982–987 Nucleic Acids Research, 1995, Vol. 23, No. 6

High-throughput DNA synthesis in a multichannel format

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Received November 21, 1994; Revised and Accepted January 24, 1995

ABSTRACT

We describe an approach to high-throughput parallel DNA synthesis in which a multiwell format is used. The reactions are carried out in open wells using an argon ambient atmosphere to prevent reagent contamination. The controlled-pore glass beads which form the substrate for synthesis are held in individual wells with high-density polyethylene filter bottoms through which reagents are drawn into a vacuum manifold. The synthesis is carried out using direct reagent dispensing into the individual reaction wells. A computer controls the sequence in which reagents are dispensed and the timing of the periodic vacuum pulses required to synthesize the desired sequence. Experiments to date have demonstrated the viability of the approach for a variety of test sequences. Results obtained with HPLC analysis demonstrate coupling efficiencies as high as 99.5% under optimized conditions. Use of the oligomers for DNA sequencing templates and as PCR primers has been demonstrated in production applications. The current instrument design consists of a series of discrete reaction chambers in a 12 channel module which can be multiplexed in a 12 × *n* format where *n* can be 1–8, i.e. 96 wells. A projected time interval for 12 parallel syntheses is 2.5 h, with 96 syntheses in 3.5 h. Because of the reduced volume of reagents required in the open well format, significant cost savings are projected.

INTRODUCTION

The availability of the phosphoramidite method for the chemical synthesis of oligonucleotides (1) has accelerated the development of a variety of recombinant DNA methodologies. Examples of procedures that depend upon the availability of custom synthetic nucleic acids include: linkers to aid molecular cloning (2); primers for site-directed mutagenesis (3); primers for DNA sequencing (4). The advent of the solid-state phosphoramidite method of DNA synthesis resulted in the availability of commercial synthesizers, which lowered the cost and increased the availability of DNA synthesis reagents. The invention of the polymerase chain reaction (PCR) and its widespread application in genomic mapping and sequencing has dramatically increased the demand for custom oligonucleotide primers. As a consequence, several limitations of commercially available instruments have become apparent. The major drawback is that for most applications, the minimum quantity of a custom oligonucleotide that can be synthesized is significantly larger than that required by the specific biological protocols. Since the reagents used in critical steps in the synthesis are extremely expensive, the cost for a specific oligonucleotide remains considerable. Other limitations include the high cost of labor to operate the machines and prepare the oligonucleotides for use, the small number of channels available on each instrument and the lack of necessary communication tools to facilitate the flow of information into and out of the instrument. These limitations become even more serious in the light of the needs of the Human Genome Project, in which individual centers are carrying out procedures such as polymorphism mapping (5,6), STS content mapping (7), radiation mapping (8) and primer walking for DNA sequencing, all of which require the production of an unprecedented number of custom oligonucleotides. Factors such as reagent costs, automation and information flow become critical issues in minimizing mapping and sequencing costs.

The automated custom DNA synthesizer which we describe was designed to preclude many of these limitations. Our instrument is distinct from the typical commercial synthesizers in that it employs an open geometry for pipetting reagents directly into small, open reaction chambers. The sensitivity of critical reagents to water vapor and oxygen is ameliorated by enclosing the reaction volume in an inert atmosphere. The direct dispensing approach allows the application of standard automation hardware for reagent dispensing, permits greater flexibility in reducing reagent usage and provides a format that is readily compatible with 96-well microtiter plate automation. The relative simplicity of the control system facilitates incorporation of the synthesizer into a network of instruments in which sequences are transmitted over network connections for minimal error propagation.

Approach

The chemistry of choice for automated DNA synthesis is the solid phase β-cyanoethyl phosphoramidite method of oligonucleotide synthesis (1). This process consists of four, individual, sequential steps which together constitute one cycle. A chain of nucleotides is assembled by repeating the cycle until a sequence of desired length has been constructed. The synthesis is performed with the

