GenoSensor Corporation

GenoSensor Water Analysis Kit

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User's Manual

Water Analysis Kit I Manual

Table of Contents

Literature Citation

When describing a procedure for publication using these products, we would appreciate that you refer to them as the GenoSensor Water Analysis Kit.

Water Analysis Kit Overview

What's in your water? –

The water we drink is put through stringent testing to make sure that it's safe for us to drink. What are those tests, and what are they testing for? The GenoSensor Water Analysis Kit will give students the opportunity to test water samples from their own environment, and through the use of polymerase chain reaction (PCR), detect the presence of bacterial contaminates.

The bacteria tested for in this kit are the same that are tested for in initial water contamination tests. Any water sample can be used, from the puddle outside to tap water.

Notes for Instructors

Kit Components and Storage Conditions (for 24 students)

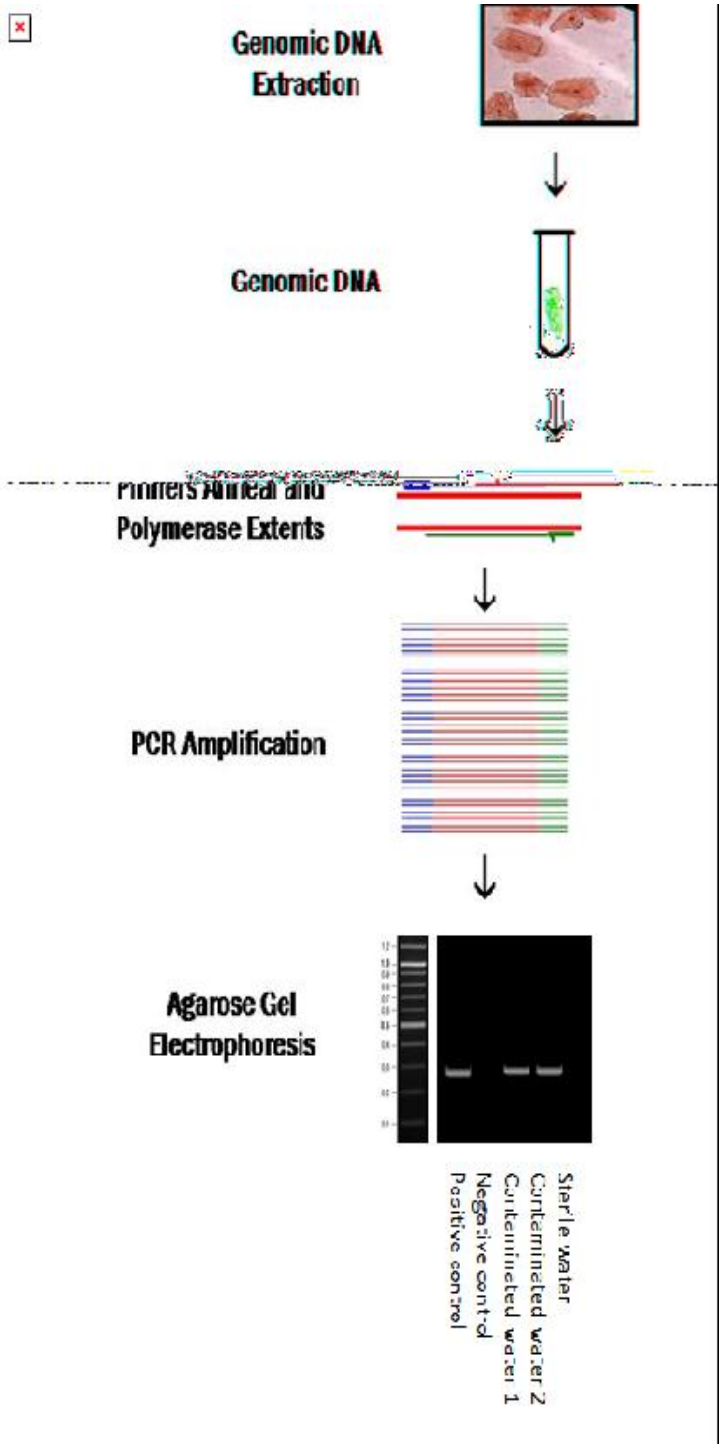
Component	Amount	Reactions	Storage
2X PCR Master Mix	300 µL	30	-20ºC
Positive Control DNA	60 µL	6	-20ºC
Negative Control (DNase/RNase-Free H_2O)	60 µL	6	-20ºC
DNA ladder	30 µL	3	-20ºC

