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Tandem oligonucleotide synthesis using linker phosphoramidites

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ABSTRACT

Multiple oligonucleotides of the same or different sequence, linked end-to-end in tandem can be synthesized in a single automated synthesis. A linker phosphoramidite [R. T. Pon and S. Yu (2004) *Nucleic Acids Res.*, 32, 623–631] is added to the 5′-terminal OH end of a support-bound oligonucleotide to introduce a cleavable linkage (succinic acid plus sulfonyldiethanol) and the 3′-terminal base of the new sequence. Conventional phosphoramidites are then used for the rest of the sequence. After synthesis, treatment with ammonium hydroxide releases the oligonucleotides from the support and cleaves the linkages between each sequence. Mixtures of one oligonucleotide with both 5′- and 3′-terminal OH ends and other oligonucleotides with 5′-phosphorylated and 3′-OH ends are produced, which are deprotected and worked up as a single product. Tandem synthesis can be used to make pairs of PCR primers, sets of cooperative oligonucleotides or multiple copies of the same sequence. When tandem synthesis is used to make two self-complementary sequences, double-stranded structures spontaneously form after deprotection. Tandem synthesis of oligonucleotide chains containing up to six consecutive 20mer (120 bases total), various trinucleotide codons and primer pairs for PCR, or self-complementary strands for *in situ* formation of double-stranded DNA fragments has been demonstrated.

INTRODUCTION

Tandem oligonucleotide synthesis is a term we introduced (1,2) to describe the synthesis of multiple oligonucleotides joined, end-to-end, through a dicarboxylic acid linker arm (Figure 1). Tandem synthesis allows more than one oligonucleotide sequence or more than one copy of a single sequence to be produced in a single automated synthesis. Upon completion of the synthesis, the oligonucleotides are released from each other by the usual cleavage and base deprotection steps.

Tandem oligonucleotide synthesis is useful because many applications require the simultaneous use of two or more oligonucleotides at a time. For example, the synthesis of double-stranded synthetic genes requires two or more self-complementary sequences to be annealed together. The widely used technique of PCR always requires two primers at a time and multiplexed PCRs (3–5) use numerous primer pairs in a single tube. It is also possible to use either pairs of forward and reverse primers (6,7), duplex primers (8,9) or strings of more than one oligonucleotide (10–12) in DNA sequencing. Sets of cooperative or helper oligonucleotides may also be used as probes (13–19) or antisense oligonucleotides (20). Recently, short synthetic double-stranded RNAs (short-interfering RNAs or siRNAs) have become very important for inducing gene silencing by RNA interference (21–23). Tandem synthesis may also be applied to the production of these double-stranded RNAs and this may help reduce the high cost and limited availability of synthetic siRNA reagents. However, our work on tandem synthesis of oligoribonucleotides will be presented in a subsequent manuscript.

When more than one oligonucleotide is going to be used, it is faster and more convenient to produce the pairs or sets of oligonucleotides as a single synthetic mixture, as long as this mixture does not require purification of the individual components. Although high-throughput DNA synthesizers can prepare 96, 384 or even 1536 syntheses in parallel using multi-well synthesis plates (24–27), each sample still needs to be configured, deprotected, desalted or purified, quantified and packaged. The final user then becomes responsible for storage of the individual oligonucleotides and the ultimate mixing of the samples. These issues may seem minor for small numbers of samples, but become more important when applied to large-scale investigations, which may use hundreds or thousands of oligonucleotide pairs or sets. In these cases, tandem synthesis can significantly reduce the post-synthesis handling steps required.

Finally, multiple copies of the same oligonucleotide can be produced in tandem to increase the amount of material

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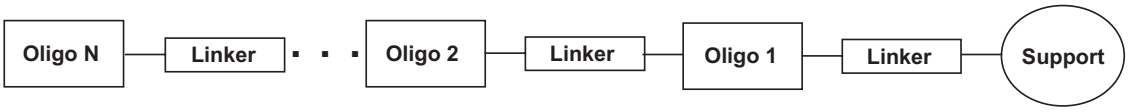


Figure 1. A string of oligonucleotides can be prepared by a single automated tandem synthesis. After synthesis, the linker to the support and the linkers joining each oligonucleotide are cleaved so that a mixture of oligonucleotides is released.

produced from a single synthesis column. This reduces both the cost of the support, which is among the most costly materials in large-scale synthesis, and the cost of labor, relative to the amount of product produced. These factors are important for the production of therapeutic oligonucleotides, which will be required in very large quantities.

A description of a method for making two oligonucleotides using a novel phosphoramidite reagent called 'Two Oligonucleotides per Synthesis' or TOPS is the most well-known early example (28) of tandem synthesis, although an earlier PCT application (29) is also available. One drawback to the TOPS reagent was the additional processing required for generating 3′-OH terminal groups, which are essential for polymerase activity, through 3-dephosphorylation of the initial product.

In our previous approach to tandem synthesis (1,2), we improved upon the tandem process by eliminating the issue of 3′-dephosphorylation. We did this by eliminating phosphoramidite coupling chemistry when adding a new oligonucleotide onto the 5′ end of an existing one. Instead, we used a procedure developed for the rapid automated derivatization of amino or hydroxyl (reusable) supports, which used a uranium coupling reagent, such as HATU, HBTU or HCTU, and 4-dimethylaminopyridine, to form ester linkages between the two oligonucleotides. Although efficient and inexpensive, this method required synthesizer modifications to include a second coupling reagent.

