Laboratory 3 - Pharmacogenetics

A person's phenotype is a direct expression of their genotype, but many environmental, cultural, and emotional factors can affect the expression of a person's genotype. Taste in particular is a complex trait, with cultural differences, mood, perceived desirability, conditioning, and other factors can all affect taste perception.

Receptors on the tongue allow you to taste five major flavors – umami, sour, sweet, salty and bitter, which is critical for nutrition and survival. Flavour is a broad phenomenon that encompasses the senses of taste, smell, and touch, enabling us to perceive sensations far more complex than the five basic tastes alone (Manley, 2015). Although human saliva naturally contains chemicals that elicit the five basic tastes: sweet-glucose, salt-sodium, umami-glutamate, sour-acids and bitter-urea, we do not detect them due to taste familiarisation. Super-tasters have an increased number of taste buds on their tongue, which intensifies all tastes, and women have more taste buds than men, with women having greater sensitivity to all tastes (Manley, 2015).

The ability to taste bitter compounds containing a thiourea moiety, such as phenylthiocarbamide (PTC) or 6-*n*-propyl-thiouracil (PROP), is primarily due to the bitter taste receptor *TAS2R38* gene located on chromosome 7. There are three missense-coding single nucleotide polymorphisms (SNPs) in this gene at positions 145 (proline or alanine); 785 (alanine or valine), and 886 (valine or isoleucine) of the open reading frame (ORF) are associated with PTC taste sensitivity (Boxer and Garneau, 2015).

Although there are eight possible amino acid combinations of these polymorphisms, only six haplotypes are commonly associated with taste sensitivity. The PAV (taster) and AVI (non-taster) are the most common haplotypes, with the others being far less common AAI ≈ AAV (intermediate taste sensitivity) >> PAI ≈ PVI (no data) >>>> AVV ≈ PVV (no data). Typically, as long as a person has at least one copy of the tasting PAV haplotype, they are able to taste bitter compounds such as PTC and PROP, however AVI homozygotes cannot taste these compounds (Boxer and Garneau, 2015). We will amplify these three specific regions of the *TAS2R38* gene that surround the three common SNPs associated with the ability to taste PTC/PROP.

As of 2013, a total of 21 SNPs had been identified in *TAS2R38*, 90% of which encode amino acid substitutions, indicating that there is a large amount of pressure from natural selection to increase the diversity of the bitter taste receptor. The majority of these variants are found in African populations, with Asians and Caucasians generally having lower diversity (Behrens et al., 2013). However, there is little data available on how exactly these variants affect taste perceptions.

The frequency of tasters for PROP and PTC varies by both race and ethnicity with the estimated frequency of PROP/PTC tasters among Caucasians being approximately 70%, whereas the taster frequency is higher in Chinese, Japanese, and sub-Saharan African populations, ranging between ~80 and 90%. Only some subgroups within India have been tested, but PROP taster frequency was shown to be <50% in these groups (Desai et al., 2011).

In this lab we will look at the genotype of the *TAS2R38* gene, as well as your ability to taste several chemicals – PTC, PROP, thiourea, and sodium benzoate. We will also taste a control strip that has no chemicals. Generally, if you can taste PTC, you will be able to also taste PROP and thiourea, but it is not a given that you will have the same level of sensitivity to each compound. These chemicals are not identical and due to the complexity of genetics, you may have a variable response, regardless of your *TAS2R38* genotype. Sodium benzoate is a common food preservative and may be tasteless, sweet, salty, bitter, or some combination of these tastes,

depending on the individual. Although the gene (or genes) conferring the ability to taste sodium benzoate is currently unknown, these strips are commonly used to assess a person's ability to taste.

1. Genotyping your *TAS2R38* gene

a) Extraction of human DNA from Saliva

NOTE: Although the *TAS2R38* gene has not been directly linked with any particularly negative health outcomes, if you do not wish to test your own DNA for any reason, please let the instructor know, and you will be provided with DNA to use in the rest of the experiments in this lab.

In today's lab, you will isolate your own genomic DNA from buccal cells. To do this, we will perform a **simple** kit-based DNA extraction. This quick protocol will be sufficient for us to amply regions of the *TAS2R38* gene.

1. Swab the inside cheek of your mouth using a sterile swab for **15 seconds**, making sure to cover the entire area of the inner cheek.

