Simple, High-Yield Syntheses of DNA Duplexes Containing Interstrand DNA-DNA Cross-Links Between an \(N^4\)-Aminocytidine Residue and an Abasic Site

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The protocol describes the preparation and purification of interstrand DNA-DNA cross-links derived from the reaction of an \(N^4\)-aminocytidine residue with an abasic site in duplex DNA. The procedures employ inexpensive, commercially available chemicals and enzymes to carry out post-synthetic modification of commercially available oligodeoxynucleotides. The yield of cross-linked duplex is typically better than 90%. If purification is required, the cross-linked duplex can be readily separated from single-stranded DNA starting materials by denaturing gel electrophoresis. The resulting covalent hydrazone-based cross-links are stable under physiologically relevant conditions and may be useful for biophysical studies, structural analyses, DNA repair studies, and materials science applications. © 2016 by John Wiley & Sons, Inc.

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**INTRODUCTION**

This unit describes methods for the high-yield preparation and purification of DNA duplexes containing interstrand cross-links at well-defined locations. These are simple, benchtop protocols that employ inexpensive, commercially available enzymes and chemicals for the post-synthetic modification of commercially available oligodeoxynucleotides (Gamboa Varela and Gates, 2015). Cross-link formation involves the reaction of an \(N^4\)-aminocytidine residue (dC\(^*\)) with an abasic site (Ap) on the opposing strand of a DNA duplex (Figs. 5.16.1–5.16.3). The resulting covalent hydrazone-based cross-links are stable under physiologically relevant conditions and may be useful for biophysical studies, structural analyses, DNA repair studies, and materials science applications.

Basic Protocol 1 describes a procedure for the installation of a dC\(^*\) residue into oligodeoxynucleotides and Basic Protocol 2 presents the preparation of Ap-containing oligodeoxynucleotides and interstrand DNA-DNA cross-link formation in a 5\(^\prime\)-32-P-
labeled oligodeoxynucleotide duplex containing the dC* and Ap residues. The preparation of an unlabeled DNA duplex containing the Ap-dC* cross-link is described in Basic Protocol 3. Basic Protocol 4 reports a method for the purification of the unlabeled duplex containing the Ap-dC* cross-link using denaturing polyacrylamide gel electrophoresis (PAGE).

CAUTION: Chemicals, solvents, and laboratory equipment should be used only after consultation with information provided by the supplier, the relevant material safety data sheet (MSDS), experienced colleagues, and local environmental and health safety personnel.

NOTE: Solvents used in the protocol were HPLC-grade. Solutions are in water unless otherwise noted.
This protocol describes the preparation of an oligodeoxynucleotide containing a single \( N^4 \)-aminocytidine (dC*) residue (Negishi et al., 1987; Gao and Orgel, 1999; Gamboa Varela and Gates, 2015). The procedure involves “post-synthetic modification” of a commercially available oligodeoxynucleotide containing a single dC residue with bisulfite and hydrazine (Fig. 5.16.1).

**Materials**

- Water (HPLC grade, Sigma-Aldrich)
- 1 M Sodium Phosphate buffer, pH 5 (reagent grade, Fisher)
- 3 M Sodium bisulfite (NaHSO\(_3\)), freshly prepared (ACS reagent grade, Sigma-Aldrich)
- 8 M Hydrazine monohydrochloride freshly prepared (reagent grade, Sigma-Aldrich)
- 0.1 mM Oligodeoxynucleotide in water
- 0.5 M Tris base (ACS reagent grade, Sigma-Aldrich)
- 0.1 M Triethylamine, freshly prepared (TEA, reagent grade, Sigma-Aldrich)
- 0.001 M Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; ACS reagent grade, Sigma-Aldrich)
- Acetonitrile (HPLC grade, Sigma-Aldrich)
- 0.01 M Ammonium acetate (BioXtra grade, Sigma-Aldrich)
- 8:2 (v:v) Methanol:water (HPLC grade, Sigma-Aldrich)
- Crushed dry ice