Another variation of multiple oligonucleotide synthesis has also been reported (30). In this different approach, the products are not linked to each other in tandem. Instead, solid-phase supports with two or more anchoring groups on the surface or mixtures of two or more supports in one column are used. Selective unblocking of the different sites on the support(s) then allows different oligonucleotides to be produced serially in a single automated run.

In this paper, we will describe how we have performed tandem oligonucleotide synthesis using 'linker' phosphoramidite reagent **4** (31,33). Linker phosphoramidites **1–3** have a cleavable linker inserted between the 3′-OH of a nucleoside and the reactive phosphoramidite group. Originally developed (32,33) to attach the first nucleoside to underivatized solid-phase supports as an alternative to diol-based 'universal' supports, these reagents yield oligonucleotides with free 3′-OH termini without any special 3′-dephosphorylation steps. Phosphoramidite **4** is a more suitable reagent for making tandem oligonucleotides because the sulfonyl diethanol group undergoes β-elimination to produce a monophosphate group, instead of an alkylated 5′-phosphodiester group (1), on the site **4** was attached to. In this paper, we show how tandem oligonucleotide synthesis with **4** can produce sets of oligonucleotides with 3′-OH and 5′-OH or 5′-phosphate ends. Such oligonucleotides can be used to prepare duplex DNAs suitable for enzymatic ligation or sets of primers suitable for PCR, sequencing or genotyping.

MATERIALS AND METHODS

General methods

Long-chain alkylamine controlled pore glass (LCAA-CPG) with either 500 or 1000 Å pores was obtained from CPG Inc. (Lincoln Park, NJ) and used either as it is, or derivatized with a nucleoside and a hydroquinone-*O*,*O*′-diacetic acid linker arm (Q-linker) (34). Either 500 or 1000 Å CPG was derivatized to ∼30–40 μmol/g for the synthesis of oligonucleotides less than ∼40–50 bases long. For longer oligonucleotides, only 1000 Å CPG was used and this was derivatized to ∼8–12 μmol/g, since we have found that reduced loadings improve long oligonucleotide synthesis. Quantitative dimethoxytrityl (DMT) analysis was performed as described previously to determine nucleoside loadings (35). Oligonucleotide synthesis was performed using ABI 394 synthesizers with eight base positions. 5-Ethylthiotetrazole (American International Chemicals, Natick, MA) was used as an activator. Nucleoside-3′-*O*-*N*,*N*-diisopropylamino-2-cyanoethyl phosphoramidite derivatives were from Transgenomic (Omaha, NE), fluorescent dye phosphoramidites were from Applied Biosystems (Foster City, CA) and linker phosphoramidites **4a–d** were synthesized as described previously (33). When terminal 5′-phosphate groups were required on the last oligonucleotide, a commercially available 5′-phosphorylating phosphoramidite (Glen Research, Sterling, VA) was used. Linker phosphoramidites **4a–d** were dissolved in anhydrous acetonitrile (0.1 M) and installed on spare base positions. No changes to conventional coupling cycles were employed, except that 2% (v/v) dichloroacetic acid (DCA) in 1,2-dichloroethane (DCE) was substituted for 5% (v/v) DCA/DCE when oligonucleotides longer than 40 bases were prepared. After synthesis, products were cleaved from the Q-linker supports by using ammonium hydroxide [2 min at room temperature (22°C)] and then deprotected by heating (16 h at 55°C). The use of Q-linker supports did not require any changes to conventional deprotection or purification protocols, other than reducing the cleavage time from 60 to 2 min (34). Oligonucleotide mass spectra were obtained by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) on a Voyager DE-STR instrument using a 3-hydroxypicolinic acid matrix. Capillary gel electrophoresis (CGE) was performed on an Agilent 3D CE instrument using PVA-coated capillaries and a polyethylene glycol matrix according to the Agilent applications note 5988-4303EN. Oligonucleotides were desalted by butanol precipitation before CGE analysis to remove low-molecular weight ions, which would interfere with the electrokinetic injection. Gene Runner v3.05 software was used to convert A_{260} amounts into nanomoles. PAGE was performed under denaturing conditions using 7 M urea and 20 or 24% polyacrylamide and an electrophoresis temperature of ∼50°C. PAGE under non-denaturing conditions was performed without urea on 20% polyacrylamide at room temperature.

Tandem synthesis of multiple 20mers (dACCTTATGTATCATACACAT)

Synthesis columns containing T derivatized, low-loaded 1000 Å CPG (~35 mg at a loading of 11 μmol/g) and the 20mer sequence was synthesized either individually (1×) as a control, or 2- (2×), 3- (3×), 4- (4×), 5- (5×) or 6-fold (6×) in tandem using phosphoramidite **4b** to begin each additional copy. The first DMT color released was collected and used to calculate the amount of initial nucleoside. After cleavage and deprotection, the crude material was quantified by using UV at 260 nm and the following A_{260} units observed: 1X, 45; 2X, 111; 3X, 125; 4X, 152; 5X, 159; and 6X, 225. The number of nanomoles of product per micromole of starting nucleoside are shown in Table S-1 (Supplementary Material). Aliquots of each crude product (0.5 A_{260} units) were analyzed by CGE to determine the amount of full-length product (Table S-1 and Figure 3). MALDI-TOF mass spectrometry (MS) was used to observe the presence of the full-length 5′-OH, $(M+H)^+$ calc. 6034.9, and 5′-phosphorylated, $(M+H)^+$ calc. 6114.9, products as follows: 1X synthesis, 6036.0; 2X synthesis, 6038.1 and 6117.7; 3X synthesis, 6035.3 and 6115.1; 4X synthesis, 6033.0 and 6112.5; 5X synthesis, 6034.7 and 6115.0; and 6X synthesis, 6033.9 and 6113.4.

