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Laboratory Exercises

Isolation of *Caenorhabditis elegans* Genomic DNA and Detection of Deletions in the *unc-93* Gene Using PCR

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PCR, genomic DNA isolation, and agarose gel electrophoresis are common molecular biology techniques with a wide range of applications. Therefore, we have developed a series of exercises employing these techniques for an intermediate level undergraduate molecular biology laboratory course. In these exercises, students isolate genomic DNA from the nematode *Caenorhabditis elegans* and use PCR to detect deletions in the *C. elegans unc-93* gene. In advance of the exercises, wild-type and three different *unc-93* deletion mutant strains are grown, harvested, and frozen by the instructor. In one approach, students isolate genomic DNA from each strain using a genomic DNA isolation kit and use agarose gel electrophoresis to analyze the DNA and to estimate its concentration. PCRs using primers directed to two different regions of the *unc-93* gene are carried out on the genomic DNA from wild-type and mutant strains, and the PCR products are analyzed by agarose gel electrophoresis. Students analyze the gel to determine the approximate location and size of deletions in the three mutant strains. Alternatively, students may lyse single nematodes and carry out PCR in one laboratory session. These exercises should be easily adaptable to detection of well characterized deletions in any organism.

Keywords: genomic DNA, PCR, *C. elegans*, *unc-93*

PCR is a powerful molecular biology technique for rapidly amplifying specific DNA sequences. PCR is widely used in various types of genetic analysis and manipulation including genetic testing, forensic DNA analysis, site-directed mutagenesis, and mutation detection [1]. For many PCR applications, genomic DNA provides the template for amplification. In addition to its uses in PCR, genomic DNA is used in construction of genomic libraries, Southern blot analysis of gene structure, and forensic DNA analysis [1]. Isolation of genomic DNA has therefore also become a routine and important procedure in molecular biology. Numerous methods exist for isolating genomic DNA, but they all have the same goal of separating genomic DNA from other cell components.

The exercises described in this report make use of genomic DNA isolation and PCR to detect deletion mutations in the *unc-93* gene of the free living soil nematode, *Caenorhabditis elegans*. *C. elegans* is a well studied model eukaryote used to investigate many aspects of genetics, development, and cell biology [1]. Wild-type nematodes move with a sinusoidal motion, but mutations in any one of a large number of genes lead to a variety of movement defects causing the animal's movement to be "uncoordinated" (thus the abbreviation "*unc*" is used for *C. elegans* genes affecting mobility). One of these genes is *unc-93* [2]. The *unc-93* gene has been cloned and sequenced and the Unc-93 protein appears to function as a regulatory subunit of a K^+ channel that coordinates muscle contraction [3, 4]. Human homologs of *unc-93* have been identified, but little information is available on their function [5, 6].

In a study that identified intragenic revertants of an *unc-93* mutation, De Stasio *et al.* [7] discovered and molecularly characterized several deletions in different regions of *unc-93* (see Fig. 1 and Table I). We present here a series of laboratory exercises for an intermediate level molecular biology laboratory course in which students detect deletions in *unc-93* using PCR. These exercises utilize an approach used to detect deletion mutations in any organism, including mutations in human genes leading to colon cancer and breast cancer [8, 9]. In addition to learning about genomic DNA isolation, PCR, agarose gel electrophoresis, and *C. elegans* as a model organism, students also learn how to prepare dilutions of DNA and how to estimate DNA concentration from gel analysis. The experimental approach described here is broadly applicable and can be adapted to any organism for which well characterized deletions exist.

EXPERIMENTAL PROCEDURES

Expected Background Skills—It is essential that students have developed proficiency in using micropipettors because reagent volumes as small as 1 μl are used to prepare PCRs and samples

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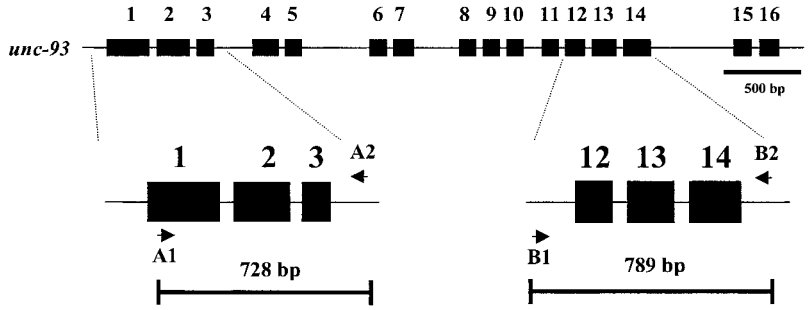

FIG. 1. ***unc-93* gene structure and location of primers used for PCR.** The sequence and structure of the *unc-93* gene were determined by Levin and Horvitz [3]. Exons are shown as *boxes*. The *lower part* of the figure shows the regions amplified by PCR, primer locations, and the predicted sizes of PCR products derived from the wild-type gene.

for gel analysis. In addition, it is helpful (but not necessary) for students to have some experience with agarose gel electrophoresis and gel analysis.

Websites—Gentra Systems, Inc. (www.gentra.com) provides dozens of protocols for using their DNA purification kits to isolate DNA from a wide array of tissues and organisms, *e.g.* nematodes, bacteria, yeast, plants, and flies. An excellent step-by-step animation of PCR can be found on the website for the Dolan DNA Learning Center at Cold Spring Harbor Laboratory (www.dnalc.org/Shockwave/pcranwhole.html). The *C. elegans* WWW server (elegans.swmed.edu) contains links to *C. elegans* methods, researchers, literature, and other information.

