STUDY OF VNTR HUMAN POLYMORPHISMS BY PCR

Ref. PCR1

1. OBJECTIVE OF THE EXPERIMENT

The objective of this experiment is to introduce students to the principles and practice of Polymerase Chain Reaction (PCR) by studying the VNTR polymorphism (D1S80 polymorphism) between individuals 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.

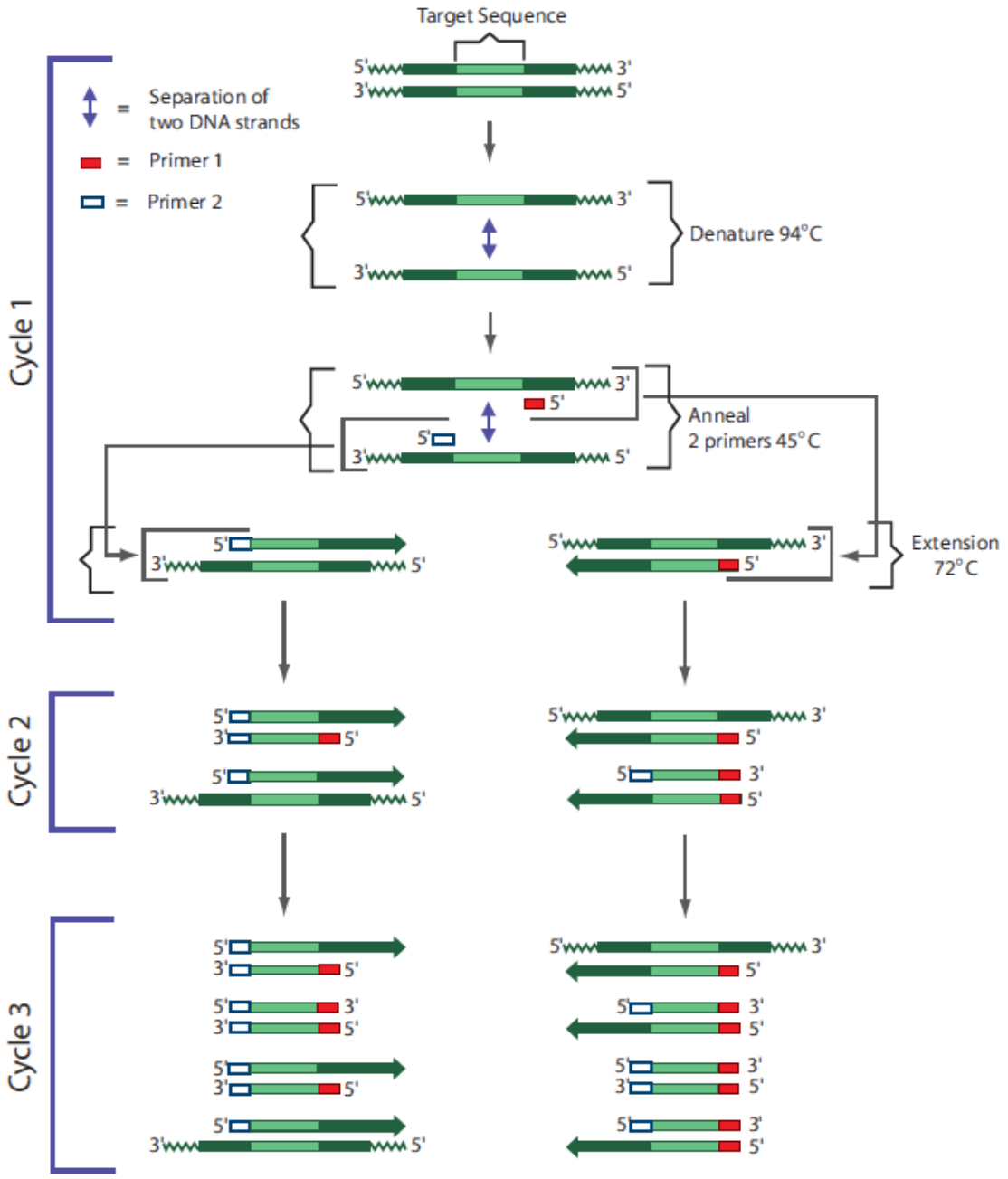

Figure 1

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.

2.2 D1S80 Human Locus

DNA polymorphisms refer to regions of chromosomes that vary widely from one individual to another. By examining several of these regions within the genomic DNA obtained from an individual, the "finger fingerprinting" of an individual can be determined.

DNA polymorphisms are used to determine paternity/maternity, identification of human remains, and the genetic basis of disease. The most usual application has been in the forensic field for the identification of possible criminals.

Different types of repetitive DNA have been found useful for fingerprinting DNA analysis: **VNTRs** or minisatellites, and **STRs** or microsatellites. By analyzing different VNTRs or STRs of the same individual, researchers can obtain unique DNA fingerprinting that will be unlike any other individual except for identical twins.

