

qPCR—MAKING OLDER TECHNOLOGY NEW AGAIN

Scientists want more from quantitative polymerase chain reactions, including more sensitivity and specificity. In addition, researchers seek new applications of this technology, such as simplifying it for point-of-care uses. As described here, a range of new products make all of these wishes come true. **by Mike May**

In Belfast, David Coulson, a postdoctoral research fellow at **Queen's University**, takes postmortem brain samples. The samples come from people who suffered from neurodegenerative diseases. Then, Coulson uses qPCR (quantitative PCR, also known as real-time PCR) to study the expression levels of various genes from the samples. “The biggest challenge in qPCR,” says Coulson, “is the analysis of the data, rather than the technique itself.”

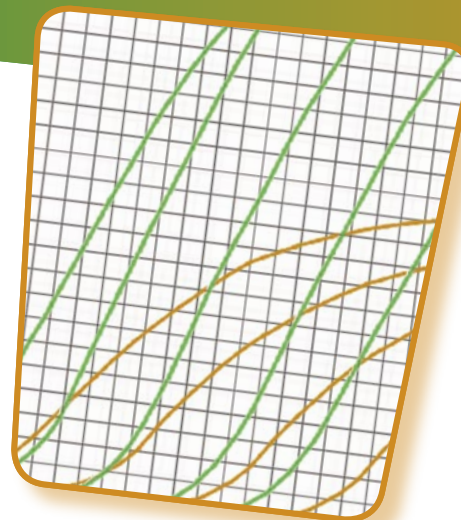
In particular, Coulson points out that normalizing qPCR data typically relies on reference genes. Ideally, these genes get expressed at constant levels, which makes them a scale of sorts for comparing the expression levels of other genes. However, says Coulson, “Certain genes that have been called reference genes are not necessarily good references.” For example, a reference gene that varies in expression introduces errors in later analysis. So before applying qPCR to comparative expression levels, researchers must validate their reference genes.

In the May 6, 2008, issue of *BMC Molecular Biology*, Coulson and his colleagues reported on applying qPCR to postmortem brain samples from people with Alzheimer's disease, Parkinson's disease, or dementia with Lewy bodies. From this work, Coulson points out that genes validated as reference genes for some disease might not work in others. “You can't predict how a disease process could affect the reference genes,” Coulson says. To keep track of reference genes, Coulson and his colleagues now use qBasePlus from **Biogazelle** in Belgium. Coulson says that this software “actually checks the stability of the reference genes as part of the downstream analysis of qPCR data.”

Like Biogazelle, many other companies hope to make qPCR more robust and easier to use. Doing that will demand improvements in reference genes and other characteristics of this research tool. As Sandrine Miller, product manager for qPCR at **Invitrogen** in Carlsbad, California, says, “This is a nice technology and one that has been in the market for some time now.” She adds, “Today, the main challenges for qPCR are sensitivity and specificity. For example, researchers might want to detect as little of a pathogen as possible or discover rare genes that other techniques can't find.” Miller also sees scientists looking for higher throughput from qPCR. In fact, most qPCR users want the same three things: sensitivity, specificity, and higher throughput.

Old and New Techniques

Although the TaqMan approach to qPCR emerged in the early 1990s, companies keep putting this relatively old technique to work on new problems. Earlier this year, for instance, **Applied Biosystems** in Foster City, California—a company that was recently acquired by Invitrogen—introduced its TaqMan MicroRNA arrays. These provide array-based qPCR. “Lots of researchers want to know which miRNAs are involved in a disease or a biological process of interest,” says Iain Russell,



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product manager of consumables at Applied Biosystems. “So we are working to put together workflows and products that provide data for many targets in parallel.” The TaqMan Human MicroRNA Array v1.0, for instance, includes 365 different miRNA assays.

Applied Biosystems also aims qPCR at mRNA. “We’ve used our algorithms to make about 800,000 mRNA assays,” says Jon Sherlock, the company’s TaqMan array product manager. That large number of assays helps researchers study a range of genes in parallel. To make the process even easier and more efficient, Applied Biosystems developed its TaqMan Express Plates. “Users can go online and select the genes of interest,” says Sherlock. “Then, we put them on a 96-well reaction plate that users just put in their own instrument.” He says that this product will make large experiments more accessible to a wider number of researchers. “Our Express Plates can also be used to validate microarray results,” Sherlock says.

