ligated into pAAV-mTTR- poly A plasmid to generate pAAV-mTTR-FluC-poly A [(Figure 2.3.7.1, Table 3, Appendix 5.3.6)].

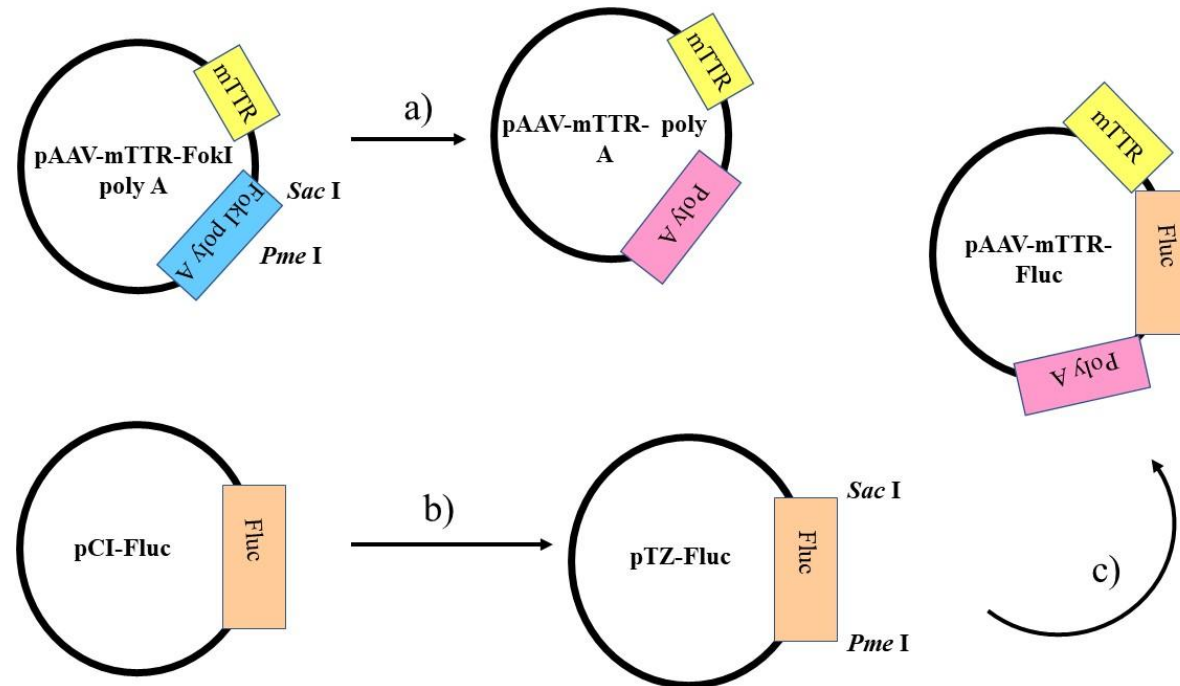


Figure 2.3.7.1: Schematic representation of the cloning strategy employed for the generation of rAAV plasmid expressing FluC. a) The previously constructed pAAV-mTTR-FokI poly A plasmid was digested with *Sac* I and *Pme* I, which removed the Fok I endonuclease domain. b) The FluC sequence was PCR amplified from pCI-FluC and cloned into pTZ57R/T plasmid, to generate pTZ-FluC. c) The FluC sequence was enzymatically digested with *Sac* I and *Pme* I. This allowed for the insertion of the FluC sequence into pAAV-mTTR-poly A, which generated pAAV-mTTR-FluC.

Table 3: Plasmids generated and used in this study.

Plasmid	Description	Source/reference
pCI-FluC	Plasmid carrying Firefly luciferase gene expressed from an mTTR promoter.	Unpublished
pTZ57R/T	TA cloning plasmid.	NewEngland Biolabs, MA, USA
pTZ-mTTR	pTZ57R/T carrying mTTR promoter sequence	This study
pVAX-CMV-TALEN(R)	Plasmid carrying the right anti-HBV TALEN expressed from a CMV promoter.	(Bloom et al., 2013)
pVAX-CMV-TALEN(L)	Plasmid carrying the left anti-HBV TALEN expressed from a CMV promoter.	(Bloom et al., 2013)
pTZ-FokI poly A	pTZ57R/T carrying mTTR promoter, Fok I and poly A sequences	This study
pAAV-CAG-GFP	An AAV genome bearing plasmid containing a CAG (CMV enhancer, chicken beta-Actin promoter and rabbit beta-Globin splice acceptor site) promoter expressing GFP.	(Mao et al., 2011)
pAAV-mTTR-FokI poly A	AAV plasmid with an mTTR promoter, Fok I endonuclease and poly A sequence.	This study
pAAV-mTTR-TALE(R)-FokI poly A	pAAV-mTTR-FokI poly A plasmid expressing Right anti-HBV TALEN from an mTTR promoter.	This study
pAAV-mTTR-TALE(L)-FokI poly A	pAAV-mTTR-FokI poly A plasmid expressing Left anti-	This study

	HBV TALEN from an mTTR promoter.	
pAAV-mTTR-FluC	An AAV plasmid expressing Firefly luciferase from an mTTR promoter	This study
pCH-9/3091	HBV replication-competent plasmid that contains a greater than genome length HBV sequence.	(Nassal et al., 1990)
pAd helper	An AAV plasmid that contains Ad genes <i>E1A</i> , <i>E1B</i> , <i>E2</i> and <i>E4</i> to provide additional helper functions	(Grimm et al., 2008)
pAAV2	An AAV plasmid that contains <i>rep</i> and <i>cap</i> genes.	(Grimm et al., 2008)

2.4 Tissue Culture methods

2.4.1 Cell lines culturing and maintenance

Human embryonic kidney cells (Hek 293 T cells), Human Hepatoma cells (Huh 7) and HepG 2.2.15 cells were maintained in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10 % foetal calf serum (Gibco, BRL, UK), penicillin (100 000 U/ml) and streptomycin (100 000 µg/ml). The cells were maintained in a humidified incubator at 37°C and 5 % CO₂. The HepG 2.2.15 cells are derived from the Human Hepatoblastoma cell line HepG2. The cell line Hep G2 was transfected with a plasmid containing a gene that confers resistance to G418 and four 5'-3' tandem copies of the HBV genome present as dimers. Cells that grew in the presence of G418 produced high levels of HBeAg and HBsAg. The HBV DNA present within these cells exists as chromosomally integrated sequences, episomally as cccDNA molecules and incomplete copies of the HBV genome (Sells et al., 1987). Cells were grown from a freezer stock by adding 1 ml of cells to 10 ml of complete medium [DMEM with 10 % FCS, penicillin and streptomycin, (Appendix 5.4.2-5.4.4)]. Cells were then centrifuged at 800 rpm for 3 mins. The supernatant was discarded, and the pellet re-suspended in 5 ml of complete medium. The cells were

added to a 25 cm² small flask. The cells were placed in the humidified incubator until they were 100 % confluent. Once confluent, cells were sub-cultured by washing with 3 ml of saline that was supplemented with 1000 × EDTA. Saline-EDTA (3 ml) was then added to the flask and incubated in the humidified incubator for 3 mins. For Huh 7 and HepG2.2.15 cells, 500 µl of saline-EDTA was mixed with 500 µl of 0.5 × Tryple express (Thermo Fisher Scientific, MA, USA, Appendix 5.4.5). After 3 mins, the cells were detached by tapping the flask. The Tryple Express was inactivated by adding complete medium to a volume of 10 ml. This was centrifuged at 800 rpm for 3 mins. The supernatant was discarded, and the pellet re-suspended in 10 ml of complete medium. The cells were transferred to a 75 cm² medium flask. The cells were grown until the desired confluency was achieved. The same procedure was followed for sub-culturing in a 175 cm² large flask, except the volumes of saline-EDTA, Tryple express and complete media was adjusted to 5 ml, 1 ml and 20 ml respectively.

2.4.2 Transfection of eukaryotic cell lines

Transfections of Huh 7 cells and HepG 2.2.15 cells were carried out with lipofectamine™ 3000 reagent (Thermofisher Scientific, MA, USA) according to the manufacturer's instructions. Huh 7 cells and HepG 2.2.15 cells were grown to 100 % confluency in a 75cm² flask and seeded in complete medium in a 24 well CellStar® plate (Huh 7 cells) and 6 well CellStar® plate (HepG 2.2.15 cells) at 60 % confluency. Cells were incubated at 37°C, 5 % CO₂ overnight. An hour prior to transfection of cells, media was replaced with antibiotic-free medium. For Huh 7 cells, each well was co-transfected with pAAV-CAG-GFP and pCH-9/3091 and the necessary plasmid (Table 3). For HepG 2.2.15 cells were transfected with pAAV-CAG-GFP and the necessary plasmids (Table 3). pAAV-CAG-GFP was included in the DNA transfection mixtures to enable the determination of the transfection efficiency using fluorescence microscopy. DNA mixes were made by adding 200 ng of DNA, 1µl/µg of P3000 reagent and OptiMEM (Life technologies, USA) to a final volume of 25 µl. A lipofectamine mix containing 1.5 µl lipofecatmine and 23.5 µl of OptiMEM was added to the DNA mixes. The mixtures were incubated at room temperature for 10 mins, and 50 µl of the transfection:DNA mixture was added to

cells. The cells were incubated at 37°C, 5 % CO₂ overnight. Thereafter the media was removed and replaced with complete medium.

2.4.3 Freezing of cells

Cells were frozen once they reached 100 % large flask (175 cm²) confluency. Cells were washed and detached as described (Section 2.4.1). After detachment, 10 ml of complete medium was added to the cells and centrifuged at 800 rpm for 3 mins. The supernatant was discarded, and the pellet re-suspended in 10 ml of freezing solution (Appendix 5.4.6). Aliquots of 1 ml were added to cryovials and stored at -80°C overnight. Thereafter they were moved to liquid nitrogen for long term storage.

2.4.4 Determination of Firefly luciferase expression from plasmids

Before application in AAV vector production, the functionality of the AAV genome and FluC gene bearing plasmids was tested *in vitro*. Firefly luciferase expression in Huh 7 cells was determined using the Dual Luciferase® Reporter Assay system (Promega, WI, USA). Briefly, Huh 7 cells were transfected using lipofecetamine™ 3000 reagent with 100 ng of pAAV-mTTR-FluC, incubated at 37°C for 48 hours. After 48 hours, the supernatant was removed from the cells and 100 µl of 1 × Lysis buffer was added. This was incubated at room temperature for 15 mins. Ten microlitres of the lysate was transferred to a 96 well luminometer plate, and 50 µl of LAR II (Luciferase Assay Substrate) was added to each well that contained a sample. The luminescence was measured using the Veritas Microplate Luminometer (Turner Biosystems, CA,USA).

2.4.5. Assessment of anti-HBV TALENs gene expression from plasmids using immunocytochemistry

To determine the expression of the anti-HBV TALENs, Huh 7 cells were seeded in a 24-well CellStar® plate and transfected using lipofectamine™ 3000 reagent (Section 2.4.2). Cells were transfected individually with 500 ng of pAAV-mTTR-TALE(R)-FokI poly A, pAAV-mTTR-TALE(L)-FokI poly A, pAAV-mTTR- FokI poly A (negative control), pVAX-CMV-TALEN(R) and pVAX-CMV-TALEN(L). The plasmids pVAX-CMV-TALEN(R) and pVAX-CMV-TALEN(L) were used as

positive controls. Transfections were performed in quadruplicate, to allow for biological replicates. After 48 hours the media was removed, and cells washed with 200 μ l of 1 $\times$ PBS (Appendix 5.4.1). The PBS was removed and 500 μ l of ice-cold methanol was added to each well and incubated for 15 mins at room temperature. The methanol was discarded, cells were washed twice with 200 μ l of 1 $\times$ PBS. The PBS was removed and 200 μ l of 0.1 % Triton-X (Appendix 5.6.1) was added to each well. The plate was incubated for 10 mins at room temperature. Cells were washed twice with 200 μ l of 1 $\times$ PBS.

The cells were blocked overnight by adding 350 μ l of 1 % Bovine Serum Albumin [BSA (Appendix 5.6.2)] and placed in a humidity chamber. The BSA was removed and 1:200 dilution of the primary mouse antibody [Ab (Sigma Aldrich. BA, GER)] that targets the Human agglutinin (HA) tag was added and incubated in a humidity chamber for 1 hour. The cells were washed twice with 200 μ l of 1 $\times$ PBS. The PBS was removed and 1:200 dilution of secondary goat anti-mouse Ab (Life Technologies, CA, USA) was added and incubated in a humidity chamber for 40 mins. The cells were washed twice with 200 μ l of 1 $\times$ PBS. The PBS was removed and 100 μ l of 1 $\times$ DAPI was added and incubated at room temperature for 2 mins. The cells were washed twice with 200 μ l of 1 $\times$ PBS. Cells were imaged using an EVOS FL microscope (Thermo Fisher Scientific, MA, USA).

2.4.6. Assessment of HBV gene silencing using an ELISA

For the assessment of HBV gene silencing by anti-HBV TALEN expressing plasmids, Huh 7 cells were seeded in a 24 well CellStar® plate. Lipofectamine™ 3000 was used to transfect cells (Section 2.4.2). As the Huh 7 cell line does not contain HBV genome within its genome, a co-transfection of pCH-9/3091 was done. The transfection mixture contained 200 ng of pCH-9/3091, 100 ng of pAAV-mTTR-TALE(R)-FokI poly A, 100 ng of pAAV-mTTR-TALE(L)-FokI poly A and 100 ng of pAAV-CAG-GFP. pAAV-CAG-GFP was included in the DNA transfection mixtures to enable the determination of the transfection efficiency using fluorescence microscopy. The transfection mixture for the negative control contained; 200 ng of pCH-9/3091, 100 ng of pAAV-mTTR- FokI poly A and a 100 ng of pAAV-CAG-GFP. For the positive control the transfection mixture contained; 200 ng of pCH-9/3091, 100 ng pVAX-CMV-TALEN(R), 100 ng pVAX-CMV-TALEN(L) and 100

ng AAV-CAG-GFP. Transfections were performed in quadruplicate, to allow for biological replicates. Media (100 μ l) was collected at day 2, day 3 and day 4 post transfection. The collected media was used to measure HBV gene silencing using the Monolisa TM HbsAg Ultra Kit (Biorad Laboratories, CA, USA) according to the manufacturer's instructions. Briefly, a 100 μ l of collected media was added to each well of a 96 well plate and 50 μ l of conjugate solution was added to each well. The plate was covered with an adhesive and incubated for 1 hour 30 mins at 37°C. After the incubation, the plate was washed by adding 4 $\times$ 100 μ l of development solution into each well using the Immuno WashTM 1575 Microplate Washer (Bio-Rad, CA, USA). The reactions were allowed to develop in the dark for 30 mins at room temperature. The reactions were stopped by adding 100 μ l of stopping solution. The optical density of the samples was measured at 490 nm and referenced at 655 nm using the iMARK Microplate Reader (Biorad Laboratories, CA, USA).

2.4.7. Assessment of plasmid derived anti-HBV TALENs toxicity in Huh 7 cells

A methyl-thiazolyl-tetrazolium (MTT) assay was performed to determine toxicity of the anti-HBV TALENs. Huh 7 cells were seeded in a 96 well CellStar® plates 24 hours prior to transfection. To confirm expression of the anti-HBV TALENs, pCH-9/3091 was added to the transfection mixture. Transfections were performed in quadruplicate, to allow for biological replicates. The transfection mixture contained 15 ng of pCH-9/3091, 15 ng of AAV-CAG-GFP, 85 ng of pAAV-mTTR-TALE(R)-FokI poly A and 85 ng pAAV-mTTR-TALE(L)-FokI poly A. For the negative control, 15 ng of pCH-9/3091, 15 ng of pAAV-CAG-GFP and 170 ng of pAAV-mTTR-FokI poly A was added. To serve as a positive control, 15 ng of pCH-9/3091, 15 ng of pAAV-CAG-GFP and 85 ng of pVAX-CMV-TALEN(R) and 85 ng of pVAX-CMV-TALEN(L) was added. To serve as a toxicity positive control, cells received 50 % dimethyl sulfoxide (DMSO). An untransfected control consisted of cells receiving no plasmid DNA or 50 % DMSO treatment. Cell viability was assessed 48 hours after transfection by adding 20 μ l of [(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) (MTT)] solution (5 mg/ml, in 1 $\times$ PBS) to each well and incubating at 37°C at 5 % CO₂ for 1 hour. Media was removed from each well and the purple formazan by-product generated was re-suspended in 200 μ l of 100 % DMSO. The optical density of the samples was measured at a wavelength of 570 nm and referenced at 650 nm using the iMARKTM microplate reader.

2.4.8. Determination of targeted cleavage of the HBV genome in HepG 2.2.15 cells

To determine whether mutations were successfully introduced by the NHEJ machinery as a result of the cleavage ability of anti-HBV TALENs, a T7E1 assay was performed. In this assay, sequences spanning the target DNA binding sites are amplified by PCR. The PCR amplicons are denatured to separate the double stranded DNA into single strands and reannealed at room temperature to allow for heteroduplex and/or duplex formation. The T7 enzyme is added, which recognizes and cleaves the generated heteroduplexes. Cleaved products are indicative of TALEN-mediated disruption to viral DNA. The HepG 2.2.15 cells were used as opposed to the Huh 7 cells as they produce high levels of targeted cccDNA as compared to a transfection of Huh 7 cells with pCH-9/3091 (Bloom et al., 2017). HepG 2.2.15 cells were seeded in a 6 well CellStar® plate. Prior to the transfections of cells, 100 µl of cell culture media was collected to determine baseline HBsAg expression levels. Cells were transfected in quadruplicate. Cells were transfected with 2300 ng of pAAV-mTTR-TALE(R)-FokI poly A, 2300 ng of pAAV-mTTR-TALE(L)-FokI and 400 ng of AAV-CAG-GFP. To serve as negative control, cells were transfected with 4600 ng of pAAV-mTTR-FokI poly A and 400 ng of pAAV-CAG-GFP. To serve as a positive control, cells were transfected with 2300 ng of pVAX-CMV-TALEN(R) and 2300 ng pVAX-CMV-TALEN(L) and 400 ng of pAAV-CAG-GFP. The cells were incubated at 32 °C at 5 % CO₂.

As HepG 2.2.15 cells are difficult to transfect, the same cells were repeatedly transfected 3 times. For the first (day 3), second (day 8) and third (day 14) transfection, 100 µl of media was removed for an ELISA (Section 2.4.6) forty-eight hours post-transfection and growth medium was replaced. On day 5 and day 10, growth media was removed, and cells washed with 500 µl of saline/EDTA. The saline/EDTA solution was removed and 500 µl of TrypLE Express was added to each well and incubated at 32 °C, 5 % CO₂ for 3 minutes. After the incubation period, 500 µl of media was added and the cells were re-suspended. The re-suspended cells were transferred into a sterile 1.5 ml microcentrifuge tubes and centrifuged at 0.2 rpm for 3 minutes. The pellet was re-suspended in 1000 µl of saline and 800 µl of cells were

stored at -80°C for gDNA extraction. The remaining cells (~200 µl) were re-seeded into the same 6-well plate and 1800 µl of complete medium was added and incubated at 32 °C (5 % CO₂). On day 16 the triple transfected HepG2.2.15 cells were harvested and stored at -80 °C until needed for gDNA extraction.

Total DNA was then extracted from the HepG 2.2.1.5 cells harvested on days 5, 10 and 16 using the Qiamp DNA Blood mini kit (Section 2.5.4). S ORF forward and S ORF reverse primers (Table 4) were used for the amplification of the target region within the S ORF. The KAPA HiFi Hotstart Ready mix (KAPA Biosystems, MA, USA) was utilized. The reaction was setup as follows: 12.5 µl of KAPA HiFi Hotstart Ready mix, 10 pmol of the forward and reverse primers, 5 µl of template DNA and dH₂O were added so that a volume of 25 µl was maintained. The PCR conditions utilized are illustrated in Table 5.

Table 4: Primers used for the amplification of the Surface ORF of the HBV genome

Primer name	Primer sequence	Description	Source/Reference
S ORF Forward	CCTAGGACCCCTTTCTCGTGT	Forward primer amplifying the S ORF target region of the HBV genome.	(Bloom et al., 2013)
S ORF Reverse	ACTGAGCCAGGAGAAACGGG	Reverse primer amplifying the S ORF target region of the HBV genome.	(Bloom et al., 2013)

Table 5: PCR cycling conditions utilized for the amplification of the Surface ORF of the HBV genome

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	2 mins	1
Denaturation	95°C	15 secs	

Annealing	58°C	15 secs	×30
Extension	72°C	15 secs	
Final extension	72°C	5 mins	1
Hold	4°C	∞	1

To determine successful PCR amplification, 3 µl of the PCR reaction was loaded onto a 1 % agarose gel (Section 2.2.3). The remaining 22 µl was purified using the Qiagen minElute Gel extraction kit as per the manufacturer's instructions (Section 2.2.4).

The T7E1 cleavage reactions were set up as follows: 450 ng of PCR amplified DNA, 1 × NEB Buffer 2 (New England Biolabs, NJ, USA) and ddH₂O to a final volume of 12 µl. The samples were denatured at 95 °C for 5 minutes, cooled to 35 °C at 0.1 °C/s and incubated at 35 °C for a further 2 minutes to allow for heteroduplex formation. Samples were cooled to 4 °C and incubated on ice for 5 minutes after which 1 µl of the T7E1 (New England Biolabs, NJ, USA) enzyme was added and incubated at 37 °C for 15 minutes. Samples were allowed to cool to 4 °C for an additional 10 minutes. Then, 2 µl of 6 × orange loading dye was added. These samples were loaded onto a 2 % agarose gel and the gel was ran at 120 V for 30 mins. The gel was imaged using UV transilluminator. Separated bands were quantified by densitometry using Image J (version 1.46). Percentage of targeted disruption was calculated using the following formula.

$$\text{Targeted cleavage} = 100 \times \left[1 - \left(1 - \frac{\text{Fraction cleaved}}{\text{Fraction uncleaved} + \text{Fraction cleaved}} \right)^{\frac{1}{2}} \right]$$

2.5 Large scale AAV production

2.5.1 Culturing of cells, transfection and harvesting of AAVs

Large scale production of AAVs expressing anti-HBV TALEN or GFP or FluC and empty vector was performed in Hek 293 T cells. Hek 293 T cells were prepared by growing up cells in 175 cm² TC flasks to 100 % confluency. Four plates were used per construct per production. A ratio of 0.25 rAAV: 1 AAV helper plasmid: 2 AAV capsid plasmid was utilized. The AAV helper plasmid contains Ad genes *E1A*, *E1B*,

E2 and *E4* to provide additional helper functions, whereas the AAV capsid plasmid contains the *rep* and *cap* genes. Hence 24.6 µg of rAAV plasmid DNA, 98.4 µg of helper plasmid and 196 µg of either AAV-2 capsid plasmid was used per four plates. The transfection DNA mix was made up to a final volume of 4 ml by adding OptiMEM (Life technologies, USA). The polyethyleneimine max (PEImax) mix for 4 plates was made by adding 1.6 ml of PEImax (Polysciences Inc, Warrington, PA) and the volume was adjusted to 4 ml using OptiMEM. The transfection DNA mix and PEImix were mixed in a ratio of 1:3, vortexed and incubated for 10 mins at room temperature. Two millilitres of DNA:PEImax mix was added to each plate in a dropwise manner and incubated for 24 hours at 37°C. After 24 hours, media was removed and supplemented with 5 % complete medium (DMEM with 5 % FCS, penicillin and streptomycin).

At 48, 72- and 96-hours media was removed from all 4 plates, placed in a clearly labelled Schott bottle and 15 ml of 5 % complete medium was added to each plate. The same bottle was used for each construct at collection timepoints and stored at 4°C. At timepoint 120 hours, the media was harvested and 5 ml of 1 × PBS (Appendix 5.4.1) was added to each plate. The cells were scraped off and placed into a 50 ml Falcon tube. The cells were centrifuged for 8 mins at 1 500 rpm at 4°C. The supernatant was discarded, and the pellet resuspended in 5 ml benzonase buffer (Appendix 5.5.2) and stored at -80°C until ready to perform freeze/thaw cycles.

Five freeze/thaw cycles were performed at 37°C and liquid nitrogen (-196°C). The cells were sonicated for 1 min 20 seconds. Benzonase (50 U/ml, Sigma Aldrich, DA, GER) was added to remove DNA and RNA. The lysate was kept at 37°C for 1 hour and vortexed at 10 min intervals. The lysate was centrifuged at 6 000 rpm for 15 mins at 4°C. The AAV containing supernatant was transferred to a 50 ml Falcon tube.

2.5.2 Precipitation of viral vectors from the supernatant

The collected supernatant (42, 72, 96, 120 hours) was filter sterilized using a 0.22 µm stericup into a 500 ml Schott bottle. About 12.5 ml of 5× PEG (Appendix 5.5.1) was added for every 50 ml of media and incubated overnight at 4°C. The samples were

centrifuged at 5 000 rpm for 30 mins at 4°C. The supernatant was discarded. The 5 ml cell lysate collected above was used to resuspend the pellet.

