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PURIFICATION TECHNIQUES

Advances in the chemistry of synthesis now allow rapid, facile and efficient production of oligonucleotides. However, purification can be time-consuming and occasionally ineffective. In some applications, there is no need for purified product. When purification is necessary, two traditional techniques are generally used: polyacrylamide gel electrophoresis (PAGE) and high performance liquid chromatography (HPLC). While the choice of technique may depend simply on previous experience or access to equipment, there are some basic advantages to both techniques.

PAGE

PAGE and its cousin capillary gel electrophoresis (CE) are probably most often used for the analysis of synthetic oligonucleotides. With UV shadowing, PAGE allows purification of small quantities of synthetic DNA and is appropriate for sequences of any length. It is especially useful for purification of longer sequences which exhibit secondary structure. However, there are several drawbacks to using PAGE for purification: it is only amenable to small-scale purification; product recovery is usually quite low; and it is time-consuming, with significant time required to cast and run the gel, as well as the time required to extract and desalt the product. In addition, it may be necessary to enrich and desalt the crude product prior to purification. CE is an excellent analytical tool but not yet a ubiquitous purification technique.

HPLC

One of the most attractive techniques for oligonucleotide purification is reverse phase (RP) HPLC. By purifying with the dimethoxytrityl (DMT) group still attached at the 5'-terminus of the synthetic oligonucleotide, failure sequences which contain no DMT groups are weakly bound to the column and easily separated from the product which is more strongly retained and eluted later. In general, a set of components with the DMT group still attached is revealed.

Normally, the product accounts for greater than 95% of these components. The buffers used in this RP HPLC technique are volatile so the need for dialysis or desalting prior to lyophilization is eliminated. After removal of the DMT group with aqueous acid, the product is obtained rapidly and in good yield. RP HPLC can also be used for the purification of detritylated oligonucleotides. The technique retains the advantage of rapid recovery of the purified product simply by lyophilization of the volatile solute. The two processes can be combined on a pH stable RP packing[1] to include elution of non-DMT-containing failure sequences from the HPLC column, followed by detritylation of the DMT-ON oligos with aqueous trifluoroacetic acid, and finally chromatographic purification of the product oligonucleotides[2]. RP HPLC purifications can be easily scaled up to large preparative columns.

Several HPLC column manufacturers have introduced ion exchange HPLC columns specifically for the analysis and purification of synthetic oligonucleotides. These columns, like capillary electrophoresis, are capable[3] of superb resolution of components in the product mixture and, consequently, provide insights into the synthesis process. Ion-exchange columns have lower capacity for oligonucleotides than RP columns. However, they are more efficient for separating full-length oligonucleotides from n-1 failure sequences. Ion exchange HPLC is often used as a second purification when highly-purified oligonucleotides are needed.

Cartridges and Columns

RP cartridges containing C18 silica gel have been popular[4] for some

1. M. Germann, R.T. Pon, and J.H. van de Sande, *Anal. Biochem.*, 1987, **165**, 399.
2. A. Le, *Rainin Dynamax Review*, 1989, **3**, 1.
3. W.J. Warren and G. Vella, *BioTechniques*, 1993, **14**, 598.
4. K.M. Lo, S.S. Jones, N.R.Hackett, and H.G. Khorana, *Proc. Natl. Acad. Sci. USA*, 1984, **81**, 2285.

time for rapid and inexpensive purification of synthetic oligonucleotides. The technique involves evaporating the ammonia used for deprotection (since the silica gel-based support is not stable to high pH), redissolving the crude mixture (with DMT group still attached), and introducing the mixture onto the cartridge. The purification procedure is similar to that described above for RP HPLC columns. The DMT groups are later removed by treatment with acetic acid prior to lyophilization to give the purified product. Several disadvantages, however, exist with this technique. During the step to concentrate the ammonia, a second base, for example, triethylamine, must be added to maintain a basic solution and avoid the loss of a small percentage of the DMT group. In addition, the acetic acid deprotection necessitates two lyophilization steps after purification and an optional extraction to remove dimethoxytritanol from the product.

These disadvantages can be overcome[5] by using a polymeric RP packing in a cartridge or syringe barrel. Because these resins are stable in the pH range 1-13, the ammonia solution, diluted with water, can be added directly to the purification matrix. After elution of failure sequences, the DMT group can be deprotected with aqueous acid. The purified product can then be eluted and isolated by lyophilization.

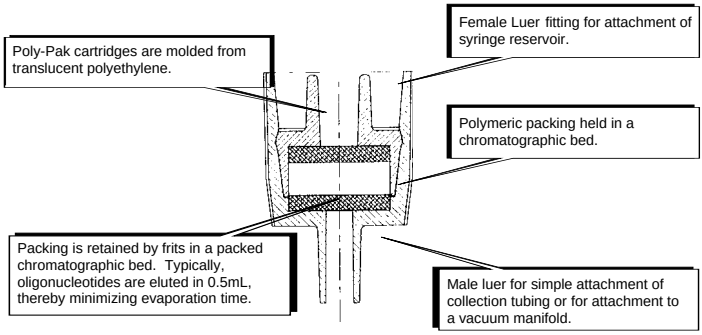
5. L.J. McBride, C. McCollum, S. Davidson, J.W. Efcavitch, A. Andrus, and S.J. Lombardi, *BioTechniques*, 1988, **6**, 362.

