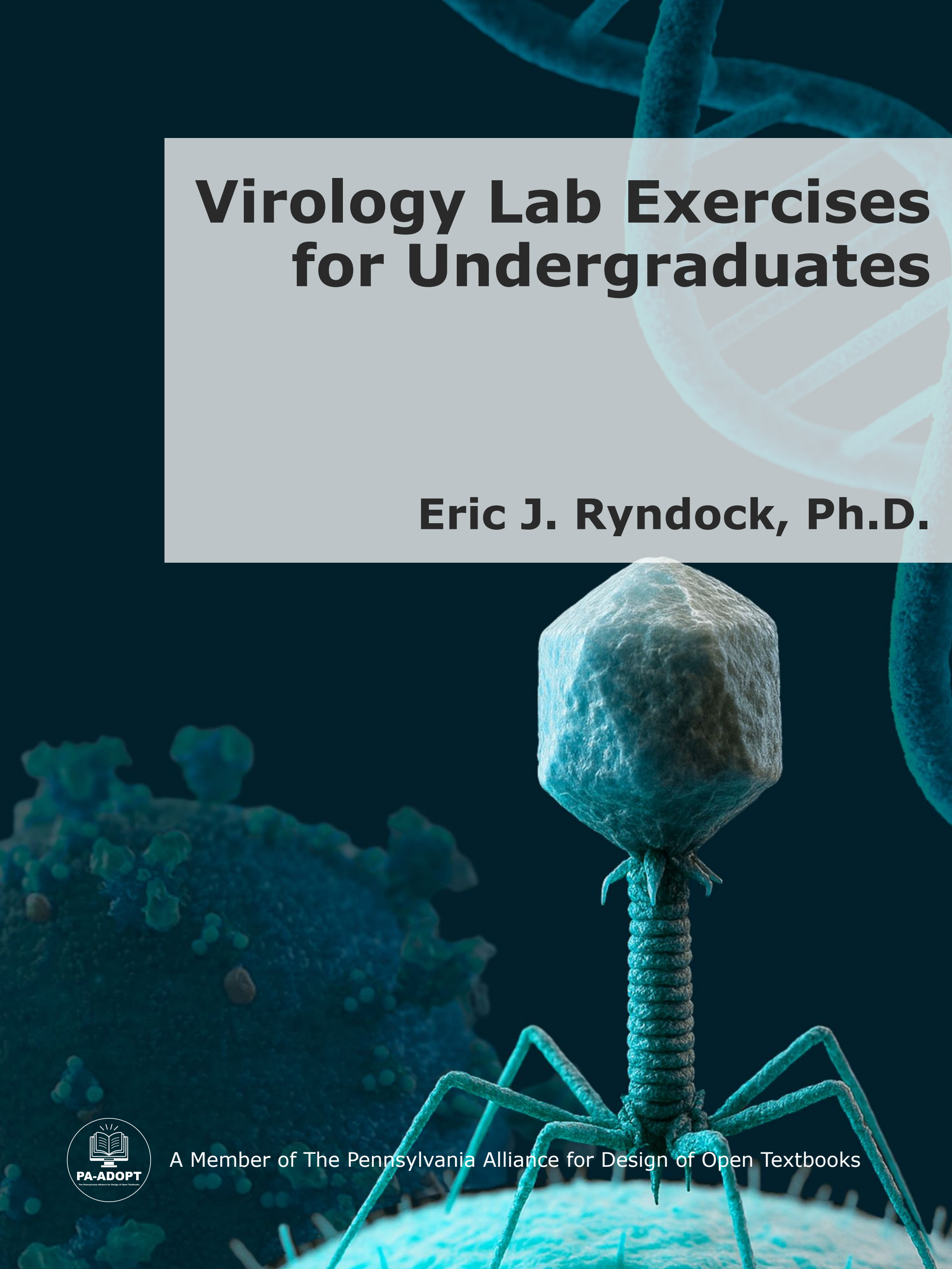
Virology Lab Exercises for Undergraduates

Eric J. Ryndock, Ph.D.



A Member of The Pennsylvania Alliance for Design of Open Textbooks

Cover image: Image by Author. Modified from Image by FusionMedicalAnimation from Unsplash.

The contents of this eTextbook were developed under a grant from the Fund for the Improvement of Postsecondary Education, (FIPSE), U.S. Department of Education. However, those contents do not necessarily represent the policy of the Department of Education, and you should not assume endorsement by the Federal Government.

About PA-ADOPT

The Pennsylvania Alliance for Design of Open Textbooks (PA-ADOPT) is made up of four participating institutions from Pennsylvania State System of Higher Education (PASSHE) that are all regional and primarily undergraduate institutions, situated in Southeastern Pennsylvania. The PA-ADOPT project addresses gaps in the open eTextbook marketplace, improve student learning, and mitigate rising student costs. PA-ADOPT was made possible by the US Department of Education Open Textbook Pilot Program.

About OER

Open Educational Resources (OER) are instructional, learning and research materials, digital or non, that open-source and in the public domain or that are licensed so that users have free and perpetual permission to engage in the following activities:

- Retain: the right to make, own, and control copies of the content
- Reuse: the right to use the content in a wide range of ways
- Revise: the right to adapt, adjust, modify, or alter the content itself
- Remix: the right to combine the original or revised content with other open content to create something new
- Redistribute: the right to share copies of the original content, revisions, and remixes with others.

About the Author

Eric J. Ryndock, Ph.D.

Dr. Eric Ryndock teaches and performs research at Millersville University. His interests encompass understanding the world of viruses. Although viruses themselves are not considered “alive,” viruses infect all living organisms. You may not be able to see them, but viruses are everywhere! We ingest viruses, their genetic material is part of our genomes, and viruses are a major driver of biological evolution. He is most interested in deciphering molecular mechanisms of viruses and designing methodologies to detect them in our environment.

Acknowledgements

The author would like to acknowledge careful editing of the lab manual by Dr. Brian S. Bowser and Dr. Christopher Stieha. Dr. Bowser is an industry scientist who has extensive experience with virology and cancer immunotherapy. Dr. Stieha is an associate professor at Millersville University with expertise in quantitive ecology and OER resources.

The author also acknowledges undergraduate research student Tatum Dolan of Millersville University. She assisted in the design and testing of the experiments in Chapter 2, which were the focus of her Honors College thesis. She intends to pursue a career as a physician.

The author also acknowledges the design of the lab manual’s cover by Helena Bleacher, an undergraduate student at Millersville University. She was previously accepted to Parson’s School of Design for illustration before shifting her focus to biological sciences. She intends to pursue a master’s degree and advance her career in genetics as a cell and gene therapy scientist.

Table of Contents

Preface

The purpose of this laboratory manual is to create a novel series of experiments for undergraduates to safely explore and learn about the field of virology. Instructors utilizing this lab manual should know that it is designed for students who have a working knowledge of cellular biology, genetics, and molecular biology. Many of the currently available virology lab manuals use experimentation that relies on expensive equipment, such as mammalian cell culture, or solely focus on one type of virus and associated techniques, such as bacteriophages. The techniques and protocols found in this manual are designed to be accessible to individuals relying on facilities found at a primary undergraduate teaching institution rather than a research-focused institution. It also exposes students to representative types of viruses that infect different hosts: bacteria, animals, and plants, while using current methods that allow students to build employable skills used in the workforce. I hope that students performing the included experiments improve their education and spark a passion for the molecular pirates that we call viruses.

Chapter 1: Bacteriophage Investigation

Laboratory #1: Growing Bacteria and Bacteriophages

Objectives

✦Familiarize yourself with aseptic technique and working with bacteria;

✦Familiarize yourself with the replication cycle of bacteriophages and the concept of host tropism;

✦Learn how to create pure cultures of the bacterial host and bacteriophage.

Outline

- Experiment 1: Isolating bacterial colonies from a bacterial culture.
- Experiment 2: Determining bacteriophage tropism and attempting to obtain a pure culture of bacteriophage.

I. Obtaining a Pure Culture of Bacterial Host

A. Introduction

Before you begin to study viruses, it is imperative that you first learn how to grow or manipulate the host cell or organism that they use for replication. The first viruses we will be working with are **bacteriophages**, a group of viruses that infect bacteria.[1] Therefore, it is important to learn how to grow their host organism, bacteria. **Bacteria** are prokaryotes, meaning they are small cells that do not have a nucleus; they can divide by the simple process of binary fission in either liquid culture media (broth) or solid media (agar). When working with bacteria, you must ensure that you have a **pure culture**, which is a bacterial sample that is growing in the absence of other species of bacteria or other organisms. To obtain a pure culture, you must use **aseptic technique**, which is a practice that guards against the introduction of unwanted organisms that can be accidentally added if you are performing microbiology procedures in a non-careful manner. Keeping your supplies near a lit Bunsen burner, wearing gloves, and only opening reagents when necessary, will limit exposure to contamination and promote an aseptic environment.

Additionally, most of the reagents we use are processed using an **autoclave**, a piece of equipment that can sterilize both liquid and solid materials using high-temperature and pressurized steam.

Your instructor will provide you with a tutorial of how the autoclave functions. Never operate the autoclave without receiving training from a faculty member.

NOTES:

1. What is the typical temperature range of an autoclave for sterilization?

2. How does an autoclave function to kill or neutralize bacteria and viruses?

3. What types of microorganisms (or other microorganism-like entities) does an autoclave struggle to kill or neutralize?

One method to produce a pure culture of a bacterial host organism is known as the **streak plate method**, where bacteria are spread across agar using a sterile loop, and are mechanically diluted until only single cells are produced. Since a single cell of a bacterium is impossible to see with the naked eye, we must allow the single bacterial cell to form a macroscopic structure known as a **colony**. Bacterial colonies are originally derived from one cell that has undergone binary fission many times, and because of that, are often referred to as a clone in molecular biology as all cells are clones of the original. Most bacterial species that will form colonies on agar will do so within 1-2 days.

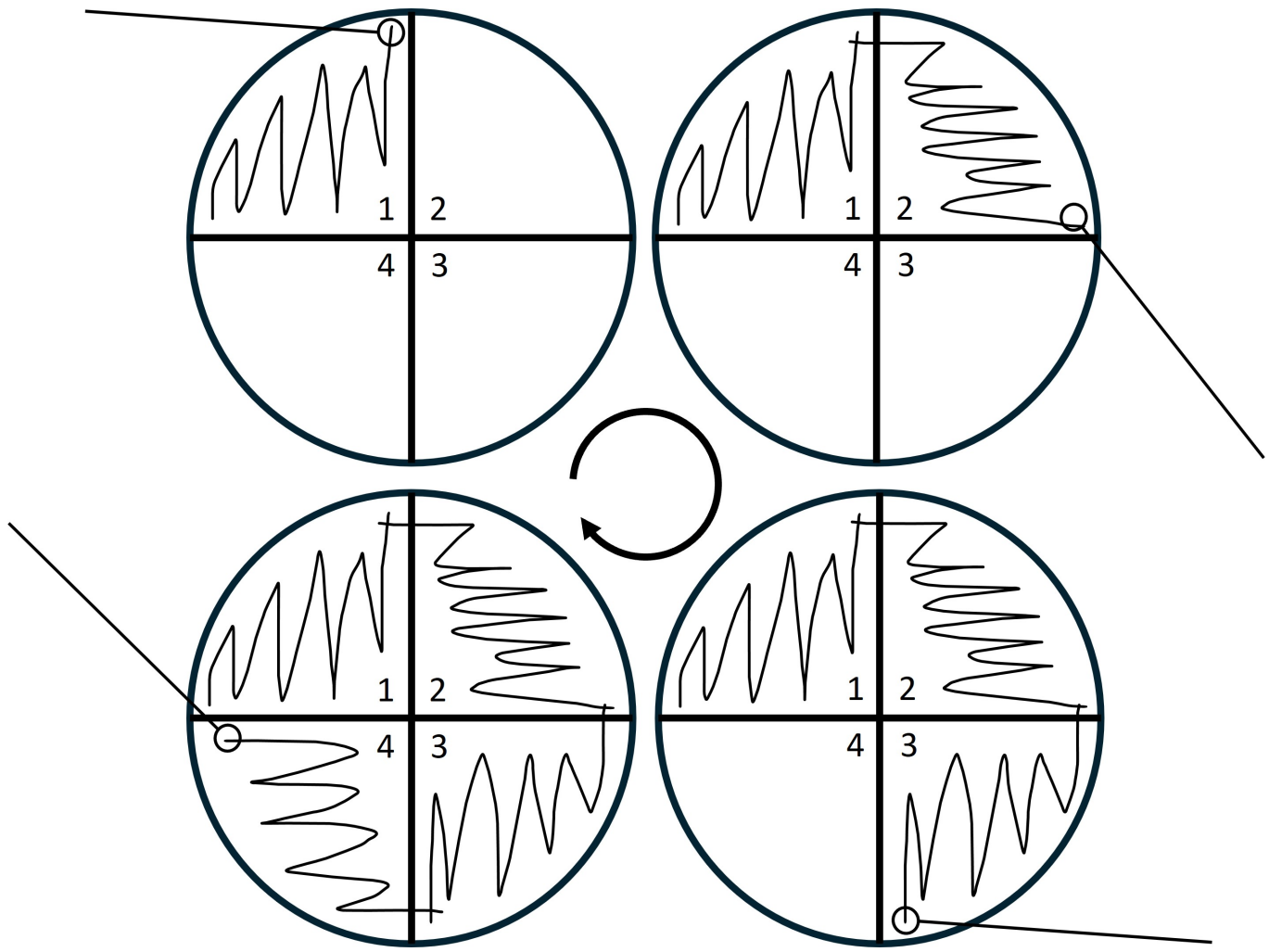


Figure 1. Streak plate method. Bacteria are mechanically diluted using the streak plate method to ensure that single colonies of bacteria are produced in either the third or fourth quadrant. The single colony can be used to obtain a pure culture of that bacterial species. Image by Author.

Your instructor will demonstrate how to prepare liquid and solid media for bacteria.

NOTES:

B. Procedure I

1. Wipe down your table space with 70% ethanol and a paper towel to create an aseptic environment.

2. Make sure all group members are wearing a lab coat and gloves.

3. On three individual tryptone agar plates, draw 2 lines using a Marker marker on the bottom side (the side connected to the agar, NOT the lid). This should create 4 equal sections (Figure 1). Additionally, write your group number, the date, and name of the bacterium being added.

4. Ignite the Bunsen burner and adjust the flame so that there is a visible blue cone (ask the instructor for assistance if needed). Sterilize your inoculating loop by placing it in the upper portion of the blue cone until the loop is glowing. Remove the loop and keep it from touching anything while it cools.

