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Fluorous Affinity Purification of Oligonucleotides

William H. Pearson,* David A. Berry, Patrick Stoy, Kee-Yong Jung, and Anthony D. Sercel

Berry & Associates, Inc., 2434 Bishop Circle East, Dexter, Michigan 48130

wpearson@berryassoc.com

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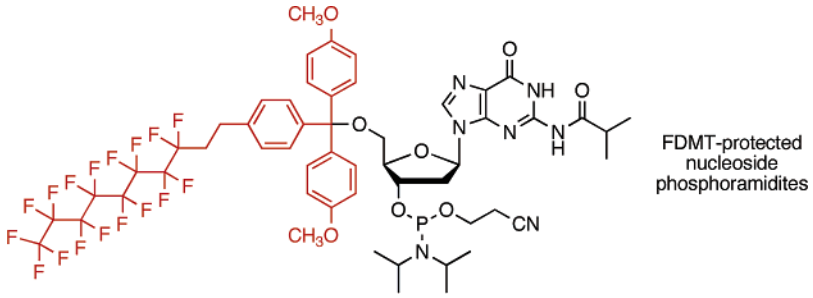

Nucleoside phosphoramidites bearing a fluorous dimethoxytrityl (FDMT) group were used to synthesize fluorous-tagged oligonucleotides, which were subjected to solid-phase extraction using a pH-stable fluorinated adsorbent. On-column detritylation afforded the purified oligonucleotides. The fluorous affinity purification method offers one-pass loading without ammonia removal, high selectivity for the removal of failure sequences, high recoveries (typically 70−100%), and the ability to purify long oligonucleotides (e.g., 50−100-mers).

Introduction

A long-standing challenge in automated oligonucleotide synthesis is product purity, an issue that has become increasingly important as the need for therapeutic oligonucleotides and long oligonucleotides[1] has arisen. Current methods[2,3] for the purification of oligonucleotides include anion-exchange (AX) chromatography, reversed-phase (RP) chromatography, polyacrylamide gel electrophoresis (PAGE), and various types of affinity chromatography. A combination of these techniques is often employed in critical cases. We describe herein the fluorous affinity purification of oligonucleotides, a rapid and convenient method that, when compared to existing methods that rely on 5′ affinity tagging, results in unusually high recoveries of materials that are free of failure sequences, even with long oligonucleotides.

While automated synthesis is the best available strategy for the production of oligonucleotides, heterogeneous mixtures of products are produced, complicating purification and limiting scale-up. These problems are exacerbated as the length of the strand increases. For example, a 20-mer (>100 chemical steps) is typically produced in 40−70% yield, depending on scale, the remaining 30−60% being a heterogeneous mixture of undesired oligonucleotides. Longer oligonucleotides (75-mer and up) are produced in low yield and low purity, limiting the utility of these potentially important materials. Above 100 nucleotides, the limits of solid-phase synthesis are soon reached as the overall yield diminishes to an unusable level while the purity continues to drop. A major class of impurities results from incomplete monomer coupling. Capping of the resultant unreacted 5′-hydroxyl groups with acetic anhydride is performed to prevent further chain elongation, ultimately producing shorter oligonucleotides known as failure sequences in the final product mixture.

A popular purification strategy uses RP adsorbent.[4,5] Reversed-phase HPLC is often performed on oligonucleotides with a 4,4′-dimethoxytrityl (DMT) group on the 5′-terminus, so-called “DMT-on” or “trityl-on” purifica-

(1) Exemplary applications of long synthetic oligonucleotides are in cloning, gene synthesis, and extended-length microarrays. See, for example: Wang, H. Y.; Malek, R. L.; Kwitek, A. E.; Greene, A. S.; Luu, T. V.; Behbahani, B.; Frank, B.; Quackenbush, J.; Lee, N. H. *Genome Biol.* **2003**, *4*, R5.

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tion, wherein the DMT group of the final nucleotide is left on during synthesis, a technique developed in 1978 by Seliger.[6] The DMT group functions as a lipophilic handle, allowing the separation of DMT-bearing oligonucleotides from those without DMT groups (e.g., failure sequences), since the DMT group imparts added hydrophobicity to the molecule and thus causes it to be more strongly retained. The DMT group is subsequently removed with acid to afford the desired oligonucleotide. Single base resolution using RP-HPLC is typically not possible on all but the shortest oligonucleotides. If that level of purification is not required, RP-HPLC may be used to purify target oligonucleotides of up to about 50 nt by the DMT-on technique (depending on the particular case), with varying degrees of success in removing failure sequences. When the oligonucleotide becomes lengthy, or if additional lipophilic modifications are present (biotin, fluorophores, quenchers, backbone and sugar modifications, etc.), the failure sequences and other undesired byproducts are more strongly retained, often running close to (or merging with) the desired product peak, making separation difficult or impossible using this technique. An additional limitation of trityl-on purification by RP-HPLC is low recovery. Researchers have explored more lipophilic trityl groups in an attempt to remove these limitation by developing stronger hydrophobic affinity interactions with the adsorbent.[7] Trityl groups bearing long alkyl chains or extended aromatic systems have been described as removable lipophilic tags for RP-HPLC.[7−9]

A particularly convenient variation of the RP purification method for purifying oligonucleotides involves disposable cartridges or small tubes containing reversed-phase adsorbent ("RP cartridge purification"),[10,11] a form of solid-phase extraction (SPE). The method is fast and does not require expensive HPLC equipment. After deprotection of the nucleobases with aqueous ammonia, the crude DMT-on solution of the oligonucleotide is passed through such a cartridge or column. Ideally, a pH-resistant polystyrene/divinylbenzene- or poly(divinylbenzene)-derived adsorbent is used. If a silica-derived adsorbent is used (e.g., C_{18}-silica RP adsorbent), the ammonia from the deprotection solution must be evaporated first to avoid degradation of the silica matrix. In either case, the DMT group allows retention of the target oligonucleotide, allowing failure sequences (which have no DMT group) to be washed off with an appropriate eluant. The DMT-on oligonucleotide can then be eluted with an eluant containing a more hydrophobic solvent such as acetonitrile, and the resultant material can be detritylated with acid. Alternatively, acid can be introduced to the cartridge or column to detritylate the bound oligonucleotide. After neutralizing and/or washing off the acid, the desired oligonucleotide is eluted. This is a quick way to remove failure sequences and other small organic molecules, remove the DMT group, and desalt the material all in one simple apparatus. RP cartridge purification is limited to relatively short oligonucleotides, typically ≤30−40-mers, since the relative hydrophobic contribution of the lipophilic DMT (or related) group diminishes as the oligonucleotide chain increases, therefore diminishing the affinity of the target compound to the adsorbent, resulting in lower overall yields and a diminished selectivity of the adsorbent for the desired oligonucleotide over failure sequences. In addition, failure sequences become the major product in the synthesis of long oligonucleotides, making it more difficult to retrieve the diminishing amount of full-length material. The presence of ammonia (from nucleobase deblocking) in the crude oligonucleotide mixture is also known to inhibit loading of the DMT-on oligonucleotide to the adsorbent, often necessitating multiple loading passes. Ammonia solutions are also commonly used in washing failure sequences from the adsorbent, which may cause premature removal of the desired material and a correspondingly lower yield. Another problem with RP cartridge purification is that oligonucleotides bearing only a 5′-*O*-DMT group (i.e., otherwise fully deprotected) are susceptible to premature detritylation, resulting in loss of material. An SPE method involving a stronger affinity interaction between the target oligonucleotide and the solid-phase adsorbent should allow higher yields and selectivities (and thus higher purities), especially with longer oligonucleotides. We report herein that fluorous affinity purification addresses these problems.

Organic molecules bearing perfluoroalkyl domains are known as fluorous molecules and may be purified by taking advantage of their affinity for perfluorinated

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(10) McBride, L. J.; McCollum, C.; Davidson, S.; Efcavitch, J. W.; Andrus, A.; Lombardi, S. J. *BioTechniques* **1988**, *6*, 362−367.

