Two complementary methods for genotyping taste receptor TAS2R38 in humans

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Synopsis:

This multi-session laboratory exercise is designed to expand the concept of genetic variation, expose students to multiple molecular techniques and underscore the importance of experimental validation in the scientific method. Briefly, students develop a hypothesis about their ability to taste certain bitter substances, isolate their genomic DNA from buccal cells, and examine a Single Nucleotide Polymorphism (SNP) of interest. They utilize both Derived Cleaved Amplified Sequence (dCAPS) and Sanger sequencing for genotyping the SNP. (Sample sequence data are provided if this methodology is not accessible or cost prohibitive). The students analyze their individual restriction and sequence data and perform direct phenotypic testing with PTC taste strips. Collection of total classroom data is used for Hardy-Weinberg calculations of expected genotype and allele population frequencies. This inquiry based laboratory exercise engages students through their desire for self-discovery without raising any serious health concerns. The exercise is designed for early to mid-level undergraduates in a basic molecular biology or genetics laboratory. The genotyping of TAS2R38 by restriction analysis is adapted from a protocol published by Merritt et al., 2008, and the experimental kit available through Carolina Biological developed in association with The Dolan DNA Learning Center, 2009. Options are provided to adapt the materials for more advanced laboratories including biochemistry laboratories.

Introduction:

This set of experiments is designed to engage students in scientific discovery by allowing them to generate a hypothesis, based on self-survey information, of their ability to taste certain bitter compounds. They test this hypothesis by using two different methods to determine their genotype for a specific taste receptor (TAS2R38) as well as direct phenotype testing. The isolation and examination of their own genetic material engages students through self-discovery, while the selection of a phenotype without clinical consequences for examination does not raise any health concerns for the student.

The students utilize the scientific method by formulating a testable hypothesis, generating experimental data, and analyzing these data to support or refute the hypothesis. Validation of experimental data using a different methodology is common in scientific practices and reaffirms the stringency of good scientific studies. The use of student's own genetic material gives each student an ownership of the project.

Students isolate genomic DNA from buccal cells using a column isolation method (QIAmp Mini DNA Kit). The genotyping is then performed using Derived Cleaved Amplified Polymorphic Sequences (dCAPS) and Sanger sequencing. The dCAPS method utilizes a mismatch primer in order to introduce a restriction enzyme site at a specific SNP site allowing for the differentiation of alleles (Neff *et al.*, 1998). Introduction of sequencing technology allows for using other applications including sequence similarity searching using BLAST (Altschul *et al.*, 1990; Ye *et al.*, 2006).

Approach/Method (Instructor Guidelines):

The method below is designed for three 3-hour laboratory periods and can be used for both majors and non-majors. The 3rd laboratory period has been adapted with some groups to incorporate a tour of the Advanced Genomics Technologies Core at the University of Vermont. We will discuss this more in Lab 3.

We have recently worked with faculty at Landmark College to incorporate this experiment into the laboratory portion of their Principles of Biology II course. The students were first and second year

students with documented learning disabilities (dyslexia, ADD, etc.). In order to meet the needs of these students, we limited the amount of time spent lecturing about the experimental protocol and provided animation resources for the faculty to share with their students before each experimental session. These have been listed in the individual laboratory sections below and included in the student manual for their review before each laboratory.

Introduction of Laboratory:

This laboratory utilizes some basic molecular biology techniques (genomic DNA isolation, PCR, restriction digestion, and Sanger sequencing) to reinforce the nature of genetic variation by elaborating the central dogma of molecular biology (DNA ➔mRNA ➔Protein) and demonstrating that a single nucleotide change can lead to a structural change in a protein affecting its function. In this case the change in function leads to an inability to taste certain bitter compounds.

This experiment is also unique in that the experimental evidence of one methodology is validated by a second. The concepts of validation of experimental data and reproducibility in scientific discovery are main principles of the scientific method. The formation of general scientific concepts and models are the result of scientific discoveries that have been reproduced and tested further in order to expand scientific knowledge.

