EXPLORING THE GENETICS OF TASTE:
SNP Analysis of the PTC gen by PCR

Ref. PCR7

1. OBJETIVE OF THE EXPERIMENT

The objective of this experiment is to introduce students to the principles and practice of the Polymerase Chain Reaction (PCR) by amplifying the TAS2R38 gene, which is responsible for the ability to detect bitter taste. Subsequently, the purification of the PCR product will be carried out and it will be digested with the restriction enzyme HaeIII to identify the presence or absence of the SNP linked to the phenotype of detecting the bitter taste in PTC papers.

2. INTRODUCTION

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.
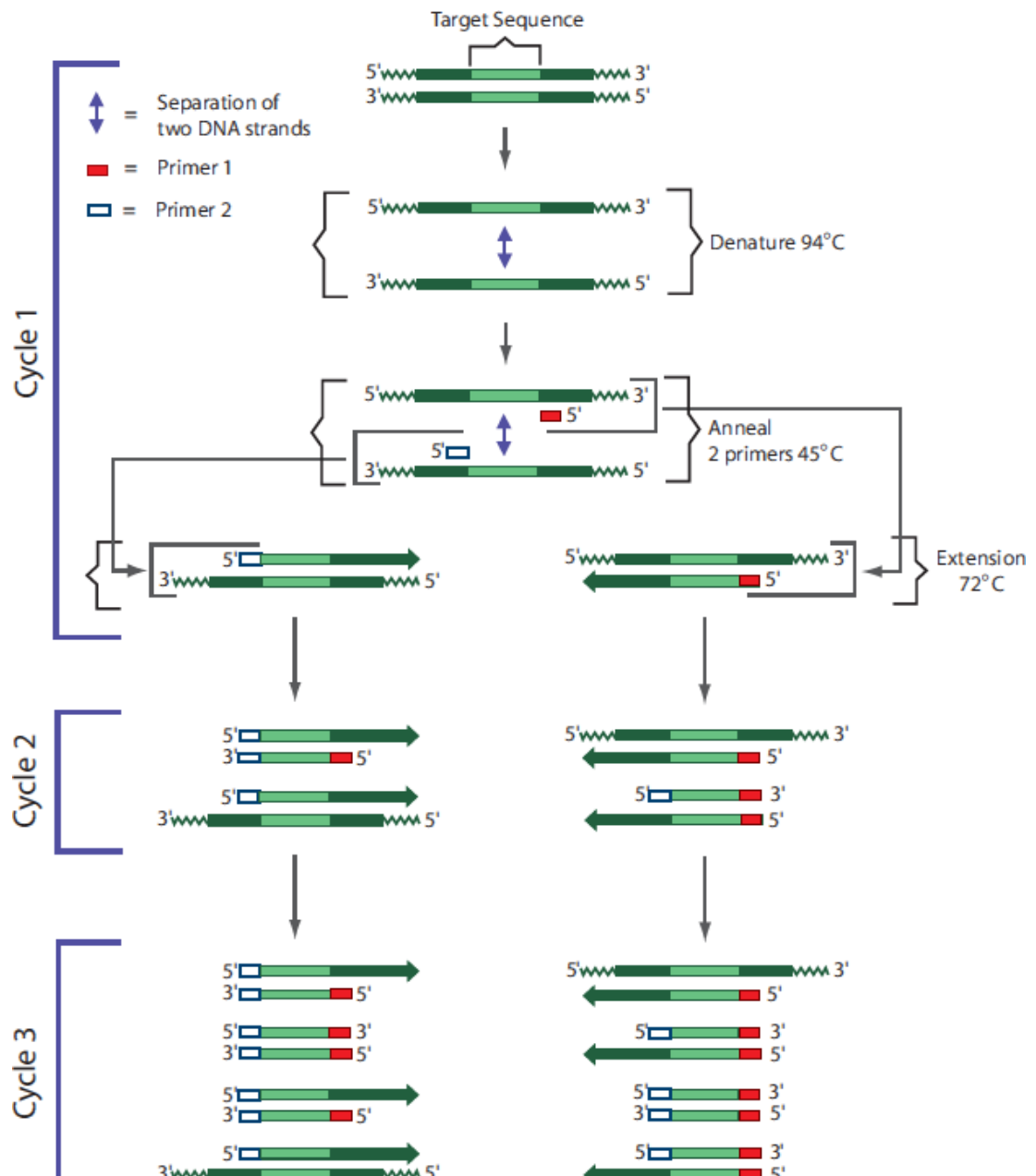
These three steps, denaturation-annealing-extension, constitute a PCR cycle. This process is repeated for 20-40 cycles by amplifying the object sequence exponentially. The PCR is carried out in a thermocycler, an instrument that is programmed for rapid heating, cooling and maintenance of the samples several times. The amplified product is then detected by removal of the reaction mixture by agarose gel electrophoresis.