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solvents or solid phases,[12−14] e.g., fluorous reversed-phase adsorbents.[15] Fluorous affinity interactions are strong and distinguishable from other types of affinity interactions (e.g., lipophilicity). In a common strategy, target molecules are retrieved from mixtures by attaching temporary fluorous tags and then employing a fluorous separation technique such as fluorous chromatography to separate the tagged molecules from other nontagged organic compounds. The interaction of a fluorous-tagged molecule with a perfluorinated surface is very strong, facilitating solid-phase extraction techniques. Most of the work in the fluorous separations area has been done with relatively small organic molecules in organic solvents. The purification of water soluble biomolecules bearing a fluorous tag should also be viable, since the fluorous affinity interaction will be enhanced by the strong hydrophobicity of the fluorous domain. Fluorous tagging or fluorous capping strategies have been used for the purification of peptides[16] and oligosaccharides.[17] After our work was complete, a report by Beller and Bannwarth described the installation of a thymidine bearing a fluorous monomethoxytrityl (FMMT) group onto two oligonucleotides (a T_{20} and a mixed-base 30-mer) for the purpose of purification using fluorous reversed-phase silica gel in SPE mode.[18] We report herein our complementary studies on the use of fluorous dimethoxytrityl (FDMT) tags for the purification of oligonucleotides. The FDMT group is most similar to the industry standard DMT group and thus is removed under milder conditions than the FMMT group, minimizing depurination. Further, the FDMT method requires only one fluorous chain (rather than two in the FMMT group) to accomplish the purification of oligonucleotides, even with long chains. The four common 5′-*O*-FDMT-deoxynucleoside phosphoramidites are synthesized, their use in preparing fluorous-tagged oligonucleotides is reported, and the fluorous purification of oligonucleotides as long as 100 base pairs is described, resulting in excellent recoveries when compared to existing methods that rely on 5′ affinity tagging.

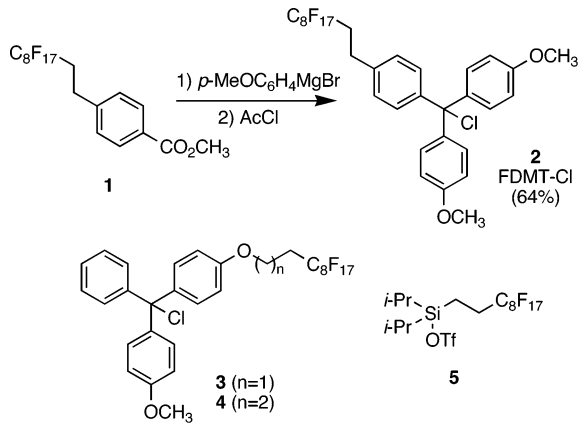

FIGURE 1. Fluorous tagging reagents.

Results and Discussion

While we are examining several strategies for the fluorous tagging of oligonucleotides, a logical first effort would be to develop a fluorous version of trityl-on RP purification. Ideally, the fluorous moiety would be incorporated into a standard DMT group in such a way that the resultant FDMT group behaves identically; the kinetics of phosphoramidite coupling and detritylation should be similar, as should the absorption spectrum of FDMT cation. Of particular concern is the effect of an electronegative perfluoroalkyl group on the chemistry of the dimethoxytrityl group. As is well-known in the fluorous field, an ethylene spacer is useful for electronic isolation of the electronegative perfluoroalkyl group from the molecule of interest. Thus, we examined fluorous DMT groups bearing a (perfluorooctyl)ethyl group at either oxygen or carbon (Figure 1). Surprisingly, a *single* perfluoroalkyl group was found to be sufficient for fluorous purification of even long oligonucleotides. Grignard reaction of commercially available **1** with *p*-methoxyphenylmagnesium bromide afforded a tertiary alcohol (not shown), which was converted to the fluorous dimethoxytrityl chloride **2** ("FDMT-Cl") in good yield. Placing a *p*-alkyl group on the DMT nucleus would normally cause an increase in trityl cation stability, but we felt that the perfluorooctyl group would counterbalance this effect (vide infra). Linking the fluorous moiety to oxygen (e.g., **3** and **4**) was less satisfactory.[19] Compound **3** was more difficult to prepare, and our experience with placing a (perfluorooctyl)ethyl group on a heteroatom[20] led us to believe this particular compound would deviate substantially from a standard DMT group. Compound **4**, where the perfluorooctyl group is further insulated from the DMT nucleus by a propyl group, was used to make nucleoside phosphoramidites and ultimately oligonucleotides. While this version of a fluorous DMT group performed well, compound **2** is preferred on

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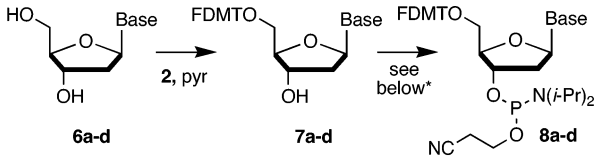
(19) (a) Compound **3**: alkylation of 4-hydroxybenzophenone with (perfluorooctyl)ethyl iodide was sluggish. (b) Compound **4**: (i) 4-hydroxybenzophenone, NaH, 3-(perfluorooctyl)propyl iodide (43%); (ii) *p*-methoxyphenylmagnesium bromide; (iii) AcCl (55%, two steps).

(20) For example (Vennall, G. Unpublished results), we have prepared fluorous versions of 4-(dimethylamino)azobenzene-4′-carboxylic acid (Dabcyl, $Me_2NC_6H_4N{=}NC_6H_4CO_2H$) and found that the absorption spectrum in methanol varies accordingly: (i) $C_8F_{17}(CH_2)_2N(Me)C_6H_4N{=}NC_6H_4CO_2H$: λ_{max} 414 nm; (ii) $C_8F_{17}(CH_2)_3N(Me)C_6H_4{-}N{=}NC_6H_4CO_2H$: λ_{max} 424 nm; (iii) $Me_2NC_6H_4N{=}NC_6H_4CO_2H$: λ_{max} 429 nm.

SCHEME 1. Synthesis of 5′-*O*-FDMT Nucleoside Phosphoramidites



Series	Base	yield of **7**	yield of **8**	phosphitidylation conditions*
a	thymine	73%	79%	A
b	N^6-benzoyladenine	91%	71%	B
c	N^4-benzoylcytosine	96%	62%	B
d	N^2-isobutyrylguanine	96%	84%	B

**phosphitidylation conditions:*
A: $(i\text{-Pr})_2NP(Cl)O(CH_2)_2CN$, $(i\text{-Pr})_2NEt$
B: $[(i\text{-Pr})_2N]_2PO(CH_2)_2CN$, tetrazole

the basis of synthetic ease. We were also able to prepare and study fluorous-tagged oligonucleotides using nucleoside phosphoramidites bearing a fluorous silyl group at the 5′-oxygen (prepared from **5**[17a,21]).

Fluorous-tagged nucleoside phosphoramidites **8a**−**d** were prepared by standard methods (Scheme 1). Fluorous dimethoxytritylation of thymidine **6a** and suitably protected forms of dA, dC, and dG (**6b**−**d**) proceeded normally to give **7a**−**d**. Phosphitylation gave the desired phosphoramidites **8a**−**d**. From a synthetic standpoint, the FDMT group was found to behave identically to a DMT group throughout this sequence. Phosphoramidites analogous to **8a** were also made using fluorous tagging agents **4** and **5** (not shown).

