Construction of novel gRNA CRSIPR-Cas 9 vectors against HPV-18 E6 and E7 oncoproteins

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Rida Mudassar

Master of Science in Healthcare Biotechnology

Registration No. 00000327488

DEDICATION

To my dear parents

for their endless love and unwavering support

To myself

for showing up, pushing through, and finishing what I started

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Starting with the name of Allah, the Most Gracious, the Most Merciful. Without His endless help, guidance, blessings, and protection, I would not have able to flourish my passion towards research and my efforts would have meant nothing. I am grateful to Allah Almighty for providing me with the opportunity, ability, and knowledge to undertake and complete this study with complete satisfaction.

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Rida Mudassar

TABLE OF CONTENTS

List of Figures

List of Abbreviations

LR-HPV	Low risk Human Papilloma virus
HR-HPV	High risk Human Papilloma virus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
gRNA	Guide RNA
crRNA	CRISPR RNA
CRT	Cisplatin Based Chemotherapy
LSIL	Low grade Squamous Intraepithelial Lesions
HSIL	High grade Squamous Intraepithelial Lesions
CIN	Cervical Intraepithelial Neoplasia
SCC	Squamous Cell carcinoma
URR	Upstream Regulatory Regions
LCR	Long Control Regions
ORF	Open Reading Frame
E	Early
L	Late
PAM	Protospacer Adjacent Motif
pRb	Retinoblastoma protein
O.D.	Optical Density

LB	Luria Broth
TAE	Tris Acetic acid EDTA
UV	Ultraviolet
NF	Nuclease Free
TALENs	Transcription Activator like effectors nucleases
ZFNs	Zinc Finger Nucleases

Abstract

High risk human papilloma viruses are the main etiological agents of cervical cancers responsible for approximately 99.7% of all cases. The persistence of viral infection is the major reason that triggers oncogenesis in host's infected cells due to the deregulation of oncogenes E6 and E7. The current treatment regimens include cisplatin base chemotherapy (CRT), radiotherapy and hysterectomy, however, these approaches have their downfalls as CRT has a recurrence rate of 25-40% and surgery results in loss of fertility. CRISPR-Cas9 therapy can prove to be an effective approach to curb viral persistence targeting HPV oncogenes at genetic level and can be an effective remedy for cervical cancer. This study employs the use of CRISPR-Cas9 gene editing system to target E6 and E7 genes of HPV-18 virus. Novel guide RNAs against oncogenes E6 and E7 genes of HPV-18 were designed and their off-targets in human genome were evaluated of CasOFFinder software. They were then cloned in pX260 CRISPR Cas 9 expression vector. The confirmation of cloning was done by sangar sequencing. The resultant E6 gRNA-CRISPR Cas9 and E7 gRNA-CRISPR Cas9 vectors can be potentially therapeutic against HPV-18 after their efficacy is evaluated *in vitro* and *in vivo.*

CHAPTER 1

Introduction

Human papilloma virus infections are caused by a family of over 200 related viruses that are dermatologically or sexually transmitted. The two groups of HPVs are low risk and high risk. Most low-risk HPVs (LR-HPV) cause no disease but some of responsible for genital or anal warts. These warts can also develop in or on the throat or mouth. High-risk HPVs (HR-HPV) are the causative agents of several cancer types, and the group consists of 14 different viruses among which HPV-16 and HPV-18 are responsible for most of these HPV-related cancers (National Cancer Institute, 2023).

High-risk HPVs are responsible for approximately 5% of all cancers all around the world indicating a significantly higher global burden of HPV-related cancers (WHO, 2022). Cervical cancer is estimated to be the fourth most frequently occurring cancer in females worldwide and almost all of these cases of cervical cancer, around 99% of cases, are associated with high-risk human papilloma virus (HR-HPV) (Meites et al., 2021).

Human papilloma viruses are small viruses that belong to the family Papovaviridae and have a conserved double-stranded DNA genome. They are non-enveloped viruses having circular genomes of ~8 kilo base pairs. The non-coding region of the genome is concentrated in 1000 bp consisting of regulatory sequences for transcription and replication of viral DNA and is called as “long control region” (LCR) or “upstream regulatory region” (URR) (Boulet et al., 2007).

The coding region of genome has 8 genes encoding for 8 proteins and is transcribed as a polycistronic mRNA. These 8 proteins can be categorized into 2 types depending upon the time of viral life cycle they are expressed in. 6 of these 8 genes are “early” (E) genes encoding for E proteins in a 4000 bp region. The 6 E proteins are E1, E2, E4, E5, E6, E7, three of which are

regulatory proteins i.e., E1, E2, E4 and the other three i.e., E5, E6, E7, are the oncoproteins. The two "late" (L) proteins, L1 and L2, are encoded from a 3000 bp region and are the structural proteins making the capsid of virus (Oyervides-Muñoz et al., 2018).

A persistent high risk HPV infection is a hallmark of carcinogenesis in the cervical epithelial cells. HPV infects the epithelial cells that produce viral particles which can spread to the basal layer. Viral replication can initiate the formation of low- and high-grade lesions that may evolve into cancer. During the viral infection cycle, the viral genome can get integrated in the host cell genome in the form of episomal DNA. This integration of HR-HPV may favor viral replication and is usually associated with a persistence infection that can also lead to malignant transformation of cells (Oyervides-Muñoz et al., 2018).

It is hypothesized that during HPV genome integration a break occurs in E2 gene that causes a loss in its function. The E2 protein has a major role as a repressor in the expression of E6 and E7 oncogenes. The loss of E2 function results in dysregulated expression of these two proteins which are majorly responsible for inducing carcinogenesis and malignant transformation in host cells. E6 and E7 gene products mainly inhibit the tumor suppressor proteins p53 and pRb respectively that are responsible for cell cycle arrest. Inhibition of these tumor suppressor genes cause uncontrolled cell divisions i.e., carcinogenesis (Boulet et al., 2007).

HPV induced cervical cancer is among one of the most neglected diseases in our country. In Pakistan, the incidence of HPV is hard to determine as the reported cases in the national cancer registries are mostly from urban areas and lack in other parts of country. Cervical cancer remains the 3rd cause of all female cancer in Pakistan and is the 3rd cause of all cancer deaths in females. Being a low-middle income country, Pakistan lacks the proper screening, prevention, and vaccination for cervical cancer and even less work has been done in studying its prevalence. However, there are a few studies that indicate a high incidence of HPV in these

cancers i.e., the incidence of co-infection of HPV-16 and HPV-18 in cervical cancer is significantly higher than that of worldwide (Bruni L et al., 2023).

Till date there is no specific targeted remedy of HPV associated cervical cancer with vaccine available that can only be administered to young females that are not sexually active i.e., 9-14 years old. The vaccination for females above 45 is available but not licensed (Khairkhah et al., 2022). As, these cancers are detected at late stage, when the infection has already surpassed the chronic persistence stage, there is a need of hour to develop novel therapies that effectively target the integrated HPV DNA from genome of human cells and efficiently eradicate the cervical cancer.

In recent years, CRISPR Cas systems have emerged as a predominant approach for genetic manipulation of defected genes associated with various diseases. Clustered regularly interspaced short palindromic repeats abbreviated as “CRISPR” is basically an arm of bacterial adaptive immune system against viral invaders (Y. Zhang & Li, 2021). The type II system comprises of a Cas 9 endonuclease and can cause double stand breaks in the target DNA sequence guided by crRNA and trRNA. (Jiang & Doudna, 2017).

The discipline of genetic engineering has been significantly redefined by the advent of research of CRISPR-Cas systems as it presents as the more robust and efficient gene editing tool when compared with the other existing ones. Not only it is much simpler to use and more flexible but also offers a relatively notable degree of precision to target genes at specific sites. The ability of these CRISPR Cas systems to demonstrate gene editing has enabled scientists to use it to target pathogenic genes of various viruses to eradicate their infection (Tripathi et al., 2022).

This study focuses on the employment of CRISPR Cas9 system against Human Papilloma virus type 18. Our study aims to develop two gRNA-guides against the E6 and E7 oncogenes of HPV-18 virus with reduced off-targets in human genome and cloning them in a mammalian

Cas9 endonuclease expression vector to obtain gRNA-CRISPR Cas9 vectors. The guides designed were 20 bp long and their off targets were analyzed in CRISPR RGEN CasOFFinder tool. The guides were then cloned in pX260 vector and the cloning was confirmed by Sanger Sequencing. The E6 and E7-gRNA-CRISPR-Cas9 vectors can be used as an effective remedy to target these viral genes knocking them out and breaking the persistence of viral infection after their efficacy is evaluated both *in vitro* and *in vivo*.

CHAPTER 2

LITERATURE REVIEW

2.1. HPV

Human papilloma viruses are small viruses belonging to family *Papillomaviridae* This diverse group of epitheliotropic viruses (Doorbar et al., 2015) having over 450 different genotypes (McBride, 2022). These small viruses appear to be approximately 52 to 60 nm in diameter under electron microscope with an icosahedral symmetry. They have a circular double stranded DNA genome that ranges from ~5 kbp to 8 kbp in length (Van Doorslaer et al., 2018) enclosed in a nonenveloped capsid (Yu et al., 2022).

2.2. Viral structure

The size of viral capsid is approximately 600Å, consisting of 72 pentamers or capsomeres of the viral major capsid protein, L1 (a total of 360 molecules of L1 protein) . It also has minor capsid protein, L2 (~12 copies) as indicated by the fine structural mapping. The dsDNA inside the capsid is also associated with the core histone protein (J. T. Schiller & Lowy, 2012; Van Doorslaer et al., 2018). The C-terminal end of the L1 protein stretches out towards the adjacent capsomeres and establishes interactions between them by forming disulfide bonds at their bases (Doorbar et al., 2015).

2.3. Genome organization and viral proteins

Transcription of all proteins of all papillomaviruses occurs from a single ORF on a single DNA strand. Typically, there are 6 to 9 proteins that are encoded from the viral genome (Van Doorslaer et al., 2018). However, all types of human papillomaviruses encode 8 proteins from 8 ORFs on their genome (Fehrmann & Laimins, 2003). Despite having differences in number and size of ORFs in the genomes of all papillomaviruses, the genomic regions can be separated into 3 distinct functional regions by two polyadenylation signals (pA). The three genomic

regions are URR (upstream regulator regions), the early gene region (E) having the pA_E signal on their promoters and the late gene region (L) having the pA_L signal on their promoters (Fig. 2.1). The URR regions in the genome provides origin of replication (Ori), the promoter regions and the binding sites for the various proteins and factors involved in replication and transcription initiation (Yu et al., 2022).

In the early stage of viral life cycle, the early proteins, E1, E2, E4, E5, E6 and E7 are expressed from the pA_E that have functional roles and in later stage of viral life cycle, the structural capsid proteins L1 and L2, are expressed from the late promoter (pA_L) (Haręża et al., 2022). Among them, there are some core genes with high degree of conservation like E1 and E2, having crucial roles in the replication process and like L1 and L2 involved the packaging of viral particles and viral entry. Other remaining genes, that present with a certain level of diversity in various virus types, like E4, E5, E6 and E7 have roles in viral pathogenesis, oncogenesis, immune evasion, and viral release from infected cells (Doorbar et al., 2012).