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3′-terminal nucleoside of the DNA chain anchored to a solid support, usually a bead of borosilicate glass. In the first step of the cycle the support-bound nucleoside is deprotected to provide a free 5′-hydroxyl group for the attachment of the second nucleotide. An excess of the second nucleotide, protected at the 5′-hydroxyl position to prevent self-polymerization and activated at the 3′-phosphate position to facilitate condensation, is then added. This results in the formation of a support-bound dimer. A capping step terminates any monomers that did not undergo condensation. Finally, the internucleotide linkage is oxidized for stability. This cycle is repeated until synthesis of the full-length oligonucleotide is completed. Since synthesis is performed with the growing DNA chain attached to a solid support, excess reagents can be removed by washing and filtration. When synthesis is complete, the fully assembled oligomer is chemically cleaved from the support and protecting groups are removed to yield a biologically active molecule. This method is readily amenable to automation, however, the sensitivity of DNA synthesis reagents to atmospheric contamination places stringent requirements on engineering design.

The greatest threat to the purity of the reagents is the uptake of atmospheric water and oxygen, which reduces phosphoramidite equivalents (9). Concentrations as low as 200 p.p.m. water will reduce phosphoramidite equivalents by 45%, which ultimately decreases coupling efficiencies (10). Due to the cyclic nature of DNA synthesis, even minimal reductions in coupling efficiencies have a cumulative effect during synthesis and result in poor overall yields of full-length oligonucleotides. It is therefore critical to the success of DNA synthesis that a water- and oxygen-free working environment be maintained.

Commercially available DNA synthesizers accomplish this by employing closed reagent delivery systems which cycle pressurized reagents through Teflon tubing, common manifolds and into small disposable reaction capsules, wherein the synthesis takes place. This design results in excess reagent consumption due to expulsion of dead volumes and rinsing of common pathways during synthesis. In addition, the complexity of the tubing network severely limits extension of the design to accommodate the simultaneous synthesis of a large number of custom oligomers.

Our approach to automated multichannel DNA synthesis employs an open reagent delivery system enclosed in an inert argon atmosphere (Fig. 1). The system consists of positively pressurized bottles that deliver reagents through separate Teflon lines to individual solenoid valves mounted on a stationary dispensing manifold. The solenoid valves are pulsed for variable time intervals in the range of milliseconds in order to dispense microliter quantities of reagents into a 12 channel, reusable Teflon reaction chamber module located below the dispensing bar. The reaction chamber module sits inside an argon bath mounted on a one-directional linear table which moves the bath and chambers in a plane underneath the dispensing bar. A computer controls the position of the reaction chambers, the amount and order in which the reagents are dispensed into the chambers and the periodic vacuum pulses which remove waste products between reactions. This segregated reagent delivery system eliminates the need for rinsing common lines and expelling dead volumes during the synthesis. As a result, an estimated 70% reduction in both reagent consumption and hazardous waste generation is achieved relative to existing commercial DNA synthesizers. The cost reductions, which result from significantly lower reagent usage, are shown in Table 1.

Table 1. Reagent consumption and costs per cycle for a 20 and a 40 nmol synthesis performed on the LBL 12 channel synthesizer

	Quantity of product	
	40 nM	20 nM
Reagent usage (µl)		
Acetonitrile	1500	1500
Tetrazole solution	50	30
Amidite solution	25	15
Iodine solution	20	20
TCA solution	240	240
Capping solution	30	30
Cost estimates ($)		
Cost per cycle	0.09	0.07
Cost per support	0.15	0.08
Cost per 21mer	2.04	1.55
Cost per base (21mer)	0.10	0.07

The 12 channel reaction chamber module (Fig. 2) was designed to significantly reduce the plumbing complexities associated with other closed-cycle systems and to provide the potential for high-throughput syntheses. The 12 individual chambers in the custom module are fitted with disposable, high-density polyethylene filters. Controlled-pore glass beads (CPG), which are used as the solid support for DNA synthesis, sit atop the filters, while the reagents are alternately dispensed and vacuum aspirated through the chambers. The chambers are serviced by a common vacuum line controlled by a single vacuum solenoid valve, so that the individual steps in DNA synthesis are carried out in parallel inside the chambers of the module. This design allows for future multiplexing of modules in a $12 \times n$ format, where n can be 1–8, i.e. 96 wells. With the intent of future robotic handling, the chambers are positioned with 9 mm center-to-center spacings consistent with standard microtiter plate well spacing.

