MORE EFFICIENT PCR? CHALLENGE ACCEPTED

Thermal cyclers are the workhorses of modern PCR, YET MANY SUFFER FROM IMPRECISE TEMPERATURE CONTROL. More reliable devices will improve experimental outcomes and reproducibility.

In May 1983, Kary Mullis, a young technician working for the Cetus Corporation, sat with idle hands and a busy mind. The laboratory he worked in was attempting to study DNA mutations linked to sickle cell anaemia, but the available techniques were both technically challenging and laborious. Mullis wanted to speed things up, and so he developed a technique that would later win him a Nobel prize.

His solution was polymerase chain reaction (PCR), a molecular technique that rapidly duplicates short, specific segments of DNA, transforming one fragment into millions of identical copies. For Mullis, PCR allowed for quick identification of DNA segments based on whether they had been amplified. However, such duplicative power has value well beyond fragment identification.

"PCR has a wide range of applications, not only in basic research, but in medical diagnostics, biopharma, forensics, environmental and water testing, and agriculture," explains Thermo Fisher's Singapore-based R&D director, Si Kee Tan.

For 40 years, this technique has helped researchers explore the world of DNA. For many outside the scientific community, PCR became a household name during the COVID-19 pandemic as it enabled fast and reliable identification of SARS-CoV-2 infections. But it's no exaggeration to say that PCR is foundational to modern molecular biology.

As such, it can be taken for granted. "It's similar to baking," says Tan, "you need to mix your ingredients in just the right proportions, select the right baking dish, and then bake it at the right temperature." However, unlike baking, the consequences of mistakes in PCR can be significant. Failure to amplify SARS-CoV-2 target genes might lead to the false conclusion that a person is COVID-free, for instance.

At the heart of PCR is the thermal cycler – its metaphorical oven. And this often-overlooked tool has had an upgrade, thanks to Tan and her colleagues, to make PCR more accessible and more reliable.

THE IMPORTANCE OF HEAT

PCR reactions are often carried out in plastic tubes or on a plate. In Mullis's early days, he would heat and cool samples by transferring tubes containing sample DNA among a series of water baths in a process that was both wet and imprecise. By contrast, thermal cyclers use electric heating and cooling components to drive the PCR reaction through each of its three phases: denaturation, annealing and extension.

During denaturation, fragments of double stranded DNA are heated until they begin to separate, creating two single strands of open DNA. Cooling initiates the annealing phase, in which synthetic DNA oligonucleotides, called primers, bind to open, complementary segments of DNA that flank the target sequence. In the extension phase, DNA polymerase binds to primer-laden DNA and use the target sequences as templates to construct complementary strands of DNA. Successive rounds of these three phases create exponential amplification of the original sequence.

OPTIMAL TEMPERATURES VARY BETWEEN PCR EXPERIMENTS, SO RESEARCHERS MUST DETERMINE THE BEST TEMPERATURE AND DURATION.

As in baking, temperature is key to PCR: high temperatures cause denaturing; cooler temperatures favour annealing; and most polymerases will only operate within a narrow temperature window somewhere between the two.

"In theory, every cycle in PCR represents a doubling of the DNA templates," Tan explains, "but if temperature is not perfectly controlled, then doubling efficiency is compromised."

Thermal cyclers thus play a pivotal role by precisely setting, holding and switching between optimal temperatures. But what are the optimal temperatures?

GUESSWORK IN OPTIMIZATION

The aim of thermal cyclers is to remove the variability in temperature control. "Thermal cycling should be the last thing you need to worry about," says Kok Shyong Chong, a product manager at Thermo Fisher. However, due to the technical challenges of rapid heating and cooling, many researchers have to spend considerable time worrying about PCR optimization.

Optimal temperatures vary between PCR experiments, so researchers must determine the best temperature and duration for each phase, and for each primer pair. Optimization involves instructing the thermal cycler to generate a heat gradient across the plate of samples, such that each row of samples experiences an incrementally warmer temperature. Based on where a sample is located on this gradient, researchers can determine the temperature it was exposed to and how that influenced the reaction's efficiency.

To establish this gradient, a typical thermocycler will use two Peltier blocks, enabling researchers to set the high temperature at one end, and the low temperature at the other.

"When you have only two Peltier blocks, you can only control two sides of the plate," explains Melike Ozturk, thermal cycler portfolio lead at Thermo Fisher. "Usually it's an 8 by 12 plate, and you can only truly control the temperatures for the first and the last columns."

What's more, although the two blocks should create a linear temperature gradient, in reality many thermal cyclers have a curved, or sigmoidal, gradient. This difference risks users miscalculating the temperatures that samples experienced and thus picking suboptimal temperatures.

"A non-linear temperature gradient is not surprising," says Chong. He likens the transfer of heat to a drop of coloured ink diffusing through a tank of water. "The ink just diffuses naturally, and you don't have much control over the layout of the colour distribution."

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▲ Temperature control is absolutely essential to efficient DNA replication in PCR.

EVOLVING TOWARDS BETTER CONTROL

To help researchers avoid these headaches, Thermo Fisher has developed VeriFlex technology — a new approach to thermal cycling that uses 3 to 6 Peltier blocks. By increasing the number of discrete heating and cooling units, this design provides researchers with more control over reaction temperatures and a better ability to achieve a true linear heat gradient during optimization.

"Each zone of the VeriFlex block is segregated and has its own microcontroller," says Chong, "meaning that each zone is like a mini thermal cycler." This design is better, says Ozturk, "because it gives you a smaller chunk of space to control, it lets you be more precise."

Furthermore, Thermo Fisher has developed an algorithm for VeriFlex that precisely predicts sample temperatures, as opposed to heating block temperatures.

"VeriFlex provides precise control over sample temperatures thanks to the data we've collected on heat transfer under different circumstances," adds Chong. "This allows us to fine-tune the algorithm according to the type of plastics and reaction volumes used." While the technology is broadly compatible, Chong notes that this optimization used plastics from Applied Biosystems, a subsidiary of Thermo Fisher, and so he "highly recommends using these plastics for consistency".

And consistency is, ultimately, the goal. "This technology gives us peace of mind, knowing that the temperature control is precise," says Tan. "It minimizes the guesswork that researchers have to do, allowing them to focus on other factors." ■

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