NOTE: Although your own saliva is not harmful to you, it must be treated as a potential biohazard, and every precaution taken to ensure it doesn't come in contact with any lab surfaces or other individuals.

2. Working over a disposable bench pad, place the swab into a 1.7 mL microfuge tube containing **500 µL** of Genomic Lysis Buffer.
3. Vigorously swirl and rub the swab against the sides of the tube to dislodge any cells.
4. Incubate at room temperature **10 minutes**.
5. Carefully press the cotton swab against the side of the tube to press out as much liquid as possible as demonstrated by your TAs. Pipette **400 µL** of lysis buffer into a **labelled** Zymo-Spin™ IICR Column in a collection tube. Avoid transferring any of the cotton swab to the column.
6. *Label the spin tube with <your name or initials>. NB: if your name is shared with one or more other students, please add a last initial or other identifier to your tube.*
7. Centrifuge at 10,000 x g for **one minute**. Discard the collection tube with the flow through.
8. *Ensure that your tubes are balanced according to the directions of your TA so that you do not break the centrifuge.*
9. Add **200 µL** of DNA Pre-Wash Buffer to the spin column and centrifuge at 10,000 x g for **one minute**. Discard flow-through.
10. Add **500 µL** of g-DNA Wash Buffer to the spin column and centrifuge at 10,000 x g for **two minutes**. Discard flow-through.
11. Transfer the spin column to a clean, **labelled**, microfuge tube.
12. *Label the microfuge tube: <your name or initials> gDNA.*
13. Add **50 µL** pre-warmed DNA Elution Buffer to the spin column.
14. Incubate **5 minutes** at **room temperature** and then centrifuge at top speed for **one minute** to elute the DNA.
15. Store your extracted DNA on ice until we are ready to use it in the next procedure.

b) Amplification of DNA via PCR

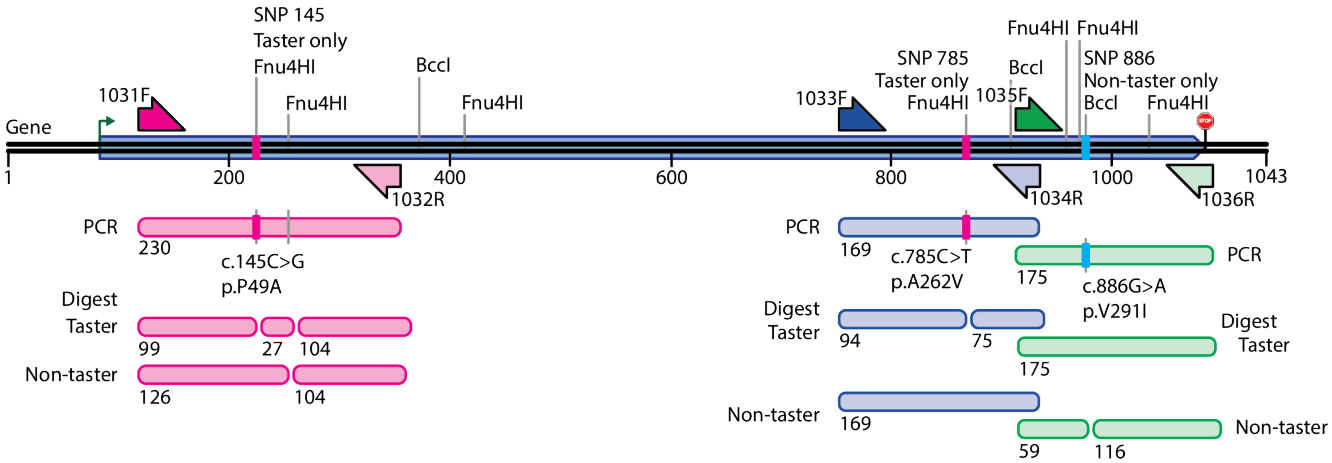
Background

In humans, *TAS2R38* comprises a single exon producing a 1143 bp mRNA that encodes a 1002 bp open reading frame (ORF). We will use specific primers to allow us to amply 3 regions of the gene, each containing a single nucleotide polymorphism (SNP) that is known to be associated with the ability to taste bitter compounds. Despite our primers having predicted annealing temperatures of ~60°C, we will be using a 68°C annealing temperature – a temperature that is at, or above the melting temperature of these primers, in order to reduce the appearance of non-specific bands – DNA that might amplify because the primers bind to a similar sequence within the genome instead of their exact match in the gene itself.