POLY-PAK™ CARTRIDGES

Glen Research has selected for use in Poly-Pak cartridges a highly purified, pH-stable polymeric resin which has been optimized for the purification of synthetic oligonucleotides. Poly-Pak cartridges exhibit the following attractive features:

1) The ability to prepare up to 20 A_{260} units from a 0.2 μmole synthesis and 50 A_{260} units from a 1 μmole synthesis. This feature is highly significant for the production of oligonucleotides destined for use in DNA amplification experiments.
2) Wash volumes are diminished to a level where the total eluent in a 40 nmole or 0.2 μmole purification is less than 15 mL. This speeds the process dramatically in comparison to other RP cartridges.
3) Product oligonucleotides are eluted in as little as 0.5 mL of aqueous acetonitrile, thereby minimizing the time needed for solvent evaporation.

The original Poly-Pak cartridges are used for purification of oligonucleotides produced on 40 nmole or 0.2 μmole scales. Poly-Pak II cartridges are for 1 μmole purifications.



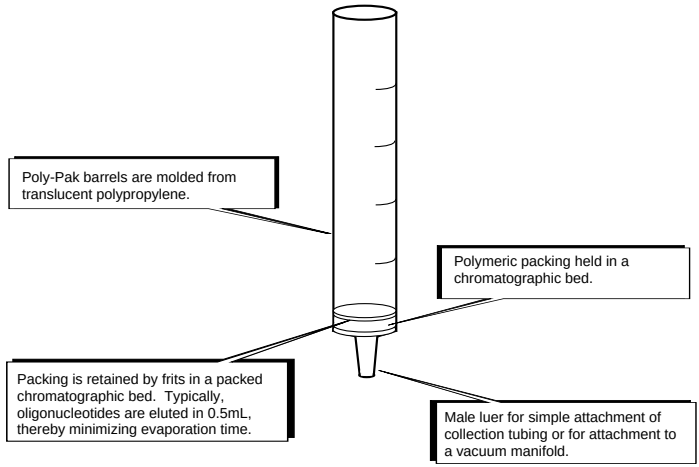
ANATOMY OF A POLY-PAK™ CARTRIDGE

Poly-Pak is a trademark of Glen Research Corporation

POLY-PAK™ BARRELS

Glen Research has selected for use in Poly-Pak barrels a highly purified, pH-stable polymeric resin which has been optimized for the purification of synthetic oligonucleotides. Poly-Pak barrels exhibit the same features as cartridges but are designed specifically for use on vacuum manifolds.

The original Poly-Pak barrels are used for purification of oligonucleotides produced on 40 nmole or 0.2 μmole scales. Poly-Pak II barrels are for 1 μmole purifications.



ANATOMY OF A POLY-PAK™ BARREL

PRINCIPLES OF POLY-PAK PURIFICATION

Automated synthesis of oligonucleotides is carried out from the 3'- to the 5'-terminus. During the synthesis process, failure sequences (caused by incomplete monomer addition) are end-capped. Assuming that end-capping is efficient, only the full-length sequence should contain a dimethoxytrityl (DMT) group at the 5'-terminus on completion of the synthesis. Since the DMT group binds strongly to reverse phase supports, the full-length sequences can be retained in a cartridge while the failure sequences are eluted. The DMT group is then removed on the cartridge and the purified product is eluted.

(Note: It is normally an option on the synthesizer to remove or to retain the acid-labile DMT group at the 5'-terminus. For the standard Poly-Pak purification procedure to operate, the synthesis MUST be carried out DMT-ON.)

Deprotection of synthetic oligonucleotides with ammonium hydroxide (30%; SG=0.88) removes the sequence from the support, while removing all base-labile protecting groups. Following this procedure, there is no need to lyophilize the ammonium hydroxide solution since the polymeric resin is stable at high pH. However, if the product has been lyophilized, simply reconstitute it in 0.1M triethylamine acetate (TEAA).

PURIFICATION OF OLIGONUCLEOTIDES (DMT-ON)

Materials	*Amount Used*	
	Poly-Pak	*Poly-Pak II*
Cartridge	1	1
HPLC grade Acetonitrile	2mL	4mL
2.0M Triethylamine Acetate (TEAA) (pH7)	2mL	4mL
Deionized Water	4mL	8mL
Ammonium Hydroxide/Water (1:10) or (1:20)	3mL	6mL
2% Trifluoroacetic Acid (TFA)/Water	2mL	4mL
20% Acetonitrile/Water	~1mL	~2mL

Procedure

The flow rate of solvents through the cartridge should be regulated at a rate of ~1-2 drops per second.

Cartridge Preparation

1. Connect a syringe to the female luer of the cartridge and have the male luer terminate in a waste vessel.
2. Flush the cartridge with 2mL *(4mL Poly-Pak II)* acetonitrile followed by 2mL *(4mL)* 2M TEAA.

Sample Preparation

3. Following synthesis, deprotect the DMT-ON oligonucleotide in ammonium hydroxide as normal. For this procedure, there is no need to lyophilize the ammonium hydroxide solution.
4. Add 3 parts deionized water to 1 part of the deprotected DMT-ON oligonucleotide in the ammonium hydroxide solution. Up to 10mL of sample solution may result.

Purification Procedure

5. Load the sample solution onto the cartridge. Collect the eluted fraction and again push it through the cartridge.

What's Happening

A good steady flow rate is usually sufficient to flush the bed of purification matrix.

