METHODS

DNA Extraction

All materials are purchased from Thermofisher Scientific unless otherwise noted, and all biohazardous materials are placed in containers and autoclaved (121°C, 20 psi, 15 mins) or bleached (10 mL *q.s.* to 100 mL with dH_2O) and then discarded. Living matter such as randomly selected leaves from plants in one of several garden areas located on campus is sampled using sterilized tweezers. Students also gather matter (such as spinach leaves) from store-bought samples. Leaves are sampled in triplicate from the same plant, placed in 1.5 mL snap-cap microcentrifuge tubes with 1 mL ultrapure MilliQ H_2O, and refrigerated at 4°C.

The following day (samples can be frozen at -20°C for longer preservation), students extract and purify DNA from the samples using a modified EtOH-precipitation-based protocol executed with P200 and P1000 micropipettes. Samples are mixed with 1mL DNA lysis buffer (5 mL 1M Tris pH 8.0, 25 mL 10% SDS [5 mL of dish soap can be used if SDS is unavailable], 10 mL 5M NaCl [Morton's iodized table salt is acceptable], 10 g store-bought unflavored meat tenderizer, 10 mL 0.5M EDTA pH 8.0, *q.s*. to 500 mL with dH_2O, mixed with magnetic stirrer), and ground with a plastic pestle for 5 mins in the 1.5 mL microcentrifuge tube. The resultant mixture is placed on a heat block at 37°C for 3 mins, mixed with 250µL protein precipitation solution (10 mL 5M NaCl, 10 g unflavored meat tenderizer, *q.s*. to 100 mL with dH_2O, mixed with magnetic stirrer), left on an ice block for 5 minutes, and centrifuged at 13,000 rpm, 22°C for 1 min.

Students remove the supernatant to another 1.5 mL microcentrifuge tube and precipitate the DNA by dripping 0.5 mL of cold (*i.e.*, stored at -20°C) 70% EtOH slowly down the tube.

DNA pellets are formed following centrifugation at 13,000 rpm, 22°C for 3 min, and the supernatant is pipetted from the pellet and discarded. To remove excess proteins, add preservative for ion-binding, and purify the DNA, students wash their pellet twice for 1 min (each time) in 1 mL of EDTA and EtOH-based "wash buffer" (250 mL 70% EtOH, 10 mL 1M Tris pH 8.0, 1 mL 0.5M EDTA, 5 mL 5M NaCl, *q.s*. to 500 mL with dH_2O, mixed with magnetic stirrer). After waiting 1 min with the pellet submerged in wash buffer, the microcentrifuge tube is centrifuged at 13,000 rpm, 22°C for 3 min and the supernatant discarded with a micropipette. Pellets are air-dried for 10 minutes, stored 1 mL of cold 70% EtOH, and refrigerated at 4°C (samples can be frozen at -20°C for longer preservation).

Polymerase Chain Reaction (PCR)

Using a thermal cycler, students implement a modified PCR protocol. Students remove their preserved samples from refrigeration, centrifuge them at 13,000 rpm, 22°C for 3 min, pipette and discard the EtOH supernatant, move their DNA pellet to a 0.2 mL thin-walled PCR tube with a micropipette tip, and add 40 µL GoTaq® Green Master Mix (400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP and 3mM MgCl2, GoTaq® DNA Polymerase is supplied in 2X Green GoTaq® Reaction Buffer at pH 8.5). The Green GoTaq® Reaction Buffer in the Master Mix also contains a compound that increases sample density, and yellow and blue loading dyes. Students then add 5 µL each of forward and reverse Rubisco Large Subunit (rbcL) primers (sequences here). PCR is run at default ("General PCR") settings, with a total reaction volume of 50 µL. PCR thin-walled tubes are labelled with student initials, placed in a covered (or parafilmed) 96-well plate, and refrigerated at 4°C (or frozen at -20°C).

