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Genetics Education

Innovations in Teaching and Learning Genetics

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Reinventing the Ames Test as a Quantitative Lab That Connects Classical and Molecular Genetics

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ABSTRACT

While many institutions use a version of the Ames test in the undergraduate genetics laboratory, students typically are not exposed to techniques or procedures beyond qualitative analysis of phenotypic reversion, thereby seriously limiting the scope of learning. We have extended the Ames test to include both quantitative analysis of reversion frequency and molecular analysis of revertant gene sequences. By giving students a role in designing their quantitative methods and analyses, students practice and apply quantitative skills. To help students connect classical and molecular genetic concepts and techniques, we report here procedures for characterizing the molecular lesions that confer a revertant phenotype. We suggest undertaking reversion of both missense and frameshift mutants to allow a more sophisticated molecular genetic analysis. These modifications and additions broaden the educational content of the traditional Ames test teaching laboratory, while simultaneously enhancing students' skills in experimental design, quantitative analysis, and data interpretation.

As called for by numerous national groups (*e.g.*, National Research Council 2003; Handelsman *et al.* 2004), biology education has moved in recent years toward the provision of research-rich environments in which undergraduate laboratories are investigative and open ended and in which quantitative skills are emphasized. While data analysis has long been a staple of student learning, recent research demonstrates that students become most engaged and learn best when they have a hand in the design of experiments as well as in the execution and analysis of resulting data (Hake 1998; Merkel 2003; Handelsman *et al.* 2007). Inquiry-based labs have been shown to improve students' research skills in biology (Myers and Burgess 2003). Further, as suggested by *BIO2010* (National Research Council 2003), biology curricula should explicitly build the quantitative skills of budding biologists. Hack and Kendall (2005) argue that biology curricula should change because current life science students must learn to use models, to apply appropriate mathematic tools and statistics to solve problems, and to manage and integrate data. That these tools are best taught in the context of biology courses themselves has been demonstrated by Metz (2008), who has shown that undergraduate biology students do not make connections between quantitative concepts taught in mathematics and statistics courses and their application to biological problems. Metz (2008) demonstrates that inclusion of quantitative and statistical analyses in biology laboratory courses led to significant gains in long-term retention of such knowledge, regardless of whether students also had taken courses in statistics.

To address the issue of connecting quantitative analysis and biological problem solving, we have extended the open-ended Ames test for the undergraduate genetics lab to allow students to practice quantitative skills during student-driven experimental design and analysis. Students bring to the lab potential mutagens of their choice, and they are charged both with creating methods to determine the number of colony-forming units (CFUs) per bacterial culture and with using that figure to determine reversion frequencies. We find that this is a difficult task for students, but having them

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conclude what sort of serial dilutions are needed, for example, is an important step in gaining a long-term understanding of the quantitative aspects of the lab and other similar analyses. This approach requires that instructors and students bring to the lab an attitude of investigation and learning rather than a sense of urgency to "do the lab" and obtain a particular result.

Importantly, this lab also fulfills a second great need within the genetics curriculum, specifically, the ability to directly connect concepts of classical genetics with those of molecular genetics. Investigating both classical and molecular genetics in the time frame of the undergraduate lab requires the use of fast-growing organisms with easily selectable phenotypes. Thus, the classic Ames test using *Salmonella typhimurium* in a reversion screen provided the starting point for the design of this lab. Marshall (2007) has published a yeast-based version of the Ames test that is also investigative in nature. Marshall's version does not include the level of quantitative analysis described here, nor does she have students perform molecular analysis of revertant genes, although her lab could be extended as we have done with Salmonella. Wessner *et al.* (2000) has described an initial qualitative spot-overlay experiment followed by a secondary dose-response experiment as a cost-effective substitute for the traditional Ames test lab. This modification increases the amount of quantitative and qualitative data generated over a period of 2–3 weeks, but it does not carry the experiment further than classical genetics. The lab described here has students carry their investigation to the molecular level, including DNA preparation, PCR, and DNA sequencing, thus connecting reversion analysis, an important and rather difficult classical genetic concept, with revertant gene sequences. The lab is cost effective and produces substantial classical and molecular genetic data in <3 weeks (two lab sessions plus sequence analysis).

Learning outcomes at the conceptual level of molecular genetics include an enhanced understanding of the fact that multiple DNA sequences can encode a functioning enzyme, and hence can confer the same phenotype, that different mutagens produce different molecular lesions, and that beneficial mutations can be produced spontaneously, as well as classical concepts of mutagenesis and reversion. Students should leave with improved understanding of molecular techniques such as DNA extraction, PCR, DNA sequencing, and sequence comparison. In addition, students' quantitative skills receive practice in the design of appropriate serial dilutions, computation and understanding of reversion frequency, use of descriptive statistics, and discussions of statistical significance.

