Evaluating and Isolating Synthetic Oligonucleotides

The Complete Guide

Applied Biosystems

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Acknowledgements

What began as a modest goal to update some of the protocols in the 1987 edition of *User Bulletin 13*, begun by Steve Lombardi and his colleagues, has grown into the substantial work you have before you. This wealth of information, suggestions and advice is obviously not the work of one, or even of a few people, but of many. We recognize and greatly appreciate the efforts of all who helped to produce the 1987 edition, especially Dr. Bill Efcavitch, Dr. Gerald Zon, Dr. Lincoln McBride and Dr. Elaine Heron.

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We hope you find this guide to be of value. After all, much of the inspiration and many of the details contained in it originated with you, the experts of DNA synthesis.

Dr. Alex Andrus

DNA Synthesis R&D Manager

Manufacturers and Suppliers

Throughout this guide, references are made to non-ABI instruments and other products used in the synthesis and isolation of oligonucleotides. Listed below for your convenience are the names, addresses and telephone numbers of these manufacturers.

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Del Mar, CA 92014
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(800) 243-2555

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WARNING	***Some chemicals used with Applied Biosystems instruments are considered hazardous. Hazards are prominently displayed on the labels of all chemicals. In addition, material safety data sheets (MSDSs) are included in all Applied Biosystems User's Manuals. MSDSs provide informtion about physical characteristics, hazards, precautions, first aid, spill clean-up and disposal procedure.*** ***Before beginning any procedure mentioned in this guide, please familiarize yourself with the MSDSs of all hazardous chemicals with which you will have contact.***

Introduction

Purpose of the Bulletin

Automated DNA synthesis today is reliable, economical and relatively simple because of advances in synthesis chemistry and technology. Oligonucleotides that once required weeks to synthesize can now be prepared efficiently and reliably in a matter of hours. The most common experiments require primers for sequencing and GeneAmp™ Polymerase Chain Reaction (PCR) applications, but hundreds of other applications require different types of synthetic oligonucleotides, including:

- RNA;
- antisensephosphate analogs;
- fluorescent dye-labeled;
- biotin-labeled;
- protein conjugates.

For some applications, economy dictates the production of small-scale oligonucleotides, while the burgeoning fields of therapeutics and diagnostics require much larger oligonucleotide quantities. Extremely long oligonucleotides have found applications as PCR templates and in gene construction.

Methods of analysis and purification are as diverse as oligonucleotide quantity and length. These methods range from none at all to exquisite new methods for high resolution characterization and ultrapurification.

At Applied Biosystems, we are committed to offering the best technical support to our users worldwide. This support includes the full spectrum of nucleic acids chemistry, from basic organic chemistry to investigation of the biological activity of the synthetic product. This publication contains all the methods of analyzing and purifying synthetic oligonucleotides with which our staff has had direct experience. We believe these methods to be the best currently available. They represent our continuing effort to make DNA synthesis a more efficient and reliable technology.

Purification and Analysis: Which Methods to Use

Because some methods of purification and analysis are more difficult and time-consuming than the actual synthesis, it makes sense to become familiar with all available options. By accurately identifying the methods necessary to obtain oligonucleotides of sufficient quantity and quality, useless expense, waste of time and costly errors can be avoided. Alternative methods are often available for analysis or purification. By evaluating the various methods described in this guide, an optimal strategy can be tailored for specific applications. Some factors to consider when devising strategies for analysis and purification are:

- oligonucleotide sequence;

- amount needed for a particular application;
- equipment necessary for the chosen method;
- time constraints;
- purity level required by the application.

The method of synthesis dramatically affects the amount of purification required to obtain high-quality product. Before the advent of solid-phase methods, syntheses were performed in solution with the product of each reaction—there are four reactions per synthesis cycle in the phosphoramidite chemistry—requiring purification.

Solid-phase synthesis eliminated the need for intermediate purification because the unreacted reagents are removed by simple filtration. In solid-phase synthesis, the 3' nucleoside is anchored to an insoluble support. Propagation is achieved by delivering synthesis reagents to the support in a stepwise fashion determined by the desired sequence and by the chemistry. Solid-phase methodology was a key advance in the development of automated DNA synthesizers.

The Advantages of Automation

Automation offers many advantages over manual solid-phase synthesis, which is rarely attempted today. The most important of these advantages are increased throughput and yield. Because Applied Biosystems DNA/RNA synthesizers can operate unattended, the number of oligonucleotides synthesized on a daily basis can easily support the needs of most laboratories. Phosphoramidite chemistry, the most widely used method of oligonucleotide synthesis, requires very precise synthesis conditions, which are more easily achieved with automated instruments. The high yields possible with this chemistry, therefore, are more consistently realized.

After its introduction in 1981, the phosphoramidite method rapidly became the DNA synthesis chemistry of choice, largely because of its inherently high coupling yields. But the synthesis chemistry alone does not guarantee good results. Chemical reactions in DNA synthesis are not perfect. Small quantities of sequences fail to undergo coupling and must be chemically capped during each base addition cycle. Without capping, these sequences would couple during subsequent steps to perpetuate shorter, failure sequences. In subsequent coupling reactions, the uncapped truncated chains would compete with the full-length oligonucleotide for phosphoramidites, diminishing coupling efficiency. Without efficient capping, a large population of n-1-mer failure sequences results, complicating analysis and purification. One of the keys to obtaining consistent high-quality product from an automated DNA synthesizer is the synthesis cycle. Every step in the cycle has been optimized for consistent success.

This guide reflects our many years of experience with oligonucleotide synthesis, as well as the incorporation of significant contributions from our customers. For the past decade, Applied Biosystems has been actively engaged in every aspect of oligonucleotide production: synthesis, analysis, purification and applications. We trust that the information contained here will assist you in attaining your goals.

Evaluation of the Crude Product

This section presents information regarding evaluation of the crude product by:

- MicroGel capillary electrophoresis (CE);
- polyacrylamide gel electrophoresis (PAGE);
- high-performance liquid chromatography (HPLC).

Postsynthesis processing, completed before analysis and purification, includes complete deprotection of the oligonucleotide, quantitation of yield and sometimes desalting.

Oligonucleotide Sequence

As a general rule, the sequence, or base content, of an oligonucleotide does not affect synthesis performance. Nor does it influence analysis and purification or selection of those methods. During synthesis, the four bases (A G C T) react at comparable rates. Little, if any, compensation for their differences is necessary. Similarly, during analysis and purification, the chemical differences imparted by the bases are not usually significant. Occasionally, some sequences may show anomalous behavior, caused in most cases by hydrogen-bonding, which creates secondary structures. The exceptions occur primarily under nondenaturing conditions such as in reverse-phase HPLC.

The relatively narrow range of chromatographic and electrophoretic differences among oligonucleotides makes this class of molecules generally straightforward to analyze and purify. Oligonucleotide length is the most discriminating factor, allowing efficient separation of product from the crude synthesis mixture.

Synthesis Yield

The theoretical yield of any oligonucleotide can be calculated by length and synthesis scale, using approximations derived from Beer's law: A= eCI (see Appendix D). The flexibility of most Applied Biosystems DNA synthesizers allows the choice of four synthesis scales without any hardware changes:

- 40 nmol;
- 0.2 µmol;
- 1 µmol;
- 10 µmol.

The smallest scale, 40 nmol, provides more than sufficient oligonucleotide for common applications such as sequencing and GeneAmp™ Polymerase Chain Reaction (PCR) primers. At the opposite end of the spectrum, the 10-µmol scale is used to produce oligonucleotides for physical studies, antisense studies, and for commercially produced probes and primers.

Table 2-1 lists the approximate yields of crude oligonucleotide from the four synthesis scales for a 20-mer sequence. The expected crude yields in the table are less than the theoretical maximum, where the synthesis chemistry is perfect and each reaction occurs at 100% yield. The theoretical maximum can be easily calculated by a simple formula: 1 µmol of single-stranded DNA contains about 10 optical density units (ODU) per base. For example, the theoretical maximum yield for synthesis of a 32-mer at the 0.2 µmol scale would be 64 ODU. The crude yield after the typical 98% average yield per cycle (71% overall yield) would be 45 ODU.

Table 2-1. Typical Crude DNA Yields of a 20-mer

Scale	ODU	Amount
40 nmol	5-10	165-330 µg
0.2 µmol	20-30	660-1000 µg
1 µmol	100	3.3 mg
10 µmol	800	26.4 mg

The actual amount of pure oligonucleotide that is attainable after purification depends on synthesis efficiency and can vary for a variety of reasons. In general, the smaller synthesis scales are more efficient, generating higher yields of pure oligonucleotide product. The approximate yields given here are applicable for both phosphorothioate and normal phosphodiester sequences.

Conversion (33 µg of oligonucleotide per ODU (A_{260nm})) between absorbance (ODU) and mass depends on an average extinction coefficient per base of 10,000. Purine-rich sequences have higher molecular extinction coefficients and therefore have higher absorbance values per unit mass. Pyrimidine-rich sequences have lower molecular extinction coefficients and therefore have lower absorbance values per unit mass.

Because the synthesis chemistry is not perfect, the expected yield is less than the theoretical maximum yield. Table 2-2 indicates the approximate crude product yield that can be expected at each scale when all instrument and reagent parameters are optimized.

Table 2-2. Approximate Yield Per Base

Scale	ODU x Number of Bases
40 nmol	0.25-0.5
0.2 µmol	1-1.5
1 µmol	5
10 µmol	40

33 µg of single-stranded DNA = 1 ODU (A_{260nm})

Enzymatic Digestion/Base Composition Analysis

An important element of oligonucleotide analysis is the integrity of the molecular structure after the rigorous chemistry of the synthesis process. Although the growing oligonucleotide is chemically protected, the very reactive synthesis chemicals can attack at various points during synthesis. Because modifications, especially of the bases, can have a profound effect on biological activity, assessing chemical authenticity is important when characterizing a crude or unpurified synthetic oligonucleotide. Modified bases can be detected by the enzymatic digestion/base compositor HPLC assay (Appendix A).

Deprotection

Complete deprotection of an oligonucleotide involves removing the protecting groups from the phosphate groups, the exocyclic amino groups on the bases, and the 5' hydroxyl moieties. Incomplete deprotection of synthetic oligonucleotides will adversely affect analysis and purification, as well as biological activity. Cyanoethyl phosphoramidite oligonucleotides only require treatment with concentrated ammonium hydroxide to simultaneously cleave the product from its support and deprotect the phosphate groups.

Deprotection of the exocyclic amines of deoxyadenosine, deoxycytidine and deoxyquanosine is achieved by incubating the sample in concentrated ammonium hydroxide for a minimum of 8 hours at 55 ºC. When using Fast Oligonucleotide Deprotection (FOD) reagents, the sample should be incubated for 1 h at 55 ºC or for 8 h at room temperature. It is important that only fresh ammonium hydroxide be used. We recommend purchase of fresh ammonium hydroxide in small bottles (500 mL or less) and storage at 4 ºC. Each bottle should be dated when opened and kept refrigerated. Ammonia bottles that have been open longer than 1 month or that have warmed to room temperature should not be used for deprotection. Also, the ammonia reagent should not be kept on the synthesizer longer than 2 weeks.

Phosphoramidite synthesis employs a capping scheme that acetylates the 5' hydroxyl moieties that have failed to undergo coupling. These truncated sequences remain capped during synthesis and do not propagate in subsequent cycles. The acetyl groups are later removed during base deprotection in ammonia, resulting in free 5' hydroxyl groups. The product oligonucleotide, which is not acetylated, has a 5' hydroxyl that is protected by a dimethoxytrityl (DMT or trityl) group. This DMT group can be automatically removed by the instrument or retained if trityl-on analysis or purification methods are used.

Manual removal of the DMT group is achieved easily by treating a dried oligonucleotide with 80% acetic acid (see Appendix B for the exact protocol). The DMT group does not interfere with quantitation by UV absorbance; however, it blocks the phosphorylation of the 5' hydroxyl during analysis by autoradiography. Note that a free 3' hydroxyl is created when the synthesized oligonucleotide is cleaved from its support. No further deprotection is required.

Composition of the Crude Reaction Mixture

The crude mixture, dissolved in ammonium hydroxide from the final deprotection, contains a variety of impurities, including extraneous ammonium salts derived from the removed protecting groups. The crude mixture also includes:

- the desired full-length oligonucleotide product;
- shorter, failure sequence oligonucleotides, resulting from incomplete coupling and subsequent capping;
- a collection of oligonucleotides resulting from other low-level side reactions that were cleaved during ammoniolysis.

For a complete description of the synthesis chemistry, see the Chemistry section of your instrument user's manual.

Mixed-Base Coupling

Syntheses that have mixed-base sites produce a more complex crude reaction mixture. Instead of one product in the mixture, there may be many, depending on the number of mixed-base sites and the number of bases at each site. This heterogeneity of mixed-base-containing oligonucleotides can present problems during analysis and purification.

An important requirement in the synthesis of mixed-base oligonucleotides is the desired equivalent incorporation of each base at a redundant, or degenerate, site where more than one base has been delivered. Competitive coupling results in equivalent amounts of a desired base in each degenerate site. Because they are different molecules, the four phosphoramidites couple at slightly different rates. The order of reactivity is T > C > G > A. Normalized to 100%, when a premixed solution of A G C T phosphoramidites are delivered from a single bottle for coupling, the relative incorporation will be approximately:

A	20%
G	26%
C	24%
T	30%

The above numbers have a range of several percent, depending on location in a sequence, age of the phosphoramidites (G degrades faster than the others), and other subtle effects. This reactivity difference is largely compensated for by the order of the phosphoramidite positions on the valve block, where A is positioned closest to the column and T farthest.

During the testing phase of new Applied Biosystems DNA synthesizers, virtually equal incorporation of the four bases has been demonstrated hundreds of times. Figure 2-1 is an HPLC chromatogram of the four-base dimer mix, resulting from concurrent delivery of A G C T phosphoramidites to a T support in the sequence 5' (AGCT) T 3'.

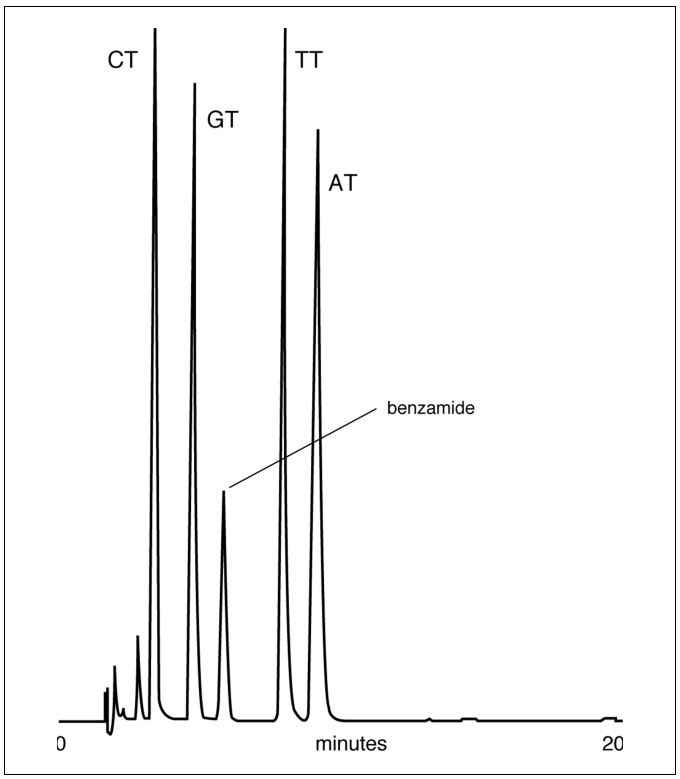


Figure 2-1. *HPLC analysis of the crude product from synthesis of 5' (AGCT) T3', on the 394, .2 µm CE cycle, trityl-off .2 µmol T WP CPG support. After cleavage and deprotection for 8 h at 55 ºC, the sample was analyzed on a Spheri-5 cartridge (220 x 4.1 mm), 1 mL/ min, 7% acetonitrile isocratic mobile phase.*

Desalting the Sample

The by-products of DNA synthesis using standard phosphoramidites other than oligonucleotides include:

- benzamide;
- isobutyramide;
- ammonium acetate;
- trace amounts of other organic impurities.

Although desalting the sample to remove these contaminants may be unnecessary for some applications, you may wish to consider desalting the crude sample before analysis and purification. At the very least, it provides for a more accurate quantitation of the oligonucleotide by UV absorbance. It also provides the opportunity to exchange the

- Oligonucleotide Purification Cartridge (OPC) with the desalting protocol;
- ethanol precipitation;
- size exclusion gel media.

The method to use depends on the quantity of oligonucleotide to be desalted and the materials and time available.

Ethanol precipitation is a quick and efficient method for desalting large quantities of oligonucleotide. Only one precipitation is needed to desalt a typical synthesis, and no carrier RNA is required. The disadvantage of this method is that recovery yields and desalting efficiency are somewhat less reproducible than with other methods. The advantage is that some of the lower molecular weight species of truncated molecules may not precipitate, so partial size fractionation can be achieved. Substitution of isopropanol for ethanol in the precipitation protocol is recommended for very short oligonucleotides (<15-mers), which may not precipitate efficiently with ethanol. A protocol can be found in Appendix C.

Dimethoxytrityl (DMT) Caution Assay

Determining the stepwise yield of coupling reactions via the DMT (trityl) cation assay is a useful though indirect indication of final product quality. It provides quick feedback regarding the performance of the DNA synthesizer. The DMT assay for determining stepwise yields is the spectrophotometric measure of the amount of DMT cation (the DMT group is the 5' hydroxyl protecting group) liberated at each 5' deprotection step in the synthesis process.

All Applied Biosystems DNA synthesizers can automatically collect, with the aid of a fraction collector, the solution containing the DMT cation produced during the detritylation steps of the synthesis cycle. We find the Isco Cygnet™ Fraction Collector convenient for one-column instruments, and the Isco Retriever II™ with a four-column adaptor suitable for multicolumn instruments. Both can accommodate various test tube sizes.

The DMT fraction is diluted to 10.0 mL (5.0 mL for syntheses at the 40-nmol scale), using 0.1 M p-toluenesulfonic acid (Aldrich, T3 592-0) in acetonitrile. The solution is thoroughly mixed and the absorbance is read in a 1.0-cm path length cuvette at 490 nm, near the absorbance maxima for the bright orange dimethoxytrityl cation.

Dilutions are required because the fractions are too concentrated for accurate results. Dilute the fractions, or aliquots of the fractions, so that the absorbance readings are within the range of linear absorbance readings; generally 0.1-1.0 is acceptable.

Total yield is calculated by converting the final DMT absorbance to a percentage of the initial DMT absorbance. The average step yield is calculated by raising the total yield to the power of the inverse number of trityl fractions, omitting the fraction collected during detritylation of the derivatized support (the first trityl collected). Details concerning the DMT cation assay can be found in Appendix F.

Synthesis Problems Detected by the DMT Assay

Understanding how to diagnose synthesis problems detected by the DMT cation assay can be very useful. Valuable instrument time can be saved because you can often make repairs or more accurately describe conditions to an Applied Biosystems technical specialist or service engineer. Familiarity with the following situations will help in the diagnosis of instrument problems.

An unusually low trityl absorbance value, corresponding to the detritylation of the derivatized support, is sometimes noted Typically, this first base-absorbance value is slightly less

than the second, as a result of spontaneous detritylation of the support bound nucleoside during storage of the column. The DMT cation cleaved in this manner is lost in the initial acetonitrile washes during the synthesis cycle, and it is not collected in the DMT cation fraction. While this has no effect on the synthesis, it does affect the trityl assay. The smallest scale, 40 nmol, does not generate sufficient DMT cation to allow for precise measurements. The 40-nmol DMT cation solutions should only be inspected for gross differences.

If an unusually low absorbance value in the trityl profile occurs during a synthesis, followed by values consistent with those prior to the low yield, one of three possible problems is indicated:

1. Only a fraction of the DMT cation solution was delivered to the collection tube in the low-value fraction. Misalignment of the fraction collector, or a mechanical problem causing the trityl solution to be delivered to waste instead of to the collection tube, would not result in poor product quality.
2. Incomplete detritylation. This could be caused by restricted delivery or flow of the detritylating agent to the column, a faulty reagent, or incomplete rinsing of the support prior to detritylation. It would affect product quality and would be detected by evaluation of the crude product.
3. A coupling failure combined with faulty capping. This would be an unlikely combination of events at only one cycle in the synthesis. It would affect product quality and could be determined with further analysis.

A coupling failure with efficient capping would be detected in the trityl assay as a large drop in absorbance at one specific fraction. Subsequent fractions would show equally low absorbance. The oligonucleotides that failed to couple in the low yield coupling reaction would be completely capped, eliminating their ability to react in subsequent coupling reactions. A failure of this type can be confirmed by the appearance of a major side product in HPLC or PAGE analysis. (For common trityl assay symptoms and causes of synthesis failure, see Appendix F.)

A coupling failure that occurs at the first coupling, noted by a large decrease in absorbance of the second trityl fraction, could be indicative of inconsistent reagent delivery (base, tetrazole or trichloroacetic acid). Most likely it stems from a failure to purge the phosphoramidite and tetrazole lines by initiating a “begin procedure” at the start of synthesis. The lines are more permeable to air or moisture than the bottles. On humid days it is necessary to purge these lines prior to synthesis when the instrument has been idle for more than six hours.

Regardless of the humidity, the instrument will need to be purged when it has been idle for more than 12 h. Purging the lines is easily accomplished by initiating a “begin procedure” prior to synthesis. Phosphoramidites have a life span of 2 weeks on an instrument with CPG (controlled-pore glass) columns, and 3 weeks on one with 40-nmol polystyrene columns. Syntheses performed with phosphoramidites older than this will probably give poor coupling yields and lower DMT cation values.

Problems Undetectable by DMT Assay

Unfortunately, the DMT cation assay will not detect some problems that may be detrimental to the final product quality. On these rare occasions, the DMT assay will not indicate

synthesis failure; however, the product will be of inferior quality, or there may be no product at all. Two undetectable problems are:

1. Inefficient oxidation. During synthesis, the trivalent phosphorus internucleotide triester must be quantitatively oxidized to its pentavalent form at each cycle. If oxidation is not complete, the residual phosphite triester will be degraded by exposure to the acidic detritylating reagent, which is the next step in the synthesis cycle. The DMT cation is liberated as usual, resulting in a normal assay. This problem usually occurs when iodine fails to deliver properly during each cycle.
2. Depurination. The cleavage of the purine bases from their deoxyribose sugars at the glycosidic bond also remains undetected by the trityl assay. It is known that the glycosidic bond of deoxyribopurine compounds, especially when derivatized with chemical protecting groups, is quite unstable in acids. Great care has been taken to develop detritylation conditions in which the degree of depurination is minimal. FOD reagents result in less depurination than standard reagents.

