ABSTRACT

Title of Document: RECOMBINANT ADENO-ASSOCIATED VIRUS-MEDIATED GENE THERAPY FOR TREATMENT OF FAMILIAL HYPERCHOLESTEROLEMIA IN RABBITS

Daniel Atlas, Elizabeth Buckshaw, David Hanna, Jennifer Lannon, Alia Mahmud, Mary Mirvis, Anna Pham, Nadira Ramnarain, Paul Randazzo, Michelle Safferman, Jeremy Zuckerberg

Directed By: Dr. Ioannis Bossis

Familial hypercholesterolemia (FH) is a genetic disorder characterized by abnormally high concentrations of low-density lipoprotein-cholesterol (LDL-cholesterol) in the blood that can contribute to heart disease. FH can result from a defect in the gene for the LDL receptor (LDL-R). FH patients lacking functional LDL-R may benefit from viral-mediated transfer of a functional copy of the open reading frame (ORF) of the LDL-R. Since a recombinant adeno-associated virus (rAAV) is not immunogenic and can be mass-produced, it shows promise for gene therapy applications. AAV6 and AAV8 have been shown to specifically transduce hepatocytes in several species, which normally remove the majority of LDL-cholesterol from the blood via LDL-R-mediated endocytosis. Because of the potential of rAAV to treat FH by delivery of a correct LDL-R ORF to hepatocytes, the liver specificity of these two AAV serotypes was evaluated. Additionally, rabbits were chosen as the animal model for this study because a specific strain of rabbits, Watanabe heritable hyperlipidemic (WHHL), adequately mimics the pathology of FH in humans. Exposure of rabbit liver to rAAV with the marker LacZ and subsequent inspection of liver tissue showed that AAV8

transduced rabbit liver more efficiently than AAV6. To assess the feasibility of producing a rAAV capable of transferring the LDL-R ORF to rabbit hepatocytes *in vivo*, rAAV8-LDL-R was mass-produced by a baculovirus system in suspension grown insect cells.

RECOMBINANT ADENO-ASSOCIATED VIRUS-MEDIATED GENE THERAPY FOR TREATMENT OF FAMILIAL HYPERCHOLESTEROLEMIA IN RABBITS

By

Team Gene Therapy

Daniel Atlas, Elizabeth Buckshaw, David Hanna, Jennifer Lannon, Alia Mahmud, Mary Mirvis, Anna Pham, Nadira Ramnarain, Paul Randazzo, Michelle Safferman, Jeremy Zuckerberg

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Advisory Committee:
Dr. Ioannis Bossis, Chair
Dr. Georgiy Belov
Dr. Robert Kotin
Dr. Douglas Powell
Yogendra Singh Rajawat
Dr. David Straney

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CHAPTER 1: INTRODUCTION

1.1 Team Gene Therapy

The Gemstone Program at the University of Maryland, College Park is a four-year multidisciplinary Honors program in which undergraduate students, as a team, create and execute a research project with the help of a faculty mentor. Students gain a unique perspective on the research process by directing all aspects of their project, from inception to completion. This longitudinal project culminates in a thesis defense and provides undergraduates a valuable experience that often focuses on science and technology in society.

Team Gene Therapy was founded in the spring of 2010 with the purpose of exploring gene therapy as a treatment of prevalent genetic diseases. More specifically, the team decided to focus on familial hypercholesterolemia (FH), a good candidate for gene therapy, due to its monogenic nature and limited current treatment options.

1.2 Prevalence of Familial Hypercholesterolemia

Heart disease, the leading cause of death in Americans, ranges from coronary artery disease to irregular heart rhythm (1). One particular metabolic disorder, FH, is an inherited genetic condition in which poor lipid metabolism predisposes individuals to severe premature cardiovascular disease (2). This disorder results in heart attacks at a young age through the elevation of blood lipoproteins, specifically low-density lipoprotein-cholesterol (LDL-cholesterol).

The most common cause of this elevation is a mutation in the LDL-

receptor (LDL-R) gene. In most countries, about one in 500 people are heterozygous for a mutation in the LDL-R gene (2). Those possessing the heterozygous mutation may suffer from heart attacks starting at the age of thirty because of an approximate two-fold increase in LDL-cholesterol levels (3). Homozygotes for the mutation, however, typically have a six- to ten-fold increase in LDL-cholesterol levels, which leads to severe atherosclerosis throughout childhood and adolescence (4). By studying the population of FH homozygotes, the manifestations of the mutant allele can be observed without any confounding effects, providing genetic proof that elevated LDL-cholesterol alone produces atherosclerosis in humans (3).

1.3 The Role of Gene Therapy in Modern Medicine

Gene therapy is an approach involving the delivery of genetic material to host cells to treat disease. Several challenges to the progress of gene therapy include limited transgene expression, toxicity, inflammatory responses, and random gene integration. Despite these existing challenges, this relatively young field is rapidly expanding.

In 1972, Dr. Theodore Friedmann and Dr. Richard Roblin first began to investigate gene therapy as a solution to address human genetic diseases (5). In 1990, the National Institutes of Health (NIH) gained approval to conduct the first experiment using gene therapy in humans. The subject was a four-year-old girl with an adenosine deaminase deficiency. Although the improvement was only temporary, the initial success further validated the approach of gene therapy (6).

The momentum gained from this study was set back by the death of a patient, Jessie Gelsinger, during Dr. James Wilson's study in 1999, calling into question the safety of gene therapy practices. As a result, numerous clinical studies were suspended pending further review of both procedural and ethical principles. Though this incident resulted in many obstacles to the progress of gene therapy, including government investigations and congressional hearings, these efforts also refined the field and ensured the safety of future patients (7).

In 2012, the European Union approved Glybera®, a gene therapy pharmaceutical for those suffering from the rare genetic disorder familial lipoprotein lipase deficiency (8). This drug treatment, produced by Amsterdam Molecular Therapeutics, is the first to be approved on a significant international scale (8). Ultimately, this approval legitimizes the potential of gene therapy as a realistic treatment option.

1.4 Animal Models Currently Used in Gene Therapy Trials

In the field of gene therapy, a wide variety of animal models ranging in complexity and applicability to humans have been used to study inherited diseases. Though mice have been commonly utilized, problems include their short lifespan and their inability to adequately mimic many human diseases and genetic backgrounds (9). To address these limitations, researchers have instead used animal models tailored to the particular disease of interest. For example, researchers have used canines, felines, equines, and bovines, in order to study hemophilia, alpha-mannosidosis, osteoarthritis, and citrullinemia, respectively (9).

The longer lifespan and similar disease etiology lend these animals to the optimal study of these diseases.

For this study, rabbits were selected as the animal model rather than mice given that rabbits are better able to represent FH in humans. The pathology of FH in a certain strain, Watanabe heritable hyperlipidemic (WHHL) rabbits, closely mimics the pathology of FH in humans. Specifically, these rabbits are genetically inbred to have defective LDL-R, which causes them to exhibit similar lipid metabolism and symptoms as those seen in humans with FH. In comparison, a knockout mouse model does not produce the LDL-R protein at all and requires a high fat diet to induce hypercholesterolemia. In humans, however, hypercholesterolemia can result from defective LDL-R alone; therefore, knockout mice are a poor representation of FH in humans. The selection of WHHL rabbits for this study proved to have the highest potential clinical applicability.

1.5 Project Overview

This study was conducted to design and mass-produce the most effective gene delivery system for transduction of the liver of the WHHL rabbit strain to treat hypercholesterolemia. First, the LDL-R mRNA of the New Zealand White (NZW) rabbit was isolated, cloned, and sequenced. Then, the most effective recombinant adeno-associated virus (rAAV) serotype for transduction and long-term expression of the NZW rabbit liver was determined via β-galactosidase assays and quantitative polymerase chain reaction (qPCR) analysis of liver samples. After determination of the optimal serotype, the rAAV serotype

of choice containing a liver-specific promoter and the LDL-R open reading frame (ORF) was mass-produced for injection into WHHL rabbits.

1.6 Research Aims

The team chose to implement a gene therapy approach in both NZW and WHHL rabbit models to study FH using the following three aims:

Aim 1: Sequencing of the full coding region of the LDL-R mRNA

In order to insert the functional wild type (WT) gene for delivery via gene therapy into the WHHL model, the complete LDL-R mRNA sequence in NZW rabbits was first determined. Previous research had attempted to sequence the gene in its entirety; however, nearly 20% of the mRNA, including the 5' untranslated region (UTR), remained unknown. Team Gene Therapy looked to discover the complete sequence to aid in development of an optimal recombinant viral vector.

Aim 2: In vivo delivery of rAAV6-LacZ and rAAV8-LacZ in NZW rabbits

Rabbits were injected with uniform concentrations of saline and either rAAV6-LacZ or rAAV8-LacZ in order to determine which serotype was most effective for liver transduction. Rabbits were sacrificed either 10 or 28 days post-injection and liver samples were analyzed for expression of LacZ and concentration of viral DNA.

Aim 3: Large-scale production of rAAV8-LSP-LDL-R

The shuttle plasmid containing the LDL-R ORF and a liver-specific promoter was prepared for rAAV8-LSP-LDL-R production by baculovirus infection of insect cells in suspension. This system produced the necessary quantity of viral particles for injection into WHHL rabbits, verified by real-time PCR.

1.7 General Research Hypothesis

Since different AAV serotypes show a wide range of tissue tropisms, we hypothesized that one serotype would more significantly transduce the NZW rabbit liver and express the marker protein β-galactosidase. We also hypothesized that shuttle plasmid transfection and recombinant viral infection would show LDL-R expression *in vitro*.

1.8 Project Significance

Determining the optimal serotype for rabbit liver transduction will aid in future gene therapy modeling in the WHHL rabbit, which is known to closely mimic FH in humans. Future implementation of rAAV with a functional LDL-R gene into WHHL rabbits would contribute to modern medicine. This study will contribute to the development of gene therapy to treat FH in higher mammals and could provide justification for further research into the efficacy of this approach in humans. A previous study has shown that gene expression in WHHL rabbits transduced with a recombinant adenovirus containing a human LDL-R

complementary DNA (cDNA) is transient, lasting less than a week (10). To improve on past studies, we suggest the following innovations to the gene delivery approach in rabbits:

A) Injection of WHHL rabbits with an AAV vector, rather than an adenovirus, carrying the LDL-R gene;

B) Transduction of rabbit hepatocytes with the rabbit LDL-R gene rather than with LDL-R from another species or chimeric constructs;

C) Mass-production of the appropriate viral vector using the baculovirus system;

D) Utilization of a promoter that allows for long-term constitutive expression.

Chapter 2: Literature Review

2.1 Genetic Disorders

Genetic disorders are those caused by defective genetic material. Such a defect can result in disease by causing a deleterious change in a biologically important molecule such as catalytic RNA or a protein (11). Some genetic diseases result in developmental defects and may not be treatable by subsequent correction of expression. For instance, correcting gene expression in individuals with Down's syndrome (trisomy 21) during adulthood is unlikely to be effective in treating the disease (12). On the other hand, some genetic disorders are caused by constitutive abnormal expression. Unlike developmental defects, diseases that result from constitutive lack of or abnormal expression may be treated by its correction. For example, factor IX deficiency, in particular, is an X-linked recessive disorder has been treated in mouse and canine models using a gene therapy approach (13).

Several causes of genetic disorders exist including chromosomal abnormalities, single gene defects, and polygenic disorders. Chromosomal abnormalities can assume different forms. Certain disorders stem from an abnormal number of chromosomes. Other abnormalities include deletions, inversions, and translocations (11). Single gene defects are caused by the inheritance of one defective gene. Methods of inheritance include dominant and recessive. In the case of dominant inheritance, only one abnormal allele may cause phenotypic expression of the disorder. Two defective alleles are required for disease manifestation in recessively inherited disorders (11). Polygenic

disorders are caused by variations in multiple genes. Contributions of multiple genes to a phenotype may cause a particular trait to show varying degrees of penetrance. While diseases resulting from chromosomal abnormalities and variations in multiple genes may not be amenable to treatment by gene therapy, gene therapy may be effective for treating individuals suffering from disorders that result from constitutive lack of expression of a single functional gene (11).

Different methods of treatment and prevention exist for these various genetic disorders. For example, for a genetic disorder that affects metabolism and disrupts the production of certain enzymes, a dietary change may be sufficient to prevent the accumulation of toxic substances that are normally broken down by these essential enzymes (14). Other treatments for genetic diseases specifically address the symptoms. For instance, a heart defect may be managed with surgery, and defective blood cell formation may be treated with a bone marrow transplant (14). These treatments do not address the disorder at the genetic level; rather, they aim to alleviate the symptoms. A different treatment strategy, however, accounts for the mutation itself by introducing genetic material via a gene therapy approach.

2.2 Familial Hypercholesterolemia

FH is an autosomal dominant genetic disorder of lipid metabolism. In many individuals with this disease, LDL-R has a diminished ability to remove LDL-cholesterol from the bloodstream (15).

2.2.1 Genetics of the Disease

Phenotypic expression of clinical hypercholesterolemia results from mutations at one of three gene loci. Most frequently, FH arises from a mutation in the gene that encodes LDL-R. This gene is located on chromosome 19p13.2 from 11,200,038 bp to 11,244,506 bp, making the gene 44,469 bp long with 18 exons (17). The full-length WT protein product is comprised of 860 amino acids (16). Over 700 variants of the gene have been shown to lead to FH (17, 18).

Mutations in the apolipoprotein B-100 gene (APOB) may also lead to FH. Located on chromosome 2p23-24, the APOB gene is 43 kb long with 29 exons. The 4,536 amino acid APOB protein serves as a ligand for LDL-R for internalization of LDL-cholesterol. Alterations of this gene can lead to hypercholesterolemia and are termed familial defective apolipoprotein B-100 (17).

Finally, FH symptoms may result from mutations in the proprotein convertase subtilisin/kexin type 9 gene (PCSK9). Located on chromosome 1p32.3, the PCSK9 gene is 25 kb long, from 55,505,149 bp to 55,530,526 bp. The protein aids in cholesterol homeostasis by binding to the epidermal growth factor-like repeat A domain of the LDL-R and inducing its degradation. (19)

Mutations in the above three loci, the LDL-R, APOB, or PCSK9 genes, can be categorized within one of the following 5 classes of mutations: (17, 18)

Class 1

The class 1 mutation is termed a null mutation in that it results in a complete lack

of production of LDL-R. This may result from a deletion of the LDL-R promoter upstream of the gene itself, or a nonsense, frameshift, or splicing mutation.

Class 2

Because the LDL-R protein is a membrane protein used for internalization of extracellular particulate matter, it must be transported to the cell surface before it is effective. Class 2 mutations result in intracellular LDL-R transport defects between the endoplasmic reticulum and the Golgi complex. Within the class 2 mutations are two further specifications termed class 2A and class 2B. In class 2A mutations, the LDL-R protein fails to be transported out of the endoplasmic reticulum. In class 2B mutations, termed "leaky" transport, the rate of LDL-R transport to the Golgi complex is significantly reduced.

Class 3

Class 3 mutations, which are most common, result in the failure of LDL-R to bind LDL-cholesterol. Such mutations are often located in the ligand-binding and epidermal growth factor precursor regions of the gene. It is important to note that WHHL rabbit strain is an apt model for the resulting disease in that they have this class of mutation.

Class 4

Class 4 mutations are the rarest of all the classes. In such cases, while the LDL-R binds to LDL-cholesterol, it cannot internalize it. Specifically, the amino acid

mutation occurs in the cytoplasmic domain or the membrane-spanning domain, preventing LDL-R collection on the cell surface and endocytosis.

Class 5

In a class 5 mutation, which is also rare, the LDL-R can bind and internalize LDL-cholesterol, but cannot dissociate from it. That is, the LDL-R cannot release the particles in the endosome. Thereby, the LDL-R is not recycled to the cell surface.

2.2.2 Prevalence of the Disorder

The prevalence of FH is different between its heterozygous and homozygous forms. Heterozygous FH is the most common inherited metabolic disorder present in one of 300 to 500 people depending on ethnicity (20). This approximate 0.2 to 0.33% occurrence pertains to people in the United States and United Kingdom (21). For certain populations, such as Chinese Canadians, French Canadians, Finns, Dutch, Icelanders, Lebanese Christians, Lithuanian Jews, and South African Afrikaners, the prevalence of heterozygous FH is higher due to the founder effect (21). The founder effect is the lack of genetic variation in a new population due to the genetic similarity among individuals that founded that small population. On the other hand, homozygous FH is much rarer, occurring one in one million people. This is because it requires two mutated alleles instead of one and because of its tendency to cause early death (20).

2.2.3 Phenotype

Individuals with FH have elevated blood levels of total cholesterol and LDL-cholesterol. Individuals who are homozygous for a polymorphism that causes a lack of LDL-R function show LDL-cholesterol levels between 650 to 1,000 mg/dL (20). Heterozygous individuals, however, have approximately 220 mg/dL of LDL-cholesterol (20). Normal levels of LDL-cholesterol are approximately 70 to 100 mg/dL of LDL-cholesterol. Total cholesterol levels in homozygous mutants are between 500 and 1,000 mg/dL. In heterozygous individuals, total cholesterol averages 325 to 450 mg/dL (21). When diagnosis or treatment is delayed or not performed, homozygous individuals develop accelerated atherosclerosis and may die in the first few years of life from coronary heart disease (20). However, blood tests report that individuals with FH maintain normal levels of triglycerides (15).

High blood LDL-cholesterol predisposes individuals to early atherosclerosis and severe premature cardiovascular disease (9). In some cases, heart attacks and death occur before the age of thirty (22). Other symptoms of FH include xanthomas over the elbows, knees, buttocks, tendons, and around the cornea of the eye, cholesterol deposits in the eyelids, and angina (15). Tendon xanthomas, specifically, occur in at least 70% of patients affected by the age of 50 (21). When coronary angiography is used, proximal obstructive lesions, coronary ectasia, thickened aortic valves, and supravalvular aortic stenosis are generally observed (21). Some of these symptoms can develop as early as infancy (22). A strong family history of high levels of total and LDL cholesterol, early heart

attacks, or therapy-resistant levels of LDL-cholesterol is a risk factor for FH (22). A clinical diagnosis can be made from these family history factors, the presence of tendon xanthomas, and high levels of LDL-cholesterol in the blood (21).

2.2.4 Role of Defects of LDL-R in FH

LDL is a chylomicron that transports cholesterol in the blood to tissues. It originates from VLDL after losing most of its triglycerides and exchanging apolipoproteins (23). LDL-cholesterol remains circulating in the bloodstream until it is taken up by LDL-R located within the plasma membrane of the liver via specific receptor-mediated endocytosis (23). The LDL-R diffuses through the plasma membrane until it associates in clathrin-coated pits. When these coated pits pinch off to form coated vesicles, LDL-cholesterol particles bound to the LDL-R in the coated pits are internalized by hepatocytes (24).

Once inside hepatocytes, the LDL-cholesterol is delivered to the early endosomes, late endosomes, and finally the lysosomes, where cholesteryl esters in LDL-cholesterol are metabolized and released as free cholesterol to be utilized as necessary (24,25). Because LDL-R is responsible for the uptake of LDL-cholesterol, it helps regulate the amount of LDL-cholesterol in the bloodstream. The LDL-R in the liver clears approximately 70% of circulating LDL-cholesterol (26). Therefore, it makes the largest contribution to the removal of LDL-cholesterol from the blood. When this function is disrupted, levels of LDL-cholesterol in the blood increase, which can cause the formation of atheromas (26).

2.2.5 Current Available Treatments

Treatments varying in degree of intensity and invasiveness are currently available for the heterozygous and homozygous forms of FH. They function by lowering the levels of LDL-cholesterol in the bloodstream and thus decreasing the possibility of atherosclerotic heart disease. One treatment option is lifestyle change; exercising as well as dieting to limit the total amount of fat to 30% of the total daily caloric intake can, to a certain point, help lower cholesterol levels (22).

The next option of treatment for FH involves drug therapy, which is often suggested in combination with a healthier lifestyle. Statins, for example, are capable of lowering the risk of cardiovascular disease by 80% when initiated during adulthood (20). These drugs are competitive inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme that catalyzes the rate-limiting step of cholesterol synthesis (21). This inhibition causes a decrease in cholesterol levels inside the cell. Liver cells respond to this decrease by increasing production of LDL-R (21). Because the mechanism by which statins lower LDL-cholesterol in the blood relies upon the functionality of LDL-R, in homozygotes, statins alone are ineffective (20). Other contraindications include statin intolerance, which can lead to severe rhabdomyolysis and kidney failure (20).

Other drugs include bile acid sequestrant resins, ezetemibe, nicotinic acid, gemfibrozil, and fenofibrate (22). These drugs can be used in combination with statins, which may lower LDL cholesterol levels by an additional 25%. These drugs also lower levels of triglycerides and lipoprotein(a), both of which

are linked to cardiovascular heart disease but are not targets of statin treatment (20). Thus, combining several drug treatments further eliminates the potential for complications from FH.

The above treatments have been sufficient for some individuals who are heterozygous for FH. However, homozygotes and some heterozygotes may require stronger treatment options to compensate for higher levels of LDL. Medicines alone are only capable of lowering LDL-cholesterol in the bloodstream of homozygous patients by at most 10% (20). As an alternative to drug therapies, these individuals may require a transplant to obtain a liver with functional LDL-R or need LDL apheresis to remove LDL-cholesterol from their blood circulation (22). LDL apheresis, though invasive, has many benefits including decreasing LDL-cholesterol levels by 50%, reducing lipoprotein(a) levels, and mitigating atherosclerosis (20).

Ultimately, the ideal FH treatment may be a gene therapy approach in order to deliver functional genes of interest to targeted cells. This strategy is promising, as it would directly address the problem of defective LDL-R.

2.3 Gene Therapy

Gene therapy is an approach that entails delivering genetic material into a patient in order to treat diseases. For example, gene therapy might entail introducing DNA encoding for a functional protein into the host's cell. Introduction of the therapeutic genetic material may require a vector, such as a viral vector. (27).

Two major classes of methods for gene therapy exist: 1) the usage of non-viral methods and 2) the usage of viral vectors.

2.3.1 Non-Viral-Mediated Gene Therapy

Non-viral-mediated gene therapy focuses on inserting and maintaining DNA into a host's cell without the use of a virus. Although non-viral vectors are less efficient at long-term foreign gene expression than viral vectors, non-viral vectors hold several advantages compared to their counterpart. These include the non-pathogenicity, non-immunogenicity, ease of use, inexpensive large-scale manufacturing, and flexibility of transgene size. Further, non-viral delivery methods pose less of a risk of integration of the gene causing a mutagenic effect (28). However, despite the advantages of non-viral-mediated gene therapy, low gene expression and transfection efficiency may limit the practical applications and commercial viability of these treatment options for genetic diseases (29).

2.3.1.1 Naked DNA

Naked DNA, which has no other additives to protect it from degradation, can either be safely injected directly into areas of the body such as skeletal muscle, skin, liver, and thyroid or be administered systemically to affect an entire system or body (30). For example, intramuscular injection of naked DNA has been used to express pharmacologically active molecules such as erythropoietin and leptin (31). Additionally, antitumor immunity has been induced by intra-tissue injection of a variety of tumor inhibitory genes (31). However, without the

assistance of a carrier vector, either the liver quickly clears the foreign DNA from the body or restriction nucleases degrade the DNA (32). In recent years various physical technological advances have improved the efficiency of DNA uptake by allowing direct penetration of DNA through the cell membrane. Naked DNA can be administered to cells via electroporation, sonoporation, or use of a gene gun. Electroporation uses pulses of high voltage to temporarily form pores in the cell membrane. Sonoporation involves ultrasonic waves rather than electricity. The use of a gene gun entails shooting gold particles coated with DNA through the cell membrane into the cytoplasm and the nucleus, bypassing the endosomal compartment (30). In general, naked DNA delivery systems are much less effective in higher primates compared to small laboratory animals.

2.3.1.2 Lipoplexes and Polyplexes

Because of the inherent problems with using viral vectors, liposome-based gene delivery, which was first created by Dr. Philip Felgner in 1987, is still one of the most promising techniques for gene delivery into cells. Lipoplexes have found the most success in treatment of cancer cells, where injections of genetic material have activated tumor suppressor control genes and decreased oncogenic activity (33). Anionic and neutral lipids were first used to construct liposomes and polymers that when combined with DNA are labeled as “lipoplexes” or “polyplexes,” respectively (33). Because the construction of anionic lipids is time and labor intensive, researchers chose to use cationic lipids instead. Cationic lipids, because of their positive charge, were first used to condense negatively

charged DNA molecules so that DNA can then be bundled into liposomes for genetic insertion. Cells take up these charged particles via endocytosis, which creates endosomes within the cell. If the membrane of the endosome does not degrade, nucleic acids cannot be released into the cytoplasm and can be easily destroyed by lysosomes before fulfilling their purpose (30). Because cationic lipids, which are toxic, do not allow for "endosomal escaping," transfection efficiency is low (33).

2.3.1.3 Inorganic Nanoparticles

Inorganic nanoparticles have also been used for gene delivery. For instance, calcium phosphates, gold, magnesium phosphates, and iron oxides can be used to deliver genetic material into cells. Nanosized inorganic materials approximately 100 nm in length have shown the capacity to trap and protect the DNA efficiently from degradation, allowing the whole DNA to escape endosomes. Additional benefits associated with inorganic particles include low manufacturing costs, resistance to microbial attack in a host, low immunogenicity, and greater ability than organics to attach to cell lines in tissue cultures, leading to greater gene transfer efficiency. However, inorganic nanoparticles exhibit no tissue specificity (34).

Although these non-viral-mediated methods have shown some effectiveness in gene delivery and advantages compared to the use of viral vectors when used *in vitro*, viral vectors currently show more promise in the field of gene therapy and effectively treating genetic diseases in animals *in vivo.*

2.3.2 Viral-Mediated Gene Therapy

It was shown in the late 1960s that polyomaviruses such as SV40 were capable of incorporating their genetic information into the genomes of target cells. More importantly, target cells retained expression of this transferred viral genome (35). This discovery opened the gateway for the use of viruses as the agent to introduce therapeutic genes to ultimately overcome genetic diseases.

Viral-mediated gene therapy involves transduction, the process in which DNA is introduced into a cell by a virus (36). After assembling the viral vector construct, it is then administered to the appropriate subject, delivering its "cargo," or the gene of interest, to the cell (37). Transduction may address a defect by providing genetic material or raising expression levels of an existing gene (37).

The most important criterion for successful implementation of gene therapy is the selection of the viral vector, as it is the vehicle used to deliver the gene of interest into the host. The viral vector construct must contain the desired gene and the essential viral sequences needed for the assembly of viral particles, with dispensable viral genes eliminated to reduce patho- and immunogenicity (38). The ideal construct fulfills the following six parameters for safe and effective delivery of the transgene (38):

1) *Adequate production*: Many viral particles are needed for sufficient transduction of target cells.

2) *Safety*: The viral vector should not elicit an immune response that would harm the target cells or host subject. Some known viruses can integrate their genetic material into the host's genome. Such insertions have been shown to cause cancer.

3) *Sustained and regulated expression of the gene of interest*: The gene of interest should be expressed for a long period of time. Brief expression of the newly introduced transgene would thus be inefficient.

4) *Targeting of the viral vectors*: Because the transduction efficiency of a particular tissue can vary dramatically among different viral vectors, it is necessary to choose one specific for the tissue to be affected.

5) *Infection of dividing and nondividing cells*: If the target organ is in a postmitotic, nondividing state, viral vectors must be capable of targeting these somatic cells.

6) *Site-specific integration*: Integration into the host genome at specific sites could enable the repair of specific mutations without random integration of gene sequences.

Discussed below are a variety of available viral vectors and the associated advantages and disadvantages of their use in gene therapy trials**.** The characteristics of these vectors are compared in Table 1.

2.3.2.1 Herpes Simplex Virus Type 1

Herpes simplex virus type 1 (HSV-1), currently the most extensively engineered herpes virus, is highly infectious and can be applied in a wide range of animal models. The virus has been classified as a feasible candidate for gene transfer *in vivo* because it remains viable after initial infection in humans and is able to stably and latently transduce non-dividing cells (39). Additionally, the large genomes of HSVs allow for the insertion of 30-40 kb of foreign genetic material, whereas other viruses such as adeno-associated viruses (AAVs) and SV40s have a maximum transgene capacity of only 4.7 kb in most cases (39). This comparatively large size allows HSV to hold at least 30 kb of non-HSV sequences representing large single genes or multiple transgenes that may be expressed simultaneously (39). Further, more than 80 HSV genes can be removed without compromising infectivity; thus, the virus is convenient for both gene deletion and replacement (40, 41). However, HSVs are unable to induce long-term expression in certain tissues, and the complexity of HSV attachment and entry causes several problems in targeting of a vector to a particular tissue (39). Currently, more clinical testing must be completed to measure the true safety and effectiveness of HSV for gene therapy. Drawbacks of HSV also include its immunogenicity and inability to be mass-produced (40, 41).

2.3.2.2 Retrovirus

Retroviruses are enveloped 8-12 kb RNA viruses carrying two identical copies of genomic RNA that resemble cellular mRNA. The virus infects host cells by entering the cytoplasm by receptor-mediated endocytosis. Reverse transcriptase is then activated to transcribe the retroviral genome into DNA, which is then inserted into the cell's chromosomes by viral integrase in order to form the provirus. The newly created viral DNA may randomly integrate into the host DNA at various locations; however, some retroviruses "prefer" certain sites for integration. This process allows for stable long-term expression, but the direct insertion of the virus' genetic information into the host chromosomes may result in oncogenesis. This type of insertion may occur in the middle of an oncogene, triggering cancer. Additionally, retroviruses other than lentiviruses require dividing cells for infectivity. The only retroviruses that have been extensively explored for gene therapy applications are lentiviruses (42).

2.3.2.2.1 Lentivirus

Lentiviruses are uniquely able to integrate their genome into the chromosomes of non-dividing cells such as neurons. They are able to infect both dividing and non-dividing cells by relying on active transport of the preintegration complex to enter the nucleus through the nuclear pores (43, 44). Lentiviruses such as human immunodeficiency virus type 1 (HIV-1) and animal lentiviruses have been studied as potential gene therapy vectors because they are less tumerogenic

than other retroviruses and tend to not insert their genetic material into promoters, but into the bodies of genes themselves (42, 43). Because they rarely integrate into transcriptional start sites, lentiviral vectors are less likely to cause cancerous cells than other retroviruses. Other advantages of lentivirus viral vectors include stable expression, transgene capacity up to approximately 8 kb, and low immunogenicity (43). However, similarly to HSV, lentiviruses have yet to be established as safe and effective to treat genetic disease in animal species. The possibility of insertional mutagenesis and pathogenic vector mutation remain obstacles to the widespread use of this vector (42, 43).

2.3.2.3 Adenovirus

Adenovirus is a non-enveloped, double-stranded DNA virus with over 50 distinct variations, divided into six subclasses. Adenovirus serotypes in subclass C have a very efficient mechanism for nuclear entry, making them popular choices for the transduction of a variety of cell types (45). Immunogenicity may prevent them from being used clinically as a viral vector. Previous trials have reported strong and even fatal immune responses to recombinant adenoviral vectors, limiting the longevity of transgene expression as well as threatening the health of the host (46). Adenoviruses have high worldwide prevalence. As a result, many humans have preexisting immunity so the potential for an immune response may preclude its use as a vector in gene therapy (47).

2.3.2.4 Adeno-Associated Virus

Adeno-associated viruses (AAVs) are non-pathogenic, single-stranded DNA viruses of the parvovirus family, which require co-infection with an adenovirus helper virus (48). The AAV genome includes two ORFs. The left ORF codes for Rep proteins for DNA replication; two promoters, *p5* and *p19*, regulate expression of the *rep* gene, which generates four replication proteins - Rep 78 and Rep 68 from *p5*, and Rep 52 and Rep 40 from *p19* (48). The right ORF contains the *cap* genes, which code for structural capsid proteins. Promoter *p40* regulates expression of the *cap* gene, which generates three structural proteins - VP1, VP2, and VP3 in approximately a 1:1:10 ratio (48).

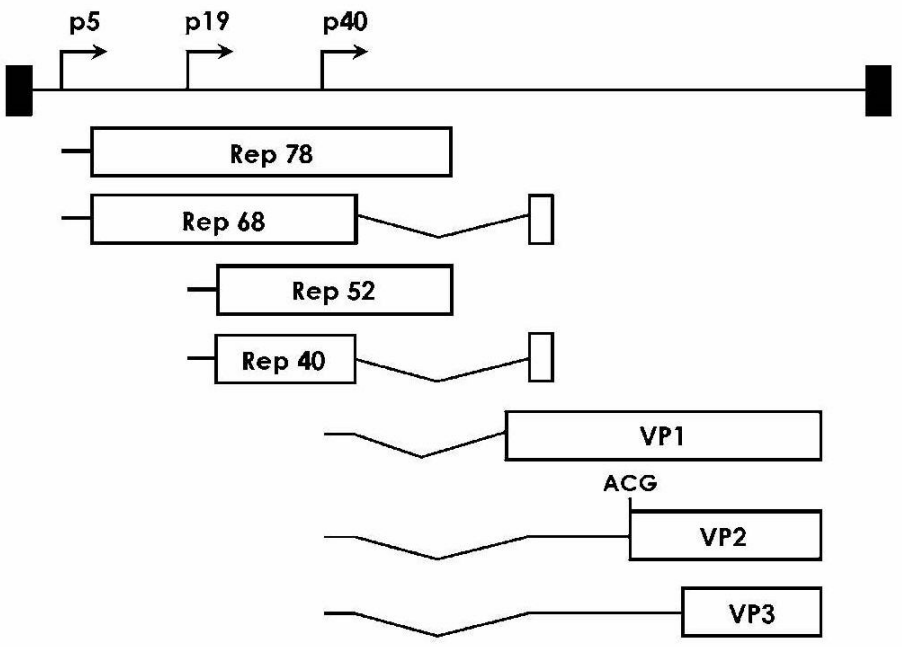


Figure 1: **Transcriptional map of AAV genome.** Map showing location of genes and promoters in adeno-associated virus genome (48).

Although various viral vector choices exist for gene therapy, clinical trials have shown AAV to be the most viable option for several reasons. AAV is the only viral vector that has been used to induce long-term expression safely in large animals, including primates both human and non-human. In the absence of significant immune responses in mammalian species, AAV effectiveness and gene expression can last several years (37). Compared to other viruses, AAV elicits less of a host immune response and provides few alterations in host gene expression during infection (49).

Additionally, AAV's non-pathogenicity, non-immunogenicity, heat stability, and resistance to changes in pH make the virus ideal for a lab setting (50). This particular virus has been shown to be safer than other viruses in that it is unable to integrate DNA into the host genome. Additionally, rAAV used in gene therapy will not become pathogenic due to its inability to replicate. Although wild type AAV may replicate in the presence of an adenovirus helper virus, rAAV lacking the rep and cap genes will not (49).

2.3.2.4.1 Adeno-Associated Virus Serotypes

Over the past few decades, at least twelve serotypes of AAV have been discovered and commonly used in research (51). Of these twelve, eight are able to infect primates. Tissue specificity varies with serotype; for example, different serotypes have been shown to target skeletal and cardiac muscle, lung, liver, retina, and brain tissues (48, 51). AAV2, the first and most popular serotype used in research, presents natural specificity for hepatocytes, neurons, and skeletal and

smooth muscle cells (52, 53). AAVs can be selected based on general tissue tropism, or engineered to have the desired tissue tropism based on the capsid serotype (54). For example, studies have shown that AAV8 vectors have been successful in transducing murine liver cells 100 times more efficiently than AAV2 vectors (55, 56). AAV6 is similar to AAV1 and AAV2, but has lower immunogencity than AAV2 and has shown proficient transduction of muscle, lung, and liver *in vivo* (57, 58). Therefore, AAV6 and AAV8 show promise as viral vectors for targeting and transducing the liver.

Viral Vector	Advantages	Disadvantages
HSV-1	- Large transgene capacity (30-40 kb) - Transduces non-dividing cells stably and latently - Allows for both gene deletion and repacement	- Difficulty maintaining long-term expression - Complexity causes problems in vector targeting
Retrovirus	- Stable expression - Low immunogenicity	- Require dividing cells for infectivity - Random integration into host DNA - High likelihood of mutagenesis and oncogenesis
Lentivirus	- Infect both dividing and non-dividing cells - Stable expression - Transgene capacity of approximately 8 kb	- Possibility of mutagenesis - Lack of safe usage in animal models
Adenovirus	- Efficient mechanism for nuclear entry - Broad host range	- High immunogenicity - Inflammatory response
AAV	- Broad tropism - Non-pathogenic - Low immunogenicity - Resistant to changes in heat, pH, and solvent - Stable, prolonged, transgene expression	- Small transgene capacity (4.7 kb)

Table 1: Comparison of viral vector choices for gene therapy.

2.4 Methods of Production of rAAV

Four rAAV production systems have been reported (59):

1) Production of rAAV in adherent HEK293 cells via expression of *rep*, *cap*, vg (viral genome), and helper adenovirus plasmids by transient transfection;

2) Production of rAAV in suspension-adapted mammalian cells infected with recombinant HSV-1 from which the *rep*, *cap*, and vg, are expressed;

3) Production of rAAV on a large scale in packaging cells infected with an adenovirus helper virus; and

4) Production in suspension-grown Sf9 via expression of *rep*, *cap*, rAAV genome from baculoviruses with which the Sf9 are infected.

The aforementioned production systems, 1, 2, and 3, suffer from one or more disadvantages. Production in adherent mammalian cells is not easily scalable. Production from a recombinant HSV is difficult due to the genomic instability of HSV. Production with an adenovirus helper virus creates the risk of contamination of AAV with toxic adenovirus. In contrast, the fourth production system described does not have these disadvantages, and can be scaled up to

produce large quantities needed for *in vivo* studies in higher mammals and humans. The recently approved Glybera was made using baculovirus technology (60). Thus, the baculovirus system may be the best reported system for large-scale production of rAAV at a good manufacturing practice level.

2.4.1 Production of rAAV in Adherent Cells

Transfection of adherent mammalian cells with a plasmid encoding the rep and cap genes, a plasmid encoding helper virus genes, and a plasmid encoding the rAAV genome is useful for the production of rAAV on the scale used for the transduction of cultured cells. In contrast, clinical applications, especially those that entail the transduction of large numbers of cells, require a much larger amount of rAAV (61). Since the number of passages transfected mammalian cells can undergo while the transfection efficiency persists is limited, increasing the amount of rAAV produced requires increasing the number of cells transfected. Thus, clinical scale production via transient transfection requires high transfection efficiency and the cumbersome technology of growing mammalian cells on a large scale. While some mammalian cell lines, such as HEK293, can be transfected efficiently, these cells are adherent, so the number of transfected cells is limited by plate area. Because the amount of transfection reagent needed increases proportionally with the number of cells to be transfected, this process may be too expensive to be used in the clinical scale production of rAAV (61).

Another difficulty in rAAV production in mammalian cells is the need for cotransfection of multiple plasmids. Even if each individual transfection is efficient, the efficiency of the cotransfection may be low. To improve cotransfection efficiency, the plasmid encoding helper virus genes can be replaced with an adenovirus helper virus (59). This approach, however, creates the risk of contamination of rAAV with adenovirus. Due to the toxicity of adenovirus, this risk may preclude clinical applications.

For small-scale production of rAAV, cultured mammalian cells can be transiently cotransfected with a helper plasmid containing the *rep* and *cap* genes and with a plasmid containing the recombinant genome to be included. Cotransfection of mammalian cells, however, is inefficient in that it uses only adherent cells (61). Therefore, although this mammalian expression system is widely used for production of small amounts of rAAV, it cannot be feasibly scaled up to produce the amounts of rAAV that will be required for studies in large mammals or clinical studies in humans.

Recently, a baculovirus/insect cell culture system has been developed by Dr. Kotin's group at NIH. Because it does not require large quantities of transfection reagents, does not rely on cotransfection of mammalian cells, entails purification by column chromatography rather than CsCl gradient centrifugation, and is amenable to automation, this system allows semi-industrial or large-industrial scale production of rAAV (61).

2.4.2 Required Levels of Viral Particles for Rabbits

Based on a rabbit's size, large quantities of rAAV are needed to adequately infect hepatocytes. Normally, ten to one thousand viral particles are required to infect a single cell because the particle-to-infectivity ratio of AAV vector preparation usually ranges from 10:1 to 1000:1 (49). The frequency of incomplete or empty viral particles, defective particles, and host cell susceptibility lends to the high ratios required for effective transduction (49). In addition, during trafficking from an endocytoplasmic vesicle to the nucleus, the vector particle may become ubiquitinated and directed to a proteasome for degradation instead of being directed to the nucleus for transgene expression (49).

A rabbit liver has approximately 10^{10} cells. Therefore, at least 10^{11} to 10^{13} viral particles, with the LDL-R cassette, would be needed to infect a large number of hepatocytes to attempt to lower LDL-cholesterol blood levels. To safely ensure an adequate number of functioning viral particles (10^{13}) reach the liver, about 10^{14} viral particles should be delivered in each rabbit. For quick and efficient large-scale production of the rAAV particles, a technique using baculoviruses can be implemented in order to attempt to achieve an observable level of expression of the LDL-R gene in the rabbits.

2.4.3 Production in Suspension-Grown Cells

Production of rAAV in suspension cells is more scalable than production in adherent cells because the number of cells can be increased proportionally to

the volume of the culture rather than to plate area (61). Two such examples include HSV-1 infection of mammalian cells and baculovirus infection of Sf9 cells.

2.4.3.1 HSV-1 Infection of Mammalian Cells

Production of rAAV from suspension-grown mammalian cells infected with a recombinant HSV containing rAAV genome is scalable but suffers from the risk of contamination of rAAV with HSV and HSV proteins. Because HSV is pathogenic and immunogenic, this risk may preclude the production of clinical grade rAAV with this system. Moreover, HSV suffers from genomic and physical instability, thus producing and storing intact recombinant HSV may be difficult (60).

2.4.3.2 Baculovirus Infection of Sf9 Cells

Alternatively, clinical-grade rAAV has been produced on a semi-industrial scale in baculovirus-infected suspension grown Sf9 cells (61). In this system, rep proteins, cap proteins, and viral genome are expressed from recombinant baculoviruses with which the Sf9 cells are infected. Originally, the *rep* genes, the *cap* genes, and the viral genome were each expressed from a separate baculovirus, but more recently *rep* and *cap* genes have been consolidated into one baculovirus so that rAAV can be produced by infection of Sf9 cells with only two

baculoviruses (61). Since a baculovirus infection can propagate throughout an insect cell culture from even a small number of initially infected cells, scaling the amount of rAAV produced in insect cells does not require a change in the amount of transfection reagent used to produce the initial baculovirus stock. Further, the baculovirus provides the helper functions needed for rAAV production, so neither an adenovirus helper virus nor a recombinant DNA construct from which helper genes are expressed is needed. Because no adenovirus helper is necessary, rAAV can be purified from the Sf9 without the risk of adenovirus contamination. Moreover, purification of rAAV from infected insect cells can be automated (61).

Infecting Sf9 cells with purified baculovirus may be difficult because of damage that the viral particles sustain due to the instability of purified baculovirus. In contrast, baculovirus is stable in living insect cells. For this reason, the baculovirus system uses titerless infected-cells preservation and scale-up (TIPS) to produce the rep and cap proteins. This system entails preserving cells infected with a baculovirus by freezing and introducing them into the culture to be infected with that baculovirus. The use of TIPS is preferable to the exposure of the suspension culture to purified baculovirus because it enables long-term preservation of the baculovirus stock and eliminates the need for determining viral titer. Because TIPS entails preserving baculovirus in frozen infected cells, it eliminates the difficulty of preserving purified baculovirus (61).

