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Appendix 1

Enzymatic Digestion/Base Composition Analysis

At Applied Biosystems we have developed a reliable and efficient assay to measure chemical authenticity. The oligonucleotide is enzymatically degraded to its constituent nucleosides with snake venom phosphodiesterase and bacterial alkaline phosphatase. The crude nucleoside mixture is then injected into a reverse-phase HPLC system that efficiently separates the desired nucleosides and any modified nucleosides. The sensitivity of the chromatography is sufficient to detect modifications at the 0.05% level (in relation to total UV absorbance).

Typical results of this assay are shown in Figure 1, which shows a chromatogram of the enzymatic digestion of a crude 61 mer. The results show little, if any, detectable modification in syntheses prepared from these phosphoramidite intermediates. Sometimes deoxyinosine (dI) is present due to the contamination of the enzyme preparations with adenosine deaminase, which converts deoxyadenosine to dI.

The enzymatic digestion of oligonucleotides and HPLC analysis of the resulting nucleosides is a proven and valuable analytical tool. The results are qualitative in detecting and characterizing chemical damage, such as base modifications. They are also quantitative in determining the nucleoside composition.

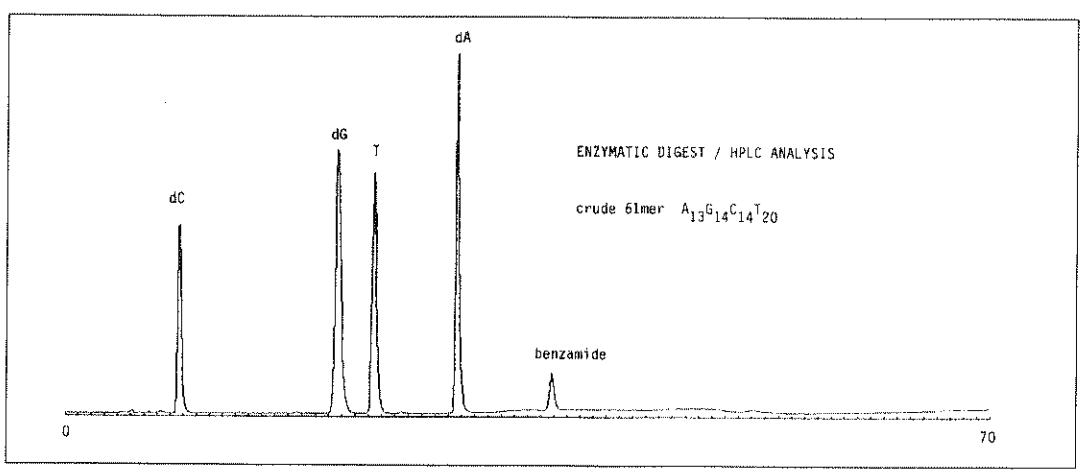

Figure 1. *Enzymatic Digest/HPLC Analysis*

Sample Preparation

Synthetic oligonucleotides (DNA or RNA) can be used in a crude or purified state. The enzymes are active under the digest conditions, with oligonucleotides dried directly from ammonia, the cleavage/deprotection reagent. Salts remaining from other sources, such as ion-exchange HPLC, may result in incomplete hydrolysis to

nucleosides and may give ambiguous results. Ethanol precipitation or desalting with the Oligonucleotide Purification Cartridge (OPC) is advised for these samples.

For the following protocol and HPLC conditions, from 0.1 to 1.0 ODU ($A_{260\,nm}$) per dried oligonucleotide is appropriate, assuming a single and total injection of the digested sample. Eppendorf tubes (1.5 mL) are convenient vessels.

Digestion

Prepare the following cocktail to digest the oligonucleotides to nucleosides. Volumes are given for a single sample. Multiply each volume by the number of samples to be analyzed. Add one to the number for an HPLC digest blank control. Pipet this total volume into a tube. For example, if analyzing 10 samples, add 11 times each volume to the mixture.

