Validation of a self-sampling device for the screening of the Human Papilloma Virus (HPV) in the Catalan Population

Bachelor's thesis

Biological Systems Engineering - EEABB

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Abstract

According to the World Health Organization, Human papillomavirus (HPV) is the most common sexually transmitted disease (STD) around the world, with more than 600 million active cases, which corresponds to 7.5% of the world's population. HPV is a considerable risk infection and is responsible for 99% of all cervical cancers. In recent years, awareness on this infection has increased as well as the protocols and methods for preventing and treating HPV infections. The present study focuses on a method for HPV screening with the use of the Evalyn Brush self-sampling device used by 44 women volunteers. For the HPV typification, DNA was extracted from Evalyn Brushes and 37 different types of the virus were analyzed by PCR followed by hybridization using nitrocellulose strips. The DNA concentration obtained from the brushes of the device validate Evalyn Brush as a good biological sample collector. Very reliable and fast results are achieved, which allow to determine infections of HPV depending on their oncological risk which can be high, medium or low. A satisfaction questionnaire allows to know the acceptance by women about the self-sampling device Evalyn Brush. The results obtained in the laboratory validate the commercial kit from Operon company for HPV screening.

Resum

Segons l'Organització Mundial de la Salut, el virus del papil·loma humà (VPH) és la malaltia de transmissió sexual (MTS) més freqüent a tot el món, amb més de 600 milions de casos actius, que correspon al 7,5% de la població mundial. El VPH és una infecció de risc considerable i és el responsable del 99% de tots els càncers de coll uterí. En els últims anys, s'ha incrementat la conscienciació sobre aquesta infecció així com els protocols i mètodes per prevenir i tractar les infeccions pel VPH. El present estudi se centra en un mètode per al cribratge del VPH amb l'ús del dispositiu d'automostreig Evalyn Brush utilitzat per 44 dones voluntàries. Per la tipificació del VPH, es va extreure ADN dels Evalyn Brushes i es van analitzar 37 diferents tipus de virus mitjançant PCR seguida d'una hibridació mitjançant tires de nitrocel·lulosa. La concentració d'ADN obtinguda dels raspalls del dispositiu validen l'Evalyn Brush com a bon recollidor de mostres biològiques. S'aconsegueixen resultats molt fiables i ràpids que permeten determinar les infeccions del VPH en funció del seu risc oncològic que pot ser alt, mitjà o baix. Un qüestionari de satisfacció permet saber l'acceptació de les dones sobre el dispositiu d'automostreig Evalyn Brush. Els resultats obtinguts en el laboratori validen el kit comercial de l'empresa Operon per al cribratge del VPH.

Resumen

Según la Organización Mundial de la Salud, el virus del papiloma humano (VPH) es la enfermedad de transmisión sexual (ETS) más frecuente en todo el mundo, con más de 600 millones de casos activos, que corresponde al 7,5% de la población mundial. El VPH es una infección de riesgo considerable y es el responsable del 99% de todos los cánceres de cuello uterino. En los últimos años, se ha incrementado la concienciación sobre esta infección, así como los protocolos y los métodos para prevenir y tratar las infecciones por VPH. El presente estudio se centra en un método para el cribado del VPH con el uso del dispositivo de automostreo Evalyn Brush utilizado por 44 mujeres voluntarias. Para la tipificación del VPH, se extrajo ADN de los Evalyn Brushes y se analizaron 37 diferentes tipos de virus mediante PCR seguida de una hibridación mediante tiras de nitrocelulosa. La concentración de ADN obtenida por los cepillos del dispositivo validan el Evalyn Brush como un buen recolector de muestras biológicas. Se consiguen resultados muy fiables y rápidos que permiten determinar las infecciones del VPH en función de su riesgo oncológico que puede ser alto, mediano o bajo. Un cuestionario de satisfacción permite conocer la aceptación de las mujeres sobre el dispositivo de automostreo Evalyn Brush. Los resultados obtenidos en el laboratorio validan el kit comercial de la empresa Operon para el cribado del VPH.

Summary

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Symbols and acronyms

bp	base pairs
DNA	Desoxyribonucleic acid
gDNA	Genomic Desoxyribonucleic acid
HPV	Human Papillomavirus
h	Hours
IARC	International Agency for Research on Cancer
Kbp	kilobase pair, equal to 1000 base pairs
LIMS	Laboratory Information Management System
min	Minutes
ml	Milliliters
µl	Microliters
ng	Nanograms
ORF	Open Reading Frame
PAP	Papanicolaou probe
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
RCT	Randomized Controlled Trial
RT-PCR	RealTime-Polymerase Chain Reaction
STD	Sexually Transmitted Disease
TBE	Tris-Borate-EDTA
V	Volts

Acknowledgements

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1. Introduction

Worldwide, cervical cancer is the fourth most frequent cancer in women and approximately 80% of them will be infected at least one time by Human papillomavirus (HPV). Nowadays, it is known that HPV is the responsible of the 99% of the total cervical cancers. In fact, there are over 200 different HPV genotypes that causes different damages in human health. Within these genotypes, two categories can be differentiated: those with a low oncogenic potential risk and those with a medium-high oncogenic potential risk [1]. HPV-16 and HPV-18 (high-risk), are the two most common genotypes and also the most oncogenic ones, which are in approximately 70% of invasive cancer cases [2]. The high-risk genotypes are generally associated with precancerous lesions and invasive cancer in late stage of the infection while low-risk genotypes are in many cases asymptomatic or benign conditions such as genital warts.

Performing an HPV test and distinguishing between the presence of low and high-risk viruses as well as the persistence of the infection, allows us to identify those patients with a potential risk of developing cervical cancer. High-risk HPV infection that persists for more than 4 years with any control or treatment increases the risk of cancer. For this reason, it is important to have a good prognosis and follow-up of every positive HPV infection cases.

1.1. Human Papillomavirus (HPV)

1.1.1. General characteristics of the virus

HPV is a double stranded DNA virus that belongs to *Papovaviridae* family and commonly infects humans. It is known that there are different genotypes of the virus that affect humans in different ways and there is a classification in order to know which incidence cause these different strains in humans. According to the International Agency for Research on Cancer (IARC), there are more than 200 types of HPV and approximately half of them are directly related to the genital tract [3].

HPV is the most common sexually transmitted disease (STD) and can infect both women and men, being able to cause cervical and other cancers, including cancer of the vulva, vagina, penis, anus or oropharyngeal cancer. There are the different types of cancer that HPV can cause with their percentages **(Figure 1).**

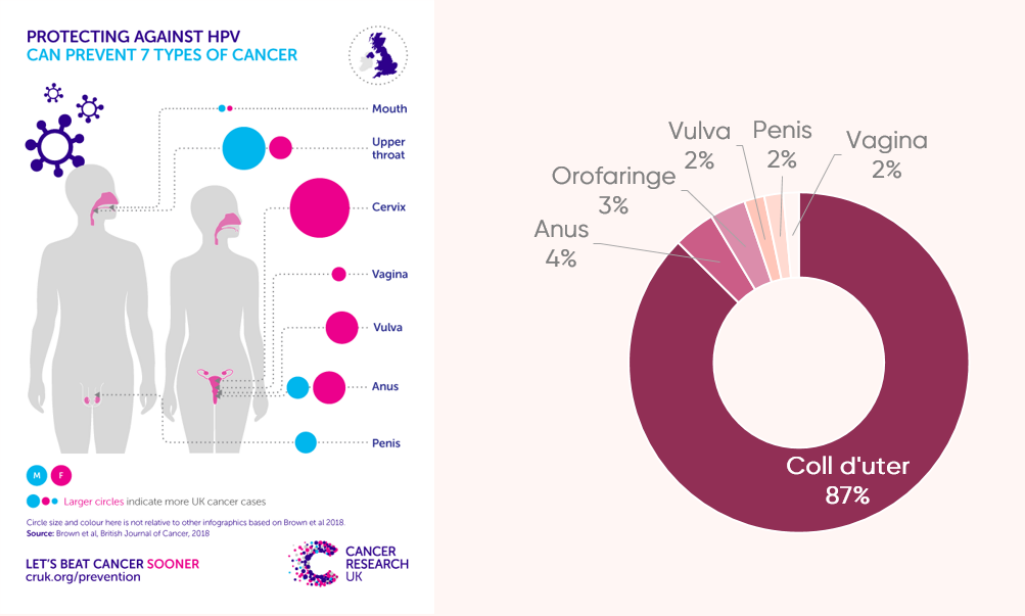


Figure 1. **HPV cancers:** On the left, different human body regions where cancer can be developed due to HPV. On the right, a graphic representation in percentages of different types of cancer that HPV infection causes [3].

1.1.2. Structure of the genome

Papillomaviruses are non-enveloped and their genome is no larger than 8 kb. All HPV genomes contains 8 open reading frames (ORF) that are all transcribed from a single DNA strand. Each ORF can be structured in three main regions; Early region, late region and long control region **(Figure 2).**

1st region: Early region (E). This region encodes proteins necessary for viral replication and has also an important role in cell transformation. These proteins are E1 and E2 that act as a factor that recognize the origin of the replication, E3, E4 that is involved in the late stages of the life cycle of the virus, E5, E6 and E7. The last two proteins target a number of negative regulators of the cell cycle: p105Rb for E6 and p53 for E7. These two proteins stimulate differentiating cells to re-enter the S phase and also facilitate stable maintenance of viral episomes.

2nd region: Late region (L). This region encodes structural (capsid) proteins necessary for virion assembly. These proteins are L1 and L2 and they assemble in capsomers.

3rd region: long control region (LCR). This region is referred to a large non-coding part and contains essential products, like cis elements, that are necessary for the replication and transcription of viral DNA [4].

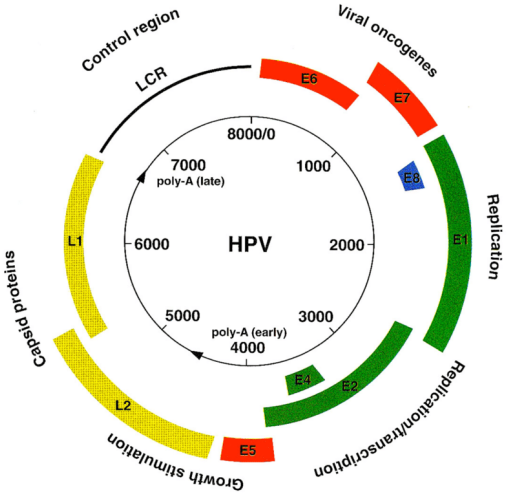


Figure 2. **HPV structure:** Circular ORF genome from HPV which illustrates the early region (E) with proteins E1, E2, E3, E4, E5, E6 and E7, the late region (L) with proteins L1 and L2 and the long control legion (LCR). Also indicates two major promoters that drive viral expression and two polyadenylation sites [4].

HPV has five major genera: α-papillomavirus, β-papillomavirus, γ-papillomavirus, mu-papillomavirus and the nu-papillomavirus. The oncogenic mucosal of the genera α is the main responsible of the major cause of cervical cancer, as well as benign genital condylomas (genital warts). On the other hand, infections of beta and gamma types are usually asymptomatic [5].

Table 1 shows a representation of some of the HPV known genotypes **(Table 1)**. Exist a classification for those genotypes which infection causes a major damage in humans and they are called high-risk types. Otherwise, for those genotypes which infection is not a significant damage, causing genital warts among other symptoms, are called low-risk types. The most carcinogenic are the genotypes HPV-16 and HPV-18 and they are common among cervical cancers [5].

Table 1. **HPV classification:** Organization of the different HPV genotypes depending on their risk and carcinogenic probability, indicating also the associated disease that these genotypes cause [5].

Human papillomavirus	Genotypes	Associated disease
High risk or oncogenic	HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59	Cervical, anal, vaginal, vulvar, penile, and oropharyngeal cancer and associated precursor lesions
Low-risk types	HPV 6, 11	Genital warts, recurrent respiratory papillomatosis
Probable carcinogenic	HPV 68	Cervical cancer
Possible carcinogenic	HPV 5, 8	Squamous cell carcinoma of the skin in patients affected by epidermodysplasia verruciformis
Possible carcinogenic	HPV 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, and 97	Uncertain

1.2. Cervical Cancer

1.2.1. History

It is nowadays known that more than 99% of cervical cancer cases are due to HPV infection, which is by far the most common sexually transmitted infection. But this was not so obvious few years before.

In the early 1980s, scientist and doctors thought that viruses could not cause cancer, and the few experts that were focusing on other viruses were investigating about the herpes simplex virus. But in the 1983, Dr. Harald zur Hausen made a proposal that caused quite a stir at the time. Dr. Hausen proposed the link between cervical cancer and HPV. In fact, he demonstrated that this type of cancer is caused by certain genotypes of papillomaviruses, assuming that if the tumor cells contained an oncogenic virus, should harbor viral DNA integrated in their genomes. Dr. Hausen finally found HPV-DNA in cervical cancer biopsies, which was the HPV-16 genotype. Since then, genotypes HPV-16 and HPV-18 have been found in about 70% of cervical cancer biopsies, and this allowed the development of HVP vaccines against these two genotypes. The discovery of HPV causing cervical cancer was the reason Dr. Hausen won the Nobel Prize in Physiology or Medicine in 2008 [6].

