

## Using PCR to Find New Gene Sequences

### INTRODUCTION

In a previous lab activity, instructions were given for designing novel PCR primers. In this lab, you will utilize a pair of novel primers to amplify a previously unidentified DNA region. Instructions are given for isolating DNA from an animal species (an insect) and a plant species. If you are successful in amplifying a good PCR product from a gene that has not previously been sequenced in a particular organism, it may be possible to have the DNA sequenced, thus adding to the knowledge base for the organism.

### PROCEDURE

Although instructions for DNA isolation are given here for both a plant and an animal, you will only work with one organism. The steps following the DNA isolation process will be identical for all DNA samples.

#### DNA Isolation for Insects

The following procedure could be used with various types of insects; however, it was originally designed for the fruit fly, *Drosophila*. For this reason, you are most likely to have success if you work with a small fly, gnat, or similar insect for this portion of the experiment (if the insect you use is significantly larger than *Drosophila*, you should scale up the reactions to adjust for the size difference). Alternatively, to obtain higher-quality DNA, your instructor may choose to have you utilize a commercial DNA extraction kit. One that has given very good results for insect DNA is a kit sold by Cartagen called Genomic DNA Extraction Kit: Arthropods (catalogue #:20810-050).

**Step 1:** Each student should isolate DNA for one or more insects as instructed. Label one microcentrifuge tube for each insect with a code number as instructed. Place one insect in each labeled tube. Note the code number of the insect(s) you are working with for future reference.

**Step 2:** Add 50  $\mu$ l of insect homogenization buffer to each tube, and grind the insect with a plastic pestle. Grind the insect as completely as possible. Use care when grinding and when removing the pestle to leave as much homogenate in the bottom of the tube as possible.

**Step 3:** Incubate the tubes for 25 minutes at approximately 30°C. This can be accomplished by placing the tubes in a floating tube "rack" in a 30°C water bath or in a heat block set for 30°C.

**Step 4:** Incubate the tubes for 1 to 2 minutes at ca. 95°C using a similar procedure. Use care when removing the tubes from the hot water.

**Step 5:** If a microcentrifuge is available, briefly spin the homogenized insects so that remaining tissues form a pellet at the bottom of the tube. Keep this solution and any dilutions made from it on ice. The supernatant from this crude homogenate can be used directly as a source of DNA in your PCR reaction.

### DNA Isolation for Plants

The following procedure was originally designed for use with the model plant *Arabidopsis*. For the best chance of obtaining good results, you should use plant material derived from young seedlings, since young plant tissues have fewer inhibitory substances in them. Alternatively, to obtain higher-quality DNA, your instructor may choose to have you utilize a commercial DNA extraction kit. One that has given very good results for plant DNA is a kit sold by Mo Bio Laboratories, Inc., called UltraClean Plant DNA Isolation Kit (catalogue #:13000-50).

**Step 1:** Each student should use a piece of plant tissue that is roughly equivalent in weight to about 10-20 mg. Depending on the size of the seedlings you are working with, this may represent a portion of a leaf, one or more entire leaves, or the entire above-ground portion of a small seedling.

**Step 2:** Place the plant material in a 1.5 mL microcentrifuge tube. Label the tube with a code number as instructed. Note the code number of the plant(s) you are working with for future reference.

**Step 3:** Use a clean plastic pestle to completely grind the tissue for about 30 seconds.

**Step 4:** Add 400  $\mu$ l of plant homogenization buffer to the plant material and grind briefly again to thoroughly mix the plant material with the solution.

**Step 5:** Close the lid of the microfuge tube and vortex it (or vigorously shake it) for about 5 seconds.

**Step 6:** Heat the sample for 5 minutes in a water bath or a heat block set for 95°C (use care to prevent exposing your skin to the extreme heat).

**Step 7:** Place the tube in a balanced microcentrifuge and spin it for 2 minutes.

**Step 8:** Without disturbing the pellet, transfer 350  $\mu$ l of the supernatant into a fresh tube (discard the old tube with remaining plant material).

**Step 9:** Add 400  $\mu$ l of isopropanol to the tube containing the supernatant.

**Step 10:** Mix the contents of the tube by inverting it several times, and then leave it at room temperature for 3 minutes.

**Step 11:** Place the tube in a balanced microcentrifuge with the hinge pointing inward (i.e., toward the inside of the rotor) and spin the tube for 5 minutes. Orienting the microcentrifuge with the hinge pointed in will ensure that the DNA pellet will form on the side of the tube opposite of the hinge (this is helpful to know when you remove the supernatant).

**Step 12:** Carefully pour off the supernatant from the tube, and then carefully remove any remaining supernatant using a micropipette. Remember that the DNA will be at the bottom of the tube on the side opposite from the hinge – be careful not to disturb it.

