**Exploring Genetic Variation in a Caffeine Metabolism Gene   
Part One: DNA Isolation and PCR**

**Background**

Ninety percent of people consume caffeine on a daily basis worldwide, making it the most commonly used stimulant. Tea is the most popular worldwide, but coffee is more commonly consumed in developed countries with 150 million regular coffee drinkers in the United States alone. Besides giving us a kickstart in the morning, coffee consumption has been linked to a decreased risk of type 2 diabetes, Parkinson’s disease, and Alzheimer’s disease, and tea drinking has been linked to a lower risk for some cancers. However, too much caffeine can also have negative effects. With regard to how caffeine affects us, some people get jumpy after drinking a single cup of coffee, while others can gulp down a Venti Americano without feeling a thing. Part of that variability is due to the development of tolerance by regular coffee drinkers (an environmental factor), but there are genetic differences in how people metabolize caffeine as well. Caffeine is primarily metabolized by the liver enzyme cytochrome P450 1A2 (CYP1A2).

**Single Nucleotide Polymorphisms (SNPs)** are single base pair mutations in a particular region of DNA. In the human genome, SNPs appear approximately every 300 bases on average. If the human genome is 3.1 billion bases, that means there are approximately 10 million SNPs! Because SNPs can occur anywhere in the genome, they can have dramatic effects on protein expression and function or no effect at all. Today we will start a multiple week lab exercise to look for a SNP in an intron of your DNA for CYP1A2. This SNP (rs762551) has been linked to how fast CYP1A2 metabolizes caffeine in those of European descent.

In today’s lab we will be isolating your DNA from cheek cells. **Detergent**, by dissolving lipids, destroys the membranes and exposes the contents of the cell and the nucleus. **Salt** makes the water solution more dense and heavy. This increased density will allow the separation of the DNA strands into the **alcohol**. Salt also neutralizes the negative charge of the DNA, making it insoluble in alcohol. Hopefully, we will have isolated enough of your DNA to amplify the caffeine intron using **PCR**. While in theory we could try to cut the gene directly from your genomic DNA, your DNA is huge, with millions of base pairs per chromosome. This is extremely unwieldy and very difficult to distinguish. However, by amplifying the region using PCR first, we target only the DNA region of interest. Furthermore, because PCR results in thousands of copies of this region, we will have much more DNA of that region to examine. Finally, we will use DNA cutting enzymes, called **restriction enzymes**, to see which SNP version you have; there are two common ones in the general population.

adapted from https://www.23andme.com/you/journal/pre\_caffeine\_metabolism/overview/

**Protocols**

**DNA Isolation**

1. You will be given about 20mL of sterile PBS (phosphate buffered saline), and you should put this in your mouth and swish vigorously for at least 60 seconds while chewing on your cheeks to isolate as many cells as possible. Spit back into the same container. ***Ideally, do this before eating, rather than immediately after a meal.*** You may do this any time during the day and store the sample in the refrigerator until our lab period. Make sure to label your tube.
2. Centrifuge your cells at approximately 4000xg for 5 minutes.
3. Remove the liquid supernatant and save the pellet. Add 25mL of PBS to wash the cells.
4. Spin again as in step 2.
5. Remove the supernatant, and resuspend the cell pellet in 1mL of PBS. Transfer the cell/PBS mix to a 2mL tube and store it on ice. Make sure to label your new tube.
6. Pellet your cells again in a microfuge at 4000xg for 5 minutes.
7. Carefully remove the supernatant, and resuspend your cells in 0.5mL Lysis Buffer (20mM Tris-HCl pH 8, 50mM NaCl, 10mM EDTA, 0.5% SDS, and 100μg/mL Proteinase K).
8. Incubate the cell lysate at 50°C for 1 hour. While you wait, work on the calculations in step 24.
9. **Make sure to wear gloves and work in the hood for steps involving phenol or chloroform.** Remove the Proteinase K by extracting the cell lysate once with phenol by adding an equal volume of phenol to the tube. Vortex the tube until it is cloudy. Microfuge it for one minute at full speed. Without disturbing the interface, use a P200 micropipet set to 200μL to carefully remove the supernatant to a new tube. Dispose of the organic phenol liquid in the liquid waste. Dispose of the tube in the phenol chloroform solid waste.
10. Extract the cell lysate once with phenol:chloroform as in step 9.
11. Extract the cell lysate once with chloroform as in step 9.
12. Determine the volume of cell lysate remaining by setting a micropipet to various volumes until you can suck up all of the liquid. Total volume: \_\_\_\_\_\_\_\_\_\_\_\_\_\_μL
13. Add 1/10 volume 3M sodium acetate. Amount: \_\_\_\_\_\_\_\_\_\_\_μL
14. Add isopropanol at a 1:1 volume from the freezer. (For example, if you have 300μL of lysate and 30μL of acetate, then add 330μL isopropanol.) Amount: \_\_\_\_\_\_\_\_\_\_\_μL
15. Invert the tube 4 to 6 times to mix and spin for 20 minutes at full speed. Make sure to spin hinge-side out so that you can find your DNA pellet. (For practical reasons, store your tube on ice until at least 5 of your classmates are ready.)
16. Carefully remove the tube from the microfuge and dump the supernatant down the sink. Alternatively, you can pipet out the supernatant if your pellet is loose.
17. Wash the side of the tube down with 0.15mL of cold 70% ethanol.
18. Spin for 5 minutes at full speed. Carefully remove the tube from the microfuge and remove all of the liquid with a P200 micropipet set to 200μL.
19. Air dry the pellet for 5 minutes.
20. Resuspend the pellet in 30μL 10mM Tris pH 8.
21. Use 2 μL to take an absorbance 260nm nanodrop reading of your DNA: \_\_\_\_\_\_\_\_\_\_\_\_\_\_ ng/μL
22. The nanodrop will also tell you how much protein is left in your sample by reading the wavelength at 280nm. The best DNA to protein ratio is to have a 260:280 ratio of 1.8. Write the 260:280 ratio of your DNA here:\_\_\_\_\_\_\_\_\_\_\_\_\_.