The optimal ratio of cells infected with baculovirus with the *rep* and *cap* genes to cells in the culture has already been established for existing stocks of frozen Sf9 containing this recombinant baculovirus. The ratio of the expression of

rep and cap proteins to the expression of rAAV genomes can affect the quality of the virus produced. Specifically, overproduction of rep and cap proteins relative to rAAV genomes will increase the fraction of empty viral particles. Since the use of frozen Sf9 infected with baculovirus with the rep and cap genes ensures that the optimal ratio of rep and cap genes to viral genomes will be produced in the suspension Sf9, TIPS eliminates the need to titer baculovirus with *rep* and *cap* genes and ensures that a large fraction of the physical rAAV particles produced in the suspension culture will contain rAAV genome (61).

Although the baculovirus genome is more stable than the HSV genome, the genomic instability of the recombinant baculovirus may make production of baculovirus with intact rAAV genome difficult. Since the recombinant baculovirus may suffer random deletions, it is important to sequence the recombinant baculovirus DNA and to verify the integrity of inverted terminal repeats (ITRs) in the recombinant baculovirus containing the rAAV genome. Because the secondary structure of the ITRs prevents PCR amplification and sequencing, a rescue assay is used to verify the integrity of the ITRs. This assay requires coinfection of Sf9 with the recombinant baculovirus containing the rAAV genome and with a bac-rep, from which two rep proteins but no cap proteins are expressed. The rep proteins produced from the bac-rep will replicate sequences flanked by functional ITRs. Thus, cells with recombinant baculovirus DNA containing a rAAV cassette with intact ITRs will accumulate copies of the rAAV genome. This replicated rAAV genome can then be detected by agarose gel electrophoresis (62).

In summary, the use of baculovirus-infected Sf9 cells for the production of rAAV at levels required for clinical trials is cost effective, reliable, non-pathogenic, and scalable. Production of rAAV in 200 liter Sf9 suspension cultures with consistently high yield has already been reported (62).

2.4.3.3 Purification of rAAV Produced Using the Baculovirus System

In order to administer the rAAV produced in insect cells to the rabbits, the rAAV must be purified from the insect cell lysate. In CsCl gradient purification, particles are separated by density in a solution of varying CsCl concentration. While this method is slow and cumbersome, it allows for separation of empty viral particles from full viral particles. Although CsCl gradient purification can be used to purify rAAV for some research applications, this technique may not be optimal for purification of rAAV for animal models or clinical applications (63). Generally, centrifugation techniques cannot be easily scaled up. rAAV can also be purified by affinity chromatography on a heparin column (58, 64). Purification using a heparin column, while fast, can suffer from low yield. Additionally, not all serotypes can bind to heparin. For the purification of some serotypes, immunochromatography may be used. For instance, if an anti-AAV6 antibody is available, it can be bound to sepharose and used for the purification of AAV6 by affinity chromatography.

2.5 Animal Models of FH

2.5.1 Presence of FH in Animal Species

The causes and effects of FH have been studied in few animal species, including rabbit, feline, and murine models. The feline model, in which cats inherit primary hyperchylomicronaemia, mimics many symptoms of FH in humans and is a possible candidate for FH research (65). However, some significant differences limit the applicability of this research to human FH. In particular, human FH primarily consists of LDL-cholesterol elevation in the bloodstream, while this cat model has elevated levels of triglyceride and cholesterol present in chylomicrons (66). Additionally, the genetics of the diseases differ: human FH is an autosomal dominant trait while the disorder in cats is autosomal recessive (67). These differences indicate that another animal model, such as mice or rabbits, may be more suitable for FH research. Furthermore, the use of companion animals such as cats and dogs for research is prohibited at the University of Maryland, thus making cats not an option for this project.

Murine models are commonly used for many types of research, including studies of heritable hypercholesterolemia and other diseases similar to FH (68). Although research involving most vertebrate animals must be approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC), the Animal Welfare Act exempts rats and mice from this regulation (69). Due to

this exemption, murine models are commonly used for research, including studies of heritable hypercholesterolemia and other diseases similar to FH (68). This model, however, is not an ideal analog of FH in humans because of intrinsic differences in the metabolic characteristics in both species (68).

One specific murine model induces hypercholesterolemia by imposing dietary changes in the subjects to include foods high in cholesterol, saturated fats, and bile acids (68). However, this diet-induced method is not the most effective, because the mice must be continuously fed this diet in order to sustain high levels of plasma LDL- cholesterol. If the diet changes, normal levels of LDL-cholesterol will promptly return in the mice subjects. For this reason, this model cannot accurately represent FH in humans, as the mice still possess the gene responsible for producing fully functional LDL-R. The hyperlipidemia is transient and self-correcting when the specialized diet is replaced with a standard diet (68).

Another form of a murine model that has been studied is the LDL-R -/- mouse, in which the mice were homozygous for the deletion of the LDL-R gene. These mice are frequently referred to as “knock-out” mice because the gene of interest has been deleted from the genome (70). Studies have shown that FH in humans causes premature atherosclerosis and highly elevated plasma levels of LDL-cholesterol, while LDL-R -/- mice have only slightly elevated LDL-cholesterol levels and little atherosclerosis. This can be attributed to the difference in the metabolism of LDL in the two species, specifically in the editing of apolipoprotein B, which transports cholesterol to tissues within the body (70). In an improved model of these LDL-R -/- mice, by additionally knocking out the

gene for the apolipoprotein B mRNA editing catalytic peptide-1 (APOBEC-1), mice have been bred with a lipoprotein profile that faithfully mimics that of humans with FH.

Figure 2 below provides a graphical comparison of lipid levels in mice of different genotypes.

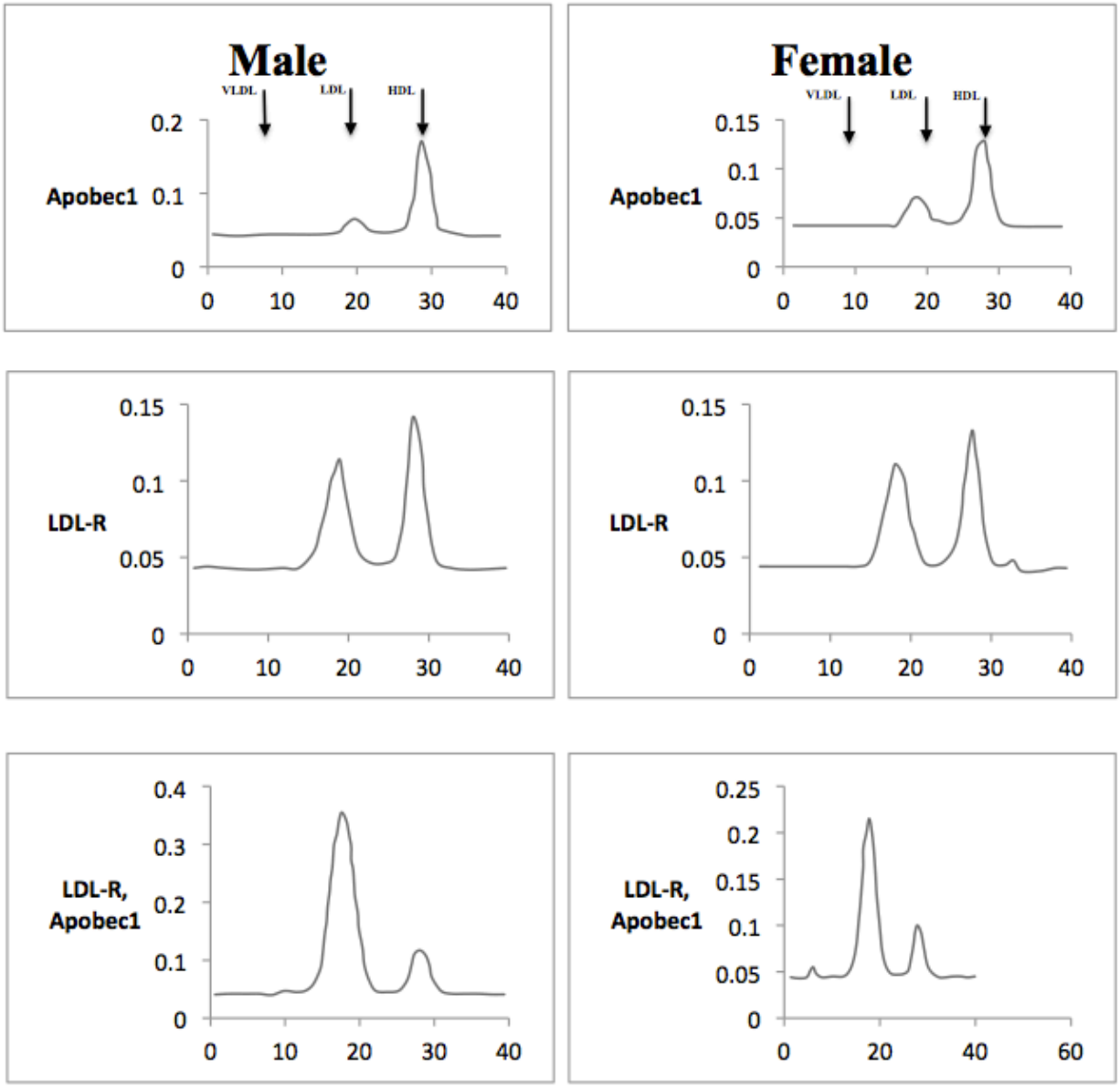


Figure 2: Plasma cholesterol distribution in knock-out mice. The measurements were found using fast performance liquid chromatography and the measurements are in OD units / fraction, with the downward pointing arrows representing the elution peak for each of the lipoprotein classes. Genotypes represented include Apobec1 -/-, LDL-R -/-, and LDL-R -/-, Apobec1 -/- (70).

Although this LDL-R -/-, Apobec -/- knock-out method induced spontaneously elevated lipid concentrations, premature atherosclerosis, and other clinical and metabolic signs similar to FH in humans, it is still not the most suitable model for studying FH (70). The knock-out mouse adequately mimics the symptoms, but not the pathology of FH because it does not possess the disease and thus LDL receptors are not synthesized at all. As previously described, humans with FH can still produce the LDL-R protein, but often times these individual's receptors insufficiently operate and cannot remove enough LDL from the bloodstream (70). Therefore, mice are not the most accurate animal model for human FH.

In comparison to the non-USDA regulated murine model, rabbits are a USDA-regulated species. Additional benefits of using rabbits as the model include their docility, non-aggression, and short vital cycles, making rabbits easy to handle and observe (71). Their size also proves advantageous in that rabbits are big enough to provide sufficient tissue samples for experimental work and small enough to be economical for studies (72) Furthermore, because the rabbit liver, at 10^{10} cells, is comparable in size to that of a human liver at 10^{11} cells, a similarly large number of viral particles is needed for adequate transduction. The WHHL rabbit strain, in particular, possesses the disease and exhibits symptoms of myocardial infarction, valvular diseases, and xanthomas. Both the phenotype and the pathology of the disease in WHHL rabbits closely mimic human FH.

Discussed below are two different rabbit species that have been selected as the animal model and the justification for their use in studies related to FH.

2.5.2 The Watanabe Heritable Hyperlipidemic Rabbit Model

The ideal animal model for FH displays genetic characteristics that are found throughout different stages of the human disease, ranging from fatty streaks in the early stages to plaques in advanced stages of disease (73). Lesions that occur in cholesterol-fed rabbits, one popular model, are different from those in humans, resembling more of a lipid-storage disorder rather than atherosclerosis (73). Such models involving induced diet changes are not optimal. Instead, the WHHL rabbit strain, which is similar both genetically and clinically to humans with FH, is the most characteristic animal model for FH.

WHHL rabbits possess a twelve-base frameshift deletion, or a deletion of four amino acids, within the cysteine-rich ligand-binding domain in the gene that encodes LDL-R (74). This specific mutation results in the production of a receptor that is the approximate molecular weight of a functional LDL-R; however, the receptor is transported to the cell surface at one-tenth the rate of the WT LDL-R. Such retardation of the transportation of the LDL-R from the endoplasmic reticulum, where the protein is made, to the Golgi, where it is modified post-translationally and sent to the plasma membrane, is due to the improper formation of disulfide bonds in the protein, leading to misfolding of the protein (74). In addition, the frameshift deletion reduces the affinity of LDL-cholesterol to its receptors so that the receptors, already few in number, that are

successfully transported to the cell's surface are less effective at removing LDL-cholesterol from the bloodstream (74). Ultimately, the cell is unable to internalize LDL-cholesterol, leading to high blood plasma LDL-cholesterol levels (75).

A similar mutation that causes 95% fewer LDL-R proteins in an individual with FH was observed in human FH patients and confirmed via S1 nuclease mapping of their LDL-R RNA (74). On a genetic level, WHHL rabbits and humans with FH are comparable. Both still produce the defective LDL-R, unlike knockouts, which do not produce the receptor protein at all.

Phenotypically, the reduction of LDL-R functionality in WHHL rabbits results in an atherosclerotic process starting *in utero*, with lesions in the aortic arch, deposition of cholesteryl esters in intimal and medial smooth muscle cells, and frequency of atherosclerotic plaques progressing with age (73, 76). Homozygous WHHL rabbits have plasma cholesterol levels reaching 900 mg/dL, the majority of which is LDL-cholesterol, and have a lifespan of two to four years compared to six years in NZW rabbits (73).

Though there are several phenotypic differences between humans with FH and WHHL rabbits, such as early distribution of lesions and involvement of the aortic valve with aortic stenosis, there are similarities as well (73). These similarities include the narrowing of the coronary ostia, with the progression of FH throughout the coronary arterial system, increased LDL-cholesterol levels in the bloodstream, and the presence of extensive tissue cholesterol deposits (73). Table 2 outlines the morphologic characteristics of homozygous and heterozygous WHHL rabbits, cholesterol-fed NZW rabbits, and atherosclerotic humans (73).

Table 2: Summary of morphologic findings in homozygous WHHL rabbits compared with cholesterol-fed WHHL heterozygous and cholesterol-fed control (New Zealand White, NZW) rabbits and humans with advanced atherosclerosis (73).

	WHHL homozygote	WHHL heterozygote	NZW	NZW regression[1]	Humans
Distribution of atherosclerosis					
			+++		
Proximal aorta	++++	++++	+	++++	++
Distal aorta	+++	+++	+++	+++	++++
			+++		
Pulmonary artery	++	++++	+	+++	+[2]
Cerebral artery	++	NE[3]	+[4]	+	+++
Coronary artery					
Epicardial	+++	+++	++	++	++++
Intramyocardial	++	+++	+++	+++	+
Involvement at flow dividers	+++	+++	+++	+++	+++[5]
Lesion types					
Fatty streaks	++	++	+	0	++
Fibrous plaques	++	+++	+	+	++
Fatty plaques	++	++	+	0	++
Complicated lesions	++++	++++	++	+++	++++
Lesion histology[7]					
			+++		
Foam cells	++	++	+	+	+
Cholesterol clefts	+++	+++	+	++++	+++
Fibrous caps	+++	+++	+	+++	+++
Calcification	+++	+++	+	++	+++
Necrosis	+++	+++	+	+	+++
Ground substance	+++	+++	++	+++	+++
Smooth muscle	++	++	+	++	++
Collagen	++	++	+++	+++	++
Myocardium					
Focal fibrosis	+++	+++	+	+	+++
Healed infarction					
Subendocardial	+++	+++	++	++	+++
Transmural	+[8]	0	0	0	+++
Extravascular lipid	+	++	+++	++	+[3]
Xanthomas	++	+	0	0	+++[3]

1 NZW regression = NZW rabbits fed 2% cholesterol for 8 weeks, followed by 5 months of normal diet (age at time of sacrifice, 12 months).
2 Associated with pulmonary hypertension in elderly patients
3 Not examined
4 From Atschul R: Selected Studies on Atherosclerosis, Springfield, IL, Charles C. Thomas, 1950.
5 Kjaernes et al. (Acta Pathol Microbiol Scand (A) 1981, 89:35) have noted a difference between human and cholesterol-fed animals for the localizationof
lipid at arterial branches.
6 Aorta.
7 Transmural infarction was found in 8 of 67 hearts from WHHL homozygotes examined by Hatanaka et al.
8 Homozygous familial hypercholesterolemia.
NZW rabbits were fed 0.5% cholesterol and 2% penut oil for 24 weeks and were 7 months of age at the time of sacrifice.
WHHL homozygous rabbits were 12 months of age.

WHHL rabbits and individuals with FH share both genetic and phenotypic characteristics, making WHHL rabbits the best representation of this disorder in humans.

2.5.3 Pathophysiology of WHHL Rabbits

WHHL rabbits are a fitting model for human FH because of the similarities between the pathophysiologies of the two organisms. In WHHL rabbits, different levels of LDL-cholesterol plasma concentration indicate differing amounts and functionalities of LDL-R since they are responsible for internalizing LDL-cholesterol. In order to determine what caused the specific effect, previous experiments involving WHHL rabbits and LDL-cholesterol have included Western blotting of hepatocytes in order to determine if proper protein expression was achieved (77). WHHL rabbits, which develop hypercholesterolemia and atherosclerosis spontaneously due to genetic and functional deficiencies of the LDL-R, present with higher concentrations of LDL-cholesterol in the blood than standard NZW rabbits.

According to a 1982 study by Havel, *et al.,* involving autoanalyzation of lipoprotein, triglyceride, and total cholesterol concentrations, female WHHL rabbits typically exhibit an LDL-cholesterol plasma concentration of 338 mg/dL as compared to the average of 11.9 mg/dL in NZW females - approximately a 30-fold increase. A 20-fold increase is observed in males with respect to LDL-cholesterol, while WHHL rabbits of both sexes have slightly lower plasma concentrations of high-density lipoprotein (HDL). Various other measurements,

including very low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) concentrations, are displayed in Table 3 below. (78)

Concentration of total cholesterol and triglycerides in plasma and lipoprotein fractions of normal and WHHL rabbits (mg/dL)

	Serum	VLDL	IDL	LDL	HDL
Total cholesterol					
Control Japanese males	38.3 ± 12.6	9.0 ± 7.8	6.7 ± 3.5	12.0 ± 3.5	7.0 ± 2.7
WHHL males	731.3 ± 53.2	91.7 ± 10.1	46.7 ± 20.0	451.3 ± 16.5	6.0 ± 2.7
Control NZW males	58.5 ± 1.9	17.0 ± 7.2	7.8 ± 3.5	11.5 ± 3.3	22.3 ± 4.4
WHHL males	487.5 ± 75.8	110.3 ± 41.5	116.3 ± 26.4	218.0 ± 68.1	8.3 ± 2.9
Control NZW females	46.8 ± 4.0	15.5 ± 4.4	8.5 ± 1.7	11.8 ± 1.3	18.0 ± 2.5
WHHL females	582.0 ± 143.3	106.0 ± 23.8	110.7 ± 21.1	338.0 ± 100.5	12.3 ± 2.5
Triglycerides					
Control Japanese males	64.3 ± 37.8	37.3 ± 30.4	7.0 ± 3.0	12.7 ± 4.0	5.0 ± 2.7
WHHL males	288.3 ± 106.1	57.0 ± 18.5	23.3 ± 12.9	162.0 ± 39.9	1.7 ± 1.1
Control NZW males	213.3 ± 27.4	128.5 ± 37.0	14.0 ± 5.8	15.5 ± 3.9	24.8 ± 4.9
WHHL males	435.0 ± 94.7	153.5 ± 48.0	78.3 ± 26.0	146.0 ± 32.8	4.8 ± 2.8
Control NZW females	108.0 ± 43.5	62.5 ± 32.5	11.8 ± 1.9	11.8 ± 1.7	24.7 ± 6.5
WHHL females	308.7 ± 128.8	124.0 ± 80.7	47.0 ± 21.7	129.7 ± 19.5	8.7 ± 1.5

Table 3. Comparison of lipid profiles between control and WHHL rabbits (2)

High blood concentration of LDL-cholesterol can contribute to the formation of atheroma. Macrophages engulf LDL-cholesterol, die, and become part of the atheroma. Advanced atheroma can result in infarction, ischemia, and aneurysms. While both alanine aminotransferase and alkaline phosphatase are found in various tissues throughout the body, elevated levels in the blood could be indicative of liver damage (79). Typically, alanine aminotransferase is found in very small concentrations in the rabbit liver. In rabbits, the normal range is 25-65IU/L (80). Significantly high levels are associated with hepatic damage and necrosis (79). However, the severity of the increase is not correlated with the severity of the disease (80). Alkaline phosphatase is expressed in the plasma membrane of hepatocyte cells that border the bile canaliculus, a structure

associated with the collection of bile. Normal levels are 10-70IU/, however its range varies widely among healthy rabbits (80). Increased levels of this enzyme can be associated with conditions of biliary stasis in which there is limited bile flow out of the liver (79).

2.6 Liver-Targeted Vector Delivery

The delivery route of the chosen vector to the targeted cells or organ can effect transduction. Several methods for gene delivery include *ex vivo, in vivo, in utero,* and surgical procedure; however, for the purpose of this study, only two methods that focus on delivering genes specifically to the liver will be discussed, hepatic portal vein injection (surgical) and peripheral ear vein injection (*in vivo).*

2.6.1 Hepatic Portal Vein Injection

The liver is one of the most complex organs for mammalian species, as it performs nearly 500 different functions including the regulation of cholesterol by synthesizing and exporting cholesterol to other cells and removing cholesterol and converting it into bile. Approximately 1.5 liters of blood/minute circulates through the liver, and the organ can hold nearly 10% of the body's total blood volume. Of the blood that passes through the liver, nearly 75% is derived from the hepatic portal vein, the blood vessel responsible for moving blood from the gastrointestinal tract to the liver. Unlike most veins, the hepatic portal vein does not deliver blood directly to the heart (81,82).

Gene delivery by hepatic venous injection occurs by first accessing and

installing ligatures, or sutures, around the jugular vein to prevent any blood flow to the area. An angiocatheter, later replaced by a balloon catheter, is then inserted into the vein and a guide wire penetrates the hepatic vein with a fluroscopic control. After gaining access to the hepatic vein, a volume of solution containing the viral vector is then injected directly into the vein (83).

Delivering a viral vector solution into the hepatic vein allows for the virus to directly target the liver without affecting numerous other organs and cells. The biggest risk with this procedure is the possibility of complications during surgery including blood loss, hepatic injury, and hemorrhage due to the force of injected fluid. A surgical procedure of this nature also requires highly skilled and trained surgeons with the appropriate lab equipment to avoid complications.

2.6.2 Ear Vein Injection

Injection of a viral vector via a peripheral vein may be the simplest method of delivering a virus to host cells. Peripheral veins are the most common access point for intravenous therapy in hospitals and paramedic services (84). If the virus has high specificity for the target organ, viral injections through peripheral veins may be appropriate as the injected serum will preferentially target the tissue of interest even though such an injection would lead this virus to pass through several organs on the way to the liver (85). Because AAV6 and AAV8 show high liver specificity, injection through a peripheral vein may be an adequate method for gene delivery to the liver (55-58). Research into the injection of vectors through the rodent tail vein causes a large volume of the injected

solution to be forced into the vena cava, creating a backup of fluid that ultimately ends up in the liver. Reports have found transgene expression levels approximately 100-fold lower in the heart, spleen, and kidney than in the liver after viral injection in rodents (85).

For rabbits in particular, the marginal ear vein, a peripheral vein, is commonly used for the removal of blood and intravenous injections (84). Preliminary research of marginal ear vein injections in rabbits has shown similar results as tail vein injections in rodents. Injections through the marginal vein are a safe and effective method of obtaining high levels of gene transfer in the liver without creating further harm to the animal (85).

Chapter 3: Isolation, Cloning, and Sequencing of the Rabbit LDL-R

3.1 Total RNA Isolation

The full-length rabbit LDL-R mRNA was isolated from liver tissue. A NZW rabbit was sacrificed after deep anesthesia, then pieces of liver tissue were collected, snap-frozen in liquid nitrogen, and stored at -80°C until further processing. To isolate total RNA, approximately 1 g of frozen rabbit liver tissue was initially ground in liquid nitrogen using a mortar and a pestle, and subsequently dissolved in 10 mL of Trizol reagent (Invitrogen, Carlsbad, CA), an acidic solution of phenol and guanidine isothiocyanate. RNA isolation was completed using the Trizol reagent and the Chomczynski and Sacchi method for the single-step RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (86). The quality and concentration of the isolated total RNA was determined by UV spectrophotometry (A260/A280) using a NanoDrop spectrophotometer. An A260/A280 ratio greater than 1.8 was considered acceptable for downstream applications.

3.2 Isolation of mRNA from Total RNA

In mammalian cells, mRNA comprises approximately 2% of total RNA. Generally, successful 5' and 3' RACE procedures require a sample enriched with mRNA. The low mRNA concentrations in the sample necessitate isolation of mRNA from total RNA. Eukaryotic mRNA has a poly-A tail whereas other eukaryotic RNAs do not. Based on this property, mRNA was separated from total RNA using an illumina Oligo(dT)-cellulose column (GE Healthcare, Carlsbad,

CA). mRNA molecules containing poly-A tails hybridize to the oligo(dT) cellulose column whereas other RNA molecules pass through the column. For every 1.2 mg of total RNA or approximately 31 A260 units, 50 μg of a mRNA-enriched sample was obtained after single passage through a column. A second passage through a column resulted in isolation of approximately 25 μg of mRNA with a purity greater than 90%.

3.3 RLM-RACE

RNA-ligase mediated rapid amplification of cDNA ends (RLM-RACE) was used to amplify the NZW LDL-R cDNA for sequencing. The ExactSTART Eukaryotic mRNA 5' & 3' RACE Kit was used (Epicentre, Madison, WI). START stands for "Selective Tagging and Amplification of RNA Transcripts." The kit uses a series of RNA-modifying enzymes and oligonucleotide adaptors to isolate and amplify a specific cDNA product through the following steps: 1) DNA synthesis by reverse transcription, 2) non-selective cDNA amplification, and 3) primer-specific cDNA amplification. This procedure allows for isolation and subsequent sequencing of the complete ORF and 5' and 3' UTR of the LDL-R mRNA.

3.3.1 mRNA Enzyme Treatment and Adaptor Ligation

Initially, RNA was treated with a series of enzymes to ensure that only full-length mRNAs with intact 5' and 3' ends will undergo reverse transcription.

Full-length mRNAs have a cap structure at the 5’ end and a poly-A tail at the 3’ end. The first enzyme, heat-labile alkaline phosphatase, was used to remove the phosphate group at the 5’ end of every uncapped mRNA strand. Alkaline phosphatase replaces this phosphate group with a hydroxyl group, preventing degraded mRNAs from participating in subsequent adaptor ligation steps. Tobacco acid pyrophosphatase was then used to remove the 5’ cap from the intact mRNAs, facilitating adaptor ligation at the 5’ end. After removal of the cap, RNA ligase was used to ligate an oligonucleotide adaptor to the 5’ end of the RNA.

3.3.2 Reverse Transcription of mRNA and Non-selective cDNA Amplification

The reverse transcription reaction used an extended oligo-dT primer consisting of an oligo(dT) sequence appended with a PCR priming site sequence at its 5′ end. The full-length cDNA molecules resulting from this reverse transcription reaction were amplified non-selectively to form a cDNA library using:

1) a forward primer located within the sequence of the 5’ RACE oligonucleotide adaptor:
(5’-ATACACATACGATTTAGGTGACACTATAG-3’), and

2) a reverse primer located within the previously described PCR priming sequence of the extended oligo-dT primer:
(5'-CTAGACTTAGAAATTAATACGACTCACTATAG-3').

3.3.3 Selective Amplification of the LDL-R cDNA

The amplified cDNA library from the previous reaction was used to selectively amplify parts of the LDL-R cDNA in three separate PCR reactions using three sets of primers:

A) The 5' end of the LDL-R mRNA was selectively amplified using the forward primer within the sequence of the 5' RACE oligonucleotide adaptor and an internal LDL-R gene-specific reverse primer (5'-AGACGTGCTCCCAGGACGAGT-3'), designed from a partial sequence of the rabbit LDL-R mRNA previously reported on Genbank.

B) The 3' end of the LDL-R mRNA was selectively amplified using the reverse primer within the sequence of the extended oligo-dT primer and an internal LDL-R gene-specific forward primer (5'-CACTGGAAATGCGACGGCCAG-3') designed from a partial sequence of the rabbit LDL-R mRNA previously reported on Genbank. The gene-specific forward primer in this reaction was upstream of the reverse primer used in the previous reaction to ensure complete coverage of the full-length LDL-R mRNA.

C) The full-length LDL-R ORF from the initiation codon to the termination codon was amplified using the forward primer

(5’-ACCGGT*GCCACC*ATGAGGACGGCGCGCTGGGT-3’) and the reverse primer (5’-TGGAGGACGACGTGGCCTGATAACTCGAG-3’) designed after sequencing the 5’ and 3’ RACE products described above. These primers also contained a Kozak sequence, as indicated by italics, and unique restrictions sites, as indicated by underlining, for convenient subsequent cloning in an expression vector. This amplification product was cloned into a pPCR-Script plasmid to verify the sequence before further downstream applications.

After purification, the amplified cDNA products from the 5’ and 3’ RACE procedures, using primer sets A and B as listed above, were cloned individually into the pPCR-script cloning vector via the process described in section 3.3.4. The sequence of the complete 5’ UTR of the LDL-R mRNA, as well as part of the ORF that was previously unknown, was obtained by sequencing the pPCR-script cloning vector containing the product from the 5’ RACE reaction. The sequence of the complete 3’UTR of the LDL-R mRNA was obtained by sequencing the pPCR-script cloning vector containing the product from the 3’ RACE reaction. All non-selective and selective amplifications described above were performed using the proof-reading polymerase pHusion (New England Biolabs, Ipswich, MA) instead of Taq polymerase, which introduces mutations at a higher rate. In addition, the use of a polymerase with proof-reading activity allows for direct cloning of the PCR products in blunt-end ligations. The PCR products from the selective amplifications were purified using a StrataPrep PCR Purification Kit

(Agilent Technologies, Santa Clara, CA) before cloning, and the integrity and quality of the PCR products were evaluated by gel electrophoresis.

3.3.4 pPCR Script Cloning

The PCR-Script Amp Cloning Kit (Stratagene, La Jolla, CA) was used to clone the LDL-R cDNA products described in section 3.3.3. The kit permits the efficient cloning of PCR fragments with a high yield and a low rate of false positives. The kit provides a linearized vector with a rare SrfI (nucleotide sequence accession #: U46017) restriction site. In the ligation reaction, the predigested vector and the PCR product are mixed at an insert-to-vector ratio of 100:1 with a ligase and the SrfI enzyme. If the vector self-ligates without the insert, it recreates the SrfI restriction site, allowing it to be redigested until it contains the insert. If the insert is ligated into the vector, the SrfI site is destroyed, and the vector can no longer be digested by the SrfI enzyme. This enhances cloning efficiency, especially with large inserts. The ligation reactions were then transformed to XL10-Gold ultracompetent bacterial cells using the heat-shock method. The transformation reactions were then plated in LB ampicillin agar plates that also contained X-Gal and isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue-white screening. After overnight incubation for colony formation, several white colonies were picked and grown in 5 mL of LB broth supplemented with 100 mg/mL ampicillin overnight at 37°C. Plasmid minipreps were performed using the alkaline lysis method and Qiagen prep columns (Qiagen, Valencia, CA). The insert cloned in the pPCR-Script plasmid was sequenced using M13 forward

and M13 reverse primers via DyeDeoxy terminator cycle sequencing with an ABI 3730 sequencer.

Chapter 4: *In Vivo* Study to Determine Optimal rAAV Serotype for Rabbit Liver Transduction

Prior to production of a recombinant virus containing the LDL-R ORF, the most efficient rAAV serotype for hepatocyte transduction and gene expression was determined in healthy NZW rabbits. Rabbits were treated with either recombinant AAV6 or AAV8, containing LacZ ORF downstream of a CMV promoter. Liver samples were then analyzed for transduction ability using qPCR and gene expression using β-galactosidase assays. The serotype that caused greatest expression levels was then chosen for mass-production. rAAV6-LacZ and rAAV8-LacZ were provided as a generous gift from Dr. Robert Kotin from the NIH.

4.1 Rabbit Care

All group members completed online BioSafety Level 2 training through the Department of Environmental Safety at the University of Maryland, as well as online rabbit care training through the American Association of Laboratory Animal Science. Group members were also trained in person on proper animal care and handling techniques by Dr. Doug Powell, Dr. Ioannis Bossis, and research specialist Yonas Araya. Animal Study Protocol #R-11-38 was submitted to the UMCP Institutional Animal Care and Use Committee and approved on August 26, 2011. Twelve 3-month-old female NZW rabbits were obtained from Charles River Laboratories in November 2011.

Rabbits were housed in room 1420S/1420T of the Gudelsky Building

(795), Department of Veterinary Medicine, University of Maryland. The rabbits were housed one per cage for the duration of the study. Rabbits were acclimated for a period of several weeks with food and water provided *ad libidum*. Fresh food and water were replenished once daily. Bedding, water, and food containers were changed weekly. Temperature, humidity, and health status were recorded daily. Rabbits were given enrichment in the form of plastic toys.

4.2 Baseline Data Collection and Analysis

Upon acclimation, rabbits were weighed to determine the amount of anesthesia necessary for surgical procedures. Initial blood samples were drawn from each rabbit before injection via the marginal ear vein and stored at -80°C, as taken from the submitted animal protocol.

4.3 Injection of rAAV6-LacZ and rAAV8-LacZ

Rabbits were randomly assigned a number 1-12 and grouped by viral serotype. Five rabbits were placed into each of two groups for rAAV6 and rAAV8 serotypes, respectively. Two rabbits were intended to be used as controls, but one died before the study began. Within each experimental group, three rabbits were sacrificed 10 days post-injection, and the remaining two were sacrificed 28 days post-injection. See Figure 4 for the rabbit group assignments.

NZW Rabbit Group Assignments

Rabbit number	AAV serotype	10-day sacrifice	28-day sacrifice
1	none (control)	died prematurely	
2	none (control)		x
3	8		x
4	8		x
5	6		x
6	6		x
7	6	x	
8	6	x	
9	6	x	
10	8	x	
11	8	x	
12	8	x	

Table 4. NZW rabbit group assignment. Rabbits were randomly assigned into five groups: two control, three rAAV6 10-day sacrifice, two rAAV6 28-day sacrifice, three rAAV8 10-day sacrifice, and two rAAV8 28-day sacrifice. Rabbit 1, designated as a control, died before the study began.

After an acclimation period of several weeks, each experimental rabbit was injected through the ear vein with $2x10^{13}$ particles of either rAAV6-LacZ or rAAV8-LacZ in 5 mL of saline. The control rabbit was injected with saline only.

4.4 Experimental Data Collection

Blood samples were taken after one week and three weeks post-injection and stored at -80°C. After the designated time period (10 or 28 days), rabbits were sacrificed. High dose anesthesia, 50 mg/kg ketamine and 15 mg/kg xylazine, was administered into the thigh muscle of the rabbit. Rabbits were then observed for movement. Once movement ceased, pupils were examined for light reactivity. Once the rabbit was confirmed to be unconscious, it was exsanguinated and then asphyxiated in a CO_2 chamber.

The liver, gall bladder, lungs, heart, ovaries, and pancreas were isolated. Each organ (except for the liver) was stored individually in 50 mL conical tubes

and frozen at -80°C immediately following removal. Carcasses were double-bagged in autoclaved bags and incinerated.

4.5 Qualitative Analysis of β-galactosidase Activity in Liver Samples

Representative liver samples from each lobe were frozen in 1.5 mL tubes for the determination of rAAV genome copy numbers and quantification of β-galactosidase expression. In addition, fresh liver samples were processed for the qualitative evaluation and visualization of LacZ staining in liver tissues. For that purpose, fresh liver from different areas of the tissue was sectioned into 1 mm slices using a tissue slicer, washed in cold phosphate buffered saline (PBS) to remove excess blood, and fixed in β-galactosidase fixative (0.5% glutaraldehyde, 2% paraformaldehyde, 0.1M phosphate buffer pH=7.3, 3mM MgCl2, 3mM EGTA, 0.01% sodium deoxycholate, 0.02% NP-40) for one hour at room temperature. Subsequently, the tissue slices were washed with a washing buffer several times for a duration of 60 minutes and finally incubated in β-galactosidase staining solution (0.1M phosphate buffer pH=7.3, 3mM MgCl2, and 0.01% sodium deoxycholate, 0.02% NP-40, 5mM ferricyanide, 5mM ferrocyanide, 1mg/mL X-gal). Pictures were taken of each sample the day after staining. The colors of liver samples treated with rAAV6-LacZ vs. rAAV8-LacZ were compared to determine which had a greater rate of viral gene expression.

4.6 Quantitative Analysis of β-galactosidase Activity in Liver Samples

Liver tissue from sacrificed animals was cut in pieces, flash frozen in liquid nitrogen, and stored at -80°C until analyzed. For the β-galactosidase assay,

liver tissue pieces were cracked with a liquid nitrogen-chilled mortar and pestle, and pieces weighing approximately 50 mg were collected for extraction. Tissue pieces were extracted with 1mL of extraction buffer (100 mM potassium phosphate buffer, 0.2% Triton X-100, 1 mM dithiothreitol, 0.2 mM PMSF, 5 μg/mL leupeptin) using a dounce homogenizer (87). The homogenized samples were then clarified by centrifugation at 10,000 x g for 10 minutes. The clarified supernatant was collected and heated at 48°C for 60 minutes to inactivate endogenous β-galactosidase activity. Total protein concentration in the samples was measured using the bicinchoninic acid (BCA) assay against a bovine serum albumin (BSA) standard (Pierce, Rockford IL). β-galactosidase activity in the samples was measured using the Galacto-Light Plus™ kit (Applied Biosystems, Bedford, MA). Galacton-Plus® is a modified X-gal molecule; when β-galactosidase cleaves the molecule, it emits light in the form of chemiluminescence, which is measured with a luminometer (Turner, Sunnyvale, CA). As the amount of β-galactosidase in the sample increases, more light will be emitted. Therefore, relative amount of chemiluminescence indicates relative amount of β-galactosidase activity in the tissues. Data were normalized based on total protein concentration and presented as relative luminescence units (RLU) per milligram of protein.

4.7 qPCR of Liver Samples

Total genomic DNA were isolated from liver samples using an autonomated Maxwell 16 DNA isolation system and Maxwell 16 purification DNA cartridges (Promega, Madison, WI). The samples were analyzed by qPCR

using spectrophotometry of cyber green dye. Four samples from different lobes of each rabbit liver were analyzed and 2 by 2 ANOVA with nested design was performed on the results. Log_{10}, log_2, inverse, and square root transformations were performed on the data and log_{10} transformation was found to best normalize the variance.

qPCR was performed on a LightCycler 2.0 (Roche Applied Science, Indianapolis, IN) using 30 ng of genomic DNA in duplicates. All reactions were performed in a 20 µl final volume containing 5 µL of template DNA, 10 µL of Premix Ex Taq (TaKaRa, Otsu, Japan), 0.4 µL of ROX Reference Dye (TaKaRa), 0.2 µmol/L of each primer, and 0.1 µmol/L of probe. rAAV copy numbers were determined using primers and a probe designed to specifically amplify part of the CMV promoter from cytomegalovirus. Genomic DNA copy numbers were determined using primers and a probe designed to amplify part of the rabbit b-actin gene. For each sample, C_t values were compared with those obtained with standard dilutions of plasmid containing either the CMV promoter or the rabbit b-actin sequence. The thermal profile for qPCR was identical for all reactions: 5 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Chapter 5: Construction and Mass-Production of rAAV8-LSP-LDL-R

5.1 Production of pFBVG-LSP-LDL-R Plasmid

Following the cloning of the full length rabbit LDL-R described earlier, an XhoI restriction enzyme site was found within the ORF. In order to subclone the LDL-R ORF from the pPCR-Script plasmid (Figure 3) to a baculovirus transfer vector, an XhoI restriction enzyme digestion was required. As such, the XhoI site within the ORF needed to be removed without changing the amino acid to maintain the integrity of the ORF upon restriction enzyme digestion. Thus, site-directed mutagenesis was performed resulting in a mutant plasmid pPCR-Script.

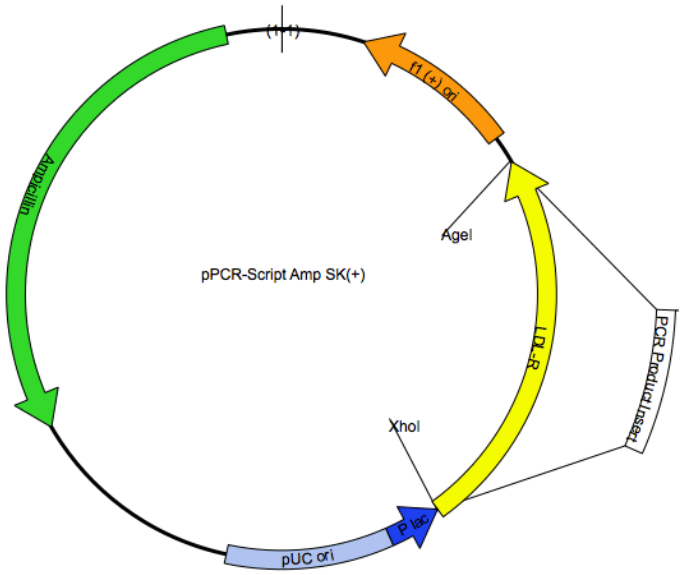


Figure 3. pPCR-Script Plasmid. This plasmid contains the LDL-R ORF with appropriate restriction sites post-site-directed mutagenesis.

To create a rAAV, the gene of interest must be inserted into a shuttle plasmid containing ITRs, a promoter, and a SV40 polyadenylation site. As such, the cytomegalovirus (CMV) promoter and LDL-R gene were subcloned into the digested pFBGR plasmid (Figure 4), creating pFBVG-CMV-LDL-R (Figure 5),

which contained all the necessary elements for the rAAV viral genome. In addition, pFBGR is a polyhedrin-based baculovirus transfer vector plasmid that allows integration of the rAAV cassette into the baculovirus genome.

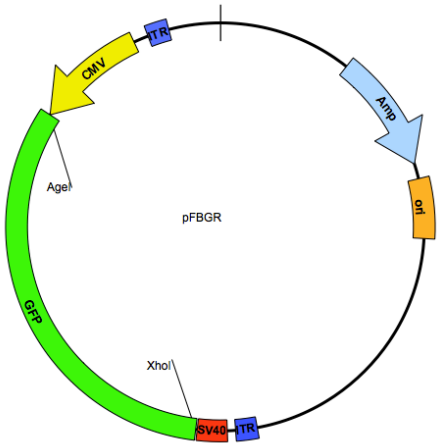


Figure 4. pFBGR plasmid. This plasmid contains the GFP ORF flanked by the XhoI/AgeI restriction sites.

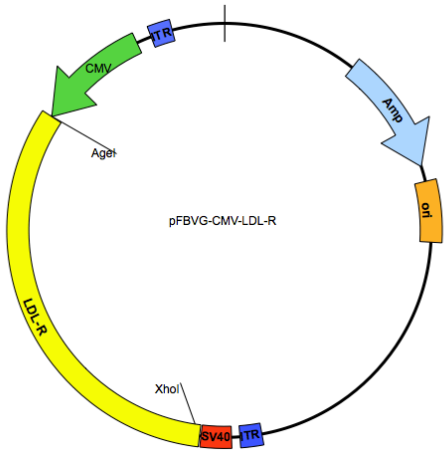


Figure 5. pFBVG-CMV-LDL-R plasmid. This plasmid contains the LDL-R ORF flanked by XhoI/AgeI restriction sites along with the CMV promoter from the pFBGR backbone.

For the purpose of creating pFBVG-CMV-LDL-R, pPCR-LDL-R and pFBGR were digested with XhoI/AgeI. The digested plasmids were then run through gel electrophoresis in order to separate the LDL-R ORF from the linear

pPCR-Script DNA, and linear pFB from the GFP ORF. The components corresponding to the cDNA of the LDL-R and the linear pFB DNA were observed under UV light from a transilluminator. The LDL-R cDNA was isolated using a QIAGEN gel extraction kit (QIAGEN, Valencia CA).