The **Variable Number of Tandem Repeats** have a sequence ranging from 7 to 100 bp in length, while the **Short Tandem Repeats** sequence is less than 2-6 bp in length. The number of repetitions can vary considerably.

The **D1S80 Human Locus** is present on chromosome 1 and contains a consensus VNTR of 16 bp (AGGACCACCAGGAAGG). The allele with the lowest number of replicates contains 14 replicates, while the allele with the most repeats contains up to 72 replicates. The most common allele contains 18 and 24 replicates, while the rarest contains 14 and 38. There is no known phenotype associated with the D1S80 locus, making it ideal to distinguish individuals only by their DNA sequence.

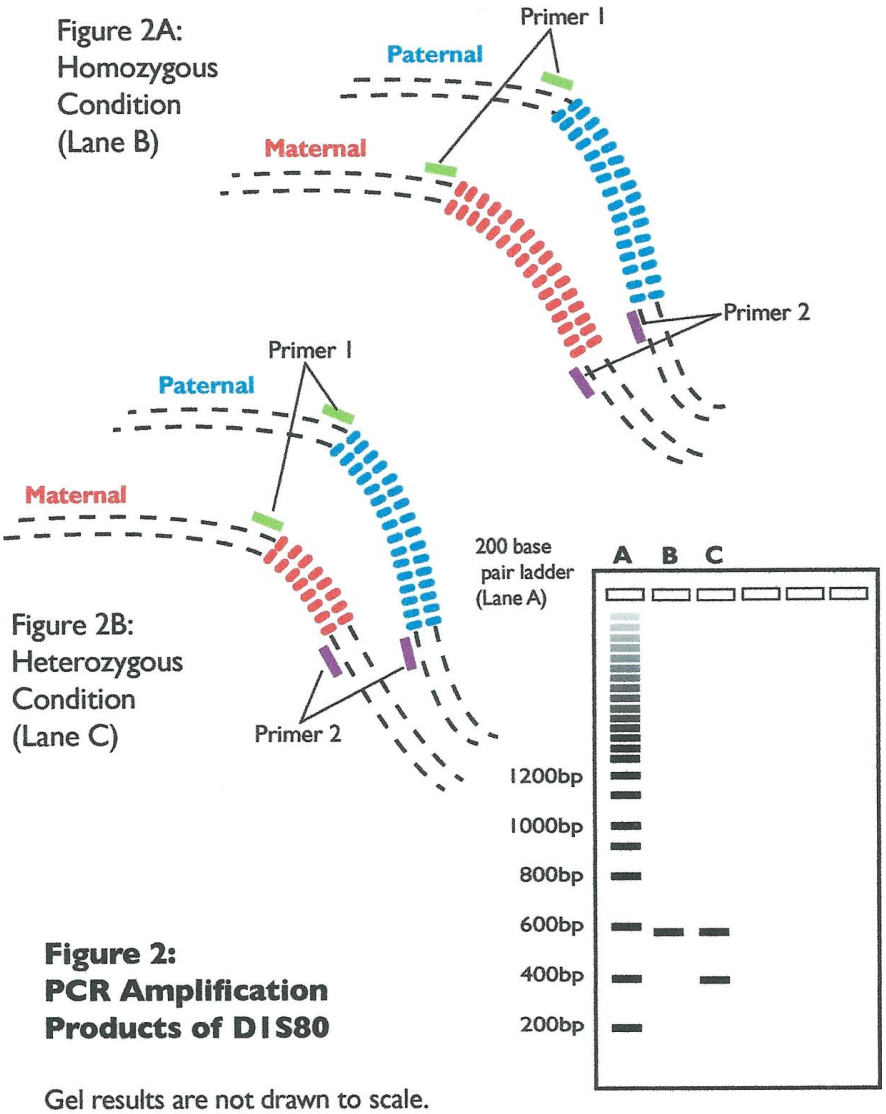

Fig. 2: An individual who is homozygous for the D1S80 locus will have the same number of replicates on both chromosomes 1, which will lead to a single band on an agarose gel after PCR. More common, a person will be heterozygous presenting different number of repeats from one chromosome to another; this will produce 2 bands on an agarose gel after the PCR. Currently, many official organisms are using STRs as they are more easily amplified and less initial DNA is required.

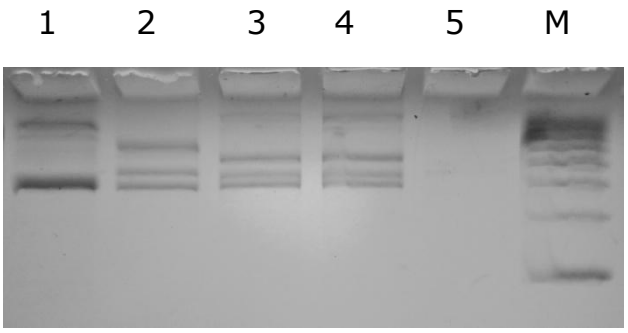

Fig. 3: PCR analysis of different individuals for the study of D1S80 polymorphism.

