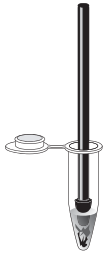

Biotechnology Explorer™

Nucleic Acid Extraction Module

Instruction Manual

Catalog #166-5005EDU

explorer.bio-rad.com

The kit is shipped at room temperature. Open immediately upon arrival and store reagents at the recommended temperature within 2 weeks of receipt.

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BIO-RAD

Dear Educator:

The foundation of molecular biology

The modern revolution in genetic engineering and molecular biology depends on obtaining pure DNA. We must be able to isolate DNA away from the nucleases, contaminants, and inhibitors that would destroy DNA or prevent us from working with it. Advances in agriculture, genetics and medicine would not be possible without the ability to obtain pure DNA.

In the Nucleic Acid Extraction Module students will extract genomic DNA (gDNA) from plants using a simple and powerful silica-binding column technique. Methods are given for measuring DNA concentration, yield and purity. The DNA is ready for applications such as cloning, PCR (polymerase chain reaction) and southern blotting.

The Nucleic Acid Extraction Module is part of the Bio-Rad Cloning and Sequencing Explorer Series (catalog #166-5000EDU) and can also be used as a stand alone kit for extracting high quality plant DNA for any use.

For more biotechnology curricula and DNA extraction kits visit explorer.bio-rad.com and look for other BioEducation instruction manuals. The Genes in a Bottle kit (catalog #166-2300EDU) is used for a classroom DNA visualization activity. The GMO Investigator kit (catalog #166-2500EDU) is used to isolate plant DNA for PCR. The Aurum™ Plasmid Mini Purification Module (catalog #732-6400EDU) is a general tool used to isolate bacterial plasmid DNA. The PCR Kleen™ Spin Purification Module (catalog #732-6300EDU) is a general tool used to isolate PCR product DNA.

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Create context. Reinforce learning. Stay current.
New scientific discoveries and technologies create more content for you to teach, but not more time. Biotechnology Explorer kits help you teach more effectively by integrating multiple core content subjects into a single lab. Connect concepts with techniques and put them into context with real-world scenarios.

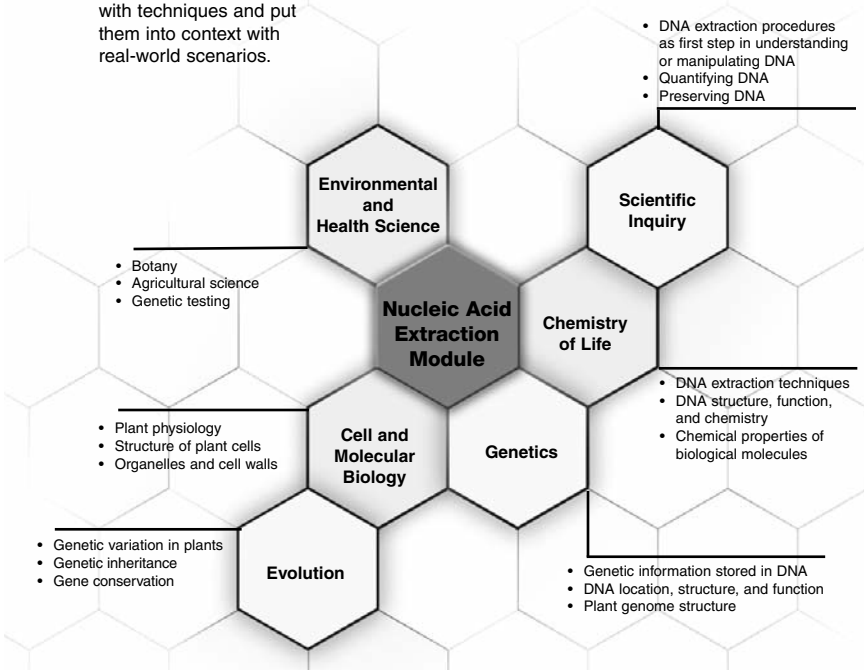


Table of Contents

Introduction

Genomic DNA extraction is a crucial technique in molecular biology and is an essential step in many downstream procedures such as cloning, polymerase chain reaction (PCR), and restriction enzyme digestions. These applications require high quality genomic DNA.

This Nucleic Acid Extraction Module utilizes a simple protocol for extracting up to 5 µg of genomic DNA from a variety of plant species using silica membrane technology and optimized aqueous reagents. In this laboratory activity, DNA is extracted from approximately 50–100 mg of plant material, and is ready for downstream applications without further manipulation, such as ethanol precipitation.

The first step in this activity is to choose an interesting plant species from which genomic DNA is extracted. Students may choose model species that plant biologists have extensively studied, such as Arabidopsis thaliana, the green alga Chlamydomonas, crop plants like rice (Oryza species), or wheat (Triticum species). Alternatively they may choose a species that has been studied less. There are over 250,000 plant species known to exist on the planet, providing plenty of options for this activity.

What Skills Do Students Need to Perform This Laboratory Activity?

This laboratory activity assumes that students and instructors have basic molecular biology skills, such as proper pipeting techniques and knowledge of molarity calculations. Bio-Rad's Biotechnology Explorer program has a full range of kits to help teach basic skills in individual laboratories.

What Is the Time-Line for Completing the DNA Extraction Protocol?

This depends greatly on the level of your students and whether additional/optional techniques and analyses are performed in addition to the basic protocol. It takes approximately 2 hr to complete the genomic DNA extraction.

Kit Inventory Checklist

This section lists equipment and reagents necessary to extract genomic DNA isolation in your classroom or teaching laboratory. Each kit contains sufficient materials for 12 student workstations, or 25 genomic DNA extractions. We recommend that students be teamed up – two to four students per workstation. Please use the checklist below to confirm inventory.

