

## One-Step PCR Sequencing

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A method is described to simultaneously amplify and sequence DNA using a new class of nucleotides containing boron. During the polymerase chain reaction, boron-modified nucleotides, i.e. 2'-deoxynucleoside 5'-a-[P-borano]-triphosphates,<sup>1,2</sup> are incorporated into the product DNA. The boranophosphate linkages are resistant to nucleases and thus the positions of the boranophosphates can be revealed by exonuclease digestion, thereby generating a set of fragments that defines the DNA sequence. The boranophosphate method offers an alternative to current PCR sequencing methods.

Single-sided primer extension with dideoxynucleotide chain terminators is avoided with the consequence that the sequencing fragments are derived directly from the original PCR products. Boranophosphate sequencing is demonstrated with the Pharmacia and the Applied Biosystems 373A automatic sequencers producing data that is comparable to cycle sequencing.

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### References

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## Automation of the Front End of DNA Sequencing

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The objective of this project is to continue developing more efficient tools and methods addressing the "front-end" processes of large-scale DNA sequencing. Our specific aims are high-throughput purification and mapping of cosmid inserts, controlled fragmentation of random inserts, direct selection vectors for cloning and sequencing, high-throughput M13 clone isolations, and high-throughput template purifications.

An approach to multi-cosmid purifications was developed using a cell-harvester and binding to GF/C glass fiber filter-bottom microtiter plates. This method proved inadequate because the yields were low and the DNA was eas-

ily fragmented. In the last year we have started examining the use of triplex-affinity capture (TAC) for this purpose as applied to BACs, based on our previous success with TAC purification and restriction mapping of cosmids (1,2).

We initially proposed to control random fragmentation for shotgun cloning using CviJ1 and its methyltransferase. Instead, we are now exploring automating it by scaled-down nebulization and parallel processing.

We have made a vector, M13-102 (3,4, patented), for facilitating construction and improving quality of M13 shotgun libraries. It allows direct selection of recombinants, dephosphorylation of inserts to reducing chimerics, contains universal primers for fluorescent sequencing, and a triplex sequence for easy TAC purification of linearized RF DNA. We also made a version of this vector, M13-100Z, which expressed the alpha-peptide of B-gal. Its utility is in flow cytometry based clone isolation. We continue to develop these vectors for multiple cloning sites, and insert flipping using in closing steps of large-scale sequencing projects.

We continue to develop high-throughput clone isolations by flow cytometric cell sorting. M13 or plasmid clones can theoretically be isolated at rates in microtiter wells at rates up to 2 per second using our present FacStar-Plus cytometer and collection assembly. Theoretical rates are much higher. This bypasses plating onto solid-media and any need for plaque/colony picking. We initially tried isolations after microencapsulation of cells in agarose gel microbeads, but with H/W and S/W improvements we can now distinguish positively selected transfected cells from background. Efficiency of sorting is very sensitive to detection efficiency. We continue to investigate different methods of fluorescence detection for various plasmid and M13 vector systems including fluorogenic substrates for B-gal, fluorescent-tagged antibodies to M13 or cell surface proteins, and green fluorescent protein as a reporter.

We have been developing a solid-phase filter plate method for M13 template purifications using carboxylated polystyrene beads (Bangs Labs, IN) for automating on the Hamilton 2200. It should process 96 samples in under 30 minutes and deliver 1-2 micrograms per sample for cycle-sequencing. This approach has proven superior to others we have tried with respect to amenability to automation (5,6).

**Ancillary projects.** We reported a method for direct fluorescence analysis of genetic polymorphisms using oligonucleotide arrays on glass supports (7), which spun off other projects including (a) enhanced discrimination by artificial mismatch hybridization (8), restriction hybridization ordering of shotgun clones, and restriction site indexing-PCR (RSI-PCR) (9, patent applied for). RSI-PCR is an alternative strategy to extra-long PCR which has application in large gap filling (>45kb) differential