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PCR Turns 25: Celebrating the Past and Anticipating the Future

By DAVID GELFAND, PhD

Introduction

Beginning with the production of the very first recombinant deoxyribonucleic acid (DNA) molecule in 1972 and continuing through the following decade, DNA-focused molecular biology researchers firmly positioned themselves on the cutting edge of scientific inquiry. With each passing day, month and year, these researchers made significant leaps forward in understanding, modifying and manipulating the key roles that DNA plays in all living organisms. Some of the key milestones from this decisive time included:

- maintenance and replication of DNA in *E. coli* (1973);
- development of the chain termination method for sequencing DNA (1977);
- production of human insulin using recombinant DNA technology (1978); and
- discovery of the first human oncogenes (1981).

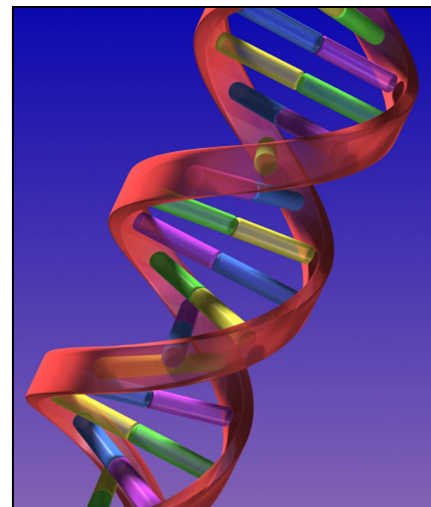
Yet, even with all of these groundbreaking developments, an enormous impediment remained in truly providing researchers the capability to unlock the scientific potential of DNA, the essential building block of life. Without the ability to significantly and specifically amplify trace amounts of DNA, it was cumbersome for researchers to obtain appropriate quantities of the

precise genetic material needed to continue and expand their cutting-edge experiments.

In early 1983, aware of the need for a solution to this fundamental limitation of genetic research, Dr. Kary Mullis, a scientist at Cetus Corporation in Emeryville, California had a simple but revolutionary thought: Perhaps researchers could mimic the body's own process of natural DNA replication in a test tube to produce large quantities of DNA for research needs. With that concept as inspiration, he began to shape the framework of a technique that would revolutionize science and leave its fingerprints on broad-reaching areas of research for decades to come. This technique, which would come to be known as polymerase chain reaction, or PCR, led to Dr. Mullis sharing the 1993 Nobel Prize in Chemistry.

Today, 25 years after its initial discovery, PCR continues to be one of science's most important molecular research tools. It has grown to enable valuable applications in such broad-reaching areas as:

- molecular diagnostics;
- gene expression;
- DNA sequencing, genotyping and human leukocyte antigen (HLA) typing;
- forensics, ecology, anthropology/evolution;
- infectious disease detection;
- blood supply safety management; and
- quantitative viral load assessment for disease management.



Furthermore, the information that PCR allows to be harvested is essential to the growing personalized medicine revolution, highlighting the continued importance of the technique in years to come.

Conception and the Early Days

The objective of PCR was to enable the quick and repeated replication of small samples of DNA — sometimes as small as a single molecule — to produce a sufficient amount of the molecular material to be studied effectively. In order to mimic the body's natural DNA replication process for this purpose, Cetus researchers relied on the activities of naturally-occurring enzymes known as polymerases. These proteins work in the body to build and repair DNA and ribonucleic acid (RNA) by constructing complementary strands based on specific DNA or RNA templates.

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Under the proper conditions and with the appropriate complementary components, researchers knew that polymerases could synthesize DNA *in vitro*. To test this theory, Cetus researchers combined a polymerase with: 1) a specific DNA sample (template) to be copied; 2) nucleotides that included the four nucleotide bases that comprise DNA (adenine, cytosine, thymine and guanine); and 3) two small synthetic DNA fragments known as a primers. By alternating the temperature of this mixture, they triggered a three-step process that created two copies of each target DNA template in the sample. The steps that have now become the fundamental components of the PCR process are:

- 1) **Denaturation** – Intense heat causes the target double-stranded DNA molecule to separate into two single strands.
- 2) **Annealing** – A temperature reduction causes the two primers to bind to the complementary single DNA strands. These primers bind at specifically-targeted points on the DNA strands, allowing the precise desired portion to be copied.
- 3) **Extension** – At the same temperature as annealing or at a second increase in temperature, the polymerase begins synthesizing the DNA copy by triggering the binding of the nucleotides to the appropriate sections of the single-stranded target DNA. The nucleotides bind according to the exact sequence of the portion of the single DNA strand. This binding occurs only along the DNA portion that falls between the two primers. The result is the creation of a copy of the original double-stranded DNA target.