We will detect the SNP associated with the taster or non-taster phenotype at each site based on the presence of absence of a Fnu4HI or BccI restriction site at the location of the SNP.

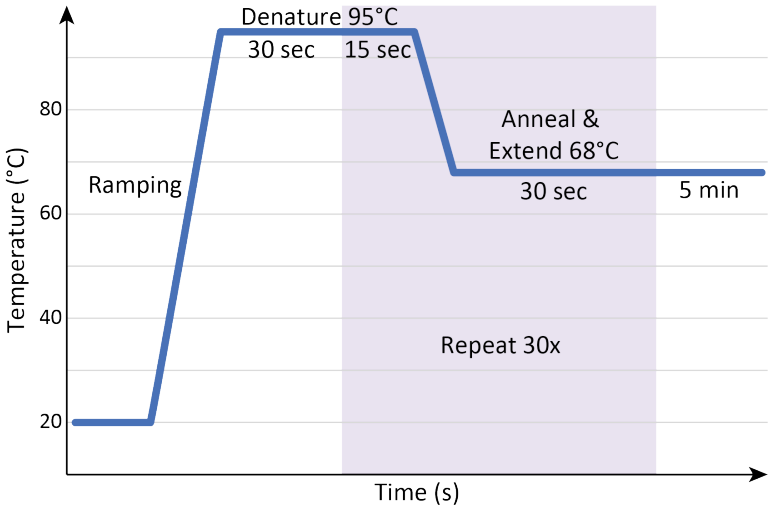
ID	SNP	Direction	Sequence (5'-3')	Length	%GC	T_m	Annealing Temp (°C)	Amplicon (bp)
1031	145	Forward	GGAGTACATTTCTGTTCATTTCAGTC CTGGAGTTTGCAGTGG	42	45	65	59	230
1032	145	Reverse	GCTGTGGTTCAGTGGTTCACTCAACT TCTGGAAGTGG	37	51	66		
1033	785	Forward	CCAGAAACTCTCGTGACCCCAGCCT GGAGG	30	63	68	59	169
1034	785	Reverse	GCCATTATCCCAACACAAACCATCAC CCCTATTTTGTCG	39	46	65		
1035	886	Forward	GGTGATGGTTTGTGTTGGGATAATG GCAGCTTGTCC	36	50	66	60	175
1036	886	Reverse	CCATTCTCAGCACAGTGTCCGGGAA TCTGC	30	57	65		

This image shows the locations of all Fnu4HI and BccI cut sites within the TAS2R38 gene, the primer binding sites, as well as the predicted PCR and restriction digestion products for our three reactions.



Experimental Design

1. You have been supplied 3 tubes of PCR master mix (black sharpie labels). The PCR tubes are labelled 1, 2, or 3 – this corresponds to SNP 145, 785, and 886 respectively. Each tube has a different set of primers so that we can amplify a specific SNP. The tube ID is VERY important for our later restriction digestions, so make sure you know which tube corresponds to which SNP.
    a. The tubes also have a 4-digit code written on the lid. Please write this code down on your worksheet so that you can identify your samples when the anonymized results are uploaded to Canvas. Your TA will also come by to record your ID code so that your work may be marked appropriately.



2. These tubes contain a mixture of all the reagents needed to perform a PCR reaction. The amounts of each component are indicated below:

Component	Final concentration	1 reaction (µL)
Nuclease-free water	---	39.75
10X ThermoPol Reaction Buffer	1X	5
10 mM dNTPs	200 µM	1
10 µM Forward Primer	0.2 µM (10 pmol)	1
10 µM Reverse Primer	0.2 µM (10 pmol)	1
Template DNA	~ < 20 ng/µL	2
10U/µL Taq DNA polymerase	0.025 U/µL	0.25
Total volume	---	50