Tandem synthesis of fluorescently labeled PCR primer pairs

Synthesis columns were packed with known amounts of 500 Å underivatized LCAA-CPG (~12–15 mg). An aliquot of 0.1 M solutions of **4** as well as the required dye phosphoramidite were installed on spare base positions. An additional 'fake' 3′-base was added to the end of the programmed sequence to compensate for the fact that no nucleoside was attached to the CPG and to begin the synthesis with the desired linker phosphoramidite. The sequences shown in Table 1 were synthesized. After cleavage and deprotection, the crude primer mixtures were analyzed by using CGE and MALDI-TOF MS (see Table 1 and Figure S-1). The primer mixtures were desalted on NAP-10 Sephadex columns (GE Healthcare) before use in PCR.

Tandem synthesis of double-stranded DNA fragments

The double-stranded fragments shown in Figure 4A–D were synthesized in the same way as described above for the multiple 20mer, using the appropriately derivatized low-loaded 1000 Å LCAA-CPG support (~30–35 mg). Single-stranded controls corresponding to the top strands of each duplex (but not 5′-phosphorylated) were prepared by conventional synthesis. After synthesis and deprotection, UV quantification of the crude material showed the following A_{260} unit amounts: duplex **A**, 97; duplex **B**, 77; duplex **C**, 124; and duplex **D**, 124. Both PAGE (Figure 5A) and CGE (Figure 5B) under non-denaturing conditions showed that the two self-complementary sequences in each tandem pair formed a duplex structure. MALDI-TOF MS, however, showed the presence of two single-stranded oligonucleotides $(M+H)^+$: duplex **A**, calc. 6026.9 and 7521.8, obs. 6023.5 and 7517.0; duplex **B**, calc. 7231.0 and 8787.6, obs. 7229.5 and 8785.6; duplex **C**, calc. 9046.0 and 10 682.8, obs. 9047.1 and 10 683.2; and duplex **D**, calc. 10 901 and 12 530, obs. 10 899.4 and 12 529.

RESULTS AND DISCUSSION

Tandem oligonucleotide synthesis involves the stepwise synthesis of a long, linear string of oligonucleotides from the surface of a solid-phase support (Figure 1). After synthesis, treatment with NH_4OH simultaneously cleaves all the linkages between the oligonucleotides and releases the products from the support. Starting the synthesis of a new oligonucleotide sequence on the 5′-terminal OH position of another oligonucleotide sequence is not difficult. These sites are similar to the surfaces of underivatized universal (36,37) or reusable (38) solid-phase supports, which contain hydroxyl linker arms. However, the linkage to each new oligonucleotide's starting point (the 3′-nucleoside) must be cleavable. Furthermore, if the oligonucleotides are to be used as primers, then a terminal 3′-OH group is required for polymerase extension.

In our previous tandem oligonucleotide syntheses (1,2), we used simple dicarboxylic acids, such as succinic, diglycolic or Q-linker, to join one oligonucleotide to the next in tandem. However, this required using a second coupling chemistry, which was not practical on most automated synthesizers. Therefore, we switched to phosphoramidite reagents, which we called 'linker phosphoramidites' for both single oligonucleotide syntheses on underivatized solid-phase supports (33) and for tandem synthesis (31). Our earliest linker

Table 1. Tandem synthesis of fluorescently labeled primer pairs synthesized on underivatized LCAA-CPG supports (bases in underlined italics denote use of linker phosphoramidites **4a–d**)

	5′-Dye-primer	5′-Phosphorylated-primer	Weight of CPG (mg)	Product (A_{260} units)	Calc. masses $[M+H]^+$	Observed masses $[M+H]^+$
A	FAM-GGA TAA CTC AAC CAC ACG TCT CT*G* (24mer)	AAG AAG TAC TGG TTG CCA ATC GT*G* (24mer)	13.3	56	7498 7820	7496.7 7818.8
B	TET-CAA CAG C*T*A TTT AAC AAG G*A* (20mer)	AGG CTA CAG TCC ATG GGA T*T* (20mer)	14.7	124	6238 6794	6238.4 6795.4
C	HEX-GA*G* TAG AGC TAC AAG ATA AAC TT*C* (24mer)	TAA CTA CA*G* GGT GTT AGA TGA ACT *C* (25mer)	12.3	98	7786 8139	7782.9 8136.7
D	HEX-TTG GTC *T*CT ATT CTC TGA ATA TTC *C* (25mer)	CTA ATT TAG AA*T* GAG AGA GGC TTC *T* (25mer)	13.6	68	7801 8309	7801.0 8309.5
E	HEX-AAT CAC AT*G* GCA AAT AAG TAC ATA *C* (25mer)	CCC TCC TCC AG*G* TAA ATC AG*C* (21mer)	14.1	93	6392 8396	6393.5 8400.0
F	HEX-ACA GAC A*G*A AAC TCA ATG AAA GC*A* (24mer)	CGA ATT CCA *A*AT CTG TTA ATT TGC *T* (25mer)	13.8	78	7687 8126	7688.3 8128.8

phosphoramidites **1–3** (Figure 2) were not suitable for tandem synthesis because they would have left an alkylated phosphate residue on the 5′ end of whatever oligonucleotide they were attached to (1). However, the improved linker phosphoramidite **4** (33) has the advantage of leaving 5′-monophosphate groups (Scheme 1) via the same β-elimination mechanism used by commercially available phosphorylating phosphoramidites (34). Unlike 3′-terminal phosphate groups, which must be removed for polymerase activity, the 5′-terminal monophosphate groups do not interfere with the biological applications and so no post-synthesis steps are required to remove them. Indeed, the 5′-phosphorylated oligonucleotides are more closely related to biologically produced nucleic acids than the 5′-non-phosphorylated sequences, which are usually synthesized.