1.5- and 2-mL polypropylene microcentrifuge tubes (Eppendorf)
- Vortex-mixer (Fisher Vortex Genie 2)
- Thermostat-controlled aluminum heating block
- C-18 Sep-Pak cartridges (Waters, 1 mL, 100 mg, cat. no. WAT023590)
- 1-mL syringes
- Speed-Vac Concentrator (SC 110, Savant)

**Preparation of a dC*-containing oligodeoxynucleotide 2**

1. To a 1.5-mL microcentrifuge tube, add 2.5 \( \mu \)L water, 2.5 \( \mu \)L of 1 M sodium phosphate buffer stock solution (pH 5), 2.5 \( \mu \)L of a 3 M stock solution in water.

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Figure 5.16.4 Sequences used in these protocols.
bisulfite, and 12.5 μL of a 8 M stock solution of hydrazine monohydrochloride in water.

2. From a stock solution of oligodeoxynucleotide 1 at a concentration of 0.1 mM transfer 5 μL into the 1.5-mL microcentrifuge tube containing the solution prepared in step 1. This brings the final reaction volume to 25 μL.

3. Agitate this mixture for 5 sec utilizing a vortex-mixer.

4. Incubate the mixture for 3 hr at 50°C in a thermostat-controlled heating block.

5. Approximately 5 min before the reaction is completed, prepare the Tris-EDTA-TEA (TET) buffer as follows. In a 2-mL microcentrifuge tube, add 300 μL water, 100 μL of a 0.5 M stock solution of Tris base, 50 μL of a 0.1 M stock solution of TEA in water, and 50 μL of a 0.001 M stock solution of EDTA. This is the TET buffer (pH ~10).

   It is important to freshly prepare the TEA stock solution as its decomposition in solution may decrease the yield of dC*. The Tris base and EDTA stock solutions can be stored at room temperature for at least 3 months.

6. When the incubation described in step 4 is complete, add 250 μL (10 volumes) of TET buffer to the 1.5-mL microcentrifuge reaction tube and mix using a vortex-mixer for 5 sec.

7. Desalt the oligodeoxynucleotide using a C-18 Sep-Pak column.

8. Prepare the C-18 Sep-Pak column using the following method:
   a. Add 1 mL acetonitrile to the column and completely elute the solvent from the column (into a waste container) by pushing air through it with a 1-mL syringe.
   b. Add 1 mL water and similarly elute to waste.
   c. Add 300 μL of 0.01 M sodium acetate solution in water and elute to waste.
   d. Add the reaction mixture (275 μL) and elute to waste.
   e. Add 1 mL of water and elute to waste. Repeat this two more times. This washes salts from the oligodeoxynucleotide.
   f. Add 1 mL of 8:2 MeOH:water solution and elute the solution into a 2-mL microcentrifuge tube. This solution contains the oligodeoxynucleotide.

   It is important that the sample is properly desalted because salt may affect the yield of the cross-linking reaction. In our hands, C-18 Sep-Pak is the best desalting method for this purpose. Ethanol precipitation or G-25 Sephadex spin columns do not desalt the sample as thoroughly.

9. Place the 2-mL microcentrifuge tube in crushed dry ice for 2 to 3 min with the cap opened.

10. Dry the sample in a Speed-vac concentrator for approximately 2 hr at 37°C.

11. The resulting dC*-containing oligodeoxynucleotide 2 can be used immediately or stored dry at −20°C for up to 16 hr.

12. When ready to use the dC*-containing oligodeoxynucleotide 2, resuspend in 250 μL of water to achieve a stock solution of approximately 1 μM for the 5′-32P-labeled cross-linking reaction (Basic Protocol 2) or 32.5 μL of water to achieve a stock solution of approximately 6 μM for the unlabeled cross-linking reaction (Basic Protocol 3).
PREPARATION OF A 5′-32P-LABELED OLIGODEOXYNUCLEOTIDE DUPLEX (5) CONTAINING THE Ap-dC* INTERSTRAND DNA-DNA CROSS-LINK

This protocol describes conversion of a 5′-32P-labeled dU-containing oligodeoxynucleotide 3 into the corresponding Ap-containing oligodeoxynucleotide 4, followed by hybridization with the dC*-containing oligodeoxynucleotide 2 and formation of the cross-linked duplex 5 (Figs. 5.16.2 and 5.16.3).