*A 2.5% agarose gel which is stained with the **DanaBlue-FlashBlue from DanaGen-Bioted** is used for the detection of amplified PCR products using genomic DNA from different individuals.*

M: Molecular weight marker.
Well 1: Homozygous for the D1S80 locus.
Well 2, 3 and 4: Heterozygotes for the D1S80 locus.
Well 5: Negative control.

It is true that more than one band is observed in both homozygotes and heterozygotes corresponding to nonspecific amplifications and the junction of the fragments generated by a larger fragment.

Agarose gel electrophoresis using standard molecular weight markers cannot be used to determine the exact number of repeats due to insufficient resolution of the agarose. However, it does serve to show clearly the different polymorphisms that will present several samples of individuals.

The allele with the lowest number of replicates contains 14 replicates, while the allele with more replicates has up to 48 replicates, so the known genotypes of the D1S80 locus may have fragments ranging from 385-815 bp. There are more than 22 known alleles being the most common allele that contains 18 and 24 replicates, while the rarest contains 14 and 38. There is no known phenotype associated with the D1S80 locus, making it ideal to distinguish people only by their DNA sequence.

Polyacrylamide gel electrophoresis is the method of choice for analysis of the D1S80 locus or capillary electrophoresis which is currently performed in most forensic identification laboratories.

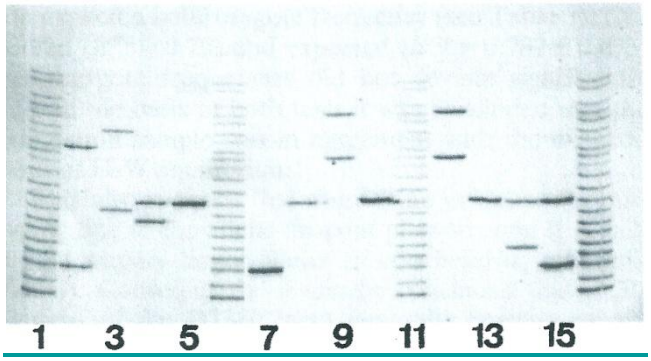

Fig. 3: Polyacrylamide gel showing different genotypes for the D1S80 locus

Well 2: Control DNA for the genotype of 18-29 fragments.
Well 3: Genotype of 23-31 fragments.
Well 4: Genotype of 22-24 fragments.
Well 5: Genotype of 24-24 fragments.
Well 7: Genotype of 18-18 fragments.
Well 8: 24-24 Fragment Genotype.
Well 9: Genotype of 28-33 fragments.
Well 10: Genotype of 24-24 fragments.
Well 12: Genotype of 28-33 fragments.
Well 13: 24-24 Fragment Genotype.
Well 14: Genotype of 20-24 fragments.
Well 15: Genotype of 18-24 fragments.

3. EXPERIMENT COMPONENTS

Enough reagents are provided for the performance of 25 individual PCRs and the production of **4 gels of 2.0-2.5% agarose electrophoresis**.

Electrophoresis buffer 10x concentrate	100 ml	
Agarose	3.0 gr	
PCR MIX	2 x 350 µl	Store at -20ºC
DNA 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.

*** The 2% agarose concentration allows a better separation of fragments less than 1000 base pairs.**

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.

3.2 PRIMERS

The primers used for the D1S80 locus are those described by Budowle et al.

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 DNA positive control**.

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

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

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

5. 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.

D1S80 PROGRAM

STEP	TEMPERATURE	TIME
Denaturation HOT STAR	95ºC	10 minutes
PCR cicles Perform 35 cicles	95ºC 67ºC 72ºC	30 seconds 30 seconds 1 minut
Final extension	72ºC	10 minutes
Final	4ºC	

6. The PCR product can be loaded directly in an agarose gel after PCR, as the red dye acts as a loading buffer.

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

8. A result similar to that observed in Figure 3 is to be obtained.

9. 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

1. El producto de la PCR puede ser sembrado directamente en un gel de agarosa después de la PCR ya que el colorante rojo actúa como tampón de carga.
2. Utilizar el método de detección o tinción del ADN que se use en el laboratorio. Le recomendamos el uso del DANABLUE-FLASHBLUE o GELSAFE, nuestros métodos no tóxicos.
3. Se ha de obtener un resultado similar al observado en la figura 1.
4. Se puede calcular la frecuencia observada de los diferentes polimorfismos en la clase.

Para cualquier duda o consulta adicional, por favor, contacte con nosotros bioted@arrakis.es