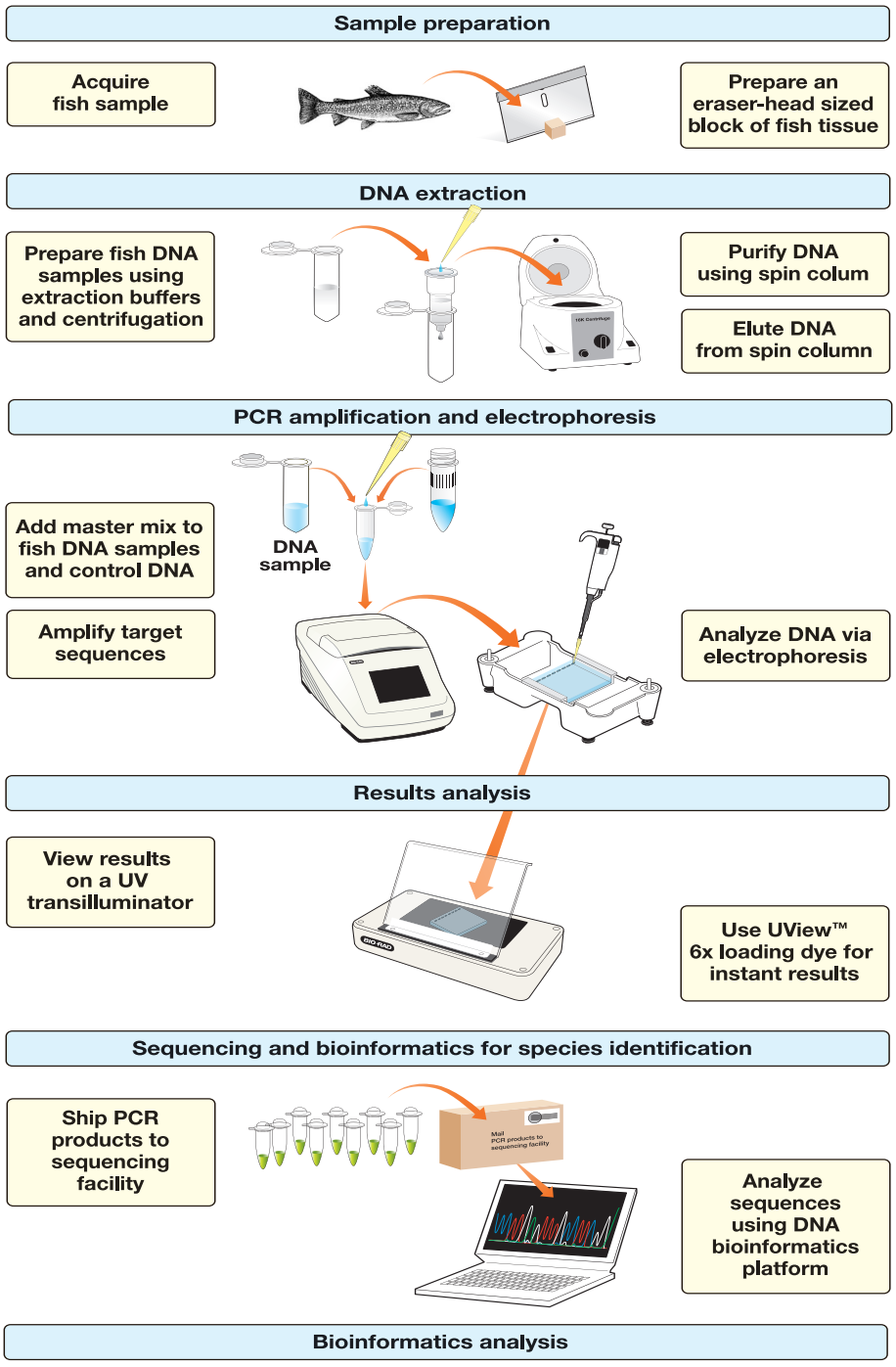
Kit Summary

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



Storage Instructions

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

Advice on Which Fish Species to Choose

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

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

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

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

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

Student Manual

Background

What Is DNA Barcoding?

