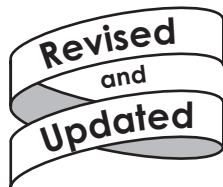




The Biotechnology Education Company ®



EDVO-Kit
951

**Water Quality Testing I:
Chromogenic Analysis of
Water Contaminants**

See Page 3 for storage instructions.

EXPERIMENT OBJECTIVE:

The objective of this experiment is to use chromogenic reagents to test simulations of water samples contaminated with known bacteria. As an extension, students will apply this test as a field activity to screen local bodies of water for the presence of coliform bacteria.

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Experiment Components

This experiment is designed for 10 student groups.

Store all components below at room temperature.

Component

Check (✓)

- Nutrient Broth (for pre-lab only)
- Coliform Detection Broth (for student experimental lab)
- Sterile water (negative control)
- *Escherichia coli* (positive control) BactoBeads™
- *Citrobacter freundii* (positive control) BactoBeads™
- ndole detection reagent

- Conical tubes
- Inoculation loops
- Microcentrifuge tubes
- Pipets

Requirements

- Bacterial incubator (optional)
- UV long wave light source (Long wave black light)
- Micropipet (5 to 50 µl)
- Disposable laboratory gloves
- Water sources in the environment
- Bottled water sources
- Disinfectant (10% bleach solution)
- Laboratory or masking tape

All 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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Background Information

WATER CONTAMINATION

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.

Contamination can enter the water supply in two major ways. 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 (Figure 1). For example, it is common practice to release certain waste materials into nearby bodies of water. This waste, called effluent, is a complex material comprising sewage and/or chemical by-products. The composition of the effluent is strictly monitored and regulated by government agencies to determine if it complies with the standards allowable for discharge. In contrast, nonpoint source pollution cannot be traced to a single, identifiable source—it often results from everyday activities. Typical nonpoint pollution arises from overflow of septic tanks, chemical spills and fertilizer-containing water runoff from farms. This “runoff” contaminates the streams, rivers, and lakes that supply our drinking water. As both sources of pollution contribute to contamination of drinking water, we must be able to effectively identify and remove these impurities.

The contaminants present in polluted water encompass a wide variety of chemical compounds, solid waste and microorganisms. Chemical contamination comprises a wide range of organic and inorganic substances, including petroleum products, industrial solvents and heavy metals. Solid waste includes paper and plastic products, fishing nets, and other marine debris. Common waterborne protists, such as *Giardia* and *Cryptosporidium*, can cause severe, life-threatening illnesses if ingested. Bacterial pathogens, such as *Salmonella* and *Shigella*, can also cause a community-wide outbreak when present in the water supply. As such, constant monitoring of water quality is vitally important to public health.

Assessment of water contamination requires both quantitative and qualitative analysis to pinpoint the location and the identity of water pollution. Effective methods for monitoring water constituents and sediments include:

- 1) Measurements of dissolved oxygen and levels of suspended sediment nutrients
- 2) Seasonal measurements of the abundance and the variety of aquatic plants and animals
- 3) General water conditions, such as pH, temperature and color
- 4) Measurements for compounds like heavy metals, pesticides and volatile organic compounds
- 5) Microbiology techniques, including culturing and plating of water-borne microbes
- 6) Molecular biology techniques, including the use of the polymerase chain reaction (PCR) to identify specific microorganisms.



Figure 1:
Different types of water pollution sources.



Background Information

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 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.

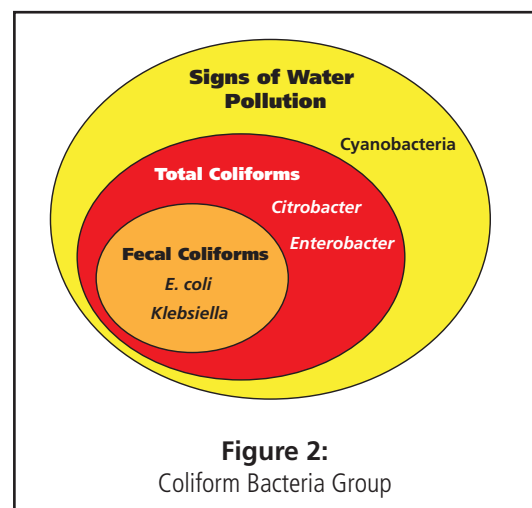
ENVIRONMENTAL TOXINS IN THE WATER SUPPLY

