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Simultaneous Synthesis of Partially Homologous Oligonucleotide Sequences

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ABSTRACT

A technique is described that allows the concurrent synthesis of homologous regions of separate oligonucleotides. The technique utilizes synthesis columns that are readily interconverted between single-chambered and dual-chambered. The regions of the oligonucleotides that differ are synthesized separately with single-chamber columns, and with the single modules joined, the homologous regions synthesized simultaneously. After synthesis, the chambers are opened separately, the solid-phase-bound oligonucleotides are placed in deprotection vials and treated as normal. Compared with standard syntheses, no decrease in yield or oligonucleotide quality was observed. This technique can result in significant savings in time and reagent costs when synthesizing a series of homologous oligonucleotides. This technique could be extended to the simultaneous synthesis of more than two oligonucleotides, possibly up to four or five oligonucleotides in an appropriate multi-chambered column.

INTRODUCTION

The synthesis of oligonucleotides by

the phosphoramidite method (5) is now a well-established and accepted technique for the synthesis of both DNA and RNA oligonucleotides. Oligonucleotides are synthesized from the 3' end, beginning with a solid-phase-bound protected nucleoside and the oligonucleotide built up sequentially by addition of protected nucleoside phosphoramidites. In our laboratory, we occasionally have the need to synthesize a number of partially homologous oligonucleotides, for example, a series of primers with identical tag sequences at one end or a series of RNA oligonucleotides incrementing in length for ribozyme cleavage studies (8). It has been possible to synthesize oligonucleotides with identical 3' ends and differing 5' ends simply by interrupting the synthesis at the point where the sequences diverge, dividing the solid-phase-bound oligonucleotides into two or three new columns and continuing each synthesis separately. Clearly, this approach is not applicable to oligonucleotides that differ at their 3' ends, unless the end user required both sequences in the same solution, as in a polymerase chain reaction (PCR). Here, we demonstrate how a simple column with two reaction chambers allows the simultaneous synthesis of homologous regions of two oligonucleotides, where the regions of homology may reside in any part of the oligonucleotides.

MATERIALS AND METHODS

Construction of Column

The standard snap-type column as supplied pre-packed by PE Applied Biosystems (Foster City, CA, USA) has 5 separate components; shown in exploded view in Figure 1. The reaction chamber is formed within the central connector enclosed at each end by the frits. Within the end caps are female luer fittings for the attachment of the delivery lines. The dual-chambered column (DCC), constructed using only components from the standard column, is shown in Figure 1. Three standard disposable columns are required to make the DCC. First, a standard frit (f-6.6) is trimmed from the standard 6.6-

mm-diameter to approximately 5.8 mm with a sharp scalpel to create f-5.8, which fits snugly inside the central chamber (connector). Second, the collars are removed from two end caps to expose the luer fittings. These luer fittings fit securely inside the connector and are pushed tight up against the f-5.8 frit. Standard frits, f-6.6, are placed into each end cap and locked in place with connectors as shown. Luer fittings are removed from two more end caps and inserted in the correct orientation into the two terminal connectors to allow attachment of the delivery lines. Constructed in this manner, the DCC has two reaction chambers connected in series with volumes of approximately 100 μL . This compares with the chamber volume of the standard column of approximately 250 μL . The DCC is converted into the single-chamber configuration (SCC) quite simply by removing one of the modified end cap assemblies, leaving the central frit, f-5.8, in place and fixing it in position and providing a connection point by placing a luer insert into the connector.

Oligonucleotide Synthesis

Oligonucleotides were synthesized using the standard 0.2- μM -scale synthesis cycle using PCR-Mate™ DNA

Synthesizer-Model 391 (PE Applied Biosystems). DNA phosphoramidite monomers and all other ancillary reagents were supplied by PE Applied Biosystems. Oligonucleotides 15A (5'-AGCCTTAAGCCTCTG-3') and 20A (5'-AGCCTTAAGCCTCTGAAGGT-3') were synthesized separately on standard 0.2- μM -scale solid-phase controlled-pore glass (CPG) columns. Oligonucleotides 15B and 20B were synthesized as follows: 10 mg of thymidine-modified CPG (0.37 μM thymidine) were placed in a column in the SCC, and the sequence 5'-GAAGGT-3' was synthesized using the 0.2- μM -scale synthesis cycle. Nine micrograms of deoxyguanosine-modified CPG (0.29 μM deoxyguanosine) were added to another chamber, the DCC was assembled and the sequence 5'-AGCCTTAAGCCTCTG-3' was synthesized in both chambers using the same synthesis cycle. Following synthesis, the contents of each chamber were dried with a flow of nitrogen through the DCC for a few minutes, then the contents of each chamber were separately decanted into glass vials and deprotected in 2 mL of aqueous ammonia (33%) at 55°C for 8 h. The ammoniac solutions were dried by vacuum-centrifugation, dissolved in 200 μL H₂O, ethanol-precipitated, washed with 80% ethanol, dried and redissolved in 200 μL H₂O.

