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DETERMINING VIRAL DETECTION EFFICIENCY OF CRISPR-CAS12A ENZYMES

TUĞBA KIRKIK

DEPARTMENT OF MOLECULAR MEDICINE AND BIOTECHNOLOGY

SUPERVISOR
Assist. Prof. SÜLEYMAN ÜÇÜNCÜOĞLU

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Supervisor	**lnstitution**	**Signature**
Dr.Öğr.Üyesi Süleyman ÜÇÜNCÜOĞLU	lstanbul Medipol University	

Members of Examination Jury

Doç.Dr. Akif AYAZ	lstanbul Medipol University
Prof. Dr. Ertuğrul KILIÇ	Istanbul Medeniyet University

The above jury decision has been approved by the Institute Board of Directors with the decision number ..... ./ ..... , ... dated ..... ./ ..... ./ .....

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TUĞBA KIRKIK

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LIST OF ABBREVIATIONS

ACS	American Cancer Society
ADB	Agarose dissolving buffer
AsCas12a	Acidaminococcus sp. BV3L6
BH	Bridge helix
Cas	CRISPR-associated proteins
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DETECTR	DNA Endonuclease Targeted CRISPR Trans Reporter
DNA	Deoxyribonucleic acid
DR	Direct repeats
ER	Early region
FDA	Food and Drug Administration
FQ	Fluorescent and a quencher
gRNA	Guide RNA
HPV	Human papillomavirus
HR-HPV	High-risk HPV
IARC	International Agency for Research on Cancer
LbCas12a	Lachnospiraceae bacterium ND2006
LCR	Long control region
LKL	Loop-lysine helix-loop
LR	Late region
LR-HPV	Low-risk HPV
NCR	Non-coding region
NTS	Non-target strand
NUC	Nuclease lobe
PAM	Protospacer adjacent motif
PCR	Polymerase chain reactions
PI	Pam-interaction

REC	Recognition lobe
RNA	Ribonucleic Acid
RPA	Recombinase polymerase amplification
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-Buffered Saline
TE	Tris-EDTA
TS	Target strand

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1.ABSTRACT

DETERMINING VIRAL DETECTION EFFICIENCY OF CRISPR-CAS12A ENZYMES

Human papillomavirus (HPV) is a virus that widely transmitted through sexual activity. It can infect oral, genital, and epithelial cells of the skin. HPV-16 and HPV-18 are significant agents in cervical cancer and responsible in many cases. In 2020, there were 2.532 patients diagnosed with cervical cancer in Turkey. Cervical cancer can be prevented or treated due to the slow progression of the infected tissue. Detection methods and screening tests that performed regularly are important. Commercial techniques used to detect human papillomavirus generally amplify the nucleic acids of the L1 region using the consensus short DNA sequences. Another method is the Pap-smear test, in which cell samples taken from the cervix are stained and imaged under a microscope. The utilization of novel CRISPR-based approaches holds promise for cost-effective, rapid, and high-accuracy test results. CRISPR-Cas is an adaptive immune system that protects bacteria and archaea from invaders. CRISPR-Cas12a shows endonuclease activity by linking to the complementary sequence of DNA with guided RNA. In this study, HPV-16 and HPV-18 patients' sample were used to target sequences were determined for both types in the L1 region of viral DNA. The guide RNA molecules were produced specifically for the target sites and were targeted with LbCas12a and AsCas12a enzymes. As a result, two crRNAs detected successfully HPV-16 and HPV-18 types.

Keywords: Cervical cancer, CRISPR-Cas12a, Detection, Human papillomavirus, L1 gene.

2.ÖZET

CRISPR-CAS12A ENZİMLERİNİN KULLANILARAK VİRAL TESPİT VERİMİNİN BELİRLENMESİ

İnsan papilloma virüsü, yaygın olarak cinsel yolla bulaşan bir virüstür. Ciltte yer alan epitel hücreler, oral ve genital mukoza bu virüs tarafından enfekte edebilir. HPV'ye ait tip 16 ve tip 18, rahim ağzı kanserinde önemli ajanlardır ve çoğu vakadan sorumludur. 2020 yılında Türkiye'de 2,532 adet hastaya rahim ağzı kanseri tanısı konmuştur. Rahim ağzı kanseri, enfekte ettiği dokuda yavaş ilerlediği için önlenebilir veya tedavi edilebilir bir kanser türüdür. Kullanılan tespit yöntemleri ve periyodik olarak yapılan testler çok önemlidir. Piyasada, insan papilloma virüsünü tespit etmek için kullanılan tekniklerde genellikle korunmuş kısa DNA dizileri ile L1 bölgesine ait nükleik asitler çoğaltılmaktadır. Bir diğer yöntem ise rahim ağzından alınan hücre örneklerinin boyanarak mikroskop altında incelendiği Pap-smear testidir. Tanı amaçlı kullanılan bu tekniklerin gerektirdiği maliyet ve analiz sonuçlarının uzun sürmesi gibi nedenlere bağlı olarak, CRISPR temelli yenilikçi tekniklerin kullanılması düşük maliyetli, hızlı ve yüksek doğrulukta tanı konulmasını sağlayacaktır. CRISPR-Cas, bakteri ve arkeleri çeşitli istilacılardan koruyan sonradan kazanılmış bir bağışıklık sistemidir. CRISPR-Cas12a, kılavuz RNA rehberliği ile tamamlayıcı DNA dizisine bağlanarak endonükleaz aktivitesi göstermektedir. Bu çalışmada, HPV-16 ve HPV-18 tiplerine sahip olan hastaların örnekleri kullanılmıştır. Viral DNA'ların L1 bölgesinde her iki tip için hedef diziler belirlenmiştir. Hedef dizilere özgü üretilen kılavuz RNA'lar, LbCas12a ve AsCas12a enzimleri ile hedeflenmiştir. Sonuç olarak, iki kılavuz RNA HPV-16 ve HPV-18'i tespit etmiştir.

Anahtar Kelimeler: CRISPR-Cas12a, İnsan papilloma virüsü, L1 geni, Rahim ağzı kanseri, Tespit.

3.INTRODUCTION AND PURPOSE

Human papillomavirus is a non-enveloped double-stranded DNA virus with an 8 kilo bases genome size and is a member of the Papillomaviridae family. The papillomaviridae family consists of five primary groups, namely Alpha (α)-, Beta (β), Gamma (γ)-, Mu (μ)-, and u (v)-papillomaviruses. There are 450 different HPV types identified in these groups (1). In the 1970s, Zur Hansen initially identified the association between HPV and cervical cancer (2). HPV has the ability to infect skin epithelial, oral, and genital mucosa. It is transmitted sexually. Most people have asymptomatic infections at some point in their lives from various HPVs, and infections are often eliminated by the immune system (3). HPV is a type-specific virus. Depending on the infection, it can induce genital warts or cancer because of the uncontrolled proliferation of oncoproteins (4). HPV is divided into two categories. High-risk HPV (HR-HPV) types infect cervical and oral mucosal tissues. HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are named as HR-HPV types. Low-risk (LR-HPV) types infect cutaneous tissues. HPV-6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 are named as LR-HPV types. The HPV genome is formed in three regions, which are the early region (ER), late region (LR), and non-coding region (NCR). In HR-HPV, E6 and E7 oncoproteins located in the ER cause degradation of tumor suppressor proteins (p53 and Rb) and lead to uncontrolled cellular proliferation (5).

Cervical cancer has the second rank in cancer-related mortality in women. HPV-16 and HPV-18 are known to be the primary factors in cervical cancer and are recognized in 75% of the total cases. In 2020, the global incidence (6) of cervical cancer was 604.127 cases and was 2.532 in Turkey (7). It is possible to prevent cervical cancer in an early stage of diagnosis. Nucleic acid amplification of HPV genome and Pap-smear tests are widely utilized for the screening of HPV. The nucleic acid amplification is carried out specifically by amplification of the L1 region. With this test, HR-HPV types can be determined as positive or negative, but qPCR is necessary to find out specifically which type it is. The pap-smear tests involve the staining of cervical tissues and imaging under a microscope (8).

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) systems serve as a defence mechanism for mobile genetic elements against of invaders. The CRISPR-Cas system consists of CRISPR arrays and

Cas genes. The CRISPR arrays include spacers derived from previous infections and direct repeats. The immune response of CRISPR-Cas systems occurs in three steps: adaptive, expression, and interference. The adaptive step involves the insertion of short foreign sequences, referred to as protospacer, into the CRISPR array. The expression (also known as crRNA biogenesis), the spacer-repeat array transcribes into a long precursor CRISPR RNA (pre-crRNA), which subsequently undergoes maturation to become short crRNAs. The mature crRNAs are identified by Cas genes. The identification process can involve single or multiple Cas genes. In the interference phase, when a phage re-attacks bacteria, the crRNAs form a complex with Cas proteins to serve as a guide to recognize the foreign genome and cleave its genome. There is a protospacer adjacent motif (PAM), which is located close to the invader's genome. PAM has 2–5 bases in length. Therefore, the crRNA-Cas complex targets the invader's genome instead of the bacterial genome. In 2020, Doudna and her team were awarded the Nobel Prize for gene editing using the CRISPR-Cas9 systems (9). CRISPR-Cas systems are divided into two classes and six subtypes (10). Class I systems have multiple effector proteins, while Class II systems have a single effector protein. Cas12a belongs to the Class II and Type V systems. Cas12a endonuclease completes its maturation process without any additional material. The PAM sequence for recognition of Cas12a is "TTTV". CRISPR-Cas12a binds to complementary DNA sequences with guide RNA (gRNA) and shows endonuclease activity. Acidaminococcus sp. (AsCas12a) and Lachnospiraceae sp. (LbCas12a) are two orthologues of the Cas12a enzyme. These enzymes are utilized in several applications (11-14). Furthermore, the Cas12a enzyme has the ability to cleave single-stranded DNA after cleavage of double-stranded target DNA. This feature of Cas12a distinguishes it from other Cas enzymes, and it can be utilized for viral detection applications (15). It is essential to develop new approaches based on CRISPR-Cas that are fast and low-cost in addition to commercial techniques. In the literature, there is no study comparing the detection activity of viruses utilizing both LbCas12a and AsCas12a enzymes. The purpose of this research was to compare the cleavage activity of the LbCas12a and AsCas12a enzymes for the detection of HPV-16 and HPV-18 types.

4.GENERAL INFORMATION

4.1.Human Papillomavirus

Human papillomavirus (HPV) belongs to the Papillomaviridae family and is a non-enveloped, circular, and double-stranded DNA virus. It is responsible for the most prevalent sexually transmitted infection. Papillomaviruses have the ability to infect both skin and mucosal tissues and evolve according to the host genome as type-specific. The Papillomaviridae family is grouped into five phylogenetic species as alpha-, beta-, gamma-, mu-, and nu papillomaviruses. To date, approximately 450 different human papillomaviruses have been identified. Some of the HPV types only cause benign lesions, such as genital warts, while the other types cause cancer because they have malignant lesions (1).

In history, amniotes were found to be infected by papillomaviruses in the Palaeozoic era, evolved in the fur and hair of mammals, and developed in their epithelial (16). In 1935, a study was published about the papillomavirus can induce the formation of skin carcinomas in rabbits. Due to the fact that it has packaged DNA with viral particles, the papillomavirus is the first DNA virus (17). In 1959, the bovine papillomavirus was discovered and associated with malignant lesions in animals (18). The effect of HPV infection on humans was discovered in the 1970s (2). Zur Hansen thought that the human papillomavirus (HPV) may be a contributing factor in the development of cervical cancer. Therefore, he published studies to confirm his hypothesis in 1983 and 1984. He isolated HPV-16 and 18 from cervical cancer lesions (19,20). In 2003, researchers analyzed skin swabs obtained from babies up to 4 years old, as well as from their parents. They found a total of 73 HPV types in healthy people, and these types belonged to beta- and gamma-papillomaviruses. They concluded that asymptomatic HPV infections in the normal epidermis are acquired during childhood (21).

4.1.1.Classification of HPV

Human papillomaviruses are classified into five primary phylogenetic genera: Alpha (α)-, Beta (β)-, Gamma (γ)-, Mu (μ)-, and Nu (ν)-papillomaviruses (Figure 4.1.1.1). Alphapapillomaviruses infect the mucosa in the anogenital epithelia.

Betapapillomaviruses infect the skin, oral cavity, nasal mucosa, and anogenital region (22).

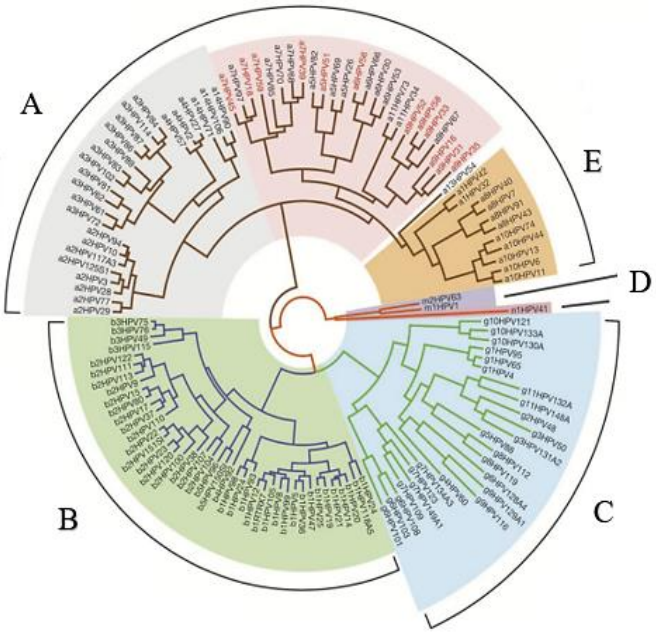

Figure 4.1.1.1. Classification of human papillomavirus. (A)Alphapapillomavirus, (B) Betapapillomavirus, (C) Gammapapillomavirus, (D) Nupapillomavirus, (D) Mupapillomavirus (23)

Classification is based on similarities in the L1 capsid protein between the HPV types. Just as L1 capsid protein is the most conservative region among other regions (mentioned in the genome structure part). The similarity ratio between the classes is at least 60%, whereas it is 90% inside a class (24). All HPVs can cause proliferative benign lesions. However, some viral types are closely associated with malignant lesions. For this reason, the International Agency for Research on Cancer (IARC) categorized HPVs as high-risk (HR) and low-risk (LR). The HR types (e.g., types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) are known to be closely associated with pre-neoplastic lesions and carcinomas, whereas the LR types (e.g., types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81) tend to cause warts (5). Alphapapillomaviruses include HR-HPV types. These types are associated with cervical, vaginal, vulva, penis, anus, head, and neck cancer (25). HPV types 16 (HPV-16) and 18 (HPV) are located in HR-HPV, and they responsible 75% of cervical cancer cases (26). Betapapillomaviruses may lead to squamous cell skin cancer and the development of UV-mediated skin cancer.

Some HPV types are classified as high-risk because they contain tumor suppressor proteins (p53 and Rb) in the E6 and E7 regions (27).

4.1.2.Epidemiology

Cervical cancer is one of the most common types of cancer, and it ranks second in cancer-related deaths in women (28). According to 2020 data, the incidence of cases was nearly 604,127 and there were 341,831 deaths (6). Despite the rising number of cases and deaths annually, cervical cancer is a preventable and treatable cancer (29). Early detection is significant for treatments. Most of the mortality rates observed in low- and middle-income countries (Figure 4.1.2.1). There are several factors, such as limited access to education, less availability of health resources, and a suboptimal hygiene area. As reported by the American Cancer Society (ACS), cervical cancer is mostly diagnosed in women between the ages of 25 and 65 (30). HPV-16 and HPV-18 are known to be major causative factors of cervical cancer and responsible for 75% of cervical cancer cases (26). According to GLOBOCAN Turkey statistics, cervical cancer ranked 19th out of 35 forms of cancer in our country in 2020, with 2,532 new diagnoses and 1,248 deaths attributable to the disease (7).

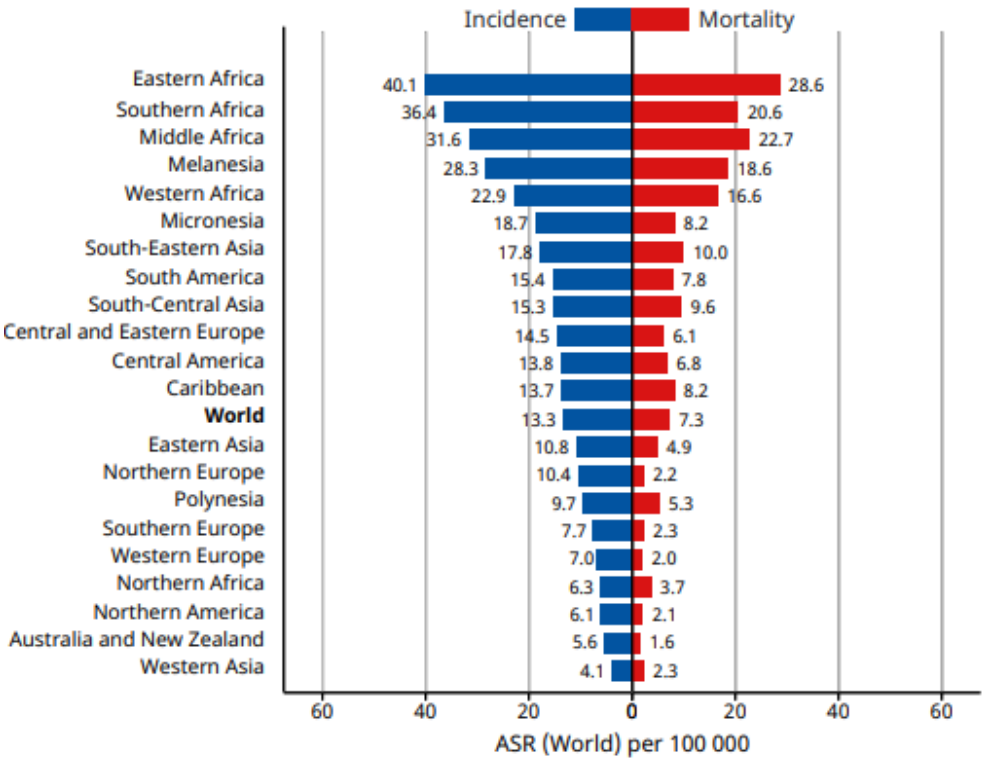

Figure 4.1.2.1. Global epidemiology of cervical cancer (31)

4.1.3.HPV genome structure

Human papillomavirus has double-stranded DNA and is approximately 8 kilo bases in length. The genome structure of HPV is categorized into three regions: early region (ER), late region (LR), non-coding region (NCR), or long control region (LCR) (Figure 4.1.3.1). ER contains E1 to E7 genes, which are responsible for viral replication. LR contains major L1 and minor L2 capsid proteins. NCR is located between the L1 and E6 regions (5).

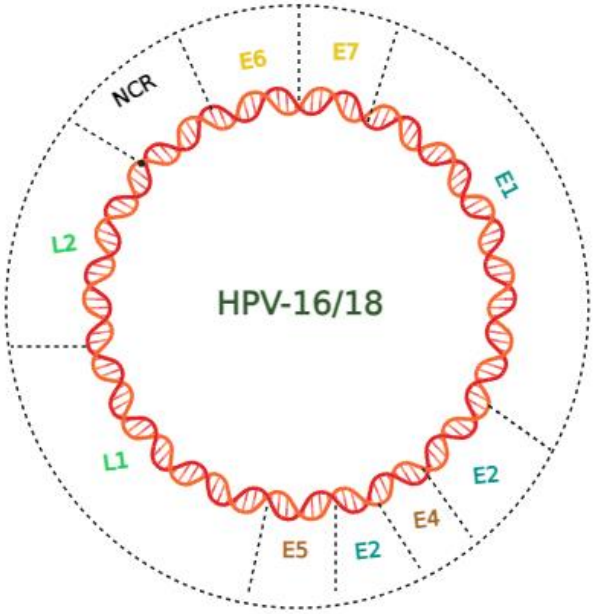

Figure 4.1.3.1. HPV16/18 genome structure

4.1.3.1.Early region (ER)

E1 and E2 proteins are essential because of their activation in early replication for infection. The E1 protein functions as a helicase enzyme and unwinds double-stranded DNA. The E2 protein involves transcription regulation regarding its DNA and protein binding domain. Moreover, E2 protein regulates E6-E7 and can bind to E1 protein. E1 and E2 proteins form a heterodimer structure, which results linking to DNA replication in host cells (32). E4 protein plays a role in the process of viral release. There are several suggestions about the E5 protein. First, it may indirectly help with genome amplification. Second, it may be an oncoprotein and down-regulate expression of the major histocompatibility complex, thereby hindering immune recognition (23,33). E6 and E7 proteins are oncoproteins found in high-risk types of HPV. E6

oncoprotein binds to tumor suppressor protein p53, inhibits cell repair before proliferation, and indirectly promotes the proliferation of cells with mutations. E7 oncoprotein binds to retinoblastoma protein (Rb) and causes the cell cycle to become uncontrolled (5). HPV has been found to be integrated into the host chromosome, where infection causes cancer in tissues. Thus, the loss of E2 expression after integration leads to the overexpression of E6 and E7 oncoproteins, which drive oncogenesis (34). The mentioned proteins often have expression in all types of HPV, but other proteins included in ER, which are E3, E5, and E8, are not expressed in all species.

4.1.3.2.Late region (LR)

Late region encodes L1 and L2 capsid proteins, which are capable of viral capsid formation. The L1 capsid protein is a highly conserved region. Therefore, the nucleotide sequence of the L1 gene is used for the classification of HPV. The nucleotide sequence similarity in the L1 gene between each discovered HPV type is less than 90% (24). L1 has a different amino acid loop between HPV types. Thus, the antibodies generated in response to certain forms of HPV types show limited interaction between distant types (35). The L2 capsid protein becomes available to bind to the extracellular matrix during infection and is cleaved by furin protease (36). Capsid proteins can self-assemble under certain conditions to form virus-like particles (VLPs). It is important to know that these VLPs form the basis of today's HPV vaccines (37).

4.1.3.3.Non-coding region (NCR)

NCR is located between the L1 and E6 regions. This region has a binding site for the E1 and E2 proteins that start viral DNA replication. It also has regulatory elements (like promoters) that control the initiation of transcription. It also has an origin of replication (ori), which is responsible for viral DNA replication in each papillomavirus genome. Their sizes differ between HPV types (5). It is 853 bp in HPV-16 and 825 bp in HPV-18. This region also contains the highest degree of variation in the viral genome (38).

