

Methods in Molecular Biology

Volume 4

New Nucleic Acid Techniques

Edited by

John M. Walker

Methods in Molecular Biology

Volume 4

New Nucleic Acid Techniques

Biological Methods

Methods in Molecular Biology

edited by ***John M. Walker***

Volume I: **Proteins, 1984**

Volume II: **Nucleic Acids, 1984**

Volume III: **New Protein Techniques, 1988**

Volume IV: **New Nucleic Acid Techniques, 1988**

Liquid Chromatography in Clinical Analysis

edited by ***Pokar M. Kabra*** and ***Laurence J. Marton, 1981***

Metal Carcinogenesis Testing

Principles and In Vitro Methods

by ***Max Costa, 1980***

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Molecular Biology**

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Nucleic Acid
Techniques**

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John M. Walker

The Hatfield Polytechnic, Hatfield, Hertfordshire, UK

Humana Press • Clifton, New Jersey

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Crescent Manor
PO Box 2148
Clifton, NJ 07015

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Printed in the United States of America.

Library of Congress Cataloging in Publication Data
Main entry under title:

Methods in molecular biology.

(Biological methods)

Includes bibliographies and indexes.

Contents: v. 1. Proteins—v. 2. Nucleic acids—v. 3. New protein techniques—v.

4. New nucleic acid techniques.

1. Molecular biology—Technique. I. Walker, John M., 1948—

II. Series.

QH506.M45 1984

574.8'8'078

84-15696

ISBN 0-89603-062-8 (v. 1)

ISBN 0-89603-064-4 (v. 2)

ISBN 0-89603-126-8 (v. 3)

ISBN 0-89603-127-6 (v. 4)

ISBN 0-89603-150-0 (v. 5)

Preface

In recent years there has been a tremendous increase in our understanding of the functioning of the cell at the molecular level. This has been achieved in the main by the invention and development of new methodology, particularly in that area generally referred to as "genetic engineering."

Although this revolution has been taking place in the field of nucleic acids research, the protein chemist has at the same time developed fresh methodology to keep pace with the requirements of present day molecular biology. Today's molecular biologists can no longer be content with being experts in one particular area alone. They need to be equally competent in the laboratory at handling DNA, RNA, and proteins, moving from one area to another as required by the problem that is being solved. Although many of the new techniques in molecular biology are relatively easy to master, it is often difficult for a researcher to obtain all the relevant information necessary for setting up and successfully applying a new technique. Information is of course available in the research literature, but this often lacks the depth of description that the new user requires. This requirement for in-depth practical details has become apparent by the considerable demand for places on our Molecular Biology Workshops held at Hatfield each summer.

Volume 2 of this series described practical procedures for a range of nucleic acid techniques frequently used by research workers in the field of molecular biology. Because of the limitations on length necessarily inherent in producing any

book, one obviously had to be selective in the choice of titles for Volume 2. The production of Volume 4 therefore allows the development of the theme initiated in Volume 2. This volume contains a further selection of detailed protocols for a range of analytical and preparative nucleic acid techniques and should be seen as a continuation of Volume 2. In particular we have introduced protocols for the rapidly developing area of plant molecular biology. Companion Volumes 1 and 3 provide protocols for protein methodology.

Each method is described by an author who has regularly used the technique in his or her own laboratory. Not all the techniques described necessarily represent the state of the art. They are, however, dependable methods that achieve the desired result.

Each chapter starts with a description of the basic theory behind the method being described. The main aim of this book, however, is to describe the practical steps necessary for carrying out the method successfully. The Methods section, therefore, contains a detailed step-by-step description of a protocol that will result in the successful execution of the method. The Notes section complements the Methods section by indicating any major problems or faults that can occur with the technique, and any possible modifications or alterations.

This book should be particularly useful to those with no previous experience of a technique, and, as such, should appeal to undergraduates (especially project students), postgraduates, and research workers who wish to try a technique for the first time.

John M. Walker

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Chapter 14

Manual Oligonucleotide Synthesis Using the Phosphoramidite Method

H. A. White

1. Introduction

Oligodeoxyribonucleotides may be synthesized on a solid support, which allows for the elongation of the chain without intermediate purifications. The exocyclic amino groups on cytosine and the purines are protected as the alkali-labile benzoyl or *iso*-butyryl amides. The 3'-OH group of the first nucleoside is attached to the solid support by a spacer chain, and the synthesis proceeds by coupling the 5'-OH group of the growing chain to the 3'-phosphorus of the monomer being added, which has its 5'-OH group protected by an acid-labile group. The phosphorus must also be protected to avoid side reactions. When the synthesis is completed, the chain must be cleaved from the support and the base, phosphorus, and terminal 5'-OH-protecting groups removed. The desired oligonucleotide is then separated from the mixture of shorter chains and modified chains (1).

