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REVISED & UPDATED

Edvo-Kit # 951

Edvo-Kit #951

Chromogenic Analysis of Water Contaminants

Experiment Objective:

In this experiment, students will test control and environmental water samples for the presence of total coliform bacteria and *E. coli* using color changing enzyme assays.

See page 3 for storage instructions.

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Table of Contents

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

Experiment Components

Components	Storage	Check (√)
• *Escherichia coli* BactoBeads™	4° C (with desiccant)	❑
• *Citrobacter freundii* BactoBeads™	4° C (with desiccant)	❑

This experiment is designed for 10 groups of students.

REAGENTS & SUPPLIES

Store all components below at room temperature.

****The Indole Detection Reagent should be stored in the dark.***

Component	Check (√)
• Coliform Detection Broth	❑
• Indole Detection Reagent*	❑
• Nutrient broth	❑
• Sterile water	❑
• 50 mL conical tubes	❑
• 15 mL conical tubes	❑
• 1.5 mL screw-cap tubes	❑
• Inoculation loops	❑
• Sterile pipets	❑

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.

Requirements *(not included in this experiment)*

- UV long wave light source (Catalog #969 recommended)
- Micropipettes (optional)
- Environmental water samples
- Water sample collection bottles (screw-cap drink bottles work well)
- Waterbath or incubator (optional)
- Laboratory gloves
- Laboratory disinfectant (10% bleach solution)

Background Information

Earth has been dubbed the blue planet because from outer space it appears as a pale blue dot and because liquid water is rare in our galaxy. However, the actual amount of fresh and available water is small (Figure 1). Moreover, these freshwater resources are under tremendous and growing pressure (Box 1). Worldwide, water is needed for energy and food production as well as for industry and mining. More basically, humans need – and many argue have a right to - daily access to safe water for drinking, food preparation, cleaning, and bathing.

Figure 1: (A) Circle representing all water in and on the earth, (B) circle representing all fresh water on earth, (C) circle representing all fresh water on earth available to plants and animals.

In 2015 the United Nation decided that ensuring universal access to water was one of its top sustainable development goals. Accomplishing this goal requires connecting individuals to water sources that are (1) physically accessible, (2) affordable, (3) reliable, and (4) safe. This remains a challenge. Threats to water access include local water scarcity, climate change, and water pollution. Water scarcity occurs when there are inadequate local water resources to match a region's water needs or when there are sufficient resources but they are poorly managed. Such scarcity issues are being exacerbated by changes in temperature and rainfall brought about by global climate change. However, even naturally available and well-managed water can become unusable when exposed to pollution.

Box 1: Earth's Water Crisis

- 75% of Earth's accessible water is devoted crop or livestock production.
- 80% of wastewater is discharged into the environment untreated.
- 1 in 9 people lacks access to clean and safe drinking water.
- 1 in 3 people lack access to a toilet.
- 2 billion people live in water basins where water use exceeds recharge.
- 160 million children live in areas of high drought severity.
- If current trends continue, two thirds of the world's population may face water shortages by 2025.

Water Pollution

Common water contaminants include chemicals (industrial solvents, salts, pesticides, and heavy metals) and physical objects (sediment, plastic products, trash). Water can also experience thermal pollution when it is used as a coolant by power plants as well as radiological pollution. Finally, water can be contaminated by biological pollutant. These are pathogenic organisms that cause diseases in humans and that are transmitted through water use.

Biological pollutants include viruses, protozoa, metazoa, algae, several species of parasitic worms, and, especially, bacteria. The vast majority of bacteria are harmless and many are even beneficial, however, some are pathogenic (Figure 2). These bacteria tend to target cells in the small and large intestines of their host and cause nausea and diarrhea. Other symptoms caused by bacterial infections are fever, fatigue, headaches, cramps, inflammation, and rashes. At their most severe, waterborne bacterial infections are responsible for potentially fatal diseases like Cholera, Typhoid, and Dysentery in humans.

The transmission of waterborne diseases from host to host is an indirect but cyclic process. A pathogenic microbe enters a water supply when the water is exposed to the fecal waste of an infected human or animal (see Box 3, The Infamous Broad Street Pump). This can occur when sewage is directly discharged into local waters, when sewer systems overflow or leak, or when rainwater sweeps in sewage from nearby livestock or wildlife. An individual then becomes infected with the pathogen by bathing, washing, or drinking contaminated water. Once infected the individual can potentially contaminate other water sources.

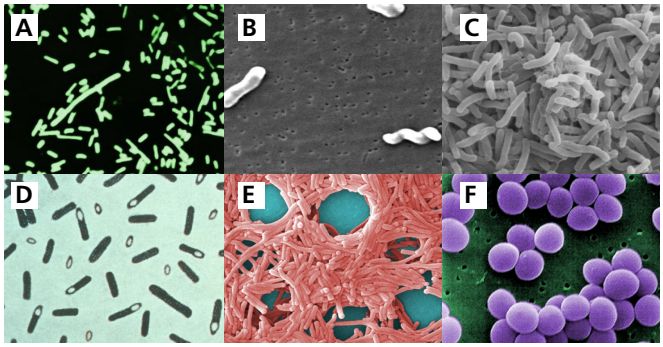


Figure 2*: Examples of dangerous waterborne bacteria and the diseases that they cause. (A) *Shigella dysenteriae*/Dysentery, (B) *Campylobacter jejuni* /Campylobacteriosis, (C) *Vibrio cholerae*/Cholera. (D) *Clostridium botulinum*/Botulism, (E) *Legionella pneumophila*/Legionnaires' Disease and Pontiac Fever, (F) *Staphylococcus aureus*/Swimmers Ear.

