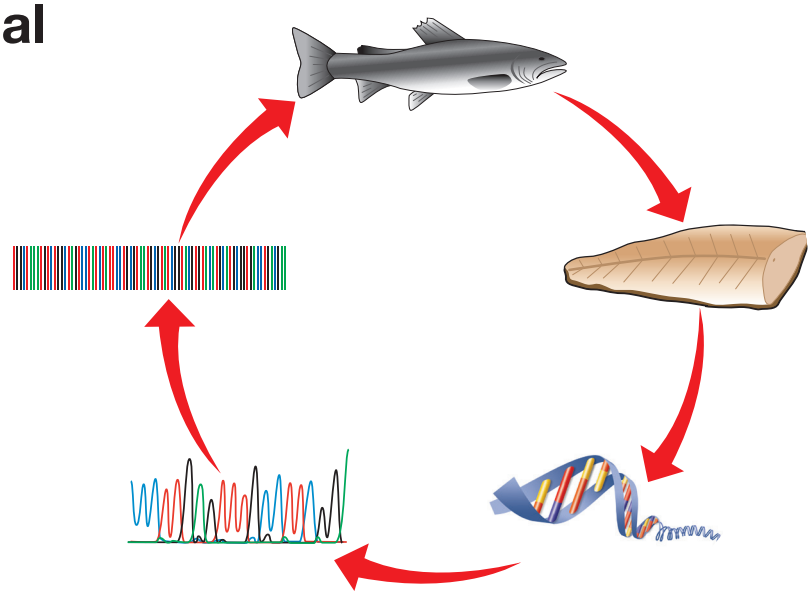
Biotechnology Explorer™

Fish DNA Barcoding Kit

Curriculum Manual

explorer.bio-rad.com

Catalog #166-5100EDU



Please visit explorer.bio-rad.com to access our selection of language translations for Biotechnology Explorer kit curricula.

BIO-RAD

To the Instructor

“Why do we have to learn this?” “Will I ever use this in real life?”

How many times have educators heard these questions? With the Fish DNA Barcoding kit, you can give your students ownership of the science they perform and help them relate scientific questions and techniques to their everyday lives. By teaching students techniques that allow them to directly investigate the world around them, you can impart relevance to what might otherwise be an isolated curriculum. The Fish DNA Barcoding kit allows students to become citizen scientists by teaching them techniques to isolate DNA from fish tissue of their choice, use the polymerase chain reaction to amplify a portion of a specific gene, analyze their PCR products using electrophoresis, and sequence the PCR product and compare that sequence to other sequences in a database.

This combination of skills can be initially performed to determine whether market substitution has occurred for the fish samples they test. Is their halibut sushi being replaced with a less expensive piece of tilapia? Where might this substitution have occurred — the wholesaler, the market, the restaurant? What are the economic, safety, and ecological impacts of market substitution? Once molecular biology skills are mastered, students can be offered the opportunity to contribute directly to the scientific knowledge base by working with scientists who are actively cataloging fish species as part of iBOL (the International Barcode of Life project). Having students connect with a scientist to receive vouchered fish samples, advocate their laboratory skills, and learn to work in collaboration encourages the development of critical communication skills.

This kit was developed in collaboration with Coastal Marine Biolabs (www.coastalmarinebiolabs.org), which is a private 501(c)(3) research-based science education organization that provides innovative laboratory- and field-based learning experiences for students. Bio-Rad thanks the organization for its invaluable insight and contribution to this curriculum.

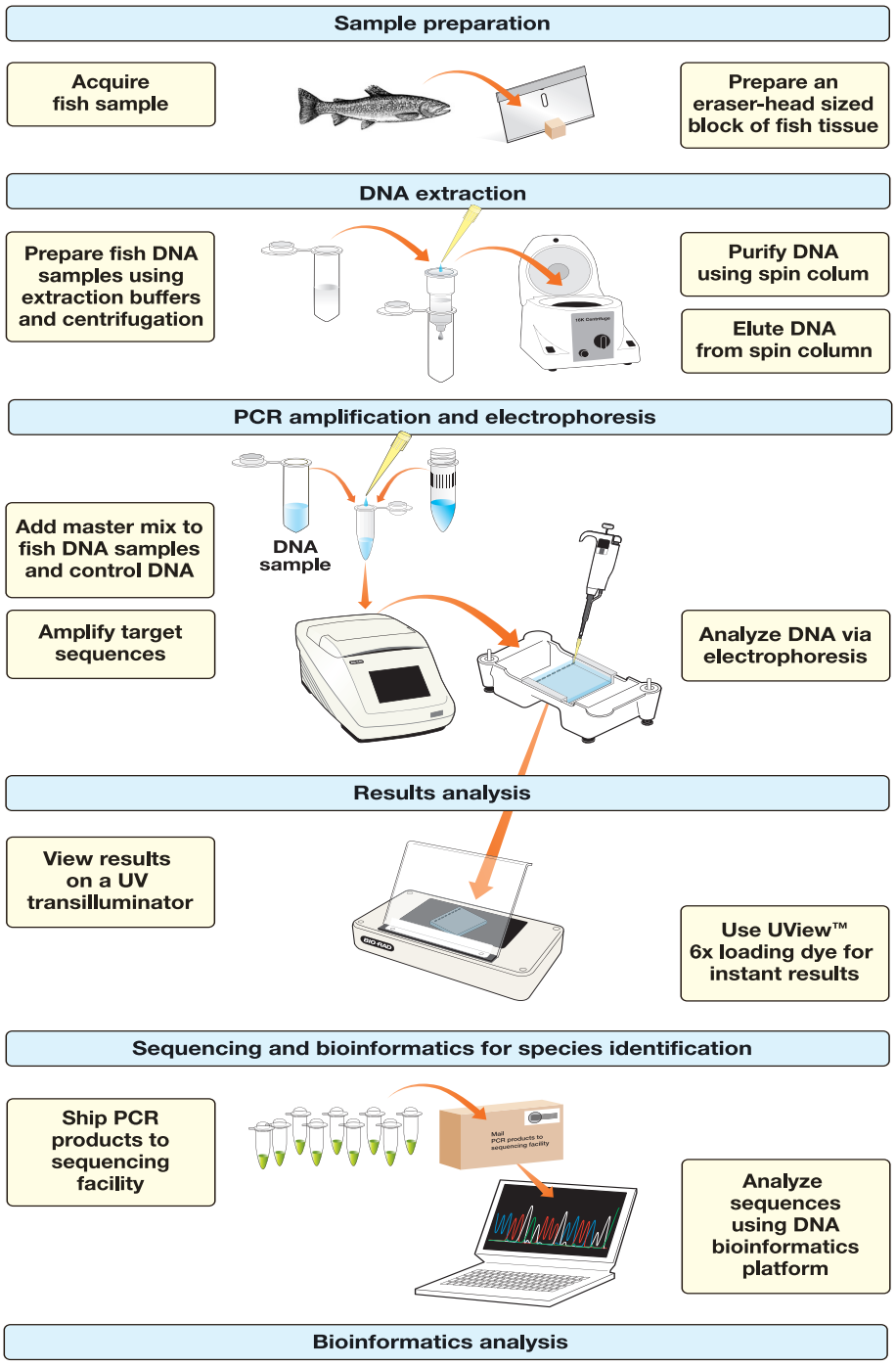
We continually strive to evolve and improve our curricula and products. As always, we welcome your stories, suggestions, and ideas!

Biotechnology Explorer Team™
Bio-Rad Laboratories
6000 James Watson Dr.
Hercules, CA 94547
Biotechnology_explorer@bio-rad.com

Table of Contents

Kit Summary

The Fish DNA Barcoding kit encompasses a laboratory workflow designed to serve eight student teams in extracting and purifying DNA from a fish sample, amplifying a region of the mitochondrial DNA, having the amplified DNA purified and sequenced, and employing bioinformatics analysis to determine the genetic identity of the original fish sample.



Storage Instructions

Open the kit box as soon as it arrives and store components at the appropriate temperatures as indicated by item packaging.

Kit Inventory Checklist

Kit Components (included)*	Quantity	(✓)
Resuspension solution, 5 ml	1	☐
Lysis solution, 5 ml	1	☐
Neutralization solution, 5 ml	1	☐
Matrix, 5 ml	1	☐
Wash buffer, 10 ml	1	☐
Spin columns	20	☐
Master mix for PCR, 1.2 ml	1	☐
Fish primer mix, 50 µl	1	☐
UView™ 6x loading dye, 0.2 ml	1	☐
pCOI control for PCR, 25 µl	1	☐
Sterile water, 2.5 ml	1	☐
PCR molecular weight ruler, 0.2 ml	1	☐
PCR tubes, 0.2 ml	50	☐
Microcentrifuge tubes, 2 ml	250	☐
Instruction manual	1	☐

* Sequencing of PCR products is a requirement to complete the DNA barcoding activity. The DNA Barcoding Sequencing module (166-5115EDU, U.S. customers only) is available for purchase separately and accomodates sequencing for up to 9 samples (8 fish + 1 positive control). Multiples may be ordered depending on the number of samples to be sequenced.

Required Accessories (not included)	Quantity	(✓)
2–20 µl adjustable-volume micropipets (166-0506EDU)	8	☐
20–200 µl adjustable-volume micropipets (166-0507EDU)	8	☐
200–1,000 µl adjustable-volume micropipets (166-0508EDU)	8	☐
2–20 µl pipet tips, aerosol barrier (211-2006EDU)	8	☐
20–200 µl pipet tips, aerosol barrier (211-2016EDU)	8	☐
100–1,000 µl pipet tips (223-9350EDU)	8	☐
Marking pens	8	☐
Razor blades or plastic knives, new and unused	16	☐
Weigh boats	16	☐
Ethanol, 95%	10 ml	☐
Graduated cylinders; 100 ml, 500 ml, 3 L	1 ea	☐
Microwave or magnetic hot plate with stir bar	1	☐
Bottle or Erlenmeyer flask, 1 L	1	☐
Distilled water	–	☐
Gloves, latex or nitrile	1 box	☐
Beakers for ice bath	1	☐

Required Accessories (not included), continued	Quantity	(✓)
50x TAE, 100 ml (166-0742EDU)	1	☐
Water bath (166-0504EDU) or dry bath (166-0562EDU)	1	☐
Fish tissue samples (grocery store, sushi restaurant, fishing trip, etc.)	1–16	☐
Microcentrifuge with variable speed setting capable of 14,000 x g (166-0602EDU)	1–4	☐
Thermal cycler (186-1096EDU)	1	☐
Molecular biology grade agarose, 5 g (161-3116EDU)	1	☐
Power supply (164-5050EDU)	2–4	☐
Horizontal electrophoresis chambers with gel casting trays and combs (166-4000EDU)	2–8	☐
Microcentrifuge tube racks (166-0481EDU)	8 racks	☐
PCR tube racks (TRC-0501EDU)	8 racks	☐
UV transilluminator or imaging system, 365 nm (166-0531EDU)	1	☐
Parafilm	1 roll	☐
Zipper sealed bag for mailing sequencing samples	1 bag	☐

Optional Accessories

Ready Gel® precast gels (161-3015EDU)
Gel staining trays, 4 (166-0477EDU)
Fast Blast™ DNA stain, 500x, 100 ml (166-0420EDU)
Lab tape

Refills Available Separately

166-5105EDU	**DNA Extraction Refill pack**, includes resuspension, lysis, and neutralization solutions, wash buffer, matrix, and 20 spin columns
166-5110EDU	**Fish DNA Barcoding Temperature-Sensitive Refill pack**, contains 2x master mix for PCR, fish primer mix, pCOI positive control DNA, UView 6x loading dye, sterile water
166-5115EDU	**DNA Barcoding Sequencing module**
166-5009EDU	**2x master mix for PCR**
166-5111EDU	**UView 6x loading dye**, 0.2 ml
166-5112EDU	**UView 6x loading dye**, 1 ml
TW1-0201EDU	**0.2 ml PCR tubes with domed caps**, natural, 1,000
223-9430EDU	**2 ml microtubes**, clear, 500

Curriculum Fit

The Fish DNA Barcoding kit aligns with a variety of courses and educational levels. Students will be most successful if they already have experience with micropipetting, gel electrophoresis, and PCR. This activity would also be suitable for students doing independent research.

Alignment

High School	Community College/ Technical College	University
AP Biology, Biotechnology, AP Environmental Science, Marine Biology, Ecology	Biotechnology, Molecular Biology, Genetics, Laboratory Methods	Biotechnology, Molecular Biology, Genetics, Biochemistry, Cell Biology

The actual laboratory procedures are routine, safe, and relatively inexpensive, provided basic laboratory equipment is available. Measures have been taken to ensure the safety of the reagents used. While proper laboratory safety techniques must always be used, the reagents provided are safe to use in the classroom. In order to complete the laboratory project in six to eight sessions, it is assumed that students meet at least once per week in a one-hour laboratory session.

Specific Objectives Met by This Project

1. Students will experience a wide range of laboratory techniques. Some of the techniques implemented in this DNA-based project are: DNA extraction, PCR, gel electrophoresis, DNA sequencing, and analysis.
2. Students will see that these individual techniques are just steps in a longer investigatory process. Few researchers can complete an entire research project in one- or two-hour laboratory sessions (the timeframe of most commercially available kits), so this multi-period project more accurately reflects what goes on in a contemporary molecular biology laboratory.
3. Students will be active participants in the process. There are opportunities during the project for students to troubleshoot or have general discussions about their results, or to make judgments about what to do next.

This exercise does not take a simple “cookbook” approach, but rather engages students in true scientific practices. Students will employ critical thinking as they carry out their investigations and use data to construct explanations for their scientific results.

Alignments

- This kit aligns with AP biology, Next Generation Science Standards (NGSS), and state standards. For detailed information of current alignments, please contact Biotechnology Explorer (explorer.bio-rad.com)

Biology Curriculum

- Through inquiry-based science students investigate relationships among fish at the genetic level by examining the *COI* gene. The highly conserved gene is part of the electron transport chain of mitochondria and is an integral part of energy flow in biological systems
- Analysis of *COI* genes in fish allows students to see how random mutations at the genetic level can lead to a diversity of species within a changing environment and opens up questions about how these errors occur in the DNA. Through the use of BLAST, a process rich in mathematical models — especially statistics — the diversity of species can be drawn out into phylogenetic trees, which help students envision the lines of descent from a common ancestor and the process of evolution

Career and Technical Skills

- Students learn to extract DNA from tissue, isolate genomic DNA using spin columns, separate DNA with gel electrophoresis, perform PCR, and carry out basic bioinformatics processes

Cross Curriculum Opportunities

- Careful selection of samples may allow students to probe economic questions about market substitution and its prevalence. This allows for a discussion about the role of biotechnology in the regulation of biological commodities and even of safety within food markets

Background for Teachers

What Is DNA barcoding?

Have you ever ordered a California roll at a sushi counter and wondered exactly what sort of seafood made up the "imitation crab" in your meal? Or have you ever been to a seafood restaurant in New England and ordered scrod and wondered what fish you were getting? Once a piece of seafood has been processed and filleted, it can be difficult to tell what species the fish is. Even if the fish was caught in the wild and not purchased from the grocery store after processing, it can sometimes be difficult to identify a fish species simply by analyzing its physical characteristics. The method of grouping organisms according to common physical characteristics, known as Linnaean taxonomy, has been around for 250 years and has long been the standard method of species identification. However, that does not mean this method is always easy or accurate.

Not only is the identification of fish species we eat important, but so is the identification of all marine species and the classification of new species. The deep sea has been called the last frontier. It is estimated that of all the species that exist in the marine environment, possibly only one third of them have been identified. UNESCO (the United Nations Educational, Scientific and Cultural Organization) even celebrated the 2012 International Day for Biological Diversity under the theme of Marine Biodiversity. For further information, please go to the UNESCO website (www.unesco.org) and search for Marine Biodiversity Day.

Up to this point, the primary form of identification and classification of new fish species has used Linnaean taxonomic methods. However, identification of species by physical characteristics and behaviors can be confounded by similarities arising from other factors. One example would be convergent evolution, where two species starting from quite different ancestors independently develop similar traits due to environmental pressures. Examples of this include quills on both porcupines and echidnas, or flight in both bats and birds. In Batesian mimicry, harmless species develop traits that resemble those of dangerous species. Examples of this include nontoxic viceroy butterflies and poisonous monarch butterflies looking like each other, or harmless milk snakes and king snakes having coloration similar to that of poisonous coral snakes.

Even after 250 years of collection, analysis, and categorization using physical characteristics of species, fewer than two million of Earth's estimated 10–50 million plant and animal species have been formally described and cataloged. With the current rate of species loss from human overpopulation, habitat destruction, pollution, and overharvesting, the rate of species disappearance threatens to outpace the rate of species discovery using this traditional classification system. The dwindling pool of experts capable of physical characterization of species makes the threat to discovery of new species even more alarming (Hebert et al. 2003).

The explosion in modern times of quick and inexpensive methods to isolate, purify, amplify, and sequence DNA has spurred development of new methods to help identify different species, whether they are fish sold at the market or newly discovered species. Using DNA-based technologies, a multinational alliance of scientists is now cataloging life using what is called a DNA barcoding system in order to accelerate the discovery of new species and develop powerful new tools to monitor and preserve Earth's vanishing biodiversity.

