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RECENTLY UPDATED!

Edvo-Kit # 953

Edvo-Kit #953

WATER QUALITY TESTING III:

Multiplex PCR Analysis for Water Contamination

Experiment Objective:

In this experiment, students will use the Polymerase Chain Reaction (PCR) to detect several waterborne microorganisms simultaneously.

See page 3 for storage instructions.

Version 953.220315

SAMPLE LITERATURE
Please refer to included weblink for correct version

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

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Experiment Components

	Components	Storage	Check (✓)
•	PCR EdvoBeads™	Room Temperature, desiccated	❑
A	Universal DNA Buffer	-20°C Freezer	❑
B	TE buffer	-20°C Freezer	❑
C	LyphoPrimer™ Mix	-20°C Freezer	❑
D	LyphoControl™ (Complete PCR Control)	-20°C Freezer	❑
E	EdvoQuick™ DNA ladder	-20°C Freezer	❑
F	Proteinase K	-20°C Freezer	❑
•	*E. coli* BactoBeads™	4°C, desiccated	❑
•	*Bacillus subtilis* BactoBeads™	4°C, desiccated	❑
•	*Serratia marcescens* BactoBeads™	4°C, desiccated	❑

Experiment #953 is designed for 6 groups of students.

LyphoPrimer™

LyphoControl™

** Each PCR EdvoBead™ contains dNTP mixture, Taq DNA polymerase buffer, Taq DNA polymerase, $MgCl_2$, and reaction buffer.*

*** LyphoPrimer™ Mix contains E. coli-specific primers, Bacillus subtilis-specific primers, and Serratia marcescens-specific primers.*

REAGENTS & SUPPLIES

Store all components below at room temperature.

- UltraSpec-Agarose™ ❑
- Electrophoresis buffer (50X) ❑
- SYBR® Safe stain ❑
- FlashBlue™ stain ❑
- Microcentrifuge tubes ❑
- 0.2 mL PCR tubes ❑
- 50 mL conical tube ❑

NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing the experiment.

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.

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Requirements

- Thermal cycler (EDVOTEK® Cat. #541-542 recommended)
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Balance
- Microcentrifuge
- Water baths 55°C and 99°C (or boiling water)
- UV Transilluminator or Blue Light visualization (EDVOTEK® Cat. #557 or Cat. #555 recommended)
- White Light Visualization system (OPTIONAL - use if staining with FlashBlue™) (EDVOTEK® Cat. #552 recommended)
- UV safety goggles
- Automatic micropipettes (5-50 µL) with tips
- Microwave
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles
- Disposable vinyl or latex laboratory gloves
- Ice buckets and ice
- Distilled or deionized water
- Isopropanol

OPTIONAL EXTENSION ACTIVITY: STANDARD PLATE COUNT

Requirements for Six Groups:

1	Cat. #615	ReadyPour™ Luria Broth Agar Base, 170 mL
2	Cat. #633	Small Petri Plates (60 x 15 mm), 20
1	Cat. #630	Microcentrifuge tubes, 500
1	Cat. #648	15 mL sterile conical tubes, bag of 25

- Sterile loops, package of 25
- Sterile Water
- Automatic micropipette and tips
- *E. coli* "contaminated" water sample (preparation on page 19)

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Background Information

Water pollution is a universal problem because clean water is essential for human health, aquatic life, and agriculture. According to the World Health Organization (WHO), contaminated drinking water is the leading cause of infectious disease on the planet, resulting in almost two million deaths each year. Individuals with weak immune systems are especially vulnerable to the contaminants in polluted water, including chemicals, solid waste, and microorganisms. Determining whether water is contaminated requires special tests to identify what kind of contaminating organisms are present and how many of them there are.

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

Contamination enters the water supply in two main ways (Figure 1). **Point source** water pollution is any contaminant that enters the water from a single, readily identifiable source, such as a manufacturing plant or water treatment facility. These contaminants are called effluent waste and contain both sewage and/or chemical by-products. Since it is common practice to release effluent waste into nearby bodies of water, water near a polluting entity is strictly monitored and regulated by government agencies. **Non-point pollution** cannot be traced to a single, identifiable source and often results from everyday activities, such as the overflow of septic tanks, soil erosion, and water run-off from farms that contains animal feces.

Both point and non-point pollution are responsible for contaminating drinking water. 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, and federal regulations have been established to keep pathogens, chemicals, and other hazardous materials out of drinking water. Since over 15 million American households are located in rural areas that rely on private, untreated wells for drinking water, the U.S. Department of Agriculture (USDA) teamed up with the Environmental Protection Agency (EPA) to improve the quality of drinking water for people in rural areas in 2011.

Although drinking water in U.S. cities is generally safe, monitoring remains necessary because our water supply can still be contaminated by corrosion in aging water pipes and other environmental factors. A 1999 EPA study revealed that maximum contaminant levels were exceeded in more than 11% of the water systems in 14 states. From 2007-2008, the Centers for Disease Control (CDC) reported 36 waterborne disease outbreaks in 23 states, which resulted in over 4,000 human illnesses.

In order to minimize contamination, the EPA has established four major guidelines for protecting drinking water:

1) Prevention – keeping contaminants from entering our drinking water,
2) Management – reducing and/or eliminating contaminants that do enter a water source,
3) Monitoring and Compliance – evaluating water quality throughout the water treatment process, and
4) Citizen Action – people in the community limiting activities that can pollute water.

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MICROORGANISMS IN THE WATER SUPPLY

Waterborne microorganisms can cause severe illness (Figure 2). For example, in 1993 the protozoa *Cryptosporidium parvum* caused gastrointestinal distress in over 400,000 individuals in Milwaukee, Wisconsin. The outbreak resulted in 4,000 hospitalizations and more than 50 deaths. *Cryptosporidium* outbreaks have become more common over the past 15 years because the organism has become resistant to water treatment disinfectants, such as chlorine.

Another waterborne protozoa, *Giardia*, is very common in the gastrointestinal systems of farm animals and household pets and will cause gastrointestinal distress in humans if the water supply is contaminated with the feces of infected animals. Although rarely life threatening, 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, such as children, the elderly, and people with AIDS or who are undergoing cancer treatment.

Although most strains of *Escherichia coli* are relatively harmless or aid digestion in human beings; certain strains of the bacterium (O157:H7 in particular) produce a potent toxin that can cause acute bloody diarrhea, kidney failure, and even death. This disease-causing strain is present in the intestines of cattle, where it is not pathogenic; but it is easily transmitted to humans if they eat undercooked beef, eat raw fruits or vegetables, or drink unpasteurized milk. Thousands of *E. coli* O157:H7 infections also occur in the U.S. each year due to contaminated water.

In addition, run-off from farms can send 0157.H7 *E. coli* into drinking water or recreational water sources such as pools, lakes, water parks, and hot tubs. *E. coli* and other bacteria can cause swimmer's ear, skin rashes, and respiratory infections in addition to gastrointestinal distress. According to the CDC, the incidence of recreational water infections (RWIs) has been steadily increasing over the past twenty years. Between 2004–2008, the number of RWI *Cryptosporidium* infections alone went up 200%. These figures may be the result of crowded public pools, code violations in pool maintenance, and/or the increased pollution of recreational lakes and streams.

METHODS TO IDENTIFY MICROBIAL WATER CONTAMINATION

Many different types of bacteria can contaminate water. Testing for all of them would be very expensive, so the first organisms scientists look for are relatively harmless, easy-to-culture microbes called coliform bacteria. Coliform bacteria are found in the digestive tract of all warm-blooded animals where they help digest food. Unfortunately, a variety of bacteria that cause human disease – such as *Escherichia, Klebsiella, Enterobacter,* and *Serratia* – are also found in mammalian digestive tracts and all of them have coliform members of their species. If a water sample is negative for coliform bacteria, scientists assume that harmful microorganisms are also absent and the water is safe to drink. If a water sample is positive for coliforms, the sample is sent to a laboratory to determine whether more dangerous microbes are present. Coliforms can also be found in soil and on plants.