PEG Purification and Submission of PCR product for Sanger Sequencing

To purify PCR fragments for sequencing, students conduct a (modified) PEG precipitation. PCR product is moved to a labelled 1.5 mL microcentrifuge tube and precipitated in 1 mL 70% EtOH, centrifuged at 13,000 rpm, 22°C for 3 mins and the supernatant discarded with a micropipette. Students then dissolve the precipitated fragments in 32 µL of MilliQ H_2O, 8 µL of 5.0 M NaCl, and 40 µL of 22% Polyethylene Glycol (PEG) 8000. They vortex their microcentrifuge tubes at medium speed for 20 seconds, leave them on an ice block for at least 20 mins, and centrifuge them at 13,000 rpm, 4°C for 10 mins. The supernatant is discarded, the pellet is dissolved in 20 µL 0.3 M NaOAc and mixed with 1 mL 95% EtOH, and the microcentrifuge tube is iced for about 15 mins. After centrifugation at 13,000 rpm, 4°C for 10 mins, the supernatant is discarded, the pellet is rinsed in 250 µL of 70% ethanol, and the tube is again centrifuged at 13,000 rpm, 4°C for 10 mins, with the resultant supernatant discarded. Pellets are air-dried for 5 minutes, eluted in 1 mL ultrapure MilliQ H_2O, and refrigerated at 4°C or -20°C. Because of possible interference with downstream applications (*i.e.*, Sanger Sequencing), we never preserve samples in TE, TAE, TBE, any EDTA-containing buffer.

In lieu of running the samples down an agarose gel with electrophoresis to confirm the presence of (usually *rbcL*-amplified) PCR product, students use a Nanodrop-Lite to evaluate the purity of their sample using A260/280 ratios (acceptable purity for Sanger Sequencing submission at >1.6). Students prepare separate tubes with 5 µL aliquots of forward primer for each sample, ensure their sample is labelled with their initials, tape the tubes to bubble wrap, and organize them in a box (snap cap tubes should be secured with parafilm if shipping materials), which is driven to the BIO5 Institute at the University of Arizona. DNA sequencing is run at

University of Arizona Genetics Core on an Applied Biosystems 3730XL DNA Analyzer with up to 600 bases of reliable sequence per read in one direction.

GEL ELECTROPHORESIS

1.Prepare TAE buffer (use 1 of the following):

a. TAE Buffer 50x Stock Recipe: 242 g tris base in double-distilled H2O, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA solution (pH 8.0)

b. Buy TAE Buffer

c. Make TAE Buffer from concentrate (10mL of concentrate, q.s. DH2O to 1L TAE)

2. Make agarose gel (use 1 of the following):

A. Melt pre-made gel in hot water bath and pour

B. Measure 0.8 agarose gel concentrate, *q.s.* DH2O to 100mL in flask, autoclave &/or microwave

3. Secure ends of cartridges, choose number of wells, and place comb over moorings in the cartridge. Wells are all the way to the left for DNA or RNA, but in the middle for other molecules where charge is uncertain. Nucleic acids are negatively charged and will run towards the cathode, so placing combs all the way to the left allows for maximum separation.

4. Pour agarose into cartridge with comb. Cover the tips of the comb, but do not pour to where the separations in the comb end.

5. Allow to sit for at least 30 minutes. Use on the same day. If not used on same day, parafilm or plastic wrap the entire cartridge with agarose gel and hold in refrigerator for 1-2 days. Alternatively, fill Ziploc bag with TAE buffer, place agarose gel in bag and hold in refrigerator for 24-36 hours.

6. Micropipette 10-20 microliters of sample into appropriate well after removing comb. Add loading dye if sample is invisible otherwise.

7. Fill gel apparatus with TAE buffer, just under the overfill line. Insert filled cartridge with gel, being sure that the cartridge is on its moorings.

8. Attach black and red cables to same color inserts on power pack. Turn on power pack after inserting cables. Set to 150 V (75 V for slower, more deliberate separations). Press “run.”

9. Turn off power pack once the samples have separated, but well before they run “off the gel.” Unplug the power pack. Pour TAE buffer into liquid (not solid) biohazard container (or sink if not a biohazard) and carefully remove cartridge. Soak in a staining tray with Fast Blast dye for 15 minutes. Slide the gel onto the gel viewer slide that corresponds to the method used (see Gel Viewer software options for details). Turn on gel viewer and enter software module. Pick correct options for coloration and viewing, save a PDF of gel, and analyze.

10. Preserve buffer “just in case” after viewing by spraying with TAE and freezing in a Ziploc bag or other holder. Alternatively, put gel back in cartridge, cover in parafilm or plastic wrap and save in refrigerator for 24 hours. Do not place used gel in a buffer-filled bag (the sample will run out into the buffer and not be preserved).