In much the same way that a UPC (universal product code) barcode can differentiate a carton of milk from a bag of carrots when both are scanned at a grocery store cash register, DNA sequences can be used to uniquely identify different species. This is the basis of DNA barcoding. DNA barcoding consists of two primary components: 1) a genetic sequence or barcode that is unique to a particular species, comparable to a commercial product's UPC code; and 2) an electronic database capable of providing the identity or name of a species by reading and matching its genetic barcode to a library of reference barcodes. That library performs a function similar to that of the grocery store's cash register computer. DNA barcoding uses a short genetic marker to identify a particular organism as belonging to a particular species. An ideal DNA barcode should be present in most of the organisms of interest, readily amplified without using species-specific PCR primers, and should exhibit relatively few nucleotide differences among members of the same species but larger variation between species.

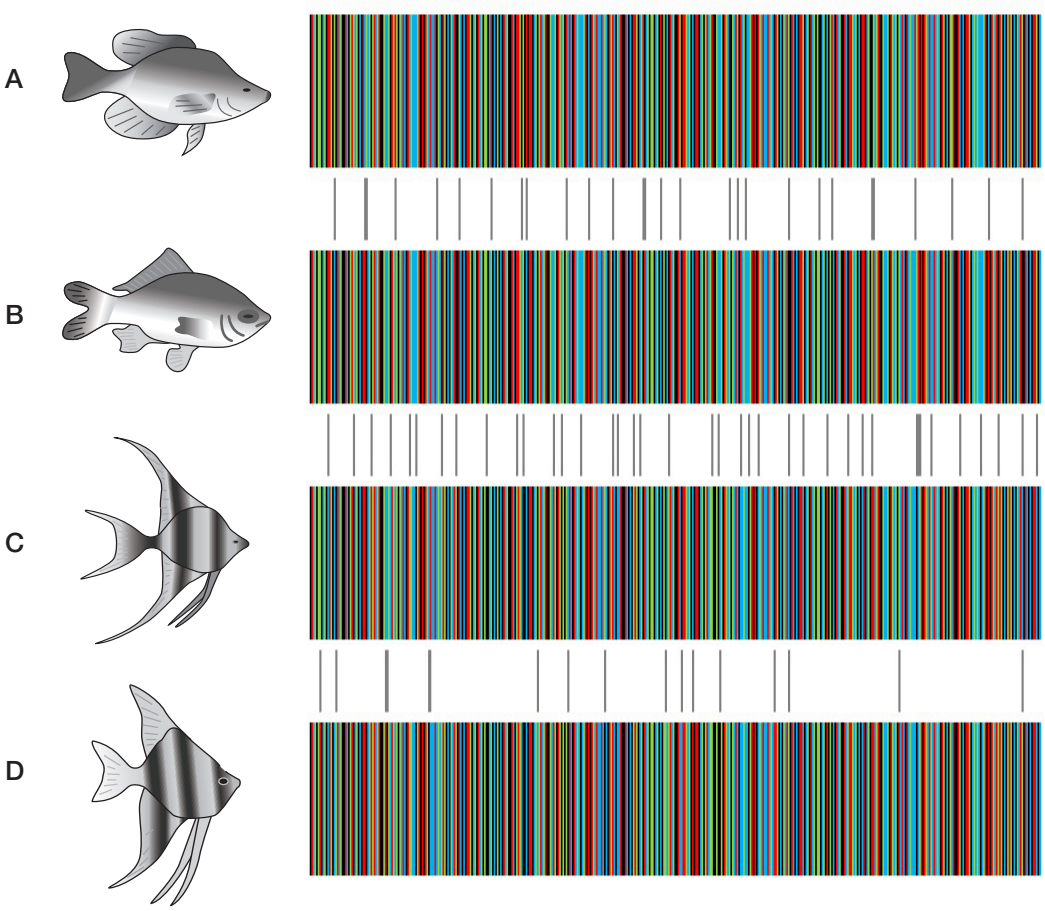


Fig. 1. Comparison of DNA barcodes. Color-coded DNA sequences serve as "DNA barcodes" for animal samples. DNA barcodes for A and B are from individuals within one genus, while the DNA barcodes for C and D are from individuals within a different genus. The grey bars interspersed between the DNA barcodes indicate nucleotide differences between the sequences. Note that there are fewer instances of nucleotide differences between members of the same genus than members of different genera.

Discrete gene loci were chosen as barcode regions for animals, plants, and fungi based on their ability to distinguish species groups within each kingdom. A 650 base pair (bp) segment of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene is the standard barcode region for animals (Stoeckle and Hebert 2008), whereas a segment of the nuclear ribosomal internal transcribed spacer region *(ITS*) is the accepted barcode region for fungi (Schoch 2012). Nucleotide sequences from two chloroplast genes — the ribulose-1,5-bisphosphate carboxylase (*rbcL*) and maturase K (*matK*) genes — are used as standard barcode regions to identify land plants (CBOL Plant Working Group 2009, Vijayan and Tsou 2010).

Table 1. Genes used to generate DNA barcodes from different taxonomic kingdoms within the Eukarya domain.

Kingdom	Gene Used for Barcode	DNA Source
Animals	Cytochrome c oxidase subunit I *(COI)*	Mitochondrion
Plants	Ribulose-1,5-bisphosphate carboxylase *(rbcL)* and maturase K *(matK)*	Chloroplast
Fungi	Nuclear ribosomal internal transcribed spacer region *(ITS)*	Nuclear ribosome

Mitochondrial DNA is an ideal choice to serve as the barcode region for animals given that sequence differences among species are much more numerous within the mitochondrial genome than in nuclear DNA. This is because mitochondrial DNA mutates at a faster rate than nuclear DNA. Additionally, the number of mitochondria can vary per cell type from just one mitochondrion to hundreds or thousands, whereas cells have only one nucleus. Thus mitochondrial DNA is more abundant than nuclear DNA in any given tissue. This facilitates recovery of more copies of the target gene from every sample.

Within the mitochondrial genome, the *COI* gene encodes subunit 1 of the cytochrome c oxidase enzyme, and it is a portion of this gene that serves as the barcode region. Cytochrome c oxidase is an enzyme found in bacteria and in mitochondria. It is the final enzyme in the electron transport chain of cellular respiration, the process by which organisms harvest energy in the form of adenosine triphosphate (ATP) from food sources.

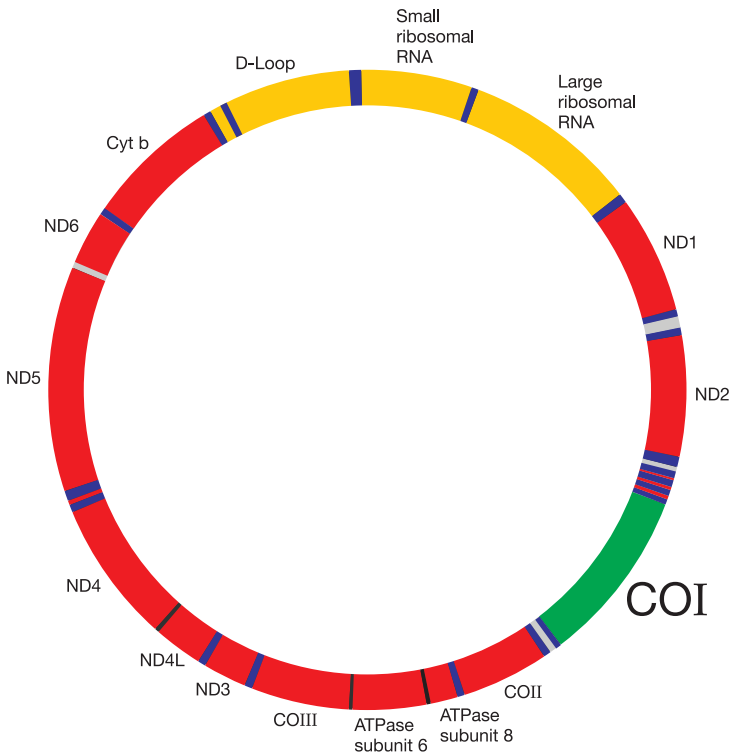


Fig. 2. Diagram of genes encoded within the mitochondrial genome. The mitochondrial genome in fish and humans is approximately 16.5 kb in length and it encodes critically important genes for oxidative phosphorylation, the process by which cellular energy is generated. The *COI* gene, a portion of which is PCR amplified in this laboratory exercise, is present within mitochondrial DNA. Mitochondrial DNA is maternally inherited in most species.

Once a DNA barcode is generated, in order to perform a barcode search it is necessary to have a searchable database that links the DNA barcodes generated by researchers from known and highly characterized biological specimens to the specimens' formal names and other important information (including data that allow the origin and current location of the source specimen to be easily tracked and verified by other researchers if necessary).

Since DNA barcodes from unknown samples will be compared against those in the database, it is critical that a) the database contains sequence data of the highest quality possible, and b) that the identity given to the species from which the reference DNA barcode is generated is assigned correctly. For example, if the code in the grocery store cash register database for button mushrooms, which are normally $2.00/lb, were accidentally switched with the code for morel mushrooms, which tend to sell for $20.00/lb, many people would be very unhappy when their button mushrooms suddenly cost ten times more than expected!

For this reason, scientists developed a highly regulated database called the Barcode of Life Data Systems (BOLD) reference library, which stores all the high-quality reference DNA barcode records. Through the BOLD Identification System (BOLD-IDS), a query (unknown or unverified) barcode sequence obtained from an unknown tissue sample or food product can be compared against reference barcode sequences contained in the BOLD reference library to determine the identity of the unknown specimen. The BOLD Systems website can be found at www.boldsystems.org.

Global Initiatives and Barcoding Applications

A particular specimen can only be identified in BOLD-IDS when reference sequences from its parent species are already represented in the BOLD reference library. The utility of DNA barcoding as a global species identification tool will therefore continue to expand as the number of reference barcodes in the BOLD database grows. At its formal launch ceremony, the International Barcode of Life (iBOL) project announced its goal to provide coverage for 500,000 species of plants and animals by 2015. To meet this landmark challenge, a global alliance of scientists has organized its barcoding activities into a number of large-scale campaigns. Each campaign coordinates the efforts of multinational teams to assemble a reference DNA barcode library for a targeted group of eukaryotic organisms. The campaigns' initial focus is on groups with the highest practical importance to humanity. Please refer to the iBOL project web page (www.ibol.org) for some examples of ongoing campaigns.

DNA Barcoding and Seafood Mislabeling

Seafood mislabeling and market substitution — the practice of incorrectly labeling or marketing seafood products — can occur at any point in the supply chain, from commercial fishing vessels to fish processing plants and commercial distribution centers to wholesale and retail fish markets to restaurants. This pervasive form of consumer fraud often conceals destructive and nonsustainable fishing activities (Jacquet 2010) and may expose consumers to potentially serious health risks (Cohen et al. 2009, Lowenstein et al. 2009).

In 2007 for example, two people in Chicago came down with symptoms of tetrodotoxin poisoning after eating soup prepared from fish purchased at a local market. Tetrodotoxin is a neurotoxin commonly found in pufferfish, the importation of which is strictly regulated by the United States Food and Drug Administration (U.S. FDA) because of the health risks associated with its consumption. Tetrodotoxin is heat stable, so it is not inactivated when tainted fish tissue is cooked. Furthermore, the minimum lethal dose of this toxin in humans is only 2 mg. Experts estimated that one of the affected persons had consumed approximately 3 mg of toxin. This person fortunately survived the bout of tetrodotoxin poisoning, but not without several weeks of rehabilitative care. The fish had been labeled headless monkfish; but DNA barcoding helped confirm that what was sold to the consumer was (potentially toxic) pufferfish, and not (nontoxic) headless monkfish, as the label had claimed (Cohen et al. 2009).

Through the coordinated efforts of an international consortium of scientists participating in the Fish Barcode of Life Initiative (FISH-BOL) and related campaigns, reference barcode records for many of the world's marine and freshwater fishes are currently represented in the BOLD reference database. As a result of this extensive coverage, BOLD-IDS is able to return accurate species identifications for many commonly available and commercially relevant fishes. Accordingly, studies of seafood mislabeling and market substitution, and their implications for food authentication, food safety, and fisheries management, represent an ideal focus for student-led inquiry.

In a 2011 market study that the media later dubbed **Sushigate**, two high school students conducted a survey of local restaurants and markets in New York City. The students collected 60 samples of fish from 14 different locations and sent the samples to be sequenced. Of the 56 samples that could be genetically identified, 13 of them (23%) were mislabeled! In quite a number of these cases, less expensive fish were represented as more expensive fish. In one instance, sushi sold as white (albacore) tuna was genetically identified as Mozambique tilapia. While the tuna sells for approximately $8.50/lb wholesale, the tilapia is only worth about $1.70/lb wholesale — quite a difference! The ability to determine the true genetic identify of the food we eat will hopefully expose fraudulent practices within the food supply chain.

More information about this wonderful example of students engaging in citizen science can be found online at phe.rockefeller.edu/barcode/sushigate.html.

Between 2008 and 2011, growing interest in DNA barcoding as a tool for detecting seafood mislabeling and market substitution motivated a number of comprehensive case studies in northeastern North America (Wong and Hanner 2008), Canada (Hanner et al. 2011), the U.S. (Daley and Abelson 2012), Spain (International Consortium of Investigative Journalists 2011), and Ireland (Miller et al. 2012). Each study involved the collection and barcode analysis of hundreds of fish specimens obtained from commercial markets, supermarkets, restaurants, and other sources. DNA barcoding revealed that 10–50% of the samples collected in these studies were mislabeled (in many cases as more expensive or desirable fish species). A number of mislabeled specimens were also identified as endangered species, underscoring the value of DNA barcoding in enforcing fishery regulations and conservation measures. In 2011, after an extensive 3-year validation study, the U.S. FDA announced its formal adoption of DNA barcoding to conduct inspections of seafood manufacturers and restaurants (U.S. FDA 2011).

Barcode Data Standards

The Fish DNA Barcoding kit and DNA Barcoding Sequencing module provide exciting new opportunities for students to conduct seafood mislabeling and market substitution studies using DNA barcoding technology. This curriculum guides students through the steps required to generate query DNA barcode sequences from fish tissue samples obtained from a variety of possible sources (supermarkets, restaurants, retail aquarium stores, etc.). Each query sequence is then analyzed, edited, and used as a search string in a customized console of BOLD-IDS to obtain the identity of the source fish species. As noted above, identifications are made by comparing a query barcode sequence to an extensive and standardized library of fish reference barcode sequences assembled through the combined activities of numerous iBOL groups.

Generating a query barcode sequence follows a relatively straightforward process. DNA is extracted from a tissue source and a ~650 bp fragment of the mitochondrial *COI* gene is amplified using PCR. The size of the resulting COI amplicon is then verified by agarose gel electrophoresis before being purified and submitted to a commercial facility for bidirectional sequencing. The sequencing facility will return two trace files containing the nucleotide sequence for the corresponding strands of the submitted DNA fragment. A suite of informatics tools is then used to analyze and edit consensus sequences assembled from the trace files.

It bears noting here that although query barcode sequences and reference barcode sequences are generated and edited in the same manner, the latter is subject to a variety of data standards established by the scientific community (Ratnasingham and Hebert 2007). For instance, a reference barcode sequence is generated from a specimen that must ultimately be deposited as a voucher in a curated collection maintained by a museum or other biorepository. If the voucher specimen represents a previously described species (as is often the case), then an expert taxonomist must verify its species name based on its morphology or provide some other form of provisional designation. The sequence of a COI reference barcode must be at least 500 nucleotides in length, contain <1% ambiguous base calls, and be devoid of stop codons, contaminating sequences, insertions, or deletions.

Reference barcode sequences are integrated into comprehensive electronic data records that contain additional mandatory information related to the source specimen and the collection event. This information includes at a minimum the original and unaltered trace files, the PCR primer sequences used to generate the reference sequence, a unique identifier for the voucher specimen and the name of the institution where it is curated, and a collection record that identifies the specimen collector, the date and location of the collection, and GPS coordinates for the collection site (Ratnasingham and Hebert 2007). These records form the basic data unit of the BOLD reference database that enables accurate species identifications to be made by its end users through BOLD-IDS. Strict compliance with data standards therefore ensures the fidelity of BOLD as a reliable species identification tool.

Engaging students in the generation of query sequences, which are exempt from these data standards, circumvents a variety of logistical and technical challenges associated with the creation of reference barcode sequences while preserving the continuity of the DNA barcoding experience. However, we anticipate that after gaining familiarity with the concepts and methods of DNA barcoding through the use of this kit, some educators may regard the experience of generating reference barcodes for the purposes of expanding the BOLD reference library as a potentially attractive extension of this curriculum, especially since students may receive authorship for the publication of reference barcode records. To assist teachers in pursuing these advanced educational endeavors, and to help them comply with current data standards, a number of scientific and educational groups operating under the auspices of the Education and Barcode of Life (eBOL) project are collaborating on the creation of advanced professional development opportunities, internet-based resources and instructional materials, and networks to facilitate the formation of educational partnerships and broaden the engagement of students in DNA barcoding.

For more information about generating reference barcodes from vouchered samples, please contact eBOL at www.EducationandBarcoding.org.

Automated DNA Sequencing Using the Sanger Procedure

DNA barcoding requires that your amplified DNA samples be sequenced. Sequencing DNA means determining the exact order of nucleotide bases, guanine (G), adenine (A), thymine (T), and cytosine (C), in a DNA molecule. DNA sequencing began in the 1970s when two research groups developed different methods for sequencing, the Maxam-Gilbert method and the Sanger method, at almost the same time. Although we take DNA sequencing for granted now and complete genomes have been sequenced in as little as a week, when researchers started sequencing DNA in the 1970s, it was a laborious process requiring the use of hazardous chemicals. After days of work, the results were relatively short sequences.