Freshly deionized water (MilliQ type)	44.0 µL
1.0 M $MgCl_2$	0.8 µL
0.5 M Tris buffer, pH 7.5	3.5 µL
Bacterial Alkaline Phosphatase (BA)	4.0 µL
Snake Venom Phosphodiesterase (SVP)	2.4 µL
Total	55.0 µL

1. Vortex the mixture and pipet 55 µL into each sample at room temperature, leaving the remaining 55 µL in the tube as the digest blank.
2. Vortex each sample and spin.
3. Incubate the sample at 37 °C for 8–24 h.

Preparation of Solutions

There are many commercial sources of the above cocktail components. The following have worked well in Applied Biosystems labs:

Snake Venom Phosphodiesterase (Pharmacia, 27-2821-01). SVP is an exonuclease that cleaves 3'-5' internucleotide phosphate bonds from the 3' terminus, yielding 5' monophosphate nucleotides. Some SVP samples contain varying amounts of adenosine deaminase activity. This results in a low level conversion of adenosine or deoxyadenosine to inosine or deoxyinosine. The enzyme comes as a lyophilized powder. Dissolve the sample to 1 mL/mg. Store at -20 °C or colder. Avoid prolonged exposure to room temperature conditions.

Bacterial Alkaline Phosphatase (Pharmacia, 27-0598-02). BAP effects the hydrolysis of 5' phosphates from the 5' monophosphate nucleotides resulting from SVP cleavage. This reagent comes as a stabilized suspension in ammonium sulfate. To prepare the stock solution for the digest protocol, centrifuge the tube to pellet the salt and remove the supernatant-containing BAP. Transfer the supernatant to a tube and dilute with

water to a final activity of 10 µL/unit. Store at -20 °C or colder. Avoid prolonged exposure to room temperature conditions prior to use.

Preparing the Digested Samples for HPLC Analysis:

1. After incubation, add 7 µL of 3 M sodium acetate and 155 µL of ethanol to each sample.
2. Vortex and freeze the samples on dry ice for 10 min, then centrifuge for 10 min.
3. Carefully remove the supernatants with a pipet and transfer them to a set of new, labeled tubes. Next, add 452 µL of ethanol to each sample.
4. Discard the original tubes, which contain the pellets. Vortex and freeze the samples on dry ice for 10 min, then centrifuge them for 10 min.
5. Again remove the supernatants, being especially careful not to disturb the pellets, and transfer them to a second set of new, labeled tubes. If necessary, leave some of the solution rather than remove any of the pellet.
6. Evaporate the samples to dryness under vacuum.

Sample injections should be spaced 85 min apart. It is advisable to run a digest blank to establish an absorbance baseline profile. The digest cocktail contains some artifacts, even after the ethanol precipitation, which appear as small peaks in the HPLC.

HPLC

Dissolve the dried sample in 60 µL of water and vortex each sample at least 30 seconds. If using an autosampler, transfer the samples to appropriate vials.

Column:	Spheri-5 RP-18, P/N 0711-0017 (cartridge)
Mobile Phases:	A: 3% acetonitrile in 0.1 M triethylammonium acetate (TEAA) 92:5:3 water/2 M TEAA/acetonitrile (v/v/v)
	B: 90% acetonitrile, 9:1 acetonitrile/water (v/v)
Sample Injection:	50 µL

Table 1. Enzymatic Digest HPLC Gradient - DNA

Elapsed Time (min)	Step Length (min)	%A	
0	5	100	Flow = 0.5 mL/min
5	30	100	Flow = 0.5 mL/min
35	30	90	Flow = 0.5 mL/min
65	5	0	Flow = 0.5 mL/min
70	2	0	Flow = 0.5 mL/min
72	15	100	Flow = 0.5 mL/min
87	2	100	Flow = 0.5 mL/min
89	10	0	Flow = 1 mL/min
99	2	0	Flow = 0 mL/min

Table 2. Enzymatic Digest HPLC Gradient - RNA

Elapsed Time (min)	Step Length (min)	%A	
0	15	100	Flow = 0.5 mL/min
15	20	100	Flow = 0.5 mL/min
35	30	90	Flow = 0.5 mL/min
65	5	0	Flow = 0.5 mL/min
70	2	0	Flow = 0.5 mL/min
72	15	100	Flow = 0.5 mL/min
87	2	100	Flow = 0.5 mL/min
89	10	0	Flow = 1 mL/min
99	2	0	Flow = 0 mL/min

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Appendix 2

Manual Detritylation of Oligonucleotides After Deprotection

Oligonucleotides can be prepared on the DNA synthesizer either trityl-on or trityl-off (trityl = DMT) by making the selection at the beginning of the synthesis. Virtually all applications require DNA to be trityl-off, or detritylated, with a free 5' hydroxyl. However, the trityl-on option is selected when purifying by HPLC or the OPC.