There are two general chemistries in use for oligonucleotide synthesis: the phosphotriester (*see* Chapter 13) and the phosphite triester. The phosphoramidite methods are versions of the phosphite triester chemistry (2). Both systems are in use for manual and automated synthesis. Many automated systems use β -cyanoethyl phosphoramidites, which simplify the deprotection of the phosphates and are very convenient for manual synthesis (3).

A synthesis starts with an appropriately base-protected [N^6 -benzoyl adenine, N^2 -isobutyryl guanine, N^4 -benzoyl cytosine (thymine is not usually protected)] nucleoside that also has its 5'-OH group protected with the dimethoxytrityl group and is coupled through its 3'-OH to controlled-pore glass support via a long-chain alkylamine arm and succinyl group (*see* Figs. 1 and 2). The elongation cycle consists of the following steps.

1. Removal of the 5'-dimethoxytrityl group with dichloroacetic acid in dichloroethane.
2. Coupling of the next monomer by adding the appropriate nucleoside 3'-O-phosphoramidite with the activating agent tetrazole.
3. Oxidation of the phosphite to phosphate with iodine/water.
4. Acetylation of any unreacted 5'-OH groups ("capping") with acetic anhydride and dimethylaminopyridine as a catalyst. The elongation cycle is repeated until the oligonucleotide is completed (Fig. 3). The efficiency of the coupling cycle is critical to the success of a synthesis. If the efficiency of the coupling cycle is 95%, the overall yield after 15 couplings is 46%, and 21% after 30 couplings.

Treatment with aqueous ammonia effects removal of the oligonucleotide chain from the support, deprotection of the bases, and removal of the β -cyanoethyl groups from the phosphates. The product may be purified either by high-performance liquid chromatography or by preparative polyacrylamide gel electrophoresis.

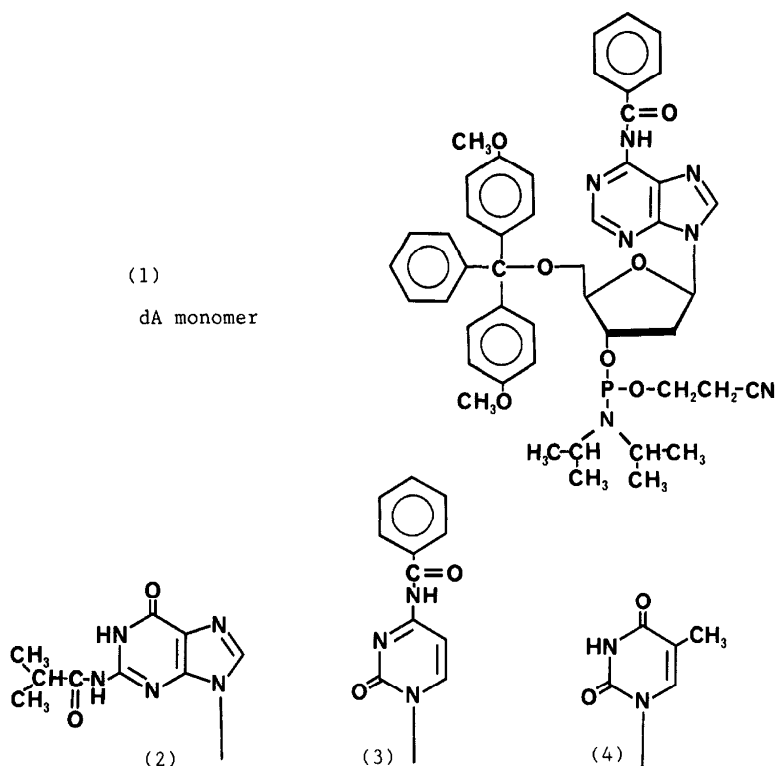


Fig. 1. (1) dA monomer [5'-O-(4,4'-dimethoxytriphenylmethyl)- N^6 -benzoyl-2'-deoxyadenosine-3'-O-(2-cyanoethyl)-di-isopropyl-amido-phosphite]. (2) Protected base, dG monomer (N^2 -isobutyryl). (3) Protected base, dC monomer (N^4 -benzoyl-). (4) Thymidine. Not usually protected.

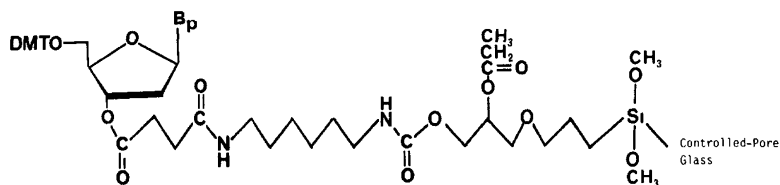


Fig. 2. Protected nucleoside linked to controlled-pore glass support with a long-chain alkylamine spacer and succinyl link.