2.5.3 Purification of AAVs by iodixanol gradient

The viral suspension was transferred into the Beckman centrifuge tube (16 × 76 mm) using a Pasteur pipette. The iodixanol gradient was setup by adding 1.5 ml of 15 %, 25 %, 40 % and 60 % iodixanol solutions into the centrifuge tube using a Pasteur pipette (Appendix 5.5.3). The Pasteur pipette was removed from the centrifuge tubes by plugging the top of the Pasteur pipette with the tip of a finger. Air bubbles were removed by tapping the centrifuge tubes gently. The centrifuge tubes were sealed and ultracentrifuged at 50 000 rpm for 2 hours at 4°C. Following centrifugation, the centrifuge tube was clamped on a retort stand. A needle (20 G) was used to punch a small hole at the top of the tube to release pressure. A syringe with a needle (20 G) was used to collect the 40 % phase (Figure 2.5.3), as the viral particles accumulate into the 40 % iodixanol phase after ultracentrifugation. Viral aliquots of 50-100 µl were stored at -80°C until required.



Figure 2.5.3: A representative of the iodoxinal fraction obtained from large scale AAV production and purification. Following ultracentrifugation, the centrifuge tube was clamped onto a retort stand. A 20 G needle was used to punch a small hole at the top of the tube to release pressure. A second 20 G needle connected to a 2 ml syringe was used to collect the 40 % phase of the iodixanol gradient, indicated by a red arrow.

2.5.4 Viral DNA isolation

The Viral DNA Qiaamp DNA extraction kit was used for the extraction of viral DNA according to the manufacturer's instructions (Qiagen, MD, USA). Briefly, 20 µl of protease was placed in a 1.5 ml Eppendorf. A viral dilution of 1:4 was made by adding 50 µl of virus in a 1.5 ml Eppendorf and topping it up to a final volume of 200 µl using 1 × PBS (Appendix 5.4.1). Buffer A1 (200 µl) was added to the viral DNA samples and mixed with the protease by pulse-vortexing for 15 secs and incubated at 56°C for 10 mins. A 100 % ethanol (200 µl) was added to the sample, mixed by pulse-vortexing for 15 secs and briefly centrifuged. The sample was added to a QIAamp mini spin column, centrifuged at 8000 rpm for 1 min. The filtrate was discarded and 500 µl of buffer AW1 was added to the column. Centrifugation at 8 000 rpm was performed for 1 min. The filtrate was discarded and 500 µl of buffer AW2 was added to the column. Centrifugation was performed at 14 000 rpm for 3 mins. The DNA was eluted by adding 200 µl of dH₂O and centrifuged at 8 000 rpm for 1 min.

2.5.5 Viral DNA quantification by quantitative PCR

The isolated viral DNA was quantified using quantitative PCR (qPCR) with mTTR promoter specific primers (Table 6). Standards were set up with dilutions prepared from the pAAV-mTTR-TALEN(R)-FokI poly A as it contained the mTTR promoter. qPCR was performed using the Faststart Essential Green master mix (Roche Holding, AG, SA) according to the manufacturer's instructions. Each reaction contained; 1× SYBR green master mix, 1 µM forward primer, 1 µM reverse primer, 1 µl of viral DNA and nuclease free water to a final volume of 20 µl. The cycling conditions used are indicated in Table 7. Viral vectors produced and listed in Table 8.

Table 6: Primers used for the quantification of AAVs using qPCR

Primer name	Primer sequence	Description	Source/Reference
mTTR forward	GCACTGGGAGGATGTTGAGT	Forward primer targeting the promoter regions of the viral vectors	(Maepa et al., 2017)

		generated	
mTTR reverse	CCCCTGTTCAAACATGTCCT	Revers primer targeting the promoter regions of the viral vectors generated	(Maepa et al., 2017)

Table 7: Cycling conditions used for viral DNA quantification by qPCR

Temperature	Time	Cycles
95°C	10 mins	1
95°C	15 secs	×40
58°C	15 secs	
72°C	15 secs	
65°C	5 secs	1
95°C	5 secs	1

Table 8: Viral vectors generated and used in this study

Capsid	Viral Vector	Description
AAV-2	AAV-mTTR-TALENR	Right anti-HBV TALEN sequence packaged into AAV-2 capsid.
	AAV-mTTR-TALENL	Left anti-HBV TALEN sequence packaged into AAV-2 capsid.
	AAV-mTTR-FluC	FluC sequence packaged into an AAV-2 capsid.
	AAV-mTTR-empty vector	mTTR promoter and poly A sequence packaged into an AAV-2 capsid to serve as a

		negative control.
	AAV-CAG-GFP	GFP packaged into an AAV-2 capsid.

2.6. Transduction of eukaryotic cell lines

Transduction of Huh 7 cells and HepG 2.2.15 cells were carried out as follows. Huh 7 cells and HepG 2.2.15 cells were grown to 100 % confluency in a 75cm² flask. The cells were seeded in complete medium in a 24 well CellStar® plate (Huh 7 cells) and 6 well CellStar® plate (HepG 2.2.15 cells) at 80 % confluency. Cells were incubated at 37°C, 5 % CO₂ overnight. To transduce cells while in suspension, the virus suspension was added to the cells during seeding and the plates were swirled to mix. To transduce attached cells, the cells were seeded to 60 % confluency in 24/48/96 well plates and incubated overnight. The media was removed the next day and 12.5 µl (for 96 well) or 50 µl (for 24 well) or 200 µl (for 6 well) viral suspension was added to the cells. Cells were then incubated at 37°C for 1 hour with swirling every 10 mins. The media was added to a final volume of 125 µl or 500 µl or 2 ml for 96/24/6 well plates respectively. Transduced cells were then incubated at 37°C, 5 % CO₂ until sampling.

2.7.1 Assessment of Firefly Luciferase gene expression by AAVs

To easily determine if the AAV vectors produced can express the transgenes under the regulation of the introduced mTTR promoter, Huh 7 cells were seeded in a 24-well CellStar® plate and transduced (Section 2.6.) with AAV-mTTR-FluC at an MOI of 1×10^5 . Cells were incubated at 37°C for 48 hours. Luminescence was measured as mentioned previously (Section 2.4.4).

2.7.2 Assessment of AAV mediated anti-HBV TALEN gene expression using an immunocytochemistry

To determine if the anti-HBV TALENs are expressed from the viral vectors generated, Huh 7 cells were seeded in a 24-well CellStar® plate. Cells were transduced (Section 2.6.) with the following viral vectors individually at an MOI of 1×10^4 : AAV-mTTR-TALENR, AAV-mTTR-TALENL, AAV-mTTR-empty vector

(negative control). All transductions were performed in triplicate. The immunocytochemical staining was performed as previously described (Section 2.4.5).

2.11.3 Assessment of HBV gene silencing by AAVs using an ELISA

To determine whether the viral vectors silenced HBV gene expression, Huh 7 cells were seeded in a 24 well CellStar® plate. As the Huh 7 cell line does not contain HBV within its genome, a transfection with pCH-9/3091 was performed 5 hours prior to cell transduction. Cells were transduced (Section 2.6.) in triplicate at an MOI of 1×10^5 . The transduction mixtures contained the following viral vectors: AAV-mTTR-TALENR and AAV-mTTR-TALENL or AAV-mTTR-empty (negative control). Media (100 µl) was collected at day 2, day 3 and day 4 post-transduction. The measurement of HBV gene silencing by ELISA was performed as previously described (Section 2.4.6).

2.11.4 Assessment of anti-HBV TALEN expressing AAVs toxicity in Huh 7 cells

An MTT assay was performed to determine toxicity of the AAVs expressing anti-HBV TALENs. Huh 7 cells were seeded in a 96 well CellStar® plate. To confirm expression of the anti-HBV TALENs from the viral vectors, pCH-9/3091 was transfected 24 hours prior to the transduction of cells. Cells were transduced (Section 2.6.) in quadruplicate with the following viral vectors at an MOI 1×10^5 : AAV-mTTR-TALENR and AAV-mTTR-TALENL. Transduction with AAV-mTTR-empty was included as a negative control. To serve as a toxicity positive control, 50 % DMSO was added to the cells. To determine whether the iodixanol present in the viral vector stocks could contribute to toxicity of the cells, 40 % iodixanol was added to the cells to serve as a control. An untransduced control consisted of cells receiving neither viral vectors, 50 % DMSO treatment or iodixanol. The determination of cell toxicity was carried out using MTT assay as previously described (Section 2.4.7).

2.11.5 Determination of AAV mediated targeted cleavage of the HBV genome in HepG 2.2.15 cells.

Hep G 2.2.15 cells were seeded in a 6 well CellStar® plate. Prior to the transduction of cells, a 100 µl of cell culture media was collected to determine baseline HBsAg expression levels. Cells were transduced (Section 2.6.) in duplicate at an MOI of $1 \times$

10⁵. To serve as a negative control cells were transduced with AAV-mTTR-Empty. Cells were transduced with AAV-mTTR-TALENR and AAV-mTTR-TALEN. A separate transduction with AAV-CAG-GFP was done to determine the transduction efficiency of the cells. The cells were incubated at 32 °C and 5 % CO₂.

To improve transduction, the same cells were transduced twice. For the first (day 3) and second (day 8) transduction, 100 µl of medium was removed for an ELISA (Section 2.4.6) forty-eight hours post-transduction and growth medium was replaced. On day 5, 800 µl of cells were harvested for gDNA extraction, while the remaining 200 µl of cells were re-seeded into the same 6-well plate. On day 10, the double transduced HepG2.2.15 cells were harvested and stored at -80 °C until needed for gDNA extraction (Section 2.5.4) and T7E1 assay was performed as described in section 2.4.8.

Chapter 3

Results

3.1 Cloning of the mTTR-FokI poly A sequence and Firefly luciferase gene into an AAV genome bearing plasmid

Anti-HBV TALENs have been previously designed to target the S ORF of the HBV genome but were expressed from a CMV promoter, which is not a liver specific promoter (Bloom et al., 2013). To ensure liver specific expression of the anti-HBV TALENs, the CMV promoter was replaced with a liver-specific, murine transthyretin promoter [(mTTR) (Costa and Grayson, 1991)]. These anti-HBV TALENs were previously cloned into a pVAX plasmid. To allow packaging in to AAV capsid, the anti-HBV TALENs were sub-cloned into an AAV ITR bearing plasmid [pAAV (Fig 3.1.1a)]. To enable convenient assessment of transgene expression a Firefly luciferase gene was also cloned downstream of an mTTR promoter in the pAAV plasmid (Fig 3.1.1b).

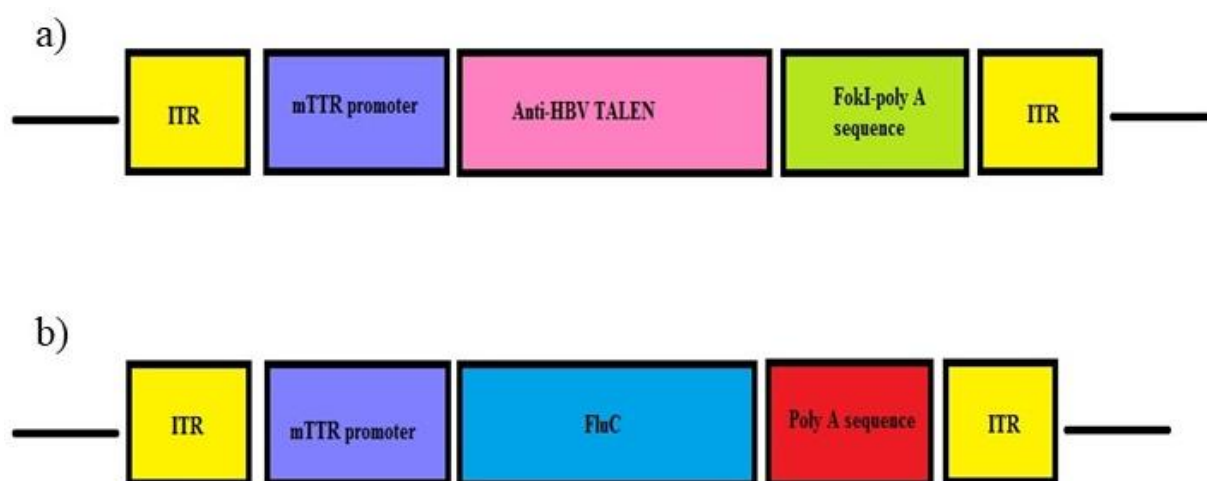


Figure 3.1.1: Generation of AAV plasmids expressing anti-HBV TALENs or FluC from an mTTR promoter. a) The anti-HBV TALEN or b) FluC sequences were inserted into an AAV plasmid to generate pAAV-mTTR-TALEN or pAAV-mTTR-FluC. The anti-HBV TALENs and FluC sequences are expressed from an mTTR promoter. The ITRs are the only *cis* elements needed for viral replication. The FokI poly A sequence consists of the FokI endonuclease, which is required for cleavage of the target DNA and a poly A sequence.

A multistep approach was adopted to clone in to the pAAV plasmid. For the construction of the AAV plasmid expressing the anti-HBV TALEN or FluC sequence, the mTTR promoter, FokI poly A sequence and FluC gene were PCR amplified from previously constructed plasmids using specific primers (Table 1). The primers were designed to amplify the mTTR promoter and FluC sequence from the pCi-FluC plasmid and the FokI poly A sequence from the pVAX-CMV-TALEN(R) plasmid (Bloom et al., 2013). As shown in Fig 3.1.2, the expected fragment sizes of 690 bp, 929 bp and 2056 bp for the mTTR promoter, FokI poly A sequence and FluC sequence respectively were obtained after PCR. The mTTR promoter, FokI poly A sequence and FluC sequence were gel extracted and ligated with an intermediary TA cloning pTZ57R/T plasmid to create pTZ-mTTR, pTZ-FokI poly A and pTZ-FluC respectively (Supp. Fig 1). One positive clone for each construct was selected and sent for sequencing by Inqaba to exclude the possibility of PCR insertions or deletions that may have occurred and to ensure that the engineered restriction sites are present. The restriction sites that were engineered into the primers used for the amplification of the mTTR, FluC and FokI poly A sequence were present and no PCR insertions or deletions were present in positive clones (Supp. Fig. 2-4).

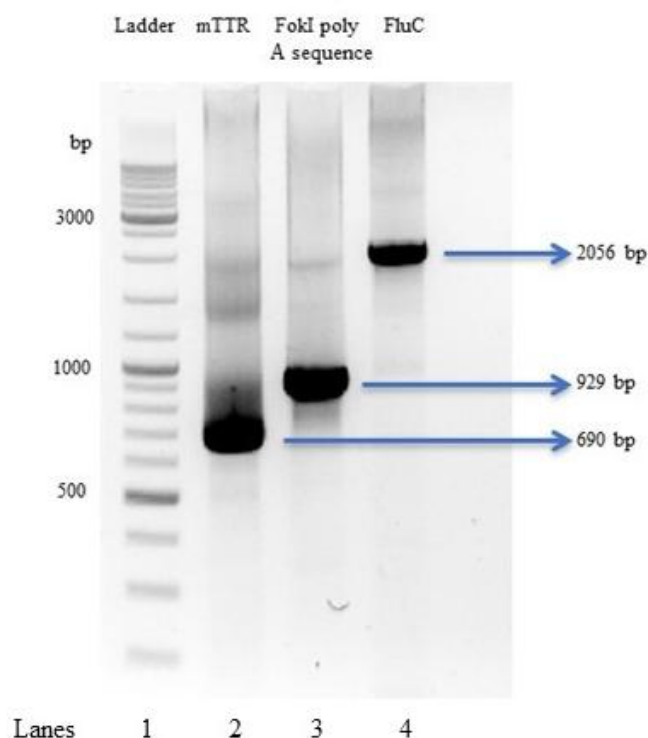


Figure 3.1.2:PCR amplification of the mTTR promoter, FokI poly A and FluC sequences. The mTTR promoter (Lane 2), FokI poly A sequence (Lane 3) and FluC gene (Lane 4) were PCR amplified using specific primers. Amplicons were then separated on a 1 % agarose gel, The O'Gene ladder (Lane 1) was loaded to determine the fragment sizes. Arrows indicate the expected bands and their respective sizes.

The FokI poly A sequence was sub-cloned from pTZ-FokI poly A into pTZ-mTTR to generate pTZ-mTTR-FokI poly A (Appendix Figure 5.3.2). After screening (not shown) and confirmation of the positive clone by digestion with *Bam* HI the expected fragments of sizes 3273 bp, 873 bp and 337 bp were obtained (Figure 3.1.3 a). The mTTR promoter-FokI poly A sequence was digested out of the pTZ-mTTR-FokI poly A plasmid using restriction enzymes *Nde* I and *Sma* I. This fragment was ligated with pAAV plasmid *Nde* I site and blunted *Mfe* I site to generate pAAV-mTTR-FokI poly A (Appendix Figure 5.3.3). After *Bam* HI digestion confirmation of the positive clone, the expected fragments of sizes 3601 bp, 873 bp and 337 bp were obtained (Fig 3.1.3 b).

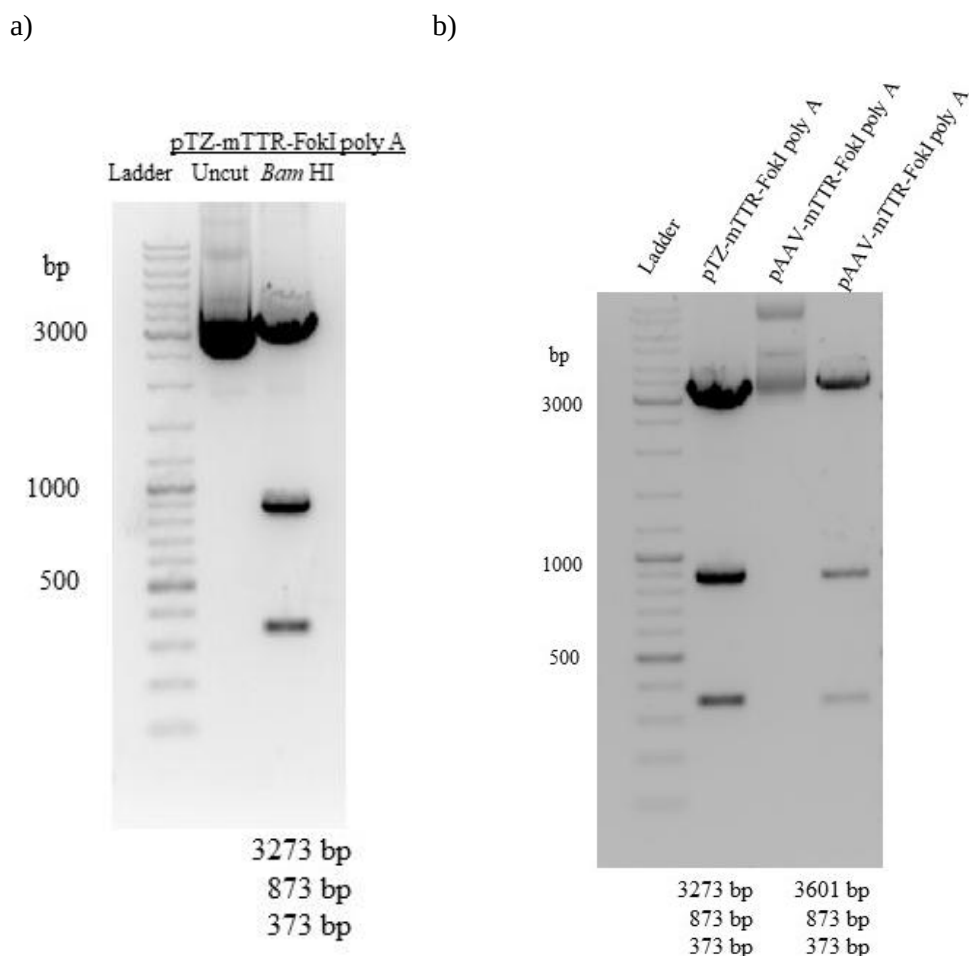


Figure 3.1.3: Generation of AAV plasmids containing the mTTR promoter and FokI poly A sequence. a) Subcloning of FokI poly A sequence from pTZ-FokI poly A with the pTZ- mTTR to generate pTZ-mTTR-FokI poly A : Lane 1: O'Gene ladder. Lane 2: uncut pTZ-mTTR-FokI poly A, lane 3: *Bam* HI digested pTZ-mTTR-FokI poly A. b) Subcloning of mTTR-FokI poly A sequence from pTZ-mTTR-FokI poly A with pAAV plasmid to generate pAAV-mTTR-FokI poly A. Lane 1: O'Gene ladder, Lane 2: *Bam* HI digested pTZ-mTTR-FokI poly A, Lane 3: Uncut pAAV-mTTR-FokI poly A, Lane 4: *Bam* HI digested pAAV-mTTR-FokI poly A. Fragments were separated on a 1 % agarose gel. Expected fragment sizes are indicated below the lanes.

To enable easy assessment of transgene expression from the mTTR promoter, the FluC sequence that was cloned into the pTZ-FluC plasmid (Supp. Fig. 1) was removed and inserted into the pAAV-mTTR-poly A plasmid, eliminating the Fok I endonuclease domain sequence to generate pAAV-mTTR-FluC (Fig 3.1.4 a, Appendix Figure 5.3.6). *Bam* HI and *Sal* I were used individually or as a combination

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to confirm positive clones. The expected fragments of sizes 3601 bp and 2588 bp were observed when using *Bam* HI, 3532 bp and 2657 bp when using *Sal* I and 3228 bp, 2284 bp, 373 bp and 304 bp when using *Bam* HI and *Sal* I together . The successful construction of pAAV-mTTR-FluC is also key for future *in vivo* studies as it enables easier live imaging of transgenic mice and reduces the number of animals that are needed for *in vivo* studies.

The fact that the two ITRs required for viral encapsidation and replication are reverse complements of each other, makes them more prone to recombination. Hence, to determine whether the ITRs for the pAAV-mTTR-FluC plasmid generated were intact, the plasmid was digested with *Eco* 88I (Fig 3.1.4 b). The restriction enzyme *Eco* 88I was used as its restriction site is present within the ITRs. The expected fragment sizes of 2680 bp, 1252 bp, 1084 bp, 608 bp and 543 bp were observed. The presence of 1252 bp and 543 bp bands indicates that both ITRs are intact and should yield full viral particles when used for vector genome packaging in to the AAV capsid.

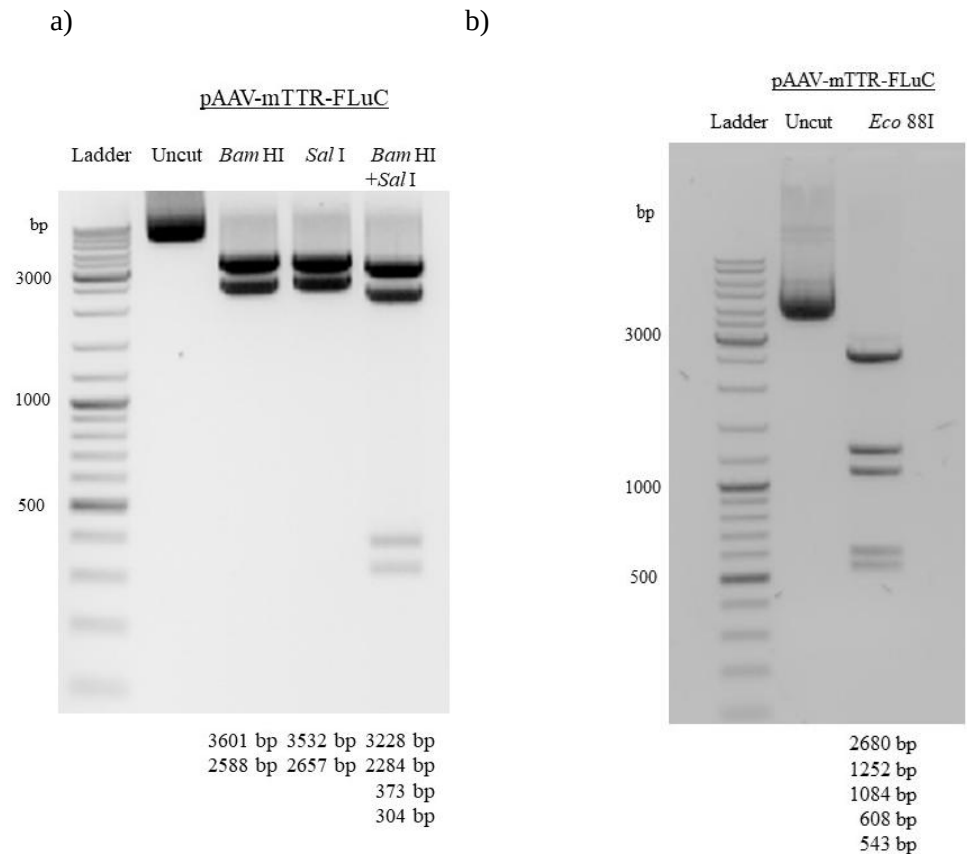


Figure 3.1.4: Generation of AAV plasmids expressing FluC. a) Confirmation of pAAV-mTTR-FluC plasmid with *Bam* HI and *Sal* I individually or in combination . Lane 1: O'gene ladder, Lane 2: Uncut pAAV-mTTR-FluC, Lane 3: *Bam* HI digested pAAV-mTTR-FluC, Lane 4: *Sal* I digested pAAV-mTTR-FluC, Lane 5: *Bam* HI and *Sal* I digested pAAV-mTTR-FluC. Fragments were then separated on a 1 % agarose gel. b) Confirmation of ITR integrity in pAAV-mTTR-FluC. Lane 1: O'gene ladder, Lane 2: Uncut pAAV-mTTR-FluC, Lane 3: *Eco* 881 digested pAAV-mTTR-FluC. Fragments were separated on a 1 % agarose gel. Expected fragment sizes are indicated below the lanes.

