PCR LAB

ACTIVITY AT A GLANCE

Goal:
Students are introduced to the Polymerase Chain Reaction (PCR) and its use as an essential laboratory procedure. They will learn about the role of PCR in this lab series and why it is necessary for the identification of *Wolbachia.* The method of PCR will be reviewed in detail. The steps of denaturation, annealing and extension performed by the PCR machine will be explained. The essentials of good pipetting skills will be stressed. This lab will present PCR as an application to identify *Wolbachia* in arthropods.

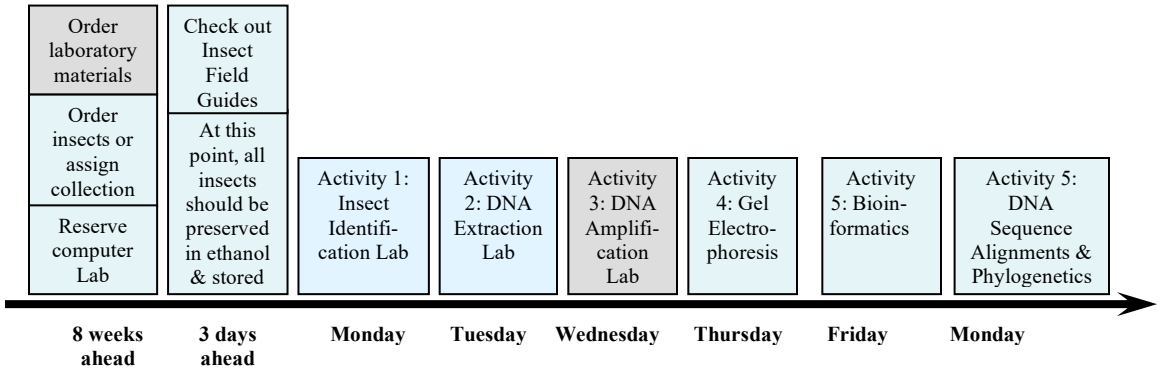
Learning Objectives:
Upon completion of this activity, students will use and understand one of the most useful biotechnology tools in the life sciences, understand DNA as the hereditary basis of life, utilize DNA as a diagnostic tool to discover microbes, and seamlessly transition their discovery-based science from organisms to molecules during this lab. Students will *amplify* DNA extracted from two morphospecies and three controls using Polymerase Chain Reaction (PCR). The piece of DNA used for identifying *Wolbachia* is the 16S rRNA gene, which encodes the RNA component of the 30S ribosomal subunit. The piece of DNA used for identifying the arthropod is the CO1 gene, which encodes the mitochondrial protein cytochrome c oxidase I.

Prerequisite Skills: Prior practice with micropipettors.

Assessed Outcomes:
Assess the student's understanding of PCR and the role it has in the identification of *Wolbachia* in arthropods.
Assess the student's ability to stay organized and successfully complete the lab.

Teaching Time: One class period

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



OVERVIEW

Most DNA analysis situations require fairly large amounts of DNA. Usually the amount in a few cells is not enough to fully analyze. A method called the polymerase chain reaction (PCR) has been developed to make many copies of DNA in a sample. PCR is essentially the microscope of the 21st century as it allows biologists to study the DNA of microorganisms that we cannot see by either eye or culture. It is revolutionizing research in microbial diversity, genetic disease diagnosis, forensic medicine, and evolution. In this portion of the lab series, you will use your samples from the DNA Extraction Lab to decipher if *Wolbachia* symbionts are present within your morphospecies. Your work could be new to science and potentially lead to new discoveries on the presence and absence of *Wolbachia* in insects. Contact The *Wolbachia* Project (wolbachiaproject@vanderbilt.edu) at Vanderbilt University for positive and negative *Drosophila* insect controls and positive control DNA samples. As in the previous lab, students should work in groups of two or more.

Goals

In this activity, we will not only seek to amplify the possible *Wolbachia* DNA, but we will also be amplifying a portion of mitochondrial DNA from the arthropod host. This second amplification is, in effect, a procedural control. Students can *hope* for a *Wolbachia* band but will be guaranteed an insect (eukaryote) band. Therefore, they can be certain that their DNA isolation and amplification was done correctly as well as having a concrete band to read on their gels.

