PCR DETECTION OF STREPTOCOCCUS SALIVARIUS AND S.ORALIS IN SALIVA SAMPLES

Ref.PCR10

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 biology and microbiology of oral cavity bacteria.

2. BACKGROUND INFORMATION

2.1 Bacteria present in oral cavity

The oral cavity is an ideal environment for the colonization of bacteria, fungi, protozoa, archaea and viruses. This is because the oral cavity is a moist and warm cavity (35°C - 37°C), which is suitable for the growth of multiple microorganisms. The oral cavity not only provides abundant and continuous flow of nutrients for the growth of bacteria, also provides saliva proteins and glycoproteins, as components of the diet such as carbohydrates. As a result, the mouth harbours more than 700 species of bacteria and a broad spectrum of viruses, archaea, fungi and protozoa. But not all organisms found in the oral cavity are in the same proportion and throughout the mouth equally, because there are different microbiological niches, depending on the amount of nutrients that reach the area, pH, amount of oxygen, strength of saliva and chewing.

Saliva is a biological fluid that cover the oral cavity. It is a hypotonic liquid respect to plasma, and we find enzymes such as amylase, components of immunity, among other molecules in his composition. Finally, saliva is an easily accessible fluid, so it can be used for the evaluation of general oral health, DNA extraction, and other utilizations.

Among the different bacteria present in the oral cavity, the genus streptococcus is the predominant. Streptococci are found in most parts of the human body and are the dominant species in the human oral cavity and in the upper respiratory tract. The species of the streptococcus genus were initially differentiated according to their haemolysis pattern that they presented in blood agar plates, being classified into β-haemolytic (generate a complete lysis), α-haemolytic (generate a partial lysis) and γ-haemolytic (non-haemolytic). Many β-haemolytic species were later differentiated from each other thanks to the study of carbohydrate antigens, being divided into group A (*S. pyogenes*) and group B (*S. agalactiae*). Historically, the oral streptococcus par excellence has been *Streptococci viridans*. *S. viridans* is a gram-positive haemolytic bacterium, which has a characteristic haemolysis, forms a green halo around it. Today the study of haemolysis is still used to classify different streptococci.

Oral streptococci were initially classified into four groups: anginosus, mitis, mutans and salivarius. While streptococci found outside the oral cavity were classified in the bovis and pyogenic groups. Recently, using more robust phylogenetic studies, they reclassified them

into 8 groups: mitis, sanguinis, anginosus, salivarius, downei, mutans, pyogenic and bovis. All of them are found in the oral cavity, less pyogenic and bovis. The mitis group is the most widespread in the oral cavity with 20 different species.

The diversity of oral microflora increases during the first months of life. The pioneer of the different species of oral bacteria is mainly streptococci, particularly *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus oralis*. These early colonizers are followed by gram negative anaerobes, such as *Pretovella melaninogenica*, *Fusobacterium nucleatum*, and *Veillonella spp*. Bacteria such as *Streptococcus mutans* and *Streptococcus sanguinis* begin to colonize the oral cavity at the time the first teeth come out, being able to attack dental tissues in order to develop and mature oral biofilms. From the first month of life to the first year and in adulthood, the oral microbiome becomes more diverse and with specific mechanisms influenced by environmental factors and the immune system.

The human microbiome project launched in 2008 was a project that tried to define the microbiome of the different areas of the human body, including the oral cavity, being the first analysis of the diversity and abundance of bacteria in humans. This project studied the diversity of the flora of the human body through sequencing methods, thus being able to identify more organisms than using classical methods. Culture of bacteria from oral cavity is a challenge, because some oral bacteria need specific nutrient requirements for their growth, others are inhibited by substances present in the culture medium or substances that are produced by other bacteria.

Oral microenvironment

The physical and biological properties of the oral cavity can be very different among people, and from here comes the great diversity of microbiological communities that have adapted to the different niches of the oral cavity. Some communities colonize the oral mucosa such as the tongue, cheeks, gums and palate, while others colonize harder areas such as teeth. Other factors that affect the composition of the community are nutrient availability and redox potentials, pH, atmospheric conditions, salinity and access to saliva.

The burden of microorganisms in the mucous membranes is relatively low due to desquamation, however, colonization increases at the sites where this process does not occur. More specifically, oral biofilms develop and mature in places of the oral cavity that are relatively protected from the mechanical actions of the tongue, cheeks, food and tooth brushing. To persist in the oral cavity, oral microorganisms adhere to the mucosa or to other organisms. This co-accession facilitates nutritional cooperation, gene transfer and cell-cell signalling. In addition, this type of growth in biofilms makes them more tolerant of environmental stress, host defences, and antimicrobial agents compared to the isolated growth of different bacteria.