The technique of Derived Cleaved Amplified Polymorphic Sequences (dCAPs) used in this experiment allows the interrogation of any known SNP by creating a unique restriction site if one is not available. The discussion of this methodology as well as advances in sequence technology can lead to larger discussion of genetic testing, personalized medicine and possible ethical and social implications.

Specific instructions for each laboratory protocol:

Pre-Lab: Hypothesis Generation

Each student will fill out a student taste survey. Instruct them to think about the food separately (without butter, salt, sugar etc.). For example, if you cover broccoli in cheese or put sour cream on a potato it can change the opinion of the food. Many tasters may choose to not eat these foods or mask the bitter taste with fat, sugar or salt. Thinking about the raw version of the foods can help with this as well. Instruct them to consider the specific taste of the food, not the texture, and to think about bitterness specifically. (For tasters, mood can also affect what they choose to eat (Laaksonen *et al.*, 2013)). It can be difficult to determine a precise phenotype. Make sure that the students know they are taking an “educated” guess to form a hypothesis by knowing their likes and dislikes.

Laboratory 1: Isolation of DNA from Buccal Cells

Suggested Animation resources

Laboratory 1

The is the DNeasy visual protocol for genomic DNA isolation (starting at 1:03 minutes end @ 4:00) https://www.youtube.com/watch?v=JAj60HTpto0&list=PLnvL-JBxB4Ysad30ymWNjwTyAW-gPfNsO&index=13

Closer look at the silica column chemistry
https://www.youtube.com/watch?v=SQvjb4vJanQ&list=PLnvL-JBxB4Ysad30ymWNjwTyAW-gPfNsO&index=11

Review of inheritance (click through animation):
http://www.dnalc.org/resources/genescreen/inheritance.html

Pipetting:
Either in an earlier lab period or in the beginning of this lab period (or both if necessary) the students should practice pipetting small volumes. We routinely provide colored water samples for pipetting. Specific pipets and volumes for this lab are p1000/400ul, 500ul and 700ul, p200 or p100/100ul, and p20/20ul.

Genomic DNA Isolation and Quantitation:
We have compared multiple methods for DNA isolation and found the use of the QIAamp DNA mini kit from Qiagen to give the most consistent yield. We also chose to use a sterile swab to isolate buccal cells vs. a rinse and spit method. The sterile swab allows for a smaller starting volume and eliminates the need for a larger centrifuge. The entire protocol can be performed with a microcentrifuge. The use of this method also exposes students to column-based DNA extractions, which are now routinely performed, and eliminates the need for students to handle phenol or chloroform in a fume hood or the instructor to deal with phenol/chloroform waste. An experimental kit is available through Carolina Biological (2009) in association with the Dolan DNA Learning Center that simplifies some of the methodology for introductory courses or high school students. The kit utilizes a Chelex isolation of genomic DNA that may be a better choice for some classes as it is less expensive. In our hands this genomic DNA is less consistent for the subsequent PCR reactions and may require some tweaking. The kit also provides a manual with useful introductory bioinformatics exercises for students.

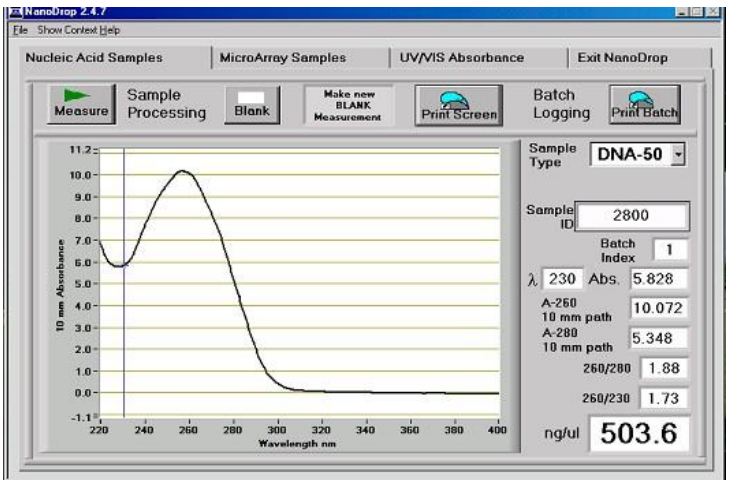
The rate-limiting step for this experiment is the coordination of the centrifugation. If a second microcentrifuge is available it can speed things up. Our latest experiment had 16 students and they finished the DNA isolation with one microfuge in 2hrs and 20 mins. (This included time for practicing pipetting and a brief overview of the day).

