Adeno-Associated Viral Vector Based Gene Therapy Product Development for Hemophilia B

Major project-II

To be submitted in partial fulfillment of the requirement

for the Degree

Of

Master of Technology

In

Industrial Biotechnology

Submitted By

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CONTENTS

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CANDIDATE'S DECLARATION

I Dhananajay Kumar, Roll No. 2K17/IBT/02 of M.Tech Industrial Biotechnology, hereby declare that the Major project –II titled **"Adeno-Associated Viral Vector Based Gene Therapy Product Development for Hemophilia B"** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associate ship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Major Project-II titled **"Adeno-Associated Viral Vector Based Gene Therapy Product Development for Hemophilia B"** which is submitted by Dhananajay Kumar, Roll No. 2K17/IBT/02, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award the Degree of Master of Technology, is a record of the project work carried out by the students under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to University or elsewhere.

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ABSTRACT

Hemophilia B is an X-linked bleeding disorder that prevents the blood from clotting normally. It is caused by mutations in the factor IX (FIX) gene leading to low levels or complete loss of FIX expression/activity. It is a rare disease that affects about 1 in 20,000 male births in the USA and approximately 400,000 individuals across the world. The severity of this disease depends on the level of FIX protein expression in the patients and has been classified as mild (5-40% FIX activity), moderate (1-5% FIX activity) or severe (< 1% FIX activity). Patients with mild disease usually bleed only after serious injury, trauma or surgery; and in such patients, the first bleeding episode usually doesn't occur until adulthood. Individuals with moderate disease, who account for about 15% of the hemophilia population, have bleeding episodes after minor injuries and often have spontaneous bleeding episodes without an obvious cause. People with severe disease also bleed following an injury and have frequent spontaneous bleeding episodes in joints and muscle. Such patients account for about 60% of the hemophilia B population (National Hemophilia Foundation, USA and Centre for Disease Control and Prevention, USA).

Replacement therapy for the missing FIX is the only drug modality currently available for the treatment of haemophilia B. It requires frequent infusion of plasma derived or recombinant FIX protein to the patients. Importantly, infusion of plasma derived FIX might entail the risk of patient infection.

This study was carried out to develop adeno associated viral vector based gene therapy product for the treatment of Hemophilia B (AAV-FIX). AAV-FIX vectors were expressed in HEK293 cells by triple plasmid co-transfection. Transfected cells were lysed to release the vector in cell lysate which was clarified and subjected to affinity based purification. The purified vectors were analysed by SDS-PAGE and ELISA for the purity and quantity.

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

LIST OF ABBREVIATION

AAV	Adeno Associated Virus
pAAV-FIX^{Pd}	Plasmid (pUC19) conatain gene of AAV and Factor Nine Padua.
pHelper	Plasmid (pUC19) conatain gene of Helper(Herpes virus).
pAAV-R2C8	Plasmid contain gene of AAV and Replication gene of serotype AAV2 and capsid gene of serotype AAV8.
Ad	Adeno virus
VP	Viral Protein
AAP	Assembly Activating Protein
Rep	Replication gene
Cap	Capsid gene
CMV	Cyto megalo virus
ITR	Inverted Terminal Repeat
SV40	Simian Virus 40
DNA	Dioxy Nucleic Acid
pUC19	Plasmid-University of California
SOC	Super optimal broth with catabolite repression
NFW	Nuclease free water
HEK	Human Embryonic Kidney
PEI	Polyethylenimine
DMEM	Dulbecco's Modified Eagle Media
FBS	Fetal bovine Serum
Peni-strep	Penicillin-streptomycin
PBS	Phosphate buffer saline
EDTA	Ethylenediaminetetraacetic acid

TAE	Tris-Acetate-EDTA
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
EtBr	Ethidium bromide
DTT	Di-thio-threitol
ELISA	Enzyme Linked Immunosorbent Assay
TMB	Tetramethylbenzidine
CFU	Colony Forming Unit
UV-VIS	Ultra Violet-Visible
O.D.	Optical Density
nm	Nanometre
ng	Nano gram
conc.	Concentration
Min.	Minute
µL	Micro liter
ml	Milli liter
gm	Gram
M	Molar
mM	Milli molar
%	Percentage
^{o}C	Degree centigrade

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL:

Hemophilia B is an X-linked bleeding disorder due to a mutation in the F9 gene encoding coagulation factor IX. In 2017 approximately 4000 in USA, 2200 in India and 1800 in China, peoples were reported as hemophilia B patients (world federation of haemophilia). FIX proteins commonly form within the liver by hepatocytes cells and releases into the blood flow. FIX is a potassium dependent serine protease that plays an important part within the blood clotting pathway. FVIII play important role in the functioning of FIX. The severity of this disease depends on the expression and amount of FIX protein within the patients. On the basis of amount of FIX protein within the patients, Patients categories into the three classes 1-serious (<1 % FIX) 2-moderate (1-5% FIX) and 3-mild (5-40% FIX).

1.2 AAV VECTOR FOR HEMOPHILIA B:

Adeno-associated virus (AAV) is a gene therapy based vector, discovered as contaminant of adenovirus. It is act as vehicle for the delivery of FIX gene to the liver cell. Where FIX gene replicate, transcript and form FIX proteins for the treatment of hemophilia B. AAV is a parvovirus and dependent on co-infection of the targeted cells for the replication by other helper virus i.e. adenoviruses. AAV vector have a small, ssDNA genome of 4.7 kilo bases with three key genes, Rep (Replication), Cap (Capsid), and AAP (Assembly associated protein). The key genes are bound by inverted terminal repeats (ITRs) from both sides. ITRs responsible for the replication and packaging of the genome of viral inside the capsid. Rep gene, which are responsible for replication and packaging of viral genome, while three Cap gene essential for the synthesis of capsid wall that protects the viral genome, as well as cell binding and internalization. AAV genome replication occurs with the help of helper virus for AAV production [1].

1.3 TRANSFECTION OF HEK-293 CELLS FOR AAV VECTOR PRODUCTION:

HEK 293 cells develop by the transfection with gene of adeno virus. Some genes of adenovirus that responsible for the transcription of AAV, present in the HEK 293 cell. For the assembly of rAAV vectors in HEK 293 mammalian cell require: (1) a recombinant vector genome i.e. pAAV-FIX^{pd} (2) pAAV-R2C8 (3) pHelper (adenovirus). Introduction of DNA into HEK 293 cells by transfection process, in which a chemical poly-ethylenimine, responsible for condensation of the DNA. The positive charge of poly-ethylenimine bind to the outer negative charge of DNA particles and provided positive charge on the outer surface of DNA that helps in the binding of negative charge of host cell during the endocytosis process [2].

In previous years, mentioned advances on the efficacy and safety of the treatment of the haemophilic patients has been obtained and implicated exclusively in western country. Thus the next aim for upcoming times is to obtain wider treatment availability at low cast. Many densely populate countries like India and China where level of hemophilic patients are larger than other countries and care of hemophilia B is far from being satisfactory. The main aim for the future is to make large availability of treatment. In the large populated countries, like China and India, the level of hemophilia care so not good and these countries are involved in the development of technology to produce recombinant factor for the cure of hemophilia B and methods developing for the transfer of gene.

The most likely progress in this field is the availability of FIX molecule with more half life; this would be a significant step forward for the treatment of hemophilia B. Many pharma companies are currently developing factors with longer half life to prevent regularly administration and reduced immunogenicity/antigenicity to minimize inhibitor development.

At presents times only drug modality available for the hemophilia B treatment. Its needs frequent infusion of plasma derived (Novo Nordisk's Rebinyn) or recombinant (Baxter's Rixubis, Pfizer's Benefix, Biogen's Aprolix) FIX protein to the patients. Significantly, infusion of plasma derived FIX would possibly entail the danger for patient infection. Currently treatment includes frequently injections of FIX

concentrate protein (i.e., 2 to 3 times in a week) into the blood vessel. Somatic gene therapy is one of the methods that offer ability to a cure by hemophilic patients by continuously production of FIX into the target cells after a one time-single dose administration of vector for hemophilia B [3, 4].

In recent study, peripheral vein administration of scAAV-FIX ^{padua} vector at a dose of 5 x 10^{11} viral genome per kilogram to 10 hemophilia B patients with the FIX coagulant and keep following up to 492 weeks to individuals (some individual followed up to, 28 to 78 weeks), resulting reduced in bleeding rate per year. Several serotype of AAV have been identified with their preferred targeting to certain cell types in humans and other model animals. Among all naturally occurring serotypes, AAV serotypes 8(AAV) show that highest preference for liver targeting transduction. Further, AAV8 serotype based gene therapy vector has shown sustained FIX expression in humans [5, 6]. Therefore, this serotype will be selected to develop AAV based FIX gene therapy vector.

1.4 VECTOR CONSTRUCTION:

Production of rAAV requires co-transfection of host HEK-293 cells with three plasmids: transfer plasmid- carrying gene of interest (e.g. FIX in the present case), helper plasmid- carrying adenoviral genes to help AAV replication and Rep-Cap plasmid- carrying genes for AAV replication and capsid formation. Constructs for these plasmid vectors have been depicted in fig. 1.1. Synthesis of these constructs was outsourced.

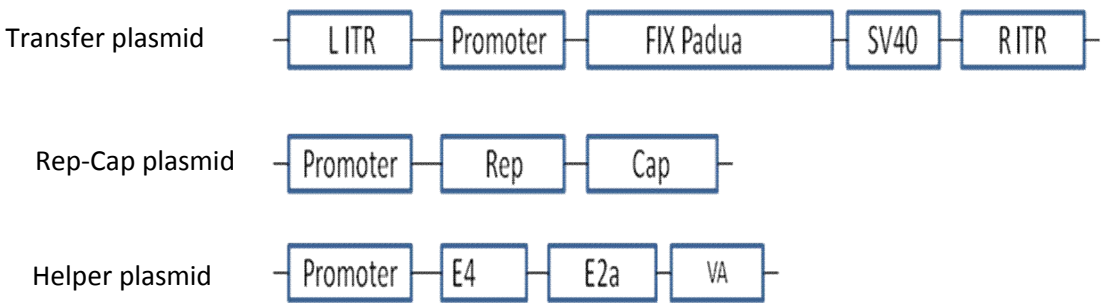


Figure 1.1: Schematic of Three Plasmid Vectors used for scAAV-FIX^{pd} production

1.5 AIM and OBJECTIVE:

Aim and objective of this work is the Development of Gene Therapy based product (AAV Vector, containing FIX gene) for Hemophilia B. The objectives of the present work can be listed as follow:

1. **Production of Plasmid DNA at Large Scale:**
    - I. Transformation of *E.coli stbl3* with pAAV-FIX^{Pd}, pHelper and pAAV-R2C8
    - II. Isolation and Purification of Plasmids DNA- pAAV-FIX^{Pd}, pHelper and pAAV-R2C8
    - III. Restriction digestion of Plasmids DNA- pAAV-FIX^{Pd}, pHelper and pAAV-R2C8
2. **Production of AAV8 Vector:**
    - I. Transfection of HEK 293 cells with three plasmids pAAV-FIX^{Pd}, pHelper and pAAV-R2C8
    - II. Harvesting of transfected HEK 293 cells
    - III. Lysis of transfected HEK 293 cells
3. **Purification of AAV8 Vector:**
    - I. Affinity chromatography
    - II. Ultrafiltration/ protein concentrate
4. **Characterization of AAV8 Vector:**
    - I. SDS-PAGE of capsid proteins of AAV8 Vector
    - II. ELISA of non-purified and purified AAV8 Vector

Summary:

In this, describes the strategy for production of an AAV based gene therapy vector for hemophilia B at the laboratory scale. The sc AAV-FIX^{Pd} vector produced using the DMEM medium (thermo Fisher Scientific) and purified by affinity chromatography using an AAV8 specific resin. Purified vectors characterized for their purity, identity as well as physical and functional titers using SDS-PAGE and ELISA methods.

CHAPTER 2

LITERATURE OF REVIEW

2. LITERATURE OF REVIEW

Hemophilia B is an X-linked bleeding disorder resulting from low level of factor IX synthesis and is due to mutations in the F9 genes. It affects one in ten thousands male births worldwide. The prevalence of hemophilia B is one in thirty thousand.

Patek, Arthur J. (In 1937), uses globulin, extracted from plasma to correct the coagulation defect. Globulin administered into muscles of haemophilic patients and observed the coagulation time, reduces little but no any phase of coagulation occurs after injection of globulin substances [7].

Biggs R, Douglas AS (1952) showed that the blood from one hemophilia B patient can be correct the blood clotting defect of other hemophilia B patients. But this exchange of blood not beneficial for the long term solutions, many patients were died after the blood exchanged from one haemophilic patients to other haemophilic patients [8].

White GC, McMillan CW (In 1982), used DNA Technology for the cloning of FVIII genes which helps in the industrial production of recombinant FVIII, two patients of hemophilia were selected for the trial of factor VIII concentrated protein for the years, resulting amount of secreted factor VIII protein increases slowly [9].

Gralnick HR, Rick ME (1983) uses Danazol for treatment of hemophilia B in patients, resulting 14 % level of factor IX increases in patients and observed rise in clotting factor activity within 6 days [10].

Rees DJ, Rizza CR,(1985) described the mutation in factor IX gene, different types of mutation involve in the hemophilia B. Point mutation was measure cause of hemophilia B, mutation in GT to TT sequence with in a junction of reading sequences[11].

Nugent DJ, Bray GL et al. (1986) observed the effect of Danazol on 7 patients of hemophilia B resulting fibrinogen decreases, no change in factor IX and finally danazol fails to increase the factor IX in patients [12].

