HIV DETECTION BY RT-PCR

Ref. PCRHIV (4 practices)

1. EXPERIMENT OBJETIVE

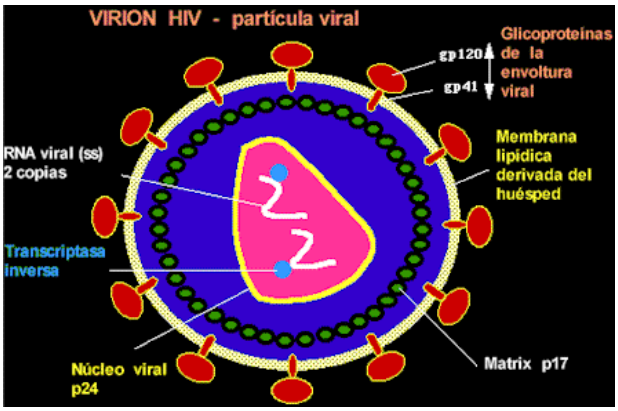
The objective of this experiment is to introduce students to the principles and practice of Polymerase Chain Reaction (PCR) as a tool for the detection of Human Immunodeficiency Virus (HIV) by PCR.

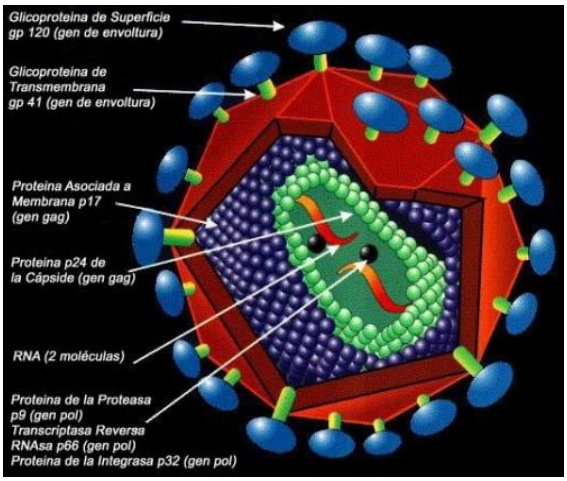
Students will acquire basic knowledge about HIV and the disease it produces, AIDS.

2. BACKGROUND INFORMATION

2.1 HIV

The **human immunodeficiency virus (HIV)** is a lentivirus (from the family Retroviridae), which causes **acquired immunodeficiency syndrome (AIDS)**.





It is formed by a spherical particle of 80-100 nm with a structure in two layers:

- The envelope is a lipid bilayer derived from the host cell where the Glycoproteins are inserted with 72 external projections. It contains the viral proteins Gp120, Gp41 and Gp17.
- The core nucleocapsid or core within which the genetic material and enzymes necessary for viral replication are found.

The genome is a single-stranded RNA made up of 2 identical strands of positive polarity. There are genes responsible for coding the components of the viral particle (structural genes) and regulating the expression of them (regulatory genes). Of the structural genes the GAG gene encodes the core proteins, the POL gene fundamentally encodes the Inverse Transcriptase and Protease and the ENV gene proteins from the viral envelope.

2.2 The disease: AIDS (Acquired Immunodeficiency Syndrome)

It is a disease caused by the **human immunodeficiency virus (HIV)**. The disease destroys the immune system gradually, which makes it more difficult for the body to fight infections.

AIDS is the sixth leading cause of death in people aged 25-44 in the United States, but in 1995 it ranked number one. Millions of people around the world are living with HIV/AIDS, including many children under the age of 15.

Common bacteria, fungi, parasites and viruses that do not usually cause serious illness in people with a healthy immune system can cause deadly diseases in people with AIDS.

HIV has been found in saliva, tears, nerve tissue, cerebrospinal fluid, blood, semen (including pre-fluid, which is the fluid that comes out before ejaculation), vaginal discharge, and breast milk. However, it has been shown that only blood, semen, vaginal flows and breast milk transmit the infection to other people.

Symptoms of AIDS

AIDS begins with an HIV infection. People infected with HIV may not have symptoms for 10 years or more, but may transmit the infection to others during this asymptomatic period. If the infection is not detected and treatment is not started, the immune system gradually weakens and AIDS develops.

Acute HIV infection progresses over time (usually a few weeks to months) to asymptomatic HIV infection (without symptoms) and then to early symptomatic HIV infection. Subsequently, it progresses to AIDS (advanced HIV infection with CD4 T-cell count below 200 cells/mm^3).

