5′-Iodination of Solid-Phase-Linked Oligodeoxyribonucleotides

This unit describes a method for converting the 5′-hydroxyl of CPG-bound oligodeoxyribonucleotides to an iodo group. This procedure is useful when an electrophilic group is desired in the terminal 5′-position of an oligodeoxyribonucleotide. Because the iodo group is an efficient leaving group in S_N2 displacement reactions, its introduction at the 5′-position allows for facile ligation to small molecules or macromolecules containing a strongly nucleophilic group. Additionally, 5′-iodo oligonucleotides can easily be converted to a wide variety of other functional groups.

Two protocols are described in this unit. For both procedures, an oligonucleotide is prepared on an automated DNA synthesizer (see *APPENDIX 3C*) and is then iodinated in-column (i.e., while still protected and immobilized on the CPG resin in the column). The first method (see Basic Protocol) describes a manual procedure performed by adding reagents to the column after it is removed from the synthesizer. The second method (see Alternate Protocol) provides an automated method performed by programming the synthesizer to perform the same iodination reaction. The manual procedure is suitable for most applications, while the automated method may be preferable for carrying out multiple iodination reactions.

CAUTION: All chemicals must be used in a fume hood by qualified individuals equipped with laboratory coats, safety glasses, and gloves.

MANUAL PROCEDURE FOR 5′-IODINATION OF OLIGODEOXYRIBONUCLEOTIDES ON A SOLID SUPPORT

BASIC PROTOCOL

This protocol outlines a method to convert the 5′-hydroxyl on protected CPG-bound oligodeoxyribonucleotides to an iodo group (Fig. 4.19.1). In this procedure, an iodination solution is passed between two syringes fitted to the ends of a DNA synthesis column containing the attached oligodeoxyribonucleotide, converting the 5′-hydroxyl to an iodo group. The 5′-iodo-modified oligodeoxyribonucleotide can subsequently be cleaved and deprotected using 28% aqueous NH_4OH.

Materials

Anhydrous *N*,*N*-dimethylformamide (DMF)
Iodination solution (see recipe)
Methylene chloride (CH_2Cl_2)
28% (v/v) ammonium hydroxide (NH_4OH)

DNA synthesizer (e.g., ABI; see *APPENDIX 3C*)
0.2 to 10 µmol DNA synthesis column with long-chain alkylamine controlled-pore glass (CPG) support
1- and 10-mL syringes
Shaker, rocker, or other agitating device
C18 reversed-phase HPLC column (*UNIT 10.5*)
Speedvac evaporator

Additional reagents and equipment for oligonucleotide synthesis (Chapters 3 and 4 and *APPENDIX 3C*) and reversed-phase HPLC (*UNIT 10.5*)

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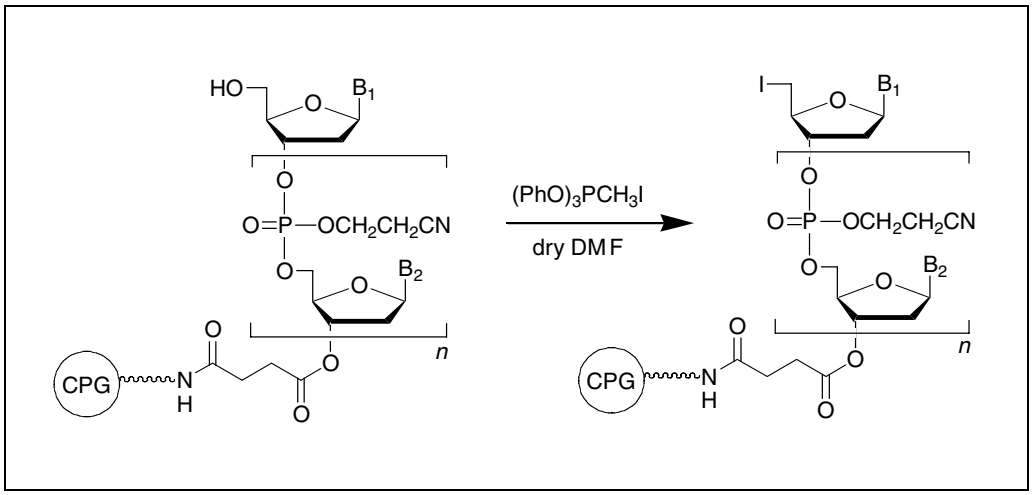