Lezoualc'h F. Boussif et al. (1995) first time described the role of polyethylenimine in the process of transfection in invitro and invivo. During their test, uses plasmid DNA for the gene transfer in cell, negative ions of polyethylenimine bind with negative charge of DNA and provide positive charges for infected to mammalian cell, resulting no any toxic effect arise during the gene transfer process[13].

Hoggan, M. D., Blacklow, N. R. et al (1996) described to the AAV virus and recognized AAV vector as a small contaminants during the preparations of adenovirus, genus of the parvoviruses[14].

Lusher JM (2000) observed the inhibitor antibody, inhibitor of factor IX in child deficient's with factor IX gene. Inactivation of inhibitor antibody was a major task for the scientist during year of 2000. It decreases the efficiency of concentrate AAV vector protein inside the cells. For the arrest bleeding episodes, uses increase doses of factor IX in inhibitor patients [15].

Xiao W (2002) explained the nuclear translocation of AAV from the cytoplasm to inside nucleus, in which AAV vector translocated followed by the endoplasmic reticulum and golgibodies [16].

Sonawane ND (2003) uses PEI-Max for the transfection of plasmid DNA into the mammalian cell line. Plasmid DNA integrate into the cell by endocytosis process and enter to nucleus resulting no any toxic effect occurs and show high efficiency of vector production [17].

JM Sommer, PH Smith et al (2003) performed the quantification of AAV virus particle. Uses direct methods for the quantification of genomic DNA and capsid proteins, compared the viral genome particle and titer of capsid by UV absorbance at 260 nm and quantative PCR (for viral genome particles) and ELISA for titre

value of capsids. Both viral genome and titre of capsid value was same using UV absorbance, qPCR and ELISA [18].

CS Manno, GF Pierce, et al (2006) transduced AAV vector containing FIX genes and studied their long term expression and immune response of host. Infused the FIX contain AAV vector through hepatic artery into patients and concluded that no any side effect occurs during the trail periods [19].

David Bruce and Tim JC Nokes [2008] uses concentrate of Prothrombin for the treatment of hemophilia B. Many management options were developed for the prevention of inhibitor antibody, each have some advantages and disadvantages both. Prothrombin complex concentrate used to prevent continuous loss of blood from the body of patients. Another method, in which alloantibody used for hemophilia B patients but they have no much effect on hemophilia B patients. Another management option was infusion of factor IX concentrate product [20].

Zincarelli C, Soltys S, (2008), analyzed the expression of AAV2 and AAV8 serotype in mice. AAV2 and 8 serotypes were developed for the target of liver cells, in which AAV2 and 8 serotypes released their genome inside the nucleus of target cells for the replication and transcription [21].

Simioni p. et al (2009) worked on the function of single point mutation (R338L) in FIX(FIX-TR338L-PADUA or FIX Padua has been shown to result in a seven time increase in activity of coagulant as compared to its wild type counterpart thus providing an opportunity to acquire therapeutic level FIX expression/activity at a lower vector dose. Lower level requirement would help lowering the immune response towards the vector and liver toxicity as well. FIX Padua was originally find in a patient with abnormality of blood coagulation and is assume to show activity of procoagulant due to an increase integration into a complex known as intrinsic tenase , a complex composed of activated factor VIII (VIIIa) and factor IX(IXa)[22].

Z. Wu, H. Yang, (2009) studied the genome size of AAV vector and their packaging limitation and examined the packed vector genome of AAV8 encoded plasmid derived vector. They use triple plasmid system to transfect HEK 293 cells, where

packaging of AAV vector occurred, and expression of gene. In their report after analysis concluded that genome of packed AAV vector never exceeded than 5.2 kilo bases. But many studied proved the size of packed genome of AAV can be exceed up to 8.2 kilo bases [23].

Robert T. Peters et al (2010) studied on the prolonged activity of FIX by uses a recombinant protein that contain a FIX molecule attached to the region of Fc of IgG, and administered intravenously in the normal rats, mice and also in mice with FIX deficient, resulting found to have extended the half life of FIX that is longer of 3 to 4 times than of rFIX [24].

Amit C. Nathwani et al (2011) worked on the persistent expression of therapeutic level of FIX in hemophilia B patients post injection with an AAV vector carrying functional FIX gene at a dose level 2 x10 ^{12} VG/kg [40] and followed to the FIX expression for more than 7 years and observed 2 to 11 % of FIX expression in all individuals that's were participate in their experiments and also concluded that vein infusion of rAAV responsible for the expression of FIX transgene to increase the phenotype of bleeding. While such clinical development has showed much promise, some limitation still exist. For example, first, about 30%-50% of individuals show immunity to the AAV, due to previous exposure of the wild type AAV, which will neutralize the efficacy of the vector to transfer the transgene to the targeted host cell. Second, the higher doses of the AAV vectors have resulted in increased level of liver enzyme levels that can be damage to the liver [25].

Amit C. Nathwani et al (2014) proofed the long term follow to ten patients with serious hemophilia B after the single high dose of AAV8 vector resulting the long term expression of factor IX and no toxic effect of AAV8 vector. In last decades investigators obtained excellent results in non-human (animals) models of hemophilia B they also obtained best result during phase 1 and phase 2 studies of somatic cell gene therapy applied in patients with fix deficiency. On other hand because of successful of two clinical studies on animal models, started clinical studies in individuals, that affected with hemophilia B, based upon the use of adeno associated virus vectors for gene transfer [26].

Mark Potter1 et al (2014) developed methods for the purification of AAV8 vector, in which SP column chromatography applied for purification of the AAV8 vector, and vector was characterized by SDS silver staining. Benzonase used for the degradation of genomic DNA of host cell in the supernatant of cells. Cells components and molecules were separated by the density gradient centrifugation methods and developed a simplified method for the purification of AAV8 vector [27].

Frauke Swieringa*, (2015), described the Rate-limiting functions of tenase complex, is activated by charged surfaces of platelets and other cell types. FIXa in tenase complex, in presence of Ca+, membrane phospholipids and co-factor FVIIIa, catalyses the conversion of procoagulant factor X into activated coagulation factor X (Xa) [28].

Joshua C Grieger, (2016) described the relationship between the HEK 293 and AAV, is a non-replicative and it is dependent upon helper viruses-adenovirus or herpes viruses for the replication in HEK 293 cells. Adeno associated virus have advantages for experimental design. Because it contain a DNA genome size of 4.7 kb, plus (+) and minus (-) strands that packaged into single particles, absence of pathogenicity and integrated in diving and cells without dividing. Transfection of the human HEK- 293 cells with AAV plasmid vectors are most useful because it express E1a/b gene of adenovirus 5, required for the replication of AAV vector) that were infected with adenovirus causes rescue and replication of infectious AAV genomes free of plasmid DNA sequences and production of adeno associated particles (infectious) [29].

Shelley A. Nass et al (2017) described the purification methods of AAV8 vector. AAV vector purified on the basis of affinity with ligand molecule by using affinity chromatography, sepharose affinity resin. Major disadvantage of the affinity chromatography is purified both empty and filled capsid protein. Separation of of both empty and filled capsid was performed by the used of Ion exchange chromatography, in which molecule separated out on the basis of charge. AAV8 vector purified by used of poros capture select affinity resin under different

purification steps- sample load, wash and elution. Purified sample applied to ultra centrifugal unit for the concentrate of purified protein [30].

George Lindsey (2017) studied on the previous and current progress of clinical trial of recombinant AAV mediated gene transfer for haemophilia B. Many sponsor worked on the treatment of hemophilia B, and focus on clinical trial. Some company completed phase1 and 2 and some ongoing phase 1. Mammalian cells and insect cell using for the production of rAAV containing FIX. Self complementary DNA sequences of FIX gene uses during ligation with plasmid DNA for the large scale production. Different companies using different dosages of rAAV for examination of their effect on the patient [31].

CHAPTER 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 LARGE SCALE PRODUCTION OF TRANSFECTED GRADE PLASMID DNA:

It includes- transformation of *E.coli stbl3* with plasmids, Miniprep and Maxiprep of plasmid DNA.

3.1.1 *E.coli stbl3* TRANSFORMATION:

Transfected grade plasmid DNA- pAAV-FIX^{pd}, pHelper and pAAV-R2C8, procured from Genscript Inc., and competent *E.coli stbl3* (One Shot™ Stbl3™, invitrogen, cat# C737303) procured from thermo fisher scientific. The competent cells were incubated with DNA in ice and treated at 42ºC in a water bath and further plunging them in ice.

MATERIALS AND METHOD:

EQUIPMENTS REQUIRED:

1. Incubator (orbitech)
2. Water bath-CORIO CD-B27 Heating Circulator with Open Bath (Julabo)
3. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
4. Sterile spreading device
5. Bio safety cabinet-300 series class II Type A2 (Thermo Fisher Scientific)

MATERIALS REQUIRED:

1. LB agar plate (with ampicillin antibiotic)
2. SOC media(invitrogen)
3. Competent cells -*E.coli stbl3*(invitrogen)

4. Transfected grade plasmid DNA- pAAV-FIX^{pd}, pHelper and pAAV-R2C8 (GenScript)
5. Ice

PROCEDURE:

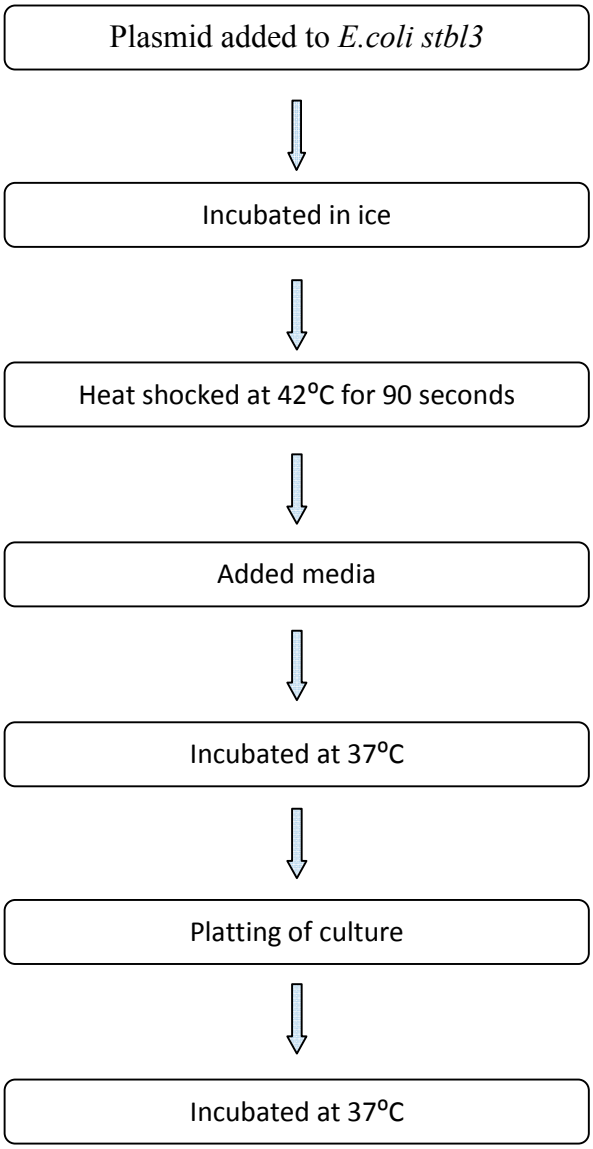


Figure 3.1: Steps of Transformation

1. Competent cell (*E.coli stbl3*) was removed from -80°C refrigerator (thermo Fisher scientific) and thawed in ice for 20 minute.

2. 20 µL *E.coli stbl3* cells mixed with 5 µL each plasmid DNA (100 ng/µL) separately into in a micro centrifuge tube and incubated for 30 minutes on ice.

3. Cells were treated at 42°C for 90 seconds in water bath.

4. Added 250 µL of without antibiotic containing SOC media to the mixture of plasmid DNA and cells then incubated for 45 min at 200 rpm.

5. After that 100µL of the transformation mixture was spread onto plate of luria burtani agar and incubated for 13 hours at 37°C.

3.1.2 MINIPREP OF PLASMID DNA:

Plasmid DNA isolated from transformed *E.coli stbl3* culture using Miniprep DNA purification kit, alkaline proteases used for the lysis of cells and eluted the plasmid DNA in buffer and precipitated by isopropanol.

