User Bulletin No. 50

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SUBJECT: HPLC Purification and Processing of 10 Micromole Synthetic Oligonucleotides

This bulletin describes procedures for isolation and purification of milligram quantities of oligonucleotides using HPLC. HPLC represents the most rapid and convenient means of purifying oligonucleotides produced with typical 10-micromole syntheses. Included are procedures for preparing the crude DNA for HPLC purification, representative chromatograms from typical and anomalous analyses, and protocols for processing and quantifying the oligonucleotide product following chromatography.

These procedures have been used with phosphoramidite and H-phosphonate-derived oligonucleotides. They should facilitate your research requiring large quantities of oligonucleotide, regardless of application.

1 Prepare the base-deprotected, crude 5'-dimethoxytrityl (DMT) DNA for initial concentration as follows:

To the DNA ammonia solution (contained in 13 x 100 mm culture tube), add ~100 µL of triethylamine (to prevent loss of the acid-labile DMT) and vortex. Concentrate the sample under a stream of N^2 (or air) for 20-30 minutes in a fume hood to remove most of the NH_3. Reduce to ¼ of the original volume. Alternatively, use a vacuum centrifuge connected to a water aspirator. Add another 100 µL of triethylamine and vortex briefly. Remove a small aliquot (5 µL is sufficient, but more may be removed if desired) and set aside to be used for an analytical HPLC injection. Concentrate the remaining sample to dryness in a vacuum centrifuge (e.g. Savant).

2 Prepare the crude 5'-DMT DNA for HPLC purification as follows:

To the tube containing the dried DNA sample, add 100 µL of triethylamine (to protect the acid labile DMT groups) followed by 1.2 mL of 0.1M triethylammonium acetate (pH 7 TEAA), vortex, and sonicate (if needed) to dissolve the DNA. Syringe filter the solution by attaching a Millipore filter (Miller-HV, 0.45 µm, Catalog No. SLHV025NS) to a 1 mL disposable syringe barrel. Allow the DNA-solution to gravity filter. Wash the test tube that contained the DNA-solution with 1 mL of 0.1M TEAA and add to the syringe filter set-up. Purge the filter by rapidly inserting the plunger (it is not necessary to allow the 1 mL of 0.1M TEAA wash to gravity filter). This will yield a final volume of ~2.0 mL (~300 µL filter hold-up).

3 Perform the HPLC Purification of the crude 5'-DMT as follows:

Depending on the oligonucleotide sequence, one of two different HPLC purification methods should be used. The first method yields good results with DNA that is unlikely to form secondary structure (e.g., duplex and/or hairpin

formation). It employs an Applied Biosystems Aquapore (Prep -10) Octyl column, catalog number 0711-0166, 10 x 250 mm.

The second method is used for oligonucleotides that are likely to form secondary structure (e.g. self-complimentary or partially self-complimentary sequences, and especially those with high G-C content). With this method it is advantageous to heat the column to melt-out any higher-order structure which otherwise would lead to a more difficult HPLC purification evidenced by giving multiple peaks. For this reason the second method takes advantage of a polymeric reverse phase column (Polypore PRP, Applied Biosystems catalog number 0712-0053, 7 x 250 mm column), which has shown very high capacity for its size. It can also be heated repeatedly without apparent damage to the column as indicated by performance.

Analytical Injection

After choosing one of these methods, inject the 5 µL aliquot onto an Aquapore prep-10 octyl column at room temperature or a Polypore PRP equilibrated to 60°C (Waters column heater, Model 1122). Use the conditions shown in Figure 1 and set the detector absorbance units full scale (AUFS) [see Trace A].

Time (min)	%Acetonitrile	%Triethylammonium Acetate, 0.1M, pH 7
0	20	80
10	30	70
25	30	70

* Flow rate for 7 x 250 mm Polypore PRP column is 3 mL/min.

* Flow rate for 10 x 250 mm Aquapore column is 11 mL/min.

Figure 1. Conditions for initial HPLC analysis of crude 5'-DMT oligonucleotide.

