A look back: long range planning

A late-night drive to Mendocino, California, USA, some 20 years ago gave a young researcher at Cetus the opportunity to dream up a solution to a problem that had been much on his mind lately—a process for generating large quantities of scarce DNA sequences. Kary Mullis had conceived the polymerase chain reaction (PCR)—that now-ubiquitous process in which a mixture of DNA, nucleotides, primers and polymerase is cycled repeatedly through temperatures appropriate for strand denaturation, primer annealing and polymerase-driven extension, leading to exponential amplification of the target sequence. "I had solved the most annoying problems in DNA chemistry in a single lightning bolt," he would later write of his revelation, with typical modesty. "I could make as much of a DNA sequence as I wanted and I could make it on a fragment of a specific size that I could distinguish easily."[1]

Of course, in those heady early days before PCR achieved full commercial exploitation, many elements of this useful technique presented quite a chore. In its earliest incarnation[2], PCR was performed by transferring samples to different heating blocks, and was driven by DNA polymerase I from *Escherichia coli*—necessitating the addition of fresh enzyme after each denaturation step, owing to the enzyme's temperature sensitivity. Within a few years, however, both of these obstacles would be overcome; programmable thermocyclers saved scientists the trouble of continuous tube trafficking and, more importantly, *Taq* polymerase—derived from the thermophilic bacterium *Thermus aquaticus*—had appeared on the scene. This polymerase had been isolated years before by researchers who had then contemplated its potential as an engine for reverse transcription, where high-temperature synthesis could eliminate the obstacles posed by RNA secondary structure[3]. But *Taq*'s ability to thrive at temperatures well over 90 °C made it a natural choice for the rigors of PCR cycling, and it performed admirably[4].

PCR promptly exploded in popularity, thanks in large part to *Taq*, which remains widely used even now. PCR, however, still had not achieved its full potential. Among other limitations, there was a definite ceiling for the size of fragments that could be readily amplified, and with the exception of a handful of reports, most scientists found it exceptionally difficult to efficiently and accurately amplify targets greater than five or six kilobases in length. One scientist, Wayne Barnes of the University of Washington, hypothesized that this might be due to errors in the extension process—the *Taq* polymerase lacks error-correcting exonuclease activity, leading to reduced accuracy over long stretches of sequence, and previous research had shown that sequence mismatches can pose an impediment to efficient *Taq* activity[5].

Fortunately, by this time *Taq* was no longer the only game in town. Researchers at Stratagene had recently purified a DNA polymerase from a different thermophile, *Pyrococcus furiosus*, and this *Pfu* polymerase possessed a 3′-to-5′ exonuclease activity that considerably improved the accuracy of PCR amplification[6]. Barnes speculated that by combining the efficient but error-prone *Taq* polymerase with the much slower but proofreading-capable *Pfu* polymerase, it might be possible to amplify far longer targets. Indeed, he found that by pooling small amounts of *Pfu* with larger quantities of *Taq* and tweaking the conditions of the denaturation step, it was possible to PCR-amplify sequences as large as 35 kilobases, confirming that even low levels of exonuclease activity from *Pfu* or other error-checking thermophilic polymerases allow primer extension to proceed beyond the mismatches that can stall *Taq*[7]. Shortly thereafter, Barnes would coauthor a follow-up paper with colleagues at Roche Molecular Systems—which now held the patent to the PCR process—in which the conditions for this dual-polymerase, 'long PCR' technique were further refined, demonstrating the potential to reliably and accurately amplify sequences tens of kilobases long from genomic templates[8].

A variety of alternative reagents and strategies for extending the effective range of PCR reactions have emerged in the meantime, but the polymerase blend still remains a popular option. Today, several different companies offer proprietary polymerase mixtures tailored for specific kinds of long-range amplification protocols, sparing modern researchers from yet one more of the hassles that has troubled their predecessors, and freeing them to preoccupy themselves with other timeless worries such as sample preparation, primer design and planning the perfect thermocycler program.

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