Laboratory Exercise

A Molecular Genetic Lab to Generate Inclusive and Exclusive Forensic Evidence: Two Suspects, a Victim, and a Bloodstained T-Shirt

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Abstract

Molecular genetic laboratory exercises can be ineffective due the student's lack of connection to the complex and sequential protocols. In this inquiry-based molecular genetic laboratory exercise, we harness students' fascination with human forensics and provide a real-life scenario using biomolecular techniques to identify "whose blood is on the t-shirt." We use fish blood to create realistic blood stains on clothing and challenge the students to use DNA analyses to clear or implicate suspects. Safety concerns are minimized through the use of fish blood, while maximizing both realism and the likelihood of student success due to fishes’ nucleated red blood cells. The goal in designing this laboratory exercise was to create a feasible protocol for large (over 300 students) second year university courses. During two 3 hour laboratory sessions, students learn and apply clean/sterile technique, DNA extraction, polymerase chain reaction, restriction fragment length polymorphisms, and agarose gel electrophoresis. The students also learn to interpret the resulting gel bands in terms of inclusive or exclusive evidence. Students have consistently ranked this lab as their favorite of five taken as part of a second year Genetics course. © 2013 by The International Union of Biochemistry and Molecular Biology, 0:00–00, 2013.

Keywords: molecular genetics; DNA extraction; polymerase chain reaction; forensics

Background

The popularity of human forensic TV series, coupled with extensive news media coverage of forensic investigations, highlights a fundamental interest many share for biological science applications in law enforcement [1, 2]. This interest can be used to encourage student engagement in laboratory or classroom exercises designed to teach molecular genetic techniques and biological principles [3]. Forensic genetics builds upon a number of diverse scientific disciplines, including molecular biology and genetics, but also principles of probability, experimental error, sample contamination, and scientific hypothesis testing (e.g. ref. 4). The laboratory described here provides technical, analytical, and critical-thinking learning opportunities for students in large, second-year undergraduate labs. Furthermore, students find the project exciting and relevant as it is designed as a forensic investigation with blood-stains, a victim, and suspects. Although a number of forensic-based laboratory exercises are described in the literature, they tend to be designed for either upper level courses (e.g. refs. 4, 5), or as intensive limited student number projects (e.g. refs. 6–8), although some paternity-based labs are applicable to large class sizes [4, 9]. It is widely acknowledged that undergraduate instruction needs to move beyond cookbook laboratory exercises, and include inquiry-based projects with unpredictable outcomes and a high potential for student personal connection [4, 10, 11]. The lab exercises described here provide such experiences and instruction.

The use of realistic crime-related scenarios to capture student interest is recognized as effective in facilitating learning molecular biology and genetics lab techniques and principles [2, 6, 8]. In this lab exercise, the opportunity for students to handle and sample an apparently human...
TABLE I

Materials (consumables and equipment) required to perform the laboratory exercise

| Basic Consumables: | 100% cotton white T-shirt, 70% ethanol, microcentrifuge tubes (1.5, 0.5, 0.2mL), pipette tips, ice buckets/ice, permanent markers, isopropanol, ddH₂O |
| Molecular Genetics Consumables: | DNA extraction: Wizard Genomic DNA Purification kit (Promega), Proteinase K (Sigma-Aldrich)  
PCR reaction: Ultratherm Taq DNA Polymerase (with buffer + MgCl₂ solution; Boca Scientific), Dilution and Storage Buffer (for 1:1 dilution Ultratherm Taq; Boca Scientific), dNTP set (Thermo Scientific), D-Loop or CO1 primers (see Figure 1; Sigma)  
Restriction enzyme reaction: 10 units/μL FastDigest Alu1 restriction enzyme (with buffer + BSA; Thermo Scientific), or Spe1-HF restriction enzyme (20 units/μL with buffer; New England Biolabs), for D-Loop or CO1 primers respectively  
Gel Electrophoresis: 1.5% Agarose B low EEO (Bio Basic), 10 mg/ml 100bp Plus DNA ladder (Fermentas), TBE buffer, Ethidium bromide (EMD Millipore), 6X Loading dye (Thermo Scientific) |
| Basic Equipment: | Micropipettors (p10, p20, p200, and p1000), heat block (0.5 mL and 1.5 mL tube blocks), vortex, PCR machine, gel electrophoresis set-up, imaging system (with UV lamp), vacuum (optional) |