MATERIAL AND METHODS:

EQUIPMENTS REQUIRED:

1. Bio safety cabinet-300 series class II Type A2 (Thermo Fisher Scientific)
2. Shaker incubator (Orbitech)
3. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
4. NanoDrop UV-Vis spectrophotometer (Thermo Scientific™)
5. Agarose gel electrophoresis chamber (*BIO-RAD*)
6. Power pac and ChemiDoc (*BIO-RAD*)

MATERIALS REQUIRED:

1. Miniprep DNA purification kit (Promega, Wizard®, Cat.# A1330)
2. Isolated colony of Transfected Bacteria (*E.coli stbl3*)
3. Autoclaved LB Broth

4. Ampicillin (Sigma Aldrich)
5. Agarose (MP Biomedical)
6. 1 kb DNA Ladder (NEB Inc.)

Table 3.1: Buffers for Plasmid DNA preparation (Miniprep):

S.No.	Buffers/ Solution	Components
1.	Lysis	NaOH (200 mM) SDS (1%)
2.	Resuspension	HCl- Tris (50mM) (pH 7.4) EDTA (15 mM) RNase A (110µg/ml)
3.	Neutralization	Guanidine hydrochloride (4.0M) potassium acetate (0.758M) glacial acetic acid (2.13M) pH 4.2
4.	TE(10X)	Tris-HCl (0.1M) (pH 7.4) EDTA (10mM)
5.	Wash buffer	Potassium acetate (162.8mM) Tris-HCl (22.7 mM) pH 7.5 EDTA (0.108 mM), pH 8.0

Table 3.2: Components of TAE Buffer:

S.No.	TAE buffers	Components	Amounts (grams or ml)
I.	50 X TAE (1000 ml)	Tris base	242.0g
		Acetic acid (glacial)	57.1 ml
		EDTA-50mM (pH 8.0)	100.0 ml
II.	1X TAE (1000 ml)	50 X TAE buffer	20.0 ml
		Purified water	980.0 ml

PROCEDURE:

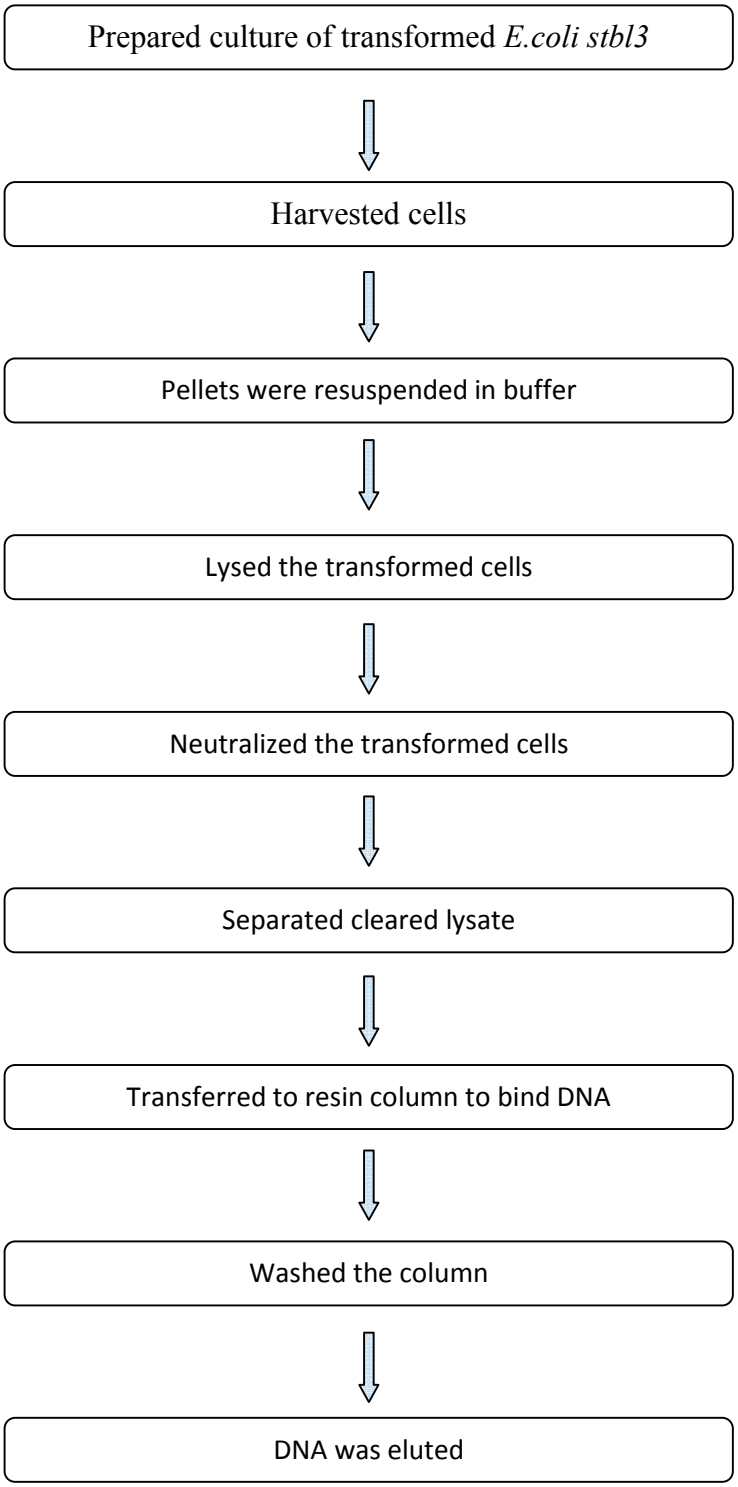


Figure 3.2: Steps of Plasmid DNA preparation (Mini prep)

CULTURE PREPARATION OF *E. coli stbl3*:

1. Cells of transformed *E. coli stbl3* were added into 7 ml of luria bertani media containing Ampicillin (1µL per ml of LB medium) and incubated at 37°C for overnight.

PREPARATION OF A CLARIFIED CELL LYSATE:

1. Cells of transformed *E. coli stbl3* were harvested at 13,000 x *g* for 7 minutes.

2. Supernatant discarded from the tube, pellet of cells resuspended in 250 µL resuspension solution and mixed it by vertaxing.

3. Lysis solution (250µL) mixed with resuspended cells and incubated at 25°C for 30 minutes.

4. Alkaline protease (10µL) was added and mixed and incubated for 8 minutes.

5. Cells were neutralized with 350µl of neutral buffer.

6. Lysate of bacteria was spinned at14000 x g for 14 minutes.

ISOLATION AND PURIFICATION:

1. Cleared lysate was passed through column inserted with collection tube and spinned at 15000 x g for 40 seconds.

2. Column was spinned after discarded the flow through and column washed with 750µL of wash solution buffer followed by spinned at 15000 x g for 1 minute at 25 °C.

3. Column was removed and discards the flow through, washed repeatly with 260 µL of wash solution buffer.

4. Centrifuged at 15000 x g for 3 minutes and transferred into centrifuge tube.

5. 100µL NFW transferred to column and spinned at 15000 x g for one minute at 4 °C. Column was discarded after centrifuged and stored tube containing eluted plasmid DNA at 4 °C.

YIELD DETERMINATION OF PLASMID DNA:

Quantification and accessed the purification of eluted DNA was done by NanoDrop UV-Vis spectrophotometer, in which 1 µL nuclease free water was used as blank and 1 µL of plasmid DNA used as sample for the quantification.

AGAROSE GEL ANALYSIS OF PLASMID DNA:

1. Agarose (0.8 %) gel was prepared with 1X TAE buffer and EtBr.

2. Gel tray along with comb putted in the electrophoresis tank and removed the comb.

3. 1kb DNA ladder and plasmid DNA was loaded into the gel as described in table 3.3 and run at 50 V and gel analyzed by using chemiDoc imaging system.

Table 3.3: Components for loading of plasmid DNA (Miniprep)

S.No.	Components (µL)	Well 1	Well 2	Well 3	Well 4
1.	1 X TAE buffer	4	4.5	4.75	4.72
2.	DNA Ladder	1	-	-	-
3.	6X loading Dye	1	1	1	1
4.	$pFIX^{Pd}$	-	0.5	-	-
5.	pHelper	-	-	0.25	-
6.	pR2C8	-	-	-	0.28
	Total	6	6	6	6

3.1.3 MAXIPREP OF PLASMID:

Cells were lysed under alkaline medium and plasmid DNA bind to resin. Buffers were used for the removal of unwanted components (impurities) except plasmid DNA. High salt buffer used for the elution of plasmid DNA. Isopropanol used for the desalting and to concentrate the plasmid DNA.

MATERIALS AND METHOD:

EQUIPMENTS REQUIRED:

1. Bio safety cabinet -300 series class II Type A2 (Thermo Fisher Scientific)
2. Shaker incubator (Orbitech)
3. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
4. Micro pipette(Thermo Scientific™)
5. NanoDrop UV-Vis spectrophotometer (Thermo Scientific™)
6. Agarose gel electrophoresis chamber (*BIO -RAD*)
7. Powerpac (*BIO -RAD*)
8. Chemi-Doc Touch Imaging System-model 1708370 (*BIO-RAD)*

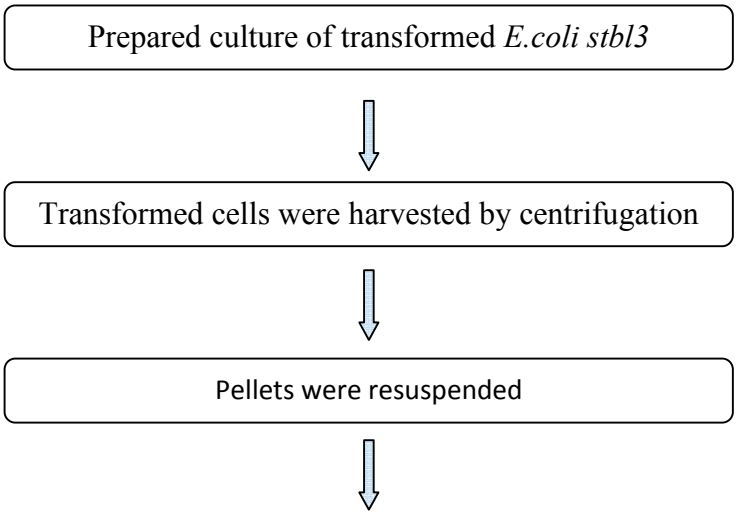
MATERIALS REQUIRED:

1. Endo free plasmid purification kit, Quigen®
2. LB agar plate of bacterial colonies
3. LB medium (Sigma Aldrich)
4. Ampicillin antibiotic (Sigma Aldrich)
5. Agarose (MP Biomedical)
6. 1X TAE buffer
7. Ethidium bromide
8. 1 kb DNA ladder and Loading dye (*BIO LAB*)

Table 3.4: **Buffers for plasmid DNA preparation (Maxiprep)**

S.No.	Buffers	Components
1.	Resuspension	Tris-Cl (0.050 M), pH 8.0 EDTA (0.010 M)
2.	Lysis	NaOH(200 mM), SDS (1%)
3.	Neutral	potassium acetate (3.2 M)
4.	Wash buffer	Acetate Potassium (1M), pH 4.9
5.	EQB	NaCl (755 mM) MOPS(0.050 M), pH 6.8 14 % isopropanol Triton x-1000 (0.15%)
6.	Elution	NaCl (1.6 M) 0.050M MOPS, pH 6.8 16% isopropanol
7.	Tris EDTA	Tris-Cl(0.010M), pH 7.5 EDTA(0.001M)

PROCEDURE:



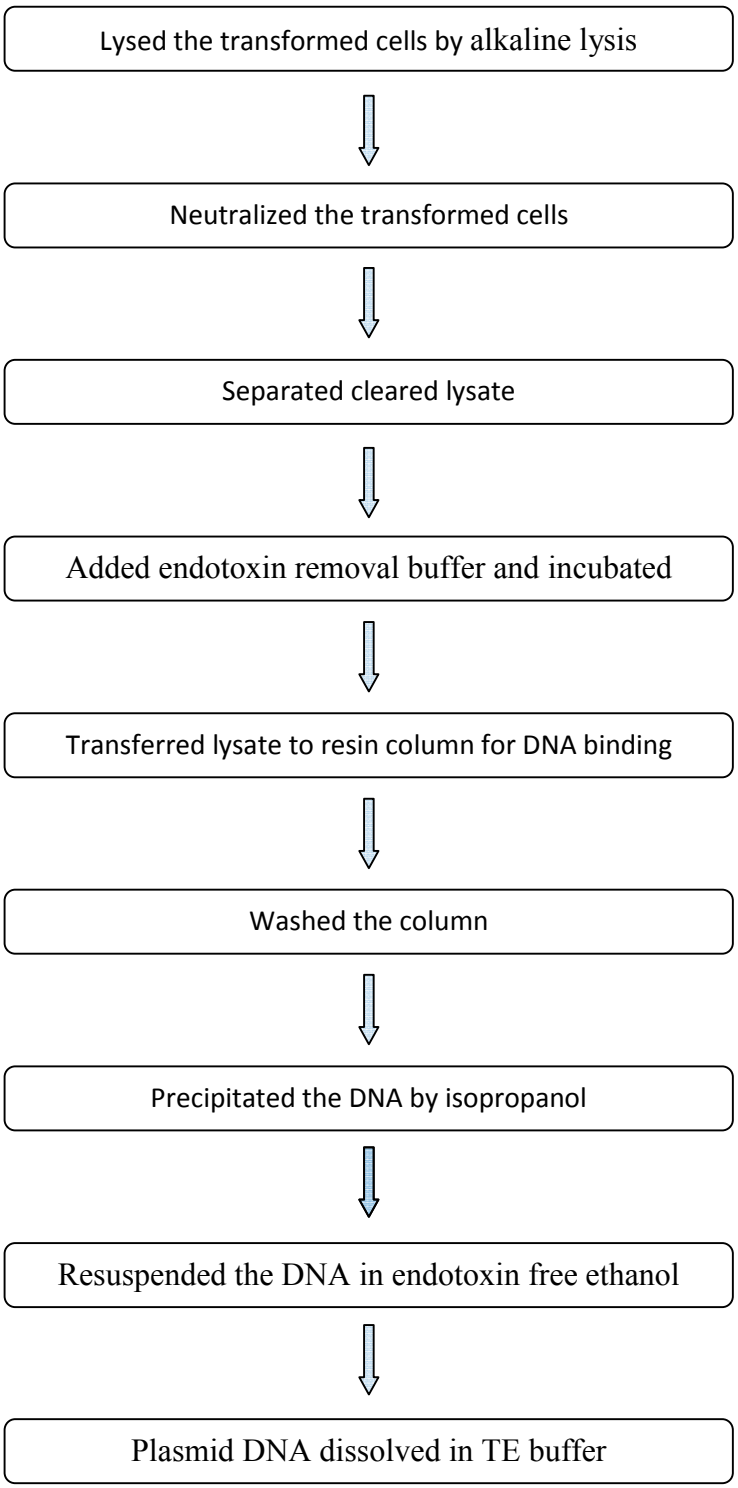


Figure 3.3: Steps of Plasmid DNA preparation (Maxiprep)

PREPARATION OF *E. coli stbl3*:

1. Cells of transformed *E. coli stbl3* were taken from plate of bacterial colonies and inoculate in a 7 ml of luria-bertani medium containing the ampicillin.