Tube ID	SNP
1	145
2	785
3	886

3. To each of the supplied tubes of PCR master mix, **add 2 µL** of your extracted DNA.
    a. **NOTE:** Be very careful while doing this – the 0.2 mL tubes are fragile and easy to break. You also need to take care that you don't fling the contents of the tube out when opening it. It is also important you practice good technique, as PCR reactions are very sensitive to contamination.
4. Carefully close each tube.
5. Bring your PCR tubes to the thermocycler at the centre bench, and place them in the machine as directed by the TAs and/or Lab Instructor.
6. Perform the cycling reaction by closing and sealing the lid and starting the P203-tas2r38 program on the machine. This program will take ~ 1 hour to complete and will amply our DNA at specific sites within the *TAS2R38* gene.

c) Restriction digestion of PCR products at SNP sites

Background

In order to tell the genotype of your DNA sample at each SNP location, we will be cutting the DNA at specific locations using restriction endonucleases (RE), specifically the enzymes Fnu4HI and BccI. These were originally

isolated from the bacteria species *Fusobacterium nucleatum* 4H and *Bacteroides caccae* (ATCC 43185) respectively and cut at specific sequences of DNA. Fnu4HI cuts at the recognition site "GC▼N▲GC", and BccI cuts several bases after its recognition site "GGATCNNNN▼N▲".

Experimental Design

1. You have been supplied 3 tubes of RE master mix. This is a mixture containing all the reagents needed to perform your restriction digest reaction, except for the DNA. The amounts of each component are indicated below:

Component	Final concentration	Fnu4HI (µL) Tube "F"	BccI (µL) Tube "B"
Nuclease-free water	---	0	0
10X rCutSmart Buffer	1X	2	2
Template DNA	<1000 ng	17	17
Fnu4HI (10 U/µL)	0.5 U/µL	1	---
BccI (10 U/µL)	0.5 U/µL	---	1
Total volume	---	20	20

2. Each tube is to analyse a specific SNP. Add 17 µL of the appropriate DNA to each RE tube (blue sharpie label for Fnu4HI, red sharpie label for BccI):
   - It is VITAL you add the correct PCR product to the appropriate tube of the restriction digest, failure to do so will RUIN your results
   - Each tube also has a 4-digit code written on the top that corresponds to your PCR reaction. Ensure that you take and use only those tubes whose code matches your PCR tubes!

RE Tube	Source DNA	Amount	Restriction Endonuclease
F1	145 PCR (tube **1**)	17 µL	Fnu4HI
F2	785 PCR (tube **2**)	17 µL	Fnu4HI
B3	886 PCR (tube **3**)	17 µL	BccI

   Each RE tube will contain a total of 20 µL after you've added the DNA.

3. Close each tube and bring to the thermocycler. Set a timer and incubate your reactions at 37°C for a minimum of 15 minutes.

4. After incubation finishes, place reactions on ice.

Assuming there is complete cutting of the DNA, specific fragment sizes will be generated after the digest is finished.

SNP	Uncut (bp)	Digested (bp)	
		Non-taster	Taster
145	230	126, 104	104, 99, 27
785	169	169	94, 75
886	175	116, 59	175

d) Polyacrylamide gel electrophoresis

Background

Gel electrophoresis is a standard lab procedure for separating DNA by size. Due to the fact that DNA is negatively charged, when exposed to an electrical field, DNA loaded into a gel matrix will move towards the positive electrode (and away from the negative electrode). In the lab, the negative cathode is commonly indicated by black cables whereas the positive anode is marked by red cables.

Shorter DNA fragments will migrate through the gel more quickly than longer ones, as the longer fragments take longer to navigate through the gel matrix. Due to this, it is possible to determine the approximate length of a DNA fragment by running it on a gel alongside a DNA ladder, which is a collection of DNA fragments of known lengths

Due to the small size of the fragments that we're generating, we will run a 10% polyacrylamide gel to better separate out these small fragments. Our gel is made, and run, using 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). Due to time constraints, and hazardous nature of liquid acrylamide, the gels have been made for you. After the gel is run, it will be stained using 1X UV-fluorescent LabSafe™ Nucleic Acid Stain (G-Biosciences) to allow us to visualize our DNA bands. This is a non-genotoxic stain (per the Ames-test, mouse marrow chromophilous erythrocyte micronucleus, and mouse primary spermatocyte chromosomal aberration tests).

Experimental Design – Sample preparation

1. For each of your **uncut PCR** products, add **6.6 µL** of 6x gel loading dye directly to the remaining PCR product in the original tube (black sharpie) – total volume after addition: 39.6 µL. Mix well by pipetting according to the directions of your TAs.