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

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

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

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

DNA Barcoding and Seafood Mislabeling

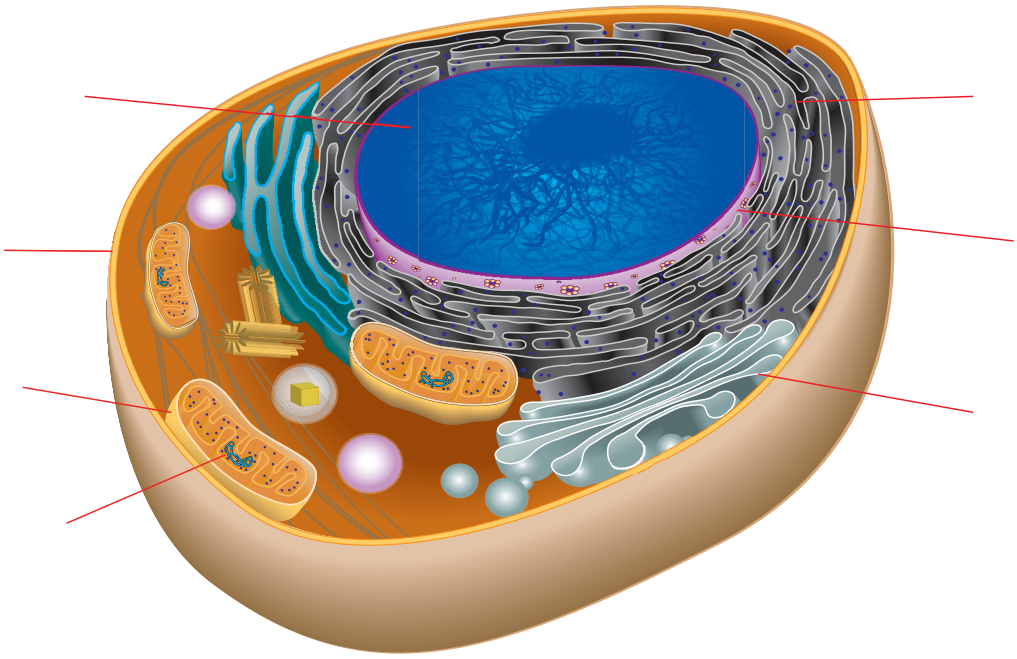
Seafood mislabeling and market substitution — the practice of incorrectly labeling or marketing seafood products — can occur at any point in the supply chain, from commercial fishing vessels to fish processing plants and commercial distribution centers to wholesale and retail fish markets to restaurants.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

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

Lesson 1: Extraction of DNA from Fish Samples

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

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



Focus Questions

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

Student Workstation

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

Common Workstation

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

Quick Guide

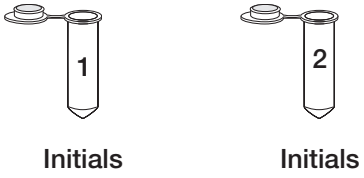
Lesson 1: DNA Extraction

Preparing Fish Samples

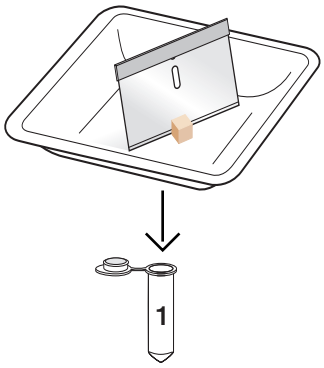
1. Label one capped 2 ml microcentrifuge tube for each of your fish samples (that is, “1” for fish sample 1, “2” for fish sample 2, etc.). Also label with your initials.

   Fish 1_____

   Fish 2_____

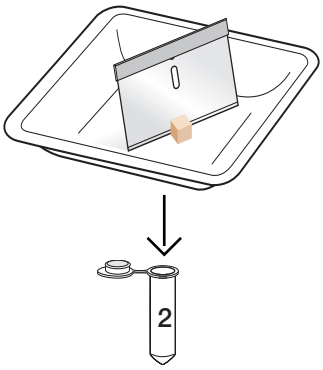


2. Cut a piece of fish muscle up to 100 mg in mass, approximately the size of a pencil eraser-head, from your first fish sample. Place the piece in a new weigh boat and slice it with a razor blade or cutting implement until finely minced. Transfer the sample into the appropriately labeled microcentrifuge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