PCR Primers

Primers to specifically amplify a 438 bp fragment of the 16S ribosomal RNA gene (ubiquitous in all *Wolbachia*) are
Wspec-F (5'−CAT ACC TAT TCG AAG GGA TAG−3') and
Wspec-R (5'−AGC TTC GAG TGA AAC CAA TTC−3').

Primers to amplify a 708 bp fragment of the CO1 cytochrome oxidase gene (ubiquitous in arthropod mitochondria) are
CO1_F: LCO1490 (5'−GGT CAA CAA ATC ATA AAG ATA TTG G−3') and
CO1_R: HCO2198 (5'−TAA ACT TCA GGG TGA CCA AAA AAT CA−3').

These primers can be provided by the Bordenstein Lab at Vanderbilt University (wolbachiaproject@vanderbilt.edu). Thanks to generous support from MiniOne, we also offer a free 2-week loaner program, including MiniOne thermal cycler and electrophoresis systems. Contact us several weeks/months in advance to coordinate shipping.

TEACHER PREPARATION

If using the MiniOne thermal cycler, download the MiniOne PCR app to your phone or mobile device. You may also ask students to do this at home.

https://theminione.com/pcr-system/minione-pcr-app/

Set up each activity station with its own set of materials as reflected below. Protocols will vary based on (i) thermal cycler and (ii) Taq polymerase. Download the correct PCR Calculator for reference.

Master Mixes

Each PCR reaction tube will contain:

Wolbachia forward primer (Wspec-F)	2 µl
Wolbachia reverse primer (Wspec-R)	2 µl
Arthropod forward primer (CO1_F)	2 µl
Arthropod reverse primer (CO1_R)	2 µl
Sterile, nuclease-free water	2.5 µl
Taq Master Mix 2X	12.5 µl
Total	23 µl

2 µl of DNA template makes a total reaction volume of 25 µl.

As you can see, pipetting such small amounts in each tube can lead to error and is quite time-intensive. We highly encourage PCR Master Mixes rather than adding each reagent to individual tubes. Determine the "best practice" for your class. Two recommended options are:

1. Teacher-prepared aliquots. The student activity below involves pre-made aliquots of primers and water. Students will combine the "Primer Mix" with a "Taq Mix" to create the final PCR Master Mix. 23ul of this mix is added to 2 ul of DNA per tube. It requires more pre-lab preparation but eliminates student error. This is recommended for general biology courses.
2. Use the PCR Calculator. This will allow students to independently set up the entire reaction. This is recommended for research courses where students will be performing multiple rounds of PCR.

Duplex Reaction

The default protocol below features a duplex reaction – both *Wolbachia* and arthropod primers are included in the same reaction. This comes with the caveat that the *Wolbachia* primers prefer a slightly higher annealing temperature (55 °C vs 49 °C). While most *Wolbachia*-infected samples will test positive at 49 °C, you may obtain brighter bands (and possibly detect weaker infections) if you run two separate reactions.

We recommend using a duplex reaction for general classes where you only intend to run the project once in the academic year. However, if students continue to test additional arthropods, you may split the PCR into two reactions. The first reaction will use only CO1 primers and a 49 °C annealing temperature. The second reaction will use only Wspec (16S) primers and a 55°C annealing temperature. Refer to individual tabs on the PCR Calculator for revised protocols.

Student Activity Sheet **Name:**_____

PCR Lab

Hypothesis: Based on extracted DNA from your sets of morphospecies and the estimated global frequency of *Wolbachia pipientis* endosymbionts, ~40%, formulate a hypothesis for your own specimens.

_____
_____
_____

MATERIALS

- ❑ 2 DNA samples from morphospecies
- ❑ 2 DNA samples from positive and negative *Drosophila* controls
- ❑ Positive DNA control
- ❑ Sterile, nuclease-free water
- ❑ Sharpie, fine tip
- ❑ Taq Master Mix
- ❑ 1 box of P200 pipet tips
- ❑ 1 box of P20 pipet tips
- ❑ P200 and P20 pipettes
- ❑ Gloves, 2 pair
- ❑ Styrofoam coffee cup with ice
- ❑ 1 rack for holding PCR tubes
- ❑ 1 Primer Mix tube of primers with
  14 µl Wspec-F primer (5 µM) *
  14 µl Wspec-R primer (5 µM) *
  14 µl CO1-F primer (5 µM) *
  14 µl CO1-R primer (5 µM) *
  17.5 µl sterile, nuclease-free water *
- ❑ 1 waste cup for tips, tubes
- ❑ Safety goggles
- ❑ Squeeze bottle of 70% ethanol
- ❑ 0.2 ml PCR tubes