MATERIALS AND METHODS

Prior to automation, the feasibility of this technique was demonstrated by hand pipetting reagents into a single channel Teflon reaction chamber located inside an argon filled glove-box. Once coupling efficiencies >99% were achieved, an automated single channel prototype was built to study optimal synthesis parameters. Standard operating conditions were established and the 12 channel instrument described above was constructed as a platform for demonstrating the possibility of parallel DNA synthesis in multiples of 12; ranging from 24 to 96 custom oligomers per synthesis.

The synthesis protocol is a direct adaptation of standard β-cyanoethyl phosphoramidite chemistry. A typical 40 nmol synthesis cycle is as follows. The beads are washed three times with 110 µl of acetonitrile (ACN) and dried using an extended vacuum pulse. Detritylation is achieved using four repetitions of a 6 s exposure to 60 µl of trichloroacetic acid, followed by four

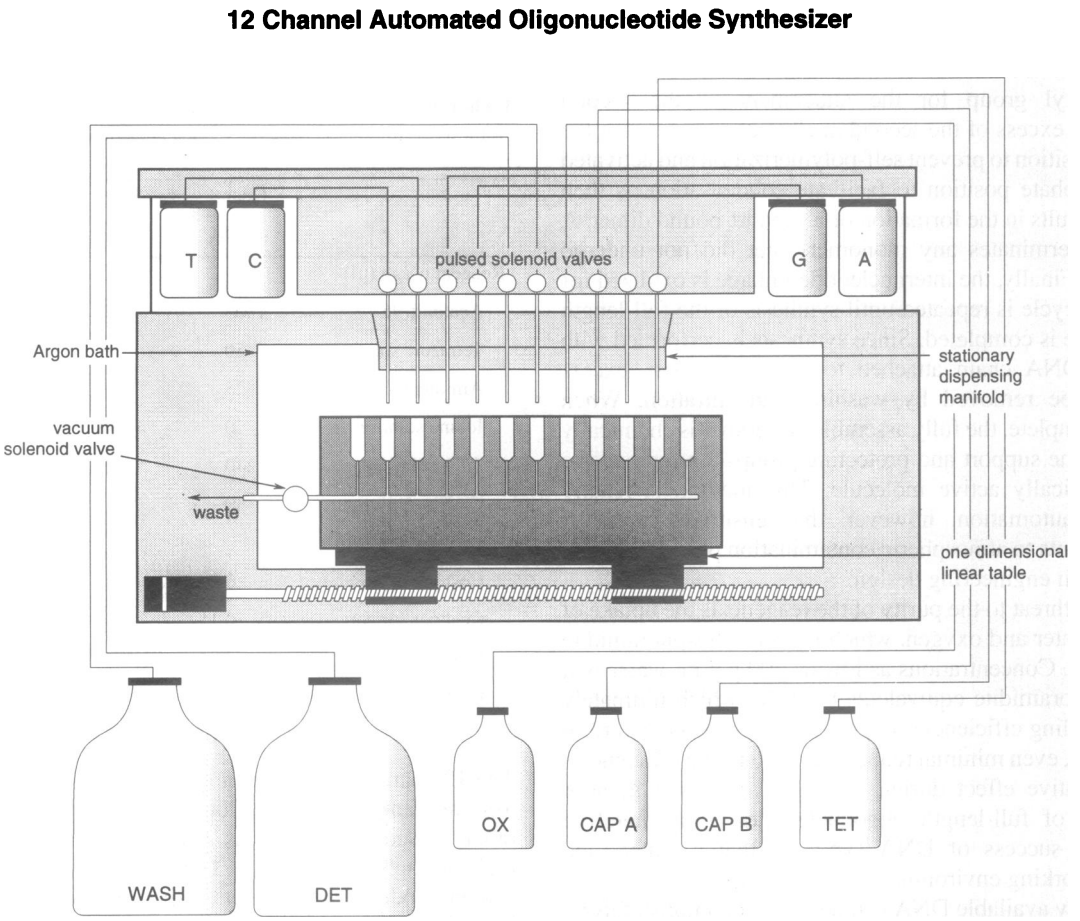

Figure 1. Schematic of the 12 channel, fully automated LBL DNA Synthesizer. Oligomers are synthesized in parallel at the rate of 12 custom 23mers/2.5 h, resulting in a production throughput of 24 custom oligomers/day.