Detritylation is conducted within the OPC purification protocol. After purification by HPLC, trityl-on oligonucleotides must be detritylated manually. The 5' dimethoxytrityl (DMT) is readily cleaved by limited exposure to mild acidic conditions, causing little or no depurination or other damage. After deprotection of the base protecting groups, oligonucleotides are far less susceptible to depurination. The dimethoxytritanol byproduct is removed in the supernatant in the following protocol.

Manual Detritylation Protocol

1. Dry the trityl-on oligonucleotide thoroughly in a small centrifuge vessel, e.g., a 0.5–2.0 mL Eppendorf-type tube. If the oligonucleotide has been purified by HPLC, several evaporations with distilled, deionized water may be necessary to remove the remaining volatile salts, e.g., triethylammonium acetate, from the mobile phase.
2. Dissolve in 30 µL/ODU of 80% acetic acid at room temperature (20 µL/ODU at large scale). Mix by vortexing and let stand for 20 min. Phosphorothioate oligonucleotides may require brief heating to attain solution.
3. Add 5 µL/ODU of 3 M sodium acetate and 100 µL/ODU of ethanol. Mix by vortexing. For very short oligonucleotides (<15-mers), isopropanol may be substituted for ethanol to ensure complete precipitation.
4. Chill the precipitating mixture at refrigerator or freezer temperatures for approximately 30 min, then centrifuge at high speed for 5 min.
5. Remove and discard the supernatant with a pipet or micropipet. Be careful not to disturb the pellet. Small quantities (less than several ODU or 100 µg) may not be visible.
6. Add 100 µL/ODU of ethanol, mix briefly and centrifuge for 1–5 min.
7. Remove and discard the supernatant again, being careful not to disturb the pellet. The oligonucleotide pellet can be dried by vacuum centrifugation.
8. Resuspend the detritylated, desalted oligonucleotide in aqueous media and quantitate by absorbance.

Appendix 3

Ethanol Precipitation of Oligonucleotides

Oligonucleotides can be isolated from impurities such as protecting group byproducts, benzamide, isobutyrylamide, short failure sequences, salts and small amounts of solvents by precipitation from alcohol and water solutions. When the reagents and conditions are well-chosen, the recovered amount of the oligonucleotide can be excellent, and can attain an enriched state of purity. Also, precipitation affords an easy opportunity to change the cation salt form associated with the oligonucleotide. As an example, the crude synthesis product is in the ammonium salt form, which may have limited solubility or inhibit some enzymes.

Precipitation Protocol

1. Dissolve the oligonucleotide in 30 µL of water (20 µL at large scale) and 5 µL of 3 M sodium acetate, per ODU of oligonucleotide.
2. Add 100 µL/ODU of ethanol, then vortex.
3. Store at refrigerator or freezer temperatures for about 30 min, then centrifuge at high speed for 5 min. For very short oligonucleotides (< 15-mers), isopropanol may be substituted for ethanol to ensure complete precipitation.
4. Remove the supernatant with a pipet or micropipet and discard, being careful not to disturb the pellet. Small quantities (less than several ODU or 100 µg) may not be visible.
5. Add 100 µL/ODU of ethanol again, mix briefly and centrifuge for 1–5 min.
6. Remove the supernatant again and discard, being careful not to disturb the pellet. The oligonucleotide pellet can be dried by vacuum centrifugation.
7. Resuspend the detritylated, desalted oligonucleotide in aqueous media and quantitate by absorbance.

Appendix 4

Quantitation of Oligonucleotides by UV Absorbance

Oligonucleotides are most accurately and conveniently quantitated by the measured absorbance of UV light of the sample in a spectrophotometer. The method is nondestructive and the sample is easily recovered.