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

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



Extracting DNA from fish samples

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

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

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

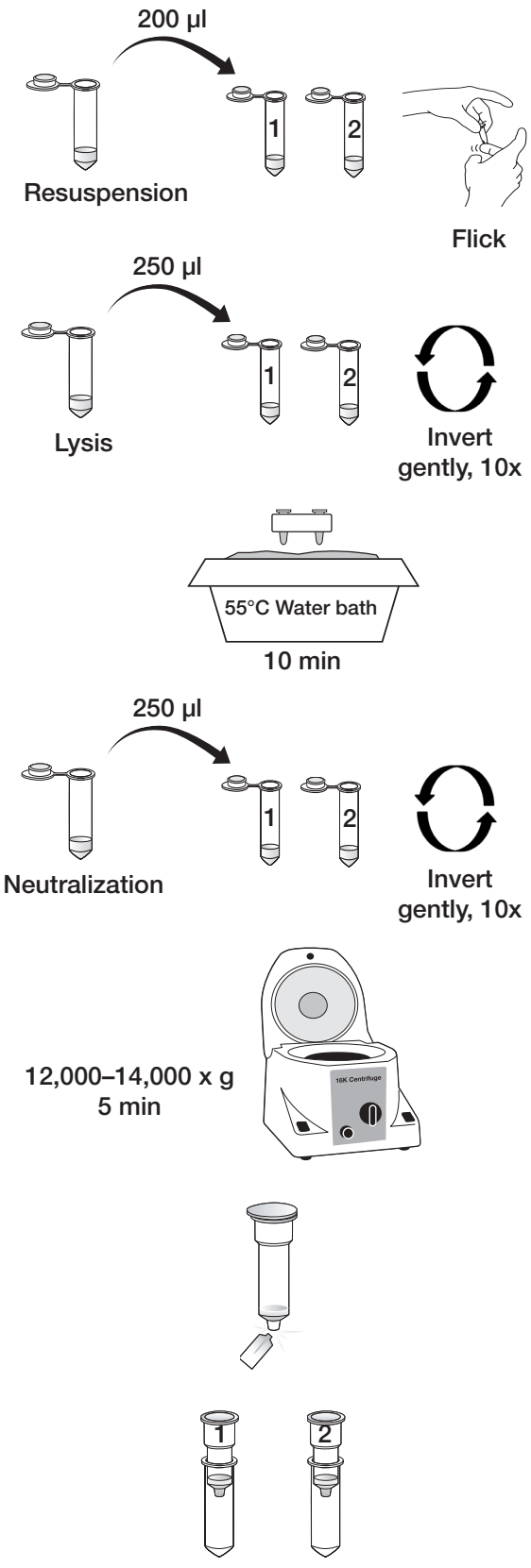
4. Add 250 µl of **Neutralization** to each microcentrifuge tube and mix gently by inverting tubes 10 times to mix contents (do not vortex). A visible cloudy precipitate may form.

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

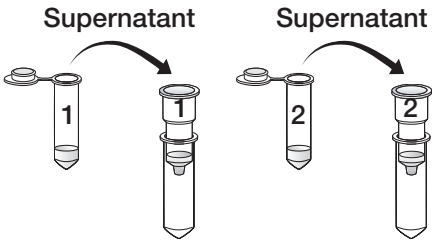
   If there are a lot of particulates remaining in the supernatant after centrifugation, centrifuge the tubes for 5 additional min.

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

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

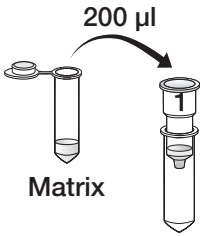


8. Transfer the entire supernatant (500–550 µl) of each fish sample from step 5 into the appropriately labeled spin column. Try not to get any of the particulates into the spin column because they will clog the column and prevent you from continuing.

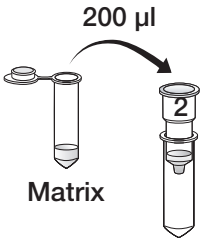


9. Thoroughly mix the tube labeled **Matrix** by vortexing or repeatedly shaking and inverting the tube to make sure particulates are completely resuspended before use.

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

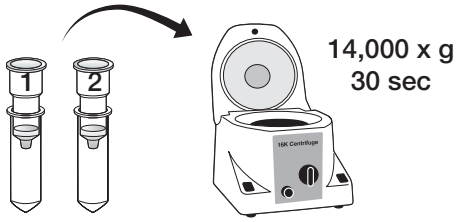


11. Using a new pipet tip, add 200 µl of thoroughly resuspended **Matrix** to the second column containing fish extract and pipet up and down to mix.



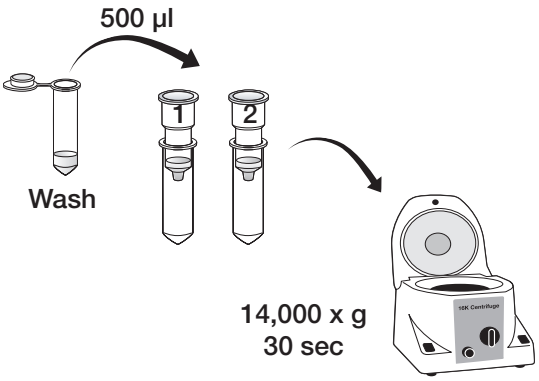
12. Centrifuge the columns for 30 sec at full speed.