Today most researchers send their samples to core university or commercial laboratory facilities where, for a nominal charge, their samples are sequenced for them using an automated sequencer. Depending on the facility, other services may also be available. These might include cleaning up PCR reactions to remove excess unincorporated nucleotides, polymerase enzyme, and buffers so that they do not interfere with the sequencing reaction. Upon receipt of the DNA samples, many sequencing facilities can provide sequence data within 24 hours! The technology used to sequence complete genomes is rapidly evolving and while it took 13 years to sequence the first human genome, the newest technologies are allowing complete genomes to be sequenced in just a few days.

The method that will be used to sequence your fish COI PCR products is a modified and automated version of the Sanger method. The steps are outlined below.

First, a single-stranded template of the DNA to be sequenced is prepared.

Next, in a reaction tube, the DNA to be sequenced is combined with several other reagents.

- Sequencing primer — starts DNA synthesis at the area to be sequenced. Sequencing primers, like primers for PCR, must be specifically designed for each particular sequencing reaction based on the sequence of the target gene. The PCR primers used for the Fish DNA Barcoding kit are composed of a universal primer sequence and a gene-specific primer sequence. This results in PCR products with the universal primer sequence at the 5' and 3' of the finished PCR products. This allows for easy sequencing of the PCR products using standard sequencing primers
- DNA polymerase
- Nucleotides

Then, modified nucleotides called dideoxynucleotides (ddNTP, dideoxynucleotide triphosphate) are added to the reaction tube. ddNTPs lack the 3'-hydroxyl group needed for elongation of the DNA molecule. The ddNTPs are also modified so that each different type (ddATP, ddGTP, ddCTP, and ddTTP) is coupled with a different fluorescent dye. This form of Sanger sequencing is commonly called dye-terminator sequencing.

DNA synthesis is then allowed to proceed in the reaction tube. During synthesis, almost all of the nucleotides that are incorporated into the new DNA strand are standard nucleotides, not the modified ddNTPs, because the standard nucleotides are in excess. However, when a ddNTP is incorporated, DNA synthesis will stop on that strand, as there is no 3'-hydroxyl to form the next phosphodiester bond. If, for example, the ddNTP incorporated into the new DNA strand is ddATP, then that DNA fragment will end with an A and have only one fluorescent marker in it, which is attached only to the ddATP molecules.

Because the sequencing reactions are always set up with both template DNA and standard nucleotides (not the fluorescently modified ddNTPs) in excess, DNA synthesis will continue until each strand incorporates a ddNTP and synthesis stops, meaning that the sequencing reaction produces fragments of all lengths and with different labels on the ends depending on which ddNTP was the final one incorporated. If either standard nucleotides or template DNA were limiting factors in the reaction, then not all possible fragments would be produced and the sequence would be incomplete.

The different-sized DNA fragments are then separated over a capillary electrophoresis instrument equipped with lasers that can detect the fluorescence of the four different dyes, which are attached to the final ddNTP (3' end) of the DNA fragment. As the DNA fragments exit the capillary electrophoresis gel, the dyes are excited by lasers and the emitted light detected. The result is a graph called a chromatogram or electropherogram, on which the bases are represented by a sequence of colored peaks. The peak height indicates the intensity of the fluorescent signal. The automated sequencer interprets the results, assigning G, A, T, or C to each peak. If the software cannot determine which nucleotide is in a particular position, it will assign the letter N to the unknown base.

As part of the DNA barcoding workflow, you will be submitting your PCR products to a sequencing facility to be sequenced. Sequencing reactions, like PCR, rely on the basic principles of DNA replication and require primers to initiate DNA replication. However, sequencing is performed in just one direction, so instead of a primer pair, sequencing makes use of a single primer per reaction. To get stronger sequence data from each fish COI PCR product, the sample PCR product will have two sequencing reactions, one run in the forward direction (from the beginning of the *COI* gene) and a second sequencing reaction in the reverse direction. These primers match part of the sequence that was included in the PCR primers that are not part of the actual *COI* gene. Ideally, these sequencing reactions will yield the same results, except that one sequence will be the reverse complement sequence of the other.

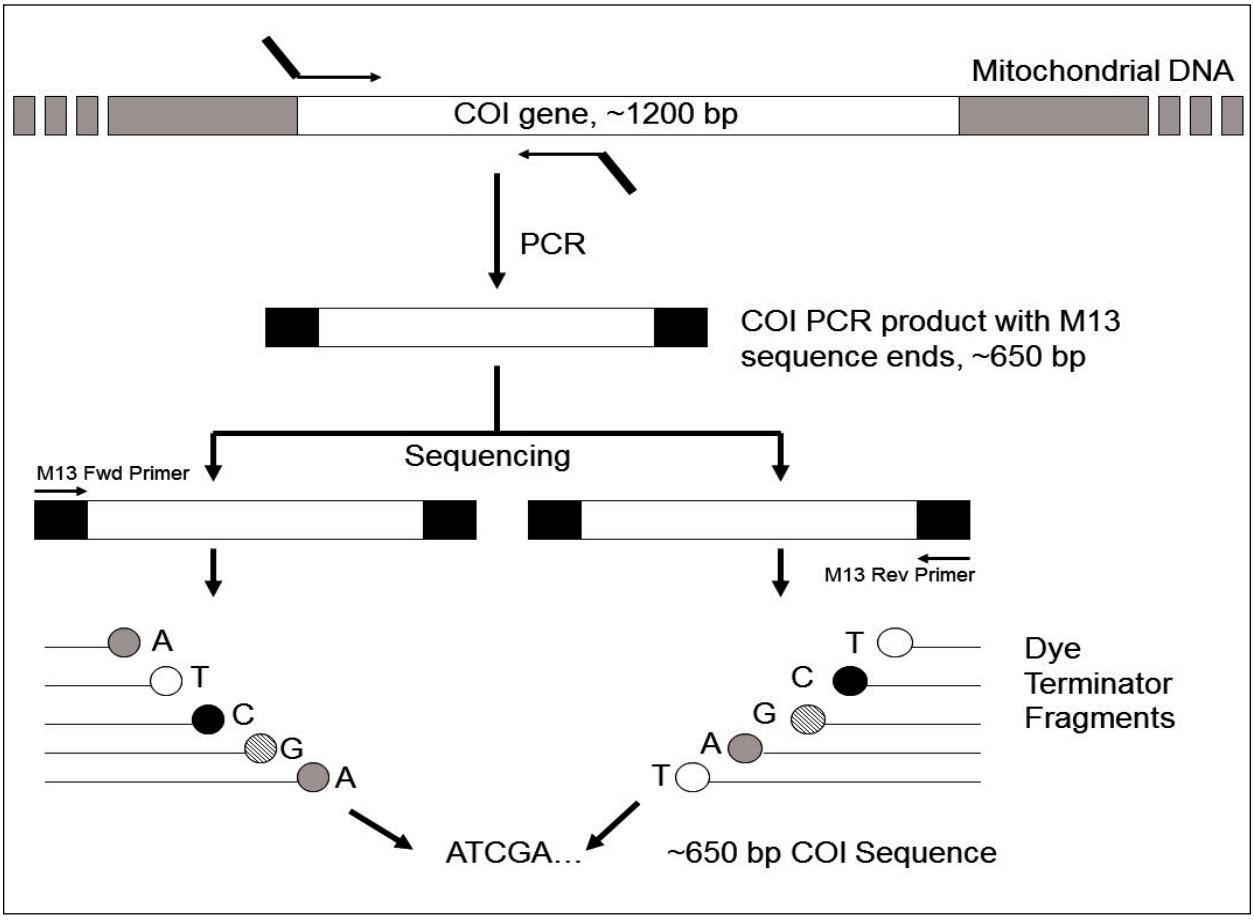


Fig. 3. During PCR, the mitochondrial *COI* gene (represented by the white bar in the mitochondrial DNA) is amplified with fish primer mix (hinged arrows). These primers contain a sequence that matches the *COI* gene (thin part of primer arrow), as well as a sequence derived from M13 DNA (thick part of primer arrow). Though the M13 sequences will not bind the initial COI template, they will be incorporated into the PCR product (depicted as black sections on the ends of the PCR product). These regions will be binding sites for the M13 sequencing primers during DNA sequencing.

It should be noted that since DNA sequencing operates on the same principles of DNA replication that PCR does, any remaining unreacted reagents from PCR can interfere with the sequencing reactions. These reagents can include nucleotides, PCR primers, Taq polymerase, and buffer. Before running sequencing reactions, these reagents are removed using size exclusion chromatography. Size exclusion chromatography is a method of separation that allows very large molecules to run through a resin while very small molecules are retained (or vice versa). This means that the large PCR product can be collected while unreacted nucleotides, unreacted PCR primers, and Taq polymerase will remain on the column. Some sequencing facilities will perform these purifications for you while others require PCR products to be cleaned before they are submitted.

References

CBOL Plant Working Group (2009). A DNA barcode for land plants. Proc Natl Acad Sci USA 106, 12794–12797.

Cohen NJ et al. (2009). Public health response to puffer fish (Tetrodotoxin) poisoning from mislabeled product. J Food Prot 72, 810–817.

Daley B and Abelson J (Dec. 2, 2012). Accountability lost in murky fish supply chain. Boston Globe, www.bostonglobe.com/business/specials/fish, accessed March 6, 2013.

Hanner R et al. (2011). FISH-BOL and seafood identification: Geographically dispersed case studies reveal systemic market substitution across Canada. Mitochondrial DNA 22(S1), 106–122.

Hebert PDN et al. (2003). Biological identifications through DNA barcodes. Proc Biol Soc 270, 313–321.

International Consortium of Investigative Journalists (Oct. 6, 2011). Hake DNA testing: How we did it. Center for Public Integrity, www.publicintegrity.org/2011/10/06/6862/hake-dna-testing-how-we-did-it, accessed March 6, 2013.

Jacquet J et al. (2010). Seafood stewardship in crisis. Nature 467, 28–29.

Lowenstein JH et al. (2009). The real maccoyii: identifying tuna sushi with DNA barcodes – contrasting characteristic attributes and genetic distances. PLoS ONE 4, e7866.

Miller D et al. (2012). Seafood mislabelling: comparisons of two western European case studies assist in defining influencing factors, mechanisms and motives. Fish and Fisheries 13, 345–358.

Ratnasingham S and Hebert PDN (2007). The barcode of life data system (www.barcodinglife.org). Mol Ecol Notes 7, 355–364.

Stoeckle MY and Hebert PDN (2008). Barcode of life. Sci Am 299, 82–86.

Schoch CL et al. (2012). Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109, 6241–6246.

U.S. FDA (last updated Oct. 28, 2011). DNA-Based Seafood Identification. www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/DNAspeciation/default.htm, accessed March 6, 2013.

Vijayan K and Tsou CH (2010). DNA barcoding in plants: taxonomy in a new perspective. Current Science 99, 1530–1541.

Wong EHK and Hanner RH (2008). DNA barcoding detects market substitution in North American seafood. Food Research International 41, 828–837.

Tips and Helpful Hints

Skills Students Need to Perform This Laboratory

The Fish DNA Barcoding kit assumes students and instructors have:

- basic molecular biology skills
- sterile technique skills
- proficiency in loading and running agarose gels
- proficiency in micropipetting with care, consistency, and accuracy

Given the complexity of the sample preparation workflow and the variations on basic PCR that are utilized in this kit, **this is not an ideal laboratory to introduce students to PCR**. Bio-Rad's Biotechnology Explorer program has a full range of kits to help teach basic skills prior to undertaking an advanced PCR laboratory such as this.

Setting Class Expectations for Success

Before starting the Fish DNA Barcoding kit workflow, it is important for the instructor to help the students understand what the curriculum involves and set expectations for the type of results they may experience along the way. This kit was not created to be a demonstration laboratory with guaranteed results for all samples, and thus may be different from labs that some students may be accustomed to performing. The Fish DNA Barcoding kit takes learning to the next level; the curriculum is an open-ended experience involving novel and real experiments. Great attention has been given to the design of the experiments with respect to controls and the creation of robust protocols to help ensure success. Specific controls have been added to the experiments to not only enable accurate interpretation of results, but also to provide a safety net so that students who do not achieve adequate results can continue with the known control and complete the workflow.

Given the sensitivity of PCR in detecting any and all DNA that is present and amplifiable by the primers used, it is of the **utmost importance to ensure that each individual fish sample is processed using a new razor blade, plastic knife, or cutting utensil**. Sequencing results in this laboratory will be of low quality if the same cutting utensil is used on several samples of fish; the negative effect on species identification will be significant. This is because DNA from several fish will be present and amplified in the PCR reactions, and thus the correct nucleotide identity at any given position during sequencing will be in question. Washing a single cutting utensil between each use is not advisable, given safety concerns with manipulating sharp, wet objects. Further, simply wiping the utensil with a towel or rinsing with ethanol after each use will not remove any DNA that is already present. See Appendix B for additional tips on sterile technique.

Note that while bacteria do not contain mitochondria, they do have a *COI* gene. Therefore, it is possible to inadvertently coamplify DNA from any bacteria present on your fish tissue during DNA extraction and PCR. Should this occur, it can be an excellent talking point about safeguards during food processing and storage, as well as the power of PCR as a detection method. To minimize possible contamination of fish DNA samples with bacterial DNA, use gloves when handling fish tissue. It is also helpful to take a piece of tissue from the interior of a tissue sample instead of the surface whenever possible to decrease the chance of amplifying surface contaminants that may be present.

Advice on Which Fish Species to Choose

The primer mix included in the Fish DNA Barcoding kit has been designed to allow amplification during PCR of a COI DNA sequence from the majority of fish, which is the step required to generate sufficient DNA for sequencing. However, though the COI protein has critical metabolic functions, there is a good deal of variation in its amino acid composition and therefore DNA sequence. This may result in some fish species having DNA that is poorly amplified or not amplified at all with the primers provided. In order to increase the odds of successful PCR amplification of the COI region from your fish DNA sample, the fish primer mix contains two sets of forward and reverse PCR primers instead of the single set used in a typical PCR reaction. Additionally, one of these primers contains several degenerate nucleotide positions, further increasing the chances of primer binding to more divergent DNA sequences. For more information on degenerate primers, see Appendix C.

The question of which type of fish sample (fresh, frozen, dried, canned, etc.) is an important one, and will impact the results obtained in this laboratory. While excellent results can be obtained with fresh, frozen, and dried fish, canned or processed fish items should be avoided as acidic conditions present during the canning process can damage DNA. High fat content (fried fish, etc.) may also inhibit DNA extraction and subsequent PCR.

Table 2. Fish samples that reliably yield mitochondrial DNA that is robustly amplified by the fish primer mix.

Very Robust	Less Robust	Difficult/Not Robust
Dried fish	Salmon	Fried fish
Catfish	Salmon roe	Canned fish
Shark	Anchovy	Sea urchin
Sturgeon	Arctic char	Red tuna
Trout	Sardine	Mussel
Rock cod		Clam
True cod		
Tilapia		
Mackerel		
Yellowfin tuna		
Sea bass		
Opah		
Imitation crab		
Flying fish roe		
Bonito flakes		

An intriguing way to introduce additional inquiry into the Fish DNA Barcoding kit laboratory is to utilize samples from different organs of a whole fish to determine which sources of tissue produce the best results. For example, muscle and gill tissues both produce very robust amplification of mitochondrial DNA. What about fins or scales?

Timeline

The entire investigation requires a minimum of four 50-minute laboratory periods or two 90-minute block lessons and one 50-minute computer laboratory period for bioinformatics analysis. Be aware that an additional 4-hour thermal cycling period is needed outside of class time. We also recommend 2–3 days of background review and lectures to prepare your students for the exercise and 1–2 days for bioinformatics sequencing data to be returned after samples are received.

Prior to Lab

- Read manual (2 hr)
- Purchase fish samples from grocery store (as needed)
- Inventory required accessories (1 hr)
- Perform instructor's advance preparation (30 min–3 hr each lab)
- Set up student workstations (30 min–1 hr each lab)

50-Minute Lessons

- Lesson 1: DNA extraction (50 min)
- Lesson 2: Set up PCR reactions (and pour gels) (50 min)
- Run PCR reactions (4 hr) — typically overnight
- Lesson 3: Gel electrophoresis (50 min)
- Lesson 4: DNA sequencing
- Wait for sequencing reaction results — 3–5 days
- Lesson 5: Bioinformatics (50 min)

Suggested Background Reading

DNA Barcoding Blog
dna-barcoding.blogspot.com

SushiGate
phe.rockefeller.edu/barcode/sushigate.html

International Barcode of Life
ibol.org

Safety Issues

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Wearing protective eyewear and gloves is strongly recommended. Students should wash their hands with soap before and after this exercise. If any solution gets into a student's eyes, flush with water for 15 minutes. Lab coats or other protective clothing should be worn to avoid staining clothes.

Instructor's Advance Preparation

This section describes preparation the instructor must perform prior to each laboratory period. If block periods are used, prepare for Lessons 1-2 and Lessons 3-4 at the same time. An estimate of preparation time is included.