Materials

0.1 mM Oligodeoxynucleotide 3 (Integrated DNA Technologies) in water
Water (HPLC grade, Sigma-Aldrich)
Uracil-DNA glycosylase reaction buffer 10× (UDG buffer; New England Biolabs)
Uracil-DNA glycosylase enzyme (UDG enzyme; New England Biolabs)
25:24:1 Phenol-chloroform-isooamyl alcohol (BioUltra for molecular biology, Sigma-Aldrich)
3 M Sodium acetate, pH 5.2 (ACS reagent grade, Sigma-Aldrich)
Absolute ethanol (200 proof, Decon Labs)
Crushed dry ice
8:2 (v:v) Ethanol:water (HPLC grade, Sigma-Aldrich)
1 M Sodium phosphate buffer, pH 5 (Fisher)
1 M Sodium chloride (NaCl; Fisher)
dC*-containing oligodeoxynucleotide 2 (from Basic Protocol 1)

1.5-mL microcentrifuge tubes
Vortex-mixer (Fisher Vortex Genie 2)
Thermostat-controlled oven-incubator set at 37°C
Benchtop centrifuge (5424, Eppendorf)
Benchtop centrifuge in cold room 4°C (AccuSpin Micro 17, Fisher Scientific)
Speed-Vac concentrator (SC 110, Savant)
Thermostat-controlled heating block

Preparation of the Ap-containing 5′-32P-labeled oligodeoxynucleotide 4

1. Label the oligodeoxynucleotide 3 using 5′-32P-label using standard procedures (Sambrook et al., 1989; see UNIT 10.4).

   NOTE: Follow all standard radiation safety protocols for your laboratory and institution.

2. Resuspend the 5′-32P-labeled oligodeoxynucleotide 3 in 50 μL of water (approximately 8 μM).

3. In a 1.5-mL microcentrifuge tube add 20 μL of water, 4 μL of UDG buffer, 12 μL of oligodeoxynucleotide 3 (100-200 K cpm), and 4 μL of UDG enzyme (20 U/mL final) for a total reaction volume of 40 μL.

4. Mix the reaction using a vortex-mixer for 10 sec.

5. Incubate for 1 hr at 37°C.


7. Centrifuge for 3 min at 21,130 × g, 24°C, using a benchtop microcentrifuge.

8. Transfer the aqueous (top) layer into a new 1.5-mL microcentrifuge tube. Proceed to ethanol precipitation and wash (steps 9 to 17) (Sambrook et al., 1989).


10. Add 200 μL (5 volumes) of cold ethanol and vortex for a few seconds.
Figure 5.16.5  Formation of the dC*-Ap cross-linked duplex 5. Oligodeoxynucleotides 2 and 4 were incubated in sodium phosphate buffer (50 mM, pH 5) containing NaCl (100 mM) at 50°C for 5 min then cooled to room temperature over the course of 5 hr. The samples were then mixed with formamide loading buffer the DNA fragments separated on a 20% denaturing polyacrylamide gel (0.4 mm thick). The labeled fragments in the gel were visualized by phosphorimager analysis. Lane 1: size marker consisting of the 32P-labeled dU-containing oligonucleotide 3. Lane 2: the Ap-containing oligodeoxynucleotide 4. Lane 3: the Ap-containing oligodeoxynucleotide treated with piperidine to induce strand cleavage (1 M, 95°C, 30 min). Lane 4: generation of cross-linked duplex 5 from the complementary oligodeoxynucleotides 2 + 4.

