

GENETIC DIAGNOSIS OF FAMILIAL HYPERCHOLESTEROLEMIA BY RFLP

Ref. PCRCOLEST (4 practices)

1. EXPERIMENT OBJETIVE

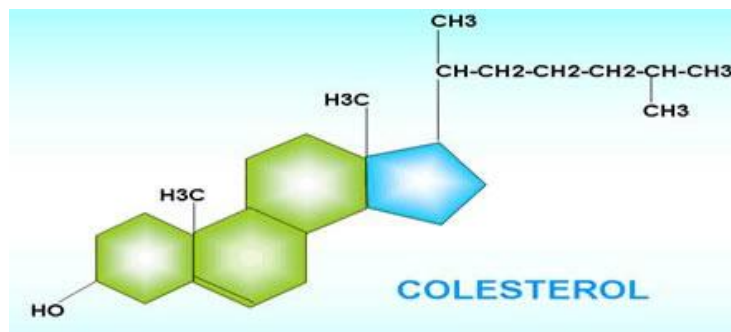
The objective of this experiment is to introduce students to the principles and practice of **Polymerase Chain Reaction (PCR)** as a tool for the genetic diagnosis of cholesterol-related diseases.

Students will acquire basic knowledge about the cholesterol molecule and possible associated cardiovascular diseases.

2. BACKGROUND INFORMATION

2.1 Cholesterol

Cholesterol is a key component of cell membranes. It regulates the fluidity of the membrane and is a precursor of steroid hormones and vitamin D. It is transported in blood as **lipoproteins**. The different types of lipoproteins and their blood levels are determining factors in the onset of **cardiovascular diseases**.



Cholesterol is a fundamental steroid component of cell membranes. It is also stored inside cells in the form of **cholesterol esters**. Its main functions derive from its role as a component of membranes and its steroidal nature, as it is a precursor to steroid hormones such as **testosterone** and **aldosterone** and vitamin D. Cholesterol comes from diet or, for the most part, from the **endogenous synthesis** in the liver. The **enzyme HMG-CoA reductase** catalyzes the first step in cholesterol synthesis.

Cholesterol is transported in blood bound to proteins (**apolipoproteins**), in the form of **cholesterol esters**, constituting **chylomicrons**, low density lipoproteins (**LDL**) and high density lipoproteins (**HDL**). LDL carries cholesterol from the liver to the tissues while HDL transports it from the tissues to the liver for excretion with bile, which is why it is sometimes referred to as "good cholesterol." Bile cholesterol can be reabsorbed again. In addition, cholesterol is a precursor of bile salts.

2.2 The disease: Familial hypercholesterolemia

Cholesterol is essential for life but an excess in serum can have negative consequences. It has been established that high amounts of cholesterol in blood, especially LDL, sometimes termed "bad cholesterol" is related to cardiovascular disease.

Elevated cholesterol levels indicate a need for cholesterol reduction through diet and other changes in lifestyle, and often the use of medication. **Statins**, which are competitive inhibitors of HMG-CoA reductase, are being used with very good results in the treatment of hypercholesterolemia.

In most cells there are LDL receptors that endocytose LDL to later release the cholesterol into the cells. LDL cholesterol is the one that accumulates in **atherosclerotic plaques**. Conversely, low levels of HDL increase the risk of cardiovascular disease. Dietary cholesterol lowers cholesterol biosynthesis in the liver, induces the formation of cholesterol esters intracellularly and decreases the synthesis of the LDL receptor. Decreasing the LDL receptor increases extracellular cholesterol which implies cardiovascular risk. There are types of hypercholesterolemia due to genetic factors. **In familial hypercholesterolemia (HF) a mutation in the LDL receptor gene causes the extracellular accumulation of LDL**, as it cannot efficiently eliminate LDL from the circulation. The result of this deficiency results in the LDL remaining in the circulation and accumulating in the arterial walls. **Patients who are heterozygous** for this mutation, therefore, possess a functional gene and have 50% of receptors that healthy individuals. **Patients who are homozygous** for this mutation, therefore, do not have LDL receptors and have extremely high blood cholesterol levels; sometimes above 600 mg/ml serum (150-200 mg is considered normal). If these patients are not treated, they usually die very young from coronary artery disease.

There are also secondary causes that cause hypercholesterolemia, such as **diabetes or liver disorders**.

