Tandem Oligonucleotide Synthesis on Solid-Phase Supports for the Production of Multiple Oligonucleotides

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More than one oligonucleotide can be synthesized at a time by linking multiple oligonucleotides end-to-end in a tandem manner on the surface of a solid-phase support. The 5′-terminal hydroxyl position of one oligonucleotide serves as the starting point for the next oligonucleotide synthesis. The two oligonucleotides are linked via a cleavable 3′-*O*-hydroquinone-*O*,*O*′-diacetic acid linker arm (*Q-linker*). The *Q-linker* is rapidly and efficiently coupled to the 5′-OH position of immobilized oligonucleotides using HATU, HBTU, or HCTU in the presence of 1 equiv of DMAP. This protocol avoids introduction of phosphate linkages on either the 3′- or 5′-end of oligonucleotides. A single NH_4OH cleavage step can simultaneously release the products from the surface of the support and each other to produce free 5′- and 3′-hydroxyl termini. Selective cleavage of one oligonucleotide out of two sequences has also been accomplished via a combination of succinyl and *Q-linker* linker arms. Tandem synthesis of multiple oligonucleotides is useful for producing sets of primers for PCR, DNA sequencing, and other diagnostic applications as well as double-stranded oligonucleotides. Tandem synthesis of the same sequence multiple times increases the yield of material from any single synthesis column for maximum economy in large-scale synthesis. This method can also be combined with reusable solid-phase supports to further reduce the cost of oligonucleotide production.

Introduction

Synthetic oligonucleotides are widely used because of their many applications in diagnostics, therapeutics, and molecular biology. Solid-phase oligonucleotide synthesis is the preferred method for the synthesis of single-stranded oligonucleotides and since the introduction of automated instrumentation based on phosphoramidite chemistry, many millions of oligonucleotides have been prepared. Demand for these materials still continues to grow rapidly. This escalating demand has led to various improvements aimed at increasing oligonucleotide synthesis productivity. Faster synthesis has resulted from shorter coupling cycles and changes to protecting groups,[1] linker arms,[2,3] and deprotection conditions.[4] New instrumentation, using 96-well plates, instead of individual synthesis columns, also allows many more oligonucleotides to be produced in parallel during each synthesizer run.[5,6]

Applications requiring synthetic oligonucleotides are very diverse, and new ones continue to be developed. However, increasingly large numbers of oligonucleotides are being used in applications that require two or more oligonucleotides at a time. The most important instance is amplification by polymerase chain reaction (PCR) where two different oligonucleotide primers are always required. Recent developments in DNA microarray technology[7] now use thousands of primer pairs for the amplification of the required cDNAs. The assembly of duplex DNA fragments is also another obvious application for pairs of oligonucleotides. Additionally, other applications that utilize multiple oligonucleotides are being developed. For example, cooperative base stacking between immediately adjacent hybridization probes has been used to enhance discrimination of single-base mismatches or assist in oligonucleotide capture.[8−12] Helper oligonucleotides increase probe signals in fluorescent in-situ hybridization assays (FISH) by opening inaccessible rRNA regions[13] and bridge oligonucleotides are required for chain reaction cloning (CRC).[14] Multiplexed primer sets for simultaneous forward and reverse

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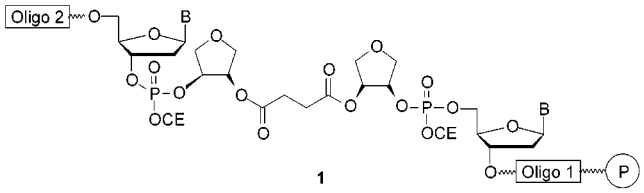
DNA sequencing,[15,16] positional sequencing by hybridization,[17] multiple PCR,[18] PCR−SSCP,[19−21] and RAPD[22−24] analysis are also used. Short duplex RNA sequences have also been used for gene inhibition by RNA interference (RNAi).[25]

Since so many applications use more than one oligonucleotide at a time, synthesis of defined mixtures of oligonucleotides, i.e., more than one oligonucleotide per synthesis, is a useful way to improve synthesis productivity. One well-established method for doing this entails substituting mixtures of individual monomer, dimer,[26] or trimer phosphoramidites[27,28] to produce mixtures of sequences with degenerate base positions or mutagenized sites. The mixture is then used as a single product. However, this technique can only make mixtures, which share a common consensus sequence.



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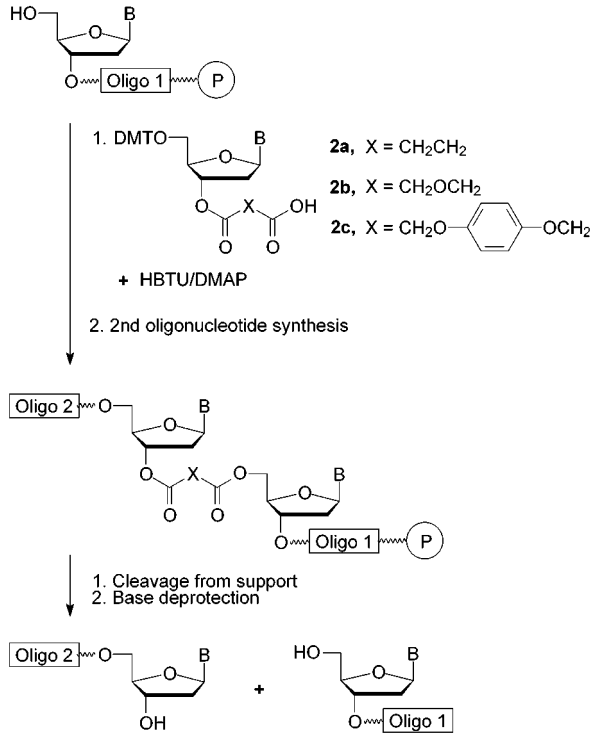
Hardy et al. have described a more versatile method for producing more than one oligonucleotide per synthesis via a linker phosphoramidite called "two oligomers per synthesis" (TOPS).[29] Using this reagent, two entirely different oligonucleotides, linked via the TOPS reagent, were prepared on a solid-phase support **1** in one continuous solid-phase synthesis. After synthesis, treatment with NH_4OH released the oligonucleotides from each other yielding a pair of primers. This method was beneficial because it doubled the number of oligonucleotides prepared from each synthesizer run without requiring any additional operator intervention. However, the TOPS reagent required an intramolecular cyclization to remove phosphate groups from the terminal 5′- and 3′-hydroxyl positions of the products. Removal of terminal phosphate groups, especially from the 3′-terminus, is an essential requirement for most applications. However, dephosphorylation requires harsh or prolonged deprotection conditions and nonquantitative dephosphorylation leaves unwanted impurities in the mixture.