Calculation and documentation of DMT cation yield data is very useful in the early diagnosis of instrument-related problems. Low DMT cation stepwise yields will inform you of inferior product quality more quickly than analysis of the oligonucleotide. The DMT cation analysis is only an indirect indication of synthesis efficiency. Analysis of the oligonucleotide mixture is the only positive confirmation of product quality.

Quantitation of the Crude Product by UV Absorbance

Quantitation of the oligonucleotide by UV absorbance is the most common and practical method of determining synthesis yield. The measurement of light absorbance by a solution sample of DNA can be converted accurately to mass, and therefore molar amounts. Comparison of the estimated theoretical yield with the measured yield is a good predictor of synthesis quality. A low overall yield may indicate

- poor coupling reactions during synthesis;
- incomplete cleavage from the support;
- inaccurate calculation from the absorbance reading.

The average absorbance maximum of the four bases is approximately 260 nm; therefore, quantitation is done at this wavelength.

Because calculating the expected absorbance from the base composition is laborious, an average extinction coefficient of 10,000 is used. This is a satisfactory approximation in a neutral pH range. The procedure for measuring the absorbance of a sample is described in Appendix D. A typical spectrum of a crude 18-mer is shown in Figure 2-2. Oligonucleotides that are very rich in either purines or pyrimidines can have absorbance maxima above or below 260 nm, depending upon the base composition. The yield of a synthesis should be verified before discarding the synthesis column. One reason for low yield may be incomplete cleavage from the solid-phase support. The most common causes of incomplete cleavage are poor delivery of ammonia to the column, or the use of ammonia that is not fresh and at maximum concentration. A manual method for treatment of a synthesis column with ammonia is described in Appendix E.

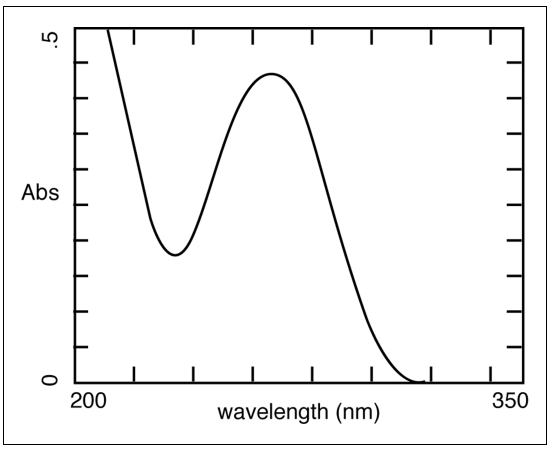


Figure 2-2. *Typical UV spectrophotometer absorbance scan*
5′ TCA CAG TCT GAT CTC GAT 3′

DNA Synthesis Impurities

Higher coupling efficiency, purer reagents, polystyrene synthesis supports, drier acetonitrile, optimized cycles and other refinements have increased DNA synthesis efficiency While these improvements have minimized failure sequences, they have not eliminated all side reactions during synthesis. These side reactions generate impurities that have been present since the beginning of automated DNA synthesis but were practically unnoticed until high-resolution analytical methods such as MicroGel capillary electrophoresis became available.

Failure sequences are shorter than the product oligonucleotide and have been, until recently, the predominant group of impurities present in the crude synthesis mixture. Most of the remaining imperfections leading to detectable impurities generate higher molecular weight oligonucleotide species, which migrate more slowly on PAGE and elute later on HPLC and MicroGel CE than does the product oligonucleotide. It is important to note that this is not a new situation resulting from impaired reagents, synthesizers, software or cycles. These impurities, termed here “n+,” have always been present and are now more noticeable because of improved analytic tools and the diminution of failure sequences. A few crude oligonucleotide mixtures have detectable n+ impurities, and the amount of n+ material is almost always less than 20% of the product oligonucleotide.

Our research indicates two primary sources of the n+ material. The predominant one is “branching” of the growing oligonucleotide, in which a phosphoramidite monomer reacts through the bases, especially the N-6 of adenosine and 0-6 of guanosine. Some branch point phosphitylations are reversed during capping, while others are stabilized by oxidation. Some branch points are cleaved at the conclusion of synthesis, during treatment with ammonia. They are relatively unstable phosphoramidite linkages, compared to the very stable, normal 3'-5' phosphodiester internucleotide linkage. However, other branch points persist, giving oligonucleotides with two 5' ends and a higher molecular weight than the desired product oligonucleotide.

Branching occurs for several reasons and under certain conditions. If the base protecting groups (A G C) are lost prematurely from the phosphoramidites, either prior to or during

synthesis, phosphitylation of the unprotected exocyclic amines can occur. The protecting groups in the FOD series are especially susceptible because of their instability. By design, the FOD reagents bear relatively unstable base protecting groups.

Standard phosphoramidites have also exhibited n+ impurities. Some of the n+ impurities may bear two or more 5' ends; therefore, they will appear overrepresented on 5' labeling kinase gels and will be enriched by 5' DMT-selective OPC purification. The DNA synthesis cycle can exacerbate n+ levels when coupling or capping times are unnecessarily long. A 15-second coupling wait and 5-second capping wait time are, sufficient and optimal for the highest purity oligonucleotides.

The other source of n+ impurities is probably the initiation and propagation of oligonucleotides from unwanted reactive sites (other than the 3' link) on the CPG support. Published reports show that the CPG surface contains reactive functionality that cannot, be irreversibly capped. During each cycle, a small population of extraneous DNA is initiated on the CPG surface and propagated with the very reactive, activated phosphoramidite. At least some of these impurities are cleaved from CPG with ammonia. These impurities have an unknown structure at the 3' end, probably 3' phosphate. Most of these support-derived impurities are shorter, lower molecular weight oligonucleotides, but some seem to be of the n+ variety. The polystyrene support allows far less unwanted growth off of the support.

Also worth mentioning is the occasional presence of another slower migrating, slower eluting impurity, a species approximately double the length of the product. The chemical basis for this “2n” impurity is a side reaction that occurs during the preparation of the 3' nucleoside succinate intermediate that is used in manufacturing the support. A small amount of this intermediate may have the succinate linkage attached through the base, rather than at the correct 3' hydroxyl site.

Oligonucleotide synthesis then proceeds through both the 5' and 3' sites, with the oligonucleotide anchored to the support through the base. Although this effect has been noted with all four bases, higher levels have been detected for A and C than for G and T. The problem arises sporadically in some shipments of CPG nudeoside from the manufacturer.

The 2n impurity has never been quantitated or judged subjectively on a gel to be more than 20% relative to the product oligonucleotide. The 2n impurity is actually 2n-1 because it bears two 5' ends. The starting 3' nucleoside is exactly in the center of this linear impurity. We isolated this impurity from the synthesis of a PCR primer, and in a controlled experiment in which the intended primer pair efficiently amplified the template, the 2n-1 impurity did not amplify by itself, nor did it inhibit amplification in the presence of the correct primers.

As a discrete, homogeneous species, the 2n-1 impurity migrates as a sharp band on PAGE and elutes as a single, well-shaped peak on reverse-phase HPLC. Perversely, OPC enriches the purified sample for the 2n-1 impurity because the sample bears two DMT groups. Kinase analysis also makes the 2n-1 impurity seem more prevalent because both 5' ends will label with ^{32}P ATP.

We are continuing to unravel the mysteries of DNA synthesis, although the remaining imperfections are increasingly subtle and of little consequence in the applications of synthetic oligonucleotides. By understanding the imperfections and limitations of the DNA synthesis chemistry, more astute choices in analysis and purification will be possible.

Analysis and Purification of Oligonucleotides by Polyacrylamide Gel Electrophoresis (PAGE)

Introduction

Polyacrylamide gel electrophoresis (PAGE) is widely used for oligonucleotide analysis and purification. The first section of this chapter discusses the basic methods (apparatus, solutions and electrophoresis conditions) required for the successful analysis and purification of oligonucleotides. Because the protocols for autoradiography, analytical ultraviolet (UV) shadowing and gel purification vary, these topics are discussed subsequently. A polymerized, cross-linked acrylamide gel matrix between two glass plates is common to all three methods.

The ends of the gel matrix are immersed in buffer chambers containing an electrolytic salt solution through which an electric field of controlled voltage or current is applied. Charged molecules migrate through the gel matrix, separating on the basis of charge and mass. The equation for electrophoretic velocity is complex, but the predominant factors effecting separation are charge and mass. Molecular shape, net hydrophobicity, size, interactions with the gel matrix and other parameters also influence the rate at which a molecule travels toward the opposite electrode.

Basic Methods

Apparatus

The most convenient and familiar format for PAGE is the slab gel apparatus. Many dimensions, shapes and configurations are available. The lengths and widths of the gel are not critical as long as the minimum length is 15–20 cm. Spacers between 0.4 and 1.6 mm are typically used. The thinnest are used for analytical gels of ^{32}P-labeled oligonucleotides, while the thickest are used for preparative gels. The most critical choice to be made is the width of the sample wells. Maximum resolution of preparative samples is obtained by having the sample band as narrow as possible. Combs that form wells approximately 1 cm wide are useful for sample loads of about 1 optical density unit (A_{260nm}). Power supplies capable of providing 3000 V and 300 mA with a constant power option are best.

WARNING ***Before conducting experiments with radioisotopes such as ^{32}P, consult your institution's Radiation Safety Officer for proper procedures in ordering, handling and disposing of radioactive materials.***

Solutions

Acrylamide Stock Solution: Prepare a stock solution of 38% acrylamide and 2% bisacrylamide. High-quality acrylamide and bisacrylamide are available from several commercial sources and can be used without further purification. To prepare this solution, weigh 380 g of acrylamide and 20 g of bisacrylamide. Dissolve with deionized water to a final volume of 1 L. Filter the solution and store it at 4 ^{o}C.

WARNING ***Acrylamide in monomeric form is a neurotoxin and a suspected carcinogen. Wear gloves and a dust mask when weighing.***

10X TBE: This solution is used in the gel itself and with dilution (1X TBE) as the electrophoresis buffer. 10X TBE is 0.89 M Tris base, 0.89 M borate and 25 mM EDTA. To prepare this buffer, weigh 108 g of tris(hydroxymethyl)aminomethane, 55 g of boric acid and 9.3 g of disodium EDTA dihydrate**.** Dissolve with deionized water to a final volume of 1 L. Store the solution at 4 °C. Filtration will prevent precipitation of TBE, adding to its shelf life. The stock is stable for many months but is susceptible to bacterial growth and should be inspected before use.

1.6% Ammonium Persulfate (APS): This is prepared as a 1.6% (w/v) solution in distilled water. Because it is the initiator of polymerization, a fresh stock should be made every week. Store it at 4 °C. Alternatively, solid ammonium persulfate may be used.

Table 3-1. Recipes for Polyacryamide Gels

Gel thickness (mm)	Total vol. (mL)	% gel (%)	40% acryl. (mL)	Urea (g)	10X TBE (mL)	APS (mL)	TEMED (µL)
0.4	100	8	20	42	10	4.4	60
				42	10	4.4	60
				42	10	4.4	60
				42	10	4.4	60
0.8	150	8	30	63	15	6.6	100
		12	45	63	15	6.6	100
		20	75	63	15	6.6	100
1.6	250	8	50	105	25	11	150
				105	25	11	150
				105	25	11	150

Recipes are for 30 x 40-cm gels.

Pouring the Gel

The glass plates should be thoroughly washed, rinsed with acetone or ethanol, dried and assembled prior to preparing the polyacrylamide solution.

1. Measure out the 40% acrylamide and 10X TBE. Bring to final volume with deionized water.

2. Add this solution and the appropriate amount of urea to a flask (with a side arm) and mix until the urea has completely dissolved. If the gel is to be used for autoradiography, filtering the solution may help prevent extraneous exposure spots on the film. For UV shadow gels, filtering is not necessary.
3. Add the APS, then mix and degas the solution under vacuum for several minutes.
4. Add the TEMED (tetramethylethylene diamine (Aldrich T2, 250-0)), mix for approximately 30 s, pour the gel (use a pipet to minimize spillage) and insert the comb. Be sure to dislodge any trapped air bubbles, especially in the wells. Wait 1-2 h before using the gel.

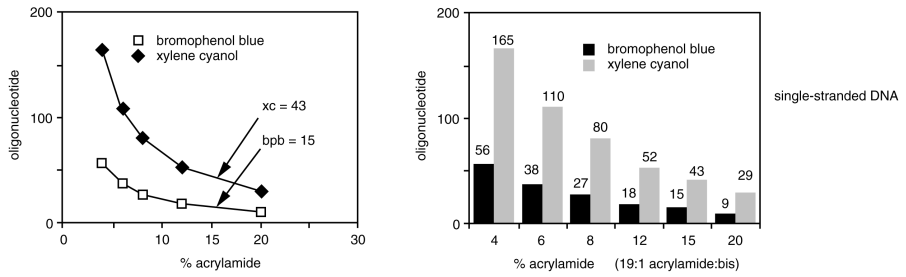
Electrophoresis Conditions

Denaturing and Tracking: Denaturing conditions should be maintained with single-stranded DNA oligonucleotides to minimize the formation of secondary structures caused by hydrogen bonding. Both the high concentration of urea (7 M) in the gel and sample loading in formamide serve as denaturants. However, secondary structure effects can be present in the gel, even in 7 M urea. Colored tracking dyes, such as bromophenol blue and xylene cyanol, can be loaded on the gel in adjoining lanes. This helps to determine the migration points of the oligonucleotides not visible during electrophoresis.

Gel Percentage and Migration Rates: PAGE is useful for the analysis and purification of oligonucleotides of any length. The gel (polyacrylamide) concentration should be varied, depending on the oligonucleotide length. Choice of gel percentage is a compromise between speed and resolution. Higher concentrations of polyacrylamide decrease the average pore size of the gel, slowing migration and increasing separation of sample molecules. According to oligonucleotide size, the recommended polyacryl-amide concentrations are:

Table 3-2. Migration Rate Table

Oligonucleotide size	Polyacrylamide concentrations (%)
<25	20
25-50	15
50-90	12
>90	8



Both the length and, to a lesser extent, the sequence of an oligonucleotide affect its mobility through the gel. The order of nucleotide migration is C>A>T>G, with C the most rapid. Oligonucleotides of the same length but with different sequences will migrate at slightly different rates. Therefore, comparison of an **oligonucleotide size marker** with the sample oligonucleotide to verify the correct length is only marginally accurate. Figure 3-1 shows the migration of different sequences of the same length in a single gel. Figure 3-2 shows a UV shadow gel of 32-mers that differ only in four bases at their 3' ends. The results clearly show the effect of base composition.

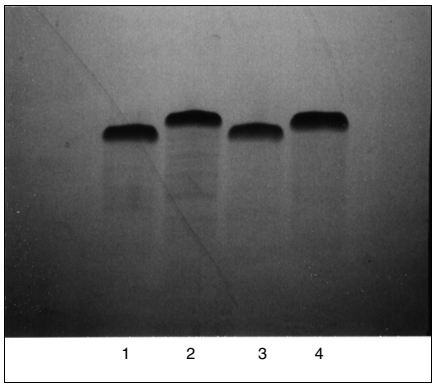


Figure 3-1. *UV shadow gels showing the migration of 18-mers of different sequence. Lane 1: $(AT)_{9^\circ}$ Lane 2: $(GT)_{9^\circ}$ Lane 3: $(CT)_{9^\circ}$ Lane 4: $(T)_{18^\circ}$*

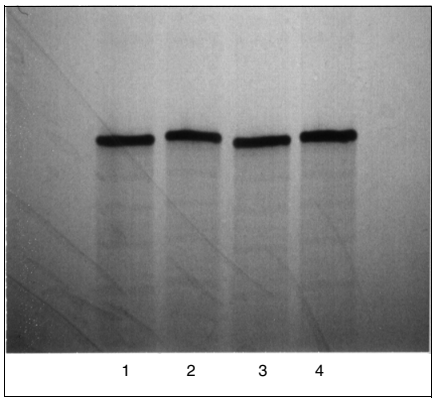


Figure 3-2. *32-mers that differ only in their 3' ends. The 3' ends are: Lane 1: AAAA. Lane 2: GGGG. Lane 3: CCCC. Lane 4: TTTT.*

Loading and Running: After the gel has been allowed to polymerize, usually for 1–2 h or longer, it is placed on the electrophoresis apparatus, and the upper and lower buffers (lX TBE) are added. Before the sample is loaded, the wells must be flushed with approximately 1 mL of 1X buffer in a syringe to remove debris and urea that can affect resolution. The gel is then prerun with a lane of bromophenol blue and xylene cyanol in formamide. Prerunning elevates the temperature of the gel, which helps to melt secondary structures and serves as visual confirmation that the gel is functioning properly.

If an oligonucleotide is suspected of forming secondary structures, heating it to 90 °C for several minutes in loading buffer and immediately placing it on ice just prior to loading

may help. Slow cooling stabilizes secondary structures. The warming of the gel and the sample serves to aid in the denaturation process. The running wattage of the gel should generate sufficient heat to warm the gel plates but allow handling for 1 min without burning the skin. Attaching a metal plate to the gel apparatus results in even distribution of the heat. This ensures against cracking the gel plates and melting the polyacrylamide. It also minimizes "smiling" or "frowning" of the gel samples.

After prerunning and just prior to loading, the sample wells are flushed a second time to remove urea. The samples are carefully loaded onto the lower surface of the sample well. The sample is dissolved in a 9:1 mixture (v/v) of formamide to 1X TBE. To avoid shearing or uneven loading, sample volume should not exceed 10 µL per 1.5-cm well for analytical size (0.4 mm) gels. A Hamilton-type syringe or a flat-tipped pipetter should be used to load the sample. Once the samples are loaded, electrophoresis should be initiated immediately to avoid diffusion.

UV Shadow Analysis

UV shadowing, the visualization technique most commonly used for purification, can also be used for analysis of crude mixtures. UV shadowing is less labor intensive than radiolabeling, and it often provides sufficient resolution for routine analysis. After electrophoresis, the gel is transferred to a fluorescent media, commonly a 20 x 20-cm plastic-wrapped preparative TLC plate and visualized under short-wavelength UV light. This method is direct, not dependent on further treatment or reactions, such as staining or labeling.

Sample Preparation

UV shadow analysis requires a higher amount of oligonucleotide than autoradiography; however, the amounts required (0.5–2 optical density units (ODU) are still only a small fraction of a typical synthesis. Desalting is not usually necessary for UV shadow analysis of oligonucleotides shorter than 50 bases. The sample may be dried down and loaded directly from the ammonia deprotect solution. Desalting sometimes reduces smearing on gels and gives a better picture. After quantitating the oligonucleotide, the following amounts should be dried down for analysis on an 0.8-mm-thick gel:

Number of Bases	ODU to be Loaded
< 25	0.5–1.7
25–50	0.8–1.4
> 50	1.5–2.0

A larger quantity of oligonucleotide may be necessary to analyze syntheses with low coupling yields.

Electrophoresis Conditions

Using the highest gel concentration for a given oligonucleotide length will maximize resolution. The width of the comb teeth should be about 1 cm. A gel length of 15–20 cm with

0.8-mm spacers is useful for the analysis of oligonucleotides up to approximately 70 bases. For oligonucleotides longer than approximately 70 bases, 5' ^{32}P end labeling and autoradiography gives better resolution.

1. When polymerization is complete (~ 1–2 h), fill the upper and lower buffer chambers with 1X TBE.
2. Prerun the gel for 30–60 min at a constant power of 15–25 W. The temperature of the gel plates should be less than 55 ×C.
3. Turn off the power.
4. Use a syringe to flush each well with approximately 1 mL of 1X TBE. Be careful to flush out all urea and gel debris.
5. Load each sample in 5–10 mL of 9:1 (v/v) of formamide to 1X TBE.
6. Load 5–10 mL of dye (bromophenol blue and xylene cyanol) in formamide into an empty outer well to aid in tracking the migration of the oligonucleotide.
7. Electrophorese at 15–25 W constant power until the desired species has migrated one-half to two-thirds the length of the gel.

The above conditions were optimized on Hoefer gel electrophoresis systems (models SE 400 and SE 600).

Visualization and Interpretation

Gently pry apart the glass plates with a nonmetallic wedge and place the gel on a fluorescent, preparative TLC plate (20 x 20 cm) that has been covered with plastic wrap. The oligonucleotides may be visualized under a short-wave UV lamp (240–300 nm). The UV-absorbing oligonucleotide appears as a shadow; it absorbs UV light and masks the emission of fluorescence in the plate. The gel may be photographed using an instant camera, black and white film, and a green filter.

Interpretation of analytical UV shadowing is subjective. Comparison with oligonucleotides in adjacent lanes of known quality and length may prove helpful.

Autoradiography

As oligonucleotide length increases, the preferred method of analysis and purification by PAGE changes. For short oligonucleotides, UV shadowing offers quick and easy high-resolution analysis. For oligonucleotides longer than approximately 70 bases, 5' ^{32}P labeling and autoradiography give better resolution of the crude mixtures.

Sample Preparation

Before the oligonucleotide undergoes electrophoresis, it must be:

1. quantitated by absorbance;
2. desalted (usually necessary only for autoradiographic analysis; see Chapter 5 and Appendix C for desalting methods);
3. evaporated to dryness.

The surface area of the sample well on a thin gel is smaller than that on a thicker gel. Consequently, normal loading of an oligonucleotide sample on a thin gel may clog it, causing much of the sample to be retained in the well. By decreasing the amount of sample loaded, the oligonucleotide mixture will enter the gel easily and give excellent resolution. Typically, 1–20 pmol of crude oligonucleotide are radiolabeled for autoradiography and loaded onto a 0.4-mm x 40-cm gel for proper resolution.

The most common method of radiolabeling is 5' end labeling. T4 polynucleotide kinase is used to catalyze the phosphorylation of the 5' hydroxyl. As shown in Figure 3-3, [γ ^{32}P] ATP serves as the phosphate donor. This method can be used at different scales, depending on the quantity and length of the oligonucleotide to be labeled. Variations are also used when phosphorylating oligonucleotides for ligations as well as for radiolabeled probes.

