DETERMINATION OF THE Rh FACTOR BY PCR

Ref.: PCR2

1. EXPERIMENT OBJECTIVE

The aim of this experiment is to introduce students to the principles and practice of the Polymerase Chain Reaction (PCR) by studying the Rh determination using the PCR technique.

2. BACKGROUND INFORMATION

2.1 PCR

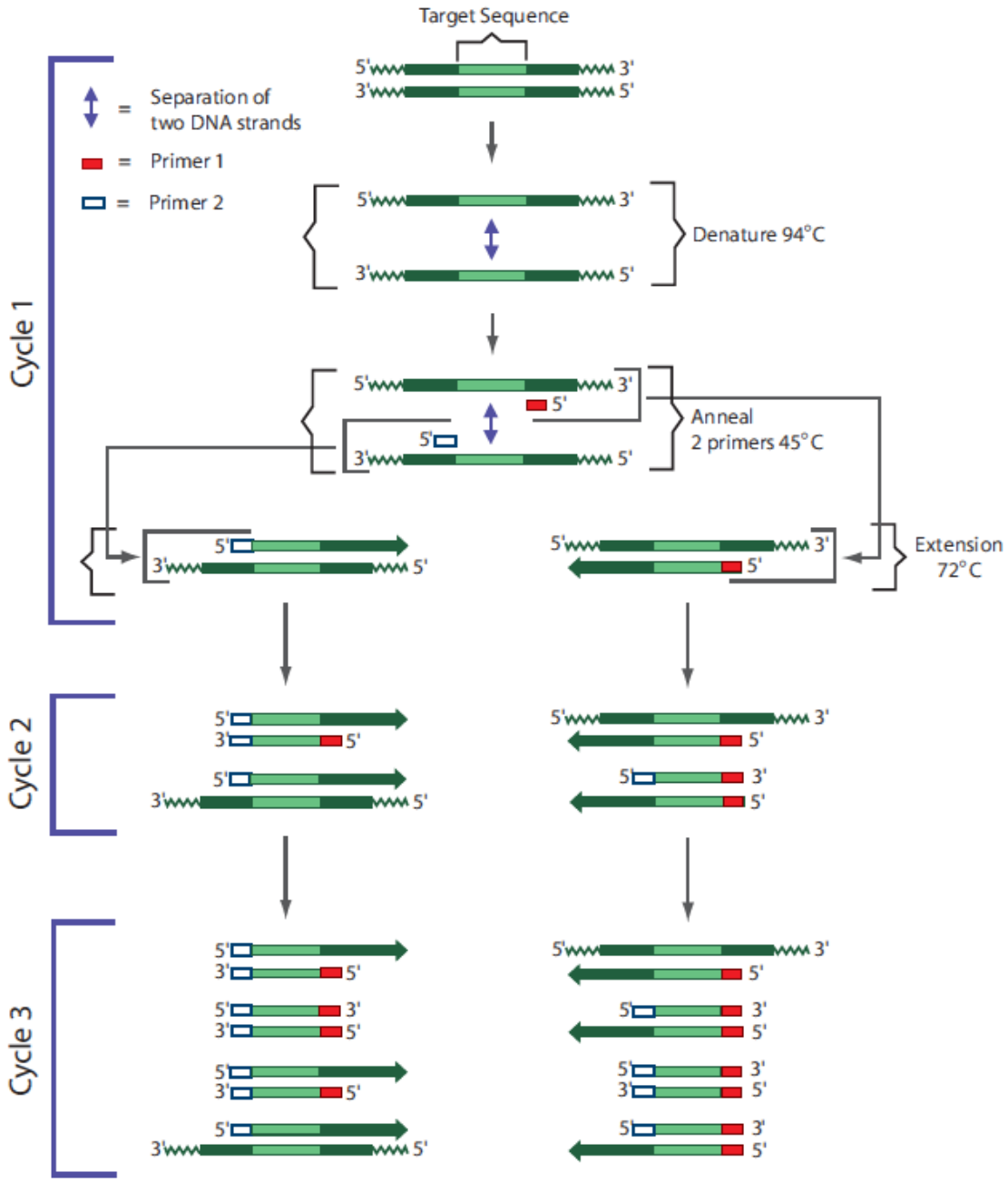
PCR has revolutionized research and diagnosis based on Molecular Biology. PCR is a simple, accurate and highly reproducible process that provides the advantage of starting with a small amount of DNA and being able to amplify it so that it has enough to perform experiments.

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 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 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 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, whereas 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 carried out in a thermocycler, an instrument that is programmed for rapid heating, cooling and maintenance of the samples several times. The amplified product is then detected by removal of the reaction mixture by agarose gel electrophoresis.

2.1 Rh factor

In 1940, Landsteiner and Wiener demonstrated that the antibodies produced against the red blood cells of the Rhesus monkeys were able to agglutinate about 85% of the blood erythrocytes of the human population.