Every single day nearly a billion people will be exposed to waterborne pathogens. Most water access programs place a particular focus on these biological pollutants because they are a significant but treatable threat. Water, Sanitation, and Hygiene (WASH) initiatives seek to simultaneously create adaptable water infrastructure, ensure access to toilets, and encourage hygienic practices like hand washing with the understanding that these three goals are interdependent. Such programs directly combat child mortality and malnutrition. In addition - because households can spend hours trying to access safe water and this chore usually falls to the children or women – WASH programs also promote gender equality, childhood education, and overall economic growth (see Box 3, A WASH Success Story).

The Biotechnology of Water Quality Testing

Preventing pathogens from contaminating safe water requires cost-effective treatment technologies, adaptable infrastructure, and basic sanitation facilities as well as continuous monitoring (see Box 3, Outbreak in Wisconsin). Such monitoring helps to determine the initial success of a project and creates provider accountability. It also means that users can be warned when accidental contamination does happen.

Monitoring water supplies for the presence of biological pollutants like bacteria can be challenging. One obstacle is that pathogenic microbes - as their name suggests - are incredibly small and can only be observed under a powerful microscope. In addition, microbial populations, and in particular bacteria, can grow or decline rapidly. This means that a very low (almost undetectable) concentration a pathogen can still pose a significant health risk. Another challenge is that bacteria are notoriously difficult to classify because they are so diverse and because they often reproduce asexually. Finally, pathogenic strains are often closely related to and almost indistinguishable from benign strains (Box 2).

Box 2: The *E. coli* strain O157:H7

The *E. coli* strain O157:H7 is the bacteria most associated with large outbreaks of food poisoning and food recalls. While the majority of *E. coli* are benign or beneficial to humans, this strain produces a toxic substance that causes severe sickness. However, O157:H7 is almost impossible to distinguish based on appearance. It does however have unique genes, antigens, and metabolic pathways.

Luckily, biotechnology offers alternatives to direct species observation. On such innovation is the use of indicator organisms as a proxy for pathogens. Indicator organisms are species whose presence in the water suggest fecal contamination and the possible presence of pathogens. Most mammals support a dense bacterial ecosystem in their colon which in turn gets transferred to their feces. In fact, one study showed that over half of the dry mass of human feces is made of bacteria! While these microbiomes are diverse (1000+ species) they tend to be dominated by between 30-40 species. Fecal indicator bacteria (FIB) belong

to this dominant group and are species that can also temporarily survive outside of their hosts. Consequently, they are consistently present in high numbers in feces-contaminated water. Ideally, FIBs are also present in the water for longer periods than pathogenic strains, safer to handle, and easy to work with in the lab.

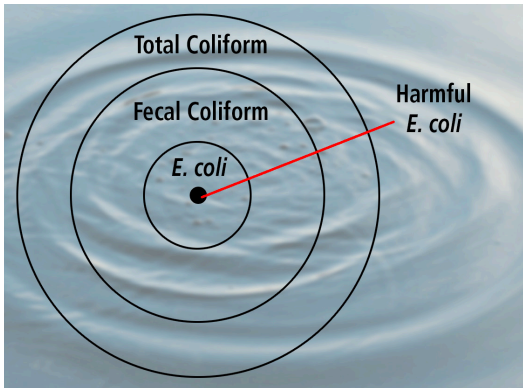


Figure 3: Coliform and *E. coli* are Indicator Organisms.

One popular group of fecal indicator bacteria (FIBs) are coliform bacteria. These are rod-shaped, gram-negative bacteria that can ferment lactose and are easy to identify based on this metabolic trait. Coliform bacteria are found in soil and water but are also always present (and abundant) in the feces of warm-blooded animals (Figure 3). Another popular FIB is *Escherichia coli* (*E. coli*). This species is primarily found in the intestinal tracts of warm-blooded animals. When water quality tests show that coliform bacteria and *E. coli* are absent, testers can conclude that pathogens are also absent. However, when a test is positive for coliform or *E. coli* then the tester must conclude that the water may be contaminated and a potential health risk.

Modern water quality tests are laboratory assays that produce easy to observe signals of a species presence. For example, scientists can isolate DNA from a water sample and then use the polymerase chain reaction (PCR) to repeatedly copy a species-specific sequence until it becomes possible to see the amplified segment on a specialized gel. Another popular test is the Enzyme-Linked Immunosorbent Assays (ELISAs) which uses the highly specific binding of proteins called antibodies to detect FIBs. Finally, there are several chromogenic assays that can confirm the presence of different bacterial species. Chromogenic tests use color change to indicate the presence of specific proteins or chemicals. In the case of water quality testing, the protein in question is an enzyme that is specific to a species or group of bacteria and that can break down a colorless indicator compound into colorful component parts (Figure 4).