Continually repeating this process will exponentially increase the number of target DNA copies. After two cycles, the result will be four copies; after three cycles there will be eight copies; and after 20 cycles there will be more than a million DNA copies. The process can be repeated as many times as necessary to achieve the desired number of DNA copies.

Early Modifications to PCR Technique

While the Cetus researchers' earliest pioneering demonstrations of PCR were a significant success, and laid the framework for one of science's most valuable breakthroughs, this work was not without problems. Most importantly, the polymerase component used in the initial PCR experiments proved to be particularly troublesome. Cetus researchers used the Klenow Fragment of the DNA polymerase I from *E. coli* as the polymerase to trigger DNA replication. However, this polymerase lacked stability at the high temperatures required for PCR's denaturation step, and thus was inactivated at this point during each PCR cycle.

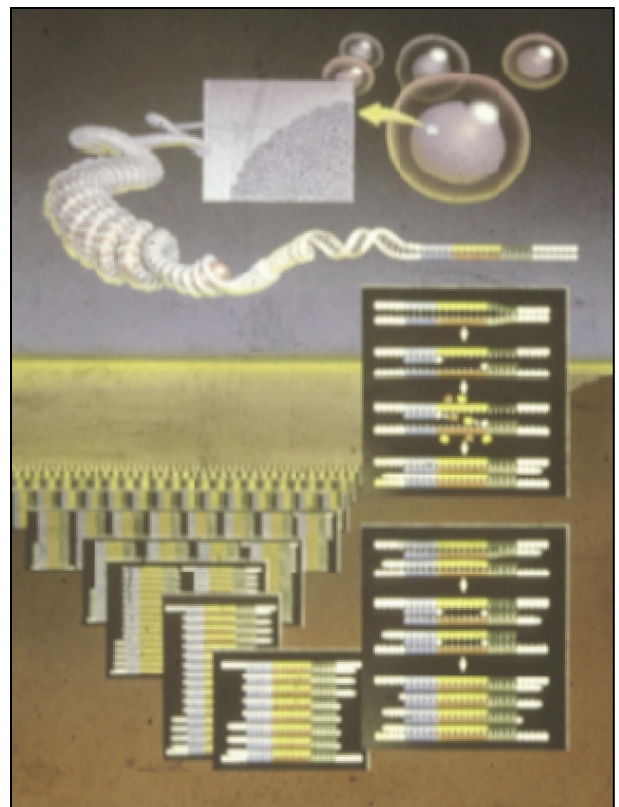
Consequently, additional polymerase was required following the denaturation step of each cycle, rendering early PCR a laborious, time-intensive and expensive endeavor. Without overcoming this issue of polymerase inactivation, PCR would have had difficulty ever realizing its true potential. Fortunately, for researchers throughout the world, there was a perfect solution waiting in the hot springs of Wyoming.

With polymerases being naturally-occurring enzymes found in all living creatures, the best avenue for identifying a polymerase that could handle the extreme PCR temperature requirements was to look to organisms living in inhospitably hot environments. Researchers had already found and studied certain microorganisms that thrived in temperatures at or exceeding 70°C. One such microorganism was the bacterium *Thermus aquaticus* (*Taq*), originally discovered in the Yellowstone National Park's thermal pools. Determined to be a potentially useful contributor to PCR, Cetus

researchers began cultivating *Taq* to isolate and attempt to purify its DNA polymerase, hoping that the enzyme would not be irreversibly inactivated by the temperatures required for DNA strand separation.

The ultimate replacement of the original PCR polymerase with *Taq* DNA polymerase proved to be a tremendous boon for PCR, allowing the entire process to be run without ever having to replenish polymerase. In addition, *Taq*'s heat-resistant DNA polymerase could be used at higher temperatures in the annealing step in the PCR process. These higher temperatures greatly increased the stringency conditions for primer binding, and thus significantly improved analytical specificity and sensitivity. Having to add enzyme only once enabled the automation of the process and led to great improvements with regard to the speed, cost and workload associated with PCR. This allowed for the widespread adoption of the technique among researchers.