Placing **7a** in 3% trichloroacetic acid in dichloromethane caused formation of the FDMT cation, which exhibited an absorption maximum at 504 nm, identical to DMT cation generated from 5′-*O*-DMT-thymidine (**9**, not shown) by the same method. Rate constants for detritylation of **7a** and **9** in 80% acetic acid-acetonitrile at 21 °C were determined by monitoring the appearance of trityl cation at 504 nm.[8n] Assuming a pseudo-first-order irreversible reaction, the apparent rate constants were found to be 4.7×10^{-4} s^{-1} and 5.9×10^{-4} s^{-1}, respectively, showing that the FDMT group is removed slightly more slowly than a DMT group. Since premature detritylation can be a problem in DMT-on purification, this may be an advantage of the FDMT group. Overall, the FDMT group behaves similarly to a DMT group in terms of trityl cation color as well as rate of detritylation.

Exemplary oligodeoxyribonucleotides (ODNs) **10**−**13** were prepared (Figure 2), where phosphoramidite **8a** was used in the last coupling step to install a fluorous-tagged thymidine at the 5′ terminus. Prior to that point, standard DMT-protected phosphoramidites were used to assemble the ODN chain. Phosphoramidites **8b**−**d** were also found to couple normally during ODN synthesis, as did the phosphoramidites derived from **4** and **5**. Cleavage of the ODNs from the solid support and nucleobase deblocking with ammonium hydroxide was carried out as usual, affording the desired oligonucleotides as mixtures containing the commonly observed byproducts (failure sequences, benzamide, etc.).

(21) Zhang, W.; Luo, Z.; Chen, C. H.-T.; Curran, D. P. *J. Am. Chem. Soc.* **2002**, *124*, 10443−10450.

10 (30-mer): 5′-**FDMT-T**TTCTCTAGACAATTGTGCAATACGTCTTT
11 (50-mer): 5′-**FDMT-T**TTCTGTTGACAATTTATCATCGGTCGTAT-AATGTGTGGAATTGGTCTTT
12 (75-mer): 5′-**FDMT-T**TTCTGGTTAAGGTGTGTATATGCTCGGC-TACTAATTAGTGAGTATTATTCTCGCTACT-ATTAACAGTTGTCTTT
13 (100-mer): 5′-**FDMT-T**TTCTGGTTAAGGTGTGTAATATGCTCAGC-TACTAATTAACAGTTGTCTAAGCTGGTTAA-CGTGAGTAATATGATCAGCTACTATTTAAC-AGTTGTCTTT

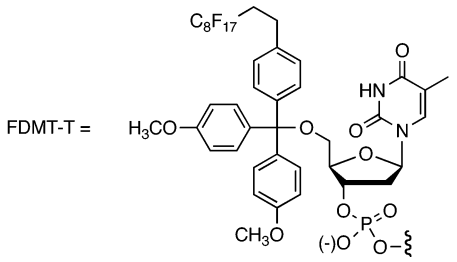


FIGURE 2. Examples of fluorous-tagged oligodeoxyribonucleotides.

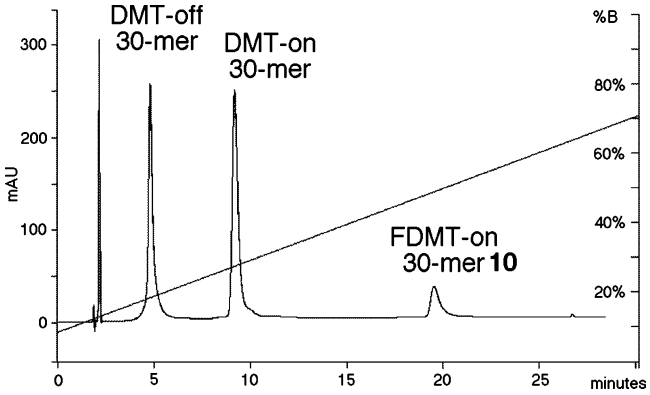


FIGURE 3. HPLC analysis of FDMT-on 30-mer **10** mixed with authentic DMT-on 30-mer and authentic DMT-off 30-mer on Fluoro*Flash* 4.6 × 150 mm column, flow rate = 1 mL/min, mobile phase A = 0.1 M triethylammonium acetate (TEAA), mobile phase B = MeCN.

Fluorous-tagged oligonucleotides were found to be highly retained on a fluorous HPLC adsorbent. To illustrate the magnitude of retention, the FDMT-tagged 30-mer **10** was mixed with authentic samples of the corresponding nonfluorous 5′-DMT-on and DMT-off oligonucleotides (prepared separately) and injected onto a fluorous silica HPLC column.[22] It should be noted that silica-based adsorbents are incompatible with ammonia; hence, evaporation of ammonia from the crude deprotected oligonucleotides was necessary prior to analysis. Figure 3 shows that the fluorous-tagged material **10** is strongly retained over the corresponding DMT-on and DMT-off 30-mers, eluting when the acetonitrile percentage neared 50% in the gradient profile. A key observation from this experiment is that, in relation to a DMT-off oligonucleotide, the FDMT-on oligonucleotide is retained much more strongly than a DMT-on oligonucleotide. With this promising initial result in hand, we proceeded to

(22) A Fluoro*Flash* 4.6 × 150 mm HPLC column was used. For SPE, both 5 μm (90 Å) and 40 μm (60 Å) Fluoro*Flash* silica gel were examined. Fluoro*Flash* is a registered trademark of Fluorous Technologies, Inc.

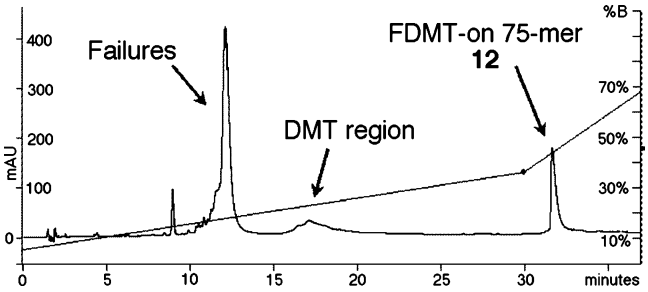


FIGURE 4. HPLC analysis of crude FDMT-on 75-mer **12** (1 μmol scale) before purification. Mobile phase A = 0.1 M aqueous TEAA; mobile phase B = acetonitrile, flow rate = 1 mL/min, column = Waters Spherisorb ODS-2 (5 μm, 4.6 × 150 mm, 1 mL/min).[23] Ammonia was evaporated before injection.

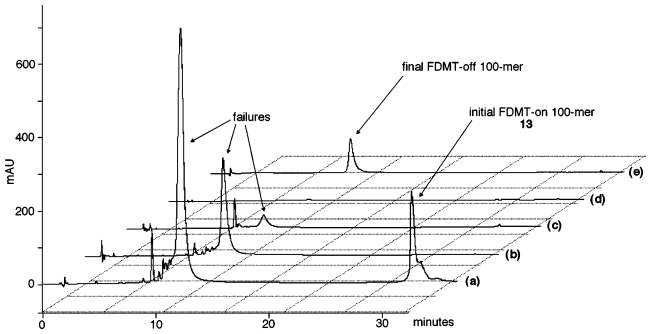


FIGURE 5. HPLC analysis during SPE purification of a 100-mer oligonucleotide (200 nmol scale). Trace (a): Crude synthesis products before purification. Trace (b): Eluate from loading of FDMT-100-mer **13** onto a Fluoro-Pak column, showing that **13** was fully loaded in one pass. Trace (c): Eluate from washing the column with 10% acetonitrile in 0.1 M TEAA followed by water, showing that the remainder of the failure sequences are eluted without eluting the fluorous-tagged oligonucleotide. Trace (d): Eluate from a second washing of the column with 10% acetonitrile in 0.1 M TEAA followed by water, showing that all of the failure sequences had been removed by the first failure wash. Trace (e): Elution of the final FDMT-off 100-mer after on-column detritylation with trifluoroacetic acid. See Figure 4 for HPLC conditions.

examine longer oligonucleotides and SPE purification techniques.

