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Ciências

Obtaining a plasmid DNA vaccine against HPV

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“I want to dedicate this work to my parents who have always supported me throughout my career”

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Resumo Alargado

Com a descoberta do genoma Humano tem sido cada vez mais fácil identificar e estudar diferentes genes implicados em diversas doenças tais como o cancro, doenças cardiovasculares, doença de Parkinson e doença de Alzheimer. Estas e inúmeras outras doenças, nomeadamente infecciosas, têm sido responsáveis pela morte de milhões de pessoas devido à ausência de um tratamento eficaz e pouco agressivo. Por esta razão, a investigação biomédica tem focado cada vez mais a procura de abordagens terapêuticas alternativas.

O desenvolvimento de vacinas de DNA tem como objetivo induzir uma resposta imunitária que permita a prevenção ou tratamento de infeções provocadas por vírus, bactérias e parasitas. Uma das estratégias preferencialmente utilizadas com esta finalidade baseia-se na utilização de DNA plasmídico (pDNA) contendo genes que codificam um antigénio. Posto isto, torna-se necessário criar estratégias eficientes de purificação para obter o pDNA puro de maneira a poder ser aplicado terapêuticamente. Assim, a cromatografia de afinidade tem sido cada vez mais aplicada como estratégia de purificação, devido à elevada seletividade obtida pela isoforma superenrolada (sc) do pDNA, permitindo não só isolar esta isoforma de outras menos eficazes biologicamente (circular aberta, linear), mas também eliminar impurezas que poderão ser tóxicas para os seres humanos. Os suportes monolíticos surgiram como uma ótima adjuvante na purificação de pDNA, juntamente com os ligandos de afinidade, pois permitem uma separação mais rápida e eficiente, assim como elevadas capacidades de ligação.

O vírus do papiloma humano (HPV) é um dos vírus sexualmente transmissíveis mais comuns que causam várias lesões epiteliais, que podem conduzir ao desenvolvimento de cancro do colo útero. Existem diferentes tipos de HPV que estão associados a diferentes tipos de riscos, mas cerca de 70% dos casos de cancro cervical são atribuídos ao HPV16 e 18 de alto risco. O passo inicial da progressão do cancro é a imortalização de células epiteliais normais. A carcinogenicidade das infeções causadas pelo HPV16 estão associadas às proteínas E6 e E7 do vírus, que atuam na desregulação do ciclo celular através da inibição das proteínas supressoras de tumores como a p53 e a proteína retinoblastoma (pRb), respetivamente.

O nosso grupo de investigação implementou a produção e purificação da vacina de pDNA sc HPV16 E6/E7, através da utilização de um monolito modificado com ligandos de arginina. Para garantir que os antigénios E6 e E7 não irão ser responsáveis pela imortalização das células transfetadas, no presente trabalho bloqueou-se o potencial oncogénico das proteínas E6 e E7 através da introdução de mutações pontuais, que à partida irão inibir as interações destas proteínas com a p53 e a pRb. A modificação do plasmídeo deu origem ao pDNA HPV16 E6/E7^{MUT}, sendo crucial a sua produção, extração e purificação para posterior aplicação

terapêutica. Assim, recorreu-se à estratégia biotecnológica implementada anteriormente pelo grupo de investigação, para o plasmídeo não mutado, tirando proveito da cromatografia de afinidade, utilizando o monolito modificado com ligandos de arginina. Posto isto, verificou-se que as condições de produção e purificação para o pDNA HPV16 E6/E7^{MUT} não mudaram significativamente em relação à estratégia já utilizada anteriormente. Foi obtida uma pureza de 100% e uma recuperação de 82%, encontrando-se dentro dos valores obtidos anteriormente para o outro plasmídeo.

Após a otimização da estratégia de purificação, foram quantificadas as impurezas, DNA genómico (gDNA), RNA, endotoxinas e proteínas, verificando-se uma diminuição significativa do teor de impurezas da amostra inicial de lisado para a amostra purificada de pDNA sc. Todos os valores de impurezas se encontram dentro dos limites recomendados pelas agências reguladoras como a FDA.

Por fim, foram realizados estudos de transfeção *in vitro* com fibroblastos de modo a avaliar a eficiência de transfeção celular e a expressão das proteínas codificadas pelos genes mutados contidos na vacina de pDNA. De modo a que estes parâmetros fossem avaliados, recorreu-se à utilização das técnicas de, transcrição reversa, seguida de reação em cadeia de polimerase em tempo real (RT-qPCR) e Western blot. Com isto, foi possível verificar que o processo de transfeção ocorreu com sucesso, observando-se um aumento do RNA mensageiro E6 e E7 que posteriormente se traduz num aumento da expressão das proteínas E6 e E7 em comparação com as células não transfetadas.

Palavras- chave

Cromatografia de afinidade, monolito de arginina; isoforma superenrolada; plasmídeo HPV16 E6/E7^{MUT}; transfeção, vacinas de DNA

Abstract

The Human papillomavirus (HPV) infection is one of the most common sexually transmitted virus and is responsible for various human epithelial lesions, including cervical cancer. The initial step for cancer progression consists in immortalization of normal epithelial cells. The carcinogenicity of HPV16 infections is associated with E6 and E7 proteins, which deregulate the host cell cycle by inhibiting tumor suppressor protein (p53) and retinoblastoma protein (pRb), respectively. DNA vaccines induce an immune response which allows the prevention or treatment of infections by viruses, bacteria and parasites. One of the best strategies used for this purpose is the plasmid DNA (pDNA). Our research group has been studying a way to effectively develop the production and purification of sc pDNA vaccine HPV16 E6/E7 through the use of a monolith modified with arginine ligands. However, it is necessary to ensure that these proteins are unable to induce cell immortalization. Thus, the E6 and E7 protein oncogenic potential was removed by introducing point mutations, which inhibit interactions of these proteins with p53 and pRb. Afterwards, affinity chromatography was used to purify sc HPV16 E6/E7^{MUT} pDNA, taking advantage of a purification strategy with arginine monolith, previously explored by our research group for the non-mutated plasmid. A purity degree > 99% and a recovery yield of 82% was achieved, which were comprised within the values obtained in our group. After optimization of the purification strategy, impurities such as genomic DNA (gDNA), RNA, endotoxin and proteins were quantified. A significant decrease in impurities of purified sc pDNA sample was observed when compared to initial lysate sample. Finally, *in vitro* transfection studies were performed to evaluate the fibroblast cell transfection efficiency and the subsequent expression of the target proteins, encoded by the genes contained in the pDNA vaccine. These results allowed us to conclude that the transfection process was successful, leading to increased E6 and E7 protein expression compared to non-transfected cells.

Keywords

Affinity chromatography, arginine monolith; DNA vaccines; supercoiled isoform; HPV16 E6/E7^{MUT} plasmid; transfection

Table of Contents

Chapter 1 - Introduction	1
1.1 Human Papillomavirus	1
1.1.1 Molecular biology and life-cycle of human papillomaviruses.....	2
1.1.2 Human papillomavirus E6 oncoprotein	4
1.1.3 Human papillomavirus E7 oncoprotein	5
1.2 Human papillomavirus 16 E6/E7 and cell immortalization	5
1.2.1 Telomerase activity and expression of the catalytic subunit hTERT	6
1.3 Human papillomavirus treatment: how far have we got?	7
1.3.1 Preventive human papillomavirus vaccine	7
1.3.2 Therapeutic human papillomavirus vaccine	8
1.4 DNA-based therapy	9
1.4.1. Gene therapy	9
1.4.2 DNA vaccination	10
1.5 Biological and non-biological vectors	12
1.5.1 Biological delivery systems	12
1.5.2 Non- biological delivery systems	13
1.6 Plasmid DNA as a non-biological delivery system	14
1.6.1 Plasmid DNA production process	14
1.6.2 Chromatographic purification.....	15
1.7 Monolithic supports: the new approach	17
1.8 Multiple applications for plasmid DNA	18
Chapter 2 - Materials and methods	19
2.1 Construction of HPV16 E6/E7 ^{MUT} pDNA.....	19
2.1.1 Transformation <i>E.coli</i> DH5a	20
2.1.2 Confirmation of the mutated fragment insertion	20
2.1.3 Production of HPV16 E6/E7 ^{MUT} pDNA	20
2.2 Purification of HPV16 E6/E7 ^{MUT} pDNA.....	20
2.2.1 Alkaline lysis and Purification with NZYTech Kit	20
2.2.2 Modified alkaline lysis	21
2.2.3 Affinity chromatography	21
2.2.4 Agarose gel electrophoresis	22
2.2.5 Plasmid quantification	22
2.2.6 Quantification of Proteins	23

2.2.7 Quantitation of genomic DNA	24
2.2.8 Endotoxin quantification	24
2.3 Cell culture	25
2.3.1 Cell transfection.....	25
2.3.2 RNA extraction.....	26
2.3.3 Protein extraction.....	26
2.4 Western blot.....	27
2.4.1 Protein quantification	27
2.4.2 Polyacrylamide gel electrophoresis	27
2.4.3 Electroblothing.....	28
2.5 cDNA synthesis	28
2.6 Reverse transcription polymerase chain reaction.....	29
2.7 Real time reverse transcription polymerase chain reaction	29
Chapter 3 - Results and Discussion	29
3.1. Construction of the HPV16 E6/E7 ^{MUT} pDNA.....	31
3.2 Transformation of cells and cloning Confirmation.....	35
3.3 Plasmid amplification in recombinant <i>E. coli</i> DH5a	35
3.4 Supercoiled pDNA purification from the clarified <i>E. coli</i> lysate.....	37
3.5 Analytical parameters for plasmid quality assessment	41
3.6.1 Reverse transcription polymerase chain reaction	45
3.6.3 Real time reverse transcription polymerase chain reaction	46
3.6.4 Western Blot	47
Chapter 4 - Conclusions and Future Perspectives.....	51
References	53

List of Figures

Figure 1- Function, size and typical structural arrangement of HPV genome	3
Figure 2 - HPV16 life cycle..	3
Figure 3- p53 degradation pathway induced by HPV16 E6 through ubiquitin/proteasome targeting.....	4
Figure 4 - HPV16 mechanism for cell immortalization	6
Figure 5- Gene therapy clinical trials in 2013.....	10
Figure 6 - Mechanisms of antigen presentation after DNA immunization	11
Figure 7- Plasmid DNA manufacture process	15
Figure 8 - Schematic representation of HPV-16 E6/E7 pDNA.....	19
Figure 9- Reference curve of plasmid DNA standards (1-50 µg/mL).	23
Figure 10 - Reference curve of protein standards (20-2.000 µg/mL).	23
Figure 11 - Reference curve of E. coli DH5α genomic DNA standards (0.005 - 50 ng/µL).....	24
Figure 12- Reference curve of endotoxin standards (0.005-0.1 EU/mL).	25
Figure 13- Reference curve of Bradford methods (0-2 µg/µL).	27
Figure 14 - DNA sequence alignment of wild-type HPV16 E6/E7 pDNA and HPV16 E6/E7 ^{MUT} pDNA	33
Figure 15 - E6 protein with required mutations	34
Figure 16 - E7 protein with required mutations	34
Figure 17 - Cloning Strategy.....	34
Figure 18 - Confirmation of the modified fragment by electrophoresis.....	35
Figure 19 - Bacterial growth curve of E.coli DH5α transformed with HPV 16 E6 / E7 ^{MUT} plasmid	36
Figure 20 - Bacterial growth curve of E.coli DH5α transformed with non-mutated plasmid ..	36
Figure 21- Chromatographic assay of lysate sample of HPV16 E6/E7 ^{MUT} pDNA with arginine monolith.....	37
Figure 22- pDNA sample injected into the arginine monolith.....	38
Figure 23- Purification of pDNA with arginine monolith	39
Figure 24- pDNA sample injected into the arginine monolith.....	39
Figure 25- Purification of pDNA with arginine monolith	40
Figure 26 - pDNA sample injected into the arginine monolith	40
Figure 27 - Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: E. coli lysate	41
Figure 28- Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: impurities fraction.....	42
Figure 29- Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: sc pDNA fraction.	43

Figure 30- Analytical chromatographic profiles of different pDNA- Blank.....	43
Figure 31- RT - PCR of mRNA E6.....	46
Figure 32 - RT- PCR of mRNA E7.....	46
Figure 33- Trend graph of expression of mRNA E6 and mRNA E7 for both mutated and non-mutated plasmids at different hours of transfection (24 hours and 48hours).	47
Figure 34- Western Blot to evaluate de expression of protein E7	48
Figure 35 - Western Blot to evaluate de expression of protein E6	48
Figure 36 - Western Blot for protein E7	48
Figure 37- Western Blot for protein E6	48
Figure 38 - Western Blot for β -actin corresponding to injected samples in Figure 35	49
Figure 39- Western Blot for β -actin corresponding to injected samples in Figure 36.	49
Figure 40 - Trend graph of E6 and E7 protein expression (in percentage) for both mutated and non-mutated plasmids at different hours of transfection (48 Hours and 72 Hours).	49
Figure 41- Western Blot for E6 protein staining.	50

List of Tables

Table 1 - Division of infections in the mucosa by HPV risk.....	1
Table 2 - The five evolutionary groups of HPV with different epithelial tropism and disease associations	2
Table 3 - HPV preventive vaccine characteristics	8
Table 4 - Advantages of DNA vaccines.....	11
Table 5 - Advantages and disadvantages of biological vectors.....	12
Table 6 - Modifications required in E6 and E7 proteins.	32
Table 7 - Purity degree and recovery yield of sc pDNA.....	44
Table 8 - Impurity levels of clarified E.coli lysate and purified sc pDNA.....	44

List of Acronyms

AEX	Anion exchange chromatography
APC	Antigen presenter cells
ASO4	3-O-desacyl-4'-monophosphoryl lipid A and aluminum hydroxide
BCA	Bicinchoninic acid
CBP	CREB Binding Proteins
CR	Conserved Regions
CREB	cAMP Response Element Binding
DC	Dendritic cells
DEPC	Diethylpyrocarbonate
DNA	Acid Deoxyribonucleic
<i>E.coli</i>	<i>Escherichia coli</i>
E6AP	E6 Association Protein
EDTA	Ethylene-diamine tetraacetic acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
gDNA	Genomic DNA
HIC	Hydrophobic interaction chromatography
HPV	Human papillomavirus
hTERT	Human telomerase reverse transcriptase
IMAC	Immobilized metal affinity chromatography
LAL	<i>Limulus</i> ameocyte lysate
LCR	Long control region
LPS	Lipopolysaccharides
MHC	Major Histocompatibility complex

mRNA	Messenger RNA
Oc	open circular
ORF	Opening reading Frames
p53	Tumor supressor protein
PBS	Phosphate buffered saline
pDNA	Plasmid DNA
pRb	Retinoblastoma protein
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Real -time reverse transcription polymerase chain reaction
Sc	Supercoild
SDS	Sodium dodecyl sulfata
SEC	Size exclusion chromatography
Tris	Tris(hydroxymethyl) aminomethane
VLP	Virus like particles

Chapter 1 - Introduction

1.1 Human Papillomavirus

The human papillomavirus (HPV) infection is one of the most common sexually transmitted diseases and causes various epithelial lesions in humans, resulting in the development of different infections in the genital tract and squamous epithelium, anogenital warts, cervical, vulval and vaginal cancer in women, as well as penile cancer in men. HPV DNA has been detected in 99.7% of patients with cervical cancer, being considered a necessary prerequisite for the disease [1]. Cervical cancer is the second most common cancer, followed by breast cancer, in young women in Europe and affects about 500,000 women each year [1,2].

Up until now, more than 150 types of human papillomavirus have been completely sequenced. These types of papillomavirus can generally be divided into five genera, based on the DNA sequence analysis, life cycle and association with diseases. The most studied are the alpha-papillomavirus genus, which infect both cutaneous and mucosal tissues and can be divided according to their risk to induce cancer as low, intermediate or high risk. As a matter of fact, 70% of cervical cancer cases are attributable to high risk HPV16 and 18 [3]. On the other hand, low risk HPV are generally associated with benign genital warts and are rarely associated with cervical cancer [4], as portrayed in Table 1.

Table 1 - Division of infections in the mucosa by HPV risk (adapted from [5]).

HPV Risk	HPV Types
Low Risk	HPV6, HPV11, HPV42, HPV43, HPV 44
Intermediate Risk	HPV31, HPV33, HPV51, HPV52, HPV58
High Risk	HPV 16, HPV 18, HPV45, HPV56

The remaining four genera, beta-papillomavirus, gamma-papillomavirus, nu-papillomavirus and mu-papillomavirus, only infect cutaneous tissue leading to different clinical situations [2,5,6], as shown in Table 2.

Table 2 - The five evolutionary groups of HPV with different epithelial tropism and disease associations (adapted from [6]).

Genus of papillomavirus	Tissues Infection	Associated disease
Alfa-papillomavirus	Cutaneous	Skin warts
	Mucosal	Low Risk HPV - genital warts High Risk HPV - cervical cancer
Beta-papillomavirus	Cutaneous	Skin cancer in immunosuppressed individuals
Gamma-papillomavirus	Cutaneous	Benign cutaneous lesions, usually detected at oral sites. Rarely found in skin cancer.
Mu-papillomavirus	Cutaneous	Benign cutaneous lesions, usually at palmar and plantar sites. Not associated with cancer.
Nu-papillomavirus	Cutaneous	Benign cutaneous lesions. Occasionally associated with cancer.