2.2 Streptococcus group

The Streptoccocus group is part of the normal flora of the mouth and upper respiratory tract and most of them are susceptible to penicillin G. Some of these species can cause endocarditis, tooth decay or disease in immunosuppressed people.

Streptococcus oralis

Streptococcus oralis is a commensal bacterium that is part of the Mitis group. *S. oralis* is a component of the human oral microbiota and the first colonizers of the oral cavity. It is a gram-positive, immobile, α-haemolytic bacterium that forms coco chains. The optimal growth conditions are at temperature between 30-35 ° C. The genome of *S. oralis* is a circular chromosome, which has a high similarity with *S. pneumoniae* and *S. mitis* chromosome. The difference with *S. pneumoniae* is that *S. oralis* has lost the pathogenicity genes.

S. oralis is an opportunistic pathogen that affects mostly immunosuppressed or hematologic cancers. It is known to cause cavities, bacterial endocarditis, respiratory distress syndrome in adults and streptococcal shock. The treatment of choice is penicillin.

Streptococcus salivarius

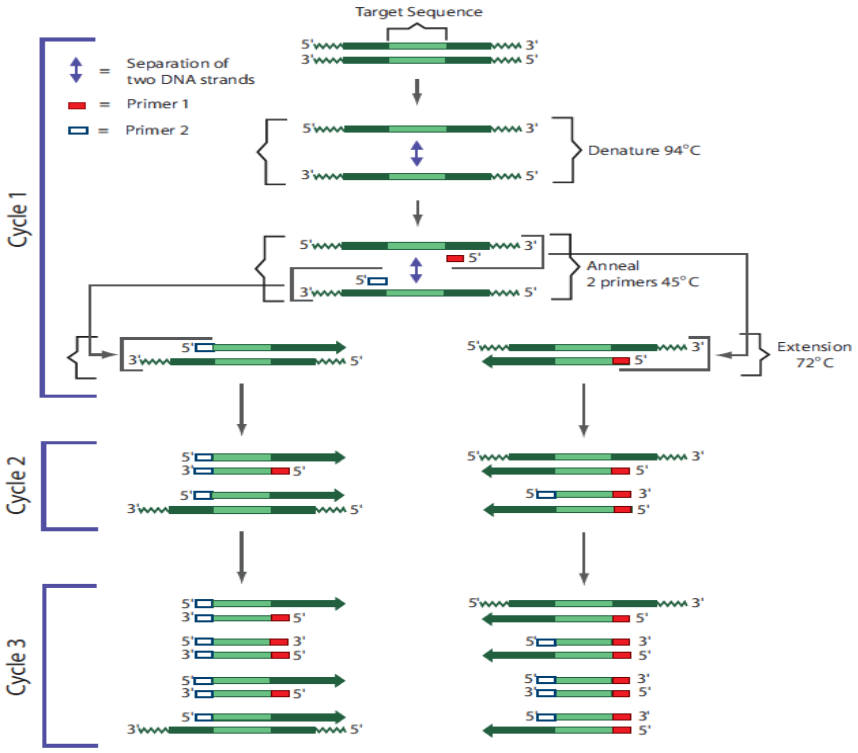
Streptococcus salivarius is a commensal bacterium. It is one of the first colonizers of the oral cavity, upper respiratory tract and intestines after birth. It is believed that it also helps homeostasis of the immune system and regulates the inflammatory response. It is an anaerobic facultative spherical gram-positive bacterium. The genome of *S. salivarius* is a circular chromosome of 2.2 million base pairs.

It is considered an opportunistic pathogen, being seen in cases of sepsis in immunocompromised patients with neutropenia, a disease associated with depletion of the white blood cell levels of organism.

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.