2. Once your **digest** has completed, add **4 µL** of 6x gel loading dye directly to the **digest** (blue or red sharpie) – total volume after addition: 24 µL. Mix well by pipetting.

3. Bring to the ice bucket at the back of the class where the gel station has been setup and fill out the gel loading sheet. Ensure that your samples are placed in the correct order!

Experimental Design – Running the gel

Due to time constraints, the instructional staff will load the gel for you, and the results will be uploaded to Canvas. The protocols are included here for you information and so that you understand how we are obtaining the final data.

1. Each gel has 15 wells, and can hold samples from two students.

2. Gels will be loaded in the following order:

1	2	3	4	5	6	7	8
Ladder	Uncut 145	Fnu4HI 145	Uncut 785	Fnu4HI 785	Uncut 886	BccI 886	blank

9	10	11	12	13	14	15
Ladder	Uncut 145	Fnu4HI 145	Uncut 785	Fnu4HI 785	Uncut 886	BccI 886

3. We will load 3 µL of NEB Low Molecular Weight DNA Ladder (150 µg total), and 15 µL of your PCR and RE digested samples.

4. The gels will be run at 80V for ~2.5 hours to ensure good separation of the bands.

5. Once the gels finish running, the gels apparatus will be disassembled and gel will be stained.

Experimental Design – Staining the gel

The instructional staff will stain and image your gels and upload the results to canvas. The protocol for the staining process is given below, but you will not need to do this step yourself due to the time constraints of the lab.

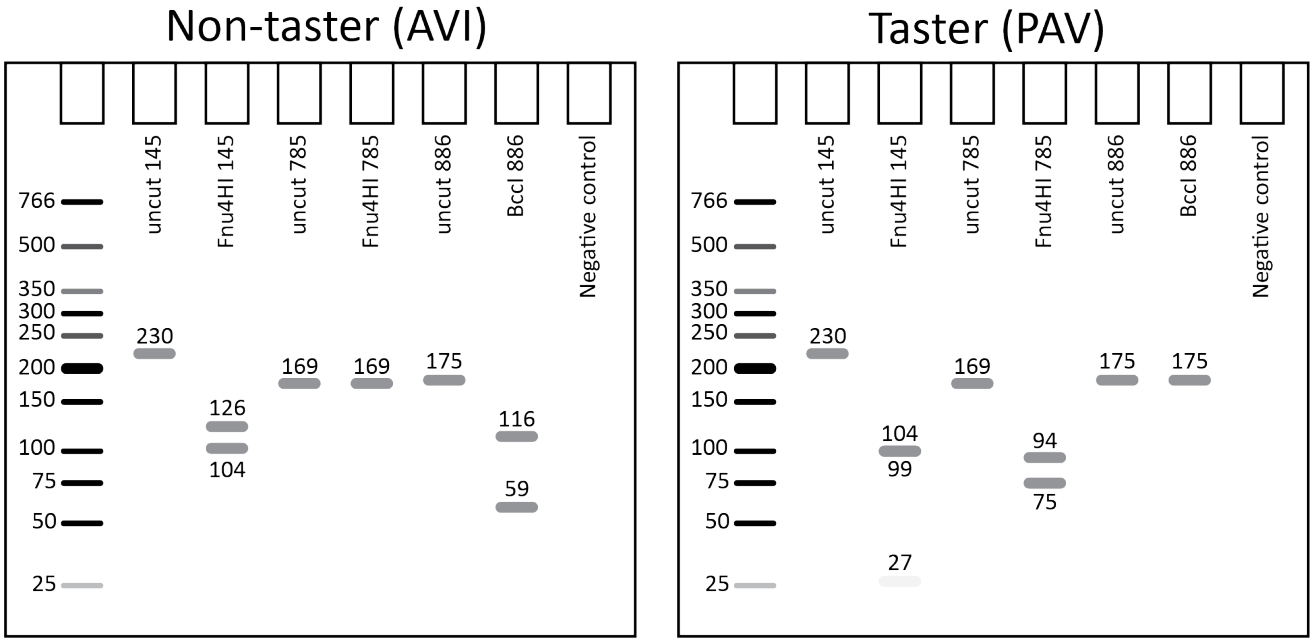
1. Submerge each gel in 10 mL of 1x LabSafe stain prepared in 1x TBE buffer.
   a. 1 µL 10 000x LabSafe + 10 mL of 1x TBE buffer