After a sample tests positive for coliform bacteria, scientists use the Polymerase Chain Reaction (PCR) test to see if dangerous microorganisms are present in the water. The PCR uses short, synthetic DNA sequences called primers that match DNA sequences in suspected bacteria. The primers fuse with very specific segments of bacterial DNA and multiply it so scientists have enough genetic material to identify exactly what kind of organisms are present. For example, if a PCR uses *Cryptosporidium*-specific primers and the test is positive, then *Cryptosporidium* must be present in the water sample.

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

Figure 2: Common Waterborne Illnesses

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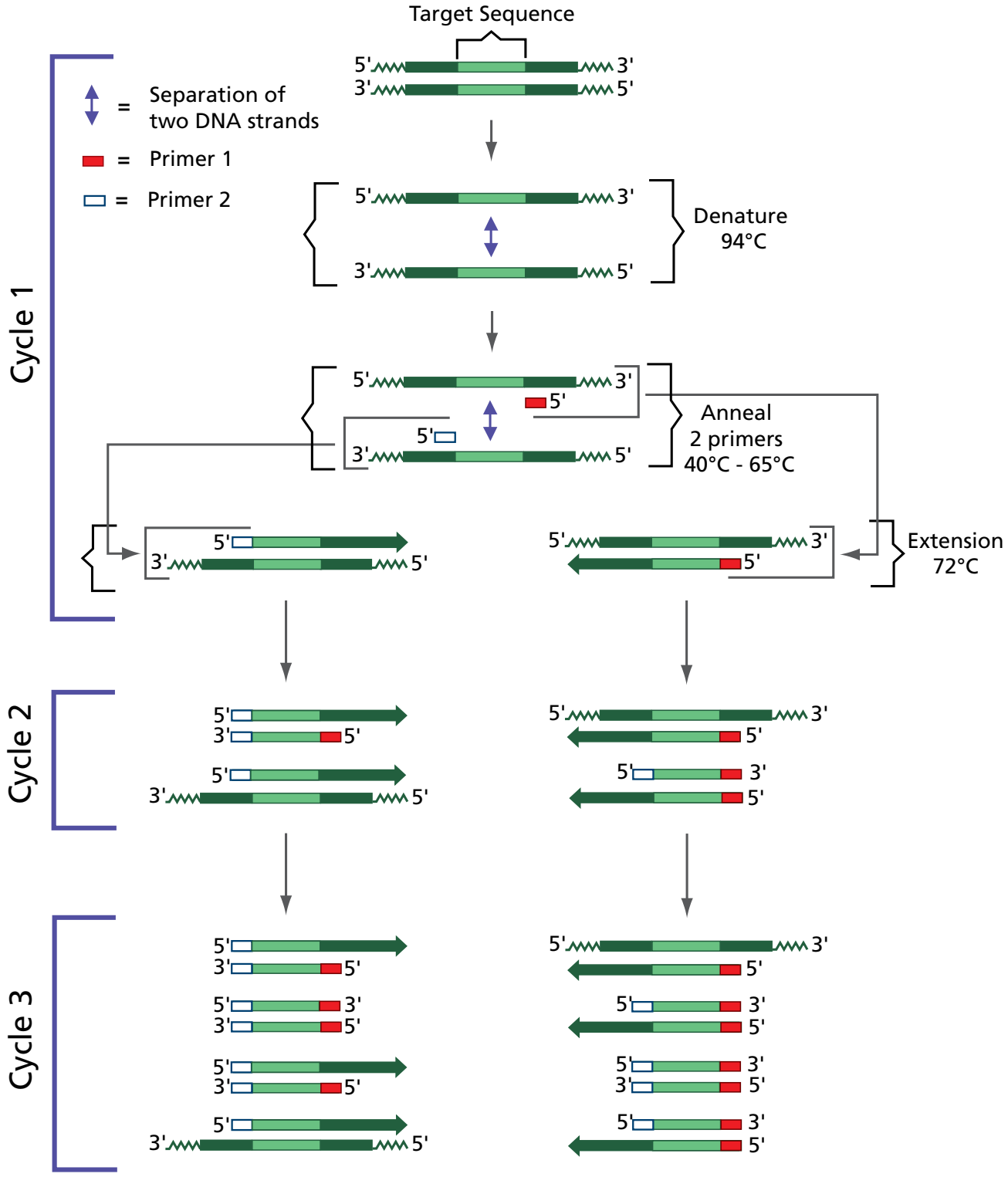

Figure 3:
DNA Amplification by the Polymerase Chain Reaction

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The first step in a PCR is extracting microbial DNA from the water sample. The extracted DNA is mixed with the primers, then heated to 94°C to unzip the DNA double helix into single strands. The sample is cooled to between 45-60°C, a temperature at which the primers can actively seek out and bond to identical sequences in the microbial DNA. This is called annealing. After annealing is complete, the temperature is raised to 72°C, which is the optimal temperature at which the primers can create a new strand of DNA. This is called extension. Each PCR cycle (denaturation, annealing, extension) takes a few minutes and doubles the amount of target DNA (Figure 3). This is called amplification and the DNA sample it produces is called an amplicon. 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 PCR machine, was created to rapidly heat and cool the samples.

PCRs can detect multiple organisms at the same time by using several primers. Each primer targets a unique, organism-specific gene that amplifies bacterial DNA at a different rate (Figure 4A). After amplification, agarose gel electrophoresis is used to separate DNA fragments according to size so they can be easily identified (Figure 4B). In this experiment, you will isolate DNA from water samples and perform PCR to detect three potential bacteria - *B. subtilis, S. marcescens,* and *E. coli.*

A

Bacteria	Size of Amplicon
B. subtilis	893 bp
S. marcescens	629 bp
E. coli	479 bp

B

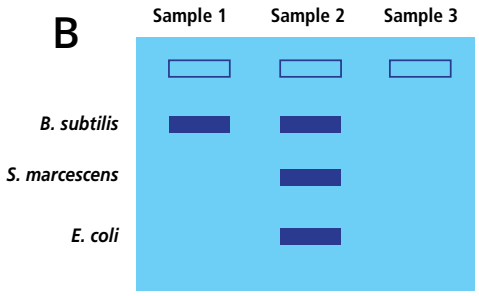

Figure 4: PCR analysis of waterborne bacteria. (A) Predicted sizes of amplicons based on DNA sequence. (B) Simulated gel result showing water samples tested by PCR.

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Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will use the Polymerase Chain Reaction (PCR) to detect several waterborne microorganisms simultaneously.

LABORATORY SAFETY

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

- Wear gloves and goggles while working in the laboratory.
- You will be using equipment that can be dangerous if used incorrectly, so use caution.
- Wear protective gloves when working with hot reagents like boiling water and melted agarose. DO NOT DRAW SAMPLES INTO PIPETS WITH YOUR MOUTH - USE MANUAL PIPET PUMPS.

Wear gloves and safety goggles

Even though the bacteria used in this experiment are not pathogenic, use standard safety measures to work with them.

- Always wear gloves and wash your hands thoroughly with soap and water after the experiment is completed.
- Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant after you complete the experiment.
- All materials – including petri dishes, pipettes, transfer pipet, loops, and tubes – that come in contact with bacteria should be disinfected BEFORE they are placed in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
  - Autoclave the items at 121°C for 20 minutes. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it on a metal tray to prevent any possibility of liquids or agar spilling into the sterilizer chamber. Tape sterilized petri dishes together and recap tubes before placing them in the garbage.
  - Soak the items in a 10% bleach solution overnight and then discard them. Wear gloves AND goggles when working with bleach.

