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Laboratory Exercise

Inquiry-Based Experiments for Large-Scale Introduction to PCR and Restriction Enzyme Digests[S]

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Abstract

Polymerase chain reaction and restriction endonuclease digest are important techniques that should be included in all Biochemistry and Molecular Biology laboratory curriculums. These techniques are frequently taught at an advanced level, requiring many hours of student and faculty time. Here we present two inquiry-based experiments that are designed for introductory laboratory courses and combine both techniques. In both approaches, students must determine the identity of an unknown DNA sequence, either a gene sequence or a primer sequence, based on a combination of PCR product size and restriction digest pattern. The experimental design is flexible, and can be adapted based on available instructor preparation time and resources, and both approaches can accommodate large numbers of students. We implemented these experiments in our courses with a combined total of 584 students and have an 85% success rate. Overall, students demonstrated an increase in their understanding of the experimental topics, ability to interpret the resulting data, and proficiency in general laboratory skills. © 2015 by The International Union of Biochemistry and Molecular Biology, 43:441–448, 2015.

Keywords: *polymerase chain reaction; restriction enzymes; undergraduate biochemistry*

Introduction

Pairing the amplification of DNA by polymerase chain reaction (PCR) with analysis using restriction endonucleases (RE) is a common approach used to illustrate both concepts in an undergraduate biochemistry lab. However, these experiments can be difficult to perform with large lab sections and in a limited time frame. At Xavier University of Louisiana, our 2 h and 50 min weekly lab period requires experiments that are quick for both students to complete and instructors to assemble. In addition, a typical semester has 3–6 class sections of the course with 20–24 students per section, and so cost-effective experiments are also a requirement. Many PCR-based experiments performed with such a large group of students can quickly become cost-prohibitive, either due to the expense associated with relatively expensive commercial reagents or the instructor time required to prepare reagents. The existing PCR/RE-based experiments in the literature designed for a more advanced course [1, 2] either were not feasible with our large class sizes [3] or did not provide an inquiry-based experience. We also noted a lack of PCR/RE experiments in the literature that are inquiry-based but that do not require students to isolate DNA as a first step.

DNA isolation is not appropriate for our student population for several reasons. Although there are a variety of commercially available kits that can be very useful for student use, our large enrollment (80–120 students per semester) makes the purchase of these kits cost prohibitive. Performing DNA isolation without these kits requires increased instructor preparation time, usually require longer periods of class time than kits, generally involves the use of hazardous reagents (e.g., chloroform and phenol), and frequently result in lower yield and/or quality of DNA [4, 5]. DNA isolation is taught in our advanced biochemistry laboratory class, which is required for all biochemistry majors. Therefore, the lack of an isolation technique in the introductory course does not negatively affect those students likely to go on to research careers.

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TABLE I *PCR amplification parameters*

	Unknown primer		*Unknown DNA*	
Step	*Temperature (°C)*	*Time (s)*	*Temperature (°C)*	*Time (s)*
Initial denaturation	95	300	95	300
Denaturation	95	60[a]	95	45[a]
Annealing	52	30[a]	52	45[a]
Extension	72[b]	300	68[b]	120
Cycles	25		25	
Final extension	72[b]	600	68[b]	300
Hold	4		4	

[a]*Any value from 30–60 s is appropriate.*
[b]*Determined by the specific polymerase used: 72°C for the ready-to-go PCR beads, 68°C for the Taq master mix.*

Many of the inquiry-based PCR experiments in the literature also incorporate some aspect of primer design by the students [6, 7]. Although primer design is an excellent topic for a biochemistry laboratory, the economic realities of teaching large classes makes purchasing student-designed primers unrealistic. As with DNA isolation, primer design is taught as part of our upper level biochemistry lab. Removing both DNA isolation and primer design from our planned experiment required that it contain an easily available source of DNA to use as the template and an inquiry-based focus that was not centered on the idea of DNA mutation.

We also wanted a short experimental series that could be modified as needed due to the availability of reagents, available instructor preparation time, requirement for students to work individually or in groups, and ensuring variety to maintain interest for both students and instructors. The use of commercial reagents allows for modularity and a minimum of instructor preparation time, but increases the per-student cost. Conversely, minimizing the use of commercial reagents reduces the flexibility and modularity of the experimental approach.

Here we report on two variations of a straightforward PCR amplification and RE procedure. Both variations are inquiry-based in that they require the students to identify an "unknown." The unknown can only be determined using both the PCR product size and the results of the RE digest, requiring students to determine all possible outcomes of the experiment and then use their results to select the appropriate unknown based on a comparison to the possible outcomes. The two approaches vary in the DNA source and PCR reagents, with one using all commercially available reagents and requiring little to no instructor preparation time while the other is considerably less expensive, but does require some preparation outside of class. Both variations were equally successful in student hands, and it is also possible to combine aspects of both (e.g., commercially available DNA with less-expensive PCR reagents) to tailor an experiment to fit almost any undergraduate biochemistry or molecular biology laboratory class.

Experimental Procedures

This mini-project is presented to the students as an exploration. They must use their final results to uncover the identity of either their DNA or the primer set used, and therefore must use critical thinking skills to evaluate their data. With the combination of possible templates and reagents we describe here, it is possible for any undergraduate laboratory with access to a thermocycler to develop a PCR/RE lab regardless of class size or available instructor preparation time.

Equipments

PCR: A standard thermocycler is required for amplification. We used the Edvocycle (Edvotek).

Restriction enzyme digest: A heat block or water bath capable of reaching 37°C is required. We used the Echotherm IC20 (Torry Pines Scientific).