Materials—Standard laboratory chemicals and equipment were obtained from Fisher, VWR, and Research Organics (Cleveland, OH); bacteriophage λ DNA was purchased from New England Biolabs (Beverly, MA); dNTPs, agarose, and DNA Micro Marker (pUC18 HaeIII) were purchased from Amresco, Inc. (Solon, OH); oligonucleotide primers for PCR were purchased from Invitrogen (Carlsbad, CA); TaqDNA polymerase, proteinase K, and BstE II DNA marker were purchased from Promega (Madison, WI); PUREGENE DNA Purification Kit (D-5500A) was purchased from Gentra Systems, Inc. (Minneapolis, MN).

Equipment and Software—The following equipment and software were used: Eppendorf 5415D microcentrifuges (Brinkmann Instruments, Westbury, NY), Ultra-Lum EB-15 UV transilluminator (Ultra-Lum, Carlsbad, CA), Dynac 420101 table top centrifuge with a swinging bucket rotor (Becton-Dickinson, Sparks, MD), Speed-Vac concentrator (Savant, Farmingdale, NY), Model 300 electrophoresis power supplies (VWR), Amplitron I and TempTronic thermal cyclers (Barnstead/Thermolyne, Dubuque, IA), MP1015 gel electrophoresis rigs and Kodak Digital Systems 1D 3.5 gel documentation and analysis software (Kodak, Rochester, NY).

Safety Issues—The primary safety issues in this exercise are associated with gel electrophoresis. Ethidium bromide (EtBr)[1] is a mutagen so it must be handled while wearing gloves. Materials contaminated with EtBr, *e.g.* gloves, gels, pipette tips, must be disposed of as hazardous waste. Gel electrophoresis buffer contaminated with EtBr should be decontaminated by absorbing the EtBr to an ion exchange resin, *e.g.* stir contaminated solution overnight with an EtBr destaining bag (Amresco, Inc.) according to manufacturer's instructions. Decontaminated buffer will still contain small amounts of EtBr, which should be disposed down a drain designated for EtBr disposal only. When viewing gels under UV illumination, it is essential that UV blocking goggles are worn and that skin exposure to UV is minimized.

Nematode Strains—The following *C. elegans* strains were used: N2 (wild type), LU55 (*unc-93*(*e1500 lr12*)), LU57 (*unc-93(e1500 lr81)*), and LU133 (*unc-93(e1500 lr28)*) [7]. In *C. elegans* nomenclature, uppercase letter and number combinations give the strain name, and lowercase letter and number combinations give the gene and allele name. All of the *unc-93* mutant strains used are revertants of *unc-93(e1500)*, a single base pair substitution in exon 9 [3]. Nematodes homozygous for the *e1500* allele alone display uncoordinated motion; this is not, however, the null phenotype [3]. Null mutations and intragenic revertants of the *e1500* allele that abolish *unc-93* gene function, like the deletion mutations used in this study, display wild-type locomotion. Thus, each of the three mutant strains used in this study contains both the *e1500* mutation and a deletion in one of the amplified regions (see Fig. 1). Nematode strains were maintained and kept in long term storage at −80 °C using standard methods [10]. All strains may be obtained from the *Caenorhabditis* Genetics Stock Center at the University of Minnesota (biosci.umn.edu/CGC/CGChomepage.htm). The sizes and approximate locations of deletions in *unc-93* used in this exercise are shown in Fig. 1 and Table I. Because the *unc-93* mutants used in this study have wild-type locomotion, there is no pedagogical reason to have students view wild-type and mutant nematodes under a microscope.

Growth and Harvesting of Nematodes—For these exercises, nematodes are prepared by the instructor in advance of class to save time and to avoid the difficulty of synchronizing nematode growth to coincide with a specific date and time. Nematodes are grown on 60-mm MYOB-agarose plates [11] that are ~80% covered with a lawn of *Escherichia coli* strain OP50 [10]. Plates are inoculated with 2–3 hermaphrodites at least 2 weeks before the exercise is scheduled to begin. Plates are incubated at 15 °C, 20 °C, or 25 °C depending on how quickly the nematodes are growing. Nematodes from one 60-mm plate are harvested for each genomic DNA preparation when nearly all of the bacterial lawn has been consumed. For each genomic DNA preparation, a plate is rinsed with 4 ml of deionized water using a 10-ml disposable sterile pipette to collect the nematodes. This step is repeated two more times, and the three rinses are pooled in a 15-ml conical polystyrene tube. Nematodes are pelleted by centrifugation in a Dynac table top centrifuge with a swinging bucket rotor at ~1000 × *g* for 3 min. Following centrifugation, the tubes are placed on ice for 30–60 s after which the supernatant is carefully removed with a 10-ml pipette to avoid disturbing the nematode pellet. Nematodes are resuspended in 1 ml of deionized water, transferred to 1.7-ml microcentrifuge tubes, and centrifuged for 1 min at top speed (13,000 rpm) in a microcentrifuge. Following centrifugation, tubes are placed on ice for 30–60 s after which supernatants are removed with a micropipettor to avoid disturbing the nematode pellet. Nematode pellets are stored at −80 °C and are stable for at least two years. Nematode pellets may be stored at −20 °C, but in our hands long term storage (>6 months) at −20 °C sometimes resulted in poor yields and degraded DNA.

