III. Amplify DNA by PCR

REAGENTS, SUPPLIES, & EQUIPMENT

For each group

Container with cracked or crushed ice

Appropriate primer/loading dye mix (25 µL) per reaction*

*DNA from specimen(s) (from part II)**

Micropipettes and tips (1-100 µL)

Microcentrifuge tube rack

Permanent marker

1 Ready-To-Go PCR Beads in 0.2- or 0.5-mL PCR tube per reaction

To share

Thermal cycler

**Store on ice*

1. Obtain PCR tube containing Ready-To-Go PCR Bead. Label the tube with your identification number.
2. Use a micropipette with a fresh tip to add 23 µL of one of the following primer/loading dye mixes to each tube. Allow the beads to dissolve for 1 minute.

   Plants: *rbc*L primers (rbcLaF / rbcLa rev)

   Fish: *COI* primers (VF2_t1/ FishF2_t1/ FishR2_t1/ FR1d_t1)

   Insects: (LepF1_t1/ LepR1_t1)

   Other animals: (LepF1_t1/ VF1_t1/ VF1d_t1/ VF1i_t1/ LepR1_t1/ VR1d_t1/ VR1_t1/ VR1i_t1)
3. Use a micropipette with fresh tip to add 2 µL of your DNA (from Part II) directly into the appropriate primer/loading dye mix. Ensure that no DNA remains in the tip after pipetting.
4. Store your sample on ice until your class is ready to begin thermal cycling.
5. Place your PCR tube, along with those of the other students, in a thermal cycler

The primer/loading dye mix will turn purple as the PCR bead dissolves.

Your teacher will prepare reactions with forward and reverse primers for a single locus

If the reagents become splattered on the wall of the tube, pool them by pulsing the sample in a microcentrifuge or by sharply tapping the tube bottom on the lab bench.

that has been programmed for 35 cycles of the following profile:

Denaturing step: 94°C 30 seconds

Annealing step: 54°C 45 seconds

Extending step: 72°C 45 seconds

The profile may be linked to a 4°C hold program after the 35 cycles have been completed.

6. After thermal cycling, store the amplified DNA on ice or at -20 °C until you are ready to continue with Part IV.

IV. Analyze PCR Products by Gel Electrophoresis

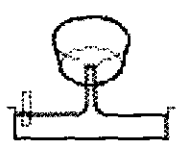
REAGENTS, SUPPLIES, & EQUIPMENT

For each group
2% agarose in 1x TBE (hold at 60°C) (50 mL per gel)
Container with cracked or crushed ice
Gel-casting tray and comb
Gel electrophoresis chamber and power supply
Latex gloves
Masking tape
Microcentrifuge tube rack
3 Microcentrifuge tubes (1.5mL)
Micropipette and tips (1–100 µL)
*pBR322/BstNI marker (20 µL per gel)**
*PCR products from Part III**

SYBR Green DNA stain (6 µL per group)
1x TBE buffer (300 mL per gel)

To share
Digital camera or photodocumentary system
Microwave
UV transilluminator <!> and eye protection
Water bath for agarose solution (60°C)

*Store on ice.



Avoid pouring an overly thick gel, which will be more difficult to visualize.

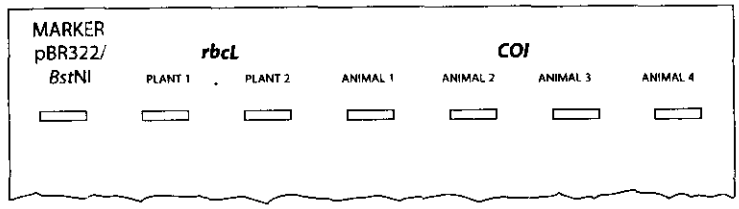
The gel will become cloudy as it solidifies.

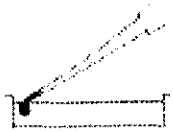
Do not add more buffer than necessary. Too much buffer above the gel channels electrical current over the gel, increasing running time.

