

EDVOTEK® • The Biotechnology Education Company®

Edvo-Kit #338

Edvo-Kit #

338

Exploring Plant Diversity with DNA Barcoding

Experiment Objective:

In this inquiry-based lab, your class will explore the genetic diversity of ten selected plants. Students will isolate plant DNA and use PCR to amplify two polymorphic regions of the chloroplast genome. Digestion of PCR products and analysis by agarose gel electrophoresis will then be used to generate unique identification profiles for each plant.

See page 3 for storage instructions.

Version 338.210628

EDVOTEK®

1.800.EDVOTEK • www.edvotek.com • info@edvotek.com

Table of Contents

Experiment Components	3
Experiment Requirements	4
Background Information	5
Experiment Procedures	
Experiment Overview and General Instructions	10
Module I: Isolation of Genomic DNA from Plants	11
Module II: Amplification of the Plant Barcode Regions	13
Module III: Electrophoresis of PCR Products	14
Module IV: Staining Agarose Gels Using FlashBlue™ (OPTIONAL)	16
Module V: Digestion of the PCR Products	18
Module VI: Electrophoresis of Restriction Digests	19
Module VII: Staining Agarose Gels Using FlashBlue™ (OPTIONAL)	19
Study Questions	20
Instructor's Guidelines	
Plant Recommendations for DNA Extraction	24
Pre-Lab Preparations	25
Experiment Results and Analysis	29
Study Questions and Answers	30
Appendices	31

Safety Data Sheets can be found on our website: www.edvotek.com/Safety-Data-Sheets

Technical Support

1.800.EDVOTEK
Mon. - Fri. 8 AM to 5:30 PM EST



Please Have the Following Info:

- Product Number & Description
- Lot Number on Box
- Order/Purchase Order #

1.800.EDVOTEK • info@edvotek.com • www.edvotek.com

www.edvotek.com



- Online Catalog
- Order Products
- Experiment Protocols
- Tech Support
- Resources!

EDVOTEK, The Biotechnology Education Company, and InstaStain are registered trademarks of EDVOTEK, Inc. PCR EdvoBead, FlashBlue and UltraSpec-Agarose are trademarks of EDVOTEK, Inc. SYBR is a registered trademark of Thermo Fisher Scientific.



1.800.EDVOTEK • Fax 202.370.1501 • info@edvotek.com • www.edvotek.com

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2016-2021 EDVOTEK, Inc., all rights reserved. Version 338.210628

Experiment Components

Component	Storage	Check (✓)
A* PCR EdvoBeads™	Room Temp., Desiccated	<input type="checkbox"/>
B Primer G mix concentrate	-20 °C Freezer	<input type="checkbox"/>
C Primer LP mix concentrate	-20 °C Freezer	<input type="checkbox"/>
D EdvoQuick™ DNA Ladder	-20 °C Freezer	<input type="checkbox"/>
E Control DNA concentrate	-20 °C Freezer	<input type="checkbox"/>
F TE Buffer	-20 °C Freezer	<input type="checkbox"/>
G Proteinase K	-20 °C Freezer	<input type="checkbox"/>
H NaCl Solution	Room Temp.	<input type="checkbox"/>
I DNA Extraction Buffer	Room Temp.	<input type="checkbox"/>
J Restriction Enzyme Reaction Buffer	-20 °C Freezer	<input type="checkbox"/>
K Restriction Enzyme Dilution Buffer	-20 °C Freezer	<input type="checkbox"/>
L <i>Alu</i> I Restriction Enzyme	-20 °C Freezer	<input type="checkbox"/>

This experiment is designed for 10 groups.

Sample volumes are very small. It is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

* Each PCR EdvoBead™ contains: dNTP Mixture, Taq DNA Polymerase Buffer, Taq DNA Polymerase, and MgCl₂

Reagents & Supplies

Store components below at Room Temperature.

Component	Check (✓)
• Micropestles with Microcentrifuge Tubes	<input type="checkbox"/>
• 10x Gel Loading Solution	<input type="checkbox"/>
• UltraSpec-Agarose™	<input type="checkbox"/>
• 50x Electrophoresis Buffer	<input type="checkbox"/>
• SYBR® Safe DNA Stain	<input type="checkbox"/>
• FlashBlue™ Liquid Stain	<input type="checkbox"/>
• Screw-top microcentrifuge tubes	<input type="checkbox"/>
• Snap-top microcentrifuge tubes	<input type="checkbox"/>
• PCR tubes, 0.2 mL	<input type="checkbox"/>

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

1.800.EDVOTEK • Fax 202.370.1501 • info@edvotek.com • www.edvotek.com

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2016-2021 EDVOTEK, Inc., all rights reserved. Version 338.210628



Requirements

- Scissors or hole punch
- Water bath (37 °C and 65 °C)
- Centrifuge (maximum speed should be 10,000xg or greater)
- Thermal cycler
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- UV Transilluminator or Blue Light visualization system (for visualizing SYBR® Safe stained gels)
- UV safety goggles
- White light visualization system (OPTIONAL for visualizing FlashBlue™ stained gels)
- Automatic micropipettes (5-50 µL, 20-200 µL, 100-1000 µL recommended) with tips
- Microwave or hot plate
- 250 mL Flasks or beakers
- Hot gloves
- Disposable laboratory gloves
- Ice buckets and ice
- Freezer
- Isopropanol
- Distilled or deionized water



Background Information

HISTORICAL WAYS TO CLASSIFY LIFE

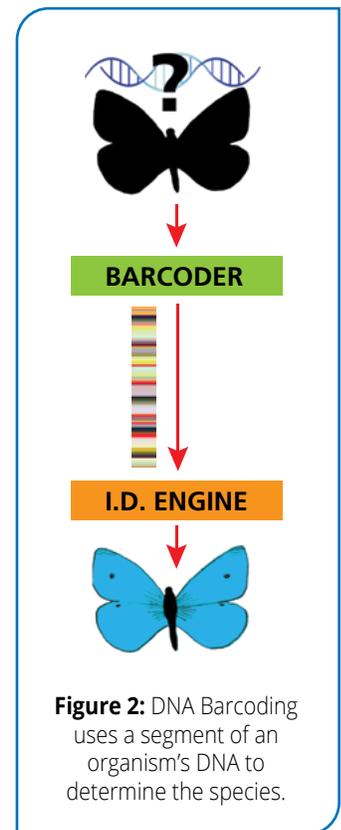
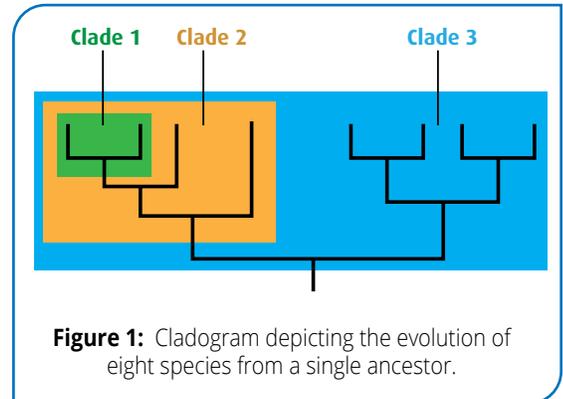
Naming and classifying organisms is part of our nature. For thousands of years, humans have been describing, discovering, and documenting the diversity of life. To date, classification systems include nearly 2 million species of animals, plants, and microorganisms. Yet experts estimate that at least 5 million species are still unknown. Each known species is classified using a binomial naming system in which the first word is the genus and the second the species. These names are used by biologists worldwide because they are short, stable, and unique. However, assigning these names can be a challenge.

Historically, morphological features such as shape, structure, color, and pattern were used to classify organisms. The science of defining and naming groups of biological organisms on the basis of shared characteristics is called taxonomy. Species are the basic unit of taxonomy and are defined as populations that could interbreed and produce fertile offspring. Closely tied to taxonomy is phylogeny. In phylogeny, scientists try to reconstruct the pattern of events that led to the diversity of life we observe today. The word phylogeny is also used to describe the hypotheses formed about the evolutionary history of taxonomic groups depicted by branches in a tree of life. When reading a phylogeny the trunk/root of the tree represents the ancestral lineage while the tips of the branches represent the descendants of that ancestor. As you move from the root to the tips you are moving through periods of time. Each division of the phylogenies (a.k.a. bifurcating tree) stands for a speciation event during which a single ancestral lineage gave rise to two or more daughter lineages. A clade is a group that includes a common ancestor and each of their descendants. Clades are nested within one another and so form a hierarchy similar to the taxonomic grouping of organisms (Figure 1).

Closely related organisms are likely to share similar morphologies. Similarities due to shared ancestry are called homologies. These show a common evolutionary history. In contrast, analogies represent similarities due to convergent evolution, and not to shared ancestry. For example, both birds and bats have adaptations that allow them to fly (i.e. wings). However, the fossil records show that both wings arose independently over time from the forelimbs of different ancestors. Distinguishing between homologies and analogies is an important part of phylogeny. The effects of the occasional analogy can be minimized by basing the clade on several complex characteristics such as the bones in the skull or a fragment of DNA.

USING MOLECULAR TECHNIQUES TO CLASSIFY LIFE

Today we are using short genetic markers in an organism's DNA to assign it to a particular species. This process is called "DNA barcoding" because the genetic marker acts like a universal product code (UPC). The UPC is used to identify retail products. This system uses 10 alternative numbers at 12 positions to generate 100 billion unique combinations. Scanning or entering in the code returns key information about an object on sale. In the same way, we can identify a specimen of interest by reading a short, standardized fragment of its DNA. This information is then compared to a database of other sequences of previously identified species. If there is a match, the investigator knows what species the specimen belongs to and can retrieve information about that species (Figure 2). If the sequences do not match, the investigator has preliminary evidence of a new species.