Kit Components	Quantity	(✔)
Lysis buffer, 20 ml	1	❒
DTT (Dithiothreitol), 0.3 g	1	❒
Wash buffer, low stringency (5x concentrate), 20 ml	1	❒
Sterile water, 2.5 ml	1	❒
Micropestles	25	❒
Aurum Mini columns, purple	25	❒
Capless collection tubes, 2.0 ml	25	❒
Microcentrifuge tubes, 1.5 ml	30	❒
Microcentrifuge tube, multicolor, 2.0 ml	60	❒
Instruction manual	1	❒

Required Accessories (not provided)	Quantity	(✔)
• 95–100% ethanol, molecular biology grade	300 ml	❒
• Distilled/deionized water	14 ml	❒
• Water bath (set at 70°C) (Catalog #166-0504EDU)	1	❒
• Balance with weigh paper or weigh boats	1 box	❒

Required Accessories (Not Provided)	Quantity	(✔)
• Microcentrifuge with variable-speed setting ≥12,000 x g (catalog #166-0602EDU)	2	❒
• 20–200 µl adjustable-volume micropipet (catalog #166-0507EDU)	12	❒
• 20–200 µl pipet tips, aerosol barrier (catalog #211-2016EDU)	1 box	❒
• 100–1,000 µl adjustable-volume micropipet (catalog #166-0508EDU)	12	❒
• 100–1,000 µl pipet tips, aerosol barrier (catalog #211-2021EDU)	1 box	❒
• Green racks, set of 5 (catalog #166-0481EDU)	12	❒
• Marking pens	12	❒
• Tubes for aliquoting (optional)	36	❒
• Razor blades or scalpels	24	❒
• –20°C freezer	1	❒
• Plants for DNA extraction (50–100 mg per plant)*	2	❒

* See section: Selecting Plant Species for Genomic DNA Isolation for tips on plant selection.

Refills Available Separately

Nucleic acid extraction kit refill, (catalog #166-5006EDU)
Includes DTT (0.3 g), lysis buffer (20 ml), low-stringency wash buffer (20 ml), sterile water (2 ml)

Aurum mini columns, 50 (catalog #732-6826EDU)

Safety Issues

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Wearing protective personal equipment such as eyewear, gloves, and labcoats should be standard laboratory practice. The lysis buffer in this product contains guanidine thiocyanate (CAS# 593-84-0) in solution. Basic laboratory practices should be followed to avoid contact with eyes, skin and clothing. Wash your hands with soap and water before and after this exercise. If any of the solution gets into eyes, flush with water for 15 minutes. If these buffers are spilled, clean with a suitable laboratory detergent and water. Avoid contact with acids or bleach as these will liberate toxic gas. Please refer to material safety data sheet for complete safety information.

Background

Why Extract Genomic DNA?

Genomic DNA isolation is the first step for many downstream procedures such as cloning, PCR, and restriction endonuclease digestions. These applications require the isolation of high quality genomic DNA.

Genomic DNA (gDNA) is found in prokaryotic (bacteria) and eukaryotic (animal, plant, and yeast) cells. In eukaryotic cells gDNA is closely associated with DNA binding proteins (histones). Therefore, when isolating DNA, one would have to separate the DNA from proteins and other components that are also found inside the cell. In addition, the DNA must be extracted carefully to ensure that it remains intact during the purification process.

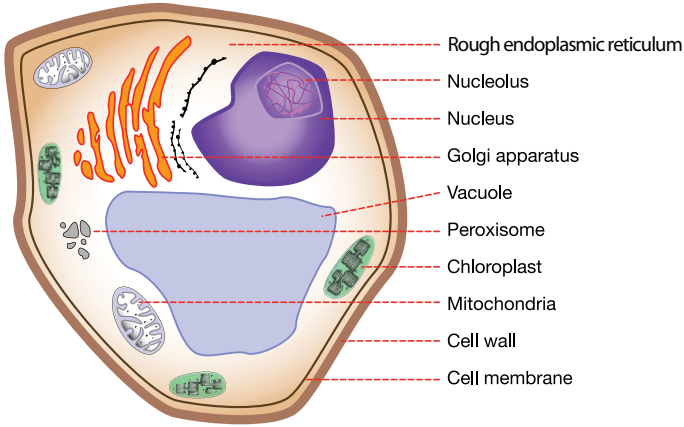
The basic steps in DNA extraction are:

1. Grow (or collect) cells/tissues from which genomic DNA is to be extracted.
2. Lyse or break open cell membranes and cell walls (if applicable) by chemical or physical means.
3. Remove cellular debris.
4. Remove remaining cellular proteins.
5. Purify DNA.

All of the steps listed above have variations depending on the cell type and the purity of the DNA needed.

Purifying Genomic DNA from Plants

Isolation of DNA from plants has complications not seen in other organisms. Plants are multicellular eukaryotic organisms with rigid cell walls composed of multiple layers of cellulose. The cell wall maintains shape and gives rigidity to plant cells. Its presence complicates DNA extraction. Plant polysaccharides, such as the cellulose from the cell wall, co-localize with DNA during the purification process. In addition, certain plants, such as many conifers and fruit trees, contain a high concentration of compounds called polyphenols. When such plants are broken down with lysis buffer, the polyphenols bind to the DNA, making it useless for further experiments.



DNA extraction from plant cells also poses the same challenges faced when extracting DNA from other cell types. Cells contain enzymes that can digest and break down the DNA during the extraction process. Membrane-bound compartments within cells have acidic contents that can also damage DNA when the contents are spilled into the cytosol during the lysis step.

