



DNA Barcoding: From Samples to Sequences

Introduction

In this lesson, students perform the wet lab experiments necessary for DNA barcoding. Beginning with a small tissue sample, students purify the DNA, perform the **polymerase chain reaction (PCR)** using *COI*-specific **primer pools**, and analyze their PCR products by **agarose gel electrophoresis**. PCR reactions that result in products of the correct size are purified and submitted for **DNA sequencing**. This DNA sequence data can be used in *Lesson Nine*, or as part of an independent project.

Learning Objectives

At the end of this lesson, students will know that:

- DNA barcoding involves multiple laboratory experiments before bioinformatics analyses are performed: **DNA purification**, polymerase chain reaction (PCR), agarose gel electrophoresis, **PCR purification**, and submission of the sample(s) for DNA sequencing.
- DNA must be purified from a tissue sample before DNA barcoding through a process involving cell **lysis** and separation of the DNA from the rest of the cell debris.
- Polymerase chain reaction (PCR) is used to **amplify** (make many copies of) a gene or region of DNA that can be used in subsequent analyses.
- Agarose gel electrophoresis is performed to confirm whether a PCR reaction was successful, resulting in a band of the appropriate size.
- The copied DNA (or PCR product) is “purified” before DNA sequencing to remove PCR **reagents** from the solution containing the copied DNA.

At the end of this lesson, students will be able to:

- Purify DNA from a small tissue sample.
- Perform PCR on the purified DNA sample.
- Use agarose gel electrophoresis to determine whether their PCR was successful.
- Prepare their PCR product for DNA sequencing (“PCR purification”).
- Develop a conceptual map that charts out the basic steps involved in many kinds of genetic research, from DNA purification to DNA sequencing and analysis.

Class Time

6 class periods of 50 minutes each:

- DNA Purification: 2 class periods
- PCR: 1 class period, plus overnight
- Agarose Gel Electrophoresis: 2 class periods
- Preparation of Samples for Sequencing: 1 class period

Prior Knowledge Needed

- DNA is the blueprint of life.
- Basic cell biology (DNA is found in the nucleus, mitochondria contain DNA and produce ATP).
- Exposure to the Bio-ITEST Advanced curriculum, *Using Bioinformatics: Genetic Research*, is highly recommended. This lab is intended to be done between *Lesson Eight: Exploring Bioinformatics Careers* and *Lesson Nine: Analyzing DNA Sequences and DNA Barcoding*. See the *Unit Overview* for additional explanations and other options for differentiation.
- Lab skills:
 - Micropipetting (required).
 - Balancing samples in a centrifuge.
 - Experience using DNA gels (helpful).

Key Concepts

- DNA barcoding involves experiments in the laboratory and on the computer.
- In the laboratory, genetic researchers must purify their DNA from a tissue sample, copy the gene or region of interest using PCR, and assess whether their PCR was successful using agarose gel electrophoresis.
- Scientific experiments build on what is **already known** about a given subject or field, using this information and observations as background when asking scientific questions. In the case of DNA barcoding, scientists use information about the expected size of the barcoding gene, and sequence information from previous experiments to design PCR **primers**.
- The DNA sequence data that results from the laboratory experiments can be used in bioinformatics analyses.

Materials

General Equipment	Quantity
Micropipettes and tips (P20, P200, and P1000) [Note: Laboratory protocols have been designed for use with traditional scientific micropipettes, or classroom micropipettes that are only adjustable in 5 µl increments.]	1 each per group (up to 1 per student)
Microcentrifuge tube racks	1 per group (up to 1 per student)
Waste buckets	1 per group
Test tube labeling pens (such as Sharpies®)	1 per group (up to 1 per student)
-20 °C Freezer to store samples [Note: Not necessary if experiments will be performed back-to-back. Purified DNA (<i>Lab 1</i>) and PCR products (<i>Lab 2</i>) are stable in the refrigerator (4°C) for up to 72 hours.]	1
High speed microcentrifuge (with a speed up to 10,000 rpm), needed for <i>Lab 1</i> and <i>Lab 4</i> [Note: A low speed microcentrifuge can be used for <i>Lab 2</i> and <i>Lab 3</i> .]	1 (up to 1 per group)
Gloves, various sizes	1 pair per student per day
Teacher Resource— <i>Aliquoting DNA Barcoding Reagents for Labs 1–4</i>	1

Lab 1: DNA Purification for DNA Barcoding	Quantity
DNA purification reagents or kit <i>Recommended: ZR Genomic DNA™-Tissue MiniPrep.</i> Available from Zymo Research. Item #D3050 (50 reactions) or D3051 (200 reactions) http://www.zymoresearch.com/	1 reaction per student
Samples (such as fish, meat, insects, etc.) [Note: See <i>Obtaining Samples for DNA Barcoding</i> below for more information.]	1 per student
Nuclease-free, ultra-pure (i.e., nano-pure) or distilled water <i>Recommended: Nuclease-Free Water.</i> Available from QIAGEN. Item #129115 (1000 ml) www.qiagen.com/	Approximately 100 µl per student
Class set of Student Handout— <i>DNA Purification for DNA Barcoding</i>	1 per student (class set)
Razor blades (or alternative means to shred tissue, such as plastic butter knives) and plastic dishes or small plates	1 per student
Vortexers [Note: Not necessary, but highly recommended.]	1–2 per class (up to 1 per group)
55°C Water bath or incubator	1
1.7 ml microfuge tubes	2 per student

Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)	Quantity
PCR reagents or kit <i>Recommended: GE Healthcare* illustra* PuRe Taq Ready-To-Go* PCR Beads 0.2 ml tube w/hinged cap.</i> Available from Fisher Scientific. Item #46-001-014 http://www.fishersci.com/	1 per student
Nuclease-free, ultra-pure (i.e., nano-pure) or distilled water <i>Recommended: Nuclease-Free Water.</i> Available from QIAGEN. Item #129115 (1000 ml) www.qiagen.com/	Approximately 50 µl per student
PCR barcoding primer pools <i>Available from NWABR –OR–</i> Primers may be ordered from a commercial producer, such as Eurofins MWG Operon. http://www.operon.com Primer sequences are available in Ivanova et al., 2007 (see <i>Resources</i>).	5.0 µl per student
Class set of Student Handout— <i>Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)</i>	1 per student (class set)
Thermocycler for PCR	1
Racks to hold 0.2 ml PCR tubes (such as empty P200 or P1000 tip box bases)	1 per student –OR– 1 per group
Ice buckets with ice	1 per group
1.7 ml microfuge tubes	1 per student

Lab 3: Analyzing PCR Results with Agarose Gel Electrophoresis	Quantity
Agarose <i>Recommended:</i> Agarose, LE Molecular Biology Grade . Available from Hardy Diagnostics. Item #C8740 (100 gm) or #C8741 (500 gm) http://www.hardydiagnostics.com	Approximately 500 mg per 2 students (i.e., 2 students per gel)
6X DNA loading dye <i>Recommended:</i> 6X DNA Loading Dye, 5 x 1 ml . Available from Fisher Scientific. Item # FERR0611, http://www.fishersci.com/ [Note: Some molecular weight standards include free 6X loading dye (see below). In addition, <i>Lab 3</i> has been designed for use with classroom micropipettes that adjust in 5 ml increments, requiring 6X loading dye to be diluted to 3X, as described in Teacher Resource— <i>Aliquoting DNA Barcoding Reagents for Labs 1–4</i> .]	2.5 µl per student
DNA molecular weight standard <i>Recommended:</i> GeneRuler 1 kb Plus DNA Ladder, ready-to-use . Available from Fisher Scientific. Item #FERSM1334, http://www.fishersci.com/	15 µl per 2 students (i.e., 15 µl per gel)
1X Tris Acetate EDTA (TAE) Buffer (“DNA Gel Buffer”) <i>Recommended:</i> 50X TAE Buffer, 1 L . Available from Fisher Scientific. Item #FERB49 http://www.fishersci.com/ [Note: Dilute 50X TEA buffer to 1X with deionized or distilled water before use.]	Approximately 50–100 ml per 2 students (i.e., 50–100 ml per gel)
Nontoxic DNA gel stain <i>Recommended:</i> Fast Blast™ DNA Stain, 100 ml . Available from Bio-Rad. Item #166-0402EDU, http://www.bio-rad.com/ [Note: Fast Blast™ DNA Stain should be prepared with deionized or distilled water.]	Approximately 50–100 ml per 2 students (i.e., 50–100 ml per gel)
Class set of Student Handout— <i>Analyzing PCR Results with Agarose Gel Electrophoresis</i>	1 per student (class set)
55°C Water bath or incubator [Note: Not necessary, but helpful to cool agarose to pouring temperature if desired.]	1
DNA gel boxes with casting stands and combs	1 per group
Power supplies [Note: Most power supplies can power up to two DNA gel boxes.]	1 per 2 groups
Erlenmeyer flasks or glass bottles for melting agarose	1 per 2 students (i.e., 1 per gel)
Microwave for melting agarose	1
Hot pads for handling hot agarose	1 per group
1.7 ml microfuge tubes	1 per student
Light Box [Note: Not necessary, but highly recommended for visualizing DNA gels stained with Fast Blast™ DNA Stain or other non-toxic stain.]	1

Lab 4: Preparation of PCR Samples for DNA Sequencing	Quantity
PCR purification reagents or kit <i>Recommended:</i> DNA Clean & Concentrator™-5 (capped columns) . Available from Zymo Research. Item #D4013 (50 reactions) or D4014 (200 reactions) http://www.zymoresearch.com/	1 reaction per student
Nuclease-free, ultra-pure (i.e., nano-pure) or distilled water <i>Recommended:</i> Nuclease-Free Water . Available from QIAGEN. Item #129115 (1000 ml) www.qiagen.com/	Approximately 30 µl per student
Class set of Student Handout— <i>Preparation of PCR Samples for DNA Sequencing</i>	1 per student (class set)
Spectrophotometer and cuvettes to measure DNA concentration (optional)	1 spectrophotometer; 1 cuvette per sample

Computer Equipment, Files, Software, and Media
Computer and projector to display PowerPoint slides. Alternative: Print PowerPoint slides onto transparencies and display with overhead projector.
<i>Wet Lab</i> PowerPoint Slides— <i>DNA Barcoding: From Samples to Sequences</i> . Available for download from the “Lessons” tab at: http://www.nwabr.org/curriculum/advanced-bioinformatics-genetic-research .
“DNA Barcoding” animation. This two-part animation introduces students to the concept of DNA barcoding (<i>Part I</i> , run time 1 minutes 35 seconds), and the laboratory and bioinformatics tools needed to obtain and analyze DNA data (<i>Part II</i> , run time 4 minutes 10 seconds). Available from the “Resources” tab at: http://www.nwabr.org/curriculum/advanced-bioinformatics-genetic-research .
Optional for Lab 2: “Polymerase Chain Reaction” video freely available from Howard Hughes Medical Institute (HHMI). Video is 1 minute 27 seconds long and requires an internet connection and speakers. Available at: http://www.hhmi.org/biointeractive/media/DNAi_PCR-Ig.wmv .
Optional for Lab 2: “Polymerase Chain Reaction (PCR)” interactive tutorial. DNAi.org contains a number of great online resources and tutorials, including one about Polymerase Chain Reaction (PCR). <ol style="list-style-type: none"> 1. Visit: http://www.dnai.org/b/index.html. 2. Select “Techniques” from the bottom menu bar. 3. Select “Amplifying” from the top menu bar. 4. From the side menu bar select “Making many copies of DNA” for a click-through 2D animation of polymerase chain reaction.
Optional for Lab 3: “How to Make and Run an Agarose Gel (DNA Electrophoresis)” video freely available on YouTube by Labtricks. Video is 2 minutes 54 seconds long. Available at: http://www.youtube.com/watch?v=2UQloYhOowM .
Optional for Lab 3: “DNA Electrophoresis Sample Loading” video freely available on YouTube from Greg Peterson, Coordinator of Biotechnology, Kirkwood Community College, Math/Science Department. Video is 3 minutes 30 seconds long. Available at: http://www.youtube.com/watch?v=tTj8p05jAFM&feature=related .
Optional for Lab 3: “Loading a Gel for Electrophoresis” video freely available on YouTube from Carolina Biologicals. Video is 5 minutes 46 seconds long. Available at: http://www.youtube.com/watch?v=h06rz8rcZpw&feature=related .
Optional for Lab 4: “Sanger Method of DNA Sequencing” video freely available from the Howard Hughes Medical Institute (HHMI). Video is 51 seconds long and requires internet connections and speakers. Available at: http://www.hhmi.org/biointeractive/dna/DNAi_sanger_sequencing.html .
Optional for Lab 4: “The Sanger Method of DNA Sequencing” by the Wellcome Trust. This animation is text based, which is useful when classroom computers are not equipped with speakers, and is freely available at: http://www.wellcome.ac.uk/Education-resources/Teaching-and-education/Animations/DNA/WTDV026689.htm .

Obtaining Samples for DNA Barcoding

Any tissue sample that contains DNA can be used for DNA barcoding. However, certain types of samples are easier for students to work with than others.

Suggested samples include:

- Small pieces of fish or shellfish from a local grocery store, market, or restaurant.
- Small pieces of meat (beef, pork, or poultry) from a local grocery store, market, or restaurant.
- Insects, reptiles, amphibians, or fish (including immature life stages such as eggs, larva, or tadpoles), collected from local ecosystems, such as parks, lakes, or oceans.
- Canned dog or cat food.
- Samples obtained from zoos, aquariums, or wildlife parks. These are often called “convenience samples,” which are left over after being collected for routine veterinary exams.

DNA can be purified from very small samples. The experiments below involve samples less than 25 milligrams (mg) in size – about half the size of a pencil eraser. While DNA is often purified from “raw” samples, DNA can be purified from cooked samples, such as canned meats, dog or cat food, or leftover meat from a meal at a restaurant.

DNA barcoding has been used by students in New York City to determine whether seafood available at markets and restaurants is correctly labeled. Other projects include identification of wildlife in local parks or streams, and the meat components of canned pet food.

Samples can be stored in the refrigerator (short-term, 1–3 days) or frozen until needed.

LAB 1: DNA Purification for DNA Barcoding

Teacher Preparation

- In advance of the laboratory experiments, have students obtain samples for **DNA purification** (see *Obtaining Samples for DNA Barcoding* above).
- Pre-heat the water bath or incubator to 55 °C. Depending upon the size of your water bath, this may take an hour or two, or even overnight.
- Load the classroom computer with the *Wet Lab* PowerPoint slides.
- Make copies of the Student Handout—*DNA Purification for DNA Barcoding*, one per student. These handouts are designed to be reused as a “class set.” Students should write their answers to questions and take notes on a separate sheet of paper or in their lab notebook, not directly on the handout.

DNA purification: Extracting the DNA from a cell, and purifying it away from the remaining cellular components.

- Teachers may wish to have their students write out the lab procedures before the activity in their lab notebooks or on a separate piece of paper as a “pre-lab” exercise, which can be used as an “entry ticket” to class. During the lab, students may check off steps as they complete them and/or describe and draw pictures of their observations during the laboratory activities.
- Review micropipetting and the use of a microcentrifuge with students, if needed.
- Set up student work stations by distributing supplies and **reagents** needed for each group, as described above under *Materials*. It is suggested that reagents from the stock bottles contained in the **ZR Genomic DNA™-Tissue MiniPrep** kit be aliquoted for student groups and labeled as described in Student Handout—*DNA Purification for DNA Barcoding*. This information is found in Teacher Resource—*Aliquoting DNA Barcoding Reagents for Labs 1–4*.

Reagents: Ingredients or components used in an experiment.