5. After the loop has cooled (30 seconds to 1 minute), carefully obtain a loopful of the appropriate bacteria culture and streak it in a zig/zag pattern across the first quadrant on the agar plate without piercing the agar (Figure 1).

6. Sterilize the loop again and contact a small area of your bacteria streak within quadrant 1 and pull it across in a zig/zag pattern across the second quadrant (Figure 1).

7. Repeat this process until all quadrants have been streaked (Figure 1).

8. Repeat this process with a new agar plate until all bacterial hosts have been streaked. Make sure to sterilize the loop between each streak, between plating each bacteria host, and after completing all of your streak plates.

9. Once you have finished and all agar plates have their lids back on, ask for the instructor’s assistance to place the agar plates, with their agar sides facing the top and their lids facing down, in a 37°C incubator. You will be analyzing your plated bacteria during Laboratory #2.

II. Tropism and Isolation of a Pure Culture of Bacteriophage

A. Introduction

Not all bacteriophages replicate in all species of bacteria. A cell, tissue, or host limitation to viral replication is known as a viral **tropism** and are the reason why some viral diseases are restricted to certain species or groups of organisms.[2] Since bacterial cells have different properties (sugars, receptors, etc.) which do not allow the bacteriophage to bind, it is important to determine the acceptable bacterial host(s) for your bacteriophage prior to doing further experiments. Your bacteriophage is known as a coliphage, called such since its preferred host is the bacterial species *Escherichia coli* (*E. coli*).

The lifecycle of bacteriophages is quite complex, but can be separated into two major processes: the lytic cycle and the lysogenic cycle (Figure 2). During the **lytic cycle**, phages initially bind to their host cell and inject their genomic contents past the membrane and inside the cytoplasm. In some cases, the viral genome will have its gene transcribed by the host cell and translated into viral proteins, which promote the creation of new **virions**. The host cell eventually lyses with the newly created virions, releasing them to infect new host cells.

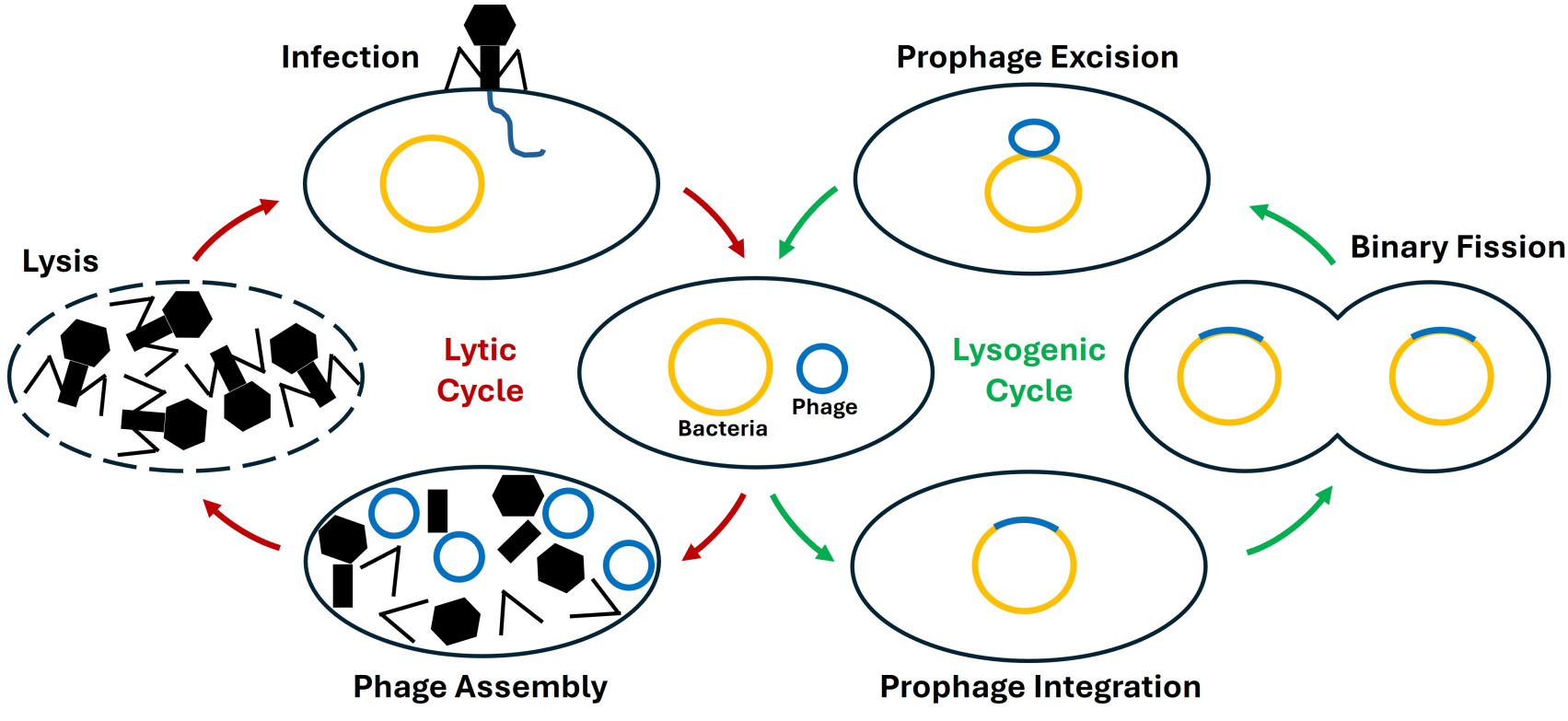


Figure 2. Replication cycle of bacteriophages. Bacteriophages utilize the lytic cycle to immediately create new virions, while the lysogenic cycle only replicates the phage genome each time the host divides without creating new virions. Phages can exit the lysogenic cycle and enter the lytic cycle under certain conditions. Image by Author.

However, some bacteriophages have evolved to induce only the lytic cycle in certain situations, and instead, undergo the lysogenic cycle. The **lysogenic cycle** occurs when certain genes of the phage genome are turned off and the phage

genome allows itself to be integrated into the host bacterial chromosome. An integrated phage genome is known as a **prophage**. There, every time the host cell divides, the phage genome is replicated. In the presence of certain stimuli, the phage genome can excise itself from the host genome and undergo the lytic cycle to produce more virions. Some phages have evolved to undergo only the lytic cycle and are therefore known as **lytic phages**. Others incorporate both cycles into their survival strategy and are known as **lysogenic phages**. Your bacteriophage is a lytic phage.

1. In situations where bacteria are resistant to antibiotics, bacteriophages can be used as an alternative therapy. Which type of phage would you expect to be most beneficial (lytic or lysogenic) for phage therapy and why?

2. Considering that bacterial cells contain exonucleases as a defense against phage DNA, why do you think many phage genomes circularize immediately after entering their bacterial host?

We will be using agar plates again to test your phage's tropism, but also to attempt to create a pure culture of your phage. To do this, you will perform the **spread plate method** for each of the bacterial hosts available, which will entail covering each plate using a liquid sample of each individual species of bacteria. You will then perform the streak plate method on this same plate, but using your bacteriophage sample. Phage that can utilize a certain bacterial host will form small clearances after lysing host cells known as **plaques**. Plaques are similar to colonies in that they originate from a single virus. Individual plaques can be isolated and used to produce a pure culture of your phage.

Your instructor will demonstrate the spread plate method for you.

NOTES:

B. Procedure II

1. On three individual tryptone agar plates, draw 2 lines using a marker on the bottom side (the side connected to the agar, not the lid). This should create 4 equal sections. Additionally, write your group number, the date, and name of the bacterium being added.

2. Using a P200 micropipette, add 100 µl of your bacterial sample to the middle of your agar plate. Eject the tip into a waste beaker.

3. Dip the angled end of a glass bacterial spreader into the beaker of ethanol and light it on fire by briefly touching it to the flame of the Bunsen burner. Wait until it cools (30 seconds) and then use it to spread the liquid bacterial culture equally over the entire plate, turning the plate as you go.

4. After the bacterial sample has dried on the plate, add 10 µl of phage sample into quadrant 1, and using an inoculation loop in the same manner as before (Figure 1), perform a streak plate method of the phage sample. Make sure to sterilize the inoculation loop when finished.

5. Repeat this process until each bacterial host plate has had the phage sample streaked out on it. Make sure to sterilize the loop between each streak and individual plates.

6. Ask for the instructor’s assistance to place the agar plates, with their agar sides facing the top and their lids facing down, in a 37°C incubator.

7. Make sure to clean up your area by placing all of your used micropipette tips into a biohazard bag and wiping down all surfaces, including glassware, with 70% ethanol. If there are any large spills of liquid bacterial cultures, notify your lab instructor so that they can use bleach on the area.

Laboratory #2: Plaque Isolation and Plaque Assay Setup

Objectives

✦Familiarize yourself with plaque morphology;

✦Learn how to pick a bacteriophage plaque to start a pure phage culture;

✦Learn how to set up a plaque assay.

Outline

- Experiment 1: Isolating bacteriophage plaques to grow phage as a pure culture.
- Experiment 2: Determining the number of bacteriophages in a sample.

I. Obtaining a Pure Culture of Bacteriophage

A. Introduction

During Laboratory #1, we began the process of isolating bacteriophage as a pure culture, which involved using the streak plate method on a bacteriophage sample across a lawn of the appropriate bacterial host. Although we used different *E. coli* hosts of the same species, genetic subtypes known as **variants** can arise that contain distinct mutations. If those distinct mutations lead to phenotypic differences, they are then referred to as **strains**. The same terminology is used for viruses. Afterwards, we expect to see individual viral clones known as plaques. Phages will only produce plaques if they are able to bind and complete the lytic cycle inside the host cell. If a bacterium allows the phage to bind, it is a **susceptible cell**. If a bacterium allows the phage to complete its replication cycle, it is a **permissive cell**. Therefore, for plaque generation, host cells must be both susceptible and permissive to the phage. Tropism can be very specific, meaning that you could see differences at the strain level of a single species of host for your phage. Answer the following questions to record your observations.

1. Which bacteriophage isolate did you use?

2. Which bacterial strains produced evidence of lysis from your bacteriophage isolate (if the answer is none, find a group that had lysis occur and use their phage to answer your remaining questions making sure to note which phage isolate you are referring to)?

3. Describe the plaque morphology: What is the diameter in mm (carefully use a metric ruler)? Does it have any additional characteristics other than being completely clear and circular (some plaques can appear cloudy)?

B. Procedure I

1. Wipe down your table space with 70% ethanol and a paper towel to create an aseptic environment.

2. Make sure all group members have a lab coat and gloves on.

3. Label a microcentrifuge tube with your phage isolate number, the date, and your lab group.

4. Ignite the Bunsen burner and adjust the flame so that there is a visible blue cone (ask instructor for assistance if needed).

5. Add 1 mL of "phage buffer" to your sterile microcentrifuge tube using a P1000 micropipette.

6. Using a sterile yellow tip and a P200 micropipette, close to the Bunsen burner, stab the top of the agar within the center of an isolated plaque. Do your best not to touch any other nearby plaques. Remember, we are trying to isolate a pure phage culture, which means we want it to originate from one plaque.

7. Next, use the same pipette tip to break up the agar plug into the phage buffer. This will help release phage from the agar plug into the buffer.

8. Close the microcentrifuge tube and briefly vortex.

9. Incubate the tube at room temperature for 30 minutes to allow the phage to diffuse into the buffer.

10. Remove the phage from the bacteria in the buffer by filtering through a 0.22 micron filter syringe. If your filter is not already attached to the syringe, remove the plunger, if present, and screw on the filter to the end of the syringe. Add the 1 mL of previously incubated phage buffer in Step 9 into the syringe with a P1000 micropipette and collect the phage in a new microcentrifuge tube by inserting and deploying the plunger of the syringe.

11. At this point the phage buffer should be free of host bacteria and agar, containing only the phage that was found in the plaque you picked. Store your purified phage sample at 4°C (ask instructor for assistance if needed).

II. Determining the Number of Bacteriophage in a Sample

A. Introduction

For lytic phages, and other viruses that lyse their host cell, you can quantify the number of functional virions by using a **plaque assay** (Figure 1).[3] The procedure requires the use of a double-layered plating technique in which hard agar serves as a base layer on the bottom, and a mixture of phage and host cells in soft agar is placed on top. The host cells can multiply on top of the hard agar, while the phage is able to infect the host cells and produce more uniform plaque morphology for counting within the soft agar. Since each plaque represents a single original phage virion, you can count the number of total phages within a specific dilution of your plated phage to determine the number of functional virions within your original virus sample. This will give you a virus count called a **titer** expressed in **plaque-forming units (PFU)**. In Laboratory #3, we will discuss the calculation of a titer and perform it on our own samples.