1.2.2. Symptoms

Cervical cancer, also called cervix cancer, affects the cervix, which is found in the lower part of the uterus. However, cancer does not appear right after the HPV infection, it takes approximately 15 to 20 years for cervical cancer to develop in women with a normal immunity system, but this period is shortened in women presenting a weakened immune system.

In early-stage cancer, main symptoms could be unusual vaginal bleeding (bleeding after sex, between your periods or even after the menopause), increased vaginal discharge and sometimes persistent back, leg and pelvic pain. As cervical cancer advances, more symptoms may appear in addition to the early-stage ones, like weight and appetite loss,

inflammation of the lower extremities of the body and other severe symptoms may appear depending in which organs the cancer has spread to.

1.2.3. Prevention

There are some ways to prevent HPV infection. The most common is by HPV vaccination and the recommendable age to get the vaccine are girls from nine to fifteen years old. In fact, HPV infections and cervical precancer have dropped since 2006, when HPV vaccines were first used in United States and from there around the world.

However, vaccines are not the only way to prevent HPV infections. As human beings, our responsibility is to make society aware of the possible risk of sexually transmitted diseases. The most important action that can be taken in order to prevent infections like HPV is the use of condoms, which decrease by a lot the possibility of being infected. It is quite important to take into account sex education in our society because a good education in early stages of life raises awareness about HPV infections. Education could be by far the best way in order to prevent in future HPV infections and potential cervical cancers.

1.2.4. Screening of cervical cancer

To give some context, the screening of HPV can be done by two different techniques: the normal cytology and HPV test.

In the 19th century, cervical cancer was described as a sexually transmitted disease and started the introduction of surgery for treating the disease. Then, in 1920, a Greek doctor called Georgios Papanicolaou developed a new technique and approximately near 1940, the screening of cervical cancer began. Later, in 1980 the linkage between HPV and cervical cancer was discovered.

Cytology or Papanicolaou probe (PAP) is a technique that a doctor performs to check for the development of cervical cancer or precancerous changes in the cells of the cervix. This type of probe consists in removing cells from the surface of the cervix and the area around

it in order to analyze them under a microscope and look for cells changes that could potentially lead to cervical cancer. It is a morphologic and diagnostic probe.

According to the European guidelines for quality assurance in cervical cancer screening [7], studies consistently show that HPV testing is more sensitive than cytology testing, in particular 23-43% more sensitive. Even though these tests are usually performed by doctors, in some countries it is also possible for patients to test themselves. Evalyn Brush is a self-sampling device that is widely used in some European countries like for example The Netherlands. In fact, the Dutch government renewed the screening protocol for cervical cancer prevention in 2017, introducing the option of a self-sampling kit as an alternative to conventional screening.

HPV tests can be done by RealTime-PCR or by PCR + Hybridization. An example of an HPV test that uses RT-PCR could be the Cobas commercial kit, from Roche Diagnostics. Evalyn Brush, the HPV testing kit reviewed in this study, uses the other method, PCR + Hybridization. PCR is needed to amplify the DNA extracted from the acquired samples, and then hybridization is performed using the Papilloma Strips by Operon company.

The *Asociación Española de Patología Cervical y Colposcopia (*AEPCC) stated that the transition to HPV screening should be an achievable goal within 3-5 years for all settings of primary cervical cancers screening. It is important to know that HPV test is a preventive probe, which means that cervical cancers could be detected before the worsening of symptoms.

The knowledge of these two different techniques is important in order to understand how cervical cancer screening started. It is also important to understand how it is managed nowadays, and try to improve and discover new techniques and protocols to achieve a better procedure in order to help the health of the population at a world level.

1.3. Self-sampling device: Evalyn Brush

Evalyn Brush is a vaginal tampon from the company Rovers Medical Devices and is one of the best self-sampling devices in the market according to the Dutch and Danish cervical screening programs. Evalyn Brush showed a DNA yield at least twice as high compared with other self-sampling devices and the DNA concentration obtained is significantly higher than other devices. The different parts of the device are shown in order to understand the functionality and structure of Evalyn Brush **(Figure 3).**

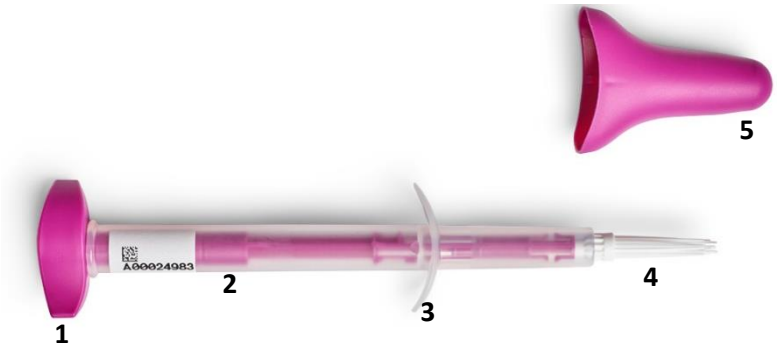


Figure 3. **Parts of Evalyn Brush:**
1. Pink plunger: Ensure the white brush **(4)** is pushed into the right position to collect the sample
2. Transparent casing: Ensures the protection of the cellular material while is being transported
3. Wings: A smooth and rounded surface to prevent injury and discomfort
4. White brush: brush with fine bristles in order to obtain the maximum amount of sample possible
5. Pink cap: Provides protection and hygiene before and after its use [8].

The point is to obtain a similar or even better sample using this device compared with the clinical collected samples while opting for an easier method for women looking for effective and optimal HPV screening.

2. Background

The present bachelor's thesis is part of a larger study and a longer duration than my stay in the company. In this way, the objectives and results are based on the data which has been collected during my stay in ADN Institut.

This project has been carried out in ADN Institut S.L, a health center specialized in the clinical and genetic diagnosis and counseling that has its own laboratory. In this project there are involved other partners that are detailed later, in the Methods section.

ADN Institut is a company that started in 2019, so it has almost been three years since its inauguration. They are specialized in genetic testing of inherited diseases, nutrition, fertility and reproduction, paternity and preventive health. Apart from genetic studies, they also offer coronavirus testing, clinical analysis like comprehensive analytical profiles, sexually transmitted diseases (STD) analyses and drug detection.

My work in this project is to validate the Papilloma Strip High+Low kit by Operon and also the validation of the self-sampling device Evalyn Brush. In order to achieve this, the tasks that I have done in ADN Institut are: gDNA extraction of human samples using manual (silica-based centrifuge columns) protocols, DNA quantification by Qubit4 fluorometer, amplification of DNA by PCR, gel electrophoresis, the analysis of 37 strains of HPV using the High+Low PapillomaStrip kit and statistical analysis of results.

3. Objectives

3.1. General

The main objective is the validation of the commercial kit Papilloma Strip for the screening of high, medium and low risk HPV genotypes in samples obtained by the patients themselves using the self-sampling device Evalyn Brush.

3.2. Specific

- Evaluation of the acceptance of the device by patients using a satisfaction questionnaire.
- Validation of Evalyn Brush as an efficient self-sampling collector to obtain an optimal DNA concentration.
- Determination of the percentage of HPV positives strains at the population level as well as the low, medium and high-risk HPV genotypes.
- Comparison of results from another study with a similar VPH detection kit in order to make a first approach about the incidence of different HPV genotypes in two different countries.

4. Materials and methods

4.1. Materials

4.1.1. Biological material

The samples used in this study correspond to the gDNA extracted from the samples obtained by the patients using the self-sampling device Evalyn Brush. These patients are women from 20 to 65 years from the gynecology and obstetrics department of *Hospital Sagrat Cor* who are volunteers to be part of this study.

4.1.2. Equipment

In this project, it is necessary the usage of different machines and equipment to develop the analysis of samples in the correct way, as well as a better efficiency of the results. There will be below a brief description of the different equipment used during the laboratory work:

- Micro-spin: It allows us to be sure to have the entire liquid on the bottom of the Eppendorf microtubes without the need of performing a centrifuge for the sample. This micro-spin can turn a maximum of 12.000 rpm.

- Mini-centrifuge: A laboratory centrifuge that allows us the good performance of the protocols like the DNA extraction for obtaining the pellet and supernatant. It can turn at a maximum speed of 13.500 rpm.

- Vortex: Necessary to mix up the mixes of the different steps in processes like the suspension of samples in PBS or the amplification of DNA by PCR.

- Thermo-shaker/thermo-block: These machines are digital dry bath and are necessary for the processes/reagents that need a thermal bath or a thermal treatment. The

thermos-blocks only generates heat without shaking and the thermos-shakers generate both of heat and shakes. Depending on the method one or other will be used.

- Qubit Fluorometric: It allows us to specifically quantify DNA and determine if we have enough nucleic acid to continue with the next step. It works with the detection of fluorescent dyes that are specific for DNA, making it even more specific than UV absorbance.

- Thermocycler (PCR): Necessary for the DNA amplification of the samples. It is a thermocycler from the company Applied Biosystems and is capable of amplifying up to 96 samples at the same time.

- Auto-LIPA (from Operon): Necessary to perform the automatic hybridization. This automatic process is done by the Auto-LIPA machine that Operon has left on loan to us in order to observe the performance of results and if it is worth it or not to buy this equipment.

- Ultraviolet ray lamp: Used to observe the results of the gel electrophoresis and also used to disinfect equipment like micropipettes, Eppendorf tubes, tips…

4.1.3. Reagents and products

4.1.3.1. gDNA extraction

For the DNA extraction we use the PureLinkGenomic DNA extraction kit from Invitrogen that consist of silica-based centrifuge columns. Different components are included in this kit in order to perform the genomic DNA extraction **(Table 2).**

Table 2. **gDNA extraction components**: Products from the PureLinkGenomic DNA extraction by Invitrogen. All these products are stored at ambient temperature except for RNase and Proteinase K when long-term storage is needed.

Product
PureLink™ Genomic Digestion Buffer
PureLink™ Genomic Lysis/Binding Buffer
PureLink™ Genomic Wash Buffer 1
PureLink™ Genomic Wash Buffer 2
PureLink™ Genomic Elution Buffer
RNase A
RT-stable proteinase K
Silica-based centrifuge columns
Collecting tubes

4.1.3.2. Quantification of DNA by Qubit fluorometer

- Qubit microtubes
- Qubit quantification kit 1x dsDNA HS Assay kit (4ºC)
- Qubit fluorometer
- DNA samples

4.1.3.3. DNA amplification by PCR

PCR kit High+Low PapillomaStrip by Operon allows us to analyze the samples using the medium-high risk HPV primers and low risk ones. Inside this kit there are also the material needed for the next step of the amplification. This project is carried out with a kit for 48 samples and their corresponding material **(Table 3).**

Table 3. **PCR reagents:** Reagents from the High+Low PapillomaStrip kit by Operon used for the PCR. There are more reagents included in this kit but only these are used for PCR.

Products
PCR Mixture
Medium-High risk primers
Low risk primers
Taq

4.1.3.4.Hybridization

For this step we will use the reagents included in the High+Low PapillomaStrip except the ones used for PCR because we already used them **(Table 4).** The content of the commercial kit used is represented in **Figure 4.**

Table 4. **Reagents from the High+Low PapillomaStrip kit:** used for the Hybridization. The channel trays in the 48 samples kit are not included and they must be purchased separately.

Products
Nitrocellulose Membranes
Denaturant
Hybridization Buffer
Wash Buffer 1
Conjugate
Wash Buffer 2
Substrate

Figure 4. **High+Low PapillomaStrip kit:** Material described in section 4.1.3.3 and 4.1.3.4.

4.1.4. Laboratory material

- Micropipettes of 0.5-10 µl, 10-100 µl, 100-1000 µl.
- Pasteur pipettes
- Eppendorf microtubes

- Micropipette tips
- Nitrile gloves
- Coolers
- Laboratory paper
- Ethanol
- Timer clock
- PBS (Phosphate Buffered Saline)

4.2. Methods

4.2.1. Work plan

In order to facilitate the screening of HPV through the analysis of samples collected with a self-sampling device, it is necessary the collaboration of four different entities.

Sagrat Cor Hospital is the entity responsible for patient recruitment (women). Patients are women who volunteered to participate in this study. They are patients form the department of Obstetrics and Gynecology from *Sagrat Cor* Hospital. It is the doctor who asks them if they want to participate in this study after performing the cytology.

Operon is responsible to provide both the reagents and the Auto-LIPA equipment. This is an important study for this company because it will show the performance and profitability of their reagents and equipment.

Rovers Medical Devices is a Dutch company in charge of providing the self-sampling devices (Evalyn Brushes) for the collection of samples.

Finally, ADN Institut is responsible to analyze and interpret the results as well as providing an appropriate laboratory to perform the HPV detection.