Procedure

Day One

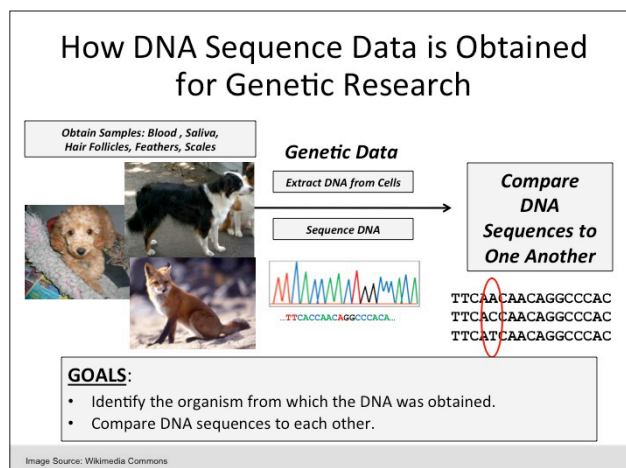
1. Explain to students the **aim of this lesson**. Some teachers may find it useful to write the aim on the board.

Lesson Aim: Obtain DNA sequence data from the barcoding **COI gene** for the sample(s) students have chosen.

COI gene: Cytochrome c oxidase subunit 1 gene.

Teachers may also wish to discuss the *Learning Objectives* of the lesson, which are listed at the beginning of this lesson plan.

2. Show **Slide #1**, “How DNA Sequence Data is Obtained for Genetic Research.” Remind students that genetic research involves obtaining samples from the species they will be studying, extracting and sequencing the DNA, and then comparing the *COI* gene sequences from their species to other species in databases like those at the National Center for Biotechnology Information (NCBI) and the Barcode of Life Database (BOLD).



Wet Lab: **Slide #1**

Polymerase chain reaction (PCR):

A scientific technique in molecular biology used to **amplify** (i.e., copy) a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Agarose gel electrophoresis:

Electrophoresis refers to the process of using an electric field to move molecules through a gel matrix. In the case of DNA, agarose is used to form the gel matrix, and the electrical current separates DNA fragments based on size, with smaller (or lower **molecular weight**) fragments moving to the positive electrode more quickly than larger fragments.

PCR purification: Extracting or purifying the PCR product (DNA fragment) away from the remaining PCR components.

DNA sequencing: The process of determining the identity and order of bases in a molecule of DNA.

Detergents: Substances (for example, soap), that contain both hydrophobic and hydrophilic regions, used to dissolve **lipids** (i.e., cell membranes).

Cell membrane: Phospholipid bilayer surrounding the cell.

Spin columns: A small column that fits inside of a microfuge tube and contains a material (such as silica) that binds to nucleic acids such as DNA. The spin column is used in conjunction with centrifugation (or vacuum pressure) to purify DNA.

Centrifugal force: The apparent force that seems to pull an object outward when the object is spun around in a circle.

Elute: To extract or remove one material from another, often by adding a solvent such as a buffer.

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From Samples to Sequences

- Obtain samples
- Purify the DNA
- Copy your gene
- Make sure you copied your gene
- Obtain DNA sequence data
- *Aquarium, zoo, grocery*
- *Lab 1: DNA Purification*
- *Lab 2: Polymerase Chain Reaction*
- *Lab 3: Agarose Gel Electrophoresis*
- *Lab 4: PCR Purification and DNA Sequencing*

3. Show *Slide #2*, “From Samples to Sequences.” Tell students that they will be performing a series of laboratory experiments. These are the same experiments that genetic researchers perform every day.
 - a. Obtain samples from the species they will be studying.
 - b. Perform **DNA purification**, which is *Lab 1*, and is the focus of this first two-day experiment.
 - c. Copy the DNA barcoding gene, *COI*, using the **polymerase chain reaction (PCR)**, which is *Lab 2*.
 - d. Confirm PCR results using **agarose gel electrophoresis** (*Lab 3*).
 - e. Prepare samples for DNA sequencing by performing a **PCR purification** experiment (*Lab 4*) and submit the samples to a **DNA sequencing** facility.
4. Show *Slide #3*, “DNA Purification Overview,” and review with students the steps involved in purifying DNA:
 - a. First, break open the cells. This is done by chopping the tissues into tiny pieces and adding **detergents** (which break down the **cell membranes**) and heat.
 - b. Second, separate the DNA from the rest of the cell debris. Genetic researchers use something called a **spin column**, which contains a membrane made of material (such as silica) that has an affinity for DNA. The DNA binds to the membrane in the spin column, and the remaining cell debris washes through the tiny holes in the membrane when the spin column is subjected to **centrifugal force** in a microcentrifuge.
 - c. Finally, **elute** (remove) the DNA from the column using a buffer. The buffer changes the pH in the spin column and causes the DNA to no longer bind to the spin column. This buffer will also keep the DNA stable for future use.

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DNA Purification Overview

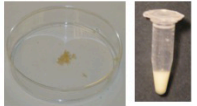


<ol style="list-style-type: none"> 1. Break open the cells. 2. Separate the DNA from the rest of the cell debris. 3. Remove DNA from the spin column and suspend the DNA in buffer for future use. 	<ul style="list-style-type: none"> • <i>Chop tissues and add detergents (disrupt cell membranes), proteinase K, and heat.</i> • <i>Small “Spin Columns” contain DNA-binding material with small holes.</i> • <i>Columns bind DNA, other cellular debris washes through column.</i> • <i>“Elute” the DNA, or remove it from the column.</i>
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Wet Lab: **Slide #3****Lysis or “to lyse”:** To break open.**Proteinase K:** Type of enzyme that breaks down proteins, including **nucleases**.**Nuclease:** Type of enzyme that breaks down nucleic acids.**Enzyme:** A type of protein that catalyzes (increases the rate of) chemical reactions. For example, ATP synthase is an enzyme that catalyzes or facilitates the creation of ATP.**Nuclease-free water:** Water that does not contain **nucleases**. Often this water has been subjected to multiple rounds of purification, including being passed through a nano-filter. It is sometimes referred to as “nano-pure” or “ultra-pure” water, as it should contain only H₂O, with no dissolved salts or other contaminants.**Buffer:** A substance used to stabilize or maintain the pH of a solution.

5. Show **Slide #4**, “DNA Purification Using ‘Spin Columns,’” which reviews the steps in DNA purification once again, with added visuals and more details about the laboratory protocol. The materials used are called “spin columns” because scientists use a microfuge to “spin” the DNA-binding columns and speed the DNA purification process. Review these steps with students:
- Day 1: Lyse** your sample (break open the cells). This step involves adding to your sample a lysis solution and **proteinase K**, an **enzyme** that breaks down other proteins. This is mixed in **nuclease-free water**. Nuclease-free water (such as distilled or nano-pure water) contains no enzymes (**nucleases**) that would break down DNA. The mixture is then heated to aid the digestion process, as proteinase K activity increases at higher temperatures.
 - Day 2:** Add your sample to the spin column to bind the DNA to the membrane.
 - Wash away the cell debris with a wash solution. For this experiment, students will perform two wash steps – one with a pre-wash solution, and one with a wash solution.
 - Elute your DNA in **buffer**.

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DNA Purification Using “Spin Columns”

<ol style="list-style-type: none"> 1. Chop up tissues and break open the cells with detergents. 2. Separate the DNA from the rest of the cell debris using spin column and centrifugation. 3. Suspend the DNA in buffer for future use. 	<p>1. Tissues Chopped and Broken Open with Detergents</p>  <p>2. Spin Column</p>  <p>3. DNA in Buffer</p> 
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Wet Lab: **Slide #4**


6. Pass out Student Handout—*DNA Purification for DNA Barcoding* and have students work through the activity in small groups of up to 4 students each.

Procedure

Day Two

7. As students enter the class, project **Slide #5**, “DNA Purification Overview,” to remind students of the purpose of today’s lab. Students lysed their cells overnight and will complete the purification today.

Wet Lab: **Slide #5**



DNA Purification Overview

1. Break open the cells.
2. Separate the DNA from the rest of the cell debris.
3. Remove DNA from the spin column and suspend the DNA in buffer for future use.

- Chop tissues and add detergents (disrupt cell membranes), proteinase K, and heat.
- Small “Spin Columns” contain DNA-binding material with small holes.
- Columns bind DNA, other cellular debris washes through column.
- “Elute” the DNA, or remove it from the column.

8. When students have completed their DNA purification, collect their samples and store them in the refrigerator (short term storage, up to 3 days) or in the freezer (long term storage).

Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)

Teacher Preparation

- Load the classroom computer with the *Wet Lab* PowerPoint slides.
- Make copies of the Student Handout—*Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)*, one per student. These handouts are designed to be reused as a class set; students should write answers to questions and take notes on a separate sheet of paper or in their lab notebook.
- Teachers may wish to have their students write out the lab procedures before the activity in their lab notebooks or on a separate piece of paper as a “pre-lab” exercise, which can be used as an “entry ticket” to class. During the lab, students may check off steps as they complete them and/or describe and draw pictures of their observations.
- Set up student work stations by distributing supplies and reagents needed for each group, as described above under *Materials*. It is suggested that reagents be aliquoted for student groups and labeled as described in Student Handout—*Preparation of PCR Samples for DNA Sequencing*. This information is found in Teacher Resource—*Aliquoting DNA Barcoding Reagents for Labs 1–4*.

- If students are not familiar with PCR, queue the classroom computer to the HHMI video, “Polymerase Chain Reaction,” and/or the DNAi.org interactive tutorial on PCR. See *Computer Equipment, Files, Software, and Media* in the *Materials* section above for URLs.

Procedure

9. Show **Slide #6**, “Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)” as students enter the room.

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Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)

1. Obtain samples.
2. Extract the DNA.
3. **Copy your gene.**
4. Make sure you copied your gene.
5. Obtain DNA sequence data.

Wet Lab: **Slide #6**

10. Show **Slide #7**, and remind students that their overall goal is to obtain DNA sequence data from the species they are studying. In the last experiment, students purified DNA from their samples. Today, they will **amplify**, or copy, the DNA barcoding gene using polymerase chain reaction (PCR).

Amplify: In PCR, to amplify is to increase in copy number.

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From Samples to Sequences

- Obtain samples
- Purify the DNA
- **Copy your gene**
- Make sure you copied your gene
- Obtain DNA sequence data

- *Aquarium, zoo, grocery*
- *Lab 1: DNA Purification*
- ***Lab 2: Polymerase Chain Reaction***
- *Lab 3: Agarose Gel Electrophoresis*
- *Lab 4: PCR Purification and DNA Sequencing*

Wet Lab: **Slide #7**

11. If students are not already familiar with PCR, it is strongly suggested that you show the online video, “Polymerase Chain Reaction,” freely available from Howard Hughes Medical Institute (HHMI). The video is 1 minute 27 seconds long and requires an internet connection and speakers. It is available at: http://www.hhmi.org/biointeractive/media/DNAi_PCR-Ig.wmv.

You may also wish to work through the “Polymerase Chain Reaction (PCR)” interactive tutorial available from DNAi.org:

- Visit: <http://www.dnai.org/b/index.html>.
 - Select **Techniques** from the bottom menu bar.
 - Select **Amplifying** from the top menu bar.
 - From the side menu bar, select **Making many copies of DNA** for a click-through 2D animation of polymerase chain reaction.
12. Show **Slide #8**, “The Power of PCR,” and emphasize to students that PCR is a very powerful technique, making it possible to copy a gene of interest many, many times. Starting with a single copy of a gene, PCR results in over a billion copies in just 30 PCR cycles, which takes about 2–3 hours. Having so many copies of a gene is what makes it possible to continue our genetic research analyses.

Wet Lab: **Slide #8**

[**Note:** During DNA purification and PCR, special purified water is used, such as deionized, “ultra-pure” or “nano-pure” water, which has been subjected to multiple rounds of purification to remove all contaminants. If nano-pure or nuclease-free water is not available, deionized or distilled water may be used.]

PCR beads: Lyophilized or “freeze-dried” PCR ingredients.

DNA template: The DNA used as instructions to make more DNA, such as in PCR.

DNA polymerase: The enzyme that assembles new DNA molecules, in the cell or *in vitro* in the laboratory, using a DNA template and **deoxyribonucleotide triphosphates (dNTPs)**.

Taq DNA polymerase: A type of heat-stable DNA polymerase, purified from the thermophilic (“heat-loving”) bacteria, *Thermus aquaticus*.

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The Power of PCR


Number of PCR Cycles (n)	Copies of DNA (2^n)
0	1
1	2
2	4
3	8
4	16
5	32
6	64
7	128
8	256
9	512
10	1024
20	1,048,576
30	1,072,741,824

13. Show **Slide #9**, which reviews the ingredients (sometimes called reagents), necessary for PCR. Tell students that they will be using **PCR beads** (as seen in the upper right corner of **Slide #9**, photographed next to the microfuge tube for reference), which contain all of the ingredients for PCR **except** the **DNA template**, primers, and nuclease-free water.

- DNA template:** This is your purified DNA sample. It is called a template because, just like with DNA replication or transcription in a cell, PCR will use your DNA to make many copies of your gene.
- Taq DNA polymerase:** This is a special DNA polymerase that is used to copy the DNA and is **heat stable**, so that the enzyme is not destroyed during the high temperatures in the PCR.
- Deoxynucleotide triphosphates (dNTPs):** Just like in your cells, these are the building blocks of the new DNA molecules.
- Primers:** These are small pieces of DNA that are specific for the DNA barcoding gene. They bind to the 5' and 3' regions of the gene to be copied, instructing the *Taq* DNA polymerase where to start DNA replication.
- Buffer and Water:** As this is a biological reaction, we must add buffers that mimic the inside of the cell for the *Taq* DNA polymerase to function properly.

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PCR Ingredients



PCR Tube & Bead 1.7 ml Microcentrifuge Tube

1. DNA "template" *Your purified DNA sample*
2. Taq Polymerase *Heat-stable DNA polymerase*
3. Deoxynucleotides (dNTPs) *Building blocks of DNA*
4. Primers *Small pieces of DNA bind to your gene*
5. Buffer and water *Maintain pH of reaction*

Wet Lab: **Slide #9****Deoxyribonucleotide triphosphates (dNTPs):**

These are the bases used for making DNA. They are abbreviated as dATP (deoxyadenosine triphosphate), dCTP (deoxycytosine triphosphate), dGTP (deoxyguanine triphosphate), and dTTP (deoxythymidine triphosphate). A mixture containing all four deoxyribonucleotide triphosphates can also be described as a "set of dNTPs."

Primers: Small pieces of DNA used to start or "prime" DNA synthesis, as in DNA replication and PCR.

Buffer: A substance used to stabilize or maintain the pH of a solution.

Primer pools: Collections or mixtures of primers, usually used in PCR.

14. Show **Slide #10**, which is a table from an important paper in the DNA barcoding scientific community (by Dr. Ivanova and colleagues).

Explain to students that this paper was written by genetic researchers who do DNA barcoding. They collected DNA sequence data of the barcoding gene from many different species, performed multiple sequence alignments to compare the sequences, and then created the many different PCR primers that correspond to each of these sequences to barcoding new samples or species. The different PCR primers are mixed together to create **primer pools**—collections of PCR primers that can be used with different types of samples for which the exact sequence of the DNA barcoding gene (and thus the corresponding PCR primers) is not known. **Primer Pool COI-2** has been used successfully for PCR with samples from mammals, fish, and insects. **Primer Pool COI-3** has been used successfully for PCR with samples from amphibians, reptiles, and mammals. Birds may also be barcoded, but students may wish to try both primer pools with bird samples. This process is explained further in the "DNA Barcoding" animation found in the *Materials* section.

