

DETECTION OF TRANSGENIC SOYA BY PCR

Ref. PCR9 (25 students)

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 genetically modified organisms.

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

2. BACKGROUND INFORMATION

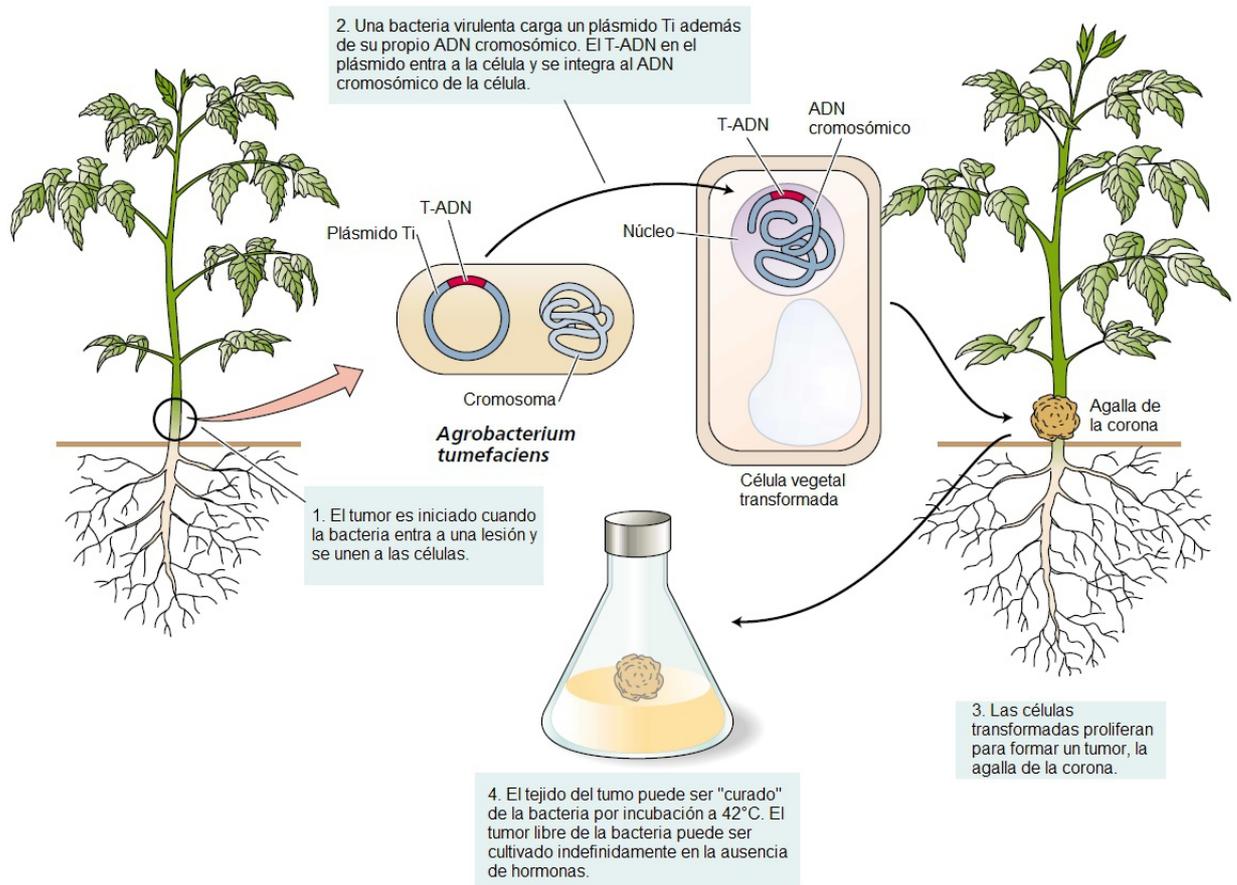
2.1 Genetically modified or transgenic organisms.

Genetic engineering has produced pest-resistant crop plants. The one that gains is the environment, because the use of pesticides is diminished; but the most paradoxical thing is that the organizations that have been dedicated to protect the environment have been those that have been noisier opposed to the introduction of these plants, which are called **genetically modified (GM)**.