In this experiment, you will be performing three chromogenic assays that test for the presence of coliform and *E. coli*. In the first test, water samples containing coliform bacteria turn blue-green while negative samples remain a light yellow. This is because coliform bacteria produce the enzyme β-D-galactosidase which breaks down the first indicator compound into an indigo-blue molecule. In the second test, water samples containing *E. coli* glow when illuminated by UV light. This is because *E. coli* produce the enzyme β-glucuronidase which hydrolyzes the second indicator compound into a blue fluorescent molecule. In the third test, a pink ring of alcohol appears at the top of samples containing *E. coil*. This is because *E. coli* contain a chain of intracellular enzymes known collectively as "tryptophanase" which digest the third indicator compound into indole, pyruvate, and ammonium. This sequence of testing is very similar to popular water quality tests used by organizations like the UN and EPA.

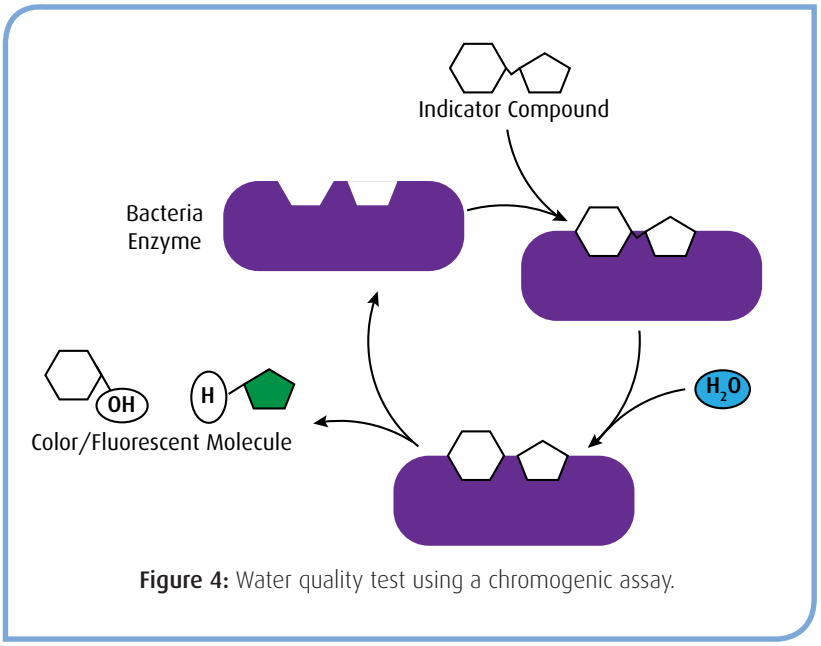


Figure 4: Water quality test using a chromogenic assay.

BOX 3: Real World Cases

The Infamous Broad Street Pump: In the summer of 1852 London was in the midst of a cholera outbreak. One of the worst-hit locations was the overcrowded suburb of Soho where 127 people had died over the course of three days. A local physician - Dr. John Snow - decided to map the cases of the outbreak and identify any patterns. The resulting map showed a strong cluster of cases around a popular public water pump with the noticeable exception of the local brewers (who drank mainly beer) and the local poor house inhabitants (who used an on-site well). Based on the map Snow suggested that cholera was caused by contaminated water (germ theory) and that, in this case, the culprit was the broad street water pump and well. Many years later it was discovered that this well was 3 feet away from a leaky private cesspool and that had likely been contaminated by a baby who had caught cholera on a family trip.

Outbreak in Wisconsin: One of the largest waterborne disease outbreaks in the US occurred in Milwaukee Wisconsin during two weeks in the early spring of 1993. During this time over 400,000 individuals became ill with stomach cramps, fever, diarrhea, and dehydration. These symptoms range in severity from mild to severe with 40,000 seeking medical attention and around 4,000 cases requiring hospitalization. The overall cost of the outbreak in medical care costs and lost productivity was estimated at $95.7 million. Investigators determined that Cryptosporidium - a parasitic protozoan - was the cause of the health emergency and were able to trace the contamination back to the Howard Avenue Water Purification Plant which had been experiencing extremely high levels of runoff due to melting snow.

A WASH Success Story: La Jagua is a small city in southern Honduras. The local school Escuele Pedro Nufio is attended by 40 students. This school had a hand pump well which was used for cleaning and drinking. However, pumping was costing the students valuable study time. More significantly, students at the school began reporting cases of diarrhea and several were diagnosed with hepatitis. In response, the community appealed to a nonprofit to help improve the health situation. Together they installed a biosand water filter for the school's classroom and latrines, a hand washing station, and an electric generator to automatically pump water. These renovations have not only provided safe drinking water but have also given students more time to study and play.