The steps in isolating genomic DNA from plants are:

1. Harvest cells	Harvest fresh plant tissue.
2. Grind cells	Physically disrupt plant cell tissue.
3. Lyse cells	Break cells open with lysis buffer containing chemicals and enzymes that disrupt cell membranes.
4. Remove cellular debris	After treatment with lysis buffer, insoluble cell components, such as the remains of the cell wall and membranes, are removed by centrifugation. The DNA is collected in the supernatant.
5. Purify DNA	Proteins and RNA remain in the supernatant with the DNA. These contaminants can be removed using protocols such as silica membrane technology, ion exchange chromatography, etc.
6. Concentrate DNA	DNA is precipitated with ice-cold ethanol in the presence of ions such as Na^+ and K^+. If there is enough DNA present, it can be seen as a white precipitate. DNA can then be pelleted by centrifugation and resuspended in a small volume of water or buffer.
7. Determine purity and concentration of DNA	The concentration of DNA can be determined by reading the absorbance of the DNA solution at 260 nm (A_{260}) in a spectrophotometer. Additionally, the ratio of the absorbance at 230, 260, 280, and 330 nm can be assessed to determine contamination with proteins, phenol, or RNA.

Selecting Plant Species for Genomic DNA Isolation

There are many techniques for extracting genomic DNA from plants, some of which are specific to a single plant type. Although every attempt has been made to make this laboratory as universal as possible, there are some plants for which this DNA extraction kit does not work. Plants with very tough extracellular matrices or cell walls, or plants with some other characteristics, may make it difficult to extract DNA using this kit.

The DNA from different species of plants may vary in quantity and in suitability for downstream applications. For example, plants that yield very little genomic DNA may amplify successfully with PCR technology, while plants that yield a lot of genomic DNA may amplify poorly.

For best results, use the following guidelines to select plants for this activity:

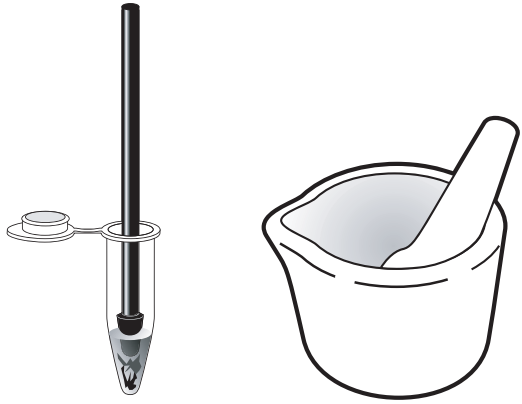
- Young leaves are the best source for genomic DNA. Because they are still growing, they have a greater ratio of nuclear to cytoplasmic volume and contain fewer chemicals that interfere with DNA isolation
- Fresh samples have higher yields of DNA than preserved or dried leaves
- While fresh tissue is best, if necessary, plant tissue can be stored for 2–3 days at 4°C in a sealed bag or tube. Do not put tissue samples into the –20°C or –80°C freezer for storage – slow freezing of tissue will disrupt cells, release DNases and may degrade the genomic DNA
- If the plant can be moved, place it in the dark for 1–2 days before harvesting tissue to reduce the amount of polysaccharides in the sample

List of Plants That Can Be Used for Genomic DNA Extraction and How They Perform When Used for PCR

Plants Known to Amplify by PCR	Plants That Do Not Amplify Well by PCR
Aluminum plant, leaves (*Pilea cadierei*)	Fern, leaves
Cabbage, leaves (*Brassica oleracea*)	Ivy, leaves (*Hedera helix*)
Carrot, root (*Daucus carota*)	Lily of the valley, leaves (*Convallaria major*)
Common sage, leaves (*Salvia officinalis*)	(*Iris douglasiana*)
Croton, leaves (*Codiaeum variegatum*)	Pacific Coast Iris, petals and leaves
Eyelash begonia, leaves	Pine, needles (*Pinus spp.*)
(*Begonia bowerae* hybrid)	Spider plant, leaves
Green bean, seed case and bean	(*Chlorophytum comosum*)
(*Phaseolus vulgaris*)	
Jade pothos, leaves (*Epipremnum aureum*)	
Lambs ear, leaves (*Stachys byzantina*)	
Lawn grass, leaves	
Parsley, leaves (*Petroselinum crispum*)	
Petunia, leaves and petals	
(*Petunia hybrida*)	
Pineapple sage, leaves (*Salvia elegans*)	
Sow thistle, leaves (*Sonchus oleraceus L.*)	
Spinach, leaves (*Spinacia oleracea*)	
Sugar cane, leaves (*Saccharum officinarum*)	
Sweet potato, tuber (*Ipomoea batatas*)	
Thyme, leaves (*Thymus vulgaris*)	
Umbrella plant, leaves (*Cyperus involucratus*)	
Wheatgrass, leaves (*Triticum aestivum*)	

Grinding and Lysing Plant Tissue

Because of their rigid cell walls, plant samples must be physically broken up or crushed prior to DNA isolation. Typically a mortar and pestle is used, though blenders and mechanical tissue grinders may also be used with some plant species. A range of tools are available for crushing different amounts of plant tissue, ranging from large porcelain mortars and pestles to small microtube grinders.



Grinding of plant tissue is frequently performed in the presence of lysis buffer as in this protocol. Alternatively, tissue can be flash frozen and ground in liquid nitrogen. If the tissue is frozen, the ground plant material must be added to lysis buffer before it can thaw. The lysis buffer normally contains a chemical called EDTA (ethylenediamine tetraacetate) that serves a dual function: it removes (chelates) magnesium ions, destabilizing the cell wall and the cell membrane, and also inhibits nucleases, enzymes that could digest the DNA.