Lesson 1: Extraction of DNA from Fish Samples

The crux of this lab is the quality and quantity of DNA extracted from your fish samples. The table on page 16 lists the reliability of different seafood samples with regard to DNA extraction and PCR amplification of the *COI* gene using the primers included in this kit; the less reliable sources may produce less robust PCR products and poorer DNA sequence. If you wish your students to have good success, use the recommended fish samples. If you want your students to have a full inquiry-based experience, using fish samples that have not been tested or that are in processed foods can be an open-ended experience.

Note: It is critical that a new cutting implement be used for each fish sample. PCR involves the amplification of DNA. If DNA from even a single cell of a contaminating sample is present due to recycling of cutting implements, pipet tips, containers, etc., it can be amplified and thus significantly affect the quality of your sequencing results and preclude definitive species identification. Wearing gloves when handling fish is recommended, as is changing gloves between specimens. Wrapping hands in plastic wrap would also work if gloves are not available.

Note: The sterile water included in the Fish DNA Barcoding kit is for setup of PCR reactions and gel electrophoresis samples only. Distilled water (supplied by the teacher) is to be used for elution of extracted DNA samples in Lesson 1.

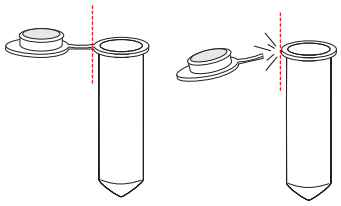
Note: Once the lysis buffer is added during DNA extraction, there is no convenient stopping point until the fish DNA is eluted from the spin purification column. If time is limited, the instructor can have the students process the fish on day 1 by cutting the fish pieces, depositing each piece into a 2 ml microcentifuge tube labeled with unique numbers for each student group, and adding resuspension buffer to the samples. The samples can then be stored overnight at 4°C. Students would then continue the extraction procedure at step 2 in the quick guide during the next lab period. Alternatively, the instructor can perform the fish preparation, although this is not the preferred method as it takes some of the sample ownership from the students and could decrease their level of engagement.

Materials Needed for Advance Preparation	Quantity	(✓)
Resuspension solution, 5 ml	1 bottle	☐
Lysis solution, 5 ml	1 bottle	☐
Neutralization solution, 5 ml	1 bottle	☐
Spin columns	16	☐
Matrix, 5 ml	1 bottle	☐
Wash buffer, 10 ml	1 bottle	☐
2 ml microcentrifuge tubes	96	☐

Materials Needed but Not Supplied	Quantity	(✓)
Ethanol, 95%	10 ml	☐
Graduated cylinder (to measure ethanol)	1	☐
Fish samples (if not provided by students)	8–16	☐
Razor blades, plastic knives, or other cutting implements (one per fish sample), new and unused	16	☐
Weigh boats	16	☐
Microcentrifuge (capable of 14,000 x g)	1–2	☐
Water bath or heat block set at 55°C	1	☐
100–1,000 µl adjustable-volume micropipet and tips	1	☐
Distilled water	5 ml	☐
Marking pens	8	☐

Procedure (estimated time: 1 hr)

1. Add 10 ml of 95% ethanol to the 10 ml of wash buffer provided with the kit.
2. Label eight 2 ml microcentrifuge tubes **Resuspension** and add 500 µl resuspension solution to each tube.
3. Label eight 2 ml microcentrifuge tubes **Lysis** and add 600 µl lysis solution to each tube.
4. Label eight 2 ml microcentrifuge tubes **Neutralization** and add 600 µl neutralization solution to each tube.
5. Label eight 2 ml microcentrifuge tubes **Matrix**. Vortex or thoroughly shake the matrix before aliquoting into the tubes. Add 500 µl of fully resuspended matrix to each tube.
6. Label eight 2 ml microcentrifuge tubes **Wash**. Ethanol should already have been added to the wash buffer. Add 2 ml of wash buffer to each tube.
7. Label eight 2 ml microcentrifuge tubes **Distilled Water**. Add 300 µl distilled water to each tube.
8. Cut caps off of thirty-two 2 ml microtubes.



9. Set the water bath or heat block to 55°C at least 30 min before the lab.
10. Set up the student workstations.
11. Set up the common workstation.

Student Workstation

Materials	Quantity	(✓)
2 ml tube with resuspension solution	1	☐
2 ml tube with lysis solution	1	☐
2 ml tube with neutralization solution	1	☐
2 ml tube with matrix	1	☐
2 ml tube with wash buffer	1	☐
2 ml tube with distilled water	1	☐
Fish samples, each in a separate weigh boat	2	☐
Razor blades, plastic knives, or other cutting implements (one per fish sample), new and unused	2	☐
Spin columns (discard caps, they will not be used)	2	☐
Empty 2 ml microcentrifuge tubes, caps cut off	4	☐
Empty 2 ml microcentrifuge tubes, caps still on	4	☐
100–1,000 µl adjustable-volume micropipet and tips	1	☐
Marking pen	1	☐

Common Workstation

Materials	Quantity	(✓)
Water bath or dry bath set to 55°C	1	☐
Microcentrifuge	2–3	☐

Lesson 2: Set Up PCR Reactions

Materials Needed for Advance Preparation	Quantity	(✓)
PCR tubes	32	☐
Sterile water (not distilled)	1 bottle	☐
2x master mix for PCR	1 vial	☐
Fish primer mix	1 vial	☐
pCOI control for PCR	1 vial	☐
Student samples from previous lab	2 tubes/station	☐

Materials Needed but Not Supplied	Quantity	(✓)
Ice baths or beakers with ice	8	☐
2–20 µl adjustable-volume micropipets	8	☐
2–20 µl pipet tips, aerosol barrier	8 racks	☐
20–200 µl adjustable-volume micropipets	8	☐
20–200 µl pipet tips, aerosol barrier	8 racks	☐
100–1,000 µl adjustable-volume micropipets	1	☐
100–1,000 µl pipet tips	1 rack	☐
Marking pens	8	☐

Procedure (estimated time: 1 hr)

Note: Add the primers to the 2x master mix for PCR and aliquot only 30 min before the lesson starts; store prepared master mix on ice.

1. Thaw the pCOI positive control for PCR and pulse-spin the tube in a centrifuge to bring all contents to the bottom. Label a 2 ml microcentrifuge tube **pCOI**. Add 20 µl of pCOI positive control DNA template to this tube.
2. Add 80 µl sterile water to the DNA in the 2 ml tube labeled **pCOI** and mix.
3. Label 8 clean 2 ml microcentrifuge tubes with a **plus sign (+).** Add 10 µl of the diluted positive control DNA from the tube labeled **pCOI** to each of the + tubes.

   This can be prepared ahead of time and stored at –20°C for 1–2 months if necessary.
4. Label eight 2 ml microcentrifuge tubes with a **minus sign (–)** and add 10 µl of sterile water to each tube.
5. Thaw the master mix and primers, mix by vortexing or flicking the tubes, and pulse-spin the tubes in a centrifuge to bring all contents to the bottom. Keep the tubes on ice.
6. Label one 2 ml microcentrifuge tube **RM** (reaction mix).
7. Add 560 µl of sterile water to the tube labeled **RM**.
8. Add 800 µl of 2x Master mix for PCR to the water in the **RM** tube and mix.
9. Add 40 µl of fish primer mix (green in color) to the master mix and water in the **RM** tube and mix. Store on ice.

   Ensure primers, master mix, and water are fully mixed and the green color of the reaction mix is uniform before proceeding.
10. Label eight clean 2 ml microcentrifuge tubes **CMM** (COI master mix). Add 150 µl of the reaction mix to each of the tubes labeled **CMM**.

11. Set up the student workstations.

Student Workstation

Materials	Quantity	(✓)
Ice bath	1	☐
Fish DNA Samples from Lesson 1	2	☐
(+) sample	1	☐
(–) sample	1	☐
CMM reaction mix	1	☐
PCR tubes	4	☐
2–20 µl adjustable-volume micropipet	1	☐
20–200 µl adjustable-volume micropipet	1	☐
2–20 µl pipet tips, aerosol barrier	1 rack	☐
20–200 µl pipet tips, aerosol barrier	1 rack	☐
Marking pen	1	☐

12. Program the thermal cycler (refer to Appendix D to program Bio-Rad's T100™ thermal cycler):

Initial denaturation	94°C	2 min
Then 35 cycles of:		
Denaturation	94°C	30 sec
Annealing	55°C	2 min
Extension	72°C	1 min
Final extension	72°C	10 min
Hold	4°C	(∞)

Preparing PCR Samples for Electrophoresis and Sequencing

Procedure (estimated time: 15 min)

Note: The students should have 40 µl PCR reactions, which will allow 5 µl to be visualized by gel electrophoresis and 30 µl to be sent for sequencing. If there is less volume than anticipated, 20 µl is the minimum amount that should be sent for sequencing.

1. Set up the student workstations.

Student Workstation

Materials	Quantity	(✓)
PCR products from previous lesson (Fish 1, Fish 2, (+), (-))	4	☐
2 ml microcentrifuge tubes	7	☐
2–20 µl adjustable-volume micropipet	1	☐
20–200 µl adjustable-volume micropipet	1	☐
2–20 µl pipet tips, aerosol barrier	1 rack	☐
20–200 µl pipet tips, aerosol barrier	1 rack	☐
Marking pen	1	☐

Lesson 3: Electrophoresis of PCR Products

Note: The UView 6x loading dye included with this kit contains a fluorescent compound that binds to DNA and allows immediate visualization of your samples with UV light (optimal wavelength 365 nm) after electrophoresis. No additional staining is required. UView 6x loading dye is nontoxic and may be disposed of according to your state's regulatory requirements. If you do not have a UV light source or would prefer to use a visual DNA stain, such as Fast Blast DNA stain (166-0420EDU), you can still do so following the use of UView 6x loading dye. However, please note that the sensitivity of UView is significantly greater than that of Fast Blast DNA stain, and PCR products may indeed be present but not detectable with Fast Blast DNA stain. Please see Appendix F for more information on Fast Blast DNA stain staining protocols.

The use of protective eye glasses, mask, and gloves is strongly recommended when operating or in the vicinity of a UV transilluminator or any ultraviolet light source.

Materials Needed for Advance Preparation	Quantity	(✓)
UView 6x loading dye	1 vial	☐
PCR molecular weight ruler	1 vial	☐
Sterile water	1 bottle	☐
PCR products for electrophoresis from previous lesson	4 per group	☐
2 ml microcentrifuge tubes	24	☐

Materials Needed but Not Supplied	Quantity	(✓)
2–20 µl adjustable-volume micropipets	8	☐
2–20 µl pipet tips, aerosol barrier	8 racks	☐
50x TAE	1 bottle	☐
Molecular biology grade agarose	1 bottle	☐
Graduated cylinders, 3 L and 500 ml	1	☐
Microwave or magnetic hot plate and stir bar	1	☐
Bottle or Erlenmeyer flask, 1 L	1	☐
Water bath at 60°C (optional)	1	☐
Gel casting trays	8	☐
Gel combs	8	☐
Lab tape for gel casting (optional)	1 roll	☐
Horizontal electrophoresis chamber	8	☐
UV transilluminator or imaging system	1	☐
Marking pens	8	☐
Power supply	2–4	☐

Procedure (estimated time: 1–3 hr)

1. Thaw the PCR molecular weight ruler and pulse-spin the tube in a centrifuge to bring all contents to the bottom.
2. Add 50 µl of UView 6x loading dye to the vial of PCR molecular weight ruler. Mix well and pulse-spin.
3. Label eight 2 ml microcentrifuge tubes **MWR** and add 25 µl of PCR molecular weight ruler to each. This can be prepared ahead of time and stored at 4°C for 1–2 months.
4. Label eight 2 ml microcentrifuge tubes **Loading Dye** and add 15 µl of UView 6x loading dye to each tube.
5. Label eight 2 ml microcentrifuge tubes **Sterile Water** and add 40 µl of sterile water to each tube.

Preparation of Agarose Gels and TAE Running Buffer

These procedures may be carried out 1–2 days ahead of time by the teacher or during class by individual student teams. Note: Convenient precast 1% agarose gels (161-3015EDU) and 50x TAE buffer (100 ml bottle, 166-0742EDU) are available from Bio-Rad. The recommended gel concentration for this application is 1% agarose. This agarose concentration provides excellent resolution and minimizes run time required for electrophoretic separation of PCR fragments.

Note: To save time, the preferred method of gel electrophoresis is to run gels at 200 V for 20 min in 0.25x TAE electrophoresis buffer. If you would rather run gels at a lower voltage for any reason, gels can be run at 100 V for 30 min in 1x TAE electrophoresis buffer. Be sure to make **1x TAE at step 7** (same recipe as in step 1) if this is your preferred method.

1. Prepare 1x TAE gel buffer. 0.5 L of 1x TAE is sufficient to make 8 agarose gels. To make 0.5 L of 1x TAE, add 10 ml of 50x concentrate to 490 ml of distilled water.
2. Make the agarose solution. To make a 1% solution, add 1 g of agarose powder per 100 ml of 1x TAE gel buffer in a heatproof container large enough to accommodate vigorous boiling (1,000 ml Erlenmeyer flask, Wheaton bottle, etc.). For 8 gels, you will need approximately 400 ml of molten agarose (4 g agarose plus 400 ml 1x TAE buffer). The agarose must be made using **TAE** gel buffer. Swirl to suspend the agarose powder in the buffer.

   Note: If using an Erlenmeyer flask, invert a 50 ml Erlenmeyer flask into the open end of the 1,000 ml Erlenmeyer flask containing the agarose. The small flask acts as a reflux chamber, allowing boiling without much loss of buffer volume by evaporation. The agarose can be melted for gel casting on a magnetic hot plate or in a microwave oven.

   Caution: Use protective gloves, oven mitts, goggles, and lab coats as appropriate while preparing and casting agarose gels. Contact with boiling, molten agarose or the vessels containing hot agarose can cause severe burns.

   Magnetic hot plate method. Add a stirbar to the flask containing agarose and buffer. Heat the mixture to boiling while stirring on a magnetic hot plate. Bubbles or foam should break before rising to the neck of the flask. Boil the solution until all of the small transparent agarose particles are dissolved. With the small flask still in place, set aside the agarose to cool to 60°C before pouring gels (a water bath set to 60°C is useful for this step).

   Microwave oven method (preferred). Place the flask or bottle containing the agarose solution into the microwave oven. Loosen the bottle cap if present. Use a medium setting and set to 3 min. Stop the microwave oven every 30 sec and swirl the flask to redistribute any undissolved agarose. This technique is the most efficient way to dissolve agarose. Alternate boiling and swirling the solution until all of the small transparent agarose particles are dissolved. With the small flask or bottle cap still in place, set aside to cool to 60°C before pouring (a water bath set to 60°C is useful for this step).

3. While agarose is cooling, place a gel comb into the appropriate slots of your gel casting tray. Gel combs should be within 2 cm of the end of the gel casting tray.
4. Pour 30–50 ml of molten agarose into the tray to a depth of approximately 0.5 cm.
5. Allow the gel to solidify at room temperature for at least 30 min.
6. Carefully remove the comb from the solidified gel. Gels can be stored wrapped in plastic wrap, sealed in plastic bags, or submerged in 1x TAE buffer for up to 2 weeks at 4°C.
7. Prepare 2.5 L of 0.25x TAE electrophoresis buffer by mixing 12.5 ml of 50x TAE with 2,487.5 ml distilled water. Mix thoroughly. 2.5 L is sufficient to run 8 agarose gels.
8. Insert the agarose gel into the electrophoresis tank and fill the tank with 0.25x TAE electrophoresis buffer until it just covers the top of the gel.
9. Set up student workstations.

Student Workstation

Materials	Quantity	(✓)
Samples labeled **E** for Electrophoresis from Lesson 2	4	☐
2 ml tube containing molecular weight ruler	1	☐
2 ml tube containing UView 6x loading dye	1	☐
2 ml tube containing sterile water	1	☐
2–20 µl adjustable-volume micropipets	1	☐
2–20 µl pipet tips, aerosol barrier	1 rack	☐
1% agarose gel	1	☐
Running buffer	250 ml	☐
Gel electrophoresis chamber	1	☐
Power supply (may be shared by multiple groups)	1	☐
Marking pen	1	☐

Common Workstation

Materials	Quantity	(✓)
UV transilluminator or imaging system	1	☐

Lesson 4: Sequencing

In order to sequence the PCR products, the remaining reagents (unreacted nucleotides, primers, Taq polymerase, and buffer) that were not used up in the PCR reaction need to be removed first. If you are using the Bio-Rad DNA Barcoding Sequencing module (166-5115EDU, U.S. customers only), both the cleanup and sequencing steps will be performed by Sequetech. Sequetech will also provide the forward and reverse sequencing primers. Therefore, all that is necessary is to place an order for sequencing on Sequetech's website and ship the samples with a printed copy of the order summary to Sequetech for processing, using the shipping envelope and shipping label provided in the DNA Barcoding Sequencing module. Please refer to the instruction manual in the DNA Barcoding Sequencing module for complete instructions.

Note: It is very important to retain the outer envelope (with yellow Bio-Rad label) of the DNA Barcoding Sequencing module kit. You will need the serial number located on the yellow Bio-Rad label to place your sequencing order with Sequetech.

However, if you plan to sequence the PCR products at a local university or a different commercial facility, you will need to check with that facility to determine if they expect the PCR products to have been cleaned up first and also if they need you to provide sequencing primers. Each sequencing facility also has different requirements for receiving samples. Instructions for sample submission can be obtained from the facility itself and are usually available on the facility's website.