4.1.4.Mechanism of HPV infection

Sexual transmission is a common way for HPV infection. During sexual activity, the squamous or mucosa epithelial tissues abraded, and HPV must reach basal cells for severe infections. HPVs just infect humans by reason of their type-specific features (39,40). The association between HPV and the host cell is done with LR capsid proteins. L1 and L2 proteins cause damage to the basal lamina and undergo a conformational change after interacting with heparin sulphate proteoglycans. Once the capsid proteins bind to the extracellular matrix, the furin protease breaks down the L2 protein. This process facilitates the transfer of the capsid to the second receptor on the keratinocyte. E1 and E2 proteins play an active role for infection to occur in the first replication. The E1 protein acts as a helicase and separates dsDNA. The E2 protein is involved in transcription regulation due to its DNA and protein-binding domain. Additionally, E2 protein is a regulator of E6-E7 and has the ability to bind to E1 protein. These two proteins make a heterodimer structure and bind to the DNA replication process of host cells (32). During HPV-DNA integration, the inhibitory effect of E2 on E6 and E7 is eliminated, leading to an increase in the expression of E6 and E7 gene products and therefore to oncogenesis (41). The E6 protein promotes cell proliferation by stimulating the degradation of the tumor suppressor p53 protein. As a result of degradation, cell cycle progression is disrupted, and tumor cell development increases (42). E7 protein is encoded by high-risk HPV types such as HPV 16 and HPV 18 and binds to the Rb tumor suppressor. Rb protein inhibits the expression of enzyme genes involved in replication (43). In HR-HPV, the E6 protein degrades p53 and the E7 protein degrades Rb, causing the cell cycle to become uncontrolled. Rapidly dividing cells allow the virus to multiply. The replicated viruses then infect other host cells (5). LR-HPV types cause genital warts. Only about 10% of these warts transmit the virus, and most disappear within 4 months. However, the deformity of genital warts may have a possible malignant transformation (44).

4.1.5.Screening methods

Cervical cancer can be prevented with regular screening. The detection of HPV infection is accomplished by the analysis of the patients' HPV genome. There are several kits that allow for the detection of viral DNA. Nucleic acid amplification

techniques are frequently used. Polymerase chain reactions (PCR) are aimed to conservative L1 region amplification. Consensus primers, such as PGMY09/PGMY1 and GP5+/GP6+, are used for the amplification of different HPV types. The COBASW 4800 test is combined with Real-Time PCR to detect HPV and is approved by the Food and Drug Administration (FDA). The test can detect 14 types of HR-HPV groups. Results can be obtained approximately 4 hours later. If a patient has any type of HR, the test is considered positive (45). Specifically, determination of HPV infection is performed with other techniques (8). Cytological techniques are quick and allow easy detection. These techniques do not harm tissue and are suitable for frequent cell sampling. Pap-smear was discovered by Dr. Papanicolau, and it shows the presence of a viral infection with staining of cervical tissues with papanicolau dye (46). However, the utilization of commercial techniques are laborious and time-consuming. Also, the qPCR includes complex protocols and costly usage of equipment (47).

4.1.6.Vaccination and current treatments

Human papillomavirus is present in epithelial cells, and it provides minimum interaction with the host immune system. These situation leads to a temporary and short-term immune response. Researchers found that virus-like particles (VLP) can be used for vaccination. VLPs are structures that do not have the infectious and oncogenic HPV genome and consist of a single structural protein. The L1 protein is capable of undergoing self-assembly to form virus-like particles. Studies with VLPs from dog, rabbit, and bovine papillomavirus have shown that each host is protected against the viral infection (48). In the following studies, VLPs eliminated most of the simple HPV infection and caused the regression of highly squamous intraepithelial lesions (49). In the 2000s, clinical trials based on HPV-16 and HPV-18 VLPs provided immunity against HPV infection and even cervical, vulva, and vaginal diseases (50).

The first commercial vaccine is Gardasil, owned by Merck & Co. and approved by the FDA in 2006. Later, Cervarix was approved in 2009. Both vaccines include the carcinogenic HPV types, specifically HPV-16 and HPV-18. Gardasil also encompasses HPV-6 and HPV-11 types of the human papillomavirus known to be responsible for the development of genital warts (51,52). The current HPV vaccine is Gardasil 9. Furthermore, apart from the first commercial version, Gardasil 9 encompasses additional HR-HPV types, namely 31, 33, 45, 52, and 58. Yeast cells (in

Gardasil) and baculoviruses (in Cervarix) are utilized for the manufacture of L1 protein. Side effects of Gardasil 9 and Cervarix are swelling and pain at the injection site and have rare serious effects (53). HPV vaccinations are recommended for females for use in girls aged 9 and above and are approved for use up to the age of 26 or 45. Currently, 125 nations (64%) have incorporated HPV vaccination programs for females, while 47 nations (24%) have incorporated programs for males (54).

Cervical cancer treatment is performed according to its progression (i.e., surgery at an early stage). Systemic platinum-based chemotherapy and radiotherapy are applied to further-stage patients. Beside these treatments, immunomodulatory vaccination can be used individually or combined with chemotherapy. Advaxis is an immunomodulatory vaccine. It is produced from Listeria monocytogenes bacteria and designed for the expression of the HPV-16 E7 protein (55). GN-00101 is another therapeutic vaccine harbouring the Mycobacterium bovis heat shock protein (Hsp65), which is covalently linked to the HPV16-E7 sequence (56).

4.2.CRISPR-Cas Systems

CRISPR-Cas is an adaptive immunity system that provides protection of mobile genetic elements from invaders. It is formed by Cas genes and the CRISPR array (Figure 4.2.1). The CRISPR array consists of a series of identical palindromic repeats, with spacers located between these repeats. These palindromic repeats are short sequences of approximately 20–40 bp and are the same when read from 5’ to 3’ on one strand of DNA and the complementary strand. Spacers are not identical, and each one is unique. Because these sequences comprise nucleic acids taken from previous invader organisms (57,58).

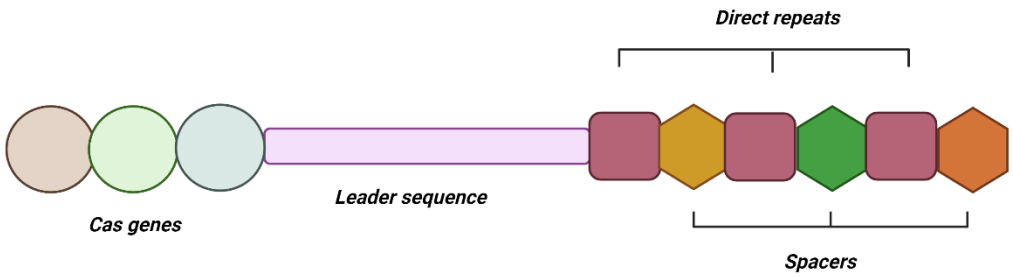

Figure 4.2.1. CRISPR array

4.2.1.History

CRISPR arrays were first observed in 1987, when a group of Japanese researchers working on iap genes in Escherichia coli (E. coli). They found short homologous repeats spaced apart by small spacers (59). In 1993, these sequences were observed in the Haloferax mediterranei archea (60). The term “CRISPR” was initially introduced in a study published by Jansen et al. in 2002. They found four different CRISPR-associated (Cas I-IV) genes in strains containing CRISPR, and these proteins had helicase and nuclease motifs (61). In 2005, published studies showed that spacer sequences are obtained from external mobile genetic elements (62-64). In 2007, the effect of the CRISPR-Cas system on the adaptive immune response was proved experimentally using Streptococcus *thermophiles*. Cells were exposed to Streptococcal phages, and most of the infected ones were dead; the rest of them survived. Upon analysis of the survived cells in CRISPR locus, they found that with the insertion of a repeat, a new spacer was integrated into the CRISPR locus (65). In 2008, researchers noticed that new spacers were based on invader phages. The spacer sequences in phages were named as “protospacer” to distinguish them from bacterial spacer sequences (66). Brouns et al. revealed that CRISPR arrays in E. coli are transcribed, make a complex with Cas genes, and serve as guide RNAs that allow them to interfere with the invader's genome (67). In 2011, non-coding trans-activating cRNA (tracrRNA) was discovered. It is a key component of the crRNA process and facilitates crRNA-Cas9 editing (68). Sapranauskas et al. transplanted CRISPR loci from Streptococcus thermophiles to E. coli. The researchers demonstrated that CRISPR systems are capable of performing genetic editing in different strains (69). In 2012, Doudna and Charpentier showed the first gene editing with the utilization of Cas9 protein derived from Streptococcus *pyogenes* (awarded the Nobel Prize in 2020) (70). In 2015, Zetche et al. identified two orthologues of Cas12a, which were AsCas12a (from Acidaminococcus sp. BV3L6) and LbCas12a (Lachnospiraceae bacterium ND2006), and their genome editing capabilities in human cells. They demonstrated that Cas12a does not include tracrRNA and recognizes T-rich PAM regions (71).

4.2.2.Mechanism of CRISPR-Cas Systems

CRISPR-Cas systems employ different mechanisms depending on the system type. Fundamentally, immune response occurs in three stages (70): adaptive, expression, and interference (Figure 4.2.2.1).

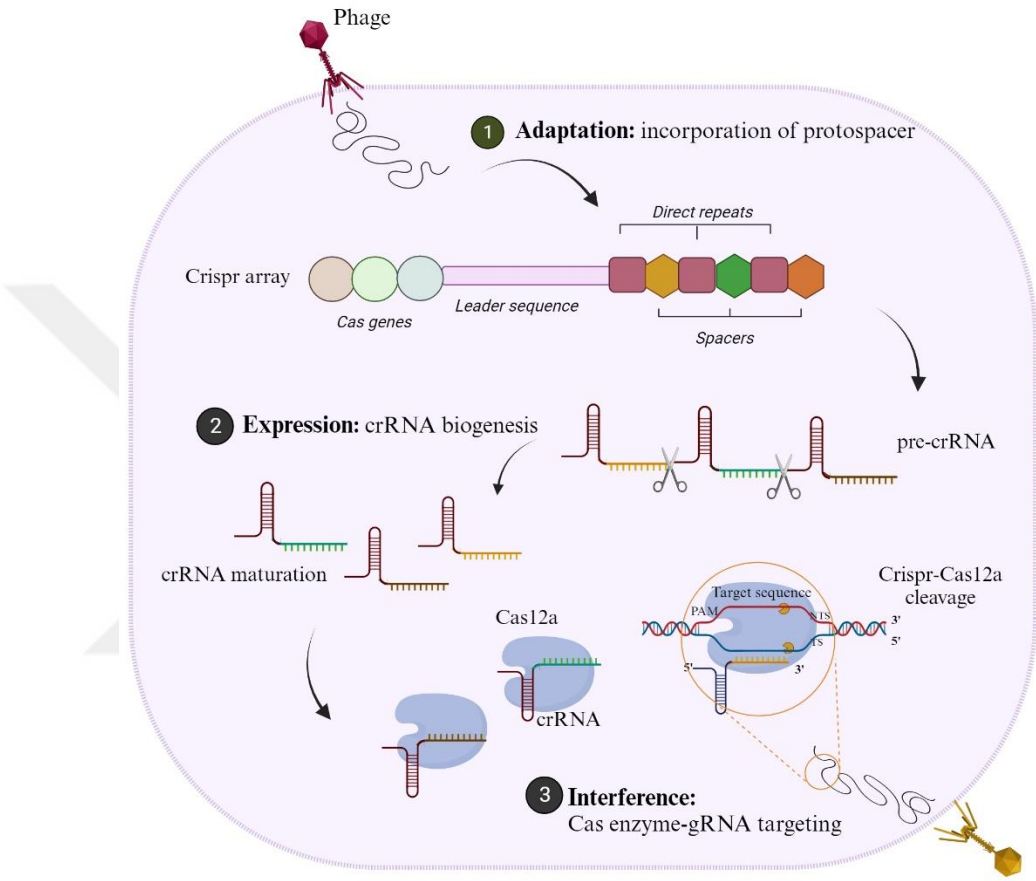

Figure 4.2.2.1. Immune response stages in CRISPR

4.2.2.1.Adaptation

Prokaryotes are frequently exposed to invaders, so the immune response must be quick, effective, and selective to prevent utilizing of resources for unnecessary threats. The adaptive phase involves the incorporation of protospacers derived from invader genomes into the CRISPR array of bacteria. CRISPR arrays are DNA sequences that consist of direct repeats (DR) and spacers. Direct repeats are typically 20–40 base pairs in length and include a palindromic structure, which allows them to form a hairpin structure upon transcription (72,73). Spacers are not identical, and each of them is unique. Because the spacers are derived from the genomes of past invaders.

Thus, CRISPR arrays create a memory for past and present invasions (74). Cas1 and Cas2 endonucleases combine to form a complex known as an adaptation complex. These endonucleases are preserved in most of the CRISPR-Cas systems, but other Cas proteins can be added in dissimilar systems. For instance, the type I CRISPR-Cas system includes the Cas3 and Cas4 adaptation complex (75), whereas the type II CRISPR-Cas system involves the Cas9-Csn2-tracrRNA adaptation complex (76). Cas1 exhibits nuclease activity by cleaving the protospacer into smaller pieces and facilitating its insertion into the CRISPR array (77). The Cas2 enzyme serves as an adaptor protein that facilitates the interaction between Cas1 proteins and enhances the stability of pre-spacer DNA (78). The insertion site of the protospacer into the CRISPR array is close to the leader sequence. The leader sequence is essential because it involves a promoter that directs transcription of the CRISPR locus into pre-crRNA. In addition to its role in transcription, the leader sequence contains recognition sites that guide the adaptation complex (79,80). Invader organisms have conservative DNA sequences of 2–5 bp in length, referred to as the protospacer adjacent motif (PAM). In this way, the adaptation complex recognizes invader's protospacer rather than its own spacer. The PAM sequence is not incorporated when protospacer are added to the CRISPR array (81).

4.2.2.2.Expression

In the expression phase, the spacer sequence, which is inserted in the adaptive phase, and the repeat sequence are transcribed into a long precursor CRISPR RNA (pre-crRNA). This pre-crRNA then undergoes maturation to become a shorter crRNA. Transcription initiates in the leader sequence. Cas6 ribonuclease is responsible for the maturation of pre-crRNA in Class I CRISPR-Cas systems (82). It is proceed by effector proteins in Class II CRISPR-Cas systems. For instance, Cas9 is for type II, Cas12 is for type V, and Cas13 is for type VI. Cas9 maturation also includes bacterial RNase III and an extra RNA species, trans-activating crRNA (tracrRNA) (83). The mature crRNAs consist of two main components: a repeat section known as tracrRNA, which is recognized by different Cas proteins, and a spacer region responsible for mediating target specificity. Maturated crRNAs consist of preserved sequences derived from direct repeats and guide sites derived from spacers. The spacer part is 17–35 bp in length and has the ability to match the protospacer sequence of the invader

genome as complementary. DR sequences bind to Cas proteins and allow ribonucleoprotein complexes with crRNA. This complex might be made up of only one protein, the Cas effector nuclease, or it could be made up of several Cas proteins (84). In this thesis, the Class 2 type V CRISPR-Cas system was used. The crRNA biosynthesis of Cas12a is discussed in the Section 4.2.3.1.

4.2.2.3.Interference

The crRNA-Cas effector complex, which formed in the expression phase, becomes a functional RNA-guided endonuclease. The protospacer sequence contains conserved sequences known as the protospacer adjacent motif (PAM) (85). The formed crRNA-Cas complex recognizes the invading organism with its PAM sequence. Subsequently, Cas proteins proceed to unwind the double-stranded DNA that is located next to the PAM site and bind as complementary base pairing between the crRNA and the protospacer (86). After binding, an R loop is formed in which crRNA is paired with DNA. The genome of the invader is cleaved by Cas endonucleases. Moreover, the fragments are used as a new source of spacers. As a result, mobile genetic elements defend themselves against future invader attacks (87).

4.2.3.Classification of CRISPR-Cas Systems

CRISPR-Cas systems are categorized into two classes, Class 1 and Class 2, and six subtypes (I-VI). Different types of CRISPR-Cas systems can exist in one organism (88). Class 1 CRISPR systems utilize multiple Cas proteins and crRNA to form an effector complex. Each subunit has a function in a multi-effector complex. Class 2 CRISPR systems utilize a single functional Cas protein together with crRNAs. This single Cas protein performs functions for target recognition and fragmentation on its own. Class 1 includes type I, III, and IV, whereas Class 2 includes type II, V, and VI Cas genes (89). The type I-II-V system is responsible for DNA editing, whereas the type VI system is responsible for RNA editing (90). Additionally, the type III system possesses the capability to edit both DNA and RNA. In type I-III, Cas6 endonuclease is responsible for the cleavage of the repeats for pre-crRNA maturation (85). The type I Cas gene involves the formation of a complex including matured crRNA, Cas3 protein, and cascade (CRISPR-associated complex for antiviral defence), which subsequently leads to the cleavage of foreign DNA. The type II Cas gene involves the

formation of a complex between matured crRNA and either Cmr/Cas10 or Csm/Cas10 proteins, which subsequently leads to the cleavage of foreign DNA. Type IV systems are characterized by the involvement of the Csf1 gene. The gene is responsible for encoding a component that is related to the Cas8 protein found in the cascade complex.

Class 2 CRISPR-Cas systems, such as CRISPR-Cas9, CRISPR-Cas12a (also known as CRISPR-Cpf1), and CRISPR-Cas13, employ a single Cas gene to defend the host from invader genetic elements. Class 2 effector proteins are less complex than Class 1 effector proteins because they perform the functions necessary for the three stages (adaptation, expression, and interference) required for the immune response, and it is important to have less complexity for gene editing (91). In the type II system, crRNA forms a ribonucleoprotein complex with Cas protein and tracrRNA, which recognize and make a cleavage in the DNA. SpCas9, which is derived from Streptococcus *pyogenes* (Sp), is commonly used in type II systems (70). Cas12a belongs to the type V system. Similar to Cas9, it has the ability to recognize DNA sequences that are complementary to the guide RNA through base pairing. Cas12a has a broad range of application areas due to its distinctive features compared to Cas9. Firstly, Cas9 requires the presence of tracrRNA and RNase III for the maturation process. In contrast, Cas12a has the ability to make the maturation process itself without any additional requirements (89). Cas9 recognizes "NGG" PAM sites and requires gene editing sites that are high in GC content (92). Cas12a recognizes "TTTV" PAM sites and requires AT-rich sites for gene editing. Promoters and introns are generally located in AT-rich sites, thus facilitating Cas12a for editing (93). Moreover, Cas12a cleavage results in staggered ends, whereas Cas9 cleavage results in blunt ends. Cas9 has two endonuclease domains, namely RuvC and HNH. Cas12a just has a RuvC endonuclease domain (94). This thesis employed two orthologs of the Cas12a enzyme, explicitly LbCas12a and AsCas12a.

4.2.3.1.Class 2-Type V Systems (Cas12a)

CRISPR-Cas12a is an endonuclease protein of Class 2 type V. The Cas12a protein was initially investigated in 2012. Researchers observed an unknown Cas structure while studying the CRISPR locus in the pathogen Francisella tularensis. This structure consisted of an adaptation complex of Cas1, Cas2, and Cas4 and an unknown Cas gene (95). The unknown Cas gene was identified as Cas12a in a report by Zetche

et al. They also found that the Cas12a protein contains 1300 amino acids. LbCas12a, AsCas12a, and FnCas12a are three homologous of the Cas12a endonucleases, derived from the Lachnospiraceae bacteria, Acidaminococcus sp., and Francisella novicida, respectively. The required PAM sequence for Cas12a is "TTTV" (V = A/C/G). The Cas12a enzyme recognizes the protospacer from the 5' PAM sequence at the interference stage. CRISPR arrays are processed into mature crRNAs that are 42–44 nucleotides in length. The Cas12a enzyme cleaves mature CRISPR RNAs (crRNAs) four nucleotides upstream of the hairpin structures that are generated by the CRISPR repeats (DR). This structure subsequently acts as a scaffold for the crRNA-Cas effector complex (96). Matured cRNAs comprise a 19-bp direct repeat and a 25-bp spacer sequence (94).

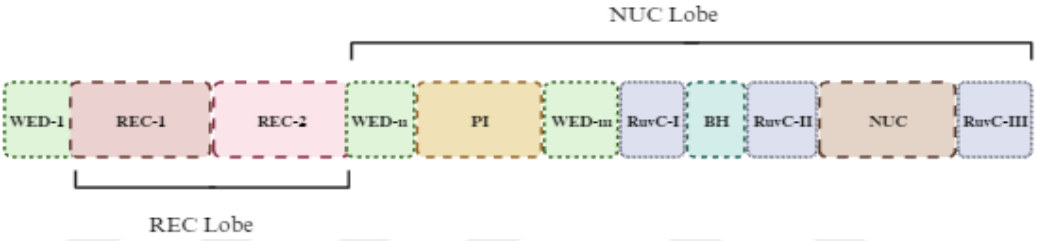

Figure 4.2.3.1.1. Cas12a domains

Cas12a is composed of two main structural components (Figure 4.2.3.1.1): the recognition lobe (REC), which is characterized by α-helical structures, and the nuclease lobe (NUC). The REC lobe has two domains, specifically REC1 and REC2. The NUC lobe contains the RuvC nuclease domain and additional domains, which are WED, the bridge helix (BH), and pam-interaction (PI) (97). The RNase region enables Cas12a to self-maturate and is located under the WED-III domain. The DNase region is located between the RuvC and NUC domains. The WED and PI domains are responsible for PAM recognition and linkage of crRNA, Cas12a, and DNA. After PAM recognition, loop-lysine helix-loop (LKL), which is located in the PI domain, incorporates the helix into PAM. Then, dsDNA unwinds and leads to hybridization of crRNA and target strand (TS) (98,99). To cleave the target strand and non-target strand by RuvC and NUC catalytic regions in the Cas12a enzyme, it is essential that these strands be positioned with 5’-3’ polarity. In the literature, it is observed that NTS has the correct position, whereas TS has the reverse polarity. Hence, the RuvC domain

exhibits cleavage activity specifically at the 18th base position on the non-target strand (NTS). Subsequently, the REC and NUC lobes of Cas12a undergo a conformational change to facilitate the entry of the target sequence (TS) into the nuclease site, ensuring proper polarity (100). NUC bends the target strand (TS) through the RuvC domain and cleavage by the RuvC domain after the 23rd base, resulting in a double-stranded staggered break in DNA. In this way, the effector protein with a single active site cleaves two strands sequentially (94).

