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# Digital PCR hits its stride

Monya Baker

As the less familiar cousin of quantitative PCR moves mainstream, researchers have more options to choose from.



A few years ago, Ramesh Ramakrishnan had to spend so much time explaining what digital PCR was that he had to rush through his explanations of applications when he gave talks at meetings. Now, he says, most audiences are at least familiar with the term, even if they have not performed the technique themselves. "It's no longer an exotic thing," says Ramakrishnan, director of R&D at Fluidigm Corporation.

The strategy for digital PCR (dPCR) has been summarized as 'divide and conquer': a sample is diluted and partitioned into hundreds or even millions of separate reaction chambers so that each contains one or no copies of the sequence of interest. By counting the number of 'positive' partitions (in which the sequence is detected) versus 'negative' partitions (in which it is not), scientists can determine exactly how many copies of a DNA molecule were in the original sample. Among other applications, researchers have used digital PCR to distinguish differential expression of alleles[1], to track which viruses infect individual bacterial cells[2], to quantify cancer genes in patient specimens[3] and to detect fetal DNA in circulating blood[4].

The concept behind digital PCR was first described in 1992 (ref. 5). A few years later, Bert Vogelstein and Ken Kinzler at Johns Hopkins University named the technique and showed that it could be used to quantify disease-associated mutations in stool from patients with colorectal cancer. But although the theory was simple, its implementation was not. Initial demonstrations were performed in commercially available 384-well plates with 5 microliters per partition, requiring volumes of reagents that would daunt most

Advances in nanofabrication and microfluidics have now led to systems that produce hundreds to millions of nanoliter- or even picoliter-scale partitions. Academic technology developers have described several implementations, but so far only a handful of companies have commercialized products or announced plans to do so (**Table 1**). Fluidigm and Life Technologies create reaction chambers within specially designed chips or plates. Bio-Rad and RainDance sequester reagents into individual droplets.

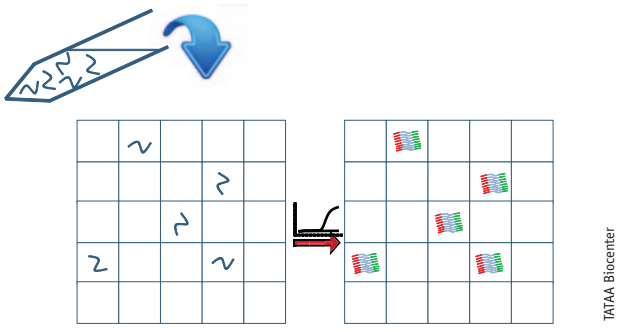
## Higher costs, higher precision

The most popular PCR technique to measure the presence and concentration of a DNA sequence is not digital PCR but its more familiar cousin, real-time quantitative PCR (qRT-PCR, or qPCR). In qPCR, DNA is copied until it produces a certain level of signal; the number of amplification cycles needed to reach this point is then used to calculate how many DNA molecules with the particular sequence were originally present relative to other DNA molecules in the sample.

Digital PCR uses the same primers and probes as qPCR but is capable of higher sensitivity and precision. In standard implementations, qPCR cannot distinguish gene expression differences or copy number variants smaller than about twofold. Identifying alleles with frequencies of less than about 1% is difficult because such tests would also dant common alleles with similar sequences. In contrast, dPCR can measure a 30% or smaller difference in gene expression, distinguish whether a variant occurs in five versus six copies and identify alleles occurring at a frequency of one in thousands. It can also be used to standardize qPCR assays.

The more partitions, the greater the resolution. "If you want to distinguish between 2 and 3 copies, you need 200 chambers. If you want to distinguish between 10 and 11, you need 8,000," explains Mikael Kubista, CEO of TATAA Biocenter, which provides services and training in both qPCR and digital PCR. In principle, one could also get similar precision by doing 8,000 replicates of qPCR, he says, but such experiments are impractical.

Jim Huggett is the science leader for nucleic acids metrology at LGC, a laboratory services and measurement standards organization in the UK. His team has directly compared the two techniques across several DNA templates and other conditions[7]. Digital PCR offers more accuracy and less ambiguity than qPCR, he says, but qPCR has enticing advantages. It is less expensive and works over a much broader



