DNA EXTRACTION LAB

ACTIVITY AT A GLANCE

Goal:
The students are introduced to DNA extraction techniques. They will isolate genomic DNA from insects and *Wolbachia,* the endosymbiotic bacteria that live within the cells of over 20% of insect species.

Learning Objectives:
Upon completion of this activity, students will transition from fieldwork and morphological classification (Lab 1) to molecular biology and biotechnology, learn about DNA as a diagnostic tool to discover unseen microbes, increase abilities in biotechnology, and understand the process of inquiry and discovery-based research. They will *isolate* total genomic DNA from morphospecies identified in the Insect Identification Lab.

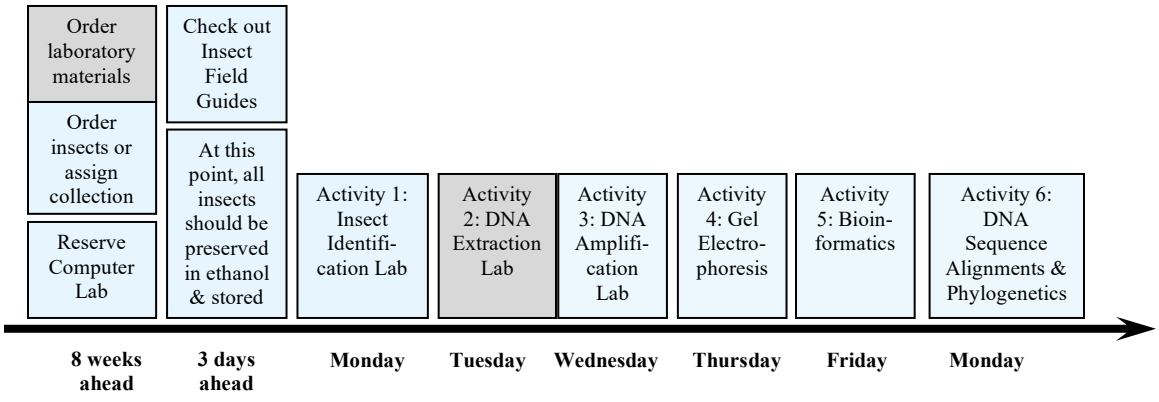
Prerequisite Skills: Prior practice with micropipettors.

Assessed Outcomes:
Assess the student's knowledge of insects infected with *Wolbachia.* They should understand that both *Wolbachia* DNA and insect DNA will be extracted from infected insects.
Assess understanding of the positive and negative control insects.
Assess ability to complete the DNA extraction.
Assess organizational skill during this lab.

Teaching Time:
90 minutes (2 class periods — optional 45 minute stopping point on page 8)

National Science Education Standards Addressed:
Unifying Concepts and Processes in Science, Science as Inquiry, Science and Technology, Life Science, Science in Personal and Social Perspectives, History and Nature of Science

Timeline for Teaching *Discover the Microbes Within: The Wolbachia Project*



OVERVIEW

In this activity, students will extract total genomic DNA from each of their two morphospecies using Qiagen's DNeasy Blood & Tissue Kit. Total genomic DNA includes DNA of the insect host as well as any symbiotic bacteria, if present. In addition to the 2 unknown morphospecies, students will also prepare positive and negative controls using *Drosophila melanogaster* fruit flies that are infected and uninfected with *Wolbachia pipientis*, respectively. The *Drosophila* controls may be obtained by contacting the Bordenstein Lab (https://my.vanderbilt.edu/discover/contact/) at least two weeks prior to beginning the lab series. Please indicate your anticipated number of student groups.

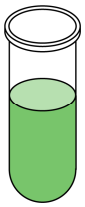
The extraction of total genomic DNA involves three distinct steps:

1. *Cell Lysis:* Students will begin by blotting the ethanol away from their insect specimens and then macerating them in a cell lysis solution (Buffer ATL). This basically breaks open cell and nuclear membranes. The dilemma here is that it also exposes DNA to proteins in the insect tissue. Therefore, the enzyme *Proteinase K* must be added to denature the proteins and keep the DNA intact. Finally, they will add ethanol to precipitate the DNA.