4.2.3.2.Viral detection with CRISPR-Cas12a

During the pandemic, it became crucial to develop detection mechanisms that provided rapid and precise results. Scientists have developed a range of CRISPR-Cas-based methods up to now (101-103). The discovery of the collateral activity of Cas12 (104) and Cas13 (105) enzymes has led to the beginning of a new era. Doudna et al. published a mechanism called "DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR)" in 2007. In this study, they combined the LbCas12a enzyme, its crRNA, target DNA, and an additional irrelevant single-stranded DNA. This ssDNA involved a fluorescent and a quencher (FQ). They showed that LbCas12a protein bound and cleaved target DNA with crRNA, and then it also cleaved the ssDNA-FQ. Further, they utilized this system for HPV-16 and HPV-18 detection (Figure 4.2.3.2.1). They determined two target sequences that differed between the two HPV types by only six base pairs. Extracted human anal swabs were used. The extracted DNA samples were amplified with isothermal recombinase polymerase amplification (RPA). Then, LbCas12a enzyme were incubated with target-specific crRNA and ssDNA-FQ probe. When LbCas12a was recognized and cleaved the target DNAs, there was a signal out due to probe degradation (104).

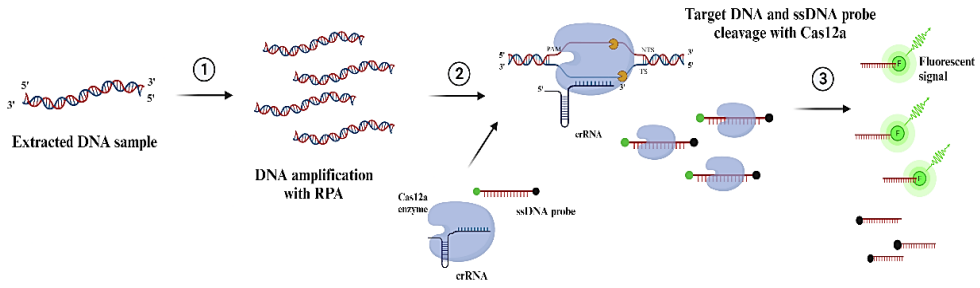

Figure 4.2.3.2.1. Mechanism of DETECTR system

5.MATERIALS AND METHOD

This study was carried out at Istanbul Medipol University Health Sciences and Technologies Research Institute (SABITA).

5.1.Materials

The materials used in the experiments are given in Table 5.1.1.

Table 5.1.1. Materials

Material	Brand	Catalog Number
Platinum SuperFi II PCR Master Mix	Thermo Fisher Scientific	12368010
MAXIscript T7 Transcription Kit	Thermo Fisher Scientific	AM1314
RNaseZap	Thermo Fisher Scientific	AM9780
Quick-DNA Miniprep Plus Kit	Zymo Research	D4068
Zymoclean Gel DNA Recovery Kit	Zymo Research	D4008
DNA Clean&Concentrator-5	Zymo Research	D4014
RNA Clean&Concentrator-5 DNase I Included	Zymo Research	R1014
HiScribe T7 High Yield RNA Synthesis Kit	New England Biolabs	E2040S
2X RNA Loading Dye	New England Biolabs	B0363S
Low Range ssRNA Ladder	New England Biolabs	N0364S
6X Purple Gel Loading Dye	New England Biolabs	B7024S
EnGen Lba Cas12a (Cpf1)	New England Biolabs	M0653S
Lambda DNA / Hind III Ladder	Hibrigen	MG-LDR-LH3-1
6x blue gel loading dye	Hibrigen	MG-YBM-01
50 bp DNA Ladder	Hibrigen	MG-LDR-50-1
1 kb DNA Ladder	Hibrigen	MG-LDR-1000-1
1 kb plus DNA Ladder	Hibrigen	MG-LDR-1000P-1
Low Range DNA Ladder	Hibrigen	MG-LDR-LW
Proteinase K	Hibrigen	MG-PASEK-01-20
Agarose	Hibrigen	MG-AGR-01-100
Gel-Safer	A.B.T.	G02-01-05
50X Tris-acetate-EDTA (TAE) buffer	A.B.T.	B045001

1X Tris-borate-EDTA (TBE)	A.B.T.	B030101
PrimeSTAR GXL Premix	Takara Bio	R051A
AsCas12a Nuclease	GenScript	Z03502
Tris-EDTA (TE) Solution 1X	WISENT INC.	809-215-LL

5.2.Methods

This study included patient samples that encompassed HPV-16 and HPV-18 types. Three target sites were identified for HPV-16, and two target sites were identified for HPV-18. The genomes of HPV-16 and HPV-18 were amplified with the polymerase chain reaction (PCR) technique. crRNA sequences were designed and produced specifically for the target regions. Cas12a targeting experiments were done using LbCas12a and AsCas12a enzymes. All oligonucleotides were synthesized by Oligomer Biotechnology Co. The general experimental design is given below (Figure 5.2.1). Human papillomavirus samples were collected from patients at Istanbul Medipol University Genetic Diseases Assessment Center. This study was approved by the Ethics Committee at Istanbul Medipol University Non-Interventional Clinical Research (Number: E-10840098-772.02-221).

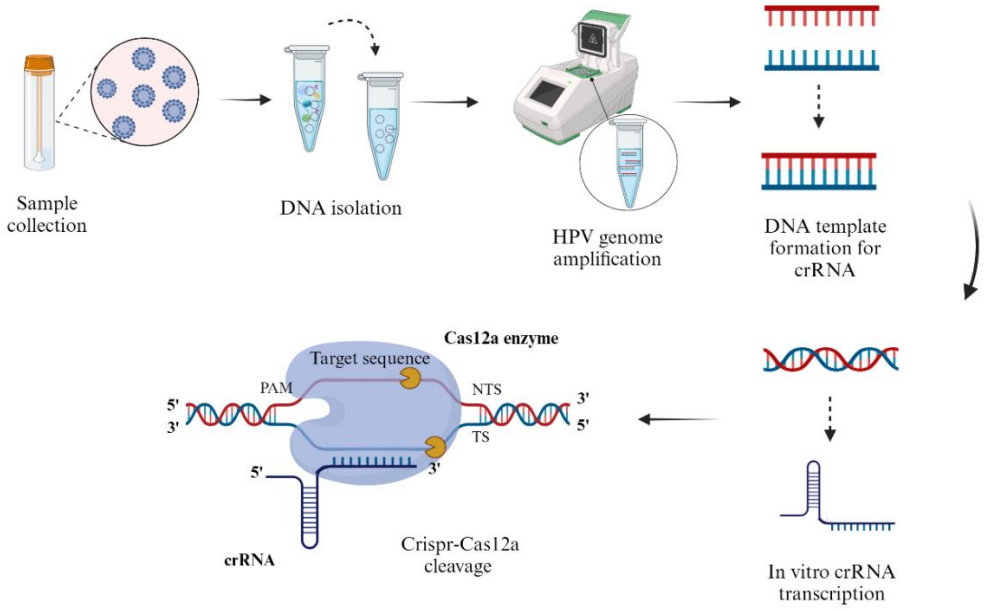

Figure 5.2.1. General scheme of the experiments

5.2.1.Determination of target sites

HPV-16 (reference sequence: NC_001526.4) and HPV-18 (reference sequence: NC_001357.1) genomes were downloaded from NCBI software. Possible target sequences were identified in L1 region with the “TTTV” PAM involved. These target sequences were analysed one by one using NCBI Blast. Three different target sequences for HPV-16 (Figure 5.2.1.1) and two different target sequences for HPV-18 (Figure 5.2.1.5) were chosen for experiments.

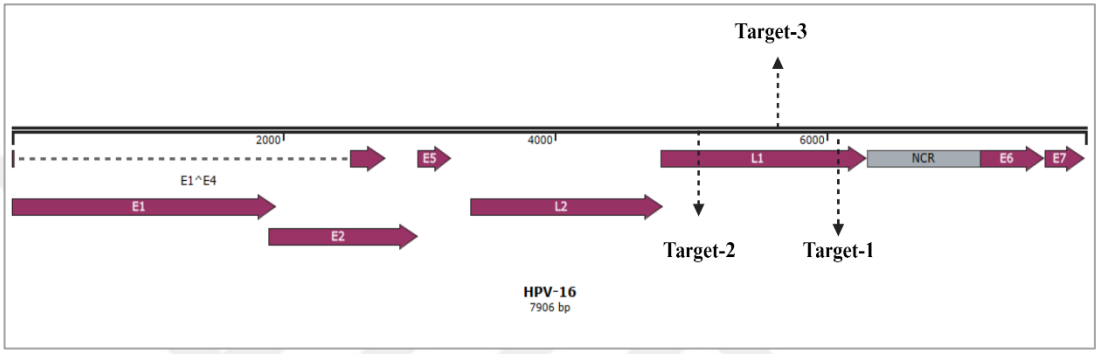

Figure 5.2.1.1. HPV-16 linearized genome (after PCR)

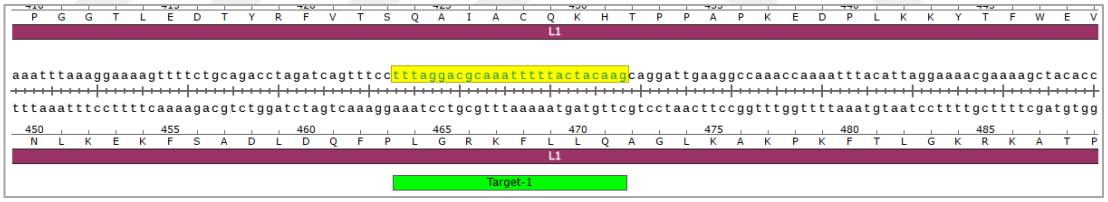

Figure 5.2.1.2. HPV-16 Target-1 location

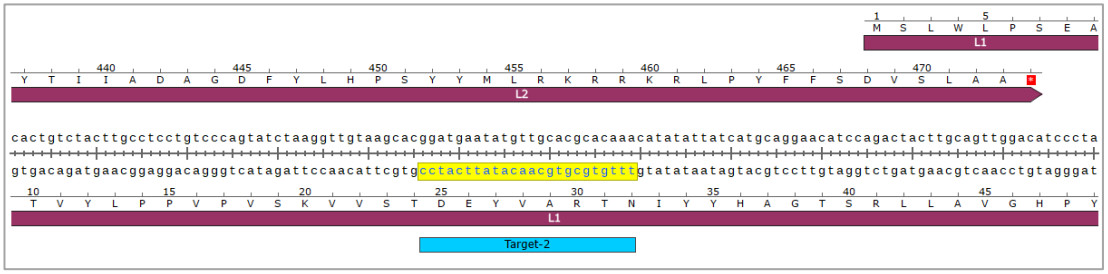

Figure 5.2.1.3. HPV-16 Target-2 location

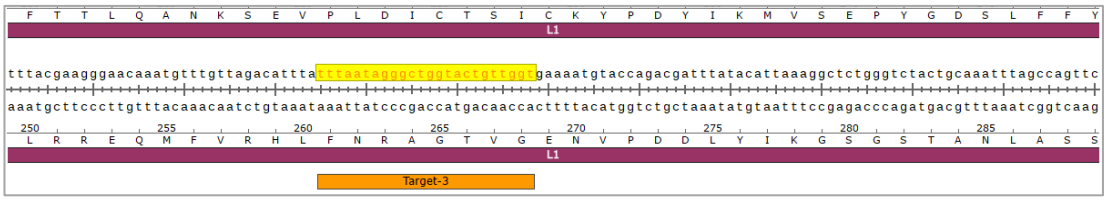

Figure 5.2.1.4. HPV-16 Target-3 location

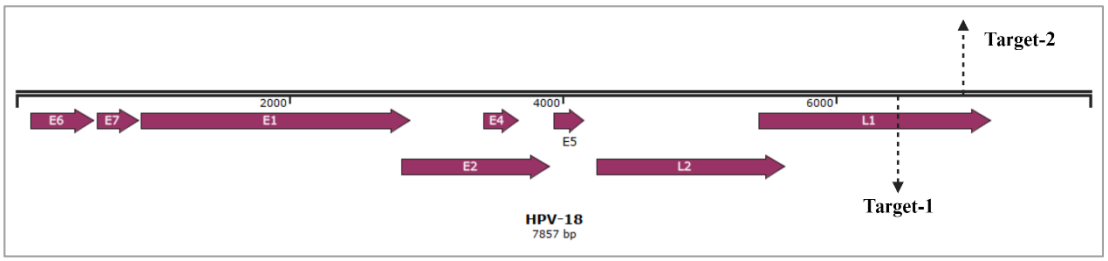

Figure 5.2.1.5. HPV-18 linearized genome (after PCR)

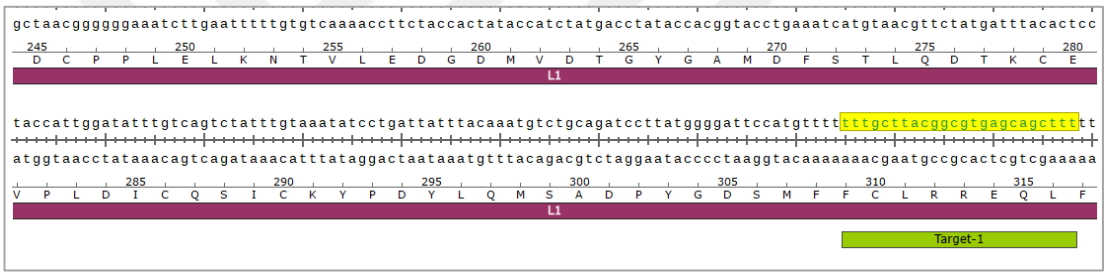

Figure 5.2.1.6. HPV-18 Target-1 location

Figure 5.2.1.7. HPV-18 Target-2 location

5.2.2.Sample collection

Vaginal swabs of cervical cancer patients were taken from Istanbul Medipol University Genetic Diseases Assessment Center. Each sample tube contained HPV-16 or HPV-18 types alone. DNA samples were isolated using Quick-DNA MiniPrep Plus kit according to the manufacturer's instructions:

1. Vaginal swabs were centrifuged at 1500 rpm for 1 min. 200 µl pellet was put into an Eppendorf tube.
2. 200 µl BioFluid&Cell Buffer and 20 µl Proteinase K was added into tube.
3. The tube was vortexed and incubated at 55℃ for 15 minutes.
4. Genomic Binding Buffer was added to solution in 1:1 ratio.
5. The mixture was transferred into the spin column and centrifuged at 12000 g for 1 min.
6. The liquid was discarded.
7. 400 µl DNA Pre-Wash Buffer was added to the spin column in a fresh collection tube and centrifuged at 12000 g for 1 min.
8. The liquid was discarded.
9. 700 µl DNA Wash Buffer was added into the column and centrifuged at 12000 g for 1 min.
10. 200 µl DNA Wash Buffer was added into the column and centrifuged at 12000 g for 1 min.
11. A new Eppendorf tube was placed in the spin column.
12. 50 µl RNase-free water was added on the top of the spin column membrane to elute DNA and centrifuged at 12000g for 2 min.
13. After purification, DNA quantifications were measured using NanoDrop One device (Thermo Fischer Scientific).

5.2.3.HPV-16 Target-1

5.2.3.1.HPV-16 genome amplification

HPV-16 genome was amplified using the polymerase chain reaction (PCR) technique. Platinum SuperFi II PCR Master Mix was used for PCR reactions. Forward and reverse primers were designed by SnapGene software. Hairpin structures and heterodimer analysis of primers were checked at IDT (Integrated DNA Technologies, Inc.). Primers are listed below.

Table 5.2.3.1.1. Primers for HPV-16 Target-1 genome amplification

Oligo Name	**Sequence (5'-3')**	**Amplicon Size**
HPV16_Forwardprimer_2023	atggctgatcctgcag	7906 bp
HPV16_Reverseprimer_2023	ggtagattatggtttctgagaac	

10 µl of PCR reaction was set up, and components were given in Table 5.2.3.1.2. Amplification was performed in thermal cycler with 3-step PCR protocol: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing at 60℃ for 10 sec and extension at 72℃ for 3 min, final extension at 72℃ for 5 min and 4℃ hold.

1% agarose gel was prepared for the separation of HPV fragments. 0.2 gr of agarose powder was put into 20 mL of 1X Tris-borate-EDTA (TBE) buffer and microwaved until the powder completely dissolved. After, 2 drops (~60 µl) of Gel-Safer were added to the solution and mixed immediately. The solution was poured into gel tray and left at room temperature until totally solidified.

When PCR was finished, 2 µl of 6X DNA loading dye was put into the PCR tube and mixed well. Total amount in the PCR tube (12 µl) and 2 µl of 1 kb DNA Ladder were loaded on the agarose gel. The gel was run at 100 V for 60 minutes. ChemiDoc MP Imaging System (BioRad) was used for visualization of PCR. Confirmed DNA was extracted from agarose gel using Zymoclean Gel DNA Recovery kit with manufacturer's instructions:

1. DNA was cut from the gel using scalpel blade under the blue light.
2. Isolated gel sample was put into an Eppendorf tube and determined the cutting gel weight as mg.
3. Agarose dissolving buffer (ADB) was added 3 times the gel volume and left at 15 min incubation.
4. The melted gel was transferred into the spin column.
5. The column was centrifuged at 16000 g for 1 min.
6. The liquid in the collection tube was discarded and 200 µl DNA wash buffer was added into the column.
7. The column was centrifuged at 16000 g for 1 min.
8. The 6. and 7. Steps were repeated.
9. The column was placed into a new Eppendorf tube and 10 µl RNase-free water was added and waited for 5 min for the filter to absorb the water properly.
10. The column was centrifuged at 16000 g for 2 min.
11. DNA concentration was measured by using NanoDrop One device (19 ng/µl)

Table 5.2.3.1.2. PCR components for total genome amplification

Reaction Component	Volume	Final Concentration
2X Platinum SuperFi II PCR Master mix	5 µl	1X
Forward primer	0.5 µl	0.5 µM
Reverse primer	0.5 µl	0.5 µM
Template DNA	0.5 µl	~60 ng*
Nuclease-free water	to 10 µl	-
Total	10 µl	

*Upon the protocol, 5-100 ng genomic DNA should be used.

For Target-1, HPV-16 L1 region to be amplified site was determined. The previously amplified total genome was used for template DNA. Forward and reverse primers were designed for Target-1 by SnapGene software. Designed primers were checked at IDT (Integrated DNA Technologies, Inc.). Primers are listed below.

Table 5.2.3.1.3. Primers used for HPV-16 L1 region

Oligo Name	Sequence (5'-3')	Amplicon Size
Hpv16_L1_forwardprimer	cgatttatacattaaaggctctg	700 bp
Hpv16_L1_reverseprimer	ttacagcttacgtttttgc	

20 µl of PCR reaction was set up and components were given in Table 5.2.3.1.4. Amplification was performed in thermal cycler with 3-step PCR protocol: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing at 60℃ for 10 sec and extension at 72℃ for 15 sec, final extension at 72℃ for 5 min and 4℃ hold.

1% agarose gel was prepared. 1 µl of PCR product, 1 µl of 6X loading dye, and 4 µl of nuclease-free water were mixed in the PCR tube. The mixture and 2 µl of low range DNA ladder were loaded in gel to confirm the length of DNA by gel electrophoresis. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using DNA Clean&Concentrator-5 kit with manufacturer's instructions:

1. 95 µl DNA Binding Buffer was added into PCR product and vortexed.
2. The mixture was put into the spin column.
3. The column was centrifuged at 16000 g at 1 min.
4. The liquid in the collection tube was discarded and 200 µl DNA wash buffer was added into the column.
5. The column was centrifuged at 16000 g at 1 min.

6. The 6. and 7. Steps were repeated.
7. The column was placed into a new Eppendorf tube and 19 µl RNase-free water was added and waited for 5 min for the filter to absorb the water properly.
8. The column was centrifuged at 16000 g at 2 min.
9. DNA concentration was measured with NanoDrop One device. (182 ng/µl)

Table 5.2.3.1.4. PCR components for L1 region amplification

Reaction Component	**Volume**	**Final Concentration**
2X Platinum SuperFi II PCR Master mix	10 µl	1X
Forward primer	1 µl	0.5 µM
Reverse primer	1 µl	0.5 µM
Template DNA	0.5 µl	~9 ng
Nuclease-free water	to 20 µl	-
Total	20 µl	

5.2.3.2.DNA template formation for crRNA

A double-stranded DNA was formed to be used as a template in crRNA transcription (Figure 5.2.3.2.1). Therefore, two single strand DNA sequences were designed. The designed sequences are given in Table 5.2.3.2.1. These sequences were annealed and blank nucleotides were filled with DNA polymerase.

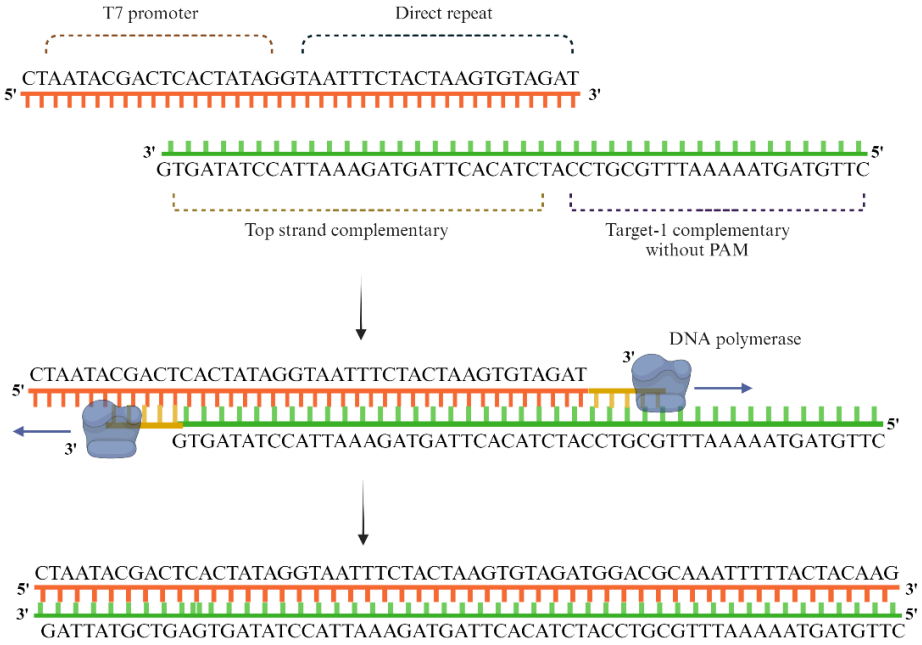

Figure 5.2.3.2.1. DNA template formation of HPV-16 Target-1

Top and bottom strands were stocked to 10 µM in nuclease-free water. 20 µl reaction was set up, and components are given in Table 5.2.3.2.2. Amplification was performed in thermal cycler with initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing at 60℃ for 10 sec and extension at 72℃ for 2 sec.

