DNA assembly: simple and super-sized

By Michael J. Haas, Senior Writer

Researchers at the **J. Craig Venter Institute** have developed an *in vitro* method for assembling DNA fragments into molecules several hundred kilobases long in a quick, one-step reaction.¹ The method should make the construction of DNA molecules and gene clusters for biosynthetic applications simpler and easier than it is with existing approaches to DNA synthesis.

Biosynthesis usually requires assembling large recombinant genes or entire gene clusters for expression in microorganisms such as yeast or bacteria. But the assembly of such large molecules is difficult to accomplish with traditional methods such as restriction cloning or specialized types of PCR that can only synthesize DNA molecules in a laborious step-wise fashion.

Last year a team at the J. Craig Venter Institute (JCVI) partially bypassed the size problem by assembling a 592 kb *Mycoplasma genitalium* genome in the yeast *Saccharomyces cerevisiae*.^{2,3} But the yields of desired molecules from engineered yeast systems were lower than those from *Escherichia coli* systems because yeast grows more slowly than bacteria.

In the current paper in *Nature Methods*, the JCVI team has taken microorganisms out of the picture entirely by developing a way to assemble large DNA molecules in a simple, one-step chemical reaction.¹ The team was led by Hamilton Smith, scientific director of synthetic biology and bioenergy at the JCVI.

The *in vitro* method could serve as a stand-alone technique for assembling DNA for small-scale applications, or the assembled DNA could be expressed in microorganisms for biosynthetic applications, according to Daniel Gibson, assistant professor of synthetic biology and bioenergy at the JCVI. Gibson was lead author on the *Nature Methods* article and the two previous papers describing the *M. genitalium* genome synthesis.

Smith's team incubated DNA fragments at 50 °C for about 15 minutes in a reagent mixture that contained three commercially available enzymes: T5 exonuclease, a high-accuracy DNA polymerase and *Taq* DNA ligase. The reaction assembled the fragments into circular DNA products that were nonreactive to the reagent mixture, thus providing a natural stopping point for the reaction without the need for human intervention.

Gibson noted that DNA intended for expression and cloning in microorganisms is usually assembled into circular molecules.

The team used the one-step method to assemble a trio of overlapping 5 kb DNA fragments into an 8 kb bacterial artificial chromosome (BAC) and successfully expressed it in *E. coli*. They also assembled DNA fragments of the *M. genitalium* genome—each about 150 kb long—into single products of up to 900 kb in length.

“Our *in vitro* recombination methods can be used to clone joined DNA molecules in *E. coli*” for faster production and higher yields of a desired product than those seen in yeast, Gibson said.

“We believe our method to be robust, as it is in routine use by numerous investigators at the JCVI,” he added.

Size matters

Practitioners in the space said the simplicity of the method and the size of DNA molecules it could produce would facilitate the engineering of microorganisms for biosynthetic and manufacturing applications.

Yi Tang, associate professor of chemical and biomolecular engineering at the **University of California, Los Angeles**, said the method would enable rapid assembly of large gene clusters. “This would be particularly useful when large, continuous DNA sequences are needed, such as in the heterologous biosynthesis of natural products and synthetic biology applications,” he said.

In December, Tang and colleagues reported engineering *E. coli* to express a fungal polyketide synthase (PKS) for the biosynthesis of polyketides.⁴

“I could envision using this method in our future work, especially in the construction of a complex biosynthetic pathway” containing multiple enzymes, he said.

Sarah O'Connor, associate professor of chemistry at the **Massachusetts Institute of Technology**, agreed with Tang that the JCVI team's method could generate the large DNA inserts often required to construct complex pathways in a host organism, thereby overcoming the technical challenges that existing methods present.

“PCR just does not work well for fragments over 5,000 base pairs” because the likelihood of introducing errors into the sequence increases with length, she said.

Also, inserts generated by existing methods may not have appropriate sites at which restriction enzymes in the host organism could clip the plasmid into the desired linear sequences. To get around this problem “we have had to cobble together a vector with multiple gene inserts and promoters—and it was a nightmare,” said O'Connor.

“The JCVI team's approach of linking together smaller fragments of DNA in high yields provides a solution to these problems,” she said.

In March, O'Connor and Weerawat Runguphan, a graduate student in her group, reported engineering and inserting a mutant plant enzyme, strictosidine synthase, into *Catharanthus roseus* to produce new indole alkaloids.⁵

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—Yi Tang,
University of California,
Los Angeles

Like O'Connor, Elizabeth Murray, president and CEO of DNA manufacturing company **Vandalia Research Inc.**, said the JCVI team's method could produce long DNA sequences with greater accuracy and efficiency than existing methods, such as synthesis and assembly of oligonucleotides.

"A problem with assembling genes from oligonucleotide synthesis is that the chemical synthesis is error prone"—a fact that makes it too costly for generating a synthetic, large BAC as the JCVI team did in their *Nature Methods* study, she said.

Murray also agreed with O'Connor that the method bypasses the problems associated with commonly used Type IIS restriction enzymes, which can only cut DNA at specific sites and do not always cut a strand to completion.

"If your sequence lacks a specific restriction site, you can synthesize a linker complex that would generate a specific site" to be cut by a particular restriction enzyme and then use the JCVI team's method to build that site into the sequence, she said.

Indeed, Gibson told *SciBX* that his team routinely incorporates *NotI* restriction sites into the designed DNA sequence.

Murray said she could envision at least two specific applications in which the JCVI team's would have an advantage over existing ones.

"This method could specifically recombine the genomes of two strains of the same bacteria to generate diversity, of individual genes or their organization within a pathway," she said. It could also be used "to create novel DNA sequences rather than just make specific sequences discovered by other means."

Murray said she was interested in trying the method in some of Vandalia's own applications. "It would have advantages for making larger clones that we are constructing for some customers, which at present require time-consuming linker construction and mapping of the DNA with restriction enzymes," she said.

Vandalia uses its PCR-based technology to manufacture gram-scale

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—Elizabeth Murray,
Vandalia Research Inc.

quantities of specific linear DNA sequences for a variety of customer applications.

"There is definitely some synergy which could come about from this [JCVI] technology, either by Vandalia creating some instrumentation or making specific vectors for its application," Murray said.

Gibson said the JCVI synthetic biology group is focused on producing a synthetic cell from the synthetic genome of *M. genitalium*,

which has the smallest genome of any known bacterium capable of independent life. "This will allow us to better understand life at the cellular level," he said. "Our *in vitro* assembly methods will be invaluable tools for accomplishing these goals."

According to Gibson, the JCVI and **Synthetic Genomics Inc.**, which funded the team's research, have filed for a patent on the one-step method reported in *Nature Methods*.

Synthetic Genomics was founded by JCVI scientists in 2005 and is developing genomics-based technologies for environmental and energy-related applications.

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