Fluorous Affinity Purification of Oligonucleotides

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User Guide for the Fluorous Affinity Purification of Oligonucleotides

Fluoro-Pak™ Columns and Fluorous Phosphoramidites

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1. Introduction

Overview. This User Guide describes a quick and simple affinity-based method for the purification of oligonucleotides that relies on the strong interaction of fluorous-tagged oligonucleotides with the fluorinated adsorbent present in Fluoro-Pak™ columns. Fluorous affinity purification is operationally similar to DMT-on purification using a reverse-phase (RP) adsorbent, e.g. RP cartridge purification.

The fluorous affinity purification method affords the following advantages:

• One-pass loading without ammonia removal.

• High selectivity for removal of failure sequences, even with long oligonucleotides. There is a large difference in the retention of fluorous and non-fluorous materials.

• Nearly quantitative recovery, even with long oligonucleotides (ca. 100-mers), free of failure sequences.

• Does not require adopting new techniques.

What does "fluorous" mean? Highly fluorinated organic compounds are both hydrophobic and lipophobic, preferring instead to associate with other fluorinated substances. For example, perfluorohexane is insoluble in both water and hexane. Organic molecules that have both an organic domain (e.g. an oligonucleotide) and a perfluoroalkyl domain (e.g., a linear perfluoroalkyl "ponytail") are known as *fluorous molecules* (not to be confused with fluorescent molecules), and may be separated from non-fluorous molecules by interaction with fluorinated separation media such as Fluoro-Pak™ columns.

Description of the fluorous purification system. For oligonucleotide purification, a fluorous tag is installed, allowing the tagged oligonucleotide to be selectively retained on the Fluoro-Pak™ column. The affinity of the fluorous tag on the oligonucleotide for the Fluoro-Pak™ adsorbent is high, much higher than the affinity of a DMT group for an RP adsorbent. The Fluoro-Pak™ adsorbent has the additional advantage that non-fluorous components of the mixture are less well retained than they would be with normal RP adsorbents. This User Guide describes the use of the fluorous purification system in a solid-phase extraction (SPE) mode, where conditions are chosen to promote complete adsorption of the fluorous-tagged oligonucleotide onto the Fluoro-Pak™ column, allowing non-fluorous impurities to be washed away. Removal of the fluorous tag then affords the desired oligonucleotide.

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The present implementation of the fluorous purification technique involves oligonucleotides bearing a fluorous dimethoxytrityl (FDMT) group at the 5' terminus ("FDMT-on"). These fluorous-tagged oligonucleotides are made using standard DMT-bearing phosphoramidites except for the last coupling step, where the appropriate 5'-*O*-FDMT nucleoside phosphoramidite (Figure 1) is used for the installation of a *single* FDMT-on nucleotide at the 5' terminus.
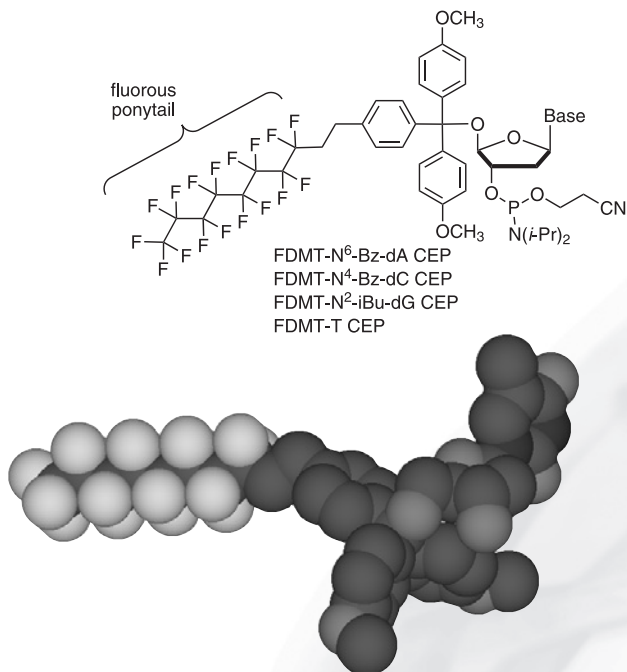

FDMT-N^6-Bz-dA CEP
FDMT-N^4-Bz-dC CEP
FDMT-N^2-iBu-dG CEP
FDMT-T CEP

Figure 1. Fluorous dimethoxytrityl (FDMT) nucleoside phosphoramidites for 5'-fluorous tagging of oligonucleotides.

