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User Bulletin: ABI 380/1 Nucleic Acid Synthesizers

Subject: OPC: Improved Purification of Long-Mers

User Bulletin - Number 52
381/380 DNA Synthesizers

OPC Purification

OPC: Improved Purification of Long-Mers

During the last few years, advances in DNA synthesis chemistry have increased efficiency and minimized side reactions. Typically, the average yield per cycle, as measured by trityl cation assay, is greater than 98%, and often greater than 99%. PE Applied Biosystems side reactions, such as base modifications, have been characterized and virtually eliminated. These improvements now allow the synthesis of short oligonucleotides (< 30-mers) which are almost free of detectable failure sequences or other impurities. Rapid, simple purification techniques, such as OPC, are sufficient for even stringent applications of the synthetic oligonucleotides.

Although synthesis of oligos less than 70 bases is considered routine and purification of these oligos can be readily accomplished using OPC (see original protocol and User Bulletin 51), the synthesis and purification of longer oligomers (>70-mers) is not as simple. For synthesis of long oligomers (even with very high synthesis efficiency) the desired, full-length product is a minor component compared to the sum of the failure sequences. Certain side reactions also become more prevalent due to increased exposure to the synthesis reagents. In particular, the acids used for detritylation (trichloroacetic or dichloroacetic acid) can promote cleavage of the purine nucleobases (A and G) from the ribose rings. During synthesis of a long-mer, the cumulative time that the bases near the 3' end are subjected to acid can be more than an hour. During this time apurinic sites can be created. These apurinic sites in the oligonucleotide undergo internucleotide cleavage during ammonia deprotection at 55 °C. When the synthesis is conducted Trityl On, in preparation for OPC purification, some of the cleavage products will bear a 5'-trityl group (see figure 1).

CPG-$^{3'}$AGTCAGTGCGCTTAGCCATAAG$^{5'}$-DMT (full length oligo)
CPG-$^{3'}$AGTC*GTGCGCTTAGCCATAAG$^{5'}$-DMT (oligo with apurinic acid)

CPG-$^{3'}$AGTC-OPO$^{-2}_{3}$+ GTGCGCTTAGCCATAAG$^{5'}$-DMT (ammonia cleavage products)

Figure 1

Apurinic cleavage can generate trityl bearing species that are less than full length. Reverse phase HPLC or OPC purification methods, which depend on trityl

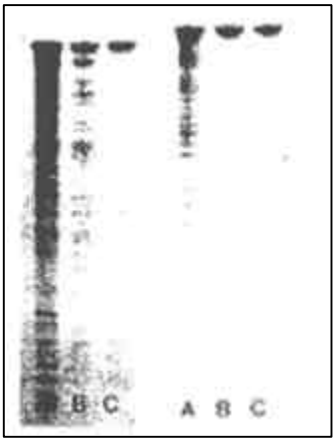
selectivity, are then less efficient in purifying the full-length oligo.

In User Bulletin 51 ("OPC Purification of Long Oligonucleotides") purification of 40- to 70-mers using the OPC cartridge was described. Purities of 82% and 96% were obtained for a 60- and 72-mer respectively. Recently, Thomas Horn and Mickey Urdea of Chiron Co. published a procedure for extending the utility of OPC to the purification of long oligonucleotides 1. They found that apurinic sites undergo internucleotide cleavage with 1 molar lysine, without cleavage of the oligonucleotide from the support. The DMT bearing cleavage fragment (2) is washed away, leaving a non-DMT bearing fragment (1) bound to the support. Following ammonia cleavage and deprotection, they used a protocol for OPC purification of long oligomers. This protocol (presented on page 3) was effective in purifying oligomers up to 118 bases in length (see figure 2).

Although depurination is a valid concern during DNA synthesis of long oligonucleotides, the degree of depurination encountered during synthesis is highly sequence and reagent dependent. Since the 3' bases of an oligo (the initial couplings) have the greatest reagent exposure, varied purine content in this area will generate varied potential for depurination. Another factor in depurination is the purity of the acid used for detritylation. Contaminants, such as water or HCI, in the trichloroacetic or dichloroacetic acid will greatly promote depurination. PE Applied Biosystems supplies high purity trichloroacetic and dichloroacetic acid reagents. In a study involving the synthesis of a 72-mer, the use of PE Applied Biosystems reagents did not promote detectable depurination. Analysis of the 72-mer synthesis, OPC purified with and without prior lysine treatment, showed no detectable purity difference. These results may not apply to all synthesis sequences and lengths, but the study demonstrates PE Applied Biosystem's commitment to synthesis reagent and product quality.

Figure 2

103-mer Study 118-mer Study



Lane A: crude.
Lane B: OPC-purified only
Lane C: lysine treated and OPC purified.

Protocol for Purification of Mers >70 Bases

1. Synthesize the oligo, using the "trityl on manual" end procedure for 380B or "trityl ON" for the 381A or the 391 (leaving the DNA on the column).
2. Dry the column by reverse flushing (function 2) for 60 seconds with argon or allow the column to air dry.
3. Remove the phosphate protecting groups: Connect one syringe (with plunger) to one end of the column. Fill the other syringe with the appropriate, following solution and use this to fill the column. The following

appropriate solutions should be handled in a fume hood: For methyl phosphoramidites: add 1 ml of thiophenol: triethylamine:dioxane (1:12 v/v/v) (ABI # 400237) and treat at room temperature for 1 hour. For cyanoethyl phosphoramidites: add 1 ml of t-butylamine:pyridine (1:9 v/v) and treat at room temperature for 1 hour (t-butylamine; Aldrich B8, 920-5 and pyridine; Aldrich 27,040-7). The solution can be periodically circulated by alternately depressing the syringe plungers.
Note: Gently depress the syringe plungers; otherwise you risk breaking the filters that hold the CPG.