   Insects are added to the ATL buffer and macerated. The ATL buffer contains SDS, sodium dodecyl sulfate. This anionic surfactant acts as a detergent and aids in cell lysis. It disrupts non-covalent bonds in proteins to denature and unfold them. The AL buffer contains the chaotropic agent guanidinium chloride. It promotes the lysis of the cell membrane, denaturation of proteins, DNA and other macromolecules. It also inactivates nucleases and promotes nucleic acid binding to pure silica material. The guanidinium interferes with hydrogen bonds, hydrophobic effects and van der Waals forces resulting in the denaturation of proteins and DNA. This leads to accumulation of the chaotropic salts in the cell lipid bilayer compromising its integrity and leading to membrane collapse. Proteinase K is added to aid in the removal of proteins. Specifically, it degrades nucleases, which may affect the DNA to be isolated.

2. *Elimination of Cellular Debris:* Once students have destroyed the hydrolytic enzymes and denatured DNA, they will begin the DNA purification process. In essence they will place the cellular components, including DNA, into a spin column and wash the spin column of all components except DNA. Upon centrifugation the material will pass through the filter, which attracts DNA and allows debris to pass through. The column is next washed using two wash buffers (AW1 and AW2).
   The DNeasy membrane combines the binding properties of a silica-base membrane with simple microspin technology. DNA absorbs to the silica membrane in the presence of a high concentration of a chaotrophic salt,

guanidinium chloride. The silica membrane has a positive charge, which binds DNA molecules through their negatively charged DNA backbone. Washing steps include the guanidinium salts and ethanol in decreasing concentrations. Column washing removes residual protein impurities with out affecting the DNA bound to the membrane. The ethanol in the wash buffer displaces the excess salts, RNA, carbohydrates and other alcohol soluble biomolecules.



3. *DNA Elution:* Students will complete the activity by removing the DNA from the filter. This is done by adding the elution buffer AE. Spinning the tube with the DNA embedded in the filter will pull the elution buffer through the matrix, thus pulling the DNA into the collection tube.

   Elution of the bound DNA is done with water or a low salt solution like TE buffer (tris, EDTA). This is because the removal of salts with the increase in water molecules unmasks charges and rehydrates the DNA as well as the surface of the silica membrane. The DNA becomes unbound and flows out of the column during the centrifugation step.

MATERIALS

- Incubator, heat block, or water bath set at 56˚C
- Vortex
- Centrifuge
- Student morphospecies
- + and – *Drosophila* controls
- Microtube Pestles (Fisher K749521-0500)
- Qiagen DNeasy Kit (69504)
- Qiagen Proteinase K (19131)
- P200 & P1000 pipets
- P200 & P1000 pipet tips
- Float racks for water bath
- Waste cups for tips, etc.
- Gloves
- Sharpies
- Tweezers
- Kimwipes
- Absolute ethanol (95–100%)
- Tube racks (VWR CBGTR-080)
- 1.5 ml microcentrifuge tubes (VWR 20170-333)

TEACHER PREPARATION

This lab requires attention to detail, but it's worth it. Aliquot all of your reagents ahead of time into labeled sets of tubes so that YOU don't get confused. Use the P1000 and P200 pipettes to aliquot the solutions into individual tubes for your students. Students will use pipettes to add the aliquoted solutions to their DNA extractions. If you do not have enough pipettes, have students share or use plastic transfer pipettes with pre-aliquoted volumes of each solution. The Qiagen DNeasy Kit contains spin columns, collection tubes, Proteinase K, and buffers ATL, AL, AW1, AW2, and AE. Note that some of these solutions require 95-100% ethanol added to them prior to the start. You will also need to order the additional items in the list above. Set up each activity station with its own set of materials as reflected on the student sheet.

Note: The Qiagen DNeasy extraction yields high quality DNA but there are a number of different extraction protocols that can be used.
See https://www.vanderbilt.edu/wolbachiaproject/resource-library/ for protocols that have been tested, including Promega Wizard SV, Bio-Rad InstaGene Matrix, and Edward's Buffer.

ACTIVITY PROCEDURE

Review the activity flow-chart on page 12 with your class and instruct them to revisit their hypothesis from the Insect Identification Lab Mini-Report. Students will work with their same partners from Lab 1 and follow the protocol outlined on the student sheet. They are encouraged to read through the procedure prior to beginning and activity in order to identify and understand the purpose of each reagent. Answers are shown below:

- Buffers ATL and AL: Lysis solutions that break open tissue, cell, and nuclear membranes
- Proteinase K: An enzyme that catalyzes the breakdown of cellular proteins by splitting them into smaller peptides and amino acids
- Ethanol: Used to precipitate DNA from the extracted material
- Buffer AW1 and AW2: Wash solutions that wash the DNA attached in the column membrane of contaminants
- Buffer AE: A solution that elutes the DNA from the membrane and allows stable storage of DNA for years in the refrigerator or freezer

Student Activity Sheet Name:_____

DNA EXTRACTION LAB

Hypothesis: Based on the background information, formulate a hypothesis about the frequency of *Wolbachia* endosymbionts in your specimens.

