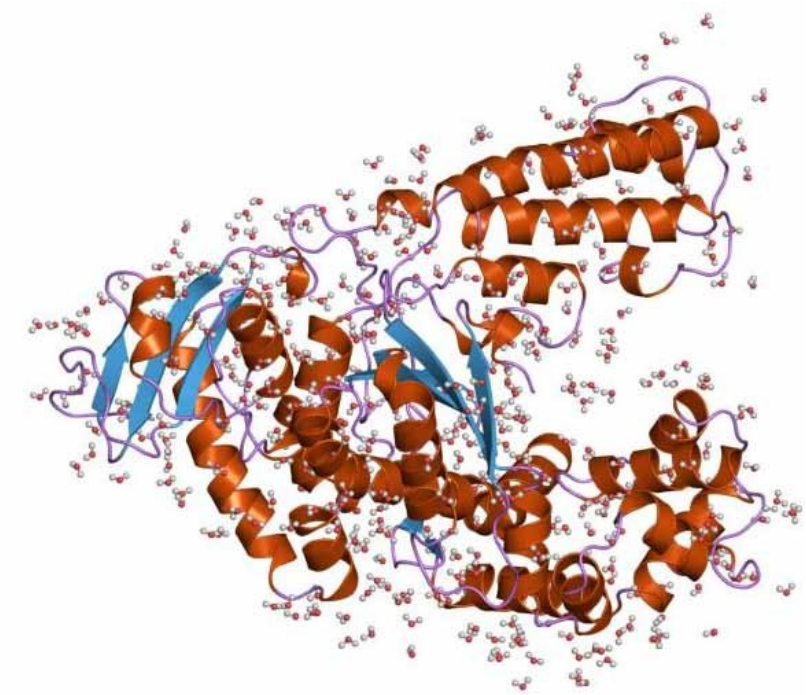
PCR: The Polymerase Chain Reaction – A Molecular Biology Revolution

My friend at school has COVID. Do I have it, too?



Molecular scale structure of the DNA polymerase enzyme from the thermophilic bacterium *Thermus aquaticus (Taq)*, isolated from Mushroom Pool, Yellowstone National Park, United States of America. *Taq* polymerase, as it is now termed, forms the heart of the polymerase chain reaction that has saved millions of lives and tremendously benefitted humanity. Credit Jawahar Swaminathan and staff at the European Bioinformatics Institute.

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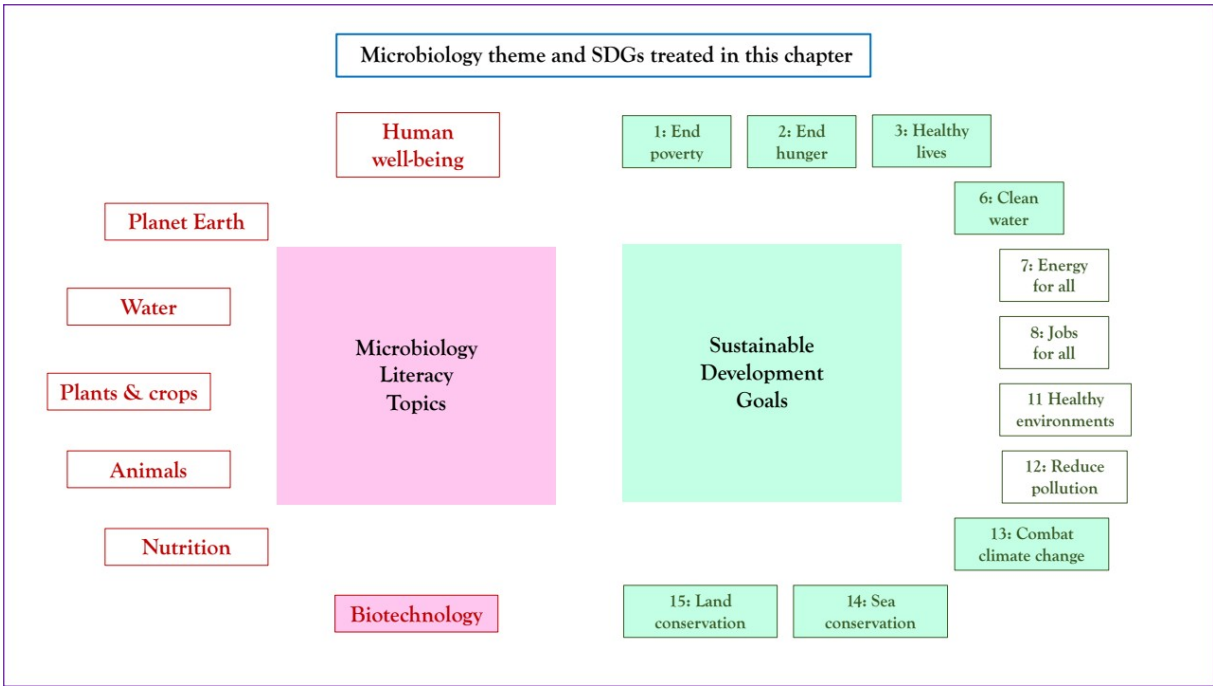
Storyline