3% agarose gel was prepared. After PCR, DNA template should be 63 bp. 1 µl of PCR product, 1 µl of 6X loading dye and 4 µl of nuclease-free water were mixed in a PCR tube. Top and bottom strands (10 µM) were also prepared as PCR product. They were loaded into the gel to confirm the PCR product. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using Zymo DNA Clean&Concentrator-5 kit with manufacturer's instructions. The concentration was 23.6 ng/µl (20ul).

Table 5.2.3.2.1. Oligoes for HPV-16 Target-1 DNA template formation

Oligo Name	**Sequence (5'-3')**
Cas12acrRNA_T7promoter_template_top	CTAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGAT
HPV_16_truncated_bottom_2023	cttgtagtaaaaatttgcgtccATCTACACTTAGTAGAAATTACCTATAGTG

Table 5.2.3.2.2. PCR components for DNA template formation

Reaction Component	**Volume**
2X Platinum SuperFi II PCR Master mix	10 µl
Cas12a_top (10 µM)	4 µl
HPV-16_bottom (10 µM)	4 µl
RNase-free water	to 20 µl
Total	20 µl

5.2.3.3.In vitro transcription of crRNA

The generated DNA was used as template for crRNA transcription. Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. NEB High Scribe T7 High Yield RNA Synthesis kit and its short RNA transcript protocol were used. 20 µl of reaction was set up into 0.2 ml RNase-free PCR tube and components are given below. The reaction was incubated at 37℃ for 4 hours in thermal cycler.

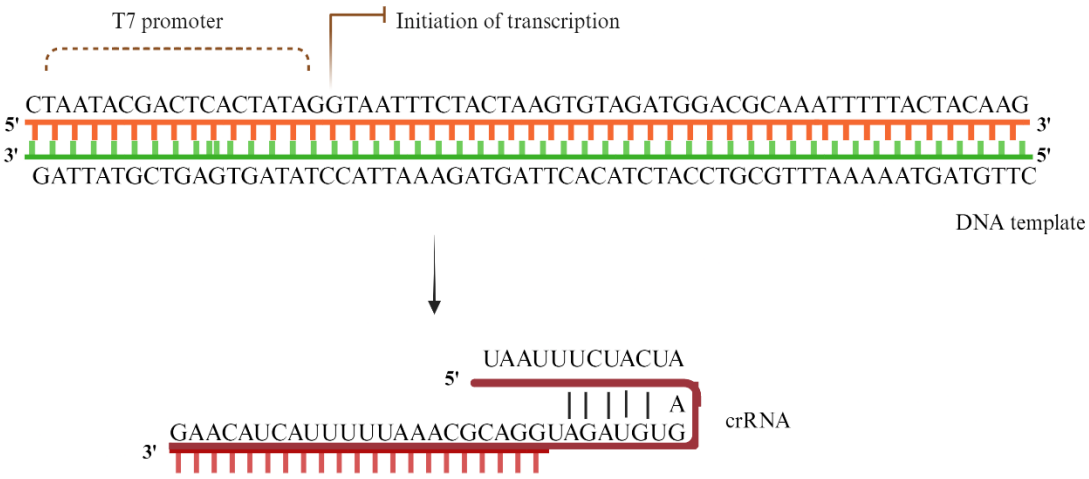

Figure 5.2.3.3.1. Transcription of HPV-16 Target-1 crRNA

After the incubation, RNA Clean&Concentrator-5 kit was used. During purification, the same conditions were provided as in the experiment to prevent contamination. Firstly, DNase I Treatment was done as described in the kit manual. The mixture was set up, and components are given in Table 5.2.3.3.2. Components were added into the transcribed tube and incubated in room temperature for 40 minutes. Therefore, short RNAs section was applied with manufacturer's instructions:

1. 50 µl ethanol and 50 µl RNA binding buffer was put into fresh RNase-free tube and mixed by vortex.
2. Mixture was transferred into the transcribed tube which DNase I treated and pipetted gently.
3. All of the amount in the tube was put into the spin column.
4. The column was centrifuged at 16000 g at 1 min.
5. The remained liquid was not discarded. 150 µl ethanol was added onto the liquid.
6. New mixture was transferred into fresh column and tube.
7. The column was centrifuged at 16000 g at 1 min. The liquid in the collection tube was discarded.
8. 400 µl RNA prep buffer was added into the column.
9. The column was centrifuged at 16000 g at 1 min. The liquid in the collection tube was discarded.
10. 700 µl RNA wash buffer was added into the column.

11. The column was centrifuged at 16000 g at 1 min. The liquid in the collection tube was discarded.
12. 700 µl RNA wash buffer was added into the column.
13. The column was centrifuged at 16000 g at 1 min. The liquid in the collection tube was discarded.
14. The column was placed into a new RNase-free Eppendorf tube and 15 µl RNase-free water was added and waited for 5 min for the filter to absorb the water properly.
15. The column was centrifuged at 16000 g at 2 min.
16. RNA concentration was measured with NanoDrop One. (9422 ng/µl)

3% agarose gel was prepared. In order to visualize RNA, 2 µl of 2X RNA loading dye and 1000 ng/µl RNA were mixed in a PCR tube and loaded into the gel. After transcription, crRNA length should be 43 bp. The length of RNA was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes.

Table 5.2.3.3.1. Transcription components of HPV-16 Target-1 crRNA

Reaction component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	8 µl*	188,8 ng*
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

*Upon protocol template DNA should be 1 µg but 384 ng was obtained after DNA template formation. To check whether less amount is worked, half of the DNA concentration was used.

Table 5.2.3.3.2. DNase-I treatment components

Reaction component	**Volume**
RNA Sample	40 µl*
DNase I	5 µl
DNA Digestion Buffer	5 µl

*Transcription was done as 20 µl but for DNase-I treatment, it recommends complete the volume to 20 µl with RNase-free water.

5.2.3.4.In vitro cleavage of L1 region using LbCas12a

In order to cleavage reactions, LbCas12a protocol was used from NEB website. As protocol, the molar ratio between Cas12a enzyme, gRNA, and target DNA should

be 10:10:1. First of all, the molar concentrations of transcribed RNA and amplified L1 target DNA were calculated below.

$$crRNA\ (\mu M) = \frac{Concentration\ of\ RNA\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times Length\ of\ RNA\ (bp)} \times 10^3$$

$$crRNA\ (\mu M) = \frac{9422\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times 43} \times 10^3 = 664\ \mu M$$

$$DNA\ (nM) = \frac{Concentration\ of\ DNA\ (\frac{ng}{\mu l})}{660\frac{g}{mol} \times Length\ of\ DNA} \times 10^6$$

$$DNA\ (nM) = \frac{182\ \left(\frac{ng}{\mu l}\right)}{660\frac{g}{mol} \times 700} \times 10^6 = 394\ nM$$

According to the protocol, 300 nM crRNA should be used. Therefore, 300 nM stock crRNA was prepared below. The calculated amount was added to a new RNase-free tube, and the rest of the volume was completed with RNase-free water.

$$M_1 \times V_1 = M_2 \times V_2$$

$$664\ \mu M \times V_1 = 0{,}3\ \mu M \times 600\ \mu l \longrightarrow V_1 = 0.27\ \mu l$$

Before starting, the experiment area was cleaned with 70% ethanol and RNase Zap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. The reaction components are given in Table 5.2.3.4.1. The reaction was set up without adding DNA to 0.2 ml RNase-free PCR tube. The solution was pre-incubated at room temperature for 15 minutes. After Cas12a-gRNA complexed during pre-incubation time, DNA was added to the reaction and incubated at 37°C for 4 hours and 65°C for 15 min (heat inactivation) in thermal cycler. Cas12a digestion is illustrated in the figure below. At the end of the 4 hours

incubation, the reaction was stopped by adding 6 µl of 6X SDS containing Purple Dye. 1% agarose gel was prepared. Each lane has 20 µl volume, so the cleavage product was loaded into two lanes. Also, substrate DNA as a control and low range DNA ladder were loaded. The gel was run at 100 V for 60 minutes and imaged on BioRad system.

Table 5.2.3.4.1. Targeting components of HPV-16 Target-1

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	3 µl
300 nM gRNA	3 µl
1 µM EnGen LbCas12a	1 µl
RNase-free water	22.77 µl
DNA	0.23 µl*

*3 nM final DNA

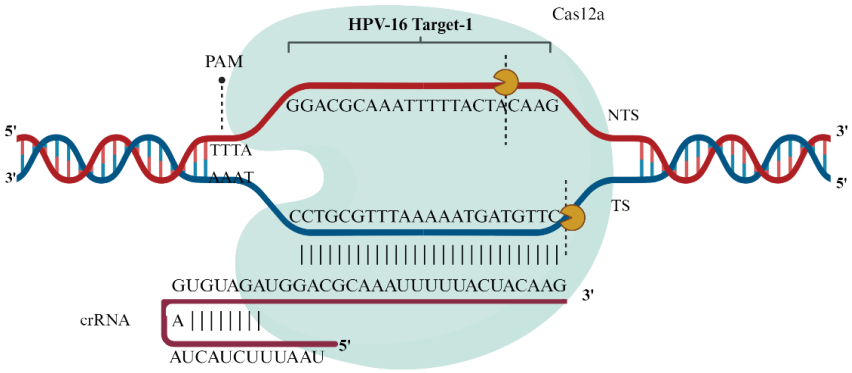

Figure 5.2.3.4.1. HPV-16 Target-1 cleavage

5.2.3.5.Troubleshooting of Target-1

The experiments described above are the optimized results. Optimization conditions are given below.

5.2.3.5.1.Total genome amplification

1. Different Tm values was carried out.

Table 5.2.3.5.1.1. PCR conditions

Initial denaturation	98°C	30 sec	1x
Denaturation	98°C	10 sec	35x
Annealing	**64°C or 58°C**	10 sec	
Extension	72°C	3 min	
Final extension	72°C	5 min	1x
	4°C	hold	

2. Two-step PCR was done in thermal cycler.

Table 5.2.3.5.1.2. PCR conditions of two-step

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing/Extension	68℃	3 min	
Final extension	72℃	5 min	1x
	4℃	hold	

3. Primer concentration was decreased to 0.25 µM.

Table 5.2.3.5.1.3. PCR components

Reaction component	**Volume**	**Final concentration**
2X Platinum SuperFi II PCR Master mix	5 µl	1X
Forward primer	0.25 µl	**0.25 µM**
Reverse primer	0.25 µl	**0.25 µM**
Template DNA	1 µl	~20 ng
Nuclease-free water	to 10 µl	-
Total	10 µl	

Table 5.2.3.5.1.4. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing	60℃	10 sec	
Extension	72℃	3 min	
Final extension	72℃	5 min	1x
	4℃	hold	

4. PrimerSTAR GXL Premix was used for genome amplification.

Table 5.2.3.5.1.5. PCR components

Reaction Component	**Volume**	**Final Concentration**
2X PrimeSTAR GXL Premix	25 µl	1X
Forward primer	0.4 µl	0.2 µM
Reverse primer	0.4 µl	0.2 µM
Template DNA	2 µl	~72 ng
Nuclease-free water	to 20 µl	-
Total	20 µl	

Table 5.2.3.5.1.6. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing	60℃	15 sec	
Extension	68℃	8 min	
Final extension	68℃	10 min	1x
	4℃	hold	

5.2.3.5.2.DNA template formation for crRNA

1. Tm value was changed in PCR.

Table 5.2.3.5.2.1. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing	**64℃/65℃/66℃**	10 sec	
Extension	72℃	2 sec	
	4℃	hold	1x

2. A different method (106) was revised for DNA template formation. Normally, direct repeats in the top strand and its complementary in the bottom strand were annealed, and blank sequences were filled with DNA polymerase. In this protocol, oligonucleotides were stocked as 100 µM in Tris-EDTA (TE) buffer. Then, they were diluted to 10 µM in Tris-Buffered Saline (TBS). 10 µl top strand and 10 µl bottom strand were added in a PCR tube. The templates were annealed at 95℃ for 2 min and cooled slowly in bench. After, the template concentration was measured in NanoDrop with 10 times diluted (64.5 ng/µl). Transcription protocol was applied as the same as In vitro transcription for crRNA formation section. 200 ng template DNA was used for transcription. It was incubated at 37℃ for 2 hours.

Table 5.2.3.5.2.2. Primers used for DNA template formation

Oligo Name	**Sequence (5'-3')**
18merT7_top	TAATACGACTCACTATAG
HPV16_ssdsDNAhybrid_crRNA_DNAtemp_bottom	tgtagtaaaaatttgcgtccATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTA

2.1. New oligoes were annealed with Platinum SuperFi II PCR Master mix.

Table 5.2.3.5.2.3. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing	60℃	10 sec	
Extension	72℃	2 sec	
	4℃	hold	1x

2.2. New oligoes were diluted to 10 µM in nuclease-free water instead of TBS. The templates were annealed with Platinum SuperFi II PCR Master mix.

Table 5.2.3.5.2.4. PCR components

Reaction Component	**Volume**
Cas12a_top (10 µM)	**6 µl**
HPV-16_bottom (10 µM)	**3 µl**
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

3. New top strand were designed.

Table 5.2.3.5.2.5. Primers used for DNA template formation

Oligo Name	**Sequence (5'-3')**
25merT7_top_2023	TAATACGACTCACTATAGTAATTTCTAC
HPV16_ssdsDNAhybrid_crRNA_DNAtemp_bottom	tgtagtaaaaatttgcgtccATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTA

5.2.3.5.3.In vitro transcription for crRNA

1. Transcription was left in overnight (16h).
2. DNA templates were heated and cooled slowly in thermal cycler;
   95℃ for 2 min | 90℃ for 2 min | 88℃ for 2 min | 85℃ for 2 min | 84℃ for 2 minutes until the temperature reaches| 37℃ for 2 min.

5.2.3.5.4.In vitro cleavage of L1 region using LbCas12a

1. 300 nM gRNA stock was renatured in thermal cycler;
   75℃ for 15 min | 74℃ for 2 min | 73℃ for 2 minutes and 1 ℃ decreased per 2 minutes| 37℃ for 2 min.
   Then, the tube was put in ice for 1 min.
2. 10:20:1 (cas12a: gRNA: target DNA) was carried out. The reaction was incubated for 2 hours.

Table 5.2.3.5.4.1. Cleavage components

Reaction component	Volume
NEBuffer r2.1 10X Reaction Buffer	3 µl
300 nM gRNA	6 µl
1 µM EnGen LbCas12a	1 µl
RNase-free water	17 µl
DNA	3 µl

5.2.4.HPV-16 Target-2

5.2.4.1.HPV-16 genome amplification

HPV-16 genomes were amplified using polymerase chain reaction (PCR). For amplification, forward primer was used from a publication (107) and reverse primer was designed by SnapGene. The designed primers' hairpin structures and heterodimer analysis were checked at IDT (Integrated DNA Technologies, Inc.) website with oligomer services. Primers are listed in the Table 5.2.4.1.1.

50 µl of PCR reaction was set up, and components are given in Table 5.2.4.1.2. Amplification was performed in thermal cycler with 3-step PCR protocol (107). 0.8% agarose gel was prepared for the separation of HPV fragments. 0.48 g of agarose powder was put into 60 mL of 1X Tris-acetate-EDTA (TAE) buffer and microwaved until the powder was completely dissolved. After, 2 drops (~60 µl) of Gel-Safer were added to the solution and mixed immediately. The solution was poured into a gel tray and left at room temperature until totally solidified.

When PCR was finished, 7 µl of 6X loading dye was put into the PCR tube and mixed well. The total amount in the PCR tube and 2 µL of HindIII DNA Ladder were loaded on agarose gel. The gel was run at 100 V for 70 minutes. ChemiDoc MP Imaging System (BioRad) was used for visualization of PCR. Confirmed DNAs were extracted from agarose gel using Zymoclean Gel DNA Recovery kit with manufacturer's instructions. The concentration was 104.5 ng/µl.

Table 5.2.4.1.1. Primers used for HPV-16 genome amplification

Oligo Name	Sequence (5'-3')	Amplicon Size
HPV16_Makale_forward	tgctgtctaaactattatgtgtgtctc	7906 bp
HPV16_Makale_reverse	atttttcaattgtttctctatttttttccac	

Table 5.2.4.1.2. PCR components for HPV-16 amplification

Reaction component	**Volume**	**Final concentration**
2X PrimeSTAR GXL Buffer	25 µl	1X
Forward primer	1.25 µl	0.25 µM
Reverse primer	1.25 µl	0.25 µM
Template DNA	1-2 µl	~30 ng
Nuclease-free water	to 50 µl	-
Total	50 µl	

Table 5.2.4.1.3. PCR conditions

Initial denaturation	98°C	30 sec	1x
Denaturation	98°C	10 sec	30x
Annealing	60°C	15 sec	
Extension	68°C	2 min	
Final extension	68°C	5 min	1x
	4°C	hold	

5.2.4.2.Template DNA formation for crRNA

In Target-1, the aim was to anneal the top and bottom strands and fill in the empty nucleotides with DNA polymerase. However, for Target-2, the top and bottom strands were designed completely and hybridized only with each other by PCR. The designed sequences are given in Table 5.2.4.2.

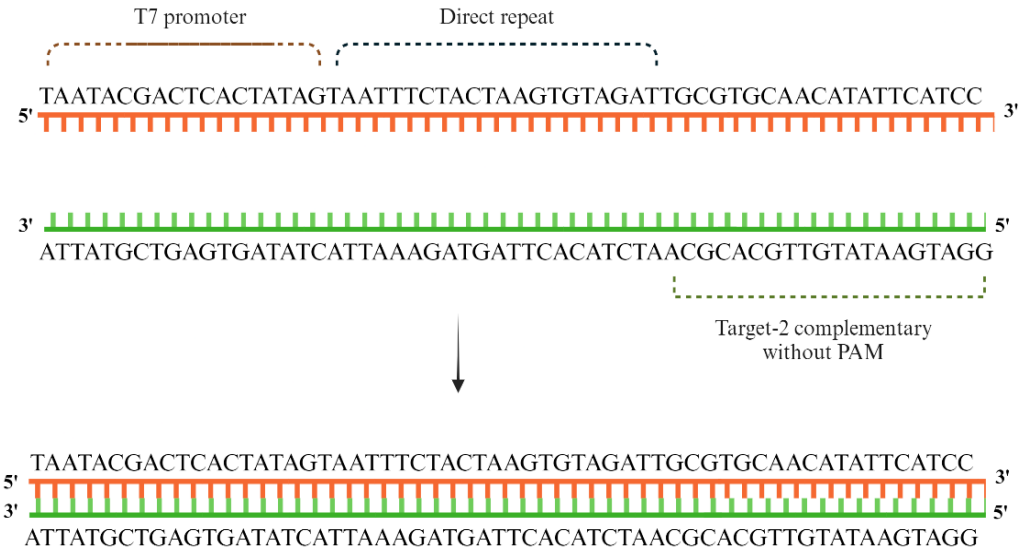

Figure 5.2.4.2.1. DNA template formation of HPV-16 Target-2

The oligoes were stocked to 10 µM in RNase-free water. 20 µl of reaction was set up, and components are given in Table 5.2.4.2.2. Amplification was performed in thermal cycler, with 2-step protocol: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing/extension at 72℃ for 2 sec and extension at 72℃ for 5 min.

3% agarose gel was prepared. After PCR, DNA templates should hybridized as 59 bp. Top and bottom strands were also loaded into the gel to confirm the PCR product. The length of DNA fragment was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using Zymoclean Gel DNA Recovery kit. The concentration was 100 ng/µl.

Table 5.2.4.2.1. Oligoes used for HPV-16 Target-2 DNA template formation

Oligo Name	**Sequence (5'-3')**
Cas12acrRNA_DR_hpv 16_target2_full_top	TAATACGACTCACTATAGTAATTTCTACTAAGT GTAGATTGCGTGCAACATATTCATCC
Hpv16crRNA_target2_ DR_bottom	GGATGAATATGTTGCACGCAATCTACACTTAGT AGAAATTACTATAGTGAGTCGTATTA

Table 5.2.4.2.2. PCR components for DNA template formation

Reaction component	**Volume**
Cas12a_full_top (10 µM)	4 µl
HPV-16_target2_DR_bottom (10 µM)	4 µl
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

5.2.4.3.In vitro transcription of crRNA

The hybridized DNA was used as template for crRNA transcription. Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. NEB High Scribe T7 High Yield RNA Synthesis kit and its short RNA transcript protocol were used. 20 µl of reaction was set up in a 0.2 ml RNase-free micro centrifuge tube, and components are given below. The reaction was incubated at 37℃ for 4 hours in thermal cycler.