Agarose gel electrophoresis: Any horizontal electrophoresis system and the appropriate power supply are required. We used the B1 mini electrophoresis system (Owl Scientific).

Course Schedule and Experimental Procedures

Class 1: Each group of two students is provided with a DNA sample at a concentration of 50–200 ng μL^{-1} and a primer mixture containing both forward and reverse primers at

TABLE II *Primers for the unknown primer identification*

Set (region)	*Forward primer*	*Reverse primer*
1 (19385–23445)	5′-AGC GTA TTA GCG ACC CAT CGT CTT-3′	5′-AAG GCA TTC CTA CGA GCA GAT GGT-3′
2 (22769–27127)	5′-CTC TGT CAG CTG CAT AAC GCC AAA-3′	5′-TGT TAT GAG CGA GGA GCG GAA GTT-3′
3 (35198–39922)	5′-AAT CGC ACT TAC GGC CAA TGC TTC-3′	5′-CAG CCA GTA GTG GGC GTT TGA TTT-3′

20 μM (each primer). Briefly, the students dilute the DNA to an appropriate working concentration (1–50 ng μL^{-1}) and combine DNA with the primer mixture to give a final mixture containing 1–50 ng DNA and 20 pmol primers (for a 25 μL PCR). The combined DNA and primers are either added to 2× Taq master mix (New England Biolabs), with the correct volume provided in instructor-prepared PCR tubes, or to a ready-to-go PCR bead (GE Healthcare) following the manufacturer's instructions. Each assembled reaction is placed in the thermocycler and amplified according to parameters shown in Table I. Reactions are stored at −20°C until the following week.

Unknown primer identification: Students are given a sample of λDNA (New England Biolabs, NCBI Reference Sequence: NC_001416.1) along with one set of the primers described in Table II.

Unknown DNA identification: Students are given one of the four different genes (HDAC8, HDAC4, HDAC7, or T4 lysozyme) in the pJExpress401 plasmid (DNA 2.0) along with primers F(CTC GAA AAT AAT AAA GGG AAA ATC AG) and R(TGG TAG TGT GGG GAC TC) (IDT DNA). Construction of these plasmids is described in detail elsewhere, but briefly the genes are *E. coli* codon-optimized sequences with an *N*-terminal NdeI restriction site and a *C*-terminal tobacco etch virus cleavage site, His_6 tag, and *C*-terminal XhoI site. HDAC4 and HDAC7 genes contain only the gene sequence for the catalytic domain of the proteins, analogous to HDAC8. The T4 lysozyme construct contains several lysine-to-arginine mutations that have no affect for the experiments described here except that AgeI sites present in the naturally occurring T4 lysozyme gene were thereby eliminated. The pJExpress constructs and the associated sequences are available upon request.

Class 2: Students split their amplified reaction into three tubes and restriction enzymes are added to two of the three tubes (Tables III and IV). Partially diluted RE mixes containing the appropriate amount of enzyme and buffer are either prepared by the instructor before class or purchased as premixed solutions (New England Biolabs) and added to the student samples after the amplified DNA is diluted. Restriction digests are allowed to proceed at 37°C for 30–45 min before the addition of 6× loading dye. The DNA is then resolved on a 0.80–1.5% agarose gel [containing GelStar (Lonza)] along with the appropriate reference ladder (e.g., 100 bp DNA ladder, New England Biolabs) in 1× TBE at 100–150 V. The gels were photographed under UV light using a Gel Logic 220 PRO system (Carestream) and the images supplied to students as .jpg files.

Results and Discussion

This experiment is designed as a two-part series primarily due to the time required for amplification. However, we have tested modifications to the annealing and extension times used when amplifying the pJExpress inserts and have found it is possible to significantly shorten the amplification time (data not shown). The first week is reserved for the PCR amplification and the second for restriction digest and electrophoresis of the resulting PCR product (Table V). Because assembling the components of PCR is not incredibly time-consuming, we use the majority of the time during the first part of this series assisting the students as they work out a set of serial dilutions in order to add the correct amount of DNA to the reaction. There is also time within this laboratory period to calculate restriction digest results for all variations of the unknown (DNA or primer) so that the instructor can provide assistance. Depending on the experience level of the students, much of this work could be done outside of lab if it is necessary to include other activities into the class.

Most of the students enroll in our course as third-year students, after having completed a year of general

TABLE III *PCR product sizes and restriction digest fragment sizes of unknown primer*

Primer set	*PCR (bp)*	*EcoRI (bp) G▼AATTC*	*HindIII (bp) A▼AGCCT*
1	4060	1841,2219	3745,315
2	4358	3335,1023	2388,1970
3	4724	3970,754	2463,1697,564

TABLE IV *PCR product size and restriction digest fragment sizes of unknown DNA*

Template	*PCR (bp)*	*AgeI (bp) A▼CCGGT*	*BstEII (bp) G▼GTNACC*
HDAC4	1783	737,722,285,39	1019,764
HDAC7	1758	N/A	863,464,306,105
HDAC8	1600	1256, 344	1112,488
T4 lysozyme variant	952	N/A	803,149

chemistry, organic chemistry, and usually a year of general biology. Typically these students have not completed any courses that cover molecular biology or biochemical methods in significant detail, although some biology majors have completed more advanced biology course that do partially overlap in content. The students are almost entirely chemistry, biochemistry, and biology majors, although premedical students from other majors occasionally enroll as well. Because our students, in particular the chemistry majors, have had very little experience with PCR or restriction digest prior to this laboratory, we find it more efficient to provide time for these calculations in class. In addition, this laboratory course operates in many ways as an independent unit from the associated lecture course, and so we must find creative ways to teach and reinforce concepts specific to the experiment within the short timeframe of each lab meeting. Students enrolled in the laboratory course are required to either enroll concurrently in the Introduction to Biochemistry lecture course or have successfully completed the lecture course. The lecture course does not expose students to the experimental approaches used in the lab but does introduce the relevant concepts (e.g., DNA structure and restriction endonucleases) prior to or concurrent with their use in lab. As the goal of this laboratory course is to provide a project-based experience for students, it is not designed or meant to reinforce lecture concepts.