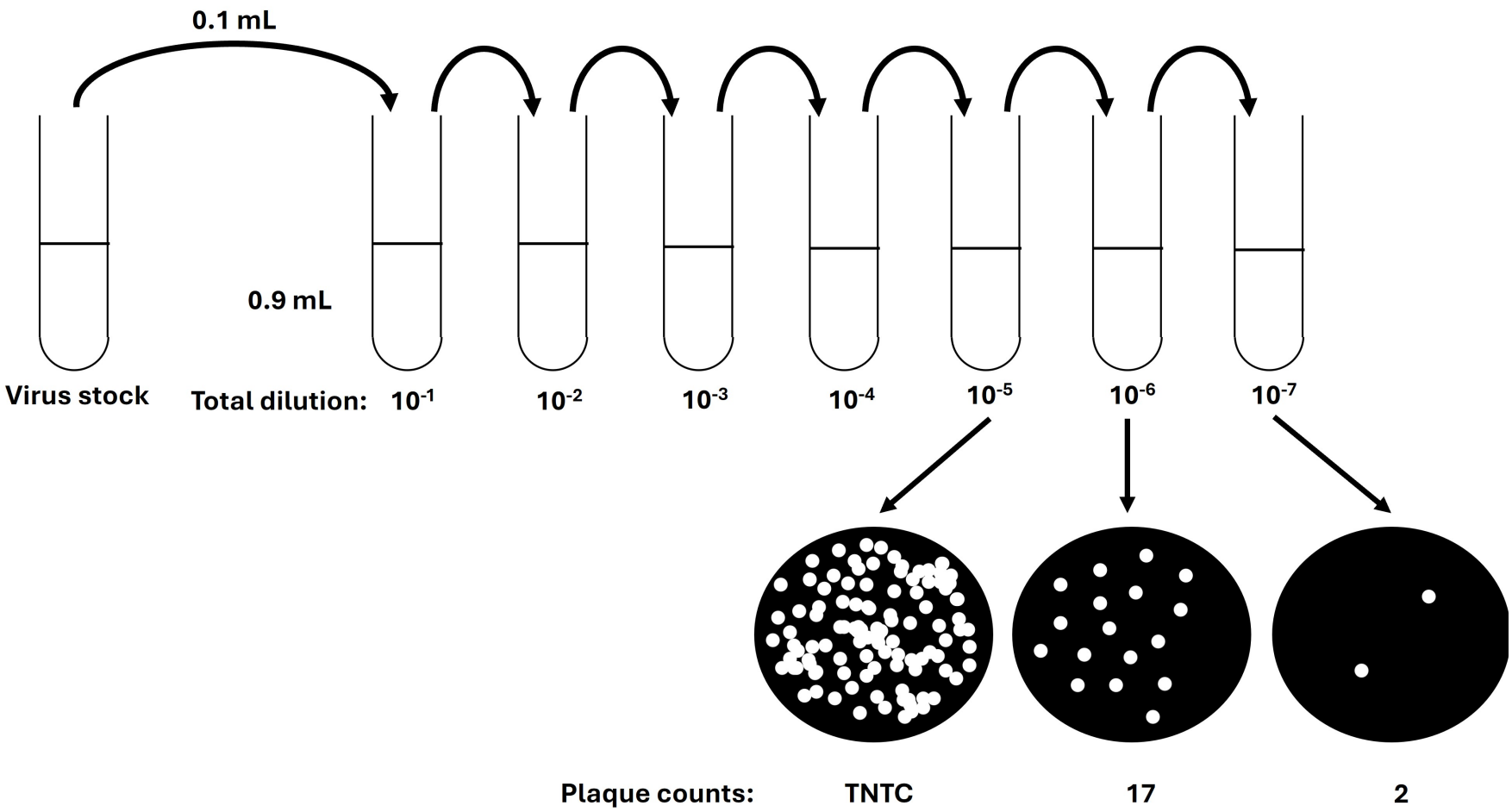


Figure 1. Plaque Assay. The plaque assay is used to count viruses that are lytic to their host cell. By plating serial dilutions of your virus sample onto host cells that support cell lysis, you can calculate how many plaque-forming units (PFU) per mL are present in your original sample (stock). Ideally you would pick a plate that has approximately 20-200 individual plaques. Plates that have indistinguishable plaques (touching each other) or plaques that are too numerous to count (TNTC) are not useful for quantitation. Image by Author.

1. Draw out your version of a flow diagram of Procedure II that we will be doing today (the instructor will draw it on board):

B. Procedure II

1. Label 9 microfuge tubes 10^{-1} to 10^{-9}.

2. Label 5 tryptone agar plates 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}.

3. With a P1000 micropipette, add 900 µL of tryptone broth to each microcentrifuge tube.

4. Perform a 10-fold serial dilution of your phage sample using your tryptone broth tubes.

5. Perform this step one tube at a time. To each 4 mL tryptone soft agar (0.75% (w/v)) tube (found in the 50°C water bath) add 200 µL of host bacteria and 100 µL of your diluted phage from the following microcentrifuge dilution tubes: 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}.

6. Rotate the tube in the palm of your hands rapidly and then pour the contents onto the appropriate agar plate.

7. Immediately swirl the soft agar contents so it coats the entire top of the plate and wait at least 20 minutes for the agar to solidify.

8. After the agar has solidified (check carefully), invert the plates and place in the 37°C incubator for 24 hours (ask instructor for assistance if needed).

Laboratory #3: Calculating PFU/mL and Bacteriophage Thermostability

Objectives

✦Learn how to quantify bacteriophages using a plaque assay;

✦Learn how to use a plaque assay within the context of an actual experiment.

Outline

- Experiment 1: Calculate the titer of your bacteriophage sample using plaque assay data.
- Experiment 2: Compare the thermostability between T2 and T4 bacteriophage.

I. Calculating Viral Titer Using Plaque Assay Data

A. Introduction

In Laboratory #2, we performed a plaque assay using our bacteriophage isolate with the intent of using that data to determine the number of viable phage in the sample, or viral titer). Answer the following questions in order calculate the titer of your phage, which will be expressed as plaque-forming units per mL (PFU/mL). Remember that each plaque that forms was originally a single virion.

1. Which bacteriophage isolate and bacterial strain did you use?

2. On which agar plates (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9}) is there evidence of lysis from your bacteriophage isolate (if the answer is none, find a group that had lysis occur and use their phage to answer your remaining questions making sure to note which phage isolate and bacterial strain you are referring to)?

3. Describe the plaque morphology: How large is it in mm (carefully use a metric ruler)? Does it have any additional characteristics other than being completely clear and circular? If you used the same isolate as the previous lab, was the plaque morphology relatively similar?

4. Find a plate(s) that has between 20-200 plaques. Counting fewer than 20 risks titer inaccuracy and counting more is too time consuming or allows for plaques to overlap each other. The plaques on the plate should be isolated and not run together. Make sure to write down the dilution of the plate (e.g. 10^{-5}) and the number of plaques counted. If you have more than one plate that was within the countable range, count both and record both. You can average the two calculations to obtain a more accurate viral titer. HINT: You can use a marker on the agar side of the plate to keep track of your counts. If there are a lot of plaques, consider dividing the plate up into quadrants to break down your counting.

5. In order to calculate the viral titer of your original sample you need to write down 3 pieces of information:

    - The number of plaques counted:
    - The dilution factor of the plate (HINT: the dilution factor is the inverse of the dilution):
    - The volume of phage dilution plated in mL:

6. Use the following equation to calculate the viral titer of your bacteriophage isolate in PFU/mL. This must be done for each countable plate. If you have more than one countable plate, you average all calculated titers to get your final titer. Record your viral titer below in PFU/mL.

$$\text{Viral titer (PFU/mL)} = \frac{(\text{number of plaques counted}) \times (\text{dilution factor})}{(\text{volume plated in mL})}$$

Viral titer (PFU/mL) =

II. Thermostability of T2 and T4 Bacteriophage

A. Introduction

While all of our bacteriophage isolates are expected to be lytic and use *E. coli* as a host organism, it is possible that they have differences in their stability in how they resist environmental pressures. This is important in the context of trying to eliminate phage in industrial settings where bacteria are used to manufacture food products or biopharmaceuticals. In other situations it can be used to understand how to store phage that might be part of an alternative therapy to cure bacterial infections in people. In this experiment, we will use our knowledge of the plaque assay to determine if two bacteriophages: T2 and T4, have different resistances to high temperature, called thermostabilities.[4]

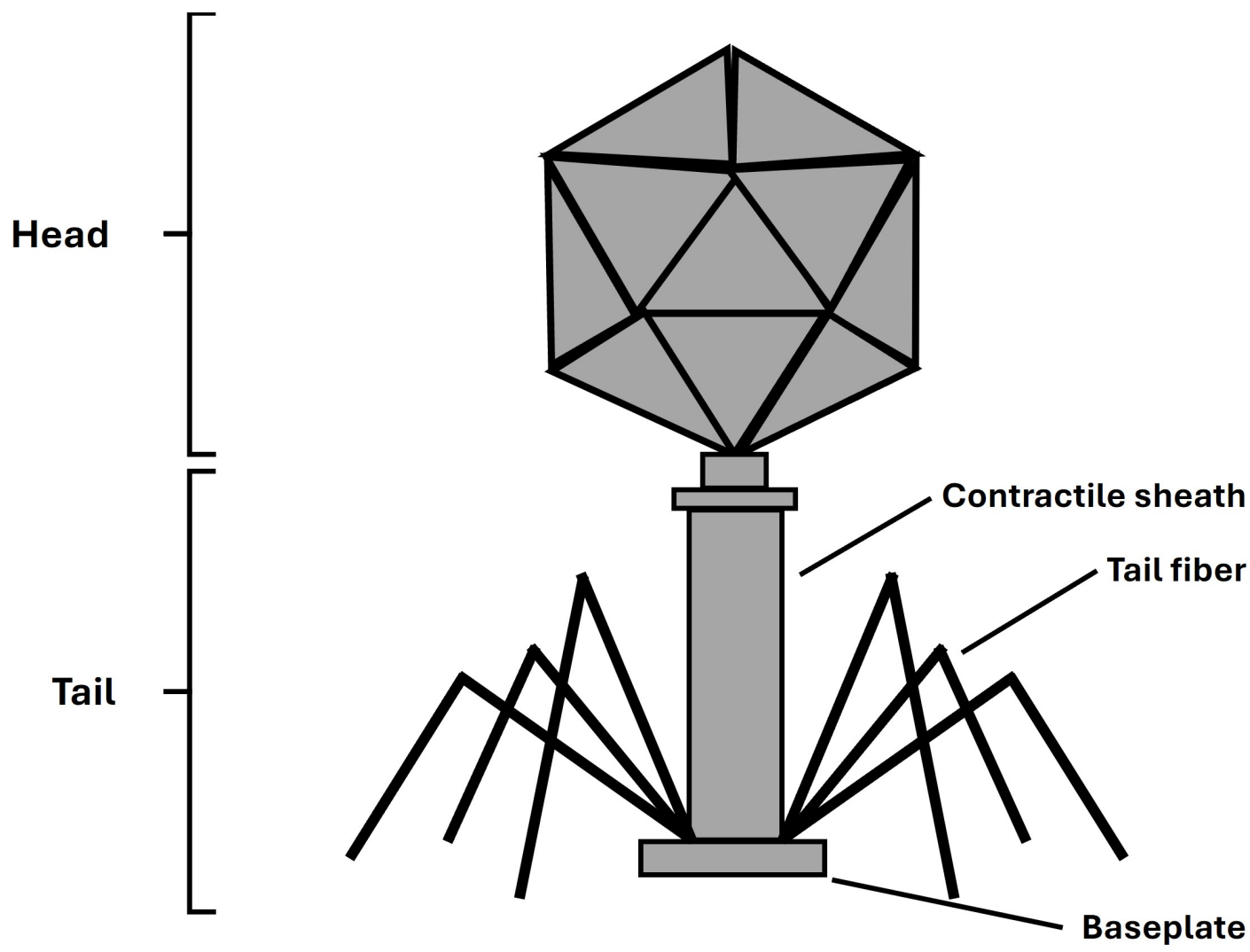


Figure 1. T-even phage structure. T-even phages are elongated in structure with a classical icosahedral head with a longer, helical symmetric tail. The tail contains substructures including the contractile sheath, base plate, and tail fibers. Image by Author.

Bacteriophages were originally numbered as they were discovered, with two general morphological phenotypes and were separated into **T-even** and **T-odd phages**. T2 and T4 are T-even phages, both belonging to viral family *Myoviridae*, which are non-enveloped viruses containing linear, double-stranded DNA (dsDNA) genomes. The T2 phage was first observed under electron microscopy (EM) in 1942 and was most famously used in the Hershey-Chase experiment to explain that DNA was the molecule of genetic inheritance in 1952.[5] The general structure of these viruses is a **head** with icosahedral symmetry that protects the viral genome and a complex tail that typically has helical symmetry with some additional structures. The **tail**, which is composed of the contractile sheath, base plate, and tail fibers, facilitate host-cell binding and delivery of the genome into the host cell. T-even phage tails contract after binding and their gnome is injected into the host cell. T-odd phage tails are non-contractile and delivery of the phage genome occurs through a series of internal conformational changes within the tail. Upon delivery of the genome to the host cell, the virus completes the lytic cycle to produce more phage virions.

B. Procedure II

1. Using a P200 micropipette, add 100 µL of T2 and T4 phage samples into two separate microcentrifuge tubes. Add cap locks to each tube.

2. In consultation with the instructor, choose one temperature to incubate your T2 and T4 phage samples at (20, 40, 60, 80, or 100°C). Each temperature will need to be accounted for at least once. Incubate the tubes for 1 hour and let cool to room temperature for at least 15 minutes.

3. Each group will be responsible for performing a plaque assay for both phages at one specific temperature. Label two sets of 9 microcentrifuge tubes 10^{-1} to 10^{-9} (one set for the T2 phage sample and one for the T4 phage sample).

4. Label two sets of 9 agar plates 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} (one for T2 and one for T4).

5. With a P1000 add 900 µL of tryptone broth to each microcentrifuge tube.

6. Perform a 10-fold serial dilution of your T2 and T4 phage sample (after temperature incubation) using your tryptone broth tubes. Make sure each tube has at least 100 µL of your phage dilution remaining.

7. Perform this step one tube at a time. To each tryptone soft agar tube (found in the 50°C water bath) add 200 µL of host bacteria and 100 µL of your diluted phage from the following microcentrifuge dilution tubes: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}.

8. Rotate the tube in the palm of your hands rapidly and then pour the contents onto the appropriate agar plate (agar plates can be found in the 37°C incubator).

9. Immediately swirl the soft agar contents so it coats the entire top of the plate and wait at least 20 minutes for the agar to solidify. Repeat for all dilutions and all phage samples.

10. After the agar has solidified, invert the plates and place in the 37°C incubator for 24 hours.

Chapter 2: Deformed Wing Virus Investigation

Laboratory #4: Extraction and Assessment of RNA From a Virus Sample

Objectives

- ✦Familiarize yourself with the materials and procedures used to safely work with RNA samples;
- ✦Learn how to extract and assess RNA samples from tissues (and viruses).

Outline

- Experiment 1: Extraction of RNA from *Apis mellifera* (European honey bee).
- Experiment 2: Concentration and purity of extracted RNA.

I. Extraction of RNA From *Apis Mellifera*

A. Introduction

While our first introduction into viruses, using bacteriophages, easily allowed us to see the manifestation of the virus in its host via the plaque assay, other viruses are not so obvious to detect in the lab. The second virus we will be working with is **deformed wing virus (DWV)**, a positive sense single stranded (+ssRNA), non-enveloped icosahedral virus belonging to the *Iflaviridae* family. DWV is one of 22 identified viruses that are able to infect *Apis mellifera*, the European honey bee, which is the bee of choice used to agriculturally generate honey and perform large-scale pollination of crops.[6] The virus can spread horizontally between adult bees and vertically from queen bee to egg, as well as through **vectors**, specifically, the small mites of the *Varroa* genus (Figure 1).[6-7] The mites enter the brood cell, which is the location where the honey bees develop. There they feed on the bee's fat tissue and undergo their own reproductive cycle to parasitize other bees.