*Sources for Figure 2:

A. 22229 lores.jpg , Dark field microscopy revealing Shigella dysenteriae bacteria. This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #22229. This image is a work of the Centers for Disease Control and Prevention, part of the United States Department of Health and Human Services, taken or made as part of an employee's official duties. As a work of the U.S. federal government, the image is in the public domain. Author: Armed Forces Institute of Pathology, AFIP, 1964.

B. Campylobacter_jejuni_5778_lores.jpg, ID#:5778 Description:This scanning electron micrograph depicts a number of Gram-negative Campylobacter jejuni bacteria, magnified 11,734x. This image is a work of the Centers for Disease Control and Prevention, part of the United States Department of Health and Human Services, taken or made as part of an employee's official duties. As a work of the U.S. federal government, the image is in the public domain. Content Providers(s):CDC/ Dr. Patricia Fields, Dr. Collette Fitzgerald Provider Creation Date:2004 Photo Credit:Janice Carr.

C. Cholera bacteria SEM.jpg Information and public domain notice http://remf.dartmouth.edu/imagesindex.html Scanning electron microscope image of Vibrio cholerae bacteria, which infect the digestive system. Zeiss DSM 962 SEM T.J. Kirn, M.J. Lafferty, C.M.P Sandoe and R.K. Taylor. The copyright holder of this work allows anyone to use it for any purpose including unrestricted redistribution, commercial use, and modification.

D. Clostridium botulinum 01.png, Obtained from the CDC [http://phil.cdc.gov/phil/home.asp Public Health Image Library]. Image credit: CDC (PHIL #2107), 1979. This image is a work of the Centers for Disease Control and Prevention, part of the United States Department of Health and Human Services, taken or made as part of an employee's official duties. As a work of the U.S. federal government, the image is in the public domain.

E. Legionella pneumophila (SEM) 2.jpg, Janice Haney Carr; provided by CDC/ Margaret Williams, PhD; Claressa Lucas, PhD;Tatiana Travis, BS - This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #11148. This image is a work of the Centers for Disease Control and Prevention, part of the United States Department of Health and Human Services, taken or made as part of an employee's official duties. As a work of the U.S. federal government, the image is in the public domain.

F. Staphylococcus aureus VISA 2.jpg, This media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #11157. This image is in the public domain and thus free of any copyright restrictions. As a matter of courtesy we request that the content provider be credited and notified in any public or private usage of this image. Content Providers(s):CDC/ Matthew J. Arduino, DRPH Photo Credit: Janice Haney Carr.

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will test control and environmental water samples for the presence of total coliform bacteria and *E. coli* using color changing enzyme assays.

LABORATORY SAFETY


Wear gloves and safety goggles

The bacterial controls supplied in this experiment are non-pathogenic. However, water samples from the environment will have an unknown microbial community. In both cases use standard safety measures including:

- Avoid contact with microorganisms by wearing gloves, goggles, and lab coats.
- Wash your hands thoroughly with soap and water after the experiment is completed.
- Wipe down the lab bench with a 10% bleach solution or other laboratory disinfectant after the experiment is completed.
- Disinfect all materials (tubes, pipette tips, loops etc.) that come in contact with bacteria before placing them in garbage either by soaking overnight in a 10% bleach solution or by autoclaving items at 121°C for 20 minutes.
- Do not drink environmental samples, even those that test negative for FIBs.

LABORATORY NOTEBOOKS

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

Before Starting the Experiment:

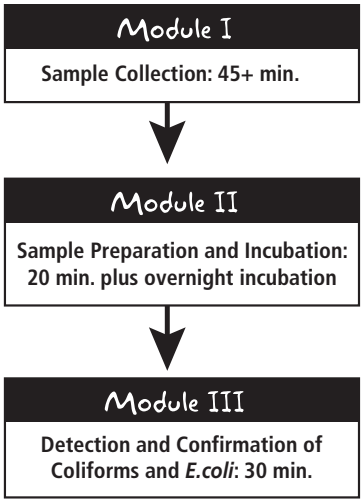
- Write a hypothesis that reflects the experiment.
- Predict experimental outcomes.

During the Experiment:

- Record your observations or photograph the results.

Following the Experiment:

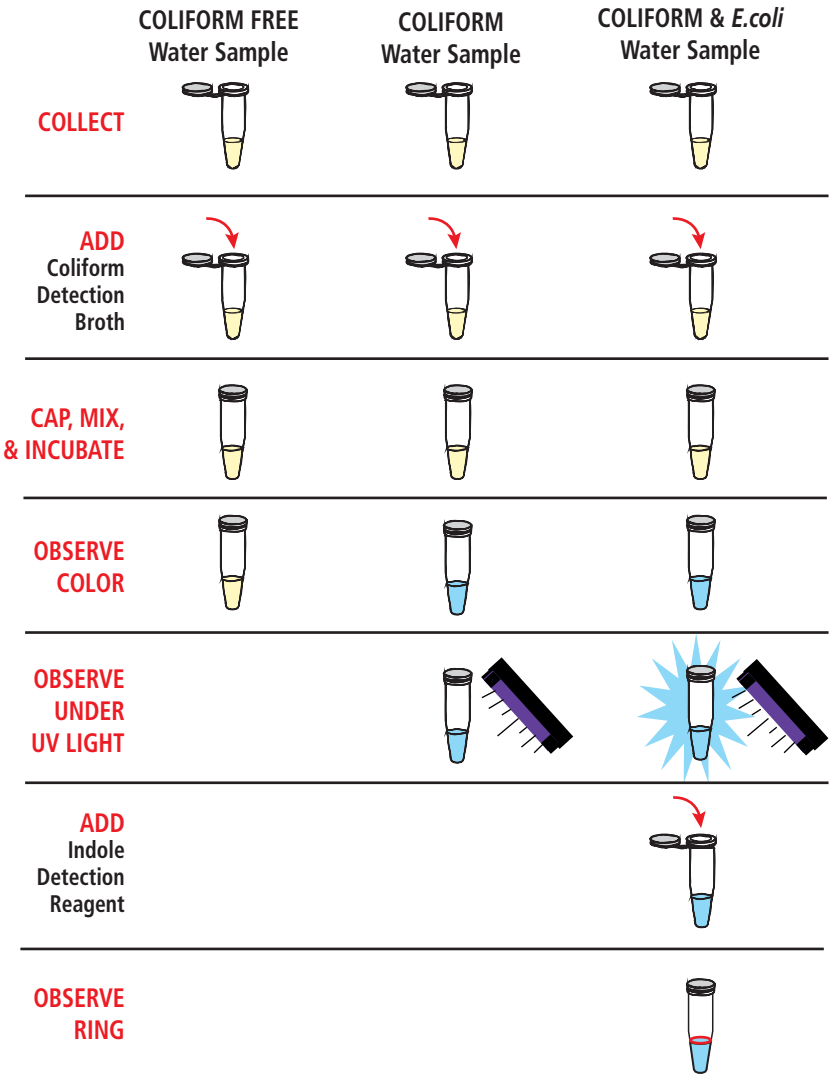
- Formulate an explanation from the results.
- Determine what could be changed if the experiment was repeated.
- Write a hypothesis that would reflect these changes.



NOTE: Experimental times are approximate.

Experiment Overview, continued

Fecal indicator 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 certain bacteria. 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), as well as the indicator molecules. As the 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, this enzyme cleaves the first indicator molecule, changing the color of the growth media from light yellow to 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 on top of the sample.



Module I: Water Collection

For this module, you will collect three environmental water samples.

1. **BRAINSTORM** with your group/class what type of samples you would like to test. **RECORD** the general locations, time points, or general descriptions of your top three.

   Water Sample 1 _____

   Water Sample 2: _____

   Water Sample 3: _____

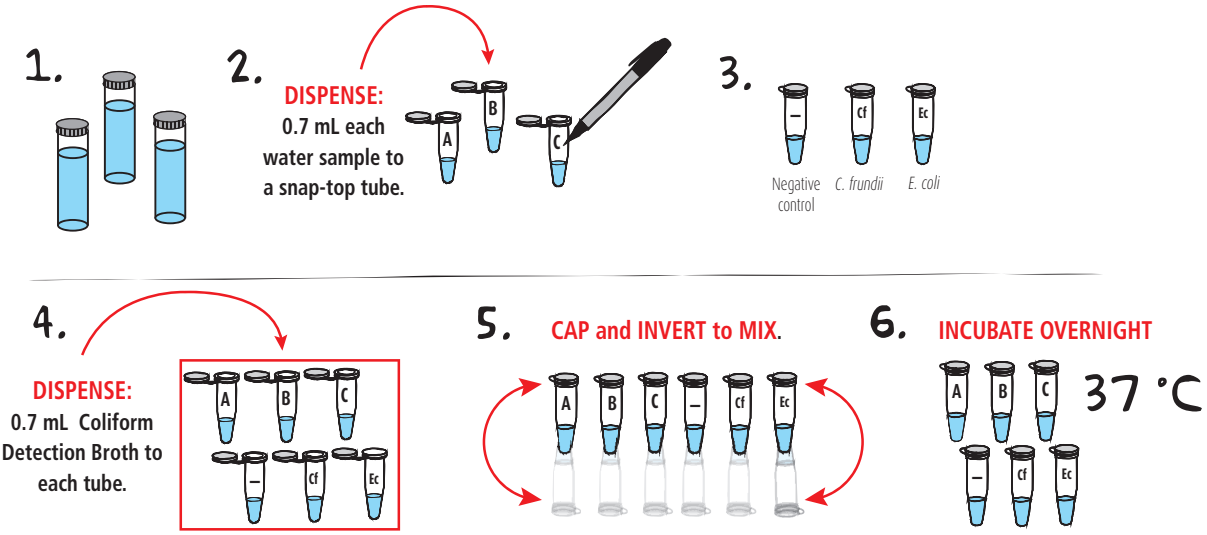
2. Wearing gloves, **COLLECT** at least 1 mL of water for each sample using a disposable plastic bottle with a resealing lid. Use Appendix A to **RECORD** relevant information about the collection site and process. ***Note: It's fine to collect a larger volume. Just remember to dispose of the additional water after the experiment.***

3. As soon as possible, **PLACE** collected samples on ice and/or **REFRIGERATE**.

OPTIONAL STOPPING POINT:
While most guidelines for water suggest testing within 24 hours of collection, we have had success carrying out Modules II and III on samples that were refrigerated within 3 days of collection and stored in the refrigerator for 1 week.

