

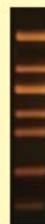


THE BIOTECHNOLOGY
EDUCATION COMPANY®

Includes EDVOTEK's All-NEW
EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

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



REVISED
&
UPDATED

Edvo-Kit #

952

Edvo-Kit #952

Water Quality Testing II: PCR-Based Testing of Water Contaminants

Experiment Objective:

In this experiment, students will monitor water quality by using the Polymerase Chain reaction to detect the presence of bacterial contamination.

See page 3 for storage instructions.

Table of Contents

	Page
Experiment Components	3
Experiment Requirements	4
Background Information	5
Experiment Procedures	
Experiment Overview	9
Module I: Isolation of Bacterial DNA from Contaminated Water	10
Module II: Amplification of <i>E. coli</i> DNA	12
Module III: Separation of PCR Products by Electrophoresis	13
Module IV: Staining Agarose Gels	15
Study Questions	17
Instructor's Guidelines	18
Pre-Lab Preparations	19
Experiment Results and Analysis	23
Study Questions and Answers	24
Appendices	25
A EDVOTEK® Troubleshooting Guide	26
B Preparation and Handling of PCR Samples With Wax	28
C Bulk Preparation of Agarose Gels	29
D Optional Extension Activity: Standard Plate Count	30

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

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Experiment Components

Includes EDVOTEK's All-NEW EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

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



Components

	Storage	Check (✓)
A PCR EdvoBeads™ <i>Each PCR EdvoBead™ contains:</i>	Room Temp., desiccated	<input type="checkbox"/>
• dNTP Mixture		
• Taq DNA Polymerase Buffer		
• Taq DNA Polymerase		
• MgCl ₂		
• Reaction Buffer		
B <i>E. coli</i> Primer Mix Concentrate	-20° C Freezer	<input type="checkbox"/>
C EdvoQuick™ DNA ladder	-20° C Freezer	<input type="checkbox"/>
D Concentrated <i>E. coli</i> DNA (Positive Control)	-20° C Freezer	<input type="checkbox"/>
E Tris buffer	-20° C Freezer	<input type="checkbox"/>
F Proteinase K	Room Temp., desiccated	<input type="checkbox"/>
G Chelating Agent	Room Temp.	<input type="checkbox"/>
H Sterile water (Negative Control)	Room Temp.	<input type="checkbox"/>
• <i>E. coli</i> BactoBeads™	4° C, desiccated	<input type="checkbox"/>

This kit contains reagents
for 25 PCR experiments.

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

REAGENTS & SUPPLIES

Store all components below at room temperature.

Component

Component	Check (✓)
• UltraSpec-Agarose™	<input type="checkbox"/>
• Electrophoresis Buffer (50x)	<input type="checkbox"/>
• 10x Gel Loading Solution	<input type="checkbox"/>
• InstaStain® Ethidium Bromide	<input type="checkbox"/>
• FlashBlue™ Stain	<input type="checkbox"/>
• Microcentrifuge Tubes	<input type="checkbox"/>
• Screw Top Microcentrifuge Tubes	<input type="checkbox"/>
• 0.2 ml PCR tubes	<input type="checkbox"/>
• 50 ml Conical tube	<input type="checkbox"/>
• Wax beads (for thermal cyclers without heated lid)	<input type="checkbox"/>

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Requirements

- Thermal cycler (EDVOTEK Cat. # 532 highly recommended)
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Waterbath (56° C and 99° C)
- UV Transilluminator or UV Photodocumentation system (if staining with InstaStain® Ethidium Bromide)
- UV safety goggles (if staining with InstaStain® Ethidium Bromide)
- White light visualization system (if staining with FlashBlue™)
- Automatic micropipets (5-50 µl) with tips
- Microwave, hot plate or burner
- Pipet pump
- 250 ml flasks or beakers
- Hot gloves
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Distilled or deionized water
- Water samples from the environment (optional)

Optional Extension Activity: Standard Plate Count

Requirements for Five Groups:

2	Cat. 615	ReadyPour™ Luria Broth Agar Base, 170 ml
3	Cat. 633	Small Petri Plates (60x15mm), 20
1	Cat. 667	Sterile loops, package of 25
1	Cat. 630	Microcentrifuge tubes, 500
1	Cat. 648	15 ml sterile conical tubes, bag of 25

- Sterile Water
- Automatic micropipet and tips
- *E.coli* "contaminated" water sample (Preparation on page 19)



Background Information

Water pollution is a major concern worldwide. While clean water is obviously important for aquatic life and agriculture, it is also essential to human health. According to the World Health Organization (WHO), contaminated drinking water is the leading cause of infectious disease in the world, resulting in almost two million deaths each year. Individuals with weak immune systems (children, the elderly and individuals afflicted with AIDS, cancer, or other diseases) are especially vulnerable to these contaminants. The contaminants present in polluted water encompass a wide variety of chemical compounds, solid waste and microorganisms. Assessment of water contamination requires both quantitative and qualitative analysis to pinpoint the location and the identity of water pollution.

Contamination enters the water supply in two main ways (summarized in Fig. 1). **Point source** water pollution is any contaminant that enters the water supply from a single, readily identifiable source, such as a manufacturing plant or water treatment facility. This waste, called effluent, is a complex material comprising sewage and/or chemical by-products. Since it is common practice to release effluent into nearby bodies of water, the composition of this wastewater is strictly monitored and regulated by government agencies. In contrast, **non-point pollution** cannot be traced to a single, identifiable source — it often results from everyday activities. Typical non-point pollution arises from overflow of septic tanks, chemical spills and water run-off from farms. This pollution contaminates the streams, rivers, and lakes that supply our drinking water.

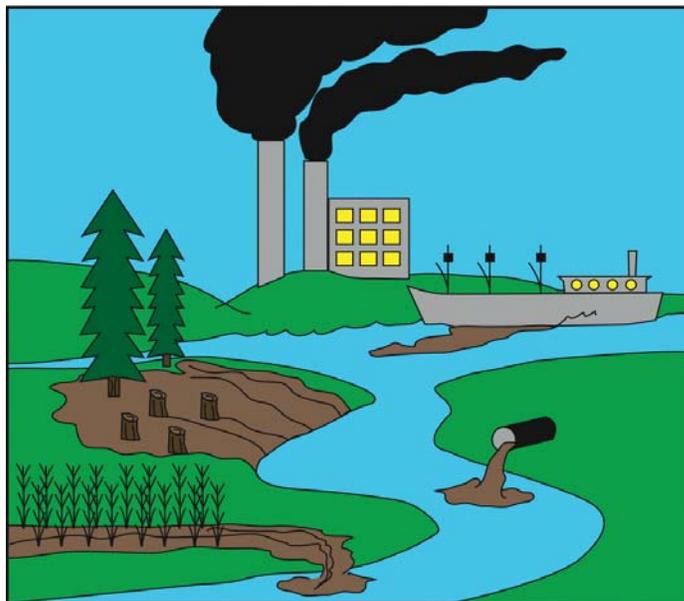


Figure 1: Point and Non-point sources of water contamination.

As both point and non-point pollution are responsible for contamination of drinking water, water treatment strategies must be able to effectively remove these impurities. Large, modern water treatment facilities have been able to supply us with safe drinking water by removing harmful disease-causing microbes and chemical contamination. In the United States and other developed countries, water treatment systems have been in place for nearly 150 years. Over time, federal regulations were established to prevent contamination of drinking water by pathogens, chemicals and other hazardous materials. Public water systems were established to provide safe drinking water to the majority of the United States. In spite of this, over 15 million American households are located in rural areas where they rely on private, untreated wells for drinking water. In 2011, U.S. Department of Agriculture (USDA) teamed up with the Environmental Protection Agency (EPA) to improve the quality of drinking water for 1.8 million residents in these rural areas.

Although drinking water in U.S. cities is generally safe, monitoring remains necessary because our water supply does develop occasional contamination. For example, corrosion in aging water supply pipelines can increase biofilm formation, resulting in increased contamination of drinking water. A 1999 EPA study revealed 14 states having more than 11% of their community water systems violating maximum contaminant levels. From 2007-2008, the Centers for Disease Control (CDC) reported 36 waterborne disease outbreaks from 23

¹ <http://www.cdc.gov/mmwr/pdf/ss/ss6012.pdf>

states, causing illness in over 4000 people. In order to minimize contamination, the EPA has established four major guidelines to protect drinking water, as follows:

- 1) Risk prevention – to prevent contaminants from entering our drinking water
- 2) Risk management – to reduce or eliminate contamination of a water source
- 3) Risk monitoring and compliance – to evaluate water quality at different stages throughout the water treatment process, including the source, the water treatment plant, and water after it has been treated and disinfected
- 4) Individual action (constant vigilance by citizens) - to protect water prior to consumption by limiting activities that can affect bodies of water.