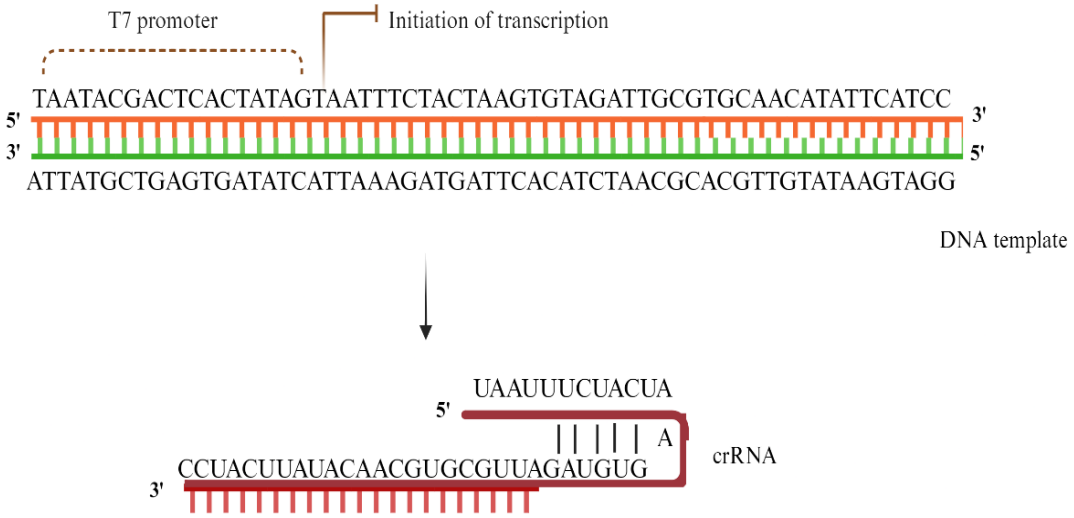

Figure 5.2.4.3.1. Transcription of HPV-16 Target-2 crRNA

After the incubation, RNA Clean&Concentrator-5 kit was used. During purification, the same conditions were provided as in the experiment to prevent contamination. Firstly, DNase I Treatment was done according to the kit manual. The mixture was set up, and the components are given in Table 5.2.3.3.2. Components were added to the transcribed tube and incubated at room temperature for 40 minutes. Therefore, a short RNA section was applied with manufacturer's instructions. After transcription, crRNA length should be 41 bp. RNA concentration was measured with NanoDrop.

3% agarose gel was prepared to visualize RNA by using 2X RNA loading dye. The length of the RNA was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes.

Table 5.2.4.3.1. Transcription components for HPV-16 Target-2

Reaction component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	10.5 µl	1 µg
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

5.2.4.4.In vitro cleavage of total genome using LbCas12a and AsCas12a

In order to perform cleavage reactions, the same protocol was used for LbCas12a and AsCas12a. First of all, the molar concentrations of transcribed RNA and amplified target DNA were calculated in below. RNA concentration was measured at 16000 ng/µl, but when loaded onto the gel it was compared with the ladder and estimated to be 2000 ng/µl.

$$crRNA\ (\mu M) = \frac{Concentration\ of\ RNA\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times Length\ of\ RNA\ (bp)} \times 10^3$$

$$crRNA\ (\mu M) = \frac{2000\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times 41} \times 10^3 = 148\ \mu M$$

$$DNA\ (nM) = \frac{Concentration\ of\ DNA\ (\frac{ng}{\mu l})}{660\frac{g}{mol} \times Length\ of\ DNA} \times 10^6$$

$$DNA\ (nM) = \frac{104.5\ \left(\frac{ng}{\mu l}\right)}{660\frac{g}{mol} \times 7906} \times 10^6 = 20\ nM$$

According to the protocol, 300 nM crRNA should be used. Therefore, 300 nM stock crRNA was prepared below. The calculated amount was added to a new RNase-free tube, and the rest of the volume was completed with RNase-free water.

$$M_1 \times V_1 = M_2 \times V_2$$

$$148\ \mu M \times V_1 = 0.3\ \mu M \times 300\ \mu l \longrightarrow V_1 = 0.61\ \mu l$$

Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. Normally, the protocol published by NEB for Cas12a cleavage reactions corresponds to final molar

concentrations of Cas12a, gRNA and target DNA in the ratio of 30:30:3. As a result of various optimizations, the molar ratio of Cas12a, gRNA and target DNA was set at 10:25:1. The reaction components are given in Table 5.2.4.4.1. 20 µl reaction was set up without adding DNA into a 0.2 ml RNase-free PCR tube. The solution was pre-incubated at room temperature for 15 minutes. After Cas12a-gRNA complexed during pre-incubation time, DNA was added to the reaction and incubated at 37°C for 1.3 hours and 65°C for 15 min (heat inactivation) in thermal cycler. Cas12a digestion is illustrated in figure below. After incubation is done, 1 µl of Proteinase K was added to tube and left at room temperature for 10 minutes. Then, 3 µl of 6X SDS containing Purple Dye was added. The same procedures were performed for AsCas12a under the same conditions. 0.8% agarose gel was prepared. The cleavage product, substrate DNA as a control and 1 kb DNA ladder, and HindIII DNA ladder were loaded. The gel was run at 100 V for 60 minutes and imaged on BioRad.

Table 5.2.4.4.1. Targeting components of HPV-16 Target-2

Reaction component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA	1.675 µl
1 µM EnGen LbCas12a/AsCas12a*	0.22 µl
RNase-free water	15.125 µl
DNA	0.98 µl**

*AsCas12a enzyme was ordered as 26.7 µM but 1 µM diluted in glycerol stock was used for cleavage reactions.
**1 nM final DNA.

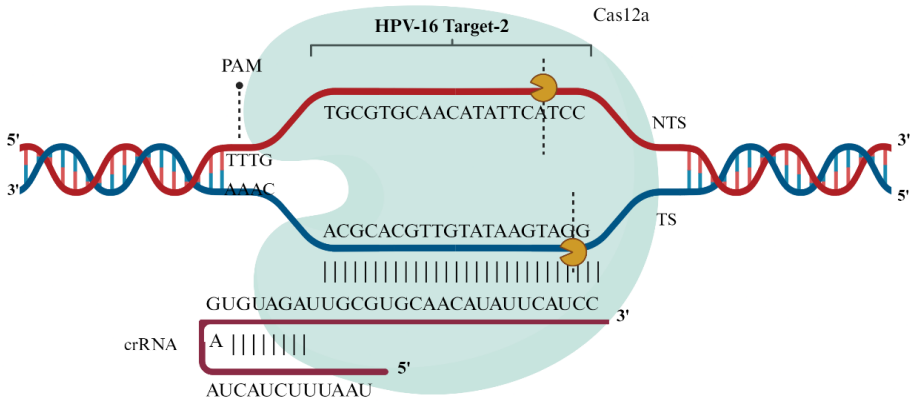

Figure 5.2.4.4.1. HPV-16 Target-2 cleavage diagram

5.2.4.5.Troubleshooting

5.2.4.5.1.Total genome amplification

1. **Table 5.2.4.5.1.1.** PCR components

Reaction Component	**Volume**	**Final Concentration**
2X Platinum Master mix	5 µl	1X
Forward primer	0.5 µl	0.5 µM
Reverse primer	0.5 µl	0.5 µM
Template DNA	1 µl	~40 ng
Nuclease-free water	to 10 µl	-
Total	10 µl	

Table 5.2.4.5.1.2. PCR conditions

Initial denaturation	98°C	30 sec	1X
Denaturation	98°C	10 sec	35X
Annealing	60°C	10 sec	
Extension	72°C	3 min	
Final extension	72°C	5 min	1X
	4°C	hold	

5.2.4.5.2.DNA template formation for crRNA

1. **Table. 5.2.4.5.2.1.** Oligoes for DNA template formation

Oligo Name	**Sequence (5'-3')**
HPV16_TARGET2_BOTTOM	GGATGAATATGTTGCACGCAATCTACAC TTAGTAGAAATTA
Cas12acrRNA_2023_T7promot er_template_top	TAATACGACTCACTATAGTAATTTCTAC TAAGTGTAGAT

Table. 5.2.4.5.2.2. PCR components

Reaction component	**Volume**
Cas12a_top (10 µM)	4 µl
HPV-16_target2_bottom (10 µM)	4 µl
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

Table 5.2.4.5.2.3. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	35x
Annealing	**56℃/60℃**	10 sec	
Extension	72℃	2 sec	
	4℃	hold	1x

1.1.**Table 5.2.4.5.2.4.** PCR components

Reaction component	**Volume**
Cas12a_top (10 μM)	4 μl
HPV-16_target2_bottom (10 μM)	4 μl
2X Pemix	10 μl
RNase-free water	to 20 μl
Total	20 μl

Table 5.2.4.5.2.5. PCR conditions

Initial denaturation	98℃	30 sec	1x
Denaturation	98℃	10 sec	30x
Annealing	60℃	15 sec	
Extension	68℃	4 sec	
	4℃	hold	1x

5.2.4.5.3.In vitro transcription of crRNA

1. Transcription was made using newly synthesized templates in Section 5.2.4.5.2. Number 1.1.

Table 5.2.4.5.3.1. Transcription components

Reaction Component	**Volume**	**Final Concentration**
RNase-free water	to 20 μl	
10X Reaction Buffer	1.5 μl	0.75X
NTP	1.5 μl each (total 6 μl)	7.5 mM each
DNA	10.5 μl	450 ng
T7 RNA Polymerase Mix	2 μl	10 mM
Total	20 l	

2. Transcription was made with Maxiscript kit.

Table 5.2.4.5.3.2. Transcription components

Reaction component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Transcription Buffer	2 µl	-
NTP(10 mM each)	1 µl each (total 4 µl)	0.5 mM each
DNA	12 µl	612 ng
(T3, T7, or SP6) Enzyme Mix	2 µl	-
Total	20µl	

5.2.4.5.4.In vitro cleavage of total genome using LbCas12a and AsCas12a

1. 10:10:3 Cas12a:gRNA:target DNA

Table 5.2.4.5.4.1. Cleavage components

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	3 µl
300 nM gRNA	1 µl
1 µM EnGen LbCas12a	0.3 µl
RNase-free water	21.03 µl
DNA	4.67 µl

1.1.Transcripts produced with Maxiscript kit were targeted in 30:30:3 ratio.

Table 5.2.4.5.4.2. Cleavage components

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	3 µl
300 nM gRNA	3 µl
1 µM EnGen LbCas12a	1 µl
RNase-free water	22.44µl
DNA	0.56

5.2.5.HPV-16 Target-3

5.2.5.1.HPV-16 genome amplification

Experiments were carried out with HPV-16 DNA produced in Section 5.2.4.1.

5.2.5.2.DNA template formation for crRNA

For Target-2, the top and bottom strands were designed completely and hybridized only with each other by PCR. The same system was used for Target-3. The designed sequences are given in Table 5.2.5.2.1.

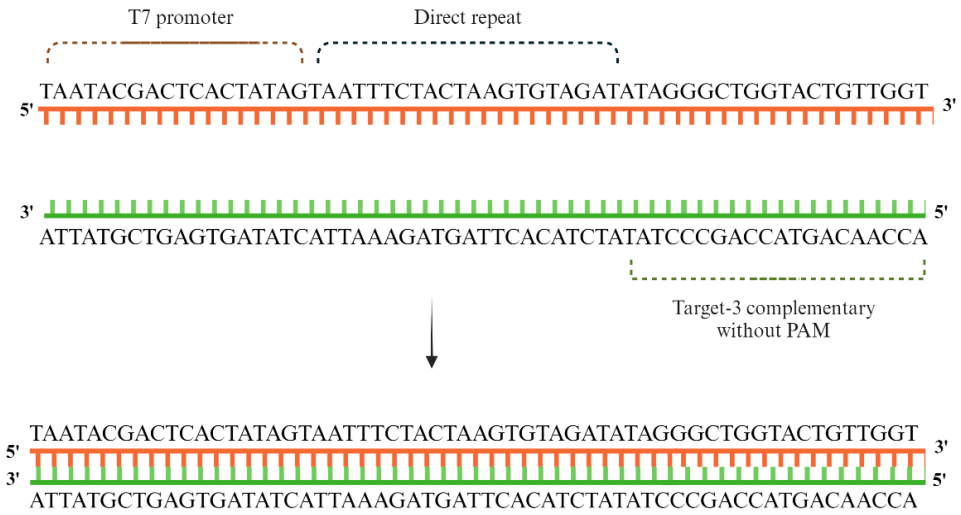

Figure 5.2.5.2.1. DNA template formation of HPV-16 Target-3

The oligoes were stocked to 10 µM in RNase-free water. 20 µl reaction was set up, and components are given in Table 5.2.5.2.2. Amplification was performed in thermal cycler with 2-step protocol: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing/extension at 73℃ for 2 sec and 4℃ for hold.

3% agarose gel was prepared. After PCR, DNA templates should be hybridized at 59 bp. Top and bottom strands were also loaded into the gel to confirm the PCR product. The length of DNA fragment was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using Zymoclean Gel DNA Recovery kit. The concentration was 44 ng/µl.

Table 5.2.5.2.1. Oligoes for HPV-16 Target-3 DNA template formation

Oligo name	**Sequence (5'-3')**
Cas12acrRNA_DR_hpv 16_target3_full_top	TAATACGACTCACTATAGTAATTTCTACTAAGT GTAGATATAGGGCTGGTACTGTTGGT
Hpv16_target3_DR_bot tom	ACCAACAGTACCAGCCCTATATCTACACTTAGT AGAAATTACTATAGTGAGTCGTATTA

Table 5.2.5.2.2. PCR components

Reaction component	**Volume**
Cas12a_full_top (10 µM)	4 µl
HPV-16_target2_DR_bottom (10 µM)	4 µl
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

5.2.5.3.In vitro transcription of crRNA

The hybridized DNA was used as template for crRNA transcription. Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. NEB High Scribe T7 High Yield RNA Synthesis kit and its short RNA transcript protocol were used. 20 µl of reaction was set up in a 0.2 ml RNase-free PCR tube and the components are given in Table 5.2.5.3.1. The reaction was incubated at 37°C for 16 hours in thermal cycler.

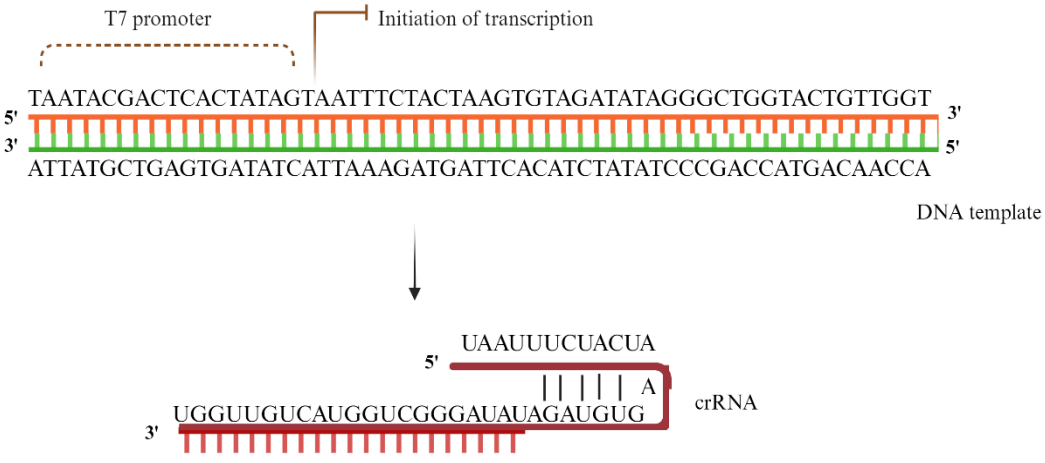

Figure 5.2.5.3.1. Transcription of HPV-16 Target-2 crRNA

After the incubation, RNA Clean&Concentrator-5 kit was used. During purification, the same conditions were provided in the experiment to prevent contamination. Firstly, DNase I Treatment was done in kit manual. The mixture was set up, and components are given in Table 5.2.5.3.1. The components were added into the transcribed tube and incubated at room temperature for 40 minutes. Therefore,

short RNA section was applied with manufacturer's instructions. After transcription, crRNA length should be 41 bp.

3% agarose gel was prepared. In order to visualize RNA, 2X RNA loading dye was used. The length of RNA was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes.

Table 5.2.5.3.1. Transcription components of HPV-16 Target-3

Reaction Component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	10.5 µl	450 ng
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

Table 5.2.5.3.2. DNase-I treatment components

Reaction Component	**Volume**
RNA Sample	40 µl*
DNase I	5 µl
DNA Digestion Buffer	5 µl

*Transcription was done as 20 µl but for DNase-I treatment, it recommends complete the volume to 20 µl with RNase-free water

5.2.5.4.In vitro cleavage of total genome using Lbcas12a and AsCas12a

In order to cleavage reactions, the same protocol was used for LbCas12a and AsCas12a. First of all, the molar concentrations of transcribed RNA and amplified target DNA were calculated below. RNA concentration was measured at 19000 ng/µl but when loaded onto the gel it was compared with the ladder and estimated to be 2000 ng/µl.

$$crRNA\ (\mu M) = \frac{Concentration\ of\ RNA\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times Length\ of\ RNA\ (bp)} \times 10^3$$

$$crRNA\ (\mu M) = \frac{2000\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times 41} \times 10^3 = 148\ \mu M$$

$$DNA\ (nM) = \frac{Concentration\ of\ DNA\ (\frac{ng}{\mu l})}{660\frac{g}{mol} \times Length\ of\ DNA} \times 10^6$$

$$DNA\ (nM) = \frac{104.5\ \left(\frac{ng}{\mu l}\right)}{660\frac{g}{mol} \times 7906} \times 10^6 = 20\ nM$$

According to the protocol, 300 nM crRNA should be used. Therefore, 300 nM stock crRNA was prepared below. The calculated amount was added to a new RNase-free tube, and the rest of the volume was completed with RNase-free water.

$$M_1 \times V_1 = M_2 \times V_2$$

$$148\ \mu M \times V_1 = 0.3\ \mu M \times 300\ \mu l \longrightarrow V_1 = 0.61\ \mu l$$

Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. Normally, the protocol published by NEB for Cas12a cleavage reactions corresponds to final molar concentrations of Cas12a, gRNA, and target DNA in the ratio of 30:30:3. As a result of various optimizations, the molar ratio of cas12a, gRNA and target DNA was set at 10:25:1. The reaction components are given in Table 5.2.5.4.1. 20 µl reaction was set up without adding DNA into a 0.2 ml RNase-free PCR tube. The solution was pre-incubated at room temperature for 15 minutes. After Cas12a-gRNA complexed during pre-incubation time, DNA was added to the reaction and incubated at 37℃ for 1.3 hours and 65℃ for 15 min (heat inactivation) in thermal cycler. After incubation is done, 1 µl of Proteinase K was added to the tube and left at room temperature for 10 minutes. Then, 3 µl of 6X SDS containing Purple Dye was added. The same procedures were performed for AsCas12a under the same conditions. 0.8% agarose gel was prepared as mentioned above. The cleavage product, substrate DNA as a control and 1 kb DNA ladder and HindIII DNA ladder were loaded. The gel was run at 100 V for 60 minutes and imaged on BioRad.

Table 5.2.5.4.1. Targeting components of HPV-16 Target-3

Reaction Component	Volume
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA	1.675 µl
1 µM EnGen LbCas12a/AsCas12a*	0.22 µl
RNase-free water	15.125 µl
DNA	0.98 µl**

*AsCas12a enzyme was ordered as 26.7 µM but 1 µM diluted in glycerol stock was used for cleavage reactions.
**1 nM final DNA

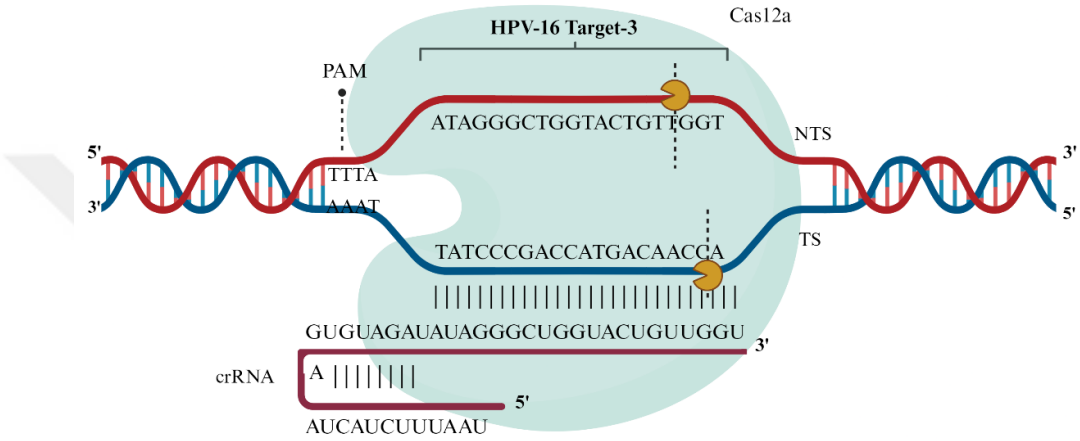

Figure 5.2.5.4.1. HPV-16 Target-3 cleavage

5.2.6.HPV-18 Target-1

5.2.6.1.HPV-18 genome amplification

HPV-18 genomes were amplified using polymerase chain reaction (PCR). Primers were designed by SnapGene. The designed primers' hairpin structures and heterodimer analysis were checked at IDT (Integrated DNA Technologies, Inc.). Primers are listed in the Table 5.2.6.1.1.

50 µl of PCR reaction was set up and components are given in Table 5.2.6.1.2. Amplification was performed in thermal cycler with 3-step PCR protocol in Table 5.2.6.1.3. 0.8 % agarose gel was prepared for the separation of HPV fragments. 0.48 g of agarose powder was put into 60 mL of 1X Tris-acetate-EDTA (TAE) buffer and microwaved until the powder was completely dissolved. After, 2 drops (~60 µl) of Gel-Safer (A.B.T) were added to the solution and mixed immediately. The solution was poured into gel tray and left at room temperature until totally solidified.

When PCR was finished, 7 µl of 6X loading dye was put into the PCR tube and mixed well. The total amount in the PCR tube and 2 µL of HindIII DNA Ladder were loaded on agarose gel. The gel was run at 100 V for 70 minutes. ChemiDoc MP Imaging System (BioRad) was used for visualization of PCR. Confirmed DNAs were extracted from agarose gel using Zymoclean Gel DNA Recovery kit with manufacturer's instructions. The concentration was 88.3 ng/µl.

Table 5.2.6.1.1. Primers used for HPV-18 genome amplification

Oligo Name	Sequence (5'-3')	Amplicon Size
hpv18_forwardprimer_all_L1_2	atgtgcctgtatacacgg	7857 bp
hpv18_reverseprimer_all_2	cccaagaggaggttaaagg	

Table 5.2.6.1.2. PCR components for HPV-18 amplification

Reaction Component	Volume	Final Concentration
2X PrimeSTAR® GXL Buffer	25 µl	1X
Forward primer	1.25 µl	0.25 µM
Reverse primer	1.25 µl	0.25 µM
Template DNA	1 µl	~20 ng
Nuclease-free water	to 50 µl	-
Total	50 µl	

Table 5.2.6.1.3. PCR conditions

Initial denaturation	98°C	30 sec	1X
Denaturation	98°C	10 sec	30X
Annealing	60°C	15 sec	
Extension	68°C	2 min	
Final extension	68°C	5 min	1X
	4°C	hold	

5.2.6.2.DNA template formation for crRNA

For HPV-18 Target-1, the top and bottom strands were designed completely and hybridized only with each other by PCR. The same system (Section 5.2.4.2.) was used for Target-1. The designed sequences are given in Table 5.2.6.2.1.