Differences in requirements may include:

- Concentration of primer
- Volume of primer
- Concentration of PCR product
- Volume of PCR product
- Premixing of primer and PCR product
- Sample shipping
- Availability of PCR cleanup as a service

If the facility does not provide PCR product cleanup, a separate activity can be run by your students to remove the unreacted PCR primers, unreacted dNTPs, Taq polymerase, and buffer using Bio-Rad PCR Kleen™ spin columns (732-6300EDU for 25 columns). If your facility does not provide sequencing primers, the DNA sequences are M13 forward (TGTAAAACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGA) for sequencing the fish DNA barcoding PCR products. Primers can be ordered from vendors such as Integrated DNA Technologies.

The Canadian Centre for DNA Barcoding (CCDB) has been validated as a sequencing provider for use by Canadian customers. If you are interested in having your PCR products sequenced at their facility, please contact the CCDB directly to make your own arrangements for ordering their services, shipping your samples, and making payments directly to the CCDB for their services.

Canadian Centre for DNA Barcoding
Biodiversity Institute of Ontario
University of Guelph
50 Stone Road East
Guelph, ON, Canada N1G 2W1
ccdb.ca
Email: info@ccdb.ca
Phone: (519) 824-4120 ext. 56393
Fax: (519) 824-5703

Sequencing Checklist

DNA Barcoding Sequencing Module	(✓)
FedEx shipping slip/billable stamp	☐
FedEx mailing envelope	☐
Sequencing ordering instructions	☐
Serial number for service redemption (located on module's yellow label)	☐

Components needed but not included in the kits	(✓)
Parafilm	☐
Zipper sealed bag for mailing sequencing samples	☐

Tasks to Perform Prior to the Laboratory

1. Fill out the order form for Sequetech on Sequetech's website: www.sequetech.com. Complete instructions are included with the DNA Barcoding Sequencing module.
2. For educators who have not purchased the DNA Barcoding Sequencing module and are using their own sequencing facility
   - Locate sequencing facility
   - Determine if you need to clean up the PCR samples before shipping them to the facility and whether the facility provides sequencing primers or not
   - If necessary, clean up PCR samples using the Bio-Rad PCR Kleen spin column kit (732-6300EDU) or comparable materials
   - Determine the required format of the samples (PCR template concentration and primer concentration and what buffer it needs to be in as well as submission in tube or microtiter plate format)
   - If necessary, purchase sequencing primers and reconstitute in the appropriate buffer at the appropriate concentration

Materials Needed for Each Workstation	Quantity	(✓)
Fish 1 SEQ tube from Lesson 3	1	☐
Fish 2 SEQ tube from Lesson 3	1	☐
(+) control SEQ tube from Lesson 3	1	☐

Materials Needed but Not Provided	Quantity	(✓)
Marking pens	8	☐
Parafilm	1 roll	☐
Zipper sealed bag	1	☐

Procedure (estimated time: 15 min)

1. Set up student workstations.

Student Workstation

Materials	Quantity	(✓)
SEQ samples (Fish 1, Fish 2, (+))	3	☐
Parafilm	3 pieces	☐
Marking pens	1	☐

Lesson 5: Bioinformatics

Download and refer to bulletin 6398 at www.bio-rad.com/fishbarcoding for full instructions to perform the bioinformatics portion of this kit.

Materials Needed but Not Provided	Quantity	(✓)
Forward and reverse sequencing files for samples	varies	☐
Computer with Internet access	8	☐

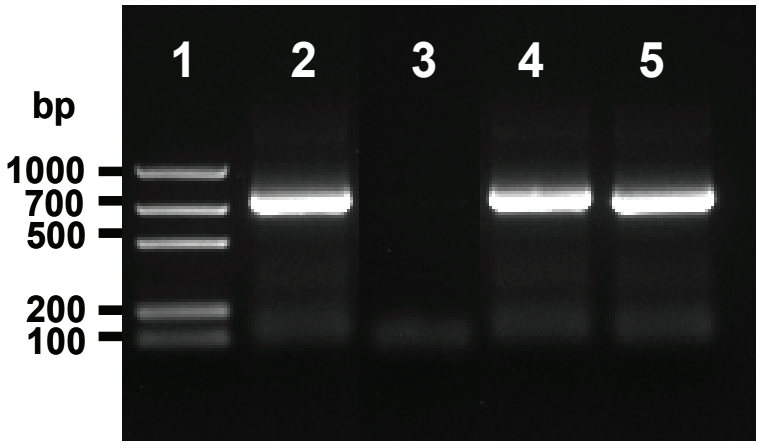
Typical Classroom Results

The lane corresponding to the positive control pCOI PCR should contain a 650 bp band. The presence of this band indicates whether a particular sample contained DNA that was amplifiable by *COI* gene–specific primers. If this lane does not contain a 650 bp band, it could be an indication that the PCR reactions were not assembled properly or that the thermal cycler was not functioning properly.

The negative control should not contain a 650 bp band. If one is present, this indicates the PCR reaction(s) became contaminated at some point during processing and that your sequencing results may be negatively impacted due to the presence of additional contaminating DNA in your samples.

The lanes with your amplified fish DNA should contain a 650 bp band that corresponds to the amplified *COI* gene, which you will go on to sequence. If there is no band present in some or all of these samples but the positive control COI PCR worked, this is an indication that something may have gone wrong during your DNA extraction procedure or that the type of fish tissue sample chosen was not optimal for this protocol. You can proceed to have the COI positive control samples sequenced and go through the bioinformatics lesson with these sequencing results. You should do so even when all student samples perform well, as the positive control DNA should yield a known result. The species identity of the positive control DNA is *Sebastes goodei*, or chilipepper rockfish.

In some or all lanes containing PCR products you may notice a faint band around 100 bp in size. This band corresponds to low level primer dimer formation during PCR.



Lane 1: PCR MW ruler

Lane 2: pCOI positive control PCR

Lane 3: Negative control PCR

Lane 4: Fish 1 PCR

Lane 5: Fish 2 PCR

Student Manual

Background

What Is DNA Barcoding?

Have you ever ordered a California roll at a sushi counter and wondered exactly what sort of seafood made up the “imitation crab” in your meal? Or have you ever been to a seafood restaurant in New England and ordered scrod and wondered what fish you were getting? Once a piece of seafood has been processed and filleted, it can be difficult to tell what species the fish is. Even if the fish was caught in the wild and not purchased from the grocery store, after processing it is sometimes difficult to identify fish species based on physical characteristics alone. The method of grouping organisms according to common physical characteristics, known as Linnaean taxonomy, has been around for 250 years and has long been the standard method of species identification. But that does not mean this method is always easy or accurate.

Not only is identification of fish species that we eat important, but so is the identification of all marine species and the classification of new species. The deep sea has been called the last frontier. It is estimated that of all the species that exist in the marine environment, possibly only one third of them have been identified.

An explosion of quick and inexpensive methods to isolate, purify, amplify, and sequence DNA has brought new methods to help identify different species; whether they are fish sold at the market or newly discovered species. Using DNA-based technologies, a multinational alliance of scientists is now cataloging life using a DNA barcoding system in order to accelerate the discovery of new species and develop powerful new tools to monitor and preserve Earth’s vanishing biodiversity.

In much the same way that a UPC (universal product code) barcode can differentiate a carton of milk from a bag of carrots when they are scanned into the cash register at a grocery store, DNA sequences can be used to uniquely identify different species. This is the basis of DNA barcoding.

DNA Barcoding and Seafood Mislabeling

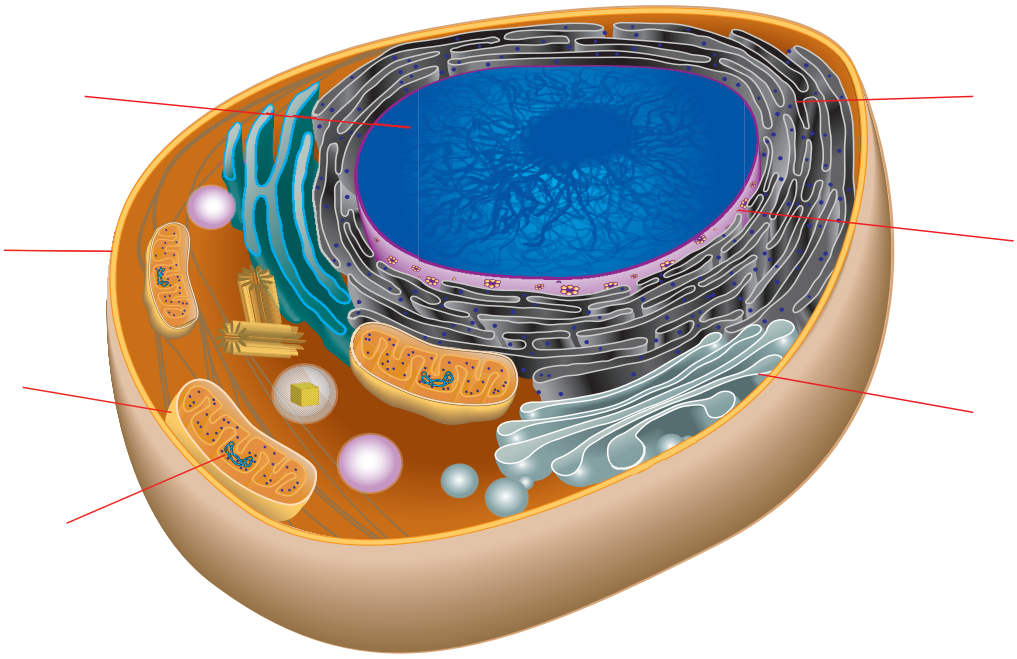
Seafood mislabeling and market substitution — the practice of incorrectly labeling or marketing seafood products — can occur at any point in the supply chain, from commercial fishing vessels to fish processing plants and commercial distribution centers to wholesale and retail fish markets to restaurants. This increasingly widespread form of consumer fraud often conceals destructive and nonsustainable fishing activities, and it may expose consumers to potentially serious health risks. In 2007, for example, two individuals in Chicago came down with symptoms of tetrodotoxin poisoning after eating soup prepared from fish purchased at a local market. Tetrodotoxin is a neurotoxin commonly found in pufferfish, the importation of which is strictly regulated by the U.S. Food and Drug Administration (U.S. FDA) because of the health risks associated with its consumption. Tetrodotoxin is heat stable, so it is not inactivated when tainted fish tissue is cooked. Furthermore, the minimum lethal dose of this toxin in humans is only 2 mg. Experts estimated that one of the affected persons had consumed approximately 3 mg of toxin. This person fortunately survived the bout of tetrodotoxin poisoning, but not without several weeks of rehabilitative care. The fish had been labeled headless monkfish, but DNA barcoding helped confirm that what was sold to the consumer was potentially toxic pufferfish and not nontoxic headless monkfish, as the label had claimed.

What is the true identity of the fish you’re holding? Is it the same as what you thought based on the packaging, menu, or your best educated guess? Let’s find out!

Lesson 1: Extraction of DNA from Fish Samples

In this lesson you will extract DNA from fish tissue obtained from a local grocery store, restaurant, fishing trip, or other source. You will begin by estimating or weighing out the proper amount of fish tissue to use and mincing it as finely as possible. After depositing it into a microcentrifuge tube, you will add a series of buffers to your sample in order to release DNA from individual cells. You will then bind the DNA to solid particles within the matrix suspension in a spin column, wash away the impurities present in the sample extract, and finally recover the purified DNA by elution into distilled water. The DNA you've extracted will be used in the next laboratory as your target DNA for PCR amplification.

Note: PCR, which will be perfomed in the next laboratory, involves amplication of DNA and therefore it is critical to use proper technique to avoid any cross contamination between fish samples during DNA extraction. Do not recycle cutting implements, pipet tips, or containers. If using gloves, change gloves in between the handling of different fish samples.



Focus Questions

1. Where is DNA found in eukaryotic cells? For reference a cell diagram is included above. Label the indicated features.
2. What parts of the cell must be broken down to extract DNA?
3. It is important to keep track of the location of the DNA at each stage of purification. For the following steps of the protocol, state whether the DNA is in the pellet, in the supernatant, bound to the column, or in the flowthrough:
   a. After centrifuging down the neutralized fish tissue lysate (pellet or supernatant).
   b. After centrifuging the supernatant through the column (column or flowthrough).
   c. After centrifuging the wash solution through the column (column or flowthrough).
   d. After centrifuging the elution solution through the column (column or flowthrough).
4. Why is it important to use a new cutting utensil for every fish sample?

Student Workstation

Materials	Quantity	(✓)
2 ml tube labeled **Resuspension**	1	☐
2 ml tube labeled **Lysis**	1	☐
2 ml tube labeled **Neutralization**	1	☐
2 ml tube labeled **Matrix**	1	☐
2 ml tube labeled **Wash**	1	☐
2 ml tube labeled **Distilled Water**	1	☐
Fish samples each in a separate weigh boat	2	☐
Razor blades, plastic knives, or other cutting implements (**Note**: it is critical to use one implement per fish sample)	2	☐
Spin columns	2	☐
Empty 2 ml microcentrifuge tubes with caps	2	☐
Empty 2 ml microcentrifuge tubes without caps	2	☐
100–1,000 adjustable-volume micropipet and tips	1	☐
Marking pen	1	☐

Common Workstation

Materials	Quantity	( )
Water bath or dry bath set to 55°C	1	☐
Microcentrifuge	1–2	☐

Quick Guide

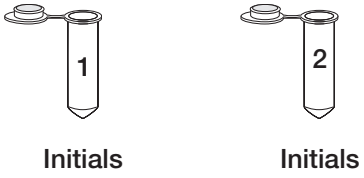
Lesson 1: DNA Extraction

Preparing Fish Samples

1. Label one capped 2 ml microcentrifuge tube for each of your fish samples (that is, “1” for fish sample 1, “2” for fish sample 2, etc.). Also label with your initials.

   Fish 1_____

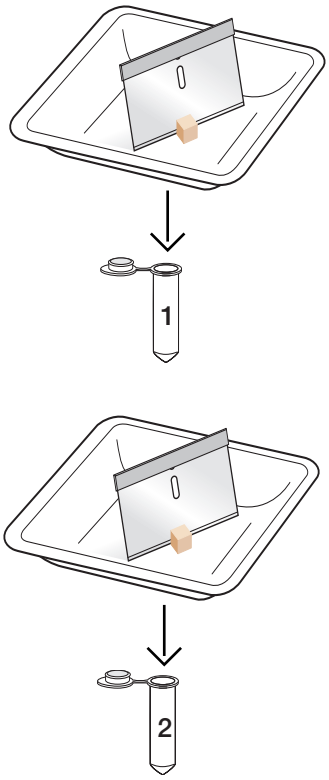
   Fish 2_____



2. Cut a piece of fish muscle up to 100 mg in mass, approximately the size of a pencil eraser-head, from your first fish sample. Place the piece in a new weigh boat and slice it with a razor blade or cutting implement until finely minced. Transfer the sample into the appropriately labeled microcentrifuge tube.

3. **Properly discard the razor blade or cutting implement.** If wearing gloves, change gloves before handling the next piece of fish. If not, wash hands thoroughly.

4. Using a new razor blade or cutting implement, cut a piece of fish muscle up to 100 mg in mass, approximately the size of a pencil eraser-head, from your second fish sample. Place the piece in a new weigh boat and slice it with a razor blade until finely minced. Transfer the sample into the appropriately labeled microcentrifuge tube. Properly discard the razor blade or cutting implement.



Extracting DNA from fish samples

1. Add 200 µl of **Resuspension** to your two microcentrifuge tubes containing minced fish and flick the tubes several times to ensure full submersion of the fish sample in the resuspension solution.

2. Add 250 µl of **Lysis** to each tube and mix gently by inverting tubes 10 times to mix contents. **Do not vortex!** Vortexing may shear genomic DNA, which can inhibit PCR amplification.

3. Incubate samples at 55°C for 10 min. The samples do not need to be shaken during incubation.

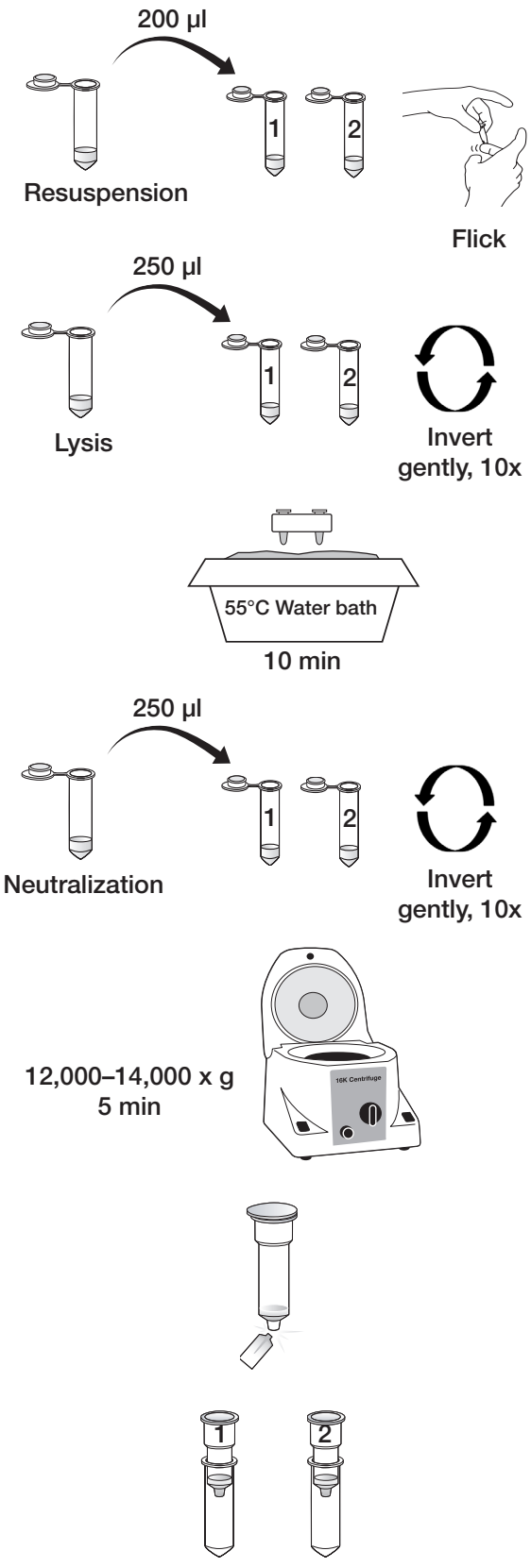
4. Add 250 µl of **Neutralization** to each microcentrifuge tube and mix gently by inverting tubes 10 times to mix contents (do not vortex). A visible cloudy precipitate may form.