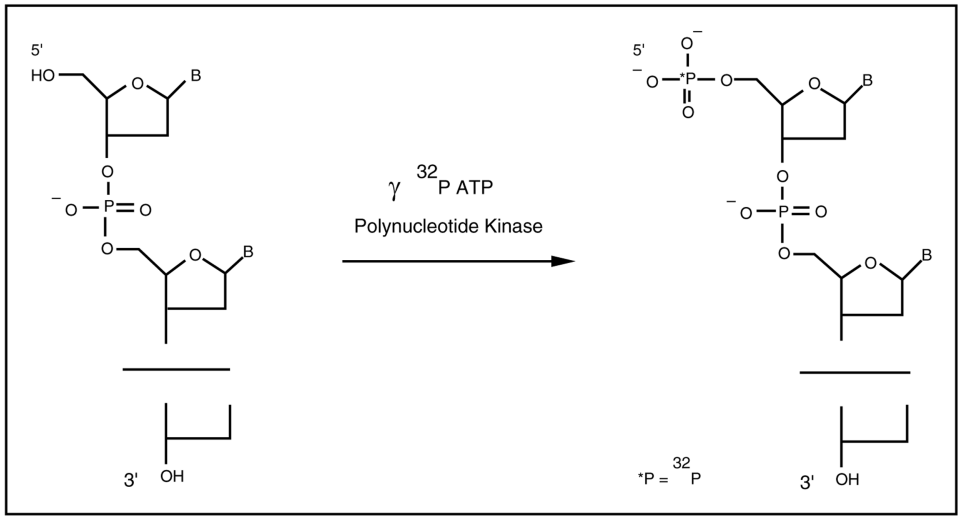


Figure 3-3. *5' ^{32}P labeling reaction*

When analyzing crude compounds, an excess of both hot and cold ATP must be present in the reaction for competitive labeling of each oligonucleotide species in a sample. At limiting ATP concentrations (an excess of oligonucleotide relative to ATP), the kinase enzyme will phosphorylate oligonucleotides of approximately 10–20 bases before phosphorylating longer sequences. The small quantity of radiolabeled ATP will not produce the excess needed for efficient phosphorylation. A proper molar excess is achieved with the addition of unlabeled ATP.

5' Radiolabeling Protocol

The oligonucleotide should be dried in an Eppendorf tube. It is usually not necessary to purify or desalt sample oligonucleotides for kinase analysis. They may be used after loading directly from the ammonia deprotection solution. Extremely long or impure syntheses may benefit from a simple ethanol precipitation or desalting operation. The amount of each sample to be labeled can vary, depending upon the activity of the ^{32}P ATP and the desired autoradiography film exposure time. The following protocol is proven and provides for analyzing a sample twice with an approximate film exposure time of 1–2 h, assuming that

the ^{32}P ATP is fresh. If the radiolabeled ATP is older than 2 weeks, twice the amount may need to be added to achieve a exposure time of 1–2 h. The half-life of ^{32}P ATP is 14.3 days.

To measure the samples, convert absorbance (ODU) to picomole amounts, using the following approximation: 1 mmol of oligonucleotide = 10 ODU/base (e.g., 10 pmol of a 50-mer = 0.005 ODU).

Number of Bases	pmol to be Labeled
< 40	5
40–70	10
> 70	20–50

It is necessary to make a serial dilution of the sample in water to avoid aliquoting sub-microliter volumes. This molar conversion from absorbance is a useful guide, although it assumes that the entire sample is full-length product oligonucleotide, which is usually not the case.

For each sample, prepare a master mix of the following:

- 4 µL of 100 µM **cold** ATP in water (400 pmol)
- 1 µL of kinase buffer (0.25 M Tris-HCl, pH-7.6; 0.1 M $MgCl_2$; 0.1 M DTT (dithiothreitol)). Kinase buffer should be stored frozen and discarded after 6 months.
- 0.2 µL of 10 mM spermidine
- 1 µL (10 units) of T4 polynucleotide kinase (New England Biolabs, 201S)
- 1.5 µL of γ ^{32}P ATP (3000 Ci (111TBq)/mmol) (NEN DuPont, NEG-002H; or Amersham, PB-168)

1. Vortex the master mix and spin it for a few minutes in a microcentrifuge, observing the appropriate radiation safety procedures.
2. Dispense 6 µL of the master mix into each sample tube.
3. Vortex, spin and heat the sample tubes at 37 °C for 45–60 min.
4. Cool, spin and add 14 µL of formamide/dye mix (0.1% bromphenol blue, 0.1% xylene cyanol) to each sample tube.
5. Vortex and spin the sample tubes. The dye-containing samples are stable. They may be stored in a refrigerator or freezer and are ready for loading on the gel.

Recommended Gel Configurations		
Plates	20–40 cm	(length)
Spacers	0.4 mm	(depth)
Wells	8–16 mm	(width)

Electrophoresis Conditions

1. Attach the gel plate assembly to the gel apparatus and clamp an aluminum plate to the surface.
2. Fill the upper and lower buffer chambers with 1X TBE.
3. Apply a constant power of ~60 W and prerun the gel for approximately 1 h. The aluminum plate should be warm but not hot.
4. Turn off the power.
5. Rinse out any urea that has diffused into the wells, using a syringe filled with 1X TBE.
6. Carefully load 10 µL of each sample and electrophorese at about 60 W constant power. When the aluminum plate reaches a stable temperature, it should be warm but not too hot to touch (approximately 55 °C). Higher temperatures, caused by too much power, may crack the glass plates, resulting in leaking and shorting. The exact run time depends on the size of the oligonucleotide, but it should be sufficient to allow the desired species to migrate approximately two-thirds of the gel length.

The above conditions were optimized on a Model SG-600-33 Adjustable Electrophoresis System (C.B.S. Scientific Co.).

Visualization and Interpretation

1. When the electrophoresis is complete, turn off the power and remove the gel plate assembly. Be careful to properly dispose of the radioactive lower buffer solution, according to Federal and local regulations.
2. Separate the glass plates by gentle prying with a nonmetallic wedge. The gel should remain attached to one of the plates, preferably the bottom one.
3. Wrap the gel with plastic wrap.

   Alternatively, lay a discarded, clean X-ray film over the gel and turn the gel over so that the film is on the bottom. Carefully remove the plate (try to prevent tears and air bubbles in the gel), leaving the gel adhering to the film. Then wrap the film in plastic wrap and smooth out most of the wrinkles.
4. Place the gel inside a light-tight film cassette containing an intensifying screen.
5. In a darkroom, lay a sheet of unexposed X-ray film on the gel.
6. Close the cassette and store it at -70 °C during exposure.

The radioactively labeled oligonucleotide molecules emit beta particles that expose the X-ray film. When the film is placed between the gel and an intensifying screen, exposure will be accelerated. The quality of results obtained from analyzing crude products by autoradiography is depicted in Figure 3-4.

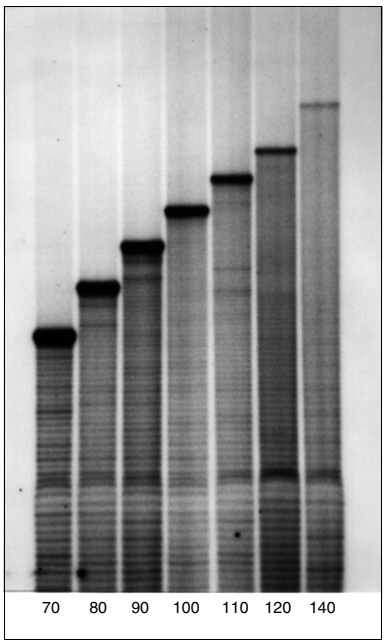


Figure 3-4. *Autoradiograph of crude oligonucleotides.*

Exposure time is critical. Remember that the total incorporated radioactivity is distributed among several (sometimes many) species, depending on the length of the oligonucleotide. An exposure time that is too long will overrepresent failure sequences because total exposure of silver grains at the product band will no longer respond to beta emissions. In other words, when a band achieves a certain blackness, it will not blacken further. The lighter bands, however, will continue to darken. This effect may mislead the viewer as to the relative amounts of product and failure sequences. An exposure that is too short will not sufficiently reveal failure sequences. Figure 3-5 shows the effect of exposure on the interpretation of results.

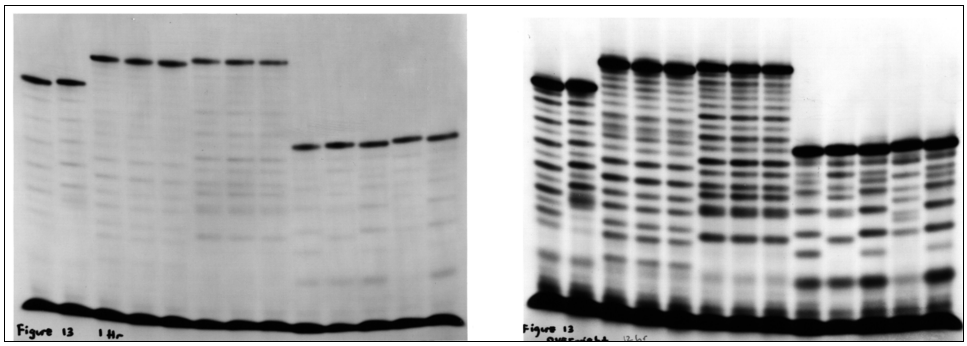


Figure 3-5. *Light (1 h) and dark (12 h) exposure of oligonucleotides of varying length and sequence.*

Interpretation of an autoradiogram is usually subjective. Visual inspection of the intensity of the major product band and comparison with the failure sequences is often sufficient. Comparison with oligonucleotides of known size and quality may also be useful.

Densitometry

Densitometry can be used to quantify bands and spots on autoradiograms and stained gels. Densitometers measure the portion of incident light that is either reflected or transmitted by a sample. Quantitation by measuring reflection is affected by small changes in surface texture, and it measures only the portion of the sample that is on the surface. For this reason, densitometers that measure reflection are usually reserved for opaque samples, such as TLC (thin-layer chromatography) plates. Other densitometers quantify light absorbance by measuring transmittance and optical density.

The densitometry signal is converted to peak format when a lane from a gel is scanned. Lane 1 of the autoradiogram in Figure 3-6 gives little information until scanned by densitometry.

The most accurate systems scan the entire gel, allowing a 3-D integration of each band. Other systems scan individual or multiple tracks on each band or lane. Because gels rarely have band widths less than 1–2 mm, a resolution of 100 µm (10 points/ mm) is usually sufficient. The system should be capable of measuring both incident and transmitted light. This prevents changes in the intensity of the incident light from distorting the data.

Problems often arise when very dark bands are seen on the film. Dark spots absorb most of the light incident upon them, which reduces the number of photons that reach the detector. This results in a low signal-to-noise ratio. A higher signal-to-noise ratio may be maintained by using a strong source of incident light, such as a laser. Other factors to consider are the optical resolution of the system and the method of analog-to-digital signal conversion. The percentage of error is lower if the transmittance is converted to optical density units before digitization.

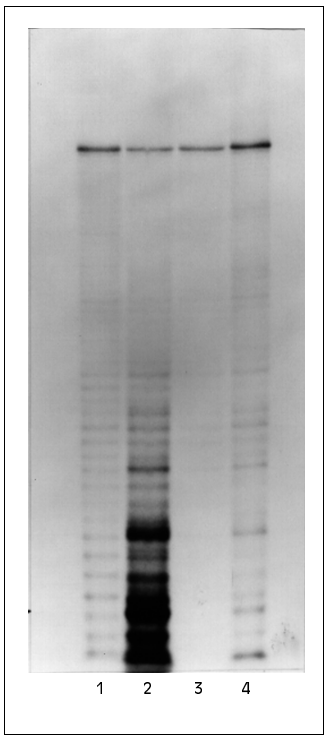


Figure 3-6. *Densitometry trace showing the quantitation of the main product and failure sequences of the 60-mer in Lane 1 of the Autoradiograph.*

Staining

Gel staining with a DNA-specific stain, such as methylene blue, may be used to visualize oligonucleotides. Staining can be particularly useful if the gel is to be preserved, but it is not recommended if the analyzed sample is to be used in subsequent applications. As an analytical tool, staining has limited sensitivity; sequences present in low concentrations may not be visible. Nevertheless, staining is more sensitive than UV shadowing and is considered easier than radiolabeling (see the 5' Radiolabeling Protocol, page 3-7).

Methylene blue and Stains-All are useful for staining oligonucleotides. For example, the gel, after electrophoresis and removal from the plates, may be soaked in a shallow pan with 200 mg of methylene blue per liter of water for approximately 15–30 min. Drain the pan and rinse the gel of all excess, unbound dye for several minutes. Stained oligonucleotides are visualized as blue bands and may be photographed under ambient light.

Ethidium bromide staining is a well-established technique for visualizing double-stranded DNA fragments. Unfortunately, it is not efficacious for visualizing short, single-stranded DNA fragments such as synthetic oligonucleotides. The UV irradiation required to visualize ethidium bromide damages DNA and compromises its usefulness. Also, the intercalation of ethidium bromide into short, single-stranded DNA is inefficient, highly sequence-dependent and sometimes undetectable in sequences shorter than 25 bases. We do not recommend ethidium bromide staining for analysis of oligonucleotides.

Purification

The goal of electrophoretic gel purification is separation of the desired product from other, contaminating oligonucleotides in the crude mixture. The key steps in this process are gel separation, UV-shadowing, product excision from the gel and recovery of the product from the gel matrix. PAGE purification is excellent for separation by length. Figure 3-7 depicts a gel of crude mixtures and their subsequently purified products.

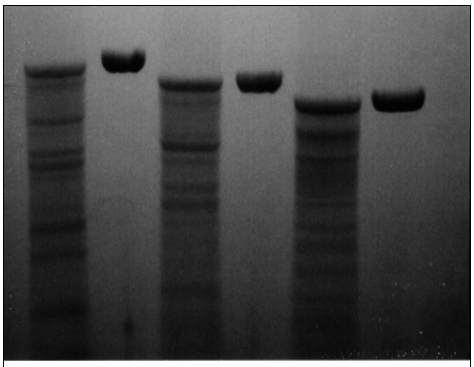


Figure 3-7. *Comparison of crude and gel-purified 85-, 75- and 65-mers. 15% polyacrylamide. Loading: 1.5 ODU of crude or 0.45 ODU of gel-purified oligonucleotide.*

The methods used in gel purification are like those used in analysis, with two major differences:

1. Preparative gels are usually thicker gels, used to accommodate the loading of a larger quantity of oligonucleotide.
2. The oligonucleotide also has to be loaded in a sufficient concentration to allow recovery of ample amounts of the desired species from the gel matrix.

Sample Preparation

DNA samples for purification are quantitated and prepared in much the same way as for analysis. In some cases, especially for long oligonucleotides, desalting is recommended before electrophoresis. Dissolve the dried oligonucleotide in loading media (9:1 (v/v) formamide/1X TBE) to a concentration of 1–2 ODU per µL. Samples that do not readily dissolve should be heated to approximately 60 °C and vortexed. To avoid possible contamination and masking of the product, do not load marker dyes with the sample. Marker dyes such as bromophenol blue and xylene cyanol should be loaded in the outer wells to aid in tracking the migration of the oligonucleotide (refer to Table 3-2).

Electrophoresis Conditions

Electrophoresis conditions for purification are similar to those for analysis. The primary differences are the gel thickness and the sample concentration. The height of the gel will depend on the length of the oligonucleotide; longer oligonucleotides require longer gels to

obtain sufficient resolution. A gel length of 15–20 cm will adequately resolve oligonucleotides up to 50 bases; for longer oligonucleotides, a 40-cm gel is recommended. The thickness of the gel and the width of the comb teeth will determine the well surface area and ultimately the amount of crude mixture that can be loaded. For example, a gel 1.5 mm thick and comb teeth 2–3 cm wide allow for the loading of 10–12 ODU (A_{260nm}) of oligonucleotide.

The increased thickness of preparative gels requires higher power settings (30–50 W constant power) and/or longer electrophoresis times to obtain maximum resolution. To achieve optimal product separation, let the sample migrate as far down the gel as possible. Because the goal is separation of the oligonucleotide from the crude mixture, it is acceptable to run the truncated sequences off the bottom of the gel. Ideally, the product band should be allowed to migrate at least two-thirds the length of the gel.

Visualization and Product Excision

Gently pry apart the glass plates with a nonmetallic wedge and place the gel on a fluorescent TLC plate covered with plastic wrap. The oligonucleotides are visualized using a short-wavelength UV lamp (approximately 240 nm). It is important that a short-wave UV lamp be used. DNA does not absorb much above 280–290 nm. Take care to minimize the exposure of the oligonucleotide to UV light, which can cause thymidine dimerization.

Figure 3-8 and Figure 3-9 are photographs of a preparative gel (before and after product band excision) illuminated by a hand-held UV light source. When excising the oligonucleotide, remember that the UV lamp must be held **directly** overhead to avoid errors. This prevents movement of the shadow from its correct position on the product band to another position that could include contaminants.

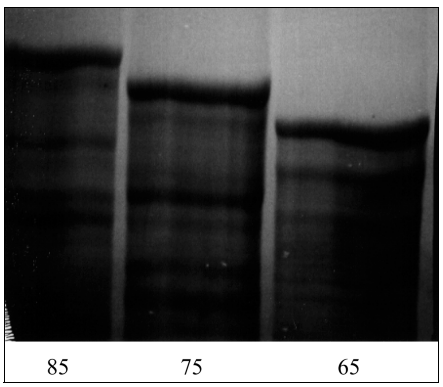


Figure 3-8. *Preparative gel. 15% polyacrylamide, 1.5-mm thick, loading 10 ODU of each crude oligonucleotide mixture.*

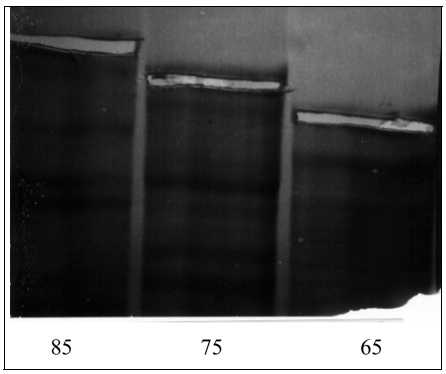


Figure 3-9. *Excision of 85-, 75- and 65-mer product bands from a preparative gel.*

The product is excised with a clean razor blade. If the oligonucleotide is not degenerate, the cuts should be slightly to the interior of the product band to eliminate contamination from failure sequences lacking only a single nucleotide. Conversely, to avoid missing some of the possible sequences in a degenerate product mixture, the cuts should be at or slightly outside the diffuse product band. The excised band is then placed in a tube or a fritted column. If more than one sequence is run on the same gel, care should be taken in handling to prevent cross-contamination.

Once the product band has been excised from the gel, it is necessary to recover the oligonucleotide from the acrylamide gel material. The two most common ways are either **soaking** or **electroelution.** Both methods are effective, but soaking is often the method of choice because it is inexpensive, easy and can be accomplished without monitoring. Product recovery yields are 10–80% of the initial oligonucleotide loading. The quantity of product recovered depends on the concentration of failure sequences in the sample. The gel slice should be soaked in at least 1 mL of any of the following extraction solutions:

- 0.5 M NaCl, 2 M triethylammonium acetate
- 50 mM triethylammonium acetate
- 0.5 M NaCl, 0.1 M Tris-HCl (pH 7.0) containing 1 mM EDTA
- Deionized water

1. Incubate the gel slice at room temperature for at least 12 h.
2. Decant and save the solution.
3. Remove dissolved urea, salts and gel debris with the Oligonucleotide Purification Cartridge (OPC) desalting protocol (see Chapter 5).

Note that the OPC desalting procedure, unlike the purification procedure, is for trityl-off oligonucleotides. As with any purification method, oligonucleotide recovery should be verified by UV absorbance.

Differences in the Purification of Long and Short Oligonucleotides

Crude samples of long oligonucleotides contain substantially more truncated sequences than do crude samples of shorter oligonucleotides because of the higher number of coupling cycles. Hence, the product is in a lower concentration relative to the crude sample of a

shorter oligonucleotide. For this reason, it is necessary to load more long oligonucleotide sample on a preparative gel to visualize the product. Because the amount of oligonucleotide that can be loaded on a gel is limited, prepurification techniques such as OPC are recommended.

Diagnosing Synthesis Problems

Oligonucleotide analysis by autoradiography or UV shadowing may be used to diagnose synthesis problems. Figure 3-10 and Figure 3-11 depict examples of "problem" syntheses. Coupling failures, such as those seen in Figure 3-10, may be caused by old tetrazole, old or wet phosphoramidites or improper flow rates.

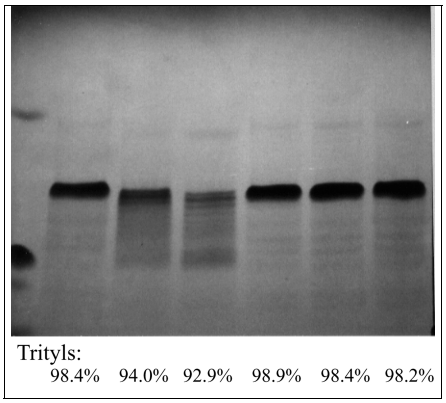


Figure 3-10. *A comparison of successful and failed 18-mer syntheses, showing the correlation between trityl and gel analysis.*

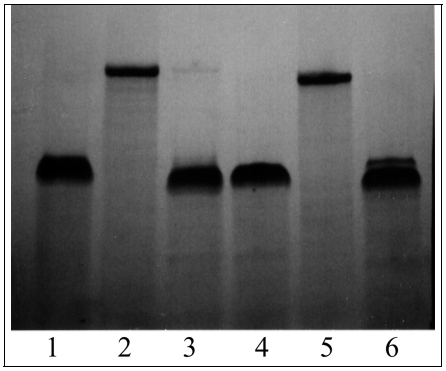


Figure 3-11. *Gel analysis of oligonucleotides from "problem" syntheses. Lane 1: 18-mer. Lane 2: 36-mer. Lane 3: 36-mer with no iodine delivery (no oxidation) after base 18 (note faint 36-mer band). Lane 4: 36-mer with a coupling failure at base 18 (tetrazole depletion) and efficient capping. Lane 5 36-mer with a coupling failure at base 19 (phosphoramidite depletion) and failure to cap. Lane 6: 18-mer with incomplete detritylation after base 6.*

Problem Areas

Sequence and base composition can have a marked effect on the electrophoretic behavior of an oligonucleotide. The most common problem associated with the sequence is secondary structure formation caused by hydrogen bonding. Secondary structures are most often, but not exclusively, associated with palindromic sequences and sequences rich in guanosine. Because secondary structures are in equilibrium with denatured molecules or other secondary structures, banding on the gel can be complex, making interpretation of the gel difficult. Figure 3-12 shows a UV shadow gel of a 20-mer that has secondary structure problems. In this figure, the 20-mer has a major band migrating at approximately the rate of a 10-mer.