Imagine going on your next family vacation and making a discovery that would forever change our world. That is what happened when Drs. Thomas and Louise Brock stopped by Yellowstone National Park, U.S.A., and observed the hot springs and their colorful microbial inhabitants during a vacation in summer 1964. Little did the Brock's and their colleagues know at the time, but their successful cultivation of *Thermus aquaticus* from Mushroom Pool in Yellowstone was about to change humanity. Not only was *T. aquaticus* one of the first thermophilic, or heat-loving, organisms grown in the laboratory, but it was also the source of the final ingredient necessary for the successful development of the polymerase chain reaction (PCR), which is now a mainstay method used in the scientific laboratory. As discussed below, the PCR has a variety of uses that continue to drive life-changing scientific discoveries, advance forensic science, and promote the development of vaccines, antibiotics, and other medicines.

Mushroom Pool, in the Lower Geyser Basin of Yellowstone National Park, as it looked on June 23, 1967. Thomas Brock, pictured near the edge of the pool, collected a sample from this spring that was the source of *Thermus aquaticus*, a heat-loving bacterium that contained the essential ingredient for the polymerase chain reaction, or PCR. Image from Thomas Brock's self-published "A Scientist in Yellowstone National Park". Importantly, approaching hot springs in Yellowstone National Park without a government-issued research permit is not only extremely dangerous it is also illegal. All visitors to the thermal features in Yellowstone must stay on a marked trail or a boardwalk or risk significant fines, injury, or even death.

The Microbiology and Societal Context

The microbiology: nucleic acids; DNA polymerase and replication; DNA amplification; extremophiles and their heat-tolerant enzymes. *Sustainability issues:* health: epidemiology, diagnostics, prophylactics, therapies; forensics; biotechnology; research.



The Polymerase Chain Reaction: The Microbiology

1. ***What is DNA?*** Before we discuss the fundamentals of the PCR and how it changed our world, let's take a moment and review a few key biological principles that inspired and allowed for the development of the PCR. All living cells contain DNA, referred to as a cell's genome. A genome is essentially an instruction manual that tells a cell what to do and how to do it. This genomic instructional manual is made up of thousands of smaller units, called genes. Each gene holds information for different traits or characteristics, such as hair color.

The genome from one type of organism is different from the genome of another type of organism, and these differences are responsible for variations in how organisms look and function. Each species of organism has their own unique genomic DNA sequence that can be used to differentiate it from another species. Even within the same species of organisms, the sequence of DNA can differ slightly. For example, each of us has genomic DNA with a sequence that varies slightly from others, and this is the reason that we each have a unique set of characteristics, including our height and the colors of our eyes. Thus, genomic DNA is like a fingerprint – a unique characteristic that defines an organism.

