DETECTION OF HYPOLACTASIA BY PCR

Ref.PCR11

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 genetically modified organisms.

Students will acquire basic knowledge about the molecular biology of the process of obtaining a GMO.

2. INTRODUCTION

2.1 PCR

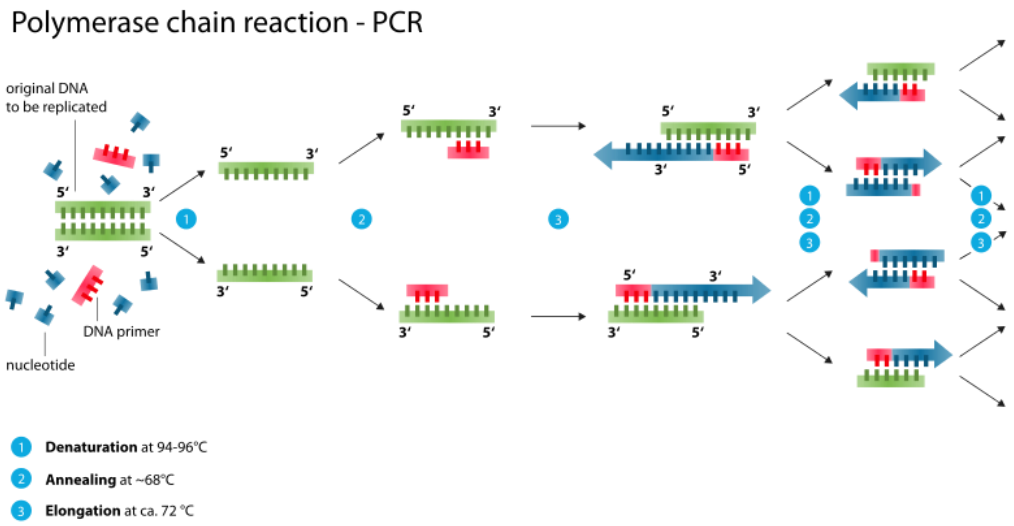
PCR has revolutionized research and diagnosis based on Molecular Biology. PCR is a simple, accurate and highly reproducible process that provides the advantage of starting with a small amount of DNA and being able to amplify it so that you have enough to perform experiments.

Many diagnostic tests have been developed, it has also been used in DNA mapping and sequencing in genome projects and is being used in forensic determinations, paternities, etc.

In all cases, DNA segments are amplified and subsequently subjected to analysis and various studies.

In a PCR reaction, the first step is preparation of DNA sample that is extracted from various biological sources or tissues. In PCR, DNA or gene to be amplified is defined as "target" and synthetic oligonucleotides used are defined as "primers". A set of 2 primers of between 20-45 nucleotides are chemically synthesized that 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, 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. PCR is performed on a thermocycler, an instrument that is programmed for rapid heating, cooling and maintenance of samples for several times. The amplified product is then detected by removal of the reaction mixture by agarose gel electrophoresis.

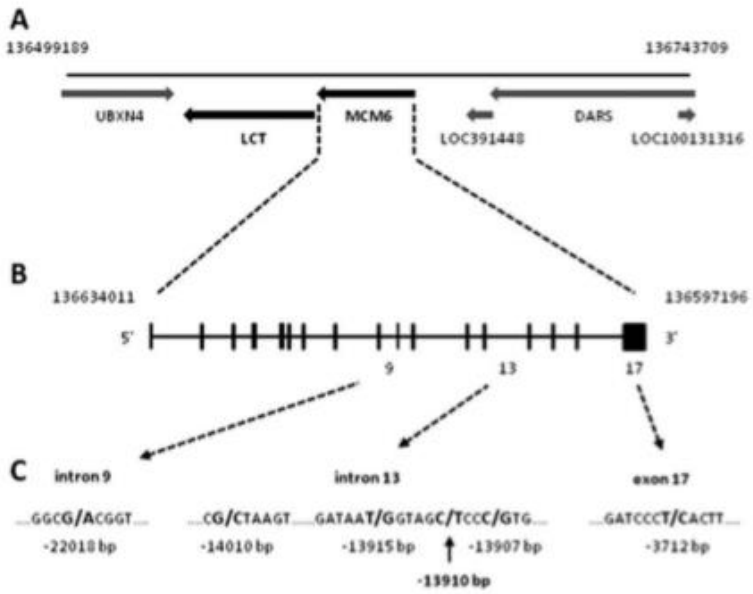
2.3 Polymorphism of lactase activity

The consumption of milk and other milk products in people with lactose intolerance can cause diarrhoea, flatulence, feeling swollen and abdominal pain. These symptoms are very similar to other pathologies that affect the gastrointestinal system, which is why it is very important to be able to make a correct diagnosis to patients with this type of symptomatology.