Calculation of the serial dilution required in order to add the appropriate amount of DNA to the PCR is by far the most difficult for our students. We assess student learning over the semester using a matched pre-/post-test system. The pre-test is given early during the first class meeting and the post-test is a subset of questions asked on a final exam near the end of the semester. All questions are short answer format and are closely matched, varying slightly in detail that do not affect the difficulty or student interpretation of the questions (e.g., different concentrations of reagents on the pre-test and post-test). In the pre-test, fewer than 25% of student can correctly apply $C_1V_1 = C_2V_2$ to solve a simple solution preparation question, despite encountering dilutions in almost every chemistry or biology course prior to our introductory biochemistry course. We attempt to overcome this roadblock by working dilutions into as many steps of our laboratory protocols as possible. We are also using the pre-test data as a baseline with which to evaluate a recently initiated process of working with faculty teaching prerequisite laboratory courses to try to identify changes that can be made in those earlier courses to improve the students' retention of content and ability to apply the concept across semesters.

In the PCR exercise, students are given a stock DNA solution that is 50–200-fold more concentrated than the desired final concentration in the reaction, which means that direct addition of the correct amount of DNA template to the PCR mixture is not possible without resorting to volumes well below 0.5 μL. The students are instructed to dilute this stock, so that a set amount (10–50 ng) of DNA can be added to the PCR in a volume of 1 μL or greater. This lab falls in week 4 in our experimental schedule, and the students are more comfortable by this point with the

TABLE V *Experimental outline*

Procedure	*Time required (hr)*
Week 1	
Calculations to determine DNA dilution	1–1.5
PCR amplification set up	0.5[a]
Week 2	
Restriction digest set up/incubation	1
Agarose gel preparation/loading	1[b]
Running/imaging agarose gel	1

[a] *Thermocycling requires about 3 h but can be done after the laboratory period has ended.*

[b] *Agarose gels can be poured during the restriction digest incubation; therefore, this time overlaps with that activity.*

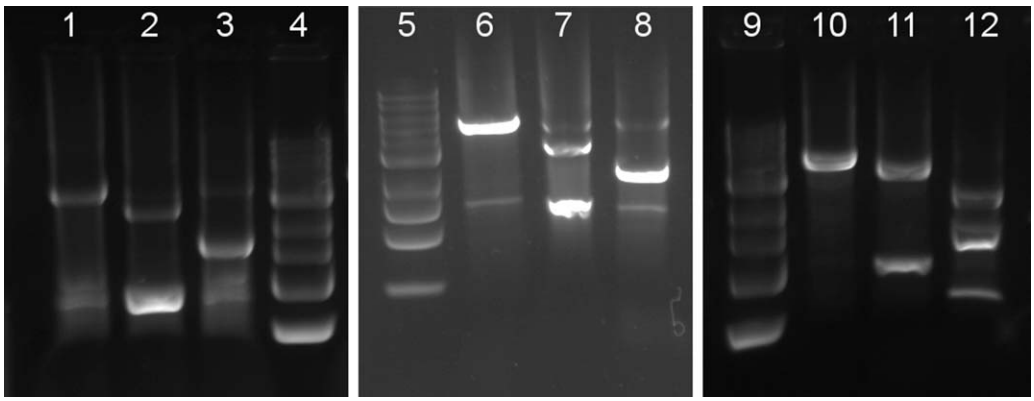

FIG 1

Student amplification and restriction digest of λDNA analyzed by agarose gel electrophoresis. Representative gels from two independent student groups are shown here. The (New England Biolabs) 1 kb DNA ladder is shown in lanes 4, 5, and 9. PCR product from amplification of λDNA using primer set 1 (lane 1), primer set 2 (lane 6), or primer set 3 (lane 10) is shown along with the respective EcoRI digest (lane 3, 7, and 11) and HindIII digest (lane 2, 8, and 12) results. After amplification, products were incubated at 37 °C with the appropriate restriction enzyme, then electrophoresed on a 0.8% agarose gel in 1× TBE for 30–45 min at 130 V. The addition of GelStar allowed for image capture under UV light using the Gel Logic camera.

standard dilution equation. However, they still struggle with both translating the results of this equation into an action and using this equation to solve a problem in which there is more than one unknown variable (i.e., the final concentration of the diluted solution and the volume of this new solution that should be added to the PCR). The majority of this laboratory period is spent assisting the students as they correctly calculate the dilution (Table V).

In the second week, students set up two separate restriction digests with their PCR product and analyze the result of these digests as well as the original PCR product on an agarose gel. Students then compare the results obtained from their gel to the possible results and determine the identity of their unknown. The emphasis on dilution is continued as students are asked to calculate the amount to 6× loading dye required to add to their digest or PCR product. By running multiple student samples on a single agarose gel, we are able to accommodate 30–36 samples (3 samples per groups and 10–12 groups) using only three mini horizontal electrophoresis systems.