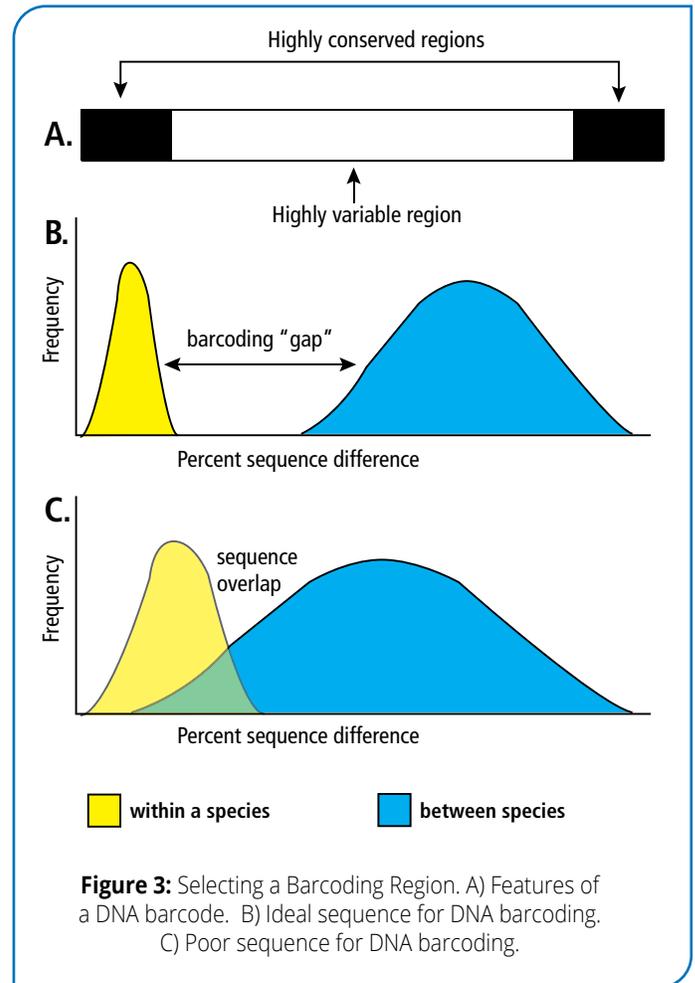


DNA barcoding represents an alternative route to taxonomic identification for situations where the morphology is inconclusive. For example, organisms at certain life stages are hard to distinguish based solely on appearance. Barcoding can help identify insects still in their egg stage and trees without leaves. DNA barcoding is also used when only a piece of the organism is present. For example, scientists have used barcoding to detect false labeling in sushi and illegal wood exports. Even under ideal circumstances morphological identification often requires considerable skill and time. Barcoding represents a way to assess biodiversity when these are not available. To accomplish this, DNA barcoding needs to find regions of DNA that distinguish between the majority of the world's species.

DNA is the hereditary material of life. It is composed of simpler units called nucleotides. There are four nucleotides — guanine (G), adenine (A), thymine (T), and cytosine (C). Billions of nucleotides link together to form a four letter code, parts of which direct protein formation. The order of this nucleotide code is carefully preserved throughout an organism's life and then passed to next generations through the process of replication. However, errors in replication can occur. Any change in the sequence of nucleotides is known as a mutation. Mutations can be as small as a single base change in the DNA sequence or as large as a multi gene duplication event or chromosomal rearrangement. These changes play a part in both normal and abnormal biological processes including evolution and cancer. Changes that occur in the reproductive cells, known as germ line mutations, are passed from parent to offspring.

Over time, the different regions of the genome change at different rates. DNA regions that code for essential traits rarely change because most mutations are selected against. These regions, known as highly conserved sequences, will be identical between many species. In contrast, highly variable regions tend to be noncoding sequences where a change in the nucleotide order is "invisible" to natural selection. These regions are physically structured so that the enzyme polymerase has a difficult time copying the DNA accurately. Such regions often differ between individuals. In between these two extremes are a full array of evolutionary rates.

In this experiment, we will distinguish between different plants by examining two chloroplast DNA regions - the first intron of the tRNA-Gly gene (Primer Set G) and the intergenic region between tRNA-Leu and tRNA-Phe (Primer Set LP). Both of these regions consist of a large non-coding region flanked by highly conserved sequences (Figure 3A). When choosing regions for DNA barcoding, it is important that the sequence within the noncoding region provides a large variation between species yet a relatively small amount of variation within a species. The area between these two types of genetic variation is known as the species gap or barcoding gap (Figure 3B). This gap enables assignment of unidentified individuals to their species with a low error rate. In contrast, the DNA sequence in an unsuitable barcoding region would show a large variation within a species and less variation between species (Figure 3C). The neighboring conserved regions are needed so that the same primers (see the next section) can be used for all specimens.



MOLECULAR METHODS USED IN DNA BARCODING

This experiment begins with DNA extraction. First cell walls, cell membranes and nuclear membranes are destroyed in a process known as cell lysis. Next, salts are added to interrupt hydrogen bonding between water and phosphate groups of the DNA backbone. This is followed by the addition of isopropanol which precipitates the DNA out of solution as a stringy white fiber. Finally, DNA is resuspended in water or TE buffer.

The next step in "reading" a DNA fragment is to target and amplify the region(s) of interest. A DNA amplification technique called polymerase chain reaction (PCR) allows a small fragment of DNA to be increased over a million fold (Figure 4). The

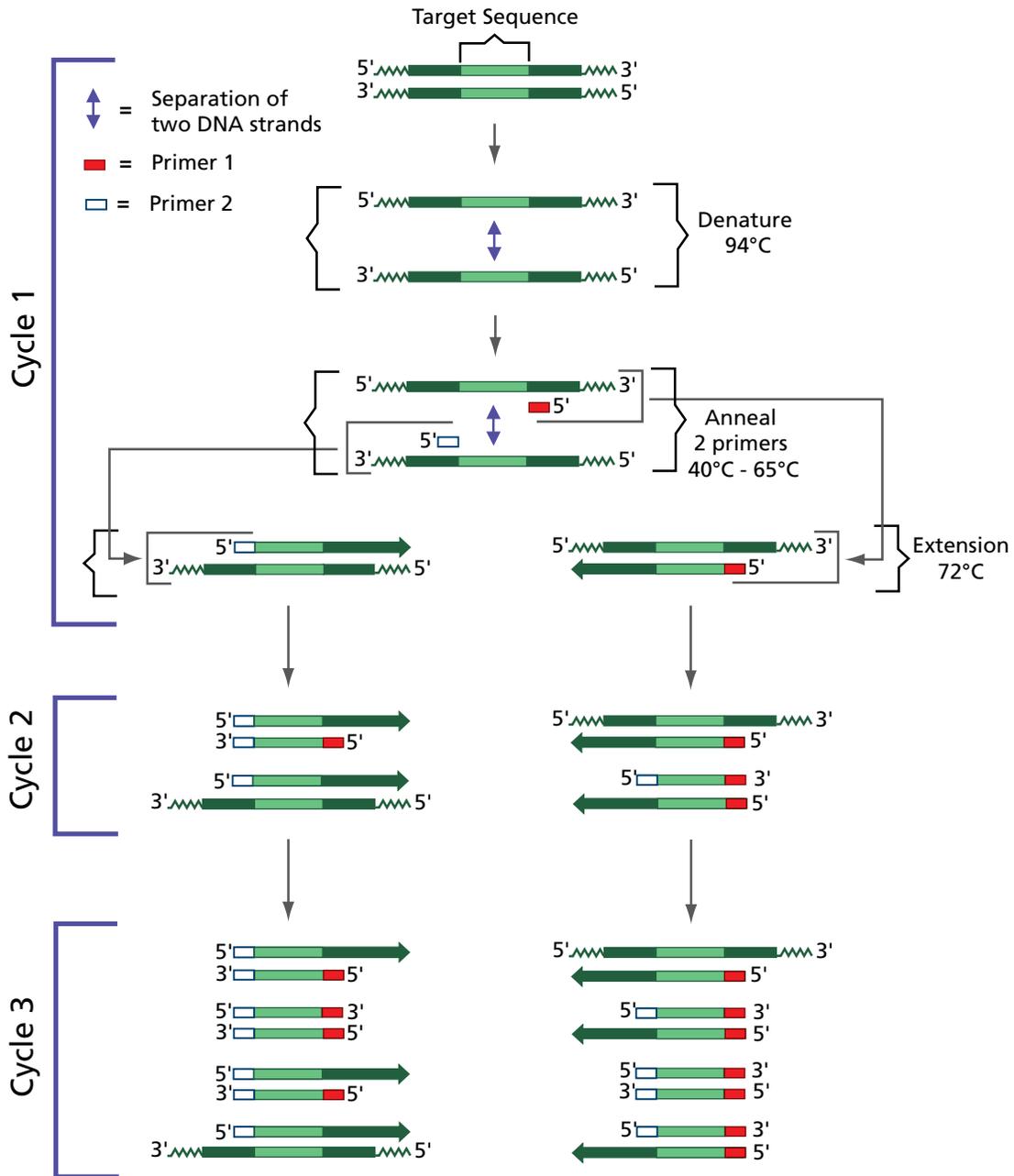


Figure 4: The Polymerase Chain Reaction – 3 cycles

enormous utility of PCR is based on its procedural simplicity and its specificity. To prepare for PCR amplification, two DNA "primers" are designed to target a specific region of the genomic sequence. Primers are synthetic oligonucleotides typically 15-30 base pairs in length that bind to the start and end of the targeted DNA region. Besides the two primers, the four deoxynucleotides (dATP, dCTP, dGTP, and dTTP) that are the precursor building blocks of DNA and a thermally stable DNA polymerase are required. DNA polymerases are enzymes that create DNA molecules by assembling nucleotides. The most commonly used DNA polymerase is the enzyme *Taq* polymerase, which is purified from the thermophilic bacterium *Thermus aquaticus* that inhabits hot springs. This enzyme is stable at near-boiling temperatures.

The PCR process requires the sequential heating and cooling of the PCR sample (primers, template, dNTPs, and *Taq*) to three different temperatures. It is efficiently performed in a thermal cycler, an instrument that is programmed to rapidly heat, cool, and maintain samples at designated temperatures for varying amounts of time. In the first step of the PCR reaction, the mixture is heated to near boiling (94 °C) to disrupt the hydrogen bonds between DNA strands. This step, which results in the complete separation of the two DNA strands, is known as "denaturing". In the second PCR step, known as annealing, the sample is cooled to a temperature between 45° - 65 °C. At this time, the two primers bind to their target complements. In the third step, of PCR, the temperature is raised to an intermediate value (usually 72 °C). During this time, known as "extension", the molecules of *Taq* polymerase bind to each DNA strand and primer complex. *Taq* then adds the four free deoxynucleotides to synthesize a new complementary strand of DNA. The order of deoxynucleotides along this strand is determined by Watson-Crick base pairing with the original strand.