After purification, the LDL-R insert and linear pFB were ligated to create the circular pFBVG-CMV-LDL-R plasmid. In order to amplify this plasmid, bacterial cells were transformed with the ligated, circular pFBVG-CMV-LDL-R. After transformation and overnight plating in ampicillin LB-agar plates, several colonies were isolated and grown overnight in 5 mL LB broth for preparation of plasmid minipreps. Plasmid minipreps were digested again with XhoI/AgeI to confirm insertion of the LDL-R ORF in the linearized pFBGR.

Following confirmation of successful insertion of the LDL-R ORF into the linearized pFBGR, a maxi-prep of the plasmid was prepared using a QIAGEN HiSpeed Plasmid Maxi-Kit (QIAGEN, Valencia CA). Integrity of the ITRs was confirmed by DNA sequencing. Expression of the LDL-R protein was confirmed by transfection of the pFBVG-CMV-LDL-R plasmid in mammalian cells (HEK293) using the FuGene HD reagent (Roche Applied Science, Indianapolis, IN). FuGene 6 is a transfection reagent composed of a blend of lipids in 80% ethanol. When mixed with DNA, FuGene HD forms a complex, which assists in transporting the DNA into mammalian cells. Two days post-transfection, total cell lysates were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting using a rabbit polyclonal antibody raised against human LDL-R (Abnova, Walnut, CA). The initial strategy

was to use this plasmid for large-scale production of rAAV-LDL-R. However, it was determined in the NZW rabbit study that expression using the CMV promoter was largely silenced 10 days post-injection and even more so 28 days post-injection. Therefore, the CMV promoter was replaced with a liver-specific promoter that is known to induce long-term expression when used within a rAAV vector (88).

5.2 Production of rAAV Via Recombinant Baculovirus/Insect Cell System

The protocol described in the following sections was performed in Dr. Robert Kotin's lab at the NIH.

5.2.1 Insertion of the rAAV-LDL-R Expression Cassette into Bacmid

In order to insert the rAAV-LDL-R expression cassette (LITR-LSP-LDL-R-polyA-RITR) construct into a bacmid, the Bac-to-Bac system was used (Invitrogen, Carlsbad, CA). Specifically, competent Bac-DH10 cells were transformed with pFBVG-LSP-LDL-R. These cells are pre-transformed with a bacmid that has a mini-*att*Tn7 target site and a helper plasmid from which transposition helper proteins are produced. Insertion of the rAAV cassette by homologous recombination occurred by transposition between this mini-*att*Tn7 site and the mini-Tn7 sites flanking the rAAV-LDL-R expression cassette in pFBVG-LSP-LDL-R. Following transformation, the Bac-DH10 cells were incubated on an agar plate with 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL X-gal, and 50 μg/mL IPTG for 72 hours at 37°C.

Because the helper plasmid, the bacmid, and pFBVG-LSP-LDL-R confer resistance to kanamycin, gentamicin, and tetracycline, these antibiotics ensure that only the transformed Bac-DH10 cells with the helper plasmid, the bacmid, and pFBVG-LSP-LDL-R can grow. Importantly, the bacmid contains a β-galactosidase ORF that is disrupted upon insertion of the construct by transposition. For this reason, only Bac-DH10 cells containing bacmid without insert had β-galactosidase expression. Because the agar plate contains X-gal and IPTG, which triggers the transcription of the β-galactosidase ORF, disruption of the β-galactosidase ORF was screened by examining colonies for color. Specifically, because β-galactosidase catalyzes the formation of a blue product from X-gal, blue colonies had bacmid that contained the intact β-galactosidase ORF and, therefore, lacked the insert. For this reason, only white colonies were picked.

5.2.2 Purification of Bacmid

Five white Bac-DH10 colonies were picked and each was grown separately overnight in 9 mL LB with kanamycin, gentamicin, and tetracycline with agitation at 37°C. Bacmid from each of these overnight cultures was purified by miniprep and screened for insert by PCR with LSP-specific primers.

5.2.3 Verification of ITR Function by Rescue Assay

In order to determine whether the ITRs were intact after transposition into the bacmid, a rescue assay was performed. Sf9 cells were cotransfected with

bacmid containing the construct and superinfected with bac-rep (recombinant baculovirus expressing rep). The resulting cells expressed the two AAV rep proteins, which, in the absence of cap proteins, replicated DNA flanked by intact ITRs without packaging. 48,72, 96 and120 hours post-infection a fraction of sample was collected and the DNA contained in the Sf9 cells was purified and run on gel electrophoresis. A band of approximately 4.2 kb composed of the amplified construct (LITR-LSP-LDL-R-RITR) was observed, indicating that the ITRs were functional. In contrast, if the ITRs were defective, the rep proteins would not have replicated the construct, so a band with the mass of the construct would not have been observed.

5.2.4 Transformation of Sf9 Cells for Production of P1 Baculovirus

In order to produce P1 baculovirus containing the rAAV-LDL-R construct, Sf9 cells were transfected with one of the bacmids determined to have rAAV-LDL-R inserted genome. To prepare Sf9 cells for transfection, 10^6 cells in 2 mL Sf9 media were allowed to attach to a well of a 6-well plate during a 30 minute incubation at 27°C. Solution A and Solution B were prepared the day of the transfection. Solution A consisted of 600 µL Grace's medium without supplement and 30 µg miniprep-purified bacmid. Solution B consisted of 600 µL Grace's medium without supplement and 30 µL CellFECTIN. Solutions A and B were mixed and incubated for 30 minutes at room temperature. Immediately before transfection, the supernatant was removed from the attached Sf9 cells, and 4.8 mL of Grace's medium were added to the mixture of Solutions A and B. 1 mL

of the resulting mixture was added dropwise to each well with attached Sf9 cells. Sf9 cells were incubated in this mixture for 5 hours at 27°C. After incubation, this mixture was removed by aspiration; the Sf9 cells were incubated in complete medium for 96 hours at 27°C. By the end of this incubation, baculovirus containing the construct was present in the supernatant.

5.2.5 Determination of Viral Titer

Since production of P2 virus requires viral titer of about 10^7 particles per mL, it was necessary to determine the titer of the P1 baculovirus in the supernatant of the Sf9 cells via a plaque assay. Serial dilutions of the P1 supernatant were placed over attached Sf9 cells that were nearly confluent. These cells were incubated for 1 hour at 27°C, and the supernatant was then removed. To prevent the diffusion of viral particles produced during the plaque assay, an agarose overlay was applied containing Sf9 medium. The Sf9 cells were incubated for 6-10 days at 27°C to allow plaque formation. To determine viral titer, the plaques were counted.

5.2.6 Titerless Infected-cells Preservation and Scale-up (TIPS)

100 mL of Sf9 culture (at $2x10^6$ per mL density) was infected with $2x10^6$ PFU of recombinant baculovirus for 72 hours at 27°C. When the diameter of the infected Sf9 cells (TIPS) reached 17-18 microns with viability 90-95% as determined using a cell-counter, they were frozen at $2x10^7$/mL. The viral titer in the TIPS was determined by plaque assay as described above, and was ensured to

be at least 10^8 PFU/mL before it was used to infect Sf9 suspension cells for rAAV production.

5.2.7 Production of rAAV from Sf9 Suspension Cells

For small-scale production of rAAV, 500 mL of Sf9 cells were infected with the baculovirus containing the rAAV-LDL-R construct and with a baculovirus containing *rep* and *cap* genes. The ratio of rep and cap proteins to rAAV genomes must be sufficiently low to minimize production of empty viral particles. In order to avoid overproduction of rep and cap proteins relative to recombinant genomes, a stock of TIPS was used with baculovirus with the genes for rep and cap that had already been optimized for rAAV production (61). The optimal ratio of Sf9 cells to TIPS had been determined to be 1:10,000. The same ratio of Bac-rep-cap TIPS was used. For this reason, 0.050 mL from both TIPS were used to infect 20 L of Sf9 culture ($2x10^6$/mL cells). The cells were grown for 72 hours at 27°C with agitation. Infected cells were monitored for diameter and viability.

To assess the quality of the rAAV produced from Sf9 cells, a small amount of virus was purified by CsCl gradient centrifugation and inspected by TEM and SDS-PAGE followed by silver stain for viral proteins. The lysate was incubated with 4% PEG8000 (polyethylene glycol) overnight with shaking at 4°C and centrifuged at 4000 rpm for 30 minutes in Sorvall GSA rotor. To purify rAAV by CsCl gradient centrifugation, the pellet was resuspended in 12 mL CsCl solution and centrifuged at 45000 rpm for 40 hours at 15°C in a T150 rotor. To

detect rAAV, the refractive index of 0.5 mL fractions of the CsCl gradient was measured. To remove CsCl from the fractions containing rAAV, these fractions were dialyzed with PBS with 2 mM $MgCl_2$. A diluted sample of recovered rAAV was then adsorbed during a 1-minute incubation to a 300-mesh formvar/carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA), negatively stained with 2% pH 7.0 phosphotungstic acid, and visualized by negative stain transmission electron microscopy (TEM).

In order to mass-produce rAAV with the construct, 20 L of Sf9 cells were infected with the baculovirus containing the rAAV-LDL-R construct and with a baculovirus containing *rep* and *cap* genes. The stock of TIPS used for small-scale production was used with the same ratio of Sf9 cells to TIPS, namely 1:10,000. Thus, 2.0 mL from both TIPS were used to infect 20 L of Sf9 culture ($2x10^6$/mL cells). The cells were grown for 72 hours at 27°C with agitation in a 25 L rocking platform bioreactor (WAVE Bioreactor System 20/50; GE Healthcare Life Sciences, Piscataway, NJ). Infected cells were monitored for diameter and viability. Five days post-infection, the cells were lysed using 1% triton x-100 for 2 to 4 hours. Both intra- and extracellular rAAV particles were recovered by processing the entire contents. Further disruption of cells was achieved by homogenization with a microfluidizer. A dual-piston mechanical cell disrupter (deBEE 1000; BEE International, South Easton, MA) operating at 15,000 psi and a 1-mm orifice reaction chamber with a flow rate of approximately 0.9 L/min solubilized the biomass and effectively lysed the cells with a single pass. Following homogenization, the lysate was returned to the bioreactor for nuclease

treatment (TurboNuclease; Acelagen, Inc., San Diego, CA) to reduce viscosity and degrade RNA, genomic DNA, baculovirus DNA, and unencapsidated vector DNA (5 U/ml at 37°C for approximately 2 hours). At the same time, PEG8000 (1.5% total) were also added for 2 hours. Following nuclease and PEG-8000 treatment, depth filtration was used for clarification (Sartopure GF + Maxicaps, 1.2 μm; Sartorius Stedim North America, Bohemia, NY) and then microfiltration using a 0.2-μm capsule filter (Sartopore 2XLG). The filtrate was collected in a sterile bioprocessing bag for further processing.

To purify the mass-produced rAAV8-LDL-R, immunoaffinity chromatography was used. The immunoaffinity medium, AVB-Sepharose (GE Life Sciences Healthcare) specifically binds several AAV serotypes, including serotypes 1, 2, 6, and 8. The filtered cell lysate containing rAAV8-LDL-R was passed through the column. The column was washed with phosphate-buffered saline (PBS) until the ultraviolet (UV) absorbance curve returned to baseline and stabilized. The adsorbed rAAV particles were eluted in acidic medium (50 mM sodium citrate adjusted with HCl to pH 3.0), and the column eluate was adjusted immediately with 1/10 volume of 1 M Tris-Cl (pH 8.0). Virus was removed from the column by three consecutive elutions in order to ensure maximum recovery.

The neutralized eluate was concentrated and diafiltrated against PBS using tangential flow filtration, TFF (100 kDa NMWCO). The final vector product was sterile-filtered (0.22-μm pore) and dispensed into sterile cryogenic storage vials (61).

5.2.8 Characterization of rAAV8-LSP-LDL-R

The total particle concentration was assessed by protein concentration assays, and the filled particle number, *i.e.*, particles that contain vector genomes (vg), was determined using two independent methods: 1) qPCR using vector-specific primers and 2) directly determining the amount of DNA extracted from the particles using SYBR Gold nucleic acid dye. After dialyzing the vector samples against PBS with 2 mM $MgCl_2$, aliquots (70 µl) were treated with proteinase K (40 µg) (Invitrogen, Carlsbad, CA) and incubated for 30 min at 56°C. The proteinase K was heat inactivated (95°C for 5 min) and the samples were slowly equilibrated to ambient temperature to allow time for annealing the complementary single-stranded vector genomes. Using a black bottom 96 wells plate, samples were diluted and added to a final volume of 200 µL of SYBR Gold solution (3 µL of SYBR Gold [Invitrogen, Carlsbad, CA], in 30 mL of fluorescent buffer—10 mM HEPES and 1 mM EDTA, pH 8.0). The fluorescent signal, proportional to the amount of DNA, was read in a Costar black bottom, 96 well plate (Corning Inc., Corning, NY) and fluorescent plate reader (485 nm/535 nm, 1 s) (VICTOR 2, Perkin Elmer, Waltham, MA). DNA reference standards were produced using dilutions of a commercially available DNA ladder (Fermantas, Glen Burnie, MD).

Real-time PCR (q-RT-PCR) was used for quantitative analysis of encapsidated vector genomes using vector specific forward and reverse primers. The primers used, forward 5'-GCCCATCTGATAGGGAATGA-3' and reverse 5'-CAAGGTCACCCCAGTTATCG-3'**,** amplified a 150 bp product, using the

following reaction conditions: initial denaturation 95°C (10 min) and 30 cycles of: 95°C (15 sec), 58°C (30 sec), 72°C (30 sec). PCR copy number standards were prepared from the pFBVG-LSP-LDL-R plasmid. All qPCR reactions were performed with a real-time, thermocycler (ICycler, BioRad, Hercules, CA) using SYBR green PCR master mix (RT^2 SYBR Green/Fluorescein Mastermix, SABiosciences, Frederick, MD). Standard curves and melting temperature curves for q-RT-PCR of rAAV8-LSP-LDL-R can be seen in appendices E.3 and E.4, respectively. SYBR

5.2.8.1 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analyses

Following fractionation and SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose membranes. Silver staining and western blotting were then performed. Membranes were incubated for 30 minutes at room temperature in blocking solution (5% fat-free milk powder diluted in PBS with Tween 20 [PBS-T] buffer). Rabbit polyclonal antiserum raised against recombinant VP1 was used at a final 1:5,000 dilution. After an hour of incubation at room temperature, the solution was removed, and the membrane was washed in PBS-T buffer (3 × 15 minutes at room temperature). The secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO), was diluted 1:5,000 and added to the solution. Following an hour of incubation, the solution was removed, and the membrane was washed 3 times for 15 minutes with PBS-T at room temperature. Light is emitted from the HRP-

catalyzed reaction with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific Pierce Protein Research Products, Rockford, IL). Images were captured using a cooled CCD camera and quantitatively analyzed using the software package provided in the G:BOX Chemi system (Syngene USA, Frederick, MD). (89)

5.2.8.2 Transmission Electron Microscopy (TEM) of AAV Particles

A diluted sample of recovered rAAV was then adsorbed during a 1 minute incubation to a 300-mesh Formvar and carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA), washed with distilled water, negatively stained with 2% pH 7.0 phosphotungstic acid, air-dried, and visualized by negative stain transmission electron microscopy (TEM) with the JEM 1200EX II (JEOL, Tokyo, Japan) (90).

Chapter 6: Results and Discussion

6.1 Isolation and Sequencing of LDL-R Gene

6.1.1 RLM-RACE

When the protocol for the 5' RLM-RACE procedure was designed, it was expected that the 5' end of the rabbit LDL-R mRNA would be similar in GC content and size to the LDL-R mRNAs of other species. However, the 5' UTR of rabbit LDL-R mRNA was found to be only 87 bp and 75% GC rich, while those of other species are approximately 200 bp and less GC rich. In addition, the N-terminal portion of the rabbit LDL-R protein is shorter in length than that of other species. Prior to this study, these properties had prevented the complete 5' UTR from being isolated and sequenced. In order to circumvent these obstacles, it was necessary to use many different combinations of primers to get a complete and accurate amplification product for sequencing. These primers as well as those used for the 3' RLM-RACE procedure and the final amplification of the full-length LDL-R mRNA are outlined in Table 5 below.

Primer #	Primer Name	Primer Sequence
1	5' RACE Forward Primer	5' ATACACATACGATTTAGGTGACACTATAG 3'
2	5' RACE LDL-R Specific Reverse Primer	5' AGACGTGCTCCCAGGACGAGT 3'
3	3' RACE Reverse Primer	5' CTAGACTTAGAAATTAATACGACTCACTATAG 3'
4	3' RACE LDL-R Specific Forward Primer	5' CACTGGAAATGCGACGGCCAG 3'
5	Full Length LDL-R ORF Forward Primer	5' ACCGGTGCCACCATGAGGACGGCGCGCTGGGT 3'
6	Full Length LDL-R ORF Reverse Primer	5' TGGAGGACGACGTGGCCTGATAACTCGAG 3'

Table 5. RLM-RACE Primer Table

Primers 2 and 4 were obtained from a partially sequenced LDL-R mRNA found on GenBank. Primers 1 and 2 were used to determine the sequence from the 5' RACE oligo acceptor to the 5' LDL-R Specific Reverse Primer. Primers 3 and 4 were used to determine the sequence from the 3' LDL-R Specific Forward Primer to the 3' RACE adaptor. Following reverse transcription and amplification using the above primers, the final amplification products were isolated and visualized using gel electrophoresis on an agarose gel under UV light using an ethidium bromide stain (see Figure 6). Figure 6A shows the 5' RACE amplification product. Less prominent bands may have been the result of variation of the 5' start site and the 3' ends. Based on the position of the prominent band on this gel relative to the ladder, this portion of the rabbit LDL-R cDNA is approximately 460 bp. Figure 6B shows the 3' RACE amplification product. Based on the position of the prominent band on this gel relative to the ladder, this portion of the rabbit LDL-R cDNA is approximately 680 bp. Both

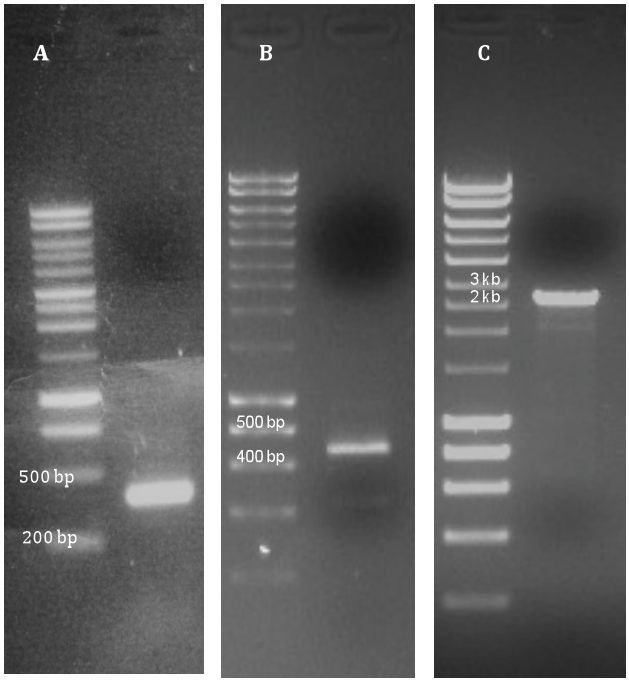


Figure 6. Isolation and sequence of the full-length rabbit LDL-R mRNA. A) 5' RACE amplification product. B) 3' RACE amplification product. C) Full-length LDL-R ORF amplification product. RACE was designed based on a partial sequence of the LDL-R mRNA deposited in GenBank. 1A shows the amplification product to be approximately 460 bp. 1B shows the amplification product to be 680 bp. 1C shows the full ORF amplification product to be 2.5 kb.

of these values were verified to be accurate using sequence analysis. These products were cloned into a pPCR-Script cloning vector and then sequenced. This preliminary sequence was used to construct primers 5 and 6 for PCR amplification and cloning of the full ORF. Figure 6C shows the full-length ORF using these two primers. Based on the position of the prominent band on this gel relative to the ladder, the full-length ORF amplification product is approximately 2.5 kb.

Primer ID Number	Primer Sequence
Seq1	5'-ATGAGTTCCAATGCCGGAAC-3'
Seq2	5'-AGTCCGATGACTTTAGCTGC-3'
Seq3	5'-GACAAGTTCAAGTGCCACAG-3'
Seq4	5'-GACAAGTTCAAGTGCCACAG-3'
Seq5	5'-TTCATGTACTGGACCGACTG-3'
Seq6	5'-ACATCGTCCTGTTCCACAAC-3'
Seq7	5'-TCCACAGCATCAACTTCGAC-3'
Seq8	5'-CCTTAACTGTCCGAGAAGTG-3'
Seq9	5'-TTGGGACAATGACACTCGTG-3'
Seq10	5'-GCTAAAGTCATCGGACTTGC-3'
Seq11	5'-GCACTTCTCGGACAGTTAAG-3'

Table 6. Primers used for Sequencing of the rabbit LDL-R

6.1.2 Sequencing and Gene Alignment

The rabbit LDL-R was sequenced using a set of primers outlined in Table 6.

Using the primers listed in table 6, the complete sequence of the rabbit LDLR mRNA was obtained (GenBank accession number: JX679211). See appendix E.1 for the complete sequence of the full length LDLR mRNA.

After sequencing the full length MRNA, nucleotide and protein sequence alignments were performed via the ClustalW algorithm with the LDL-R mRNA

sequences of other mammalian species to evaluate for conservation. These alignments were done using the mRNA sequences for the human, mouse, ovine and bovine LDL-R reported on Genbank. Alignments were completed using Multalin software (91). See appendix E.2 for the nucleotide sequence alignment. In this alignment, text in red indicates areas of complete consensus. Blue text indicates an area of low consensus or discrepancy between sequences. Black text represents an area of neutral consensus. From this alignment, it is apparent that despite the wide range of species, there are several areas in this alignment of high consensus. Percent identity was calculated by dividing the number of pairs in the alignment comprised of identical nucleotides by the length of the alignment. Percent identities for pairwise alignments between species ranged from 26.6% (mouse and rabbit) to 95.5% (ovine and bovine). This indicates a moderate level of conservation at the nucleotide level for this gene among several mammalian species. For the interest of the study, the rabbit and the human LDL-R mRNA display 46.6% identity. The human full length mRNA is substantially larger than the rabbit mRNA (approximately 5.3 kb versus 3.2 kb respectively). At the ORF level (initiation to termination codon) the two sequences are 75.5% identical. The 5' UTR is smaller in the rabbit compared to other species including humans (87 bp versus 187 bp for the rabbit and human respectively). However, the 5' UTR of the rabbit gene is similar in length to the 5' UTR of both the bovine and ovine LDL-R mRNA. Additionally, the 5' UTR in the rabbit LDL-R mRNA has a 75.9% GC content compared to what is found in other species (approximately 60% GC on average). The high GC content region also extends in the N-terminal

portion of the ORF. This may explain the failure in previous attempts to clone and sequence the full length rabbit LDL-R mRNA. In total, the rabbit LDL-R mRNA is 66.9% GC, whereas the human LDL-R mRNA is 54.4% GC. The 3' UTR is considerably larger in humans compared to other mammalian species including the rabbit (534 bp vs 2.5 kb for the rabbit and human respectively). These observations are expected due to the low degree of conservation usually displayed in the 5' UTR and 3' UTRs among species.

A protein alignment using the clustalW algorithm was also performed using the rabbit, human, mouse, ovine, and bovine LDL-R amino acid sequences. This alignment (Figure 7) indicates several areas of high consensus and conservation. Percent identities for pairwise alignments between species ranged from 73.1% (mouse and rabbit) to 96.4% (ovine and bovine). Percent similarities for pairwise alignments between species ranged from 83.0% (mouse and rabbit) to 97.9% (ovine and bovine). Percent similarity was calculated by dividing the number of pairs of aligned amino acid residues that are comprised of two residues similar to each other by the number of residues in the alignment. Similar residues may not be identical, but any difference would not be expected to have a large effect on the overall function of the protein. For the interest of the current study, the alignment of the rabbit and human LDL-R amino acid sequences indicated 75.6 % identity and 84.4 % similarity. Portions of this alignment have been highlighted to indicate their functional importance in the rabbit LDL-R protein. Yellow highlighted text represents the ligand-binding domain of the LDL-R including the seven cysteine rich repeats. The alignment of the ligand-binding

domains of the human and rabbit LDL-R indicates 74.8% identity and 84.4% similarity. Turquoise highlighted text represents the Epidermal Growth Factor (EGF) homology domain. The alignment of the EGF homology domain of the human and rabbit LDL-R indicates 84.0% identity and 91.2% similarity. Green highlighted text and gray highlighted text represent the O-linked sugar domain and the trans-membrane domain respectively. However, these portions of the alignment are too short to yield any significant information from an in-depth analysis of the identity and similarity percentages. The portion of the alignment corresponding to the site of the twelve-base deletion responsible for the WHHL phenotype is double underlined.

Figure 7. Protein alignment for the rabbit, human, mouse, ovine, and cow LDL-R

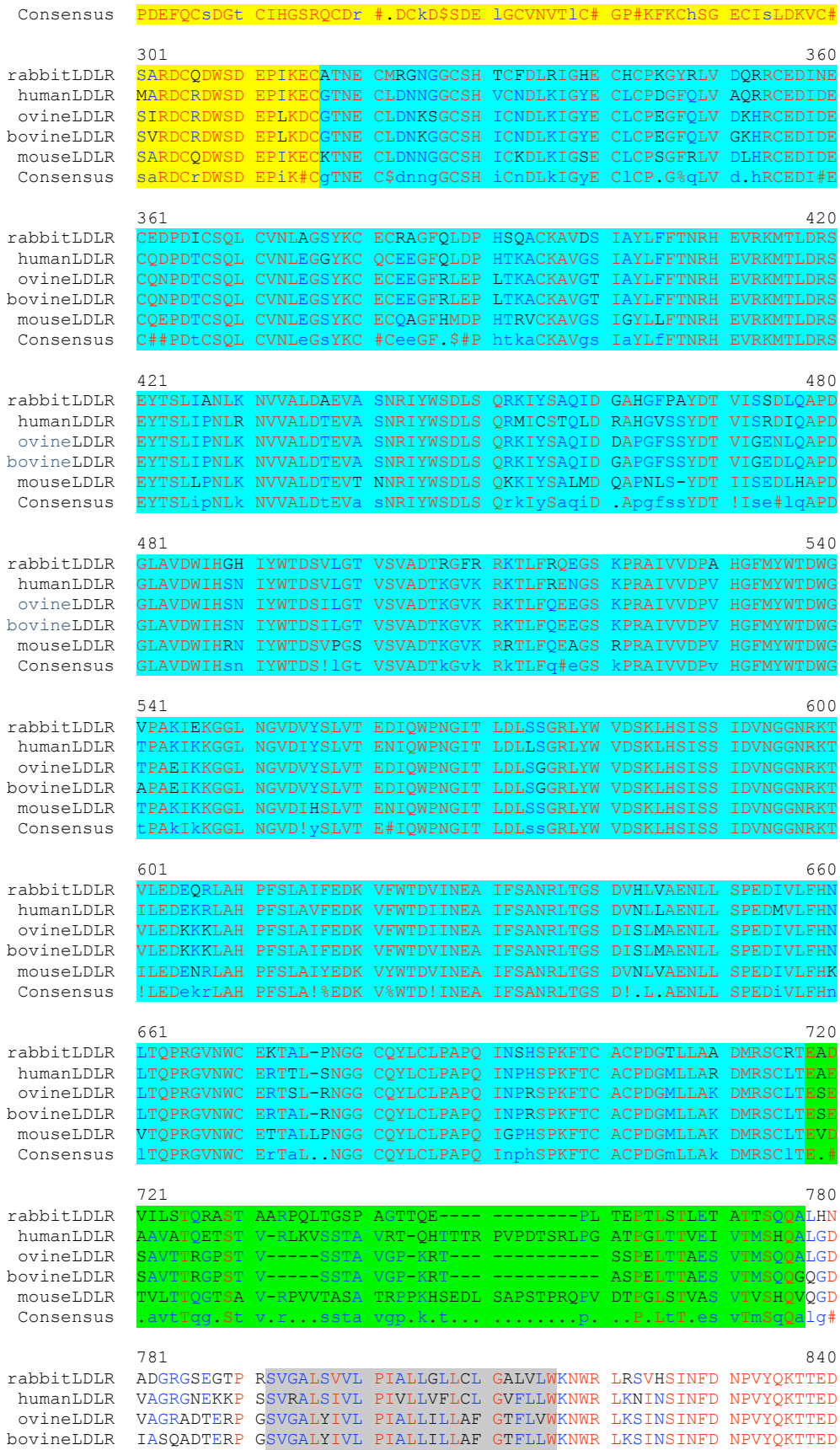
```
            1                                                          60
rabbitLDLR      MRTARW VLGLLLAAAA GAAAGDKCGR NEFQCRNGKC ISYKWVCDGS SECQDGSDEW
 humanLDLR  MGPWGWKLRW TVALLLAAA- GTAVGDRCER NEFQCQDGKC ISYKWVCDGS AECQDGSDES
 ovineLDLR  MRLAGWGLRW AIALLIAAG- EAAVEDNCAR NEFQCRDGKC ISYKWVCDGT AECQDGSDES
bovineLDLR  MRLAGWGLRW AIALLIAVG- EAAVEDNCGR NEFQCQDGKC ISYKWVCDGT AECQDGSDES
 mouseLDLR  MSTADLMRRW VIALLLAAA- GVAAEDSCSR NEFQCRDGKC IASKWVCDGS PECPDGSDES
 Consensus  m..agw.lRW .iaLLlAaa. gaAveD.C.R NEFQCr#GKC IsyKWVCDGs aECqDGSDEs

            61                                                        120
rabbitLDLR  EQTCMSLTCK SDDFSCGGRL NRCIPGHWKC DGQQDCEDGS DELGCAPKTC SQDEFRCAEG
 humanLDLR  QETCLSVTCK SGDFSCGGRV NRCIPQFWRC DGQVDCDNGS DEQGCPPKTC SQDEFRCHDG
 ovineLDLR  QETCKSVTCK MGDFSCGGRV NRCISESWRC DGQKDCENGS DEEGCPPKTC SQDEFRCNDG
bovineLDLR  QETCKSVTCK MGDFSCGGRV NRCISGSWRC DGQVDCENGS DEEGCSPKTC SQDEFRCNDG
 mouseLDLR  PETCMSVTCQ SNQFSCGGRV SRCIPDSWRC DGQVDCENDS DEQGCPPKTC SQDDFRCQDG
 Consensus  q#TC.SvTCk sg#FSCGGRv nRCIp.sWrC DGQvDC##gS DE.GCpPKTC SQD#FRC.#G

            121                                                       180
rabbitLDLR  ACISRLFACD GEPDCPDGSD EASCAPSTCG PAHFRCNSSS CVPALWACDG EPDCDDGSDE
 humanLDLR  KCISRQFVCD SDRDCLDGSD EASCPVLTCG PASFQCNSST CIPQLWACDN DPDCEDGSDE
 ovineLDLR  KCIAPKFVCD LDLDCLDGSD EASCPMPTCS PANFQCNSSM CIPQLWACDG DPDCDDGSDE
bovineLDLR  KCIAPKFVCD LDLDCLDGSD EASCPMPTCG PANFQCNSSM CIPQLWACDG DPDCDDGSDE
 mouseLDLR  KCISPQFVCD GDRDCLDGSD EAHCQATTCG PAHFRCNSSI CIPSLWACDG DVDCVDGSDE
 Consensus  kCIsp.FvCD .#.DClDGSD EAsCp..TCg PA.FqCNSS. C!PqLWACDg #pDCdDGSDE

            181                                                       240
rabbitLDLR  WPARCGAR-- PSPQPGRGPC SRHEFHCGSG ECVHASWRCD GDADCRDGSD ERDCAAATCR
 humanLDLR  WPQRCRGLYV F--QGDSSPC SAFEFHCLSG ECIHSSWRCD GGPDCKDKSD EENCAVATCR
 ovineLDLR  WPKHCGSPHP SGPPKDDNPC SALEFHCGSG ECIHSSWRCD SDPDCKDKSD EENCAVATCR
bovineLDLR  WPKHCGTPHP SGPLQDNNPC SALEFHCGSG ECIHSSWHCD HDPDCKDKSD EENCAVATCR
 mouseLDLR  WPQNCQGRDT AS-KGVSSPC SSLEFHCGSS ECIHRSWVCD GEADCKDKSD EEHCAVATCR
 Consensus  WP..Cg.... ..p..d..PC SalEFHCgSg EC!HsSWrCD gdpDCkDkSD EenCAvATCR

            241                                                       300
rabbitLDLR  PDEFQCSDGT CIHGSRQCDQ QQDCGDMSDE VGCVNVTLCE GPDKFKCHSG ECISLDKVCN
 humanLDLR  PDEFQCSDGN CIHGSRQCDR EYDCKDMSDE VGCVNVTLCE GPNKFKCHSG ECITLDKVCN
 ovineLDLR  PDEFQCSDGT CIHGSRQCDR EPDCKDLSDE LGCVNVTLCE GPNKFKCHSG ECISLDKVCN
bovineLDLR  PDEFQCSDGT CIHGSRQCDR EPDCKDLSDE LGCVNVTLCE GPNKFKCQSG ECISLDKVCN
 mouseLDLR  PDEFQCADGS CIHGSRQCDR EHDCKDMSDE LGCVNVTQCD GPNKFKCHSG ECISLDKVCD
```


Consensus PDEFQCsDGt CIHGSRQCDr #.DCkD$SDE lGCVNVTlC# GP#KFKChSG ECIsLDKVC#
301 360
rabbitLDLR SARDCQDWSD EPIKECATNE CMRGNGGCSH TCFDLRIGHE CHCPKGYRLV DQRRCEDINE
humanLDLR MARDCRDWSD EPIKECGTNE CLDNNGGCSH VCNDLKIGYE CLCPDGFQLV AQRRCEDIDE
ovineLDLR SIRDCRDWSD EPLKDCGTNE CLDNKSGCSH ICNDLKIGYE CLCPEGFQLV DKHRCEDIDE
bovineLDLR SVRDCRDWSD EPLKDCGTNE CLDNKGGCSH ICNDLKIGYE CLCPEGFQLV GKHRCEDIDE
mouseLDLR SARDCQDWSD EPIKECKTNE CLDNNGGCSH ICKDLKIGSE CLCPSGFRLV DLHRCEDIDE
Consensus saRDCrDWSD EPiK#CgTNE C$dnngGCSH iCnDLkIGyE ClCP.G%qLV d.hRCEDI#E
361 420
rabbitLDLR CEDPDICSQL CVNLAGSYKC ECRAGFQLDP HSQACKAVDS IAYLFFTNRH EVRKMTLDRS
humanLDLR CQDPDTCSQL CVNLEGGYKC QCEEGFQLDP HTKACKAVGS IAYLFFTNRH EVRKMTLDRS
ovineLDLR CQNPDTCSQL CVNLEGSYKC ECEEGFRLEP LTKACKAVGT IAYLFFTNRH EVRKMTLDRS
bovineLDLR CQNPDTCSQL CVNLEGSYKC ECEEGFRLEP LTKACKAVGT IAYLFFTNRH EVRKMTLDRS
mouseLDLR CQEPDTCSQL CVNLEGSYKC ECQAGFHMDP HTRVCKAVGS IGYLLFTNRH EVRKMTLDRS
Consensus C##PDtCSQL CVNLeGsYKC #CeeGF.$#P htkaCKAVgs IaYLfFTNRH EVRKMTLDRS
421 480
rabbitLDLR EYTSLIANLK NVVALDAEVA SNRIYWSDLS QRKIYSAQID GAHGFPAYDT VISSDLQAPD
humanLDLR EYTSLIPNLR NVVALDTEVA SNRIYWSDLS QRMICSTQLD RAHGVSSYDT VISRDIQAPD
ovineLDLR EYTSLIPNLK NVVALDTEVA SNRIYWSDLS QRKIYSAQID DAPGFSSYDT VIGENLQAPD
bovineLDLR EYTSLIPNLK NVVALDTEVA SNRIYWSDLS QRKIYSAQID GAPGFSSYDT VIGEDLQAPD
mouseLDLR EYTSLLPNLK NVVALDTEVT NNRIYWSDLS QKKIYSALMD QAPNLS-YDT IISEDLHAPD
Consensus EYTSLipNLk NVVALDtEVa sNRIYWSDLS QrkIySaqiD .ApgfssYDT !Ise#lqAPD
481 540
rabbitLDLR GLAVDWIHGH IYWTDSVLGT VSVADTRGFR RKTLFRQEGS KPRAIVVDPA HGFMYWTDWG
humanLDLR GLAVDWIHSN IYWTDSVLGT VSVADTKGVK RKTLFRENGS KPRAIVVDPV HGFMYWTDWG
ovineLDLR GLAVDWIHSN IYWTDSILGT VSVADTKGVK RKTLFQEEGS KPRAIVVDPV HGFMYWTDWG
bovineLDLR GLAVDWIHSN IYWTDSILGT VSVADTKGVK RKTLFQEEGS KPRAIVVDPV HGFMYWTDWG
mouseLDLR GLAVDWIHRN IYWTDSVPGS VSVADTKGVK RRTLFQEAGS RPRAIVVDPV HGFMYWTDWG
Consensus GLAVDWIHsn IYWTDS!lGt VSVADTkGvk RkTLFq#eGS kPRAIVVDPv HGFMYWTDWG
541 600
rabbitLDLR VPAKIEKGGL NGVDVYSLVT EDIQWPNGIT LDLSSGRLYW VDSKLHSISS IDVNGGNRKT
humanLDLR TPAKIKKGGL NGVDIYSLVT ENIQWPNGIT LDLLSGRLYW VDSKLHSISS IDVNGGNRKT
ovineLDLR TPAEIKKGGL NGVDVYSLVT EDIQWPNGIT LDLSGGRLYW VDSKLHSISS IDVNGGNRKT
bovineLDLR APAEIKKGGL NGVDVYSLVT EDIQWPNGIT LDLSGGRLYW VDSKLHSISS IDVNGGNRKT
mouseLDLR TPAKIKKGGL NGVDIHSLVT ENIQWPNGIT LDLSSGRLYW VDSKLHSISS IDVNGGNRKT
Consensus tPAkIkKGGL NGVD!ySLVT E#IQWPNGIT LDLssGRLYW VDSKLHSISS IDVNGGNRKT
601 660
rabbitLDLR VLEDEQRLAH PFSLAIFEDK VFWTDVINEA IFSANRLTGS DVHLVAENLL SPEDIVLFHN
humanLDLR ILEDEKRLAH PFSLAVFEDK VFWTDIINEA IFSANRLTGS DVNLLAENLL SPEDMVLFHN
ovineLDLR VLEDKKKLAH PFSLAIFEDK VFWTDIINEA IFSANRLTGS DISLMAENLL SPEDIVLFHN
bovineLDLR VLEDKKKLAH PFSLAIFEDK VFWTDVINEA IFSANRLTGS DISLMAENLL SPEDIVLFHN
mouseLDLR ILEDENRLAH PFSLAIYEDK VYWTDVINEA IFSANRLTGS DVNLVAENLL SPEDIVLFHK
Consensus !LEDekrLAH PFSLA!%EDK V%WTD!INEA IFSANRLTGS D!.L.AENLL SPEDiVLFHn
661 720
rabbitLDLR LTQPRGVNWC EKTAL-PNGG CQYLCLPAPQ INSHSPKFTC ACPDGTLLAA DMRSCRTEAD
humanLDLR LTQPRGVNWC ERTTL-SNGG CQYLCLPAPQ INPHSPKFTC ACPDGMLLAR DMRSCLTEAE
ovineLDLR LTQPRGVNWC ERTSL-RNGG CQYLCLPAPQ INPRSPKFTC ACPDGMLLAK DMRSCLTESE
bovineLDLR LTQPRGVNWC ERTAL-RNGG CQYLCLPAPQ INPRSPKFTC ACPDGMLLAK DMRSCLTESE
mouseLDLR VTQPRGVNWC ETTALLPNGG CQYLCLPAPQ IGPHSPKFTC ACPDGMLLAK DMRSCLTEVD
Consensus lTQPRGVNWC ErTaL..NGG CQYLCLPAPQ InphSPKFTC ACPDGmLLAk DMRSClTE.#
721 780
rabbitLDLR VILSTQRAST AARPQLTGSP AGTTQE---- --------PL TEPTLSTLET ATTEQQALHN
humanLDLR AAVATQETST V-RLKVSSTA VRT-QHTTTR PVPDTSRLPG ATPGLTTVEI VTMSHQALGD
ovineLDLR SAVTTRGPST V-----SSTA VGP-KRT--- ---------- SSPELTTAES VTMSQQALGD
bovineLDLR SAVTTRGPST V-----SSTA VGP-KRT--- ---------- ASPELTTAES VTMSQQGQGD
mouseLDLR TVLTTQGTSA V-RPVVTASA TRPPKHSEDL SAPSTPRQPV DTPGLSTVAS VTVSHQVQGD
Consensus .avtTqg.St v.r...ssta vgp.k.t... ....p. ..P.LtT.es vTmSqQalg#
781 840
rabbitLDLR ADGRGSEGTP RSVGALSVVL PIALLGLLCL GALVLWKNWR LRSVHSINFD NPVYQKTTED
humanLDLR VAGRGNEKKP SSVRALSIVL PIVLLVFLCL GVFLLWKNWR LKNINSINFD NPVYQKTTED
ovineLDLR VAGRADTERP GSVGALYIVL PIALLILLAF GTFLVWKNWR LKSINSINFD NPVYQKTTED
bovineLDLR IASQADTERP GSVGALYIVL PIALLILLAF GTFLLWKNWR LKSINSINFD NPVYQKTTED

```
 mouseLDLR  MAGRGNEEQP HGMRFLSIFF PIALVALLVL GAVLLWRNWR LKNINSINFD NPVYQKTTED
 Consensus  .agrg.ee.P .svgaLs!vl PIaLl.lL.l G.fllWkNWR Lks!nSINFD NPVYQKTTED

            841                                                 888
rabbitLDLR  EVHICRSQDG YTYPSRQMVS LEDDVA
 humanLDLR  EVHICHNQDG YSYPSRQMVS LEDDVAALSI VLPIVLLVFL CLGVFLLW
 ovineLDLR  EVHICRSQDG YTYPSRQMVS LEDDAA
bovineLDLR  EVHICRSQDG YTYPSRQMVS LEDDVA
 mouseLDLR  ELHICRSQDG YTYPSRQMVS LEDDVA
 Consensus  EvHICrsQDG YtYPSRQMVS LEDDvA.... .......... ........
```

The percent identity for the nucleotide alignment was significantly lower than that of the protein alignment. However, this is expected in genes that are constantly selected for or in genes in which a homozygous mutation is lethal. If mutations result in a lethal protein product, then most surviving mutations would be expected to be synonymous. Though the EGF homology domain was the portion that showed the most similarity between the two sequences, the ligand-binding region and, more specifically, the site of the deletion for WHHL rabbits were highly similar between the two species, lending merit to the use of the WHHL rabbit as an animal model for FH. Despite a relatively high degree of conservation, the human and the rabbit protein display significant variation. Therefore using the rabbit as a model for FH and gene therapy likely requires the use of the rabbit sequence in the recombinant viral vector choice, rather than the human sequence or a chimeric sequence.

6.2 Data from *in vivo* Studies

In order to determine the most effective serotype for transduction and expression in rabbit hepatocytes, qualitative and quantitative assays were performed. Liver samples isolated from NZW rabbits injected with either rAAV6-LacZ or rAAV8-LacZ were used for these assays. First, to qualitatively show

observable differences in functionality of each serotype, a β-galactosidase staining assay was performed. Once these differences were confirmed, an assay measuring relative expression of β-galactosidase was performed in order to quantitatively show differences in functionality. qPCR was also performed in order to show the transduction efficiency of each virus and the maintenance of viral DNA in liver cells over time.