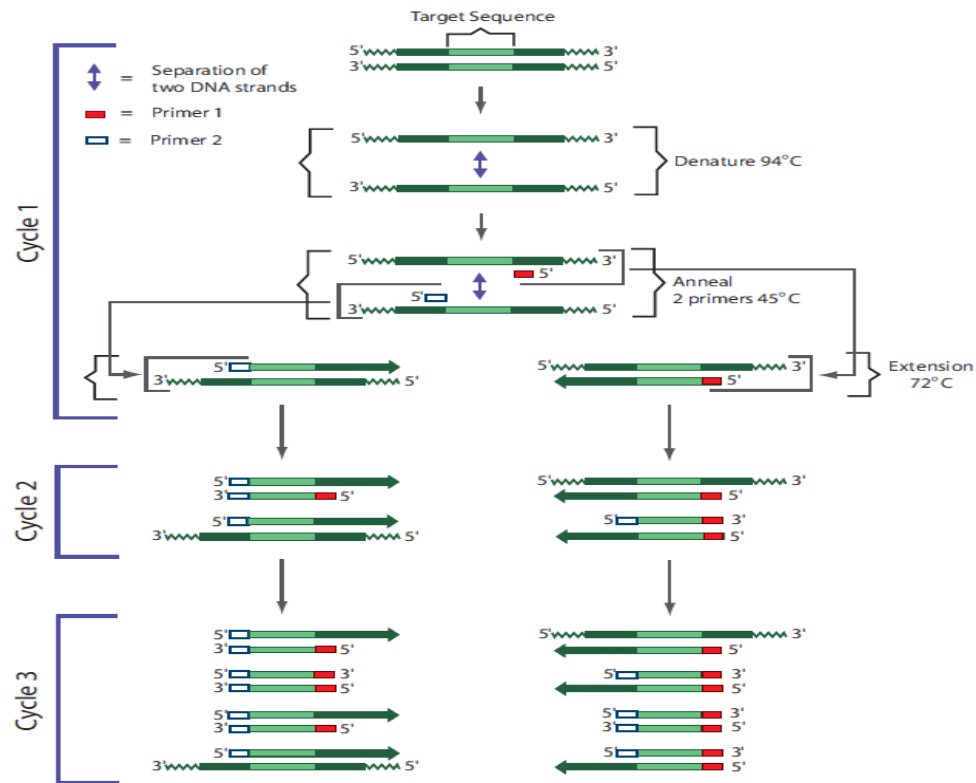
2.3 RFLP and PCR analysis

A mutation can be detected by genetic diagnoses based on **restriction fragment length polymorphism (RFLP)**, i.e. there are variations in the locus of restriction in DNA regions of healthy individuals versus diseased individuals.

In RFLP analyzes, a specific region of DNA within or near the disease-causing gene is **first amplified using PCR**,

A) 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 of 2 primers, between 20-45 nucleotides, 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 μ l. 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.



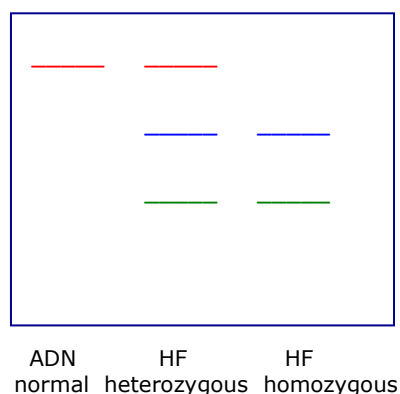
These three steps, denatured-annealing-extension, constitute a PCR cycle. This process is repeated for 20-40 cycles by amplifying the object sequence exponentially. The PCR is performed on a **thermocycler**, an instrument that is programmed for rapid heating, cooling and maintenance of the samples for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

B) In this case, followed by the PCR reaction, the amplified region is digested with specific restriction enzymes. **If the patient has a mutation of the LDL receptor gene, the pattern of digestion that can be observed on an agarose gel is different from that observed in healthy patients.**

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 NONTOXIC dyes that will migrate in the agarose gel as if they were DNA fragments resulting from the digestion of the amplified fragment with restriction enzymes.

STATEMENT OF FACTS

We have the Smith family where the father and the mother are heterozygous for the HF mutation, have 2 children and will be made a genetic diagnosis by RFLP of the HF mutation to establish the degree to which the disease can suffer.



From the result observed in the agarose gel, it will be possible to establish if the patients are normal, heterozygous or homozygous for the HF mutation. The DNA of patients who are heterozygous for the mutation will have one normal allele and one mutated allele. Homozygous individuals will have the 2 mutated alleles.

3. 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

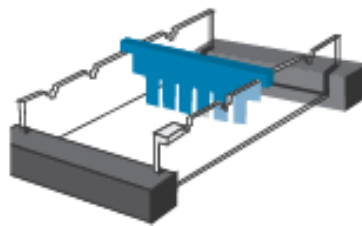
Add 450 ml of distilled water to each 10x Electrophoresis Buffer container to make 2 x 500 ml of 1x Electrophoresis Buffer which is the Working Buffer.