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Genetic Researchers Developed Primers for DNA Barcoding

Table 1 PCR primer sets or cocktails used to amplify either 16S rDNA or COI, M13 tails are highlighted when present (*indicates original reference for the unaltered version of each primer)

Name	Ratio	Cocktail name / Primer sequence 5'-3'	Product / primer position	References
16S			2974-3546	
16Sae-3'	1	CGCTGTTTCTTCAAAAGAC	2954-2973	Palumbi (1996)
16Sae-5'	1	CGCTGTTTCTTCAAAAGAC	3546-3547	Palumbi (1996)
COI-1			6472-7126	
VF1a	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	This study
FR1a	1	CACTCTAGGCTTCTTCAAAAGATTTGG	7152-7127	This study
COI-2			6472-7129	
VF1a	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Helbert et al. 2004
VF1b	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1c	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1d	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1e	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1f	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1g	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1h	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1i	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1j	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1k	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1l	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1m	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1n	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1o	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1p	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1q	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1r	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1s	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1t	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1u	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1v	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1w	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1x	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1y	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1z	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
COI-3			6472-7126	
VF1a	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1b	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1c	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1d	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1e	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1f	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1g	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1h	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1i	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1j	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1k	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1l	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1m	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1n	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1o	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1p	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1q	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1r	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1s	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1t	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1u	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1v	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1w	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1x	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1y	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006
VF1z	1	TTCCTCCACACCAACCAAGGATTTGG	6446-6471	*Ivanova et al. 2006

Credit: Ivanova et al. 2007. Universal primer cocktails for fish barcoding. *Mol Ecol Notes*.

Wet Lab: **Slide #10**

15. Tell students to note on a sheet of paper or in their lab notebook which primer pool they will be using in this experiment, based on the type of sample they are working with.

[Note: Do not mix primer pool COI-2 and COI-3 together in a single PCR reaction.]

Thermocycler: Type of machine used to automate polymerase chain reaction that cycles through all of the temperatures required to complete the PCR.

16. Pass out Student Handout–*Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)*. Have students work through the activity in small groups of up to 4 students each. If students are working with many different types of samples, have those working with the same primer pools work together (i.e., all of the students using Primer Pool COI-2 for fish in one group, and all of the students using Primer Pool COI-3 for reptiles in another group). Alternatively, if pairs or groups of students are working with the same sample (i.e., the same organism from which the DNA was purified), one or more members of the group may try the PCR with one primer pool (Primer Pool COI-2), while the other member(s) of the group try the PCR with the other primer pool (Primer Pool COI-3).
17. Once students have assembled their PCR, help them place their PCR tubes in the **thermocycler**. Teachers are encouraged to create a template or table of their thermocycler for students to record where they placed their tubes, such as the ones shown in **Figure 1**. Note that different types of thermocyclers have capacities for different numbers of PCR tubes (usually 24, 36, or 96).

	1	2	3	4	5	6
A	JT1	JT2	JT0	DK1	DK2	DK0
B	CM1	CM2	CM0	GF1	GF2	GF0
C	SA1	SA2	SA0	MM1	MM2	MM0
D	FA1	FA2	FA0	TY1	TY2	TY0

	1	2	3	4	5	6	7	8	9	10	11	12
A	JT1	JT2	JT0		DK1	DK2	DK0		SA1	SA2	SA0	
B	CM1	CM2	CM0		GF1	GF2	GF0		FA1	FA2	FA0	
C									TY1	TY2	TY0	
D					MM1	MM2	MM0					
E									DO1	DO2	DO0	
F	LE1	LE2	LE0									
G												
H												

Figure 1: Examples of Tables or Templates of Student Samples in a 24-tube (left) or 96-tube (right) Thermocycler. Note that Each Cell Contains the Initials and Sample Number of Different Students.

18. The PCR experiment in the thermocycler will likely run for 2–3 hours. Most machines have an option to keep samples at 4°C indefinitely at the end of the PCR reaction run, so that the reaction may be run overnight or over the weekend. Tubes can be removed from the machine and placed in the refrigerator (short term, up to 3 days) or freezer (long term, more 3 days).

Lab 3: Analyzing PCR Results with Agarose Gel Electrophoresis

Teacher Preparation

- Load the classroom computer with the *Wet Lab* PowerPoint slides.
- Make copies of the Student Handout—*Analyzing PCR Results with Agarose Gel Electrophoresis*, one per student. These handouts are designed to be reused as a class set; students write answers to questions and take notes on a separate sheet of paper or in their lab notebook.
- Teachers may wish to have their students write out the lab procedures before the activity in their lab notebooks or on a separate piece of paper as a “pre-lab” exercise, which can be used as an “entry ticket” to class. During the lab, students may check off steps as they complete them and/or describe and draw pictures of their observations.
- If students are not familiar with agarose gel electrophoresis, queue your computer to one or more of the following tutorials:
 - “How to Make and Run an Agarose Gel (DNA Electrophoresis)” by labtricks.com (4:54 min) includes how to calculate percent agarose, make and pour an agarose gel, a close-up of sample loading on the gel, and running the gel: <http://www.youtube.com/watch?v=2UQloYhOowM>.
 - “Loading a Gel for Electrophoresis” by Carolina Biologicals (5:46 min) includes different types of pipettes used and a close-up of the proper techniques to load a pre-poured agarose gel. [Note: The first 2:50 min of the video covers loading a gel with a micropipette.]: <http://www.youtube.com/watch?v=h06rz8rcZpw&feature=related>.
 - “DNA Electrophoresis Sample Loading” from Greg Peterson, Coordinator of Biotechnology, Kirkwood Community College, Math/Science Department, includes pouring the agarose gel, preparing the DNA samples for loading on the gel, and close-ups of the “do’s and don’ts” of loading DNA samples on gels, including a number of common mistakes. [Note: The first 1:00 min of the video moves quickly, and the voice-over does not precisely match the video content; however, the sample loading examples, beginning at 1:04 min, are extremely helpful. If this video is not shown to students, teachers may want to review it to anticipate and evaluate common gel loading problems encountered by students.]: <http://www.youtube.com/watch?v=tTj8p05jAFM&feature=related>.
- Set up student work stations by distributing supplies and reagents needed for each group, as described above under *Materials*. It is suggested that reagents be aliquoted for student groups and labeled as described in Student Handout—*Analyzing PCR Results with Agarose Gel Electrophoresis*. This information is found in Teacher Resource—*Aliquoting DNA Barcoding Reagents for Labs 1–4*.
- **Visualizing DNA Gels with Fast Blast™ DNA Stain.** DNA gels can be stained quickly (i.e., in less than 20 minutes) with 100X Fast Blast™ DNA stain or overnight with 1X Fast Blast™ DNA stain. Students are instructed in Student Handout—*Analyzing PCR Results with Agarose Gel Electrophoresis* to

[Note: Students often confuse the tubes for the loading dye and the **molecular weight standard**. Both are in small, 1.7 ml microfuge tubes, and both are blue. Teachers may wish to only set out the loading dye, and hand out the molecular weight standard after all samples have been prepared. If students have no bands in their molecular weight standard lane, they may have loaded dye instead of the molecular weight standard. If there are many bands in their sample wells, they may have mixed their samples with molecular weight standard instead of loading dye.]

[Note: Fast Blast™ DNA Stain can be re-used up to 7 times.]

load 10 µl of the PCR reaction on their gel, which should contain a sufficient quantity of DNA to visualize using either staining protocol. Alternative DNA gel stains (such as Carolina Blue) could also be used. For more details, see the manufacturer's instructions.

➤ Quick Stain with 100X Fast Blast™ DNA stain:

- i. Dilute the Fast Blast stock solution 1:5 (final is 100X) with deionized water, such as 100 ml 500X Fast Blast in 400 ml water.
- ii. Stain each gel in a staining tray, Tupperware®, or other similar container in 40–50 ml of 100X Fast Blast stain (enough to completely cover the gel) for 2–3 minutes. Do not stain for more than 3 minutes.
- iii. Rinse each gel for 10 seconds in 500–700 ml of clean, warm (40–55°C) tap water in a 1-liter beaker or similar container.
- iv. Wash each gel for 5 minutes in 500–700 ml of clean, warm (40–55°C) tap water in a 1-liter beaker or similar container. Place gel and water on a rocking platform during the wash, or gently agitate the container every 30–60 seconds.
- v. Repeat the 5 minute wash step with fresh, warm water.
- vi. Visualize results by placing gels on a white light box, or in sandwich-sized zip-top plastic bags and holding them up to a light. Gels in plastic bags may also be scanned and printed for student records.

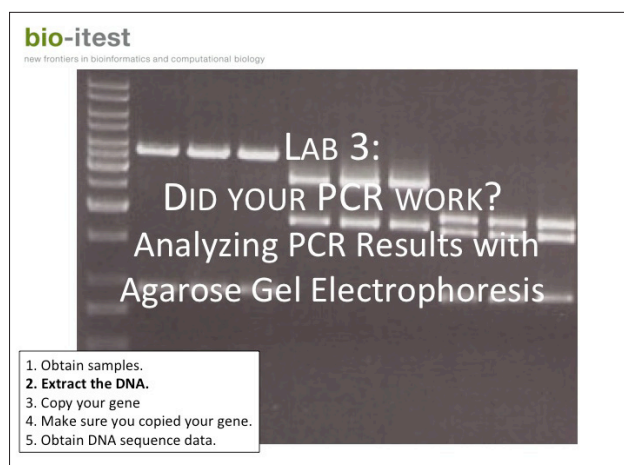
➤ Overnight Stain with 1X Fast Blast™ DNA stain:

- i. Dilute the Fast Blast stock solution 1:500 (final is 1X) with deionized water, such as 1 ml of 500X Fast Blast in 499 ml water.
- ii. Stain gels overnight in 1X stain in a gel staining tray (i.e., plastic tub or Tupperware®), rocking or agitating occasionally if possible. You should begin to see DNA bands after 2 hours, but at least 8 hours of staining is recommended for complete visualization.
- iii. Visualize results as described above under the Quick Stain procedure, *Step vi*.

Procedure

19. Show **Slide #11**, “Lab 3: Did Your PCR Work? Analyzing PCR Results with Agarose Gel Electrophoresis” as students enter the room.

Wet Lab: **Slide #11**



20. Show **Slide #12**, and remind students that their overall goal is to obtain DNA sequence data from the species they are studying. In the last experiment, students copied, or amplified, the barcoding gene from their purified DNA samples. Today, they will use agarose gel electrophoresis to determine whether their PCR was successful.

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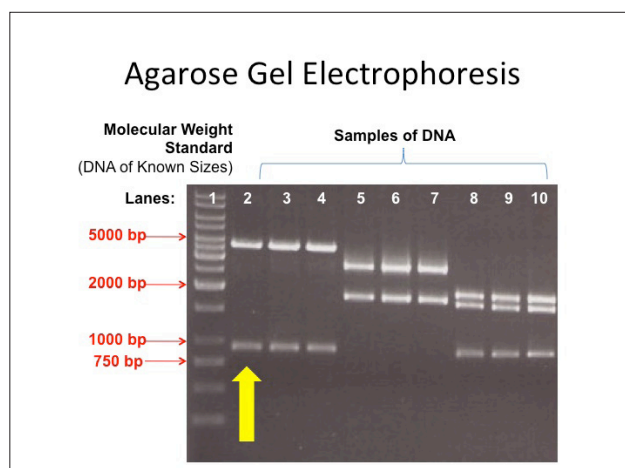
From Samples to Sequences

- Obtain samples
- Purify the DNA
- Copy your gene
- Make sure you copied your gene**
- Obtain DNA sequence data

- Aquarium, zoo, grocery*
- Lab 1: DNA Purification*
- Lab 2: Polymerase Chain Reaction*
- Lab 3: Agarose Gel Electrophoresis*
- Lab 4: PCR Purification and DNA Sequencing*

Wet Lab: **Slide #12**

21. Show **Slide #13**, and tell students that agarose gel electrophoresis is a method used by scientists to separate DNA molecules by size. DNA is placed at the top of the gel, which is a solid, porous material. An electric current is passed through the gel, and the negatively-charged DNA at the top of the gel moves toward the positively-charged electrode at the bottom of the gel.



Wet Lab: **Slide #13**

22. Walk through the gel image on **Slide #13** and highlight these key points with students:

- Each DNA sample is in a different vertical lane.
- Every DNA gel includes a **molecular weight standard**, which contains DNA bands of known size that scientists use to estimate the size of the DNA bands in their sample. In this gel, the molecular weight standard is in Lane 1.

Molecular weight standard:

Sometimes called a “molecular weight marker” or a “DNA ladder” and abbreviated “MW”, this is a mixture of DNA fragments of known size used to identify the approximate size of a molecule run on a gel, using the principle that molecular weight is inversely proportional to migration rate through a gel matrix. Therefore, when used in gel electrophoresis, standards effectively provide a logarithmic scale by which to estimate the size of the other fragments (providing the fragment sizes of the standard are known). Standards are loaded in lanes adjacent to sample lanes before the run commences.

Sometimes the amount of DNA in each band of the molecular weight standard is also known, so that scientists can estimate the amount of DNA in their samples. This is covered in more detail in **Slide #19**.

- c. The DNA samples are in Lanes 2–10.
 - d. DNA gels are stained, in this case with a chemical called ethidium bromide, to visualize the DNA.
 - e. Examples of the sizes of four molecular weight standard bands are shown: 5000 base pairs (bp), 2000 bp, 1000 bp, and 750 bp.
23. Ask students to estimate the sizes of the bands in Lane 2 (yellow arrow). The bottom band is approximately 900 bp, while the band above it is approximately 4500 bp. [**Note:** Lanes 2, 3, and 4 contain bands of approximately 900 and 4500 bp.]
24. Show **Slide #14**, which reviews the steps involved in agarose gel electrophoresis: making the gel, preparing your samples, loading your samples onto the gel, running the gel, and visualizing the gel. If your students have experience making and running agarose gels, you may wish to skip to *Step #33*. If your students do not have experience making and running agarose gels, you may wish to show one or more of the tutorial videos listed above under *Lab 3: Teacher Preparation*.

Wet Lab: **Slide #14**

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Agarose Gel Electrophoresis

1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
4. Run the gel.
5. Visualize the gel.

25. Show **Slide #15**, which reviews the first step: making the agarose gel. Tell students that agarose is made from a chemical called agar that is isolated from seaweed. Agar is also used as a thickening agent, and is what gives many consumer products their characteristic texture, including many sauces, puddings, and custards. The final product is similar in consistency to Jell-O® and, as with Jell-O®, the agarose is melted and poured into a mold. A small comb is placed in the liquid to form the holes or **wells** into which the DNA will be loaded.

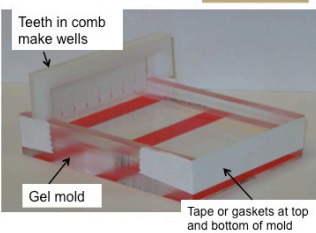
Wells: Holes in a gel into which samples are placed or “loaded.”

Wet Lab: **Slide #15**

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Agarose Gel Electrophoresis

1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
4. Run the gel.
5. Visualize the gel.



Agarose melted in buffer to pour in mold

26. Tell students that the size of the pores in the DNA gel (determined by the amount of agarose used) influences how quickly (or slowly) the DNA will move through the gel. Scientists usually make agarose gels with 0.8% to 2.5% agarose. Today, students will be making and running a 1% agarose gel.