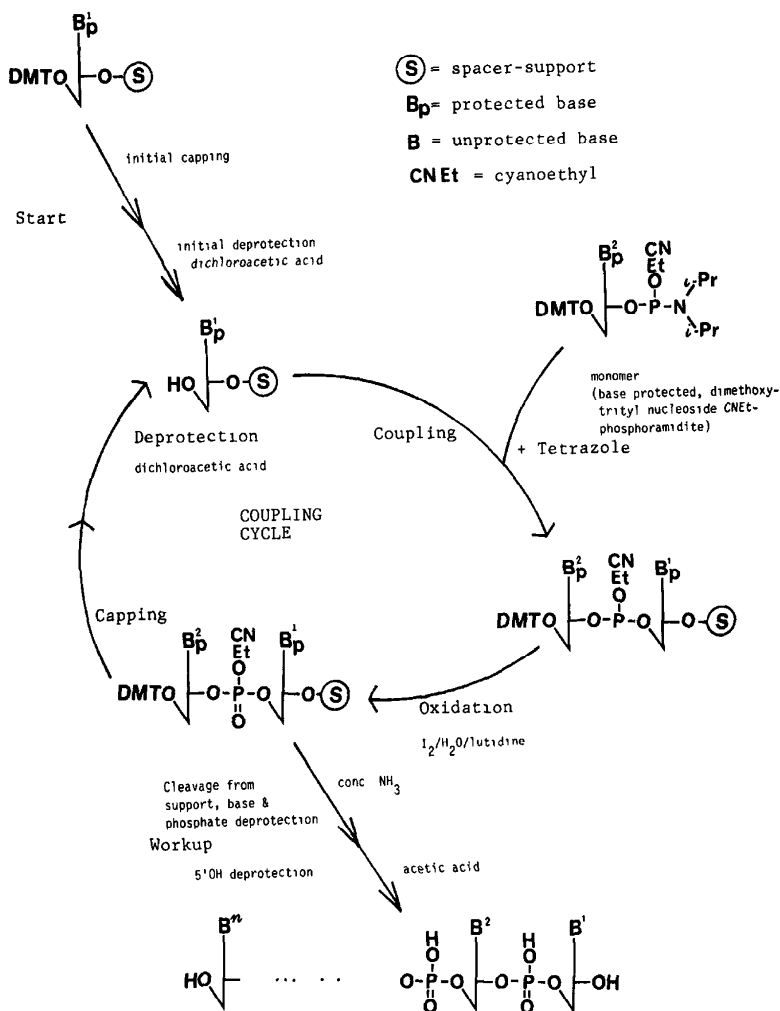


Fig. 3. Steps in the synthesis.

There are many versions of the phosphoramidite protocol. The one described below is fairly typical and is suitable for manual synthesis of oligonucleotides up to 30 or so nucleotides long on a scale of 1–5 μmol (starting). Many workers carry out the synthesis in a sintered funnel, but the flow apparatus

described below is satisfactory and requires less room and fewer manipulations.

2. Materials

2.1. Reagents

All reagents must be of the highest quality available since solid-phase methods may "concentrate" the effects of trace impurities. Many protocols for the preparation and purification of the solvents, protected deoxyribonucleoside-3'-O-cyanoethyl phosphoramidites, and other reagents are in the literature (for example, *see* ref. 4), but most workers will find it more convenient to purchase these materials. They are available from, among others, BDH Chemicals, Ltd. (Broom Road, Poole, Dorset, BH12 4NN, England) and Cruachem, Ltd. (11 Napier Square, Livingston, EH54 5DG, Scotland).

The deoxynucleoside phosphoramidites and the derivatized supports must be stored desiccated and are usually stored at -20°C , although they are stable at room temperature. It is critical that they be kept dry and not exposed to acid vapors. They are available in septum-sealed bottles.

1. Tetrazole must be of the highest purity (sublimed) and stored in sealed amber bottles. It should be microcrystalline in appearance and should not leave a residue when dissolved in acetonitrile (0.5 mol/L).
2. Dichloroacetic acid should be of high purity and kept anhydrous.
3. Aqueous ammonia ($\approx 35\%$ w/w) should be BDH "Aristar" grade or equivalent and must be kept tightly sealed at $0-5^{\circ}\text{C}$ to avoid loss of ammonia.
4. Argon may be standard "industrial" grade. This is normally oxygen-free and perfectly dry. As a precaution, most workers pass it through a drying tube of silicagel (previously dried *in vacuo* over P_2O_5).

