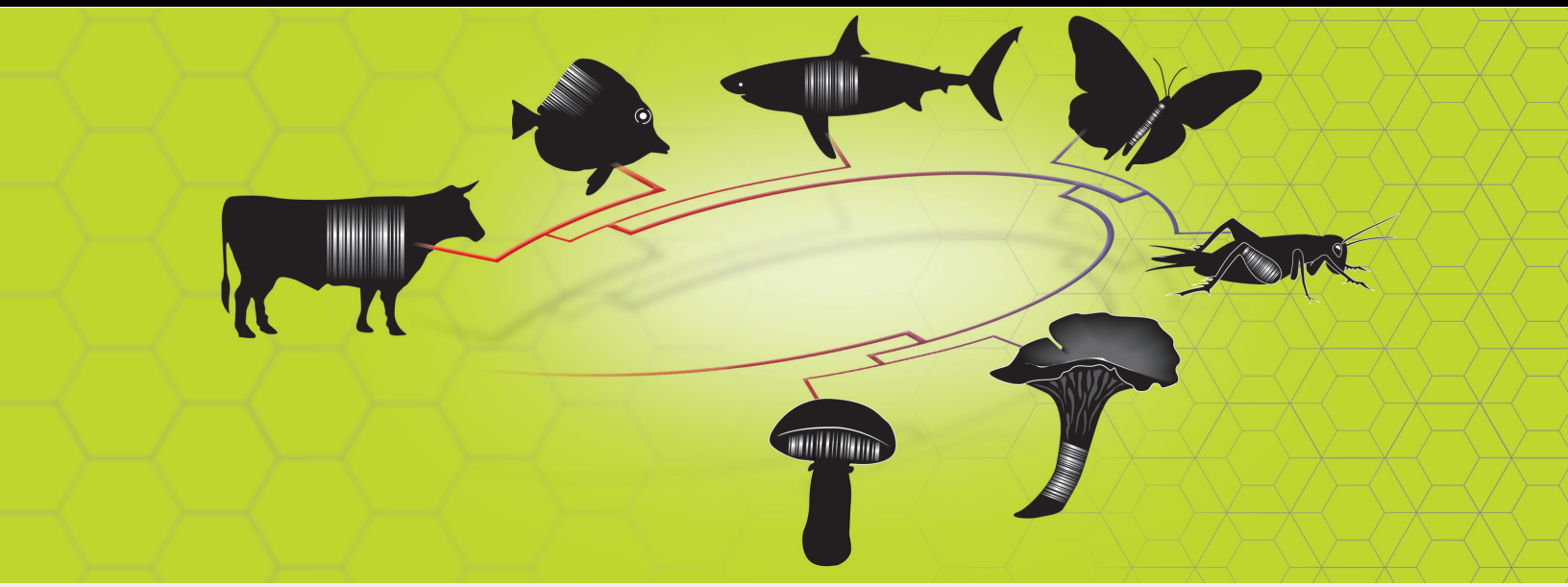
BIO-RAD Explorer



DNA Barcoding Kit

Catalog #17007432EDU, 17007366EDU, and 17007154EDU

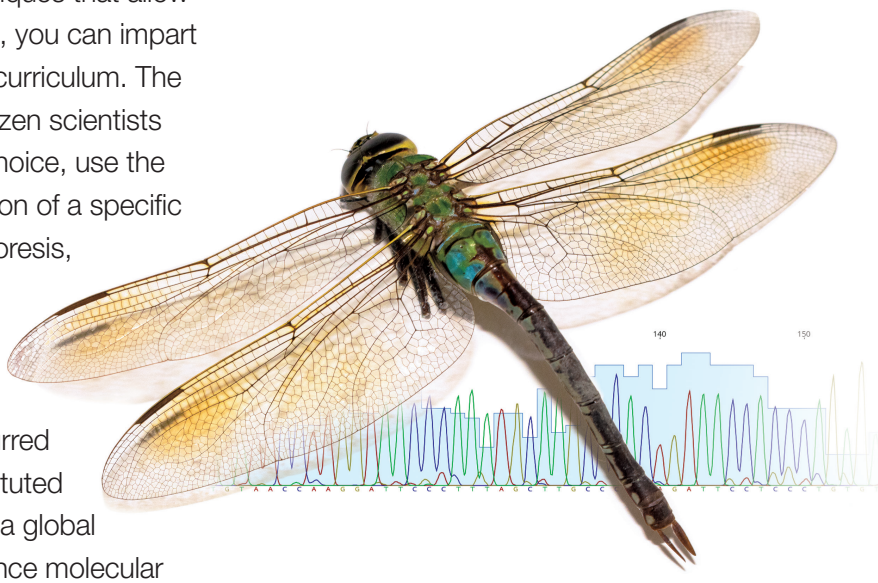
Instructor Guide

For technical support, call your local Bio-Rad office, or in the U.S. call **1-800-4BIORAD** (1-800-424-6723) option 2.

BIO-RAD

Dear Instructor

How many times have you heard students say: Why do we have to learn this? Will I ever use this in real life? With the 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 DNA Barcoding Kit allows students to become citizen scientists as they isolate DNA from tissue samples of their choice, use the polymerase chain reaction (PCR) to amplify a portion of a specific gene, analyze their PCR products using electrophoresis, sequence the PCR product, and compare that sequence to other sequences in a database.



This combination of skills can be performed to determine whether a market substitution has occurred in which a less expensive product has been substituted for one that is more expensive, or to participate in a global effort to catalog the biodiversity of planet Earth. Once molecular biology skills are mastered, students may contribute directly to the scientific knowledge base by working with scientists who are actively cataloging species as part of the International Barcode of Life project (iBOL). Having students connect with a scientist to receive vouchered 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 (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 activity.

We continually strive to evolve and improve our lessons and products. We welcome your stories, suggestions, and ideas!

Bio-Rad Explorer Team
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Kit Storage

When you receive the DNA Barcoding Kit or PCR Module:

1. Note storage location and record the batch numbers from the product labels.
2. Store the **DNA Extraction Module** at room temperature.

3. Store the **PCR Module(s)** refrigerated at –20°C.

4. Visit **bio-rad.com/barcoding** to download the instructor and student guides as well as the separate Bioinformatics Guide.

Technical Support is available at support@bio-rad.com or 1-800-4BIORAD, option 2.

Safety Guidelines

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 min. Lab coats or other protective clothing should be worn to avoid staining clothes.

Always use proper safety precautions when collecting and processing samples. Some organisms, including animals and fungi, are harmful and/or poisonous if handled improperly. Improper handling may result in serious injury or death. It is recommended that students work only with samples that have been positively identified as nontoxic, or otherwise use samples from a reliable retailer such as a grocery store. Use appropriate personal protective equipment minimally including gloves and safety goggles. Use extreme caution when using cutting utensils to prevent injury.