5. Centrifuge the tubes for 5 min at top speed (12,000–14,000 x g) in the microcentrifuge. A compact pellet will form along the side of the tube. The supernatant contains the DNA.

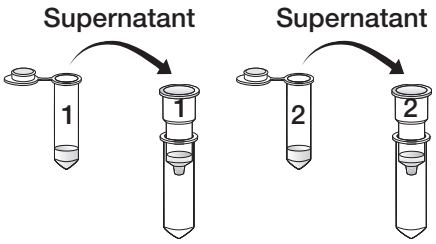
   If there are a lot of particulates remaining in the supernatant after centrifugation, centrifuge the tubes for 5 additional min.

6. **Snap (do not twist!)** the bottoms off of the spin columns and insert each column into a capless 2 ml microcentrifuge tube.

7. Label one spin column **1** for Fish 1 and a second spin column **2** for Fish 2. Also label the columns with your initials.

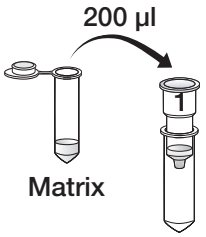


8. Transfer the entire supernatant (500–550 µl) of each fish sample from step 5 into the appropriately labeled spin column. Try not to get any of the particulates into the spin column because they will clog the column and prevent you from continuing.

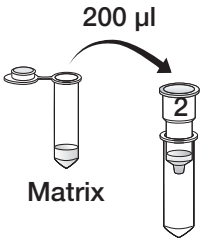


9. Thoroughly mix the tube labeled **Matrix** by vortexing or repeatedly shaking and inverting the tube to make sure particulates are completely resuspended before use.

10. Add 200 µl of thoroughly resuspended **Matrix** to the first column containing fish extract and pipet up and down to mix.

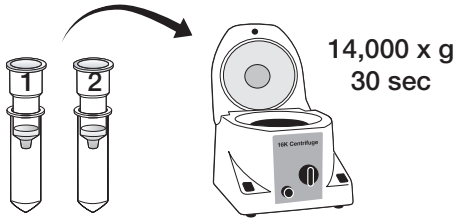


11. Using a new pipet tip, add 200 µl of thoroughly resuspended **Matrix** to the second column containing fish extract and pipet up and down to mix.



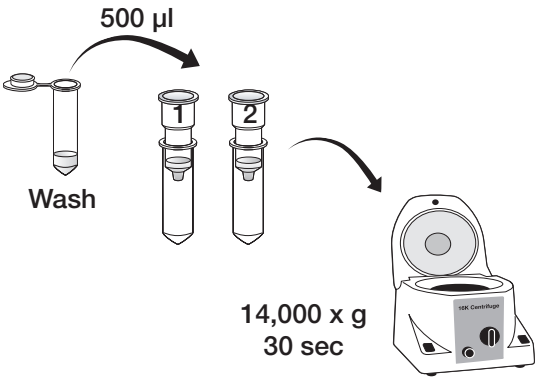
12. Centrifuge the columns for 30 sec at full speed.

Take care to spin the column for only 30 sec. Drying the matrix completely at this point will result in loss of DNA.



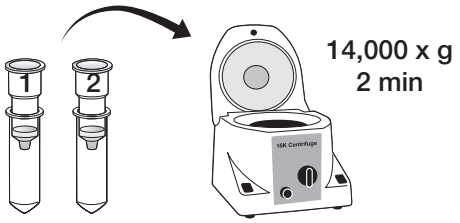
13. Remove the spin column from the 2 ml microcentrifuge tube, discard the flowthrough at the bottom of the 2 ml tube, and replace the spin column in the same tube. Add 500 µl of **Wash** and wash the matrix by centrifugation for 30 sec.

Take care to spin the column for only 30 sec. Drying the matrix completely at this point will result in loss of DNA.



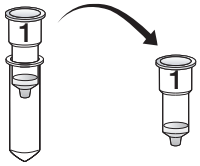
14. Repeat step 13 to wash samples again.

15. Remove the spin column from the 2 ml microcentrifuge tube, discard the flowthrough at the bottom of the 2 ml tube, and replace the spin column in the same tube. Centrifuge columns for a full 2 min to remove residual traces of ethanol and dry out the matrix.

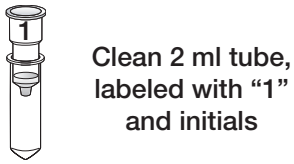


16. Label two clean 2 ml capless microcentrifuge tubes with your fish sample name and your initials.

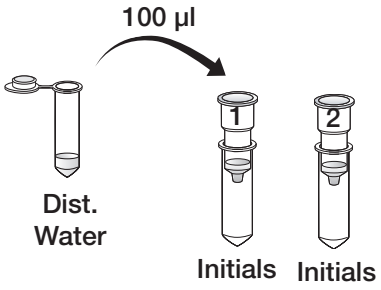
17. When your 2 min spin is completed, remove the spin columns and discard the 2 ml microcentrifuge wash tubes.



18. Place the spin column for each sample into a new capless 2 ml microcentrifuge tube from step 16.

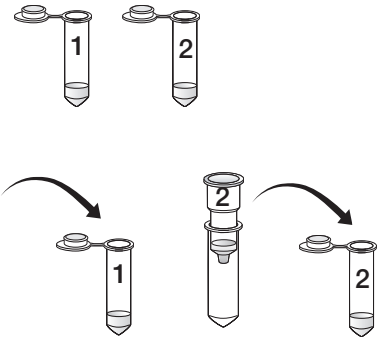


19. Using a fresh pipet tip for each sample, add 100 µl of distilled water to each spin column, being careful not to touch the resin. Elute the DNA by centrifuging for 1 min at full speed.



20. Label two clean 2 ml microcentrifuge tubes (with caps) **Fish 1** and **Fish 2** and your initials.

21. Transfer the eluted DNA into the appropriately labeled 2 ml microcentrifuge tube with caps and store the DNA at 4°C until you are ready to proceed.



Lesson 2: Set Up PCR Reactions

In the last laboratory, you extracted DNA from fish tissue. In this lab, you will prepare those samples and some additional experimental control samples for the polymerase chain reaction (PCR).

PCR is DNA replication in a test tube. PCR allows you to amplify specific regions of DNA and make millions of copies of the target sequence. Your experiment in this lab is to make enough copies of the COI target sequence so that the resulting PCR product can be visualized by gel electrophoresis and also be submitted for DNA sequencing in a later lesson.

Your Task for This Lesson

For this experiment you will set up a PCR reaction for each fish DNA extract you generated in the last laboratory, as well as two control PCR reactions. One control PCR reaction will be the positive control, which will use pCOI plasmid DNA as your target sequence. The other reaction will be a negative control, which will use water instead of target DNA. Since water should not have any DNA in it, there should be no amplification of any target sequence in this sample. By having a known control that you are sure should not amplify the COI target sequence, you can tell if your PCR reactions have been contaminated by DNA containing the *COI* gene.

Focus Questions

1. How do researchers target the portion of DNA to be amplified during PCR?
2. What two aspects of primer design have been used in this laboratory to ensure successful DNA amplification from a wide variety of fish samples?
3. Do you expect the pCOI plasmid to generate a PCR product? What about the negative control? Why or why not?
4. Why is it important to use aerosol filter tips when setting up a PCR reaction?

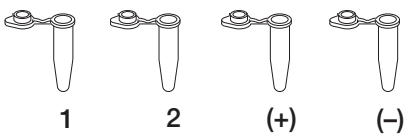
Student Workstation

Materials	Quantity	(✓)
Ice bath	1	☐
Fish DNA samples	2	☐
(+) sample	1	☐
(–) sample	1	☐
CMM reaction mix	1	☐
PCR tubes	4	☐
2–20 µl adjustable-volume micropipet	1	☐
20–200 µl adjustable-volume micropipet	1	☐
2–20 µl pipet tips, aerosol barrier	1 rack	☐
20–200 µl pipet tips, aerosol barrier	1 rack	☐
Marking pen	1	☐

Quick Guide

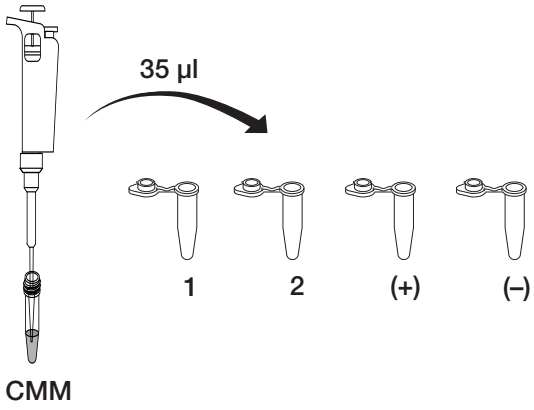
Lesson 2: PCR Amplification of DNA

1. Label four PCR tubes with your initials and the sample name (1 for fish sample 1, 2 for fish sample 2, (+) for the PCR positive control DNA, (–) for the PCR negative control). Keep the tubes on ice for the remaining steps.

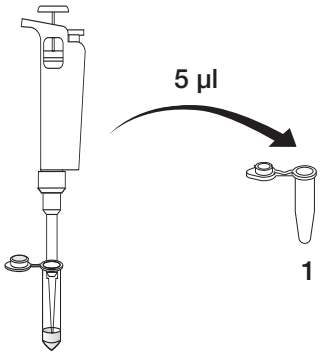


2. Using a fresh aerosol filter pipet tip each time, add 35 µl of **CMM** (COI master mix) reaction mix to each PCR tube, capping each tube immediately after the addition of liquid.

3. Using a fresh aerosol filter pipet tip for each tube, add 5 µl of the appropriate DNA sample directly into the CMM liquid in each PCR tube as indicated by the labels on the tubes, and pipet up and down to mix. Recap each tube immediately after adding DNA.



Tube Name	Master Mix DNA
1	35 µl CMM, 5 µl fish sample 1
2	35 µl CMM, 5 µl fish sample 2
(+)	35 µl CMM, 5 µl (+) sample
(–)	35 µl CMM, 5 µl (–) sample



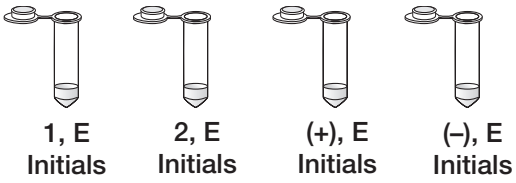
4. When instructed, place the PCR tubes in the thermal cycler and run the program with the following cycling conditions:

    1. 94°C – 2 min
    2. 94°C – 30 sec
    3. 55°C – 2 min
    4. 72°C – 1 min
    5. Repeat steps 2–4 35x
    6. 72°C – 10 min
    7. 4°C – hold

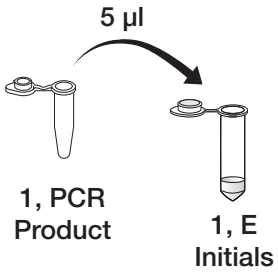
    Store tubes at 4°C after thermal cycling is complete.

Preparing PCR Samples for Electrophoresis and Sequencing

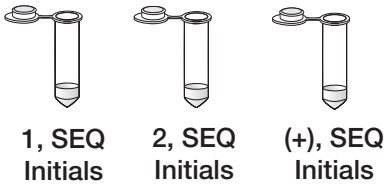
1. Label four 2 ml microcentrifuge tubes with both your initials and **E**. E stands for electrophoresis. Now label one of these tubes **Fish 1**, one tube **Fish 2**, one tube **(+)**, and one tube **(–)**.



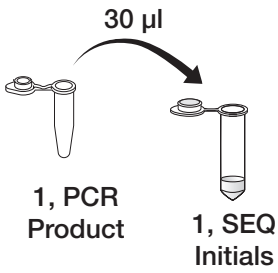
2. Remove 5 µl from each PCR reaction and deposit into the 2 ml microcentrifuge tube corresponding to that sample.



3. Label three 2 ml microcentrifuge tubes with both your initials and **SEQ**. SEQ stands for sequencing. Now label one of these tubes **Fish 1**, one tube **Fish 2**, and one tube **(+)**. You will not be sequencing your negative control sample.



4. Remove 30 µl from each PCR reaction and deposit into the 2 ml microcentrifuge tube corresponding to that sample.



5. Store all samples at 4°C until you are ready to proceed with electrophoresis and sequencing.

Lesson 3: Gel Electrophoresis

In the last laboratory, you sought to amplify a portion of the mitochondrial *COI* gene from your fish DNA samples and included control PCR reactions to aid in the analysis of gel electrophoresis results you will obtain in today's lab. Gel electrophoresis will allow you to determine the success of your PCR reactions by visualizing the size of your amplified DNA.

The expected band size that corresponds to your successfully amplified *COI* gene PCR product is approximately 650 bp. You may also notice an additional band less than 100 bp in size. This band corresponds to unincorporated primers from your PCR reaction, which can stick to each other in what is known as a primer dimer. A molecular weight ruler (DNA standard) has been provided so that you have a reference sample containing several DNA molecules with known molecular weights. Using this standard for comparison, you can estimate the size of your PCR product.

The UView 6x loading dye you will add to each of your samples contains a fluorescent compound that binds to DNA. During gel electophoresis it will comigrate with your DNA and allow your DNA to be visualized with UV light. No additional staining of the gel is required for visualization of your results.

Consult with your educator about the use of appropriate personal protective equipment prior to using any UV light source.

Focus Questions

1. What is the purpose of the agarose gel?
2. What purpose(s) does the UView loading dye serve?
3. What do think the results should look like for each sample?

Student Workstation

Materials	Quantity	(✓)
Electrophoresis samples labeled **E** from Lesson 2	4	☐
2 ml tube containing molecular weight ruler	1	☐
2 ml tube containing UView 6x loading dye	1	☐
2 ml tube containing sterile water	1	☐
2–20 µl adjustable-volume micropipets	1	☐
2–20 µl pipet tips, aerosol barrier	1 rack	☐
1% agarose gel	1	☐
Running buffer	250 ml	☐
Gel electrophoresis chamber	1	☐
Power supply (may be shared by multiple groups)	1	☐
Marking pen	1	☐

Common Workstation

Materials	Quantity	(✓)
UV transilluminator or imaging system	1	☐

Quick Guide

Lesson 3: Gel Electrophoresis

1. Retrieve the 5 µl samples of PCR products (4 samples) from 4°C. To each one, add 5 µl of sterile water. Use a new pipet tip each time.

2. Add 2 µl of UView 6x loading dye to each sample, using a new pipet tip each time. Mix samples well and pulse-spin.

3. Set up your gel electrophoresis apparatus as instructed.

4. Load the agarose gel in the following lane order and volumes, using a new pipet tip each time:

   Lane Sample

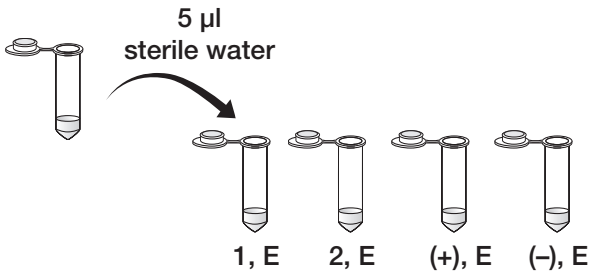
   1 – EMPTY
   2 – EMPTY
   3 – 20 µl PCR molecular weight ruler
   4 – 12 µl (+) E
   5 – 12 µl (–) E
   6 – 12 µl 1 E
   7 – 12 µl 2 E
   8 – EMPTY

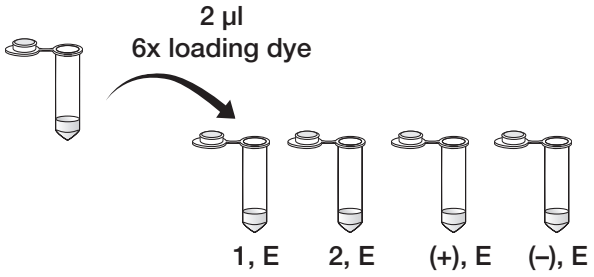
5. Ask your instructor whether the electrophoresis buffer your electrophoresis units contain is 0.25x TAE or 1x TAE.