One student accidently threw out their 100ul eluate. We were able to isolate enough material to continue by conducting a second elution of the QIAamp column using 50ul of elution buffer. We have also collected second swabs for a back-up prep in case there was a problem with the initial DNA isolation. These can be stored at -20°C for several weeks if necessary.

DNA Quantitation:

We routinely perform NanoDrop analysis in our facility and provide a trace of the results to the students during the second laboratory period (Figure 1). If the institution we are visiting has a NanoDrop or spectrophotometer available, we will perform the quantification with the students if there is adequate time at the end of the laboratory period. If a NanoDrop

Figure 1- NanoDrop Trace



or spectrophotometer is unavailable, 10-20 ul of the genomic DNA eluate can be run on an agarose gel by the instructor or in a separate laboratory with the students to determine the amount of genomic DNA to use in the subsequent PCR reactions. Typically either 5 or 10 ul of the student's genomic DNA sample is used for PCR in laboratory 2.

Optional Task:
Explore the SNP database at NCBI http://www.ncbi.nlm.nih.gov/snp
Overview of SNPs http://www.ncbi.nlm.nih.gov/books/NBK174586/

Single Nucleotide Polymorphisms are single base-pair mutations that occur in DNA. They occur every ~300 bps in the human genome. That means that there are over 10 million SNPs in the human genome. Some of these are of no consequence while others can have a dramatic effect on protein expression or function.

In the SNP database search for TAS2R38. The first 3 hits correspond to the three main SNPs C145G, C785T and G886A affecting PTC tasting ability (files rs713598, rs141972905 and rs 10246939 respectively). Clicking on rs713598 will open a file for the first SNP, which the students will be studying in this experiment. There is a lot of information within this file. Below we suggest few items to focus on.

1. The Gene Model(s) block gives a quick overview of the nucleic acid and amino acid changes associated with this SNP.

2. Below this there are two contig maps that include the TAS2R38 gene. Use the slide tool above the map to show how these maps are interactive. Zoom out to view the entire gene and show the other SNPs in the gene.

3. The Fasta sequence shows the SNP represented by a green S.

4. Below the Fasta sequence is an NCBI resource list. The OMIM file 607751.0001 is a great link for an overview of the gene and the different alleles.

5. The Population Diversity section shows a schematic of observed genotype and allele frequencies. It is important to look at the sample number when weighing the results. An optional task or project is to explore the possible reasons for the variation between populations (more literature is provided in laboratory 3 - optional task #2).

Laboratory 2: PCR Amplification of Taste Receptor

Suggested Animation resources:

Laboratory 2
These are somewhat repetitive but they are short and provide some different information on PCR amplification.

Two videos https://www.youtube.com/watch?v=2KoLnIwoZKU
https://www.youtube.com/watch?v=iQsu3Kz9NYo
This video describes the reagents and has an interactive animation to follow:
http://learn.genetics.utah.edu/content/labs/pcr/

Pipetting
Before students begin setting up the PCR reaction we have them practice pipetting 1ul of a 50% glycerol solution. We do not add dye to this solution and we have them demonstrate their pipetting technique to each other and an instructor. This will help them more accurately pipet very small volumes of enzymes for this and subsequent experiments.

Nomenclature
We do not mention the term Restriction Fragment Length Polymorphism (RFLP) or PCR-RFLP in the student manual but suggest that this term is discussed in relation to CAPS and dCAPS. CAPS and PCR-RFLP are the same technique; where the area around a SNP is PCR amplified and then digested at a naturally occurring differential restriction site. dCAPs is a modification of this technique; where the introduction of a mismatch primer creates a useful (differential) restriction site. dCAPS is a powerful and low cost technique that allows for restriction site analysis at any SNP.