These three steps--denaturation, annealing, and extension - make up one PCR "cycle." Each cycle doubles the amount of the target DNA. Calculated mathematically, if the cycle is repeated n times the number of copies will be an exponential enlargement of 2^n . For example, ten cycles will produce 2^{10} or 1024 copies. The PCR process is repeated for 20-40 cycles, theoretically amplifying the target sequence to millions of copies. In practice, the amount of product often reaches a maximum after about 35 cycles. This is due to depletion of the reaction components and loss of DNA polymerase activity.

Once the desired fragment of DNA has been amplified we use restriction enzymes to detect variations in the nucleotide order. Restriction enzymes are endonucleases that catalyze the cleavage of the phosphate bonds within both strands of DNA. The distinguishing feature of restriction enzymes is that they only cut at very specific sequences of bases. Recognition sites are often symmetrical, i.e., both DNA strands in the site have the same base sequence when read 5' to 3'. Such sequences are called palindromes. A single base change in the recognition palindrome results in the inability of the restriction enzyme to cut the DNA at that location. This will alter the length and number of DNA fragments generated after digestion. A sequence variation of this type is called a Restriction Fragment Length Polymorphism, or RFLP.

In Figure 5A, we have two DNA samples prepared from plant tissue that differ by a single nucleotide base pair known as a single nucleotide polymorphism or a SNP. In this case, the restriction enzyme *AluI* cuts between the G and C in the sequence "AGCT". Because DNA from the first sample has two recognition sequences for the restriction enzyme, it

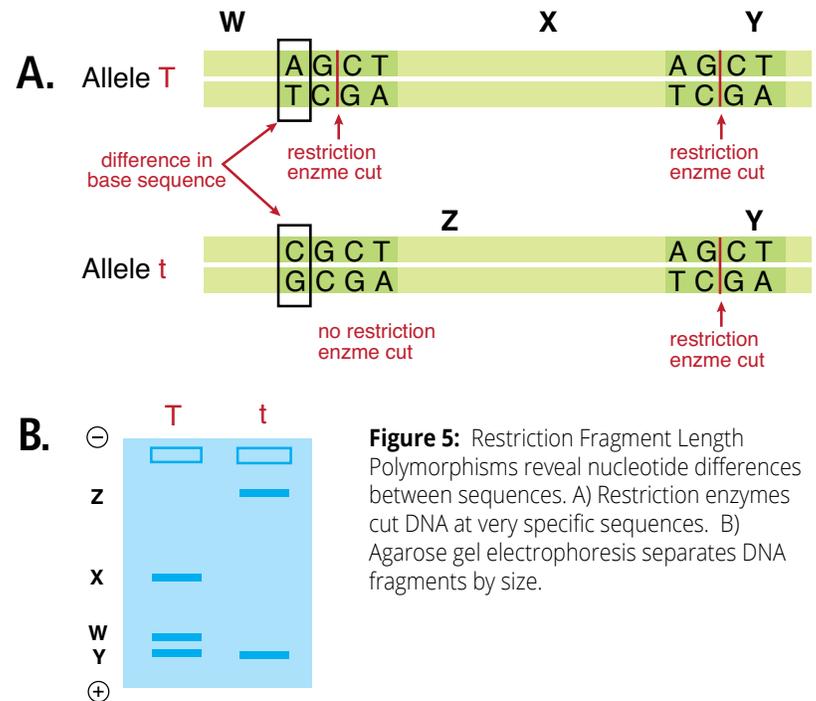


Figure 5: Restriction Fragment Length Polymorphisms reveal nucleotide differences between sequences. A) Restriction enzymes cut DNA at very specific sequences. B) Agarose gel electrophoresis separates DNA fragments by size.

is cleaved in two places yielding three fragments. In contrast, DNA from the second sample has one recognition sequence, which yields two restriction fragments.

Following amplification and digestion, the PCR-RFLP fragment products are visualized and sized using gel electrophoresis. In gel electrophoresis the product is added into depressions (or "wells") within a gel, and then an electrical current is passed through the gel. Because the sugar-phosphate backbone of DNA has a strong negative charge, the current drives the DNA through the gel towards the positive electrode. On the molecular level, the gel has small channels through which the DNA can pass. Small DNA fragments move through these holes easily, but large DNA fragments have a more difficult time squeezing through the tunnels. Because molecules with dissimilar sizes travel at different speeds, they become separated and form discrete "bands" within the gel. The banding patterns are determined by the fragments generated from the restriction digest (Figure 5B). After the current is stopped, the bands can be visualized using a stain that sticks to DNA.

DNA barcoding is based on the idea that inside the genome of every single organism there is a unique identifier that when "scanned" returns the species that the organism belongs to. This identifier is made up of a combination of single nucleotide polymorphisms (SNPs) that are constant within a species but vary between species. Each nucleotide is considered a character that can be used to differentiate between species. In theory, random combinations of the four possible nucleotides at fifteen positions would create one billion unique codes – enough to describe the 7 to 50 million species that currently exist on Earth. In this experiment we use PCR-RFLP analysis of two chloroplast regions to reveal SNPs that can be used to describe common plants.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this inquiry-based lab, your class will explore the genetic diversity of ten selected plants. Students will isolate plant DNA and use PCR to amplify two polymorphic regions of the chloroplast genome. Digestion of PCR products and analysis by agarose gel electrophoresis will then be used to generate unique identification profiles for each plant.

LABORATORY SAFETY:

Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!



- Wear gloves and goggles while working in the laboratory.
- Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose.
- DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
- Always wash hands thoroughly with soap and water after working in the laboratory.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

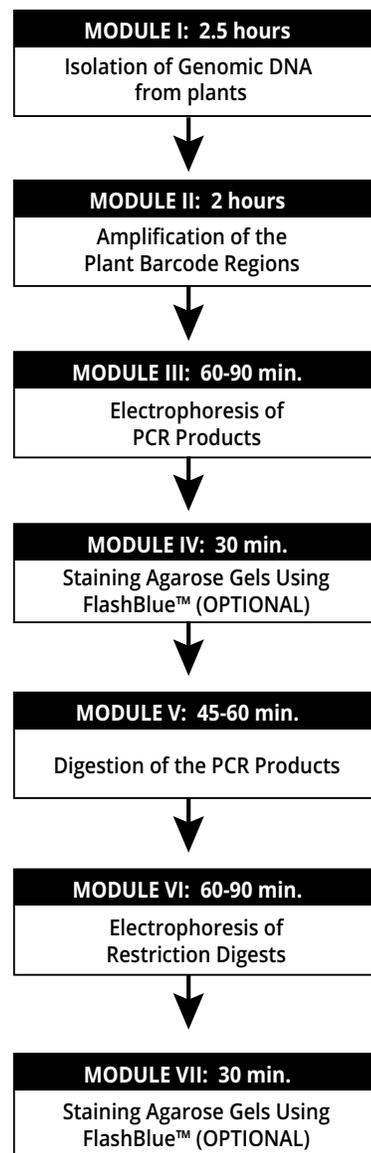
- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

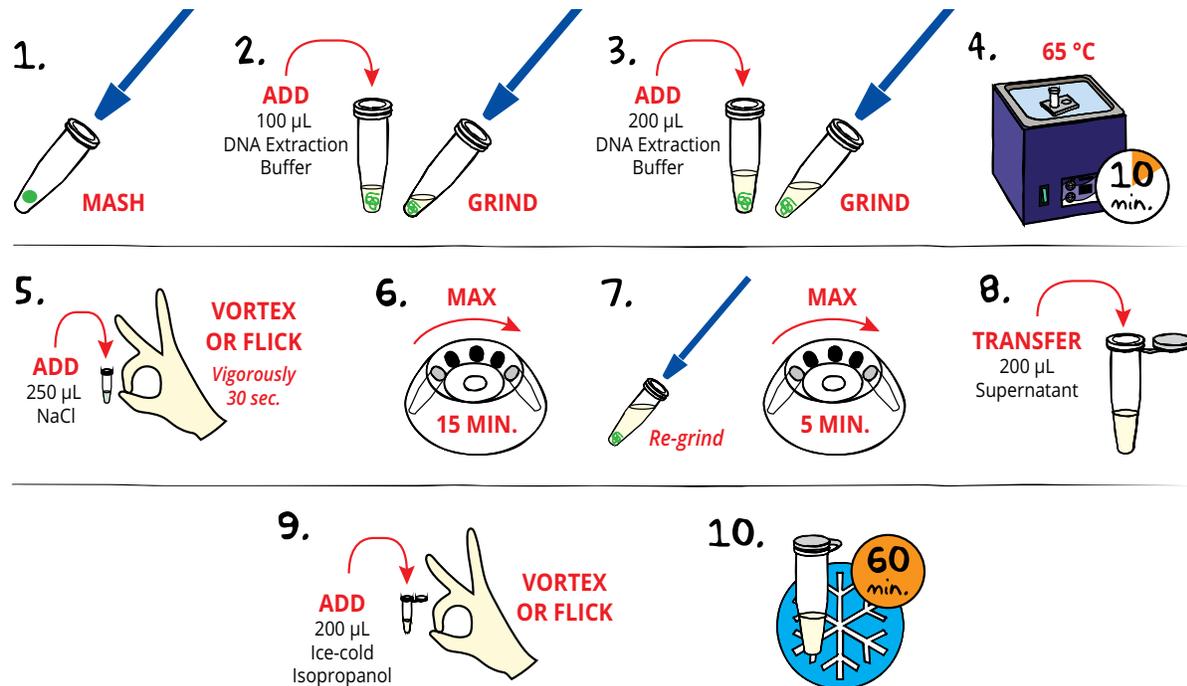
- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.