Lysis buffer must have buffering capacity to maintain the overall pH of the lysate in the presence of the acidic compounds that are released from subcellular organelles. Most lysis buffers are prepared at pH 8.0 using Tris (hydroxymethyl aminomethane), a commonly used buffer in molecular biology protocols.

The plant cell membrane is a selectively permeable plasma membrane, allowing some molecules to pass through and blocking others. The plasma membrane is composed of phospholipids and proteins. The cell membranes need to be disrupted, and this can be done with detergents or chaotropic agents. Detergent breaks up cell membranes by removing lipid molecules from the membranes. The choice of detergent depends on the application. Ionic detergents, such as SDS (sodium dodecyl sulfate) or Sarkosyl (*N*-Laurylsarcosine), are commonly used, but non-ionic detergents such as Triton X-100 may also be used. Non-ionic detergents are milder than ionic detergents and usually leave proteins intact and functional. Chaotropic agents disrupt proteins and membranes by destabilizing their three dimensional structure. Frequently the lysis buffer also contains a reducing agent such as DTT or β-mercaptoethanol to further denature proteins by reducing disulfide bonds.

The classical method of DNA extraction from plants uses a non-ionic detergent called CTAB (cetyl trimethyl ammonium bromide) to lyse the plant cells and precipitate the DNA, leaving most polysaccharides in solution.

Specialized techniques have been developed to overcome the challenges faced when isolating genomic DNA from some plants. Lysis buffer containing PVP (polyvinylpyrrolidone) and a high concentration of salt works best for isolating DNA from plants with high levels of polyphenols, including grape species, many fruit trees, and conifers. PVP binds to the polyphenols, preventing them from complexing with the DNA, and the high salt reduces the co-precipitation of polysaccharides with the DNA.

Common Methods of DNA Extraction

After cell lysis and centrifugation to remove cellular debris, proteins and RNA are still present in the supernatant along with the DNA. There are several methods to remove these contaminants. It is important to remember that during the purification process, the lysate should never be vortexed or mixed vigorously, since this would result in breaking or shearing of the large molecules of genomic DNA into smaller pieces. Intact, full-length DNA molecules are required for subsequent steps, such as PCR. If DNA

becomes sheared, the gene of interest may be broken into multiple pieces, and therefore may not be amplifiable.

The following are examples of common techniques that are used for purifying genomic DNA:

- **Silica-binding-based purification** — DNA binds strongly to silica in the presence of high concentrations of chaotropic salts, such as guanidine, that disrupt hydrophobic interactions. The mechanism of binding is not fully understood. However, one theory is that binding is due to the exposure of anions on the DNA and silica as a result of dehydration by the salts. The phosphates on the DNA bind to the silica through the formation of a cation bridge formed by the salt. The DNA can be released from the silica by reducing the salt concentration. This chemistry is the basis for many of the commercially available kits for DNA purification, including the Nucleic Acid Extraction Module used for this activity.
- **Ion exchange chromatography** — Purification of DNA with ion exchange chromatography uses a positively-charged resin or other matrix, usually in a glass or plastic chromatography column. DNA and RNA are both negatively charged and bind to the positively charged matrix through ionic interactions. Components of the cell lysate that are not negatively charged do not bind to the matrix and are discarded.

  Molecules bound to the matrix can be removed (eluted) by increasing the salt concentration of the solution on the column. Molecules are eluted based on the strength of their binding to the column, with more weakly bound molecules eluting at a lower salt concentration than more strongly bound molecules. Using this differential elution, DNA can be separated from proteins and RNA.

- **Organic extraction** — DNA can be purified in a two-step process of enzyme treatment and organic extraction. Proteins in the cell lysate are degraded by treatment with proteases, enzymes that break proteins into small pieces. Besides getting rid of protein contaminants, this step also degrades enzymes called nucleases that might destroy the target DNA. Proteinase K is one of the most commonly used proteases, degrading most proteins and inactivating enzymes under a broad range of conditions.

  After cellular proteins have been degraded, organic extraction is used to precipitate proteins in the lysate. Phenol or a combination of phenol and chloroform causes proteins to coagulate at the interface of the organic and the aqueous solutions. The proteins are then removed by centrifugation, leaving RNA and DNA in the aqueous solution.

- **RNA Removal** — RNA is often removed from gDNA preparations by treating the sample with enzymes called ribonucleases (RNases) that selectively degrade RNA without damaging the DNA. Ribonuclease A, a nuclease that cleaves only single-stranded RNA molecules, is commonly used.

Concentrating the Extracted DNA

Many protocols result in a DNA solution that is too dilute for experimental purposes, thereby requiring further concentration of the DNA. The most common methods use either ethanol or isopropanol in the presence of a high salt concentration. DNA precipitates in the presence of high salt concentrations (for example, NaCl) and either ethanol or isopropanol. The cations in the salt neutralize the charge on the phosphate backbone of the DNA and allow DNA molecules to come close together. Normally, in aqueous solution, the strong negative charges of DNA molecules repulse each other. The ions do not bind strongly to DNA in aqueous solution, but in the presence of an organic solvent, such as an alcohol, DNA-cations form a tight complex and precipitate out of solution. If there is enough DNA present, a white precipitate should be visible, but DNA may very well be present even if a precipitate is not observed.

The precipitated DNA is pelleted by centrifugation, after which the pellet is washed at least once with 70–80% ethanol to remove any remaining salt. Note that 100% ethanol cannot be used for the wash because salt will not dissolve in pure ethanol. Nor can the ethanol concentration be <70%, as the DNA might become resuspended with the salt and be lost. After the washes, the DNA pellet is dried to evaporate any remaining ethanol and then resuspended either in water or the desired buffer.