One method to identify the presence of viruses is the detection of the viral genome. Since DWV has an RNA genome, we will extract RNA from locally collected honey bees to determine if they are infected with DWV. This is accomplished by mechanically breaking open honey bee tissue and cells using a bead homogenizer and extracting total RNA using the RNeasy Mini Kit (Qiagen). The RNA extraction kit is designed to separate all cellular components away from RNA so that you end up having a pure RNA sample.

Since RNA is easily degraded by enzymes known as **RNases** in the environment, our collected honey bees have been stored at -70°C and after RNA extraction, we must store the purified RNA at -70°C, too. When working with RNA, you will need to exercise extreme caution so your sample does not become contaminated or degraded. Your bench, micropipettes, and racks should be vigorously cleaned with 70% ethanol. You should then re-clean all items with a spray designed to eliminate RNases such as RNase-Away. An additional safeguard is to only use aerosol-barrier pipette tips, which are pipette tips with a small filter inside that prevent contaminants from migrating from the barrel of the micropipette into the sample area of the pipette tip. Additionally, all tubes have been autoclaved to ensure they are RNase-free. Unless you are performing a step in a protocol that prevents it, always keep RNA samples on ice.

Figure 1. Image showing honey bee with *Varroa* mite on its thorax. Photo by Scott Bauer courtesy of USDA Agricultural Research Service.

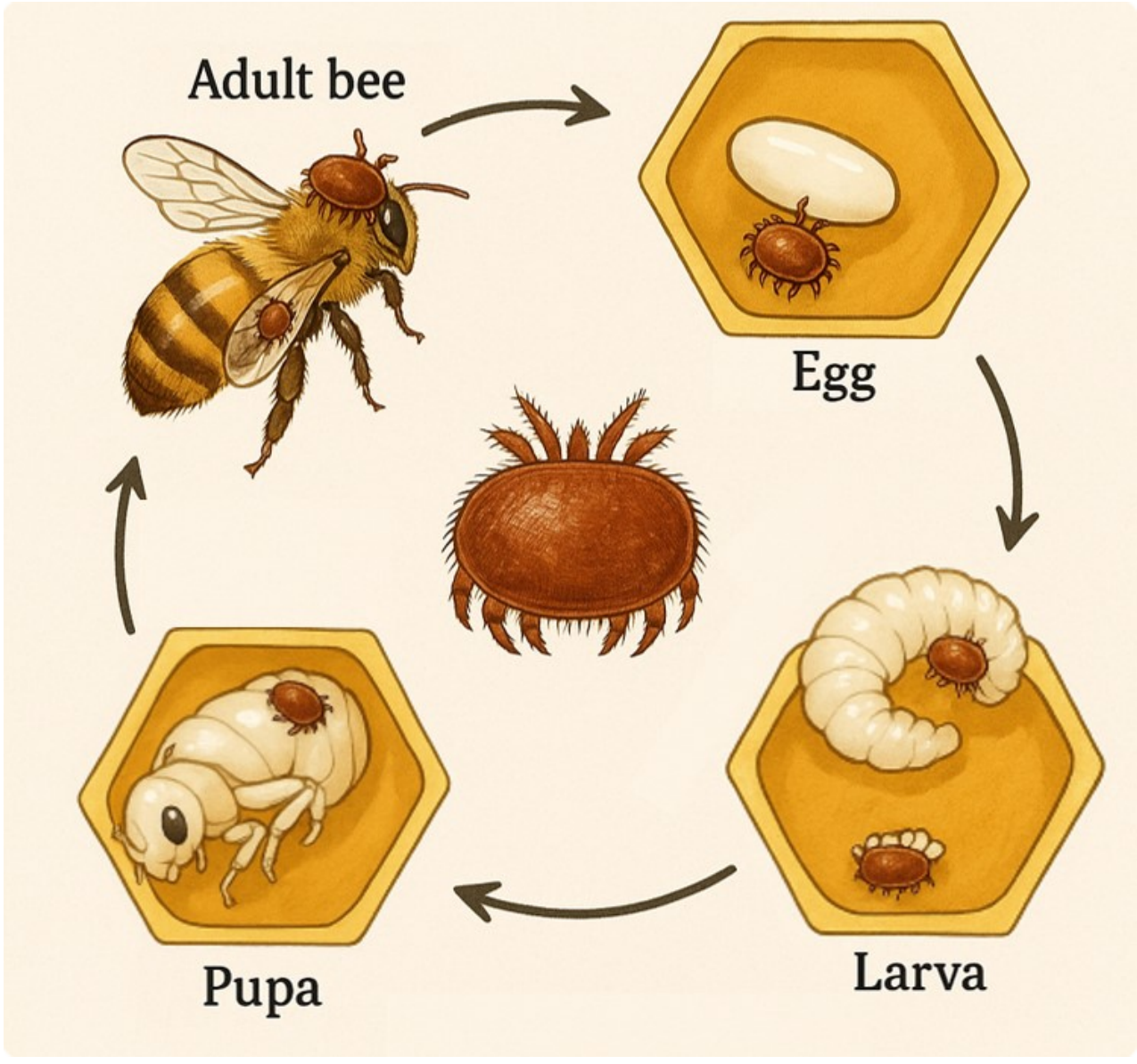


Figure 2. The life cycle of the honey bee with *Varroa* mites. Mites feed on the honey bee as it develops from egg, larva, pupa, and adult. Image by Author.

B. Procedure I

1. Wipe down your table space, micropipettes, forceps, and tube racks with 70% ethanol and then again with RNase decontamination solution.

2. Make sure all group members have gloves on. In this lab be very aware to not touch extraneous items.

3. You will be given two tubes, each with one collected honey bee. If there is any information written on the tube, please write that down in your notebook.

4. Carefully move each honey bee into a separate bead homogenizer tube containing 5-6 triple-washed zirconium beads. Some bees will have pollen attached to their legs, that is ok. Wipe down your forceps with 70% ethanol between honey bee samples.

5. Homogenize your honey bee samples using the bead homogenizer. The instructor will guide you through that process.

6. After homogenization of your honey bee samples, use your P1000 micropipette to add 600 µl of RLT buffer (lysis buffer) to each sample and vortex for 15 seconds.

7. Centrifuge samples in a microcentrifuge at max speed for 3 minutes.

8. Carefully transfer the supernatant of each sample into a clean microcentrifuge tube (make sure to estimate the volume of supernatant added to each tube using your micropipette).

9. Use your P1000 micropipette to add 1 volume of 70% ethanol to your samples (e.g. add 450 µl of 70% ethanol into 450 µl of sample).

10. Place the spin column into the collection tube and then use your P1000 micropipette to transfer up to 700 µl of samples into the spin column. Spin samples in a microcentrifuge at max speed for 15 seconds. If you have more than 700 µl of volume for a sample, you will have to repeat this process by first emptying the collection tube and adding more volume into the spin column, centrifuging the samples again.

11. Discard liquid in the collection tube and use a P1000 micropipette to add 700 µl of RW1 buffer to the spin column and centrifuge as before.

12. Discard liquid in collection tube and use a P1000 micropipette to add 500 µl RPE buffer to the spin column and centrifuge as before.

13. Discard liquid in collection tube and use a P1000 micropipette to add 500 µl RPE buffer to the spin column and centrifuge at max speed, but for 2 minutes.

14. Move spin column to a new microcentrifuge tube. Be careful not to get RPE buffer onto the spin column, as the column should be as dry as possible.

15. Using a P200 micropipette, add 50 µl of RNase-free water directly to the filter within the spin column. Let the water absorb to filter for 1 minute then centrifuge at max speed for 1 minute.

16. Neatly label your tubes with your group number, sample number, and date. Place your tube on ice.

II. Determining RNA Concentration and Purity

A. Introduction

Spectrophotometers are instruments that pass varying wavelengths of light through a solution and determine the absorbance of that sample at each of those wavelengths, creating an absorbance spectrum. Using the Beer-Lambert law, the concentration of the sample can be determined. Many spectrophotometers require large volumes of sample to make accurate measurements; however, recent technological advances have created **micro-volume spectrophotometers** that allow quantification of nucleic acids in a 1-2 µL sample.

Nucleic acids have a peak absorbance at ~260 nm (A260), while proteins have a peak absorbance at ~280 nm (A280). By determining the A260/A280 ratio of a sample, the relative purity can be assessed. An A260/A280 ratio of ~1.8 is generally accepted as "pure" DNA, while "pure" RNA is ~2.0. Additionally, many contaminants during nucleic acid isolation (e.g. TRIzol, phenol, guanidinium HCl, etc.) have peak absorbance close to A230. By determining the A260/A230 ratio of a sample, the extent of chemical contaminants can be assessed. An A260/A230 ratio of "pure" DNA or RNA is expected to be in the range of 2.0-2.2.

B. Procedure II

1. Turn on the micro-volume spectrophotometer, allowing it to warm up (ask instructor when it is ready to use) and select “RNA” as the sample.

2. Clean the pedestal surface with a dry wipe.

3. Use RNase-free water as a blank and pipet 2 µl of it onto the pedestal and hit the button to blank the sample.

4. You only blank once, therefore for each subsequent sample you must wipe off the blank with a dry wipe and then pipet 2 µl of your sample onto the pedestal and hit measure.

5. Repeat this process until all lab groups have recorded their RNA sample concentration and purity in both samples. Use Table 1. and Table 2. to record your results.

Table 1. Spectrophotometer analysis of your first bee’s RNA.

Sample:	Measurement
Concentration (ng/µL)	
A260/A280	
A260/A230	

Table 2. Spectrophotometer analysis of your second bee’s RNA.

Sample:	Measurement
Concentration (ng/µL)	
A260/A280	
A260/A230	

6. Place your samples within the tube rack that your instructor will place in the -70°C ultra freezer.

Laboratory #5: cDNA Synthesis of RNA

Objectives

✦Familiarize yourself with the materials and procedures used to synthesize cDNA from RNA

Outline

• Experiment 1: cDNA synthesis of extracted RNA from honey bees.

I. cDNA Synthesis of an RNA Sample

A. Introduction

The synthesis of DNA from an RNA template is mediated by a process called **reverse transcription**, which produces complementary or copy DNA (cDNA).[8] **Reverse transcriptases (RTs)** are the enzymes that facilitate reverse transcription and use an RNA template and a short DNA primer complementary to the 3' end of the RNA to direct the synthesis of the first strand cDNA in a 5’ to 3’ direction, which can be used directly as a template for the **Polymerase Chain Reaction (PCR)**. Primers for reverse transcription include oligo-dT, random hexamer, or gene-specific (Figure 1). **Gene-specific primers** are usually a luxury item and are not typically used, but they specifically bind to an RNA target so that cDNA synthesis only occurs for those specific RNA molecules with bound primers. **Oligo-dT primers** consist of a long chain of thymine bases that are complementary to the poly(A) tails found on messenger RNA (mRNA). Due to this cDNA synthesis of RNA with poly(A) tails is enhanced, while other RNA targets such as ribosomal RNA (rRNA), is prevented. In our case, we are interested in cDNA synthesis of the RNA genome of DWV and honey bee mRNA; therefore we will be using **random hexamers**, which are 6-base sequences that can randomly bind to all RNA molecules regardless of their sequence or presence of a poly(A) tail. In most workflows, RNA samples are first treated with **DNase I**, an endonuclease which eliminates **genomic DNA (gDNA)** prior to the cDNA synthesis reaction. This ensures that all DNA at the end of cDNA synthesis is derived from the RNA sample and not gDNA contamination.

Many RTs are available from commercial suppliers. Avian Myeloblastosis Virus (AMV) Reverse Transcriptase and Moloney Murine Leukemia Virus (M-MuLV, MMLV) Reverse Transcriptase are RTs that are commonly used in molecular biology protocols. The cDNA synthesis kit we will be using is ProtoScript II

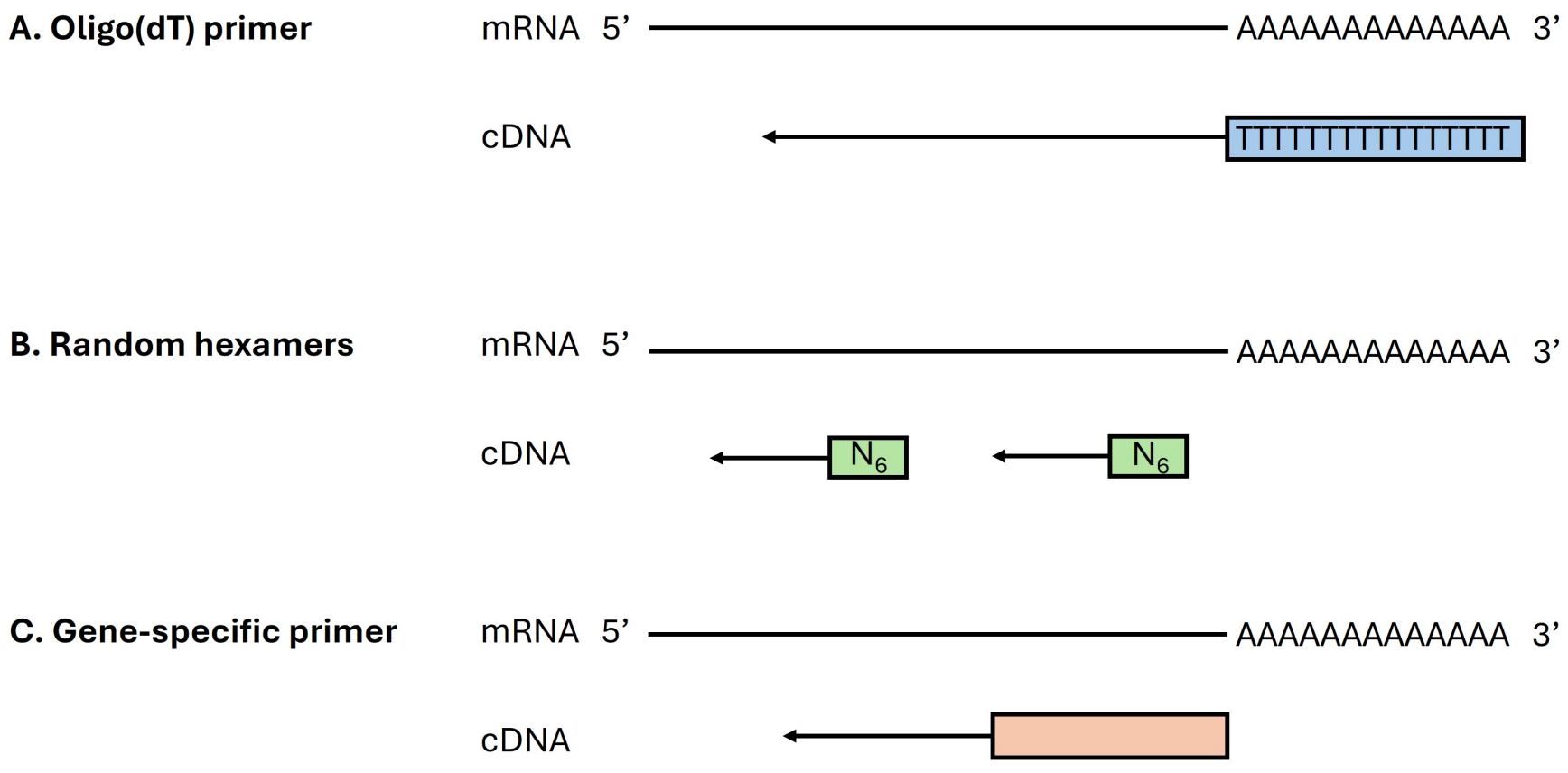


Figure 1. A variety of short DNA primers can be used to initiate cDNA synthesis of RNA. All work with reverse transcriptase: A. Oligo-dT B. Random hexamer C. gene-specific. Image by Author.