27. Show **Slide #16**, which reviews the second step in the process of agarose gel electrophoresis: sample preparation. A small volume of the **PCR product** is mixed with a loading buffer – a concentrated solution containing a dye and glycerol which makes the DNA both visible (which helps you load it on the gel) and heavy enough to sink into the well of the gel. The final volume is achieved by adding a small amount of water, if needed.


PCR product: The DNA copied or “amplified” during PCR.

Wet Lab: **Slide #16**

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Agarose Gel Electrophoresis

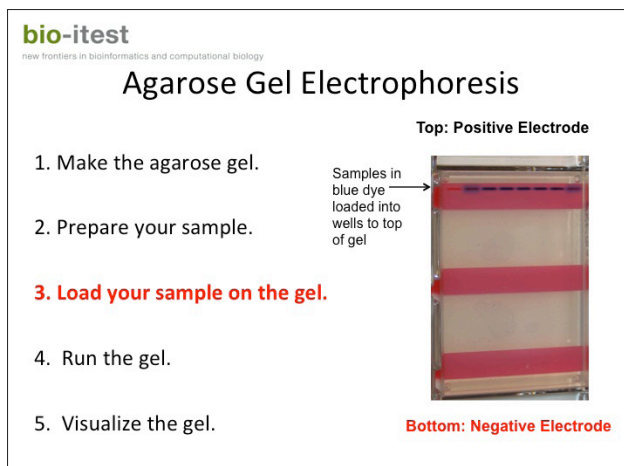
1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
4. Run the gel.
5. Visualize the gel.



- 10 μ l of DNA
- Loading Buffer
 - 6X concentrated
 - Adds blue color
 - Usually contains glycerol (helps samples “sink” into wells“)

28. Show **Slide #17**, which shows the samples loaded into the wells at the top of the gel. This scientist used a micropipette to carefully load her DNA sample into the wells.

Wet Lab: **Slide #17**



Gel box: Apparatus in which an agarose gel is run.

Power supply: Used to create the electrical field to which the DNA will be subjected during agarose gel electrophoresis.

[**Note:** Some teachers (and scientists!) use the phrase “run towards red” as a reminder that the DNA is loaded at the top of the gel, near the **black** (negative) electrode, so that the DNA will “**run towards [the] red**” (positive) electrode at the bottom of the gel.]

[**Note:** Many gel boxes can be set for “constant volts” **or** “constant amps” (as Watts = Amperes x Volts). In this case, be sure that “constant volts” is selected.]

29. If students are not already familiar with agarose gel electrophoresis, it is strongly suggested that you show one or all of the tutorials listed in the *Materials* section at the beginning of this lesson, particularly those sections related to sample loading. “DNA Electrophoresis Sample Loading” from Greg Peterson, Coordinator of Biotechnology, Kirkwood Community College, Math/Science Department, contains the most detailed information about what to do (and what not to do) when loading DNA samples onto agarose gels.

30. Show **Slide #18**, which illustrates the components of the **gel box** necessary to run the agarose gel. Review each component with your students:

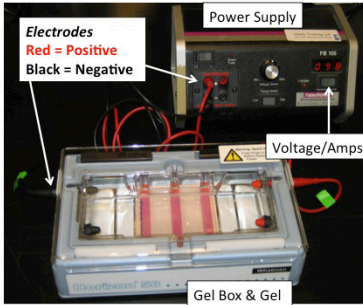
- The agarose gels are run in a gel box, which is the clear plastic case shown in the photo.
- The **power supply** generates the electrical current needed to move the DNA through the gel.
- The red, positive electrode connects the power supply to the bottom of the gel box, while the black, negative electrode connects the power supply to the top of the gel box.
- Note that the DNA is negatively charged, and will therefore move toward the positive electrode at the bottom of the gel.
- The power supply displays how many volts and amperes (amps) are being run through the gel box. It is very important never to run the gel with more volts or amps than instructed.

Wet Lab: **Slide #18**

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Agarose Gel Electrophoresis

1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
- 4. Run the gel.**
5. Visualize the gel.



Electrodes
Red = Positive
Black = Negative

Power Supply

Voltage/Amps

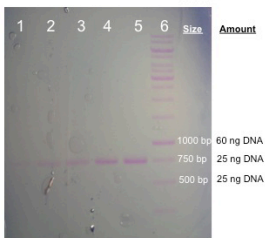
Gel Box & Gel

Wet Lab: **Slide #19**

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Agarose Gel Electrophoresis

1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
- 4. Run the gel.**
5. Visualize the gel.



Molecular Weight Standard

Lane	Size	Amount
1	1000 bp	60 ng DNA
2	750 bp	25 ng DNA
3	500 bp	25 ng DNA
4		
5		
6		

Separating pieces of DNA based on size.

31. Show **Slide #19**, which shows another DNA gel stained with Fast Blast™ (from Bio-Rad Laboratories). This is the stain recommended for use with student gels. Review the following information with your students:
- Once the electrical field has been applied to the gel box from the power supply, the DNA molecules will “run” through the gel, migrating to the positive electrode at the bottom of the gel. The rate of migration is based on their size. Small pieces of DNA, being less massive, will migrate more quickly, while larger molecules will travel more slowly.
 - The molecular weight marker in this gel is in Lane 6. The sizes of the different bands in the molecular weight marker are shown on the right, and are provided in the information from the molecular weight manufacturer. The smaller (lower molecular weight) bands are at the bottom of the gel, and the larger (higher molecular weight) bands are at the top of the gel.
 - In this example, the amount of DNA in each molecular weight standard band is also known, and can be used to calculate the amount of DNA in your samples. Knowing the amount of DNA in your sample is important for subsequent experiments, such as DNA sequencing.

- d. Ask students to estimate the size of the sample bands in the sample lanes (Lanes 2–5). These bands are just below the 750 bp band in the molecular weight standard lane, and are estimated to be between 650–700 bp. [**Note:** These are PCR samples from a DNA barcoding experiment with salmon.]
- e. Ask students to estimate the amount of DNA in the sample lanes, using the known quantity of DNA in the molecular weight standard lane that is **closest in size to the DNA sample bands** (i.e., 750 bp, 25 nanograms (ng) of DNA).
 - **Lane 1:** Approximately 25 ng. The band is about the same intensity as the 750 bp band in the molecular weight standard lane.
 - **Lane 2:** Approximately 50 ng. The band is about twice the intensity of the 750 bp band in the molecular weight standard lane.
 - **Lane 3:** Approximately 75 ng. The band is about three times the intensity of the 750 bp band in the molecular weight standard lane.
 - **Lane 4:** Approximately 100 ng. The band is about four times the intensity of the 750 bp band in the molecular weight standard lane.
 - **Lane 5:** Approximately 125 ng. The band is about five times the intensity of the 750 bp band in the molecular weight standard lane.

32. Show **Slide #20**, which illustrates some of the different methods used to visualize DNA gels: ethidium bromide with UV (ultraviolet) light or Fast Blast™ blue stain (from Bio-Rad Laboratories) with visible (white) light. While the methods may differ, the results are the same: the stain binds to the DNA in the molecular weight standard and sample lanes on the gel, and then the stain is visualized by shining light through the gel.

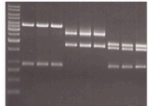
Wet Lab: **Slide #20**

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Agarose Gel Electrophoresis

1. Make the agarose gel.
2. Prepare your sample.
3. Load your sample on the gel.
4. Run the gel.
- 5. Visualize the gel.**

Ethidium Bromide & UV Light



Fast Blast™ Stain & Visible (White) Light



33. Pass out Student Handout–*Analyzing PCR Results with Agarose Gel Electrophoresis*. Have students work through the activity in the same small groups from Lab 2.

34. Optional Graphing Extension Activity:

Students can graph the log of the molecular weight of each band in their molecular weight standard (y-axis) against the distance each band traveled, in centimeters or millimeters (x-axis), either by hand or in a graphing program like Microsoft® Excel. Students can then determine the exact molecular weight of their PCR product band(s) by measuring the distance traveled by their DNA bands, and either plotting it on their graph or using their regression equation to solve for Y (molecular weight).

Lab 4: Preparation of PCR Samples for DNA Sequencing

Teacher Preparation

- Load the classroom computer with the *Wet Lab* PowerPoint slides.
 - Make copies of the Student Handout—*Preparation of PCR Samples for DNA Sequencing*, one per student. These handouts are designed to be reused as a class set; students should write answers to questions and take notes on a separate sheet of paper or in their lab notebook.
 - Teachers may wish to have their students write out the lab procedures before the activity in their lab notebooks or on a separate piece of paper as a “pre-lab” exercise, which can be used as an “entry ticket” to class. During the lab, students may check off steps as they complete them and/or describe and draw pictures of their observations.
 - Queue your computer to one of the following tutorials on DNA sequencing:
 - “The Sanger Method of DNA Sequencing” by the Wellcome Trust. Freely available at:
<http://www.wellcome.ac.uk/Education-resources/Teaching-and-education/Animations/DNA/WTDV026689.htm>.
 - “Sanger Method of DNA Sequencing” video freely available from the Howard Hughes Medical Institute (HHMI). Video is 51 seconds long and requires an internet connection and speakers. Available at:
http://www.hhmi.org/biointeractive/dna/DNAi_sanger_sequencing.html.
 - Set up student work stations by distributing supplies and reagents needed for each group, as described above under *Materials*. It is suggested that reagents from the stock bottles contained in the **DNA Clean & Concentrator™-5 (capped columns)** kit be aliquoted for student groups and labeled as described in Student Handout—*Preparation of PCR Samples for DNA Sequencing*. This information is found in Teacher Resource—*Aliquoting DNA Barcoding Reagents for Labs 1–4*.
35. Show **Slide #21**, “Preparation of PCR Samples for DNA Sequencing,” as students enter the room.

Wet Lab: **Slide #21**

[**Note:** DNA sequencing is more sensitive at detecting DNA than many of the DNA gel staining techniques used in classrooms. Therefore, students who do not see a DNA band on their agarose gel in *Lab 3* are still encouraged to proceed with *Lab 4* and submit their purified DNA sample for DNA sequencing.]

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Preparation of PCR Samples for DNA Sequencing

1. Obtain samples.
2. Extract the DNA.
3. Copy your gene.
4. Make sure you copied your gene.
5. **Obtain DNA sequence data.**

36. Show **Slide #22**, and remind students that their overall goal is to obtain DNA sequence data from the species they are studying. In the last experiment, students analyzed their PCR products using agarose gel electrophoresis. Today, they will prepare their PCR samples for DNA sequencing.

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From Samples to Sequences

- | | |
|-----------------------------------|---|
| • Obtain samples | • <i>Aquarium, zoo, grocery</i> |
| • Purify the DNA | • <i>Lab 1: DNA Purification</i> |
| • Copy your gene | • <i>Lab 2: Polymerase Chain Reaction</i> |
| • Make sure you copied your gene | • <i>Lab 3: Agarose Gel Electrophoresis</i> |
| • Obtain DNA sequence data | • <i>Lab 4: PCR Purification and DNA Sequencing</i> |

37. Show one of the DNA sequencing tutorials to familiarize students with the process of DNA sequencing, listed above under *Lab 4: Teacher Preparation*.

38. Show **Slide #23**, and review with students the components and logic of DNA sequencing. DNA sequencing is an adaptation of PCR, and has many of the same components:

- a. **DNA Template:** The student's PCR product.
- b. **Taq DNA polymerase:** This is a special DNA polymerase that is used to copy the DNA and is heat stable, so that the enzyme is not destroyed by the high temperatures during the PCR.
- c. **Primers:** These are small pieces of DNA that are specific to the sample being sequenced. They bind to the 5' and 3' regions of the gene to be copied, instructing the Taq DNA polymerase where to start DNA replication.
- d. **Buffer and water:** As this is a biological reaction, we must add buffers that mimic the inside of the cell for the Taq DNA polymerase to function properly.

Dideoxyribonucleotide triphosphates (ddNTPs): Dideoxyribonucleotide triphosphates (abbreviated ddNTPs) are similar to the normal **deoxyribonucleotide triphosphates** that are used for making DNA with one change: they are missing the **3' hydroxyl (-OH) group on the deoxyribose sugar**.

Chromatogram: A chromatogram is a type of data file produced by a DNA sequencing instrument.

39. Finally, show students **Slide #24**, “PCR Purification,” and explain to them that because DNA sequencing is so similar to the PCR experiment they performed, they must remove all of **their** PCR ingredients before sending samples for sequencing. To do this, they perform a PCR purification, which is very similar to DNA purification, but with fewer steps, as there are no cells to break open!

- **Step 1:** Mix the DNA (PCR product) with DNA binding buffer.
- **Step 2:** Bind the DNA to the spin column and wash away the PCR ingredients.
- **Step 3:** Elute the DNA off the spin column.

Wet Lab: **Slide #24**

40. Pass out Student Handout—*Preparation of PCR Samples for DNA Sequencing*. Have students work through the activity in the same small groups from *Lab 2* and *Lab 3*.

[**Note:** You do not need to add any primers to the sequencing reactions if you use the suggested commercial facilities. The barcoding primers include adapters that permit direct sequencing using standard M13 Forward (“M13F”) and M13 Reverse (“M13R”) primers. Most DNA sequencing facilities provide the standard M13F and M13R primers free of charge.]

Submitting Samples for DNA Sequencing

Purified PCR products can be sent for DNA sequencing at a commercial DNA sequencing facility. Suggested facilities include:

Eurofins MWG Operon

<http://www.eurofinsdna.com/products-services/custom-dna-sequencing.html>

Sequencing through Operon is performed via pre-purchase of barcoded DNA sequencing tubes, which are activated online after receipt. Samples to be sequenced are placed in the tubes, and the barcode and sample name are entered online. The tubes are then sent via FedEx® to the Operon sequencing facility, and DNA sequence data is returned to customers via email. The cost of FedEx® shipping is included in the cost of the sequencing (approximately \$5 per reaction as of August 2012). Samples are stable at room temperature for a few days (during shipment). Be sure to record each sample name and the DNA barcode from each Operon DNA sequencing tube before submission.

Seattle Biomedical Research Institute (Seattle BioMed, Seattle, WA)

<http://www.seattlebiomed.org/dna-sequencing-and-fragment-analysis>

Sequencing through Seattle BioMed is performed in 0.2 ml tubes similar to the tubes used for PCR in *Lab 2*. Tube ordering information is available on the Seattle BioMed DNA Sequencing Facility webpage. Once customers get an account, samples are prepared per Seattle BioMed instructions, and sent to Seattle BioMed via U.S. mail, FedEx®, or UPS®. Samples are stable at room temperature for a few days (during shipment). When DNA sequence results are ready, customers are notified by email that their data is available for download from the password-protected Seattle BioMed server. Cost for nonprofit/academic institutions is \$10 per reaction as of November 2012.

Closure

41. Summarize the lessons of this unit:
- Students purified DNA from samples of their choice, copied the barcoding gene using polymerase chain reaction (PCR), analyzed their PCR products using agarose gel electrophoresis, and prepared their samples for DNA sequencing.
 - This constitutes the **wet lab** portion of the DNA barcoding process. The next step is to analyze the DNA sequence data using the tools of bioinformatics.

Glossary

Agarose gel electrophoresis: Electrophoresis refers to the process of using an electric field to move molecules through a gel matrix. In the case of DNA, agarose is used to form the gel matrix, and the electrical current separates DNA fragments based on size, with smaller (or lower **molecular weight**) fragments moving to the positive electrode more quickly than larger fragments.

Amplify: In PCR, to amplify is to increase in copy number.

Buffer: A substance used to stabilize or maintain the pH of a solution.

Cell membrane: Phospholipid bilayer surrounding the cell.

Centrifugal force: The apparent force that seems to pull an object outward when the object is spun around in a circle. This is the force that creates a **pellet** in the bottom of a microfuge tube during centrifugation or forces material through a **spin column**.