3.2 Assessment of luciferase expression from pAAV-mTTR-FluC plasmid

To determine the activity of the mTTR promoter and poly A sequence in liver derived cells, Huh 7 cells was transfected with pAAV-mTTR-FluC plasmid in quadruplicate. After 48 hours, cell lysates were used to measure luciferase activity, using a luciferase assay. A significant expression of Firefly luciferase was observed ($p = 0.04$) relative to the untransfected cells (Fig 3.2). These findings suggest that mTTR promoter and poly A sequence cloned are functional in Huh 7 cells. Hence the right and left anti-HBV TALEN sequences can now be cloned into the pAAV-mTTR-FokI poly A plasmid.

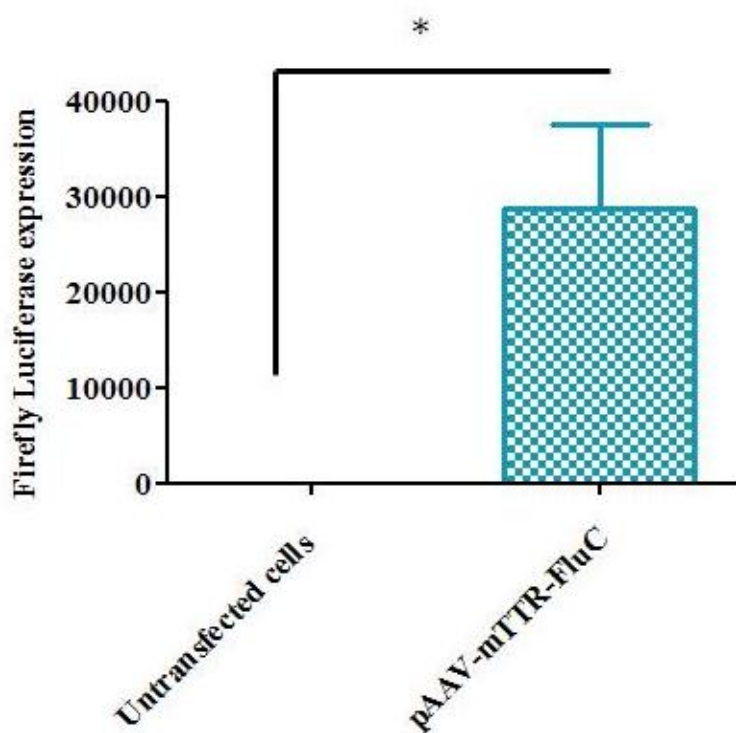


Figure 3.2: Plasmid encoded Firefly luciferase expression under the control of an mTTR promoter. Huh 7 cells were transfected with pAAV-mTTR-FluC. A luciferase assay was performed 48 hours post transfection, Two tailed and paired T-Test was used to determine the statistical significance, * indicates significant FluC gene expression with $p = 0.04$. Error bar indicates the normalized standard error of the mean (SEM,n=4).

3.3 Construction of anti-HBV TALEN expressing AAV genome bearing plasmids

The generated pAAV-mTTR-FokI poly A plasmid was used for the cloning of the right or left anti-HBV TALEN encoding sequences. To achieve this, the pAAV-mTTR-FokI poly A plasmid was digested with restriction enzymes *Sac* I and *Spe* I. The anti-HBV TALEN encoding sequences were digested out with the same restriction enzymes from the pVAX-CMV-TALEN plasmids (Appendix Figure 5.3.1). The resulting plasmids generated were pAAV-mTTR-TALE(R)-FokI poly A and pAAV-mTTR-TALE(L)-FokI poly A expressing the right and the left TALEN respectively (Fig 3.3.1, Appendix Figure 5.3.4). Following confirmation of clones with *Bam* HI digestion the expected fragment sizes for the right and left anti-HBV TALEN plasmids of 3601 bp, 2750 bp and 873 bp were observed, whereas the pAAV-mTTR-FokI poly A empty plasmid yielded fragment sizes of 3601 bp, 873 bp and 373 bp.

To determine whether the ITRs for the TALEN encoding AAV plasmids generated were intact, the plasmids were enzymatically digested with *Eco* 88I (Fig 3.3.2). The expected fragment sizes of 2680 bp, 1173 bp and 936 bp for pAAV-mTTR-FokI poly A empty plasmid were observed. Both pAAV-mTTR-TALEN(R)-FokI poly A and pAAV-mTTR-TALEN(L)-FokI poly A generated fragments sizes of 3115 bp, 2680 bp and 1347 bp as expected. The additional top band after *Eco* 88I digestion is indicative of undigested or partially digested plasmid. The presence of 1173 bp and 936 bp for pAAV-mTTR-FokI poly A empty plasmid and 3115 bp and 1347 bp for pAAV-mTTR-TALEN(R)-FokI poly A and pAAV-mTTR-TALEN(L)-FokI poly A indicates that the ITRs are intact.

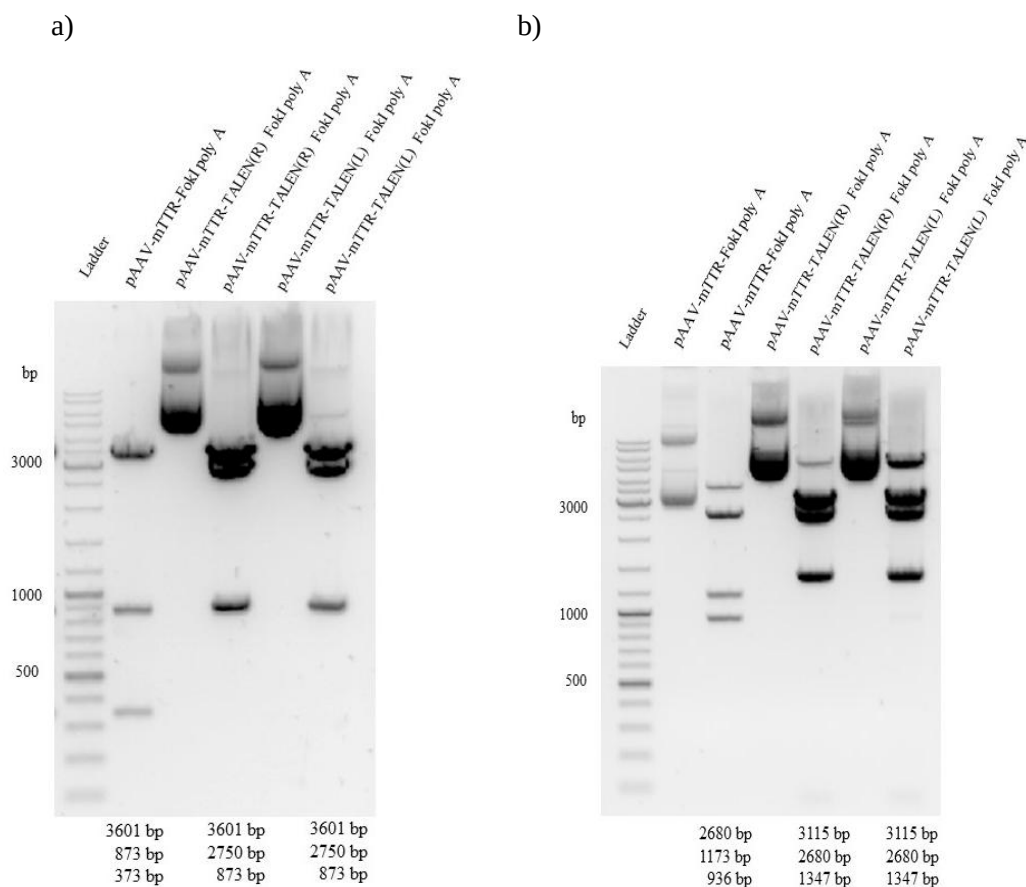


Figure 3.3.1: Generation of AAV plasmids expressing anti-HBV TALENs. a) Generation of pAAV-mTTR-TALE(R)-FokI poly A and pAAV-mTTR-TALE(L)-FokI poly A. Lane 1: O'gene ladder, Lane 2: *Bam* HI digested pAAV-mTTR-FokI poly A empty plasmid, Lane 3: Uncut pAAV-mTTR-TALEN(R)-FokI poly A, Lane 4: *Bam* HI digested pAAV-mTTR-TALEN(R)-FokI poly A, Lane 5: Uncut pAAV-mTTR-TALEN(L)-FokI poly A, Lane 6: *Bam* HI digested pAAV-mTTR-TALEN(L)-FokI poly A. b) Confirmation of ITR integrity in pAAV-mTTR-FokI poly A empty plasmid, pAAV-mTTR-TALE(R)-FokI poly A and pAAV-mTTR-TALE(L)-FokI poly A. Lane 1: O'gene ladder, Lane 2: Undigested pAAV-mTTR-FokI polyA empty plasmid, Lane 3: *Eco* 88I digested pAAV-mTTR-FokI polyA empty plasmid, Lane 4: Uncut pAAV-mTTR-TALEN(R)-FokI poly A, Lane 5: *Eco* 88I digested pAAV-mTTR-TALEN(R)-FokI poly A, Lane 6: Uncut pAAV-mTTR-TALEN(L)-FokI poly A, Lane 7: *Eco* 88I digested pAAV-mTTR-TALEN(L)-FokI poly A. Fragments were separated on a 1 agarose gel. Expected fragment sizes are indicated below the lanes.

As mentioned previously the right and left anti-HBV TALEN encoding sequences are similar except for the RVDs present at position 12 and 13. This makes it difficult to discriminate between the two in case they were mixed during handling. A digestion of the anti-HBV TALEN encoding sequence with *Bgl* I results in a distinct banding pattern between the right and left anti-HBV TALEN encoding sequence, which allows for discrimination between the two sequences. To distinguish the right and left anti-HBV TALEN encoding sequences from each other, the 2301 bp fragments containing only the TALEN encoding sequence were digested and purified out of pAAV-mTTR-TALE(R)-FokI poly A or pAAV-mTTR-TALE(L)-FokI poly A plasmids with restriction enzymes *Nhe* I and *Bam* HI (Fig 3.3.3 a). The purified fragments were digested with *Bgl* I and compared to the fragments purified from the previously generated pVAX-CMV-TALEN(R) or pVAX-CMV-TALEN(L). Same as the fragment obtained from pVAX-CMV-TALEN(R), the fragment purified from pAAV-mTTR-TALE(R)-FokI poly A gave the expected fragments of sizes 1100 bp, 710 bp and 610 bp after *Bgl* I digestion. Similarly, fragments from pAAV-mTTR-TALE(L)-FokI poly A and pVAX-CMV-TALEN(L) gave the same banding pattern of 1800 bp and 510 bp fragments detected on the gel (Fig 3.3.3 b). To further confirm that the RVD encoding sequences present in the right and left anti-HBV TALEN genes were different and comparable to that of the positive controls, sequencing was performed (Supp. Fig. 5-8). The right and left anti-HBV TALENs contain different nucleotide encoding RVDs that allow for discrimination between the two. It is difficult to sequence the entire anti-HBV TALEN gene due to the repetitive nature of the TALEN encoding sequence. Therefore, only the first 4 nucleotide encoding RVDs were sequenced. The nucleotide sequences obtained were converted into amino acids. As expected, the first 4 RVDs present in the right anti-HBV TALEN are: NI, HD, HD, NG and the first 4 RVDs present in the left anti-HBV TALEN are: HD, HD, NG, NK. Based on the results in (Fig 3.3.3) and the sequencing results, the right and left anti-HBV TALENs cloned into pAAV plasmid are correct and can be used for *in vitro* studies.

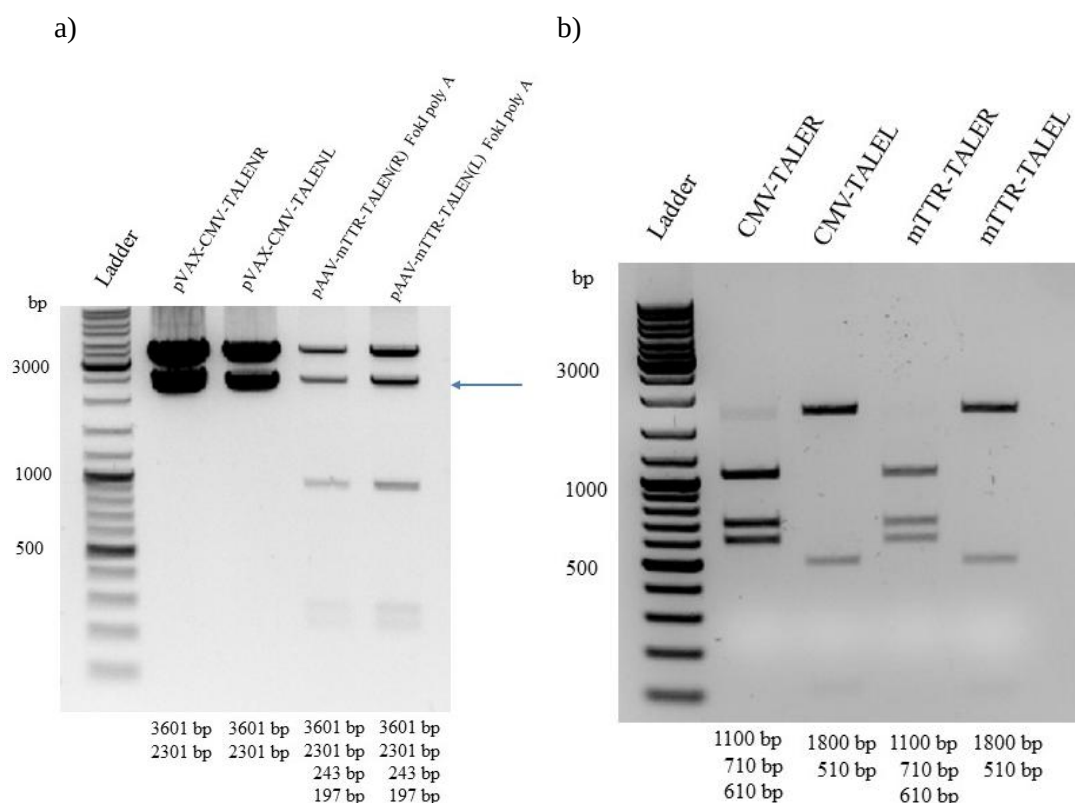


Figure 3.3.2: Enzymatic digestion of TALE encoding sequence to distinguish between the right and left anti-HBV TALE sequence. a) Purification of the right and left anti-HBV TALE containing fragments from pAAV plasmids or pVAX plasmids by *Nhe* I and *Bam* HI digestion. Lane 1: O'gene ladder, Lane 2: Digested pVAX-CMV-TALEN(R) Lane 3: Digested pVAX-CMV-TALEN(L), Lane 4: Digested pAAV-mTTR-TALEN(R)-FokI poly A Lane 5: Digested pAAV-mTTR-TALEN(L)-FokI poly A. b) Digestion of the 2301 bp extracted right and left TALE encoding sequences with *Bgl* I. Lane 1: CMV-TALER, Lane 2: CMV-TALEL, Lane 3: mTTR-TALER, Lane 4: mTTR-TALEL. Fragments were resolved on a 1 % agarose gel. Expected fragment sizes are indicated below the lanes.

3.4 Characterisation of AAV plasmids carrying anti-HBV TALENs encoding sequences in mammalian cell lines

3.4.1 Detection of anti HBV-TALENs expression from plasmids

To determine expression of the anti-HBV TALENs, Huh 7 cells were transfected with pAAV plasmid or pVAX plasmid carrying anti-HBV TALEN encoding sequences. Expression of the anti-HBV TALENs from pAAV plasmids were compared to those from pVAX plasmid, which served as a positive control. A transfection with pAAV-

Chapter 3- Results

CAG-GFP was performed to ensure transfection of the cells was successful. All Transfections were performed in quadruplicate to control for variability.

Immunofluorescence detection was performed using a monoclonal antibody that bound to the hemagglutinin epitope (HA) tag linked to the TALEN encoding sequence. A fluorescently labelled secondary antibody was added to allow for the detection of the HA tag and DAPI was used to stain the nucleus. Transfection of the Huh 7 cells were successful as GFP was detected (Supp. Fig. 9). The faint green observed in cells transfected with pAAV-mTTR-FokI poly A represent a non-specific background, hence there was no detection of HA tag as expected. The DAPI staining images are different between the single-channel and merged images as the exposure had to be adjusted for each image to achieve the desirable outcome (Fig 3.4.1). The HA tag was detected from cells transfected with either pAAV plasmid or pVAX positive control plasmid expressing the anti-HBV TALEN. In our hands the left anti-HBV TALENs consistently expressed greater than the right anti-HBV TALENs, so the exposure between the images are different. However, these results indicate that the pAAV plasmids constructed are able to express anti-HBV TALENs.

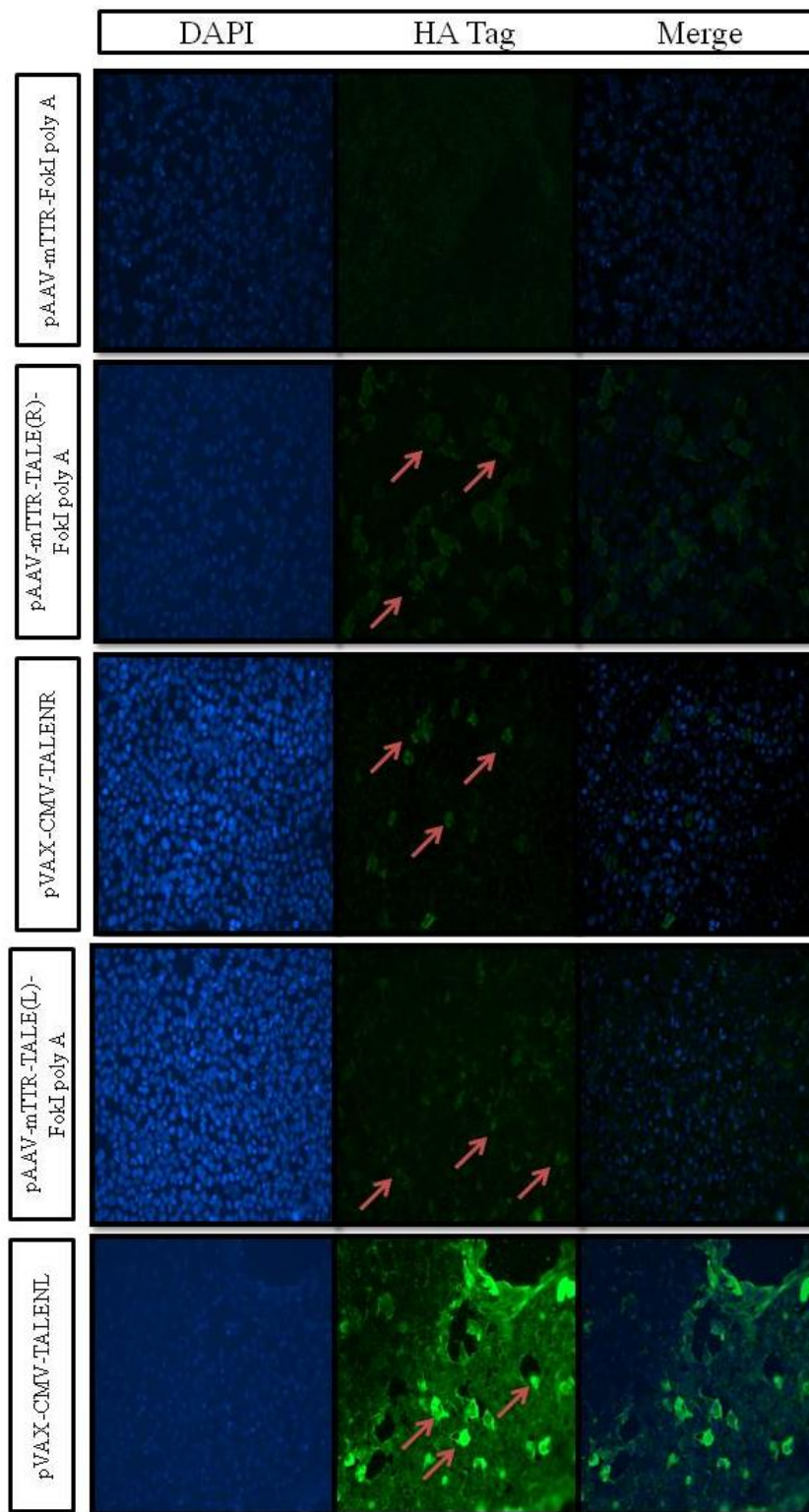


Figure 3.4.1: Detection of plasmid derived anti-HBV TALENs in Huh 7 cells. Immunofluorescence detection of the anti-HBV TALENs performed in Huh 7 cells. Huh 7 cells were transfected with pAAV/pVAX plasmid containing the right or left anti-HBV TALEN gene. DAPI was used to stain the nucleus. Cells were visualized using a fluorescence

microscope at 20 × magnification. Images depicted above are a representation of several images from different fields and wells. Red arrows indicate cells containing the HA tag.

3.4.2 HBsAg gene silencing in Huh 7 cells by anti-HBV TALENs expressed from plasmids

Once expression of the anti-HBV TALENs was detected using plasmids, HBsAg levels were assessed in Huh 7 cells following co-transfection with pcH-9/3091, a HBV replication competent plasmid and pAAV-mTTR-TALEN or pVAX-CMV-TALEN or pAAV-mTTR-FokI poly A. A GFP expressing plasmid was included as the third plasmid to assess transfection efficiency. The pVAX-CMV-TALENR and pVAX-CMV-TALENL transfection served as the positive control, whereas the pAAV-mTTR-FokI poly A plasmid transfection served as the negative control. Transfections were performed in quadruplicate to control for variability. Transfection efficiency was evaluated visually 48 hours post transfection and compared across the reactions, however absolute quantification was not performed (Supp. Fig. 10). Medium was collected on day 2, day 3 and day 4 post transfection and analysed for HBsAg levels.

The pAAV-mTTR-TALENs were able to achieve about 60 % ($p=0.005$, 3-fold change) gene silencing at day 2 and this was increased to 80 % ($p=0.0002$, 5-fold change) on day 3 and remained the same at day 4 ($p=0.0003$, 5-fold change). This HBsAg reduction was comparable to those obtained from cells transfected with pVAX-CMV-TALEN positive control plasmids (Fig 3.4.2). This indicates that the pAAV carrying the anti-HBV TALENs are capable of targeting the S ORF of the HBV genome.

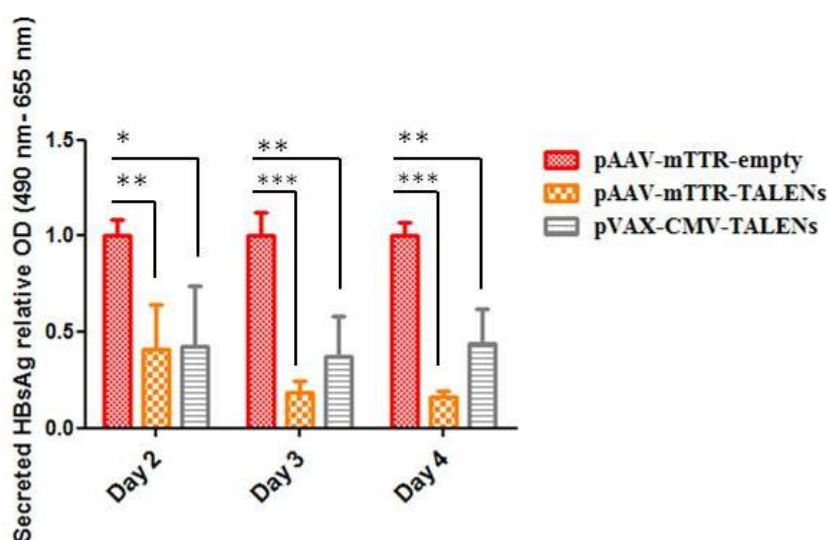


Figure 3.4.2: Assessment of plasmid derived TALEN-mediated knockdown of HBsAg in Huh 7 cells. To assess anti-HBV TALEN efficiency, Huh 7 cells were co-transfected with pCH-9/3091, pAAV-CAG-GFP and either pVAX-CMV-TALEN plasmids or pAAV-mTTR-TALEN plasmids or pAAV-mTTR-FokI poly A. HBsAg levels in the supernatants were measured two, three- and four-days post-transfection. Transfections were performed in quadruplicate. Statistical significance was determined by paired two tailed T-test: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ ns- non-significant. Error bars indicate the standard error of the mean (SEM, $n=4$).