According to Beer's law: A = eCl:

A = absorbance;

e = molar extinction coefficient;

C = concentration (mol/L);

l = path length (cm), typically 1 cm.

The conditions are defined at a specific wavelength, temperature and media, all of which influence "e". The purine and pyrimidine bases of DNA and RNA strongly absorb light with maxima near 260 nm. A useful approximation is e = 10, 000 for each of the four bases. The bicyclic purines, deoxyadenosine and deoxyguanosine, absorb more strongly (higher extinction coefficients) than the monocyclic pyrimidines, deoxycytidine and thymidine. Using this and other approximations, absorbance can be translated to mass and concentration of oligonucleotides.

An ODU is the absorbance of a 1-mL solution, typically in water, measured at 260 nm in a 1-cm path-length cuvette. One ODU represents approximately 33 µg of single-stranded oligodeoxynucleotide (DNA). For example, 1 mg of an oligonucleotide is about 30 ODU. Conversely, for concentration purposes, 1 µmol of oligonucleotide will absorb 10 ODU/base. For example, 0.2 µmol of an 18-mer would be approximately 36 ODU. Figure 1 shows a spectrophotometric scan in the wavelength range of absorbance by oligonucleotides.

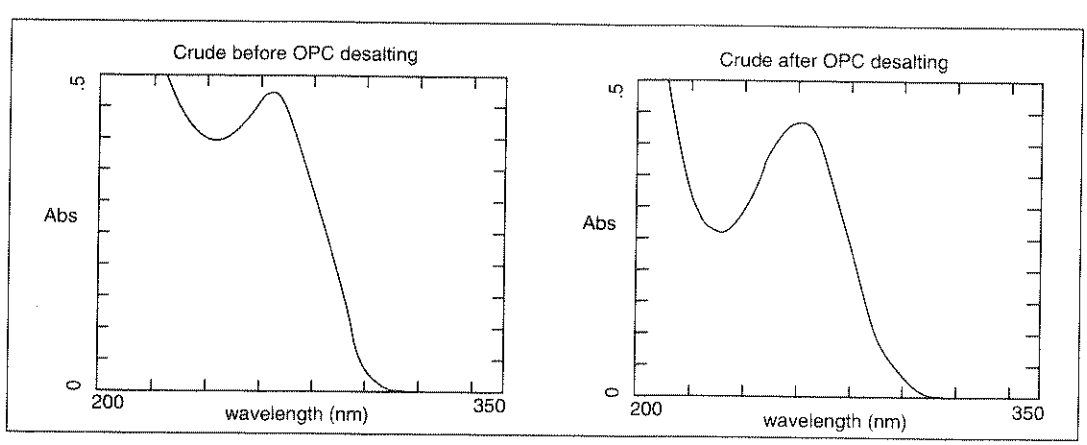

Figure 1. *200–350-nm scan of a typical oligonucleotide*

Appendix 5

Manual Cleavage and Deprotection of Oligonucleotides

The information and procedures provided in this appendix are required for manual cleavage and deprotection of oligonucleotides made on the 381A and 391 PCR-MATE synthesizers, or when one of the standard ending procedures is not used.

Reagents, Solvents and Apparatus

Reagent-grade, concentrated ammonium hydroxide is provided in the installation reagent kit. Additional quantities should be purchased from a local supplier. Small DNA collection vials with a rubber-lined screw cap are available from ABI (P/N 400048) or can be purchased from a local source. However, Teflon-lined caps should be used with the vials because rubber-lined caps can leach contaminants into the DNA-ammonium hydroxide solution. They can be ordered from Wheaton, Inc. (P/N 240408, size 13-425).

Double Syringe Method (for Manual Ammonia Cleavage)

1. Attach an empty luer tip syringe, with plunger fully inserted, into one end of the synthesis column.

IMPORTANT	**The concentration of ammonia is critical. Use fresh, concentrated ammonium hydroxide that has been opened less than 1 month.**

2. Load 2 mL of concentrated ammonia into another luer tip syringe and attach it to the other end of the column.
3. Holding one syringe in each hand, carefully inject the reagent through the column to the empty syringe and return the reagent through the column several times.
4. Allow it to stand for at least 1 h at room temperature.
5. Drain all the reagent into one syringe, then detach and eject the contents carefully into an appropriate vial to achieve complete deprotection by heating.

