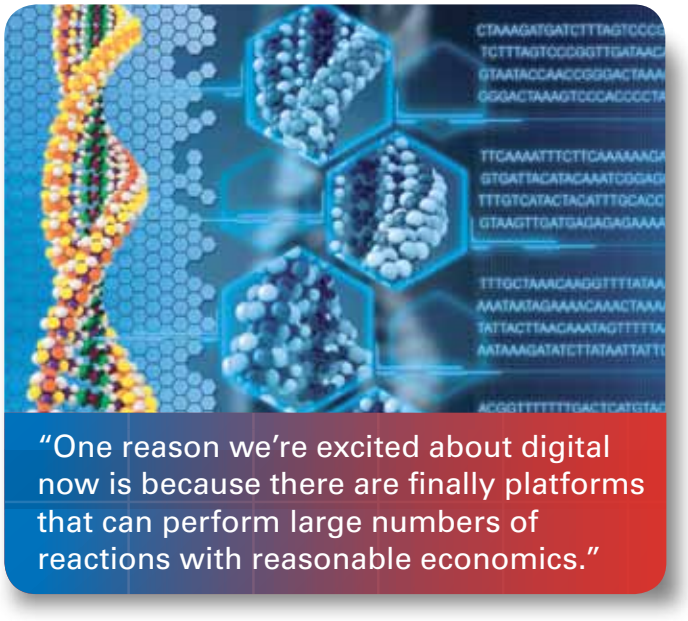
qPCR Innovations and Blueprints

Quantitative PCR users can rapidly generate large amounts of high-quality data with new instruments and products made possible by microfluidics and miniaturization technology. These platforms are the tools for developing techniques that require extremely high throughput and sensitivity such as digital PCR and single-cell analysis. Researchers are adopting these methods to ask sophisticated questions about genetics and cancer biology as well as to develop novel research and diagnostic assays. As qPCR innovators explore new frontiers and everyday users venture into more complicated workflows, international groups of industry and academic partners are keeping us on the path of best practices. Two consortia are generating guidelines on the qPCR process—from experimental design and pre-analysis sample collection, to processing data and publishing results. The guidelines are blueprints that ensure reproducibility, validity, and transparency. **By Chris Tachibana**



"One reason we're excited about digital now is because there are finally platforms that can perform large numbers of reactions with reasonable economics."

The **Gene Quantification** website is like a 7-Eleven store for quantitative polymerase chain reaction (qPCR): a one-stop shop for news, advice, and product links. Its curator is Michael W. Pfaffl, a professor in the physiology department at **Technical University of Munich,** who has been developing qPCR methodology for more than a decade. Pfaffl sees qPCR innovation in three areas: platforms, technologies, and physiology.

"Innovations in platforms are in high throughput and miniaturization," says Pfaffl, and a major factor is microfluidics technology—running submicroliter reactions in submillimeter spaces. The RainStorm platform in **RainDance Technologies'** RDT 1000 produces picoliter droplets at a rate of 10 million per hour by running aqueous samples through a stream of oil. Samples in the droplets undergo PCR amplification on a disposable chip that allows more than 2.5 million parallel reactions. Using completely different microfluidics technology, the **Fluidigm** BioMark HD runs PCR reactions in soft rubber chips that control the flow of picoliter reaction mixes with pressure-activated valves, "like a hose on a driveway that you drive across," says Fluidigm Chief Executive Officer Gajus Worthington. The highest capacity chip runs 96 reactions on 96 samples for 9,216 assays in just a few hours.

You don't need microfluidics to run thousands of simultaneous assays, though. A more traditional high throughput option, specialized for detection of cancer-related genes or microRNAs, is the **WaferGen** SmartChip System. The system's metal-alloy chips run as many as 5,184 qPCR reactions in about two hours, with instruments and software for loading, amplifying, and analyzing cDNA samples in SmartChip Panels. These come preloaded with primers for expression profiling up to 1200 human microRNAs or 1,250 human cancer-related genes, with controls. Panels can also be made with custom configurations of WaferGen pre-validated or customer-specified primers.