**Step 13:** Air dry the pellet for about 10 minutes to allow any remaining isopropanol to be completely evaporated.

**Step 14:** Add 100  $\mu\text{l}$  of TE buffer to the tube and dissolve the DNA pellet by repeatedly aspirating the solution up and down. Ensure that any visible pellet at the bottom of the tube becomes completely resuspended.

**Step 15:** Keep this solution and any dilutions made from it on ice. The supernatant from this crude homogenate can be used directly as a source of DNA in your PCR reaction.

### PCR Reactions

From this point on, both types of DNA can be treated in the same manner (i.e., using the same volumes, reaction times, etc.). A standard process for setting up and running PCR reactions is given below, but first, three factors — DNA concentration, annealing temperature, and using control primers — must be considered.

#### DNA Concentration

Your PCR reactions should ideally contain about 1  $\mu\text{g}$  or less of template DNA (however, reactions often work fine with higher concentrations). If a high-quality spectrophotometer is available, you may directly quantitate the concentration of a dilution of your DNA sample (your instructor will show you how to use the spectrophotometer). You can then dilute your DNA to approximately 0.5  $\mu\text{g}$  per  $\mu\text{l}$  (and use 2  $\mu\text{l}$  in your PCR reactions).

Remember that the concentration you obtain from the spectrophotometer analysis will indicate the concentration of your dilute solution, so you will need to multiply this number by your dilution factor to obtain the concentration of your full-strength DNA solution.

Even if you know the DNA concentration of your sample, it is usually wise to run each PCR condition (each pair of primers) in several concentrations, including a negative control with no DNA. This way, at least one concentration should be in the proper range for good PCR amplification (it is difficult to obtain reliable spectrophotometric measurements of DNA). After your DNA is diluted to 0.5  $\mu\text{g}/\mu\text{l}$ , make six PCR reactions for each DNA sample with 0 (negative control), 0.5, 1.0, 1.5, 2.0, and 3.0  $\mu\text{l}$  of DNA solution per reaction. You will add 3.0, 2.5, 2.0, 1.5, 1.0, and 0  $\mu\text{l}$  of distilled water respectively to each tube to bring the total of each tube to 3.0  $\mu\text{l}$  of DNA/water solution.

If you cannot quantitate your DNA sample, make a 10-fold dilution and a 100-fold dilution of your original sample, and make the following eight conditions (adding appropriate volumes of distilled water to bring your sample up to 3  $\mu\text{l}$ ): 0  $\mu\text{l}$  (negative control), 3.0  $\mu\text{l}$  of the 100-fold dilution, 3.0  $\mu\text{l}$  of the 10-fold dilution, and 0.5, 1.0, 1.5, 2.0, and 3.0  $\mu\text{l}$  of the original sample.

In the next section, you will consider adding several annealing temperatures for each DNA concentration. Since the number of possible reactions could become quite large, your instructor may decide to have you reduce the number of DNA concentrations you will test.

### Annealing Temperature

You will base the annealing temperature for your PCR reaction on the temperature that was specified in the primer design program when the primer sequences were generated. The program that generated these sequences used known rules to find sequences that would anneal specifically at the temperature specified. However, the calculations yield only theoretical optimal primers for the specified temperatures. Often, the true optimum annealing temperature for the selected primers is 1° or 2° above or below the predicted optimum temperature. For this reason, for each PCR condition (i.e., for each pair of primers and for each DNA concentration), you should test several annealing temperatures. Some PCR thermal cyclers have a gradient block, which will allow an investigator to test several annealing temperatures at the same time (the block can provide a range of temperatures over its surface). However, if your PCR thermal cycler does not have this feature, you will have to run a separate PCR reaction for each annealing temperature you use.

Verify with your instructor the number of annealing temperatures you should use. Now, take a minute to make sure that you know how many PCR tubes you will be preparing to test your primers (consider both DNA concentrations and annealing temperatures). In the space below, use the headings shown to create a table with three columns. This table will show code designations for each PCR tube (which you will use when you label your PCR tubes), details regarding the DNA concentration for each tube, and the annealing temperatures that will be tested in each case. Since it may be necessary to test a fairly large number of conditions, your instructor may have you plan and test these conditions as one group or as part of several large student groups.

**PCR Tube Code Designation**

**Notes on DNA Concentration**

**Annealing Temperature**

### Using Control Primers

If your PCR reactions produce appropriately sized bands in an electrophoresis gel, you will know that your DNA isolation technique was good and that your primers were correctly designed. However, if no bands are apparent, this alone will not indicate whether your DNA was of poor quality or, alternatively, whether your primers were not correctly designed (or possibly whether the annealing temperatures were all too high or too low). To identify the source of error if your PCR reaction fails to generate a product, you should use a control primer pair known to amplify a PCR product correctly for the DNA you are using.