blood-stained shirt provides immediate focus for the students, especially as the instructors explain to the students the hazards of human blood-borne diseases and the necessity for proper handling to avoid human (i.e. their own) DNA contamination. Although we developed a crime scenario based on a murder, instructors can modify the basic crime scenario to appeal to their own tastes, ranging from more dramatic to perhaps more realistic. This lab exercise is based on DNA extraction from a forensic source (blood-stain), followed by polymerase chain reaction (PCR) amplification of a DNA fragment and restriction endonuclease digest of the fragment to provide diagnostic restriction fragment length polymorphisms (RFLPs). The purpose of the lab exercise described here is twofold: 1) introduce the students to issues surrounding the collection of DNA-based evidence for forensic applications; and 2) provide students with hands-on experience in basic and advanced molecular genetic techniques, including the generation and interpretation of data.

DNA extraction is the first step in most modern molecular genetic methods; however, technical difficulties often complicate DNA recovery. Perhaps one of the best known situations where DNA must be extracted under problematic conditions is in forensics, where samples for DNA samples recovered from crime scenes tend to be small and potentially degraded (e.g. bloodstains, saliva, hair, semen; ref. 12). This lab includes DNA extraction (using a commercially available DNA extraction kit) from bloodstains found on a T-shirt following basic sterile (clean) techniques and evidence-tracking protocols. However, the handling of human (or mammalian) blood for the extraction of DNA has serious safety issues [13] and is technically difficult, making it beyond the scope of most first- or second-year undergraduate laboratories. The novel aspect of our approach is to use fish blood rather than human tissue. Although in this lab we use a mtDNA marker, our experience is that the nucleated red blood cells in fish blood increase the success of DNA extraction even from small or degraded samples. Safety concerns are minimal as there are no known fish blood-borne diseases or parasites dangerous to humans.

Although a variety of scenarios can be devised to pique the student’s interest, our scenario is simple: We explain to the students that a murder has been committed, and a blood-stained T-shirt was found at the bottom of a trash can in the victim’s house. The suspects in the crime are two neighbors (suspect 1 and suspect 2), one of whom maintains that the blood is the victim’s own as she suffered a severe nosebleed the previous day while gardening. The students are provided with high-quality PCR product generated from DNA taken from the two suspects and the victim. The goal of the lab is for the students to extract forensic-quality DNA from the exhibit (i.e. the bloody T-shirt) and use PCR and restriction enzyme digestion to test for a match with the known source PCR products using advanced molecular genetic methods.

The results of the DNA analysis of the suspects and the victim may indicate where the blood (and the T-shirt) likely came from—if the bloodstain DNA matches one of the suspects, that individual is implicated as having been at the crime scene. This information is not conclusive but it is sufficient to determine whether an individual should remain in the group of possible suspects (i.e. providing inclusive evidence) or whether the individual can be removed as a possible suspect (i.e. providing exclusive evidence) based on this evidence [14]. If the evidence is inclusive (i.e. the blood matches the suspect), the suspect may be guilty, but further evidence is required since more than one individual may have DNA that matches the signature of the blood on the T-shirt. On the other hand, if the evidence is exclusive, then that individual can be ruled out with confidence and, generally, courts prefer exclusive evidence.
Materials and Preparation