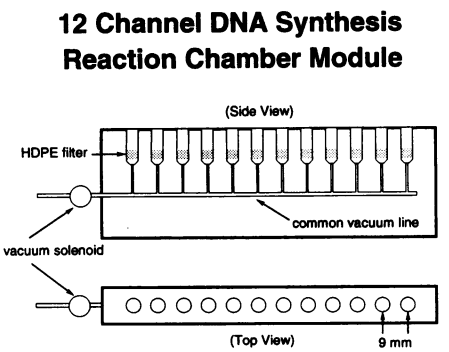

Figure 2. Schematic of the 12 channel DNA synthesis reaction chamber module used in the multichannel automated LBL DNA synthesizer. Working volume of each chamber is 230 µl.

washes with ACN. Coupling begins by dispensing 25 µl of one of the four phosphoramidite bases onto the beads, followed immediately by 50 µl of tetrazole. After 1 min, the beads are washed twice with ACN. Capping is accomplished with a 6 s exposure to 30 µl of a 1:1 mixture of the two capping solutions, followed by two washes with ACN. The final step of oxidation is carried out with a 15 s exposure to 20 µl of iodine solution. The beads are given a final series of five washes with ACN to end the cycle or to prepare for the following cycle. The reagents are dispensed in a jet that wets the beads during each step and mixes the reagents during the coupling and capping steps. The synthesis time for 12 custom 23mers is 2.5 h.

The DNA synthesis reagents used for testing and development were supplied by Applied Biosystems. During production runs, reagents supplied by Glen Research performed equally well. The acetonitrile was low water (0.001% H_2O) from Baxter chemicals (no. 015-4). This reagent was used for all washes and for dissolving the phosphoramidites. The concentration of the phosphoramidites used was 0.05 M, which provides a 30-fold molar excess of phosphoramidite to support-bound nucleoside.

Pre-synthesis operations, such as filter cutting and bead loading, are currently performed manually. The filter material is standard 1/16 in. X-tra fine (15–25 µm pore) high-density hydrophobic polyethylene sheet supplied by Porex Technologies. Filters are cut and installed in the reaction chambers using a custom 3 mm diameter cutting tool. Bulk 500 Å pore size CPG beads are loaded into the chambers using a fixture that reproducibly meters the volume of beads delivered to each chamber. A typical volume of beads for a 20 nmol synthesis is ~1.6 mm^3, which corresponds to ~0.7 mg beads. The amount of beads dispensed is reproducible to within ± 0.1 mg. Post-synthesis oligonucleotide cleaving and deprotecting are also

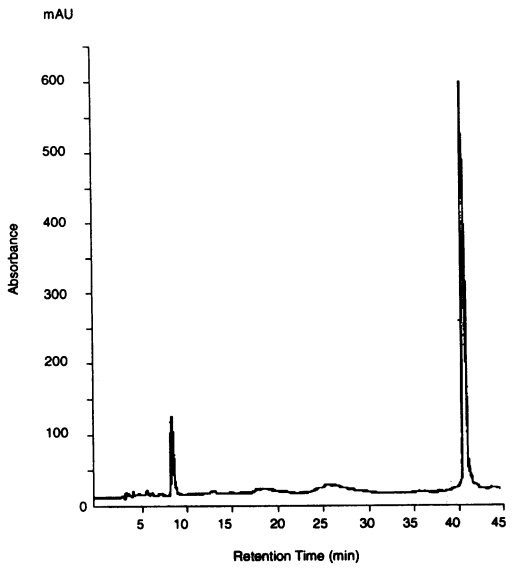

Figure 3. HPLC chromatogram of a mixed base 20mer synthesized on a fully automated single channel synthesizer. The sequence is 5′-tgc ctc gga cat taa gtg gg-3′. The peak at 8.7 min is due to the benzamide protecting group and the peak at 41 min represents the trityl-bearing product. Chromatography was performed at 1 ml/min with the following gradient: 0 min, 5% buffer B; 22 min, 12% buffer B; 40 min, 40% buffer B. Buffer A was 0.1 M triethylammonium acetate (pH 6.9). Buffer B was 100% acetonitrile. Absorption λ = 254 nm, Average coupling efficiency was 99.2%.

performed manually off-instrument using custom fixtures. This approach increases synthesizer throughput by making the synthesizer immediately available for the next production run. In order to cleave and deprotect the oligonucleotides, the reaction chamber module is removed from the synthesizer and inverted to transfer the beads into 1 ml glass vials. After the addition 0.5 ml 30% NH_4OH, the vials are capped and heated for 1 h at 85 °C. The vials are then chilled, uncapped and the oligomers are evaporated to dryness using a 12 nozzle drying manifold. The manifold introduces a gentle flow of air into each vial which dries the oligonucleotides within 20 min. The total time required to synthesize, cleave, deprotect and dry 12 custom 23mers is 4 h.