The contribution of *Taq* DNA polymerase to the fundamental PCR process



The Various Cycles of PCR.

cannot be overstated. This fact was evidenced in 1989 by Science magazine that awarded the first “Molecule of the Year” designation to *Taq* DNA polymerase, “...the DNA polymerase molecule that drives the [PCR] reaction.”

Since a significant amount of molecular research also entails the study of RNA as opposed to DNA, researchers required early versions of PCR to possess the capability to study RNA. However, since RNA is not an efficient template for *Taq* DNA polymerase, a creative solution was required. To resolve this issue, researchers elected to reverse transcribe (*i.e.*, copy) the RNA back to a DNA strand. Generating this complementary copy of DNA allowed researchers to then conduct standard PCR to unlock the clues of the original RNA sample. This process, known as reverse transcription PCR (RT-PCR), was achieved through the use of the enzyme known as reverse transcriptase.

RT-PCR rapidly became a critical research tool, particularly with the discoveries of thermoactive and thermostable reverse transcriptases that markedly improved the analytical specificity and sensitivity. Also, the cost of RT-PCR was reduced since only a single enzyme was required. The process would prove valuable in the decades following its development as researchers began to focus more and more on gene expression (measurement of mRNA) as a means to diagnose disease and develop novel treatments.

Early Applications for PCR

During the earliest days following the discovery of PCR, the technique quickly became central to enabling some groundbreaking applications. One specific individual for whom PCR proved to be critically important was Gary Dotson, the first person in the world to be exonerated of a criminal conviction by DNA evidence. The ability of PCR to replicate minute concentrations of DNA enabled law enforcement officials to positively exclude Dotson as the source of a semen stain in connection to a rape for which he was

falsely accused.

In 1989, more than ten years after being sentenced for the rape, Dotson walked out of prison a free man thanks to the power of PCR. Ever since, PCR has played a leading role in the field of forensics, helping to convict the guilty and, perhaps more importantly, clear the innocent. According to the Innocence Project,¹ DNA testing to date has exonerated 233 people in the United States, including 17 who served time on death row.

Infants born to mothers with human immunodeficiency virus (HIV) constitute another group impacted by PCR’s initial discovery. PCR’s ability to identify and replicate the smallest amount of DNA allowed researchers to quickly and easily determine the presence of even low concentrations of the HIV virus in these infants. In fact, two of the first groups to enter into PCR technology licensing agreements with Cetus Corporation were reference laboratories involved in screening infants of HIV-positive mothers.

Another almost immediate application of the PCR technique involved the identification of genetic mutations that caused or contributed to specific human diseases. The first genetic application of PCR to a disease was the detection of the mutation in the β -globin gene responsible for causing sickle cell anemia. This provided researchers and physicians with a powerful new tool in diagnosing and treating many genetic diseases. With the undeniable power of PCR, the ability to identify those molecular contributors to disease that reside in DNA became a reality, laying the founda-

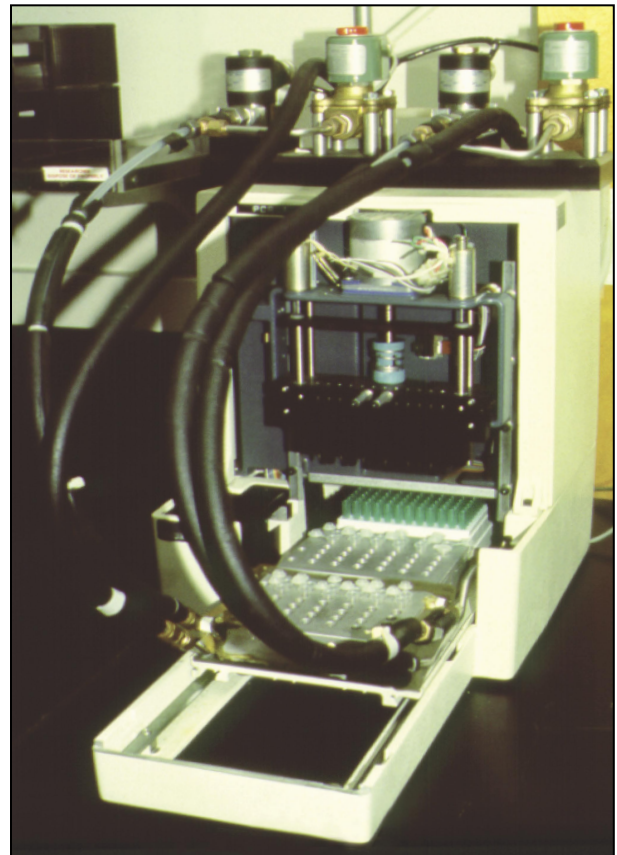
tion for future applications in the area of screening for genetic diseases.