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



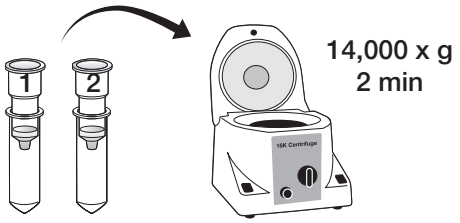
13. Remove the spin column from the 2 ml microcentrifuge tube, discard the flowthrough at the bottom of the 2 ml tube, and replace the spin column in the same tube. Add 500 µl of **Wash** and wash the matrix by centrifugation for 30 sec.

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



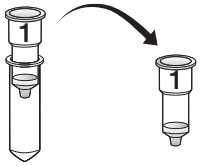
14. Repeat step 13 to wash samples again.

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

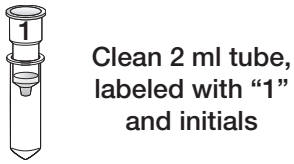


16. Label two clean 2 ml capless microcentrifuge tubes with your fish sample name and your initials.

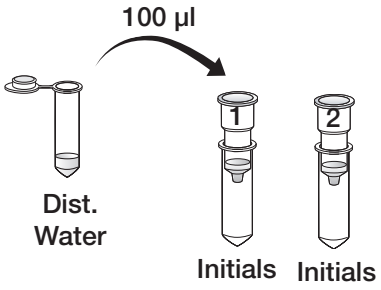
17. When your 2 min spin is completed, remove the spin columns and discard the 2 ml microcentrifuge wash tubes.



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

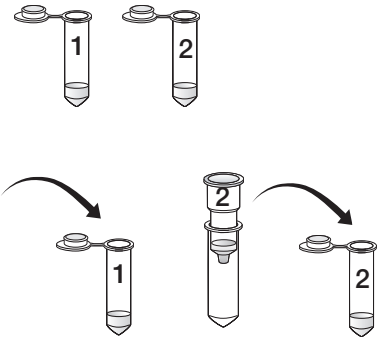


19. Using a fresh pipet tip for each sample, add 100 µl of distilled water to each spin column, being careful not to touch the resin. Elute the DNA by centrifuging for 1 min at full speed.



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

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



Lesson 2: Set Up PCR Reactions

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

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

Your Task for This Lesson

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

Focus Questions

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

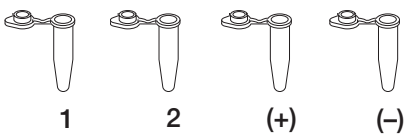
Student Workstation

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

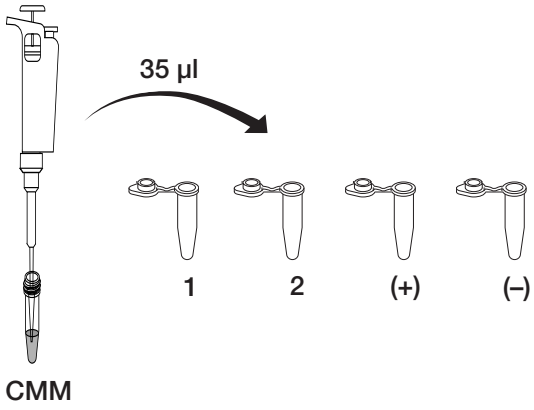
Quick Guide

Lesson 2: PCR Amplification of DNA

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

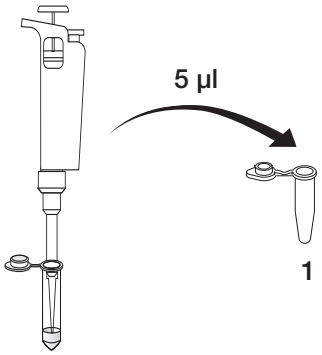


2. Using a fresh aerosol filter pipet tip each time, add 35 µl of **CMM** (COI master mix) reaction mix to each PCR tube, capping each tube immediately after the addition of liquid.



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

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



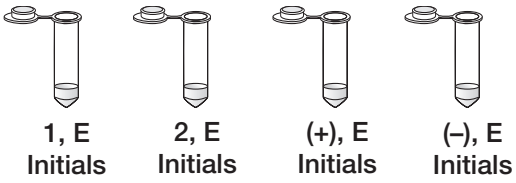
4. When instructed, place the PCR tubes in the thermal cycler and run the program with the following cycling conditions:

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

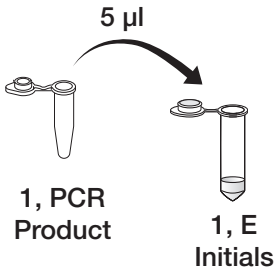
    Store tubes at 4°C after thermal cycling is complete.