The original Ames test (Ames 1979) is a reversion screen using Salmonella strains with a His^- phenotype due to mutations in *HisD* or *HisG*. These Salmonella are plated on histidine-deficient media. Mutagens or other compounds are then added to the plates using one of several application procedures and the number of revertant His^+ colonies is enumerated. We report here protocols for adding a quantitative component to this lab in which both spontaneous and mutagen-induced reversion frequencies are experimentally derived. Traditional pedagogical applications of the Ames test end with students simply comparing the numbers of revertant colonies produced by different substances. This process does not allow quantitative comparison between mutagenesis of strains carrying different His^- mutations. In addition, when students carry out a traditional Ames test lab, they do not determine which DNA sequence changes confer revertant phenotypes and thus cannot infer which mutation mechanisms acted on the strains or fully understand the concept of reversion.

Recently, some research laboratories have used DNA sequence analysis in conjunction with the traditional Ames test (Levine *et al.* 1994; Abu-Shakra *et al.* 2000) and found strong evidence of substantial sequence variation among revertants of a given strain (Koch *et al.* 1994). We report here a new protocol that allows students to analyze the molecular lesions that confer the His^+ phenotype in revertants of strains carrying either a missense or frameshift His^- mutation in *HisG* or *HisD*, respectively. The variability in the molecular lesions between these revertants will give students concrete, experimentally derived examples of the connection between phenotype and genotype by showing students that there are many ways that gene sequences can evolve to confer a specific phenotype. Students will also find that treatment with known mutagens preferentially induces specific types of mutations (*e.g.*, transitions and deletions) that correlate to the mutagenic properties of these substances (Takiya *et al.* 2003). Data produced in this lab can also provide a starting point for discussions of mutation mechanisms, DNA repair, and molecular evolution.

MATERIALS AND METHODS

Strains and media: Salmonella strains TA98, TA100, and TA102 were obtained from BioReliance. Strains TA1535 and TA1538 were obtained from the American Type Culture Collection. Only strains TA1535 and TA1538 are used in the final teaching laboratory, as they are readily available and produce consistent results easily analyzed by undergraduates. Overnight cultures were grown in 10 ml of L medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose) at 37°. Nutrient-rich plates contained L medium with 1.5% bacto agar; nutrient-deficient plates contained VBM (0.02% $MgSO_4$*$7H_2O$, 0.2% citric acid, 1.0% K_2HPO_4, 0.35% $NaNH_4HPO_4$, 1.5% bacto agar, 2.0% dextrose). Three milliliters of overlay agar (9.7×10^{-11}% L-histidine, 1.1×10^{-3}% biotin, 0.55% agar, 0.45% NaCl) was used to plate Salmonella strains.

Week 1: CFU determination and Ames test—one 3-hr lab

Determining CFUs in bacterial cultures: To allow students to quantify reversion frequencies, students determine the CFU

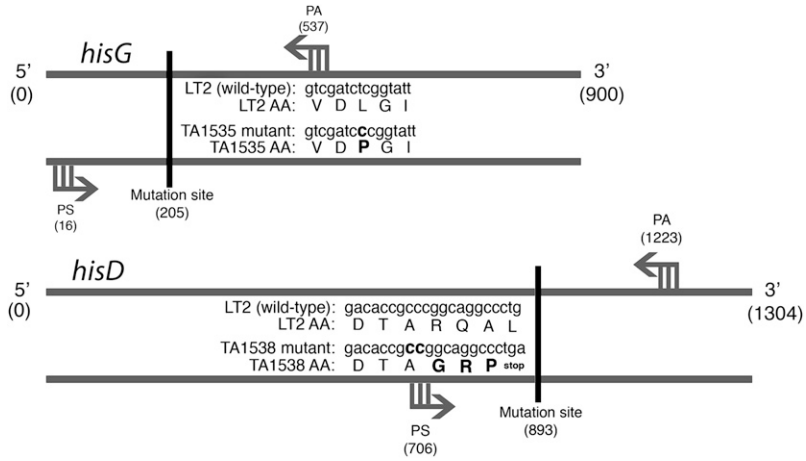

FIGURE 1.—The *hisG* and *hisD* genes of *S. typhimurium* and the location and sequence of the mutation sites found in TA1535 and TA1538, respectively. Amino acid (AA) sequences are shown under the base sequences. Mutations conferring the His$^-$ phenotype are shown in boldface type and are surrounded by bases and AAs to indicate the effect of the mutation on the protein sequence. PS and PA denote binding sites for sense and antisense primers used in PCR. Base locations are relative to the first base of the respective coding regions.

content of their overnight cultures. Students are told to expect 2×10^8–2×10^9 CFUs/ml in overnight cultures of Salmonella. Students must determine how to appropriately and accurately dilute their cultures to reach 100 colonies/plate, taking into account the expected bacterial concentration and the volume to be plated. Students typically plate 100 μl of two dilutions, between 1×10^{-6} and 5×10^{-7}, on nutrient-rich agar. Plates are incubated for 24 hr at 37° after which students count individual colonies. Students must then use this information to determine how many His$^-$ bacteria were plated in their Ames test.