A DIGITAL REVOLUTION

High throughput capability is driving the development of digital PCR, named for its binary output—a yes or no answer to the question "Did the reaction contain the target sequence?" Digital PCR allows absolute instead of relative quantification, eliminating the need for standard curves or endogenous controls. "You partition samples to the point where a reaction either has a template molecule or it doesn't," explains Ramesh Ramakrishnan, director of molecular biology and assay development at Fluidigm. End-point PCR is conducted on an array of diluted samples, "then you figure out the starting concentration of the sample by counting positives after the partitioning." Ramakrishnan's group published a 2010 *PLoS One* proof-of-concept study on haplotyping, or determining the sequence of alleles along a chromosome. They found that digital PCR can be used to show that specific alleles are linked—meaning they are on the same chromosome—if the alleles are co-amplified in a single-molecule reaction. The alleles tested by the group included one that modifies the severity of sickle cell anemia.

Another application of digital PCR is cell-free, noninvasive fetal diagnostic assays using nucleic acids from maternal blood samples. In 1997, Yuk Ming Dennis Lo, director of the **Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong,** found that fetal DNA could be detected in maternal blood plasma, and surprisingly, about 5–10 percent of DNA from the mother's plasma is fetal. "We can easily detect the sex of the baby using Y chromosomal DNA in the blood," he says. Then, for a male fetus, his group established prenatal diagnosis principles using single-molecule counting by digital PCR for recessive, X-linked diseases such as hemophilia. If a woman carries one normal and one affected allele for the hemophilia gene, Lo's digital PCR assay will show a balance between the normal allele on one X chromosome and the disease allele on the other X. When she is carrying a male fetus, which has one X

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and one Y chromosome, the inherited allele for the hemophilia gene—normal or affected, will stand out as overrepresented in the maternal plasma. The same principle can be applied to Down syndrome (trisomy 21) detection for overrepresentation of chromosome 21 sequences relative to sequences on other chromosomes.

Digital PCR is a rapid and sensitive method for detecting chromosome or gene copy number variations, says Ramakrishnan. Another application is monitoring targeted drug treatments, such as with the breast cancer drug herceptin, which targets the HER2 receptor. Tumor cells become resistant to the drug as the copy number of the HER2 gene increases. "With microarrays, you're doing well if you can see two-fold differences in copy number," says Ramakrishnan. "With real-time PCR, a two-fold difference is easy, but seeing the difference between two- and three-fold, or three- and four-fold is difficult." As long as enough molecules are sampled for good statistics, he says, digital PCR allows simple counting against a reference sequence, so subtle differences in ratios can be detected, such as 3-to-1 versus 4-to-1. Digital PCR is also a sensitive method for detecting low levels of pathogens or contaminants in a dilute sample.

Paul Pickering, head of the Digital PCR Business Unit for **Life Technologies,** says that digital PCR is not a new idea, but is now feasible with the availability of high throughput instruments like the OpenArray Real-Time PCR Platform, a system capable of simultaneously running up to three plates, each with 3,072 wells of 33-nanoliter reaction size, for more than 36,000 data points per day. Imagine trying to do that with 384-well plates, says Pickering. "One reason we're excited about digital now is because there are finally platforms that can perform large numbers of reactions with reasonable economics." Life Technologies offers a kit of plates, software and a proprietary master mix optimized to facilitate digital PCR.

LOOKING AT INDIVIDUALS INSTEAD OF POPULATIONS

New qPCR technologies let researchers ask increasingly sophisticated physiological questions. Besides detecting nucleic acids in blood or urine, a hot topic at qPCR meetings is single-cell analysis. Fluidigm's Worthington says that current gene profiling methods for tissue or culture samples are "like getting a combined score for all kids and all subjects in a classroom, instead of individual grades by subject. Many differences can't be seen unless you test individuals instead of populations." Because it enables this level of analysis, single-cell qPCR is gaining much interest from the cancer biology community.