Module I: Isolation of Genomic DNA from Plants



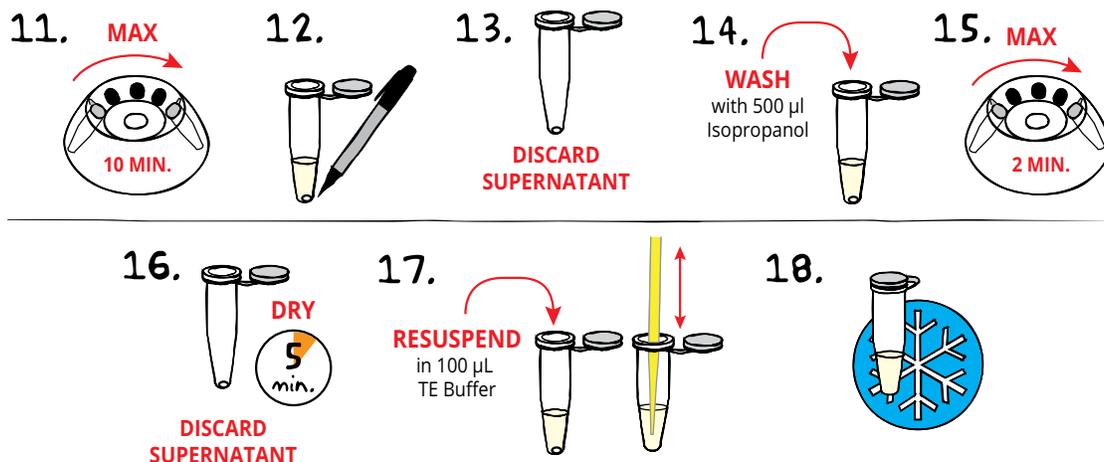
- Using scissors or a hole punch, **HARVEST** a ~1x1x.01 cm³ piece of tissue from a plant into a screw-top microcentrifuge tube with pestle. Use the pestle to partially **MASH** the tissue for 10 seconds. For plant recommendations, see page 24.
- ADD** 100 µL of DNA Extraction Buffer to each tube and continue grinding the tissue for 10 seconds.
- ADD** an additional 200 µL of DNA Extraction Buffer to each tube and grind the tissue again for 10 seconds.
- LABEL** the tube with your group name or number. **INCUBATE** the tubes in a water bath at 65 °C for 10 min.
- ADD** 250 µL NaCl solution to each tube and mix well by vortexing or flicking for 30 seconds.
- CENTRIFUGE** the plant tissue lysate at full speed (10,000xg) for 15 minutes.
- RE-GRIND** the pelleted material in each tube and **CENTRIFUGE** the tube at full speed (10,000xg) for 5 minutes.
- Carefully **TRANSFER** 200 µL of the supernatant into a fresh, labeled 1.5 mL snap-top microcentrifuge tube being careful not to disturb the pellet. Discard the tubes with pellets.
- PRECIPITATE** the DNA from the supernatant by adding 200 µL of ice-cold isopropanol. **MIX** well by vortexing or flicking the tube.
- INCUBATE** the tube in the freezer for at least one hour. For convenience, samples can be left in the freezer overnight.



OPTIONAL STOPPING POINT:

Store the plant tissue lysate at -20 °C if it will not be used immediately.

Module I: Isolation of Genomic DNA from Plants, continued



11. **INSERT** the tube in the rotor so that the hinge is facing towards the outside edge. **CENTRIFUGE** the sample for 10 minutes at full speed. The precipitated plasmid DNA will form a small, white pellet at the bottom of the hinge side of tube after centrifugation.
12. After centrifugation, a very small DNA pellet should be visible at the bottom of the tube. With a marker, **CIRCLE** the location of the DNA pellet.
13. Carefully **REMOVE** and **DISCARD** all the supernatant, leaving the pelleted DNA at the bottom of the tube. Take care to avoid the pellet when removing the supernatant, as it may come loose.
14. **WASH** the pellet with 500 µL of isopropanol.
15. **CENTRIFUGE** the sample at full speed for 2 minutes.
16. Carefully **REMOVE** and **DISCARD** the supernatant and allow the DNA pellet to dry for 5 minutes.
17. Completely **RESUSPEND** the pellet in 100 µL of TE by pipetting up and down several times or by vortexing vigorously.
18. **PLACE** tubes in ice. **PROCEED** to Module II: Amplification of the Plant Barcode Regions.



OPTIONAL STOPPING POINT:

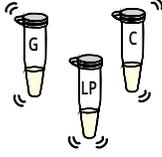
Store the DNA at -20 °C if it will not be used immediately.

Module II: Amplification of the Plant Barcode Regions

1. LABEL **2. ADD** **3. GENTLY MIX** **4. QUICKLY SPIN** **5. PCR**

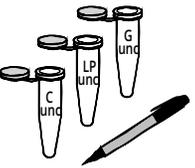
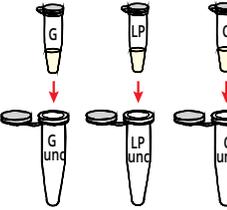
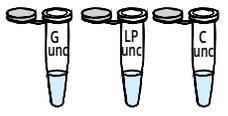


All Tubes:	1 PCR EdvoBead™
Tubes LP, G:	5 µL Extracted DNA
Tube C:	5 µL Control DNA
Tube G:	20 µL G Primer Mix
Tube LP:	20 µL LP Primer Mix
Tube C:	20 µL Selected Primer Mix





6. **7. LABEL** **8. TRANSFER** 5 µl uncut DNA **9. ADD** 5 µL 10X gel loading dye & 15 µL distilled water **10. PROCEED** to Module III. Keep uncut PCR products frozen or on ice.


NOTE: Not all groups will perform a control reaction. In this case, proceed with the PCR for your extracted DNA samples only.

- LABEL** three 0.2 mL PCR tubes "G", "LP", and "C" plus your group name or number.
- To each tube, **ADD** one PCR EdvoBead™. **ADD** 5 µL of extracted DNA to tubes G and LP and 5 µL control DNA to tube C. Next, **ADD** 20 µL of "G primer mix" to the tube G, 20 µL of "LP primer mix" to tube LP, and 20 µL of the selected primer mix to tube C.
- MIX** the PCR samples gently. Make sure the PCR EdvoBead™ is completely dissolved.
- Quickly **CENTRIFUGE** to collect the sample at the bottom of the tube.
- AMPLIFY** DNA using PCR.
PCR Cycling Conditions:
 Initial denaturation: 94 °C for 5 minutes
 94 °C for 30 seconds
 55 °C for 30 seconds
 72 °C for 90 seconds } 35 cycles
 Final Extension 72 °C for 4 minutes
- PLACE** tubes on ice.
- LABEL** three 0.5 mL microcentrifuge tubes "G unc", "LP unc", and "C unc". These tubes will contain the uncut PCR products that are analyzed in Module III.
- TRANSFER** 5 µL of uncut PCR product into the appropriate tube.
- ADD** 5 µL 10X Gel Loading Dye and 15 µL distilled water to each uncut PCR product.
- PROCEED** to Module III (Electrophoresis of PCR Products) with the samples in tubes G unc, LP unc, and C unc. The samples in the PCR tubes should be frozen or kept on ice until they are used in Module V.

NOTES

Step 1:

Tube C is a PCR positive control and will include the control DNA handed out by your instructor and either the G or LP primer.

Step 2:

Primer set G amplifies the tRNA-Gly gene. Primer set LP amplifies the intergenic region between tRNA-Leu and tRNA-Phe.

Step 5:

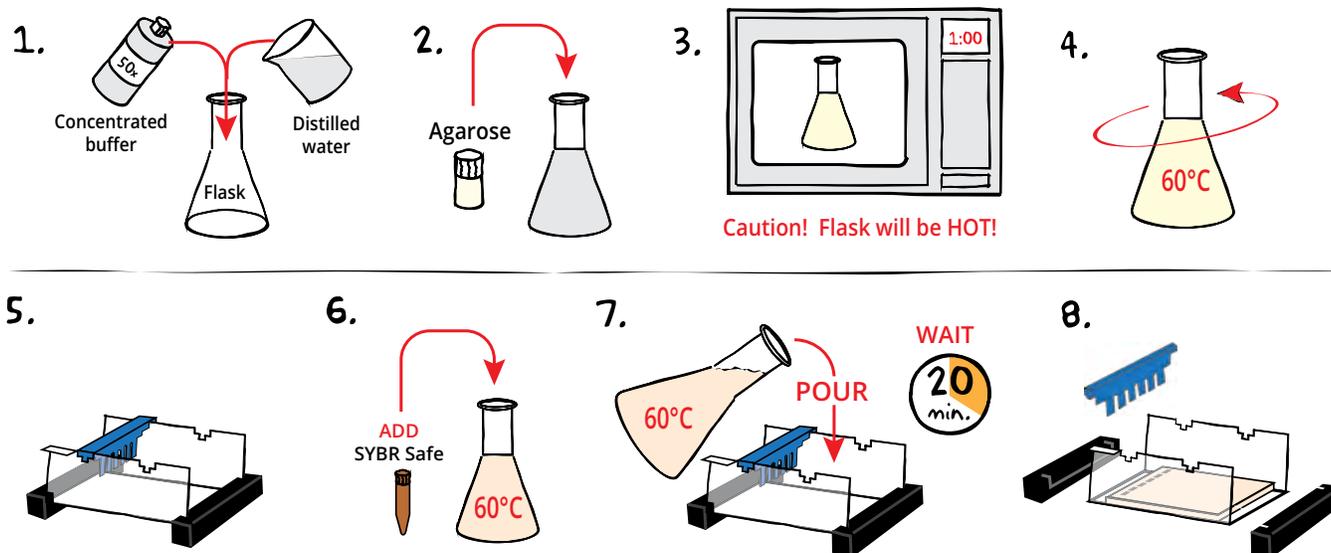
Samples can be frozen following Step 5. Be sure to thaw the samples completely before proceeding to Step 6.



OPTIONAL STOPPING POINT:

All six samples may be stored at -20 °C for electrophoresis and restriction digest at a later time.