Scheme 1. Tandem Synthesis without Phosphate Linkages



Since removal of terminal 3′-phosphate groups is essential for priming DNA polymerases, we have developed a much simpler strategy that completely eliminates the undesirable phosphate linkages. Instead of an expensive phosphoramidite-based linker reagent, we utilized an easily accessible nucleoside-3′-*O*-carboxylate in a scheme analogous to the derivatization of amino[30] or hydroxyl supports[31] (Scheme 1). However, the 5′-terminal hydroxyl position of an existing oligonucleotide now serves as the starting point for the new synthesis. Each new oligonucleotide sequence is linked through two easily cleavable ester linkages and there are no phosphate groups between sequences. This approach utilizes a 3′-*O*-hydroquinone-*O*,*O*′-diacetic acid linker (*Q-linker*) and eliminates the need for removal of unwanted 3′- or 5′-phosphate groups. Faster deprotection is also possible, if labile protecting groups are used, since the rate-limiting dephosphorylation step is no longer required. The number of oligonucleotides that can be prepared in a single tandem synthesis is also not limited, except by the general restraints of support pore size, depurination, and coupling efficiency, which affect all solid-phase syntheses.

In this manuscript we demonstrate how tandem synthesis of multiple oligonucleotides on a single solid-phase support can be applied to produce sequencing or PCR primers, duplex DNA strands, and sets of 5′-fluorescently labeled PCR primers suitable for automated genotyping. In these applications, synthesis in tandem allows more oligonucleotides to be prepared from each instrument setup and reduces the number of individual samples that

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must be deprotected, purified, analyzed, and packaged after each synthesis. It also provides end users with fewer individual samples that must be handled and stored. Tandem synthesis can also be used to prepare multiple copies of the same sequence in a single run. This is important for the large-scale synthesis of antisense and other pharmaceutically important oligonucleotides, where the yield of product from each synthesis column is important. Finally, we also demonstrate how tandem synthesis using a combination of succinyl **2a** and *Q-linker*[3] **2c** arms can allow selective cleavage and isolation of different oligonucleotides without chromatography or electrophoresis.

Materials and Methods

General Methods. Nucleosides **2a**−**c** were prepared and attached to controlled pore glass supports (CPG) according to previously described procedures.[3,32] Long-chain alkylamine (LCAA) and glycerol CPG supports were obtained from CPG, Inc. (Lincoln Park, NJ). Solid-phase DNA synthesis was performed using an Applied Biosystems 394 DNA synthesizer with eight base positions. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) **6** was obtained from Perseptive Biosystems (Framingham, MA), *O*-benzotriazoly-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) **7** was from Quantum Biotechnologies (Montreal, QC, Canada), and *O*-(1*H*-6-chlorobenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU) **8** was a donation from Luxemburg Industries (Tel-Aviv, Israel). Phosphorothioates were prepared using Beaucage reagent (3*H*-1,2-benzodithiol-3-one 1,1-dioxide) purchased from RI Chemicals (Orange, CA). Fluorescent dye phosphoramidites were purchased from Applied Biosystems (Foster City, CA). Capillary gel electrophoresis (CGE) was performed using replaceable HP Polymer A (28% poly(ethylene glycol)) in HP PVA coated capillaries on an HP 3D capillary electrophoresis instrument. Microsatellite analysis was performed on an ABI 377 DNA sequencer using ABI GeneScan software. MALDI-TOF mass spectra were obtained from a 3-hydroxypicolinic acid/ammonium citrate matrix using a Voyager STR mass spectrometer in positive-ion linear mode. Quantitative analysis of dimethoxytrityl colors was performed using 5% dichloroacetic acid/1,2-dichloroethane (±5% accuracy).

Automated Coupling of Nucleosides 2 to Glycerol−**CPG Supports and Immobilized Oligonucleotides.** A solution of nucleoside **2** (0.2 mmol) and diisopropylethylamine (0.2 mmol, 35 μL) in anhydrous acetonitrile (2 mL) was installed on spare base position 7 of the DNA synthesizer. A solution of HATU, HBTU, or HCTU coupling reagent (0.2 mmol) and 4-(dimethylamino)pyridine (0.2 mmol, 24.4 mg) in anhydrous acetonitrile (2 mL) was installed on spare base position 8. A custom function to simultaneously deliver both reagents at positions 7 and 8 was programmed. A custom begin procedure, using the above custom function, delivered both nucleoside and coupling reagent solutions to the synthesis column (4.0 s). After a variable wait step (see Table 1), the column was rinsed, unreacted sites were capped with Cap A + B acetic anhydride/*N*-methylimidazole capping reagents (5 min), and then automated oligonucleotide synthesis was performed without further modification.

Tandem Oligonucleotide Synthesis. The first oligonucleotide sequence was prepared using a prederivatized LCAA-CPG support according to conventional practice with the terminal 5′-dimethoxytrityl group removed (Tr-Off). Automated cleavage was not selected. After completion of the synthesis, the column was left on the synthesizer and the second oligonucleotide sequence was programmed. The synthesis was then started using the above automated begin procedure to add the appropriate 3′-terminal nucleoside **2** to the 5′-hydroxyl position of the existing support-bound sequence(s). Trityl colors were collected and quantitatively measured to estimate the yield of each nucleoside addition as well as average phosphoramidite coupling yields. The tandem synthesis procedure was repeated as many times as necessary to prepare the desired string of oligonucleotides.