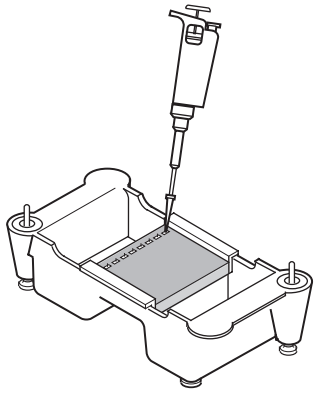
   If your buffer is 0.25 x TAE, run the gel at 200 V for 20 min.

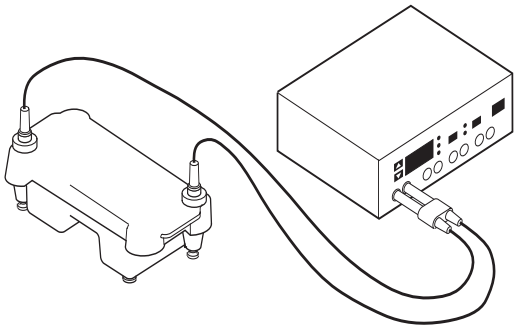
   If your buffer is 1x TAE, run the gel at 100 V for 30 min.

6. Visualize the gel on a UV transilluminator or imaging system. No gel staining is required as the loading dye contains a fluorescent compound that will allow visualization of DNA with UV light.









Lesson 4: Sequencing

In this DNA sequencing stage, you will be submitting your PCR products to a sequencing facility to be purified and sequenced. Sequencing reactions, like PCR, rely on the basic principles of DNA replication and also require primers to initiate DNA replication. However, sequencing is performed in just one direction. So instead of a primer pair, sequencing makes use of a single primer per reaction. To aid in getting as much data as possible from each fish COI PCR product, the sample PCR product will have a sequencing reaction run in each direction. One sequencing reaction will run in the forward direction (from the beginning of the *COI* gene) and a second sequencing reaction will run in the reverse direction. Ideally, these sequencing reactions will yield the same results, with one being the reverse complement sequence of the other. Running both reactions also aids in determining the sequence at the beginning and end of the COI PCR product, since these areas are typically problematic and the beginning sequence for the forward sequencing reaction is the end sequence for the reverse sequencing reaction.

Once you obtain these sequences, you will use bioinformatics tools to analyze the quality of your data, assemble a consensus sequence from your high-quality sequencing results, and search the Barcode of Life Database (BOLD) to determine what the closest match is for your fish sample.

Focus Questions

1. Why is it necessary to have PCR products purified before sequencing? (Hint: Think about how sequencing works and what might interfere with that if left over from PCR).
2. What primers will be used to sequence your PCR product? Where are those sequences on your PCR product? How did they get there?
3. What steps of the process so far may impact the quality of the sequence that will be generated? How would each step impact the sequence quality?

Student Workstation

Materials	Quantity	(✓)
SEQ samples from Lesson 2	3	☐
Marking pen	1	☐
Parafilm	1 piece per microcentifuge tube	☐

Quick Guide

Lesson 4: Sequencing

1. Parafilm your capped **Fish 1 SEQ, Fish 2 SEQ,** and **(+) SEQ** tubes thoroughly to prevent leakage while shipping.

2. Record the sample names on your tubes and make sure these match the names your instructor is submitting to the sequencing facility. This is the only way you can identify the correct sequencing data file for each sample.

3. Give your samples to your instructor for shipment to the sequencing facility.

Appendices

Appendix A:

Glossary of Terms

Aliquot — verb form: the division of a quantity of material into smaller, equal parts. Noun form: one of a number of small, equally divided parts.

Annealing — binding of single-stranded DNA to complementary DNA sequences. Oligonucleotide primers bind to single-stranded (denatured) template DNA.

Base call — reading a DNA sequencing chromatograph and assigning a base to each peak.

Base pairs — complementary nucleotides held together by hydrogen bonds. In DNA, adenine is linked by two hydrogen bonds with thymine (A-T) and guanine is linked with cytosine by three hydrogen bonds (G-C). Because of the three H-bonds between G and C (compared to the two between A and T), the G-C bonding is stronger than the A-T bonding.

Biorepository — a repository (place or building where things are stored) for biological materials. Biorepositories collect, process, preserve, store, and distribute specimens to support future scientific investigation. They may also manage and retain collections of specimens from many diverse organisms.

Chromatogram — also known as electropherogram, or trace file. A visual representation of the signal peaks detected by a sequencing instrument. The chromatogram contains information on the signal intensity as well as the peak separation time.

COI — abbreviation for cytochrome c oxidase subunit I. This gene is located in a cell's mitochondrial DNA and it encodes a protein within an enzyme complex that is involved in the electron transport chain of cellular respiration. This is the process by which organisms harvest energy, in the form of ATP, from food sources.

Consensus sequence — a sequence derived from the alignment of similar DNA, RNA, or protein sequences. Normally, each position in the consensus sequence is determined by the base or amino acid that predominates in that position in the majority of the aligned sequences. Consensus sequences can be used to design primers for PCR.

Contig — a sequence that has been constructed by comparing and merging the information from sets of overlapping DNA segments.

Degenerate primers — a mixture of PCR primers that are similar but not identical. They may be designed based on a consensus sequence derived from similar organisms, with substitutions of different bases at one or more locations in the primers.

Denaturation — with respect to DNA, separation of complementary strands of DNA into single-stranded DNA. Denaturation of DNA is also sometimes referred to as "melting." *In vivo*, DNA is denatured by enzymes. But in PCR, DNA is denatured by heat.

DNA barcode — a short, standardized gene region represented by its constituent nucleotide sequence. DNA barcodes exhibit fewer nucleotide differences among members of the same species and larger differences between members of different species groups. A 650 bp segment of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene is the standard barcode region for animals, whereas a segment of the nuclear ribosomal internal transcribed spacer region (*ITS*) is the accepted barcode region for fungi. Nucleotide sequences from two chloroplast genes — the ribulose-1,5-bisphosphate carboxylase (*rbcL*) and maturase K *(matK)* genes — are used as standard barcode regions to identify land plants. A query DNA barcode is a barcode sequence that is unknown or unverified and is obtained from a tissue sample or food product of unknown origin. A reference DNA barcode is a barcode sequence from a known source that has been extensively verified through numerous criteria, including taxonomic verification and vouchering processes (see Voucher definition for more information).

dNTPs — commonly used abbreviation for all four deoxynucleotide triphosphates (dATP, dTTP, dGTP, and dCTP) used in synthesizing DNA.

ddNTPs — commonly used abbreviation for dideoxynucleotide triphosphates (ddATP, ddTTP, ddGTP, and ddCTP) which are modified nucleotides that serve as chain terminating inhibitors of DNA polymerase during DNA sequencing.

Electropherogram (aka trace file) — see chromatogram.

Electrophoresis — a technique for separating molecules based on their relative migrations in an electric field. DNA and RNA are usually separated using agarose gel electrophoresis, and proteins are separated using a polyacrylamide matrix (PAGE or SDS-PAGE).

Elute — to remove adsorbed material from an adsorbent (that is, a column filter or matrix) by the addition of a solvent.

Extension — the phase of PCR amplification during which the DNA polymerase synthesizes a new DNA strand that is complementary to the template strand by incorporating dNTPs that are complementary to the template DNA.

Genomic DNA (gDNA) — all of the chromosomal DNA found in a cell or organism.

Lysis — the process of rupturing a cell to release its contents. Once lysed, the mixture of the cell and lysis solution is called a lysate.

Master mix — a premixed reagent solution for chemical or biological reactions. A PCR master mix contains all components needed for PCR (dNTPs, primers, buffer, salts, DNA polymerase, and Mg^{2+}) except for the template DNA.

Matrix — for the purposes of this kit, the matrix suspension contains particulates that will bind any DNA present in the supernatant of the centrifuged fish tissue lysates and will allow other impurities present in the lysate to be washed away.

Neutralization — a step during DNA extraction that entails the addition of a neutralizing salt solution that counteracts the effects of an alkaline lysis solution.

Nucleotide — a fundamental unit of DNA and RNA. Molecules comprising a sugar, a phosphate group, and one of four bases: adenine, guanine, cytosine, and thymine (DNA) or uracil (RNA).

Oligonucleotide (oligo) — a short segment (often 10–30 bases) of DNA or RNA that is usually made synthetically. Frequently used as primers for PCR or sequencing.

PCR — polymerase chain reaction. A technique for rapidly creating multiple copies of a segment of DNA using repeated cycles of DNA synthesis.

Pellet — the insoluble precipitate that occurs on the bottom or side of a tube following centrifugation.

Primer — a short, single-stranded oligonucleotide designed to bind DNA template strands at the end of the sequence of interest and serve as the starting point for DNA synthesis. Primers can be single-stranded either DNA or RNA.

Primer dimer — in a PCR reaction, primers with enough complementary sequences may stick to each other, causing bands of approximately 100 base pairs when visualized by electrophoresis.

Quality score (or value) — a numerical value used in DNA sequencing data indicating the confidence level for base calls. A higher quality value means higher confidence that the base call is correct. A lower quality value indicates the base call is less reliable.

Sequence — the ordered list of bases that make up a DNA strand. When linked with a chromatogram, this would be considered a read.

Supernatant — the liquid that remains above a solid residue or precipitate following precipitation, centrifugation, or other process.

Taq DNA polymerase — a DNA polymerase that is stable at high temperatures. Taq DNA polymerase is commonly used in PCR. The enzyme was originally isolated from the thermophilic bacterium *Thermus aquaticus*, which can tolerate high temperatures.

Template DNA — the target DNA that contains the sequence to be amplified by PCR.

UPC — abbreviation for universal product code. A UPC contains a unique combination of bars and spaces that distinguishes each product sold by a company. No two products share the same barcode.

Voucher — a specimen archived in a permanent collection (usually in a museum, biorepository, or other institution with a mandate to preserve materials indefinitely).

Vortex — induction of a jarring circular motion using a vortexer machine (#166-0610EDU). Vortexing is typically used to aid in the resuspension of insoluble material within a liquid suspension.

Appendix B:

PCR Amplication and Sterile Technique

PCR is a powerful and sensitive technique that enables researchers to produce large quantities of specific DNA from very small amounts of starting material. Because of this sensitivity, contamination of PCR reactions with unwanted DNA is always a potential problem. Therefore, utmost care must be taken to prevent cross-contamination of samples. Steps to prevent contamination and failed experiments include:

1. **Use filter-type pipet tips.** The end of the barrels of micropipets can easily become contaminated with aerosolized DNA molecules. Pipet tips that contain a filter at the end can prevent aerosol contamination from micropipets. DNA molecules within the micropipet cannot pass through the filter and contaminate PCR reactions. Xcluda® aerosol barrier pipet tips (211-2006EDU and 211-2016EDU) are ideal pipet tips to use in PCR reactions. For this laboratory aerosol barrier tips should be used for PCR.
2. **Aliquot reagents.** Sharing of reagents and pipetting multiple times into the same reagent tube can easily introduce contaminants into your PCR reactions. When at all possible, divide reagents into small aliquots for each team or, if possible, for each student. That way, if one aliquot of a reagent does become contaminated, then only a minimal number of PCR reactions will become contaminated and fail.
3. **Change pipet tips.** Always use a new pipet tip when entering a reagent tube for the first time. If a pipet tip is used repeatedly, contaminating DNA molecules on the outside of the tip will be transferred to other solutions, resulting in contaminated PCR reactions. If you are at all unsure whether your pipet tip is clean, err on the safe side and discard the tip for a new one. The price of a few extra tips is a lot smaller than the price of failed reactions.
4. **Use good sterile technique.** When opening tubes or pipetting reagents, leave the tubes open for as little time as possible. Tubes that are open and exposed to the air can easily become contaminated by aerosolized DNA molecules. Go into reagent tubes efficiently, and close them as soon as you are finished pipetting. Also, try not to pick tubes up by the rim or cap, as you can easily introduce contaminants from your fingertips.
5. Bleach at a concentration of 10% destroys DNA, so wiping down surfaces and rinsing plastic pipet barrels, mortars, and pestles with 10% bleach can get rid of any surface DNA contamination that may arise.

Appendix C:

Degenerate Primers

Normally PCR primers are unique sequences of nucletotides, designed to match the known sequence of the target DNA. When the sequence of the template DNA is not known, there are several alternative approaches for primer design. One approach is to take advantage of genetic homology among closely related organisms. For example, the target DNA may not have been sequenced in the species of interest, but the gene may have been sequenced in several other species. Genes that code for the same protein in different organisms are likely to have sequences that are conserved, very similar, or even identical in the different species. These conserved sequences usually code for parts of the protein that are essential for function; in other words, mutations in these areas are likely to be detrimental to the organism, so evolution discourages any changes.

If genomic DNA (gDNA) or messenger RNA (mRNA) sequences from similar species are aligned, a consensus sequence can be derived. The consensus sequence may be exactly the same in all species, or it may have one or more bases that vary among the species. For example, a consensus sequence could be represented by A-C-T-G-G-N-T-T-A-C-C-G, where A, C, G, and T represent the bases that are the same in all of the species compared, and N represents a base that varies in different species. In other words, the base at the N position might be G, C, A, or T.

Since the goal of PCR is to amplify the DNA region of interest, primers are designed to bracket that region. Once the primers have been designed based on the consensus sequences derived from other organisms, it is possible that they will have enough complementarity with the target DNA to bind during the annealing step. However, to increase that probability, one or more bases within the primers may be replaced with the other three bases, bringing degeneracy, or wobble, to the primer sequences. (This is also described as introducing wobbles into the primer; the higher the degeneracy, the more wobbles.) In a simplified example, if the consensus sequence is NATC, the set of degenerate primers would be AATC, TATC, GATC, and CATC.

However, in many cases not all of the bases are used as substitutes for the variable base. To increase the probability that the primer will anneal to the target DNA, the variable base may be replaced with a similar base. For example, if the variable base is a T, it might be replaced only with C (the other pyrimidine). The International Union of Biochemistry (IUB) offers a code to tell whoever is synthesizing the primers which bases to substitute at each variable position in the table on the next page:

Table 3. Oligonucleotide International Union of Biochemistry (IUB) codes for mixed (wobble) bases.

IUB Code	Bases	Derivation of IUB Code
N	A/G/C/T	Any
K	G/T	Keto
S	G/C	Strong
Y	T/C	Pyrimidine
M	A/C	Amino
W	A/T	Weak
R	G/A	Purine
B	G/T/C	–
D	G/A/T	–
H	A/C/T	–
V	G/A/C	–

Degeneracy is achieved by having multiple bases introduced at specific base positions during the manufacture of the oligonucleotides (oligos). Oligos are short individual DNA sequences that are usually manufactured synthetically. Typically, a primer is a single oligo sequence; however, degenerate primers are composed of multiple oligo sequences. Degenerate primers therefore allow binding to a greater number of related target sequences that exhibit a small amount of sequence diversity.

Appendix D:

Programming Instructions for T100 Thermal Cycler

Though the instructions below appear to skip over the 72°C extension step during the 35 rounds of thermal cycling, they in fact do not. The T100 thermal cycler's touch screen technology makes it possible to skip programming that step because we are not changing the default settings of the machine.

T100 Thermal Cycler

Program the T100 (necessary only the first time you perform the lab)

Turn on the T100 by flipping the power switch at the rear of the machine

Select **New Protocol**

Press **50 µl** on top right of the screen

Enter **40**

Press **OK**

Program the Initial Denaturation

Press **95°C** in column 1

Enter **94**

Press **OK**

Press **3:00** in column 1

Enter **200**

Press **OK**

Program the 35 PCR Cycles

Press **95°C** in column 2

Enter **94**

Press **OK**

Press **0:30** in column 2

Press **OK**

Press **55°C** in column 3

Press **OK**

Press **0:30** in column 3

Enter **200**

Press **OK**

Press **34X** in column 5

Enter **35**

Press **OK**

Program the Final Extension

Press **5:00** in column 6

Enter **1000**

Press **OK**

Save the Protocol

Press **Save** on the lower menu bar of the screen

Enter **BARCODE**

Press **Save**

Press **Home** on the lower menu bar of the screen

Run the BARCODE Program

Press **Saved Protocols**

Press **Main** in the Folders column

Press **BARCODE** in the Files column

Press **Run** on the lower menu bar of the screen

Press **OK**

Appendix E:

Run Agarose DNA Gels in 20 Minutes

Bio-Rad's Biotechnology Explorer R&D team has developed a new electrophoresis buffer formula. Using a reduced concentration of running buffer (0.25x TAE) and higher voltage (200 V), any agarose gel can be run 33% faster. Advantages of this new formula include:

- Excellent gel resolution
- Minimal run time
- Fast separation of DNA in gels of any agarose gel concentration (0.8–4.0%)
- Compatibility with all Bio-Rad Biotechnology Explorer program kits

TAE buffer is provided as a 50x concentrate that can be mixed with distilled water to yield the necessary concentrations for making agarose gels and electrophoresis running buffer.

Use 1x TAE to make agarose gels:

350 ml of 1x TAE is enough to make eight 7 x 10 cm agarose gels. To make 350 ml of 1x TAE from a 50x TAE concentrate, add 7 ml of concentrate to 343 ml of distilled water. Detailed instructions for making agarose gels can be found in individual kit instruction manuals.