It is recommended that students form 6 groups, and 4 students in each group. Each group has one positive and one negative control sample from the kit, representing contaminated and non-contaminated water samples. Two additional water samples in each group are collected by students as unknown samples for testing.

Additional Required Materials

- ❑ Ice
- ❑ Heat block or water bath and thermometer
- ❑ PCR thermal cycler
- ❑ PCR tubes and tube racks
- ❑ Microcentrifuge
- ❑ Microcentrifuge tubes and tube racks
- ❑ Vortexer (optional)
- ❑ Micropipettes (p10, p200) and tips
- ❑ Gel electrophoresis equipment
- ❑ Gel electrophoresis supplies: agarose, TBE buffer, DNA loading buffer, running buffer, gel dye (e.g., SYBR Safe, Gel Red)
- ❑ Gel transilluminator (e.g., UV light box, Gel Documentation System)

Figure 1. Water Analysis Experiment Overview



Pre-lab preparation for DNA isolation and PCR (for 24 students)

1. **Students collect their own water samples and it is fun!** It is recommended that students form 6 groups, and 4 students in each group. Each group has one positive and one negative control sample from the kit, representing contaminated and non-contaminated water samples. Two additional water samples in each group are collected by students as unknown samples for testing.
2. Set heat block or water bath to 95ºC. It is recommended that you follow the manufacturer's recommendations to ensure a good heat transfer (e.g., adding water or sand).
3. Set up PCR thermal cycler with the PCR profile, from page 11.
4. Thaw 2X PCR Master Mix on ice.
5. **Before opening the 2X PCR Master Mix tube, spin in a microcentrifuge for 10 seconds at 6,000 rpm, or greater. Vortex for 10 seconds, then spin again for 10 seconds. Place 2X PCR Master Mix back on ice until ready to use.**
6. Label 6 microcentrifuge tubes "MM", one tube for each team. After performing step #4, aliquot 10 µL of the 2X PCR Master Mix into each of the tubes.
7. Store the "MM" samples on ice, along with any leftover 2X PCR Master Mix. Each kit contains enough 2X PCR Master Mix for 24 reactions. In class, students will combine 10 µL 2X PCR Master Mix + 10 µL of template for a final PCR volume of 20 µL.
8. Each group performs PCR on one positive (contaminated) and one negative (non-contaminated) sample, plus two samples collected by each group.

Agarose Gel Electrophoresis

- Electrophoresis reagents are not provided in the kit. Please refer to the Additional Required Materials list, on page 4.
- Best results are obtained by adding DNA dye (i.e. Gel Red or SYBR Safe) to molten agarose.
- Note: Some DNA dyes are light sensitive and care must be taken to avoid exposing the agarose gel to light when storing and running the gel. It is, therefore, recommended that you refer to the manufacturer's instructions for your particular type of DNA dye.
- Enough DNA ladder has been provided, in the kit, to load 3 lanes with 10 µL of ladder per each well.

- After PCR, load 10 µL of positive control reaction product into gel lanes.
- After PCR, load 10 µL of negative control reaction product into gel lanes.
- Loading dye added to the sample ensures the sample will sink to the bottom of the well.
- After PCR, load as much as 20 µL of each student PCR reaction product into a designated lane. The amount of sample that a well can hold depends on the size of the comb used in making the gel. For example, the maximum well loading volume per well for a 12-toothed comb is 20 µL. Each student should keep track of the lane location containing their own sample.

Shipping, Storage and Safety

Shipping and Storage

GenoSensor Water Analysis kits are shipped on blue ice. Components should be stored at temperatures shown in the above table. Under proper storage conditions, components are stable for 1 year from the date received. Expiration dates are also noted on product labels.

