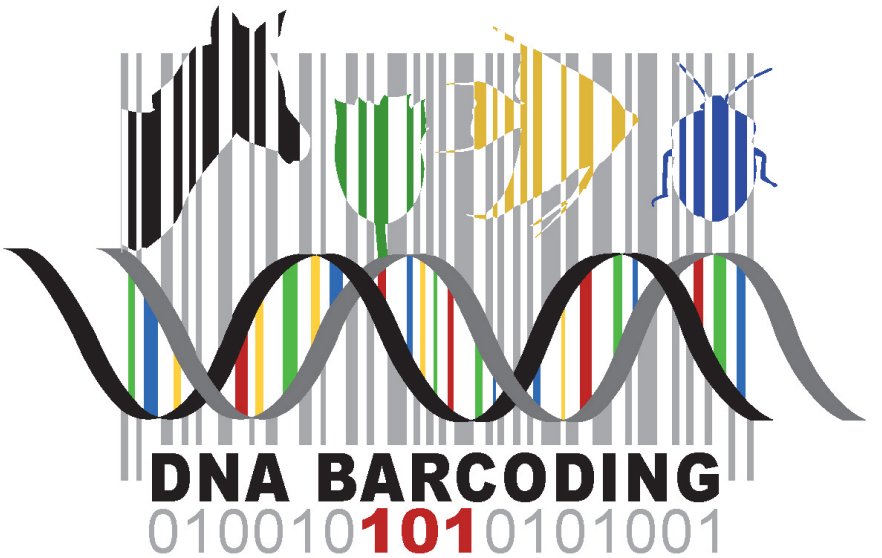


Using DNA Barcodes to Identify and Classify Living Things

www.dnabarcoding101.org

CSH Cold Spring Harbor Laboratory
DNA Learning Center

LABORATORY

Using DNA Barcodes to Identify and Classify Living Things

OBJECTIVES

This laboratory demonstrates several important concepts of modern biology. During this laboratory, you will:

- Collect and analyze sequence data from plants, fungi, or animals—or products made from them.
- Use DNA sequence to identify species.
- Explore relationships between species.

In addition, this laboratory utilizes several experimental and bioinformatics methods in modern biological research. You will:

- Collect plants, fungi, animals, or products in your local environment or neighborhood.
- Extract and purify DNA from tissue or processed material.
- Amplify a specific region of the chloroplast, mitochondrial, or nuclear genome by polymerase chain reaction (PCR) and analyze PCR products by gel electrophoresis.
- Use the Basic Local Alignment Search Tool (BLAST) to identify sequences in databases.
- Use multiple sequence alignment and tree-building tools to analyze phylogenetic relationships.

INTRODUCTION

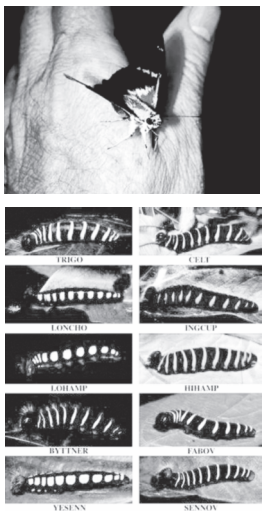
Taxonomy, the science of classifying living things according to shared features, has always been a part of human society. Carl Linneas formalized biological classification with his system of binomial nomenclature that assigns each organism a genus and species name.

Identifying organisms has grown in importance as we monitor the biological effects of global climate change and attempt to preserve species diversity in the face of accelerating habitat destruction. We know very little about the diversity of plants and animals—let alone microbes—living in many unique ecosystems on earth. Less than two million of the estimated 5–50 million plant and animal species have been identified. Scientists agree that the yearly rate of extinction has increased from about one species per million to 100–1,000 species per million. This means that thousands of plants and animals are lost each year. Most of these have not yet been identified.

Classical taxonomy falls short in this race to catalog biological diversity before it disappears. Specimens must be carefully collected and handled to preserve their

distinguishing features. Differentiating subtle anatomical differences between closely related species requires the subjective judgment of a highly trained specialist—and few are being produced in colleges today.

Now, DNA barcodes allow non-experts to objectively identify species—even from small, damaged, or industrially processed material. Just as the unique pattern of bars in a universal product code (UPC) identifies each consumer product, a "DNA barcode" is a unique pattern of DNA sequence that can potentially identify each living thing. Short DNA barcodes, about 700 nucleotides in length, can be quickly processed from thousands of specimens and unambiguously analyzed by computer programs.



DNA Barcoding revealed that what was once thought to be one species of butterfly is really ten species with caterpillars that eat different plants.

The International Barcode of Life (iBOL) organizes collaborators from more than 150 countries to participate in a variety of "campaigns" to census diversity among plant, fungi, and animal groups—including ants, bees, butterflies, fish, birds, mammals, mushrooms, and flowering plants—and within ecosystems—including the seas, poles, rain forests, kelp forests, and coral reefs. The 10-year Census of Marine Life, completed in 2010, provided the first comprehensive list of more than 190,000 marine species and identified 6,000 potentially new species.

There is a surprising level of biological diversity, literally in front of our eyes. For example, DNA barcodes showed that a well-known skipper butterfly (*Astraptes fulgerator*), identified in 1775, is actually ten distinct species. DNA barcodes have revolutionized the classification of orchids, a complex and widespread plant family with an estimated 20,000 members. The urban environment is also unexpectedly diverse; DNA barcodes were used to catalogue 54 species of bees and 24 species of butterflies in community gardens in New York City.

DNA barcodes are also used to detect food fraud and products taken from conserved species. Working with researchers from Rockefeller University and the American Museum of Natural History, students from Trinity High School found that 25% of 60 seafood items purchased in grocery stores and restaurants in New York City were mislabeled as more expensive species. One mislabeled fish was the endangered species, Acadian redfish. Another group identified three protected whale species as the source of sushi sold in California and Korea. However, using DNA barcodes to identify potential biological contraband among products seized by customs is now well established.

DNA barcoding relies on short, highly variably regions of the genome. Although there is no universal barcode, a growing list of variable regions can help differentiate species from diverse taxonomic groups. With thousands of copies per cell, mitochondrial and chloroplast sequences are readily amplified by polymerase chain reaction (PCR), even from very small or degraded specimens. Regions of chloroplast genes, including *rbc*L—RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) large subunit—and *mat*K—maturase K—are used for barcoding plants. The most abundant protein on earth, RuBisCo catalyzes the first step of carbon fixation, while maturase K encodes for a protein that assists with RNA editing. A region of the mitochondrial gene *COI* (cytochrome c oxidase subunit I) is used for barcoding animals. *COI* is involved in the electron transport phase of respiration. Thus, many genes used for barcoding are involved in the key reactions of life: storing energy in carbohydrates and releasing it to form ATP. *COI* in fungi and lichens is difficult to amplify, insufficiently variable, and some fungal

groups lack mitochondria. Instead, the nuclear internal transcribed spacer (ITS), a variable region that surrounds the 5.8s ribosomal RNA gene, is targeted. Like organelle genes, there are many copies of ITS per genome, and the variability in fungi and lichens allows for their identification. The ITS region is also used for barcoding plants, as certain taxa are particularly hard to identify using rbcL and matK. Additionally, various organisms require more taxa-specific primers for identification. For instance, green macroalgae lack *mat*K and are difficult to barcode with *rbc*L and ITS. For these plants, another chloroplast gene, *tuf*A, which codes for elongation factor Tu (EF-Tu) involved in protein synthesis, is often used. DNA barcoding to the species level is sometimes difficult with a single barcode, as species may share identical barcodes. For these, using multiple barcoding regions can help differentiate closely related species.

This laboratory uses DNA barcoding to identify plants, fungi, or animals—or products made from them. First, a sample of tissue is collected, preserving the specimen whenever possible and noting its geographical location and local environment. A small leaf disc, a whole insect, or samples of muscle are suitable sources. DNA is extracted from the tissue sample, and the barcode portion of the *rbc*L, *COI*, or *ITS* gene is amplified by PCR. The amplified sequence (amplicon) is submitted for sequencing in one or both directions.

The sequencing results are then used to search a DNA database. A close match quickly identifies a species that is already represented in the database. However, some barcodes will be entirely new, and identification may rely on placing the unknown species in a phylogenetic tree with near relatives. Novel DNA barcodes can be submitted to GenBank® (www.ncbi.nlm.nih.gov).

FURTHER READING

Benson D.A., Cavanaugh M., Clark K., Karsch-Mizrachi I, Lipman D.J., Ostell J., Sayers E.W. (2013). *Nucleic Acids Res*. GenBank®. 41(D1): D36–D42.

Hebert P.D., Cywinska A., Ball S.L., deWaard J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270(1512): 313-21.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H., Hallwachs W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proc Natl Acad Sci U S A*. 101(41):14812-7.

Hollingsworth P.M. et al (2009). A DNA barcode for land plants. *Proc Natl Acad Sci U S A* 106(31): 12794-7.

Ratnasingham, S., Hebert, P.D.N (2007). Barcoding BOLD: The Barcode of Life Data System. *Molecular Ecology Notes* 7(3): 355-64.

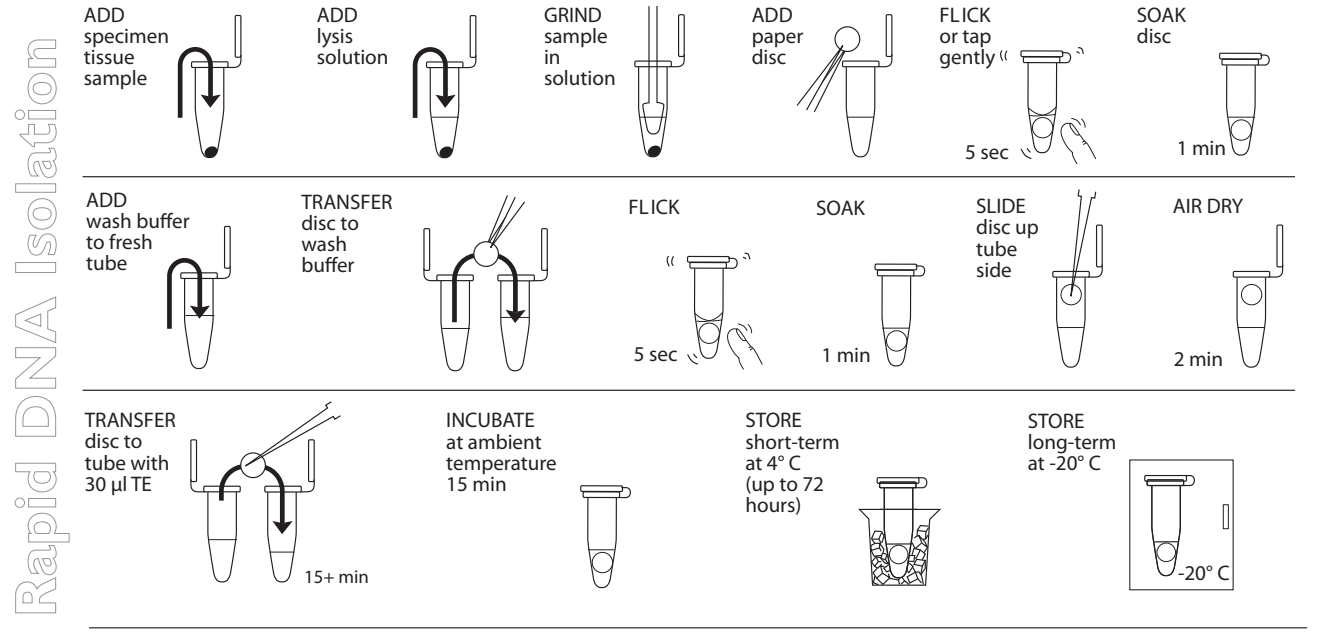
Stoeckle M. (2003). Taxonomy, DNA, and the Bar Code of Life. *BioScience* 53(9): 2-3.

Van Den Berg C., Higgins W.E., Dressler R.L., Whitten W.M., Soto-Arenas M.A., Chase M.W. (2009) A phylogenetic study of laeliinae (*Orchidaceae*) based on combined nuclear and plastid DNA sequences. *Annals of Botany* 104(3): 417-30.

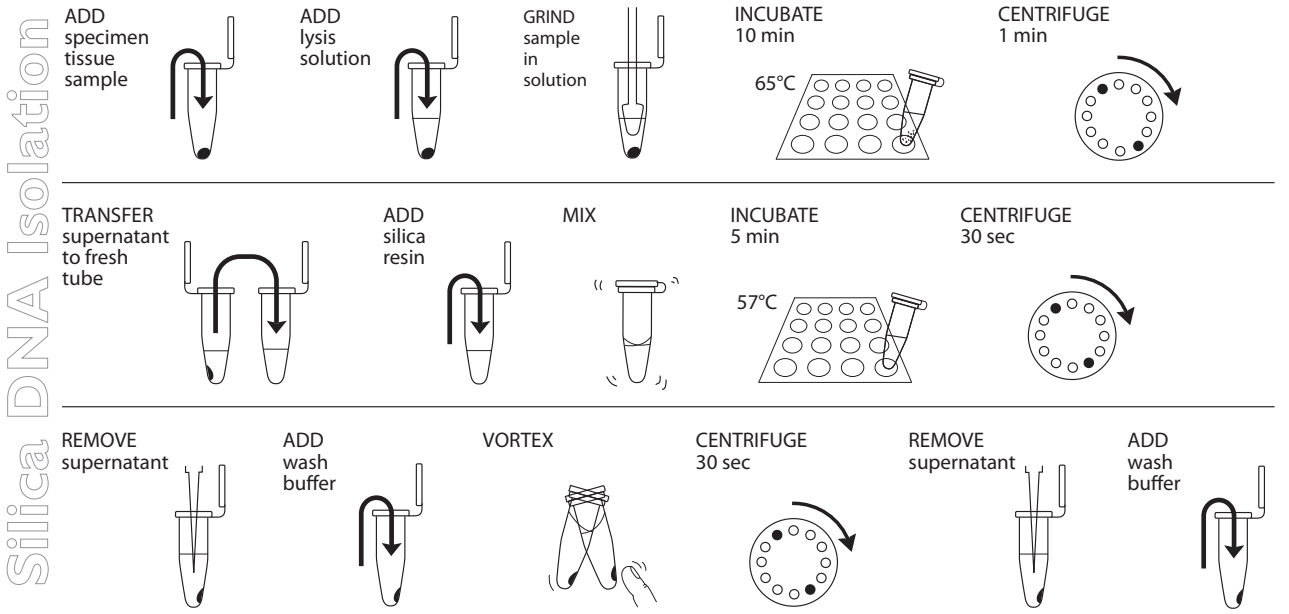
OVERVIEW OF EXPERIMENTAL METHODS

I. COLLECT, DOCUMENT, AND IDENTIFY SPECIMENS

IIa. ISOLATE DNA: RAPID DNA ISOLATION



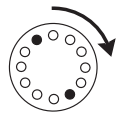
b. ISOLATE DNA: SILICA DNA ISOLATION



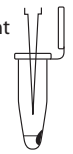
IIb. ISOLATE DNA: SILICA DNA ISOLATION, continued

VORTEX

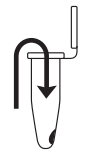
CENTRIFUGE
30 sec



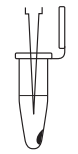
REMOVE
remaining
supernatant



ADD
dH_2O

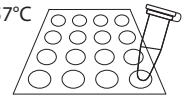


MIX
by
pipetting
in and out

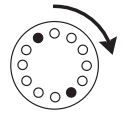


INCUBATE
5 min

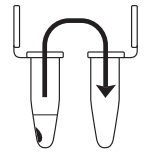
57°C



CENTRIFUGE
30 sec



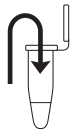
TRANSFER
supernatant
to fresh
tube



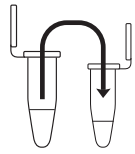
STORE
at -20 °C

III. AMPLIFY DNA BY PCR

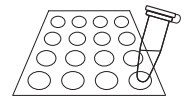
ADD
primer
mix



TRANSFER
DNA to
PCR tube

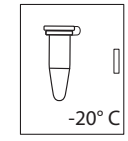


AMPLIFY
in thermal
cycler



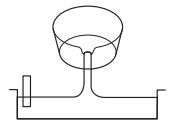
STORE
at 4° C

STORE
long-term
at -20° C



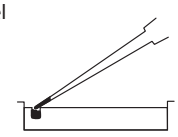
IV. ANALYZE PCR PRODUCTS BY GEL ELECTROPHORESIS

POUR
gel

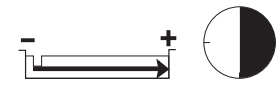


SET
20 min

LOAD
gel

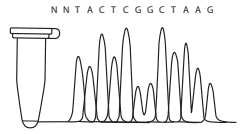


ELECTROPHORESE
130 volts
30 min

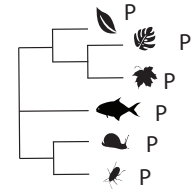


SEQUENCE PCR PRODUCT AND ANALYZE RESULTS

SEND
sample
for
sequencing



ANALYZE
results
using
bioinformatics



EXPERIMENTAL METHODS

I. Collect, Document, and Identify Specimens

The DNA isolation and amplification methods used in this laboratory work for a variety of plants, fungi, and animals—and many products derived from them.