Cartridge Preparation

1. *We use 5mL disposable syringes.*
2. *The acetonitrile washes organic residues from the resin and wets it, while the TEAA acts as an ion pairing reagent to enhance the binding of the oligonucleotide to the resin.*

Sample Preparation

3. *For this procedure to be successful, the synthesis must have been carried out DMT-ON. The base labile protecting groups must have been removed with ammonium hydroxide.*
4. *The volume of diluted ammonium hydroxide applied to the cartridge has little or no effect on the purification. (If the ammonium hydroxide solution has been lyophilized, reconstitute the pellet in 3mL of 0.1M TEAA.)*

Purification Procedure

5. *During the loading process, the DMT-ON oligonucleotides tend to stick to the polymeric packing while the failure sequences do not.*

6. Flush the cartridge with 3mL *(6mL)* of ammonium hydroxide (1:20) for oligos ≤35mer, or ammonium hydroxide (1:10) for oligos >35mer.
7. Flush the cartridge with 2mL *(4mL)* of deionized water.
8. Detritylate the support-bound oligonucleotide by flushing the cartridge with 2mL *(4mL)* of 2% TFA.
9. Flush the cartridge with 2mL *(4mL)* deionized water.
10. For oligos >50mer, repeat steps 6 and 7.)
11. Elute the purified, detritylated oligonucleotide by flushing the cartridge with 20% acetonitrile. Collect the eluted fractions. The first 4 drops of eluent can be discarded and the product is normally in the next 4 to 6 drops.
12. Determine the A_{260} units and store any unused oligonucleotide as a lyophilized solid at -20°C.

Note:
If the oligonucleotide is to be stored for a period longer than a few months, elute the product with water:acetonitrile:ammonium hydroxide (90:10:1). The ammonium salt of the oligonucleotide is stable in storage.

6. This wash flushes the remainder of the failure sequences from the cartridge.
7. Water flushes excess ammonium hydoxide from the cartridge.
8. A faint pink/orange band may be observed in the cartridge.
9. Water flushes excess 2% TFA. The DMT group remains adsorbed to the resin.
(10. Longer oligonucleotides may contain contaminating sequences produced by depurination of dA sites. These shorter sequences are truncated at the 3' terminus and are removed to an extent in these additional steps.)
11. Rather than count drops, it may be convenient to elute with 0.5mL (1mL) of 20% acetonitrile. A further 1mL (2mL) of eluent may be collected for security until the A_{260} units are determined. An unusual sequence may elute in the 1mL (2mL) wash.
12. Poly-Pak purification should yield on a 0.2 µmole scale, 10-20 A_{260} units, and Poly-Pak II on a 1 µmole scale, 40-70 A_{260} units, depending on the sequence.

Note:
We thank Bruce Kaplan of City of Hope, Duarte, CA for suggesting an alternative elution of product to give better stability in storage.

Note: *Volumes for Poly-Pak II cartridges on a 1 µmole scale are shown in parenthesis.*

LARGE SCALE PURIFICATION (DMT-ON)

The packing material used in Poly-Pak cartridges is available in bulk and may be used for large scale purification of oligonucleotides. The particle size distribution of the packing is 7-38 µm and filter funnels capable of retaining such small particles must be selected. The following volumes and quantities are approximate and should be optimized for the actual purification being undertaken.

Materials	*Amount Used per 10 µmoles of Synthesis*
Poly-Pak™ Packing	2.5g
HPLC grade Acetonitrile	15mL
2.0M Triethylamine Acetate (TEAA) (pH7)	15mL
Deionized Water	15mL
Ammonium Hydroxide/Water (1:10)	25mL
2% Trifluoroacetic Acid (TFA)/Water	15mL
20% Acetonitrile/Water	~10mL

The following general procedure is repeated at each step. Slurry and stir the packing with each solvent in 3 portions. Apply vacuum to the funnel to wash and filter the packing.

1. Prepare the packing by slurrying and washing with Acetonitrile then with 2M TEAA. This is analogous to Steps 1 and 2 on Page 8.
2. Prepare the sample solution as described in Steps 3 and 4 on Page 8.
3. Slurry the sample solution with the conditioned packing and filter. Collect the filtrate and apply it to the packing a second time. Collect and retain the filtrate for analysis.
4. Slurry and wash the packing with dilute ammonium hydroxide, water, 2% TFA, and water, in a manner analogous to steps 6-9 on Page 9. Keep each wash solution separate and retain them until a satisfactory mass balance has been achieved.
5. Elute the purified oligonucleotide with 20% Acetonitrile/Water.

Analysis of mass balance can be determined by UV spectroscopy but critical solutions should also be analyzed by HPLC. These notes are provided as guidelines but each large scale purification must be accompanied by careful analysis.

PURIFICATION OF DYE-LABELLED OLIGONUCLEOTIDES

In general terms, carry out the synthesis of oligonucleotides using 5'-fluorescent dye labels, e.g., 5'-Fluorescein (6-FAM), 5'-Hexachloro-Fluorescein (HEX) or 5'-Tetrachloro-Fluorescein (TET), using the standard DMT-ON synthesis cycle. (These products do not have a DMT-group to remove). For oligonucleotides prepared with dye amidites containing a DMT group (e.g., fluorescein and acridine) or an MMT group (e.g., Cy-3™ and Cy-5™) use the DMT-OFF cycle. (Cy-3 and Cy-5 are further protected with a monomethoxytrityl (MMT) group. The absorbance of the MMT cation (yellow) is different from the DMT cation (orange) so absorbance-based trityl monitors will detect it incorrectly as a low coupling. Conductivity monitors will interpret the release more accurately.)