2. Incubated at 37°C at 200 rpm for 14 hours.

3. 250 µL of ampicillin was added into 250 ml of autoclaved LB medium in conical flask.

4. 500 µL of culture added to 250 ml of luria-bertani medium containing the ampicillin, incubate for 12 hours at in shaker incubator at 200 rpm.

PLASMID DNA PREPARATION:

1. Flasks of overnight grow culture (transformed *E. coli stbl3*) were removed from the incubator and transfer the culture into falcon tubes and centrifuged at 7000 x g for 20 minutes at 3°C.

2. Pellet of cells resuspended in 10 ml of resuspension buffer containing RNase A and Lyses Blue reagent after supernatant discarding.

3. Cells were lysed with 10 ml lysis buffer and incubated at 25°C.

4. The filter cartridges were prepared by capped the outlet of cartridges and kept in falcon tube.

5. 12 ml neutral buffer mixed with lysate and filtered.

6. Filtered lysate equilibrated with 2.5 ml of buffer equilibration followed by mixing and incubate for 35 minutes in ice.

7. In mean time filter column was equilibrated with 12 ml buffer equilibration.

8. The filtered lysate was transferred to equilibrated column, leave for some time for passed through the resin of column under the flow of gravity and washed the column with buffer wash.

9. After washed column, 20 ml buffer elution passed to the column and collected eluted DNA into a tube followed by precipitated with 12 ml isopropanol and spinned at 13000 rpm for 25 minutes at 3 °C.

10. DNA pellet dissolved in 5 ml 75% ethanol (endotoxin free) and centrifuged at 15,000 x *g* for 15 minutes at 3 °C.

11. DNA pellet dried at room temperature and dissolved with 500 µL TE buffer.

YIELD DETERMINATION OF PLASMID DNA:

Concentration of DNA was determined at wavelength of 260 nm by using 1 µL of TE buffer as a blank and 1 µL of DNA (concentration 1:10).

ELECTROPHORESIS OF PLASMID DNA:

Table 3.5: Components for loading of plasmid DNA (Maxiprep):

S.No.	Components (µL)	Well 1	Well 2	Well 3	Well 4
1.	1 X TAE buffer	4	1.91	1.62	3.6
2.	DNA Ladder	1	-	-	-
3.	6X loading Dye	1	1	1	1
4.	$pFIX^{Pd}$	-	3.09	-	-
5.	pHelper	-	-	1.38	-
6.	pR2C8	-	-	-	1.40
	Total	6	6	6	6

1. Agarose gel (0.8 %) prepared with 1 X TAE buffer and EtBr.

2. Gel tray along with comb putted in the tank of electrophoresis and removed comb from the gel.

3. 1 kb DNA ladder and plasmid DNA were loaded as described in the table 3.5 and run at 50V and analyzed under chemiDoc imaging System.

3.2 RESTRICTION DIGESTION of pFIX^{pd}, pHelper and pR2C8:

EQUIPMENTS REQUIRED:

1. Agarose gel electrophoresis unit *(BIO-RAD)*
2. Chemi-Doc Touch Imaging System-model 1708370 (*BIO-RAD)*
3. Dry water bath(Thermo Fischer Scientific)

MATERIALS REQUIRED:

1. HindIII-HF (NEB Inc.)
2. Sal-I-HF (NEB Inc.)
3. Cut smart buffer (NEB Inc.)
4. Nuclease free water (Promega Inc.)
5. 6x loading dye (NEB Inc.)
6. 1x DNA Ladder (NEB Inc.)
7. Plasmids (pFIX^{pd}, pHelper and pR2C8)

PROCEDURE:

1. 2 µg of each plasmid DNA were digested with restriction enzyme as mention in table 4.5 reaction mixture and incubated at 40°C for 60 minutes.
2. Reaction was inactivated at 80 °C for 20 minutes.
3. Digested plasmid DNA loaded to 0.8% agarose gel and run at 50 Volt.
4. Gel was analyzed under chemiDoc imaging System.

Table 3.6: **Reaction Mixture for Restriction Digestion:**

S.No.	Components	pFIX^{pd}	pHelper	pR2C8
1.	Nuclease free water (µL)	2.5	22.5	21.5
2.	10 Cut smart buffer(µL)	4	4	4
3.	Plasmid DNA(µL)	33	13	14
4.	HindIII-HF (µL)	0.5	0.5	-
5.	Sal-I -HF(µL)	-	-	0.5
	Total reaction volume (µL)	40	40	40

3.3 TRANSFECTION OF HEK-293 CELLS FOR VECTOR PRODUCTION:

Adherent HEK-293 (ATTC-1573) cells revived into complete medium contain DMEM (sigma aldrich) + FBS (gibco) + penicillin –streptomycin (sigma Aldrich) and subcultured 3 times in a week. Transfection of HEK-293 with transfected grade plasmid DNA uses PEI-Max (1 mg/ml, *polysciences, Inc.)*, a chemical reagent that help in introduction of plasmid DNA into cell and harvested cell using centrifuge and lyses the cell with liquid nitrogen and heat treatment at 37 °C.

3.3.1 HEK-293 CELL REVIVAL/VIAL THAW:

EQUIPMENTS REQUIRED:

1. Water bath-CORIO CD-B27 Heating Circulator with Open Bath (Julabo)
2. Bio safety cabinet (Thermo Fisher Scientific)
3. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
4. Phase contrast microscope (moticAE31E)
5. CO_2 incubator (Thermo Fisher Scientific)

MATERIAL REQUIRED:

1. HEK-293 cell (ATTC-1573)
2. Complete DMEM medium [DMEM (gibco) + FBS (gibco) + penicillin – streptomycin (sigma aldrich), 100: 10: 0.5].
3. T- flasks (coring, Sigma Aldrich)
4. Serological pipette(eppendrof)
5. Hemacytometer

Table 3.7: **Components of Complete DMEM medium:**

S.No.	Components	Volume (ml)
1.	DMEM	1000
2.	FBS (10% of DMEM)	100
3.	Penicillin-streptomycin (0.5% of DMEM and FBS)	5.5

Note: complete DMEM media was preapared and filtered through nalgene® vacuum filtration system (0.2µm pore size).

PROCEDURE:

1. 18 ml of DMEM complete added in a 75 cm^2 T-flask and kept it into CO_2 incubator.

2. Frozen cells (HEK-293) were thawed into water bath.

3. Vial was wiped with 70% ethanol outside the bio safety cabinet.

4. Vial contents HEK-293 cells mixed with 9.0 ml of complete DMEM and spinned at 125 x g for 420 seconds.

5. Supernatant discarded aseptically and pellet dissolved in complete DMEM.

6. T-flask containing complete medium was removed from 5% CO_2 incubator.

7. The resuspended cell pellet was added in the flask containing complete DMEM and incubated in CO_2 incubator.

3.3.2 SUB-CULUTRING OF HEK-293 CELLS:

EQUIPMENTS REQUIRED:

1. Water bath- CORIO CD-B27 Heating Circulator with Open Bath (Julabo)
2. Bio-safety cabinet -300 series class II Type A2 (Thermo Fisher Scientific)
3. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
4. Phase contrast microscope (moticAE31E)
5. Incubator- Large-Capacity Reach -In CO_2 (Thermo Fisher Scientific)
6. Vi-CELL XR (Beckman Coulter)

MATERIALS REQUIRED:

1. Culture of HEK-293 cell
2. Complete DMEM medium [DMEM(gibco)+ FBS(gibco) + penicillin – streptomycin(gibco), 100 : 10 : 0.5]
3. TrypLE™ express enzyme (gibco)
4. Tissue-culture treated flasks (coring, sigma aldrich)
5. Serological pipette (Eppendrof)
6. DPBS buffer (autoclaved, filtered, pH 7.2)

Table 3.8: Composition of 1X DPBS (pH 7.2)

S.No	Components	Amounts
I.	Sodium chloride	8.0g
II.	Potassium chloride	0.2 g
III.	Phosphate disodium	1.44 g
IV.	Phosphate monosodiun	0.29 g
V.	Purified water	Upto 1000 ml

Note: Four components were dissolved in purified water and filter through 0.2 μm filter membrane under vacuum pressure, measured pH and autoclaved.

PROCEDURE:

1. Flask of HEK-293 cells was removed from 5% CO2 incubator to observe their confluency by phase contrast microscope and discarded whole growth medium from the flask.

2. Cells were washed with 15ml of DPBS and discarded DPBS from flask after washing the cells.

3. 1 ml of trypsin was added in flask and spreading it at the surface of adherent cells by swirling the flask, then kept back into CO2 incubator for 3 minutes.

4. Trypsinizes flask was removed from CO_2 incubator and observed detached cellsthe 8 ml complete DMEM added to flask and pipetted to remove clumping of cells.

5. Aliquot 0.5 ml of cells was taken from the flask for the cell counting through hemacytometer (mixed cells and trypsin in 1:2 ratios and loaded in hemacytometer and observed to cell under phase contrast microscope), remaining cells were spinned at 200 x g for 5 minute.

6. Pellet of HEK 293 resuspended in complete DMEM then added in the flask containing fresh complete DMEM and incubated in CO_2 incubator.

3.3.3 TRASFECTION OF HEK-293:

MATERIALS REQUIRED:

1. Subcultured cells of HEK-293 (AATC Inc)
2. TryLE express (gibco, Lot#1868752)
3. OPTI-MEM media (1X), (gibco, lot#1929953)

4. pAAV-FIX^{pd}
5. pHelper
6. pAAV-R2C8

- (Genscript)

7. PEI-Max reagent (1 mg/ml, *polysciences, Inc.)*
8. Complete Growth Medium [DMEM (sigma ,Lot#RNBG8075) + FBS (gibco, Lot# 2039224) + Penicillin–Streptomycin (Sigma#P0781, Lot# 058M4848V)]

PROCEDURE:

1. Day 1, Seed of HEK-293 packaging cells at 10 x10^6 cells per flask were prepared in complete DMEM growth medium in 175 cm^2 T-flask and incubated in CO_2 incubator for 20 hours.

2. Day 2, media was aspirated, and added 30 ml fresh complete DMEM containing FBS and penicillin –streptomycin antibiotics and incubated in CO_2 incubator for 3 hours.

3. Transfection mixture was prepared as mention in table 3.9 (A) and (B).

4. Mixture was incubated at room temperature in bio safety cabinet for 20 minutes.

5. 597.36 µL of transfection mixture was added into the each T-flask containing HEK-293 packaging cells and incubated for 18 hours.

6. Day 3, all media was removed from the flasks and added equal volume (30ml) of fresh prepared complete DMEM in each flask and incubated for 48 hours (Day 4, only cells were monitored for their confluency).

7. Day 5, transfected HEK-293 cells were harvested.

Table 3.9: **Components for Transfection mixture:**

(A)

Plasmids	Size (kb)	Amount of DNA for 20 flask	Concentration of DNA(µg/µL)	Required volume (µL) =Amount of DNA/ concentration
pAAV-FIX^{Pd}	5.25	105	0.646	162.53
pAAV-Helper	12.30	246	1.444	170.36
pAAV-R2C8	7.34	146.8	1.426	102.94
Total	24.89	497.8		453.83

(B)

Components for transfection	Volume (µL)
DNA	453.83
Opti-MEM	10,000
PEI-max	1493.4
Total (for 20 flask)	11,947.23
For 1 flask	597.36

3.3.4. HARVESTING OF TRANSFECTED HEK-293 FOR PROTEIN EXPRESSION:

1. Flasks containing transfected cells were taken from 5% CO_2 incubator and removed all media content from flask and washed with PBS buffer.

2. 2 ml of TryLE express was added in each flask and spread it on the surface of adherent cells and kept again in the CO_2 incubator for 3 minute.

3. Flasks were removed from incubator and tapping on the base for the detachment of cells. Cells were observed under phase contrast microscope.

4. 8 ml DMEM complete added and gentle pipetting to cells for removal of clumping of cells and spinned at 3000rpm for 13 minutes.

5. Supernatant removed from tube and washed the pellet with 25 ml of DPBS buffer followed by spinned at 3000 rpm for 13 minutes.

6. Supernatant removed from tube and invert tube on the lint free paper for the removal of all the content of DPBS and pellet stored for lysis.

3.3.5. LYSIS OF TRANSFECTED HEK-293 FOR THE EXPRESSION OF PROTEIN (FREEZE /THAW METHOD):

1. Pellet of transfected HEK-293 cells were dissolved in 40 ml (2ml/Flask) of autoclaved DPBS and added EDTA free protease inhibitor (one tablet/ 7ml of DPBS) (cOmplete^{TM}, Sigma Aldrich).

2. Cells were incubated in liquid nitrogen, until the frozen of the cell.

3. After frozen of cells, cells were thawed in water bath until the removal of frozen of the cell.

4. Step 2 and 3 were repeated and spinned at 3000 rpm for 13 minute at 4 ^{o}C.

5. 8 µL of nuclease added to the supernatant and incubated for 1 hour in water bath (at 37^{o}C).