Use special caution when working with hot molten agar. Heat-resistant gloves and other standard personal protective equipment, including goggles and a laboratory coat, are recommended. Visit **explorer.bio-rad.com** to access Safety Data Sheets for individual products.

Kit Components*

Each kit contains materials for eight student workstations.

Item	Quantity
DNA Extraction Kit	
Resuspension solution	5 ml
Lysis solution	5 ml
Neutralization solution	5 ml
Matrix	5 ml
Wash buffer	10 ml
Spin columns	20
2 ml capped microcentrifuge tubes	250
Fish PCR Module	
Fish Primer Mix	50 µl
UView 6x Loading Dye and Stain	200 µl
COI-ITS PCR Control DNA	20 µl
Sterile water	2.5 ml
PCR MW Ruler	200 µl
PCR tubes	50
Mammals, Insects, and Fungi PCR Module	
Mammals and Insects Primer Mix	50 µl
Fungi Primer Mix	50 µl
UView 6x Loading Dye and Stain	200 µl
COI-ITS PCR Control DNA	20 µl
Sterile water	2.5 ml
PCR MW Ruler	200 µl
PCR tubes	50

* Materials for sequencing are not included. Sequencing of PCR products is a requirement to complete the DNA barcoding activity. See Preparation Instructions for information about sequencing options.

DNA Extraction Module

Fish PCR Module

Mammals, Insects, and Fungi PCR Module

Required materials not included in this kit

Item	Quantity
Equipment	
Thermal cycler with at least 32 wells	1
100–1000 µl adjustable-volume micropipet and aerosol barrier tips	8
20–200 µl adjustable-volume micropipet and aerosol barrier tips	8
2–20 µl adjustable-volume micropipet and aerosol barrier tips	8
Horizontal gel electrophoresis chamber with gel casting tray and comb	2–8
Power supply	1–4
Dry bath or water bath	1
Microcentrifuge, ≥14,000 x g	1
UV transilluminator or imaging system	1
Microwave oven or magnetic hot plate with stir bar	1
Graduated cylinders, 100 ml, 500 ml, and 3 L, for preparing buffers	1 each
Bottle or Erlenmeyer flask, 1 L	1
Erlenmeyer flask, 1 L, for preparing agarose	1
Razor blade or plastic knife, new and unused	16
Weigh boat	16
Microcentrifuge tube rack	8
PCR tube rack	8
Crushed ice and container (e.g., ice bucket or styrofoam cup)	8
Permanent marking pen	8
Reagents and Consumables	
Tissue samples for barcoding	2 or more
Distilled water	750 ml
Ethanol, 95%	10 ml
Zipper sealed bag for mailing sequencing samples	1
Parafilm	
Lab tape	