11. Place the 1.5-mL microcentrifuge tube in crushed dry ice for 55 min.
12. Centrifuge for 45 at 17,000 × g, 4°C, using a benchtop centrifuge.
13. Remove the supernatant and discard.
14. Add 90 μL of 8:2 ethanol:water to the microcentrifuge tube and vortex for a few seconds.
15. Centrifuge for 30 min at 17,000 × g, 4°C, using a benchtop centrifuge.
16. Remove the supernatant and discard.
17. Dry in a Speed-vac concentrator for 3 min at room temperature (23°C).

*Do not dry the mixture at 37°C, as this can cause cleavage of the oligodeoxynucleotide at the Ap site (Gates et al., 2004; Gates, 2009).*
**Preparation of the cross-linked 5′-32P-labeled oligodeoxynucleotide duplex 5**

18. Resuspend the Ap-containing oligodeoxynucleotide 4 in 40 μL of water (approximately 2.5 μM)

19. To a 1.5-mL microcentrifuge tube add 4.5 μL water, 1 μL of a 1 M stock solution (pH 5) of sodium phosphate buffer, 2 μL of 1 M stock solution of NaCl in water, 10 μL of the dC*-containing oligodeoxynucleotide 2, and 2.5 μL of the Ap-containing oligodeoxynucleotide 4 (approximately 30-50 K cpmp)

20. Mix the solution on a vortex-mixer for 20 sec.

21. Incubate in a heating block for 5 min at 50°C.

22. Remove the aluminum heating block containing the reaction tube from the heating source and allow it to cool to room temperature (over the course of approximately 5 hr).

23. The Ap-dC* cross-link is formed during this incubation typically in yields of 88% to 95%. For many purposes, the cross-linking reaction mixture may be used without purification, especially because the cross-linked duplex is well resolved from the uncross-linked material on a denaturing polyacrylamide gel (Fig. 5.16.5).

   *It is important that the incubation time does not exceed 5 hr because unreacted Ap-site undergoes cleavage and a low molecular weight cross-link begins to form [see Supporting Information of Gamboa Varela and Gates, 2015]). The cross-linked DNA duplex 5 can be stored in solution for 1 day at −20°C. The cross-linked DNA also can be ethanol precipitated and stored dry up to 3 days at −20°C. Radiolytic degradation of the DNA can occur if the 5′-32P-labeled material is stored for long periods of time.*

**PREPARATION OF AN UNLABELED DNA DUPLEX CONTAINING THE INTERSTRAND Ap-dC* CROSS-LINK**

Some applications may require the preparation of larger quantities of cross-linked duplexes that do not bear the 5′-32P-label present in Basic Protocol 2. Accordingly, the protocol below describes preparation of an unlabeled DNA duplex 8 containing the interstrand Ap-dC* cross-link, prepared by hybridization of the Ap-containing oligodeoxynucleotide 7 with the dC*-containing oligodeoxynucleotide 2.

**Materials**

- 0.1 mM Oligodeoxynucleotide 6 (Integrated DNA Technologies) in water
- Water (HPLC grade, Sigma-Aldrich)
- Uracil-DNA glycosylase “10×” reaction buffer (UDG buffer; New England Biolabs)
- Uracil-DNA glycosylase enzyme (UDG enzyme; New England Biolabs)
- 25:24:1 Phenol-chloroform-isooamyl alcohol (BioUltra for molecular biology, Sigma-Aldrich)
- 1 M Sodium phosphate buffer, pH 5 (Fisher)
- 1 M Sodium chloride (NaCl; Fisher)
- dC*-containing oligodeoxynucleotide 2 (from Basic Protocol 1)
- 3 M Sodium acetate, pH 5.2 (Sigma-Aldrich)
- 1.5 mL microcentrifuge tubes
- Vortex-mixer (Fisher Vortex Genie 2)
- Thermostat-controlled oven-incubator set at 37°C
- Benchtop centrifuge (5424, Eppendorf)
- Benchtop centrifuge in cold room 4°C (AccuSpin Micro 17, Fisher Scientific)
Preparation of the Ap site-containing unlabeled oligodeoxynucleotide 7
1. In a 1.5-mL microcentrifuge tube add 10 μL of oligodeoxynucleotide 6 (a 0.1 mM stock solution in water), 4 μL UDG buffer, 21 μL water, and 5 μL UDG enzyme (25 U).
2. Mix for 30 sec using a vortex-mixer.
3. Incubate for 1 hr at 37°C.
4. Add 40 μL of phenol:chloroform:isoamyl alcohol and vortex for a few seconds.
5. Centrifuge for 3 min at 21,130 × g, 24°C, using a benchtop centrifuge.
6. Remove the aqueous layer and ethanol precipitate as described in Basic Protocol 2 (steps 9 to 16).
7. Dry the Ap-containing oligodeoxynucleotide 7 in a Speed-vac concentrator for 2 to 3 min at room temperature (23°C).