Reverse-phase high performance liquid chromatography (HPLC) of trityl-on oligomers was used to assess the quality of our synthetic oligonucleotides. Trityl-on HPLC utilizes the difference in hydrophobicity between the trityl-on and non-trityl-bearing species. Under certain solvent conditions, a reverse-phase column will easily separate the two types of oligonucleotides. The tritylated product, being more hydrophobic, will be retained longer on the column than the truncated sequences. The average coupling efficiency of an oligonucleotide can be calculated using the following formula: average coupling efficiency = $R^{(1/n-1)}$, where R is the ratio of the area of the full-length product peak to the sum of the areas of all the peaks, n is the number of nucleotides in the oligonucleotide and $n - 1$ is the number of synthesis cycles performed. All analyses were performed on a Hewlett Packard series 1050 HPLC using a Vydac 0.46 × 25 cm C_{18}, 5 μm particle size column.

RESULTS AND DISCUSSION

Early experiments involved the manual synthesis of poly(T) homopolymers to demonstrate the efficacy of DNA synthesis

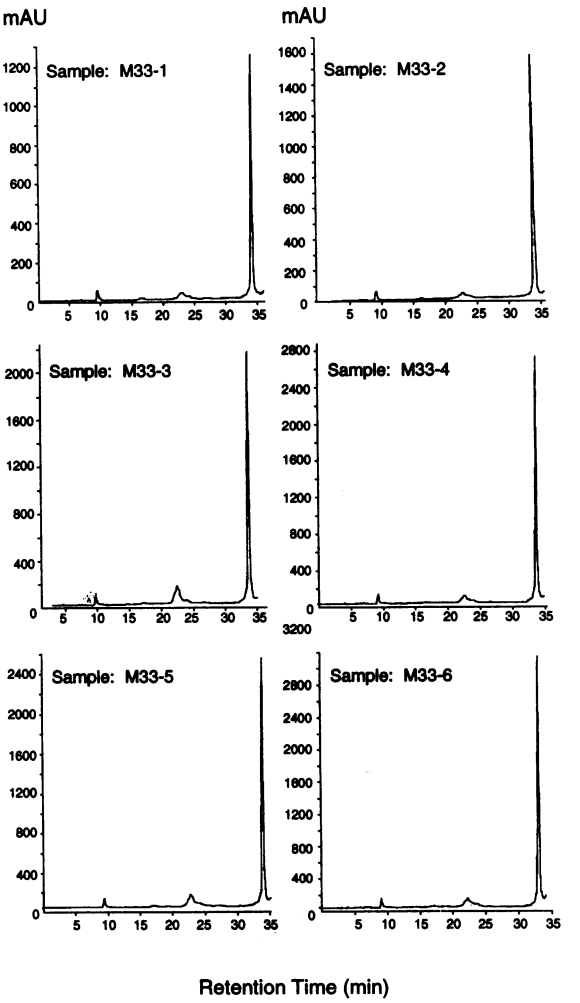

Figure 4. This figure shows the relative absorbances versus retention times on HPLC chromatograms of six out of 12 identical mixed base 20mers synthesized on the fully automated 12 channel LBL DNA synthesizer. The sequence is 5′-tgc ctc gga cat taa gtg gg-3′. Chromatography was performed at 1 ml/min with the following gradient: 0 min, 5% buffer B; 22 min, 12% buffer B; 40 min, 40% buffer B. Buffer A was 0.1 M triethylammonium acetate (pH 6.9). Buffer B was 100% acetonitrile. Absorption λ = 254 nm. Average coupling efficiency varied from 98.0 to 99.7% for the 12 reactions.