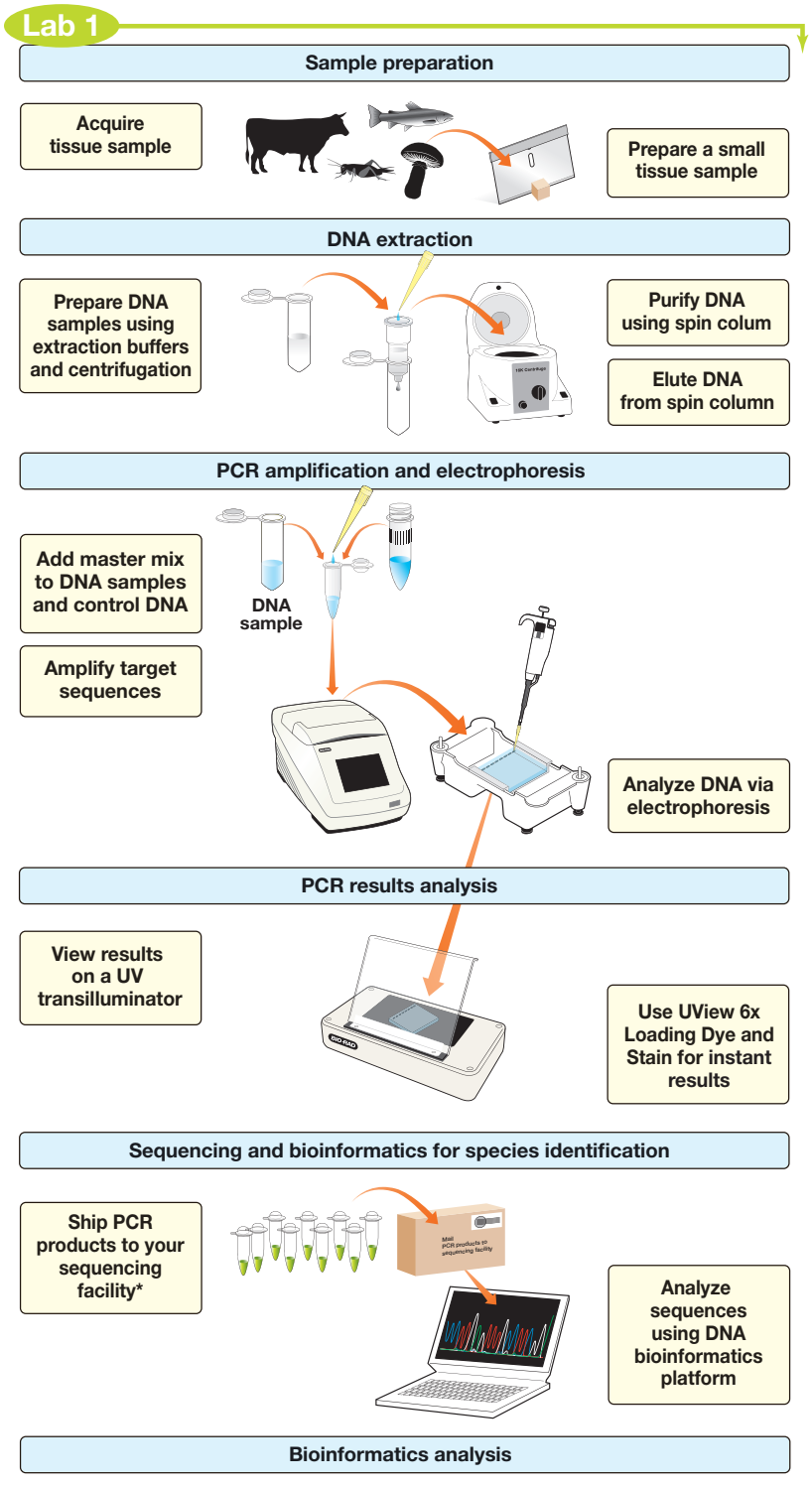
Ordering Information

Catalog #	Description
Kits and Refill Packs	
12016408EDU	**DNA Extraction Module**
1665015EDU	**DNA Extraction Reagent Pack**
12016300EDU	**Fish PCR Module**
17007432EDU	**Fish DNA Barcoding Kit**
17007153EDU	**Fish DNA Barcoding Kit with Agarose and TAE Buffer**
12016353EDU	**Mammals, Insects, and Fungi PCR Module**
17007366EDU	**Mammals, Insects, and Fungi DNA Barcoding Kit**
17007135EDU	**Mammals, Insects, and Fungi DNA Barcoding Kit with Agarose and TAE Buffer**
17007154EDU	**Fish, Mammals, Insects, and Fungi DNA Barcoding Kit**
17007155EDU	**Fish, Mammals, Insects, and Fungi DNA Barcoding Kit with Agarose and TAE Buffer**
Reagents and Consumables	
1665009EDU	**2x Master Mix for PCR**
1665111EDU	**UView 6x Loading Dye and Stain**, 0.2 ml
1665112EDU	**UView 6x Loading Dye and Stain**, 1 ml
2239430EDU	**EZ Micro Test Tubes**, 500
TWI0201EDU	**0.2 ml PCR Tubes with Domed Caps**, clear, 1,000
1613015EDU	**1% TAE Mini ReadyAgarose Precast Gel**, 7.1 x 10 cm, 8-well
7326300EDU	**PCR Kleen Spin Columns**, 25
Equipment and Laboratory Supplies	
1660506EDU	**Professional Adjustable-Volume Micropipet**, 2–20 µl
1660507EDU	**Professional Adjustable-Volume Micropipet**, 20–200 µl
1660508EDU	**Professional Adjustable-Volume Micropipet**, 100–1,000 µl
1861096EDU	**T100 Thermal Cycler**
1664000EDU	**Mini-Sub Cell GT Cell**
1645050EDU	**PowerPac Basic Power Supply**
1660562EDU	**Digital Dry Bath**, 120 V
1660504EDU	**Temperature-Controlled Water Bath**, 120 V
12011919EDU	**Mini Centrifuge**, 100–240 V
1660602EDU	**Model 16K Microcentrifuge**, 120 V
1660531EDU	**UView Mini Transilluminator**
1660610EDU	**BR-2000 Vortexer**, 120 V
1660477EDU	**Gel Staining Tray**, pack of 4
1660481EDU	**Green Racks**, set of 5
TRC0501EDU	**96-Place PCR Tube Rack and Cover**, set of 5, multicolor

Visit **explorer.bio-rad.com** for a full list of 220–240 V equipment.

Kit Activity Overview

The DNA Barcoding Kit encompasses a laboratory workflow designed to serve eight student groups in extracting and purifying DNA from a tissue sample, amplifying a region of DNA suitable for barcoding, sequencing the amplified DNA, and using bioinformatics analysis to determine the genetic identity of the original sample.



* Sequencing not included with this kit.

Activity Timelines

This activity is designed to take four approximately 50-min or two approximately 90-min laboratory periods plus one 50-min computer laboratory period for the bioinformatics analysis after sequencing is complete. Tables 1 and 2 provide estimated timelines. Sequencing PCR samples typically takes 1–2 days after they are received by the sequencing facility. Consult with your sequencing provided for time estimates. An additional 4-hour thermal cycling period is needed outside of class.

Table 1. Suggested timeline for 90-min or longer class periods.

	Class Period 1	Class Period 2	Class Period 3
In-class work	**Lesson 1:** DNA Extraction **Lesson 2:** PCR Amplification	**Lesson 3:** Gel Electrophoresis **Lesson 4:** DNA Sequencing	**Lesson 5:** Bioinformatics Analysis
Out-of-class work	Run PCR program	Send samples for sequencing	

Table 2. Suggested timeline for 50-min class periods.

	Class Period 1	Class Period 2	Class Period 3	Class Period 4	Class Period 5
In-class work	**Lesson 1:** DNA Extraction	**Lesson 2:** PCR Amplification	**Lesson 3:** Gel Electrophoresis	**Lesson 4:** DNA Sequencing	**Lesson 5:** Bioinformatics Analysis
Out-of-class work		Run PCR program		Send samples for sequencing	

Curriculum Fit

Required prior knowledge and skills

- How to use a micropipet consistently and accurately
- How to perform agarose gel electrophoresis and interpret gel results
- Key steps and purpose of the polymerase chain reaction (PCR)
- How to maintain sterility when handling samples

Concepts, topics, and skills

- **DNA extraction** — students will practice extracting DNA from tissue and isolating genomic DNA using spin columns from one of several organisms.
- **PCR** — students will set up and run their own PCR reactions to amplify the DNA barcoding regions of their chosen samples.
- **DNA sequencing** — following extraction and amplification, students will have their DNA samples sequenced and will interpret the results.
- **Genetics workflow** — students will see that these individual techniques are steps in a longer 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.
- **DNA barcoding** — students will investigate the relationship among species at the genetic level by examining specific barcoding regions of genomic DNA. These highly conserved regions are integral to the survival of the respective species. DNA barcoding analysis allows students to see how random mutations at the genetic level lead to a diversity of species.
- **Bioinformatics** — students will use bioinformatics tools, including BLAST, to process their sequencing data and generate consensus sequences, query DNA sequence databases to find matches, assess the reliability of the matches based on alignment details, and make conclusions about the identity of their samples.

Preparation Instructions

See Table 3 for an overview of the preparation instructions.

Table 3. Instructor preparation overview.

Preparation Step	Time Required	When to Begin Preparation
Prepare for DNA sequencing	As needed	Several weeks before the lessons, in case additional materials are required
Prepare and dispense solutions	1 hr	Up to 4 weeks before Lesson 1 DNA Extraction, except for the wash buffer and reaction mix(es)
Acquire tissue samples	As needed	Any time before Lesson 1 DNA Extraction
Preprogram the thermal cycler	10 min	Any time before Lesson 2 PCR Amplification
Prepare agarose gels and TAE buffer	1–2 hr	Up to 1 week before Lesson 3 Gel Electrophoresis
Download, review, and prepare the Bioinformatics Guide	2 hr	Any time before Lesson 5 Bioinformatics
Prepare student workstations	10–30 min for each lesson	Just before each lesson
Prepare reaction mix(es)	10 min	Just before Lesson 2, PCR Amplification

1. Prepare for DNA sequencing several weeks before the lessons.

The services and requirements of a sequencing facility will impact the necessary final preparation of student DNA samples and may require the purchase of additional materials. Identify the sequencing facility you will use several weeks before this activity.

i. Locate a sequencing facility. Local universities often have sequencing facilities, and commercial sequencing services can be found in most regions.

ii. Determine the sample requirements for the sequencing facility. Some facilities may provide services to preprocess your samples. Requirements may include:

- **PCR sample cleanup** —clean up the PCR samples to remove the remaining PCR reagents, such as PCR primers, which will compromise the sequencing results. This step is always necessary, but some facilities may offer this as a service. If your sequencing facility does not offer PCR sample cleanup, use a PCR cleanup kit such as Bio-Rad's PCR Kleen Spin Column Kit (catalog #7326300EDU) to prepare samples prior to sending them to the sequencing facility.
- **PCR template concentration and volume** — adjust the concentration and volume as required by the sequencing facility. For this activity, it is generally sufficient to assume the final samples are 10 ng/µl with a 0.7 kb amplicon.
- **Sequencing primers** — request or purchase M13 forward (TGTAAAACGACGGCCAGT) and M13 reverse (CAGGAAACAGCTATGA) primers. If your sequencing facility does not offer standard sequencing primers, purchase sequencing primers (for example, from Integrated DNA Technologies) and reconstitute them in the appropriate buffer and at the appropriate concentration and volume as required by the sequencing facility. Ask the facility whether the primers should be premixed with samples or shipped separately.

2. Prepare and dispense solutions up to four weeks before Lesson 4.

Reagents, except the reaction mix of PCR master mix and primers, may be prepared up to 4 weeks before Lesson 1 and stored refrigerated at 4°C. The PCR master mix plus primers solution should be prepared no more than 30 minutes before the students prepare their PCR samples.

Note: Use the included sterile water only to set up PCR reactions and gel electrophoresis samples. Use distilled water for all other purposes unless otherwise noted.

Note: The included UView 6x Loading Dye and Stain is a nontoxic stain that allows for immediate visualization of your samples with ultraviolet (UV) light after electrophoresis.

Prepare and dispense solutions into labeled tubes as specified in Table 4 below.

To help prevent cross-contamination, label tubes and dispense one solution at a time. Be sure to use a new pipet tip for each solution.

Table 4. Reagent preparation and dispensing instructions.

Tube Type and Quantity for 8 Lab Workstations	Tube Label	Tube Contents and Preparation Instructions	Storage
For Lesson 1 DNA Extraction from Tissue Samples			
8 microcentrifuge tubes with caps, 2.0 ml	**R**	500 µl resuspension solution	Up to 4 weeks at room temp.
8 microcentrifuge tubes with caps, 2.0 ml	**Lys**	600 µl lysis solution	Up to 4 weeks at room temp.
8 microcentrifuge tubes with caps, 2.0 ml	**N**	600 µl neutralization solution	Up to 4 weeks at room temp.
8 microcentrifuge tubes with caps, 2.0 ml	**X**	500 µl resuspended matrix **Preparation:** Before dispensing, resuspend the resin matrix by shaking, stirring manually, or vortexing	Up to 4 weeks at room temp.
8 microcentrifuge tubes with caps, 2.0 ml	**Wash**	2 ml wash buffer and ethanol solution **Preparation:** Before dispensing, add 10 ml of 95% ethanol to the bottle of wash buffer, replace the cap, and shake to mix.	1–2 days at room temp.
8 microcentrifuge tubes with caps, 2.0 ml	**dH2O**	300 µl distilled water	Indefinitely
For Lesson 2 PCR Amplification			
8 microcentrifuge tubes with caps, 2.0 ml	**+**	10 µl prepared positive control DNA stock (**+S**) **Preparation:** Pulse-spin the COI-ITS PCR Control DNA. To a new 2.0 ml microcentrifuge tube labeled **+S** (positive PCR control DNA stock), add 20 µl COI-ITS PCR Control DNA and 80 µl sterile water. Thoroughly mix.	Up to 4 weeks at 4°C
8 microcentrifuge tubes with caps, 2.0 ml	**–**	10 µl sterile water	Up to 4 weeks at 4°C
8 microcentrifuge tubes with caps, 2.0 ml	**FishRM** **MIRM** **FunRM** (label according to which primer set(s) you are using)	150 µl prepared reaction mix stock (**FishS**, **MIS**, and/or **FunS**) **Preparation:** NO MORE THAN 30 MIN BEFORE PCR ACTIVITY, thaw, vortex, and pulse-spin the 2x master mix and primer mix(es). To a new 2.0 ml microcentrifuge tube labeled **FishS** (fish reaction mix stock), **MIS** (mammals and insects reaction mix stock), or **FunS** (fungi reaction mix stock), add 560 µl sterile water, 800 µl 2x master mix, and 40 µl corresponding primer mix. Thoroughly mix.	Do not store. Use within 30 min.
For Lesson 3 Gel Electrophoresis			
8 microcentrifuge tubes with caps, 2.0 ml	**MWR**	25 µl prepared molecular weight ruler **Preparation:** Before dispensing, add 50 µl UView 6x Loading Dye and Stain to the PCR MW Ruler. Thoroughly mix and pulse-spin.	Up to 4 weeks at 4°C
8 microcentrifuge tubes with caps, 2.0 ml	**LD**	15 µl UView 6x Loading Dye and Stain	Up to 4 weeks at 4°C
8 microcentrifuge tubes with caps, 2.0 ml	**sH2O**	40 µl sterile water	Up to 4 weeks at 4°C

3. Acquire tissue samples any time before the lessons.

Source tissue samples or have your student source tissue samples any time before the activity. To avoid spoilage and contamination, store tissue samples frozen and then thaw the day of Lesson 1.

The success of this laboratory is highly dependent on the quality and quantity of DNA extracted from your samples. You may choose to provide samples for your students or have them gather their own. Table 5 lists which samples reliably produce high quality DNA samples and PCR results. For a more open-ended inquiry experience for your students, use species not listed. See Teaching Tips and Notes for more information and suggestions.

Avoid fur, feathers, exoskeleton, bones, or other keratinous or chitinous samples. Also avoid canned or processed samples, as acidic conditions can damage DNA. High fat-content samples (for example, fried foods) may also inhibit DNA extraction and subsequent PCR. Fresh, frozen, or dried samples can produce excellent results.

Table 5. Samples, by species, that reliably produce successful results.

Robust		Less Robust	Difficult/Not Robust
Insects			
Beetles Moths Butterflies	Bees Wasps Flies	Spiders	Molted exoskeletons Cocoons Gastropods
Mammals and Birds			
Cow Pig Sheep Deer	Pheasant Duck	Elk Turkey	Ground meat Fur Feathers Bones
Fungi			
Fresh mushroom caps		Dried mushrooms are less robust than fresh mushrooms, but can still produce good results	Older mushrooms, due to the risk of contamination
Fish			
Dried fish Catfish Shark Sturgeon Trout Rock cod True cod Tilapia	Mackerel Yellowfin tuna Sea bass Opah Imitation crab Flying fish roe Bonito flakes Shrimp	Salmon Salmon roe Anchovy Arctic char Sardine	Fried fish Canned fish Sea urchin Red tuna Mussel Clam

4. Preprogram the thermal cycler with the program in Table 6 any time before Lesson 3 PCR Amplification.

Table 6. Thermal cycler parameters for PCR amplification.