MICROORGANISMS IN THE WATER SUPPLY

Common waterborne microorganisms can cause severe illness when present in the water supply (Fig. 2). For example, in 1993, the protozoa *Cryptosporidium parvum* caused gastrointestinal distress in over 400,000 individuals in Milwaukee, Wisconsin. The outbreak resulted in 4000 hospitalizations and more than 50 deaths. *Cryptosporidium* outbreaks have become more common over the past 15 years, as the protozoa have become resistant to some water treatment programs (e.g. chlorination).

Another waterborne protozoa, *Giardia*, will also cause severe gastrointestinal distress in humans. *Giardia* is very common in the gastrointestinal system of farm animals and household pets. Over the course of its life cycle, *Giardia* forms chlorine-resistant cysts that are shed in the feces of an infected animal. The protozoa are then transmitted to humans from water contaminated by animal feces. Although rarely life threatening, this infection (giardiasis) causes severe dehydration, resulting in more than 4,600 U.S. hospital visits per year. The effects of this disease are more severe in individuals with compromised immune systems (e.g., AIDS and cancer patients, elderly persons).

Certain species of bacteria will also cause illness when present in drinking water. For example, most strains of *Escherichia coli* are relatively harmless – in fact, certain strains of *E.coli* are found in the human large intestine, where they aid in digestion. However, certain strains of *E.coli* (in particular, O157:H7) have acquired the activity to produce a potent toxin that can cause acute bloody diarrhea, kidney failure and even death. This bacterium is commonly present in the intestine of cattle, where it is not pathogenic. However, it is easily transmitted to humans through ingestion of undercooked beef, unpasteurized milk or raw produce. Furthermore, as the bacterium is present in fecal matter, it can enter the water supply in run-off from farms. It is estimated that thousands of *E.coli* (O157:H7) infections occur in the U.S. each year due to contaminated drinking water.

These same microorganisms often contaminate recreational water sources like pools, lakes, water parks and hot tubs, where they can cause recreational water illnesses (RWI) like swimmer's ear, skin rashes, respiratory infections and gastrointestinal distress. According to the CDC, the incidence of RWIs has been steadily increasing over the past twenty years. For example, from 2004 – 2008, the number of *Cryptosporidium* infections caused by recreational water increased over 200%. In part, this may be the result of poor water quality -- crowded of public pools, code violations in pool maintenance, and/or increased pollution of recreational lakes and streams.

Disease	Pathogen
Cholera	Bacteria - <i>Vibrio cholera</i>
Typhoid Fever	Bacteria - <i>Salmonella typhi</i>
Giardia	Protozoa - <i>Giardia lamblia</i>
Cryptosporidiosis	Protozoa - <i>Cryptosporidium parvum</i>
Hemolytic-uremic syndrome	Bacteria - <i>E. coli</i> (O157:H7)
Dysentery	Bacteria - <i>Shigella dysenteriae</i>
Legionnaires' disease	Bacteria - <i>Legionella pneumophila</i>
Hepatitis A	Viral – <i>Hepatitis A</i>
Guinea worm disease	Nematode - <i>Dracunculus medinensis</i>

Figure 2: Common Waterborne Illnesses

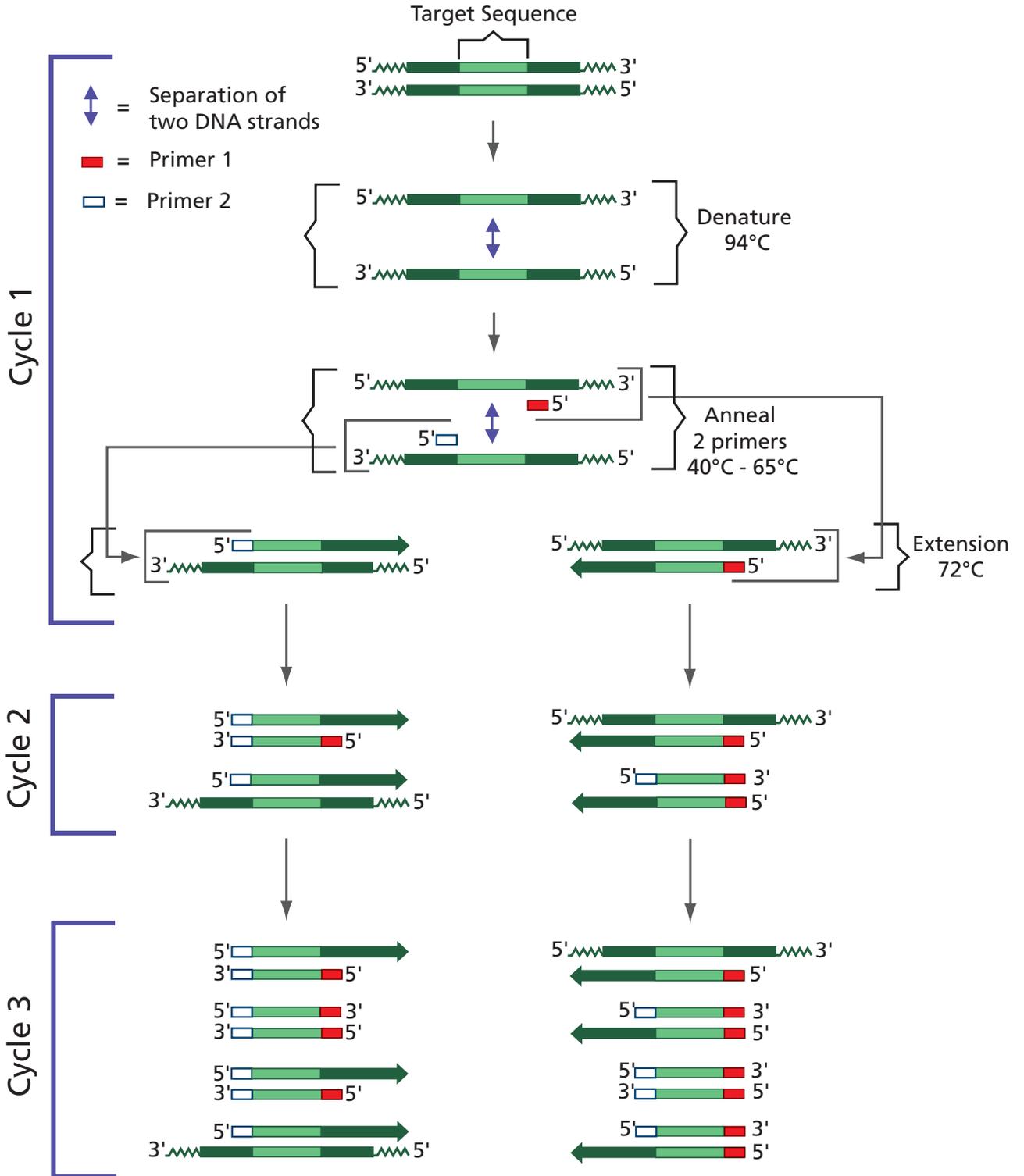


Figure 3:
DNA Amplification by the Polymerase Chain Reaction

METHODS TO IDENTIFY MICROBIAL WATER CONTAMINATION

To identify water contamination in both drinking and recreational water, scientists look for specific microbes called coliform bacteria first. These microbes represent a group of relatively harmless bacteria of the genera of *Escherichia*, *Klebsiella*, *Enterobacter*, and *Serratia* that normally inhabit the gastrointestinal tract of animals and humans. The presence of these organisms in a water sample typically warns of fecal contamination. If a water sample is negative for coliform bacteria, it is assumed that harmful microorganisms are also absent, and the water is deemed safe for drinking. If a water sample is positive for coliforms, the sample is then sent to a laboratory to determine whether more dangerous pathogens are present.

After a sample tests positive for coliform bacteria, scientists will use the polymerase chain reaction (PCR) to quickly detect any dangerous microorganisms that may also be present in the water. PCR can detect a particular pathogen using short, synthetic DNA molecules called primers that target a pathogen-specific DNA sequence for amplification. In this way, PCR can identify exactly which organisms are present in the sample. For example, if a PCR product is produced using *Cryptosporidium*-specific primers, then the microorganism must be present in the water sample.