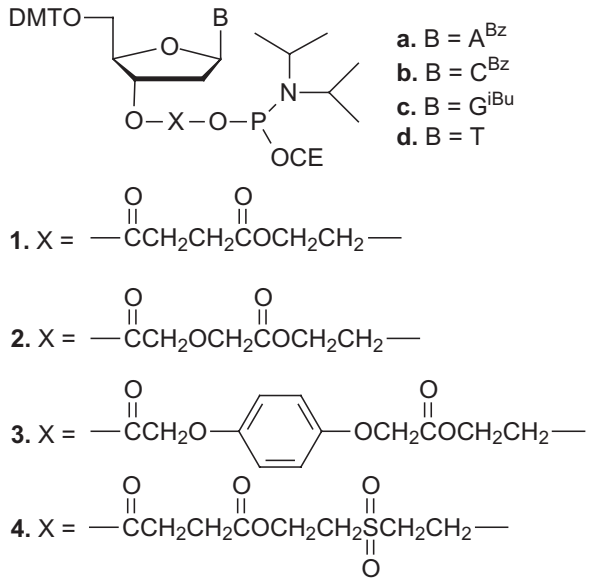
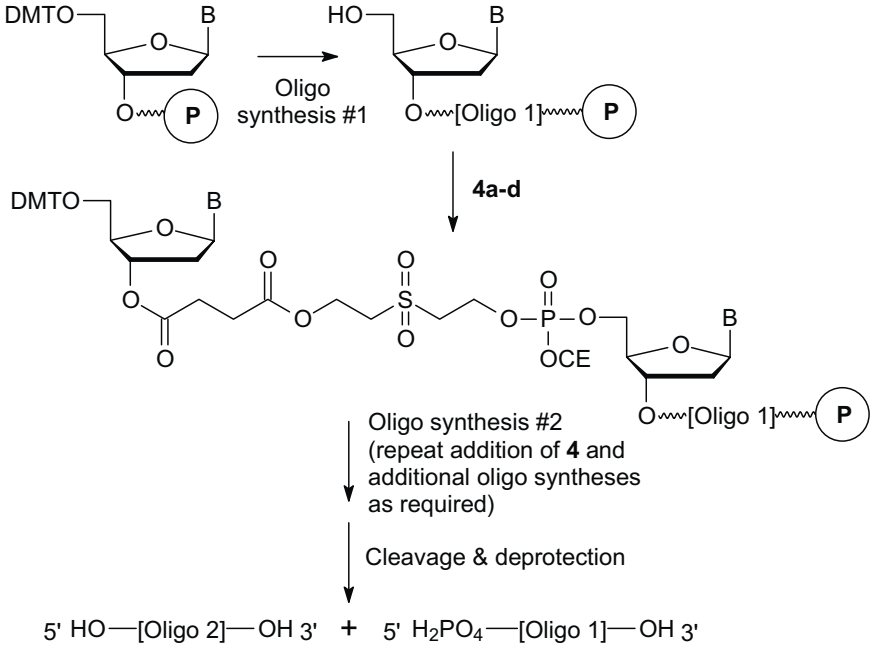


Figure 2. Structures of linker phosphoramidite reagents have a cleavable linkage between the protected nucleoside and the phosphoramidite group.

Using linker phosphoramidite **4** one can synthesize long strings of tandem oligonucleotides in a single automated synthesis. The maximum length of oligonucleotide string, which can be made is limited by the same factors that limit the length of individual oligonucleotides, namely coupling efficiency, extent of depurination or base modification, and support pore size. Although, solid-phase synthesis can produce oligonucleotides of 100 or more bases (our laboratory routinely makes oligonucleotides up to ∼140 bases in length), it is important to examine whether the quality of oligonucleotides produced in tandem is comparable to oligonucleotides produced individually.

To examine this issue, we prepared the 20mer sequence dACCTTATGTATCATACACAT individually (1×) and in tandem syntheses where the sequence was repeated 2-fold (2×, equivalent to a 40mer), 3-fold (3×, equivalent to a 60mer), 4-fold (4×, equivalent to an 80mer), 5-fold (5×, equivalent to a 100mer) and 6-fold (6×, equivalent to a 120mer). The tandem syntheses were performed using our preferred protocols for long oligonucleotide synthesis. These included the use of low loading (10 μmol/g) wide-pore (1000 Å) LCAA-CPG and a detritylation reagent with 2% instead of 5% (v/v) DCA in DCE solution. Introduction of the linker phosphoramidite was easily accomplished by using one of the four spare base positions available on the ABI 394 DNA synthesizer.

