10: Polymerase Chain Reaction (PCR) locus PV92

Polymerase Chain Reaction or PCR allows important amplification (a million times) of a single DNA fragment. This method is widely used in molecular biology, medical diagnosis, the determination of microorganisms and even criminology. Kary Mullis, in the 1980s, invented the PCR. For this discovery, he obtained the Nobel Prize in Chemistry in 1993. The discovery of thermoresistant DNA polymerases (Taq polymerase) isolated from bacteria (*Thermus aquaticus*) living in extreme conditions permitted the automatization of the PCR.

Themes: PCR, structure of human genome, genotyping, criminology, DNA and electrophoresis.

PCR

Principle of the PCR:
A PCR reaction requires:

1) DNA to amplify (called the template)
2) Primers
3) Desoxynucleotides triphosphates (dATP, dCTP, dGTP, dTTP)
4) A thermoresistant DNA polymerase (Taq polymerase for instance)
5) Magnesium (Mg^{2+}) necessary for the activity of the Taq polymerase

These five elements are mixed in an Eppendorf tube and are then processed to different temperature cycles.

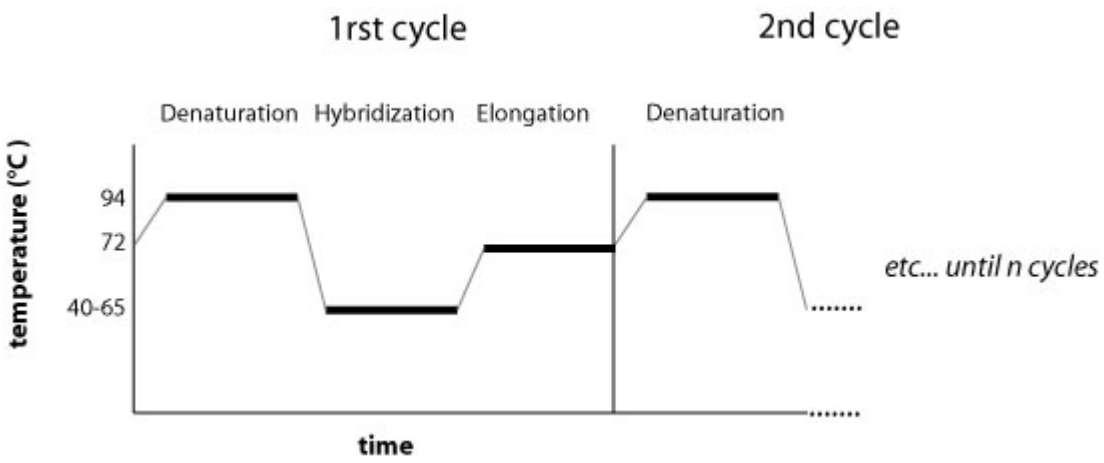
1rst step: **Denaturation** (94°C)
The DNA template (double-stranded DNA) is heated up to 94°C. The two strands of the DNA are separated from one another. We call this step the denaturation of DNA.

2nd step: **Hybridization** (40-65°C)
The temperature is lowered, the primers will hybridize to the target sequence of the single-stranded DNA by complementarity.