TATAA Biocenter

Digital PCR works by diluting a sample into many partitions and counting up

**Table 1** | Commercial digital PCR offerings

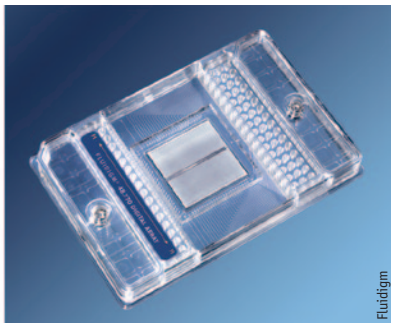
| Vendor | Instruments and list price | Consumables and list price | Number and volume of partitions | Volumes required | qPCR capacity | Multiplexing |
|---|---|---|---|---|---|---|
| Fluidigm Corporation | BioMark HD: $200,000–$250,000 | 12 arrays per chip[a] (765 wells per array): $400 per chip (works in both EP1 and BioMark) | 12-inlet chip: 9,180 partitions, 6 nl per partition | 12-inlet chip: 8 μl of mix, ~4 μl of sample; 57% analyzed[b] | Yes | Can use up to 5 colors to detect 5 targets (assumes 5th color is ultraviolet) |
| | EP1: $100,000–$150,000 | 48 arrays per chip[a] (770 wells per array): $800 per chip (works in both EP1 and BioMark) | 48-inlet chip: 36,960 partitions, 0.85 nl per partition | 48-inlet chip: 4 μl of mix, ~2 μl of sample[b] | No | Can use up to 5 colors to detect 5 targets |
| Life Technologies | OpenArray RealTime PCR System and QuantStudio 12K Flex instrument: $140,000 and $90,000–$190,000, respectively | OpenArray plates[a] (64 holes per subarray): $150 per plate | Varies; 3,072 partitions per plate, 48 subarrays per plate, 33 nl per partition (machines run 3–4 plates at once) | 100 μl of sample per plate (across 48 arrays) | Yes | Uses 2 colors of probes to detect 2 targets |
| Bio-Rad Laboratories | QX100 ddPCR System (machines to generate and read droplets): $89,000 | 8 samples per chip (14,000–16,000 droplets per sample): $3 per sample | Up to 96 samples per run (assumes manual pipetting into PCR plate); 1,344,000 partitions per run (assuming separate thermocycler runs 12 chips at once), 1 nl per partition | Up to 9 μl per sample (20,000 droplets made); an average of 70% read | No | Uses 2 colors to detect 2 targets |
| RainDance[c] | RainDrop Digital PCR (machines to generate, collect and read droplets): $100,000 | 8 samples per chip (up to 10,000,000 droplets per sample): $10–$30 per sample | 8 samples per run; up to 80,000,000 partitions per run, 5 pl per partition | 5–50 μl per sample | No | Uses 2 colors, but can use varying concentrations of probes to detect up to 10 targets |

[a]Arrays can hold separate samples, or the same sample can be spread over multiple arrays. [b]For rare allele analysis, protocols are available to eliminate the dead volume. [c]Plans full commercial launch later this year.

dynamic range than digital PCR. For example, it can determine that transcripts of one gene are as much as a billion times more abundant than transcripts of another gene.

Also, qPCR experiments can routinely analyze hundreds of sequences per sample run. Eventually, Kubista believes that it will be possible to multiplex dPCR to examine perhaps as many as 100 reactions at once, but no one would consider measuring large numbers with digital PCR today, he says. And qPCR is already well-integrated into many researchers' labs. "We've been developing workflows for qPCR for 20 years." In contrast, the first full conference dedicated to applications of digital PCR is scheduled for October of this year (see http://www.healthtech.com/digital-pcr).

MicroRNA researcher Muneesh Tewari at the Fred Hutchinson Cancer Research Center uses digital PCR in situations where absolute quantification is important, such as when detecting low-abundance RNA. One advantage of digital PCR is that with more partitions, a greater volume of a dilute RNA sample can be analyzed, he says.




Also, digital PCR does not require the calibration and internal controls necessary for qPCR. Instead, counts from replicate wells can simply be added together. "Thinking in terms of absolute copies is so intuitive," he says. Nonetheless, his lab currently performs more qPCR experiments than digital PCR experiments. qPCR has lower cost and higher throughput, says Tewari, and his staff is more familiar with it.

That's a typical situation, says Paul Pickering, head of the digital PCR business unit at Life Technologies. "Most customers are seeking to do RT-PCR and then, in the situations that they need it, they'll deploy digital PCR." In those cases, he says, "there are four attributes that customers value: sensitivity, specificity, precision of the answer and the fact that you can get an absolute count without needing to reference any other material."