4. Remove the solution and wash 5 times with acetonitrile.
5. Allow the column to air dry or blow the column dry with argon.
6. Prepare a 1M lysine solution pH = 9 (adjust pH with NaOH if necessary) (L-lysine monohydrochloride; Sigma # L5626, Aldrich #L460-5).
7. Place 2 ml of the lysine solution into a 16 X 100 mm test tube. Connect a single syringe to one side of the synthesis column and a male-to-male luer fitting (ABI p/n 110127) to the other. Insert the column-syringe-luer apparatus into the test tube and draw the lysine solution into the column. Incubate at 55 °C for 90 minutes by inserting the test tube and contents into a heating block. The lysine solution should be periodically circulated (every 30 minutes). Maintaining the column assembly allows the use of your 380 B to automatically cleave the DNA from the column.
Note: the luer fitting of the syringe must be located in the center of the syringe barrel to allow easy insertion into the test tube.
8. After incubation in the lysine solution, wash the column 5 times with water.
9. Cleave the DNA from the column: For a 381 A synthesizer: manually cleave the DNA from the column using concentrated ammonia and the procedure described in your manual. For a 380 B synthesizer: automatically cleave the DNA by placing the column back on the synthesizer and programming a single base, trityl-on synthesis for the column (since the first base of each sequence is column derived, no base coupling will occur, and since it is a trityl-on synthesis, no TCA wash will occur. The synthesizer will default directly to the programmed end procedure).
10. Deprotect the exocyclic amines:
Using fresh ammonia stock, bring the volume of ammonia in the collectionvial to 3 mL and incubate the ammonia solution at 55 °C for 8 to 12 hours.
11. Proceed to OPC purification as previously described in User Bulletin #51 (the protocol from UB #51 has been attached for your convenience).

OPC Purification Protocol

(from PE Applied Biosystems User Bulletin #51)

Solutions Needed:

- HPLC grade acetonitrile, 5 mL
- 2.0 M triethylamine acetate (Part No. 400613), 5 mL
- Deionized water, 1 mL
- 1.5 M ammonium hydroxide, 15 mL (1:10 dilution of conc. ammonium hydroxide in deionized water)
- 2% trifluoroacetic acid, 5 mL (1:50 dilution of Neat TFA Part No. 400137)
- 20% v/v acetonitrile in deionized water, 1 mL

1. After completion of a trityl-on synthesis, cleave the oligonucleotide from the support and deprotect following normal protocols for the synthesis method utilized.
2. Connect an all polypropylene syringe (Aldrich Zl 1686-6), an OPC cartridge, and male-to-male Luer tip as series. Make sure all fittings are snug. The OPC cartridge may be immobilized with a laboratory clamp.
3. Flush the cartridge with 5 mL HPLC grade acetonitrile, followed by 5 mL

2.0 M triethylamine acetate. Remove the syringe from the OPC cartridge before removing the plunger; then re-insert the syringe barrel prior to th next addition.

4. Dilute an aliquot containing ~ 20 O.D. units of the crude, deprotected oligonucleotide still in conc. ammonia with one-third volume of deionized water. The final volume of the solution should be 1 to 4 mL.
   Important: Keep the flow rate at 1 to 2 drops per second for all subsequent reagent additions.
5. Place this solution (step 4) in the syringe and slowly push it through the cartridge. Save the eluted fraction, place it in the syringe, and gently push it through the cartridge. This will load 1 to 5 O.D. units of the crude oligonucleotide (depending on length, sequence, and synthesis quality) onto the cartridge.
6. Slowly wash the cartridge with 3 x 5 mL 1.5M ammonium hydroxide.
7. Flush cartridge with 2 x 5 mL deionized water.
8. Detritylate the OPC-bound oligonucleotide with 5 mL of the 2% trifluoroacetic acid solution. Gently push ~ 1 mL through the cartridge, incubate for 5 minutes, then gently flush the remaining TFA solution through the cartridge.
9. Flush cartridge with 2 x 5 mL deionized water.
10. (For sequences up to 40 bases, add this step) Gently push through the cartridge 1 x 5 M ammonium hydroxide, followed by 2 x 5 mL deionized water.
11. Elute the purified, detritylated oligonucleotide by slowly washing the cartridge with 1 mL of the 20% acetonitrile solution.
12. Determine the O.D. units at 260 nm with an aliquot of the eluate from step 10.
13. Store any OPC-purified oligonucleotide as a dry solid at -20 °C.

Helpful Hints:

- Store the remaining crude solution in ammonia. This will not harm the product in any way and will save you time.
- Use fresh ammonia for cleavage on the instrument and for deprotection at 55 °C to acquire optimum separation.
- Store the TEA-Ac and 15M ammonium hydroxide at 4 °C. Make the 1.5M solution of ammonium hydroxide daily, as needed.

Note:

- Don't be concerned that TFA will harm your sample. Once deprotected, the bases are 10 times less susceptible to depurination.
- The 260 nm/280 nm ration of purified synthetic DNA is highly sequence dependent and may differ from the typical 1.8 value associated with genomic DNA isolated from a natural source.