** Volumes may vary if setting up more than 6 reactions*

INTRODUCTION

In this activity, you will learn what Polymerase Chain Reaction (PCR) does, how it works, and why it is useful to research in the biological sciences. You will use PCR to make many copies of *Wolbachia* DNA (if present) and arthropod DNA from the extracted DNA of the two selected specimens and control insects. You will also amplify a previously extracted DNA sample which is positive for *Wolbachia* and a water sample. The piece of DNA used for identifying *Wolbachia* is a region that codes for a small subunit of the ribosomal RNA (16S rRNA) that is unique to *Wolbachia*. The piece of DNA used for identifying arthropod DNA is a region that codes for the cytochrome oxidase I protein in animal mitochondria (CO1).

The protocol refers to three different mixes:

1. **Primer Mix** – this includes all four primers and water
2. **Taq Master Mix** – this includes Taq polymerase, dNTPs, $MgCl_2$, and buffer
3. **PCR Master Mix** – you will combine the Primer Mix and Taq Master Mix to produce the "PCR Master Mix"; this will be added to your PCR tube along with DNA

PREPARATION

The thermal cycler should be programmed for the optimum settings below.

Promega GoTaq*

(Standard protocol for most Taq mixes)

1 cycle
2 min. @ 94° C

30 Cycles
30 sec. @ 94° C
45 sec. @ 49° C
1 min. @ 72° C

1 cycle
10 min. @72° C
Hold @4° C

MiniOne Taq

1 cycle
1 min. @ 94° C

30 Cycles
10 sec. @ 94° C
20 sec. @ 49° C
1 min. @ 72° C

1 cycle
2 min. @ 72° C
MiniOne will automatically hold @ 4° C

* When programming the thermal cycler, select 'Initial denaturation' *and* 'Final extension'

GE illustra PuReTaq Ready-To-Go PCR Beads

(Refer to product information for protocol)

1 cycle
2 min. @ 94° C

30 Cycles
30 sec. @ 94° C
45 sec. @ 55° C
1 min. @ 72° C

1 cycle
10 min. @72° C
Hold @4° C

PROCEDURE

Label Tubes

1. Remove all unnecessary items at your lab station. Clean all surfaces by wiping down with 70% ethanol.
2. Collect six 0.2 ml PCR tubes, number and label them with your initials.

> Note that you will use 6 tubes because a previously purified sample of *Wolbachia* DNA has been included as a positive control and water has been included as a negative control.

Tube #	Tube Contents
1	
2	
3	− Control
4	+ Control
5	*Wolbachia* DNA
6	Water

Prepare a PCR Master Mix

3. Obtain a pre-made Primer Mix from your teacher or create one by adding the following reagents to a 1.5 ml microcentrifuge tube. Use a new pipette tip for each reagent. (Note: If you have more than 6 reactions, use the PCR Calculator)

Primer Mix for 6 reactions:

Wspec-F	14 µl
Wspec-R	14 µl
COl-F	14 µl
CO1-R	14 µl
Water	17.5 µl
Total	73.5 µl

4. Add 87.5 µl of Taq Master Mix to your Primer Mix. (Note: If you have more than 6 reactions, use the PCR Calculator)

5. Briefly vortex or mix by pipetting up and down.

Set up the PCR Reaction

6. Place the labeled PCR tubes on ice. Add 23 µl of PCR Master Mix to each tube. Keep tubes on ice to keep polymerase from activating once you add the Taq mix.

7. Add 2 µl of DNA template from each sample to its correlating tube. ***Be sure to change the pipette tips for each DNA template!***

8. Cap and gently tap the bottom of each tube to mix the components. If drops are still on the side of the tube, spin very gently in a centrifuge for a couple of seconds at a time, until all liquids are collected in the bottom. Place your six tubes with labels (initials and number) into the thermal cycler. Once everyone has prepared their samples, the thermal cycler can be turned on. If using MiniOne, observe the PCR program as it cycles.

9. Clean up your lab station and wipe surfaces with ethanol.

10. When the thermal cycler is done, store the samples in the 4 °C refrigerator.

11. Proceed to the Gel Electrophoresis Lab.