Your collection of specimens may support a census of life in a specific area or habitat, an evaluation of products purchased in restaurants or supermarkets, or may contribute to a larger "campaign" to assess biodiversity across large areas. It may make sense for you to use sampling techniques from ecology. For example, a quadrat samples the plant and/or animal life in one square meter (or ¼ square meter) of habitat, while a transect collects samples along a fixed path through a habitat. A "Hula Hoop" can be used as an acceptable substitute for a quadrat.

Use common sense when collecting specimens. Respect private property; obtain permission to collect in any location. Respect the environment; protect sensitive habitats, and collect only enough of a sample for barcoding. Do not collect specimens that may be threatened or endangered. Be wary of poisonous or venomous plants and animals. Consult your teacher if you are in doubt about the safety or conservation status of a potential specimen. You will also need high quality photographs of your organisms and a small sample for classical taxonomic analysis and to act as a reference sample if you plan to submit your data to GenBank®.

Do not take more sample than you need. Only a small amount of tissue is needed for DNA extraction—a piece of plant leaf about ⅛- to ¼-inch diameter or a piece of animal or fungal tissue the size of a grain of rice.

Minimize damage to living plants by collecting a single leaf or bud, or several needles. When possible, use young, fresh leaves or buds. Flexible, non-waxy leaves work best. Tougher materials, such as pine needles or holly leaves, can work if the sample is kept small and is ground well. Dormant leaf buds can often be obtained from bushes and trees that have dropped leaves. Fresh, frozen leaves work well. Dried leaves and herbarium samples are variable.

Avoid twigs or bark. If woody material must be used, select flexible twigs with soft pith inside. As a last resort, scrape a small sample of the softer, growing cambium just beneath the bark. Roots and tubers are a poor choice, because high concentrations of storage starches and other sugars can interfere with DNA extraction.

For fungi, obtain fruit bodies (such as mushrooms) when possible, as DNA is easier to obtain from fruiting bodies than mycelia. Only include multiple fruiting bodies in the same sample when they are clearly growing together and appear similar, and avoid contamination by other fungi. Fresh samples work well for DNA isolation, while dried samples give variable results. Fungal fruiting is weather and climate dependent, so their abundance will vary.

Small invertebrate animals, such as insects, can be collected whole and euthanized in a kill jar by placing them in a freezer for several hours. Samples of muscle tissue can be taken from animal foods—such as fish, poultry, or red meat. Internal organs and bone marrow are also good sources of DNA. Fresh and frozen samples, and those preserved in ethanol, work well. However, bone, skin, leather, feather, dessicated, and processed samples are challenging.

SUPPLIES, & EQUIPMENT

To Share

Collection tubes, jars, or bags
Tweezers, scalpel, and scissors
Smartphone with camera or digital camera with GPS (optional)
Field guide or taxonomic key
Organism/documentation sheet
Pen/pencil
Clipboard
Ruler

Optional

1- or ¼-meter quadrat or similar
Transect (measuring tape)
Graph paper

1. Collect specimens according to a strategy or campaign outlined by your teacher. "Field Techniques Used by Missouri Botanical Garden" has many good methods for collecting and preparing plant specimens: http://www.mobot.org/MOBOT/molib/fieldtechbook/pdf/handbook.pdf.
2. Use a smartphone or digital camera to photograph your specimen in its natural environment, or where it was obtained or purchased.
   a. Take wide, medium, and close-up views.
   b. Include a person for scale in wide and medium shots. Include a ruler or coin for scale in close-ups, or place specimen on graph paper with defined grid size.

Please be aware that details described in steps 3 and 4 may change as the devices, software, and websites develop over time.

3. A global positioning system (GPS)-enabled phone or camera stores latitude, longitude, and altitude coordinates along with other metadata for each photo. Visualize or extract this geotag information:
   a. In Apple *iPhoto*, click on "i" (image properties) to plot the photo on a map. Click on "Photo," then "Show extended photo info" to find GPS coordinates.
   b. *GeoSetter*, photo metadata freeware for PCs, will plot your photo on a map.
   c. In Google *Picasa* photo editor, click on "i" to find GPS coordinates.
   d. Your smartphone's manual should explain how to use the GPS feature to obtain coordinates.

A smartphone app can continuously record your location, making it easy to document a collection trip or a sampling transect.

   e. Many smartphones also have apps that make it easy to harvest GPS coordinates.
   f. Record this information along with other metadata on the organism documentation sheet (available on www.dnabarcoding101.org/resources/).
4. Share your collection location by dropping a pin on a Google map:
   a. Sign in to or create a *Google Maps* account.
   b. Create and name a new map.
   c. Zoom in as much as possible on the collection location.
   d. Click on the pin icon to create a pin, then click the collection location.
   e. Give a title to the pin, and add any collection notes in the description field.
   f. To add a link to a photo or other url, click on the picture icon under the "Rich text" option.
   g. Click on "Done" to save your pin drop.

h. Click on "Collaborate" or "Share" to share your map with others.

5. Use a field guide or taxonomic key to identify your specimen as precisely as possible: kingdom > phylum > class > order > family > genus > species. Taxonomic keys for local plants, fungi, or animals are often available online, at libraries, or from universities, natural history museums, and botanical gardens.

   For example:

   Plants: Tropicos (http://tropicos.org)
   Animals: Integrated Taxonomic Information System (http://www.itis.gov)

6. Check to see if your specimen is represented in the Barcode of Life Database, BOLD (www.boldsystems.org) or GenBank® (www.ncbi.nlm.nih.gov):

   a. Search by entering genus and species names in the search bar at top right. If the species is represented in the database, the "Taxonomy Browser" will list the number and sources of specimen records.

   b. Click on "Download Public Sequences" for a fasta file of available barcode sequences.

   c. Click on "Taxonomy Browser" at top left to explore barcode records by group.

7. Use tweezers, scalpel, or scissors to collect a small sample of tissue.

8 Freeze your sample at -20°C until you are ready to begin Part II.

IIa. Isolate DNA from Plant, Fungal, or Animal Samples

REAGENTS, SUPPLIES, & EQUIPMENT

For each group
(for isolating DNA from 2 samples)

Lysis solution [6 M Guanidine Hydrochloride GuHCl] (120 µL)
Wash buffer (480 µL)
TE buffer (75 µL)
Specimen tissue sample(s) (from Part I)
Whatman No.1 Chromatography paper discs (2, 3-mm diameter)

To share

6 microcentrifuge tubes (1.5 mL)
Micropipettes and tips (2–1000 µL)
Permanent marker
2 Sterile plastic pestles
Microcentrifuge tube rack
2 Sterile tweezers
Possible: 2 Razor blades, scissor, tweezer, or 2, 10-µL pipette tips for tissue removal from specimen
For sample storage: 96–100% Ethanol or freezer

The rapid DNA extraction is inexpensive, fast, and does not require a water bath or centrifuge. It also works with many sample types.

Tissue should be no larger than a grain of rice. Using more than the recommended amount can affect amplification.

1. Obtain plant, fungal, or animal tissue ~10 mg or ⅛- to ¼-inch diameter by removing a piece of the tissue with a razor blade, clean tweezers, scissors, or back of a 10-µL pipette tip to enable efficient lysis. If you are working with more than one sample, be careful not to cross-contaminate specimens. (If you only have one specimen, make a balance tube with the appropriate volume of water for centrifugation steps.) Be sure to preserve the remainder of the organism at -20°C or in 96–100% Ethanol.

Lysis solution dissolves membrane-bound organelles including the nucleus, mitochondria, and chloroplast.

2. Place tissue in a clean 1.5-mL tube labeled with a sample identification number.
3. Add 50 µL of lysis solution to each tube.

Grinding breaks up cell walls and other tough material. Once ground, the sample should be liquid, but there may be some particulate matter remaining.

4. Twist a clean plastic pestle against the inner surface of the 1.5-mL tube to *forcefully* grind the tissue for at least 2 minutes. Use a clean pestle for each sample. Ensure the sample is ground into fine particles.

Whatman chromatography paper binds the DNA, helping separate DNA from contaminants.

5. For each sample, use a separate sterile tweezer to add one 3-mm diameter disc of Whatman No. 1 Chromatography paper to the lysed extract. Tap or flick the tube gently to ensure the disc is fully submerged in the extract. Allow the disc to soak in the extract for 1 minute.

Wash buffer will remove contaminants that can inhibit PCR while the DNA remains bound to the paper.

6. While the disc is soaking, add 200 µL of wash buffer to a clean 1.5-mL tube labeled with the sample identification number.

Discard/set-aside the tweezer following Step 7. Use of the tweezer to transfer the disc in future steps will contaminate the disc with impurities that may affect PCR.

7. Remove the disc from the extract using a sterile tweezer or pipette tip and transfer the disc into the fresh tube containing wash buffer. Tap or flick the tube to mix for 5 seconds, then allow the disc to sit in the wash buffer for 1 minute.

Ethanol in the wash buffer can inhibit PCR, so drying the paper after the wash step is required.

8. Use a sterile pipette tip to gently drag the disc out of the wash buffer and up the tube wall to dry at the top of the tube. Ensure that little to no debris is attached to the disk. Allow the disc to air dry for 2 minutes to evaporate the ethanol on the disc.
9. While the disc is air-drying, add 30 µL of TE to a clean 1.5-mL tube labeled with the sample identification number.
10. Once dry, carefully transfer the disc using a sterile tweezer or pipette tip into the

fresh tube containing 30 µL of TE. Allow the disc to soak for a minimum of 15 minutes at ambient temperature (soaking the disc overnight at 4° C is optimal) to elute the purified DNA.

11. The disc in TE can be stored at 4° C temporarily or frozen at -20° C for long-term storage until ready to begin Part III; ensure that the disc has incubated at ambient temperature for at least 15 minutes before storage at 4° C or -20° C. In Part III, you will use 2 µL of DNA for each PCR reaction. This is a crude DNA extract and contains nucleases that will eventually fragment the DNA at room temperature. Keep the sample cold to limit this activity.

IIb. Isolate DNA from Plant, Fungal, or Animal Samples

REAGENTS, SUPPLIES, & EQUIPMENT

For each group (for isolating DNA from 2 samples)

- Lysis solution [6 M Guanidine Hydrochloride GuHCl] (720 µL)
- Silica resin (8 µL)
- Specimen tissue sample(s) (from Part I)
- Wash buffer (2400 µL)
- Distilled water or TE buffer (240 µL)

To share

- Container with cracked or crushed ice
- Microcentrifuge
- Microcentrifuge tube rack
- 6 microcentrifuge tubes (1.5 mL)
- Micropipettes and tips (2–1000 µL)
- Permanent marker
- 2 Plastic pestles
- Vortexer (optional)
- Water bath or heating block at 65°C and 57°C
- Possible: 2 Razor blades, scissor, tweezer, or 2, 10-µL pipette tips for tissue removal from specimen
- For sample storage: 96–100% Ethanol or freezer

This DNA extraction method is inexpensive and has the advantage of working reproducibly with almost any kind of plant, fungus, or animal specimen.

Tissue should be no larger than a grain of rice. Using more than the recommended amount can affect amplification.

1. Obtain plant, fungal, or animal tissue ~10 mg or ⅛- to ¼-inch diameter by removing a piece of the tissue with a razor blade, clean tweezers, scissors, or back of a 10-µL pipette tip to enable efficient lysis. If you are working with more than one sample, be careful not to cross-contaminate specimens. (If you only have one specimen, make a balance tube with the appropriate volume of water for centrifugation steps.)

Lysis solution dissolves membrane-bound organelles including the nucleus, mitochondria, and chloroplast.

Grinding the tissue breaks up the cell walls and other tough material. When fully ground, the sample should be liquid, but there may be some particulate matter remaining.

2. Place sample in a clean 1.5-mL tube labeled with an identification number.
3. Add 300 µL of lysis solution to each tube.
4. Twist a clean plastic pestle against the inner surface of the 1.5-mL tube to *forcefully* grind the tissue for 2 minutes. Use a clean pestle for each tube if you are doing more than one sample.
5. Incubate the tube in a water bath or heat block at 65° C for 10 minutes.
6. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for one minute at maximum speed to pellet debris.
7. Label a clean 1.5-mL tube with your sample number. Transfer 150 µL of the supernatant (clear solution above pellet at bottom of tube) to the fresh tube. Be careful not to disturb the debris pellet when transferring the supernatant. Discard old tube containing the debris.

Silica resin is a DNA binding matrix that is white. In the presence of the lysis solution the silica resin binds readily to nucleic acids.

Centrifugation pellets the silica resin, which is now bound to nucleic acid. The pellet will appear as a tiny teardrop-shaped smear or particles on the bottom side of the tube underneath the hinge.

8. Add 3 µL of silica resin to tube; ensure silica resin is mixed and homogenous. Close tube and mix well by flicking or vortexing (solution will turn cloudy, but silica will settle shortly after). Close and incubate the tube for 5 minutes in a water bath or heat block at 57° C.
9. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin. Use a micropipette with fresh tip to remove all supernatant, being careful not to disrupt the white silica resin pellet at the

bottom of the tube.

10. Add 500 µL of ice cold wash buffer to the pellet. Mix well by pipetting up and down (or by closing the tube and flicking or vortexing) to resuspend the silica resin.

Wash buffer removes contaminants from the sample while nucleic acids remain bound to the resin. The silica resin is not soluble in the wash buffer. The silica resin may stay as a pellet or break up during the washing.

11. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the resin. Use a micropipette with fresh tip to remove all supernatant, being careful not to disrupt the white silica resin pellet at the bottom of the tube.

Washing twice is much more effective than washing once with twice the volume.

12. Once again, add 500 µL of ice cold wash buffer to the pellet. Close tube and mix well by vortexing or by pipetting up and down to resuspend the silica resin.

13. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the silica resin.

There will be approximately 50 µL of supernatant remaining after the brief spin to be removed. In the presence of water or TE buffer, nucleic acids are eluted from the silica resin.

14. Use a micropipette with fresh tip to remove the supernatant, being careful not to disrupt the white pellet at the bottom of the tube. Spin the tube again for ~15 seconds to collect any drops of supernatant and then remove these with a micropipette.

15. Add 100 µL of distilled water (or TE buffer) to the silica resin and mix well by vortexing or by pipetting up and down. Incubate the mixture at 57°C for 5 minutes.

