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EDVOTEK AT-HOME:

Water Quality Testing III:

Multiplex PCR Analysis for Water Contaminants

Experiment Objective:

In this experiment, students learn how Polymerase Chain Reaction (PCR) is used to detect several waterborne microorganisms simultaneously.

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Instructions

1. Carefully **READ** the background.

2. **HIGHLIGHT** any words or phrases that you don't know. **LOOK UP** the definition, and **WRITE** the definition into the margins.

3. **UNDERLINE** important information about the experiment.

4. Use this information to **FILL IN** the role of each experimental component.

5. **READ** through the experiment, pausing to **ANSWER QUESTIONS** about what is happening.

6. **ANALYZE** the results.

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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.

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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EAH_water.200330

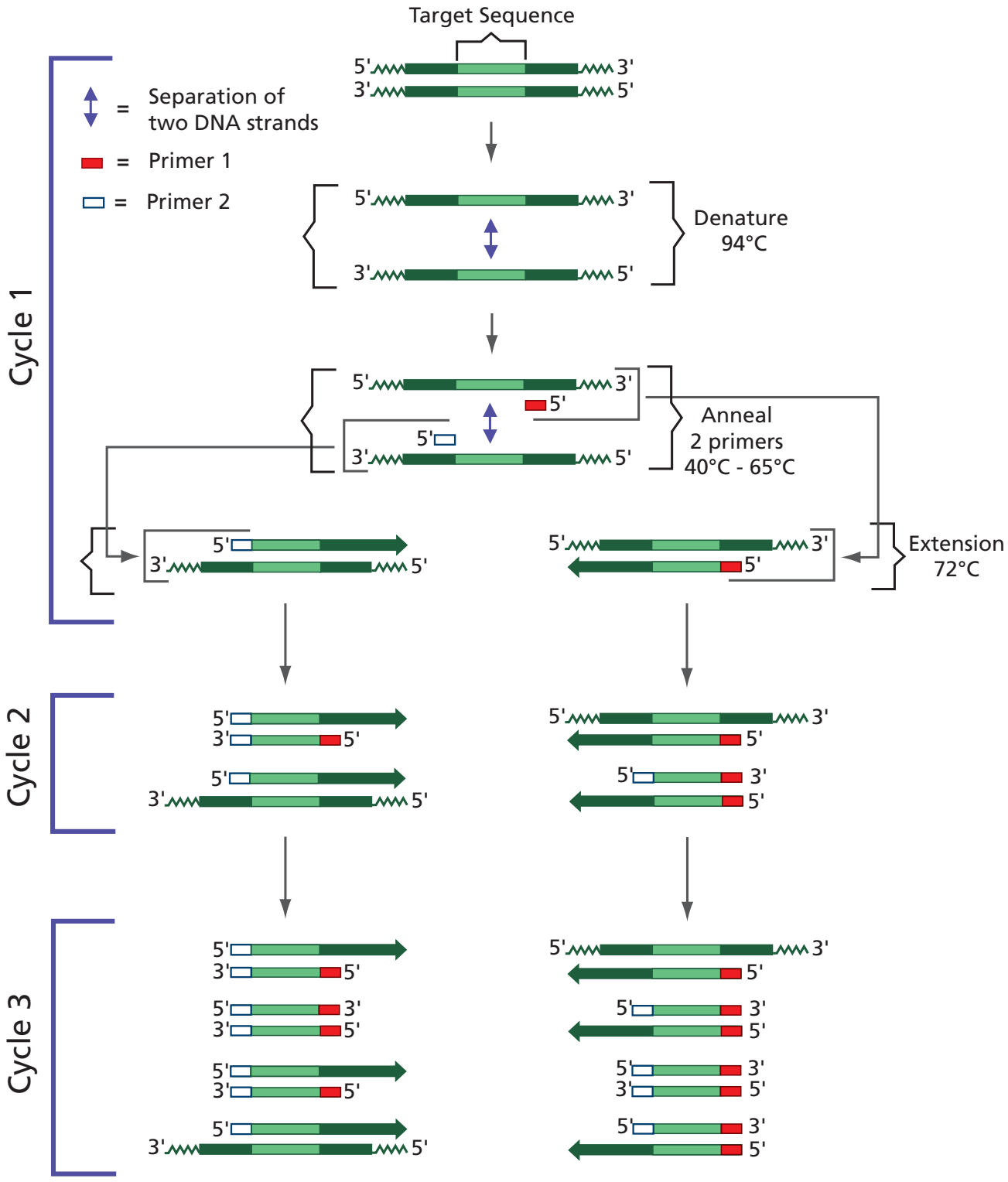

Figure 3:
DNA Amplification by the Polymerase Chain Reaction

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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.