Reverse Transcriptase (New England BioLabs) which contains a recombinant M-MuLV reverse transcriptase with reduced RNase H activity, the activity that degrades RNA complexed with other nucleic acids, and increased thermostability. It can be used to synthesize first strand cDNA at higher temperatures than the wild-type MMuLV. The enzyme is active up to 50°C, providing higher specificity, higher yield of cDNA and more full-length cDNA product, up to 12 kb in length.

The use of engineered RTs improves the efficiency of full-length product formation, ensuring the copying of the 5' end of the mRNA transcript is complete, and enabling the propagation and characterization of a faithful DNA copy of an RNA sequence. The use of the more thermostable RTs, where reactions are performed at higher temperatures, can be very helpful when dealing with RNA that contains high amounts of secondary structure.

1. What are the advantages and disadvantages of the three different primers associated with cDNA synthesis of RNA?

2. What enzyme is used to eliminate gDNA within RNA samples destined for cDNA synthesis and why would gDNA present a problem in downstream applications like PCR?

3. Convert the RNA sequence listed below to the sequence that would be created after the cDNA synthesis reaction. Also draw an arrow in the direction that the cDNA synthesis would occur.

    - 5’ AUGUGCAGUUACGUAUAGUCUAGUCUAAAAAAA 3’

B. Procedure I

1. Wipe down your table space, micropipettes, forceps, and tube racks with 70% ethanol and then again with RNase decontamination solution.

2. Prepare RNA for cDNA synthesis first by removing honey bee gDNA via DNase I treatment. First, review the concentration and purity of your RNA samples from Laboratory #4 and consult with the instructor on whether your sample is acceptable for cDNA synthesis. Ideally, you want to treat up to 10 µg of RNA with DNase I prior to cDNA synthesis. Add all reagents to a microcentrifuge tube using a P20 and P200 micropipette (Table 1).

- Unless your RNA concentration was above 200 ng/µL, plan on adding your entire sample (48 µL) to the reaction. Remember, RNA samples from different bees go into separate reactions.

Table 1. DNase I Treatment

Component	Volume
RNA (max of 10 µg)	__ µL
DNase I Reaction Buffer (10X)	10 µL
DNase I Enzyme (1x)	1 µL
Nuclease-free water	to 100 µL
TOTAL	**100 µL**

3. Incubate at 37°C for 10 minutes.

4. Add 1 µL of 0.5 M EDTA with a P20 micropipette and mix (to a final concentration of 5 mM).

5. Add cap locks to your tubes and heat inactivate DNase I by incubating at 75°C for 10 minutes.

6. Prepare your RNA for the cDNA synthesis reaction by adding the following components in a new tube using a P20 micropipette (Table 2).

Table 2. cDNA Synthesis Part I

Component	Volume
DNase I-treated RNA (max of 1 µg)	6 µL
Random Primer Mix (60 µM)	2 µL

7. Incubate for 5 minutes at 65°C to reduce RNA secondary structure. Spin down briefly in a microcentrifuge and place immediately on ice.

8. Add the following components to the tube using a P20 micropipette to complete the 20 µL reaction (Table 3).

Table 3. cDNA Synthesis Part II

Component	Volume
ProtoScript II Reaction Mix (2X)	10 µL
ProtoScript II Enzyme Mix (10X)	2 µL

9. Incubate at 25°C for 5 minutes.

10. Incubate at 42°C for 1 hour.

11. Add cap locks to your tubes (if you removed them) and heat inactivate RT by incubating at 80°C for 5 minutes.

12. The cDNA should be stored at -20°C in a labeled 1.5 mL tube.

Laboratory #6: RT-PCR

Objectives

✦ Familiarize yourself with the materials and procedures used in PCR to copy DNA.

Outline

- Experiment 1: RT-PCR of viral and non-viral cDNA targets in honey bees.

I. RT-PCR of a cDNA Sample

A. Introduction

Polymerase chain reaction (PCR) is a method used to amplify DNA, typically from samples where the target DNA is of low abundance. When the DNA target is a copy of an RNA target (cDNA), such as what we created during Laboratory #5, the method is known as **reverse-transcriptase PCR (RT-PCR)**, due to the required cDNA synthesis step that uses the RT enzyme to synthesize the cDNA.

PCR relies on a key enzyme called DNA polymerase that polymerizes DNA from a DNA template. The **DNA polymerase** used in PCR must also be heat-stable since some of the PCR steps will reach 95°C. This is achieved by using a DNA polymerase from a bacterium called *Thermus aquaticus*, which lives in very hot environments. The enzyme is usually referred to as *Taq* polymerase when listed as a PCR reagent. However, DNA polymerase cannot synthesize new DNA without short, single-strand segments of DNA that are complementary to the DNA target known as **primers**. The primers we will use to detect the DWV target cDNA are listed in Table 2. They will bind to the target DNA when it becomes single-stranded and serve as the site of DNA synthesis. Each DNA target requires a pair of primers, which work together to amplify the region of target DNA between them, which is known as the **amplicon**.

Other components within the PCR reaction include free nucleotides, buffer, and input DNA. **Free nucleotides** are the monomers that DNA polymerase will use to build new copies of the target DNA molecule. Just like all enzymes, DNA polymerase has an optimum pH and requires the assistance of cofactors such as Mg^{2+}. The **buffer** solution keeps the pH of the reaction constant and provides necessary enzyme cofactors. **Input DNA** is simply the DNA found within the

sample added to the PCR reaction, which may or may not contain the target DNA.

PCR reactions are placed in a machine called a **thermocycler** that changes temperature conditions during the PCR reaction. Once the reaction is assembled and placed inside a thermocycler, a PCR program instructs the thermocycler to change temperatures, which is necessary to carrying out the three major steps of the PCR reaction: 1. Denaturation 2. Annealing and 3. Extension (Figure 1). During **denaturation**, the reaction is heated almost to boiling, which breaks the hydrogen bonds connecting the nitrogenous bases, making a double stranded DNA molecule into two single-stranded molecules. Afterwards, the reaction is cooled to allow for **annealing**, where new hydrogen bonds form between the complementary primers and single-stranded target DNA molecules. The last step,

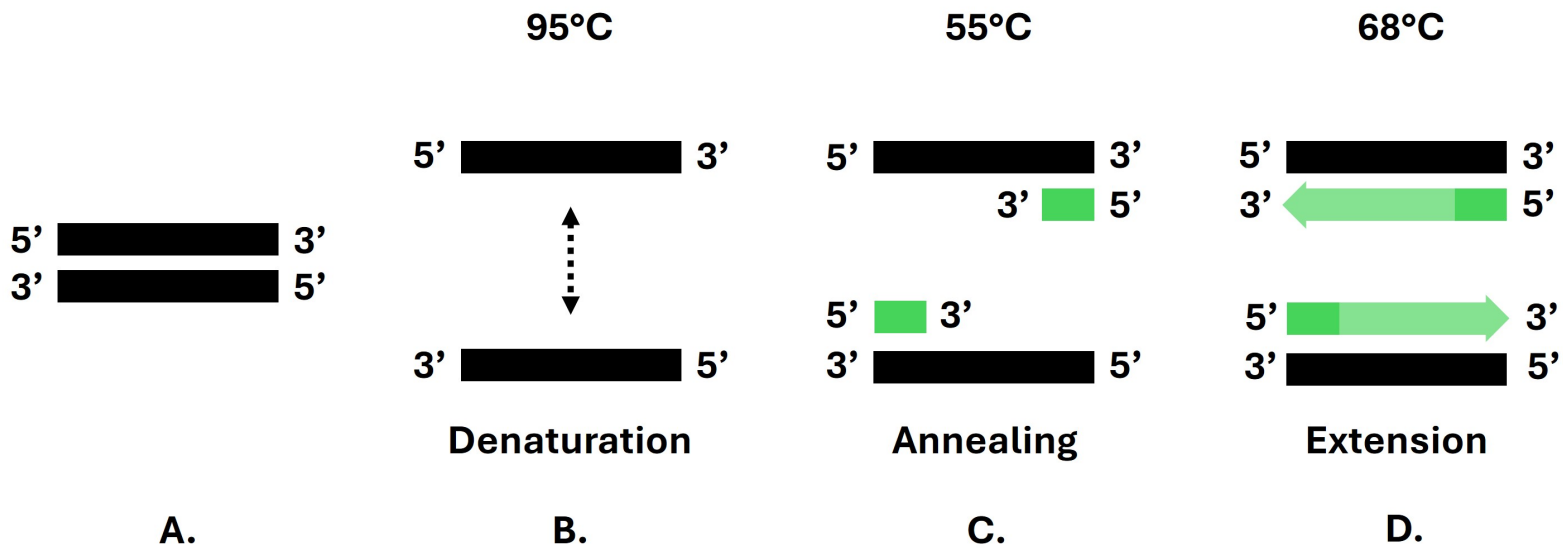


Figure 1. The major steps of a PCR reaction. A. The input DNA (black) prior to any temperature changes by the thermocycler. B. In denaturation, the thermocycler heats the PCR reaction to high temperatures, disrupting the hydrogen bonds between the two DNA strands, creating single stranded DNA strands. C. In Annealing, the thermocycler reduces the temperature of the PCR reaction, allowing the pair of PCR primers (dark green) to bind to the complementary regions of the target DNA. D. In Extension, the thermocycler increases the temperature so that DNA polymerase becomes active and adds free nucleotides (light green), extending the DNA from each PCR primer, producing the amplicon. Image by Author.

extension, occurs at a slightly higher temperature than the annealing step, where the DNA polymerase is active, but the primers remain bound to the target DNA. In this step, DNA polymerase builds a new copy of DNA off of the bound primer in a 5’ to 3’ direction, using the target DNA as a template.

1. One of the controls we will utilize in our PCR reaction will be a tube where no input DNA is added, known as the **no template control (NTC)**. What do you think this controls for?

2. We will also be using a second control, where we will be using primers against an abundant honey bee (*Apis*) cDNA target called beta-actin (Table 2). What do you think this controls for?

B. Procedure I

1. Thaw your cDNA samples, mix, and spin them down briefly in the microcentrifuge.

2. For each cDNA sample you will set up three 25 µL reactions using a P20 micropipette, each in a separate PCR tube. The reagent and volumes in each tube are provided below (Tables 1, 3-4). This can be done at room temperature, because we are using a "hot start" DNA polymerase, which will only become active once it has experienced temperatures at or above 45°C due to a bound inhibitor that is released upon heating.

Table 1. No Template Control (NTC)

Component	Volume
5 µM Forward Primer (DWV)	1 µL
5 µM Reverse Primer (DWV)	1 µL
Hot Start 2X Master Mix	12.5 µL
Nuclease-free water	10.5 µL
TOTAL	**25 µL**

Table 2. Primer Sequences

Name	Sequence
Forward DWV	5'-ATCAGCGCTTAGTGGAGGAA-3'
Reverse DWV	5'-TCGACAATTTTCGGACATCA-3'
Forward *Apis beta-actin*	5'-AGGAATGGAAGCTTGCGGTA-3'
Reverse *Apis beta-actin*	5'-AATTTTCATGGTGGATGGTGC-3'

Table 3. Deformed Wing Virus (DWV) Experimental

Component	Volume
5 µM Forward Primer (DWV)	1 µL
5 µM Reverse Primer (DWV)	1 µL
cDNA sample	1 µL
Hot Start 2X Master Mix	12.5 µL
Nuclease-free water	9.5 µL
TOTAL	**25 µL**

Table 4. Apis beta-actin RNA Control

Component	Volume
5 µM Forward Primer (*beta-actin*)	1 µL
5 µM Reverse Primer (*beta-actin*)	1 µL
cDNA sample	1 µL
Hot Start 2X Master Mix	12.5 µL
Nuclease-free water	9.5 µL
TOTAL	**25 µL**

3. Mix and spin down each tube briefly in the microcentrifuge.

4. With assistance from your instructor, use the thermocycler to set up the following conditions:

Table 5. PCR Program

Step	Details
Initial denaturation	1 min @ 95 °C
Denature	30 sec @ 95 °C
Anneal	1 min @ 55 °C
Extension	1.5 min @ 68 °C
Final Extension	5 min @ 68 °C
Hold	∞ @ 12 °C

Note: The Denature through Extension steps are repeated for an additional 39 cycles.

5. Your instructor will remove the tubes from the thermocycler after the cycle has completed and place them at -20°C.

Laboratory #7: Agarose Gel Electrophoresis

Objectives

✦Familiarize yourself with the materials and procedures used in agarose gel electrophoresis of DNA products.

Outline

- Experiment 1: Agarose gel electrophoresis and downstream analysis of PCR products.

I. Agarose Gel Electrophoresis of PCR Products

A. Introduction

In the previous lab we performed a PCR on our cDNA samples. Although each sample had its own cDNA, two separate reactions were carried out using two different sets of primers. One pair was designed against the genome of DWV and is expected to yield an amplicon of 702 **base pairs (bp)**, while the other pair was designed against an mRNA of honey bee beta-actin and is expected to yield an amplicon of 181 bp. Our next step is to determine if our honey bee samples are positive for DWV.