Chromatogram: A chromatogram is a type of data file produced by a DNA sequencing instrument.

COI gene: Cytochrome c oxidase subunit 1 gene.

Deoxyribonucleotide triphosphates (dNTPs): These are the bases that are used for making DNA. They are abbreviated as dATP (deoxyadenosine triphosphate), dCTP (deoxycytosine triphosphate), dGTP (deoxyguanine triphosphate), and dTTP (deoxythymidine triphosphate). A mixture containing all four deoxyribonucleotide triphosphates can also be described as a “set of dNTPs.” See also **Dideoxyribonucleotide triphosphates (ddNTPs)** and **DNA sequencing**.

Detergents: Substances (for example, soap) that contain both hydrophobic and hydrophilic regions, used to dissolve **lipids** (i.e., cell membranes).

Dideoxyribonucleotide triphosphates (ddNTPs): Dideoxyribonucleotide triphosphates (abbreviated ddNTPs) are similar to the normal **deoxyribonucleotide triphosphates** that are used for making DNA with one change: they are missing the **3' hydroxyl (-OH) group on the deoxyribose sugar**. The **3' hydroxyl group** is necessary for DNA synthesis because **DNA polymerase** builds a chain of DNA by catalyzing the formation of a **phosphodiester bond** between the first phosphate group on the new dNTP and the 3' hydroxyl group at the end of the DNA strand. If the last dNTP in a DNA strand is missing the 3' hydroxyl group, DNA polymerase will be unable to add a new base and DNA synthesis will stop. When ddNTPs are used for automated DNA sequencing, they are also labeled with a fluorescent dye. When the names of these bases are abbreviated, an extra “d” is added to indicate that they are missing the 3' hydroxyl group. The abbreviations for these bases are: ddATP, ddCTP, ddGTP, and ddTTP. The normal bases are dATP, dCTP, dGTP, and dTTP.

DNA polymerase: The enzyme that assembles new DNA molecules, in the cell or *in vitro* in the laboratory, using a DNA template and **deoxyribonucleotide triphosphates (dNTPs)**.

DNA purification: Extracting the DNA from a cell, and purifying it away from the remaining cellular components.

DNA sequencing: The process of determining the identity and order of bases in a molecule of DNA. A common method for sequencing DNA involves: purifying DNA from a sample, making a copy of that DNA *in vitro* (PCR), separating the new DNA molecules by their size, and identifying the base at the end of each DNA molecule by measuring the intensity of the fluorescent signal. This entire process is commonly known as **Sanger sequencing** after Fred Sanger, the biochemist who developed the method for using **dideoxyribonucleotide triphosphates (ddNTPs)** in conjunction with regular deoxynucleotides (dNTPs) to create DNA molecules of random sizes. Automated DNA sequencing instruments use capillary electrophoresis to separate the differently sized molecules of DNA. Capillary electrophoresis separates DNA molecules in a small capillary tube instead of in an agarose gel. Automated DNA sequencing instruments also contain a laser that excites the fluorescent dye attached to each DNA base, instruments that capture and measure the intensity of fluorescence, and software for processing the fluorescent signal and creating a **chromatogram**. A key point to note is that the DNA

bases that are measured are produced by synthesizing new DNA *in vitro*, and might contain differences from the original sequence in the sample due to errors during DNA synthesis. Scientists use chromatogram-viewing programs like FinchTV to view and analyze their chromatograms and associated DNA sequence data. They use sequence assembly programs to reconstruct a model of the original sequence. For more on DNA sequencing, see also *Lesson Nine*.

DNA template: The DNA used as instructions to make more DNA, such as in PCR.

Elute: To extract or remove one material from another, often by adding a solvent such as a buffer.

Enzyme: A type of protein that catalyzes (increases the rate of) chemical reactions. For example, ATP synthase is an enzyme that catalyzes or facilitates the creation of ATP.

Flow-through: Liquid that passes through a spin column into the collection tube.

Gel box: Apparatus in which an agarose gel is run.

Golgi apparatus or Golgi body: A membrane-bounded cellular organelle that is involved in modifying proteins and transporting them to their final cellular destination.

Lysis or “to lyse”: To break open.

Lipids: Organic compounds that are non-polar; typically comprise the cell membrane.

Molecular weight standard: Sometimes called a “molecular weight marker” or “DNA ladder” and abbreviated “MW,” this is a mixture of DNA fragments of known size used to identify the approximate size of a molecule run on a gel, using the principle that molecular weight is inversely proportional to migration rate through a gel matrix. Therefore, when used in gel electrophoresis, standards effectively provide a logarithmic scale by which to estimate the size of the other fragments (providing the fragment sizes of the standard are known). Standards are loaded in lanes adjacent to sample lanes before the run commences.

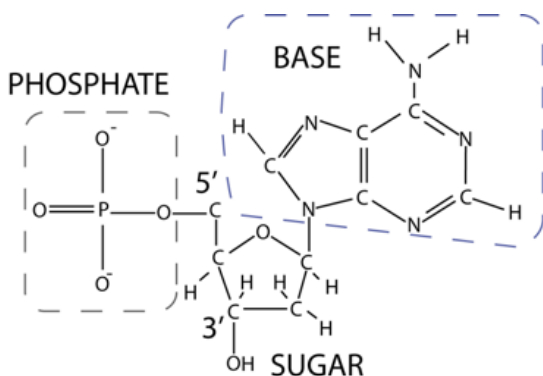
No-cap collection tube: Type of microfuge tube used to hold a spin column and collect the **flow-through**.

Nuclear membrane: Phospholipid bilayer that surrounds the nucleus.

Nuclease: Type of enzyme that breaks down nucleic acids.

Nuclease-free water: Water that does not contain **nucleases**. Often this water has been subjected to multiple rounds of purification, including being passed through a nano-filter. It is sometimes referred to as “nano-pure” or “ultra-pure” water, as it should contain only H₂O, with no dissolved salts or other contaminants.

Nucleotide: Nucleotides are the building blocks of DNA. Each nucleotide contains a five-carbon sugar, a base, and a phosphate group attached to the 5' carbon of the sugar. In DNA, the sugar is deoxyribose and the base can be adenine, cytosine, thymine, or guanine. Note the 5' and 3' labels on the sugar molecule.



PCR beads: Lyophilized or “freeze-dried” PCR ingredients.

PCR product: The DNA copied or “**amplified**” during PCR.

PCR purification: Extracting or purifying the PCR product (DNA fragment) away from the remaining PCR components.

Pellet: After centrifugation, the pellet is the material found at the bottom of the tube.

Phosphodiester bond: A phosphodiester bond is a strong covalent bond between a phosphate group and two five-carbon ring carbohydrates. **DNA polymerase** catalyzes the formation of phosphodiester bonds between the **3’ hydroxyl group** on the deoxyribose at the end of a DNA strand and the phosphate group attached to the 5’ carbon of the deoxyribose on the new DNA **nucleotide**.

Polymerase Chain Reaction (PCR): A scientific technique in molecular biology used to **amplify** (i.e., copy) a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Power supply: Used to create the electrical field to which the DNA will be subjected during agarose gel electrophoresis.

Primer pools: Collections or mixtures of primers, usually used in PCR.

Primers: Small pieces of DNA used to start or “prime” DNA synthesis, as in DNA replication and PCR.

Proteinase K: Type of enzyme that breaks down proteins, including **nucleases**.

Reagents: Ingredients or components used in an experiment.

Sanger sequencing: A method of **DNA sequencing** that uses **deoxyribonucleotides (dNTPs)** and **dideoxyribonucleotide triphosphates (ddNTPs)**. The ddNTPs end DNA synthesis at random positions, and the resulting fragments can be analyzed to determine the exact DNA sequence. For more on Sanger sequencing, see also **DNA sequencing**.

Spin column: A small column that fits inside of a microfuge tube and contains a material (such as silica) that binds to nucleic acids such as DNA. The spin column is used in conjunction with centrifugation (or vacuum pressure) to purify DNA.

Supernatant: After centrifugation, the supernatant is the liquid found above the **pellet**.

Taq DNA polymerase: A type of heat-stable DNA polymerase, purified from the thermophilic (“heat-loving”) bacteria *Thermus aquaticus*.

Template: See **DNA template**.

Thermocycler: Type of machine used to automate polymerase chain reaction that cycles through all of the temperatures required to complete the PCR.

Total DNA: All of the DNA found in a cell, including the DNA in the nucleus and in the mitochondria.

Wells: Holes in a gel into which samples are placed or “loaded.”

3’ hydroxyl group: During DNA synthesis, **DNA polymerase** catalyzes the formation of a **phosphodiester bond** between the 3’ hydroxyl group on the deoxyribose at the end of a DNA strand and the phosphate group attached to the 5’ carbon of the deoxyribose on the new DNA **nucleotide**. See also **DNA sequencing**.

Resources

The Howard Hughes Medical Institute (HHMI) **Bioactive** website has a large collection of useful videos and animations on a number of topics, including DNA, evolution, infectious disease, biodiversity, and cancer. These can be freely accessed online at: <http://www.hhmi.org/biointeractive/video/index.html>.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H. and Hebert, P.D.N. Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes*. 2007; 7: 544-548.

See also the **Materials** section for a number of helpful videos and online resources.

Credit

The authors wish to thank Wikimedia Commons for some of the images and definitions found in this lesson.



Lab 1: DNA Purification for DNA Barcoding

Student Researcher Background:

What is DNA Purification?

DNA purification is the process of separating nucleic acids (DNA) from the rest of the cell. This multi-step process involves breaking open the cell by disrupting the cell membrane, binding the DNA to a small membrane contained in a **spin column**, and washing away the cellular membranes and organelles. For DNA barcoding, you will isolate **total DNA**—the DNA in the nucleus and the DNA in the mitochondria.

Why Purify DNA?

DNA, including the sequence of DNA, is the primary focus of genetic research. Purification of DNA is the first laboratory experiment performed during the process of genetic research. This DNA is used in later experiments, such as polymerase chain reaction (PCR) and DNA sequencing. During the next two days, you will be performing the same DNA purification experiments that genetic researchers perform every day.

Spin columns: A small column that fits inside of a microfuge tube and contains a material (such as silica) that binds to nucleic acids such as DNA. The spin column is used in conjunction with centrifugation (or vacuum pressure) to purify DNA.

Day 1 Procedure:

The goal for *Day 1* is to begin to **lyse**, or break open, the cells in your sample. Lysing the cells is the first step in purifying the DNA away from the rest of the cell. The DNA purification experiment will be completed on *Day 2*.

1. Label a 1.7 ml microfuge tube with your sample name, your initials, and the date.
2. Take up to 25 mg of your tissue sample and cut it or shred it into **tiny** pieces. Do not use more than 25 mg of tissue. **25 mg is about half the size of a pencil eraser.** Ask your teacher how to cut up the sample (such as with a razor blade or butter knife).
3. Put your sample pieces into your labeled 1.7 ml microfuge tube.
4. Add 95 μ l of nuclease-free water (labeled "Water") to your tube.
5. Add 95 μ l of 2X Digestion Buffer (labeled "Digest") to your tube.

Digestion **buffer** contains **detergents** that will help break up and dissolve away the cell membrane (the phospholipid bilayer surrounding the cell), just like the soap dissolves fats and grease when you wash dishes.

6. Add 10 μ l of **Proteinase K** (labeled "Prot K") to your tube.

When something ends in "-ase," it's an **enzyme**—a protein that has a specific function or job. A proteinase breaks down proteins,

Buffer: A substance used to stabilize or maintain the pH of a solution.

Detergents: Substances that contain both hydrophobic and hydrophilic regions, used to dissolve lipids (for example, soap).

including proteins called **nucleases**. Nucleases break down nucleic acids like DNA, which we don't want to happen! By adding Proteinase K to our experiment, we can prevent any nucleases from breaking down our DNA because Proteinase K will break down proteins like nucleases.

7. Make sure that the lid of your microfuge tube is closed tightly, and mix the contents of your tube thoroughly by vortexing for 10 seconds.

If you don't have a vortexer, you can mix your tube by flicking it with your finger and tapping it on the table or lab bench top, repeating every few seconds. However, if you don't vortex your sample, you need to be sure to mix it **very** well by hand for 1–2 minutes. The more you mix, the better your lysis will be, and the more DNA you will be able to purify.

8. Incubate your microfuge tube at 55°C until the tissue is completely lysed. Talk to your teacher; she or he may want you to incubate your tube overnight.

Heat will help the digestion buffer dissolve the cell membrane, just like you use warm water to wash dishes. Lysis may take 1–3 hours, and is okay to leave overnight.

Where is the DNA?

Figure 1.1 shows a simple eukaryotic cell, with a nucleus, mitochondria, and **Golgi apparatus**. For simplicity, the other organelles are not shown. Now, at the end of *Day 1*, you have begun to break open or lyse the cells, and the DNA, still wrapped in the **nuclear membrane** (the phospholipid bilayer that surrounds the nucleus) or inside the mitochondria, is floating in the buffer in your microfuge tube. [**Note:** Illustrations are not to scale.]

Golgi apparatus or Golgi body:

A membrane-bounded cellular organelle that is involved in modifying proteins and transporting them to their final cellular destination.

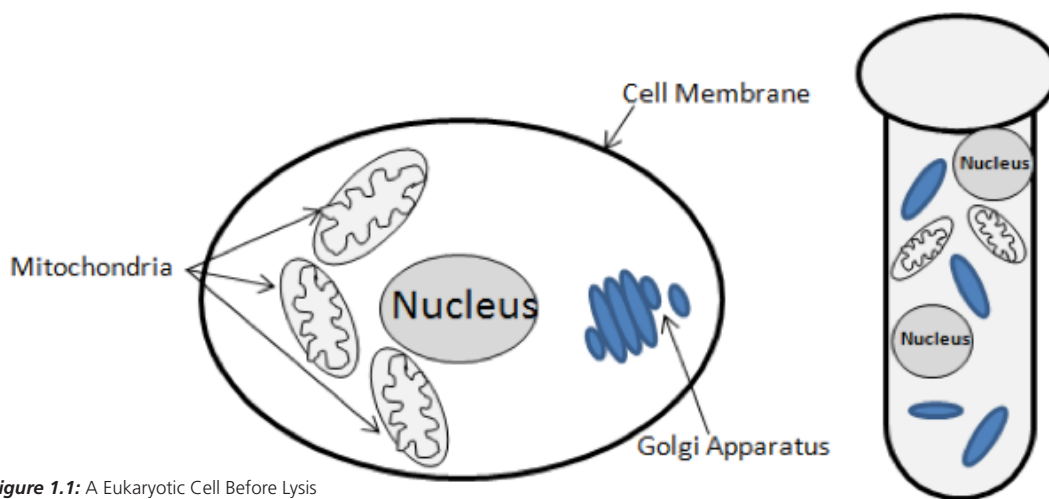


Figure 1.1: A Eukaryotic Cell Before Lysis (Left) and After Lysis (Right).



Question 1. On a separate piece of paper or in your lab notebook, describe what happened to the cell membrane. Where did it go?

Day 2 Procedure:

The goal for *Day 2* is to finish the DNA purification. The cell has been lysed, or broken open, on *Day 1*. Now you will need to separate the DNA from the rest of the cell debris.

9. Make sure that the lid of your microfuge tube containing your sample is closed tightly, and vortex the tube containing your sample for 15 seconds.

If you don't have a vortexer, you can mix your tube by flicking it with your finger and tapping it on the table or lab bench top, repeating every few seconds. However, if you don't vortex your sample, you need to be sure to mix it **very** well by hand for 1–2 minutes. The more you mix, the more DNA you will be able to purify.