1.1.1 Molecular biology and life-cycle of human papillomaviruses

The structural and genomic organization of all the known papillomaviruses are similar. HPV is a small double-stranded DNA virus with an icosahedral capsid, which belongs to the *papillomaviridae* family and contains nearly 7900 base pairs (bp) [5,7]. Its genome contains six early open reading frames (ORFs) and two late ORFs. The early segment encodes E1, E2, E4-E7 proteins, which are involved in genome replication and in the early segment control (Figure 1). E1 and E2 are regulators of viral transcription and replication, while E6 and E7 are oncoproteins that interfere with cell cycle by degradation or inactivation of tumor suppressor proteins, such as p53 and retinoblastoma protein (pRb), respectively [7,8]. The late segment is responsible for encoding capsid structural proteins. L1 is composed of 72 capsomers and L2 is a minor component present in the center of the virion. Transcription and replication of capsid proteins are mostly regulated via cis-responsive elements in a long control region (LCR) located between the L1 and E6 genes, while some other regulatory elements are positioned within genes. The LCR size can change in different HPV [2,5,8].

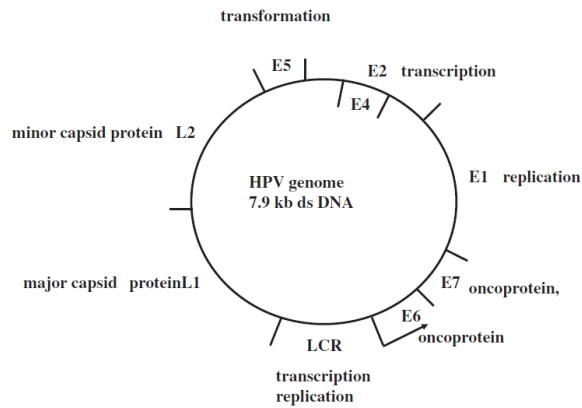


Figure 1- Function, size and typical structural arrangement of HPV genome [8].

HPV life cycle is directly associated to epithelial cell differentiation, as shown in Figure 2. Viral infection occurs through microtraumas to the epithelium, leading to the basal cell exposure and consequent virus entry. Upon basal layer access, HPV genome is established as an episome, with approximately fifty copies per cell, replicating simultaneously with the host cell DNA. Establishment of the HPV genomes is related to E6, E7 and E5 oncoproteins expression as well as E1 and E2 proteins, which are responsible for the replication process. During cell division, basal cells migrate from the basal layer to the suprabasal layer, where differentiation is initiated. Unlike uninfected cells, the HPV-infected cells enter the S phase of the cell cycle, resulting in the amplification of thousands of viral genome copies per cell. Concomitantly, E1 and E4 protein synthesis occurs together with the capsid protein expression, L1 and L2, leading to virion assembly. Subsequently, the virion is released through shedding of the upper layer of the epithelium [9,10].

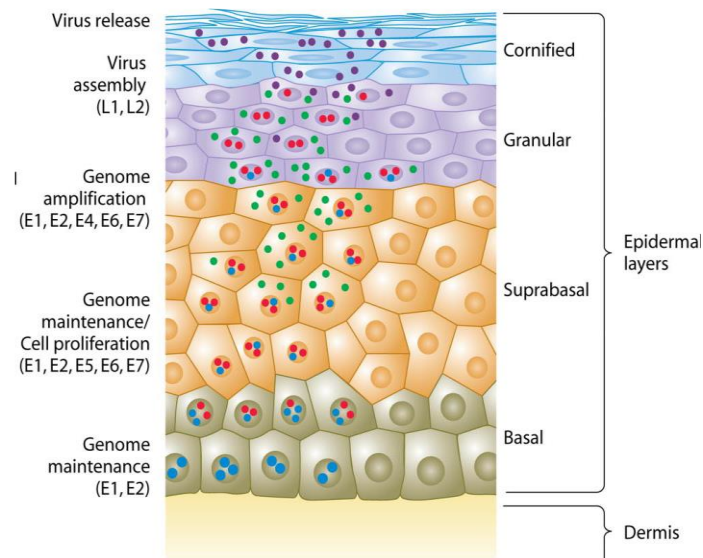


Figure 2 - HPV16 life cycle. The colors show the type of protein that is involved in the cycle: Blue - E1 and E2; red - E5, E6, E7; Green - E4; Purple - L1 and L2 [10].

1.1.2 Human papillomavirus E6 oncoprotein

The E6 oncoprotein contains 150 amino acids with a molecular weight of 16-18 kDa, which presents four motifs with two cysteines (Cys-XX-Cys), capable of forming two domains linked to zinc and enabling important functions such as the activation of transcription, transformation, immortalization and association with cellular proteins [11]. One of the main functions of the E6 protein is the degradation of p53 protein, which represents a key step in cervical carcinogenesis. This cellular protein is a transcription factor that ensures the integrity of the genome, preventing cell division through a mechanism called apoptosis, in response to cellular stress, DNA damage, or hypoxia. The degradation of p53 occurs via E6 binding to ubiquitin ligase, known as the E6-associated protein (E6AP), as portrayed in Figure 3. The complex E6/E6AP binds to the central region, called the p53 core domain, targeting p53 for proteasome degradation [12].

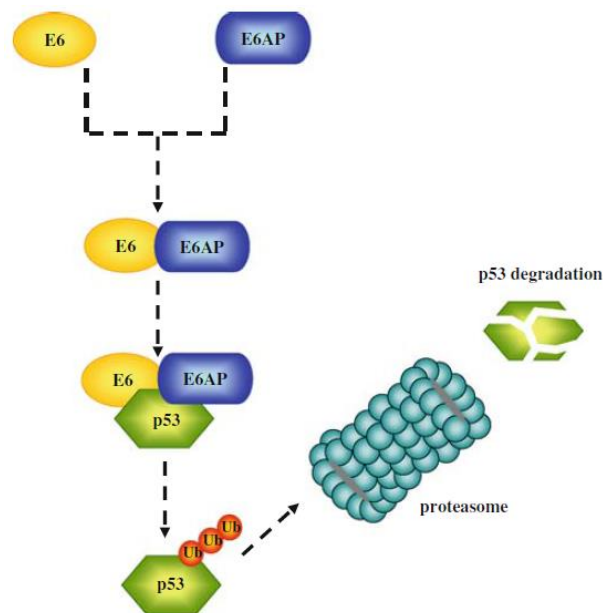


Figure 3- p53 degradation pathway induced by HPV16 E6 through ubiquitin/proteasome targeting [12].

Phosphorylation is a prevalent mechanism by which transcription factors are regulated in response to cellular signals. The first transcription factor whose activity was shown to be regulated by phosphorylation is the cyclic AMP response element (CRE)-binding protein (CREB). E6 can repress transcription by a mechanism that is not fully understood yet, the structure of CREB complexed with its co-activator the CREB-binding protein (CBP) [13,14] and p300, protein transcriptional regulators which play important roles in modulation of cellular responses [13,14]. E6 binding to the CBP prevents p53-mediated acetylation by CBP, reducing its affinity for DNA binding sites. The complex CBP/p300 activates CREB-dependent transcription through the recruitment of RNA helicase A, an RNA polymerase II complex component, to a promoter which contains the functional CRE site. E6 can compete with RNA helicase A for the same binding site, thereby preventing its activation. The repression of the

transcriptional machinery affects different target genes, particularly those involved in cytokine and immune signaling, allowing the virus to obtain other surviving mechanisms [16].

E6 protein also has the ability to activate the catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), whose function is to add repetitions of hexamers on chromosome telomeric ends, playing an important role in the development of cancer cells [17].

1.1.3 Human papillomavirus E7 oncoprotein

The high-risk HPV E7 protein is a nuclear protein composed with approximately 100 amino acids. E7 incorporates three conserved regions (CR), CR1, CR2 and CR3. The CR1 is required for cellular transformation and degradation of pRb, but does not contribute directly to pRb binding. CR2 contains a binding sequence region for pRb. CR3 is a terminal domain which is involved in the association of pRb and other host cell proteins, metal binding and can also function as a dimerization domain [11,16].

The main function of E7 oncoprotein is associated to the pRb repression, facilitating the progression of the cell cycle through G1 to S phase transition promotion. The pRb is hypo-phosphorylated during the G₀ and G₁ phase and becomes highly phosphorylated during stage S, G₂ and M, in normal cells. When the pRb is in a hypo-phosphorylated state, it strongly binds to the E2F transcription factor, repressing the transcription of genes necessary for DNA synthesis. Binding of E7 protein to hypo-phosphorylated pRb prevents the pRb binding to the E2F transcription factor, promoting cell cycle continuity in differentiated epithelial cells, allowing the replication of HPV genes [9].

The E7 interacts with two other proteins of the same family of pRb, called “pocket” protein, namely p107 and p130, which negatively regulate the transcription of E2F. The p130/E2F complexes are more prevalent in differentiated or quiescent cells, while p107/E2F and Rb/E2F are most abundant in cells entering the G₁ and S phases. Both p130 and p107 are necessary for the regulation of several genes. E7 interacts with these genes via CR2 domain, leading to uncontrolled differentiation and cellular proliferation, thereby providing a more favorable environment for viral replication [16].

1.2 Human papillomavirus 16 E6/E7 and cell immortalization

The initial step of cancer progression is the immortalization of normal epithelial cells [19]. The carcinogenicity of HPV16 infections is associated to high risk E6 and E7 proteins function in the deregulation of the cell cycle by p53 and pRb impairment, respectively. These two

high-risk proteins are sufficient to induce cell immortalization, when continuously expressed [9]. Telomerase is overly expressed in most human cancers and represents an important role in cell immortalization. Furthermore, the inactivation of p53 and Rb proteins is highly associated with telomerase activation. As a matter of fact, inactivation of both proteins by HPV proteins and telomerase activation through E6 viral protein induction of hTERT expression are required for immortalization of human epithelial cells in culture [20].

1.2.1 Telomerase activity and expression of the catalytic subunit hTERT

Telomerase is a specialized reverse transcriptase enzyme which synthesizes the repeated telomeric DNA sequences at the ends of chromosomes. The telomerase activity is absent in normal somatic human cells, resulting in the progressive shortening of telomeres in each cell division [21]. This lack of activity leads to a progressive erosion of telomeric DNA at the ends of chromosomes, leading to incomplete DNA replication and contributing to chromosomal instability and senescence. Thereby, the telomeres shortening is responsible for regulating the normal life time of cells [14,20].

Telomerase is active in more than 90% of immortal and cancer cells. Its activity is directly associated to the expression of the catalytic subunit hTERT. In figure 4 is shown a possible mechanism for increased levels of this protein through HPV16 E6 interaction with hTERT proximal promoter, which contains two E-boxes [23]. These E-boxes have binding sites for c-myc, which is a strong activator of the hTERT promoter. HPV16 E6 and c-myc cooperatively have the ability to activate the hTERT promoter *in vivo*. This activation helps to maintain sufficient telomere length for cell survival by increasing the levels of DNA and chromosomal mutations, leading to cell immortalization [6,10,12]. Given the prevalence of high-risk HPV infection, regulation of hTERT levels could inhibit the progression of HPV-induced cancer [19].

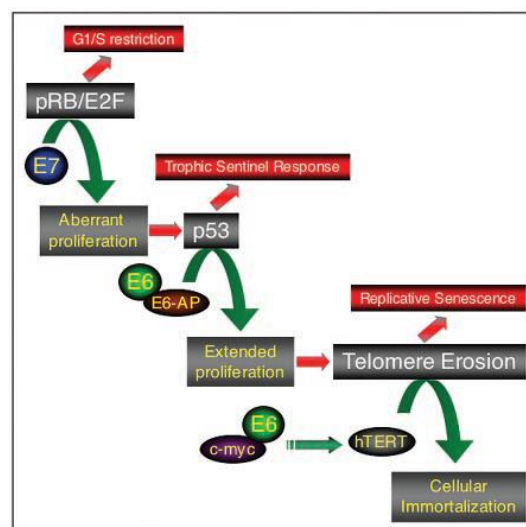


Figure 4 - HPV16 mechanism for cell immortalization [23].

1.3 Human papillomavirus treatment: how far have we got?

The main purpose of any cancer treatment is to effectively kill most of the cancer cells without provoking serious damage to the healthy surrounding tissue. Nowadays, there are several techniques for the treatment of cancer such as chemotherapy, radiotherapy and immunotherapy. Nevertheless, these conventional treatments show an inability to target cancer cells with enough efficiency to produce high levels of direct killing of malignant cells [25].

Among all HPV types known until now, HPV16 and HPV18 are found most frequently in cervical cancer, underlining the need for preventive and therapeutic HPV vaccines development for the treatment of this pathology [26].

Currently, vaccines are available for many serious human pathogens such as bacteria and viruses, and for about half of all human parasites [27]. Preventive HPV vaccines play a crucial role in cervical cancer control. This vaccination system aims to prevent HPV infection by inducing a neutralizing antibody response. However, the high prevalence of virus infection and high cost of vaccines have proven to be a major obstacle [26]. The development of an effective therapeutic vaccine would most likely have a good impact on the mortality and morbidity associated to HPV infections, emphasizing the urgent need to explore this therapeutic pathway [26].

DNA vaccines have shown great potential as therapeutic vaccines against HPV, particularly in the treatment of lesions associated with this virus, arising as a response to cervical cancer mortality. In addition to being less expensive and having an easier manufacturing process, DNA vaccines present the ability to induce an immune response in T and B cells, which could represent an advantage for the development of therapeutic DNA vaccines against HPV [26].

1.3.1 Preventive human papillomavirus vaccine

Preventive vaccination, as the name implies, prevent HPV infections by inducing a neutralizing antibody response. Most vaccines use virions to induce the production of antibodies. Since it is difficult to obtain sufficient quantities of HPV virions in cultured cells to induce a host response, it is necessary to find other alternatives. L1 protein production was able to overcome this hurdle. This protein generates a capsid structure, similar to that found in the virion, being used as virus-like particles (VLP). These DNA-free particles are responsible for the development of a strong immune response and the encoding gene can be cloned into microorganisms, such as yeast or baculovirus, where L1 proteins can be expressed [2,24].

Nowadays, two preventive HPV vaccines are available on the market, as shown in Table 3, named Gardasil and Cervarix. Gardasil is a quadrivalent VLP vaccine derived from HPV6, 11, 16 and 18. Its function is to protect from possible infections by most clinically important HPV types, such as benign HPV6 and 11 for genital warts and HPV16 and 18 for cervical cancer. On the other hand, Cervarix vaccine is a bivalent VLP derived from HPV16 and 18. This vaccine is administered with an adjuvant, AS04 (3-O-desacyl-4'-monophosphoryl lipid A and aluminum hydroxide), which is able to induce higher and long-lasting immune responses [24,26]. Both vaccines are administered as a series of three intramuscular injections over a period of six months and are addressed to adolescent girls who, preferably, have not engaged in sexual activity. The restriction of the target population of these vaccines and the fact of being inefficient when the infection already occurred, represent the main disadvantages of these vaccines [2]. Another disadvantage presented by conventional vaccines is the high prevalence of HPV infections worldwide. Taking into consideration that over 80% of cervical cancer cases occur in developing countries, the investment in better storage and distribution conditions would be crucial for widespread use of preventive HPV vaccines. Nonetheless, effective HPV eradication would only be possible over a considerable amount of time, due to persisting HPV infections [26].

Table 3 - HPV preventive vaccine characteristics (adapted from [2]).

Vaccine	Gardasil	Cervarix
Valence	Quadrivalent	Bivalent
Antigens in vaccine	Capsid proteins of HPV 6,11,16 and 18	Capsid Proteins of HPV 16 and 18
Host for antigen	Yeast	Baculovirus
Age range (Females)	9-45 years	10-25 years
Diseases prevented	Cervical cancers and their precursor lesions, most genital warts, laryngeal papillomas	Cervical cancers and their precursor lesions, subset of head and neck cancers

1.3.2 Therapeutic human papillomavirus vaccine

The available preventive vaccines against HPV were developed with the intent of preventing the infection spreading, leading to specific antibody production against L1 protein. However, this mechanism is not viable when the individual has already been infected with HPV. Therapeutic vaccines have been developed with the intent of promoting tumor regression and to eradicate residual cancer. In order to develop effective therapeutic DNA vaccines,

adequate target antigens must be chosen to effectively produce an immune response against the intended threat. The E6 and E7 HPV proteins are tumor-specific antigens, since both are solely expressed in tumor cells. Both proteins are essential for cellular transformation and are continuously expressed in malignant cells, therefore representing ideal targets for therapeutic vaccination against HPV [26].

However, E6 and E7 oncogenic potential should be extinguished before being used as antigens for DNA vaccines. This elimination of the tumorigenic potential is achieved by introducing point mutations, which will inhibit the E6 and E7 interactions with p53 and pRb. The mutations are performed through major rearrangement of gene sequences, disrupting ligand binding domains. Different cell immortalization studies are crucial before clinical application, in order to confirm the deletion of the oncogenic potential [29].

1.4 DNA-based therapy

In the 1980's began the application of recombinant DNA and gene cloning technology. The increase of genomic data in the 1990s contributed to the establishment of several genes as disease causing factors, allowing to exploit the potential of new therapies based on gene modification [25]. The discovery of the human genome has provided an important support in the identification of genes implicated in several diseases, including cancer, Parkinson's disease, Alzheimer's disease and cardiovascular disorders, leading to the development of DNA-based therapies, which are mainly focused on the modification of potential disease-causing genes [30]. Genes are selected and modified, ensuring the specificity in the control of the disease state. One of the biggest advantages of using DNA-based therapy is the selective recognition of molecular targets, conferring greater specificity to the treatment. This therapy is usually used to attenuate the disease at an early stage, thus preventing its progression.

In DNA-based therapy, different types of genetic material can be used, such as plasmids, oligonucleotides, ribozymes, DNazymes, aptamers and small interfering RNAs [31].

1.4.1. Gene therapy

Gene therapy is an approach used to treat serious diseases, which can be acquired or inherited, correcting their genetic causes by altering deformed genes or by introducing genes whose function might be irregular [32]. About 60% of clinical gene therapy trials in progress worldwide are involved with cancer treatment, followed by cardiovascular and monogenic diseases treatment, as shown in Figure 5 [33]. According to the US Food and Drug Administration (FDA), gene therapy products mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome, and they are administered as nucleic acids, viruses, or genetically engineered microorganisms.