Preparing PCR Samples for Electrophoresis and Sequencing

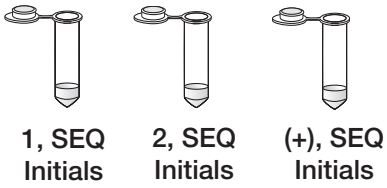
1. Label four 2 ml microcentrifuge tubes with both your initials and **E**. E stands for electrophoresis. Now label one of these tubes **Fish 1**, one tube **Fish 2**, one tube **(+)**, and one tube **(–)**.



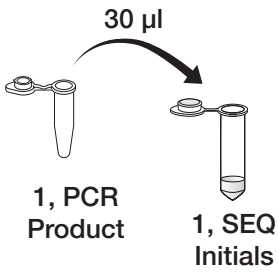
2. Remove 5 µl from each PCR reaction and deposit into the 2 ml microcentrifuge tube corresponding to that sample.



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



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



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

Lesson 3: Gel Electrophoresis

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

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

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

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

Focus Questions

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

Student Workstation

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

Common Workstation

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

Quick Guide

Lesson 3: Gel Electrophoresis

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

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

3. Set up your gel electrophoresis apparatus as instructed.

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

   Lane Sample

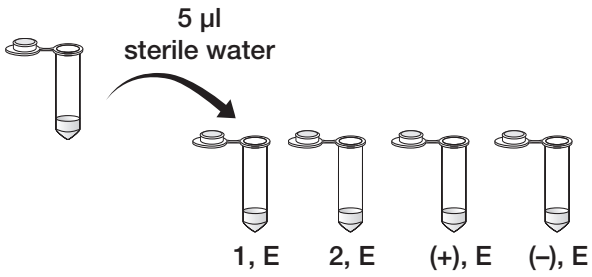
   1 – EMPTY
   2 – EMPTY
   3 – 20 µl PCR molecular weight ruler
   4 – 12 µl (+) E
   5 – 12 µl (–) E
   6 – 12 µl 1 E
   7 – 12 µl 2 E
   8 – EMPTY

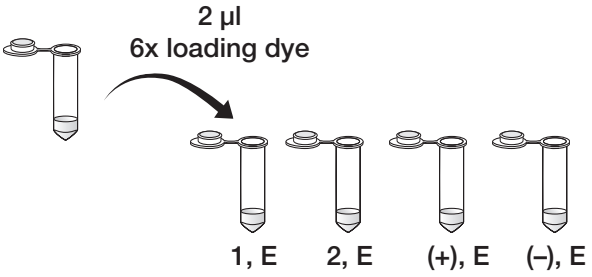
5. Ask your instructor whether the electrophoresis buffer your electrophoresis units contain is 0.25x TAE or 1x TAE.

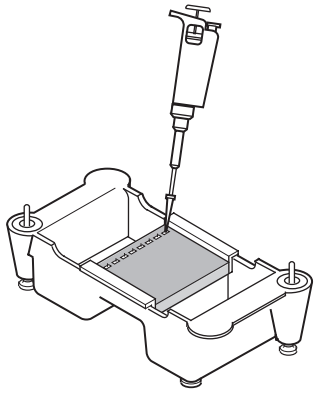
   If your buffer is 0.25 x TAE, run the gel at 200 V for 20 min.

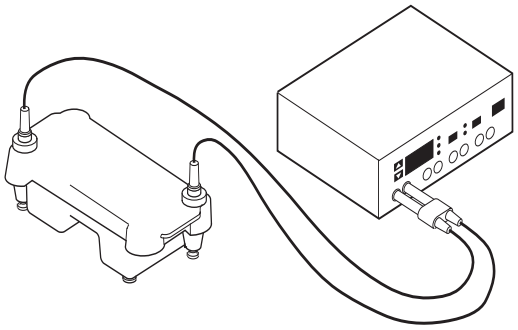
   If your buffer is 1x TAE, run the gel at 100 V for 30 min.

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









Lesson 4: Sequencing

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

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

Focus Questions

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

Student Workstation

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

Quick Guide

Lesson 4: Sequencing

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

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

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

Appendices

Appendix A:

Glossary of Terms

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Electropherogram (aka trace file) — see chromatogram.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Appendix B:

PCR Amplication and Sterile Technique

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

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

Appendix C:

Degenerate Primers

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

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

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

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

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

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

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

Appendix I:

Extension Topics

1. Inheritance of Mitochondrial DNA

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

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

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

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

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

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

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

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

References for Extension Topics

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