It’s not just about looking at more genes or more miRNAs, though, because tools from Applied Biosystems also focus on using smaller samples. “Using large amounts of tissue tends to mask local effects,” explains Sherlock. “Looking at smaller samples provides more accurate results, especially for more localized effects.”

As scientific questions move toward smaller samples, researchers want to examine differences between smaller pieces, down to cells or even organelles. This leads to the study of single-cell heterogeneity. “The simplest example of this is genetic differences from one cell to another,” says Frank Feist, executive director for the Munich-based **Advalytix** business at **Olympus**. Such variations could be used in many ways, including telling scientists more about disease. For example, Advalytix’s customers focus on immunology, stem cells, and oncology. “They’re trying to see if the degree of heterogeneity has clinical relevance,” says Feist, “and there is a strong hypothesis that it is relevant.”

Single-cell heterogeneity could be unveiled with qPCR. In the past, however, qPCR lacked the sensitivity to work with single cells, which are also difficult to handle. Advalytix’s AmpliGrid slide improves qPCR sensitivity and makes it easy to handle single cells, according to Feist. “The AmpliGrid’s flat glass surface contains 48 lithographically defined reaction sites that each hold a single 1-microliter drop within a hydrophobic ring,” explains Feist. “Researchers deposit single cells into these reaction sites using flow cytometry, laser capture microscopy, or micromanipulation with up to 100 percent accuracy.” Then, researchers perform the reverse transcription step of the qPCR reaction on the AmpliGrid, which Feist says “improves sensitivity of the overall qPCR reaction because the inert glass surface does not absorb any template and the 1-microliter volume makes it stochastically more likely for template and polymerase to react.” After reverse transcription, the product is diluted and further analyzed with standard qPCR.

Cross-Platform Products

For any scientific technology, it can be difficult to combine the right parts from various companies. In some cases, scientists simply purchase both the platform and reagents from one vendor. On



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the other hand, mixing vendors might create better performance in some situations. For instance, Invitrogen developed its new EXPRESS qPCR Supermix reagents for high performance on virtually all qPCR instruments. In addition, Invitrogen’s SuperScript VILO cDNA synthesis kit—included in the EXPRESS qRT-PCR reagents—was developed to provide greater sensitivity and linearity in qRT-PCR. “VILO uses SuperScript III in a novel formulation to extend the linearity of synthesis and generate four-fold greater yields of cDNA—enabling the end-users to use more cDNA in their downstream qPCR reaction without inhibition and giving much earlier detection in qRT-PCR, typically at least two cycles earlier,” says Miller.

Agilent Technologies’ Stratagene Products Division located in La Jolla, California, also makes reagents for qPCR, such as its new Brilliant II Fast reagents. “It runs much faster,” says David Kerry, Stratagene’s product manager for genomics. “Standard qPCR runs take 65 to 90 minutes, depending on the gene, the size, et cetera. Using Brilliant II Fast, a standard 40 cycles can be performed in as little as 48 minutes.” Such an increase in speed might not matter much with small experiments. “However, if you’re looking to perform hundreds if not thousands of reactions, the extra time can add up,” says Kerry. “Therefore, the faster you perform the reactions, the faster you can get to the results that you want to investigate.”

Faster reactions also mean better productivity. Rachel Formosa, Stratagene’s product line manager for genomics, says, “In surveys we’ve done of customers, we’ve found that about 70 percent of qPCR instruments have four or more users, so they want others getting done quickly.” The Brilliant II Fast reagents also help researchers follow the results of a run more quickly. “With Brilliant II,” says Formosa, “you start seeing the gene of interest in qPCR three to four cycles earlier than with other reagents. That’s a 10-fold difference in detection.”

Reagents for qPCR come in various forms. **Integrated DNA Technologies (IDT)** in Coralville, Iowa, for example, recently released its Ultramer long oligonucleotides, which can be used with qPCR. John Havens, IDT’s business development manager, says, “These DNA oligomers can be made with 60–200 bases, and they are fully quality-control tested with electrospray mass spectroscopy. This makes sure that the product has the right identity, as measured by mass with an accuracy of plus or minus 0.02 percent.”