Step	Temperature, °C	Duration
Initial denaturation	94	2 min
35 cycles of:		
Denaturation	94	30 sec
Annealing	55	2 min
Extension	72	1 min
Final extension	72	10 min
Hold	12	(∞)

5. Prepare agarose gels and TAE buffer up to one week before Lesson 3 Gel Electrophoresis.*

i. Prepare eight 1% agarose gels with 8 wells each. It is essential to use 1x TAE (not 0.25x TAE) to make these gels. See Appendix A for gel casting instructions.

ii. Prepare 2.2 L of 0.25x TAE electrophoresis buffer by adding 11 ml of 50x TAE to 2,200 ml of distilled water to use with the Fast DNA Gel Protocol.*

Note: The correct volume of distilled water would be 2,189 ml, but the difference does not affect results.

* This activity is designed to take advantage of Bio-Rad's Fast Gel Protocol using 0.25x TAE running buffer and higher voltages. See Appendix A for details and alternative electrophoresis options with preparation instructions.

6. Download and prepare the Bioinformatics Guide.

The Bioinformatics Guide includes both instructor preparation instructions and a student guide to perform bioinformatics using the Barcode of Life Data Systems **Student Data Portal (BOLD-SDP)**.

Visit **bio-rad.com/barcoding** to download Bulletin 6398, the Bioinformatics Guide.

7. Prepare student workstations.

Use Tables 7–14 to set up workstations just before each lesson

Table 7. Lesson 1 DNA Extraction student workstation.

Materials	Quantity
Resuspension buffer (**R**)	500 µl
Lysis buffer (**Lys**)	600 µl
Neutralization buffer (**N**)	600 µl
Matrix (**X**)	500 µl
Wash buffer (**Wash**)	2 ml
Distilled water (**dH2O**)	300 µl
Empty 2 ml microcentrifuge tubes with caps	2
Empty 2 ml microcentrifuge tubes with caps removed	4
Spin column	2
Bulk tissue sample	2
Empty, clean weigh boat	2
Razor blade, plastic knife, or other clean cutting utensil (**Note:** it is critical to use one utensil per sample)	2
100–1,000 µl adjustable-volume micropipet and tips	1
Marking pen	1

Table 8. Lesson 1 DNA Extraction common workstation.

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

Table 9. Lesson 2 PCR Amplification student workstation.

Materials	Quantity
Positive PCR control (**+**)	10 µl
Negative PCR control (**–**)	10 µl
DNA extract samples from Lesson 1	2
Appropriate reaction mix (**FishRM**, **MIRM**, or **FunRM**)	150 µl
PCR tube	4
2 ml microcentrifuge tube with cap	7
2–20 µl adjustable-volume micropipet and aerosol barrier tips	1
20–200 µl adjustable-volume micropipet and aerosol barrier tips	1
Ice bath with crushed ice	1
Marking pen	1

Table 10. Lesson 2 PCR Amplification common workstation.

Materials	Quantity
Thermal cycler with at least 32 wells	1

Table 11. Lesson 3 Gel Electrophoresis student workstation.

Materials	Quantity
Electrophoresis samples, labeled **E**, from Lesson 2 (**1E**, **2E**, **+E**, **–E**)	4
Molecular weight ruler (**MWR**)	25 µl
UView 6 x Loading Dye and Stain (**LD**)	15 µl
Sterile water (**sH2O**)	40 µl
1% agarose gel	1
TAE running buffer	250 ml
2–20 µl adjustable-volume micropipet with aerosol barrier tips	1
Horizontal gel electrophoresis chamber	1
Power supply (may be shared)	1
Marking pen	1

Table 12. Lesson 3 Gel Electrophoresis common workstation.

Materials	Quantity
UV transilluminator or imaging system	1

Table 13. Lesson 4 Sequencing student workstation.

Materials	Quantity
Sequencing samples, labeled **SEQ**, from Lesson 2 (**1SEQ**, **2SEQ**, **+SEQ**)	3
Marking pen	1
Parafilm pieces	3

Table 14. Lesson 5 Bioinformatics student workstation.

Materials	Quantity
Forward and reverse sequencing files for student samples	Varies
Computer with internet access	8

Teaching Tips and Notes

Lesson 1 DNA Extraction

Preventing cross-contamination is of utmost importance in this activity. Use the following precautions to minimize the chance of contamination. Additional information about sterile technique can be found in Appendix B

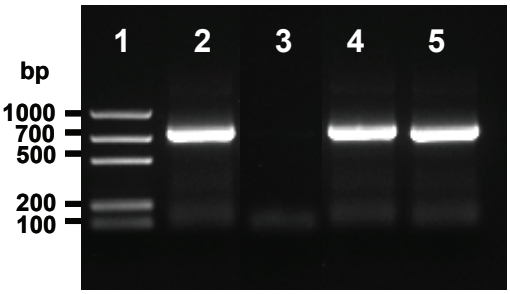
- New, clean, and DNA-free cutting utensils are required for each sample. Do NOT cross-contaminate samples. The DNA from a single contaminating cell can ruin results. Use gloves and change them between specimens
- Wiping a utensil and rinsing in ethanol between samples will NOT sufficiently remove DNA present on a cutting utensil
- While bacteria do not contain mitochondria, they do contain a *COI* gene, which will coamplify with sample DNA if the samples are contaminated. Taking samples from the interior of bulk animal tissue can reduce the chances of contamination with bacteria
- Once lysis buffer is added during DNA extraction, there is no convenient stopping point until the DNA is eluted from the spin purification column. If you choose to stop before adding the lysis buffer, be sure student samples are labeled with their initials, and store them refrigerated at 4°C for up to 1 week
- **Option:** introduce additional inquiry into this laboratory activity by using samples from different organs from an organism to determine which sources of tissue produce the best results. For example, the muscle and gill tissues of a fish both produce very robust amplification of mitochondrial DNA. What about fins or scales?