Chemical contamination includes volatile organic components (like industrial solvents), petroleum products (like gasoline, oil and diesel), insecticides, fertilizers and heavy metals. For example, the introduction of untreated sewage or fertilizer-containing water runoff from farms to the water supply results in the over-enrichment of nutrients. The response of the aquatic environment to this influx of nutrients is known as eutrophication. The increased nitrogen and phosphate levels in water promote the excessive growth of algae, in a phenomenon known as an algal bloom. The rapidly growing algae utilize important nutrients in the environment and lower oxygen levels in water, thus affecting the viability of plants and animals that also live in this ecosystem. This results in decreased biodiversity in a particular environment. Since most instances of eutrophication are man-made, public awareness can prevent the destruction of these fragile aquatic environments.

METHODS TO IDENTIFY MICROBIAL WATER CONTAMINATION

To identify water contamination, scientists first look for microbes that may be used as markers for contamination in both drinking and recreational water. For example, it has been established that high levels of Cyanobacteria (often called blue-green algae) correlate with poor water ecosystems, as they are often involved in eutrophication of aquatic ecosystems. In addition, water is also checked for coliforms, a group of relatively harmless bacteria that may indicate the presence of more harmful organisms. Examples of coliform bacteria include *Escherichia*, *Klebsiella*, *Enterobacter*, and *Serratia* (Figure 2). These bacteria normally inhabit the gastrointestinal tract of animals and humans. Therefore, the presence of these organisms in water 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. However, if a sample tests positive for coliform bacteria, it is sent to a laboratory that specializes in analyzing whether dangerous pathogens are present.

Contaminants in water are detected by a variety of experimental approaches. Biotechnology has impacted public health in various areas that include medicine, food production, bioremediation, genomics,



Background Information

and ecology. The discovery of the polymerase chain reaction (PCR) has facilitated molecular biology based diagnostics. PCR-based identification and typing requires a small numbers of a particular microbe for DNA extraction and detection. This allows for early detection where the concentrations of microbial contaminants are extremely low. The requirements for PCR are a water sample, from which the microbial DNA is extracted, a specific set of two primers, the four-deoxytriphosphate nucleotides, the reaction buffer and the heat-stable *Taq* DNA polymerase. DNA primers are designed to recognize a specific microbial gene in the extracted DNA of a particular organism.

This experiment is an adaptation of a U.S. Environmental Protection Agency (EPA) approved test that detects simultaneously total coliform and *E.coli*. *Citrobacter*, a group of coliform bacteria, bare a strong resemblance to *E.coli* in morphology. However, *Citrobacter* are usually longer, and have a rod-shaped appearance (Figure 3). Students will first test the reagents using control cell lines with known Type I bacteria. As an extension they will test water bodies in the environment for the presence of contamination.

The test is based on two chromogenic substrates which are converted into a visible or fluorescent colors. Unlike the PCR test, this detection method does not require equipment or previous experience in microbiology or molecular biology. It is easily adaptable to field work. It requires a 24-hour incubation at 37° C or 48 hours at room temperature to obtain results. Water samples are tested for the presence or absence of total Coliform and *E.coli* by the addition of bacterial nutrients that will enhance their growth. A specific detergent is also added to largely inhibit the growth of accompanying flora especially gram positive organisms.

Two colorimetric reagents, one which is specific for coliform and the other for *E.coli*, are added to the incubation medium for differential and simultaneous detection of total Coliform and *E.coli*. Both reactions can accurately serve as early detection methods of water pollution. As the concentrations of Coliform and *E.coli* increase, the color intensity will also increase which makes them useful reagents to quantitate the amount of Coliform or *E.coli* in water.

The first colorimetric reagent is cleaved by total Coliform including *E.coli*. These bacteria release enzymes in the medium. Upon cleavage, an indigo-blue chromophore is generated which will turn to a visible blue-green color in the presence of the yellow medium. Typically 90 to 95% of the investigated *E.coli*-strains are positive in this assay. This activity is less common in other bacterial species and can therefore be used as a preliminary differential test for *E.coli*.