using direct dispensing of reagents into an open Teflon chamber surrounded by an argon atmosphere. Once coupling efficiencies >99% were achieved, studies involving the synthesis short A, C, T and G homopolymers were conducted using the automated single channel synthesizer. Optimal reaction times, reagent concentrations and amounts and solvent wash conditions for each of the four bases were determined. Using an optimized synthesis protocol, we were able to synthesize homopolymers having chromatograms which showed average coupling efficiencies ≥99%. To test the reliability of these conditions on all coupling combinations, a mixed base 20mer consisting of all possible binary combinations of the four bases was synthesized. A chromatogram of the oligomer (Fig. 3), when analyzed, yielded an average coupling efficiency of 99.2%. Figure 4 shows chromatograms of six out of 12 identical mixed base 20mers synthesized simultaneously on the 12 channel DNA synthesizer. A single sequence was chosen to highlight chamber-to-chamber

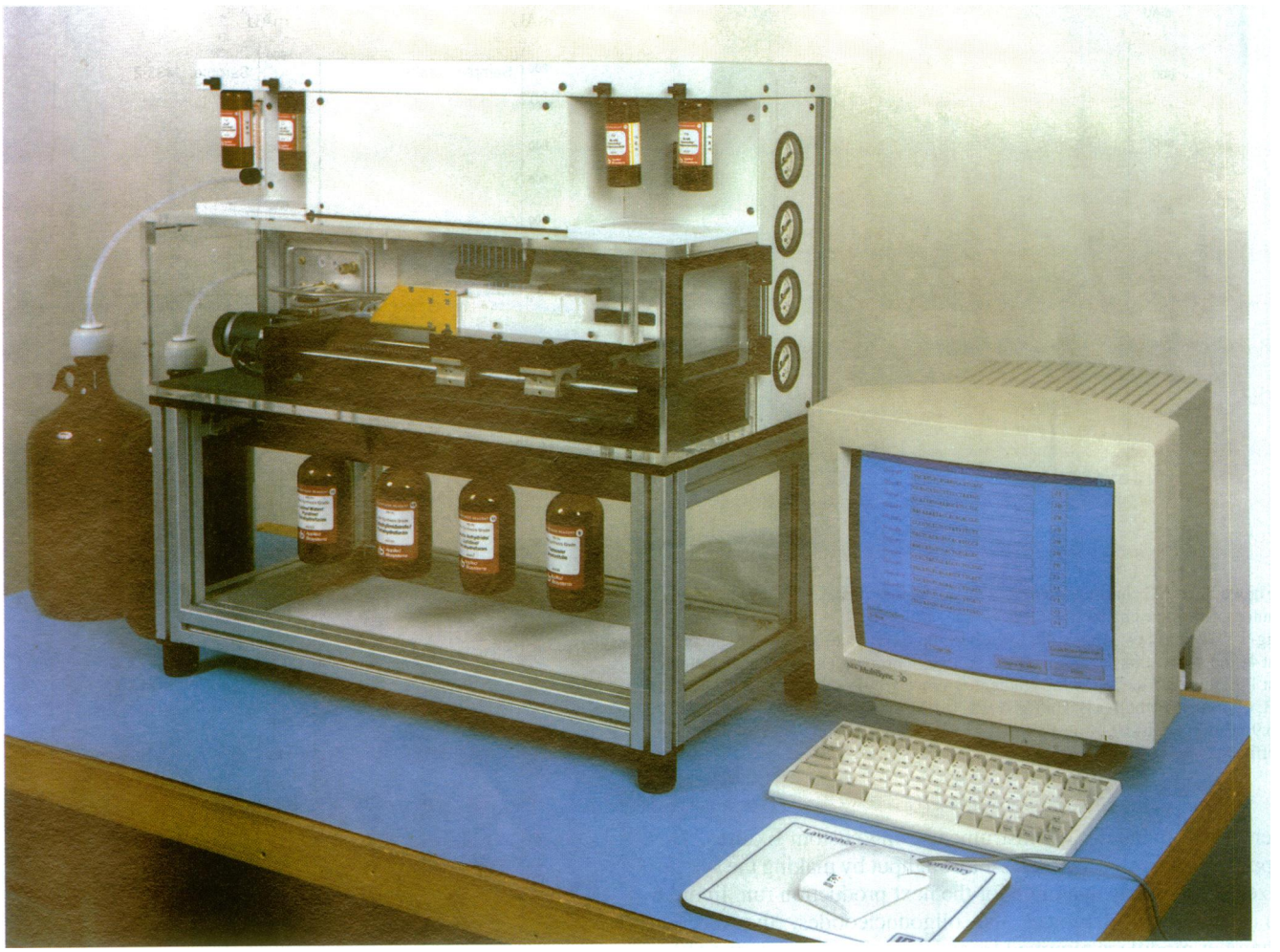

Figure 5. Photograph of the existing 12 channel instrument based on the approach described in the text. The overall dimensions of the instrument are length 73 cm, height 69 cm and depth 38 cm.

reproducibility. These and similar results demonstrate that we can routinely obtain 98.5% coupling efficiencies for 40 nmol scale syntheses for oligomers of typical primer length.

The biological viability of our synthetic oligonucleotides was verified by synthesizing custom oligomers for use as PCR and sequencing primers. Sets of identical primers were synthesized on the LBL 12 channel synthesizer and on an ABI 394 DNA synthesizer and then subsequently run in parallel PCR and sequencing reactions. In all cases, the LBL primers equaled or surpassed the performance of the primers synthesized on the ABI synthesizer, as indicated by PCR and sequencing reactions. The early prototype of the 12 channel DNA synthesizer has successfully synthesized 240 oligonucleotides used as PCR primers for biologists here at the LBL Human Genome Center. The second and most recent version of the 12 channel synthesizer (Fig. 5) is now operational and is capable of providing PCR and sequencing primers at a production rate of 120 custom oligomers per working week and is the primary source of production oligonucleotides used at this facility.

Future developments