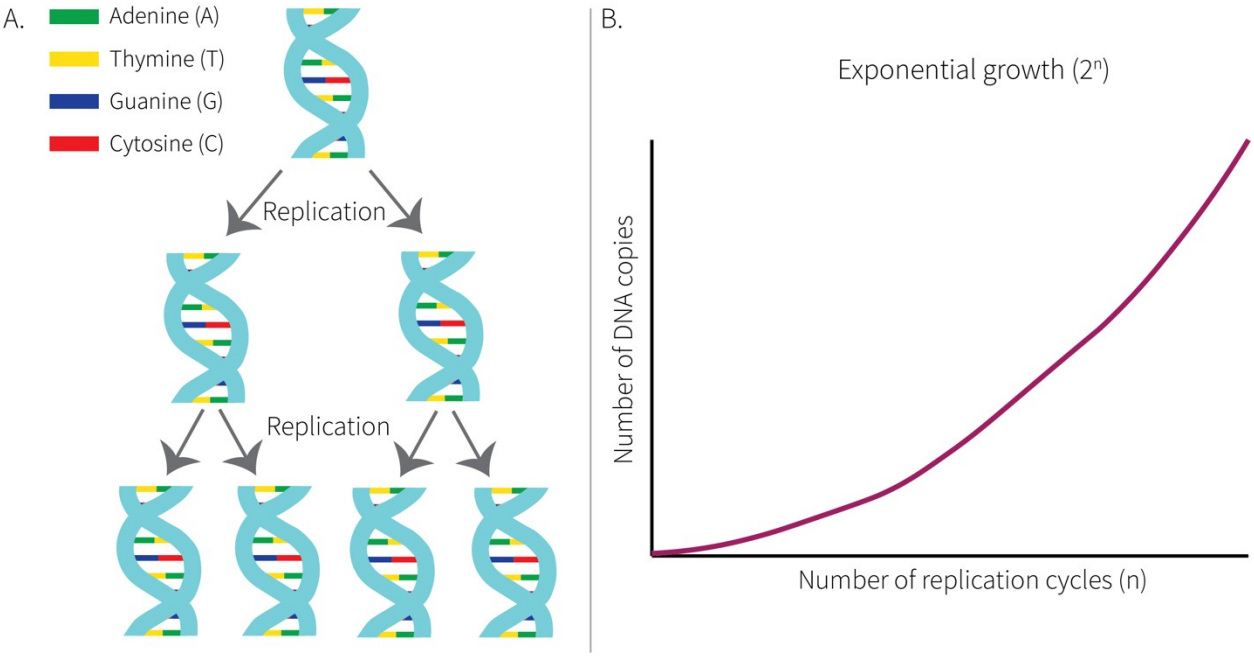
Scientists have long thought that traits and characteristics were inherited from an organism's parents, but it was not until the 1950s that the actual structure of DNA was determined. DNA is double-stranded: think of a ladder, but one that twists in the form of a

helix. The side supports of the ladder are made up of so-called sugar phosphate backbones, the components of which are constant throughout the DNA molecule. However, the rungs of the ladder do not consist of a single piece of wood or metal but rather of two pieces. In the case of DNA, each rung is made up of two compounds, called bases, that vary up and down the ladder and provide the information that is encoded in the genes.

There are four bases of different sizes in DNA: adenine (A), cytosine (C), guanine (G), and thymine (T). These bases have two key properties: Adenine (A) on one strand chemically forms hydrogen bonds with thymine (T) in the other strand, whereas cytosine (C) in one strand chemically forms hydrogen bonds with guanine (G) in the other strand. *A sticks to T, and G sticks to C.* These pairings are specific, and other combinations are not possible.

Secondly, although individual bases have different sizes, the base pairs A+T and G+C occupy identical spaces, which means that the rungs of the ladder – A+T or G+C – always have the same length – the side supports are always the same distance from one another, as in all good ladders. This pattern of base pairing – the complementarity of the two strands of the DNA double helix – represents a zipper-like structure. This finding by Drs. James Watson and Francis Crick was rewarded with the Nobel Prize.

2. ***How does DNA replicate?*** The complementarity of the two DNA strands neatly provides a mechanism for duplication of the DNA molecule by DNA polymerase because separation of the two strands allows them to be exactly copied, with an A on the strand to be copied always specifying a T on the new strand being synthesized. Each strand serves as a *template* for the synthesis of a new daughter strand.



Structure and replication of DNA. (A) The DNA double helix undergoing replication. After one round of replication a single copy of DNA becomes two. After a second round of replication, these two copies become four, and so on. Each round of replication results in a doubling of the number of copies, known

as exponential growth and in this case 2^n, where the *2* corresponds to the two strands of DNA and *n* is the number of rounds of replication, depicted in **(B)**.

The enzyme that copies DNA strands is called DNA polymerase and it is found in all forms of life. From a biological perspective, this makes intuitive sense because we know that all cells need to have a genome so, before a cell divides, its genome must be perfectly copied in order that each daughter cell receives a copy during the process of cell division.

Prior to DNA polymerase attaching to and copying DNA in a cell, the strands of DNA must be separated or "unzipped" by specialized enzymes called helicases. Once the double strand of DNA is separated into its two strands, each strand is copied by DNA polymerase to form a total of four single strands of DNA, or two double strands of DNA. This is what happens in the cell before it divides, so that each daughter cell receives a daughter genome.

3. ***The need to replicate DNA in the lab.*** Despite the advantage of using DNA to identify different species or even individuals within the same species, there is an inherent problem with this approach: the amount of DNA in a cell is really, really small. An average microbial cell only contains several femtograms of DNA and a human cell only contains several picograms of DNA. For perspective, a small paperclip weighs close to a gram. Thus, we would need to take the DNA from about a quadrillion (1,000,000,000,000,000 or 10^{15}) microbial cells or about a trillion (1,000,000,000,000 or 10^{12}) human cells (roughly all the cells in an average human body) to generate a gram of DNA. While scientists do not necessarily need a gram of DNA for most of their experiments, they do need a lot more than a femtogram or a picogram. As such, scientists need a way to increase – to amplify – the amount of DNA from a cell without changing the sequence of that cell's DNA: they need to replicate it in the laboratory.