"Tumors are not one kind of cell," says Mikael Kubista, chief executive officer of the qPCR training and contract services company **TATAA Biocenter,** and head of the **Department of Gene Expression Profiling, Biotechnology Institute, Academy of Sciences, Czech Republic.** Single-cell qPCR promises a more nuanced analysis of the genetics and gene expression of biopsy cells, he says. Several systems can now collect individual cells for analysis, including laser microdissection of cells from solid tissue, or capturing single cells in suspension by fluorescence-activated cell sorting or micromanipulation with glass capillaries the width of an individual cell. The next step is addressing technical issues such as the best way to lyse a single cell and reverse transcribe its approximately one picogram of mRNA with minimal loss and variability. New analysis methods are needed to distinguish genuine cell-to-cell differences from technical variation and noise. With the right methods and markers, however, Kubista says that single-cell expression profiling could detect cancer stem cells, which have the potential for self-renewal and differentiation and to cause relapse and metastasis. "Expression profiling on 10,000 cells won't pick up the signature of a few tumor stem cells," he says. However, single-cell assays might be able to detect cells undergoing an epithelial-to-mesenchymal transition with a loss of cell-cell adhesion and increase in mobility that is associated with tumor invasiveness. Single-cell qPCR requires an extremely careful workflow, though. "You have such tiny amounts, if you make a mistake, your sample is gone," says Pfaffl.

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RainDance Technologies
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TATAA Biocenter
www.tataa.com

Technical University of Munich
www.tum.de

Thermo Fisher Scientific
www.thermo.com

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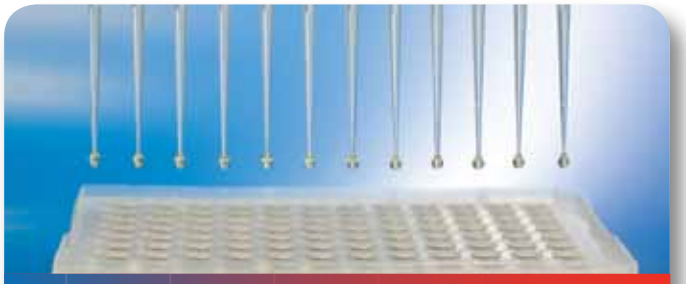
qPCR GUIDELINES (THERE'S AN APP FOR THAT)

Attention to workflow, from experimental design to sample processing to final analysis, is increasingly important as researchers push the boundaries of qPCR, yet strive to stay within the lines of reliability, reproducibility, and validation. Years of discussion on these issues are coalescing into universal guidelines for qPCR best practices. "We're entering a phase where standardization is important for making more reliable, robust assays," says Kubista.

An example is the four-year European Union "Standardisation and improvement of generic Pre-analytical tools and procedures for In-vitro DIAgnostics" (SPIDIA) initiative. This consortium of academic and industry partners and the European Committee for Standardization (CEN) is generating evidence-based guidelines and tools for standardizing the pre-analysis process, such as ways to collect samples that preserve gene expression profiles as they were in vivo and to minimize ex vivo biomolecular synthesis, degradation, and modification. The project is coordinated by **Qiagen,** and Uwe Oelmueller is Qiagen's SPIDIA coordinator and vice president of Research and Development, Molecular Diagnostic Sample Preparation. "Qiagen is in charge of overall project management, coordinating the activities of the 16 partners," he says, "and we also do science—development and discovery work." Of the €13 million continued »



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budget, €4 million comes largely from the industry partners, says Oelmueller. The project is a natural fit with Qiagen's products for sample preparation that preserve bioanalyzable profiles, and all consortium companies benefit from the networking and product development opportunities. Guideline releases have been ongoing, from the SPIDIA consortium's published recommendations to standards that require approval by 31 European countries, which will take several years to finalize.