Inducing mutagenesis via the Ames test: To produce His$^+$ revertants, 100 μl of a TA1535 or TA1538 overnight culture is mixed with 3 ml of top agar and immediately plated on VBM. After allowing the top agar to harden, 10 μl of a potentially mutagenic substance is pipetted onto a sterile 0.65-cm filter paper disc. Pairs of students are encouraged to test the mutagenicity of two substances they bring to the lab as well as both control substances; solid substances such as food products are placed in a student-determined portion of water and blended. We obtained the best results when no more than a half-volume of water was used to blend solids. The disc is then placed, mutagen side down, on the center of the top agar overlay. Potentially mutagenic test substances that are not water soluble are plated via direct application to the center of the plate. Positive controls include NaN_3 at 0.05 mg/ml for strain TA1535 and 4NOP at 0.5 mg/ml for strain TA1538, although both mutagens are tested with each strain. Test substances, such as uncooked foods, that might contain substantial amounts of other microorganisms can be autoclaved or filter sterilized, if sufficiently liquid, before application. Alternately, the potential mutagen can be plated directly without Salmonella on VBM to confirm the absence of these potential contaminants. In our experience, contaminating bacteria are seen at very low frequency and are easily distinguished from the Salmonella colonies. Plates are incubated at 37° for 48 hr. Revertant colonies are counted in a manner that does not contaminate the plates. Students circle the revertant colony that they would like to use for molecular analysis and plates are stored at 4°. Instructors may want to provoke a discussion concerning how one would rigorously determine the mutation spectrum of a substance, leading students to realize that spontaneously derived mutants will also be present at lower frequency in any mutation-induced collection of revertants. In a research setting, it might therefore be best to sequence every revertant from a single plate and compare the mutations observed to those seen from spontaneously generated revertant sequences. To give the students more control over the lab experiment, however, one independent revertant colony identified by each student is picked with a sterile inoculating loop and grown overnight in 10 ml of L medium prior to the next lab.

Week 2: Characterizing molecular lesions conferring a His$^+$ phenotype—one 2-hr lab

To isolate genomic DNA from liquid cultures of Salmonella, Qiagen DNeasy mini-prep spin columns were used per manufacturer's instructions. We designed primers to amplify 491- and 487-bp fragments that flank His$^-$ mutations of *hisG46* (in TA1535) and *hisD3052* (in TA1538), respectively. Base-pair assignments denote position relative to the start codon of the respective His coding region (Figure 1): *hisG* (sense)—5′-CGC TTTACGCATAGCT-3′ (bp 16); *hisG* (antisense)—5′-AGCTTC AAGCGTCGC-3′ (bp 537); *hisD* (sense)—5′-CCGTCTGAAG TACTG-3′ (bp 706); and *hisD* (antisense)—5′-TCAATGGTT GATGCC-3′ (bp 1223). Each thermocycler reaction contains 21 μl nuclease-free water, 1 μl each of sense and antisense primers for the appropriate gene (at 12.5 pmol/μl), 2 μl template DNA, and 25 μl of clear Promega master mix (3 mM $MgCl_2$, 400 μM dNTPs, and 50 units/ml Taq polymerase). The optimal annealing temperatures for *hisG* and *hisD* primers necessitate separate thermocycling for each set. Thermocycler programming is 5 min at 94°, 30 cycles of 2 min at 94°, 30 sec at 38° (*hisG* primers)/44° (*hisD* primers), 2 min at 72°, and a final elongation time of 20 min at 72°. Following PCR amplification, amplicons are purified using Qiagen QIAquick spin columns; gel electrophoresis using 1.5% agarose with TBE can be performed to confirm the presence of desired fragments. Purified amplicons (10–20 ng) and 3- to 5-pmol sense primer were sent to the Yale University DNA facility for sequencing. Sequence data were analyzed using MacVector and compared to both the sequences from Salmonella LT2 (wild type) and TA1535/TA1538 (NCBI). For class use, DNA sequences can be compared using NCBI's BLAST engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Full text lab handouts may be obtained from http://www.lawrence.edu/fast/DESTASIE/protocols.html.

RESULTS

Bruce Ames and colleagues produced a number of Salmonella strains with mutations in the *his* operon, two of which we recommend for use in the undergraduate genetics lab. We identified strains with different His$^-$ mutations so students could see for themselves how secondary mutations lead to reversion of a missense *vs.* a