6.2.1 β-galactosidase Staining Assay

Liver sections from transfected rabbits were fixed and incubated in a β-galactosidase staining solution containing 1 mg/mL X-gal. Intensity of blue color produced by cleavage of X-gal is proportional to the concentration of β-galactosidase, and therefore the expression levels of the viral LacZ gene. The samples shown below are representative of rAAV8-LacZ and rAAV6-LacZ treatment after 10 days. Qualitatively, there was distinctly higher β-galactosidase activity with rAAV8-LacZ treatment as displayed by the accumulation of the blue X-gal substrate (Figure X). This observation suggests that rAAV8 is a more efficient viral vector in targeting rabbit liver than rAAV6. No visual development of β-galactosidase was seen in rabbits treated with rAAV8-LacZ or rAAV6-LacZ after 28 days (data not shown).

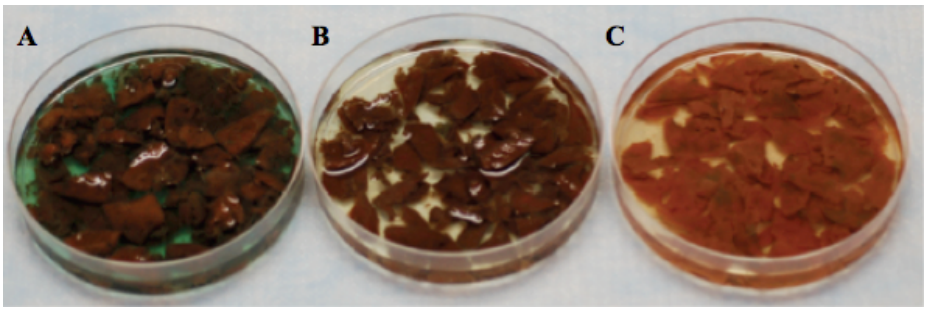


Figure 8: β-galactosidase staining of liver sections. The samples shown in the figure above represent liver slices isolated from three different rabbits. (A) shows representative liver samples after 10 days from rabbits injected with the rAAV8-LacZ, and (B) is representative of liver samples after 10 days from rabbits injected with rAAV6-LacZ. (C) is from the control rabbit after 28 days with no viral injection. Results shown are after overnight incubation in β-galactosidase staining solution.

6.2.2 Quantitative Detection of β-galactosidase Activity

A quantitative assay was performed to measure β-galactosidase expression. Samples were taken from five lobes of each rabbit liver. Assays were performed twice on each sample and the mean was taken to obtain the data shown in Tables 7 and 8. After performing the BCA assay to determine total protein concentration in each sample, 10 μL of sample was used for each luminescence reading. This resulted in a total protein mass of 10-20 μg per sample, which was in the targeted linear range based on standard curves. Based on the measured volume, total protein concentration per sample, and luminescence data, we obtained data in relative luminescence units per mg of protein, which is representative of relative β-galactosidase activity in each tissue sample.

Rabbit ID Number	Virus Serotype	Sacrifice Time	Relative Luminescence Units per mg of Total Protein				
			Lobe 1	Lobe 2	Lobe 3	Lobe 4	Lobe 5
10	AAV8	10 days	98457	54478	115642	39724	63245
11	AAV8	10 days	124693	87426	103071	61301	109117
12	AAV8	10 days	28415	74516	93476	64512	33419
3	AAV8	28 days	3014	1742	2104	3451	1076
4	AAV8	28 days	784	1428	1911	845	741

Table 7: β-galactosidase assay of NZW rabbits treated with rAAV8-LacZ. 5 NZW rabbits were injected with rAAV8-LacZ. Rabbits were sacrificed after 10 or 28 days. Galacto-light Plus assay was performed on samples from five lobes of each liver. Samples were run in duplicates and the numbers shown are the average of the two outputs. Data shown represents the relative luminescence units per mg of total protein (total protein concentration was determined from the BCA assay).

Rabbit ID Number	Virus Serotype	Sacrifice Time	Relative Luminescence Units per mg of Total Protein				
			Lobe 1	Lobe 2	Lobe 3	Lobe 4	Lobe 5
7	AAV6	10 days	14076	9652	16102	4129	7415
8	AAV6	10 days	2489	9651	4187	3258	6145
9	AAV6	10 days	1784	2105	3014	1784	2479
5	AAV6	28 days	184	196	321	85	142
6	AAV6	28 days	78	112	154	104	118

Table 8: β-galactosidase assay of NZW rabbits treated with rAAV6-LacZ. 5 NZW rabbits were injected with rAAV6-LacZ. Rabbits were sacrificed after 10 or 28 days. Galacto-light Plus assay was performed on samples from five lobes of each liver. Samples were run in duplicates and the numbers shown are the average of the two outputs. Data shown represents the relative luminescence units per mg of total protein (total protein concentration was determined from the BCA assay).

For the purpose of statistical analysis, data was divided into four groups: AAV6 sacrificed after 10 days (rabbits 7, 8, and 9), AAV6 sacrificed after 28 days (rabbits 5 and 6), AAV8 sacrificed after 10 days (rabbits 10, 11, and 12), and AAV8 sacrificed after 28 days (rabbits 3 and 4). Preliminary analysis on raw data with Bartlett's test for equal variance yielded a significant result, which meant the untransformed data was not homogeneous in variance. After $\log_{10}$ transformation, Bartlett's test yielded a non-significant result, indicating that the variances for the

transformed data were homogeneous, which allowed for ANOVA to be performed. The following Figure 9 and Table 9 show the distribution of the raw and transformed data and the statistical analysis performed (Newman-Keuls Multiple Comparison Test).

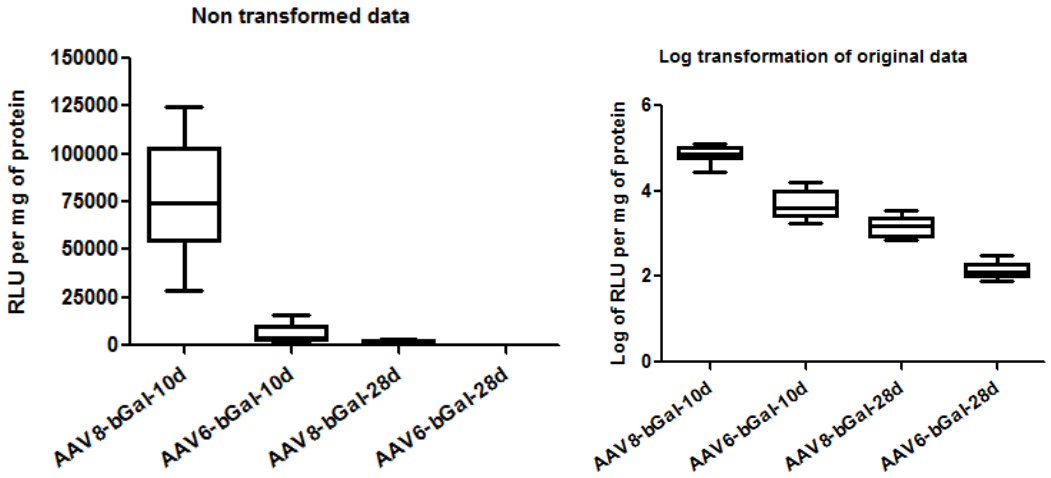


Figure 9. β-galactosidase assay of liver tissue post-infection. Raw and log-transformed β-galactosidase assay data post-infection with AAV6 and AAV8 serotypes. Samples were collected at either 10 or 28 days after treatment.

Compared Serotypes		q	Significant? P< 0.05
AAV6 10 days	AAV6 28 days	21.18	Yes
AAV8 10 days	AAV8 28 days	23.33	Yes
AAV6 10 days	AAV8 10 days	18.57	Yes
AAV6 28 days	AAV8 28 days	13.21	Yes

Table 9. Newan-Keuls Multiple Comparison Test on β-Galactosidase Assay Data. ANOVA was performed on log-transformed data. Further analysis shows significant differences between all experimental groups.

ANOVA was performed on the transformed data and indicated a significant difference between groups. Further analysis by Newman-Keuls Multiple Comparison Test showed significant differences between all groups, including AAV6 at 10 days and AAV8 at 10 days; AAV6 at 28 days and AAV8 at

28 days; AAV6 at 10 days and AAV6 at 28 days; and AAV8 at 10 days and AAV8 at 28 days.

Luminescence per mg of liver tissue in rabbits exposed to AAV8 was significantly greater than in rabbits exposed to AAV6 at both time points. This indicates that AAV8 had greater viral expression in liver tissue than AAV6. A significant decrease in luminescence data between 10 days and 28 days for rabbits treated with both serotypes indicates that viral expression decreased over time. This suggests that the host cell silenced transgene expression between 10 and 28 days for both serotypes.

6.2.3 qPCR Analysis of Liver

To quantify the degree of transduction and maintenance of the viral transgene in the liver, qPCR was performed on liver samples. Samples were taken from four different lobes of the liver of each rabbit. qPCR was performed twice on each sample and the average was taken to obtain the data shown in Tables 10 and 11. qPCR was used with a primer specific to the CMV promoter, a viral promoter not otherwise present in mammalian cells. The data in the tables therefore represent the number of AAV genomes per diploid rabbit genome. The sensitivity of the assay was 0.01 copies per rabbit diploid genome.

Rabbit ID Number	Virus Serotype	Sacrifice Time	# AAV genomes per diploid rabbit genome			
			Lobe 1	Lobe 2	Lobe 3	Lobe 4
10	AAV8	10 days	21.4	3.45	6.78	11.8
11	AAV8	10 days	7.45	0.92	5.21	1.47
12	AAV8	10 days	1.93	17.1	9.45	2.75
3	AAV8	28 days	0.85	1.75	16.2	4.23
4	AAV8	28 days	3.15	0.45	7.56	11.47
2	CONTROL	28 days	<0.01	<0.01	<0.01	<0.01

Table 10: qPCR of NZW rabbits treated with rAAV8-LacZ. 5 NZW rabbits were injected with rAAV8-LacZ and 1 with saline solution only (control). Rabbits were sacrificed after 10 or 28 days. qPCR was performed on samples from four lobes of each liver using a CMV promoter-specific primer. Samples were run in duplicates and the numbers shown are the average of the two outputs. Data shown represents the number of CMV promoters per diploid rabbit genome. Sensitivity of the assay was 0.01 copies per diploid genome.

Rabbit ID Number	Virus Serotype	Sacrifice Time	# AAV genomes per diploid rabbit genome			
			Lobe 1	Lobe 2	Lobe 3	Lobe 4
7	AAV6	10 days	0.06	0.09	0.19	0.24
8	AAV6	10 days	0.27	<0.01	0.13	0.05
9	AAV6	10 days	0.57	0.17	0.06	0.42
5	AAV6	28 days	0.14	0.07	0.41	0.26
6	AAV6	28 days	0.25	0.03	<0.01	0.34
2	CONTROL	28 days	<0.01	<0.01	<0.01	<0.01

Table 11: qPCR of NZW rabbits treated with rAAV6-LacZ. 5 NZW rabbits were injected with rAAV6-LacZ and 1 with saline solution only (control). Rabbits were sacrificed after 10 or 28 days. qPCR was performed on samples from four lobes of each liver using a CMV promoter-specific primer. Samples were run in duplicates and the numbers shown are the average of the two outputs. Data shown represents the number of CMV promoters per diploid rabbit genome. Sensitivity of the assay was 0.01 copies per diploid genome.

For the purpose of statistical analysis, data was divided into four groups: AAV6 sacrificed after 10 days (rabbits 7, 8, and 9), AAV6 sacrificed after 28 days (rabbits 5 and 6), AAV8 sacrificed after 10 days (rabbits 10, 11, and 12), and AAV8 sacrificed after 28 days (rabbits 3 and 4). Preliminary analysis on raw data with Bartlett's test for equal variance yielded a significant result, which meant the untransformed data was not homogeneous in variance. After $\log_{10}$

transformation, Bartlett's test yielded a non-significant result, indicating that the variances for the transformed data were homogeneous, which allowed for ANOVA to be performed. The following Figure 10 and Table 12 show the distribution of the raw and transformed data and the statistical analysis performed (Newman-Keuls Multiple Comparison Test).

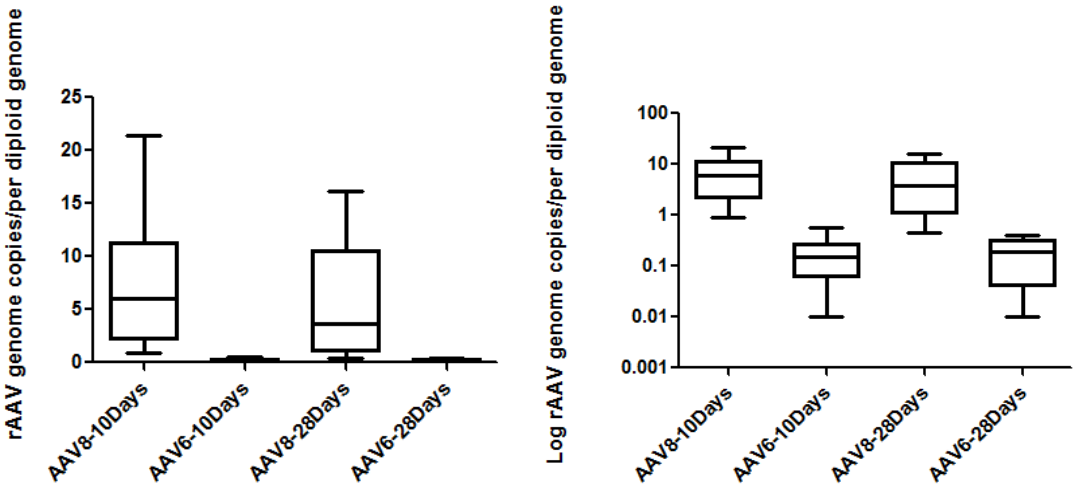


Figure 10. qPCR of liver tissue post-infection. Raw and log-transformed qPCR data post-infection with AAV6 and AAV8 serotypes. Samples were collected at either 10 or 28 days after treatment.

Compared Serotypes		q	Significant? P<0.05
AAV6 10 days	AAV6 28 days	0.0002958	No
AAV8 10 days	AAV8 28 days	1.255	No
AAV6 10 days	AAV8 10 Days	5.784	Yes
AAV6 28 days	AAV8 28 days	3.576	Yes

Table 12. Newan-Keuls Multiple Comparison Test on qPCR Data. ANOVA was performed on log-transformed data. Further analysis shows significant differences between AAV6 and AAV8 at both time points.

ANOVA was performed on the transformed data and indicated a significant difference between groups. Further analysis by Newman-Keuls

Multiple Comparison Test showed significant differences between all groups, including AAV6 at 10 days and AAV8 at 10 days; and AAV6 at 28 days and AAV8 at 28 days. No significant difference was found between AAV6 at 10 days and AAV6 at 28 days; or between AAV8 at 10 days and AAV8 at 28 days.

The number of viral genomes per diploid rabbit genome in liver of rabbits exposed to AAV8 was significantly greater than that of rabbits exposed to AAV6 at both time points. This indicates that AAV8 had greater viral transduction in liver tissue than AAV6. No significant change in the number of viral genomes between time points for both serotypes indicates that viral DNA was maintained over time. This suggests that viral genomes were not destroyed by host immune response between 10 and 28 days.

6.3 Production of pFBVG-LSP-LDL-R Plasmid

Prior to restriction enzyme digestion with XhoI/AgeI for ligation of the LDL-R ORF into pFB, site directed mutagenesis was performed to destroy a XhoI site within the LDL-R ORF between nucleotides 2505 and 2510. Below is a segment from nucleotides 2478 to 2429 containing the XhoI site.

CAGGAGCCAGGACGGCTACACCTACC C<u>CT CG</u>**A** <u>G</u>AC AGATGGT CAGCCTGGAG
P R D

<u>CTCGAG</u> = XhoI site
A = site directed mutagenesis

A/C site directed mutagenesis at nucleotide 2509 destroyed the XhoI site, but maintained the arginine residue and the integrity of the amino acid sequence. The following forward and reverse A/C primers were synthesized with a 5' phosphor group (Integrated DNA Technologies, Coraville, IA):

5’ CGA CAG ATG GTC AGC CTG GAG 3’ (Forward primer A/C)
3’ CGA GGG GTA GGT GTA GCC G 5’ (Reverse primer A/C)

The pPCR-Script-LDL-R plasmid was used as the template in the PCR with these primers. After running the PCR reaction, the solution was treated with the DpnI restriction enzyme to degrade the template plasmid, leaving the linear mutant PCR product. After purification of the product, the DNA was self-ligated at the open 5’ phosphate ends with DNA ligase to obtain mutant pPCR-Script-LDL-R without the XhoI restriction site. The cloning vector containing the LDL-R insert, pPCR- Script, was digested with AgeI/XhoI to remove the LDL-R insert for ligation into pFB. Although two bands were expected, only one thick band was observed in the gel (Figure 11) due to insufficient resolution. The size of the LDL-R insert and that of the pPCR-Script plasmid backbone differed by only 100 bp. Since the LDL-R insert was 2.6 kb compared to the plasmid backbone of 2.7 kb, the bottom portion of the observed band was hypothesized to be the insert. The bottom portion of the band was, therefore, excised, and the DNA was extracted and ligated into digested pFB. The resulting plasmid was named pFBVG-CMV-LDL-R (Figure 12)

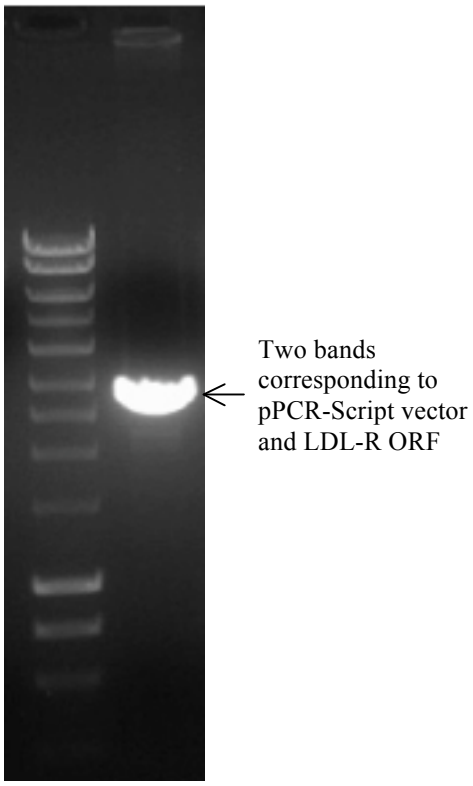


Figure 11: Gel electrophoresis of digested pPCR-Script plasmid, containing the LDL-R ORF. The resulting fragments were nearly identical in size, creating one thick band. LDL-R ORF: 2.7 kB, pPCR-Script backbone: 2.6 kB. The bottom portion was used for downstream procedures

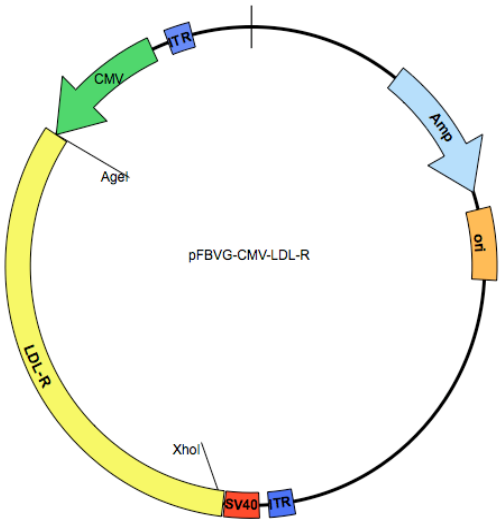


Figure 12: pFBVG-CMV-LDL-R plasmid, containing the LDL-ORF, flanked by AgeI and XhoI restriction sites, and CMV promoter. This plasmid was constructed via ligation of LDL-ORF and pFB plasmid backbone.

This new plasmid was identical to pFBGR with the viral genome insert replacing the GFP ORF. In order to exclude the possibility that the subcloning plasmid backbone was inserted into pFB, the plasmid was screened for insert by restriction digestion following bacterial transformation and purification. Six of the 7 miniprep products showed the expected restriction pattern (Figure 13). Subsequently, two miniprep products that showed the expected restriction pattern were screened by PCR with primers specific for the insert (Figure 14).

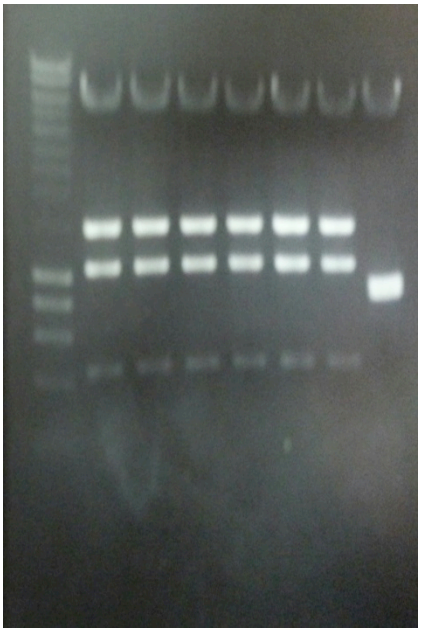


Figure 13: Gel electrophoresis of purified plasmids taken from bacterial colonies. 6 of 7 bacterial colonies confirmed the presence of the LDL-R insert.

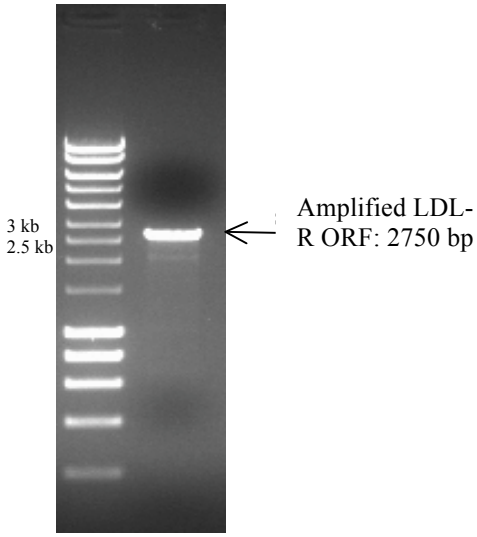


Figure 14: Gel electrophoresis of purified PCR product taken from bacterial colony. The presence of the correct insert was confirmed by the observation of a 2750 bp band, corresponding to the LDL-R ORF.

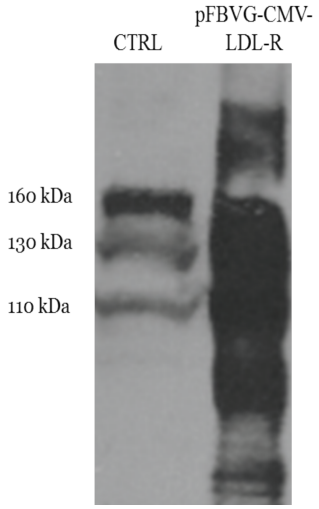


Figure 15: Western blot of HEK 293 transfection by pFBVG-CMV-LDL-R . HEK293 cells expressed LDL-R gene

Expression of LDL-R was confirmed by transfection of HEK293 cells with pFBVG-CMV-LDL-R and Western blot (Figure 15).

Since β-galactosidase expression diminished significantly after 10 days and was non-existent 28 days post-infection due to CMV silencing, a liver-specific promoter was chosen that would not be silenced in hepatocytes for long-term expression of LDL-R. To replace the CMV promoter in pFBVG-CMV-LDL-R with the liver-specific promoter HCR-ApoE-hAAT (hepatic control region apolipoprotein enhancer/alpha1-antitrypsin), a DNA cassette was first synthesized containing the liver-specific promoter flanked by AgeI and SpeI sites (GenScript, Piscataway, NJ). The synthesized construct was provided in a pUC57 vector (pUC57-LSP). Double digestion of this plasmid with AgeI/SpeI and ligation to

digested pFBVG-CMV-LDL-R yielded the pFBVG-LSP-LDL-R plasmid (Figure 16).

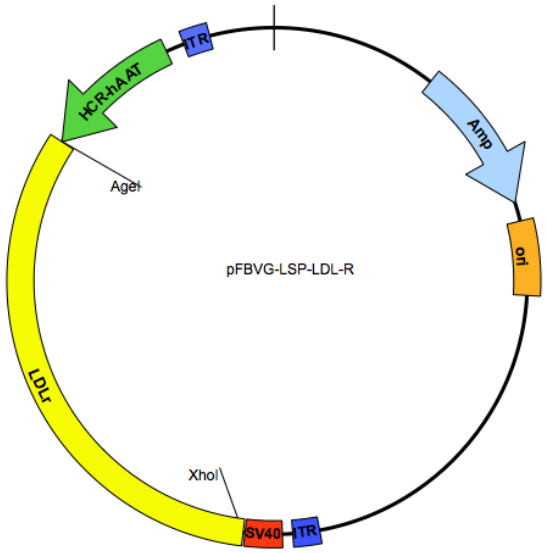


Figure 16: pFBVG-LSP-LDL-R plasmid. The CMV promoter was replaced with the liver-specific HCR-ApoE-hAAT promoter by ligation of pFBVG-CMV-LDL-R and pUC57-LSP.

Expression of pFBVG-LSP-LDL-R into two hu 3B, and Western blot (Figure 17).

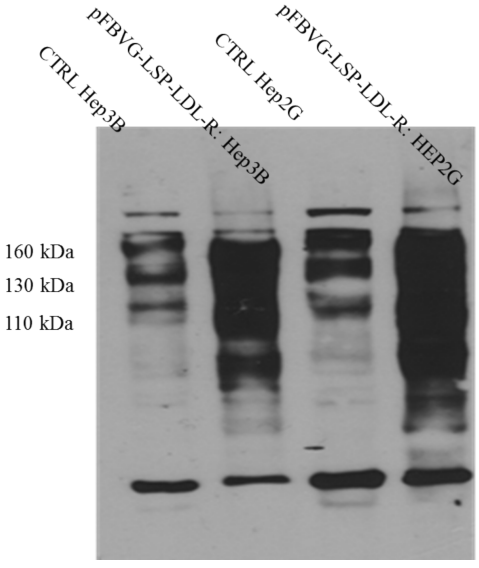


Figure 17: Western blot of HEP3B and HEP2G transfection by pFBVG-LSP-LDL-R . HEK3B and HEK2G cells expressed LDL-R gene.

Shown below (Figure 18) is the sequence of the artificially synthesized cassette containing the convenient restriction sites and various promoter elements. As shown, the p10 promoter is enclosed within the region between the AgeI and XhoI restriction sites. Upon digestion at those sites and insertion of the LDL-R ORF to pFB, this p10 promoter was removed. As an insect cell promoter, p10 is responsible for expression of LDL-R upon transfection of Sf9 insect cells with the pFBVG-LSP-LDL-R plasmid. Prior to mass production of rAAV-LDL-R, expression of LDL-R needed to be confirmed in insect cells to verify the integrity of the ORF. Therefore, it was necessary to reinsert this promoter into the pFBVG-LSP-LDL-R. To that end, the plasmid containing the HCR-ApoE-hAAT promoter was constructed to contain the p10 promoter as well.

ACTAGTCCCTAAAATGGGCAAACATTGCAAGCAGCAAACAGCAAACACACA
GCCCTCCCTGCCTGCTGACCTTGGAGCTGGGGCAGAGGTCAGAGACCTCTCTGGGCCC
ATGCCACCTCCAACATCCACTCGACCCCTTGGAATTTCGGTGGAGAGGAGCAGAGGT
TGTCCTGGCGTGGTTTAGGTAGTGTGAGAGGGTCCGGGTTCAAAACCACTTGCTGGGT
GGGGAGTCGTCAGTAAGTGGCTATGCCCCGACCCCGAAGCCTGTTTCCCCATCTGTAC
AATGGAAATGATAAAGACGCCCATCTGATAGGG*AATGACTCCTTTCGGTAAGTGCAGTG*
GAAGCTGTACACTGCCCAGGCAAAGCGTCCGGGCAGCGTAGGCGGGCGACTCAGATCCCA
GCCAGTGGACTTAGCCCCTGTTTGCTCCTCCGATAACTGGGGTGACCTTGGTTAATATTCAC
CAGCAGCCTCCCCCGTTGCCCCTCTGGATCCACTGCTTAAATACGGACGAGGACAGGGCC
*CTGTCTCCTCAGCTTCAGGCACCACCACTGACCTGGGACAGTGAATC*CTCTAAGGTAAAT
ATAAAATTTTTAAGTGTATAATGTGTTAAACTACTGATTCTAATTGTTTCTCTCTTTTA
GATTCCAACCTTTGGAACTGAGCTAGCTCGCGA*TACGGACCTTTAATTCAACCCAACACA*
ATATATTATAGTTAAATAAGAATTATTATCAAATCATTTGTATATTAATTAAAATACTATACTGTA
*AATTACA*GAATTCGTTTAAACGGATCC ACCGGT

Figure 18: HCR-ApoE-hAAT and p10 promoters. Red: HCR and SV40 intron. *Italics: hAAT and p10* promoters*. Underline: unique AgeI and SpeI restriction sites

6.4 Rescue Assay

Coinfection of Sf9 cells with baculovirus containing rAAV genome and with bac-rep resulted in the production of a 4.3 kb single stranded DNA, which was visualized by 1% agarose gel electrophoresis (Figure 19). Because rep

proteins replicate sequences flanked by ITRs, the presence of this DNA is evidence that the ITRs of the rAAV are functional and resulted in the rep protein-mediated replication of the rAAV genome. Moreover, the fact that the size of the replicated DNA is the size of the rAAV genome further supports the conclusion that the rAAV genome was replicated. Since the rAAV genome can be replicated by the rep proteins only if its ITRs are intact, the production of a 4.3 kb DNA is evidence that the ITRs are intact.

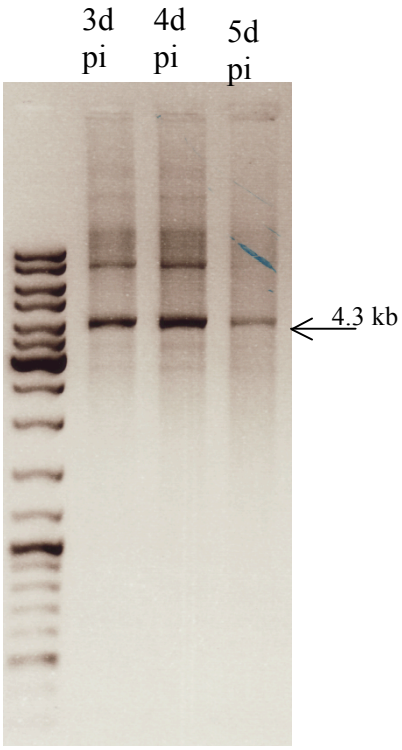


Figure 19. Rescue assay to verify ITR integrity. Sf9 were coinfected with recombinant baculovirus with rAAV genome and with a recombinant baculovirus from which AAV rep proteins were expressed. DNA from Sf9 was purified 72 hours after infection and visualized on a 1% agarose gel.

6.5 Examination of Fractions Eluted from AVB Column by Silver Staining, qPCR, and Direct DNA Binding

In order to detect rAAV eluted from AVB column, the absorbance at 280 nm of eluted material from three consecutive elutions was monitored (Figure 20). Additionally, viral proteins from the fraction with highest Abs<280> were

detected by SDS-PAGE followed by silver stain (Figure 21). The most intense bands detected by silver stain had the molecular weights of VP1, VP2, and VP3. For this reason, the fraction was concluded to contain purified rAAV.

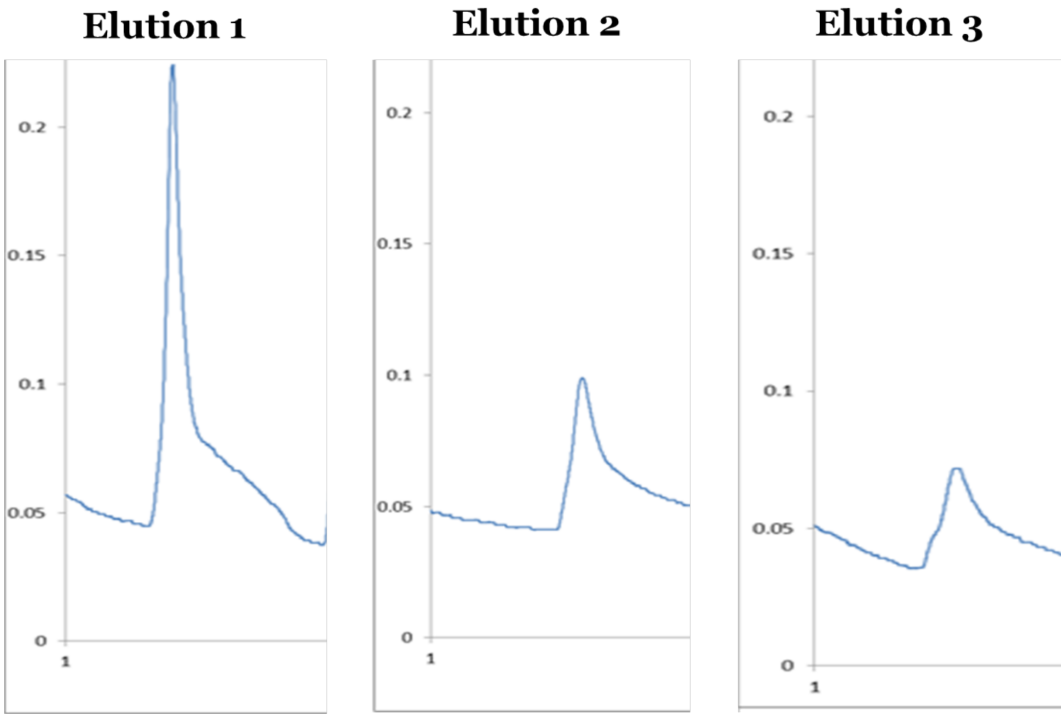


Figure 20. Absorbance profiles of three consecutive elutions from AVB column. Sf9 cultures were homogenized, filtered, and passed over an AVB column. Abs<280> of three consecutive elutions was measured.

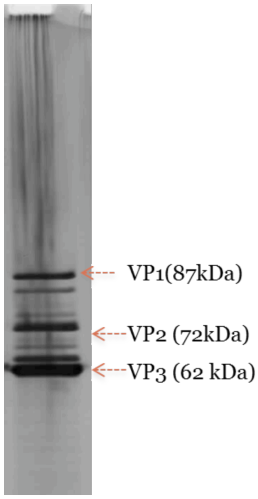


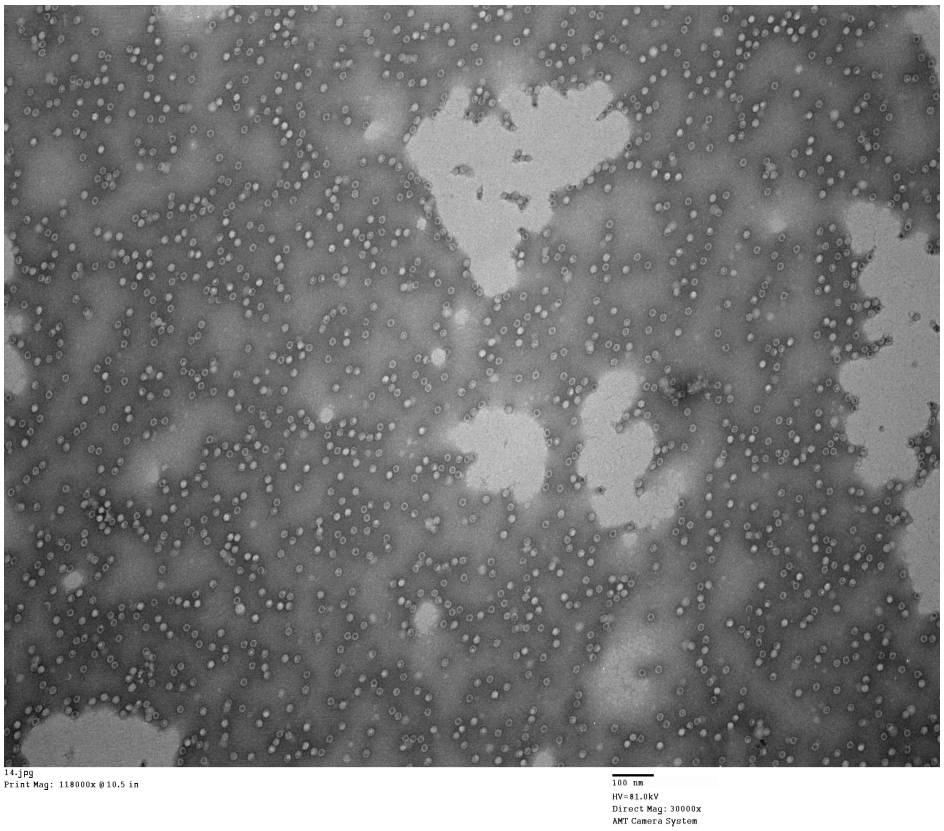
Figure 21. SDS-PAGE and silver stain of eluted fraction with greatest Abs<280>. Material eluted from AVB column was separated by size by SDS-PAGE and detected by silver stain

To quantitatively assess the DNA content of the vector particles, qPCR and DNA direct flourscent binding assays were performed. Both assays yielded similar results. According to qPCR analysis, $1.5X10^{14}$ viral particles contained the 150bp sequence amplified by

PCR. This analysis was corroborated by measurement of relative fluorescence from the binding of DNA to SYBR Gold nucleic acid dye indicated that $9.7X10^{13}$ viral particles contained nucleic acid. The similarity between the number of viral genomes calcuted from qPCR data and from DNA direct binding data indicates that a sufficient quantity of vector particles containing viral genome were mass-produced (see Appendix E.5).

6.6 Examination of Purified rAAV by TEM

Examination of purified rAAV by TEM shows that the physical particles have the size and shape of AAV and that a large fraction of these particles contain nucleic acid (Figure 22).



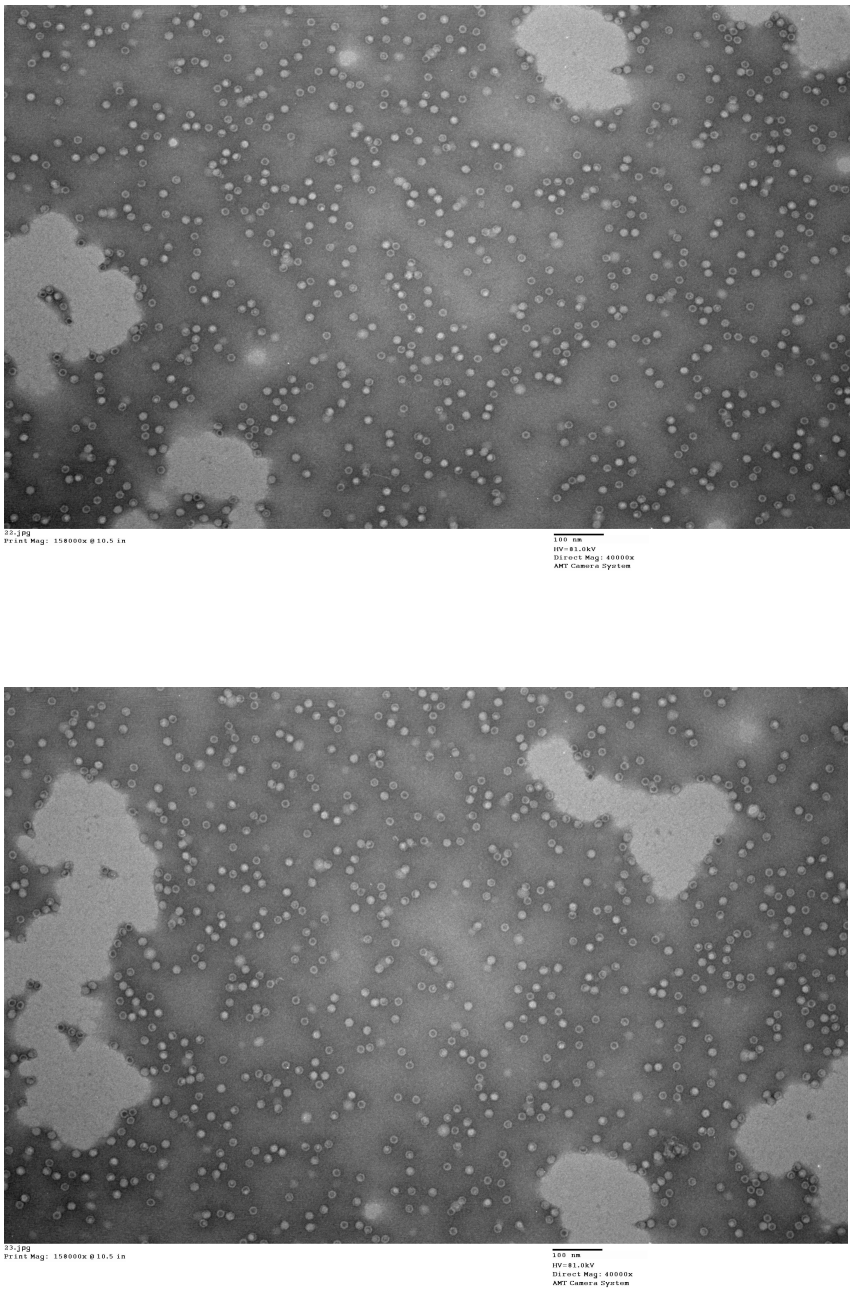


Figure 22. TEM image of purified rAAV. Diluted rAAV purified by CsCl gradient was mounted onto 300-mesh formvar/carbon-coated grids, negatively stained with pH 7.0 phosphotunstic acid, and visualized by TEM.

Chapter 7: Conclusions and Future Directions

7.1 Significance of Sequencing the Complete NZW Rabbit LDL-R mRNA

Although expression of the human LDL-R has been induced in WHHL rabbits by a viral vector in order to treat the rabbit's hypercholesterolemia, the immunogenicity of the human LDL-R in rabbits may have prevented the long-term effects of this expression from reflecting the effects of expression of LDL-R in humans (92). Thus, the use of the WHHL rabbit as an animal model for exploring the treatment of FH by transfer of an LDL-R ORF may require the expression of the rabbit LDL-R rather than the human LDL-R. Since the WHHL rabbit is an excellent animal model for FH, the safety and effectiveness of transfer of the LDL-R ORF to rabbit hepatocytes may be useful in developing gene therapy as a possible treatment for FH in humans.

Prior to this study, the 5' UTR of the NZW LDL-R mRNA was unknown. With the complete structure of the fully functional NZW LDL-R mRNA, any significant differences occurring between the NZW and WHHL genomes in the 5' UTR can be investigated and evolutionary relationships can be assessed. Additionally, research involving the NZW rabbit LDL-R mRNA can be more effective due to the enhanced ability to predict structure, make primers, and identify mutants.

7.2 Significance of Determining AAV Specificity of Rabbit Liver

Determining liver-specificity of AAV serotypes is important for assessing the feasibility of rAAV-mediated gene transfer in rabbit hepatocytes. Because

rAAV8 transduced the liver more efficiently and caused greater transgene expression levels than rAAV6, rAAV8 should be considered for future gene transfer into rabbit hepatocytes *in vivo.* Since many cases of FH result from a defect in the LDL-R, this disease may be amenable to treatment by transfer of the LDL-R ORF to hepatocytes. AAVs may be useful for gene therapy in humans because they are non-immunogenic non-pathogenic and because they can be mass-produced. For these reasons, exploring liver-specificity of AAV may be useful for developing a treatment for humans suffering from FH. Since previous literature does not address CMV silencing in rabbit hepatocytes, the observed silencing indicates that future *in vivo* studies should involve mammalian tissue-specific promoters rather than viral promoters.

7.3 Significance rAAV8-LSP-LDL-R Mass Production

The livers of large mammals, including humans, contain trillions of cells. For this reason, even with a high transduction efficiency, transfer of LDL-R ORF via a rAAV to a large fraction of liver cells requires semi-industrial production of rAAV. Moreover, the clinical use of rAAV requires preparation of rAAV without the risk of contamination with toxins or pathogens. Production of rAAV with an adenovirus helper virus or with a recombinant HSV creates the risk of contamination with pathogens, and purification by CsCl creates the risk of contamination with CsCl, which is toxic. Inspection of rAAV8-LSP-LDL-R by TEM and western blot shows that the large-scale production of rAAV8 by the baculovirus system and the subsequent purification by affinity chromatography is

feasible. Because it does not create the risk of contamination, this system for rAAV production may allow the production of quantities of rAAV-LSP-LDL-R sufficient for treatment of FH by transfer of the LDL-R ORF to human liver cells.