These biotechnological products can be used to modify cells *in vivo* or to transfect cells *ex vivo*, before administrating to the recipient [33].

The ideal transfection system should fulfil various aspects such as: easy and low cost production; ability to transport nucleic acids, regardless of its size, and deliver the gene to specific cell types; inability to generate strong immune responses; ability to induce sustained expression, by integrating a specific region of the genome or remain in an episomal position [32].

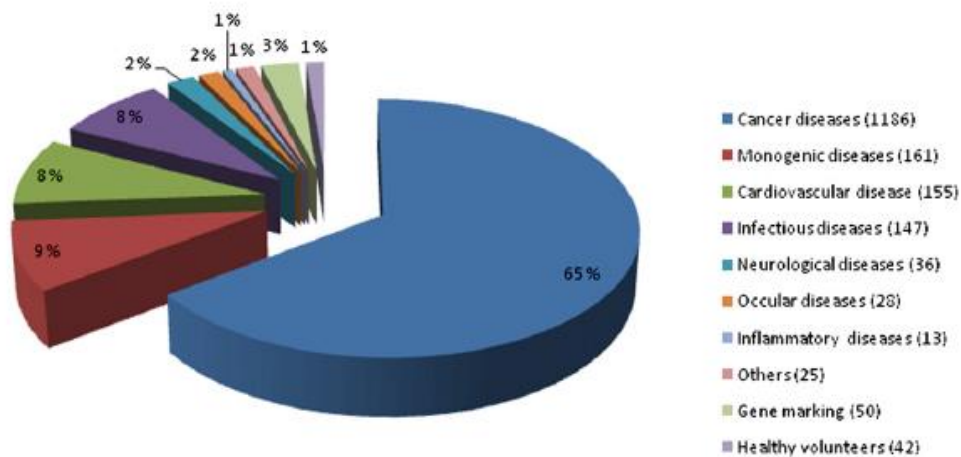


Figure 5- Gene therapy clinical trials in 2013 [33].

1.4.2 DNA vaccination

Since the vaccination concept was demonstrated over 200 years ago by Jenner, where it was proved that prior exposure to cowpox could prevent infection by smallpox, the development and widespread use of vaccines against a variety of infectious agents have been a great triumph of medical science over the last century [34].

Millions of adults and children die each year due to infectious diseases because no effective vaccines have been developed so far. The use of DNA vaccines technology to produce an immune response is relatively new. One of the best strategies for this purpose is the use of plasmid DNA, which encodes specific pathogenic proteins, known as antigens, and leads to humoral and cellular immune response against disease-causing parasites, bacteria and viruses [35].

Upon cell transfection with a DNA vaccine, the host cell machinery recognizes the plasmid DNA and transcribes it into specific antigens, as portrayed in Figure 6 [35]. Since these antigens are recognized as foreign, they are processed as peptides and end up binding to the major histocompatibility complex I and II (MHC I and MHC II), which will in turn present these

peptides to antigen presenting cells (APC), such as dendritic cells (DC). Consequently, T-cell and B-cell are activated, leading to production of antibodies and memory cells [36].

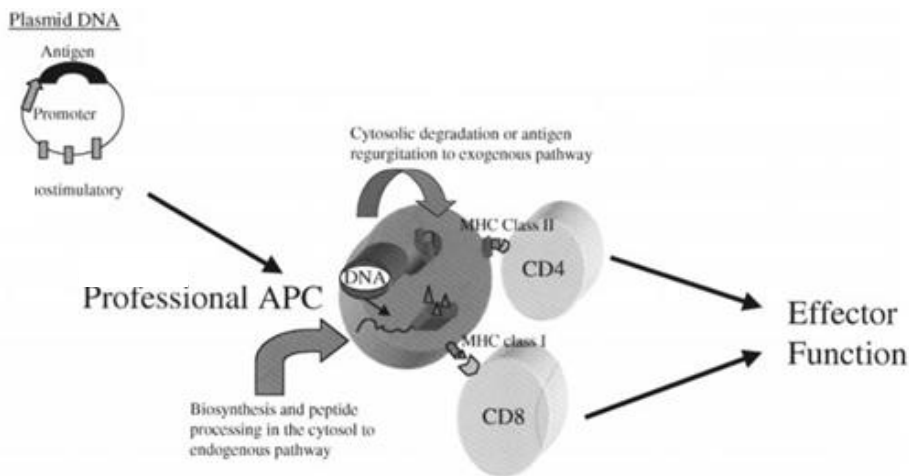


Figure 6 - Mechanisms of antigen presentation after DNA immunization (adapted from [34]).

DNA vaccination provides several important advantages, for instance the stimulation of both types of immunity when compared to conventional protein vaccines, which only stimulate antibody responses [32,35]. Also, these vaccines have several economic, accessibility and manufacturing advantages over conventional vaccines, as summarized in Table 4 [24,36].

Table 4- Advantages of DNA vaccines (adapted from [26]).

Advantages	Attributes
Design	<ul style="list-style-type: none"> Can generate effective cytotoxic T lymphocyte and antibody responses Can be engineered to express tumor antigenic peptides or proteins Enables prolonged expression of antigens and enhances immunologic memory
Safety	<ul style="list-style-type: none"> Unable to revert into virulent forms, unlike live vector-based vaccines Capacity for safe and effective repeated administration No significant adverse effects in any clinical trials
Stability	<ul style="list-style-type: none"> Temperature-stable Long shelf life
Manufacture	<ul style="list-style-type: none"> Suitable for large scale production at high purity Rapid production and formulation Easy to store and transport

1.5 Biological and non-biological vectors

The extracellular environment is unfavorable for cell entry of foreign DNA, limiting transfection efficacy rates. To overcome this hurdle, great advances have been made in the past years regarding the development of new strategies to efficiently deliver therapeutic DNA into cells. These delivery systems can be divided into two groups: biological and non-biological systems [29,37].

1.5.1 Biological delivery systems

Viruses have acquired several biological properties over the years, which allow them to effectively recognize and transfer DNA molecules to cells with great ease [25]. For this reason, the attenuated virus have been largely used as viral delivery systems for therapeutic purposes. Thus, the viral genome is modified to reduce its virulence and to include the intended transgene and take advantage of its infection mechanism, cell entry and release of the desired content. Afterwards, the gene of interest enters the nucleus and is integrated into the host genome, resulting in its transcription. Different types of viral vectors can be used, as shown in the table 5, presenting different advantages and disadvantages [29,38]. Among the viral vectors adenovirus, adeno-associated virus, lentivirus and retrovirus, the most widely used are the adenovirus and retroviruses [25]. Despite the wide application of viral vectors, it has been proven that they can induce cell toxicity [31].

Table 5 - Advantages and disadvantages of biological vectors (adapted from [38,39]).

Delivery System	Advantages	Disadvantages
Adenovirus	High transfection efficiency Transfects many cell types (proliferating and nonproliferating cells)	No targeting Strong immune response Short duration of expression Insert size limited to 4-5 Kb
Adeno-associated virus	Prolonged expression Low immunogenicity Transduction does not require cell division	No targeting Causes mutagenesis Insert size limited 5 Kb Difficult production
Lentivirus	Transfects proliferating and nonproliferating cells Transfects hematopoietic cells	Can origin human immunodeficiency virus Limited storage Insert size limited to 8 kb
Retrovirus	High transfection efficiency Prolonged expression Large insert size (8-12 kb) Low immunogenicity	Only transfects proliferating cells Causes mutagenesis No targeting
Bacteria	Easy manufacture, innate tropism for specific target tissues	Low stability and biosafety

Another option of biological delivery system is the use of bacteria, firstly described in the early 1980's [42], using a continuous engineering process to overcome barriers associated with the *in vitro* and *in vivo* application. The DNA transfer into mammalian cells by bacteria consists of a process named bactofection [42]. In addition to the easy manufacture and application, the carrier bacteria can also provide the necessary costimulatory effects when used as live vaccines. Furthermore, intracellular bacteria can be applied to deliver eukaryotic expression plasmids to introduce relevant transgenes into somatic tissues to treat, cure or prevent diseases that result from genetic disorders [41].

Escherichia coli (*E. coli*), a rod-shaped gram-negative bacteria that is approximately 2 microns in length and 0.5 microns in diameter, is one of the most studied bacteria and has been identified as an ideal gene carrier since most of its strains are harmless and have been extensively studied and described [42].

The use of bacterial systems for gene therapy has the added advantage that bacteria often have an innate tropism for specific target tissues. In most reported cases, disruption of the bacterial carriers, with subsequent release of the DNA, occurs either spontaneously within the infected cell or is facilitated by treatment with antibiotics or use of specific auxotrophic mutants. However, bactofection presents decreased stability and biosafety, which leads to less efficacy, when compared with non-biological delivery systems [41].

1.5.2 Non- biological delivery systems

Gene delivery using nonviral approaches has been extensively studied as a basic tool for intracellular gene transfer and gene therapy [43]. The existing vectors (naked DNA, cationic liposomes, etc) are far from being perfect delivery systems. Non-biological systems can solve some of the problems related to toxicity that, otherwise, biological vectors present. These vectors are characterized by the lack of immune response, being relatively safe and easily produced in large-scale, at reduced cost. Due to its stability, non-biological vectors have the ability to transfer different transgenes of different sizes that can be stored for a long time. Unfortunately, their low transfection efficiency limits their use in large amounts [23,30,38].

Non-biological delivery systems can be divided into two groups according to the DNA delivery method. One of such is the physical methods group, in which DNA is delivered to their target by physical forces such as ultrasound, electroporation, hydrodynamic injection, laser, and mechanical energy, to create temporary weak points in the target cell membrane, weakening it and making it more permeable to entry of the desired transgene by diffusion [30,41,42]. This process is done without using any vehicle, unlike the chemical methods, which take advantage of using a vehicle that can be prepared by various chemical reactions to cross cell membrane and, for some cases, to assist in the transport of the DNA into the nucleus.

Chemical vectors are proposed as promising alternatives to the biological vectors. The strategy further studied for non-viral delivery is the introduction of DNA into condensed particles by using cationic lipids or cationic polymers. Consequently, these particles are taken up by cells via endocytosis, macropinocytosis, or phagocytosis as intracellular vesicles, from which a small fraction of the DNA is released into the cytoplasm and migrates into the nucleus, giving place to transgene expression [43]. In our research group we have conducted various studies with nanoparticles, such as chitosan nanoparticles for the delivery of p53 supercoiled plasmid DNA [45]

1.6 Plasmid DNA as a non-biological delivery system

Plasmid DNA is a circular molecule which presents a closed structure of double-stranded DNA. Each of these chains is a linear polymer of deoxyribonucleotides linked via phosphodiester bonds, due to negatively charged phosphate groups ($\text{pH} > 4$). The inside of the double helix is highly hydrophobic due to the packaging of aromatic bases [35,44]. The pDNA backbone can be rolled up in the space, forming a conformation called supercoiled (sc). A fraction of pDNA molecules may also exist in the open circular (oc) or linear [46] isoforms. The pDNA molecules are considered replicons due to its ability to replicate autonomously in an appropriate host [37].

The pDNA vectors have been considered safer, easier to produce and simpler to use than the biological vectors, which has led to an increasing demand for high quantity production of highly pure pDNA, for future therapeutic applications.

1.6.1 Plasmid DNA production process

The pDNA manufacture can be divided into two different stages, as demonstrated in the figure 7: upstream processing including the fermentation and downstream processing. The first step begins with the construction and selection of appropriate expression vectors and production of microorganisms, followed by selection and optimization of fermentation conditions. Once these conditions are established and the cell growth is attained, it is necessary to isolate and purify the target molecule [47].

This DNA vector contains several basic elements to replicate in a microbial host, such as an origin of replication effective for cloning in the host and an antibiotic resistance gene for growth selection. In addition, the pDNA also contains the eukaryotic expression cassette composed by a strong promoter for eukaryotic cell expression, a polyadenylation termination sequence and the gene encoding the antigen of interest [48].

Generally, pDNA is produced in a recombinant *E. coli* host by fermentation and obtained through an alkaline lysis process, which is followed by pDNA primary isolation and purification. The primary isolation process comprises the concentration and clarification steps, which contribute to the reduction of sample volume and also impurities, such as gDNA, RNA, proteins and endotoxins. Finally, chromatography is used as a purification step to separate the different isoforms (sc, oc and linear) and the remaining impurities that share structural and physicochemical similarities, fulfilling the requirements of regulatory agencies [45,47].

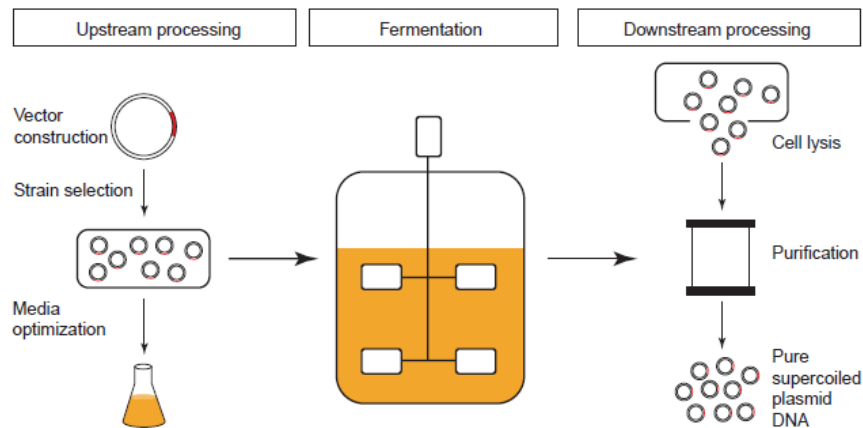


Figure 7- Plasmid DNA manufacture process [47].

1.6.2 Chromatographic purification

Chromatography is a crucial biotechnological area which provides different strategies for the separation of a target molecule from an extract with different impurities. Several chromatographic strategies are used after clarification and concentration techniques, where the impurity content has been reduced, but not all allow to reach the required purity. In a pDNA vaccine production process, chromatographic purification is required for the complete removal of impurities and for the purity evaluation [37]. Purification must include the separation of the sc pDNA from the other isoforms while removing impurities such as RNA, protein, gDNA and endotoxins. There are various specifications for the therapeutic use of pDNA. According to regulatory agencies, the solution must be clear and colorless, the sc pDNA content must be higher than 97%, proteins must be undetectable by BCA method, RNA must be undetectable by electrophoresis, the gDNA content must be lower than 2ng/ μ g plasmid assessed by real time reverse transcription polymerase chain reaction (RT-qPCR) and endotoxins must be lower than 0.1 EU/ μ g plasmid by LAL assay [48,49].

Liquid chromatography plays an important role in the purification of pDNA as well as in the control of its purity, as an analytical tool. Thus, to achieve pDNA purification, different parameters must be taken into account, namely the structure, size and conformation of pDNA, the rheological properties (e.g. viscosity) of the lysates as well as the diversity of

biomolecules [37]. There are numerous liquid chromatographic techniques which take advantage of different pDNA characteristics to accomplish its separation, such as size exclusion chromatography (SEC), anion exchange chromatography (AEX), hydrophobic interaction chromatography (HIC) and affinity chromatography [50].

The SEC is based on the size of the molecules present in the lysate. A lysate is a complex mixture of different molecules with different molecular masses. The reduction of plasmid hydrodynamic radius, due to supercoiling phenomenon, is the basis for its selective separation from different DNA molecules, with baseline separation of RNA and other smaller impurities such as endotoxins and proteins. Typically, large molecules such as gDNA are excluded from the particles, eluting at first, followed by the relaxed and then by the sc pDNA conformations. Smaller molecules such as RNA, oligonucleotides, endotoxins and salts are retarded in bead pores, being eluted at the end [37]. This method is considered an ideal polishing step, allowing the removal of residual contaminants (RNA, endotoxins, proteins, gDNA). The disadvantage of this type of chromatography is the limited pDNA selectivity, hindering sc pDNA purification in a single step [37,52].

AEX is based on the interaction between pDNA negatively charged phosphate groups and positively charged groups in a stationary phase. This technique allows the separation of all isoforms (sc, oc, linear) due to their different conformations, which leads to different local load densities. Thus, isoforms have different retention times along an increasing salt gradient [37]. However, similar chemical and physical molecule composition might prevent efficient sc pDNA separation since this biomolecule can have the same elution profile [52].

The HIC principle takes advantage of the hydrophobicity of the molecules, using high salt concentrations for its retention. Elution is done by decreasing the salt concentration of the mobile phase, thereby weakening hydrophobic interactions. For nucleic acids, the separation occurs by exploiting the hydrophobicity differences between the pDNA, single stranded DNA, RNA and endotoxins. The use of high quantities of salt represents a main disadvantage for this method, due to environmental impact associated and high costs [52].

Affinity Chromatography is a technique used for the selective purification of biomolecules based on the exploitation of affinity interactions between the target molecule and the immobilized ligand. Affinity chromatographic methods in general do not require further purifying steps, thereby presenting increased yields. One limitation usually associated with this purification strategy is the fragility and low binding capacity of the biological ligands [35,50]. Nevertheless, synthetic ligands were then developed to reconcile the selectivity of the natural ligands with high capacity, durability and cost. Different affinity chromatography strategies can be applied in pDNA purification, such as immobilized metal affinity

chromatography (IMAC), triple-helix affinity chromatography, protein-DNA affinity chromatography and amino acid-DNA affinity chromatography [50].

IMAC takes advantage of the affinity interaction between the metal ions (immobilized on the support by the chelating compound) and target molecules, thus allowing high efficiency in the separation of target molecules from other contaminants [37]. Different affinity interactions have been demonstrated between several molecules and immobilized metal ions, which can be presented in the following order: endotoxins > RNA > pDNA. However, the main disadvantage of IMAC is that the effective separation of pDNA from gDNA is only possible if the latter's structure is destabilized, exposing bases and reinforcing interactions [52].