When we want to replicate DNA to obtain large quantities, we do the same thing but in the "test tube" (i.e., outside of the cell), and we do not carry out one round of replication each time but rather multiple rounds: through a second round of copying, two double strands of DNA become four double strands, and, after a third round of copying, four double strands of DNA become eight double strands. This is called an exponential function and represents a doubling in the number of the DNA strands during each round of replication.

4. ***DNA strand separation in the test tube.*** Scientists have also learned that an efficient way to unzip the strands of DNA is by simply increasing the temperature to near boiling, or ~94°C (201°F). This is the preferred method of separating DNA strands in a test tube in the laboratory since it does not require a special helicase enzyme. However, these temperatures are hard on proteins and can cause them to denature and become non-functional. For example, when you cook a chicken egg (~71°C, ~160°F), the proteins and enzymes that form the transparent liquid components denature to form a white, opaque, more firm, solid texture that can be eaten. This also means that the egg itself can no longer develop into a chick, since those proteins and enzymes are denatured and no longer functional. A similar thing happens to a typical DNA polymerase enzyme when it is subjected to heating to 94°C – it becomes denatured and non-functional.

But this created a dilemma for scientists: in order to amplify DNA by carrying out multiple rounds of replication with DNA polymerase, they first needed to heat the mixture to separate the strands. But, this essential heat step denatured and deactivated the DNA

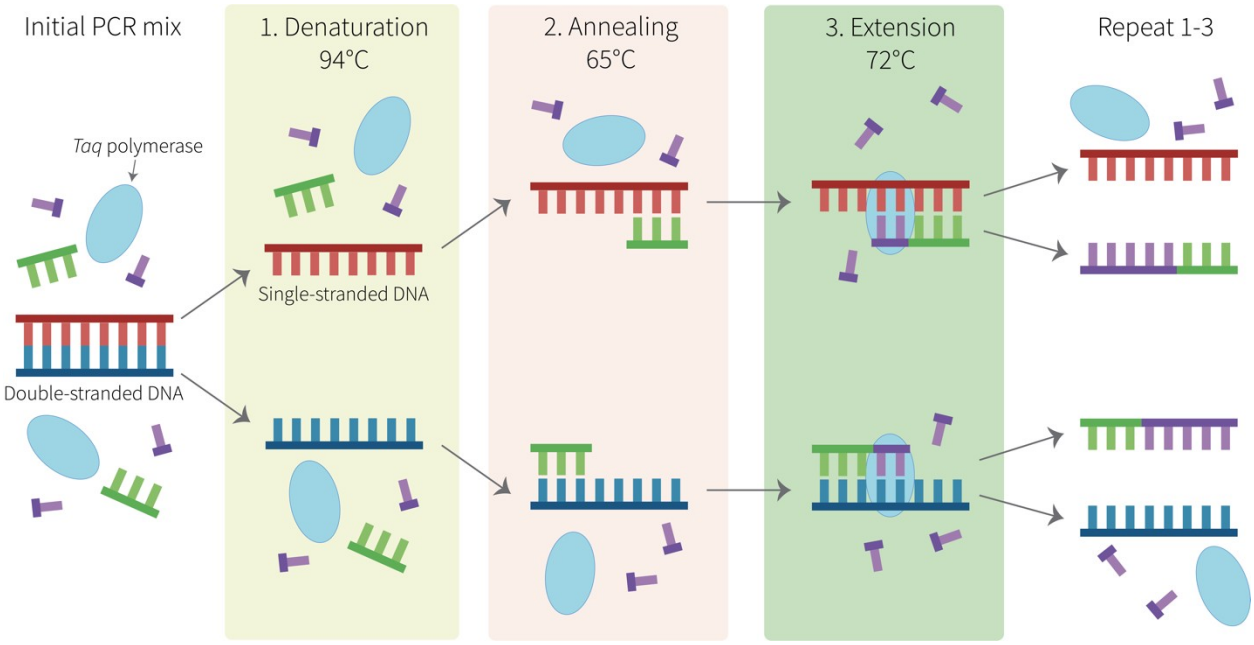
polymerase itself. Thus, for each doubling of DNA – each replication cycle – scientists had to add more DNA polymerase, a costly and time demanding task. What were scientists to do?

