PCR BACTERIAL 16S RNAr GENE

Ref. PCR16S

1. EXPERIMENT OBJETIVE

The objective of this experiment is to introduce students to the principles and practice of Polymerase Chain Reaction (PCR) by amplifying a fragment of the bacterial 16S rRNA gene using the PCR technique.

It is not necessary to isolate the DNA from bacteria, since a bacterial DNA sample is supplied to carry out the amplifications.

2. BACKGROUND INFORMATION

2.1 PCR

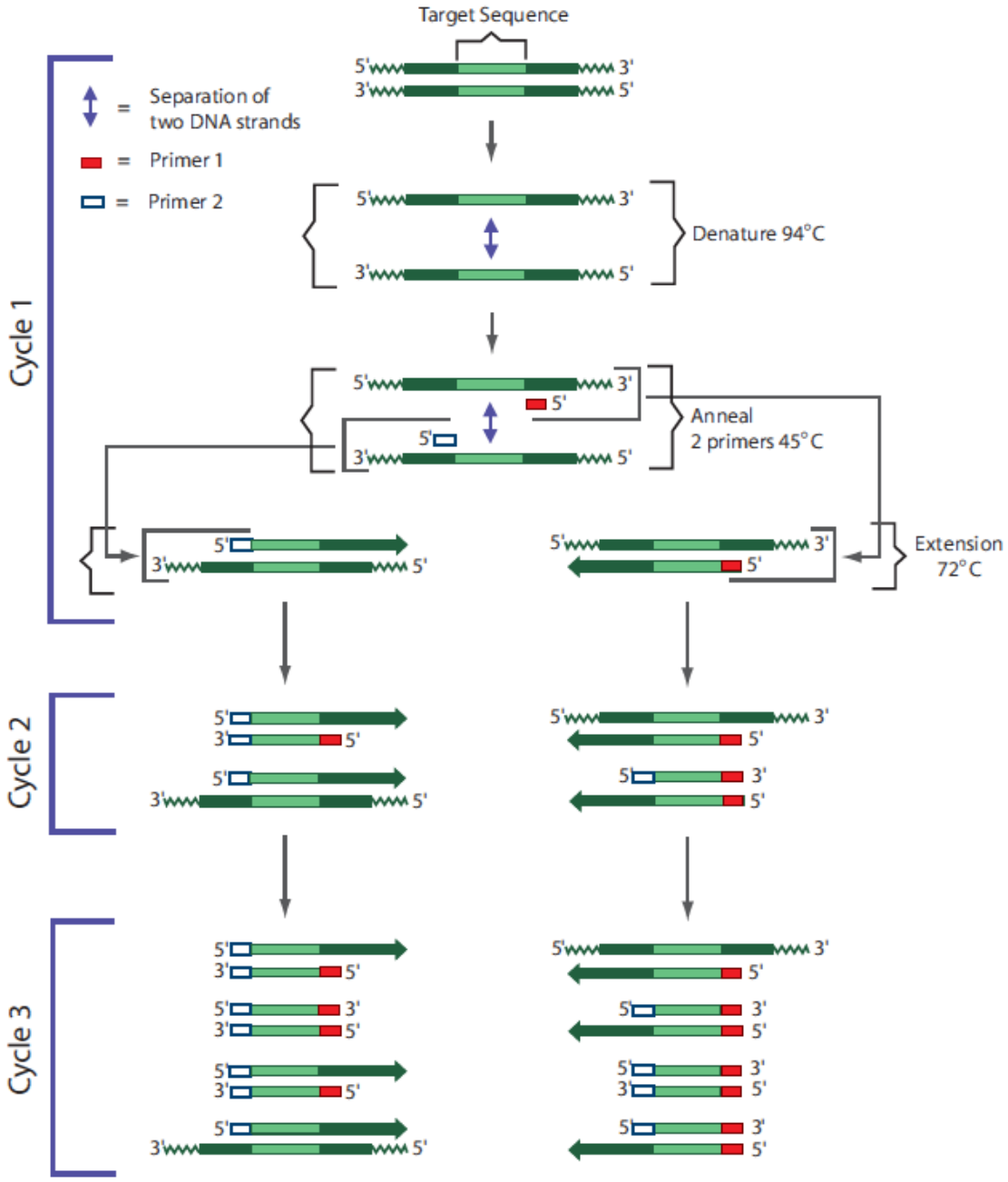
PCR has revolutionized research and diagnosis based on Molecular Biology. PCR is a simple, accurate and highly reproducible process that provides the advantage of starting with a small amount of DNA and being able to amplify it so that it has enough to perform experiments.

A large number of 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, clinical diagnosis, etc.

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

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 of 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 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, denaturation-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.

2.2 16s ribosomal RNA

It is a polyribonucleotide of approximately 1500 nucleotides, encoded by the rrs gene, also called **16S ribosomal DNA** (16S rDNA), from which phylogenetic and taxonomic information can be obtained from prokaryotes.