Examination of the crude synthesis mixture obtained from a nonoptimal preparation of the FDMT-on 75-mer **12** (Figure 4) showed an interesting additional benefit of fluorous tagging.[23] In addition to high selectivity in retention of the fluorous-tagged 75-mer over nonfluorous-tagged failure sequences, separation from residual *DMT-bearing* materials was observed. These impurities may result from the incomplete and/or reversible nature of detritylations during the synthesis (which uses DMT-protected monomers until the last coupling). In traditional DMT-on RP-HPLC, such impurities may be overlooked, since they have similar HPLC mobility to the desired DMT-on strand. Shifting the desired strand to a longer retention time offers the advantage that DMT-on impurities, if present, may be removed along with failure sequences.

The utility of the fluorous method for oligonucleotide purification was further illustrated using SPE. Using the fluorous-tagged oligonucleotides shown in Figure 2, SPE purification using commercial fluorous silica gel was examined.[22] Loading the crude ammonia-containing oligonucleotide solutions was unsatisfactory, probably due to degradation of the silica-based adsorbent by ammonia. Removal of the ammonia prior to loading was an improvement, but the recovery of purified oligonucleotides was low. We therefore identified an adsorbent specifically optimized for the purification of fluorous-tagged oligonucleotides, namely Fluoro-Pak adsorbent.[24] This material has fluorinated organic groups bound to an ammonia-stable polymeric resin. Optimization of pore and particle size was necessary for the best SPE performance. To illustrate this method, fluorous affinity purification of the 100-mer oligonucleotide **13** in SPE mode was followed by HPLC (Figure 5). This oligonucleotide was prepared on a 0.2 μmol scale, installing a fluorous-tagged thymidine in the last step using the phosphoramidite **8a**. Cleavage from the support with ammonium hydroxide at room temperature followed by deblocking the nucleobases with ammonium hydroxide at 55 °C gave a solution of the crude products in ammonium hydroxide solution. HPLC analysis of this mixture showed the FDMT-100-mer to be well-separated from failure sequences and other synthesis byproducts [trace (a) in Figure 5]. The following protocol was used for fluorous trityl-on SPE purification of this material: A Fluoro-Pak column containing 100 mg of adsorbent[25] was conditioned by passing through 2 mL of acetonitrile, 2 mL of 0.1 M aqueous triethylammonium acetate (TEAA), and 2 mL of a salt-based loading buffer (to enhance binding).[26] The crude ammonium hydroxide solution was diluted with an equal volume of loading buffer, then passed through the Fluoro-Pak column in a dropwise fashion. Analysis of the eluate [trace (b)] showed that all of the FDMT-100-mer **13** had been loaded onto the column in one pass, leaving much of the undesired materials unbound. Washing the column first with 2 mL of 10% acetonitrile in 0.1 M TEAA and then with water (these two steps constituting a "failure wash") led to the elution of the remainder of the failure sequences without eluting the bound fluorous oligonucleotide [trace (c)]. A second failure wash verified that all of the failure sequences had been removed while the fluorous oligonucleotide remained bound [trace (d)]. This step is not necessary in a routine purification. The retained fluorous-tagged oligonucleotide was then subjected to on-column detritylation by passing through 3 mL of 3% aqueous trifluoroacetic acid (TFA), 1 mL of 0.1

(23) Fluorous-tagged oligonucleotides were also found to be well-retained on traditional reversed-phase (RP) adsorbents such as C_{18} silica; hence, we often use RP-HPLC to analyze fluorous-tagged compounds. It is known that fluorous tags are nonpolar and can be useful in separations using RP adsorbents. However, for maximum selectivity and generality, separations based on the retention of fluorous materials on fluorous adsorbents is the best choice. See ref 15b.

(24) Fluoro-Pak is a trademark of Berry & Associates, Inc.

(25) The results reported herein involved 100 mg of adsorbent for the purification of both 200 nmol and 1 mmol synthesis products, but we have recently found that 75 mg and 150 mg Fluoro-Pak columns are best matched for these two synthesis sizes.

(26) Diluting the ammonia solution with 5% aqueous DMF containing 100 mg/mL NaCl was found to enhance the rate of binding of the fluorous-tagged oligonucleotides to the Fluoro-Pak adsorbent. While the thermodynamics of binding are favorable, the kinetics of binding are slow without this modification. Salt solutions are known to improve binding to RP cartridges. See, for example: (a) *User Guide: Trityl-on Oligonucleotide Purification*; Varian Analytical Instruments: Palo Alto, CA. (b) Reddy, M. P.; Farooqui, F., US Patent 5,770,723, June 23, 1998.

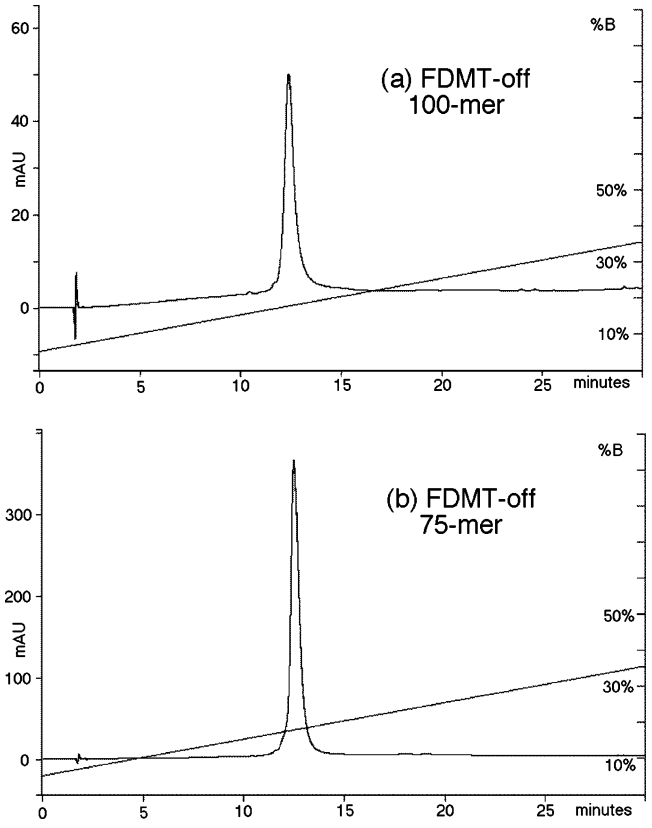


FIGURE 6. HPLC analysis of final, detritylated 100-mer (trace a) and 75-mer (trace b). See Figure 4 for HPLC conditions.

M TEAA to neutralize the acid, and 1 mL of water to remove the excess buffer. Final elution of the trityl-off, fully deprotected 100-mer was accomplished by passing through 1 mL of 10% aqueous acetonitrile, leaving the fluorous trityl byproducts on the column. HPLC analysis of the eluate showed a single peak for the resultant 100-mer [trace (e) in Figure 5 and Figure 6a]. Based on an estimate of the amount of fluorous-tagged 100-mer in the original crude mixture by HPLC, an approximately quantitative recovery of the detritylated 100-mer was obtained from the purification process, producing 6 optical density units (ODU, A_{260} units) of purified material from this particular 0.2 μmol synthesis. Other runs with several different 0.2 μmol synthesis batches of **13** gave 3−6 ODU of purified detritylated 100-mer, representing approximately 76−100% recovery based on estimation of the amount of **13** in the crude mixtures. When considering the HPLC traces shown in Figure 6, the reader should be aware that while the fluorous tagging/purification technique is useful for isolating full-length material without contamination by failure sequences, the resultant fluorous-purified material is still a mixture of products that are not easily resolved by RP-HPLC. This is true for any method that relies on installation of an affinity tag at the 5′ terminus of the oligonucleotide (e.g., DMT-on purification).[4−9] The single peak shown undoubtedly contains, in addition to the desired full-length product, a number of other materials such as deletion oligonucleotides (*n*-1, *n*-2, etc.), since the final phosphoramidite coupling attaches a fluorous-tagged nucleotide to a *preexisting distribution* of the desired chain plus deletion materials, again, a limitation of any such affinity-tagging method.[4−9] Deletion mutations arise from (for example) incomplete capping, which leads to extension of undesired truncated chains. These deletions are not easily resolved by RP-HPLC. Although the fluorous method does not remove deletion mutations (or any other chains that participate in the final coupling with the fluorous phosphoramidite), its ability to remove all failure sequences in one simple SPE operation from even long oligonucleotides is significant, since these contaminants are the major synthesis product in such cases.