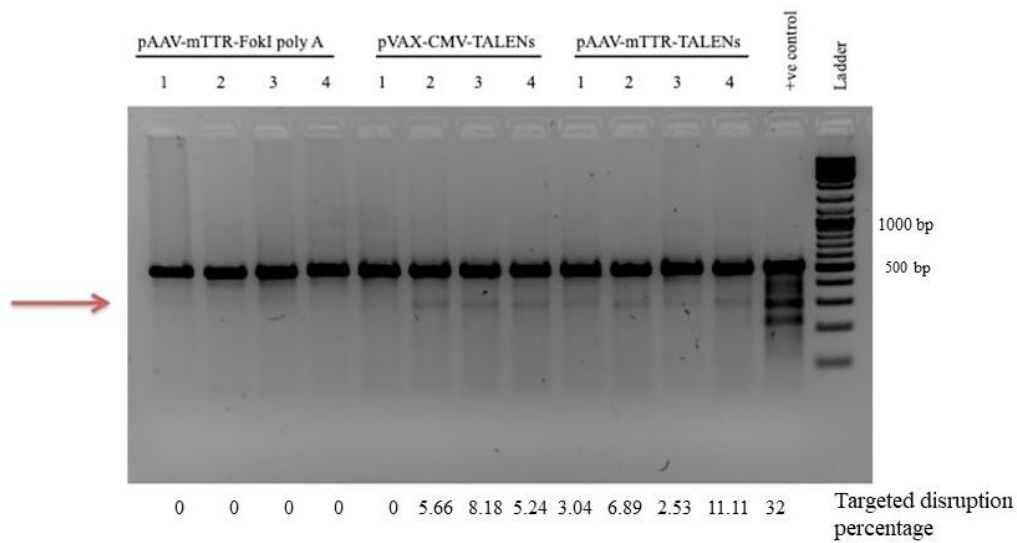
3.4.3 Anti-HBV TALEN mediated on target cleavage of HBV DNA

To assess the efficacy of anti-HBV TALENs in targeted disruption of viral DNA, HepG 2.2.15 cells were used. These cells contain a stable integrated HBV sequence that constitutively maintains HBV viral replication. The HepG 2.2.15 cells were transfected with pAAV-mTTR-TALENs or pVAX-CMV-TALENs, which served as the positive control or pAAV-mTTR-FokI poly A, which served as a negative control. A transfection with pAAV-CAG-GFP was done to evaluate transfection efficiency visually and compared across the reactions, however absolute quantification was not performed (Supp. Fig. 11). Transfections were performed in quadruplicate to control for variability. To examine targeted mutagenesis mediated by anti-HBV TALENs, total DNA was extracted from the triple transfected HepG 2.2.15 cells. The DNA extracted was PCR amplified using primers that amplify the S ORF (Table 8) to produce a 499 bp fragment. A PCR band at 499 bp was generated for pAAV-mTTR-TALENs, pAAV-mTTR-FokI poly A and pVAX-CMV-TALENs transfections, which were of the expected fragment size (Supp. Fig. 12). Site-specific TALEN-mediated cleavage

was assessed using a T7E1 assay. The T7-treated samples were resolved on a 2 % agarose gel and the density of the separated bands were calculated to determine the percentage of targeted disruption. A positive control for targeted disruption was generated by mixing wild-type and mutant PCR amplicons of HBx sequences in equal amounts before denaturation.

If the anti-HBV TALENs had caused any mutagenesis at the target site, the T7 enzyme will cleave the annealed amplicon into two smaller DNA products of similar size, appearing as a single band (Fig 3.4.3 a). The T7E1 assay indicates that the cells transfected with pAAV-mTTR-FokI poly A did not demonstrate any cleavage as a single band at 499 bp was visible indicating the presence of homoduplexes of DNA. The positive control demonstrated cleavage activity of 32 % as there were three bands present representing uncleaved and two smaller cleavage products. This indicates that the T7E1 enzyme is working as expected but lower than 50 % cleavage activity was obtained. The pVAX-CMV-TALENs and pAAV-mTTR-TALENs were able to cause targeted mutagenesis at the target site. This is shown by the presence of a band smaller than 499 bp representing two cleavage products of smaller size. The pVAX-CMV-TALENs and pAAV-mTTR-TALENs demonstrated comparable cleavage activity. The average cleavage ability of the pVAX-CMV-TALENs is 4.77 % and 5.89 % for the pAAV-mTTR-TALENs. The data indicates that the pAAV-mTTR-TALENs are causing cleavage at the intended target sites and are working as they are meant to. The lower than expected T7E1 cleavage activity of pVAX-CMV-TALENs and pAAV-mTTR-TALENs is likely due to low transfection efficiency. The HepG 2.2.15 cells are difficult to transfect and require multiple transfections to obtain the desired outcome. No T7E1 reactions did not show any cleavage as expected (Figure 3.4.3.b). This data correlates with the decrease in HBsAg levels demonstrated by the pAAV-mTTR-TALENs and the pVAX-CMV-TALENs.

a)



b)

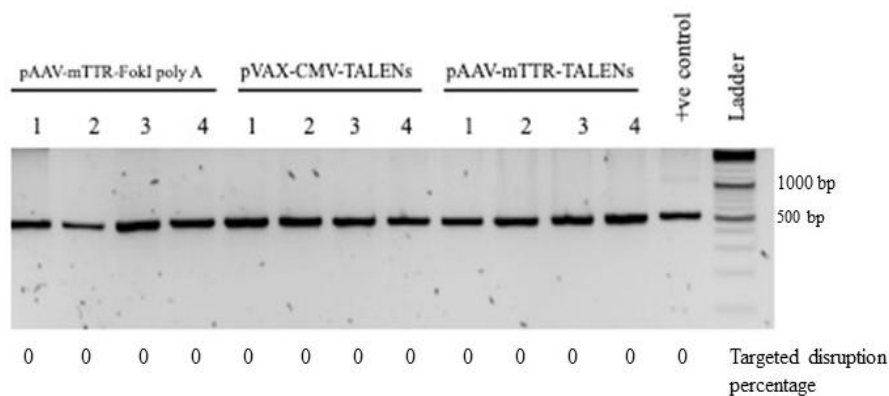


Figure 3.4.3: Anti-HBV TALEN mediated HBV genome cleavage in HepG 2.2.15 cells. Total DNA was extracted from HepG 2.2.15 cells that were transfected in quadruplicate. PCR amplification of S TALEN target sites was carried out and a T7E1 assay was conducted on each of the samples. Both T7 treated **(a)** and untreated **(b)** amplicons were resolved on a 2 % agarose gel and the O'Gene ladder was loaded to determine the fragment sizes. Arrow shows the cleavage product. Percentage of targeted disruption obtained from each transfection is indicated below the lanes.

3.4.4 Viability of Huh 7 cells transfected with plasmids expressing anti-HBV TALENs

To ensure that the anti-HBV TALEN mediated inhibition of HBsAg levels and cleavage were as a result of the TALENs and not toxicity to the cells, an MTT assay was performed. This assay works on the ability of viable cells with an active metabolism to convert MTT into a purple formazan product that can be detected at an absorbance reading of 570 nm. Huh 7 cells were co-transfected with pCH-9/3091, pAAV-CAG-GFP and pAAV-mTTR-TALENs or pVAX-CMV-TALENs or pAAV-mTTR-FokI poly A. The cells were treated with 50 % DMSO, which served as a positive control for toxicity and cells that were untreated, served as a negative control for toxicity. Target plasmid pCH-9/3091 was included to assess TALEN activity by HBsAg ELISA (data not shown). To control for variability, transfections were performed in quadruplicate.

Treatment of cells with DMSO indicated great levels of toxicity as compared to the untreated cells [(p = 0.05, 5-fold change) (Fig 3.4.4)]. The pAAV-mTTR-TALENs and pVAX-CMV-TALENs are comparable to the pAAV-mTTR-FokI poly A. The pAAV-mTTR-FokI poly A is comparable to the untreated control. This indicates that the anti-HBV TALENs are not toxic to the cells and any decrease in HBsAg levels and cleavage is as a result of the HBV genome cleavage by anti-HBV TALENs.

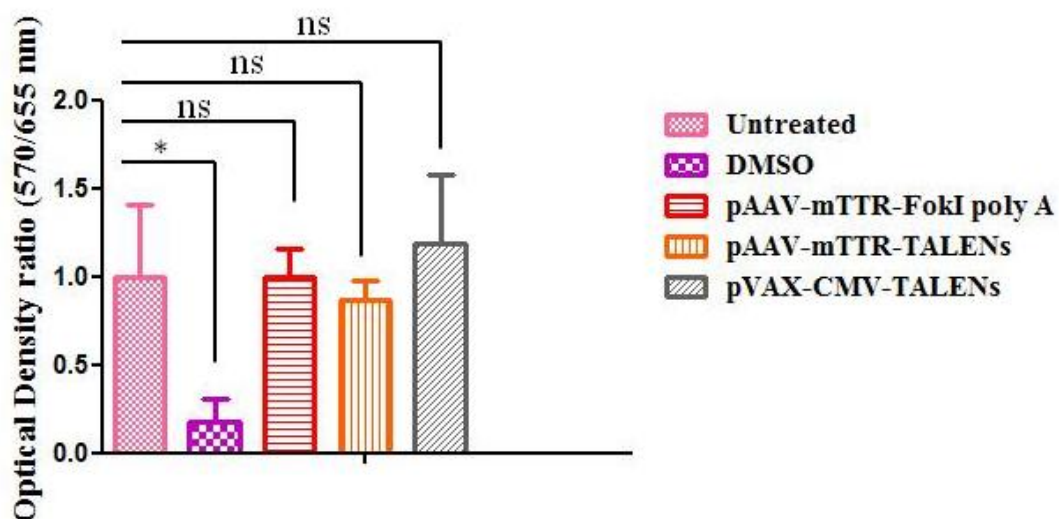


Figure 3.4.4: Assessment of anti HBV TALENs toxicity in Huh 7 cells. To assess potential toxicity of anti HBV TALENs, an MTT assay was conducted 48 hours after treatment of Huh 7 cells with pAAV-mTTR-FokI poly A or pAAV-mTTR-TALENs or pVAX-CMV-TALENs or DMSO.

Reactions were performed in quadruplicate. Two tailed, paired T-test was used to determine statistical significance: *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ ns- non-significant. Error bars indicate the Standard error of the mean (SEM) (n=4).

3.5 **Production and characterisation of anti-HBV TALEN expressing AAVs**

3.5.1 AAV production, purification and quantification

The characterization of the pAAV plasmids constructed demonstrated that they are fully functional and can be used to produce AAVs. Hence, the pAAV right and left anti-HBV TALEN encoding sequences were packaged into an AAV-2 capsid to generate AAV-mTTR-TALENR and AAV-mTTR-TALENL respectively. To serve as a negative control, the TALE deficient sequence from pAAV-mTTR-FokI poly A was also packaged into an AAV-2 capsid to generate AAV-mTTR-empty. The AAV expressing luciferase from mTTR promoter or GFP from a chicken beta actin promoter was also produced using pAAV-mTTR-FluC or pAAV-CAG-GFP plasmid for easy assessment of transgene expression and transduction efficiency respectively. The isolated viral DNA from the viral vectors generated were quantified using qPCR with mTTR promoter specific primers (Table 4). Standards were setup with dilutions from pAAV-mTTR-TALEN(R)-FokI poly A as it contained the mTTR promoter. The viral titers obtained for each viral vector are depicted in Table 9. The expected titers from viral vector preparation are around 10^{13} copies/ml. The average titers obtained for the majority of the vectors were around $4.01 \times 10^{11} - 3.65 \times 10^{12}$ copies/ml with only AAV-mTTR-FluC giving 2.08×10^{13} copies/ml, hence an MOI (1×10^5), which is 10-fold lower than the optimal MOI (1×10^6) was used for all the experiments. This is due to viral titers obtained from viral production being too low to yield enough viral particles for the optimal MOI. Multiple viral productions were performed but this did not help as undiluted virus suspension in idioxanol is toxic to the cells.

Table 9: Viral vector titers of AAVs stocks used.

Viral Vector	Plasmids used	Viral stock number	Titers per ml	Average titers per ml
AAV-mTTR-TALENR	pAAV-mTTR-TALEN(R)-FokI-poly A pAAV2 pAd helper	1	2.89×10^{11}	4.01×10^{11}
		2	3.2×10^{11}	
		3	5.95×10^{11}	
AAV-mTTR-TALENL	pAAV-mTTR-TALEN(L)-FokI-poly A pAAV2 pAd helper	1	4.09×10^{10}	5.05×10^{11}
		2	4.76×10^{11}	
		3	1.01×10^{12}	
AAV-mTTR-empty	pAAV-mTTR-FokI poly A pAAV2 pAd helper	1	3.51×10^{11}	9.755×10^{11}
		2	1.6×10^{12}	
AAV-mTTR-FluC	pAAV-mTTR-FluC pAAV2 pAd helper	1	2.08×10^{13}	2.08×10^{13}
AAV-mTTR-GFP	pAAV-CAG-GFP pAAV2 pAd helper	1	3.65×10^{12}	3.65×10^{12}

3.5.2 Luciferase expression from AAVs

A luciferase assay was conducted to determine if the AAV vectors produced can express their transgenes under the regulation of the introduced mTTR promoter. Huh 7 cells were transduced at an MOI of 1×10^5 with AAV-mTTR-FluC. After 48 hours, cell lysates were used to measure the luciferase activity.

A significant expression of FluC was observed with AAV-mTTR-FluC ($p= 0.006$) relative to the uninfected cells (Fig 3.5.2). These findings suggest that the vectors produced can express transgenes under the regulation of the mTTR promoter sequence introduced.

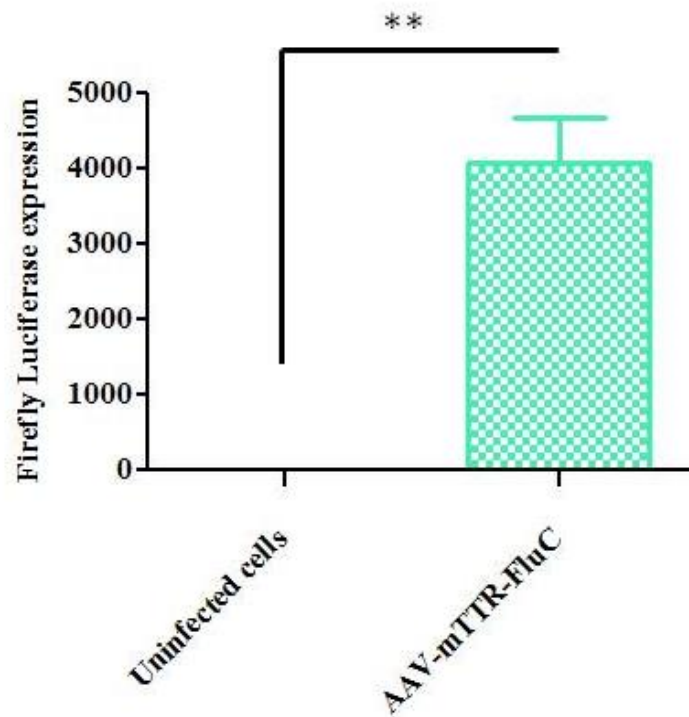


Figure 3.5.2: AAV mediated Firefly luciferase expression under the control of an mTTR promoter. Huh 7 cells were transduced with AAV-mTTR-FluC. A luciferase assay was performed 48 hours post transduction. Two tailed paired T-test was used to determine statistical significance: ** indicates significant FluC gene expression with $p = 0.006$. Error bar indicates the normalized Standard error of the mean (SEM, $n=4$).

3.5.3 Viability of Huh 7 cells transduced with AAVs expressing anti-HBV TALENs

As low viral vector titers were obtained and the fact that both left and right TALEN encoding AAVs had to be combined, a vector volume higher than 10 % of the culture media was used to infect the cells. Hence, to determine whether this high volume of 40 % iodixanol in which the vectors are suspended in or whether the viral vectors generated resulted in toxicity to the cells, an MTT assay was performed. To confirm expression of the anti-HBV TALENs from the viral vectors and to ensure that transfection was successful, pCH-9/3091 and pAAV CAG GFP were co-transfected 24 hours prior to the transduction of cells. Cells were transduced at an MOI of 1×10^5 with AAV-mTTR-TALENR and AAV-mTTR-TALENL or AAV-mTTR-Empty. Uninfected cells served as a negative control. DMSO was utilized as a positive control for toxicity. To test iodixanol toxicity, cells were also treated with 40 % iodixanol. To ensure reproducibility transductions were performed in quadruplicate.

A significant decrease ($p = 0.008$, 10-fold change) in the viability of cells were noticed when cells were treated with DMSO. There was also a decrease in the viability of cells when the cells were infected with AAV-mTTR-TALENs ($p = 0.006$, 3-fold change). However, this was similar to cells treated with 40 % iodixanol only ($p = 0.002$, 2-fold change). Showing that the toxicity was as a result of iodixanol and not the vectors. Hence, to account for this, the volume of the 40 % iodixanol in all the viral suspensions used was kept the same in all the experiments. The AAV-mTTR-TALENs gave comparable results to the AAV-mTTR-empty vector. Showing that the cell viability reduction observed is not as a result of anti-HBV TALEN expression. Overall, this indicates that the viral vectors generated are not toxic to the cells and any therapeutic benefits associated with these viral vectors are as a result of the anti-HBV TALENs (Fig 3.5.3).

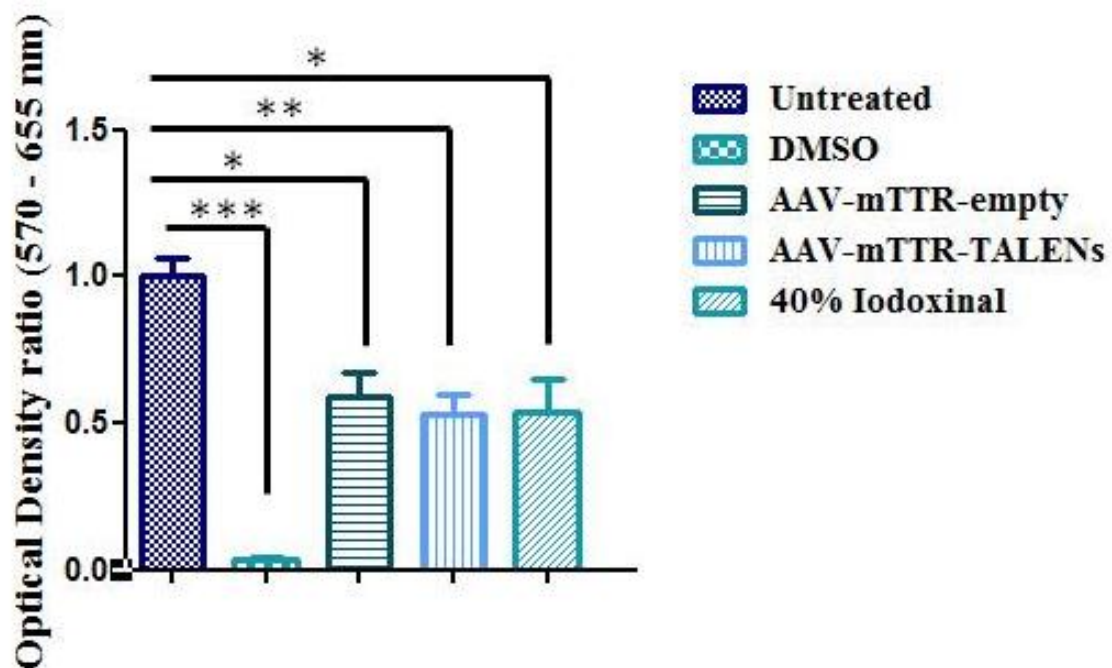


Figure 3.5.3: Assessment of cell viability following transduction with anti-HBV TALENs expressing viral vectors. Huh 7 cells were co-transfected with pCH-9/3091 and pAAV-CAG-GFP 24 hours pre-transduction. Cells were then transduced with AAV-mTTR-empty vector or AAV-mTTR-TALENs. Untreated cells served as a negative control for toxicity, whereas cells treated with DMSO served as a positive control for toxicity. A treatment of cells with 40 % iodixanol was used to determine if the 40 % iodixanol contributed to the toxicity of cells. Transfections and transductions were performed in quadruplicate. Two tailed paired T-test was used to determine statistical significance; *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ ns- non-significant. Error bars represented as Standard error of the mean (SEM, $n=4$).

3.5.4 Assessment of AAV derived anti-HBV TALEN expression, HBsAg gene silencing and on-target cleavage of HBV DNA

To assess HBV gene expression inhibition by AAVs, a co-transfection with pCH-9/3091 and pAAV CAG-GFP was performed 5 hours prior to cell transduction with AAVs. GFP expression was assessed 24 hours post transfection visually and compared across the reactions, however absolute quantification was not performed (Supp. Fig. 13). Transduction with AAV-mTTR-empty was included as a negative control. AAV-CAG-GFP was used separately to determine the transduction efficiency at 48 hours post-transduction (Supp. Fig 14). Based on the toxicity that the volume of 40 % iodixanol contributes to cells, the volume of 40 % iodixanol was kept the same when the cells were transduced with AAV-mTTR-TALENs or AAV-mTTR-empty. Transductions were performed in triplicate to ensure reproducibility. Media was collected on day 2, day 3 and day 4 post-transduction and analysed for HBsAg levels.

As compared to empty vector, the AAV-mTTR-TALENs were able to significantly decrease HBsAg levels at 2 days post transduction by 55 % [(Fig 3.5.4 a) ($p = 0.006$, 2-fold change)]. However, a significant increase in HBsAg levels were observed at day 3 ($p = 0.13$, 1-fold change) and day 4 ($p = 0.3$, 1-fold change) as compared to day 2 for AAV-mTTR-TALENs. Due to the lower MOI used, this might have been as a result of gene expression from the dominant untargeted HBV genome. This was supported by lack of immunocytochemical HA tag staining in Huh 7 cells (Supp. Fig. 16) and cleavage activity below detection by T7E1 assay with DNA isolated from Hep G 2.2.15 cells (Supp. Fig. 15) transduced at MOI of 1×10^5 . AAVs tend to work better *in vivo* than *in vitro*. Hence *in vivo* studies will be carried out in the future.

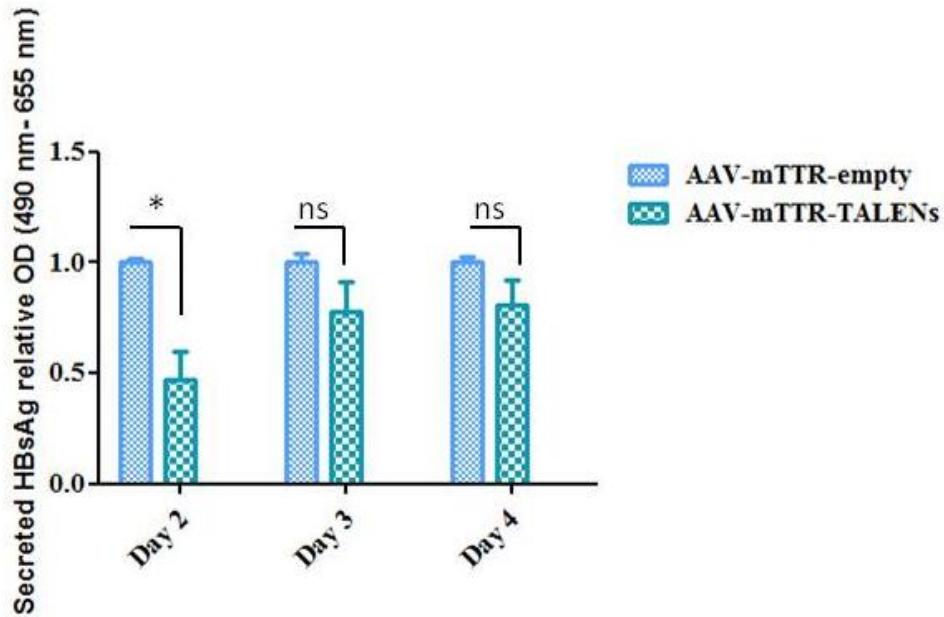


Figure 3.5.4: Assessment of viral vector derived TALEN-mediated knockdown of HBsAg expression in Huh 7 cells. To assess anti-HBV TALEN efficiency, huh 7 cells were co-transfected with pCH-9/3091 and pAAV-CAG-GFP 5 hours pre-transduction of cells with viral vectors AAV-mTTR-TALENs. A transduction with AAV-mTTR-empty was done as a negative control. HBsAg levels in the supernatants were measured two, three- and four-days post-transduction. Transductions were performed in triplicate. Two tailed paired T- test was used to determine significance, * $P \leq 0.05$ ns- non-significant, Error bars represented as Standard error of the mean (SEM, $n=4$).