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. After amplification, agarose gel electrophoresis is used to separate DNA fragments according to size so they can be easily identified. 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.*

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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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Experiment Components

Fill in the purpose of each experimental component.

Component	Purpose
• PCR EdvoBeads™:	_____
dNTP Mixture	_____
Taq DNA polymerase	_____
MgCl2	_____
• DNA Lysis Buffer	_____
• Proteinase K	_____
• Primer Mix	_____
• PCR Control	_____
• DNA Ladder	_____
• UltraSpec-Agarose®	_____
• Electrophoresis Buffer	_____
• SYBR® Safe Stain	_____

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Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students learn how Polymerase Chain Reaction (PCR) is used to detect several waterborne microorganisms simultaneously.

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.

Following the Experiment:

- Formulate an explanation from the results.
- Explain how you would use this technique in your neighborhood.

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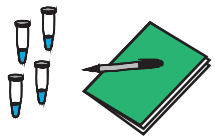
Module I: Isolation of Bacterial DNA from Contaminated Water

1.



OBTAIN the four water samples and **RECORD** their identities 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.

STOP **ANSWER QUESTION BELOW.**

4.

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

5.

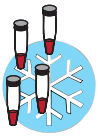
MIX the samples by vortexing or flicking the tubes for 20 seconds.

6.

INCUBATE all four samples in a 99 °C water bath for 5 minutes.

STOP **ANSWER QUESTION BELOW.**

7.



PLACE all the samples on ice. The extracted DNA is now ready for Module II: Multiplex PCR Amplification of Water Contaminants.

STOP **ANSWER QUESTION BELOW.**

#3 Q: What would happen if you used the same pipette tip for all the samples?

#6 Q: What is happening to the cells during these incubations?

#7 Q: Is there anything else besides DNA in the sample? If so, why can PCR still be done efficiently?

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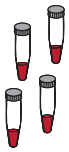
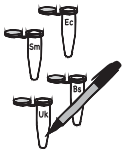
Module II: Multiplex PCR Amplification of Water Contaminants

1.



OBTAIN your four extracted DNA samples from Module I:
- *E. coli*
- *S. marcescens*
- *B. subtilis*
- Unknown

2.



LABEL four fresh 0.2 mL snap-top PCR tubes with the samples' names and your initials.

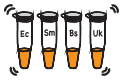
3.

To the fresh "*E. coli*" tube, **ADD** 20 µL primer mix, 5 µL extracted *E. coli* DNA, and a PCR EdvoBead™. **REPEAT** this step for each of the remaining three samples.

STOP **ANSWER QUESTION BELOW.**

4.

Gently mix

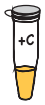


MIX the PCR samples. Make sure the PCR EdvoBeads™ are completely dissolved.

5.

CENTRIFUGE the samples for a few seconds to collect the sample at the bottom of the tubes.

6.



Positive Control Reaction

OBTAIN a tube of "Positive Control Reaction". 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 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).

STOP **ANSWER QUESTION BELOW.**

8. After PCR, **PLACE** the tubes on ice. **PROCEED** to Module III: Separation of PCR Products by Electrophoresis. STOP **ANSWER QUESTION BELOW.**

#3 Q: In your own words, what is the role of the extracted DNA, the primers, and PCR Bead in the reaction?

#7 Q: Explain the reasoning behind the temperature choice for each step?

#8 Q: What would happen if you ran the samples on a DNA gel before PCR?

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Module IV: Separation of DNA Fragments by Electrophoresis

In Module IV, you will perform agarose gel electrophoresis on both of your DNA samples – the digested and undigested PCR products from Module III. Once the electrophoresis has completed you will visualize the DNA bands to determine your *TAS2R38* genotype.

1.

CREATE 1X buffer and **MIX** with agarose powder in a 250 mL flask (see Table A).

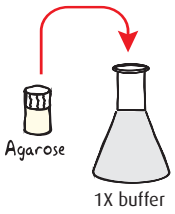
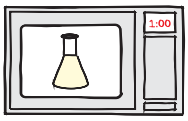


Table A — Individual 1.0% UltraSpec-Agarose™ Gel with Diluted SYBR® Safe Stain

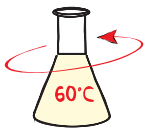
Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Add DILUTED SYBR® (Step 5)
7 x 7 cm	0.5 mL	24.5 mL	0.25g	25 mL	25 µL
7 x 14 cm	1.0 mL	49.0 mL	0.50 g	50 mL	50 µL

2.