The triple-helix affinity chromatography is based on the recognition of specific DNA sequences immobilized (oligonucleotides) to form a triple helix with the target biomolecule at acidic pH. This technique presents several disadvantages such as its relatively low recovery (32%) due to the involvement of strong interactions, and triple helix formation process is very slow, resulting in long chromatographic runs [52].

Affinity chromatography using amino acids was firstly introduced by Vijayalakshmi and co-workers for the purification of immunoglobulins and a wide variety of proteins, confirming its great potential in purification [52]. Amino acid ligands have been widely used in biotechnological applications, since they are natural compounds that can be safely used in pharmaceutical applications [50]. Over time, our research group has developed several studies using various amino acids as affinity ligands on agarose supports for the purification of pDNA. Histidine-, lysine- and arginine-agarose matrices were successfully used in the sc pDNA isolation, proving the involvement of specific and selective interactions between this molecule and the amino acid ligands. All these three affinity matrices allowed the recovery of sc pDNA with a high purity degree, according to the recommendations. However, histidine and lysine supports showed low recovery yields (around 40%), while arginine-support demonstrated better selectivity and a higher recovery yield (79%) [49,51,52]. Although amino acid ligands present great specificity and recognition ability for sc pDNA, these conventional stationary phases have some limitations, such as low diffusivity and capacity [55].

1.7 Monolithic supports: the new approach

Monoliths have emerged as a very good solution for the purification of pDNA. Nowadays, monoliths are considered the fourth generation of the chromatographic stationary phases. These innovative supports resemble a disc with multiple stacked membranes, but actually consist in a single polymerized unit [55-58]. The pore size depends of the polymerization conditions, namely temperature, and can be adjusted according to the intended application. For the successful implementation of porous stationary phases in chromatography, an

association between pore size of the solid particle and the diameter of the target molecules should be taken into consideration. Frequently, superporous particles have large connecting flow pores with sizes in the range of 1/4 to 1/20 of the overall particle diameter (particle average size 106-180 μm). Consequently, these structural characteristics have significant relevance in pDNA chromatography owing to the accessible surface area provided by superpores, while pDNA is excluded from the smaller pores [56].

A major advantage of monoliths is the acceleration of mass transfer within the flow channels and the absence of accessible diffusive pores. The monoliths are permeable, allowing for a high availability and accessibility of the surface area and an uniform migration. Due to these factors, monoliths present greater capacity and resolution, when compared to conventional supports [59]. These supports present high recovery yields coupled with high purity percentages and can also be used for analytical methods, thereby standing out as a good alternative to conventional supports [60].

Our research group has been conducting different chromatographic studies for sc HPV16 E6/E7 pDNA vaccine purification, making use of monoliths modified with different amino acids [61]. Arginine monolith purification strategy initially presented a good purity percentage but also demonstrated a relatively low recovery yield [49]. However, through the use of experimental design, the recovery yield was increased from 39% to 83.5%, maintaining the high purity grade [62].

1.8 Multiple applications for plasmid DNA

DNA vaccines approach has shown to be safer and more stable than conventional vaccines. The use of plasmids, as therapeutic instruments, is safer, since they are not live organisms and do not replicate in the recipient, avoiding the risk of reversing to a disease-inducing state. The only concerns are associated with the possible integration into the genome and the development of the immune response [63].

Thus, this work was planned with the intent of designs, produce and purifying the HPV16 E6/E7^{MUT} sc pDNA from a clarified lysate. The plasmid used in previous works [49], [62] was modified to include point mutations in E6 and E7 target genes that have been shown to impair the interaction between E6 and E7 proteins with p53 and pRB tumor suppressors, respectively, reducing its oncogenic potential. Furthermore, a number of different techniques are described to further analyze *in vitro* transfection efficiency of the designed DNA vaccine and its ability to correctly express the target proteins. These studies are absolutely essential to assure the safety and efficacy of this therapy, allowing the progress to *in vivo* trials.

Chapter 2 - Materials and methods

2.1 Construction of HPV16 E6/E7^{MUT} pDNA

The 7.9 kbp HPV16 E6/E7 plasmid (p1321) was obtained from Addgene (Cambridge, USA). This vector encodes for the human HPV-16 E6/E7 proteins. The vector contains the human beta-actin mammalian expression promoter and the ampicillin resistance gene as shown in Figure 8.

This plasmid was modified by including several point mutations, to prevent the interaction between E6 and E7 proteins with p53 and pRB. The modification was performed by Nzytech (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal). Briefly, the E6 and E7 genes were removed from the pDNA wild-type and were replaced by the synthesized mutated genes. The first 7 amino acids of protein E6 were eliminated and leucine at position 57 was mutated to glycine. Also, cysteine was mutated at position 113 to arginine. On the other hand, for the E7 protein, histidine at position 2 was mutated to proline, cysteine at position 24 was mutated to glycine and glutamic acid at position 46 was mutated to alanine.

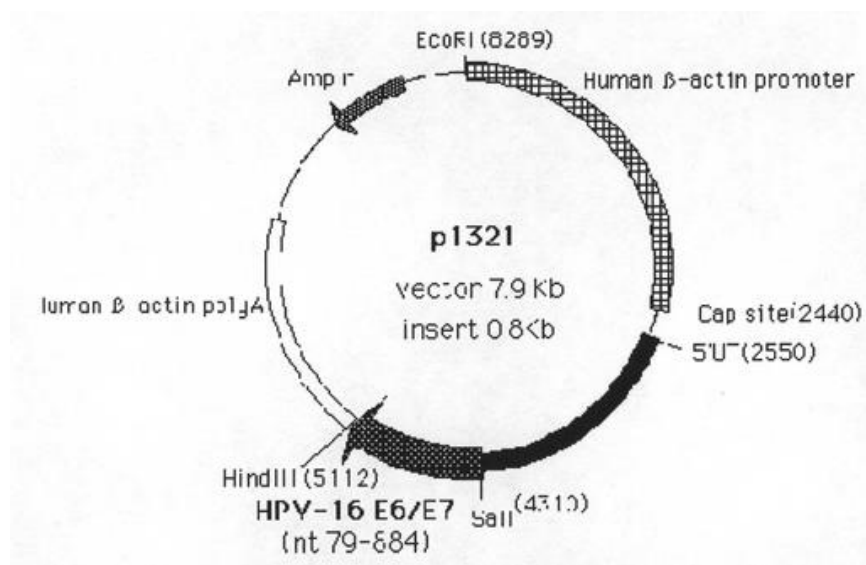


Figure 8 - Schematic representation of HPV-16 E6/E7 pDNA (plasmid 8641 from Addgene [74]).

2.1.1 Transformation *E. coli* DH5a

E. coli DH5a competent cells were transformed with the plasmid 1321 HPV16 E6/E7^{MUT}, following the heat shock protocol (30 s in the 42 °C water bath). The transformed cells were grown overnight at 37 °C in plates with LB-Agar medium containing ampicillin and colonies were screened for the presence of the construct HPV16 E6/E7^{MUT} plasmid. Subsequently, some colonies were inoculated in LB medium and grown at 37 °C and 250 rpm overnight. The HPV16 E6/E7^{MUT} plasmid was isolated from bacteria and purified using the NZYMiniprep Kit (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal).

2.1.2 Confirmation of the mutated fragment insertion

To confirm the insertion of the desired modified fragment into the vector, enzymatic digestion with restriction enzymes was performed. Initially, plasmid sample was digested with HindIII restriction enzyme (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal), according to the procedure recommended by the manufacturer. In short, up to 1 µg of DNA is used with 1 µL of HindIII enzyme, 2 µL 10 x NZYtech Buffer, completing to a 20 µL volume with sterilized ultrapure water. Then, this mixture is incubated at 37 °C for 1 hour, until total linearization of the plasmid is achieved. Then, Sall restriction enzyme (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal) was applied using the same procedure. Finally, agarose electrophoresis was performed to verify the presence of the correct fragment size

2.1.3 Production of HPV16 E6/E7^{MUT} pDNA

The HPV16 E6/E7^{MUT} plasmid was amplified in *E. coli* DH5a cells, by performing a fermentation at 37 °C, under constant agitation at 250 rpm, using Terrific Broth medium (20 g/L Tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017M KH₂PO₄ and 0.072M K₂HPO₄) supplemented with 100 µg/mL ampicillin. Bacterial growth was carried out until the OD₆₀₀ ~ 7 be achieved, followed by centrifugation at 4500 g for 10 minutes for cell recovery. The pellets were stored at -20 °C.

2.2 Purification of HPV16 E6/E7^{MUT} pDNA

2.2.1 Alkaline lysis and Purification with NZYTech Kit

Plasmid DNA was obtained by alkaline lysis and purification using the kit NZYMaxiprep (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal) following the manufacturer's instructions. The binding and elution of pDNA to NZYtech anion columns was promoted under appropriate low-salt and pH conditions. Proteins, RNA and impurities were removed by

washing, using a solution with an average content of salt. After washing, pDNA was eluted with a high salt solution and concentrated by isopropanol precipitation. Finally, the native pDNA (sc and oc isoforms) was used as control for cell transfection tests.

2.2.2 Modified alkaline lysis

In the cases that a lysate sample was required, bacterial cell pellets obtained in the production step, were lysed using a modified alkaline lysis process, as described by Diogo *et al.*, 2000 [64]. Thus, bacterial pellets of 250 mL of medium were resuspended in 20 mL of solution I (50mM glucose, 25mM tris-hydroxymethyl aminomethane (Tris) and 10 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0). Alkaline lysis was conducted by adding 20 mL of solution II (200 mM NaOH and 1% (w/v) sodium dodecyl sulfate (SDS), and incubating for 5 minutes at room temperature. Following, the solution II neutralization was accomplished by adding 20 mL of solution III (3 M potassium acetate, pH 5.0) and incubating for 20 minutes on ice. Removal of cellular debris, gDNA and proteins was done by two centrifugations at 20 000 g, for 30 minutes, at 4 °C with an Allegra TM 25R centrifuge (Beckman Coulter). The concentration and impurity reduction was performed according to a previously published method by Diogo *et al.*, 2000 [64]. In short, the nucleic acids in the supernatant were precipitated by adding 0.7 volumes of isopropanol, followed by 30 minutes incubation on ice. Recovery of the precipitate was obtained by centrifuging for 30 minutes at 16 000 g (4 °C). Pellets were dissolved in 2 mL of 10 mM Tris-HCl, pH 8.0. Then, RNA and protein precipitation was carried out by adding ammonium sulfate to pDNA solution up to a final concentration of 2.5 M. After 15 minutes of incubation on ice, impurities were removed by 20 minutes centrifugation at 16 000 g (4 °C). The supernatant was recovered and the sample was desalted by passing through a PD10 desalting column (GE Healthcare Life Science, GmbH) according to the manufacturer's instructions, using 10 mM Tris-HCl and 10mM EDTA pH 8.0 as elution buffer.

2.2.3 Affinity chromatography

Chromatographic assays were performed for the purification of sc pDNA isoform of HPV16 E6/7^{MUT} from the lysate sample obtained by the modified alkaline lysis step. These experiments were carried out in an AKTA Purifier system (GE Healthcare Bioscience Uppsala, Sweden), which consists in a compact separation unit coupled with a personal computer equipped with software UNICORN 5.11. An arginine monolith, with column bed volume of 0.34 mL and presenting an average pore size of 1500 nm, was prepared and provided by BIA Separations (Adjdovscina, Slovenia). The purification strategy used had already been optimized by our research group [49]. Sodium chloride solutions (NaCl) (Panreac, Barcelona, Spain) and Tris (Merck, Darmstadt, Germany) were prepared with ultra-pure deionized water,

purified with a Milli Q Water System (Millipore, Billerica, MA). Buffers were prepared and then filtered through a membrane with a pore size of 0.20 μm (Sheicher and Shuell, Bioscience GmbH Dassei, Germany), followed by ultrasonic degassing. In short, the monolith was equilibrated with 550 mM NaCl in Tris-EDTA buffer pH 8.0, using a flow rate of 1 mL/min. The sample was injected using a 500 μL loop, under the same conditions described above. Ionic strength was increased up to 750 mM NaCl in Tris-EDTA buffer pH 8.0 and then to 800 mM in Tris-EDTA buffer pH 8.0. Finally, ionic strength was increased up to 1 M NaCl in Tris-EDTA buffer pH 8.0. All assays were performed at room temperature and the absorbance monitored at 260 nm. All fractions were recovered according to the obtained chromatogram, concentrated and desalted with Vivaspin centrifugal concentrators (Vivaproducts, Littleton, MA, USA), 800 g at 4 $^{\circ}\text{C}$ in 3-18K Sigma centrifuge (Sigma Laborzentrifugen GmbH, Germany) for subsequent electrophoretic analysis. The purified sc isoform fraction was also used for quantitative analysis of impurities (gDNA, proteins and endotoxins). At the end of all experiments the monolith and the AKTA Purifier system were washed with water Milli Q.

2.2.4 Agarose gel electrophoresis

The pooled fractions from each chromatographic assay were analyzed by horizontal electrophoresis in 15 cm long 0.8% agarose gel (Hoefer, Holliston, MA, USA) stained with Greensafe Premium (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal) (0.016 $\mu\text{L}/\text{mL}$). Electrophoresis was performed at 120 V for 30 minutes with TAE buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0). The gel was visualized under ultraviolet light in a system in FireReader (Uvitec Cambridge, Cambridge, UK). Hyper ladder I (Biolone, London, United Kingdom) was used as a marker for DNA molecular weight.

2.2.5 Plasmid quantification

The evaluation of sc pDNA purity and recovery was accomplished through the application of a modified quantification method, established by our group, using the CIMac[™] pDNA analytical column [49]. First, a calibration curve was performed using pDNA standards (1 to 50 $\mu\text{g}/\text{mL}$) as portrayed in Figure 9. These standards were prepared by dilution of the highest pDNA concentration with 200 mM Tris-HCl pH 8.0. CIMac[™] pDNA analytical column was equilibrated with 600 mM NaCl in 200 mM Tris-HCl (pH 8.0). After injection of 20 μL of plasmid sample at 1 mL/min, a linear gradient from 600 mM to 700 mM NaCl in 200 mM Tris-HCl (pH 8.0) was applied during 10 minutes, leading to elution of all pDNA species. The pDNA concentration in each sample was calculated using the previously constructed calibration curve, and the respective purity was assessed by the ratio of sc pDNA peak area and the total area of all peaks.

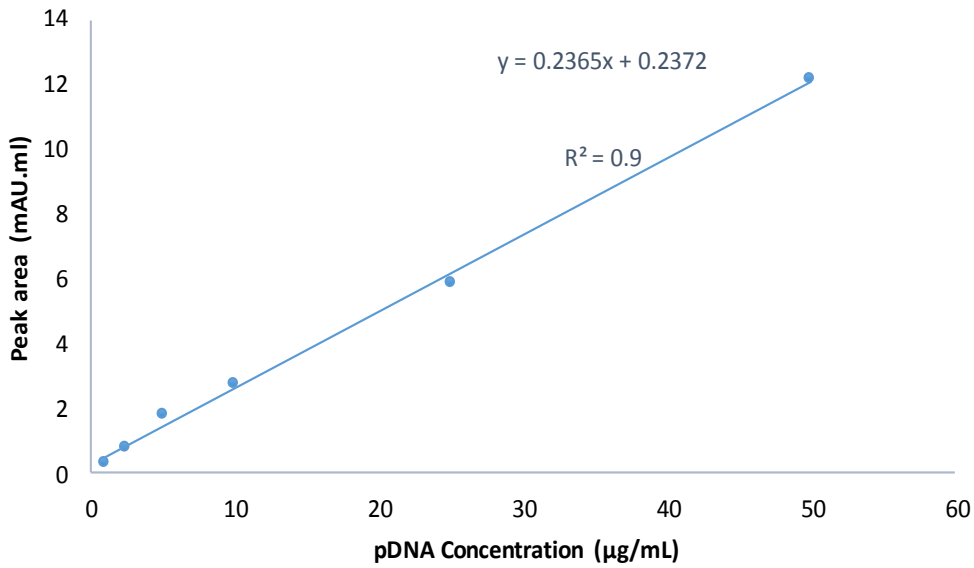


Figure 9- Reference curve of plasmid DNA standards (1-50 µg/mL).

2.2.6 Quantification of Proteins

The protein content was evaluated by BCA method (bicinchoninic acid) using a protein assay kit from Pierce (Rockford, USA). Briefly, 25 µL of each sample was added to the microplate, followed by the addition of 200 µL BCA reagent. After 30 minutes incubation at a temperature of 37 °C, the absorbance was read at 562 nm in an Anthos 2020 microplate reader (Biochrom, Cambridge, United Kingdom). A calibration curve with protein standards was constructed, using Bovine Serum Albumin (BSA) (20-2.000 µg/mL), as shown in Figure 10.

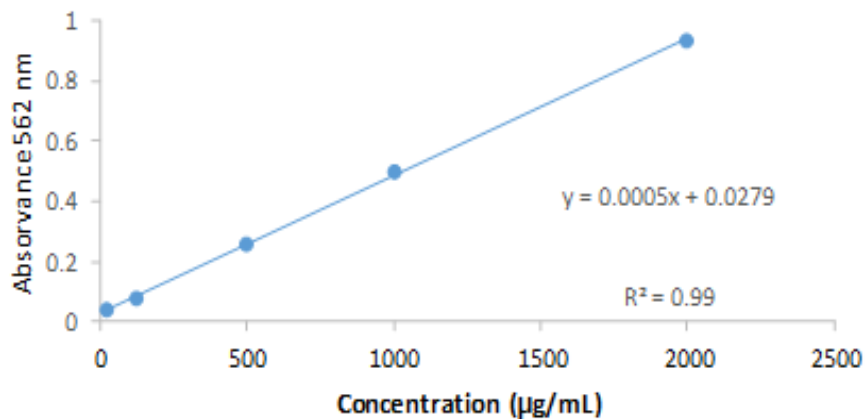


Figure 10 - Reference curve of protein standards (20-2.000 µg/mL).