LABORATORY NOTEBOOKS

Record the following in your laboratory notebook or on a separate worksheet.

Before Starting the Experiment:

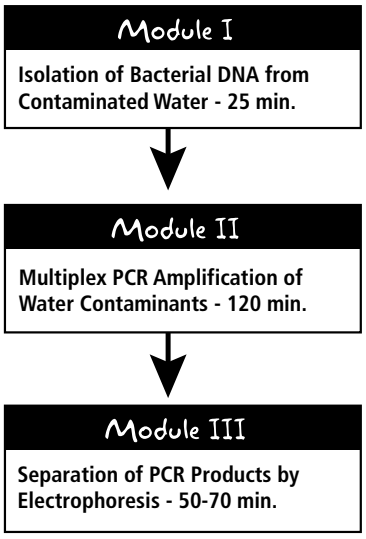
- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record your observations or photograph the results.

Following the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed if the experiment was repeated.
- Write a hypothesis that would reflect these changes.



NOTE: Experimental times are approximate.

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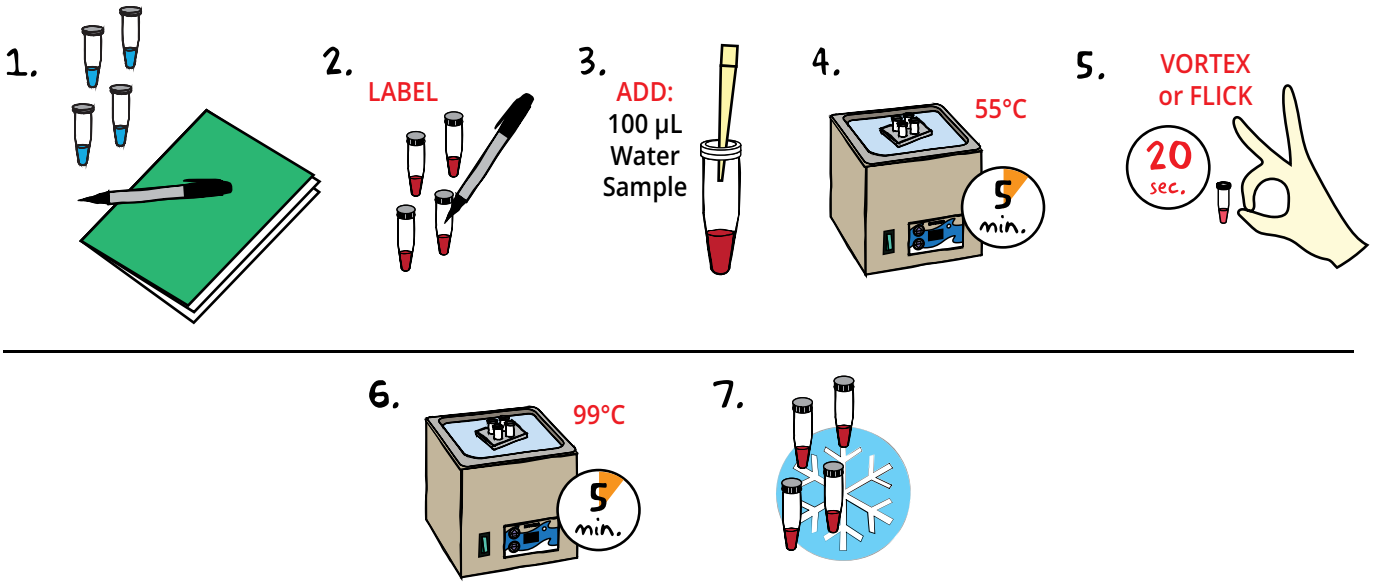
Module I: Isolation of Bacterial DNA from Contaminated Water



1. **OBTAIN** the four water samples from your instructor. **RECORD** the identities of each sample in your lab notebook (*E. coli, Serratia marcescens, Bacillus subtilis*, and the unknown water sample).

2. **OBTAIN** four 1.5 mL screw-top tubes containing Lysis Buffer. **LABEL** the tubes with the identities of your water samples.

3. **ADD** 100 µL of each water sample to the appropriate tube of Lysis Buffer and **MIX** by pipetting up and down for 5 seconds. ***NOTE: Take care to avoid cross-contamination between samples.***

4. **INCUBATE** all four samples in a 55°C water bath for 5 minutes.

5. **MIX** the samples by vortexing or flicking the tubes for 20 seconds.

6. **INCUBATE** all four samples for 5 minutes in a 99°C water bath or in boiling water on a hot plate.

7. **PLACE** all the samples on ice or into a tube rack to cool. The extracted DNA is now ready for Module II: Multiplex PCR Amplification of Water Contaminants.

OPTIONAL STOPPING POINT:
The samples may be stored at -20°C for amplification at a later time.

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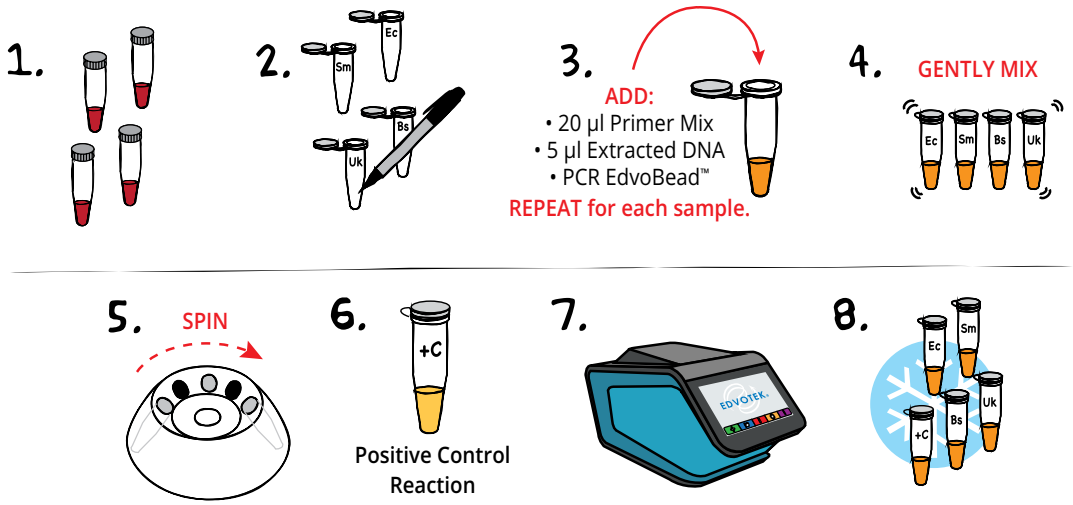
Module II: Multiplex PCR Amplification of Water Contaminants



1. **OBTAIN** your four red extracted DNA samples from Module I (*E. coli, Serratia marcescens*, *Bacillus subtilis,* and the unknown).
2. **LABEL** four fresh 0.2 mL snap-top PCR tubes with the samples' names and your initials.
3. To the 0.2 mL PCR tube labeled "*E. coli*", **ADD** 20 µL yellow primer mix, 5 µL red extracted *E. coli* DNA, and a PCR EdvoBead™. If mixed correctly, the final solution will be light orange. **REPEAT** this step for each of the remaining three samples.
4. **MIX** the PCR samples. Make sure the PCR EdvoBeads™ are completely dissolved.
5. **CENTRIFUGE** the samples for a few seconds to collect the sample at the bottom of the tubes.
6. **OBTAIN** a tube of "Positive Control Reaction" from your instructor. The positive control contains primers, template DNA and PCR components, and is ready for PCR amplification.
7. **AMPLIFY** the DNA using PCR.
   PCR cycling conditions:
   - Initial denaturation 94°C for 300 seconds (5 minutes).
   - 94°C for 30 seconds
   - 54°C for 30 seconds
   - 72°C for 60 seconds

   } 30 cycles
   - Final Extension 72°C for 240 seconds (4 minutes).
8. After PCR, **PLACE** the tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis.

NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing the experiment.

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

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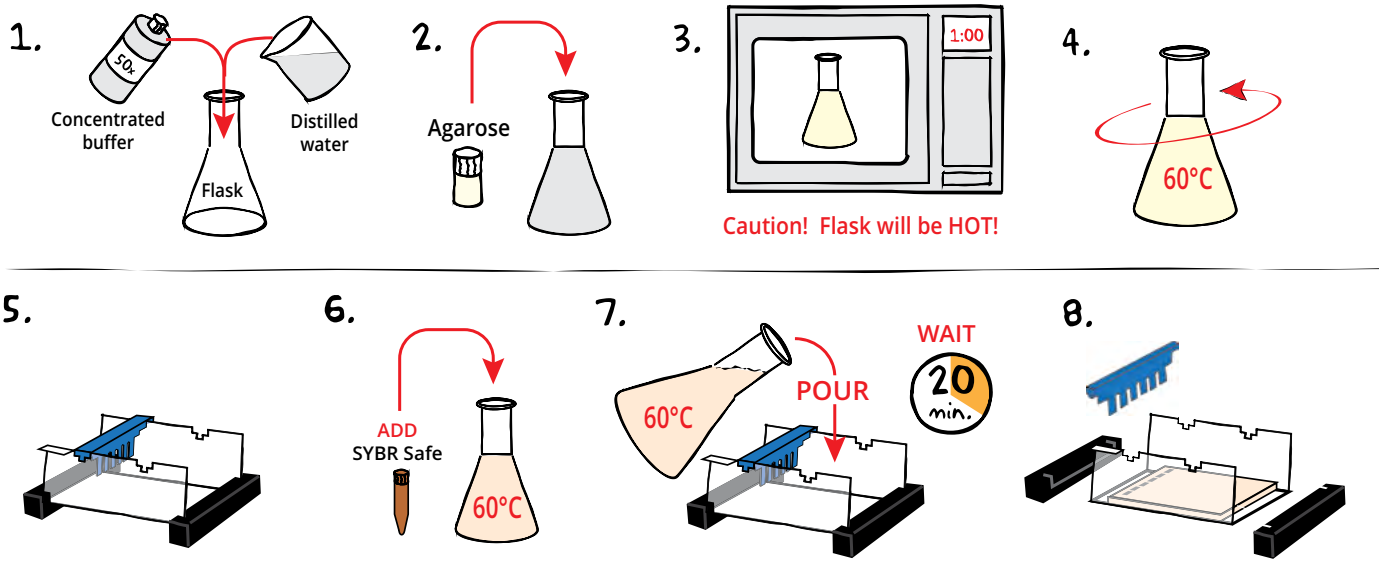
Module III: Separation of PCR Products by Electrophoresis



1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **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).
4. **COOL** agarose to 60°C with careful swirling to promote even dissipation of heat.
5. 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.
6. Before casting the gel, **ADD** ***diluted SYBR® Safe*** to the molten agarose and swirl to mix (see Table A).
7. **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.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Wear gloves and safety goggles

IMPORTANT:

14 x 7 cm gels are recommended. Place the 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

Table A — **Individual 1.0% UltraSpec-Agarose™ with SYBR® Stain**

Size of Gel Casting tray	Concentrated Buffer (50x) +	Distilled Water +	Amt of Agarose =	TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.5 mL	29.5 mL	0.3 g	30 mL	30 µL
10 x 7 cm*	1.0 mL	49.0 mL	0.5 g	50 mL	50 µL
14 x 7 cm	1.2 mL	58.8 mL	0.6 g	60 mL	60 µL

** Recommended gel volume for the EDGE™ Integrated Electrophoresis System.*

NOTE:
The listed volumes of SYBR® Safe are for stain that has already been diluted by the instructor.

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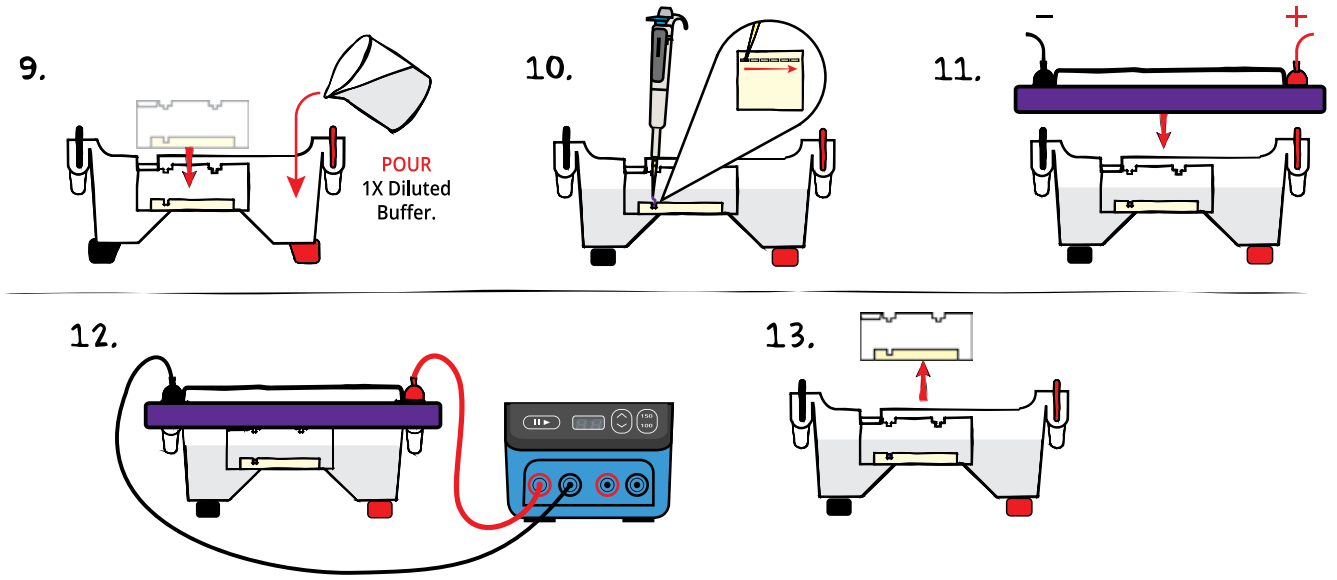
Module III: Separation of PCR Products by Electrophoresis, continued



9. **PLACE** the gel (on the tray) into the electrophoresis chamber. **COVER** the gel with 1X electrophoresis buffer (See Table B for recommended volumes). The gel should be completely submerged.
10. **LOAD** the entire volume (25 µL) into the well in the order indicated by Table 1, right.
11. **CHECK** that the gel is properly oriented, then **PLACE** the safety cover onto the chamber. Remember, the DNA samples will migrate toward the positive (red) electrode.
12. **CONNECT** the leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines).
13. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

Table 1: Gel Loading Table

Lane	Sample Name
1	EdvoQuick™ DNA Ladder
2	*E. coli*
3	*Serratia marcescens*
4	*Bacillus subtilis*
5	Unknown
6	Positive Control

OPTIONAL STOPPING POINT:
Gels can be stored for several days. Place gel in a watertight plastic bag with 2 mL of electrophoresis buffer and store in the refrigerator.