Figure 4.19.1 Iodination of solid-phase-linked oligodeoxyribonucleotides. Abbreviations: B_1 and B_2, thymine and/or *N*-protected nucleobases; CPG, long-chain alkylamine controlled-pore glass (resin).

Synthesize oligonucleotide and obtain control sample

1. Using a DNA synthesizer, synthesize 0.2 to 10 µmol of CPG-bound protected oligodeoxyribonucleotide with the 5′-*O*-(4,4′-dimethoxytrityl) group removed (DMTr-OFF; see *APPENDIX 3C*).

2. Open DNA synthesis column, remove and set aside ~1 mg of resin, then close column.

   This sample of the resin will be cleaved and deprotected without undergoing the iodination reaction and thus can be used as an HPLC reference to determine the retention time of the starting material and estimate the retention time of the 5′-iodinated product.

Dehydrate resin

3. Attach an empty 1.0-mL syringe to one end of the DNA synthesis column. To the other end, attach a 1.0-mL syringe filled with anhydrous DMF.

4. Slowly push DMF through column to the other syringe.

   This step should remove any water that may be adsorbed to the resin that would otherwise react with the water-sensitive iodination reagent.

Iodinate oligodeoxyribonucleotide

5. Remove the DMF-filled syringe and quickly replace with a 1.0-mL syringe filled with iodination solution.

6. Push iodination solution back and forth between the two syringes several times.

7. Seal the junctions between the column and the syringes with Parafilm to prevent leakage.

8. Put on shaker, rocker, or other agitating device for 15 to 20 min at room temperature.

   If the 5′-end of the oligonucleotide contains a bulky group, it may be desirable to increase the reaction time (see Critical Parameters and Troubleshooting for details).

9. Unwrap the column and use the syringes to remove the iodination solution from the column.

10. Using a 10-mL syringe, push 5 to 10 mL DMF through the column, followed by a few milliliters of CH_2Cl_2. Blow-dry beads using air.

    The CH_2Cl_2 is not mandatory, but it facilitates drying of the CPG beads, making it easier to remove them from the column.

Cleave and deprotect iodinated deoxyribonucleotide

11. Open column and transfer beads to a sealed vial for deprotection.
12. Cleave and deprotect the iodinated product and the sample of starting material (from step 2) by soaking each resin in 28% NH_4OH (~1 mL for 1-µmol column) at room temperature for 18 to 24 hr.
13. Remove ammoniacal solution, rinse beads twice with small portions (e.g., 0.25 mL for 1-µmol column) of deionized water, combine portions, and evaporate liquids in a Speedvac evaporator.
14. Analyze purity by HPLC using a C18 reversed-phase column (*UNIT 10.5*).

AUTOMATED PROCEDURE FOR 5′-IODINATION OF OLIGODEOXYRIBONUCLEOTIDES ON A SOLID SUPPORT

ALTERNATE PROTOCOL

This protocol involves the use of a DNA synthesizer to perform the same iodination reaction described in the Basic Protocol. The advantage of this automated procedure is that the iodination reaction can be conveniently run on the DNA synthesizer as a 15-min procedure following DNA synthesis. Depending on the number of columns on the particular synthesizer, multiple reactions may be run simultaneously. Although the iodination reagent is not harmful to the synthesizer, the reagent must be filtered prior to use to ensure that no particulates clog the lines. Because of this additional step, it is often more convenient to use the manual procedure if only one or two iodination reactions will be performed. If multiple reactions are to be performed, the automated procedure is generally faster and more convenient.