2.2 Analysis of the SNP of the PTC gene

Four letters of nucleotides specify the genetic code: A (adenine), C (cytosine), T (thymine) and G (guanine). A point mutation occurs when one nucleotide is replaced by another nucleotide, for example when an A is replaced by a C, T or G (**Figure 1**). When said mutation is present in at least 1% of the population, it is known as **Single Nucleotide Polymorphism or SNP (pronounced "snip")**. A SNP can also occur when a single base pair has been removed or added to a sequence.
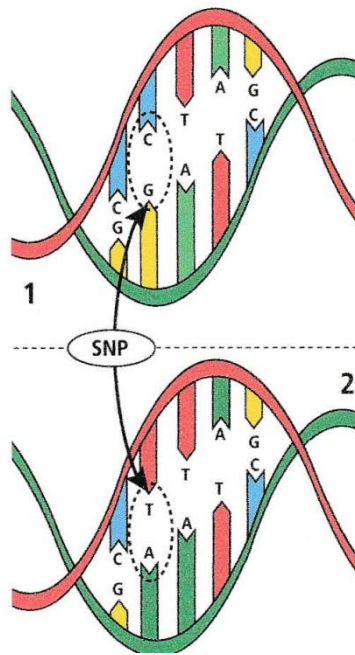
Figure 1

SNPs are the most common type of genetic variation among people. **They occur more frequently in the non-coding regions of genes and in regions between genes**. Although these SNPs do not translate into amino acids, they can affect protein production through gene splicing, transcription factor binding or non-coding RNA.

A SNP can also occur in the coding sequence of a gene, where it can affect the protein product of that gene. For example, sickle cell anemia occurs because of a single nucleotide polymorphism because the hydrophilic glutamic acid amino acid will be replaced by the hydrophobic amino acid valine in the hemoglobin chain of ß-globin.

In this practice, students will isolate the DNA from their saliva and use it to carry out the PCR reaction of the TAS2R38 gene to analyze the presence or absence of the SNP that occurs at position 145 of this gene for sensitivity to a compound that it has a bitter taste, PTC (phenylthiocarbamide).

There is variability in the population in the sensitivity to the bitter compound PTC. This fact was discovered in 1931 in a series of events that imply on the one hand a scientific curiosity and on the other hand a safety issue in the laboratory.

A chemist named Arthur Fox was mixing a powdered chemical when he accidentally left some of the dust released into the air. A close colleague exclaimed how bitter the dust tasted, but Fox (who was closer to the chemist) did not detect any bitter taste. Interested, both men took turns to try the chemical.

Fox continued to find the insipid chemical, while his colleague found it bitter. Then, Fox tested a large number of people. Again he found a mixture of "tasters" and "non-tasters" and published his findings. This attracted the interest of the geneticist L. H. Snyder, who tested the compound in families and formulated the hypothesis that the taster/non-taster status was genetically determined.

The ability to detect the PTC compound is linked to the presence of the Taste 2 receptor member of protein 38 that is encoded by the TAS2R38 gene. The TAS2R38 gene has two alleles: the dominant allele (T), which confers the ability to detect the bitter taste of the PTC, and the non-tasting recessive allele (t). A person inherits a copy of the gene from each of their parents. The combination of these different alleles within an individual is known as a genotype, which in turn dictates the phenotype: in this case, whether an individual is a "taster" or "non-taster" of the bitter taste. PTC tasters have one of two possible genotypes; or they are homozygous dominant and have two copies of the allele

(TT), or are heterozygous and have a T allele and a non-dominant allele t (Tt). The "non-tasters" are recessive homozygotes and have two copies of the recessive allele (tt). Within the general population, approximately 70% of the people evaluated can detect the bitter taste of the PTC, while the other 30% cannot.

Sequence analysis along the coding region of the TAS2R38 gene revealed that the tasting and non-tasting PTC alleles of the bitter taste differ by 3 amino acids due to SNPs in 3 different locations (**Table 1**). There are 5 versions worldwide: AVI, AAV, AAI, PAV, PVI, so called because of the combination of amino acids present in the gene. The two most common haplotypes are AVI and PAV, which represent non-tasters and tasters, respectively. Changes in the amino acid sequence alter the shape of the receptor protein, which determines the strength with which it can bind to the PTC. Since all people have two copies of each gene, combinations of variants of the bitter taste gene determine if someone finds the PTC intensely bitter, somewhat bitter or tasteless. This can be quantified approximately by means of a taste test or characterized with greater precision by determining the nucleotides at positions 145, 785 and 886.