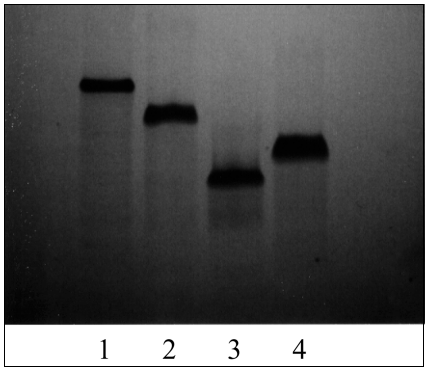


Figure 3-12. *An example of the effects of secondary structure on oligonucleotide migration. Lane 1: 25-mer. Lane 2: 20-mer 5' $(AGTC)_5$ 3'. Lane 3: 20-mer with secondary structure 5' GGT GGC CAC CAT GGT GGC CC 3'. Lane 4: 15-mer.*

The mixed base sites of oligonucleotides are often referred to as degenerate or redundant. In the synthesis of mixed probes of high degeneracy, the inherent complexity of the crude product will reduce resolution. Because of base composition differences between the oligonucleotides in the mixture, the product band will appear broader than normal. Figure 3-13 shows a highly degenerate 20-mer in the crude and purified state. Lanes 3 and 4 show the slight PAGE analytical difference between an incompletely deprotected (lane 3) and fully deprotected oligonucleotide (lane 4).

Sample preparation can also affect gel migration. Samples with the DMT group on the 5' end will not end label with T4 polynucleotide kinase. Hence, product quality will appear minimal at best by kinase analysis. DMT-bearing oligonucleotides analyzed on UV shadow gels will show a slightly retarded product band. Some detritylation may occur during sample preparation and electrophoresis. Compared to deprotected samples, samples with base-protecting groups remaining will appear as slightly higher molecular weight products because the base-protecting groups hinder migration. Additionally, the base-protecting groups hamper the ability of T4 polynucleotide kinase to end label the product molecules.

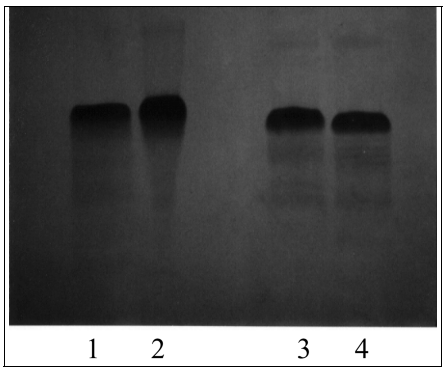


Figure 3-13. *Examples of the effects of degeneracies and of incomplete base deprotection on UV shadow gel analysis. Lane 1: crude 20-mer with 16 fourfold degenerate positions. Lane 2: Same 20-mer after OPC purification. Lane 3: 18-mer with incomplete deprotection (stored at -20 °C immediately after cleavage). Lane 4: Same 18-mer after 8 h of deprotections at 55 °C.*

To achieve complete quantitative base deprotection, samples should be incubated for a minimum of 8 h at 55 °C in concentrated, fresh ammonia. Oligonucleotides synthesized with Fast Oligonucleotide Deprotection (FOD) reagents require 1 h at 55 °C in concentrated ammonia for complete deprotection. Excessive salt in the oligonucleotide sample may affect migration and may interfere with enzymatic labeling reactions. Fresh kinase reaction buffer should be used because it contains DTT (see the 5' Radiolabeling Protocol, page 3-7). The loss of DTT through degradation over time can strongly affect 5'-labeling efficiency.

Other sample-related problems are caused by:

- inaccurate quantitation of the sample;
- inactive enzyme;
- inaccurate ATP concentrations.

There is a marked effect on the results obtained when enzymatically phosphorylating with kinase under limiting ATP concentrations. If radioactive ATP is the sole source of ATP, there will not be sufficient reagent to use as the phosphorylating agent. Under these conditions, the enzyme will preferentially label shorter oligonucleotides. By adding **cold** (unlabeled) ATP to the reaction mixture, efficient phosphorylation can be accomplished independent of the length of the molecules.

References

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Frank, R.; Koster, H. “DNA Chain-Length Markers and the Influence of Base Composition on Electrophoretic Mobility of Oligodeoxyribonucleotides in Polyacrylamide Gels.” *Nucleic Acids Research.* 1979, *6*(6), 2069–2087.

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Analysis and Purification of Oligonucleotides by High-Performance Liquid Chromatography

Introduction

Separation of oligonucleotides from associated impurities by high-performance liquid chromatography (HPLC) occurs primarily because of differences in net hydrophobicity (reverse-phase) and charge (ion-exchange). Interactions between the sample (solute) molecules and the stationary adsorbent packed in the column determine the rate and order of elution. The composition of the liquid mobile phase can be altered (gradient) or held constant (isocratic).

The mobile phase is predominantly aqueous, except for organic solvents or ionic salts that compete with the sample for interactive adsorbent sites or otherwise influence elution of the sample. Components of the mobile phase may also act as denaturants by minimizing hydrogen bonding of oligonucleotides with certain sequences into intra- or intermolecular conformations known as secondary structures.

HPLC is a well-established method for the analysis and purification of synthetic oligonucleotides. The advantages of HPLC are:

- level of automation;
- quantitation of the separated products;
- ease of pure product recovery;
- high resolution;
- sensitivity;
- inherent versatility;
- parameters that can be tailored for each separation.

Many HPLC adsorbent mediums are available for the evaluation and purification of oligonucleotides, but the two most prominent are **reverse-phase** and **ion-exchange** column materials. As solutes for separation, oligonucleotides are hydrophilic, charged and water soluble.

Reverse-phase (RP) columns separate oligonucleotides by differences in hydrophobicity. The solid particles within the RP column are usually silica-bonded with hydrocarbon chains via silanol linkages. The polar, aqueous, mobile phase and the hydrophobic adsorbent are considered to be in the **reverse** phase, as opposed to conventional, normal-phase chromatography, which uses organic solvents and underivatized silica gel.

Ion-exchange columns separate oligonucleotides primarily by their charge. The particles are also typically inorganic silica, derivatized with charged groups such as alkyl ammonium (typically weak or strong anion exchangers).

Because of the resolution limitations of both types for longer oligonucleotides, HPLC is a useful system for the reliable analysis and purification of oligonucleotides up to about 50 bases. Beyond approximately 50 bases, the single-base resolving power is lost. Longer oligonucleotides are best analyzed by electrophoretic methods such as slab gel (PAGE) or MicroGel capillary electrophoresis. Of all methods currently available, HPLC has the largest capacity for oligonucleotide purification.

Chromatography System

In general, an HPLC system consists of the following:

- injector (manual or autosampler);
- binary pumping system;
- UV detection system;
- chart recorder, integrator or data acquisition software;
- gradient controller;
- fraction collector.

An HPLC system is analogous to audio/video equipment. The components can be custom assembled in a modular format or purchased as a total system. In configuring the system, it is imperative that void volumes be minimized, especially for analytic scale and microbore HPLC. Most systems are configured primarily for analysis; therefore, void volumes and mixing points are often minimized only up to the flow cell of the detector.

Nucleic acids, both DNA and RNA, absorb light strongly at about 260 nm because of their chromophoric, conjugated pyrimidine and purine ring systems. Detecting and quantitating the absorbance of ultraviolet light can be done precisely.

For preparative work, void volumes and mixing points must be minimized between the flow cell and point of collection. A finite period of time is required for a compound to pass from the detector to the collection port, which can be determined by:

- injecting any UV-absorbing compound;
- collecting the fractions at specific time points;
- measuring the absorbance of the fractions individually.

This time interval is the lag before the samples should be collected. It corresponds to the fraction containing the strongest UV absorbance minus the maximum peak time on the chart recorder. For the most accurate preparative work, this time interval should not exceed 10–20 s. It is also helpful to have a digital readout of the absorbance in real time. This can be extremely important when purifying complex mixtures in which the points of collection play a key role in obtaining pure material.

HPLC purification differs from analysis in the following ways:

- Typically, a larger column and higher flow rates are used for isolating milligram amounts of oligonucleotide.
- The resolution requirements are less strict for purification than for analysis.
- Because the amount of product needed for most applications is quite small, only a portion of the peak need be collected.

Hence, good collection techniques and postpurification analysis can routinely produce pure product, even when the analytic chromatogram shows a complex mixture.

As a general guide, HPLC instrumentation capable of both analysis and purification of up to several milligrams per injection should have the following features and specifications for use with oligonucleotides:

Injector:	Manual (syringe injection) or autosampler.
Pumps:	At least two, capable of precise metered flow from 0.1 to 5.0 mL/min.
Gradient controller and mixer:	Both programmable and capable of storing user-defined gradient programs.
Detector:	Preferably a variable wavelength from 190 to 600 nm. Most oligonucleotides are detected at 260 nm.
Data system:	An integrating recorder is required, preferably a data system that stores multiple runs and allows redisplay and reformatting of data.

Proper HPLC Technique and Maintenance

Modern HPLC is meant to be an automated, largely unattended operation; however, compared to PAGE (polyacrylamide gel electrophoresis), the hardware is relatively complex. Nevertheless, observing a few proven HPLC techniques will ensure consistent, optimal results and a minimum of down time.

Sample preparation:	Filter or centrifuge samples to remove insoluble particulate matter.
Pump maintenance:	Clear all salts and buffers from the pumps at the end of a session. Maintain the mobile phases under a positive pressure of about 5 psi of an inert gas, such as helium.
Data storage:	Store chromatograms in personal computer-based systems.
Mobile phase:	Filter and degas all mobile phases.
Guard columns:	Mounted in-line between the injector and the column, a guard column can extend column lifetime by trapping insoluble impurities and chemically damaging species that may be present in the sample.

Reverse-Phase Chromatography

Oligonucleotides may be applied to reverse-phase HPLC columns in either of two forms: tritylated (trityl-on) or nontritylated (trityl-off). The large, hydrophobic dimethoxytrityl (DMT) group may be left on or removed from the 5' end of the oligonucleotide at the option of the user. Highly efficient syntheses (high coupling yield, minimum side reactions, complete capping and detritylation) give crude oligonucleotides with more of the desired full-length product bearing a DMT group.

The retention time differences between tritylated and nontritylated oligonucleotides in the crude oligonucleotide mixture are quite large. Because reverse-phase columns have greater

capacity for tritylated oligonucleotides, this is the preferred method of purification. Also, the trityl-on species usually follows a predictable pattern of analysis, which allows for the efficient separation of the tritylated product oligonucleotide from the non-tritylated failure sequences. However, the trityl group is labile, and preserving it quantitatively on the oligonucleotide during sample preparation can be difficult. For this reason, and because of its greater resolution, trityl-off chromatography is preferred for analysis of crude oligonucleotide mixtures. Each method will be discussed separately.

Trityl-On Chromatography

Theoretically, when all the reactions involved in an oligonucleotide synthesis have worked properly, the only compound bearing the terminal 5' DMT group is the full-length product oligonucleotide. All other species in the crude reaction mixture will have been acylated at the 5' position during capping. Upon deprotection in ammonia, these acylated chains will have free 5' hydroxyl groups.

Trityl-on HPLC utilizes the difference in hydrophobicity between the trityl- and nontrityl-bearing species. Under certain gradient and mobile-phase conditions, a reverse-phase column will easily separate the two types of oligonucleotide. The more hydrophobic tritylated product will be retained longer on the column than the shorter, nontritylated components of the crude reaction mixture.

A typical trityl-on chromatogram is shown in Figure 4-1, using the conditions and gradient of Table 4-1. The initial peak appearing at 5 min retention time is benzamide, the by-product from deprotection of the deoxycytosine and deoxyadenosine bases. Isobutyryl amide from the deoxyguanosine base does not have a chromophore and will not appear in the chromatogram. The next group of peaks appearing between 14–18 min are the trityl-off failure sequences. Any full-length, desired-sequence product oligonucleotide, which has inadvertently lost its trityl group, will also appear in this region. The major peak occurring at 30 min is the tritylated product.

The number of peaks in the region where trityl-bearing compounds elute indicates that the tritylated species is not homogeneous. The heterogeneity of tritylated species is a consequence of the imperfections of DNA synthesis. Resolution by HPLC of the tritylated species in the synthesis mixture is often not very efficient. The hydrophobic interaction between the DMT group and the adsorbent is dominant.

The trityl-on oligonucleotides in the mixture are often not well-separated by length. Longer trityl-on oligonucleotides have less overall hydrophobicity than do shorter ones because of the increased number of charges on the larger compound. They also usually have shorter retention times. By comparison, the differences in retention time between tritylated and nontritylated oligonucleotides are very large.

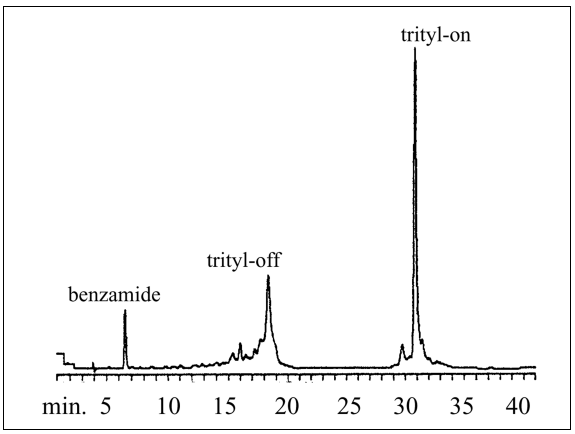

Figure 4-1. *Typical analytic trityl-on HPLC*

Sample Preparation

When reverse-phase, trityl-on chromatography is used to evaluate and purify synthetic oligonucleotides, it is necessary to leave the final 5' DMT group attached to the oligonucleotide. This can be done by choosing the trityl-on end function of the synthesizer. After treatment with ammonia at 55 °C for 8 h, the oligonucleotide will be completely deprotected except for the DMT group. The Fast Oligonucleotide Deprotection (FOD) reagents require only 1 h at 55 °C or 8 h at room temperature for deprotection.

The ammonium hydroxide used for cleavage and deprotection is then removed by evaporation, usually by vacuum centrifuge. It is important to note that the DMT group is unstable to heat and acid. Therefore, evaporation should be done at room temperature, and a drop of triethylamine should be added periodically throughout the evaporation process as a precaution. After the sample is dried, it should be redissolved immediately in 1 mL of 0.01 M triethylammonium bicarbonate (TEAB), pH 7.0, and 5 µL of triethyl-amine. Samples can be stored at -20 °C for up to 2 years with minimal detritylation.

Filtering or centrifuging HPLC samples prior to injection is always advised, especially if any cloudiness appears. Keep in mind that cellulose or glass filters will irreversibly bind some of the sample. Many suitable filters and microfiltration devices are available for filtering HPLC samples.

To demonstrate that HPLC sufficiently resolves different DMT-bearing species, several compounds were synthesized and separated in an Applied Biosystems lab. Figure 4-2 shows the sequence and retention times of three trityl-on oligonucleotides: an 18-, 30- and 44-mer. The 18- and 30-mers show sharp product peaks, but the 44-mer shows a broad product peak.

Analysis of each by PAGE showed excellent purities. This suggests that much of the resolving capacity of HPLC is lost for oligonucleotides longer than 30 bases, at which point the product peaks tend to broaden. When mixed together as trityl-on oligonucleotides, the 44-mer elutes first, followed by the 30-mer and finally the 18-mer.

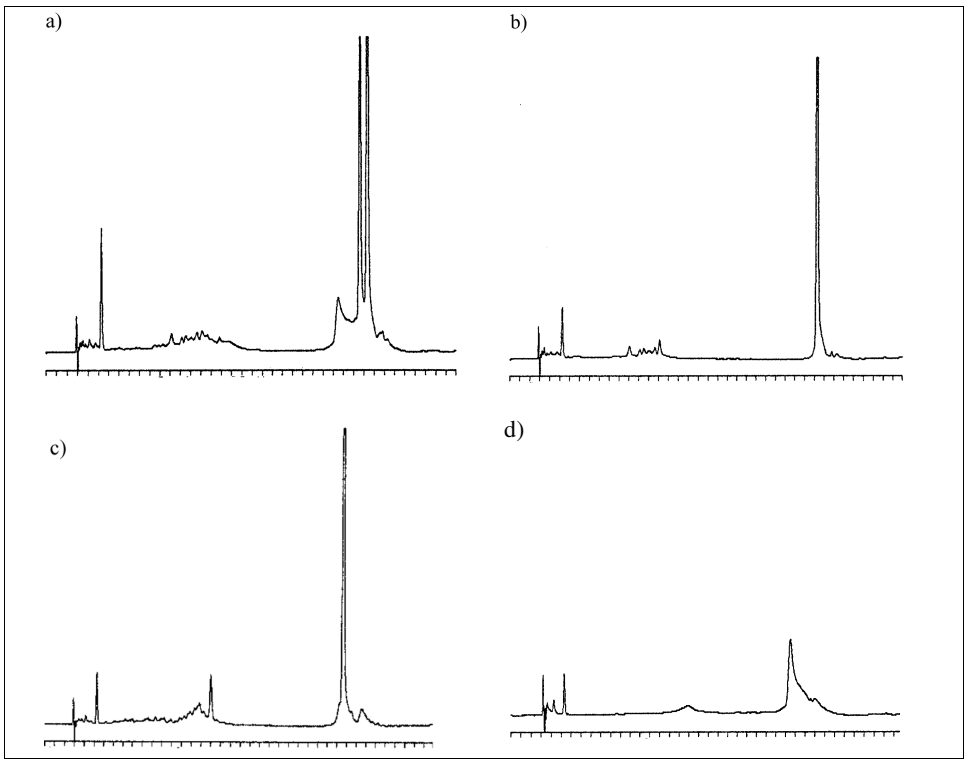

Figure 4-2. *(a) Mix of 18-, 30- and 44-mer trityl-on oligonucleotides: (b) 18-mer 5′ TCA CAG TCT GAT CTC GAT 3′. (c) 30-mer 5′ ATC CAG TCT ATT AAT TGT TGC CGG GAA GCT 3′. (d) 44-mer 5′ CAG GAA ACA GCT ATG ACC ATG ATT ACA CTG GCC GTC GTT TTA CT 3′*

Analysis and purification of trityl-on oligonucleotides is accomplished with an octylsilyl C-8 column, such as Aquapore® RP-300 (ABI P/N 0711-0059), or a stronger hydrophobic C-18 octadecylsilyl column, such as the Spheri-5® RP-18 (ABI P/N 0711-0117). A standard-size cartridge (4.6 x 220 mm) is suitable for analysis of about 0.3–1.0 optical density units (ODU) of crude mixtures. Smaller bore (2.1-mm) or even microbore (1.0-mm) columns offer some advantages in speed and reduced mobile-phase volumes. The column also appears to perform well for separating other tritylated species in the mixtures.

For purifications, up to about 10 ODU can be applied with some loss of resolution. A larger (7 x 250-mm) column (ABI P/N 0712-0017) provides better separation for larger loadings. To purify the entire product contained in a 10-µmol-scale synthesis, repeated preparative injections may be required even when using a larger column.

The mobile phase most often used for reverse-phase HPLC analysis and purification of oligonucleotides is an increasing gradient of acetonitrile (B) in 0.1 M triethylammonium acetate (TEAA), pH 7.0 (A). Most crude reaction mixtures in the 15–40 nucleotide range can be analyzed and purified, using the linear gradient system in Table 4-1. This gradient and flow rate are not suitable for large-scale purification.

This simple gradient is useful for trityl-on and trityl-off oligonucleotide analyses and purifications. Tritylated oligonucleotides will elute at 25–35 min under these conditions. The retention time for trityl-off oligonucleotides will be 12–15 min.

Table 4-1. Reverse-Phase Gradient

Start Time	% B (acetonitrile) at start time
0.0	8
24	20
34	40

Mobile Phases: A = 0.1M TEAA
B = acetonitrile

Flow rate: 1 mL/min

4.6 x 220-mm cartridge

For more hydrophobic oligonucleotides, it may ultimately be necessary to operate at 50% acetonitrile. The conditions may also need to be altered for large sequences, or for sequences in which one base predominates. Base composition and sequence length will affect mobility retention time. The retention time of tritylated oligonucleotides will usually decrease as chain length increases.

When the proper conditions for a purification have been determined by an analytical run, examine the chromatogram for the best possible collection points. Because the unwanted trityl-on peaks will usually elute later in the chromatogram and will appear as separate peaks or as shoulders of the main peak, it is important to know when to start and stop collecting. In general, collection can stop when the absorbance is 20% below peak maximum on the back end of the peak. Figure 4-3 depicts a chromatogram of an oligonucleotide purified by this collection process.

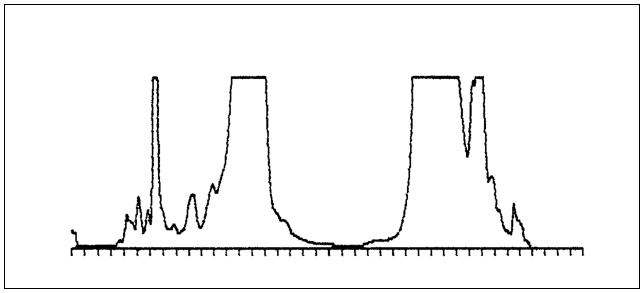

Figure 4-3. *Trityl-on preparative chromatogram*

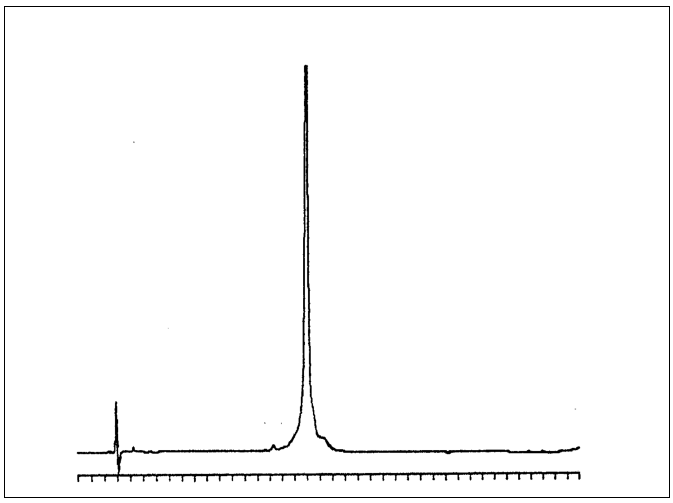

Figure 4-4. *Analytical trityl-off chromatogram of purified oligonucleotide from Figure 4-3*

After collection of the purified product from trityl-on chromatography, the oligonucleotide must be detritylated to render a biologically active compound. The first step is to evaporate the solution under vacuum. The evaporated sample, which could appear as an oil, is treated with approximately 20 µL of 80% acetic acid per ODU of oligonucleotide at room temperature for 20 min (see Appendix B). This acidic treatment does not cause depurination because the bases are much more stable to acid after deprotection. An equal volume of ethanol is added, and the solution is evaporated. Several coevaporations with distilled, deionized water may be necessary to remove the remaining volatile salts. After purification, the oligonucleotide from Figure 4-3 was detritylated and precipitated. Analysis of the product is shown in Figure 4-4.

The hydrolyzed trityl group can be removed by any of the following methods:

- Oligonucleotide Purification Cartridge (OPC, see Chapter 5);
- organic/aqueous extraction;
- ethanol precipitation (see Appendix C);
- desalting on a gel size-exclusion matrix.