Key PCR Enhancements

As PCR was rapidly adopted by research laboratories around the world in the late 1980s and 1990s, innovators in the field began to spearhead critical enhancements to the fundamental technology. These improvements to PCR were designed to simplify the process and enable the execution of newer, more revolutionary research. While there have been countless incremental enhancements since PCR’s discovery, three of the most important and empowering were: 1) introduction of the thermocycler; 2) discovery of new thermostable polymerases; and 3) the creation of real-time PCR.

The Role of the Thermocycler

While the engineering and science groups at Cetus began developing thermocyclers in 1985, the first commercially available thermocycler



The very first thermocycler (built at Cetus in 1984) for Klenow-mediated PCR. (On display at the Smithsonian Institution.)

appeared in early 1988, a quantum leap forward for the PCR process. Designed to automatically and precisely regulate the temperature changes required for PCR, thermocyclers allowed for complete walk-away automation of the process for the very first time. With these instruments, researchers were able to program PCR experiments and then focus on other activities while the PCR work was performed.

This advancement turned PCR from a laborious and time-consuming operation to a simple process and fundamental component of all molecular biology laboratories. Simply put: *Taq* DNA polymerase and thermocyclers afforded all researchers, regardless of workload, realistic access to PCR technology.

Discovery of New Thermostable Polymerases

The discovery of a variety of new thermostable polymerases by scientists in the Program in Core Research at Roche Molecular Systems enabled broad application of PCR in answering a variety of emerging scientific questions. In fact, these new enzymes and applications permitted the construction of a whole new conceptual framework for the postulation of new questions not previously imaginable. Two examples that directly led to the human genome sequencing project include: 1) a new mutant form of *Taq* DNA *pol* *AmpliTaq*[®] DNA Polymerase FS, which dramatically changed automated DNA sequencing chemistry and enabled high-throughput capillary sequencing; and 2) *AmpliTaq* Gold[®] DNA Polymerase, a new “Hot Start” form of *Taq* DNA *pol*. Additionally, other enzymes and enzyme blends led to the amplification of 20–40 kb segments of DNA in addition to the achievement of exceptionally high-fidelity in PCR.

Real-Time PCR

In the early years of PCR, the technique simply operated in a qualitative fashion. If a particular segment of DNA was present in a sample, PCR would replicate it to a concentration at which it could be detected. This helped confirm or deny the presence of a spe-

cific molecular target without providing any insight into exactly how much of it resided in the original sample. For simple identification objectives related to pathogen detection — gene sequencing, forensic medicine and genetic disease diagnosis — these capabilities were more than sufficient. Some of the most important work enabled by qualitative PCR included: a) the much-publicized mapping of the human genome; and b) ongoing safety monitoring of donated-blood supplies through the identification of debilitating viruses such as HIV and hepatitis C.

However, in those instances in which researchers would benefit from not only identifying DNA but also from measuring its concentration, early PCR was not up to the challenge. This limitation provided innovative researchers an opportunity from which real-time PCR was born.

Real-time PCR (or quantitative PCR, *i.e.*, qPCR) was first described in the early 1990s as a process through which a targeted DNA strand is amplified and quantified simultaneously. Real-time PCR functions in much the same way as traditional PCR, except that an indication of the concentration of amplified DNA is provided at the end of each cycle. This is accomplished through the incorporation of either fluorescent dyes or fluorogenic (optical) oligonucleotide probes in the PCR process. Both result in a measurable fluorescence that is indicative of DNA concentration. Real-time PCR's ability to quantify DNA levels opened up entirely new avenues of investigation for researchers and include gene expression, molecular diagnosis, and therapeutic target identification and validation, among others.

Today's PCR and the Next Wave of Innovation

As important a role as PCR has played in the past 25 years, the technology's crowning achievement may only now be coming to fruition. The fundamental ability to peek into the DNA of individuals has provided researchers with the tools needed to realize the promise of personalized medicine.