5. ***What is the connection between extremophilic microorganisms and DNA amplification?*** In the mid-1980s, several groups of scientists were working to develop a way of amplifying an organism's DNA. About the same time that scientists were developing methods to copy DNA, Dr. Brock and his colleagues had isolated the first thermophilic microorganisms, including *T. aquaticus*, from hot springs in Yellowstone. These organisms not only tolerated high temperatures (>90°C), but they grew best at those extreme temperatures. At the time, no one suspected that any organism could thrive at these temperatures. For these cells to grow at high temperature, they need to be able to effectively copy their DNA at these temperatures. This meant that their DNA polymerase should remain functional at high temperature.

Scientists proceeded to isolate the DNA polymerase from *T. aquaticus* (called *Taq* polymerase) and test its utility replicating DNA through the repeated cycles of DNA strand unzipping and copying. Remember that to unzip, or denature, DNA, it must be heated to temperatures much higher than the body temperature of humans or other animals (see PCR Figure below). So, for a DNA polymerase enzyme to function during each step of DNA replication, it would need to withstand exceedingly high temperatures. Remarkably, *Taq* polymerase remained functional each time the DNA was heated to 94°C to separate the strands prior to replication, meaning that new DNA polymerase did not need to be added after each denaturation step, so the reaction could be automated, and numerous cycles (rounds) could be run in sequence. It is this DNA polymerase enzyme that gave birth to the polymerase chain reaction: the PCR.

6. ***How does the PCR work?*** DNA polymerases cannot initiate the synthesis of a complementary DNA on a naked template DNA strand: they can only extend an existing complementary piece of DNA. The cell solves this by providing a short piece of DNA (or RNA), called a primer, on the template. Scientists figured out that by adding to the DNA mix a short, synthetic primer that could be extended by DNA polymerase, a particular region of DNA could be specifically targeted for precise amplification of a single gene of interest.

Soon, specialized instruments that could rapidly heat and cool reactions were invented, allowing for the exponential replication of 1 molecule of double stranded DNA into nearly 500 million identical copies in just 30 cycles of PCR that together took less than a few hours. Further biotechnological advances have allowed for optimization of the PCR through modification of the *Taq* enzyme and improved reaction conditions, thereby increasing sensitivity and efficiency of the reaction. This modern-day PCR technology has ushered in a new generation of applications.



PCR. Through a series of heating and cooling steps and with the addition of *Taq* DNA polymerase and primers, DNA can be replicated exponentially through the polymerase chain reaction (PCR). The first step of the PCR is to denature the double-stranded DNA into single-stranded DNA. This allows for short DNA sequences, called primers, to attach, or anneal, at 65°C to the single-stranded DNA in the second step. Finally, the *Taq* DNA polymerase extends the complimentary single-stranded DNA starting at the primers (step 3). This is repeated upwards of 30 cycles to generate millions of copies of a single DNA fragment.

7. ***Societal Impacts: How does PCR impact our daily lives?*** The PCR is an essential tool in clinical, pharmaceutical, forensic, and research laboratories because it can be used to create millions of identical copies of a gene or DNA fragment. In some cases, clinicians and researchers want to simply determine whether the gene is present in a sample or not. However, to do this, they need a sensitive way to detect a gene even if it is present in extremely low numbers. This approach might be used to identify an infectious microbe in the body of a sick patient. This use of PCR might enable the monitoring of the spread of an emerging contagious disease or to detect biothreat agents or antimicrobial resistance genes. In other cases, clinicians and researchers use the PCR to create enough identical copies of a gene to enable the determination of the sequence of that gene to identify an organism or individual. This is common in the research laboratory where scientists are aiming to describe the number of sequence variants of a gene (termed diversity) in a sample. Still in other cases, researchers use PCR to generate enough identical copies of a gene so that it can be effectively used in molecular biology applications to modify what an organism can do. Let's take a closer look at a few key examples of these applications.

- ***Medicine.*** One of the most substantive ways that the PCR has impacted human society is in its use in the pharmaceutical industry. For example, it is estimated that nearly 6% of the world's population is affected by diabetes, a condition where the body does not produce adequate amounts of the hormone insulin to regulate the amount of glucose in a person's blood stream. This disease is especially prevalent among low income and impoverished

populations, meaning that any treatment for this disease would not only need to be widely available but also low cost.

The primary treatment for diabetic patients is to provide them with insulin made from a source from outside of their body. In the past, insulin was obtained from the pancreas (the organ that makes insulin) from cows or pigs. However, today, nearly 500 million people are afflicted with diabetes while there are only ~ 1 billion cows and ~700 million pigs on the planet that could serve as a source of insulin. There are simply not enough cows or pigs on Earth to provide enough insulin to provide daily doses to diabetic individuals.