Chapter 4

Discussion

Hepatitis B infection is a global health problem infecting approximately 2 billion people worldwide, with around 240 million chronic carriers (WHO, 2017). Valuable progress has been made in vaccine and antiviral development, but no reliable cure exists for the treatment of HBV infection. The inability to achieve a functional cure for chronic HBV infection is the persistence of cccDNA and the failure of current antiviral therapy to eliminate it (Allweiss and Dandri, 2017). Gene editing has recently shown promise as a means of inactivating cccDNA to achieve a cure [reviewed in (Ely et al., 2016)]. The goal of using engineered nucleases for the treatment of HBV infection, is that the cleavage of the viral DNA will result in mutations, as it is repaired by the error prone NHEJ repair pathway. Anti-HBV TALENs targeting the S, C, P and X ORFs of the HBV genome have successfully been designed within the AGTRU. These anti-HBV TALENs demonstrated optimal cleavage activity in the Hep G 2.2.15 cell line, without any measurable cytotoxicity (Bloom et al., 2013). The lack of a safe and efficient delivery system for the anti-HBV TALENs remains the obstacles for the translation of this therapeutic into clinics. This study focused on achieving liver specific expression of the anti-HBV TALENs and obtaining a safe delivery system.

4.1 Successful construction of pAAV plasmids containing anti-HBV TALENs

As HBV infects the liver, optimal expression of the therapeutic transgene (anti-HBV TALENs) within the liver is required for targeting HBV. The use of liver specific promoters such as mTTR, has been shown to improve the expression of anti-HBV sequences and result in silencing of HBV gene expression. Replacement of a CMV promoter with an mTTR promoter in Helper dependent Adenovirus (HDAd) carrying anti-HBV pri-miR, resulted in expression and processing of anti-HBV pri-miR being more sustained (Mowa et al., 2014a). An mTTR promoter has been used to test the expression of anti-HBV pri-miR but the use of this promoter to express anti-HBV TALENs has not been investigated. After cloning the mTTR promoter and FokI poly A sequence, the activity of the mTTR promoter in Huh 7 cells were tested using a luciferase assay. A significant expression ($p= 0.04$) of the Firefly luciferase was observed (Fig 3.2). The results confirmed that the mTTR promoter is active and can be used to drive the expression of anti-HBV TALENs. Following the insertion of the right and left anti-HBV TALENs into an pAAV plasmid, their expression in Huh 7 cells were

determined by detection of the HA tag present in the TALE sequence (Fig 3.4.1). The right and left anti-HBV TALENs were detected in Huh7 cells. These results are comparable with the right and left anti-HBV TALENs sequences present in pVAX plasmid (Bloom et al., 2013). When the functionality of plasmid derived anti-HBV TALENs was investigated *in vitro*, about 80 % gene silencing (Fig 3.4.2) and an average of 5.89 % target cleavage (Fig 3.4.3) was observed. The HBsAg gene silencing and cleavage activity was comparable to the pVAX control. However, the cleavage activity observed here was lower than that observed by Bloom et. al, as the cleavage obtained when using pVAX plasmids resulted in 35 % targeted cleavage (Bloom et al 2013). As the targeted cleavage percentage in the T7E1 cleavage control was lower than the expected 50 %, the overall lower cleavage percentages observed might have been as a result of lower T7E1 cleavage activity. Despite the lower cleavage activity observed, the results indicate that the right and left anti-HBV TALENs have successfully been cloned into an AAV plasmid, serving their intended function and these plasmids can be used for the production of viral vectors

4.2 AAV mediated delivery of anti-HBV TALENs

One of the crucial aspects in attaining successful gene therapy is categorized into efficiency of transport, unloading into target cells, activity, immune activation, regulatory concerns and commercialization (Ramamoorth and Narvekar, 2015). Despite the availability of effective anti-HBV TALENs, gene editing technologies are hampered by difficulties with adequate delivery efficiency. Viral vectors are optimal vehicles for gene transfer because of their ability to efficiently transduce host cells. The removal of the replicative and pathogenic ability of viruses and their ability to carry their transgene make them suitable as gene delivery vehicles (Nayak and Herzog, 2010). AAVs are the preferred method for delivering genes to target cells due to mild immune response induced, non-pathogenicity of the wild type virus and ability to transduce a broad range of cells. AAVs are promising vectors for many applications of gene therapy, but a drawback of these vectors is the limited size of the transgenes they can accommodate. DNA components encoding dimeric anti-HBV TALENs exceed the packaging capacity of 4.5 kb. As a result, two vectors are required to deliver sequences that express a complete anti-HBV TALEN dimer. AAVs containing anti-HBV TALENs were successfully produced in this study (Table 9). Quantification by qPCR indicated that the total viral particles of the most purified viruses were around 4.01×10^{11} – 3.65×10^{12} copies/ ml. This is lower than the expected viral titers of 10^{13} copies/ ml.

The possible reasons resulting in the lower viral titers obtained could be due to the following: low viability of Hek 293 T cells following transfection with the different rAAV plasmids and the 5× PEG utilized was not of molecular biology grade and could have played a major role during the precipitation process. No reports exist on the ability of AAVs expressing anti-HBV TALENs. Anti-HBV TALENs could contribute to the low viability of cells, which resulted in lower than expected viral titer. However, all these factors remain to be investigated. The results obtained were enough to carry out further characterizations but limited the MOI to 1×10^5 that could be used to transduce cells. Confirming the activity of the mTTR sequence cloned, transduction of Huh 7 cells with AAV expressing luciferase resulted in a significant amount of firefly luciferase expression (Fig 3.5.2). The construction of viral vectors containing firefly luciferase is key for the assessment of transgene expression *in vivo* by bioluminescence imaging for long term studies. This makes it unnecessary to kill animals at each timepoint, reducing the requirement of animals needed for the study (Zincarelli et al., 2008).

AAV-mTTR-TALENs, were able to achieve about 55 % HBsAg gene silencing at 2 days post transduction. However, on day 3 and day 4 post-transduction, HBsAg levels significantly increased. Also, no TALEN expression (Supp. Fig 16) or targeted mutagenesis (Supp. Fig 15) was observed when using AAV-mTTR-TALENs. Possible reasons for these observations could be that because of the lower MOI used the population of cells that have been targeted by anti-HBV TALENs are smaller than those that have not been targeted. Thus, the cleavage is below detection by T7E1 assay and gene expression from larger untargeted population outnumber the targeted population. The possibility of one viral vector containing only either a right or left anti-HBV TALEN entering the cell may also limit the cleavage mediated mutagenesis. To assess if the latter is the case, two vectors carrying different reporter genes e.g. GFP and mCherry can be used to transduce cells simultaneously followed by assessment of transgene expression by Fluorescence imaging or Flow cytometry in the future. In our hands AAVs are usually effective *in vivo* as compared to *in vitro* studies. This was observed in Mowa, et al, where greater gene-silencing was observed *in vivo* when using AAVs containing pri-miR (Mowa et al., 2014b). Hence *in vivo* studies will be conducted in the future to further characterize the viral vectors produced.

All together these results indicate that while the pAAV containing the anti-HBV TALENs work as expected, the delivery of these anti-HBV TALENs using AAVs require further optimization.

4.3 Safety of anti-HBV TALENs

One of the issues surrounding the utilization of engineered sequence specific nucleases is the potential to cleave non-specifically (Stone et al., 2016). Unintended cleavage by these nucleases is seen as a major contributor to cell toxicity. Cleavage at unintended sites within the genome may lead to genome instability, unwanted chromosomal rearrangements and cell death (Scott et al., 2014). A high level of specificity is a crucial feature required by these sequence specific nucleases. TALEN specificity and versatility is mediated by the well-characterised DNA-binding domain that can be engineered to target a wide range of DNA sequences (Miller et al., 2007) whereas the mutagenic efficiency of TALENs is dependent on catalytic activity of the FokI nuclease domain (Bibikova et al., 2001). TALENs are unique in that they require dimerization of the right and left FokI nuclease domain to fuse, for cleavage at the target site to occur. With the incorporation of the mTTR promoter, expression of the transgene should only occur in the liver. This aids in reducing the off-target expression of the anti-HBV TALENs.

Existing data does not suggest that the TALENs used in this study are associated with any toxicity to cells (Bloom et al., 2013). This correlates with results obtained in this work. The pAAV-mTTR-TALENs did not demonstrate any toxicity to cells after an MTT assay was conducted (Fig 3.4.4). This indicates that any therapeutic benefit associated with the anti-HBV TALENs are not as a result of cell death. When viral vectors were used, cell toxicity was observed. However, this was as a result of 40 % iodixanol in which the viral vectors are suspended in (Fig 3.5.3). The iodixanol toxicity comes from the fact that low viral titers were obtained during viral purification (Table 9), which resulted in larger volumes of viral vectors (higher than 10 % media) being utilized. To account for the cytotoxic effects associated with the 40 % iodixanol, the volume of 40% iodixanol was kept consistent when using the different viral vectors to transduce cells. Administration of viral vectors can lead to the activation of the innate and adaptive immune response against the viral particles or gene products and result in toxicity (Nayak and Herzog, 2010). However, AAVs are a weak immunogen as microarray studies have shown that AAV does not elicit strong type 1 IFN

responses as seen with Ads vectors (McCaffrey et al., 2008). This study supports the lack of AAV toxicity as all vectors used were not different to 40 % iodixanol only treated cells in the MTT assay.

Conclusion

Developing a curative approach to target HBV cccDNA is crucial in overcoming the battle to combat chronic HBV infection. Current treatments are ineffective as they are incapable of permanently disrupting cccDNA. This dissertation is the first to report on the incorporation of the anti-HBV TALENs into an AAV-2 capsid, under the control of a liver specific promoter. The anti-HBV TALENs targeted the S ORF of the HBV genome. The anti-HBV TALENs as a plasmid or viral vector mediated inhibition of HBsAg *in vitro* with minimal toxicity observed. Targeted mutagenesis was observed when using anti-HBV TALENs as a plasmid in HepG 2.2.15 cell line, indicating that the anti-HBV TALENs functioned as expected. Further investigation of the viral vectors is required to achieve the optimal results. Nevertheless, this study has identified AAV-2 viral vectors as promising delivery tools for anti-HBV TALENs delivery.

Future work

The next step of the study would be the incorporation of the anti-HBV TALENs that target the C ORF of the HBV genome into AAV-2. The experiments performed in this study will be done to determine the efficiency, safety and effectiveness of the core anti-HBV TALENs as plasmids and viral vectors. Once the core anti-HBV TALENs have been well characterized *in vitro*, *in vivo* work will be carried out in transgenic mice. A well-suited vector for *in vivo* studies (AAV-8) that target the S and C ORF of the HBV genome will be produced and characterised *in vivo*. The efficiency and safety of the viral vectors will be tested short term (1 month) and long term (~ 1 year) *in vivo* studies.

Chapter 5

Appendix

5. 1 Bacterial culture solutions and recipes

5.1.1 Luria Bertani (LB) medium

Ten grams of Bacto-tryptone, 5 g yeast extract and 5 g NaCl were dissolved in a litre of distilled water. The medium was autoclaved for 20 minutes at 121°C and 1kg/cm².

5.1.2 Luria Bertani agar (LA) medium

Ten grams of Bacto-tryptone, 5 g yeast extract, 5 g NaCl and 10 g Agar was added to a litre of distilled water. The medium was autoclaved for 20 minutes at 121°C and 1kg/cm². The medium was cooled to approximately 55°C and the desired antibiotic (100 µg/ml ampicillin or 50 µg/ml kanamycin) was added. The medium was poured into petri dishes and allowed to set.

5.1.3 Ampicillin stock (100 µg/ml)

A gram of ampicillin (ParsReac Applichem Inc, MO, USA) was weighed out and added to 3 ml of sterilized dH₂O and 7 ml of 100 % ethanol. The solution was filter sterilized and stored at -20°C.

5.1.4 Kanamycin stock (50 µg/ml)

Kanamycin (0.25 g) was weighed out and added to 5 ml of sterilized dH₂O. The solution was filter sterilized. Due to kanamycin being light sensitive, foil was used to cover the aliquots and stored at -20°C.

5.1.5 Transformation buffer

A 100 ml stock solution was prepared as follows: 100 mM CaCl₂ (1.47 g), 10 mM Pipes and 15 % glycerol (15 ml). The pH was adjusted to 7.0 using NaOH. Sterilized dH₂O

was added to maintain a volume of 100 ml. The solution was autoclaved for 20 minutes at 121°C and 1kg/cm². The transformation buffer was stored at 4°C until needed.

5.2 Plasmid DNA isolation buffers

5.2.1 Solution 1 (Re-suspension solution)

A litre of re-suspension solution was prepared by adding 6.06 g Tris Base, 3.72 g EDTA and 800 ml dH₂O. The pH was adjusted to 8.0 using NaOH. The volume was adjusted to a litre using dH₂O. The solution was autoclaved for 20 minutes at 121°C and 1kg/cm². The solution was allowed to cool before 100 mg RNaseA was added. The solution was stored at 4°C.

5.2.2 Solution 2 (Lysis solution)

One litre of lysis solution was prepared by adding 8.0 g NaOH pellets, 500 ml dH₂O and 10 g SDS. The volume was adjusted to a litre. The solution was autoclaved for 20 minutes at 121°C and 1kg/cm² and stored at room temperature.

5.2.3 Solution 3 (Neutralizing solution)

One litre of neutralizing solution was prepared by adding 294.5 g potassium acetate and 500 ml dH₂O. The pH was adjusted to 5.5 with glacial acetic acid and adjusted to 1 litre with dH₂O. The solution was autoclaved for 20 minutes at 121°C and 1kg/cm² and stored at 4°C.

5.2.4 QBT buffer (Equilibration buffer)

One litre of equilibration buffer was prepared by adding 43.83 g NaCl and 10.46 g MOPS (free acid) in 800 ml dH₂O. The pH was adjusted to 7.0 using NaOH. A 150 ml IsoH and 15 ml 10 % Triton-X-100 solution were added. The volume was adjusted to 1L using dH₂O.

5.2.5 QC buffer (Wash buffer)

A litre of QC buffer was prepared by adding 58.44 g NaCl and, 10.46 g of MOPS and dissolving it in 800 ml dH₂O. The pH was adjusted to 7.0 using NaOH and 150 ml

isopropanol was added. The volume was adjusted to a litre using dH₂O. The solution was autoclaved for 20 minutes at 121°C and 1kg/cm² and stored at room temperature.

5.2.6 QF buffer (Elution buffer)

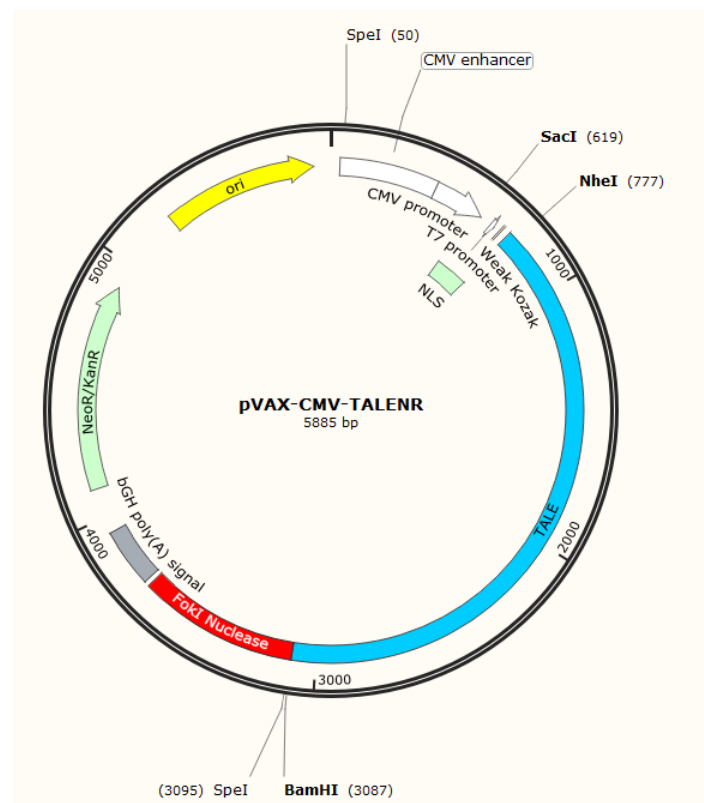
A litre of QF buffer was prepared by adding 73.05 g NaCl, 6.06 g of Tris Base, which was dissolved in 800 ml dH₂O. The pH was adjusted to 8.5 using HCl. A 150 ml isopropanol was added, and the volume was adjusted to a litre using dH₂O.

5.2.7 1 × TAE gel electrophoresis running buffer

A 50 × TAE stock was prepared by mixing 242 g Tris Base, 57.1 ml glacial acetic acid and 100 ml of 0.5M EDTA. The volume was adjusted to 1L with dH₂O.

5.3 Plasmid maps

a)



b)

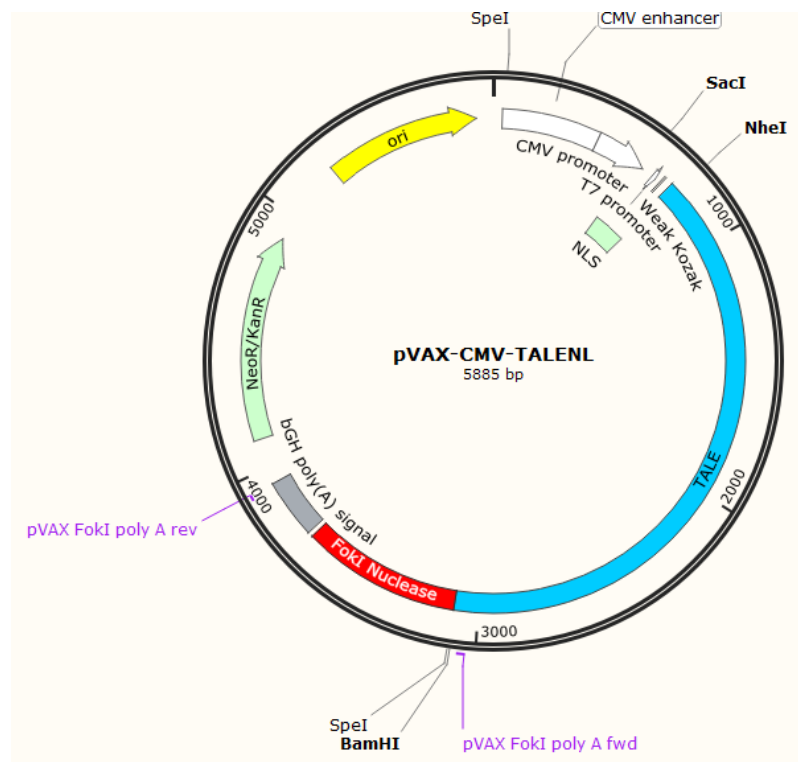


Figure 5.3.1: pVAX-CMV-TALENs plasmid map. A) pVAX-CMV-TALENR b) pVAX-CMV-TALENL. Depicted on the above map is the CMV enhancer, CMV promoter, anti-HBV TALE sequence, FokI endonuclease domain and poly A signal. The primers pVAX-FokI poly A forward and pVAX-FokI poly A reverse is the primer set used for the amplification of the FokI poly A sequence.

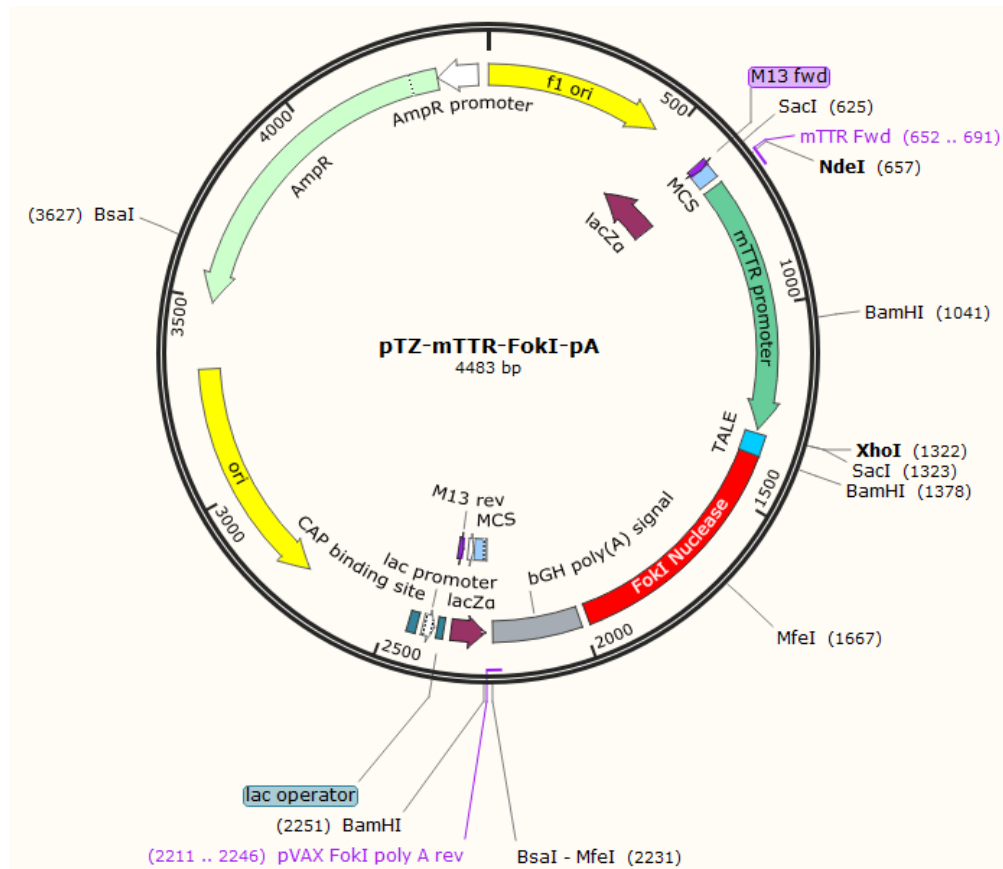


Figure 5.3.2: pTZ- mTTR- FokI poly A plasmid. Depicted on the above map are the mTTR promoter, FokI endonuclease domain and poly A signal. Illustrated are the restriction enzymes used for the ligation of the mTTR promoter and FokI poly A sequence. Restriction enzymes *Nde* I and *Bsa* I were utilized for the ligation of the mTTR promoter and FokI poly A sequence into pAAV plasmid.

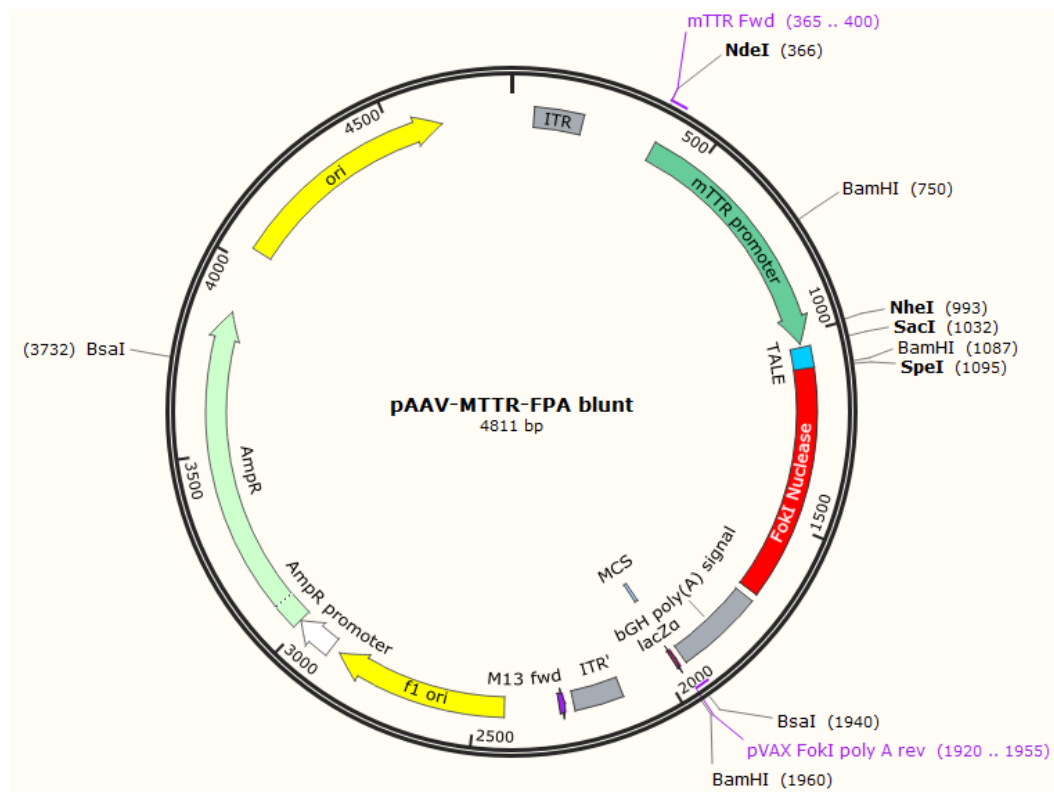


Figure 5.3.3: pAAV-mTTR-FokI poly A plasmid. Depicted on the above map are the two ITRs which are the only cis elements needed for viral replication, the mTTR promoter, FokI endonuclease domain and poly A signal. The restriction enzymes *Sac* I and *Spe* I were utilized for the insertion of the right and left anti-HBV TALE sequence.