10. Add 700 μ l of Genomic Lysis Buffer (labeled "Lysis") to your sample, and mix thoroughly by vortexing.

If you do not have a vortexer, you can mix your tube by flicking it with your finger and tapping it on the table or lab bench.

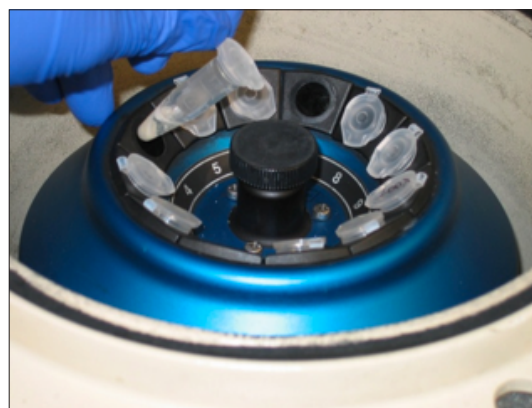
This Genomic Lysis Buffer also contains detergents. On *Day 1*, you dissolved the cell membrane. Now you are dissolving the nuclear membrane that surrounds the nucleus, and the inner and outer mitochondrial membranes to release the mitochondrial DNA.



Question 2. On a separate piece of paper or in your lab notebook, describe what your sample looks like.

How to Load Samples in a Microcentrifuge

When loading microfuge tubes in a microcentrifuge, always place the tubes with the hinge of the lid facing "out" or "up" (below, left). That way, when centrifugation is complete, the **pellet** will always be located on the outer-most portion of the tube (below, right). This is especially important when the pellet is small or hard to see.



11. Centrifuge (or “spin”) your tube at 10,000 revolutions per minute (rpm) for one minute. See “How to Load Samples in a Microcentrifuge” for more information.

We are using the **centrifugal force** of the centrifuge to push all of the cell debris, like the membranes, organelles, and proteins, to the bottom of the microfuge tube. Your DNA will be in the liquid, or **supernatant**, above the cell debris found in the **pellet**, as seen in **Figure 1.2**. [Note: If your centrifuge doesn’t go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]



Figure 1.2: Spinning the Microfuge Tube Separates the Supernatant From the Pellet.

12. When you are done “spinning,” handle the microfuge tube **very** gently so that you don’t dislodge any pellet of insoluble debris at the bottom of the tube. Hold the tube at eye level to see if you have a pellet. If you don’t have a pellet of debris at the bottom of your tube, that is a good sign that your tissue is completely lysed!

13. Obtain a spin column and a no-cap collection tube from your teacher.

Spin columns contain a tiny piece of material in the bottom that binds to your DNA. This holds the DNA in the column while the rest of the liquids wash away into the collection tube. The liquid that passes through the columns is called the **flow-through**. Spin columns are often placed in **no-cap collection tubes** to collect the flow-through before discarding it. No-cap collection tubes are essentially microfuge tubes with the lids cut off, so the lids won’t get in your way. **Be careful when handling the spin column and no-cap collection tube** as they are not attached to each other (as seen in **Figure 1.3**).

Centrifugal force: The apparent force that seems to pull an object outward when the object is spun around in a circle. This is the force that creates a pellet in the bottom of a microfuge tube during centrifugation, or forces material through a spin column.

Supernatant: After centrifugation, the supernatant is the liquid found above the pellet.

Pellet: After centrifugation, the pellet is the material found at the bottom of the tube.

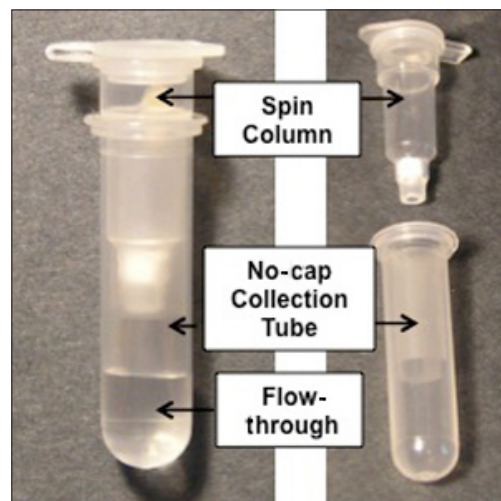


Figure 1.3: Spin Columns are Placed in No-cap Collection Tubes.

14. Place the spin column in the no-cap collection, and label the lid of your spin column with your initials. You need to be able to keep track of your sample in the centrifuge. Otherwise, they all look alike.
15. Using a P1000 micropipette dialed to 600 μ l, carefully remove the supernatant, or liquid, from your sample tube, being careful not to touch the pellet of cell debris (which may or may not be visible at the bottom of the tube).
16. Add the supernatant to your spin column, being careful not to touch the DNA-binding membrane found at the bottom of the spin column.
17. Centrifuge your spin column at 10,000 rpm for one minute. This will bind the DNA to the spin column, and the rest of the liquid will flow through the spin column into the no-cap collection tube.
- [**Note:** If your centrifuge doesn't go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
18. You should see flow-through liquid in your no-cap collection tube. If you don't see this liquid, centrifuge your spin column again. Discard the flow-through and the no-cap collection tube. (See **Figure 1.3**).
19. Place the spin column **in a new no-cap collection tube**.
20. Add 200 μ l of Pre-Wash Buffer (labeled "Pre-Wash") to your spin column. This will help prepare your sample for the wash step below.
21. Centrifuge your sample at 10,000 rpm for one minute.
- [**Note:** If your centrifuge doesn't go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
22. Add 400 μ l of g-DNA Wash Buffer (labeled "Wash") to your spin column. This will wash away any remaining material other than your DNA.
23. Centrifuge at 10,000 rpm for one minute. This will help remove any of the residual Wash Buffer.
- [**Note:** If your centrifuge doesn't go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
24. Label a new 1.7ml microfuge tube with your name, date, and sample name.
25. Place the spin column **in the newly labeled microfuge tube** from Step #24.
26. Add 100 μ l of DNA Elution Buffer (labeled "Elute") directly into the spin column but **do not touch the bottom of the spin column with your pipette tip**.
- Eluting** means making the DNA release from the spin column into your microfuge tube. The elution buffer changes the pH in the spin column so the DNA will no longer bind to the column material.
27. Close the lid of your spin column, and leave the lid of your 1.7 ml microfuge tube open. (See **Figure 1.4**).

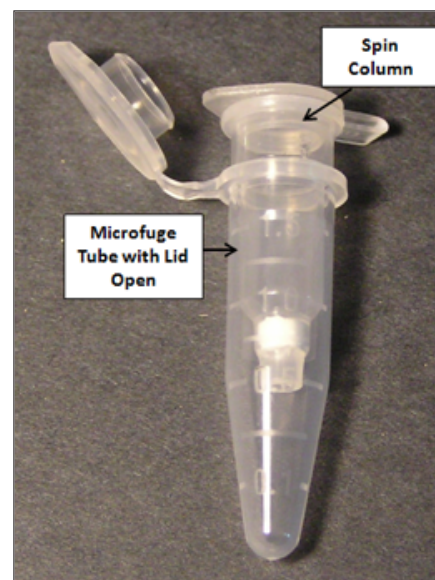


Figure 1.4: Close the Lid of the Spin Column and Leave the Lid of the Microfuge Tube Open.

28. Incubate your spin column and microfuge tube at room temperature for 5 minutes.
29. Centrifuge your spin column and microfuge tube for 1 minute at 10,000 rpm to elute the DNA from the spin column into the microfuge tube. (See **Figure 1.5**).
30. Discard the spin column and close the lid of your microfuge tube.
31. Use your DNA immediately or store on ice (short-term, for up to 2 hours) or in the freezer (long term).



On your separate sheet of paper or in your lab notebook, answer each of the following questions:

Question 3. What did you do in this laboratory experiment and why?

Question 4. What skills did you learn or practice?

Question 5. List at least three types of samples from which you could purify DNA, based on what you have learned about tissues in your classes. What type of cells or tissues could you **not** purify DNA from?



Figure 1.5: Spinning Elutes the DNA from the Spin Column.

Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)

Student Researcher Background:

What is Polymerase Chain Reaction (PCR)?

Polymerase Chain Reaction or “PCR” is a powerful technique used by many scientists to **amplify** (or “copy”) a particular gene or region of DNA in a test tube. Starting with only a few copies of DNA, PCR uses the same basic ingredients as DNA replication in the cell and results in **billions** of copies of your specific gene. **All PCR experiments contain a few vital components:**

- A **DNA template**, which is DNA used as instructions to make more DNA. You will use the DNA that you purified in *Lab 1* as a template to amplify your gene of interest (the DNA barcoding gene).
- A **DNA polymerase**, which is an enzyme that assembles new DNA molecules. You will use a special kind of DNA polymerase called **Taq** polymerase to copy your gene.
- Forward and reverse **primers**, or small pieces of DNA used to start or “prime” DNA synthesis, which bind specifically to the 5’ and 3’ sequences on either side of the DNA barcoding gene. These primers tell the DNA polymerase where to start copying your gene.
- **Deoxynucleotide triphosphates (dNTPs)**, which are the building blocks of DNA, and are used to build the new DNA molecules during your PCR.
- **Buffer** (usually in a 10X concentrate, which you dilute with pure water) that helps to stabilize the pH of the solution and reproduce the conditions in a cell so that the **Taq** DNA polymerase can function.

Today you will be using **PCR beads** (**Figure 2.1**), which are tiny white beads that contain all the ingredients for your PCR (freeze-dried or “lyophilized”) **except** the DNA template, primers, and nuclease-free water.

How Do You Know Which Primers to Use?

Scientific experiments build on what is already known. In order to make the **Taq** DNA polymerase specifically copy **your** gene and not just any piece of DNA, you need to use primers that are known to bind to the DNA right next to the gene you want to copy. Genetic researchers who use DNA barcoding have developed primers specific for the *COI* gene. You will be using these same mixtures of many different primers called **primer pools**. Each primer in the primer pool was originally derived from the DNA barcoding sequence from one or more animals. By using a pool of primers instead of single forward and reverse primers, we are increasing the likelihood that one or more primers will work for your species of interest. Your teacher can help you decide which primer pool to use with your sample.

Primer Pool COI-2 is used for mammals, fish, or insects.

Primer Pool COI-3 is used for amphibians and reptiles, and can also be used for mammals.

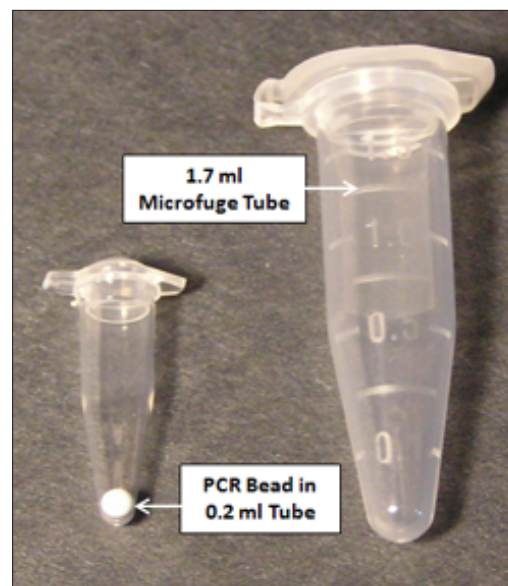


Figure 2.1: PCR Bead in a 0.2 ml Tube Compared to a 1.7 ml Microfuge Tube.

Primer pools: Collections or mixtures of primers, usually used in PCR.

Procedure:

[Note: Work in groups of up to 4 students.]



1. Obtain ice in your ice container. PCR primers should be kept cold.
2. PCR tubes are very small and do not permit complicated labels. *On a separate sheet of paper or in your lab notebook, make a table like the one below (Table 2.1) to list each DNA sample purified in Part 1 by you and your group members, and the number that you will use to label your PCR tubes. **Your PCR tube should also include your initials**, such as “JT” in the examples below.*

Table 2.1: DNA Samples and PCR Test Tube Labels:

PCR Tube Label	Species (or sample number) from which DNA was purified
*1	
*2	
*3	
*4	
* Be sure to include your initials in addition to the tube number.	
Example:	
JT1	Joe Ting's Salmon DNA from Puget Sound, Washington

3. The PCR tubes are the small, 0.2 ml clear tubes provided by your teacher (See Figure 2.1). Each tube contains a small white bead that includes the *Taq* DNA polymerase, deoxyribonucleotides (dNTPs), and buffer. Obtain up to five tubes for your group: one for each student, and one for the negative control.
4. Label each PCR tube as you described in Table 2.1 (for example, “JT1” or “JT2”).
5. Label one PCR tube with one of your group member’s initials and a “0” (for your group’s Negative Control, for example, “JT0”).
6. Obtain a 1.7 ml microfuge tube for each member of your group and label this tube as you described in Table 2.1 (for example, “JT1” or “JT2”).
7. Obtain a 1.7 ml microfuge tube for your group and label it with the initials and “0” for your Negative Control (as in Step #5 above).
8. Briefly “spin down” each tube of PCR primers using a microcentrifuge. This will force all of the liquid into the bottom of the tubes to ensure accurate measurement with your micropipette.

9. Add each of the following components to your 1.7 ml microfuge tube, in the order listed below. Try to keep the components on ice as much as possible.
 - a. 40.0 μ l of nuclease-free water (labeled "Water")
 - b. 5.0 μ l of your purified DNA
 - c. 5.0 μ l of COI primer pool (labeled "COI-2" or "COI-3" depending upon pool)
10. Have one member of your group prepare the PCR mixture for the Negative Control by adding each of the following ingredients to the 1.7 ml Negative Control microfuge tube ("0") for your group.
 - a. 45.0 μ l of nuclease-free water ("Water")
 - b. 5.0 μ l of COI primer pool (labeled "COI-2" or "COI-3" depending upon pool)
11. Mix your 1.7 ml microfuge tube containing your PCR mixture by closing the lid and flicking the tube with your finger.
12. Spin down the contents of your 1.7 ml microfuge tube with your PCR mixture.
13. Repeat the mixing and spinning steps above for your Negative Control tube ("0").
14. Add all 50 μ l of your PCR mixture from your 1.7 ml microfuge tube to your labeled 0.2 ml PCR tube.
15. Have one member of your group add all 50 μ l of the Negative Control PCR mixture from the Negative Control "0" 1.7 ml microfuge tube to the corresponding Negative Control "0" 0.2 ml PCR tube for your group.
16. Working with your teacher and the rest of your class, put all 5 of your group's tubes into the **thermocycler**.

The PCR reaction conditions are as follows:

1 cycle:	94 °C for 2 minutes	[initial denaturation]
35 cycles:	94 °C for 30 seconds	[denaturation]
	52 °C for 40 seconds	[annealing]
	72 °C for 1 minute	[elongation]
1 cycle:	72 °C for 10 minutes	[final elongation]
Overnight:	4 °C	

Thermocycler: Type of machine used to automate polymerase chain reaction that cycles through all of the temperatures required to complete the PCR.



On your separate sheet of paper or in your lab notebook, answer each of the following questions:

Question 1. What did you do in this laboratory experiment and why?

Question 2. What skills did you learn or practice?

Question 3. What are we testing for in the Negative Control PCR? What do you expect to happen in this PCR?

Lab 3: Analyzing PCR Results with Agarose Gel Electrophoresis

Student Researcher Background:

What is Agarose Gel Electrophoresis?

Agarose gel electrophoresis is a procedure used to determine the presence and size of pieces of DNA. By analyzing the results of PCR on an agarose gel, genetic researchers are able to determine: (1) whether the PCR reaction was successful, indicated by the presence of a DNA band on the gel; and (2) whether the PCR reaction resulted in a PCR of the expected size or molecular weight.