In some populations, a proportion of adult individuals have maintained the ability to hydrolyse lactose. This persistence of lactase is due to a single nucleotide variant with autosomal dominant inheritance. This nucleotide change is 100 base pairs from the lactase gene regulatory region (LCT). This regulatory region is located approximately 14kbp upstream of the lactase gene, in an intronic region that helps transcribe the lactase gene. The change of a single nucleotide was discovered in 2002, it is a change in -13910 C>T and it i0s present in 80-90% of the northern European population. Other variants associated with lactase resistance have also been discovered such as -13915 T>G which is present in Sudanese, Kenyans, Ethiopians and populations in the Middle East. Another is the -13907 C>G present in Sudanese and Kenyans.

The inability to digest lactose in adults has been associated with the C/C genotype, that is homozygous to cytosine, in this SNP. Several methods have been described to detect the lactose persistence gene variant. We will perform the detection of this single nucleotide variant through the PCR technique and subsequently a digestion with the restriction enzyme FaqI, in order to identify the different genotypes.

The human genome consists of 2.9 billion base pairs of DNA. Of this total, only 2% consists of exons which code for proteins. Introns and other non-coding sequences may have functions that are yet to be discovered, although many others seem not to have them. Many of these sequences are repeated hundreds or thousands of times throughout the genome representing just over 20% of the human genome.



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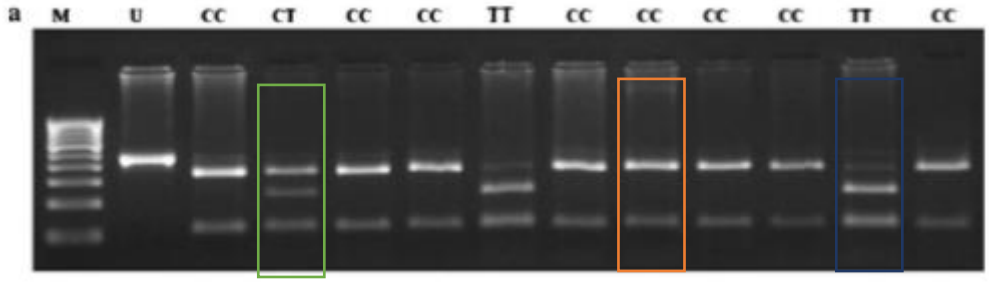
SNPs associated with lactase persistence. Map of the human chromosome 2q21 region that contains the human lactase gene and the minichromosome 6 gene.The intron-exon organization of the MCM6 gene. The location of the SNP is given in base pairs with respect to the initiation codon of the lactase gene.

PCR detection of lactase gene

Primers used for PCR reaction amplify lactase gene, with which we obtain a 448bp amplification fragment. Subsequently, digestion with restriction enzyme FaqI results in the different molecular weight fragments depending on the genotype present. In case of being homozygous C/C, two fragments are obtained in digestion, one of 350bp and another of 100bp, on the other hand, in case of being homozygous for T/T we also obtain two fragments, one of 250 and one of 100bp, finally if it is heterozygous C/T three fragments are obtained, 350bp, 250bp and 100/98bp.



Agarose gel analysis of the lactase gene. Analysis of the lactase gene in different subjects for SNP C>T. We can see the heterozygous marked in green, where three bands of different molecular weight are seen, 350bp, 250bp and 100bp. On the other hand, in orange the homozygous for C/C, in this case only two bands appear one of 350bp and one of 100bp. And finally, in blue the homozygous for T/T where two bands appear one of 250bp and another of 100bp.

3. EXPOSITION OF FACTS

Approximately 1.5ml of saliva will be collected from students in the corresponding saliva sample collection tube. It is recommended that you have not eaten anything during the 30 minutes prior to sampling. To do this, pass tongue with up-down movements through the walls of cheeks, jaws and palate to collect the cells.