2.2.7 Quantitation of genomic DNA

The evaluation of gDNA was performed using real-time polymerase chain reaction (PCR) in a PCR system multicolor IQS (BioRad), as previously described [87]. Sense (5'-ACACGGTCCAGAACTCCTACG-3') and antisense (5'-CCGGTGCTTCTTCTGCGGGTAACGTCA-3') primers were used to amplify a fragment of 181 base pairs of the 16S ribosomal RNA gene. PCR amplicons were quantified by following changes in the fluorescence of the Sybr Green I dye (Thermo Scientific, Molecular Biology, USA). A calibration curve was constructed through serial dilutions of *E. coli* DH5 α gDNA (0.005 to 50 ng/mL), purified with gDNA Wizard purification kit (Promega), as shown in Figure 11.

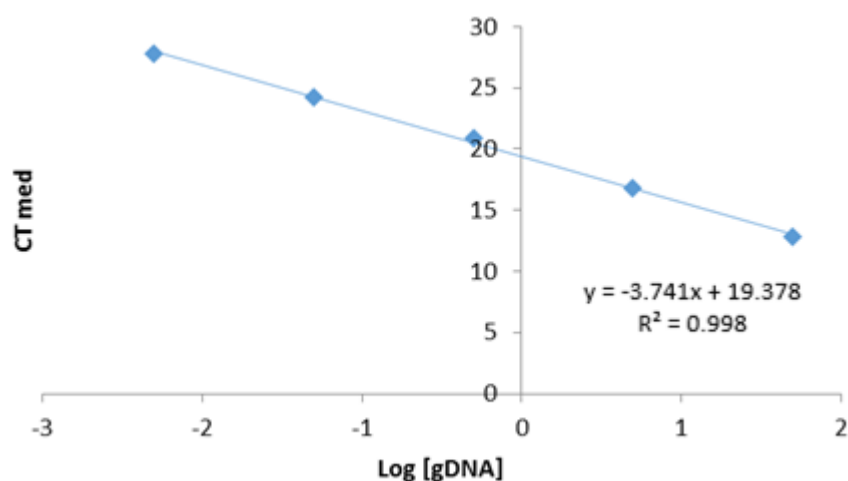


Figure 11 - Reference curve of *E. coli* DH5 α genomic DNA standards (0.005 - 50 ng/ μ L).

2.2.8 Endotoxin quantification

Endotoxin contamination was evaluated using lysate assay kit ToxinSensorTM chromogenic Limulus ameocyte (LAL) from GenScript (US, Inc.) according to the manufacturer's instructions. As shown in Figure 12, the calibration curve was constructed with stock solutions, provided with the kit (0.005-0.1 EU/mL). This procedure was performed inside a laminar flow cabinet, to guarantee the sterility of the quantification process. Kit samples for analysis were diluted in non-pyrogenic water, which was also used as a blank.

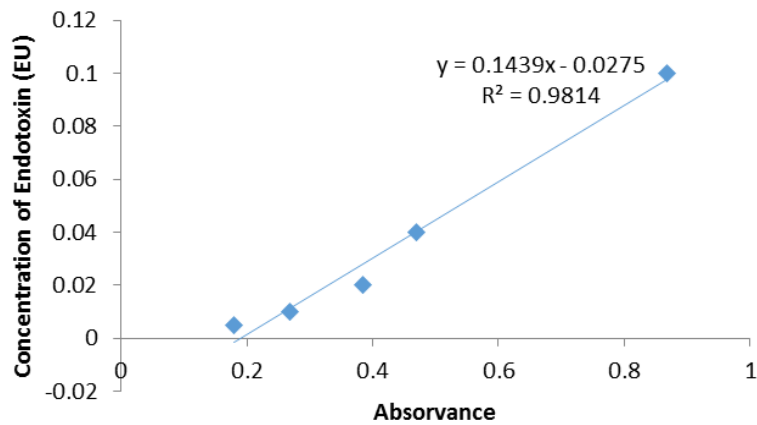


Figure 12- Reference curve of endotoxin standards (0.005-0.1 EU/mL).

2.3 Cell culture

Fibroblast cells (FB) were cultured in Dulbecco's Modified Eagle Medium (DMEM)-F12 High Glucose (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v:v) of fetal bovine serum (FBS) and a mixture of penicillin (100 µg/mL) and streptomycin (100 µg/mL). All cells were grown at 37 °C in a humidified atmosphere with 5% of CO₂ in air.

2.3.1 Cell transfection

The intent of this work was to evaluate the expression of the E6 and E7 proteins by comparing different pDNA isoforms and preparation methods of HPV16 E6/E7^{MUT} plasmid, in order to identify the most effective procedure. Thus, the transfection protocol was performed with Lipofectamine 2000 (Invitrogen, Life Technology, USA) according to manufacturer's instructions, in order to transfect FB cells with sc HPV16 E6/E7^{MUT} pDNA obtained by affinity chromatography and with pre-purified pDNA (sc and oc) obtained by NZYTech kit. Summing up, the FB cell line was cultured until reach a confluence of 80% to 90%. Then, complete medium was substituted by medium without antibiotic and FBS and cells were incubated for 24 hours. Thereafter, Lipofectamine 2000 (Invitrogen, Life Technology, USA) reagent and different plasmid samples were incubated at room temperature for 45 minutes, mixing every 15 minutes, and added to the wells, followed by 6 hours of incubation. Hereafter, the cell medium without antibiotic and FBS was replaced with the respective medium with antibiotic, followed by 24, 48 or 72 hours of incubation. Cells intended to be used for RNA extraction were cultured in 12-wells, while cells planned to be used for protein extraction and then western blot were cultured in 6-wells.

2.3.2 RNA extraction

Transfected cells and controls were used for RNA extraction by adding 250 µL of Tri (Grisp, Porto, Portugal) to each well (12 well plates). The samples were either immediately stored at -80 °C or used to RNA extraction. The samples were incubated 5 minutes at room temperature, followed by adding 50 µL of chloroform and vigorous mixing, leading to the separation of the different biomolecules present in the sample. Then, incubation was carried out for 10 minutes at room temperature, followed by centrifugation at 4 °C for 15 minutes at 12 000 RCF in a Hettich Mikro 200R centrifuge (Hettich Andreas GmbH, Tuttlingen, Germany). Thereafter, the clear and aqueous phase was carefully pipetted, to recover all RNA species without destabilizing other biomolecules and contaminating the retrieved sample. RNA was precipitated by adding 125 µL of ice-cold isopropanol, followed by gentle shaking and an incubation of 10 minutes on ice. Centrifugation was performed for 15 minutes at 4 °C and 12 000 RCF. The pellet was washed with 125 µL of 75% ethanol in Diethylpyrocarbonate (DEPC) water to remove possible organic compounds and centrifuged for 5 minutes at 12 000 RCF. Finally, the supernatant was discarded and the pellet was resuspended in 20 µl DEPC water. An electrophoresis in 1% agarose gel was carried out to confirm the success of the technique. Sample quantification was performed with Nanophotometer (Implen GmbH, Munich, Germany).

2.3.3 Protein extraction

Transfected and control cells were also submitted to protein extraction. Briefly, cells were scrapped with cell scrapper and phosphate buffered saline (PBS) and divided in microtubes. Then, the microtubes were centrifuged in a Hettich Mikro 200R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 11500 rpm, for 7 minutes, 4 °C. The supernatant was discarded and 500 µL of PBS were added, followed by centrifugation in the same conditions. Afterwards, supernatant was discarded again and complete lysis medium (25 mM Tris (pH 7.4), 2.5 mM EDTA, 2% Triton X-100, 2.5 mM EGTA, 1 mM PMSF, 10 µL/mL complete EDTA free protease inhibitor cocktail (Roche, Indianapolis, USA)) was added according to pellets volume, followed by 10 minutes of incubation on ice and vortex. This procedure was performed three times. The resulting proteins were used to perform western blot technique.

2.4 Western blot

2.4.1 Protein quantification

The Bradford micro-assay from BioRad (Hemel Hempstead, UK) was used to measure the protein concentration. In summary, duplicates of mixing 1 μL of sample, 159 μL of MiliQ water and 40 μL of Biorad reagent were prepared in a microplate for each sample. Upon 15 minutes of incubation at room temperature, absorbance was recorded at 595 nm in an Anthos 2020 microplate reader (Biochrom, Cambridge, United Kingdom). Afterwards, a calibration curve was constructed, using Bovine Serum Albumin (BSA) from Sigma-Aldrich (St. Louis, MO, United States of America) as a protein standard (0-2 $\mu\text{g}/\mu\text{L}$), as depicted in Figure 13.

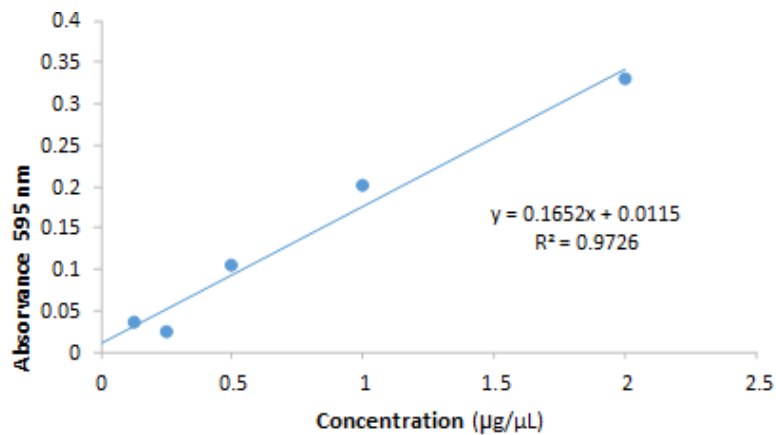


Figure 13- Reference curve of Bradford methods (0-2 $\mu\text{g}/\mu\text{L}$).

2.4.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed with pooled fractions of proteins of transfected and control cells, using 12.5% polyacrylamide resolving gel and 4.7% polyacrylamide stacking gel. The samples were prepared with 50 μg of each protein sample, whose volume was calculated through the calibration curve constructed by protein quantification, and 5 μL of loading buffer, followed by 10 minutes of denaturing at 100 $^{\circ}\text{C}$. The molecular weight marker used was NZYColour Protein Marker II (NZYTech, Lda. - Genes and Enzymes, Lisbon, Portugal). Afterwards the electrophoresis was carried out at 110 V, for 90 to 120 minutes. The resulting electrophoresis gel was visualized or used for electroblotting.

2.4.3 Electroblothing

Protein gel electrotransfer is based on the transference of total proteins of an electrophoresis gel to a polyvinylidenedifluoride membrane. Electroblothing was carried out through a BioRad system with protein gel resulting from previous electrophoresis. Membrane activation was performed with sheer methanol, followed by MiliQ water equilibration. Electrotransfer was performed at 0.75 mA and 250 V for 40 minutes. Afterwards, the membrane was incubated 1 hour at room temperature with 5% BSA, under constant agitation. Afterwards, the membrane was washed with Tris-buffered saline solution with 0.1% Tween 20 (TBS-T 0.1%) for 15 minutes, cut-out and incubated at 4 °C, overnight, with mouse anti- β -actin monoclonal antibody (Sigma-Aldrich, MO, United States of America), and at room temperature for 2 hours with mouse anti-E6 IgG monoclonal antibody (Santa Cruz Biotechnonology, Heidelberg, Germany) and mouse anti-E7 IgG monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany), separately. Hereafter, the membranes were washed 3 times with TBS-T 0.1% for 15 minutes and incubated at room temperature with goat anti-mouse IgG polyclonal antibody (Santa Cruz Biotechnology Heidelberg, Germany) for 1 hour, followed by washing for 15 minutes with TBS-T 0.1%. Then, membranes were incubated for 5 minutes with ECF (GE Healthcare, Buckinghamshire, United Kingdom) and were visualized in BioRad FX-Pro-plus (Bio-Rad, Hemel Hempstead, UK).

2.5 cDNA synthesis

To assess mRNA expression of target proteins it is necessary to synthetize cDNA from the extracted RNA. Thus, 1 μ g of total RNA from non-transfected and transfected cells (24h-48h post transfection), was reversely transcribed into cDNA with RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Molecular Biology, USA). According to the manufacturer's instructions, 1 μ L of Random Primers was added, followed by H₂O adding to a final volume of 12 μ L. The samples were incubated for 5 minutes at 65 °C in a thermal cycler T Professional Basic Gradient (Biometra GmbH, Göttingen, Germany). Afterwards, 4 μ L of reaction buffer, 1 μ L of Ribolock RNase Inhibitor, 2 μ L dNTP mix and 1 μ L of Revertaid M-MuPVRT were added by this exact order. Then, cDNA synthesis reaction was incubated for 5 minutes at 25 °C, followed by 60 minutes at 42 °C and, finally for 5 min at 70 °C, in the thermal cycler. Finally, the resultant cDNA was stored at -20 °C.

2.6 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed by using the thermal cycler Gradient Basic Professional T (Biometra GmbH, Goettingen, Germany). RT-PCR was performed by adding 1 μ L cDNA to a reaction with 0.1 EU/ μ L Taq polymerase, 2 mM MgCl₂, 0.16 μ M of each forward and reverse primer. Primers were designed specifically for E6 (Fw: 5'- CAG GAG CGA AGA AAG CCC TT -3', Rv: 5- ATA GTC TAC CAG CTC ACG TCG T -3), and E7 (Fw: 5'- TCC ACA AGC AGC TGG ACC GGA -3', Rv: 5'- GCA CAC AAT TCC TGT TAG GCC CAT -3'), with resulting amplicons of 117 bp and 154 bp, respectively. The following cycle conditions were used: 95 °C for 5 minutes, 20 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute and final extension at 72 °C for 5 minutes.

2.7 Real time reverse transcription polymerase chain reaction

RT-qPCR was performed using the CFX connect Real-time PCR detected system (Biorad). First, a reaction mix was prepared with 0.31 μ M of forward primer, 0.31 μ M reverse primer and 10 μ L Syber Green (Thermo Scientific, Molecular Biology, USA). Primers used for E6 and E7 amplification were the same used for RT-PCR. GAPDH (Fw: 5'- TGA CGT GCC GCC TGG AGA AA - 3', Rv: 5'- AGT GTA GCC GCC CAA GAT CAG CTT -3') was used as the housekeeping gene. RT-qPCR was performed in 96 well plates using 1 μ g cDNA as template. Samples were initially denatured at 95 °C for 3 minutes followed by 35 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. Melting curve was performed with a starting temperature of 65 °C to a final temperature of 95 °C, with 0.5 °C increments for 5 seconds.

Chapter 3 - Results and Discussion

Cancer and infectious diseases are responsible for millions of deaths every year due to lack of effective treatments, underlining the growing need for new biomedical and biotechnological approaches. DNA vaccines arise as a possible therapeutic tactic, being able to drive all immune responses against diseases caused by parasites, bacteria and viruses [35].

Chromatographic strategies are important not only to separate different pDNA isoforms (sc, oc, linear) but also to eliminate toxic impurities that may harm humans. The application of amino acids as affinity ligands in purification strategies has increased in the past years. Also, monoliths have emerged as advantageous supports for pDNA purification, allowing faster separation, more efficiency and a higher resolution capacity [60].

In our research group, a purification strategy has been established using an arginine monolith for the purification of sc HPV16 E6/E7 pDNA [47,57]. The objective of this work was to prepare, produce and purify the sc HPV16 E6/E7^{MUT} pDNA by using the purification strategy already studied. Additionally, a characterization was performed to verify the impurities of the obtained sc pDNA to guarantee its suitability as a therapeutic target, by fulfilling the requirements proposed by the regulatory agencies, as FDA. Finally, *in vitro* transfection studies were performed to evaluate the expression of the target proteins as a therapeutic target and to compare with non-mutated sc HPV16 E6/E7 pDNA, which has no altered DNA sequences.

3.1. Construction of HPV16 E6/E7^{MUT} pDNA

The E6 and E7 viral oncoproteins are responsible for oncogenic character of HPV. E6 promotes the degradation of p53, which indirectly leads to activation of telomerase, and E7 is responsible for pRB inactivation. Together, these oncogenic changes lead to inactivate the apoptosis pathway and to increased cell proliferation, which in turn will lead to cell immortalization. Even though HPV preventive vaccines have shown success in immunizing against the virus, there are no HPV vaccines currently available for HPV infection treatment [65].

To address this problem, inactivation of several specific regions responsible for oncogenic transformation and subsequent immortalization were performed, by NZYtech, to E6 and E7 genes included in HPV16 pDNA, through insertion of point mutations which impair the interaction between E6 and E7 proteins with tumor suppressor proteins. These mutations eliminate the oncogenic function of the target proteins, while maintaining its antigenicity towards the immune system, allowing to induce a therapeutic effect against HPV.

Thereby, as portrayed in Table 6, the first 7 amino acids of protein E6 were eliminated and leucine at position 57 was mutated to glycine. Also, cysteine was mutated at position 113 to arginine. On the other hand, for the E7 protein, histidine at position 2 was mutated to proline, cysteine at position 24 was mutated to glycine and glutamic acid at position 46 was mutated to alanine (Table 6).

Table 6- Modifications required in E6 and E7 proteins.