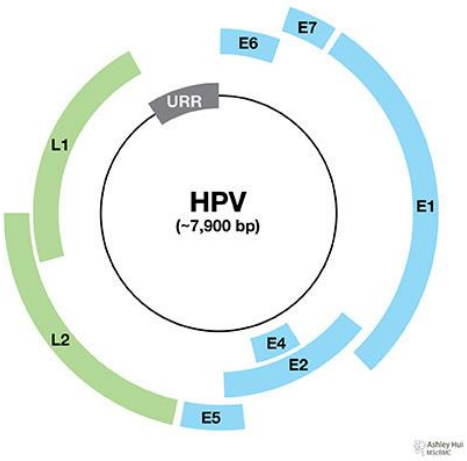


Figure 2.1. HPV genome.
Circular HPV genome indicating the early (E) and late (L) genes and the upstream regulatory region (URR)

E1 and E2 proteins work together to regulate viral replication and transcription. The initiation of replication occurs by the attachment of E2 proteins at the binding site of Ori. E2 protein then further recruits E1 proteins progressively in a step-by-step manner that results in the formation

of E1 double hexamer complex at the binding site. This complex act as a viral specific DNA helicase that unwinds the DNA in both directions after which cellular replication factors and polymerases can bind to DNA to initiate replication (Bhattacharjee et al., 2022). E2 protein also acts as a transcriptional repressor of viral genome by binding to specific sites on viral DNA in promoter region (Haręża et al., 2022).

E4 protein is expressed from an E1^E4 mRNA transcript. E4 ORF is present in E2 ORF in a different reading frame and is transcribed from early regions, from start codon of E1, in late stage of viral life cycle. The splicing and fusion of the first few codons of E1 and E2 mRNA results in E1^E4 mRNA transcript from which the protein is translated. E4 functions to disrupt the keratin of cellular cytoskeleton to ease the viral release from the cell and causes cell cycle arrest, thereby contributing to viral transmission (Doorbar, 2013; Wang et al., 2011).

E5, E6 and E7 are the oncoproteins that have role in oncogenesis in infected host cells by binding to various cellular tumor suppressor proteins and pro-apoptotic proteins and thereby causing inhibition of cell cycle arrest and cell immortalization (Yu et al., 2022).

2.4. Classification of HPV

HPVs are classified on the basis of their DNA sequence of L1 ORF into 5 genera which further have different types of viruses. Each individual type of virus has at least 10% or more than 10% dissimilarity among their L1 DNA sequences (Chen et al., 2018) The viruses of different genera differ from each other by more than 40% dissimilarity among their L1 genomic sequences. The five genera are named as alpha-papillomavirus, beta-papillomavirus, gamma-papillomavirus, mu-papillomavirus, and nu-papillomavirus (Bzhalava et al., 2015).

HPV types can also be categorized into low-risk (LR) and high-risk (HR) types, based on their potential to cause oncogenesis. LR-HPVs are usually associated with causing cutaneous and anogenital warts (Kombe Kombe et al., 2021). HR-HPVs are a sub-group of limited number of

viruses (~15) belonging to genus alpha-papilloma virus associated with different types of cancers including invasive carcinomas (including head, genital, cervical and neck cancers) (de Martel et al., 2020; Sabatini & Chiocca, 2020). These HR HPV types are responsible for around 99.7% of cases of squamous cell carcinoma as indicated by epidemiological studies. 72% of all the cervical cancers, caused by HR-HPVs, are found be predominantly caused by only two types, i.e., HPV-16 and HPV-18 (Haręża et al., 2022).

2.5. HPV-18 infection and cervical cancer

A significant proportion of infections caused by HPVs have the likelihood to become undetectable within 1 to 2 years. The risk of developing precancerous conditions of cervical intraepithelial neoplasia (CIN) notably increases in women who experience a persistent infection of an oncogenic HPV (Chan et al., 2019). Squamous cell carcinoma (SCC) represents the most predominant histological type of cervical oncogenicity caused by HPVs while adenocarcinomas and adenosquamous carcinomas are also frequently observed. The precursor lesions that give rise to SCC can be classified by squamous intraepithelial lesion, SIL (low grade and high grade) system (Groves & Coleman, 2015). They can also be divided into three-grade cervical intraepithelial neoplasia, CIN (CIN 1, CIN 2, CIN 3) systems (Zhou et al., 2023). Low grade SIL generally happens to fall under the category of CIN grade 1 indicating the acute non-neoplastic HPV infections with a very low potential to malignancy development and significant HPV self- replication ability (productive infection). Whereas high grade SIL lesions fall under CIN grade 2/3 and are characterized by the occurrence of an abortive viral infection cycle due to deregulated early gene expression (non-productive infection) with a higher potential to develop invasive carcinomas (Chan et al., 2019; Zhou et al., 2023).

The infection cycle of HPV starts when it enters the host's epithelial keratinocytes in the basal layer of cervical stratified epithelium (usually in the transformation zone). Micro-wounding or abrasions that are caused by scratching or during sexual contact is the most typical way of viral

exposure to the basal epithelial cells that are mitotically competent (Ozbun, 2019). The viral gene expression program is dependent on the differentiation process of host epithelial cells for the completion of its life cycle (Crosbie et al., 2013). The virion entry in the host cell is receptor mediated involving a complex formation between the viral particles and the molecules present on the cell surface of basal epithelium like integrins and heparan sulfate proteoglycans (Campos, 2017; Haręża et al., 2022). Viral major and minor coat proteins L1 and L2) are involved in facilitating the entry of virus through a process that resembles micropinocytosis (Campos, 2017).

The early infection phase in the basal epithelial cells is associated with a maintenance of 50-200 viral episome copies per cell by amplification of viral genome at low-level and does not depend on host cell cycle. The virus can undergo two pathways of infections i.e., productive, and unproductive pathway (Fig. 2.2) (Burd & Dean, 2016).

Both LR and HR HPVs can adapt the productive infection pathway in the superficial epithelial layers of cervix and cannot lead to oncogenesis as the cell death of infected keratinocytes, that are terminally differentiated at the epithelial surface, occurs and they are eliminated from the body naturally. At this stage of infection, the DNA of virus can be present both extracellularly and intracellularly when virions are released from surface of epithelium and can be disseminated to other persons (Groves & Coleman, 2015; Schiffman & Wentzensen, 2013). As the suprabasal differentiating cells are bound to get matured and undergo senescence, they do not natively exhibit the expression of the replication machinery that the virus needs to survive. Therefore, HPVs have evolved to produce two oncogenes E6 and E7 that delay the cell cycle arrest by triggering cellular proliferation and by putting a check on apoptosis and thus letting the cells allow replication and subsequently release of virions when they die (Crosbie et al., 2013). This infection cycle of viruses is associated with the development of low grade squamous intraepithelial lesions (LSIL) (Groves & Coleman, 2015).

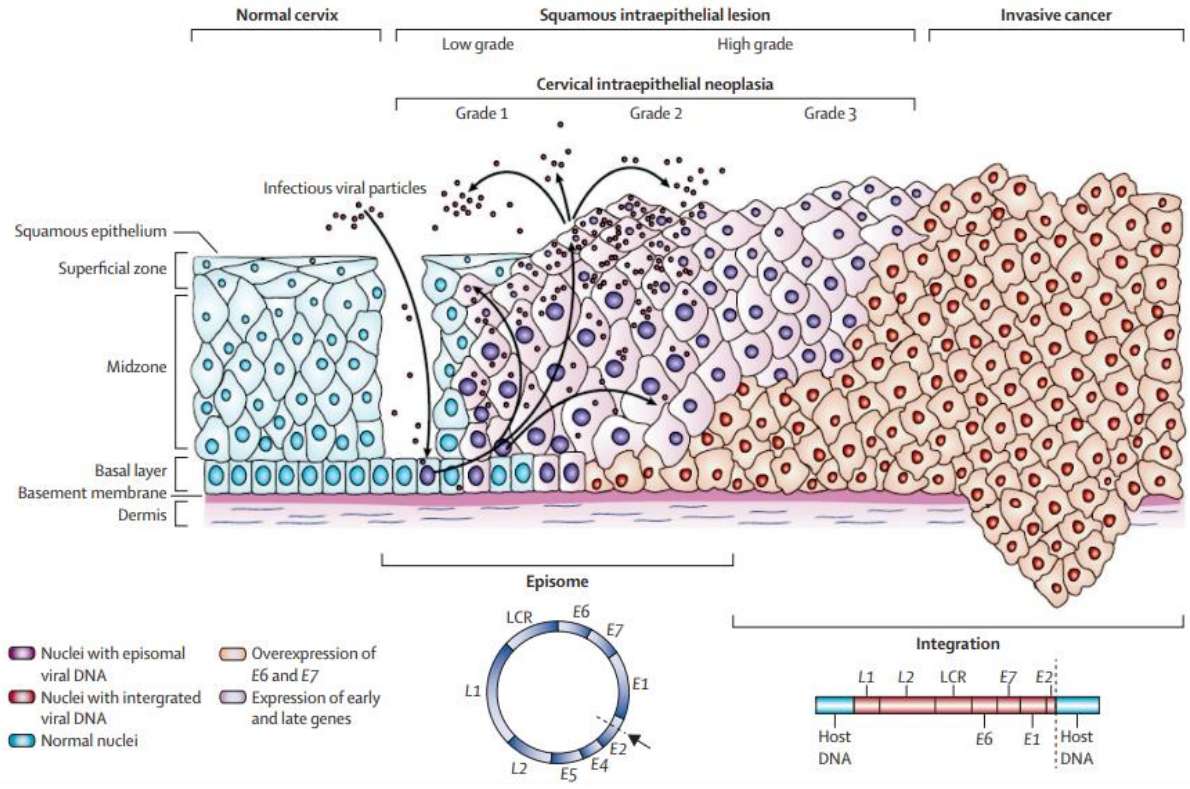


Figure 2.2. The infection cycle of HPV virus leads to oncogenesis.
Viral DNA is present in various forms at different stages of infection at stages of infection. The viral replication and release of viral particles is maximum low grade squamous intraepithelial lesion (LSIL) indicating a productive infection while the high-grade squamous intraepithelial lesions (HSIL) are characterized by the integration of viral genome causing uncontrolled cell division leading to invasive cancer (Crosbie et al., 2013)

Non-productive infection results from the deregulation of viral gene expression. In certain HR-HPV types the E6 and E7 proteins are so potently causing the hindrance in cell cycle arrest events that the cells having viral infection never undergo complete development or differentiation and continue their proliferation without apoptosis. The persistence of infection leads to invasive carcinogenesis. The presence of genomic abnormalities in high-grade lesions due to continuous cellular proliferation facilitates the ultimate integration of viral DNA in the host's genome which further increases instability of chromosomal regions and increases the activation of viral oncogenes triggering neoplastic transformation in cervical cells. Nonetheless, there are certain cases of cancer in which the viral genome still exists in episomal form (Burd & Dean, 2016; Lintao et al., 2022). Integration is a chance event that can be a consequence of inflammation caused by infection as inflammation induces double stand breaks

in the genomic DNA by generation of reactive oxygen species (ROS) (Williams et al., 2011). The deregulation in viral gene expression results from the complete deletion of disruption of E2 gene when the viral DNA integrates in host genome. As E2 is the negative regulator of E6 and E7 genes, loss of E2 protein results in the overexpression of these two viral oncogenes (Fig. 2.3) . E6 functions by preventing the cellular apoptosis and senescence when it binds and causes the degradation of p53, a tumor suppressor protein via a ubiquitin-pathway while E7 binds to retinoblastoma protein (Rb) releasing the E2F transcription factor that is involved in activation of transcription of S-phase genes that further take part in cell cycle entry leading to cell immortalization (Klingelhutz & Roman, 2012; RUTTKAY-NEDECKY et al., 2013)

