This Week's Citation Classic®

Messing J, Crea R & Seeburg P H. A system for shotgun DNA sequencing. *Nucl. Acid. Res.* 9:309-21, 1981. [Dept. Bacteriology, Univ. California, Davis, CA] **Messing J** & **Vieira J.** A new pair of M13 vectors for selecting either DNA strand of double digest restriction fragments. *Gene* 19:269-76, 1982. [Dept. Biochemistry, Univ. Minnesota, St. Paul, MN] **Yanisch-Perron C, Vieira J & Messing J.** Improved Ml3 phage cloning vectors and host strains: nucleotide sequences of the M13mp and pUC vectors. *Gene* 33:103-19, 1985. [Dept. Biochemistry, Univ. Minnesota, St. Paul, MN]

DNA purification has been essential for studies in molecular biology, in particular for DNA sequencing, probing, and mutagenesis. By amplifying DNA in *E. coli,* by either the M13mp or pUC derived systems, physical separation techniques became redundant. Creating cloning sites by site-directed mutagenesis has led to the construction of cloning vectors with new marker systems and multiple cloning sites (MCS) to enhance recombinant DNA techniques and to facilitate DNA sequencing with universal primers. (The *SCI*® indicates that these three papers have been cited in more than 2, 180, 2, 455, and 5,000 publications, respectively.]

From "Too Trivial" to a Classic

Joachim Messing
Waksman Institute
State University of New Jersey
Rutgers
P.O. Box 759
Piscataway, NJ 08855-0759

Although our 1981 paper has been cited frequently, it was first rejected for publication in the *Proceedings of the National Academy of Sciences* because the reviewers regarded the work as too trivial. It was subsequently published in *Nucleic Acids Research* with some delay, but it caught public attention for a number of reasons. A more detailed review of events is presented in a later paper.[1] The idea came up in 1974, when I attended one of the first meetings on the use of restriction endonucleases in molecular biology. This meeting was organized by Walter Fiers, Marc van Montagu, and Jeff Schell in Gent. It was clear that DNA sequencing would be essential for the study of any gene. Sanger's laboratory was sequencing phage ϕX174 by a primer walking method, which was limiting as a general sequencing method in two respects. First, it was cumbersome to isolate a new primer for every sequencing reaction, which could be overcome by using a universal primer and a unique insertion site for DNA to be sequenced. Second, the phage was not suitable as a vector because viral genes could neither be deleted and replaced, nor could DNA be added, because it needed to fit into an icosahedron coat. Since I worked in P.H. Hofschneider's laboratory, where a different single-stranded DNA phage contained in a filamentous particle was discovered in 1963,[2] I saw an opportunity to circumvent the second problem by developing phage M13 into a cloning vehicle.

The development of M13 into a cloning vehicle was based on a shotgun cloning approach and a new reporter gene system. Since most viral sequences are essential, cloning was done not by cutting the phage DNA with an enzyme recognizing a unique site, but rather one that could cut at 10 different sites. The marker system is based on the repressible lactose operon where β-galactosidase is monitored by a non-invasive color reaction in situ. Since such a histochemical monitoring system does not require selective growth of recombinants, advantage could be taken of the plaque-forming capability of phage-infected cells. However, rather than using entire genes as markers, only the portion encoding the amino terminal region and the repressible control region was cloned; the rest was provided *in trans* by the host of the phage.[3]

A mutant phage, with a unique EcoRI cleavage site in the amino terminal region of the *lacZ* gene, was isolated by using the specificity of DNA cleavage of the restriction enzyme and the ability of transfected cells to discriminate between linear and circular molecules to select NMU mutated M13 DNA.[4] When I did clone a small piece of synthetic DNA containing a *Hin*dIII site into the unique EcoRI site, even small in-frame insertions allowed the continued use of the marker gene. This proved that multiple cloning sites could be created in the same location, which is the basis of today's polylinkers.[5]