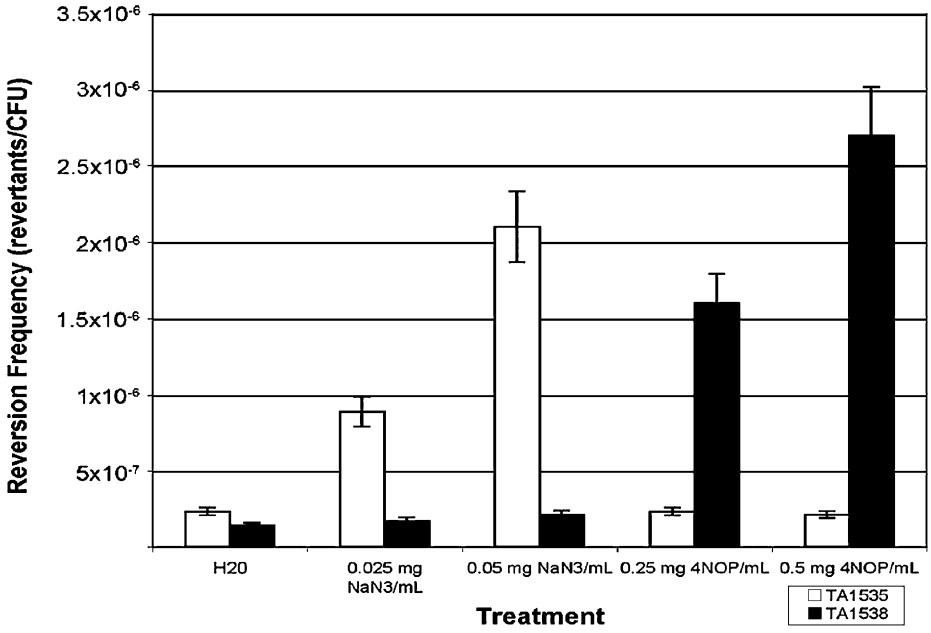

FIGURE 2.—Reversion frequency, measured in revertants/CFU, of TA1535 and TA1538 after exposure to H_2O (negative control) and indicated concentrations of NaN_3 and 4NOP. $n = 15$; error bars, 1 SE.

frameshift mutation. After experimenting with five Salmonella strains—TA98, TA100, TA102, TA1535, and TA1538—we identified two strains for which assays provided reproducible, countable numbers of spontaneous revertant colonies from easily measured quantities of overnight cultures. In three replicate experiments, both TA100 and TA102 produced >100 very large spontaneous revertants per plate; the excessive number and size of colonies produced made accurate counting difficult as colonies often merged. Strains TA98, TA1535, and TA1538 each produced between 10 and 50 spontaneous revertants per plate. TA1535, containing a missense T → C allele of *hisG* (*hisG46*), and TA1538, containing a −1 frameshift mutation in *hisD* (*hisD3052*), were selected for use, and all data that follow are derived from experiments with these two strains.

To accurately calculate reversion frequencies, experiments were conducted to determine the average CFU content of overnight cultures. Overnight cultures of TA1535 consistently grew to a slightly higher density than did TA1538, with an average of 4.5×10^8 compared to 3.9×10^8 CFUs/ml, respectively ($n = 36$). TA1535 and TA1538 colonies looked similar after ~24 hr of incubation. Excessive incubation periods (>24 hr) produced large colonies in both strains, which merged and made colony counting difficult.

It is desirable to have students use known mutagens as positive controls that have the lowest possible toxicity to humans and that have different mutation spectra. NaN_3 and 4NOP were identified for this purpose. NaN_3 is known to induce missense mutations (OLSEN *et al.* 1993) while 4NOP primarily induces deletions and insertions (AMES *et al.* 1975). Because TA1535 contains a missense allele (of *HisG*) and TA1538 contains a frameshift allele (of *HisD*), each mutagen should produce a different reversion frequency with the two Salmonella strains.

As expected, NaN_3 and 4NOP did produce revertants at very different frequencies in the two Salmonella strains used; each treatment exceeded only the spontaneous reversion rate in the expected strain. The spontaneous reversion rate of TA1535 was 2.3×10^{-7}/CFU while TA1538 spontaneously reverted at a rate of 1.4×10^{-7}/CFU (Figure 2). After testing mutagen concentrations ranging from 1.0 to 0.0001 mg/ml, we used concentrations that yielded reversion rates significantly higher than the spontaneous rate, but for which the number of revertant colonies per plate could be enumerated by hand. Significant increases in the reversion rate to 8.9×10^{-7} and 2.1×10^{-6} revertants/CFU were observed in TA1535 after treatment with 0.025 and 0.05 mg NaN_3/ml, respectively. Similarly, treatment of TA1538 with 0.25 and 0.5 mg 4NOP/ml produced significant increases in the reversion rate to 1.6×10^{-6} and 2.7×10^{-7} revertants/CFU, respectively. Treatment of TA1535 with 4NOP and of TA1538 with NaN_3 produced reversion rates that were virtually identical to the spontaneous reversion rate for each strain. Students will note that colonies of strain TA1535 are larger than those of TA1538. Students should also note that, after treatment with 4NOP, strain TA1538 produces large numbers of small colonies that aggregate around the center of the plate, indicating the lower diffusion rate of 4NOP. At the recommended concentrations, kill zones surrounding the filter paper disc will not be observed with either strain.

It should be noted that the calculated reversion frequencies will be more rigorously quantitative for the spontaneous reversion frequency and comparisons between mutagens more difficult. Mutagen-induced frequencies should be considered conservative estimates due to possible differences in mutagen diffusion rates through the top agar. In our hands, water-soluble sub-

TABLE 1

Different environmental substances qualitatively tested for potential mutagenicity