This lab exercise requires a minimum of two laboratory sessions for completion. During the first lab session (Day 1) DNA extraction from a forensic source (bloodstain) is followed by polymerase chain reaction (PCR) amplification of a DNA fragment. In the second lab session (Day 2) restriction endonuclease digests of the PCR products are performed to provide diagnostic RFLPs for analysis. The logistic bottleneck for the lab instructor may be obtaining fresh blood to spatter the T-shirt; Chinook salmon blood is available from a commercial salmon farm in British Columbia, Canada ($50 + shipping for 10 mL, yellowislandaquaculture@gmail.com) and we used it for both staining the T-shirt, and for DNA extraction during lab preparation (victim’s DNA sample). For the suspects’ DNA, we used Bull trout and Cutthroat trout DNA, primarily because we have those species in our lab and they produce different sized fragments for the D-loop (control region) gene in this lab, providing a simple and relatively fool-proof genetic marker for individual identification. However, we provide primer sequences, mtDNA fragment sizes, restriction enzymes, and RFLP patterns for two genes, D-loop (control region), and Cytochrome Oxidase I (CO1) using 24 fish species (Fig. 1).

we do not explicitly discuss the mechanism of RFLP variation (i.e. DNA sequence variation) as it is the subject of an earlier lab; however, we encourage instructors to introduce RFLPs to their students in either the lab or lecture. The logistic bottleneck for the lab instructor may be obtaining fresh blood to spatter the T-shirt; Chinook salmon blood is available from a commercial salmon farm in British Columbia, Canada ($50 + shipping for 10 mL, yellowislandaquaculture@gmail.com) and we used it for both staining the T-shirt, and for DNA extraction during lab preparation (victim’s DNA sample). For the suspects’ DNA, we used Bull trout and Cutthroat trout DNA, primarily because we have those species in our lab and they produce different sized fragments for the D-loop (control region) gene in this lab, providing a simple and relatively fool-proof genetic marker for individual identification. However, we provide primer sequences, mtDNA fragment sizes, restriction enzymes, and RFLP patterns for two genes, D-loop (control region), and Cytochrome Oxidase I (CO1) using 24 fish species (Fig. 1).
Thus various sources of fish blood may be substituted into this lab (depending on availability). DNA samples from fish that are commonly available (e.g. fish counter in the grocery store or at the pet store) can also be used as long as PCR primers exist that will amplify all three species reliably and result in differentiated RFLPs. Using mtDNA makes the likelihood of successful extraction and PCR amplification higher [2], even in the inexperienced hands of second-year undergraduate students. Instructors working with other species or other mtDNA fragments can substitute as they see fit; however, we caution that this lab becomes much more problematic if species without nucleated blood cells are used, or if single-copy nuclear DNA fragments are used.

**Student Preparation and Safety Precautions**

The laboratory instructor provides an introduction to the Molecular Biology of DNA extraction, PCR, restriction endonucleases, and agarose gel separation of double stranded DNA fragments before students engage in these protocols. In addition, the instructor emphasizes the importance of sterile technique and outlines three reasons for using this technique: 1) to avoid infection from blood-borne diseases (the students are not specifically informed that it is not human blood but if students request this information, an explanation is provided indicating the importance of using sterile techniques with all types of blood samples); 2) to avoid contaminating the sample with their own DNA; and 3) to avoid degrading the DNA from their own DNA-degrading enzymes (DNAse found on their hands). The students should be provided with a lab protocol prior to performing the laboratory exercise. While the use of fish blood minimizes the safety risks associated with this lab, there are general safety issues that should be addressed prior to starting this lab (these may have already been addressed). This includes the importance of sterile technique at all stages of the lab exercise, including protection from mutagenic ethidium bromide (due to intercalation with human DNA) and contamination of samples. Care when using hot surfaces (heat block and thermal cycler) and the hazards of using UV light (producing gel images) should be identified. We have found it beneficial to encourage review of distributed materials prior to the lab through the inclusion of a short prelab quiz.

**Methods**

This lab requires two 3 hour lab periods and includes five basic tasks:

1. Excise blood stain from cloth.
2. Extract DNA from blood stain using a commercial kit.
3. Amplify DNA fragments using Polymerase Chain Reaction (PCR).
4. Cut the amplified DNA fragments with a restriction enzyme.
5. Run the cut DNA fragments on an agarose gel to determine the source of the blood stain.