Preparation of the cross-linked duplex 8
8. Add 10 μL water to the freshly prepared Ap-containing oligodeoxynucleotide 7.
9. Add to the microcentrifuge tube containing the Ap-oligodeoxynucleotide 7, 5 μL of the 1 M stock solution of NaCl and 2.5 μL of 1 M sodium phosphate buffer.
10. Resuspend the dC*-containing oligodeoxynucleotide 2 in 32.5 μL water and vortex vigorously for 30 sec.
11. Transfer the dC*-containing oligodeoxynucleotide 2 to the microcentrifuge tube containing the Ap-oligodeoxynucleotide.
12. Incubate in a thermostat-controlled heating block for 5 min at 50°C.
13. Remove the aluminum heating block containing the reaction tube from the heating source and allow it to cool to room temperature (over the course of approximately 5 hr).

The cross-linked DNA can be ethanol precipitated (see Basic Protocol 2, steps 9 to 16) and stored dry at −20°C until purification.

PURIFICATION CROSS-LINKED DUPLEX 8 USING PAGE
This protocol describes the purification of the cross-linked duplex 8 via denaturing PAGE. Basic procedures for gel electrophoresis have been summarized previously (Sambrook et al., 1989; see UNIT 10.4).

The procedure described here is for the purification of approximately 1 nmol of cross-link 8. The procedure can be utilized for reactions up to 20 nmol of cross-link.

CAUTION: Gel electrophoresis involves the use of a high voltage power source. Use a gel apparatus with proper safety designs after receiving proper safety training.

Materials
- 20% denaturing polyacrylamide solution (19:1 acrylamide/bis-acrylamide, 8 M urea; Fisher)
- N,N′,N″-Tetramethylethylenediamine (TEMED; BioReagent, for molecular biology; Sigma-Aldrich)
10% (w/v) Aqueous ammonium persulfate (for molecular biology, for electrophoresis; Sigma-Aldrich)
Tris-borate-EDTA buffer, 1× (TBE buffer; BioReagent, for molecular biology; Sigma-Aldrich)
Formamide loading buffer (see recipe) (17.75 M formamide, deionized, Calbiochem), 0.01 M EDTA (Sigma-Aldrich) with bromophenol blue dye (ACS reagent, Sigma-Aldrich)
Elution buffer (0.2 M NaCl, 0.001 M EDTA, pH 8)
Crushed dry ice
Water (HPLC grade, Sigma-Aldrich)
Glass gel plates (16 × 19.7 cm) with 2-mm thick spacers and 12-well comb
1.5- and 2-mL microcentrifuge tubes
100-mL beaker and magnetic stir bar
20-mL disposable syringe and 18-G, 1.5-in. needle
Electrophoresis power source (2060-FBS, E-C Apparatus Corporation)
Vortex-mixer (Fisher Vortex Genie 2)
All-Purpose Laboratory Wrap (Saran Wrap, Fisherbrand)
UV lamp and silica gel TLC plate impregnated with UV-254 fluorophore (Sigma-Aldrich)
Disposable razor blade
Glass rod
Poly-Prep Chromatography column (spin column, Bio-Rad Laboratories)
Clinical centrifuge (spin-bucket, IEC)
C-18 Sep-Pak cartridges (1 mL, 100 mg, Waters, cat. no.WAT023590)
Speed-vac Concentrator (SC 110, Savant)