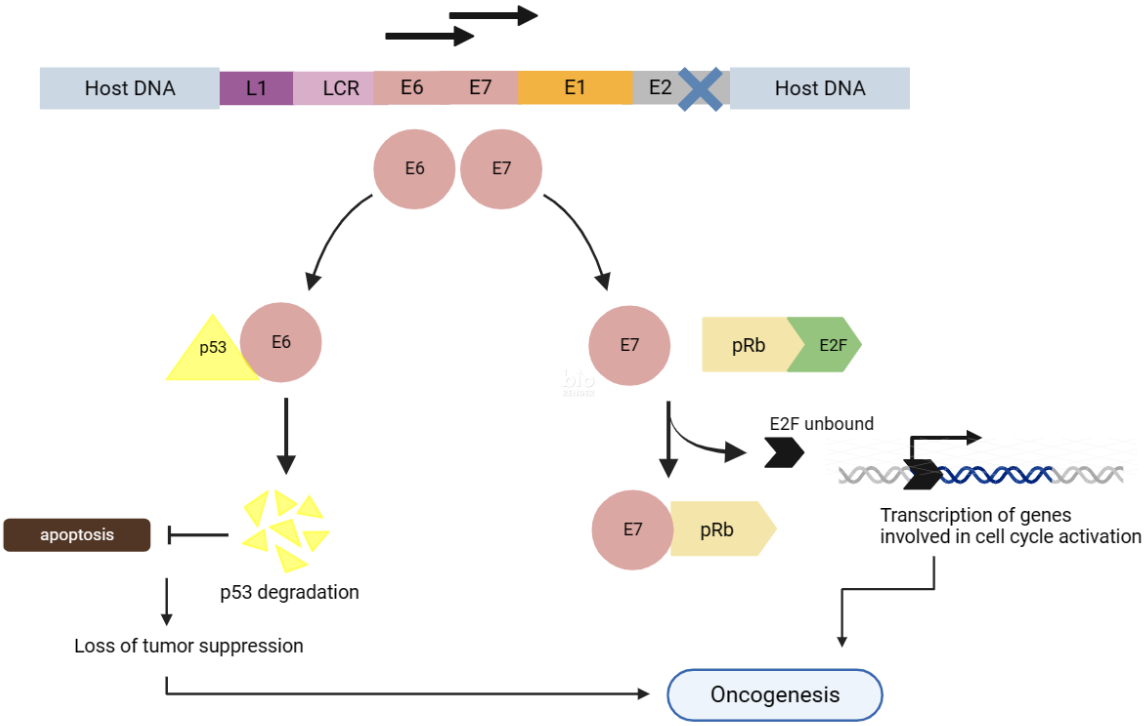


Figure 2.3. HPV oncogenes.
E6 and E7 are HPV oncogenes that by binding to p53 and pRb results in malignant transformation of cells as p53 and pRb are the tumor suppressor genes and wok by inhibiting apoptosis and binding to E2F to stop transcription of cell cycle activation genes.

2.6. Epidemiological Burden of Cervical Cancer

HR-HPVs infection leading to oncogenesis impose significant burden on health facilities. Not only in women, but also in men, HR-HPVs cause serious health complications and cancers. These viruses are etiological agents of about 3% of all cases of cancers that occur in females

and approximately 2% of all male cancers in United states. As reported by CDC, an estimated number both women and men that are affected by HPV related cancers is 570,000 and 60,000 in US respectively (National Cancer Institute, 2023).

WHO has classified cervical cancer as a major public health problem being the 4th most frequently occurring cancer in women worldwide and devised various global strategies for its elimination. In 2020, there were ~604000 new cases and ~340000 deaths from cervical cancer reported with 90% of these deaths occurring in developing and under-developed countries (WHO, 2022). The presence of HR-HPV DNA has been reported to be found in 99.7% of cervical cancers with HPV 16 and 18 being the most common two types account for 70% of all cervical cancer cases globally. HPV 18 is the second most common etiological agent occurring in all invasive cancer (Chan et al., 2019; S. Zhang et al., 2020). According to ICO/IARC report published in 2023, among the 604,147 invasive cervical cancer cases that are diagnosed annually around the globe, 14.2% are caused by HPV-18. HPV-18 is the cause of 11.6% of all squamous cell carcinomas and 34.9% of all adenocarcinomas of cervix (Bruni L et al., 2023).

In Pakistan, among the women population of 73.8 million aged 15 years and older, 5008 are diagnosed with this type of cancer and with a mortality rate of 3197 deaths annually positioning this disease as the 3rd most frequently occurring cancer among women. Among all these cases, 88.1% are caused by HPV 16/18 and approximately 0.5% of the general women population are estimated to be a carrier of these infectious viruses at a given time (Bruni L et al., 2023).

2.7. Current treatment regimens

The current therapeutic approaches employed to treat cervical cancer includes the radiation therapy as well as radical or complete hysterectomy procedures (surgery) along with the administration of adjuvant or neoadjuvant chemotherapy (Khairkhah et al., 2022).

Conventionally, the cisplatin-based chemotherapeutic (CRT) approach has proven to be effective in the management of benign to advanced metastasized cervical cancer in women (Marchetti et al., 2020). The standard therapy for invasive cervical cancer is considered to be CRT with a definitive radiation therapy but has a downfall of relatively high recurrence rates of cancer i.e., 25-40% even after adequate administration (Marth et al., 2018). Hysterectomy has adverse effects on fertility therefore resulting in various illnesses including both mental and physical ailments that consequently imposes a significant damage to the well-being of patients and their quality of life (De Felice et al., 2018).

For prophylactic measures against HPVs, there are three vaccines, prequalified and approved by WHO and US Food and Drug administration, namely Ceravix, Gardasil and Gardasil 9 which are bivalent, tetravalent, and 9-valent vaccines respectively. All of them provide protection against two most common HPV oncogenic types 16/18 and are administered intramuscularly with 3 doses given over a period of 6 months (J. Schiller & Lowy, 2018). Numerous clinical trials and various surveillance studies have provided convincing evidence that supports the safety of HPV vaccinations and prove their efficacy in preventing HPV infections, precancerous lesions and HPV associated malignancies when administered before viral exposure (Lei et al., 2020; WHO, 2022).

2.8. CRISPR cas system and its application in treatment of viral infections

CRISPR stands for “clustered regularly interspaced short palindromic repeats” and these along with the CRISPR-Cas associated proteins are a major revolution in the field of genetic engineering. Since the discovery of this system, this technology has proved to be a major success for gene editing. The CRISPR-Cas system is a part of bacterial immune system against bacteriophages or foreign nucleic acids and is implicated in many biological aspects for genome editing. 6 types of these CRISPR-Cas systems have been identified up till now that can be classified in two classes (Xu & Li, 2020).

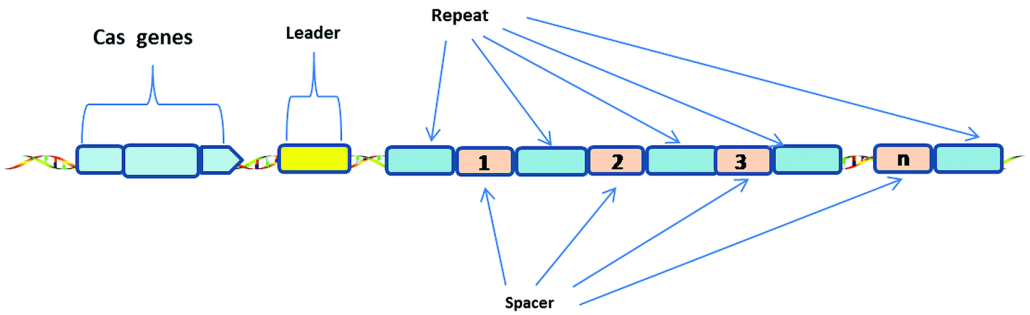


Figure 2.4. The CRISPR locus.
The CRISPR locus has different sequence elements, i.e., Cas genes for Cas 1, Cas 2 and Cas 9. The spacers are the sequences of viral invaders integrated between direct repeats. Leader sequences have some regulatory roles (J. Zhang et al., 2018).

The CRISPR-Cas9 is a type II system, belonging to class 2, and works by directing a complex of gRNA-Cas9 enyzme to the targeted site upstream to a seed sequence also known as protospacer adjacent motif (PAM) (Karimian et al., 2019). The gRNA is a single chimera consisting of crRNA and tracrRNA transcribed individually from different CRISPR sites.

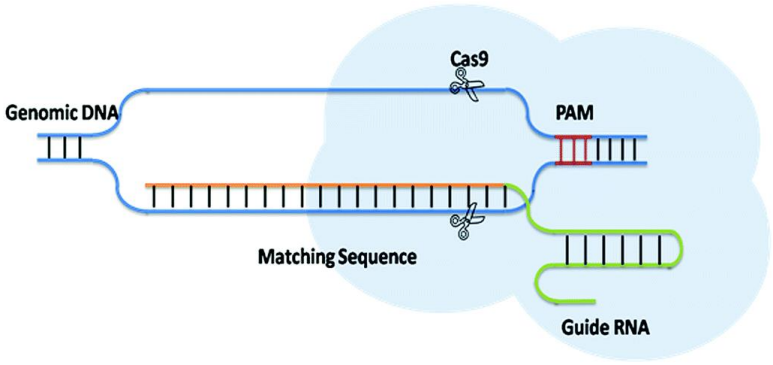


Figure 2.5 Principle of CRISPR Cas system.
gRNA which is a chimer of tracrRNA (green part of strand) and crRNA (orange part of strand) guides the Cas9 endonuclease to target adjacent to PAM to include dsDNA breaks.

The type II system consists of an array of spacer sequences, which are the sequences of invaders integrated in the system by Cas 1 and Cas 2 enzymes and separated with each other by direct repeats. The genes of Cas enzymes are located on either side of the CRISPR array (Wei et al., 2022). The spacers transcribe the precrRNAs further processed to crRNAs. Another site upstream to the CRISPR array transcribes the tracrRNA (Kim et al., 2017). The crRNA and tracrRNA complex (gRNA) guide the Cas9 to target site to cause doubles stranded breaks in DNA of invading agent (F. Zhang et al., 2014). The ds DNA breaks are repaired by NHEJ or

by homologous recombination (HDR) . NHEJ is error prone, thus cause a mutation on the targeted site while a desired DNA segment can also be introduced at the targeted DNA site via homologous direct repair (HDR) (Ceccaldi et al., 2016).

During the last decade, the CRISPR-Cas 9 technology has been used extensively to target DNA by combining crRNA and trRNA in form of single guide (sg) RNA chimera and cause double stranded DNA breaks in various prokaryotic and eukaryotic systems for editing or therapeutic purposes. In virology research, it is mainly used for editing of specific viral genes to get a know-how of its function, for diagnosis purposes or to target viral genomes to block the viral replication as a therapeutic approach (Tripathi et al., 2022). The effectiveness of this CRISPR Cas 9 technology as a therapeutic agent for various DNA viruses like HBV, HPV, EBV, HSV and some RNA viruses like Influenza, SARS, and HIV at its double stranded proviral stage too with minimum off-target effects has already been tested (Bayat et al., 2018). HPV genome, like other DNA viruses has also been targeted via a CRISPR-Cas 9 system, to develop a novel therapeutic for HPV induced carcinoma as an alternative for chemotherapy that has many side effects most important of them being the toxicity to normal cells too and the cancer cells developing resistance against them. Moreover, no available chemotherapeutic drug can completely eradicate the viral genome possessing the ability to get integrated in human DNA (latency) and circumvent the host immune system.

There are many studies in which oncogenes E6 and E7 of HPV-16 and HPV-18 have most commonly been targeted. The use of CRISPR-Cas 9 is more ideal as a targeted gene therapy causing the point mutations in the oncogenes in DNA and inhibiting their expression in comparison with other available therapeutic approaches (Wei et al., 2022).

CHAPTER 3

METHODOLOGY

3.1. Selection of suitable vector

For the expression of gRNA and Cas 9 enzyme in mammalian cells, a vector a suitable expression vector was selected. While deciding on which vector to choose, it was made certain that it possessed all the necessary features which are described below.