Module III: Electrophoresis of PCR Products



NOTE: If you are casting your own gels, review the following instructions. If you are using pre-cast gels, proceed to Step 8.

- DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
- MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
- DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
- COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
- While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch. **NOTE: This experiment requires 6 wells.**
- Before casting the gel, **ADD diluted SYBR® Safe** to the molten agarose and swirl to mix (see Table A).
- POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
- REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.



Reminder:

Two groups will share one 2.0% agarose gel with at least six sample wells for electrophoresis. We strongly recommend 7x10 cm or 7 x 14 cm gels for this experiment.

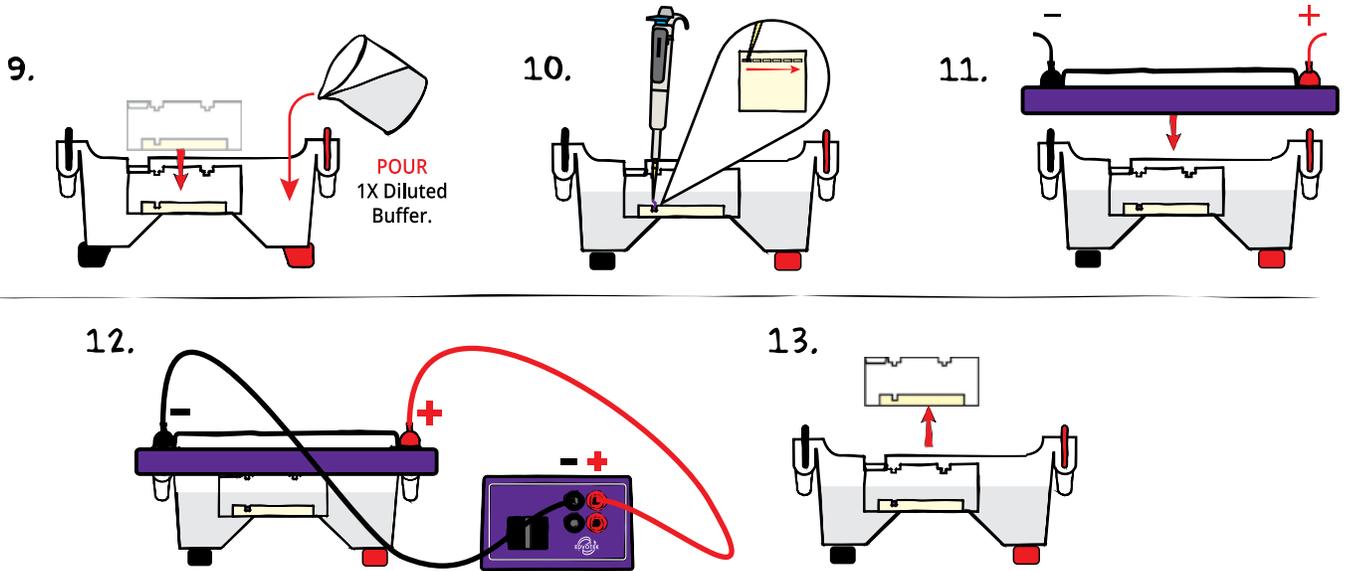
Table A

Individual 2.0% UltraSpec-Agarose™ Gel with SYBR® Safe Stain

Size of Gel Casting tray	Concentrated Buffer (50x)	Distilled Water +	Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.5 mL	24.5 mL	0.5 g	25 mL	25 µL
10 x 7 cm*	0.8 mL	39.2 mL	0.8 g	40 mL	40 µL
14 x 7 cm	1.0 mL	49 mL	1.0 g	50 mL	50 µL

* Recommended gel volume for the EDGE™ Integrated Electrophoresis System.

Module III: Electrophoresis of PCR Products, continued



9. **PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **LOAD** 25 µL of each sample into the well in the order indicated by Table 1.
11. **PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

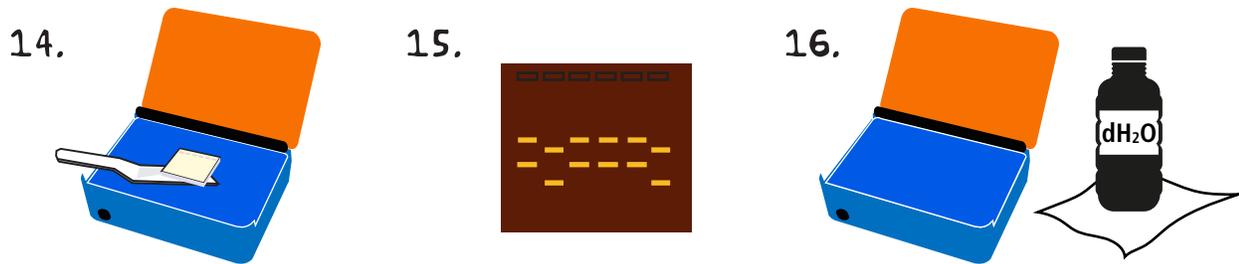
Reminder:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

Lane	Recommended	Sample Name
1	EdvoQuick™ DNA Ladder	
2	Control PCR Product (G or LP region)	
3	G PCR Product (Group 1)	
4	LP PCR Product (Group 1)	
5	G PCR Product (Group 2)	
6	LP PCR Product (Group 2)	

EDVOTEK Model #	Total Volume Required	50x Conc. Buffer	Dilution + Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

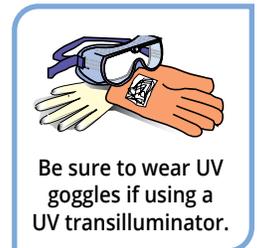
Volts	Electrophoresis Model	
	EDGE	M12 & M36
	Min/Max (minutes)	Min/Max (minutes)
150	15/30	20/40
125	N/A	30/50
100	30/40	40/60

Module III: Electrophoresis of PCR Products, continued



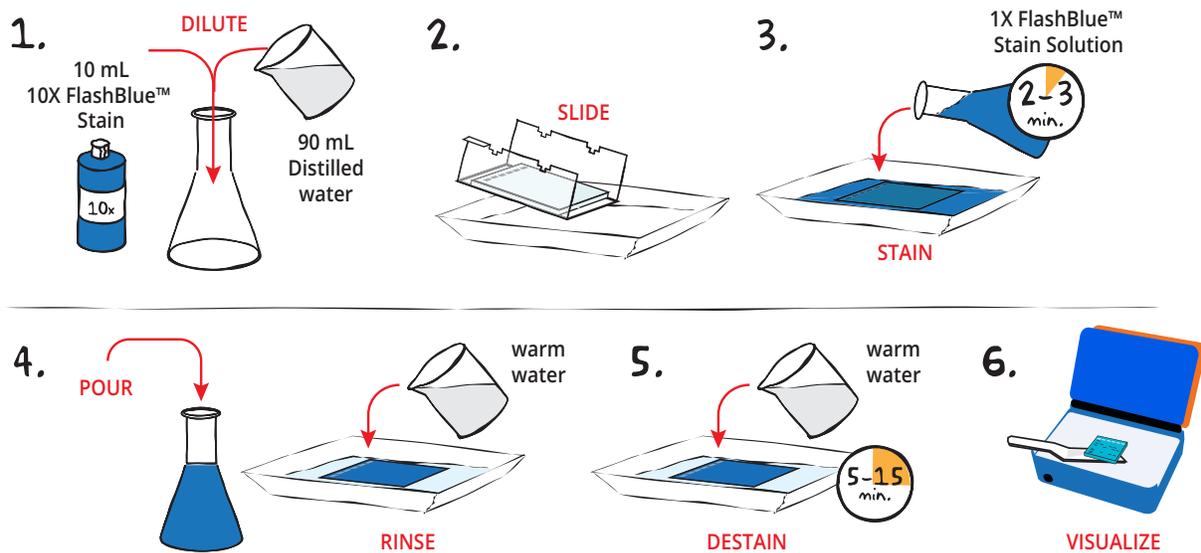
VISUALIZING THE SYBR[®] GEL

14. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator.
15. **TURN** the unit on. DNA should appear as bright green bands on a dark background. **PHOTOGRAPH** results.
16. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.



Module IV: Staining Agarose Gels with FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. *IF staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.*



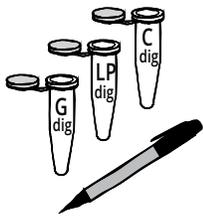
- DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
- REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
- COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
- POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45°C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
- COVER** the gel with clean, warm water (40-45°C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
- Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

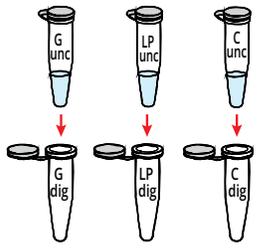


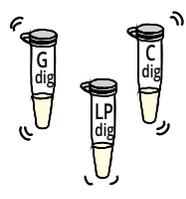
ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

- DILUTE** 1 mL of 10X FlashBlue™ stain with 149 mL distilled water.
- COVER** the gel with diluted FlashBlue™ stain.
- SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
- Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

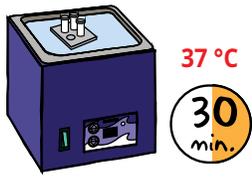
Module V: Digestion of the PCR Products

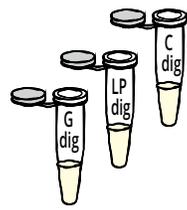
1. LABEL 

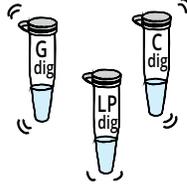
2. TRANSFER 15 μ L uncut DNA. 
ADD 5 μ L Enzyme reaction buffer and 5 μ L AluI enzyme

3. GENTLY MIX 

4. SPIN 

5.  37 °C 30 min.

