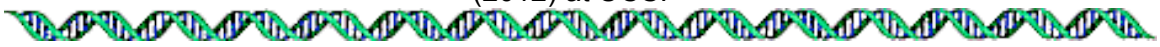
Testing for the PTC gene with PCR

Adapted from Emily Fox (Bio 100A Fall '04) and Mark Kunitomi (Bio 100A Fall '03), now (2012) at UCSF



A. Objectives

At the end of this lab, students will be able to

- Explain the purpose of amplifying DNA and to list applications of PCR
- define the terms "genotype" and "phenotype" and to understand how they relate to their ability to taste a bitter substance
- describe the components of a PCR reaction and what happens at each temperature during cycling
- predict where a restriction enzyme will cut, given the cut site and target sequence
- explain the principles of gel electrophoresis and how DNA molecules can be separated and visualized

B. Before coming to lab

1. Read the background for this lab exercise and answer the three "Test your understanding" questions and, if you can, fill out the worksheet in part E.
2. Watch the animation on PCR (link provided on the laboratory website)

C. During Lab

3. You are testing your own genome for the PTC gene, but you should work in sync with another student to avoid mistakes.
4. Follow the procedure outlined in part F.

D. Background:

For today's lab we will be using a technique called cleaved amplified polymorphic sequence (CAPS) analysis to determine student genotypes for the PTC gene. The PTC gene encodes a taste receptor that is responsible for the ability to taste PTC. Two common **alleles** (what does this mean?) are found in most human populations.

Why can some people taste PTC and others cannot?

The answer lies in our genetics and more specifically in the sequence of one particular gene, TAS2R38 (the PTC gene). Below is an example of two sequences of the PTC gene, one from a person who can taste PTC and the other from someone who cannot.

Nucleotide sequence of TASTER PTC gene

ATGTTGACTCTAACTCGCATCCGCACTGTGTCCTATGAAGTCAGGAGTACATTTCT
GTTCATTTCAGTCCTGGAGTTTGCAGTGGGGTTTCTGACCAATGCCTTCGTTTTCTT
GGTGAATTTTTGGGATGTAGTGAAGAGGCAGGCACTGAGCAACAGTGATTGTGTG
CTGCTGTGTCTCAGCATCAGCCGGCTTTTCCTGCATGGACTGCTGTTCCTGAGTGC
TATCCAGCTTACCCACTTCCAGAAGTTGAGTGAACCACTGAACCACAGCTACCAAG
CCATCATCATGCTATGGATGATTGCAAACCAAGCCAACCTCTGGCTTGCTGCCTGC
CTCAGCCTGCTTTACTGCTCCAAGCTCATCCGTTTCTCTCACACCTTCCTGATCTGC
TTGGCAAGCTGGGTCTCCAGGAAGATCTCCCAGATGCTCCTGGGTATTATTCTTTG
CTCCTGCATCTGCACTGTCCTCTGTGTTTGGTGCTTTTTTAGCAGACCTCACTTCAC

AGTCACAACTGTGCTATTCATGAATAACAATACAAGGCTCAACTGGCAGAATAAAG
ATCTCAATTTATTTTATTCCTTTCTCTTCTGCTATCTGTGGTCTGTGCCTCCTTTCCT
ATTGTTTCTGGTTTCTTCTGGGATGCTGACTGTCTCCCTGGGAAGGCACATGAGGA
CAATGAAGGTCTATACCAGAAACTCTCGTGACCCCAGCCTGGAGGCCCACATTAAA
GCCCTCAAGTCTCTTGTCTCCTTTTTCTGCTTCTTTGTGATATCATCCTGTGCTGCC
TTCATCTCTGTGCCCCTACTGATTCTGTGGCGCGACAAAATAGGGGTGATGGTTTG
TGTTGGGATAATGGCAGCTTGTCCCTCTGGGCATGCAGCCATCCTGATCTCAGGC
AATGCCAAGTTGAGGAGAGCTGTGATGACCATTCTGCTCTGGGCTCAGAGCAGCC
TGAAGGTAAGAGCCGACCACAAGGCAGATTCCCGGACACTGTGCTGA