**Set up PCR**

1. Dilute your DNA to 10ng/μL in 10mM Tris pH 8 if necessary. You will use this as your template.
2. Each reaction will contain the following. Fill in column 3.

|  |  |  |  |
| --- | --- | --- | --- |
| **Reagent and concentration** | **Final Concentration** | **Amount Needed in 50μL** | **Amount for a MasterMix** |
| Template DNA (10ng/μL) | 50ng |  |  |
| 10mM Forward primer | 0.2mM |  |  |
| 10mM Reverse primer | 0.2mM |  |  |
| 25mM MgCl2 | 1.5mM |  |  |
| 10X Taq Buffer | 1X |  |  |
| 10mM dNTPs | 0.2mM |  |  |
| Water |  |  |  |
| Taq DNA polymerase (5 units/μL) | 1.25 units |  |  |

1. In order to simplify this process, we should make a MasterMix so that the amounts added are more consistent. In this case, everything but the unique DNA template will be mixed into one tube. We will make 2 MasterMixes for the class, so take the volumes for each reagent in the table and multiply by 8. Fill in the column on the far right. This will give us enough to use for half of the class plus some extra to account for pipetting errors. Put all these components in a tube, flick the tube several times to mix, and store the MasterMix on ice.
2. Each student should combine \_\_\_\_\_μL of the MasterMix with 50ng of template DNA in a well-labeled PCR tube. The reactions will be cycled using the parameters indicated below and stored in the freezer.
3. Next week, we will analyze your sequences using restriction enzymes and agarose gel electrophoresis.

|  |  |  |  |
| --- | --- | --- | --- |
| **Stage** | **Temp** | **Time** | **# of Cycles** |
| Initial Denaturation | 94°C | 5 min | 1 |
| Denaturation | 94°C | 30 sec | 35 |
| Annealing | 58°C | 30 sec |
| Extension | 68°C | 1 min |
| Final Extension | 68°C | 5 min | 1 |
| Hold | 4°C | Indefinitely | |

**Concept Check:**

1. Describe the purpose of each of the chemicals in the Lysis Buffer indicated in step 7.
2. What is the purpose of the phenol-chloroform extractions?
3. The sequences of your primers are as follows:

**h.caffeine.Forward: 5’- GAGAGCGATGGGGAGGGC -3’**

**h.caffeine.Reverse: 5’- CCCTTGAGCACCCAGAATACC -3’**

What is the Tm for each?

1. You can find the sequence of the SNP by going here: <http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs762551>

Copy the sequence below (you may copy/paste on your computer or print it out), and highlight the sequences that will be amplified by the stated primers. (Hint: remember that your reverse primer is always the reverse and complement of the gene sequence you are reading.) How big is the total piece that will be amplified?