_____
_____
_____

MATERIALS (per group of two students)

- ❑ 2 different morphospecies
- ❑ + and – Drosophila controls
- ❑ Gloves
- ❑ Sharpie
- ❑ Tweezers
- ❑ 1 box Kimwipes
- ❑ 1 box of P200 pipet tips
- ❑ 1 box of P1000 pipet tips
- ❑ P200 and P1000 pipets
- ❑ 1 waste cup for tips & tubes
- ❑ 4 microtube pestles
- ❑ 1 tube of Proteinase K (120 µl)
- ❑ 1 tube of Buffer ATL (1.1 ml)
- ❑ 1 tube of Buffer AL (1 .2 ml)
- ❑ 2 tubes of Buffer AW1 (1.5 ml each)
- ❑ 2 tubes of Buffer AW2 (1.5 ml each)
- ❑ 1 tube of Buffer AE 600 µl)
- ❑ 1 tube of ethanol (95–100% – 1.2 ml)
- ❑ 4 spin columns
- ❑ 12 empty 1.5 ml microcentrifuge tubes
- ❑ 1 tube rack
- ❑ Safety goggles

INTRODUCTION

In this activity, you will:

- Isolate total genomic DNA from morphospecies identified in the Insect Identification Lab
- Isolate DNA from infected (+) and uninfected (–) *Drosophila* controls.
- Learn about DNA as the molecular basis of heredity and the interactions of bacterial and animal organisms.
- Learn about cell structures and the nature of scientific knowledge

In this activity, you will extract total genomic DNA from each of your morphospecies using Qiagen's DNeasy Blood & Tissue Kit. Total genomic DNA includes DNA of the insect host as well as any symbiotic bacteria *Wolbachia*, if present. In addition to the two unknown morphospecies, you will also prepare positive and negative controls using *Drosophila melanogaster* fruit flies that are infected and uninfected with *Wolbachia*, respectively. Review the activity flow-chart on page 12 and work with the same partners from Lab 1. Read through the procedure prior to beginning the activity in order to identify and understand the purpose of each reagent.

BEFORE YOU BEGIN

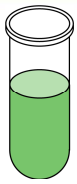
After the teacher reviews the entire procedure, note the purpose of each reagent:

- Buffers ATL and AL:
  _____
- Ethanol:
  _____
- Buffer AW1:
  _____
- Buffer AW2:
  _____
- Buffer AE:
  _____

The extraction of total genomic DNA involves three distinct steps:

1. Cell Lysis: You will begin by blotting the ethanol away from the insect specimens and then macerating them in a cell lysis solution, Buffer ATL. This basically breaks open cell and nuclear membranes that prevent you from extracting DNA from cells. The dilemma here is that it also exposes DNA to proteins in the insect tissue that could break up the DNA. Therefore, the enzyme *Proteinase K* must be added to denature the proteins and keep the DNA intact. Finally, you will add ethanol to precipitate the DNA.

2. Elimination of Cellular Debris: Once you have destroyed the hydrolytic enzymes and precipitated DNA, you will begin the DNA purification process. In essence you will place the cellular components, including DNA, into a spin column and wash the spin column of all components except DNA. Upon centrifugation the material will pass through the filter, which attracts DNA and allows debris to pass through. This will be followed by two wash steps with two separate buffers, AW1 and AW2.



3. DNA Elution: You will complete the activity by removing the DNA from the filter. This is done by adding the elution buffer AE. Spinning the tube with the DNA embedded in the filter will pull the elution buffer through the matrix, thus pulling the DNA into the collection tube.

PROCEDURE

Preparation

1. In the chart below note the contents of what you will put in each tube.

Tube #	Contents (Voucher #)
1	
2	
3	– *Drosophila* control
4	+ *Drosophila* control

2. Collect four 1.5 ml microcentrifuge tubes. Using a Sharpie marker, number them 1–4 along with your initials.

Cell Lysis

IT IS IMPORTANT TO DO STEP 3 AS RAPIDLY AS POSSIBLE! MACERATED TISSUE RELEASES DNases WHICH LEAD TO A RAPID BREAKDOWN OF DNA.