Preparation of denaturing polyacrylamide gel
1. Assemble the glass plates for the gel, sealing the sides and bottom to prevent leaking.
2. In a 100-mL beaker equipped with a magnetic stir bar, pour 40 mL of 20% polyacrylamide solution containing 8 M urea.
3. Add TEMED (20 to 25 μL) to the stirred 100-mL beaker from step 2.
4. Add 200 μL of aqueous ammonium persulfate to the stirred 100-mL beaker from step 3.
5. Pour the mixture into the gel plate assembly.
6. Insert a 12-well comb between the plates assembly.
7. Allow gel polymerization to occur (1.5 to 2 hr at room temperature).
8. After polymerization is complete, remove the comb and bottom spacer slowly, and wash out the wells with a 10 mL solution of 1× TBE using a 20-mL disposable syringe equipped with a disposable needle.
9. Mount the plate assembly onto the gel stand. Fill the top and bottom wells of the gel stand with 1× TBE.
10. Before loading the samples, electrophorese the gel for 30 min at 300 V.

Separation of cross-linked DNA from uncross-linked DNA on the gel
11. Add 20 μL formamide loading buffer to the dry cross-link 8 sample.
12. Vortex and spin down for 10 sec.
13. Wash the gel wells once again with 10 to 15 mL 1× TBE.
Preparative gel purification of duplex 8 containing the Ap-dC* cross-link. Oligonucleotides 6 and 7 were incubated in sodium phosphate buffer (50 mM, pH 5) containing NaCl (100 mM) at 50°C for 5 min then cooled to room temperature over the course of 5 hr. The samples were mixed with formamide loading buffer and the DNA fragments resolved on a 20% denaturing polyacrylamide gel (2 mm thick). The unlabeled fragments in the gel were visualized by UV-shadowing. Lane 1: size marker consisting of the unlabeled dU-containing oligonucleotide 6. Lane 2: the Ap-containing oligonucleotide 7 treated with piperidine (1 M, 95°C, 30 min) to induce strand cleavage at the Ap site. Lane 3: the dC*-containing oligonucleotide 2. Lane 4: generation of cross-linked duplex 8 from the complementary oligonucleotides 2 + 7.

14. Load the sample onto the wells of the gel.
15. Rinse the sample tube with 10 µL formamide loading buffer.
17. Load the sample onto the same well as in step 14.
18. Electrophorese the gel at 300 V.
19. Disconnect the gel from the power source once the dye has traveled 12 to 14 cm from the well (approximately 4 hr).
20. Remove the plate assembly from the stand and separate the plates carefully and remove the gel from both plates.

Isolation of cross-linked DNA from gel
21. Wrap the gel with plastic Saran wrap and place on top of a large silica gel TLC plate containing UV-254 fluorophore.
22. Illuminate the gel with a handheld UV-254 lamp onto the gel to “UV-shadow” the DNA bands in the gel (Sambrook et al., 1989).

   The DNA bands will appear as purple bands against the light green fluorescence of emitted by the TLC plate. The cross-linked DNA will appear approximately 5- to 7-cm from the wells (Fig. 5.16.6).

   Minimize the amount of time the DNA is exposed to the handheld UV light. UV light can cause DNA damage.

23. Cut the band from the gel with a disposable razor blade and transfer the gel slice into a 2-mL microcentrifuge tube.

Extraction of cross-linked DNA from gel slice and desalting

24. Crush the gel slice with the tip of a glass rod and add 1 mL elution buffer.

25. Shake the sample utilizing a vortex mixer for at least 1 hr.

26. Centrifuge the 2-mL microcentrifuge tube for 2 min at 21,130 g, 24°C.

27. Transfer the liquid supernatant to an assembled spin-column with a 2-mL microcentrifuge receiving tube at the bottom.