2. Incubate gel at room temperature with gentle shaking for 20 minutes in the dark.

3. Briefly rinse the gel in RO H_2O, and image the gel. If there is high background, gel may be de-stained in 1x TBE for ~10 minutes at room temperature with gentle shaking in the dark

Interpretation of your results

Possible results:
Your results will vary depending on your genotype. The most common non-taster and taster genotypes have been shown below, but it is possible to have mix of taster and non-taster SNPs in the gene, so your genotype may not match these examples exactly.



2. Your bitter tasting phenotype

Experimental Design – Taste test

1. Fill out the section on the worksheet about taste preferences of various foods, and from this predict if you think you'll be a bitter taster or not.

2. Thoroughly wash your hands with soap and water, then obtain the Control, PTC, PROP, thiourea, and sodium benzoate taste-test strips from your TAs and/or Lab Instructor

3. Starting with the control, touch the taste test strip to your tongue. Record your observations on your lab worksheet.

3. References

Behrens M, Gunn HC, Ramos PCM, Meyerhof W, and Wooding SP (2013) Genetic, Functional, and Phenotypic Diversity in TAS2R38-Mediated Bitter Taste Perception. *Chemical Senses* **38**:475–484.

Boxer EE, and Garneau NL (2015) Rare haplotypes of the gene TAS2R38 confer bitter taste sensitivity in humans. *Springerplus* **4**:505.

Desai H, Smutzer G, Coldwell SE, and Griffith JW (2011) Validation of Edible Taste Strips for Identifying PROP Taste Recognition Thresholds. *Laryngoscope* **121:**1177–1183.

Klenz J, Chow M, and Fontana T (2015) Are You a Hidden Heterozygote? Use of PCR to Genotype Brown vs. Blue-Eye-Color Alleles. *Tested Studies for Laboratory Teaching, Proceedings of the Association for Biology Laboratory Education* **36**:61.

Manley KJ (2015) Taste genetics and gastrointestinal symptoms experienced in chronic kidney disease. *Eur J Clin Nutr* **69**:781–785, Nature Publishing Group.

Rudbeck L, and Dissing J (1998) Rapid, Simple Alkaline Extraction of Human Genomic DNA from Whole Blood, Buccal Epithelial Cells, Semen and Forensic Stains for PCR. *BioTechniques* **25:**588–592, Future Science.

PHAR 203 – Laboratory 3 Worksheet Name:

Laboratory 3 – Pharmacogenomics Worksheet [30 points total]
Due: In lecture, the day after lab.

Although the TAS2R38 *gene has not been directly linked with any particularly negative health outcomes, if you do not wish to test your own DNA for any reason, please let the instructor know, and you will be provided with purified DNA to use in the rest of the experiments in this lab.*

1. Whose DNA did you use for this exercise? _____ **[1]**

2. What was the unique ID written on your PCR and digest tubes? _____ **[1]**

3. PCR is very sensitive to contamination. What control should you run whenever doing a PCR? **[1]**

4. Tasting Survey - Please rate on a scale of 1-5 (**1 Delicious**, 2 Like, 3 Meh, 4 Don't like, **5 Disgusting**, NA unknown). Consider these flavours as individual foods. Take into consideration whether you have always liked these. Were you a picky kid, or are there foods that you have just learned to like? **[1]**

Bitter melon (苦瓜) *	1	2	3	4	5	NA
Brussels sprouts*	1	2	3	4	5	NA
Broccoli, raw*	1	2	3	4	5	NA
Cabbage, raw*	1	2	3	4	5	NA
Carrot	1	2	3	4	5	NA
Chocolate, dark*	1	2	3	4	5	NA
Coffee, strong/black*	1	2	3	4	5	NA
Corn	1	2	3	4	5	NA
Eggplant	1	2	3	4	5	NA
Grapefruit juice*	1	2	3	4	5	NA
Green bean	1	2	3	4	5	NA
Kale*	1	2	3	4	5	NA
Matcha (抹茶)*	1	2	3	4	5	NA
Orange Juice	1	2	3	4	5	NA
Peas	1	2	3	4	5	NA
Potato	1	2	3	4	5	NA
Pu'er tea (普洱茶)*	1	2	3	4	5	NA
Red Radish*	1	2	3	4	5	NA
Tofu	1	2	3	4	5	NA
Tonic water*	1	2	3	4	5	NA
Turnip*	1	2	3	4	5	NA

5. Foods marked with an asterisk are reported to be bitter to some people, or are known to contain thiourea containing compounds. **[2]**
    a) How many "bitter foods" did you rate with a 4 or 5?

    b) Based on this result, do you think you might be a taster?