Representation of the entities that collaborate with each other in order to have a good communication and logistics throughout the project **(Figure 5).**

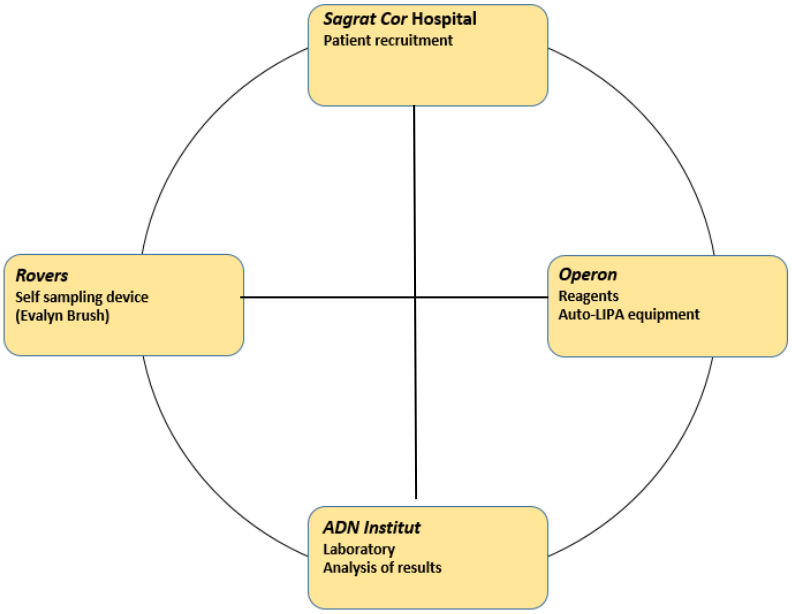


Figure 5. **Work plan:** Representation of the different entities which are part of this study and their role. Every line represents the communication and contact between one entity and other.

4.2.2. Arrival of samples

This is the beginning of the analysis process. In ADN Institut laboratory, a package arrives with the informed consent (which is crucial to treat the samples legally), the satisfaction questionnaire and the Evalyn Brush with the biological sample extracted.

The satisfaction questionnaire used consist of 23 questions in which volunteers must mark a single answer. It is divided into different sections: general characteristics, device characteristics, instruction sheet, screening test and knowledge of cancer prevention (this questionnaire is attached as an annex, page 65). Then, it is time to transfer the answers of the questionnaire to an excel sheet in order to analyze and interpret the satisfaction of the self-sampling device.

The next step is to register the patients in two different softwares that ADN Institut uses in order to have control of their patients. This software are NetClinicas and Zendo LIMS (Laboratory Information Management System).

NetClinicas is a software specialized in clinics and the main objective of this program is to have the patient's information well organized. The registration of the patient to NetClinicas is done using the information of the informed consent document, which consists in the name and last name of the patient, their identity card number, the date of birth and a personal email in order to send at the end the respective results of the HPV analysis.

Zendo Lims allows to have a control of the processes and protocols which are being used in the laboratory. In this way, a constancy of what is being done in every step is made. For example, if a DNA extraction is being performed and difficulties are encountered during this process, it is noted in this software to know what has happened during the analysis.

Once the patient is registered in this two different softwares, it is proceeded to incubate the Evalyn Brush samples in PBS **(Figure 6)**. First step is to remove the pink cap from the Evalyn Brush and make sure that the sample is clean and there are no inhibitory components like blood. If that happens, the white brush will move it into a 3 ml of PBS falcon tube (normal volume is 5ml of PBS). Then, the pink plunger is pushed up in order to get out from the transparent casing the white brush. The biological sample is in the white brush, so, with the help of tweezers the white brush is removed and moved to a falcon tube of 50 ml with 5 ml of PBS already inside the tube. Then, these samples can hold up well in the fridge at 5ºC for next one or two weeks.

Figure 6. **Evalyn Brush in PBS:** Process of inserting the sample from Evalyn Brush into a falcon tube with 5ml of PBS with the help of non-reusable tweezers.

4.2.3. PapillomaStrip Test (by Operon)

The detection of HPV infection in this study will be done with the PapillomaStrip test by Operon Company. It is a test based on the principle of reverse hybridization and allows a qualitative detection in DNA samples from cervico-uterine smears.

This high + low PapillomaStrip kit allows the detection of these following HPV genotypes:

High risk: 16, 18, 31, 33 ,35, 39, 45, 51, 52, 56, 58 and 59.

Medium risk: 26, 53, 66, 67, 68, 69, 70, 73 and 82

Low risk HPV types: 6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 70, 71, 72, 74, 81, 83, 84 and 94.

The kit consists of three main steps:

1) DNA extraction: DNA purification and quantification protocols.

2) Amplification by PCR: This kit has the reagents for the amplifications of regions E6-E7 from all the medium-high and low risk genotypes. It is a fact that the expression of E6-E7 genes is associated with carcinogenesis, this is why these two regions are described as an important biological and pathological information. There is one primer for each HPV type,

so you make sure to avoid problems during the amplification process. In this process there is also the amplification of a control gene, GADPH control in the case of low-risk genotypes and β-globine control in the case of medium-high genotypes.

3) Hybridization: In this process, the product from the amplification attaches in a specific binding with nylon membranes. These membranes or nitrocellulose strips have the control gene (GADPH or β-globine depending on the HPV type), a developing control and control lines. This control helps us understand and interpret the results since it is a qualitative test. The hybridization is performed using a conjugate (streptavidin-peroxidase), followed by the addition of a substrate that generates a blue precipitate.

Once these processes are finished, it is time to interpret the results. The nitrocellulose strip should render in all cases the control gene in order to obtain valid results. The results are interpreted with the aid of two charts, one for medium-high types and other for low types. Different results at the end of the process are represented below **(Figure 7).**

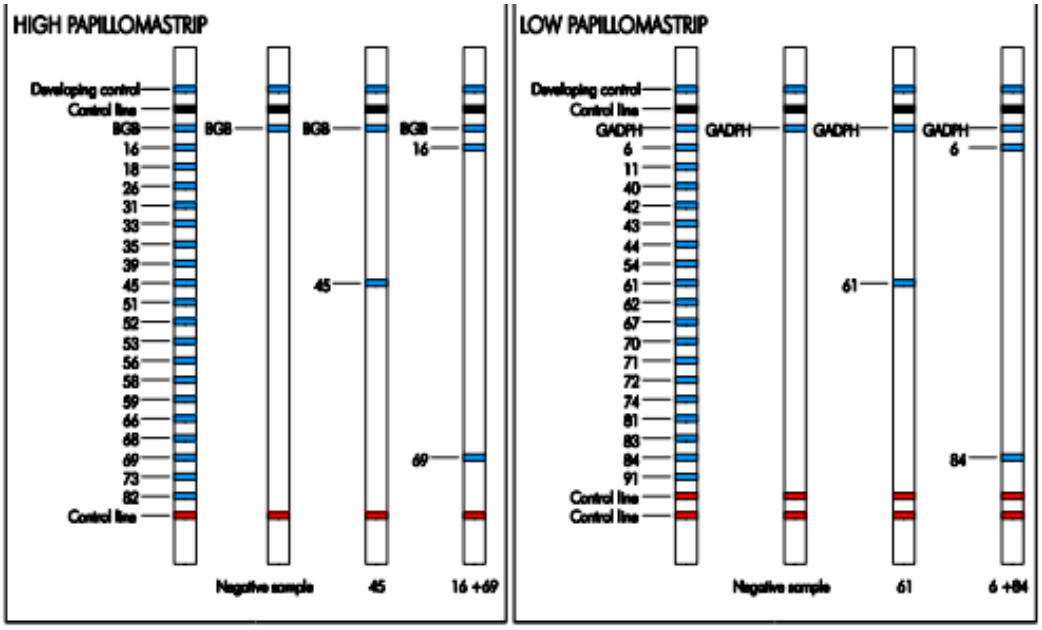


Figure 7. **Examples of different results:** On the left, examples from the medium-high HPV types. There is an example for negative sample showing only the control gen band, a positive sample by genotype 45 and other positive sample by genotypes 16 and 69. On the right, examples from the low HPV types. There is a negative sample as before but changing the control gene (GADPH) and adding one extra control line, a positive sample by genotype 61 and other positive sample by the genotypes 6 and 84 [9].

4.2.4. Genomic DNA extraction using PureLinkGenomic DNA kit by Invitrogen

Once the samples are in the falcon tube with the PBS, the genomic DNA extraction is carried out. The commercial kit used for this protocol is the PureLinkGenomic by Invitrogen **(Figure 8)**. This kit is based on the silica-based columns. These columns are responsible for absorbing the genomic DNA thanks to the silica gel that allows a good purification of the DNA. The different steps in this method allow the purification of the products or compounds that are not desirable in order to perform, for example, a PCR.

Before the use of columns, it is necessary to do a sample preparation and pretreatment in order to obtain a good yield and a desired concentration of DNA (ng/µl). The first step is to take 500 ml from the falcon tubes with the samples and introduce them into an Eppendorf microtube (1.5 ml).

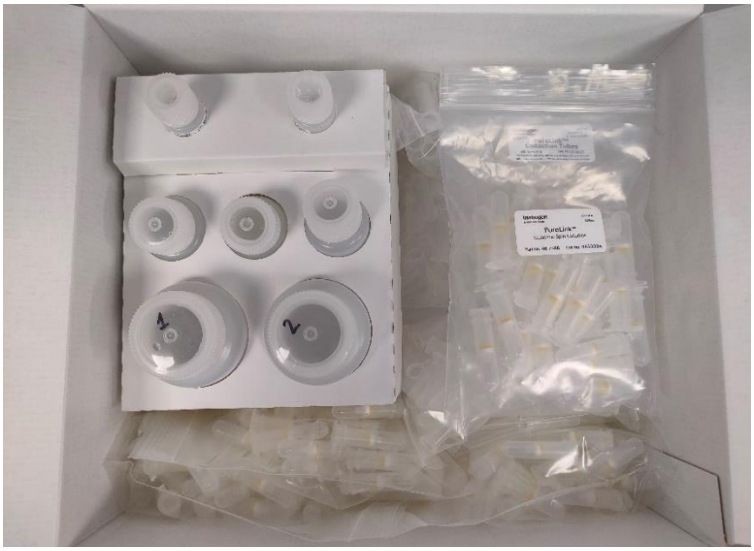


Figure 8. **Content of PureLinkGenomic kit:** On the left, the different reagents used in the process. On the right, the silica-based columns.

Then, these microtubes with samples are centrifuged at 10000 rpm for 5 minutes in order to obtain a good amount of pellet. After that, 300 µl are discarded from the microtubes to do a resuspension of the pellet in the 200 µl left. This is done in order to have a higher concentration of the sample. Then, 20 µl of proteinase K and RNase are added with 200 µl of the Genomic Lysis Buffer. Proteinase K is used to digest protein and break down

damaging proteins. RNase is used to break down contaminating RNA during the DNA isolation. Genomic Lysis buffer is a specialized and optimized Lysis buffer that improves the activity of proteinase K and delete possible protein contaminations. Afterwards, an incubation is required at 55 ºC from 30 min to 1 hour **(Figure 9).**

Figure 9. **Thermo-block/Thermo-shaker:** used to perform the incubation at 55 ºC.

Once the incubation is finished, 200 µl of pure ethanol are added in the Eppendorf microtubes, this step allows to eliminate the solvation shell that surrounds the DNA. Then, it is time to transfer all the content (650 µl more or less) from the microtubes to the silica-based columns. Columns make a series of centrifugations and washes with the different buffers of the kit **(Figure 10)**. At the end of the two washes, it is time to add the 75 µl of elution buffer to the columns in order to obtain our desired product (genomic DNA). Elution buffer remove the nucleic acid material from the silica-based column after it has been captured. This silica column is placed in a well identified and labeled Eppendorf microtubes. A centrifugation is performed in order to obtain our DNA inside the Eppendorf and discard the silica-based column.

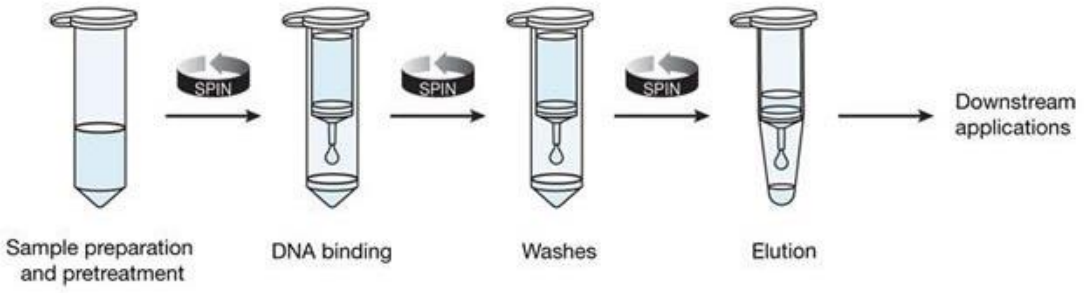


Figure 10. PureLink spin column-based purification method [10].