2.2. Solvents

1. Acetonitrile must be of "HPLC" grade or equivalent and must be *anhydrous* for the coupling reaction. Since anhydrous acetonitrile is time-consuming to prepare and costly to purchase, it is economical to use analytical reagent or HPLC grade acetonitrile when possible during the synthesis. Anhydrous acetonitrile can be prepared by distillation of HPLC-grade acetonitrile from calcium hydride (2) and should be stored in septum-capped bottles under dry argon. Anhydrous acetonitrile in septum-sealed bottles is available from Cruachem. Note that acetonitrile is highly flammable and is toxic by ingestion, skin absorption, and inhalation.
2. 1,2-Dichloroethane should be rendered anhydrous by first passing it through a column of basic alumina, followed by distillation from phosphorus pentoxide. It should be stored sealed under dry argon. Note that dichloroethane is highly flammable and toxic by inhalation and may cause skin irritation. A satisfactory grade is available from Cruachem.
3. Tetrahydrofuran and 2,6-lutidine must be anhydrous for use in the capping step. These may be purchased (Cruachem) or prepared by distillation from calcium hydride. Store both in amber bottles under dry argon. Both solvents are toxic. Tetrahydrofuran is highly flammable and may form explosive peroxides.
4. Other reagents (acetic anhydride, acetic acid, dimethylaminopyridine) should be of analytical reagent-grade quality.

2.3. Solutions

2.3.1. Coupling Mixture

1. Monomer: 0.2 mol/L of the 5'-dimethoxytrityl deoxyribonucleoside 3'-O-(*N,N*-diisopropylamino)-cyanoethyl

Table 1
Monomer Molecular Weights and Acetonitrile Volumes

Monomer, base-protected space nucleoside cyano- ethyl phosphoramidites	Molecular weight	Acetonitrile needed to make 0.2 mol/L solution, mL/g
T Dimethoxytrityl-T-	744.83	6.7
C Dimethoxytrityl-BzdC-	833.92	6.0
A Dimethoxytrityl-BzdA-	857.95	5.8
G Dimethoxytrityl-iBudG-	839.93	6.0

phosphoramidites in anhydrous acetonitrile. Table 1 gives the molecular weights of the four monomers and the volumes of acetonitrile required to prepare the 0.2 mol/L solutions (5). If kept anhydrous (stored under dry argon), these monomer solutions are stable for several days.

2. Tetrazole: 0.5 mol/L tetrazole (mol wt, 70.06) in anhydrous acetonitrile. This solution should be kept under dry argon and is stable for several days.

The two solutions listed above are mixed immediately before use. Note: the solutions described in Table 1 may be purchased from Cruachem, Ltd. and are stable for several months if stored in amber glass bottles (except for the iodine solution). It is good practice to replace these solutions after they have been in use 3–4 wk as a precaution.

2.3.2. Oxidation

Iodine: 0.1 mol/L iodine (sublimed) in 4:2:1 tetrahydrofuran/2,6-lutidine/water (filter after preparation). The iodine

solution may throw a precipitate on standing, so should be freshly prepared each day. It is therefore convenient to store the iodine solution in two parts: iodine in tetrahydrofuran and lutidine/water. These are mixed before use. Different workers use slightly different iodine mixtures; there seems to be little difference among them.

2.3.3. Capping

1. 6.5% (w/v) 4,*N,N*-dimethylaminopyridine in anhydrous tetrahydrofuran.
2. Acetic anhydride/2,6-lutidine/tetrahydrofuran (1:1:8)

These solutions are mixed immediately before use.

2.3.4. Deprotection

1. 3% (v/v) Dichloroacetic acid in 1,2-dichloroethane.

2.4. Apparatus

2.4.1. Flow Apparatus

The apparatus consists of a small chromatography column (6.5 mm bore; 50 mm long) fitted with a septum injector that can be connected to up to six reagent bottles (five are used in this method) via a six-position rotary selector valve. Figure 4 shows the layout of the flow apparatus. The support rests on a PTFE fritted disk at the bottom of the column. The column height can be varied to allow for different amounts of support. This apparatus may be obtained from Omnifit Ltd. (51 Norfolk Street, Cambridge, CB1 2LE, England) (catalog no. 3600 DNA Synthesis Kit) or a similar type of apparatus from Cruachem.

Dry argon is used to pressurize the reagent reservoirs via a pressure regulator and an eight-way distribution manifold (unused positions are blocked off or may be used to repressurize septum-sealed solvent bottles). The selector valve allows each reservoir to be connected in turn to the synthesis column.

Addition of two-way valves between the rotary valve and the column allows for use of two or more columns for multiple synthesis (*see* Chapter 13). Solvents and reagents leave the column via a length of narrow-bore PTFE tubing (which provides a reasonable pressure drop) and a cutoff valve ("exit valve").