6. ADD 5 μ L Gel Loading Solution 

7. GENTLY MIX 

8. PROCEED to Module VI.

- LABEL** three 0.5 mL snap top microcentrifuge tubes "G dig", "LP dig", and "C dig".
- For PCR product G, **TRANSFER** 15 μ L of DNA from the G PCR reaction in Module II, 5 μ L of Enzyme Reaction Buffer and 5 μ L of AluI enzyme. Repeat for LP PCR product and for the control PCR product.
- Gently **MIX** the restriction digestions by gently tapping the tube.
- Quickly **CENTRIFUGE** to collect sample at the bottom of the tube.
- INCUBATE** reaction tubes in a 37 °C water bath for 30 minutes.
- ADD** 5 μ L of 10x gel loading solution to reaction tubes G dig, LP dig, and C dig to stop the reactions.
- CAP** the tubes and **MIX** by tapping.
- PROCEED** to Module VI: Electrophoresis of Restriction Digests.



OPTIONAL STOPPING POINT:

The digested samples may be stored at -20 °C for electrophoresis at a later time.

Module VI: Electrophoresis of Restriction Digests and Module VII: Staining Agarose Gels with FlashBlue™ (OPTIONAL)

Two groups will share one 2.0% agarose gel with at least six sample wells for electrophoresis. For best results, we recommend using a 10 x 7 cm or 14 x 7 cm gel. Refer to pages 14-17 (Modules III & IV) for detailed instructions about casting agarose gels and performing electrophoresis.

LOAD 25 µL of each DNA sample into the wells in the following order.

Gel 2: RFLP Analysis to Detect Barcode Polymorphisms

Lane	Recommended	Sample Name
1	EdvoQuick™ DNA Ladder	
2	Control <i>AluI</i> Digested Product (G or LP region)	
3	G <i>AluI</i> Digested Product (Group 1)	
4	LP <i>AluI</i> Digested Product (Group 1)	
5	G <i>AluI</i> Digested Product (Group 2)	
6	LP <i>AluI</i> Digested Product (Group 2)	

Study Questions

1. List and describe the four major steps in the PCR-RFLP process. What is the most likely cause of the Restriction Fragment Length Polymorphisms?
2. Why did we choose DNA markers that contain highly conserved regions on both ends?
3. What are some uses of DNA barcoding?



Instructor's Guide

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of Components and Requirements on pages 3 and 4 to ensure that you have all the necessary components and equipment.

Some modules of this experiment may require multiple laboratory periods. The experiment can be temporarily stopped after the completion of each module or at designated stopping points. Student results will not be compromised if the experiment is paused at these times. Please consider this in the planning and the implementation of this experiment.

APPROXIMATE TIME REQUIREMENTS		
Module	PreLab	Experiment
I	45 min.	2.5 hours
II	45 min.	2 hours
III	15 min.	45 min.
IV	10 min.	30 min.
V	35 min.	45-60 min.
VI	15 min.	45 min.
VII	10 min.	30 min.

Technical Support

1.800.EDVOTEK

Mon. - Fri. 8 AM to 5:30 PM EST



Please Have the Following Info:

- Product Number & Description
- Lot Number on Box
- Order/Purchase Order #

1.800.EDVOTEK • info@edvotek.com • www.edvotek.com

www.edvotek.com

- Online Catalog
- Order Products
- Experiment Protocols
- Tech Support
- Resources!



1.800.EDVOTEK • Fax 202.370.1501 • info@edvotek.com • www.edvotek.com

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2016-2021 EDVOTEK, Inc., all rights reserved. Version 338.210628



ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Prior to starting this experiment, carefully check the list of Components and Requirements on pages 3 and 4 to ensure that you have all the necessary components and equipment.

Preparation For:	What to do:	When:	Time Required:
Module I: Isolation of Genomic DNA from Plants	Prepare DNA Extraction buffer	No more than one hour before the experiment.	10 min.
	Prepare and aliquot reagents	Up to one day before performing the experiment.	30 min.
	Equilibrate waterbaths	One hour before performing the experiment.	5 min.
Module II: Amplification of the Plant Barcode Regions	Prepare and aliquot reagents	One day to 30 minutes before performing the experiment.	30 min.
	Program thermal cycler	One day to 30 minutes before performing the experiment.	15 min.
Module III: Electrophoresis of PCR Products	Prepare diluted electrophoresis buffer & SYBR® Safe Stain	Up to one day before performing the experiment.	10 min.
	Aliquot reagents OR prepare molten agarose and pour gels	One day to 30 minutes before performing the experiment if preparing as a batch beforehand.	5 - 45 min.
Module IV: Staining Agarose Gels with FlashBlue™ (OPTIONAL)	Prepare staining components	The class period or overnight after the class period.	10 min.
Module V: Digestion of the PCR Products	Prepare and aliquot reagents	One day to 30 minutes before performing the experiment. IMPORTANT: Prepare the diluted restriction enzyme no more than one hour before performing the experiment.	30 min.
	Equilibrate waterbaths	One hour before performing the experiment.	5 min.
Module VI: Electrophoresis of Restriction Digests	Prepare diluted electrophoresis buffer	Up to one day before performing the experiment.	10 min.
	Aliquot reagents OR prepare molten agarose and pour gels	One day to 30 minutes before performing the experiment.	5 - 45 min.
Module VII: Staining Agarose Gels with FlashBlue™ (OPTIONAL)	Prepare staining components	The class period or overnight after the class period.	10 min.

Pre-Lab Preparations: Module I - Isolation of Genomic DNA From Plants

This module takes three and a half hours. If you cannot perform this entire module in one class period, the experiment can be stopped following Step 10. Samples can be stored in the freezer (-20 °C) for up to one week. The first ten steps will take approximately two hours and the last eight steps will take thirty minutes.

Plant samples can be provided by the instructor or students can bring in samples they have selected. Please note that plant DNA extraction can be challenging - plant cell walls are difficult to disrupt and secondary compounds (tannins, phenolics, and complex polysaccharides) can remain in the lysate and inhibit downstream reactions. This kit can be tried with any plant material. However, we recommend avoiding dried herbs and spices. We also recommend harvesting the tissue from leaves when possible, rather than from roots or stems. For your convenience, we have provided a list of commonly available fruits and vegetables and our success at extracting DNA from them on page 24.

DAY OF THE LAB

1. Set filled water bath to 65 °C
2. Preparation of DNA Extraction Buffer.
NOTE: Prepare no more than one hour before starting the experiment.
 - a. Add 100 µL of DNA Extraction Buffer (I) to each tube of Proteinase K (G) and allow the samples to hydrate for several minutes. After the samples are hydrated, pipet up and down several times to thoroughly mix the material.
 - b. Transfer the entire amount of rehydrated proteinase K solution back to the bottle of DNA extraction Buffer (I).
 - c. Invert the bottle several times to mix.
3. Aliquot the following reagents into 0.5 mL tubes.
 - a. 400 µL of DNA Extraction Buffer.
 - b. 300 µL of NaCl solution (H).
 - c. 750 µL Isopropanol. Stored on ice.
 - d. 120 µL of TE Buffer (F).
4. Aliquot 750 µL Isopropanol into a 1.5 mL tube. Store on ice until needed.

FOR MODULE I Each group should receive:

- 400 µL of DNA Extraction Buffer
- 300 µL of NaCl solution
- 750 µL ice-cold Isopropanol.
- 120 µL of TE Buffer.
- One 1.5 mL snap-top microcentrifuge tube
- One Screw-top microcentrifuge tube
- One micropestle

Plant Recommendations for DNA Extraction

Common Names	Latin Name	Extraction Success
Apple	<i>Malus domestica</i>	Recommended
Artichokes	<i>Cynara cardunculus</i>	Recommended
Arugula	<i>Eruca sativa</i>	Highly Recommended
Asparagus	<i>Asparagus officinalis</i>	Highly Recommended
Basil	<i>Ocimum basilicum</i>	Recommended
Bay leaves	<i>Laurus nobilis</i>	Recommended
Beet leaves	<i>Beta vulgaris</i>	Highly Recommended
Blueberry	<i>Vaccinium sp.</i>	Recommended
Broccoli	<i>Brassica oleracea</i>	Recommended
Brussel Sprout	<i>Brassica oleracea</i>	Recommended
Carrots	<i>Daucus carota</i>	Recommended
Celery	<i>Apium graveolens</i>	Recommended
Cilantro	<i>Coriandrum sativum</i>	Not Recommended
Corn	<i>Zea mays sp.</i>	Highly Recommended
Cucumber	<i>Cucumis sativus</i>	Recommended
Dill	<i>Anethum graveolens</i>	Recommended
Eggplant	<i>Solanum melongena</i>	Not Recommended
Garlic	<i>Allium sativum</i>	Recommended
Ginger	<i>Zingiber officinale</i>	Recommended
Green beans	<i>Phaseolus vulgaris</i>	Not Recommended
Honeyew	<i>Cucumis melo</i>	Recommended
Lemon Grass	<i>Cymbopogon sp.</i>	Recommended
Lettuce	<i>Lactuca sativa</i>	Recommended
Marjoram	<i>Origanum majorana</i>	Not Recommended
Mint	<i>Mentha sp.</i>	Recommended
Onion	<i>Allium cepa</i>	Recommended
Orange	<i>Citrus sinensis</i>	Recommended
Oregano	<i>Origanum vulgare</i>	Not Recommended
Peas	<i>Pisum sativum</i>	Highly Recommended
Pepper	<i>Capsicum annuum</i>	Recommended
Pinapple	<i>Ananas comosus</i>	Recommended
Potato	<i>Solanum tuberosum</i>	Not Recommended
Sage	<i>Salvia officinalis</i>	Not Recommended
Shallot	<i>Allium cepa</i>	Recommended
Spinach	<i>Spinacia oleracea</i>	Recommended
Tarragon	<i>Artemisia dracunculus</i>	Not Recommended
Thyme	<i>Thymus vulgaris</i>	Recommended
Tomato	<i>Solanum lycopersicum</i>	Highly Recommended
Watercress	<i>Nasturtium officinale</i>	Highly Recommended
Watermelon	<i>Citrullus lanatus</i>	Recommended
Yellow Squash	<i>Cucurbita pepo</i>	Highly Recommended

Pre-Lab Preparations: Module II - Amplification of the Plant Barcode Regions

PREPARATION OF THE PLANT BARCODE PRIMERS

1. Thaw the G and LP Primer mix on ice.
2. Add 1 mL of TE Buffer (F) to each tube of Primer Mix Concentrate (B & C). Cap tubes and mix.
3. Label ten 0.5 mL microcentrifuge tubes "G primer" and ten 0.5 mL microcentrifuge tubes "LP primer".
4. Aliquot 45 μ L of the diluted primer mix into the appropriate microcentrifuge tubes. Place the tubes on ice until they are needed.
5. Distribute tubes of diluted G and LP primer to each group.

