

DETECTION OF HEPATITIS B VIRUS BY PCR

Ref. PCRVHB (4 practices)

1. EXPERIMENT OBJETIVE

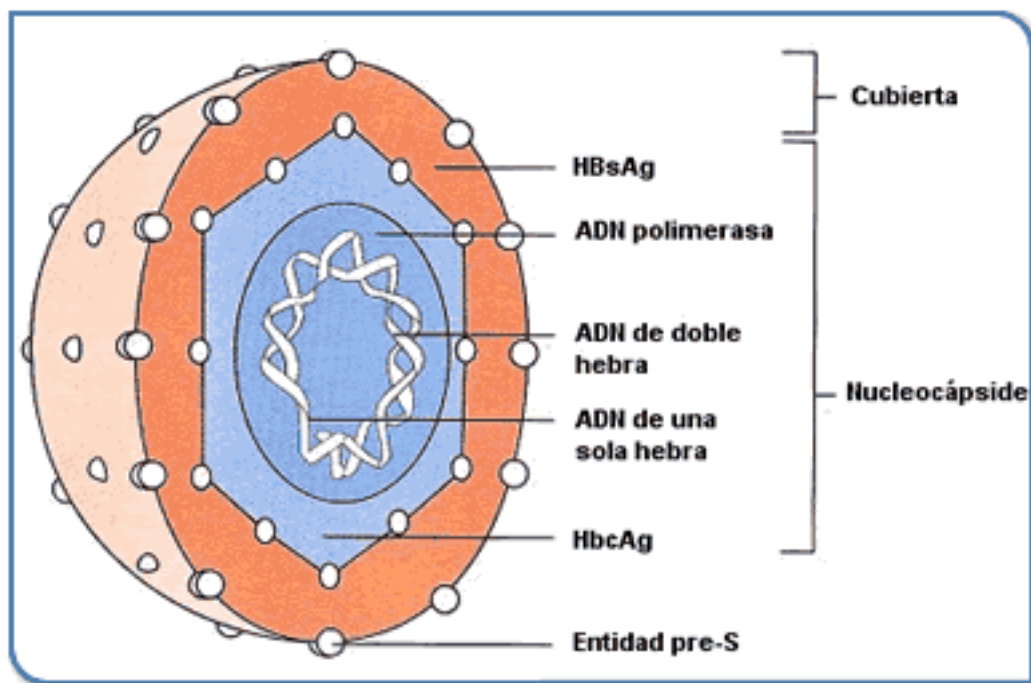
The aim of this experiment is to introduce students to the principles and practice of **Polymerase Chain Reaction (PCR)** as a tool for the detection of **Hepatitis B virus (HBV)** by PCR.

Students will acquire basic knowledge about HBV and the disease it produces, **Hepatitis B**.

2. BACKGROUND INFORMATION

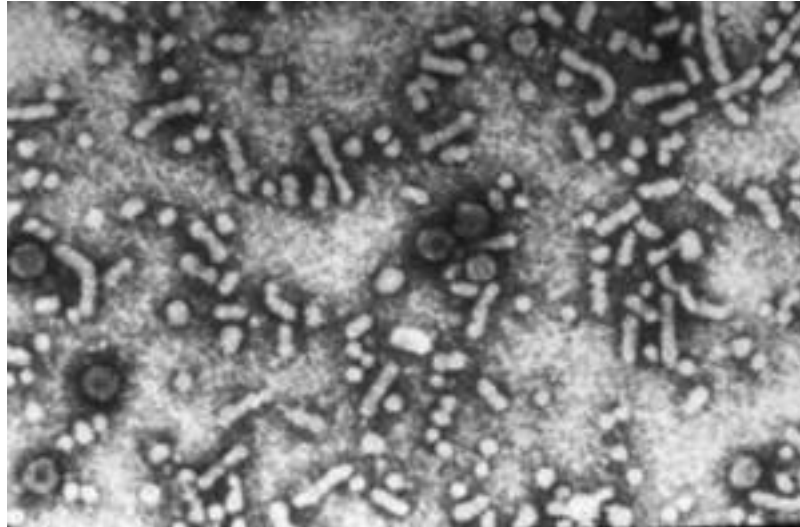
2.1 Hepatitis B Virus

Hepatitis B virus (HBV) is a hepadnavirus (*hepa* means that it replicates in the liver, *dna* indicates that its genome consists of DNA). HBV is transmitted through the blood or body fluids; mainly infects the liver producing inflammation (hepatitis) that destroys hepatocytes and may alter liver function.