Table B — **1x Electrophoresis Buffer (Chamber Buffer)**

EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL

Table C — **Time and Voltage Guidelines (1.0% Agarose Gel)**

	Electrophoresis Model EDGE™ (10 x 7 cm gel)	M12 (14 x 7 cm gel)
Volts	Min/Max (minutes)	Min/Max (minutes)
150	15/25	30/40
100	25/35	40/50
75	NA	50/60

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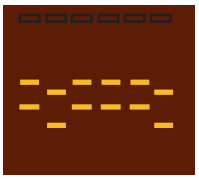
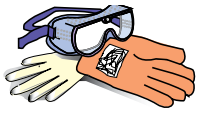
Module III: Separation of PCR Products by Electrophoresis, continued

14. 

15. 

16. 

VISUALIZING THE SYBR® GEL



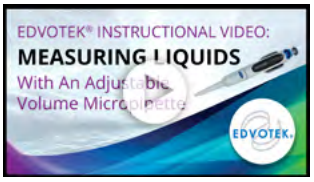
Be sure to wear UV goggles if using a UV transilluminator.

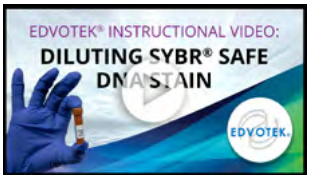
14. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator and turn the unit on. **ADJUST** the brightness to the desired level to maximize band visualization. DNA should appear as bright green bands on a dark background.

15. **PHOTOGRAPH** results.

16. **REMOVE** and **DISPOSE** of the gel and **CLEAN** the transilluminator surfaces with distilled water.

Related EDVOTEK® Instructional Videos





www.youtube.com/edvotekinc

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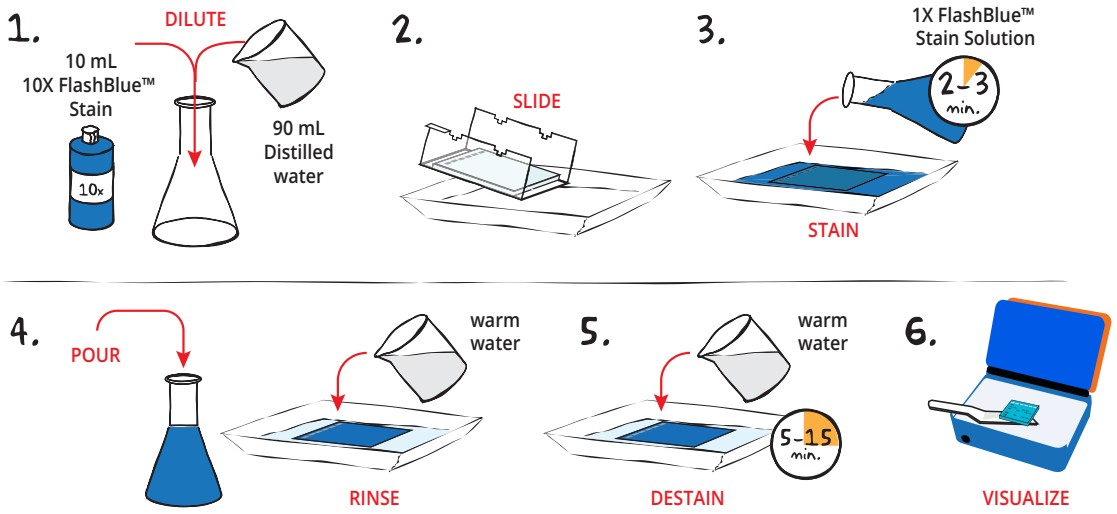
Module IV: Staining Agarose Gels with FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. ***IF staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.***



1. **DILUTE** 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.
2. **REMOVE** the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.
3. **COVER** the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**
4. **POUR** the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45°C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.
5. **COVER** the gel with clean, warm water (40-45°C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.
6. 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.

Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 149 mL distilled water.
2. **COVER** the gel with diluted FlashBlue™ stain.
3. **SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

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Study Questions

1. What is non-point water pollution? Why is it important to be aware of this type of pollution?
2. List and describe the three steps of PCR. How can PCR be used to monitor water contamination?
3. What are coliform bacteria? If a water sample tests positive for coliforms, what action should be taken and why?
4. What is *Giardia*?

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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 and DNA extraction solutions.	No more than one hour before class.	45 min.
	Prepare and aliquot Lysis Buffer.	Prepare on the day the students will be performing the experiment OR freeze for up to one week.	15 min.
	Preheat water baths.	Anytime before performing the experiment.	15 min.
Module II: Multiplex PCR Amplification of Water Contaminants	Program thermal cycler.	Anytime 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 Product by Electrophoresis	Prepare 1X Electrophoresis buffer and dilute SYBR® Safe.	Up to one week before performing the experiment.	45 min.
	Prepare molten agarose and pour gel.		
Module IV: Staining Agarose Gels with FlashBlue™ (OPTIONAL)	Prepare staining components.	The class period or overnight after the class period.	10 min.

NOTE:
The PCR cycling conditions have changed. Before running the experiment, confirm that the program matches the settings below:

- Initial denaturation 94°C for 300 seconds (5 minutes).
- 94°C for 30 seconds
- 54°C for 30 seconds } 30 cycles
- 72°C for 60 seconds
- Final Extension 72°C for 240 seconds (4 minutes).

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Pre-Lab Preparations: Module I

Divide the students into six groups. Each group will amplify DNA from 4 water samples and 1 positive control. If your thermocycler cannot hold 30 tubes, the student samples can be stored at -20°C, both before and after performing PCR, to allow for batches of reactions to be run. (If freezing your samples, we recommend adding the PCR EdvoBead™ immediately before performing PCR for best results.) Stopping points are marked in the student protocol.

ISOLATION OF DNA FROM BACTERIAL CULTURES

FOR MODULE I
Each Group should receive:

4	0.5 mL screw-cap tubes containing contaminated water samples (one of each)
4	1.5 mL screw-top tubes containing Lysis Buffer, on ice

NOTE:
The PCR experiment will produce positive results with very small numbers of bacteria. Take care to avoid cross-contamination during sample preparation.

Prepare the Contaminated Water Samples:

1. Label three 50 mL conical tubes with *"B. subtilis", "E. coli", and "S. marcescens"*. These will be the "contaminated" water samples.
2. Add 15 mL of distilled or deionized water to each of the three conical tubes.
3. Using a sterile loop, transfer five *B. subtilis* BactoBeads™ to the appropriately labeled conical tube. Cap and mix gently. Repeat this step for the other two cultures (*E. coli* and *S. marcescens)*, being sure to use a fresh sterile loop for each of the bacteria. ***NOTE: Take care to avoid cross-contamination.*** Shake to dissolve beads.
4. Incubate the three "contaminated" water samples for 15 minutes at room temperature. Samples can be placed on a shaking platform if available.
5. Prepare a mixture of bacteria for the "Unknown" by combining 3 mL of each bacteria in a 50 mL conical tube. Alternatively, collect a water sample from a local pond or stream. ***Please note: Samples collected from local water sources may not contain any of the bacteria identified by this experiment.***
6. Label and dispense 200 µL of each sample into the appropriately labeled 0.5 mL screw-cap tubes. Be sure to make six complete sets, one for each group.
   NOTE: If needed, the samples can be stored in the refrigerator after the incubation for up to a week. Wrap the lids in tape or parafilm to avoid contamination.