The rRNA folds into a secondary structure, characterized by the presence of double-stranded segments, alternating with single-stranded regions. In eukaryotes the 18S rRNA is the macromolecule equivalent. Their sequences are highly conserved, presenting regions common to all organisms, but contain variations that concentrate specific areas.

Sequence analysis of 16S rRNAs from different phylogenetic groups revealed the presence of one or more characteristic sequences that are termed **signature oligonucleotides**. These are short specific sequences that appear in all members of a particular phylogenetic group and never (or only rarely) are present in other groups, including the closest ones.

Comparison of 16S rRNA sequences (or the genes encoding them) allows establishing the phylogenetic relationships between prokaryotic organisms. In clinical microbiology the molecular identification based on 16S rDNA is mainly used for bacteria whose identification by another type of technique is impossible, difficult or time consuming.

The molecular method of bacterial identification by sequencing the 16S rDNA includes three steps:

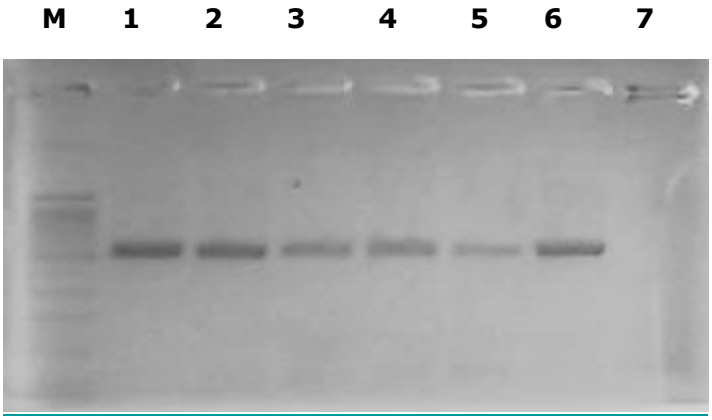
A) PCR amplification of the gene from the appropriate sample. **What we will do in this practice**.

B) Determination of the nucleotide sequence of the PCR product.

C) Analysis of the sequence.

Amplification of the 16S rDNA is achieved by a thermocycler thanks to the polymerase chain reaction (PCR). As the substrate, DNA purified from a pure culture of the pathogen is normally used. Alternatively, the DNA may be obtained directly from the clinical sample.

In this practice we will perform the amplification of the 16S rDNA gene using universal primers that will produce a fragment of 466 base pairs, such as template DNA can be used bacterial DNA by the students isolated from some bacterial culture or bacterial DNA that is supplied as a positive control.



PCR analysis of different individuals for the amplification of the bacterial 16S rRNA gene.

A 1.5% agarose gel is used which is stained with DanaGlue-Bioted DanaBlue for the detection of amplified PCR products using bacterial DNA.

Marker: DanaMarker Shumman.
Well 1 to 6: Amplification from different samples.
Well 7: Negative control.

3. EXPERIMENT COMPONENTS

Enough reagents are provided for carrying out 25 individual PCRs and the production of 4 gels of 1.5% agarose electrophoresis.

COMPONENT		STORE
10x Concentrated electrophoresis buffer	100 ml	
Agarose	2,5 gr	
PCR MIX	700 µl	at -20ºC
Positive control bacterial DNA	25 µl	at -20ºC

10x electrophoresis buffer to make 2 x 500 ml of 1x electrophoresis buffer which is the working buffer.

3.1 PCR Mix

Ready for use, it allows amplifying any fragment from DNA, so that the user only has to add the sample of the isolated DNA.

4. EXPERIMENT PROCEDURES

4.1 DNA Extraction

The step prior to any genetic study is usually the isolation of genomic DNA, this can be done in different ways (home methods, commercial kits, etc.) and from different samples (blood, tissue, bacteria, etc.).

To carry out this practice, it is not necessary to extract the DNA of the students, as it supplies a sample of bacterial DNA to perform the practice, although bacterial DNA can be used by students isolated from bacterial cultures.

4.2 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 bacterial DNA for each PCR reaction.

   IMPORTANT: **Prepare a negative amplification control** by placing **2.5 µl of nuclease-free water** instead of DNA, to see if the reagents, micropipettes or tips may be contaminated with DNA.

REAGENTS	VOLUME
PCR MIX	**22,50 µl**
Bacterial DNA (100-250 ng)	2,5 µl
Total Volume	**25 µl**

2. Mix well.
3. For those thermal cyclers that do not have a heated lid, add 25 µl of mineral oil to prevent evaporation.

16S ARNr PROGRAM

STEP	TEMPERATURE	TIME
Initial denaturation	**95ºC**	**10 minutes**
PCR cycles **Carry out 35 cycles**	**95ºC** **55ºC** **72ºC**	**30 seconds** **30 seconds** **45 seconds**
Final extension	**72ºC**	**10 minutes**
Final	**4ºC**	

4. The PCR product can be seeded directly onto an agarose gel after PCR, since the MIX contains red fill buffer.

5. Use the method of detection or staining of the DNA used in the laboratory. We recommend the use of **DANABLUE** or GELSAFE, our non-toxic methods.

6. A similar result to that observed in the figure should be obtained.

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