Hydrolysis of a second indicator dye yields a blue fluorescence when illuminated with UV-long light (black light.) The indole reagent is used to confirm the presence of *E.coli* in a sample that shows positive fluorescence. Addition of a few drops of the indole detection reagent forms a distinctive red ring at the top of the solution which positively confirms the presence of *E.coli*.

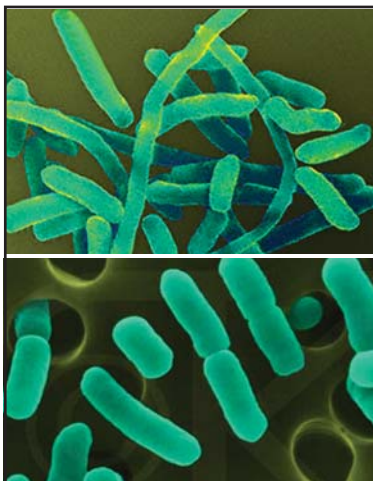


Figure 3:
Citrobacter freundii (top) and
Escherichia coli (bottom)



Experiment Overview and General Instructions

EXPERIMENT OBJECTIVE

The objective of this experiment is to use chromogenic reagents to test simulations of water samples contaminated with known bacteria. As an extension, students will apply this test as a field activity to screen local bodies of water for the presence of coliform bacteria.

EXPERIMENT OVERVIEW

Coliform bacteria can be identified using a simple chromogenic (color-changing) assay. The assay takes advantage of specific substrates that are converted to colors (visible or fluorescent) by enzymes that are specific to coliform bacteria. As the concentration of coliform bacteria in the sample increases, the intensity of the color change also increases, making this assay a way to estimate the amount of total coliform bacteria in water.

A nutrient broth is added to the water sample to promote the growth of coliform bacteria. This broth also contains a detergent to inhibit the growth of non-coliform microbes (in particular, gram-positive bacteria), and two special indicator molecules. One molecule identifies the presence of total coliform bacteria, whereas the other molecule specifically identifies *E.coli*.

As the coliform bacteria grow and divide, they release specific enzymes into the nutrient medium. The first enzyme is present in all coliform bacteria (including *E.coli*). When present in the media, the enzyme cleaves the first indicator molecule, changing the color of the growth media from light yellow to bright blue-green.

An *E.coli*-specific enzyme cleaves the second indicator molecule, producing a molecule with bright blue fluorescence when illuminated with long-wave UV light (i.e. a black light). To further confirm the presence of *E.coli*, the Indole test is performed. In the presence of *E.coli*, the addition of the Indole reagent will produce a distinctive red ring will form on top of the growth media.

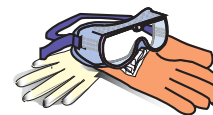
IMPORTANT

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

1. When working in this lab, it is important to avoid infecting yourself or others. Avoid all direct contact with microorganisms.

Wear lab coats, gloves and goggles.

2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. **DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.**
4. While wearing gloves, clean your work area with antibacterial agents (examples include dilute bleach solution or isopropyl alcohol) provided by your instructor.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.



Experiment Overview and General Instructions

EXPERIMENT FLOW CHART

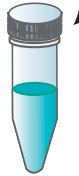
These procedures should be performed for all controls and samples.



Mixture of 0.7 ml water sample and 0.7 ml coliform detection broth will yield yellow color.

Incubate at 37°C overnight.

For safety - Do Not Open caps after Coliform detection broth is added to the water sample.

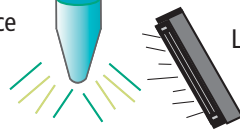


Blue-Green color confirms presence of coliforms.



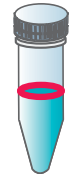
If color remains yellow, water is free of coliforms.

Blue-Green with fluorescence suggests presence of *E. coli*.



Long wave U.V. light (black light)

Add indole reagent.



Indole red ring formation confirms presence of *E. coli*.

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Module I: Testing Laboratory Water Samples**Each student group requires the following:**

- Tube of coliform detection broth (7 ml)
- Sterile water for negative control
- Control for *Citrobacter* water sample
- Control for *E.coli* water sample
- 6 Microcentrifuge tubes
- Pipets

A classroom pipetting station will have:

- Indole detection reagent
- Transfer pipet

Each student group will prepare a total of 6 sample tubes. Three tubes will be used for laboratory sample testing and the other three will be used for environmental sample testing.