28. Spin using a spin-bucket centrifuge for 10 min at level 5.

29. Desalt the resulting liquid using a C-18 Sep-Pak column (see Basic Protocol 1, step 8).

30. Put the eluted solution in crushed dry ice for 2 to 3 min.

31. Evaporate to dryness via Speed-vac concentrator at room temperature (approximately 2 to 3 hr).

   After this point, the cross-link is ready to use in other experiments. The dry cross-link can be stored for at −20°C long periods of time, the longest we have stored it is 2 months and have not observed significant degradation during this time.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Formamide loading buffer

   17.75 M formamide, deionized (Calbiochem)
   0.01 M EDTA (Sigma-Aldrich)
   0.06 Bromphenol blue dye (ACS reagent, Sigma-Aldrich)
   Store up to 6 months at 4°C

COMMENTARY

Background Information

Interstrand DNA-DNA cross-links are often studied as critical, medicinally relevant DNA lesions generated by anticancer chemotherapeutic agents such as cis-platin, carboplatin, bendamustine, or chlorambucil (Povirk and Shuker, 1994; Rajski and Williams, 1998; Schärer, 2005; Cheson and Leoni, 2011; Zhu et al., 2013; Johnson et al., 2014). In addition, there is evidence that some as-yet-unidentified endogenous DNA cross-link(s) may drive human aging (Niedernhofer et al., 2005; Bergstrahl and Sekelsky, 2007). Accordingly, there is substantial interest in the structure, occurrence, biochemical processing, and biological properties of interstrand DNA-DNA cross-links. Structural and biochemical studies can benefit from methods for the high yield preparation of structurally well-defined DNA cross-links. In addition, the facile methods for the preparation of interstrand cross-links may facilitate the construction of stable DNA nanostructures in the field of materials science and nanomedicine (Rajendran et al., 2011; Chen and Schuster, 2013).
The reactions of duplex DNA with bifunctional electrophiles such as anticancer cross-linking drugs or mutagenic agents like acrolein occurs with low sequence selectivity (Kohn et al., 1987; Povirk and Shuker, 1994; Kozekov et al., 2010). Furthermore, only a small number (1% to 3%) of the initial alkylation events go forward to generate interstrand cross-links (Rink et al., 1993; Povirk and Shuker, 1994; Kozekov et al., 2010). As a result, treatment of mixed sequence DNA with these agents typically generates intractable mixtures of cross-linked duplexes in very low yields. This is usually unacceptable from a preparative perspective. Alternatively, elegant syntheses of structurally defined cross-linked duplexes have been devised (Balkrishnen et al., 1996; Gao and Orgel, 1999; Manoharan et al., 1999; Harwood et al., 2000; Noll et al., 2001; Nakatani et al., 2002; Schärer, 2005; Hong et al., 2006; Hentshel et al., 2012; Carrette et al., 2013; Nishimoto et al., 2013; O’Flaherty et al., 2013; Ye et al., 2013; Gruppi et al., 2014; Haque et al., 2014; Mukherjee et al., 2014; Pujari et al., 2014; Tomás-Gamasa et al., 2014), but many of these multi-step organic reaction sequences may not be practical in many of the biochemical and materials science laboratories with interests in cross-linked DNA. In the work described here, we sought a simple, benchtop procedure for the synthesis of DNA duplexes containing a site-specific interstrand cross-link that employs inexpensive commercially available enzymes, chemicals, and oligodeoxyribonucleotides. The protocol for post-synthetic modification of the oligodeoxyribonucleotides and cross-linking uses procedures and equipment that are common in many laboratories. The cross-linking reaction exploits the facile post-synthetic generation of dC* and Ap in oligodeoxyribonucleotides (Lindahl et al., 1977; Negishi et al., 1987; Varshney and van de Sande, 1991; Gao and Orgel, 1999) to access one of the original forms of “click chemistry”, hydrazone formation (Wang and Canary, 2012; Kool et al., 2013), and generates high yields of cross-link at a single defined location in a DNA duplex. If purification is desired, the cross-linked material is readily separated from uncross-linked oligodeoxyribonucleotides using denaturing gel electrophoresis. The general methods described here can also be used for the preparation and isolation of Ap-derived cross-links involving the native nucleobases guanine and adenine (Dutta et al., 2007; Johnson et al., 2013; Price et al., 2014; Catalano et al., 2015; Price et al., 2015; Yang et al., 2015).