Almost all people infected with HIV, if not treated, will contract AIDS. There is a small group of patients in whom AIDS develops very slowly or never appears. These individuals are called patients without progression of the disease and many seem to have a genetic difference that prevents the virus from causing damage to their immune system.

The symptoms of AIDS are mainly the result of infections that do not usually develop in people with a healthy immune system. These are called **opportunistic infections**. In people with AIDS, HIV has damaged the immune system, making them very susceptible to such opportunistic infections.

Common symptoms are:

- Chills
- Fever
- Rash
- Sweating (particularly at night)
- Swollen lymph nodes
- Weakness
- Weight loss

Note: In the beginning, HIV infection may not produce any symptoms. However, some people do experience flu-like symptoms with fever, rash, sore throat, and swollen lymph nodes, usually 2 to 4 weeks after contracting the virus. This is called acute retroviral syndrome. Some people with HIV remain for years without symptoms between the time they are exposed to the virus and when they develop AIDS.

AIDS transmission routes

The virus can spread (transmit):

- Through sexual contact: including oral, vaginal and anal sex.
- Through blood: via blood transfusions (now very rare) or for sharing needles.
- From mother to child: A pregnant woman can transmit the virus to her fetus through shared blood circulation, or a nursing mother can pass it on to her baby through breast milk.

Other methods of virus spread are infrequent and include accidental needle injury, artificial insemination with infected donated semen, and transplants of infected organs.

HIV infection is not spread by:

- Casual contact as a hug
- Mosquitoes
- Participation in sports
- Touching things that have been touched previously by a person infected with the virus
- AIDS is not transmitted to a person who **DOES** blood or organs. People who donate organs never come into direct contact with recipients. In the same way, someone who donates blood never has contact with the recipient. Sterile needles and instruments are used in all these procedures.

However, HIV can be passed on to the person **RECEIVING** blood or organs from an infected donor. To reduce this risk, blood banks and organ donation programs conduct minute examinations of donors, blood, and tissue.

Among those at highest risk for HIV are:

- Drug addicts who share needles to inject drugs
- Infants born to mothers with HIV, when not receive HIV treatment during pregnancy
- Persons engaged in unprotected sex, especially with individuals who have other high-risk behaviours, who are HIV positive or have AIDS
- People who received blood transfusions or blood products between 1977 and 1985 (before screening for the virus became common practice)
- Sexual partners of people who engage in high-risk activities (such as injecting drug use or anal sex)

Diagnosis of AIDS

In most cases immunoenzymatic techniques (EIA, ELISA) are used in **a blood sample**. If the result is positive, a more specific technique is used to confirm the result, with Western Blot being the most commonly used method.

In addition, lymphocyte counts are performed to detect the involvement of the immune system, and since 1995 a technique has been developed, **demonstrating the viral genome using molecular biology techniques (PCR)**, which allowed the measurement of the amount of HIV virus in the blood, which in turn is a reflection of the amount of virus that exists in the whole organism.

Treatment of AIDS

At this time, there is no cure for AIDS. However, several treatments are available that can help keep symptoms at bay and improve the quality and length of life of those who have already developed symptoms.

When applying the PCR technique for HIV frozen blood samples from 10 years or more before it was seen that of those with very few virus (low viral load), only 10% had developed AIDS, whereas people who had large numbers of virus (high viral load) in the blood had developed AIDS and died mostly.

Up to 1995, a series of drugs called Viral Inverse Transcriptase Inhibitors (RETROVIR, VIDEX, HIVID) were available which, either separately or in combination, had a weak and transient effect on the HIV virus, delaying the onset AIDS in an infected person at most 2 years; if used in the AIDS phase delayed death in 1 or 2 years.

This is because the virus is able to become resistant to these drugs because it is changing (mutating) each time it reproduces (replicates); Of course, people who have a lot of viruses have a higher rate of replication (and resistance) and the prognosis is worse than if they have few viruses. It was also seen that with the above drugs it was possible on average to divide by 10 or 50 the amount of virus from the blood, which, in a person who had, for example, 300,000 viruses per millilitre, is an insignificant and insufficient reduction to prevent progression to AIDS.

A series of drugs called **Viral Protease Inhibitors** (NORVIR, INVIRASE, CRIXIVAN) appeared in 1995-1996, which, in combination with the above, are able to divide the viral load by 1,000 or more; in some patients they succeed in making these viruses disappear from the blood and, keeping the treatment several years, may perhaps completely eliminate the virus from the organism.