Trityl-Off Chromatography

In trityl-off HPLC, the last DMT group on the 5' end of the oligonucleotide is removed prior to ammonia deprotection, usually on the DNA synthesizer. The differences between oligonucleotides of similar length and sequence that contribute to separation are very subtle, although smaller oligonucleotides usually elute faster than longer oligonucleotides. Product identification is not as obvious or predictable as in trityl-on HPLC. However, resolution is usually excellent, making this a viable approach for routinely analyzing oligonucleotides and purifying small quantities.

Single-base resolution can often be achieved, at least to 25 bases and sometimes longer, depending on the sequence. The advantages of this method are:

- ease of sample preparation without the need to preserve the DMT;
- recovery of the purified compound in a ready-to-use form.

The optimal column for trityl-off HPLC (Aquapore RP-300) has a moderately hydrophobic, octylsilyl-bonded phase and a large average pore size of 300 Å. These features are ideal for rapid, efficient trityl-off oligonucleotide analysis.

A typical chromatogram is shown in Figure 4-5. The major peak at 14.5 min is the product, an 18-mer. This peak is surrounded by minor peaks that constitute the various failure sequences and by-products of DNA synthesis. Identifying these peaks by relative retention times is very difficult because of the complex effect of base composition on retention times. Compounds of shorter length than the product usually elute faster than the product. Retention time is based on net surface hydrophobicity. Benzamide appears here at 5.9 min.

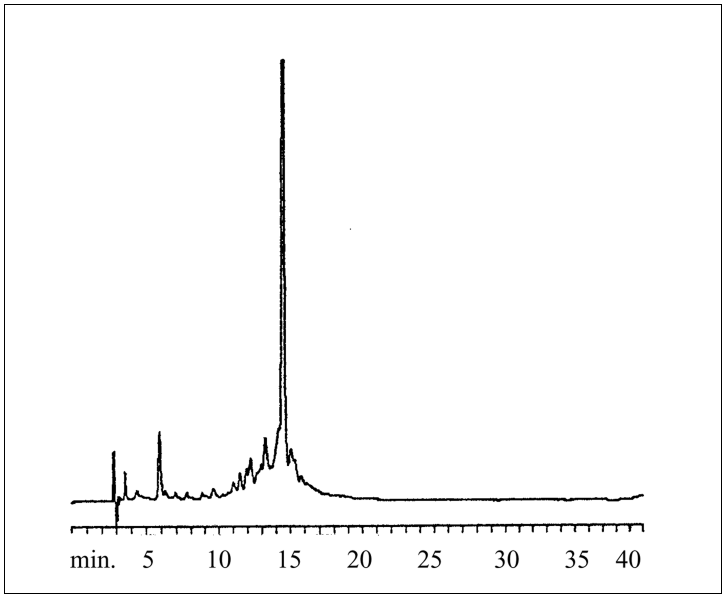

Figure 4-5. *Typical trityl-off analytic chromatogram, 18-mer*

Sample Preparation

Sample preparation is much simpler with trityl-off, reverse-phase HPLC than with trityl-on HPLC. Because the product does not have a trityl group, it is much more stable during preparation for analysis and purification. The ammonia solution containing the crude mixture can be easily and quickly evaporated to dryness in a vacuum centrifuge, most rapidly with mild heat (35–40 °C) and without the addition of triethylamine. Alternatively, samples can be dried under a stream of inert gas such as argon or nitrogen. After evaporation, the oligonucleotide sample can be redissolved in distilled, deionized water and stored in a refrigerator or freezer indefinitely. A small precipitate may be present in the sample upon reconstitution. It can easily be removed by simple centrifugation or filtration.

Figure 4-6 depicts HPLC resolution of four crude oligonucleotides having differing lengths. The chromatogram shows resolution of the four components (18-, 29-, 57-and 72-mers) that were mixed. This demonstrates the resolving power of trityl-off HPLC for oligonucleotides of differing lengths.

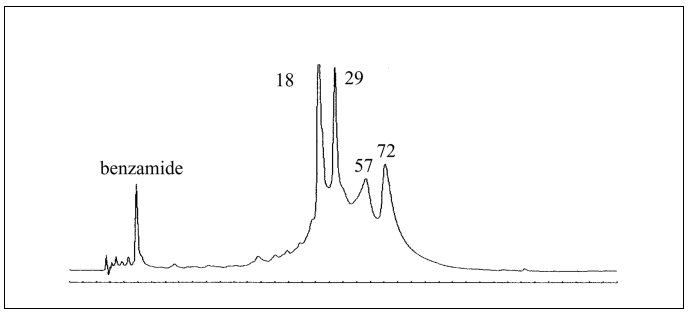

Figure 4-6. *Mix of four oligonucleotides*

Evaluation of the Analytic Chromatogram and Purification

Using the conditions described in Table 4-1, about 1% of the crude mixture from a 1-µmol synthesis (10 µL) can be injected for analysis. Detection at 0.1 ODU AUFS (absorbance units full-scale) is typical for routine examination of the chromatogram. Under most circumstances, the oligonucleotide will elute when the mobile phase composition reaches 10-15% acetonitrile, depending upon the sequence and length.

As with the trityl-on method, the collection process is very important. Because the impurities can elute both before and after the product peak, great care must be taken to decide where collection should begin and end. The collection in a chromatogram should begin and end at 50% of peak maximum.

If the analysis reveals a more complex mixture, purification is more complex. It is important to remember that, for most molecular biology applications, only small quantities of pure product are required. Hence, purity is more important than quantity in the collection process.

Problems that arise when using trityl-off chromatography to purify deoxyguanosine-rich sequences, or oligonucleotides that have mixed sites or regions of self-complementarity, are similar to those that arise when using trityl-on HPLC. Self-complementary oligonucleotides can elute as multiple peaks, often with unpredictable retention times.

Workup of the Final Product

Because the purified product in trityl-off chromatography does not have a 5' DMT group, sample workup is simple. The solution containing the pure product can be evaporated under vacuum with mild heat (35–40 °C) and used directly for many applications. If a white film is found on the bottom of the vial after the sample evaporates, it is probably residual triethylammonium acetate.

The presence of ammonium salts can inhibit some enzymatic processes. Addition of a small amount (~100 µL) of distilled, deionized water and re-evaporation will remove these salts. Alternatively, the purified product can be ethanol precipitated with concomitant counterion exchange (see Appendix C for the protocol).

Limitations of Reverse-Phase HPLC

The major limitations of both types of reverse-phase HPLC relate to the length of the oligonucleotide and to occasional secondary structures formed by inter- and intrastrand base pairing, which persist because of the nondenaturing conditions. The percentage of full-length product in the synthesis product mixture is inversely proportional to the length of the oligonucleotide. Therefore, purification of long oligonucleotides is complicated by their low concentration in the crude mix. The ability of reverse-phase adsorbents to resolve oligonucleotides diminishes as oligonucleotide length increases. Because of their size, longer oligonucleotides cannot enter the pores of the solid-phase matrix where the majority of the bonded phase exists. The longer oligonucleotide thus has a limited number of sites with which to interact on the support, and this affects the capacity of the column. Longer oligonucleotides that differ in length by a single base exhibit small differences in hydrophobicity, resulting in convergence of retention times and loss of resolution.

Reverse-phase HPLC may also not be effective with oligonucleotides that are rich in deoxyguanosine because the chromatographic conditions described are not sufficiently denaturing. Hence, oligonucleotides bearing many deoxyguanosine residues, especially those with four contiguous deoxyguanosines that interact by stacking, will behave like self-complementary oligonucleotides. If the number of deoxyguanosines is sufficiently large, the chromatogram will often have no major peaks whatsoever.

Secondary structure problems can occur with oligonucleotides of any length. This sequence-dependent phenomenon affects resolution because the secondary structure can mask differences in the hydrophobicity of chemically similar compounds to the extent that separation of these species becomes virtually impossible. Also, the individual compounds elute as broader peaks, further complicating separation. The degree of secondary structure can be so great (as with palindromic sequences) that the product will elute as a series of peaks. This series represents an oligonucleotide that has adopted a number of stable conformations.

Polymeric Reverse-Phase HPLC

As an alternative to hydrocarbon-bonded silica columns, a useful addition to HPLC column technology is polymeric adsorbents, such as highly cross-linked, rigid polystyrene beads. Polymeric columns offer the following advantages:

- stability at high temperature, pH extremes and aggressive chemical mobile phases;
- longer lifetimes than their silica counterparts;
- very high capacity for trityl-on oligonucleotides.

Also, because the high pH of the mobile phase and the heat of the columns are denaturing, most secondary structure problems are eliminated.

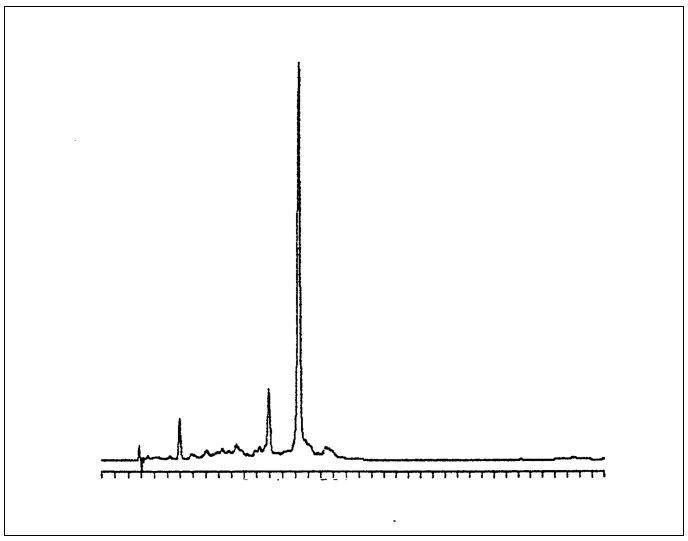

Figure 4-7. *Chromatogram of a typical trityl-off oligonucleotide analyzed by a polymeric reverse-phase column (available from the Hamilton Co., Reno, Nevada).*

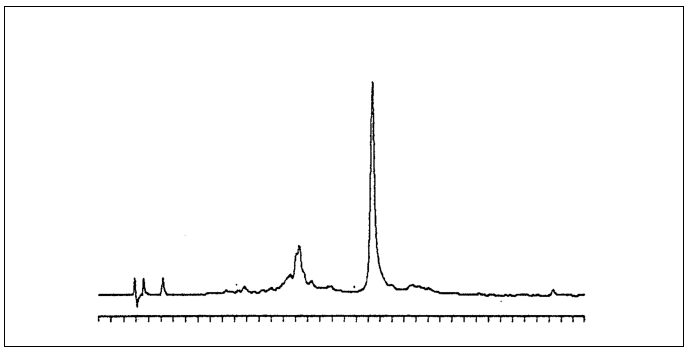

Figure 4-8. *Preparative run of a trityl-on oligonucleotide by a polymeric reverse-phase column. Sample preparation and product workup are similar to those for silica-column, reverse-phase.*

Ion-Exchange HPLC

Ion-exchange HPLC is another efficient technique for analysis and purification. Separation is based largely on charge. Each oligonucleotide in the crude mixture has a different net charge, which is determined by **base length** (the number of phosphate groups in the molecule) and **base composition** (the respective charges on the bases).

It would be advantageous to neutralize the positive charges on the bases by raising the pH of the mobile phase, but most solid-phase supports are silica-based and degrade at pH 8 or greater. Base composition, therefore, can affect the ion-exchange separation process.

Separation of the crude mixture is accomplished by slowly increasing the ionic strength (concentration) of the mobile phase. By doing so, the longer, more highly charged oligonu-

cleotides will elute later than the shorter ones. Minimizing secondary structures is an important requirement of this method.

Phosphate groups masked by secondary structures will not exhibit the effect of a full charge and will deviate from the predictable size-dependent elution pattern. Secondary structure effects can be minimized by adding a strong denaturant such as formamide to the mobile phase, or by heating the column. These measures are not necessary for most oligonucleotides.

Sample preparation for ion-exchange chromatography is the same as for trityl-off methods and is much simpler than the methods used for trityl-on chromatography. Because the oligonucleotide to be applied to ion-exchange HPLC does not have a trityl group, it is more stable during preparation. Oligonucleotides separate by charge and increasing size, with the product peak eluting last because it is the most highly charged.

Resolution decreases as longer oligonucleotides are separated because the relative difference in charge between longer oligonucleotides is less than between shorter oligonucleotides. Also, as the length of the oligonucleotide increases, inevitable peak broadening results. When identical mobile-phase conditions are used, the retention times of oligonucleotides in ion-exchange HPLC will usually be quite reproducible and predictable. Sequence length, therefore, can be reliably confirmed.

This method utilizes a standard 4.6 x 220-mm anion-exchange column, such as Aquapore AX-300 (ABI P/N 0711-0077). The mobile phase is 20% acetonitrile in a potassium dihydrogen and monohydrogen phosphate buffer system, pH 6.7. A standard gradient is 100-400 mM of the phosphate buffer over 30 min at a flow rate of 1.5 mL/min. Oligonucleotides greater than 25 bases will probably need a higher concentration of phosphate buffer. Other salts and solvents are sometimes used. Halide salts can corrode metal surfaces and should be avoided.

Because most reaction contaminants will elute before the product peak, it is advisable to collect most of the sample from the back side of the peak during preparative runs. A conservative collection process is recommended. Start collection when the absorbance is about 50% of maximum and continue through the peak until the absorbance has decreased to 50%. Complex mixtures require a more conservative approach.

Oligonucleotides purified by ion-exchange HPLC must be desalted. On a mass basis, a great excess of nonvolatile salts will be collected with the oligonucleotide product. Desalting can be achieved by various procedures. For amounts up to several milliliters of collected product, the OPC desalting protocol is preferred. Gel size-exclusion chromatography can also be used.

HPLC of Oligonucleotide Conjugates

Covalent attachment of reporter groups—fluorescent dyes, biotin, proteins, etc.—can be made at virtually any site on the oligonucleotide. These include the 5' and 3' ends, through the bases and through an internucleotide phosphate modification. Analysis and purification are feasible for most of these derivatized oligonucleotides, and only slight modifications to the HPLC methods are necessary.

The retention time and elution pattern of conjugated oligonucleotides will be affected by the hydrophobicity of the attached species. Biotin, for example, especially when attached with a long linker arm, imparts significant additional hydrophobicity and greatly increases

retention time. Biotinylated oligonucleotides elute later than their corresponding 5' hydroxyl sequences.

Figure 4-9 shows an 18-mer that was conjugated with a biotin through the 5' end. The biotinylated product elutes at 22.0 min. A small amount of 5' hydroxyl oligonucleotide is present as the smaller preceding peak at 18.5 min. Benzamide is present at 5.0 min. HPLC conditions were according to Table 4-1.

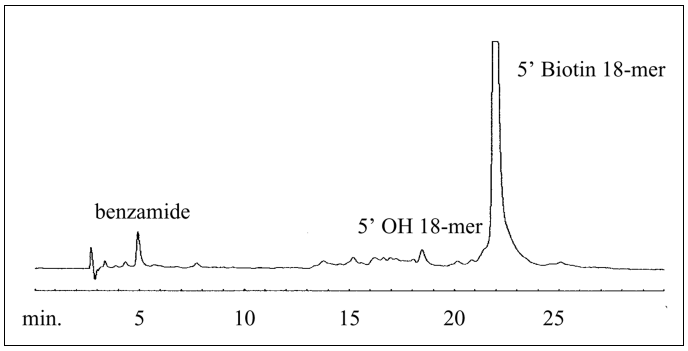

Figure 4-9. *Typical chromatogram of biotinylated oligonucleotide.*

Fluorescent dyes, most commonly attached through the 5' terminus, impart additional hydrophobicity to the oligonucleotide. The dye itself often carries a charge. Like biotinylated oligonucleotides, fluorescent dye-labeled oligonucleotides elute later than their 5' hydroxyl counterparts. Detection at the fluorescence emission wavelength is a powerful adjunct for detection. Figure 4-10 shows a typical chromatogram of an incomplete labeling reaction to prepare a 5' 5-carboxyfluoroscein (FAM) 18-mer. The FAM dye 18-mer elutes at 22.7 min, and the unlabeled 18-mer elutes at 17.4 min.

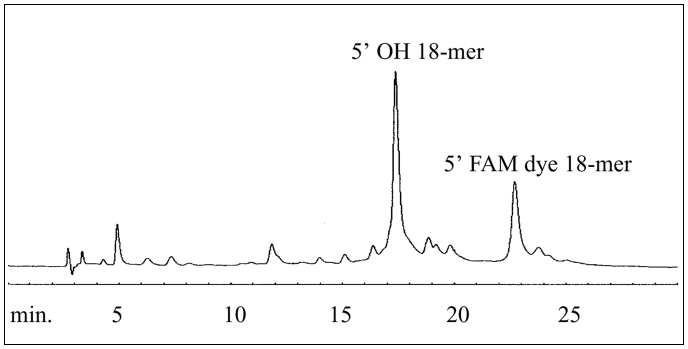

Figure 4-10. *Typical chromatogram of a 5' fluorescent dye-labeled oligonucleotide.*

Further Analysis of HPLC-Purified Oligonucleotides

The purified sample may be analyzed for contaminants by ^{32}P labeling, followed by gel electrophoresis and autoradiography, or by HPLC. If further purification is necessary, we recommend an alternative method that will separate the mixture using a different set of parameters. This should help ensure that a pure compound is isolated.

If only one type of chromatography is available, changing the gradient slightly and taking more conservative collections will give better results. A combination of different purification techniques is the best approach to acquiring consistently pure oligonucleotides, regardless of their length and sequence.

Conclusion

HPLC is a powerful technique for the analysis and purification of DNA. Oligonucleotides purified by HPLC have been used for all applications. The preceding data demonstrate that the analysis and purification of oligonucleotides can be accomplished reliably and efficiently with several HPLC procedures. The HPLC methods for increasing chromatographic resolution are constantly improving, especially for longer oligonucleotides. For large-scale purification, HPLC is clearly the method of choice.

References

Maisano, F.; Parente, D.; Velati Bellini, A.; Carrera, P.; Zamai, M.; Grandi, G. *BioChromatography.* 1989, *4*(5), 279–281.

Newton, P. "Complex Biological Matrices: Column Capacity and Separation Strategy," *LC•GC*. 1990, *8*(2), 116–122.

Zon, G.; Thompson, J.A. "Isolation, Purification and Analysis of Oligodeoxyribonucleotides." *BioChromatography*. 1986, 1(1), 22–32.

The Oligonucleotide Purification Cartridge

Scope and Applications

Since its introduction in 1988, the Oligonucleotide Purification Cartridge (OPC, ABI P/N 400771) has been used with excellent results. Designed specifically for rapid, easy purification of synthetic oligonucleotides, the OPC provides the level of purity required for such common applications as sequencing primers, Polymerase Chain Reaction (PCR) primers and hybridization probes. No sample preparation is needed.

The method is based on a small, syringe-mounted cartridge containing an adsorbent material with a strong and specific affinity for dimethoxytrityl (DMT) oligonucleotides. The ammonia solution of the crude DMT oligonucleotide is applied directly to the cartridge. Only the DMT oligonucleotide is retained. By-products, failure sequences not bearing a DMT group, and other impurities are not retained and are eluted. The DMT group of the OPC-bound oligonucleotide is removed with a mild acid solution, and the purified, detritylated oligonucleotide is eluted with approximately 1 mL of a 20% acetonitrile solution. The entire operation requires 15–20 min.

Many OPC operations can be conducted simultaneously with the use of a simple vacuum manifold (see DNA Synthesis User Bulletin 59, March 1991). Efficient, reliable purifications are achieved with oligonucleotides of any length.

Purification Quality

The success of OPC purification depends on the homogeneity of the trityl species in the crude DNA. Many factors determine the degree of trityl homogeneity found in a crude sample:

- Synthesis efficiency of the detritylation, capping and oxidation steps is essential to trityl homogeneity and to OPC success.
- Low coupling efficiency is not a concern when capping is quantitative.
- Any process that leads to internucleotide cleavage, such as depurination, contributes to trityl heterogeneity and detracts from OPC purification.
- Extraneous growth off the synthesis support may also generate truncated DMT oligonucleotides.

Purification Quantity

Quantity is also important to OPC purification. The salient factors determining oligonucleotide quantity are:

- oligonucleotide length;
- quantity of the DMT oligonucleotide;

- loading solution composition.

The OPC yield is determined by the quantity of DMT oligonucleotide that is able to bind. The affinity of the DMT oligonucleotide for the OPC is higher for short oligonucleotides because they have more DMT character than do long oligonucleotides. While a 20-mer may yield 5 optical density units (ODU) of purified product, a 100-mer may yield only 1 ODU. Obviously, if less DMT oligonucleotide is loaded than the OPC can retain, the OPC is not fully utilized. Applying DMT oligonucleotide in excess of OPC capacity helps ensure that the maximum yield of purified DNA will be attained.

Finally, ammonia in the loading solution limits the binding capacity of DMT oligonucleotides, but it is necessary for purification and for denaturation. The Manual Purification Protocol on page 5–6 gives specific details on maximizing yield without compromising quality.

Functions of the Protocol Steps

Each step of the OPC protocol has a distinct and important function. The initial acetonitrile wash cleanses the OPC cartridge of any contaminants and wets the support with a water-soluble organic solvent to allow aqueous solutions to penetrate the OPC support. Therefore, we recommend that the acetonitrile prewash always be done immediately prior to OPC purification.

The 2 M triethylammonium acetate (TEAA, ABI P/N 400613) wash is necessary to remove the acetonitrile that would prevent the DNA from binding to the OPC. It also prepares the support with TEAA, which helps bind DNA.

Loading is accomplished from an aqueous ammonia solution of the crude DNA. Ammonium hydroxide provides a denaturing environment that keeps complementary non-DMT oligonucleotides from hybridizing with the DMT oligonucleotide. This is most important when using the OPC to purify sequences that have a large self-complementary component. Ammonia also prevents binding of non-DMT oligonucleotides on the OPC adsorbent. Non-DMT oligonucleotides have a substantial affinity for the TEAA-treated OPC absorbent in the absence of ammonia.

A loading solution of 50% water/concentrated ammonium hydroxide (v/v) can denature and prevent binding of nontrityl-bearing DNA while still allowing ~5 ODU of trityl-bearing DNA to be retained on the nonpolar support. The spent loading solution should be collected and saved; it may still contain a significant amount of trityl-bearing product, which can be purified with subsequent OPC operations.

Two or three dilute ammonia washes follow loading to ensure that all non-DMT oligonucleotides have been removed. The water wash removes residual ammonia to prevent neutralizing the trifluoroacetic acid (TFA; ABI P/N 400137) that follows. A 2% solution of TFA in water detritylates the retained DNA. This detritylation can sometimes be observed as a slight orange or pink blush on the support.