TABLE 1. Results of Fluorous Affinity Purification of Oligonucleotides 10−13 with On-Column Detritylation

oligonucleotide[a,b]	A_{260} units (purified)	recovery[c] (%)
30-mer **10**	11−12	75−88
50-mer **11**	9−16	60−100+
	28[d]	71[d]
75-mer **12**	9−11	100+
100-mer **13**	3−6	76−100

[a] A variety of synthesis runs of each oligonucleotide were used. [b] Unless otherwise noted, all purifications were done on 200 nmol synthesis results. [c] The percentage of fluorous-tagged oligonucleotide was estimated by HPLC integration of all oligonucleotides in the crude synthesis product. A percentage of the total initial A_{260} units was thus estimated, from which the recovery of purified, detritylated oligonucleotide was calculated using the final A_{260} unit value. Some values were greater than 100%, likely reflecting the significant error involved in the HPLC integration method for estimating the amount of initial fluorous-tagged oligonucleotide. The recovery values listed in the table must be considered to be approximate due to the limitations of integrating HPLC peaks in complex crude mixtures. [d] A 1 μmol synthesis product was used on 100 mg of adsorbent.[25]

Using the same protocol, fluorous SPE purification of multiple synthesis batches of **10**-**12** gave the detritylated oligonucleotides with excellent recoveries (Table 1; see also Figure 6b for an HPLC trace of purified, detritylated 75-mer derived from **12**). HPLC showed that the above SPE protocol was indeed general, removing all failure sequences prior to on-column detritylation. The amount of oligonucleotide reported in Table 1 (in A_{260} units) will vary significantly from case to case, since it is a function of the quality of the synthesis and the chain length. The more significant data is the recovery: of the available fluorous-tagged oligonucleotide, much of it is recovered in detritylated form after purification, free of failure sequences. This is in contrast to results with DMT-on SPE purification, where both recoveries and the selectivity for removal of failure sequences diminish with increasing oligonucleotide length.

Conclusions

Installation of a fluorous tag onto the 5′ end of an oligonucleotide provides a useful handle for its purification. Of the various implementations of this strategy that are being examined, our initial work using a fluorous dimethoxytrityl group imparts high selectivity in HPLC and SPE separations. Fluorous-tagged materials are highly retained in preference to nonfluorous materials such as failure sequences and DMT-bearing impurities. In SPE mode, complete removal of failure sequences is possible, even with longer oligonucleotides, where such impurities are the major products of nucleic acid synthesis, a result that is not possible to achieve with DMT-

on purification technology. One-pass loading, ammonia compatibility, and on-column detritylation are convenient aspects of this method. High recoveries of failure-free oligonucleotides are observed, even with long oligonucleotides, perhaps due to a combination of the high affinity of the fluorous-tagged oligonucleotide for the Fluoro-Pak adsorbent and the absence of premature detritylation due to the slightly higher stability of the FDMT group. While no method that relies on a 5′ affinity tag can remove deletion mutations or other undesired 5′-hydroxyl-bearing chains that are present when the tag is installed, the fluorous affinity method is useful for removing other impurities, especially the major products (i.e., failure sequences) encountered in the synthesis of long oligonucleotides synthesis. For critical nucleic acid applications such as therapeutics where further purification is required to remove deletion mutations (e.g., anion-exchange HPLC), the fluorous method should be useful as a simple prepurification. For less critical applications, especially the increasing number of applications requiring longer oligonucleotides, the fluorous method offers a way to isolate most or all of the full-length material synthesized, free of failure sequences.

We are currently developing other fluorous-tagged reagents for oligonucleotide purification, including RNA phosphoramidites, modified nucleoside phosphoramidites and fluorous versions of popular labeling reagents.[20]

Experimental Section

General Methods. HPLC analyses of phosphoramidites were performed isocratically using a Waters Spherisorb ODS-2 C_{18} column (5 μm, 4.6 × 150 mm) and the mobile phase (1 mL/min) indicated. HPLC analyses of oligonucleotides were performed on a gradient instrument using (unless otherwise noted) a Waters Spherisorb ODS-2 C_{18} column (5 μm, 4.6 × 150 mm) and the mobile phases (1 mL/min) indicated. ^{13}C NMR spectra of fluorous-tagged compounds are listed as "Partial", since the fluoroalkyl carbons are highly split by the fluorine atoms, and thus are not readily identified. ^{1}H- and ^{13}C NMR spectra of fluorous phosphoramidites are also partially reported due to the added complexity of diastereomers.

4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]-trityl Chloride (2, FDMT-Cl). A solution of methyl 4-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)benzoate **1** (4.85 g, 8.3 mmol, Fluorous Technologies, Inc.) in THF (24 mL) was added over 15 min to an ice-cold solution of 4-methoxyphenylmagnesium bromide (40.6 mL of a 0.5 M solution in THF, 20.3 mmol) in dry THF (41 mL). After the mixture was warmed to rt for 1 h, it was poured into ice−water (50 mL) and extracted with ethyl acetate (50 mL). The organic phase was dried (sodium sulfate) and concentrated in vacuo to afford 5.90 g (94%) of 4,4′-dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl alcohol as a pale amber glass that crystallized upon standing. This material was sufficiently pure to be used in the next step. A sample was purified by chromatography on silica gel (10% ethyl acetate in hexanes), mp 103−105 °C.

Acetyl chloride (8.25 mL, 24.6 mmol) was added to a suspension of 4,4′-dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl alcohol (5.9 g, 7.7 mmol) in cyclohexane (60 mL) and the mixture heated at reflux for 1 h. After being cooled to rt, the solution was concentrated to half volume in vacuo, diluted with pentane (25 mL), and then cooled on an ice bath for 0.5 h. The resulting fine white crystals were collected, washed with pentane (10 mL), and dried overnight in vacuo to give 4.08 g (68%) of the title compound as a white powder: mp 136−138 °C; ^{1}H NMR (500 MHz, CD_3CN) δ 2.39 (2 H, m), 2.92 (2 H, m), 3.80 and 3.82 (6 H, 2 s), 6.81−7.15 (12 H, m); partial ^{13}C NMR (500 MHz, CD_3CN) δ 26.2, 33.0, 55.5 (2 x), 113.2, 113.5, 127.8, 128.0, 128.4, 129.3, 130.3, 131.2, 137.9, 138.8, 159.3. Anal. Calcd for $C_{31}H_{22}O_2F_{17}Cl$: C, 47.43; H, 2.82. Found: C, 47.09; H, 3.00.

5′-*O*-[4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl]thymidine (7a). 4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl chloride (**2**, FDMT-Cl, 3.94 g, 5.02 mmol) was added in three equal portions over 1 h to a solution of thymidine (**6a**, 1.00 g, 4.37 mmol) in dry pyridine (30 mL) at rt. After 18 h, methanol (5 mL) was added. After 15 min, the mixture was concentrated in vacuo and partitioned between ethyl acetate and brine. The organic layer was dried (sodium sulfate) and concentrated to afford an oil, which was purified by silica gel chromatography (60:1 dichloromethane/methanol) to give 3.09 g (73%) of the title compound as a light beige glass: ^{1}H NMR (500 MHz, CD_3CN) δ 1.46 (3 H, s), 2.29−2.49 (4 H, m), 2.88 (2 H, t, J = 8.7 Hz,), 3.36 and 3.46 (2 H, AB_q, J_{AB} = 10.4 Hz, and J_{AX} = 2.5 Hz, J_{BX} = 2.5 Hz), 3.77 and 3.78 (6 H, s), 4.11 (1 H, m), 4.59 (1 H, m), 6.47 (1 H, t, J = 7.6 Hz), 6.82−7.38 (12 H, m), 7.63 (1 H, s), 9.95 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN) δ 12.0, 26.1, 32.9 (t), 41.1, 55.3, 63.9, 72.1, 85.1, 111.2, 113.5, 158.9, 164.4. Anal. Calcd for $C_{41}H_{35}N_2O_7F_{17}$: C, 49.91; H, 3.96; N, 2.87. Found: C, 49.70; H, 3.56; N, 2.83.