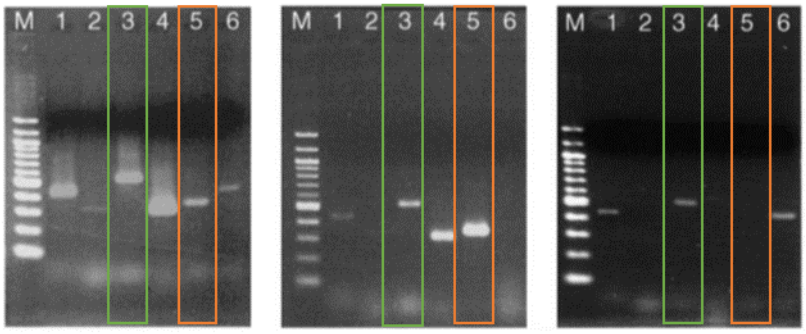
A typical PCR reaction contains template DNA, Taq polymerase and the 4 dNTPS in an appropriate reaction buffer. The total reaction volume is 25-50 µl. In the first step of the PCR reaction, the complementary strands of DNA are separated (denatured) from each other at 94°C, while the Taq polymerase remains 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.

PCR detection of Streptoccus oralis and Streptococcus salivarius

The primers that are used for the PCR reaction, on one hand, amplify the specific genes for the *S. oralis* bacteria, and, on the other hand, the specific *S. salivarius* genes. In this case, if there is presence of *S. oralis* in the saliva, a 374 bp fragment will amplify us, while if there is presence of *S. salivarius*, a 544 bp fragment will be amplified.



a. Patient no. 2 b. Patient no. 4 c. Patient no. 9

Agarose gel analysis of *S. salivarius* and *S. oralis*. The amplification of the different primers has been performed in several patients and independently. Corresponding to well number 3 the amplification for *S. salivarius* (marked in green) and number 5 for *S. oralis* (marked in orange). We can observe that three patients analysed in the image present *S. salivarius* in their saliva, while for *S. oralis* the patient 9 does not present this type of streptococcus in his saliva sample.

3. STATEMENT OF FACTS

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

4. EXPERIMENT COMPONENTS

COMPONENT		STORE
10x Concentrated electrophoresis buffer	100 ml	
Agarose	6.0 gr	
Mix PCR detection *S. salivarius* and *S. oralis*	2 x 350 µl	at -20ºC
Positive control DNA *S. salivarius* and *S. oralis*	10 µl	at -20ºC
Gelsafe DNA staining	25 µl	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 EXTRACTION OF DNA FROM SALIVA

Each student will collect a sample of their own saliva in a collection tube of saliva or glass **We RECOMMEND use our DANAGEN SALIVA kit.**

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 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 2,5 µl of positive DNA that we give in the 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.

REAGENTS	VOLUME
MIX PCR	**22,5 µl**
DNA (100-250 ng)	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 5 minutes at 94ºC, then program 35 specific cycles of each product to be amplified.

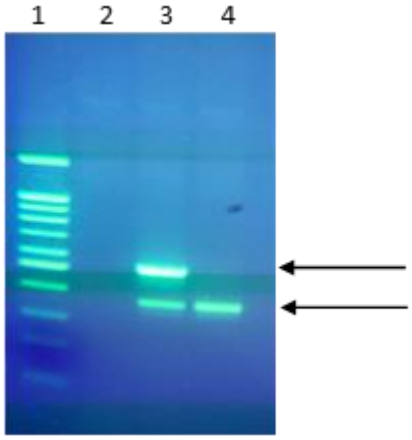
PCR PROGRAM

STEP	TEMPERATURE	TIME
Denaturetion HOT STAR	**94ºC**	**5 minutes**
PCR cycles **Carry out 35 cycles**	**98ºC** **66ºC** **72ºC**	**10 seconds** **60 seconds** **45 seconds**
Final extension	**72ºC**	**5-10 minutes**
Final	**4ºC**	

5. The PCR product can be load directly into **a 2.0% agarose gel** after PCR, as the red dye acts as a loading buffer.

6. Use the method of DNA detection or staining used in the laboratory. We recommend using the GELSAFE supplied with the kit.

6. PRACTICE RESULTS



2% Agarose gel

1: Molecular weight marker
2: Negative control
3: Sample 1 of saliva
4: Sample 2 of saliva

An example of the amplification result of the negative controls for the amplification is presented (check that the PCR reagents were not contaminated by DNA) and two saliva samples. In the first saliva sample (3) it is seen that there is amplification for the two streptococci studied, a band of 544 and 374 bp respectively appearing, while the second saliva sample only presents *S. oralis* in his saliva, thus seeing only 374 bp band amplified.

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. What type of streptococcus do you find in your saliva sample?

Those with two lines in the agarose gel will be positive for the two streptococci studied, while those who only have one line will have to see which of the two lines is whether the one with the highest molecular weight or the lowest in order to identify streptococcus.

4. How many people in the group of students who have done the practice have both types of streptococci in their saliva sample? And how many only one of them?

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.