Additional Materials *(also see Basic Protocol)*

Glass wool or line filter (e.g., preparation and delivery line filter from ABI)
Empty DNA synthesis column (e.g., 1000-Å CPG column; ABI)
10-mL syringes
Clean, oven-dried phosphoramidite bottle compatible with synthesizer
Reagent bottle compatible with synthesizer, filled with anhydrous DMF

Synthesize oligonucleotide

1. Using a DNA synthesizer, synthesize 0.2 to 10 µmol CPG-bound protected oligodeoxyribonucleotide with the 5′-*O*-DMTr group removed (see *APPENDIX 3C*).

   Some resin (≤1 mg) may be removed at this point to use as a reference in HPLC.

Filter iodination solution for use in synthesizer

2. Filter iodination solution under anhydrous conditions as follows. Insert a small filter or glass wool into an empty DNA synthesis column. Take up iodination solution in a 5- or 10-mL syringe, and attach it to the column/filter. Attach another syringe to the opposite end of the column. Push solution through column/filter into the opposite syringe.

   Generally, each reaction requires ≤1 mL of iodination solution.

   The Preparation and Delivery Line Filter from ABI is the ideal size and shape to fit into a 1000-Å ABI CPG column.

   If the solution contains a large quantity of particulates, the filter may become clogged. If this happens, it may be necessary to remove the syringes (while making sure to minimize exposure to the air) and wash the filter. To wash it, simply push dry DMF through the column filter in the opposite direction from that in which the iodination solution was flowing.

   The above filtration system is only one of a variety of ways that the filtration can be performed.

Table 4.19.1 IodoCycle Program for ABI 392 and 394 Synthesizers[a]

Step	Function	Time (sec)
1	Begin	
2	Prep DMF	15
3	DMF to waste	4
4	DMF to column	15
5	Reverse flush	10
6	Block flush	4
7	Phos prep	3
8	Iodo to column	20
9	DMF to waste	3
10	Block flush	4
11	Wait	300
12	Reverse flush	10
13	Iodo to column	20
14	DMF to waste	3
15	Block flush	4
16	Wait	300
17	DMF to column	15
18	Flush to waste	10
19	DMF to column	20
20	Flush to waste	10
21	DMF to column	20
22	Reverse flush	10
23	Block flush	4
24	CH_3CN to waste	3
25	CH_3CN to column	20
26	Reverse flush	10
27	CH_2Cl_2 to waste	3
28	CH_2Cl_2 to column	20
29	Reverse flush	10
30	Block flush	4
31	End	

[a]Modified from Miller and Kool (2002) with permission from the American Chemical Society.

3. Attach a needle to the syringe containing the iodination solution.
4. Inject the iodination solution into an oven-dried and septum-sealed phosphoramidite bottle.

Prepare synthesizer for iodination reaction

5. Write an iodination procedure for the DNA synthesizer containing the following steps:
   a. Rinse beads with anhydrous DMF.
   b. Send iodination solution to column.
   c. Wait (5 min).
   d. Flush iodination solution to waste.
   e. Send iodination solution to column.

f. Wait (5 min).

g. Rinse with anhydrous DMF.

An example of a functional procedure for ABI 392 and 394 synthesizers is shown in Table 4.19.1.

6. Install the iodination reagent into one of the phosphoramidite positions and install a bottle of anhydrous DMF into one of the reagent positions on the DNA synthesizer.

7. Edit sequence: enter a single base as the sequence.

If this is not done, the iodination cycle will repeat itself for each base in the sequence.

Iodinate oligodeoxyribonucleotide

8. Start synthesis.

Due to differences between synthesizers, it is recommended that the column be observed while the cycle is run the first few times to make sure the "iodination solution to column" and "wash" sequences are of the correct length of time. A period of 20 to 30 sec for "iodination solution to column" should be sufficient to completely fill the columns on an ABI 392 or 394 synthesizer.

Cleave and deprotect iodinated deoxyribonucleotide

9. After the synthesis is complete, transfer the CPG resin to a sealed vial. Cleave and deprotect the iodinated product by soaking the resin in 28% NH_4OH (~1 mL for 1-µmol column) at room temperature for 18 to 24 hr.