Before PCR can be performed, microbial DNA must be purified from the water sample. Next, the extracted DNA is mixed with the primers, a thermostable DNA polymerase (*Taq*) and nucleotides. Then, the mixture is heated to 94° C to denature the DNA (i.e., unzip the DNA double helix into single strands). Next, the sample must be cooled to between 45-60° C, a temperature at which the primers can begin to base-pair (or “anneal”) to their target DNA sequences. Finally, after annealing is complete, the temperature is raised to 72° C, the optimal temperature at which *Taq* polymerase will extend the primer to synthesize a new strand of DNA. Each PCR “cycle” (denaturation, annealing, extension) doubles the amount of the target DNA in less than five minutes (summarized in Figure 3). In order to produce enough DNA for analysis, twenty to forty cycles may be required. To simplify this process, a specialized machine, called a “thermal cycler” or a “PCR machine”, was created to rapidly heat and cool the samples.

In this experiment, we will be monitoring water quality by performing an experiment that detects the coliform bacteria *E.coli*. Microbial DNA is extracted from the environment sample and analyzed using PCR. After amplification, the PCR products will be analyzed using agarose gel electrophoresis to separate DNA fragments according to size. If the water sample is contaminated by *E.coli* bacteria, a 1475-bp segment of the *E.coli* genome will be amplified by PCR and detected on the gel. The absence of this band suggests that the water is free from coliform bacteria.



Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will monitor water quality by using the Polymerase Chain reaction to detect the presence of bacterial contamination.

LABORATORY SAFETY:

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

1. Wear gloves and goggles while working in the laboratory.
2. Exercise caution when working in the laboratory – you will be using equipment that can be dangerous if used incorrectly.
3. Wear protective gloves when working with hot reagents like boiling water and melted agarose. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. The bacteria used in this experiment are not considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
 - A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
 - B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
 - Autoclave at 121° C for 20 minutes. Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
 - Soak in 10% bleach solution. Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
 - C. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.



LABORATORY NOTEBOOKS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

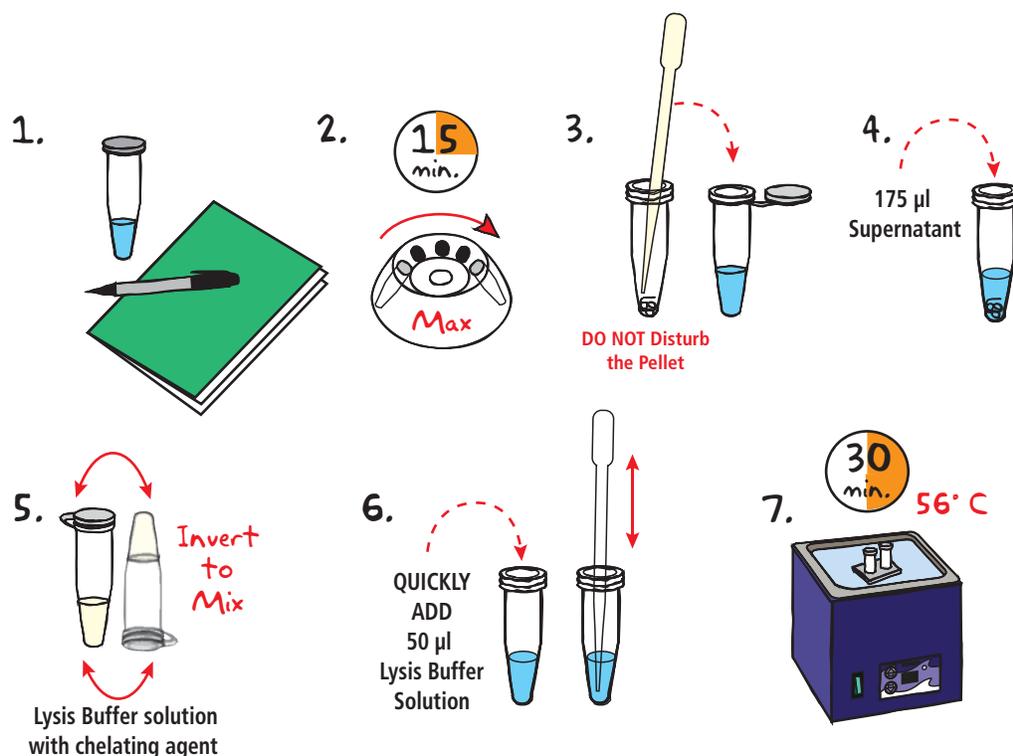
During the Experiment:

- Record (draw) your observations, or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

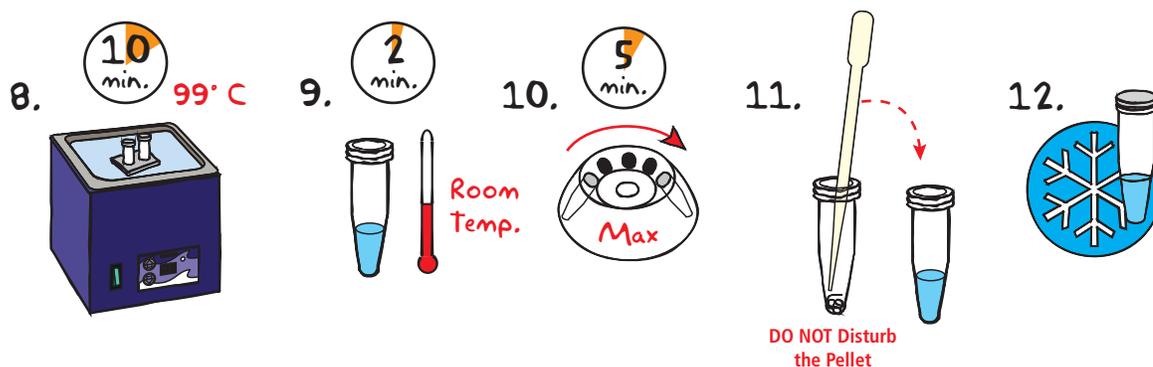
Module I: Isolation of Bacterial DNA from Contaminated Water



1. **OBTAIN** a water sample in a screw-cap microcentrifuge tube from your instructor. **RECORD** the identity of the sample (control *E.coli* OR environmental water sample) in your lab notebook.
2. **CENTRIFUGE** the water sample for 15 minutes at maximum speed to pellet the cells.
3. **REMOVE** the supernatant and place it in a separate, labeled tube. **DO NOT DISTURB THE CELL PELLETT!**
4. **RESUSPEND** the cell pellet in 175 µl of the supernatant by pipetting up and down or by vortexing vigorously. **DISCARD** the remaining supernatant.
5. **MIX** the lysis buffer solution with the chelating agent by inverting several times. To better mix the solution, cut several millimeters off of a micropipet tip to enlarge the opening. **MIX** the solution by pipetting up and down using this special tip.
6. Quickly **ADD** 50 µl of the lysis buffer solution to the tube containing the bacterial cell suspension. **MIX** by pipetting up and down or by vortexing vigorously.
7. **INCUBATE** the sample in a 56° C waterbath for 30 minutes.

continued

Module I: Isolation of Bacterial DNA from Contaminated Water, continued



8. **MIX** the sample by vortexing or flicking the tube. **INCUBATE** the sample in a 99°C water bath for 10 minutes.
9. **ALLOW** the sample to cool at room temperature for 2 minutes.
10. **CENTRIFUGE** the samples at maximum speed for 5 minutes.
11. Carefully **TRANSFER** just the supernatant into a fresh, labeled microcentrifuge tube. Avoid the pellet at the bottom of the tube. After removing the supernatant, **DISCARD** the tube with the pellet.
12. **PLACE** tube on ice.
13. **PROCEED** to Module II: Amplification of *E.coli* DNA.



OPTIONAL STOPPING POINT:

The extracted DNA may be stored at -20° C for amplification at a later time.