Preparative Injections

Set the UV detector to 280 nm (it is also recommended to use a preparative flow cell) and 2.0 AUFS. When using the Aquapore column at room temperature (see above gradient, flow rate ≈ 11 mL/min), one-half of a 10 µmol synthesis can be injected without experiencing “overloading”. The Polypore PRP column has enough capacity to purify about one-third of a 10 µmol synthesis (the Polypore PRP column can be used at room temperature in place of the Aquapore). If time is not a factor, better results may be obtained by injecting smaller amounts and by diluting each injection with as much 0.1M TEAA as possible, depending on injection loop size.

Although the purpose of heating the Polypore PRP column is to melt-out secondary structure, multiple peaks which usually are not present in the analytical trace may appear when performing preparative injections. Such multiple peaks should be individually collected (see Trace B) and re-analyzed under the same conditions by directly injecting 25-30 µL of the collectate at 254 nm, 0.1 AUFS (see Traces C and D). Often these multiple peaks are the same desired product and will give essentially identical retention times (see C and D). However, one or more of these fractionated peaks may be undesired material (see Trace E-H).

Concentrate the collectate (pooled if warranted) under a stream of N^2 (or air) for ~30 minutes to remove most of the acetonitrile. Transfer the solution to a large test tube(s) and concentrate to dryness in a vacuum centrifuge.

Dissolve the resultant DNA residue in several mL's of H_2O and transfer to a "Pyrex" or "Kimex" test tube (13 x 100 mm). Concentrate to dryness in a vacuum centrifuge.

4 Remove the 5'-DMT as follows:

Dissolve the resultant DNA residue in 3 mL 80% glacial acetic acid. Let stand at room temperature for 10 minutes. Freeze the reaction mixture on dry-ice and concentrate to dryness in a vacuum centrifuge.

5 Extract the 5'-HO-DNA with ethyl acetate to remove DMT-OH and other organic impurities as follows:

Dissolve the resultant DNA residue in 1 mL of H_20. Add 1 mL of ethyl acetate and vortex. Allow the organic layer to separate from the aqueous layer containing the DNA and possible HPLC column particulates (centrifuge if necessary). Remove the organic layer with a Pasteur pipet and discard. Repeat the extraction procedure two more times. During the third and final extraction, centrifuge for 5-10 minutes to separate organic and aqueous phases well enough to spin down particulates in the aqueous portion. Remove the organic layer, then carefully remove the aqueous, DNA-containing layer from any particulates spun to the bottom. Transfer to a clean test tube. Concentrate to dryness in a vacuum centrifuge.

6 *Precipitate the 5'-HO-DNA with ethanol to remove triethylammonium acetate and residual organic impurities as follows:

Dissolve the resultant DNA by sonicating and/or warming (50-60°C) in 300 µL of 1.0 M NaCl. If the DNA fails to dissolve, check the pH and, if necessary, adjust to either neutral or slightly basic by adding a small amount of NaOH (5 µL portions of 5N). Precipitate the DNA by adding 1 mL of 200 proof ethanol drop-wise while vortexing. Chill on ice for ~30 minutes. Centrifuge for ~5 minutes. Carefully remove supernatant and save for later OD measurement. Repeat the precipitation procedure using double the amounts of 1 M NaCl and ethanol. Bring the sample to dryness in a vacuum centrifuge.

*For tetramers and smaller oligos precipitation may not be possible. In these cases run a size exclusion column using Bio-Gel™ P-2, 200-400 mesh (Bio-Rad Laboratories, catalog no. 150-0150) instead of precipitation.

7 OD measurement:

Dissolve the resultant DNA residue in 2 mL of H_20. Remove a 10 µL aliquot for OD measurement. If any particulates remain, centrifuge for 5-10 minutes and carefully transfer the supernatant to a clean test tube (syringe filtering may lead to contamination due to lubricants used on the syringe plunger). Bring the 10 µL aliquot to 1 mL with H_2O and measure OD_{260}. Multiply the number obtained by 200 to determine the OD-units contained in the bulk sample. Concentrate the bulk sample to dryness in a vacuum centrifuge. Discard the OD aliquot or use for HPLC compositional analysis[1] after digestions with snake venom phosphodiesterase and alkaline phosphatase.

Check the supernatant of the precipitation procedure (obtained in step 6) for non-recovered DNA by bringing the volume to 3 mL with H_2O and removing a 150 µL aliquot. Dry the aliquot using a stream of N^2. Dissolve in 1 mL of H_2O and measure OD_{260}. Usually there is very little material (a few OD's) in the supernatant, which is then discarded.