Table 1. Nucleoside Loadings through Ester Linkages to Glycerol-CPG

nucleoside	coupling reagent + DMAP (1:1)	nucleoside loading (μmol/g) 0 s[a]	60 s	150 s	300 s	600 s
2a	HATU	4	32	46	56	63
2a	HBTU	3	11	18	29	40
2a	HCTU	8	27	36	38	56
2b	HATU	48	52	54	60	62
2b	HBTU	6	25	32	42	46
2b	HCTU	21	38	42	40	43
2c	HATU	43	79	80	80	80
2c	HBTU	22	48	64	71	70
2c	HCTU	52	64	72	71	76

[a] Wait time after delivery of nucleoside and coupling reagent solutions to synthesis column.

Cleavage from the Support. Oligonucleotide phosphodiester sequences prepared with succinic, diglycolic, or *Q-linker* arms were automatically cleaved from the supports and from each other using respective automatic end procedures with 3, 15, and 60 min NH_4OH treatments. Oligonucleotide phosphorothioate sequences made with the *Q-linker* were subjected to a 15 min ammonium hydroxide cleavage step because of the slower release of phosphorothioates.[33] For the selective cleavage experiment, the synthesis column was removed from the synthesizer and manually treated with NH_4OH (2 mL) for 2 min. The eluant was collected and then the column was treated with a second portion of NH_4OH (2 mL) for 60 min. The second eluant was then collected separately. After the cleavage step, all products were deprotected by heating in NH_4OH (55 °C, 16 h), evaporated to remove ammonia, redissolved in water, and quantitated at 260 nm by UV spectroscopy.

Synthesis on Reusable Supports. The ISIS 2302 20-mer phosphorothioate sequence, dGCCCAAGCTGGCATCCGTCA, was prepared on reusable glycerol-CPG supports using the recycling procedure previously described.[33] This procedure was similar to conventional synthesis except that labile *Q-linker* chemistry was used in combination with chloroacetic anhydride as a capping reagent. After tandem synthesis, the products were cleaved from the support (NH_4OH, 15 min) and the supports were regenerated by treatment with 0.05 M K_2CO_3 in methanol (5 min). After being washed with anhydrous acetonitrile, the synthesis columns were reused.

Results and Discussion

Dicarboxylic acids, such as succinic **3**, diglycolic **4**, or *Q-linker* **5**, have been frequently used as linker arms to attach the first nucleoside to the surface of amino or hydroxyl derivatized supports.[34] A wide variety of methods are available for producing the required amide and ester linkages to these linkers. Recently, we utilized peptide synthesis coupling reagents to greatly speed-up these reactions and allow automatic on-line derivatization of supports.[30] Although these reagents are typically used to produce amide bonds, we found that addition of 1 equiv of 4-(dimethylamino)pyridine (DMAP) also allowed rapid ester formation.[31] This reaction was used as

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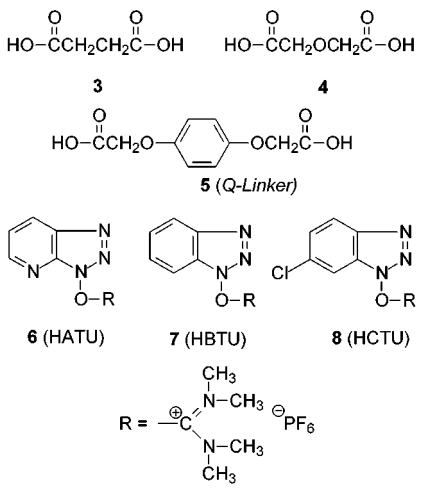
part of a process for reusing hydroxyl derivatized solid-phase supports, such as glycerol−CPG.[33]



During our work with reusable hydroxyl supports, we realized that the terminal 5′-OH group of a support bound oligonucleotide was not different than any other surface hydroxyl group when the automated esterification reaction[31] was performed. Thus, it seemed reasonable that multiple oligonucleotides linked together in tandem could be prepared. However, unlike initial nucleoside derivatization of a support where esterification to every site is not necessary, a coupling between oligonucleotides needs to be highly efficient. Otherwise, the yield of subsequent oligonucleotides will be low.

To optimize the coupling conditions, we installed underivatized glycerol-CPG containing columns on an ABI 394 DNA synthesizer. Acetonitrile solutions of 5′-dimethoxytrityl-N^4-benzoyl-2′-deoxyadenosine containing either 3′-*O*-succinic, diglycolic, or *Q-Linker* arms (**2a**−**c**, B = A^{Bz}) and either HATU **6**, HBTU **7**, or HCTU **8** and DMAP (1:1) were installed on spare base positions. An automated "begin" procedure simultaneously filled the column with both solutions (4.0 s). A wait time of either 0, 60, 150, 300, or 600 s was implemented to allow the esterification to proceed. The column was then automatically rinsed, treated with capping reagents, and detritylated. Quantitative dimethoxytrityl group analysis allowed the nucleoside loading to be determined (Table 1).

The results showed a marked difference in the reactivity of the three linker arms, with the fastest and best results produced by the nucleoside with the *Q-linker* **2c**. This linker arm was found to be the most efficient in providing complete derivatization of all of the surface glycerol sites on the support (70−80 μmol/g) in the shortest time (60 s). Nucleoside loadings obtained with the diglycolic and succinic acid linkers were significantly lower, especially when less than 300 s was allowed for the coupling.

The choice of coupling reagent also had a marked effect on the speed and extent of the esterification. The best results were obtained when HATU **6** and DMAP were employed. With this reagent combination and **2c**, all of the surface sites were esterified in 60 s. Nucleoside loadings of up to 60 μmol/g could also be obtained with **2a** and **2b**, if longer coupling times were allowed. However, despite its efficiency, HATU is a relatively expensive reagent and so we were also interested in evaluating a newly developed coupling reagent, HCTU **8**, and comparing it with the widely used and less expensive HBTU **7**. Rapid coupling was observed with HCTU and DMAP, but the loadings achieved, especially with **2a** and **2b**, were not quite as high as those obtained with HATU. With HCTU and **2c**, complete esterification required only 150 s. Coupling reactions with HBTU and DMAP were the slowest, although with **2c**, complete esterification was still possible in 300 s.