Safety Warnings and Precautions

This product is intended for educational use only. It is not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals. Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practices should handle these products. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves. Exercise caution to avoid contact with skin or eyes. If contact should occur, wash immediately with water and follow your laboratory safety protocols. Safety Data Sheets for products are available upon request.

Student Guide

Introduction

Is your water safe to drink? What are indicators that would make you think it is or is not? Clarity, odor, color. Where did it come from? The source of your water is a good indicator of whether it is safe to drink or not. Is it pure? We try not to drink unpurified water, but occasionally our water can become contaminated. The water we drink is put through stringent testing to make sure that it's safe for us to drink. What are those tests, what are they testing for?

Coliform bacteria is a broad term for bacteria that are rod-shaped, Gram negative, non-spore forming and the ability to break down lactose. *Escherichia coli* is the hallmark example. *E. coli* co-evolved with mammals, evidence of this is the ability to break down lactose (the sugar in milk). All mammals produce milk for their young, at birth the baby acquires its gut flora from its mom. *E. coli* can be found in soil, on vegetation, and in the gut of warm blooded animals. It's an ecosystem we are indirectly part of. A rabbit eats plants with *E. coli*, the rabbit fertilizes the ground, plants grows on the feces, another rabbit eats the lettuce and the cycle continues and co-evolves. Coliforms are not necessary harmful, but they are indicator organisms. Their presence implies the water system might be compromised, which could be caused by any number of things.

In this lab, you will be performing a water analysis that is commonly used in small communities to test their drinking water. You will be testing your own water samples for the presence of bacteria.

Before you start, remember, this experiment is primarily for educational purposes and is not meant to replace or challenge official water testing methods. If your drinking water tests positive for bacteria, you might consider notifying your water company, however, keep in mind bacteria are everywhere and could have contaminated your test results indirectly (e.g., not wearing gloves when handling the water samples, allowing the water to sit exposed).

Water Analysis Kit Protocol

Pre-lab Preparation

1. Refer to Pre-lab Preparation on page 6.
2. Bring your own samples! At least two water samples should be collected by each group.
3. Important! Before proceeding with the 2X PCR Master Mix, refer to 2X PCR Master Mix Preparation protocol, on page 6. Keep 2X PCR Master Mix on ice when not in use.

DNA Preparation

Wear gloves and handle solutions carefully.

1. Pipette out 50 µL of your water sample into an appropriately labeled 1.5 mL microcentrifuge tube. If preparing the positive and negative control, this step can be skipped.
2. Place the sample in the heat block or water bath to incubate at 95°C for 1 minute. If using a water bath, make sure the tubes are tightly sealed and not fully submerged, to avoid contamination. Heating causes the cells to lyse (split open) exposing the DNA and making the DNA accessible to PCR components.
3. Immediately place on ice for 5 minutes.
4. Spin in a microcentrifuge for 10 seconds, to reintegrate condensation that has collected in the cap back into the sample.
5. Place the sample on ice until ready to proceed.

PCR Reaction

Wear gloves and handle solutions carefully.

1. Prepare and label a small PCR tube with your initials. Label both the top and side of the PCR tube to ensure clarity.
2. Before opening the 2X PCR Master Mix tube, ensure that 2X PCR Master Mix has been prepared according to the protocol on page 6 and is on ice.
3. As directed by your instructor, label and prepare PCR tubes for controls. Follow the "Control Samples PCR Mixture" table below.
4. Add 10 µl of “2X PCR Master Mix” and 10 µL from your sample prepared above to the labeled PCR tube for a total of 20 µL as indicated in the table below. (Note: It is preferred that the PCR reaction mix preparation is done on ice).
5. Mix the 20 µL PCR reaction mixture by pipetting in and out with the pipette, and the close the lid tightly.
6. Store the sample on ice until it is ready to be loaded into the thermal cycler.

