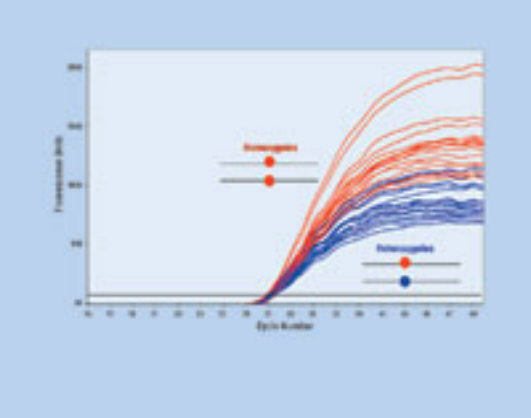
Genomics & Proteomics

PCR Leaves its Teen Years, and Lingering Questions, Behind

Discovered almost two decades ago, PCR has retained its place as the top technique for cyclic amplification of DNA segments. Despite improved methodologies, it remains a confounding process for many researchers. Here are some key questions still surrounding PCR.

By Bill Schu, Senior Editor

Polymerase chain reaction (PCR) is like the duct tape of genomics, an all-purpose tool with surprising flexibility and longevity. For the uninitiated, PCR multiplies a single, microscopic strand of DNA billions of times within a few hours, a process that has applications for gene amplification, copy-number analysis, gene expression analysis, genotyping, forensic investigation, and microbiological diagnosis.



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Symmetric PCR applied to single cell analysis results in too much scatter to distinguish alleles using a single probe. (Source: Lawrence Wangh, MD, Brandeis University)

Although improved machines and techniques have shaved time and complexity off the process, PCR still has a seemingly endless ability to confound. And of course, the process for amplifying RNA—reverse-transcription PCR—is a completely different process. (See "Tackling High Variability in Gene Expression Studies," p. 30.)

Here, we present some lingering questions about PCR, and provide an inside look at what researchers are doing to answer them.

1. Why is a 20-year-old technology still so difficult to figure out?

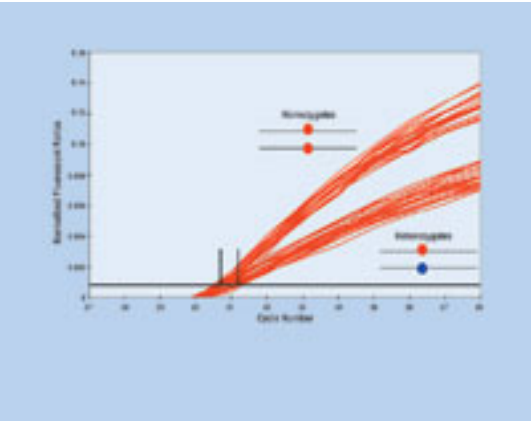
"For many people, PCR is still a really daunting process," says Gregory Shipley, PhD, director of quantitative genomics at the University of Texas Health Sciences Center, Houston. Shipley is a member of the Association for Biomolecular Resource Facilities (ABRF), which facilitates the use of nucleic acid-based technologies, and Texas is one of a handful of PCR core facilities around the country. "We've spent a lot of time reverse engineering how PCR machines really work and what the accompanying software was telling us. But most people haven't done that. People come up to me and say, ‘We're going to start a PCR core. We know how to sequence, so we're good!' And

I'll say, ‘Good luck with that!' Starting from scratch is tough."

According to Shipley, part of the difficulty stems from the number of variables involved. In quantitative PCR (qPCR), the conversion of a fluorescent signal from reactions into a usable value by calculation from a standard curve, for example, researchers must know which primers to use, which probes to use, how to optimize reagent concentrations and determine cycling conditions, and how to select a good amplicon site. That's a lot to wade through, even for researchers who are familiar with the process. "There's a lot of work that goes into setting up an assay before you even turn on primer express," Shipley says.

"Nonspecific amplification due to mis-priming is a problem that plagues symmetric PCR and has manifestations like primer-dimer [nontarget amplification products caused by homologies within primers]," says Lawrence Wangh, PhD, associate professor of biology, Brandeis University, Waltham, Mass. "It is the reason why you have to take great care in primer design."

"The biggest bottleneck in PCR," says Shipley, "is that people don't know what they're doing. They get data and they think it means something, and they throw it out there in the literature. In the old days, when journals were going to publish qPCR data, they'd say, ‘Where's the Northern that proves this is right?' That was stupid, but they didn't know better then. But now, it's gone the other way. ‘Oh, it's qPCR data! It must be grand!' And they accept it carte-blanche. That's crap, too."



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LATE-PCR genotyping using a single-probe based on kinetics or quantitative end-point analysis results in much less scatter. (Source: Lawrence Wangh)

Marcia Holden, PhD, biologist at the National Institute for Standards and Technology (NIST), Gaithersburg, Md., points to another bottleneck: accuracy. "For a long time, PCR was a qualitative measurement. Of course the quality of even those qualitative measurements, whether or not a peak shows up, can be dependent on how well the instrument is performing," says Holden. "Yet quality becomes even more critical for quantitative PCR. There are just endless places for things to go wrong."