PCR Amplification of a 221 bp fragment of the TAS2R38 gene
The student manual mentions that the TAS2R38 gene consists of one exon allowing for primer design without the concern of intron sequences. It is important for students to understand this concept in relation to future genomic PCR experiments they may conduct.

A mismatch primer is used to create a new restriction site for differentiation between the two alleles. Figure 1 in the student manual provides a model for the students. Review with your students why the reverse primer will be used for sequence analysis and not the forward primer (cleavage site too close to the forward primer).

We routinely use EX-TAQ from Takara for amplification of the PCR product. Each student team (2-3 students) works together to create the master mix. The student protocol is designed for teams of three students to prepare a 4X master mix. This master mix is designed to have each students use 5 ul of their DNA prep in the PCR reaction. (If student DNA concentrations are low we routinely double the amount of DNA adjusting the master mix accordingly).

With smaller groups of students each student can prepare a 4X master mix and set up their reaction plus positive and negative control reactions. We have found the preparation of individual controls to be wasteful in large groups and we do not usually run these controls on the gels to minimize the number of gels needed. We do discuss the reasons for positive and negative controls in our overview of PCR.

Note: If positive and negative PCR controls are set up these should be added to the gel in laboratory session 3. The positive control (TT or Tt) should be subjected to HaeIII digestion before gel electrophoresis to confirm that the enzyme is working properly.

Note: There is a naturally occurring restriction enzyme site from bp 141-145 (Fnu4HI GCNCG) that can distinguish the polymorphism c145g (pro49ala). However, the Fnu4HI enzyme is quite expensive and requires a different set of primers for more information please see Merrit *et al*., 2008.

Check with the specific sequencing facility for PCR reaction and reverse primer concentrations and amounts to be sent to for sequencing analysis. We work with the Advanced Genome Technologies Core (AGTC) facility at UVM for Sanger sequence analysis. They provide us with files containing the electropherogram and the sequence for further analysis. If sequencing of student samples is cost

prohibitive, sample sequence data can be distributed to students after restriction digest analysis for interpretation in lab 3. This will change some of the timing suggested in the student manual for lab 3. (Find sample files of all genotypes in supplementary materials containing electropherograms and sequence).

Optional Task
BLAST the primer sequences to determine their specificity. **BLAST** for **B**asic **L**ocal **A**lignment **S**earch **T**ool is an algorithm for comparing biological sequence information (nucleotide or amino-acid sequences). A BLAST search compares a query sequence with a library or database of sequences, and identifies sequences that resemble the query sequence above a certain threshold. Use the sequences below.

Forward Primer (without mismatch) – 5' CCTTCGTTTTCTTGGTGAATTTTTGGG ATGTAGTGAAGAGGCGG 3'

Reverse Primer (reverse complement sequence) – 5'GATGATTGCAAACCAAGCCAACCT 3'
Go to the NCBI BLAST site http://blast.ncbi.nlm.nih.gov/Blast.cgi

Under the Basic BLAST select nucleotide blast. Paste one of the above sequences into the query box.

Under Choose Search Set – Database select the Human genomic + transcript box.

Click the blue BLAST button at the bottom of the page.

A report will be generated within a short amount of time. In the top right hand corner of the report are two recourses for how to read and interpret the report.

Experimental Option or Follow-up
There is tremendous variation in PTC-sensitivity among tasters (Blakeslee, 1932). Many studies have looked at the three main SNPs C145G, C785T and G886A (protein changes - P49A, A262V, and V296I respectively) to explain some of these differences (reviewed in Wooding, 2006). Emerson, (2012) provides an experimental protocol to amplify and sequence the entire TAS2R38 gene in order to further investigate taste variation at this level. Briefly, primers are used to PCR amplify the entire TAS2R38 gene. The PCR product is then sequenced with both primers and the genotype of all 3 SNPs can be determined. The phenotypic difference between tasters can then be compared to all three SNPs to see if there is a correlation.