3.4 AAV8 VECTOR PURIFICATION :

3.4.1 CHROMATOGRAPHY -AFFINITY:

EQUIPMENTS REQUIRED:

1. Akta Pure150 (GE Healthcare Life Sciences)
2. Tricorn 5/200 column (GE Healthcare Life Sciences)
3. Magnetic stirrer(Eppendorf)
4. Weighing balance(Sartorius)
5. pH meter (Lab India)
6. Vacuum /pressure pump(PALL Corporation)
7. Bottle top filtration unit(Thermo Fisher Scientific)

MATERIALS REQUIRED:

1. POROS Capture Select AAV8 Affinity Resin (Thermo Fisher Scientific)
2. Buffer equilibration –buffer phosphate (50mM)+ 250 mM NaCl, (pH 7)
3. Buffer wash - buffer phosphate (50mM) + 1M NaCl, (pH 6.9)
4. Elution buffer – 100mM citric acid (pH 2.2) and Trisodium citrate 50 mM (pH 3.3
5. Filter paper (0.8, 0.4 and 0.2µm)-Merck Inc.

Table 3.10: 2M Tris (Neutralization buffer) pH 7.0

S.No	Components	Amounts
1.	Tris base	60.57 gm
2.	Purified water	Upto 250 ml

Note: Tris base was dissolved in purified water and make up volume 250 ml and maintained pH 7.0 by the addition of 1M HCl.

Table 3.11: Buffers for purification of AAV8 vector:

S.No.	Buffers/ Solution	Components	Amounts (Grams or ml)
1.	50mM phosphate buffer + 250mM NaCl pH 7.0 (volume 2000 ml)	Sodium phosphate monobasic monohydrate	3.58 g
		Sodium phosphate dibasic anhydrous	10.56 g
		Sodium chloride	29.2 g
		Purified water	Upto 2000 ml
2.	50mM phosphate buffer + 1M NaCl pH 6.9 (volume 2000 ml)	Sodium phosphate monobasic seven hydrate	3.58 g
		Sodium phosphate dibasic anhydrous	10.56 g
		Sodium chloride	117 g
		Purified water	Upto 2000 ml
3.	50mM Trisodium citrate (pH 3.3) volume 1000ml	Trisodium citrate	14.7 g
		Purified water	Upto 1000 ml
4.	100mM of citric acid (pH 2.2) volume 1000ml	Citric acid monohydrate	21.014 g
		Purified water	Upto 1000 ml

Note: All components of buffer dissolved in purified water and makeup the final volume and filtered with 0.2 µm filter paper under vacuum pressure.

METHOD:

Purification of AAV8 vector was performed by using affinity Chromatography, column (Tricorn 5/200, GE Healthcare Life Sciences) filled with POROS Capture Select AAV8 Affinity Resin (Thermo Fisher) along with Akta Pure150 (GE Healthcare Life Sciences). Lysate protein Sample was pass through column at flow rate 1.3 ml/min, with 0.5 MPa of column pressure, and the column wash 50mM phosphate buffer + 250 mM NaCl (pH 7), 50mM phosphate buffer + 1 mM NaCl

(pH 6.9) and finally elution of protein was performed with Trisodium citrate 50 mM (pH 3.3) and 100mM of citrate buffer (pH 2.2).

COLUMN PACKAGING:

1. 10 ml of POROS capture resin was transfer in a new tube.

2. 10 ml of purified water mixed properly and kept at 25 ºC for gravity settling of resin. Supernatant was discarded and again repeats steps 2 two times.

3. Supernatant was discarded and resin filled into the Tricorn 5/200 empty column.

4. Column was qualified by the passing of 0.1M sodium chloride to the column (flow rate of 1ml/min).

Table 3.12: Parameters of affinity chromatography:

1.	Column Resin volume	4 ml
2.	Column Flow rate	1ml/min
3.	Column pressure	0.5 MPa
4.	Absorbance (UV)	280nm

Table 3.13: Phases of Affinity Purification of AAV8 vector:

S.No.	Phases	Volume (ml)
1.	Water for injection	20
2.	Equilibration	20
3.	Sample load	40
4.	Post load Wash 1(Low salt)	20
5.	Post load Wash 2(High salt)	20
6.	Equilibration wash	20
7.	Elution	40

PURIFICATION PROCEDURE:

1. Lysate protein sample was filtered with 0.8, 0.4 and 0.2 micrometer filter paper under vacuum pressure pump.

2. Column was equilibrated with 20 ml of both equilibration buffer- Phosphate buffer (50mM) + 250mM NaCl, 50mM Phosphate buffer + 1M NaCl with a flow rate of 1mL/min.

3. Lysate sample was loaded to the column (flow rate of 1ml/min).

4. Column was washed with 50mM Phosphate buffer + 250mM Nacl buffer, 50mM Phosphate buffer + 1M NaCl and again with 50mM Phosphate buffer + 250mM NaCl after sample load.

5. Protein was eluted with 50mM trisodium citrate, pH 3.3 and 100mM citric acid, pH 2.2 by passing through the column.

6. Eluted protein was neutralized immediately with 2M Tris buffer and maintained the pH 7.0 of eluted protein.

3.4.2 ULTRAFILTRATION/ CONCENCTARTE OF ELUTED PROTEIN:

MATERIAL AND EQUIPMENTS:

1. Centrifuge (Sorvall™ ST 16 Thermo Scientific™)
2. Centrifugal Filter -30kDa (Amicon® Ultra-4)
3. DPBS buffer

PROCEDURE:

1. Eluted sample was loaded in filter device of centrifugal filter.

2. Spinned at 13,000 rpm for 20 minutes.
3. Filtered permeate discarded from the centrifuge tube.
4. Added DPBS buffer to filter device and spinned at 11,000 rpm for 15 min.
5. Protein sample collected from filter device for the analysis.

3.5 CHARECTERIZATION OF AAV8 VECTOR

3.5.1 SDS-PAGE:

Concentrated protein was reduced by sample buffer laemmli and DTT followed by heat treatment at 90 °C for 12 minutes. Reduced concentrated Proteins were differentiated by SDS-PAGE (4-15%) and stained with silver staining kits followed the manufacturer's protocol and bands were visualized under chemiDoc.

EQUIPMENTS REQUIRED:

1. SDS-PAGE assembly (Mini-PROTEAN®, *BIO-RAD*)
2. Ultra rocker (*BIO-RAD*)
3. Chemi-Doc Touch Imaging System-model 1708370 (*BIO-RAD)*

MATERIALS and REAGENTS REQUIRED:

1. SDS-PAGE precast gel
2. 1 X Tris/glycin running buffer
3. 250kDa protein ladder
4. Sample buffer laemmli and DTT (*BIO-RAD*)
5. Titriplex sodium EDTA
6. Sodium Carbonate
7. Sodium Thiosulphate
8. Silver Nitrate
9. Methanol
10. Acetic acid

Table 3.14: Components of SDS running buffer

S.No.	Running buffers	Components	Amounts (grams or mL)
1.	10 X running buffer (1000 ml)	Tris base	30.0 g
		glycin	144.0g
		SDS	10 g
2.	1X running buffer (1000 ml)	10 X running buffer	100 ml
		Purified water	900 ml

Table 3.15: List of Silver staining reagents and preparation:

S. No.	Reagents	Preparation
1.	Solution- Fixing	50 ml of methanol + 10 ml of acetic acid + 40 ml of filtered Purified water
2.	5% methanol	5 ml methanol + 95 ml of filtered Purified water
3.	0.02% Sodium Thiosulphate	Dissolved 20 mg sodium thiosulphate GR in required amount of filtered Purified water and makeup the volume upto 100 ml
4.	0.2% Silver Nitrate(staining solution)	Dissolved 200mg Silver Nitrate GR in required amount of filtered Purified water and make up the volume upto 100 ml
5.	3% Sodium Carbonate(Developing Solution)	Dissolved 3 gram Sodium Carbonate in 80 ml filtered Purified water. Added 50µL of solution of formaldehyde and 2 ml of Sodium thiosulphate (0.02%) and maked up the volume to 100ml
6.	1.4% Sodium EDTA (Stop solution)	Dissolved 1.4 gram Titriplex Sodium EDTA in required amount of filtered Purified water and make up the volume to 100ml

PROCEDURE:

A. SAMPLE PREPARATION:

1. DTT (Dithiothreitol) was added in 4X Laemmli sample buffer to a final concentration of 50 mM.

2. 100 µL sample buffer containing SDS and DTT was added into 100 µL of each concentrated protein (elute 1 and elute 2) and incubated at 95 °C for 10 minutes.

3. Reduced concentrated protein was loaded into wells of 4-15% Precast gel and run at 100 Volt power.

B. SILVER STAINING:

1. After completion of run time, gel was taken out and put in the fixing solution (100 ml) for 30 minutes on the gel rocker.

2. Fixing solution was discarded and added 100 ml of 5% methanol solution and kept on gel rocker for 15 minutes.

3. 5% methanol solution was discarded and given two washes with filtered Purified water for 5 minutes each on a gel rocker.

4. Discarded the water and added 100 ml of 0.02% sodium thiosulphate and incubate at gel rocker for 2 minutes.

5. Sodium thiosulphate was discarded and washed twice the gel with purified water for 1 minute.

6. Gel was incubated with 100 ml of 0.2% Silver nitrate for 25 minute gel rocker.

7. After completion of incubation Silver nitrate solution was discarded and washed two times the gels with purified water of 1 minute each followed by addition of the developing solution (Sodium Carbonate + Formaldehyde solution + Sodium thiosulphate) till the bands of interest are well developed.

8. Discarded the developing solution and added 100 ml of stop solution (1.4 %Sodium EDTA).

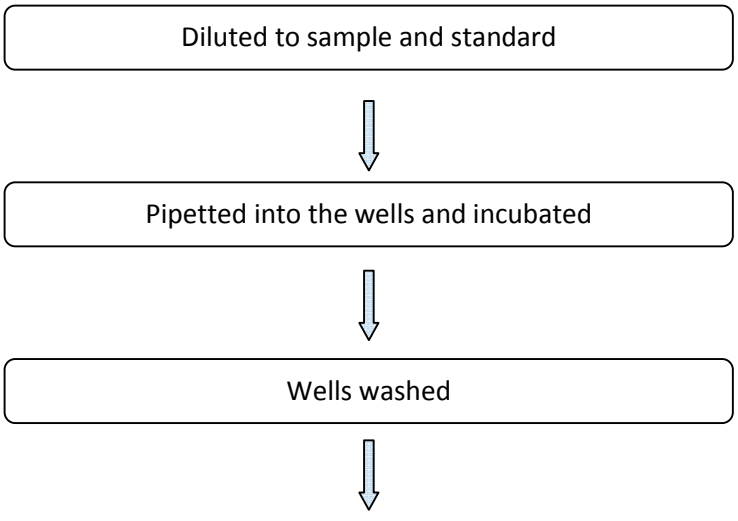
3.5.2 TITRATION OF AAV8 VECTOR:

Titration of concentrated protein of rAAV8 vector was determined by use of AAV8 Titration ELISA kit, procures from progen Pvt Inc., and followed the manufacture's protocol.

MATERIALS REQUIRED:

1. AAV 8 ELISA Titration kit, (PROGEN, Lot#170007)
2. Multi channel micropipette
3. Sterile pipette tips
4. Distilled water
5. Vials for specimen dilutions

PROCEDURE:



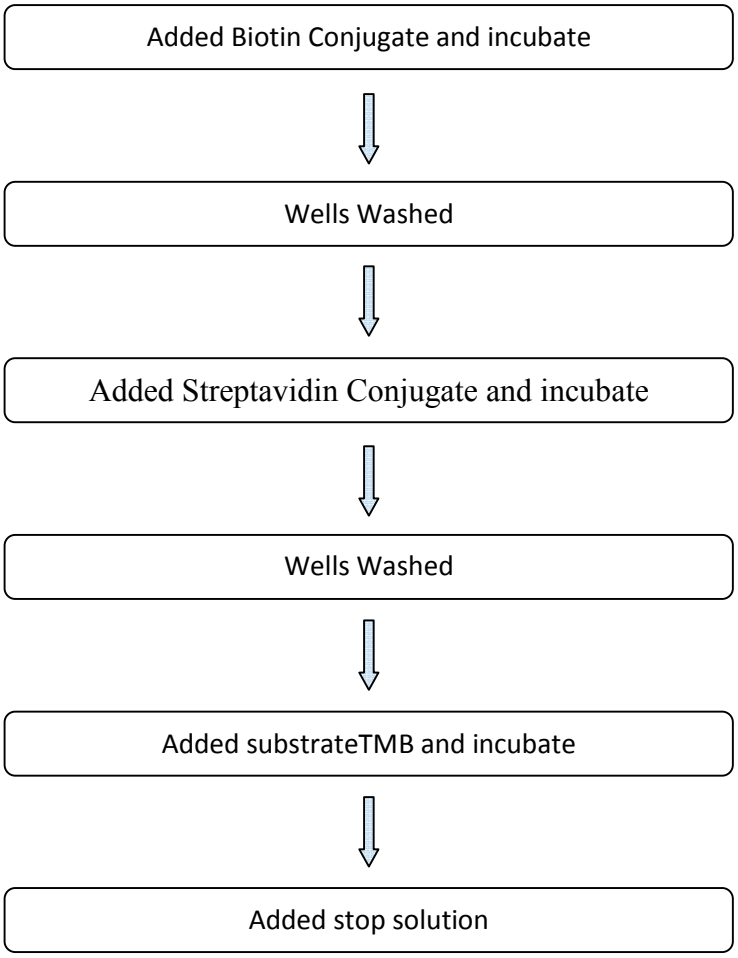


Figure 3.4: Steps of ELISA

Table 3.16: Template for ELISA Assay:

	1	2	3
A	Blank	Input (1:1000)	Elute 2(1:10000)
B	Control 1:1	Input (1:1000)	Elute 2(1:10000)
C	1:2	Elute1 (1:10)	
D	1:4	Elute1 (1:100)	
E	1:8	Elute1 (1:1000)	
F	1:16	Elute 1 conc. (1:10000)	
G	1:32	Elute 2 (1:1000)	
H	1:64	Elute 2 (1:1000)	

1. 100 µL sample buffer (as a blank) was pipetted in the well of plate and 100 µL of serial dilutions of Standard and specimen into the wells of the plates and sealed with adhesion foil and at 37°C plate was incubated for 1 hour.