We decided that the optimum reagents were nucleosides with the *Q-linker* arm **2c** since they were much more reactive than nucleosides **2a**,**b**. Additionally, the solubility of thymidine nucleoside **2c** in acetonitrile was superior to **2a** (B = Thy). Nucleosides **2c** also allowed a much shorter ammonium hydroxide treatment to be used to cleave the oligonucleotides from the support[3] than would have been the case with **2a** (2 min vs 60 min). For reasons of economy, we elected to use HBTU in subsequent experiments and allowed 5−10 min for the coupling to proceed. However, if faster coupling reactions or less reactive nucleosides are required, then either HATU or HCTU could be employed.

Tandem synthesis was first demonstrated by making a series of oligodeoxyadenosine sequences. An initial hexanucleotide $d(Ap)_5A$ was prepared on an LCAA-CPG support, prederivatized with **2c**, using regular synthesis conditions. However, instead of being cleaved from the support, the detritylated support-bound hexanucleotide was used in a second synthesis. A new nucleoside **2c** (B = A^{Bz}) was automatically added to the 5′-OH terminus, followed by nine automated phosphoramidite coupling cycles to produce $d(Ap)_9A$ linked to the initial hexanucleotide. A third $d(Ap)_{13}A$ oligonucleotide was then added to the support-bound 10 and 6-mers by repeating the procedure. Thus, a string of 30 consecutive bases, consisting of 6, 10, and 14-mers were prepared on a single synthesis column. The yield of each of the two esterification steps was estimated by collecting and measuring the trityl colors. This showed that the addition of **2c** was essentially quantitative (99.8−100%). The synthesis column was then subjected to an automated NH_4OH cleavage step that simultaneously released the oligonucleotides from the support and each other. After overnight deprotection, capillary gel electrophoresis (CGE) of the crude product clearly showed the presence of three different oligonucleotides (Figure 1a) in a ratio of 1:2:2.8. This ratio was not much different than the 1:1.7:2.3 ratio expected for 6, 10, and 14-mers. MALDI-TOF mass spectrometry (Figure 1b) of the crude mixture also clearly showed the presence of three products with the masses expected for 6-, 10-, and 14-mers and confirmed the complete removal of all linking and protecting groups.

Tandem synthesis of a 24 base-long M13 sequencing primer (dCGCCAGGGTTTTCCCAGTCACGAC) was performed on 1000 Å CPG columns. The test sequences were prepared either 1× (control), 2×, 3×, or 4× in tandem. Thus, oligonucleotides strings of either 24, 48, 72, or 96 bases in length were prepared. Relative to the control synthesis, the tandem syntheses produced 1.5−2.5 times as much crude product and the purity of the tandem synthesized material (66−69% full-length) was again slightly greater than the single synthesis (61%). The slight increase in overall yield of the tandem 24-mers can probably be attributed to the fact that coupling efficiency becomes slightly greater as the coupling site becomes further removed from the surface of the support. Mass