One method that will be useful to answer this question, is agarose gel electrophoresis, a rapid, convenient way to analyze DNA preparations that contain fragments between approximately 100 and 10,000 bp in length. It is particularly useful for estimating purity and size of plasmid DNA preparations, the size of PCR reaction product(s), and the size(s) of DNA fragments generated from restriction digests.

Agarose gel electrophoresis separates mixtures of molecules based on size after the sample is added to a depression within the aqueous agarose gel matrix, called a well, and then generating an electrical current through the gel (Figure 1). Molecules in the sample will be attracted to either the positive or negative pole of the electric field depending on their charge. When the sample is composed of DNA molecules, the negatively-charged DNA will migrate toward the positive pole. Small DNA molecules migrate rapidly through the agarose gel matrix, whereas larger DNA molecules are hindered by the sieve-like nature of the agarose. Thus, DNA molecules of different sizes migrate at rates that are inversely-proportional to their size. The sizes of different DNA fragments in the

mixture can be determined by comparison to a sample containing DNA molecules of known sizes, referred to as a **DNA ladder**.

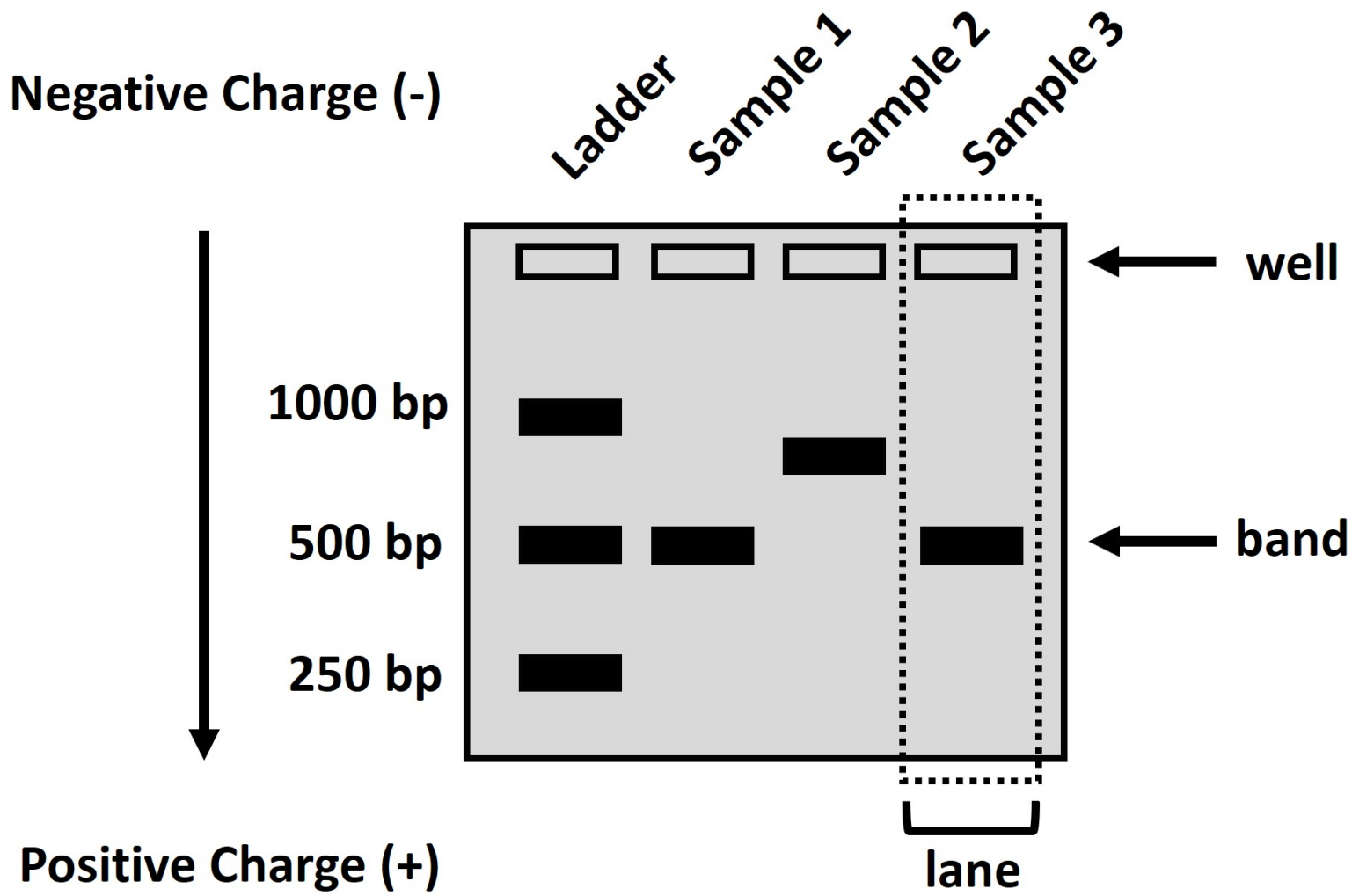


Figure 1. Agarose gel electrophoresis of DNA. DNA samples are loaded in the wells of the agarose gel and separated by size after applying electric current. Negative charge is applied near the well, repelling the negatively charged DNA samples through the gel toward the positively charged end. Larger fragments move slower through the gel matrix, while smaller fragments move faster. A DNA ladder, containing fragments of known size is used as a comparison to determine the sizes of unknown fragment lengths in other DNA samples. Image by Author.

Agarose gels are typically run with either a **Tris-acetate-EDTA (TAE)** or **Tris-borate-EDTA (TBE) buffer**. The borate in TBE buffer is a potent inhibitor of many enzymes, which can be beneficial for preserving the integrity of nucleic acids during electrophoresis. However, if DNA fragments are excised from a TBE gel, they are much more difficult to purify and carry-over as the borate can inhibit downstream cloning enzyme steps. Thus, gel extraction of nucleic acids is carried out almost exclusively using TAE gels. When resolving smaller DNA fragments, TBE buffer is often used, as it tends to resolve bands better than TAE and has better buffering capacity. For separation of larger DNA fragments, TAE typically gives better resolution.

The percentage of agarose can also be varied to more accurately resolve DNA fragments. Agarose gels are typically 0.5% to 2% agarose. Lower percentage

gels are used to resolve larger DNA fragments, while higher percentage gels are used to resolve smaller fragments.

The DNA molecules are normally not visible in the agarose matrix; thus, a fluorescent dye is typically added to the gel to specifically stain the nucleic acids and allow you to visualize the DNA bands. Additionally, the intensity of staining is proportional to the mass of DNA in each band. Two commonly used DNA stains are **ethidium bromide (EtBr)** and **SYBR green I**, which bind to DNA. While EtBr is routinely used in academic labs as a DNA stain, it is a mutagen, and any compound that binds to DNA and causes mutations should be treated with caution.

1. Draw a diagram of an agarose gel with an appropriately sized DNA ladder illustrating the bands you would expect to find in a cDNA sample that is DWV-positive and the bands you would expect to find in a cDNA sample that is DWV-negative. Make sure to use two lanes for each sample, one to show the bands for the DWV-specific primer set and a second to show the bands for the beta-actin-specific primer set.

2. We also created a no template control (NTC) reaction using the DWV primer set in the previous lab. Would we expect to find bands in this sample assuming our experiment worked as expected? Why?

3. Although we didn’t use this control, some experimenters choose to set up a reaction with a DNA template, but without primers. Would we expect to find bands in this sample? Why?

B. Procedure I

1. Using gloves, prepare the gel tray by sealing the Plexiglas gel tray with rubber gaskets at both ends. Repeat this procedure for how many gels you need to create (check with instructor). Ideally, we want to run DWV and *beta-actin* PCR samples on different gels. Also, make sure to include space for a DNA ladder on each gel.

2. Select a comb or combs with the desired number of wells and insert them into the appropriate slot(s) on the gel tray (check with instructor).

3. Make ~40 ml of a 1.0 % (w/v) solution of agarose in 1X TBE buffer in a 250 ml Erlenmeyer flask for each gel (check calculations with instructor). Place a dampened paper towel on top of the flask.

4. Heat the mixture just to boiling using the microwave. Examine the flask and swirl to mix the contents (do this slowly & carefully -- see note below); continue microwaving and boiling, with occasional swirling, until all the agarose is dissolved. BE CAREFUL HANDLING MICROWAVED AGAROSE -- IT IS EASY TO BURN YOURSELF. Microwaved agarose can become superheated and boil over once it is removed from the microwave and mixed. Wear protective hand mitts and swirl the flask slowly to mix.

5. Add ethidium bromide (to 1X concentration) to the slightly cooled gel. Ethidium bromide-contaminated tips should go into their own waste container. Carefully pour the melted agarose solution into the gel tray until it reaches the top of the tray and allow the gel to solidify for at least 20 minutes. While the gel is solidifying, we will prepare our samples and make a plan on how to arrange them within the gel.

6. Add 5 µL of 6x loading dye to each of your PCR samples. Vortex to mix and briefly spin down the tubes.

7. When your gel is solidified, first remove the comb by wetting the top of the gel with TBE buffer (use a P1000 micropipette) and then pull the comb up gently. Carefully remove the gel tray from the gel box, taking care not to let the gel slide out of the tray.

8. Place the gel(s) in the electrophoresis chamber(s) so that the wells will be closest to the negative electrode (black). Fill the buffer chambers with 1X TBE buffer until the top of the gel is just covered.

9. Add 5 µL of ethidium bromide to the well of buffer closest to the positive electrode (red). EtBr-contaminated tips should go in their own waste container. Note: Ethidium bromide has a positive charge and therefore runs opposite that of DNA on the gel. Adding a little ethidium bromide to the running buffer near the positive electrode helps prevent "washing out" on the bottom of your gel.

10. Plan how you are going to load your samples and the lanes of ladder on the gel and create a key to the wells of the gel.

11. Carefully pipette 10 µL of the DNA ladder into the appropriate wells.

12. Carefully pipette 15 µL of each of your samples into the correct wells in the gel.

13. After all wells have been loaded, carefully slide on the lid of the gel box and connect the electrical leads to the power supply (black to black and red to red). Make sure the electrodes are oriented correctly (wells at negative (black) electrode, DNA will migrate to the positive (red) electrode). Run the gel at 120V (constant voltage) for approximately 45-60 minutes until the loading dye is approximately 2/3 of the way down the gel.

14. After turning off the power to the power supply, remove the gel tray from the apparatus and place in a designated container for travel. All gels will be imaged using the ChemiDoc Imaging system.

15. Turn on the ChemiDoc and slide a gel out of the gel tray and onto the UV tray of the ChemiDoc.

16. Slide the UV tray into the ChemiDoc and image the gel using the ethidium bromide setting. Save the image of the gel to an external drive. Repeat steps 14 and 15 until all gels have been imaged. All images will be digitally uploaded for student access.

Chapter 3: Zucchini Yellow Mosaic Virus Investigation

Laboratory #8: Testing Viruses Using ELISA

Objectives

✦Familiarize yourself with plant viruses and their modes of transmission;

✦Compare and contrast mechanical vs. aphid transmission of plant viruses.

Outline

- Experiment 1: Determination of virus using a lateral flow ELISA.
- Experiment 2: Mechanical transmission of ZYMV.
- Experiment 3: Preparation of aphid transmission of ZYMV.

I. Lateral Flow ELISA Test for ZYMV

A. Introduction

Zucchini Yellow Mosaic Virus (ZYMV) is a non-enveloped, positive-sense, single stranded RNA (ssRNA) virus which affects cucurbits.[9-10] *Cucurbitaceae* is a family of agricultural plants which includes zucchini, squash, melons, and cucumber. A crop infected with ZYMV is identified largely by the spotted yellowing of the leaves of the crop as well as stunting the growth of the plant, including the fruit (Figure 1). However, other viruses such as **squash mosaic virus (SqMV)** cause similar pathology. These viruses affect the ability of the plant to gain nutrients through photosynthesis and negatively impacts the growth and yield of the crop. Thus, a fruit grown from these affected plants are deemed not marketable. This causes major financial loss to the agriculture industry, as cucurbits account for approximately $1.5 billion USD per year within the USA alone.

Figure 1. Pathology of ZYMV in an infected squash plant showing leaf discoloration. Image by Author.

The spread of this virus is caused by many transmission scenarios including vertical transmission to seeds, mechanically through farm equipment, and through small insect vectors known as aphids. We will start a multi-week experiment in an attempt to fulfill **Koch's postulates** by tying a specific virus to the pathology we exhibit in the plant. In order to determine which virus (ZYMV or SqMV) is present in the plant, we will perform an **enzyme-linked immunosorbent assay (ELISA)** on infected plant tissue in the form of a **lateral flow test** (Figure 2). These tests are commonly found in other applications such as testing for pregnancy hormones in humans and utilize a combination of antibodies to bind target antigen in the sample and control antigen located within the test strip. In our case, the antigen being tested will be the viral capsid protein.