The method described here requires five solvent bottles. If the Omnifit 3600 DNA Synthesis Kit is used, an additional 250-mL solvent bottle with a three-valve cap will be needed, as well as an additional Omnifit HP tube end fitting (2100), a 2310 gripper fitting, and a 2320 sealing plug. It is wise to stock up on PTFE tubing and Omnifit connector spares since inevitably some will be lost or damaged in the assembly. We have found that it is better to avoid the O-ring type fittings. Those supplied with apparatus should be replaced with PTFE cone-type fittings or with the flangeless high-pressure fittings.

The apparatus should be assembled as described in the instructions and shown in Fig. 4. The argon plumbing should be thoroughly flushed with argon before the solvent bottles are connected. It is convenient to connect (and label) the rotary valve with "STOP" at the twelve o'clock position (using an Omnifit 2320 sealing plug) and the solvents/reagents connected in the following order (clockwise): *reagent grade* acetonitrile; iodine/lutidine/water; dichloroacetic acid; dichloroethane; *anhydrous* acetonitrile. The solvent bottles must be thoroughly cleaned and dried before filling. Reagent grade acetonitrile, anhydrous acetonitrile, and dichloroethane should be placed in the liter bottles and the 250-mL bottles used for the dichloroacetic acid and the oxidizing mixture. If the humidity is high, it is wise to fill the anhydrous acetonitrile and dichloroethane bottles under a blanket of dry argon.

It is convenient to connect a septum-sealed bottle of anhydrous acetonitrile to the argon distribution manifold using a short syringe needle. This bottle is used as a source of acetonitrile for making up the tetrazole and monomer solutions. The risk of moist air entering the bottle is minimized if it is always pressurized with argon.

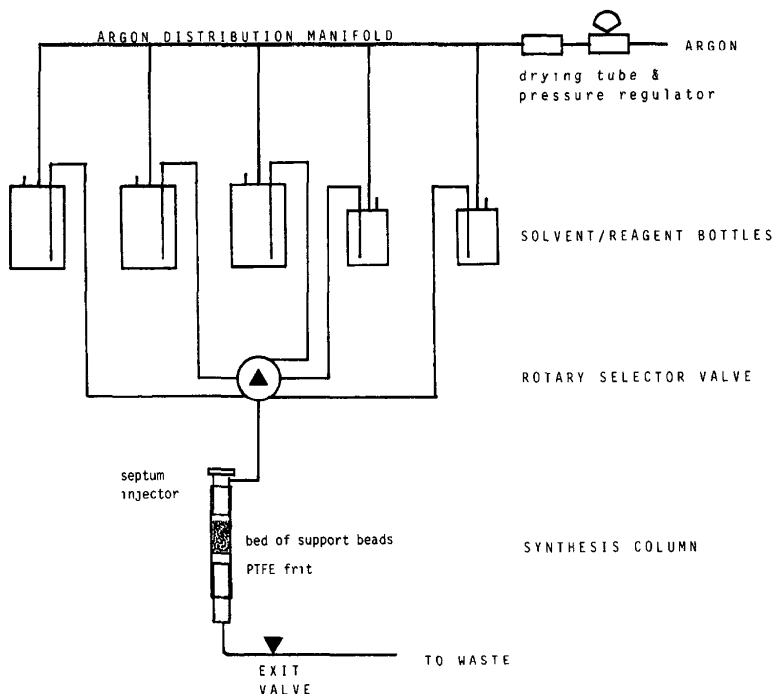


Fig. 4. Flow apparatus.

2.4.2. Syringes and Needles

Syringes with PTFE-tipped plungers—such as the Hamilton "1000 series" "gastight" syringes—are essential. Minimum requirements are for three 500- μ L, one 1-mL, one 2.5-mL, and one 5-mL syringes.

All of the syringes should be equipped with needles designed for *septum penetration*. It is preferable to use needles with a *side hole* (such as the Hamilton style 5 point), since these minimize damage to the apparatus septum.

Syringes should be stored filled with anhydrous acetonitrile during a synthesis when not actually in use.

2.4.3. Mixing Vials

Vials with conical interiors and PTFE-lined septum caps are used for mixing the monomer with tetrazole for each coupling and for preparing the capping reagent mixture. Vials such as the Pierce "Reacti-Vials" are ideal (Pierce and Warriner Ltd., 44 Upper Northgate Street, Chester, CH1 4EF, England). The 0.3- or 1-mL size serves for preparing the monomer/tetrazole mixture, and the 3- or 5-mL size serves for the capping mixture. It is important that the vials be scrupulously cleaned and dried. They should be stored in a desiccator over silica gel that has previously been dried *in vacuo* over phosphorus pentoxide.