CAUTION:

All sample and reagent additions should be performed under semi-sterile conditions. Reaction microcentrifuge tubes should not be opened after samples are added and capped.

A. SETTING UP LABORATORY WATER SAMPLES

1. Label three microcentrifuge tubes as follows:
 - "Neg. Ctl"
 - "*Citrobacter*"
 - "*E.coli*"
2. To the tube labeled "Neg. Ctl", add 0.7 ml of sterile water.
3. To the tube labeled "*Citrobacter*", add 0.7 ml of Control for *Citrobacter* water sample.
4. To the tube labeled "*E.coli*", add 0.7 ml of Control for *E.coli* water sample.
5. Add 0.7 ml of Coliform Detection Broth to each of the three microcentrifuge water sample tubes.
6. Incubate all three tubes at 37°C overnight or at room temperature for 48 hours.

B. OBSERVATION OF CONTROLS AFTER OVERNIGHT INCUBATION

1. Examine and record your observations of the 3 tubes. If the detection broth has changed to a blue-green color, it confirms the presence of coliform.
2. Use a long wave UV light (black light) to examine the tubes. Shine the light away from your eyes onto the tubes. A blue-green color with fluorescence indicates the presence of *E.coli*.
3. Add 2 drops of the Indole detection reagent to each tube that has a blue-green color with fluorescence. **Do not mix or disturb!** The formation of a red ring at the top of the medium will confirm the presence of *E.coli*.



Protect eyes from UV light by wearing UV protective goggles!

Module II: Testing Water Samples from the Environment and/or Liquid Food Products

CAUTION:

All sample and reagent additions should be performed under semi-sterile conditions. Reaction microcentrifuge tubes should not be opened after water samples are added and capped.

A. SETTING UP TEST WATER SAMPLES

See Flow Chart on page 8.

1. Place 0.7 ml of three (3) collected samples into separate microcentrifuge tubes. Clearly label the tubes indicating their contents.
2. Use a sterile pipet to dispense 0.7 ml of Coliform Detection Broth into each of the three microcentrifuge tubes containing the collected samples.
3. Cap the tubes and mix well.
4. Incubate at 37°C overnight, or at room temperature for 48 hours.

B. OBSERVATION OF COLLECTED SAMPLES AFTER OVERNIGHT INCUBATION

1. Examine and record your observations of the 3 tubes. If the detection broth has changed to a blue-green color, it confirms the presence of coliform.
2. Use a long wave UV light (black light) to examine the tubes. Shine the light away from you onto the tubes. A blue-green color with fluorescence indicates the presence of *E.coli*.
3. Add 2 drops of the Indole detection reagent to each tube that has a blue-green color with fluorescence. Do not mix or disturb! The formation of a red ring at the top of the medium will confirm the presence of *E.coli*.

Examples of Test Samples:

- Clear water samples from natural water bodies. Examples of environmental water sources include ponds, lakes, rivers, or puddles.
- Various bottled water samples.
- Well or tap water. (The medium contains reagents for chlorination neutralization.)
- Clear liquid food products such as white grape and apple juices.
- Bottled juices that have been stored at room temperature or 37°C overnight (capped and uncapped).



Experiment Results & Analysis

INTERPRETATION OF ANTICIPATED RESULTS

1. Water Control

There should be no change in the color of coliform nutrient broth which is a yellow color. If contamination was introduced during the preparation of the control, there may be a slight turbidity in the medium.

2. Total Coliform

Coliform is a collective term for a number of bacteria associated with water contamination. The ones most commonly used as indicators of fecal water contamination are *Klebsiella*, *Enterobacter*, *Citrobacter*, and *E.coli*.

Color change of the yellow broth to **blue-green**, even if only in the upper portion, confirms the presence of the coliform group of bacteria. The blue pigment generated by bacterial cleavage of the detection reagent, when mixed with the yellow medium, will yield a blue-green color.

3. *E. coli*

E. coli will also yield a blue green color. Look for fluorescence in the blue-green broth using UV light (black light) in front of the bottle. Shine the UV light towards the sample and away from your eye. A bluish fluorescence indicates presence of *E. coli* due to the cleavage of the *E. coli*-specific fluorescent reagent. Typically, 90 to 95% of the investigated *E. coli* strains are positive for this assay. This activity is much less common in other bacterial species and therefore is used as a preliminary differential test for *E. coli*.