PCR Reaction Mixture Tables

Student Sample PCR Mixture	
2X PCR Master Mix	10 µL
Prepared Water Sample	10 µL
Volume total	20 µL

Control Samples PCR Mixture			
Positive Control		**Negative control**	
2x PCR Master Mix	10 µL	2x PCR Master Mix	10 µL
Positive control DNA	10 µL	Negative Control	10 µL
Volume total	20 µL	Volume total	20 µL

Positive and negative controls give guidelines or boundaries to the experimental results. The positive control will show a result for water that is contaminated with *E. coli* and/or coliform bacteria. The negative control show the result if no DNA is present for the PCR.

The DNA ladder is a standard which is used like a ruler to show the approximate size of the DNA fragment migrating through the gel during electrophoresis.

PCR Parameters

Program your thermal cycler as follows:

1. 94ºC – 2 minutes
2. 94ºC denaturing – 20 seconds}
3. 58ºC annealing – 20 seconds} **repeat steps 2, 3, & 4 for 40 cycles**
4. 72ºC extension – 20 seconds}
5. 72ºC – 5 minutes
6. 4ºC – finished / hold

Agarose Gel Electrophoresis

General Procedure, detailed instructions should be given by instructor

1. Prepare 0.8 – 1% agarose.
2. For staining, use a DNA dye which is added directly to the molten agarose. For light sensitive dyes, it may be necessary to keep the gel in the dark during gelation. Follow the manufacturer's instructions.
3. Set up electrophoresis apparatus and pour the molten agarose for gelation.

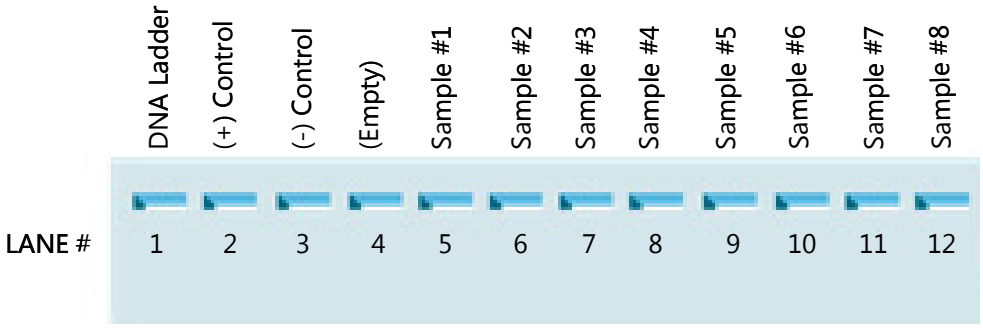
Loading the Gel

1. In a properly labeled microcentrifuge tube, add at least 10 µL of your digested PCR product.
2. Add DNA loading dye, according to your instructor's directions. Loading dye is added to the sample to make sure that the sample will sink to the bottom of the well and properly enter the agarose gel. Mix by gently pipetting up and down.
3. Following your instructor's directions, carefully pipette at least 10 µL of your sample into your assigned well. Be sure to record which well holds your sample.

Figure 2. Sample Gel Setup and Loading. Three 12-well mini-gels are enough to accommodate 24 students (6 teams) plus 6 control reactions; run 2 teams per gel.

Lane 1:	10 µl DNA Ladder
Lane 2:	10 µl Positive Control
Lane 3:	10 µl Negative Control
Lane 4:	Up to 20 µl Your own sample 1
Lane 5:	Up to 20 µl Your own sample 2
Lanes 6 - 9	Repeat lanes 2-5 for the next group

Or alternatively, load as below:



4. Place the lid on the electrophoresis chamber, connect to the power supply, and run at ~100V for ~20 minutes; stop before the loading dye has run off gel. If you

are using a light sensitive DNA dye, it may be necessary to run the gel in the dark - consult the manufacture's instructions.

5. Visualize under UV light and record the results manually or photographing.
6. Confirm that the negative control does not have bands (i.e., no bacteria present).
7. Compare individual experimental bands to positive control DNA.

Results and Discussion

1. What do the bands on the gel tell you?
2. What does it mean if no bands are present for a sample?
3. What purpose do the positive and negative control samples serve? Did they yield the expected results?
4. What further steps might you take to verify your results?