Scheduling—The genomic DNA isolation and PCR exercises are carried out over four laboratory periods, whereas the combined lysis/PCR protocol requires only two periods. Some parts of the exercises do not require the entire laboratory period (4 h) or have long incubation or electrophoresis steps. Therefore, we use these open time periods for various purposes such as reviewing protocols, discussing results of current and previous exercises, lecturing, and carrying out parts of unrelated laboratory exer-

[1] The abbreviations used are: EtBr, ethidium bromide; TE, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

cises. The approximate time required for each part of these exercises and the length of open time periods are indicated below:

Part I: Isolation of Genomic DNA—3.5–4 h total with 1 h open during the proteinase K digestion early in the protocol and 1 h open during the DNA hydration step at the end of the protocol. Alternative A: the instructor can set up the proteinase K digestion, thereby saving 1 h. Alternative B: students may be dismissed at the start of the DNA hydration step which can be completed by the instructor, thereby saving 1 h.

Part II: Gel Electrophoresis of Genomic DNA—2 h total for gel and sample preparation, electrophoresis, and photography, with 45 min open during gel electrophoresis. Alternative: the instructor can provide a premade gel, and the period can begin with students preparing samples for electrophoresis, thereby saving 20–30 min.

Part III: PCR Amplification of the unc-93 Gene—(*a*) 1 h to prepare PCRs using isolated genomic DNA; this does not include the time that the samples spend in the thermal cycler. (*b*) 2.5 h for the combined lysis/PCR protocol. Alternative: the instructor can begin the 60 °C incubation for the combined lysis/PCR protocol, thereby saving 1 h.

Part IV: Gel Electrophoresis of PCR Products—2 h total for gel and sample preparation, electrophoresis, and photography with 45 min open during gel electrophoresis. Alternative: the instructor can provide a premade gel, and the period can begin with students preparing samples for electrophoresis, thereby saving 20–30 min.

Isolation of Genomic DNA—At the start of the exercise, students (working in groups of two) are provided with four tubes of frozen nematodes (strains N2, LU55, LU57, and LU133). Tubes containing mutant strains are coded with numbers or letters to disguise their identity from the students. Genomic DNA is isolated using a PUREGENE DNA purification kit (Gentra Systems, Inc.) according to manufacturer's instructions with slight modifications. The precise composition of the buffers in the kit is proprietary. To avoid contamination, reagents used for genomic DNA isolation are aliquoted in advance of the laboratory period.

1. Suspend each nematode pellet in 600 μl of Cell Lysis Solution, which contains Tris, EDTA (to chelate Mg^{2+} ions, which are required for activity of DNases), and SDS (to lyse cells and denature proteins). Invert tubes several times to mix.

2. Add 6 μl of proteinase K (20 mg/ml stock in 50 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$) to each tube. Mix by inverting the tubes 25 times.

3. Incubate the tubes at 55 °C for 60 min, and invert the tubes every 10–15 min to mix. Treatment with proteinase K is necessary because *C. elegans* is covered by a thick protein cuticle; the protease destroys the cuticle and releases the components of the cells.

4. Add 3 μl of RNase A Solution (4 mg/ml) to each tube; mix by gently inverting the tubes 25 times, and incubate at 37 °C for 15 min. RNase degrades RNA in the cell lysate.

5. Cool the tubes at room temperature for 5 min. Add 200 μl of protein precipitation solution (ammonium acetate) to each tube. Mix by vortexing on high for 20 s. At high concentrations, ammonium acetate causes proteins to denature and precipitate.

6. Incubate the tubes on ice for 5 min. During this time a white protein precipitate should form. Pellet the precipitated proteins by centrifugation at 4 °C in a microcentrifuge for 3 min at 13,000 rpm. During this spin, set up and label microcentrifuge tubes containing 600 μl of isopropanol (one tube for each tube of nematode lysate).

7. After centrifugation, carefully decant the supernatant from each tube into a microcentrifuge tube containing 600 μl of isopropanol. Mix by gently inverting 50 times. DNA is insoluble in isopropanol and the high salt concentration provided by the ammonium acetate added earlier. Precipitated genomic DNA should be visible as thin white threads.

8. Pellet the DNA by centrifugation at room temperature in a microcentrifuge for 1 min at 13,000 rpm. The DNA should be visible as a very small white pellet in each tube.

9. Being careful to avoid the pellet, transfer most of the supernatant to clean microcentrifuge tubes with a 1000-μl pipettor, briefly (for ~10 s) centrifuge the tubes with the DNA pellets, and remove the remaining supernatant with a smaller bore pipette tip. If the pellet is still visible in the original tubes, the saved supernatant can be discarded. Invert the tubes with the DNA pellets onto a Kimwipe, and let the pellets drain for 1 min.

10. Add 600 μl of 70% ethanol to each pellet, and gently invert the tubes a few times to wash the pellet. Pellet the DNA by centrifugation at room temperature in a microcentrifuge for 3 min at 13,000 rpm.

11. Being careful to avoid the pellet, transfer most of the supernatant to clean microcentrifuge tubes with a 1000-μl pipettor, briefly (for ~10 s) centrifuge the tubes with the DNA pellets, and remove the remaining supernatant with a smaller bore pipette tip. The pellet will be loose at this point, so be careful to check that the pellet is still visible in the tube. If the pellet is still visible in the original tubes, the supernatant can be discarded. Invert tubes with the DNA pellets onto a Kimwipe, and let the pellets drain for 1 min.

12. Dry the pellets for 1–2 min under vacuum using a SpeedVac. Alternatively, tubes with the lids open, can be placed at 37 °C for 5–10 min or until pellets are dry.