Measuring DNA Concentration, Yield, and Purity

Once purified, the concentration, yield, and purity of DNA can be determined by measuring the absorbance of the DNA solution at 260 nm (optical density) or OD_{260} (also referred to as A_{260}) using a spectrophotometer. A solution with an OD_{260} of 1 contains approximately 50 µg/ml of DNA.

The purity of the DNA can also be estimated with spectroscopy.

- A pure preparation of DNA has a ratio of OD_{260}: OD_{280} of 1.8. If the OD_{260}:OD_{280} is <1.8, the DNA is probably contaminated with protein (if the ratio is >1.8, the DNA may be contaminated with RNA)
- A ratio of OD_{230}: OD_{260} >0.5 indicates contamination with phenol
- Absorbance at 330 nm is caused by light scattering and indicates the presence of particulate matter in the sample

Note that the genomic DNA isolated using the nucleic acid extraction module contains RNA. Therefore, reading the absorbance at 260 nm using spectrometry does not provide an accurate concentration value. In order to get a reliable concentration measurement, contaminating RNA can be first removed by treating with RNase A. Alternatively, a fluorometer, which specifically quantifies double stranded nucleic acids, can be used to accurately measure the concentration of the genomic DNA that is obtained (see Appendix A).

Other Cellular DNA in Plants

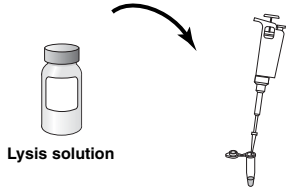
In addition to genomic DNA, plant cells contain DNA in their mitochondria (referred to as mtDNA for mitochondrial DNA). Green plants also have DNA within their chloroplasts called cpDNA (for chloroplast DNA; also abbreviated ctDNA). Refer to Appendix B for detailed information on non-genomic DNA found inside plants.

Genomic DNA Isolation – Quick Guide

Stage 1

1. Label one 1.5 ml flip-top micro centrifuge tube with your initials and plant name for each plant sample.

2. Pipet 200 µl of lysis solution into each tube.

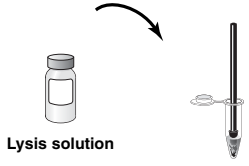


3. For each plant weigh 50–100 mg of plant material and record the weight.

4. For each plant, use a razor blade or scalpel to chop the plant material into 1–2 mm pieces. Add the chopped material to the lysis solution.

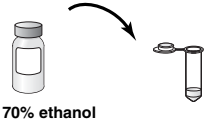
5. For each plant, use a micropestle to grind the plant material for at least 3 min.

6. Once a homogenous lysate has been generated (i.e. 'chunks' of plant material are no longer visible), add 500 µl of lysis solution to the lysate and grind further if homogeneity has not yet been achieved.

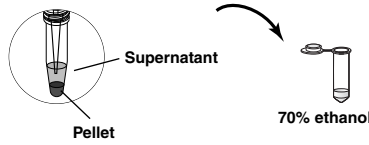


7. Cap microcentrifuge tube and centrifuge 5 min at top speed.

8. For each plant sample label a new flip-top microcentrifuge tube with your initials and plant name. Add 500 µl of 70% ethanol into each tube.

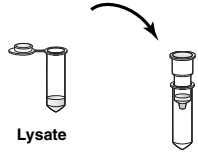


9. Retrieve samples from microcentrifuge and add 400 µl of the supernatant to the 70% ethanol in the appropriately labeled tube, taking care not to disturb the pellet. Pipet up and down to mix lysate and ethanol.



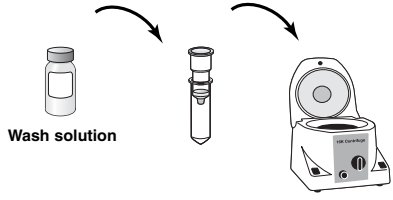
10. Label the top edge of a mini-column for each plant and place columns in 2 ml capless collection tubes.

11. For each sample, pipet 800 µl of cleared plant lysate into the appropriate column.



12. Centrifuge columns for 1 min. Discard flow-through from collection tube and replace column in collection tube.

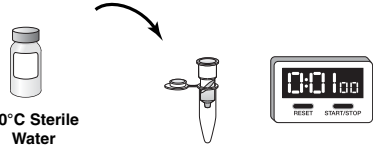
13. Add 700 µl of wash buffer to each column. Spin full speed for 1 min. Discard flow-through. Repeat for **two** more times for a total of **3 washes**.



14. After final wash step, discard flow-through and replace columns in capless collection tubes and dry columns by spinning for 2 min at full speed in microcentrifuge.

15. Transfer each column to a fresh, appropriately labeled, capped microcentrifuge tube.

16. Add 80 µl of 70°C sterile water to the bed of each column. Let sit for 1 min.



17. Spin column in tube for 2 min.

18. Discard column, cap labeled tube containing purified nucleic acid and store at –20°C.

Starting the Laboratory

Timeline

Day 1. Estimated preparation time: 1 hour

Read through protocol.

Obtain plant samples (see suggested plant samples in Background section).

Day 2. Estimated preparation time: 2 hours

Perform genomic DNA isolation.

Day 3. (Optional)

Determine DNA concentration, yield, and purity using a spectrophotometer. If desired, analyze genomic DNA samples on agarose gel. See Appendix A for more detail on optional activities.

Materials Required

Listed are materials and reagents required at the workstations prior to beginning the exercise.

Common Equipment Required	Quantity	(✔)
• Balance	1	❒
• Weigh paper/boats	1 per plant sample	❒
• Water bath at 70°C (for warming the sterile water used for DNA elution)	1	❒
• Microcentrifuge	1	❒

Each student team requires the following items for DNA extraction from 2 plants. Aliquotting reagents for individual student workstations is up to the instructor's discretion.