For long-term storage it is recommended DNA samples be stored in TE buffer (Tris/EDTA). Tris provides a pH 8.0 environment to keep DNA and RNA nucleases less active. EDTA further inactivates nucleases by binding cations required by nucleases.

16. Place your tube and those of other groups in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 30 seconds at maximum speed to pellet the silica resin.

Transferring silica resin to the PCR reaction in Part III can inhibit the PCR amplification.

17. Label a clean 1.5-mL tube with your sample number. Transfer 50 µL of the supernatant (clear solution) to the fresh tube. Be careful not to disturb the pellet when transferring the supernatant. Discard old tube containing the silica resin.

18. Store your sample on ice or at -20°C until you are ready to begin Part III. In Part III, you will use 2 µL of DNA for each PCR reaction. This is a crude DNA extract and contains nucleases that will eventually fragment the DNA at room temperature. Keep the sample cold to limit this activity.

IIc. Isolate DNA from Animal Samples using Qiagen® DNeasy Blood and Tissue Kit

REAGENTS, SUPPLIES, & EQUIPMENT

For each group
(for isolating DNA from 2 samples)

Qiagen® DNeasy Blood & Tissue Kit, including
- Buffer ATL (430 µL)
- Buffer AL (480 µL)
- Proteinase K (50 µL)
- Buffer AW1 (1200 µL)
- Buffer AW2 (1200 µL)
- Buffer AE (240 µL)
- 2 DNeasy Mini spin columns plus 4 additional collection tubes (2 mL)

96–100% Ethanol (480 µL)*
Specimen tissue sample(s) (from Part I)
4 microcentrifuge tubes (1.5 mL)

To share

Container with cracked or crushed ice
Microcentrifuge
Micropipettes and tips (2–1000 µL)
Permanent marker
Water bath or heating block at 56° C
Vortexer (optional)
Microcentrifuge tube rack
Possible: 2 Razor blades, tweezers, or scissor for tissue removal from specimen
For sample storage: 96–100% Ethanol or freezer

*additional ethanol needed for initial use of kit

This DNA extraction method uses a commercial kit. Although it is more expensive than the rapid or silica protocols, it has the advantage of working reproducibly with dry and fatty animal specimens. It also works well with fresh animal tissues.

Prior to beginning:

- Buffers ATL and AL may form a precipitate upon storage. If necessary, warm to 56° C until the precipitate has fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. **Before using for the first time, add the appropriate amount of 100% ethanol as indicated on the bottle to obtain a working solution.**

Tissue should be no larger than a grain of rice. Using more than the recommended amount can affect amplification.

1. Obtain animal tissue ~10 mg or ⅛- to ¼-inch diameter by removing a piece of the tissue with a razor blade, clean tweezers, or scissors to enable efficient lysis. (If you only have one specimen, make a balance tube with the appropriate volume of water for centrifugation steps.)

Lysis solutions ATL and AL open tissues and dissolve membrane bound organelles including the nucleus and mitochondria.

2. Place tissue into a clean 1.5-mL microcentrifuge tube labeled with an identification number.
3. Add 180 µL of buffer ATL to each tube. Use different pipette tip for each sample.

Proteinase K rapidly digests protein, including enzymes that digest DNA.

4. Add 20 µL Proteinase K (20 mg/mL) to each tube. Use a different pipette tip for each sample.
5. Thoroughly mix tube for 5 seconds: securely grasp the upper part of the tube, and vigorously hit the bottom end with the index finger of the opposite hand. Alternatively, use a vortex if available.
6. Incubate at 56° C for at least one hour (recommended 3 hours to overnight) in a water bath or on a rocking platform (incubator) until the sample is completely lysed. Samples may appear viscous after incubation.
7. Remove from incubator and thoroughly mix by hand or vortex (if available) for 15 seconds.

8. Add 200 µL of buffer AL to each tube, then mix thoroughly by hand or vortex (if available) for 5 seconds.

Nucleic acid pellets are not soluble in ethanol and will not dissolve during washing.

9. Add 200 µL of 96–100% ethanol to each tube, then mix thoroughly by hand or vortex (if available) for 5 seconds.
10. From the mixture in step 7, transfer the entire solution (including precipitate, ~600 µL in total volume) to a DNeasy Mini spin column labeled with the identification number. Spin column should be placed in a 2-mL collection tube.
11. Place your tubes in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spin centrifuge).
12. Dispose of the collection tube containing the flow-through and put the column in a clean 2-mL collection tube.

Buffers AW1 and AW2 (step 16) are wash solutions that wash away contaminants from the DNA.

13. Add 500 µL of buffer AW1 to the spin column.
14. Place your tubes in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge for 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spin centrifuge).
15. Dispose of the collection tube containing the flow-through and put the column in a clean 2-mL collection tube.
16. Add 500 µL of buffer AW2 to the spin column.
17. Place your tubes in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge 3 minutes at ≥20,000 x g (14,000 rpm for an Eppendorf mini spin centrifuge).
18. Dispose of the collection tube containing the flow-through.

Remove the spin column carefully so it does not come into contact with the flow-through.

19. Place spin column in a clean 1.5-mL microcentrifuge tube labeled with the identification number.

Buffer AE elutes the DNA from the spin column membrane into the microcentrifuge collection tube and allows stable storage of the DNA.

20. Add 100 µL of buffer AE directly to the center of the the membrane of the spin column.
21. Incubate samples for 5 minutes at room temperature.
22. Place your tubes in a balanced configuration in a microcentrifuge, with cap hinges pointing outward. Centrifuge 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spin centrifuge).
23. Discard spin column but keep your 1.5-mL microcentrifuge tube containing the eluted DNA.
24. Store your sample on ice or at -20° C until you are ready to begin Part III. In Part III, you will use 2 µL of DNA for each PCR reaction. This is a crude DNA extract and contains nucleases that will eventually fragment the DNA at room temperature. Keep the sample cold to limit this activity.

IId. Isolate DNA from Plant and Fungal Samples using Qiagen® DNeasy Plant Kit

REAGENTS, SUPPLIES, & EQUIPMENT

For each group
(for isolating DNA from 2 samples)

Qiagen® DNeasy PlantKit, including:
- Buffer AP1 (960 µL)
- RNAse A (10 µL)
- Buffer P3 (320 µL)
- Buffer AW1 (~1620 µL)
- Buffer AW2 (2400 µL)
- Buffer AE (240 µL)
- 2 QIAshredder Mini spin columns
- 1 DNeasy Mini spin column
- 6 2-mL Collection tubes

96–100% Ethanol (quantity varies)*
6 microcentrifuge tubes (1.5 mL)
Specimen tissue sample(s) (from Part I)

To share

Container with cracked or crushed ice
Microcentrifuge
Micropipettes and tips (2–1000 µL)
Permanent marker
Plastic pestle
Water bath or heating block at 42°C and 65°C
Vortexer (optional)
Microcentrifuge tube rack
Possible: 2 Razor blades, tweezers, or scissor for tissue removal from specimen
For sample storage: 96–100% Ethanol or freezer

*additional ethanol needed for initial use of kit

This DNA extraction method uses a commercial kit. Although it is more expensive than the rapid or silica protocol, it has the advantage of working reproducibly with difficult plant or fungal specimens.

Prior to beginning:

- Buffer AP1 and Buffer AW1 concentrate may form precipitates upon storage. If necessary, warm to 65°C to dissolve (before adding ethanol to Buffer AW1). Do not heat Buffer AW1 after ethanol has been added.
- Buffer AW and Buffer AW1 are supplied as concentrates. **Before using for the first time, add the appropriate amount of 100% ethanol as indicated on the bottle to obtain a working solution.**

1. Make sure the sample is totally dry.
2. Obtain plant or fungal tissue ~10 mg or ⅛- to ¼-inch diameter by removing a piece of the tissue with a razor blade, clean tweezers, scissors, or back of a 10-µL pipette tip to enable efficient lysis. (If you only have one specimen, make a balance tube with the appropriate volume of water for centrifuge steps.)

Tissue should be no larger than a grain of rice. Using more than the recommended amount can affect amplification.

3. Place sample in a clean microcentrifuge 1.5-mL tube labeled with an identification number.
4. Twist a clean plastic pestle against the inner surface of the 1.5-ml tube to *forcefully* grind the tissue for 1 minute. Use a clean pestle for each different sample.
5. Briefly centrifuge tubes for 15 seconds at maximum speed to disrupt the static electricity.
6. Add 400 µL of AP1 and 4 µL of RNase A solution (100 mg/ml). Use a different pipette tip for each sample.
7. Securely grasp the upper part of the tube, and vigorously hit the bottom end with the index finger of the opposite hand to ensure the tissue is fully hydrated. Alternatively, vortex (if available) for 5 seconds.

Grinding the plant tissue breaks up the cell walls. When fully ground, the sample should be a green, fine liquid. There may be some particulate matter remaining.

The enzyme RNase A digests ribonucleic Acid (RNA) that could interfere with PCR.

8. Incubate for 10 minutes in a 65° C waterbath. Mix by inverting tubes 2 or 3 times during incubation.

Adding Buffer P3 precipitates detergent, proteins, and polysaccharides.

9. Add 130 µL Buffer P3 to each tube. Use a different pipette tip for each sample. Mix by inverting.
10. Incubate for 5 minutes on ice.
11. Centrifuge the lysate for 5 minutes at ≥20,000 x g (14,000 rpm for an Eppendorf mini spin centrifuge).
12. Pipette the lysate into a QIAshredder Mini spin column placed in a 2-mL collection tube labeled with the identification number. Use a different pipette tip for each sample.
13. Centrifuge for 2 minutes at ≥20,000 x g (14,000 rpm for an Eppendorf mini spin centrifuge).
14. Transfer the flow-through into a new microcentrifuge tube without disturbing the pellet if present. You should have about 450 µL flow-through to transfer. In some cases, less flow-through is is recovered. If this happens, determine the volume of flow-through necessary for the next step. Use a different pipette tip for each sample.
15. Add 1.5 volumes of Buffer AW1 to the cleared lysate, and mix immediately by pipetting. For example: if 450 µL of lysate is recovered, add 675 µL of Buffer AW1. Adjust the amount of Buffer AW1 according to the volume of lysate recovered.
16. Transfer 650 µL of the mixture into a DNeasy Mini spin column placed in a 2-mL collection tube labeled with the identification number. Use a different pipette tip for each sample.
17. Centrifuge for 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spin centrifuge). Discard the flow through and 2-ml collection tube. Repeat this step with the remaining sample.

Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.

18. Place the DNeasy Mini spin column into a new 2-ml collection tube labeled with the identification number. Add 500 µL Buffer AW2 to the spin column.
19. Centrifuge for 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spincentrifuge). Discard the flow through and reuse the collection tube for step 15.
20. Add another 500 µL of Buffer AW2. Centrifuge for 2 minutes at ≥20,000 x g (14,000 rpm for an Eppendorf mini spin centrifuge).
21. Transfer the DNeasy mini spin column to a new, 1.5-mL microcentrifuge tube labeled with the identification number. Transfer the spin column from the collection tube to the microcentrifuge tube carefully, so the column does not come into contact with the flow-through.

Buffer AE elutes DNA from the membrane and allows stable storage of DNA.

22. Add 100 µL of Buffer AE to the center of the DNeasy spin column membrane for elution. Incubate for 5 minutes at room temperature.
23. Centrifuge for 1 minute at ≥6000 x g (8000 rpm for an Eppendorf mini spin centrifuge). Discard DNeasy Mini spin column and keep microcentrifuge tube with the eluted DNA.
24. Store your samples on ice or at -20°C until you are ready to begin with Part III. In Part III, you will use 2 µL of DNA for each PCR reaction. This is a crude DNA extract and contains nucleases that will eventually fragment the DNA at room temperature. Keep the sample cold to limit this activity.

III. Amplify DNA by PCR

To amplify a DNA barcode region, choose the most appropriate set of primers for each sample. The table below lists available primer sets, the type of organism they target, and the PCR protocol for each set. For information on the primer sets, go to page 49 or http://www.dnabarcoding101.org/lab/planning-prep.html#prseq.

REAGENTS, SUPPLIES, & EQUIPMENT

Per group (for amplifying 2 samples)

For **Ready-To-Go PCR Bead** Amplification:
- 2 Ready-To-Go PCR Beads in 0.2- or 0.5-mL PCR tubes
- Appropriate primer/loading dye mix (50 µL; 23 µL per reaction)*

For **NEB *Taq* 2X Master Mix** Amplification:
- Master Mix (30 µL; 12.5 µL per reaction)*
- Appropriate primer/loading dye mix (25 µL; 10.5 µL per reaction)*

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

To share

Container with cracked or crushed ice
Micropipettes and tips (2–100 µL)
Microcentrifuge tube rack
PCR tube rack
Permanent marker
Thermal cycler

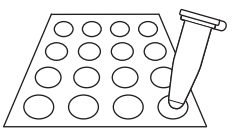
**Store on ice*

Instructions for Ready-to-Go PCR Bead amplificaton are included here; for NEB *Taq* 2X Master Mix Amplification follow directions in Planning and Prep, page 51.

1. Obtain PCR tube containing Ready-To-Go PCR Bead containing dehydrated *Taq* polymerase, nucleotides, and buffer. Label the tube with your identification number.
2. Use a micropipette with a fresh tip to add 23 µL of the appropriate primer/loading dye mix to each tube (refer to primer table below and on the next page). Allow the beads to dissolve for 1 minute.
3. Use a micropipette with fresh tip to add 2 µL of your DNA (from Part II) directly into PCR tube with primer and polymerase mixture. 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 that has been programmed with the appropriate PCR protocol.

If the reagents become splattered on the wall of the tube, pool them by briefly spinning the sample in a microcentrifuge (with tube adapters) or by sharply tapping the tube bottom on the lab bench.

To use adapters, "nest" the sample tube within sequentially larger tubes: 0.2 mL within 0.5 mL within 1.5 mL. Remove caps from tubes used as adapters.



Primers	Profile
Plant *rbc*L primer set: (rbcLaF / rbcLa rev)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 94°C 15 seconds Annealing step: 54°C 15 seconds Extending step: 72°C 30 seconds One final step to preserve the sample: 4°C *ad infinitum*
Plant *mat*K primer set: (matK-3F / matK-1R)	Initial step: 94° C 3 minutes 41 cycles of the following profile: Denaturing step: 94° C 30 seconds Annealing step: 48° C 40 seconds Extending step: 72° C 1 minute Additional extending step: 72° C 10 minutes One final step to preserve the sample: 10°C *ad infinitum*

Primers	Profile
Plant Plant-ITS primer set: (nrITS2-S2F / nrITS2-S3R)	Initial step: 95° C 2 minutes and 30 seconds 35 cycles of the following profile: Denaturing step: 95° C 30 seconds Annealing step: 56° C 30 seconds Extending step: 72° C 30 seconds Additional extending step: 72° C 10 minutes One final step to preserve the sample: 10°C *ad infinitum*
Plant (algae-specific) *tuf*A primer set: (tufA_F / tufA_R)	Initial step: 94° C 4 minutes 35 cycles of the following profile: Denaturing step: 94° C 1 minute Annealing step: 54° C 30 seconds Extending step: 72° C 1 minute One final step to preserve the sample: 10°C *ad infinitum*
Vertebrate (non-fish) Vertebrate primer cocktail: (VF1_t1/VF1d_t1/VF1i_t1/ VR1d_t1/VR1_t1/VR1i_t1)	Initial step: 94°C 1 minute 35 cycles of the following profile: Denaturing step: 94°C 15 seconds Annealing step: 54°C 15 seconds Extending step: 72°C 30 seconds One final step to preserve the sample: 4°C *ad infinitum*
Vertebrate (fish) Vertebrate primer cocktail: (VF2_t1 / FishF2_t1 / FishR2_t1 / FR1d_t1)	Initial step: 94° C 1 minute 35 cycles of the following profile: Denaturing step: 94° C 15 seconds Annealing step: 54° C 15 seconds Extending step: 72° C 30 seconds One final step to preserve the sample: 4°C *ad infinitum*
Invertebrate *COI* primer set: (LCO1490 / HC02198)	Initial step: 94° C 1 minute 35 cycles of the following profile: Denaturing step: 95° C 30 seconds Annealing step: 50° C 30 seconds Extending step: 72° C 45 seconds One final step to preserve the sample: 4° C *ad infinitum*
Fungi ITS primer set: (ITS1F / ITS4)	Initial step: 94° C 1 minute 35 cycles of the following profile: Denaturing step: 94° C 1 minute Annealing step: 55° C 1 minute Extending step: 72° C 2 minutes One final step to preserve the sample: 4°C *ad infinitum*
Fungi (lichen-specific) ITS primer set: (ITS1F_(Gad) / ITS4)	Initial step: 94° C 1 minute 35 cycles of the following profile: Denaturing step: 94° C 30 seconds Annealing step: 54° C 30 seconds Extending step: 72° C 45 seconds One final step to preserve the sample: 4° C *ad infinitum*

6. After PCR, 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

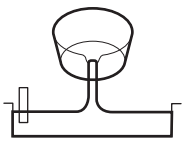
For each group with two samples

- 2% agarose in 1x TBE (hold at 60°C) (~50 mL per gel)
- pBR322/*Bst*NI marker (20 µL per gel) or 100-bp ladder (5 µL per gel)*
- PCR products from Part III*
- SYBR Green DNA stain (10 µL)
- 1x TBE buffer (~300 mL per gel)

*Store on ice.