Module II: Sample Incubation and Preparation

In this module you will prepare three environmental samples and three control water samples (negative, C. freundii, and E. coli). C. freundii is a non-pathogenic, gram negative bacteria that belongs to the family Enterobacteriaceae that is used as a general indicator of coliform bacteria. The E. coli used is a non-pathogenic strain and safe to use as a classroom control. The negative sample is sterilize water.



1. Before beginning this module **PROCURE** three environmental water samples. Remember to use gloves when collecting or handling these samples, as their bacterial content is unknown.
2. **DISPENSE** 0.7 mL of each water sample into a snap top tube. **LABEL** each tube with your group ID and with a number or acronym that identifies the original bacteria source.
3. **COLLECT** the negative control, *C. freundii* sample, and *E. coli* sample.
4. Using a sterile pipet tip, **DISPENSE** 0.7 mL of Coliform Detection Broth into all six microcentrifuge tubes.
5. **CAP** the tubes and **MIX** well by inverting each tube several times.
6. **INCUBATE** all six samples in a 37° C incubator overnight (18-24 hours). If an incubator is unavailable incubate at room temperature for 48 hours or until the two bacterial controls become cloudy and blue-green in color.

Module III: Detection and Confirmation of Coliforms and *E. coli*

In this module you will observe your samples for color change that indicates the presence of the enzyme β-D galactosidase (indicates coliform bacteria) and for blue fluorescence that indicates the presence of the enzyme β-D glucuronidase (indicates E.coli). The presence of E. coli in color changing & fluorescing samples will be confirmed with an additional Indole test.

1. **OBSERVE** your samples for any color change. If the detection broth has changed to a blue-green color, it confirms the presence of coliform.
2. Use a long wave UV light to **OBSERVE** your samples for fluorescence. If the detection broth fluoresces blue, it confirms the presence of *E. coli* bacteria. ***NOTE: The plastic tubes may be slightly fluorescent under certain UV lights. Compare samples to the negative control to determine this base fluorescence.***
3. **RECORD** these results below. You may also wish to photograph each tube under visible and UV light.
4. **ADD** 2 drops of indole detection reagent to those tubes that had a blue-green color change and fluorescence. DO NOT mix or invert these tubes after the indole has been added.
5. **OBSERVE** your samples for a red-ring at the top of the medium. A red ring offers additional confirmation of the presence of *E. coli.*
6. **RECORD** these results.
7. Which fecal indicator bacteria are present in each sample?
8. If there are positive environmental samples answer the following: (a) How might this body of water become contaminated? (b) Are there any additional tests that you would like to carry out? (c) Is this a public health concern? If so who should you notify?

Sample	Solution Color (yellow or blue-green)	Appearance under UV Light (fluorescent or non-fluorescent)	Indole Test Results (red ring or no red ring)	Coliform Bacteria Present?	*E.coli* Bacteria Present?
Negative Control					
Coliform Positive Control					
***E.coli* Positive Control**					
Environmental Sample 1					
Environmental Sample 2					
Environmental Sample 3					

Study Questions

1. In 2010 the UN recognized the right to safe and clean drinking water as a human right. However, several counties including the United States abstained from this vote. Why do you think these countries may have been hesitant to classify water access as a right? Do you agree or disagree?

2. Choose a biological pollutant and find out:

   a. Full species name:
   b. Kingdom:
   c. What disease it causes:
   d. Symptoms of the disease:
   e. The number of individuals infected last year:
   f. Suggested treatments and prevention methods:

3. Why do primary water quality test focus on indicator species rather than pathogenic species?

4. How would you treat a sample of water that tested positive for both E. coli and coliform differently than a sample that tested positive for coliform but negative for E. coli? Why? Why is it unlikely that you would have a sample that is positive for E. coli but not coliform bacteria?

5. What is a chromogenic assay? Brainstorm several advantages and disadvantages of using chromogenic assays when testing water quality.

6. Optional: Every location has its own unique set of water resources and water needs. Research local water use and see if you can find out what percentage goes to the following activities: irrigation, public supply, aquaculture, mining, industrial, thermoelectric power. Next, see if you can find out where this water originally comes from. If you are in the United States, both the EPA and U.S. Geological Service have state and county data about water sources and water use.