**Day 1**

1. Collection of Blood Stain: The students use gloves and sterile technique when handling the blood stains, and the potential for sample contamination is explained. The students will only need a small piece (5 × 5 mm²) of blood stained cloth, and it should be shredded (to allow for better digestion of the blood cells) and then transferred to a 1.5 mL snap cap microcentrifuge tube. The importance of using sterile technique should be emphasized.
2. Digestion of blood cells and extraction of DNA: We suggest using a commercially available kit (e.g. Wizard Genomic DNA Purification kit [16]) for the DNA extraction,
as it is relatively inexpensive, effective, and uses no hazardous reagents. However, any DNA extraction technique will likely work.

3. PCR Amplification of blood-extracted DNA: The students perform two PCR amplifications: the first is on the DNA they extracted from the blood stain (this can be done in duplicate for smaller classes) and the second is on a “negative control” (water). A third reaction can also be run, a “positive control” containing a previously prepared PCR product that will amplify even under normally restrictive PCR conditions. The instructor should explain the importance of a negative control (and positive control), not only for forensics (where they are critical), but also in Molecular Biological research in general. Since PCR is sensitive to small amounts of DNA (and the Ultratherm Taq polymerase we use has very high processivity), some of the students may well get a band in their negative control, which can serve as an important learning experience. We provide the students with prepared solution containing most of the components for the PCR (buffer, MgCl₂, dNTPs, and water), lab instructors add the Taq (due to associated errors in transferring small amounts and high cost), and students add the primers (see Fig. 1) and DNA (or negative control; Table II). The purpose and function of each of the key components (e.g. free nucleotides, Taq polymerase, primers (free 3'-OH ends)) should be discussed. Once these reactions have been prepared, the students load their tubes in the PCR machine. The details of the PCR program used for this lab are described to the students and, after students leave, the PCR protocol is run and the PCR products are stored frozen for the second lab session. Throughout the laboratory exercise, students are advised of the importance of proper labeling for forensic evidence.

Day 2

4. Restriction enzyme reaction: The students prepare six 0.5 mL tubes labeled as: 1) bloodstain, 2) negative control, 3) victim, 4) suspect 1, and 5) suspect 2. We supply each student with PCR amplified fragment from the victim and the two suspects (for tubes 3, 4, and 5). Each tube receives 10 μL of the appropriate PCR, along with water (7.0 μL), buffer (2.0 μL), and the restriction enzyme (1.0 μL) that was selected to provide different RFLPs in the three individual’s samples, i.e. three different fish species). The restriction enzyme reactions are incubated at 50°C for 40 min, after which 2.0 μL of agarose gel loading dye is added.

5. Run restriction enzyme reactions on agarose gel to visualize fragments: We provide prepared 1.5% agarose gels containing Ethidium Bromide (or there is the option of using less toxic alternatives such as Gel Red®) for the students to load their samples. After a molecular size standard (3 μL) is loaded in the first lane, a student loads 15 μL from each restriction reaction in the following five lanes, using care to keep track of the location of each sample. This is repeated by subsequent students until <5 lanes remain, one of which will be loaded with the molecular size standard. The gels are run for 40 min at 140 V and then photographed under UV light. Student pairs are each provided with a printed gel image for use in their analysis and lab report/notebook.


Results and Discussion

This lab has been offered and refined over the last 10+ years to maximize the likelihood of student success. Typically lab sections have over 70% success in amplifying the bloodstain-extracted DNA and producing a usable gel image. Essentially, students use the gel image to determine whether the blood stain was from the victim or one of the suspects (1 or 2) and decide the implications of their own outcome. Students engage in a comparison and discussion of their results with other groups in their lab section. For students who have some success but produce “problem” outcomes that are inconsistent with other groups, discussions identifying possible technical errors (e.g., contamination, gel loading error, PCR failure) provide a positive learning experience. Furthermore, although the potential for contamination is high, we have found that relatively few students experience negative control PCRs with discernable bands (<5%). However, when this occurs, it is useful as a valuable discussion point and learning opportunity that has relevance beyond forensic genetics and this lab exercise. In our labs we highlight the observed variation among students’ results—emphasizing the importance of experimental variation and the implications of such variation for molecular genetic applications involving potential criminal prosecution. For example, differences in band intensity or band presence/absence likely reflect efficacy of DNA extraction, whereas band presence in negative controls signals contamination and thus a compromised experiment. Figure 2 shows gel images for student outcomes using the two recommended mtDNA fragments. The differences in RFLP patterns among the victim and two suspects is obvious for contamination and thus a compromised experiment. Figure 2 shows gel images for student outcomes using the two recommended mtDNA fragments. The differences in RFLP patterns among the victim and two suspects is obvious for contamination and thus a compromised experiment. Figure 2 shows gel images for student outcomes using the two recommended mtDNA fragments. The differences in RFLP patterns among the victim and two suspects is obvious for contamination and thus a compromised experiment.