The experience with two years of use is very good, with reductions of mortality of more than 50%, recovery of the lost T4 lymphocytes and marked improvement of the symptoms of the disease.

These treatments are relatively well tolerated and are administered by mouth (they do not need to be injected). Although these treatments are very expensive (a protease inhibitor plus two transcriptase inhibitors suppose a year more than 6000 euros), in Spain they are covered by the Social Security and, above all, the lives saved and the saving that they produce in expenses hospitalizations amply offset their economic value.

Prevention of AIDS

Sexually:

- Having sexual abstinence (not having sex)
- Through the practice of safe sex, that is to say, without penetration (kissing, caressing, hugging, autoeroticism or masturbation)
- Using a condom in every sexual relationship

By blood:

- Using blood and derivatives that have been previously analyzed and are free from virus
- Recommending Injectable Drug Users to use a new needle and syringe in each application wash and/or boil them.
- Using latex or polyurethane gloves whenever blood or secretions are handled body fluids

Perinatally:

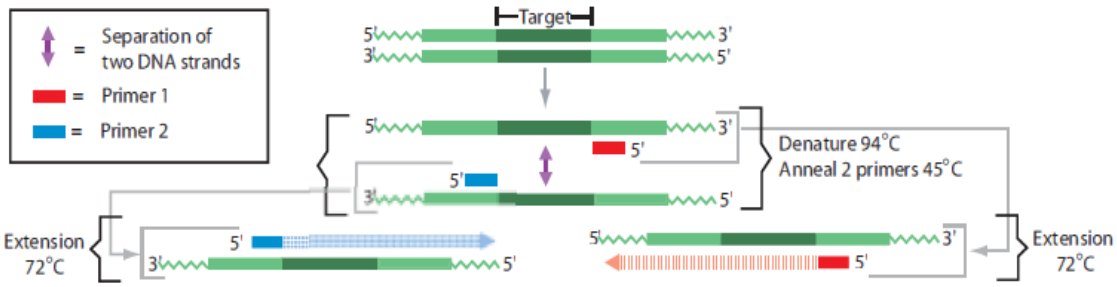
- Offering 100% HIV screening to pregnant women, free of charge, voluntary and confidential in health services throughout the country.

2.3 RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) is a variant of PCR, a laboratory technique commonly used in molecular biology to generate a large amount of DNA copies, a process called "**amplification**". In RT-PCR, however, a strand of RNA is retrotranscripted into complementary DNA (cDNA) using an enzyme called **reverse transcriptase** and the result is amplified in a traditional PCR. **PCR in Reverse Transcription** should not be confused with real-time PCR, quantitative PCR or Q-PCR, which is sometimes mistakenly abbreviated as RT-PCR.

Exponential amplification by PCR in Reverse Transcription is a highly sensitive technique, which can detect a very low RNA copy number. The main uses of RT-PCR are related to the field of **Molecular Diagnosis** and to scientific research. It can be used as a method of molecular gene detection to study the genome of RNA viruses such as retroviruses (such as HIV) or influenza virus (influenza virus).

One of the most important features is that in the RT-PCR process, the generated cDNA no longer carries the introns that would have the original DNA. Thus, in expressing the cDNA product of the RT-PCR, a mRNA formed exclusively by exons will be generated. This has enabled this technique to be one of its most important uses: to insert eukaryotic genes into prokaryotic organisms.



These three steps, denatured-annealing-extension, constitute a **PCR cycle**. This process is repeated for 20-40 cycles by amplifying the object sequence exponentially. The PCR is performed on a **thermocycler**, an instrument that is programmed for rapid heating, cooling and maintenance of the samples for several times. The amplified product is then detected by removal of the reaction mixture by agarose gel electrophoresis.

In this practice a simulated PCR will be performed since the instrument to carry out the PCR has a very high cost, for that it will be used NON TOXIC dyes that will migrate in the agarose gel as if they were of the amplified DNA fragments.

3. STATEMENT OF FACTS

Pedro Polimerasa has had several heterosexual relationships without any protection and with unknown people, which is why he decided to have an AIDS test. Blood was drawn and tested positive for ELISA.

The next step they are going to do at the Hospital Nuestra Señora de E. coli where they take their case is a PCR to confirm if it is definitely a positive result.

They use a commercial kit of the house NORGEN BIOTEK, this kit allows to make the extraction of the RNA from the serum and in turn to realize the RT-PCR for the detection of HIV.

This kit allows amplification of a **fragment of 297 bp of HBV**, in addition the PCR reaction generates a fragment of 499 bp, this amplification is very important that it is present in all samples, since it is used as amplification control, that is, it is a control that the PCR reaction works.