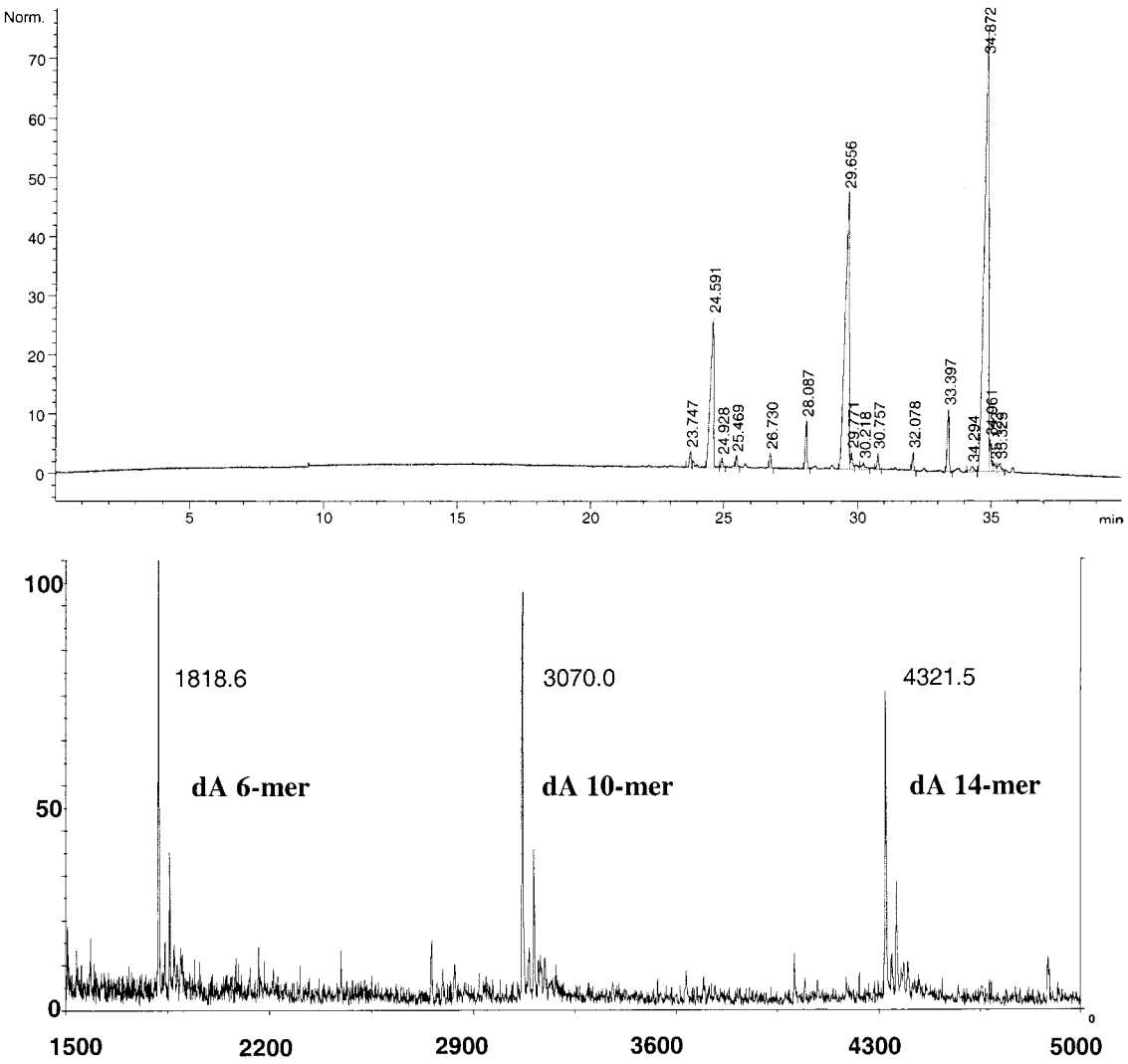


Figure 1. (a) CGE analysis of the crude product containing $d(Ap)_5A$ (24.59 min), $d(Ap)_9A$ (29.66 min), and $d(Ap)_{13}A$ (34.87 min). **(**b) MALDI-TOF mass spectrometry of three oligonucleotides prepared by tandem synthesis: $d(Ap)_5A$, $(M + H)^+$ calcd 1817.3, obsd 1818.6; $d(Ap)_9A$, $(M + H)^+$ calcd 3070.1, obsd 3070.0; and $d(Ap)_{13}A$ $(M + H)^+$ calcd 4321.5, obsd 4321.5. An $(M + K)^+$ ion also appears for each oligonucleotide.

spectrometry also confirmed the complete removal of all protecting and linking groups.

A duplex DNA fragment containing the two complementary oligonucleotide 20-mers: dTAATACGACTCACTATAGGG and dCCCTATAGTGAGTCGTATTA was prepared in a single tandem synthesis. Trityl analysis indicated that the yield of **2c** ($B = A^{Bz}$) was 99.6% and the average coupling yields for the phosphoramidite additions during the first and second oligonucleotide syntheses were 99.5% and 99.6% respectively. For comparison, each 20-mer was also prepared individually. After cleavage and deprotection, the crude products were analyzed by CGE (Figure 2). The individually prepared 20-mers migrated as expected for single-stranded DNA with almost identical migration times (43 min). However, the crude product from the tandem synthesis showed a much slower product migrating at 67 min, corresponding to a double-stranded 20 base-pair fragment. The duplex fragment was 85% of the crude product and no impurities greater than 1% were present with migration times corresponding to the single-stranded 20-mers. The double-stranded nature of the tandem synthesized product was also confirmed by polyacrylamide electrophoresis under both denaturing and nondenaturing conditions.

One possibility with tandem synthesis is the sequential decrease in the number of 5′-OH ends which occurs when phosphoramidite coupling yields are less than 100%. This means that oligonucleotides produced early in the synthesis will always be present in greater amounts than oligonucleotides produced later. Fortunately, when used in PCR the primers are usually present in large excess and their uneven distribution is not significant. For example, previously reported PCR primers prepared by tandem synthesis with the TOPS reagent did not show any problems.[29] However, we were interested in knowing whether the amplification protocols commonly used for genotyping were sensitive to this imbalance between primers. In particular, since automated genotyping is widely used, we wanted to verify the utility of tandem synthesis method for preparing fluorescently labeled primers for automated microsatellite analysis.

We prepared three pairs of PCR primers by tandem synthesis for automated genotyping of mouse genomic DNA. In each tandem synthesis the unlabeled oligonucleotide was synthesized first on the support (primer 1) and the labeled oligonucleotide was made second (primer 2). The very last coupling step used either 6-FAM, HEX, or TET fluorescent dye phosphoramidites

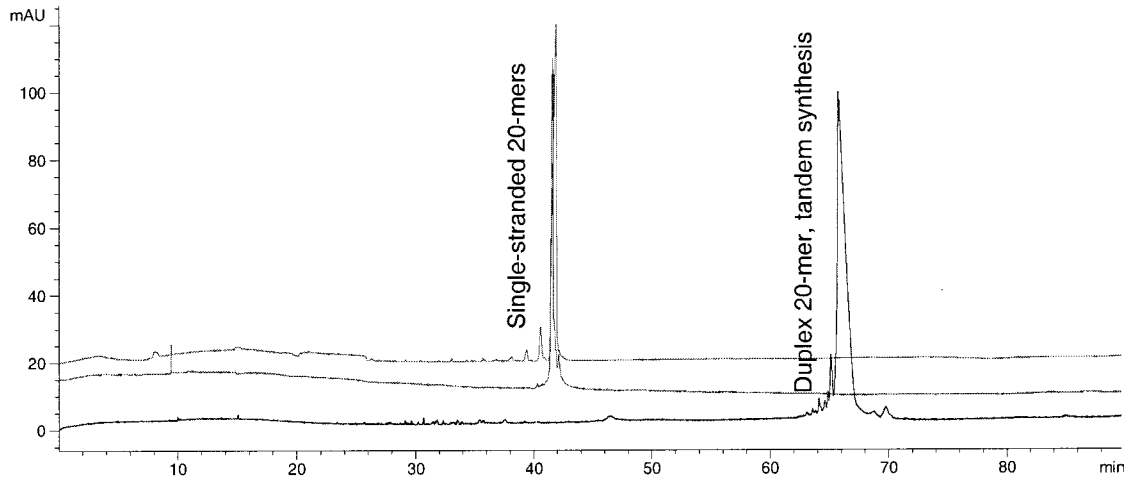


Figure 2. CGE analysis of the crude double-stranded 20-mer product dTAATACGACTCACTATAGGG/dCCCTATAGTGAGTCGTATTA produced by tandem synthesis (bottom trace, 67 min). The single-stranded oligonucleotides from individual syntheses are shown in the top two traces (43 min).

to add the fluorescent label to the second primer. The tandem oligonucleotides were then cleaved from the support and deprotected. One portion of each product was subjected to only a minimal cleanup (desalting on Sephadex) and another portion was purified by preparative polyacrylamide gel electrophoresis (PAGE) to yield each individual primer as a single-band product.