To overcome this limitation, scientists used the PCR and other molecular biology tools to turn microbial cells into insulin production factories. The genes that are required to produce insulin were copied by the PCR and introduced into the bacterium *Escherichia coli* and the fungus *Saccharomyces cerevisiae*. These genetically modified organisms (GMOs) are easy and inexpensive to grow in large quantities. This means that these GMOs can be used to produce large amounts of insulin for relatively low cost. We owe a debt of gratitude to the PCR, in particular *Taq* polymerase, for making it possible to generate synthetic insulin to treat the global diabetes epidemic.

- ***Pathogen ecology and disease epidemiology.*** Between December 2019 and April 2022, nearly 500 million people were infected by a novel coronavirus (SARS-CoV-2), resulting in over 6 million deaths, and counting. We all remember this as the COVID-19 pandemic. However, how do clinicians and scientists know how many cases of COVID-19 have occurred globally? In other words, where did this number of estimated infections come from?

The SARS-CoV-2 virus is an RNA-virus, meaning that its genome is made of single stranded RNA rather than DNA. (Note: most scientists do not refer to viruses as living organisms because they cannot replicate on their own, thus the earlier statement that all living organisms have DNA genomes remains true). The most sensitive way to detect SARS-CoV-2 is to first convert the RNA of the virus into DNA through a process called reverse transcription. Then, clinicians and scientists apply the PCR to copy the SARS-CoV-2 genetic material to levels high enough to be identified in the samples.

If you have had a PCR-based COVID test, you know these samples typical come from your nasopharynx, or your nose. This test is so sensitive that it can detect the presence of as few as 100 SARS-CoV-2 RNA molecules per milliliter of specimen. In this case, PCR is being used to identify and help stop the spread of the deadly virus that causes COVID-19. Of course, PCR is key to identifying new variants of concern of pathogens, mutants that have important new properties, such as heightened virulence or transmissibility, and was crucial for monitoring and modelling the successive waves of COVID-19 variants.

PCR was used as a diagnostic tool long before COVID-19; it is also commonly used to identify the presence of other human pathogens such as *Chlamydia trachomatis* (a bacterium that causes the sexually transmitted disease chlamydia), *Streptococcus* (the bacterium that causes strep throat), HIV (the virus that causes acquired immunodeficiency syndrome or AIDs), *Mycobacterium tuberculosis* (the bacterium that causes tuberculosis), *Neisseria gonorrhoeae* (the bacterium that causes the sexually transmitted disease gonorrhea), and influenza (the virus that causes the flu), among countless others. Once detected, appropriate treatments can be quickly administered to control these infections. Our ability to rapidly detect and identify infectious

agents using the PCR has had a huge impact on how clinicians treat diseases and public health agencies monitor and control their spread through our communities.

- ***Forensic science: the PCR fingerprint.*** PCR also plays an important role in criminal justice, particularly for forensic scientists. Forensic scientists are tasked with collecting and analyzing evidence from crime scenes, including the possible presence of fingerprints on surfaces that may have been touched. Oftentimes, the evidence that is collected is not in and of itself capable of being used to identify a criminal or a victim. For example, a hair left at a crime scene on its own cannot be used to identify someone since there are only so many varieties and colors of hair. The same is true for blood collected at a crime scene. Further, those hair and blood samples could be from an entirely different type of animal! Fortunately for forensic scientists, the root of a hair (where it attaches to your skin) and blood contain cells, and these cells contain genomic DNA unique to the individual who left them. However, criminals are often keen to not leave evidence behind and thus samples of their hair and/or blood are likely to be minimal.

To overcome this limitation, forensic scientists use the PCR to create millions of identical copies of the DNA from those pieces of evidence. With millions of identical DNA copies, forensic scientists can obtain the DNA sequence from the piece of evidence and compare it to DNA sequences obtained from suspects to find a match. Remarkably, the use of the PCR by forensic scientists has assisted in thousands of criminal investigations and prosecutions of perpetrators. It has also been used to prove that a suspect is innocent of a crime. In both instances, it has been used to retrospectively identify perpetrators/show innocence in old cases that have been re-opened (i.e., cold cases).

- ***Basic research.*** The PCR is routinely used by the research community, and perhaps nowhere has it made more of an impact than on environmental microbiology. Environmental microbiologists have long known that the organisms that can be grown in the laboratory represent just a fraction of the organisms that are known to exist in nature. But it was not until the PCR was applied to DNA obtained from environmental samples that they were able to prove this unequivocally.

