GENETIC DIAGNOSIS OF HEREDITARY CANCER

Ref. PCRCAN (4 practices)

1. EXPERIMENT OBJETIVE

The aim of this experiment is to introduce students to the principles and practice of **Polymerase Chain Reaction (PCR)** as a tool for the genetic diagnosis of cancers with an inherited component.

Students will acquire basic knowledge about the molecular biology of cancer by studying the case of a **tumour suppressor gene** such as **p53**.

2. BACKGROUND INFORMATION

2.1 Tumour suppressor genes, p53

Many factors have been identified that contribute to the onset of cancer, including exposure to certain carcinogens in our diet and environment. In addition, some forms of cancer that have a family tendency have been found. **This group of cancers appear attached to the inheritance of mutated suppressor genes, such as p53**.

Although familial cancers collectively constitute a small fraction of the known cancer total, they are presented as a dominant inheritance model. Mutations that are directly inherited correspond to **mutations in the germ line**; these mutations can be detected in familial pedigrees. A second type of mutation, known as **somatic mutation**, has no direct genetic cause and is acquired during the life of the individual.

With the advancement of applications from molecular biology to medicine, gene maps and their location on chromosomes has been used **as a tool for the identification of predisposition to various diseases**. The procedure for obtaining such information includes DNA extraction and mutation analysis in hot areas for cancer related genes, **such as p53**.

The study of inherited cancers has contributed to molecular biologists that study cancer have the opportunity to look for genes that are critical in the normal development of cells and carcinogenesis. At the molecular level, cancer formation is characterized by alteration in both dominant **oncogenes** and **tumour suppressor genes,** such as p53. These tumour suppressor genes give rise to proteins that limit cell growth. In contrast, oncogenes are involved in promoting cell growth.

Currently, p53 tumour suppressor protein has become the focus of several biological studies of cancer; there is much interest in understanding how this gene works in normal cells compared to cancer cells. This gene is found on the short arm of chromosome 17 and codes for a nuclear phosphoprotein. In a normal cell it functions as a cell regulator.

Currently there is evidence that normal p53 is a protein that binds to DNA in a specific sequence that acts as a transcription regulator. If you have a mutation, you will lose this function of binding to DNA and will promote uncontrolled cell growth and therefore function as an oncogene.

For a tumour suppressor gene such as p53 to play a role in the transformation into cancer, it is necessary for both alleles to be mutated.

The p53 protein has 3 domains. The first contains the amino region which contains the transcription activating region. The second is the central region where most of the **"hot zones"** of mutations are located. These zones are called so because they are the zones where the mutations are detected with a high frequency, within this region there are 5 subregions where the point mutations in human cancers. The third domain is the carboxyl section containing the oligomerization sequences.

Examples of hot zones include codons 165 and 175 in exon 5; 196 and 213 in exon 6; 245 and 248 in exon 7; 273 and 282 in exon 8; all within the p53 protein. Several of these mutations give rise to p53 proteins with altered conformation; this can lead to changes that will result in an increase in the stability of mutant proteins and their ability to bind to normal p53 proteins so that they will inactivate them.

It is of special interest to comment that there is a correlation between the place of the mutation and the tumour it produces. An example is the mutation at amino acid 175 which is common for colon carcinoma but rarely seen in lung carcinoma.

2.2 The disease: Li-Fraumeni syndrome

Li-Fraumeni syndrome (LFS) is a rare autosomal dominant disease that affects young patients and is a predisposition to develop a wide range of tumours.

The historical and classical definition is based on familiar criteria, for example in the observation of a sarcoma in a patient under 45, who has either a first-degree relative who has developed any type of cancer before the age of 45, or a second-degree relative who has had cancer or sarcoma before the age of 45. It is difficult to estimate the incidence of this rare disease, since its definition raises a problem of nosological classification. The most characteristic tumours are osteosarcomas, soft tissue sarcomas, breast cancer in young subjects, leukaemia/lymphomas, brain tumours and adenocarcinoma; however, any type of tumour can be observed. **A germline mutation of the p53 gene is found in 70% of the families with LFS**, as well as in some families or patients with disease patterns suggestive of the syndrome, without strictly meeting the criteria. The risk of developing cancer for a patient with a deleterious mutation in the p53 gene is 15% at age 15, 80% at 50 years of age women, and 40% at men of the same age; the significant difference between sexes is explained almost entirely by breast cancers. The risk of developing a second cancer, especially a radiation-induced cancer, is high. Genetic counselling is difficult, due to the broad spectrum of tumours and their appearance at any age, especially during childhood.