Module II: Amplification *E.coli* DNA



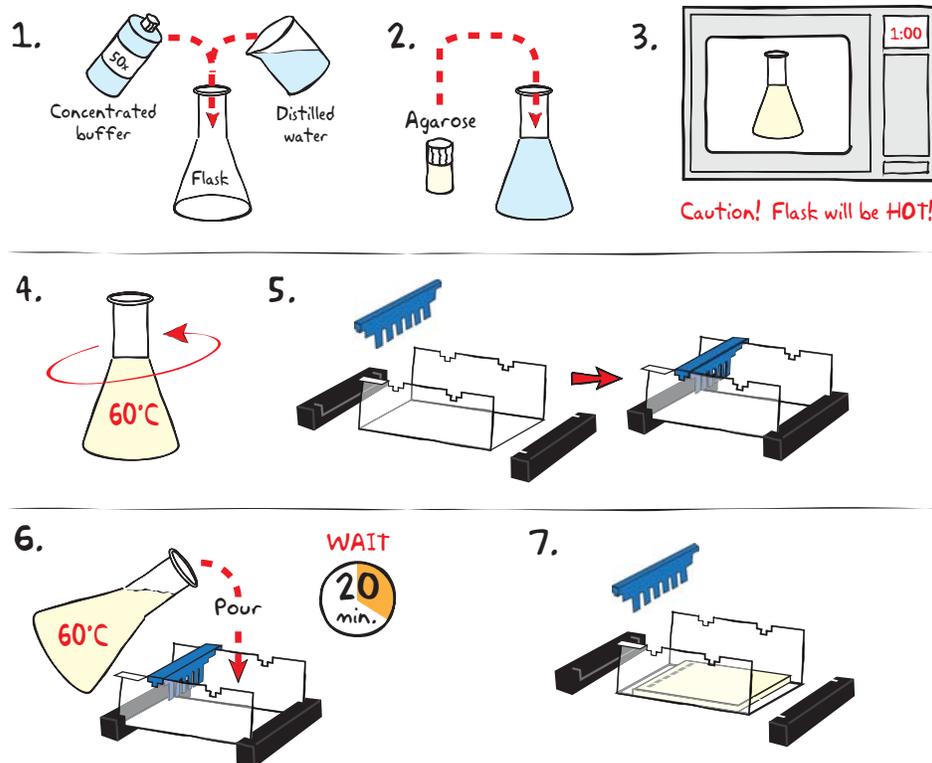
1. **LABEL** a 0.2 ml PCR tube with the sample and your initials
2. **ADD** 20 µl primer mix, 5 µl extracted DNA and the PCR EdvoBead™ to the labeled 0.2 ml tube.
3. **MIX** the PCR sample. Make sure the PCR EdvoBead™ is completely dissolved.
4. **REPEAT** steps 1 through 3 with the control sample provided by your instructor.
5. **CENTRIFUGE** the sample for a few seconds to collect the sample at the bottom of the tube.
6. **AMPLIFY** DNA using PCR
PCR cycling conditions:
 - Initial denaturation 95° C for 5 minutes
 - 94° C for 45 seconds
 - 50° C for 45 seconds
 - 72° C for 90 seconds
 } 30 cycles
 - Final Extension 72° C for 7 minutes
7. After PCR, **ADD** 5 µl of 10x Gel Loading Solution to the sample. **PLACE** tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis.



OPTIONAL STOPPING POINT:

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

Module III: Separation of PCR Products by Electrophoresis



IMPORTANT:

7 x 14 cm gels are recommended. Place well-former template (comb) in the first set of notches.

If you are unfamiliar with agarose gel prep and electrophoresis, detailed instructions and helpful resources are available at www.edvotek.com



Wear gloves and safety goggles

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

Table A Individual 1.0% UltraSpec-Agarose™ Gel

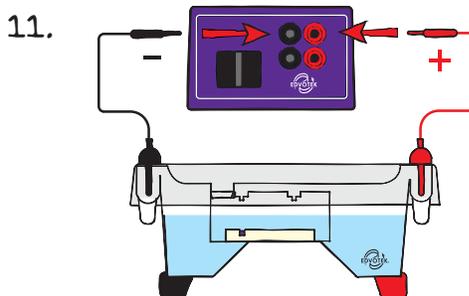
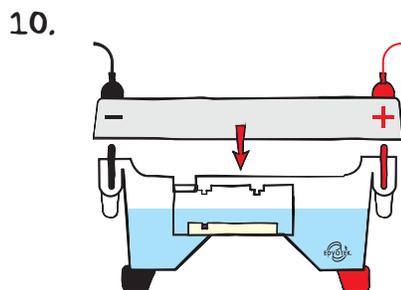
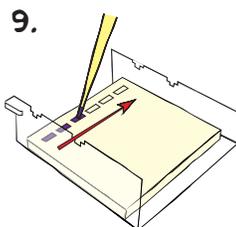
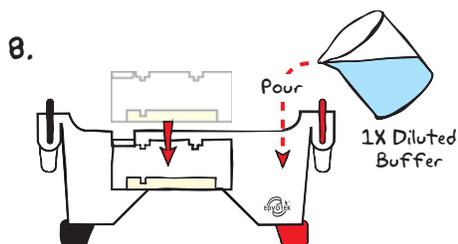
Size of Gel Casting tray	Concentrated Buffer (50x)	Distilled Water	Amt of Agarose	= TOTAL Volume
7 x 7 cm	0.5 ml	24.5 ml	0.25g	25 ml
7 x 14 cm	1.0 ml	49.0 ml	0.50 g	50 ml

Module III: Separation of PCR Products by Electrophoresis

Includes EDVOTEK's All-NEW EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

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



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

- PLACE** gel (on the tray) into electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
- LOAD** the entire volume (30 µl) into the well in the order indicated by Table 1, right.
- PLACE** safety cover. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
- CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
- After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to **STAINING** the agarose gel.

Lane	Sample Name
1	EdvoQuick™ DNA Ladder
2	<i>E. coli</i> DNA (Positive Control)
3	Sterile Water (Negative Control)
4	DNA extracted from water contaminated in the laboratory or control sample
5	DNA extracted from environmental water sample

Table 1: Sample Table

Table B

1x Electrophoresis Buffer (Chamber Buffer)

EDVOTEK Model #	Total Volume Required	50x Conc. Buffer	Dilution + Distilled Water
M6+	300 ml	6 ml	294 ml
M12	400 ml	8 ml	392 ml
M36	1000 ml	20 ml	980 ml

Table C

Time and Voltage Guidelines (1.0% - 7 x 14 cm Agarose Gel)

Volts	Recommended Time	
	Minimum	Maximum
125	55 min.	1 hour 15 min.
70	2 hours 15 min.	3 hours
50	3 hours 25 min.	5 hours

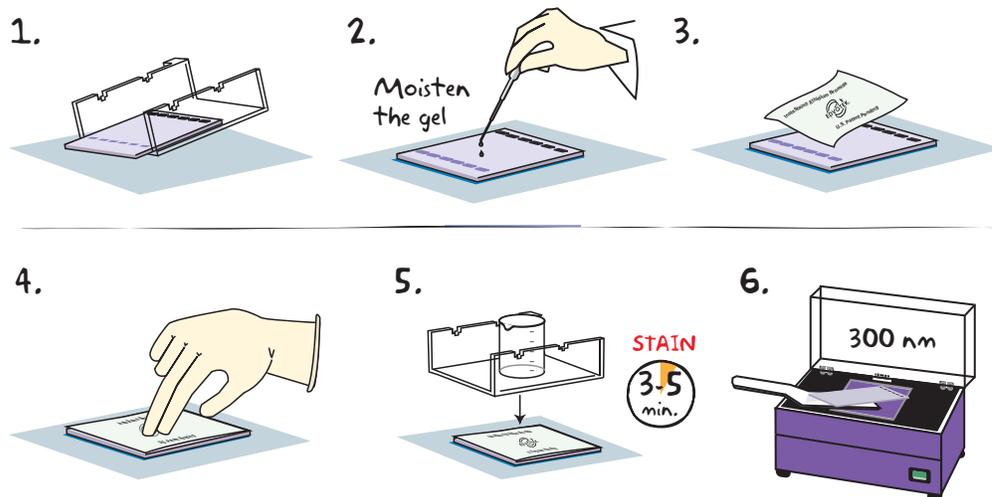


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Module IV-A: Staining with InstaStain® Ethidium Bromide