CGE analysis of the crude products showed the expected products were the major components in each crude mixture (Figure 3a−c). However, in the case of the HEX labeled primer pair, a third significant peak arising from poor coupling of the 5′-TET fluorescent label was also present. As expected, the primers were in unequal ratios. The primers in the FAM primer pair were the closest to equal with a 54:47 ratio of primer 1 to primer 2. However, the TET and HEX primer pairs produced respective ratios of only 65:35 and 61:27.

The PCR primer pairs were used to amplify known microsatellite markers from (C57BL6/J × NOD) mouse genomic DNA. Amplification was performed using either the crude mixture obtained from the tandem synthesis (unequal amounts of primer) or with the individually purified and recombined primers (equal amounts of each primer). A multiplexed amplification reaction, which included all three primer pairs in a single tube, was also performed. The amplified products were applied to a 377 DNA sequencer and analyzed by GeneScan software. In each case, there was no significant difference between the results obtained from the crude primer pairs and the purified and reconstituted primer pairs. Thus, automatic fluorescent genotyping was insensitive to the PCR primers present in different amounts.

Tandem oligonucleotide synthesis can also be applied to the synthesis of multiple copies of the same oligonucleotide sequence. This provides two clear advantages. First, the amount of product obtained from any given synthesis column is increased in proportion to the number of tandem syntheses. This is important because during large-scale synthesis the single-most expensive material is the solid-phase support. Thus, tandem synthesis reduces the relative cost of the support by allowing more product to be produced per column. Second, tandem synthesis allows instrumentation to operate longer without manual intervention. The products prepared in this manner may be pharmaceutically important nucleic acids or shorter fragments, such as dimers and trimers, which will be used as block synthons for future syntheses.[35,36]

A triple tandem synthesis of three $d(Ap)_5A$ hexanucleotides was performed to determine the utility of this approach. In this synthesis, the yield of each addition of **2c** was nearly quantitative. After cleavage and deprotection the amount of crude material was normalized to A_{260} units per gram of support. The crude product collected from the triple tandem synthesis was compared to the crude product produced from a single conventional synthesis. As expected, the amount of crude material produced by the tandem synthesis (5460 A_{260} units/gram) was approximately 3 times as much as produced from a single synthesis (1580 A_{260} units/gram). Also, of interest was that CGE analysis indicated that the purity of the crude hexanucleotide produced in tandem (91%) was slightly better than the crude hexanucleotide (85%) obtained from a single synthesis.

A short phosphodiester sequence, dCGGTA, which has been shown to induce apoptosis,[37] was also selected for tandem oligonucleotide synthesis. This sequence was prepared either 1× (control), 5×, 8×, or 10× in a single tandem synthesis on a 1 μmol scale. The 1×, 5×, 8×, and 10× syntheses produced 800, 4640, 6300, and 6550 A_{260} units/gram, respectively. Relative to the single synthesis, the amount of crude product obtained increased by factors of 5.8, 7.9, and 8.2 times for the 5×, 8×, and 10× tandem syntheses, respectively. Thus, for this short sequence, an 8× tandem synthesis produced the same amount of material as 8 individual syntheses, but with only 1/8 the number of columns or instrument setups.

Tandem synthesis was also demonstrated on a phosphorothioate 20-mer sequence of pharmaceutical importance (ISIS 2302, dGCCCAAGCTGGCATCCGTCA). For comparison, a single control synthesis produced 10 900 A_{260} units of crude product per gram of support. Tandem synthesis was performed to make two copies of the sequence per synthesis. However, for maximum economy, the synthesis was also performed on a reusable glycerol-