All of these dye-labelled oligonucleotide can be deprotected at room temperature in concentrated ammonium hydroxide for a time appropriate for the monomers used. A minimum of 24 hours at RT is required for oligos containing ibu-protected dG residues. 6-FAM labelled oligos can be heated in ammonium hydroxide as normal. For the other dyes, monomers with protecting groups which can be removed in *2 hours or less* at 55°C are preferred. Do not exceed this time or temperature when using HEX or Cy-5 labelled oligos. Remove the ammonia immediately after deprotection.

PURIFICATION OF OLIGONUCLEOTIDES (DYE-ON)

Materials	*Amount Used*	
	Poly-Pak	*Poly-Pak II*
Cartridge	1	1
HPLC grade Acetonitrile	2mL	4mL
2.0M Triethylamine Acetate (TEAA) (pH7)	2mL	4mL
Deionized Water	4mL	8mL
8% Acetonitrile/0.1M TEAA (pH7)	3mL	6mL
20% Acetonitrile/Water	~2mL	~4mL

Procedure

Sample Preparation

1. Dry the dye-labelled oligonucleotide and dissolve the residue in 1mL 0.1M Triethylammonium acetate (TEAA).

Cartridge Preparation

2. Connect a syringe (we use 5mL disposable syringes) to the female luer of the cartridge and have the male luer terminate in a waste vessel.
3. Flush the cartridge with 2mL *(4mL)* acetonitrile followed by 2mL *(4mL)* 2M TEAA.

Purification Procedure

4. Load the sample solution from step 1 onto the cartridge. Collect the eluted fraction and again push it through the cartridge.
5. Flush the cartridge with 3mL *(6mL)* 8% acetonitrile in 0.1M TEAA.
6. Flush the cartridge with 2mL *(4mL)* of deionized water.
7. Elute the purified oligonucleotide by flushing the cartridge with ~1mL *(~2mL)* 20% acetonitrile. Collect the eluted fractions.

What's Happening

Sample Preparation

1. The volume of sample solution applied to the cartridge has little or no effect on the purification.

Cartridge Preparation

2. The acetonitrile washes organic residues from the resin and wets it, while the TEAA acts as an ion-pairing reagent to enhance the binding of the oligonucleotide to the resin.

Purification Procedure

4. During the loading process, the dye-labelled oligonucleotides tend to stick to the polymeric packing while the failure sequences do not.

5. The 8% acetonitrile wash flushes the remainder of the failure sequences from the cartridge. This step was devised by researchers at Applied Biosystems Division of Perkin Elmer.

6. Water flushes excess of the above solution from the cartridge.

7. It may be prudent to elute with 1mL (2mL) of 20%

Use the color of the product to determine the extent of elution from the cartridge.

8. Determine the A_{260} units and store any unused oligonucleotide as a lyophilized solid or in neutral aqueous media at -20°C.

Note:

Dye-labelled oligonucleotides should be stored in the dark, either dry or in neutral aqueous media at -20°C. Do not store the crude dye-labelled oligonucleotide in ammonia solution.

acetonitrile. A further 1mL (2mL) of eluent may be collected for security until the A_{260} units are determined. These unusual oligos may elute slowly but their color, when present, is useful in determining the extent of elution.

8. *Poly-Pak purification should yield on a 0.2 µmole scale, 10-20 A_{260} units, and Poly-Pak II on a 1 µmole scale, 40-70 A_{260} units, depending on the sequence.*

Note: *Volumes for Poly-Pak II cartridges on a 1 µmole scale are shown in parenthesis.*

DESALTING OF OLIGONUCLEOTIDES

Poly-Pak cartridges may be conveniently used for desalting oligonucleotides which have been purified by ion exchange HPLC or by gel electrophoresis.

Materials	*Amount Used*	
	Poly-Pak	*Poly-Pak II*
Cartridge	1	1
HPLC grade Acetonitrile	2mL	4mL
2.0M Triethylamine Acetate (TEAA) (pH7)	2mL	4mL
0.1M Triethylamine Acetate (TEAA) (pH7)	2mL	4mL
50% Acetonitrile/Water	~1mL	~2mL

Procedure

Cartridge Preparation

1. Prepare the cartridge as described for DMT-ON purification.

Desalting Procedure

2. Load the solution containing the oligonucleotide onto the cartridge.
3. Flush the cartridge with 3mL *(6mL)* of 0.1M TEAA.
4. Elute the desalted oligonucleotide by flushing the cartridge with 0.5mL *(1mL)* of 50% acetonitrile.
5. Determine the A_{260} units. Store any unused oligonucleotide as a lyophilized solid at -20°C.

What's Happening

Desalting Procedure

2. For this procedure to be successful, the oligonucleotide must be applied to the cartridge in an aqueous solution or a solution containing less than 5% organic solvent. The volume of the sample solution is not critical but it is always prudent to keep the unretained solvent until the purified product is quantified.

3. 0.1M TEAA removes the salts from the cartridge.

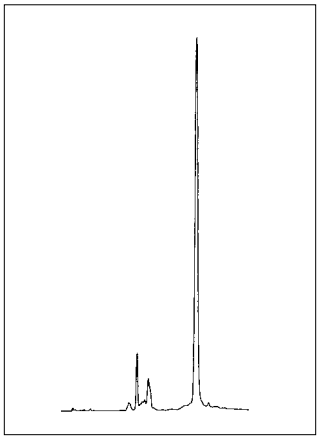
4. The product should elute totally in 0.5mL (1mL) of 50% acetonitrile.