1. Pipet 180 microliters (µl) of buffer ATL into each tube to macerate the insects in.
2. Place the small insect or abdomen of a larger insect into the buffer (**no larger than 2 mm^2**) of tube 1 with tweezers. If the insect is preserved in ethanol, blot it thoroughly dry on a Kimwipe. Blot the ethanol away from your + and – *Drosophila* controls as well.
3. Take tube 1 and macerate **THOROUGHLY** using a microtube pestle.
4. **IMMEDIATELY** add 20 µl of Proteinase K (destroys DNases that break down DNA).
5. Add 200 µl of buffer AL (lysis buffer to break open cells).
6. Mix by vortexing for 10 seconds or inverting 25 times. ***(Do not pre-mix Proteinase K and Buffer AL, they must be added separately).***
7. Repeat steps 2–6 with the other three samples. Be sure to use a different pestle and pipet tips for each tube.
8. Incubate for at least 10 minutes at 56º C.
9. If the solutions are thoroughly homogenized, proceed to Step 10. If there are still solid particles:
    a. Centrifuge tubes for 1 minute to pellet cellular debris.
    b. Transfer each supernatant to a new tube and label accordingly. Use this tube going forward.
10. Add 200 µl of Ethanol (95–100%) to each tube. This will precipitate DNA from the extracted material.
11. Vortex.

***OPTIONAL STOPPING POINT. STORE TUBES AT 4° C OVERNIGHT.**

Cellular Debris Removal

1. Collect four DNeasy spin columns fitted in four 2.0 ml collection tubes and label the lids of the spin columns 1–4 with your initials.
2. Pipet the liquid from tube 1 of the above steps (with or without exoskeleton) into the DNeasy Mini spin column #1. Using a new pipet tip for each transfer, repeat this process with the four other tubes. Make sure to keep tube numbers consistent.
3. Centrifuge for 1 minute at 6000 g or 8,000 rpm. The DNA is now caught in the filter of the spin column. Discard the flow through waste into the 2.0 ml collection tubes in the waste bucket.
4. Place the spin column containing the DNA from tube 1 in the same emptied 2.0 ml collection tube.
5. Repeat for your other three tubes, remembering to label.
6. To each, add 500 µl of Buffer AW1. This is a wash buffer that washes the DNA.
7. Centrifuge for 1 minute at 6000 g or 8000 rpm.
8. Again, discard the flow through waste in the 2.0 ml collection tubes in the waste bucket and place the DNeasy Mini spin column from tube 1 into the same emptied 2 ml collection tube labeled "1"; repeat for your other three tubes.
9. Add 500 µl of Buffer AW2 (a second wash buffer) to each of your four tubes and centrifuge for 3 minutes at 20,000 g or 13,000 rpm (or max speed if your centrifuge doesn't go that high)*. Discard flow-through and collection tubes. This step is also removing the ethanol.
10. Place your spin columns into 1.5 ml microcentrifuge tubes. Again, be sure to label the lids of each tube 1–4 and include your initials this time. These will contain your purified DNA samples. There will be two lids, one from the spin column and another from the collection tube.

    ***Note—if the centrifuge you are using cannot attain this speed, you can allow the tube to air dry for 5 minutes. This will evaporate the ethanol.**

DNA Elution and Dilution

1. Pipet 100 µl of Buffer AE directly onto the membrane. This is an elution buffer that rinses the DNA off the spin column filter and into the 1.5 ml tube.
2. Incubate at room temperature for 1 minute.
3. Centrifuge (6000g or 8,000 rpm) for 1 minute to elute.
4. Discard the spin column and KEEP the labeled 1.5 ml tube.
5. Store the eluted DNA frozen at –20º C until PCR.

DNA Isolation Flow Chart

1 2 3 4

↓

Add ATL and macerate

↓

Add Proteinase K and AL

↓

Vortex and incubate

↓

Add Ethanol (Stopping point here and store in 4°C)

↓

Spin through a filter and discard flow-through

↓

Wash with Buffer AW1

↓

Spin and discard flow through

↓

Wash with Buffer AW2

↓

Spin and discard flow through

↓

Add Elution Buffer, spin, and keep flow through

Please answer the following questions:

1) Briefly outline the steps required to isolate DNA, taking the cellular structures into consideration.

2) What are the cellular structures and elements that serve as a barrier to getting your DNA out of the cell and intact?

3) After completion of DNA extraction, what did the DNA look like?

4) Research scientists who are trying to determine the identification of a new flu virus would perform a DNA extraction from human tissues. Will the method used to isolate DNA be similar or different from the method you used to isolate insect and *Wolbachia* DNA?

5) What is the purpose of isolating the DNA? What is your next step in determining the frequency of *Wolbachia*?