7.4 Future Studies

The rAAV8-LSP-LDL-R particles will be injected into WHHL rabbits in order to evaluate the effects of viral-mediated transfer of the LDL-R ORF into hepatocytes on blood LDL levels. In order to assess the safety and effectiveness of this treatment for FH in WHHL rabbits, the blood concentrations of HDL, LDL, alkaline phosphatase, and alanine aminotransferase will be measured at multiple time points post-exposure to rAAV. Additionally, liver samples will be observed under a fluorescent microscope to phenotypically analyze its morphology. Specifically, the degree of inflammation of the tissue sample will first be determined in order to evaluate the presence or severity of necrosis and possible tissue changes. To detect cholestasis, the hepatocytes will be observed for any foamy swelling or pigment changes, while potential scarring will be observed to determine if fibrosis has occurred (93). The duration of the infection and of transgene expression will be determined by qPCR with primers specific to the rAAV genome and by western blot with an anti-LDL-R antibody, respectively.

Transfer of the LDL-R ORF into WHHL rabbit hepatocytes may decrease blood LDL-cholesterol levels via expression of functional LDL-R. Because the phenotype of FH in the WHHL rabbit is similar to that of FH in humans,

successful treatment of WHHL rabbits with rAAV8-LSP-LDL-R would provide evidence that viral-mediated transfer of the LDL-R ORF may be a feasible treatment for FH in humans.

Because intracellular cholesterol homeostasis is necessary for the survival of hepatocytes, maintaining physiological LDL-R expression levels may be necessary for long-term infection. Regulatory elements that induce abnormally high LDL-R expression or that are not sensitive to intracellular cholesterol concentration may cause pathological accumulation of cholesterol in infected cells. Transfer of the LDL-R gene to hepatocytes may induce physiological and cholesterol-sensitive expression of LDL-R and, for this reason, may be more effective than transfer of the LDL-R ORF with a liver-specific promoter in long-term treatment of hypercholesterolemia resulting from LDL-R deficiency. However, at present there are no widely used viral-mediated gene therapy approaches that can deliver pieces of genomic DNA as large as the human LDL-R gene (~44 kb).

Finally, the immunological response to a gene therapy treatment must be considered. The introduction of any foreign protein, particularly the mass of protein present in 10^{13} viral particles as generated in this study (0.1 mg), is certain to pose immunological risks to the host as well as to the success of the treatment. Rabbits are known to be highly immunocompetent, making studies of immune response especially important. Successful completion of these studies in rabbits would contribute to transition of this gene therapy approach as a potential treatment option for FH in humans.

Appendices

Appendix A: Isolation and Sequencing of LDL-R Gene

A.1 Total RNA Isolation

The following protocol was performed with TRIzol Reagent Kit (cat# 15596-026), purchased from Invitrogen

1. Store 1g of rabbit tissue at -80°C

2. Homogenize sample by adding 10ml of Trizol reagent (Invitrogen, Carlsbad, CA) and grinding tissue with mortar and pestle

3. Transfer tissue sample to a 2ml low-retention tube and pass sample through a sterile, disposable needle 10 times in order to fragment high-molecular components such as DNA

4. Let homogenate sit at room temperature for 5 minutes

5. Add 200μL chloroform and vortex vigorously for 15 seconds

6. Let homogenate sit at room temperature for 3 minutes

7. Centrifuge sample at 12,000 x g for 15 minutes at 4°C

8. Transfer upper aqueous phase to new 1.5mL RNase-free tube

9. Add 5mL isopropanol to isolated aqueous phase

10. Incubate at room temperature for 10 minutes

11. Centrifuge at 12,000 x g for 10 minutes at 4°C

12. Remove supernatant from tube, leaving only the RNA pellet

13. Wash pellet with 10mL of 75% ethanol

14. Centrifuge sample at 7500 x g for 5 minutes at 4°C and discard the wash

15. Resuspend RNA pellet in RNase-free water by passing solution up and down several times through pipette tip

16. Incubate in heat block at 60°C for 10 minutes

A.2 mRNA Isolation from Total RNA

The following protocol was performed with an Illumina oligo(dT)-cellulose column (cat# 27-5543-02), purchased from GE Healthcare

1. Load 1mL 0.1M NaOH onto assembled columns and let it drain completely

2. Equilibrate the column with 4mL of binding buffer (10mM Tris-HCl, 1mM EDTA, 0.3M NaCl at room temperature) in 1mL aliquots

3. Load 1mL of binding buffer onto column and allow 0.5mL to pass through.

4. Add 3mL of binding buffer to isolated total RNA pellet

5. Heat RNA solution in a 70°C water bath for 5 minutes and chill on ice for 5 minutes

6. Load dissolved RNA solution into column under gravity flow and wash with 4mL of binding buffer

7. Elute mRNA with 1.5mL elution buffer (10mM Tris-HCl, 1mM EDTA) and collect the eluate in a 15mL sterile centrifuge tube

8. Equilibrate the column with 4mL binding buffer

9. Heat the sample from step 8 in a 70°C water bath for 5 minutes and then chill on ice for 5 minutes

10. Incubate RNA sample at room temperature for 20 minutes, then add 90µL of 5M NaCl.

11. Immediately load RNA into column and was with 4mL binding buffer

12. Repeat Step 8

13. Add 90µL of 5M NaCl and 3mL of ethanol to RNA

14. Centrifuge sample at 7,000 x g for 2 minutes at 4°C

15. Add 1mL of 75% ethanol to sample and repeat step 15

16. Remove supernatant and dry pellet at room temperature

17. Dissolve pellet in DEPC-treated 1mM EDTA and store at -20°C

A.3 Isolation and Amplification of cDNA

The following protocol was performed with the ExactSTART Eukaryotic mRNA 5'-&3'-RACE Kit (cat# ES80910), purchased from Epicentre.

A.3.1 Alkaline Phosphatase Treatment

1. Combine 10µL APex reaction buffer, 5µL APex heat-labile alkaline phosphatase, nuclease-free water, and RNA sample to achieve a total reaction volume of 100µL

2. Incubate reaction at 37°C for 15 minutes

3. Add 1µL of glycogen, 100µL of 1:1 phenol: chloroform

4. Centrifuge mixture at 10,000 x g for 1 minute. Collect and save the aqueous phase in a new tube

5. Add 100µL of nuclease-free water and repeat step 4, and combine aqueous phase with that of step 4

6. Add 200µL of 24:1 chloroform: isoamyl alcohol to combined aqueous phases and repeat step 4

7. Add 20µL of 3M sodium acetate and 100µL of isopropyl alcohol to the tube containing the combined aqueous phases and repeat step 4

8. Incubate tube on ice for 20 minutes

9. Centrifuge sample at 10,000 x g for 15 minutes at 4°C

10. Remove and discard the supernatant

11. Wash pellet with cold 80% ethanol and repeat steps 9 and 10

12. Dry pellet and Resuspend RNA in 7.5µL nuclease free-water

A.3.2 Tobacco Acid Pyrophosphatase Treatment

1. Mix the following in a PCR tube and place on ice: 1µL TAP buffer, 0.5µL RiboGuard RNase inhibitor, 7.5µL RNA sample, and 1µL TAP enzyme

2. Incubate mixture at 37°C for 30 minutes

3. After incubation period, keep mixture at room temperature and proceed immediately to Part C

A.3.3 5'-RACE Acceptor Oligo Ligation

1. Assemble the following reagents in the order given: 10µL TAP-treated RNA from Part B Step 3, 4µL nuclease-free water, 2µL RNA ligase buffer, 1µL TAP STOP buffer, 1µL 5'-RACE acceptor oligo, 1µL 2mM ATP solution, and 1µL T4 RNA ligase

2. Incubate reaction at 37°C for 30 minutes and proceed immediately to Part D

A.3.4 First-Strand cDNA Synthesis

1. Add the following reagents in a reaction tube at room temperature: 20µL of mixture from Part B Step 2, 14µL nuclease-free water, 1µL cDNA synthesis primer, 2µL dNTP Premix, 2µL MMLV RT buffer, and 1µL MMLV reverse transcriptase

2. Incubate reaction at 37°C for 1 hour

3. Incubate reaction at 85°C for 10 minutes

4. Add 1µL of RNase solution to reaction and incubate at 55°C for 5 minutes

5. Store reaction tube at 55°C until PCR amplification in Part E

A.3.5 Second-Strand cDNA Synthesis and PCR Amplification

1. Add the following reagents to reaction tube at 55°C: 41µL of mixture from Part D Step 4, 18µL nuclease-free water, 5µL PCR primer 1, 5µL PCR primer 2, 30µL FailSafe PCR premix E, 1µL pHusion (New England Biolabs, Ipswich, MA) DNA polymerase

2. Cycle the sample in a thermocycler under the following conditions: 95°C for 30 seconds, followed by 20 cycles of: 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 3 minutes, and hold at 4°C

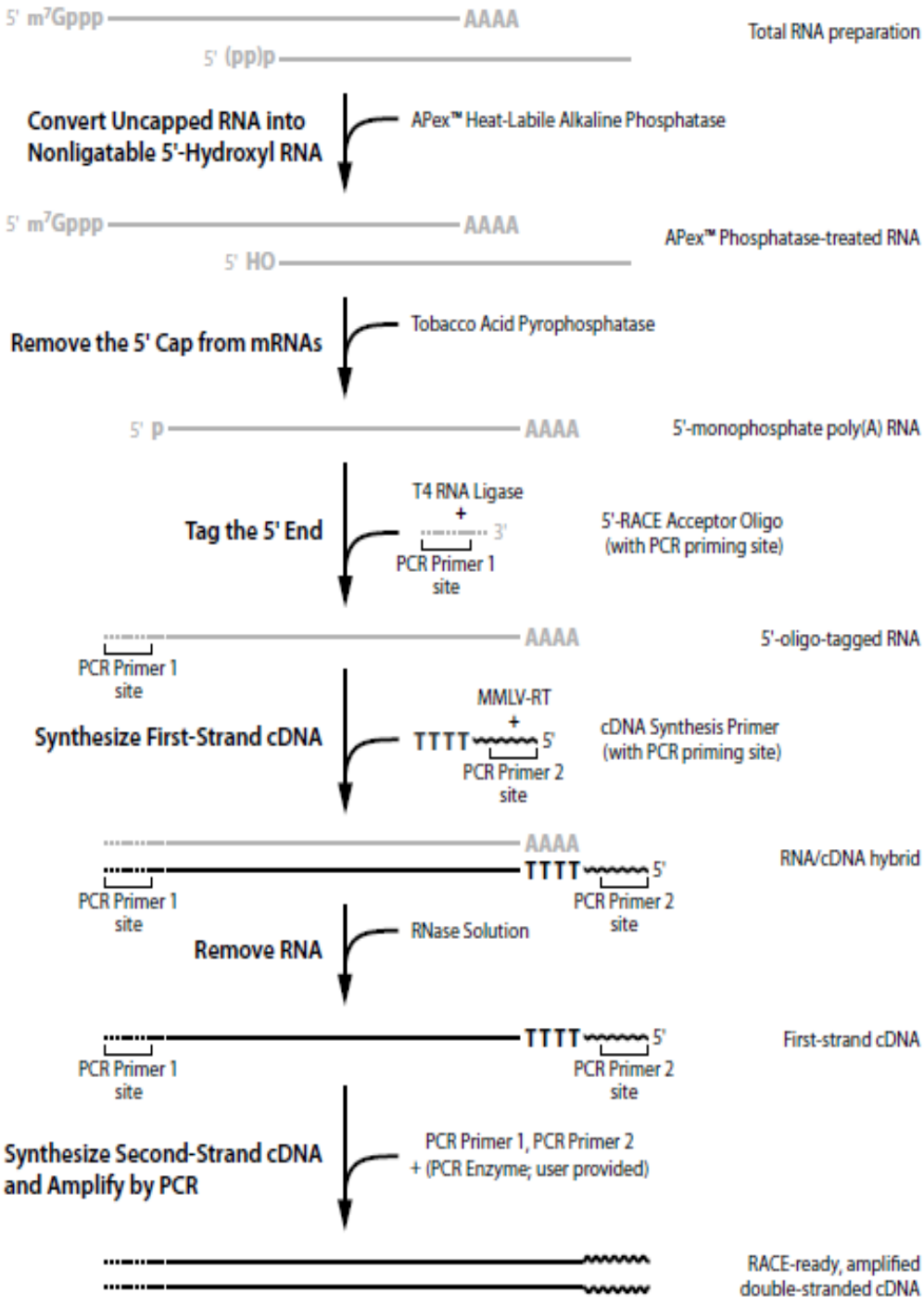


Figure 23: Overview of 5'-&3'-RACE Kit Procedure

A.4 Purification of PCR Product

The following protocol was performed with a StrataPrep PCR Purification Kit (cat#211188) purchased from Agilgent Technologies

1. Add a volume of DNA-binding solution equal to the volume of the aqueous portion of the PCR product to a microcentrifuge tube and mix solution

2. Transfer mixture to the microspin cup that is seatd in a 2mL receptacle tube

3. Microcentrifuge the mixture at maximum speed for 30 seconds

4. Remove and retain the microspin cup and discard DNA-binding solution

5. Mix 20mL of 100% ethanol with 5X PCR buffer

6. Add 750µL of mixture from step 5 to microspin cup

7. Repeat step 3 and discard the wash buffer

8. Transfer microspin cup to a new 1.5mL microcentrifuge tube and discard receptacle tube

9. Add 50µL elution buffer directly onto top of fiber matrix located at the bottom of the microspin cup

10. Incubate at room temperature for 5 minutes

11. Repeat step 3 and discard the microspin cup

A.5 1% Agarose Gel Electrophoresis

1. Mix 400ml TAE buffer with 4g agarose in a flask

2. Microwave mixture for 5 minutes, while stirring occasionally

3. Assemble gel tray while the mixture cools down

4. Pour mixture into gel tray

5. Add 4 µL ethidium bromide and let mixture sit for approximately 1 hour

6. Load samples into gel
    a) Combine 20µL of sample with 4µl 5X buffer
    b) Add 20µL into each well of gel using fine tipped pipettes

c) Add 12µL of marker/ladder

7. Run the gel at 110 volts for 25 minutes

8. View gel in transilluminator

A.6 Cloning of LDL-R cDNA products

The following protocol was performed with the PCR-Script Amp Cloning Kit (cat#400771) purchased from Stratagene

1. To prepare the ligation reaction, add the following components in order in a 0.5mL microcentrifuge tube: 1µL of pPCR-Script AMP SK(+) cloning vector, 1µL PCR-Script 10x reaction buffer, 0.5µL of 10mM rATP, 4µL of blunt-ended PCR product, 1µL of *Srf* I restriction enzyme, 1µL of T4 DNA ligase, 1.5µL distilled water

2. Gently mix ligation reaction and incubate at room temperature for 1 hour

3. Heat mixture at 65°C for 10 minutes

4. Store mixture on ice until ready to perform the transofration into the XL10-Gold Kan ultracompetent cells

5. Thaw the ultracompetent cells on ice

6. Gently mix the cells and aliquot 40µL of cells into each of 3 chilled 14mL round-bottom tubes

7. Add 1.6µL of XL10-Gold β-mercaptoethanol mix to 40µL of competent cells

8. Gently swirl the contents of the tube and incubate the cells on ice for 10 minutes

9. Add 2µL of mixture from step 4 to the tube from step 8 and gently swirl

10. Incubate tube on ice for 30 minutes

11. Preheat NZY^+ broth in a 42°C water bath for later use

12. Heat tubes in a 42°C water bath for 30 seconds

13. Incubate tubes on ice for 2 minutes

14. Add 0.45mL of broth from step 11 to each tube and incubate tubes at 37°C for 1 hour with shaking at 225-250 rpm

15. Spread 100µL of 2% X-gal and 100µL of 10mM IPTG on LB-ampicillin agar plates 30 minutes prior to plating the transformations

16. Plate 200µL of the transformation reactions and the transformation reactions containing the pUC18 control plasmid using a sterile spreader

17. Incubate plates overnight at 37°C. Colonies containing plasmids with inserts will be white.

18. Extract white colonies for examination

A.7 Plasmid Miniprep

The following protocol was performed using the QIAprep Spin Miniprep Kit (cat# 27104) purchased from QIAGEN

1. Centrifuge 3mL of overnight bacterial culture sample at 8000rpm for 3 minutes at room temperature

2. Resuspend bacterial cells in 250µL resuspension buffer (P1) and transfer to a microcentrifuge tube

3. Add 250µL lysis buffer (P2) to solution and mix thoroughly for no more than 5 minutes

4. Add 350µL neutralization buffer (N3) to solution and mix thoroughly

5. Centrifuge mixture for 10 minutes at 13,000rpm

6. Extract the supernatant from step 5 by decanting or pipetting into the QIAprep spin column

7. Wash the spin column with 0.5mL binding buffer (PB), centrifuge for 1 minute, and discard the flow-through

8. Wash the spin column with 0.75mL wash buffer (PE), centrifuge for 1 minute, and discard the flow-through

9. Transfer spin column to a 1.5mL microcentrifuge tube and add 50µL elution buffer (EB) to center of spin column

10. Let sample stand for 1 minute and centrifuge for 1 minute

Appendix B: Production of pFVG-lacZ and pFBVG-CMV-LDL-R plasmids

B.1 Site-Directed Mutagenesis of XhoI within LDL-R ORF

The reagents used in the following protocol were purchased from Integrated DNA Technologies

1. Combine the following at room temperature: 0.5µL forward primer, 0.5µL reverse primer, 0.25µL of 40mM dNTP mix, 1.25µL of 10x Pfu DNA polymerase buffer, 1µL of LDL-R ORF, 0.25µL of Pfu DNA polymerase, and 8.75µL of distilled water.

2. Run the following PCR program: a) 5 minutes at 95°C b) Repeat 18x: 50 seconds at 95°C, 50 seconds at 60°C, and 1 minute at 68°C
   c) 7 minutes at 68°C

3. Add 0.25µL of DpnI to reaction mixture and incubate for 1 hour at 37°C

B.2 Double Digestion of pPCR-LDL-R and pFBGR

1. Combine the following elements: Total 20 µL
   a) 9µL H20
   b) 2µL 10X buffer
   c) 2µL BSA
   d) 5µL DNA sample
   e) 1µL XhoI restriction enzyme
   f) 1µL AgeI restriction enzyme

2. Incubate mixture for 3 hours at 37°C

B.3 Extraction of LDL-ORF and pFBGR DNA sample from Gel

The following protocol was performed with a QIAquick Gel Extraction Kit (cat#28704), purchased from QIAGEN

1. Excise DNA fragment from agarose gel with a scalpel

2. Weigh the gel in a tube. Add 3 volumes of Buffer QG to 1 volume of gel

3. Incubate at 50°C for 10 minutes and vortex the tube every 2 minutes\

4. After the gel has dissolved completely, add 10µL 3M sodium acetate until the mixture turns yellow

5. Add 1 gel volume of isopropanol to sample and mix

6. Place the QIAquick spin column in the provided 2mL collection tube

7. Apply sample to the spin column and centrifuge for 1 minute

8. Discard the flow-through

9. Add 0.5mL Buffer QG to spin column and centrifuge for 1 minute

10. Repeat step 8 and add 0.75mL Buffer PE to spin column

11. Centrifuge the spin column for 1 minute at 17,000 x g

12. Place QIAquick spin column into a clean 1.5mL microcentrifuge tube

13. Add 50µL Buffer EB to QIAquick membrane and centrifuge column for 1 minute.

B.4 DNA Ligation of pFB and LDL-R ORF

1. Combine the following in an eppendorf tube:
    a) 1µL DNA ligase buffer
    b) 1µL T4 DNA ligase
    c) 1µL of pFB linear DNA
    d) 7µL of LDL-R ORF

2. Store in 16°C environment overnight

3. The following day, combine 4µL of ligation sample with 60µL E. coli competent cells

4. Put sample on ice for 30 minutes

5. Heat-shock bacteria in 42°C heat block for 40 seconds

6. Put sample on ice for 2 minutes

7. Add 350mL of SOC recovery media to bacterial mixture

8. Put on agitator for 1 hour at 37C

9. Transfer 100µL of bacteria to plate and spread

B.5 Plasmid Maxiprep

This protocol was performed with a QIAGEN Plasmid Plus Maxi Kit (cat#12963) purchased from QIAGEN

1. Centrifuge 250mL of overnight LB culture at 6000 x g for 15 minutes at 4°C
2. Resuspend the bacterial pellet in 10mL resuspension buffer (P1)
3. Add 10mL lysis buffer (P2) to sample, mix, and incubate at room temperature for 5 minutes
4. During incubation, screw the cap onto the nozzle of the QIAfilter cartridge
5. Add 10mL neutralization buffer (P3) to the mixture, and mix thoroughly
6. Pour lysate into the barrel of the QIAfilter cartridge and incubate at room temperature for 5 minutes
7. Equilibrate a HiSpeed tip with 10mL equilibration buffer
8. Remove cap from QIAfilter cartridge nozzle and insert plunger into cartridge
9. Filter cell lysate into equilibrated HiSpeed tip
10. Wash the HiSpeed tip with 60ml wash buffer (QC)
11. Elute DNA with 15mL elution buffer (QF)
12. Add 10.5mL isopropanol to lysate, mix, and incubate for 5 minutes
13. During incubation, remove plunger and attach QIAprecipitator Module onto cartridge nozzle
14. Transfer the eluate-isopropanol mixture into the syringe and insert the plunger
15. Filter the mixture through the QIAprecipitator using constant pressure
16. Remove QIAprecipitator from syringe and take out the plunger. Reattach the QIAprecipitator and add 2mL 70% ethanol to syringe
17. Remove QIAprecipitator from syringe and take out the plunger. Reattach the QIAprecipitator, insert the plunger, and dry the membrane by forcefully pressing air through the QIAprecipitator
18. Dry the nozzle of the QIAprecipitator

19. Remove the plunger from a new 5mL syringe, attach the QIAprecipitator and hold it over a 1.5mL collection tube
    a) Add 1mL buffer TE to syringe
    b) Insert plunger and elute DNA into collection tube using constant pressure

20. Remove QIAprecipitator from syringe, pull out plunger, and reattach QIAprecipitator to the 5mL syringe

21. Transfer the eluate from step 19 to the 5mL syringe and elute a second time into the 1.5mL collection tube

B.6 Transfection of HeLa Cells

1. Seed 5 x 10^4 cells/35mm dish in 3mL complete medium 24 hours prior to transfection

2. Add 97µL serum-free medium into bottom of 7mL Bijou tube

3. Pipette 3µL of FuGene 6 reagent into medium without allowing any contact with walls of plastic tube

4. Gently flick the tube to mix the solution and incubate for 15 minutes at room temperature

5. Add 1µg DNA to diluted FuGene reagent

6. Gently flick the tube to mix the solution and incubate for 30 minutes at room temperature

7. Add solution to HeLa cells in a dropwise manner. Gently swirl the wells to ensure even distribution over the entire plate surface

8. Incubate cells at 37°C for 2 hours

B.7 Western Blot

1. Obtain protein lysate
    a) Keep cells on ice
    b) Resuspend cell pellets in extraction buffer
    c) Sonocate cells with decontaminated sonocator tip
    d) Centrifuge cells at 16rpm for 10 minutes

2. Add 100µL blue buffer and 5µL mercaptoethanol to samples

3. Heat samples in 95°C heat block for 5 minutes

4. During heating, prepare 10% Tris-glycine gel
    a) Wash wells with distilled water
    b) Fill gel cartridge with tris-glycine buffer

5. Load wells with either 7µL of marker, 7µL of control, and 7µL of each sample

6. Run the gel at 30amps for 1 hour

7. Prepare membrane and sponges for blotting
    a) Place sponges and a membrane in two separate beakers, each containing 1400mL transfer buffer
    b) Squeeze sponges inside buffer to remove any air bubbles
    c) Pour buffer into a large plastic container

8. Place the cartridge with the gel still on it into transfer buffer and gently rock it to see if the gel will come off of the cartridge on its own. Allow it to sit in buffer for 10 minutes.

9. Assemble the transfer apparatus
    a) Position one sheet of filter paper underneath the gel, while still in the buffer
    b) Gently lift the gel and filter paper and place them on top of 3 sponges
    c) Place soaked membrane on top of the gel and use pipet to remove air bubbles
    d) Place second piece of filter paper on top of membrane and remove air bubbles
    e) Place 3 more sponges on top of filter paper
    f) Place apparatus into vertical gel cartridge

10. Add transfer buffer to gel cartridge and run the gel at 25 volts in 4°C

11. Apply primary antibody Anti-LDLr pAb (Abnova cat#PAB8804)
    a) Wash with western blot buffer and rock apparatus gently

12. Apply secondary antibody Bovine anti-rabbit igG-HRP (Santa Cruz Biotechnology cat# sc-2370)
    b) Wash with western blot buffer and rock apparatus gently

B.8 Enhanced Chemiluminscence

1. Cut a piece of sheet protector to the proper size and place it in the metal developing cartridge

2. Dry membrane from western blot apparatus

3. Place membrane inside sheet protector

4. Put minimal amount of signing reagent (developing peroxide solution) onto membrane until the entire membrane is covered

5. Let membrane sit for 2 minutes and pipette any excess liquid

6. Close sheet protector and tape it into the x-ray film cartridge

7. Take cartridge to the dark room and activate the developing machine

8. Cut a corner of the film to distinguish which way the samples are oriented on the film in comparison to the membrane from the western blot

Appendix C: *In vivo* Study to Determine Optimal rAAV Serotype for Rabbit Liver Transduction

C.1 Guidelines of Ethical Treatment and Euthanasia

All research conducted using animal models must meet clear and strict and specific guidelines outlined by the American Veterinary Medical Association (AVMA), including proper and humane euthanasia. As described by the AVMA, it is the duty of every veterinarian to show the highest level of respect to animals and ensure that their death's for research be as painless and distress free as possible. While it is not always possible to euthanize an animal with complete absence of pain or discomfort, the AVMA requires that euthanasia techniques result in a rapid loss of consciousness, followed by cardiac arrest, and then ultimately a loss of brain function (29).

In its latest report on euthanasia guidelines, the AVMA describes two different categories for euthanasia methods: acceptable and conditionally acceptable. As described in the 2007 report, "acceptable methods are those that consistently produce a humane death when used as the sole means of euthanasia; conditionally acceptable methods are those techniques that by the nature of the technique or because of greater potential for operator error or safety hazards might not consistently produce humane death or are methods not well documented in the scientific literature" (29).

For the purpose of study, the following acceptable methods, in regards to rabbit models, were examined (28, 29).

1) *Barbiturate Overdose* - Barbiturates are drugs that are able to depress the central nervous system (CNS) and slowly can induce euthanasia by

shutting down the cerebral cortex and creating a loss of consciousness in animal species. An overdose of these drugs create deep anesthesia, followed by apnea, loss of control of the respiratory system, and finally cardiac arrest. Barbiturates are best administered intravenously and can be an effective method of euthanasia because of the minimal pain experienced by animals, as well as their cheap expense compared to other methods.

2) *Inhalent Anesthetic Overdose* - Several inhalent anesthetics exist to euthanize animal species including ether, halothane, methoxylfurane, desflurane, and isoflurane; however, studies have shown that halothane has been found to be the most effective and safe inhalent anesthetic when trying to induce anesthesia quickly. In order for inhalent anesthetics to function, animals must be placed in a closed container with either a gauze soaked in the chosen anesthetic or the anethesthic transferred into the container by a vaporizer. Of the two available options, vaporizing is the least preferred method because of the longer induction time and the commonality of animals struggling and becoming irritated and anxious during vaporization. Inhalent anesthetics are an effective agent for smaller animals (typically 7 kilograms or less) and are considered safe in laboratory settings as most available inhalent anesthetics are both nonflammable and nonexplosive.

3) *Carbon Dioxide (compressed tanks only)* When used with the right equipment, carbon dioxide (CO2) can be an effective method of

euthanizing small laboratory animals such as rats, mice, chickens, and rabbits. However, small burrowing animals such as rabbits of the species Oryctolagus experience some complications during exposure to CO2, as these animals have the physiologic mechanisms for dealing with hypercapnia, or loss of consciousness due to too much carbon dioxide in the bloodstream. Thus, researchers must use a sufficient concentration of carbon dioxide to kill the animal by hypoxemia. The only allowable technique of administering carbon dioxide to animals is through gas cylinders, as the chambers allow the exchange of the gas to be accurately regulated. CO2 is also a popular euthanasia method because of the gas is inexpensive, nonexplosive, nonflammable, and nearly harmless to personnel staff.

4) *Carbon Monoxide* - A colorless, odorless gas, carbon monoxide (CO) can block the uptake of oxygen by binding to hemoglobin and cause fatal hypoxemia. CO remains both nonflammable and nonexplosive as long as the gas concentration remains below 10%. However, regardless of the gas's concentration it can still be extremely dangerous for personnel working with it due to its toxicity and difficult to detect. An exhaust system is required to prevent humans from exposure that can create a health hazard. The gas itself creates a loss of consciousness in animals with minimal discomfort and an absences of pain. Concentrations between four to six percent are often used to ensure that death occurs quickly.

5) *Potassium Chloride in Conjunction with General Anesthesia* - Intravenously or intracardially injecting a supersaturated solution of potassium chloride into animals has been deemed an appropriate method of creating cardiac arrest and ultimately death. AVMA does note however that it is unacceptable to apply this method to an unanaesthetized animal. Animals must be under anesthesia and have a loss of consciousness, reflex muscle response, and response to noxious stimuli. The injection of the cardiotoxic potassium ions (administered 1 to 2 mmol/kg of body weight) will cause cardiac arrest in the subjects. Although potassium chloride is not a controlled substance and thus easy to obtain in research facilities, personnel must be trained and knowledgeable inanesthetic techniques to be able to appropriately administer the substance. Often times potassium chloride in conjunction with general anesthesia is not a chosen method for euthanasia because of the substance's ability to create rippling of muscle tissue and clonic spasms after the injection occurs.

C.2 Approved IACUC protocol detailing rabbit care, blood withdrawal, viral injection, rabbit sacrifice, and end state tissue collection

UMCP Institutional Animal Care & Use Committee
Animal Study Protocol

*Please note that 'animal' is used to generically define all vertebrates covered by AWR and PHS Policy.

PART I: CORE INFORMATION (Sections A - F required for all protocols)
SECTION A: ADMINISTRATIVE

A1. PERSONNEL INFORMATION

Principal Investigator: Dr. Ioannis Bossis | Dept/Div: Veterinary Medicine | Bldg/Office #: 795/1415
Office Phone: 3013148042 | Lab Phone 3013142587 | E-mail Address bossisi@umd.edu
Project Title: AAV6/8-mediated gene transfer of the low-density lipoprotein receptor (LDL-R) in the liver of the heritable hyperlipidemi
Funding Source(s) Gemstone | Has a grant been submitted for this work? ○ NO ◉ YES (It is optional to attach proposal.)
Protocol Number: (Assigned by IACUC) | Previous Protocol Number: (Renewals Only):

A2. INTERACTION WITH ANIMALS

○ None. This is an observational study. No animal holding, housing, or management will occur under this protocol. (Section O is not required.)
◉ This is a study where animals will be held, housed, managed, or controlled. (Section O is required.)

A3. KEY PERSONNEL

NOTE: A Personnel Qualification Form (Part I: Section F) must be completed for every member of the research group listed on this protocol. The form should illustrate skills or training necessary for the roles specified below (e.g., surgeon, anesthetist, phlebotomist, breeder, provides husbandry, observer, etc.).

	NAME	ROLE IN PROJECT
Add Name	Bossis, Ioannis	Principle Investigator
Add Name	Powell, Doug	Attending Veterinarian,Trainer and surgeon
Add Name	Rajawat, Yogendra	Graduate Research Assistant
Add Name	Atlas, Dan	Researcher
Add Name	Buckshaw, Liz	Researcher
Add Name	Hanna, David	Researcher
Add Name	Lannon, Jennifer	Researcher
Add Name	Mahmud, Alia	Researcher
Add Name	Mirvis, Mary	Researcher
Add Name	Pham, Anna	Researcher
Add Name	Ramnarain, Nadira	Researcher
Add Name	Randazzo, Paul	Researcher
Add Name	Safferman, Michelle	Researcher
Add Name	Zuckerberg, Jeremy	Researcher

A4. COLLABORATING (INCLUDES SUB-CONTRACTING) INSTITUTIONS

Will any facilities other than University of Maryland facilities (main campus or Research and Education Centers) be used for animal use activities (e.g., housing, experimentation, observation, or procedures)?

Version 2009.01

(•) No. All work will be performed in University of Maryland facilities.
( ) Yes. Work will be performed at another institution. **STOP.** Please contact the IACUC Manager before you fill out this form.

A5. CONTINUING RESEARCH

Does this application continue research conducted on a current or previous protocol?

(•) No. This is a new protocol. ( ) Yes. This protocol is the 3 year *de novo* review of a currently active protocol.

A6. SUPPLEMENTAL SECTIONS INCLUDED IN THIS APPLICATION (Select all that apply.)

(Please fill out those supplemental sections that pertain to the nature of your research.)

- [X] Section G: Hazardous Agent Use* - i.e. biological, chemical, radiological, rDNA (*only those agents used on animals)
- [X] Section H: Chemical Restraint for Non-surgical procedures
- [ ] Section I: Survival Surgical Procedures
- [ ] Section J: Non-Survival Surgical Procedures
- [X] Section K: Non-surgical Procedures
- [ ] Section L: Field Capture / Field Studies
- [ ] Section M: Breeding colonies (Including Genetically Engineered Animals)
- [ ] Section N: Antibody Production
- [X] Section O: Special Instructions for Emergency Animal Care REQUIRED for all animals held on campus.
- [ ] Section P: PI Managed Animal Facility (holding longer than 12 hours)
- [ ] Section Q: Exemptions to The Guide or Exemptions to Routine Animal Care Procedures

Previous Protocol Number:
(Renewals Only):

SECTION B: ANIMAL USE JUSTIFICATION

B1. PURPOSE

Describe in lay terms the purpose and goals of this animal use study. Discuss the potential scientific benefit with respect to human or animal health, the advancement of knowledge, or the good of society.
Avoid the use of jargon and define ALL acronyms/abbreviations.

Familial hypercholesterolemia (FH) is a genetic disorder caused by mutations in the LDL-R gene and causes individuals to have abnormally high levels of cholesterol in the blood. Our goal is to determine if gene therapy can treat FH in humans. Viral-mediated gene therapy holds the potential for a effective treatment by inserting a functional gene for transcription. Transfer of a functional LDL-R transgene by recombinant Adeno-Associated virus-6 or 8 (rAAV6/8) will increase LDL-R activity and therefore decrease blood Cholesterol concentration. The Watanabe Heritable Hyperlipidemic Rabbits (WHHL) is an excellent animal model because it mimics the condition in humans. If successful, this treatment could potentially be used in treating this fatal disorder in humans. Before doing the experiments to WHHL rabbits, we are proposing to do pilot experiments on New Zealand White Rabbits using rAAV6/8 expressing Alkaline Phosphatse (rAAV6/8-AP) to determine Viral doses and to establish experimental end points.
In recent Years, Adeno-Associated virus(AAV) vectors have received much attention in the field of gene therapy for their ability to establish long-lasting transgene expression in a variety of tissues. AAV vectors have a number of appealing qualities for gene therapy including lack of pathogenicity and toxicity, no autonomous replication ability (non-replicative), absence of viral coding sequences from the vector genome, ability to infect dividing and non-dividing cells, highly efficient transduction of various cell types and low intrinsic adjuvant properties that prevent activation of innate immunity and rapid immunoclearance of the viral vector. Owing to unique combination of attractive features, recombinant AAVs (rAAVs) are one of the most promising viral vectors for gene therapy. A vast number of experimental animals (mice, dogs, pigs, nonhuman primates), and several clinical trials in humans, have provided convincing results for the efficacy and safety of this genetic delivery system.

B2. LITERATURE SEARCH FOR ALTERNATIVES TO PAINFUL PROCEDURES

Does the study include procedures that have the potential for producing pain (see instructions)?

(•) No. There is no potential for pain.
( ) Yes. There is potential for pain.

B3. ANIMAL USE JUSTIFICATION (check all that apply)

The justification for using live vertebrate animals rather than alternative means of achieving the research goal is: (check all that apply.)

- [X] The complexity of the processes being studied cannot be duplicated or modeled in non-vertebrate systems because:

There are no non-vertebrate species that mimic this disorder. There is no natural model of FH in mice. Genetically modified mice with inactivated LDLR gene are available. However, this model doesn't reflect the condition in humans

with FH, where the LDLR is produced but has reduced functionality. In addition, the size and anatomy of mice is very different from that in humans, and experiments will have diminished predictive value. There are rat models that could potentially be used but are ultimately inappropriate because in rats and mice the hypercholesterolemia is induced and not genetic in origin.

☐ There is not enough information known about the processes being studied to design nonliving models. Explain:

☐ Other (explain):

B4. SPECIES JUSTIFICATION (address each species individually)

Species: Rabbit

This species was selected for the study because of the following attributes (select all that apply):

☒ A large database exists allowing comparisons with previous data.

Explain: Literature search conducted on Pubmed with key words Familial hypercholesterolemia and Animal models (215 Results), FH and Mice (201 Results), FH and Rats (90 Results), FH and Rabbits (211 Results) verified that WHHL rabbits are the natural animal models that mimic human disease. Recent Manuscript (Journal of Biomedicine and Biotechnology Volume 2011,2011, Article ID 406473) (http://www.hindawi.com/journals/jbb/2011/406473/#B8) describes that mice and rats are inappropriate because of marked differences in lipoprotein metabolism, pathophysiological findings of atherosclerosis.

☒ The anatomy or physiology is uniquely suited to the study proposed.

Explain: The WHHL rabbits have the same genetic disorder seen in affected humans in which a defective LDL receptor is produced. Lipoprotein metabolism in rabbits closely resembles that in humans. However, representative laboratory animals such as mice and rats have very different lipoprotein metabolism from that in humans.

☒ This is the lowest species on the phylogenetic scale that is suitable for the proposed study.

Explain: The only other species that exhibits a disorder similar to that found in humans is a cat model. However, our policy is to avoid research using companion animals.

☐ Other attributes. *(details required)*.

Add Species

B5. NUMBER JUSTIFICATION (address each species individually)

Species: New Zealand White Rabbits

The number of animals requested for this protocol is based on the following (select all that apply):

☒ A statistical estimate of the number required to achieve statistical significance.

Explain: The sample size for the in vivo gene therapy study (5 rabbits per group) was determined assuming: alpha = 0.05 (two sided), beta = 0.20 (one sided), a standard deviation in responses equal to one third the mean response (as derived from previously published studies), and a desired power which will allow detection of a 5-fold increase in liver concentration of recombinant alkaline phosphatase in animals receiving recombinant AAV from that of the reference group. The two-tailed unpaired Student t test, the Mann-Whitney U test or the Fisher exact test will be used to assess the significance of differences in response between animals receiving different viral doses and control group.

☐ The estimated minimum number necessary to achieve the goals of the study in the absence of a statistical estimate.

☒ The number necessary to obtain sufficient tissue or other material for testing or analysis.

Explain: The number of rabbits proposed in this study are required for adequate determination of the optimum gene therapy strategy using adeno-associated viral vector. These animals will be used for pilot experiments to determine the viral dose and Bioavailability.

☒ The number required to provide sufficient technical training or practice for the number of trainees expected.

Explain: The number of rabbits proposed in this study will also be used for technical training of undergraduate students listed in this protocol. Students will be train to restrain,Intravenous fluid administration,blood collection and finally organ collection after proper anesthesia.

☐ Other *(details required)*.

Add Species

B6. ANIMAL NUMBERS

Adult and/or juvenile/larval animals only:
NOTE: If this is a 3 year renewal protocol and there are animals remaining on the expiring protocol, the number of animals remaining on the expiring protocol (and transferring to the new protocol) must included in the number of animals requested under the new protocol. Animals on the expiring protocol will be transferred to the new protocol upon activation of the new protocol.

Species: Rabbits | Age or weight range: 3-6 months | Sex and strain/stock: F/New Zealand White

TOTAL# required for 3-year protocol: 40

Categorize animals by the most severe pain they will experience using USDA Categories: *See IACUC website for examples of painful procedures.*

Category C: (Non-Painful Procedures)	Category D: (Procedures using anesthesia/analgesia)	Category E: (Painful procedures without anesthesia/analgesia)
40	40	

Source of the animals:
NOTE: If transferred from another protocol, provide PI name and protocol number. Charles River Laboratories

Add Species

Will embryonic and/or neonate animals be used at any time during this protocol?

- ○ No. Only censused animals will be used. See IACUC policy on *Accounting for Animals in Census.*
- ○ Yes. Embryos (avian, aquatic, or mammal) and/or preweaning neonates will be used.

B7. OVERVIEW OF EXPERIMENTAL DESIGN AND ANIMAL USE TIMELINES

Provide a brief (250 words or less) summary of the overall experimental design of this proposal. The description should define animal groups, group sizes, and how each group will be tested or used. It should not include a detailed review of surgery or other activities, but should include the use of any unique drugs or practices: *Animal numbers described in timeline must be consistent with section B.6.*

New zealand white (NZW) rabbits will be used for pilot experiments to determine the optimal dosage of recombinant AAV-6 (rAAV-6-AP, AAV-6 expressing Alkaline Phosphatase) and time points required for optimum virus dissemination in various organs and transduction efficiency to liver . Injection of recombinant virus will be performed through the ear vein. NZW rabbits will be divided in 6 groups, 3 groups for two time points.
Control Rabbit→ 5 NZW rabbits
10^13 rAAV → 5 NZW rabbits
10^14 rAAV → 5 NZW rabbits
10^15 rAAV → 5 NZW rabbit
Total= 20
As there are two time points.
15X 2 = 40 NZW rabbits in total.

Virus dissemination in various organs and transduction efficiency with recombinant virus will be evaluated 3 and 6 weeks post-delivery. Tissues will be collected from euthanized experimental animals. We anticipate that most virus will end up in the liver, which is the target organ. Five control rabbits(will not receive virus), Five rabbits will receive 10^13, Five will receive 10^14, and Five will receive 10^15 viral particles. The virus will be diluted in normal saline and sterilized with filtration through 0.22 micron millipore filters. 1-10mL (No more than 10 ml of saline per rabbit) of volume depending on dosing, will be injected per rabbit. We will perform this experiment to determine optimal dosing and dilution on New Zealand White Rabbits. After the NZW rabbits have been injected, blood samples will be collected every week (for 3 and 6 weeks) , also through the ear vein. Less than one milliliter of blood will be collected from each rabbit each week.

The use of NZW rabbits will also aid in training the undergraduate students.

Describe the anticipated sequence of experimental events (timeline) such as breeding, preparation of animals, surgery, testing procedures, collection of tissues, euthanasia, etc.:

Upon receiving, the animals will be given seven days for acclimation to the animal facilities. After this period, injection of viral particles will take place. A week after initial injections, blood samples will be drawn weekly until the end of the experiment. At the end of each experiment, rabbits from all the groups will be euthanized and several tissues will be collected.
All virus injection will be performed through the marginal ear vein. No more than 10 ml of normal saline solution containing virus will be injected per rabbit. Intravenous (IV) injections will be administered following the procedures described below.

1) Rabbits will be taken out from cages using standard operating procedure for restraining, and immediately will be put in restraint box.
2) After proper restraining, we will administer sedatives. Sedative will be administered 15 to 30 minutes prior of the procedure. Light anaesthetics/Sedative used will be Ketamine (25 mg/kg)/Xylazine(5 mg/kg) IM. Rabbits will be tranquilized to reduce handling stress, enhance vasodilation and prevent injury.
3) We will first locate the marginal ear vein. Gentle stroking and tapping of the ear will make the vein more visible. We will disinfect the injection site, and insert a catheter using 22-24 gauge butterfly needle into the vein at a slight angle. We will aspirate/draw blood to make sure it is properly inserted. Butterfly needle will be properly secured with adhesives and clips.We will use the lateral saphenous vein in case of problems with ear veins.