10. Remove iodination solution and DMF from synthesizer. To ensure that the iodination reagent does not damage or contaminate the synthesizer, replace the iodination reagent bottle with one containing anhydrous DMF and use the manual functions to briefly rinse the lines.

The unused iodination solution can be stored in the phosphoramidite bottle (see recipe for storage conditions).

11. Analyze purity using HPLC with a C18 reversed-phase column (*UNIT 10.5*).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Iodination solution

0.226 g (0.5 mmol) methyltriphenoxyphosphonium iodide [$(PhO)_3PCH_3I$; Aldrich; 0.5 M final]

0.85 ml anhydrous *N*,*N*-dimethylformamide (DMF)

Prepare fresh or store up to 3 months at –70°C

If the solution will be used on a DNA synthesizer, it should be filtered before use (see Alternate Protocol).

$(PhO)_3PCH_3I$ reacts quickly with water and is light sensitive. Minimize exposure of the reagent to the atmosphere while weighing, and promptly flush the vial and the original reagent bottle with N_2 or argon when finished.

It is convenient to prepare the iodination solution during oligonucleotide synthesis. If the solution will be used within a few hours of its preparation, there is no need to protect it from light. If the iodination solution has been frozen, always refilter (see Alternate Protocol, step 2) after thawing.

$(PhO)_3PCH_3I$ is often the consistency of mud or a thick oil. Although this makes it more difficult to manipulate, it generally still works well.

COMMENTARY

Background Information

In 1997, Xu and co-workers described a DNA-ligation method which allowed for the efficient ligation of a 5′-iodo-modified oligodeoxyribonucleotide with a 3′-phosphorothioated oligonucleotide (Xu and Kool, 1997). In the presence of a complementary template, ligation proceeded efficiently under mild conditions. The product of this ligation method is an oligonucleotide identical to natural DNA with the exception that one oxygen atom in the phosphodiester backbone is replaced by a sulfur atom; it therefore retains most of the chemical properties of natural DNA (Xu and Kool, 1998). The 5′-iodo-modified oligomers were prepared on a DNA synthesizer using a previously synthesized 5′-iodo-dT phosphoramidite. Unfortunately, 5′-iodo-modified phosphoramidites are time-consuming and expensive to synthesize, so the utility of this ligation system was significantly limited.

It was subsequently found that the 5′-iodo-oligodeoxyribonucleotides are in fact accessible via a much simpler, more direct route (Miller and Kool, 2002). The protocols described in this unit are based on this new approach, in which 5′-iodination is carried out directly on the DNA column, before deprotection and cleavage. The protecting groups used on the DNA bases and backbone effectively prevent the very reactive $(PhO)_3PCH_3I$ from reacting at any site other than the 5′-hydroxyl. This is true when using both Glen Research's Sterling 2-cyanoethyl (CE) phosphoramidites (5′-DMTr-dA^{Bz}, 5′-DMTr-dC^{Bz}, and 5′-DMTr-dG$^{i\text{-}Bu}$) or their Ultramild CE phosphoramidites (5′-DMTr-dA$^{i\text{-}Bu}$ or 5′-DMTr-dA^{AcOPh}, 5′-DMTr-dG$^{AcOPh\text{-}i\text{-}Pr}$, and 5′-DMTr-dC^{Ac}). Also, it was found that when deprotection and cleavage are carried out at room temperature for 24 hr, very little hydrolysis of the iodo group occurs. This new method of iodinating the 5′-end of oligodeoxyribonucleotides effectively makes the 5′-iodo/3′-phosphorothioate ligation system accessible to anyone with access to synthetic DNA. Unfortunately, it was found that both 2′-*O*-*tert*-butyldimethylsilyl (TBDMS) and 2′-*O*-triisopropylsilyloxymethyl (TOM) protecting groups on protected oligoribonucleotides are cleaved by both $(PhO)_3PCH_3I$ and another iodinating system, PPh_3/I_2/imidazole in DMF (Kool and Miller, unpub. observ.).

