

Synthesis and Purification in a Single Column on a High-Throughput Automated Oligonucleotide Production System

J. Baier, J. Kaufman, G. Mason, J. Wang, P. Wright and A. Andrus

Perkin-Elmer/Applied Biosystems Division, Foster City, CA, USA

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ABSTRACT

A new oligonucleotide production system, the ABI 3948, was developed to automate the entire process: sequence entry, synthesis, cleavage, deprotection, purification, quantitation, sample collection and data management. Up to 48 primer-length, high-purity oligonucleotides can be produced on the ABI 3948 in a 24-h period of unattended operation. Synthesis, cleavage and purification all occur within the unique polystyrene-based OneStep[®] column. Oligonucleotides, analyzed by MicroGel[®] capillary electrophoresis, anion-exchange HPLC, polyacrylamide gel electrophoresis and enzymatic digestion/HPLC routinely demonstrate >90% product purity. Stringent PCR and automated fluorescent sequencing and genetic analysis experiments show the benefits of combined synthesis and purification of oligonucleotides.

INTRODUCTION

The expanding scope of genome sequencing projects and a growing array of polymerase chain reaction (PCR) applications are generating greater demand for oligonucleotide primers. With annual worldwide oligonucleotide production in the millions and growing, new automated systems are required. The current generation of DNA synthesizers may be inadequate to provide the dozens of oligonucleotides that a laboratory or facility requires each day. In addition to throughput, an evolved system requires rapid production, low costs, high purity and minimal labor/expertise. To achieve high primer throughput, current synthesizers often require around-the-clock attention to remove completed syntheses and to enter and begin new sequences. Also, crude oligonu-

cleotides require further labor-intensive, post-synthesis operations, such as cleavage, deprotection, analysis, purification and quantitation.

The ABI 3948 automates the entire process of sequence and data entry, synthesis and all post-synthesis tasks (cleavage from the support, deprotection, purification, yield measurement and sample collection) to produce up to 48 primer-length oligonucleotides in 24 h. The purified oligonucleotides are ready for immediate use in a solution of measured concentration.

MATERIALS AND METHODS

The ABI 3948

The ABI 3948 (Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA, USA) (Figure 1) is a floor-standing, self-contained instrument under Macintosh[®] computer control (Apple Computer, Cupertino, CA, USA). Up to 48 color-coded OneStep[®] columns (PE/ABI) are placed on the circular turntable to begin synthesis (Figure 2). In between the porous frits is high cross-link, 1000Å pore size, non-swelling polystyrene (13). The beads are a mixture of 50–100 nmol of 3' nucleoside succinate for synthesis and underivatized polystyrene for 5' dimethoxytrityl (DMT) selective purification of the product (12). The colors correspond to the 3' nucleoside of the sequence: A (green), G (yellow), C (red) and T (blue).

A rotating turntable provides sequential processing of samples in sets of three OneStep columns. The turntable has 16 spatially indexed positions, each with three column ports.

The plumbing consists of three independently controlled and monitored liquid delivery systems. Reagents are delivered in parallel through three clamping-jaw, fluid delivery heads. At the start, the turntable rotates the first set of three columns to the synthesis fluid head. The fluid head clamps down on the columns and synthesis is initiated (Figure 3). The synthesis reactions (coupling, capping, oxidation and detritylation) are conducted in normal fashion. The 3.5-min synthesis cycle time per monomer addition is near the minimum that allows for completion of the chemical reactions. The Fast-Phoramidite™ (PE/ABI) set of 3' phosphoramidite nucleoside monomers (A^{bz}, G^{dmf}, C^{bz} and T) affords highly efficient synthesis and 1-h deprotection at 65°C in concentrated NH₄OH (2,4,15). Phosphoramidite monomers can be installed directly as dry powders and automatically diluted and mixed with dry acetonitrile, according to the AutoDilute bottle procedure.

After the first set of three oligonucleotides has completed synthesis, the turntable rotates one position, moving the first set to the cleavage fluid head and the second set of three columns to the synthesis fluid head. While synthesis begins for the second set, NH₄OH is delivered to the cleavage head, cleaving the first set from the support in 45 min. The cleaved oligonucleotides are sent through Teflon® tubing wrapped in coils around a heated cylinder. The temperature is elevated to 65°C from ambient for 60 min to remove the nucleobase and phosphate protecting groups.



Figure 1. The ABI 3948 Nucleic Acid Synthesis and Purification System.