Prepare the Bacterial Lysis Reagents:

1. Add 1 mL of Universal DNA Buffer (A) to a 50 mL conical tube. Add 19 mL distilled water and mix well. Label the tube as "Lysis Buffer".
2. Resuspend each of the two tubes of Proteinase K with 50 µL of diluted Lysis Buffer from step 1. Allow the Proteinase K to rehydrate for one minute before mixing the sample thoroughly.
3. Add all of the dissolved Proteinase K back to the conical tube of Lysis Buffer.
4. Pipette 0.8 mL of the Lysis Buffer into 24 appropriately labeled 1.5 mL screw-top tubes. Each group will need 4 tubes for Module I.
   NOTE: Store prepared Lysis Buffer on ice for use on the same day, or freeze until needed.

Prepare the Water Baths:

You will need two water baths for Module I, one at 55°C and another at 99°C. If a second water bath is not available, you may substitute a beaker of boiling water or dry bath.

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Pre-Lab Preparations: Module II

FOR MODULE II
Each Group should receive:

4	1.5 mL screw-top tubes containing red extracted DNA (from Module I)
4	0.2 mL snap-top PCR tubes
1	100 µL Primer Mix
4	PCR EdvoBeads™
1	Tube of Positive Control Reaction

MULTIPLEX PCR AMPLIFICATION OF WATER CONTAMINANTS

The PCR primers are provided as a lyophilized mixture that must be rehydrated by the instructor before performing the experiment. The PCR EdvoBeads™ can be distributed prior to setting up the PCR – students or instructors can gently transfer the PCR EdvoBeads™ using gloved hands. Alternatively, beads can be gently "poured" from the vial into individual PCR tubes. After distributing the beads, it is important to close the PCR tubes securely to prevent the beads from absorbing moisture and becoming hard to resuspend prior to the experiment. ***NOTE: The PCR EdvoBeads™ are fragile, use care to not crush the beads while transferring to a PCR tube.***

This kit features the NEW EDVOTEK® LyphoControl™ and LyphoPrimer™. The reagents are also color coded so that a correctly assembled PCR reaction should appear orange in color. These innovations will help ensure experimental success.

LyphoPrimer™

LyphoControl™

Preparation of the Multiplex Primer Mix:

1. Thaw the TE buffer (B). Mix well before using.
2. Before preparing the primer mix, make sure the solid material is at the bottom of tube of LyphoPrimer™ Mix (C). If not, centrifuge the tube at full speed for 20 seconds or tap the tube on the lab bench.
3. Dilute the LyphoPrimer™ by adding 1 mL of TE Buffer to the tube. Cap and mix well and place on ice. The solution should be clear and yellow in color, and no solid pieces should remain.
4. Pipette 100 µL of the diluted Primer Mix into six labeled snap-top microcentrifuge tubes. Label these tubes “Primer Mix". Distribute one tube per group.

Preparation of the Control DNA:

1. Add 150 µL of TE buffer (B) to the tube containing LyphoControl™ Complete PCR Control (D). Pipette the solution up and down to mix.
2. Pipette 25 µL of the diluted Positive Control into six 0.2 mL PCR tubes (one for each group).
   NOTE: The LyphoControl™ already contains all necessary PCR components and does not need a PCR Edvobead™.

Additional Materials:

- Distribute four fresh 0.2 mL snap-top PCR tubes and four PCR EdvoBeads™ per group.

PCR Amplification:

The Thermal cycler should be programmed as outlined in Module II in the Student’s Experimental Procedure.

- Accurate temperatures and cycle times are critical. A pre-run for one cycle (takes approximately 3 to 5 min.) is recommended to check that the thermal cycler is properly programmed.
- For thermal cyclers that do not have a heated lid, it is necessary to place a layer of wax or mineral oil above the PCR reactions in the microcentrifuge tubes to prevent evaporation. Visit www.edvotek.com for more information.

NOTE:
PCR Cycling Conditions have changed. Please review your PCR program before performing the experiment.

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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 14 x 7 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 its own individual gel prior to conducting the experiment (see Module III in the Student's Experiment Procedures). 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 B).

SYBR® Safe Stain Preparation
Prepare diluted SYBR® Safe by adding 400 µL of 1X electrophoresis buffer to the tube of concentrated SYBR® Safe and tapping the tube several times to mix. For individual gel preparation, each group will need 50 µL of the diluted SYBR® Safe for a 14 x 7 cm gel. For Batch Gel Preparation, you will use the entire tube of SYBR® Safe (see Appendix B).

Preparing Gels in Advance
Gels may be prepared ahead and stored for later use. Solidified gels can be stored for up to a week in the refrigerator in plastic bags containing a small amount of buffer to prevent drying. We recommend adding only 2 mL of buffer to the bag to prevent SYBR® Safe Stain from diffusing out of the gel.

Do not store gels at -20° C because freezing will destroy them.

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.

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

If students do not know how to use micropipettes, we recommended 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
- Diluted SYBR® Safe Stain (25 µL)
- EdvoQuick DNA ladder (30 µL)

Additional Materials
Each 1.0% gel should be loaded with the EdvoQuick™ DNA ladder and PCR reactions from one student group.

- Pipette 30 µL of the EdvoQuick™ DNA ladder (E) into labeled microcentrifuge tubes and distribute one tube of EdvoQuick™ DNA ladder per gel.

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Pre-Lab Preparations: Module IV

STAINING AGAROSE GELS WITH FLASHBLUE™ (OPTIONAL)

Wear gloves and safety goggles

FlashBlue™ can be used as an alternative or in addition to SYBR® Safe in this experiment. If only staining with FlashBlue™, you can omit SYBR® Safe from the gel preparation. However, FlashBlue™ is less sensitive than SYBR® Safe and will take a longer time to obtain results. Alternatively, gels can be visualized first with SYBR® Safe and then with FlashBlue™.

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 develop in the destaining solution, resulting in dark blue DNA bands that contrast with a uniformly light blue background. A white light box is recommended for visualizing gels stained with FlashBlue™. (EDVOTEK® Cat. #552 or Cat. #557 is recommended.)

- Stained gels may be stored in destaining liquid for several weeks if they are refrigerated, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels should be discarded in the garbage and destaining solutions should be disposed of down the drain.

FOR MODULE IV
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

Photodocumentation of DNA (Optional):

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

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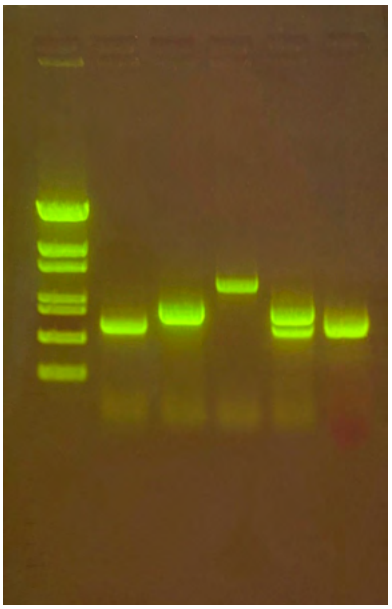
Experiment Results and Analysis



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	Extracted DNA - *E. coli*	479
3	Extracted DNA - *Serratia marcescens*	629
4	Extracted DNA - *Bacillus subtilis*	893
5	Unknown - *S. marcescens & E. coli*	629, 479
6	Positive Control	479

NOTE: *This gel shows representative results for the samples provided in the experiment. If environmental samples are used for the unknown, it is possible they may not contain any of the bacteria identified by this experiment. In this case, no amplicons would be produced by PCR.*

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

Appendices

A EDVOTEK® Troubleshooting Guide

B Bulk Preparation of Electrophoresis Buffer and Agarose Gels

C 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.	Store Lysis Buffer on ice after preparation or freeze until needed.
	Water bath not at proper temperature.	Use a thermometer to confirm water bath set point.
	Not enough DNA.	Repeat the extraction. Be careful that you do not aspirate pellet. Try pelleting more bacteria.