## Digital PCR on chips

In 2006, Fluidigm became the first company to commercialize digital PCR. It offers two systems that mix samples with reagents, partition the reaction mix-

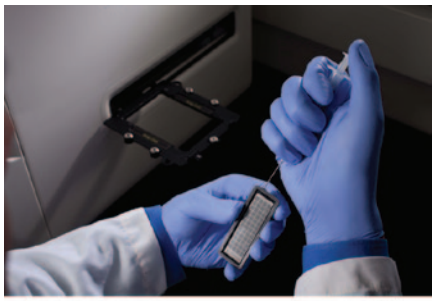
results within each partition. The simpler, cheaper EP1 machine detects only endpoints, that is, whether or not a reaction has occurred. The BioMark HD System, which also performs qPCR, can be set to monitor the course of a reaction and provide data that can eliminate false positives. Both systems use chips containing sophisticated microfluidics and tiny valves that partition samples into about 800 reactions, with either 12 or 48 samples per chip. If researchers want to run more reactions per sample, they can just double up arrays within chips or even double up on chips, says Ramakrishnan.

The company has developed another chip called the 200K with hundreds of thousands of partitions, and has licensed separate technology for chips with as many as a million partitions. However, plans to commercialize these technologies are on hold pending greater demand. "We can go up in terms of partitions, but we haven't found a huge pull from the market in going to that higher density," says Ramakrishnan.

Life Technologies began offering digital PCR in 2009 after acquiring long-time collaborator BioTrove. It now sells two machines that can be used for both digital PCR and qPCR, the OpenArray and QuantStudio 12K Flex. These mix samples with reagents, load mixtures into reaction chambers, run amplification cycles and monitor reactions as they occur. The machines rely on plates that are roughly the size of a microscope slide and are essentially highly engineered peg boards with nano-sized holes; capillary forces and careful placement of hydrophilic and hydrophobic surfaces hold samples in place.

The OpenArray machine holds up to three plates, each containing 48 arrays with 64 partitions apiece. QuantStudio holds up to four plates and can also accept formats used in high-throughput qPCR experiments: TaqMan Array cards as well as 96- and 384-plate formats. "What we found is that a lot of our customers want the capability [for digital PCR] but aren't ready to jump in and say that that's the only thing that they have to do," says Pickering. Buying a machine that can do both, he says, is similar to the decision to purchase a hybrid gas and electric automobile rather than an electric-only vehicle.

Bio-Rad



Life Technologies

Life Technologies' QuantStudio System for digital PCR and qPCR.

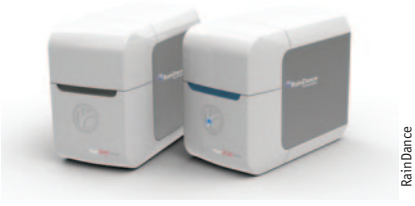
## Digital PCR in droplets

Companies like Bio-Rad and RainDance sell machines that cannot perform qPCR but which offer many more partitions. In droplet digital PCR, reaction chambers are separated not by the walls of a well but by carefully titrated emulsions of oil, water and stabilizing chemicals. First, samples are put into a machine where they are mixed with all the necessary reagents and dispersed into tiny droplets. The droplets for each sample are transferred into tubes that can be placed in a thermocycler for to a droplet reading machine, which functions like a flow cytometer to analyze each droplet for whether or not a reaction has occurred.

QuantaLife launched the first commercial digital droplet PCR system a year ago. In December 2011, the company was acquired by Bio-Rad Laboratories for $162 million, with promises for more cash if products hit certain milestones. All along, the goal was to develop an instrument that was both inexpensive and easy to use, says Bio-Rad marketing manager Mike Lucero, who was an early employee of QuantaLife. "We have two rules at the company: no chips and no lasers." He's betting that the low cost of consumables will set the company apart, he explains, holding up a clear, lightweight strip studded with sets of cups for holding collections of droplets, each narrower than a toothpaste cap. "This is less than $10," he says. "And it's because we took the time and effort to make it out of plastic."

Getting the chemistry for the droplets right was crucial, says Ben Hindson, one of QuantaLife's original employees and now a senior principal scientist at Bio-Rad. The droplets produced must remain a uniform size even if the temperature fluctuates slightly as they are generated. What's more, the droplets cannot burst or coalesce during handling, thermocycling and reading, and they also must maintain biocompatible conditions that support PCR. It takes 25 minutes to generate droplets for 96 samples, says Hindson, and one person running the system can analyze 3 sets of 96 samples a day. The technology has begun to appear in independent research; in a high-profile paper combining genomic, transcriptomic, proteomic and metabolomic data, digital droplet PCR developed by QuantaLife was used to detect differential expression of a variety of alleles[1].