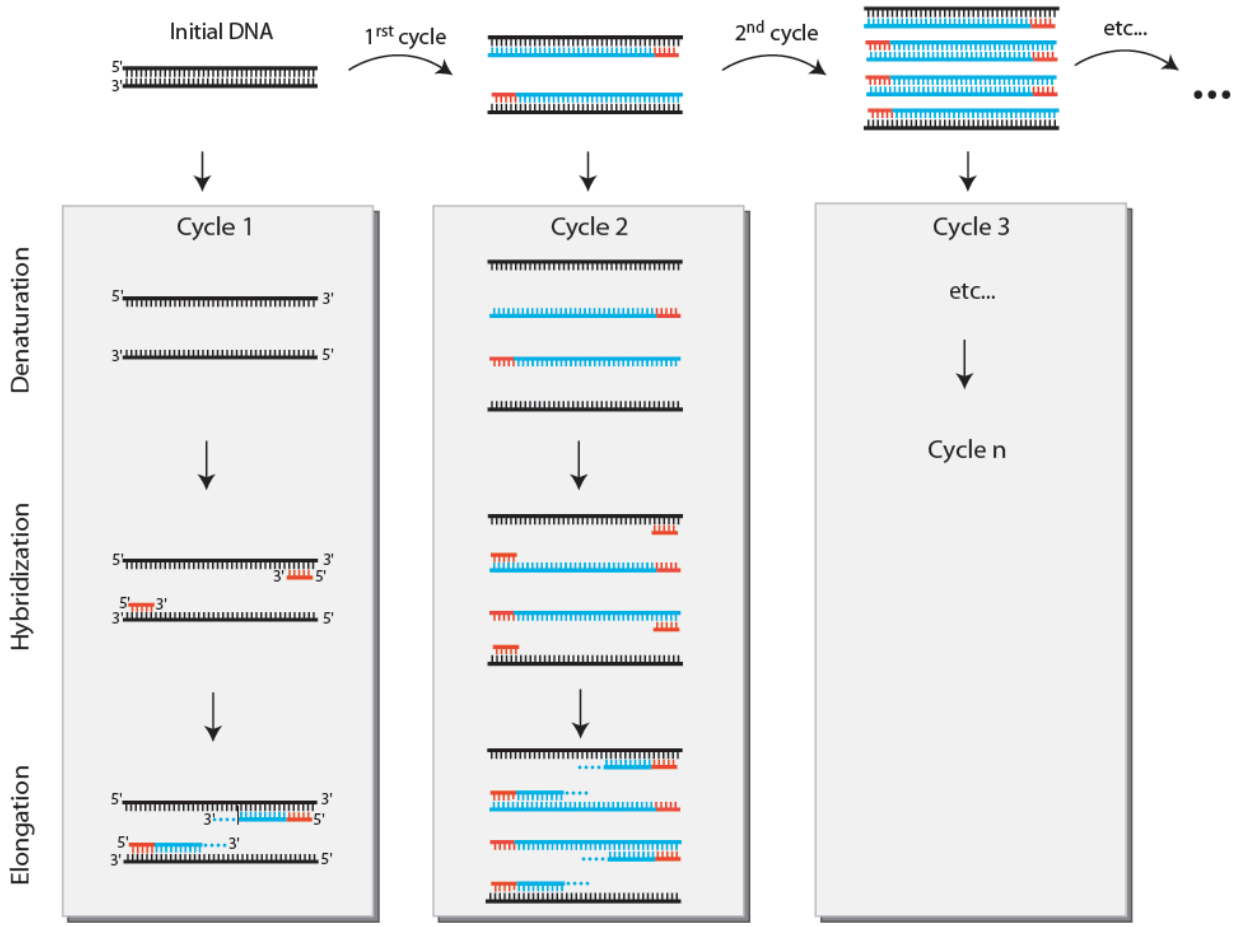
3rd step: **Elongation** (72°C)
At this temperature, the Taq polymerase synthesizes complementary DNA strands by adding dNTPs right after the primers. The elongation time depends on the size of the DNA fragment to amplify and on the enzyme polymerization speed. (The Taq polymerase adds 1000 nucleotides per minute).

These three steps constitute the first cycle of the PCR. At the end of it, we have doubled the number of initial target DNA fragment. Usually, 30 cycles are required in a classical PCR experiment. Therefore, after 30 PCR cycles, we will have amplified 2^n times our target DNA.

Here are represented the different steps in a PCR cycle:

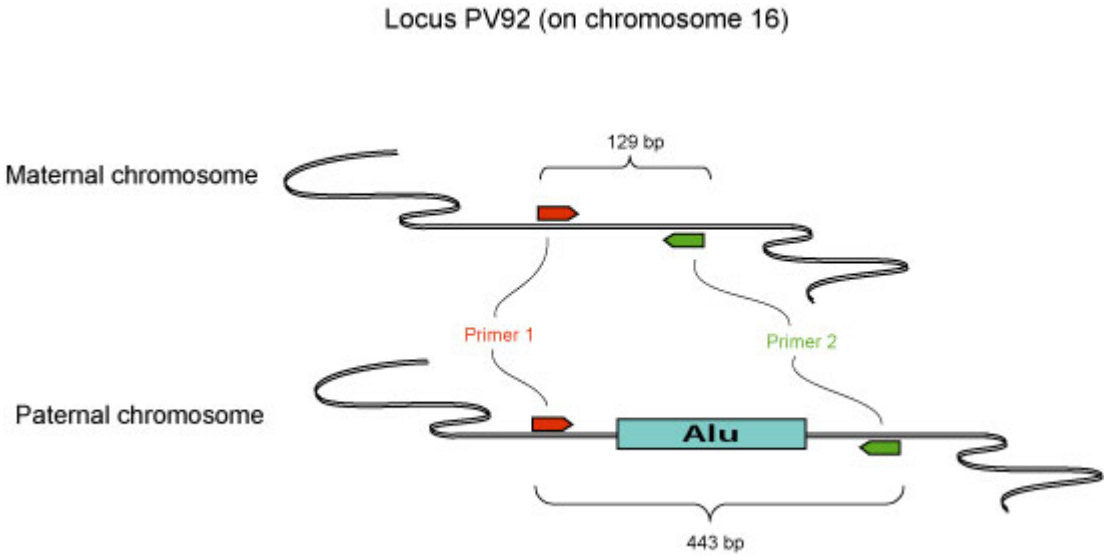


PCR amplification mechanism:



Alu sequences

Human DNA is composed of a great number of repeated sequences which represent more than 50% of the genome. Alu sequences are one of the most abundant repetitive DNA. They are approximately 300bp in length and were found in the Primate kingdom. They were named after the Alu I restriction enzyme site that can be found in the Alu sequence. These sequences are spread all over the genome thanks to the retrotransposition process that uses intermediate RNA. These elements contribute to the evolution of the genome, its architecture but can also be the cause of several genetic diseases. Alu retrotranspositions still continues in the youngest subfamilies but is a rare event. The insertion of a few Alu sequences has occurred recently (about 1 million years ago) and cannot be found in every Human being. This polymorphism may be used to study differences between populations. For instance, the locus PV92 localized on the chromosome 16 may or may not contain an Alu sequence, depending on the person considered: Some person possess an Alu sequence on both of their chromosome 16 (+/+), only on one chromosome (+/-) or have none (-/-).



Experiment

Protocol:

1) DNA extraction from mouth epithelial cells

- Put 500 µl of water in an Eppendorf tube.
- Rub inside of your cheek with a cotton swab to obtain a good amount of epithelial cells.

- Soak the swab in the tube containing 500 µl of water. Turn and press the swab in the inner surface of the tube.
- Wring well the swab when you take it out of the tube.
- Centrifuge the tube 2 min at the maximal speed to let the cells fall at the bottom of the tube.
- Take out the supernatant with the P1000. Be careful not to suck the pellet (leave it at the bottom of the tube).
- Add 0.1 ml of the NaOH solution 200 mM on the pellet. Resuspend it using the vortex or with the pipette P1000.
- Close the tube and incubate it at 95°C during 10 min in the heating block.
- Vortex a little
- Add 0.1 ml HCl 200mM in your tube.
- Add 0.1 ml Tris-HCl (pH 8.5) 200mM in your tube.
- Your DNA preparation is ready and can either be stored to the freezer or directly used for the following steps of the experiment.

2) Amplification by PCR

- Prepare the mix of reagents for the PCR right before use. This mix contains 2x Taq mix (Taq polymerase, dNTPs, polymerization buffer and red loading buffer), the primers and some water.

For a class of 16 pupils for instance, we count 16 reactions plus a negative and a positive control. In the end, we have 18 reactions. In order to make easier the pipetting and to have enough mix of reagents in case of mistake, we prefer to prepare a mix for 24 reactions in total.

	1 reaction	**24 reactions**
2 x Taq mix (red)	12.5 µl	300 µl
Primers mix	5 µl	120 µl
DMSO	1.25 µl	30 µl
H2O	1.25 µl	30 µl

- The mix is prepared in a 1.5 ml Eppendorf tube just before use. Mix the solution in the tube with your pipette (by careful aspiration and release).
- Share 20µl of the reagent mix out in the little PCR tubes on which you will have written (on the side of it) the initials of the person to be tested.
- In your little tube, add 5µl of your DNA solution. Close the tube.
- In the little PCR tube bound to be the negative control, add 5µl of water instead of your DNA solution. Close the tube.

- In the little PCR tube bound to be the positive control, add 5µl of the DNA B2B. Close the tube.
- By following the how to use instructions of the PCR machine, put the tubes in the machine and start the program PV92.
- The program lasts about 1h30. It has the following cycles:

94°C, 5 min
94°C, 30 s
54°C, 30 s
72°C, 30 s } 40 cycles
72°C, 5 min

- At the end of the PCR cycles, the tubes can be stored in the freezer.
- Stop the PCR machine following the given instructions.