2. After completion of incubation, contents of well were discarded and again added 200 µL of buffer-wash to each well and incubated for 5 seconds, after 5 seconds of incubation discarded the contents of each well. Washing step was repeated two times.

3. 100 µL of biotin conjugate was added in each well.

4. Wells were sealed by adhesion foil and incubate at 37°C for 1 hour.

5. Again washed the wells as step 2.

6. 100 µL of streptavidin conjugate was added into wells and sealed with adhesion foil and at 37°C incubated the plate for 1 hour.

7. Washing steps were repeated again according to step 2.

8. 100 µL of TMB substrate was added in the wells and at 25°C incubated the plate for 15 minutes.

9. 100 µL stop reagents were mixed into wells to stopped colour reaction and measured the intensity of colour at 450 nm in an ELISA reader.

CHAPTER 4

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSIONS

4.1 LARGE SCALE PRODUCTION OF TRANSFECTED GRADE PLASMID DNA:

4.1.1 TRANSFORMATION:

Competent *E.coli stbl3* cells were procured from Invitrogen, for the transformation with three transfected grade plasmid DNA as- pAAV-FIX^{Pd}, pHelper and pAAV-R2C8. Procure the Miniprep kit from promega Inc. and Maxiprep kit from Quagen Inc for preparation of plasmid DNA were prepared followed by the manufacture's protocols.

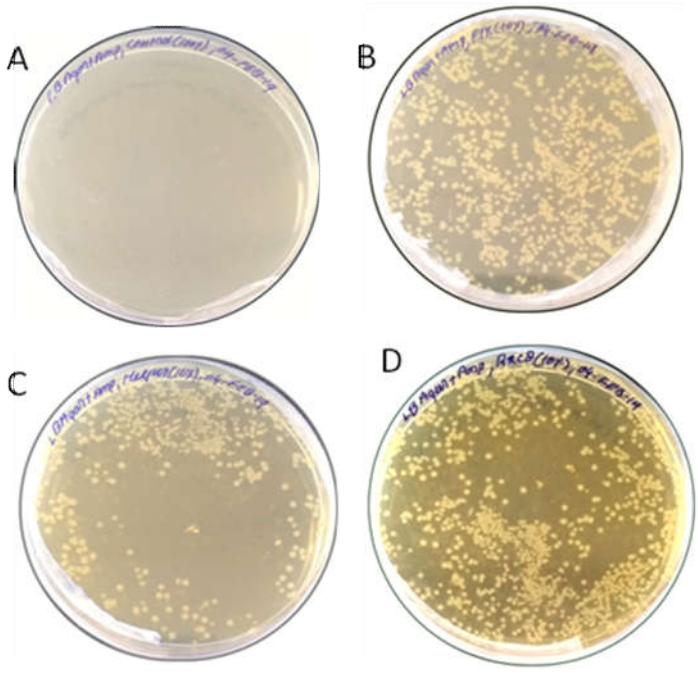


Figure 4.1: Transformed colonies of *E.coli stbl3.* A-control, B- pAAV-FIX^{pd}, C-pHelper and D- pAAV-R2C8

Table 4.1: Number of Transformed colonies of *E.coli stbl3*

Plates	Number of transformed colonies	Transformation efficiency (cfu/ µg)
A (control)	000	000
B (pAAV-FIX^{Pd})	895	4.47 x 10^5
C (pHelper)	322	4.81 x 10^5
D (pAAV-R2C8)	963	1.61 x 10^5

Transformation of *E.coli stbl3* is required for the production of three plasmids i.e. pAAV-FIX^{Pd}, pHelper and pAAV-R2C8. *E.coli stbl3* act as competent cell to take up the plasmid DNA from their surrounding environment during the heat shocked process, pore of membrane of *E.coli stbl3* open for a minute and DNA enter to inside the cells, and closed pore when cells put in the cool environment i.e. in ice. The transformed cells spreaded on the plates (separate for each plasmid DNA) and incubated at 37°C for the overnight, resulting a numbers of colonies of transformed *E.coli stbl3* were grow in each plates. After completion of incubation times plates were removed from the incubator and cells were counted manually. The transformation efficiency was calculated by following this formula as mention below-

Efficiency of Transformation = Number of colonies/ amount of DNA (ng)
X 1000ng/µg

3.1.2 PLASMID DNA PREPARATION:

Each plasmid DNA i.e. pAAV-FIX^{Pd}, pHelper and pAAV-R2C8 were prepared, followed by miniperp and maxiprep, in which culture of transformed *E.coli stbl3* cells of 5 ml for miniprep and 250 ml for maxiprep prepared. Cells were harvested, isolated and purified by manufacturer protocol as mention in method section

concentration and purity of plasmid DNA were determined by use of NanoDrop UV-VIS spectrophotometer as mention in table 4.2 and 4.3.

Table 4.2: Concentration of plasmid DNA (Miniprep)

Plasmid Vector Mini preps		
Plasmid	**Conc. (ng/µl)**	**A260/A280**
pAAV-FIX^{Pd}	337.3	1.91
pHelper	781.8	1.86
pAAV R2C8	705.7	1.86

Table 4.3: Concentration of plasmid DNA (Maxiprep)

Plasmid Vector Maxi preps		
Plasmid	**Conc. (ng/µl)**	**A260/A280**
pAAV-FIX^{Pd}	646.0	1.93
pHelper	1444.0	1.91
pAAV-R2C8	1426.0	1.90

Agarose gel electrophoresis of each plasmid DNA were performed for the size determination of plasmid DNA and also for to confirmed the purity of plasmid DNA. 0.8 % of agarose gel (prepared in 1X TAE buffer) was used for analysis. 1kb DNA ladder and palsmid DNA were mixed separetly with loading dye and loaded into the wells of Agarose gel and run at 50 V. After completion of agarose gel run, gel was observed under chemi doc imaging system (UV light). Three bands of each plasmid DNA were formed as-linear (top), open circular (middle) and supercoiled (lower).

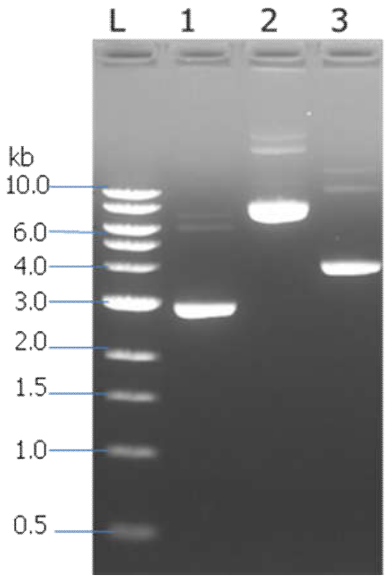


Figure 4.2: A- Gel of pAAV-FIX^{Pd}, pHelper and pAAV-R2C8 plasmid DNA (Mini prep)

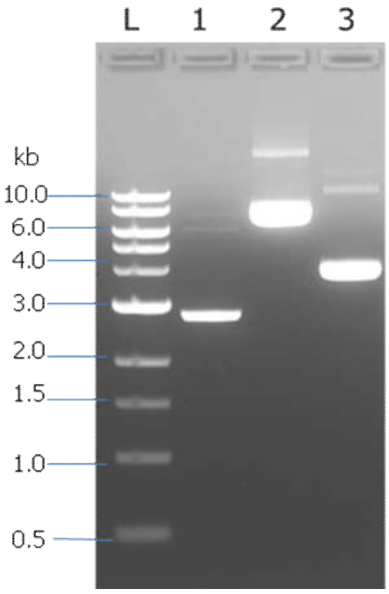


Figure 4.2: B-Gel of pAAV-FIX^{Pd}, pHelper and pAAV-R2C8 plasmid DNA (Maxi prep)

3.1.3 RESTRICTION DIGESTION of pAAV-FIX^{Pd}, pHelper and pAAV-R2C8:

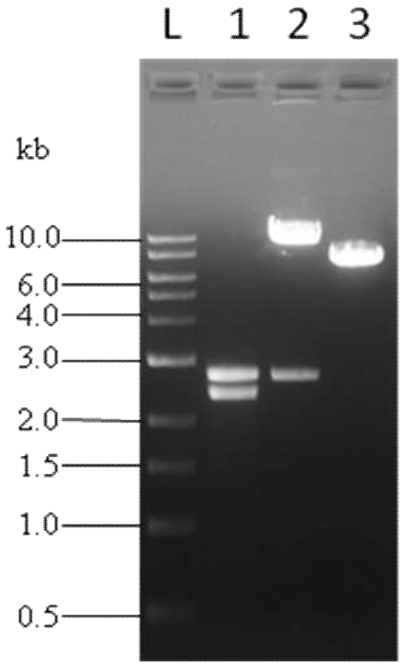


Figure 4.2: C- Gel of Digested pAAV-FIX^{Pd}, pHelper and pAAV-R2C8

Figure 4.2: Gel of pAAV-FIX^{Pd}, pHelper and pAAV-R2C8 plasmid DNA: Plasmid DNAs were isolated from *E. coli stbl3*. In both Fig. A (miniprep) and B (maxiprep) well indicates- L-1 kb ladder, 1- pAAV-FIX^{Pd}, 2-pHelper and 3-pAAV-R2C8. C – L- 1kB ladder, well 1, 2, and 3 are digested pFIX^{Pd}, pHelper and pR2C8 with Hind III-HF, Hind III-HF, and Sal-I-HF respectively.

Size of fragments of each gene- FIX, Helper and R2C8 were confirmed by the restriction digestion with enzymes. Restriction site of Hind III-HF presented in both plasmid DNA as well as in gene of interest so overall two restriction site of Hind III-HF was existed in each pAAV-FIX^{Pd} and pHelper plasmids. Resulting two bands one of back bone i.e.pUC19 and another one i.e. gene of interest were formed. But in case of pAAV-R2C8 only one restriction site of Sal-I-HF present resulting one band formed.

4.2 TRANSFECTION OF HEK-293 USING PEI- MAX:

Aim of this work was to develop pure rAAV8 with more titer using PEI transfectants for the transfection of HEK-293 cells. Initially, HEK293 cells were procured from AATC (American type culture collection), and grow in complete DMEM medium (DMEM+ FBS+ penicillin-streptomycin) as mention in procedure. The viability of cells was maintained for the high transfection efficiency and rAAV production and cells was observed regularly by use of phase contrast microscope. PEI Max has more efficiency of plasmid DNA delivery in HEK-293 cells. HEK-293 well tolerated to PEI-Max. PEI Max less expensive than other chemical transfectants and no clump formation of cells during transfection periods. Optimal ratio of plasmids and transfection reagents are major requirement in the process of rAAV production. The adherent HEK-293 cells grow in complete DMEM and seeded 10 x 10^6 /ml in 175 cm^2 T-flasks. 25µg DNA per flask subjected to PEI Max in a ratio of 1:3 and incubated for 20 minute at room temperature then transferred into Culture of HEK-293 cells. Many study show that 1:3 ratios of DNA and PEI Max are suitable for transfection and it help to the generation of genomes of vector [50]. Plasmid DNA diluted with reduced Serum Media.