Instructor's Guide

For Module I:

Student groups can be responsible for collecting their own environmental samples (Module I). Alternatively, you can collect water samples beforehand and have groups select a subset to test during class. If time is limited, this experiment can also be done using only the control samples or by preparing mock environmental samples using the surplus control solutions. Enough reagents and plastics are included for 60 water samples (3 environmental samples + 3 controls x 10 groups). Below are some examples of possible collection sets:

FOR MODULE I
Each group will need:
- Gloves
- Water collection bottles

- **Water collected from various locations:** This can be exploratory (e.g. is there coliform contamination in the stream by my house? the water puddle in the school parking lot? the duck pond at the local park?) OR it can be more hypothesis driven (e.g. is the chance of contamination higher upstream or downstream of town? in streams with or without riparian buffer strips? in popular or private beaches?). For the latter, consider gathering supplemental information about local land use. The USGS, EPA, and your state's Department of Natural Resources all have detailed land-use datasets. Another place to start is the map software of your favorite search engine.

- **Drinking water from wells or taps:** In the US, public water systems are required to deliver safe drinking water 365 days a year, 24 hours a day. When fecal coliform bacteria or *E.coli* is confirmed a health advisory alert is immediately issued. However, citizen scientist tests can complement public monitoring. When testing tap water, consider also testing the faucet itself by washing or soaking it and then testing the wash water.

- **A time series:** Coliform bacteria are living organisms whose population can vary tremendously in size over time. For example, the occurrence of coliform bacteria is significantly higher in the summer when higher water temperatures promote fast bacterial growth. Coliform contamination can also spike after a rainstorm. This is particularly true in localities with combined sewer systems.

- **Before, during and after water purifications:** Water purification is an area of rapid technological advancement. Test a treatment (adding bleach, boiling, distilling, changing the pH, straining through sediment or charcoal layers, exposing to sunlight or UV light, using commercial counter top and straw filters, etc.) OR have students develop their own purification method. To create a large mixed culture of *C. freundii* and *E. coli* combine one BactoBead™ of each in 30 mL of LB medium in a sterile 50 mL container. Incubate in a shaking incubator at 37° C for 6 hours at 240 rpm or in a non-shaking incubator at 37° C for 12 hours. For additional Luria broth see item #611.

- **Contaminated food or beverages:** Sicknesses caused by bacteria-contaminated food sources like beef, eggs, lettuce, etc. have been increasingly in the news. The same reagents used for coliform detection in water and can also be used for food quality tests. For food testing we suggest macerating 1 g of food in 10 mL of sterile water, incubating the mixture overnight, and then using the supernatant.

Warning: The bacterial controls supplied in this experiment are non-pathogenic. However, water samples from the environment will have an unknown microbial community. In both cases use standard safety measures including wearing gloves while handling water samples and washing hands afterwards.

For Module II:

Prepare Controls

1. Prepare the negative control samples by aseptically dispensing 0.7 mL of sterile water to ten snap top tubes labeled "Sterile Water (Negative Control)".
2. Aseptically dispense 15 mL of nutrient broth to two 50 mL conical tubes.
3. Label one tube *C. freundii*. Using a sterile loop transfer two *C. freundii* Bactobeads™ to this tube. Cap and shake well.
4. Label one tube *E. coli*. Using a sterile loop transfer two *E.coli* Bactobeads™ to this tube. Cap and shake well.
5. Allow both tubes to incubate at room temperature for 10 minutes.
6. Dispense 0.7 mL of *C. freundii* to ten snap top tubes labeled "Control for Citrobacter".
7. Dispense 0.7 mL of *E. coli* to ten snap top tubes labeled "Control for *E. coli*".

NOTE: Control samples can be prepared up to a week before Module II. Keep prepared samples in the fridge to limit bacterial growth.

FOR MODULE II
Each group will need:
- 3 Empty snap-top tubes
- 3 Environmental water samples
- 1 Snap-top tube of control for Citrobacter
- 1 Snap-top tube of control for *E. coli*
- 1 Snap-top tube of sterile water (negative control)
- 15 mL tube containing 5 mL Coliform Detection Broth
- Pipette and tips (or 6 sterile transfer pipets)

WARNING: The chromogenic tests performed in this experiment are sensitive. Avoid contamination of both control and environmental water samples by using basic aseptic techniques. These include preparing the negative control sample first, limiting air exposure by keeping tube lids on whenever possible, cleaning the lab bench with a general sterilizer before Module II, and using gloves.

Distribute Coliform Detection Broth

1. Label 10 sterile 15 mL conical tubes "Coliform Detection Broth" (or "CDB").
2. Aliquot 5 mL of the broth into each tube.

For Module III:

1. Make sure students have access to a long wave UV light.
2. Make sure students have access to cameras for result pictures.
3. Set up a pipetting station for the Indole Detection reagent with 10 plastic transfer pipets and the Indole solution provided in this kit. Because the reagents used in this solution can smell, this station is best located in a well ventilated place.