Antibodies produced were shown to target a molecule called the Rhesus antigen (Rh), individuals who possessed this molecule were named Rh positive and the remaining 15% were named Rh negative.

The Rh locus consists of 2 structural genes: D and CcEe, which encode the D polypeptide chain and the C/c and E/e proteins, respectively.

The presence or absence of the D gene in the genome determines the genetic basis of Rh-positive / Rh-negative blood group polymorphism. Therefore, individuals are classified as Rh+ if they contain D antigen on the membrane of their erythrocytes. However, the Rh system is much more complex, and up to 47 different Rh antigens have been described.

The D antigen is highly immunogenic and induces an immune response in many Rh- individuals when they receive a transfusion with Rh+ blood. For this reason, routine checks on blood donors and individuals undergoing transfusion are routinely performed in many countries, so that Rh- patients receive only D negative products.

The Rh system has also been implicated in conditions such as hemolytic disease of the newborn, autoimmune hemolytic anemias, and non-immune haemolytic reactions. Haemolytic disease of the newborn occurs when a Rh-mother carries an Rh+ fetus, despite fetal erythrocytes being separated from the mother's circulation by the placenta, during pregnancy, fetal erythrocytes may escape to the mother circulation where they are considered as foreign and produce an immune response. Repeated pregnancies cause a high level of antibodies in the mother that can cross the placenta, reach the fetal circulation and react with the erythrocytes of the fetus causing its death. To avoid this, an immunosuppressive therapy is administered to the mother.

In this practice, the students will isolate the DNA from their saliva and use it to carry out the PCR reaction. **The basis of this practice is the amplification of a fragment of the D gene**. Amplification of this fragment will indicate that the person is Rh+. Because the D and CcEe genes are highly homologous, it is possible to design primers that pair in a region of the D gene and also to a region of the CcEe gene (see Figure 1).

Thus, all DNA samples serve as a template for amplification. **Rh+ individuals will produce 2 fragments of different sizes (1200bp and 600bp) and Rh- individuals a single band corresponding to the CcEe gene fragment (1200bp)**.