5. Up to 70 A_{260} units can be desalted on Poly-Pak cartridges.

Note: *Volumes for Poly-Pak II cartridges on a 1 μmole scale are shown in parenthesis.*

PURIFICATION OF UNMODIFIED OLIGONUCLEOTIDES

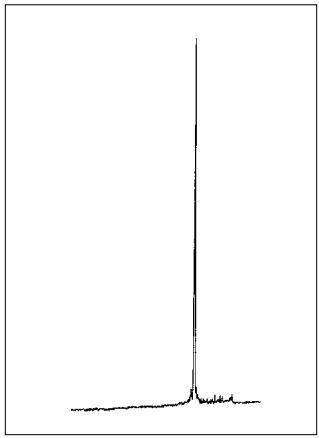
The ability of Poly-Pak cartridges to produce high quality oligonucleotides is demonstrated in the purification of a 21-mer. Figure 1 contains the RP HPLC trace of the crude DMT-ON oligonucleotide after deprotection. In Figure 2, the deprotected 21-mer has been purified on a Poly-Pak cartridge and isolated in 4 drops of aqueous acetonitrile. The analysis in Figure 2 is carried out using capillary gel electrophoresis (CE).



Time	0.1M TEAA(%)	Acetonitrile(%)
0	97	3
5	97	3
20	75	25
30	75	25

Column: Spherisorb ODS-2(150X4.6mm)
Flow Rate: 1mL/min.
Detector: UV at 254nm

Figure 1: *DMT-ON Chromatography by RP HPLC .*



Capillary: Beckman eCAP ssDNA gel
Length: 30cm
Field: 11.1kV
Temp.: 30°C
Flow Rate: 1mL/min.
Detector: UV at 254nm
Buffer: Tris-borate-urea

Figure 2: *DMT-OFF Capillary Electropherogram of Poly-Pak purified oligonucleotide.*

PURIFICATION OF AMINO-MODIFIED OLIGONUCLEOTIDES

Amino-modification is an important technique for the preparation of labelled oligonucleotides for use in DNA sequencing, amplification, and in the production of diagnostic probes. A popular method for attachment of an amino group to a synthetic oligonucleotide is the use of a 5'-amino-modifier containing the monomethoxytrityl (MMT) protecting group. These amino-modifiers are specifically designed to take advantage of RP purification techniques, including cartridge purification.

The Poly-Pak procedure for purification of amino-modified oligonucleotides is the same as that described beginning on Page 8. Two minor exceptions are worthy of note:

1. When the amino-modifier contains the thermally labile MMT protecting group, carry out the deprotection at 40°C for 24 hours.
2. During the detritylation (Step 8, Page 9), pass 2% TFA through the Poly-Pak three times and leave to react for 2 minutes each time. A yellow color may become visible in the cartridge.

The chromatogram in Figure 3 demonstrates the efficiency of this technique.

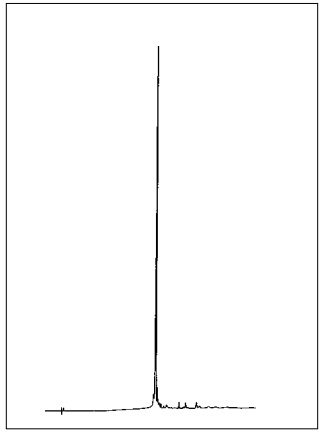


Figure 3: *RP HPLC of an amino-modified oligonucleotide purified as described. Column , flow rate and detector are as described in Figure 1, Page 15.*

3'-Amino-Modifier CPG has gained popularity for the direct synthesis of oligonucleotides containing an amino group at the 3'-terminus. Similarly, Amino-Modifier-dT is used to introduce amino groups at specific points in oligonucleotides. When synthesized DMT-ON, these 3'-amino-oligonucleotides can be purified on a Poly-Pak cartridge using the procedure described on Page 8.

PURIFICATION OF OLIGOPHOSPHOROTHIOATES

Oligophosphorothioates (S-Oligos) have shown potential as therapeutic agents due to the nuclease resistance of the internucleotide linkages. These modified oligos can be produced using a variety of techniques by phosphoramidite chemistry and also conveniently by H-phosphonate chemistry. Whatever the synthetic route, the product S-Oligos can be quickly purified on Poly-Pak cartridges. The multiplicity of the DMT-ON product (Figure 4) is caused by diastereomers at the phosphorothioate linkage adjacent to the DMT group. The product analyzed by RP HPLC is shown in Figure 5.

Oligophosphorothioates can be further analyzed by PAGE, CE, or ion exchange HPLC following the conditions described[1].