Position nucleotide	Change in nucleotide No tasters > Tasters	Change in codon No tasters > Tasters	Change in Amino Acid No tasters > Tasters
145	G > C	GCA > CCA	Alanine > Proline
785	T > C	GTT > GCT	Valine > Alanine
886	A > G	ATC > GTC	Isoleucine > Valine

Table 1. Relationship of variations in specific locations in the TAS2R38 gene with the ability to detect the bitter taste

One way to detect a SNP is to use restriction enzymes. Restriction enzymes are endonucleases that catalyze the cleavage of phosphate bonds within both strands of DNA. The distinctive feature of restriction enzymes is that they only cut very specific base sequences. These recognition sites are generally 4 to 8 base pairs in length and the division occurs within or near the site. The recognition sites are often symmetric, that is, both strands of DNA at the site have the same sequence of bases when they are read 5 'to 3'. Such sequences are called palindromes. A single base change in the recognition palindrome results in the inability of the restriction enzyme to cut the DNA at that location. This will alter the length and number of DNA fragments generated after digestion.

These fragments can be separated according to their length by agarose gel electrophoresis. **The process of enzymatic digestion followed by electrophoresis is often referred to as restriction fragment length polymorphism (RFLP) analysis.**

In the example of the PTC gene, **the enzyme HaeIII** only cuts the tasting allele (T) with the ability to detect the bitter taste (5'-GGCG-GCCACT-3 '). The polymorphism present in the non-tasting allele that does not have the ability to detect the bitter taste (5'-GGC-GGGCACT-3 ') occurs as a change in a single base precisely at the recognition site of the restriction enzyme, **so that HaeIII cannot digest the non-taster DNA (t)** (**Figure 2**).

| Tasters Homozygotes (TT)	Tasters Heterozygotes (Tt)	Non- tasters (tt)
1. DNA Extraction from saliva	1. DNA Extraction from saliva	1. DNA Extraction from saliva
2. PCR amplification of PTC gene region **GGCGGCCACT** 221 pb PCR product	2. PCR amplification of PTC gene region **GGCGGCCACT** 221 pb PCR product + **GGCGGGCACT** 221 pb PCR product	2. PCR amplification of PTC gene region **GGCGGGCACT** 221 pb PCR product
3. Restriction digestion with HaeIII **GGCGG CCACT**	3. Restriction digestion with HaeIII **GGCGG CCACT** + **GGCGGGCACT**	3. Restriction digestion with HaeIII **GGCGGGCACT**
4. Agarose gel electrophoresis **Fragment of 44 pb** **Fragment of 177 pb**	4. Agarose gel electrophoresis **Fragment of 44 pb** **Fragment of 177 pb** **Fragment of 221 pb**	4. Agarose gel electrophoresis **Fragment of 221 pb** **(it is not cut)**
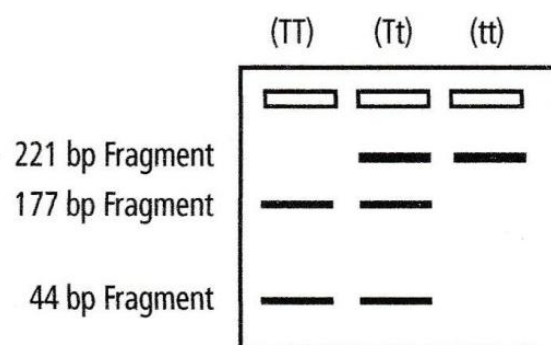
Figure 2. Analysis of genotypes of the PTC gene

3. EXPERIMENT COMPONENTS

Sufficient reagents are supplied for performing 25 individual PCRs and the performance of 4 electrophoresis gels in 2.5 or 3% agarose.

Concentrated electrophoresis buffer 10x	100 ml	
Agarose	6.0 gr	
Gene PTC MIX PCR	2 x 350 µl	Store at -20ºC
GELSAFE DNA staining	25 µl	Store at +4ºC
PCR Fragment Purification Kit	25 samples	
FastDigest Green Buffer	70 µl	Store at -20ºC
Hae III restriction enzymes	70 µl	Store at -20ºC
Positive Control homozygous DNA taster	15 µl	Store at -20ºC
Control Paper without flavor	25 units	
PTC Control Paper (bitter taste)	25 units	

10x electrophoresis buffer to make 2 x 500 ml of 1x electrophoresis buffer, which is the working buffer.
3.1 MIX POLYMERASE HOT STAR
A Hot Star polymerase ready for use 2x is used, which allows amplifying any fragment from DNA, so that the user only has to add water. An activation step of 10 minutes at 95ºC is required so that non-specific products are eliminated as "primers-dimers". It also contains a red dye that allows easy visualization and direct seeding in 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 DNA positive control of homozygous taster**.