13. Add 250 μl of DNA hydration solution (which contains Tris and EDTA) to each pellet. Incubate the tubes at 65 °C for 45–60 min to dissolve the pellets. Periodically tap the tubes to disperse the DNA.

14. After pellets have dissolved, store DNA at 4 °C because genomic DNA, which has a high molecular weight, may shear into smaller fragments as a result of freezing and thawing. DNA is analyzed by gel electrophoresis during a later laboratory period.

Gel Electrophoresis of Genomic DNA—To avoid contamination, λ DNA and DNA markers are aliquoted in advance of the laboratory period. In addition, λ DNA is diluted to 50 ng/μl from a 500 ng/μl stock. The high concentration stock is fairly viscous, which can cause pipetting errors by students. Students work in groups of two with one student preparing the gel and the other preparing the λ DNA dilutions.

1. Prepare a 0.8% agarose gel containing 0.5 μg/ml EtBr using standard precautions for handling EtBr.

2. While the gel is being made, prepare the samples to be electrophoresed. Uncut λ DNA (~48,000 bp) at a concentration of 50 ng/μl will be used to estimate the concentration and size of the genomic DNA. Prepare tubes containing 50, 100, and 250 ng λ DNA, and adjust the volume of each sample to 10 μl with 10 mM Tris, pH 8.0, 1 mM EDTA (TE). Add 2 μl of 6× loading dye (15% Ficoll Type 400, 0.25% bromphenol blue in deionized water) to each tube. In addition, prepare λ BstE II marker (1 μl of marker, 9 μl of TE, and 2 μl of 6× loading dye).

3. Prepare three different concentrations of each genomic DNA. Tubes containing genomic DNA must be mixed thoroughly by tapping and inverting before pipetting the genomic DNA. Prepare tubes containing 2, 4, and 8 μl of each genomic DNA, and adjust the volume of each sample to 10 μl with TE. Add 2 μl of 6× loading dye to each tube.

4. Load samples onto the gel, and electrophorese at 150 V (constant voltage) for 45 min. Photograph the gel under UV transillumination.

5. For each set of genomic DNA samples, identify a λ DNA band with an intensity similar to that of one of the bands for each genomic DNA. This will reveal the approximate mass of genomic DNA present in a given sample, which, when divided by the volume of the genomic DNA sample, yields the approximate concentration of the genomic DNA. See "Results" for a sample calculation.

PCR Amplification of the unc-93 Gene—To avoid contamination, all reagents are aliquoted in advance of the laboratory pe-

riod. Immediately before the class period, TaqDNA polymerase, supplied at a concentration of 5 units/μl, is diluted to 1 units/μl in 1× TaqDNA polymerase buffer (10× TaqDNA polymerase buffer is supplied with TaqDNA polymerase) before being aliquoted to each group. The following oligonucleotides are used as primers:

A1: 5′-CGAGAGGAGTACACGCCGG-3′
A2: 5′-TCCTCGGCCATTGTCGCT C-3′
B1: 5′-TGCCTACTTCCAGTCTTCTCT-3′
B2: 5′-CGGAGCGTTTTCAATTGC C-3′

For ease of use, primers can be diluted to the same concentration before the class period; in this case each primer is diluted to 30 μM. Other stock reagents are 25 mM $MgCl_2$, dNTPs (mixture contains 25 mM of each dNTP), and 10× TaqDNA polymerase buffer. Students prepare premixes of reagents to minimize the amount of pipetting. Genomic DNA preparations should have concentrations in the range of 12.5–50 ng/μl. Amplification may not be successful using genomic DNA preparations with higher or lower concentrations.

1. Prepare premixes for amplification of regions A and B of the *unc-93* gene. For premix A, mix 5.83 μl of primer A1, 5.83 μl of primer A2, 2.8 μl of dNTPs, 42 μl of $MgCl_2$, and 35 μl of 10× TaqDNA polymerase buffer. This is sufficient for preparing seven PCRs. Premix B is prepared in the same way, except that primers B1 and B2 are substituted for primers A1 and A2.

2. Assemble five PCRs for each premix in 500 μl of microcentrifuge tubes. Four of the tubes will contain genomic DNA (one tube for each of the four nematode strains being examined). Omit genomic DNA from one tube to serve as a negative control to test for contamination of other reagents with target DNA sequence. For the experimental samples, add, in order, 33.9 μl of sterile water, 13.1 μl of premix, and 2.0 μl of genomic DNA. For the negative control, add 35.9 μl of sterile deionized water and 13.1 μl of premix. Gently mix the contents of each tube.

3. To each tube, add 1.0 μl of TaqDNA polymerase and thoroughly mix the contents of each tube by swirling the mixture with the pipette tip used to add the polymerase while pipetting up and down. Close the tube, and tap it several times to mix ingredients. Briefly (for ~10 s) centrifuge the tubes in a microcentrifuge. Final concentration of components in each 50-μl reaction: genomic DNA, 0.5–1.0 ng/μl (25–50 ng total); each dNTP, 200 μM; each primer, 0.5 μM; 50 mM KCl; 10 mM Tris (pH 9.0 at 25 °C); 0.1% Triton X-100; 3 mM $MgCl_2$; TaqDNA polymerase, 0.02 units/μl (1 unit total).

4. If using a thermocycler without a heated lid, add 50 μl of light mineral oil to each tube. The mineral oil will float on top of the reaction and will prevent evaporation.