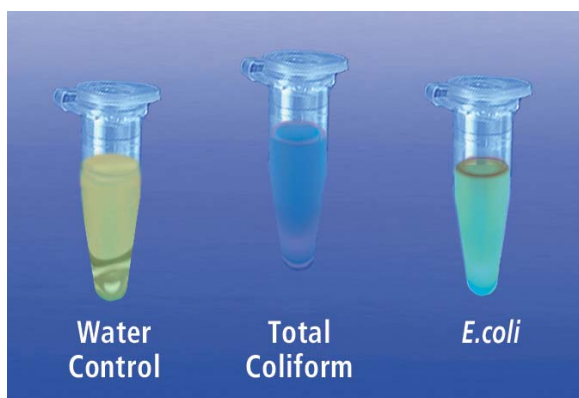
4. Confirmation of *E. coli*

Other species of bacteria may also give a positive fluorescence. The indole reagent is used to distinguish *E. coli* from other bacteria. Add 2 drops of the reagent to the broth suspected to contain *E. coli*. A **red ring** at the top of the broth indicates indole production and confirms the presence of *E. coli*.

The color of the medium should be a straw yellow color. Coliform will convert the added blue detection reagent to yield a blue-green color. If *E. coli* is present, the bluegreen color will also fluoresce under long wave U.V. light (black light). Formation of a red ring upon addition of the indole reagent will confirm the presence of *E. coli*.



Protect eyes from UV light by wearing UV protective goggles!



Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What are the four U.S. Environmental Protection Agency (EPA) guidelines to protect our drinking water supply?
2. What bacteria generally constitutes fecal Coliform?
3. What is eutrophication, and why does it occur?
4. What is meant by "Non point" pollution?
5. Why is the indole reagent used to detect *E.coli* in this water detection test?



Pre-Lab Preparations

DAY BEFORE THE LAB:

Preparation of the Control for Coliform Water Samples

1. Aseptically dispense 15 ml of Nutrient Broth (not Coliform Detection Broth) to each of two 50 ml conical tubes. Label one of the tubes "Control for *Citrobacter* water sample" and the other tube "Control for *E.coli* water sample".
2. To set up "Control for *Citrobacter* water sample", use a sterile loop and transfer one (1) *C.freundii* Bactobead™ to this tube. Cap the tube and discard the loop into disinfectant or place into an autoclave bag for decontamination. Shake the tube to resuspend the cells.
3. To set up the "Control for *E.coli* water sample", use a new sterile loop and repeat the inoculation process using one (1) *E.coli* Bactobead™. Remember to discard the loop into disinfectant or place into an autoclave bag for decontamination.
4. Place both tubes in a 37°C incubator or on a 37°C shaker platform overnight (18-24 hours).
5. After the incubation period is complete, you should notice an obvious turbid growth in each culture. Students will use these cultures to inoculate their Coliform Detection Broth.
6. On the day of the lab, set up stations with the cultures or dispense 1 ml of culture for each student group.

DAY OF THE LAB:

Each group will prepare six (6) test samples.

Three (3) laboratory prepared samples will include: 1) Sterile water, 2) Control for *Citrobacter* water sample; 3) Control for *E.coli* water sample.

Three (3) additional 0.7 ml samples will be obtained from the environment or from other water-based products, such as juices or various bottled waters, which may prove to have interesting possibilities.

1. Label 10 sterile 15 ml conical tubes (for 10 student groups) "Coliform Detection Broth". Aliquot 8 ml of the Coliform Detection Broth™ into each tube.
2. Label 10 microcentrifuge tubes "water" and aliquot 0.7 ml of sterile water into each of the tubes.
3. A designated class station should be set up with the Indole detection reagent and a plastic transfer pipet.

A classroom pipetting station will have:

- Indole detection reagent
- Transfer pipet

Each Student/group requires the following:

- Tube of coliform detection broth (7 ml)
- Sterile water for negative control
- Control for *Citrobacter* water sample
- Control for *E.coli* water sample
- 6 Microcentrifuge tubes
- 6 Pipets
- Long wave U.V. light (black light)

Each student group will prepare a total of 6 sample tubes. Three tubes will be used for laboratory sample testing and the other three will be used for environmental sample testing.

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