Substances tested A–C	Substances tested D–Z
Artificial vanilla flavoring	Deodorant
Artificial sweetner	Diet cola
Bread crust	Ethidium bromide
Burnt bread crust	Hair dye
Burnt hamburger	Hand sanitizer
Carmex	Organic coffee (caffeinated)
Celery	Peanut butter
Cigar tobacco	Rat poison
Cigar ashes	Stone-ground mustard
Chewing tobacco	Super glue
Coffee (caffeinated)	Tea (caffeinated)
Cooked hamburger	Tea leaves (caffeinated)

stances appear to diffuse uniformly from the centrally placed discs, as indicated by uniform distributions of revertants across the plate. Students will, however, note that 4NOP does not fully diffuse across the plate (it is minimally water soluble) and revertants will be clustered within an ~3-cm radius of the disc. This fact does not diminish the importance of the quantitative data analysis as one can still rigorously compare the response of the two different Salmonella strains to 4NOP and see that reversion frequencies differ substantially. Students should be led to understand that reversion frequencies of 4NOP and any other less soluble substances should be reported as minimal reversion frequencies as CFUs plated outside the diffusion zone will not have been exposed to mutagen, while bacteria within the diffusion zone may be exposed to a gradient of mutagen. It is a good lesson for students to realize that even quantitative results are subject to bias of various sorts and must be based on experiments where as many variables as possible are controlled. Students should be encouraged to brainstorm other methodologies that would ensure uniform distribution of mutagens, such as inclusion in the top agar before plating (although the increased temperature here might be problematic). We note that we do not use other methodologies in the teaching lab because we prefer to minimize the spread of toxic mutagens to glassware that is handled by undergraduates. The disc method keeps the mutagen application in a defined space with minimal "spread" around the lab.

To determine whether students will see significant mutagenesis after treatment with substances of their choice, we subjected 24 substances to a qualitative assay (Table 1) using our updated protocol. Perishable food products were plated on VBM without Salmonella strains to confirm that they did not contain other microorganisms; only bread and chewing tobacco produced bacterial colonies. Two application procedures were used for each substance, and treatments that produced at least a 50% increase in the reversion frequency were quantitatively tested for potential mutagenicity (Figure 3). Five substances were found to be potentially mutagenic in at least one strain (Figure 3). Each treatment produced significant mutagenesis in TA1538, but only direct application of melted Carmex lip balm induced mutagenesis in both strains. Carmex treatment increased the reversion frequency to 5.3×10^{-7} revertants/CFU in TA1535 and 6.4×10^{-7} revertants/CFU in TA1538.

We started a database of various mutations that confer the His^+ phenotype, to which students will add data. Genomic DNA was prepared from TA1535 and TA1538 as described and sequenced to confirm the integrity of our initial strains. The resulting sequences were exactly

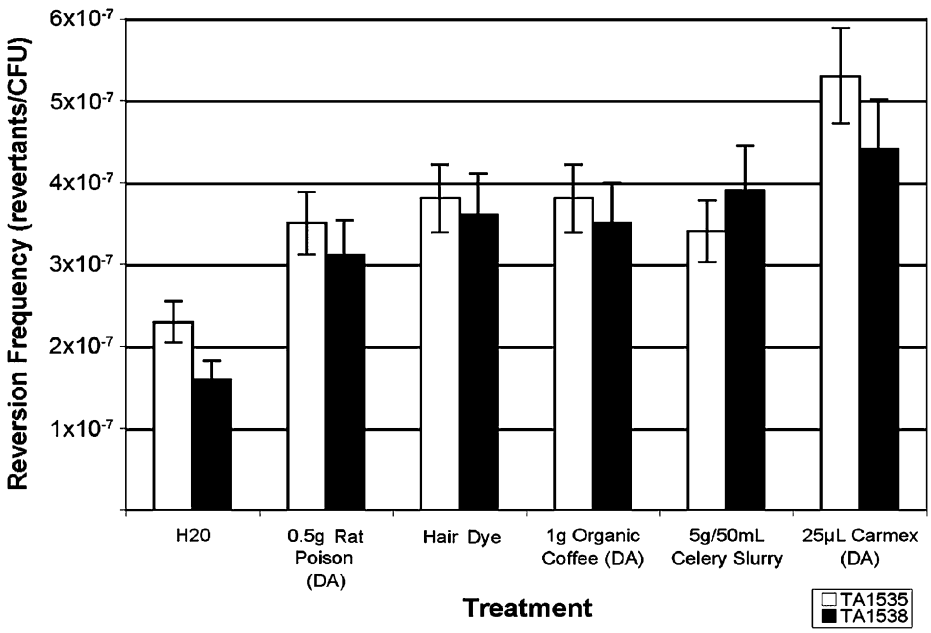

FIGURE 3.—Reversion frequency, measured in revertants/CFU, of TA1535 and TA1538 after exposure to H_2O (negative control) and various suspected environmental mutagens. Application procedure was 10 µl on filter paper or direct application (DA). $n = 15$ for H_2O treatments and $n = 3$ for all other treatments. Error bars, 1 SE.

TABLE 2

Different molecular lesions conferring the His$^+$ phenotype in strain TA1535 after treatment with various substances

Treatment	C → T	C → A
H_2O	3	2
NaN_3	2	0
Organic coffee	0	1
Hand sanitizer	2	0

as described (NCBI accession J01804). Five spontaneous revertants and at least five mutagen-induced revertants from each strain were prepared from two separate experiments and were sequenced to determine typical mutation tendencies. Variation in the kinds of mutations conferring the His$^+$ phenotype was observed (Table 2 and Table 3).