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Appendix A
EDVOTEK® Troubleshooting Guides

PCR AND ELECTROPHORESIS

PROBLEM:	CAUSE:	ANSWER:
There is very little liquid left in tube after PCR.	Sample has evaporated.	Make sure the heated lid reaches the appropriate temperature.
		If your thermal cycler does not have a heated lid, overlay the PCR reaction with wax (see Appendix B for details).
		Make sure students close the lid of the PCR tube properly.
	Pipetting error.	Students should pipette 20 µL primer mix and 5 µL extracted DNA into the 0.2 mL tube. The sample should be light green.
The ladder, control DNA, and bacterial PCR products are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		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.
		The proper buffer was not used for gel preparation. Make sure to use 1x Electrophoresis Buffer.
	The gel was not stained properly.	Ensure that SYBR® Safe was added to the gels before electrophoresis. 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.
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.
Individual DNA bands were not visible.	Tracking dye should migrate at least 6 cm from the wells to ensure adequate separation.	Be sure the gel runs 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.	Store gels in a minimal amount of buffer, 1-2 ml, to prevent diffusion of the DNA. Re-stain the gel with FlashBlue™.

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Appendix B

Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities that the whole class can share. Leftover 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 1X Electrophoresis Buffer

50x Conc. Buffer	+	Distilled Water	Total Volume 1X Buffer
60 mL		2,940 mL	3000 mL (3 L)

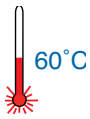
BATCH AGAROSE GELS (1.0%)

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

Table E
Batch Preparation of 1.0% UltraSpec-Agarose™

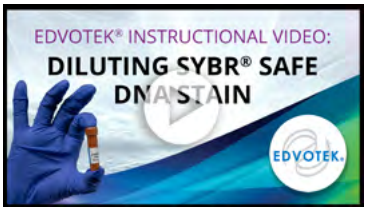
50x Conc. Buffer	+	Distilled Water	+	Amt of Agarose	=	Total Volume
6.0 ml		294 ml		3.0 g		300 ml
8.0 ml		392 ml		4.0 g		400 ml

1. Use a 500 mL flask to prepare the 1X electrophoresis buffer.
2. Pour the measured UltraSpec-Agarose™ into the prepared buffer. Refer to **Table E** for the mass. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.
4. 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.
5. 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.



6. Add the entire volume of ***diluted*** SYBR® Safe, prepared on page 21, to the cooled agarose.
7. 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 14 x 7 cm gel. ***For this experiment, 14 x 7 cm gels are recommended.***
8. 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. Alternatively, gels can be stored in water-tight plastic bags with 2 mL of 1X electrophoresis buffer for up to 1 week in the refrigerator.

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.



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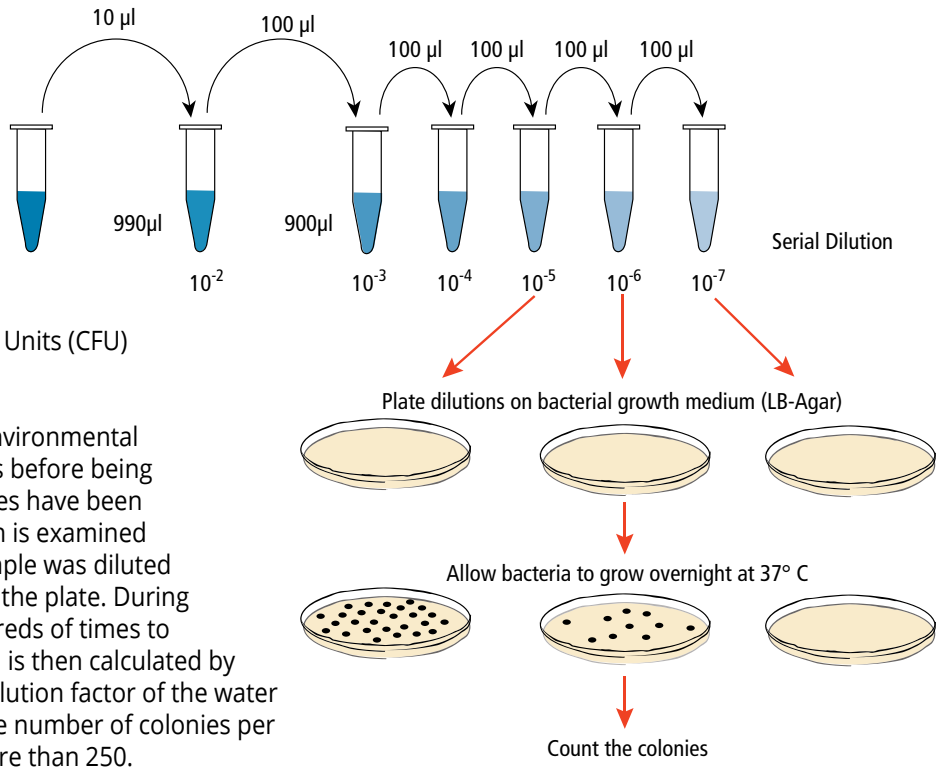
Appendix C

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 several times 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: $\dfrac{\text{(\# of colonies)}}{\text{(dilution factor) (volume plated)}}$

In this extension activity, students will determine whether the traditional Standard Plate Counts can detect bacterial contaminants in a water sample better than PCR-based methods. 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.

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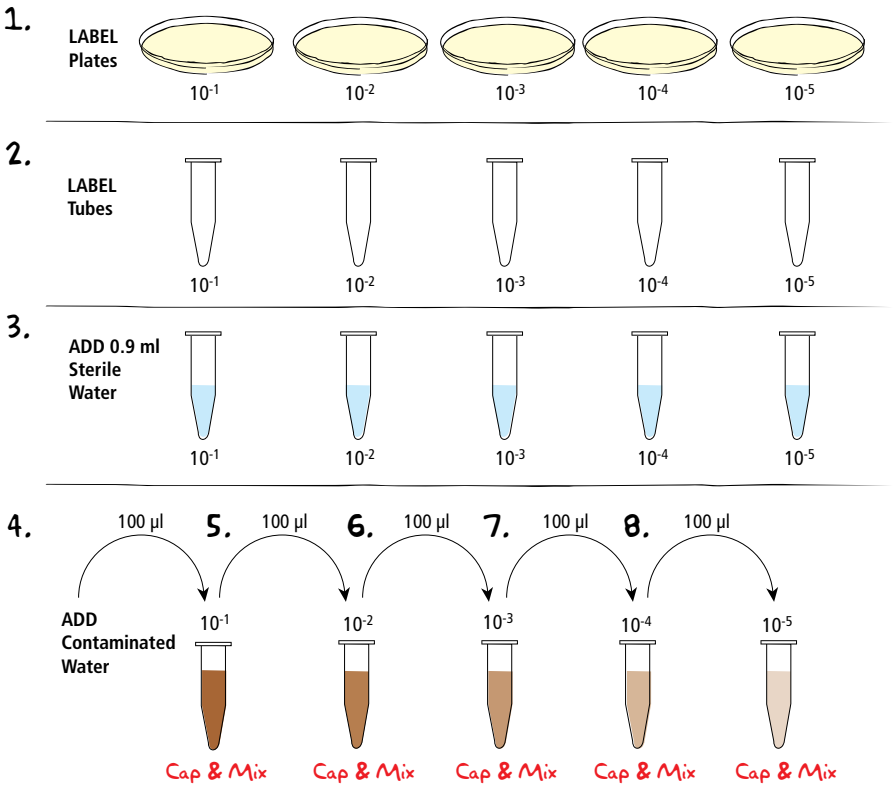
Appendix C

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}, and 10^{-5}.
2. **LABEL** five 1.5 mL microcentrifuge tubes as follows: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}.
3. **ADD** 0.9 mL of sterile water to each of the labeled microcentrifuge tubes.
4. **PIPETTE** 100 µL of contaminated water into the tube labeled 10^{-1}. **CAP** and **MIX**.
5. Using a fresh pipet or tip, **PIPETTE** 100 µL of the 10^{-1} sample into the tube labeled 10^{-2}. **CAP and MIX**.
6. Using a fresh pipet or tip, **PIPETTE** 100 µL of the 10^{-2} sample into the tube labeled 10^{-3}. **CAP and MIX**.
7. Using a fresh pipet or tip, **PIPETTE** 100 µL of the 10^{-3} sample into the tube labeled 10^{-4}. **CAP and MIX**.
8. Using a fresh pipet or tip, **PIPETTE** 100 µL of the 10^{-4} sample into the tube labeled 10^{-5}. **CAP and MIX**.