- An origin of replication
- Multiple cloning sites (MCS)
- Selectable markers
- Mammalian expression cassettes for Cas9 enzyme
- Mammalian expression promotor for gRNA

3.2. Sequence retrieval for E6 and E7 genes

The sequences for the E6 and E7 genes of HPV-18 were retrieved from NCBI data base in FASTA format.

3.3. gRNA designing

for gRNA designing against the two target genes E6 and E7 were designed on IDT gRNA designing tool software. The software gives all the possible gRNAs in the input DNA sequence. The sequences for E6 and E7 genes were added in the input bar to get the gRNA sequences that were ~20 bp long. Following are the characteristics that a gRNA oligonucleotide must possess:

- It should have a GC content between 40-80%.and should not have any secondary structures.
- The target sequence of gRNA must be adjacent to a protospacer adjacent motif (PAM) which is 5’ NGG 3’ for streptococcus pyogenes Cas 9.
- The gRNA must be 17-24 bp long.

- The sequence of gRNA must be specific to reduce off-target editing effects i.e., the sequence should not be anywhere in the whole genome.

Even if gRNA is specific, it can bind to sequences in the genome with one or more base pair mismatches (off-targets). Therefore, after selection of suitable gRNA sequences for both genes E6 and E7, they were checked for having any off targets in the human genome through a CRISPR RGEN tools software (CasOFFinder). This tool gives the sequences in the whole genome that have the possibility be targeted by the gRNA besides the target sequence. The gRNA sequences were entered into the input bar and the off targets were assessed by adjusting the mismatch criteria to 3 base pairs and bulge criteria to 0 bulges.

The gRNA sequences were also validated by OligoCalc software for any secondary structures like hairpin formation and for the presence of any self-annealing or self-complementary properties.

As, the vector pX260 utilized the CRISPR Cas system ll having a separate tracrRNA cassette, 10 extra nucleotides were also added at the start of gRNA before cloning because after its transcription, the first 10 base pairs from the resultant crRNA are cut off when it is processed before the formation of crRNA-tracrRNA complex (sgRNA) (Cong et al., 2013).

3.4. In silico cloning

In silico cloning was done in the SnapGene software. Double digestion of pX260 vector was done by Bbs1 enzyme that generated sticky ends. The base pairs complementary to the sticky ends were added in the double stranded DNA form of gRNA and it was ligated in the vector.

gRNA oligonucleotides, in DNA form (forward and reverse oligonucleotides), were ordered after *in silico* cloning from Macrogen.

3.5. Plasmid recovery

The plasmid was received, adsorbed on a Whattman filter paper. To recover the plasmid, the circles on the filter paper containing plasmid were cut with a clean razor blade and immersed in NF water. It was kept at 65-70°C for 10-15 min that released the plasmid in water, 2-3µl of which was used in transformation.

3.6. Electrocompetent cells preparation of DH5α

Competent cells were prepared by inoculating a single colony of DH5α from a freshly streaked plate in flask containing Luria broth. The incubation of flask was done at 37°C overnight in a shaking incubator. The next day 1ml of that primary culture was transferred to a flask containing 50ml Luria broth (secondary inoculum). The shaking incubation at 37°C was given again for 2-3 hours or until the O.D. reached 0.4-0.5. The bacterial culture was then moved to already chilled falcon tubes and incubated on ice for 20 min. After that, the bacterial culture in the flacons was subjected to centrifugation at 5000 rpm for 10 mins at 4°C. Supernatant was removed carefully, and the pallet was dissolved in 10 ml of 10% sterile glycerol solution by vortexing and then 30 ml of 10% glycerol was further added to make the total volume up to 40 ml. Centrifugation at 4°C was done again for 10 min at 5000 rpm. The supernatant was discarded, and the pallet was washed in 20 ml of 10% glycerol solution at same conditions. The step was repeated with 10 ml of glycerol. After all, the pallet was resuspended in 0.5 ml of 10% glycerol solution and aliquots of 50µl in microfuge tubes were kept at -80°C after snap freezing with liquid nitrogen.

3.7. Electroporation

Electroporation was performed in 2mm cuvettes. The 50 ml aliquots of electrocompetent cells were taken out from the -80°C freezer and were thawed on ice. 3-5µl (5ng) of the plasmid recovered from filter paper was added to the electrocompetent cells on ice. The total of 55µl of competent cell and plasmid mix was transferred to the chilled cuvettes that were washed

properly and sterilized by exposure to UV for 20 mins as well as by freeze sterilization at -20°C. The cuvette was put in the electroporator after removing the excess moisture from outside of cuvette by a tissue wipe. The cells were pulsed at 2.5kV for 5 milliseconds for the plasmid DNA to pass into the cells. The cuvette was removed from the electroporator and 1ml of LB broth was immediately added to recover the cells from electric shock. The negative control cells with no plasmid were also pulsed in electroporator and 1ml of LB broth was also added to them. They were transferred to microfuge tubes which were then given incubation in a shaking incubator at 37°C for 1 hour. 200µl of inoculum was poured on the LB agar plates containing 200µg/ml of ampicillin and spread evenly with a spreader. The control inoculum was spread on both ampicillin positive and negative plates. The plates were given incubation for 16 hours at 37 °C.

3.8. Extraction and storage of plasmid

A single isolated colony from the ampicillin positive plate (plasmid + cells) cultured after electroporation was inoculated in labelled flask containing 25 ml of Luria Broth with 200ug/ml of ampicillin. The flasks were kept in shaking incubator at 37°C for 16 hours (overnight culture). Plasmids were extracted from the overnight bacterial culture by plasmid miniprep. The same culture was also used for glycerol stock preparation of plasmids.

3.8.1. Glycerol stocks preparation

For this purpose, 500 ml of bacterial culture incubated overnight was put in 500 ml of sterilized 50% glycerol solution in a labelled cryovial and thoroughly mixed by pipetting up and down. The cryovial was kept in at -80°C refrigerator.

3.8.2. Plasmid Miniprep

Plasmid was extracted from bacterial culture by alkaline lysis method in a small microfuge tube. Three solutions used in plasmid miniprep, i.e., resuspension solution Ⅰ, denaturation solution Ⅱ and neutralization solution Ⅲ were prepared before miniprep and autoclaved

except for solution Ⅱ. Solution Ⅱ was prepared fresh before miniprep and sterilized through syringe filtration. The overnight bacterial culture was taken in sterilized microfuge tubes (1.5 ml in each) and was centrifuged at 13000 rpm for 2 mins. The supernatant was discarded completely and carefully by pipetting without disrupting the pallet. 150 µl of ice-cold solution Ⅰ was added to the tubes and the pallet was dissolved by vortexing. 300 µl of freshly prepared solution Ⅱ, that causes the lysis of cells, was added, and mixed by inverting the tubes a few times. The liquid became clear. The tubes were kept on ice for not more than 3 mins. 250 µl of solution Ⅲ was added to the tubes for the precipitation of chromosomal DNA and denatured proteins and mixed by inverting them 5 to 6 times. The appearance of the liquid at this stage became cloudy due to white precipitates of chromosomal DNA and proteins. The microfuge tubes were given an incubation at 4°C by keeping them on ice for 10 min. The microfuge tubes were then centrifuged at 4°C at 13000 rpm for 15 mins in a refrigerated centrifuge. The clear supernatant consisted of plasmid DNA and the denatured protein debris and chromosomal DNA got settled in the form of pallet. The supernatant was carefully picked with a micropipette and transferred to another sterilized centrifuge tube. Two volumes of chilled absolute ethanol were added for precipitation of plasmid DNA and centrifuged at 13000 rpm at 4°C for 15 min. The supernatant was discarded, and the pallet was washed again by 70% ethanol again in a centrifuge for 10 mins at 4°C. The supernatant was discarded again, and the residual ethanol was air dried completely. The pallet (not usually visible) was resuspended in 50µl of NF water and stored at -20°C.

3.9. Plasmid DNA quantification and agarose gel electrophoresis

Plasmid DNA was quantified in a nanodrop spectrophotometer. 1-2µl of plasmid DNA sample was used for this purpose in a Colibri microvolume spectrophotometer® following instructions guide of user manual. Plasmid DNA was also run on 1% agarose gel electrophoresis for further

confirmation. For preparation of 1% agarose gel, 0.5g of agarose was weighed and added to 50ml of 1X TAE buffer (tris acetic acid EDTA) freshly diluted from a 50X TAE stock. After the dissolution of agarose by boiling, the mixture was allowed to cool down after which ethidium bromide was added to it as a staining agent and mixed completely. The gel casting tray was assembled with a comb and the mixture was poured in the tray. After the gel solidified, the comb was removed, and the tray was put in a gel tank filled with 1X TAE buffer. The samples were mixed with 6X loading dye and loaded in the wells of gel. 1 kb ladder was also loaded as a reference. The gel was run for 55 min at 82V.

3.10. PCR confirmation of plasmid pX260

For confirmation of plasmid, PCR was done. For this purpose, a set of froward and reverse primers was designed. A GC content of 40-60%, a melting temperature within a range of 5°C of each other, and a length of 18-25bp were the points that were kept in mind while designing primers. The primers were also validated for the presence of any secondary structures (hairpin or loop formation), self-annealing or self-complementarity on OligoCalc (website). The sequences of the primers are given below.

Forward primer (Ex Pf)	5’ GGGTAGTTTGCAGTTTTAAA 3’
Reverse primer (Ex Pr)	5’ CTAGAGCCATTTGTCTGCAG 3’

A conventional thermocycler by Applied Biosystems was used for PCR amplification. The contents of the reaction mixture prepared for PCR are as follows.

2X Fermentas Master mix	12.5µl
NF water	9.5µl
Forward primer	1µl
Reverse primer	1µl
Plasmid DNA	1µl
Total volume	25µl

The reaction mixture was put in the thermocycler after spinning down all the contents for 15-20 seconds and the profile for amplification was set at 95°C for the initial denaturation for 5 min and then 35 cycles of denaturation at 95°C for 45 sec, followed by annealing at 55°C for 45 sec and elongation at 72°C for 45 sec. Lastly, a final extension at 72°C was given for 10 mins. After amplification, the 10µl of the reaction mix with 3µl loading dye was run on 2% agarose gel for gel electrophoresis to analyze the PCR reaction. A 50 bp ladder was used as a reference. The gel was visualized in a UV illuminator.

3.11. Restriction digestion of vector for confirmation and cloning

Further confirmation of plasmid was also done by restriction digestion by Thermofisher Scientific EcoR1 enzyme (10U/µl). Restriction digestion of vector for purpose of cloning was done by Thermofisher Bpil (Bbs1) enzyme (10U/µl). Both digestions were carried out separately. 1ul of enzyme was used for 1000 ng (1µg) of plasmid in a 20µl reaction mix. The contents of the digestion mix used are as follows.

Bpil (Bbs1 enzyme)/EcoR1	1µl
10X Buffer G	2µl
Plasmid (1000ng)	1µl
NF water	16µl
Total volume	20µl

After spinning it down to mix all the components, the digestion mix was given incubation at 37°C for 16 hours. After 16 hours, the microfuge tube was put at 65°C in a water bath for 20 mins. This was done for the heat inactivation of restriction enzyme to prevent its star activity. It was stored at -20°C before its use in ligation. For the confirmation of restriction digestion, the reaction mix was also run for agarose gel electrophoresis on a 0.7% gel in TAE buffer with an uncut control plasmid with a 1kb ladder as a reference. The gel was visualized in a UV illuminator.