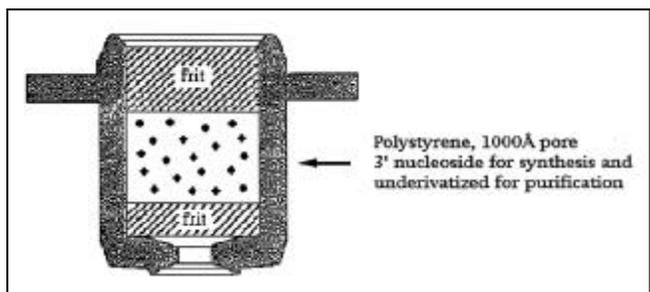


Figure 2. OneStep Synthesis and Purification Column.

After deprotection is complete, the turntable rotates again, bringing the first set to the purification fluid head, the second set to the cleavage fluid head and the third set to the synthesis fluid head. At this point, nine oligonucleotides are in production. The temperature is lowered to ambient in the deprotection coils and the first set of crude, deprotected oligonucleotides pass through the initial OneStep columns, where they are purified by an oligonucleotide purification cartridge (OPC)-type protocol (2). The crude 5' DMT oligonucleotides in NH₄OH slowly pass through the OneStep columns, where the polystyrene in the column acts as a hydrophobic affinity medium. The 5' DMT group allows only the correct sequence to be retained in the column by selectively binding to the polystyrene. Failure sequences and other impurities are washed away with water. Detritylation is conducted with 3% trifluoroacetic acid (TFA), followed by a water wash and elution of the purified oligonucleotide in 1 mL of 20% acetonitrile. The presence of acetonitrile has been shown to allow full activity in DNA sequencing and PCR. The dual role of the OneStep column allows for simple and inexpensive oligonucleotide synthesis and purification.

Purified oligonucleotides pass through a UV flow cell for quantitation by A₂₅₄ absorption before robotic-dispensing to a 48-position collection rack with microplate dimensions. The robotic dispensing tip and other common flow paths are rinsed thoroughly to prevent cross-contamination of samples.

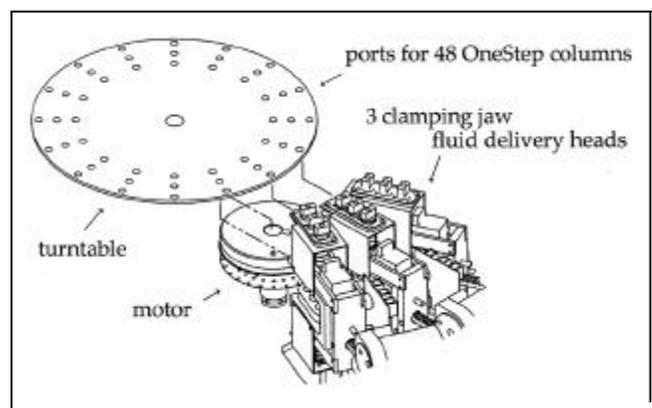


Figure 3. ABI 3948 Turntable and Jaw Assembly.

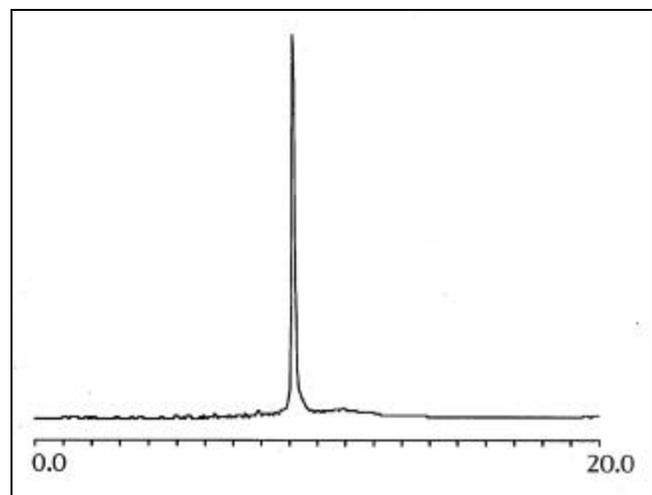


Figure 4. Anion-exchange HPLC analysis of 25-mer, 5' AGG ATT CCT GTA ACA ACA AGT CAG G 3', ABI 3948 synthesis and purification, 95% integrated product area.

The products are quantitated by real-time integration of total A₂₅₄ absorbance in the flow cell. Accuracy of ±15% is attained within the range of 0.5 to 3 optical density units (ODU). This sequential, parallel processing continues for about 24 h until all 48 samples are finished. The purified oligonucleotides are ready for immediate use by pipetting from the measured and recorded concentration. The collection format is of microplate dimensions, which provides immediate use and integration with other automated sampling devices. Typical yield is about 2 ODU (60 µg, 10 nmol [20-mer]). The automated purification protocol requires minimal additional time or expense.