The first difficulty in working with this technique is the introduction of the desired DNA fragment (useful gene) into the plant cell, and then into the plant genome.

Gill disease causes the formation of an irregular "tumour" in the stem of the plants, known as gill. The cause is a common soil bacterium called ***Agrobacterium tumefaciens***, which opportunely infects plants where it has been damaged by, say, the biting of herbivorous insects. The bacterial parasite carries out the attack by building a tunnel through which it deposits a packet of its own genetic material into the plant cell. The packet consists of a DNA fragment that is carefully extracted from a special plasmid and then wrapped in a protective cover before being sent through the tunnel. Once delivered the DNA packet, it integrates, as would the viral DNA, into the DNA of the host cell. However, unlike a virus and once it has been lodged, this DNA fragment does not make more copies of itself. Instead, it produces plant growth hormones and specialized proteins that serve as nutrients for the bacteria, simultaneously favouring the division of plant cells and bacterial growth and creating a closed circuit of positive intercommunication: growth hormones cause cells to multiply and in each cell division the invading bacterial DNA is copied together with that of the host cell, so that more and more bacterial nutrients and plant growth hormones are produced.

The consequence of this frenzy of uncontrolled growth is the appearance in the plant of an irregular mass, the gill, very useful for the bacteria because it constitutes a kind of factory in which the plant is forced to produce precisely what the bacterium needs, and even in larger quantities.



Agrobacterium is a prefabricated transfer system for introducing foreign DNA into plants, a natural genetic engineer. So that a gene of choice could be inserted into the **Agrobacterium** plasmid and then transferred to the plant cell, so that when the genetically modified bacterium infected the host, it would insert the chosen gene into the chromosome of the plant cell.

As the debate about GM foods revolves around us, it is important to understand that our practice of taking foods that have been genetically modified is actually thousands of years old. In fact, both our domestic animals, the origin of the meat we eat, and the crop plants that supply us grains, fruits and vegetables, are genetically far removed from their wild ancestors.



The effect of centuries of artificial selection: the corn and its wild ancestor teosinte to the left.

Many of the wild ancestors of crop plants offered relatively little to the first farmers: they were difficult to grow and their production was scarce. For agriculture to work, it was necessary to change it. Early farmers understood that the desirable characteristics were maintained from generation to generation involved an inborn modification (we would say genetics). In this way began the huge agricultural program of our ancestors. The activity depended on an artificial selection, according to which the farmers only raised those individuals who had the desired traits, for example, the cows that produced more milk. In effect, farmers did what nature does in the course of natural selection: choosing from the range of genetic variations available, in order to ensure that the next generation is enriched with those that are better adapted to the consumption, in the case of the farmers, and the survival, in the case of the nature. Biotechnology has offered us a way to generate desired variations, so we do not have to wait for them to appear naturally; is not, of itself; more than the last of a series of methods that have been used to *genetically modify* our food.

2.2 Genetically modified soya (RR soya).

The use of pesticides is widespread to eradicate insects and weeds that can compromise the annual harvest. Without knowing exactly the risks that the use of them can entail. For this reason, companies like Monsanto create varieties of insecticide resistant plants, in order to reduce their use. In addition, ensure the cultivation and harvest.

One of these crops is RR or Roundup Ready soya or 40-3-2 soya. It is a variety of soybeans which has incorporated a gene for resistance to glyphosate herbicide by transgenesis. This resistance gene comes from a soil bacterium, *Agrobacterium*.

This resistance helps the soybean plant survive the use of the herbicide glyphosate, which is used against weeds. Therefore, it helps the soybean crop to resist without having to discuss the use of nutrients, soil and sun with weeds. Optimizing the crop and reducing the use of pesticides.

Glyphosate acts in all plant species by inhibiting the activity of enzymes that synthesize the aromatic amino acids, necessary to do photosynthesis. Unable to synthesize these amino acids, plants die or slow their growth considerably. Glyphosate-resistant transgenic soybeans contain an enzyme from bacteria responsible for the synthesis of aromatic amino acids and which is also resistant to glyphosate.