Figure 1. Schematic diagram of standard PE Applied Biosystems synthesis column (SC) in exploded perspective and the DCC in exploded perspective on the left and in cross-section on the right.

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Analysis

Ten-microliter samples of the oligonucleotides were diluted to 1200 μL , and UV spectra were measured. The concentrations were determined using the extinction coefficients at 260 nm of $1.53 \times 10^5 \text{ L/mol/cm}$ and $2.16 \times 10^5 \text{ L/mol/cm}$ for the 15-mers and 20-mers, respectively (13).

Samples of each oligonucleotide (0.3 optical density units) were diluted to about 6 μL with 95% formamide loading dye and electrophoresed in a 15% polyacrylamide gel containing 7 M urea. The oligonucleotides were visualized by UV shadowing (7); no significant difference was observed between the pairs of 15-mers and 20-mers (data not shown).

Capillary electrophoresis were kindly performed by Dr. Geoffrey Burge of Beckman Instruments (Gladesville, NSW, Australia) using a P/ACE™ 2100 with UV detector fitted with an eCAP™ DNA capillary (Beckman Instruments). The voltage differential was 14 kV, injection was electrophoretic and Tris-borate-urea buffer (100 mM Tris-borate, pH 8.3, 7 M urea) was used.

RESULTS AND DISCUSSION

The development of oligonucleotide synthesis in the last decade has concentrated on two main areas. That is, (i) the synthesis of large amounts of oligonucleotides and derivatives for use in therapeutic applications (1,2,12,15) and (ii) the synthesis of multiple oligonucleotides in parallel synthesizers (3,9). For small laboratories with relatively small synthesis capacities, it can be a source of irritation to have to independently synthesize a number of essentially homologous oligonucleotides. In response, we have developed methods to allow the simultaneous synthesis of those homologous portions using a single-column synthesizer.

Oligonucleotides that differ only at their 5' ends are simply synthesized as a batch in a single column up to their point of divergence, then the dried solid phase is redistributed to new columns and the different ends are completed. However, by separating the synthesis column into two compartments, homol-

ogous regions can be concurrently synthesized irrespective of where they occur in the sequences. The design of the DCC allows the user to quickly convert between single-chamber and dual-chamber mode, synthesizing regions of different sequence separately then combining the chambers to simultaneously synthesize homologous regions.

The DCC described here is prepared simply using the PE Applied Biosystems snap columns as described in Materials and Methods. Each chamber has a volume of approximately 100 μL , and the total column volume is similar to the standard column, so that no modification of the standard synthesis cycle is required. The integrity of the chambers is maintained by the tightness of the fit of the luer fittings inside the connector. The DCCs constructed in this laboratory have been pressure tested. All were able to withstand a pressure differential of at least 1.5 atmospheres (atm) without loss of integrity of the reaction chambers. In comparison, the pressure regulator that delivers reagents to the column is typically set at around 4 psi, which is only about 0.25 atm.

The criteria to judge the success of

an oligonucleotide synthesis is the quality of the product. The quality of the syntheses described here have been evaluated by polyacrylamide gel electrophoresis (PAGE) and capillary electrophoresis (CE). PAGE analysis (not shown) of the two sets of oligonucleotides produced no discernible differences. Analysis by CE (Figure 2) showed minor differences in the amount of shorter, failure sequences; although, in this example, the oligonucleotides 15B and 20B synthesized simultaneously contained a lower proportion of failure sequences than the control oligonucleotides 15A and 20A.

The technique should be able to be expanded to the simultaneous synthesis of more than two oligonucleotides. It would require the construction of a more sophisticated multi-chambered column in which each of individual reaction chambers could be added to or removed from the reagent flow path at appropriate times. Clearly there would be limits to the number of reaction chambers that could be connected in such a series and still have efficient syntheses occur in each chamber. We expect that the limits would be determined

Figure 2. Staggered capillary electrophoretograms of the oligonucleotides 15A, 15B, 20A and 20B.

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by the resistance to the reagent flow and the total volume of the flow path.

A number of related strategies have been described using a variety of synthesis chemistries. Derivatized paper discs, which were manually moved between columns, have been used with both the phosphite triester and H-phosphonate chemistries (6,10,11). A method using commonly available derivatized CPG solid phase has been described for H-phosphonate chemistry in which the solid phase was contained within cartridges that were manually sorted and rearranged after each coupling step as required (14). Another strategy using phosphoramidite chemistry and CPG has been described in which the solid phase is contained within porous Teflon® wafers, which are shuffled between four columns, each of which is dedicated to coupling a specific nucleotide (4). The advantage of the strategy described here is that,

unlike all of the methods described above, only commonly available reagents and components are used. Therefore anybody with access to a single-column instrument and PE Applied Biosystems synthesis columns could easily apply this technique.

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