Two different reversion mutations were found in a 2-base region of *hisG* in strain TA1535 (Table 2), directly reverting the original His$^-$ mutation (site 2) or changing the C 1 base upstream (site 1). Reversion to the LT2 (wild-type) sequence of *hisG*, a C → T transition at site 2 was the most common mutation (40% of sequenced *hisD* alleles), replacing the mutant proline with wild-type leucine. Three isolates of a C → A transversion were also observed in TA1535 at site 2, encoding a histidine at the same site of the enzyme. A revertant found after treatment of TA1535 with hand sanitizer included a C → T transition at site 1 in two revertant colonies, and an identical mutation was observed in one spontaneous revertant colony. These alleles substitute a serine for the mutant proline.

A great deal of variation was observed in the *hisD* base sequence of TA1538 revertant alleles. The site of indel mutations (insertions and deletions) spanned a 30-base region encompassing the original mutation site. A sampling of these mutations is presented (Table 3). 4NOP was the only treatment that produced the same mutation twice. The mutations conferring the His$^+$ phenotype in TA1538 are best characterized by the net effect of the insertions and deletions found in each allele, relative to the wild-type (LT2) *hisD* sequence (Table 4). All revertant alleles did restore reading frame, but some added new amino acids as well. The most common mutation was a net deletion of 3 bases, which restored the correct reading frame (50% of alleles). Students will find it interesting that none of the revertant colonies of TA1538 contained a wild-type sequence for the *hisD* gene. A sequence database, including sequence of wild-type alleles, is available at http://www.lawrence.edu/fast/DESTASIE/protocols.html.

We optimized the protocols for characterizing molecular lesions to maximize the success rate. We define final success as the generation of a positive sequencing result (base sequence) that could yield a His$^+$ phenotype. Agarose gel electrophoresis was performed after each intermediate step leading to base sequence analysis (genomic DNA preparation, PCR amplification, and PCR product purification) to ensure the presence of desired products. After all of these treatments, DNA fragments of the appropriate size (Figure 4) were observed from DNA preparations from revertant strains that were prepared with our optimized protocol and the appropriate primer set 100% of the time.

DNA concentrations of the final purified PCR products were calculated by both gel electrophoresis and spectrophotometric analysis at 260 nm. Typical DNA concentrations were found to be between 5 and 20 ng/μl. To prevent lab congestion, save time, and reduce material expenses, students do not need to determine the DNA concentration of their final product. Instead, all DNA concentrations are assumed to be 10 ng/μl after PCR amplicon purification, and samples were prepared as per the off-site sequencing facility's recommendation. Of 38 samples prepared in this manner and sequenced, only 1 did not produce a positive result, and a positive result was obtained after this sample was sequenced again.

DISCUSSION

We designed and field-tested a 2-week investigative lab for an intermediate-level undergraduate course in genetics using the classical Ames test as a starting point.

TABLE 3

Samples of insertion and deletion reversion mutations of TA1538 and the net effect of these molecular lesions

Treatment	DNA sequence (base 880–910)	bp insertion	bp deletion	Net Δ
LT2 (wild type; none)	cgcgcggacaccgcccggcaggccctgagc	0	0	0
TA1538 (His$^-$ mutant; none)	cgcgcggacaccgcc ggcaggccctgagcg	0	1	−1
H_2O (spontaneous revertant)	cgcgcggacaccgcc ggcc**a**ggccctgagc	1	1	0
4NOP	c ggacaccgcc**gc**ggcaggccctgagcgcc	2	5	−3
Organic coffee	cgcgcggacaccgcc ggcag**tagaacgtca**	10	1	9

A 30-base sequence is shown from base 880, relative to the start codon of *hisD*. All bases are contiguous; gaps indicate deletions of bases relative to LT2 sequence. Boldface letters indicate insertion. Net Δ, net effect of insertions and deletions in number of bases relative to LT2.

TABLE 4

The net effect of different molecular lesions conferring the His^+ phenotype in strain TA1538 after treatment with various substances relative to wild-type LT2 sequence

Treatment	Deletion of 3	Deletion of >10	Insertion
H_2O	2	1	2
4NOP	2	0	0
Organic coffee	0	0	1
Artificial vanilla	1	1	0
Hand sanitizer	1	1	0

The lab allows students to play a role in experimental design, and it is open ended and discovery based. Students produce both quantitative data that can be statistically compared and qualitative sequence data that can be analyzed for effect on amino acid code and reading frame. The main advantages to the enhanced lab are its emphasis on quantitative analysis and the explicit connection made between reversion phenotype and gene sequences.

We have increased the efficacy, rigor, and quantitative aspects of the lab compared to the usual implementation of the classic Ames test. Students directly measure, on the basis of serial dilutions of their own design, CFUs in overnight cultures to calculate reversion frequencies rather than simply count revertant colonies. This is a classical genetic measurement and one that undergraduates often have difficulty grasping. The technique of serial dilution is often equally mysterious to students. Measuring and calculating both dilutions and using them to calculate frequencies aids student understanding of these quantitative techniques (see assessment discussion below).

The classic Ames test was further extended to include a molecular genetic investigation, allowing students to see firsthand the direct connection between a phenotype (His^- and His^+) and DNA sequences. Students choose a revertant colony for study, extract genomic DNA, use PCR to amplify large regions of the affected genes, use a core facility to produce DNA sequence, align and analyze the resulting sequences, and correlate to phenotype. The procedures that we designed have been 97% effective in producing usable molecular data (base sequences). DNA preps using Qiagen's DNAeasy kit, PCR done with Promega's clear master mix, and the indicated thermocycling have never failed with our protocols, obviating the need for an intermediate step of gel electrophoresis. DNA sequences and chromatograms arrive from core facilities as electronic files, and freely available software is used to align and compare sequences. Students will still need to use their own common sense, however, when comparing frameshift alleles, as the simple alignment tool does not always "see" insertions or deletions as well as the human eye.