E6 protein modification		E7 protein modification	
Position and exchange	Modification	Position and exchange	Modification
MHQKRTA7	Delete	H2P	CAT to CCT
L57G	TTA to GGA	C2AG	TGT to GGT
C113R	TGT to CGT	E46A	GGA to GCA

Figure 14 represents the DNA sequence alignment of wild-type HPV16 E6/E7 pDNA and HPV16 E6/E7^{MUT} pDNA.

```

HPV16_E6/E7_wt      ATGCACCAAAAAGAGAACTGCAATGTTTCAGGACCCACAGGAGCGACCCAGAAAGTTACCA 60
HPV16_E6/E7_mutant -----GTCGACATGTTTCAGGACCCACAGGAGCGACCCAGAAAGTTACCA 39
*****

HPV16_E6/E7_wt      CAGTTATGCACAGAGCTGCAAACAACTTACATGATATAATATTAGAATGTGTGTACTGC 120
HPV16_E6/E7_mutant CAGTTATGCACAGAGCTGCAAACAACTTACATGATATAATATTAGAATGTGTGTACTGC 99
*****

HPV16_E6/E7_wt      AAGCAACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCATAGTA 180
HPV16_E6/E7_mutant AAGCAACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATGGATTGCATAGTA 159
*****

HPV16_E6/E7_wt      TATAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAGTTTTATTCTAAAATT 240
HPV16_E6/E7_mutant TATAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAGTTTTATTCTAAAATT 219
*****

HPV16_E6/E7_wt      AGTGAGTATAGACATTATTGTTATAGTTTGTATGGAACAACATTAGAACAGCAATACAAC 300
HPV16_E6/E7_mutant AGTGAGTATAGACATTATTGTTATAGTTTGTATGGAACAACATTAGAACAGCAATACAAC 279
*****

HPV16_E6/E7_wt      AAACCGTTGTGTGATTGTTAATTAGGTGTATTAACTGTCAAAAGCCACTGTGTCTCTGAA 360
HPV16_E6/E7_mutant AAACCGTTGTGTGATTGTTAATTAGGTGTATTAACCGTCAAAAGCCACTGTGTCTCTGAA 339
*****

HPV16_E6/E7_wt      GAAAAGCAAAGACATCTGGACAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACC 420
HPV16_E6/E7_mutant GAAAAGCAAAGACATCTGGACAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACC 399
*****

HPV16_E6/E7_wt      GGTTCGATGTATGCTTGTGGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGTAATCA 480
HPV16_E6/E7_mutant GGTTCGATGTATGCTTGTGGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGTAATCA 459
*****

HPV16_E6/E7_wt      TGCATGGAGATACACCTACATTGCATGAATATATGTTAGATTGCAACCAGAGACAACTG 540
HPV16_E6/E7_mutant TGCCTGGAGATACACCTACATTGCATGAATATATGTTAGATTGCAACCAGAGACAACTG 519
***

HPV16_E6/E7_wt      ATCTCTACGGTTATGAGCAATTAATGACAGCTCAGAGGAGGAGGATGAAATAGATGGTC 600
HPV16_E6/E7_mutant ATCTCTACGGTTATGAGCAATTAATGACAGCTCAGAGGAGGAGGATGAAATAGATGGTC 579
*****

HPV16_E6/E7_wt      CAGCTGGACAAGCAGAACCCGGACAGAGCCCATTAACAATATTGTAACCTTTTGTGCAAGT 660
HPV16_E6/E7_mutant CAGCTGGACAAGCAGCACCGGACAGAGCCCATTAACAATATTGTAACCTTTTGTGCAAGT 639
*****

HPV16_E6/E7_wt      GTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTCGTACTTTGGAAG 720
HPV16_E6/E7_mutant GTGACTCTACGCTTCGGTTGTGCGTACAAAGCACACACGTAGACATTCGTACTTTGGAAG 699
*****

HPV16_E6/E7_wt      ACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATTAATCTA 780
HPV16_E6/E7_mutant ACCTGTTAATGGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCATTAATCTA 759
*****

HPV16_E6/E7_wt      CCATGGCTGATACCTGCAGGTACCGGATCCGGTACCGAGCTCGAATTCAGCTTTGGAATT 840
HPV16_E6/E7_mutant CCATGGCTGATACCTGCAGGTACCGGATCCGGTACCGAGCTCGAATTCAGCTT----- 813
*****

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Figure 14 - DNA sequence alignment of wild-type HPV16 E6/E7 pDNA and HPV16 E6/E7^{MUT} pDNA. Legend: E6 mutations E7 mutations Sall /HindIII restriction sites.

Figures 15 and 16 represent the comparison between mutated and wild-type E6 and E7 proteins, respectively.

MFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVDFAFRDGCIVYRDGNPYAVCDKCLKFYISKISEYRHY
 CYSLYGTTLEQQYNKPLCDLLIRINCINRQKPLCPEEKQRHLDDKKQRFHNIIRGRWTGRCMSSCCRSSRTRRETQL-

E6wt	MHQKRTAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVDFAFRDLCIV	60
E6mutant	-----MFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQLLRREVDFAFRDGCIV	53
	*****	***
E6wt	YRDGNPYAVCDKCLKFYISKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRINCQKPLCPE	120
E6mutant	YRDGNPYAVCDKCLKFYISKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRINCINRQKPLCPE	113
	*****	*****
E6wt	EKQRHLDDKKQRFHNIIRGRWTGRCMSSCCRSSRTRRETQL-	158
E6mutant	EKQRHLDDKKQRFHNIIRGRWTGRCMSSCCRSSRTRRETQL-	151

Figure 15 - E6 protein with required mutations.

The synthesized gene was digested by Sall and HindIII restriction enzymes, and then cloned into vector p1321, as shown in Figure 17, becoming designated as HPV16 E6/E7^{MUT} pDNA.

MPGDTPTLHEYMLDLQPETTDLYGYEQLNDSSEEEDEIDGPAGQAAPDRAHYNIVTFCKCDSTLRRCVQSTHVDIRTL
 EDLLMGTGLGIVCPICSQKP-

E7wt	MHGDTPTLHEYMLDLQPETTDLYGYEQLNDSSEEEDEIDGPAGQAAPDRAHYNIVTFCK	60
E7mutant	MPGDTPTLHEYMLDLQPETTDLYGYEQLNDSSEEEDEIDGPAGQAAPDRAHYNIVTFCK	60
	* *****	
E7wt	CDSTLRRCVQSTHVDIRTL	98
E7mutant	CDSTLRRCVQSTHVDIRTL	98

Figure 16 - E7 protein with required mutations.

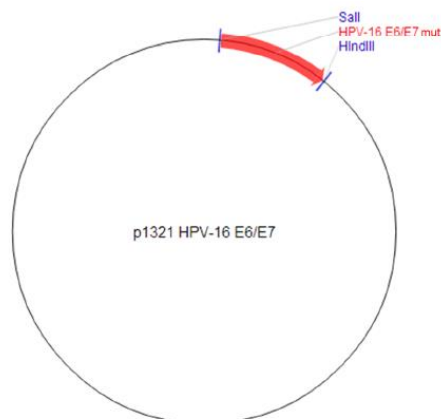


Figure 17 - Cloning Strategy.

3.2 Transformation of cells and cloning Confirmation

Hereafter, HPV16 E6/E7^{MUT} pDNA was transformed by our research group in *E. coli* DH5a bacteria, following a heat shock protocol, as described above. In order to confirm the presence of the desired modified fragment, an enzymatic digestion with the two restriction enzymes used for construction of the vector was performed. Lane 1 of Figure 18 corresponds to the HPV16 E6/E7^{MUT} pDNA sample extracted from *E. coli* before enzymatic digestion. Firstly, HindIII enzyme was applied for plasmid linearization, as portrayed in lane 2 of Figure 18. Afterwards, Sall restriction enzyme was used to obtain the desired fragment, as pictured in lane 3 of Figure 18. The molecular weight of the electrophoretic band confirms that it is the desired fragment with 819 bp, synthesized by NZYtech.

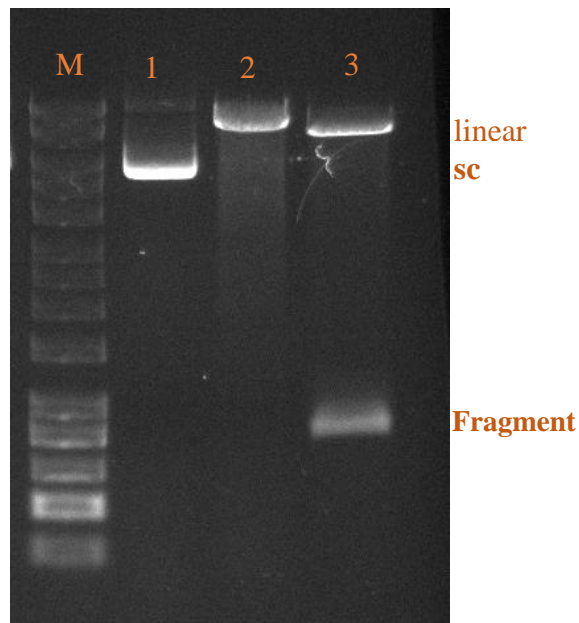


Figure 18 - Confirmation of the modified fragment by electrophoresis. M -Marker; 1 - Initial Sample; 2 - Sample digested with HindIII (linearized); 3- Sample digested with Sall (Linearized + Fragment 819 bp).

3.3 Plasmid amplification in recombinant *E. coli* DH5a

E. coli bacteria have been the most extensively used host in biotechnological industry to obtain large amounts of recombinant DNA. The study of pDNA vaccines as preventive and therapeutic approaches against several diseases, such as cancer, has widely increased [66]. After transformation, HPV16 E6/E7^{MUT} pDNA was amplified in *E. coli* DH5a by fermentation. About 10 hours of growth were necessary to obtain an OD₆₀₀ of approximately 7. The growth was interrupted at this stage to favor higher sc pDNA isoform content compared to other undesired isoforms (oc, linear). It is extremely important to assure sc isoform is present in

majority, since it has been shown that this isoform is therapeutically more active than others (oc, linear) [54].

Comparing both growth curves of HPV16 E6/E7^{MUT} pDNA (Figure 19) and non-mutated HPV16 E6/E7 pDNA (Figure 20), it is observed an exponential growth of about 6 hours, followed by a phase of latency. In HPV16 E6/E7 pDNA curve, between 4 and 5 hours of growth, it is observed a higher growth phase, followed by latency. Therefore it can be concluded that both plasmids have a similar growth curve, suggesting that the pDNA modifications required to synthesis of the mutated fragment did not impair the bacterial growth.

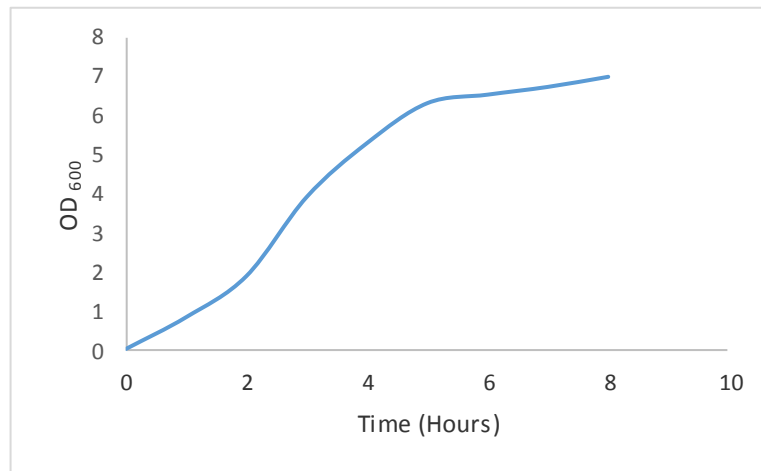


Figure 19 - Bacterial growth curve of *E.coli* DH5a transformed with HPV 16 E6 / E7^{MUT} plasmid.

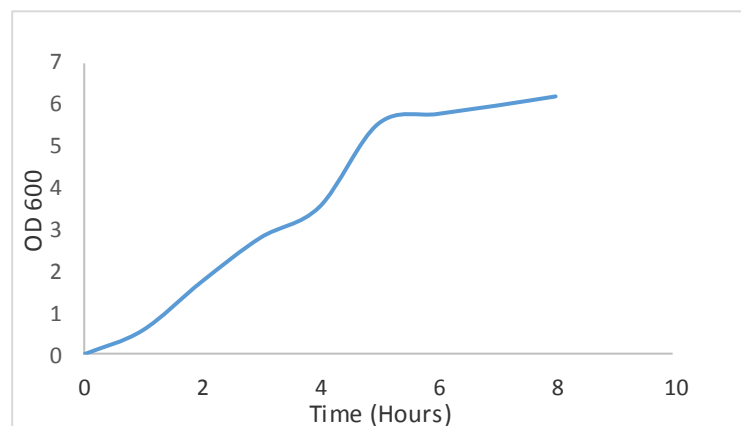


Figure 20 - Bacterial growth curve of *E.coli* DH5a transformed with non-mutated plasmid.

3.4 Supercoiled pDNA purification from the clarified *E. coli* lysate

The purification strategy used had already been defined by our research group for wild-type HPV16 E6/E7 pDNA. However, it was necessary to assess if the pDNA modifications due to synthesis of the mutated fragment could impair the selectivity of the previously established chromatographic strategy with the arginine monolith. The aim of the first experiment (Figure 21) was to verify the behavior of the lysate sample of HPV16 E6/E7^{MUT} pDNA with arginine monolith, applying the same elution strategy previously described, and thus compare if the elution profile was similar to that already obtained with the non-mutated plasmid [49]. Thereby, the monolithic column was initially equilibrated with 550 mM of NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0), at room temperature and flow rate of 1 mL/min. After injection of the lysate sample (500 μ L), a first peak was obtained during the flowthrough of unbound species. Then, the ionic strength of elution buffer was increased to 740 mM NaCl and then to 790 mM NaCl in order to partially elute different species in the second and third peaks, respectively. The chromatographic run was concluded through a final elution step using 1 M NaCl buffer to recover the strongly bound species.

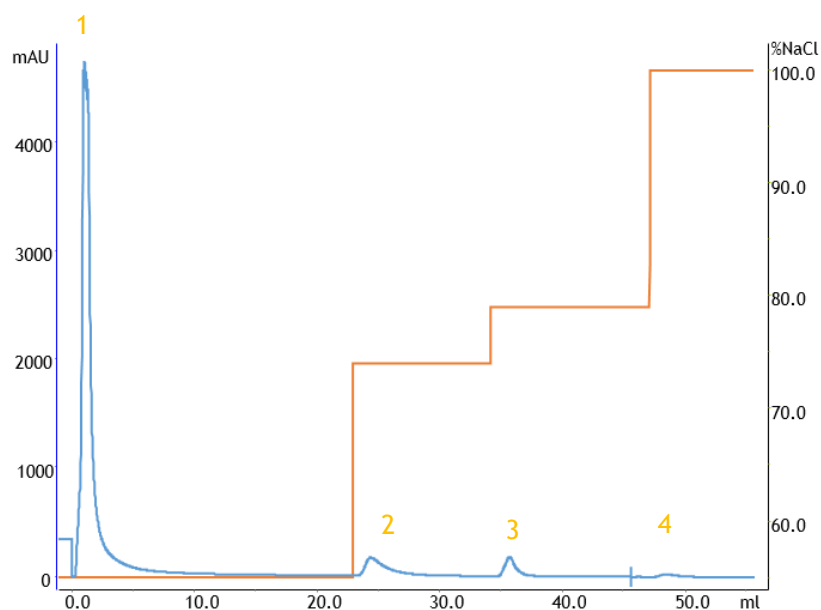


Figure 21- Chromatographic assay of lysate sample of HPV16 E6/E7^{MUT} pDNA with arginine monolith. Elution was performed at 1 mL/min by stepwise gradient of 550 mM, 740 mM, 790 mM and 1 M of NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0), as represented by the orange line. UV detection at 260 nm. Injection volume was 500 μ L.

To establish a correlation between chromatographic peaks and several species present in the lysate sample, an agarose gel electrophoresis was performed (Figure 22). The results show that most part of RNA was eluted in the first peak and in the second together with pDNA (lanes 1 and 2), although vestigial amounts are also present in the third and fourth peaks (lanes 3 and 4). The third lane revealed that most sc pDNA was eluted in the third peak, but also oc pDNA and RNA are present. The last step with 1 M NaCl was applied to remove the remaining molecules, where there is a small loss of sc pDNA. According these results, some elution improvements should be performed in order to purify and isolate all the sc pDNA.

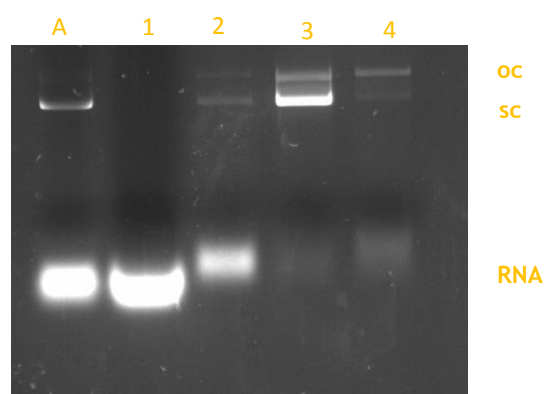


Figure 22- pDNA sample injected into the arginine monolith. A: initial sample; 1- 550 mM NaCl of impurities as RNA; 2- 740 mM NaCl where there elution the isoform sc, oc and RNA 3- 790 mM NaCl where there elution of isoform sc, oc and RNA 4- 1M NaCl where there elution of the isoform sc, oc and RNA.

Since the isolation of sc pDNA was unsuccessful under these chromatographic conditions, some optimization of elution strategy was performed and a new purification strategy was achieved, as presented in Figure 23. Initially, arginine monolith was equilibrated with 550 mM of NaCl, at room temperature and flow rate of 1 mL/min. After injection of the lysate sample, a first peak was eluted during the flowthrough. Afterward, a NaCl stepwise gradient was performed to 750 mM, 790 mM and 1 M of NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0). The several obtained peaks were analyzed by agarose gel electrophoresis.

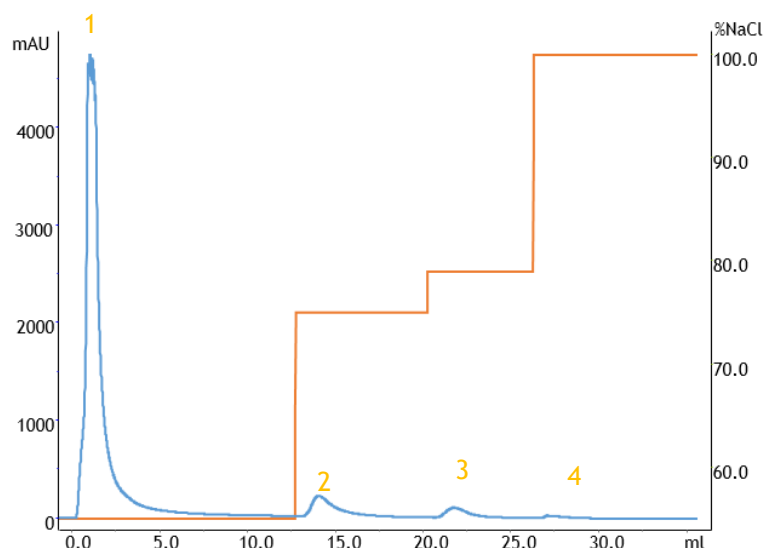


Figure 23- Purification of pDNA with arginine monolith. Elution was performed at 1 mL/min by stepwise gradient of 550 mM; 750mM; 790mM and 1M the NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0), as represented by the orange line. UV detection at 260 nm. Injection volume was 500 μ L.

The results presented in Figure 24 show that most part of RNA was eluted in the first peak and a small amount in the second peak (lanes 1 and 2). As demonstrated in lane 3 of electrophoresis, the sc pDNA eluted in the third peak is isolated. However a small amount was retained at 790 mM, being eluted in the fourth peak at 1 M NaCl (lane 4).

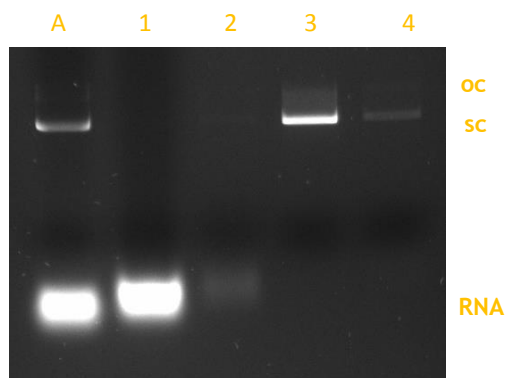


