Clever PCR: more genotyping, smaller volumes

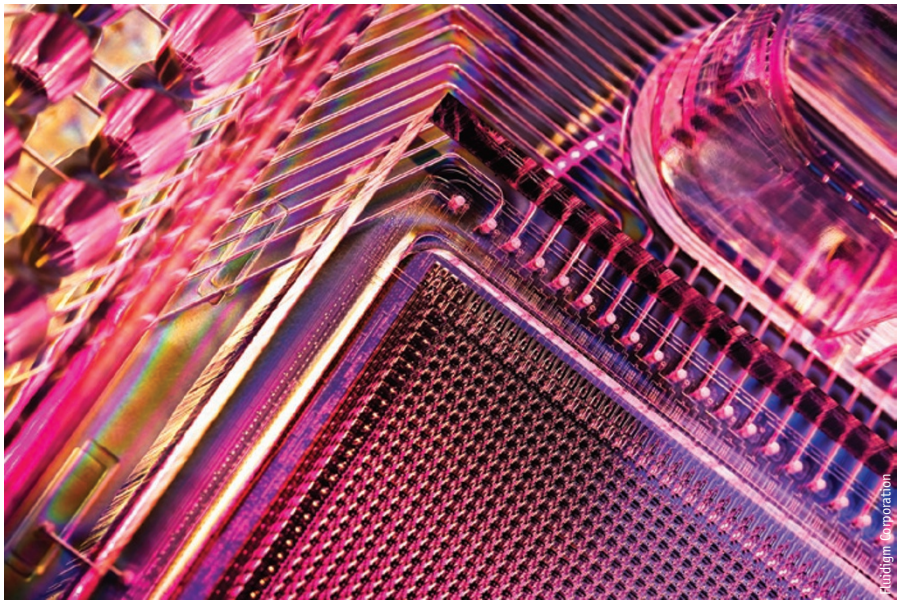
Monya Baker

With microfluidics and multiplexing, researchers can get more information from PCR products in less time and with fewer reagents.

"Where PCR is really going," says Olivier Harismendy at the University of California, San Diego, "is parallelization and miniaturization." Indeed, researchers are making use of a wide variety of materials and applications toward these goals.

Miniaturizing PCR protocols offers a range of benefits, says Bruce Gale, who directs the Utah State Center of Excellence for Biomedical Microfluidics. "As soon as you go to microfluidics, you can bump up the speed and precision because you use small volumes," he says. Other advantages include portability plus the ability to work with smaller samples and fewer reagents. Growing enthusiasm for shrinking volumes is being felt in the industry, says Jeremy Gillespie, group product manager at Thermo Fisher Scientific. Scientists are increasingly interested in buying smaller amounts of reagents because they are using smaller volumes in their assays, he says.

Most microfluidics chips conduct PCR using minute volumes of highly dilute samples, relying on fluorescence (or melting-point temperatures) to determine the presence and quantities of a genetic sequence in a sample. Many applications involve finding some version of a needle inside some version of a haystack: rare mutations among lots of wild-type DNA or fetal DNA in a maternal blood sample, for example. Some applications look at relative amounts of DNA sequences, such as copy-number variation, allele ratios or microbe sampling. Newer PCR applications use microfluidics techniques as a preparative technology for next-generation sequencing. These sequester DNA in tiny reaction vessels for PCR amplification, then collect the amplicons for subsequent analysis.



The Access Array chip from Fluidigm can amplify 48 genomic regions from 48 samples.

Microfluidics for next-generation sequencing

With each run of a sequencer potentially generating data for 100 gigabases, "you've got to think about how to design experiments so as not to waste data, because it's costly," says Daniel J. Turner, head of sequencing technology development at the Wellcome Trust Sanger Institute. "The two things that affect how much sequencing you have to do to see what you need to see are how specific your sequence enrichment is and how uniform your data are," explains Turner.

Sequencing capacity is wasted when some genomic regions are amplified more than others. "When you say you want 20-fold, you want 20-fold everywhere," says Harismendy. "If you want to sequence all the exons at 20-fold coverage, you don't want some at 100-fold or at fivefold," he adds. If some exons in a study are only present at fivefold coverage, then an analysis can only claim fivefold coverage. Compared with other technologies for massively parallel PCR, he says, microfluidics platforms excel at uniformity because the products of PCRs do not compete with each other.