Since the 18S ribosomal gene is highly conserved, primers for this gene can be generated that should amplify products in virtually all eukaryotic organisms. As a control for this experiment, you should run duplicate samples of all of the DNA concentrations listed in the table earlier, using a pair of 18S ribosomal gene primers as a control. A single optimum annealing temperature can be determined for these primers; therefore, you should only need to run one annealing temperature condition for this primer pair. This primer pair should amplify a product in one to several of your PCR tubes (based on correct DNA concentration) if your DNA sample is sufficiently pure and undegraded. If you get good bands with this control primer pair, but not with your other primer pair, then it is probable that either your experimental primers are not complementary with the sequence in your template DNA, or the optimum annealing temperature is actually lower or higher than the temperatures you tried with your experimental primers.

### Preparing Standard PCR Reactions

**Step 1:** Add appropriate amounts of template DNA and distilled water (as needed) to labeled tubes, as described earlier. Each tube should contain a total of 3  $\mu$ l of DNA/water solution. Keep your PCR tubes on ice during this process, until you place them in the DNA thermal cycler (for longer storage, they can be kept refrigerated until thermal cycling).

**Step 2:** Add 22  $\mu$ l of PCR “master mix” to each of the DNA samples. There will be two master mixes, one for your experimental primers and one for the 18S ribosomal gene controls. Be sure to add the correct mix to each tube.

The PCR master mix contains the following components: A buffer, a thermostable DNA polymerase, KCl and MgCl<sub>2</sub> (to serve as cofactors for the polymerase), a dNTP mix, and each of the primers specific for the particular PCR reaction.

**Step 3:** If your thermal cycler requires oil (your instructor will advise you of this), add 1 to 2 drops of mineral oil to the side of the PCR tube. It will form a layer over the aqueous PCR mixture.

If you added oil, “burst”-spin the PCR tube in a microcentrifuge to completely separate the aqueous PCR mix from the oil. *Caution:* Place PCR tubes within the “larger” microcentrifuge tubes *with the microcentrifuge tube caps cut off* within the microcentrifuge; otherwise, the PCR tubes will not remain in the rotor. Spin the tubes for several seconds.

**Step 4:** Your instructor will set up the thermal cycler to conduct PCR.

### Electrophoresis of PCR Samples

**Note:** This will be done on a different day from the preceding procedures.

**Step 1:** Add 5  $\mu$ l of a 6X loading dye to the aqueous portion of each PCR sample. Burst-spin as done in Step 3 (above) to separate the oil from the dyed aqueous solution. The dye serves both to make

your sample more viscous and colored (to facilitate gel loading) and to provide a visible reference to determine how long to conduct electrophoresis.

**Step 2:** Set your micropipette for the volume indicated by your instructor (this volume differs with gel type). Carefully submerge the tip of your micropipette below the oil layer (if present), and slowly depress the plunger, expelling small air bubbles into the dyed aqueous solution. Then *slowly* release the plunger, aspirating the measured amount of the dyed PCR solution. Raise the tip above the oil layer and move the tip around the upper wall of the tube to remove excess oil adhering to the outside of the tip.

**Step 3:** Add your sample to one well in the gel (note which well[s] you load), by submerging the tip below the buffer solution and directly into the opening of a well. Do not expel the solution until you are sure that your tip is inserted into a well, and do not insert your tip too far into the well (be careful to avoid puncturing the bottom of the gel with your pipette tip). When you expel the sample into the well, you should be able to see the colored solution settling into the well and filling it. One student should also load one lane on each gel with a molecular marker for size determination.

**Step 4:** After the gel is loaded, your instructor will set the voltage. The gel should be allowed to run until the dye front is close to the opposite end of the gel.