We have tested two different protocols, with each having an advantage if supply funds and/or instructor preparation time is limited. There are other possible variations that exist with our tested reagents as described below, all of which require students to determine how to prepare appropriate reaction mixtures, predict all possible outcomes, and interpret their results to match to one of those outcomes. Both protocols produce reliable results and only 10–15% of students end the series without collecting usable data, due primarily to student errors when setting up the PCR. If a student has a successful PCR, then there is an over 95% success rate with restriction digest. Errors during PCR are unavoidable and can generally be attributed to a mistake during the addition of DNA and/or primers to the reaction. Student calculations are checked in order to ensure they have a correct dilution calculation, but as mentioned above, many still struggle with translating this calculation into an action. It is likely that the success rate could be brought to nearly 100% if there was an opportunity for students to repeat the PCR and analysis, paying closer attention to the assembly of the reaction the second time. However, this solution or any other sort of "practice" run of the experiment to eliminate these mechanical errors does not fit within our limited time available for the experiment.

Unknown Primer Set

Here the students are all given a sample of λDNA and a primer set designed to amplify one of the three different regions (Table II). The lab protocol (Supporting Information) supplies them with the region amplified by each primer set as well as the EcoRI and HindIII sites in the entire λDNA genome. Students calculate the expected product sizes from each of the three primer sets as well as the possible EcoRI and HindIII fragments that would result from each amplified region (Table III). This setup has the advantage of requiring no instructor time to prepare DNA as λDNA is commercially available from a variety of sources. This version of the experiment was used in five semesters with 15 sections containing a total of 288 students. As shown in Fig. 1, the identification of the primer set used cannot be determined by observing only the PCR product size. Although none of the products are of identical size, they are similar enough (Table III) that students are not able to verify identity when observing only their results (compare lanes 1, 6, and 10 in Fig. 1). The results of the EcoRI digest provide some information to students with primer set 2, as the expected bands are slightly smaller than those with either primer set 1 or 3 (Table III). However, because all EcoRI digests are expected to give two

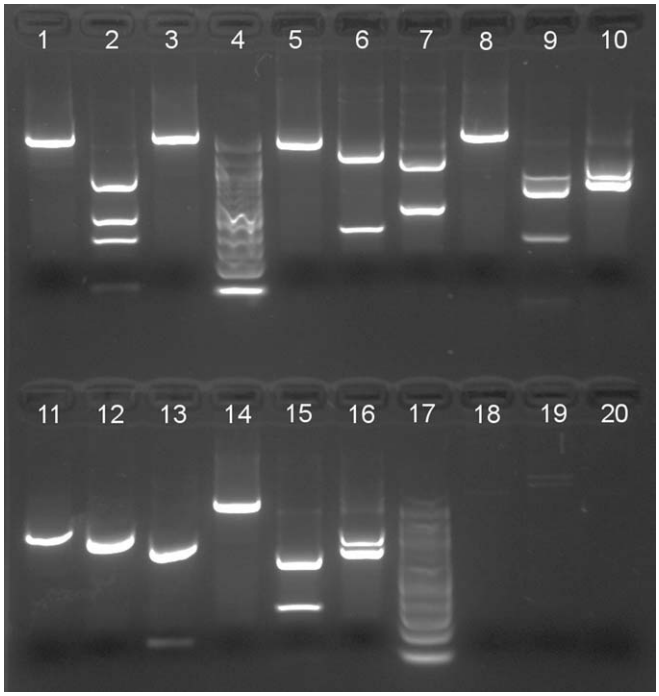

FIG 2

Student amplification and restriction digest of pJExpress clones analyzed by agarose gel electrophoresis. Gel shown contains results from six independent student groups. The (New England Biolabs) 100 bp DNA ladder is shown in lanes 4 and 17. PCR product from amplification of HDAC7 (lane 1), HDAC8 (lane 5), HDAC4 (lanes 8 and 14), or T4 lysozyme variant (lane 11) is shown along with the respective AgeI digest (lane 3, 6, 9, and 15, and 12) and BstEII digest (lane 2, 7, 10, and 16, and 13) results. After amplification, products were incubated at 37 °C with the appropriate restriction enzyme, then electrophoresed on an 1.5% agarose gel in 1× TBE for 25–35 min at 150 V. The addition of GelStar allowed for image capture under UV light using the Gel Logic camera.

products, it is impossible to use these results alone to determine the primer set identity (Fig. 1, compare lanes 7 and 11), although the bands obtained from EcoRI digest of the region amplified with primer set 1 often do not separate well (Fig. 1, lane 3). Students can begin to make an initial determination of their primer set based on careful examination of the product size and EcoRI digest results. The HindIII digest results of each amplified region are different enough that they allow for a final confirmation (Fig. 1, compare lanes 2, 8, and 12). As with the EcoRI digest, one HindIII digest results in similar sized bands that do not separate well (Fig. 1, lane 8).

The choice to amplify these particular regions of λDNA was based on the identification of common restriction sites, but with different digest pattern resulting with each region. This is a very flexible experimental design as there are many other regions of λDNA that could be amplified in a similar experiment and even different restriction enzymes that could be used in our amplified region (e.g., BglII). The commercial availability of λDNA makes it a good DNA source for teaching labs that do not have the means or time available to isolate plasmid DNA from bacterial cultures.