1. Seal the ends of the gel-casting tray with masking tape, or other method appropriate for the gel electrophoresis chamber used and insert a well-forming comb.
2. Pour the 2% agarose solution into the tray to a depth that covers about one-third the height of the comb teeth.
3. Allow the agarose gel to completely solidify; this takes approximately 20 minutes.
4. Place the gel into the electrophoresis chamber and add enough 1x TBE buffer to cover the surface of the gel.
5. Carefully remove the comb and add additional 1x TBE buffer to fill in the wells and just cover the gel, creating a smooth buffer surface.
6. Use a micropipette with a fresh tip to transfer 5 µL of each PCR product (from part III) to a fresh 1.5mL microcentrifuge tube. Add 2 µL of SYBR Green DNA stain to tube.
7. Add 2 µL of SYBR Green DNA stain to 20 µL of pBR322/*Bst*NI marker.
8. Orient the gel according to the diagram on the following page, so the wells are along the top of the gel. Use a micropipette with a fresh tip to load 20 µL of pBR322/*Bst*NI size marker into the far left well.

A 100-bp ladder may also be used as a marker.

9. Use a micropipette with a fresh tip to load each sample from Step 6 in your assigned wells, according to the following diagram:

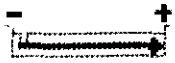




Expel any air from the tip before loading, and be careful not to push the tip of the pipette through the bottom of the sample well.

The samples you load may not be exactly the same as those shown.

10. Store the remaining 20 µL of your PCR product on ice or at -20°C until you are ready to submit your samples for sequencing.



Transillumination, where the light source is below the gel, increases brightness and contrast.

11. Run the gel for approximately 30 minutes at 130V. Adequate separation will have occurred when the cresol red dye front has moved at least 50 mm from the wells.

12. View the gel using UV transillumination. Photograph the gel using a digital camera or photodocumentary system.

RESULTS AND DISCUSSION

I. Think About the Experimental Methods

1. Describe the purpose of each of the following steps or reagents used in DNA isolation (Part II or Part IIa of Experimental Methods):

   i. Collecting new leaves or leaf buds.

   ii. Using only a small amount of tissue.

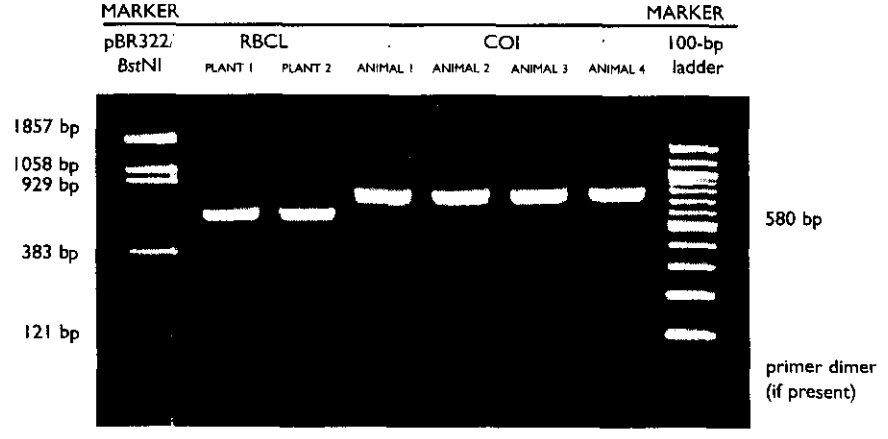
   iii. Grinding tissue with pestle.

   iv. Nuclei lysis buffer or Edward's buffer.

   v. Heating or boiling.

II. Interpret Your Gel and Think About the Experiment

1. Observe the photograph of the stained gel containing your PCR samples and those from other students. Orient the photograph with the sample wells at the top. Use the sample gel shown below to help interpret the band(s) in each lane of the gel.



2. Locate the lane containing the pBR322/*Bst*NI markers on the left side of the gel. Working down from the well, locate the bands corresponding to each restriction fragment: 1857, 1058, 929, 383, and 121 bp. The 1058- and 929-bp fragments will be very close together or may appear as a single large band. The 121-bp band may be very faint or not visible.
3. Looking across the gel at the PCR products, do the bands all appear to be the same bp size and intensity?

Additional faint bands at other positions occur when the primers bind to chromosome loci other than the intended locus and give rise to "nonspecific" amplification products.

4. It is common to see a diffuse (fuzzy) band that runs ahead of the 121-bp marker. This is "primer dimer," an artifact of the PCR that results from the primers overlapping one another and amplifying themselves.
5. Which samples amplified well, and which ones did not? Give several reasons why some samples may not have amplified; some of these may be errors in procedure.

If you have a very faint product or none at all, your teacher will help you decide if your sample should be sent for sequencing.

6. Generally, DNA sequence can be obtained from any sample that gives an obvious band on the gel.