Technical Background Information

Introduction to PCR

In 1983, during his time working at Cetus Corporation, Kary Mullis developed a technique that has changed the field of genetics and biological science in general. This revolutionary process was termed “polymerase chain reaction,” or PCR. He earned the Nobel Prize in Chemistry in 1993 due to his innovation. His technique enabled researchers, besides a few expert microbiologists, to amplify DNA. Before that, amplification of DNA was extremely difficult and time consuming. Now, scientists in any field can incorporate molecular biology into their research with PCR.

Currently, PCR is used in a wide variety of areas, for example, gene mapping, DNA sequencing, gene expression, gene detection, forensics, criminal investigation, medical diagnostics, and genome sequencing. Very few of these applications were practically possible before PCR. The experiment does require an initial investment in specially made machinery, but with the proper equipment, nearly anyone can perform a successful PCR experiment without significant cost.

PCR and Biotechnology — Revolutionizes an Entire Research Community

PCR is capable of producing large amounts of targeted DNA from an extremely small amount of starting material, known as your template. DNA can be obtained from any cell such as blood cells, hair cells, cheek cells etc., and after proper treatment to isolate DNA, PCR can be applied to create millions of copies of virtually any desired DNA sequence. That is one of the most significant powers of PCR, specificity. Although you may put an entire genome worth of DNA into a PCR, it amplifies the exact piece of DNA desired and leaves the rest out.

The basic components of PCR are: reaction buffer, deoxyribonucleoside triphosphates (dNTPs) of adenine, guanine, thymine, and cytosine, DNA polymerase, two DNA oligonucleotide primers, and DNA template (starting material).

PCR Makes Use of Two Basic Processes in Molecular Genetics

1. Complementary DNA strand hybridization

For DNA to be amplified, one must have a known sequence which flanks the gene of interest upstream and downstream. These sequences are used to create what are known as 'oligonucleotide primers,' meaning a short ~20 base pair nucleotide sequence which is used as a starting point for DNA replication. The primers are said to be complementary to their target regions, so they will anneal (attach) to those regions specifically. Primers are required by DNA polymerase because it cannot add nucleotides without a preexisting chain.

Complementary Strand Hybridization occurs when two different oligonucleotide primers anneal to each of their respective complementary base pair sequences on the template. They are designed specifically to anneal at opposite ends of opposite strands of the specific sequence of DNA that is desired to be amplified.

2. DNA strand synthesis via DNA polymerase

In a PCR, a special type of DNA polymerase is used that is able to withstand the temperature fluctuations required for thermal cycling. Most DNA polymerases cannot tolerate the high temperatures and fluctuations from ~60ºC-94ºC. The breakthrough in PCR came with the isolation of DNA polymerase from a thermophilic bacterium known as Thermus aquaticus. This bacterial species lives in high temperature steam vents and therefore its DNA polymerase evolved to withstand extremely high temperatures.

During PCR, DNA is synthesized and multiplies by 2 each cycle, thus the growth of DNA copy # over the reaction is exponential. In theory, after 30 cycles there will be 2^{30}. This is over a billion copies of DNA. Yielding this amount of DNA allows the possibility of visualization through a variety of means. One of the most popular visualization methods is agarose gel electrophoresis.

PCR Stages

The machinery required to perform PCR is known as a thermal cycler. The thermal cycler enables the steps of PCR to be automated. The reaction involves a repetitive series of cycles, each of which consists of template denaturation, primer annealing, and extension of the annealed primer by Taq DNA polymerase. Before beginning DNA amplification, genomic DNA is prepared from students' cells. The students' DNA is then added to a mixture of the necessary reagents: oligonucleotide primers, thermostable DNA polymerase (Taq), the four deoxynucleotides (A, T, G, C),

and reaction buffer. These reagents are pre-mixed as a 2X PCR Master Mix in the Water Analysis profiling kit. The tubes are placed into the thermal cycler. These thermal cyclers contain an aluminum block that holds the samples and can be rapidly heated and cooled across extreme temperature differences. The rapid heating and cooling of this thermal block is called temperature cycling or thermal cycling.

The first step of the PCR temperature cycling procedure involves heating the sample to 94°C. At this high temperature, the template strands separate. This is called the denaturation step.