In Europe, the laws and the permissibility of GM foods are very strict, with very few varieties of GM foods allowed in Europe. In addition, the product obtained from these varieties is normally used as animal feed. In this case, RR GM soybeans are allowed for cultivation in Europe as well as in Spain since 2017.

2.3 The MG food debate.

The cultivation of genetically modified plants by human intervention is being highly debated and very resist by environmental organizations. They consider it a threat for human health, environment and familiar or sustainable agriculture. Most scientific studies support their use and the experience of their use for decades. On the other hand, it is true that developing countries, and the United States also, are massively turning to the cultivation of this type of crops, with economical purpose.

To see which of the controversies or reluctance people have about transgenics, we will analyse some more familiar sentences:

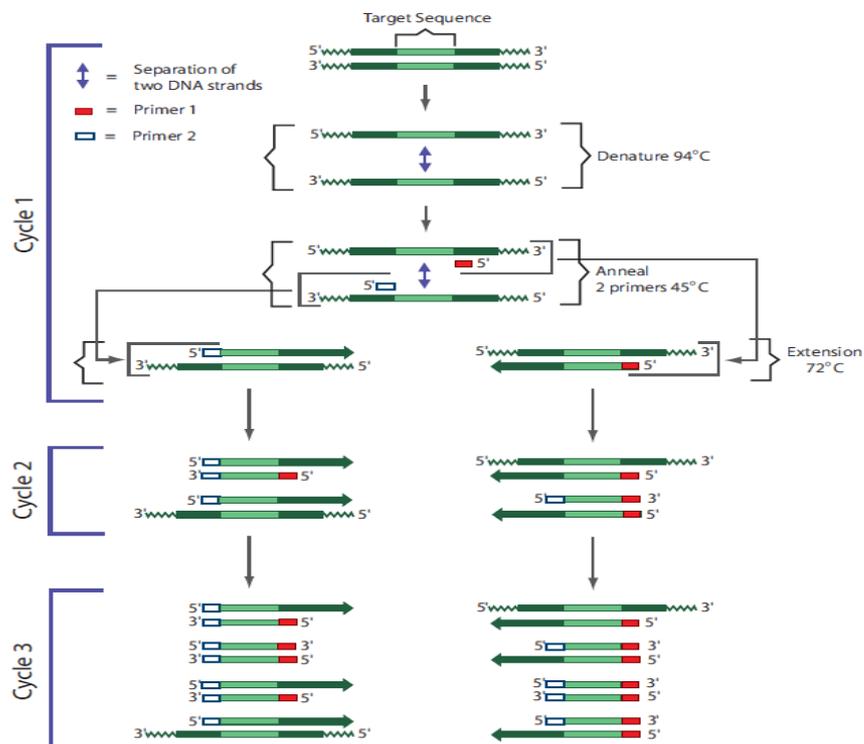
- It is not natural. Currently no one can take a strictly natural diet. Ancient farmers often crossed different species, creating entirely new ones with no direct equivalents in nature. Wheat, for example, is the result of a series of crosses. In this way, our wheat is a combination of the characteristics of several ancestors that perhaps nature would never have invented.

- Produce allergens and toxins in our food.
- It is not known if these species are more invasive. As it is a controlled crop in order to collect it is not true that they will invade areas that do not belong to them.
- It will cause resistance to herbicides. That is, the surrounding plants that we want to eradicate will grow and become resistant because of the gene present in these transgenic plants.

2.4 PCR analysis.

In a PCR reaction, the first step is the preparation of the DNA sample that is extracted from various 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 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 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.

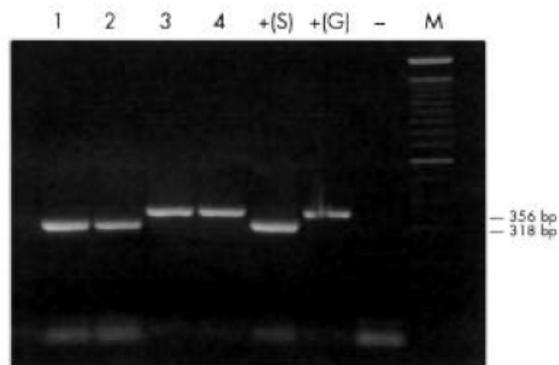


These three steps, denatured-annealing-extension, constitute a PCR cycle. This process is repeated for 20-40 cycles by amplifying the object sequence exponentially. The PCR is performed on a thermocycler, an instrument that is programmed for rapid heating, cooling

and maintenance of the samples for several times. The amplified product is then detected by removal of the reaction mixture by agarose gel electrophoresis.