2.4.4. Deprotection Flask

Removal of the base-protecting groups, as well as cleavage from the support and deprotection of the phosphates, is accomplished by heating with concentrated ammonia solution. Considerable pressure is developed when the vessel is heated. We have found it best to use a heavy-walled 50-mL flask with a ground-glass stopper. Stopper and flask each have four hooks so the stopper can be wired in place.

3. Method

3.1. General Approach

The key to a successful synthesis lies in organization and attention to detail. The synthesis steps are:

1. Preparation of the support and apparatus.
2. Initial capping.

3.1.1. Coupling Cycle

1. Removal of the 5'-OH group ("deprotection") with dichloroacetic acid solution.

2. Washing.
3. Addition of the next nucleotide ("coupling") by injection of the monomer/tetrazole mixture.
4. Washing.
5. Oxidation of the phosphityl group.
6. Washing.
7. Capping of unreacted 5'-OH groups with acetic anhydride and catalyst mixture.
8. Washing.
9. Repeat steps 1–8.
10. Workup: deprotection of phosphates, bases, cleavage from support, and removal of 5' terminal dimethoxytrityl group.

We have found that a fairly simple recording system is effective in ensuring that the synthesis is carried out without omission or duplication of steps. The sequence is written on a blackboard. We then cross each nucleotide out *twice*—once when the monomer has been injected and again when we have noted the release of the orange-colored dimethoxytrityl cation.

The method is described for a 1.5- μ mol synthesis (i.e., start with 1.5 μ mol of the 3' immobilized nucleoside). Preparation of an oligonucleotide in the 15–30 nucleotides range on this scale should afford between 50 μ g and 2 mg of product depending upon the chain length and sequence. This will be in the range of 1–50 A_{260} units (an A_{260} unit is that amount of an oligonucleotide that gives an absorbance of 1.0 at 260 nm when dissolved in water and read in a 1-cm cuvet).

3.2. Preparation for Synthesis

1. Weigh out the appropriately derivatized controlled-pore glass support to give 1.5 μ mol of the 3' end nucleoside. For controlled-pore glass beads with a long chain alkylamine/succinyl-linked nucleoside, typical loadings are in the range of 20–50 μ mol/g. With a loading of 30 μ mol/g, 50 mg would be required for a 1.5- μ mol synthesis.

2. With the exit valve off, carefully moisten the PTFE fritted disk with reagent-grade acetonitrile (avoiding wetting the column walls).
3. Carefully pour in the support and add about 0.5 mL of acetonitrile with a Pasteur pipet. Stir the support briefly to remove air bubbles.
4. Adjust the position of the column base and PTFE fritted disk so that there will be a space of about 1–2 mm between the top of the support bed and the septum injector.
5. Next gently fill the column with acetonitrile to the top. Remove the septum cap from the flow injector and *gently* push it into the top of the column. (Take care that the displaced acetonitrile does not spurt!) When the septum injector is clamped home, replace the septum cap.
6. Open the exit valve and turn on the reagent-grade acetonitrile.
7. The general procedure for selecting a reagent or solvent is to rotate the rotary valve to the correct position, then open the argon supply valve to the bottle (each bottle has three valves: fluid, argon, and vent) and finally open the bottle fluid valve. At the end of the period the rotary selector valve should be turned first: the bottle fluid and argon valves are then turned off. We have found it best to turn off the argon to each bottle when it is not in use. The argon flow rate is low, so it is remotely possible for some vapor phase mixing to occur between bottles when fluid is withdrawn from one if this precaution is not taken.
8. Adjust the argon pressure until the flow rate through the system is 1 mL/min with acetonitrile (the flow rate with dichloroethane will be higher). The pressure should be about 6 psi.
9. Allow reagent grade acetonitrile to flow for a minimum of 1 min and then switch to anhydrous acetonitrile and wash 2 min.
10. Carry out the Capping step described below. This blocks any free amino or hydroxyl groups on the support. Wash

the support 2 min with reagent grade acetonitrile and 2 min with dichloroethane.

3.3. *Performing the Synthesis*

3.3.1. *Injection Procedure*

All reagent injections should be made as follows:

1. With the exit valve closed and the rotary valve on STOP, loosen the septum cap by one-half to one turn.
2. Insert the syringe needle fully and then tighten the cap (it should not be overtightened).
3. Open the exit valve and slowly inject the syringe contents over 15–30 s.
4. Close the exit valve, loosen the cap, withdraw the syringe, and retighten the cap.