To share

- Container with cracked or crushed ice
- Gel-casting tray and comb
- Gel electrophoresis chamber and power supply
- Latex gloves
- Masking tape
- Microcentrifuge tube rack
- PCR tube rack
- 3 Microcentrifuge tubes (1.5mL)
- Micropipette and tips (1–100 µL)
- Digital camera or photodocumentary system
- Microwave
- UV <!> or LED transilluminator and eye protection
- Water bath for agarose solution (60° C)

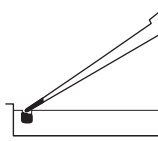


Avoid pouring an overly thick gel, which makes visualization of the DNA more difficult.

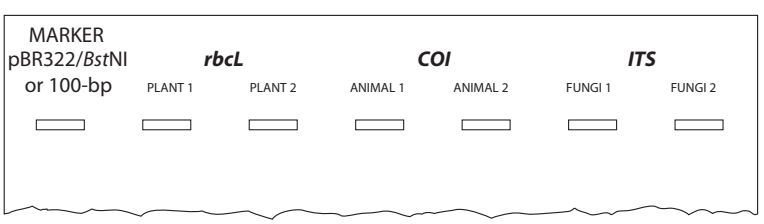
The gel will become cloudy as it solidifies.

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 just enough 1× TBE buffer to cover the surface of the gel.
5. Carefully remove the comb and add additional 1× 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 1.5-mL microcentrifuge tube. Add 2 µL of SYBR Green DNA stain to each tube with 5 µL of PCR product. *Do not* add SYBR Green directly to the tubes containing the full 25 µL of PCR product from part III; **SYBR Green interferes with the sequencing reaction.**
7. Add 2 µL of SYBR Green DNA stain to 20 µL of pBR322/*Bst*NI marker or 5 µL of 100-bp marker.
8. Orient the gel according to the diagram below, 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 or 5 µL of 100-bp marker into the far left well.
9. Use a micropipette with a fresh tip to load each sample from Step 6 in your assigned wells, similar 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.
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 or LED transillumination. Photograph the gel using a digital camera or photodocumentary system.

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

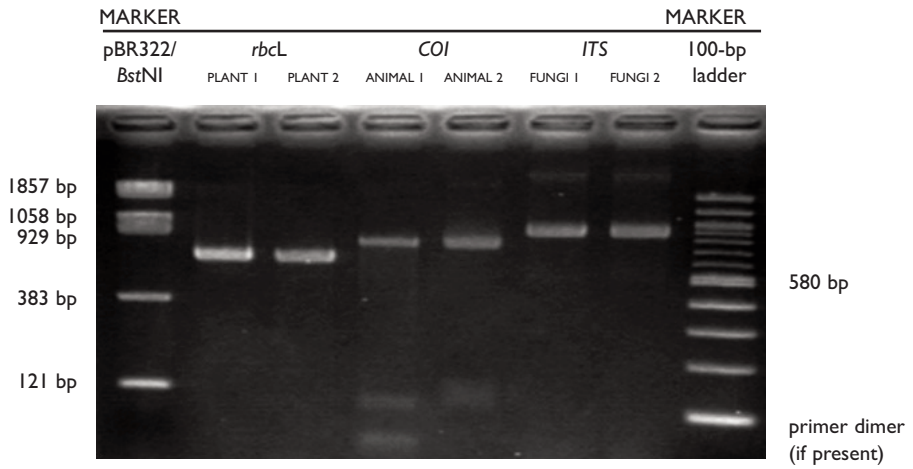
RESULTS AND DISCUSSION

I. Think About the Experimental Methods

1. Describe the effect of each of the following steps or reagents used in DNA isolation (Part I of Experimental Methods):
   i. Collecting fresh or dried specimens
   ii. Using only a small amount of tissue
   iii. Grinding tissue with pestle
   iv. Lysis solution
   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 on the next page 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.



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.

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.

3. Looking across the gel at the PCR products, do the bands all appear to be the same bp size and intensity?
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.
6. Generally, DNA sequence can be obtained from any sample that gives an obvious band on the gel.

BIOINFORMATICS

I. Use BLAST to Find DNA Sequences in Databases (Electronic PCR)

1. Perform a BLAST search as follows:
   a. Do an Internet search for "ncbi blast."
   b. Click the link for the result BLAST: *Basic Local Alignment Search Tool.* This will take you to the Internet site of the National Center for Biotechnology Information (NCBI).
   c. Under the heading "Web BLAST," click "Nucleotide BLAST."
   d. Enter the primer set you used into the "Enter Query Sequence" search window. These are the query sequences.

The following primers were used in this experiment:

Plant *rbc*L gene
- rbcLa f — 5'- ATGTCACCACAAACAGAGACTAAAGC-3' (forward primer)
- rbcLa rev — 5'- GTAAAATCAAGTCCACCRCG-3' (reverse primer)

Plant *mat*K gene
- matk-3F — 5'- CGTACAGTACTTTTGTGTTTACGAG-3' (forward primer)
- matk-1R — 5'- ACCCAGTCCATCTGGAAATCTTGGTTC-3' (reverse primer)

Plant ITS region
- nrITS2-S2F — 5'- ATGCGATACTTGGTGTGAAT-3' (forward primer)
- nrITS2-S3R — 5'- GACGCTTCTCCAGACTACAAT-3' (reverse primer)

Plant *tuf*A gene
- tufA_F — 5'- TGAAACAGAAMAWCGTCATTATGC-3' (forward primer)
- tufA_R — 5'- CCTTCNCGAATMGCRAAWCGC-3' (reverse primer)

Vertebrate (non-fish) *COI* gene
- VF1_t1 — 5'-TCTCAACCAACCACAAAGACATTGG-3' (forward primer)
- VR1d_t1 — 5'-TAGACTTCTGGGTGGCCRAARAAYCA-3' (reverse primer)

Vertebrate (fish) *COI* gene
- VF2_t1 — 5'-CAACCAACCACAAAGACATTGGCAC-3' (forward primer)
- FishR2_t1 — 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3' (reverse primer)

Invertebrate *COI* gene
- LCO1490_F — 5'-GGTCAACAAATCATAAAGATATTGG-3' (forward primer)
- HC02198_R — 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (reverse primer)

Fungi *ITS* region
- ITS1 F — 5'-TCCGTAGGTGAACCTGCGG-3' (forward primer)
- ITS4 R — 5'-TCCTCCGCTTATTGATATGC-3' (reverse primer)

Fungi (lichen-specific) *ITS* region
- ITS1F_(Gad) — 5'-CTTGGTCATTTAGAGGAAGTA-3' (forward primer)
- ITS4 R — 5'-TCCTCCGCTTATTGATATGC-3' (reverse primer)

   e. Omit any non-nucleotide characters from the window because they will not be recognized by the BLAST algorithm.

f. Under "Choose Search Set," select "Nucleotide collection (nr/nt)" from the pull-down menu.

g. Under "Program Selection," optimize for "Somewhat similar sequences (blastn)."

h. Click "BLAST." This sends your query sequences to a server at the National Center for Biotechnology Information in Bethesda, Maryland. There, the BLAST algorithm will attempt to match the primer sequences to the DNA sequences stored in its database. A temporary page showing the status of your search will be displayed until your results are available. This may take only a few seconds or more than a minute if many other searches are queued at the server.

---

2. The results of the BLAST search are displayed in three ways as you scroll down the page:

   a. First, a *Graphic Summary* illustrates how significant matches, or "hits," align with the query sequence. **Why are some alignments longer than others?**

   b. This is followed by *Descriptions of sequences producing significant alignments*, a table with links to database reports.

      - The accession number is a unique identifier given to a sequence when it is submitted to a database, such as GenBank®. The accession link leads to a detailed report on the sequence.
      - Note the scores in the "E Value" column on the right. The Expectation, or E, value is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the E value, the higher the probability that the hit is related to the query. For example, an E value of 1 means that a search with your sequence would be expected to turn up one match by chance.
      - **What is the E value of your most significant hit, and what does it mean? What does it mean if there are multiple hits with similar E values?**
      - **What do the descriptions of significant hits have in common?**

   c. Next is an *Alignments* section, which provides a detailed view of each primer sequence (*Query*) aligned to the nucleotide sequence of the search hit (*Subject*). Notice that hits have matches to one or both of the primers. For example:

	Forward Primer	*Reverse Primer*
Plant	nucleotides 1-26	nucleotides 27-46
Vertebrate (non-fish)	nucleotide 1-25	nucleotides 26-53
Fish	nucleotides 1-25	nucleotides 26-51
Fungi	nucleotide 1-19	nucleotides 20-39
Invertebrate	nucleotides 3-25	nucleotides 26-51

---

3. Predict the length of the product that the primer set would amplify in a PCR reaction (*in vitro*).

   a. In the *Alignments* section, select a hit that matches both primer sequences.

   b. **Which nucleotide positions do the primers match in the subject sequence?**

c. The lowest and highest nucleotide positions in the subject sequence indicate the borders of the amplified sequence. Subtracting one from the other gives the difference between the coordinates.

d. However, the PCR product includes both ends, so add 1 nucleotide to the result that you obtained in Step 3.c. to determine the exact length of the fragment amplified by the two primers.

e. **What value do you get if you calculate the fragment size for other species that have matches to the forward and reverse primer? Do you get the same number?**

---

4. Determine the type of DNA sequence amplified by the primer set:

   a. Click on the accession link (beginning with "*ref*") to open the data sheet for the hit used in Question 3 above. Accession Numbers will be linked next to "Sequence ID".

   b. The data sheet has three parts:

      - The top section contains basic information about the sequence, including its basepair (bp) length, database accession number, source, and references to papers in which the sequence is published.
      - The bottom section lists the nucleotide sequence.
      - The middle section contains annotations of gene and regulatory FEATURES, with their beginning and ending nucleotide positions ("xx..xx"). These features may include genes, coding sequences (CDS), regulatory regions, ribosomal RNA (rRNA), and transfer RNA (tRNA).

   c. Identify the feature(s) located between the nucleotide positions identified by the primers, as determined in 3.b. above.

II. Determine Sequence Relationships Using the Blue Line

The following directions explain how to use the Blue Line of *DNA Subway* (https://dnasubway.cyverse.org) to analyze novel DNA sequences generated by a DNA sequencing experiment. If you did not sequence your own DNA sample, you can follow these directions to use DNA sequences produced for other students. You can find supplementary instructions by clicking on the "manual" link on the *DNA Subway* homepage.

DNA Subway is an intuitive interface for analyzing DNA barcodes. Generally, you progress in a stepwise fashion through the button "stops" on each "branch line." An "R" indicates that analysis is available. A blinking "R" indicates an analysis is in process. A "V" means that results are ready to view.

You can analyze relationships between DNA sequences by comparing them to a set of sequences you have compiled yourself, or by comparing your sequences to others that have been published in databases such as GenBank® (National Center for Biotechnology Information). Generating a phylogenetic tree from DNA sequences derived from related species can also allow you to draw inferences about how these species may be related. By sequencing variable sections of DNA (barcode regions)

you can also use the Blue Line to help you identify an unknown species, or publish a DNA barcode for a species you have identified, which is not represented in published databases like GenBank® (www.ncbi.nlm.nig.gov/genbank).

---

1. Create a *DNA Subway* Project and Upload DNA Sequences

Note: Only registered users submitting novel, high-quality sequences will be able to submit sequence to GenBank.

a. Log in to *DNA Subway* at dnasubway.cyverse.org. If you do not have an account, you will need to register first to save and share your work.

b. Select "Determine Sequence Relationships" (Blue Line) to begin a project.

c. Under "Select a project type" > "Barcoding", create a project by selecting *rbc*L (plants), *COI* (animals), 16S (bacteria), or *ITS* (fungi). If you are analyzing a barcode region that is not listed, select "DNA" under "Select Project Type" > "Phylogenetics".

d. "Select Sequence Source" provides several ways to obtain sequences for barcode analysis. Select the most appropriate way to upload your data from the following four options:

- *Upload sequence(s) in ab1* (files ending with .ab1) *or FASTA* format. Click "Browse" to navigate to a folder on your desktop or drive containing your sequence(s). Select a sequence by clicking on its file name. Select more than one sequence by holding down the ctrl key while clicking file names. Once you have selected the sequences you want, click "Open".
- *Enter a sequence in FASTA format*. Below is an example of this format. The ">" symbol demarcates the sequence name. The sequence is started on the next line.

  >sequence name
  atcgccccttaatattgcctt…..

- *Import a sequence/trace from the DNALC*. If your DNA sample was sequenced by GENEWIZ, your sequence data will be automatically uploaded to this database. Search for your tracking number and click on the linked tracking number. Select one or more files from the list. Click to "Add selected files".
- *Select a sample sequence*. If you do not have a file, you may select any of the available sample sequences.

e. Provide a title in the *Name Your Project* section.

f. Write a short description of your project in the *Description* section (optional).

g. Click "Continue" to load the project into *DNA Subway*.

---

2. View and Build Sequences

There are many plants, animals, and fungi which do not have a documented barcode sequence. For instance, there are an estimated 350,000 species of angiosperms (flowering plants), but as of July 2018 there were only about

270,000 *rbc*L angiosperm sequences in GenBank®. For other species, diversity in the barcode sequences are not well characterized. This means that there are opportunities to submit novel sequences and contribute to the global barcoding effort. Only samples that have high quality sequence for both the forward and reverse reads are good enough to ensure a low error rate and can be published to GenBank®, so the sequence quality must be checked. Sequences for which there is only one high quality read are not considered high enough quality to publish. These sequences and those with no high quality sequence can still be analyzed even though the results are not publishing quality.

a. On the *Assemble Sequences* branch line, Click "Sequence Viewer" to display the sequences you have input in the project creation section. If you did not upload trace files, you can scroll to see the sequence. If you uploaded trace files, click on the file names to view the trace files.