Materials Needed for Each Workstation	Where Provided	Quantity
Razor blades or scalpels	Instructor's own	2
Microcentrifuge tubes, 1.5 ml	Kit	2
Microcentrifuge tubes, multicolor, 2 ml	Kit	4
Capless collection tubes, 2 ml	Kit	2
Micropestles	Kit	2
Sterile water (at 70°C)	Kit	200 µl*
Lysis buffer (prepared with DTT)	Kit	2 ml*
1x wash buffer (with 95–100% ethanol added)	Kit	5 ml*
Aurum Mini columns, purple	Kit	2
1,000 µl adjustable micropipet	Instructor's own	1
Tips for 1,000 µl adjustable micropipet	Instructor's own	As needed
200 µl adjustable micropipet	Instructor's own	1
Tips for 200 µl adjustable micropipet	Instructor's own	As needed
Marking pen	Instructor's own	1
70% ethanol	Instructor's own	2 ml
Plant samples	Collected by students	2
Tube racks	Instructor's own	1

* Represents the amount to be aliquoted for each workstation if the instructor chooses to do so.

Advance Preparation for Day 2

Objectives	Obtain plant material
	Warm the sterile water and prepare for use during the elution step
	Prepare lysis buffer
	Prepare the wash buffer
Time required	30 min

Procedures

1. Obtain plant materials. The best results will be obtained from young, fresh leaves. However other parts of plants have been used with success. It is important not to put the fresh plant tissue in the freezer. If tissue needs to be stored-store at 4°C for as short a time as possible and ensure tissue is kept moist.
2. Set water bath to 70°C.
3. Place tubes of sterile water in the water bath at 70°C. The warmed water is going to be used for eluting the genomic DNA.
4. Prepare lysis buffer.

   Add 0.3 g of DTT to 20 ml of lysis buffer for a final concentration of 100 mM DTT. Once DTT has been added to the lysis buffer, it should be stored at –20°C and is stable for up to 2 months. If longer storage is required, prepare only enough lysis buffer for the current laboratory activity and store the remaining lysis buffer at room temperature and the remaining DTT at 4°C.
5. Prepare wash buffer.

   Add 95–100% ethanol to the 5x wash buffer. The amount of ethanol to add is indicated on the wash buffer label. The wash buffer can be stored at room temperature for up to 1 year once ethanol has been added.
6. Prepare 70% ethanol.

   Add 14 ml of 95–100% ethanol to 6 ml of distilled/deionized water.

Detailed Protocol for Genomic DNA Extraction

Safety – Protective eye goggles, gloves, and lab coats should be worn in the laboratory at all times. Proper safety precautions, including no eating or drinking in the lab, should always be practiced. Refer to the Safety section for more information.

Ensure that sterile water for elution of DNA is at 70°C.

Select 1 or 2 plants from which DNA will be extracted. The younger the plant material, the better the DNA yield will be. If necessary, clean plant material to remove soil or debris.

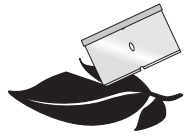
1. Label each 1.5 ml microcentrifuge tube with your initials and plant name.
2. Pipet 200 µl of lysis buffer into each 1.5 ml microcentrifuge tube.
3. Weigh 50–100 mg of each plant material. Record the weight of each plant material.

   Note: For most leaves 50–75 mg of tissue is sufficient. For plant tissue that is high in water content such as cabbage leaves, use 75–100 mg.

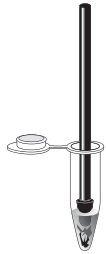
Name of Plant	Part of Plant Used (Leaf, Root, etc)	Weight (mg)

4. For each plant, use a razor blade or scalpel to cut the material into small pieces (less than 1–2 mm in diameter).

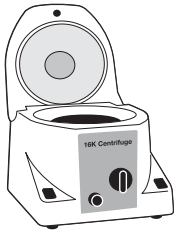
   Note: Use a new razor blade or scalpel for each plant type used to avoid contaminating samples. Add the chopped plant material into a microcentrifuge tube containing 200 µl of lysis buffer.



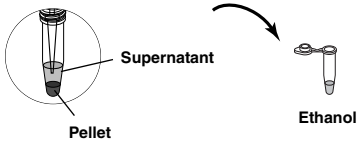
5. For each plant, use a clean micropestle to grind the plant material for at least 3 minutes. Be careful not to let lysis buffer spill over the side of the tube, which would result in loss of sample. Move the pestle up and down and twist it to ensure thorough grinding of the plant sample. If the plant material compacts at the bottom of the tube, use a clean pipet tip to dislodge it and continue grinding. Check to make sure that the material has been ground to very fine particles (that is, particles difficult to see by eye, rather than in visible chunks), even if this requires further grinding.



6. Once a homogeneous lysate has been generated, add an additional 500 µl of lysis buffer. Continue grinding using the micropestle until the lysate is homogeneous.
7. Cap (close) the microcentrifuge tube and place it in a microcentrifuge. Make sure all the tubes are in a balanced arrangement in the rotor. If sharing with other students, accommodate classmates' tubes to ensure economic use of the microcentrifuge. Centrifuge at full speed for 5 minutes at room temperature.



8. While the tubes are centrifuging, add 500 µl of 70% ethanol into one labeled colored microcentrifuge tube for each plant extract.
9. Retrieve the samples from microcentrifuge. For each sample, carefully remove 400 µl of supernatant (taking care not to disturb the pellet) and add it to the 500 µl of 70% ethanol in the appropriately labeled tube. Avoid transferring any solid plant material to the ethanol; if necessary, recentrifuge the lysate. Using a p1000 pipet, pipet up and down to thoroughly mix the lysate and ethanol into a homogeneous solution. Cap tubes.