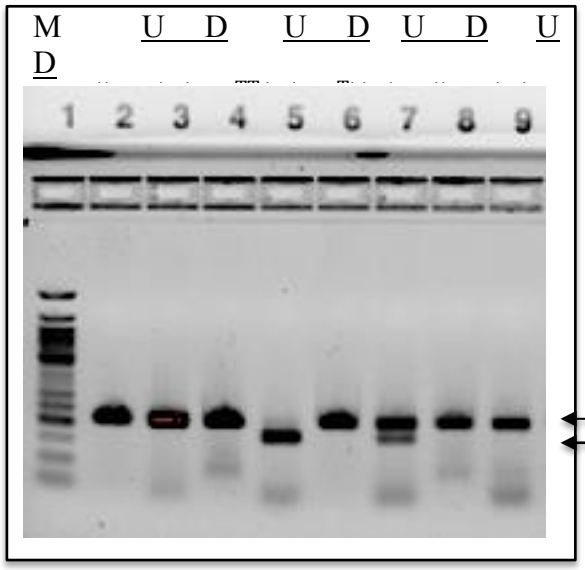
Lab 3: Genotype and Phenotype Determination

Suggested Animation resources

Laboratory 3
https://www.dnalc.org/view/15923-Cycle-sequencing.html
https://www.youtube.com/watch?v=oG7ob-MtO8c - (Review of Hardy-Weinberg equation with examples)

Restriction Digestion and Gel Analysis
We have found a one-hour incubation with HaeIII to be efficient for complete digestion of the PCR samples. The incubation time can be adjusted to meet the needs of the lab. If there is a problem with the restriction digestion of the PCR product, the sequence analysis will confirm the genotype and allow

Figure 2– Restriction Digest on E-gel



the opportunity to discuss the usefulness of experimental validation especially in more complex experiments. (If the provided sequence files are used to match the genotype demonstrated by restriction analysis it is important that the digest is incubated for at least one hour). We have found the use of the E-Gel® electrophoresis system (Invitrogen) convenient for traveling to colleges. The premade gels run quickly and reduce exposure of students to Ethidium Bromide. Figure 2 shows HaeIII restriction digest of 212 bp PCR products. Uncut (U) and digested (D) PCR products are show from 1 homozygous taster (TT), 2 homozygous non-tasters (tt) and a heterozygote (Tt). A figure of the NEB Quick-Load® 50pb DNA Ladder is provided in the student manual. If a different Ladder is used this should be modified.

Optional Tour of Sequencing Facility

If possible we conduct Laboratory 3 at the University of Vermont (UVM). This allows for a tour of the Advanced Genome Technologies Core (AGTC) facility at UVM. We can schedule this before or after the wet bench work or during the restriction enzyme digestion depending on time restrictions. We have found that touring of core facilities used in this and other laboratories to be exciting and a great learning opportunity for students at smaller colleges. We have also included this experience for the students by scheduling a 4th "laboratory" day or if the course schedule is inflexible inviting students to visit the facility at their convenience

PTC Taste Test

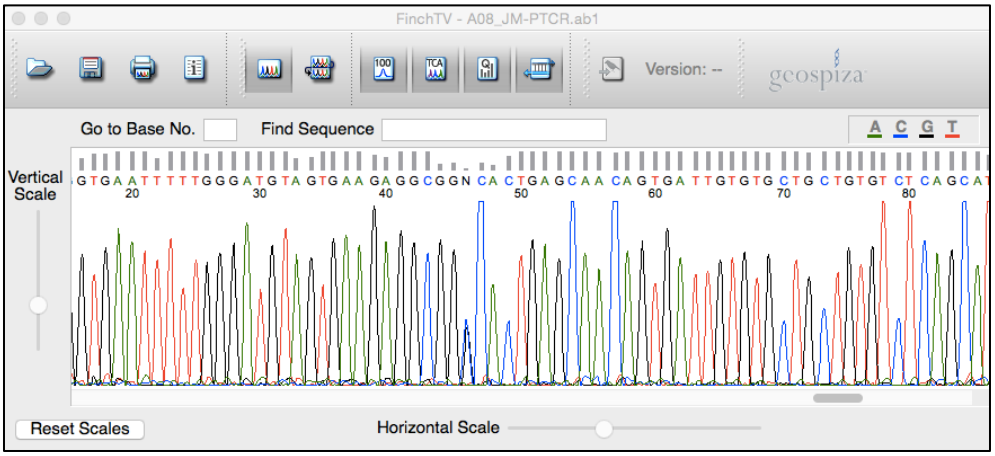
The protocol is written to use the PTC strips without a negative control of paper strips. We have found it is very obvious whether a student is a taster or not. Control strips can be generated or purchased (ordering information provided in Instrumentation and Materials section).