Fluidigm launched one such microfluidic platform, its Access Array, in the fall of 2009. The chip uses a matrix that guides samples and reagents into tiny chambers. "You take 48 samples and 48 primer pairs, and it makes every combination and does PCR, and then you can pump the samples back out," explains Ken Livak, technology developer at Fluidigm Corporation. During the PCR a DNA barcode is added to each sample so that the 2,304 amplified

The stand-alone thermocycler from Fluidigm conducts PCRs on a microfluidics chip.

products can be pooled and shuttled into any next-generation sequencer. Separate chips can provide unique sets of barcodes so that PCR products from multiple chips can be pooled and sequenced together. The Access Array Chip excels in producing uniform numbers of the desired amplicons, says Livak. "You get a 'tight' range so that you don't have to go way deep [into the sequence] without missing anything," he adds.

Johan den Dunnen heads the Genome Technology Center at Leiden University Medical Center in the Netherlands, where his team uses Fluidigm's products as well as an approach called hybridization capture (**Box 1**). The larger or more complex the targeted regions are, he says, the more likely he is to go with hybridization, but the Fluidigm PCR approach offers several advantages. Even without any optimization, he says, it has "nice uniform enrichment" compared to hybridization techniques. Plus, with hybridization capture, researchers only find out that targeted regions were missed after a sequencing run has been completed. In contrast, Fluidigm's arrays can show whether a target region was enriched before sequencing, allowing his team to decide whether or not to run a sample.

RainDance Technologies has commercialized an instrument that uses microdroplets to efficiently prepare samples for next-generation sequencing. The core of RainDance's technology is the creation and combination of two large sets of tiny, precisely aliquoted drops, each about the size of a typical eukaryotic cell. In one set, each drop contains a specific pair of PCR primers designed to target a region of interest. In the other set, each drop contains a large portion of genomic DNA to be amplified along with the enzymes and other reagents necessary for PCR. A specially designed chip merges the drops at a rate of about 3,000 per second, all the while ensuring that the drops combine in a constant one to one ratio.

Harismendy recently completed a collaboration with RainDance showing how the technology can be used for target capture. He and colleagues reported using this approach to examine 435 exons in

BOX 1 PCR PROBES FOR MULTIPLEXING

Several techniques allow researchers to do massively parallel sequencing without using microfluidics[4,5]. These often use long, cleverly designed targeting primers or probes aimed at capturing rather than amplifying desired regions.

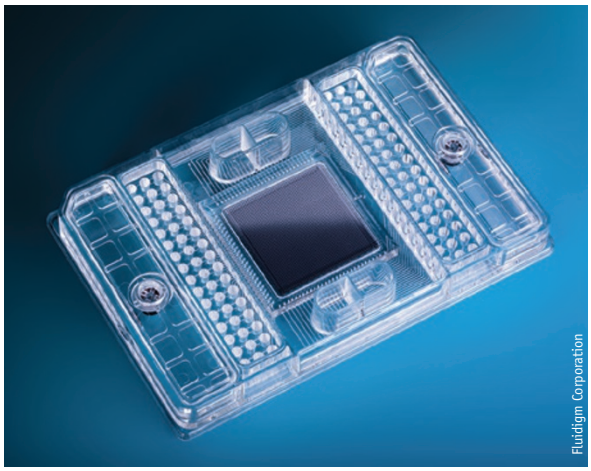
In the circularization approach, primers have varying target-specific sequences and common linking sequences. When added to genomic DNA along with ligase, these primers circularize desired DNA regions (leaving non-circularized bits to be cleared away by nucleases) and also add common DNA tags that allow every sequence to be read by a single set of primers. "We've done 100,000 simultaneous captures in the same tube, and they don't interfere with each other," says Jay Shendure of the University of Washington. Though Shendure uses molecular inversion probes (also called padlock probes) that he helped develop as a graduate student, conceptually similar reagents will soon be available commercially. Olink Genomics, for example, is developing what it terms the Selector Technology, which works on DNA that has first been cleaved by restriction enzymes.

Other widely used methods are based on hybridization, which can occur either on arrays or in solution. In this approach, desired genomic regions are captured either in solution followed by PCR or on arrays and fed into appropriate next-generation sequencers.