Structure of HBV

When intact HBV particles (also known as Dane particles) are examined under an electron microscope, they appear as 42 nm diameter spheres. Each complete virus particle consists of an inner core (**nucleocapsid**) surrounded by an outer protein coat or shell.



Electronic photograph of viral particles of Hepatitis B

The **nucleocapsid** measures approximately 27 nm in diameter and contains:

- Circular DNA, partially double-stranded containing the genetic information necessary for viral replication
- DNA polymerase that catalyzes the production of DNA
- Two proteins: HBcAg (core antigen)

The main components of the virus cover are:

- A protein known as **surface antigen or HBsAg** covering almost 80% of the coating. Minor variations in the genetic material responsible for the coding of HBsAg have been identified and they generate the production of different subtypes. These are called adw, ayw, adr and ayr, where a, d, w, y, and r refer to the antigenic determinants in the HBsAg surface protein. It is important to note that because the antigenic determinant "a" is common to all known subtypes, exposure to an HBsAg subtype that generates the development of protective antibodies will protect against all HBsAg subtypes.
- Other 2 proteins: pre-S1 and pre-S2, which make up the pre-S entity.

HBV particles

During a hepatitis B virus infection, hepatocytes produce large amounts of surface antigen. However, only small amounts of HBsAg combine with nucleocapsids to form complete virus particles. The rest, for the most part, is released into the bloodstream as small pathognomonic spherical particles and filaments; these particles are not infectious because they do not contain DNA. HBsAg functions as an important serological marker for HBV infection.

Replication of hepatitis B virus

When a person becomes infected with HBV, the virus invades the hepatocytes where it replicates. Nucleocapsids are produced in the hepatocyte cytoplasm, while the complete HBV particles, the Dane particles, are produced in intracellular membranes. The hepatocytes then secrete the Dane particles and are released into circulation. They can infect nearby hepatocytes or pass to a new host through the blood or other body fluids.

2.2 The disease: Hepatitis B

Hepatitis B is a liver disease caused by the **hepatitis B virus (HBV)**. Hepatitis B virus was the first hepatitis virus to be identified. It is a disease that affects 300 million people in the world and it is estimated to be responsible for between 250,000 and 500,000 deaths a year.

Hepatitis B is endemic in China and other parts of Asia. Most infections occur in that region during childhood, and 8%-10% of the adult population is chronically infected. Liver cancer caused by hepatitis B is one of the top three causes of cancer in man, and is also a major cause of cancer in women in that region.

There are also high rates of chronic infection in the Amazon basin and in Eastern and Central Europe. It is estimated that 2%-5% of the general population of the Middle East and the Indian subcontinent suffer from chronic infection. In Western Europe and North America, the population with chronic infection does not reach 1%.

Most people who get the hepatitis B virus recover without consequences. This form of infection, which lasts less than 6 months, is known as **acute hepatitis B**. In contrast, when the infection lasts for more than 6 months, it is known as **chronic hepatitis B**. Approximately 5% of adults who acquire the infection develop the chronic form. The probability of developing chronic hepatitis B depends on the age and immune status (defences) of the subject, being greater when acquired in childhood than when acquired as an adult.

The clinical manifestations of hepatitis B virus infection are very varied, and it is important to emphasize that this infection frequently cannot give any symptoms for many years, which does not necessarily mean that the infection is controlled. The damage caused by hepatitis B virus in the liver is also variable and depends on liver repair ability and the body's ability to control infection. The most important consequences of this infection in the long term are the development of **hepatic cirrhosis** and **hepatocellular carcinoma**.

In the last time a series of new alternatives of treatment of the disease have been developed. On the other hand, there is a highly effective and safe vaccine to prevent infection.

Symptoms of hepatitis B

- **Acute Hepatitis B**

The symptoms of acute hepatitis B occur after 1 to 4 months of acquisition of the virus. Many people may not have any symptoms. Symptoms include:

- Fatigue
- Decreased appetite (anorexia)
- Nausea
- Jaundice or yellowing of the skin
- Choloria
- Pain in the upper right side of the abdomen
- Pain or swelling of the joints

These symptoms usually disappear within 3 months.

A very low proportion of people with acute hepatitis B (0.1 to 0.5%) develop a more severe form of the disease characterized by liver failure (fulminant hepatitis).