Agarose is a powdered material made from seaweed. After mixing the agarose powder in buffer and heating it to melting in the microwave, the liquid is poured into a small mold (similar to the procedure used when making Jell-O®). A comb is placed in the liquid to make small holes or **wells** in which to put the DNA. After placing the gel in the gel box (see **Figure 3.1**), DNA samples are mixed with loading dye and a single DNA sample is loaded into each well of the gel. Loading dye is often concentrated, such as 3X or 6X, and is diluted with water and/or your DNA sample. An electric current is then passed through the gel; since DNA is negatively charged, it flows through the gel to the positive electrode (anode). The rate at which the DNA moves through the gel is determined by its size or molecular weight—smaller molecules travel more quickly, and larger molecules travel more slowly.

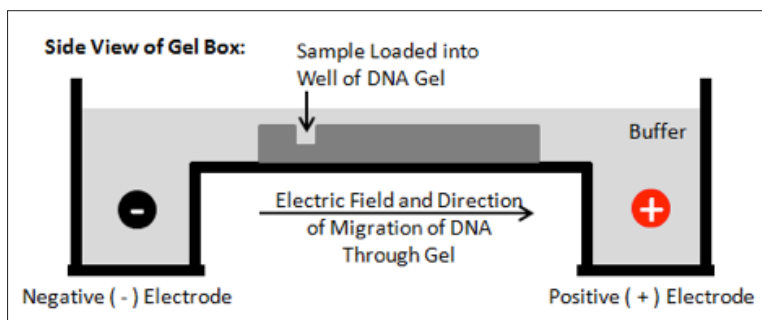


Figure 3.1: Running a DNA Gel.

How Do You Know the Molecular Weight of Your DNA Samples?

By including a known **molecular weight standard** on your gel, you can determine the size of your DNA bands or **PCR products**. Molecular weight standards, often abbreviated "MW," contain pieces of DNA of known sizes. By comparing your DNA band to the band(s) of the molecular weight standard, you can estimate the size and concentration of your DNA band(s). You can also make a graph of the molecular weight standards relative to the distance each molecular weight standard band traveled through the gel. This allows for a more accurate estimation of the size of your DNA band(s).

Agarose gels are stained to visualize the DNA bands they contain—both the molecular weight standards and your PCR products. Some stains, such as ethidium bromide, can be very toxic. Other stains such as Fast Blast™ made by Bio-Rad Laboratories are much safer for student researchers to use.

Molecular weight standard:

Sometimes called a "molecular weight marker" or "DNA ladder" and abbreviated "MW," this is a mixture of DNA fragments of known size used to identify the approximate size of a molecule run on a gel, using the principle that molecular weight is inversely proportional to migration rate through a gel matrix. Therefore, when used in gel electrophoresis, standards effectively provide a logarithmic scale by which to estimate the size of the other fragments (providing the fragment sizes of the standard are known). Standards are loaded in lanes adjacent to sample lanes before the run commences.

PCR product: The DNA copied or "amplified" during PCR.

PART I: Pouring a DNA Gel

[**Note:** Work in groups of up to 4 students (the same students who performed the PCR together). If your teacher has already poured your DNA gels, skip *Part I* and go to *Part II*.]

- Obtain the following items:
 - 1 tube of powdered agarose (500 mg)
 - 50 ml DNA Gel Buffer
 - Erlenmeyer flask or glass bottle
 - Labeling tape
- Use the labeling tape to label your flask or bottle with your group's name or number.
- Combine the agarose and buffer in your flask or bottle and gently mix them together by swirling.
- Microwave the mixture on high for 1 minute. Check the liquid to see if all of the agarose has melted. If the agarose hasn't completely melted yet, microwave again for 20 seconds, and check again. Repeat microwaving for 20 seconds at a time until all the agarose has melted.
- Set the melted agarose aside where it can cool until it is only warm to the touch, usually about 55–60°C. Your teacher may have you place your melted agarose in a water bath to cool.
- Ask your teacher about the proper procedure for pouring a gel with your gel apparatus. This will usually involve the following steps (also shown in **Figure 3.2**):
 - Blocking the top and bottom ends of a gel casting tray with tape, blocks, or a rubber gasket.
 - Pouring the warm agarose gel mixture into the mold.
 - Placing the well comb into the notches on the gel casting tray. Combs typically have 6, 8, or 14 "teeth" to create "wells" in the gel in which to load your DNA.
 - Waiting for the gel to solidify completely.
- You can store your gel in the gel box or in the refrigerator for up to 2 weeks, as long as the gel is completely submerged in DNA Gel Buffer.

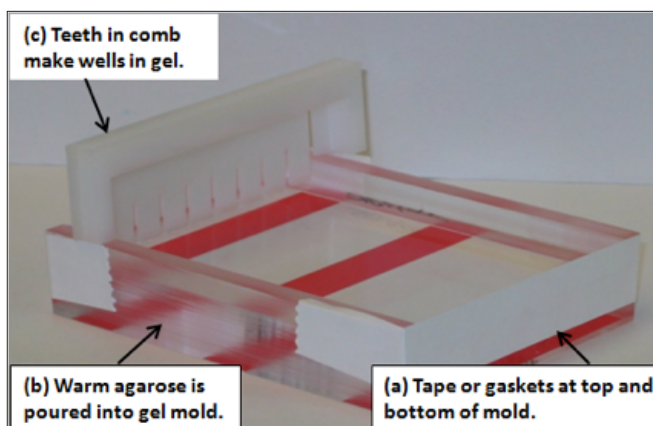


Figure 3.2: Procedures for Pouring an Agarose DNA Gel.

PART II: Running a DNA Gel

- Obtain an ice bucket and ice, and keep all of your tubes on ice as you work.
- Label a 1.7 ml microfuge tube with the following information:
 - "PCR"
 - Your name
 - The date
- Transfer all 50 µl of your PCR from the small 0.2 ml PCR tube to your new labeled 1.7 ml microfuge tube, as seen in **Figure 3.3**.

11. To prepare your DNA (PCR) sample for the gel (see **Figure 3.3**):

- Label a new 1.7 ml microfuge tube with your name and the word "gel."
- Add 5.0 μ l of 3X loading dye (labeled "3X") to your new "gel" microfuge tube.
- Add 10.0 μ l of your PCR sample to your "gel" microfuge tube.

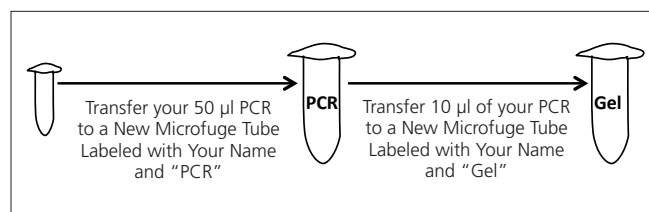


Figure 3.3: Transferring Your PCR Reaction to Microfuge Tubes.

12. Have a member of your group prepare the PCR Negative Control (tube "0") for the gel:

- Label another new 1.7 ml microfuge tube with your group's name, "negative control," and "gel."
- Add 5.0 μ l of 3X loading dye (labeled "3X") to the negative control "gel" microfuge tube.
- Add 10.0 μ l of the negative control PCR sample "0" to the negative control "gel" microfuge tube.

13. Cap the "gel" microfuge tubes, and mix them by either flicking the tube with your finger and tapping the tube on your bench top, or by vortexing and then spinning the tubes in a microcentrifuge.



14. In your lab notebook or on a separate sheet of paper, make a table similar to **Table 3.1** to list where you will load each sample from your group on your DNA gel. You will also be loading molecular weight standards on each gel. Molecular weight standards (sometimes abbreviated "MW") contain pieces of DNA of known sizes (see **Figure 3.5** at the end of this handout). This will help you determine whether your PCR reactions produce DNA bands of the expected size (about 650 base pairs). **Be sure to include your molecular weight standards in your table and on your gel!**

[**Note:** The Well Number is the slot on the gel in which you will load your samples (see **Figure 3.4**). Most gels have 8 wells, but some have only 6, and others have up to 20. Check to see how many wells are in your gel.]

15. Put your gel in your gel box and add enough DNA Gel Buffer to the gel box to just cover your gel.

Remember: "Run Towards Red."

Be sure that the top of your DNA gel (where the wells are) is near the negative, or black, electrode on the gel box, and the bottom of your gel is near the positive, or red, electrode. That way the negatively-charged DNA will always "run towards red."

Well Number	Sample Name
1	
2	
3	
4	
5	
6	
7	
8	
Example:	
1	Molecular Weight Standard
2	Joe Ting's Salmon PCR-1

Table 3.1: Samples Loaded onto DNA Gel

16. Load the 15 μ l of your PCR “gel” sample into the appropriate well of your gel, using **Table 3.1** as your guide.

Figure 3.4 illustrates how to place your pipette tip *just above the well* before releasing the sample into the well. It is important not to push your pipette tip too deeply into the well, or you risk poking it through the bottom of the well and creating a leak.

17. Have one member of your group load 15 μ l of molecular weight standard in the appropriate well of your gel, using **Table 3.1** as your guide.

18. Have another member of your group load the 15 μ l of the Negative Control “gel” sample in the appropriate well of your gel, using **Table 3.1** as your guide.

19. Plug one end of each of the electrode cords into your gel box, and plug the other ends into the power supply. Be sure to connect the black cords to the black electrodes and the red cords to the red electrodes.

20. Turn on the gel power supply to approximately 100 volts. Be sure the power supply is registering volts, and not amps. On some gel boxes, there is a button or switch to select “constant volts” or “constant amps;” you should select “constant volts.”

21. Keep an eye on your gel, or set a timer. Most gels run for about 40–50 minutes, though smaller gels may run faster. As the gel runs, the DNA loading dye will be visible in the gel. You should stop the gel by turning off the power supply when the bottom blue band of loading dye is about 1–2 centimeters from the bottom of the gel (about $\frac{3}{4}$ of the way down the gel).

22. Your instructor will tell you how to visualize your gel.

23. Review **Figure 3.5**, which shows the size of each DNA band in your molecular weight standard.

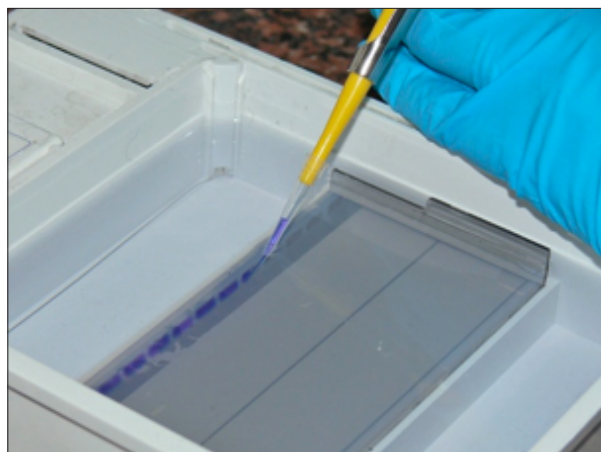


Figure 3.4: Loading Samples into a Well. Image Source: Navaho, Wikimedia Commons.

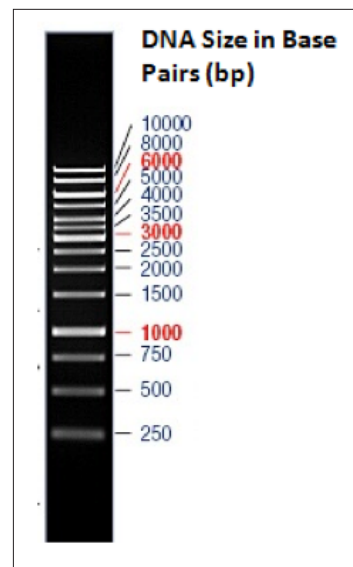


Figure 3.5: Molecular Weight Standard (1 kb DNA ladder from Fermentas). Image Source: Thermo Scientific.



On your separate sheet of paper or in your lab notebook, answer each of the following questions:

Question 1. What did you do in this laboratory experiment and why?

Question 2. What skills did you learn or practice?

Question 3. Was your PCR successful? Did you have a DNA band in your sample well? If so, what was the approximate size of your DNA (also called your “PCR product”)?



Lab 4: Preparation of PCR Samples for DNA Sequencing

Student Researcher Background:

What is PCR Purification and Why Do We Need To Do It?

If your PCR reaction was successful, the next step is to perform **PCR purification**: removal of all of your PCR ingredients, leaving only your DNA to be sequenced by a DNA sequencing facility. If your PCR ingredients were left in with your DNA sample, they would interfere with the PCR reaction performed for the DNA sequencing (described below). PCR purification is very similar to DNA purification: the DNA binds to a spin column containing a DNA-binding membrane, and all other components are washed away before the DNA is eluted from the spin column.

Sanger sequencing: A method of DNA sequencing that uses deoxyribonucleotides (dNTPs) and **dideoxyribonucleotide triphosphates (ddNTPs)**. The ddNTPs end DNA synthesis at random positions, and the resulting fragments can be analyzed to determine the exact DNA sequence.

What is DNA Sequencing?

DNA sequencing is the process of determining the exact order of the nucleotide bases—adenine (A), thymine (T), guanine (G), and cytosine (C)—in a particular piece of DNA. This is often performed at DNA sequencing facilities by laboratory technicians who are trained to work with the large machines necessary for this procedure. **Sanger sequencing** is a method of DNA sequencing used by many genetic researchers today, and is very similar in many ways to the polymerase chain reaction that you performed.

PART I: PCR Purification

1. Add 80 μ l of DNA binding Buffer (labeled “Buffer”) to the microfuge tube labeled “PCR” from *Lab 3* that contains the remaining 40 μ l of your PCR product.
[**Note:** Do not purify your Negative Control.]
2. Place one spin column in a no-cap collection tube.
3. Label the lid of the spin column with your name or initials.
4. Transfer the approximately 120 μ l of your PCR + DNA Binding Buffer sample to the spin column. Be careful when handling the spin column and no-cap collection tube, as the two are not attached to one another.
5. Close the lid of the spin column and centrifuge (or “spin”) your sample at 10,000 rpm for one minute.
[**Note:** If your centrifuge doesn’t go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
6. Add 200 μ l of Wash Buffer (labeled “Wash”) to your spin column.
7. Centrifuge your sample at 10,000 rpm for one minute.
[**Note:** If your centrifuge doesn’t go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
8. Discard the flow-through from your no-cap collection tube and put your spin column back in the no-cap collection tube.
9. Add another 200 μ l of Wash Buffer (labeled “Wash”) to your spin column.
10. Centrifuge your sample again at 10,000 rpm for one minute.
[**Note:** If your centrifuge doesn’t go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]

11. Discard the flow-through from your no-cap collection tube and put your spin column back in the no-cap collection tube.
12. Centrifuge your sample at 10,000 rpm for one minute to remove any residual Wash Buffer from the DNA binding membrane in the spin column.
[**Note:** If your centrifuge doesn't go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
13. Label a new 1.7 ml microfuge tube with your name, the date, and your sample name.
14. Place your spin column in the labeled 1.7 ml microfuge tube from *Step #13*.
15. Add 30 μ l of nuclease-free water (labeled "Water") to the membrane of your spin column, making sure not to touch the membrane at the bottom of the spin column with your pipette tip.
16. Centrifuge your microfuge tube and spin column at 10,000 rpm for one minute.
[**Note:** If your centrifuge doesn't go as fast as 10,000 rpm, centrifuge on maximum speed for 5 minutes.]
17. Discard your spin column.
18. Close the lid of your 1.7 ml microfuge tube with your purified PCR sample and keep the tube on ice, or store it in the freezer, as instructed by your teacher.