IMPORTANT	**Use a tightly sealed DNA collection vial that can withstand positive pressure. The vial must also have a Teflon-lined cap. Rubber-lined caps have contaminants that leach out of the cap liner during base deprotection. Remember that the product DNA is now in solution and no longer bound to the support. Save the column until the cleavage is confirmed.**

6. If you are using standard phosphoramidites, remove the exocyclic amine base-protecting groups (benzoyl and isobutyryl) by heating the vial of DNA at 55 °C for 8–15 h.

   If you are using FOD phosphoramidites, remove the exocyclic amine base-protecting groups (dimethylformamidine and isobutyryl) by heating the vial of DNA at 55 °C for 1 h or by allowing it to remain at room temperature for 8 h.

   In either case, longer heating is advisable if the ammonium concentration is questionable.

7. After completing deprotection, cool the ammonium hydroxide-DNA solution and perform the appropriate analysis or purification step:

   - When purifying by OPC, dilute the trityl-on oligonucleotide with water, and then load it directly onto the cartridge. No other preparation is needed.
   - If the DMT group was removed previously as a part of the synthesis cycle, the DNA is ready for analysis and/or purification by PAGE or ion-exchange HPLC.
   - When purifying by trityl-specific, reverse-phase HPLC, remove the ammonia by vacuum. To prevent accidental detritylation, keep the solution basic by adding 1 drop of distilled triethylamine every 10 min. Also, avoid heating the sample.

After collection and concentration of the product from reverse-phase HPLC, perform the following procedures:

1. Detritylate the dried sample by dissolving it in 200–500 µL of 80% acetic acid. Because the acetic acid is an aqueous solution, the trityl cation will react with water to form tritanol and will not become orange.
2. After 20 min, add an equal volume of 95% ethanol and lyophilize the sample. The dried sample is lyophilized from ethanol until no acetic acid remains. The hydrolyzed DMT group and remaining salts can be removed from the vial by methods such as the OPC desalting procedure.

Appendix 6

Dimethoxytrityl (DMT) Cation Assay/ Common Trityl Assay Symptoms and Causes of Synthesis Failure

Introduction

Determining the stepwise yield of coupling reactions during synthesis is a convenient indicator of the performance of an automated DNA synthesizer. The standard assay for determining stepwise yield is to spectrophotometrically measure the amount of DMT cation liberated at each 5' deprotection step in the synthesis process. The DMT group is the 5' hydroxyl protecting group.

All Applied Biosystems DNA synthesizers can automatically collect, with the aid of a fraction collector, the solution containing the DMT cation during the detritylation step of the synthesis cycle. The group is completely ionized in the acidic solution in which it was cleaved. The yield can be accurately measured (± 0.5%) at 498 nm.

This assay is valid only when used in conjunction with a synthesis cycle utilizing a capping procedure that quantitatively blocks unreacted oligonucleotide chains. If these chains are not capped, the assay will give incorrectly high yield data because of the coupling and subsequent detritylation of the n- sequences.

If the DMT cation measures greater than 98%, the instrument is probably performing properly. Measurement of the DMT cation will detect problems such as improper flow rates, "wet" acetonitrile, or empty reagent bottles. Each can reduce the stepwise yield by as much as 5%—a figure that is difficult to detect visually. Also, when a problem in the synthesis process has occurred, whether instrument-related or chemical, it is usually much easier and quicker to detect if the DMT cation results have been quantified and documented.

The Assay Procedure

It is difficult to recommend a standard procedure for the DMT cation assay because of the various fraction collectors, spectrophotometers and other equipment used. We find the Isco Retriever II, with a four-column adapter, suitable for two- and four-column instruments. Both can accommodate several test tube sizes, such as a graduated 15-mL tube (VWR, 21054-008). Each sample should be diluted with proper mixing to 10 mL with 0.1 M toluene sulfonic acid monohydrate in acetonitrile. The samples are then mixed and the absorbance is read at 498 nm.