- **Chronic hepatitis B**

Chronic hepatitis B is often asymptomatic or only manifested by nonspecific symptoms such as tiredness or decreased appetite. Occasionally exacerbations of the inflammatory activity of the liver occur which can lead to exacerbations of symptoms. To the extent that infection causes major damage to the liver, symptoms of liver cirrhosis may develop.

Hepatitis B transmission pathways

The hepatitis B virus is transmitted through contact with blood or contaminated body fluids. Transmission routes include:

- **Sex:** Probably the most common form of infection. Transmission may be through both heterosexual and homosexual relationships.
- **Blood transfusions:** It is currently a form of transmission practically non-existent due to routine blood tests that are used for transfusions.
- **Perinatal transmission:** It consists of the transmission of hepatitis B virus from mother to child, usually close to the time of delivery. It is an important route of infection in countries of high prevalence such as China.
- **Injectable drugs:** The use of contaminated syringes and/or needles is an important route of infection.
- **Tattoos or piercings** made with non-disposable material.
- **Close contact:** Infection can occur if blood from an infected person comes in contact with mucous membranes (eyes, mouth, genitals) or with small wounds from another person. This happens, for example, when you share a razor blade, a toothbrush or a nail clipper.
- **Medical procedures:** The hepatitis B virus can be transmitted by contaminated instruments during invasive medical procedures such as surgeries if the necessary precautions are not followed.

Diagnosis of hepatitis B

Hepatitis B virus infection is usually diagnosed in a person who has symptoms of acute hepatitis or through investigation of abnormal liver tests in a patient without symptoms. In any case, the doctor will question the patient about risk factors for acquiring the virus and will look for physical signs that may lead to the presence of cirrhosis of the liver.

Because many liver diseases may have clinical manifestations similar to hepatitis B, usually the laboratory tests are the ones that give the definitive diagnosis.

- **Aminotransferases:** Also known as transaminases, they are tests that allow to estimate the degree of hepatic inflammation. ALT (alanine transferase or SGPT) and AST (aspartate transferase or SGOT) can rise to values above 1000 U/L in acute hepatitis and range from the normal range (less than 40 U/L) to several hundred chronic hepatitis.
- **Bilirubin:** Bilirubin is a breakdown product of red blood cell hemoglobin that is eliminated by the liver. Its elevation indicates a more important failure of hepatic excretory capacity and manifests as jaundice.
- **Albumin:** Albumin is the major plasma protein and is produced in the liver. Its decrease usually indicates significant liver damage.
- **Prothrombin time:** Prothrombin is a protein produced by the liver used for coagulation. Its measurement is expressed as a percentage of the normal value or as INR (international normalized ratio). The normal INR is 1. As prothrombin production decreases the INR increases.
- **Viral markers:** The hepatitis B virus can be detected through a series of tests that directly detect virus-produced proteins (antigens) or the body's immune response against the virus (antibodies). Hepatitis B surface antigen (HBsAg) is present in both acute and chronic infection. Their stay for more than 6 months defines chronic hepatitis B. Anti-core antibodies may be of the IgG or IgM type (*anti-HBc IgM*). The presence of anti-HBc IgM generally indicates an acute infection. Detection of the **antigen e** (HBeAg) is an indicator of active infection and viral replication. Its detection is important during the treatment, since its disappearance indicates that the viral replication has been controlled. In some patients there may be variants of the virus that undergo a mutation (pre-core mutants) and do not produce HBeAg, despite active infection.
- **Viral load:** The detection and quantification of viral DNA (genetic material) is an excellent way to monitor the degree of viral replication. It is frequently used to diagnose and monitor response to therapy.

- **Liver biopsy:** Obtaining a small piece of liver for microscopic analysis is an excellent way to determine the degree of damage to the liver, important for deciding the therapy. See information on liver biopsy.

Treatment of hepatitis B

Acute hepatitis B does not require specific treatment, since 95% of adults recover spontaneously. It is important to remember that contacts of the person with acute hepatitis B should be evaluated and eventually vaccinated. Acute hepatitis B is highly contagious, so steps should be taken to avoid transmission.

People who develop chronic hepatitis B should be evaluated by a doctor experienced in the management of this disease (gastroenterologist or hepatologist). Treatment decisions are individualized. The goal of treatment is to keep controlled replication of the virus to prevent progressive damage to the liver.