4. EXPERIMENT PROCEDURES

4.1 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.

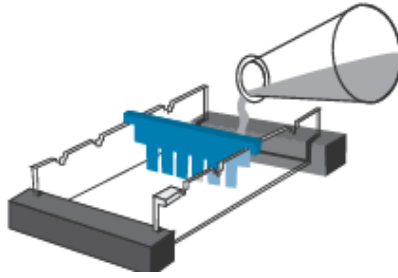
For 7 x 10 cm gels: Add **42 ml of 1x electrophoresis buffer** plus **0.40 g of agarose**, stir the mixture to dissolve the agarose clumps.

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.

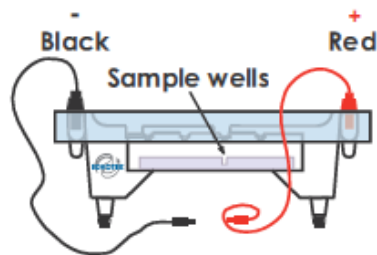


6.b) Allow the gel to solidify. To accelerate the process, the gel can be planted and then put it in a refrigerator (if the electrophoresis is performed the next day, keep the gel at 4°C).

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.

4.2 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.

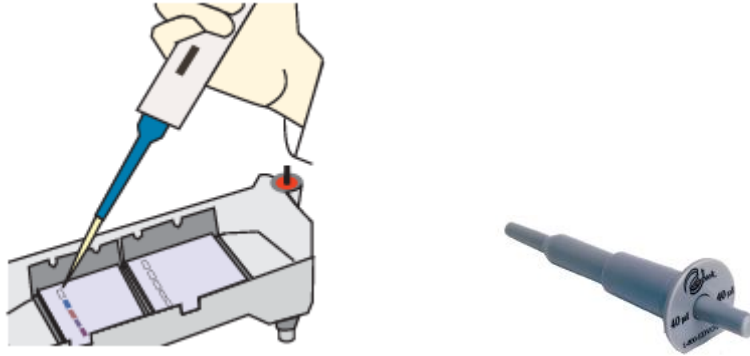
A) Electrophoresis samples

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	BLACK	DNA CONTROL (WITHOUT DISEASE)
2	RED	HF POSITIVE CONTROL
3	LILAC	FATHER
4	BLUE	MATHER
5	YELLOW	SON 2
6	WHITE	SON 1

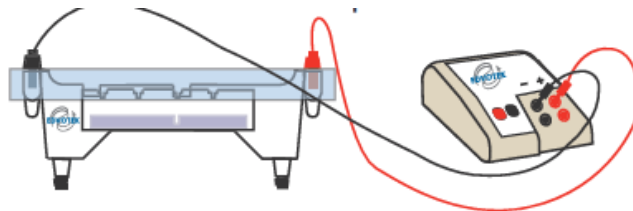
2.b) Load 20 microliters of each sample, using the fixed volume micropipette with a pipette tip supplied.



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).

5. PRACTICE RESULTS

DNA HF Father Mother Son 1 Son 2
control control



We can clearly observe the pattern of bands after PCR amplification plus digestion with restriction enzymes that parents are heterozygous, while in offspring one of the son is homozygous for the mutated allele and the other homozygous for the normal allele.

6. QUESTIONS AND ANSWERS ABOUT THE PRACTICE

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

1. What is Familial Hypercholesterolemia?

It is a dominant genetic disease in which one or more mutations exist the receptor gene for LDL.

2. Since both parents are heterozygous, what is the probability that the children are affected by the disease or not?

As the mutation is dominant there is a 50% probability that the children are heterozygous and 25% that they are totally affected by the disease (homozygous).

3. In what degree are children 1 and 2 affected?

Child 1 is not affected by the disease by having 2 good copies of the gene, while son 2 is homozygous and therefore is totally affected by the disease.

4. What is an RFLP analysis and how can it be used to look for genetic diseases?

This technique makes it possible to establish a correlation between the difference in the number of restriction sites and a certain disease. This will produce differences in the length of the PCR amplified fragments located near the gene that produces a disease when being digested with specific restriction enzymes.

5. Why are there 2 different primers in PCR?

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

6. Why is it so important to avoid having high blood cholesterol levels?

Excess cholesterol tends to accumulate on the inside and outside of the arteries forming plaques that can lead to blocking the arteries resulting in heart attacks or stroke when blood flow is blocked.

7. What are statins?

It has recently been discovered that statins inhibit the synthesis of cholesterol in the liver and thereby lower blood cholesterol levels.

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