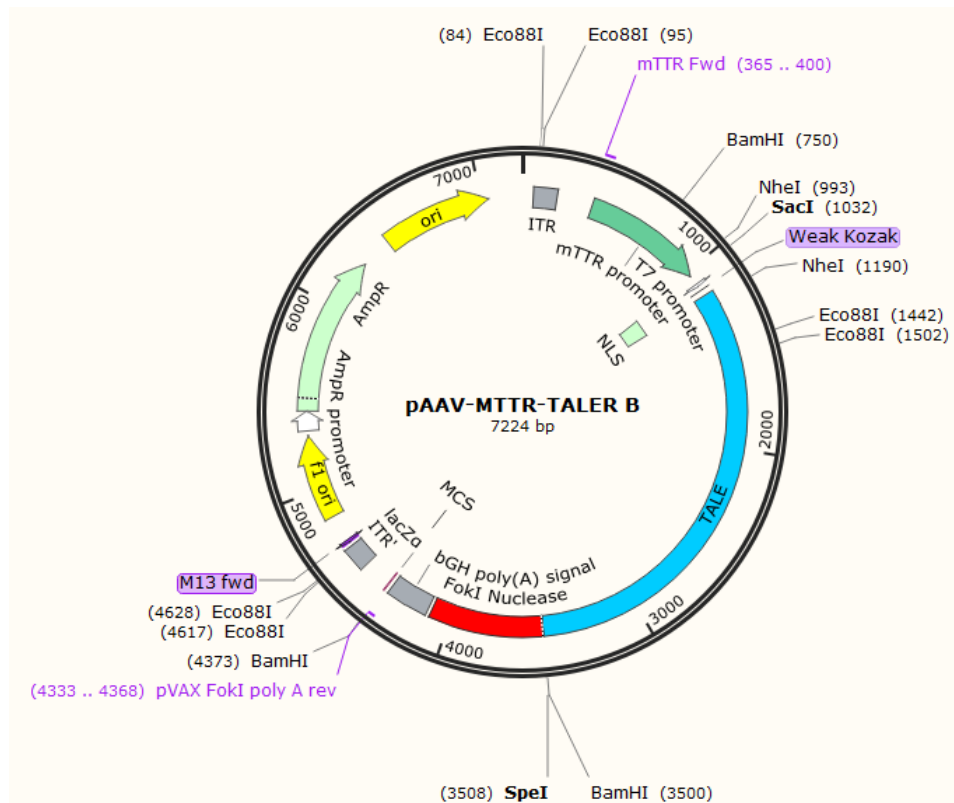


Figure 5.3.4: pAAV-mTTR-TALE(R)-FokI poly A plasmid. Depicted on the above map are the two ITRs, the mTTR promoter, right anti-HBV TALE sequence, FokI endonuclease domain and poly A signal.

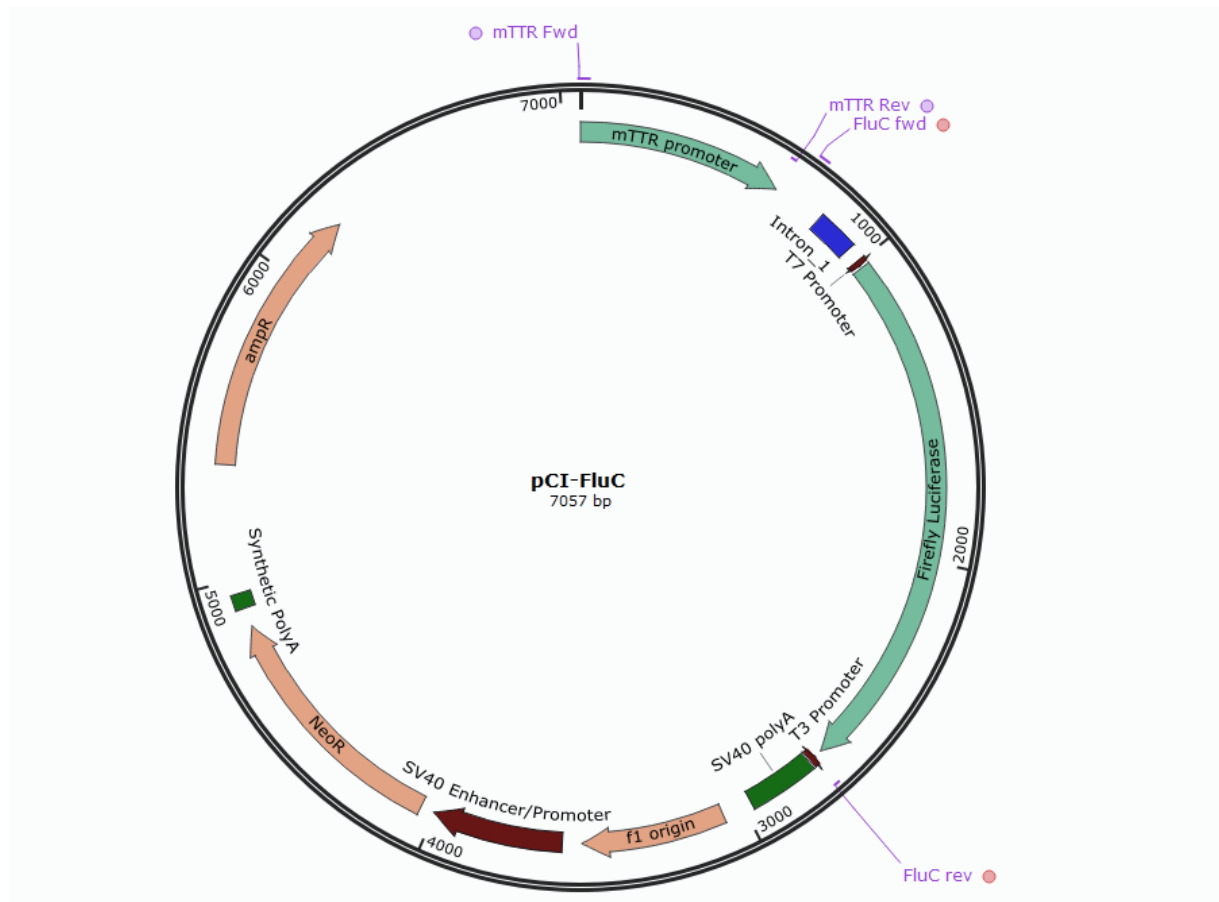


Figure 5.3.5: pCI-FluC plasmid. Depicted on the above map are the mTTR promoter, Firefly luciferase (FluC) gene and poly A sequence. The mTTR promoter was PCR amplified using primers mTTR fwd and mTTR reverse. The FluC gene was PCR amplified using primers FluC fwd and FluC reverse. The mTTR promoter and FluC gene was utilized for the construction of pAAV-mTTR-FokI poly A plasmid and pAAV-mTTR-FluC plasmid respectively.

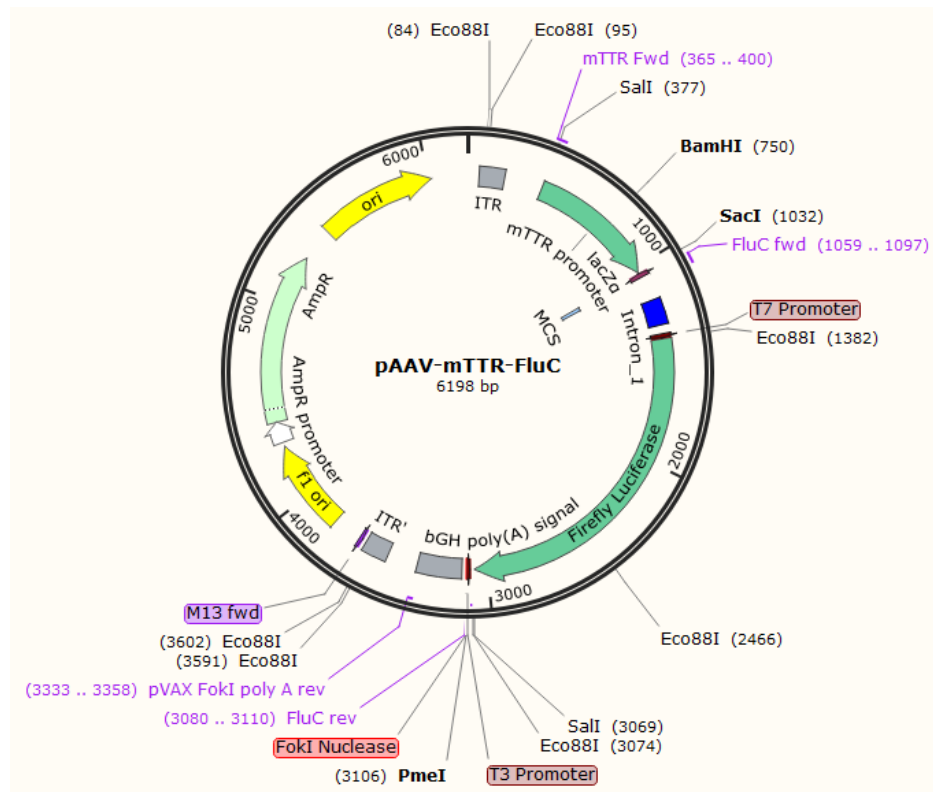


Figure 5.3.6 : pAAV-mTTR-FluC plasmid. Depicted above are the two ITRs, the only cis elements needed for viral replication, the mTTR promoter, FluC gene and poly A sequence. Restriction enzymes Sac I and Pme I were utilized for the insertion of the FluC gene into the pAAV-mTTR-poly A plasmid to generate pAAV-mTTR-FluC.

5.4 Tissue Culture

5.4.1 1 × phosphate buffer saline (PBS)

One Phosphate Buffer Saline (PBS) tablet (Sigma Aldrich, BA, GER) was dissolved in 500 ml of reverse osmosis (RO) water. The PBS was sterilized by autoclaving.

5.4.2 DMEM stock

Five sachets of DMEM powder, high glucose or low glucose, (Life technologies, CA, USA) and 18.5 g of NaHCO₃ was dissolved in 4750 ml of SABAX water. The pH was adjusted to 6.7-6.8 using HCl. The stock was filter sterilized using VacuCap 90 PF (PALL Life Sciences, NY, USA) filter device, into 500 ml autoclaved schott bottles. The sample was taken and incubated overnight at 37°C to observe contamination.

5.4.3 1000 × penicillin/streptomycin (100U/ml/ 100 µg/ml)

Streptomycin (1.5 g) and 0.9 g penicillin were dissolved in 15 ml PBS. The solution was filter sterilized and stored in 0.5 ml aliquots at -20°C.

5.4.4 Fetal Bovine serum (FBS)

Heat inactivated FBS was filter sterilized, aliquoted into 50 ml working stocks and stored at -20°C.

5.4.5 0.5 × TrypleE_{TM}Express

TrypleE_{TM} [(1X) (Life Technologies, CA, USA)] was diluted 2-fold using Saline-EDTA solution. The solution was filter sterilized and stored at 4°C.

5.4.6 Freezing solution

A 10 ml freezing solution was made by adding 5.6 ml complete media, 3.4 ml FBS and 1ml dimethyl sulphoxide (DMSO).

5.5 Large scale viral production reagents

5.5.1 5 × Polyethylene glycol stock

Polyethylene glycol (PEG, Sigma Aldrich. BA, GER) stock was made by adding 250 g PEG and 57.5 g NaCl. Distilled water was added so that a total volume of 500 ml was achieved. The solution was autoclaved at 121°C for 30 minutes. The solution was stored at 37°C until needed.

5.5.2 Benzonase buffer

A litre of benzonase buffer was prepared by adding 50 ml of 1M Tris HCl (pH 8.5) and 0.19g 2mM MgCl₂. The volume was adjusted to a litre using dH₂O. The solution was autoclaved at 121°C for 20 minutes and stored at 4°C until required.

5.5.3 Iodixanol Solutions

Four iodixanol solutions were made each at different percentages. The percentages were: 60 %, 40 %, 25 % and 15 %. The 60 % iodixanol solution was prepared by adding 60 ml iodixanol (Optiprep, Progen, Biotechnik, GmbH) and 2.5 µl/ml phenol red (Sigma Aldrich). The 40% iodixanol was prepared by adding 30 ml iodixanol and 15 ml PBS-MK. The 25 % iodixanol was prepared by adding 20 ml iodixanol, 28 ml PBS-MK and 120 µl phenol red. The 15 % iodixanol solution was prepared by adding 12 ml iodixanol and 36 ml PBS-MK-NaCl.

5.5.4 PBS-MK

A litre of PBS-MK was made by adding 1× PBS tablet in 900 ml of dH₂O and autoclaving to sterilize the solution. After sterilization, 0.1 g 1mM MgCl₂ and 0.19 g 2.5mM KCl was added to the solution. To sterilize the solution filtration was utilized.

5.5.5 PBS-MK-NaCl

To make 500 ml of PBS-MK-NaCl, 500 ml of PBS-MK was made. To the solution, 58.44 g 1M NaCl was added. The solution was sterilized by filtration.

5.6 Immunocytochemistry

5.6.1 0.1% Triton X

A 0.1 % solution of TritonX was made by adding 10 µl of Triton X in 10 ml of PBS. The solution was mixed by vortexing

5.6.2 Bovine Serum Albumin

A 1 % BSA solution was made by adding 0.1 g BSA and adding it to 10 ml of PBS. The solution was mixed by vortexing.

5.7 O'gene ladder



Chapter 6

Supplementary Results

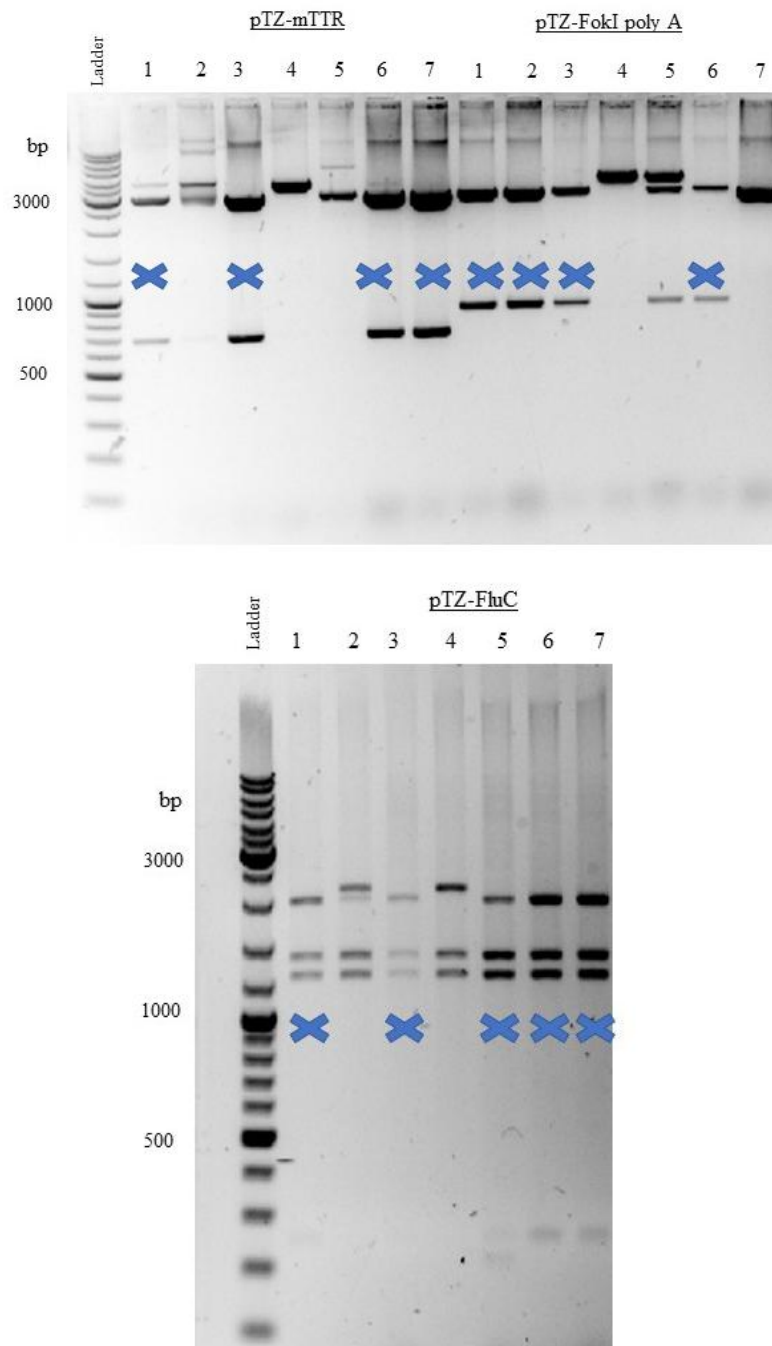


Figure 1: Screening of pTZ-mTTR, pTZ-FokI poly A and pTZ-FluC clones.

The mTTR promoter, FokI poly A sequence and FluC gene were gel extracted and ligated into an intermediary TA cloning pTZ57R/T plasmid to create pTZ-mTTR, pTZ-FokI poly A and pTZ-FluC respectively. Positive clones were detectable after screening with *Sal* I and

Bam HI for pTZ-mTTR and pTZ-FokI poly A respectively, which resulted in the expected bands of sizes 2887 bp and 681 bp for pTZ-mTTR and 2944 bp and 873 bp for pTZ-FokI poly A respectively. Screening with *Bg* I and *Pst* I for pTZ-FluC resulted in clones that gave expected fragment sizes of 2012 bp, 1413 bp, 1267 bp and 252 bp. Amplicons were then separated on a 1 % agarose gel. The O'Gene ladder was loaded to determine the fragment sizes. Positive clones are indicated by a blue cross.

Chapter 6-Supplementary Results

	
	10 20 30 40 50
Reference	CSKWRTWMGW CGTCAYTACT AGGGAAGCTT GCATGCAGGC CTCTGCAGTC
Forward	-----C CAWAGMTATG CTCTG-AAAT
Reverse	CSKWRTWMGW CGTCAYTACT AGGGAAGCTT GCATGCAGGC CTCTGCAGTC
	
	60 70 80 90 100
Reference	GACGGGCCCCG GGATCCGATT GATCCTCGAG CTCGGTACCT CGCGAATGCA
Forward	GATCCATATG AGATCTG-TC GACA---AAA TTTTATCGAT CACGAGACTA
Reverse	GACGGGCCCCG GGATCCGATT GATCCTCGAG CTCGGTACCT CGCGAATGCA
	
	110 120 130 140 150
Reference	-TCTAGATTG ATCGCTAGCT CAC-CATGGT GGCAGGAGCT TGTGGATCTG
Forward	GCCTYGATCG AG-GTCAATT CACGCGAGTT AATAATTACC AGCGCGG--G
Reverse	-TCTAGATTG ATCGCTAGCT CAC-CATGGT GGCAGGAGCT TGTGGATCTG
	
	160 170 180 190 200
Reference	TGTGACGGCT TCTCCTGGTG AAGGGGCTTT TATACCCCTT CCTTCCAACC
Forward	CCAAATAAAT AATCC--GCG AGGGG----- CAGGTGACGT TTGCCAGCG
Reverse	TGTGACGGCT TCTCCTGGTG AAGGGGCTTT TATACCCCTT CCTTCCAACC
	
	210 220 230 240 250
Reference	CAGGCTGCTG ATCCCTGCCA AGCTGACTCC AAACCTGCTG ATTCTGATTA
Forward	CGCGCTGGTA ATTATTAACC TCGCGAATAT TGATTCTGA-G GCCGCGATTG
Reverse	CAGGCTGCTG ATCCCTGCCA AGCTGACTCC AAACCTGCTG ATTCTGATTA
	
	260 270 280 290 300
Reference	TTGACTTAGT CAACAAAAGG AGAATAAGTA ACCTACACAA ATATGAACCT
Forward	CCGCAATCGC GAGGGGCGAG TGACCTT-TG CCCAGCGCGC GTTCGCCCCG
Reverse	TTGACTTAGT CAACAAAAGG AGAATAAGTA ACCTACACAA ATATGAACCT
	
	310 320 330 340 350
Reference	TGCCTAGGGA GATTAG--AG TATCGGAACA CTCGCTCTAC GAAATGTGCA
Forward	CCCCGGACGG TATCGATAAG CTTAGGAGCT TGGGCTGCAG GTCGAGGGCA
Reverse	TGCCTAGGGA GATTAG--AG TATCGGAACA CTCGCTCTAC GAAATGTGCA
	
	360 370 380 390 400
Reference	GACAGACGGG GATCCTCTAG A--GCTACCT GCTGATCGCC CGGCCCTGT
Forward	CTGGGAGGAT GTTGAGTAAG ATGGAAACT ACTGAT---- -GACCCCTGC
Reverse	GACAGACGGG GATCCTCTAG A--GCTACCT GCTGATCGCC CGGCCCTGT
	
	410 420 430 440 450
Reference	TCAAACATGT CTAATACTC TGTCTCTGCA AGGGTCATCA GTAGTTTTC
Forward	AGAGACAGAG TATTAGGACA TGTTTGAACA GGGGCCG--- GGCGATCAGC
Reverse	TCAAACATGT CTAATACTC TGTCTCTGCA AGGGTCATCA GTAGTTTTC
	
	460 470 480 490 500
Reference	ATCTTACTCA ACATCCTCCC AGTGCCCTCG ACCTGCAGCC CAAGCTCCTA
Forward	AGGTAGCTCT AGAGGATCCC CGT-CTGTCT GCACATTTTC TAGAGC---G
Reverse	ATCTTACTCA ACATCCTCCC AGTGCCCTCG ACCTGCAGCC CAAGCTCCTA

Reference	AGCTTATCGA	TACCGTCCGG	GGCGGGGCGA	ACGCGCGCTG	GGCAAAGGTC	
Forward	AGTGTTCGGA	TACTCT----	-----	AATCTCCCTA	GGCAAGGTTTC	
Reverse	AGCTTATCGA	TACCGTCCGG	GGCGGGGCGA	ACGCGCGCTG	GGCAAAGGTC	
						
		560	570	580	590	600
Reference	ACCTGCCCCCT	CGCGATTGCG	GCAATCGCGG	CCTCGAATCA	ATATTCGCGA	
Forward	ATAT-----	-----TTGTG	TAGGTTACTT	ATTCTCCTTT	-TGTTGACTA	
Reverse	ACCTGCCCCCT	CGCGATTGCG	GCAATCGCGG	CCTCGAATCA	ATATTCGCGA	
						
		610	620	630	640	650
Reference	GGTTAATAAT	TACCAGCGCG	CGCTGGGCAA	ACGTCACCTG	CCCCTCGCGG	
Forward	AGTCAATAAT	---CAGAATC	AGCAGGTTTG	GAGTCA--G	CTTGGCAGGG	
Reverse	GGTTAATAAT	TACCAGCGCG	CGCTGGGCAA	ACGTCACCTG	CCCCTCGCGG	
						
		660	670	680	690	700
Reference	ATTATTTATT	TGGCCCGCGC	TG-GTAATTA	TTAACTCGCG	TGAATTGACC	
Forward	ATCAGCAGCC	TGGGTGGA	GGAGGGGGTA	TAAAAGCCC-	---CTTCACC	
Reverse	ATTATTTATT	TGGCCCGCGC	TG-GTAATTA	TTAACTCGCG	TGAATTGACC	
						
		710	720	730	740	750
Reference	TCGATCGAGG	CTAGTCTCGT	GATCGATAAA	ATTTTGTCGA	CA-GATCTCA	
Forward	AGGA--GAAG	CCGTCACACA	GATCCACAAG	CTCCTGCCAC	CATGGTGAGC	
Reverse	TCGATCGAGG	CTAGTCTCGT	GATCGATAAA	ATTTTGTCGA	CA-GATCTCA	
						
		760	770	780	790	800
Reference	TATGGATCAA	TCTAGATGCA	TTCGCGAGGT	ACCGAGCTCG	AGGWYMMWGC	
Forward	TAGCGATCAA	TCTAGATGCA	TTCGCGAGGT	ACCGAGCTCG	AGGATCAATC	
Reverse	TATGGATCAA	TCTAGATGCA	TTCGCGAGGT	ACCGAGCTCG	AGGWYMMWGC	
						
		810	820	830	840	850
Reference	GSATCCCYGG	KGACMATCGA	CTGCAGAGGC	CTGCATGCAA	GCTTTCCTTA	
Forward	GGATCCC--G	GGCCCCTCGA	CTGCAGAGGC	CTGCATGCAA	GCTTTCCTTA	
Reverse	GSATCCCYGG	KGACMATCGA	CTGCAGAGGC	CTGCATGCAA	GCTTTCCTTA	
						
		860	870	880	890	900
Reference	TAGTGAGTCG	TATTAGAGCT	TGGCGTAATC	ATGGTCATAG	CTKGKTTTTC	
Forward	TAGTGAGTCG	TATTAGAGCT	TGGCGTAATC	ATGGTCATAG	YTKKTTTTC	
Reverse	TAGTGAGTCG	TATTAGAGCT	TGGCGTAATC	ATGGTCATAG	CTKGKTTTTC	

Figure 2: Sequence alignment of the cloned mTTR amplicon.