The existing system has demonstrated the capability for high-throughput synthesis of oligomers at a greatly reduced cost. As noted earlier, the design lends itself to extension to a two-dimensional format in which $12 \times n$ oligomers can be simultaneously synthesized. Although a capability for 96-well synthesis was our original goal, our experience to date has demonstrated that there are distinct advantages to this incremental approach, since the current needs of our in-house sequencing effort seldom require more than 24 oligomers/day. As the need for additional custom oligomers increases, the production capacity can be scaled up correspondingly by expanding to the multichamber format. The proposed 96 channel DNA synthesizer will have the capacity to synthesize oligomers in multiples of 12 up to a maximum of 96. Projected synthesis times range from 1.7 h for 12 custom 21mers to 3.5 h for 96 custom 21mers.

With the reduction in reagent costs in the current design, the limiting cost factors for DNA synthesis are now associated with the labor-intensive pre-processing steps of filter cutting and bead loading and in the post-processing procedures of cleaving and deprotecting. It typically requires 10 min to prepare the chambers for synthesis and 20 min of operator time to cleave, deprotect, dry and resuspend the oligomers using the procedure described above. We are currently designing an automated version of the manual bead dispenser which will not only facilitate operator-free chamber preparation, but also allow accurate dispensing of fewer

beads for synthesis of smaller quantities of oligomers. A fully automated cleaving, deprotecting and drying station is also being constructed, which will reduce post-processing operator time to 5 min. In addition, we will begin testing Beckman's UltraFAST DNA synthesis chemistry (acetyl-protected deoxyC phosphoramidites) (11) on our 12 channel synthesizer, which, if successful, would decrease the total synthesis and processing time for 12 custom 23mers from 4 to 3 h.

Figure 5 is a photograph of the most recent version of the 12 channel system. It has been fabricated using commercially available translation-stage hardware, dispensing valves, digital I/O cards for the PC controller and interconnect plumbing. Refinements to the design are currently being implemented. Hardware modifications are focused on the design of a two-dimensional stage for adaptation to the multichannel, $n \times 12$ format. Software projects include the design of an improved user interface for more convenient operation, including a capability for transmission of sequences via a network connection. The ability for software tracking of reagent consumption is also under development.

ACKNOWLEDGEMENTS

The authors would like to acknowledge several useful discussions with Dr Robert Kaiser of the University of Washington, Seattle. This work was supported by the Director, Office of Energy Research, Office of Health and Environmental Research, Human Genome Program of the US Department of Energy under Contract no. DE-AC03-76SF00098. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the US Department of Energy to the exclusion of others that may be suitable.

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