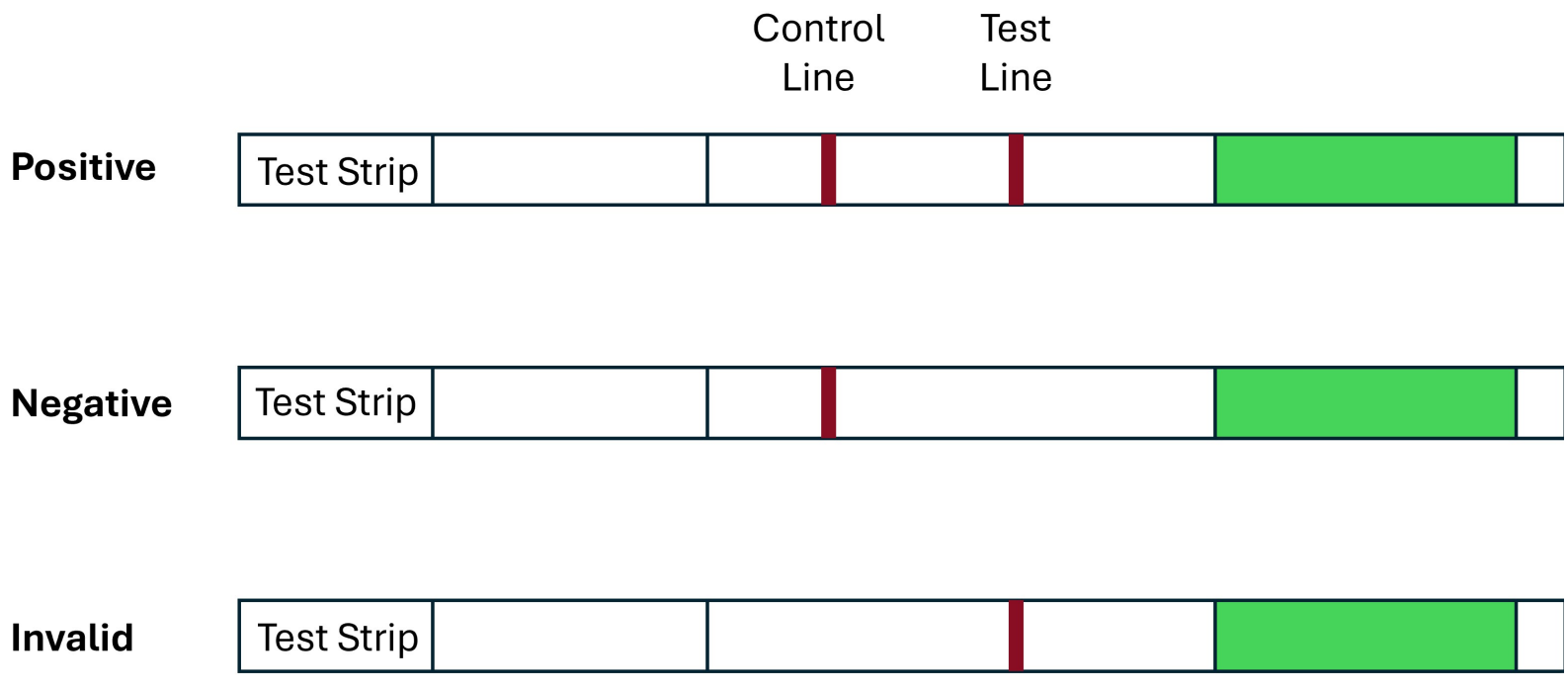


Figure 2. Diagram of Agdia platform ELISA test strip. This shows the three scenarios: positive result, negative result, and invalid result based on the appearance of the test and control lines. Image by Author.

B. Procedure I

1. Remove two samples of approximately 2.5 cm^2 region of virally affected leaf tissue (if no symptoms are present then simply take from any area of the leaf) from each respective plant. Work as a class to ensure all plants are tested at least once.

2. Cut open the top of two sample bags. The bags include sample buffer, make sure not to spill the buffer.

3. Place each leaf sample into individual sample bags.

4. Using the mesh lining of the bag, move the bag between your fingers, grinding the leaf samples into the buffer. An appropriately ground leaf will result in the buffer turning green.

5. Once the leaf tissue is thoroughly ground into the buffer, allow the bag to sit for at lest 3 minutes. This will settle large particles to the bottom of the bag.

6. Vertically place one ELISA test strip for ZYMV into one sample bag and another test strip for SqMV into the second sample bag so that the strips are just touching the buffer. Take care to only touch the top of the white portion of the strip. The strip is to be inserted into the bag with the green end facing down.

7. Incubate the ELISA test strips in the buffer for up to 30 minutes until bands are visible. Samples with higher titers of virus may be visible in as little as 5 minutes.

8. Use the diagram (Figure 2) to determine which virus has infected the zucchini plant. Record you results below as well as a description of the plant pathology (feel free to take a photo using your phone). Compare what you see against the provided non-infected plant.

II. Mechanical Transmission of Plant Viruses

A. Introduction

Mechanical transmission of plant viruses is a common occurrence in agriculture. Most notably this happens when farming equipment (hand tools or engine-powered) transfers the virus from infected plants to non-infected plants. This usually requires a wounding event, which leads to the breaking of plant cell walls. This mode of transmission stresses the importance of preventing cross-contamination in agriculture by sanitizing equipment when able.

We will be simulating mechanical transmission of our isolated virus by grinding up infected leaf tissue in phosphate buffered saline in the presence of activated carbon and silicon carbide. Activated carbon will be used to prevent our plant from getting secondary infections during the wounding process, as well as removing potential compounds that could inhibit viral infection. Silicon carbide will act as an abrasive in the procedure, leading to the wound in the leaf of the infected plant that will facilitate mechanical infection.

Procedure II

1. Remove one moderately sized leaf displaying signs of viral pathology from the ZYMV-infected plant.
2. Add the leaf to a mortar and pestle.
3. Add a few mL of phosphate buffered saline.
4. Add a small amount of both activated carbon and silicon carbide.
5. Grind up the leaf using the mortar and pestle. If the mixture becomes too dry add more buffer. If the mixture becomes too wet, add more of the powders. You are aiming to have a dark green to black liquid that is lightly viscous.
6. Each group will receive two 1-week old zucchini plants with visible true leaves. The plants have been heavily hydrated, which is important to the mechanical infection procedure. Using plant labels, label one “non-infected” and the other “infected.” Make sure to include your lab group’s name as well as today’s date.
7. Using a gloved hand, dip your thumb and index finger into the mixture and gently rub both cotyledons of your “infected” plant. The cotyledons are the first leaves that appear after a seed sprouts. Done correctly, the cotyledons will not be torn, but appear darkened on the surface of the leaf.

III. Aphid Transmission of Plant Viruses

A. Introduction

Aphid transmission of plant viruses occurs when aphids, that feed on plant juices, move from an infected plant to a non-infected plant during feeding.[11] Virus travels (and attaches to) the feeding mouthpart of the aphid, called the **stylet**. Aphids exhibit **parthenogenesis**, a form of asexual reproduction, in which the female aphids do not need sperm to create viable embryos. This allows for them to reproduce at alarming rates!

We will be observing the ability of the melon aphid (*Aphis gossypii*) to transmit our virus from an infected plant to a non-infected plant. This aphid species is known to transmit ZYMV.

B. Procedure III

1. Add one infected plant to an insectarium. Make sure the pot is set inside another shallow container to catch water from watering.

2. Add 15-20 melon aphids to the insectarium.

3. Allow the aphids to feed on the infected plant for 1 week.

Laboratory #9 and #10: Confirmation of Mechanical Infection and Quantitation of Plant Virus Pathology

Objectives

✦Determine if our ZYMV experiment fulfilled Koch's postulates;

✦Determine percent chlorosis in the leaves of a ZYMV-infected plant;

✦Evaluate changes in plant growth during a ZYMV infection.

Outline

- Experiment 1: Confirmation of a ZYMV mechanical infection using lateral flow ELISA
- Experiment 2: Quantification of chlorosis in leaves of ZYMV-infected plant.
- Experiment 3: Comparing above ground mass of control and ZYMV-infected plants.

I. Confirmation of ZYMV Mechanical Infection Using Lateral Flow ELISA

A. Introduction

In Laboratory #8, your lab group mechanically infected one out of two healthy 1-week old zucchini plants. The purpose of this was an attempt to fulfill **Koch's postulates**, a series of steps that 19th Century physician Robert Koch developed to establish a connection between pathogens and the diseases they cause.

Koch's postulates state in order to link a pathogen to its disease you must:

1. Identify a pathogen within a diseased host (and not healthy hosts).
2. Culture or isolate the pathogen from the diseased host.
3. Inoculate a new healthy host with the cultured/isolated pathogen.
4. Identify the same pathogen again within the previously healthy (and now diseased) inoculated host.

There are scenarios where fulfilling Koch’s postulates is difficult or impossible. For example, some pathogens grow poorly outside of living organisms, and in the case of human pathogens, it would be unethical to inoculate healthy humans with a pathogen that would cause them harm. At this point in the experiment, your group has attempted to satisfy Koch’s postulates 1-3, but need to re-examine the inoculated zucchini plant to confirm ZYMV is present to satisfy postulate 4. To do so we will be testing the plant again for ZYMV, but in this case we will also test the control plant as a negative control using the ZYMV-specific ELISA.

B. Procedure I

1. Remove one sample of approximately 2.5 cm^2 region of leaf tissue from your control and ZYMV-inoculated plant (we will use the term “inoculated” and not “infected” until we confirm the presence of the virus is present post-inoculation. Ideally, remove your control leaf tissue first so that you limit viral contamination of that sample. Wash your hands before you collect your inoculated sample.

2. Cut open the top of two sample bags. The bags include sample buffer, make sure not to spill the buffer.

3. Place each leaf sample into individual sample bags.

4. Using the mesh lining of the bag, move the bag between your fingers, grinding the leaf samples into the buffer. An appropriately ground leaf will result in the buffer turning green.

5. Once the leaf tissue is thoroughly ground into the buffer, allow the bag to sit for at least 3 minutes. This will settle large particles to the bottom of the buffer.

6. Vertically place one ELISA test strip for ZYMV into each sample bag so that the strips are about ¼ inch into the buffer. Take care to only touch the top of the white portion of the strip. The strip is to be inserted into the bag with the green end facing down.

7. Incubate the ELISA test strips in the buffer for up to 30 minutes until bands are visible. Samples with higher titers of virus may be visible in as little as 5 minutes.

8. Use the previous diagram to determine which virus has infected the zucchini plant. Record you results below as well as a description of the plant pathology (feel free to take a photo using your phone). Compare what you see against a non-infected plant.

II. Quantifying Chlorosis in ZYMV-Infected Leaves

A. Introduction

While observing symptoms of disease is important, it is equally important to measure them. In order to quantify the area of a leaf that is displaying chlorosis, it is most easily accomplished using image processing software. We will be using the freeware program ImageJ to accomplish this task on our own ZYMV-infected leaves.[12]

You can download the ImageJ software for your specific operating system here: ImageJ Download

B. Procedure II

1. Using a smart phone, take a picture of a representative leaf from your control plant and one from your ZYMV-infected plant. You will remove a leaf from each plant, place it on a sheet of white paper with a metric ruler in a well-lit space, and take the image one at a time.

2. Transfer the files to your computer and open the "control leaf" file by selecting FILE and OPEN in ImageJ (make sure to choose the correct file).

3. After opening the image you will see that the image was taken next to a ruler. Select the STRAIGHT LINE button and while holding the SHIFT key on your keyboard draw a straight line that reaches two whole numbers along the centimeter portion of the ruler. You have now created a line across a specific measurement.

4. Select ANALYZE and then select SET SCALE. Within the box that appears change the "known distance" to the length in centimeters of the line you drew across the ruler. Also change the "unit of length" to cm and check GLOBAL.

5. Save a copy of the scaled image by selecting FILE and choosing SAVE AS. Name the file "Control Leaf Scaled".

6. Next, select IMAGE then ADJUST and then COLOR THRESHOLD. You should now see a new box appear with various color meters for Hue, Saturation, and Brightness.

7. Make sure that the setting "dark background" at the bottom of the box is not selected.

8. The goal of the next step is to have the ImageJ select the entire leaf without any background. ImageJ default selection color is red, so we want to have the entire leaf red. You can do this by adjusting the "Hue" and "Brightness" max levels.

9. Record the Hue max level that selected the entire control leaf but not the background here:

10. Close the "control leaf" file and open up the "diseased leaf" file selecting FILE and OPEN in ImageJ.

11. After opening the image you will see that the image was taken next to a ruler. Select the STRAIGHT LINE button and while holding the SHIFT key on your

keyboard draw a straight line that reaches two whole numbers alone the centimeter portion of the ruler. You have now created a line across a specific measurement.

12. Select ANALYZE and then select SET SCALE. Within the box that appears change the “known distance” to the length in centimeters of the line you drew across the ruler. Also change the “unit of length” to cm and check GLOBAL.

13. Save a copy of the scaled image by selecting FILE and choosing SAVE AS. Name the file “Disease Leaf Scaled”. You will use this image twice, so keep it handy.

14. Next, select IMAGE then ADJUST and then COLOR THRESHOLD. You should now see a new box appear with various color meters for Hue, Saturation, and Brightness.

15. Make sure that the setting “dark background” at the bottom of the box is not selected.

16. The goal of the next step is to have the ImageJ select the entire leaf without any background. ImageJ default selection color is red, so we want to have the entire leaf red. You can do this by adjusting the “Hue” and “Brightness” max levels. ***However, in this case you must use the “Hue” max level that was written down for the control leaf.

17. Afterwards select PROCESS then BINARY and then MAKE BINARY. This will make your image black and white.

18. If you end up with a black leaf on a white background you are good to go. If you end up with a white leaf on a black background select IMAGE and then LOOK UP TABLES and then INVERT LUT, which should fix the issue. Alternatively you can select EDIT and then INVERT.

19. Use the RECTANGLE TOOL button to draw a rectangle around your leaf.

20. Select ANALYZE and then SET Measurement and make sure to check AREA and LIMIT TO THRESHOLD.

21. Select ANALYZE and then Particles. In the menu set Size to “0.20”. Under SHOW selected the pull down menu for OUTLINES. Check DISPLAY RESULTS and make sure nothing else is selected. After clicking OK the area measurement will appear. Record the total are in cm^2:

22. Save a copy of the image by selecting FILE and choosing SAVE AS. Name the file “Binary Total Leaf Area”.

23. Close the current file and reopen up the “Diseased Leaf Scaled” file selecting FILE and OPEN in ImageJ.

24. Next, select IMAGE then ADJUST and then COLOR THRESHOLD. You should now see a new box appear with various color meters for Hue, Saturation, and Brightness.

25. Make sure that the setting “dark background” at the bottom of the box is not selected.

26. The goal of the next step is to have the ImageJ select only the leaf area depicting chlorosis without any background. ImageJ default selection color is red, so we want to have the entire leaf red. You can do this by adjusting the “Hue” and “Brightness” max levels. ***However in this case you must use the “Hue” max level of 55. The RGB setting at 55 is our lab standard for chlorosis yellowing hue.

27. Afterwards select PROCESS then BINARY and then MAKE BINARY. This will make your image black and white.

28. If you end up with a black leaf on a white background you are good to go. If you end up with a white leaf on a black background select IMAGE and then LOOK UP TABLES and then INVERT LUT, which should fix the issue.

29. Use the RECTANGLE TOOL button to draw a rectangle around your leaf.

30. Select ANALYZE and then SET Measurement and make sure to check AREA and LIMIT TO THRESHOLD.

31. Select ANALYZE and then Particles. In the menu set Size to “0.20”. Under SHOW selected the pull down menu for OUTLINES. Check DISPLAY RESULTS and make sure nothing else is selected. After clicking OK the area measurement will appear. Record the total are in cm^2:

32. Save a copy of the image by selecting FILE and choosing SAVE AS. Name the file “Binary Diseased Leaf Area”.

33. To Calculate percent chlorosis in your leaf by dividing the area of your diseased leaf by the area of your total leaf and multiple by 100.

III. Measurement of Above-Ground Mass in ZYMV-Infected Plants

A. Introduction

Considering ZYMV pathology causes plants to have altered metabolism, which could negatively impact plant growth. Your lab group will measure control vs. ZYMV-infected plant above-ground mass.