Alignment of mTTR reference sequence with mTTR forward and reverse sequences from the pTZ MTTR plasmid. The mTTR sequence was sequenced using universal primers. There was 100 % complementarity and the engineered restriction sites were present.

Chapter 6-Supplementary Results

	
	10 20 30 40 50
Reference	CCAACTAGTC AAAAGTGAAC TGGAGGAGAA GAAATCTGAA CTTCGTCATA
Forward	CCAACTAGTC AAAAGTGAAC TGGAGGAGAA GAAATCTGAA CTTCGTCATA
Reverse	CCGATTGAYC GGTCTCCAAT TGCCATAGAG CCCACCGCAT CCCCAGCATG
	
	60 70 80 90 100
Reference	AATTGAAATA TGTGCCTCAT GAATATATTG AATTAATTGA AATTGCCAGA
Forward	AATTGAAATA TGTGCCTCAT GAATATATTG AATTAATTGA AATTGCCAGA
Reverse	CCTGCTATTT TCTTCCAAT CCTCCCCCTT GCTGTCCTGC CCCACCCAC
	
	110 120 130 140 150
Reference	AATTCCACTC AGGATAGAAT TCTTGAAATG AAGGTAATGG AATTTTTTAT
Forward	AATTCCACTC AGGATAGAAT TCTTGAAATG AAGGTAATGG AATTTTTTAT
Reverse	CCCCCAGAAT AGAATGACAC CTACTCAGAC AATGCGATGC AATTCCTCA
	
	160 170 180 190 200
Reference	GAAAGTTT-- -ATGGATATA GAGGTAAACA TTTGGGTGGA TCAAGGAAAC
Forward	GAAAGTTT-- -ATGGATATA GAGGTAAACA TTTGGGTGGA TCAAGGAAAC
Reverse	TTTTATTAGG AAAGGACAGT GGGAGTGGCA CCTTCCAGGG TCAAGGAAGG
	
	210 220 230 240 250
Reference	CG-GACGGAG CAATTTATAC TGTCGGATCT CCTATTGATT ACGGTGTGAT
Forward	CG-GACGGAG CAATTTATAC TGTCGGATCT CCTATTGATT ACGGTGTGAT
Reverse	CACGGGGGAG GGGCAAACAA CAGATGGCTG GCAACTAG-- AAGGCACAGT
	
	260 270 280 290 300
Reference	CGTGGAT-AC TAAAGCTTAT AGCGGAGGTT ATAATCTGCC AATTGGCCAA
Forward	CGTGGAT-AC TAAAGCTTAT AGCGGAGGTT ATAATCTGCC AATTGGCCAA
Reverse	CGAGGCTGAT CAGCGGGTTT AAACGGGCCC TTAAAAGTTT ATCTCGCC--
	
	310 320 330 340 350
Reference	GCAGATGAAA TGCAACGATA TGTCGAAGAA AATCAA--A CACGAAACAA
Forward	GCAGATGAAA TGCAACGATA TGTCGAAGAA AATCAA--A CACGAAACAA
Reverse	GTTATTAAAT TTCCGTCTCA CTTCCTCTAA GGTTAATGTG CCGGCTTTAA
	
	360 370 380 390 400
Reference	ACATATCAAC CCTAATGAAT GGT-----GGAA AGTCTATCCA
Forward	ACATATCAAC CCTAATGAAT GGT-----GGAA AGTCTATCCA
Reverse	TCATTTCTCC ACCAATTAAA AGCTCTTCTA CACTAAGAAC AGCTCCATTA
	
	410 420 430 440 450
Reference	TCTTCTGTAA CGGAATTTAA GTTTTTAT-T TGTGAGTGGT CACTTTAAAG
Forward	TCTTCTGTAA CGGAATTTAA GTTTTTAT-T TGTGAGTGGT CACTTTAAAG
Reverse	CAATTAGTGA TATGATTTAA TCGTGTAAGC TGAGCTTTGT AGTTTCCTTTT
	
	460 470 480 490 500
Reference	GAAACTACAA AGCTCAGCTT ACACGATTAA ATCATATCAC TAATTGTAAT
Forward	GAAACTACAA AGCTCAGCTT ACACGATTAA ATCATATCAC TAATTGTAAT
Reverse	AAAGTGACCA CTCACAAAT- AAAAACTTAA ATTCCGTTAC AGAAGATGGA

	510	520	530	540	550
Reference	GGAGCTGTTT	TTAGTGTAGA	AGAGCT--TT	TAATTGGTGG	AGAAATGATT
Forward	GGAGCTGTTT	TTAGTGTAGA	AGAGCT--TT	TAATTGGTGG	AGAAATGATT
Reverse	TAGACTTTCC	ACCATTTCATT	AGGGTTGATA	TGTTTGTTTC	GTGTTTGATT

					
	560	570	580	590	600
Reference	AAAGCCGGCA	CATTA---A	CCTTAGAGGA	--AGTGAGAC	GGAAATTTAA
Forward	AAAGCCGGCA	CATTA---A	CCTTAGAGGA	--AGTGAGAC	GGAAATTTAA
Reverse	TTCTTCGACA	TATCGTTGCA	TTTCATCTGC	TTGGCCAATT	GGCAGATTAT

				
	610	620	630	
Reference	TAACGGCGAG	ATAAACTTTT	AAGGGCCCGT	TTAA-
Forward	TAACGGCGAG	ATAAACTTTT	AAGGGCCCGT	TTAAA
Reverse	AACCTCCGCT	ATAAGCTTTA	GTATCCACGA	TCACA

Figure 3: Sequence alignment of FokI poly A cloned amplicon.

Alignment of FokI poly A reference sequence with -FokI poly A forward and reverse sequences from the pTZ FokI poly A. The FokI poly A sequence was amplified using universal primers. There was 100 % complementarity and the engineered restriction sites were present.

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		10	20	30	40	50
Reference	AGCCTACCGT	GGTGTTCGTT	TCCAAAAAGG	GGTTGCAAA-	--AAATTTTG	
Forward	-----CYAW	GCTGAKYACT	GAATTGATCG	AGCTCCACAG	TTAAATTGCT	
Forward In	-----	ACGGATYGT	TC-AAAAAGG	GGTTGCAAA-	--AAATTTTG	
Reverse	-----CTAR	WCGGAYGTCW	YTACAGGGAA	GCTTGCATGC	AGGCCTCTGC	
Reverse In	GMGCTCTTCA	TTCAATCTAT	ACATTAAAGAC	GACTCGAAAT	CCACATATCA	
		60	70	80	90	100
Reference	AACGTGCAAA	AAAAGCTCCC	AAT----CAT	CCAAAAAATT	ATTAT-CATG	
Forward	AAC--GCAGT	CAGTGCTTCT	GACACAACAS	YYTCGAACTT	AAGCTGCAGT	
Forward In	AACGTGCAAA	AAAAGCTCCC	AAT----CAT	CCAAAAAATY	ATTAT-CATG	
Reverse	AGTCGACGGG	CCCGGGATCC	GATTGATCGT	TT--AAACCC	CTCACTAAAG	
Reverse In	AATATCCGAG	TGTMGYMWAC	A-----	TTCCAAAACC	GTGATGGAAT	
		110	120	130	140	150
Reference	GATTCT---A	AAACGGATTA	CCAGGGATTT	CAGTCGATGT	ACACGTTCTG	
Forward	GACTCTCTTA	AGGTAGCCTT	GCAGAAGTTG	GTCTGAGAGC	ACTGGGCAGG	
Forward In	GATTCT---A	AAACGGATTA	CCAGGGATTT	CAGTCGATGT	ACACGTTCTG	
Reverse	GG-----	AAGCGGCCGC	CCGGG--TCG	ACTCTAGTTT	ACACGGCGAT	
Reverse In	GG-----	-AACAA-CAC	TTAAA----	ATCGCAGTAT	CCGGAATGAT	
		160	170	180	190	200
Reference	CACATCTCAT	--CTACCTCC	CGGTTTTAAT	GAATACGATT	TTGTGCCAGA	
Forward	TAAGTATCAA	GGTTACAAGA	CAGGTTTAAG	GAGACCAATA	--GAAACTGG	
Forward In	CACATCTCAT	--CTACCTCC	CGGTTTTAAT	GAATACGATT	TTGTGCCAGA	
Reverse	C---TTTCCG	---CCCTTCT	TGGCCTTTAT	GAGGATCTCT	CTGATTTTTTC	
Reverse In	TTGATTGCCA	A-AAATAGGA	TCTCTGGCAT	GCGAGAATCT	CACGCAGGCA	
		210	220	230	240	250
Reference	GTCCTTCGA-	--TAGGGACA	AGACAATTGC	ACTGATCATG	AACTCCTCTG	
Forward	GCTTGTCGA-	--GACAGAGA	AGACTCTTGC	GTTTCTGATA	GGCACCTATT	
Forward In	GTCCTTCGA-	--TAGGGACA	AGACAATTGC	ACTGATCATG	AACTCCTCTG	
Reverse	TTGCGTCGAG	TTTTCCGGTA	AGACCTTTCG	GTACTTCGTC	CACAAACACA	
Reverse In	GTTCTATGAG	--GCAGAGCG	ACACCTTTAG	GCAGACCA--	-GTAGATCCA	
		260	270	280	290	300
Reference	GATCT---AC	TGGTCTGCCT	AAAGGTGTCTG	CTCTGCCTCA	TAGAACTGCC	
Forward	GGTCTT--AC	TGACATCC--	----ACTTTG	CCTTTCTCTC	CACAGGTGTC	
Forward In	GATCT---AC	TGGTCTGCCT	AAAGGTGTCTG	CTCTGCCTCA	TAGAACTGCC	
Reverse	ACTCCTCCGC	GCAACTTTTT	CGCGGTTGTT	ACTTGACTGG	CGACGTAATC	
Reverse In	GAGGA---GT	TCATGATCAG	TGCAATTGTC	TTGTCCCTAT	CGAAGGACTC	
		310	320	330	340	350
Reference	TGCGTGAGAT	TCTCGCATGC	CAGAGATCCT	ATTTTGGCA	ATC-AA-ATC	
Forward	CACTCCAGT	TCA---ATTA	CAGCTCTTAA	GGCTAGAGTA	CTT-AATACG	
Forward In	TGCGTGAGAT	TCTCGCATGC	CAGAGATCCT	ATTTTGGCA	ATC-AA-ATC	
Reverse	CACGATCTCT	TTTTCCGTCA	TCGTCTTTCC	GTGCTCCAAA	ACA-ACAACG	
Reverse In	TGGCACAAAA	TCGTA-TTCA	TTAAAACCGG	GAGGTAGATG	AGATGTGACG	
		360	370	380	390	400
Reference	ATTCCGGATA	CTGCGAT--T	TTAAGTGTTG	TTCCATTCCA	TCACGGTTTT	
Forward	ACTCACTATA	GGCTAGC--C	TCGAGGATTG	GGG-ACCCTG	CGCTGAACAT	
Forward In	ATTCCGGATA	CTGCGAT--T	TTAAGTGTTG	TTCCATTCCA	TCACGGTTTT	
Reverse	GCGGCGGGAA	GTTCAACGGC	GTCATCGTCG	GGAAGACCTG	CGACACCTGC	
Reverse In	AACGTGTACA	-TCGACTGAA	ATCCCTGGTA	ATCCGTTTTA	GAATCCATGA	

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		410	420	430	440	450
Reference	G--GAATGTT	TACTACACTC	GGATATTTGA	TATGTGGATT	TCGAGTCGTC	
Forward	G--GAAGACG	CCAAAAACAT	AAAGAAAGGC	CCGGCGCCAT	TCTATCCGCT	
Forward In	G--GAATGTT	TACTACACTC	GGATATTTGA	TATGTGGATT	TCGAGTCGTC	
Reverse	GTCGAAGATG	TTGGGGTGT	GGA----GC	AAGATGGATT	CCAATTCAGC	
Reverse In	TAATAATTTT	TTGGATGATT	GGGAGCTTTT	TTTGCACGTT	CAAAATTTT	
		460	470	480	490	500
Reference	TTAATGTATA	GATTTGAAGA	AGAGCTGTTT	CTGAGGAGCC	TTCAGGATTA	
Forward	GGAAG--ATG	GAACCGCTGG	AGAGCAACTG	CATAAGGCTA	TGAAGAGATA	
Forward In	TTAATGTATA	GATTTGAAGA	AGAGCTGTTT	CTGAGGAGCC	TTCAGGATTA	
Reverse	GGGAGCCACC	TGATAGCCTT	TG--TACTT	AATCAGAGAC	TTCAGGCGGT	
Reverse In	TGCAACCCCT	TTTTGGAAAC	GA---ACAC	CACGGTAGGC	TGCGAAATGC	
		510	520	530	540	550
Reference	CAAGATTCAA	AGTGCCTGTC	TGGTGCCAAC	CCTATTCTCC	TTCTTCGCCA	
Forward	CGCCCTGGTT	CCTGGAACAA	TTGCTTTTAC	AGATGCACAT	ATCGAGGTGG	
Forward In	CAAGATTCAA	AGTGCCTGTC	TGGTGCCAAC	CCTATTCTCC	TTCTTCGCCA	
Reverse	CAACGATGAA	---GAAGTGT	TCGTCTTCGT	CCCAGTAAGC	TATGTCTCCA	
Reverse In	CCATACTGTT	---GAGCAAT	TCACGTTCAT	TATA--AATG	TCGTTCGCGG	
		560	570	580	590	600
Reference	AAAGCACTCT	GATTGACAAA	TACGATTTAT	CTAATTTACA	CGAAATTGCT	
Forward	ACATCACTTA	CGCTGAGTAC	TTCGAAATGT	CCGTTCCGTT	GGCAGAAGCT	
Forward In	AAAGCACTCT	GATTGACAAA	TACGATTTAT	CTAATTTACA	CGAAATTGCT	
Reverse	GAA----TGT	AGCCATCCAT	C---CTTGT	CAATCAAGGC	GTTGGTCGCT	
Reverse In	GCGCAACTGC	AACCTCCGATA	AATAACGCGC	CCAACACCGG	CATAAAGAAT	
		610	620	630	640	650
Reference	TCTGGTGGCG	CTCCCCTCTC	TAAGGAAGTC	GGGGAAGCG-	-GTTGC--C	
Forward	AT--GAAACG	ATATGGGCTG	AATACAAATC	ACAGAATCGT	CGTATG--C	
Forward In	TCTGGTGGCG	CTCCCCTCTC	TAAGGAAGTC	GGGGAAGCG-	-GTTGC--C	
Reverse	TCCGGATTGT	TTACATAACC	GGACATAATC	ATAGGACCT-	--CTCA--C	
Reverse In	TGAAGAGAGT	TTTCACTGCA	TACGACGATT	CTGTGATTG	TATTACAGCC	
		660	670	680	690	700
Reference	AAGAGGTTCC	AT--CTGCCA	GGTATCAGGC	AAGGATATGG	GCTCACTGAG	
Forward	AGTGAAAACT	CT--CTTCAA	TTCTTTATGC	CGGTGT-TGG	GCGCGTTA--	
Forward In	AAGAGGTTCC	AT--CTGCCA	GGTATCAGGC	AAGGATATGG	GCTCACTGAG	
Reverse	ACACAGTTTC	CC--TCTTTG	ATTAACGCCC	AGCGTTTT--	-CCCGGTAT-	
Reverse In	ATATCGTTTC	ATAGCTTCTG	CCAACCGAAC	GGACATTT--	-CGAAGTACT	
		710	720	730	740	750
Reference	ACTACATCAG	CTATTCTGAT	TACACCCGAG	GGGGATGATA	A-ACCGGGCG	
Forward	--TTTATCGG	A-GTTGCAGT	TGCGCCCGCG	AACGACATTT	ATAATGAACG	
Forward In	ACTACATCAG	CTATTCTGAT	TACACCCGAG	GGGGATGATA	A-ACCGGGCG	
Reverse	-CCAGATCCA	CAACCTTCGC	TTCAA-AAA	TGGAACAAC	TTACCGACCG	
Reverse In	-CAGCGTAAG	TGATGTCCAC	CTCGAT-ATG	TGCATCTGTA	AAAGCAATTG	
		760	770	780	790	800
Reference	CGGTCGGTAA	AGTTGT-TCC	ATTTTTTGAA	GCGAAGGTTG	TGGATCTGGA	
Forward	TGAAATGCTC	AACAGTATGG	GCATTTTCGCA	GCCTA--CCG	TGGTGTTCGT	
Forward In	CGGTCGGTAA	AGTTGT-TCC	ATTTTTTGAA	GCGAAGGTTG	TGGATCTGGA	
Reverse	CGCCCGGTTT	A-TCATCCCC	CTCGGGTGTA	ATCAGAATAG	CTGATGTAGT	
Reverse In	TTCCAGGAAC	CAGGGCGTAT	CTCTT-CATA	GCC----TTA	TGCAGTTGCT	

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	810 820 830 840 850
Reference	TACCGGGAA- ---AACGCT GGGCGTTAAT CAA-AGAGGC GAACTGTGTG
Forward	TTCCAAAAA- ---GGGGTT GCA-AAAAAT TTT-GAACGT GCAAAAAAAG
Forward In	TACCGGGAA- ---AACGCT GGGCGTTAAT CAA-AGAGGC GAACTGTGTG
Reverse	CTCAGTGAGC CCATATCCTT GCCTGATACC TGGCAGATGG AACCTCTTGG
Reverse In	CTCCAGCGGT TCCATCTTCC AGCGGATAGA ATGGCGCCGG GCCTTTCT--
	
	860 870 880 890 900
Reference	TGAGAGGTCC TATGATTATG TCCGGTTATG TAAAC-AATC CGGAA-GCGA
Forward	CTCCCAATCA TCCAAAAAAT TATTATCATG GATTCTAAAA CGGA---TTA
Forward In	TGAGAGGTCC TATGATTATG TCCGGTTATG TAAAC-AATC CGGAA-GCGA
Reverse	CAACCGCTTC CCCGACTTCC TTAGAGAGGG GAGCGCCACC AGAA---GCA
Reverse In	TTATGTTTTT GCGTCTTCC ATGTTTCAGCG CAGGGTCCCC AATCC--TCG
	
	910 920 930 940 950
Reference	CCAACGCCTT GA-TTGACAA GGATGGATGG CTACATTCTG GAGACATAGC
Forward	CCAGGGATTT CAGTCGATGT ACACGT-TCG TCACATCTCA TCTACCTC-C
Forward In	CCAACGCCTT GA-TTGACAA GGATGGATGG CTACATTCTG GAGACATAGC
Reverse	-----ATTT CGTGTAATT AGATAAATCG -TATTTGTCA ATCAGAGTGC
Reverse In	AGGCTAGCCT ATAGTGAGTC GTATTAAGTA --CTCTAGCC TTAAGAGCTG
	
	960 970 980 990 1000
Reference	TTACTGGGAC GAAGACGAAC ACTTCTT-CA TCGTTGACCG CCTGAAGTCT
Forward	CGGTTTTAAT GAATACG--- ATTTTGTG-TG CCAGAGTCCT TCGATAGGGA
Forward In	TTACTGGGAC GAAGACGAAC ACTTCTT-CA TCGTTGACCG CCTGAAGTCT
Reverse	TTTTGGCGAA GAAGGAGAAT AGGGTTGGCA CCAGCAGCGC ACTTTGAATC
Reverse In	TAATTGAACT GGGAGTGGAC ACCTGTGGAG AGAAGGGCAA AGTGGATGTC
	
	1010 1020 1030 1040 1050
Reference	CTGATTAAGT ACAAAGGCTA TCAGGTGG-- -CTCCCGCTG AATTGGAA-T
Forward	CAAGACAATT GCACTGATCA TGAAC-T-- -CTCTGGATC TACTGGA--T
Forward In	CTGATTAAGT ACAAAGGCTA TCAGT--G-- -CTCCCGCTG AATTGGAAAT
Reverse	TTGTAATCCT GAAAGGCTCC TCAGAAAC-- -AGCTTCTTC TTTCAAATCT
Reverse In	AGTAAGACCA ATAGTGCCCTA TCAGAAACGC AAGAGTCTTT CTCTGTCTCG
	
	1060 1070 1080 1090 1100
Reference	CCATCTTGCT CCAACACCCC AACATCTTCG ACGCAGGTGT CGCAGGTCTT
Forward	CTGCCTAAAG GTGTCGCTCT GCCTTCATAG ACCTGCCTGC GTGAGATTCT
Forward In	CCATCTTGCT C-AACACCCC AACATCTTCG ACGCAG-TGT CGCAGGTCTT
Reverse	ATACATTGAT ACGACTTCCG AATCCACAT AT-CAAATAT CCGAGTGTAG
Reverse In	ACAAGCCCAG TTTTCTATTG GATCTCTTTA AC-CTGTCTT GTAACCGTTG
	
	1110 1120 1130 1140 1150
Reference	-CCCGACGA- TGACGCCGGT GAACTTCCC GCGCCGTTGT TGTTTTGGAG
Forward	-----CGCA TGCCA--GAA GATCCTATTG TGACAATCAA TTCATTCCCG
Forward In	TCCCGACGAA TGACGCCGGT GA-CTTCCCG ACGTCGTG-- TGTTT-GCAG
Reverse	-----TAAT CATTCCCAA ACCGTGAATG GAATTGACCA CACTTAAAT
Reverse In	-----ATACT TACTGCCAG TGTCTTCACG ACTACTTCTG CAGCTACCTT
	
	1160 1170 1180
Reference	CACGGAAAGA CGATGACGGA AAAAGAGATC G
Forward	CAT-ACCTGC GAAT----- -
Forward In	CACGAAAGAC GAATGACGGC AAAAATRT-- -
Reverse	CGCAGTATTC CGGGAAAKG- ----- -
Reverse In	AAGAAGAGT- ----- -

Figure 4: Sequence alignment of FluC cloned amplicon

Alignment of FluC reference sequence with FluC forward and reverse sequences from pTZ Fluc. Universal primers were used to sequence the FluC sequence as well as intermediate FluC primers. The sequences generated from intermediate FluC primers are illustrated as Forward In and Reverse In respectively. There was 100 % complementarity and the engineered restriction sites were present.



Figure 5: Sequence alignment of the right TALE sequence from pAAV-mTTR-TALEN(R)- FokI poly A

Sequence alignment of the right TALE encoding sequence with a reference sequence. The nucleotides sequences generated were converted into amino acids. The first 4 RVDs present in the right anti-HBV TALEN are highlighted by a red box.

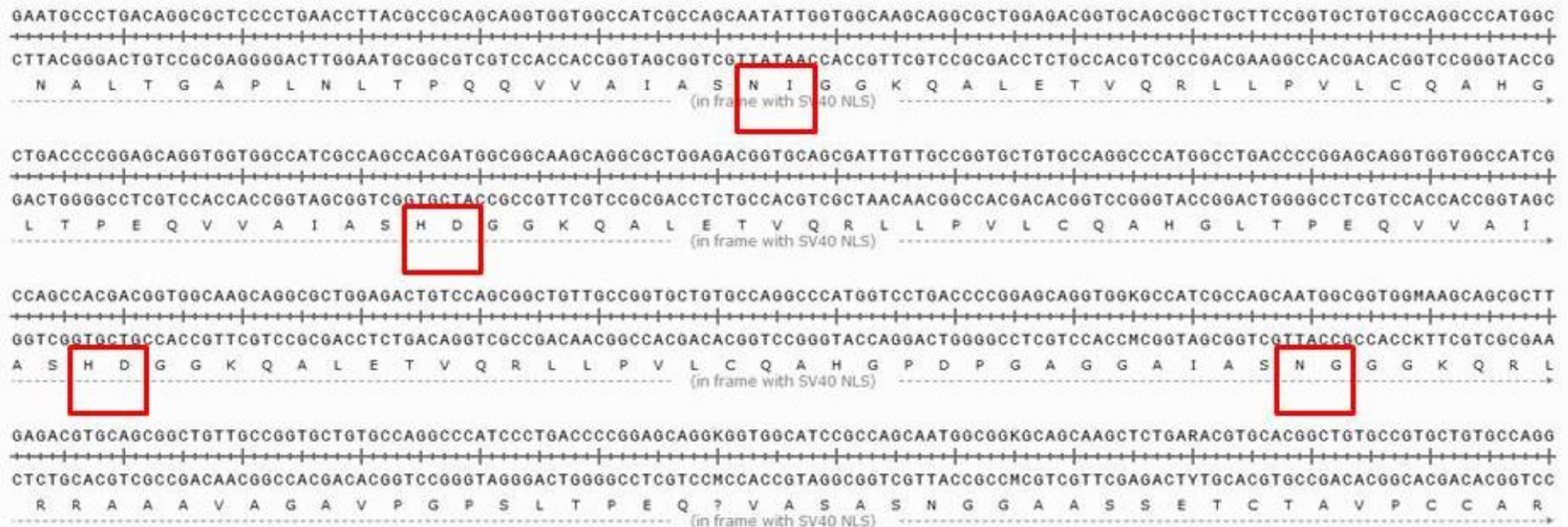


Figure 6: Sequence alignment of the right TALE sequence from pVAX-CMV-TALEN(R)

Sequence alignment of the right TALE encoding sequence with a reference sequence. The nucleotides sequences generated were converted into amino acids. The first 4 RVDs present in the right anti-HBV TALEN are highlighted by a red box.