Preferred Method

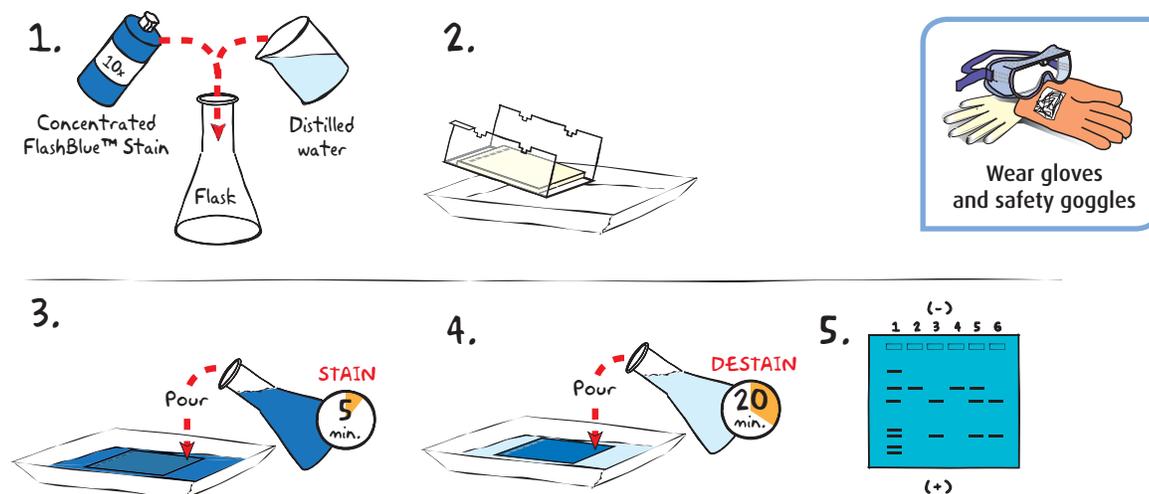


1. Carefully **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray on to a piece of plastic wrap on a flat surface. **DO NOT STAIN GELS IN THE ELECTROPHORESIS APPARATUS.**
2. **MOISTEN** the gel with a few drops of electrophoresis buffer.
3. Wearing gloves, **REMOVE** and **DISCARD** the clear plastic protective sheet from the unprinted side of the InstaStain® card(s). **PLACE** the unprinted side of the InstaStain® Ethidium Bromide card(s) on the gel. You will need 2 cards to stain a 7 x 14 cm gel.
4. With a gloved hand, **REMOVE** air bubbles between the card and the gel by firmly running your fingers over the entire surface. Otherwise, those regions will not stain.
5. **PLACE** the casting tray on top of the gel/card stack. **PLACE** a small weight (i.e. an empty glass beaker) on top of the casting tray. This ensures that the InstaStain® Ethidium Bromide card is in direct contact with the gel surface. **STAIN** the gel for 3-5 minutes.
6. **REMOVE** the InstaStain® Ethidium Bromide card(s). **VISUALIZE** the gel using a mid-range ultraviolet transilluminator (300 nm). DNA should appear as bright orange bands on a dark background.



BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!

Module IV-B: Staining with FlashBlue™



- DILUTE** 10 ml of 10x concentrated FlashBlue™ with 90 ml of water in a flask and **MIX** well.
- REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off of the casting tray into a small, clean gel-staining tray.
- COVER** the gel with the 1x FlashBlue™ stain solution. **STAIN** the gel for 5 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 5 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
- TRANSFER** the gel to a second small tray. **COVER** the gel with water. **DESTAIN** for at least 20 minutes with gentle shaking (longer periods will yield better results). Frequent changes of the water will accelerate destaining.
- Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Alternate Protocol:

- DILUTE** one ml of concentrated FlashBlue™ stain with 149 ml dH₂O.
- COVER** the gel with diluted FlashBlue™ stain.
- SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.

Study Questions

1. Name three water-borne microorganisms that can cause disease in humans. What are some symptoms of infection by these microorganisms?
2. What are the four guidelines established by the EPA to prevent water contamination?
3. List and describe the three steps of PCR. How can PCR be used to monitor water contamination?
4. What is non-point water pollution? Why is it important to be aware of this type of pollution?

Instructor's Guide

OVERVIEW OF INSTRUCTOR'S PRELAB PREPARATION:

This section outlines the recommended prelab preparations and approximate time requirement to complete each prelab activity.

Preparation For:	What to do:	When:	Time Required:
Module I: Isolation of Bacterial DNA from Contaminated Water	Prepare contaminated water samples	No more than one hour before class	45 min.
	Preheat waterbath	At least 30 min. before performing the experiment	15 min.
	Prepare Lysis Buffer	No more than thirty minutes before experiment is performed.	10 min.
Module II: Amplification of <i>E.coli</i> DNA	Program thermal cycler	Any time before performing the experiment.	15 min.
	Prepare and aliquot various reagents (Primer, DNA template, ladder, etc.)	One day to 30 minutes before performing the experiment	30 min.
Module III: Separation of PCR Products by Electrophoresis	Prepare diluted TAE buffer	Up to one hour before performing the experiment.	45 min.
	Prepare molten agarose and pour gel		
Module VII: Staining Agarose Gels	Prepare staining components	Up to 10 min. before the class period.	10 min.

Pre-Lab Preparations

This kit contains enough reagents to analyze 25 samples using PCR. We recommend splitting the class into 12 groups as follows:

Set A (6 groups):

Module I: These students will extract DNA from water contaminated by *E.coli* in the laboratory (see below).

Module II: These students will perform PCR using the Negative Control (sterile water).

Set B (6 groups):

Module I: These students will extract DNA from water collected from the environment.

Module II: These students will PCR using the Positive Control (purified *E.coli* DNA).

In Module III, six agarose gels will be prepared. Two student groups will share one gel. Each gel should include the samples from Set A and Set B.

MODULE I: ISOLATION OF DNA FROM BACTERIAL CULTURES

Prepare the Contaminated Water Samples:

1. Label a 50 ml conical tube with "*E.coli*".
2. Add 15 ml of distilled or deionized water to the conical tubes.
3. Using a sterile loop, transfer five *E.coli* BactoBeads™ to the labeled conical tube. Cap and mix gently.
4. Incubate the "contaminated" water sample for 15 minutes at room temperature. Samples can be placed on a shaking platform if available.
5. Label and dispense 1.5 ml of each sample into an appropriately labeled 1.5 ml screw-cap tubes.

FOR MODULE I

Each Student Group will need:

- 1 1.5 ml screw-cap tube containing *E.coli* contaminated water (Set A)
OR
Water collected from the environment (Set B)
- 1 0.5 ml snap-top tube containing Lysis Buffer
- 2 2 ml screw-cap tubes

Prepare the Bacterial Lysis Reagents

(Prepare no more than one hour before starting the experiment).

1. Add 50 μ l of Tris buffer (E) to each of the tubes containing Proteinase K. Allow the Proteinase K to rehydrate for a minute before mixing the sample thoroughly by pipetting up and down
2. Add all of the dissolved Proteinase K to the tube containing the chelating agent (G).
3. Add an additional 1.5 ml of the Tris buffer (E) to the tube containing the chelating agent and Proteinase K.
4. Mix the Tris buffer solution with the chelating agent by inverting several times. To better mix the solution, cut several millimeters off of a micropipet tip to enlarge the opening. Mix the solution by pipetting up and down using this special tip.
5. Dispense 130 μ l of the solution into thirteen 0.5 ml snap-top tubes labeled "Lysis Buffer". Mix the solution thoroughly between each aliquot. At this point, the lysis buffer can no longer be stored. It should be used as soon as possible.

Pre-Lab Preparations

MODULE II: AMPLIFICATION OF *E. coli* DNA

Preparation of the *E. coli* Primer Solution

1. Thaw the Primer Mix Concentrate on ice.
2. Add 1 ml of Tris buffer (E) to the tube of Primer Mix Concentrate (B). Cap tube and mix.
3. Aliquot 60 μ L of the diluted Primer Mix into 13 labeled 0.5 ml snap-top microcentrifuge tubes.

Preparation of the *E. coli* DNA (Positive Control)

1. Thaw the tube of Control DNA Concentrate on ice.
2. Add 20 μ L of Tris buffer to the tube containing the Control DNA Concentrate. Pipet up and down to mix.
3. Dispense 6 μ L of the diluted control DNA into six 0.5 ml microcentrifuge tubes. Label these tubes "+ control".

Preparation of the Negative Control

1. Dispense 7 μ L of the sterile water into six 0.5 ml microcentrifuge tubes. Label these tubes "- control".

PCR Amplification

The thermal cycler should be programmed for the following cycles:

- Initial Denaturation: 95° C for 5 min.
- 30 cycles: 94° C for 45 sec, 50° C for 45 sec, 72° C for 90 sec.
- Final Extension: 72° C for 7 min.

At the completion of the cycling, the thermal cycler can be programmed to hold the samples at 4° C overnight.

FOR MODULE II

Each Group should receive:

- 1 2 ml screw top tube containing extracted DNA (Module I)
- 1 0.5 ml snap-top tube containing positive control *E. coli* DNA (Set A) OR the negative control sterile water (Set B)
- 1 0.5 ml snap-top tube containing *E. coli* primer solution
- 2 PCR EdvoBeads™
- 2 0.2 ml snap-top PCR tubes

Pre-Lab Preparations

MODULE III: SEPARATION OF PCR PRODUCTS BY ELECTROPHORESIS

Preparation of Agarose Gels

This experiment requires one 1.0% agarose gel per student group. For best results, we recommend using a 7 x 14 cm gel. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation:

Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module VI in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water and agarose powder.

