PCR: Thirty-five years and counting

In the 35 years since its invention, polymerase chain reaction (PCR) has become a standard technique in laboratory biology, but scientists continue to find groundbreaking—and even life-saving—applications for it. **By Alan Dove**

In May 1983, Kary Mullis, then a scientist at the Cetus Corporation in Emeryville, California, synthesized some oligonucleotides, mixed them with a small amount of template DNA, and added a polymerase enzyme and a few other reagents. After a series of incubations, the polymerase had copied the template many times over in a chain reaction, just as Mullis had hoped.

Mullis eventually moved away from the scientific mainstream, and Cetus was swallowed by a series of other companies, but PCR persisted. Like the DNA templates it targets, PCR has been replicated, amplified, and extended into laboratories worldwide. Dedicated thermal cyclers and related tools flood the market, vendors sell a wide selection of thermostable polymerases, and undergraduates worldwide learn the technique in introductory biology courses.

Now marking its 35th anniversary, PCR has become a ubiquitous laboratory tool. Nonetheless, researchers, engineers, and physicians are still finding ways to propel it into new territories. A sampling of a few of these efforts shows just how far PCR's reach has grown: from dairies to clinics, and from classrooms to outer space.

The launch sequence

The primary hardware component of PCR is the thermal cycler, a machine capable of heating and cooling sample tubes rapidly, and maintaining them at precise temperatures for specific periods of time. Though three decades of development and competition have improved these machines immensely, they are still relatively heavy, power-hungry, and expensive. As a result, one of the defining techniques of modern molecular biology has remained stubbornly inaccessible to educators and unusable in many remote locations.

"PCR is one of the most important [research] technologies, and yet it is one of the most limiting when you're outside of the lab because of the size and cost" of the equipment, says Zeke Alvarez-Saavedra, geneticist and cofounder of **MiniPCR** in Cambridge, Massachusetts. Frustrated by PCR's persistent immobility, Alvarez-Saavedra teamed with molecular neurobiologist Sebastian Kraves in 2013 to build a portable thermal cycler.

Most thermal cyclers control their temperatures using Peltier junctions, thermoelectric devices that can switch rapidly between heating and cooling. Unfortunately, Peltier junctions are inefficient, and the components required to operate them keep PCR machines heavy and greedy for electricity.

Alvarez-Saavedra and Kraves took a different approach, heating the samples with a thin-film resistive heater similar to the window defrosters found in cars. For cooling, the team uses a simple fan. A microcontroller drives the heating, cooling, and incubation cycles. The simpler design made the machine much smaller and lighter than ordinary thermal cyclers, and brought other benefits too. "When you make something smaller, it has less parts and the power supply is smaller ... so that helps reduce the cost," says Alvarez-Saavedra.

As they had hoped, the low-cost MiniPCR system immediately appealed to schools. "Going through high school without being able to get close to biotechnology was kind of a pain point for us [when we were] becoming scientists, so we wanted to do away with those barriers," says Kraves. The company has also developed a small, simple agarose gel electrophoresis system as a companion product. A complete kit with the MiniPCR thermal cycler, gel system, and accessories sells for less than USD 1,000, putting it well within the budgets of many school systems.

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Though schools have become a major market for MiniPCR, others have been quick to adopt the platform as well. Kraves says field scientists, animal breeders, and food companies have all put the system to use, often finding it cheaper and faster to do their own PCR-based assays on the spot rather than send samples to a remote lab.

The most surprising call, though, came from engineers working on the International Space Station. "We never really designed the technology to be space-friendly, so we were surprised when the space agencies ... told us it was very well designed for space flight," says Kraves. The MiniPCR system is now the core component of the ongoing Genes in Space competition, where secondary school students propose PCR-based experiments that are then carried out in microgravity by astronauts on the space station.