3.3.2. *Coupling Cycle*

3.3.2.1. Deprotection

1. Turn on the dichloroacetic acid and allow it to flow. Insert the waste tube into a collection vessel containing about 1 mL of 10% (w/v) trichloroacetic acid. Observe the bed closely. When the orange color at the top of the bed begins to diminish, switch to dichloroethane.
2. It is important to keep the exposure to the acid to a minimum. Purines and pyrimidines differ slightly in rate of deprotection, so this step is best controlled visually. The time will be on the order of 30 s.
3. Continue collection of the effluent until the bed is free of color. The samples should be diluted with 10% trichloroacetic acid in dichloroethane. Read at 417 nm ($\epsilon = 224,000$) or 504 nm ($\epsilon = 507,000$) (6).
4. Wash for 1 min with dichloroethane and for 2 min with anhydrous acetonitrile. The synthesis may be interrupted at this point. If the interruption is longer than a few

minutes, wash with anhydrous acetonitrile again before the coupling step.

3.3.2.2. Coupling

1. Inject 0.18 mL of 0.5 mol/L tetrazole in acetonitrile into a dry, argon-filled, septum-sealed vial (a 0.3- or 1.0-mL "Reacti-vial").
2. Inject 0.15 mL of the appropriate phosphoramidite solution (0.2 mol/L in acetonitrile) into the tetrazole solution and mix. It is essential to minimize exposure to water vapor and oxygen at this stage.
3. Rapidly withdraw the vial contents into the phosphoramidite syringe and inject it into the column. Close the exit valve and allow a total of 3 min for the coupling reaction. Rinse the syringe thoroughly with acetonitrile.
4. Wash with reagent grade acetonitrile for 2 min.

3.3.2.3. Oxidation

1. Allow the iodine/tetrahydrofuran/lutidine mixture to flow for 1 min.
2. Wash with reagent grade acetonitrile for 2 min and with anhydrous acetonitrile for a further 2 min. Check that no iodine color remains. If it does, prolong the wash.

3.3.2.4. Capping

1. Add 0.25 mL of acetic anhydride/lutidine to a septum-sealed vial and inject 0.75 mL dimethylaminopyridine solution into it and mix. Withdraw the contents and inject into the column. Allow 2 min reaction time.
2. Wash for 2 min with anhydrous acetonitrile and 2 min with dichloroethane.
3. This cycle is repeated until the chain is completed. The capping step may be omitted after the last oxidation. Allow a final 2-min wash with dichloroethane.

3.3.3. Workup of the Oligonucleotide

1. Extrude the support (washing with dichloroethane using a Pasteur pipet) into a small glass sinter-based funnel (a funnel 20 x 20 mm with a 6-mm diameter fine glass sinter base works well). Draw the solvent off and wash three times with small volumes of diethyl ether. Draw air through the support until it is dry.
2. Transfer the dry support to a 50-mL round-bottom flask and add 5 mL of concentrated aqueous ammonia. Grease (using a nonsilicone grease) the upper part of the ground-glass stopper and seal the flask. Use rubber bands to hold the stopper tightly in place (use a length of light wire—those sold for sealing plastic bags for use in domestic freezers are ideal—as a precaution).
3. Heat the flask to 50°C for 16 h.
4. Cool the flask and open cautiously. Allow the ammonia to vent off for about 30 min assisted by a gentle stream of air.
5. Filter the contents into a 100-mL round-bottom flask using a small glass sinter funnel (as above), and wash the reaction flask and the support with 3 x 2 mL water. Rotary evaporate to dryness using a bath set at 35–40°C. Some syntheses give products that foam badly during rotary evaporation. In such cases, transferring to a 250-mL flask helps.
6. Add 5 mL of acetic acid-water (80 mL of glacial acetic acid + 20 mL of water) to the flask to remove the terminal dimethoxytrityl group (color will not be visible). Allow to stand for 30 min at room temperature.
7. Add 5 mL of water and 10 mL of diethyl ether and shake vigorously. Remove the ether (upper) layer with a Pasteur pipet, and repeat the extraction once.
8. Rotary evaporate to dryness. Add 5 mL of water and again evaporate to dryness. Repeat this procedure until there is no smell of acetic acid. Two to three evaporations should be sufficient.

9. The oligonucleotide is now ready for purification and should be taken up in an appropriate volume of water (usually 1–2 mL) and is best stored at -20°C .

3.3.4. Purification

For many applications, preparative polyacrylamide gel electrophoresis is the method of choice for convenient purification (*see* Chapters 13 and 15). If kinase labeling is used for analytical purposes (7,8), it is best to freeze dry the product mixture two or three times. This removes volatile materials that may inhibit the kinase.

For greater chemical purity, HPLC is employed. As a first purification step, an anion-exchange method is best. This separates mainly on the basis of chain length—the product peak is the last to be eluted with a salt gradient. Reverse-phase methods are preferred as a second purification (9). Reverse-phase methods are described in Chapter 13.

3.4. Ion-Exchange HPLC Protocol

Any HPLC machine capable of gradient programming and monitoring at 260 nm will serve. An analytical-size column is adequate to purify the small amounts of oligonucleotide needed for many applications (1–10 A_{260} units).