Comparison to DMT-on purification. RP cartridge purification of DMT-on oligonucleotides is limited to relatively short oligomers, typically ≤30-40-mers, since the relative hydrophobic contribution of the lipophilic DMT group diminishes as the oligonucleotide chain increases, therefore diminishing the affinity of the target compound to the adsorbent. This results in lower overall yields and a lower selectivity of the adsorbent for the desired DMT-on oligonucleotide over non-DMT failure sequences. While DMT-on RP purification of long oligonucleotides may provide some material, yields are low and failure sequences are likely to be a significant contamination. Another problem with DMT-on purification is that the ammonia present in the crude oligonucleotide (from nucleobase deblocking) is 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. All of these limitations are overcome using fluorous affinity purification, since the affinity of the fluorous-tagged oligonucleotide for the Fluoro-Pak™ adsorbent is so strong.

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2. Preparation of Fluorous-Tagged Oligonucleotides

Of the various methods for installing a fluorous tag onto an oligonucleotide, the most common is to use one of the four basic FDMT-bearing 2'-deoxyribonucleoside phosphoramidites in the last coupling step (see Figure 1). See www.berryassoc.com for other fluorous reagents and their specific installation protocols.

To install a fluorous phosphoramidite at the 5' terminus:

Since the FDMT nucleoside phosphoramidite is installed only once (i.e., at the 5' terminus), install it in a custom amidite position and use normal DMT phosphoramidites in the other four positions.

Dilution: Dilute the FDMT nucleoside phosphoramidite with anhydrous acetonitrile to the standard concentration required for your synthesizer.

Coupling protocol: Standard coupling times work well, but maximum yields are obtained if the coupling is run for ≥3 minutes. Times up to 15 minutes may be used, but are not necessary.

Final deblocking: The synthesis should be performed in the trityl-on mode in order to retain the FDMT group at the 5' terminus.

Cleavage from the solid support and nucleobase deprotection: No changes from standard protocols are required. It is not necessary to remove the excess ammonia. Indeed, retaining the ammonia is preferable to keep the solution basic and retain a maximum amount of FDMT-on material.

3. Fluoro-Pak™ Columns

Berry & Associates' Fluoro-Pak™ adsorbent has fluorinated organic groups bound to a pH-stable polymeric resin. Multiple pore and particle sizes have been evaluated in order to provide optimal performance with reasonable back-pressure.

Two columns are available:

Fluoro-Pak™ Columns: Each column contains 75 mg of adsorbent, and may be used for up to 0.2 micromole purifications.

Fluoro-Pak™ II Columns: Each column contains 150 mg of adsorbent, and are designed for 1 micromole purifications.

Both columns use 3 mL tubes with an open top and a male Luer fitting at the bottom. A snap-in female Luer adaptor for the top of the column is available for users who wish to employ a syringe for elution.

Please inquire for bulk adsorbent, alternate column formats, or Fluoro-Pak™ HPLC columns.

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4. Fluorous Purification Protocol

Solutions may be passed through the column using air pressure from a syringe or an air line. Alternatively, a vacuum box may be used to draw the solutions through the column. The following protocol illustrates the syringe method.