4.2.5. Quantification of DNA (Qubit fluorometer)

Qubit fluorometer is a device designed to measure with a high precision the quantity of DNA, RNA and protein present in a sample using the highly sensitive Qubit essays. The concentration or the quality of the target molecule (DNA in this case) in the sample is reported by a fluorescent colorant which emits a signal when it bonds it to the target, in order to minimize the effects of the non-desirable products on the result **(Figure 11).**

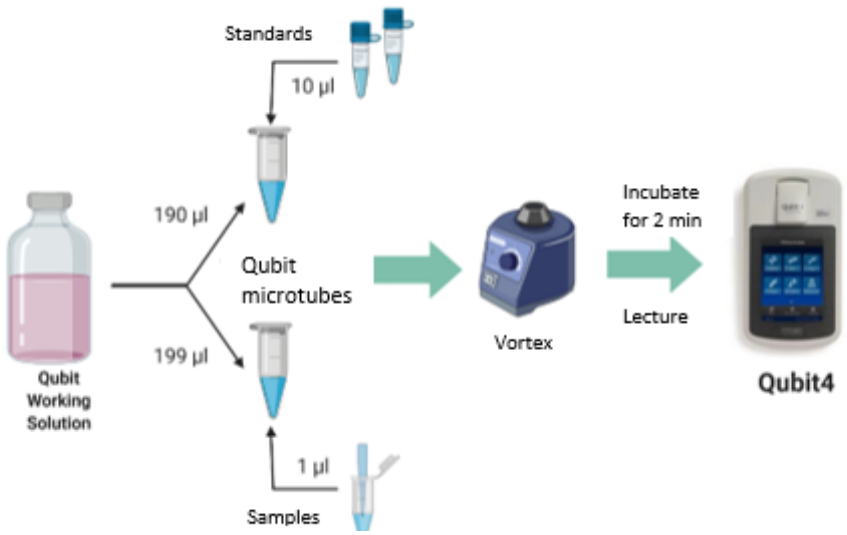


Figure 11. Procedure to perform a DNA quantification.

First step is to temper reagents from the kit 30 minutes before use. For each quantification it is needed 1 Qubit microtube plus two extras to read the standards of quantification included in the kit. Standards allow to calibrate Qubit making a very low quantification for one standard and other very high for the other standard.

When the reagents are temperate, the qubit samples and standard are prepared in a Qubit microtube. For the sample, 190 µl of Qubit Working Solution and 1 µl of sample are added. For standards A and B, 190 µl of Qubit Working Solution and 10 µl of the corresponding standards are added. A briefly vortex is performed and then the microtubes are incubated 2 minutes at the room temperature inside the racks **(Figure 12)**. Finally, the microtubes with standards are introduced inside the Qubit fluorometer and then it is time to read the standards. Once the standards are correct, it is turn to quantify the samples and then proceed to their storage at 4ºC.

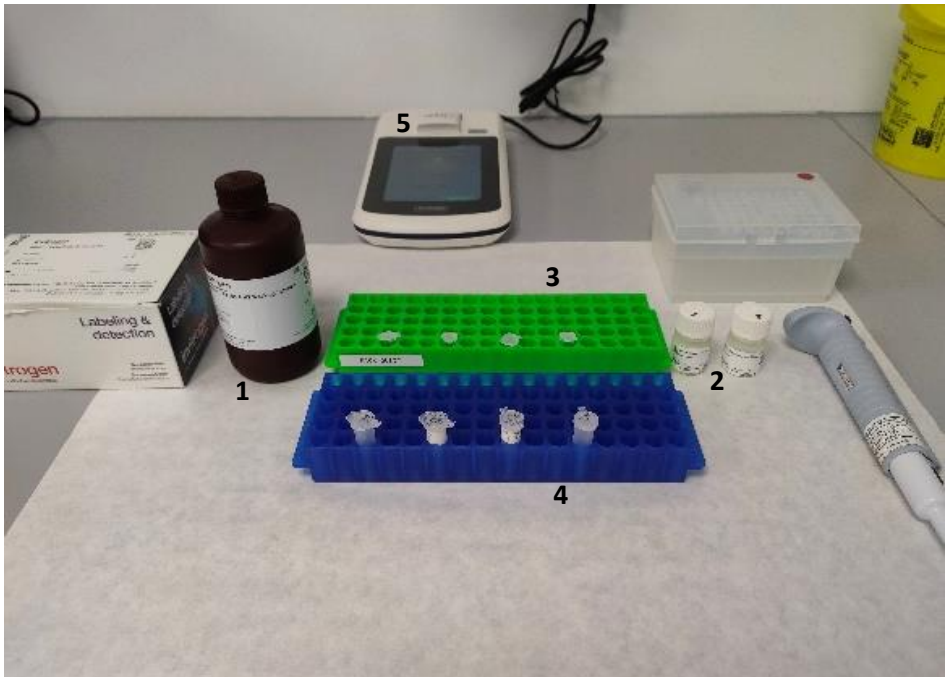


Figure 12. Quantification of the genomic DNA using Qubit 4:
1. Qubit Working Solution
2. Standards (low and high concentration)
3. Green rack with Qubit microtubes
4. Blue rack with DNA extracted in Eppendorf microtubes
5. Qubit 4 Fluorometer

4.2.6. Polymerase Chain Reaction (PCR) using High+Low Primers from Operon

Polymerase Chain Reaction (PCR) is a technique that allows the amplification of a specific sequence of DNA using the ability of DNA polymerase to synthesize a strand of DNA complementary to the offered template strand. In fact, it is a process capable of amplifying a single DNA molecule into millions of copies in a short time. In order to achieve this, the performance of PCR needs the following compounds:

- DNA template. Our samples with the genomic DNA that contains the target sequence.
- DNA Polymerase. Corresponds to the "engine" of the PCR. In this case the DNA Polymerase used is Taq Polymerase.
- Primers. Essential if you want to amplify a specific region of your sequence. There are high and low risk primers in order to amplify the regions that are related to HPV.
- dNTPs (Deoxynucleoside triphosphates). Their function is to expand the growing DNA strand with the help of Taq Polymerase, they serve as building blocks for new DNA strands.
- Buffers and cofactors. The best-known cofactor is $MgCl_2$ (Magnesium Chloride). This compound is an essential ingredient for the PCR master mix and its function is to enhance the enzymatic activity of DNA Polymerase to boost DNA amplification.

At the end, the PCR consist of different cycles with temperature changes and different durations done by a thermocycler. The PCR design depends on the study and the specific target to amplify. The different steps of PCR are:

- Initial Denaturation
- Denaturation: The double-stranded DNA templates are heated to separate the strands
- Hybridation/Annealing: short DNA molecules (primers) bind to flanking regions of the target DNA
- Elongation/Extension: DNA polymerase extends the 3' end of each primer along the template strands.
- Final Elongation/extension

In the figures below, you can find the main PCR steps **(Figure 13)**, and a real design of the PCR protocol used for this study **(Figure 14).**

Denaturation, annealing and extension steps are repeated ("cycled") 25-50 times to exponentially produce exact copies of the target DNA. As it is explained, every PCR design is different depending on the study and the conditions or compounds. In this case, the protocol used is the HPV OPERON protocol, which Operon has designed for this type of analysis.

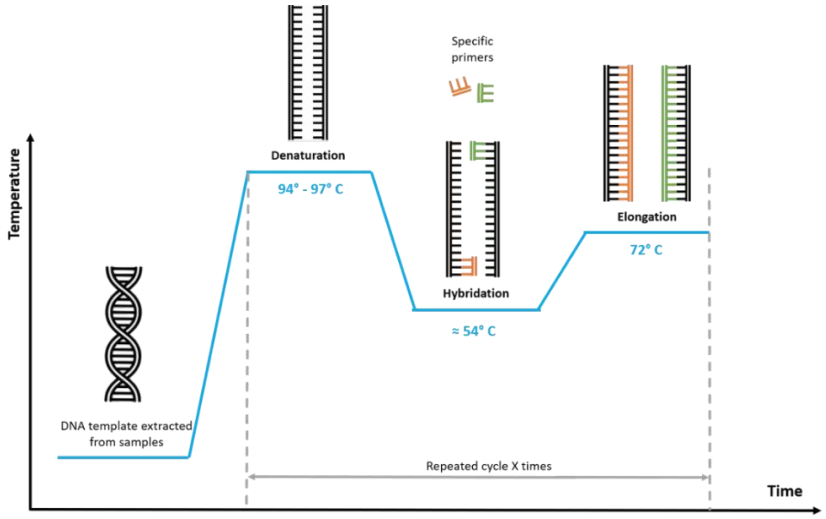


Figure 13. Different steps of PCR [11].

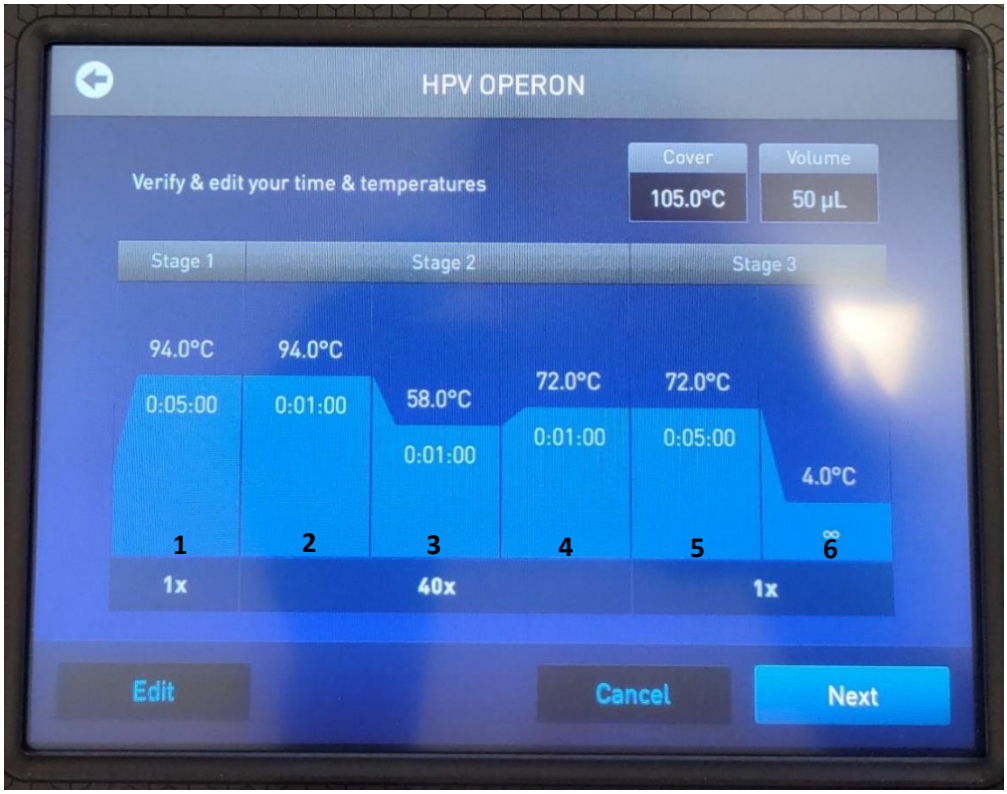


Figure 14. HPV OPERON PCR design for the High+Low risk HPV screening.

1. Initial Denaturation – 94 ºC – 5 min
2. Denaturation – 94 ºC – 1 min
3. Annealing – 58 ºC – 1 min
4. Extension – 72 ºC – 1 min

} X 40 cycles

5. Final Extension – 72 ºC – 5 min
6. Infinite Hold – 4ºC

$$\text{Total PCR time} = 5\ \text{min} + (1 + 1 + 1)\ min * 40 + 5\ \text{min} = 130\ \text{min} = 2\text{h}\ 10\ \text{min}$$

In this case, it is used the high and low risk primers for the screening of HPV. Also, there is the master mix and TaqPolymerase. At the end, these three different compounds will be mixed together in one Eppendorf tube, one for the high-risk mix and other for the low-risk mix. In order to know the volumes required to perform the PCR, equation 1 will be used.

$$Ci * Vi = Cf * Vf \quad (1)$$

It is necessary to determine the initial volume of each component from their corresponding concentrations. Next step is to isolate the variable Vi as it is represented in equation 2.

$$Vi = \frac{Cf * Vf}{Ci} \quad (2)$$

Where:

- Ci is the stock concentration
- Vi is the initial volume of the component or the volume to add to form the mix
- Cf is the work concentration
- Vf is the final volume of the mixture

The next step is to calculate the necessary volumes for each product **(Table 5).**

Table 5. **High/Low PCR mixes:** volumes to transfer in Eppendorf tubes to do High and Low mixes for one sample

Products	Stock concentration	Work concentration	Volume
Master Mix	10 mM	7.5 mM	38 µl
Medium-High/Low risk primers	10 µM	1 µM	5 µl
Taq	12 U/ µl	0.48 U	2 µl
TOTAL			**45** µL

If there is more than one sample to amplify, the number of the samples are multiplied by the quantity needed to do the mix. After that, there will be two Eppendorf tubes; High-Medium Mix and Low Mix. Then, 45 µl are transferred in PCR microtubes (200 ml) previously labeled and inside coolers **(Figure 15).** The total volume needed for performing the PCR for HPV screening is 50 µl, so the 5 µl remaining will be from the DNA samples or nuclease-free water in case of controls. Once there is 50 µl in PCR microtubes, a vortex and spin are performed and then they are ready to enter in the PCR process. The total time of PCR reaction is 2:10 hours. Once the process is finished, the PCR microtubes are stored in the refrigerator at 4 ºC.