The syntheses were monitored by quantitative DMT analysis of the first and last couplings of each 20mer block in the tandem syntheses. These colors indicated that the coupling



Scheme 1. Synthesis scheme for preparing strings of oligonucleotides linked in tandem via linker phosphoramidite **4**. For simplicity, only two oligonucleotides are shown. However, the number of oligonucleotides which can be produced is only limited by the maximum number of bases in the total string which can be produced.

efficiency of the linker phosphoramidite was ~97–99% per insertion and overall, the tandem syntheses had average coupling efficiencies of >99%. However, the coupling efficiencies determined by DMT analyses tend to overestimate the actual performance. Therefore, after cleavage and deprotection, the crude products were also analyzed by CGE. No products longer than 20 bases were observed, confirming the quantitative cleavage of the linkages between the tandem oligonucleotides. Although, the CGE resolution did not resolve the 20mer products having 5′-OH and 5′-PO_4 ends (see MALDI-TOF MS results, given in the next column), the single base resolution allowed the amount of full-length product (overall yield) in each crude synthesis product to be quantitatively measured. The average coupling efficiency of each synthesis was then calculated from the amount of full-length product.

The results in Table S-1 (Supplementary Material) show the amount of crude material produced (in nmol) normalized to 1 μmol of starting nucleoside, which allowed the product amounts produced by the various tandem syntheses to be compared relative to each other and a single non-tandem synthesis. The amount of crude product produced, relative to a single synthesis, increased almost linearly for tandem syntheses of 3-, 4- and 6-fold. Relative amounts for the 2- and 5-fold syntheses were somewhat less, for reasons not clear. In general, however, because coupling efficiency is not 100%, the amount of each additional synthesis added in tandem is expected to be less than the previous synthesis.

Most importantly, the CGE analyses (Figure 3) showed little difference and the quality of all the 20mer produced was comparable. The amount of full-length product ranged from ~76 to 85% and corresponded to average coupling efficiencies of 98.6–99.1%. These results were similar to values generally obtained in routine oligonucleotide synthesis in our laboratory. Although the 3-, 4-, 5- and 6-fold results showed a slightly increased N-1 impurity relative to the individual and 2-fold syntheses, this was not enough to significantly affect their use in most biochemical applications.

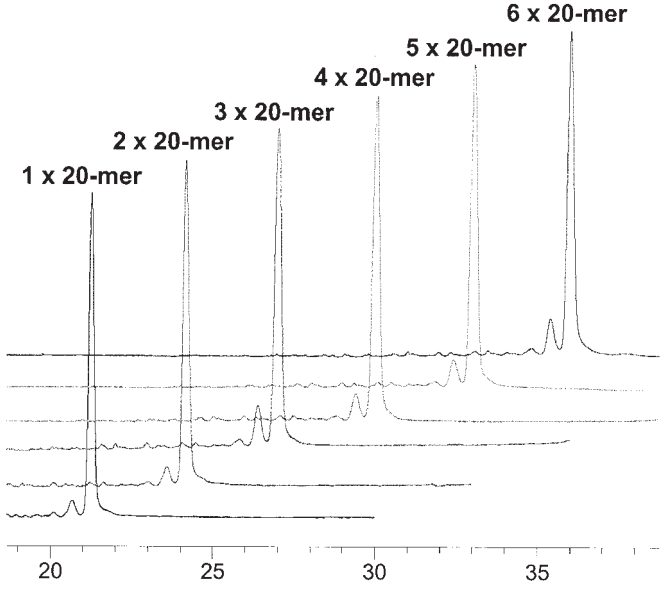


Figure 3. Results from capillary gel electrophoresis of crude 20mer produced individually by a conventional synthesis (1× 20mer) and 2-fold (2×, equivalent to a 40mer), 3-fold (3×, equivalent to a 60mer), 4-fold (4×, equivalent to an 80mer), 5-fold (5×, equivalent to a 100mer) and 6-fold (6×, equivalent to a 120mer) tandem syntheses.

In addition, each synthesis product was checked by MALDI-TOF MS to verify the sequence identity and the complete removal of all protecting and linking groups. The MS results also confirmed the expected presence of a mixture of 5′-OH ($[M+H]^+$ = 6035) and 5′-phosphorylated ($[M+H]^+$ = 6115) oligonucleotides in the tandem products, in the approximately 1:1, 1:2, 1:3, 1:4 and 1:5 ratios expected for the 2-, 3-, 4-, 5- and 6-fold tandem syntheses, respectively.

All of the above results indicated that the synthesis of strings of oligonucleotides in tandem could produce material of quality, which was useful, if not comparable with single synthesis, even when the total string length was up to 100 or 120 bases. This is certainly sufficient to produce most primer pairs, which generally have individual lengths of only 20–30 bases. Furthermore, although we demonstrated up to a 6-fold tandem synthesis, applications which require production of large amounts of oligonucleotides should only need to perform a 2-fold tandem synthesis to halve the relative cost of the support. When only two copies per synthesis were produced, we found no difference in quality.