Which begs the question…

2. Is there any such thing as absolute quantitative PCR?

Most PCR experts agree that PCR reactions cannot be precisely quantitated. According to Shipley, "The word ‘absolute' got stuck on this method a long time ago, and it is the biggest misnomer. Nobody in the universe can do absolutely quantitative PCR. We cannot put a real honest-to-God number on anything. There is no absolute, and every time someone says in the literature that they're doing absolute quantification, I shudder."

As an example, Shipley describes a recent article in the journal Biotechniques. "There's some really nice qPCR data back there, it shows some nice data [from the ABI Prism] 7900, and it's analyzed totally wrong." This occurs all the time, says Shipley. "It just shows you that there's no quality control."

When things go wrong during PCR amplification, the quality of DNA cloning and protein functional analysis suffer, and blips in the polymerase can easily be confused with genuine mutations. "We sent out an assay that I have for human beta-actin to several labs around the world," says Shipley. "We told the researchers to just run it the way they normally do on whatever machine they normally use, with whatever chemistry they use. It was amazing the disparity in the quality of data that came back. Very few people were able to actually get the 'right' answer, if you will."

NIST's Holden has similar stories, including an instance in which two laboratories collaborated on a PCR project, with different instrumentation and different people running the assays. "To save money, they purchased a batch of fluorescent probe and the primers. One lab reconstituted them and then shipped half of it to the other lab," she says. "They both ran these assays more or less the same way, but each with their own batch of test materials, and they got very different answers. Their precision within each lab was pretty good, but the answers were quite different."

One of Holden's recent studies reached the conclusion that, although there are many different real-time amplification platforms, the results of DNA measurements from experiments performed on different platforms are not necessarily comparable, and results may not be repeatable within instruments.
Findings such as these have researchers asking…

3. Why aren't there established standards for PCR?

The inaccuracies illustrated above have spurred NIST to try to establish some standards for qPCR. In a recent study, Holden and colleagues found that "the results of DNA measurements from experiments performed on different platforms are not necessarily comparable, and results may not be repeatable within instruments." The authors concluded that "in order to deliver useful, informative genetic tests, standardization of real-time PCR detection platforms to provide repeatable, reliable results is warranted alongside a better understanding of inter-assay and intra-assay repeatability."

"The problem with the available assays is not that they don't work," says Shipley. "The problem is that they don't have any standards. And a lot of them are really long and don't lend themselves to standards being made. That's good for the assay but bad for academics who like to put real statistics on things."

NIST has now begun a host of activities around developing standards for forensic analysis—work that Holden thinks could also be important for qPCR. "We're hoping that we can identify perhaps some factors that are more important than others that contribute to the accuracy of qPCR measurements," she says. "We've asked people to document the piece of equipment they're using.

When was the last time it was maintained, how they handled the material, the amount of time after they prepared their material before they ran their assays."

NIST is currently in the second phase of a pilot study designed to determine the factors that impact the quality and accuracy of qPCR measurements. According to Holden, "We got back the results of the first pilot study about a year ago, we analyzed it, made some modifications in the study, and sent out the second batch of material, which we hope to have analyzed by April. They can use any platform they want, they can use any type of probe that they want for detection. The idea is to see if we can determine where there are pitfalls and where the biggest inaccuracies are."

Holden says that she and colleagues at LGC, London, hope to have standard reference materials in publishable form this year. Researchers could use the material to monitor the performance of their equipment or the accuracy of the technician who's making the measurements, or the number of other things that impact on these measurements, like calibration of their pipetters.

"By doing this on a regular basis, one could establish a bar for performance of one's equipment," says Holden. "If you run the assay on one piece of equipment, does that make a difference? If you use one probe type over another, does that make a difference? I'm not sure if we're going to have enough data to really come up with a definitive answer to that, but hopefully we'll see some tendencies."

Beyond that, NIST is looking at a host of other things related to PCR. "We're working on issues of fluorophores, the different styles of fluorescent probes that are used in qPCR," says Holden. "We're looking at things like that with respect to things like TaqMan, molecular beacons and hybrid probes that are in between."

As work on qPCR standards progresses, innovators are looking to the next generation of PCR and asking themselves…

4. Which recent PCR innovation is poised to make the biggest difference in genomics research?

There are many promising advances, most of which are tweaks of existing machinery, techniques, or priming strategies. "Hairpin" PCR, which completely separates genuine mutations from polymerase misincorporations in a sample and produces a double record of the sequence by copying both strands in one pass, is

PCR SNAGS

Faint or no signal from sample DNA

- Sample contains PCR inhibitor (i.e., heme compounds, EDTA, or certain dyes)
- Sample DNA is degraded
- Insufficient sample DNA added because of inaccurate quantitation
- Incorrect pH
- Primer choice not optimal (i.e.: primers may be annealing to sites of template secondary structure or may have internal secondary structure)
- Tm of primers is lower than expected

Poor yield for multiplex PCR

- Non-optimal thermal cycling parameters
- Competition from mispriming and other competing side reactions

Yield gets progressively poorer for successive PCR amplifications performed over time

- Expired or mishandled reagents

Inconsistent yields with control DNA

- Combined reagents not spun to bottom of PCR sample tube
- Combined reagents left at room temperature or on ice for extended periods of time

one. Another is PCR-on-a-chip, a microfluidic technology in which the heating and cooling of samples takes place in micro-etched channels on a plate. (See our April 2004 article, "PCR-on-a-Chip Downsizes Even as It Grows Up.")