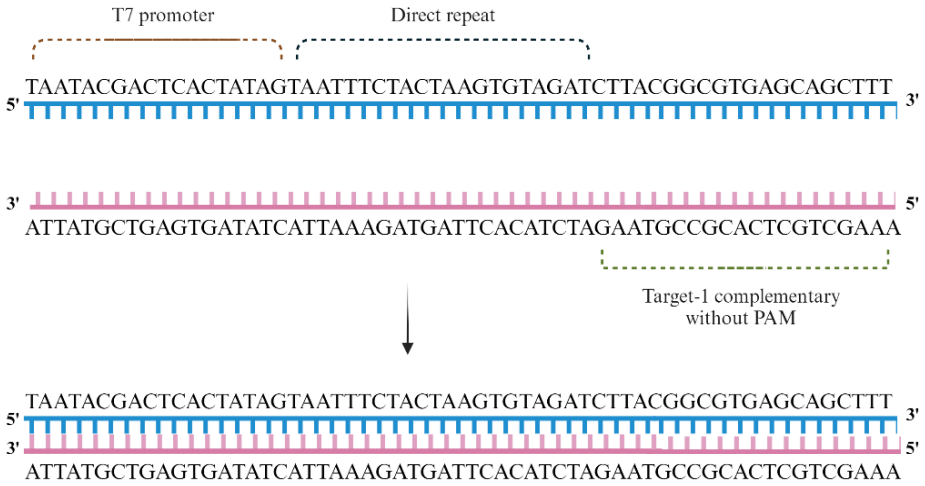

Figure 5.2.6.2.1. DNA template formation of HPV-18 Target-1

The oligoes were stocked to 10 µM in RNase-free water. 20 µl reaction was set up, and components are given in Table 5.2.6.2.2. Amplification was performed in thermal cycler with 2-step PCR protocol: initial denaturation at 98℃ for 30 sec, 35 cycles of denaturation at 98℃ for 10 sec, annealing/extension at 72℃ for 2 sec and 4℃ for hold.

3% agarose gel was prepared. After PCR, DNA templates should be hybridized at 59 bp. Top and bottom strands were also loaded into the gel to confirm the PCR product. The length of the DNA fragment was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using Zymoclean Gel DNA Recovery kit. The concentration was 81.3 ng/µl.

Table 5.2.6.2.1. Oligoes for HPV-18 Target-1 DNA template formation

Oligo Name	**Sequence (5'-3')**
Cas12acrRNA_DR_hpv18_target1_full_top	TAATACGACTCACTATAGTAATTTCTACTAAGTGTAGATCTTACGGCGTGAGCAGCTTT
Hpv18crRNA_target1_DR_bottom	AAAGCTGCTCACGCCGTAAGATCTACACTTAGTAGAAATTACTATAGTGAGTCGTATTA

Table 5.2.6.2.2. PCR components

Reaction Component	**Volume**
Cas12a_full_top (10 µM)	4 µl
HPV-18_target1_DR_bottom (10 µM)	4 µl
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

5.2.6.3.In vitro transcription of crRNA

The hybridized DNAs were used as templates for crRNA transcription. Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. NEB High Scribe T7 High Yield RNA Synthesis kit and its short RNA transcript protocol were used. 20 µl of a reaction was set up to a 0.2 ml RNase-free PCR tube and components are given in Table 5.2.6.3.1. The reaction was incubated at 37℃ for 16 hours in thermal cycler.

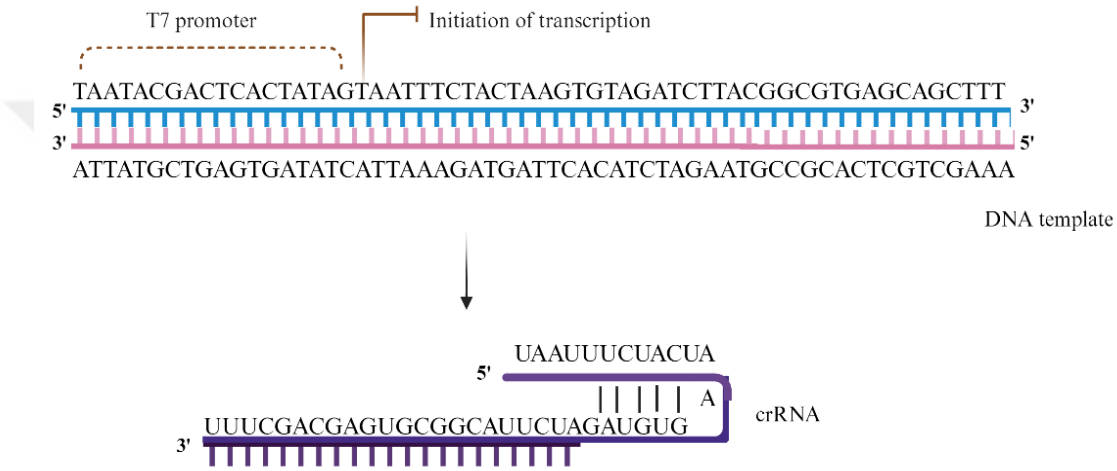

Figure 5.2.6.3.1. Transcription of HPV-18 Target-1 crRNA

After the incubation, RNA Clean&Concentrator-5 kit was used. During purification, the same conditions were provided in the experiment to prevent contamination. Firstly, DNase I Treatment was done according to the kit manual. The mixture was set up, and components are given in Table 5.2.6.3.1. Components were added to the transcribed tube and incubated at room temperature for 40 minutes. Therefore, short RNA section was applied with manufacturer's instructions. After transcription, crRNA length should be 41 bp. RNA concentration was measured with NanoDrop.

3% agarose gel was prepared. In order to visualize RNA, 2X RNA loading dye was used. The length of RNA was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes.

Table 5.2.6.3.1. Transcription components of HPV-18 Target-1

Reaction Component	Volume	Final Concentration
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	10 µl	813 ng
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

Table 5.2.6.3.2. DNase-I treatment components

Reaction Component	Volume
RNA Sample	40 µl*
DNase I	5 µl
DNA Digestion Buffer	5 µl

*Transcription was done as 20 µl but for DNase-I treatment, it recommends complete the volume to 20 µl with RNase-free water

5.2.6.4.In vitro cleavage of total genome using LbCas12a and AsCas12a

In order to cleavage reactions, the same protocol was used for LbCas12a and AsCas12a. First of all, the molar concentrations of transcribed RNA and amplified target DNA were calculated below. RNA concentration was measured at 15000 ng/µl but when loaded onto the gel it was compared with the ladder and estimated to be 1000 ng/µl.

$$crRNA\ (\mu M) = \frac{Concentration\ of\ RNA\ \left(\frac{ng}{\mu l}\right)}{330\frac{g}{mol} \times Length\ of\ RNA\ (bp)} \times 10^3$$

$$\text{crRNA}\ (\mu\text{M}) = \frac{1000\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{330\frac{\text{g}}{\text{mol}} \times 41} \times 10^3 = 74\ \mu\text{M}$$

$$\text{DNA}\ (\text{nM}) = \frac{\text{Concentration of DNA}\ (\frac{\text{ng}}{\mu\text{l}})}{660\frac{\text{g}}{\text{mol}} \times \text{Length of DNA}} \times 10^6$$

$$\text{DNA}\ (\text{nM}) = \frac{88.3\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{660\frac{\text{g}}{\text{mol}} \times 7857} \times 10^6 = 17\ \text{nM}$$

According to the protocol, 300 nM crRNA should be used. Therefore, 300 nM stock crRNA was prepared below. The calculated amount was added to a new RNase-free tube and the rest of the volume was completed with RNase-free water.

$$M_1 \times V_1 = M_2 \times V_2$$

$$74\ \mu M \times V_1 = 0.3\ \mu M \times 300\ \mu l \longrightarrow V_1 = 1.22\ \mu l$$

Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. Normally, the protocol published by NEB for Cas12a cleavage reactions corresponds to final molar concentrations of Cas12a, gRNA, and target DNA was set at 30:30:3. As a result of various optimizations, the molar ratio of Cas12a, gRNA, and target DNA was set at 10:25:1. The reaction components are given in Table 5.2.6.4.1. 20 µl reaction was set up without adding DNA to a 0.2 ml RNase-free PCR tube. The solution was pre-incubated at room temperature for 15 minutes. After Cas12a-gRNA complexed during pre-incubation time, DNA was added to the reaction and incubated at 37°C for 1.3 hours and 65°C for 15 min (heat inactivation) in thermal cycler. After incubation is done, 1 µl of Proteinase K was added into tube and left at room temperature for 10 minutes. Then, 3 µl of 6X SDS containing Purple Dye was added. The same procedures were performed for AsCas12a under the same conditions. 0.8% agarose gel was prepared. The cleavage product, substrate DNA as a control, 1 kb DNA ladder and HindIII DNA ladder were loaded. The gel was run at 100 V for 60 minutes and imaged on BioRad.

Table 5.2.6.4.1. Targeting components of HPV-18 Target-1

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA	1,675 µl
1 µM EnGen LbCas12a/AsCas12a*	0,22 µl
RNase-free water	15,97 µl
DNA	1,14 µl**

*AsCas12a enzyme was ordered as 26.7 µM but 1 µM diluted in glycerol stock was used for cleavage reactions.
**1 nM final DNA

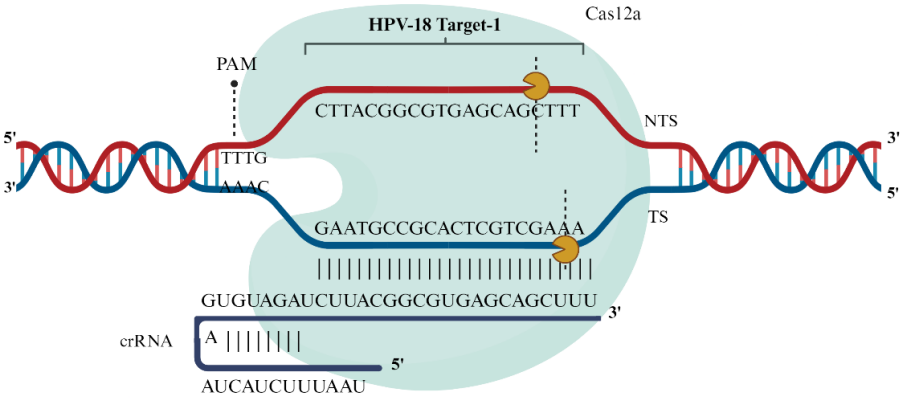

Figure 5.2.6.4.1. HPV-18 Target-1 cleavage

5.2.6.5.Troubleshooting of Target-1

5.2.6.5.1.Genome amplification

1. L1 region was amplified using total amplified HPV-18 genome.

Table 5.2.6.5.1.1. Primers were used for L1 region amplification

Oligo Name	**Sequence (5'-3')**	**Amplicon Size**
hpv18_forwardprimer_all_L1_2	**atgtgcctgtatacacgg**	1171 bp
hpv18_L1_reverseprimer	**ataattgattatgccagcaaaca**	

Table 5.2.6.5.1.2. PCR components

Reaction Component	**Volume**	**Final Concentration**
2X Platinum SuperFi II PCR Master mix	5 µl	1X
Forward primer	0.5 µl	0.5 µM
Reverse primer	0.5 µl	0.5 µM
Template DNA	0.5 µl	~45 ng
Nuclease-free water	to 10 µl	-
Total	10 µl	

Table 5.2.6.5.1.3. PCR conditions

Initial denaturation	98°C	30 sec	1x
Denaturation	98°C	10 sec	35x
Annealing	60°C	10 sec	
Extension	72°C	25 sec	
Final extension	72°C	5 min	1x

5.2.6.5.2.DNA template formation for crRNA

1. **Table 5.2.6.5.2.1.** Oligoes for DNA template formation

Oligo Name	**Sequence (5'-3')**
Cas12acrRNA_T7promoter_template_top	CTAATACGACTCACTATAGGTAATTTCTACTAAGTGTAGAT
hpv18_truncated_bottom	AAAGCTGCTCACGCCGTAAGATCTACACTTAGTAGAAATTA

Table 5.2.6.5.2.2. PCR components

Reaction Component	**Volume**
Cas12a_top (10 µM)	2 µl
HPV-18_bottom (10 µM)	2 µl
2X Platinum SuperFi II PCR Master mix	5 µl
RNase-free water	to 10 µl
Total	10 µl

Table 5.2.6.5.2.3. PCR conditions

Initial denaturation	98°C	30 sec	1x
Denaturation	98°C	10 sec	35x
Annealing	56°C	10 sec	
Extension	72°C	2 sec	
	4°C	hold	1x

5.2.6.5.3.In vitro transcription of crRNA

1. Overnight transcription was done.

Table 5.2.6.5.3.1. Transcription components

Reaction Component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	8 µl	100 ng
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

5.2.6.5.4.In vitro cleavage

1. 30:30:3 cleavage ratio was done for L1 region.

Table 5.2.6.5.4.1. Cleavage components

Reaction component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	3 µl
300 nM gRNA	3 µl
1 µM EnGen LbCas12a	1 µl
RNase-free water	17 µl
DNA	1.71 µl

5.2.7.HPV-18 Target-2

5.2.7.1.HPV-18 genome amplification

Experiments were carried out with HPV-18 DNA produced in Section 5.2.6.1.

5.2.7.2.DNA template formation

A double-stranded DNA was formed to be used as a template in crRNA transcription (Figure 5.2.7.2.1). Therefore, two single strand DNA sequences were designed. The designed sequences are given in Table 5.2.7.2.1. These sequences were annealed and blank nucleotides were filled with DNA polymerase.

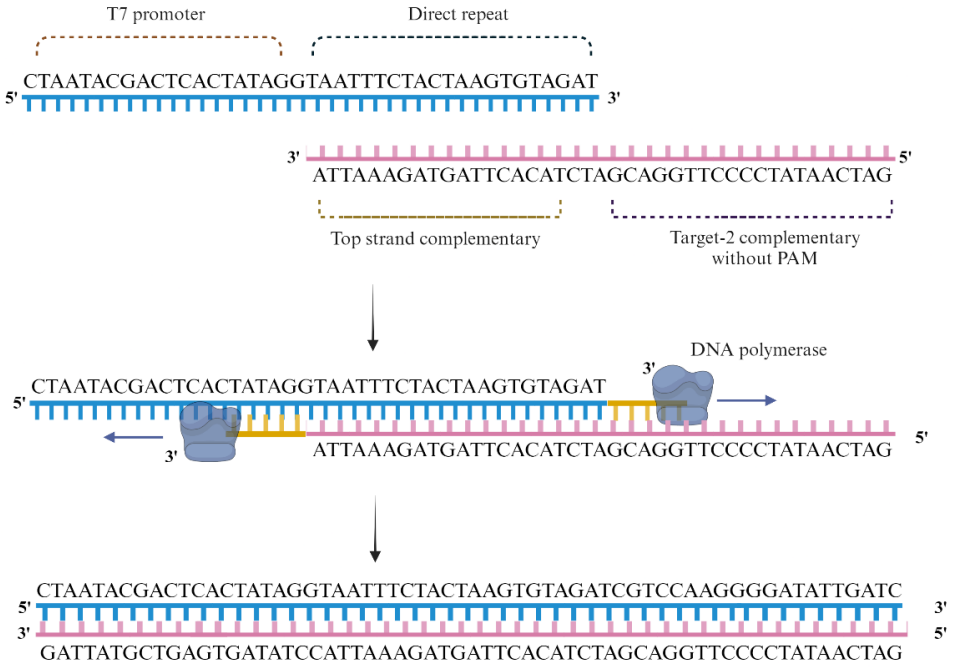

Figure 5.2.7.2.1. DNA template formation of HPV-18 Target-2

Top and bottom strands were stocked to 10 µM in nuclease-free water. 20 µl reaction was set up, and components are given in Table 5.2.7.2.2. Amplification was performed in thermal cycler with initial denaturation at 98°C for 30 sec, 35 cycles of

denaturation at 98℃ for 10 sec, annealing at 60℃ for 10 sec and extension at 72℃ for 2 sec.

3% agarose gel was prepared. After PCR, DNA template should be 61 bp. 20 µl PCR product and 4 µl 6X loading dye were mixed in a PCR tube. Top and bottom strands (10 µM) were also prepared as PCR product. They were loaded into the gel to confirm the PCR product. The gel was run at 100 V for 60 minutes. The confirmed DNA was purified using Zymoclean Gel DNA Recovery kit with manufacturer's instructions. The concentration was 20 ng/µl.

Table 5.2.7.2.1. Oligoes for HPV-18 Target-2 DNA template formation

Oligo Name	**Sequence (5'-3')**
Cas12acrRNA_T7promoter_ template_top	CTAATACGACTCACTATAGGTAATTTCTACT AAGTGTAGAT
HPV18_tatget2_bottom	ATTAAAGATGATTCACATCTAGCAGGTT CCCCTATAACTAG

Table 5.2.7.2.2. PCR components

Reaction component	**Volume**
Cas12a_top (10 µM)	4 µl
HPV-18_bottom (10 µM)	4 µl
2X Platinum SuperFi II PCR Master mix	10 µl
RNase-free water	to 20 µl
Total	20 µl

5.2.7.3.In vitro transcription of crRNA

The generated DNA was used as template for crRNA transcription. Before starting, the experiment area was cleaned with 70% ethanol and RNaseZap. During the experiment, mask, filtered tips, RNase-free tubes, and RNase-free water were used to prevent RNase contamination. NEB High Scribe T7 High Yield RNA Synthesis kit and its short RNA transcript protocol were used. 20 µl of reaction was set up in a 0.2 ml RNase-free PCR tube and components are given in Table 5.2.7.3.1. The reaction was incubated at 37℃ for 16 hours in thermal cycler.

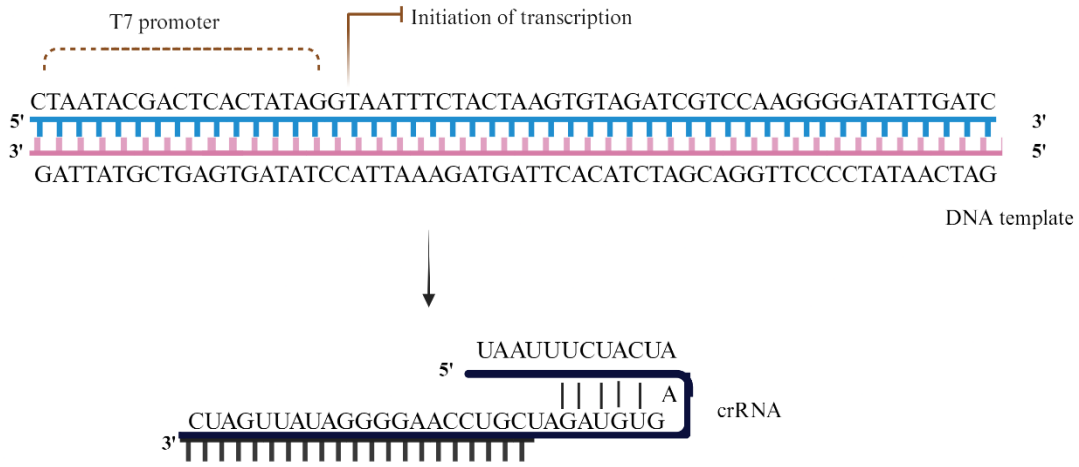

Figure 5.2.7.3.1. Transcription of HPV-18 Target-2 crRNA

After the incubation, RNA Clean&Concentrator-5 kit was used. During purification, the same conditions were provided in the experiment to prevent contamination. Firstly, DNase I Treatment was done according to the kit manual. The mixture was set up and components are given below. Components were added to the transcribed tube and incubated at room temperature for 40 minutes. Therefore, short RNA section was applied with manufacturer's instructions. RNA concentration was measured with NanoDrop One. (16000 ng/µl)

3% agarose gel was prepared. In order to visualize RNA, 2 µl of 2X RNA loading dye and 1000 ng/µl RNA were mixed in a PCR tube and loaded into the gel. After transcription, crRNA length should be 41 bp. The length of RNA was confirmed by gel electrophoresis. The gel was run at 100 V for 60 minutes.

Table 5.2.7.3.1. Transcription components of HPV-18 Target-2

Reaction component	**Volume**	**Final Concentration**
RNase-free water	to 20 µl	
10X Reaction Buffer	1.5 µl	0.75X
NTP	1.5 µl each (total 6 µl)	7.5 mM each
DNA	10.5 µl*	210 ng*
T7 RNA Polymerase Mix	2 µl	10 mM
Total	20 µl	

*Upon protocol template DNA should be 1 µg but 384 ng was obtained after DNA template formation. To check whether less amount is worked, half of the DNA concentration was used.

Table 5.2.7.3.2. DNase-I treatment components

Reaction component	**Volume**
RNA Sample	40 µl*
DNase I	5 µl
DNA Digestion Buffer	5 µl

*Transcription was done as 20 µl but for DNase-I treatment, it recommends complete the volume to 20 µl with RNase-free water.