Figure 24- pDNA sample injected into the arginine monolith. A: initial sample; 1- 550 mM NaCl of impurities as RNA; 2- 750 mM NaCl where there elution RNA 3- 790 mM NaCl where there elution of isoform sc pDNA 4- 1M NaCl where there elution of the isoform sc pDNA.

With the purpose of minimize the sc pDNA loss, and thereby increase its recovery percentage, new chromatographic adjustments were performed (Figure 25). Foremost, the monolithic column was equilibrated at room temperature with 550 mM of NaCl, with a flow rate of 1 mL/min and the lysate sample was injected, being obtained a first peak of unbound species. Then, the ionic strength of elution buffer was increased to 750 mM NaCl, and then to 800 mM NaCl and finally to 1 M NaCl to remove all the remaining molecules bound to arginine monolith.

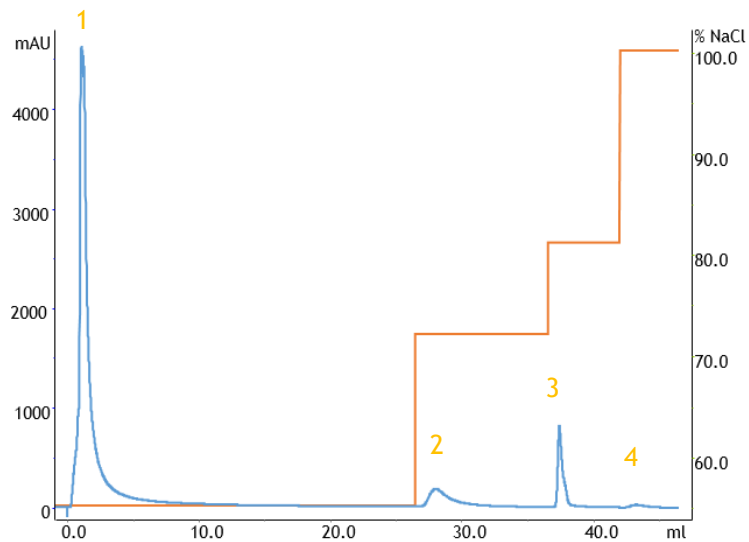


Figure 25- Purification of pDNA with arginine monolith. Elution was performed at 1 mL/min by stepwise gradient of 550 mM; 750 mM; 800 mM and 1M the NaCl in 10 mM Tris-HCl and 10 mM EDTA buffer (pH 8.0), as represented by the orange line. UV detection at 260 nm. Injection volume was 500 μ L.

Analysis from agarose gel electrophoresis showed that the increase of ionic strength allowed the recovery and isolation of the sc pDNA isoform in the third peak (lane 3 of Figure 25), free from other nucleic acids (namely oc pDNA isoform, gDNA and RNA). On the other hand, RNA species were eluted in the first, second and fourth peaks (lanes 1, 2 and 4 of Figure 25). By considering the obtained results, it can be concluded that sc pDNA loss was successfully minimized. Therefore, it was verified, through small adjustments, that arginine monolith is a good purification strategy to isolate the sc HPV16 E6/E7^{MUT} pDNA from lysate sample, as before demonstrated for the non-mutated pDNA [49,62].

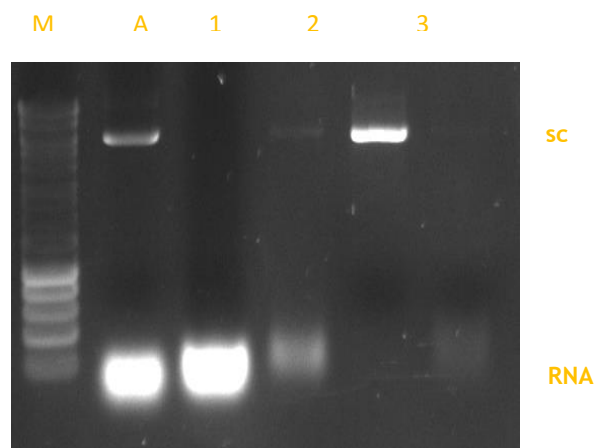


Figure 26 - pDNA sample injected into the arginine monolith. M- marker; A: initial sample; 1- 550 mM NaCl of impurities as RNA; 2- 750 mM NaCl where there elution RNA and a very little sc pDNA 3- 800 mM NaCl where there elution of isoform sc pDNA 4- 1M NaCl where there elution of RNA.

3.5 Analytical parameters for plasmid quality assessment

To further advance into *in vitro* and *in vivo* studies, the purified sc pDNA sample previously obtained must be free of impurities such as proteins, RNA, gDNA and endotoxins. According to regulatory agencies such as FDA, the solution must be clear and colorless, the sc pDNA content must be higher than 97%, proteins must not be detectable by BCA method, RNA must not be detectable by electrophoresis, the gDNA content must be lower than 2 ng/ μ g plasmid by real time PCR quantification and endotoxins levels must be lower than 0.1 EU/ μ g plasmid by LAL assay [48,49].

First of all, quantification and purity assessment of sc pDNA sample was carried out by a modified analytical method established by our research group, using CIMac™ analytical column [49]. Briefly, this method allows to quantify the purity degree and the recovery yield of sc pDNA sample, by comparing the chromatographic profile of the purified sample with the initially injected sample and using a calibration curve (1-50 μ g/mL). Initial lysate sample and pDNA samples resulting from the previously described chromatographic run were injected into the column. All the chromatographic runs were carried out with linear gradient from 600 mM to 700 mM NaCl in 200 mM Tris-HCl buffer pH 8.0 for 10 minutes. In Figure 27 it is represented the chromatogram obtained by injecting the complex lysate sample and, as it can be observed, two peaks were obtained, corresponding to different types of molecules. The first peak corresponds to the impurity content that did not bind to the analytical column, while the second peak represents the sc pDNA isoform.

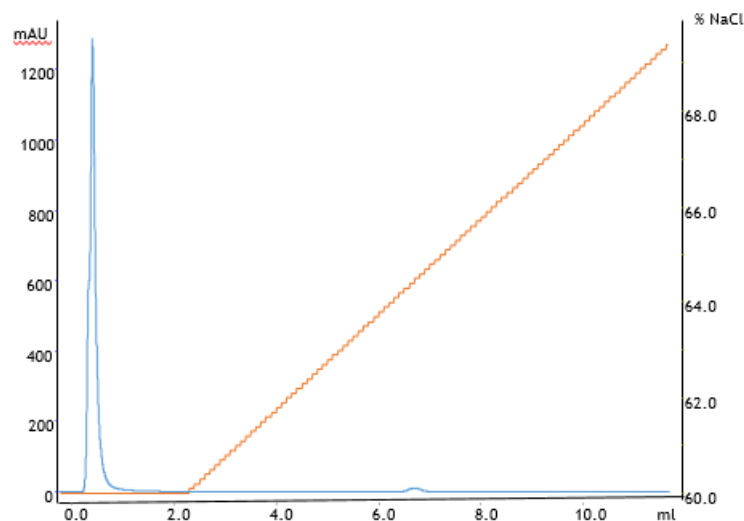


Figure 27 - Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: *E. coli* lysate.

In Figure 28 it is portrayed the chromatogram obtained for the fraction previously pooled out at 550 mM of NaCl during the purification run. By comparing the first peak in Figure 27 and the chromatogram in Figure 28, it can be observed a strong similarity between both peaks, suggesting that this analyzed sample is composed by only impurities and did not contain pDNA molecules.

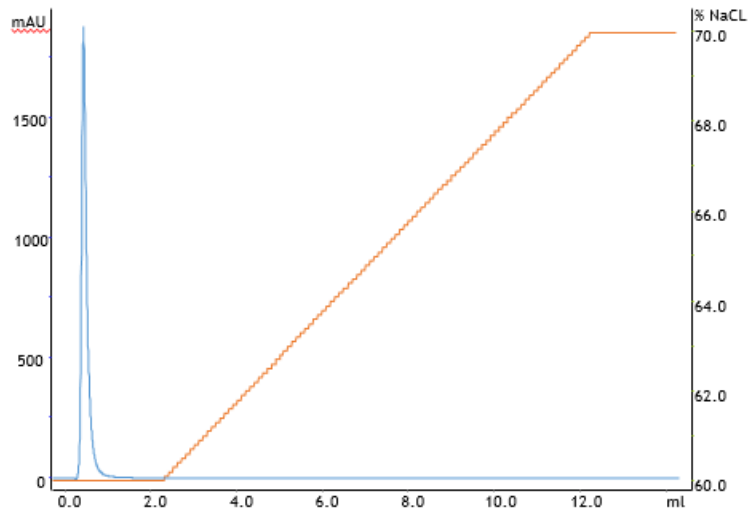


Figure 28- Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: impurities fraction.

By comparing the analytical chromatogram depicted in Figure 29, correspondent to the fraction previously pooled out at 790 mM of NaCl, with the analytical chromatogram of the lysate sample (Figure 27), it is evident that this sample contained a huge amount of sc isoform with probably a satisfactory purity degree since it presents a much smaller first peak. Moreover, the first peak in this chromatographic run can be due to the presence of remaining impurities or due to a residual effect produced by salt interference, since the sample without salt is injected into the analytical column after the equilibration step with 600 mM of NaCl.

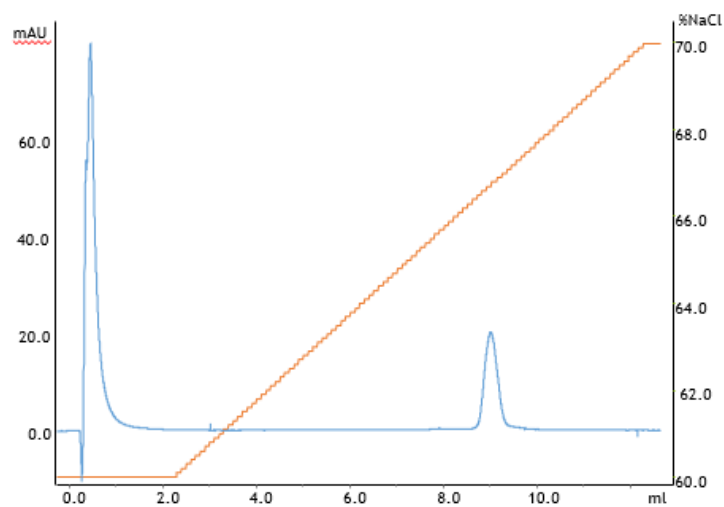


Figure 29- Analytical chromatographic profiles of different pDNA-containing samples recovered throughout the purification process: sc pDNA fraction.

Thereafter, a blank assay was performed by injection of 200 mM Tris-HCl buffer pH 8.0 to assess the buffer salt interference, as shown in Figure 30. The resulting peak area was deducted in all chromatograms of analyzed samples.

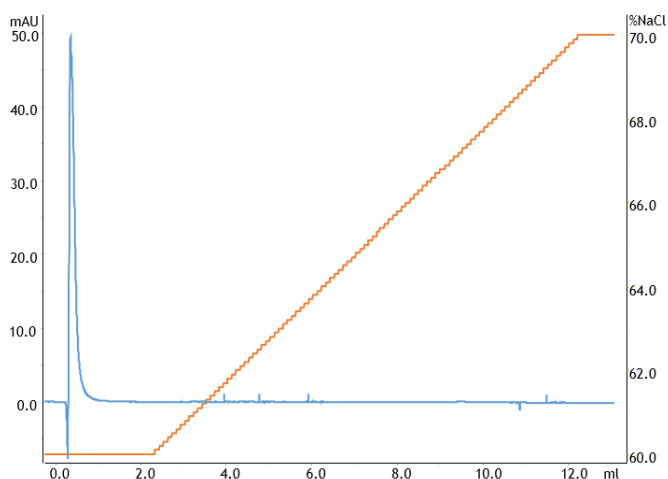


Figure 30- Analytical chromatographic profiles of different pDNA- Blank.

To calculate the purity degree of sc pDNA isoform, a ratio between the peak area corresponding to sc isoform and the total peak areas was determined. As shown in Table 7, the recovered sc pDNA sample presented >99% purity. On the other hand, recovery yield was calculated by performing a ratio between sc pDNA content present in the initial sample and the content of sc pDNA fraction from the purified fraction. Taking into consideration the

calibration curve, it was calculated the sc pDNA mass present in the lysate sample (6.45 μg) and in the recovered sc pDNA sample (5.344 μg). Then, the ratio between both masses was calculated to retrieve the recovery yield. As portrayed in Table 7, the purified sc pDNA sample presented a recovery yield of 82.85%. The comparison between the yield obtained in the present work with the recovery yield obtained in different purification strategies established in our research group for sc pDNA isolation with arginine monolith (recovery yield 39%), allowed to observe that it is similar to the value obtained with the optimization strategy developed by experimental design (83.5%) [47,57].

Table 7 - Purity degree and recovery yield of sc pDNA.

Sample	Concentration of sc pDNA ($\mu\text{g/mL}$)	Purity (%)	Recovery yield (%)
Clarified <i>E.coli</i> Lysate	12.90	4.9	-
Sc pDNA	26.71	99	82.85

Biotechnological products to be therapeutically applied must not contain any traces of protein, because proteins may lead to strong immune responses and biological reactions [67]. To assess protein contamination within the purified sc pDNA sample, BCA method was performed as described in the materials and methods section. As shown in Table 8, sample lysate presented 12,6 $\mu\text{g/mL}$ of protein and no traces of protein were found in the purified sc pDNA sample, fulfilling the regulatory agency recommendations.

Table 8 - Impurity levels of clarified *E.coli* lysate and purified sc pDNA

Sample	Protein ($\mu\text{g/ml}$)	Endotoxin		gDNA	
		(EU/mL)	(EU/ μg pDNA)	(ng/mL)	(ng/ μg pDNA)
Clarified <i>E.coli</i> Lysate	12.6	255.83	19.83	29.31	2.27
sc pDNA	Undetectable	0.26	0.009	0.38	0.014

On the other hand, elimination of gDNA is very important due to the presence of bacterial oncogenes, which can be activated in the transfected cells and lead to tumor development [67]. In addition, the host gDNA can also be integrated in the eukaryotic DNA, inducing fever and allergic or inflammatory reactions [68]. For gDNA quantitation, real-time PCR was performed as described above. As represented in Table 8, sample lysate presented 2.27 ng gDNA/ μg pDNA while purified sc pDNA sample presented 0.014 ng gDNA/ μg pDNA. Thus, it can be concluded that sc pDNA purified sample presents a gDNA value within the limits established by regulatory agencies. These results show the arginine monolith purification efficiency in the recovery of pure sc pDNA.

Endotoxins or lipopolysaccharides (LPS) are highly negatively charged and are present in the wall cell of gram-negative bacteria such as *E.coli* [67]. Since this bacterium was used as a host and endotoxins can lead to nonspecific activation of the immune system, stimulation of excess cytokine production, induction of shock syndrome and decreased transfection efficiency *in vitro* studies [69], it is crucial to guarantee that endotoxin content is decreased. To quantify endotoxins, LAL assay was performed as described above. As depicted in Table 8, lysate sample presented 19.83 EU/ μ g pDNA while purified sc pDNA sample presented 0.009 EU/ μ g pDNA. These results highlight once again the efficiency of this chromatographic technique, considering that the endotoxin content of purified sc pDNA meets the values recommended by regulatory agencies. Comparing the impurity amount present in the purified sc HPV16 E6/E7^{MUT} pDNA with the impurity amount obtained in the purified sc pDNA HPV 16 E6/E7 it is concluded that it was possible to obtain lower levels of endotoxins and gDNA [49]. Given that, the purification strategy used to obtain both plasmids was the same, these small purity degree differences could be explained by the adjustments made in the gradient.

3.6 Transfection efficiency

3.6.1 Reverse transcription polymerase chain reaction

In order to verify transfection and gene transcription efficiency of sc HPV16 E6/E7^{MUT} pDNA, some *in vitro* transfection experiments were performed and accompanied by RT-PCR. Fibroblast cells were cultured and transfected with sc HPV16 E6/E7^{MUT} pDNA, purified by arginine monolith. After 48 hours of transfection the transfected cells and non-transfected cells were used for RNA extraction, as described above. Amplification of E6 and E7 transcripts was performed by RT-PCR with specifically designed primers. As depicted in Figures 31 and 32, both E6 and E7 transcripts were found only in transfected cells. Both electrophoretic bands correspond to the intended amplicon molecular weight, 117 bp for E6 transcript and 154 bp for E7 transcript. These data suggest that transfection procedure and gene transcription occurred correctly, ie the pDNA successfully entered the cell and reached the nucleus, where it was transcribed in mRNA E6 and mRNA E7.

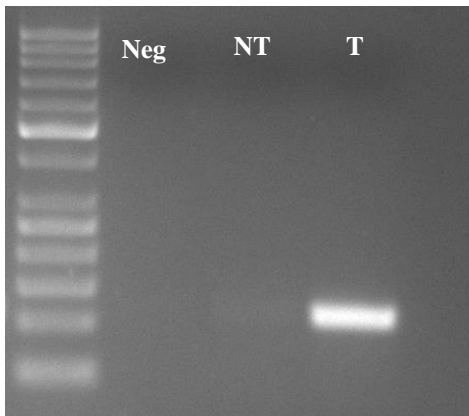


Figure 31- RT - PCR of mRNA E6. First lane: negative control; second line: non transfected cells and third line: transfected cells (117 bp).

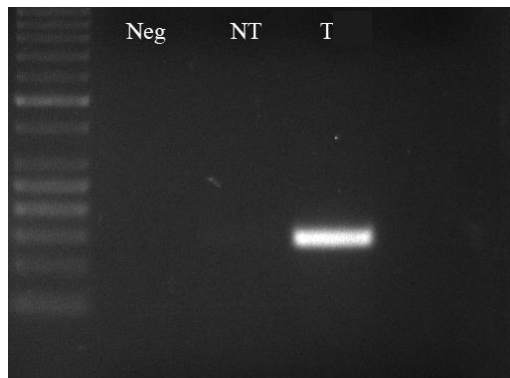


Figure 32 - RT- PCR of mRNA E7. First lane: negative control; second line: non transfected cells and third line: transfected cells (154 bp).

3.6.3 Real time reverse transcription polymerase chain reaction