3.12. Oligonucleotide annealing

The forward and reverse oligoes of gRNA for E6 and E7 gene (in DNA form) were annealed in a thermocycler. 1ul from 100 mM concentrations of both forward and reverse oligoes for E6 gRNA were added to 8µl of NF water. Same was done for E7 gRNA. After spinning down, the tubes were put in a thermocycler. The profile was set at the initial incubation at 37°C for 30 mins and then denaturation at 95°C for 5 mins. The temperature was then ramped down to 25°C slowly at the rate of 5°C per min. The heterodimer formation was confirmed by running a gel electrophoresis on 2% 1X TAE agarose gel and by visualizing in a UV illuminator. These heterodimers of gRNA sequences of E6 and E7 were used as an insert to be cloned in the vector.

3.13. Ligation of gRNA Insert in Vector Backbone

To have gRNA expression in the mammalian cells along with the Cas 9 enzyme, the E6 and E7 gRNA sequence in the heterodimers form were inserted in the px260 plasmid separately by using a Thermofisher Scientific T4 DNA Ligase. The annealed oligoes mix of E6 gRNA sequence was diluted in a ratio of 1:100 with NF water. The concentration of diluted E6 heterodimer and the Bbs1 digested vector was quantified by nanodrop spectrophotometer. For ligation, a molar ratio of insert to vector used was optimized at 20:1. The concentration of the E6 heterodimer and digested vector used in a molar ratio of 20:1 in the ligation mix was calculated by NEB ligation calculator and the volumes to be used were adjusted. For 200ng of vector, the quantity of insert to be used was 16ng. Following were the contents of the ligation mix prepared.

T4 DNA Ligase	1µl
2X Ligase Buffer	2µl
Insert (16ng)	4µl
Vector (200ng)	4µl
NF water	9µl
Total volume	20µl

The vector and the insert were mixed in a microfuge tube at first and briefly incubated in water bath at 42°C. After incubation, the water bath was turned off and was allowed to cool down at room temperature slowly. The remaining contents were added then. After spinning it down the ligase mix for 15-20 sec, it was incubated at 4°C for 16 hours (overnight). The next day, heat inactivation of the ligase mix was done at 65°C for 20 mins in a water bath prior to its transformation in competent cells. After heat inactivation, the mix was stored at -20°C until transformation. The gRNA heterodimer for E7 gene was also ligated in pX260 vector in the same way.

3.14. Heat shock Competent cells preparation DH5α

Competent cells for heat shock transformation were prepared by inoculating a single colony of DH5α from a freshly streaked plate in flask containing Luria broth. The flask was given incubation at 37°C overnight in a shaking incubator. The next day 1ml of that primary culture was transferred to a flask containing 50ml Luria broth (secondary inoculum). It was given shaking incubation at 37°C again for 2-3 hours or until the O.D. reached 0.5. The culture was then moved to already chilled falcon tubes and kept on ice for 20 min. It was given 4°C centrifugation at 6000rpm for 10 mins. after discarding the supernatant, the pallet was dissolved gently in 10 ml of 0.1 M $CaCl_2$. It was again centrifuged at 6000 rpm for 10 mins at 4°C. The supernatant was discarded again, and the pallet was resuspended in 1 ml of 0.1 M $CaCl_2$ + 15% glycerol solution, 50 µl of which was aliquoted further in labelled microfuge tubes. The tubes were dipped in liquid nitrogen for snap freezing. The heat shock competent cells were stored at -80°C till they were used for heat shock transformation.

3.15. Heat shock transformation

The heat inactivated ligation mixture was transformed in the DH5α competent cells by heat shock transformation. Three aliquoted microfuge tubes of heat shock competent cells were taken out from -80°C refrigerator and were thawed on ice. 5µl of ligation mixture for E6 gRNA

was added to a tube and 5µl from the E7 gRNA ligation mixture was added to the other one. The third microfuge tube was used as a control with nothing added to it. The three of them were kept on ice for 30 mins. The cells were given a heat shock at 42ºC for 90 sec in a water bath. The cells were then immediately put back on ice to recover from heat shock for 5 mins. 1ml of room temperature was added to the tubes and they were put on the shaking incubator at 37ºC for 1 hour. After 1 hour, 200 µl of the culture from the tubes with ligation mix was spread on separate ampicillin positive plates. 200µl of culture from control tube was also spread on ampicillin positive plate and on ampicillin negative plate as well. The plates were put in incubator at 37°C for 16 hours.

3.16. Confirmation of ligation by sequencing analysis

The single isolated colonies from the plates that were cultured with bacterial cells transformed with E6 and E7 ligation mix were inoculated in flasks (one single colony in one flask) containing sterilized LB broth and put at 37°C in a shaking incubator for 16 hours. The next day, the plasmid DNA from the cultures was extracted and PCR was done using the isolated plasmids as templates with Ex Pf and Ex Pr, the forward and reverse primers in the thermocycler with same conditions as described in section 3.10. PCR reaction was analyzed on gel electrophoresis. The desired bands of PCR products were cut from the gel by a sterile blade and were purified using a Thermofisher Scientific Gel Purification kit according to manual.

The purified PCR products were analyzed by Sanger Sequencing that confirmed the successful ligation of sequences of gRNA for E6 and E7 genes in the pX260 vector.

CHAPTER 4

RESULTS

4.1. Vector selection

The vector chosen was pX260-U6-DR-BB-DR-Cbh-NLS-hSpCas9-NLS-H1-shorttracr-PGK-puro (Cong et al., 2013). It was ordered from DNA custom technologies and consisted of all the required sequence elements and fulfilled all the criteria that were taken into consideration while selecting a suitable vector for the gRNA and CRISPR-Cas9 expression in mammalian cells (Fig. 4.1). Following are the major features of pX260 vector.

- The vector has an origin of replication (F1) for its propagation in bacterial cells.
- The vector has multiple cloning sites for the desired sequence to be cloned in a particular position. For gRNA sequence insertion, it has restriction sites for Bbs1 enzyme.
- The vector has selectable markers to allow the growth of only those bacterial and mammalian cells that have taken up the plasmid after transformation and transfection respectively. It has an expression cassette for ampicillin resistance gene for selection bacterial cells and puromycin resistance gene for its selection in mammalian cells.
- The vector has an expression cassette for spCas-9 gene under chicken β-actin promotor. It also has a U6 promotor upstream to the cloning site for successful gRNA expression as well as the tracrRNA sequence under an H1 promotor.

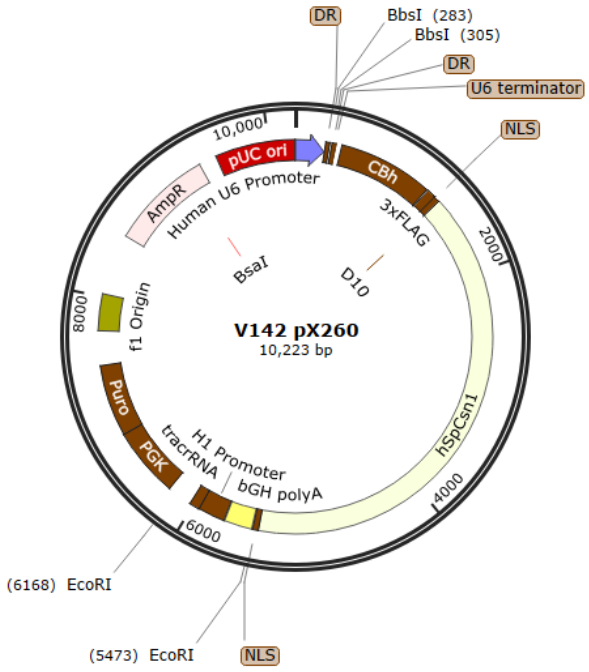


Figure 4.1. pX260 vector.
The vector consists of all the necessary sequence elements.

4.2. In silico gRNA designing

4.2.1. E6 and E7 gene sequence retrieval

The sequence for the two HPV-18 genes, E6 and E7, was retrieved from NCBI in FASTA format. E6 gene is 477 bp long while E7 gene is 318 bp long (Fig.4.2). Both genes are involved in oncogenesis in infected cells by binding to p53 and pRb respectively and causing inhibition of cell cycle arrest.

NCBI Reference Sequence: NC_001357.1

GenBank Graphics

>NC_001357.1:105-581 Human papillomavirus - 18, complete genome
ATGGCGCGCTTTGAGGATCCAACACGGCGACCCTACAAGCTACCTGATCTGTGCACGGAACTGAACACTT
CACTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAGTATTGGAACTTACAGAGGTATTTGAATT
TGCATTTAAAGATTTATTTGTGGTGTATAGAGACAGTATACCCCATGCTGCATGCCATAAATGTATAGAT
TTTTATTCTAGAATTAGAGAATTAAGACATTATTCAGACTCTGTGTATGGAGACACATTGGAAAAACTAA
CTAACACTGGGTTATACAATTTATTAATAAGGTGCCTGCGGTGCCAGAAACCGTTGAATCCAGCAGAAAA
ACTTAGACACCTTAATGAAAAACGACGATTTCACAACATAGCTGGGCACTATAGAGGCCAGTGCCATTCG
TGCTGCAACCGAGCACGACAGGAACGACTCCAACGACGCAGAGAAACACAAGTATAA

NCBI Reference Sequence: NC_001357.1

GenBank Graphics

>NC_001357.1:590-907 Human papillomavirus - 18, complete genome
ATGCATGGACCTAAGGCAACATTGCAAGACATTGTATTGCATTTAGAGCCCCAAAATGAAATTCCGGTTG
ACCTTCTATGTCACGAGCAATTAAGCGACTCAGAGGAAGAAAACGATGAAATAGATGGAGTTAATCATCA
ACATTTACCAGCCCGACGAGCCGAACCACAACGTCACACAATGTTGTGTATGTGTTGTAAGTGTGAAGCC
AGAATTGAGCTAGTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAGCAGCTGTTTCTGAACACCC
TGTCCTTTGTGTGTCCGTGGTGTGCATCCCAGCAGTAA

Figure 4.2. E6 and E7 genes.
The sequences for E6 and E7 genes were retrieved from NCBI with the accession number of HPV-18 complete genome sequence i.e., NC_001357.1

4.2.2. gRNA oligonucleotide design

SpCas9 enzyme requires a seed region (protospacer adjacent motif or PAM) of 3 base pairs i.e., 5' NGG 3' downstream to the target site in genomic DNA. The IDT crRNA designing tool software gave all possible gRNA for the input gene sequence adjacent to the PAM region. The gRNAs for E6 and E7 (Fig 4.3) were the ones with a suitable off target as well as on target score.