(encouraging mispriming and other primer artifacts)
• Combined reagents not thoroughly mixed Primers not uniformly suspended before adding to reaction mixture. (Primers can aggregate and settle to the bottom of the tube.)

(Source: Greenwood Molecular Biology Facility, Honolulu, Hawaii)

"One of the future directions is multiplexing," says the University of Texas' Shipley, wherein multiple PCR reactions are performed in a single microfluidic channel. "The machines we have now are getting better and better at multiplexing up to five colors for most of the newer ones. I think nucleic acids are going to come in, and that will allow for much more multiplexing. I don't know how it will work, there will have to be some sort of hybridization thing, and hybridization is always fraught with problems."

Stratagene, La Jolla, Calif., for example, recently launched a new real-time PCR system called the Mx3005P. The design of the Mx3005P allows researchers the ability to conduct five-way multiplexing and simultaneously test five different targets in a single test tube.

Most researchers agree that new machines from Agilent Technologies, Applied Biosystems, BioTrove, and Stratagene, among others, are very effective, but costs can still be prohibitive. "Let's say you buy the best least-expensive machine out there, which would probably be the [MX30005P] from Stratagene, which has really good software," says Shipley. "Up front, you've got to come up with $25,000, and [additional funds] for a service contract. And then you have to pay the reagent costs, and so forth. Do you know how many assays I can run at $200 for 20 samples? We would never get to $25,000."

Linear-after-the-exponential PCR (LATE-PCR), a new offshoot technique co-invented by Brandeis' Wangh, is an advanced form of asymmetric PCR that achieves reliable asymmetric amplification by using several innovations in the design of primers and using an improved thermal cycle. "If you generate single-stranded product, it accumulates throughout the reaction, and you don't necessarily have to probe it during the annealing step," says Wangh. "With conventional molecular beacons [from Public Health Research Institute] and even with TaqMan probes [from Applied Biosystems, Foster City, Calif.] probe/target annealing takes place during the annealing step, because the probe has to bind at a temperature above the primers. We realized that we could introduce a lower temperature-detection step, one that either is at the same point in the thermal cycle as the annealing step but is introduced after the limiting primer runs out, or is after the extension step. In either case, you have separated annealing temperature from detection temperature."

LATE-PCR creates a temperature space that is there purely for detection, allowing probes to bind to a single-stranded target and melt off without interfering or being degraded during extension. "In the case of low-temperature molecular beacons, the loop is shorter and the stem is shorter, which

makes them more allele-discriminating," says Wangh. "That's a major breakthrough. In LATE-PCR, the Ct value of the reaction depends on the initial number of target genomes, just like in symmetric PCR. But, regardless of how much you start with, all LATE-PCR reactions eventually generate a lot of single-stranded product, and you can take that product use it for cycle sequencing directly, without any cleanup. We call this 'Dilute-'N-Go' sequencing."

Wangh has also developed a class of reagents called "Elixirs," which interact with the components of a reaction in a unique way that prevents mis-priming. "They are a substitute for Hot Start," Wangh says. "But unlike Hot Start, which is present at time zero and then gone after you heat the reaction the first time, Elixirs persist throughout the reaction. They eliminate mis-priming when you mix components together, but they also eliminate mis-priming once the genomic strands are separated. They suppress primer-dimer, and they improve the construction of multiplex reactions, in which you have many components.

"In general, the whole field of cell biology, and PCR in particular, is moving toward smaller and smaller amounts of starting material," Wangh continues. "There is great utility and advantage to that, but you face the challenges that are inherent to symmetric PCR. In particular, if you have to take more cycles to reach Ct, the chances of mis-priming and amplifying junk increase, and these unwanted products compete with your amplicon. The only way to solve that problem in symmetric PCR is to spend a great deal of time optimizing reactions. LATE-PCR makes designing highly specific primers logical, and Elixirs make optimizing reactions faster and easier. We routinely carryout reactions started with only a few DNA or RNA molecules recovered from single cells using our PurAmp method."

"LATE-PCR is quite an interesting concept," says Shipley. "You're talking about disproportionate primer concentrations giving supposedly more accurate results. That's intriguing. But among the members of the nucleic acid research group within the ABRF, we're all just doing TaqMan chemistry or using SYBR. I find LATE-PCR very intriguing, and it's something that should be put out there for people interested in qPCR. But it isn't something any of us are doing yet, quite frankly."

Shipley says that time constraints are one thing that has kept innovation in PCR on the back burner. "I'm skeptical until I see it work. I'm running a core facility, and I don't have time to mess around with this stuff. It's one of those things where you can get yourself in kind of a rut, where you're into a production line mode, and to change something as drastically as that . . . how do I sit down and [quality-check] 600 assays for LATE-PCR? I don't know."

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