PHAR 203 – Laboratory 3 Worksheet Name:

6. Bitter compound taste test – for each of the taste test strips **rate how bitter** you find the strip on a scale from 1 to 5, where 1 is no bitterness (it tastes like wet paper) and 5 is extremely bitter (disgusting).

   The chemicals used in the tasting strips are non-hazardous, non-alcoholic, and chemically synthesized (not derived from any animals or plants). NB: If you didn't use your own DNA for this exercise, also record the taste data for the person's whose DNA you used. **[1]**

						Donor phenotype (if used)
Control	1	2	3	4	5	_____
PTC	1	2	3	4	5	_____
PROP	1	2	3	4	5	_____
Thiourea	1	2	3	4	5	_____

7. Sodium benzoate is a common food preservative and may be tasteless, sweet, salty, bitter, or some combination of these tastes. What did it taste like to you? **[1]**

8. The Fnu4HI restriction endonuclease has the recognition site 5'-GCNGC-3'. At the location of SNP 145, this enzyme will only cut the taster genotype. Below is a small portion of the TAS2R38 taster sequence. The number given at the start (136) indicates that the first base shown (A) is nucleotide 136 of the coding sequence of the gene. **[4]**

   136 5'- AAG AGG CAG CCA CTG AGC -3' 153

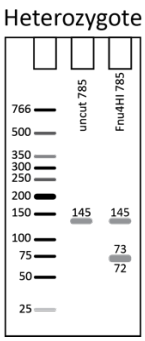
   a) Draw a box around the restriction site.

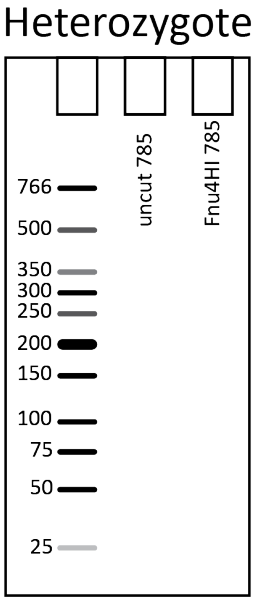
   b) Indicate the location of the SNP.

   c) What would this base be the non-taster genotype?

   d) The taster genotype encodes a proline at the site of the SNP. What is the codon for proline that is used here? What amino acid and codon are present in the non-taster?

9. If you are homozygous for each of the SNPs we test, your restriction fragment pattern may not match what the diagram of possible results in the lab manual. What would you expect to see if you were heterozygous for the most common taster/non-taster genotypes? Draw the banding pattern for SNP 785 on the gel image provided at the right. **[3]**





PHAR 203 – Laboratory 3 Worksheet Name:

Use the image of your DNA samples that has been uploaded to canvas to answer the following questions, as well as the information provided in lab and the lab manual.

10. What bands did you see in your SNP 145 uncut PCR and Fnu4HI digest lanes? Were any bands brighter than the others? [1]

11. What genotype (DNA base), amino acid, and phenotype does your SNP 145 pattern match? [3]

12. What bands did you see in your SNP 785 uncut PCR and Fnu4HI digest lanes? Were any bands brighter than the others? [1]

13. What genotype (DNA base), amino acid, and phenotype does your SNP 785 pattern match? [3]

14. What bands did you see in your SNP 886 uncut PCR and Fnu4HI digest lanes? Were any bands brighter than the others? [1]

15. What genotype (DNA base), amino acid, and phenotype does your SNP 886 pattern match? [3]

16. From your data, what is your SNP profile for the TAS2R38 gene in terms of amino acids (e.g. PAV/PAV, AAV/PVV, etc)? **[1]**

17. What is a possible reason why the phenotype and genotype data for an individual might not match, other than errors in performing any of the protocols? **[1]**