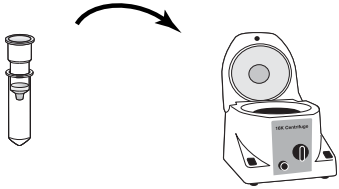


Note: If a precipitate is visible (a common occurrence when extracting DNA from starchy sources such as potatoes), spin the tube for an additional 5 minutes in a microcentrifuge at full speed to pellet the precipitated starch and use the supernatant for the next step.

10. Label the top edge of two purple mini DNA extraction columns with your initials and plant names. Place each column into a 2 ml capless collection tube.
11. For each sample, transfer 800 µl of cleared lysate and ethanol mixture to each column.

    This step binds DNA to the column.

Note: Be sure that there is no plant material in the lysate that is added to the column. If necessary, centrifuge the tube again to pellet any plant material prior to loading the supernatant on the column.



12. Place the capless collection tube containing the column into the microcentrifuge. Make sure that the microcentrifuge is balanced. Centrifuge for 1 minute at full speed at room temperature. Discard the flow-through from the collection tubes.

Note: If some of the supernatant does not pass through column, centrifuge again for 1 minute. If there is still supernatant remaining in the column, carefully remove the excess supernatant with a pipet and discard it, taking care not to disrupt the column bed. Further centrifugation will not help. Some plant samples can block the column with either carbohydrate or pigments. Even when blockage occurs, it is likely that some of the DNA has bound to the column matrix and is sufficient for the rest of the experiment.

13. Add 700 µl of wash buffer to each column. Centrifuge at full speed at room temperature for 1 minute. Discard the flow-through. Repeat the wash step two more times for a total of 3 washes. Check the appropriate box after completing each wash step.

    ❒ Wash 1

    ❒ Wash 2

    ❒ Wash 3

14. After the final wash step, discard the flow-through and place each DNA extraction column back in its capless collection tube. Dry columns by centrifuging for 2 minutes at full speed at room temperature. This step is vital to ensure that none of the wash buffer contaminates the DNA sample.

    ❒ Final spin

15. Transfer each DNA extraction column to a clean, appropriately labeled, colored microcentrifuge tube.

16. Obtain the sterile water from the 70°C water bath. Immediately pipet 80 µl of the warmed sterile water onto the membrane at the bottom of each column, making sure that the water wets the column bed. Leave for 1 minute at room temperature to allow the water to saturate the membranes in the column.

17. Place the column, still in the microcentrifuge tube, into the microcentrifuge. Orient the loose cap of the microcentrifuge tube downwards, towards the center of the rotor, to minimize friction and damage to the cap during centrifugation. Centrifuge at full speed at room temperature for 2 minutes.

    This step elutes DNA from the column.

18. Remove the column from the microcentrifuge. Cap microcentrifuge tube containing the gDNA and store at –20°C. Be sure that your tubes are labeled as gDNA with your initial, plant name, and date.

Focus Questions for DNA Extraction

1. Where is DNA found in eukaryotic cells?
   (Hint: think of different kinds of cells.)

2. What parts of the cell must be broken down to extract DNA?
   (Hint: think about cell structure.)

3. Why is it more difficult to extract DNA from plants?

4. Why are young plants the best source for DNA?

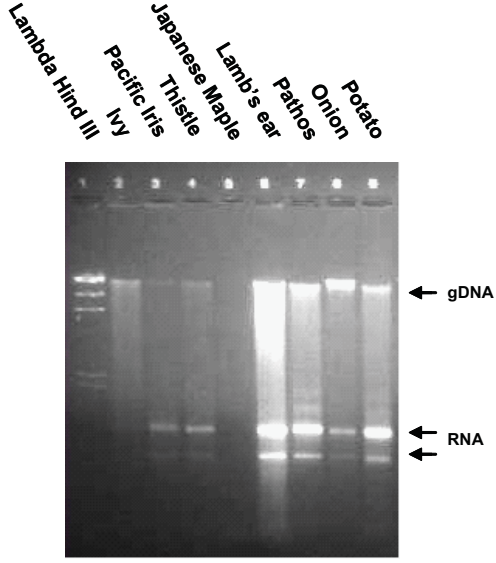
5. Briefly explain how you will achieve the basic steps in the DNA extraction.

Appendix A
Optional Activities

Depending on time available, you can analyze your genomic DNA sample prior to proceeding to a subsequent activity, such as PCR. This may include agarose gel electrophoresis, fluorometry, or spectroscopy.

Performing Agarose Gel Electrophoresis

Optional analysis of the final product may be performed using agarose gel electrophoresis. You can load ~10 µl of genomic DNA on a 0.8–1% agarose gel and electrophorese the sample at 75 volts for 1 hour. Genomic DNA is seen as a faint band at the top of the gel. RNA appears as two major bands much further down the gel. Note: Some plant extractions may not yield sufficient DNA to be visible on a gel. However, even if the DNA is not visible on the gel, it is likely to amplify when used for polymerase chain reaction (PCR).



Measuring DNA Concentration, Yield, and Purity by Fluorometry

A fluorometer, which specifically quantifies double stranded nucleic acids, can be used to accurately quantify the DNA. DNA concentrations can also be assessed by reading the absorbance at 260 nm (A_{260}) using spectrophotometer; however, because RNA is present in the sample, a spectrophotometer does not provide an accurate reading of DNA concentration. To get a more accurate reading, the contaminating RNA can be removed using RNase I, followed by ethanol precipitation to remove nucleotides. Bio-Rad's VersaFluor™ fluorometer (catalog #170-2402EDU) and DNA Quantitation Kit (catalog #170-2480EDU) can be used to accurately quantify DNA concentrations.