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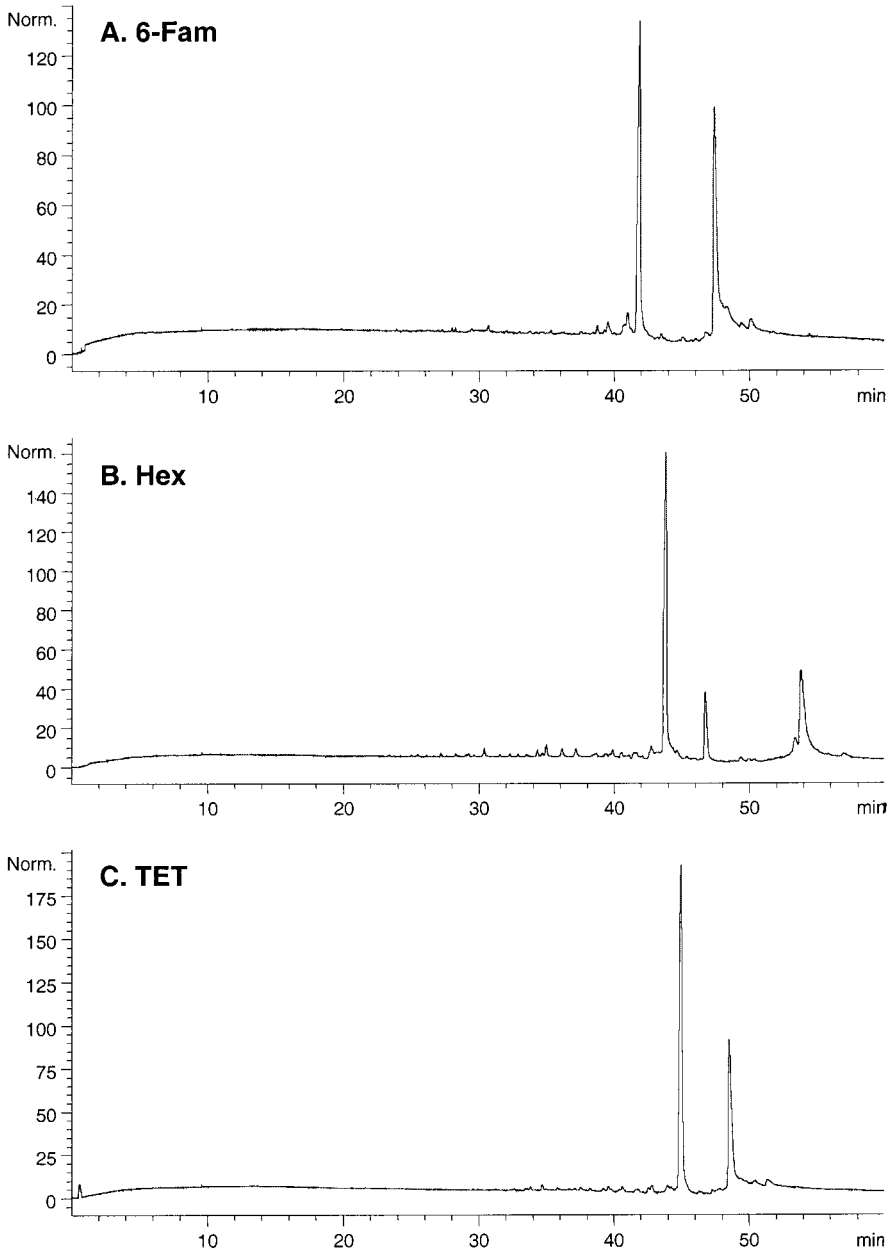
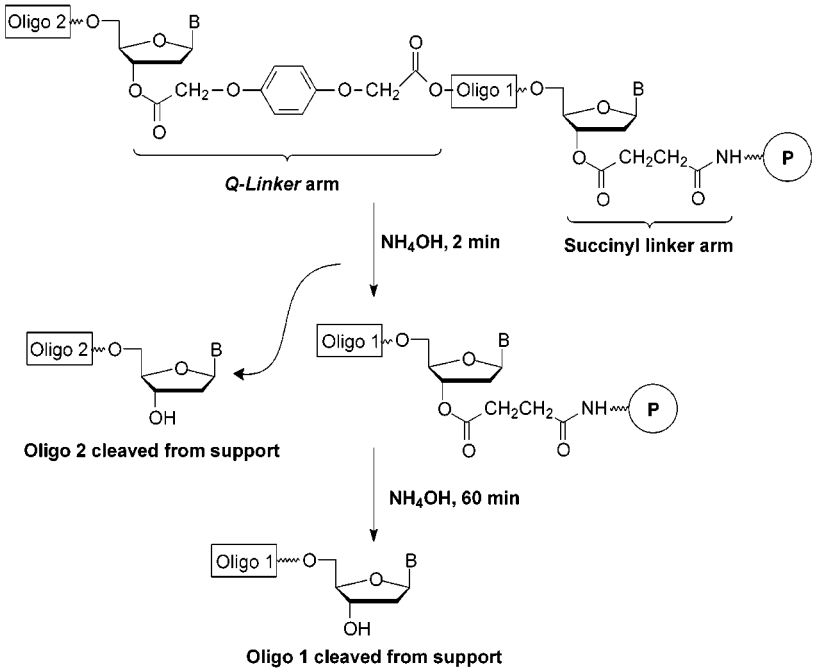


Figure 3. Fluorescent primer pairs for automated microsatellite analysis. (A) 6-FAM-labeled primer pair, dCACGGGTGCTCTATTTGGAA 20-mer with 5′-OH end (41.9 min) and dAGTCAGTCAGGGCTACATGATG 23-mer with 5′-FAM dye label (47.4 min). Ratio of 20-mer to 23-mer = 1.2:1.0. (B) HEX-labeled primer pair, dCATGAATAAGAACGAAAAGGGC 22-mer with 5′-OH end (43.8 min), dGTAGGAGAGAACAACTGTCTTCTGC 26-mer with 5′-HEX dye label (53.8 min), and product from incomplete HEX labeling (46.7 min). Ratio of 22-mer to labeled 26-mer = 2.6:1.0. (C) TET-labeled primer pair, dTATCCAACACATTTATGTCTGCG 23-mer with 5′-OH end (44.9 min) and dAGAGTTTGGTCTCTTCCCCTG 22-mer with 5′-TET dye label (48.5 min). Ratio of 23-mer to 22-mer = 1.8:1.0.

CPG support and after each tandem synthesis, the columns were regenerated and reused.[33] Thus, the ISIS 2302 sequence was made two times in tandem and then the column was regenerated and a second double tandem synthesis was performed. The crude product produced from the two double tandem syntheses (i.e., four syntheses) on one column was 34 900 A_{260} units/gram or an increase of 3.2 times from the single control synthesis. The double tandem synthesis was also performed on another reusable column, but this time the column was reused five times, i.e., five double tandem syntheses totaling 10 syntheses were performed. In this case, 77 000 A_{260} units/gram were obtained, or 7.1 times more material than produced from a single synthesis. Therefore, tandem synthesis can be easily combined with our process for recycling and reusing synthesis columns to significantly lower the cost of support for large-scale oligonucleotide synthesis. However, for best results, synthesis conditions need to be optimized for maximum coupling efficiency because the amount of tandem product is dependent on the overall yield of the first synthesis.