Nucleotide sequence of NON-TASTER PTC gene

ATGTTGACTCTAACTCGCATCCGCACTGTGTCCTATGAAGTCAGGAGTACATTTCT
GTTCATTTCAGTCCTGGAGTTTGCAGTGGGGTTTCTGACCAATGCCTTCGTTTTCTT
GGTGAATTTTTGGGATGTAGTGAAGAGGCAGGCACTGAGCAACAGTGATTGTGTG
CTGCTGTGTCTCAGCATCAGCCGGCTTTTCCTGCATGGACTGCTGTTCCTGAGTGC
TATCCAGCTTACCCACTTCCAGAAGTTGAGTGAACCACTGAACCACAGCTACCAAG
CCATCATCATGCTATGGATGATTGCAAACCAAGCCAACCTCTGGCTTGCTGCCTGC
CTCAGCCTGCTTTACTGCTCCAAGCTCATCCGTTTCTCTCACACCTTCCTGATCTGC
TTGGCAAGCTGGGTCTCCAGGAAGATCTCCCAGATGCTCCTGGGTATTATTCTTTG
CTCCTGCATCTGCACTGTCCTCTGTGTTTGGTGCTTTTTTAGCAGACCTCACTTCAC
AGTCACAACTGTGCTATTCATGAATAACAATACAAGGCTCAACTGGCAGAATAAAG
ATCTCAATTTATTTTATTCCTTTCTCTTCTGCTATCTGTGGTCTGTGCCTCCTTTCCT
ATTGTTTCTGGTTTCTTCTGGGATGCTGACTGTCTCCCTGGGAAGGCACATGAGGA
CAATGAAGGTCTATACCAGAAACTCTCGTGACCCCAGCCTGGAGGCCCACATTAAA
GCCCTCAAGTCTCTTGTCTCCTTTTTCTGCTTCTTTGTGATATCATCCTGTGTTGCC
TTCATCTCTGTGCCCCTACTGATTCTGTGGCGCGACAAAATAGGGGTGATGGTTTG
TGTTGGGATAATGGCAGCTTGTCCCTCTGGGCATGCAGCCATCCTGATCTCAGGC
AATGCCAAGTTGAGGAGAGCTGTGATGACCATTCTGCTCTGGGCTCAGAGCAGCC
TGAAGGTAAGAGCCGACCACAAGGCAGATTCCCGGACACTGTGCTGA

Can you see the difference? Neither can we, so by using a bioinformatics tool called Clustal W2 we can align the two sequences to see where they are the same and where they are different.

Results from ClustalW2

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99.8% identity in 1002 nt overlap
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Below is the part of the sequence that is different for the two alleles:

Taster

GCCCACATTAAAGCCCTCAAGTCTCTTGTCTCCTTTTTCTGCTTCTTTGTGATATCATCCT
GTGCTGCCTTCATCTCTGTGCCCCTACTGATTCTGTGGCGCGACAAAATAGGGGTGATG

Non-Taster

GCCCACATTAAAGCCCTCAAGTCTCTTGTCTCCTTTTTCTGCTTCTTTGTGATATCATCCT
GTGTTGCCTTCATCTCTGTGCCCCTACTGATTCTGTGGCGCGACAAAATAGGGGTGATG

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Based on this alignment, we can see that out of the 1002 base pairs encoding the PTC gene, only one is different between the taster and the non-taster. This change in one base causes a single amino acid change in the protein that PTC encodes, and this small change in the protein differentiates between those who can taste PTC and those that cannot.

So how do we test this?

In order to do any testing of the gene, we will need a lot of DNA, and more specifically, a lot of DNA from just the PTC gene region that we are interested in. Our first step is to isolate a small amount of DNA and to amplify the region containing the PTC gene with a technique known as the Polymerase Chain Reaction (PCR).

PCR

PCR is a technique used to amplify a single or a few copies of a piece of DNA in an exponential manner to generate many copies. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences complementary to the target region along with a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified (see Figure 1).