   - The DNA sequencing software measures the fluorescence emitted in each of four channels—A, T, C, G—and records these as a trace, or electropherogram. In a good sequencing reaction, the nucleotide at a given position will be fluorescently labeled far in excess of background (random) labeling of the other three nucleotides, producing a "peak" at that position in the trace. Thus, peaks in the electropherogram correlate to nucleotide positions in the DNA sequence.
   - A software program called *Phred* analyzes the sequence file and "calls" a nucleotide (A, T, C, G) for each peak. If two or more nucleotides have relatively strong signals at the same position, the software calls an "N" for an undetermined nucleotide.
   - *Phred* also examines the peaks around each call and assigns a quality score for each nucleotide. The quality scores corresponds to a logarithmic error probability that the nucleotide call is wrong, or, conversely, to the accuracy of the call.

***Phred* Score**	**Error**	**Accuracy**
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

   - The electropherogram viewer represents each *Phred* score as a blue bar. The horizontal line equals a *Phred* score of 20, which is generally the cut-off for high-quality sequence. Thus any bar at or above the line is considered a high-quality read. **What is the error rate and accuracy associated with a *Phred* score of 20?**
   - Every sequence "read" begins with nucleotides (A, T, C, G) interspersed with Ns. In "clean" sequences, where experimental conditions were near optimal, the initial Ns will end within the first 25 nucleotides. The remaining sequence will have very few, if any, internal Ns. Then, at the end of the read, the sequence will abruptly change over to Ns.

- Large numbers of Ns scattered throughout the sequence indicate poor quality sequence. Sequences with average *Phred* scores below 20 will be flagged with a "Low Quality Score Alert." You will need to be careful when drawing conclusions from analyses made with poor quality sequence. **What do you notice about the electropherogram peaks and quality scores at nucleotide positions labeled "N"?**
- **Note:** The exclamation icon (!) indicates poor quality sequence.

b. Use the "X" and "Y" buttons to adjust the level of zoom. You can undo zooming by pressing the "Reset" button.

c. Examine the quality of the sequence(s). Any sequence for which the forward or reverse has the warning icon indicating a low quality score is not of good enough quality to publish and any determination of novelty will be tentative as sequencing errors could appear to be novel polymorphisms.

d. Click "Sequence Trimmer" to trim your sequences; this automatically removes Ns from the 5' and 3' ends of selected sequences. Click again to view the trimmed sequences. **Why is it important to remove excess Ns from the ends of the sequences?**

e. If you wish to view trimmed sequences, click on the file name.

---

3. Pair and Build Consensus for Forward and Reverse Reads

a. Click "Pair Builder" to pair your forward and reverse reads. If you have two reads for a sample, pair the sequences by checking the box to the right of each read for the sample. By default, *DNA Subway* assumes that all reads are in the forward orientation, and displays an "F" to the right of the sequence. If any sequence is not in that orientation, click the F to reverse complement the sequence. The sequence will display an "R" to indicate the change. (Reverse complementing involves reversing the order of the reverse read and then changing the bases to their complementary bases. In this way, the two sequences should be identical, and should mostly overlap.)

b. Check the square boxes next to the reads, and a dialogue box will appear asking if you wish to designate the sequences as a pair. Alternatively, Click "Try auto pairing" to pair sequences which have identical sample names, but appended with an F or R based on sequencing direction.

c. Click "Save" to save your pair assignments.

d. Once you have created sequence pairs, click "Consensus Editor" to make a consensus sequence from both sequences in the selected pairs. To examine the consensus sequence click "Consensus Editor" again, and then click on the link to the pair you wish to examine. **How does the consensus sequence optimize the amount of sequence information available for analysis? Why does this occur?**

e. If there are any mismatched nucleotides between the first and second sequence, these will be highlighted yellow in the consensus editor window. **Do differences tend to occur in certain areas of the sequence? Why?**

- A dash (–) is used to represent a gap in the data. In our consensus editor, the dash is used to "pad" the alignment between the forward and reverse sequences. A dash is a useful feature in an alignment because one of the possible mutations that could differentiate two related sequences is an insertion or deletion. In our case, misalignments between a forward and reverse read from the same sample are due to sequencing error. Since they are sequences from the same sample, they should be identical.
- One recommendation on trimming at the beginning of the sequence is to trim up to the last position where one sequence has an "N" or a "–" within the first 50 or so bases. Starting from the right, you can also trim the sequence starting at the first "N" or "–" you find 75 or so base pairs from the end of the read. These recommendations are only rules of thumb. You will have to choose how strictly you wish to trim. If a trim on either end is more than 100bp, you may have to consider the effects of discarding large amounts of sequence. Trimming 200 bp in total represents 1/3 of our approximately 600 bp of sequence.

f. Large numbers of yellow mismatches—especially in long blocks—may indicate that you have incorrectly paired sequences from two different sources (organisms), or that you failed to reverse complement the reverse strand.
   - Return to *Pair Builder* to check your pairs and reverse complements.
   - Click the red "x" to redo a pairing, and toggle "F" and "R" settings, as needed.

g. A large number of mismatches in properly paired and reverse complemented sequences indicate that one or both sequences is of poor quality. Often, one of the sequencing reactions produces a high quality read that can be used on its own. To determine this:
   - Examine the distribution of Ns to see if they are mainly confined to one of the two sequences.
   - Examine the electropherograms to see if one of the two sequences is of good quality.
   - If one of the sequences seems of good quality, return to *Pair Builder*, and click the red x to undo the pairing.

h. Few or no internal mismatches indicate good quality sequence from forward and reverse reads. If you like, you can check the consensus sequence at yellow mismatches and override the judgment made by the software:
   - Click a highlighted mismatch to see the electropherograms and graphic summarizing *Phred* scores for each read.
   - Click the desired nucleotide in the black rectangle to change the consensus sequence at that position. You should only change the consensus if you have a strong reason to believe the consensus is wrong.
   - Click the button to "Save Change(s)."

4. BLAST (Basic Local Alignment Search Tool) Your Sequence

   A BLAST search can quickly identify any close matches to your sequence in sequence databases. In this way, you can identify an unknown sample to the genus or species level. It also provides a means to add samples for a phylogenetic analysis.

   a. On the *Add Sequences* branch, click "BLASTN". Then, click the "BLAST" button next to the sequence you want to query against DNA databases.

   b. The returned list has information about the 20 most significant alignments (hits):

      - Accession number, a unique identifier given to each sequence submitted to a database. Prefixes indicate the database name—including gb (GenBank®), emb (European Molecular Biology Laboratory), and dbj (DNA Databank of Japan).
      - Organism and sequence description or gene name of the hit. Click the genus and species name for a link to an image of the organism, with additional links to detailed descriptions at Wikipedia and Encyclopedia of Life (EOL).
      - Several statistics allow comparison of hits across different searches. The number of mismatches over the length of the alignment gives a rough idea of how closely two sequences match. The Bit Score formula takes into account gaps in the sequence; the higher the score the better the alignment. The Expectation or E-value is the number of alignments with the query sequence that would be expected to occur by chance in the database. The lower the E-value, the higher the probability that the hit is related to the query. For example, an E value of 0 means that a search with your sequence would be expected to turn up no matches by chance. **Why do the most significant hits typically have E-values of 0?** (This is not the case with BLAST searches with primers.) **What does it mean when there are multiple BLAST hits with similar E values?**
      - Examine the last column in the report called "Mismatches." For barcodes, this is an informative column, with the best hits being those with the lowest number of mismatches. Note that hits with low numbers of mismatches can sometimes be lower on the list, as the bit scores are used to arrange the hits in the table. High bit scores can occur when the alignment length is longer, even when there are more mismatches than for other hits.
      - If there are zero mismatches between your sequence and a BLAST result, it is unlikely that your sequence is unique. Instead, the identical sequences probably match because they are in the same taxonomic group as your sample. Check to see if the matching sequences are from species that seem reasonable for your sample. If your best matches include some mismatches, you may have identified a novel barcode. The more mismatches you find, the more likely that your sequence is unique, especially in regions of the sequence with high quality scores. However,

sequencing errors could explain the difference, so it will be important to reexamine the trace files at any sites with mismatches to ensure that the consensus at those locations is of high quality.

c. Add BLAST sequence data to your phylogenetic analysis by checking the box(es) next to any accession number(s), then clicking on "Add BLAST hits to project" at the bottom of the BLAST results window.

---

5. Add Sequences to Your Analysis

   a. Click "Upload Data" to add additional sequence data to your analysis without starting a new project. Use "Upload Sequence(s)" to upload *ab1* trace files or *FASTA*-formatted sequences stored locally on your computer; Use "Enter Sequences(s)" to paste or type sequences in *FASTA* format.

   b. If you would like to import sequences from non-local sources you can use "Import Sequence" to search a sequence database using a sequence identifier. For GenBank® sequences you can search by Accession number. Search BOLD by species name, or search the DNALC sequence database by tracking number for sequences you processed with GENEWIZ through the DNALC system.

   c. If your sequence is high quality and had no hits with zero mismatches, you may use NCBI BLAST to confirm that the sequence is novel. Click on the BLASTN button and then double-click on the sequence (the actual nucleotides) that you identified as possibly novel to select them. Right-click (PC) or command-click (Mac) and then select copy to move the sequence to your clipboard.

      - In a web browser go to http://blast.ncbi.nlm.nih.gov. From this page click on "Nucleotide BLAST."
      - Paste your sequence into the "Enter Query Sequence" window under "Enter accession number(s), gi(s), or FASTA sequence(s)."
      - Under "Program Selection" select "Highly similar sequences (megablast)"; next click "BLAST."
      - On the results page you will get a list of results very similar to what was returned by *DNA Subway*.
      - Scrolling down the page, you will find alignments of your sequence (Query) to the sequences from the closest matches in GenBank® (Sbjct).
      - Analyze the results of the BLAST search, which are displayed in three ways as you scroll down the page:
        - First, a graphical overview illustrates how significant matches (hits) align with the query sequence. Matches of differing lengths are indicated by color-coded bars. For barcoding results, it is likely that most matches will be red, indicating high scores, and cover most of the width of the table, showing matches that span the length of your query sequence.
        - This is followed by a table with "Descriptions of sequences producing significant alignments" much like the table for BLAST results in *DNA Subway*.

- Next is an "Alignments" section, which provides a detailed view of each primer sequence ("Query") aligned to the nucleotide sequence of the search hit ("Sbjct," "subject").
- From the table, identify any matches that are 100% identical or any matches with high identity that appear to represent species or sequences you have not identified previously in *DNA Subway*. Select these sequences by clicking on the box to the left of each hit. After selecting sequences, click Download, ensure *FASTA* (complete sequence) is selected, and then click Continue.
- Open the resulting *FASTA* file (named seqdump). *Double-click* the sequences to select them all, then *right-click* (PC) or *command-click* (Mac) and *select* copy to move the sequence to your clipboard. Add these sequences to your project using the Upload Data function, as in step 1.

- Click "Sequence Viewer" back on *DNA Subway*, and view the trace file for the forward read of your query sequence. Locate the position on your table where the query sequence differed from the GenBank® match. Determine if the nucleotides you identified as different were of high quality (e.g. not sequencing errors). Because of sequence trimming, you may have to search for the polymorphic site, as the numbers from the BLAST alignment and in the trace file may not correspond.

d. You may also choose to search for your sequence at the International Barcode of Life (IBOL) database, BOLD (Barcode of Life Online Database); their records are not all in GenBank®.

- Click the BLAST button and then *double-click* the nucleotides for the sequence you are analyzing. *Right-click* (PC) or *command-click* (Mac) and then *select* copy to move the sequence to your clipboard.
- In a web browser go to http://boldsystems.org. Click on the menu in the top right-hand corner of the webpage. Select "Identification". This will bring you to the "Identification Engine" page.
- Select the tab that corresponds to the appropriate kingdom for the sample (animal, plant, or fungal).
- Under the Animal Identification [COI] tab, select "Species Level Barcode Records." On the Fungal Identification [ITS] tab, select "ITS Sequences." On the Plant Identification [*rcb*L & *mat*K] tab, select "Plant Sequences."
- Paste the sequence into the search box labeled "Enter sequences in fasta format"; next click "Submit."
- Again, a results table is produced. The column labeled "similarity" indicates how similar your sequence was to the records in the BOLD, with a 100% match indicating they were exact matches. Some records in BOLD are not public, or are not accompanied by species-level identifications. Scrolling down the list of matches you will see a pairwise alignment of your sequence (Query) to the matched sequences (Subj). Once again, identify any new hits that may be identical to your sequence. For published hits,

you can download the sequence by clicking the link to the right of "Published," then clicking "FASTA" and saving the file. This FASTA file can be uploaded, as described above, in step 1.

e. Back in *DNA Subway*, click "Reference Data" (optional) to include additional sequences. Depending on the project type you have created, you will have access to additional sequence data that may be of interest. For example, if you are doing a DNA barcoding project using the *rbc*L gene, samples of *rbc*L sequence from major plant groups (Angiosperms, Gymnosperms, etc.) will be provided. Choose any data set to add it to your analysis; you will be able to include or exclude individual sequences within the set in the next step.

---

6. Analyze Sequences: Select and Align

Unknown samples can potentially be identified to the species level by a BLAST search. In this case, a phylogenetic analysis adds depth to your understanding by showing how your sequence fits into a broader taxonomy of living things. If your BLAST search fails to identify your sequence, phylogenetic analysis can usually identify it to at least the family level.

a. Click "Select Data" to display all the sequences you have brought into your analysis, including "User data," "BLAST hits", or "Reference data". Check off sequences you wish to include in an alignment. In general, to determine the relationship of your sequence to species with known barcodes, it is best to concentrate on similar sequences. For instance, you should align sequences from samples that you believe are the same species and any close matches from database searches. You may also use the "Select all" feature to include all sequences; to deselect all sequences, click "Select all" a second time. You may run new alignments or download different sequences at any time after selecting a new set of sequences.

   - To download selected sequences to a FASTA file click the "Download" button and save the resulting file.
   - Once you have selected the sequences you wish to align, you must click "Save Selections" in the blue dialog box that appears when you make any selections.

b. Click "MUSCLE" to generate the multiple sequence alignment. This software will align all sequences that were included in the "Select Data" step. Click "MUSCLE" again to open the created multiple alignment. MUSCLE is a software tool that takes several DNA sequences and repositions them (adding gaps where necessary) to generate a multiple sequence alignment (the alignment of 3 or more DNA sequences). Assuming the DNA sequences share a common origin, alignments of DNA sequence can reveal mutations between different sequences, including insertions, deletions, or single nucleotide polymorphisms (SNPs).

   - Click the "Trim Alignment" button to trim the alignment to a region where all the selected DNA sequences overlap. Without this trimming step, those missing regions of sequences would be interpreted by the phylogenetic tree building algorithm as true deletions in the sequence,

rather than missing data. In this step, we are trimming again to account for the BLASTN matches we introduced, which may have different sequence lengths from the data we generated. **Why is it important to remove sequence gaps and unaligned ends?**

- Scroll through your alignments to see similarities between sequences. Sequence Conservation displays a histogram across the displayed sequences. At positions where most nucleotides are the same, the histogram approaches 100%; dips in the histogram are more variable regions. Sequence Variation displays the nucleotides that occur at that position relative to the consensus sequence; the colors of the bars (Green = A, Red = T, Black =G, Blue =C) display what alternative nucleotide(s) appear at that position. The Consensus bar is light gray in positions where all sequences shown contain the same nucleotide; colors appear to indicate any nucleotide position that is not at 100% consensus with other aligned sequences. Missing sequence is indicated in each row by a dark grey block on either end of the sequence. Light gray spaces indicate agreement with the consensus sequence.
- Note that the 5' (leftmost) and 3' (rightmost) ends of the sequences are usually misaligned, due to gaps (-) or undetermined nucleotides (Ns). **What causes these problems?**
- Note any sequence that introduces large, internal gaps (-----) in the alignment. This is either poor quality or unrelated sequence that should be excluded from the analysis. To remove such unrelated sequences, return to Select Data, uncheck that sequence, and save your change. Then click "MUSCLE" to recalculate.