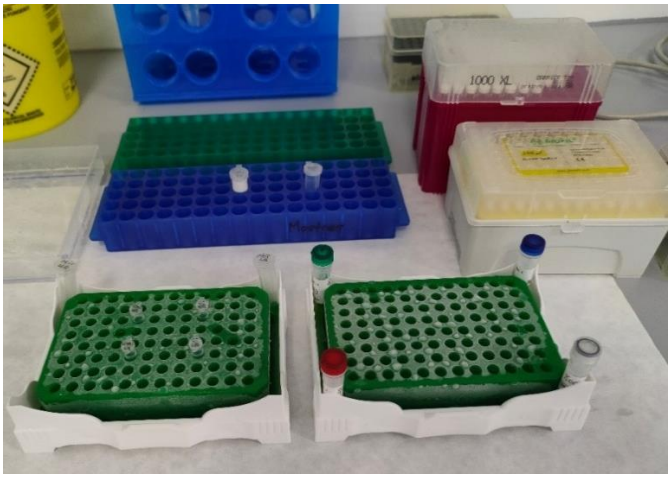


Figure 15. **PCR method:** Bottom left; PCR microtubes (200 ml), bottom right; Reagents described in table 5, top left; blue rack with the sample and H_2O free nuclease for controls, top right; tips with filter.

4.2.7. Gel electrophoresis

This technique is used in order to separate DNA fragments of varying sizes, from 100 bp to 25 kb. Gel electrophoresis allows to know if PCR has been done correctly. For example, if there was some contamination from other samples during the PCR process, this technique would reveal the contamination in the negative controls and another PCR should be performed. **Figure 16** represents the different materials used for this method.

DNA molecules are pushed by an electrical field through a gel made from agarose and TBE that contains small pores. DNA molecules goes through the negative pole to the positive pole, due to the negative charge of the DNA. Agarose is a natural polymer and has a several properties that make it useful as a gelling agent. TBE (Tris-Borate-EDTA), is a buffer used in gel electrophoresis because it helps molecules go through the gel and maintains the pH of the gel.

The first step to make this gel is to determine which concentration of agarose it is wanted. In many cases, the desired concentration is 1 % gel, so 0.5 g of agarose and 50 ml of TBE are needed. These two products are added to an Erlenmeyer flask. Then the mixture must melt by heating with a microwave. Few periods of 30 seconds heating and removing the

flask and swirling the contents to mix well until the agarose is completely dissolved. Next, a DNA gel stain is added into the mixture for visualizing DNA when the process is finished.

Afterwards, when the mixture has cooled, it is poured into the gel mold with a correspondent comb (this comb is needed to make the holes to put the samples once the mixture is gelled). Later, when mixture is gelled, the comb is removed and is placed in the gel box. Once the gel is placed, all the box must be covered with TBE to perform a good electrophoresis. Then, it is time to place the PCR product on each of the holes that comb made in the gel but one hole is reserved for the ladder (this ladder is used to know the sizes of the bands). Finally, the box is covered and the process starts with 100 V and 45 min.

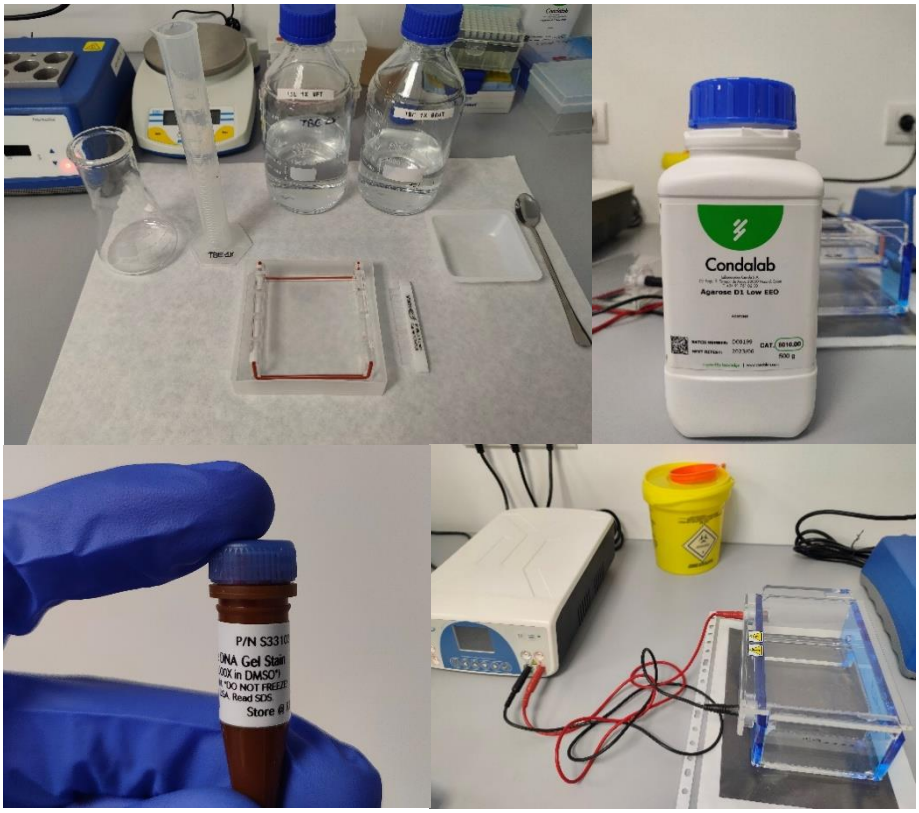


Figure 16. **Gel electrophoresis material:** Top left; materials used for gel electrophoresis, top right; agarose used in this technique, bottom left; DNA gel stain, bottom right; gel box.

When time has passed, comes the step of visualizing and observing the result of the electrophoresis. The gel is removed from the gel box and it is transferred into an ultraviolet ray lamp that will show the result very clearly **(Figure 17).**

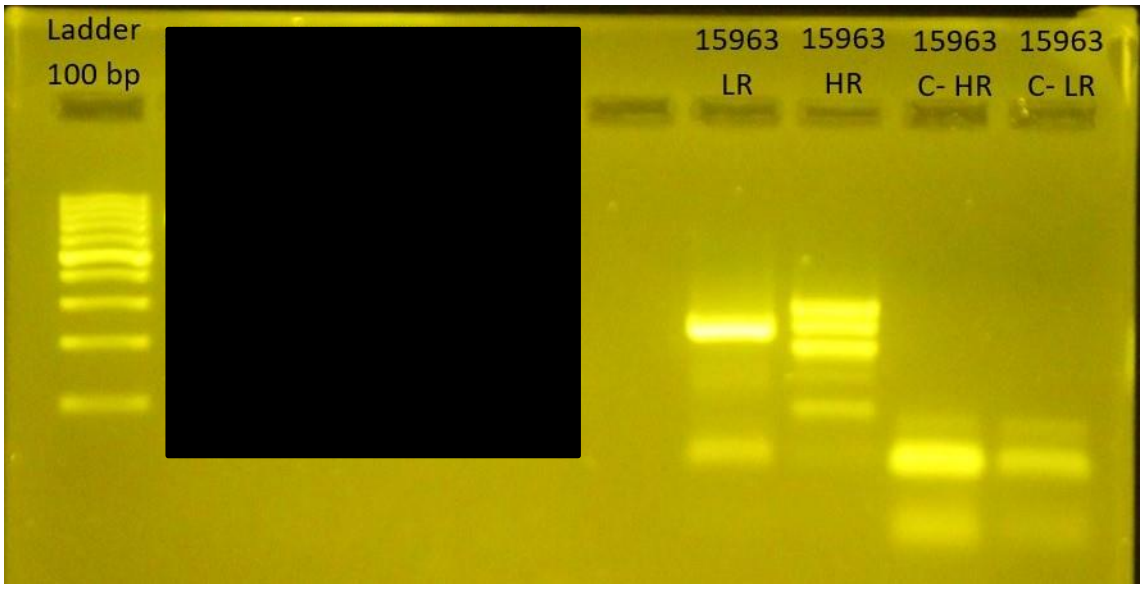


Figure 17. **Result of gel electrophoresis technique:** On the left, ladder of 100 bp. On the right, 15963 LR (low risk) 15963 HR (high risk) are samples from this study. 15963 C- HR and C- LR correspond to the negative controls of high and low risk.

DNA samples should have at least one band and negative controls should have one strong band and other weak bands. The good performance of the PCR is confirmed and we can proceed to the next step.

4.2.8. Hybridization of PCR product using Auto-LIPA robot

As it is detailed before, the reagents and materials used in the hybridization process are the Papilloma Strip Test for HPV screening from Operon and the Auto-LIPA robot. This technique allows us to detect if there is an HPV infection and if it is the case, the type of such HPV infection. Auto-LIPA robot is an automatic machine capable of doing all the different steps that a hybridization needs. **Figure 18** and **Figure 19** represent the aspect of the Auto-LIPA robot in different stages of this method.

Hybridization is a natural process that consist of double-stranded DNA molecule that bind to one another, and when DNA is replicated this new strand hybridizes to the old strand. This is the basis of this technique, make small pieces of DNA designed to screen for the presence or absence of certain DNA or RNA molecules in the cell, in this case from HPV.

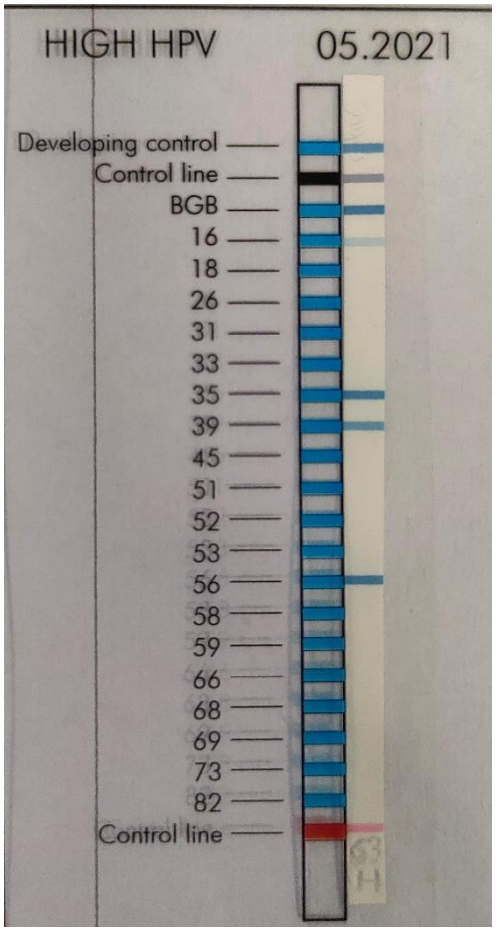
Figure 18. **AutoLIPA robot from Innogenetics**: There is a waste deposit, 6 dispensing tubes with distilled water bottles, two water tanks and the top part reserved for the tray with the nitrocellulose strips.

First of all, this robot needs cleaning before and after use. The 6 dispensing tubes or channels are put in distilled water in order to clean the inside of the tubes. Once the cleaning process is done the remain water drops to the waste deposit. Then, the different tubes are removed from the distilled water and the bottles are stored for the final cleaning at the end of the process. Next step is to put in the correct order the different reagents from the Papilloma Strip kit from Operon as it is detailed in section 3.2.3.4. First channel will be for Hybridization Buffer, second channel for Wash buffer 1, third channel for Wash Buffer 2, fourth channel for conjugate, fifth channel for distilled water and the last channel for substrate. Once the different reagents are prepared, the machine needs to heat the first and second channel for a correct performance of the technique that lasts more or less 30 minutes. This is a good moment to prepare the tray with the nitrocellulose strips with our samples and their negative controls.

Figure 19. AutoLIPA dispensing tubes + reagents: Different dispensing tubes from AutoLIPA robot with their corresponding reagents.

The first step to prepare the tray is to temper at room temperature the denaturant and samples. In the meantime, it is a good moment to label the tray and the nitrocellulose strips. In the kit there are two types of nitrocellulose, one from the low-risk primers and another for the medium/high-risk primers. In this case, for each sample there will be two strips and a negative control for low risk and also for high risk. The strips are labeled also in pencil in order to avoid losing traceability of the samples. Once the strips and the tray are labeled, it is time to place the strips in each position of the corresponding trays with the help of tweezers. Afterwards, 12.5 µl of denaturant are added at the top of the bucket. It is crucial that the drop of denaturant does not touch the strips. Then, it is time to add 12.5 µl of PCR product in each position. This step is delicate since the samples must be homogenized with the denaturant by slowing mixing up and down with a micropipette in order to avoid DNA contaminations in the other positions of the tray. Finally, it is left to incubate at room temperature for 10 minutes (**Figure 20).**

Figure 20. **Hybridization method:** On the left, the aspect of the tray with the different strips and their correspondent mixture of denaturant + PCR product (green color). On the right, me performing this step.



Once the incubation time is finished, the tray is placed on the top of AutoLIPA and the lid is lowered to avoid contaminations. The total time of the hybridization is approximately 2 hours and 15 minutes. At the end of the process, the tray is removed from the robot and the nitrocellulose strips are removed from the tray and are placed between papers to dry them. Finally, the strips are sticked to a paper to interpret the result of the amplified strains. With the help of low and high risk plasticized guides. It must be verified that negative control is not contaminated with either human DNA or any HPV strain as well as the intern controls of the strips **(Figure 21).**

Figure 21. **Nitrocellulose strip final result**: the different bands and the plasticized guide to interpret the HPV strains. In this case, this sample is positive for strains HPV-16, HPV-35, HPV-39 and HPV-56.