- **General measures**

Patients with chronic hepatitis B should receive the hepatitis A vaccine if they are not immune. It is recommended to avoid the consumption of alcohol and medicines that are not clearly necessary. Overweight and obesity can be factors that contribute to liver damage (see diet) causing fatty liver. In patients with cirrhosis, abdominal ultrasound is usually recommended and alpha-fetoprotein levels are measured every 6 months.

- **Antiviral treatment**

There are at least 3 treatment options for chronic first-line hepatitis B, including the interferon and the oral antiviral *Entecavir* and *Tenofovir*. The decision about when to start the treatment and what type of medication to use should consider all the clinical and laboratory history of the patient and is usually a decision shared between the doctor and the patient. It is very important to consider that the hepatitis B virus can mutate by developing changes in the structure of the enzyme polymerase that make oral antiviral ineffective (resistance). To avoid resistance, it is essential that the patient has excellent adherence to the treatment.

- **Interferon:** Interferon alpha is a substance normally produced by the body's immune cells against infections, particularly viral ones. This medicine is used in subcutaneous (under the skin) injections. A formulation called **pegylated interferon** or **peginterferon** is now preferred which allows administration once a week. The duration of treatment is between 4 and 12 months. It is a treatment that can have many adverse effects, but has the advantage that when a response is achieved, it is usually sustained over time. It should not be used when the patient has decompensate cirrhosis.
- **Entecavir:** *Entecavir* is a potent antiviral drug - a nucleoside analogue - whose main advantages are its potent antiviral activity and resistance development. It is well tolerated. Because it has been shown to have activity against the HIV virus, it should not be used in people co-infected with HIV if they are not on antiretroviral therapy (see co-infection with hepatitis B-HIV).
- **Tenofovir:** It is a nucleotide analogue that was initially developed as a treatment against HIV virus. Like *Entecavir*, it is a high-potency drug, and it has a low-potency resistance. It is very well tolerated.
- **Other antiviral drugs:** **Lamivudine** is a medicine given orally in doses of 100 mg daily. This compound directly inhibits the virus by interfering with viral replication mechanisms. It is a very well tolerated drug, with almost no adverse effects. The major drawback of this treatment is that it needs to be used for long periods of time and can cause the emergence of resistant virus (mutation of the YMDD polymerase region), which are associated with a lack of response to treatment. **Adefovir** works similarly to *Lamivudine*, inhibiting viral polymerase. It is well tolerated in general; however it has the potential to damage renal function, so it is monitored with periodic exams. Its advantage over *Lamivudine* is that the possibility of generating resistant mutants is much lower. **Emtricitabine** and **Telbivudine** are oral antiviral that may also be used, but are not considered as first-line drugs when used as monotherapy.

- **Liver transplantation:** It is a treatment option for some patients when decompensate cirrhosis has been established. Liver transplantation for people with hepatitis B is more complex than for other indications, as it requires expensive treatments to control replication of the virus after transplantation.

Prevention of hepatitis B

The hepatitis B vaccine is the building block of prevention of this disease. OMS recommends that it be administered all infants.

The vaccine can be integrated into the vaccine schedule and given in three to four doses. In areas where HBV transmission from mother to child is frequent, the first dose should be given as soon as possible after birth (within the first 24 hours).

Complete vaccination induces antibodies reaching protective concentrations in more than 95% of infants, children and young adults. The protection lasts for at least 20 years and possibly persists for a lifetime.

All children and adolescents below the age of 18 who have not been vaccinated before should be vaccinated. High-risk populations should also be vaccinated, in particular:

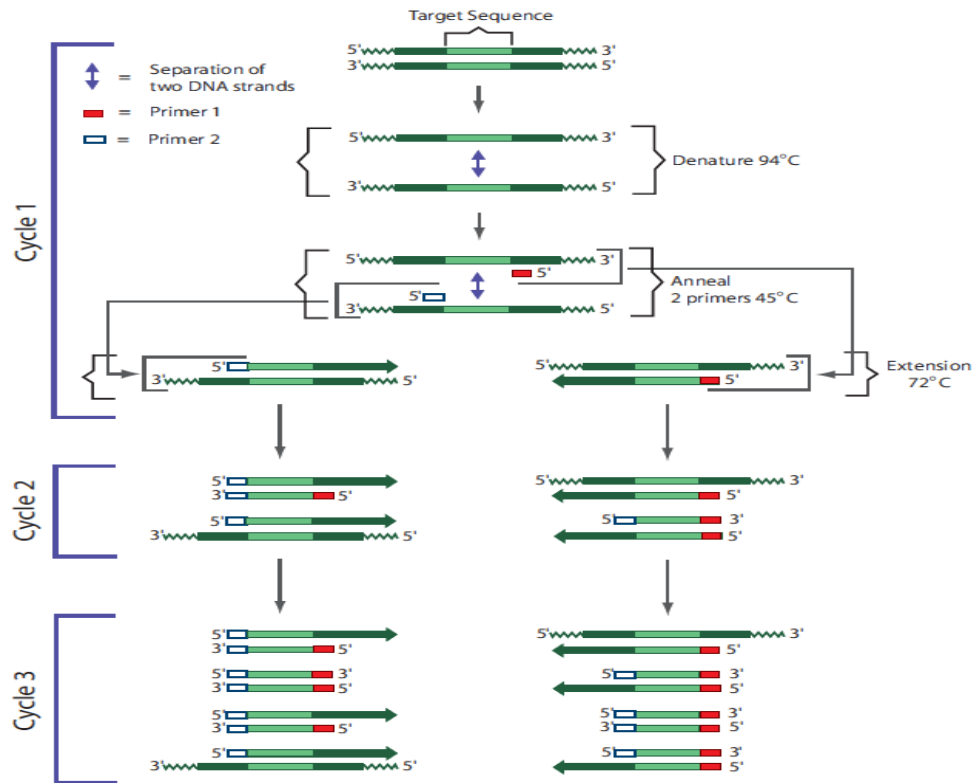
- People with high-risk sexual behavior;
- Domestic contacts and contacts of infected persons;
- Injecting drug users;
- Patients who need frequent transfusions of blood or blood products;
- Recipients of solid organ transplants;
- Individuals at work risk of HBV infection, such as healthcare professionals, and
- international travelers to countries with high rates of HBV infection.

2.3 PCR

PCR has had an extraordinary impact on several aspects of biotechnology.

In a PCR reaction, the first step is the preparation of the DNA sample that is extracted from several biological sources or tissues. In PCR, the DNA or gene to be amplified is defined as "target" and the synthetic oligonucleotides used are defined as "primers". A set consists of 2 primers of between 20-45 nucleotides that are chemically synthesized to correspond to the ends of the gene to be amplified. Each primer binds to one end of each DNA strand and is the starting point of the amplification.

A typical PCR reaction contains template DNA, Taq polymerase and the 4 dNTPS in an appropriate reaction buffer. The total reaction volume is 25-50 µl. In the first step of the PCR reaction, the complementary strands of DNA are separated (**denatured**) from each other at 94°C, while the Taq polymerase remains stable. In the second step, known as **annealing**, the sample is cooled to a temperature between 40-65°C allowing hybridization of the 2 primers, each to a strand of the template DNA. In the third step, known as **extension**, the temperature is raised to 72°C and the Taq polymerase adds nucleotides to the primers to complete the synthesis of a new complementary strand.



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

María Lasosa works at the Barcelona Blood and Tissue Bank as a quality technician. Her mission is to carry out the different tests to ensure that the donated blood donations are safe and suitable for transfusions.

Today it is going to re-test 4 samples that went wrong in the first control, these samples were positive for the hepatitis B virus, therefore, it is necessary to perform a control by PCR to assure the quality of the blood and on the other hand if they give positive for HBV to inform donors.

It uses a commercial kit from the house NORGEN BIOTEK, this kit allows the extraction of DNA from the serum and in turn performs the PCR for the detection of HBV.

This kit allows to amplify a **750 bp fragment of HBV**, in addition the PCR reaction generates a 300 bp fragment, this amplification is very important that it is present in all the 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 μ l	1	
Tips rack	1	
Samples microtubes	6	at 4°C

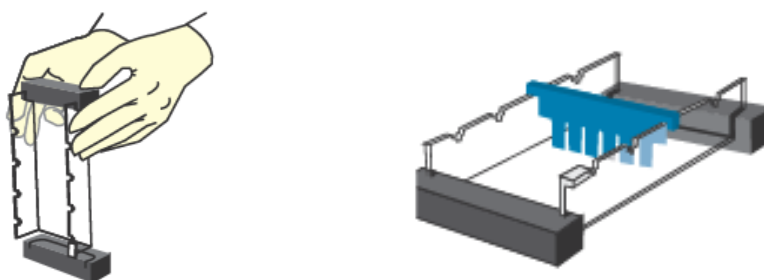
Add 450 ml of distilled water to each 10x Electrophoresis Buffer container to make 2 x 500 ml of 1x Electrophoresis Buffer which is the Working Buffer.