5) Gradually (within 10-15 min), we will administer the normal saline containing virus.
6) After administration, we will apply pressure at vein using the cotton swab proximal to injection site and release the butterfly needle.
7) We will apply pressure in the site to create hemostasis (clothes pin or cotton swab) for at least 2 minutes. The ear will be flipped back and forth to assure that bleeding will not re-start.
8) Rabbits will be monitored and we will only release animal from restrainer when bleeding has completely stopped and no swelling or hematoma is formed. We will move animals to their cage for recovery. Animals will be monitored until recumbent, however, an animal regaining consciousness will potentially struggle.
9) After IV injections, rabbits will be monitored twice daily.

Ear vessels in the rabbit (central ear artery or marginal ear veins) are readily accessible and will be used for collecting blood. While collecting the weekly blood samples, we will withdraw blood using the following procedure.
1) Rabbits will be taken out using the proper restraining procedure.
2) Rabbits will be tranquilized to reduce handling stress, enhance vasodilation and prevent injury. The Ketamine (25 mg/kg)/Xylazine(5 mg/kg) IM will be used as recommended.
3) If required rabbits will be wrapped in a large cloth to avoid inadvertent movement. Restraint can cause stress, therefore the duration of restraint will be minimized.
4) Place the rabbit in the restrainer.
5) The ear will be warmed by gentle rubbing or tapping the area with finger to dilate the vessel.
6) Clean the skin with alcohol (70% alcohol) and all the procedures will be carried out aseptically.
7) Holding the needle with two fingers, penetrate the vein or artery with a small 20-24 gauge needle (depending on size of rabbit) with the bevel up. Sampling of blood from the vein will be performed as close to the base of the ear as possible, whereas sampling from the artery will be performed nearer to the tip of the ear. Additional attempts will be made distally toward the ear tip for the vein and proximally toward the base for the artery.We will use the lateral saphenous vein in case of problems with ear veins.
8) We will allow the arterial blood to drip from the needle hub and free catch it in an appropriate collection tube.
9) Approximately 1ml blood will be collected using 20-24 gauge needle syringe.
10) In order to minimise damage to the ear the number of attempts to take a blood sample will be minimized (no more than three needle sticks in any one attempt).
11) After collecting the desired amount, blood flow will be stopped by applying finger pressure on soft tissue placed at the blood sampling site to create hemostasis (clothes pin or cotton swab) for at least 2 minutes.
12) Flip the ear back and forth to assure that bleeding will not re-start.
13) Only release animal from restrainer when bleeding has completely stopped and no hematoma is formed.
14) We will move animals to their cage for recovery. Animals will be monitored until recumbent, however, an animal regaining consciousness will potentially struggle.
14) Log necessary information in the Rabbit Use Record.
15) Check rabbit within 15 min upon returning to cage.

At the end of each experiment (3 weeks and 6 weeks) all rabbits will be euthanized using higher dose of anaesthetics (Ketamine (35-50 mg/kg)/Xylazine(5-15 mg/kg) and exsanguination after higher dose of anaesthetics . After euthanasia, tissue samples will be collected from all rabbits by opening the abdominal cavity aseptically. After liver sample collection,carcass will be disposed off (for incineration) as recommended by DES,UMCP with the help of animal facility manager/research specialist Yonas Araya.

SECTION C: SPECIAL CONCERNS FOR ANIMAL USE

C1. ANIMAL HOUSING and PROCEDURE LOCATIONS (UMCP)

Specify building and room number for each planned activity below (address each species individually). Section O required.

Species: **New Zealand White**

ACTIVITY		BUILDING	ROOM
Housing/Holding	Greater than 12 hours	795	1420S/1420T
	Less than 12 hours		
Survival Surgery	Pre-operative preparation		
	Surgery		
	Post-operative care		
Non-Survival Surgery	Pre-operative preparation		
	Surgery		
Nonsurgical Procedures (ie. Behavior testing)			
Euthanasia (including Tissue Harvesting)			
Imaging			
Breeding Colony Activities			

Add Species

C2. SPECIAL HUSBANDRY REQUIREMENTS

Are there any special husbandry needs? Note that special husbandry needs that are approved must be implemented through direct arrangements with DLAR or Facility Manager of the relevant housing facility.

- (•) No. There are no special husbandry requirements.
- ( ) Yes. There are special husbandry needs. (Section Q must be filled out.)

C3. PHYSICAL RESTRAINT

Will the proposed research require the use of physical restraint (other than short-term hand-held) of awake animals?

- ( ) No. Physical restraint of awake animals will not exceed short-term hand restraint.
- (•) Yes. Physical restraint must be used.

Please address C3a - f below.

C3a. Briefly describe or identify the restraint device:

Commercially Available Rabbit Restraint Boxes will be used.

C3b. Briefly describe the procedure for restraining the animal:

To remove a rabbit from its cage, we will grasp the loose skin around the neck and pull the rabbit to the front of the cage. Using the other hand, we will support the rabbit's hind quarters and lift it out of the cage. After we have the rabbit out of the cage, tuck the rabbit into the cradle of one arm. If at any time the rabbit begins to struggle, either we will place it down or hold it close to the body and re-establish hold. If the rabbit is held properly, it will feel secure and will not struggle.
When restraining a rabbit on the tabletop, place one hand around the rump and rest other hand across the shoulders. If more restraint is needed, a commercial restraint device will be used, such as restraint box.
When placing the rabbit back into the cage, first we will place the hind end inside the cage. This will prevent the rabbit from trying to leap into the cage.

C3c. State the duration of the restraint period:

Rabbits will be restraint for IV injections (15 min.-45 min) and for weekly bleeding (10-30 min.).

C3d. Describe the plan for observation of the animal during the period of restraint:

We will monitor activity of every animal. We will Observe each animal for 'normal' behavior. Normal being defined as "alert, active and responsive to stimuli. In case of sedation we will monitor Heart rate,Respiration rate, various reflexes (Paw pinch,Corneal reflexes).

C3e. Describe the plan for animal care and support during the period of restraint to ensure comfort and well-being:

All animals will be handled very gently and will be restraint following proper restraining procedures. Restraint can cause stress, therefore the duration of restraint will be minimized. If required rabbits will be wrapped in a large cloth to avoid inadvertent movement and hypothermia.

C3f. Describe the procedure for conditioning the animal to the restraint device and procedure so as to minimize potential animal distress during restraint:

Afetr initial procurement rabbits will be acclimatize to the cages. Restraining the rabbits will be accomplish using specially designed restraint boxes To decrease stress rabbits will be accustomed to the restraint box before starting actual research protocol. Rabbit will panic if its head is restrained and it feels it can back out of the box therefore we will secure the hindquarters first,and then the head. If any point the rabbit panics while in a restraint box,release all restraints immediately (releasing the head first).

C4. WITHHOLDING OF ANESTHETICS OR ANALGESICS

Does this protocol involve procedures that are expected to cause pain, but for which pain-relieving anesthetics and/or analgesics will not be provided? (A literature search must be conducted for any painful procedures.) *All numbers must be consistent with B.6 and B.7.*

- ( ) No. There are no painful procedures (i.e., no greater pain than would be expected from simple injections).
- (•) No. Anesthetics and/or analgesics will be provided for pain relief.
- ( ) Yes. This protocol includes painful procedures for which anesthetics and/or analgesics must be withheld.

C5. ANTICIPATED COMPLICATIONS (This section required for all LD/ID/MI studies.)

Do you anticipate any animal health complications (e.g. local or systemic infection, physical or physiological impairment, heavy tumor burden, tumor necrosis, malnutrition, dehydration, etc.) arising from the experimental procedures or animal manipulations in this protocol?

- (•) No. Animal health complications are not expected.
- ( ) Yes. Animal health complications may occur. Plan of care must include humane endpoints.

C6. ADMINISTERED SUBSTANCES

- ( ) No. No other substances will be given to animals.
- (•) Yes. Administered substances are listed below. Justification is needed for all non-pharmaceutical grade substances.

List substance, dose or concentration, route (to include osmotic pumps, via headposts, etc), volume, frequency, site and needle size.

Substance 1

rAAV-6-AP will be administered intravenously. Dose will be 10^13 or 10^14 or 10^15 viral particles per animal.

Additional Substance

SECTION D: EUTHANASIA & DISPOSITION

D1. EUTHANASIA: (consistent with AVMA Guidelines for Euthanasia)

Please indicate the role of euthanasia in the proposed activity:

- [x] Animals will be euthanized as part of the experimental protocol. (Specify method below.)
- [ ] Euthanasia is not planned, but will be performed to prevent animal distress. (Specify method below.)
- [ ] Euthanasia will not be performed.

Please specify the method(s) of euthanasia below. Use a separate line for each species studied under this protocol.

Generally Acceptable Methods Of Euthanasia *(injection, inhalation, physical under anesthesia)*

	Species	Method	Anesthetic or Injectable Agent / Dose / Route of Administration or MS222 Concentration
Add Species	New Zealand White	Exsanguination under anesthesia	50 mg/kg Ketamine and 15 mg/kg xylazine

NOTE: If CO2 or isoflurane euthanasia was selected for any species, a secondary method to ensure non-recovery is required. Please select the secondary method(s) that will be used to ensure euthanasia:

- [ ] Bilateral thoracotomy
- [ ] Decapitation
- [ ] Tissue / organ collection.
- [ ] Other method to ensure death Specify:

Conditionally Acceptable Methods Of Euthanasia *(physical without anesthesia)*

	Species	Method	Scientific Justification for Procedure without Anesthesia
Add Species			

D2. FINAL DISPOSITION OF ANIMALS

Indicate the method(s) of terminating responsibility for the live animals (select all that apply):

- [x] Euthanized by methods specified in section D1 above (Euthanasia).
- [ ] Live animals returned to production / breeding unit.
- [ ] Live animals transferred to alternate protocol #
- [ ] Other (specify):

D3. FINAL DISPOSITION OF TISSUES, FLUIDS, OR CARCASSES

Indicate the method(s) of disposing of the carcasses and surplus tissues or fluids (select all that apply):

- [x] Carcasses of non-biohazardous dead animals will be disposed of by DES.
- [ ] Non-radioactive tissues or fluids will be disposed of by the PI.
- [ ] Radioactive carcasses, tissues, or fluids will be disposed of by DES.
- [ ] Other (specify):

E1. TRANSPORTATION

Indicate the method(s) of disposing of the carcasses and surplus tissues or fluids (select all that apply):

- [ ] No transportation of animals will occur once they are on campus.
- [ ] All transportation will conform to UMCP transportation guidelines.
- [ ] Other (specify):

PRINCIPAL INVESTIGATOR AGREEMENT

This agreement signifies that you (as PI) have read and understood your responsibilities to operate as the Principal Investigator. The concurrent signatures signify that the appropriate individuals have been contacted as to conducting this research on campus. The agreement may be signed electronically as part of this form or a copy may be signed manually and sent separately from an electronically submitted protocol application.

- ☒ I acknowledge responsibility for the procedures and care of animals used in this protocol. I will conduct all work in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, USDA regulations (9 CFR Parts 1, 2, 3), the Federal Animal Welfare Act (7 USC 2131 et. Seq.), the *Guide for the Care and Use of Laboratory Animals,* and policies set forth by the University of Maryland IACUC.
- ☒ I have determined that the research proposed is not unnecessarily duplicative.
- ☒ I confirm that all individuals on this protocol are participating in an appropriate Occupational Health & Safety Program. (Note: The UMCP Animal Handler Health Review forms are located at http://www.health.umd.edu/forms/animalhandlerform609.pdf; participation in an Occupational Health Program is mandatory for those with direct animal contact). Labs should also have DES Chemical Hygiene Plan.
- ☒ I authorize individuals listed on this application to conduct procedures involving animals and I accept responsibility for their oversight in the conduct of this proposal.
- ☒ I confirm that all individuals listed on this protocol as working with animals have completed the Animal User training or will be required to do so before being permitted to begin work with animals. Further, I certify that those individuals are properly trained, or will receive such training prior to working with animals, in all areas relevant to their assigned work with animals
- ☒ For animals held in a UMCP operated facility, I understand that in cases of necessary medical treatment, UMCP University veterinarians are authorized to provide the treatment required to sustain life, or if that is not possible, to prevent distress and pain by humane euthanasia. I recognize that the veterinary staff will contact me as soon as possible using the emergency contact information that I provide in this application, but I understand that such contact may not always be possible prior to providing treatment or performing euthanasia.
- ☒ I will notify the IACUC regarding any unexpected study results that negatively impact the welfare of the animals, including but not limited to those that require veterinary care or treatment not described in the approved protocol.
- ☒ For animals held in a UMCP operated facility or used on the UMCP campus, I will notify a University veterinarian and the IACUC when unanticipated pain or distress, unexpected morbidity, or unanticipated mortality occurs with animals approved for use under this protocol.
- ☒ I will obtain approval from the IACUC before initiating any change in the study design or procedures by submitting a request for minor or significant change as appropriate. I understand that work performed without IACUC approval cannot be published with certification of IACUC approval and may result in federally-required reporting of non-compliance.
- ☒ For all USDA Category D (anesthesia / analgesia provided to relieve potential pain) and USDA Category E (pain not relieved by anesthesia / analgesia) animal use procedures, I certify that I have reviewed the pertinent scientific literature and the sources and/or databases noted in this application and found no scientifically acceptable alternative to any of those procedures that would result in less pain or distress.

PI Name: Dr. Ioannis Bossis Date:

Project Title: AAV6/8-mediated gene transfer of the low-density lipoprotein receptor (LDL-R) in the liver of the heritable hyperlipidemic

Principal Investigator signature: Date:

Chair Signature: Date:

DES Signature (If applicable): Date:

Facility Manager Signature (If applicable): Date:

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Bossis,I oannis Day Phone #: 3013148042

Dept / Div: Veterinary Medicine Office/Lab #: 795/1413 E-mail Address: bossisi@umd.edu

Highest Degree Earned:

☐ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☒ PhD ☐ Other (specify):

UMCP Relationship:

☒ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☐ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

● This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

● Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

Dr. Ioannis Bossis has more than 25 years of experience working with animals. Dr Bossis has a Ph.D in animal science, and was a staff scientist at NIH responsible for the animal studies of a branch. He will be involved in all the procedures including anesthesia, IV virus administraion, blood collection, tissue harvesting and euthanasia. The PI will be personally involved in training all the undergraduate students involved in the project.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

On line training done for handling laboratory animals with AALAS
completed UMD PI/Animal User training in April, 2011

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Powell, Douglas Day Phone #: (301) 405-4921

Dept / Div: Attending Vet, UMD Office/Lab #: CARF/1101 E-mail Address: dpowell@umd.edu

Highest Degree Earned:

☐ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☒ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☒ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☐ Undergraduate Student ☒ Other (specify): UMCP head attending veterinarian

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

● This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

● Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

Dr. Powell is the head attending veterinarian at UMCP. He has vast experience as a professional and lab animal veterinarian for more than 30 years. He will be involved in all the procedures including anesthesia and virus delivery by catheterization. Dr. Powell will also train all the undergraduate students involved in the project.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Rajawat,Yogendra Day Phone #: 3013142587

Dept / Div: Veterinary Medicne Office/Lab #: 795/1415 E-mail Address: yrajawat@umd.edu

Highest Degree Earned:

☐ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☒ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☒ Graduate Student ☐ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

● This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

● Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

Yogendra is a trained DVM and has more than 12 years of experience working with animals. He will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including anesthesia, virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

On line training done for handling laboratory animals with AALAS
completed UMD PI/Animal User training in year 2008,year 2010 and recently in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Atlas, Daniel Day Phone #: 610-390-1416

Dept / Div: Office/Lab #: E-mail Address: datlas@umd.edu

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

● This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

● Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Daniel will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Buckshaw, Liz Day Phone #: 443-472-3063

Dept / Div: Office/Lab #: E-mail Address: lizbuckshaw@verizon.net

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

● This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

● Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Liz will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Hanna, David Day Phone #: 443-690-9626

Dept / Div: Office/Lab #: E-mail Address: davidhanna13@gmail.com

Highest Degree Earned:

☐ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☐ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, David will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Lannon, Jenny Day Phone #: 4104282913

Dept / Div: Office/Lab #: E-mail Address: jnlannon@hotmail.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Jenny will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Mahmud, Alia Day Phone #: 240-533-1003

Dept / Div: Office/Lab #: E-mail Address: alia.mahmud@gmail.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Alia will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Mirvis, Mary Day Phone #: 570-498-4008

Dept / Div: Office/Lab #: E-mail Address: mirvis.mary@gmail.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Mary will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Pham, Anna Day Phone #: 240-731-9120

Dept / Div: Office/Lab #: E-mail Address: csianna7@yahoo.com

Highest Degree Earned:

☐ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☐ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Anna will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Ramnarain, Nadira Day Phone #: 410-599-6474

Dept / Div: Office/Lab #: E-mail Address: nramnarain91@gmail.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Nadira will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Randazzo, Paul Day Phone #: 240-277-6729

Dept / Div: Office/Lab #: E-mail Address: prandazz@umd.edu

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Paul will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Safferman, Michelle Day Phone #: 443-621-7882

Dept / Div: Office/Lab #: E-mail Address: msafferman@yahoo.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

○ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

○ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Michelle will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

SECTION F: PERSONNEL QUALIFICATIONS FORM (PQF)(Cover each individual listed in Section A3)

F1. PERSONAL INFORMATION

Name (Last, First): Zuckerberg, Jeremy Day Phone #: 443-271-2863

Dept / Div: Office/Lab #: E-mail Address: jzuckerberg@ymail.com

Highest Degree Earned:

☒ High School ☐ Associate ☐ BA / BS ☐ MA / MS ☐ MD / DVM / DDS ☐ PhD ☐ Other (specify):

UMCP Relationship:

☐ Faculty ☐ Staff ☐ Post-doctoral ☐ Visiting Scientist ☐ Off campus Associate

☐ Graduate Student ☒ Undergraduate Student ☐ Other (specify):

PI statement: ○ This individual **WILL NOT HAVE** animal contact. (No further information is required.)

◉ This individual **WILL HAVE** animal contact. (Complete the remainder of this form.)

If this individual WILL HAVE animal contact, have they completed the PI/Animal Users training class?

○ No. Animal users must schedule and complete training before initiating any animal activities.

◉ Yes. The individual completed the PI/Animal Users training class.

What experience do you have to perform the procedures and use the techniques required of you in this protocol? (Be specific.)

After Proper training, Jeremy will be monitoring and handling the animals on daily basis. He will also be involved in all the procedures including virus delivery, blood collection, tissue harvesting, euthanasia and disposal of carcass.

If the protocol requires **specific skills that are not listed above** please detail those here along with the training received and whether or what level of proficiency the individual has with the specific techniques.

If training in specific skills is needed (as checked above) or if you will be engaged in procedures for which you are not presently proficient, indicate who will provide the necessary training and who will provide oversight until you have achieved proficiency.

Training will be provided by Dr. Ioannis Bossis and Dr. Doug Powell.
completed UMD PI/Animal User training in April,2011.

Add another PQF form

C.3 Qualitative Analysis of β-galactosidase

1. Dissect liver out from rabbit and section into several 1mm slices using a tissue slicer

2. Wash tissue samples in 1L of cold PBS solution (80g NaCl, 2g KCl, 11.5g Na_2HPO_4, 2g KH_2PO_4)

3. Fix the thin sections in β-galactosidase fixative (0.5% glutaraldehyde, 2.0% paraformaldehyde in 0.1M phosphate buffer containing 3mM Mgcl2 and 3mM EGTA) on ice for 30-60min

4. Wash three times in β-galactosidase wash buffer (2 mM MgCl2 and 0.01% sodium deoxycholate in 0.1M phosphate buffer) for 15 minutes at room temperature, with gentle rocking

5. Incubate tissues in β-galactosidase staining solution (25 mg/ml Xgal in dimethylformamide, 50 mM potassium ferrocyanide 0.1M sodium phosphate buffer of pH 8.0, and 50 mM potassium ferricyanide in 0.1M sodium phosphate buffer of pH 8.0) at 37°C overnight

6. Monitor staining and take pictures of tissue samples

C.4 Quantitative Analysis of β-galactosidase

C.4.1 Preparation of Samples

1. Wash tissues in PBS

2. Suspend samples in lysis solution (100mM potassium phosphate, 0.2% Triton X-100, 1mM DTT, 0.2mM PMSF, and 5µg/mL leupeptin) for 5 minutes on ice

3. Collect supernatant in microtubes and incubate at 48°C for 60 minutes

C.4.2 Determination of Total Protein Concentration

The following protocol was performed with Peirce BCA Protein Assay Kit (cat#23225), purchased from Thermo Scientific

1. Pipette 25µl of each standard or unknown sample into microplate well

2. Add 200µL of the working reagent to each well and mix plate thoroughly on a plate shaker for 30 seconds

3. Cover and incubate tubes at 37°C for 30 minutes

4. Cool plates to room temperature

5. Set the spectrophotometer to 562nm, and measure the absorbance of all the samples within 10 minutes

6. Subtract the average 562nm absorbance of the blank standard sample from the 562nm absorbance of all other individual standards and unknown samples

7. Prepare a standard curve by plotting the average blank-corrected 562nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

C.4.3 Determination of β-galactosidase Concentration

The following protocol was performed with Galacto-Light Plus Kit (part#T9003), purchased from Applied Biosystems

1. Dillute Galacton-Plus substrate 1:100 with reaction buffer

2. Transfer 5µL of extract to microplate wells

3. Add 70µL of reaction buffer to wells and incubate for 1 hour

4. Place plate in luminometer and inject 100µL of accelerator

5. Read signal after a 2 second delay

C.5 DNA Isolation from Liver Samples and Purification

The following protocol was performed with the Maxwell 16 MDx DNA Purification Cartridge Kit (cat#AS3000), purchased from Promega

1. Add 500µL of nuclease-free water to each tube of lyophilized proteinase K, and gently swirl to dissolve

2. Dispense solution into smaller aliquots and store at -20°C

3. Scrape 5µm sections of sample of interest into microtubes

4. Briefly centrifuge samples and collect sample at bottom of tubes

5. Incubate samples overnight at 70°C

6. Add 2 volumes of lysis buffer to each sample

7. Vortex microtubes

8. Turn on Maxwell 16 MDx Instrument

9. Verify that the Home screen indicates “SEV”. Press “Run/Stop” to continue

10. Select “DNA” and “Tissue DNA” protocol

11. Transfer cartridges containing samples and plungers from the preparation rack onto Maxwell 16 platform

12. Place 1 blue elution tube for each cartridge into elution tube slots

13. Add 300µL of elution buffer to each elution tube

14. Press “Run/Stop” button to begin purification

15. Follow on-screen instructions to transfer data

16. Press “Run/Stop” to stop process

17. Remove elution tubes, cartridges, and plungers from platform

C.6 qPCR of Liver Samples

1. Combine the following: 5 µl of template DNA, 10 µl of Premix Ex Taq (TaKaRa), 0.4 µl of ROX Reference Dye (TaKaRa), 0.2 µmol/l of each primer, and 0.1 µmol/l of probe, and 4µL nuclease-free water

2. Set the following thermal profile for all reactions with 30ng of genomic DNA in duplicates using the Lightcycler 2.0 machine: 95°C for 5 minutes, followed by 40 cycles of 15 seconds at 95°and 1 minute at 60°C

Appendix D: Production of rAAV Via Recombinant Baculovirus/Insect Cell System

The following protocols were performed with the Bac-to-Bac Baculovirus Expression System (cat#10359-016), purchased from Invitrogen, and were conducted at the NIH under the guidance of Dr. Robert Kotin.

D.1 Insertion of LITR-LP-LDLr-polyA-RITR into Bacmid

1. Thaw on vial of DH10BAC competent cells on ice
2. Dispense 50µL of cells into 15mL rounded polypropylene tubes
3. Add 50ng of plasmid DNA to cells and gently mix
4. Incubate on ice for 30 minutes
5. Heat-shock cells for 45 seconds at 42°C
6. Chill on ice for 2 minutes
7. Add 450µL of S.O.C. medium and shake in 37°C incubator for 4 hours
8. Plate 100µL of dilution mixture on LB agar plate containing 50µg/ml kanamycin, 7µg/mL gentamicin, 10µg tetracycline, 100µg/ml Blou-gal, and 40µg/m: IPTG
9. Incubate for 72 hours at 37°C
10. Streak a single white colony on a fresh plate containing the same components from step 8 and incubate overnight at 37°C

D.2. Isolation of Recombinant DNA from Bacmid

1. Harvest 1.5mL bacterial cells by centrifugation at 9,000 x g for 15 minutes
2. Discard the supernatant and add 0.4mL resuspension buffer to pellet. Transfer cell suspension to centrifuge tube
3. Add 1.5mL lysis buffer and invert tube 5x
4. Add 2.1nL precipitation buffer, invert tube 5x and centrifuge mixture at 8,000rpm for 10 minutes

5. Discard the supernatant and resuspend DNA in 70% ethanol
6. Centrifuge sample at 8,000 rpm for 5 minutes at 4°C
7. Discard the supernatant, air-dry pellet for 10 minutes
8. Dissolve pellet in 200µL TE Buffer
9. Store bacmid DNA at 4°C

D.3 Isolation of P1 Baculovirus

1. Seed 2mL of Sf9 cells ($1x10^6$) into each well of a 6-well plate
2. Incubate for 30 minutes at room temperature
3. Mix 600µL of Grace's medium without supplement and 30µg Bacmid DNA from protocol D.2
4. Prepare a separate solution of 600µL of Grace's medium without supplement and 30µg CellFectin
5. Combine the solutions from steps 3 and 4 and mix gently
6. Incubate for 30 minutes at room temperature
7. Add 4.8mL of Grace's medium to combined mix and remove supernatant from 6-well plate
8. Add 1mL of mix into each will in a drop-wise manner
9. Incubate 6-well plate for 5 hours at 27°C
10. Remove lipid mix, add 2mL of complete medium, and incubate for 96 hours at 27°C
11. Harvest the supernatant, which contains the P1 Baculovirus

D.4 Production of P2 Baculovirus

1. Plate 100mL of Sf9 suspension cells ($2x10^6$) and incubate at room temperature for 1 hour
2. Infect cells with 1mL P1 Baculovirus
3. Incubate cells for 72 hours at 27°C
4. Collect 2mL of medium containing virus and transfer to microtube
5. Centrifuge sample at 500 x g for 5 minutes
6. Harvest P2 Baculovirus supernatant and store in dark bottle at 4°C

D.5 Double Infection and Viral Purification by Affinity Chromatography

1. Dilute stock of TIPS to 1:10,000
2. Infect 200mL of Sf9 cells ($2x10^6$) with diluted TIPS cells
3. Place suspension culture on shaker for 5 days at 135 rpm and 28°C
4. Add 1% of triton and 0.075M final NaCl
5. Homogenize sample with deBEE 1000 homogenizer using a D10 nozzle and process pressure at 15Kpsi
6. Centrifuge sample at 12,000 x g for 45minutes
7. Filter 0.2µM of the supernatant
8. Add 10µg/mL of turbonuclease (cat#N0103M, Accelagen)
9. Incubate sample for 2 hours at 37°C
10. Add 50% PEG to supernatant until the final concentration of cells is 4%
11. Place sample on shaker overnight at 4°C
12. Centrifuge sample at 4,000rpm for 30 minutes
13. Resuspend the pellet with 12mL of CsCl solution with a reflective index=1.372

14. Ultracentrifuge sample in TI50 rotor at 45,000rpm, at 15°C for 40 hours

15. Harvest rAAV by fractionating the gradient with 0.5mL fractions

16. Pool the fractions that have a reflective index around 1.372

17. Dialyze the fractions containing rAAV with PBS and 2mM $MgCl_2$

18. Perform western blot analysis to confirm correct protein expression

Appendix E: Data

E.1 *Oryctolagus cuniculus* Low Density Lipoprotein Receptor mRNA, Complete cds

```
   1 gtgcactcct cgaagcccga ccgcccgcac gcacgctacg gctgggctgc gggtcccgga
  61 gcggcactga cggcggaggc tcccacgatg aggacggcgc gctgggtcct cggcctgctc
 121 ctggccgccg ctgccggggc tgcagcaggg gacaagtgtg gccggaatga gttccaatgc
 181 cggaacggga agtgtatctc ctacaagtgg gtgtgtgacg gcagctccga gtgtcaggac
 241 ggctcggacg agtgggagca gacctgcatg tctctcacct gcaagtccga tgactttagc
 301 tgcggcggcc gcctgaaccg ctgcatcccc gggcactgga aatgcgacgg ccagcaggac
 361 tgcgaggacg gctccgacga gctgggctgc gcgcccaaga cgtgctccca ggacgagttc
 421 cgctgcgccg agggcgcgtg catctcccgg ctgttcgcct gcgacgggga gccggactgc
 481 cccgacggct cggacgaggc ctcgtgcgcg ccgtccacct gcggccccgc ccacttccgg
 541 tgcaacagct cctcctgcgt ccccgcgctg tgggcctgcg acggcgagcc ggactgcgac
 601 gatggctccg acgagtggcc ggcgcgctgc ggcgcccgcc ccagcccgca gcccggccgc
 661 gggccctgct cccgccacga gttccactgt ggcagcggcg agtgcgtgca cgcgagctgg
 721 cgctgcgacg gcgacgccga ctgcagggac ggctcggacg agcgcgactg cgccgcggcc
 781 acgtgccgcc cggacgagtt ccagtgctcg gacgggacgt gcatccatgg cagccggcag
 841 tgtgaccagc agcaggactg cggggacatg agcgacgagg tgggctgcgt caacgtgaca
 901 ctgtgcgagg ggcccgacaa gttcaagtgc cacagcgggg agtgcatctc cctggacaaa
 961 gtgtgcaact ccgccaggga ctgccaggat tggtcagacg agcccatcaa agagtgcgcg
1021 accaatgagt gcatgcgggg caacggaggc tgctcccaca cctgcttcga cctcagaatc
1081 ggccacgagt gtcattgtcc caaaggctac cggctggtgg accagcgacg ctgcgaagat
1141 atcaatgagt gtgaggaccc cgacatctgc agccagctgt gtgtgaacct ggcgggcagc
1201 tacaagtgcg agtgccgggc cggcttccag ctggaccccc acagccaggc ctgcaaggcc
1261 gtggactcca tcgcctacct cttcttcacc aaccggcacg aggtgcgcaa gatgaccctg
1321 gaccgtagcg agtacacaag cctcatcgcc aacctcaaga acgtggtggc cctggacgcg
1381 gaggtggcca gcaaccgcat ctactggtcg gacctgtccc agcgcaagat ctacagcgca
1441 cagatcgacg gggcgcacgg cttccccgcc tacgacaccg tcatcagcag cgacctgcag
1501 gcccccgatg ggctggctgt ggactggatc cacggccaca tctactggac agactccgtg
1561 ctgggcaccg tgtccgtggc cgacaccagg gggttcagga ggaagacact gttccggcag
1621 gaaggctcca agcccagggc catcgtggtg gaccccgcgc acggcttcat gtactggacc
1681 gactggggcg tccccgccaa gatcgagaaa gggggcctga acggcgtgga cgtctactcc
1741 ctggtgaccg aggacatcca gtggcccaat ggcatcaccc tggatctttc cagcggccgc
1801 ctctactggg tggactccaa gctgcactcc atctccagca tcgacgtcaa cgggggcaac
1861 cggaagacgg tgctggagga cgagcagcgg ctggcgcacc ccttctctct ggccatcttt
1921 gaggacaaag tgttctggac ggacgtcatc aacgaagcca tcttcagtgc caaccgcctc
1981 accggctccg acgtccacct ggtggccgag aacctgctgt ccccggagga catcgtcctg
2041 ttccacaacc tcacgcagcc cagaggggtg aactggtgcg agaagacggc cctccccaac
2101 ggcggctgcc aatacctgtg cctgccggcc ccacagatca atagccactc gcccaagttc
2161 acctgcgcct gccccgacgg cacgctgctg gccgcggaca tgcggagctg ccgcacagag
2221 gccgacgtga tcctgagcac ccagagggcg tcgacggccg ctcggccgca gctcacgggc
2281 agccctgccg gtaccacaca ggagcccctg accgagccca cgctcagcac cttggagacg
2341 gcgaccacgt cccagcaagc cctgcacaac gccgacggcc gaggcagcga ggggacgccc
2401 aggagcgtgg gggccctgtc cgtcgtcctg cccatcgcgc tgctgggcct gctctgcctc
```

2461 ggcgccctgg tcctgtggaa gaactggcgg ctccgcagcg tccacagcat caacttcgac
2521 aacccggtct accagaagac cacggaggac gaggtgcaca tctgcaggag ccaggacggc
2581 tacacctacc cctcgagaca gatggtcagc ctggaggacg acgtggcctg agccgccgcc
2641 tggatctcgc cgccacccgc gggcctgagc cgccgccgcc gccgcctgcc cgggccgtgt
2701 tttatatatt tattcgcccg ggatggggcc ggctgctctc tggacagagg ggggcccggg
2761 cctccctgca ctgcgcccgc gctcatctca ggagctccgt gtttacctcg ccggttgccc
2821 ttaactgtcc gagaagtgcc tgcgcccgcc cacgccaggt ccctggtccc cggcagcgtg
2881 gggcgggggc tgggcggagc gcggcccggg cgtgcatggc gttttagctt tgcacctcgc
2941 aagccgcgcg agtctgtgac gacatttgca ctttgtgcgt ttctggagac gctgagcaca
3001 tgtatagaaa ttatttattt ttgctaacgc tggctgctgt gtgcagcggc gcggggccgc
3061 ccgtgggggg gtgggggagg gctccgtgcc gcctttgaac cactgtatag agagttttta
3121 tagcctgaag gggtccctgt ggttgattaa acttctttaa cgagt

E.2 Nucleotide Alignment for the Rabbit, Human, Mouse, Ovine, and Bovine LDL-R mRNA