Researchers led by Nick Papadopoulos and Bert Vogelstein at Johns Hopkins University recently reported surprising heterogeneity in different tissues of the same individual[6]. To obtain their results, the researchers used a combination of hybridization and primer design to accomplish 1,000-fold coverage of the mitochondrial genome. In one set of experiments, researchers used three different sets of primers (segregated into separate vessels to avoid interference during PCRs) to amplify separate but overlapping regions of the mitochondrial genome. In a second set of experiments, the researchers made biotinylated DNA probes specific for the mitochondrial genome, used these to pull out desired sequences from whole genome DNA, amplified those and fed them into an Illumina sequencer. "If you want to get the mitochondrial DNA, sequencing the whole genome is just wasteful," says Papadopoulos. High-throughput microfluidics techniques seem like good technologies, he says. "However, for mtDNA they can be an overkill," he adds. Nonetheless, complete, thorough coverage is essential; with the appropriate primers on hand, Papdopolous thinks his team will continue to use both the biotinylation capture and standard PCR approaches: "When you have the same results from two different methods it's really more believable."

Most researchers, however, will end up using just one approach, and each has pluses and minuses. Hybridization approaches may capture many unwanted sequences and offer less uniformity than PCR. However, they are readily scalable, and there is no need to use more DNA even when larger regions of the genome are captured. The circularization approaches usually capture more than 90% of the desired sequences and can capture at least 25-fold more sequences than currently available microfluidic approaches from RainDance and Fluidigm, but some sequences are amplified to a much greater degree than others.

As technology develops, of course, optimal approaches change. Eventually, the cost of capturing targets for sequencing might outstrip the costs of sequencing the whole genome, or single-molecule sequencing could make some target-capture strategies obsolete.

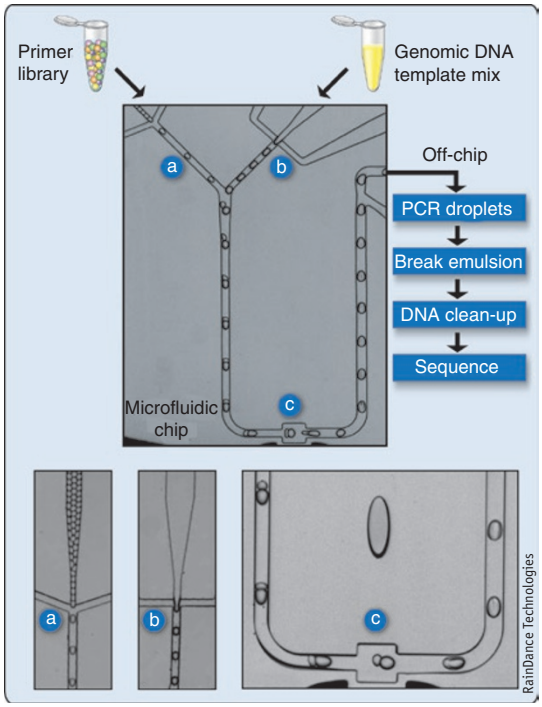


Samples loaded into the Access Array Integrated Fluidic Circuit are amplified, barcoded and pumped out again for analysis.

samples from six people[1]. They chose regions that included repetitive elements, varying amounts of G+C content and other sequence features that often cause variation in amplification. The results showed accuracy and coverage equivalent to what they would have obtained if they ran all the PCRs in 1.5 million separate 20-microliter tubes, but they used a fraction of the reagents and disposables. "It was so obvious that it was a big savings and it's also much easier," says Harismendy, who is not affiliated with RainDance. "There's little handling. You just put your [primer] library on one side and your template [DNA] on the other side," he adds. The droplets for each sample are then transferred into a single PCR tube that is placed into a thermocycler to allow PCRs to run their course; after a sufficient number of amplification cycles, the oil-water emulsion that keeps droplets separate is broken and barcodes can be ligated to PCR products, which are then ready to go into any of the next-generation sequencers.

An advantage of microfluidics systems is that they can work reliably with whole genome–amplified DNA without becoming biased to certain alleles. This could be particularly useful for researchers who may only have tiny amounts of starting DNA, says Darren R. Link, a cofounder of RainDance. "They can do the [whole-genome amplification], use RainDance technology and still have sample remaining," he says. RainDance's platform currently allows about 4,000 primer pairs to be used at one time, but improved efficiencies to be launched this summer will expand the instrument's range to around 20,000 primer pairs.

Nonetheless, researchers like Wellcome's Turner are currently using multiplexing rather than microfluidics approaches because the former are capable of pulling down tens of thousands of genomics regions at once (**Table 1**). When RainDance expands its primer capacity in the summer of 2010, the technology will start to become competitive with multiplexing, Turner says, though he thinks the projects he is running will likely require coverage of many more regions. He is quick to point out that the decision about which preparative technology works best depends on the numbers of samples as well as the number and type of sequences to be studied.