5′-*O*-[4,4′-Dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)-trityl]thymidine, 3′-[(2-Cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (8a). A mixture of 5′-*O*-[4,4′-dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl]thymidine **7a** (1.98 g, 2.03 mmol) and *N*,*N*-diisopropylethylamine (1.1 mL, 820 mg, 6.09 mmol) in anhydrous THF (70 mL) was cooled to 0 °C and treated with chloro(2-cyanoethoxy)(diisopropylamino)phosphine (680 μL, 720 mg, 3.05 mmol) in a dropwise fashion. After 5 h at rt, the mixture was diluted with ethyl acetate (150 mL) and the resultant solution was washed with 5% aqueous $NaHCO_3$ (150 mL), dried over Na_2SO_4, and concentrated in vacuo at ≤30 °C. Chromatography of this residue on 60 g of silica gel (previously deactivated with triethylamine, elution with 1:2 then 1:1 ethyl acetate/hexanes) gave 1.90 g (79%) of the title compound as an off-white crisp foam. HPLC analysis (10:1 acetonitrile/0.1 M TEAA) showed two diastereomers in a ratio of 52:48 (t_R = 5.4 and 6 min) and a total purity of >99%: ^{1}H NMR (500 MHz, CD_3CN) δ 1.03−1.17 (14 H, m), 1.48 and 1.50 (3 H, 2 d, J = 0.9 Hz), 2.35−2.45 (4 H, m), 2.52 and 2.64 (2 H, 2 t, J = 6.0 and 6.0 Hz), 2.90 (2 H, m), 3.28 and 3.30 (2 H, 2 m), 3.54−3.76 (2 H, m), 3.75 and 3.76 (6 H, 2 s), 4.04−4.10 (1 H, m), 4.61−4.63 (1 H, m), 6.25 (1 H, dd, J = 14.3 and 7.1 Hz), 6.85−7.47 (13 H, m), 9.14 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN; diastereomers) δ 12.4, 21.1, 21.2, 24.9, 25.0, 44 1, 44.1, 56.0, 59.5, 59.6, 64.2, 64.3, 85.4, 85.5, 74.0, 74.4, 111.4, 111.5, 114.2, 119.4, 119.6 (CN), 159.9, 164.9; ^{31}P NMR (500 MHz, CD_3CN) δ 148.5, 148.6 (2 s, 53:47 ratio). Anal. Calcd for $C_{50}H_{52}N_4O_8F_{17}P$: C, 50.42; H, 4.40; N, 4.70. Found: C, 50.14; H,4.40; N, 4.71.

***N*^{6}-Benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxyadenosine (7b).** 4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl chloride (**2**, FDMT-Cl, 3.50 g, 4.46 mmol) was added in three equal portions over 1 h to a solution of commercial *N*^{6}-benzoyl-2′-deoxyadenosine (**6b**, 1.45 g, 3.88 mmol) in dry pyridine (25 mL) at rt. After 18 h, methanol (5 mL) was added. After 15 min, the mixture was concentrated in vacuo and partitioned between ethyl acetate and brine. The organic layer was dried (sodium sulfate) and concentrated to afford an oil, which was purified by silica gel chromatography (50:1 dichloromethane/methanol) to give 2.48 g (91%) of the title compound as an amber glass: ^{1}H NMR (500 MHz, CD_3CN) δ 2.36 (2 H, m), 2.58 (1 H, m,), 2.88 (2 H, m), 3.01 (1 H, m), 3.75−3.79 (7 H, 2 s and AB_q), 3.95 (1 H, AB_q, J = 12.9 and 1.8 Hz), 4.73 (1 H, m), 4.73 (1 H, m), 6.49 (1 H, t, J = 6.4 Hz), 6.80−8.03 (17 H, m), 8.16 (1 H, s), 8.74 (1 H, s), 9.08 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN) δ 26.1, 32.9 (t), 40.9, 55.4, 63.9, 84.9, 89.4, 113.5, 158.8, 165.2.

Anal. Calcd for $C_{47}H_{38}N_3O_7F_{17}$: C, 52.28, H, 3.55; N, 3.89. Found: C, 52.14; H, 3.84; N, 4.08.

***N*⁶-Benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxyadenosine, 3′-[(2-Cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite (10b).** A solution of *N*⁶-benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxyadenosine (**7b**, 42.1 g, 38.3 mmol) in anhydrous dichloromethane (900 mL) was treated sequentially with 2-cyanoethyl tetraisopropylphosphorodiamidite (15.9 mL, 15.1 g, 50.0 mmol) and 1*H*-tetrazole (1.07 g, 15.3 mmol) at rt. After 4 h, the mixture was washed with 5% aqueous sodium bicarbonate, dried over sodium sulfate, and concentrated in vacuo at ≤30 °C. Chromatography of this residue on silica gel (previously deactivated with triethylamine, elution with 1:1 then 2:1 ethyl acetate/hexanes containing 0.5% triethylamine) gave 35.5 g (71%) of the title compound as an off-white crisp foam. HPLC analysis (10:1 acetonitrile/0.1 M triethylammonium acetate) showed two diastereomers in a ratio of 57:43 (t_R = 4.15 and 5.15 min) and a total purity of >97.5: ^{1}H NMR (500 MHz, CD_3CN) δ 1.07−1.19 (14 H, m), 2.42 (2 H, m), 2.54 and 2.65 (2 H, 2 t, J = 6.0 and 6.0 Hz), 2.60 (1 H, m), 2.86 (2 H, m), 3.08 (1 h, m), 3.30 (2 H, m), 3.60−3.83 (2 H, m), 3.73 (3 H, 2 s), 4.19−4.23 (1 H, m), 4.90 (1 H, m), 6.44 and 6.45 (1 H, 2 dd, J = 5.8 and 5.8 Hz), 6.76−7.99 (17 H, m), 8.25 and 8.26 (1 H, 2 s), 8.57 (1 H, s), 9.33 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN; diastereomers) δ 21.1, 25.0, 44.1, 56.0, 59.6, 85.65, 84.75, 86.36, 86.59, 114.1, 119.5, 119.6, 159.7; ^{31}P NMR (500 MHz, CD_3CN): δ 148.6, 148.7 (2 s, ratio = 59:41). Anal. Calcd for $C_{57}H_{55}N_7O_7F_{17}P$: C, 52.50; H, 4.25; N, 7.52. Found: C, 52.19; H,4.37; N, 7.73.