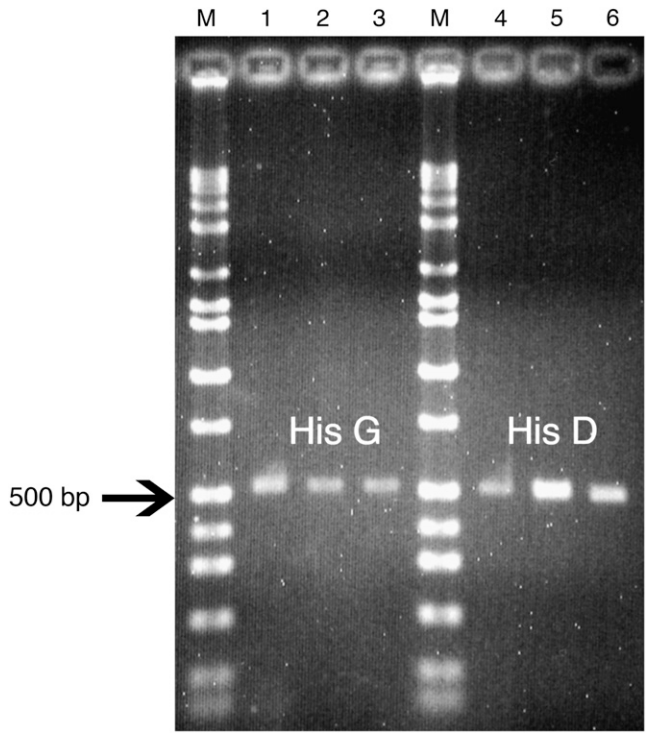

Figure 4.—Agarose gel (1.5%) of purified PCR products. Lanes 1–3 were amplified with the *hisG* primer set, and lanes 4–6 were amplified with the *hisD* primer set. M, Hi-Lo DNA marker; 1–3, TA1535 overnight culture, spontaneous revertant, and 0.05 mg/ml NaN_3-induced revertant, respectively; 4–6, TA1538 overnight culture, spontaneous revertant, and 0.5 mg/ml 4NOP-induced revertant, respectively.

We suggest the use of Salmonella strains with different His^- mutations so students can observe for themselves the connection between DNA sequence and phenotype, the types of lesions induced by different mutagens, and how the secondary mutations lead to reversion of a missense *vs.* a frameshift mutation. Use of both NaN_3 and 4NOP as positive controls allows students to discover that mutagens can have different mutation spectra. TA1535 is not significantly reverted by 4NOP. NaN_3 does not produce significant revertants of TA1538, demonstrating that different types of mutations are needed for reversion in these two strains. When students receive and analyze their revertant sequences, they will note that 4NOP does indeed produce specific frameshift mutations and that NaN_3 exposure is correlated with missense mutations.

This lab lends itself very well to written analysis of the data in manuscript form. To maximize student learning, class data should be pooled to some degree so that variability in overnight culture concentration is obvious and the need to account for it by calculating reversion frequency, rather than by simply comparing numbers of revertant colonies directly, is clear. In addition, a larger data set will allow students to see firsthand that (1) reversion in each strain requires a different type of revertant mutation, (2) different mutagens and the

spontaneous mechanisms have particular mutation spectra, and (3) multiple DNA sequences can encode a functional enzyme. We suggest that instructors begin a database of revertant sequences generated by their classes to which each year's class will add data. Instructors could manipulate data release to have students compare data sets of different sizes such that students can see how collecting larger data sets allows scientists to see trends more clearly.

The learning outcomes from this lab are numerous and instructors can use data generated in this lab at many different points in a typical genetics course. The use of model organisms as test subjects due to the universality of DNA structure and function should be made explicit at the outset of the lab. The concept of reversion is often difficult for students to grasp, but adding the molecular analysis to this lab makes it clear that multiple types of mutations can create a functional gene product from the original His^- genotype. Instructors can then build on student knowledge from this lab when introducing the concept of suppressor screens. The connection between phenotype, genotype, and alleles will be explicit and tangible since students will have produced data at all three levels themselves. Instructors may then make connections between the gene sequences and protein structure, molecular evolution, and mechanisms of mutation as they desire. They can refer to this lab when discussing the importance of DNA repair (these strains have a *uvrB* mutation, which eliminates the excision repair mechanism) and the error-prone nature of DNA replication and repair.

Further extension of this lab is possible. S9 rat liver extract can be incorporated into the histidine-deficient media to mimic the metabolic action of the mammalian liver (with additional expense). Students could design the PCR primers and optimize PCR or they could see their PCR amplicon in an agarose gel prior to or after amplicon purification.