Smartphone PCR

MiniPCR was the first company to get PCR into space, but they are not the only one trying to make the technique cheaper and more portable. **Ahram Biosystems** in Seoul, South Korea, sells the Palm PCR miniaturized thermal cycler, which is similar to the MiniPCR device. Meanwhile, **Biomeme** in Philadelphia, Pennsylvania, is taking the portable PCR idea to a new level, extending it to include real-time PCR.

In real-time or quantitative PCR (qPCR), experimenters use fluorescent markers to monitor the progress of PCR amplification continuously. By measuring the rate at which new DNA copies appear, researchers can calculate how many template molecules must have been in the starting sample. They can also read results directly from the PCR reaction tubes without having to run agarose gels. All that monitoring and calculating, however, requires substantial computing power and additional equipment, making qPCR even more complicated and expensive than standard PCR.

At Biomeme, cofounders Marc DeJohn, Jesse van Westrienen, and Max Perelman thought that an unrelated trend might help bring qPCR out of the lab. "We thought, 'hey, everybody's got a smartphone in their pocket,' [and] we didn't really see anybody using them for molecular diagnostics," says van Westrienen. While addressing the same engineering problems MiniPCR faced with its thermal cycler, Biomeme also tackled the difficulties of adding optical sensors via a smartphone application that can both control and monitor qPCR reactions, and shelf-stable reagent kits for sample preparation.

For field use, "sample prep is arguably the most difficult thing ... and one of our focuses from day one has been to develop a very simple product that doesn't require any expertise or special lab equipment," says van Westrienen. The result is a catalog of kits designed for different applications, each with freeze-dried reagents and primers premeasured in sample tubes.

Perelman says that by using one of the company's kits, a minimally trained experimenter can turn a crude sample into a set of qPCR reactions ready for the thermal cycler in a few minutes. The machine itself has a dock compatible with standard smartphone connectors. Biomeme's web-based data portal can store the resulting raw data, which researchers can analyze online or download to their own computers.

At about USD 4,000, the current Biomeme device is considerably more expensive than MiniPCR's system, but Perelman argues that it offers additional utility: "You can complete everything in under an hour... just left swipe or right swipe in the app and [you're] able to see the amplification in real time." Perelman adds that the company is now working with defense and law enforcement users as well as field scientists and food companies.

The quick and the dead

While portable PCR is expanding the technique's reach dramatically, researchers worldwide continue to push its capabilities forward in countless incremental ways as well. The problem of distinguishing live from dead bacteria provides a good example.

PCR is exquisitely sensitive and specific for detecting nucleic acid sequences, but simply knowing that a particular DNA or RNA sequence is present doesn't prove it's associated with a live organism. That's a major problem in the food industry, where both pasteurized and unpasteurized foods will test positive for pathogenic bacteria by PCR, even though the bacteria are safely dead in the former and dangerously alive in the latter. As a result, food labs have long relied on relatively slow, cumbersome culture assays for definitive testing.

DNA crosslinking reagents that can only penetrate dead bacteria provided the first major advance in this field. Crosslinking prevents the dead bacteria's DNA from amplifying in the subsequent PCR reaction. The first generation of these reagents was hard to work with, requiring a darkroom and careful cold storage. More recently, Takashi Soejima and colleagues at **Morinaga Milk Industry** in Kanagawa, Japan, have developed stable, light-tolerant compounds that similarly target dead bacteria. The team's most recent work involved cont.>

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palladium-based reagents that selectively interfered with PCR amplification of DNA from dead, but not live bacteria. Combining the palladium reagents with qPCR yielded a test that could replace the older, more expensive techniques, streamlining production in dairies and other food facilities (*1*).