1. Equilibrate a Whatman "Partisil 10 SAX" column (5 mm $\times$ 25 cm) at 1.5 mL/min with 0.003 mol/L KH_2PO_4 , pH 6.3, in 6:4 formamide:water (adjust the pH before addition of the formamide—reagent-grade formamide is satisfactory, but the potassium dihydrogen phosphate must be of very high purity, such as BDH "Aristar" grade).
2. Inject up to 100 μL of the crude product, and run a linear gradient between 0.003 and 0.21 mol/L KH_2PO_4 .
3. The elution profile should show a pattern of small peaks that precede the large product peak. A typical elution requires 30–40 min. One or two runs suffice to collect sufficient product.

4. The formamide is removed on a gel-filtration column (Biogel P2 or Sephadex G15) run with 20% (v/v) ethanol (redistilled or BDH "Aristar" grade) in water. The ethanol/water is removed by rotary evaporation.

4. Notes

1. The density of argon is its chief advantage over nitrogen. Oxygen-free nitrogen will serve instead.
2. The volume of monomer and tetrazole solutions lost through the syringe dead volume is significant. To compensate for this, it is necessary to prepare an extra 30 μmol of phosphoramidite for every two to four withdrawals to be taken from a bottle.
3. Controlled-pore glass is the support of choice for most syntheses. Other types of silica support (such as "Frac-tosil" and "Vydac") are used in some methods.
4. Depurination, particularly of the benzoyl-protected adenine, occurs during the acid-deprotection step. It is therefore important to keep the exposure to the acid to a minimum. Purine (particularly adenine)-rich sequences are more difficult to synthesize successfully for this reason—especially if the 3' end is purine-rich. The more acid-labile "pixyl" (9-phenylxanthen-9-yl) group is sometimes employed in place of the dimethoxytrityl group to reduce the exposure to acid conditions. Protecting groups for adenine that do not lead to depurination are being developed.
5. The orange color of the dimethoxytrityl cation is sometimes somewhat fugitive. Detergent or solvent residues in the collection vessel may cause loss of color. A synthesis should not be abandoned on the basis of a single low measurement of the dimethoxytrityl release. If, however, color release from the *support bed* is not observed, a further treatment with dichloroacetic acid is worth a try, but the synthesis may have failed. Low yields toward the end of a synthesis may often be acceptable since very small amounts of product are usually sufficient.

6. The terminal dimethoxytrityl group may be left on the oligonucleotide if reverse-phase HPLC is to be used. This very apolar group will shift the product peak away from the others. It is removed after collection of the peak, and the oligonucleotide is rechromatographed. Other workers favor removal of the final dimethoxytrityl before the ammonia treatment.
7. "Mixed chains" are often required if an amino acid sequence is being used to generate an oligonucleotide probe. In such a case some positions in the oligonucleotide sequence may be two, three, or four different bases. Rather than make each chain separately, it is usual to use the appropriate monomer mixture (equimolar) at the coupling step for these positions. This is often effective if a family of eight or 16 chains is being made. The risk increases as the number of chains increases. There is no certainty that each monomer is coupling with equal frequency, so some sequences may be present in vanishingly small yields. Those chains rich in adenine may be lost through depurination. It is best to make the members of a group eight at a time rather than all at once.
8. Only two problems with the flow apparatus are likely to appear:

If a large bubble forms at the top of the support bed, it will impede reagent access. The bubble can be removed as follows: on an acetonitrile wash step set the exit valve to OFF and rotary valve to STOP. Fill a 500- μ L syringe about half full of acetonitrile and inject slowly to compress the bubble. Then slowly withdraw the syringe plunger; the bubble should enter the syringe. Before withdrawing the syringe, set the rotary valve to acetonitrile. Open the exit valve after removing the syringe, and repeat if required.

Fragments of septum may block the column and cause the flow to slow or stop. The septum injector must then be removed (exit valve closed) and a needle or fine wire used to remove the offending fragment. This usually intro-

duces an air bubble, which should be removed as above. An extra anhydrous acetonitrile wash should be used to remove any water that may have entered. It is wise to replace the septum every 10 or so nucleotides to avoid this problem.

9. Two common reasons for utter failure of a synthesis are amines in the solvents and entry of water. If yields diminish greatly with each coupling, water entry is the likeliest problem. The dead volume in the septum injector may trap some iodine solution and allow water to bleed during the coupling. The acetonitrile washes and the acetic anhydride in the capping step usually prevent this problem. Should it occur, the washes may be extended and the capping mixture injection begun before the syringe is fully inserted so the acetic anhydride washes the dead space. When water entry is not the source of low yields, the solvents should be replaced or repurified.

Acknowledgment

Cruachem Ltd. kindly provided samples of their cyanoethyl phosphoramidites for testing this protocol.

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