Batch Gel Preparation:

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

Preparing Gels in Advance:

Gels may be prepared ahead and stored for later use. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks.

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

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

Additional Materials:

Each 1.0% gel should be loaded with the EdvoQuick™ DNA ladder and PCR reactions from one or two student groups.

- Aliquot 30 μl of the EdvoQuick™ DNA ladder (C) into labeled microcentrifuge tubes and distribute one tube of EdvoQuick™ DNA ladder per gel.

NOTE:

Accurate pipetting is critical for maximizing successful experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students are unfamiliar with using micropipets, we recommend performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

FOR MODULE III Each Group should receive:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- EdvoQuick DNA ladder (30 μl)

NOTE:

QuickGuide instructions and guidelines for casting various agarose gels can be found on our website.

www.edvotek.com/quick-guides

Pre-Lab Preparations

MODULE IV: STAINING

InstaStain® Ethidium Bromide (*PREFERRED METHOD*)

InstaStain® Ethidium Bromide provides the sensitivity of ethidium bromide while minimizing the volume of liquid waste generated by staining and destaining a gel. An agarose gel stained with InstaStain® Ethidium Bromide is ready for visualization in as little as 3 minutes! Each InstaStain® card will stain 49 cm² of gel (7 x 7 cm). You will need 2 cards to stain a 7 x 14 cm gel.

Use a mid-range ultraviolet transilluminator (Cat. #558) to visualize gels stained with InstaStain® Ethidium Bromide. **BE SURE TO WEAR UV-PROTECTIVE EYEWEAR!**

- Standard DNA markers should be visible after staining even if other DNA samples are faint or absent. If bands appear faint, repeat staining with a fresh InstaStain card for an additional 3-5 min. If markers are not visible, troubleshoot for problems with electrophoretic separation.
- Ethidium bromide is a listed mutagen. Wear gloves and protective eyewear when using this product. UV protective eyewear is required for visualization with a UV transilluminator.
- InstaStain® Ethidium Bromide cards and stained gels should be discarded using institutional guidelines for solid chemical waste.

FlashBlue™

FlashBlue™ can be used as an alternative to Ethidium Bromide in this experiment. However, FlashBlue™ is less sensitive than InstaStain® Ethidium Bromide and will take a longer time to obtain results.

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

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

Photodocumentation of DNA (Optional)

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

FOR MODULE IV-A Each Group should receive:

- 2 InstaStain® cards per 7 x 14 cm gel



Wear gloves and safety goggles

FOR MODULE IV-B Each Group should receive:

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

Experiment Results and Analysis



Includes EDVOTEK's All-NEW EdvoQuick™ DNA Ladder

- Better separation
- Easier band measurements
- No unused bands

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



Lane	Sample Name	Molecular Weight (bp)
1	EdvoQuick™ DNA Ladder	---
2	<i>E.coli</i> DNA (Positive Control)	1475
3	Sterile Water (Negative Control)	---
4	DNA extracted from water contaminated in the laboratory	1475
5	DNA extracted from environmental water sample*	1475

Note – Environmental samples may or may not contain *E. coli* bacteria. As such, no DNA would be amplified in water samples that do not contain *E. coli* bacteria.

In some samples, a diffuse, small-molecular weight band, known as a "primer dimer", may be present below the 200 bp marker (not observed in photo shown). This is a PCR artifact and can be ignored. Other minor bands may also appear due to nonspecific primer binding and the subsequent amplification of these sequences.

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

Appendices

- A EDVOTEK® Troubleshooting Guide
- B Preparation and Handling of PCR Samples With Wax
- C Bulk Preparation of Agarose Gels
- D Optional Extension Activity: Standard Plate Count

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

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Appendix A

EDVOTEK® Troubleshooting Guides

DNA EXTRACTION

PROBLEM:	CAUSE:	ANSWER:
Poor DNA Extraction	Samples not mixed well enough during extraction	In addition to flicking the tube, vortex or pipet up and down to mix the sample.
	Proteinase K inactive because it was prepared too far in advance.	Prepare Proteinase K within 30 minutes of use.
	Water bath not at proper temperature	Use a thermometer to confirm water bath set point.
	Not enough DNA	Repeat extraction with more bacteria.
There is sediment in the extracted DNA	The chelating agent was transferred to the tube.	Centrifuge the sample again and move supernatant to a fresh tube. Take care to avoid pellet.
The extracted DNA is very cloudy	Cellular debris from pellet transferred to tube	Centrifuge sample again and move supernatant to a fresh tube. Take care to avoid pellet.
	Cellular debris not separated from supernatant	Centrifuge sample again. If possible, centrifuge at a higher speed. Move cleared supernatant to a fresh tube.

Appendix A

EDVOTEK® Troubleshooting Guides

PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after PCR	Sample has evaporated	<p>Make sure the heated lid reaches the appropriate temperature.</p> <p>If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax (see Appendix B for details)</p> <p>Make sure students close the lid of the PCR tube properly.</p>
	Pipetting error	Make sure students pipet 20 µL primer mix and 5 µL extracted DNA into the 0.2 mL tube.
The ladder, control DNA, and bacterial PCR products are not visible on the gel.	The gel was not prepared properly.	<p>Ensure that the electrophoresis buffer was correctly diluted.</p> <p>Gels of higher concentration (> 0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of "clumps" and glassy granules before pouring gels.</p> <p>The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.</p>
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
After staining, the ladder and control PCR products are visible on the gel but some bacterial samples are not present.	Bacterial DNA sample was not concentrated enough.	Poor DNA extraction. Repeat Module I (Isolation of Bacterial DNA)
	Bacterial DNA sample was degraded.	If DNA is not used right after extraction, store sample at -20°C.
	Wrong volumes of DNA and primer added to PCR reaction.	Practice using pipettes
	The chelating agent was transferred to the PCR sample.	The chelating agent inhibits the activity of <i>Taq</i> polymerase. Repeat PCR, but avoid any sediment at bottom of tube.
Some bacterial samples have more/less amplification than others.	Concentration of DNA varies by sample.	There is an inherent variability in the extraction process.
Low molecular weight band in PCR samples	Primer dimer	Low concentration of extracted DNA in PCR reaction.
DNA bands were not resolved.	Tracking dye should migrate at least 6 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 6 cm before staining and visualizing the DNA (approximately one hour at 125 V).
DNA bands fade when gels are kept at 4°C.	DNA stained with FlashBlue™ may fade with time	Re-stain the gel with FlashBlue™

Appendix B

Preparation and Handling of PCR Samples with Wax

ONLY For Thermal Cyclers WITHOUT Heated Lids, or Manual PCR Using Three Waterbaths

Using a wax overlay on reaction components prevents evaporation during the PCR process.

How to Prepare a Wax overlay

1. Add PCR components to the 0.2 ml PCR Tube as outlined in Module II.
2. Centrifuge at full speed for five seconds to collect sample at bottom of the tube.
3. Using clean forceps, add one wax bead to the PCR tube.
4. Place samples in PCR machine and proceed with Module II.

Preparing PCR Samples for Electrophoresis

1. After PCR is completed, melt the wax overlay by heating the sample at 94° C for three minutes or until the wax melts.
2. Using a clean pipette, remove as much overlay wax as possible.
3. Allow the remaining wax to solidify.
4. Use a pipette tip to puncture the thin layer of remaining wax. Using a fresh pipette tip, remove the PCR product and transfer to a new tube.
5. Add 5 µl of 10x Gel Loading Buffer to the sample. Proceed to Module III to perform electrophoresis.

Appendix C

Bulk Preparation of Agarose Gels

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

Bulk Electrophoresis Buffer

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

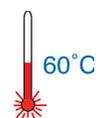
Table D Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 ml		2,940 ml	3000 ml (3 L)

Batch Agarose Gels (1.0%)

For quantity (batch) preparation of 1.0% agarose gels, reference Table E.

- Use a 500 ml flask to prepare the 1X Diluted gel buffer.
- Pour the measured UltraSpec-Agarose™ into the prepared buffer. Refer to Table E for the mass. Swirl to disperse clumps.
- With a marking pen, indicate the level of solution volume on the outside of the flask.
- Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
- Cool the agarose solution to 60° C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.
- Dispense the required volume of cooled agarose solution for casting each gel. Measure 25 ml for each 7 x 7 cm gel, 50 ml for each 7 x 14 cm gel. ***For this experiment, 7 x 14 cm gels are recommended.***
- Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Then proceed with preparing the gel for electrophoresis.