In the first application, scientists extracted DNA from Obsidian Pool, a hot spring in Yellowstone National Park. The small amount of DNA that was obtained was used in a PCR to generate enough copies of a gene to allow for its sequencing. What the scientists discovered was an entirely new world of microorganisms (identified by their unique DNA gene sequence) that was previously unknown to science. The sheer enormity of the newfound biodiversity is equivalent to walking outside and discovering plants (phylum Plantae), which represent a single phylum of organisms, for the first time but doing this close to a dozen times – nearly 20 new bacterial and archaeal phyla were discovered in that single hot spring sample.

In the three decades since its first use in environmental microbiology, DNA from thousands of samples from across the world have been subjected to PCR and sequencing and, amazingly, scientists are continuing to discover new microorganisms. These studies have revolutionized our understanding of the evolution of life on Earth and the role of microorganisms in the global biogeochemical cycles that sustain plant, animal, and human health. And let's not forget, each one of these newly identified organisms could host the next major discovery, just like *T. aquaticus* and the PCR.

Relevance for Sustainable Development Goals and Grand Challenges

- **GOALS 1&2: End poverty and hunger.** The human population is increasing at a dramatic rate and our civilization faces a daunting task of feeding the 8 billion humans that are alive today (2022). One proposed solution is to incorporate genetically modified organisms (GMOs) with enhanced traits into common agricultural practices. These GMO crops have been designed to be resistant to pathogens, such as insects and viruses, tolerant of herbicides used to kill competing weeds, and to have increased yields. Importantly, all these traits theoretically can help to lower the cost of food production and increase crop yields. Development of GMO crops relies heavily on molecular biology tools including the PCR to introduce specific genes encoding those traits and to detect and confirm that those specific genes have been correctly inserted into a plant's genome, such as herbicide or pathogen resistance.
  Scientists have also been working to genetically engineer or modify food crops to be more nutritious. For example, golden rice is a genetically modified variety of white rice that has been engineered to produce a precursor to vitamin A, called beta-carotene. In many regions of the world, including sub-Saharan Africa and South Asia, vitamin A deficiency is highly prevalent and can cause blindness and increased risk of mortality in children. Natural sources of vitamin A include animal products, leafy greens, sweet potatoes, carrots, tomatoes, and bell peppers, which are not readily available in these poverty-stricken regions. Thus, several decades of molecular research that has relied on PCR has been dedicated to developing golden rice as an inexpensive alternative to combat global vitamin A deficiency.

- **GOAL 3: Good health and well-being.** As our global climate continues to change and the world's population expands, infectious diseases are becoming increasingly more prevalent and rampant. The PCR provides a rapid and specific method for detecting and identifying infectious agents, such as viruses and Bacteria. During the COVID-19 pandemic, the PCR has been instrumental in identifying new variants and helping scientists better understand how the virus is transmitted from animal to person, from person to person, and how variants impact disease outcomes. Having more knowledge of how viruses spread, or their epidemiology, will help scientists and governments implement policies that may help stop the spread of the next pandemic.

- **GOAL 6: Clean water and sanitation.** As populations began to grow rapidly around the turn of the 19th century, so did the prevalence of deadly diseases such as typhoid, cholera, and yellow fever. Scientists eventually linked the pathogens that cause these diseases with municipal drinking water supplies. To traditionally track the pathogen load in drinking water supplies, technicians would see how many microorganisms would grow on a petri plate. However, this process takes 24-48 hours to complete, which could result in contaminated water reaching households before pathogens were detected.

Furthermore, many pathogens do not grow on petri plates and may go undetected. In more recent history, scientists have developed PCR-based methods to detect and identify pathogens. This approach is rapid and can provide much higher sensitivity than culture-based techniques in as little as a couple hours, which has helped to reduce the frequency of water-borne disease transmission. As a notable example, scientists are now using PCR-based methods to detect and track the prevalence of pathogens that cause COVID19 and polio on municipal wastewaters. These efforts are allowing scientists to track, in near real-time, pathogen burdens in cities.

- **GOAL 13: Climate action.** Greenhouse gases, including carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O), trap heat in our atmosphere leading to rising temperatures on Earth. Microorganisms are involved in cycling these greenhouse gases through several specific processes mediated by different enzymes. By using the PCR, scientists can quantify and determine the prevalence and activity of the genes involved in microbial greenhouse gas consumption and production. Through this microbial involvement in greenhouse gas cycling can be linked to different environmental factors, such as moisture, sunlight, temperature, and nutrient loading. Furthermore, organisms or genes correlated with key processes can be identified for targeted investigation. With a better understanding of the factors that control microbial cycling of greenhouse gases in hand, scientists can better predict the rates of these processes and can make recommendations to maximize the consumption and minimize the production of greenhouse gases to reduce overall emissions, helping to mitigate climate impacts on ecosystems.