The forensic genetic lab was first introduced into the second year Genetic labs at the University of Windsor in 2002, and it has consistently ranked as the favorite lab of the five completed by students each year, with annual polled averages of 3.95–4.43 [of a possible 5 (highest rating on a Likert scale)]. Initially, the lab was developed for ~90 students, but the lab (and course) has grown in popularity to over 300 students. However, the logistics of the lab preparation are such that they have accommodated that growth. Indeed substantially larger numbers of students could be accommodated with this lab given sufficient lab space and equipment.

The forensic genetics lab provides a number of important learning outcomes, including both technical and more conceptual learning opportunities (Table III). The lab has served to provide focused and exciting technical instruction in molecular genetic techniques that are valuable for the students in later courses, undergraduate student research (e.g., honors theses), and even eventual job opportunities or graduate school. The conceptual learning objectives (Table III) provide valuable tools for future student success in genetic and molecular biology, but also for unrelated courses that rely on hypothesis testing, data interpretation, and problem solving.

Overall, the forensic genetic lab described here does capture the imagination of the students, and the opportunity for them to learn and apply advanced molecular genetics lab techniques is exciting for students. This laboratory exercise could be expanded (if time permits) or altered to expand on the topics described here or introduce other molecular, biochemical, or analytical topics. For example, discussion of variation in blood cell types (e.g., nucleated versus non-nucleated; we do discuss with students the fact that in mammals [including humans] only the relatively rare white blood cells contain nuclear DNA), ethidium bromide DNA staining chemistry, DNA sequence variation in populations, and statistical analysis could be added to this

| TABLE III Learning outcomes for forensic genetic lab, organized into Technical, Comprehension, Analytical, and Conceptual categories of research skills (note: Communication skills are associated with all Comprehension and Analytical skills) |
|---|---|
| Skill |
| Technical |
| Analytical, Conceptual |
| Comprehension, Analytical |
| Conceptual, Comprehension |
| Technical |
| Communication |

At the end of the laboratory exercise, the student will be able to…

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Perform relevant molecular genetic techniques including:

- a. using sterile technique
- b. extracting DNA using a Wizard kit
- c. preparing solutions for PCR
- d. preparing restriction enzyme reactions

Analyze and interpret gel image, including potential sources of error

Describe sequence and importance of steps/solutions in protocol

Form conclusions (including exclusive versus inclusive evidence), with justification

Perform responsibly in laboratory environment, using safe practice

Communicate scientific knowledge and arguments orally and in written form (through classroom discussions and assignment completion)
exercise. Many students have contacted the authors to comment on how unusual and exciting this lab was for them, and how it helped them understand the power and pitfalls of molecular genetic methods and interpretation.

Assessment

We assess the student’s grasp of the methodological and conceptual components of this lab using an in-lab assignment, as well as questions on a laboratory exam. The assignment includes submission of labeled gel image and matching, multiple choice, and short answer questions; questions address techniques and protocols, application (to other scenarios), and interpretation of results. We encourage in-lab discussion of broader implications of this laboratory exercise, and the potential for the inclusion of specific discussion materials such as published papers and media reports of actual forensic DNA analysis cases is present. This exercise also lends itself to more intensive assessment techniques such as formal lab reports, research papers, forensic reports etc. should student numbers, resources, and other logistics permit.

REFERENCES