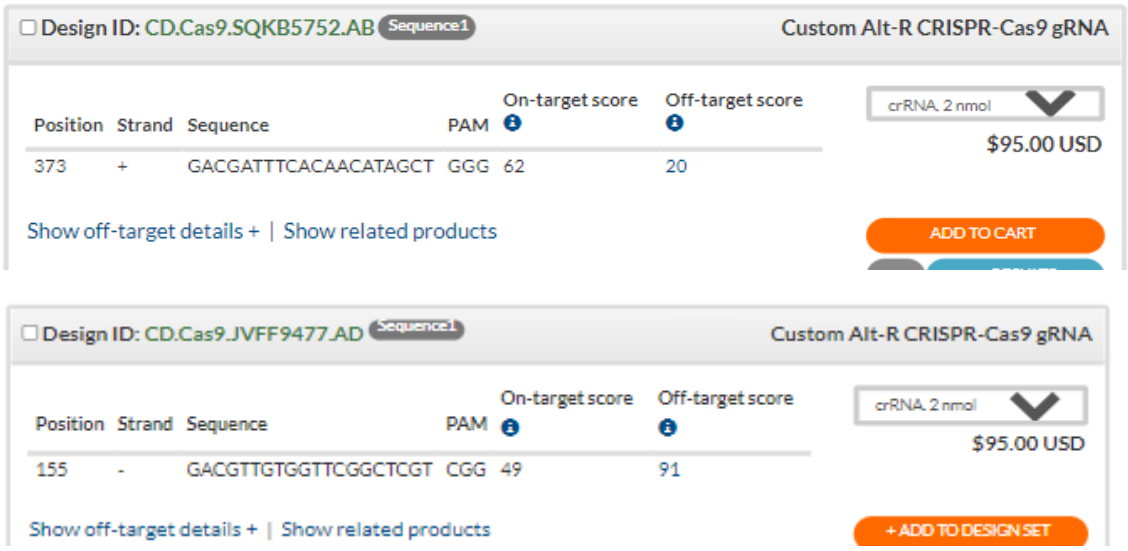


Figure 4.3. gRNA for E6 (above) and E7 (below) genes from IDT crRNA design tool

The off targets for these gRNA sequences were further analyzed on CRISPR RGEN tools software (CasOFFinder) for both E6 and E7 genes. The most suitable gRNA sequences at 373 nt position (+) strand for E6 and at 155 nt position (-) strand for E7 were selected as they had 40-70% GC content and low off targets. The E6 gRNA has 2 off-targets and E7 gRNA has no off-targets with 3 mismatches. The final sequences of gRNA for both genes after adding 10 any nucleotides (N) to their 5' ends are mentioned as follows:

gRNA E6	5' GTAATAGAAC**GACGATTTCACAACATAGCT** 3'
gRNA E7	5' GACATTGTGT**GACGTTGTGGTTCGGCTCGT** 3'

4.2.3. In silico cloning of Vector and gRNA

The gRNA sequence was cloned in the pX260 vector on SnapGene software before ordering and proceeding for wet lab procedures for cloning. After successful in silico cloning, the pX260 vector and gRNAs were ordered. The gRNAs were ordered in form to forward and reverse DNA oligos. The sequences of these oligos are mentioned in the following table.

E6 gRNA antisense (36bp)	5' TAAAACAGCTATGTTGTGAAATCGTCGTTCTATTAC 3'
E6 gRNA sense (36bp)	5' AAACGTAATAGAACGACGATTTCACAACATAGCTGT 3'
E7 gRNA sense (36bp)	5'AAACGACATTGTGTGACGTTGTGGTTCGGCTCGTGT 3'
E7 gRNA antisense (36bp)	5' TAAAACACGAGCCGAACCACAACGTCACACAATGTC 3'

Both gRNAs were reverse translated to double stranded DNA with its sequence being on the anti-sense strand. The overhangs for the Bbs1 restriction sites were added to both DNA sequences on either end then they were cloned separately in a Bbs1 digest vector. The sequences of gRNAs cloned downstream to human U6 promoter and between direct repeats are shown in Fig. 4.4 and 4.5 for E6 and E7 respectively.

Figure 4.4. Cloned E6 gRNA in pX260.
E6 gRNA cloned between the direct repeats of pX260 in dsDNA form. Red nucleotides indicate the overhangs added to the sequence for cloning in BBs1 restriction sites.

Figure 4.5 . Cloned E6 gRNA in pX260.
E7 gRNA cloned between the direct repeats of pX260 in dsDNA form. Red nucleotides indicate the overhangs added to the sequence for cloning in BBs1 restriction sites.

4.3. Propagation of pX260

The pX260 vector received from DNA custom technologies was propagated by electroporation in electrocompetent E.coli DH5α cells and then culturing them on ampicillin positive plates. Results showing pulse generated in electroporator (Fig.4.6.).

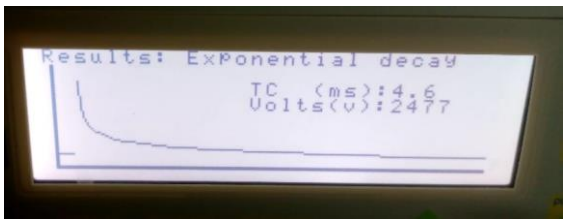


Figure 4.6. Pulse generated during electroporation.
2.5kV of current given at a time interval of ~5 seconds results in successful electroporation of plasmid.

The presence of colonies on ampicillin positive plate (Fig. 4.7) indicates successful electroporation along with a negative control electroporated without plasmid.

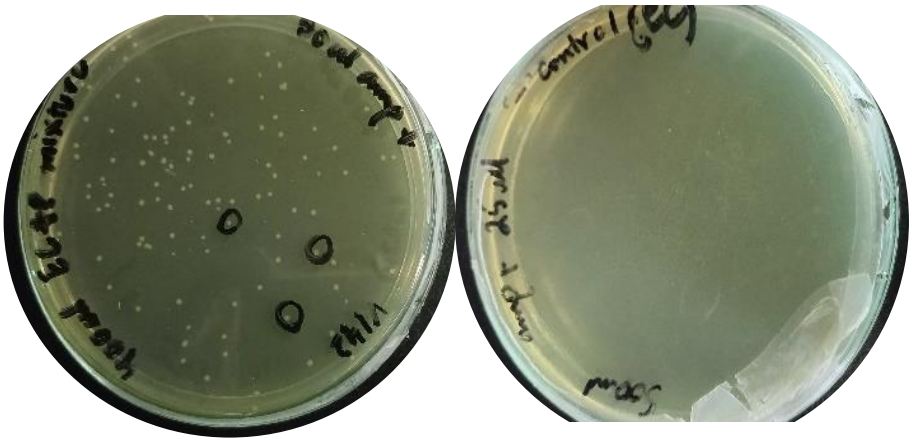


Figure 4.7. pX60 positive colonies.
pX260 positive colonies (left) and negative control cells electroporated without plasmid (right) on ampicillin positive plates.

4.4. Quality and quantity assessment of plasmid after miniprep

The plasmid was extracted from overnight inoculum of transformed colonies in LB broth by alkaline lysis method. Gel electrophoresis after plasmid miniprep was performed visualized in the UV transilluminator (Fig 4.8) indicated bands at ~8000 bp with 1kbp ladder as reference confirming the isolation of plasmid (~10,000 bp) in desired super coiled form which appears below its original size due to supercoils.

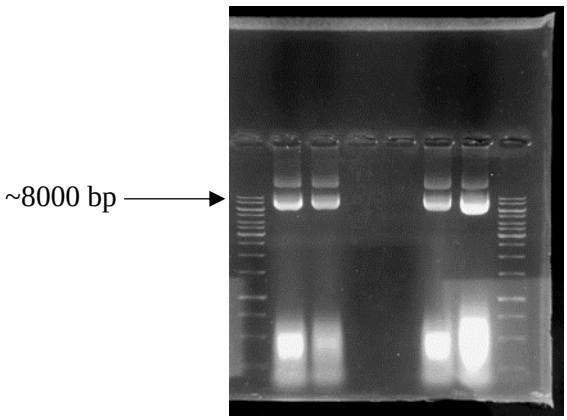


Figure 4.8. Gel electrophoresis of pX260 plasmid.
Supercoiled plasmid bands at ~8000bp with a 1kbp DNA marker on 1% TAE agarose gel.

The quantification by nanodrop spectrophotometric analysis was also done and reveals a good concentration of 1949.98ng/µl as well a good purity as indicated by A260/A3280 ratio of 1.97 Fig. 4.9.

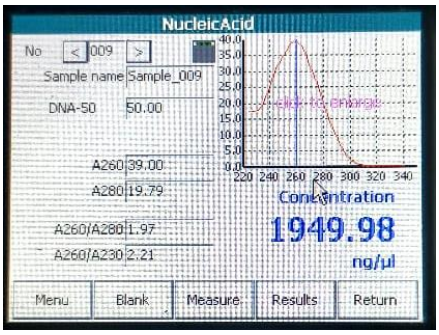


Figure 4.9 DNA quantification
Purity and concentration of plasmid. A good A260/A280 and A230/A260 ratio of 1.97 and 2.21 respectively on nanodrop spectrophotometer indicates the purity of plasmid.

4.5. Confirmation of plasmid

After extraction of plasmids, the confirmation was done by PCR and restriction digestion. pX260 plasmid has two restriction sites for EcoR1 enzyme at nt position 5473 and 6168. The restriction digested product, along with uncut vector as a control generated two bands at 695 bp and 9528 bp position below the 3rd well from right clearly under the UV transilluminator when analyzed on gel resulting from the cuts at the aforementioned positions indicated in Fig.4.10.

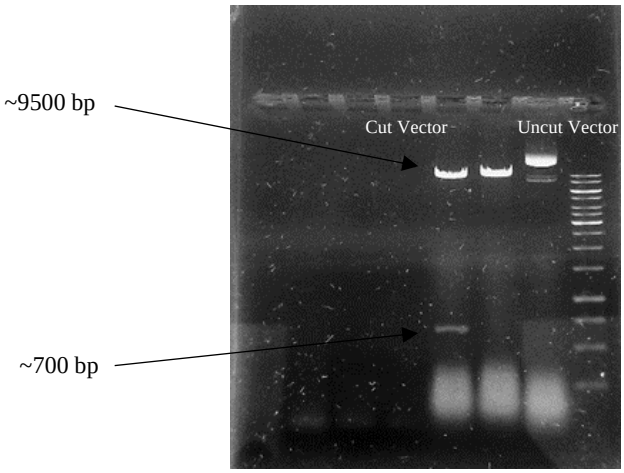


Figure 4.10. Restriction digestion of pX260 by EcoR1.
The two bands a 695 bp and 9528 bp after the plasmid is cut by EcoR1 at two different sites and run on 1% agarose gel with 1kb DNA marker.

Also, the PCR reaction with the external primers showed a distinct band of 247 bp when analyzed on 2% agarose gel and visualized under transilluminator indicated in Fig.4.11.

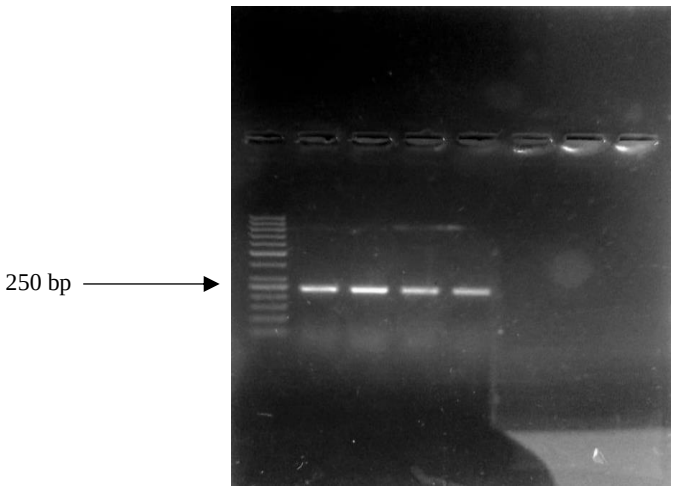


Figure 4.11. PCR confirmation with external primers.
The PCR of pX260 with external primers resulted in a 247 bp amplicon that can be visualized on 2% 1XTAE agarose gel with 50bp DNA marker.

4.6. Restriction digestion with Bbs1 enzyme

For the purpose of cloning, the vector must be cut open with sticky ends where the insert can get ligated. Therefore, the plasmid was digested with Bbs1 enzyme. The restriction sites for Bbs1 enzyme are located on the 283 and 305 nt positions. The restriction digestion with Bbs1 enzyme for 16 hrs resulted in the generation of a band at 10,201 bp and the small band of 22 bp was run off the gel when visualized under transilluminator indicated in Fig. 4.12.