5. Add tubes containing premix A to a thermal cycler programmed as follows: 1 cycle: 95 °C, 5 min; 35 cycles: 95 °C, 1.5 min; 54 °C, 2 min; 72 °C, 3 min; 1 cycle: 72 °C, 5 min to ensure complete synthesis of PCR products; hold at 4 °C after final step. Add tubes containing premix B to a thermal cycler programmed as follows: 1 cycle: 95 °C, 5 min; 35 cycles: 95 °C, 1.5 min; 56 °C, 2 min; 72 °C, 3 min; 1 cycle: 72 °C, 5 min to ensure complete synthesis of PCR products; hold at 4 °C after final step.

6. Following completion of the program, the instructor or students remove tubes from the thermal cyclers for storage at −20 °C.

Combined Lysis/PCR Protocol—If the goal of the lab investigation is limited to understanding PCR and electrophoretic analysis of products, the genomic DNA preparation may be omitted, and single nematodes may be lysed and subjected to PCR in a single tube during one period, using a method modified from Williams [12]. As above, all PCR reagents are aliquoted in advance of the laboratory period. The concentrations of primers and other reagents are the same as above except that the TaqDNA polymerase added is at a concentration of 5 units/μl.

1. Nematodes are grown as above, and single adult hermaphrodites are placed in 2.5 μl of lysis buffer (50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM Mg Cl_2, 0.45% Nonidet P-40, 0.45% Tween 20, 0.01% (w/v) gelatin. Autoclave and store in aliquots at −20 C; just before use add proteinase K to 60 μg/ml) [12]. Best results are obtained if 2.5 μl of lysis buffer is placed in the cap of a labeled PCR tube with the cap still attached to the tube. A single nematode is then added to the lysis buffer using a platinum pick under a dissecting microscope. The tubes are closed and are placed upside down on ice until all of the desired tubes contain a nematode.

2. Brief centrifugation to pellet the nematode and lysis buffer is followed by incubation for at least 15 min (or up to 1 week) at −70 °C. The instructor should complete this step prior to the laboratory period. Students then complete the rest of the procedures.

3. Incubate tubes at 60 °C in a thermocycler for 1 h. If using a thermocycler without a heated lid, add 50 μl of light mineral oil to each tube prior to the 60 °C incubation.

4. Incubate tubes at 95 °C for 15 min to inactivate proteinase K. Transfer samples to ice until PCR mix is added.

5. Prepare premixes for amplification of regions A and B of the *unc-93* gene. For premix A, mix 5.83 μl of primer A1, 5.83 μl of primer A2, 2.8 μl of dNTPs, 42 μl of $MgCl_2$, 35 μl of 10× TaqDNA polymerase buffer, 3.5 μl of TaqDNA polymerase, and 237.5 μl of sterile deionized water. This is sufficient for preparing seven PCRs. Premix B is prepared in the same way, except that primers B1 and B2 are substituted for primers A1 and A2.

6. Add 47.5 μl of premix to tubes containing lysed wild-type and mutant worms (four tubes total) and to one tube containing 2.5 μl of sterile water (and 50 μl of light mineral oil if using a thermal cycler without a heated lid) as a negative control. When adding premix to tubes containing mineral oil, insert the pipette tip below the mineral oil layer, dispense the premix, and thoroughly mix the contents of each tube by swirling the mixture with the pipette tip used to add the polymerase while pipetting up and down. If mineral oil is not used, mix by gentle tapping. In either case, briefly centrifuge the tubes in a microcentrifuge. Final concentration of components in each 50-μl reaction: each dNTP, 200 μM; each primer, 0.5 μM; 50 mM KCl; 10 mM Tris (pH 9.0 at 25 °C); 0.1% Triton X-100; 3 mM $MgCl_2$; TaqDNA polymerase, 0.05 unit/μl (2.5 units total).

7. Add tubes containing premix A to a thermal cycler programmed as follows: 1 cycle: 94 °C, 5 min; 30 cycles: 94 °C, 1 min; 57 °C, 1 min; 72 °C, 1 min; 1 cycle: 72 °C, 10 min to ensure complete synthesis of PCR products; hold at 4 °C after final step. The same conditions are used for both premix A and premix B.

8. Following completion of the program, the instructor or students remove tubes from the thermal cyclers for storage at −20 °C.

Optional Modifications to PCR Protocols—To make this exercise more investigative, additional PCRs can be prepared in which different student groups omit other ingredients, substituting sterile deionized water as controls. For example, omitting one or both primers will demonstrate whether nonspecific priming comes from the genomic DNA preparation. Students may also test the effects of varying the concentrations of $MgCl_2$ (*e.g.* 0–3.0 mM), primers (*e.g.* 0.1–1.0 μM), dNTPs (*e.g.* 50–500 μM), genomic DNA (0–5.0 ng/μl), TaqDNA polymerase (0–5 units/reaction), and annealing temperature (*e.g.* 50–60 °C). For very thorough discussions of the role of each of these components in PCR and the effect of varying their concentrations, see Refs. 13 and 14.

Gel Electrophoresis of PCR Products—To avoid contamination, DNA markers are aliquoted in advance of the laboratory period. Students work in groups of two with one student preparing the gel and the other preparing the PCRs and DNA markers for electrophoresis.