FOR MODULE II

Each group should receive:

- 2 PCR tubes and 2 PCR EdvoBeads™
- 45 μ L Primer G
- 45 μ L Primer LP
- 6 μ L Control DNA and extra PCR reagents (optional)
- 20 μ L 10X Gel loading sol.
- 50 μ L distilled water

PREPARATION OF CONTROL DNA

1. Thaw the tube of Control DNA Concentrate (E) on ice.
2. Add 40 μ L of TE Buffer (F) to the tube containing Control DNA Concentrate. Pipet up and down to mix.
3. Aliquot 6 μ L of the diluted DNA control into the appropriate microcentrifuge tubes. Place the tubes on ice until they are needed.
4. Distribute tubes of diluted DNA. Each control will be shared by two groups

ADDITIONAL MATERIALS

- Dispense 20 μ L of 10X Gel Loading Solution per 0.5 mL microcentrifuge tube. Label these 10 tubes "10x Solution". Distribute one tube per student group
- Dispense 50 μ L of distilled water per 0.5 mL microcentrifuge tube. Label these 10 tubes "Water". Distribute one tube per student group.
- Each group will also receive two PCR tubes and two PCR EdvoBeads™.
- Groups receiving the control DNA should receive an extra PCR tube and PCR EdvoBead™. Alternatively, the instructor can prepare the additional control samples and distribute to students after PCR.

PROGRAMMING THE THERMAL CYCLER

The Thermal cycler should be programmed as outlined in Module II in the Student's Experimental Procedure.

Pre-Lab Preparations: Module III - Electrophoresis of PCR Products

AGAROSE GEL ELECTROPHORESIS

This experiment requires a 2.0% agarose gels. Each gel can be shared by 2 student groups. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Prepare SYBR® Safe Stain

1. Prepare 1x Electrophoresis Buffer by combining 20 µL of 50X Concentrated Buffer with 980 µL of distilled water.
2. Add 500 µL of the 1X buffer from step 1 to the tube of SYBR® Safe and mix by tapping the tube several times. The diluted SYBR® Safe Stain is now ready to be used during agarose gel preparation.

Individual Gel Preparation

Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Part 1 in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water, agarose powder, and *diluted* SYBR® Safe Stain.

Batch Gel Preparation

To save time, a larger quantity of agarose solution can be prepared for sharing by the class. See Appendix B.

Preparing Gels in Advance

Gels may be prepared ahead and stored for later use. Solidified gels can be store in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels.

Do not freeze gels at -20° C as freezing will destroy the gels.

Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

Additional Materials

Each 2.0% gel should be loaded with the EdvoQuick™ DNA ladder.

- Aliquot 25 µL of the EdvoQuick™ DNA ladder (D) into labeled microcentrifuge tubes and distribute one tube of EdvoQuick™ DNA ladder per gel.

Visualizing SYBR® Safe-Stained Gels

SYBR® Safe is a DNA stain that fluoresces when bound to double-stranded DNA, allowing us to visualize our samples. This DNA stain is compatible with both UV and blue-light transilluminators. For best results, we recommend the TruBlu™ 2 Blue Light Transilluminator ([Cat #557](#)).

FOR MODULE III Each Group Will Need:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- Diluted SYBR® Safe Stain
- EdvoQuick™ DNA Ladder (25 µL)

Pre-Lab Preparations: Module IV - Staining Agarose Gels with FlashBlue™ (OPTIONAL)

STAINING WITH FLASHBLUE™ (OPTIONAL)

FlashBlue™ stain is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to “equilibrate” in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background. A white light box (Cat. #552) or the white light feature of the TruBlu™ 2 (Cat. #557) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.

PHOTODOCUMENTATION OF DNA (OPTIONAL)

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.



FOR MODULE IV

Each Group Will Need:

- 10 mL 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

Pre-Lab Preparations: Module V - Digestion of PCR Products

DAY OF THE LAB

1. Set filled water bath to 37 °C.
2. Thaw the Restriction Enzyme Reaction Buffer (J) and the Restriction Enzyme Dilution Buffer (K). Place on ice.
3. Within 30 minutes of starting the Module V experiment, remove the restriction enzyme from the freezer and place on ice. Gently tap the tube on the tabletop or centrifuge it to collect the enzyme at the bottom of the tube.
4. Dilute the restriction enzyme in the Restriction Enzyme Dilution Buffer.
 - a. Add 200 µL of Restriction Enzyme Dilution Buffer to the tube containing the concentrated *AluI* restriction enzyme (L).
 - b. Mix the tube for 30 seconds (vortex or tap bottom of the tube) and set on ice for 1 min. Be sure to mix the diluted enzyme well as the concentrated enzyme solution contains glycerol and is very viscous. At this point, the enzyme can no longer be stored.
 - c. Dispense 20 µL of the *AluI* Restriction Enzyme to 0.5 mL microcentrifuge tubes. Label these tubes "*AluI*".
 - d. Place the tubes on ice until they are needed.
5. Also aliquot the following reagents into labeled 0.5 mL microcentrifuge tubes.
 - a. Dispense 20 µL Restriction Enzyme Reaction Buffer (J) per group.
 - b. Dispense 20 µL of 10x Gel Loading Solution per group.
6. If necessary, the samples can be frozen overnight after Module V, step 5. Thaw completely before proceeding to Module V, step 6.

FOR MODULE V

Each group should receive:

- 20 µL Restriction Enzyme Reaction Buffer
- 20 µL *AluI* Restriction Enzyme
- 20 µL 10x Gel Loading Solution

NOTE:

Sample volumes are very small. It is important to quickly spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

Pre-Lab Preparations: Module VI - Electrophoresis of Restriction Digests

For this module, two groups will share one 7 x 14 cm 2.0% agarose gel with at least five sample wells for electrophoresis. See pages 14-17 (Module III) and Appendix C for detailed preparation information.

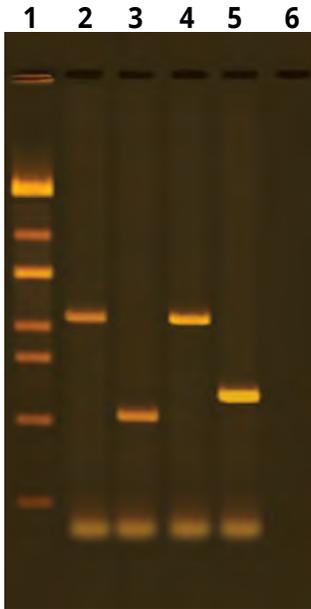
Before electrophoresis, two groups sharing a gel will receive 25 µL of the EdvoQuick™ DNA ladder. Reagents will be distributed when electrophoresis is performed.

Metric rulers and semi-log graph paper (semi-log) will be needed if the students are calculating the restriction fragment sizes by creating a linear standard curve (Appendix D). However, the students should be able to estimate the sizes of the DNA fragments by visual inspection of the gel or a photograph of the gel.



Experiment Results and Analysis

GEL A - RESULTS AFTER PCR AMPLIFICATION



Student results, including amplified product sizes and banding patterns, will vary from these results depending on which plant was chosen. These gels represent results from the DNA barcoding experiment using corn (*Zea mays sp.*, lanes 2 and 3) or tomato (*Solanum lycopersicum*, lanes 4 and 5) as the starting material. For reference, the control DNA included with this kit was extracted from corn.

Gel A shows the results after PCR amplification of the two barcoding regions. In general, the region amplified using "G primer mix" (the first intron of the tRNA-Gly gene) will be around 750 bp (lanes 2 and 4). The region amplified using the "LP primer mix" (intergenic region between tRNA-Leu and tRNA-Phe) will vary in size depending on species (lanes 3 and 5).

Includes EDVOTEK's All-NEW EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

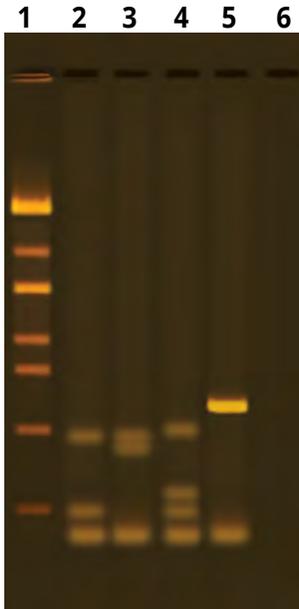
EdvoQuick™ DNA ladder sizes:
2640, 1400, 1100, 700, 600, 400, 200



TABLE 1: Gel Loading

Lane	Sample
1	EdvoQuick™ DNA Ladder
2	Corn, tRNA Gly, undigested
3	Corn, tRNA Leu-Phe, undigested
4	Tomato, tRNA Gly, undigested
5	Tomato, tRNA Leu-Phe, undigested

GEL B - RESULTS AFTER RESTRICTION DIGEST OF PCR PRODUCTS



Gel B shows the results after the PCR products are digested with restriction enzyme *AluI*. After electrophoresis, several bands of different sizes are present in the samples containing corn DNA (lanes 2 and 3). This suggests that multiple *AluI* cut sites are present within these regions. In contrast, while the first intron of the tomato tRNA-Gly gene ("G Primer Mix") has several *AluI* cut sites (lane 4), the intergenic region between tRNA-Leu and tRNA-Phe ("LP Primer Mix") does not appear to have any *AluI* cut sites (lane 5).