Gene expression studies have provided drug developers with an understanding of how disease operates at its most basic level. The combination of that basic understanding and data generated by screening an individual's unique genetic make-up allows scientists to develop specifically-targeted treatments that are safe and effective for specific patients.

Today, this promise is already being realized through therapeutics designed to target the molecular contributors of disease. Drugs such as Gleevec[®] and Erbitux[®] have already been approved in the United States and abroad, and countless other personalized medicines populate the pipelines of pharmaceutical and biotechnology companies. Without PCR's fundamental ability to amplify DNA, none of this cutting-edge research would be possible.

However, despite the continued integral role that PCR plays in genetic research, the process continues to have specific limitations. Two of the most critical shortcomings of PCR — as we know it today — are low throughput and high cost. These drawbacks render PCR impractical for such cutting-edge research projects as whole genome analysis and other high-throughput studies. Accordingly, important emerging areas of research including high-throughput gene expression and genotyping cannot be fully pursued with existing PCR technology. With PCR's impressive track record of innovation and continuous enhancement, researchers should be up to the task of developing new solutions to address these current shortcomings. In fact, one such product that may be able to overcome these specific limitations is already in development.

SmartChip[™] Real-Time PCR System

The SmartChip Real-Time PCR System is designed as the first whole genome, high-throughput gene expression real-time PCR platform with the goal of delivering significant speed and cost advantages to researchers in the gene expression and genotyping markets. The fundamental premise of the system lies in enhancing the sensitivity and accuracy of current real-time PCR technology by combining it with the

high-throughput capability and cost-efficiencies of existing microarrays.

Ultimately, this marriage of technology and capabilities can significantly increase current throughput levels while offering discovery and validation in a single step. The result will be the ability to conduct gene expression research at a fraction of the time and cost currently produced by existing instrument systems. For example, whole genome assay time may be significantly reduced with the SmartChip system, relative to the several days or months required with microarrays or real-time PCR, respectively.

This decrease in research time has broad economic implications across the drug development industry, particularly for pharmaceutical companies seeking avenues for reducing time to market for its therapeutics. Further, this level of throughput has the potential to dramatically impact the cost of such assays.

In addition, the SmartChip system incorporates a number of key ease-of-use features including content-ready, high-density chips containing 30,000–100,000 nano-wells with gene panels optimized for cancer, toxicology and whole genome applications. At the same time, the system will only require a very small sample size as compared to other technologies and platforms. It will offer real-time detection and sophisticated readout options while

assuring detection sensitivity and temperature uniformity across chips.

The SmartChip system, which is being developed by Fremont, California-based WaferGen Biosystems, is currently involved in an alpha testing program being conducted at the University of Pittsburgh Medical School. The company expects that the platform may be available to researchers through an early access program in 2009.

Summary

Looking back over the past 25 years of scientific breakthroughs, it is difficult not to view PCR as one of the industry's most vital contributing technologies. From its conception, early experimentation, and development by researchers at Cetus Corporation—and subsequently at Roche Molecular Systems—to its recent contributions to evolution and analysis of DNA samples from extinct organisms, to the birth of personalized medicine, PCR has served as one of molecular research's most important tools.

As PCR continues to contribute to the rapidly growing collection of knowledge related to the molecular makeup and origins of humans, the technology will continue to evolve to meet the expanding needs of researchers. Therein lies the beauty of this simple yet elegant technique.

ABOUT THE AUTHOR

David Gelfand, PhD, is one of the pioneers of the PCR technique and is credited with developing *Thermus aquaticus* DNA polymerase and thermostable reverse transcriptases while at Cetus Corporation in the 1980s. In 1990, Dr. Gelfand received the US Distinguished Inventor Award for his groundbreaking work in isolating and purifying *Taq* DNA polymerase. He and his colleagues in the Program in Core Research at Roche Molecular Systems discovered and developed many additional designer DNA polymerases for high-temperature reverse transcription PCR, automated DNA sequencing, and novel genotyping applications.

REFERENCE

1. The Innocence Project is a litigation and public policy organization dedicated to exonerating wrongfully-convicted people through DNA testing. <<http://www.innocenceproject.org/Content/351.php>>.

SmartChip™ is a trademark of WaferGen Biosystems, Inc.

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