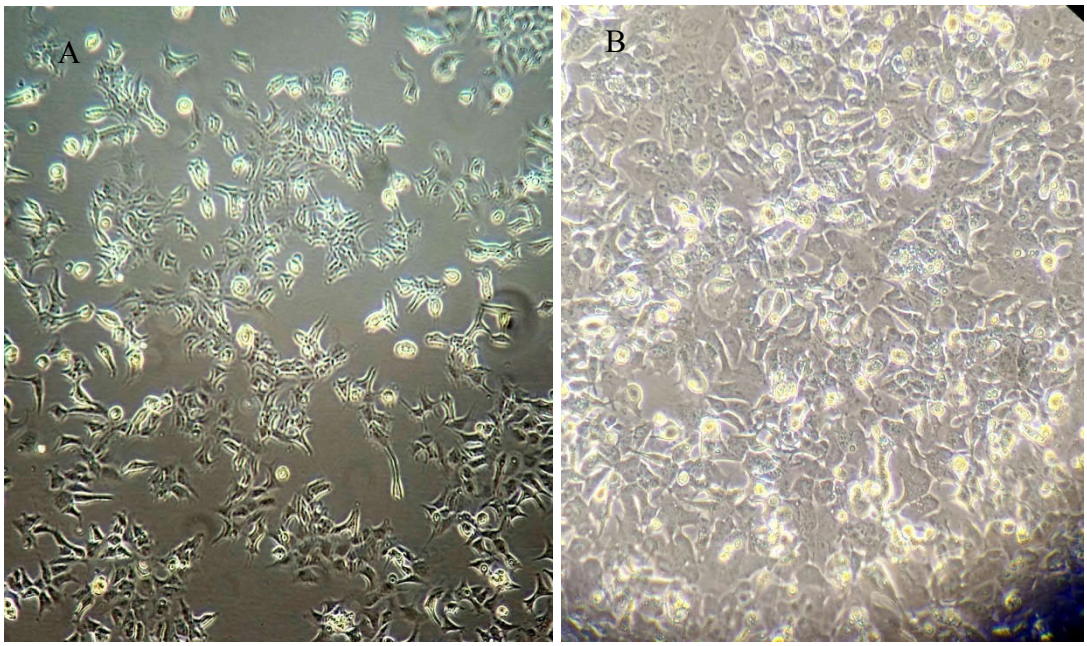
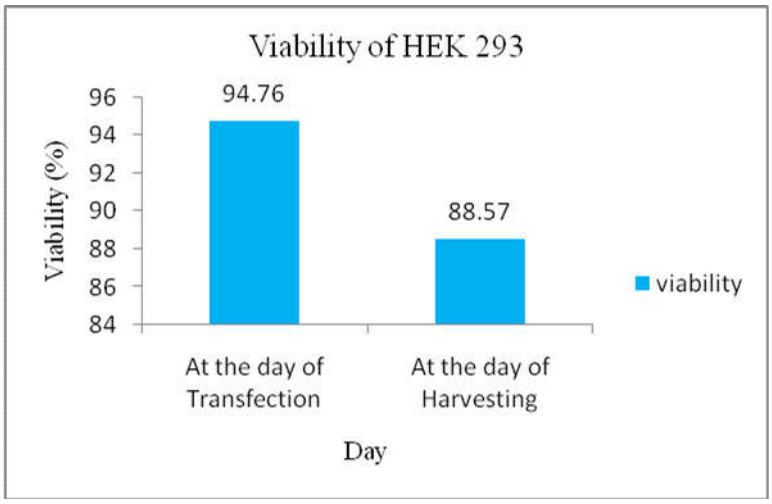


Figure 4.3: Morphology of HEK-293- (A) Pre-Transfection, (B)-Post-Transfection

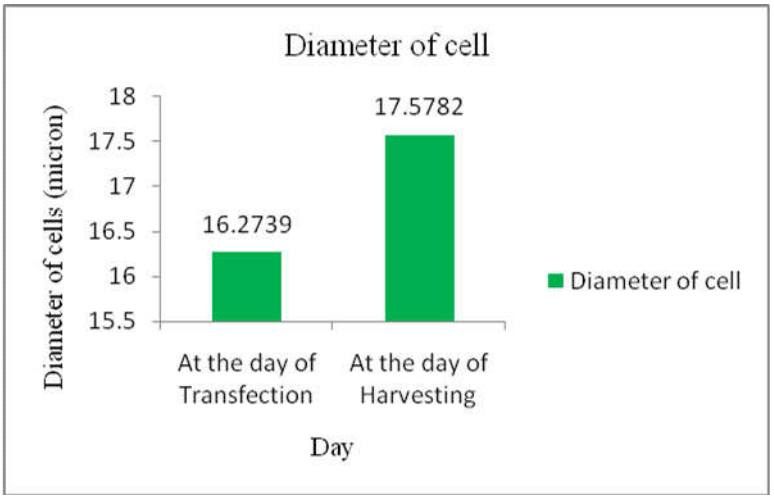
Table 4.4: Viability, Total cells of HEK 293 during Transfection:

S.No.	Days	Viability of cell (%)	Total cells (10^{6})	Diameter of cell (micron)
1.	At the day of Transfection	94.76	16.2739	16.2739
2.	At the day of Harvesting	88.57	17.5782	17.5782

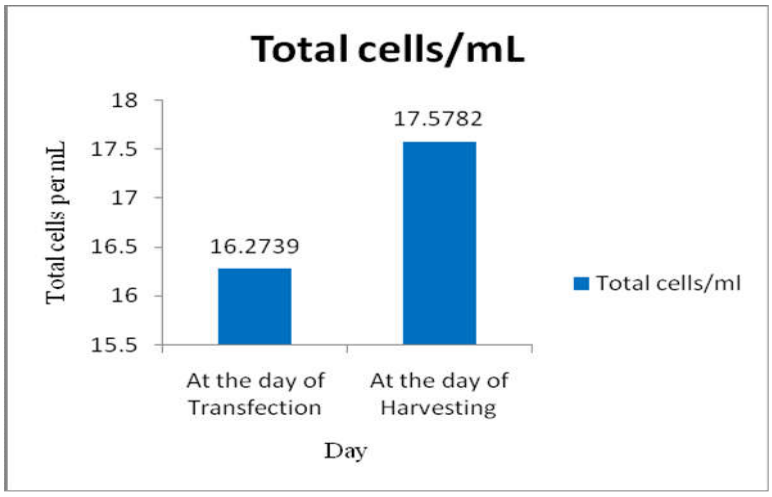
Viability of HEK 293 cells essential for the experiments work. HEK 293 cells viability was maintained by 5 % CO_2 at 37°C and media exchanged after 80% confluency. During transfection process confluency increased and due to this some cells was died under less surface area. Addition of plasmid DNA into the cells causes increased diameter of each HEK 293 cell. Total number of cells also increases during the transfection periods.



Graph 4.1(A): Viability of HEK293



Graph 4.1 (B): Diameter of HEK 293



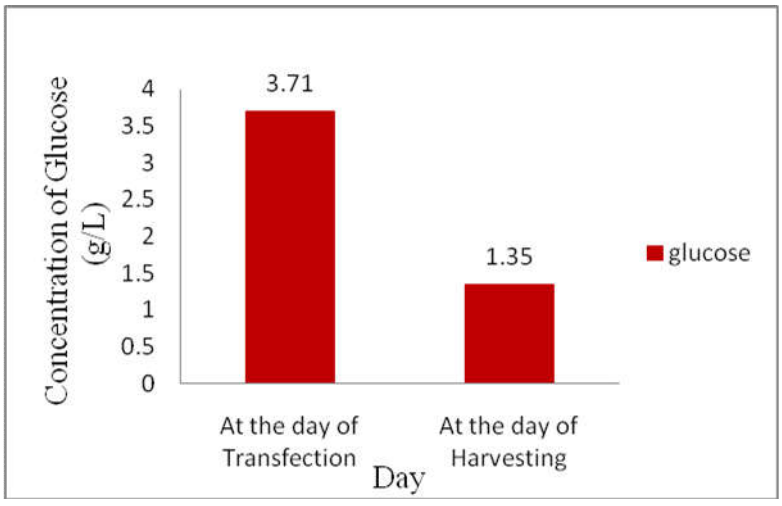
Graph 4.1(C): Total cells of HEK 293

Graph 4.4: HEK 293 Cell: (A) Viability of HEK-293 cells were decreases during the transfection process while (B) and (C) Diameter of the cells and total number of cells increases.

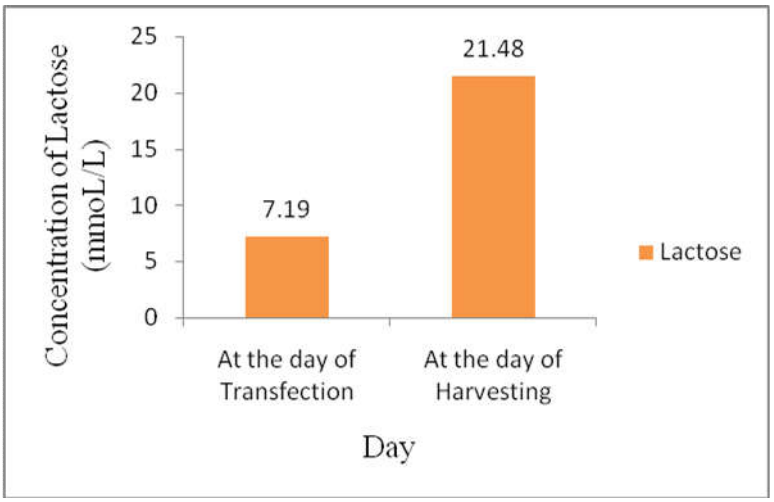
DMEM enriched medium contain carbohydrates, amino acids and vitamins. HEK-293 cells uses glucose as a carbon source from the DMEM medium and oxidises under the aerobic conditions and produces lactose molecule in the DMEM. When cells grown, concentration of glucose decreases from the DMEM medium. Growth rate of cells decreases due to lactose production. Concentration of lactose increases until glucose decreases in medium.

Table 4.5: Concentration of Glucose and Lactose during Transfection:

S. No.	Day	Concentration of Glucose (mg/L)	Concentration of Lactose(mmoL/L)
1.	At the day of Transfection	3.71	7.19
2.	At the day of Harvesting	1.35	21.48



Graph 4.2: A- Glucose Consumed by HEK-293



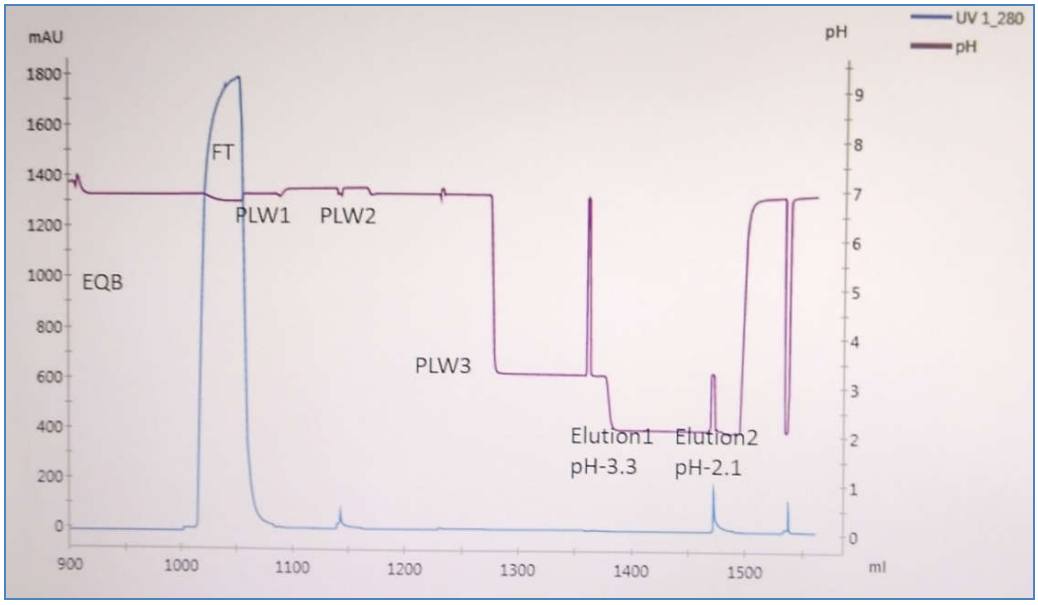
Graph 4.2: B- Lactose produced by HEK-293

Graph 4.2: The concentration of glucose and lactose was measured by use of Cedex Bio-analyzer (Roche Custom Biotech). A-at the day of transfection (before transfection) the concentration of glucose and lactose was 3.71 g/L and 7.19 mmol/L respectively, while at the day of harvesting of transfected HEK-293 cells was the concentration of glucose and lactose was 1.35g/L and 21.48 mmol/L respectively.

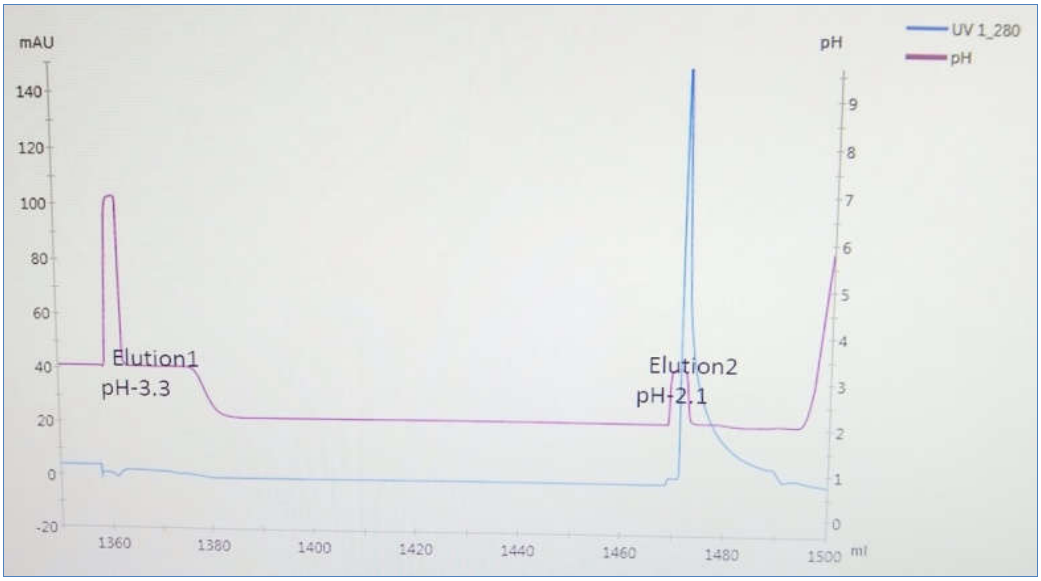
4.3 AAV 8 VECTOR PURIFICATION:

In this purification method "POROS AAV8 Capture Select" Affinity matrices, was used for the purification, it have yield improved recoveries of approximately 80% .In this proteins releases by lysed. HEK-293 cell passed through POROS AAV8 capture select affinity column and washed followed by elution of protein with specific buffer with different pH buffer. Akta pure 150 (unicorn 7.0.1 software) and Tricorn 5/200 column, was used during the purification of AAV8 Vector protein. Resin affinity chromatography column was equilibrated and sample passed flow through the column and due to presence of contaminants in sample absorption was increased. And absorption was down during the three post liquid wash of column by using wash buffer pH 7. Eluted the protein using the elution buffer 1 (Trisodium citrate pH 3.2) and elution buffer 2 (100 mM citric acid, pH 2.1) through the column.