To test the efficacy of this lab, 10 summer research students were recruited to field-test the lab; 6 of these students had taken a genetics course the previous year (and performed a traditional, qualitative Ames test) and the rest had not. Students were given two pretests: a self-assessment of knowledge and a problem-based objective test in which half of the questions tested quantitative skills (*e.g.*, dilutions, frequency calculations) and the rest covered concepts that connect classical and molecular genetics (*e.g.*, what is a reading frame?). A short prelab lecture on the development and utility of the Ames test and the concept of reversion analysis preceded implementation of the lab. Students had no difficulty following the procedures, although many needed help in determining how to prepare and use serial dilutions, in spite of having done lab-based research full time for the previous 10 weeks. Three days later, students collected data and determined reversion frequencies. To shorten the volunteer time needed,

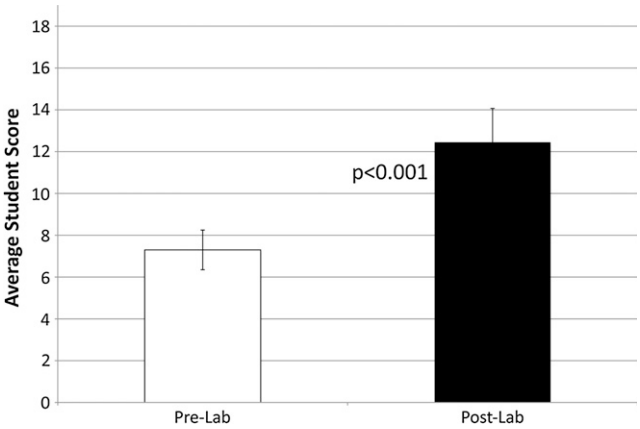

FIGURE 5.—Average student scores on an objective assessment of concepts and quantitative skills before and after completing the laboratory exercise. Sample questions include "Describe how to use serial dilution to produce 100 bacterial colonies on a plate when the starting bacterial overnight culture is expected to contain 10^6 bacteria per ml. Assume you place 100 μl of your final dilution on the agar plate" and "What is meant by 'reading frame'?" Scores are out of 19 possible points. $n = 10$. Error bars = 1 SD.

students were provided with sequence data for four mutant alleles and asked to compare them to wild-type sequence and determine the molecular basis of the revertant phenotype. Each pair of students was asked to summarize their findings orally. Students were especially interested in the fact that a simple base substitution mutation produced a His^+ reversion in TA1535, but complex indels were required to restore the reading frame and confer the His^+ phenotype in TA1538. Following the summaries, the students completed the post-tests and a series of questions evaluating the lab directly.

Pre- and post-test scores demonstrated students' marked improvement in understanding both the quantitative and the molecular aspects of the lab (Figure 5). Pre- and post-tests were coded and mixed by a third party prior to scoring. Pretest scores indicated that even students who had taken a genetics course the previous year had difficulty with the quantitative analysis and serial dilutions associated with this lab, skills not used in the traditional Ames test that they had used the previous year. In spite of the fact that one of our introductory biology labs has students undertaking serial dilutions (with explicit directions not designed by the students), no student scored perfectly on this question on the pretest, underscoring the need to have students practice this skill repeatedly and to design the dilutions themselves with a particular goal in mind. Paired scores were raised by an average of 6 points of 19 total (Figure 5). Average pre- and post-test scores were significantly different ($P < 0.001$, paired two-sample *t*-test).

Self-assessment of student knowledge and skills also increased from pre- to post-lab. Students anonymously rated their understanding of 12 concepts or skills on a

five-point scale (1, very poor understanding; 2, poor understanding; 3, neutral understanding; 4, good understanding; 5, excellent understanding), including such questions as "I feel comfortable calculating mutation frequencies" and "I understand how to use and analyze a DNA sequence." Students' confidence increased after completion of the lab and its analysis; ratings increased by an average of 1.6 points on the five-point scale. The differences between average pre- and post-lab responses for each question were all significantly different ($P < 0.05$, paired two-sample t-test), with the exception of the question "I understand how to use and analyze DNA sequence" ($P < 0.07$). Student responses were abnormally high to this question in the prelab self-assessment (3.8), but nonetheless, an increase (to 4.8) was observed after the lab and analysis.

Anonymous student assessment of the lab itself was very positive. Average responses to questions such as "this lab should be part of the Genetics curriculum," and "Other students would benefit from the skills learned in this lab" were rated, on average, 4.4 and 4.5 on a five-point scale (1, strongly disagree; 2, somewhat disagree; 3, neutral; 4, somewhat agree; 5, strongly agree). In space reserved for written comments about the lab, students wrote "Having my own input, 'my mutagen,' I was more excited to learn the results and why they occurred," and "I like the addition of sequencing, using my own potential mutagen." Students also commented on the skill-building aspects of the lab: "The addition of the quantitative component enhanced [my] understanding of the concept of reversion frequency" and "[There were] a variety of techniques from microbiology, molecular biology, and genetics. This lab really brings it all together."

In conclusion, we developed a new multi-week investigative lab that addresses two very important issues in a genetics curriculum. The lab adds rigorous quantitative analysis as well as molecular genetics to the classic Ames test. The lab has been well received by students and has been shown in a pilot study to improve student skills and knowledge on an objective test as well as student confidence on the basis of self-assessments.

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