Lesson 2 PCR Amplification

- **Prepare the reaction mixes no more than 30 min before PCR activity.**
- The students should have 40 µl PCR reactions, which allow 5 µl for gel electrophoresis and 30 µl to be sent for sequencing plus some extra. If there is less final volume than anticipated, 20 µl is the minimum amount that should typically be sent for sequencing

Lesson 3 Gel Electrophoresis

- Typical classroom results:
  - Lane 1: PCR MWR
  - Lane 2: COI-ITS PCR Control DNA — a 650 bp band, corresponding to amplification by either COI-specific primers or ITS-specific primers, indicates that PCR was successful. If this band is absent, then the PCR reaction was unsuccessful
  - Lane 3: PCR negative control — no visible bands in this lane indicates that contamination of the PCR samples is unlikely. If there are bands in this lane, then PCR samples may have been contaminated



  - Lane 4: Sample 1 PCR product — if DNA extraction and PCR were successful, an appropriately sized band will be visible in this lane. If no band is present, then something may have gone wrong during the DNA extraction.
  - Lane 5: Sample 2 PCR product — same as sample 1 PCR product
- 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

Lesson 4 Sequencing

- Whether student PCR reactions were successful or not, proceed with sequencing of the control DNA not only as a substitute for students whose PCR was unsuccessful, but also because the positive PCR control DNA should yield a known result. The identities of the species in the positive PCR control DNA are *Sebastes goodei*, the chilipepper rockfish, and *Mochella sextelata*, the common morel

Lesson 5 Bioinformatics

- Visit **bio-rad.com/barcoding** to download Bulletin 6398, the Bioinformatics Guide
- The Bioinformatics Guide includes complete instructor preparation instructions and a student guide to perform bioinformatics using the Barcode of Life Data Systems Student Data Portal (BOLD-SDP)

Instructor Background

DNA Barcoding

It can be difficult to identify a 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. Confounding factors such as convergent evolution, where two species starting from quite different ancestors independently develop similar traits due to environmental pressures, make Linnaean taxonomical classification difficult. Examples include quills on both porcupines and echidnas, wings on bats and birds, or biomimicry such as nontoxic viceroy butterflies resembling poisonous monarch butterflies.

Even after 250 years of collection, analysis, and categorization of species using physical characteristics, fewer than two million of Earth's estimated 10–50 million plant and animal species have been formally described and cataloged. With the increases in human population, habitat destruction, pollution, and overharvesting, the rate of species loss threatens to outpace the rate of species discovery using this traditional classification system.

The explosion of faster and cheaper technology to isolate, purify, amplify, and sequence DNA has spurred the development of new methods to help identify different 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 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 scanned at the grocery store, DNA sequences can be used to uniquely identify different species. 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 UPC; and 2) a 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 a grocery store's cash register computer. DNA barcoding uses a short genetic marker to identify an individual organism as belonging to a particular species. An ideal DNA barcode should be present in most 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.

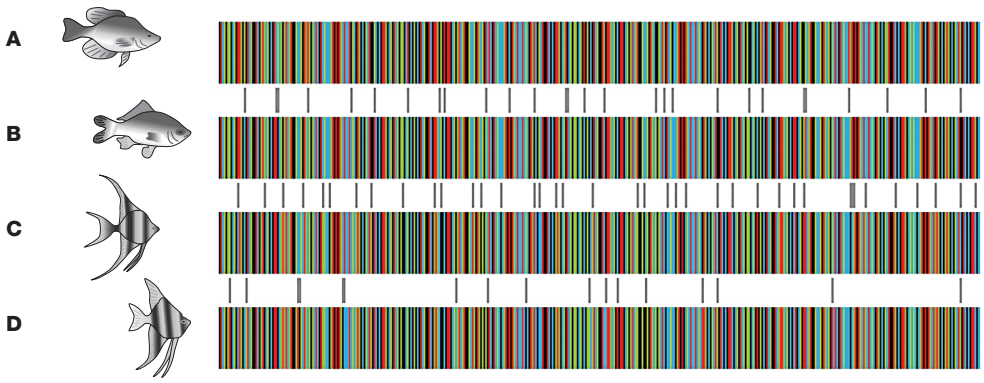


Fig 1. Comparison of DNA barcodes. Color-coded DNA sequences serve as "DNA barcodes" for animal, plant, and fungal samples. DNA barcodes A and B are from individuals within one genus, while the DNA barcodes for C and D are from individuals within a different genus. The gray bars between the DNA barcodes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

DNA Barcode Loci Selection

Discrete gene loci were chosen as barcode regions for animals, plants, and fungi based on their ability to distinguish species groups within each kingdom as shown in Table 15. A 650 base pair (bp) segment of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene is the standard barcode region for animals (Hebert 2003, 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 15. 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

Within the mitochondrial genome, the *COI* gene encodes subunit I 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.

In eukaryotes, the *ITS* region is between the two genes that encode the large ribosomal and small ribosomal RNAs. RNA polymerase transcribes the two genes and the spacer region together as a single transcript and the spacer is later removed by endonucleases during assembly of the ribosome subunits.

Within the chloroplast genome, *rbcL* encodes the ribulose-1,5-bisphosphate carboxylase, also known as rubisco, which catalyzes the first major step of carbon fixation during photosynthesis, while *matK* encodes maturase K, a conserved protein involved in intron splicing.

DNA Barcoding Reference Databases

Once a DNA barcode is generated, a searchable database is necessary to link the DNA barcodes to verified and highly characterized biological specimens. 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 possible quality, and b) that species identity is correctly assigned to reference sequences. Similarly, if the code in the grocery store cash register database linked to button mushrooms, which are normally $2.00/lb, were accidentally switched with the code for the morel mushrooms, which tend to sell for $20.00/lb, many people would be very unhappy when their button mushrooms suddenly cost ten times what was 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 boldsystems.org.

Global Initiatives and Barcoding Applications

The utility of DNA barcoding as a global species identification tool will continue to expand as the number of reference barcodes in the BOLD database grows. At its formal launch ceremony in 2010, the International Barcode of Life (iBOL) project announced its goal to provide coverage for 500,000 species of plants and animals by 2015. Its goal as of 2019 is to barcode 2.6 million species by 2026. To meet these landmark challenges, 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 was on groups with the highest practical importance to humanity. Refer to the iBOL project web page (ibol.org) for some examples of ongoing campaigns.

Barcode Data Standards

Although query barcode sequences and reference barcode sequences are generated and edited in the same manner, the latter are subject to a variety of data standards established by the scientific community (Ratnasingham and Hebert 2007). For instance, the specimen from which a reference barcode sequence is generated 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, 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 and enable accurate species identifications to be made by end users of BOLD-IDS. Strict compliance with data standards ensures the fidelity of BOLD as a reliable species identification tool.

Having students generate query sequences, which are exempt from these data standards, circumvents many logistical and technical challenges associated with creating reference barcode sequences while preserving the DNA barcoding experience. However, after gaining familiarity with the concepts and methods of DNA barcoding, some educators may choose to extend this curriculum to include generating reference barcodes for the BOLD reference library, especially since students may receive authorship for the publication of reference barcode records. To assist educators 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. Visit EducationAndBarcoding.org for more information.