Use 0.25x TAE to make electrophoresis running buffer:

A 2.5 L volume of 0.25x TAE buffer is required to run eight 7 x 10 cm agarose gels. To make 2.5 L of 0.25x TAE from a 50x TAE concentrate, add 12.5 ml of concentrate to 2.49 L of distilled water. To make 2.5 L of 0.25x TAE from a 1x TAE solution, add 625 ml of 1x TAE to 1,875 ml of distilled water.

Note: Do not use 0.25x TAE to make agarose gels; doing so can lead to a loss of DNA resolution.

To run gels:

Place the gel in an electrophoresis chamber and cover it with 0.25x TAE; ensure the gel is submerged. Run gels at 200 V for no more than 20 min. Monitor gel loading dye progress to get a relative idea of electrophoresis progress.

Appendix F:

Staining with Fast Blast DNA Stain

The sensitivity of the UView™ 6x loading dye provided with the Fish DNA Barcoding kit is significantly greater than Fast Blast DNA stain, and thus UView is the preferred method for visualizing DNA. If Fast Blast stain is used, please be aware that PCR products may indeed by present but not visible.

Preparation for Staining Agarose Gels

Fast Blast DNA stain is provided as a 500x concentrate that must be diluted prior to use. The stain can be used as a quick stain when diluted to 100x to allow the visualization of DNA within 15–20 minutes, or can be used as an overnight stain when diluted to 1x. Fast Blast DNA stain is a convenient, safe, and nontoxic alternative to ethidium bromide for the detection of DNA. Fast Blast DNA stain contains a cationic compound that belongs to the thiazin family of dyes. The positively charged dye molecules are attracted to and bind to the negatively charged phosphate groups on DNA. The proprietary dye formula stains DNA deep blue in agarose gels and provides vivid, consistent results.

Warning

Although Fast Blast DNA stain is nontoxic and noncarcinogenic, latex or vinyl gloves should be worn while handling the stain or stained gels to keep hands from becoming stained blue. Lab coats or other protective clothing should be worn to avoid staining clothes. Dispose of the staining solutions according to protocols at your facility. Use either 10% bleach solution or 70% alcohol solution to remove Fast Blast DNA stain from most surfaces. Verify that these solutions do not harm the surface prior to use.

Preparation for Overnight Staining Protocol (Recommended)

To prepare 1x stain (for overnight staining), dilute 1 ml of 500x Fast Blast DNA stain with 499 ml of distilled or deionized water in an appropriately sized flask or bottle, and mix. Cover the flask and store at room temperature until ready to use.

Preparation for Quick Staining Protocol

To prepare 100x stain (for quick staining), dilute 100 ml of 500x Fast Blast DNA stain with 400 ml of distilled or deionized water in an appropriately sized flask or bottle and mix. Cover the flask and store at room temperature until ready to use. Destaining requires the use of at least one large-volume container, capable of holding at least 500 ml, at each student workstation. 100x Fast Blast DNA stain can be reused at least seven times. Please note, in contrast to 1% agarose gels, 3% agarose gels require 5 min staining, prior to destaining in warm water. Due to the high percentage of agarose, gels stained by this quick method may take longer to destain to a satisfactory level than 1% agarose gels. Multiple washes with warm tap water will assist the destaining of these gels.

Appendix G:

Calculating G Force vs RPM (revolutions per minute)

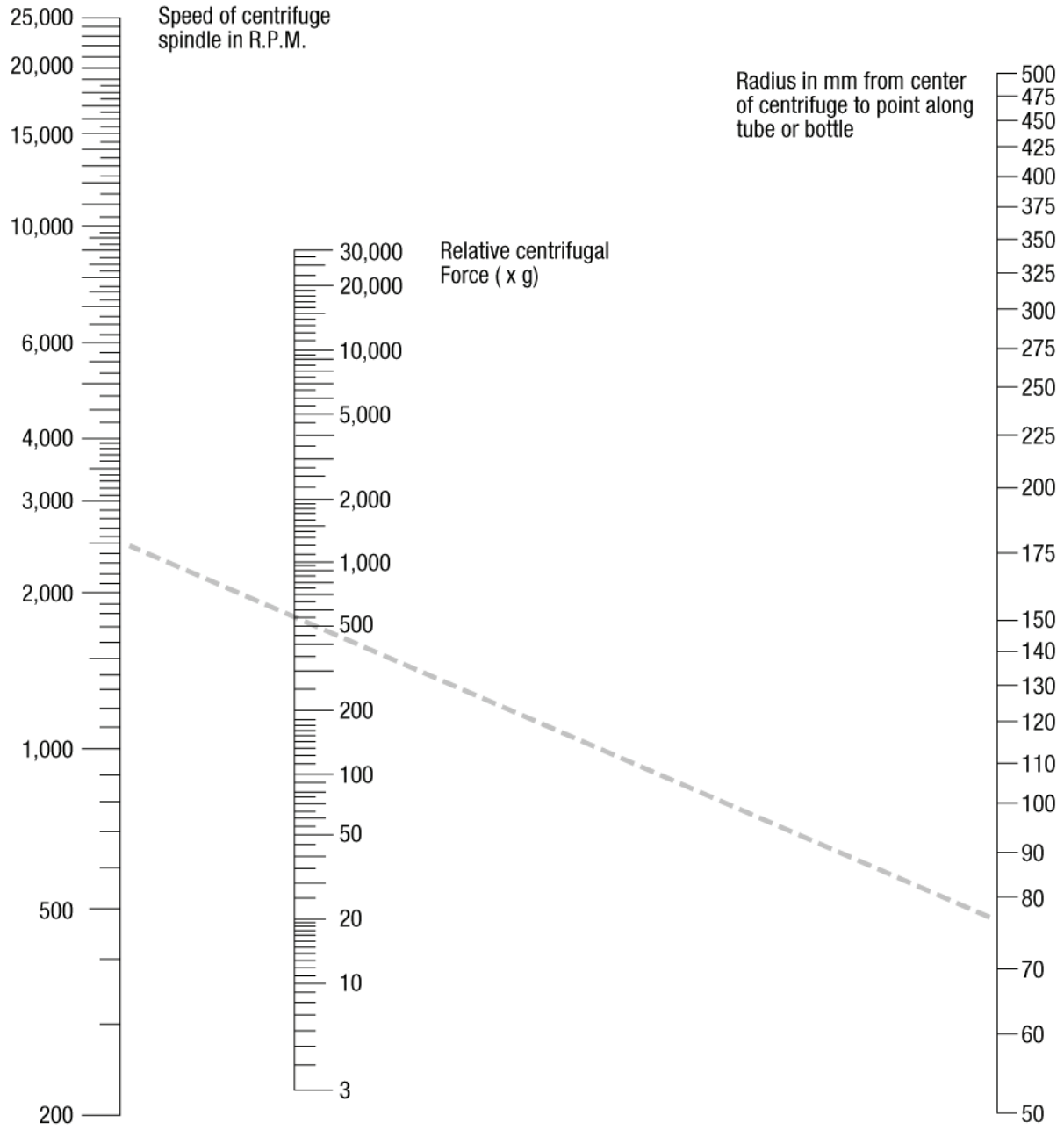
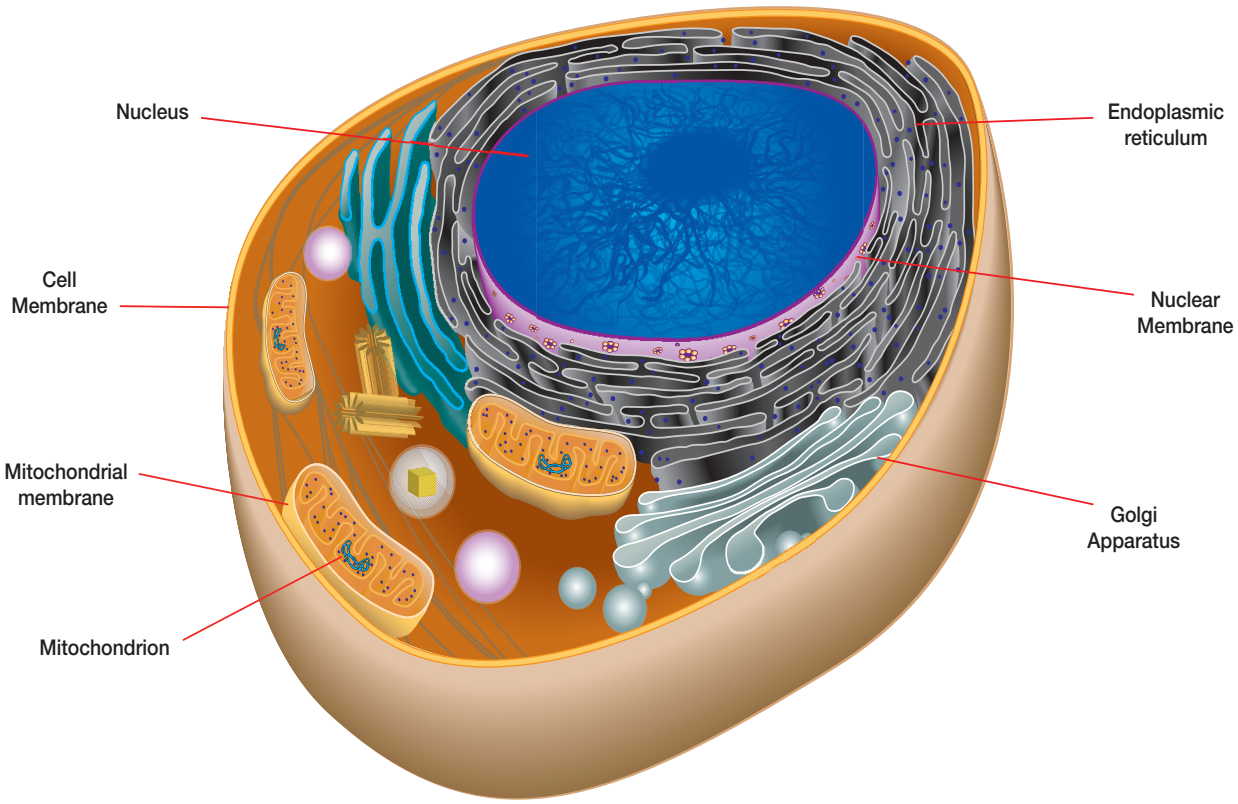


Chart provided by Corning Incorporated, Life Sciences, Lowell, MA USA.

Appendix H:

Answers to Focus Questions



Lesson 1

1. Nucleus, mitochondrion, chloroplast
2. Cell wall (plant), cell membrane, nuclear membrane, mitochondrial membrane
   a. Supernatant
   b. Column
   c. Column
   d. Flowthrough
3. Because contaminating DNA will be amplified during PCR, which will cause mixed sequencing data and will prevent definitive species identification.

Lesson 2

1. By using primers designed to bind to a particular DNA sequence.
2. Multiple primer sets, degenerate primers
3. pCOI plasmid should generate a PCR product because it has the *COI* gene cloned into a vector backbone. The water control should not generate a PCR product as there should be no DNA in it.
4. Aerosol tips are important when setting up PCR reactions because they prevent carryover of aerosolized samples when pipetting between different tubes.

Lesson 3

1. The agarose gel acts as a sieve for DNA to migrate according to its size when an electrical field is applied to it.
2. The UView loading dye weighs down the DNA sample so that it stays in the well after loading. It also contains a fluorescent compound that allows the DNA to be visualized with UV light.
3. The positive control sample should generate a 650 bp band following PCR. The negative control (water) sample should not generate a 650 bp band following PCR. Both fish samples should generate a 650 bp band following PCR.

Lesson 4

1. PCR products must be purified before sequencing because primers and oligonucleotides present for the PCR reaction can interfere with sequencing, which requires different primers and precise ratios of oligonucleotides.
2. M13 forward and M13 reverse primers will be used to sequence the PCR products. These sequences are found at the very ends of the PCR products, and were incorporated during PCR amplification using the PCR primers.
3. Steps that could impact the quality of the sequence obtained (and prevent poor sequencing results):
    - Handling the fish properly (changing gloves) and not cross-contaminating samples
    - Cutting each fish with a new cutting implement
    - Not reusing pipet tips between samples
    - Using aerosol pipet tips for PCR

Appendix I:

Extension Topics

1. Inheritance of Mitochondrial DNA

For quite a long time it was thought that mitochondrial DNA (mtDNA) was inherited strictly through the maternal line, and for most species this is the case. What are some reasons that mtDNA is predominantly maternally inherited? There are several current hypotheses. One is that of simple dilution (a spermatocyte contains only 100 to 1,000 mitochondria, whereas an oocyte (egg cell) contains between 100,000 and 1,000,000 mitochondria). Another thought is that mtDNA from the spermatocyte fails to enter the oocyte upon fertilization, or that the sperm mtDNA may enter the oocyte but is degraded afterwards.

Can you think of an instance in which mtDNA is not maternally inherited? During asexual reproduction there are no separate mother and father from whom the offspring would inherit genetic material, and thus the mtDNA can come from only a single source.

Interestingly, reports of occasional paternal transmission of mtDNA do exist, though evidence suggests these events are rare. This phenomenon has been observed in a single case in humans (Schwartz and Vissing 2002), and has also been discovered in fruit flies (Kondo et al. 1992), honeybees (Meusel and Moritz 1993), and cicadas (Fontaine et al. 2007). Paternal inheritance of mtDNA has also been observed in the coastal redwood *Sequoia sempervirens* (Neale et al. 1989). mtDNA inheritance from both parents occurs more regularly in some bivalves, an example of which is mussel (Hoeh et al. 1991).

2. Barcoding of Species from Other Domains: Bacteria and Archaea

Aren't prokaryotes some of the most diverse organisms on the planet? What genetic locus is used for classification of these organisms?

Prokaryotes exhibit some of the most complex biodiversity on Earth, and there is great interest in designating a proper DNA barcode region to classify them. Despite this enthusiasm, efforts have been hampered by the fact that prokaryotic genomes can be highly variable, even within a single species. This is primarily due to the fact that prokaryotes can participate in horizontal gene transfer. While the transmission of genetic material from parent to offspring as a result of reproduction (sexual or asexual) is termed vertical gene transfer, horizontal gene transfer refers to the exchange of genetic material between organisms in the absence of reproduction. Horizontal gene transfer can occur between different species, and even between different evolutionary domains, such as between bacteria and archaea (Koonin et al. 2001). This additional mode of genetic information exchange can afford organisms evolutionary advantages, such as antibiotic resistance in the case of bacteria, and also leads to substantially greater sequence diversity between organisms — even organisms within the same species. This complicates our ability to determine a suitable DNA barcode region for species classification. In fact, it is widely accepted that sequence analysis of several genes will be required for genetic classification of prokaryotes. Some genes currently used for this are 16s rRNA (small subunit ribosomal RNA), *cpn60* (60 kDa chaperonin), *mutS* (DNA mismatch repair protein mutator S), and *gyrB* (DNA gyrase subunit B).

How do prokaryotes transfer genetic material horizontally? Among the several mechanisms are:

1. **Transformation** — the process of uptake and expression of foreign DNA. Transformation is a commonly used technique in biotechnology for targeted gene or protein expression (see pGLO™ Bacterial Transformation kit, #166-0003EDU).
2. **Transduction** — a process during which bacterial DNA from one individual is transferred to another through a viral intermediate (that is, bacteriophage infection).
3. **Bacterial conjugation** — the process by which transfer of genetic information from one bacterium to another occurs through direct physical contact.

References for Extension Topics

Fontaine KM, Cooley, JR, Simon, C (2007). Evidence for paternal leakage in hybrid periodical cicadas (Hemiptera: Magicicada spp. PLoS One. 9, e892.

Hoeh WR, et al. (1991). Heteroplasmy suggests limited biparental inheritance of Mytilus mitochondrial DNA. Science 251, 1488–1490.

Kondo R, et al. (1992). Further observation of paternal transmission of Drosophila mitochondrial DNA by PCR selective amplification method. Genet Res 59, 81–84.

Koonin EV et al. (2001). Horizontal gene transfer in prokaryotes: quantification and classification. Annu Rev Microbiol 55, 709–742.

Meusel MS and Moritz RF (1993). Transfer of paternal mitochondrial DNA during fertilization of honeybee (Apis mellifera L.) eggs. Curr Genet 24, 539–543.

Neale DB, et al. (1989). Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. Proc Nat Acad Science 86, 9347–9349.

Schwartz M, Vissing J (2002). Paternal inheritance of mitochondrial DNA. N Engl J Med 347, 576–580.

BIO-RAD

Bio-Rad
Laboratories, Inc.

Life Science
Group

Web site *www.bio-rad.com* ***USA*** *800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *01 877 89 01* ***Belgium*** *09 385 55 11* ***Brazil*** *55 11 5044 5699*
Canada *905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *44 52 10 00* ***Finland*** *09 804 22 00*
France *01 47 95 69 65* ***Germany*** *089 31 884 0* ***Greece*** *30 210 9532 220* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300*
Israel *03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *03 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *0318 540666*
New Zealand *64 9 415 2280* ***Norway*** *23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04*
Singapore *65 6415 3188* ***South Africa*** *27 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *08 555 12700* ***Switzerland*** *026 674 55 05*
Taiwan *886 2 2578 7189* ***Thailand*** *800 88 22 88* ***United Kingdom*** *020 8328 2000*

10028899 Rev A US/EG *Sig 1212*