4. COMPONENTS

10X Concentrated electrophoresis buffer	100ml	
Agarose	6,0gr	
Mix PCR detection lactase	2 x 350 µl	Conserve at -20ºC
Positive control of DNA gene lactase in homozygosis (C/C)	10 µl	Conserve at -20ºC
Positive control of DNA gene lactase in heterozygosis (C/T)	10 µl	Conserve at -20ºC
GELSAFE DNA staining	25 µl	Conserve at +4ºC
Purification kit of extraction products of PCR	25 samples	
Restriction enzyme Faq I	25 µl	Conserveat -20ºC
FastDigest Green Buffer	50 µl	Conserve at -20ºC
SAM	37,5 µl	Conserve at -20ºC
Water, nuclease free	425 µl	Conserve at -20ºC

10X electrophoresis buffer to prepare 1X electrophoresis buffer which is the working buffer to make the gels and that of the cuvette.

5. EXPERIMENT PROCEDURES

5.1 EXTRACTIONOF DNA FROM SALIVA

4 work groups will be carried out.

Each of them or in pairs will collect their own saliva sample in a saliva collection tube (supplied). In addition, a positive DNA control will be attached with the kit. It is recommended to use the DANAsaliva extraction kit for the extraction of DNA from saliva.

5.2 PCR REACTION

NOTE:Always use filter tips and change tips every time an action is taken to avoid contamination that can lead to false results.

1. Use 2,5 µl (100-250 ng) of the DNA from each DNA extraction.

IMPORTANT:

a) **Prepare a negative amplification control** by placing 2,5 µlof nuclease-free water instead of DNA, to see if reagents or micropipettes and tips may be contaminated with DNA, do not amplify nothing.

b) **Prepare two positive amplification control** by placing 2,5 µl of controls DNA that you have in this kit.

2. The typical concentrations of the primers and parameters used will depend on each system used. A typical final concentration of primers is 0.5 µM.

Reactivos	Volumen

Mix PCR	22,5 µl
DNA (100-250ng)	2,5 µl
Total volume	25 µl

3. Mix well; the red dye included in the polymerase facilitates the process.
4. Perform the amplification process.

IMPORTANT:

For activation of the "HOT STAR" Polymerase it is necessary to program an initial denatured step of 3 minutes at 95ºC, then program the 35 specific cycles of each product to be amplified.

PCR lactase program

Step	Temperature	Time
Denaturalization Hot Star	95ºC	3 minutes
PCR cycles Carry out 35 cycles	94ºC 60ºC 72ºC	30 seconds 30 seconds 30 seconds
Final extension	72ºC	5 minutes
Final	4ºC	

5.2 DNA PURIFICACTION OF PCR PRODUCTS

Once PCR is finished we will proceed to purify the amplified fragment of 448 bp, for this we will use a kit used in DANAGENE Clean PCR research kit that allows the elimination of polymerase, nucleotides, salts, etc. and stay only with the fragment of 448 bp so that when we do the digestion with the Faq I enzyme it is not inhibited by presence of other components.

1. **Add 150 µl binding** buffer to the microtube containing **25 µl of the PCR.** Mix well by pipetting.

2. **Transfer the sample (175 µl) to a spin column.** Place the spin column in a collection tube.VERY IMPORTANT, place the 175 µl in the middle of the white membrane

3. **Centrifuge for 1 minute at 10,000-12,000 rpm.**

4. **Remove the filtrate and add 700 µl of Wash Buffer.** Centrifuge for 1 minute at 14,000 rpm

5. **Remove residual ethanol** by centrifugation for 3 minutes at 14,000 rpm.

6. Place the spin column in a new 1.5 ml microtube and add **25 µl of Elution Buffer** pre-heated at 70 ° C exactly in the centre of the white membrane (touch the tip without breaking the membrane)

7. **Incubate for 2 minutes and centrifuge for 1 minute at 14,000 rpm. Now the microtube contains the DNA to digest with Faq I.**

5.3 DIGESTION BY RESTRICTION ENZYME Faq I

To perform digestion by the restriction enzyme FaqI place the following volumes in a 1.5 ml microtube.