Sanger Sequencing

For use in barcoding, amplified DNA samples must 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. In the 1970s, two research groups simultaneously developed different methods for DNA sequencing: the Maxam-Gilbert method and the Sanger method. At that time, sequencing was a laborious process requiring the use of hazardous chemicals. Even after days of work, only relatively short sequences were obtained.

Today, most researchers send their samples to core university or commercial laboratory facilities where, for a nominal charge, their samples are sequenced using an automated sequencer. Depending on the facility, other preliminary services may also be available such as 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 a few days or less.

Sanger sequencing, a commonly used sequencing technique, is a type of cycle sequencing in which numerous cycles of primer extension are used with modified nucleotides to determine the sequence. 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 sequencing reaction based on the sequence of the target gene.
- DNA polymerase
- Nucleotides
- Modified nucleotides called dideoxynucleotides (ddNTP, dideoxynucleotide triphosphate) which lack the 3'-hydroxyl group needed for elongation of the DNA molecule. Each type (ddATP, ddGTP, ddCTP, and ddTTP) is commonly coupled with a different fluorescent dye.

Then, DNA synthesis proceeds in the reaction tube.

During synthesis, most 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. When a ddNTP is incorporated, DNA synthesis will stop on that strand, as there is no 3'-hydroxyl to form the next phosphodiester bond. Each synthesized strand will include only one ddNTP and therefore will have only one fluorescent marker. 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. Since the inclusion of a ddNTP is random and rare compared to the inclusion of a standard nucleotide, the sequencing reaction produces fragments of all lengths and with different labels on the ends depending on which ddNTP was the final one incorporated.

The different-sized DNA fragments are then separated by a capillary electrophoresis instrument equipped with lasers to detect the fluorescence of the four different dyes that 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 students' 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 higher quality sequence data from each PCR product, the PCR samples will be sequenced using two sequencing reactions, one run in the forward direction and a second in the reverse direction. The sequencing primers match sequences at the tail ends of the PCR primers that are not part of the actual target genes. Ideally, each sequencing reaction will yield the same result, but in reverse complement.

The primers included in the PCR modules have been designed to amplify barcoding genes for a wide range of species and generate sufficient DNA for sequencing. However, despite using conserved genes for barcoding, there is enough sequence variability across species that PCR amplification using the provided primers may still result in poor or no amplification. To increase the likelihood of successful PCR amplification, the primer sets contain degenerate nucleotide positions, further increasing the chance of primer binding. For more information about degenerate primers, see Appendix C.

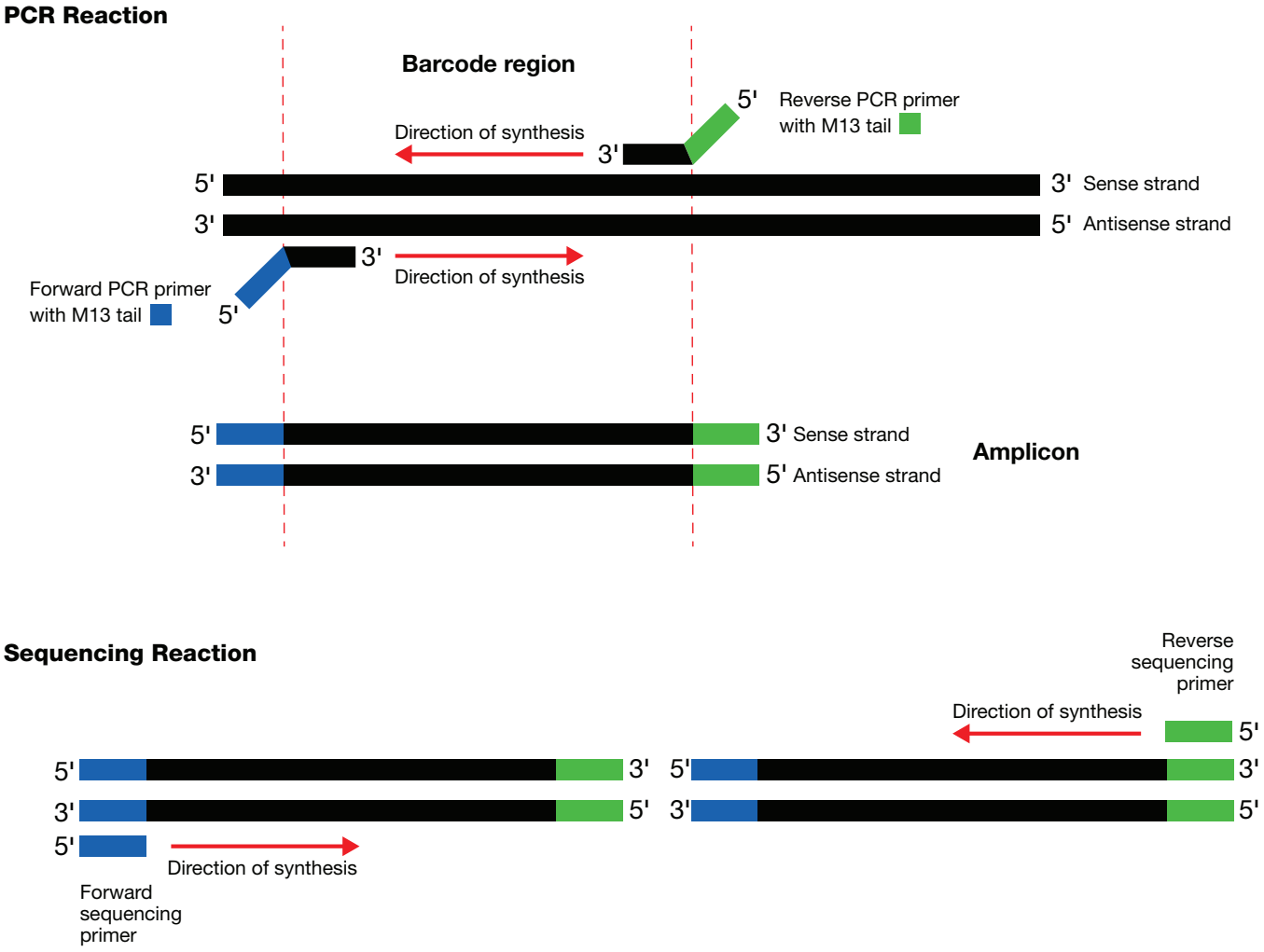


Fig. 2. Amplification and sequencing of a barcoding region using the polymerase chain reaction. PCR amplification of a portion of DNA uses a pair of primers together in the same reaction resulting in an amplicon (double-stranded PCR product) of defined length. The PCR primers have tails that are not part of the target, but produce an amplicon with an M13 primer sequence at each end. Sequencing reactions also use a template of double-stranded DNA, however, sequencing reactions use only one primer per reaction and create single strands of DNA terminated by ddNTPs.