1. Prepare a 1.2% agarose gel containing 0.5 μg/ml EtBr using standard precautions for handling EtBr.

2. Remove 10 μl from each PCR reaction to a fresh microcentrifuge tube containing 2 μl of 6× loading dye. To minimize the amount of mineral oil transferred from reactions overlaid with mineral oil, do not depress the plunger on the micropipettor until the pipette tip has been pushed to the bottom of the tube. The

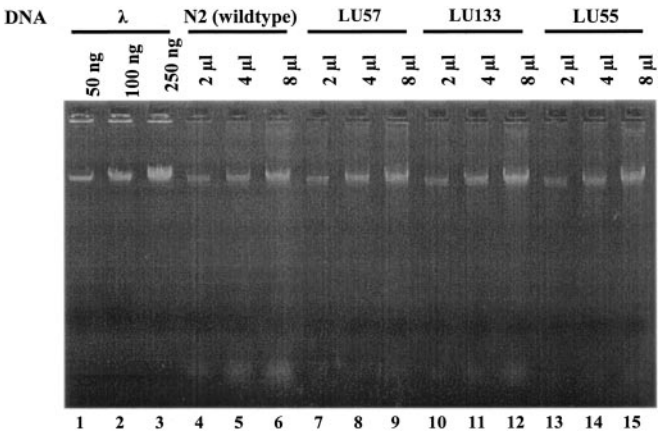

FIG. 2. **Agarose gel analysis of *C. elegans* genomic DNA.** The indicated volumes of genomic DNA and masses of bacteriophage λ DNA were analyzed on a 0.8% agarose gel. λ DNA (~48,000 bp) is included as both a mass and molecular weight standard. Results of a student gel are shown.

aqueous phase (which contains the PCR products) is the lower phase, and the mineral oil is the upper phase. In addition, prepare λ BstE II marker (1 μl of marker, 9 μl of TE, and 2 μl of 6× loading dye) and DNA Micro Marker (1 μl of marker, 9 μl of TE, and 2 μl of 6× loading dye).

3. Load samples onto the gel and electrophorese at 150 V (constant voltage) for 45 min. Photograph the gel under UV transillumination. Use the molecular weight markers to estimate the size of PCR products produced in each reaction.

RESULTS

Following isolation, *C. elegans* genomic DNA is analyzed by agarose gel electrophoresis to assess the purity, integrity, and concentration of the DNA (Fig. 2). The genomic DNA in these samples is quite pure with respect to the presence of RNA; only a small amount of degraded RNA is visible near the bottom of the gel in most lanes. In some preparations, significant amounts of RNA contamination are visible as an intense smear typically running below the 702-bp λ BstEII marker (data not shown). Genomic DNA is visible in all lanes as a moderately sharp band migrating to approximately the same position as uncut λ DNA indicating that it has a size greater than or equal to ~50-kb. The compactness of the genomic DNA bands indicate that the DNA is not degraded; degraded DNA appears as a smear trailing into lower molecular weight regions of the gel. The concentration of the genomic DNA is estimated by visually comparing genomic DNA band intensities with the intensities of known masses of uncut λ DNA. For example, the band present in 8 μl of N2 genomic DNA (Fig. 2, *lane 6*) is comparable in intensity to the band present in the 100-ng λ DNA band (Fig. 2, *lane 2*). Therefore, ~100 ng of N2 DNA are contained in 8 μl, yielding a concentration estimate of 12.5 ng/μl. Genomic DNA preparations typically have concentrations in the range of 12–25 ng/μl for a total yield of 3–6 μg of DNA. Finally, this analysis also allows assessment of student pipetting technique. A progressive increase in band intensity should be observed with increasing mass of λ DNA and increasing volume of genomic DNA.

Two different regions of the *unc-93* gene are amplified from genomic DNA from wild-type and *unc-93* mutant strains (Fig. 1), and PCR products are analyzed by agarose

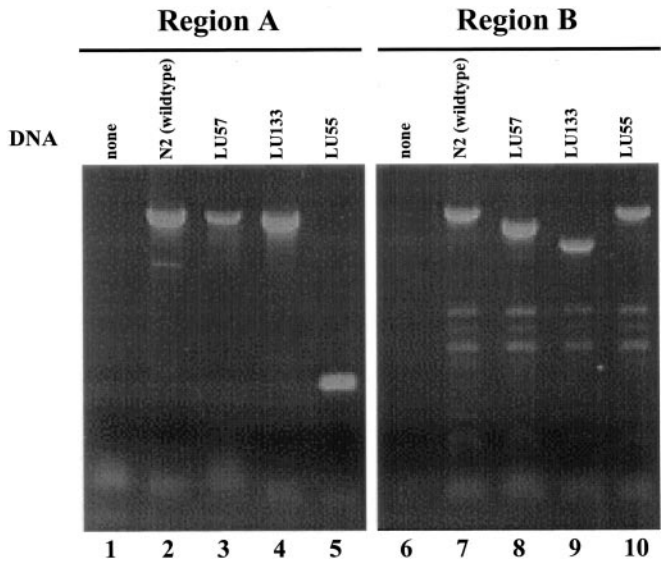

FIG. 3. **Agarose gel analysis of PCR amplification of the *unc-93* gene.** PCRs containing genomic DNA from the indicated strains and using primer pairs A1-A2 and B1-B2 to amplify region A and region B, respectively, of the *unc-93* gene were carried out as described under "Experimental Procedures." PCR products were analyzed on a 1.2% agarose gel. Results of a student gel are shown.

gel electrophoresis (Fig. 3). Expected PCR products are visible as the most intense bands in each lane. A few nonspecific amplification products are visible, especially in the region B amplifications. We consistently observe more nonspecific amplification of region B than of region A (compare *lanes 7–10* with *lanes 2–5* in Fig. 3); the reason for this is not known. All lanes contain a diffuse, low molecular weight band that is presumably primer dimers. As expected, PCRs from which genomic DNA was omitted do not contain amplification products, indicating that the bands are products of amplification from the indicated genomic DNA and not from contamination of the reagents used in the PCRs. Deletions are clearly visible in region A for LU55 (Fig. 3, *lane 5*) and in region B for LU57 (Fig. 3, *lane 8*) and LU133 (Fig. 3, *lane 9*) when compared with the wild-type amplification products (Fig. 3, *lanes 2* and *7*). Molecular weight analysis confirms that the PCR products and deletions match the predicted sizes and locations (Table I). We obtain similar results using the combined lysis/PCR protocol (data not shown).