Critical Parameters

In the preparation of the dC*-containing oligodeoxyribonucleotide, removal of bisulfite at the end of the procedure is critical, as the cross-link yield will be greatly reduced or hindered completely if residual bisulfite remains during the cross-linking reaction. We find that desalting of dC*-containing oligodeoxyribonucleotides using a C-18 Sep-Pak cartridge is superior to other methods such as ethanol precipitation and Sephadex G-25 spin columns. Another important consideration is the design of the dC*-containing oligonucleotide. We have designed our oligonucleotides to contain only a single dC residue (and, therefore, only a single dC* residue) in the strand complementary to the Ap-containing oligonucleotide. In an oligonucleotide with multiple dC residues, presumably all would be transformed to dC* upon treatment with bisulfite/hydrazine. We have not explored cross-link formation when other dC* distal residues are present. We suspect that cross-linking would proceed normally in such a setting. It is critical that the Ap-containing oligodeoxyribonucleotide is used immediately after it is prepared as it is prone to cleavage, especially in the single-strand form.

Troubleshooting

The Ap-dC* cross-link can be formed in oligodeoxyribonucleotide duplexes of various lengths. However, the melting temperature of the oligodeoxyribonucleotides needs to be taken into consideration as shorter strands will need longer incubation times and/or lower incubation temperatures. In general, yields are likely to be compromised if the cross-linking reactions are carried out at or above the melting temperature of the duplex. The melting temperatures of Ap-containing duplexes are substantially lower than that of a fully paired duplex of similar length (Vesnaver et al., 1989; Sági et al., 2001). Use of low temperatures to favor hybridization of short duplexes may slow the cross-linking reactions. For example, formation of the Ap-dC* cross-link required incubation for 4 days at 4°C to obtain 40% yield in a 12 base pair duplex. The location of the dU residue in the oligodeoxyribonucleotide also can affect yield of Ap generation by UDG. dU residues near the end of oligodeoxyribonucleotides are less effective substrates for UDG (Varshney and van de Sande, 1991). The Ap-dC* cross-link in duplex DNA is thermally reversible (Gamboa Varela and Gates, 2015). That is, the cross-link can be broken by heating the duplex to its melting point, but the
cross-link is then spontaneously regenerated upon cooling and rehybridization.

**Anticipated Results**

With the protocols provided, cross-linked duplexes can be obtained in yields of 90% or better. If desired, the cross-linked duplexes can be easily purified by gel electrophoresis.

**Time Considerations**

The cross-links can be obtained in 1 to 3 days. In approximately 5 to 6 hr, the dC*-containing oligodeoxyxynucleotide can be obtained and the dried sample stored at −20°C for 16 hr (overnight), then resuspended in water and utilized in the cross-linking reaction. The Ap-containing oligodeoxynucleotide can be prepared from the corresponding dU-containing oligodeoxynucleotide in a few hours. The Ap-oligodeoxynucleotide should be used immediately in the cross-linking reaction. The cross-linking reaction takes approximately 1 hr of set up time and 5 hr of incubation. Gel purification of the cross-linked duplex requires approximately 8 hr. The gel slices containing the cross-linked duplex can be frozen overnight and extraction of the material from the gel continued on the next day.

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**Literature Cited**


interstrand DNA-DNA cross-links forged by reaction of an abasic site with the opposing guanine residue of 5'-CAP sequences in duplex DNA. J. Am. Chem. Soc. 135:1015-1025. doi: 10.1021/ja308119q.


Vesnaver, G., Chang, C.-N., Eisenberg, M., Groll- man, A.P., and Breslauer, K.J. 1989. Influence of abasic and anucleosidic sites on the stability, conformation, and melting behavior of a


Methods for Cross-Linking Nucleic Acids

5.16.15