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.

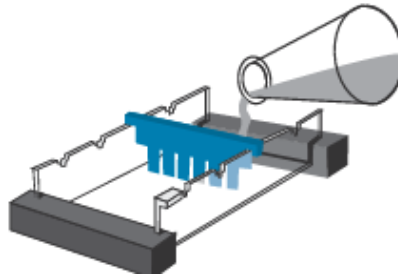
For 7 x 10 cm gels: Add **42 ml of 1x electrophoresis buffer** plus **0.40 g of agarose**, stir the mixture to dissolve the agarose clumps.

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.

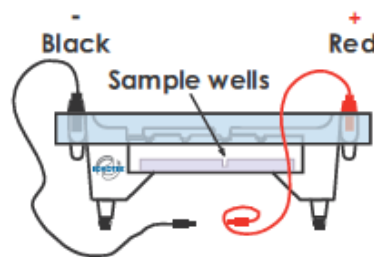


6.b) Allow the gel to solidify. To accelerate the process, the gel can be planted and then put it in a refrigerator (if the electrophoresis is performed the next day, keep the gel at 4°C).

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.

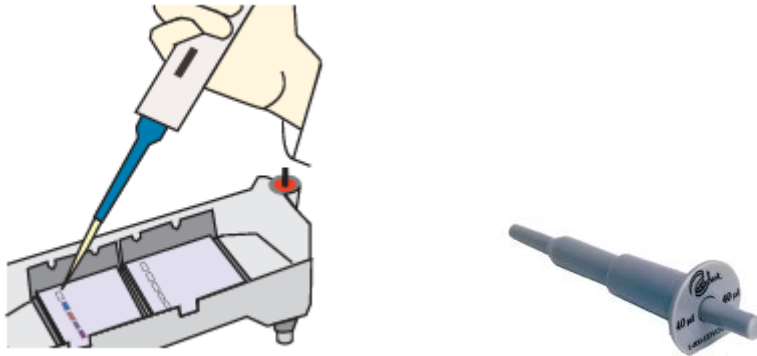
A) Electrophoresis samples

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) Six different samples presented in 6 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	DONOR 1
4	BLUE	DONOR 2
5	YELLOW	DONOR 3
6	WHITE	DONOR 4

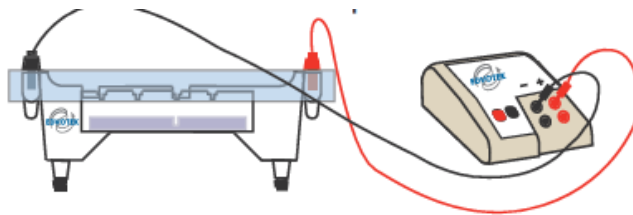
2.b) Load 20 microliters of each sample, using the fixed volume micropipette with a pipette tip supplied.



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).



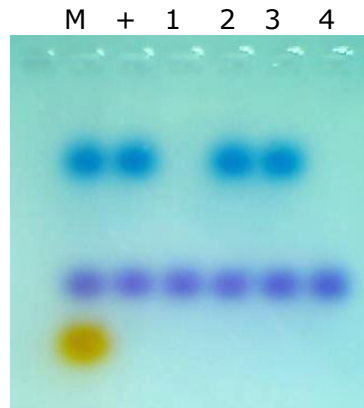
3.b) Set the power supply **at 75 volts (30 minutes) or 150 volts (20 minutes)**. **Watch that the dyes do not come out of the gel.**

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 (750 bp corresponding to the HBV fragment, 300 bp corresponding to the PCR control, 200 base pairs).

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

1, 2, 3 and 4: 4 different blood donors.

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 replication in cells?**

In replication (DNA synthesis in cells) a large amount of proteins are involved in all stages of cell division and synthesis and the reactions are carried out at 37°C (body temperature). In the PCR, only the synthesis is carried out and it is realized at non-physiological temperatures.

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. **What donors have tested positive for HBV?**

Donors 2 and 3

5. **Is the blood of these donors fit for transfusion?**

Do not.

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

It's a RNA virus.

7. **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.

8. **What does the presence of a 300 bp band indicate in all reactions?**

It indicates that in all cases the reaction of the PCR has been working, this is very important in those cases in which the band of 700 bp is not observed, in this way false negatives are ruled out, the HBV band has not been amplified but if the control of the PCR.

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