No surveillance measures can be considered effective with the exception of those aimed at breast cancers in women over 20 years of age.

Li-Fraumeni syndrome is caused by germline genetic alterations in the tumour suppressor gene p53, located in section 13.1 of the short arm of chromosome 17 (17p13.1). This gene consists of 11 exons and encodes a 2.8 kb mRNA that gives rise to a protein of about 20 minutes of half life that is mainly located in the nucleus, although it can also be detected in the plasma in phase G1 and during the synthesis of DNA, since the function of p53 is to control the cell cycle during the G1 phase by transactivating genes encoding proteins with growth suppressing activity. However, p53 is not necessary for the normal growth and functioning of the cell, **but to suppress the development of tumours**. In situations of hypoxia or DNA damage, p53 concentrations increase rapidly, paralyzing the cell cycle in the G1 phase, allowing the cell to have time to repair the DNA and, if it is not possible, to induce apoptosis.

Between 73-88% of the germline mutations occurring in the p53 gene are clustered between exons 5, 6, 7, and 8 that correspond to the central region of p53. It is a highly hydrophobic and highly conserved region that exhibits DNA binding activity and interacts with the target sequences of the genes that will undergo transcriptional transactivation.

Thus, genetic alterations in the p53 tumour suppressor gene contribute to the development of different cancers. Firstly, germinal mutations generate predisposition to the early onset of various tumours in Li-Fraumeni syndrome, as described above. But sometimes somatic mutations are also present that can lead to the appearance of tumours. In these cases, the prevalence of areas where tumours develop varies with respect to Li-Fraumeni syndrome. The cancers most frequently caused by somatic mutations in the p53 gene - in proportion - include ovarian, colorectal, oesophagus, head and neck cancer, larynx, lung, pancreas, etc. In general, these mutations are more frequent in the more aggressive subtypes.

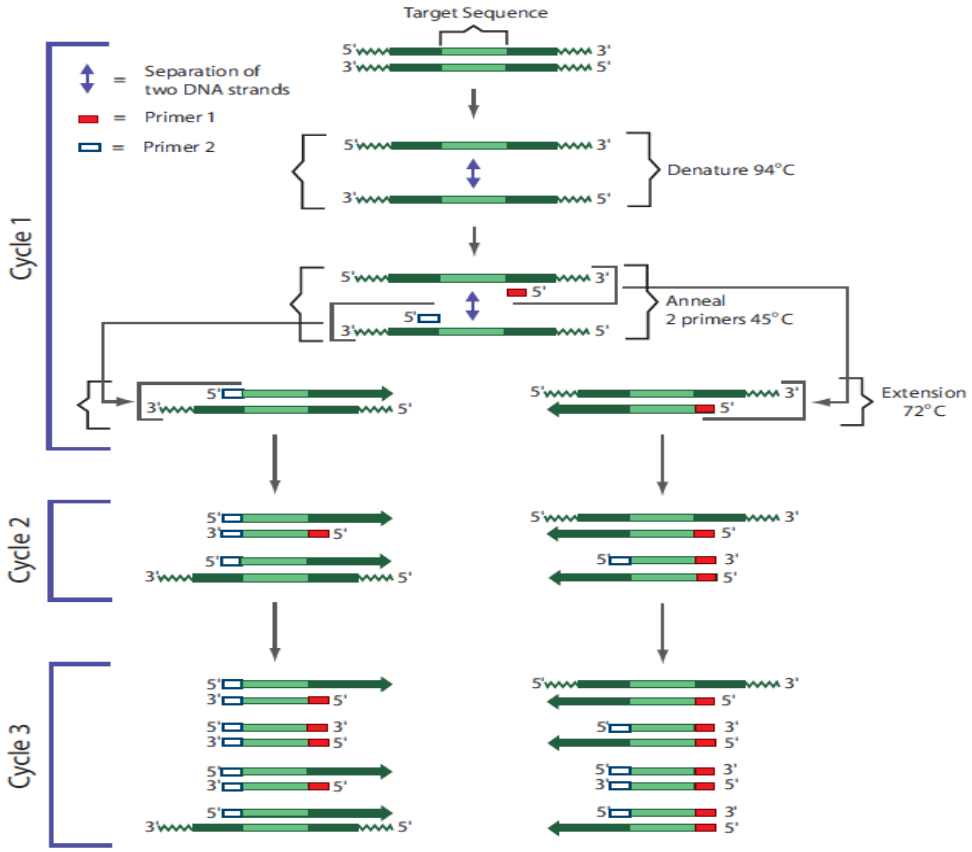
The heterogeneity of the symptoms and tumours associated with the Li-Fraumeni syndrome is the reason that the specialists think that this syndrome is underdiagnosed, as well as that its diagnosis is later than it would be desirable. Molecular biology techniques, especially those based on sequencing, allow for a diagnosis more efficient and safe; and at the same time early and earlier, and sometimes allow its association with certain prognoses.