Total input volume was 40 ml that passes through the column and after post load wash the elution volume was 25ml. Eluted volume was concentrated by use of 30 kDa centrifuge filter unit that removes protein of less than 30 kDa size. Buffer exchanged with DPBS buffer to maintain the pH of elute. 500 µL of final volume of concentrated protein of elute 1 and elute 2 were prepared by buffer exchange process.



Graph 4.3 (A)



Graph 4.3 (B)

Graph 4.3: Chromatogram of Affinity Chromatography- (A) and (B)-Resin affinity column chromatography profile of AAV8 vector purification. Blue line: OD_{280} (mAU), purple line: measured the pH.

Column resin was tagged with variable region of specific antibody that binds non-covalently with receptor of capsid of AAV8 vector and retain for some times in the column, remaining components of sample were washout by the low salt and high salt wash buffers. During the elution period, elution buffer (citric acid, low pH 2.3) passed through the column when citric acid interact with resin resulting created acidic environment surrounding, under low pH (high H^+ ions) a conformational changed in the binding of resin antibody and receptor of capsid, resulting under high H^+ ions bonds between resin antibody and capsid's receptor breakdown and capsids protein eluted out from the column.

4.4 SDS-PAGE ANALYSIS OF AAV8 CAPSID PROTEINS:

Purity of concentrated protein was analyzed by SDS-PAGE (4-15% precast gel procured from Bio-Rad) and silver staining, in which the concentrated purified protein was reduced by SDS and DTT, DTT breakdown the disulfide bond between proteins while SDS denatured to protein and provided negative charge on the protein molecules, at 95 ºC for 10 minutes, resulting capsid of AAV8 vector go to breakdown into three major capsid proteins are VP1, VP2, and VP3. For the confirmation of presence and purity of capsid protein in the sample, reduced concentrated proteins were loaded into the wells of SDS-PAGE where three capsid proteins moves down according to the their molecular weight in the presence of electric field. After completion of run, gel was washed with solution and stains with silver nitrate for the detection of bands of proteins in gel, silver nitrate bind with proteins and develop colour. Sizes of three capsid proteins were determined by 250 kDa dual colour marker. In lane 1, 2 and 3 bands were appears as blurry it means some contaminants available after purification. While the in lane 4, 5 and 6, three cleared bands of sizes- 87 kDa, 72kDa and 61 kDa of VP1, VP2 and VP3 respectively were formed, it means in elute 2 contains purify AAV8 vector greater than elute 1.

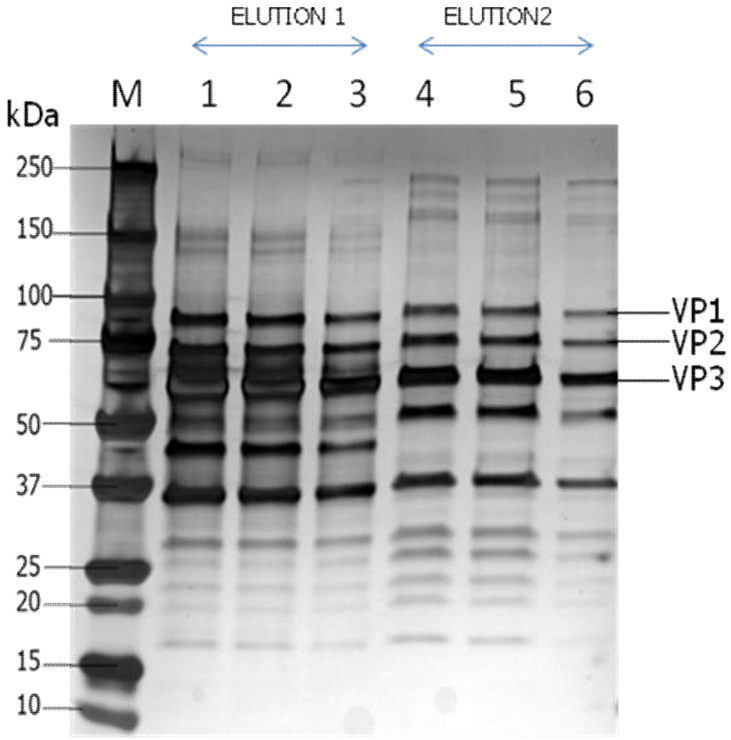
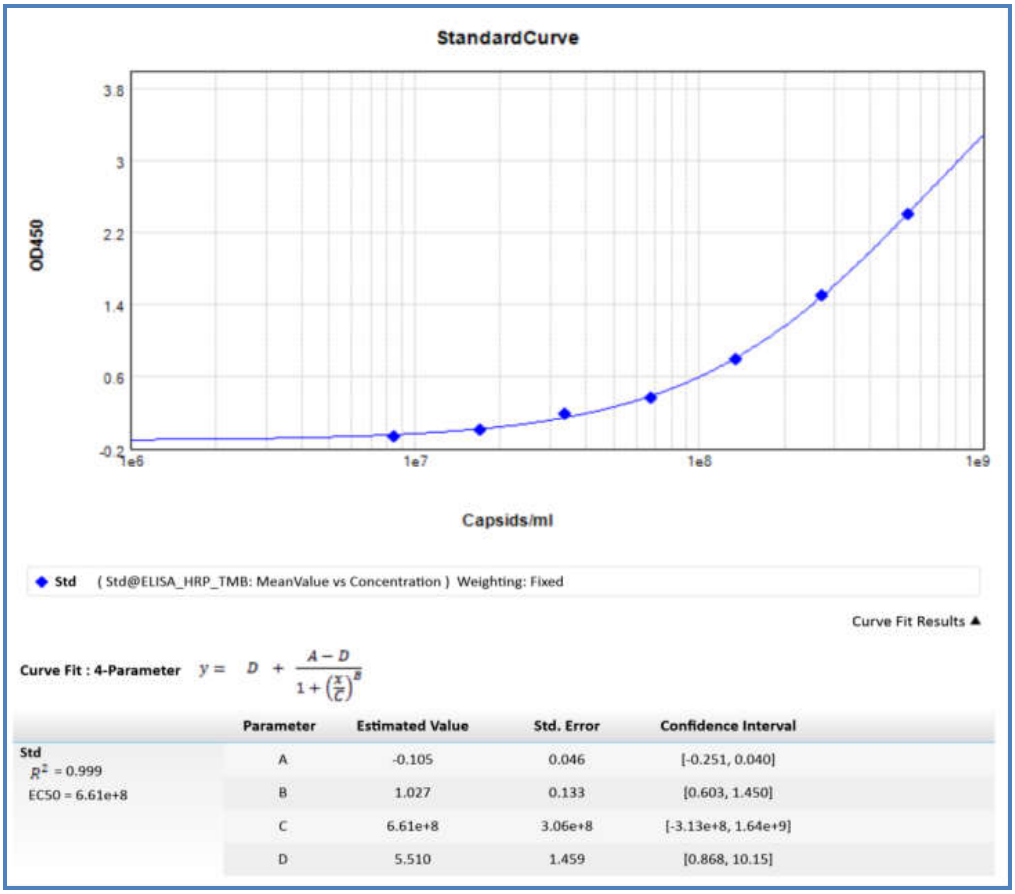


Figure 4.4: SDS-PAGE of AAV8 Capsid Proteins: M- 250 kDa Marker. Lane 1, 2 and 3 contain 40, 20, and 10 µL of elute 1 respectively. Lane 4, 5 and 6 contain 40, 20, and 10 µL of elute 2 respectively.

4.5 TITRATION OF AAV VECTOR PARTICLES:

Titration of AAV8 vector particle in the sample was determined by the use of Titration AAV8 ELISA kit (Progen). The different dilution of purified concentrated protein sample and unpurified protein sample were pepetting into the well of micro titre plate that was already coated with a monoclonal antibody specific for capture of AAV8 capsid, as mention in method. The quantity of AAV8 vector was determined by the use of 4 parameter standard curve, used for the higher complexity between antigen and antibody. The used ELISA was sandwich ELISA in which specific Monoclonal antibody of AAV8 already coated onto the wells of plates by the manufacturer. Standard AAV8 and samples was loaded into the separate wells of plates and incubated, during this periods monoclonal antibody binds with the AAV8 vector presents in the sample, the unwanted components were removed by the

washing two time with assay buffer. The anti-AAV8-biotin conjugate antibody binds with the AAV8 particle, and started sandwich process in which two antibodies binds to AAV8 particles from both side.



Graph 4.4: Standard curve of AAV8 vector

Table 4.6: Concentration of standard AAV8 vector

Sample	Well	Standard value Concentration units/ml	O.D.	Avg O.D.
1	B1	5.4E+08	2.407	2.407
2	C1	2.7E+08	1.513	1.513
3	D1	1.35E+08	0.792	0.792
4	E1	67500000	0.373	0.373
5	F1	33750000	0.19	0.19
6	G1	16875000	0.018	0.018
7	H1	8437500	-0.059	-0.059

Table 4.7: Concentration (Titre) of AAV8 vector:

Sample	O.D	Result	Mean Result	Adjusted Conc. (units/ml)
Elute 1 (1:10)	3.491	1.16E+09	1.16E+09	1.16E+10
Elute 1 (1:100)	3.491	1.16E+09	1.16E+09	1.16E+11
Elute 1 (1:1000)	3.491	1.16E+09	1.16E+09	1.16E+12
Elute 1 (1:10000)	3.491	1.16E+09	1.16E+09	1.16E+13
Elute 2 (1:1000)	3.491	1.16E+09	1.16E+09	1.16E+12
	3.491	1.16E+09		1.16E+12
Elute 2 (1:10000)	3.491	1.16E+09	1.16E+09	1.16E+13
	3.491	1.16E+09		1.16E+13
Elute 2 (1:100000)	3.384	1.07E+09	1.1E+09	1.07E+14
	3.445	1.12E+09		1.12E+14
Input (1:1000)	3.352	1.05E+09	1.02E+09	1.05E+12
	3.289	1E+09		1.00E+12

Streptavidin-HRP conjugate bind with the biotin strongly. When substrate TMB (tetramethylbenzidine) were added to each wells of plates resulting blue colour developed due to the reaction between enzyme HRP (horse radish peroxidise) and substrate TMB. Reaction was stopped by stop solution (160mM sulphuric acid), yellow colour developed after addition of stop solution. Intensity of colour measured at 450nm by ELISA reader. AAV8 particles were presented in input (pre-purification), concentrated elute 1 and elute 2 (post-purification) samples. The concentration of AAV8 vector in sample (input, pre-purification, $1:10^3$), was 1.05E+12 units/ml, while concentration of AAV8 vector (Elute2 concentrate, $1:10^4$) was 1.16E+13 units/ml as mention in table 4.7.

CONCLUSIONS:

In this study, HEK-293 cells transfected with three transfected grade plasmid DNA as pAAV-FIX^{pd}, pHelper and pAAV-R2C8 for the development of the AAV vector containing FIX gene for the treatment of Hemophilia B, a genetic disorder. Three plasmids were produce at large scale using transformation of *E.coli stbl3*. HEK-293 cells were revive, seeded and sub cultured in complete DMEM medium and transfected with three transfected grade plasmid DNA using PEI-Max as a transfected chemical reagent that allow integration of Plasmids DNA from outside to inside of the cell. The harvest transfected HEK-293 cells were lysed by freez/thaw for the releasing of AAV vector from inside to outside of cells. Protease inhibitor and nuclease were used for the degradation of protease enzyme and genomic DNA of HEK-293 cells. AAV8 Vector purified by using affinity chromatography and ultra filtration methods. Characterization of AAV8 was performed by using SDS-PAGE and sandwich ELISA. Purity (qualitative analysis) of purified AAV8 vector was confirmed by SDS-PAGE, three bands of purified AAV8 vector capsid protein VP1, VP2 and VP3 of 87 kDa,72 kDa and 61 kDa, were formed. The quantitative of purified AAV8 vectors particles were determined by ELISA, resulting 1.16E+13 units/ml of AAV8 vectors were presented in the purified concentrate protein sample.

FUTURE PERSPECTIVE:

The main objective of the work adeno associated viral vector based gene product development for the hemophilia B followed by large scale plasmid production, sub culturing and transfection of adherent HEK 293 cells for the vector production. Second objective to the purification of AAV8 vector by affinity chromatography and characterization of AAV8 vector containing FIX gene by SDS-PAGE and ELISA has been accomplished. Future prospective such as-

1. Western blots for the confirmation of presence of AAV8 vector in purified sample.
2. Gel exclusion chromatography for separation of proteins based on molecular weight.
3. Ion exchange chromatography to the separation of empty and filled (with gene of interest) capsid of AAV8 vector
4. Quantative analysis of genomic DNA of HEK 293 cell and FIX gene inside the capsid of AAV8 vector by quantative PCR.
5. Analysis of filled (with gene of interest) and empty capsid of AAV8 by transmission electron microscopy.
6. Transfection of suspension HEK 293 with plasmid DNA and comparative analysis of AAV produce by adherent and suspension HEK 293.

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