RT-qPCR allows precise quantification of specific nucleic acids in a complex mixture even if the starting amount of material is at a very low concentration. This is accomplished by monitoring the amplification of a target sequence in real-time using fluorescent technology [70-72]. The first step in the RT-qPCR reaction is to selectively convert only the RNA molecules that correspond to protein-encoding genes into cDNA. The RNA that encodes the protein sequence is called mRNA and it is purified and extracted as a fraction of total cellular RNA from a cell collection. Experimentally, the process of reverse-transcription has many variations, but the essential step is the conversion of mRNA into a cDNA template in a reaction catalyzed by an RNA-dependent, DNA polymerase enzyme called reverse-transcriptase [70]. Fibroblast cells were cultured and transfected with both plasmids, sc HPV16 E6/E7 pDNA and sc HPV16 E6/E7^{MUT} pDNA, purified by arginine monolith and a native pDNA sample purified with a commercial kit was used as a control. After 24 and 48 hours of transfection, the transfected cells were used for RNA extraction and cDNA synthesis, as described above. To evaluate the mRNA expression at different transfection times, a suitable internal standard is required to control the variability between samples called 'housekeeping'. The housekeeping gene serves as internal reference between different samples and helps normalize for experimental error. As housekeeping, it was used the GAPDH. As can be seen in RT-qPCR, once again it was confirmed that the transfection procedure and gene transcription occurred correctly, the pDNA successfully entered the cell and reached the nucleus, where it was transcribed in mRNA E6 and mRNA E7 (Figure 33). There is an increase in both mRNA transcripts of the E6 and E7 proteins at 48 hours. This behavior is similar in both mutated and

non-mutated plasmids, proving that the mutations included in E6 and E7 genes did not change the expression and recognition of respective mRNAs.

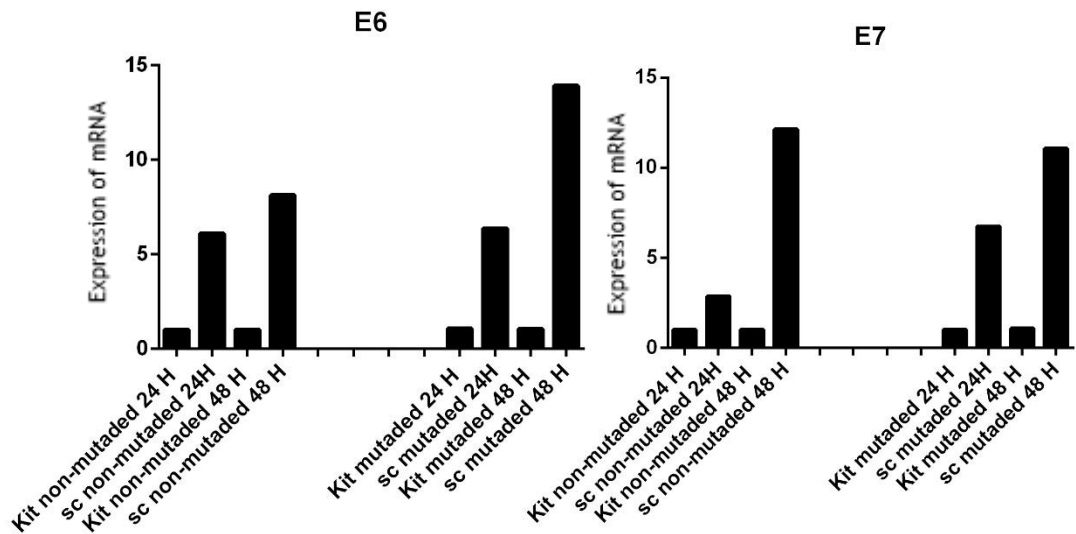


Figure 33- Trend graph of expression of mRNA E6 and mRNA E7 for both mutated and non-mutated plasmids at different hours of transfection (24 hours and 48hours).

3.6.4 Western Blot

Western blot is used to separate and identify proteins. In this technique, the protein of interest is separated based on molecular weight through gel electrophoresis. These results are then transferred to a membrane that will be incubated with primary antibodies specific to the protein of interest. The unbound antibodies are washed off and after some procedures, the bound antibodies are then detected by developing the film. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present [73].

After evaluation of presence and quantification of target transcripts, correct translation into protein was evaluated. Following 48 hours of transfection, the cells transfected with both mutated and non-mutated plasmids, and the non-transfected cells were submitted to protein extraction. Making use of western blot technique, a comparison between sc pDNA transfected cells and non-transfected cells was performed. As shown in Figures 34 and 35, increased expression of E6 and E7 proteins was verified in transfected cells.

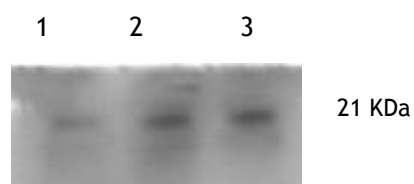


Figure 34- Western Blot to evaluate de expression of protein E7. Lane 1: control group; lane 2: sc pDNA HPV16 E6/E7; lane 3: sc pDNA HPV16 E6/E7^{MUT}.



Figure 35 - Western Blot to evaluate de expression of protein E6. Lane 1: control group; lane 2: sc pDNA HPV16 E6/E7; lane 3: sc pDNA HPV16 E6/E7^{MUT}.

Subsequently, it is necessary to assess which is the ideal transfection time to get a higher protein expression of E6/E7. Hence, following 48 hours and 72 hours of transfection, sc pDNA transfected cells and non-transfected cells were submitted to protein extraction to compare protein expression levels. Looking at Figure 36 and 37, it can be observed that for both plasmids the protein staining is identical for transfected cells, while no band is presented in the control group, as previously suggested by RT- PCR data. These data also suggest that E6 and E7 protein expression is more intense after 48 hours, decreasing after 72 hours.

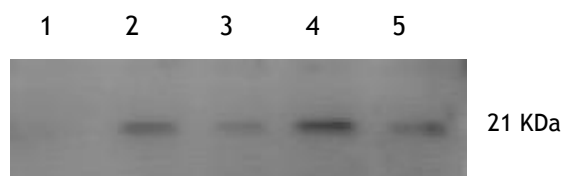


Figure 36 - Western Blot for protein E7. 1- Control group; 2 - sc pDNA HPV 16 E6/E7 after 48 hours of transfection; 3 - sc pDNA HPV 16 E6/E7 after 72 hours of transfection; 4- sc pDNA HPV16 E6/E7^{MUT} after 48 hours of transfection; 5- sc pDNA HPV16 E6/E7^{MUT} after 72 hours of transfection.

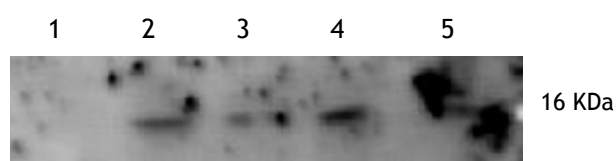


Figure 37- Western Blot for protein E6. 1- Control group; 2 - sc pDNA HPV 16 E6/E7 after 48 hours of transfection; 3 - sc pDNA HPV 16 E6/E7 after 72 hours of transfection; 4- sc pDNA HPV16 E6/E7^{MUT} after 48 hours of transfection; 5- sc pDNA HPV16 E6/E7^{MUT} after 72 hours of transfection

Normalization with β -actin housekeeping (Figure 38 and 39) was also performed. A trend graph (Figure 40) was prepared to demonstrate the difference in expression of E6 and E7 proteins at 48 and 72 hours after transfection. In general, the results revealed that there is a significant increase in protein expression at 48 hours of transfection, as before demonstrated by western blot.

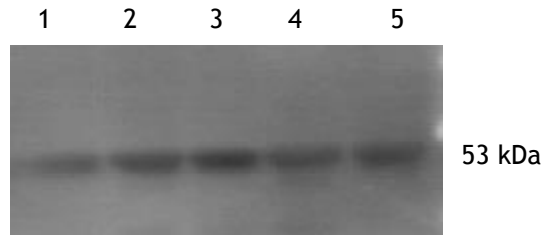


Figure 38 - Western Blot for β -actin corresponding to injected samples in Figure 35 1- Control group; 2 - sc pDNA HPV 16 E6/E7 after 48 hours of transfection; 3 - sc pDNA HPV 16 E6/E7 after 72 hours of transfection; 4- sc pDNA HPV16 E6/E7^{MUT} after 48 hours of transfection; 5- sc pDNA HPV16 E6/E7^{MUT} after 72 hours of transfection.

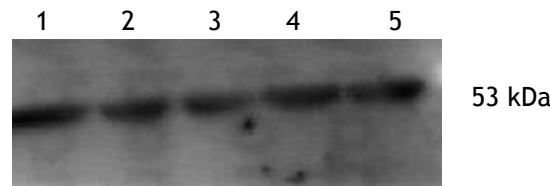


Figure 39- Western Blot for β -actin corresponding to injected samples in Figure 36 1- Control group; 2 - sc pDNA HPV 16 E6/E7 after 48 hours of transfection; 3 - sc pDNA HPV 16 E6/E7 after 72 hours of transfection; 4- sc pDNA HPV16 E6/E7^{MUT} after 48 hours of transfection; 5- sc pDNA HPV16 E6/E7^{MUT} after 72 hours of transfection.

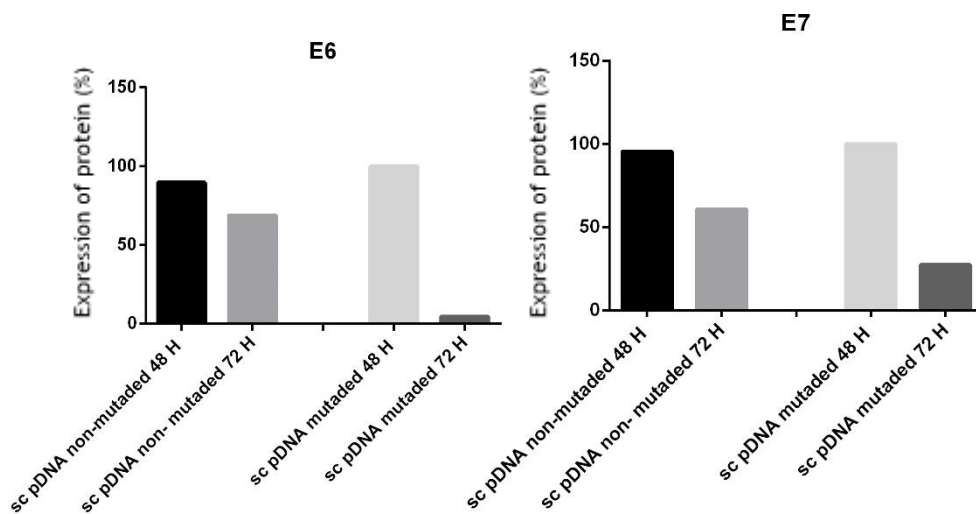


Figure 40 - Trend graph of E6 and E7 protein expression (in percentage) for both mutated and non-mutated plasmids at different hours of transfection (48 Hours and 72 Hours).

Afterwards, comparison between different pDNA samples was performed to verify the added value of the purification strategy established by our research group, when compared the transfection efficiency of sc pDNA sample purified by arginine monolith and the pDNA sample purified by a commercial kit (NZYMaxiprep), as well as the efficiency of sc pDNA isoform when compared with oc pDNA isoform. Following 48 hours of transfection, transfected cells with different pDNA samples (sc isoform, oc isoform, pDNA kit) and non-transfected cells were submitted to protein extraction for further application in western blot. This experience was performed for E6 protein staining. As depicted in Figure 41, the purified sc pDNA isoform demonstrated increased transfection efficiency, towards other isoforms and purification methods, as already anticipated.

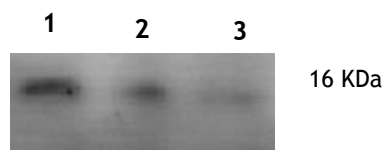


Figure 41- Western Blot for E6 protein staining. First lane- sc pDNA; second lane - oc pDNA; third lane - commercial kit.

Chapter 4 - Conclusions and Future Perspectives

Nowadays, DNA vaccines have been widely studied not only to prevent but also for the treatment of several infection diseases. To ensure the vaccine biosafety and therapeutic application, it is necessary to study and implement suitable purification strategies. Our research group has effectively developed a purification method for sc pDNA vaccine of HPV16 E6/E7, through the use of a modified arginine monolith. However, in the present work, the oncogenic potential of E6 and E7 viral proteins was removed through specific mutations in order to avoid the immortalization of transfected cells. After genes modification, *E. coli* transformation and pDNA production, sc HPV16 E6/E7 pDNA was purified by adjusting the purification method already implemented by our research group. The purification strategy used allowed to recover 82.85% of the sc pDNA isoform with a purity of >99%, which is consistent with what has already been published by our research group.

Then, the assessment of several impurities was performed to underline the efficiency of arginine monolith purification. A significant decrease in the impurity content of the purified sc pDNA sample, when compared to the initial sample, was demonstrated. The obtained impurity levels obtained were the following: Proteins - undetectable; Endotoxins- 0.009 EU/ μ g pDNA and gDNA - 0.014 ng/ μ g pDNA.

Afterwards, *in vitro* transfection studies were conducted using FB cells, in order to evaluate the transfection and expression efficiency of sc HPV16 E6/E7^{MUT} pDNA. Firstly, RT-PCR was performed to evaluate the presence and expression of E6 and E7 mRNA. Amplification of both transcripts was verified in transfected cells. Then, the correct E6 and E7 protein expression was confirmed by Western blot. Moreover, the evaluation of E6 and E7 protein expression was performed to compare cells transfected with sc HPV16 E6/E7 pDNA and sc HPV16 E6/E7^{MUT} pDNA samples and non-transfected cells, with the intent of assuring that expression of mutated pDNA was not altered by the inserted mutations. Also, several transfection hours were tested, such as 48 hours and 72 hours. Normalization with β -actin allowed to verify increased protein expression in transfected cells. The obtained data suggested that the protein expression achieved for both pDNAs is identical and after 48 hours of transfection, more protein expression is observed. As a preliminary study, a comparison between different pDNA isoforms (sc and oc) and pDNA samples obtained by different purification methods (arginine monolith and commercial kit) for the E6 protein staining was performed. The transfected cells with purified sc pDNA presented increased protein expression, suggesting that this molecule presents high transfection efficiency. In the future, this experiment should be repeated for E7 protein staining.

The analysis of transcription efficiency between the different pDNA samples was accessed by RT-PCR and RT-qPCR. The results confirm that the transfection procedure and gene

transcription occurred correctly, the pDNA successfully entered in the cell and reached the nucleus, where it was transcribed in mRNA E6 and mRNA E7. There is an increase in both transcripts of the E6 and E7 protein at 48 hours in sc HPV16 E6/E7 and sc HPV16 E6/E7^{MUT}.

In the future, the expression of the E6 and E7 proteins by immunohistochemistry techniques should be quantified. Also, transfection studies with a primary line of dendritic cells could provide an insightful perspective of antigen presentation activity. Cell immortalization studies to compare the wild-type HPV16 E6/E7 pDNA with HPV16 E6/E7^{MUT} pDNA would also be interesting to guarantee cell immortalization impairment. For that, it would be interesting to study telomerase activity through quantification of its catalytic subunit hTERT by using a RT-qPCR probe, as well as to evaluate if the p53 and pRB expression levels were reestablished by western blot and apoptosis evaluation by flow cytometry.

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