Typically, about 50% yield (not corrected coupling efficiency) is obtained. For example, a 10 µmol synthesis of 5'-DMT (AT)5 afforded 500 OD_{260} units of

purified product, (AT)5, which has a mM extinction coefficient given by (5 x 14)+(5 x 7) = 105.

Note For purines A and G use 14, and for pyrimidines C and T use 7.

Since 1 μmol in 1 mL is 1 mM, 10 μmol OD (AT)5 in 1 mL would give 10 x 105 = 1,050 OD_{260} units. Thus, the yield of product was (500/1,050) x 100 = 48%.

References

1 Eadie, J. S., McBride, L. J., Efcavitch, J. W. Hoff, L.B., and Cathcart, R. Analytical Biochemistry **165**, 442-447 (1987)

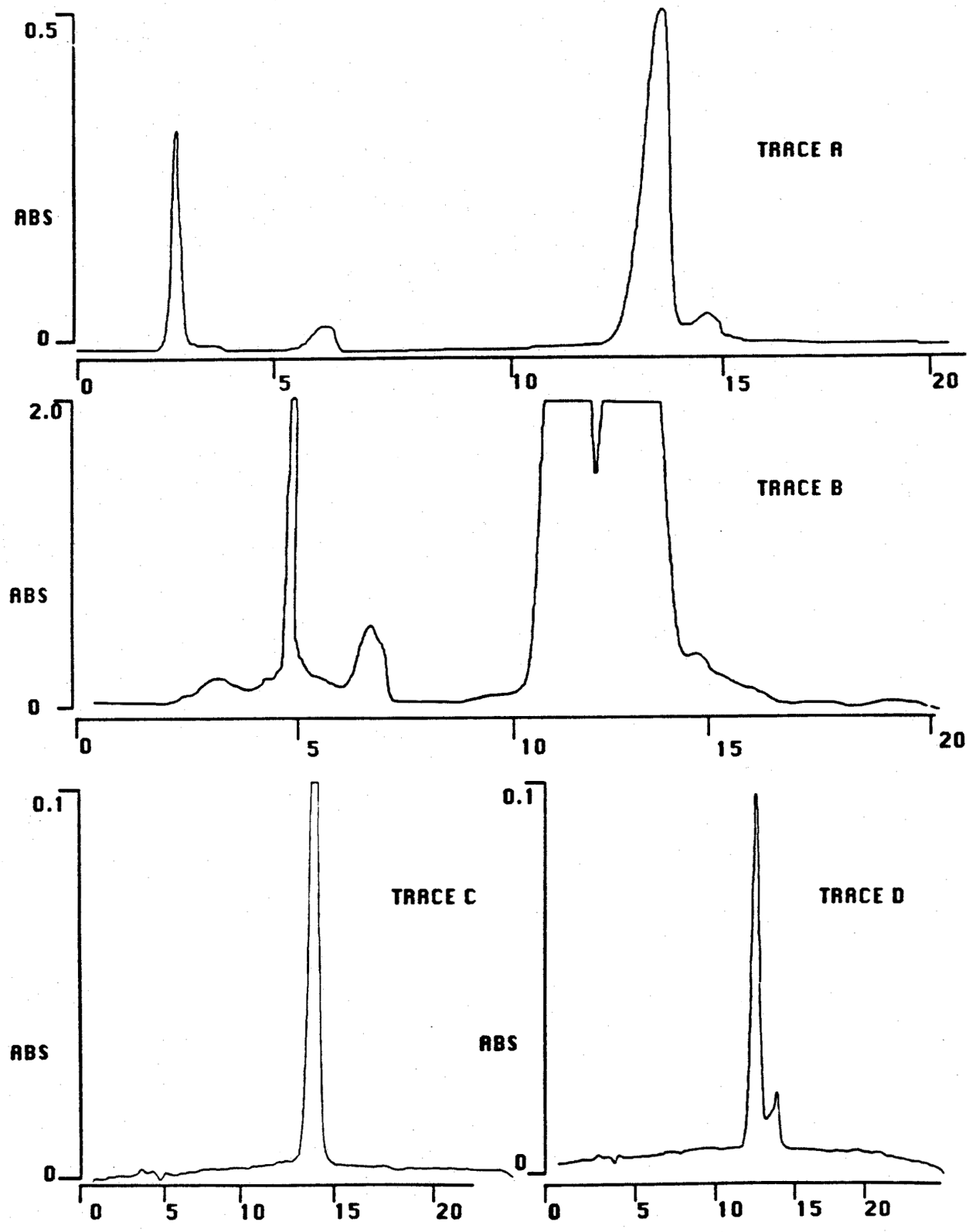

Figure 2. Traces A, B, C, D

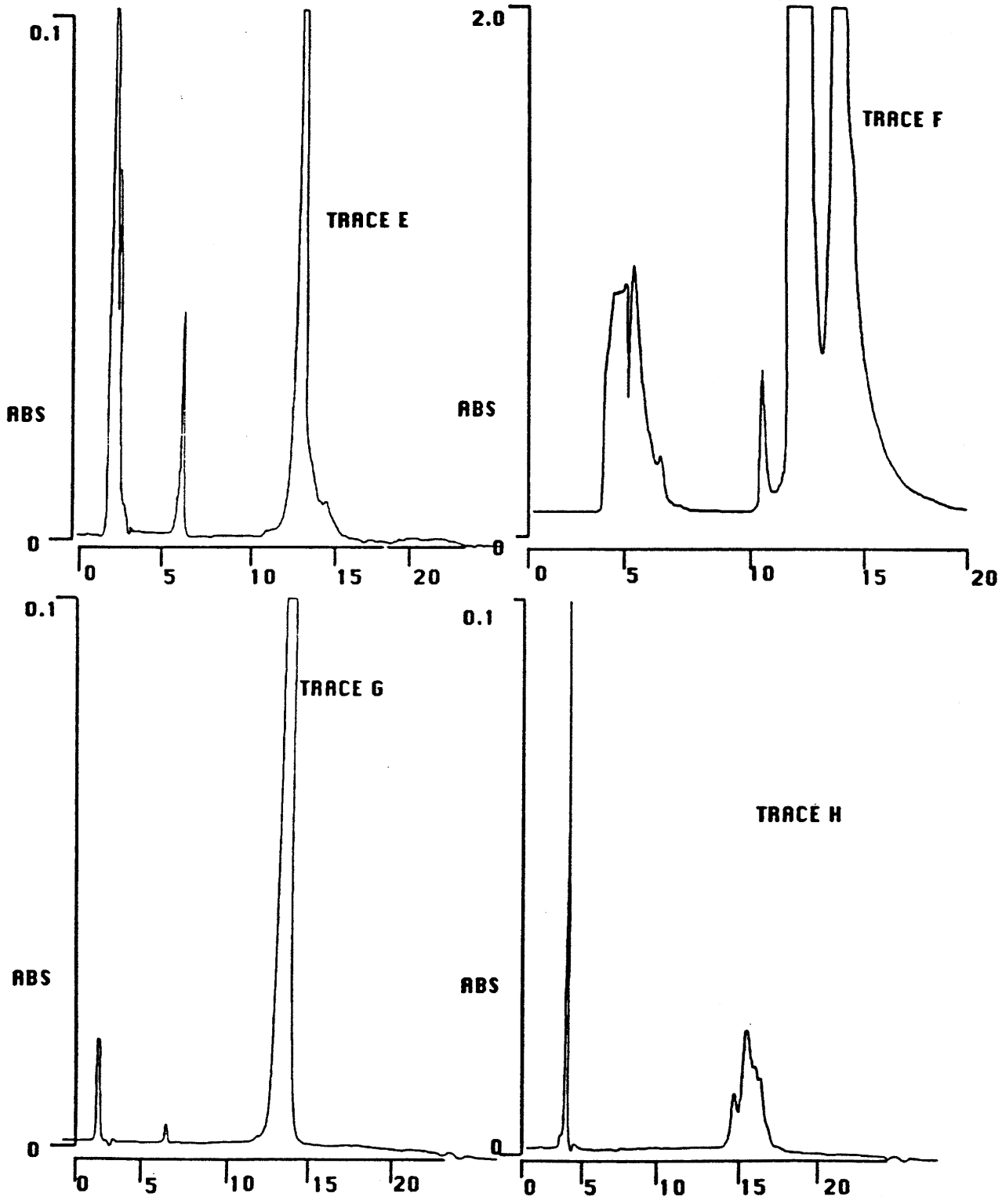

Figure 3. Traces E, F, G, H

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