4. EXPERIMENT COMPONENTS

COMPONENT		STORE
10x Concentrated electrophoresis buffer	2 x 50 ml	
Agarose	1.75 gr	
Micropipette 20 µl	1	
Tips rack	1	
Samples microtubes	4	at 4ºC

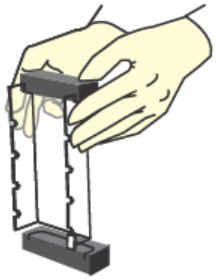
Add 450 ml of distilled water to each 10x Electrophoresis Buffer container to make 2 x 500 ml of 1x Electrophoresis Buffer which is the Working Buffer.

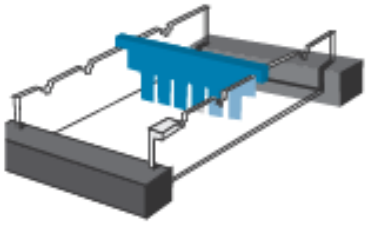
5. EXPERIMENT PROCEDURES

5.1 Agarose gel preparation

A) Mold preparation

Take the mold to make the gels and close the ends with the stops so that the agarose does not go out. Then place the comb to form the wells.





B) Agarose gel preparation

1.b) Use a 100 ml beaker or erlenmeyer to prepare the gel solution.

2.b) **For 7 x 7 cm gels**: Add **32 ml of 1x electrophoresis buffer** plus **0.30 g of agarose**, stir the mixture to dissolve the agarose clumps.

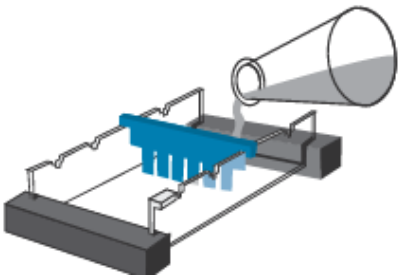
For 7 x 10 cm gels: Add **42 ml of 1x electrophoresis buffer** plus **0.40 g of agarose**, stir the mixture to dissolve the agarose clumps.

Make sure the 450 ml of distilled water has been added to the 10x Electrophoresis Buffer

3.b) Heat the mixture to dissolve the agarose. The fastest method is the use of a microwave, a heating plate can also be used, in both cases, in order for the agarose to dissolve **the solution must be brought to boiling point**. The final solution should appear clear without apparent particles.

4.b) **Cool** the agarose solution to about 55°C (to accelerate the process can be cooled by placing the container under a water tap and shaking). If there is excessive evaporation of the liquid, add electrophoresis buffer.

5.b) Add the agarose solution to the mold.

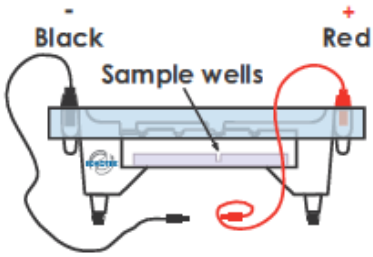


6.b) Allow the gel to solidify. To accelerate the process, the gel can be planted and then put it in a refrigerator (if the electrophoresis is performed the next day, keep the gel at 4ºC).

C) Gel preparation for electrophoresis

1.c) After the gel has solidified carefully remove the stops.

2.c) Place the gel in the electrophoresis chamber correctly oriented with the wells closest to the negative pole (black color).



3.c) Fill the electrophoresis chamber with **300 ml of 1x electrophoresis buffer**. ***The electrophoresis buffer can be used for 2 electrophoresis practice. Once the electrophoresis is finished, store this used buffer in a different container; don't mix a electrophoresis buffer new with one used buffer.***

4.c) Ensure that the gel is completely covered with tampon.

5.c) Remove the comb that has formed the wells very carefully to do not break any well.

6.c) Proceed to the load of the gel and carry out the electrophoresis.

5.2 Gel load and electrophoresis

Note: If you are unfamiliar with loading agarose gels, it is advisable to practice load before performing the experiment, or carry out the complete experiment before doing it with the students.

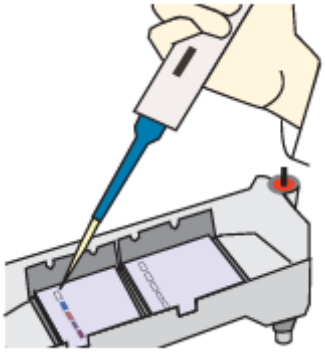
A) Electrophoresis samples

Check the volume of the all samples. Sometimes small drops of the sample may be on the walls of the microtubes. Make sure that the entire amount of sample is uniform before loading the gel. Centrifuge briefly the sample microtubes, or tap microtubes over a table to get the entire sample in the bottom of the microtube.