2.3 PCR analysis

The detection of any mutation associated with the Li-Fraumeni syndrome or any localized somatic mutation in the p53 gene by **PCR amplification followed by direct sequencing**. For the detection of germ mutations associated with Li-Fraumeni syndrome, it is recommended to start the study by exons 5, 6, 7 and 8 where most of the alterations are located, with the possible reduction of time and cost that it entails in the majority of the cases.

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, 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, 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.

In this practice a simulated PCR will be performed since the instrument to carry out the PCR has a very high cost, for that it will be used NONTOXIC dyes that will migrate in the agarose gel as if they were of the DNA fragments resulting from the digestion of the amplified fragment with restriction enzymes.

STATEMENT OF FACTS

Nelia Hurtado, 36 years old, found a small irregular mass after her routine breast palpation, as her mother had undergone a mastectomy. The diagnosis was breast cancer. Part of the oncologist's job is to ask about cancer in family history.

After consulting with her mother, we observed that the paternal family is free of cancer while in the maternal family it is observed that there have been several cases of cancer. This leads the oncologist to think of a possible presence of Li-Fraumeni syndrome. In this case, a secondary diagnostic test is usually performed. **Nelia provides a blood sample and tumour biopsy to perform DNA analysis for the p53 gene**.

Normally the procedure is to amplify the gene using PCR followed by some of the different methods to detect the presence of a point mutation in the known "**hot zones**".

In this simulation experiment, the amplified DNA of the p53 gene obtained from Nelia will be digested with a restriction enzyme that recognizes the mutated sequence and will result in a pattern of bands in an agarose gel electrophoresis. In parallel, the same will be done with a normal (healthy) amplified DNA control that will provide us with a different band pattern.

Nelia has 3 children under the age of 15, if the presence of Li-Fraumeni syndrome is detected; it will be advisable to study the children to see if they have it and, if it is necessary, to establish control measures.

3. EXPERIMENT COMPONENTS

COMPONENT		STORE
10x Concentrated electrophoresis buffer	2 x 50 ml	
Agarose	1.75 gr	
Micropipette 20 µl	1	
Tips rack	1	
Samples microtubes	4	at 4ºC

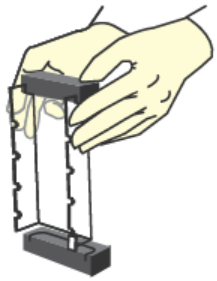
Add 450 ml of distilled water to each 10x Electrophoresis Buffer container to make 2 x 500 ml of 1x Electrophoresis Buffer which is the Working Buffer.

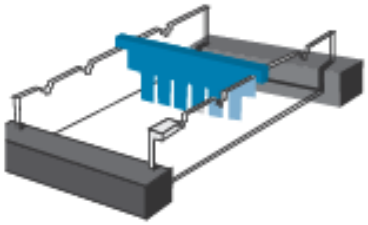
4. EXPERIMENT PROCEDURES

4.1 Agarose gel preparation

A) Mold preparation

Take the mold to make the gels and close the ends with the stops so that the agarose does not go out. Then place the comb to form the wells.