---

7. Analyze Sequences: Create a Phylogenetic Tree

   a. A phylogenetic tree is a graphical representation of relationships between taxonomic groups. In this experiment, a gene tree is determined by analyzing the similarities and differences in DNA sequence. **What assumptions are made when one infers evolutionary relationships from sequence differences?**

   b. Click "PHYLIP ML" to generate a phylogenetic tree using the maximum likelihood method. Click "PHYLIP ML"again and a tree will open in a new window.

   c. For "Select Outgroup" function, select the species that is the least closely related species in your selection of species. Note: determining the outgroup might require background research. The outgroup will be different depending on the sequences being compared.

   d. Look at your tree.

      - Trees consist of branch tips that are labeled with the name of the sequence and/or organism you analyzed. Two branches are connected to each other by a node. A node represents the point at which descendants from an inferred common ancestor diverged into different lineages.

- The length of each branch is a measure of the evolutionary distance from the ancestral sequence at the node. Species or sequences with short branches from a node are closely related, while those with longer branches are more distantly related.
- A group formed by a common ancestor and its descendants is called a clade. Related clades, in turn, are connected by nodes to make larger, less closely-related clades.
- Generally, the clades will follow established phylogenetic relationships ascending from genus > family > order > class > phylum. However, gene and phylogenetic trees do disagree on some placements, and much research is focused on "reconciling" these differences. **Why do gene and phylogenetic trees sometimes disagree?**

d. Find and evaluate your sequence's position in the tree.
   - If your sequence is closely related to any of the reference or uploaded sequences, it will share a single node with those species.
   - If your sequence is identical to another sequence, the two will diverge directly from the node without branches.
   - If your sequence is distantly related to all of the species in your tree, your sequence will sit on a branch by itself—with the other sequences grouping together as a clade.
   - Look at the scientific names of sequences within the most closely associated clade. If all members share the same genus name, you have identified your sequence as belonging to that genus. If different genus names are represented, check and see if they belong to the same family or order.

e. Return to the menu, and click on "PHYLIP NJ" to generate a phylogenetic tree using the neighbor joining method. **How does it compare to the maximum likelihood tree? What does this tell you?**
   - To find the most likely tree and determine the reliability of the branches in this tree, NJ in *DNA Subway* uses bootstrapping, or resampling of the sequence data. Bootstrapping is a computational technique for assessing the accuracy of a statistical estimate. In bootstrapping, the columns in the sequence alignment are randomly resampled over and over to make many new alignments – 100 for NJ in DNA Subway – and these alignments are used to construct NJ trees. The final tree represents the "most likely" tree and shows the confidence of relationships with bootstrap levels. Each bootstrap value is the number of times that particular relationship appears in the 100 resampled trees. The values do not represent the distance between sequences. Instead, a higher value indicates that a branch of the tree is well supported, while low values indicate that the relationships are less certain. In general bootstrap values above 70 might be considered as plausible given the data, and above 95 can be considered "correct."

f. If neither tree places your sequence within an identifiable clade—or if that clade is only at order level—you will need to add more sequences that may

increase the resolution of your analysis. Return to Step 5, and add more reference sequences or obtain sequences within the order or family clade that contained your sequence. Then repeat Steps 6-7 to select, align, and generate trees from your refined data set.

---

8. Exporting Sequences to GenBank®

   If you do not identify any identical hits through searches in *DNA Subway*, GenBank®, and BOLD, and you have determined that your sequence is of high quality, you may have a novel sequence.

   Once you have identified a potentially novel sequence there are additional steps that you can take, including publishing your sequence to GenBank® through *DNA Subway*. It is not required that a sequence be novel to publish it to GenBank®. However, discretion should be used, and sequences that are already present in GenBank® multiple times for a particular species or without vetted metadata (definitive species identification, collection information, etc.) should not be published.

   Note: Only high quality consensus sequences that have been generated by a submitter, and which have not been previously submitted can be exported to GenBank®.

   a. Click "Export to GenBank®" in the project window.
   b. Click "New submission." (If you are working with an animal sample, you need to specify if it is from a vertebrate, invertebrate, or echinoderm) then Click "Proceed."
   d. If you have already collected information of your samples in the DNALC Barcoding Samples Database, write the sample's code number. Its information will be retrieved automatically. If not, you can enter the sample information manually in the next step; click "Continue."
   e. Verify and fill in the information required in the "Specimen info" window; click "Continue".
   f. Add photos of the sample if you have any available.
   g. Verify your submission information, make any appropriate changes if necessary, and finally click "Submit." You will receive a notification that your sequence has been submitted to NCBI and a specialist there will check it. If your submission passes NCBI's verification procedure, you will receive a notification that your sequence has been published in GenBank®.

ANSWERS TO RESULTS AND DISCUSSION QUESTIONS

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 fresh or dried specimens**

   Fresh samples are easier to isolate DNA from than other samples, so they maximize the chances of success. Dried specimens are common for plant and fungal collections and often contain intact DNA, although this DNA can be more difficult to isolate. Other samples, such as those that are processed or degraded, can have less intact DNA or PCR inhibitors.

   ii. **Using only a small amount of tissue**

   Using a small amount of tissue reduces carry-forward of PCR inhibitors present in the sample. These include metal ions (plants and animals), polysaccharides, and secondary metabolites (plants).

   iii. **Grinding tissue with pestle**

   Grinding disrupts plant cell walls and animal chitin or connective tissue. It also produces small clumps of cells that are more easily lysed to release DNA.

   iv. **Lysis solution**

   GuHCl lysis solution is a chaotropic agent, which interferes with hydrogen bonds and other interactions that stabilize stuctures. This dissolves the cell membrane and membrane-bound organelles (nucleus, mitochondria, chloroplast, etc.). In addition, GuHCL denatures biomolecules by disrupting hydrogen bonds with water surrounding them. This allows positively charged ions to form a salt bridge between the negatively charged silica and the negatively charged DNA backbone in high salt concentration.

   v. **Heating or boiling**

   Heating to 65°C with the GuHCl lysis solution helps to break down the cell and nuclear membranes and also denatures enzymes that can degrade the purified DNA. Heating to 57°C helps with the binding and release of DNA to the silica resin in the presence of the GuHCl lysis solution and distilled water respectively.

II. Interpret Your Gel and Think About the Experiment

3. **Looking across the gel at the PCR products, do the bands all appear to be the same bp size and intensity?**

   rbcL, COI, and *ITS* primers amplify differently sized products that migrate to different positions on the gel. However, each barcode primer set is optimized to amplify the same region across a range of species. Although the size of products for each primer can vary, the majority of PCR products will be of similar basepair size and, therefore, will migrate to the same position on the gel. However, the intensity of staining (thickness of bands) will vary between reactions. This is related to the mass of DNA product produced by the PCR reaction and the volume of the reaction that is successfully loaded in the well.

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.**

   It may be difficult to extract enough DNA from tough leaves or dry materials. Some primer sets may not work with certain groups of organisms; for example, *rbc*L primers work less well with non-vascular plants (mosses and liverworts).

   Major problems in PCR amplification typically occur at several points in the procedure: a) grinding step did not sufficiently disrupt the tissue, b) supernatant transferred after protein precipitation carried forward too many inhibitors, c) the nucleic acid pellet was lost after the precipitation step, or d) the small volume of DNA template was not pipetted directly into the PCR reaction (it was left in pipette or on wall of PCR tube).

ANSWERS TO BIOINFORMATICS QUESTIONS

I. Use BLAST to Find DNA Sequences in Databases (Electronic PCR)

2.a. **Why are some alignments longer than others?**

The main difference in length occurs between hits that align to both primers versus those that align only to the forward or reverse primer. The lengths and colors of the alignment bars tell how much of your query matched sequences in the database. Where the forward and reverse primer matches, you will see a black vertical line between the forward and reverse primer in the graphic summary. Typically, most of the significant alignments will have complete matches to the forward and reverse primers.

2.b. **What is the E value of the most significant hit and what does it mean? What does it mean if there are multiple hits with similar E values?**

The lowest E value obtained for a match to both primers should be in the range of 0.001 to 2e-04, or 0.0002. This might seem high for a probability, but in fact each of these values means that a match of this quality would be expected to occur by chance less than once in this database! For example, a score of 0.33 would mean that a single match would be expected to occur by chance once in every three searches. E values are based on the length of the search sequence, and thus the relatively short primers used in this experiment produce relatively high E values. Searches with longer primers or long DNA sequences return E values with smaller values. Multiple hits with similar E values are from closely related species.

What do the descriptions of significant hits have in common?

For the plant primers, the sequence sources should all be chloroplast genomes. For the vertebrate, fish, and invertebrate primers, the hits should all be mitochondrial genomes. For the fungi primers, the hits should all be to the nuclear internal transcribed spacer of the 5.8s ribosomal RNA gene.

3.b. **Which nucleotide positions do the primers match in the subject sequence?**

The answers will vary for each hit and primer set.

For *Silene Conoidea* (NC_023358.1), the plant primers match 43684–43709 and 43111–43130, respectively.

For *Pucrasia macrolopha* (NC_020587.1), the vertebrate (non-fish) primers match 6589–6613 and 7272–7298 respectively.

For *Mallotus villosus* (NC_015244.1), the fish primers match 5556–5584 and 6233–6258 respectively.

For *Candida orthopsilosis* (NC_018301.1), the fungi primers match 344066–344085 and 344559–344577 respectively.

For *Choristoneura longicellana* (NC_019996.1), the invertebrate primers match 1474–1498 and 2155–2180 respectively.

3.e. **What value do you get if you calculate the fragment size for other species that have matches to the forward and reverse primer? Do you get the same number?**

The length range of the products produced from the primers will be between 450 to 800 nucleotides.

For the plant primers, using *Silene Conoidea* (NC_023358.1) as an example gives 43709 – 43111 = 599 nucleotides. These are the absolute nucleotide coordinates for this blast hit, and the total length will vary. The range in possible lengths should be between 550 and 600 nucleotides.

For the vertebrate (non-fish) primers, *Pucrasia macrolopha* (NC_020587.1) as an example gives 7298 – 6589 = 710 nucleotides.

For the fish primers, *Mallotus villosus* (NC_015244.1) as an example gives 6258 – 5556 = 703 nucleotides.

For the fungi primers, *Candida orthopsilosis* (NC_018301.1) as an example gives 344577 – 344066 = 512 nucleotides.

4.c. **Identify the feature(s) located between the nucleotide positions identified by the primers, as determined in 3.b. above.**

Depending on the hit, the name of features may vary. However, for plant primers, the feature is usually a gene named *rbc*L that codes for a product called "ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit." For the vertebrate (non-fish), fish, and invertebrate primers, the feature is usually a gene named *COI* or COXI, which codes for cytochrome C oxidase subunit I. For the fungi primers, the feature is usually the nuclear internal transcribed spacer (*ITS*), a variable region that surrounds the 5.8s ribosomal RNA gene.

II. Identify Species and Phylogenetic Relationships Using *DNA Subway*

2.a. **What is the error rate and accuracy associated with a *Phred* score of 20?**

A *Phred* score of 20 equals 1 error in 100 or 99% accuracy.

What do you notice about the electropherogram peaks and quality scores at nucleotide positions labeled N?

At N positions, peaks representing different nucleotides have similar amplitudes (heights) and overlap, or no single peak rises above the background of lower amplitude peaks. Quality scores are very low.

2.b. **Why is it important to remove excess Ns from the ends of the sequences?**

Each N is scored as a misalignment, causing experimental sequences to appear to be less related to reference sequences than they actually are. This will significantly impact tree building, potentially placing related sequences in different clades.

3.e. **How does the consensus sequence optimize the amount of sequence information available for analysis? Why does this occur?**

The consensus sequence extends the length of the sequence and improves the accuracy of the sequence in regions where one read is of low quality. Sequence immediately following each primer has many errors and this sequence should be trimmed from the results. The read from the opposite strand usually extends into this region and provides data for the sequence at either end of the amplicon that would otherwise be lost. Also, the sequence quality can be low at different positions because of high GC content or other characteristics of the DNA. Often,

the sequence quality from one direction is better than from the other direction. By selecting the best sequence for these regions, the overall quality of the consensus will be better than either forward or reverse sequences.

3.f. **Do differences tend to occur in certain areas of the sequence? Why?**

Differences cluster at the 5' and 3' ends because the sequence quality at the ends is poor.

4.b. **Why do the most significant hits typically have E values of 0? (This is not the case with BLAST searches with primers.) What does it mean when there are multiple BLAST hits with similar E values?**

The lower the E value, the lower the probability of a random match and the higher the probability that the BLAST hit is related to the query. Searching with a long (500 bp or more) barcode sequence increases the number of significant alignments with high scores compared to searches with short primers. It is common to have multiple hits with identical or very similar E values. Of course, identical matches to the same species would be expected to have an E value of zero. However, other hits with 0 or very low E values are often found for members of the same genus. In some families of plants, fungi, or animals, the barcode regions used in this experiment are not variable enough to make a conclusive species determination. Similar E values would also be obtained when two sequences have the same number of sequence differences, but at different positions.

6.b. **What causes these problems?**

The quality of sequences may be low at either end, contributing to gaps and Ns, and the length of the sequences in the databases may also be of different lengths, which can lead to gaps.

Why is it important to remove sequence gaps and unaligned ends?

Gaps and unaligned ends are scored as mismatches by the tree-building algorithms, making sequences appear less related than they actually are, forcing related sequences into different clades.

7.a. **What assumptions are made when one infers evolutionary relationships from sequence differences?**

The major assumption is that mutations occur at a constant rate; the "molecular clock" provides the measure of evolutionary time. Since branch lengths of a phylogenetic tree represent mutations per unit of time, an increase in the mutation rate at some point in evolutionary time would artificially lengthen branch lengths. If the barcode region mutates more frequently in one clade, then a larger number of differences would be incorrectly interpreted as increased phylogenetic distance between it and other clades. Also, although there is a chance that any given nucleotide has undergone multiple substitutions (for example A>T>C or A>T>A), tree-building algorithms only evaluate nucleotide positions as they occur in the sequences being compared. If the sequences being evaluated do not include a variation that happened during evolution, it will not be taken into account, and the algorithm will assume the minimum number of substitutions. Since the chance of multiple substitutions increases over time, the

phylogenetic tree will tend to overestimate relatedness between distantly related species that diverged extremely long ago.

Why do gene and phylogenetic trees sometimes disagree?

Traditional phylogenetic trees are primarily based on morphological (physical) features. Related clades share morphological features by descent from a common ancestor. However, unrelated groups may develop a similar morphological feature when they independently adapt to similar challenges or environments. (For example, bats and birds have wings, but this feature arose independent of a common ancestor.) Gene trees can call attention to situations—at many taxonomic levels—where morphological similarities have been misinterpreted as a close phylogenetic relationship. Also, gene trees may identify new species that cannot be differentiated by morphology alone.

7.e. **How does it compare to the maximum likelihood tree? What does this tell you?**

The trees will likely have a different arrangement of nodes and place some sequences on different nodes. This tells you that there are multiple possible solutions for most phylogenetic trees, and different algorithms will calculate different optimum trees.

PLANNING AND PREPARATION

The following table will help you to plan and integrate the different experimental methods.