Unknown DNA

Here the students are given one of four pJExpress plasmids containing different inserts, and a common set of primers able to amplify all inserts. As with the unknown primer experiment, students use the list of possible amplification sites to calculate the product sizes and the RE sites (AgeI and BstEII, see Table IV and Supporting Information) in each possible product. This version of the experiment has been used in five semesters with 15 sections and a total of 296 students. Figure 2 shows the representative gel from student-run reactions containing each of the possible DNA templates. The pJExpress plasmids were amplified with both 2× mix and the ready-to-go PCR bead. The choice of 2× master mix over the ready-to-go PCR bead is dependent on the supply budget and time available for preparation by the instructor. Both reagents work equally well with a variety of template/primer combinations (data not shown).

As with the unknown primer experiment, the identification of the unknown DNA cannot be made from the product size alone (Fig. 2). Amplification of three out of the four templates (HDAC4, HDAC7, and HDAC8) results in products that differ in size by <100 bp (Table IV). These products look identical on the 1.5% agarose gel (compare lanes 1, 5, and 8 in Fig. 2). The fourth template, T4 lysozome, does give a product size that can be distinguished from the rest. However, this difference is only immediately apparent when the product (Fig. 2, lane 11) is resolved on a gel that also contains one of the HDAC products (Fig. 2, lane 14). Restriction digest of the products allows student to narrow down their unknown, either by observing a lack of an AgeI site within their product (HDAC7 and T4 lysozyme in Fig. 2 lanes 3 and 12, respectively) or comparing the AgeI digest results. As shown in Fig. 2, the products obtained from AgeI digest of HDAC4 (lane 9) and HDAC8 (lane 6) are of very different sizes. The BstEII digest results can be used to confirm the identity of the unknown DNA as all four regions result in different restriction patterns (Fig. 2, compare lanes 2, 7, 10, and 13). An example of the reproducibility of these results among different groups of students can be seen by comparing lanes 8–10 and 14–16 in Fig. 2, as both were obtained from the use of HDAC4 DNA. Finally, Fig. 2 also illustrates an unsuccessful student experiment (lanes 18–20). Very faint bands can be observed in lanes 18 and 19, indicating that the DNA was either diluted too much prior to amplification or while adding the 6× loading dye. Our choice of genes and plasmid was based on material that was conveniently available; the approach described here should be applicable to a wide range of alternate

TABLE VI *Student assessment results*

Question	*Unknown primer set* (n = *58*)		*Unknown DNA* (n = *296*)	
	Pre-test (%)	*Post-test (%)*	*Pre-test (%)*	*Post-test (%)*
Diluting by mixing of two solutions	16	81	16	87
Calculation of PCR product size given information about plasmid, gene, and primers	19	94	17	93

All pre-test to post-test differences significant at $p < 0.001$ *by paired* t*-test.*

vectors and/or genes provided that a simple set of restriction enzymes can be used to differentiate the constructs.

Interpretation of Results

Although the experiment has a high success rate, the success rate of unknown identification by the students is slightly lower, with 80% of students correctly identifying their unknown. Errors in identification are due to an inaccurate estimation of band sizes or a lack of clarity about how to interpret the gel image. We have attempted to address the latter by urging the students to calculate all possible band sizes, then discussing how those might appear on the gel. Both experimental variations provide a chance to discuss the limitations of agarose gel electrophoresis. For example, both experimental approaches result in bands that do not separate well using an agarose percentage compatible with the full range of sizes (e.g., 2388 and 1970 bp, or 722 and 737 bp). There are also bands that are too small (e.g., 39 bp) to be visible on the gel. Even with these limitations, the digest results are clear enough to allow identification of the unknown. Very few unknowns can be identified on the basis of the PCR product size alone, which requires that students use all their results in the determination of the unknown.

Student Performance

To assess student learning about the concepts involved in the experimental process (rather than simply whether or not they were able to correctly identify the unknown, which is assessed through written reports), during the final semester of implementing the unknown primer experiment, we began utilizing open-ended questions on a pre-test given the first day of the semester and matched questions on the final exam (neither pre-tests nor final exams are returned to students). Students were judged as understanding the concept if their final answer contained no more than one minor error unrelated to the overall concept (e.g., arithmetic error after a completely correct written setup). Prior to this experiment, students have only limited knowledge of how to calculate the product size from amplification of a specific region or the band that would result from digestion with a specific RE. By the end of the semester, student's performance on this type of question has improved significantly (Table VI). The improvement in this type of question can be attributed entirely to this experiment. Students also improved substantially in their understanding of how to execute dilution calculations (Table VI), although this effect can only be partially attributed to this experiment due to the use of dilution calculations throughout the semester. Either experimental approach is equally effective at achieving these learning gains.

An anonymous survey was used to collect student opinions about the experimental series at the end of the semester and was completed by the majority of students for each of six semesters (one semester of the unknown primer set approach and five semesters of the unknown DNA approach). The responses indicate that nearly half of all

TABLE VII *Student survey results*

How would you describe the gain you made in your: (n = *261*)	*No gain*	*Little gain*	*Moderate gain*	*Good gain*	*Great gain*
Understanding of techniques to characterize nucleic acids (%)	7	15	32	31	15
Numerical problem-solving skills (%)	2	13	27	36	22
Ability to interpret experimental results (%)	2	8	27	40	22

students feel that they have gained a "good" or "great" improvement in their understanding of relevant techniques (Table VII), which can be attributed entirely to this experiment. Additional related questions, which are also impacted by the other experiments during the semester, indicate that most students feel they have made "good" or "great" gains in their ability to solve numerical problems and interpret experimental results. Students' comments on open-ended questions on the survey also reflect gains in learning and appreciation for the inquiry-based approach. For example, "This course has helped me understand that biochemistry is essential to understanding genetics, which is what I research outside of school." Another student commented, "It gave more in depth understanding of biological systems rather than this is DNA and this is RNA." And a third, "This gave me hands-on experience on how to analyze DNA which I thought was awesome." In addition, some students also explicitly commented on improvements in understanding of dilutions, such as, "This course has made dilution calculations very clear for me. Even though I had already taken the General Chemistry Labs, I was not so informed about dilution calculations until I took this class."