### Analysis of PCR Products

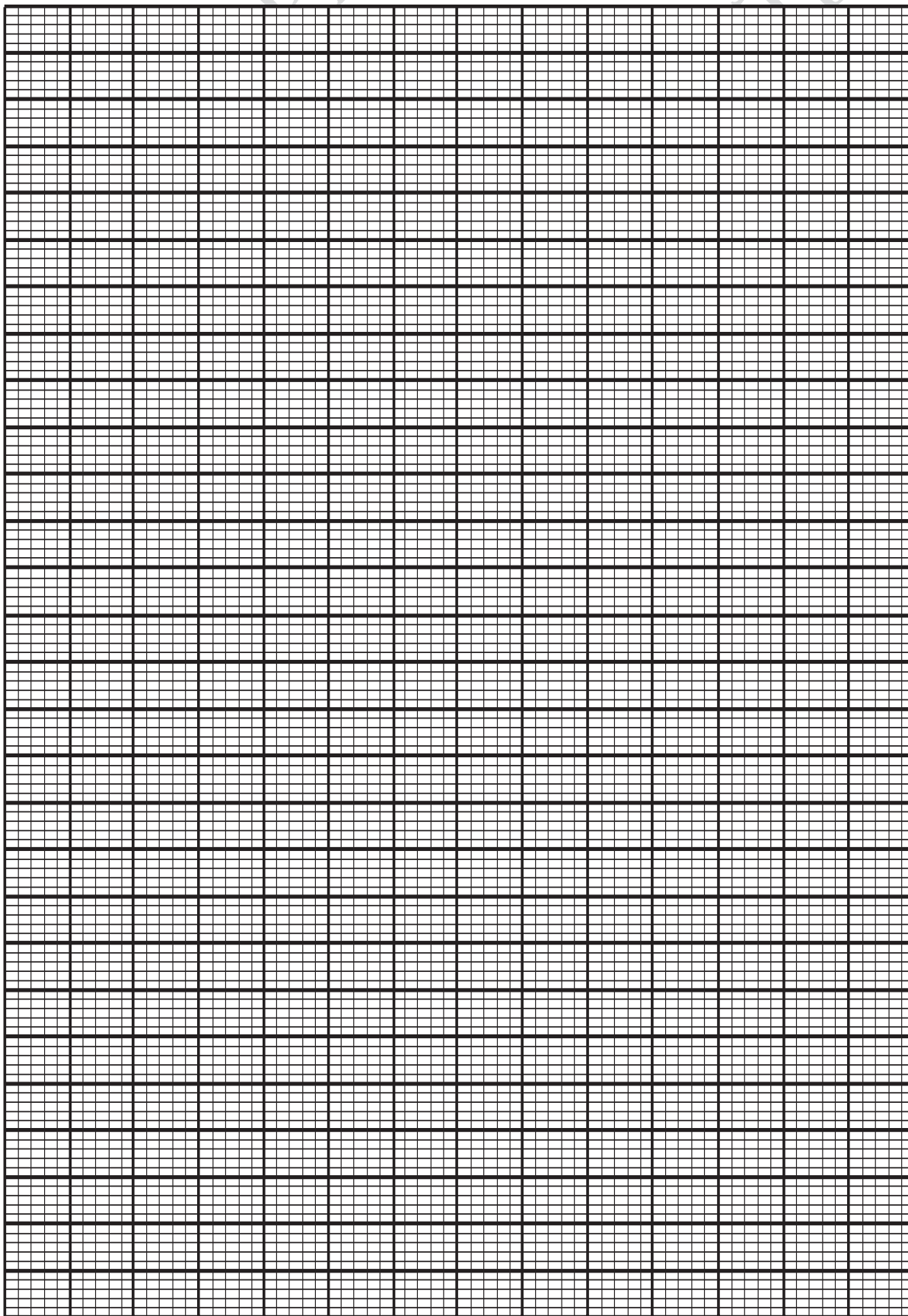
**Step 1:** View the gel(s) on an ultraviolet light box, or use a gel documentation system to photograph the gel (wear gloves when handling the gel — it contains a potent mutagen, ethidium bromide). If you view the gel directly (rather than viewing an image of the gel), *wear appropriate plastic eye protection*. Since prolonged exposure to UV light may also harm the skin, students may wish to wear a plastic face shield and appropriate clothing to cover exposed skin.

If your PCR experiment is successful and some of your reactions produce clear, obvious bands in the electrophoresis gel, you should estimate the size of the PCR products. To do this, take measurements of how far each band migrated from the well. Measure the distance from the well to the center of the band on the gel that represents your PCR product. Also measure the distances between the well and each of the bands in the lane that contains the reference DNA. Your instructor will tell you the sizes of the DNA fragments represented by each band in the reference DNA lane.

**Step 2:** To estimate the size of the DNA you isolated, compare the distance traveled by the band representing the PCR product to that of each of the reference bands. Do this by plotting a standard curve for the reference DNA fragments on semilog graph paper and comparing the measurement for your PCR product to these. Plot the sizes of the reference fragments (in base pairs) on the log scale axis and the distances migrated by the bands on the standard scale axis. You should be able to draw a straight line passing through (or nearby) each of the points. Now use the standard curve to estimate the size of the PCR product you generated.

**Q1** How large was the PCR product you generated? Hand in a copy of your standard curve showing distance migrated by your PCR product and its estimated size on the graph paper on page 62.

If this product is of the expected size, you may have isolated a new gene sequence! The next step, if time and resources permit, may be to have your PCR product sequenced, thus contributing to the knowledge base for this organism.



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