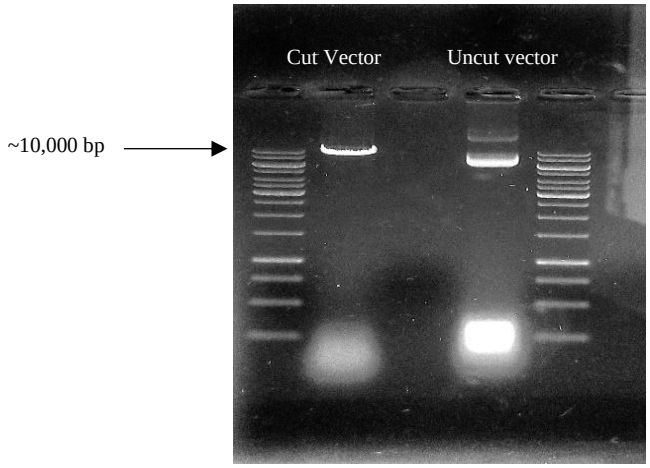


Figure 4.12. Restriction digestion of pX260 by Bbs1.
The band a 10,201 bp and the plasmid is cut by Bbs1 at two different sites compared with the uncut control plasmid at 8000 bp.

4.7. Heterodimer confirmation of oligonucleotide annealing

For generation of a double stranded DNA insert for gRNA that is to be cloned in the vector, the forward and reverse oligos after were annealed as mentioned in section 3.12 and visualized under UV transilluminator after running them on 2% agarose gel electrophoresis (Fig. 4.13).

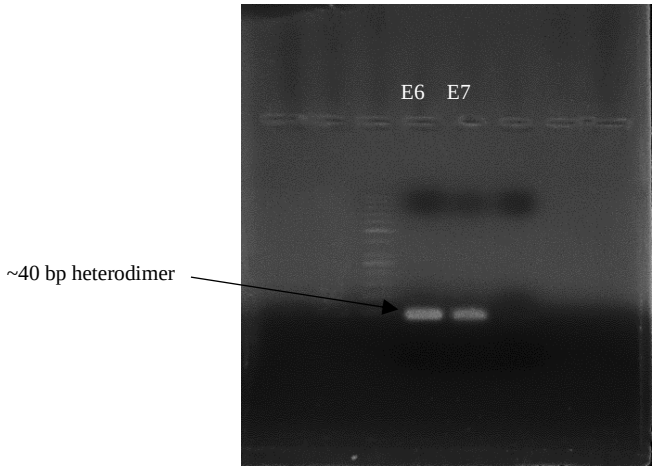
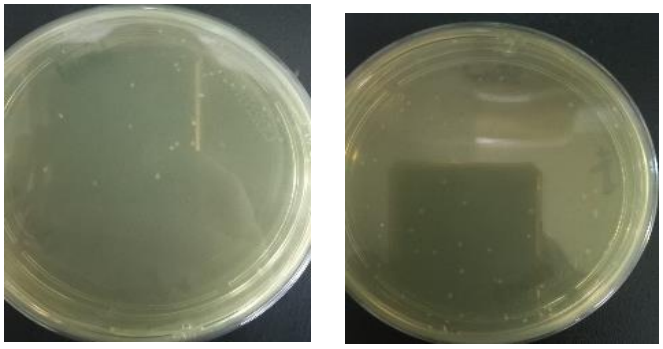


Figure 4.13. Dimerization of sense and antisense oligos of gRNAs.
The heterodimer formation confirmed on gel electrophoresis at the position below 50bp band (~40bp).

4.8. Heat shock transformation of ligated vectors

The ligation was confirmed when the ligase mix after heat inactivation was directly transformed in the heat shock competent E.coli DH5alpha cells. The transformed cells when cultured on ampicillin positive plates resulted in colonies that contained the ligated plasmid. The presence of colonies on ampicillin positive plates for the ligated gRNA-CRISPR Cas 9 vector for E6 and E7 genes are shown in Fig 4.14.



E6 ligation colonies E7 ligation colonies

Figure 4.14. Ligation confirmation after Heat-shock transformation
The ligase mix transformed in heat shock competent cells resulted in the formation of distinct isolated colonies after 16 hr incubation at 37°C on ampicillin positive plates.

4.9. Confirmation of ligation by PCR and Sanger sequence analysis

The overnight cultures from the isolated colonies on both E6 and E7 plates were subjected to miniprep and the confirmation of isolated E6- and E7- gRNA-CRISPR Cas 9 vectors was done by PCR amplification by external primers. PCR reaction was analyzed by gel electrophoresis. The bands obtained (Fig. 4.15) were cut with a sterile blade and the PCR product was purified with a Thermofisher Scientific Gel Purification Kit according to manual.

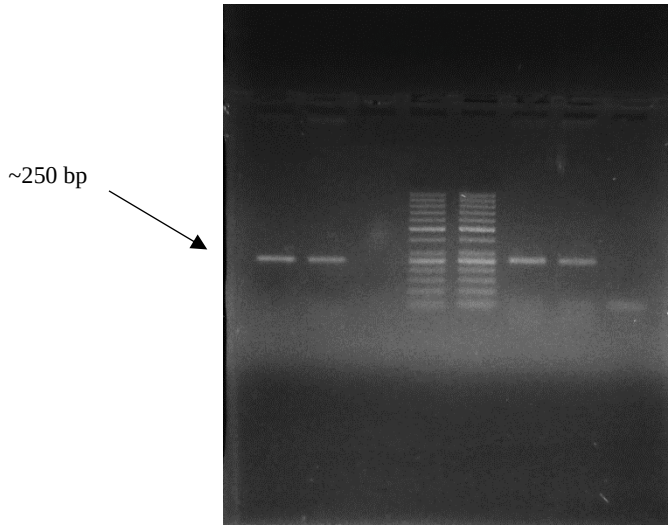


Figure 4.15. PCR amplification with external primers.
Ligated E6 (Left) and E7 (right) gRNA-CRISPR Cas9 vectors confirmed by PCR resulting in ~250 bp bands with a 50bp DNA marker.

The purified products were sent to Eurofins for Sanger sequencing. The chromatogram for the amplified regions of both vectors confirmed the successful cloning of both gRNAs in pX260 vector Fig 4.16 and 4.17. Successful cloning was confirmed when the sequence of vectors was aligned with the intended sequence of vectors ligated *in silico.* The vectors were named as E6 gRNA pX260 and E7 gRNA pX260.

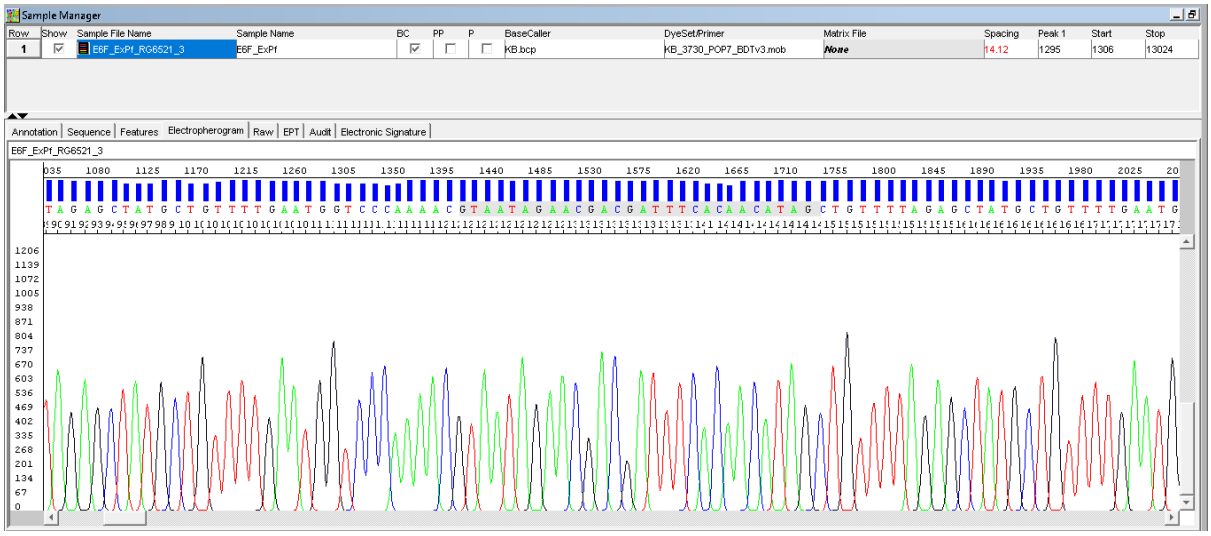


Figure 4.16. Electropherogram for E6 gRNA CRISPR cas 9 vector

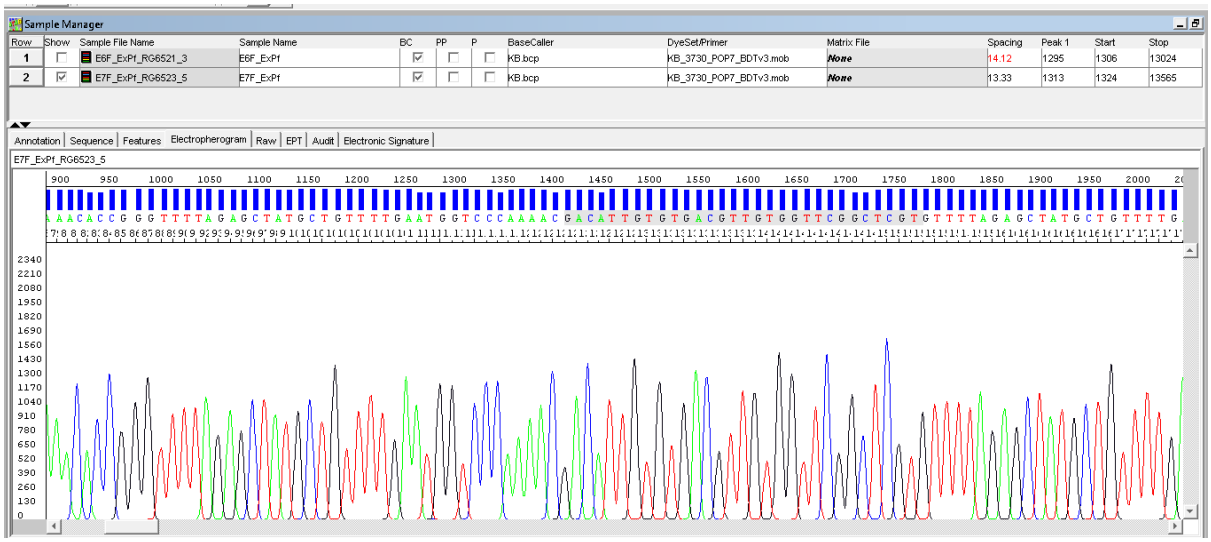


Figure 4.17. Electropherogram for E7 gRNA CRISPR cas 9 vector

CHAPTER 5

DISCUSSIONS

As discussed earlier, cervical cancer is a global health threat worldwide contributing to a significant number of cases diagnosed every year and has a high mortality rate. Many of these cases are caused by high risks HPVs with HPV-16 and HPV-18 being the elusive etiological agents. The deregulated expression of oncogenes of HPV due to persistence infection are the main trigger for malignant transformation and the cervical cancer cells exhibit an over dependence on these oncogenes wherein any perturbation of modification in these genes can have deleterious impacts on cancer cell health. The standard treatment is cisplatin-based chemotherapy, surgical hysterectomy, radiation therapy and various immunotherapeutic drugs are also administered to manage cervical cancer. But none of them targets viral pathogenic genes specifically being the only reason to contribute to the risk of recurrence rate that is extremely high around 25-40% (Khairkhah et al., 2022). Therefore, a specific targeted treatment is the need of the hour for complete eradication of viral infection that breaks the persistence resulting in cancer.

The deployment of CRISPR-Cas9 technology has opened newfound avenues in the realm of genetic engineering presenting the ability to selectively manipulate genes with unparallel accuracy. In this study, the focus was on the construction of gRNA-CRISPR-Cas 9 vectors to target the E6 and E7 oncogenes of HPV-18 with the goal to establish a potent approach for combating viral infection effectively. The core findings of the study were the designing and assembling of gRNA vectors selectively targeting the crucial segments of HPV genome.