In addition to their usefulness in DNA ligation reactions, the 5′-iodo oligomers are also good intermediates for making other types of 5′-modifications. The 5′-iodo group is easily converted to thiols, amines, azides, thioethers, thiocyanates, and selenides while the oligomer is still attached to resin (Kool and Miller, unpub. observ.). 5′-Thiols and amines are convenient handles for attaching isothiocyanates, succinimidyl esters, maleimides, iodoacetamides, and other commercially available labels. Alternatively, thiol-containing moieties will react directly with 5′-iodo oligomers, forming very stable thioethers.

Critical Parameters and Troubleshooting

The most important key to success in running this reaction is to keep the iodination reagent anhydrous. The large excess of reagent called for in this protocol should temper this potential problem. Care should nevertheless be taken to minimize the amount of time the reagent is exposed to the air, and the reagent and iodination solution should always be stored under an inert gas.

Another important point is that the reaction time may need to be increased when bulkier groups are in the 5′-position. For example, when pyrene is in the 5′-position, the iodination reaction requires ~25 min for completion. Additionally, if there is a modified base containing a strong nucleophile near the 5′-end, it may displace the iodo group, cyclizing the oligomer either during synthesis or during deprotection. This is often observed when the 5′-base is a purine, in which the N3 nitrogen attacks the 5′-carbon, resulting in some cyclized material (Dimitrijevich et al., 1979). This cyclized byproduct may be hydrolyzed back to the 5′-hydroxy starting material upon treatment with NH_4OH.

Anticipated Results

The hydrophobicity of the iodo group allows for easy separation of 5′-modified oligodeoxyribonucleotides from their 5′-hydroxy starting materials via reversed-phase HPLC (*UNIT 10.5*). For example, using a gradient of 0% to 20% CH_3CN in 50 mM triethylammonium acetate (TEAA), pH 7.0, over a 20-min period to elute the crude reaction mixture from an analytical C18 reversed-phase column, iodinated trimers elute 5 min later than their corresponding starting materials, while iodinated 13-mers elute ~2 min after their respective starting materials (see Fig. 4.19.2). A chromatogram of the de-

protected 5′-hydroxyl starting material should therefore give an indication of when the iodinated product will come off of the column. Also, knowledge of the starting material retention time allows for calculation of percent conversion to the iodinated product. Electrospray ionization (ESI) mass spectrometry (*UNIT 10.2*) should be used to verify the identity of the product.

The success of this iodination reaction appears to depend on the identity of the 5′-base, but not the overall base composition, of a particular oligomer. Generally, 5′-pyrimidines afford the highest yields because they do not cyclize as easily as purines. Yields of 70% to 90% are typical for pyrimidines and 50% to 80% for purines, based on total integration of HPLC chromatograms (Miller and Kool, 2002, and unpub. observ.). Because the starting material will contain failure sequences from incomplete phosphoramidite coupling during oligomer synthesis, HPLC chromatograms will