Sequence Analysis

Sample sequencing data for all genotypes are included in the supplementary materials. The data includes electropherograms and the linear sequences (Figure 3). These data can be viewed by downloading free sequence viewing software. We recommend the viewer from FinchTV http://www.geospiza.com/Products/finchtv.shtml. The sequence information can be used for sequence similarity searching using nucleotide BLAST (blastn) to identify the sequence and conduct an alignment to identify any nucleotide differences between the student sequence and sequences in the reference database. It is important to remember that this sequence is generated with the reverse primer so it will be the reverse sequence. The  button will create the forward sequence.

Below is the sequence of a heterozygote with an N at position 46, where both C and G nucleotides are present at this site.

Figure 3 - Electropherogram

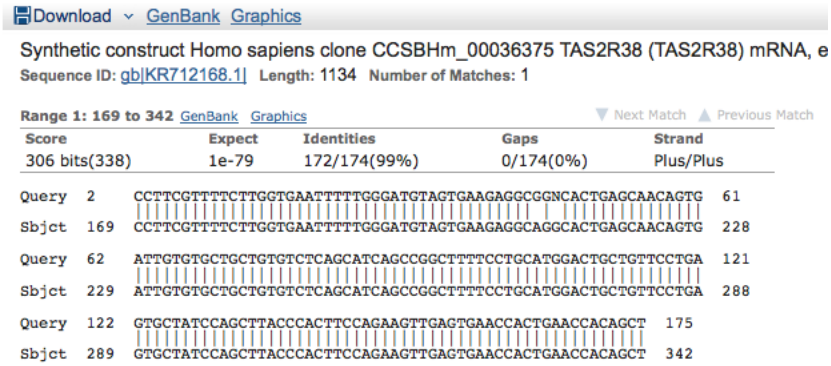


Sequence files in supplementary materials non-taster (tt) MT and RD, Taster homozygote (TT) AM and TH, Taster heterozygote (Tt) JM and WE. An overview of interpreting electropherograms can be found at: http://www.udel.edu/dnasequence/Site/Interpreting_Electropherograms.html

BLAST - alignment

In FinchTV, you can directly query the student sequence against a reference database using BLAST (blastn) via the edit tab. Figure 4 shows the first alignment result. A gap in sequence alignment is seen where the modified primer generated the restriction enzyme site in tasters (A to G). A second gap is seen at the SNP site. An N (no base clearly distinguishable) is called by the sequencer because both a C and G nucleotide is present at this site (heterozygote).

Figure 4 – TAS2R38 Sequence Alignment



Hardy-Weinberg Equations

A worksheet for Hardy-Weinberg Equations is included in the supplementary material. The YouTube video listed as supplementary material provides a clear overview of the Hardy-Weinberg Principle. The published ratios predict 70-75% tasters and 25-30% non-tasters in the USA. These frequencies vary in populations as discussed briefly in the overview of the SNP database (optional task in laboratory 1). More literature information is provided in optional task 2 below. Our most recent experiment conducted on 29 students and instructors resulted in 21Tasters (3 TT, 18 Tt) and 8 non-tasters (8 tt)

(72.4% Tasters and 27.6% Non-tasters). These results can be merged with smaller student group results to strengthen the Hardy-Weinberg Equations.