Table E Batch Preparation of 1.0% UltraSpec-Agarose™						
Amt of Agarose	+	50x Conc. Buffer	+	Distilled Water	=	Diluted Buffer (1x)
3.0 g		6.0 ml		294 ml		300 ml
4.0 g		8.0 ml		392 ml		400 ml



NOTE:

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

NOTE:

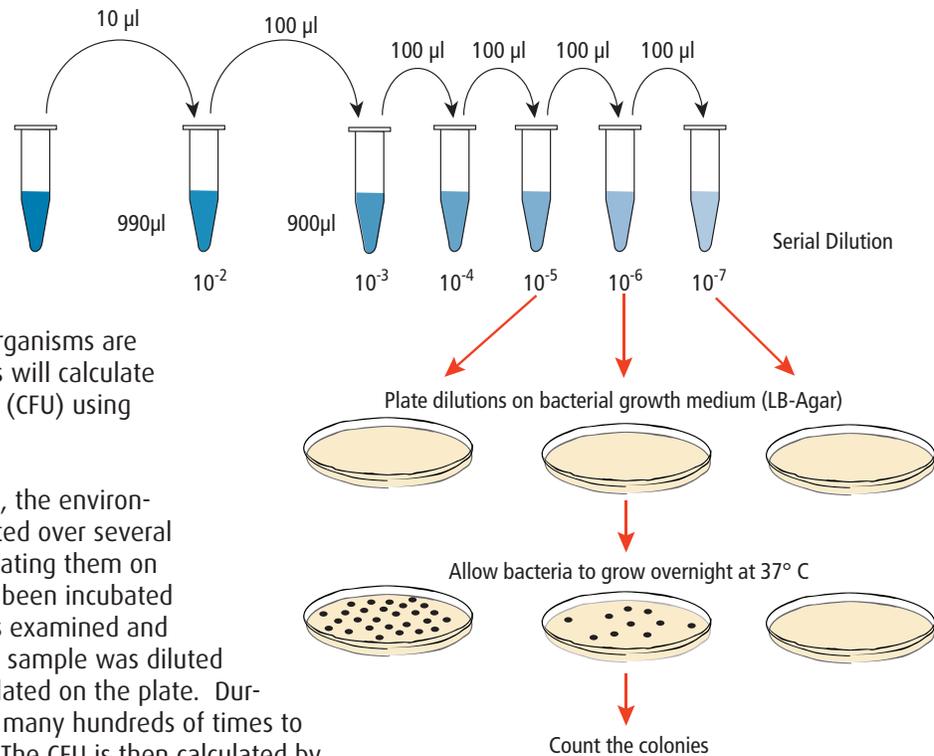
QuickGuide instructions and guidelines for casting various agarose gels can be found on our website.
www.edvotek.com/quick-guides

Appendix D

Optional Extension Activity: Standard Plate Count

In the main experiment, PCR is used to identify which (if any) microorganisms are present in a water sample. One drawback to using PCR for water quality testing is that this technique will amplify any DNA in the sample, even if it comes from dead or lysed bacteria. In order to accurately determine how many viable microorganisms are present in a water sample, scientists will calculate the number of Colony Forming Units (CFU) using the Standard Plate Count.

To perform the Standard Plate Count, the environmental water sample is serially diluted over several orders of magnitude before being plating them on nutrient agar. After the plates have been incubated for 24 hours, the microbial growth is examined and each colony is counted. If the water sample was diluted properly, single microbes will be isolated on the plate. During the incubation, each cell divides many hundreds of times to form visible masses called colonies. The CFU is then calculated by multiplying the number of colonies by the dilution factor of the water sample. For a valid Standard Plate Count, the number of colonies per plate should be not less than 25 and not more than 250.

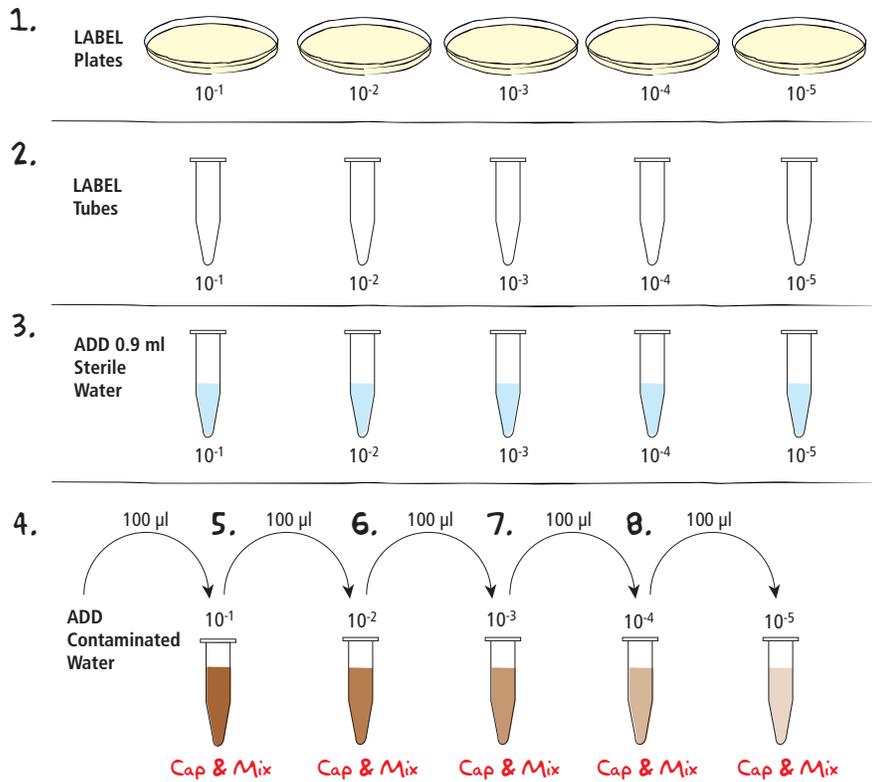


Formula: $\frac{\text{(\# of colonies)}}{\text{(dilution factor) (volume plated)}}$
--

In this extension activity, students will compare the traditional Standard Plate Count to PCR-based methods of microbe detection to detect and monitor the presence of bacterial contaminants in the water supply. Each group will isolate bacterial DNA from one of the diluted *E.coli* water samples. The dilutions will be made before starting Module I: DNA Extraction. To ensure that each dilution is represented in the PCR results, each group is assigned one of the five dilution samples for DNA extraction.

Appendix D

Optional Extension Activity: Standard Plate Count



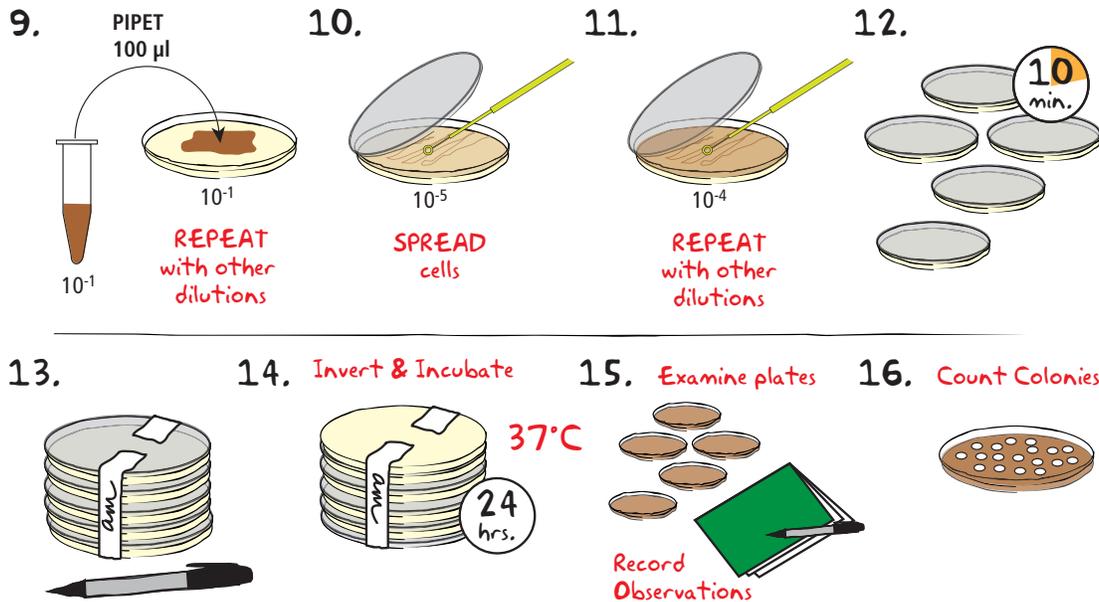
Student Protocol: Serial Dilution and Plating of Contaminated Water Samples