Although the DNA no longer has a trityl group, a secondary affinity mechanism retains the DNA as a triethylammonium salt on the OPC at neutral and low pH. Please note that no depurination can be detected by base composition analysis in a pure oligonucleotide subjected to normal OPC conditions (3–5 min, 2% TFA in water) nor in one subjected to extreme conditions (1 h, 2% TFA in water).

A water wash following detritylation removes residual TFA to prevent it from contaminating the final product. It also prepares longer oligonucleotides (greater than 40 bases) for the additional wash with dilute ammonia.

The dilute ammonia wash removes the shorter, previously trityl-bearing oligonucleotides, while allowing the longer oligonucleotides to be retained. Tritylated short oligonucleotides may result from:

- internucleotide cleavage;
- growth off the support;
- slight inefficiencies in synthesis chemistry.

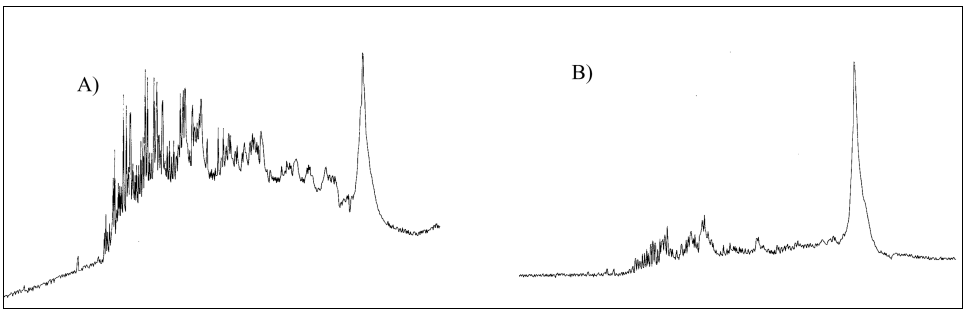

Figure 5-1. *Purification with the OPC using the long oligonucleotide protocol (step 7a) works well, even for 120-mers. Figure 5-1 shows a MicroGel capillary electrophoresis comparison (ABI Model 270A) of a crude (A) and an OPC-purified (B) 120-mer (synthesized on the Model 394, 40-nmol polystyrene, 0.05 M phosphoramidites, version 1.01, primer cycle).*

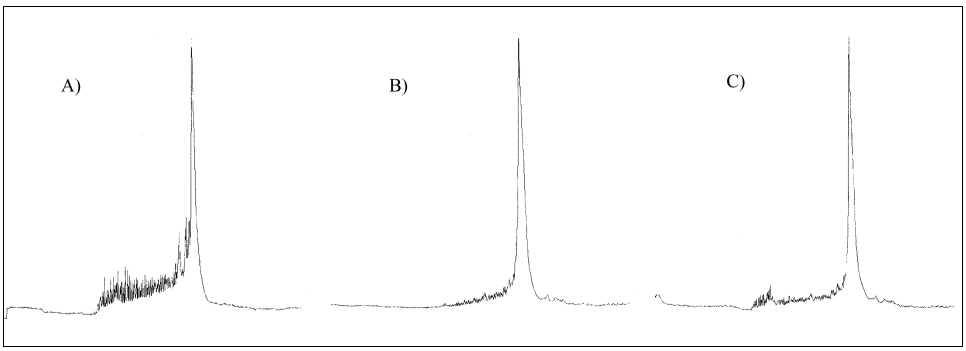

Figure 5-2. *MicroGel capillary electrophoresis comparison (ABI, Model 270A) of a 72-mer: (A) crude; (B) purified with the standard OPC protocol (i.e., without step 7a). Note that the short failure sequences left by the standard protocol (B) are removed by the additional wash found in the long oligonucleotide protocol (C).*

The success of this ammonia wash depends not on trityl selectivity, but on the length of the individual oligonucleotides. A water wash follows to remove residual ammonium hydroxide. Elution of the product occurs with 20% acetonitrile in water. Because the cleaved DMT remains on the OPC, it does not contaminate the product.

OPC Reuse

Reuse of any purification matrix can lead to problems with carryover and sample contamination. Avoiding these problems is especially important in synthetic DNA applications because of the potential for errant priming and hybridization. Analytic HPLC studies demonstrate that approximately 5% of an oligonucleotide product is carried over as a contaminant into a second, different oligonucleotide sequence upon reuse of the OPC. Also, the DMT that is released upon TFA treatment is very strongly bound to the OPC matrix. This results in diminished capacity and selectivity for DMT oligonucleotides. It is strongly recommended that the OPC be disposed of after a single use.

Phosphorothioate DNA

The OPC method is very efficient for purifying the antisense, phosphate DNA analog, phosphorothioate oligonucleotide. Because phosphorothioates are more hydrophobic than normal oligonucleotides, 1 mL of 35% acetonitrile in water is required to elute them.

RNA

The OPC is useful for **desalting** synthetic RNA oligonucleotides. To minimize phosphate migration, which gives rise to 2'–5' phosphate linkages, the final step in RNA deprotection should be the 2' desilylation (see DNA Synthesis User Bulletin No. 53, December 1989). RNA syntheses, therefore, should be conducted trityl-off. RNA cannot be purified by the OPC as can DNA, but it can be desalted. For efficient separation of the crude, fully deprotected RNA from the tetrabutylammonium salts after desilylation, use the OPC desalting protocol.

The OPC purification protocol can be used for any labeled oligonucleotide that has a DMT group, which can be removed during the protocol. Labeled oligonucleotides without a DMT group may be OPC-desalted. Examples of oligonucleotide labels include biotin, acridine and fluorescent dyes.

Labeled Oligonucleotides

The OPC purification protocol can be used for any labeled oligonucleotide that has a DMT group, which can be removed during the OPC protocol. Labeled oligonucleotides without a DMT group may be OPC-desalted. Examples of oligonucleotide labels include biotin, acridine and fluorescent dyes.

Desalting

Unlike the OPC purification protocol, the desalting protocol can yield up to 50 ODU of desalted oligonucleotide because no ammonia is used. Desalting removes most of the nonoligonucleotide impurities. Figure 5-3 shows the change in the UV spectra of an oligonucleotide after OPC desalting. The desalting protocol is useful for:

- trityl-off oligonucleotides;
- RNA oligonucleotides, after desilylation;

- polyacrylamide gel purification extracts;
- anion-exchange HPLC fractions.

Triethylammonium is the counter ion, bound to DNA that has been desalted or purified by the OPC. If it is necessary to remove the triethylammonium counter ion, ethanol precipitation with 1.0 M sodium chloride is advised.

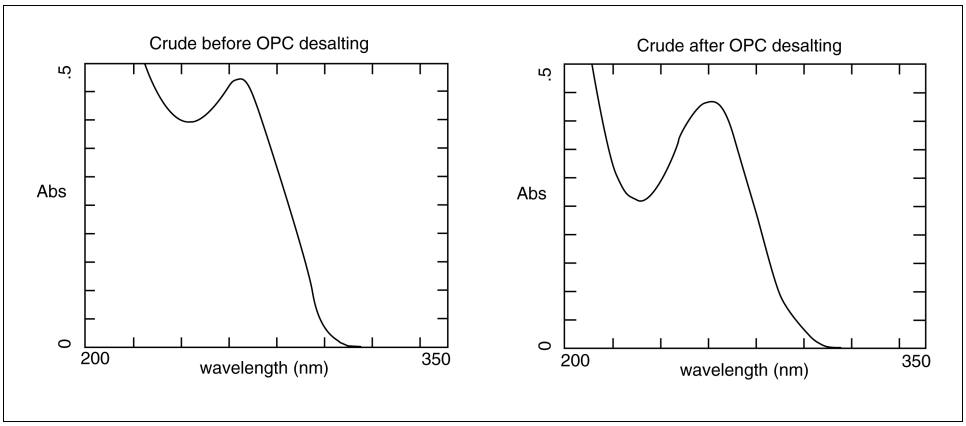

Figure 5-3. *UV spectra of a crude and an OPC-desalted 18-mer*

DNA Synthesis User Bulletin 59 details many improvements designed to make OPC the preferred oligonucleotide purification method. They include:

- increased capacity;
- faster operation;
- multiplexed operation;
- purification of bigmers;
- RNA;
- DNA analogs (e.g., phosphorothioates).

Ammonia in the loading solution of the oligonucleotide provides the desired non-denaturing media but limits the binding capacity of DMT oligonucleotides. The current protocol printed on the OPC package specifies adding one-third volume of water to the crude oligonucleotide/ammonia solution to yield 1–5 ODU (A_{260nm}/mL) of purified, detritylated oligonucleotide. If more water is used, or if the ammonia is partially removed after several minutes in a vacuum centrifuge, the binding capacity of the DMT oligonucleotide will increase to yield about 10 ODU of purified, detritylated oligonucleotide.

Manual Purification Protocol

1. Pass 5 mL of acetonitrile through the OPC to waste.
2. Pass 5 mL of 2 M TEAA through the OPC to waste.
3. Dilute the ammonia solution containing the oligonucleotide with an equal volume of water, e.g., 1 mL of each. Pass the diluted solution through the OPC at a rate of about 1 drop per second and collect the eluate.

4. Pass the eluate through a second time. Collect and save the eluate for subsequent OPC purifications.
5. Pass 5 mL of dilute ammonia through the OPC to waste, followed by 10 mL of water.
6. Fill the syringe barrel with 5 mL of 2% TFA in water and pass a portion through the OPC to waste. Let stand 3–5 min before passing the remainder through to waste.
7. Pass 10 mL of water through the OPC to waste.
    a. **This step applies *only* to oligonucleotides greater than 40 bases**. Pass 5 mL of dilute ammonia (1.5 M = 9:1 H_20:concentrated ammonia (v/v)) through the OPC to waste followed by 5 mL of water.
8. Elute by drop with 1 mL of 20% acetonitrile (ABI P/N 400314) in water (v/v, 35% acetonitrile for phosphorothioate DNA) and collect the purified oligonucleotide in a suitable (e.g., 1.5-mL Eppendorf) vial.

Multiplexed Vacuum Manifold Protocol

This protocol uses an inexpensive, solid-phase extraction device to conduct many OPC purifications simultaneously. It offers increased throughput and entails fewer manual operations (see Figure 5-4).

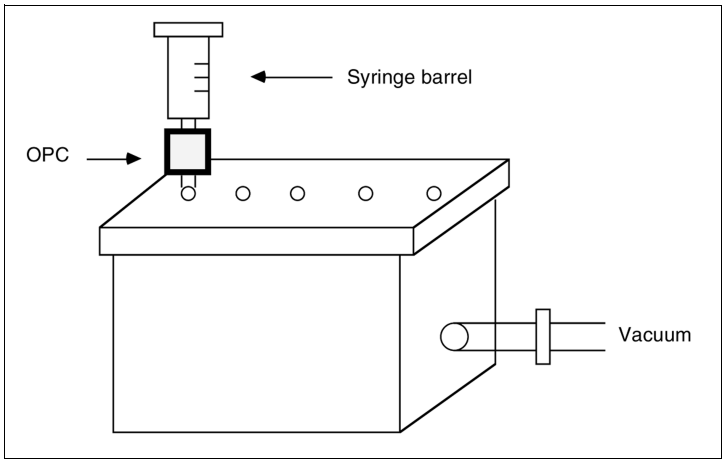

Figure 5-4. *OPC Vacuum Manifold*

The following vacuum manifolds are manufactured by Analytichem (a division of Varian Associates, Inc.) and are ideally suited for multiplexed OPC processing:

Vac Elut®:	Catalog number 1223-4036 (10-place manifold, 12 x 75-mm test tube rack)
Vac Elut SPS 24®:	Catalog number 1223-4022

Both units have been tested at Applied Biosystems laboratories and gave excellent results, with no cross-contamination of samples. Other commercially available manifolds may work equally well. Consider the following factors in selecting a manifold:

- inertness of the manifold to ammonia and TFA;
- size of the available collection vials;
- convenience of collection or removal of the washes;
- desired throughput.

Recommended Protocol for the Analytichem/Varian Manifolds

Preparation

1. Equip the vacuum manifold with an in-line trap for waste and a water aspirator pump or small vacuum pump.
2. For each oligonucleotide to be purified, connect a male-male luer adapter to one end of an OPC and a 5-mL plastic syringe barrel to the other.
3. Insert the free end of the luer-luer adapter into a luer fitting at the top of the manifold.
4. Plug all remaining sites. (Vac Elut 10: Remove the test tube rack. Vac Elut SPS 24: Place the clean test tubes into the test tube rack in anticipation of the loading step. Rotate the top to the waste position.)
5. Turn on the vacuum.

Procedure

1. Wash each OPC with 5 mL of acetonitrile.

   A wash step consists of filling the syringe barrel (which serves as a solvent reservoir) with 5 mL of the wash solvent and having the vacuum draw the wash through the OPC. Except where otherwise stated, the vacuum does not need to be regulated to reduce the flow rate.
2. Wash with 5 mL of 2 M TEAA and turn off the vacuum. Remember to vent, as necessary. (Vac Elut 10: Place the rack containing clean 12 x 75-mm test tubes into the box. Vac Elut SPS 24: Rotate to the collect position. Make sure the test tubes are in place.)
3. Load each oligonucleotide, dissolved in 1 mL of concentrated ammonia and 1 mL of water, into the syringe barrels. Turn the vacuum on but regulate it to approximately 2 in. Hg, using the bleed or the quick release valve. A low flow rate is essential to avoid cross-contamination of the samples.
4. Return the oligomer solution to the barrel and reload it onto the cartridge with the vacuum set at about 2 in. Hg. (Vac Elut 10: Remove the rack. Vac Elut SPS 24: Rotate to the waste position.)
5. Wash the OPC with 5 mL of 1.5 M (dilute) ammonia, then wash it twice with 10 mL of water.
6. Detritylate by pulling 1–2 mL of a 5-mL, 2% TFA solution through the OPC. Then vent the system and turn off the vacuum. Allow the remaining 3 mL of 2% TFA to fill the OPC. Let it stand for approximately 5 min to detritylate completely. Pull any remaining TFA solution through the OPC.

7. Wash twice with 5 mL of water.

    a. **This step applies *only* to oligonucleotides greater than 40 bases**. Wash with 5 mL of 1.5 M ammonia, followed by two 5-mL water washes.

8. Elute with 1 mL of 20% acetonitrile in water (v/v, 35% acetonitrile for phosphorothioate DNA) with the vacuum set at about 2 in. Hg. (Vac Elut 10: place the rack into the box again with clean 12 x 75-mm test tubes. Vac Elut SPS 24: Rotate to the collection position. Make sure that clean test tubes are in place.)

Desalting Protocol

1. Pass 5 mL of acetonitrile through the OPC to waste followed by 5 mL of 2 M TEAA.
2. Dissolve the oligonucleotide in 1–3 mL of aqueous solution (e.g., 0.1 M TEAA). The loading solution should not contain organic solvents or ammonium hydroxide.
3. Pass the diluted solution through the OPC at a rate of about 1 drop/s.
4. Collect the eluate and pass it through a second time.
5. Pass 15 mL of 0.1 M TEAA through the OPC to waste.
6. Elute dropwise with 1 mL of 50% acetonitrile in water (v/v) and collect the desalted oligonucleotide in a suitable (e.g., a 1.5-mL Eppendorf) vial.

Analysis of Oligonucleotides by MicroGel Capillary Electrophoresis

Introduction

MicroGel capillary electrophoresis is a powerful new method for the analysis of oligonucleotides that offers the following advantages:

- dramatically decreased analysis time;
- excellent resolution;
- in-capillary detection;
- reduced sample requirements;
- automation.

Capillary electrophoresis (CE) on the Model 270 (A or A/HT) is already established as an important analytical tool for other biomolecules such as proteins, peptides and high-molecular-weight, double-stranded nucleic acids. By using special polymeric gel-filled capillaries, the CE method has been extended to single-stranded oligonucleotides.

As with polyacrylamide slab gel electrophoresis (PAGE), DNA separates primarily and predictably in a gel-filled capillary by the ratio of mass to charge under the influence of an electric field. The elution pattern is the same: small oligonucleotides followed by the largest, usually the product. Single-base resolution beyond 100 bases can often be attained. The analysis, called an electropherogram, is **quantitative**. It can be displayed, stored, integrated and printed like HPLC chromatograms. Slab gels (PAGE), on the other hand, are visualized at a single time point in the analysis, do not easily yield to integrative quantitation, and are thus a **qualitative** method of analysis.

The resolution of MicroGel CE surpasses that of current techniques—HPLC and PAGE—for oligonucleotide analysis. The combination of gel materials and heating of the capillary confers a significant denaturing effect for predictable elution patterns devoid of secondary structure artifacts. MicroGel capillaries can sustain multiple injections, depending on storage and handling conditions. This chapter examines several parameters affecting oligonucleotide analysis:

- resolution limits;
- analysis time;
- sample preparation and concentration;
- reproducibility;
- electrophoresis settings;
- adaptability to automation.

Capillary Electrophoresis System

The system used for MicroGel capillary electrophoresis is the Model 270A, or the Model 270A/HT with a high-throughput autosampler. Figure 6-1 shows the instrument configuration, consisting of a high-voltage power supply, with electrodes immersed in static buffer reservoirs. The ends of the fused silica, gel-filled capillary are mounted in the buffer reservoirs, and the capillary window is positioned at the detector site. A variable-wavelength UV/VIS detector collects light passed directly through the capillary. This produces the typical HPLC-type analog voltage signal to be used by the integrator, chart recorder or data system, which can be processed by an A/D converter.

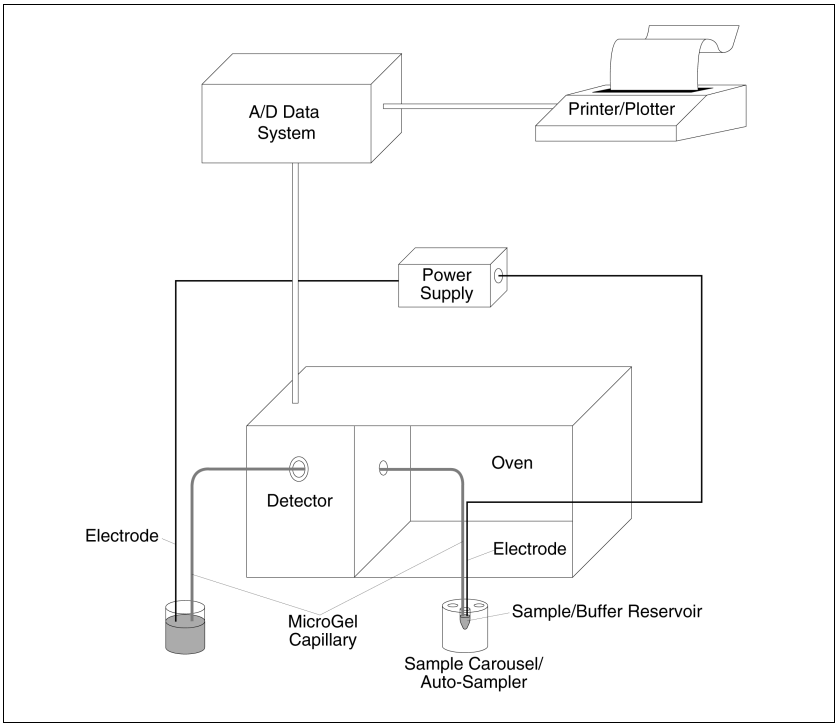

Figure 6-1. *MicroGel/Model 370 A/HT Capillary Electrophoresis System*

Capillaries

The MicroGel capillary is a thin, 50-cm, fused-silica tube, coated on the outside with polyimide. The small internal diameter of the capillary (50 µm) allows fast and efficient heat dissipation, permitting the use of high field strengths to achieve rapid analysis times. At the detection point, the polyimide coating is removed, producing a clear window with a length of approximately 5 mm. The gel concentration is constant throughout and is identical for all capillaries, as has been demonstrated by extensive quality testing.

MicroGel material is a viscous, chain-entangled elastic polymer that has hydrophilic and denaturing properties. Polyacrylamide, the traditional polymer used for slab electrophoretic analysis of nucleic acids, has also been extensively investigated as a gel matrix for capillary electrophoresis. The commercial development of polyacrylamide capillaries and their use, even by experts, have been hampered because of instability problems. It is clear that when

urea is present, capillary stability is compromised, and the capillary will have a short shelf life. Acrylamide/urea capillaries also must be refrigerated off the instrument when not in use.

Although MicroGel capillaries do not contain conventional chemical denaturants such as urea or formamide, hydrogen bonding between the bases is almost totally disrupted upon interaction with the MicroGel matrix and the elevated capillary temperature. MicroGel capillaries are sufficiently stable to endure many sample injections. Typically, 50–100 analyses per MicroGel capillary can be attained if proper handling and analysis conditions are observed. The buffer chambers and the MicroGel capillaries are filled with a Tris-phosphate buffer (for the exact formulation, see the MicroGel product insert).

It is important to change the buffers after every few hours of electrophoresis time, especially on the sample side. With most systems, this is the smaller volume buffer chamber, which undergoes ion depletion during the course of electrophoresis. Changes in ionic strength or pH of the buffer or sample solutions have dramatic effects on elution time, resolution and peak size.

Relative Elution Times

Analysis times are reduced by increasing field strength and capillary temperature, and by decreasing capillary length. When a reference marker is included in the same run as the unknown sample, a relative elution time may be calculated by dividing the elution time of the unknown oligonucleotide peak by the elution time of the reference peak. This relative elution time is very reproducible and may be used to predict the elution time of the same oligonucleotide in subsequent runs. Elution time and pattern will vary slightly, depending on the sequence.

The elution time variability of oligonucleotides using MicroGel capillaries is approximately 1.0% relative standard deviation (RSD) when an internal standard is included with the sample in a run. Accurate peak area quantitation with an internal standard is still limited because of possible differences in molar extinction coefficients. Injection anomalies can be caused by sample conductivity differences or changes in electrode alignment. For further discussion of this topic, see CE Application Note 13: *Factors Influencing Reproducibility on the Model 270A Capillary Electrophoresis Instrument*.

Installation

Installation of a MicroGel capillary takes only a few minutes on the Model 270A or 270A/HT (see CE User Bulletin 2: *Installing a Capillary in the 270A/HT*). Setup and maintenance are faster and easier than with the more labor-intensive analytical methods such as HPLC or PAGE. The capillary oven chamber can be precisely temperature-controlled between 5 °C above ambient and 60 °C. MicroGel capillaries can be used at temperatures between 30 and 50 °C. Temperatures higher than 50 °C may degrade the gel capillary matrix. All examples shown here were conducted at 40 °C. Elevated temperature can often improve resolution by disrupting secondary structures, which can lead to extra or broadened peaks.