Optional Tasks:

1. Within a more advanced molecular or biochemistry class, explore the predicted 3D protein structures of TAS2R38 bitter receptors encoded for by different alleles. The papers by Tan *et al*., (2012) and Floriano *et al*., (2006) predict the 3D structure of the PTC receptor as compared to other seven-transmembrane domain G protein-coupled receptors. These receptors may confer differential sensitivity to various ligands (Emerson, 2012; Henkin & Gillis, 1977; Tharp *et al.*, 2005). This exercise requires the instructor to have knowledge in the area of protein modeling.
2. Delve deeper into the potential natural selection mechanisms that have allowed the maintenance of both alleles and the differences in PTC taste sensitivity around the world (Cavalli-Sforza *et al.*, 1994; Wooding, 2006; Wooding *et al.*, 2004). This could include further expansion of the understanding of population genetics and differential selective pressure in various environments. The following sites provide some interesting information.
   http://www.nidcd.nih.gov/health/statistics/smelltaste/Pages/global.aspx
   http://www.tastescience.com/abouttaste4.html

Lab Safety:

These labs require instruction on how to work with proteinase K, ethidium bromide and UV light as well as a discussion of sterile technique to avoid contamination of the samples. If using E-gels the exposure to ethidium bromide is minimized significantly. The E-gel waste (ethidium bromide) should be disposed of properly. (Ordering information for the SYBR-safe E-gel system is also provided in the Instrumentation and Materials section). Students will be working with their own cheek cell materials lowering concerns regarding infectious material. Cell waste should be disposed of properly. Students should wear lab coats, gloves, and eye protection during these experiments.

Concern about the safety of the PTC tasting strips has been questioned by Texley *et al*., 2004, however further evaluation by Merrit *et al*., 2008 compares the toxicity of PTC to common table salt and they state "We calculate that the 230 mg of NaCl (salt) in a vending machine bag of potato chips is about 100 times more toxic than the .007 mg of PTC in a taste paper." There has been 75 years of PTC research without any evidence of toxicity associated with PTC taste paper (Merritt *et al.*, 2008; Wooding, 2006).

Student Evaluation:

Students can be evaluated in several ways. Upper level students are asked to write a full laboratory paper outlining the development of their hypothesis, the methods used to test their hypothesis, results of the experiment for their individual sample and for the entire class, followed by conclusions including a discussion of the expected and observed allele frequencies based on Hardy-Weinberg principles.

The student manual provides areas for the addition of student data and questions for testing student interpretation and understanding of the experiment and data. These can be collected for evaluation. Worksheets for Hardy-Weinberg equations are also provided.

Justification:

This inquiry-based laboratory engages students through their desire for self-discovery. Students conduct a self-evaluation in order to develop a hypothesis about their ability to taste bitter compounds in foods (those containing a thiourea moiety). In the process of testing their hypothesis they perform several

molecular biology techniques, learn about genetic variation in humans, and gain an understanding of the relationship between genetic equilibrium and evolution. The laboratory reinforces the nature of genetic material and patterns of inheritance by the demonstration that a single nucleotide change can result in a phenotypic change.

The core competencies covered in this lab are directly related to the scientific method including; observational strategies, hypothesis testing, experimental design, evaluation of experimental evidence and experimental validation through multiple techniques. Each student develops a hypothesis based on their food preferences and predicts the possible genotypes related to the expected phenotype. Students then harvest their own DNA, perform PCR amplification of the gene of interest and conduct dCAPS analysis of the PCR product. A portion of the PCR reaction is also used for Sanger sequencing analysis. The generation of sequence information provides an introduction to sequence viewer software and BLAST searches. An analysis of the group data allows the introduction of Hardy-Weinberg distribution frequencies expected in populations.

Upper level students are expected to strengthen their scientific writing skills by generating a formal laboratory report, and all students are expected to answer specific questions within their student manual using correct scientific language as well as demonstrating their ability to communicate scientific concepts through accompanying worksheets. This laboratory can be used to discuss genetic testing including what should be tested, who should be tested, and the potential social impacts of testing.

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