4.1 Materials needed:
See Section 5 for solution preparation.

Quantity	Item
1	Fluoro-Pak™ or Fluoro-Pak™ II column
1	Luer adaptor (available with columns)
1	3 mL PE/PP syringe with male Luer tip and needle
1	20 mL vial for catching waste
2 mL*	acetonitrile (MeCN)
3 mL*	0.1 M aqueous TEAA (triethylammonium acetate)
2-5 mL*	Loading Buffer (Berry & Associates # LB-7100, or see "Solutions" below)
3 mL*	3% aqueous TFA (trifluoroacetic acid)
2 mL*	10% MeCN in 0.1 M aqueous TEAA
1 mL*	20% MeCN in water

*Note: Similar volumes are used for both column sizes.

4.2 Sample preparation

Without removing the ammonia used in the deblocking step, dilute the crude deprotected FDMT-on oligonucleotide with an equal volume of Loading Buffer. The final sample volume should be 2-6 mL.

4.3 Column setup and working with solutions

Clamp the Fluoro-Pak™ tube in a vertical position with a 20 mL vial underneath to catch waste. Attach a Luer syringe adaptor (Figure 2) to the top of the tube. The hole in the Luer adaptor allows the addition of liquids to the column via syringe needle (Figure 3), obviating the need to remove and replace the Luer adaptor each time a new solution is added. After introducing the desired solution to the top of the column, remove the syringe needle, attach the syringe barrel (filled with air) directly to the Luer adaptor, and apply air pressure using the plunger (Figure 4). Do not attempt to use the syringe to both introduce a solution and pressurize the column. A flow rate of 2 seconds per drop should be used except during loading, which is best accomplished with a slower flow rate (see below). Compressed air or a vacuum manifold may also be used to elute the column.

NOTE: Rinse the syringe and needle with water before handling a new solution, or use a new syringe.

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Figure 2. Snap-in Luer adaptor for syringe use. Available with 10-pack of Fluoro-Pak™ columns.

Figure 3. Introducing a solution to the column. Use a syringe with needle to pass the solution through the hole in the Luer adaptor.
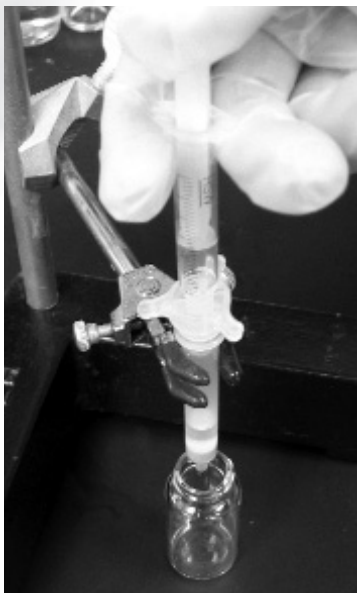

Figure 4. With the desired solution already present in the column, use a syringe to pressurize the column with air, thereby eluting the solution.

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4.4 Conditioning the column

Pass the following through the Fluoro-Pak™ column to waste at a flow rate of 2 seconds per drop:

1. 2 mL of MeCN

2. 2 mL of 0.1 M aqueous TEAA

3. 2 mL of Loading Buffer

The first 2-3 drops of MeCN may be discolored.

4.5 Loading the FDMT-on oligonucleotide

Using the sample as prepared in 4.2 above, pass it through the pre-conditioned column at a flow rate of **5 seconds per drop,** conditions that allow one-pass loading.

It is essential that the Loading Buffer is used and a slow loading rate is employed. While the interaction of the fluorous-tagged oligonucleotide for the Fluoro-Pak™ adsorbent is thermodynamically favorable, the kinetics of adsorption are important. Faster flow rates necessitate multiple loading passes, and the absence of Loading Buffer is detrimental to binding.

HPLC analysis shows that most of the failure sequences do not load and are thus found in the waste, whereas little or no fluorous-tagged material remains unbound. The following step assures that any remaining failure sequences are removed from the column.

4.6 Eluting remaining failure sequences

Pass the following through the Fluoro-Pak™ column to waste at a flow rate of 2 seconds per drop:

1. 2 mL 10% MeCN in 0.1 M aqueous TEAA

2. 2 mL of water

The acetonitrile/TEAA solution selectively washes the remaining non-fluorous compounds (failure sequences) from the resin, leaving the FDMT-on oligonucleotide bound, even with 100-mers. This is in contrast to DMT-on RP purification, where failure sequences and DMT-on material are retained to an increasingly similar degree as the chain length increases.