4.3. Statistical methods

The statistical methods used in the present study are univariate descriptive statistics, numerical summaries and graphics in order to represent all the data obtained throughout the project.

5. Results and discussion

Before showing the results and their corresponding analysis, I have to put in context the project limitations and weaknesses that have been presented along my stay in the project.

This is a project with an estimated duration of two years. I started at the beginning and it was supposed to be an average of 20 samples per week. However, sample recruitment at the beginning was quite low. Samples were coming from patients of *Hospital Sagrat Cor* and it was obviously optional to take part in this study. In the present project, a total of 44 samples (14 of them from the Hospital Sagrat Cor and 30 from routine patients of ADN Institut) were used.

So, the following results are not definitive and they can be considered as a first approach to the evolution that maybe it will have two years from now. Hence, the analysis of the results is elaborated with the data available and the total number of individuals is 44 for samples analyzed and 14 for the total number of satisfaction questionnaires answered.

It is also important to remark that on the analysis of samples there is no information about patient's age. In this way, the analysis of results will be based only on the possible HPV infections, ignoring the ages of patients.

Due to the small set of data that a sample of volunteers has provide, it does not allow to go much further than making a descriptive analysis of this data. Selection methods vary greatly in ease and cost-effectiveness. The effects of selection factors associated with subjects' recruitment into studies can introduce bias and seriously limit the generalizability of results [12]. As this project have not a random sample of the desired population under study, it is not suitable to introduce inferential statistics.

5.1. Validation of Evalyn Brush by satisfaction questionnaire

The total answers in the satisfaction questionnaire are 14 (n = 14). It should be noted that this is not a significant and representative sample due to the low number of answers. However, it can be useful to study the behavior of the acceptance by patients of the Evalyn Brush and observe if there is any trend in favor of using the device.

All the questions from this questionary are described in the **Appendix.**

First question to analyze is: "Are you used to using a tampon? "(**Figure 22)**. In this question, there are four different answers; Yes, No, Sometimes and Always. To avoid redundancies, answers Yes, Sometimes and Always have all been brought together in a single answer of yes. In this way, there will be only yes and no answers for this question. This is due to an incongruity in the questionnaire and will be modified to continue with the study.

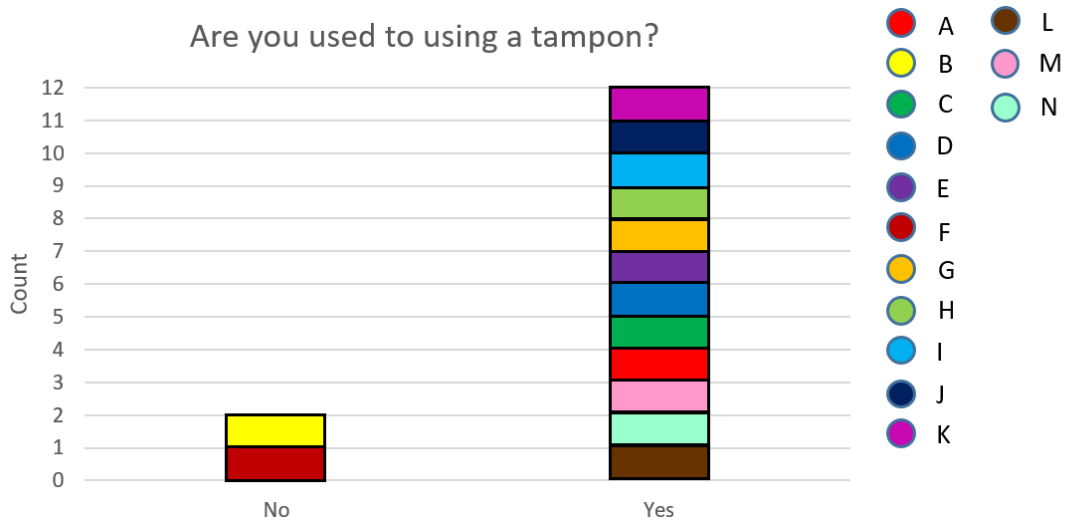


Figure 22. **Satisfaction questionnaire 1:** Representation of every answer for the question; "Are you used to using a tampon?" by their corresponding age. (n = 14).

As it can be observed, the majority of women are used to use a tampon. Just women B and F have answered no. These results will be useful when compared to the following question to study.

Next question to study is: "In general, how did you find the performance of the device?" **(Figure 23)**. This question has three different answers; Difficult, Easy and Very easy. Now we will be able to determine the ease of using the device, the relationship between being used to using tampons and how they find the device.

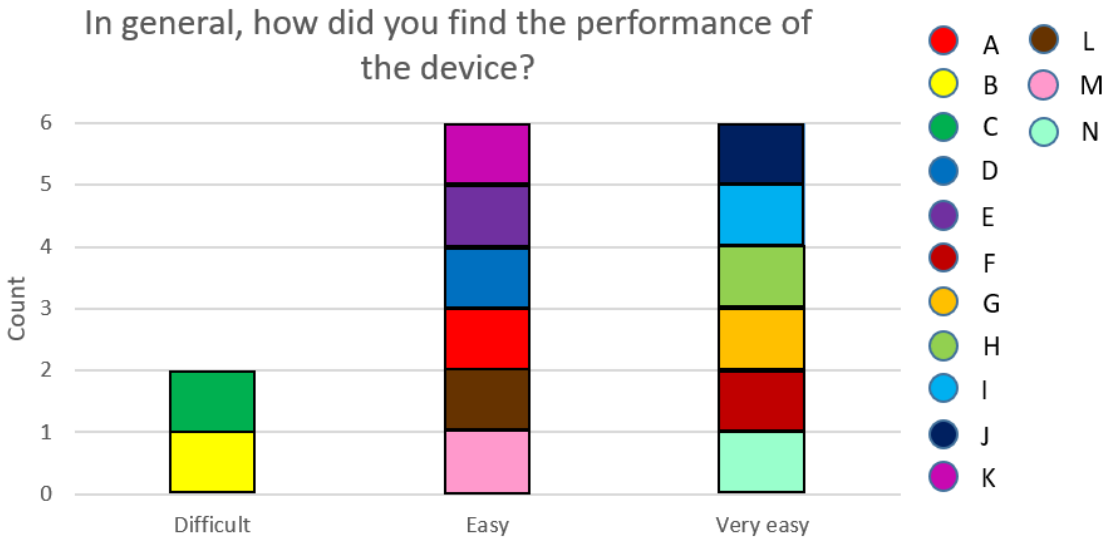


Figure 23. **Satisfaction questionnaire 2:** Representation of every answer for the question; "In general, how did you find the performance of the device?" by their corresponding age. (n = 14).

As it can be observed, woman B who have answered that they are not used to using tampon is the same who considered the usage of the device to be difficult. It can be related to the fact that she is not used to using it and therefore it becomes more difficult to use the Evalyn Brush. Woman C said that it was difficult but in the other question answered that she was used to using tampons. This is something to take into account, when the study will reach the number of samples wide enough to draw some conclusions, it will be necessary to see if this phenomenon is repeated or if they are separated cases.

In addition, woman F who answered that she is not used to tampons found it very easy to use the device. This is the contrary case of the above commented, so the same as the other, this it could be a single case or maybe at the end there is a tendency of this behavior. Either way, the fact that a person who is not familiar with such devices/operations finds that it is

a very easy device to use, indicates that the steps to follow for its operation are clear enough and easy to follow them.

The third and fourth question to analyze from the satisfaction questionnaire are; "Confidence that the test was performed correctly" with these answers; Medical sampling, self-sampling, both and NS/NC and "Which screening was easier for you to perform?" with these answers; Doctor, self-sampling and NS/NC. Answers with NS/NC mean that they did not answer this question for some reason. Women are forced to give a single answer, so that is why there is the option to put both in the questionnaire. Percentages of women's answers of these two questions have been carried out **(Figure 24) (Figure 25).**

This analysis will help understand if women who used Evalyn brush are confident about whether the sample collection has been carried out correctly and also to know if they consider this self-sampling method easier than the typical cytology performed by the doctor.

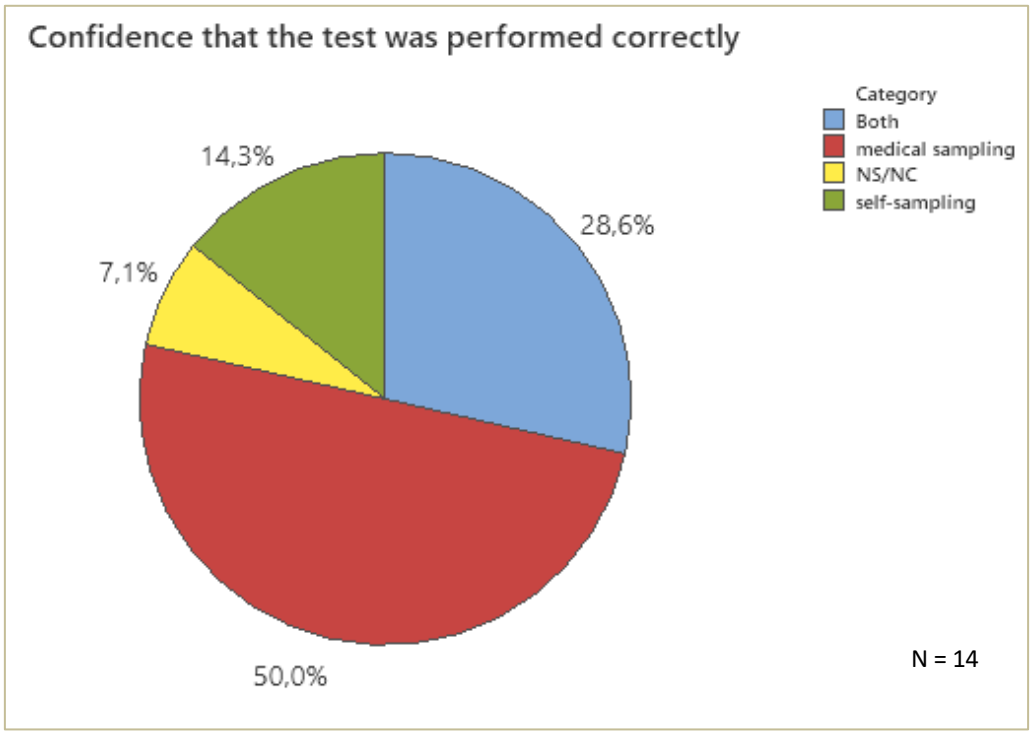


Figure 24. **Satisfaction questionnaire 3:** Circular graphic representing the answers form the question; "Confidence that the test was performed correctly". (N = 14).

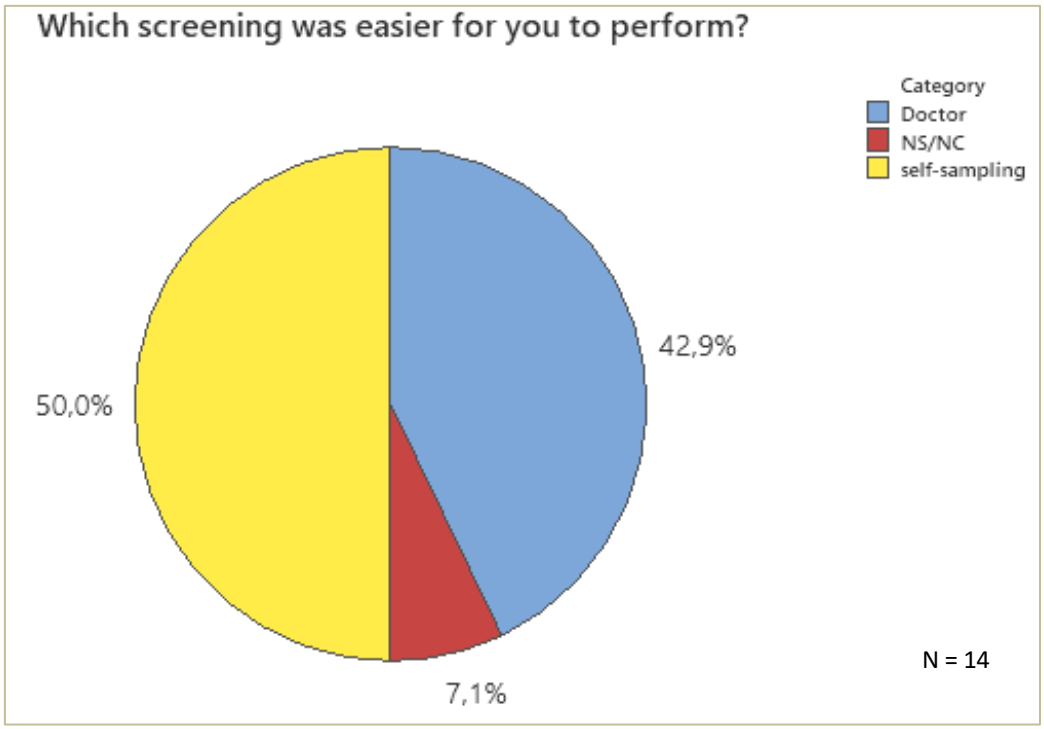


Figure 25. **Satisfaction questionnaire 4:** Circular graphic representing the answers form the question; "Which screening was easier for you to perform?". (N = 14).