RainDance creates tiny droplets as vessels for PCR amplification.

Table 1 | Preparative DNA technologies for next-generation sequencing

Approach	Vendors and products	Regions targeted	Approximate cost	Approximate amount of starting DNA
Separate PCRs in picoliter droplets	RainDance	4,000 regions per sample (going up to 20,000 in summer 2010)	$225,000 for instruments; $350 per chip	1.5–2 micrograms
Separate PCRs in nanoliter microchambers	Fluidigm	48 regions for each of 48 samples (2,304 reactions per chip)	$79,000 for instruments; $350 per chip	0.05 micrograms
Multiplex PCR using circularization	Olink Genomics' Selector Technology	100–2,000 regions per sample	To launch later in 2010	0.5 micrograms
Hybridization capture	In solution: Agilent's SureSelect febit's HybSelect Roche NimbleGen's SeqCap EZ Exome On array: Agilent's CaptureArray Roche NimbleGen's Sequence Capture Others	Hundreds of thousands (up to 30 megabases, or the entire exome) per sample	Around $500–$2,000; varies considerably with product and purchase volume	3–5 micrograms

Little solutions

Still, there is more to genotyping than sequencing. In addition to its gene expression products, Fluidigm already offers microfluidics chips that can analyze copy-number variations and single-nucleotide polymorphisms. Both analyses run on the BioMark instruments also used for gene expression analyses. These chips have an optically clear top through which fluorescence signals corresponding to DNA content can be read. Because the chip is designed to interface with standard laboratory equipment, researchers can load the chip with whatever primers and probes they want. "The chip works like a microtiter plate," explains Robert Jones, executive vice president of research and development at Fluidigm. "You can buy assays from a number of vendors, you can put them in the part of the chip just like you would a plate, you put the samples in, and the system does all the PCR," says Jones.

RainDance plans to launch several additional applications for its microdroplets: deep resequencing and methylation analysis and, in the future, single cell analysis. Other technologists are also creating applications based on the ability to make droplets with precisely controlled volumes. Researchers led by Richard Mathies at University of California, Berkeley recently reported a microfluidic technique that can be used to detect one dangerous *Escherichia coli* O157 pathogen in a background of 100,000 harmless bacterial cells[2]. First, forward primers specific to each cell type to be analyzed are attached to thousands of 34-micrometer beads, so that every bead can bind DNA from the cell types expected in the sample. These beads, along with the cells to be analyzed, are diluted into nanoliter droplets produced by a microfabricated emulsion generator array. Each droplet contains PCR reagents, including reverse primers labeled with a unique fluorescent dye for each cell type. Researchers can readily control the creation, loading and transport of intact droplets into standard tubes and the thermocycling used for PCR, explains lead author Yong Zeng. After a sufficient number of PCR cycles, the beads are analyzed by flow cytometry. The fluorescence signal allows researchers to track the distribution of the rare cells as well as count the droplets containing no, one or multiple cells.

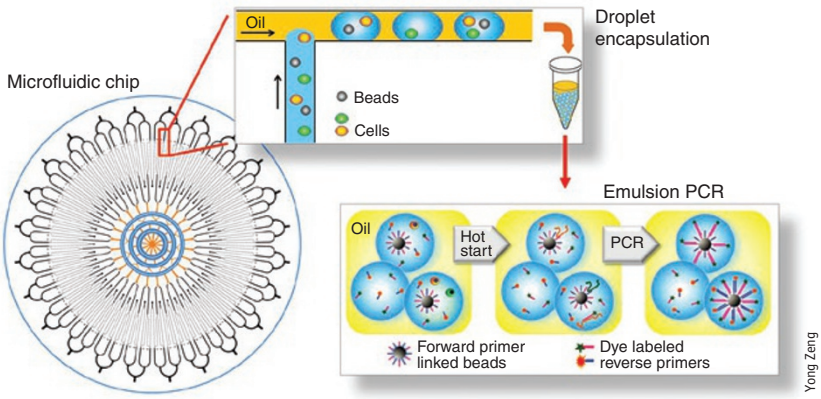
Most of these 'digital PCR' applications rely on having just one analyte per sample and thus require excessive dilutions, but the ability to make droplets with extremely precise volumes circumvents that requirement. As all the droplets are the same size, explains coauthor Richard Novak, statistical analyses can be used to calculate absolute numbers and frequencies of rare cells in a population. Novak and Zeng believe perhaps as many as 20 separate fluorophores could be used in future versions of this technique, allowing the detection of rare mutations in clinical samples and perhaps even the co-occurrence of mutations in a single cell.