The water washes the buffer out of the resin prior to the acid-catalyzed detritylation in the next step.

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4.7 On-column detritylation

Pass the following through the Fluoro-Pak™ column to waste at a flow rate of 2 seconds per drop:

1. 3 mL 3% aqueous TFA

2. 1 mL of 0.1 M aqueous TEAA

3. 1 mL of water

The TFA cleaves the FDMT group from the full-length oligonucleotide. A faint yellow color may appear on the column due to the trityl cation, but the absence of coloration should not be taken as evidence of a problem. Due to the nature of the Fluoro-Pak™ adsorbent, the bright orange color of the trityl cation is not observed as it is in DMT-on RP purification.

The TEAA wash neutralizes the residual TFA and ensures that the detritylated oligonucleotide has lipophilic triethylammonium counterions and is thus retained on the support during the subsequent water wash, which removes excess TEAA buffer.

4.8 Elution of the final detritylated oligonucleotide

Pass the following through the Fluoro-Pak™ column at 2 seconds per drop (it is important to be patient!), collecting the eluate in an appropriate sample tube:

1 mL of 20% acetonitrile in water

Determine the optical density units at 260 nm to quantify the final oligonucleotide. Use as-is or lyophilize for short-term storage.

A further 1 mL of 20% acetonitrile may be collected and assayed at 260 nm in the event of an unusually strongly retained oligonucleotide. Also, a higher concentration of acetonitrile (e.g. 30-50%) may be used in the first elution if a smaller final volume is required. The fluorous trityl by-product from detritylation is retained on the column, even with high acetonitrile concentrations (50+%).

If the oligonucleotide needs to be stored, add 100 mL of 10x TE buffer (100 mM Tris, 10 mM EDTA, pH 8) and store at 4 °C for a ready-use solution, or freeze at -20 °C for longer periods.

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5. Solutions

For all solutions, HPLC grade water, DMF, and acetonitrile are recommended. Molecular biology grade sodium chloride (e.g. Sigma #S3014) should be employed.

5.1 Loading buffer

Use Berry & Associates Loading Buffer (Part No. LB-7100), or prepare as follows: (a) Dissolve 20 g of sodium chloride in about 100 mL of water in a 200 mL volumetric flask. (b) Add 10 mL of *N,N*-dimethylformamide (DMF). (c) Dilute the resultant solution to 200 mL with water.

5.2 0.1 M Aqueous triethylammonium acetate (TEAA) buffer

Dilute 10 mL of commercial 2.0 M TEAA buffer (e.g. from Glen Research) to 200 mL with water.

5.3 3% Aqueous trifluoroacetic acid (TFA)

Add 3 mL of protein sequencing grade trifluoroacetic acid (caution!) to about 50 mL of water in a 100 mL volumetric flask. Dilute to 100 mL with water.

5.4 Failure wash buffer (10% acetonitrile in 0.1 M TEAA)

Add 10 mL of acetonitrile to a volumetric flask and dilute to 100 mL with 0.1 M TEAA buffer.

5.5 Elution solvent (20% acetonitrile in water)

Add 20 mL of acetonitrile to a volumetric flask and dilute to 100 mL with water.

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6. Frequently Asked Questions

6.1 What happens if I allow the column to go dry?

Ideally, solutions should be eluted until the meniscus falls to the top of the adsorbent bed and no further, but control experiments show that running the column to dryness does not significantly affect overall performance.

6.2 Can the Fluoro-Pak™ columns be reused?

The columns are meant to be disposed after each use. Fluorous trityl by-products are retained on the column, and it is difficult to assure that there will be no contamination in multiple runs.