There are so many advancements made in the field of gene therapy and so many approaches to target pathogenic genes have been optimized by scientists. A few of them being the mega nucleases, zinc finger nucleases and TALENs. Mega nucleases are the restriction enzymes that

are modified genetically possessing the ability to locate extended segments of DNA sequences ~14-40 bp and bind to them specifically targeting that genomic region. But mega nucleases have major setbacks as each one recognized only specific DNA sequence decreasing the likelihood of finding a mega nuclease from 100s of naturally occurring one that specifically targets desired gene locus limiting scientist to engineer only a few specific genomes (Silva et al., 2011). Zinc finger nucleases (ZFNs) when used in a combinatorial approach of 6-7 zinc fingers assembly complexed with Fok1 endonuclease also resulted in specific targeting of a 18-24 bp stretch. transcription activator-like effector (TALE) proteins worked in the same way recognizing a single nucleotide base pair and a coupling assembly of TALE with Fok1 nuclease generated a functional programmable nuclease (TALEN). Scientists were successful in significantly enhancing the efficiency of genome editing by sequential developments regarding artificially engineered mega nucleases, ZFNs and TALENs, however there were still certain limitations in using these tools (Gaj et al., 2013). The process of targeting various genomic sites demanded the redevelopment of another fresh group of proteins. The limited adoption of these tools throughout the scientific community can therefore be attributed to the challenges in protein engineering necessitated by these techniques.

The latest progress in the elucidation of CRISPR-Cas systems in the field of genetic biomedicine has opened doors to facilitate the modification of genes with significant efficacy and cost effectiveness. It is simpler to use as it employs the strategy of RNA-DNA interaction to target genes rather than protein-DNA interactions (Adli, 2018). The current study entails a meticulous selection procedure of gRNAs for the computational prediction and designing gRNAs incorporating several aspects like efficiency and *in silico* evaluation of off-target effects. The software tool used for designing gRNAs was IDT crRNA deign tool that gave all the possible gRNA for the input target sequence and predicts their off-target and on-target scores. The selected gRNAs have a comparable off-target and on target score. The further

evaluation of off targets was analyzed on CasOFFinder CRISPR RGEN tools. The gRNAs used in the underlying study have 2 off-targets for E6 gRNA and no off-targets for E7 gRNA.

The pX260 plasmid used in the study utilized a tracrRNA of 89 nucleotides expressed under a U6 promotor and presents another cassette for the expression of 30 nucleotide spacer under another U6 promotor. This strategy is reported to better in editing efficiency as compared to the strategy where tracrRNA is expressed from gRNA scaffold and the resultant is a chimeric gRNA (crRNA + tracrRNA). The requirement of PAM (Cong et al., 2013).

The gRNAs were subsequently cloned in the Bbs1 restriction sites of pX260 and sanger sequencing further validated the successful incorporation of gRNAs in the vectors.

Nevertheless, the effective application of CRISPR-Cas9 technology in targeting HPV oncogenes is not without its share of obstacles. One significant obstacle that arises is the possibility of off-target effects, even in cases when gRNAs have been meticulously designed and chosen. The occurrence of off-target effects may give rise to inadvertent modifications in genes that are not the intended targets, which might possibly result in unfavourable cellular outcomes. Hence, it is crucial to conduct a comprehensive evaluation and verification of the specificity of the chosen gRNAs prior to contemplating any potential clinical implementations. The development of novel methods for designing gRNA and ways to enhance specificity might potentially address this challenge to a greater extent.

Chapter 6

CONCLUSIONS

In summary, our research makes a valuable contribution to the growing field of CRISPR-Cas9 applications by establishing the vectors that can target E6 and E7 oncogenes. The successful design of the guides, evaluation of off-targets *in silico* and construction of the vectors in wet lab that are validated by sequencing can be a candidate to be a therapeutic in replacement of CRT and radiotherapy for cervical cancer caused by HPV-18. The ongoing advancement of genetic engineering has presented a great opportunity for CRISPR-Cas9 to significantly impact cancer therapy. However, the assessment of this technology *in vitro* to test its efficacy and its use in clinical settings necessitates collaborative efforts across several disciplines to effectively convert research discoveries into practical solutions.

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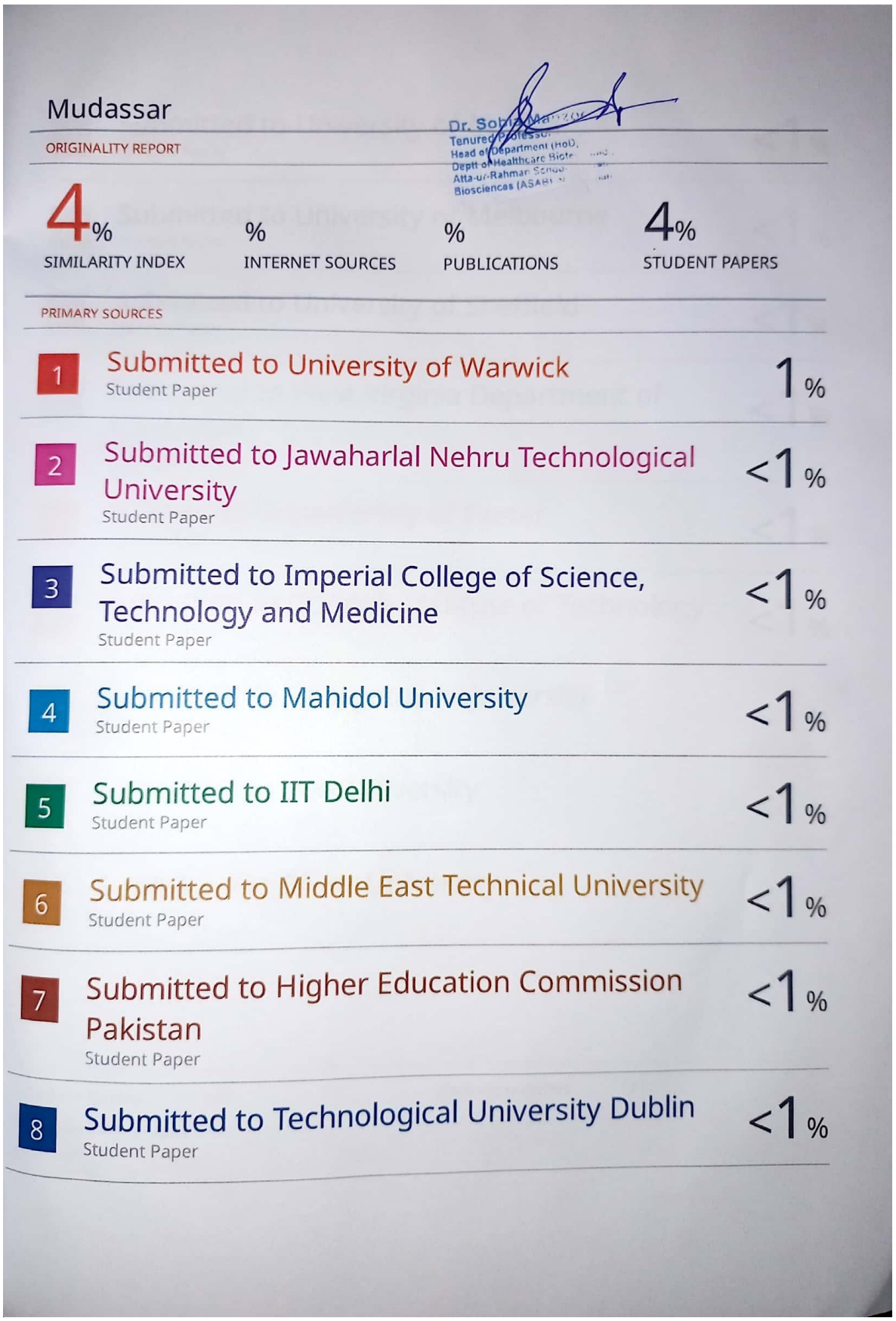

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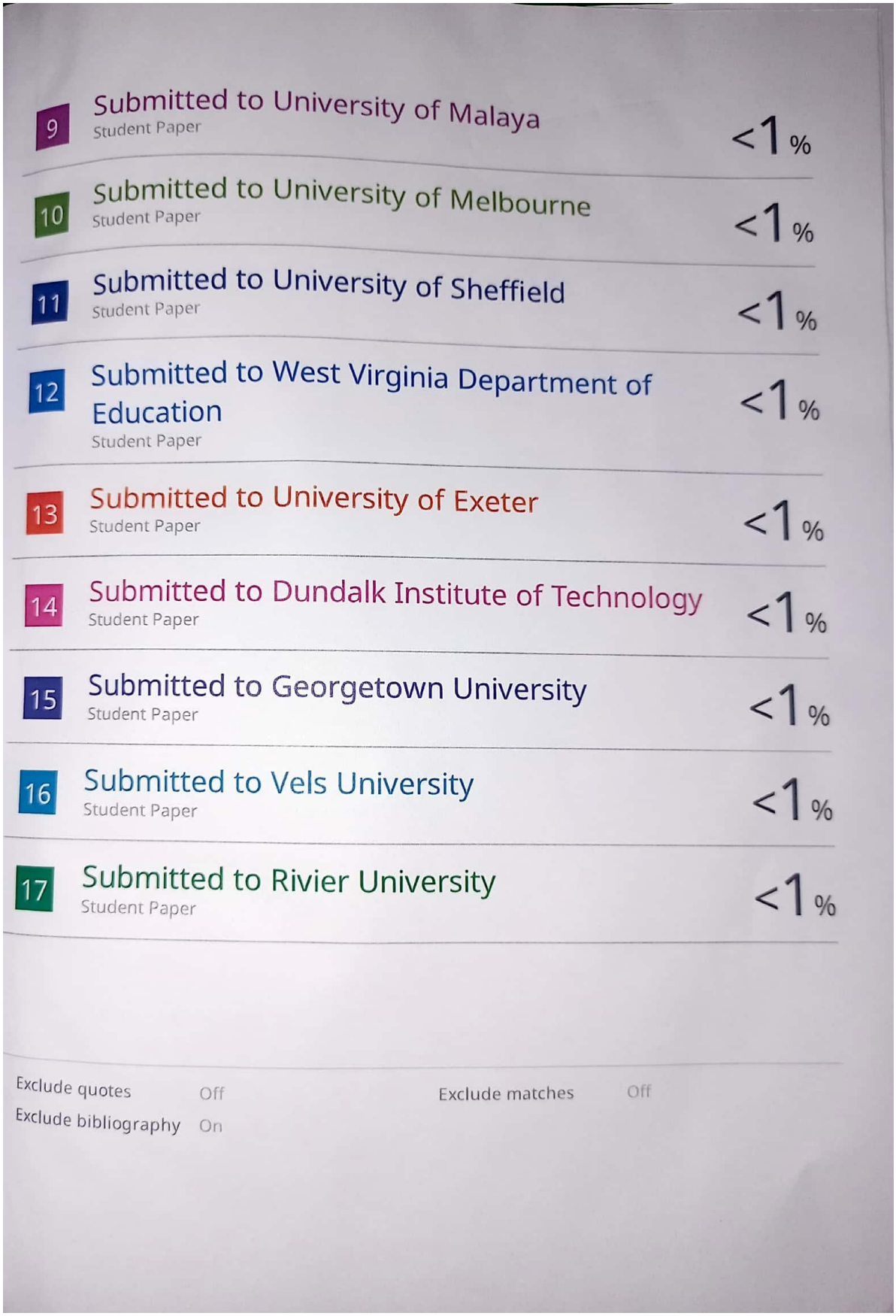

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