PART II: Preparing Your PCR Sample for DNA Sequencing

The barcoding primers have been specifically designed to work with standard sequencing primers called M13F (M13 forward) and M13R (M13 reverse), which are provided free of charge by most DNA sequencing facilities. Many sequencing facilities require specific DNA concentrations, DNA sequencing tubes, and sequencing tube labeling. Check with your sequencing facility to see what they require.

Determining DNA Concentration:

Ideally, DNA concentration is determined using a spectrophotometer. If a spectrophotometer is available, ask your teacher for the correct protocol. If a spectrophotometer is not available, you can estimate your DNA concentration using your molecular weight standard from your DNA gel in *Lab 3*. (See **Figure 4.1**).

For example, we know from the product information provided with our molecular weight standard that the 3rd band from the bottom of the gel in **Figure 4.1** is 750 base pairs (bp) and contains 25 nanograms (ng) of DNA.

To estimate the amount of DNA present in your PCR product, you should use the band closest in size to your PCR product.

For example, if a 700 bp PCR product is approximately four times as bright as the band at 750 bp in **Figure 4.1**, **one can estimate that there is about 100 ng of DNA in the PCR product band** ($4 \times 25 \text{ ng} = 100 \text{ ng}$).

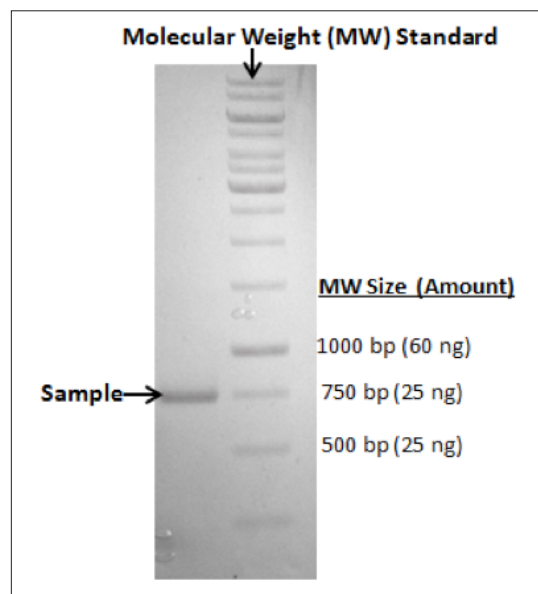


Figure 4.1: Molecular Weight Standard Used to Calculate Amount of DNA per Band.



On your separate sheet of paper or in your lab notebook, answer each of the following questions:

Question 1. Which band in your molecular weight standard is closest in size to your PCR product, and how much DNA is in that molecular weight standard band?

Question 2. Is your PCR product band brighter than the band in the molecular weight standard, as bright, or less bright?

Question 3. Based on your answers to Questions #1 and #2, how much DNA would you estimate is present in 10 µl of your PCR product? (Remember, you added 10 µl of your PCR product to the agarose gel.)

To estimate the concentration of DNA in the example of the PCR product discussed above in **Figure 4.1**:

100 ng DNA / 10 µl of PCR product loaded onto the gel = 10 ng/µl or 10 µg/ml.



On your separate sheet of paper or in your lab notebook, answer each of the following questions:

Question 4. What is the estimated concentration of your PCR product (in ng/µl)? Show your work.

Question 5. What did you do in this laboratory experiment and why?

Question 6. What skills did you learn or practice?

Question 7. How is this PCR purification similar to the DNA purification that you performed? How is it different?

If you will be sequencing your DNA through the company Eurofins MWG Operon, follow these instructions:

19. Obtain an Operon DNA Sequencing Tube from your teacher.
20. In your lab notebook or on a separate sheet of paper, write down the **barcode number** found on the side of your sequencing sample tube and the name of the sample that you will be sequencing (**Figure 4.2**).



Figure 4.2: Record the Barcode Number Found on the Side of the Operon DNA Sequencing Tube.

21. Add 30 μ l of your purified PCR product to your sequencing sample tube by ***gently but firmly pushing the pipette tip through the gasket on the lid of the tube*** (see **Figure 4.3**). After putting your sample in the tube, carefully pull your pipette tip out of the tube, leaving the gasket in place.
22. Give your DNA sequencing tube containing your sample to your instructor.



Figure 4.3: Pipetting the Sample into the Operon Sequencing Tube by Gently Forcing the Pipette Tip through the Gasket.



Lab 1: DNA Purification for DNA Barcoding Teacher Answer Key

1. On a separate sheet of paper or in your lab notebook, describe what happened to the cell membrane. Where did it go?

The cell membrane was digested or broken down by the detergents in the lysis solution. Pieces of the membrane are floating around in the buffer in the microfuge tube with the DNA.

2. On a separate sheet of paper or in your lab notebook, describe what your sample looks like.

If students began with a sample of the appropriate size (i.e., not too big), the cells should be completely lysed, and the samples should be clear, indicating that all of the membranes have been dissolved.

3. What did you do in this laboratory experiment and why?

Students purified DNA from sample X to perform DNA barcoding. Students may go into greater detail about the specific steps of the protocol: breaking open the cells, dissolving the membranes, and removing the DNA from the rest of the cell debris. The important thing is for students to understand that genetic research starts with DNA, and we must have DNA purified away from the other cellular components for subsequent experiments.

4. What skills did you learn or practice?

This question is designed to help students identify laboratory skills that they can list on a resume and/or college application.

- Handling samples
- Pipetting
- Microcentrifugation
- DNA purification using spin columns

5. List at least three types of samples from which you could purify DNA, based on what you have learned about tissues in your classes. What type of cells or tissues could you **not** purify DNA from?

This question is designed to help students realize that DNA is present in, and can be isolated from, many different types of cells/tissues. Common samples include:

- Muscle
- Blood
- Saliva
- Shaft of bird feathers (analogous to the root of a mammalian hair)

- Skin (if they contain the basal layer of the epidermis, which gives rise to new skin cells; the topmost layers of skin cells or stratum corneum are dead and contain little DNA)
- Fish scales

If students have learned about blood cells in their classes, they should know that red blood cells do not have nuclei and therefore are not a source of DNA. However, whole blood also contains white blood cells, which are often used for DNA purification.



Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)

Teacher Answer Key

On your separate sheet of paper or in your lab notebook, answer each of the following questions:

1. What did you do in this laboratory experiment and why?

Copied the DNA barcoding gene, *COI*, from the purified DNA obtained in Laboratory Experiment 1. This PCR product will be used in subsequent experiments, including agarose gel electrophoresis and DNA sequencing.

2. What skills did you learn or practice?

This question is designed to help students identify laboratory skills that they can list on a resume and/or college application.

- Handling samples
- Pipetting
- Performing a Polymerase Chain Reaction

3. What are we testing for in the Negative Control PCR? What do you expect to happen in this PCR?

Students are adding only water to their Negative Control PCR – no DNA. They are testing for DNA contamination of any of their PCR reagents, such as the primer pool and/or the nuclease-free water. They do not expect anything to happen in this PCR as there should not be any DNA present to copy.



Lab 3: Analyzing PCR Results with Agarose Gel Electrophoresis

Teacher Answer Key

On your separate sheet of paper or in your lab notebook, answer each of the following questions:

1. What did you do in this laboratory experiment and why?

Analyzed the results of our PCR reactions using agarose gel electrophoresis. Gel electrophoresis is used to separate and visualize pieces of DNA by comparing them to a known DNA Molecular Weight Standard.

2. What skills did you learn or practice?

This question is designed to help students identify laboratory skills that they can list on a resume and/or college application.

- Handling samples
- Pipetting
- Pouring, loading, running, and analyzing DNA gels
- Optional: Graphing agarose gel electrophoresis results

3. Was your PCR successful? Did you have a DNA band in your sample well? If so, what was the approximate size of your DNA (also called your "PCR product")?

The answer to this question will vary by student. If the PCR was successful, they should have a single band of approximately 650 bp in their sample well, but no bands in their negative control well.



Lab 4: Preparation of PCR Samples for DNA Sequencing Teacher Answer Key

On your separate sheet of paper or in your lab notebook, answer each of the following questions:

1. Which band in your molecular weight standard is closest in size to your PCR product, and how much DNA is in that molecular weight standard band?

Students should use either the 500 bp band or the 750 bp band. Both contain 25 ng of DNA.

2. Is your PCR product band brighter than the band in the molecular weight standard, as bright, or less bright?

Student answers will vary by experiment.

3. Based on your answers to *Questions #1* and *#2*, how much DNA would you estimate is present in 10 μ l of your PCR product? (Remember, you added 10 μ l of your PCR product to the agarose gel.)

Student answers will vary by experiment, but should be consistent with answers to *Questions #1* and *#2*. For example, if the student's PCR product is half as bright as the 500 bp band containing 25 ng of DNA, the student should estimate that their DNA band contains approximately 12.5 ng of DNA.

4. What is the estimated concentration of your PCR product (in ng/ μ l)? Show your work.

Student answers will vary by experiment, but should be consistent with answers to *Questions #1–3*. For example, if the student estimated that their DNA band contained approximately 12.5 ng of DNA (from *Question #3*):

$12.5 \text{ ng DNA} / 10 \mu\text{l of PCR product loaded onto the gel} = 1.25 \text{ ng}/\mu\text{l or } 12.5 \mu\text{g/ml}$.

5. What did you do in this laboratory experiment and why?

Purified the PCR product away from the PCR reaction components like primers, buffer, and DNA polymerase; estimated the concentration of the PCR product.

6. What skills did you learn or practice?

This question is designed to help students identify laboratory skills that they can list on a resume and/or college application.

- Handling samples
- Pipetting
- PCR product purification

- Laboratory math
- Analyzing DNA gel results, including estimating DNA concentration
- Diluting samples

7. How is this PCR purification similar to the DNA purification that you performed? How is it different?

The ultimate goal of DNA purification and PCR purification is the same: to obtain purified DNA for future experiments. In both cases, students are separating DNA from other contaminants or components using a spin column (i.e., DNA-binding membrane). As in DNA purification, in PCR purification, samples are loaded onto a DNA-binding spin column, washed, and then eluted (removed) from the column for future use.

These two experimental techniques have key differences. In DNA purification, there are additional steps involved compared to PCR purification, including lysis of the cell, removal of cell debris, and two different wash steps (a pre-wash and a wash). In the PCR purification, there is no cell to lyse. In DNA purification, the contaminants, or things being removed from the DNA, include cellular organelles and membranes. In PCR purification, the contaminants, or things being removed from the DNA, are the components of the PCR (*Taq* DNA polymerase, unused primers, unused dNTPs, and buffer). Students may also note that the DNA recovered in the DNA purification is **total** cellular DNA (i.e., nuclear and mitochondrial DNA), and the DNA is eluted in Elution Buffer, while the DNA recovered in the PCR purification is the amplified (or copied) PCR product, and the DNA is eluted in pure water.



Aliquoting DNA Barcoding Reagents for Labs 1–4

[**Note:** The following tables assume that students will be working in groups of 4.]

Lab 1: DNA Purification for DNA Barcoding

Reagent Name	Tube Label	Quantity	Volume (μl) Needed per Student	Volume (μl) Needed per Group of 4	Total Volume (μl) Aliquoted per Tube
2X Digestion Buffer	Digest	1 per group	95	380	400
Water	Water	1 per group	95	380	1000*
Proteinase K	Prot K	1 per group	10	40	45
Genomic Lysis Buffer	Lysis	1 per student	700	2800	3000 (use a 5 ml or 15 ml tube)
DNA “Spin” Column	None	1 per student	N/A	N/A	N/A
No-cap Collection Tube	None (clear)	2 per student	N/A	N/A	N/A
Pre-Wash Buffer	Pre-Wash	1 per group	200	800	850
Wash Buffer	Wash	2 per group	400	1600	1650
DNA Elution Buffer	Elute	1 per group	100	400	450

* The same tube of nuclease-free or ultra-pure water can be used for all experiments. Have students keep this tube after each lab.

Lab 2: Copying the DNA Barcoding Gene Using Polymerase Chain Reaction (PCR)

Reagent Name	Tube Label	Quantity	Volume (μl) Needed per Student	Volume (μl) Needed per Group of 4	Total Volume (μl) Aliquoted per Tube
Water	Water	1 per group	40	200***	1000*
Primer Pool COI-2	COI-2	1 per group	5	25**	30
Primer Pool COI-3	COI-3	1 per group	5	25**	30
0.2 ml PCR Tubes with Beads	None	5 per group	N/A	N/A	N/A

* The same tube of nuclease-free or ultra-pure water can be used for all experiments. Have students keep this tube after each lab.

** 5 μl per student PCR (20 μl) plus 5 μl for each group's negative control.

*** 40 μl for each student PCR (160 μl) plus 40 μl for each group's negative control.

Preparing PCR Primers:

If you are purchasing your own primers to create primer pools, follow the protocol outlined in Ivanova et al., 2007 (see *Resources*). Contact NWABR if you need assistance.

If you have received PCR primer pools from NWABR, they will arrive as 10 μM stock solutions. Either dilute and aliquot the primers immediately, or store stocks in the freezer straight away for later use. To dilute 10 μM stock solutions of primer pools to the working concentration of 2 μM used by students, dilute primers 1:5 (i.e., 1 μl primer pool per 4 μl nuclease-free water). For a class of 30 students, dilute 50 μl of primer pool stock solution into 200 μl nuclease-free water, and then aliquot 30 μl per group of 4 students.

Like all small pieces of linear DNA, PCR primers are sensitive to multiple rounds of freezing and thawing. It is recommended that you aliquot the “mother tube” of primer pools into multiple “daughter tubes,” with each daughter tube containing enough primer pool to complete one academic year of lab activities. That way, each tube will only have to be frozen and re-thawed once.

Lab 3: Analyzing PCR Results with Agarose Gel Electrophoresis

Reagent Name	Tube Label	Quantity	Volume (μl) Needed per Student	Volume (μl) Needed per Group of 4	Total Volume (μl) Aliquoted per Tube
Water	Water	1 per group	10	50**	1000*
3X DNA Loading Dye (blue)	3X	1 per group	5	25**	30
Molecular Weight Marker	MW	1 per group	N/A	15 μl	18 μl
Agarose	Agarose	1 per group	N/A	500 mg	500 mg

* The same tube of nuclease-free or ultra-pure water can be used for all experiments. Have students keep this tube after each lab.

** For each student's PCR and each group's Negative Control.

Preparing 3X DNA Loading Dye:

Most DNA loading dye is supplied as a 6X solution. This lab has been designed to be compatible with classroom micropipettes that only adjust in 5 μl increments. Therefore, the 6X DNA loading dye should be diluted 1:1 with deionized or distilled water (such as 100 μl 6X dye to 100 μl water) before aliquoting for student use.

6X DNA loading dye is stable at room temperature for over a month. 3X DNA loading dye should be stored in the freezer until needed.

Lab 4: Preparation of PCR Samples for DNA Sequencing

Reagent Name	Tube Label	Quantity	Volume (μl) Needed per Student	Volume (μl) Needed per Group of 4	Total Volume (μl) Aliquoted per Tube
DNA Binding Buffer	Buffer	1 per group	80	320	350
DNA "Spin" Column	None	1 per student	N/A	N/A	N/A
No-cap Collection Tube	None	1 per student	N/A	N/A	N/A
Wash Buffer	Wash	1 per group	400	1600	1700
Water	Water	1 per group	30	120	1000*
DNA Sequencing Tubes	None	1 per student	N/A	N/A	N/A

* The same tube of nuclease-free or ultra-pure water can be used for all experiments. Have students keep this tube after each lab.