Set Up PCR Reactions

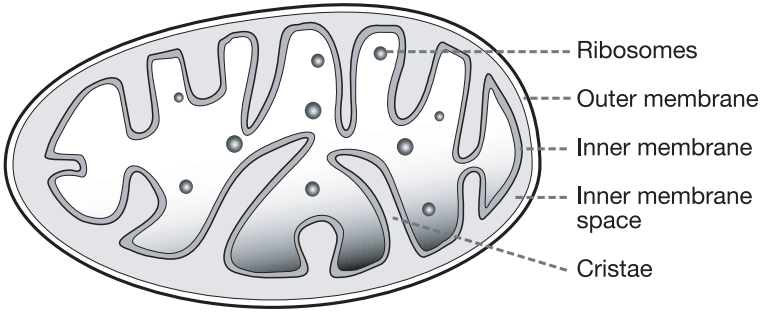
Genomic DNA can be used for PCR reactions. The exact quantity of gDNA per reaction should be optimized for each particular application. A good starting point is 25 ng of gDNA or, if the gDNA concentration has not been quantified, 5 µl per reaction.

Bio-Rad's GAPDH PCR Module (catalog #166-5010EDU) contains all the reagents and instructions required to perform a 2 step nested PCR to amplify a portion of the glyceraldehyde 3' phosphate dehydrogenase gene from plant genomic DNA derived using this Nucleic Acid Extraction Module.

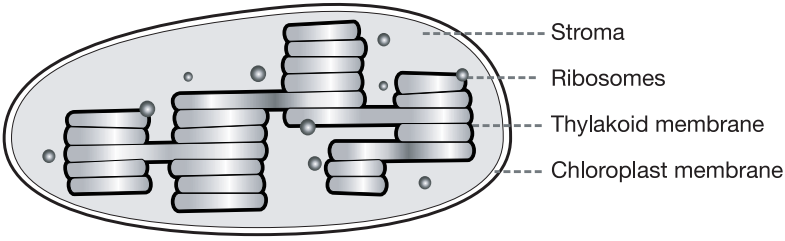
Appendix B
Other Cellular DNA

Besides genomic DNA, plant cells contain DNA in their mitochondria (referred to as mtDNA for mitochondrial DNA). Green plants also have DNA within their chloroplasts called cpDNA (for chloroplast DNA; also abbreviated ctDNA). When genomic DNA is isolated from plant samples using the DNA Extraction Module, the purified sample also contains mtDNA and cpDNA.

Mitochondria are subcellular organelles, the number of which is directly related to the metabolic activity of the cell, i.e., the higher the metabolic activity, the more mitochondria are present. Mitochondria are the cell's "power plants," producing ATP to energize cellular activities. Mitochondria have two membranes, an outer membrane that is freely permeable to small molecules and an inner membrane that acts as a permeability barrier.



The inner mitochondrial membrane is the site of many of the mitochondrial metabolic reactions. Within the inner membrane is the matrix that contains ribosomes and mtDNA. Plant mtDNA is variable in size and ranges from 200,000 to 2,500,000 base pairs! The proteins synthesized in the mitochondria are only a small number of those needed for the metabolic activities of the mitochondria. Therefore, most proteins used in mitochondria are encoded on the nuclear DNA, synthesized in the cytosol, and imported into the mitochondria.



In addition to mtDNA, cpDNA is found in chloroplasts in green plants. Chloroplasts have a number of similarities to mitochondria. They have two outer membranes, the inner of which acts as a permeability barrier. Within the outer membranes is a fluid-filled compartment called the stroma that contains the thylakoid membrane, ribosomes, starch granules, and the circular molecules of cpDNA. The thylakoid membrane forms a separate membrane-bound compartment within the chloroplast, a series of flattened stacks of interconnected vesicles. The stacks are called grana and are the site of photosynthesis. The cpDNA molecules average in size from 120,000–200,000 base pairs and contain over 100 genes; however, they do not produce many of the proteins needed in the chloroplast. Similar to the mitochondria, chloroplasts import many proteins that are encoded by nuclear DNA and synthesized in the cytosol. Interestingly, the enzymes needed for replication of cpDNA must themselves be imported from the cytosol into the chloroplast.

Appendix C
Instructor's Answer Guide

1. Where is DNA found in eukaryotic cells? (Hint: think of different kinds of cells.)

   DNA is found in the nucleus and in the mitochondria of all eukaryotes and also in plant plastids, including chloroplasts.

2. What parts of the cell must be broken down to extract DNA? (Hint: think about cell structure.)

   The cell, nuclear, and organelle membranes must be ruptured and cellular enzymes that would destroy DNA or bind DNA must be denatured. (There is no nuclear membrane in prokaryotes.)

3. Why is it more difficult to extract DNA from plants?

   Plants have a rigid cell wall that is difficult to break down, they have large vaculoles containing acids that could damage DNA, and may contain polyphenols and other inhibitory compounds that can bind DNA, preventing its use downstream.

4. Why are young plants the best source for DNA?

   Young plant leaves are still growing, have a greater nuclear to cellular volume, and have fewer chemicals that interfere with DNA isolation.

5. Briefly explain how you will achieve the basic steps in the DNA extraction.

   Students may purchase plant material from the store or collect samples from the field. Cell membranes and cell walls will be disrupted by grinding with micropestles and disrupted chemically by lysis buffer. Lysis buffer will also denature cellular proteins. Centrifugation will remove cellular debris and silica columns will be used to purify DNA.

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