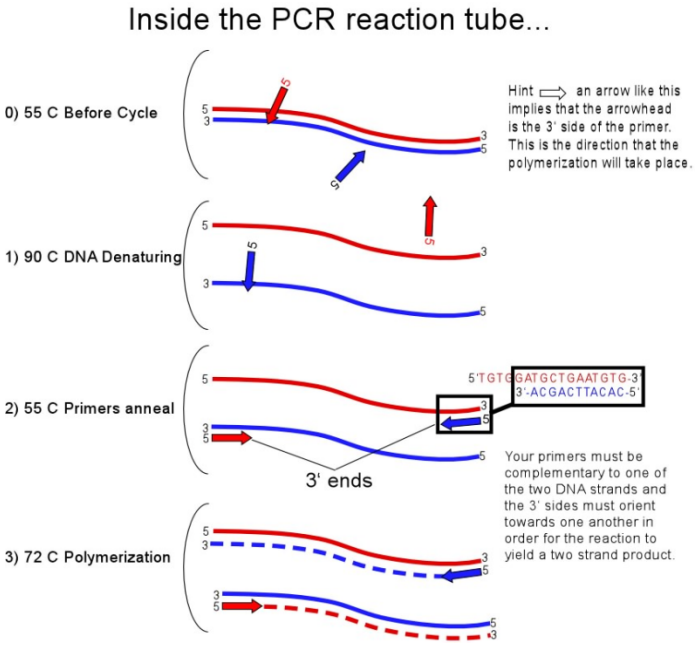

Figure 1: Reactions inside the PCR tube

Components Required for PCR

1. Buffer
   Provides a proper chemical environment for optimum activity and stability of the DNA polymerase.
2. DNA Polymerase
   DNA polymerase is an enzyme that catalyzes the polymerization of dNTPs to DNA. We will be using Taq DNA Polymerase today.
3. Template
   The target that will be used to generate new DNA from.
4. Primers
   Primers guide the DNA polymerase to where it should begin polymerization
5. dNTPs
   The building blocks of DNA used by DNA polymerase

Typically, the thermo-cycling profile of a PCR reaction consists of a cycle of 3 different temperature steps which are repeated between 20 and 30 times, depending on how much product DNA is desired. **For each cycle the amount of DNA from the region that is being amplified is theoretically doubled.** The cycling is often preceded by a single temperature step (called *hold*) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The cycle generally consists of these three steps:

1. Denature (~90°C)
   - The double-stranded DNA template molecule is made single-stranded. The temperature for this step is typically in the range of 95-100°C, near boiling. The high heat breaks the hydrogen bonds between the strands.
2. Anneal (~55°C)
   - In this annealing step the temperature is much lower ~60°C, allowing the primers to bind to the single-stranded DNA template.
3. Extension (~72°C)
   - The DNA polymerase enzyme adds bases complementary to the template to the bound primers As amplification proceeds, the DNA sequence between the primers doubles after each cycle. Following thirty such cycles, a theoretical amplification factor of one billion is attained.

Test your understanding:

1. What question do we try to answer with this lab?
2. What is the template DNA we are using for the PCR reaction?
3. What exactly will we amplify in the PCR reaction?

E. PCR Reaction setup - worksheet

Taq DNA polymerase requires specific conditions to work optimally. Please fill out the worksheet below to determine what quantity of each component should be added. We will be making a 50 µl reaction.

- Remember the standard way to calculate dilution is $C_1V_1 = C_2V_2$
- (C = concentration, V = volume)
- 1 mM (milliMolar) is equal to 1000 µM (microMolar)

Input this into your Lab Notebook

Component	Final Concentration	Amount to Add (50µl reaction)
5X Standard *Pfu* Reaction Buffer	1X	
10 mM dNTPs	200 µM	
10 µM Forward Primer	0.2 µM	
10 µM Reverse Primer	0.2 µM	
DNA	variable	6 µl
Pfu DNA Polymerase (1 unit / µl)	1 unit/50 µl PCR	
water		
	Total	50 µl

F. Procedure

DNA extraction

1. Label your tube of Chelex buffer/Proteinase K
2. Use a sterile loop to gently scrape the inside of your cheek
3. Put the loop in the Chelex buffer/Proteinase K, and mix to release cells
4. Give your tube to the instructor
5. When the whole class is ready, the tubes will be incubated at 56°C for 30 min
6. (Introduction to PCR during incubation)
7. Vortex tube and spin down briefly in centrifuge
8. Incubate tube at 100°C for 8 min
9. Centrifuge, max speed, 5 min
10. Place tubes on ice

PCR reaction

11. Label a single PCR tube for your reaction
12. Follow the PCR reaction table to add appropriate volumes of each reagent to your tube (keep everything on ice!)
13. When the whole class is ready, we will load the tubes in the thermocycler and run the following program:

First:	40 cycles of:	Then:
98°C, 10 min	72°C, 30 sec	72°C, 5 min
60°C, 5 min	95°C, 20 sec	10°C, hold
	60°C, 20 sec	

G. Review

What was the purpose of _____ in our PCR experiment.

1. using chelex/proteinase K buffer
2. heating cells and chelex/proteinase K buffer to 56 °C for 30 minutes?
3. heating cells and chelex/proteinase K buffer to 100 °C for 8 minutes?
4. centrifuging cells and chelex
5. keeping cells on ice
6. using primer mix
7. using our own cells
8. using Taq polymerase
9. using deoxynucleotides
10. using ATP (in reaction buffer)
11. using MgCl (in reaction buffer)
12. using buffer
13. heating the DNA up to over 90.0 °C?
14. What component of the PCR reaction ensures that we amplify only DNA from the PTC gene?
15. Explain what is happening at each temperature in the PCR reaction: 95°C, 55°C, 72°C, and 4°C.
16. Distinguish between a gene and an allele.
17. How many alleles can we have per gene?
18. How many alleles can exist in a population for a given gene?