TABLE 2: Gel Loading

Lane	Sample
1	EdvoQuick™ DNA Ladder
2	Corn, tRNA Gly, <i>AluI</i> digest
3	Corn, tRNA Leu-Phe, <i>AluI</i> digest
4	Tomato, tRNA Gly, <i>AluI</i> digest
5	Tomato, tRNA Leu-Phe, <i>AluI</i> digest

**Please refer to the kit
insert for the Answers to
Study Questions**

Appendices

- A EDVOTEK® Troubleshooting Guide
- B Bulk Preparation of Agarose Gels
- C Data Analysis Using a Standard Curve

Safety Data Sheets can be found on our website: www.edvotek.com/Safety-Data-Sheets

Technical Support

1.800.EDVOTEK

Mon. - Fri. 8 AM to 5:30 PM EST



Please Have the Following Info:

- Product Number & Description
- Lot Number on Box
- Order/Purchase Order #

1.800.EDVOTEK • info@edvotek.com • www.edvotek.com

www.edvotek.com

- Online Catalog
- Order Products
- Experiment Protocols
- Tech Support
- Resources!



1.800.EDVOTEK • Fax 202.370.1501 • info@edvotek.com • www.edvotek.com

Duplication of any part of this document is permitted for non-profit educational purposes only. Copyright © 2016-2021 EDVOTEK, Inc., all rights reserved. Version 338.210628



Appendix A

EDVOTEK® Troubleshooting Guides

DNA EXTRACTION AND RESTRICTION DIGESTION

PROBLEM:	CAUSE:	ANSWER:
Poor DNA Extraction	Samples not mixed well enough during extraction	In addition to flicking the tube, vortex or pipet up and down to mix the sample.
	Proteinase K inactive because it was prepared too far in advance.	Prepare Proteinase K within one hour of use.
	Water baths not at proper temperature	Use a thermometer to confirm water bath set point.
	Not enough DNA	Extracting DNA from some plants can be challenging. Refer to page 24 for some recommendations.
The extracted DNA is very cloudy	Cellular debris from pellet transferred to tube	Centrifuge sample again and move supernatant to a fresh tube. Take care to avoid pellet.
	Cellular debris not separated from supernatant	Centrifuge sample again. If possible, centrifuge at a higher speed. Move cleared supernatant to a fresh tube.
The PCR products did not digest	The restriction enzymes were not active	Be sure that the restriction enzymes were diluted in the correct buffer (K). For optimal activity, prepare the enzymes within 30 minutes of use.
	There were no cut sites in this region	This may happen in some plant species depending on the DNA sequence.
There are bands on my gel that can't be explained by the restriction digests	Some bands may represent partially digested DNA.	The sample was not digested at the right temperature
		The sample was not digested for the appropriate amount of time
I can't see any bands after running the gel of PCR products	Low level of amplification	Proceed to restriction digest (Module V). If DNA extraction was inefficient, PCR may not have produced enough DNA to visualize when only running 5 μ L of the sample.



Appendix A

EDVOTEK® Troubleshooting Guides

PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after PCR	Sample has evaporated	Make sure the heated lid reaches the appropriate temperature.
		Make sure students close the lid of the PCR tube properly.
	Pipetting error	Make sure students pipet 20 μ L primer mix and 5 μ L extracted DNA into the 0.2 mL tube.
The ladder, control DNA, and student PCR products are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Gels of higher concentration (> 0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of "clumps" and glassy granules before pouring gels.
		The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
After staining the gel, the gel background is very dark.	The gel needs to be destained longer.	Submerge the gel in distilled or deionized water. Allow the gel to soak for 5 minutes.
After staining, the ladder and control PCR products are visible on the gel but some student samples are not present.	Student DNA sample was not concentrated enough.	Extracting DNA from some plants can be challenging. Refer to page 24 for some recommendations.
	Student DNA sample was degraded.	If DNA is not used right after extraction, store sample at -20°C.
	Wrong volumes of DNA and primer added to PCR reaction.	Practice using micropipets
Some student samples have more/less amplification than others.	Concentration of DNA varies by sample.	There is an inherent variability in the extraction process.
Low molecular weight band in PCR samples	Primer dimer	Low concentration of extracted DNA in PCR reaction.
DNA bands were not resolved.	To ensure adequate separation, make sure the tracking dye migrates at least 3.5 cm on 7 x 7 cm gels and 6 cm on 7 x 14 cm gels.	Be sure to run the gel the appropriate distance before staining and visualizing the DNA.
DNA bands fade when gels are kept at 4°C.	DNA stained with FlashBlue™ may fade with time	Re-stain the gel with FlashBlue™

Appendix B

Bulk Preparation of Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

Bulk Electrophoresis Buffer

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

Table D Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 ml		2,940 ml	3000 ml (3 L)

Batch Agarose Gels (2.0%)

For quantity (batch) preparation of 2.0% agarose gels, see Table E.

- Use a 1 L flask or bottle to prepare the diluted gel buffer.
- Pour 10 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
- With a marking pen, indicate the level of solution volume on the outside of the flask.
- Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
- Cool the agarose solution to 60 °C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.

Table E Batch Prep of 2.0% UltraSpec-Agarose™		
Amt of Agarose	+	Diluted Buffer (1x)
10.0 g		500 mL



- Add the entire tube of **diluted SYBR® Safe** stain (see page 26) to the cooled agarose and mix well.

- Dispense the required volume of cooled agarose solution for casting each gel. Measure 25 mL for a 7 x 7 cm tray, 40 mL for a 10 x 7 cm tray, and 50 mL for a 14 x 7 cm tray. **NOTE: For this experiment, 10 x 7 cm or 14 x 7 cm gels are recommended.**
- Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

NOTE:

The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

Appendix C

Data Analysis Using a Standard Curve

Agarose gel electrophoresis separates biomolecules into discrete bands, each comprising molecules of the same size. How can these results be used to determine the lengths of different fragments? Remember, as the length of a biomolecule increases, the distance to which the molecule can migrate decreases because large molecules cannot pass through the channels in the gel with ease. Therefore, the migration rate is inversely proportional to the length of the molecules—more specifically, to the \log_{10} of molecule's length. To illustrate this, we ran a sample that contains bands of known lengths called a "standard". We will measure the distance that each of these bands traveled to create a graph, known as a "standard curve", which can then be used to extrapolate the size of unknown molecule(s).

1. Measure and Record Migration Distances

Measure the distance traveled by each Standard DNA Fragment from the lower edge of the sample well to the lower end of each band. Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

Measure and record the migration distances of each of the fragments in the unknown samples in the same way you measured the standard bands.

2. Generate a Standard Curve.

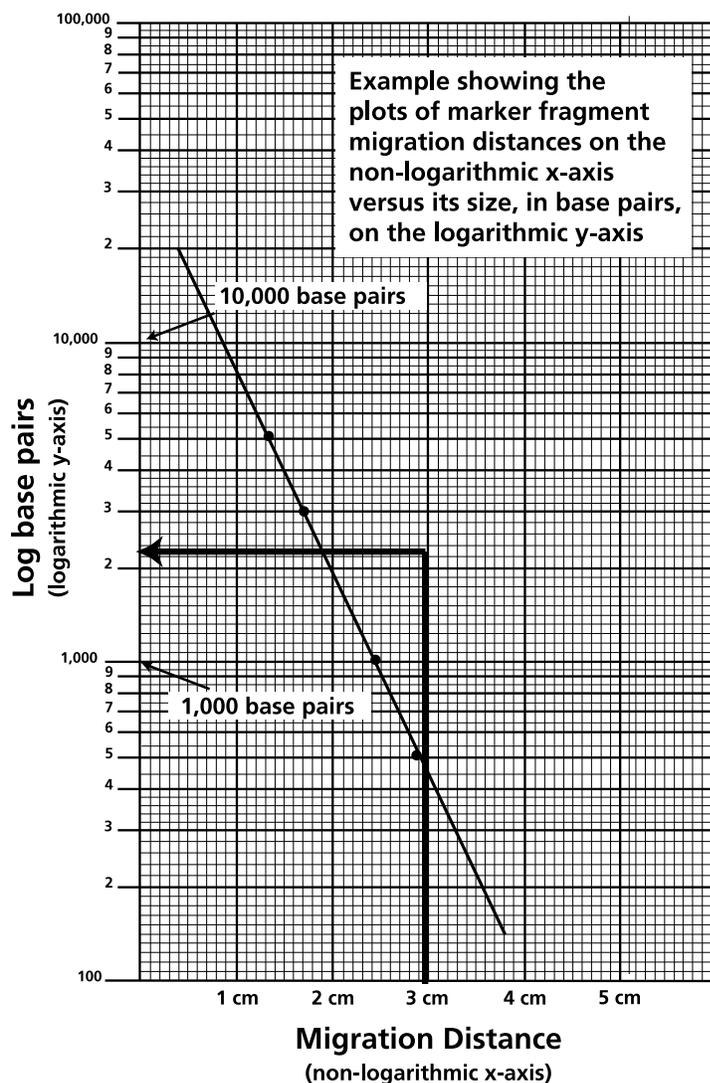
Because migration rate is inversely proportional to the \log_{10} of band length, plotting the data as a semi-log plot will produce a straight line and allow us to analyze an exponential range of fragment sizes. You will notice that the vertical axis of the semi-log plot appears atypical at first; the distance between numbers shrinks as the axis progresses from 1 to 9. This is because the axis represents a logarithmic scale. The first cycle on the y-axis corresponds to lengths from 100-1,000 base pairs, the second cycle measures 1,000-10,000 base pairs, and so on. To create a standard curve on the semi-log paper, plot the distance each Standard DNA fragment migrated on the x-axis (in mm) versus its size on the y-axis (in base pairs). Be sure to label the axes!



Figure 6:

Measure distance migrated from the lower edge of the well to the lower edge of each band.

Figure 7: Semilog graph example



Appendix C: Data Analysis Using a Standard Curve, continued

After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 7 for an example).

3. Determine the length of each unknown fragment.

- a. Locate the migration distance of the unknown fragment on the x-axis of your semi-log graph. Draw a vertical line extending from that point until it intersects the line of your standard curve.
- b. From the point of intersection, draw a second line, this time horizontally, toward the y-axis. The value at which this line intersects the y-axis represents the approximate size of the fragment in base pairs (refer to Figure 7 for an example). Make note of this in your lab notebook.
- c. Repeat for each fragment in your unknown sample.

**Includes EDVOTEK's All-NEW
EdvoQuick™ DNA Ladder**

- Better separation
- Easier band measurements
- No unused bands

EdvoQuick™ DNA ladder sizes:
2640, 1400, 1100, 700, 600, 400, 200