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)	2,50 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.

PTC PROGRAM

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

4.3 Purification PCR fragment

Once the PCR has finished we will proceed to the purification of the amplified fragment of 221 pairs of bases, for this we will use a kit used in research DANAGENE CLEAN PCR Kit that allows to eliminate the polymerase, surplus nucleotides, salts, etc. and to stay with only the fragment of 221 bp so that when we carry out the digestion with the enzyme HaeIII it is not inhibited by the presence of other components.

1. **Add 150 µl binding buffer** to the microtube containing the **25 µl of the PCR**. Mix well by pipetting.

2. **Transfer the sample (175 µl) to a spin column**. Place the spin column in a collection tube. **VERY IMPORTANT: place the 175 µl right on the center of the white membrane**.

3. **Centrifuge for 1 minute at 10,000-12,000 r.p.m.**

4. **Remove the filtrate and add 700 µL of Wash Buffer**. Centrifuge for 1 minute at 14,000 r.p.m.

5. **Remove the residual ethanol** by centrifugation for 3 minutes at 14,000 r.p.m.

6. Place the spin column in a new 1.5 ml microtube and add **25 µl of Elution Buffer** pre-heated to 70 ° C **exactly in the center of the white membrane (touch with the tip without breaking the membrane)**.

7. **Incubate for 2 minutes and centrifuge for 1 minute at 14,000 r.p.m**. **Now the microtube contains the DNA to be digested with Hae III.**

4.4 Digestion with the restriction enzyme Hae III

1. Take out the FastDigest Green Buffer microtube from the freezer and let it thaw. The restriction enzyme Hae III will always be in the liquid state and should always be kept in the freezer, only take out when using it and return it quickly to the freezer.
Centrifuge to collect the drops that may remain on the walls.

2. The reactions we are going to prepare will have a total volume of 30 microliters.

REACTION	VOLUMEN
Purified DNA fragment (step 7)	25 µl
FASTGREEN Green plug	2.5 µl
Hae III Red plug	2.5 µl

3. Incubate in a water bath at 37ºC for at least 15 minutes.

4. Seed the samples in a **3% agarose gel** and perform the electrophoresis. It is not necessary to add a loading buffer since the Fastgreen used contains green.

VERY IMPORTANT:

a) The percentage of agarose must be very high since there is a very small fragment of 44 bp and it is necessary to separate well the fragments of 177 and 221 base pairs.

b) The electrophoresis time will be longer than normal to be able to separate the bands of 221 and 177 base pairs well in the case of heterozygous tasters.

c) The use of a molecular weight marker is recommended to verify the sizes of the fragments.

5. PRACTICE Results
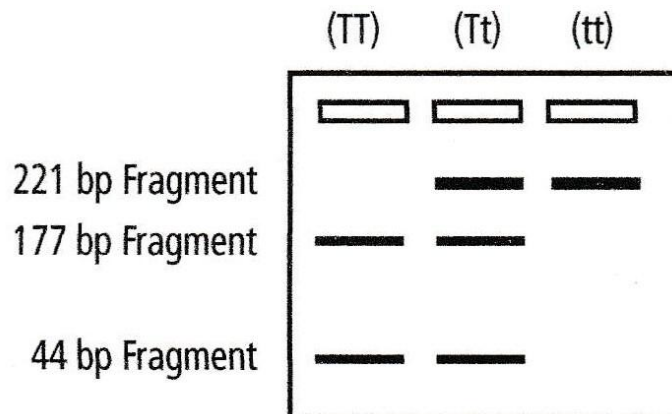
TASTERS of the PTC bitter taste:
Homozygous tasters (TT): fragments of 177 and 44 base pairs. Both copies of the gene contain the polymorphism, allowing to be cut by the enzyme HaeIII.

Heterozygous tasters (Tt): 1 allele remains uncut (221 bp) since it does not contain the polymorphism and the other allele is cut to contain the polymorphism, generating fragments of 177 and 44 base pairs.

NOTE: the band of 44 base pairs is very dim in the heterozygotes as it is a small size fragment.

NO-TASTERS of the PTC bitter taste:
Homozygous recessive (tt): remains uncut by the enzyme HaeIII since no allele contains the polymorphism.

Once the genotype of each student has been determined, we will check the phenotype by means of the unflavored control papers and the PTC papers (bitter taste) that are supplied; if the genotype has been well determined, it must match the ability to distinguish the bitter taste or not from the PTC

GENOTYPE	CONTROL PAPER	PTC PAPER
TT	Without taste	Intense bitter taste
Tt	Without taste	Medium bitter taste
tt	Without taste	Without taste