B) Agarose gel preparation

1.b) Use a 100 ml beaker or erlenmeyer to prepare the gel solution.

2.b) **For 7 x 7 cm gels**: Add **32 ml of 1x electrophoresis buffer** plus **0.30 g of agarose**, stir the mixture to dissolve the agarose clumps.

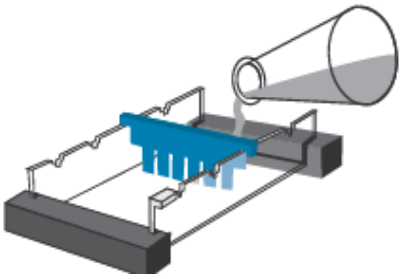
For 7 x 10 cm gels: Add **42 ml of 1x electrophoresis buffer** plus **0.40 g of agarose**, stir the mixture to dissolve the agarose clumps.

Make sure the 450 ml of distilled water has been added to the 10x Electrophoresis Buffer

3.b) Heat the mixture to dissolve the agarose. The fastest method is the use of a microwave, a heating plate can also be used, in both cases, in order for the agarose to dissolve **the solution must be brought to boiling point**. The final solution should appear clear without apparent particles.

4.b) **Cool** the agarose solution to about 55°C (to accelerate the process can be cooled by placing the container under a water tap and shaking). If there is excessive evaporation of the liquid, add electrophoresis buffer.

5.b) Add the agarose solution to the mold.

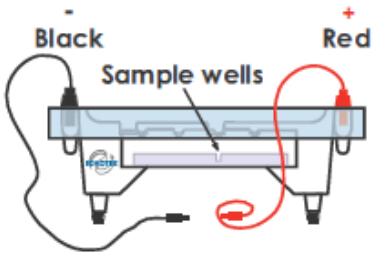


6.b) Allow the gel to solidify. To accelerate the process, the gel can be planted and then put it in a refrigerator (if the electrophoresis is performed the next day, keep the gel at 4ºC).

C) Gel preparation for electrophoresis

1.c) After the gel has solidified carefully remove the stops.

2.c) Place the gel in the electrophoresis chamber correctly oriented with the wells closest to the negative pole (black color).



3.c) Fill the electrophoresis chamber with **300 ml of 1x electrophoresis buffer**. ***The electrophoresis buffer can be used for 2 electrophoresis practice. Once the electrophoresis is finished, store this used buffer in a different container; don't mix a electrophoresis buffer new with one used buffer.***

4.c) Ensure that the gel is completely covered with tampon.

5.c) Remove the comb that has formed the wells very carefully to do not break any well.

6.c) Proceed to the load of the gel and carry out the electrophoresis.

4.2 Gel load and electrophoresis

Note: If you are unfamiliar with loading agarose gels, it is advisable to practice load before performing the experiment, or carry out the complete experiment before doing it with the students.

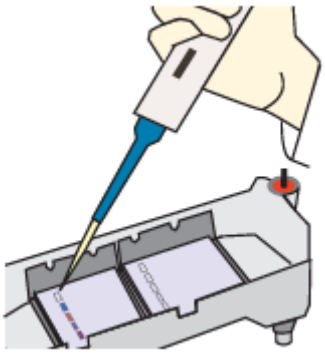
A) Electrophoresis samples

Check the volume of the all samples. Sometimes small drops of the sample may be on the walls of the microtubes. Make sure that the entire amount of sample is uniform before loading the gel. Centrifuge briefly the sample microtubes, or tap microtubes over a table to get the entire sample in the bottom of the microtube.

1.a) Five different samples presented in 5 tubes of a different color each one are supplied, loading the samples in the following order:

WELL	SAMPLE	DESCRIPTION
1	**GREEN**	MOLECULAR WEIGHT MARKER
2	**BLACK**	DNA CONTROL (NORMAL TISSUE)
3	**RED**	NELIA BLOOD DNA (NORMAL TISSUE)
4	**LILAC**	NELIA TUMOR DNA FROM BIOPSIA
5	**BLUE**	NORMAL BREAST TISSUE DNA FROM BIOPSIA

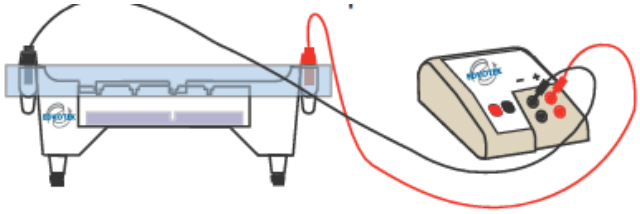
2.b) Load 20 microliters of each sample, using the fixed volume micropipette with a pipette tip supplied.



