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June 14, 2011

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By Monica Heger

This article was originally published June 13.

Combining the throughput of pyrosequencing with the sensitivity of fluorescent detection, researchers at Harvard University have designed a new sequencing technology that they believe will eventually enable fast, high-throughput, cheap sequencing with long read lengths.

Dubbed fluorogenic pyrosequencing, the technique is "pyrosequencing-like," but relies on fluorescence-based detection instead of the luminescence and electrochemical detection methods used in other pyrosequencing methods.

Publishing a proof of principle this week in *Nature Methods*, senior author Sunney Xie told *In Sequence* that the technology is "highly scalable and versatile."

Xie added that the team is "exploring our options" for commercialization but would not comment further.

The method is based on pyrosequencing, used by both Roche's 454 GS systems and the Ion Torrent PGM. While those methods use luminescence and electrochemical detection, respectively, the Harvard method takes a page from short-read sequencing platforms and incorporates fluorophores into the detection process, "combining benefits of both classes of clonal sequencers," according to the authors of the paper.

The team loads primed DNA templates into a microfluidic device, and then adds non-fluorescent, terminal phosphate-labeled fluorogenic nucleotides, or TPLFNs. DNA polymerase triggers primer extension and phosphatase digests a non-fluorescent intermediate, which then generates fluorophores contained within the microfluidic wells. The fluorophore is detected by a charge-coupled device camera.

Within the microfluidic device, individual DNA templates are isolated into microreactors. Each contains about 5,000 copies of a primed DNA template, and the reactor is kept cool until the DNA template, polymerase, phosphatase, and one of the four TPLFNs are all loaded. Then the temperature is raised to start the sequencing reaction.

To test the methodology, the team designed three random DNA templates. In the first template, the method was error free to 36 bases, with one error in the 42-base read. In the second 41-base template, it was error free the entire length of the read, and in the third, the method was error-free only to about 30 bases, and had six errors in the entire 40-base read.

An analysis of the performance over all three reads found that the system had a single-read raw accuracy of at least 99 percent to a read length of 30 bases. Beyond 30 bases, the accuracy decreased due to signal decay and dephasing.

"Despite having similar chemistry, fluorogenic pyrosequencing has the potential for much lower reagent costs than conventional pyrosequencing because of the relatively small DNA copy number (thousands versus ~10 million for pyrosequencing) and feature size required in fluorescence-based approaches," the authors wrote. "At the same time, TPLFN chemistry is considerably simpler than that used in commercial fluorescence-based platforms," they said.

They added that the signal decay they observed could be due to DNA loss resulting from misincorporation, primer-template melting, digestion by enzymatic impurities, or DNA dissociation. The dephasing, meantime, could be the result of incomplete extension for a subpopulation of DNA templates, or "carry-forward," in which incomplete washing causes some DNA templates to advance ahead of others.

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"Commercial sequencing platforms use computer algorithms to model and correct for dephasing in combination with sophisticated image processing, leading to increases in read length," they note. "We expect these improvements to our sequencing chemistry, wash-cycle efficiency, and data analysis to yield increases in read length and accuracy," though they did not provide estimates for the read length or accuracy they expect to reach in future versions of the system.

'A Lot of Head Room'

The Harvard researchers are developing the technology with a three-year, $2 million grant under the National Human Genome Research Institute's "$1,000 Genome" program that was awarded in 2009 (*IS 10/13/2009*).

Tim Harris, director of the applied physics and instrumentation group at the Howard Hughes Medical Institute and former director of research at Helicos BioSciences, said that he was impressed by how far the team has come in such a short amount of time.

They've been working on the method for "a couple of years, and they already have a really amazing performance level," he said. "That says to me that there's probably a lot of head room here. They could probably ramp the performance up a lot, both in terms of read length and error rate."

He noted, however, that the method will still have the same problems with sequencing through homopolymer regions that other pyrosequencing methods, like 454, have.

On the other hand, a key advantage of the fluorogenic method is that it has the potential to lower the cost of pyrosequencing. The microreactors would be less than $1 each and the reagents would be relatively inexpensive because the method uses only one-color fluorescence detection.

Harris said that commercializing the method could pose some hurdles, though, because there is "a fair bit of overlap" with other commercial pyrosequencing methods and within the phosphate-labeled nucleotide space.

It may be "difficult to bring the technology to the market independently of those that have already captured some of the IP that they overlap with," he said.

"Low-cost sequencing is a crowded space," he added, noting that Xie and his team would have to identify a specific niche. "You have to convince your backers that not only will you be able to get IP that won't be sued, but you have to convince them that you can find a space in the market with significant profit potential."

He said that because the method is intrinsically inexpensive, a good niche would likely be in clinical applications.

Xie added that he and his coauthors — Peter Sims, William Greenleaf, and Haifeng Duan — are now trying to optimize the method for single-cell sequencing, which he thought would be particularly useful for cancer research. Additionally, he said he is interested in coupling the sequencing method with a microfluidics-based sample prep method to simplify and miniaturize the sample prep process.

"We're very excited about what we can do with it," he said.

Xie did not disclose a timeline for the next steps of development, but said that his lab is exploring its options, including forming commercial partnerships and licensing the technology.

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