Quantitatively, the stepwise yield is determined by dividing the last absorbance value, or the lowest, by the second detritylation, or the highest. This value represents the overall yield. When it is multiplied exponentially by the reciprocal of the number of couplings, the stepwise yield is obtained. The first base detritylation is not relevant because no coupling has yet occurred. The DMT absorbance of each subsequent base should show a steady decline. Rising or fluctuating absorbance values may indicate synthesis problems or faulty assay technique.

Table 1 shows theoretical data from an assay of the synthesis of a 15-mer in which the yield at each step was exactly 98.0%. In this simplified example, the average stepwise yield is equivalent to the result calculated from the overall yield. In actual cases, these numbers will differ slightly. Because the DMT assay depends upon small differences between large numbers, there are significant statistical fluctuations.

Expected stepwise yields from this assay should be in the 98 + 0.5% range. Yields below 97% indicate possible synthesis difficulties that may affect final product quality. However, stepwise yields in the expected range do not ensure product quality. There are factors that occasionally may result in inadequate quality, despite the fact that stepwise yields are high. Hence, there can be false positives when using this assay alone.

The only way to confirm product quality is to evaluate the crude oligonucleotide either by PAGE, gel capillary electrophoresis, or one of the several HPLC procedures. The DMT assay used in conjunction with either PAGE, CE or HPLC will ensure product quality while minimizing the time required for analysis and purification.

When time does not permit reading every sample, inspect it to determine gross changes.

Table 1. Theoretical Results from the DMT Cation Assay
DMT Yield Sample Calculation

Number (n)	Base	Absorbance (A)
1	Support T	
2	A	0.274
3	G	0.271
4	C	0.269
5	T	0.262
6	G	0.258
7	A	0.251
8	A	0.247
9	C	0.243
10	T	0.243
11	G	0.242
12	C	0.233
13	T	0.226
14	A	0.221
15	G	0.217
16	C	0.210
17	A	0.204
18	G	0.198

Y = Overall yield = lowest/or highest $A_n/A_2 = Y$

$0.198/0.274 = 0.723$ (72%)

Y_s = Stepwise yield = overall yield$^{1/\text{couplings}}$ $Y_s = Y^{1/n}$

$0.723^{1/16} = 0.98$ (98.0%)

Common Trityl Assay Symptoms and Causes of Synthesis Failure

Symptoms	Possible Causes	Action
Low trityl volume and colorless	• Poor TCA delivery	• Check TCA delivery
Low trityl volume, but dark or normal color trityl	• Poor acetonitrile delivery	• Check acetonitrile delivery
Normal trityl volumes, but consistently low coupling yields	• Wet HPLC-grade acetonitrile or water in phosphoramidites and/or tetrazole.	• Replace acetonitrile with a water content <300 ppm (0.003%). Replace phosphoramidites and/or tetrazole
	• A leak is allowing atmospheric water vapor into plumbing	• Check instrument for leaks
	• Poor delivery of phosphoramidites or tetrazole	• Check phosphoramidites and tetrazole deliveries
Normal trityl volumes with normal color in first trityl, but colorless trityls with addition of other bases	• Instrument was idle for more than 6 h, and the phosphoramidite purge was not performed	• Perform phosphoramidite purge prior to a run when the instrument is idle for more than 6 h
	• Wet HPLC-grade acetonitrile or water in phosphoramidites and/or tetrazole	• Replace acetonitrile with a water content <300 ppm (0.003%). Replace phosphoramidites and/or tetrazole
Random low trityl color	• Problems in collecting, diluting, or mixing trityls	• Check fraction collector alignment. Check diluting and mixing procedures
	• Simultaneous coupling and capping failure	• Check the reagent delivery system for leaks or restrictions
	• Incomplete detritylation	• Check TCA delivery
Normal trityls, but DNA product is a "smear" on a gel	• Poor iodine delivery	• Check iodine delivery
Normal trityls, but DNA product contains many "failure bands" on a gel	• Poor capping reagent delivery	• Check capping reagent (11 and 12) delivery
Good trityls but no bands on gel	• Poor ammonium hydroxide delivery	• Check ammonium hydroxide delivery
	• Ammonium hydroxide not sufficiently concentrated	• Store ammonium hydroxide at 4 °C and replace it on the instrument weekly