After producing multiple copies of the same sequence, we evaluated the method for producing multiple copies of different sequences. One set of experiments produced libraries of trinucleotide codons by tandem synthesis. A tandem synthesis of pAA*T*CC*T*GG*T*TTT (where p = 5′-phosphate group introduced by a 5′-phosphorylating phosphoramidite reagent and *T* is **4d**) produced a mixture of the four phosphorylated trinucleotide codons: pAAT, pCCT, pGGT and pTTT. Electrophoresis on 24% polyacrylamide/7 M urea gels of the tandem mixture against individually prepared trinucleotides, showed three major bands consisting of pGGT, pAAT + pTTT (co-migrating) and pCCT. MALDI-TOF MS of the mixture was expected to easily confirm the presence of all four trinucleotides. Unfortunately, however, the strong tendency of oligonucleotides, especially thymidine sequences to form adducts with Na^+ and K^+ complicated the interpretation. Although pCCT ($[M+H]^+$ = 901.6), pAAT ($[M+H]^+$ = 949.6) and pGGT ($[M+H]^+$ = 981.6) were all easily observed, no $[M+H]^+$ peak for pTTT was observed. Instead, the sodium ($[M+Na]^+$ = 953.6) and potassium ($[M+K]^+$ = 969.9) adducts were found. A second tandem synthesis to prepare a library of all 20 trinucleotide codons by making pAA*A*AA*C*AC*T*AT-*C*AT*G*CA*G*CA*T*CC*G*CG*T*CT*G*GA*A*GA*C*GC*T*GG*T*GT*T*TA-*C*TC*T*TG*C*TG*G*TTC (where p = 5′-phosphate group introduced by a 5′-phosphorylating phosphoramidite reagent and *A*, *C*, *G* and *T* are **4a–d**) was also successful. However, as expected, the mixture of 20 trinucleotides was too complex to characterize completely by electrophoresis or MALDI-TOF MS.

After the tandem synthesis of the trinucleotides, we prepared several pairs of longer oligonucleotides (20mer to 25mer), which were intended for automated genotyping assays (Table 1). We also used this experiment to confirm the utility of linker phosphoramidites **4a–d** as a means of introducing the very first nucleoside onto underivatized LCAA-CPG supports. As we have noted previously, such supports represent the simplest possible 'universal' supports (33). Thus, each tandem synthesis performed two linker phosphoramidite additions,

one coupling to begin the first primer on an underivatized LCAA-CPG support, and a second coupling to begin the second (5′-dye-primer) sequence on the 5′ end of the first primer. Unlike characterization of the above trinucleotide codon libraries, it was extremely easy to distinguish between the 5′-fluorescently labeled primer and the 5′-phosphorylated primer by gel electrophoresis with UV shadowing, even when the primers were of the same length. We also used MALDI-TOF MS to confirm the presence of the two expected products from each tandem synthesis, free of all protecting and linking groups. We then analyzed the crude reaction products by CGE analysis (Figure S-1). In each case there were two dominant products. After correcting for the different extinction coefficients of each sequence (the contribution of the fluorescent dye to absorbance at 260 nm was ignored), we used the peak areas to estimate that the ratio of 5′-dye labeled primer to 5′-phosphorylated primer was no <0.9–1. Since PCR amplification protocols do not require the primers to be in precise ratios, these crude product mixtures were quite satisfactory for automated genotyping.

Next, we investigated the potential of tandem oligonucleotide synthesis to prepare double-stranded DNA fragments in a 'one-pot' synthesis. This strategy was interesting because the self-complementary oligonucleotides would spontaneously hydrogen-bond into the correct duplex structures as soon as the protecting groups were removed. We designed four pairs of double-stranded fragments, ranging in size from 44 to 76 bases (Figure 4A–D). A four base overhang was also included so that we could ligate the 44 base duplex (duplex **A**) onto the other three duplexes. Each tandem synthesis was prepared using our long oligonucleotide synthesis protocol. Single oligonucleotides corresponding to the top strand 24mer, 28mer, 34mer and 40mer shown in Figure 4A–D (non-phosphorylated) were also synthesized for comparison. Coupling yields were monitored by DMT analysis and were ~99% for both the regular phosphoramidites and the linker phosphoramidite **4d**. After synthesis, cleavage and deprotection, the crude products were analyzed by MALDI-TOF MS. In each case, the crude material showed two ions corresponding to the two separate single-stranded oligonucleotides. The MALDI-TOF MS was not expected to show ions corresponding to double-stranded DNA (39) and this was indeed the case.

The presence of double-stranded DNA in the crude material was verified by analyzing aliquots of each tandem synthesis by PAGE using both non-denaturing (Figure 5A) and denaturing

A
```
5′-pCGGCATATGGACGCGTCATGGAGA-3′                (24-mer)
         ||||||||||||||||||||                    +
     3′-TATACCTGCGCAGTACCTCT-5′                 (20-mer)
```

B
```
5′-pGCCGAAAGGTAATGTTGGAGTGACTTGC-3′            (28-mer)
         ||||||||||||||||||||||||||              +
     3′-TTTCCATTACAACCTCACTGAACG-5′              (24-mer)
```

C
```
5′-pGCCGACCGTATAGTAGGAGACGTTGGTGGGCGAT-3′      (34-mer)
         ||||||||||||||||||||||||||||||||        +
     3′-TGGCATATCATCCTCTGCAACCACCCGCTA-5′        (30-mer)
```