Red: high consensus Black: neutral consensus Blue: low consensus

```
           1                                                          60
rabbitmRNA
 humanmRNA  CTCTTGCAGT GAGGTGAAGA CATTTGAAAA TCACCCCACT GCAAACTCCT CCCCCTGCTA
 mousemRNA                                             GCAGACTCCT CCCCCGCCTG
 ovinemRNA  CGCGTCC--- ---------- ---------- ---------- ---------- ----------
bovinemRNA  CGCGTCC--- ---------- ---------- ---------- ---------- ----------
 Consensus  c.c.t.c... .......... .......... .......... .......... ..........

          61                                                         120
rabbitmRNA
 humanmRNA  GAAACCTCAC ATTGAAATGC TGTAAATGAC GTGGGCCCCG AGTGCAATCG ----------
 mousemRNA  GAAACCTCGC CCCTAGTACT GGGAATGACT CTGGGCGTGC GGCGTAGTTT GCAGCCGGGA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            121                                                        180
rabbitmRNA                                              GTGCACTCC TCGAAGCCCG
 humanmRNA  ---------- ---------- ---------- ---------- --------CG GGAAGCCAGG
 mousemRNA  CACCGTGAGG CTTGCGAGCC CAGATTCGCA GCCGAGACAC CGTGGGGCCC GCGATCCAGT
 ovinemRNA  ---------- ---------- ---------- ---------- --TGGCCGCG CGAAGCCGGG
bovinemRNA  ---------- ---------- ---------- ---------- --GGGCCGCG CGAAGCCCGG
 Consensus  .......... .......... .......... .......... ..tgg.c.Cg .gaAgcC.gg

            181                                                        240
rabbitmRNA  ACCGCCCGCA CGCACGCTAC GGCTGGGCTG CGGGTCCCGG AGCGGCACTG ACGGCGGAGG
 humanmRNA  GTTTCCAGCT AGGACACAGC AGGTC--GTG ATCCGGGTCG GGACAC-TGC CTGGCAGAGG
 mousemRNA  GTTTGCAGCG GGAACATTTC GGGGTCTGTG ATCCGAGTGA GGACGC-AAC GCAGAAGCTA
 ovinemRNA  GGTGCCAGAG CGGTCACTGC CAGCGGACAG CGGCCTGTGC CGGGGCGTGA CAAGCGGAGG
bovinemRNA  GTTTCCAGAG CGGTCACTGC GAGCGGACAG CGGCCTGGGC CGGGGCGTGA CAAGCGGAGG
 Consensus  gtttcCaGcg cGgaCactgC ggg.gg.ctG cggc..gtg. .G.ggC.tg. c.aGcgGagg

            241                                                        300
rabbitmRNA  CTCCCACGAT GAGGACGGCG ---------- --CGCTGGGT CCTCGGCCTG CTCCTGGCCG
 humanmRNA  CTGCGAGCAT GGGGCCCTGG GGCTGGAAAT TGCGCTGGAC CGTCGCCTTG CTCCT---CG
 mousemRNA  -----AGGAT GAGCACCGCG GATCTGATGC GTCGCTGGGT CATCGCCCTG CTCCT---GG
 ovinemRNA  CTGGGATCAT GAGGCTCGCG GGCTGGGGAC TGCGCTGGGC CATCGCCCTG CTCAT---CG
bovinemRNA  CTGGGATCAT GAGGCTTGCG GGCTGGGGGC TGCGCTGGGC CATCGCCCTG CTCAT---CG
 Consensus  ctg.gA.cAT GaGgcccgcG ggctgg...c tgCGCTGGgc CaTCGcCcTG CTCcT...cG
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```
            301                                                         360
rabbitmRNA  CCGCTGCCGG GGCTGCAGCA GGGGACAAGT GTGGCCGGAA TGAGTTCCAA TGCCGGAACG
 humanmRNA  CCGCGGCGGG GACTGCAGTG GGCGACAGAT GCGAAAGAAA CGAGTTCCAG TGCCAAGACG
 mousemRNA  CTGCTGCCGG AGTTGCAGCA GAAGACTCAT GCAGCAGGAA CGAGTTCCAG TGTAGAGACG
 ovinemRNA  CTGCGGGGGA GGCTGCAGTG GAAGACAACT GTGCGAGAAA TGAGTTTCAG TGCCGAGATG
bovinemRNA  CTGTGGGGGA GGCTGCAGTG GAAGACAACT GTGGGAGAAA CGAGTTTCAG TGCCAAGATG
 Consensus  CtGcgGcgGg ggcTGCAGtg GaaGACaa.T Gtgg.aGaAA cGAGTTcCAg TGccgagAcG

            361                                                         420
rabbitmRNA  GGAAGTGTAT CTCCTACAAG TGGGTGTGTG ACGGCAGCTC CGAGTGTCAG GACGGCTCGG
 humanmRNA  GGAAATGCAT CTCCTACAAG TGGGTCTGCG ATGGCAGCGC TGAGTGCCAG GATGGCTCTG
 mousemRNA  GAAAATGCAT CGCTAGCAAG TGGGTGTGCG ATGGCAGCCC CGAGTGCCCG GATGGCTCCG
 ovinemRNA  GGAAATGCAT CTCCTATAAG TGGGTTTGTG ATGGGACCGC CGAGTGCCAA GACGGCTCTG
bovinemRNA  GGAAATGCAT CTCCTATAAG TGGGTTTGTG ATGGGACCGC CGAGTGCCAA GACGGCTCTG
 Consensus  GgAAaTGcAT CtCctacAAG TGGGT.TGtG AtGGcAgCgC cGAGTGcCag GAcGGCTCtG

            421                                                         480
rabbitmRNA  ACGAGTGGGA GCAGACCTGC ATGTCTCTCA CCTGCAAGTC CGATGACTTT AGCTGCGGCG
 humanmRNA  ATGAGTCCCA GGAGACGTGC TTGTCTGTCA CCTGCAAATC CGGGGACTTC AGCTGTGGGG
 mousemRNA  ATGAGTCCCC AGAGACATGC ATGTCTGTCA CCTGTCAGTC CAATCAATTC AGCTGTGGAG
 ovinemRNA  ATGAATCCCA GGAGACGTGT AAGTCTGTCA CCTGCAAGAT GGGGGACTTC AGCTGTGGGG
bovinemRNA  ATGAATCCCA GGAGACATGC AAGTCTGTCA CCTGCAAGAT GGGGGACTTC AGCTGTGGGG
 Consensus  AtGAgTccca ggAGAC.TGc atGTCTgTCA CCTGcaAgtc cggggAcTTc AGCTGtGGgG

            481                                                         540
rabbitmRNA  GCCGCCTGAA CCGCTGCATC CCCGGGCACT GGAAATGCGA CGGCCAGCAG GACTGCGAGG
 humanmRNA  GCCGTGTCAA CCGCTGCATT CCTCAGTTCT GGAGGTGCGA TGGCCAAGTG GACTGCGACA
 mousemRNA  GCCGTGTCAG CCGATGCATT CCTGACTCCT GGAGATGTGA TGGACAGGTA GACTGTGAAA
 ovinemRNA  GCCGTGTCAA CCGCTGCATT TCGGAGTCCT GGAGATGCGA TGGCCAGAAG GACTGCGAGA
bovinemRNA  GCCGTGTCAA CCGCTGCATT TCGGGGTCCT GGAGATGCGA TGGCCAGGTG GACTGCGAGA
 Consensus  GCCGtgTcAa CCGcTGCATt cC.gagtcCT GGAgaTGcGA tGGcCAggtg GACTGcGAga

            541                                                         600
rabbitmRNA  ACGGCTCCGA CGAGCTGGGC TGCGCGCCCA AGACGTGCTC CCAGGACGAG TTCCGCTGCG
 humanmRNA  ACGGCTCAGA CGAGCAAGGC TGTCCCCCCA AGACGTGCTC CCAGGACGAG TTTCGCTGCC
 mousemRNA  ATGACTCAGA CGAACAAGGC TGTCCCCCCA AGACGTGCTC CCAGGATGAC TTCCGATGCC
 ovinemRNA  ATGGCTCGGA CGAGGAAGGC TGTCCCCCCA AGACGTGCTC CCAGGATGAG TTCCGTTGCA
bovinemRNA  ATGGCTCGGA CGAAGAAGGC TGTTCCCCCA AGACGTGCTC CCAGGATGAG TTCCGCTGTA
 Consensus  AtGgCTC.GA CGAgcaaGGC TGtcCcCCCA AGACGTGCTC CCAGGAtGAg TTcCGcTGc.

            601                                                         660
rabbitmRNA  CCGAGGGCGC GTGCATCTCC CGGCTGTTCG CCTGCGACGG GGAGCCGGAC TGCCCCGACG
 humanmRNA  ACGATGGGAA GTGCATCTCT CGGCAGTTCG TCTGTGACTC AGACCGGGAC TGCTTGGACG
 mousemRNA  AGGATGGCAA GTGCATCTCC CCGCAGTTTG TGTGTGATGG AGACCGAGAT TGCCTAGATG
 ovinemRNA  ATGATGGCAA GTGCATTGCC CCGAAGTTCG TCTGTGACTT GGACCTGGAC TGCTTAGATG
bovinemRNA  ATGACGGCAA GTGCATTGCC CCGAAGTTCG TCTGTGACTT GGACCTGGAC TGCTTAGATG
 Consensus  a.GAtGGcaa GTGCATctCc CcGcaGTTcG tcTGtGAct. gGAcC.gGAc TGCttaGAtG


            661                                                         720
rabbitmRNA  GCTCGGACGA GGCCTCGTGC GCGCCGTCCA CCTGCGGCCC CGCCCACTTC CGGTGCAACA
 humanmRNA  GCTCAGACGA GGCCTCCTGC CCGGTGCTCA CCTGTGGTCC CGCCAGCTTC CAGTGCAACA
 mousemRNA  GCTCTGATGA GGCCCACTGC CAGGCCACCA CTTGTGGCCC CGCCCACTTC CGCTGCAACT
 ovinemRNA  GCTCGGATGA GGCGTCCTGC CCCATGCCCA CCTGCAGCCC TGCCAACTTC CAATGCAACA
bovinemRNA  GCTCGGATGA GGCGTCCTGC CCCATGCCCA CCTGCGGCCC TGCCAACTTC CAGTGCAACA
 Consensus  GCTCgGAtGA GGCctccTGC ccg.tgccCA CcTGcgGcCC cGCCaaCTTC CagTGCAACa

            721                                                         780
rabbitmRNA  GCTCCTCCTG CGTCCCCGCG CTGTGGGCCT GCGACGGCGA GCCGGACTGC GACGATGGCT
 humanmRNA  GCTCCACCTG CATCCCCCAG CTGTGGGCCT GCGACAACGA CCCCGACTGC GAAGATGGCT
 mousemRNA  CATCCATATG CATCCCCAGT CTTTGGGCCT GCGACGGGGA TGTCGACTGT GTTGACGGCT
 ovinemRNA  GCTCCATGTG CATCCCCCAG CTCTGGGCCT GCGACGGCGA CCCCGACTGC GACGATGGCT
bovinemRNA  GCTCCATGTG CATCCCCCAG CTCTGGGCCT GCGATGGCGA CCCCGACTGT GACGATGGCT
 Consensus  gcTCCat.TG CaTCCCCcag CT.TGGGCCT GCGAcggcGA ccccGACTGc GacGAtGGCT
```

```
            781                                                        840
rabbitmRNA  CCGACGAGTG GCCGGCGCGC TGCGGCGCCC GCCCCA---- --GCCCGCAG CCCGGCCGCG
 humanmRNA  CGGATGAGTG GCCGCAGCGC TGTAGGGGTC TTTACGTG-- ----TTCCAA GGGGACAGTA
 mousemRNA  CCGATGAGTG GCCACAGAAC TGCCAGGGCC GAGACACGGC ---CTCCAAA GGCGTTAGCA
 ovinemRNA  CAGACGAGTG GCCAAAGCAC TGCGGGAGCC CCCACCCATC AGGCCCCCCG AAGGACGACA
bovinemRNA  CAGATGAGTG GCCAAAGCAC TGCGGGACCC CCCACCCATC AGGCCCCCTG CAGGACAACA
 Consensus  C.GAtGAGTG GCCa.aGcaC TGcgggggcC .ccaC.c..c ..gcccccag ..gGacagca

            841                                                        900
rabbitmRNA  GGCCCTGCTC CCGCCACGAG TTCCACTGTG GCAGCGGCGA GTGCGTGCAC GCGAGCTGGC
 humanmRNA  GCCCCTGCTC GGCCTTCGAG TTCCACTGCC TAAGTGGCGA GTGCATCCAC TCCAGCTGGC
 mousemRNA  GCCCCTGCTC CTCCCTGGAG TTCCACTGTG GTAGCAGTGA GTGTATCCAT CGCAGCTGGG
 ovinemRNA  ACCCCTGCTC GGCCCTCGAG TTCCACTGCG GCAGTGGCGA GTGCATCCAC TCCAGCTGGC
bovinemRNA  ACCCCTGCTC GGCCCTCGAG TTCCACTGCG GCAGTGGCGA GTGCATCCAC TCCAGCTGGC
 Consensus  gcCCCTGCTC ggcCctcGAG TTCCACTGcg gcAGtgGcGA GTGcaTcCAc tccAGCTGGc

            901                                                        960
rabbitmRNA  GCTGCGACGG CGACGCCGAC TGCAGGGACG GCTCGGACGA GCGCGACTGC GCCGCGGCCA
 humanmRNA  GCTGTGATGG TGGCCCCGAC TGCAAGGACA AATCTGACGA GGAAAACTGC GCTGTGGCCA
 mousemRNA  TCTGTGACGG CGAGGCAGAC TGCAAGGACA AGTCAGATGA GGAGCACTGC GCGGTGGCCA
 ovinemRNA  GTTGCGACAG TGACCCTGAC TGCAAGGACA AGTCTGACGA GGAGAACTGC GCTGTGGCCA
bovinemRNA  ATTGCGACCA TGACCCTGAC TGCAAGGACA AGTCTGACGA GGAGAACTGC GCTGTGGCCA
 Consensus  gcTGcGAcgg tGaccC.GAC TGCAaGGACa agTCtGAcGA GgagaACTGC GCtGtGGCCA

            961                                                       1020
rabbitmRNA  CGTGCCGCCC GGACGAGTTC CAGTGCTCGG ACGGGACGTG CATCCATGGC AGCCGGCAGT
 humanmRNA  CCTGTCGCCC TGACGAATTC CAGTGCTCTG ATGGAAACTG CATCCATGGC AGCCGGCAGT
 mousemRNA  CCTGCCGACC TGATGAATTC CAGTGTGCAG ATGGCTCCTG CATTCACGGT AGCCGCCAGT
 ovinemRNA  CGTGCCGGCC TGATGAATTC CAGTGCTCTG ATGGGACCTG CATCCATGGC AGCCGGCAGT
bovinemRNA  CGTGCCGGCC TGATGAATTC CAGTGCTCGG ATGGGACCTG CATCCATGGT AGCCGGCAGT
 Consensus  CgTGcCG.CC tGAtGAaTTC CAGTGctC.G AtGGgaccTG CATcCAtGGc AGCCGgCAGT

            1021                                                      1080
rabbitmRNA  GTGACCAGCA GCAGGACTGC GGGGACATGA GCGACGAGGT GGGCTGCGTC AACGTGACAC
 humanmRNA  GTGACCGGGA ATATGACTGC AAGGACATGA GCGATGAAGT TGGCTGCGTT AATGTGACAC
 mousemRNA  GTGACCGTGA ACATGACTGC AAGGACATGA GCGACGAGCT CGGCTGCGTC AATGTGACAC
 ovinemRNA  GCGACAGGGA GCCTGACTGT AAGGATCTGA GTGACGAGCT GGGCTGCGTC AACGTGACTC
bovinemRNA  GCGACAGGGA GCCTGACTGT AAGGATCTGA GTGACGAGCT GGGCTGCGTC AACGTGACTC
 Consensus  GtGACcgggA gcatGACTGc aaGGAcaTGA GcGAcGAgcT gGGCTGCGTc AAcGTGACaC

            1081                                                      1140
rabbitmRNA  TGTGCGAGGG GCCCGACAAG TTCAAGTGCC ACAGCGGGGA GTGCATCTCC CTGGACAAAG
 humanmRNA  TCTGCGAGGG ACCCAACAAG TTCAAGTGTC ACAGCGGCGA ATGCATCACC CTGGACAAAG
 mousemRNA  AGTGTGATGG CCCCAACAAG TTCAAGTGTC ACAGTGGGGA GTGCATCAGC TTGGACAAGG
 ovinemRNA  TTTGCGAGGG GCCCAACAAG TTCAAGTGCC ACAGCGGCGA GTGCATCTCC CTGGACAAAG
bovinemRNA  TTTGTGAGGG GCCCAACAAG TTCAAGTGCC AAAGCGGTGA GTGCATCTCC CTGGACAAAG
 Consensus  t.TGcGAgGG gCCCaACAAG TTCAAGTGcC AcAGcGG.GA gTGCATCtcC cTGGACAAaG


            1141                                                      1200
rabbitmRNA  TGTGCAACTC CGCCAGGGAC TGCCAGGATT GGTCAGACGA GCCCATCAAA GAGTGCGCGA
 humanmRNA  TCTGCAACAT GGCTAGAGAC TGCCGGGACT GGTCAGATGA ACCCATCAAA GAGTGCGGGA
 mousemRNA  TGTGCGACTC CGCCCGCGAC TGCCAGGACT GGTCGGATGA GCCCATCAAG GAGTGCAAGA
 ovinemRNA  TGTGCAACTC CATCAGGGAC TGCCGGGACT GGTCGGACGA GCCCCTCAAG GACTGCGGGA
bovinemRNA  TGTGCAACTC CGTCAGGGAC TGCCGGGACT GGTCGGACGA GCCCCTCAAG GACTGTGGGA
 Consensus  TgTGCaACtc cgccaGgGAC TGCCgGGAcT GGTCgGAcGA gCCCaTCAAg GAgTGcggGA


            1201                                                      1260
rabbitmRNA  CCAATGAGTG CATGCGGGGC AACGGAGGCT GCTCCCACAC CTGCTTCGAC CTCAGAATCG
 humanmRNA  CCAACGAATG CTTGGACAAC AACGGCGGCT GTTCCCACGT CTGCAATGAC CTTAAGATCG
 mousemRNA  CCAACGAGTG TTTGGACAAC AATGGTGGCT GTTCCCACAT CTGCAAGGAC CTCAAGATTG
 ovinemRNA  CCAATGAGTG TCTGGACAAC AAGAGCGGCT GCTCTCACAT CTGCAATGAC CTCAAGATCG
bovinemRNA  CCAACGAGTG TCTGGACAAC AAGGGCGGCT GCTCTCACAT CTGCAATGAC CTCAAGATCG
 Consensus  CCAAcGAgTG t.TGgacaaC AA.gGcGGCT GcTCcCACat CTGCaatGAC CTcAagATcG
```

```
            1261                                                        1320
rabbitmRNA  GCCACGAGTG TCATTGTCCC AAAGGCTACC GGCTGGTGGA CCAGCGACGC TGCGAAGATA
 humanmRNA  GCTACGAGTG CCTGTGCCCC GACGGCTTCC AGCTGGTGGC CCAGCGAAGA TGCGAAGATA
 mousemRNA  GCTCTGAGTG CCTGTGTCCC AGCGGCTTCC GGTTGGTGGA CCTCCACAGG TGTGAAGATA
 ovinemRNA  GCTACGAGTG CCTGTGTCCT GAAGGCTTCC AGCTAGTGGA CAAGCACAGA TGCGAAGATA
bovinemRNA  GCTACGAGTG CCTGTGTCCC GAAGGCTTCC AGCTAGTGGG CAAGCACAGA TGTGAAGATA
 Consensus  GCtacGAGTG cCtgTGtCCc gaaGGCTtCC aGcTgGTGGa CcagCacaGa TGcGAAGATA

            1321                                                        1380
rabbitmRNA  TCAATGAGTG TGAGGACCCC GACATCTGCA GCCAGCTGTG TGTGAACCTG GCGGGCAGCT
 humanmRNA  TCGATGAGTG TCAGGATCCC GACACCTGCA GCCAGCTCTG CGTGAACCTG GAGGGTGGCT
 mousemRNA  TTGACGAGTG TCAGGAGCCA GACACCTGCA GCCAGCTCTG TGTGAACCTG GAAGGCAGCT
 ovinemRNA  TCGATGAGTG TCAGAACCCT GACACCTGCA GCCAGCTCTG CGTGAACCTC GAGGGCAGCT
bovinemRNA  TCGATGAGTG TCAGAACCCT GACACCTGCA GCCAGCTCTG CGTGAACCTC GAGGGCAGCT
 Consensus  TcgAtGAGTG TcAGgAcCC. GACAcCTGCA GCCAGCTcTG cGTGAACCTg GagGGcaGCT

            1381                                                        1440
rabbitmRNA  ACAAGTGCGA GTGCCGGGCC GGCTTCCAGC TGGACCCCCA CAGCCAGGCC TGCAAGGCCG
 humanmRNA  ACAAGTGCCA GTGTGAGGAA GGCTTCCAGC TGGACCCCCA CACGAAGGCC TGCAAGGCTG
 mousemRNA  ACAAGTGTGA GTGCCAGGCC GGCTTCCACA TGGACCCACA CACCAGGGTC TGCAAGGCTG
 ovinemRNA  ACAAATGCGA GTGCGAAGAG GGCTTCCGGC TGGAGCCCCT CACCAAGGCC TGCAAGGCCG
bovinemRNA  ACAAATGCGA GTGTGAAGAG GGCTTCCGGC TGGAGCCCCT CACCAAGGCC TGCAAGGCCG
 Consensus  ACAAgTGcgA GTGcgagGa. GGCTTCCagc TGGAcCCCCa CAccaaGGcC TGCAAGGCcG

            1441                                                        1500
rabbitmRNA  TGGACTCCAT CGCCTACCTC TTCTTCACCA ACCGGCACGA GGTGCGCAAG ATGACCCTGG
 humanmRNA  TGGGCTCCAT CGCCTACCTC TTCTTCACCA ACCGGCACGA GGTCAGGAAG ATGACGCTGG
 mousemRNA  TGGGCTCCAT AGGCTATCTG CTCTTCACCA ACCGCCACGA GGTCCGGAAG ATGACCCTGG
 ovinemRNA  TGGGCACCAT CGCCTACCTC TTCTTCACCA ACCGCCATGA AGTGAGGAAG ATGACTCTGG
bovinemRNA  TGGGCACCAT CGCCTACCTC TTCTTTACCA ACCGCCACGA AGTCAGGAAG ATGACTCTGG
 Consensus  TGGgCtCCAT cGcCTAcCTc tTCTTcACCA ACCGcCAcGA gGTcaGgAAG ATGAC.CTGG

            1501                                                        1560
rabbitmRNA  ACCGTAGCGA GTACACAAGC CTCATCGCCA ACCTCAAGAA CGTGGTGGCC CTGGACGCGG
 humanmRNA  ACCGGAGCGA GTACACCAGC CTCATCCCCA ACCTGAGGAA CGTGGTCGCT CTGGACACGG
 mousemRNA  ACCGCAGCGA GTACACCAGT CTGCTCCCCA ACCTGAAGAA TGTGGTGGCT CTCGACACGG
 ovinemRNA  ACCGAAGCGA GTATACCAGC CTCATCCCCA ACCTTAAGAA TGTGGTTGCC CTGGACACGG
bovinemRNA  ACCGAAGCGA GTACACCAGC CTTATCCCCA ACCTTAAGAA CGTGGTCGCC CTGGACACGG
 Consensus  ACCG.AGCGA GTAcACcAGc CTcaTCcCCA ACCT.AaGAA cGTGGT.GCc CTgGACaCGG

            1561                                                        1620
rabbitmRNA  AGGTGGCCAG CAACCGCATC TACTGGTCGG ACCTGTCCCA GCGCAAGATC TACAGCGCAC
 humanmRNA  AGGTGGCCAG CAATAGAATC TACTGGTCTG ACCTGTCCCA GAGAATGATC TGCAGCACCC
 mousemRNA  AGGTGACCAA CAATAGAATC TACTGGTCCG ACCTGTCCCA AAAAAAGATC TACAGCGCCC
 ovinemRNA  AGGTGGCCAG TAACAGGATC TACTGGTCTG ACCTGTCCCA GAGGAAGATC TACAGTGCCC
bovinemRNA  AGGTGGCCAG TAACAGGATC TACTGGTCTG ACCTGTCCCA GAGGAAGATC TACAGTGCCC
 Consensus  AGGTGgCCAg cAAcaG.ATC TACTGGTCtG ACCTGTCCCA gag.AaGATC TaCAGcgCcC

            1621                                                        1680
rabbitmRNA  AGATCGACGG GGCGCACGGC TTCCCCGCCT ACGACACCGT CATCAGCAGC GACCTGCAGG
 humanmRNA  AGCTTGACAG AGCCCACGGC GTCTCTTCCT ATGACACCGT CATCAGCAGA GACATCCAGG
 mousemRNA  TGATGGACCA GGCCCCTAAC TTGTC---CT ACGACACCAT CATCAGTGAG GACCTGCATG
 ovinemRNA  AGATCGATGA CGCCCCCGGC TTCTCCTCCT ACGACACCGT CATCGGTGAG AATCTCCAGG
bovinemRNA  AGATCGACGG AGCCCCCGGC TTCTCCTCCT ACGACACCGT CATTGGCGAG GATCTCCAGG
 Consensus  aGaTcGAcgg .GCcCccggC tTctCctcCT AcGACACCgT CATcaGcgag gAccTcCAgG

            1681                                                        1740
rabbitmRNA  CCCCCGATGG GCTGGCTGTG GACTGGATCC ACGGCCACAT CTACTGGACA GACTCCGTGC
 humanmRNA  CCCCCGACGG GCTGGCTGTG GACTGGATCC ACAGCAACAT CTACTGGACC GACTCTGTCC
 mousemRNA  CCCCTGACGG GCTGGCGGTA GACTGGATCC ACCGCAACAT CTACTGGACA GATTCAGTCC
 ovinemRNA  CCCCCGACGG GCTGGCGGTG GACTGGATCC ACAGCAACAT CTACTGGACC GACTCCATCC
bovinemRNA  CCCCCGATGG GCTGGCGGTG GATTGGATCC ACAGCAACAT CTACTGGACC GACTCCATCC
 Consensus  CCCCcGAcGG GCTGGCgGTg GAcTGGATCC ACaGCaACAT CTACTGGACc GAcTCcgTcC
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            1741                                                        1800
rabbitmRNA  TGGGCACCGT GTCCGTGGCC GACACCAGGG GGTTCAGGAG GAAGACACTG TTCCGGCAGG
 humanmRNA  TGGGCACTGT CTCTGTTGCG GATACCAAGG GCGTGAAGAG GAAAACGTTA TTCAGGGAGA
 mousemRNA  CAGGCAGCGT ATCTGTGGCT GACACCAAGG GCGTAAAGAG GAGGACACTG TTCCAAGAGG
 ovinemRNA  TGGGCACCGT CTCCGTGGCT GACACCAAAG GGGTGAAGAG GAAGACACTG TTCCAGGAGG
bovinemRNA  TGGGTACCGT CTCCGTGGCT GACACCAAAG GTGTGAAGAG GAAGACGCTC TTCCAGGAGG
 Consensus  tgGGcAccGT cTCcGTgGCt GAcACCAagG G.gTgAaGAG GAagACacTg TTCcaggAGg

            1801                                                        1860
rabbitmRNA  AAGGCTCCAA GCCCAGGGCC ATCGTGGTGG ACCCCGCGCA CGGCTTCATG TACTGGACCG
 humanmRNA  ACGGCTCCAA GCCAAGGGCC ATCGTGGTGG ATCCTGTTCA TGGCTTCATG TACTGGACTG
 mousemRNA  CAGGGTCCAG ACCCAGAGCC ATCGTAGTGG ACCCTGTGCA TGGCTTCATG TACTGGACAG
 ovinemRNA  AAGGCTCCAA GCCCACGGGCC ATTGTGGTTG ATCCTGTCCA CGGCTTCATG TATTGGACTG
bovinemRNA  AAGGCTCCAA ACCCACGGGCC ATTGTGGTCG ATCCCGTCCA TGGCTTCATG TATTGGACTG
 Consensus  aaGGcTCCAa gCCaaGgGCC ATcGTgGTgG AtCCtGt.CA tGGCTTCATG TAcTGGACtG

            1861                                                        1920
rabbitmRNA  ACTGGGGCGT CCCCGCCAAG ATCGAGAAAG GGGGCCTGAA CGGCGTGGAC GTCTACTCCC
 humanmRNA  ACTGGGGAAC TCCCGCCAAG ATCAAGAAAG GGGGCCTGAA TGGTGTGGAC ATCTACTCGC
 mousemRNA  ATTGGGGAAC ACCCGCCAAG ATCAAGAAAG GGGGTTTGAA TGGTGTGGAC ATCCACTCAC
 ovinemRNA  ACTGGGGCAC CCCTGCCGAG ATCAAGAAGG GGGGCCTCAA CGGCGTGGAC GTTTACTCGC
bovinemRNA  ACTGGGGCGC TCCTGCCGAG ATCAAGAAGG GGGGCCTCAA CGGCGTGGAC GTTTACTCGC
 Consensus  AcTGGGGcac .CCcGCCaAG ATCaAGAAaG GGGGccTgAA cGGcGTGGAC gTctACTCgC

            1921                                                        1980
rabbitmRNA  TGGTGACCGA GGACATCCAG TGGCCCAATG GCATCACCCT GGATCTTTCC AGCGGCCGCC
 humanmRNA  TGGTGACTGA AAACATTCAG TGGCCCAATG GCATCACCCT AGATCTCCTC AGTGGCCGCC
 mousemRNA  TGGTGACCGA AAACATCCAG TGGCCAAATG GCATCACACT AGATCTTTCC AGTGGCCGTC
 ovinemRNA  TGGTGACCGA GGACATCCAG TGGCCCAATG GCATCACTCT GGATCTTTCT GGAGGCCGCC
bovinemRNA  TGGTGACCGA GGACATCCAG TGGCCCAACG GCATCACTCT AGATCTTTCT GGCGGCCGCC
 Consensus  TGGTGACcGA ggACATcCAG TGGCCcAAtG GCATCAC.CT aGATCTttcc aG.GGCCGcC

            1981                                                        2040
rabbitmRNA  TCTACTGGGT GGACTCCAAG CTGCACTCCA TCTCCAGCAT CGACGTCAAC GGGGGCAACC
 humanmRNA  TCTACTGGGT TGACTCCAAA CTTCACTCCA TCTCAAGCAT CGATGTCAAC GGGGGCAACC
 mousemRNA  TCTATTGGGT TGATTCCAAA CTCCACTCTA TCTCCAGCAT CGATGTCAAT GGGGGCAATC
 ovinemRNA  TCTACTGGGT CGACTCCAAA CTGCACTCCA TCTCCAGCAT CGATGTCAAC GGAGGGAACC
bovinemRNA  TCTACTGGGT CGACTCCAAA CTGCACTCCA TCTCCAGCAT CGATGTCAAT GGGGGGAACC
 Consensus  TCTAcTGGGT .GAcTCCAAa CTgCACTCcA TCTCcAGCAT CGAtGTCAAc GGgGGcAAcC

            2041                                                        2100
rabbitmRNA  GGAAGACGGT GCTGGAGGAC GAGCAGCGGC TGGCGCACCC CTTCTCTCTG GCCATCTTTG
 humanmRNA  GGAAGACCAT CTTGGAGGAT GAAAAGAGGC TGGCCCACCC CTTCTCCTTG GCCGTCTTTG
 mousemRNA  GGAAAACCAT TTTGGAGGAT GAGAACCGGC TGGCCCACCC CTTCTCCTTG GCCATCTATG
 ovinemRNA  GGAAGACCGT GCTGGAGGAC AAGAAGAAGC TGGCACACCC CTTCTCCTTG GCCATCTTTG
bovinemRNA  GGAAGACCGT GCTGGAGGAC AAGAAGAAGC TGGCGCACCC CTTCTCTTTG GCCATCTTTG
 Consensus  GGAAgACCgT gcTGGAGGAc gAgaAgagGC TGGC.CACCC CTTCTCctTG GCCaTCTtTG

            2101                                                        2160
rabbitmRNA  AGGACAAAGT GTTCTGGACG GACGTCATCA ACGAAGCCAT CTTCAGTGCC AACCGCCTCA
 humanmRNA  AGGACAAAGT ATTTTGGACA GATATCATCA ACGAAGCCAT TTTCAGTGCC AACCGCCTCA
 mousemRNA  AGGACAAAGT GTATTGGACA GATGTCATAA ACGAAGCCAT TTTCAGTGCC AATCGACTCA
 ovinemRNA  AGGATAAAGT ATTTTGGACG GATATTATCA ACGAAGCCAT TTTTAGTGCC AACCGCCTCA
bovinemRNA  AGGATAAAGT ATTTTGGACG GATGTTATCA ACGAAGCCAT TTTTAGTGCC AACCGCCTCA
 Consensus  AGGAcAAAGT aTttTGGACg GAtgTcATcA ACGAAGCCAT tTTcAGTGCC AAcCGcCTCA

            2161                                                        2220
rabbitmRNA  CCGGCTCCGA CGTCCACCTG GTGGCCGAGA ACCTGCTGTC CCCGGAGGAC ATCGTCCTGT
 humanmRNA  CAGGTTCCGA TGTCAACTTG TTGGCTGAAA ACCTACTGTC CCCAGAGGAT ATGGTTCTCT
 mousemRNA  CGGGTTCAGA TGTGAATTTG GTGGCTGAAA ACCTCTTGTC CCCGGAGGAC ATTGTCCTGT
 ovinemRNA  CAGGCTCAGA CATCAGTCTG ATGGCAGAAA ACCTGTTATC GCCGGAGGAC ATTGTCCTTT
bovinemRNA  CAGGTTCAGA CATCAGCCTG ATGGCAGAAA ACCTGTTATC GCCGGAGGAC ATTGTCCTTT
 Consensus  CaGGtTCaGA cgTcaaccTG .TGGC.GAaA ACCTgtTgTC cCCgGAGGAc ATtGTcCT.T
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            2221                                                                2280
rabbitmRNA  TCCACAACCT CACGCAGCCC AGAGGGGTGA ACTGGTGCGA GAAGACGGCC CTCCCC---A
 humanmRNA  TCCACAACCT CACCCAGCCA AGAGGAGTGA ACTGGTGTGA GAGGACCACC CTGAGC---A
 mousemRNA  TCCACAAGGT CACACAGCCT AGAGGGGTGA ACTGGTGTGA GACAACAGCC CTCCTCCCCA
 ovinemRNA  TCCACAACCT CACGCAGCCG AGAGGAGTGA ACTGGTGTGA GAGGACTTCC CTCCGC---A
bovinemRNA  TCCACAACCT CACGCAGCCG AGAGGGGTGA ACTGGTGTGA GAGGACTGCC CTCCGC---A
 Consensus  TCCACAAccT CACgCAGCC. AGAGGgGTGA ACTGGTGtGA GAggAC.gCC CTccgC...A

            2281                                                                2340
rabbitmRNA  ACGGCGGCTG CCAATACCTG TGCCTGCCGG CCCCACAGAT CAATAGCCAC TCGCCCAAGT
 humanmRNA  ATGGCGGCTG CCAGTATCTG TGCCTCCCTG CCCCGCAGAT CAACCCCCAC TCGCCCAAGT
 mousemRNA  ATGGTGGTTG CCAGTACCTG TGCCTGCCCG CCCCACAGAT CGGTCCCCAC TCGCCCAAAT
 ovinemRNA  ATGGCGGCTG CCAGTACCTG TGTCTGCCGG CCCCACAGAT CAACCCCCGC TCACCCAAGT
bovinemRNA  ATGGTGGCTG CCAGTACCTG TGTCTGCCGG CCCCACAGAT CAACCCCCGC TCACCCAAGT
 Consensus  AtGGcGGcTG CCAgTAcCTG TGcCTgCCgG CCCCaCAGAT CaacccCCaC TCgCCCAAgT

            2341                                                                2400
rabbitmRNA  TCACCTGCGC CTGCCCCGAC GGCACGCTGC TGGCCGCGGA CATGCGGAGC TGCCGCACAG
 humanmRNA  TTACCTGCGC CTGCCCGGAC GGCATGCTGC TGGCCAGGGA CATGAGGAGC TGCCTCACAG
 mousemRNA  TCACCTGCGC CTGCCCTGAT GGCATGCTGC TGGCCAAGGA CATGCGGAGC TGCCTCACAG
 ovinemRNA  TCACCTGTGC CTGCCCCGAT GGCATGCTGC TGGCCAAGGA CATGAGAAGC TGCCTCACAG
bovinemRNA  TCACGTGTGC CTGCCCCGAC GGCATGCTGC TGGCCAAGGA CATGAGAAGC TGCCTCACAG
 Consensus  TcACcTGcGC CTGCCCcGAc GGCAtGCTGC TGGCCaaGGA CATGaGgAGC TGCCtCACAG

            2401                                                                2460
rabbitmRNA  AGGCCGACGT GATCCTGAGC ACCCAGAGGG CGTCGACGGC CG-------- --CTCGGCCG
 humanmRNA  AGGCTGAGGC TGCAGTGGCC ACCCAGGAGA CATCCACCGT CAGGCTAAAG GTCAGCTCCA
 mousemRNA  AAGTCGACAC TGTACTGACC ACCCAGGGGA CATCCGCCGT CCGGCCTGTG GTCACCGCAT
 ovinemRNA  AGTCTGAATC TGCAGTGACC ACTCGAGGAC CCTCCAC--- ---------G GTCAGCTCGA
bovinemRNA  AGTCTGAATC TGCAGTGACC ACCCGAGGAC CCTCCAC--- ---------G GTCAGCTCGA
 Consensus  AggctGA..c tgcagTGacC ACcCagggg. C.TCcaC.g. c........g gtCagctC.a

            2461                                                                2520
rabbitmRNA  CAGCTCACGG GCAGCC---- ---------- ---------C TGCCGGTACC ACACAGGAGC
 humanmRNA  CAGCCGTAAG GACAC---AG CACACAACCA CCCGACCTGT TCCCGACACC TCCCGGCTGC
 mousemRNA  CAGCTACCAG GCCACCGAAG CACAGTGAGG ATCTCTCAGC TCCCAGTACT CCTAGGCAGC
 ovinemRNA  CAGCTGTGGG GCC------- ---------- ---------- ---------- -----GAAGC
bovinemRNA  CAGCTGTGGG GCC------- ---------- ---------- ---------- -----GAAGC
 Consensus  CAGCtgt.gG Gcc.c..... .......... .......... t.cc...ac. .c...G.aGC

            2521                                                                2580
rabbitmRNA  CCCTGACCGA GCCCACGCTC AGCACCTTGG AGACGGCGAC CACGTCCCAG CAAGCCCTGC
 humanmRNA  CTGGGGCCAC CCCTGGGCTC ACCACGGTGG AGATAGTGAC AATGTCTCAC CAAGCTCTGG
 mousemRNA  CTGTGGACAC CCCAGGGCTC AGCACAGTGG CGTCAGTGAC AGTGTCCCAC CAAGTCCAGG
 ovinemRNA  GCACATCCAG CCCTGAGCTC ACCACAGCCG AGTCGGTGAC AATGTCCCAA CAAGCCCTGG
bovinemRNA  GCACGGCCAG CCCTGAGCTC ACCACAGCCG AGTCGGTGAC AATGTCCCAA CAAGGCCAGG
 Consensus  cc..ggcCa. cCCtg.GCTC AcCACagtgG aGtcgGtGAC aatGTCcCA. CAAGccCtGg

            2581                                                                2640
rabbitmRNA  ACAACGCCGA CGGCCGAGGC AGCGAGGGGA CGCCCAGGAG CGTGGGGGCC CTGTCCGTCG
 humanmRNA  GCGACGTTGC TGGCAGAGGA AATGAGAAGA AGCCCAGTAG CGTGAGGGCT CTGTCCATTG
 mousemRNA  GTGACATGGC TGGCAGAGGG AATGAGGAGC AGCCACATGG TATGAGGTTC CTGTCCATCT
 ovinemRNA  GCGATGTTGC CGGCCGAGCG GACACGGAGA GGCCCGGGAG CGTGGGTGCC CTGTACATCG
bovinemRNA  GTGACATTGC CAGCCAAGCG GACACGGAGA GGCCCGGGAG CGTGGGTGCC CTATACATCG
 Consensus  gcgAcgttGc cgGCcgAGgg aacgaGgaGa .GCCc.ggaG cgTGgGggcc CTgTcCaTcg

            2641                                                                2700
rabbitmRNA  TCCTGCCCAT CGCGCTGCTG GGCCTGCTCT GCCTCGGCGC CCTGGTCCTG TGGAAGAACT
 humanmRNA  TCCTCCCCAT CGTGCTCCTC GTCTTCCTTT GCCTGGGGGT CTTCCTTCTA TGGAAGAACT
 mousemRNA  TCTTCCCTAT TGCACTGGTT GCCCTCCTTG TCCTTGGGGC CGTCCTGCTG TGGAGGAACT
 ovinemRNA  TCCTCCCCAT TGCACTGCTC ATCCTCCTGG CCTTCGGGAC CTTCCTCGTC TGGAAGAACT
bovinemRNA  TCCTCCCCAT CGCACTGCTC ATCCTCCTGG CCTTCGGAAC CTTCCTCCTC TGGAAGAACT
 Consensus  TCcTcCCcAT cGcaCTgcTc gtCcTcCT.g .CcTcGGggc CtTccTccT. TGGAaGAACT
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            2701                                                        2760
rabbitmRNA  GGCGGCTCCG CAGCGTCCAC AGCATCAACT TCGACAACCC GGTCTACCAG AAGACCACGG
 humanmRNA  GGCGGCTTAA GAACATCAAC AGCATCAACT TTGACAACCC CGTCTATCAG AAGACCACAG
 mousemRNA  GGCGGCTGAA GAACATCAAC AGCATAAACT TTGACAACCC AGTCTACCAG AAGACCACAG
 ovinemRNA  GGCGGCTGAA GAGCATCAAC AGCATCAACT TCGACAACCC TGTGTACCAG AAAACCACGG
bovinemRNA  GGCGGCTGAA GAGCATCAAC AGCATCAACT TCGACAACCC CGTGTACCAG AAGACCACGG
 Consensus  GGCGGCTgaa gAgCaTCaAC AGCATcAACT TcGACAACCC .GTcTAcCAG AAgACCACgG

            2761                                                        2820
rabbitmRNA  AGGACGAGGT GCACATCTGC AGGAGCCAGG ACGGCTACAC CTACCCCTCG AGACAGATGG
 humanmRNA  AGGATGAGGT CCACATTTGC CACAACCAGG ACGGCTACAG CTACCCCTCG AGACAGATGG
 mousemRNA  AGGACGAGCT CCACATTTGC CGAAGCCAGG ATGGCTATAC CTACCCCTCA AGACAGATGG
 ovinemRNA  AGGACGAGGT CCACATCTGC CGCAGCCAGG ACGGCTACAC CTACCCCTCG AGACAGATGG
bovinemRNA  AGGATGAGGT CCACATCTGC CGCAGCCAGG ACGGCTACAC CTACCCCTCG AGACAGATGG
 Consensus  AGGAcGAGgT cCACATcTGC cgcAgCCAGG AcGGCTAcAc CTACCCCTCg AGACAGATGG

            2821                                                        2880
rabbitmRNA  TCAGCCTGGA GGACGACGTG GCCTGAGCCG CCGCCTGGAT CTCGCCGCC- ----------
 humanmRNA  TCAGTCTGGA GGATGACGTG GCGTGAACAT CTGC------ ---------- ----------
 mousemRNA  TCAGCCTGGA GGACGATGTG GCATGAGCAG CCGGGAGAG- ------CCGT CTCTTTCCGG
 ovinemRNA  TCAGCTTGGA GGATGACGCG GCATGAGCTG CTGCCTTGAG TCCTCTCCCT CCCTGGAACC
bovinemRNA  TCAGCTTGGA GGATGACGTG GCGTGAGCTG CTGCCTTGAG TCCTCTCCCT CCCCCGAACC
 Consensus  TCAGccTGGA GGAtGAcGtG GC.TGAgC.g CtGcct.ga. ..c.c.ccct c.c.......

            2881                                                        2940
rabbitmRNA  ---------- ---------- ---------- -------ACC CGCGGGCCTG AGCCGCCGCC
 humanmRNA  ---------- ---------- ---------- ---CTGGAGT CCCGTCCCTG CCCAGA----
 mousemRNA  GATCCATTGC CAAGCTTAGG CAGAAAAGAC ACTCTCTCCA GACCTCCCCA TCCAGC----
 ovinemRNA  TGCCGCTGGT CACTCAAAGG CAGCAAAAAC ACTTTCTCCC CCGACCCTTG ACCTGCCCCC
bovinemRNA  TGCTGCCAGT CACTCAAAGG CAGCAAGAAC ACTTTCTCCC CTGACCCTTG ACCTGCTCCC
 Consensus  ........g. ca..c..agg cag.aa..ac act.tctccc c.c..cCctg acC.Gc..cc

            2941                                                        3000
rabbitmRNA  GCCGCCGCCT G--------- ---------- ---------- ---------- ----------
 humanmRNA  -ACCCTTCCT GAGACCTCGC CGGCCT-TGT TTTATTCAAA GACAGAGAAG ACCAAAGCAT
 mousemRNA  -ACTGGTCCT GCCACCTCCC TGGGCTCTGT GTTGCTCAAA GCAAGATAAG AGCAAAGCTG
 ovinemRNA  AACCCTTCCT GAGACCTCCG CGCCAAAAGC ACTGTTGGGG ACTGCAGCC- ------TGTC
bovinemRNA  AACCCTTCCT GAGACTTCCG CGCCAAAAGC GCTGTTGGGG ACTGCCGCCC AGGCACTGTC
 Consensus  .aCccttCCT Gagacctcc. cg.c....g. ..tgtt.... .c...ag... a...a...t.

            3001                                                        3060
rabbitmRNA  ---------- ---------- ---------- -CCCGGGCCG TGTTTTATAT ATTTATTCGC
 humanmRNA  TGCCTG---- ---------- ---------- -CCAGAGCTT TGTTTTATAT ATTTATTCAT
 mousemRNA  GGCTGGGGGC CAAGCTCAGC TGCCTGTCTG CCCCAGGTTC TGTTTTATAT ATTTATTGTC
 ovinemRNA  TGCCTGTCTG ---------- ---------- -CCAGAGCTT TGTTTTATAT ATTTATTTTT
bovinemRNA  TGCCGGTCTG ---------- ---------- -CCAGAGCTT TGTTTTATAT ATTTATTTTT
 Consensus  tgcc.g.... .......... .......... .CCagaGctt TGTTTTATAT ATTTATT.tt

            3061                                                        3120
rabbitmRNA  CCGGGATGGG GC-------- ---------- ---------- ---------- ----------
 humanmRNA  CTGGGAGGCA GAACAGGCTT CGGACAGTGC CCATGCAATG GCTTGGGTTG GGATTTTGGT
 mousemRNA  TGGGGA--CA GAAAAGGCTA CTGGCTGTGC TTGAAATTCG AATTCTGCCT GGGAATTTTT
 ovinemRNA  CTGGGAGGCA GAACAGGCAT CAGACGGCAC CCACGGGCAG CAGTTGGGTT GCTTTTTTTT
bovinemRNA  CTGGGAGGCA GAACAGGCTT CAGACGGTGC CCACGGGCGG CAGTTGCGTT GCTTTTTTTT
 Consensus  ctGGGAggca Gaacaggctt c.gac.gtgc cca.g....g .a.t.gg.tt g..ttttttt

            3121                                                        3180
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TTC--TTCCT TTCCTCGTGA AGGATAAGAG AAACAGGCCC GGGGGGACCA GGATGACACC
 mousemRNA  TTT--TTTCT CCCATCTTCA TTTCCTTGGA AGCCAAATAG G--------- ----------
 ovinemRNA  CTTCCTTTCT TTACATGTGA AGGAGAAAGG CAGAACAGCT GGGGAATCTG ----------
bovinemRNA  CTTCCTTTCT TCACACGTGA AGGAGAAAGG CAGAACAGCT GGGGAATCTG ----------
 Consensus  .tt..tttct t..c.cgtga agga.aa.gg .a..a.a.c. gggg...c.. ..........
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            3181                                                       3240
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TCCATTTCTC TCCAGGAAGT TTTGAGTTTC TCTCCACCGT GACACAATCC TCAAACATGG
 mousemRNA  -------CTG TCCCAGAAGC TTCTGAATTC TACTCTTTTC AACACTAAC- ----------
 ovinemRNA  -----TCTTC TCCGGGAATC TCTGAGCTGC TCTCCTTCTA GACACACTCC TTCCT-----
bovinemRNA  -----TCCTC TCCGGGAATC TCTGAGCTGC TCTCCTTCTA GACACACTCC TCCCT-----
 Consensus  .....t.ctc tcc.ggaa.c t.tgag.t.c tctccttct. gacaca.tcc t.........

            3241                                                       3300
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----CGGCTG
 humanmRNA  AAGATGAAAG GGGAGGGGAT GTCAGGCCCA GAGAAGCAAG TGGCTTTCAA CACACAACAG
 mousemRNA  ACGGAGGAGG AAGAGACCCA CCCAGGCAGA GAGAAGCCTG CTGG-----A CATGAGACAG
 ovinemRNA  --GGAGGGAA TGGCAGACAG ACACGGAGCC GGAAAGCTAG TGGCTTTGAA CGCACGAAAG
bovinemRNA  --GGAGGGAA TGACGGAGGG ACGTGGAGCC GGAAAGCTAG TGGCTTTGAA TGCACGAGAG
 Consensus  ..ggagg.a. .gg.gg.... .c..gg..c. g..aagc.ag tggcttt.aa c.cacgacaG

            3301                                                       3360
rabbitmRNA  CTCTCTGGAC AGAGGGGGGC CCGGGCCTCC ---------- ----CTGCAC TGCGCCCGCG
 humanmRNA  CAGATGGCAC CAACGGGACC CCCTGGCCCT GCCTCATCCA CCAATCTCTA AGCCAAACCC
 mousemRNA  CAGGTACTAC TGACCGGGTC CTGGGTACC- ---------- ---------A TAGCCAGTCC
 ovinemRNA  CAGGTGCTAC CCACAGGGTC CCAGGTGGCT GCTGTTGCCA CCTACCCTCT AGCCCAGGCC
bovinemRNA  CAGGTGCTAC CCACAGGGTC CCAGGTGGCT GCTGTTGCCA CCCACCCTCT AGCCCAGGCC
 Consensus  CaggtgctAC c.Ac.GGgtC Cc.gGt..Ct gc.....cca cc.acc.... agcccaggCc

            3361                                                       3420
rabbitmRNA  CTCATCTCAG GAGCTC-CGT GTTTACCT-- ---------- ---------- ----------
 humanmRNA  CTAAACTCAG GAGTCAACGT GTTTACCTCT TCTATGCAAG CCTTGCTAGA CAGCCAGGTT
 mousemRNA  CTAACCTCAG GACTTCCTGC ATTGACCTT- ---------- ---------- ----------
 ovinemRNA  CCACACTCAG GACTGAACGT GTTTACTTCT GCCAGGCAAG CCTGC----- --GGGATGGC
bovinemRNA  CCACACTCAG GACTGAACGT GTTTACTTCT GCCAGGCAAG CCTGC----- --GGGATGGC
 Consensus  CtaaaCTCAG GAct.aacGt gTTtACcTct .c.a.gcaag cct.......  ..g..a.g..

            3421                                                       3480
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  AGCCTTTGCC CTGTCACCCC CGAATCATGA CCCACCCAGT GTCTTTCGAG GTGGGTTTGT
 mousemRNA  ---------- ---------- ---------- -CATCCCAGA GCCTTCCTGC CTGACTCTGC
 ovinemRNA  CAGAATTGGT CTGCCACCCC CTGGTCAGGA TCAAACTAGG GTTTTCCAAG CAGGGTTTGT
bovinemRNA  CAGAGTTGGT CTGCCACCCC CTGGTCAGGA TCAAACTAGG GTTTTCCAAG CTGGGTTTGT
 Consensus  .....ttg.. ctg.cacccc c...tca.ga .caa.c.ag. gt.ttcc.ag ctgggtttgt

            3481                                                       3540
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ACCTTCCTT- --AAGCCAGG AAAGGGATTC ATGGCGT-CG GAAATGATCT GGCTGAATCC
 mousemRNA  CGCCCCCATC AGAAGCCAGG AAAGTGACTC GTGACATTCG GTACTGATCT TGCTGGATCT
 ovinemRNA  CCTCCACTT- --AAGCCAGG AGGGGACCCT GGGTCCAACC TTCCAGGAGT GGCACCCGCT
bovinemRNA  CCTCCACTT- --AAGCCAGG AGGGGACCCT GGGTCCAACC TTCCAGGAGT GGCGCCCGCT
 Consensus  cc.cc.ctt. ..aagccagg a..gg..c.. g.g.c...c. .t.c.g...t ggc.....ct

            3541                                                       3600
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GTGGTGGCAC CGAGACCAAA CTCATTCACC AAATGATGCC ACTTCCCAGA ----------
 mousemRNA  TGAGGCCGGC AGCTGCCAGT GATGGATACC CGCTCATGCC TTTGCCAAAA CGTCACCCGT
 ovinemRNA  GAA------- -----CCCTA TTCATTCACC AAAACCCAGA TCATCCCCAG AGAAA-----
bovinemRNA  GAA------- -----CCCTA TTCATTCACC AAAACCCCGA TCATCCCCAA AGAAA-----
 Consensus  g.a.......  .....cc..a .tcattcacc aaa.c..... tc.tccc.aa .g..a.....

            3601                                                       3660
rabbitmRNA  ---------- ---------- --CGCCGGTT GCCCTTAACT G---TCCGAG AAGTGCCTGC
 humanmRNA  --------GG CAGAGCCTGA GTCACTGGTC ACCCTTAAT- ---ATTTATT AAGTGCCTGA
 mousemRNA  TCCTGGAGGG ACAAGCCCAA GTCGCCATTC TCCCTTAAT- ---ATTTATC AAGTGCCTGA
 ovinemRNA  ---------- ACAAATTCGA GCCACCAGTC GCCCTTAACG TACATGTATT AAGTGCCTGA
bovinemRNA  ---------- ACAAATGCGA GCCACCAGTC GCCCTTAACG TTCATGTATT AAGTGCCTGA
 Consensus  .......... acaa...cga g.CaCcagTc gCCCTTAAc. ...aT.tatt AAGTGCCTGa
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            3661                                                       3720
rabbitmRNA  GCCCGCCCAC GCCA------ ---------- ---------- ---------- ----------
 humanmRNA  GACACCCGGT TACCTTGGCC GTGAGGACAC GTGGCCTGCA CC-------- ----------
 mousemRNA  GACAACTGGT TGCCTTGCTC ---AGGAGTC CTGGCCTGCT CA-------G TGTCCTGCTG
 ovinemRNA  GACCCCC--T TACTTTGTGC GTGAAGATGA GCAGTTCCCA CCTCTAACT- -ATCCGTCTG
bovinemRNA  GACCCCC--T TACTTTGTGC GTGAAGATGA GCAGCTCCCA CCTCTAACTG TATCCTTCTG
 Consensus  GaCccCc..t taC.ttg..c gtga.ga... g..gc...ca cc........ ..tcc..ctg

            3721                                                       3780
rabbitmRNA  ---------- GGTCCCTGGT CCCCGGCAGC GTG------- ---------- ----------
 humanmRNA  --CAGGTGTG GCTGTCAGGA CACCAGCCTG GTGCCCATCC TCCCGACCCC TACCCACTTC
 mousemRNA  CTCAGGGGTG GCCGGCAGGA CCCCTGTCCT GTGCCCAGCC CCCGAGTCTC CGAGTGAGGC
 ovinemRNA  TCTGGGAGTG GGTATCTGGA CCCCATCCCT G--------- ---------- ----------
bovinemRNA  TCCGGGAGTG GGTGTCTGGA CCCCAGCCCC G--------- ---------- ----------
 Consensus  ..c.gg.gtg GgtgtCtGGa CcCCagccc. Gtg....... .......... ..........

            3781                                                       3840
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CATTCCCGTG GTCTCCTTGC ACTTTCTCAG TTCAGAGTTG TACACT---- --GTGTACAT
 mousemRNA  --TTGCACTT CTCTCTGCGC ACTTTTCCCG TTCTGTGCTG GGCACCCACG TGGTCCACAT
 ovinemRNA  ---------- --CCCCTTGC ACTTTTTCAG TTCAGGGTCG TACACT---- --GTGTAAAG
bovinemRNA  ---------- --CCCCTTGC ACGTTTCCAG TTCAGGGTCG AACACT---- --GCGTAAAG
 Consensus  .......... ..c.ccttgc actttt.cag ttcag.gt.g .acact.... ..gtgta.a.

            3841                                                       3900
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TTGGCAT--- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TTGTACTCCT AGGTTGCACT GACCCTGGTG CATTGGATGG GACCTCAGGC CGGAAGTGGC
 ovinemRNA  TCGACATTCT A--------- ---------- ---------- ---------- ----------
bovinemRNA  TCGACATTCT A--------- ---------- ---------- ---------- ----------
 Consensus  t.g.cat.ct a......... .......... .......... .......... ..........

            3901                                                       3960
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CCCGAATCAT TGACCACAGA AGATAATTGC CAGGCTCCAA GCATCCATTG CCTTCTTCAG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            3961                                                       4020
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- --TTGTGTTA TTATTTTGCA CTGTTTTCTG
 mousemRNA  TACTGGGGGG AACTCAGGGC CTCTGTCTGG TGTTTAGTTA GTCTGTTTGT TTGTTTGTTT
 ovinemRNA  ---------- ---------- ---------- -------TTA TTATTTTGCA CTACTTTCCG
bovinemRNA  ---------- ---------- ---------- -------TTA TTATTTTGCA CTACTTTCCG
 Consensus  .......... .......... .......... .......tta ttattttgca ct..tttc.g

            4021                                                       4080
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TCGTGTGTGT TGGGATGG-- ---------- ---------- ------GATC CCAGGCCAGG
 mousemRNA  GTTTGTTTGT TTGGTGGTTT CTGGCTCTGC ACAGCAGAGT TGTTTAGCTC CTCTGGTAAC
 ovinemRNA  TTCTGGTTCC CTTCTGGCTG CA-------- --AAGAGTCT GGGTTGATGA TTGGTACCAA
bovinemRNA  TTCTGGTTCC CTTCTGGCTG CA-------- --AAGAGTCT GGATTGATGA CTGGTACCGA
 Consensus  tt.tg.tt.. .t..tgg.t. c......... ..a..ag..t .g.tt..... ct.g..c...

            4081                                                       4140
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GAAAGCCCGT GTCAATGAA- ---------- --TGCCGGGG ACAGAGAGGG GCAGG-----
 mousemRNA  GTCATCCTCT CTCACAGAGA CATCCAGCCT GCTGAAGGGG AGAGATGGGG CGAGGGGAGG
 ovinemRNA  CCTGGCCC-- ---------- ------CTCT CCCAGCATGT AGGGAGATGT GTGTGCGGTG
bovinemRNA  CCCGGCCCTT CTCCCAGACC CGGCCTCTCT CCCAGCACGT AGGGAGACCT GTGCGCGGTG
 Consensus  ....gccc.t .tc...ga.. .........ct .c...c..g. ag.gaga.g. g...g.g..g
```