B. Procedure III

1. Using scissors, cut each of your plants (control and ZYMV-infected) at ground level at the base of the stem.

2. Weigh each plant and record their mass.

    Control plant mass (g):

    ZYMV-infected plant mass (g):

3. To enhance our data collection, we will share data across all lab groups so that we have large sample sizes of both control plants and ZYMV-infected plants.

Glossary

- **agarose gel electrophoresis**: a technique used to separate and analyze nucleic acid fragments based on their size.
- **amplicon**: the amplified product of nucleic acid that results from PCR.
- **annealing**: the second step of PCR, following denaturation, where the temperature is lowered to allow for primers to bind to the target DNA.
- **aphid transmission**: the process where aphids facilitate the transfer of plant viruses from infected plants to healthy plants using their piercing mouthparts during feeding.
- **aseptic technique**: a set of practices and procedures used to limit the risk of contamination by harmful or unwanted microorganisms.
- **autoclave**: a machine that creates an environment of increased pressure and temperature to sterilize instruments and reagents.
- **Bacteria**: one of the domains of life consisting of microscopic, single-celled organisms that populate virtually all areas of the world.
- **bacteriophage (phage)**: viruses that infect bacteria.
- **colony**: a visible mass of bacterial cells that all originate from the same single cell.
- **copy DNA (cDNA)**: a complementary DNA copy of an RNA molecule synthesized by the enzyme reverse transcriptase.
- **deformed wing virus (DWV)**: a positive, single-stranded RNA virus that infects certain insects and exhibits pathology in honey bees.
- **DNA polymerase**: an enzyme that synthesizes DNA using a DNA template.
- **DNase I**: an enzyme that degrades DNA.
- **denaturation**: the first step of PCR, where temperature is raised to separate double-stranded DNA into single strands.
- **enzyme-linked immunosorbent assay (ELISA)**: a technique that uses antibodies to detect various soluble substances such as proteins or other antibodies.

- **ethidium bromide (EtBr)**: a dye commonly used to visualize nucleic acid that fluoresces under UV light.
- **extension**: the last step of PCR that allows DNA polymerase to synthesize a new DNA strand starting at the primers that bound to the target DNA during annealing.
- **gene-specific primers**: short, single-stranded DNA sequences designed to only bind to the nucleic acid of interest for later amplification.
- **genomic DNA (gDNA)**: the complete set of DNA found within the cells of an organism.
- **head**: the protein structure of a bacteriophage, also known as a capsid, that encases and protects the viral genome.
- **Koch's postulates**: a set of criteria used to establish a relationship between a disease and the microorganism that causes it.
- **lateral flow test**: a type of ELISA where the liquid sample runs through an absorbent test strip that displays colored lines at specified locations on the strip indicating positive or negative results.
- **lysogenic cycle**: a viral replication cycle, often exhibited in bacteriophages, where the viral DNA integrates into the genome of the host after infection, is replicated as the host genome is replicated, and the host cell survives.
- **lysogenic phage**: bacteriophages that can participate in the lysogenic cycle, also known as temperate phages.
- **lytic cycle**: a viral replication cycle, often exhibited in bacteriophages, where the virus infects, replicates, and new phages leave the host cell once it is destroyed.
- **lytic phage**: bacteriophages that exclusively participate in the lytic cycle.
- **mechanical transmission**: the process where non-living devices and surfaces, such as agricultural equipment, transfer plant viruses from infected plants to healthy plants.
- **micro-volume spectrophotometer**: a spectrophotometer that can accommodate microliter-sized volumes.

- **oligo-dT primers**: short, single-stranded DNA sequences composed of deoxythymidine nucleotides designed to bind to poly(A) tails of messenger RNAs to facilitate cDNA synthesis.
- **parthenogenesis**: a form of reproduction where offspring develop from an unfertilized egg and is considered a type of asexual reproduction which only requires the female for genetic contribution.
- **permissive cell**: a cell that allows a virus to replicate and produce new virions.
- **plaque**: a visible clearing on a monolayer of cells where a virus has originally infected, killed a single host cell, and spread new virions to neighboring cells.
- **plaque assay**: a method used to measure the number of infectious virions in a serial dilution of a virus sample by counting plaques.
- **plaque-forming units (PFU)**: the unit of measure used when referring to the number of infectious viral particles within a virus sample measured by plaque assay.
- **Polymerase Chain Reaction (PCR)**: a technique used to amplify specific DNA sequences, creating millions of copies of the target DNA sequence.
- **prophage**: the genome of a bacteriophage after it has integrated into the genome of the host cell.
- **pure culture**: growth of a single species of microorganism in laboratory culture media.
- **random hexamers**: short, single-stranded DNA sequences, composed of six randomized nucleotides designed to bind nucleic acid targets, often used to facilitate cDNA synthesis.
- **reverse transcription**: the process where the enzyme reverse transcriptase synthesizes DNA from an RNA template and is often referred to as cDNA synthesis if found in a molecular biology method.
- **reverse transcriptase (RT)**: an enzyme that synthesizes DNA from an RNA template.
- **reverse transcriptase PCR (RT-PCR)**: a technique that combines reverse transcription and PCR to detect RNA.

- **RNase**: an enzyme that degrades RNA.
- **spectrophotometer**: a device that measures the intensity of light at different wavelengths as it passes through a solution, often used to determine the concentration of nucleic acids or proteins.
- **spread plate method**: a method used to isolate and quantify microorganisms by spreading a small volume of diluted sample evenly across the surface of an agar plate.
- **strain**: a microorganism with distinct genetic changes within its genome that lead to distinct physical properties.
- **streak plate method**: a method used to isolate microorganisms from a mixed cultured by mechanically diluting organisms across the surface of an agar plate.
- **stylet**: the sucking mouthparts of an aphid, which are used to feed on the sap of plants.
- **susceptible cell**: a cell that can be infected by a virus.
- **SYBR green I**: a fluorescent dye commonly used to visualize nucleic acid that preferentially binds to double-stranded DNA.
- **tail**: the protein structure of a bacteriophage, that is responsible for recognizing the host cell and delivering the viral genome.
- **titer**: a general measurement of the concentration of virus in a sample.
- **tropism**: the specific cell type, tissue, or species that a virus can infect.
- **T-even phage**: a type of bacteriophage, labeled with an even number, consisting of an elongated head structure.
- **T-odd phage**: a type of bacteriophage, labeled with an odd number, consisting of a compressed head structure.
- **Tris-acetate-EDTA (TAE) buffer**: a buffer commonly used in agarose gel electrophoresis, typically for resolving larger DNA fragments.
- **Tris-borate-EDTA (TBE) buffer)**: a buffer commonly used in agarose gel electrophoresis, typically for resolving smaller DNA fragments.
- **variant**: a microorganism with distinct genetic changes within its genome.

- **vector**: an organism that transmits a virus from one host to another.
- **virion**: the fully-formed infectious viral particle and a term often used when a virus is extracellular or having just been assembled in an infected cell.
- **zucchini yellow mosaic virus (ZYMV)**: a positive, single-stranded RNA virus that infects certain plants and exhibits pathology in cucumbers, squash, and melons.

Instructor Notes

Laboratories #1-3

In order to set up the Bacteriophage Investigation Labs #1-3, the author uses the following specific host organisms and bacteriophages from Carolina Biological Supply: *Escherichia coli* strain B, *Escherichia Coli* strain C, *Micrococcus luteus*, T2 phage, T4r phage, and φX174 phage. All bacteria can be grown overnight on tryptone agar using a 37°C incubator or tryptone broth using a 37°C shaking incubator. Bacteria and phage were chosen due to accessibility and biosafety considerations.

The following general reagents and equipment are also used and can be purchased from multiple vendors:

1. Bunsen burner

2. Micropipettes (P20, P200, and P1000) and corresponding micropipette tips

3. Inoculating loop

4. Plate spreader

5. 100 mm petri dishes (sterile)

6. Snap-capped 14 mL round-bottom polypropylene test tubes (sterile)

7. 0.22 micron filter syringe

8. 70% ethanol

9. 1.5 mL microcentrifuge tubes (sterile)

10. Tryptone broth

11. Tryptone soft agar (0.75% agar w/v)

12. Tryptone hard agar (1.5% agar w/v)

13. Phage buffer (50 mM Tris-HCl, 100 mM NaCl, 8 mM $MgS0_4$; sterile)

14. Autoclave

15. Shaking incubator

16. Incubator

17. Lab coat

18. Lab gloves

19. Vortex

Laboratories #4-7

In order to set up the Deformed Wing Virus Investigation Labs #4-7, the author uses honey bees collected by local apiarists and some from hives maintained by his university using killing jars with 70% isopropyl alcohol. *Varroa* mites will release from honey bees during this process and can serve as great demos while viewed under a stereo microscope. Make sure to store all samples that will be used for RNA isolation in a -70°C ultra freezer. If an instructor does not have access to honey bee populations infected with deformed wing virus, custom DNA oligonucleotides can be ordered from various providers who specialize in customer nucleic acid synthesis using the reference genome retrieved from the GenBank database (Accession No. PV664207.1). Since these would already be cDNA samples, you can integrate them just prior to the PCR step in Lab #6 as control samples or add them into student experimental cDNA samples to simulate a positive sample. Other more specific reagents include RNeasy Mini Kit (Qiagen), ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs), Hot Start *Taq* 2X Master Mix (New England Biolabs), and the primer sequences listed in Lab #6 Table 2.

The following general reagents and equipment are also used and can be purchased from multiple vendors:

1. Micropipettes (P20, P200, and P1000) and corresponding micropipette tips

2. Forceps

3. Triple washed zirconium beads (3.0 mm)

4. Bead homogenizer

5. Microcentrifuge

6. RNase decontamination solution

7. 70% ethanol

8. 1.5 mL microcentrifuge tubes (sterile)

9. Micro-volume spectrophotometer

10. DNase I enzyme and corresponding buffer

11. Heat blocks or water baths

12. Ice and containers for ice

13. Agarose

14. Agarose gel electrophoresis box

15. UV gel box illuminator or similar gel scanner with UV light

16. Ethidium bromide

17. Gel loading dye

18. DNA standards/ladder (100-1,000 bp range)

19. Lab gloves

20. 0.2 mL PCR tubes

21. Thermocycler

Laboratories #8-10

In order to set up the Zucchini Yellow Mosaic Virus Investigation Labs #8-10, the author uses a viral isolate of zucchini yellow mosaic virus and squash yellow mosaic virus generously shared from the lab of Dr. Christina Rosa at Penn State University, University Park, Pennsylvania. Virus is propagated in black beauty cultivar zucchini plants showing true leaves using the exact protocol found in Lab #8 and stored in whole leaf tissue in a -70°C ultra freezer. These viruses can be

used together to create a co-infection in a plant. Instructors should reach out to their local plant pathology departments at local universities or governmental institutions such as USDA-ARS rather than commercial retailers for virus samples. Other plant viruses that cause mosaic pathology in their corresponding host plant leaves can also be substituted to create a similar lab experience. Melon aphids (*Aphis gossypii*) were generously shared from the lab of Michelle Heck at Cornell University, Ithaca, New York. Aphids are housed in insectariums and fed black beauty cultivar zucchini plants under grow lights with a 12 hour day and 12 hour dark cycle. When propagating aphids for experiments with viruses, care must be taken by the instructor to follow all governmental guidelines. The use of aphids in this investigation can be considered optional if such control is impractical at the instructor's institution. Instructors must plan on a timeline that allows 2 weeks to pass between Lab #8 and #9/10 to facilitate infection. Other more specific reagents include the Agdia ImmunoStrip for ZYMV (Agdia Inc.) and the ImageJ software which can be downloaded as directed in Lab #9/10. This lab takes up 2 lab periods to allow students to process all leaves digitally. Students must image all leaves and complete plant weighing during Lab #9 to maintain consistent timing of pathology.

The following general reagents and equipment are also used and can be purchased from multiple vendors:

1. Micropipettes (P20, P200, and P1000) and corresponding micropipette tips
2. Activated carbon powder
3. Silica carbide powder
4. Phosphate buffered saline
5. Lab gloves
6. Black beauty cultivar zucchini seeds
7. Potting soil
8. Pots and dishes to catch water
9. Mortar and pestle
10. Grow lights
11. Insectariums rated for aphid size or smaller

Literature Cited

1. Duckworth DH. Who discovered bacteriophage? J Hist Med Allied Sci. 1976;31(4):418-430.

2. Allen JM, Guyre CA. An undergraduate laboratory activity demonstrating bacteriophage specificity. *Am Biol Teach*. 1994;56(7):426-428.

3. Dreyer G, Campbell-Renton ML. The quantitative determination of bacteriophage activity and its application other than the study of the Twort-D'Herelle phenomenon. J *Pathol Bacteriol*. 1933;36(3):399-423.

4. Sun X, et al. Isolation, characterization and application of bacteriophage PSDA-2 against *Salmonella Typhimurium* in chilled mutton. *Food Sci Nutri*. 2020;8(9):4698-4707.

5. Hershey AD, Chase M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol*. 1952;36(1):39-56.

6. Chen YP, Pettis JS, Collins a, Feldlaufer MF. Prevalence and transmission of honeybee viruses. Apple Environ Microbiol. 2006;72(1):606-611.

7. Derso S, Zemene M, Bogale B, Belete S, Melaku S, Hailu H. A review on varroa mites of honey bees. *Acad J Entomol*. 2015;8(3):150-159.

8. Chen, YP, Higgins JA, Feldlaufer MF. Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). *Appl Environ Microbiol*. 2005;71(1):436-441.

9. Desbiez C, Lecoq H. Zucchini yellow mosaic virus. *Plant Pathol*. 2003;46(6):809-829.

10. Ivanov KI, Eskelin K, Lohmus A, Makinen K. Molecular and cellular mechanisms underlying potyvirus infection. *J Gen Virol*. 2014;95(7):1415-1429.

11. El-Wareth A, Hamada M, Ahmed HM. Cotton aphid (*Aphis gossypii* Glover) and green peach aphid (*Myzus persicae* Sulzer) efficiency of zucchini yellow mosaic virus potyvirus (ZYMV) transmission on squash plants at Farouk Governorate. *Egypt Academy J Bill Sci G Microbiol*. 2017;9(2):21-29.

12. Pride L, Vallad G, Agehara S. 2020. How to measure leaf disease damage using image analysis in ImageJ: HS1382, 9/2020. EDIS. 2020(5). doi:10.32473/edis-hs1382-2020