***N*⁴-Benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxycytidine (7c).** 4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl chloride (**2**, FDMT-Cl, 2.97 g, 3.78 mmol) was added in three equal portions over 1 h to a solution of commercial *N*⁴-benzoyl-2′-deoxycytidine (**6c**, 1.09 g, 3.29 mmol) in dry pyridine (25 mL) at rt. After 18 h, methanol (3 mL) was added. After 15 min, the mixture was concentrated in vacuo and partitioned between ethyl acetate and brine. The organic layer was dried (sodium sulfate) and concentrated to afford an oil, which was purified by silica gel chromatography (50:1 then 25:1 dichloromethane/methanol) to give 3.41 g (96%) of the title compound as a light beige glass: ^{1}H NMR (500 MHz, CD_3CN) δ 2.30−2.42 (3 H, m), 2.75 (1 H, ddd, J = 13.6, 6.0 and 6.0 Hz), 2.89 (2 H, t, J = 8.5 Hz), 3.44 and 3.51 (2 H, AB_q, J_{AB}=10.9 Hz, and J_{AX} = 3.0 Hz, J_{BX} = 3.5 Hz), 3.79 (6H, s), 4.16 (1 H, m), 4.59 (1 H, dd, J = 10.9 and 5.6 Hz), 6.29 (1 H, t, J = 5.4 Hz), 6.18−7.85 (18 H, m), 8.39 (1 H, d, J = 7.5 Hz), 8.85 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN) δ 26.1, 32.9, 42.3, 55.4, 96.8, 113.1, 155.5, 162.5, 165.5. Anal. Calcd for $C_{47}H_{38}N_3O_7F_{17}$: C, 52.28; H, 3.55; N, 3.89. Found: C, 52.14; H, 3.84; N, 4.08.

***N*⁴-Benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxycytidine, 3′-[(2-Cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite (8c).** A solution of *N*⁴-benzoyl-5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-2′-deoxycytidine (**7c**, 54.7 g, 50.7 mmol) in anhydrous dichloromethane (1 L) was treated sequentially with 2-cyanoethyl tetraisopropylphosphorodiamidite (21.0 mL, 19.9 g, 66.1 mmol) and 1*H*-tetrazole (1.42 g, 20.3 mmol) at rt. After 3 h, the mixture was washed with 5% aqueous sodium bicarbonate, dried over sodium sulfate, and concentrated in vacuo at ≤30 °C. Chromatography of this residue on silica gel (previously deactivated with triethylamine, elution with 1:2 then 1:1 ethyl acetate/hexanes containing 0.5% triethylamine) gave 40.0 g (62%) of the title compound. HPLC analysis (15:1 acetonitrile:0.1 M triethylammonium acetate) showed two diastereomers in a ratio of 70:30 (t_R = 5.74 and 6.35 min) and a total purity of >98%: ^{1}H NMR (500 MHz, CD_3CN) δ 0.15−1.18 (14 H, m), 2.40 (2 H, m), 2.38 and 2.57 (2 H, 2 m), 2.54 and 2.64 (2 H, 2 t, J = 6.0 and 6.0 Hz), 2.92 (2 H, t, J = 8.3 Hz), 3.41 (2 H, m), 3.58−3.70 (2 H, m), 3.76 (3 H, s), 3.77 (3 H, s), 4.14−4.16 (1 H, m), 4.65−4.67 (1 H, m), 6.11−6.17 (1 H, 2 dd, J = 6.6 and 4.6 Hz), 6.86−7.95 (18 H, m), 8.25 and 8.30 (1 H, 2 d, J = 7.5 and 7.5 Hz), 9.18 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN; diastereomers) δ 21.1, 25.0, 44.1, 56.0, 59.5, 59.7, 72.4, 72.5, 86.3, 86.5, 87.6, 87.8, 114.3, 119.5, 119.6, 159.9, 163.7; ^{31}P NMR (500 MHz, CD_3CN) δ 148 8 (s). Anal. Calcd for $C_{56}H_{55}N_5O_8F_{17}P$: C, 52.55; H, 4.33; N, 5.47. Found: C, 52.44; H,4.68; N, 5.76.

5′-*O*-[4,4′-Dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-*N*²-isobutyryl-2′-deoxyguanosine (7d). 4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl chloride (**2**, FDMT-Cl, 2.47 g, 3.44 mmol) was added in three equal portions over 1 h to a solution of commercial *N*²-isobutyryl-2′-deoxyguanosine (**6d**, 1.07 g, 2.99 mmol) in dry pyridine (25 mL) at rt. After 18 h, methanol (3 mL) was added. After 15 min, the mixture was concentrated in vacuo and partitioned between ethyl acetate and brine. The organic layer was dried (sodium sulfate) and concentrated to afford an oil, which was purified by silica gel chromatography (50:1 then 25:1 dichloromethane/methanol) to give 3.11 g (96%) of the title compound as light beige glass: ^{1}H NMR (500 MHz, CD_3CN) δ 1.07 (3 H, d, J = 6.7 Hz), 1.33 (3 H, d, J = 6.8 Hz), 1.24 (1 H, m), 2.30−2.46 (3 H, m), 2.72−2.92 (3 H, m), 3.29 and 3.34 (2 H, AB_q, J_{AB} = 10.1 Hz, and J_{AX} = 3.7 Hz, J_{BX} = 3.7 Hz), 3.73 (3 H, s), (3H, s), 4.13 (1 H, m), 4.71 (1 H, m), 6.19 (1 H, dd, J = 6.5 and 6.5 Hz), 6.74−7.38 (12 H, m), 7.83 (1 H, s, 8-H), 9.13 (1 H, br s), 12.16 (1 H, br s); partial ^{13}C NMR (500 MHz, CD_3CN) δ 18.9, 19.0, 26.1, 32.9 (t), 55.4, 64.3, 113.4, 156.1, 158.8, 180.2. Anal. Calcd for $C_{45}H_{40}N_5O_7F_{17}$: C,49.78; H, 3.71; N, 6.45. Found: C, 49.68; H, 4.10; N, 6.59.

5′-*O*-[4,4′-Dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-*N*²-isobutyryl-2′-deoxyguanosine, 3′-[(2-Cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite (8d). A solution of 5′-*O*-[4,4′-dimethoxy-4″-(1*H*,1*H*,2*H*,2*H*-perfluorodecyl)trityl]-*N*²-isobutyryl-2′-deoxyguanosine (**7d**, 51.8 g, 47.7 mmol) in anhydrous dichloromethane (1 L) was treated sequentially with 2-cyanoethyl tetraisopropylphosphorodiamidite (19.7 mL, 18.7 g, 62.0 mmol) and 1*H*-tetrazole (1.34 g, 19.1 mmol) at rt. After 4 h, the mixture was washed with 5% aqueous sodium bicarbonate, dried over sodium sulfate, and concentrated in vacuo at ≤30 °C. Chromatography of this residue on silica gel (elution with 10:2:1 chloroform/hexanes/triethylamine) gave 35.6 g (58%) of the title compound as an off-white crisp foam whose purity was found to be >99% by HPLC. Impure fractions were combined and evaporated to give another 17 g of material, which was rechromatographed, eluting with 5:5:1 hexanes/ethyl acetate/triethylamine followed by ethyl acetate containing 0.5% triethylamine to afford another 15.7 g (26%; combined yield 84%) of the title compound, again as an off-white crisp foam. HPLC analysis (10:1 acetonitrile/0.1 M triethylammonium acetate) showed two diastereomers in a ratio of 48:52 (t_R = 5.67 and 6.15 min) and a total purity of >99: ^{1}H NMR (500 MHz, CD_3CN) δ 1.06−1.17 (21 H, m), 2.41−2.65 (5 H, m), 2.84−2.88 (3 H, m), 3.31 (2H, m), 3.56−3.78 (2 H), 3.74 (6 H, 3 s), 4.20 (1 H, m), 4.68 (1 H, m), 6.23 and 6.24 (1 H, 2 dd, J = 6.6 and 6.6 Hz), 6.75−7.33 (12 H, m), 7.81 and 7.82 (1 H, 2 s); partial ^{13}C NMR (500 MHz, CD_3CN; diastereomers) δ 19.2, 19.3, 19.4, 24.9, 25.0, 33.0 (t), 44.1, 56.0, 59.3, 65.0, 65.1, 85.3, 85.4, 86.9, 87.0, 114.0, 119.6, 119.7, 156.4, 159.8, 180.9, 181.0; ^{31}P NMR (500 MHz, CD_3CN) δ 148.4, 148.6 (2 s ratio = 50:50). Anal. Calcd for $C_{54}H_{57}N_7O_8F_{17}P$: C, 50.40; H, 4.47; N, 7.62. Found: C, 50.25; H,4.79; N, 7.84.