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            4141                                                        4200
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- -------TTG ACCGGGACTT CAAAGCCGTG ATCGTGAATA
 mousemRNA  GATACACGCA TGGTTTCTCA GGTGTGGTGG CACTTGGCTT TAATCCTATG CTCAAGAGGA
 ovinemRNA  GC-------- ---------- -------TTG ATCATGACTT CAAAGCCAAG ATTACGACTA
bovinemRNA  GC-------- ---------- -------TTC ATCATGACTT CAAAGCCAAA ATTACGACTA
 Consensus  g......... .......... .......ttg a.c.tgactt caaagcca.g at.a.ga.ta

            4201                                                        4260
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TCGAG----- ---------- ---------- ---------- ---------- ----------
 mousemRNA  AGAGGTGGGC ACATGTCTGA GGTCCAAGCC TACATAGTGA GACCTTATAT ATCTAAAAAC
 ovinemRNA  GATGATCTG- ---------- ---------- ---------- ---------- ----------
bovinemRNA  GACGATCTG- ---------- ---------- ---------- ---------- ----------
 Consensus  ...g.t..g. .......... .......... .......... .......... ..........

            4261                                                        4320
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CAAAAGAAAT CCCAGACTAT TTGGTGGGAC TTGGAAGAAT ACTTAGAAAT TGCCACAGAT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4321                                                        4380
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TTGTCACATG GGTAACCTAA GAAGACAGTG GTGGCCTTAC TTGGATTCCT GGGTAGATCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4381                                                        4440
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  ACCTGTCCAC TACTCAAGAC ACAAGGGCAC ACTGCCCAGC CCACTGGGAC ACTTGTAAAG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4441                                                        4500
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- --AACTGCCA TTGTCGTCTT TATGTCCGCC CA--------
 mousemRNA  AGCTCTGTAA TGTGCCGATG GCACCTGGCT CGGTTTTCAT TCTGTATATT CAAGGATATC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4501                                                        4560
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- -----CCTAG TGCTTCCACT TCTATGCAAA
 mousemRNA  ACACATATGT ATTAAATCTA TTTATTTTTG CAAACCCTGA TTGCTGCACC TCTCTGCAAT
 ovinemRNA  ---------- ---------- ---------- -----CCTAC TGTTTCCACT TCTAGGCAGA
bovinemRNA  ---------- ---------- ---------- -----CCTAC TGTTTCCACT TCTAGGCAGA
 Consensus  .......... .......... .......... .....ccta. tg.ttccact tcta.gca.a

            4561                                                        4620
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TGCCTCC--- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TTCTCCAGGT TAGGCTGGGG TGATACTCTG GGATGCTCTG TATACAAAGA TGATTTGAAT
 ovinemRNA  GGCCACTG-- ---------- ---------- ---------- ---------- ----------
bovinemRNA  GGCCACTG-- ---------- ---------- ---------- ---------- ----------
 Consensus  .gcc.c.g.. .......... .......... .......... .......... ..........
```

```
            4621                                                        4680
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GGGCTGATCT CAGGCCTGTC CTGTGCGTTG CTTTGAGTGG GTGGGAACTG CTTTGAAACC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4681                                                        4740
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CTTGTTCAGA TGTTTTTATA GGCTGAAAAT ATCATACTGT GATGGATTAA ATTCTTTTTT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4741                                                        4800
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- -------AAG CCATTCACTT CCCCAATCTT G--------- ----------
 mousemRNA  GGAAAAAAAA AAAAAAAAAA GCAGACTCCT CCCCCGCCTG GAAACCTCGC CCCTAGTACT
 ovinemRNA  ---------- --------AG CCATTCATAT CCCC------ ---------- ----------
bovinemRNA  ---------- --------AG CCATTCATAT CCCC------ ---------- ----------
 Consensus  .......... ........ag ccattca..t cccc...... .......... ..........

            4801                                                        4860
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GGGAATGACT CTGGGCGTGC GGCGTAGTTT GCAGCCGGGA CACCGTGAGG CTTGCGAGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4861                                                        4920
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CAGATTCGCA GCCGAGACAC CGTGGGGCCC GCGATCCAGT GTTTGCAGCG GGAACATTTC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4921                                                        4980
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ----TCGTTG ATGGGTATGT GTTTAAAACA TGCACGGTGA GGCCGGGCGC AGTGGCTCAC
 mousemRNA  GGGGTCTGTG ATCCGAGTGA GGACGCAACG CAGAAGCTAA GGATGAGCAC CGCGGATCTG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            4981                                                        5040
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GCCTGT---- ---------- ---------- ---------- ---------- ----------
 mousemRNA  ATGCGTCGCT GGGTCATCGC CCTGCTCCTG GCTGCTGCCG GAGTTGCAGC AGAAGACTCA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5041                                                        5100
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGCAGCAGGA ACGAGTTCCA GTGTAGAGAC GGAAAATGCA TCGCTAGCAA GTGGGTGTGC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
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            5101                                                        5160
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GATGGCAGCC CCGAGTGCCC GGATGGCTCC GATGAGTCCC CAGAGACATG CATGTCTGTC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5161                                                        5220
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- --AATCCCAG CACTTTGGGA GGCCGAGGCG GGTGGATCAT ----------
 mousemRNA  ACCTGTCAGT CCAATCAATT CAGCTGTGGA GGCCGTGTCA GCCGATGCAT TCCTGACTCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5221                                                        5280
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGGAGATGTG ATGGACAGGT AGACTGTGAA AATGACTCAG ACGAACAAGG CTGTCCCCCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5281                                                        5340
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- -----GAGGT CAGGAGATCG AGACCATC-- ----------
 mousemRNA  AAGACGTGCT CCCAGGATGA CTTCCGATGC CAGGATGGCA AGTGCATCTC CCCGCAGTTT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5341                                                        5400
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- -CTGGCTAAC
 mousemRNA  GTGTGTGATG GAGACCGAGA TTGCCTAGAT GGCTCTGATG AGGCCCACTG CCAGGCCACC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5401                                                        5460
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ACGTGAAACC CCGTC----- ---------- ---------- ---------- ----------
 mousemRNA  ACTTGTGGCC CCGCCCACTT CCGCTGCAAC TCATCCATAT GCATCCCCAG TCTTTGGGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5461                                                        5520
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGCGACGGGG ATGTCGACTG TGTTGACGGC TCCGATGAGT GGCCACAGAA CTGCCAGGGC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5521                                                        5580
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CGAGACACGG CCTCCAAAGG CGTTAGCAGC CCCTGCTCCT CCCTGGAGTT CCACTGTGGT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
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            5581                                                    5640
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- --------TC TACTAAAAAT
 mousemRNA  AGCAGTGAGT GTATCCATCG CAGCTGGGTC TGTGACGGCG AGGCAGACTG CAAGGACAAG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5641                                                    5700
rabbitmRNA  ---------- ---------- ---GGGCGGG GGCTGGGCGG AGCGCGGCCC GGGCGTGCAT
 humanmRNA  ACAAAAAATT AGCCGGGCGT GGTGGCGGGC ACCTGTAGTC CCAGCTACTC GGGAGGCTGA
 mousemRNA  TCAGATGAGG AGCACTGCGC GGTGGCCACC TGCCGACCTG ATGAATTCCA GTGTGCAGAT
 ovinemRNA  ------GACA AGCCCGTCAT GGTTGGCTGG TGTCCATCTG AAACACGCAT GGCCGGTCTA
bovinemRNA  ------GACA AGCCCATCAT GGTTGGCTGG TGTCCATCTG AAACATGCAT GGCAGGTCTG
 Consensus  ......ga.. agccc..c.t ggtgGgc.gg tgccga.ctg a.a.atgC.. Ggg.Gg.c..

            5701                                                    5760
rabbitmRNA  GGCGTT---- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GGCAGGAGAA TGGTGTGAAC CCGGGAAGCG GAGCTTGCAG ---------- ----------
 mousemRNA  GGCTCCTGCA TTCACGGTAG CCGCCAGTGT GACCGTGAAC ATGACTGCAA GGACATGAGC
 ovinemRNA  CCCTGTAAGA T--------- ---------- ---------- ---------- ----------
bovinemRNA  CCCTGTAAGA T--------- ---------- ---------- ---------- ----------
 Consensus  ggCtgta..a t......... .......... .......... .......... ..........

            5761                                                    5820
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GACGAGCTCG GCTGCGTCAA TGTGACACAG TGTGATGGCC CCAACAAGTT CAAGTGTCAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5821                                                    5880
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------T GAGCCGAGAT TGCGCCACTG CAGTCCGCAG TCTGGCCTGG
 mousemRNA  AGTGGGGAGT GCATCAGCTT GGACAAGGTG TGCGACTCCG CCCGCGACTG CCAGGACTGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5881                                                    5940
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GCGACAGAGC GAGACTCCGT CTCAAAAAAA AAAAACAA-- ---AAAAAAA CCATGCATGG
 mousemRNA  TCGGATGAGC CCATCAAGGA GTGCAAGACC AACGAGTGTT TGGACAACAA TGGTGGCTGT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            5941                                                    6000
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TGCATCAGCA GCCCATGGC- ---------- ---------- ---------- ----------
 mousemRNA  TCCCACATCT GCAAGGACCT CAAGATTGGC TCTGAGTGCC TGTGTCCCAG CGGCTTCCGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6001                                                    6060
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TTGGTGGACC TCCACAGGTG TGAAGATATT GACGAGTGTC AGGAGCCAGA CACCTGCAGC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
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            6061                                                       6120
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CAGCTCTGTG TGAACCTGGA AGGCAGCTAC AAGTGTGAGT GCCAGGCCGG CTTCCACATG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6121                                                       6180
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ----CTCTGG CCAGGCATGG CGAGGCTGAG GTGGGAGGAT GGTTT----- ----------
 mousemRNA  GACCCACACA CCAGGGTCTG CAAGGCTGTG GGCTCCATAG GCTATCTGCT CTTCACCAAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6181                                                       6240
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ------GAGC TCAGGCATTT GAGGCTGTCG TGAGCTATGA TTATGCCACT GCTTTCCAGC
 mousemRNA  CGCCACGAGG TCCGGAAGAT GACCCTGGAC CGCAGCGAGT ACACCAGTCT GCTCCCCAAC
 ovinemRNA  ------GTGC TCTGGGCGCA GGAATTTCAT GGGATTCATT AAACAAGAAT GCTTTC----
bovinemRNA  ------GTGC TCTGGGCTCG GGAATTTCAT GGGATTCATT AAACAAGAAT GCTTTC----
 Consensus  ......g.gc tc.gg..... g....t..a. .g.a.t.a.t a.ac.aga.t gctttc....

            6241                                                       6300
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CTGGGCAACA TAGTAAGACC CCA------- ---------- ---------- ----------
 mousemRNA  CTGAAGAATG TGGTGGCTCT CGACACGGAG GTGACCAACA ATAGAATCTA CTGGTCCGAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6301                                                       6360
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  -TCTCTTAAA AAATGAATT- ---------- ---------- ---------- ----------
 mousemRNA  CTGTCCCAAA AAAAGATCTA CAGCGCCCTG ATGGACCAGG CCCCTAACTT GTCCTACGAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6361                                                       6420
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  ACCATCATCA GTGAGGACCT GCATGCCCCT GACGGGCTGG CGGTAGACTG GATCCACCGC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6421                                                       6480
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------T GGCCAGACAC AGGTGCCTCA CGCCTGTAAT CCCAGCACTT TGGGAGGCTG
 mousemRNA  AACATCTACT GGACAGATTC AGTCCCAGGC AGCGTATCTG TGGCTGACAC CAAGGGCGTA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6481                                                       6540
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  AGCTGGATCA CTTGAGTTCA GGAGTTGGAG ACCAGGCCT- ---------- ----------
 mousemRNA  AAGAGGAGGA CACTGTTCCA AGAGGCAGGG TCCAGACCCA GAGCCATCGT AGTGGACCCT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
```

```
            6541                                                        6600
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ----GAGCAA CAAAGCGAGA
 mousemRNA  GTGCATGGCT TCATGTACTG GACAGATTGG GGAACACCCG CCAAGATCAA GAAAGGGGGT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6601                                                        6660
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TCCCATCTCT ACAAAAACCA AAAAGTTA-- ---------- ---------- ----------
 mousemRNA  TTGAATGGTG TGGACATCCA CTCACTGGTG ACCGAAAACA TCCAGTGGCC AAATGGCATC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6661                                                        6720
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  ACACTAGATC TTTCCAGTGG CCGTCTCTAT TGGGTTGATT CCAAACTCCA CTCTATCTCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6721                                                        6780
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- -------AAA ATCAGCTGGG TACGGTGGCA CGTGCCTGTG
 mousemRNA  AGCATCGATG TCAATGGGGG CAATCGGAAA ACCATTTTGG AGGATGAGAA CCGGCTGGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6781                                                        6840
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ATCCCA---- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CACCCCTTCT CCTTGGCCAT CTATGAGGAC AAAGTGTATT GGACAGATGT CATAAACGAA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6841                                                        6900
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ------GCTA CTTGGGAGGC TGAGGCAGGA
 mousemRNA  GCCATTTTCA GTGCCAATCG ACTCACGGGT TCAGATGTGA ATTTGGTGGC TGAAAACCTC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6901                                                        6960
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  GGATCGCCTG AGCCCAG--- ---------- ---------- ------GAGG TGGAGGTTGC
 mousemRNA  TTGTCCCCGG AGGACATTGT CCTGTTCCAC AAGGTCACAC AGCCTAGAGG GGTGAACTGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            6961                                                        7020
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  AGTGAGCCAT GATCGAGCCA CTGCACT--- ---------- -------CCA GCCTGGGCAA
 mousemRNA  TGTGAGACAA CAGCCCTCCT CCCCAATGGT GGTTGCCAGT ACCTGTGCCT GCCCGCCCCA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
```

```
            7021                                                    7080
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CAGATGAAGA CCCTATTTCA GAAATACAAC T--------- ---------- ----------
 mousemRNA  CAGATCGGTC CCCACTCGCC CAAATTCACC TGCGCCTGCC CTGATGGCAT GCTGCTGGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7081                                                    7140
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ------ATAA AAAAATAAAT AAATCCTCCA GTCTGGATCG
 mousemRNA  AAGGACATGC GGAGCTGCCT CACAGAAGTC GACACTGTAC TGACCACCCA GGGGACATCC
 ovinemRNA  ---------- ---------- ---------- -----TGAAA TTGCCACACA TATCATAT--
bovinemRNA  ---------- ---------- ---------- -----TGAAA TTACCACACA TATCATAT--
 Consensus  .......... .......... .......... .....tgaa. t.accac.ca ....a.at..

            7141                                                    7200
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TTTGACGGGA CTTCAG---- ---------- ---------- ---------- ----------
 mousemRNA  GCCGTCCGGC CTGTGGTCAC CGCATCAGCT ACCAGGCCAC CGAAGCACAG TGAGGATCTC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7201                                                    7260
rabbitmRNA  ---------- ---------- --------TT AGCTTTGCAC CTCGCAAGCC GCGCGAGTCT
 humanmRNA  ---------- GTTCTTTCTG AAATCGCCGT GTTACTGTTG CACTGATGTC CGGAGAGACA
 mousemRNA  TCAGCTCCCA GTACTCCTAG GCAGCCTGTG GACACCCCAG GGCTCAGCAC AGTGGCGTCA
 ovinemRNA  ---------- ---------- --------TT ATGACCACTG GGGGGGTGGG GGTCGGGTGG
bovinemRNA  ---------- ---------- --------TT ATGACCACTG GGGGG-TGGG GGTTGGATGG
 Consensus  .......... .......... ........tt at.acc.ctg ggcggatg.c ggt.G.gtc.

            7261                                                    7320
rabbitmRNA  GTGACGACAT ---------- ---------- ---------- ---------- ----------
 humanmRNA  GTGACAGCCT CCGTCAGACT CCCGCGTGAA ---GATGTCA CAAGGGATTG GCAATTGTCC
 mousemRNA  GTGACAGTGT CCCACCAAGT CCAGGGTGAC ATGGCTGGCA GAGGGAATGA GGAGCAGCCA
 ovinemRNA  GGGGCAGCCT CCCATGGAAA C--------- ------ATCT GAGTTGATTG GCTACCGTAC
bovinemRNA  GGAGCAGCCT CCCATGGAAA C--------- ------GTCT GAGTGGGTTG ACTACTGTAC
 Consensus  GtgaCagccT ccca..ga.. c......... ......gtc. gag.ggattg gc.ac.gt.c


            7321                                                    7380
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CCAGG----- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CATGGTATGA GGTTCCTGTC CATCTTCTTC CCTATTGCAC TGGTTGCCCT CCTTGTCCTT
 ovinemRNA  AGCC------ ---------- ---------- ---------- ---------- ----------
bovinemRNA  AACC------ ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7381                                                    7440
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GGGGCCGTCC TGCTGTGGAG GAACTGGCGG CTGAAGAACA TCAACAGCAT AAACTTTGAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7441                                                    7500
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  AACCCAGTCT ACCAGAAGAC CACAGAGGAC GAGCTCCACA TTTGCCGAAG CCAGGATGGC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
```

```
            7501                                                    7560
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TATACCTACC CCTCAAGACA GATGGTCAGC CTGGAGGACG ATGTGGCATG AGCAGCCGGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7561                                                    7620
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- GACAAAACAC TGTGTCCCCC
 mousemRNA  AGAGCCGTCT CTTTCCGGGA TCCATTGCCA AGCTTAGGCA GAAAAGACAC TCTCTCCAGA
 ovinemRNA  ---------- ---------- ---------- ---------- -ACAGAACAC GG-CACGGC-
bovinemRNA  ---------- ---------- ---------- ---------- -ACAGAACAC GGGCACCCC-
 Consensus  .......... .......... .......... .......... .aca.aacac .g.c.cc.c.

            7621                                                    7680
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CC-------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CCTCCCCATC CAGCACTGGT CCTGCCACCT CCCTGGGCTC TGTGTTGCTC AAAGCAAGAT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7681                                                    7740
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  AAGAGCAAAG CTGGGCTGGG GGCCAAGCTC AGCTGCCTGT CTGCCCCAGG TTCTGTTTTA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7741                                                    7800
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TATATTTATT GTCTGGGGAC AGAAAAGGCT ACTGGCTGTG CTTGAAATTC GAATTCTGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7801                                                    7860
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGGGAATTTT TTTTTTTCTC CCATCTTCAT TTCCTTGGAA GCCAAATAGG CTGTCCCAGA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........


            7861                                                    7920
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  AGCTTCTGAA TTCTACTCTT TTCAACACTA ACACGGAGGA GGAAGAGACC CACCCAGGCA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            7921                                                    7980
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GAGAGAAGCC TGCTGGACAT GAGACAGCAG GTACTACTGA CCGGGTCCTG GGTACCATAG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
```

```
            7981                                                    8040
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CCAGTCCCTA ACCTCAGGAC TTCCTGCATT GACCTTCATC CCAGAGCCTT CCTGCCTGAC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8041                                                    8100
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- --AGTGCAGG GAACCGTGAT AAGCCTTTCT GGTTTCGGAG
 mousemRNA  TCTGCCGCCC CCATCAGAAG CCAGGAAAGT GACTCGTGAC ATTCGGTACT GATCTTGCTG
 ovinemRNA  ---------- ---------- ----TGCCGG AGACTGTGAA CACCTTTTCT CTTTCTGAAG
bovinemRNA  ---------- ---------- ----TGCCGG AGACTGTGAA CACCTTTTCT CTTTCTGAAG
 Consensus  .......... .......... ....tgc.gg ..ac.gtga. .a.c.tttct ..tt.tg.ag

            8101                                                    8160
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  CACGTAAATG CGTCCCTG-- --------TA CAGATAGTGG GGA------- ----------
 mousemRNA  GATCTTGAGG CCGGCAGC-- --------TG CCAGTGATGG ATACCCGCTC ATGCCTTTGC
 ovinemRNA  CATGTAAATA ACACCCCCAC CACCGCCATG CAGACAGGGG GGA------- ----------
bovinemRNA  CATGTAAATA ACACCCCCAC CACCGCCGTG CAGACAGTGG GGA------- ----------
 Consensus  catgtaaat. .c.ccc.c.. ........tg caga.agtgg gga....... ..........

            8161                                                    8220
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CAAAACGTCA CCCGTTCCTG GAGGGACAAG CCCAAGTCGC CATTCTCCCT TAATATTTAT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8221                                                    8280
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CAAGTGCCTG AGACAACTGG TTGCCTTGCT CAGGAGTCCT GGCCTGCTCA GTGTCCTGCT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........


            8281                                                    8340
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  GCTCAGGGGT GGCCGGCAGG ACCCCTGTCC TGTGCCCAGC CCCCGAGTCT CCGAGTGAGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8341                                                    8400
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CTTGCACTTC TCTCTGCGCA CTTTTCCCGT TCTGTGCTGG GCACCCACGT GGTCCACATT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8401                                                    8460
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGTACTCCTA GGTTGCACTG ACCCTGGTGC ATTGGATGGG ACCTCAGGCC GGAAGTGGCC
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......- .......- .......- .......- .......- .......-
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            8461                                                    8520
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CCGAATCATT GACCACAGAA GATAATTGCC AGGCTCCAAG CATCCATTGC CTTCTTCAGT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8521                                                    8580
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- --------TT
 humanmRNA  ---------- ---------- ---------- ---------- -----TTTTT TGTTATGTTT
 mousemRNA  ACTGGGGGGA ACTCAGGGCC TCTGTCTGGT GTTTAGTTAG TCTGTTTGTT TGTTTGTTTG
 ovinemRNA  ---------- ---------- ---------- ---------- -----TTTTT TGTTATGTTT
bovinemRNA  ---------- ---------- ---------- ---------- -----CTTTT TGTTATGTTT
 Consensus  .......... .......... .......... .......... .....ttttt tgttatgtTt

            8581                                                    8640
rabbitmRNA  GCACTTTGTG CGTTTCTGG- ---------- ---------- ---------- ----------
 humanmRNA  GCACTTTGTA TATTGGTTG- ---------- ---------- ---------- ----------
 mousemRNA  TTTGTTTGTT TGGTGGTTTC TGGCTCTGCA CAGCAGAGTT GTTTAGCTCC TCTGGTAACG
 ovinemRNA  GCACTTTGTA TATTTGTTG- ---------- ---------- ---------- ----------
bovinemRNA  GCACTTTGTA TATTTGTTG- ---------- ---------- ---------- ----------
 Consensus  gcacTTTGTa tatTtgTtg. .......... .......... .......... ..........

            8641                                                    8700
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TCATCCTCTC TCACAGAGAC ATCCAGCCTG CTGAAGGGGA GAGATGGGGC GAGGGGAGGG
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8701                                                    8760
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  ATACACGCAT GGTTTCTCAG GTGTGGTGGC ACTTGGCTTT AATCCTATGC TCAAGAGGAA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8761                                                    8820
rabbitmRNA  ---------- ---------- ---------- AGACGCTGAG CACATGTATA GAAAT-----
 humanmRNA  ---------- ---------- ---------- AAACTGTTAT CACTTATATA TATATATATA
 mousemRNA  GAGGTGGGCA CATGTCTGAG GTCCAAGCCT ACATAGTGAG ACCTTATATA TCTAAAAACC
 ovinemRNA  ---------- ---------- ---------- AAACATTTCT CACTTACGTG TATATATAT-
bovinemRNA  ---------- ---------- ---------- AAACATTTCT CACTTATGTG TATATATAT-
 Consensus  .......... .......... .......... AaAca.Ttat caCtTataTa tatAtatat.


            8821                                                    8880
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  AAAAGAAATC CCAGACTATT TGGTGGGACT TGGAAGAATA CTTAGAAATT GCCACAGATT
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            8881                                                    8940
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  TGTCACATGG GTAACCTAAG AAGACAGTGG TGGCCTTACT TGGATTCCTG GGTAGATCCA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........
```
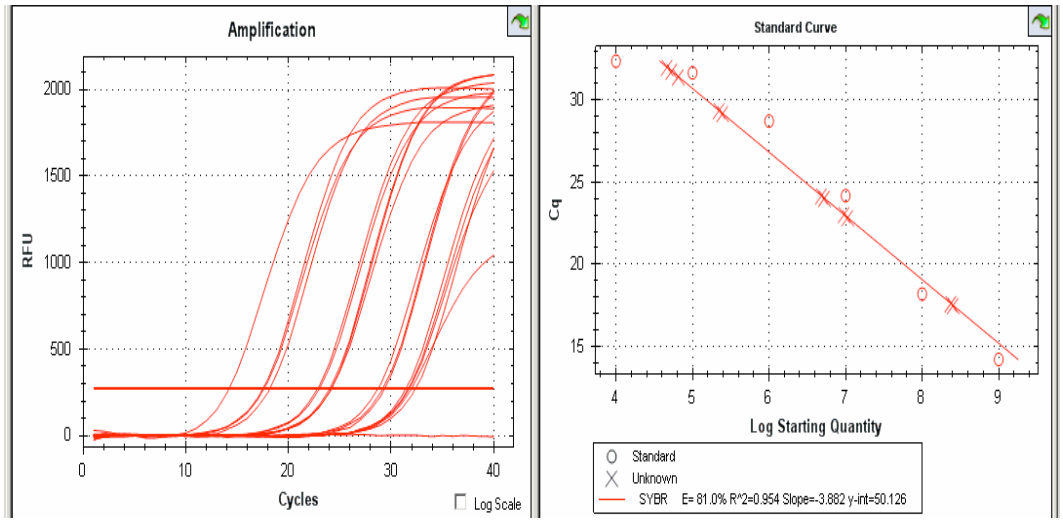
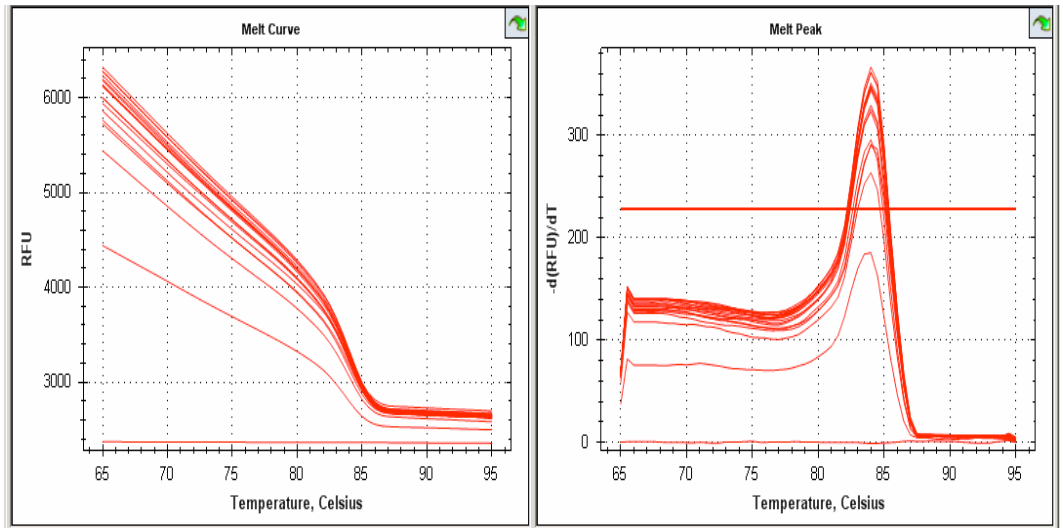

```
            8941                                                    9000
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 mousemRNA  CCTGTCCACT ACTCAAGACA CAAGGGCACA CTGCCCAGCC CACTGGGACA CTTGTAAAGA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            9001                                                    9060
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  ---------- ---------- ---------- ---------- ---------- --------CA
 mousemRNA  GCTCTGTAAT GTGCCGATGG CACCTGGCTC GGTTTTCATT CTGTATATTC AAGGATATCA
 ovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
bovinemRNA  ---------- ---------- ---------- ---------- ---------- ----------
 Consensus  .......... .......... .......... .......... .......... ..........

            9061                                                    9120
rabbitmRNA  ---------- -------TAT TTATTTTTGC TAACGCTGGC TGCTGTGTG- ----------
 humanmRNA  CACATATATA TAAAATCTAT TTATTTTTGC AAACCCTGGT TGCTGTATTT GTTCAGTGAC
 mousemRNA  CACATATGTA TTAAATCTAT TTATTTTTGC AAACCCTGAT TGCTGCACCT CTCTGCAATT
 ovinemRNA  ---ATAAAAG AAAGATCTAT TTATTTTTGC AAATCCTGGT TGCTGCAA-- ----------
bovinemRNA  ---A--AAAG AAAGATCTAT TTATTTTTGC AAATCCTGGT TGCTGCAA-- ----------
 Consensus  ...ata.a.. .aa.atcTAT TTATTTTTGC aAAccCTGgt TGCTGca... ..........

            9121                                                    9180
rabbitmRNA  ---------- ---------- ---------- ---------- ---------- ----------
 humanmRNA  TATTCTCGGG GCCCTGTGTA GGGGGTTATT GCCTCTGAAA TGCCTCTTCT TTATGTACAA
 mousemRNA  TCTCCAGGTT AGGCTG---- --GGGTGATA CTCTGGGA-- ------TGCT CTGTATACAA
 ovinemRNA  ---------- ---------- ---------- -------AGA TGCCTCGTCT TTCTGTACAA
bovinemRNA  ---------- ---------- ---------- -------AGA TGCCTCGTCT TTCTGTACAA
 Consensus  .......... .......... .......... .......a.a tgcctc.tct tt.tgtacaa

            9181                                                    9240
rabbitmRNA  ---------- ---------- ---------- ---------- ------CAGC GGCGCGGGGC
 humanmRNA  AGATTATTTG CACGAACTGG ACTGTGTGCA ACGCTTTTTG GGAGAATGAT GTCCCCGTTG
 mousemRNA  AGATGATTTG AATGGGCTGA TCTCAGGCC- ---------- ---------T GTCCTGTGCG
 ovinemRNA  AGCTTAGTTG AGCGTGCCGT GCACAGCCCT C----TTCAA GAGAATCAGG TTCTATGTTG
bovinemRNA  AGCTTATTTG AGCGTGCCGT GCACAGCCCT C----TTCAA GAGAATCAGG TTCTGTGTTG
 Consensus  ag.ttatttg a.cg.gc.g. .c.cag.cc. .....tt... g...a.cag. gtC...gttg

            9241                                                    9300
rabbitmRNA  CGCCCGTGGG GGGGTGGGGG AGGGCTCCGT GCCGCCTTTG AACCACTGTA TAGAGAGTTT
 humanmRNA  TATGTATGAG TGGCTTCTGG GAGATGGGTG TCACTTTTTA AACCACTGTA TAGAAGGTTT
 mousemRNA  TTGCTTTGAG TGGGT----- ------GGGA ACTGCTTTGA AACCCTTGTT CAGATGTTTT
 ovinemRNA  TATATATGAG TTGCTTTTTG AGGATGGGTG TCGCTTTTTA AACCACTGTA TAGAATGTTT
bovinemRNA  TATATATGGG TTGCTTTTTG AGGATGGGTG TCGCTTTTTA AACCACTGTA TAGAATGTTT
 Consensus  tat.taTGaG tgGcTt.t.g aggatgggtg tC.cttTTta AACCacTGTa tAGAa.gTTT

            9301                                                    9360
rabbitmRNA  TTATAGCCTG AAGGGG-TCC CTGTGGTTGA TTAAACTTCT TTAACGAGT
 humanmRNA  TTGTAGCCTG AA-TGTCTTA CTGTGATCAA TTAAATTTCT TAAATGAACC AATTTGTCTA
 mousemRNA  TATAGGCTGA AAATATCATA CTGTGATGGA TTAAATTCTT TTTTGGAAAA AAA------A
 ovinemRNA  TTATAGCCTG AA-TGCCTTA CTGTGATCAA TTATATTTCT TAAATAAAGT ATTTGACT--
bovinemRNA  TTATAGCCTG AA-TGCCTTA CTGTGATCGA TTACATTTCT TAAATAAAGT ATTTGACT--
 Consensus  TtataGCctg AA.tg.ctta CTGTGaTcgA TTAaAtTtcT TaaatgAa.. a.tt......

            9361  9370
rabbitmRNA
 humanmRNA  AAAAAAAAAA
 mousemRNA  AAAAAAAAAA
 ovinemRNA  -AAACCGGAA
bovinemRNA  -AAATCGGAA
 Consensus  .aaa....aa
```

E.3 Amplification and Standard Curve of qPCR Analysis of Purified rAAV8-LSP-LDL-R



E.4 Melting Temperature Curve of qPCR Analysis of Purified rAAV8-LSP-LDL-R



E.5 Average Flourescence from DNA Direct Binding to SYBR Gold

Direct DNA Measurement Report

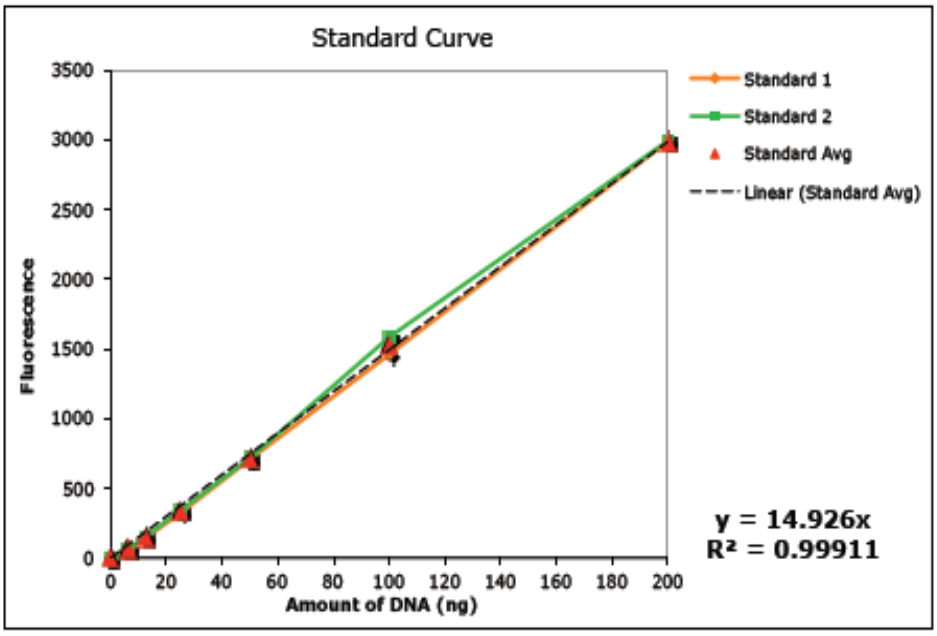
Project/Title: **AAV8-LDLR (GEMS)**

Date of Measurement: 3/1/2013
Investigator:

Notes:

Titer(s):

Sample	Volume (ml)	Vg/ml	Total (vg)
AAV8-LDLR	150	6.4671E+11	**9.70E+13**



Appendix F: Budget

Order Date	Catalog #	Name	Unit Size	Price	Quantity	Total Price (excluding S+H)	Supplier	Shipping Price
4/3/11	M0530S	Phusion® High-Fidelity DNA Polymerase	100 units	$103.00	2	$206.00	New England BioLabs Inc.	$19.00
4/3/11	R0552S	AgeI	300 units	$65.00	1	$65.00	New England BioLabs Inc.	
6/9/11	552846	Bright Linearized Baculovirus DNA	2.5 µg	$343.00	1	$343.00	BD Biosciences	$30.00
6/9/11	PAB8804	LDL-R Antibody	100 µg	$313.20	1	$313.20	Acris Antibodies Inc.	$32.00
10/25/11	Strain code: 052	Female New Zealand White (NZW) Rabbit	1 rabbit	$116.15	12	$1,393.80	Charles River	$265.76
10/10/2011, 10/21/11, 11/17/11, 12/20/12 2/1/12	5326	Rabbit Feed – Rabbit Diet HF	N/A	$19.35	20	$387.00	Quality Lab	$22.00
10/10/2011, 10/21/11, 11/4/11, 11/17/11, 12/20/12, 2/1/12	N/A	Bedding – Paper Chip SSP brand	N/A	$13.15	28	$368.20	Quality Lab	$25.00
11/4/12	E2HL-100	EnzyChrom™ AF HDL and LDL/VLDL Assay Kit	N/A	$359.00	1	$359.00	BioAssay Systems	$34.00
11/4/12	3460-08	MaxDiscovery™ Alanine Transaminase (ALT) Color Endpoint Assay Kit	N/A	$268.00	2	$536.00	Bio Scientific	$25.00
11/4/12	Z5030033	Alkaline Phosphatase Assay Kit	N/A	$395.00	1	$395.00	BioChain	$37.00
11/4/12	G5635-1KU	β-Galactosidase from *Escherichia coli*	N/A	$64.90	1	$64.90	Sigma-Aldrich	$8.00
2/1/11	19-098-930	Fisherbrand* Polylatex Shoe Cover Size XL	N/A	$51.39	2	$102.78	Fisher Scientific	$5.00
6/21/11	12658-027	Sf-900™ III SFM (1X), liquid	100 mL	$67.50	5	$337.50	Life Technologies – Invitrogen	$47.97
11/15/11	19-057-416A	Kimberly-Clark Professional A10 Light-Duty Lab Coat	50 units	$117.66	1	$117.66	Fisher Scientific	$11.00
TOTAL PRICE = $5,550.77								

List of Abbreviations

AAV6: Adeno-Associated Virus Serotype 6

AAV8: Adeno-Associated Virus Serotype 8

CAP: Capsid protein

CMV Promoter: Cytomegalovirus promoter

cDNA: Complementary DNA

FH: Familial hypercholesterolemia

HCR-ApoE-hAAT promoter: Hepatic control region Apolipoprotein enhancer/alpha1-antitrypsin promoter

HDL: High-density lipoprotein

IACUC: Institutional Animal Care and Use Committee

ITR: Inverted terminal repeat

LDL-R: Low density lipoprotein receptor

ORF: Open reading frame

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

rAAV: Recombinant adeno-associated viral vector

qPCR: Quantitative-polymerase chain reaction

q-RT-PCR: Quantitative-real time-polymerase chain reaction

REP: Replication protein

RLM-RACE: RNA ligase mediated rapid amplification of cDNA ends

RT-PCR: Reverse transcription-polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

UTR: Untranslated region

WHHL: Watanabe Heritable Hyperlipidemic Rabbit**s**

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