DISCUSSION

The exercises described here have been used in more than 10 different sections of three different laboratory courses at two predominantly undergraduate institutions over a period of five years and have yielded good results for most students. In the most recent semester in which these exercises were used, 11 of 12 students rated both the genomic DNA isolation and PCR exercises as "excellent," with the remaining student rating them as "good" ("fair" and "poor" were the other choices).

The genomic DNA isolation protocol allows discussion of a variety of concepts associated with nucleic acid purification such as cell disruption techniques, protein denaturation by SDS, enzymatic removal of RNA, differential precipitation (elimination of proteins by "salting out" fol-

TABLE I
C. elegans strains, sizes and locations of unc-93 deletions, and predicted sizes of unc-93 PCR products

Strain	Deletion size and location	Predicted sizes of PCR products	
		Region A	Region B
		bp	
N2 (wild type)		728	789
LU57	78 bp, Region B	728	711
LU133	173 bp, Region B	728	616
LU55	517 bp, Region A	211	789

lowed by precipitation of DNA in alcohol and high salt). In addition, when the genomic DNA is precipitated, it is almost always visible as thin, thread-like fibers, allowing students to see DNA in a form other than a pellet at the bottom of a centrifuge tube or an ethidium-bromide stained band on a gel. Other benefits of having students prepare a relatively large amount of genomic DNA is that they have plenty of DNA to share with students whose preparations are not as good or to save for use in another semester if needed. This exercise can be simplified by skipping the genomic DNA isolation exercise and instead using a single-nematode PCR protocol [12]. This avoids the significant effort and cost (primarily the cost of agarose in the growth medium) to grow and harvest nematodes for genomic DNA preparation, and it shortens the exercise to two laboratory periods.

Some genomic DNA preparations contain RNA in substantial amounts, which fortunately does not seem to interfere with PCR. The presence of RNA is probably caused by inaccurate pipetting of RNase or insufficient mixing of the lysate following RNase addition. During one semester we observed low yield and degradation of genomic DNA, which we later attributed to thawing and refreezing of nematode pellets that occurred when the door of the −20 °C freezer was left ajar on several occasions. As a result, we now store nematode pellets at −80 °C and have not subsequently encountered that problem. The most common pitfall that occurs is poor pipetting technique during preparation of λ DNA and genomic DNA dilutions. This leads to a failure to see the expected progressive increase in band intensity with increasing mass of DNA which in turn leads to difficulty in estimating the concentration of genomic DNA in a sample.

The PCR portion of the protocol requires pre- and post-laboratory discussion of the principles behind nucleic acid amplification and the effect of the concentration of each reaction component and the temperature cycle conditions on yield and specificity [13, 14]. The key theoretical concepts that students must grasp are exponential amplification of the target DNA sequence and selection of that sequence by primers flanking the sequence. Exponential amplification can be effectively described using an animation of PCR like that found on the website of the Dolan DNA Learning Center at Cold Spring Harbor Laboratory (www.dnalc.org/Shockwave/pcranwhole.html). To emphasize the considerations that go into primer selection, students can be given the *unc-93* genomic sequence and asked to design primers that could be used to detect insertions or deletions in particular regions of *unc-93*. The pros and cons of using primers that bind to exons for some applications (*e.g.* more sequence conservation for cross-species amplification for single nucleotide polymorphism mapping) *versus* introns for other applications (*e.g.* screening for mutations that affect phenotype) can be discussed. Students can also use standard formulas to calculate the melting temperature (T_m) of the primer-template hybrid [13, 14]. These T_m values are needed to estimate the annealing temperature to use (*e.g.* annealing temperature is usually 5–10 °C below the calculated T_m), although it must be made clear that the optimal annealing temperature must be empirically determined [13, 14]. Calculation of T_m for different primer sequences should demonstrate the relationship between G-C content and T_m, and a discussion of the basic concepts of nucleic acid hybridization should readily reveal the importance of using primers closely matched with respect to T_m. The effect of annealing temperature on PCR can be demonstrated experimentally by having students test different annealing temperatures in the exercises described in this paper and discussing the results (*e.g.* an annealing temperature that is too low can lead to nonspecific amplification, whereas an annealing temperature that is too high can lead to reduced yield or failure to amplify). At Lawrence University, the primer design exercise is extended by having students design primers that could be used for PCR mutagenesis. Specifically, students design a mutagenic primer that contains an epitope tag, a stop codon, and a restriction site and a second non-mutagenic primer that straddles a nearby restriction site. Students must determine which should be the upstream and downstream primer, which strand each primer must bind to, and the order of required elements in the mutagenic primer. In addition, they work with the codon bias of *C. elegans* to design the epitope tag. With this addition, the lab teaches the basics of PCR as well as its application to screening and site-directed mutagenesis. We have found that students develop a much deeper understanding of PCR when they are required to design primers from scratch.