Experiment Part	Day	Time	Activity	
I. Collect, Document, and Identify Specimens	-1	varies	**Lab:**	Collect tissue or processed material
II. Isolate DNA from Plant, Fungal, or Animal Samples	1	30-60 min	**Pre-lab:**	Aliquot* distilled water or TE, lysis solution, wash buffer, and silica resin (silica protocol); hole punch Whatman No. 1 chromatography paper (rapid protocol)
				Set up student stations
		30/80 min	**Lab:**	Isolate DNA (rapid/silica)
III. Amplify DNA by PCR	2	15 min	**Pre-lab:**	Prepare and aliquot* primer mix
				Set up student stations
		10 min	**Lab:**	Set up PCR reactions
		70–150 min	**Post-Lab:**	Amplify DNA in thermal cycler
IV. Analyze PCR Products by Gel Electrophoresis	3	30 min	**Pre-lab:**	Dilute TBE electrophoresis buffer
				Prepare agarose gel solution
				Set up student stations
		30 min	**Lab:**	Cast gels
		45+ min		Load DNA samples into gels
				Electrophorese samples
				Photograph gels

*Suggested volumes account for pipetting error and include about 20% additional reagent per tube.

Collecting Samples

Brainstorm in class to identify one or more organized campaigns that students can be involved with. Students may select samples of their own choosing; this can be done as a homework assignment or in class if the season permits. Alternatively, teachers can provide samples.

Obtain permission to collect on private property, parks, or nature preserves.

One application of DNA barcoding is to survey species from a particular location or habitat. Since accounting for every plant and animal in a habitat is usually impossible, samples are collected to generally represent the habitat. A common sampling unit is a quadrat, a 1-meter square frame that is laid over the ground and from which each different plant and animal is collected for barcoding. Quadrats make it possible to compare samples from different locations or habitats. Nets are useful for collecting flying insects or swimming invertebrates. A sample of freshwater or marine organisms can be strained from a defined amount of water.

Plants and Fungi

Avoid collecting woody parts, which are difficult to break up, and starchy storage tissue, which includes metabolites that may interfere with PCR. If fresh green plants are not available, DNA is readily isolated from frozen or dried material. Students may also use items from the grocery store. Fresh produce works well, and many processed foods containing plant material will also work. It is difficult to isolate DNA from fatty or oily foods, such as peanut butter.

For fungi, obtain fruit bodies (such as mushrooms) when possible. Avoid contamination by other fungi, such as moldy mushrooms or multiple species growing together. Fresh samples from soft mushrooms work well for DNA isolation, while dried samples and hard fungi give variable results. Fungal fruiting is weather and climate dependent, so their abundance will vary, although both fresh and dried mushrooms are readily available from stores.

Animals

Insects offer great opportunities for barcoding. A kill jar is a simple and humane way to collect and kill insects. Make a kill jar from a wide-mouth plastic jar with tight fitting lid. Cut enough discs of paper towel to make a ½-inch stack in the bottom of the jar, then soak the toweling with acetone (nail polish remover). Keep the jar tightly capped, away from flames. Alternatively, kill insects by placing them in the freezer for at least one hour. Larger animals may be safely sampled without injury by isolating DNA from hair, feathers, or dung. Hair roots (follicles) and flesh scraped from the base of the feather shaft are reliable sources. Fresh meats and fish from the grocery are good sources of DNA for barcoding. Many processed foods, and food scraps obtained at no cost, are good sources of DNA.

Isolating DNA

Refer to table below to determine the appropriate isolation method and PCR primers for different samples.

Sample Type	**Isolation Method** $= Cost per sample, % = PCR success rates (for first choice primers), N/A: not applicable				**Recommended Primers for PCR**	**DNA Extraction and Primer Tips**
	IIa. Rapid DNA Isolation $ Small Effort	**IIb. Silica DNA Isolation** $$ Medium Effort	**IIc. QIAGEN DNeasy Blood and Tissue Kit** $$$ Large Effort	**IId. QIAGEN DNeasy Plant Kit** $$$ Large Effort	The first primer listed is recommended. Use alternate primer(s) if PCR fails.	Small sample sizes are required (approximately the size of a grain of rice). 3-mm tissue punch devices, such as surgical biopsy punches, may help to standardize sample preparation. Commercial kits are recommended for difficult (like highly processed, dry, and fatty) samples.
Plants (Terrestrial)	85%	85%	N/A	85%	1: *rbc*L 2: *matK* 3: plant *ITS*	Soak dry samples in water prior to DNA isolation. DNA extraction is more difficult from needles, seeds, bark, roots, waxy leaves, and cones. *rbc*L and *matK* may not resolve the sample to the species level. Plant-ITS is an alternative but fewer reference sequences exist in sequence databases.
Plants (Aquatic)	50%	50%	N/A	70%	1: *rbc*L 2: *matK* 3: plant *ITS*	*rbc*L and *matK* may not resolve the sample to the species level. Plant-ITS is an alternative but fewer reference sequences exist in sequence databases.
Plants (Algae-specific)	30%	40%	N/A	80%	1: *rbc*L 2: *tuf*A 3: plant *ITS*	These primers are recommended for macroscopic green algae.
Invertebrates (Terrestrial)	60%	70%	80%	N/A	1: Invertebrate *COI*	For larger organisms, remove a small portion (example: abdomen of an ant, leg of a mosquito or spider).
Invertebrates (Freshwater)	30%	60%	70%	N/A	1: Invertebrate *COI*	
Invertebrates (Marine)	30%	40%	70%	N/A	1: Invertebrate *COI* 2: *LepF1_t1*/ *LepR1_t1*	The rapid and silica isolation methods do not work across a broad spectrum of marine invertebrates; the Qiagen DNeasy Blood and Tissue kit is recommended.
Vertebrates (Fish)	35%	70%	80%	N/A	Vertebrate (fish) cocktail *COI*	
Vertebrates (Non-Fish)	60%	70%	80%	N/A	Vertebrate (non-fish) cocktail *COI*	
Fungi	70%	70%	N/A	80%	Fungi *ITS*	
Fungi (lichen-specific)	70%	70%	N/A	90%	Fungi (lichen-specific) *ITS*	Pre-wash samples with 100% acetone to remove lipids and polyphenol contaminants and let them dry for several hours before DNA isolation.

Part IIa works well for plant and terrestrial invertebrates, with an affordable and very rapid isolation method using Whatman paper.

Part IIb works well for plant, fungal, or animal samples and uses an affordable silica resin DNA isolation method.

Parts IIc and IId work well for animal, or plant or fungal samples, but use more expensive reagents from Qiagen® DNeasy Blood and Tissue Kit catalogue number 69506 (250 preps) or Qiagen® DNeasy Plant Kit, catalogue number 69106 (250 preps). These are recommended for difficult samples or for samples for which IIa or IIb fail to give DNA that amplifies.

Paper Discs

Whatman No.1 chromatography paper (Carolina Biological Item # 689110)
3-mm hole punch
100% EtOH

1. Clean a 3-mm hole punch with 100% EtOH, allow to dry. It is recommended that this hole punch is dedicated solely to creating the discs.
2. Use hole punch to punch discs (1 per sample) from the Whatman No.1 chromatography paper.
3. Store the discs in a sterile container, such as a 1.5-mL microcentrifuge tube.

Ethylenediaminetetraacetic Acid (EDTA) (0.5 M, pH 8.0)

Makes 100 mL.
Store at room temperature (indefinitely).

1. Add 18.6 g of EDTA (disodium salt dihydrate, MW 372.24) to 80 mL of deionized or distilled water.
2. Adjust the pH by slowly adding ~2.2 g of sodium hydroxide (NaOH) pellets (MW 40.00); monitor with a pH meter or strips of pH paper. (If neither is available, adding 2.2 g of NaOH pellets will make a solution of ~pH 8.0.)
3. Mix vigorously with a magnetic stirrer or by hand.
4. Add deionized or distilled water to make a total volume of 100 mL of solution.
5. Make sure that the bottle cap is loose and autoclave for 15 min at 121°C.
6. After autoclaving, cool the solution to room temperature and tighten the lid for storage.

Note: Use only the disodium salt of EDTA. EDTA will only dissolve after the pH has reached 8.0 or higher.

6 M Guanidine Hydrochloride Solution

Makes 100 mL.
Store at room temperature (for 6 months).

1. Dissolve 57.32 g of guanidine hydrochloride (m.w. = 95.53) in 50 mL of deonized or distilled water.
2. Add deionized or distilled water to make a total volume of 100 mL of solution.

Silica Resin Solution

Makes 50 mL.
Store at 4°C.

1. Dissolve 25 g of silicon dioxide (m.w. = 60.08) in 35 mL of deionized or distilled water.
2. Add deionized or distilled water to make a total volume of 50 mL of solution.

Note: The silica resin must be rinsed with 50 mL distilled water 3-4 times by centrifugation prior to bringing the total volume to 50 mL.

Sodium Chloride (NaCl) (5 M)

Makes 500 mL.
Store at room temperature (indefinitely).

1. Dissolve 146.1 g of NaCl (MW 58.44) in 250 mL of deionized or distilled water.
2. Add deionized or distilled water to make a total volume of 500 mL of solution.

Tris/EDTA (TE) Buffer

Makes 100 mL.
Store at room temperature (indefinitely).

1. In a 200-mL beaker mix the following:

   99 mL of deionized or distilled water
   1 mL of 1 M Tris pH 8.0
   200 µL of 0.5 M EDTA
2. Mix well.

Tris-HCl (1 M, pH 8.0 and 8.3)

Makes 100 mL.
Store at room temperature (indefinitely).

1. Dissolve 12.1 g of Tris base (MW 121.10) in 70 mL of deionized or distilled water.
2. Adjust the pH by slowly adding concentrated hydrochloric acid (HCl) for the desired pH listed below.

   pH 8.0: 5.0 mL
   pH 8.3: 4.5 mL
3. Monitor with a pH meter or strips of pH paper. (If neither is available, adding the volumes of concentrated HCl listed here will yield a solution with approximately the desired pH.)
4. Add deionized or distilled water to make a total volume of 100 mL of solution.
5. Make sure that the bottle cap is loose and autoclave for 15 min at 121°C.
6. After autoclaving, cool the solution to room temperature and tighten the lid for storage.

Note: A yellow-colored solution indicates poor-quality Tris. If your solution is yellow, discard it and obtain a Tris solution from a different source. The pH of Tris solutions is temperature dependent, so make sure to measure the pH at room temperature. Many types of electrodes do not accurately measure the pH of Tris solutions; check with the manufacturer to obtain a suitable one.

Wash Buffer

Makes 500 mL.
Store at -20°C (indefinitely).

1. Combine the following:

   Deionized or distilled water, 234 mL
   1 M Tris (pH 7.4), 10 mL
   5 M NaCl, 5 mL
   0.5 M EDTA, 1 mL
   100% Ethanol, 250 mL

2. Mix thoroughly.

Primer Strategy and Design

DNA barcoding relies on finding a universal chromosome location (locus) that has retained enough sequence conservation through evolutionary history that it can be identified in many organisms, but that also has enough sequence diversity to differentiate organisms to at least the family level. Regions of the chloroplast *rbc*L gene, mitochondrial *COI* gene, and nuclear *ITS* region generally fulfill these requirements.

Primers are designed to target conserved sequences that flank the variable barcode regions. However, even the conserved flanking regions have accumulated enough sequence differences over evolutionary time that it is impossible to identify universal primer sets that will work across all taxonomic groups of plants and animals. Thus, barcode primers often need to accommodate sequence variation, or degeneracy, at one or several nucleotide positions.

W	=	A or T	B	=	C or G or T
S	=	G or C	D	=	A or G or T
M	=	A or C	H	=	A or C or T
K	=	G or T	V	=	A or C or G
R	=	A or G	N	=	A or C or G or T
Y	=	C or T			

The degeneracy problem is often solved when the oligonucleotide primers are synthesized. Traditionally, a mixture of primers is synthesized—each having a different nucleotide in any of the variable positions. However, synthetic nucleotides are now available that pair with multiple nucleotides and can be incorporated at variable positions in a single primer.

The table below shows the letter abbreviation given for degenerate nucleotides. For example, a primer with the sequence 'ATCC**R**' contains both ATCC**A** and ATCC**G**.

Even degenerate primers cannot ensure amplification in taxonomic groups in which all or part of a particular primer sequence is deleted. Thus, broad surveys of unknown plants or animals typically employ multiple primer sets against slightly different flanking regions, which are combined in a multiplex PCR reaction.

The *rbc*L primer set used in this laboratory will work well for most green plants. We suggest starting with one of three *COI* primer sets for animals: one for fish, one for vertebrates, and one for other invertebrates (DMI). For fungal species, use the *ITS* primer set, which has the highest chance of success for identifying a broad range of fungi. These primer sets will not uniformly work across all groups. If the primers in this laboratory do not work with a group of organisms you are studying, consult the primer list (http://www.boldsystems.org/views/primerlist.php) at the Barcode of Life Online Database web site for alternatives.

Barcode Primer Sequences

Plant Primer Sets

5'-TGTAAAACGACGGCCAGTATGTCACCACAAACAGAGACTAAAGC-3' (forward primer–rbcLaf-M13)
5'-CAGGAAACAGCTATGACGTAAAATCAAGTCCACCRCG-3' (reverse primer–rbcLa-revM13)
or
5'-TGTAAAACGACGGCCAGTCGTACAGTACTTTTGTGTTTACGAG-3' (forward primer–matk-3F-M13)
5'-CAGGAAACAGCTATGACACCCAGTCCATCTGGAAATCTTGGTTC-3' (reverse primer–matk-1R-revM13)
or
5'-TGTAAAACGACGGCCAGTATGCGATACTTGGTGTGAAT-3' (forward primer–nrITS2-S2F-M13)
5'-CAGGAAACAGCTATGACGACGCTTCTCCAGACTACAAT-3' (reverse primer–nrITS2-S3R-revM13)
or
5'-TGTAAAACGACGGCCAGTTGAAACAGAAMAWCGTCATTATGC-3' (forward primer–tufA_F-M13)
5'-CAGGAAACAGCTATGACCCTTCNCGAATMGCRAAWCGC-3' (reverse primer–tufA_R-revM13)

Vertebrate (Fish) Cocktail

5'-TGTAAAACGACGGCCAGTCAACCAACCACAAAGACATTGGCAC-3' (forward primer–VF2_t1)
5'-TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC-3' (forward primer–FishF2_t1)
5'-CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA-3' (reverse primer–FishR2_t1)
5'-CAGGAAACAGCTATGACACCTCAGGGTGTCCGAARAAYCARAA-3' (reverse primer–FR1d_t1)

Vertebrate (Non-fish) Cocktail

5'-TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG-3' (forward primer–VF1_t1)
5'-TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG-3' (forward primer–VF1d_t1)
5'-TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG-3' (forward primer–VF1i_t1)
5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA-3' (reverse primer–VR1d_t1)
5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA-3' (reverse primer–VR1_t1)
5'-CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA-3' (reverse primer–VR1i_t1)

Invertebrate Primer Set

5'-TGTAAAACGACGGCCAGTGGTCAACAAATCATAAAGATATTGG-3' (forward primer LCO1490)
5'-CAGGAAACAGCTATGACTAAACTTCAGGGTGACCAAAAAATCA-3' (reverse primer HC02198)

Fungi Primer Set

5'-TGTAAAACGACGGCCAGTTCCGTAGGTGAACCTGCGG-3' (ITS1 F)
5'-CAGGAAACAGCTATGACTCCTCCGCTTATTGATATGC-3' (ITS4 R)

Fungi (Lichen) Primer Set

5'-TGTAAAACGACGGCCAGTCTTGGTCATTTAGAGGAAGTA-3' (ITS1F_(Gad))
5'-CAGGAAACAGCTATGACTCCTCCGCTTATTGATATGC-3' (ITS4)

Ready-to-Go PCR Beads

Ready-To-Go™ PCR beads limit reagent waste and optimize PCR reactions in a classroom setting. Each bead contains reagents so that when brought to a final volume of 25 µL, the reaction contains 2.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 µM of each dNTP.