D
```
5′-pGCCGAGTAAGAAGTAAGATAACTACTGCACAGGTAAGGTG-3′  (40-mer)
         ||||||||||||||||||||||||||||||||||||||    +
     3′-TCATTCTTCATTCTATTGATGACGTGTCCATTCCAC-5′   (36-mer)
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Figure 4. Tandem synthesis of duplex DNA fragments (*T* is the linker phosphoramidite **4d**). (**A**) A 44 base-long synthesis of a 24/20mer duplex. (**B**) A 52 base-long synthesis of a 28/24mer duplex. (**C**) A 64 base-long synthesis of 34/30mer duplex. (**D**) A 76 base-long synthesis of a 40/36mer duplex.

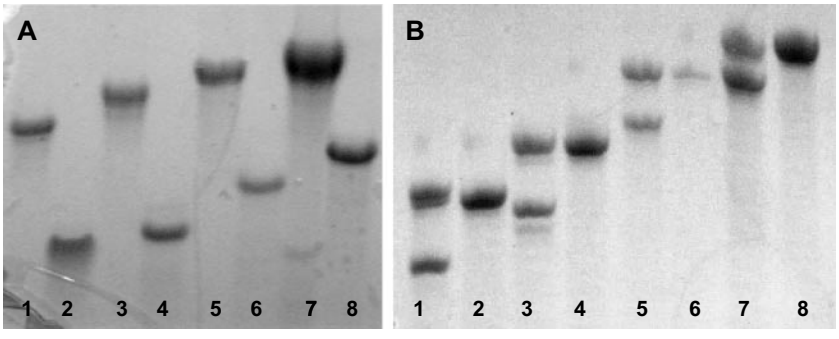

Figure 5. PAGE of double-stranded oligonucleotides (crude products) made from the tandem syntheses in Figure 4. (**A**) Non-denaturing conditions (20% polyacrylamide, no urea, room temperature). (**B**) Denaturing conditions (24% polyacrylamide, 7 M urea, 50°C). Lane 1, 24/20mer duplex **A**; lane 2, single-stranded 24mer (top strand only); lane 3, 28/24mer duplex **B**; lane 4, single-stranded 28mer (top strand only); lane 5, 34/30mer duplex **C**; lane 6, single-stranded 34mer (top strand only); lane 7, 40/36mer duplex **D**; and lane 8, single-stranded 40mer (top strand only).

(Figure 5B) conditions. Under non-denaturing conditions, the products of tandem synthesis appeared as a single band which migrated much more slowly than just the top strand by itself (synthesized separately). This marked difference in mobility, as well as the difference in ethidium bromide staining confirmed the double-stranded structure. However, when the products were electrophoresed under non-denaturing conditions (7 M urea and 50°C), there was a marked change. The tandem products now appeared as two distinct bands, with one band the same as the separately synthesized top strand. This gel experiment clearly confirmed that the two separate oligonucleotides were synthesized in each tandem synthesis.

The compatibility of the phosphorylated 5′ ends of the overhanging duplexes with T4 polynucleotide ligase was confirmed by combining the 24/20mer duplex **A** (with a dCGGC overhang) with the 40/36mer duplex **D** (with a dGCCG overhang) in the presence of the ligase. Gel electrophoresis under non-denaturing conditions showed the disappearance of the shorter duplex and the appearance of a new 60 bp ligation product.

The crude products from the four tandem syntheses were also analyzed by capillary gel electrophoresis under non-denaturing conditions (polyethylene glycol at 30°C). For comparison, the single-stranded top strands, which were synthesized separately, were also analyzed (Figure 6). These analyses showed that almost all the materials in the crude tandem synthesis products migrated as a peak, which was slightly broader and much slower than the single-stranded sequences. The presence of excess single-stranded oligonucleotide was only apparent in one tandem synthesis (Figure 6C) and then it was only a minor impurity. However, in most cases the double-stranded product peak showed small amounts of what might be either peak artifacts (owing to the non-denaturing conditions) or unresolved impurities eluting either just before or just after the main peak. If greater purity is required, then purification by denaturing high-performance liquid chromatography (40) might be possible. This is a relatively new technique that can separate duplex DNA fragments containing single base mismatches (heteroduplexes) from perfectly matched duplexes (homoduplexes) by taking advantage of the different melting and elution properties of partially mismatched strands under ion-pairing reversed-phase chromatographic conditions. This technique was originally developed for high-speed mutation detection but instruments for oligonucleotide purification are also available. However, we lacked such equipment and were unable to evaluate it.

In general, however, tandem oligonucleotide synthesis of more than one unique sequence, i.e. PCR primers, multiplexed

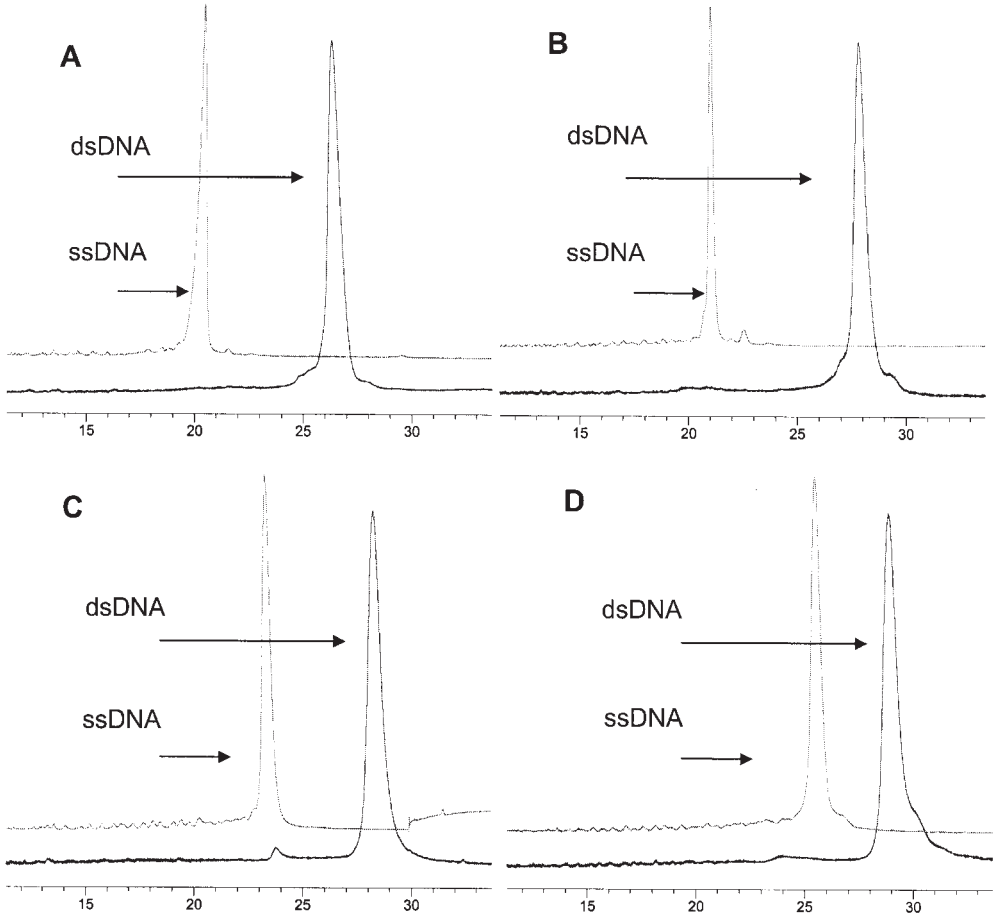

Figure 6. Capillary gel electrophoresis analyses of crude products from tandem oligonucleotide synthesis under non-denaturing conditions. (**A–D**) The duplexes **A–D** (dsDNA) whose structures depicted in Figure 4A–D are shown. In each panel, a separately synthesized single-stranded oligonucleotide (ssDNA) corresponding to the top strand of each duplex is also shown.