1. **LABEL** five nutrient agar plates as follows: 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ & 10⁻⁵.
2. **LABEL** five 1.5 ml microcentrifuge tubes as follows: 10⁻¹, 10⁻², 10⁻³, 10⁻⁴ & 10⁻⁵.
3. **ADD** 0.9 ml of sterile water to each of the labeled microcentrifuge tubes.
4. PIPET 100 µL of contaminated water into the tube labeled 10⁻¹. **CAP and MIX.**
5. Using a fresh pipet or tip, **PIPET** 100 µL of the 10⁻¹ sample into the tube labeled 10⁻². **CAP and MIX.**
6. Using a fresh pipet or tip, **PIPET** 100 µL of the 10⁻² sample into the tube labeled 10⁻³. **CAP and MIX.**
7. Using a fresh pipet or tip, **PIPET** 100 µL of the 10⁻³ sample into the tube labeled 10⁻⁴. **CAP and MIX.**
8. Using a fresh pipet or tip, **PIPET** 100 µL of the 10⁻⁴ sample into the tube labeled 10⁻⁵. **CAP and MIX.**

continued

Appendix D

Optional Extension Activity: Standard Plate Count



9. **PIPET** 100 μL of the 10^{-1} sample onto the center of the LB agar plate labeled 10^{-1} . **REPEAT** this with each of the four remaining dilutions.
10. Starting with the most dilute sample (10^{-5}), **SPREAD** the cells over the entire plate using an inoculating loop. To prevent contamination when plating, **DO NOT** set the lid down on the lab bench. Instead, lift the lid of the plate just enough to allow for spreading. Be careful to avoid gouging the agar plate with the loop.
11. Using the same loop, **SPREAD** the next four dilutions as described in Step 10. Be sure to move from most dilute to most concentrated (10^{-4} , 10^{-3} , 10^{-2} , 10^{-1}).
12. **COVER** the plates and **WAIT** five to ten minutes for the cell suspension to be completely absorbed by the agar.
13. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the plates with your initials or group number.
14. **PLACE** the plates in an inverted position (agar side on top) in a 37°C incubator overnight (24 hours).
15. **EXAMINE** dilution plates. **RECORD** your observations in your lab notebook. Make note of size, color, and appearance of bacterial colonies.
16. **COUNT** the colonies on the plates. Only plates containing 25 – 250 colonies will be used to calculate the CFU. Use the appropriate dilution plate, **CALCULATE** the concentration of bacteria (CFU) using the formula below:

Formula: $\frac{\text{\# of colonies}}{(\text{dilution factor})(\text{volume plated})}$
--

Appendix D

Optional Extension Activity: Standard Plate Count

INSTRUCTOR'S GUIDE: SERIAL DILUTION AND PLATING OF CONTAMINATED WATER SAMPLES

If performing this extension activity with your class, each group should isolate bacterial DNA from one of their diluted water samples. The dilutions will be made before starting Module I: DNA Extraction. To ensure that each dilution is represented, **ASSIGN EACH GROUP** one of the five dilution samples for DNA extraction.

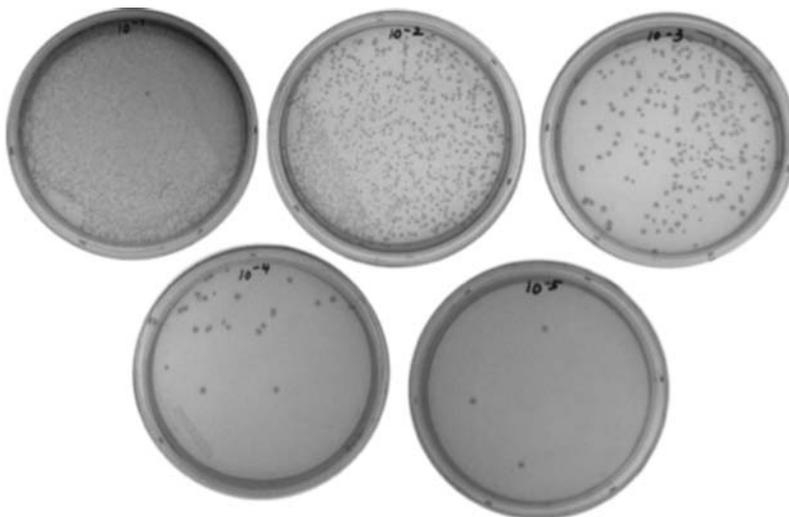
Experiment Time Table (Plate count ONLY. Refer to page 18 for Modules I-IV)

Preparation for:	What to do:	When:	Time required:
Serial Dilution and Plating of Contaminated Water Samples	Pour LB agar plates	2-7 days before use	One hour
	Aliquot sterile water	Up to 15 minutes before class	15 minutes
	Perform serial dilution	Before DNA extraction	30 minutes
	Experiment analysis	24h after plating cells.	50 minutes

Requirements for Five Groups:

- 2 Cat. 615 ReadyPour™ Luria Broth Agar Base, 170 ml
 - 3 Cat. 633 Small Petri Plates (60x15mm), 20
 - 1 Cat. 667 Sterile loops, package of 25
 - 1 Cat. 630 Microcentrifuge tubes, 500
 - 1 Cat. 648 Sterile conical tubes, 15 ml Bag of 25
- Sterile Water
 - Automatic micropipet and tips
 - *E. coli* "contaminated" water sample (Preparation on page 19)

RESULTS:



With PCR, students should be able to detect microbial DNA in each of the diluted water samples (main experiment). The relative intensity of the PCR products should be relatively consistent, though the most dilute samples may appear lighter. In contrast, with the Standard Plate Count, students will see the colony number decreasing as the relative dilution increases. This will allow the students to calculate the number of viable microorganisms present in the water sample.

Appendix D

Optional Extension Activity: Standard Plate Count

PRE-LAB PREPARATIONS:

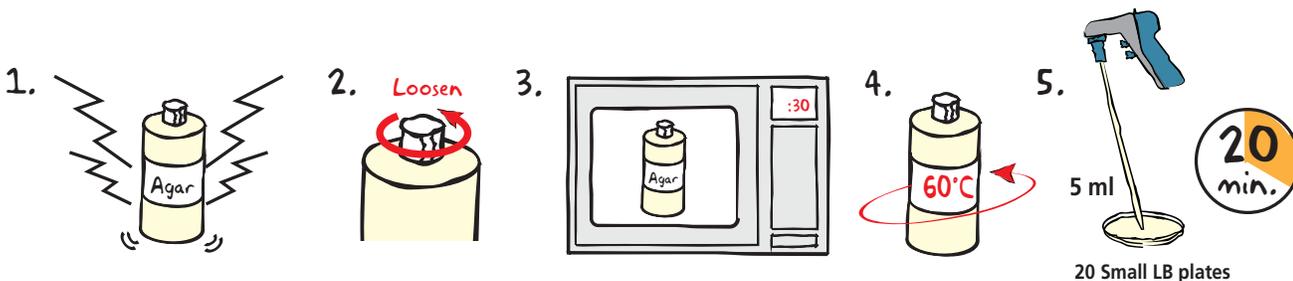
DISPENSE 6 ml sterile water into a 15 ml conical tube.

PREPARE one per student group.

Each Student Group Will Receive:

- 1 Contaminated water sample (*E.coli*)
- 5 Small LB-agar Plates
- 1 15 ml conical tube containing 6 ml sterile water
- 5 1.5 ml snap-top microcentrifuge tubes
- 1 Sterile loop

POURING PLATES



One bottle of ReadyPour™ medium will make 30 small LB plates. 60 small LB plates (Cat. 633) are necessary for this experiment.

1. **BREAK** solid ReadyPour™ medium into small chunks by vigorously squeezing and shaking the plastic bottle.
2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ medium bottle. This allows the steam to vent during heating.

CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.

3. **MICROWAVE** the ReadyPour™ medium on high for 30 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).
4. **COOL** the ReadyPour™ to 60°C with careful swirling to promote even dissipation of heat.
5. Using a fresh 10 ml pipet, **POUR** 5 ml of the medium into the small petri plates. **WAIT** at least twenty minutes for the LB-agar to solidify. For optimal results, leave plates at room temperature overnight.



Wear Hot Gloves and Goggles during all heating steps.

Store the plates at room temperature for no more than two days. Plates should be placed in a sealable plastic bag to ensure that they do not dry out. If plates are prepared more than two days in advance, they should be stored inverted in a plastic bag in the refrigerator (4° C). Warm the plates in a 37° C incubator for 30 minutes before use.