Due to the low quantity of answered questionnaires collected (14), we cannot stand in the percentages as they are not significant but an overall assessment can be done. In **Figure 24,** it can be observed that the half of the women consider that they have more confidence on the medical sampling (cytology). However, a great part of the population consider that they have more or the same confidence using the self-sampling method. These results are the expected as Evalyn Brush is something new and has not been tested in this country compared to countries such as the Netherlands. In a Randomized Controlled Trial (RCT) performed in the Netherlands, a total of 26409 women had a PAP test after an individual invitation and then a reminder of invitation was sent to them again in a letter for regular cytology screening or an HPV self-sampling kit at home. The response rate in the group who received the self-sampling kit was significantly increased compared to the group who received reminder invitation for a PAP test [13]. These types of results give evidence of the confidence that women have in self-sampling devices instead of a regular cytology.

On the other hand, in **Figure 25,** the majority of women have found self-sampling easier than the cytology performed by the doctor. This can have some factors involved such the sensation of pain, the feeling of privacy or shame among others. Each woman presents different feelings and emotions during the performance of both methods and by consequence the results of these sensations do not follow a common behavior.

To sum it all up, the Evalyn Brush can be validated as a good device for the screening of HPV in this first step of the study, and the evolution of the device acceptance grows positively due to the good feelings that women reflected in this first satisfaction questionnaires. In general, it is an easy-to-use device according to the assessments of the women who have used it.

5.2. Validation of Evalyn Brush as sample collector and genomic DNA extraction to obtain an optimal DNA concentration.

As discussed above, gel electrophoresis started a few weeks after starting to analyze the samples. Therefore, the evaluation of valid samples was determined from a range of DNA concentration, a cut-off value of 20 ng/µl which, if not exceeded or equaled, was considered an invalid sample.

The percentage of valid samples has been qualified (indicator of how many patients collected their samples correctly). In the present project, there was different condition samples which had to be taken into account as they may have presented problems when performing the DNA extraction or the quantification of DNA.

These different conditions could be:

- The brush from the self-sampling device is clean and with clear visible signs of biological sample on it. The procedure of this type of samples is normal and no preventive and corrective action has been taken
- The brush from the self-sampling device is clean but there are no clear visible signs of biological sample on it. The procedure of this type of samples is to transfer them into falcon tubes in 3 ml of PBS instead of 5 ml. This is done to concentrate the sample on the PBS and achieve an optimal concentration to perform the PCR
- The brush from the self-sampling device shows signs of blood. It is known that blood can affect in the DNA extraction protocol since the blood could inhibits the reaction of digestion. The procedure of this type of sample is the same as in the previous one. In addition, in the DNA extraction explained in section 4.2.4, more than one centrifuge is performed to ensure that all the content pass through the silica-based column.

If the DNA concentration obtained is higher or equal to 20 ng/µl (this value is the minimum to ensure a good PCR), then the sample is considered valid.

The method to determine the DNA concentration is the Qubit 4 explained in section 4.2.5. So, 100% of the total samples analyzed (N=44) are considered valid as they have all exceed the 20 ng/µl using the lectures from the Qubit. Even with samples with less biological sample than normal or those with blood, Evalyn Brush has been performed well enough.

To put some context into the results obtained, the Evalyn Brush bibliography corroborated that with Evalyn Brush, the large-scale self-sampling implementation performed in Denmark showed a valid sample in 99.69% of the cases [14]. In the present project, this value has been the 100% of the total samples that have arrived at the laboratory.

5.3. Screening of HPV strains using High+Low PapillomaStrip kit

In this section, what is meant to do is interpret and describe all the different results obtained. In this way, a better interpretation of the results will be performed, being able to know the total positives and negatives samples in every oncogenic risk type, the most frequent HPV genotypes in positives samples and the most frequent oncogenic risk types in the total samples analyzed. In addition, a comparison with another study using a similar protocol will be carried out.

After performing the DNA extraction and quantification and PCR, a gel electrophoresis is carried out to determine if the methodology described has been successful. However, at the beginning of the study, gel electrophoresis was not performed as it was thought that it was not necessary. It was a few months later that gel electrophoresis began to be done. This is the reason to have one single gel electrophoresis result as it is represented in **Figure 17.** Once the gel electrophoresis result is considered correct, samples can proceed with the methodology. Final step will be the interpretation of nitrocellulose strips.

As it is mentioned before, the final number of samples analyzed is 44. The nitrocellulose strips are photographed in order to have a multimedia test of the results and also to upload them in Zendo LIMS **(Figure 26).**

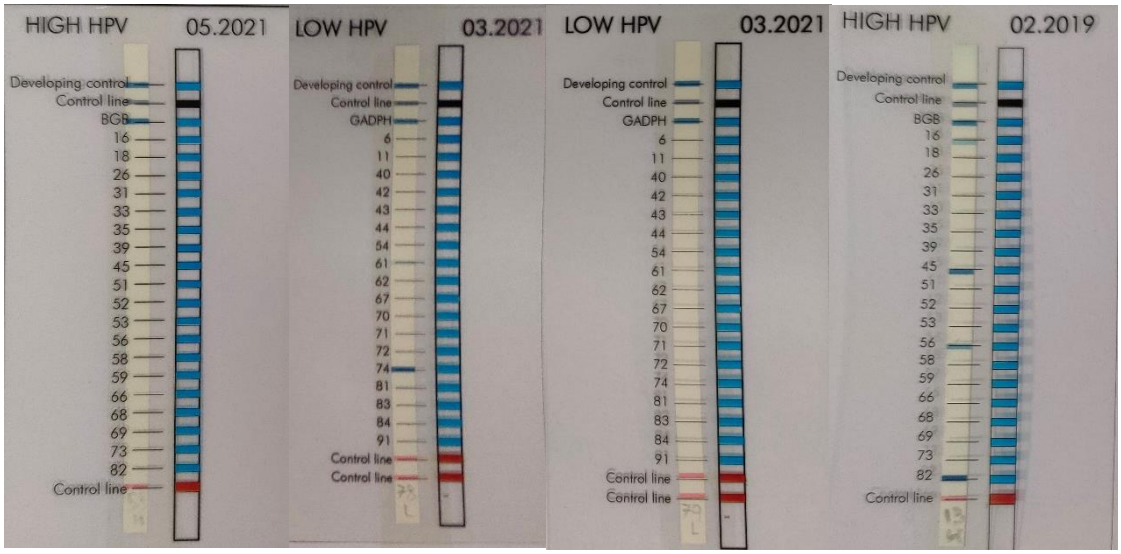


Figure 26. **Results from different nitrocellulose strips**: From left to right; Negative for high-risk, Positive for low-risk (HPV-61, HPV-74 strains), Negative for low-risk, Positive for high-risk (strains HPV-16, HPV-43, HPV-56 and HPV-82).

To keep track of the results obtained they are registered in a computer, which will be very useful when the interpretation of them will be carried out. In this case, with the 44 samples analyzed and registered in the computer, a representation of the results of every sample indicating if it is positive or negative has been made **(Table 6)**. As previously mentioned, there are only 14 samples coming from the study and all of them are complete test, which means that the analysis is made for medium-high risk and also low-risk strains. However, there are some samples that only medium-high risk strains have been analyzed. This is because some of the private tests that patients have done with ADN Institut only covered the medium-high risk with the money that they paid. That is the reason that there is N/A (No Apply) in the low-risk column.

Table 6. Results of the total samples analyzed.

Individuals	High risk	Medium risk	Low risk
#1	Negative	Negative	Positive (42)
#2	Positive (31,39,51,59)	Positive (53)	Positive (84)
#3	Positive (31,51,59)	Positive (53)	Positive (43,84)
#4	Positive (39,45,58)	Positive (66,67)	Positive (6,43,54,84,91)
#5	Positive (35)	Negative	Positive (54,74)
#6	Positive (51)	Negative	Positive (61)
#7	Positive (51,56)	Positive (68)	Positive (54,74)
#8	Negative	Negative	N/A
#9	Positive (45)	Negative	N/A
#10	Negative	Negative	N/A
#11	Positive (56)	Negative	N/A
#12	Negative	Negative	Negative
#13	Negative	Negative	N/A
#14	Negative	Negative	N/A
#15	Negative	Negative	Negative
#16	Negative	Negative	N/A
#17	Negative	Negative	N/A
#18	Negative	Negative	Negative
#19	Positive (59)	Negative	Positive (62)
#20	Positive (51,59)	Negative	Positive (74)
#21	Negative	Negative	Negative
#22	Negative	Negative	N/A
#23	Negative	Negative	N/A
#24	Negative	Negative	N/A
#25	Negative	Negative	N/A
#26	Positive (16,52,56)	Positive (68)	Positive (42,43,54,62,74)
#27	Positive (56)	Negative	Positive (61)
#28	Negative	Positive (67)	Positive (42,54)
#29	Negative	Negative	Negative
#30	Negative	Negative	Negative
#31	Positive (59)	Negative	Negative
#32	Positive (39,51,52,56,59)	Positive (53,73)	Positive (54)
#33	Negative	Positive (68)	Positive (42,74)
#34	Negative	Positive (66)	Positive (42,74)
#35	Negative	Negative	Positive (44)
#36	Negative	Positive (53)	Negative
#37	Positive (56)	Negative	Positive (42)
#38	Positive (16,45,56)	Positive (82)	N/A
#39	Positive (18,56)	Negative	N/A
#40	Positive (59)	Positive (68)	N/A
#41	Positive (58)	Negative	Positive (6,61,74,81)
#42	Negative	Negative	Negative
#43	Positive (52)	Positive (70)	Positive (62)
#44	Positive (16,35,39,56)	Positive (67,70)	Positive (6,54,84)

A representation of all the results from all the samples with their corresponding risk test has been made **(Figure 27).**

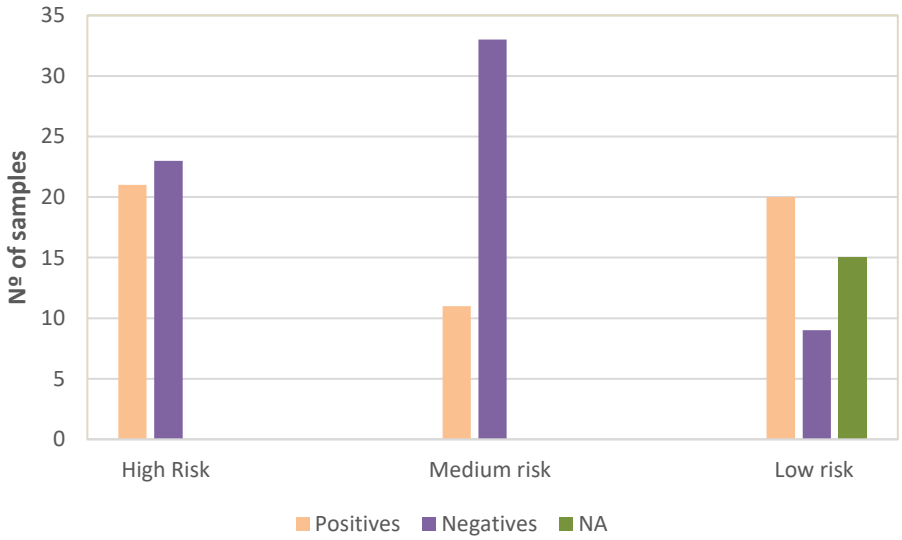


Figure 27. Graphic representation of the total samples analyzed: Summary of the results by counting positives, negatives and NA of every different oncogenic risk tests. (N = 44)

From all the 44 total samples, 21 are positives and 23 negatives for high risk. In an orientate way, at the end of the study, more or less 50 % of the total samples will be positives for at least one high risk strain.

About 33 of samples have tested negative and 11 positives in the case of medium risk test.

At low-risk we found 20 positives, 9 negatives and 15 NA. This last comes because not all the private tests performed at ADN Institut are complete (which means high+low test), sometimes patients want to know if there is some trouble with the infection of the most carcinogenic HPV strains (high risk) and they are not interested in the low-risk strains.

The main idea is to group the total positive samples in this project and see the prevalence of the total strains analyzed with the PapillomaStrip kit.

By doing this, we know the frequencies of the strains with positive result and also which ones are the most common among the total samples analyzed **(Figure 28)**. It is important

to emphasize that there are only 44 samples and the number is low to make strong and definitive conclusions. This analysis will be useful to determine the first steps and the evolution or trend that the results follow from this first analysis until the end.