5.2.7.4.In vitro cleavage of total genome using LbCas12a

In order to cleavage reactions, LbCas12a protocol was used from NEB website. As protocol, the molar ratio between Cas12a enzyme, gRNA, and target DNA should be 10:10:1. RNA concentration was measured at 16000 ng/µl, but when loaded onto the gel it was compared with the ladder and estimated to be 300 ng/µl. First of all, the molar concentrations of transcribed RNA and amplified total DNA were calculated below.

$$\text{crRNA}\ (\mu\text{M}) = \frac{\text{Concentration of RNA}\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{330\,\frac{\text{g}}{\text{mol}} \times \text{Length of RNA (bp)}} \times 10^3$$

$$\text{crRNA}\ (\mu\text{M}) = \frac{300\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{330\,\frac{\text{g}}{\text{mol}} \times 41} \times 10^3 = 22.2\ \mu\text{M}$$

$$\text{DNA}\ (\text{nM}) = \frac{\text{Concentration of DNA}\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{660\,\frac{\text{g}}{\text{mol}} \times \text{Length of DNA}} \times 10^6$$

$$\text{DNA}\ (\text{nM}) = \frac{54\ \left(\frac{\text{ng}}{\mu\text{l}}\right)}{660\,\frac{\text{g}}{\text{mol}} \times 7857} \times 10^6 = 10.4\ \text{nM}$$

According to the protocol, 300 nM crRNA should be used. Therefore, 300 nM stock crRNA was prepared below. The calculated amount was added to a new RNase-free tube and the rest of the volume was completed with RNase-free water.

$$M_1 \times V_1 = M_2 \times V_2$$

$$22.2\ \mu M \times V_1 = 0{,}3\ \mu M \times 300\ \mu l \longrightarrow \quad V_1 = 4\ \mu l$$

Before starting, the experiment area was cleaned with 70% ethanol and RNase Zap. During the experiment, mask, filtered tips, RNase-free tubes and RNase-free water were used to prevent RNase contamination. The reaction components are given in Table 5.2.7.4.1. The reaction was set up without adding DNA to a 0.2 ml RNase-free PCR tube. The solution was pre-incubated at room temperature for 15 minutes. After Cas12a-gRNA complexed during pre-incubation time, DNA was added to the reaction and incubated at 37°C for 1.3 hours and 65°C for 15 min (heat inactivation) in thermal cycler. Cas12a digestion as illustrated in figure below. At the end of the incubation, the reaction was stopped by adding 3 µl of 6X SDS containing Purple Dye. 0.8% agarose gel was prepared. The cleavage product, substrate DNA as a control and HindIII DNA ladder were loaded. The gel was run at 100 V for 60 minutes and imaged on BioRad.

Table 5.2.7.4.1. Targeting components of HPV-18 Target-2

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA	2 µl
1 µM EnGen LbCas12a	0.67 µl
RNase-free water	9.73 µl
DNA	5.6 µl*

*3 nM final DNA

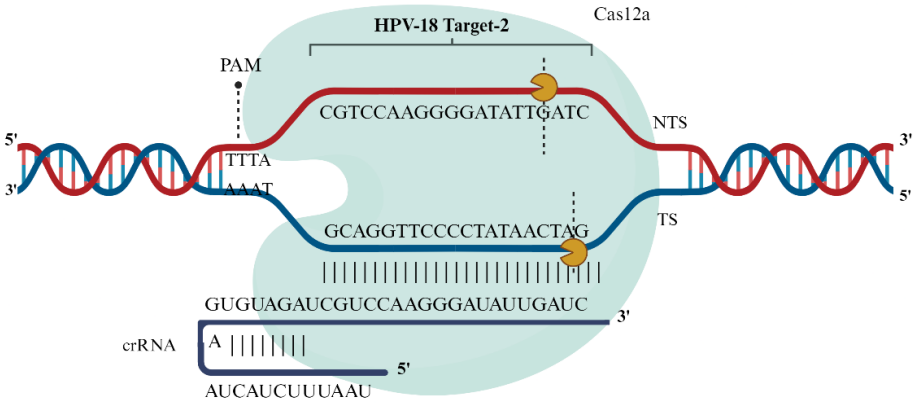

Figure 5.2.7.4.1. HPV-18 Target-2 cleavage

6.RESULTS

Cervical cancer can be prevented if it is detected at an early stage. Therefore, it is essential to develop rapid and accurate detection methods. The CRISPR-Cas system is an adaptive immune system that protects mobile genetic elements from phages. The Cas12a enzyme is able to indiscriminately cleavage, so it is commonly used for nucleic acid detection. In this thesis, it was aimed at the detection of HPV-16 and HPV-18, which lead to cervical cancer, using LbCas12a and AsCas12a enzymes. Firstly, samples were taken from patients with HPV-16 and HPV-18 types. Specific gRNAs were designed to be related to determined target sites and targeted with Cas12a enzymes. As a result, Target-3 of HPV-16 enabled the detection of HPV with both enzymes. Target-1 of HPV-18 showed activity only with the AsCas12a enzyme. The results of this thesis research are shown below.

6.1.Amplification of HPV-16 and HPV-18 from isolated DNA samples

Samples were taken from the Istanbul Medipol University Genetic Diseases Assessment Center. DNA was isolated (mentioned in Section 5.2.2) and amplified with the PCR technique. Firstly, total genome amplification of HPV-16 was performed using the Platinum SuperFi II master mix, as mentioned in Section 5.2.3.1. After PCR, DNA length (~7.9 kb) was confirmed in an agarose gel (Figure 6.1.1). For Target-1, HPV-16 L1 region to be amplified was determined and amplified with PCR. DNA length (~700 bp) was confirmed in agarose gel (Figure 6.1.2). For Target-2, new primers were designed (Table 5.2.4.1.1). Total genome amplification of HPV-16 was performed with PrimeSTAR GXL Premix, as mentioned in Section 5.2.4.1. After PCR, DNA length (~7.9 kb) was confirmed in agarose gel (Figure 6.1.3).

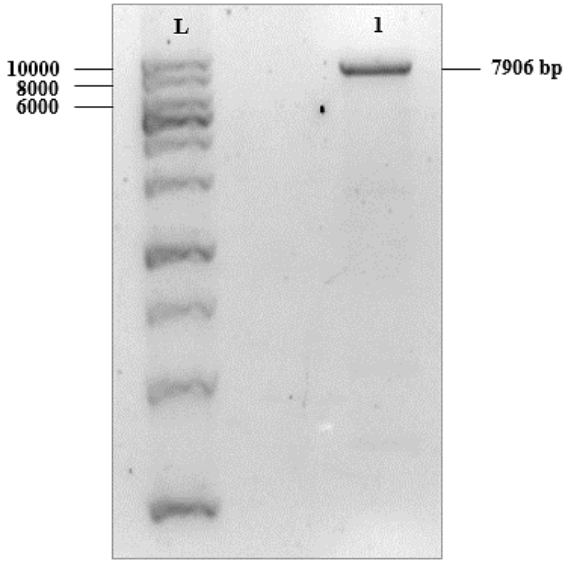

Figure 6.1.1. Total genome amplification of HPV-16 (7.9kb) with Platinum SuperFi II master mix. (L) 2 µl of 1 kb DNA ladder and (1) 10 µl of PCR product with 2 µl of 6x DNA loading dye were loaded into wells on a 1% agarose gel with a running time of 60 min at 100V.

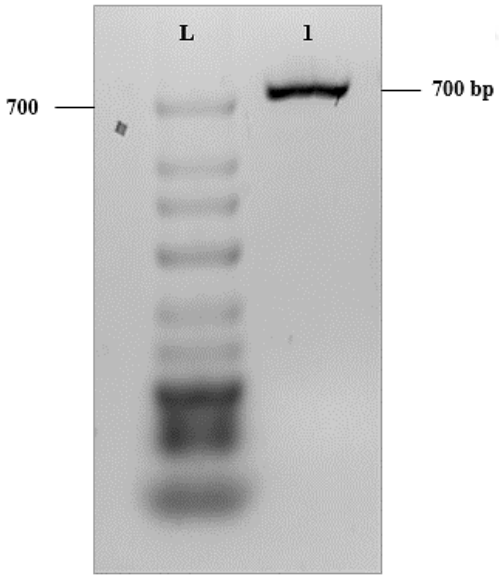

Figure 6.1.2. Specific site amplification in L1 region (~700 bp). (L) 2 µl of low range DNA ladder and (1) 1 µl of PCR product with 1 µl of 6x DNA loading dye were loaded into wells on a 1% agarose gel with a running time of 60 min at 100V.

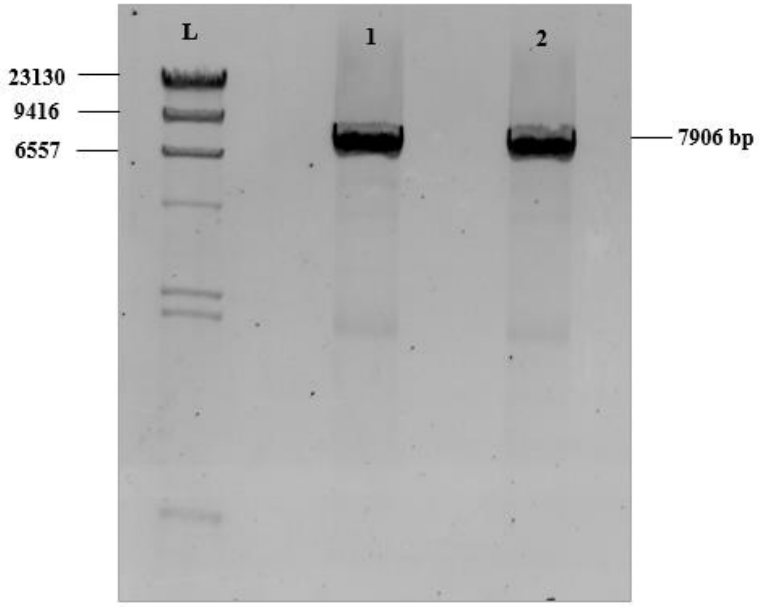

Figure 6.1.3. Total genome amplification of HPV-16 (7.9 kb) with PrimeSTAR GXL Premix. (L) 2 µl of HindIII DNA ladder and (1), (2) 50 µl of PCR product with 7 µl of 6x DNA loading dye were divided into two well on a 0.8% agarose gel with a running time of 70 min at 100V.

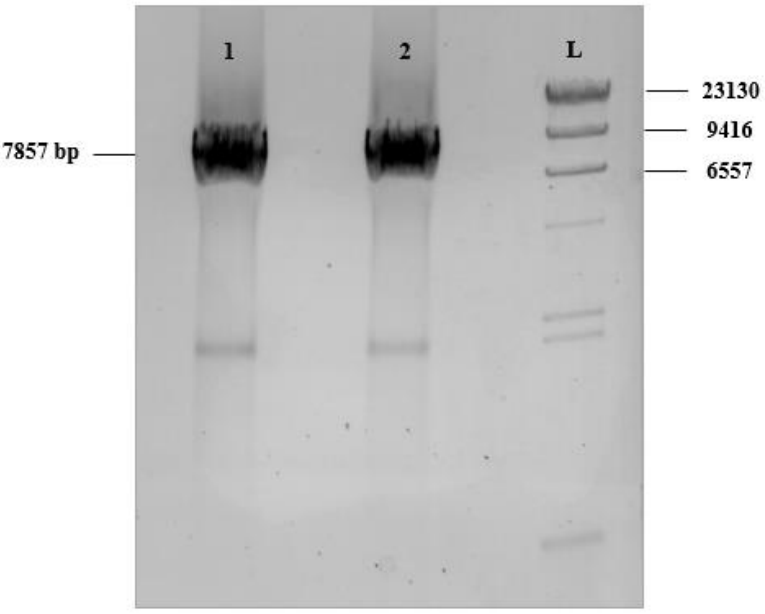

Figure 6.1.4. Total genome amplification of HPV-18 (7.8 kb) with PrimeSTAR GXL Premix. (L) 2 µl of HindIII DNA ladder and (1), (2) 50 µl of PCR product with 7 µl of 6x DNA loading dye were divided into two well on a 0.8% agarose gel with a running time of 70 min at 100V.

6.2.Formation of target-specific crRNAs

crRNA production was performed in two stages. Firstly, double-stranded DNA templates were formed. Target-specific top and bottom strands were designed. These sequences were annealed, and blank nucleotides were filled with DNA polymerase (Section 5.2.3.2) for HPV-16 Target-1 and HPV-18 Target-2. Annealed top and bottom strands were designed for the rest of the targets and hybridized (Section 5.2.4.2). Secondly, DNA templates were transcribed with T7 polymerase and converted to target-specific crRNAs. All of the agarose gels were prepared at 3% and run at 100V for 60 min. The outcomes of HPV-16 and HPV-18 are given below:

- HPV-16 Target-1: 63 bp in length DNA template and 43 bp in length crRNA were formed. **(Figure 6.2.1)**
- HPV-16 Target-2 and Target-3: 59 bp in length DNA template and 41 bp in length crRNA were formed. (**Figure 6.2.2. and 6.2.3)**
- HPV-18 Target-1: 59 bp in length DNA template and 41 bp in length crRNA were formed. (**Figure 6.2.4)**
- HPV-18 Target-2: 61 bp in length DNA template and 41 bp in length crRNA were formed. (**Figure 6.2.5)**

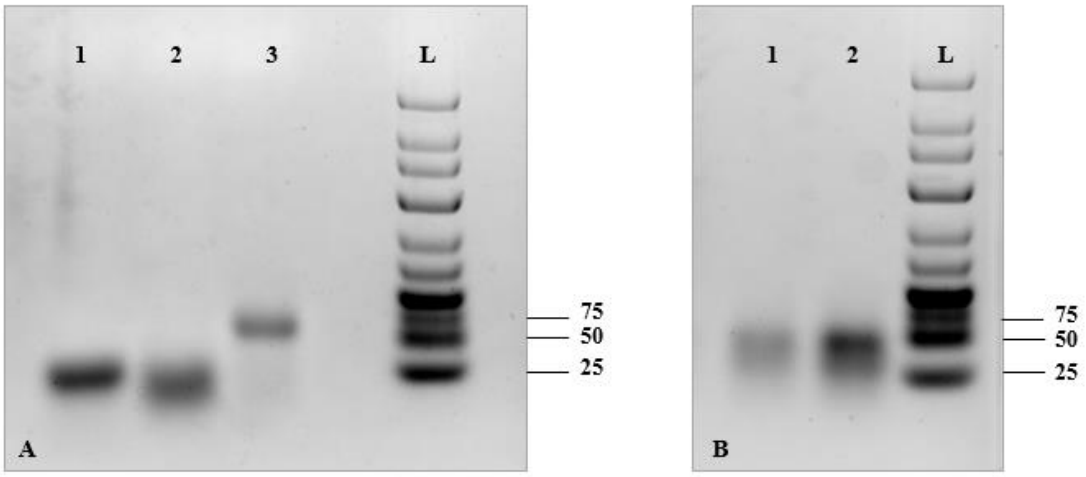

Figure 6.2.1. (A) DNA template formation and (B) transcription of HPV-16 Target-1. (A)(L) 2 µl of low range DNA ladder, (1) 1 µl of Cas12a top strand, (2) 1 µl of bottom strand of Target-1, and (3) 1 µl of PCR product with 1 µl of 6x DNA loading dye. (B)(L) 2 µl of low range DNA ladder, (1) 500 ng and (2) 1000 ng cRNA on agarose gel.

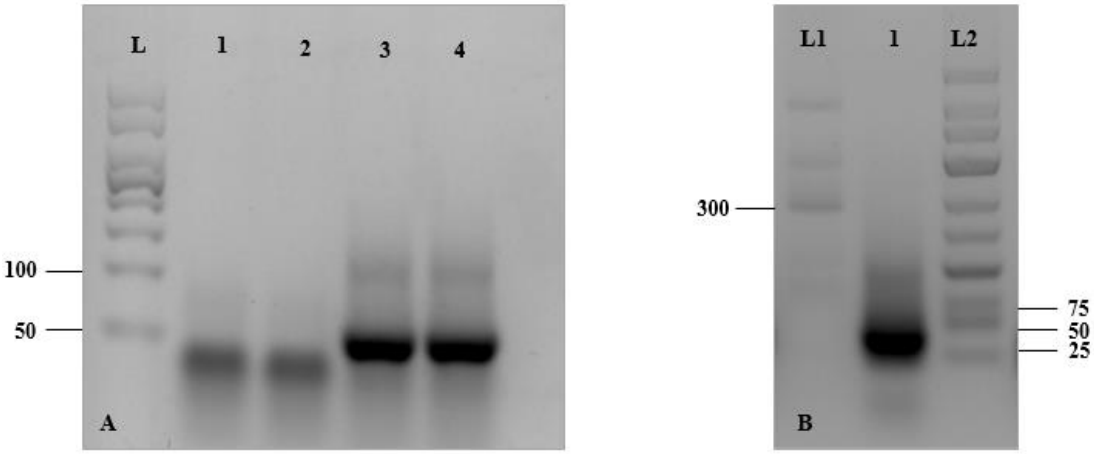

Figure 6.2.2. (A) DNA template formation and (B) transcription of HPV-16 Target-2. (A)(L) 2 µl of 50 bp DNA ladder, (1) 1 µl of Cas12a top strand, (2) 1 µl of bottom strand of Target-2, and (3),(4) 20 µl of PCR product with 4 µl of 6x DNA loading dye were divided into two wells. (B)(L1) 1 µl of low range ssRNA ladder and 5 µl of 2x RNA dye were mixed, (L2) 2 µl of low range DNA ladder, and (1) 1 µl of transcribed crRNA on agarose gel.

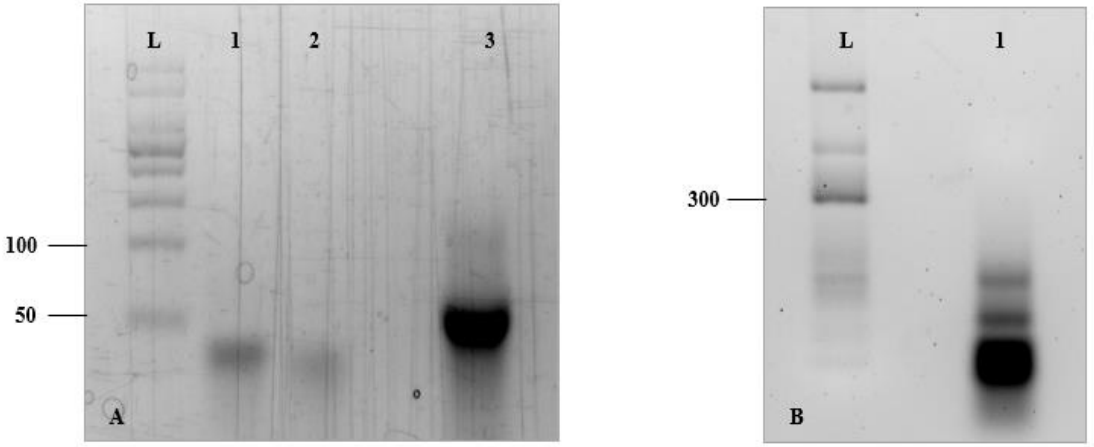

Figure 6.2.3. (A) DNA template formation and (B) transcription of HPV-16 Target-3. (A)(L) 2 µl of 50 bp DNA ladder, (1) 1 µl of Cas12a top strand, (2) 1 µl of bottom strand of Target-3, and (3) 20 µl of PCR product with 4 µl of 6x DNA loading dye. (B)(L) 1 µl of low range ssRNA ladder and 5 µl of 2x RNA dye were mixed, and (1) 1 µl of transcribed crRNA on agarose gel.

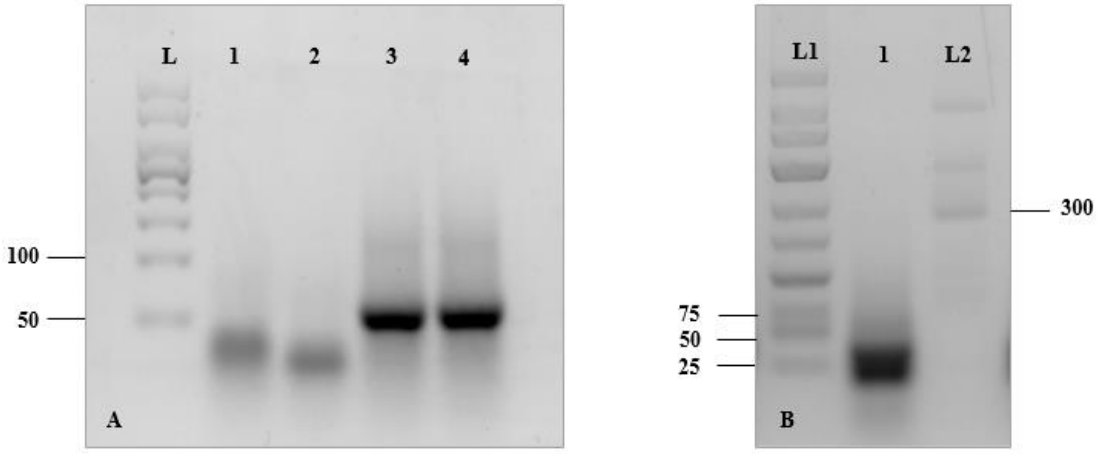

Figure 6.2.4. (A) DNA template formation and (B) transcription of HPV-18 Target-1. (A)(L) 2 µl of 50 bp DNA ladder, (1) 1 µl of Cas12a top strand, (2) 1 µl of bottom strand of Target-1, and (3),(4) 20 µl of PCR product with 4 µl of 6x DNA loading dye were divided into two wells. (B)(L1) 2 µl of low range DNA ladder, (1) 1 µl of transcribed crRNA and (L2) 1 µl of low range ssRNA ladder and 5 µl of 2x RNA dye were loaded on agarose gel.

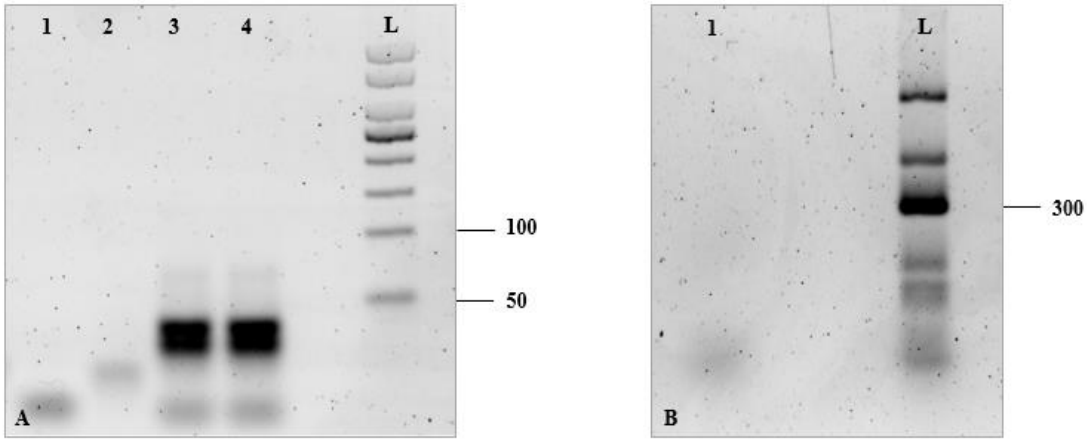

Figure 6.2.5. (A) DNA template formation and (B) transcription of HPV-18 Target-2. (A)(L) 2 µl of 50 bp DNA ladder, (1) 1 µl of Cas12a top strand, (2) 1 µl of bottom strand of Target-2, and (3),(4) 20 µl of PCR product with 4 µl of 6x DNA loading dye were divided into two well. (B)(1) 1 µl of transcribed crRNA, and (L) 1 µl of low range ssRNA ladder and 5 µl of 2x RNA dye were loaded on agarose gel.