- **GOAL 14: Life below water.** Marine ecosystems are undergoing rapid change as Earth's climate changes on a global scale. Loss of biodiversity is a major concern as the habitats for marine organisms change more quickly than species can adapt. The PCR is an essential tool for surveying biodiversity at the genetic level by amplifying genes common to all organisms in preparation for DNA sequencing. This process provides a catalog of all organisms present in a sample collected from seawater, the seafloor, or on individual organisms like corals, sharks, and whales. In the past, this approach has primarily focused on cataloging the microorganisms present in a sample. However, in recent years, scientists have begun to develop methods of cataloging macroorganisms that have recently travelled through a body of water, such as fish or sharks, by using a method called *environmental PCR* or *ePCR*. By comparing these "snapshots" of biodiversity with those collected in the past or in different regions, scientists can better understand what factors might be leading to extinction events and loss of biodiversity.

- **GOAL 15: Life on land.** Like the approaches used for cataloging microorganisms present in marine samples (see **Goal 14**), the PCR is frequently used in terrestrial samples such as soils, permafrost, lakes, and streams to amplify genetic markers of biodiversity. In a classic example, scientists use PCR on DNA recovered from hair that has been collected on traps placed in forests to track the migration and intermingling of populations of animals, such as the grizzly bear. In addition, the PCR can be used to

identify and track diseases or invasive animals/plants that might be decimating populations of native animals and/or plants, helping scientists and epidemiologists determine ways to stop the spread of harmful diseases and organisms. Invasive organisms are those that spread rapidly and can rapidly alter or destroy a healthy, functioning ecosystem. By using eDNA, scientists can track the spread of invasive species, particularly in aquatic systems.

Potential Implications for Decisions

1. ***Individual***
    - **a.** Disease/pathogen identification
    - **b.** Antibiotic resistance

2. ***National Policies***
    - **a.** Pathogen detection and identification in municipal water supplies
    - **b.** Surveys of biodiversity and factors leading to loss of biodiversity
    - **c.** Consider policies for genetically modified organisms in agriculture
    - **d.** Consider policies for invasive species monitoring using eDNA

Pupil Participation

1. ***Class Discussion***

a. Using the PCR, it is possible to quickly screen wastewater for the presence of disease-causing pathogens, such as COVD-19, polio, or monkeypox. What are some ethical concerns for using this biotechnological approach to screen the wastewater from individual homes or apartment buildings as a means for tracking the spread of infectious diseases? Is it possible that certain demographic groups might be subject to discrimination based on increased detection of certain pathogens in wastewater?

b. Aside from seawater or soils, what are some other environments where you might want to use the PCR to identify organisms that might be present?

2. ***Exercises***

a. Can you calculate the number of copies of a gene that would be present after 30 rounds of the PCR? Assume you are starting with just a single copy of a gene.

The Evidence Base, Further Reading, and Teaching Aids

The polymerase chain reaction fact sheet. https://www.genome.gov/about-genomics/fact-sheets/Polymerase-Chain-Reaction-Fact-Sheet

COVID-19 and PCR testing. https://my.clevelandclinic.org/health/diagnostics/21462-covid-19-and-pcr-testing

The PCR protocol. https://www.addgene.org/protocols/pcr/

Pace, N R. A Molecular View of Microbial Diversity and the Biosphere. 1997. Science. https://www.science.org/doi/full/10.1126/science.276.5313.734

Dougherty M M, Larson E R, Renshaw M A, Gantz C A, Egan S P, Erickson D M, David M. Lodge. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. 2016. Journal of Applied Ecology. https://besjournals.onlinelibrary.wiley.com/doi/10.1111/1365-2664.12621

Glossary

Cultivation—the process of growing a single organism in isolation, often in a laboratory setting
Polymerase chain reaction (PCR)—a technique used to replicate, or amplify, short pieces of DNA
DNA—deoxyribonucleic acid, a biomolecule present in all living organisms that contains the genetic instructions, or blueprint, for that organism's characteristics
Genome—the full collection of DNA present in an organism
Gene—a single unit of DNA that is defined by a sequence of bases, or nucleotides, that can be used by a cell as the instructions for making a specific protein
DNA polymerase—an enzyme that creates new DNA by adding nucleotides
Thermophilic—describing a heat-loving organism