FOR MODULE III
Each group will need:
- Control and environmental samples from Module II
- Access to camera
- Access to a UV light
- Access to the Indole pipetting station

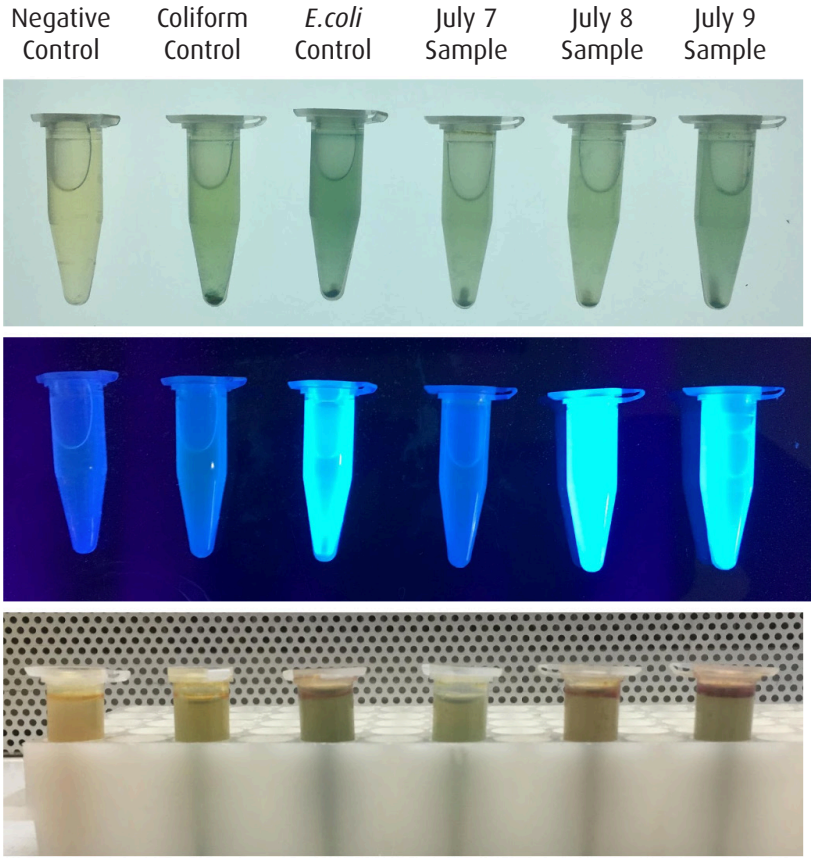
Experiment Results and Analysis

Three samples were collected over the course of three days in early July. On the second day a large rainstorm occurred one hour before the sample was collected. Samples were collected from the same location (right) and at the same time of day. They were stored in 50 mL conical tubes. Depending on the collection day they were kept in the refrigerator for 4, 3, or 2 days before being tested.

Environmental samples collected prior to the rain storm tested positive for coliform but not *E. coli*. This suggests that there is an active bacteria population in the stream which includes coliform species but that the stream did not initially have biological contamination. However, immediately after the storm and 24 hours later the testing indicated that both coliform and *E. coli* were present. This suggests fecal contamination.

Sample	Solution Color	Appearance under UV Light	Indole Test Results	Coliform Bacteria Present?	*E. coli* Bacteria Present?
Negative Control	yellow	Non-fluorescent	No Red Ring	No	No
Coliform Positive Control	green/ blue-green	Non-fluorescent	No Red Ring	Yes	No
E. coli Positive Control	Blue-green	Fluorescent	Red Ring	Yes	Yes
July 7 Sample (ES1)	green/ blue-green	Non-fluorescent	No Red Ring	Yes	No
July 8 Sample (ES2)	green/ blue-green	Fluorescent	Red Ring	Yes	Yes
July 9 Sample (ES3)	Blue-green	Fluorescent	Red Ring	Yes	Yes

Experiment Results and Analysis, continued



Answer to Step 8:

Both July 8 and July 9 samples were positive for biological contamination. The cause of this contamination was likely the rain storm and the source may be either runoff from the surrounding area or overflow from the sewer system. Further testing is suggested for both samples to determine if any pathogenic species are present and to estimate the bacteria load/concentration. Because this stream is not used for drinking or washing it is not a significant health concern. However, the local government could be informed and a warning sign posted.

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

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Appendix A
Data Collection Sheet

Water Sample Name: _____ ID:_____

A. SITE INFORMATION	
Location (Address or Latitude/Longitude)	
Sample Point (e.g. kitchen tap, small stream ~ 1 meters across, eastern shore of freshwater lake etc.)	
Surrounding Land Use (forest, pasture, cropland, urban, golf course, marsh etc.)	
Surrounding Vegetation (grasses, trees, shrubs or species names when possible)	

B. COLLECTION INFORMATION	
Date Collected	
Time Collected	
Volume Collected	
Collector(s)	

C. WATER INFORMATION	
Water temperature	
Velocity (circle one)	Still Slow Fast
Clarity (circle one)	Clear Slightly Turbid Turbid Very Turbid
Additional Observations (odor, surface oils, debris, trash)	

D. WEATHER INFORMATION	
Air Temperature	
Cloud Cover	
Wind	
Recent Precipitation	

E. PROCESSING INFORMATION	
Transportation Conditions	
Storage Conditions/Time	
Water Tests (check all that apply)	o FIB (Coliform & *E. coli*) o Dissolved Oxygen Levels o Seminent Levels o pH o Aquatic Life Inventory o Chemical (Nitrates, Heavy Metals, Ammonia)

Please include any additional notes or photographs on the back of this page.