Given a single site in a vector for cloning DNA to be sequenced by the Sanger method, it was obvious that always the same restriction fragment could be used as a primer.[6] Still, there were two problems. Sequencing gels limit the amount of information obtained from a single primer reaction. Therefore, a primer walking method was employed in the case of ϕX174. To take advan-

tage of M13 cloning, however, the emphasis needed to be shifted from the isolation of multiple primers to the isolation of multiple templates. The latter is much faster, since M13 single-stranded DNA is secreted in about 1,000 copies from an intact cell, making its purification simple and adaptable to small growth volumes of infected cultures. Therefore, the solution had to be a method of random cloning of small overlapping DNA fragments into a single site. Although DNA sequencing by M13 cloning was now possible, preparation of the primer from the plasmid was still cumbersome. When my friend, Peter H. Seeburg, mediated a temporary stay in Howard Goodman's laboratory in San Francisco in the spring of 1978 before going to Davis, we both tried to persuade Howard to commission the synthesis of a universal primer to the newly founded company of Collaborative Research. After Howard checked the price, he came back and told us it would cost half a year's postdoctoral salary and was out of the question. However, after Peter moved to the newly founded Genentech company in south San Francisco, Roberto Crea, who was responsible for the synthesis of the insulin coding region, was asked to synthesize a universal primer and an adapter to enlarge the number of sites for M13 cloning. While waiting for the synthetic DNA, I used our old method[4] to eliminate restriction sites, like *Bam* HI and *Acc* I, located in viral genes that would interfere with the polycloning site. An additional problem was posed by the NIH Recombinant Advisory Committee in the use of the common host strains for M13. A new series of *E. coli* strains with my initials that met the containment criteria of the NIH and still were suitable for the *lac* detection system were prepared.[5] After Roberto supplied the right oligonucleotides, I replaced the old cloning sites with the new ones. The use of chemical synthesis for primers and larger polycloning sites concluded this first phase of combining the Sanger method with M13 cloning, resulting in our 1981 *Classic* paper.

Making my strains available even before publication, without any restrictions, demonstrating the method's potential by sequencing cauliflower mosaic virus (8031 bp) within three months,[7] sending protocols with the strains,[8] and our Apple // computer software[9] helped spread the technique. Companies like BRL and New England Biolabs helped in promoting it. In addition, Sanger's laboratory used the method instantly, sometimes even before I was able to publish the new strains and how to use them.

Actually, the citation frequency could have been even higher if Jeff Vieira and I would not have kept publishing additional improvements. These papers replaced the 1981 paper, although a great number of their citations are also due to the pUC cloning system.[10] This became clear last year when I read about a statistic in *The Scientist®,* which found that my papers during 1981 to 1988 were cited about eight times more frequently than the *Classic* 1981 paper.[11] Another factor is typical for aged methods and materials. In recent years, most papers using the methods and the materials either leave out the reference or quote the distributors like BRL or the Maniatis book. On the other hand, a better way of looking at it is that probably almost all of the refrigerators in the 10,000 to 20,000 molecular biology laboratories around the world have a piece of my vectors or polycloning sites stored and have them ready for interesting experiments.

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3. **Messing J, Gronenborn B, Müller-Hill B & Hofschneider P H.** Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hind*II fragment of the *lac* regulatory region in the M13 replicative form in vitro. *Proc. Nat. Acad. Sci. USA* 74:3642-6, 1977. (Cited 520 times.)
4. **Gronenborn B & Messing J.** Methylation of single-stranded DNA in vitro introduces new restriction endonuclease cleavage sites. *Nature* 272:375-7. 1978. (Cited 200 times.)
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9. **Larson R & Messing J.** Apple II computer software for DNA and protein sequence data. *DNA* 2:31-5. 1983.
10. **Vieira J & Messing J.** The pUC plasmids. an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259-68. 1982. (Cited 3.720 times.)
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