It is too early to consider commercialization of this device, says Zeng, but the droplet protocol should translate readily to other laboratories, provided they have access to microfabrication facilities. "The current limitation for translating microfluidics to biological labs is the cost of fabrication and the expertise."

Much of the cost of a microfluidics chip depends on sophisticated lithography used to create nanoscale pumps and valves. This means that the chips can simultaneously conduct dozens of assays on dozens of samples in little time, so companies can charge hundreds of dollars for a chip. Gale, however, hopes to take microfluidics to the other end of the market. This year he reported the use of small plastic disposable disks for certain types of genotyping[3]. Each disk contains a thousand nanoliter-sized wells that dilute material from a single sample. The sample is brought into chambers not with the standard pumps and valves, but by spinning the disk on its axis, filling wells by centrifugal force. In a prototype, Gale's research team showed each PCR cycle could be completed in about half a minute. The amount of amplification in the wells is detected by fluorescence and indicates how much of a particular DNA sequence a sample

RainDance Technologies

RDT 1000 instrument conducts massively parallel PCRs in picoliter droplets.



A microfabricated emulsion generator array from the Richard Mathies lab at the University of California, Berkeley uses PCR to find rare cells.

contained. Analysis took just over half an hour, including image analysis. Though the device is still being optimized, Gale imagines that versions of it could be used to find rare cell types or mutations in both environmental samples and bodily fluids. Too young to even have a website, start-up company Espira aims to commercialize these disks. The goal is to pair a $20 camera with PCR chips that cost just a few pennies.

The scope of other PCR-based applications is tremendous, Gale says. The hope is that the advance of new technologies can work with microfluidic PCR amplifications in much the same way as it has worked in sequencing. In other words, as capacity goes up, costs and size come down.

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SUPPLIERS GUIDE: COMPANIES OFFERING PRODUCTS FOR PCR, TARGET ENRICHMENT AND NEXT-GENERATION SEQUENCING ANALYSIS

Company	Web address
454 Life Sciences, a Roche company	http://www.454.com/
Affymetrix	http://www.affymetrix.com/
Agilent Technologies	http://www.agilent.com/
Ambry Genetics	http://www.ambrygen.com/
Applied Biosystems (part of Life Technologies)	http://www.appliedbiosystems.com/
Biosearch Technologies	http://www.biosearchtech.com/
Bioteam	http://www.bioteam.net/
Complete Genomics	http://www.completegenomics.com/
DNAStar	http://www.dnastar.com/
Epicentre Biotechnologies	http://www.epibio.com/
Eppendorf	http://www.eppendorf.com/
EurekaGenomics	http://www.eurekagenomics.com/
Eurofins MWG Operon	http://www.eurofinsdna.com/
febit	http://www.febit.com/
FlexGen	http://www.flexgen.nl/
Fluidigm	http://www.fluidigm.com/
Genomatix	http://www.genomatix.de/
GenomeQuest	http://www.genomequest.com/
GenScript	http://www.genscript.com/
Geospiza	http://www.geospiza.com/
Helicos Biosciences	http://www.helicosbio.com/
Illumina	http://www.illumina.com/
Intelligent Bio-Systems	http://www.intelligentbiosystems.com/
Invitrogen	http://www.invitrogen.com/
LingVitae	http://www.lingvitae.com/
OGT	http://www.ogt.co.uk/
Olink Bioscience	http://www.olink.com/
Open Genomics (part of Agilent)	http://www.opengenomics.com/
Oxford Nanopore Technologies	http://www.nanoporetech.com/
Partek	http://www.partek.com/
Qiagen	http://www.qiagen.com/
R&D Systems	http://www.rndsystems.com/
RainDance Technologies	http://www.raindancetechnologies.com/
Roche NimbleGen	http://www.nimblegen.com/
Rubicon Genomics	http://www.rubicongenomics.com/
Sigma-Aldrich	http://www.sigmaaldrich.com/
ThermoFisher Scientific	http://www.thermofisher.com/
USB (sells Affymetrix products)	http://www.usbweb.com/