In summary, we have developed two inquiry-based experimental approaches for introducing students to the principles of PCR and restriction digests, avoiding simple cookbook methods while still retaining adequate simplicity to be applicable to large course sections managed by individual instructors. Both approaches significantly improve students' ability to apply relevant concepts and solve related problems. The framework we describe here could also be used in a more advanced laboratory course with students performing the isolation of the pJExpress variants from *E. coli*, by simply adding a DNA isolation step prior to PCR.

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PCR (unknown primer set)

Objectives: A) To amplify a region of DNA using PCR.

Introduction:

In the first half of this experiment, you will explore the use of polymerase chain reaction (PCR) to amplify a region of DNA. During class, your instructor will give you additional information about the DNA you will be amplifying. In the second half, you will analyze your product using restriction endonucleases (also called restriction enzymes or REs). Your instructor will discuss aspects of both labs in the first pre-lab lecture. You should read over the protocols for both labs and the assigned readings in the lab manual before beginning in order to understand the sequence of events.

PCR amplification of λDNA:

You will be using Ready-To-Go PCR beads to set up your reactions. These thermostable beads contain all components needed for the reaction with the exception of the DNA template and primers. Each group will receive one tube containing a PCR bead. Only your final mixture of DNA and primers should be added to this tube.

You will also be given a primer mixture containing both a forward and reverse primer, each at a concentration of 20 μM. Next week, you will analyze your PCR product to identify your unknown DNA fragment. Your instructor will provide you with the information necessary to calculate the products that might result from this week's PCR.

1. Obtain an aliquot of DNA from your instructor. This DNA is at a concentration of 200 ng/μL.
2. Calculate the dilution needed to add 50 ng of DNA to your PCR with a final volume of 30 μL. (You will need to make a serial dilution in order to pipet accurately.)
3. Obtain a tube of 20 μM unknown primer mixture from your instructor.
4. Calculate the volume needed to add 25 pmol of mixture to your PCR.
5. In a separate tube, mix DNA, primers, and MilliQ H_2O to a final volume of 30 μL.
6. Add 25 μL of the mix to the tube containing the PCR bead. Ensure the bead is at the bottom of the tube before adding your solution, and do not touch the bead with your pipet tip.
7. Mark your drawer number on the top of the PCR tube.
8. Place your reaction into the thermocycler and make a note of the program used in your lab notebook.
9. Determine the expected product size(s) for your reaction. Next week, you will analyze your PCR product and RE digest using an agarose gel and determine the fragment size(s).

Restriction Analysis of DNA (unknown primer set)

Objectives: A) To analyze an amplified region of using restriction enzymes. B) To identify the DNA amplified based on the PCR and restriction digest results.

Introduction:

In today's experiment, DNA amplified in the previous class will be digested with a restriction enzyme. The restriction pattern then will be electrophoretically analyzed to identify your amplified DNA. We will use two Type II restriction enzymes; the specific information about these enzymes and the location of the RE sites in the amplified DNA was provided to you.

As an aid in determining the length of time the gel should be run, a tracking dye will be added to the samples. The mixture contains bromophenol blue (BPB; dark blue color) and xylene cyanol (XC; medium sky blue). The BPB runs at a rate that is approximately equal to a 500-bp DNA fragment; XC's migration is similar to a 4000-bp fragment.

Restriction digest of amplified λDNA:

1. Obtain one tube of each of the restriction enzymes from your instructor. This tube contains the enzyme, buffer, and water in a volume of 15 μL.
2. Retrieve your PCR tube from the previous class.
3. Allow tubes to thaw on your bench if they are still frozen.
4. In a clean microcentrifuge tube, mix 5 μL of your PCR product with 15 μL of MilliQ H_2O.
5. Next, carefully add 5 μL of your PCR product to each tube containing the enzyme mixture. The final volume of the reactions will be 25 20 μL.
6. Place the tubes containing the enzyme mixtures + DNA (or water) in the heat block set at 37˚C.
7. Your reaction will need to incubate for 30 minutes. While you are waiting, your instructor will demonstrate how to pour an agarose gel. Make sure to note the details of preparing the gel in your notebook.
8. After 30 minutes, remove your enzyme digests from the heat block.
9. Add 4 μL 6X loading dye to both enzyme digests and the tube prepared in step 7.
10. Load 20 μL of each tube in separate wells of the agarose gel. (Your instructor will demonstrate how to load the gel).
11. Load (or have your instructor load) the appropriate amount of DNA ladder.
12. Run the gel at 150 V. Your instructor will monitor the progress of the blue dye front as it migrates down the gel and will notify you when the samples have been electrophoresed for the appropriate amount of time. You should make a note of how far the dye front has progressed down the gel at this point.
13. Visualize the gel using the Gel Logic camera as demonstrated by your instructor.
14. Use the restriction digest pattern and size of your PCR product to determine which DNA fragment was amplified in the previous class.

PCR (unknown DNA)

Objectives

A) To amplify a region of DNA using PCR. B) To learn how to calculate fragment sizes of digested DNA.

