Genotyping Caffeine Metabolism

Adapted from:
Julie Millard, Tenzin Passang, Jiayu Ye, Gabrial Kline, Tina Beachy, Victorica Hepburn, and Edmund Klinkerch. (2018). Genotype and Phenotype of Caffeine Metabolism: A Biochemistry Laboratory Experiment. *Journal of Chemical Education* **95:** 1856-1860.

Yvelande Zephyr and Susan Walsh. (2015). Laboratory Exercise: Exploring Genetic Variation in a Caffeine Metabolism Gene. Genetics Society of America Peer-Reviewed Education Portal (GSA PREP)

Introduction

Caffeine is the most widely used psychoactive drug in the world, with the majority of the world's population consuming it on a daily basis. In addition to its activity as a central nervous system stimulant, caffeine is also used clinically to treat premature neonatal apnea and to enhance the analgesic effect of pain relievers such as acetaminophen. Despite its widespread use, individuals respond very differently to caffeine, with some people experiencing anxiety after a single cup of coffee and others consuming several cups even in the evening without any sleep disturbances. Both environmental and genetic factors are believed to modulate individual responses to caffeine.

One factor influencing caffeine's biological effects is its lifetime in the body, mediated principally by the liver enzyme cytochrome P450 1A2 (CYP1A2). The activity of CYP1A2, which also metabolizes many other substrates, can vary between individuals by more than 10-fold. Both genetics and personal habits, such as smoking, contribute to this variation. In this biochemistry laboratory experience, you will characterize CYP1A2 genotype and phenotype of test subjects to examine the role of this enzyme in caffeine metabolism.

In this experiment, caffeine metabolism genotyping is performed for CYP1A2*1F, a site containing a C→A single-nucleotide polymorphism (SNP [rs762551]; CCCGGG→CACGGG) that has been reported to affect the activity of the enzyme. In addition to being associated with higher CYP1A2 activity, the AA genotype has also been linked to a lower risk of myocardial infarction and a higher enhancement of athletic performance upon caffeine ingestion. Genotyping of DNA from saliva samples is achieved via Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) analysis.

PCR-RFLP analysis involves making billions of copies of CYP1A2 DNA via PCR. Subsequently, the CYP1A2 copies are reacted with a restriction enzyme (ApaI) which cuts DNA specifically at the sequence CCCGGG. ApaI will not cut the single-nucleotide polymorphism sequence CACGGG. The DNA digestion fragments will be resolved on an agarose gel. Since you are a diploid organism the options are as follows:

Genotype	**Fragments**	**Phenotype**
CCCGGG/CCCGGG	254 bp; 489 bp	Normal Caffeine Metabolism
CACGGG/CCCGGG	254 bp; 489 bp; 743 bp	Intermediate Caffeine Metabolism
CACGGG/CACGGG	743 bp	Fast Caffeine Metabolism

An uncut, negative control will be resolved and should be 743 bp. A SacI digested, positive control will be resolved and should be at 263 bp and 480 bp.

Purification of total DNA from animal saliva using the DNeasy® Blood & Tissue Kit

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- Buffer AL or Buffer AW1 are not compatible with bleach.

Procedure

1. **Collect 1 ml saliva.**

   Note: Ensure that the animal from which the sample was taken has not eaten any food in the 30 min prior to sample collection.

2. **Add 4 ml PBS (50 mM Potassium phosphate, 150 mM NaCl, pH 7.2) to the sample and centrifuge at 1800 x *g* for 5 min**.
3. **Carefully decant the supernatant. Resuspend the pellet in 180 µl PBS.**
4. **Add 25 µl proteinase K solution and 200 µl Buffer AL (without added ethanol). Mix thoroughly by vortexing, and incubate at 56°C for 10 min.**

   It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

5. **Add 200 µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing.**

   It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

6. **Pipet the mixture from step 5 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.**
7. **Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow- through and collection tube.**
8. **Place the DNeasy Mini spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x *g* (14,000 rpm).

9. **Place the DNeasy Mini spin column in a clean 1.7 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**
10. **Use the Nanodrop spectrophotometer to determine the [DNA] in ng/uL.**
11. **Dilute the DNA to 10 ng/uL using dH_2O.**

Polymerase Chain Reaction (PCR) Amplification of DNA Region Containing Single-Nucleotide Polymorphism (SNP) [rs762551].

Mix the following components in this order in a PCR tube:

- 10 uL dH_2O
- 5 uL 10 ng/uL template DNA
- 5 uL 2 uM Forward Primer (5'-GAGAGCGATGGGGAGGGC-3')
- 5 uL 2 uM Reverse Primer (5'-CCCTTGAGCACCCAGAATACC-3')
- 25 uL 2x ReadyMix Taq PCR Reaction Mix (0.06 units/uL Taq DNA Polymerase; 20 mM Tris-HCl; 100 mM KCl; 3 mM $MgCl_2$; 0.002% gelatin; 0.4 mM dNTP, stabilizers)

Run each tube through the following thermocycle program:

1. 94°C for 5 minutes
2. 94°C for 30 seconds
3. 58°C for 30 seconds
4. 68°C for 1 minute
5. Goto #2 34 times
6. 68°C for 5 minutes
7. Hold at 4°C

Samples will be stored at 4°C until the next lab meeting.

Restriction Fragment Length Polymorphism (RFLP) Analysis

Add 25 uL of dH_2O to three 1.7 mL microfuge tubes. Divide the PCR reaction product by adding 15 uL to each of the three 1.7 mL microfuge tubes. Add 5 uL of 10x CutSmart Buffer (NEB) to each tube. Mix and spin. Add 5 uL dH_2O to one tube; 5 uL SacI (20 units) to the second tube; and 5 uL ApaI (20 units) to the third tube. Place the tube containing additional water on ice for one hour. Place the tube containing SacI at 37°C for one hour. Place the tube containing ApaI at 25°C for one hour.

Pour a 1.2% TAE-agarose gel. Add the appropriate amount of 6x Gel Loading Dye (NEB) to each reaction tube. Load 20 uL of each reaction and 100 bp DNA ladder (NEB) to the gel. Electrophoresis at 50 V for approximately an hour. Image the gel with the BioRad gel imager.

Post-Lab

Attach a well labeled image of your DNA gel. Add a figure caption communicating the major conclusions.