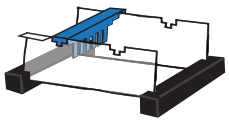
DISSOLVE the agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved and clear like water).

3.



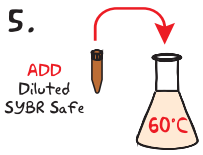
COOL the agarose to 60°C by carefully swirling the flask to promote even dissipation of heat.

4.



While the agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the comb in the appropriate notch.

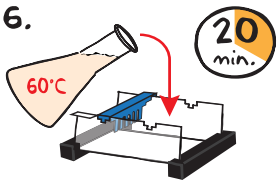
5.



Before casting the gel, **ADD** diluted SYBR® Safe to the cooled molten agarose and swirl the flask to mix (see Table A).

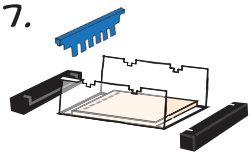
ANSWER QUESTION BELOW.

6.



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.

7.



REMOVE the end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

ANSWER QUESTION BELOW.

#5 Q: Why is SYBR® added to the cooled (60°C) agarose?

#7 Q: In your own words, explain what is happening to the gel. What does the cooled gel look like on a microscopic level?

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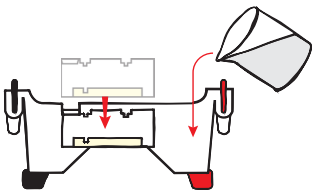
Module IV: Separation of DNA Fragments by Electrophoresis, continued

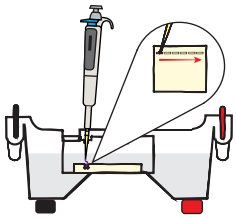
8.



PLACE the gel (on the tray) into an electrophoresis chamber. **COVER** the gel with 1X TBE electrophoresis buffer. The gel should be completely submerged.

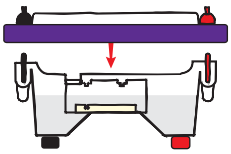
STOP **ANSWER QUESTION BELOW**

9.



LOAD the entire sample (25 µL) into the wells in consecutive order.

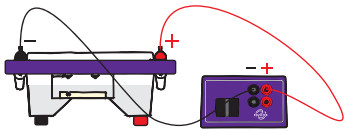
10.



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.

STOP **ANSWER QUESTION BELOW**

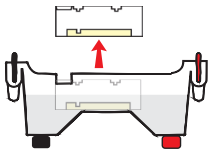
11.



CONNECT the leads to the power source and **PERFORM** electrophoresis.

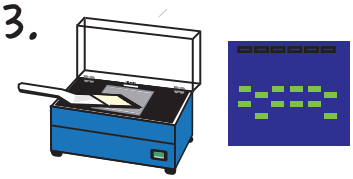
STOP **ANSWER QUESTION BELOW**

12.



After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

13.



SLIDE the 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.

STOP **ANSWER QUESTION BELOW**

#8 Q: What is the role of the buffer in the tank?

#10 Q: Which electrode will the DNA migrate toward and why? What will happen if the gel is placed in the tank backwards?

#11 Q: What would happen if the gel was run for a shorter amount of time? Longer?

#13 Q: Why is a blue light needed to view the DNA? How is the blue light able to illuminate the DNA bands?

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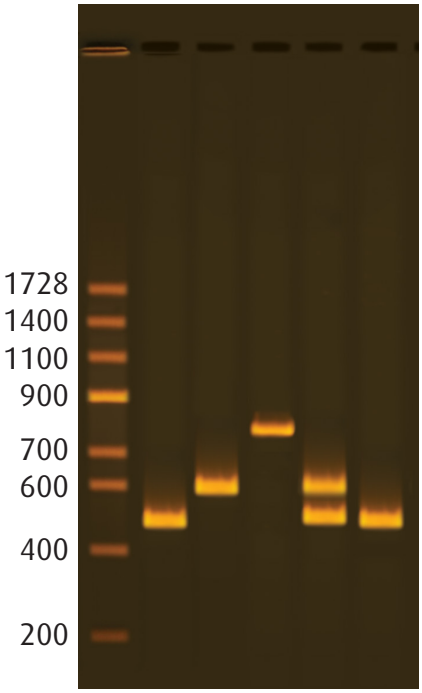
Experiment Results and Analysis

EXAMINE the gel results below and **ANSWER** the following questions.