Establishing a Method

The Model 270 allows control of many variables affecting analysis. Parameters such as resolution, speed and sample effects can be optimized. Because both the injection and elution of the oligonucleotide occur under the influence of a precisely controlled electric field, settings are important. The capillary establishes an electrical circuit at very high voltage and very low current. Voltage is held constant and the current may change as a function of resistance. The following are recommended method parameters:

Gel Capillary Electrophoresis Parameters

Detector:	Wavelength = 260 nm
Rise time:	1 or 0.5s
Range:	0.01
Zero:	yes (auto-zero at the beginning of each run)
Sample:	Time: 5 s
Voltage:	-5 kV
Temperature:	40 °C
Time:	Run Time: 22 min
Voltage:	-15 kV
Temperature:	40 °C

Data Acquisition/Integrator Parameters

Acquisition time:	15–50 min
Sampling rate:	5 points/s
Signal:	0–1 V
Integration format:	baseline-to-baseline or peak-to-peak
Area Reject:	10,000

The data acquisition system used to process the data shown here is the Model 1020S PE/Nelson Personal Integrator with a Panasonic KX-P1080i dot-matrix printer and a Hewlett Packard Model 7550A Graphics Plotter.

Sample Preparation

Single-stranded synthetic DNA or RNA oligonucleotides of 4–150 bases can be effectively analyzed by MicroGel CE. Various oligonucleotide analogs can also be evaluated, including phosphorothioates. Analysis can be done directly from crude reaction mixtures containing ammonium hydroxide, or from lyophilized (dried in a vacuum centrifuge) samples

dissolved in water. No spurious peaks caused by ammonium hydroxide will be seen on the electropherogram, but changes to the sample pH may inhibit sample introduction.

Introduction of an oligonucleotide onto the capillary occurs by **electrokinetic** injection, whereby the highly charged sample is induced to migrate into the capillary inlet under the influence of an electric field. This is different from the physical placement of a sample onto an HPLC column, or the physical loading of the sample in a well at the top of the gel during PAGE analysis. The electrokinetic injection voltage is less than the running voltage, with a duration of 3–20 s. The amount of oligonucleotide sample that enters the capillary increases, although not linearly, with concentration, injection duration and voltage. It can be influenced, however, by the presence of other salts that alter the conductivity of the sample solution.

Usually, the crude sample is sufficiently free of salts to allow the proper conductance for adequate loading. Prior to MicroGel CE analysis, samples containing inhibiting levels of salt and other contaminants should either be purified (with the Oligonucleotide Purification Cartridge (OPC), for example) or should undergo a desalting procedure such as OPC or precipitation (see Appendix C). The presence of small amounts of organic solvents does not affect injection. Samples that have been purified by HPLC and eluted in salt-containing media can be analyzed directly if they do not contain excessive salt, which limits electrokinetic loading. If salt contamination is suspected, a desalting procedure may be necessary.

Oligonucleotide concentrations of approximately 0.1–0.5 optical density units (3–15 µg) per mL of deionized water are appropriate. Nonionic solutions containing, for example, urea or formamide can also be used at comparable concentrations. At least 20 µL of sample solution (about 0.004 optical density units (ODU) of DNA) in a 500-µL microcentrifuge (Eppendorf style) tube is required to ensure adequate immersion of the capillary tip and electrode during injection (see Figure 6-2). Several repetitive injections can be made from this amount of sample. After a few injections, however, the sample introduced onto the capillary will diminish, resulting in smaller peaks due to depletion of the sample and to the carryover of salts from the buffer.

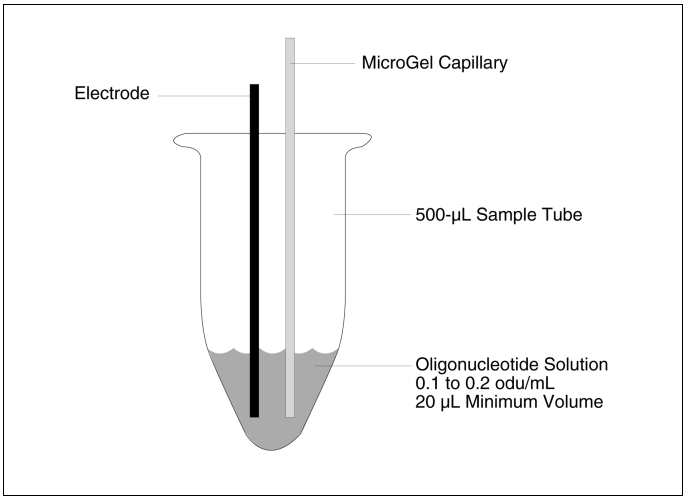

Figure 6-2. *Sample tube with capillary and electrode.*

Examples

Electropherograms generated from gel-filled capillaries present graphic and highly resolved representations of oligonucleotide purity. Quantitative results can be obtained from peak areas by using a data system. As with any chromatographic data, reliable conventions must be developed to obtain consistent interpretations. Variables, such as attenuation, rise time and peak width on a data acquisition system, can dramatically affect purity analysis. Once data acquisition parameters are set, consistent sample concentration, injection duration and injection voltage should also be set. Generally, synthetic yields are consistent enough that analysis conditions can be held constant. Large oligonucleotides often require higher sample concentrations or increased injection times, due to reduced product yields. Run times also need to be increased for longer oligonucleotides.

Figure 6-3 shows an electropherogram of a typical crude oligonucleotide, an 18-mer produced under standard synthesis conditions. The sample was prepared after measuring the absorbance of an aliquot at 260 nm. After chilling the ammonia solution on ice, an aliquot containing 0.1 ODU was transferred to a 500-µL microcentrifuge tube and dried. After 200 µL of water was added and mixed by vortexing, the sample was positioned in the autosampler and electrokinetically injected. Using the sample parameters given above, the electropherogram and integration report show that the product, the largest peak eluting at 12 min, constitutes 83.6% of the total integrated area.

Interpreting Product Purity

The integrated percent of the product peak is often cited as a measure of purity by MicroGel CE and by HPLC. It should be noted that only a single wavelength, typically 260 nm, is monitored. Some impurities may not absorb at 260 nm and therefore will be undetected. Also, the relative extinction coefficients of all species in the sample are not corrected. Integration parameters also influence quantitation based on threshold reject values, peak identification format, etc.

The purity shown in Figure 6-3 would indicate excellent performance for this oligonucleotide in most, if not all, applications including Polymerase Chain Reaction (PCR), sequencing and probe experiments. The sample components that elute prior to the product are failure sequences, sometimes referred to as “n-” species, whereas the desired, full-length product is called the “n” peak or band. Later-eluting impurities arise from other synthesis imperfections, comprised of either higher molecular weight or less charged species. These are termed “n+”.

This elution pattern is, of course, consistent with slab gel electrophoresis (PAGE), in which the components, n-, n and n+ are viewed as bands. Figure 6-3 shows several discrete n- peaks and virtually no n+ peaks. Since the detectability limit by UV shadow or staining detection with PAGE is about 0.05 ODU, these low-level impurities may not be apparent. In addition, the high resolution of gel capillary electrophoresis is required to separate impurities from the product. Figure 6-4 shows a different synthesis of the same 18-mer sequence with a greater amount of n+ impurities and an integrated product peak area of 65.7%. The relative purity of the oligonucleotides in Figure 6-3 and Figure 6-4 cannot be compared by PAGE analysis.

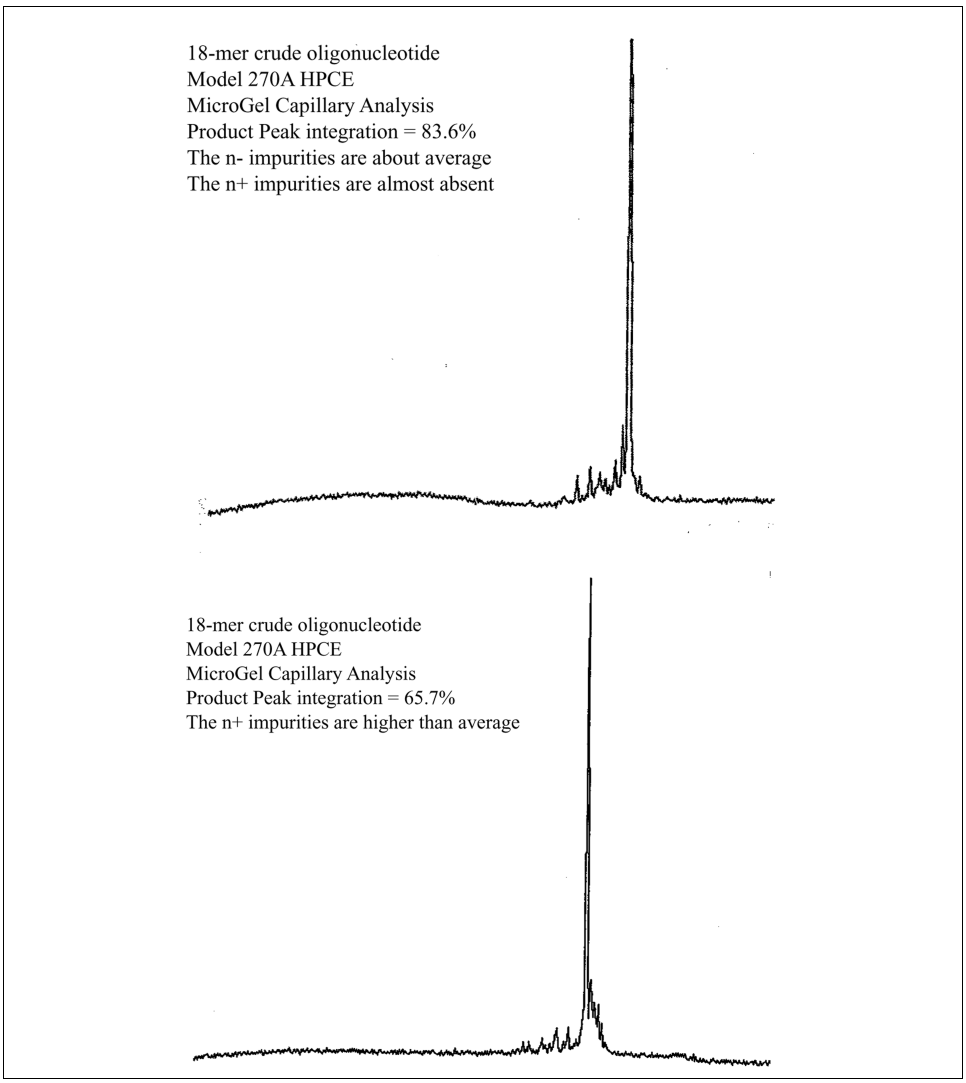

Figure 6-3.
Figure 6-4. Electropherograms of a crude 18-mer 5' TCA CAG TCT GAT CTC GAT 3'

Resolution and Oligonucleotide Length

As oligonucleotide length increases, resolution decreases. The size of the product peak (n) relative to impurities (n-) decreases also. This is simply a function of the less-than-perfect synthesis chemistry. At typical 98% stepwise efficiency, the yield of a 20-mer will be approximately 68%. At this same efficiency, the yield of a 40-mer will be 45%. As a consequence, longer oligonucleotides are less pure, and the product is less distinct by any analytical method. By injecting more sample, either with a higher concentration, longer injection time or higher injection voltage, the capillary may be overloaded, resulting in loss of resolution.

Figure 6-5 shows a 29-mer with a relatively high amount of n+ impurities. Figure 6-6 is a 65-mer with a product peak integration of 19%. Note that in this electropherogram the absorbance remains high during elution of all components. This pattern is indicative of an overloaded capillary, which may be difficult to avoid when analyzing impure or long oligonucleotides. The 72-mer in Figure 6-7 is of higher purity: 51% product peak integration. The absorbance signal returns to the baseline during elution of all sample components.

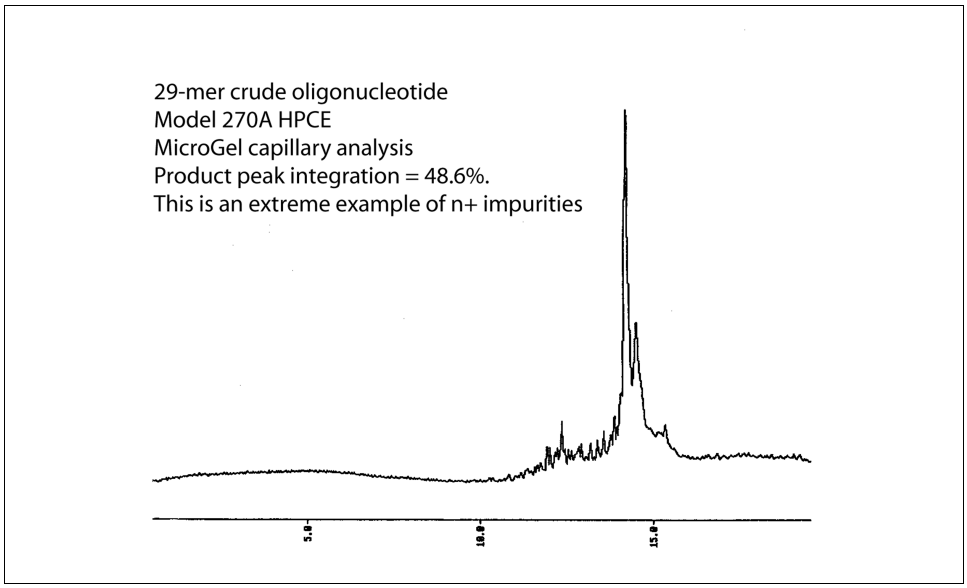

Figure 6-5. *29-mer sequence: 5' CCA TGA AGC TTT GAC CAT GAA AAT GGA GA 3'*

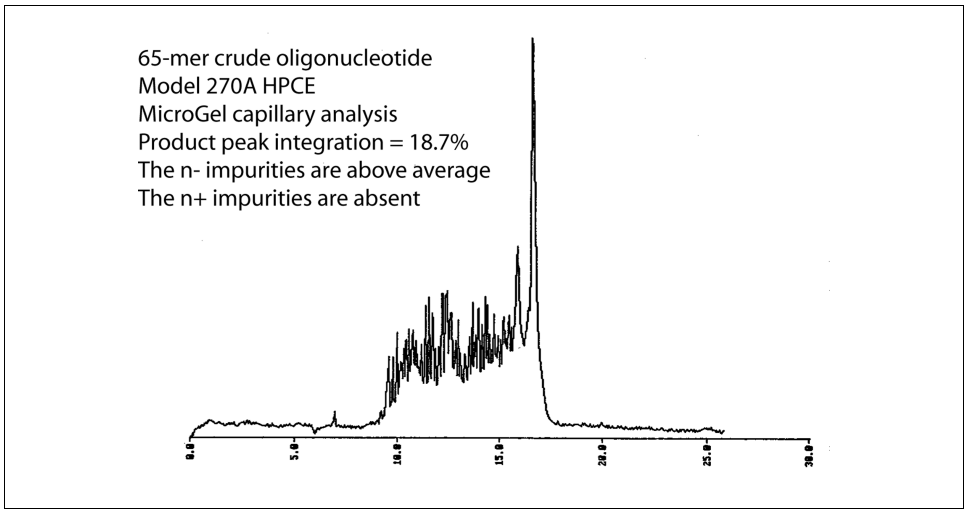

Figure 6-6. *65-mer sequence: 5' TCT CTG CGC GAC GTT CGC GGC GGC GTG TTT GTG CAT CCA TCT GGA TTC TCC TGT CAG TTA GCT TT 3'*

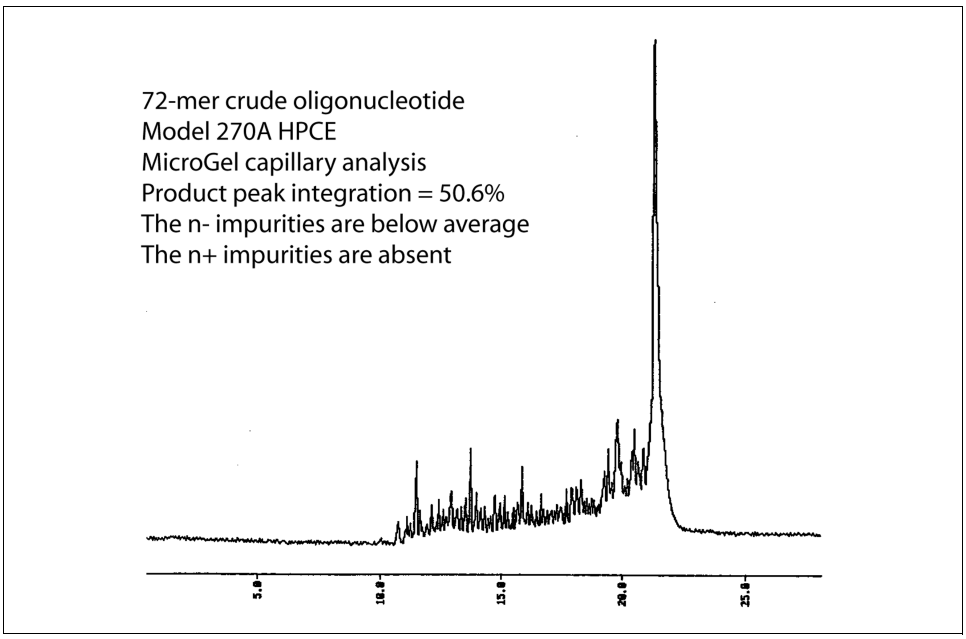

Figure 6-7. *72-mer sequence: 5' AGG GCC GAG CGC AGA AGT GGT CCT GCA ACT TTA TCC GCC TCC ATC CAG TCT ATT AAT TGT TGC CGG GAA GCT 3'*

Even longer oligonucleotides will show single-base resolution when critical parameters such as concentration, injection time and injection voltage are optimized, often empirically. The 120-mer shown in Figure 6-8 gives a quantitative assessment of purity unavailable by any other method for such a long oligonucleotide. The integrated product purity of 10% is consistent with a stepwise efficiency of approximately 98%, probably the maximum that can realistically be attained. Higher trityl measurements are sometimes possible. They are augmented, however, by side reactions that contribute to the trityl release but detract from product purity.

As an illustration of the resolving power of MicroGel CE, the 120-mer in Figure 6-8 and a 119-mer (same sequence as the 120-mer minus the third base from the 3' terminus) were mixed and analyzed. Figure 6-9 is the electropherogram showing useful separation of the two long oligonucleotides, differing in length by only one base. Considering that both oligonucleotides were in a crude state of approximate 10% purity, this method demonstrates excellent resolution.

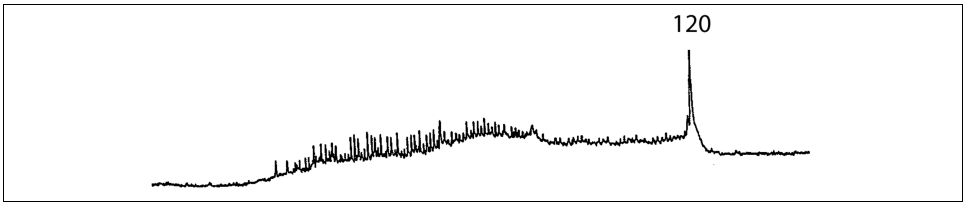

Figure 6-8. *120-mer sequence: 5' CAA CAG GGG ATT TGC TGC TTT CCA TTG AGC CTG TTT CTC TGC GCG AGG TTC GCG GCG TGT TTG TGC ATC CAT CTG GAT TCT CCT GTC AGT TAG CTT TTC ACA GTC TGA TCT CGA TAT 3'*

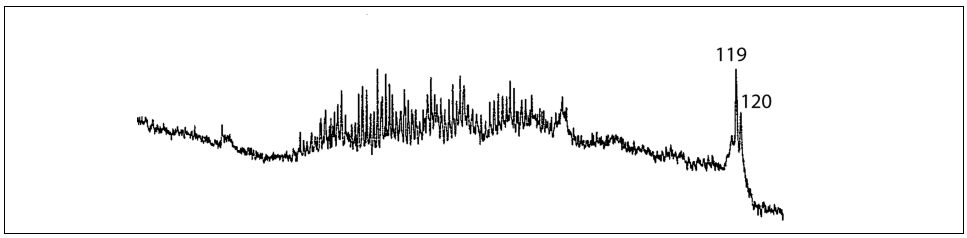

Figure 6-9. *Mixture of 120-mer and 119-mer*

MicroGel CE separation of oligonucleotide occurs primarily by length. The MicroGel matrix is an entangled polymer in which the large oligonucleotide molecules must migrate through pores in response to the electric field. Their shape, charge and hydrophobicity also influence elution patterns.

As an example, a mixture of 18-mers sharing the same base composition ($A_4G_3C_5T_6$) are separated in Figure 6-10. Although they have the same molecular weight and net charge, a different sequence order of the three bases at the 5' end allows separation. Achieving separation between oligonucleotides of the same length, much less the same base composition, is not a typical occurrence. However, this example shows the significance of the chemical interaction between the gel matrix and oligonucleotides.

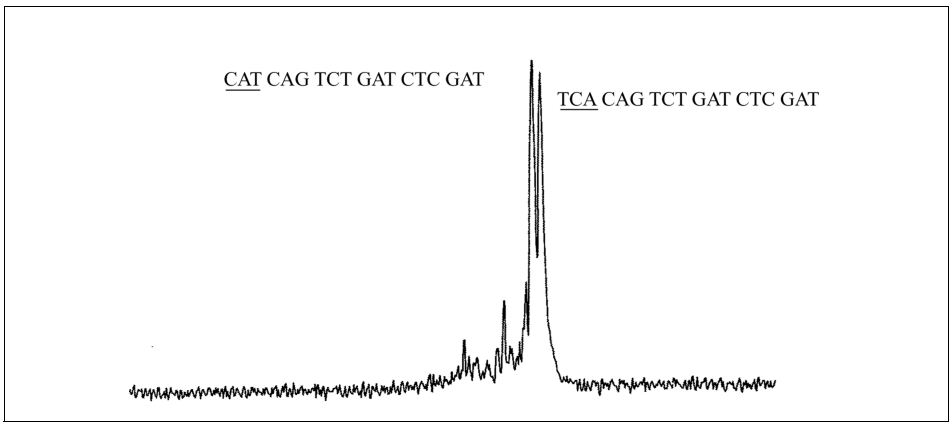

Figure 6-10. *Mixture of two 18-mers*

Trityl-On Oligonucleotides

Trityl-on oligonucleotides can be separated easily from trityl-off oligonucleotides by gel capillary analysis. The presence of the very hydrophobic 5' dimethoxytrityl (DMT) group interacts strongly with the MicroGel matrix, which retards elution. The elution time difference between trityl-on and trityl-off oligonucleotides by capillary gel electrophoresis is less pronounced than in reverse-phase HPLC, but more so than in PAGE. The considerations that apply in HPLC for preserving the trityl group on the sample apply also in CE analysis.

Acidic solutions must be avoided to prevent detritylation. Adding a few microliters of triethylamine to the sample before concentrating it under vacuum may help. Figure 6-11 shows a mixture of trityl-on and trityl-off oligonucleotides of the same length and se-

quence. The trityl-on oligonucleotides elute later due to the hydrophobic interaction with the gel matrix and increased molecular mass.