1.a) Four different samples presented in 4 tubes of a different color each one are supplied, loading the samples in the following order:

WELL	SAMPLE	DESCRIPTION
1	**GREEN**	MOLECULAR WEIGHT MARKER
2	**RED**	POSITIVE CONTROL
3	**LILAC**	PATIENT 1 (PEDRO POLIMERASA)
4	**BLUE**	PATIENT 2

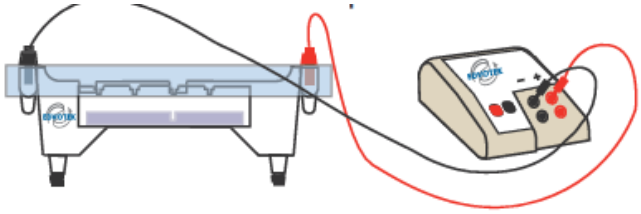
2.b) Load 20 microliters of each sample, using the fixed volume micropipette with a pipette tip supplied.



B) Carry out electrophoresis

1.b) After the samples have been loaded, place the electrophoresis apparatus cover on the electrode terminals carefully.

2.b) Insert the plug of the black cable into the black input of the power supply (negative input). Insert the red cable plug into the red input of the power supply (positive input).



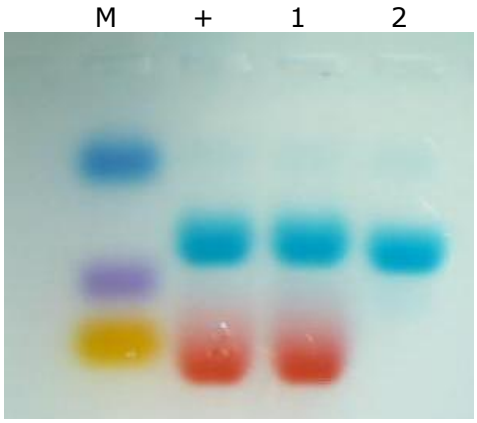
3.b) Set the power supply **at 75 volts (30 minutes) or 150 volts (20 minutes)**. **Watch that the dyes do not come out of the gel.**

4.b) After 10 minutes the separation of the dyes will begin to be observed.

5.b) After the electrophoresis is finished, **turn off the power supply**, disconnect the cables and remove the cover.

6.b) Place the gel in a white light transilluminator (if not available, a sheet of white paper may also be used).

6. PRACTICE RESULTS



M: The molecular weight marker has 3 fragments (750pb corresponding to the light blue band, 450bp corresponding to the intense blue band, 300bp corresponding to the yellow band).

+: Positive control, useful to control that the PCR reaction works.

Patient 1: Bands of 297 and 499 bp.

Patient 2: Band of 499 bp.

7. QUESTIONS AND ANSWERS ABOUT THE PRACTICE

A series of questions can be asked of students about the practice:

1. **What is the difference between the PCR reaction and the RT-PCR?**

The main difference is that by RT-PCR RNA can be amplified by a previous step using an enzyme called **Reverse Transcriptase** that passes the RNA to cDNA.

2. **What is the function of the 4 nucleotides (dATP, dCTP, dGTP, dTTP) in a PCR reaction**?

The 4 dNTPs are the components of DNA. For DNA synthesis a template DNA and 2 primers are required, the opposite strand of the template is synthesized following the Watson-Crick base pairing rule.

3. **Why are there 2 different primers?**

They present a different sequence that coincides with the beginning and end of the gene or sequence to be amplified (template DNA).

4. **Is HIV a DNA or RNA virus?**

It's a RNA virus.

5. **What is PCR positive control for?**

In all PCR it is necessary to include a positive control, to that reaction DNA fragments of HIV are added and tells us how a positive result should come out.

6. **What does the presence of a band of 499bp indicate in all reactions?**

It indicates that in all cases the reaction of the PCR has worked, this is very important in those cases that the band of 297 bp is not observed, in this way false negatives are ruled out, the HIV band has not been amplified but if the control of the PCR.

7. **Is Pedro Polimerasa carrying HIV?**

Yes, he is HIV-positive.

8. **How could you have avoided getting HIV?**

By conducting safe sex practices, using condoms.

For any further questions or queries, please contact us info@bioted.es