The thermal cycler then rapidly cools to 60°C to allow the primers to anneal to the separated template strands. This is called the annealing step. The two original template strands may reanneal to each other or compete with the primers for the primers complementary binding sites. However, the primers are added in excess such that the primers actually out-compete the original DNA strands for the primers' complementary binding sites.

Lastly, the thermal cycler heats the sample to 72°C for Taq DNA polymerase to extend the primers and make complete copies of each template DNA strand. This is called the extension step. Taq polymerase works most efficiently at this temperature. Two new copies of each complementary strand are created. There are now two sets of double-stranded DNA (dsDNA). These two sets of dsDNA can now be used for another cycle and subsequent strand synthesis. At this stage, a complete temperature cycle (thermal cycle) has been completed.

Each step takes about 30 seconds to 1 minute, and this process continues for roughly 30-40 cycles depending on how the user has programmed the thermal cycler. Each step is repeated in that order each cycle until it is completed. At the end, the product is put on hold at a low temperature, generally 4°C, until the user is ready to proceed to the analysis of the product.

Check your local drinking water system. Based on the federal Safe Drinking Water Act (SDWA), all public water systems (PWS) must periodically check their water supply for possible breaks or leaks that can contaminate the water supply. Every community has to submit an annual report called consumer confidence report. Check yours here http://water.epa.gov/drink/local/index.cfm .

Coliform Bacteria

It is not feasible to scan for all pathogens (pathogens are disease carrying organisms) instead we detect Coliforms. One regulation to the previously stated act is TCR "Total Coliform Rule" that enforces a Maximum Contaminant Level (MCL). Coliforms are not necessary harmful, they are an indicator organism. Their presence implies your water system might be compromised, which could be caused by a number of things.

"Coliform bacteria" is a broad term for bacteria that are rod-shaped, Gram negative, non-spore forming and the ability to break down lactose. *Escherichia coli* is the hallmark example. *E. coli* co-evolved with mammals, evidence of this is the ability to break down lactose (the sugar in milk). All mammals produce milk for their young, at birth the baby acquires its gut flora from its mom. *E. coli* can be found in soil, on vegetation, and in the gut of warm blooded animals. It's an ecosystem we are indirectly part of. A rabbit eats plants with *E. coli*, the rabbit fertilizes the ground, plants grows on the feces, another rabbit eats the lettuce and the cycle continues and co-evolves.

This experiment is primarily for educational purposes and is not meant to replace or challenge official water testing methods. If your drinking water is positive for bacteria, consider notifying the water company, but keep in mind bacteria are everywhere and could have contaminated your test results indirectly. Be careful to wear gloves, and avoid letting the water sit too long exposed.

Troubleshooting

Symptom	Possible causes	Solutions
No amplification product	Questionable template quality	Analyze starting material
	Inhibitory Substance in reaction	Decrease sample volume
	Insufficient cycle #	Run additional cycle
	Incorrect thermal cycler program	Verify times and temperatures
	Errors in heat block incubation	Calibrate heating block, use sand or water to maximize contact with tube for proper heat transfer
	Contaminated tubes/solutions	Autoclave tubes and use filter tips
	Primer annealing temperature too high	Lower annealing temperature in 2º increments
Weak bands/faint signal	Low concentration of DNA template	Increase swabbing time, thoroughly swab.
	DNA Dye degradation during preparation	Light sensitive dyes should be kept in the dark during gel preparation. Prepare in dark room or place a box over the electrophoresis apparatus during gelation and electrophoresis.
	Expired, contaminated or degraded DNA dye	Verify that the DNA dye has not degraded in storage, been contaminated or expired.
Non-specific amplification product	Premature Taq-polymerase replication	Mix solutions on ice, place reaction directly to 94º thermal cycler
	Primer annealing temperature too low	Raise annealing temperature in 2º increments
	Insufficient mixing of reaction solution	Mix solutions thoroughly before beginning the reaction
	Exogenous DNA contamination	-Wear gloves -Use dedicated area for sample preparation -Use non-aerosol tips

Technical Service

Contact Us

For more information or technical assistance, please call, write, fax, or email.

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