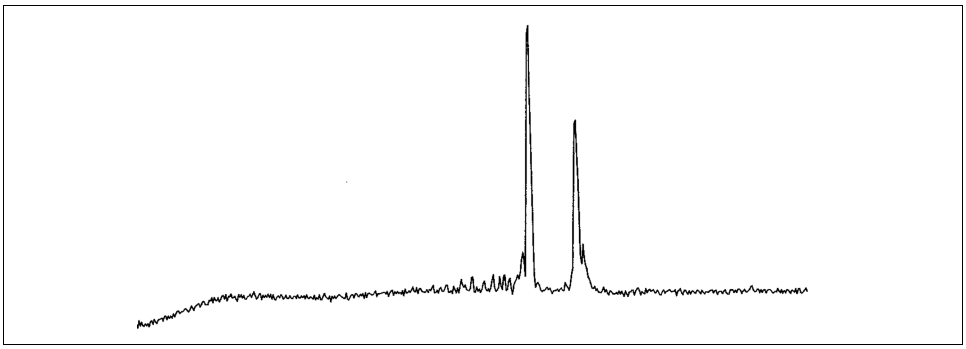

Figure 6-11. *Mixture of 5' DMT and 5' OH 18-mers 5' TCA CAG TCT GAT CTC GAT 3'*

Labeled Oligonucleotides

Oligonucleotides are now often derivatized postsynthesis and labeled by covalent attachments with other molecules to serve an expanding variety of novel applications. The most common site of attachment is the 5' end. The phosphoramidite reagent Aminolink 2 (ABI P/N 400808) can be used as the final base on the DNA synthesizer, producing a nucleophilic amine group after cleavage and deprotection. The 5' amino-linked oligonucleotide can be analyzed and purified by the same methods as underivatized 5' hydroxyl oligonucleotides. The aminohexyl phosphate moiety confers a slight retarding effect during electrophoresis relative to the 5' hydroxyl moiety.

Gel capillary electrophoresis is a convenient method for assessing the efficiency of the Aminolink 2 coupling and for gauging the purity of the crude amino-linked product. Figure 6-12 shows a mixture of 5' hydroxyl and 5' amino-linked oligonucleotides. Their identities were established by injections of purified oligonucleotides and mixtures at other ratios (data not shown). The amino-linked oligonucleotides consistently elute later than their 5' hydroxyl counterpart sequences. Further purification at this stage may not be necessary.

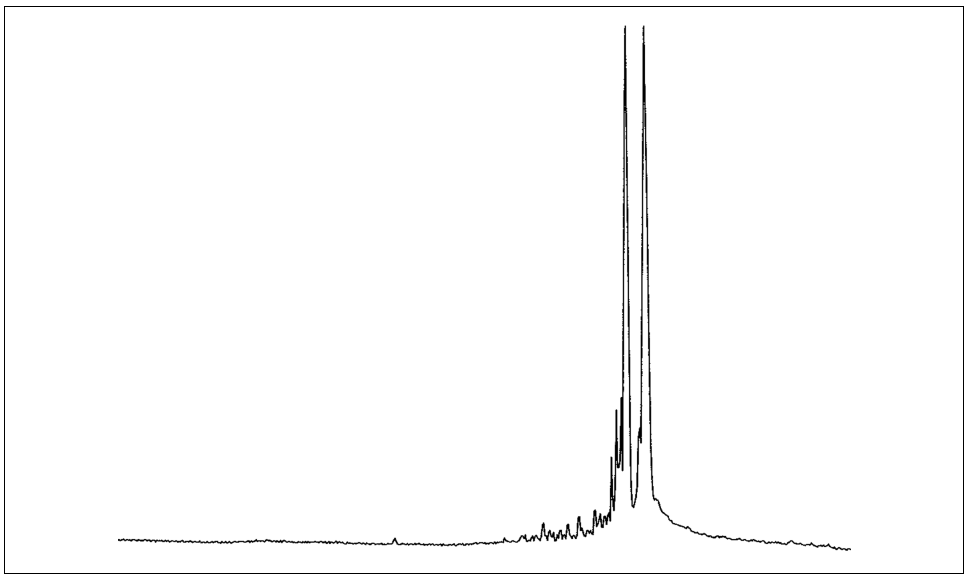

Figure 6-12. *Mixture of 5' OH and 5' aminohexylphosphate 5' TCT AAA ACG ACG GCC AGT 3'*

Amino-linked oligonucleotides are then reacted further in solution, usually with an active ester molecule such as NHS-biotin or NHS-fluorescent dye, to make biotinylated and fluorescent dye-labeled oligonucleotides. Biotinylated oligonucleotides, for example, can be made by reaction of the amino-linked oligonucleotide with a large excess of the NHS-biotin (e.g., Sulfo-Biotin, Pierce Chemical Co.) reagent. Careful purification is necessary to remove unreacted biotin and organic solvents, base and salts present in the coupling reaction.

Alternatively, biotinylated oligonucleotides may be conveniently prepared on the DNA synthesizer with biotin phosphoramidite reagents available from many sources. Figure 6-13 shows an OPC-purified, biotinylated 25-mer. The biotin phosphoramidite used here contains a trityl group, which allows efficient purification by OPC even when coupling efficiency is low. The corresponding unlabeled 25-mer elutes one minute earlier (data not shown). Some versions of biotin phosphoramidites allow multiple incorporation of biotin throughout the oligonucleotide or at the 3' end. Biotinylated oligonucleotides elute significantly later than their 5' hydroxy counterparts because of the hydrophobic nature and mass addition of the biotin and linker moieties.

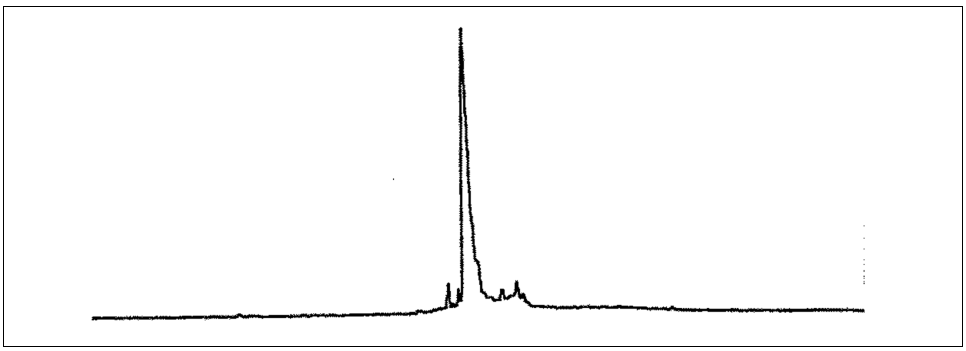

Figure 6-13. *OPC-purified 5' biotin AGG CGA GCA GAA GTG TCC TGC ACT T 3'*

By similar procedures, a variety of active-ester fluorescent dyes are available to construct fluorescent dye-labeled oligonucleotides. A crude 5' 5-carboxy, fluorescein-labeled 18-mer is shown in Figure 6-14. Like biotin, fluorescent dyes retard the electrophoretic velocity of oligonucleotides, while otherwise behaving normally and exhibiting well-formed peaks. The tallest peak in Figure 6-14 is the unlabeled 5' OH 18-mer, which failed to couple with the fluorescent dye compound. The dye-labeled product follows, slightly resolved into two peaks, reflecting a diastereomeric carbon in the linker moiety.

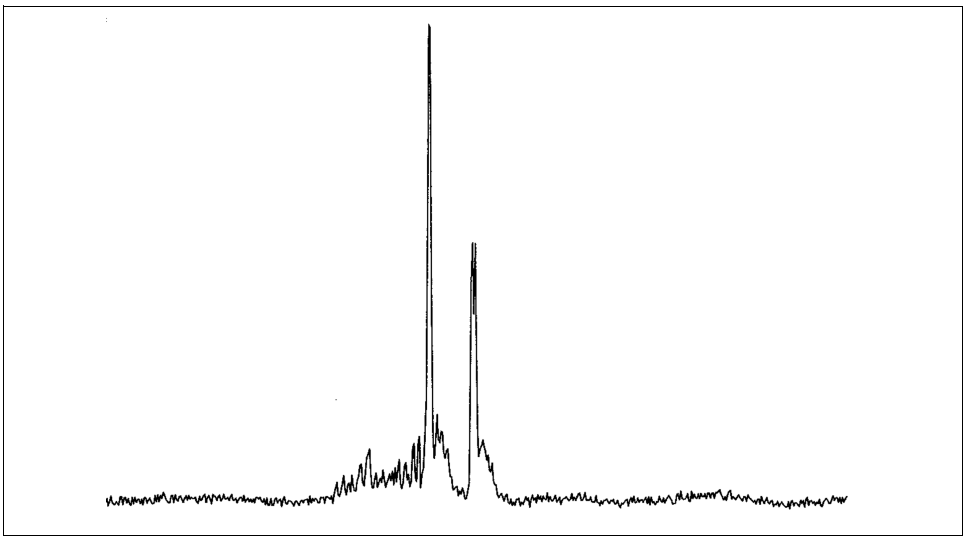

Figure 6-14. *Mixture of 5' OH and 5' (5-FAM-linker) 5' TGT AAA ACG ACG GCC AGT 3'*

RNA

Oligoribonucleotides (RNA) are excellent substrates for gel capillary electrophoresis. Because they are more hydrophilic, they elute slightly earlier than their DNA counterparts. RNase degradation does not seem to be significant when the RNA sample is dissolved in purified filtered water. The considerations and separation parameters that pertain to DNA also pertain to RNA. Because of the lower synthesis efficiency, higher costs and more stringent applications associated with RNA, the high-resolution quantitative analysis that gel

capillary electrophoresis provides is especially important. Figure 6-15 shows an electropherogram of a crude RNA 22-mer.

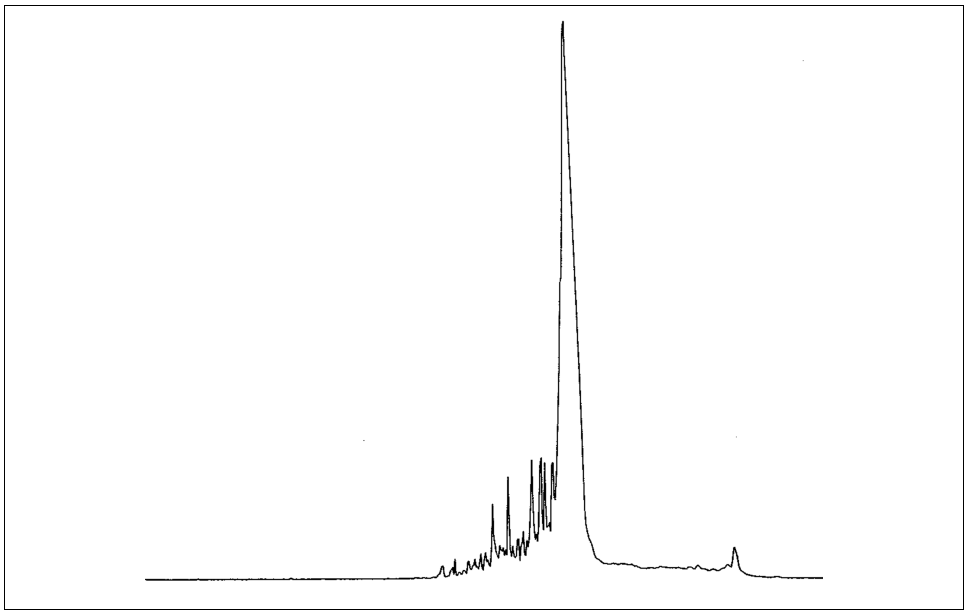

Figure 6-15. *Crude RNA 22-mer: 5′ AUA AUG GUU UGU UUG UCU UCG U 3′*

Phosphorothioate Oligonucleotides

Internucleotide phosphate analogs are being intensively studied for their inhibition of gene expression and resistance to nuclease degradation. These experiments are most frequently conducted with phosphorothioate oligonucleotides (usually referred to as antisense oligonucleotides), where one of the nonbridging oxygen atoms of the internucleotide phosphate has been replaced with a sulfur atom. Synthesis of the phosphorothioate analogs is very efficiently conducted on the DNA synthesizer with the usual phosphoramidite chemistry, using tetraethylthiuram disulfide (TETD; ABI P/N 401147) as a sulfurizing agent instead of the iodine oxidizing reagent.

Phosphorothioate oligonucleotides are stable, easily handled compounds. They are significantly more hydrophobic than their phosphodiester, oxygen-containing counterparts. Also, the sulfurizing reaction is not stereospecific at the chiral phosphorous center, yielding a large number of chemically distinct, diastereomeric products. The net result, upon analysis by any of the common methods, is a slight broadening of the product peak or band.

It should be emphasized again that gel capillary electrophoresis is a very useful technique for assessing the purity of phosphorothioate oligonucleotides, either in their crude or purified state. Oligonucleotides containing both phosphodiester and phosphorothioate linkages may be easily prepared in a single synthesis operation (see DNA User Bulletin 58: *Sulfurization with TETD: Phosphorothioate Oligonucleotide Synthesis via Phosphoramidite Chemistry*, February 1991).

Figures 6-16–6-18 show three electropherograms of a 25–mer sequence, consisting of phosphodiester and phosphorothioate linkages in three arrays. Figure 6-16, the sequence

with a single phosphorothioate linkage, shows the narrowest peaks and the earliest elution time of the product. Figures 6-17–6-18 show the effects of many phosphorothioate linkages: broadened peaks, lower resolution and slightly increased elution times.

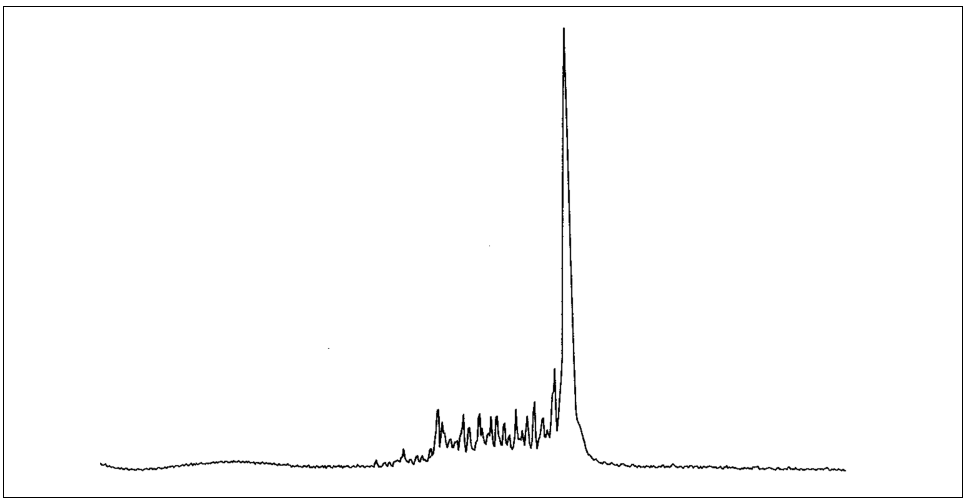

Figure 6-16. *1 P-S, 23 P-O 5′ AGT CAG TCA GTC A_sGT CAG TCA GTC T 3′*

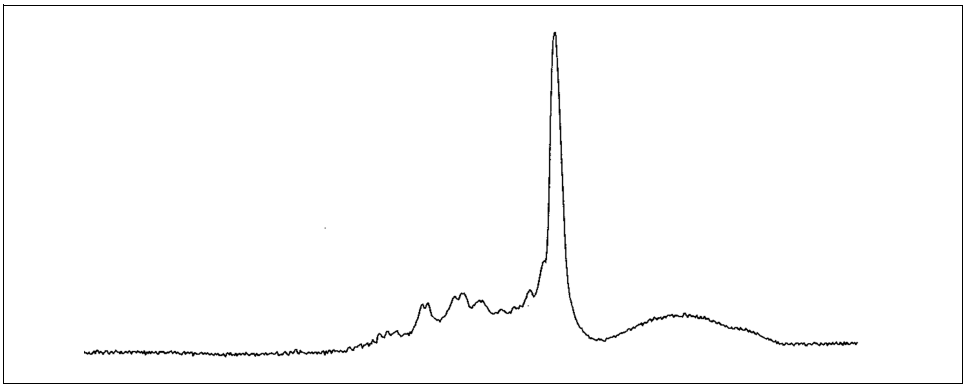

Figure 6-17. *12 P-S, 12 P-O 5′ $A_oG_oT_o$ $C_oA_sG_s$ $T_sC_sA_o$ $G_oT_oC_o$ $A_sG_sT_s$ $C_sA_oG_o$ $T_oC_oA_s$ $G_sT_sC_s$ T 3′*

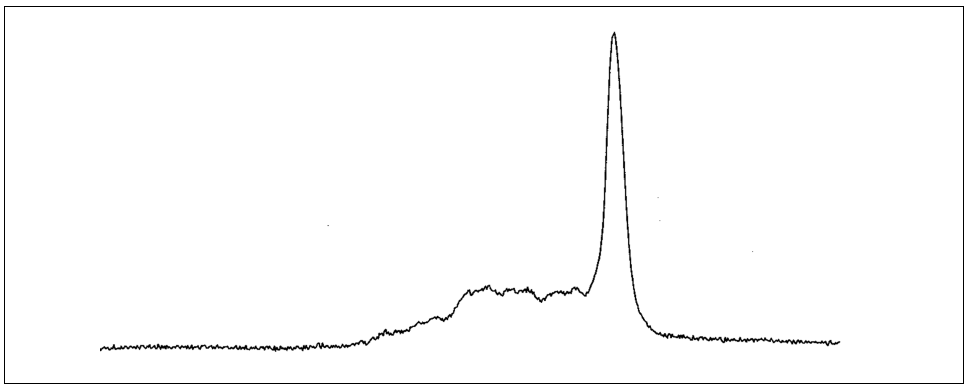

Figure 6-18. *23 P-S, 1 P-O, 5' AGT CAG TCA GTC A_oGT CAG TCA GTC T 3'*

Conclusion

MicroGel CE offers a direct method of evaluating the purity of synthetic oligonucleotides. Under high-voltage conditions, the denaturing gel matrix produces predictable elution patterns for DNA, DNA analogs, RNA and their labeled counterparts. Coupled with an appropriate data system, integrator or chart recorder, MicroGel CE offers the advantages of both PAGE and HPLC. With its high resolution, speed and ease of automation, MicroGel CE is well-suited for oligonucleotide analysis.

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Appendix A

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 A-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.

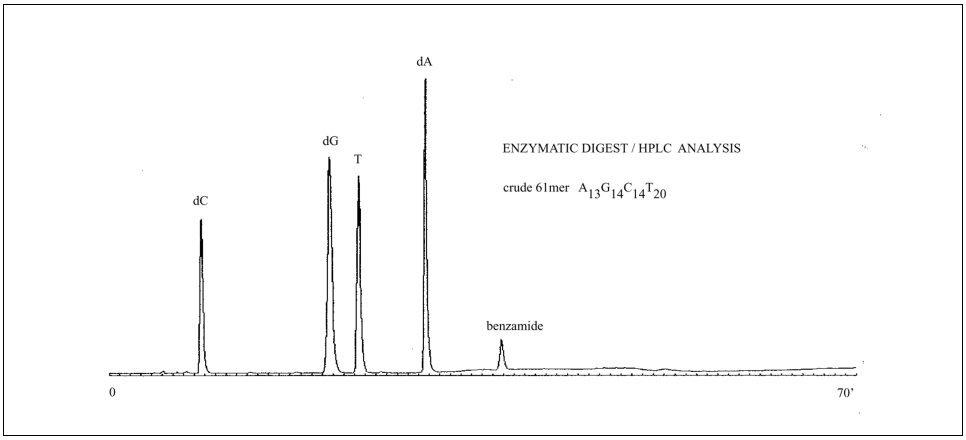

Figure A-1. *Enzymatic Digest/HPLC Analysis*

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 deoyxadenosine 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 A-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 A-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 B

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 by-product 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 C

Ethanol Precipitation of Oligonucleotides

Oligonucleotides can be isolated from impurities such as protecting group by-products, 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 D

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 mmol of oligonucleotide will absorb 10 ODU/base. For example, 0.2 mmol of an 18-mer would be approximately 36 ODU. Figure D-1 shows a spectrophotometric scan in the wavelength range of absorbance by oligonucleotides.

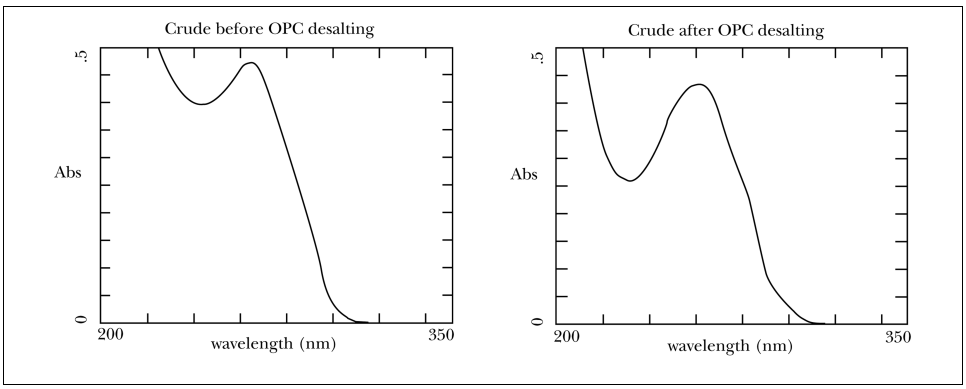

Figure D-1. *200–350-nm scan of a typical oligonucleotide*

Appendix E

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 F

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 F-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 F-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$^{1/couplings}$ $Y_s = Y^{1/n}$

$0.723^{1/16} = 0.98$ (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. • A leak is allowing atmospheric water vapor into plumbing • Poor delivery of phosphoramidites or tetrazole	• Replace acetonitrile with a water content <300 ppm (0.003%). Replace phosphoramidites and/or tetrazole • Check instrument for leaks • 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 • Wet HPLC-grade acetonitrile or water in phosphoramidites and/or tetrazole	• Perform phosphoramidite purge prior to a run when the instrument is idle for more than 6 h • Replace acetonitrile with a water content <300 ppm (9.003%). Replace phosphoramidites and/or tetrazole
Random low trityl color	• Problems in collecting, diluting, or mixing trityls • Simultaneous coupling and capping failure • Incomplete detritylation	• Check fraction collector alignment. Check diluting and mixing procedures • Check the reagent delivery system for leaks or restrictions • 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 • Ammonium hydroxide not sufficiently concentrated	• Check ammonium hydroxide delivery • Store ammonium hydroxide at 4 °C and replace it on the instrument weekly

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