In 2009, a set of qPCR best-practice guidelines was published by an international consortium led by Stephen A. Bustin, professor of molecular science, **Barts and the London School of Medicine and Dentistry, University of London.** The *m*inimum *i*nformation for publication of *q*uantitative real-time PCR *e*xperiments (MIQE, or "mikey") guidelines are a blueprint for good assay design, and they standardize the information submitted with publications. Developed in part after considerable variability was found in clinical qPCR assay results, the guidelines include a checklist of items to include with any publication using qPCR, and cover everything from sample collection and experimental design, to data analysis and reporting results. Guideline developers are urging journals to include MIQE compliance in their publication requirements. Some MIQE guidelines are simple, for example, using the term RT-PCR for reverse transcription-qPCR, and qPCR for quantitative real-time PCR, which might not include reverse transcription. Others require more effort from investigators, such as supplying primer or amplicon sequences. It's worth it, though. "A researcher following the guidelines is virtually guaranteed to end up with an efficient PCR assay and is encouraged to provide all assay details to enhance the transparency of experiments," says Bustin. And to make MIQE easy and fun, there's an iPhone app with links to references and screens to tick off checklist items.

ADDITIONAL RESOURCES

Gene Quantification Web Pages
www.gene-quantification.info

MIQE App
itunes.apple.com/app/miqe-qpcr/id423650002?mt=8

MIQE Guidelines
www.clinchem.org/cgi/content/full/55/4/611

SPIDIA
www.spidia.eu

Companies are now helping investigators follow MIQE guidelines. Ian Kavanagh, senior research and development manager at **Thermo Fisher Scientific** says the Solaris qPCR gene expression assays were developed with input from the MIQE guideline authors. The assays offer predesigned, optimized primers and probes that detect all known splice variants of mRNAs in the human and mouse genomes. An essential component of the MIQE guidelines is knowing your amplicon, and Kavanagh says, "Solaris fully discloses all primer and probe sequences so customers can do their own bioinformatics searches to see what the amplicon looks like."

On the data-crunching end, MIQE compliance is a feature of qbase^{PLUS}, third-party qPCR data-analysis software that is compatible with more than 95 percent of qPCR instruments on the market. It originated as freeware developed by Jan Hellemans and Jo Vandesompele, researchers at **Ghent University** in Belgium, who started the qPCR software and services company **Biogazelle** in 2007. Vandesompele says qbase^{PLUS} "uses only peer-reviewed quantification models for relative quantification, efficiency correction, inter-run calibration and error propagation, and stores and exports the assay information that is required for MIQE guidelines." This information includes primer sequences, target information, and PCR efficiency.

The MIQE guidelines are a work in progress. An update was published in March 2011 and guideline developers are constantly receiving feedback from researchers. Some find the checklist daunting, but Kavanagh says, "every scientist should have the information it asks for in their laboratory notebooks anyway, if they've done their experiments correctly."

THE FUTURE: LEAVING IT TO THE PROS

Of course, one way to ensure high-quality qPCR is to let the pros do it. Kubista predicts developments in qPCR "similar to oligonucleotide synthesis and sequencing, which were first set up in academic labs, then in core labs. Today, it's cheaper to send samples to a specialized provider with high throughput instrumentation and quality control programs, so everything is according to guidelines and best practices. Within five years I believe we will have this change in qPCR." Kubista is banking on this trend with the TATAA Biocenter. In addition to offering training in basic qPCR, including hands-on workshops, and courses in experimental design, sample preparation, standardization, quality control, data mining, and data analysis, the TATAA Biocenter performs qPCR contract services, from study design to post-PCR analysis. Biogazelle also provides contract services, including expression profiling for human and mouse microRNAs and long non-coding RNAs, and WaferGen offers proof-of-principle expression profiling or single-nucleotide polymorphism genotyping on customer-supplied samples.

With the economies of scale and quality assurance that core facilities or contract research organizations can provide, outsourcing qPCR work is tempting. Researchers still need to understand good data handling and experimental design, though, says Kubista. "The user doesn't need to be a statistician, but has to understand the purpose of different controls and replicates." It's a lot to keep track of, with technological advances leading researchers in many directions; but a regular stop at the Gene Quantification website will keep you stocked up on current qPCR news and developments.

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