Havens points out that such oligomers make useful positive controls for qPCR. “You could synthetically make one [continued >](#)

Genomics

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of these to represent the target of interest in a genomics sample to verify that your reactions are working properly," he says. Moreover, a researcher could make Ultramers with slight variations at specific points to see if that impacts the results from qPCR. "This would make sure that allele-specific qPCR is designed correctly," explains Havens. If researchers want to test a string of variations, IDT will provide Ultramers in 96-well plates. Getting such long oligos, though, does increase the price per base when compared to the price of shorter oligos, since the technology for making the Ultramers is more costly.

When needed, Ultramers can also be made as dual-labeled probes. IDT will make these with a fluorophore on each end or with a fluorophore on one end and a quencher on the other.

Increasing the Take

To increase the amount and quality of information gathered with qPCR, researchers also want multiplexing. "When you look at the expression of single genes in individual tubes," says Ilgar Abbaszade, strategic marketing manager for gene and protein sciences at **Promega** in Madison, Wisconsin, "you can get errors in comparing them because of differences in the reagent concentrations in the different tubes due to pipetting mistakes, for example. You can even get differences based on the location of the well, because of slight differences in heating and cooling conditions in the different wells." If the expression levels of different genes can be measured in the same tube—multiplexed—then some of those errors can be minimized. "More than 35 percent of the people I ask say that they want to multiplex, but only a few of them do it," says Abbaszade.

To help more scientists multiplex qPCR, Promega commercialized its Plexor qPCR System. "It is as simple as the dye-based systems,"

says Abbaszade, "but at the same time it is as specific as probe-based assays." Promega also provides software that helps customers design multiplexed qPCR assays. "This software is free on our website," says Abbaszade, "and it's free even for people who don't use Plexor. The software helps scientists design primers that work in the same tube with no interference." Then, customers can order the primers that they designed. Abbaszade adds that this system should work with any genes.

Multiplexing qPCR could also be used to make more-powerful diagnostic tools. "You could combine the expression data from several genes, and use that to develop tests to diagnose certain diseases," Abbaszade says. "Multiplexed qPCR assays could also, for example, help to evaluate the potency of stem-cell lines, and that usually depends on the expression level of more than one gene. Researchers could use Plexor to combine that all in one test."

Sometimes, applications of qPCR depend more on simplicity than anything else. For instance, if a company wants to use qPCR to develop a product that could be used by a wide range of people—including ones with no training in molecular biology—the qPCR process must be as easy and foolproof as possible. In Cardiff, Wales, scientists at **Q Chip** make that kind of qPCR tool. This company developed a proprietary system that "lets us make microspheres that are all the same size and have the same content," says Jo Daniels, Q Chip's chief scientific officer. "It's a reliable way to make very uniform spheres from any polymer."

This company's ReaX product is a bead—about a thousand micrometers in diameter—that includes qPCR assays. "This product stabilizes the contents of the beads," says Daniels. "It also makes it so that basically anyone can do qPCR. You just need the DNA, and the beads can be analyzed with any kit on any platform." The Q Chip beads come in plates, and a user just pulls back the strip across the wells, and pipettes in the DNA.

In fact, extending qPCR to new users is part of the point behind Q Chip. "We want to push qPCR toward point of care," says Mark Barry, Q Chip's chief executive. "There will be lots of applications in the future for clinicians, soldiers, et cetera, who will need easy-to-use and reliable assays, and ReaX provides just that." Q Chip also provides its proprietary MicroPlant for companies that want to use Q Chip's technology to make their own ReaX products. "By using Q Chip's MicroPlant under license," says Barry, "companies can manufacture their own beads and assays."

Overall, new qPCR tools—from turning up the capabilities of TaqMan to developing entirely new products, such as reagents or beads—make this technology faster, simpler, and more sensitive. That already makes qPCR more powerful in the lab, and it could eventually end up in the clinic, too.

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DOI: 10.1126/science.opms.p0800028