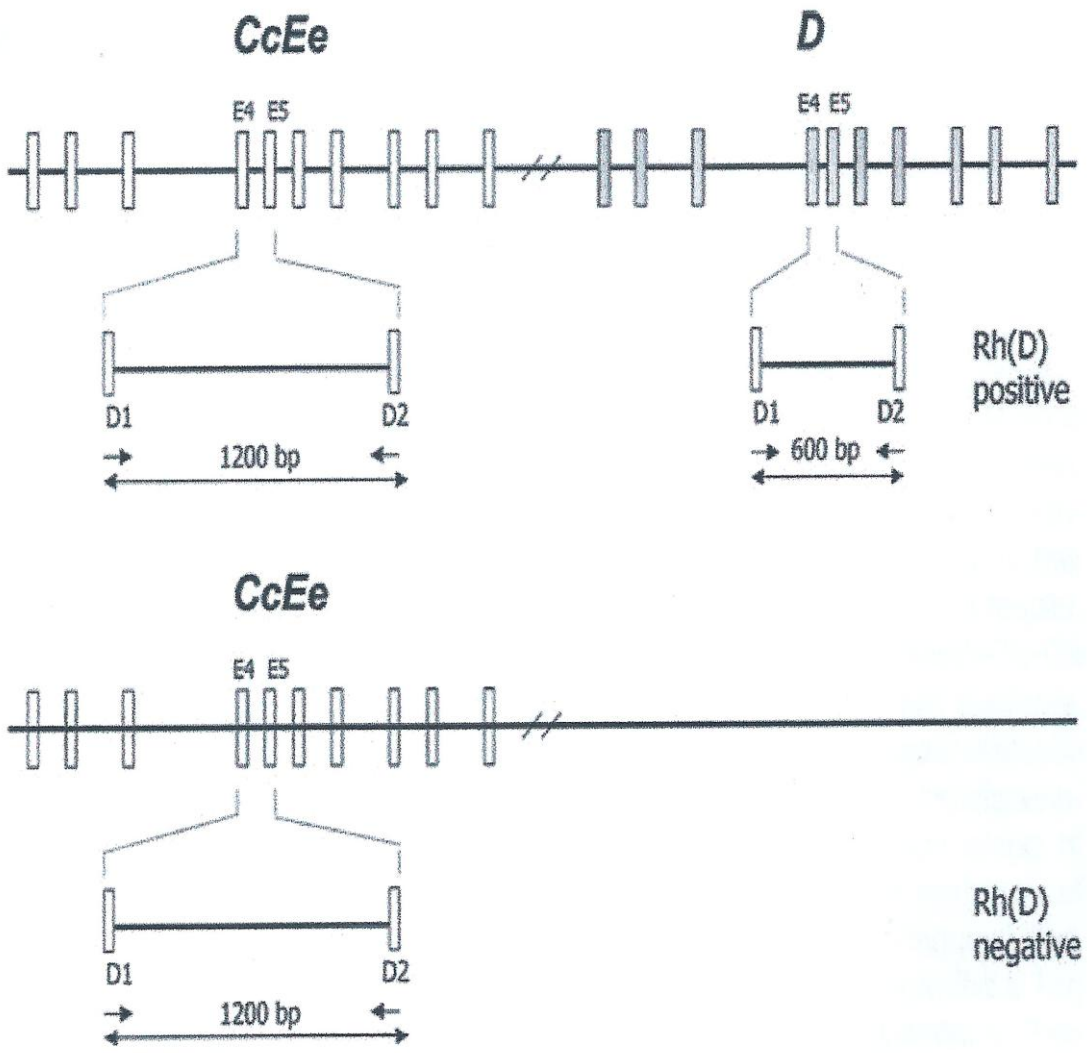


Figure 1

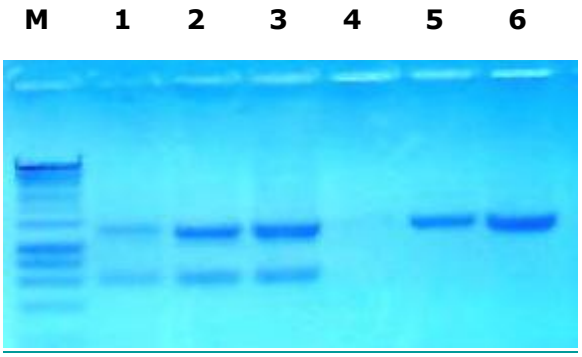


Figure 2

Fig. 2: PCR analysis for the Rh determination of different individuals.
A 1% agarose gel stained with the **DanaBlue of DanaGen-Bioted** is used for the detection of amplified PCR products using genomic DNA from different individuals and using the Rh-Forward and Rh-Reverse primers.
Marker: DanaMarker BEETHOVEN.
Well 1: Rh +
Well 2: Rh +
Well 3: Rh +
Well 4: Negative control.
Well 5: Rh-
Well 6: Rh-

3. EXPERIMENT COMPONENTS

Enough reagents are provided for the performance of 25 individual PCRs and the production of 4 gels of 1% agarose electrophoresis.

Electrophoresis buffer 10x concentrate	100 ml	
Agarose	1.5 gr	
PCR MIX	2 x 350 µl	Store at -20ºC
Rh+ positive control	20 µl	Store at -20ºC

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

3.1 MIX POLYMERASE HOT STAR

The polymerase is 2x concentration ready for use, which allows amplification of any fragment from DNA. The user only has to add water. **A 10 minute activation step is required at 95°C** so that non-specific products as "primers-dimers" are removed. It also contains a **red dye** that allows easy visualization and direct seeding into the gel without the need to mix with a loading buffer.

4. PRACTICE

4.1 DNA extraction

The previous step 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, etc.).

For the practice of this practice it is recommended that the source of the DNA comes from the **student's saliva**, since it is the most accessible source of DNA and does not pose any risk, such as blood draw. To this end, the use of **DANAGENE SALIVA KIT** is recommended, which allows the genomic DNA to be obtained from a saliva sample or oral smear.

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 each student's DNA for each PCR reaction.
IMPORTANT:
a) **Prepare a negative amplification control** by placing **2.5 µl of nuclease-free water** instead of DNA, this is to know if reagents or micropipettes and tips may be contaminated with DNA. In the negative control, nothing is to be amplified.
B) **Prepare a positive amplification control** by placing **2.5 µl of the Rh+ positive control** instead of the DNA.

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

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.

2. Mix well; the red dye included in the polymerase facilitates the process.

3. For thermocyclers that do not have a heated lid, add 25 µl of mineral oil to prevent evaporation.

4. Perform the amplification process.
IMPORTANT: For the activation of the "HOT STAR" Polymerase, it is necessary to program an initial denaturation step of 10 minutes at 95ºC, then program the 30 or 40 specific cycles of each product to be amplified.

Rh PROGRAM

STEP	TEMPERATURE	TIME
Denaturation HOT STAR	95ºC	10 minutes
PCR cicles Perform 35 cicles	95ºC 64ºC 72ºC	30 seconds 30 seconds 45 seconds
Final extension	72ºC	10 minutes
Final	4ºC	

5. The PCR product can be loaded directly in an 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 the use of DANABLUE or GELSAFE, our non-toxic methods.

7. A result similar to that observed in Figure 2 is to be obtained.

8. The observed frequency of the different polymorphisms in the class can be calculated.

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