6.3 Are deletion sequences removed?

Fluorous affinity purification involves the selective retention of any oligonucleotide that has a 5'-FDMT group. It is ideal for separating these compounds from the failure sequences that result from incomplete coupling throughout the synthesis, i.e., those that are successfully capped by acetic anhydride and thus cannot couple further. However, it should be recognized that there are still undesired FDMT-on oligonucleotides in the final crude synthesis product, and these will not be discriminated by fluorous affinity purification. For example, incomplete capping is a common problem in oligonucleotide synthesis. Uncoupled material that is also uncapped leaves a 5'-hydroxyl group that then is extended in the next coupling cycle to produce a final oligonucleotide with a deleted base. This n-1 strand (or n-2, etc.) will lead to a final FDMT-on oligonucleotide that differs from the target strand by one or more base. Nonetheless, failure sequences (which are removed by fluorous affinity purification) are a major contaminant, especially with longer oligonucleotides, where they are the major products of the synthesis.

6.4 How can I assay the amount of FDMT-on oligonucleotide present in my crude synthesis product?

Analytical RP-HPLC can be used, since FDMT-on oligonucleotides are also well-retained on RP adsorbents. Removing the ammonia is required before injection onto a silica-based column. For example, an FDMT-on 75-mer elutes at about 30-33 minutes from a 4.6 x 150 mm C_{18} RP silica column using a flow rate of 1 mL/min and a gradient of 5% to 35% acetonitrile/0.1 M TEAA over 30 min followed by ramping to 80% acetonitrile/0.1 M TEAA over 10 min (See Figure 5). Under these conditions, failure sequences elute at about 10-12 minutes. Some DMT-bearing materials at around 15-20 minutes may be observed, especially for long oligonucleotides. These impurities result from the incomplete and/or reversible nature of detritylations during synthesis. In traditional DMT-on purifications, these impurities are often not noticed, since they appear in the same reagion as the final DMT-on strand. Thus, a further advantage of the fluorous affinity purification method is potential resolution from DMT-on impurities as well as failure sequences.
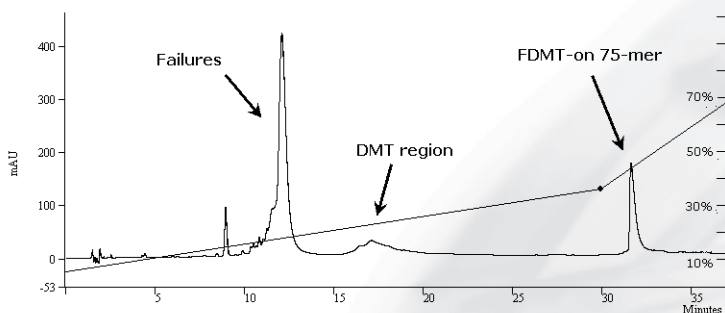

Figure 5. HPLC analysis of a crude mixed-base 75-mer (1 micromole scale) before fluorous affinity purification. Mobile A = acetonitrile; mobile B = 0.1 M aqueous TEAA, flow rate = 1 mL/min, column = 4.6 x 150 mm C_{18} RP silica.

6.5 Can I use HPLC to purify the FDMT-on oligonucleotide and remove the trityl later? Does the FDMT group behave like a DMT group in detritylations?

If you wish to use HPLC to isolate FDMT-on oligonucleotides and detritylate later, an RP HPLC column may be used, or for maximum selectivity, contact Berry & Associates about a Fluoro-Pak™ HPLC column. Yields of FDMT-on oligonucleotides will be diminished, since the FDMT group is lost to some degree upon manipulation, just as a DMT group is.

After purification, the FDMT group may be removed using the same conditions (e.g. aqueous acetic acid) that are used for DMT removal. The kinetics of removal of the FDMT and DMT groups have been measured and are essentially the same; the DMT group comes off 1.2 faster than the FDMT group in 80% acetic acid/acetonitrile. The UV absorbance maximum of the FDMT cation (504 nm) is the same as the DMT cation under these conditions.

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6.6 What sort of yields may I expect from fluorous affinity purification?