Getting the drop on cancer

Researchers and equipment makers have also made more radical modifications to PCR by borrowing technologies from other fields. One such effort yielded droplet digital PCR (ddPCR), which combines aspects of fluorescence-activated cell sorting with conventional PCR. Though the protocol for ddPCR is somewhat complicated, it's also become highly automated. Indeed, **Bio-Rad** in Hercules, California, now sells complete ddPCR machines that can run the entire procedure automatically. In these systems, a sprayer separates a prepared PCR reaction into thousands of nanoliter-size droplets, then keeps the droplets separate during the thermal cycling steps. Each droplet hosts its own series of amplifications, which the machine then sorts with fluorescent markers to detect target sequences.

ddPCR has proven particularly useful for detecting scarce targets in samples with very high background levels of nontarget DNA, such as DNA tumor markers circulating in patient blood samples. The technique has become robust enough that at least one company, **Biodesix** in Boulder, Colorado, now offers clinical tests called "liquid biopsies," based on ddPCR. Using a small blood sample, the Biodesix test can quickly identify the precise mutations in a lung cancer patient's tumor, allowing doctors to pick the most effective drugs for that tumor type.

"Once we know the exact mutations, there are targeted therapies that can make a difference in their outcome, and that's ... shifting the entire paradigm of how we treat patients," says Mark Bowling, Paul R. and Kathryn M. Hettinger Walker Distinguished Professor of Clinical Oncology at **East Carolina University** in Greenville, North Carolina, and an early adopter of the new test. While conventional biopsy analysis and sequencing can yield the same information, the Biodesix test is much faster. "We can get test results back within three days," says Bowling. Standard biopsy testing can take over two weeks. Bowling says the difference can literally be life or death for patients whose tumors will respond to targeted therapies.

Because it's based on PCR, the Biodesix test has room to expand, as drug makers release more genetically targeted treatments. However, Bowling says that even with the limited selection of current targeted chemotherapy drugs, knowing a patient's exact tumor type already changes his team's treatment strategy about one third of the time.

Building a better cycle

Clinical tests have always been a major focus for PCR developers, but sometimes the technique's requirements have held it back. The first proof-of-principle publication describing Mullis' PCR technique demonstrated how it could be used to identify the hemoglobin mutation responsible for the sickle-cell trait (*2*). Unfortunately, PCR remains too expensive for many clinical labs in sub-Saharan Africa, where sickle-cell anemia is endemic.

Even as PCR continues to expand into new areas, these and other limitations have inspired numerous researchers to develop alternative nucleic acid amplification techniques. Many of these methods operate with similar principles but offer different sets of benefits. Multiple displacement amplification, which uses a highly processive polymerase, has become a standard technique in genomic sequencing, while rolling-circle amplification, which can copy a target sequence repeatedly onto a single strand of DNA, has found wide use in biochemistry.

Meanwhile, loop-mediated isothermal amplification (LAMP) has become a popular choice for researchers who want to get DNA-based tests into rural settings. As its name implies, LAMP uses an enzyme that can open and repeatedly copy a DNA molecule at a constant temperature, eliminating the need for expensive thermal cyclers.

That's appealing for companies like **LaCAR MDx Technologies** in Liège, Belgium, which is trying to develop clinical tests for use in less-developed countries. "We were just looking for a way to make molecular genetic testing easier. You could use some cartridge or [PCR device], but then you need to have expensive materials and complicated technology," says Arnaud Allaer, LaCAR's CEO.

Instead, the company began working with LAMP, and quickly discovered that it was extremely robust. Besides operating without a thermal cycler, the company's LAMP-based assays can identify point mutations in a gene from fresh or frozen blood samples, or even dried blood spots on blotter paper, without requiring a DNA extraction step. Finally fulfilling the promise of the original PCR paper, LaCAR has now developed an inexpensive, robust test for the sickle-cell trait based on their findings. "We have done one clinical trial at the University of Liège, [and] we'll do one also in the Congo," says Allaer (*3*).

Whether they're making portable thermal cyclers, using cutting-edge PCR tests for rapid cancer diagnosis, or exploring other techniques to break free of PCR's limitations, experts in the field are optimistic about the next 35 years of DNA amplification. "It's an exciting time," says Bowling.

References

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