6.3.Detection of HPV-16 and HPV-18 using CRISPR-Cas12a

Human papillomavirus is divided into three regions: early, late, and non-coding. In comparison to other regions, the L1 region, which is encoded by the late region, is highly conservative. HPV classification is based on the difference in L1 regions from other types. Therefore, target sites were determined in the L1 region (Section 5.2.1). There was no cleavage obtained with crRNAs of HPV-16 Target-1 (Figure 6.3.1) and HPV-18 Target-2 (Figure 6.3.5). Interestingly, only a single cleaved fragment was observed after targeting with the crRNA of HPV-16 Target-2 (Figure 6.3.2). The crRNA of HPV-16 Target-3 detected HPV-16 effectively. Additionally, AsCas12a showed higher cleavage activity than LbCas12a (Figure 6.3.3). The crRNA of HPV-18 Target-1 also showed cleavage activity with AsCas12a. There was no cleavage obtained with LbCas12a (Figure 6.3.4).

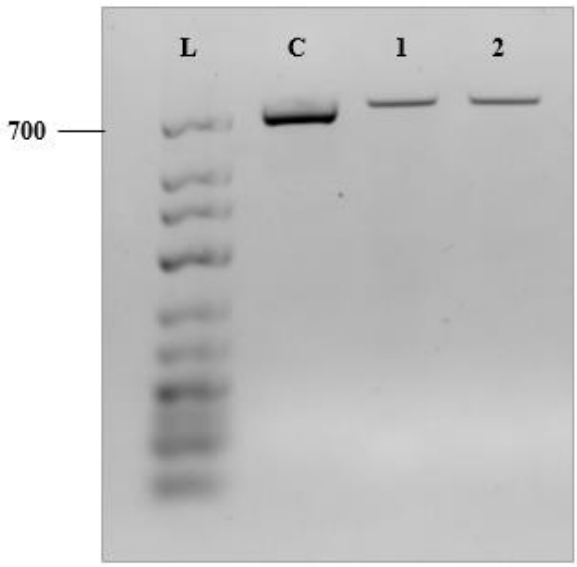

Figure 6.3.1. HPV-16 Target-1 cleavage in L1 region using LbCas12a. (L) 2 µl of low range DNA ladder, (C) HPV-16 L1 region as a control, and (1),(2) 30 µl of cleavage product with 6 µl of 6x SDS containing Purple Dye were divided into two wells on 1% agarose gel.

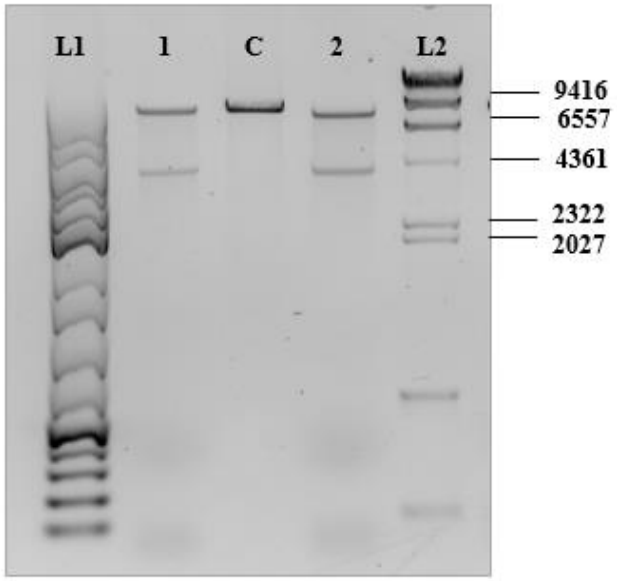

Figure 6.3.2. HPV-16 Target-2 cleavage in total genome using LbCas12a and AsCas12a. (L1) 2 µl of 1 kb plus DNA ladder, (C) HPV-16 DNA as control, and 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with (1) LbCas12a, (2) AsCas12a and (L2) 2 µl of HindIII DNA ladder were loaded on 0.8% agarose gel.

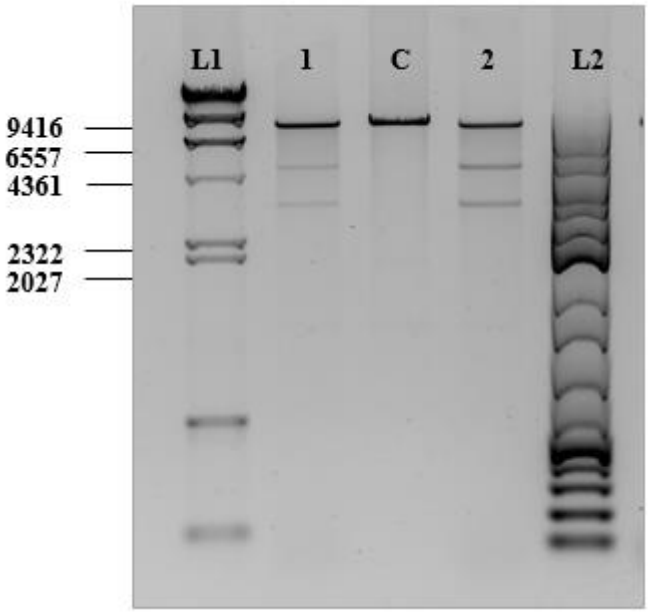

Figure 6.3.3. HPV-16 Target-3 cleavage in total genome using LbCas12a and AsCas12a. (L1) 2 µl of HindIII DNA ladder, 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with (1) LbCas12a, and (2) AsCas12a, (C) HPV-16 DNA as a control, and (L2) 2 µl of 1 kb plus DNA ladder were loaded on 0.8% agarose gel.

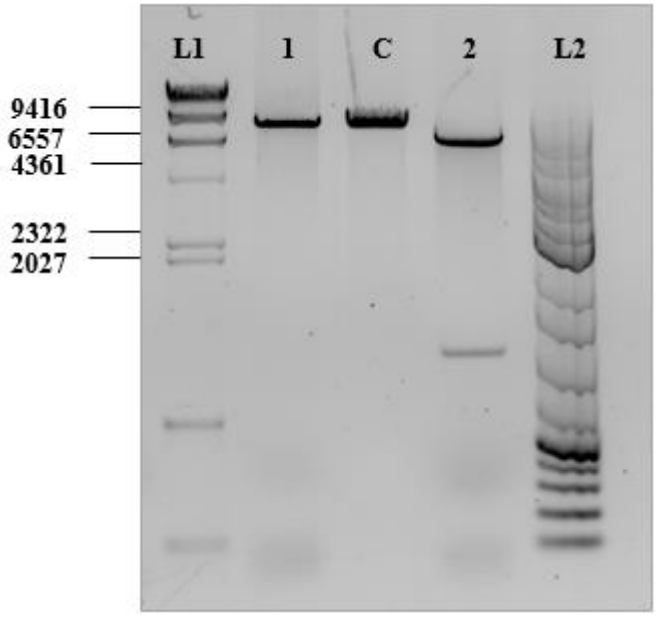

Figure 6.3.4. HPV-18 Target-1 cleavage in total genome using LbCas12a and AsCas12a. (L1) 2 µl of HindIII DNA ladder, 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with (1) LbCas12a, and (2) AsCas12a, (C) HPV-18 DNA as control, and (L2) 2 µl of 1 kb plus DNA ladder were loaded on 0.8% agarose gel.

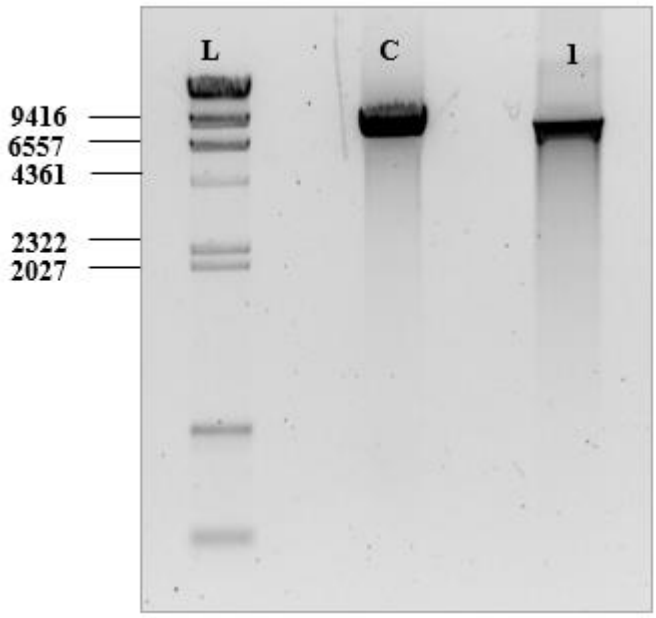

Figure 6.3.5. HPV-18 Target-2 cleavage in total genome using LbCas12a. (L) 2 µl of HindIII DNA ladder, (C) HPV-18 DNA as control, and (1) 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with LbCas12a were loaded on 0.8% agarose gel.

6.4.Specificity of Target-3 (HPV-16) and Target-2 (HPV-18) for detection

In order to show the produced crRNAs were type-specific, the crRNA of Target-3 was targeted with the HPV-18 genome (Table 6.4.1), and the crRNA of Target-2 was targeted with the HPV-16 genome (Table 6.4.2). The reaction was incubated at 37℃ for 1.3 hours and 65℃ for 15 min (heat inactivation) in thermal cycler. After incubation, 1 µl Proteinase K was added into tube and left at room temperature for 10 minutes. Then, 3 µl 6X SDS containing Purple Dye was added. The same procedures were performed for AsCas12a under the same conditions. The gel was run at 100 V for 60 minutes and imaged on BioRad. There was no cleavage observed, and it was confirmed that the produced crRNAs were type-specific (Figure 6.4.1).

Table 6.4.1. Cleavage components for HPV-18 Target-1

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA (HPV-16 Target-3)	1,675 µl
1 µM EnGen LbCas12a/AsCas12a*	0,22 µl
RNase-free water	14,965 µl
DNA (HPV-18)	1,14 µl**

*AsCas12a enzyme was ordered as 26.7 µM but 1 µM diluted in glycerol stock was used for cleavage reactions.**1 nM final DNA

Table 6.4.2. Cleavage components for HPV-16 Target-3

Reaction Component	**Volume**
NEBuffer r2.1 10X Reaction Buffer	2 µl
300 nM gRNA (HPV-18 Target-1)	1,675 µl
1 µM EnGen LbCas12a/AsCas12a*	0,22 µl
RNase-free water	15,125 µl
DNA (HPV-16)	0,98 µl**

*AsCas12a enzyme was ordered as 26.7 µM but 1 µM diluted in glycerol stock was used for cleavage reactions.
**1 nM final DNA

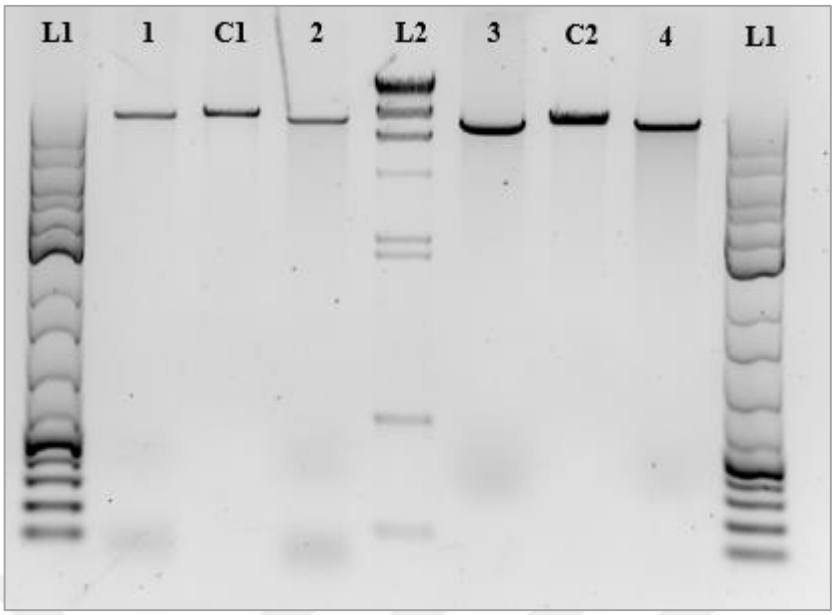

Figure 6.4.1. Specify of crRNAs for HPV detection.(L1) 2 µl of 1 kb plus DNA ladder, 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with (1) Target-2-crRNA-LbCas12a, and (2) Target-2-crRNA-AsCas12a, (C1) HPV-16 DNA as control, and (L2) 2 µl of HindIII DNA ladder, 20 µl of cleavage product with 3 µl of 6x SDS containing Purple Dye with (3) Target-3-crRNA-LbCas12a, (4) Target-2-crRNA-AsCas12a and (C2) HPV-18 DNA as control were loaded on 0.8% agarose gel.

7.DISCUSSION AND CONCLUSION

Cervical cancer is one of the most prevalent cancer type among women. High-risk types of HPV, especially HPV-16 and HPV-18, are the main causes of cervical cancer. Early detection, frequent screening tests, and HPV vaccinations are critical to cancer prevention. It is essential to develop rapid, sensitive, and low-cost detection methods. Cas12a enzyme, which is part of the Class 2 type V CRISPR system, is widely utilized in nucleic acid detection due to the following characteristics: Recognition of T-rich regions, which facilitates gene editing, and cleavage of any specific ssDNA after target site cleavage (93,104).

In this study, the most prevalent types of cervical cancer cases, HPV-16 and HPV-18, were detected using the CRISPR-Cas12a system. For both types, a total of 5 target sites were identified in L1 region. Five crRNA sequences specific to these target

regions were generated. Targeting experiments were performed with LbCas12a and AsCas12a enzymes. Two of the crRNAs were successfully detected in HPV-16 and HPV-18.

Firstly, HPV samples were isolated. These samples were amplified using the polymerase chain reaction (PCR) technique. Multiple experimental optimizations were conducted to achieve proper amplification of human papillomavirus (HPV). Two different primer concentrations (0.5 μM and 0.25 μM) were used. Lower primer concentrations produced DNA with the correct size and fewer side bands. The use of higher primer concentrations may lead to primer dimers between themselves or other DNA locations in HPV genome. It can result in the generation of side bands and formation of unexpected DNA lengths. Then, annealing temperature was optimized. Among the three temperatures (58°C, 60°C and 64°C), the optimal temperature was 60°C. Three-step and two-step PCR protocols were performed. After two-steps PCR, the product was shown in a lower size but product of the three-step PCR was in the correct size. Two different enzymes (Platinum SuperFi II and PrimeSTAR GXL Premix) were utilized for genome amplification. Although the Platinum SuperFi II enzyme successfully amplified DNA after several optimizations, reproducibility was not achieved in other experiments. PrimeSTAR GXL Premix showed a higher DNA concentration and it was reproducible. Platinum SuperFi II was only used to amplify HPV-16 Target-1, whereas PrimeSTAR GXL was utilized for all other genome amplifications. For HPV-16 Target-2 and Target-3 genome amplification, forward primer were sourced from publication (107), whereas reverse primer were custom-designed. Pone et al were performed full-circle PCR for HPV-16 genome. They were used four primer sets to create short and long amplicons. Afterwards, they were overlapped of these amplicons. In this study, two of primer sets were used and amplified with standard PCR methodology.

Secondly, DNA templates were synthesized for crRNA transcription. The template formation protocol was designed by my advisor, Assist. Prof. Süleyman ÜÇÜNCÜOĞLU. Three approaches were utilized for DNA template formation. Initially, a top strand was designed which included T7 promoter and direct repeat sequences. A bottom strand was designed to include reverse complementary of each identified target sequence without PAM and the complementary of the direct repeat

sequence. Therefore, these two strands were annealed, and the blank parts were filled with DNA polymerase. In another approach, top and bottom strands were designed as blank parts filled with DNA polymerase. These strands were hybridized with PCR. In the final approach, a protocol (106) was revised. Top strand was concluded to contain only T7 promotor sequence, and bottom strand involved complementary T7 promoter, direct repeats and reverse complementary of target sites. These two strands were hybridized in thermal cycler and transcribed directly. HPV-16 Target-1 and HPV-18 Target-2 DNA templates were synthesized with the first approach. HPV-16 Target-2/3 and HPV-18 Target-1 DNA templates were formed with the second approach. The third one was carried out for optimization. Among these three approaches, the second one showed the best results. DNA templates for HPV-16 Target-2, Target-3 and HPV-18 Target-1 were formed at the proper length. The templates were observed in lower length. The next step was in vitro transcription of crRNAs. The generated DNA templates for each target from the previous step were utilized as templates for their transcription. Two different transcription kits (MAXIscript T7 Transcription Kit and HiScribe T7 High Yield RNA Synthesis Kit) were used. Side bands were observed in all of the transcripts. The higher RNA concentration was observed when using HiScribe T7 High Yield RNA Synthesis Kit (NEB).

Finally, detection trials of two types of HPV (HPV16/18) were accomplished using five different crRNAs. Target-3 crRNA produced for HPV-16 successfully detected HPV-16 with LbCas12a and AsCas12a enzymes. A single cleaved fragment was observed with Target-2 crRNA. There was no cleavage activity with the utilized crRNA of Target-1. The detection of HPV-18 was observed with the crRNA of Target-1 only when the AsCas12a enzyme was used. Beside this, the LbCas12a enzyme did not exhibit any cleavage activity. There was no cleavage with the crRNA of Target-2. The reason for the ineffective Target-2 cleavage with perfectly matching crRNA could be derived from the RNA structures. The variation in cleavage activity in five crRNAs may be associated with template DNA formation or Cas12a enzymes may have different sensitivities depending on the target sites. Three approaches were utilized for template DNA formation. Even if there are a few missing nucleotides after formation, it is not possible to distinguish them in the agarose gel. Due to this loss, the cleavage efficiency may have decreased. Creutzburg et al. (108) demonstrated that alterations

in nucleic acid position in crRNA had a direct impact on the efficiency of cleavage activity.

In addition to that, the observation of non-target side DNA fragment with the LbCas12a for Target-2 could be a result of the complete digestion of shorter target side DNA. In order to determine the possibility of this hypothesis, further experiments should be performed. For example, targeting a circular DNA with the same target sequences will help to reveal the faith of non-target side DNA fragment. Even though the linear DNA targeting results were interesting and puzzling which may lead a new feature of LbCas12a, it is not in the scope of this study.

In conclusion, this thesis study revealed that two important agents in cervical cancer can be detected in a simple, affordable, and type-specific way based on the CRISPR-Cas12a system. Moreover, this method can facilitate the detection of other types of HPV or various types of cancers with alterations in crRNAs.

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Konu: Etik Kurulu Kararı

BAŞVURU BİLGİLERİ	**ARAŞTIRMANIN AÇIK ADI**	Determining Viral Detection Efficiency by Using Various CRISPR-Cas12a Enzymes (Çeşitli CRISPR-Cas12a Enzimleri Kullanılarak Viral Tespit Verimliliğinin Belirlenmesi)			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACI UNVANI/ADI/SOYADI	Tuğba KIRKIK			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ UZMANLIK ALANI	Yüksek Lisans Öğrencisi			
	KOORDİNATÖR/SORUMLU ARAŞTIRMACININ BULUNDUĞU MERKEZ	İstanbul			
	DESTEKLEYİCİ	-			
	ARAŞTIRMAYA KATILAN MERKEZLER	TEK MERKEZ ☒	ÇOK MERKEZ ☐	ULUSAL ☒	ULUSLARARASI ☐

	Belge Adı	Tarihi	Versiyon Numarası	Dili
Değerlendirilen Belgeler	ARAŞTIRMA PROTOKOLÜ/PLANI			Türkçe ☐ İngilizce ☐ Diğer ☐
	OLGU RAPOR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
	BİLGİLENDİRİLMİŞ GÖNÜLLÜ OLUR FORMU			Türkçe ☐ İngilizce ☐ Diğer ☐
Karar Bilgileri	**Karar No: 12**	**Tarih: 05/01/2023**		
	Yukarıda bilgileri verilen Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu başvuru dosyası ile ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş ve araştırmanın etik ve bilimsel yönden uygun olduğuna **"oybirliği"** ile karar verilmiştir.			

İSTANBUL MEDİPOL ÜNİVERSİTESİ GİRİŞİMSEL OLMAYAN KLİNİK ARAŞTIRMALAR ETİK KURULU	
BAŞKANIN UNVANI / ADI / SOYADI	Dr. Öğr. Üyesi Mahmut TOKAÇ

Unvanı/Adı/Soyadı	Uzmanlık Alanı	Kurumu	Cinsiyet		Araştırma ile ilişki		Katılım *		İmza
Dr. Öğr. Üyesi Mahmut TOKAÇ	Tıp Tarihi ve Etik	İstanbul Medipol Üniversitesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Prof. Dr. Mete ÜNGÖR	Endodonti	İstanbul Medipol Üniversitesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Doç. Dr. Mehmet Kemal ÖZDEMİR	Elektrik ve Elektronik	İstanbul Medipol Üniversitesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Doç. Dr. İlknur KESKİN	Histoloji ve Embriyoloji	İstanbul Medipol Üniversitesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Doç. Dr. Devrim TARAKCI	Fizyoterapi ve Rehabilitasyon	İstanbul Medipol Üniversitesi	E ☒	K ☐	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Dr. Öğr. Üyesi Neziha HACIHASANOĞLU ÇAKMAK	Biyokimya	İstanbul Medipol Üniversitesi	E ☐	K ☒	E ☐	H ☒	E ☒	H ☐	E imzalıdır
Dr. Öğr. Üyesi Pakize YİĞİT	Sayısal Yöntemler/ Biyoistatistik	İstanbul Medipol Üniversitesi	E ☐	K ☒	E ☐	H ☒	E ☐	H ☒	Katılmadı

* :Toplantıda Bulunma

Girişimsel Olmayan Etik Kurulu Sekreteri
Bilge KAYA