Lab Introduction

The experiment this week and next week are linked. This week, you will explore the use of polymerase chain reaction (PCR) to amplify a region of DNA. The following week, you will analyze your PCR product using restriction endonucleases (also called restriction enzymes or REs). You should read over the introductions and protocols for both labs and the assigned readings in the textbook before beginning this experiment in order to understand the sequence of events.

You will be using a 2X Taq polymerase master mix to set up your reactions. This master mix contains contain all components needed for the reaction (polymerase, dNTPs, buffer) with the exception of the DNA template and primers. You will add a primer mixture (containing both a forward and a reverse primer) and template DNA containing an unknown gene to the master mix and then perform a PCR to amplify the template DNA.

Next week, you will analyze your PCR product to identify your unknown gene. The information in Table 1 is required to determine both the size of your possible PCR products and the possible fragments that result for each template.

Table 1. Base pair positions of primers and restriction enzyme sites for each of the possible genes.

Gene	Forward primer	Reverse primer	AgeI sites	BstEII sites
A	1281	3064	2003, 2288, 2327	2045, 4741
B	1281	3019	-	2144, 2450, 2555, 4696
C	1281	2881	2537	2393, 4558
D	1281	2233	-	2084, 3910

Reagents and Supplies

Ultrapure H_2O: available in 15 mL tubes on the stock bench.

PCR tubes: available on the stock bench.

DNA template, primer mixture, and 2X PCR mix: your instructor will provide these after you complete the necessary calculations.

Experiment Safety

Closed top shoes and appropriate clothing required. Lab coat recommended.

Experiment Protocol

As with the previous experiment, values given below as _____ are values that you should fill in **before** class as part of your pre-lab. Values given as [_____] (a blank in square brackets) are values that you will fill in **during** class, based on information provided to you by your instructor or results from earlier experimental steps. This is the last time you will be reminded to fill in ____ values before class (in your lab notebook), but should always do so for all remaining experiments this semester.

PCR Amplification of DNA

1. You will be given an aliquot of 5 µL DNA by your instructor. This DNA is at a concentration of [___] ng/µL. (Hint: flip over your 96-place rack to find holes appropriate for these small tubes.)
2. Calculate the dilution needed to add [___] ng of DNA to your PCR with a final volume of 25 µL.

You will need to make a serial dilution in order to pipet accurately, because the volume of DNA to be added to the reaction is very small (< 1 µL). The volumes given to you in this protocol are **not** important for this calculation except as limits to how much you have available; pay careful attention to your units.

In this serial dilution, you need to make a more dilution solution of DNA so that the volume of solution to be added to the PCR is a measurable quantity, while still delivering the correct number of ng. To make this solution, you will perform a normal dilution ($C_1V_1=C_2V_2$), but the only value this is pre-determined is the concentration of the DNA stock you are given (C_1). You will have to make a decision about what concentration you want your more dilute DNA stock to be (C_2), and then choose an appropriate V_1 (at least 1 µL but no more than the volume of the original solution that you have) and solve for V_2. Your C_2 should be chosen such that when you use this new solution to add obtain the correct number of ng of DNA, the volume required is at least 1 µL but also less than half the volume of the PCR reaction.

3. You will be given a tube containing 5 µL of [___] µM primer mixture by your instructor.
4. Calculate the volume needed to add [___] pmol of mixture to your PCR. (Hint: this is very similar to the template calculation. Again pay careful attention to your units.)
5. Calculate the amount of 2X Taq master mix needed for a 25 µL PCR: ___ µL.
6. Calculate how much ultrapure H_2O will be required to reach a final volume of 25 µL once the DNA, primer, and Taq master mix are combined: [____] µL.
7. Obtain tubes of 2X Taq master mix, DNA template, and primer mix from your instructor. The tube of 2X Taq master mix already contains the appropriate volume, so you should directly add the reagents to it (do not pipet out of the 2X Taq tube).
8. Prepare your serial dilution of your DNA template, and the primer stock if necessary.
9. Label the top of the PCR tube containing the Taq master mix with your drawer number.
10. Add the calculated volumes of ultrapure H_2O, diluted DNA template, and primer mixture to the tube containing the Taq master mix.
11. Place your reaction into the thermocycler. Be sure that you have a record of the temperature and time for each step of the PCR in your notebook. When the reaction finishes, your instructor will store the PCR tube containing your reaction at -20 °C until your next class.
12. Complete the clean-up as described below.
13. Determine the expected product size(s) for your reaction before you leave class today (see below). Next week, you will analyze your PCR product and RE digest using an agarose gel and determine the actual fragment size(s).

Clean-up

Discard all PCR tubes (including remaining template and primer mixture) and micropipettor tips in the trash. Return the 15 mL tube of ultrapure water to the stock bench. Return all other used items to the appropriate drawers.

Data Analysis

Complete this data analysis in class if time permits.

Calculation Examples

1. The base pair positions given in Table 1 can be thought of as positions along a line. The forward and reverse primers mark the beginning and end of the gene, respectively. To illustrate this, consider the example of gene E, which has a forward primer position of 3500 and a reverse primer position of 5000. We can visualize the DNA as a line, such that:

```
|                                          |----------gene---------|          |
1                                        3500                    5000       6000
```

2. Calculating the size of the gene is just a matter of subtracting the start position (3500 bp) from the end position (5000 bp): 5000 bp - 3500 bp = 1500 bp. This is the expected size of the PCR product for gene E.