The short answer is that this method will recover most or all of the available full-length material. The actual amount isolated will depend on the overall synthesis yield, which is subject to the quality of the synthesis as well as the length of the oligomer, since the yield decays as chain length increases.

Berry & Associates have examined a variety of oligonucleotides in lengths ranging from 30 to 100 nucleotides. In each case, HPLC was used to estimate the amount of fluorous-tagged oligonucleotide as a percentage of the total oligonucleotide content, and the final yields of purified, detritylated product reflected 70-100% recovery of the oligonucleotide derived from the FDMT-on material in the crude mixture. For example, from a 0.2 micromole synthesis of a sample mixed-base 100-mer, it was estimated that six A_{260} units of the crude material could be assigned to FDMT-on material, the rest being failure sequences, which are the major products of a long synthesis. After Fluoro-Pak™ purification, six A_{260} units of purified, detritylated oligonucleotide were isolated, reflecting a quantitative recovery of available material. Other examples on 0.2 micromole scale include 9-16 A_{260} units (60-100%) of a 50-mer and 9-11 A_{260} units (nearly quantitative) of a 75-mer. See Figure 6 for an RP-HPLC trace of the final 75-mer. About 30 A_{260} units were recovered from a 1 micromole synthesis of a 100-mer, and 44 A_{260} units from a 75-mer. Again, the amount of oligonucleotide recovered will depend on the quality of the synthesis, so it important to realize that the fluorous affinity purification method will recover a high level of full-length material, whether that amount is large or small.
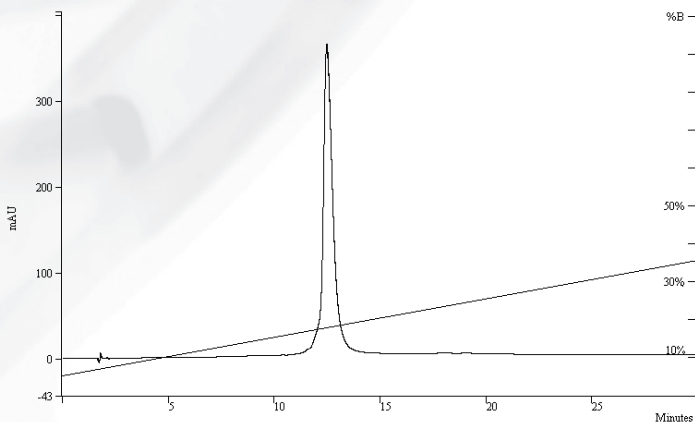

Figure 6. HPLC analysis of a mixed-base 75-mer after fluorous affinity purification (FDMT removed by on-column detritylation). Mobile A = acetonitrile; mobile B = 0.1 M aqueous TEAA, flow rate = 1 mL/min, column = 4.6 x 150 mm C_{18} RP silica.

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7. Ordering Information

Item	Catalog No.	Size/pack
Phosphoramidites*		
FDMT-N^6-Bz-dA CEP	FL-1000	
		100 µmol
		250 µmol
		500 µmol
FDMT-N^4-Bz-dC CEP	FL-1100	
		100 µmol
		250 µmol
		500 µmol
FDMT-N^2-iBu-dG CEP	FL-1200	
		100 µmol
		250 µmol
		500 µmol
FDMT-T CEP	FL-1300	
		100 µmol
		250 µmol
		500 µmol
Columns		
Fluoro-Pak™ columns	FP-7210	Pack of 10
	FP-7210-A	Pack of 10
	(with Luer adaptor)	
Fluoro-Pak™ II columns	FP-7220	Pack of 10
	FP-7220-A	Pack of 10
	(with Luer adaptor)	
Solutions		
Loading Buffer	LB-7100	200 mL

* See www.berryassoc.com for other fluorous reagents.

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8. Legal

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In addition, the use of these products is licensed under U.S. Patents 6,156,896; 5,859,247; and 5,777,121 and one or more pending patents owned or controlled by Fluorous Technologies, Inc.

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