Finally, it is also possible to perform tandem oligonucleotide synthesis using different linker arms. For example, a relatively stable succinic acid linker arm can be used to attach the first nucleoside to the support and a more easily hydrolyzed *Q-linker* arm can be used to attach the second oligonucleotide. Selective cleavage of the different linker arms will then allow easy separation of the products (Scheme 2). This approach was demonstrated by preparing the 17 base-long sequence dG-

Scheme 2. Selective Cleavage of Tandem Oligonucleotides



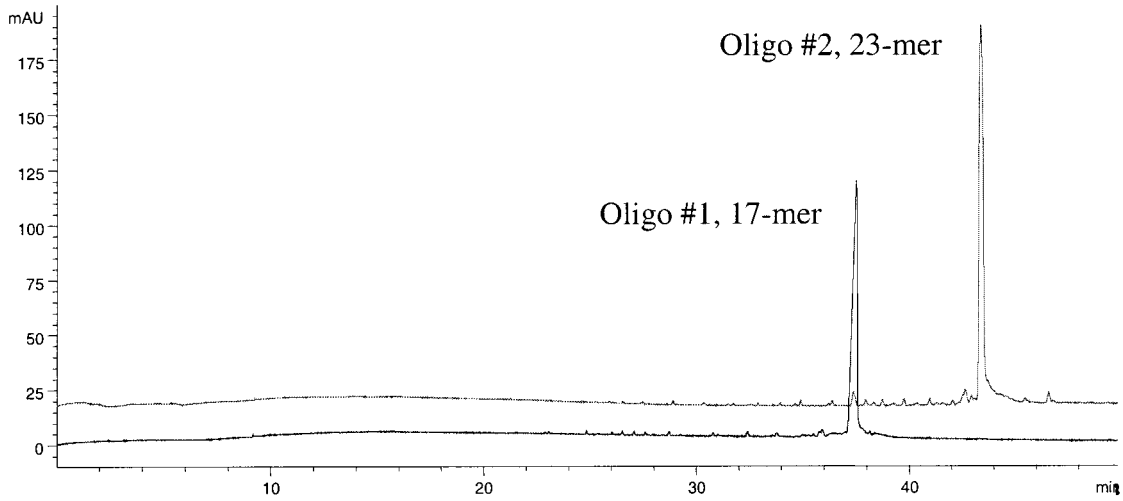


Figure 4. CGE analysis of two oligonucleotides synthesized in tandem and separated by selective cleavage from the support. Synthesis 1: dGTAAAACGACGGCCAGT 17-mer (bottom trace, 37.8 min). Synthesis 2: dCGCCAGGGTTTTCCCAGTCACGA 25-mer (top, 43.4 min).

TAAAACGACGGCCAGT (Oligo 1) on a conventional synthesis column containing a succinyl linker arm. Trityl analysis showed an initial nucleoside loading of 39 μmol/g and average coupling yields of 98%. Then, nucleoside **2c** (B = A^{Bz}) with the *Q-linker* arm was added. Trityl analysis at this point showed a loading of 28 μmol/g. The 23 base-long sequence dCGCCAGGGTTTTCCCAGTCACGA (Oligo 2) was then synthesized in tandem. Trityl analysis indicated 99% average coupling efficiency for the second oligonucleotide.

The synthesis column was treated with NH_4OH (2 min) to cleave Oligo 2 from the support (92 A_{260} units). A second NH_4OH treatment (60 min) released Oligo 1 (116 A_{260} units). After deprotection, CGE analysis (Figure 4) of the crude products showed only a trace (2.3%) of Oligo 1 contaminating the Oligo 2 product. This cross-contamination was less than the amount of N-1 failure sequence (2.6%) and is not significant for many oligonucleotide applications. CGE analysis of the crude product containing Oligo 1, showed no detectable cross-contamination from Oligo 2 and confirmed that the *Q-linker* is completely cleaved within 2 min.

The selective cleavage and separation of the above oligonucleotides does not require any chromatography or electrophoresis. Instead, the method is very simple and automation of the separation only requires addition of an appropriate switching valve or fraction collector to collect the appropriate ammonium hydroxide fractions. Therefore, tandem synthesis on appropriately modified synthesizers can also be applied to the high-throughput synthesis of individual oligonucleotides as well as mixtures of oligonucleotides.

One other benefit of having a synthesizer setup to perform tandem oligonucleotide synthesis is the elimination of pre-derivatized supports. Instead, the first nucleoside of the first sequence can be automatically added to the surface of an inexpensive underivatized amino[30] or hydroxyl support[31,33] by running the same nucleoside

addition cycle used to begin a tandem synthesis. On 96-well plate synthesizers all of the wells can be filled with the identical, i.e., universal, underivatized supports so pre-sorting of supports into the correct positions is eliminated. This strategy is superior to use of existing "Universal" supports which add the first nucleoside as a phosphoramidite, because no postsynthesis dephosphorylation is required to generate terminal 3′-hydroxyl groups.[34]

Only a few accounts of uronium coupling reagents have been reported[38] for oligonucleotide synthesis.[39−41] However, uronium coupling reagents in DMF solution are well established for solid-phase peptide synthesis[42] and acetonitrile solutions of these reagents are completely compatible with DNA synthesis. The HBTU/DMAP solutions have been stable for at least one week on our synthesizers. Therefore, the method of tandem synthesis is compatible with current oligonucleotide synthesis protocols and does not require major modifications other than the automated nucleoside addition.

Tandem synthesis of multiple oligonucleotides is quite practical because both the chemistry and instrumentation for making oligonucleotides of 100 or more bases are well established. This is more than sufficient to allow 1−2 pairs of primer-length oligonucleotides (typically 20−25 bases) to be prepared. Most significantly, the avoidance of phosphate-based linking groups eliminates terminal phosphate groups and all of the problems with postsynthesis dephosphorylation are completely removed. Therefore, no modifications are needed to the postsynthesis workup and deprotection of the products, and the method is compatible with fast deprotection schemes.

Tandem synthesis, with the appropriate software, will allow synthesizers to produce at least twice as many oligonucleotides per run before manual intervention is required. Handling and labor costs for all postsynthesis steps, such as deprotection, quality control, and packaging will be reduced and end-users will benefit from having fewer samples to store and work with. Although, these benefits may seem relatively minor when small numbers of oligonucleotides are involved, modern applications require increasing large numbers of oligonucleotides. It is not uncommon for individual projects to require thousands of oligonucleotides and many synthesis facilities make over 10 000 sequences per week. It is therefore critical, that as many steps as possible be automated so that manual intervention and processing operations are minimized as much as possible.

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Supporting Information Available: CGE, MALDI-TOF, and microsatellite analysis results for oligonucleotides synthesized in tandem as well as the custom "begin" procedure used to perform nucleoside addition on the ABI 394 DNA synthesizer. This material is available free of charge via the Internet at http://pubs.acs.org.

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