Continued

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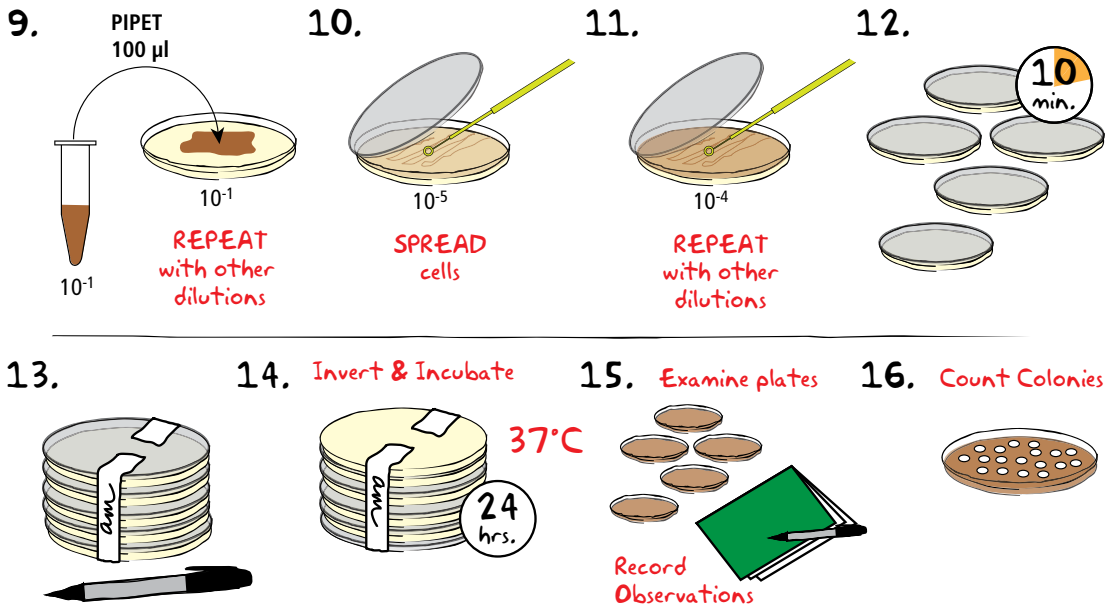
Appendix C

Optional Extension Activity: Standard Plate Count



9. **PIPETTE** 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 spread the cells over the agar. Be careful not to gouge the agar 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. Note the 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: $\dfrac{\text{(Number of colonies)}}{\text{(dilution factor) (volume plated)}}$

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Appendix C
Optional Extension Activity: Standard Plate Count

INSTRUCTOR'S GUIDE

Serial Dilution and Plating of Contaminated Water Samples

If you perform 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, or the undiluted water sample, for DNA extraction in Module I.

Additional Requirements:

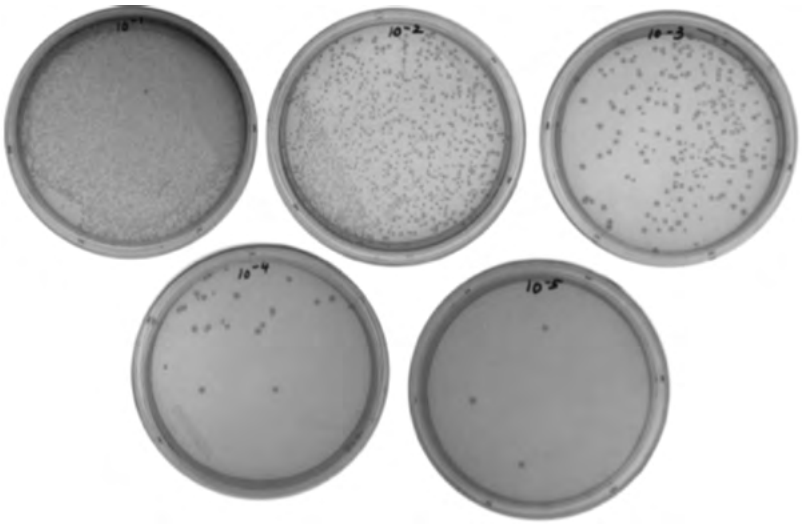
1 Cat. #615 ReadyPour™ Luria Broth Agar Base, 170 mL bottle
2 Cat. #633 Small Petri Plates (60 x 15 mm), package of 20
1 Cat. #667 Sterile loops, package of 25
1 Cat. #630 Microcentrifuge tubes, package of 500
1 Cat. #648 Sterile conical tubes, 15 mL, bag of 25

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

Experiment Time Table (Plate count times ONLY)
Refer to times on 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

RESULTS



The PCR 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.

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Appendix C

Optional Extension Activity: Standard Plate Count

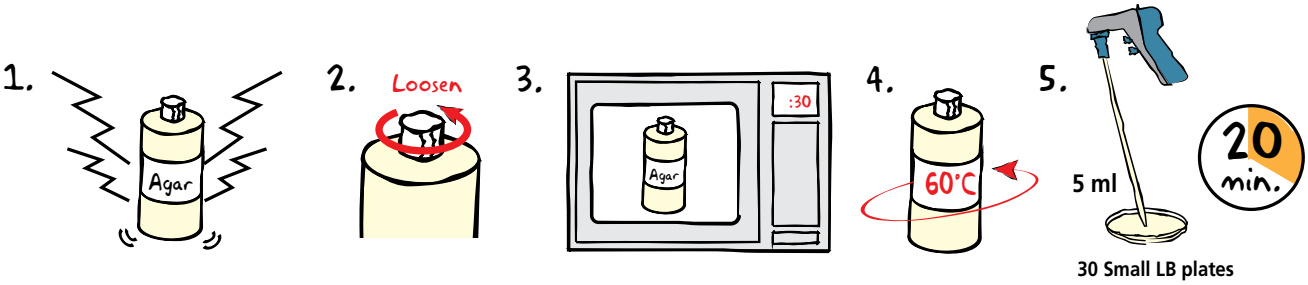
PRE-LAB PREPARATIONS FOR EXTENSION ACTIVITY

DISPENSE 6 mL sterile water into a 15 mL conical tube. **PREPARE** one tube per student group.

Each Student Group Will Receive:

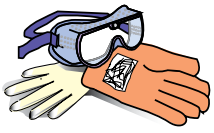
1	Contaminated water sample (*E. coli*)
5	Small LB-agar Plates
1	15 mL conical tube containing 6 mL sterile water
5	1.5 mL snap-top microcentrifuge tubes
1	Sterile loop

POURING PLATES



30 Small LB plates

One bottle of ReadyPour™ medium (Cat. #615) will make 30 small LB plates (Cat. #633). Each student group will require 5 plates.



Wear Hot Gloves and Goggles during all heating steps.

1. **BREAK** the 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.

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.

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