Precise and economical delivery of reagents is achieved throughout the system by fluid sensors positioned at critical points in the liquid delivery lines. The fluid sensors are placed on the delivery lines downstream of the OneStep columns and detect the liquid/gas interface in the delivery lines. Feedback control shuts off flow when the minimum sufficient amount of reagent has been delivered and precisely positioned. Conventional synthesizers utilize only timed deliveries and require excess reagent to compensate for flow-rate variations. Optimized positioning of small liquid volumes, especially critical with the phosphoramidite nucleosides and tetrazole activator during coupling, is attained by a combination of timed deliveries and direct detection of reagents. Fluid sensors and the low dead-volume of the OneStep columns allow a reagent cost reduction of up to 50% compared with other DNA synthesizers. The waste is directed into a 10-L organic waste container and a 4-L halogenated waste container. Both are enclosed in a secondary containment tray in the lower cabinet. Segregation of the halogenated and nonhalogenated wastes allows for economical disposal.

ABI 3948 Software

A dedicated Macintosh computer application controls and monitors all ABI 3948 functions. Sequences in batch format can be entered from a variety of text applications, over a network and from distant locations by fax and e-mail (optional equipment required). An integrated database and a customizable order-entry template allow for complete logging, tracking, billing and record keeping with automatic documentation of each run. The graphical user interface makes set-up and operation simple with operator prompts, point-and-click com-

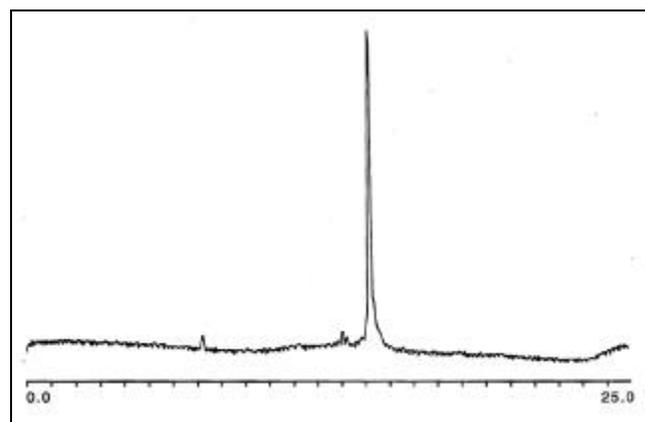


Figure 5. MicroGel™ Capillary electrophoresis of 26-mer 5' [TET] ATC CCC TCC CCC ATG CCA TCC TGC GT [TAMRA] 3'. ABI 3948 synthesis and purification. Yield: 0.7 ODU (20 µg, 2700 pmol)

mands and real-time status screens. For example, the oligonucleotides in a run are sorted according to sequence length. The program assigns the samples for fastest production and informs the operator how to place the color-coded OneStep columns on the turntable (Figure 3). In addition to the optimized protocols, for flexible programming, procedures and cycles can be edited. Password protection gives complete security.

RESULTS AND DISCUSSION

Oligonucleotide Analysis Data

Typical primer-length (approximately 20 nucleotides [nt]) oligonucleotides produced on the ABI 3948 are practically free of impurities, due to high synthesis efficiency, purification and desalting (Figure 4). Longer oligonucleotides can also be made on the ABI 3948, with 50 nt being the longest that has received extensive testing. Oligonucleotides were checked for incomplete deprotection, base modifications and correct base composition by enzymatic (snake venom phosphodiesterase/bacterial alkaline phosphatase) digestion and HPLC analysis of the resulting deoxynucleosides (3,7).

A variety of labeling and specialty reagents can be employed in the four smaller (12 mL) monomer positions. Biotin, Aminolink 2™ (5' amino; PE/ABI), Phosphalink™ (5' and 3' phosphate; PE/ABI) and fluorescent dye [6-FAM], [TET], [HEX] phosphoramidites (1,14) couple with high efficiency. The double-label 26-mer (Figure 5) was synthesized on a [TAMRA] (tetramethylrhodamine) polystyrene support for 3' labeling and with [6-FAM] (6-carboxyfluorescein) phosphoramidite for the 5' label. The 5' fluorescent dye is

quenched by energy transfer to [TAMRA] when the oligonucleotide is intact and single-stranded. The TaqMan™ (5' exonuclease assay) probe (PE/ABI) was used in real-time detection, quantitative PCR assays measuring fluorescence upon *Taq* DNA polymerase displacement and exonucleolytic cleavage during PCR (5,8,10,11). Cleavage/deprotection was conducted with tert-butylamine/water/methanol to preserve the [TAMRA] dye. The purification protocol was slightly modified to ensure adequate yield and purity for fluorescent dye-labeled oligonucleotides. To ensure there is no carryover or cross-contamination of samples from common liquid flow path elements, homopolymers (A₁₀, G₁₀, C₁₀ and T₁₀) were made in the same set of three and in previous and succeeding sets. Enzymatic digestion (3,7) and HPLC analysis showed none of the erroneous deoxynucleosides within the limits of detection (0.01%). PCR primers of a common template were similarly produced on the ABI 3948. No amplification products were detected due to spurious, contaminating primers.