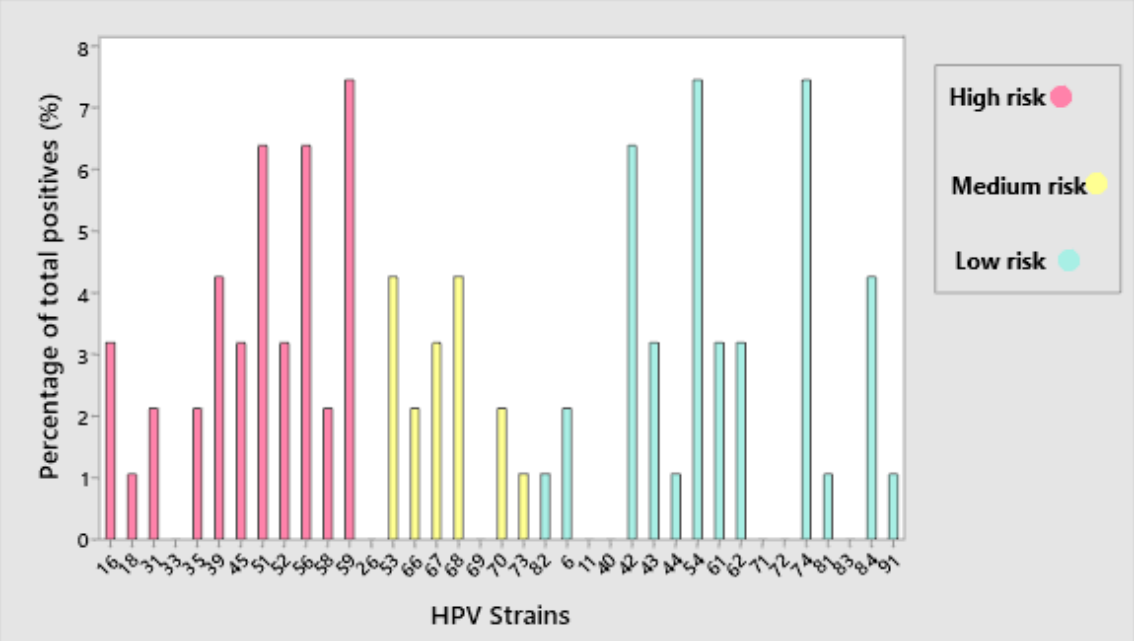


Figure 28. Percentage of every HPV strain of the total positive's samples. (N = 44)

The most frequent strains are HPV-59, HPV-54 and HPV-74. It is quite important to take into account that one of them (HPV-59) is a high oncological risk strain, which means that the probability of having cervical cancer increases. At this point, it is very recommendable to visit the gynecologist as soon as possible. As this graphic shows, more than 7 % of the total positive samples are from a high-risk strain. However, the other most common strains are low oncological risk. This type of HPV variant is less urgent to treat but it should not be underestimated since this type of infection could cause genital warts or even develop cervical cancer if time passes without any medical supervision.

The most oncogenic HPV strains are HPV-16 and HPV-18. The results are a 3.25 % of the total positives from strain HPV-16 and 1 % from strain HPV-18. These two genotypes are the ones with the most oncological risk in humans. That is why these type of studies and experiments are important, because most of the women who have participated in this

study were unaware that they had this infection and will now be able to go to their medical center and try to find the best solution possible. So, in this first analysis of the study which the human sample is 44, it can be concluded that more than 4 % of the total positives will be infected by the strain HPV-16 and HPV-18, which is quite high due to the severity of these two infections.

11 of the 12 high-risk genotypes, 6 of the 9 medium-risk genotypes and 12 of the 18 low-risk genotypes have been detected. It is the 91.6 % of the high-risk variants detected in front of the 66.6 % of the low-risk. The hybridization kit used has a sensitivity of 100 % in every HPV genotype (high, medium and low risk). However, this kit includes all high-risk strains but does not include all the medium and low risk strains. This could be a reason for this percentages obtained.

Another point to highlight is the genotypes with such a high frequency among the total positives but do not become the ones with the highest frequencies. These are the HPV-51 and HPV-56 which corresponds to high-risk and HPV-42 which correspond to low-risk. Keeping in mind that it is a very premature phase of the study and firm conclusions cannot be drawn, as it can be seen the variants of high oncological risk do not have low percentages. In fact, if the sum of all the percentages separated by the oncological risk types is made, it turns out that high-risk genotypes are approximately the 41 %, medium-risk the 17 % and low-risk the 42 % of the total positive samples.

In order to contextualize this data and compare the incidence of different HPV genotypes in two different countries, they will be compared with another real study. This study was carried by The National Health and Nutrition Examination Survey (NHANES) [15]. The representative sample for this study is females aged 14 to 59 from the United States. The sample recruitment was done by a self-collected vaginal swap specimen, which were analyzed for HPV DNA polymerase chain reaction followed by type-specific hybridization (Roche prototype line blot assay). With such a high similarity of the method for HPV screening carried by this project and the NHANES study, a better comparison and

discussion of the results can be made. The total number of the population for NHANES project was n = 1921. The representation of the prevalence (number of cases presenting HPV infection divided by the number of individuals that make up the group or population at a given time) in all samples analyzed has been made **(Figure 29).**

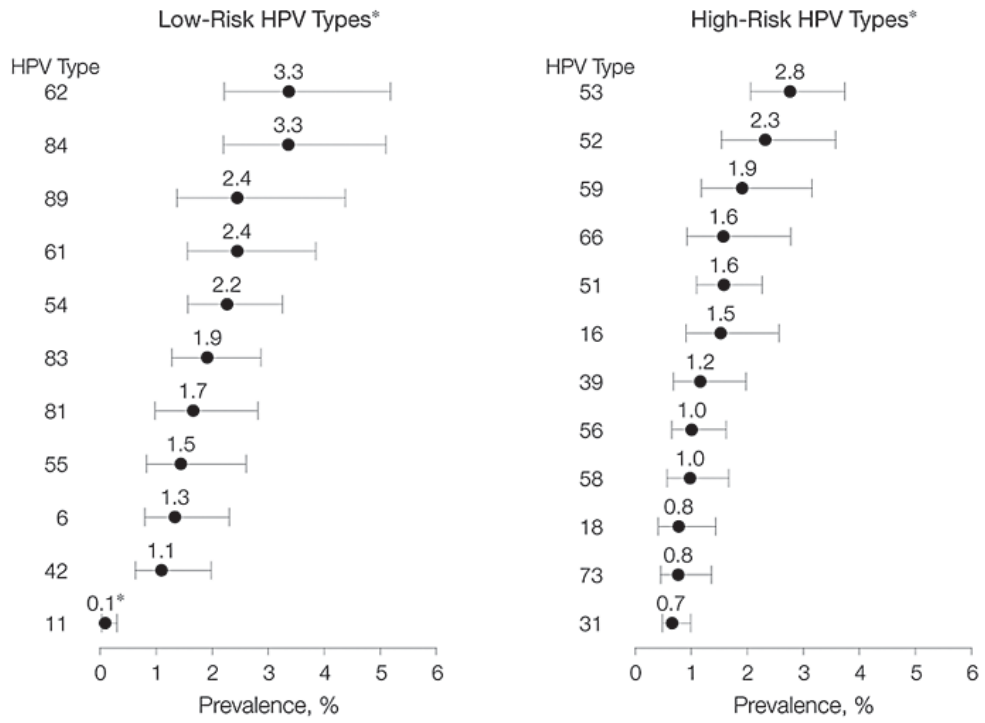


Figure 29. Prevalence of HPV types among females aged 14 to 59 years. (n = 1921) [15].

The results of NHANES study corroborated that the prevalence of high-risk HPV types was 15.2% and low-risk HPV types was 17.8%. The most common HPV types detected were HPV-62, HPV-84, HPV-53, HPV-89 and HPV-61. HPV-16 was detected in the 1.5% and HPV-18 in the 0.8% of females aged 14 to 59 years. Also, the 60.1% of the females infected with HPV had only 1 HPV type detected, 23.9% had 2 types and 16% had 3 or more types detected.

In **Figure 28**, the percentage of every HPV type of the total positives is represented, and prevalence is not represented. However, prevalence of high, medium and low-risk types can be calculated from the results shown in **Table 6.** Prevalence of high-risk types is 45.45%,

medium-risk types 31.81% and low-risk types 76.92%. The most common HPV types detected were HPV-59, HPV-54, HPV-74, HPV-51, HPV-56 and HPV-42. HPV-16 was detected in 3.25% and HPV-18 in the 1% of the total samples analyzed. In addition, the 27.27% of the females infected with HPV had only 1 HPV type detected, 15.9% had 2 types and 18.18% had 3 or more types detected.

Very significant differences between these prevalence of both studies can be observed. The difference of the total number of samples analyzed (1921 samples from the NHANES study in front of 44 samples from the present study) can be an important reason for the significative differences. However, in both studies the higher prevalence is from the low-risk HPV types. Related to the most common HPV types detected, in the NHANES study three out of five (HPV-62, HPV-84 and HPV-61) are low-risk types, one out of five is medium-risk type and HPV-89 (which the screening with the kits in which the present study was performed is not included) is low-risk as well. In the present study, three out of six (HPV-54, HPV-74 and HPV-42) are low-risk types and three out of six (HPV-59, HPV-45 and HPV-56) are high-risk types. Hence, there is a kind of behavior about low-risk types which are the ones that are more prevalent in both studies and also the types that have been detected the most. The percentage of HPV-16 and HPV-18 are especially similar in both studies despite the wide difference in the number of samples analyzed. The behavior that is deduced from the NHANES study is that those female with an HPV infection of only 1 HPV type are the ones with the highest percentage in all the samples followed by those with 2 HPV types and then those with 3 or more HPV types.

This comparison is a first approximation of the HPV incidence between two different countries. As this present project is not finished, no definitive conclusions can be made and are only based on the first results of the study.

6. Conclusions

According to the results obtained in the research and development processes, the following conclusions can be drawn:

- The commercial kit Papilloma Strip from the company Operon S.A. for the screening of high, medium and low risk HPV genotypes seems to be effective. With this kit, 44 samples have been successfully analyzed.
- The acceptance of the self-sampling device Evalyn Brush by the patients who have been part of the study (the number of satisfaction questionnaires filled out is 14), has generally been positively assessed.
- Evalyn Brush has been validated in the process of obtaining biological samples and also to obtain an optimal DNA concentration to carry out the PCR. All samples tested have given a DNA concentration higher than the minimum to perform the PCR.
- Evalyn Brush has been validated in the manual genomic DNA extraction using PureLinkGenomic commercial kit from the company Invitrogen.
- The first approach of the comparison between the present study and an NHANES study has been useful to evaluate the similarities and differences between HPV incidence between two different countries. Definitive conclusions cannot be drawn due to the low number of samples analyzed in the present study.

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8. Appendix: Satisfaction Questionnaire

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VPH test control

CUESTIONARIO DE SATISFACCIÓN

1. Características generales

a) Edad: ____

b) Educación
- ☐ Escuela Primaria Obligatoria
- ☐ Escuela Secundaria Obligatoria
- ☐ Bachillerato
- ☐ Grado de formación profesional
- ☐ Universidad o más

c) ¿Ha tenido hijos?
- ☐ Sí
- ☐ No

d) ¿Está familiarizada con el uso de tampón?
- ☐ Sí
- ☐ Siempre
- ☐ A veces
- ☐ No

e) Edad primera relación sexual: ____

2. Características del dispositivo

En base a su experiencia con el dispositivo:
a) ¿Cómo encontró la longitud del dispositivo?
- ☐ El dispositivo era demasiado largo
- ☐ El dispositivo presentaba la medida justa
- ☐ El dispositivo era demasiado corto
- ☐ Otras

b) ¿Cómo encontró el tamaño del dispositivo?
- ☐ El dispositivo era demasiado grande
- ☐ El dispositivo presentaba la medida justa
- ☐ El dispositivo era demasiado pequeño
- ☐ Otras

c) En general, ¿cómo encontró el funcionamiento del dispositivo?
- ☐ Muy fácil de usar
- ☐ Fácil de usar
- ☐ Difícil de usar
- ☐ Muy difícil de usar

3. Hoja de instrucciones

En referencia a la interpretación de la hoja de instrucciones, usted las encontró:
- ☐ Muy fáciles de entender
- ☐ Fáciles de entender
- ☐ Difíciles de entender
- ☐ Muy difíciles de entender

4. Test de cribado

4.1 Comparando las sensaciones que ha vivido durante el estudio, con qué cribado experimentó más las siguientes emociones:

a) Vergüenza
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

b) Dolor
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

c) Incomodidad
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

d) Molestias
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

e) Relajación
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

f) Confianza de que el test se realizó correctamente
- ☐ Durante el muestreo médico
- ☐ Durante el auto-muestreo

4.2 En base a su experiencia con el estudio:
a) ¿Qué cribado le resultó más fácil de realizar?
- ☐ El realizado por el médico
- ☐ El realizado durante el auto-muestreo

b) ¿Estaría dispuesta a realizarse la toma de muestra en el futuro?
- ☐ Definitivamente sí
- ☐ Seguramente sí
- ☐ Seguramente no
- ☐ No

5. Grado de conocimiento en la prevención del cáncer

5.1 ¿Conoce otras campañas de detección precoz de cáncer?
- ☐ Sí
- ☐ No

5.2 ¿Considera la detección precoz de cáncer de cuello de útero conveniente?
- ☐ Sí
- ☐ No

5.3 ¿Conoce la citología cervical o prueba de Papinocolau?
- ☐ Sí
- ☐ No

5.4 ¿Sabe que cérvix es sinónimo de cuello uterino?
- ☐ Sí
- ☐ No

5.5 ¿Conocía el virus de papiloma humano?
- ☐ Sí
- ☐ No

5.6 ¿Conocía que el virus de papiloma humano es causante de cáncer?
- ☐ Sí
- ☐ No