Fast Gel Protocol and Electrophoresis Preparation Instructions

See **bio-rad.com/barcodingvideos** for how-to videos on casting, loading, and running agarose gels.

Fast Gel Protocol

There are multiple ways to hasten visualization of DNA bands on an agarose gel using modified conditions and alternative reagents. Options, as well as the required materials and protocols, are provided below.

1. Cast 1% agarose gels with 1x TAE buffer.
2. Prepare 0.25x TAE electrophoresis buffer.
3. Prepare DNA samples for electrophoresis using 6x Loading Dye and Stain.
4. Load samples and run the gel using conditions in Table 16.

Table 16. Electrophoresis options.

Electrophoresis Buffer and Voltage	Electrophoresis Time, min
0.25x TAE at 300 V*	10
0.25x TAE at 200 V	20
1x TAE at 100 V	30

* Requires power supply capable of voltages of 300 V, such as the PowerPac Basic Power Supply (catalog #1645050EDU).

5. After gel electrophoresis, visualize instantly with a UV transilluminator or a gel imaging system.

Preparing Agarose Gels

Cast either eight 7 x 7 cm gels with one 8-well comb for eight workstations or four 7 x 10 cm gels with two 8-well combs each to be shared between two workstations. Table 17 provides measurements for a variety of options.

Table 17. Volumes and quantities of reagents for agarose gels.

Number of Gels	1	4	8	16
1% TAE agarose gel (7 x 7 cm) — serves one workstation				
Purified water, ml	39	156	312	624
50x TAE, ml	0.8	3.2	6.4	12.8
Agarose, g	0.4	1.6	3.2	6.4
Total volume of molten agarose, ml	40	160	320	640
1% TAE agarose gel (7 x 10 cm) — serves two workstations				
Purified water, ml	49	196	392	784
50x TAE, ml	1.0	4.0	8.0	16.0
Agarose, g	0.5	2.0	4.0	8.0
Total volume of molten agarose, ml	50	200	400	800

Prepare molten agarose

1. Add the appropriate amount of agarose powder and then the liquids to a suitable container; fill to less than 50% of the container volume. Swirl to mix.

   Note: If using an Erlenmeyer flask, invert a small 25 ml beaker over the opening to minimize evaporation. If using a bottle, loosen the cap so that air and steam can escape.

2. Place the agarose solution in the microwave. Microwave for 3 min. Continue to heat in 30 sec increments until the solution boils and all agarose has completely dissolved. Use caution when swirling as this may cause superheated agarose to suddenly boil over.

   Caution: Always wear heat-protective gloves, goggles, and a lab coat while preparing agarose gels. Hot molten agarose can cause severe burns.

3. Let the agarose cool to about 60°C before pouring the gels.

Cast agarose gels

There are a variety of ways to cast agarose gels. This section outlines the tape method. Consult the instruction manual for your horizontal electrophoresis system for alternative methods.

1. Firmly seal the ends of a gel tray with standard laboratory tape (not regular sticky tape).

2. Place the comb in the appropriate slot in the gel tray. If pouring a double-well gel, place a comb at one end of the tray and another in the middle.

3. Once the molten agarose has cooled to 60°C or less, pour enough agarose to cover the gel comb teeth or to a depth of 0.5–0.75 cm.

4. Allow the gel to solidify at room temperature for 10–20 min.

5. Carefully remove the comb(s) and the tape.

6. Store gels in a sealable plastic bag at room temperature for up to 1 day or in the refrigerator for up to 1 week.

Preparing TAE Buffer

Conventionally, 1x TAE buffer is used both for gel casting and as running buffer. The electrophoresis time can be greatly reduced by instead running the gels with 0.25x TAE buffer at 200 or 300 V. When using this faster protocol, gels should still be cast using 1x TAE buffer.

1. Combine distilled water with the volume of 50x TAE buffer indicated in Table 18 and mix well.

   Note: If you are using 0.25x TAE buffer sequentially between classes, ensure the buffer is at or below room temperature before using. If the buffer starts out warm, it may become hot enough to melt the agarose gel during a high-voltage run.

Table 18. Volumes and quantities of reagents for electrophoresis buffer.

Number of Electrophoresis Chambers	1	4	8	16
0.25% TAE Buffer				
Distilled water, ml	274	1,094	2,189	4,378
50x TAE, ml	1.4	5.6	11	22
Total volume of 0.25% TAE buffer, ml	275	1,100	2,200	4,400

PCR Amplification 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:

- **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 (2112006EDU and 2112016EDU) are ideal pipet tips to use in PCR reactions. For this laboratory, aerosol barrier tips should be used for PCR.
- **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 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.
- **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.
- **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.

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.

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 it 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 are the same in all of the species being compared, and N represents a base that varies between the 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, a set of primers may be used where one or more bases within the primers is replaced with other 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.

In practice, the full set of degenerate primers could be added to a PCR reaction. However, if there are multiple wobble bases in a primer sequence, the total combinations can quickly add up. Therefore, in many cases, not all of the bases are used at each variable base. Selecting which bases to include in the final set of primers is usually based on maintaining the highest probability that the primer will anneal to the target DNA. In some situations, 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 as shown in Table 19.

Table 19. 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.

Extension Topics

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). Inheritance of mtDNA from both parents occurs more regularly in some bivalves, an example of which is mussel (Hoeh et al. 1991).

Extension References

Fontaine KM et al. (2007). Evidence for paternal leakage in hybrid periodical cicadas (Hemiptera: *Magicicada* spp.). PLoS One 2, 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.

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

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 because 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 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). However, whole genome sequencing is now cheap and rapid enough that it is more common and even expected for some applications. How do prokaryotes transfer genetic material horizontally? Among the several mechanisms are:

Transformation — the process of uptake and expression of foreign DNA. Transformation is a commonly used technique in biotechnology for targeted gene or protein expression.

Transduction — a process during which bacterial DNA from one individual is transferred to another through a viral intermediate (that is, bacteriophage infection).

Bacterial conjugation — the process by which transfer of genetic information from one bacterium to another occurs through direct physical contact.

Extension References

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

Resources

Visit **bio-rad.com/barcoding** for additional classroom resources

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, bostonglobe.com/business/specials/fish, accessed Jun 18, 2021.

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 Sci 270, 313–321.

International Consortium of Investigative Journalists (Oct. 6, 2011). Hake DNA testing: How we did it. Center for Public Integrity, publicintegrity.org/2011/10/06/6862/hake-dna-testing-how-we-did-it, accessed June 18, 2021.

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. (2011). 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 (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 Feb. 23, 2018). DNA-Based Seafood Identification. fda.gov/food/science-research-food/dna-based-seafood-identification, accessed June 18, 2021.

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 Res Int 41, 828–837.

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