Oligonucleotide Applications Data

The ABI 3948 DNA Synthesis and Purification System (PE/ABI) produces both unlabeled and fluorescent dye-labeled purified primers that can be successfully employed in a variety of sequencing and PCR applications. Experimental results are equal to, or better than, data generated using crude primers synthesized on other systems. In stringent applications such as multiplex PCR using genomic DNA, purified fluorescent-dye primers from the ABI 3948 gave equivalent results to HPLC-purified primers from other synthesizers. Other PCR applications that have successfully utilized ABI 3948 primers include rtPCR, plasmid PCR and microsatellite analyses of 2- and 4-bp repeats.

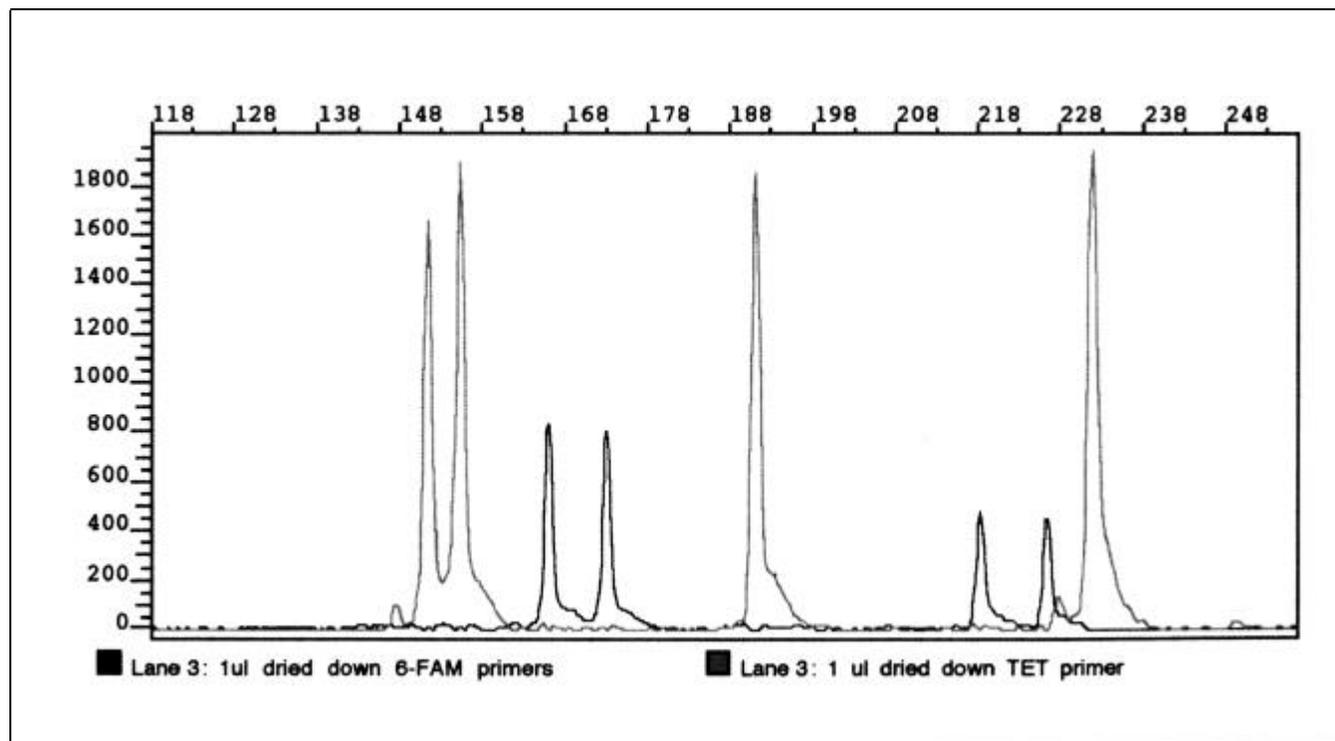


Figure 6. Results of multiplex PCR using four ABI 3948 primer pairs show heterozygosity at each locus amplified. Each primer pair consists of one fluorescent dye labeled [6-FAM] (blue) or [TET] (green) and one unlabeled primer. Electrophoresis by ABI PRISM 373 DNA Sequencer, 24-cm, well-to-read gel. Analysis by ABI PRISM 672 GeneScan Software.

3101. Applied Biosystems Division of The Perkin-Elmer Corp. is ISO 9001 certified.

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Address correspondence to Alex Andrus, Perkin-Elmer/ Applied Biosystems Division, 850 Lincoln Centre Dr., Foster City, CA 94404, USA. Internet: andrusan@perkin-elmer.com