PCR detection GM soya (RR soya)

The primers used for the PCR reaction, on the one hand, amplify endogenous regions of soybeans, in this case the soy lectin gene (Le1) that will give rise to a 318 bp fragment, and, on the other hand, will also amplify the foreign genes introduced in the process of formation of the transgenic, in this case the glyphosate resistance gene of *Agrobacterium tumefaciens* (EPSPS) that will give a fragment of 356 bp.



Agarose gel analysis of PCR products from different foods containing soya. The amplification of the normal and transgenic gene has been carried out separately, being the amplification products of the lectin gene, 1 and 2 (fragment of 318 bp), while 3 and 4 correspond to the transgenic gene (fragment of 356 bp). The foods studied are chocolate soy drink (1 and 3) and miso paste (2 and 4). These two foods have both normal and transgenic soya. On the other hand, they have performed a positive control of the normal gene (+ (S)), a positive transgenic control (+ (G)) and a negative control (-), where it is seen that there has been no amplification.

3. STATEMENT OF FACTS

We will go to the market to buy different food products containing soya to see if it belongs to a normal variety or transgenic.

For this the first thing we will do will be the DNA extraction of the different selected foods: 1. Green soya; 2. Powdered soya milk. Other products with soya can also be used (it is important to work with pulverized samples, for example, using a grinder). **SEE EXTRACTION PROTOCOL (ANNEX 1).**

Next, we will use the PCR MIX for the detection of RR transgenic soya.

4. EXPERIMENT COMPONENTS

COMPONENT		STORE
10x Concentrated electrophoresis buffer	100 ml	
Agarose	6.0 gr	
Corn samples for extraction	4 samples	
DNA food extraction Kit	For 25 samples	
Mix PCR detection of transgenic corn	2 x 350 µl	at -20°C
Positive control normal DNA	10 µl	at -20°C
Positive control transgenic DNA	10 µl	at -20°C
Gelsafe DNA staining	25 µl	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 EXTRACTION OF DNA FROM FOOD CONTAINING MAIZE

There will be 4 working groups.

Four food samples containing maize are supplied and the presence of normal or transgenic maize DNA is to be detected: 1. Green soya; 2. Powdered soya milk B; 3. Soya cookies; 4. Soya protein. Other products with soya can also be used (it is important to work with pulverized samples, for example, using a grinder). **SEE EXTRACTION PROTOCOL (ANNEX 1).**

For the extraction of the samples supplied, use 100 mg and for the samples to be evaluated, start with 200 mg.

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 5 µl (100-250 ng) of the DNA from each DNA extraction.

IMPORTANT:

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

REAGENTS	VOLUME
MIX PCR	20 µl
DNA (100-250 ng)	5 µl
Total volume	25 µl

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

IMPORTANT:

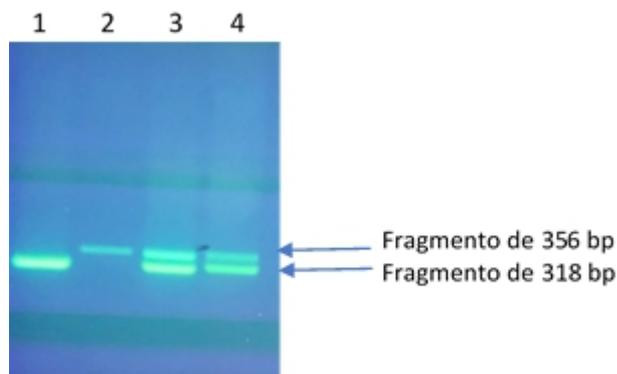
For activation of the "HOT STAR" Polymerase it is necessary to program an initial denatured step of 3 minutes at 95°C, then program the 35 specific cycles of each product to be amplified.