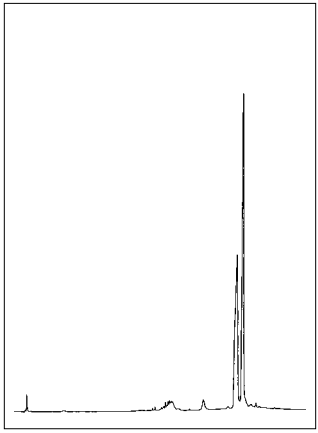


Figure 4: *RP HPLC of a DMT-ON oligophosphorothioate, using the conditions shown in Figure 1, Page 15 but increasing the acetonitrile content to 40% in the same time.*

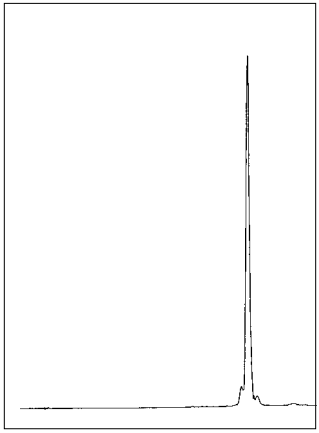


Figure 5: *RP HPLC of a DMT-OFF oligophosphorothioate, following the conditions shown in Figure 1, Page 15.*

1. B.J. Bergot and W. Egan, *J. Chromatography*, 1992, **599**, 35.

PURIFICATION STRATEGY

The purification of modified oligonucleotides using RP cartridges can be very effective as long as the strategy has been well conceived. The development of Poly-Pak cartridge purifications should be carried out in conjunction with RP HPLC. With a properly developed strategy, the method should be analogous to an affinity purification rather than a true chromatographic technique. In other words, you must find conditions where the desired product sticks to the cartridge while all impurities can be washed off and then change the conditions to elute the purified product.

In developing a Poly-Pak purification, first check the RP HPLC of the product mixture. The desired product should elute from a RP HPLC gradient with a retention difference of at least 7% and preferably 10% acetonitrile.

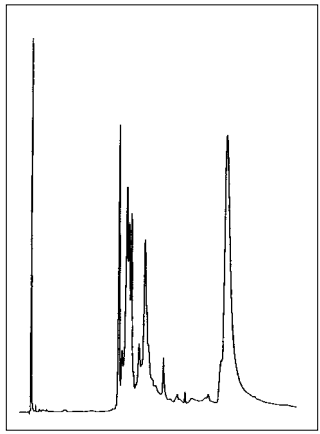


Figure 6: *RP HPLC of a crude oligonucleotide modified at the 5' terminus with thiol-modifier C6 S-S following the conditions shown in Figure 1, Page 15.*

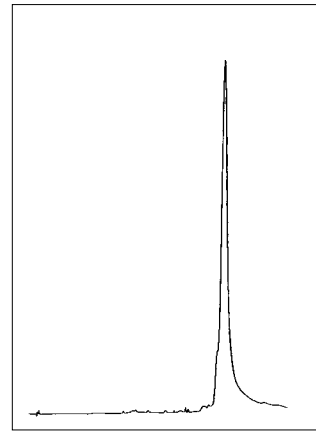


Figure 7: *RP HPLC of the DMT-on oligonucleotide shown in Figure 6 modified at the 5' terminus with thiol-modifier C6 S-S and following Poly-Pak purification.*

To illustrate purification strategy using Poly-Pak cartridges, the purification of oligos modified using 5'-Thiol-Modifier C6 S-S is described. This is a 3 step process. Step 1 purifies the DMT-ON oligonucleotide with the disufide group intact. Step 2 cleaves the disulfide linkage. Step 3 purifies and desalts the 5'-thiol oligonucleotide. Figures 6, 7 and 8 illustrate the progress of the purification and desalting steps.

Step 1
Purify the DMT-ON oligonucleotide as described on Pages 8 and 9 with the exception that the detritylation steps (8 and 9) are omitted. The purified DMT-ON oligonucleotide is eluted with 1 mL of 20% acetonitrile and there is no need to evaporate the solvent.

Step 2
Add the eluate from the cartridge to an equal volume of a solution of dithiothreitol (DTT) (0.2M DTT in 0.1M phosphate buffer, pH=8.3-8.5). Leave at RT for 0.5h to cleave the disulfide.

Step 3
Add 2 volumes of water to the incubation solution from Step 2. Desalt the oligonucleotide on a Poly-Pak cartridge, as described on Page 14, but eluting with 20% acetonitrile. In this way, the DMT containing C6 thiol will remain bound to the cartridge while the oligonucleotide containing the 5'-thiol elutes from the cartridge. The two minor impurities shown are non-nucleosidic and do not affect thiol-specific conjugation.

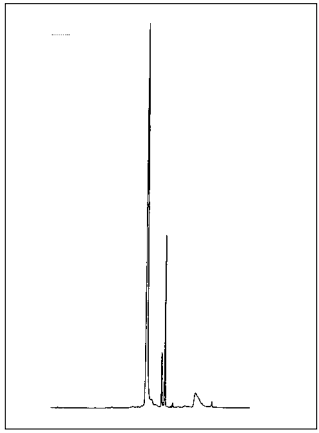


Figure 8: *RP HPLC of the thiol-modified oligonucleotide shown in Figure 7, desalted using a Poly-Pak cartridge.*

PURIFICATION OF BIOTINYLATED OLIGONUCLEOTIDES

In the years since we introduced our range of Amino-Modifiers, it has become clear that their most common use is as precursors for the introduction of biotin by reaction with the appropriate biotin NHS ester. While these methods have proved to be relatively successful, the only satisfactory means of purifying biotinylated oligonucleotides produced in this manner is by RP HPLC. The process is time-consuming and suffers from the possibility of cross-contamination which is potentially troublesome in PCR applications.

CE phosphoramidites based on a branched hydrocarbon backbone terminating in a DMT group and with a linkage to biotin extending away from the branching site have now been introduced. These products can be added in multiple steps at the 3' or 5' terminus, can be inserted at any position in the sequence, or can be added once at the 5' terminus for subsequent RP purification.