3) Preparation of an agarose gel

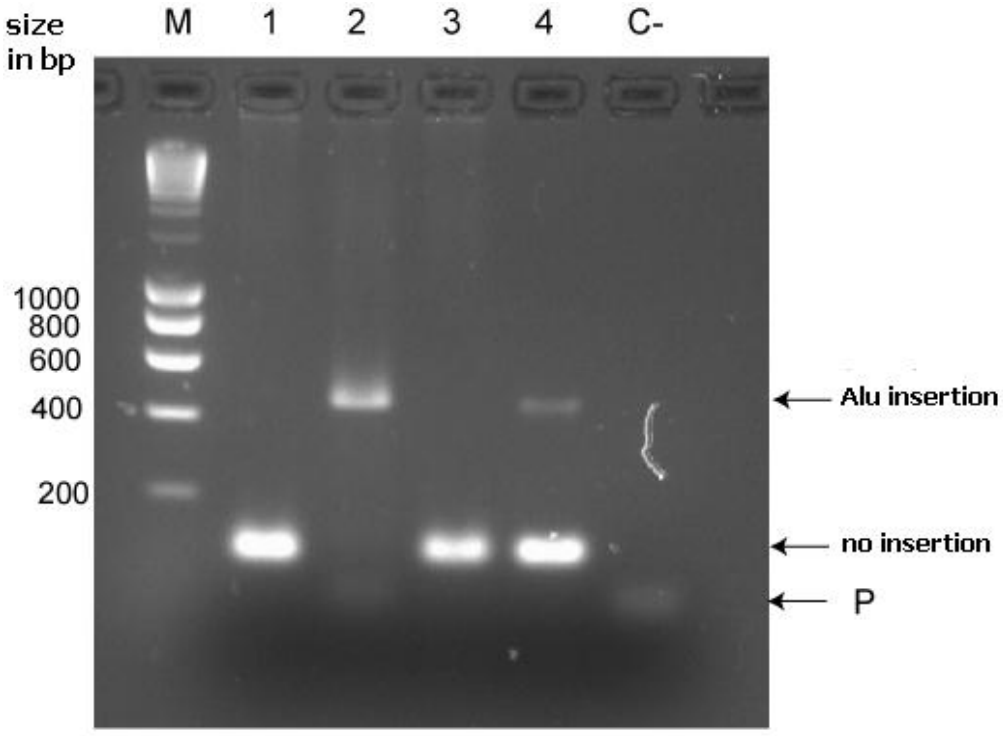
Agarose gel is a matrix which separates DNA molecules by size with an electric field. Small fragments migrate more quickly than bigger ones. The distance between DNA bands of a given length is determined by the percent agarose in the gel. In our present experiment, we decide to use a 1.5% agarose gel.

- Weigh 1.05g of agarose and put it in a 250mL Erlenmeyer.
- Add 70mL of electrophoresis buffer (TBE 1x)
- Boil the mixture (microwave oven or Bunsen burner) to dissolve the agarose until the solution becomes clear. Be careful that the solution does not boil over during the heating step! If there are still some agarose at the bottom of the Erlenmeyer, boil the mixture again.
- Let the solution cool down to about 60°C at room temperature. Stir or swirl the solution while cooling.
- Add 7µL SYBR-safe (ultra-fluorescent dye used to visualize the DNA) and stir the solution.
- Pour the solution into the gel rack. Insert the comb in the rack to form wells (slots to load the DNA preparation). Be careful not to pour a hot agarose solution because it will put the rack out of shape.
- When the gel has cooled down and become solid, carefully remove the comb. Remove a band of gel at each side of your electrodes (upper and lower sides of the rack).
- The gel must be completely covered with TBE.

4) Genotype analysis on the gel

- Take your tubes back. They already contain the loading buffer (red) and are ready to be put on the gel.
- Load on the gel: - first well: 5µL DNA ladder (molecular weigh marker)
  - second and following wells: 20µL of your sample
  - penultimate well: negative control
  - last well: positive control
- Switch on the generator and put the gel to migrate at 100V (maximal amperage).
- When the red dye has reached the bottom of the gel, switch off the generator.
- Let the gel cool down a bit (you can even put it in the fridge for a couple of minutes) so that the intensity of the signal will be higher.
- Take the gel and observe the bands under the blue lamp.
- Take a picture of it.

5) Results and discussions



M: Molecular weigh marker
Columns 1 to 3: genotypes from three distinct persons (1 is -/-, 2 is +/+, 3 is -/-)
Column 4: positive control (+/-)
C-: negative control (no DNA). This control allows us to check the absence of DNA contaminations.
P: primers

- Have a look at your genotype and the other persons'.
- Calculate the frequences of the class **genotypes** and **alleles**.
- Calculate the **Hardy-Weinberg** value for the class.

Material

- P20 pipettes with their sterile tips
- P200 pipettes with their sterile tips
- P1000 pipettes with their sterile tips
- Sterile swabs or ear cotton
- Gloves
- Microcentrifuge
- Heating block (95°C)
- Eppendorf tubes (1.5mL)
- PCR tubes (0.2mL)
- PCR machine
- Sterile water
- NaOH (200mM)
- HCl (200mM)
- Tris-HCl pH 8.5 (200mM)
- 2x Taq mix (including Taq DNA polymerase, dNTPs and the buffer)
- Primers
- Agarose
- Electrophoresis or gel rack
- Buffer for migration TBE 1x
- SYBR-Safe
- Blue lamp (DNA detection)
- Molecular weigh marker
- DNA B2B (C+)
- DMSO

Material storage

- The 2x Taq mix is stored at -20°C and thaw it on the ice (or briefly at room temperature). You should keep it in the freezing block during the manipulations and put it back at -20°C after use.
- The SYBR-Safe is stored in the fridge (+4°C). You need to take it out of the fridge at least 30min before you use it. This way, it will be soluble enough for the experiment.
- The DNA marker (DNA B2B = positive control) and the primers are stored at -20°C. They are thawed at room temperature and put back at -20°C after use.
- The rest of the material can be kept at room temperature.