TRANSGENIC PCR PROGRAM

STEP	TEMPERATURE	TIME
Denaturetion HOT STAR	95°C	3 minutes
PCR cycles Carry out 35 cycles	95°C	45 seconds
	60°C	45 seconds
	72°C	25 seconds
Final extension	72°C	10 minutes
Final	4°C	

- The PCR product can be load directly onto a **3.0% agarose gel** after PCR, as the red dye acts as a loading buffer.
- Use the method of DNA detection or staining used in the laboratory. We recommend using the GELSAFE supplied with the kit.

6. PRACTICE RESULTS



3% Agarose gel

- Positive control of normal DNA
- Positive control of transgenic DNA (PCR with only primer transgenic)
- Positive control of transgenic DNA (PCR with both primers)
- Positive control transgenic DNA.

An example of a result of the amplification of the normal positive DNA and transgenic DNA controls provided in the kit is presented. We can observe in the case of normal soya only one band of 318 bp and in the case of transgenic soya the presence of the 2 bands, the band that share with normal soya, that is 318 bp, and the specific band at 356 bp.

7. QUESTIONS AND ANSWERS ABOUT THE PRACTICE

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

1. 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.

2. 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).

3. Which products we have purchased are normal and which GMOs?

The corn flour of brand B is transgenic while the rest of products containing corn, this is not modified.

4. Explain how a normal variety of a transgenic variety of Bt maize differs in an agarose gel?

The PCR reaction contains 2 sets of different primers, one amplifying a fragment of a gene from the maize invertase that will be present in all samples, and the other set will amplify a fragment of the exogenous gene of the *Bacillus thuringiensis* toxin which will also be present present in transgenic varieties.

5. What is your opinion on genetically modified or transgenic foods?

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

ANNEX 1

Protocol for extracting DNA from 100-200 mg of foods containing corn

Given the wide variety of samples that comprise maize-containing foods, it is difficult to present a universal protocol for all samples.

The main and most important step to obtain good yields is a good break and homogenization of the sample that will be specific for each type of sample. In all cases and for greater effectiveness liquid nitrogen should be used to spray the sample.

In solid powder samples (flours, etc.) homogenize with a hand electric homogenizer; in large solid samples (corn flakes, chocolate, cookies, etc.) use a coffee grinder to spray a large sample and then weigh the required amount of powder.

1. Weigh 100-200 mg of the sample into a 2.0 ml microtube and add **1.2 ml of CTAB-1 Buffer + 25 µl Proteinasa K**. Vortex vigorously. **In the samples supplied in the kit weigh only 100 mg.**
2. **Incubate at 65°C for 30 minutes.** Repeat vortex several times during incubation.
3. **Centrifuge at 14,000 rpm for 5-10 minutes.** A pellet will appear and a layer of grease on the surface, introduce the pipette tip through this surface layer, trying to collect only **500 µl of supernatant** which is the transparent liquid with color (avoid taking pellet and surface layer) and place in a microtube 1.5 ml.
4. Add **250 µl of Union Stool Buffer** to the 500 µl supernatant. Vortex vigorously
5. Add **all** in a microcolumn spin with his collection tube. **Centrifugate at 10.000 rpm for 60 seconds.** Eliminate collection tube.
6. Place the Spin microcolumn into a new collection tube and add **500 µl of Disinhibition Buffer** to the reservoir. **Centrifuge at 12,000 rpm for 60 seconds.** Remove the liquid.
7. Add **700 µl of Wash Buffer** to the Spin microcolumn reservoir. **Centrifuge at 12,000 rpm for 60 seconds.** Remove the liquid.
8. **Centrifuge at maximum speed for 2 minutes to remove residual ethanol.**
9. Remove the collection tube and insert the Spin microcolumn into a 1.5 ml microtube. Add **150 µl of Elution buffer** (preheated to 70°C) in the centre of the white membrane. Incubate for 2 minutes.
10. **Centrifuge at full speed for 60 seconds.** The microtube now contains the DNA.