B) Carry out electrophoresis

1.b) After the samples have been loaded, place the electrophoresis apparatus cover on the electrode terminals carefully.

2.b) Insert the plug of the black cable into the black input of the power supply (negative input). Insert the red cable plug into the red input of the power supply (positive input).



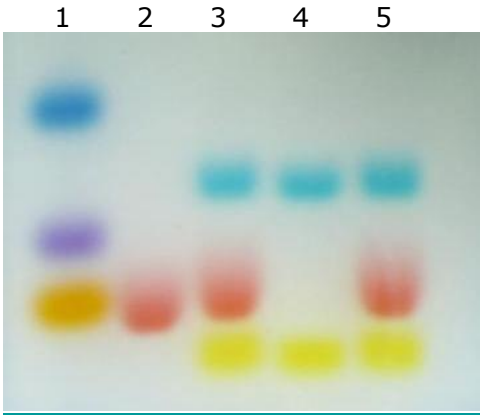
3.b) Set the power supply **at 75 volts (30 minutes) or 150 volts (20 minutes)**. **Watch that the dyes do not come out of the gel.**

4.b) After 10 minutes the separation of the dyes will begin to be observed.

5.b) After the electrophoresis is finished, **turn off the power supply**, disconnect the cables and remove the cover.

6.b) Place the gel in a white light transilluminator (if not available, a sheet of white paper may also be used).

5. PRACTICE RESULTS



1: Molecular weight marker.

2: Normal DNA for the p53 gene, the allele contains no restriction sites for the enzyme.

3: Nelia blood (normal tissue) DNA. It is observed that it has a normal and a mutated allele for the p53 gene.

4: DNA tumour from the Nelia biopsy showing the band pattern for the mutated p53 gene.

5: Normal breast tissue DNA from the biopsy. It is observed that it has a normal and a mutated allele for the p53 gene.

6. QUESTIONS AND ANSWERS ABOUT THE PRACTICE

A series of questions can be asked of students about the practice:

1. What is the difference between tumour suppressor gene and oncogene?

Tumour suppressor genes such as p53 give rise to normal proteins that limit cell growth. On the contrary the oncogenes promote the cellular growth. If there are mutations in any of them can lead to uncontrolled cell growth (cancer).

2. Why does the Nelia tumor sample show a pattern with less bands than the blood sample?

The Nelia blood sample contains bands representing one normal allele and one second that is mutated for the p53 gene, hence the pattern of bands is the sum of both. The tumor represents that both alleles are mutated resulting in a pattern of bands that is a subset of that of blood.

3. What is the purpose of normal control?

In all experiments, especially in biomedical analyzes, controls are necessary to obtain results that are not artefacts or are misleading.

4. 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.

5. Why are there 2 different primers in PCR?

They present a different sequence that coincides with the beginning and end of the gene or sequence to be amplified (template DNA).

6. Would you do a genetic analysis of this type to know if you are predisposed to have cancer?

More information to comment to the students

Between 5 and 10% of tumours are due to the inheritance of a genetic mutation, but to investigate this type of cancer means to advance in the knowledge of all the others. The most frequent are the breast and colon.

Studies on hereditary cancer face several questions: how to interpret mutations of key cancer genes? What other genetic causes or do not influence the appearance of these tumours? What is the relationship between the colon cancer that the father develops and the breast cancer that his daughter suffers? Why has the cancer appeared in the 30-year-old son rather than the 60-year-old father? How do genetic changes occur and what roles do those most subtle in the proliferation of cancer cells? And, how we could apply the new technologies to the sequencing of these mutations, and above all, to know how to interpret them?

Providing answers to these unknowns and others will mean being able to develop more effective and safer treatments than to offer the patient, and give the family alternatives for prevention.

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