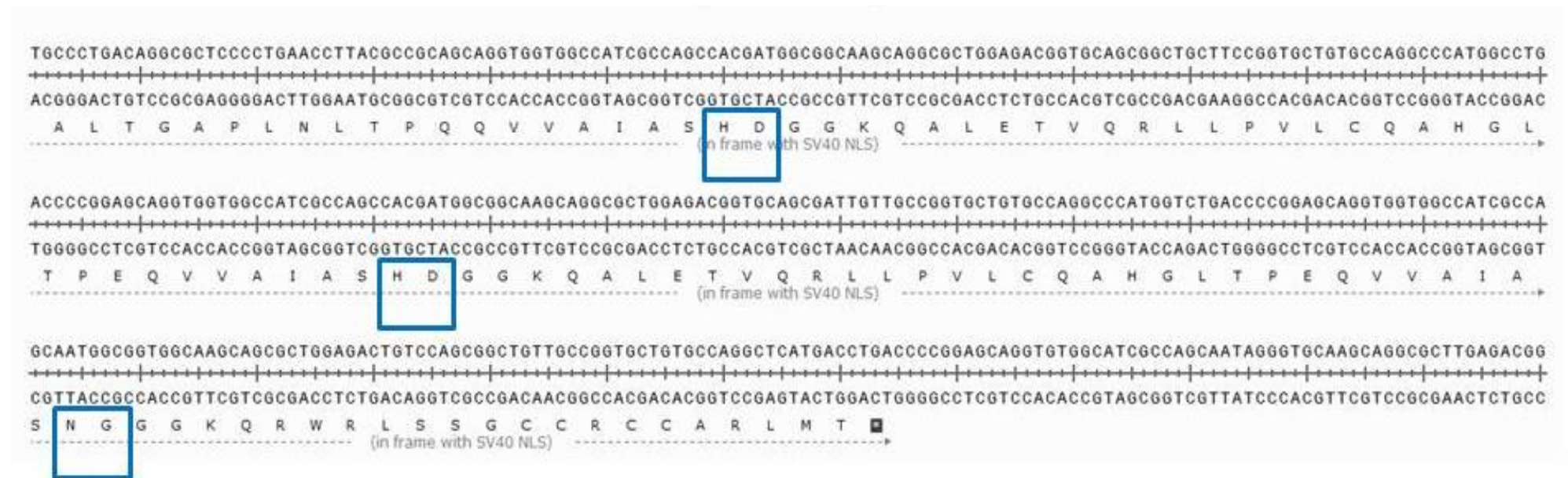


Figure 7: Sequence alignment of the left TALE sequence from pAAV-mTTR-TALEN(L)- FokI poly A

Sequence alignment of the left TALE encoding sequence with a reference sequence. The nucleotides sequences generated were converted into amino acids. The first 4 RVDs present in the left anti-HBV TALEN are highlighted by a blue box.

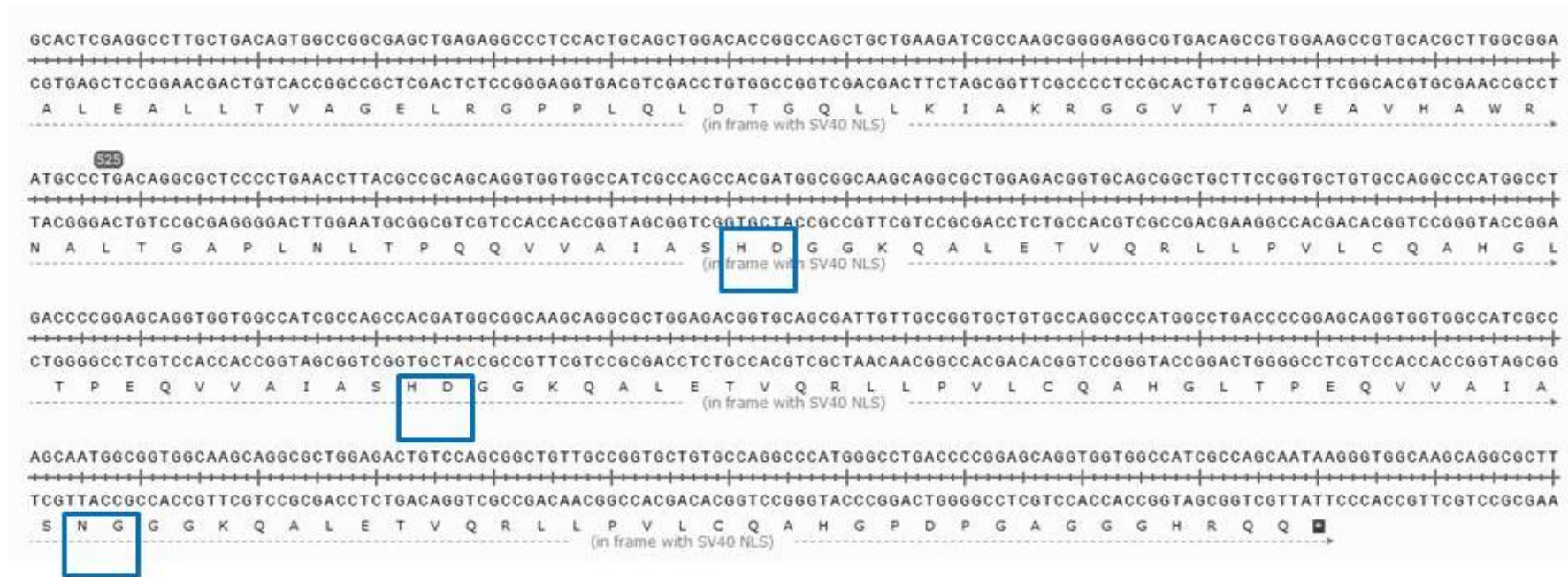


Figure 8: Sequence alignment of the left TALE sequence from pVAX-CMV-TALEN(L)

Sequence alignment of the left TALE encoding sequence with a reference sequence. The nucleotide sequences generated were converted into amino acids. The first 4 RVDs present in the left anti-HBV TALEN are highlighted by a blue box.

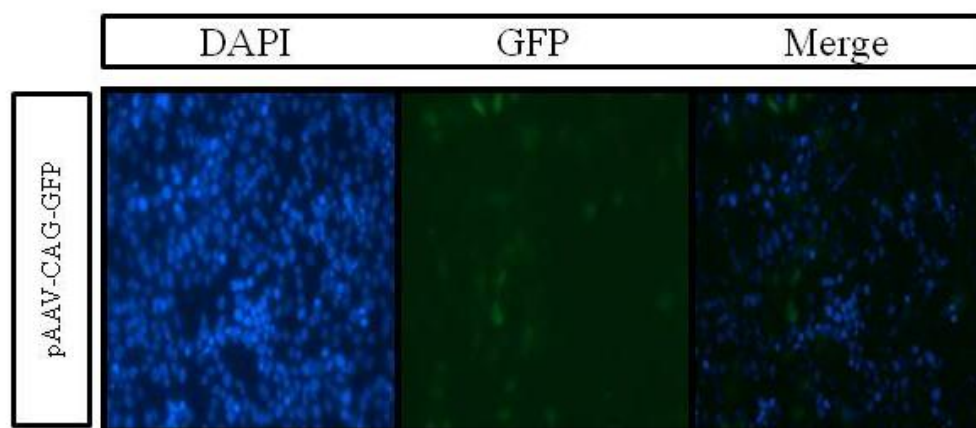


Figure 9: Transfection efficiency of pAAV-mTTR-TALENs and pVAX-CMV-TALENs in Huh 7 cells before Immunocytochemical staining. Assessment of the transfection efficiency for Immunocytochemistry was evaluated using the pAAV-CAG-GFP plasmid. The pAAV-CAG-GFP plasmid was included as a control. GFP expression was visualised using fluorescence microscopy at 20× magnification. DAPI was used to stain the nucleus. Images depicted above are a representation of several images from different fields.

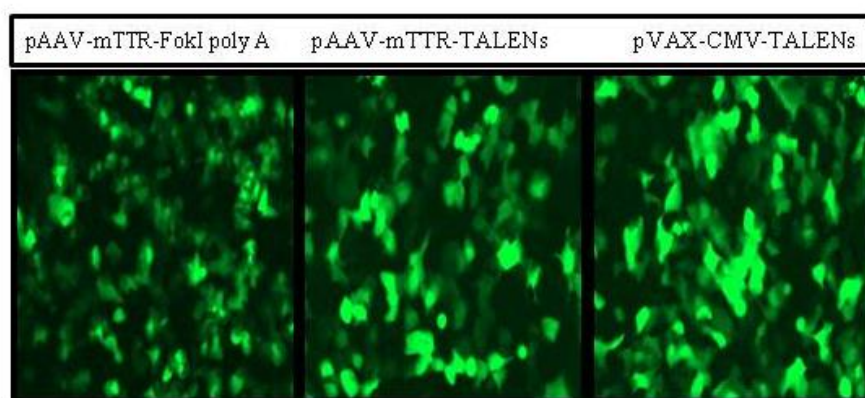


Figure 10: Transfection efficiency of pAAV-mTTR-TALENs and pVAX-CMV-TALENs in Huh 7 cells before ELISA. Assessment of the transfection efficiency for ELISA was evaluated using the pAAV-CAG-GFP plasmid. The pAAV-CAG-GFP plasmid was included in the individual transfection mixes. GFP expression was visualised using fluorescence microscopy at 20× magnification. Images depicted above are a representation of several images from different fields.

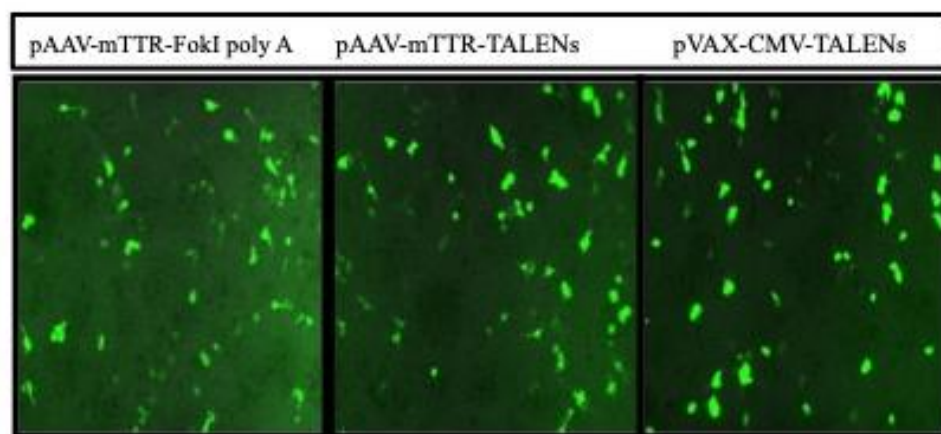


Figure 11: Transfection efficiency of pAAV-mTTR-TALENs or pVAX-CMV TALENs in HepG 2.2.15 cells before T7E1 assay. Assessment of the transfection efficiency in triple transfected HepG2.2.15 cells was evaluated by co-transfecting cells with pAAV-CAG-GFP. GFP expression was visualised 48 hours post-transfection using fluorescence microscopy at 20× magnification. The images represent day 14 of triple transfected HepG2.2.15 cells.

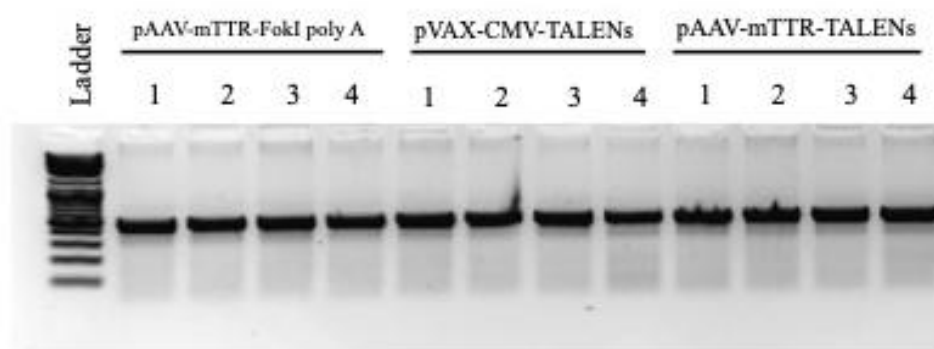


Figure 12: PCR amplification of S TALEN target site from HepG 2.2.15 cells for T7E1 assay.

HepG.2.2.15 cells were transfected in triplicate with the various anti-HBV TALEN plasmids. Total DNA from HepG2.2.15 cells transfected were extracted and amplified. PCR amplification produced the expected band of 499 bp. Amplicons were then separated on a 1 % agarose gel. The O'Gene ladder was loaded to determine the fragment sizes.

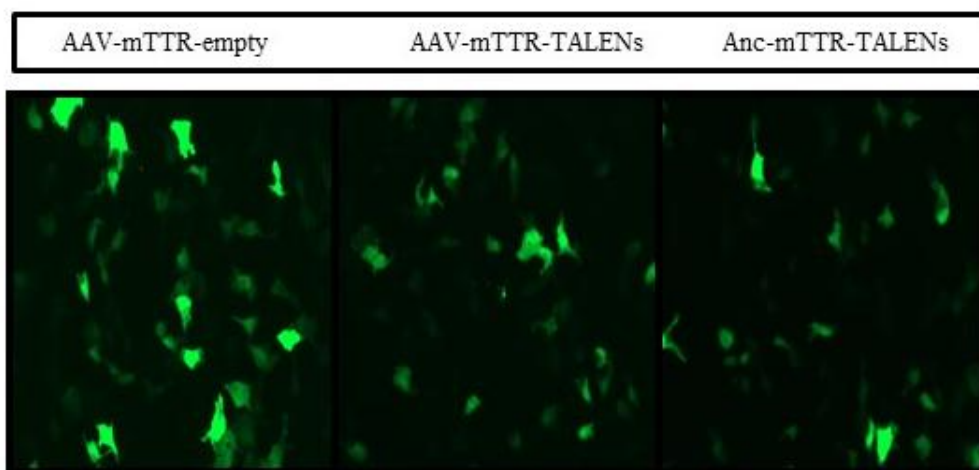


Figure 13: Transfection efficiency of Huh 7 cells before AAV transduction. Assessment of the transfection efficiency for ELISA was evaluated using the pAAV-CAG-GFP plasmid. The pAAV-CAG-GFP plasmid was included in the transfection mix of pCH-9/3091 to determine the transfection efficiency of cells. GFP expression was visualised using fluorescence microscopy at 20× magnification. Images depicted above are a representation of several images from different fields.

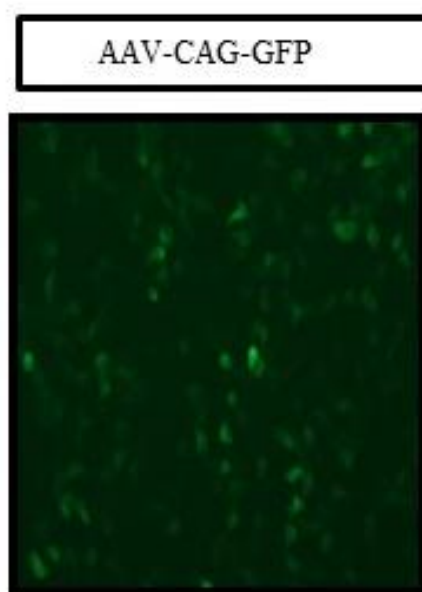
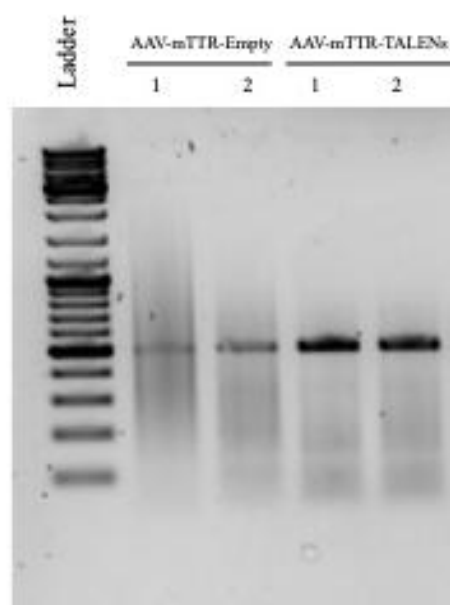
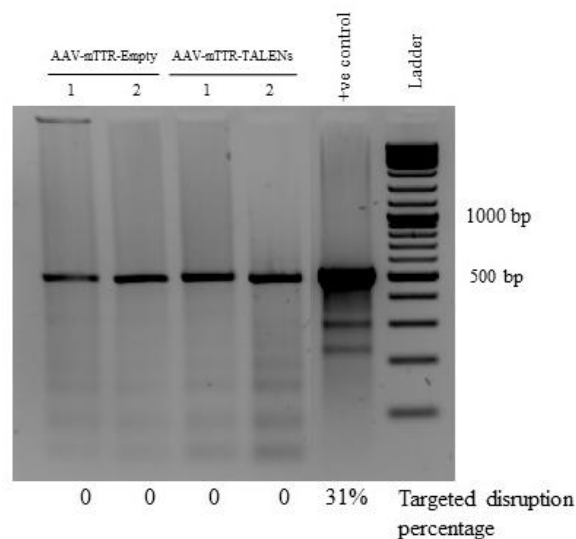


Figure 14: Transduction efficiency of Huh 7 cells before an ELISA. Assessment of the transfection efficiency for ELISA was evaluated using the AAV-CAG-GFP viral vector. GFP expression was visualised using fluorescence microscopy at 20× magnification. Images depicted above are a representation of several images from different fields.

a)



b)



c)

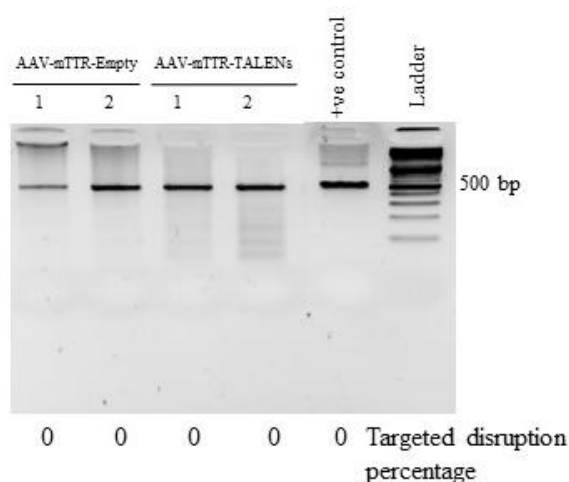


Figure 15: PCR amplification of S TALEN target site from HepG 2.2.15 cells transduced with AAVs and assessment of vector mediated HBV genome cleavage.

a) HepG.2.2.15 cells were transduced in duplicate with the viral vectors containing the anti-HBV TALEN sequence. Total DNA from HepG2.2.15 cells transfected were extracted and amplified. PCR amplification produced the expected band of 499 bp indicated by an arrow. Amplicons were then separated on a 1 % agarose gel. The O'Gene ladder was loaded to determine the fragment sizes. PCR amplification of S TALEN target sites was carried out and a T7E1 assay was conducted on each of the samples. The viral vectors with (b) and without (c) T7E1

treatment are shown. Amplicons were resolved on a 2% agarose gel and the O'Gene ladder was loaded to determine the fragment sizes. Targeted disruption obtained from each transduction is indicated below the lanes.

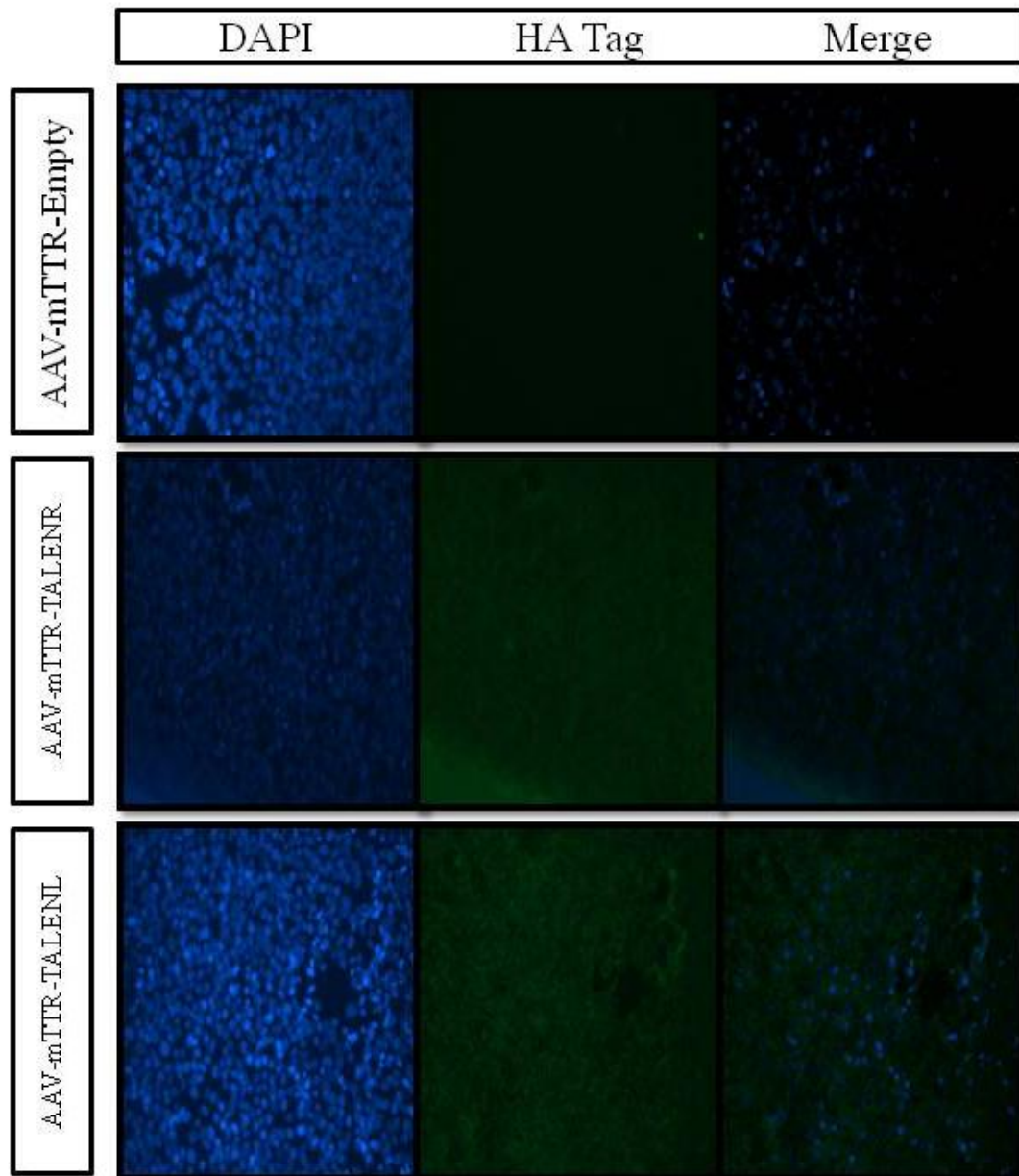


Figure 16: Detection of vector derived anti-HBV TALENs in Huh 7 cells. Immunofluorescence detection of the anti-HBV TALENs performed in Huh 7 cells. Huh 7 cells were transduced with AAV-mTTR-empty, AAV-mTTR-TALENR and AAV-mTTR-TALENL. DAPI was used to stain the nucleus. Cells were visualized using a fluorescent microscope at 20 × magnification. Images depicted above are a representation of several images from different fields and well.

Chapter 7

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Chapter 7- Reference

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