The chromatogram shown in Figure 9 demonstrates the use of a biotin phosphoramidite to label the 5' terminus of an oligonucleotide. The biotinylated oligonucleotide was simply purified by the DMT-ON technique on a Poly-Pak cartridge, using the procedure described on Page 8. This product would certainly be of sufficient purity for use as a biotinylated PCR primer or probe.

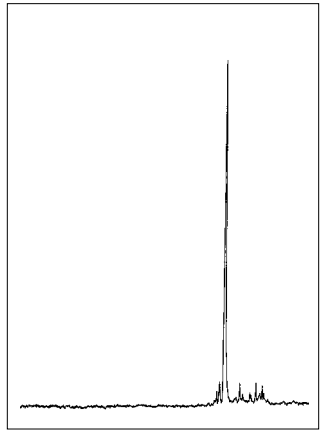


Figure 9: *CE of a Poly-Pak purified biotinylated oligonucleotide, following the conditions shown in Figure 2, Page 15.*

PURIFICATION OF DYE-LABELLED OLIGONUCLEOTIDES

Most oligonucleotides dye-labelled at the 5' terminus can be purified on Poly-Pak cartridges in a manner analogous to the DMT-ON procedure. The purification is possible when the fluorescent group is sufficiently lipophilic. In these cases, the dye-labelled oligonucleotides bind sufficiently to the packing to allow the failure sequences to be washed away preferentially. Oligonucleotides labelled at the 5' terminus with fluorescent dyes, poly-aromatic hydrocarbons and steroids have been purified in this way on Poly-Pak cartridges. Biotin is not appropriate for this procedure and must be purified DMT-ON, as described on Page 20.

Figures 10 and 11 show oligonucleotides labelled at the 5'-terminus with 5'-fluorescein (6-FAM) and Cy-3 CE phosphoramidites and purified using the procedure described on Page 11.

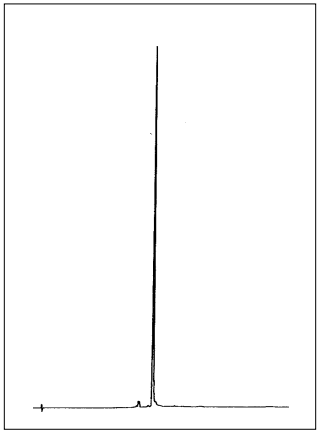


Figure 10: *RP HPLC of a FAM labelled oligonucleotide, using the conditions shown in Figure 1, Page 15.*

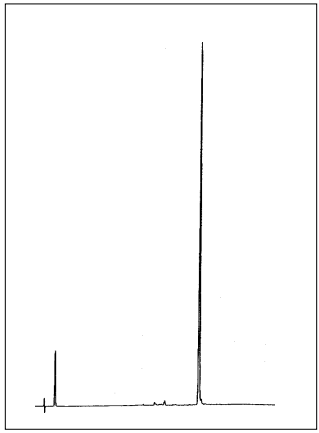


Figure 11: *RP HPLC of a Cy-3 labelled oligonucleotide, following the conditions shown in Figure 1, Page 15.*

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SOLUTION PREPARATION

The following solutions may be prepared by the user. Always wear gloves and protective eye wear while handling corrosive organic liquids. Read and take heed of the information in Material Safety Data Sheets supplied by the manufacturers.

1. 2M Triethylamine Acetate

Ingredients:

Glacial Acetic Acid	120mL
HPLC Grade Water	605mL
Triethylamine	275mL
Total Volume	1L

Add the glacial acetic acid to the water. With vigorous stirring, slowly add the triethylamine. (Note that triethylamine must be of the highest quality and must be colorless.) The triethylamine will form an upper layer and slowly will be absorbed into the aqueous acetic acid. The mixture will become quite warm. When the final solution has cooled, measure the pH and adjust, if necessary, to 7.0 to 7.5 with glacial acetic acid or triethylamine.

2. Ammonium Hydroxide/Water (1:10)

Add one volume Ammonium Hydroxide (SG 0.88) to ten volumes of HPLC Grade Water.

3. 2% TFA/Water

Add two volumes of trifluoroacetic acid to 98 volumes of HPLC Grade Water.

WHY IS MY YIELD NOT HIGHER?

This is probably the most commonly asked question and usually follows a purification which yields a lower amount of product than expected. First of all, Poly-Pak cartridges have a finite capacity for oligonucleotides and this is approximately equivalent to 0.3-0.4 μmoles. A 0.2 μmole synthesis should be completely purified by a single cartridge, whereas a 1 μmole synthesis can be purified using a Poly-Pak II cartridge or barrel. Assuming a high quality 0.2 μmole DNA synthesis, a 20mer should yield about 30 A_{260} units of crude and purification should provide at least 10 A_{260} units of product. The remainder of the UV absorbing material is in the ammonium hydroxide loading solution which contains virtually no DMT-ON product and the diluted ammonium hydroxide wash which contains a small amount of failure sequences as well as some DMT-ON product.

If the yield is substantially lower, it is necessary to examine the DNA synthesis to determine if that was problematical. If the DMT yields were low, the amount of available DMT-ON product will be substantially lowered, as will the number of crude A_{260} units. Even if DMT yields seem to have been satisfactory, other synthesis factors can contribute to a low yield. The only way to obtain a satisfactory answer is to measure the A_{260} units in the crude product and to analyze this by RP HPLC.

WHAT PURITY CAN I EXPECT FROM A POLY-PAK?