3. AgeI cuts this template at 2200 bp. Because 2200 bp is outside of the gene (it is less than 3500 bp), digesting with AgeI will not affected the product, and a single DNA fragment of 1500 bp would still be observed.

4. BstEII cuts this template at 3700 bp, 4200 bp, and 5500 bp. 5500 bp is outside the gene (it is greater than 5000 bp), so it can be ignored. However, 3700 bp and 4200 bp are within the gene, so BstEII will cut this gene into smaller pieces at this two positions:

```
  |-------------------|----------------------------gene-------------|-----------------------------------------------|
3500              3700                                4200                                      5000
```

5. Because BstEII cuts the gene in two places, three fragments will result: 200 bp (from 3700 bp - 3500 bp), 500 bp (from 4200 bp - 3700 bp), and 800 bp (from 5000 bp - 4200 bp).

Calculation of Digest Fragment Sizes

1. Calculate the product size for each of the four genes based on the information given in Table 1.
2. Calculate the fragment sizes that result when each of the four genes are digested with AgeI based on the information given in Table 1. (A "-" in the table indicates that the restriction enzyme does not cut the template DNA at any position.)
3. Calculate the fragment sizes that result when each of the four genes are digested with BstEII based on the information given in Table 1.
4. Ensure that the answers to your calculations are recorded in your notebook.

Restriction Analysis of DNA (unknown DNA)

Objectives

A) To analyze an amplified region of using restriction enzymes. B) To identify the DNA amplified based on the PCR and restriction digest results.

Lab Introduction

In today's experiment, DNA amplified last week will be digested with a restriction enzyme. The restriction pattern then will be electrophoretically analyzed to identify your amplified DNA. We will use two Type II restriction enzymes; the specific information about these enzymes and the location of the RE sites in the amplified DNA was provided to you previously so that you could predict the size of the fragments.

As an aid in determining the length of time the gel should be run, a tracking dye will be added to the samples. The mixture contains bromophenol blue (BPB), which is a dark blue color. The BPB runs at a rate that is approximately equal to a 500 bp DNA fragment. In addition, some of the samples will contain additional tracking dyes of various colors and migration speeds.

Reagents and Supplies

Ultrapure H_2O: available in 15 mL tubes on the stock bench.

1.5 mL microcentrifuge tubes, 6X loading dye: available on the stock bench.

PCR from last class, GelStar, restriction enzymes, 100 bp ladder, agarose, 1X TBE, gel rigs: your instructor will provide these as needed.

Nitrile gloves: available on the benches.

Insulating gloves: stored on the microwave.

Experiment Safety

Closed top shoes and appropriate clothing required. Lab coat recommended. GelStar binds to DNA and so is a potential carcinogen, and gloves should be worn when handling solutions or gels containing GelStar. Use caution and appropriate hand protection when handling boiling agarose.

Experiment Protocol

Restriction Digest of DNA

1. Retrieve your PCR tube.
2. Allow tubes to thaw on your bench if they are still frozen.
3. In two clean microcentrifuge tubes, mix 4.00 µL of your PCR product with 20.5 µL of ultrapure H_2O. In a third clean microcentrifuge tube, mix 4.00 µL of your PCR product with 21.0 µL of ultrapure H_2O.
4. Label the tube with 21.0 µL of H_2O "control", and label the other two tubes with the names of the restriction enzymes to be used. Be sure to also put your drawer number or initials on these tubes so that you will be able to identify them.
5. Bring the two tubes labeled with the restriction enzyme names to your instructor. Your instructor will add 0.5 µL of the appropriate restriction enzyme to each tube.
6. Use a micropipette set to 20 µL to mix the solution. To do this, pipette up 20 µL once, then pipette the solution back into the same tube. Repeat 2-3 times, trying to avoid introducing bubbles. Once the solution is uniformly pale green, you can stop mixing.

7. Place all three tubes in the heat block set at 37˚C for 30 minutes.
8. Your reaction will need to incubate for 30 minutes. While you are waiting, you should prepare your agarose gel (step 1 of the next section).
9. After 30 minutes, remove your enzyme digests from the heat block.

DNA Gel Electrophoresis

1. Your instructor will demonstrate how to pour an agarose gel. Make sure to note the details of preparing the gel in your notebook. Before the gel is poured, 0.5 µL of GelStar will be added to allow the DNA to be viewed under a UV light.
2. Add ___ µL 6X loading dye to all three tubes, such that the final concentration of dye is 1X.
3. Use a micropipette set to 20 µL to mix the solution. To do this, pipette up 20 µL once, then pipette the solution back into the same tube. Repeat 2-3 times, trying to avoid introducing bubbles. Once the solution is uniformly blue, you can stop mixing.
4. Load 20 µL of each tube in separate wells of the agarose gel. (Your instructor will demonstrate how to load the gel).
5. Load (or have your instructor load) the appropriate amount of DNA ladder.
6. Run the gel at 150 V for approximately 30 minutes. Your instructor will monitor the progress of the blue dye front as it migrates down the gel and will notify you when the samples have been electrophoresed for the appropriate amount of time. You should make a note of how far the dye front has progressed down the gel at this point.
7. Visualize the gel using the Gel Logic camera as demonstrated by your instructor.

Clean-up

Discard all PCR, microcentrifuge tubes, micropipettor tips, unused 6X dye, and nitrile gloves in the trash. Return the 15 mL tube of ultrapure water to the stock bench. Return all other used items to the appropriate drawers.

Data Analysis

Complete this analysis before leaving class, if there is time remaining.

1. Use the restriction digest pattern and size of your PCR product to determine which DNA fragment you amplified.