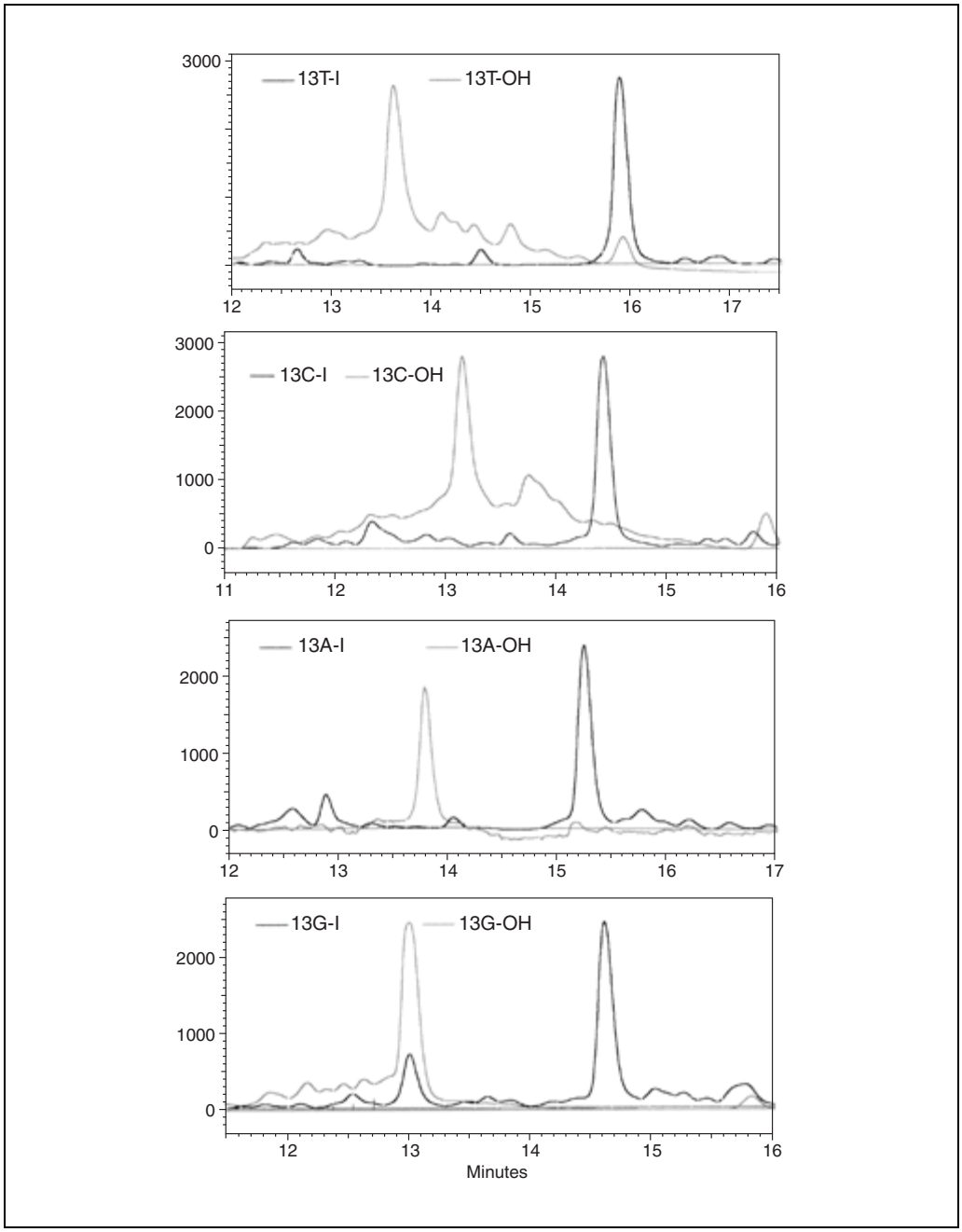

Figure 4.19.2 RP-HPLC profiles of 5′-d(NGTAGGCAAGAGT) before and after 5′-iodination. From top to bottom, N = T, C, A, and G, respectively. Reprinted from Miller and Kool (2002) with permission from the American Chemical Society.

contain multiple peaks. However, a single predominant product peak with a longer retention time should be observed when using a reversed-phase column.

Time Considerations

The manual procedure described in the Basic Protocol can be completed in less than an hour. The automated procedure requires more time to set up the reactions, but once the reagent and synthesizer are prepared, many reactions can be performed quickly. For example, if a 4-column synthesizer is used with a 15-min iodination cycle, 16 reactions can be performed per hour.

Literature Cited

Dimitrijevich, S.D., Verheyden, J.P.H., and Moffatt, J.G. 1979. Halo sugar nucleosides. 6. Synthesis of some 5′-deoxy-5′-iodo and 4′,5′-unsaturated purine nucleosides. *J. Org. Chem.* 44:400-406.

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Key References

Miller and Kool, 2002. See above.

Reports a method to iodinate the 5′-carbon of oligodeoxyribonucleotides on a solid support.

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