Water nuclease free	17 µl
10x FastDigest Green Buffer	2 µl
20x SAM (1.0mM)	1,5 µl
DNA	15-20 µl
FastDigest enzyme	1 µl

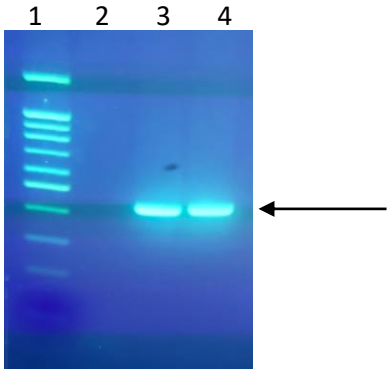
Mix and perform a spin. Incubate at 37°C for 30 minutes in a water thermostat or in a heat block. At the end of this time, incubate 5 minutes at 65°C to inactivate the enzyme.

When placing the FastDigest Green Buffer it is not necessary to load sample buffer to run it on a 2% agarose gel, since the buffer already serves us for it.

Note: if when introducing a little product of digestion by enzymes you observe that this is not deposited in the bottom of the well, and it goes out. You can solve the problem by adding 2.5 µl of loading buffer. This is because in the previous purificationethanol has not been correctly removed from the sample.

6. RESULTS

Amplification results of lactase gene by PCR

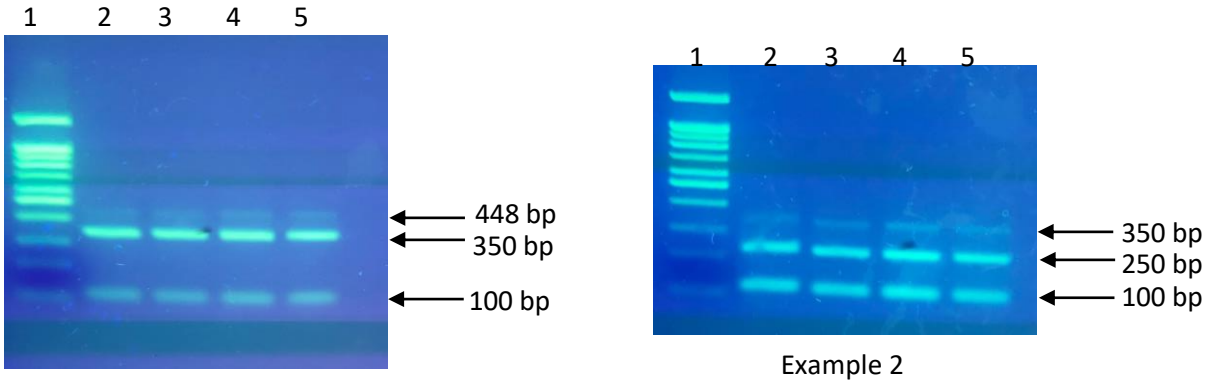


Agarose gel2%

1: Molecular weight marker
2: Negative control
3: Sample1 amplification gene lactase
4: Sample 2 amplification gene lactase

An example of the result of the amplification of the lactase gene with a negative control is presented in well 2 where no type of amplification result is seen, and two samples in wells 3 and 4, where the amplification has been performed of the lactase gene and a 448bp amplification band appears. Finally, in well 1 we can see the molecular weight marker to be able to correctly identify the weight of the band.

Results of digestion gene of lactase



Example 1 Example 2

Agarose gel 2%

Distribution of samples are the same in these two gels, but the samples are not the same for the two gels.

1: Molecular weight marker
2: Sample 1
3: Sample 2
4: Sample 3
5: Sample 4

Two examples of the result of the digestion of the PCR products performed with the restriction enzyme FaqI are presented. In Example 1, we have 4 samples and a molecular weight marker to identify the bands that appear. We can see in 4 cases that 2 very marked bands appear while a very thin third band of greater molecular weight appears. The band of greater molecular weight corresponds to the band of 448 bp, it is a product of the PCR that has not been digested. On the other hand, the two other bands that appear in all samples are the 350 bp and 100 bp band, in this case samples correspond to homozygous genotype for nucleotide C. In example 2, we also have 4 samples and a marker of molecular weight. We can see that in 4 cases three bands appear. These bands correspond to molecular weights of 100 bp, 250 bp and 350 bp. That is because samples of example 2 are heterozygous for the lactose SNPs, and therefore do not present problems in the digestion of this sugar.

7. QUESTIONS AND ANSWERS ABOUT THIS PRACTICE

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

1. **What is the role 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 template chain is synthesized following the Watson-Crick base pairing rule.

2. **Why are there 2 different primers or "primers"?** They have a different sequence that coincides with the beginning and end of the gene or sequence to be amplified (template DNA)
3. **How many people in the group of students who have done the practice have hypolactasia?**

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