The lyophilized *Taq* DNA polymerase in the bead becomes active immediately upon addition of the primer/loading dye mix and template DNA. In the absence of thermal cycling, "nonspecific priming" at room temperature allows the polymerase to begin generating erroneous products, which can show up as extra bands in gel analysis. Therefore, work quickly. Be sure the thermal cycler is set and have all experimenters set up their PCR reactions as a coordinated effort. Add primer/loading dye mix to all reaction tubes, then add each student template, and begin thermal cycling as quickly as possible. Hold reactions on ice until all student samples are ready to load into the thermal cycler.

NEB *Taq* 2× Master Mix

The NEB Taq 2× master mix is a cost-effective alternative to PCR beads and works well in a classroom setting. Taq 2× Master Mix is an optimized ready-to-use solution containing *Taq* DNA Polymerase, dNTPs, $MgCl_2$, KCI and stabilizers. The Master Mix is used at a 1× final concentration with DNA template and primers in a total reaction volume of 25 µL. It is stable for fifteen freeze-thaw cycles when stored at -20°C or for three months at 4°C, so for frequent use, an aliquot may be kept at 4°C.

Primer/Loading Dye Mix (for Ready-to-Go PCR Beads)

The primer/loading dye mix customizes the PCR reaction for DNA barcoding. The mix incorporates the appropriate primer pair (0.26 picomoles/µL of each primer), 13.8% sucrose, and 0.0081% cresol red. The inclusion of the loading dye components, sucrose and cresol red, allows the amplified product to be directly loaded into an agarose gel for electrophoresis.

Makes enough for 50 reactions. Store at –20°C for 1 year.

1. Mix in a 1.5-ml tube:

   640 µL of distilled water
   460 µL of Cresol Red Loading Dye (see recipes below)
   20 µL of 15 pmol/µL 5' primer
   20 µL of 15 pmol/µL 3' primer

(For multiplex primers, add 20 µL of each primer, and reduce volume of distilled water by 20 µL for each additional primer.)

Primer/Loading Dye Mix (for NEB Taq 2× Master Mix (#M0270)

The primer/loading dye mix customizes the PCR reaction for DNA barcoding. The mix incorporates the appropriate primer pair (0.526 picomoles/µL of each primer), 13.8% sucrose, and 0.0081% cresol red. The inclusion of the loading dye components, sucrose and cresol red, allows the amplified product to be directly loaded into an agarose gel for electrophoresis.

Makes enough for 50 reactions. Store at –20° C for 1 year.

1. Mix in a 1.5-ml tube:

   600 µL of distilled water
   460 µL of Cresol Red Loading Dye (see recipes below)
   40 µL of 15 pmol/µL 5' primer
   40 µL of 15 pmol/µL 3' primer

(For multiplex primers, add 40 µL of each primer, and reduce volume of distilled water by 40 µL for each additional primer.)

Alternative PCR protocol for NEB *Taq* 2× Master Mix

1. Obtain a PCR tube and use a micropipette with a fresh tip to add 12.5 µL of the master mix to each tube.
2. Use a micropipette with a fresh tip to add 10.5 µL of the appropriate primer/loading dye mix (for NEB *Taq* 2× Master Mix) to each tube. Mix well by pepetting up and down.

Plantcocktail:	*rbc*L primers (rbcLaF / rbcLa rev)
Fungi cocktail:	*ITS* primers (ITS1F/ITS4)
Fish cocktail:	*COI* primers (VF2_t1/ FishF2_t1/ FishR2_t1/ FR1d_t1)
Vertebrate (non-fish):	(VF1_t1/ VF1d_t1/ VF1i_t1 / VR1d_t1/ VR1_t1/ VR1i_t1)
Invertebrate cocktail:	(LCO1490/ HC02198)

3. Use a micropipette with fresh tip to add 2 µL of DNA (from Part II) directly into the appropriate primer/loading dye mix. Ensure that no DNA remains in the tip after pipetting. Mix well by pipetting up and down.
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 that has been programmed with the appropriate PCR protocol.

1% Cresol Red Dye

Makes 50 mL.
Store at room temperature (indefinitely).

1. Mix in a 50-mL tube:

   500 mg cresol red dye
   50 mL of distilled water

Cresol Red Loading Dye

Makes 50 ml.
Store at –20°C (indefinitely).

1. Dissolve 17 g of sucrose in 49 mL of distilled water in a 50-mL tube.
2. Add 1 mL of 1% cresol red dye and mix well.

Centrifuging PCR Tubes

Remove caps from 1.5-mL tubes to use as adapters in which to centrifuge 0.5-mL PCR tubes used for PCR amplification. Two adapters are needed to spin 0.2-mL PCR tubes—a capless 0.5-mL PCR tube is nested within a capless 1.5-mL tube.

Thermal Cycling

Amplification of *rbc*L and *COI* is simplified by the large number of chloroplast and mitochondrial genomes, which are present at 100-1,000s of copies per cell. *ITS* is also present at high copy number in most fungi. Thus, the barcode regions are amplified more readily than most nuclear loci and small amount of specimen collected

provides enough starting template to produce large quantities of the target sequence, reducing the concentration of contaminants that might inhibit PCR. The recommended amplification times and temperatures will work adequately for most common thermal cyclers, which ramp between temperatures within a single heating/cooling block. IMPORTANT: Follow manufacturer's instructions for Robocycler or other brands of thermal cyclers that physically move PCR reaction tubes between multiple temperature blocks. These machines have no ramping time between temperatures, and may require longer cycles.

Troubleshooting for Failed PCR Reactions

When PCR reactions fail, there are many possible reasons. A common source of difficulty is low quality DNA caused by using too much sample for the isolation. If you suspect your students have used too much material and their PCR failed, consider re-isolating the DNA with the standard protocol while ensuring the students use less material. For dried, degraded, or processed samples, PCR may fail due to low yield, in which case using the appropriate alternative method may allow amplification. For the silica isolation, other possible sources of trouble include evaporation of ethanol from the wash buffer, which can be avoided by storing the wash buffer at low temperature in a sealed container, and failing to remove wash buffer before elution, which can be avoided by carefully removing the wash buffer and drying briefly before elution. Mixing the DNA/silica mixture during incubation may also increase yields.

The PCR may also fail due to changes in the sequence at the primer binding sites, making it impossible to amplify even with high quality DNA. Consulting the literature on the taxa you are studying may help determine whether this is the case. It may also help to re-amplify after lowering the annealing temperature a few degrees, as this may allow the primers to anneal even if the primer binding sites have mutated.

Agarose (2%)

Makes 200 mL.
Use fresh or store solidified agarose for several weeks at room temperature.

1. To a 600-mL beaker or Erlenmeyer flask, add 200 mL of 1x TBE electrophoresis buffer and 4 g of agarose (electrophoresis grade).
2. Stir to suspend the agarose.
3. Dissolve the agarose using one of the following methods:
   - Cover the flask with aluminum foil and heat the solution in a boiling water bath (double boiler) or on a hot plate until all of the agarose is dissolved (~10 min).
   - Heat the flask uncovered in a microwave oven at high setting until all of the agarose is dissolved (3–5 min per beaker).
4. Swirl the solution and check the bottom of the beaker to ensure that all of the agarose has dissolved. (Just before complete dissolution, particles of agarose appear as translucent grains.) Reheat for several minutes if necessary.
5. Cover the agarose solution with aluminum foil and hold in a hot water bath (at ~60°C) until ready for use. Remove any "skin" of solidified agarose from the sur- face before pouring the gel.

Notes: Samples of agarose powder can be preweighed and stored in capped test tubes until ready for use. Solidified agarose can be stored at room temperature and then remelted over a boiling water bath (15–20 min) or in a microwave oven (3–5 min per beaker) before use. When remelting, evaporation will cause the agarose concentration to increase; if necessary, compensate by adding a small volume of water. Always loosene cap when remelting agarose in a bottle.

Gel Electrophoresis

CAUTION: Be sure to electrophorese only 5 µL of each amplified product. The remaining 20 µL must be retained for DNA sequencing: 10 µL for the forward read and, potentially, 10 µL for the reverse read.

Plasmid pBR322 digested with the restriction endonuclease *Bst*NI is an inexpensive marker and produces fragments that are useful as size markers in this experiment. The size of the DNA fragments in the marker are 1,857 bp, 1,058 bp, 929 bp, 383 bp, and 121 bp. Use 20 µL of a 0.075 µg/µL stock solution of this DNA ladder per gel. Other markers or a 100-bp ladder (5 µL of 50 µg/mL solution per gel) may be substituted.

View and photograph gels as soon as possible after electrophoresis or appropriate staining/destaining. Over time, the small-sized PCR products will diffuse through the gel and the bands they form will lose sharpness.

DNA Sequencing

DNA sequencing of the *rbc*L, *COI* or *ITS* amplicon is required to determine the nucleotide sequence that constitutes the DNA barcode. The forward, the reverse, or both DNA strands of the amplified barcode region may be sequenced. A single, good-quality barcode from the forward strand is sufficient to identify an organism. The majority of database sequences are from the forward strand, so sequencing only the forward strand reduces sequencing costs and simplifies analysis. If you only do a forward read, save the remaining 10 µL of amplicon. If the forward read fails, and time permits, you can send the remainder out to sequence the reverse strand.

However, bi-directional sequencing is important for several reasons. 1) A reverse sequence may provide a readable barcode when the forward sequence fails. 2) Good forward and reverse reads can be combined to produce a consensus sequence that extends the read up to 40 or more nucleotides. This is because the primer itself is not sequenced for either strand, and additional nucleotides downstream from the primer are typically unreadable. Thus, good forward and reverse primers complement these missing sequences, adding most of the primer sequences on either end. 3) One direction may provide a read through a region that is refractory to sequencing in the other direction, such as a homopolymeric region containing a long string of C residues. Thus, the insurance provided by bi-directional sequencing may be worth the added cost, especially if you have need to complete an analysis in a limited time.

Sequencing different barcode regions—*rbc*L, *COI,* and *ITS*—and using degenerate and multiplex primers complicate DNA sequencing. Strictly speaking, each different primer would need to be provided for forward and reverse sequencing reactions. As a work-around for this problem, the primers used in this experiment incorporate a universal M13 primer sequence. In addition to a sequence specific to the *rbc*L, *COI* or *ITS* barcode locus, the 5' end of each primer has an identical 17 or 18 nucleotide sequence from the bacteriophage vector M13.

In the traditional approach to genome sequencing, genomic DNA is cloned into an M13 vector. Then a universal M13 primer is used to sequence the genomic insert just downstream from the primer. This same strategy is used in sequencing *rbc*L, *COI* and *ITS* barcodes in this experiment. During the first cycle of PCR, the M13 portion of the primer does not bind to the template DNA. However, the entire primer sequence is covalently linked to the newly-synthesized DNA and is amplified in subsequent rounds of PCR. Thus, the M13 sequence is included in every full-length PCR product. This allows a sequencing center to use universal forward and reverse M13 primers for the PCR-based reactions that prepare any *rbc*L, *COI,* or *ITS* amplicon for sequencing.

The sequence of the M13 forward and reverse primers are:

M13F: TGTAAAACGACGGCCAGT
M13R: CAGGAAACAGCTATGAC

Using Genewiz DNA Sequencing Services

We recommend using GENEWIZ, Inc. for DNA barcode sequencing. GENEWIZ has optimized reaction conditions for producing the barcode sequences in this laboratory and produces excellent quality sequence with rapid turnaround—usually within 48 hours of receipt of samples. GENEWIZ sequences are automatically uploaded to the DNALC's *DNA Subway* website.

Before submitting samples for sequencing, consult the GENEWIZ guide.

Getting Started: GENEWIZ DNA Sequencing Services

1. Go to www.GENEWIZ.com and click "Register" to create a user account.
2. You will receive an Activation email at the email address used as the Username from GENEWIZ, and you will need to verify the email address by clicking on the activation link provided in the message.
3. After signing in to your user account, update your Profile, found under the My Account heading.
4. When creating your profile enter your institution name followed by "**-DNALC**" (very important!), then your personal information so invoices are sent to the correct location.
5. When prompted to select the "Methodologies Used", select "Sanger Sequencing".
6. Obtain a valid Purchase Order number from your purchasing department or use a valid credit card. Contact GENEWIZ for pricing information.

Preparing Your PCR Product

7. If you have not sequenced samples at GENEWIZ before, consult their detailed guide at:

   https://www.genewiz.com/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines
8. Verify that you have obtained PCR product of the correct length and with visible concentration on an agarose gel.
9. Prepare 8-strip, 0.2-ml PCR tubes appropriate for the number of samples you wish to submit. If you will be submitting a large number of samples (≥48), you can submit up to 94 PCR products per 96-well plate.
10. You will need to have 10 µL of PCR product for each sequencing reaction. Samples do not need to be split into two tubes to receive forward and reverse sequencing. For example: if you have 8 samples that need forward and reverse sequencing (16 reactions in the chart), you can send the samples in 8 tubes as long as you list all forward reads first, followed by all reverse reads.

Submitting a Sample for Sequencing

11. Log in to your user account to place your sequencing order.
12. Select "SANGER SEQUENCING" from the list.
13. Select "PCR Product – Un-Purified."
14. Select "Custom for the Service Type."
15. For sequencing priority, select "Standard."
16. For DNA concentration, it is best to send in a gel image with representative samples alongside a marker. This will be used by GENEWIZ to calculate the correct amount of clean-up reagents to use and the amount of product to use in the sequencing reaction. If a gel image is not supplied, GENEWIZ will use our default amount for setting up the sequencing reactions (5 ng/µL).
17. You can choose either the online form or upload an Excel form. If you choose to use the online form, you

will need to enter the number of samples.

Filling Out the Sample Form

18. Enter a sample name for each sample. This could be a number or initials, etc.
19. For DNA Length (vector + insert in bp)," enter 501–1000 for *rbc*L, *COI*, and *ITS*.
20. Leave the My Primer section empty. For GENEWIZ Primer: select "M13F" to sequence the forward strand, and "M13R" to sequence the reverse strand.

    To create a consensus barcode sequence, each sample should be sequenced in the forward and reverse direction. To do so, you will need to enter all of the sample information for the reverse reads after all sample information for the forward reads. Example table:

Sample					Primer					
Plate	Tube	Sample #	DNA Name *	Length (bp) *	My Primer *	Lib.	Edit	GENEWIZ Primer Selecti	Difficult Templ.	Notes
1	A:1	1	EXAMPLE	ex:4000-6000	ex:Mine			ex:T7	ex:Hairpin	
1	ML1	1	1-barcode	501-1000		+		M13F		
1	ML2	2	2-barcode	501-1000		+		M13F		
1	ML3	3	3-barcode	501-1000		+		M13F		
1	ML4	4	1-barcode	501-1000		+		M13R		
1	ML5	5	2-barcode	501-1000		+		M13R		
1	ML6	6	3-barcode	501-1000		+		M13R		

21. Click "Save & Review."
22. Carefully review your form, then click "Add to Cart."
23. Enter your payment information, and click "Check Out."
24. Review your order, then click "Submit."

Shipping Samples to GENEWIZ

1. Print a copy of the order form, and mail it along with your samples.
2. Be sure that the tubes are labeled exactly the same in the gel photo and on the order form. Failure to do so may delay sequencing or make it impossible to complete. Email DNALCSeq@cshl.edu if you need help.
3. Ship your samples via standard overnight delivery service (Federal Express, if possible).
4. Pack your samples in a letter pack or small shipping box, padding samples to prevent too much shifting. Room temperature shipping—with no ice or ice pack—is expected. PCR products are stable at ambient temperature, even if shipped on a Friday for Monday delivery.
5. Address the shipment to GENEWIZ at the following location:

   GENEWIZ, Inc.
   115 Corporate Blvd.
   South Plainfield, NJ 07080

   Additional GENEWIZ shipping options:

   You may be able to reduce shipping costs by using a GENEWIZ drop box. Call 1-877-436-3949 to find out if one is available in your area.