Students can be told about the effects of varying the concentrations of different PCR ingredients and the physico-chemical reasons for these effects [13, 14], but the lesson will be much more effective if they are allowed to vary the concentrations of some of the ingredients and to observe directly the effects on yield and specificity of PCR. For example, Mg^{2+} is an essential cofactor for most DNA polymerases, so a low Mg^{2+} concentration will reduce polymerase activity leading to low yield. However, if the concentration of Mg^{2+} is too high, double-stranded DNA molecules are stabilized. This reduces the denaturation of template molecules, leading to reduced yield, and can stabilize binding of primers to partially complementary sequences leading to increased formation of nonspecific amplification products. dNTPs are obviously required for DNA synthesis, so low dNTP concentrations will reduce yield. However, dNTPs chelate Mg^{2+}, so high dNTP concentrations reduce the effective Mg^{2+} concentration leading to reduced yield as described above. Primer concentrations are critical as well; excessive concentration of primers can lead to extensive production of primer dimers and can reduce the specificity of amplification by binding of primers to non-complementary sites.

The most common difficulty encountered with the PCR portion of the exercises described here is nonspecific amplification (Fig. 3, *lanes 7–10*). Given that nonspecific PCR products are usually much fainter than specific products and that students know the expected size of the wild-type PCR products, it is usually trivial for students to identify the specific amplification products. In fact, we believe that there is some benefit to the presence of nonspecific amplification because it allows an instructor to reinforce to students the universal challenge of developing a PCR assay with the desired degree of specificity and yield. Students occasionally encounter samples that fail to amplify; during a recent semester, two PCRs of 48 conducted (not including negative controls) failed to produce the expected product probably because of improper pipetting or inadequate mixing of the reaction. Less frequently, we have observed reduced yield of PCR products with primers that are three years old or older; therefore we recommend ordering new primers every two to three years.

Following in-class analysis of the data, students are instructed to prepare a report combining results and analysis of genomic DNA isolation and PCR. Their reports are expected to include the following data and analysis: photographs of the gels of genomic DNA and PCR products, a statement of the estimated concentration of each genomic DNA and one example of how they calculated the concentrations, the estimated size (in bp) of the genomic DNA, a table listing the observed sizes (in bp) of the PCR products (data in this table are generated using Kodak gel analysis software), and a table listing the location (region A or B) and approximate size (in bp) of the deletion in *unc-93* in each mutant strain. For the genomic DNA portion of the exercise, students are expected to comment on the purity, integrity, and yield of the DNA, the quality of their pipetting in preparing λ DNA and genomic DNA dilutions, and the likely accuracy and reliability of their concentration and size estimates. For the PCR portion of the exercise, students are expected to comment on specificity and yield of the PCR products, the presence of nonspecific amplification products, the appearance and meaning of results in the negative control lanes, and differences between the observed and expected sizes of the wild-type PCR products. The effect of variations in reaction conditions (*e.g.* different annealing temperatures, omission of one or both primers, different concentrations of reaction components) can also to be described along with the underlying physico-chemical reasons for the observed effects (*e.g.* increased annealing temperature reduced yield by reducing the amount of primer bound to target DNA molecules; nonspecific amplification products produced in the absence of oligonucleotide primers were probably caused by annealing of small fragments of degraded genomic DNA to random sites in the genomic DNA).

Another common student problem is confusion of mass and concentration. For example, students will sometimes use units of mass (*i.e.* ng) to describe the estimated concentration of genomic DNA rather than units of concentration (*i.e.* ng/μl). Some students are confused by the experimental error in determining the size of DNA molecules by agarose gel electrophoresis. In addition, some forget that each mutant strain has a deletion in only one of the tested regions of the *unc-93* gene. For example, if the PCR product of region B from LU55 has an observed size of 765 bp instead of the expected 789 bp (Table I), some students will report that LU55 has a 24-bp deletion even though their gel clearly shows that LU55 has a large deletion in region A (Fig. 3, *lane 5*) and therefore cannot also have a deletion in region B. Thus, it is important that students be familiar with the experimental error inherent in agarose gel electrophoresis and that they be sure to use all of the information available to them to analyze their data.

The exercises described here are used in intermediate level laboratory courses at Lawrence University (BIOL 354 Molecular Biology) and at John Carroll University (BL 215 Introduction to Biotechnology). At Lawrence, BIOL 354 students have already had some experience with DNA cloning in a prerequisite course and so are better able to understand a more sophisticated presentation of PCR. The PCR exercise follows a two-week open-ended exercise in which students are expected to produce a restriction map given an "unknown" plasmid and four restriction enzymes. The PCR exercise thus builds on the restriction mapping experience and allows a subsequent detailed discussion of the uses of PCR for restriction fragment length polymorphism detection in the context of forensic analysis, genetic screening, and population biology. At John Carroll, BL 215 is a stand-alone laboratory techniques course and is the first laboratory exposure to DNA technology for nearly all of the students in the course. Therefore, the PCR exercise is placed at the end of the course to allow students to develop micropipettor skills and a good understanding of less technically demanding DNA techniques (*e.g.* plasmid DNA isolation, restriction mapping, DNA cloning) before moving on to a technique as conceptually and technically complex as PCR.

In summary, we have developed a series of linked exercises that introduce students to three widely used molecular biology techniques (genomic DNA isolation, PCR, and gel electrophoresis) and a commonly used model organism (*C. elegans*). These exercises mimic a standard research approach used to detect mutations in any organism, including humans [8, 9]. In addition, these exercises can by adapted to any organism (Gentra DNA purification kits can be used to isolate DNA from a wide variety of organisms such as yeast, plants, *Drosophila*, and *E. coli*) and to any gene in which deletions have been characterized.

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