primers or duplex structures, should only be considered when the application required is tolerant of minor impurities. This is because the effort to purify individual products from the mixture of full-length and N-1, N-2, etc. oligonucleotides, if possible at all, will outweigh the time saved through tandem synthesis. Thus good synthesis practices must be maintained, otherwise poor syntheses will produce unsatisfactory mixtures. However, it is well established that the quality of routinely synthesized 'crude' oligonucleotides is quite sufficient for most biochemical applications and the majority of small-scale oligonucleotides are used with only a simple desalting step for purification. In these cases, tandem synthesis can produce usable products at lower costs than individual syntheses.

In the case of therapeutic oligonucleotides, both the quality and the cost of production are important. Although, we have demonstrated the concept of tandem synthesis in up to a 6-fold synthesis of 20mer, large-scale synthesis is unlikely to be employed for more than a 2-fold tandem synthesis. This is because yield decreases exponentially with the overall length, while phosphoramidite costs remain the same. However, with high-coupling efficiencies (99.0–98.5%), the yield of a 2-fold tandem synthesis of 20mer will only be ~9–13% less than the yield from two individual 20mer syntheses. Both syntheses consume almost the same amount of phosphoramidite (one extra coupling with **4** is required for tandem synthesis) but this is offset by a 50% savings in the cost of support, which is a more expensive component of the total cost.

In summary, we have shown that linker phosphoramidite reagents provide a simple and efficient method for making multiple oligonucleotides in a single solid-phase synthesis. Tandem oligonucleotide synthesis can produce high-quality strings of oligonucleotides which are comparable to the quality of individual oligonucleotides. Since the solid-phase synthesis of oligonucleotides of 100 bases or more in length is well established, tandem synthesis of primer pairs, sets of cooperative oligonucleotides or self-complementary duplexes of up to ~100 bases in total length proceeds very well. Tandem synthesis may also be advantageous to produce multiple copies of the same sequence for large-scale applications, although the reagents described in this manuscript introduce 5′-phosphate groups on some of the products. If 5′-non-phosphorylated oligonucleotides are required, tandem synthesis via ester linkages (2) and not phosphate linkages should be used.

Our tandem syntheses of duplex DNA fragments demonstrated that such structures would spontaneously form once protecting groups were removed and that the phosphorylated 5′ ends produced were compatible with ligase. Although, there are limited applications where short double-stranded oligodeoxyribonucleotides are required, the same is not so for short double-stranded oligoribonucleotides. siRNAs are double-stranded RNAs made of synthetic 21–25 base-long oligoribonucleotides, which have recently become very widely used as gene silencing reagents (21–23). Such reagents are particularly suitable for tandem oligonucleotide synthesis because RNA synthesis entails an additional 2′-deprotection step. Tandem RNA synthesis would eliminate the need to perform the deprotection steps on each separate strand. The 5′-terminal phosphate group introduced on one strand by tandem synthesis is also beneficial since the RNA interference mechanism requires a 5′-terminal phosphate or 5′-phosphodiester for activity (41). Tandem synthesis of both siRNA strands in a single tube may also reduce the risk of accidental degradation by RNAses, since the double-stranded material that forms upon deprotection is less susceptible to degradation than single-stranded RNA. However, to be satisfactory tandem RNA synthesis requires high-coupling efficiencies, since 42 base-long strings rather than 21mer need to be synthesized. Efficient deprotection methods that are not affected by secondary structure are also required. We are working toward these goals and will report on this in a future manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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