Poly-Pak cartridges and barrels are not very intelligent. They really do not understand which of the oligonucleotides offered up to them is the desired product. They are only capable of separating DMT-ON oligonucleotides from non-DMT containing failure sequences since the polymeric packing has a high affinity for the lipophilic DMT group. Although a stepwise gradient of increasing organic content may be successful in some separations, the Poly-Pak purification strategy is better understood if looked on as an affinity-type purification. If the product quality is poor after Poly-Pak

purification, all of the components formerly had a DMT group on them.

How can that happen? Unfortunately, we do not understand this fully. However, we do know that a well used and maintained synthesizer tends to produce high quality products after Poly-Pak purification while an infrequently used synthesizer with older reagents installed tends to produce poorer quality oligonucleotides even after Poly-Pak purification. Several mechanisms may be implicated in the formation of DMT-ON oligonucleotides which are not the desired length: Incomplete capping leads to sequences with point deletions; Incomplete oxidation also leads to sequences with point deletions; Depurination of dA sites leads to chain scission on deprotection and oligonucleotides truncated at the 3' terminus; Incompletely capped CPG gives rise to maverick sequences also truncated at the 3' terminus; and Unreversed chain branching at dG sites leads to higher molecular weight products.

Without evading the question any longer, a good synthesizer will yield oligos after Poly-Pak purification which are >95% pure by CE or ion-exchange HPLC and which give clean and accurate results in their intended usage. A stressed synthesizer will yield a product which is upgraded in quality but which is only around 90% pure.

CAN POLY-PAK CARTRIDGES BE REUSED?

The answer to this question is a qualified yes. We would not recommend their reuse for the purification of PCR primers even though non-specific binding is very low. However, they may certainly be reused for the purification of further amounts of the same oligonucleotide. Just start the process from the beginning. With each use, yield will drop and backpressure will increase.

HOW ABOUT C18 SILICA CARTRIDGES?

C18 silica RP cartridges are used successfully for the purification of synthetic oligonucleotides. We even use them here (from Alltech)

since they are available with up to 0.9g of packing material which gives a good capacity. They are not, however, pH stable and at the very least the ammonium hydroxide must be evaporated first.

CAN THIS PROCESS DAMAGE OLIGOS?

Not at all! The polymeric packing, frits and cartridge body are totally inert. However, the solutions must be prepared using the best quality reagents. Procedures for the formulation of the reagents are described on Page 23. The two reagents which must be pure are triethylamine and trifluoroacetic acid. If you forget to dilute the trifluoroacetic acid, it will destroy the DNA!

HOW CAN LARGE NUMBERS OF SAMPLES BE PROCESSED?

Poly-Pak cartridges can be used manually or on a vacuum manifold whereas Poly-Pak barrels are designed for use on vacuum manifolds only. Using a vacuum manifold, up to 12 (or even 24) samples can be processed in parallel. We dispense the liquids from wash bottles and, in this way, 12 samples can be processed in about 20 minutes. Most users load the samples onto and elute the product from the cartridges by hand and use the manifold for washes only. Others load and elute on the manifold. Experimentation will determine which mode is best for you.

WHEN DO I USE CARTRIDGES AND WHEN BARRELS?

Cartridges are useful at any time since they can be used individually by hand or attached to a vacuum manifold for parallel sample processing. Barrels are really only intended for parallel processing on a vacuum manifold. Barrels are a little cheaper since they are less complex to produce and they do contain a little more packing material so isolated yields may be about 25% higher using them. Traces of packing material may occasionally leak from barrels since the frit system is a simple press fit. If this happens, the traces of packing can be centrifuged and separated. To our knowledge,

packing has never leaked from the new design of Poly-Pak cartridge.

HOW DO POLY-PAK CARTRIDGES COMPARE WITH COMPETING PRODUCTS?

We think Poly-Pak cartridges are the best of this type and we are totally unbiased. Seriously though, all cartridges containing similar polymeric matrices will purify oligonucleotides to the same extent. The main differences are in convenience of use. Poly-Pak cartridges contain very fine polydivinylbenzene beads which we believe have the highest affinity for DMT-ON oligonucleotides. Poly-Pak cartridges contain the packing in a tight bed allowing smaller volume washes than some competitive products. Finally, Poly-Pak cartridges have comfortable backpressure. We believe this all contributes to making Poly-Pak cartridges the very best polymeric reverse phase cartridge.

TROUBLESHOOTING

Call or fax with DMT yields and RP HPLC data on the crude DMT-ON product, if possible, and crude and purified A_{260} units. The more data you can provide, the more likely we are to be able to troubleshoot the problem.

Item	Catalog No.	Pack
Packing and Cartridges		
Poly-Pak™ Packing Material	60-1000-05 60-1000-25	5g 25g
Poly-Pak™ Cartridges	60-1100-01 60-1100-10	each Pack/10
Poly-Pak™ Barrels	60-2100-30	Pack/30
Poly-Pak™ II Cartridges	60-3100-01 60-3100-10	each Pack/10
Poly-Pak™ II Barrels	60-4100-30	Pack/30
Reagents and Kits		
Wash Solvent Kit *(Sufficient for use with up to 10 cartridges)*	60-4900-01	each
2.0M Triethylammonium Acetate (TEAA) HPLC grade	60-4110-52 60-4110-57 60-4110-60	200mL 450mL 960mL
2% Aqueous Trifluoroacetic Acid	60-4040-57	450mL
Vacuum Manifold		
12-Port Vacuum Manifold *(Includes glass chamber with rack to hold collection tubes, aluminum lid with 12 shut-off valves)*	60-9000-01	each