Relative Rates of Detritylation of 5′-*O*-[4,4′-Dimethoxy-4″-[4-(1*H*,1*H*,2*H*,2*H*)-perfluorodecyl]trityl]thymidine (7a) and 5′-*O*-(4,4′-Dimethoxytrityl)thymidine (9). The procedure of Takenaka and co-workers was used.[8n]

Determination of λ_{max} of the Trityl Cations. The absorption spectra of the trityl cations derived from **7a** and **9** were determined as follows: A solution of 3.2 mg of **7a** in 3% trichloroacetic acid (TCA) in dichloromethane was allowed to stand for 5 min and then diluted to 1/32 of the original concentration with 3% TCA/dichloromethane. The absorption spectrum showed λ_{max} = 504 nm (21 °C). Similarly, 1.4 mg of

9 was subjected to the same procedure, and the resultant sample also showed $\lambda_{max} = 504$ nm (21 °C).

Rate Constant for Detritylation of 7a. A stock solution of **7a** (1 mg/mL) in acetonitrile was prepared. A reference cuvette was filled with 2 mL of 80% acetic acid/acetonitrile. A sample cuvette was charged with 1.94 mL of the same solution, and the absorbance was zeroed against the reference cuvette. To this was added 63 μL of the stock solution of **7a**, mixing well by drawing the solution into the pipet and discharging it repeatedly. The change in absorbance at 504 nm (versus the reference cuvette) was monitored against time at 21 °C. Twenty-seven data points were taken between 90 and 7200 s. The data were treated as a pseudo-first-order irreversible reaction, and the rate constant was determined using the equation $\ln(1 - A_t/A_\infty) = -kt$, plotting $\ln(1 - A_t/A_\infty)$ versus time in seconds, resulting in a linear plot ($R^2 = 0.9977$) with the negative of the slope revealing $k = 4.75$ s^{-1}; $t_{1/2} = 1459$ s.

Rate Constant for Detritylation of 9. A stock solution of **9** (1 mg/mL) in acetonitrile was prepared. A reference cuvette was filled with 2 mL of 80% acetic acid/acetonitrile. A sample cuvette was charged with 1.94 mL of the same solution, and the absorbance was zeroed against the reference cuvette. To this was added 63 μL of the stock solution of **9**, mixing well by drawing the solution into the pipet and discharging it repeatedly. The change in absorbance at 504 nm (versus the reference cuvette) was monitored against time at 21 °C. Twenty-five data points were taken between 60 and 4500 s. The data were treated as a pseudo-first-order irreversible reaction, and the rate constant was determined using the equation $\ln(1 - A_t/A_\infty) = -kt$, plotting $\ln(1 - A_t/A_\infty)$ versus time in seconds, resulting in a linear plot ($R^2 = 0.9962$) with the negative of the slope revealing $k = 5.89$ s^{-1}; $t_{1/2} = 1176$ s.

Synthesis of Oligonucleotides. Oligonucleotides were prepared on an Expedite 8909 synthesizer using standard 2-cyanoethyl-*N,N*-diisopropylphosphoramidite chemistry. With the exception of fluorous phosphoramidites, all reagents, phosphoramidites, and solid supports were purchased from Glen Research Corp. Standard 5′-*O*-DMT phosphoramidites were used to prepare the oligonucleotide up through the penultimate monomer, after which the appropriate 5′-*O*-FDMT phosphoramidite was used to install the final fluorous-tagged nucleotide. Nucleobase protecting groups were benzoyl for dA and dC, and isobutyryl for dG. The syntheses were carried out on either 0.2 μmol or 1 μmol scale using 1000 Å CPG solid supports bearing a 3′-linked 5′-*O*-DMT-thymidine, with the exception of 100-mer synthesis, which was carried out on 2000 Å support. Reagents recommended by the manufacturer were employed, with the exception of the substitution of THF/pyridine/Ac_2O for Cap A and 16% *N*-methylimidazole/THF for Cap B. The manufacturer's protocols, dilutions, and coupling times were used, except that capping was performed for 75 s and fluorous phosphoramidites were coupled for ≥3 min. Syntheses were performed in the trityl-on mode in order to retain the final FDMT group, and the oligonucleotides were cleaved from the CPG support with concentrated ammonium hydroxide at rt for 1 h (3 mL total, pushed through in four equal portions, 15 min each). Nucleobase deprotection was accomplished by heating the resultant ammonium hydroxide solution at 55 °C for 16−24 h. Yields were determined by UV absorbance at 260 nm according to standard practice.

Fluorous Purification of Oligonucleotides: General Procedure. A Fluoro-Pak tube (Berry & Associates, Inc.) containing 100 mg of a pH-stable, fluorinated polymeric adsorbent was used in the following steps, wherein a 200 nmol synthesis product was purified. More recent studies have shown that 75 mg of adsorbent behaves identically; commercial Fluoro-Pak columns contain 75 mg of adsorbent for purifying up to 200 nmol, whereas Fluoro-Pak II columns contain 150 mg of adsorbent, and are useful for the purification of up to 1 μmol of oligonucleotide. *Solutions were passed through the tube at a flow rate of 2 s per drop (unless otherwise noted)* with pressure from a disposable PE/PP syringe or a compressed gas line (air or inert gas), or using vacuum via a commercial vacuum box. It is important to adhere to flow rate directions. "Loading buffer" refers to 100 mg/mL NaCl in water containing 5% *N,N*-dimethylformamide. TEAA refers to triethylammonium acetate. A specific example of the purification protocol is given below, wherein the fluorous-tagged 100-mer **13** was subjected to purification. Before purification, analysis of the crude mixture by UV measurement at 260 nm and HPLC integration indicated that about 6 optical density units (ODU) of the total mixture could be assigned to the fluorous-tagged 100-mer **13**.

Fluorous Affinity Purification Protocol. (1) To condition the adsorbent, the following were passed through the tube to waste: 2 mL of acetonitrile, 2 mL of 0.1 M TEAA, and 2 mL of loading buffer (see above). (2) Without removing the ammonia used in the deblocking step, the crude deprotected oligonucleotide **13** (200 nmol scale) was diluted with an equal volume of loading buffer. The resultant solution was passed through the Fluoro-Pak column at a rate of *5 s per drop*. The eluate was collected and analyzed by HPLC (Figure 5b), showing that the fluorous oligonucleotide had been fully retained by the adsorbent, whereas a substantial amount of the nonfluorous-tagged materials had eluted. The flow rate during loading is important. Other experiments showed that a loading rate of 2 s/drop could be employed, which loaded most of the fluorous oligonucleotide. In those cases, passing the eluate through the tube a second time assured complete binding. (3) 2 mL of 10% acetonitrile in 0.1 M TEAA followed by 2 mL of water was passed through the tube. The combined eluates were collected and analyzed by HPLC (Figure 5c), showing that failure sequences were eluted without stripping the fluorous-tagged oligonucleotide from the column. In a separate experiment, two 2 mL elutions with 10% acetonitrile in 0.1 M TEAA were carried out before the water wash to verify that the failure sequences were entirely removed in the first 2 mL elution (Figure 5d). (4) 3 mL of 3% aqueous TFA (trifluoroacetic acid) was passed through the tube to waste, followed by 1 mL of 0.1 M TEAA and 1 mL of water. (5) One milliliter of 10% aqueous acetonitrile in water was passed through the tube, collecting the eluate in an Eppendorf tube. Flow rates faster than 2 s/drop are not recommended. Other experiments showed that a smaller volume (about 600 μL) could be used if 20−30% aqueous acetonitrile was used instead. Analysis by HPLC (Figure 5e and Figure 6a) showed one peak. UV measurement at 260 nm showed that 6 ODU of purified oligonucleotide was obtained, representing an approximately quantitative recovery of the detritylated 100-mer.

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