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

1. What bacteria were in the unknown sample?

2. If you used water from a local stream and no bacteria showed up, does that mean the stream is safe to drink from?

3. Using the graph paper on the next page, create a standard curve based on the ladder. Known base pair lengths should be graphed against how far the DNA fragment migrated on the gel.

4. Measure the length that each bacterial DNA migrated in the gel. Using the standard curve, determine how large each DNA fragment is.

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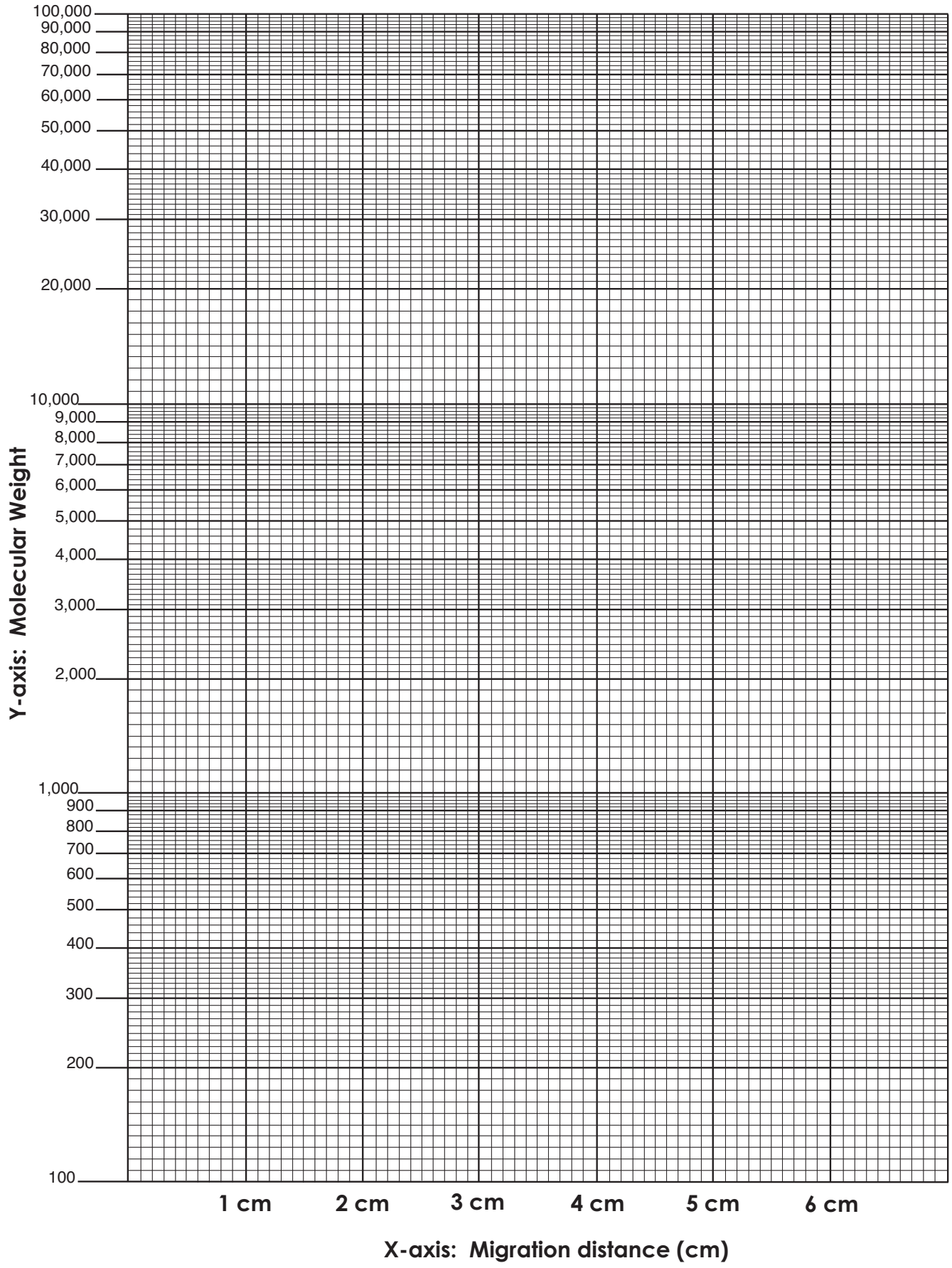

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