Another digital PCR system has been developed by RainDance and is scheduled to launch later this year. The machines in



RainDance

RainDrop Source and RainDrop Sense machines

The most common applications of digital PCR at the TATAA Biocenter are standardizing qPCR assays, detecting copy number variations, detecting rare mutations and distinguishing differences between expression of nearly identical alleles, says Mikael Kubista.

this system generate and read millions of picoliter-sized droplets, a feature that not only allows scientists to identify rarer alleles but also alleviates some of the need to dilute samples carefully. "With all those droplets, we can deal with a wide variety of different concentrations," explains company co-founder Darren Link. He dismisses competitors' claims that millions of partitions are more than most scientists will need. "Too many is never a problem, especially when you are talking about expression analysis," he says. "You don't want to run titrations to find the sweet spot of the dynamic range." Link also emphasizes that the system does not require any manual pipetting as droplets are moved between machines that make droplets, perform thermocycling and analyze droplets.

Researchers at RainDance and the University of Strasbourg and University Paris-Descartes reported that they could detect one mutant *KRAS* gene within 200,000 wild-type *KRAS* genes when the former was diluted into genomic DNA. The seven most common *KRAS* mutations were screened in two multiplex experiments: one examining the wild-type allele along with four mutations and one with the wild type alongside three mutations[8]. At AACR, RainDance presented results detecting cancer mutations in patient serum.

### Thinking digitally

Digital PCR may not require the same kind of calibration and controls as qPCR, but there is still plenty of scope for artifacts, says Huggett. Working in tiny volumes and with single-molecule concentrations is a complicated engineering feat. "dPCR is at an early stage, so my advice would be to proceed with caution and be careful of sweeping statements," he says. For example, some researchers believe that digital enzyme-inhibiting substances that occur in some samples. For qPCR, the problem is that inhibitors increase the number of amplification cycles required to reach a given signal. But even though digital PCR does not count cycles, inhibitors could still be a problem if they cause false negatives by preventing reactions from occurring at all.

Some factors are particularly important to consider with digital PCR, says Kubista. "For example, it is really critical that the assay is well-performing, that you are confident that if there is a single target molecule [you] will see it. Not all assays are that good." In addition, researchers need to make sure that multiple sequences of interest do not appear on the same piece of DNA; otherwise, they cannot be separated into different partitions. (Also, if the positive partitions are clustered together rather than randomly dispersed, there is probably an issue with sample loading or analysis.)

"At the moment," says Jim Huggett, "digital PCR is a specialist approach that is much more costly than qPCR, and qPCR is suitable for the vast majority of applications."

Specificity is also an issue. Many assays will amplify products other than the sequence of interest, particularly if pseudogenes are present. Understanding rates of false positives is crucial when hunting for rare alleles. In these cases, most partitions will not contain the molecule of interest, and the number of false positives could dwarf the number of true positives. For these reasons, Kubista recommends a variety of control experiments. His center offers a kit called ValidPrime that amplifies just one copy of a gene per haploid genome and can be used to assess specificity.

Special consideration is warranted for the rarest alleles. If a sequence is only going to occur once in 50 microliters, says Pickering, it's essential to analyze more than 50 microliters of the sample. "No matter what the technique, if you haven't sampled enough volume to get what you're looking for, you're not going to detect it."

In applications for quantifying more abundant molecules, such as detecting expression, researchers generally need to get a rough estimate of the concentration of their target of interest in order to make appropriate dilutions. Otherwise, too many partitions will contain multiple copies. (Statistics can compensate for this, but only to a limited extent.) If every partition shows a reaction, researchers cannot calculate the concentration of the original molecules, explains Kubista. "You get the best use of the chip by having 80% positive [partitions]. If the number rises above 90%, precision drops."

Monitoring the 'response curve', or how levels of DNA change over the course of amplification, can help eliminate false readings caused by nonspecific labeling of DNA sequences—a benefit that companies such as Fluidigm and Life Technologies, which provide such data, are keen to point out. Advocates of droplet digital PCR, however, believe that accurate measurements can be made with endpoint data alone, and cite the advantages of a greater number of partitions. "For allele-specific experiments, you may get false positives, but you can quantitate what that false positive rate is rather than infer from a curve," says Hindson.

Researchers should also consider all the steps that occur before digital PCR begins, says Kubista. As samples are processed, material is lost. Running controls in which a sequence is spiked in before processing can help determine how much sample is necessary, he says.

Although the experts urge care in designing digital PCR experiments, they are enthusiastic about its potential. As the technology matures and the costs come down, more researchers will learn to ask questions only digital PCR can answer, says Kubista. "There are a few applications today, and there will be more tomorrow."

**Monya Baker is technology editor for *Nature* and *Nature Methods* (m.baker@us.nature.com).**

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