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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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Abstract

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.

Keywords

DNA cross-link; abasic; oligonucleotide; DNA repair; nanomaterials; click chemistry; hydrazone

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, bench-top 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 (Figures 1-3). 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'-³²P-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.

BASIC PROTOCOL 1

PREPARATION OF AN OLIGODEOXYNUCLEOTIDE CONTAINING AN *N*⁴-AMINOCYTIDINE RESIDUE (dC*)—This protocol describes the preparation of an oligodeoxynucleotide **2** (Figure 4) containing a single *N*⁴-aminocytidine (**dC***) residue (Gamboa Varela and Gates, 2015; Gao and Orgel, 1999; Negishi et al., 1987). The procedure involves “post-synthetic modification” of a commercially available oligodeoxynucleotide **1** containing a single dC residue with bisulfite and hydrazine (Figure 1).

Materials: Water (HPLC grade, Sigma-Aldrich)

1 M Sodium Phosphate buffer, pH 5 (reagent grade, Fisher)

3 M Sodium bisulfite (NaHSO₃), freshly prepared (ACS reagent grade, Sigma-Aldrich)

8 M Hydrazine monohydrochloride freshly prepared (reagent grade, Sigma-Aldrich)

0.1 mM Oligodeoxynucleotide **1** (Integrated DNA Technologies) 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)

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 cc, 100 mg, cat # WAT023590)

Crushed dry ice

Speed-Vac Concentrator (SC 110, Savant)

Preparation of a dC*-containing oligodeoxynucleotide 2

1. To a 1.5 mL Eppendorf tube, add 2.5 μ L of water, 2.5 μ L of sodium phosphate buffer 1 M stock solution (pH 5), 2.5 μ L of a 3 M stock solution in water of sodium bisulfite, and 12.5 μ L 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 μ L into the 1.5 mL Eppendorf containing the solution prepared in step 1. This brings the final reaction volume to 25 μ L.
3. Agitate this mixture for 5 s utilizing a vortex-mixer.
4. Incubate the mixture at 50 °C for 3 h in a thermostat-controlled heating block.
5. Approximately 5 min before the reaction is completed; the Tris-EDTA-TEA (TET) buffer needs to be prepared.
 - a. In a 2 mL Eppendorf tube, add 300 μ L of water, 100 μ L of a 0.5 M stock solution of Tris base, 50 μ L of a 0.1 M stock solution of TEA in water, and 50 μ L 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 μ L (10 volumes) of TET buffer to the 1.5 mL Eppendorf reaction tube and mix using a vortex-mixer.
7. Desalt the oligodeoxynucleotide using a C-18 Sep-Pak® column (1 cc, 100 mg, cat #WAT023590).
8. The C-18 Sep-Pak® column is prepared with the following method:
 - a. Add 1 mL of 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 of water and similarly elute to waste.
 - c. Add 300 μ L of 0.01 M sodium acetate solution in water and elute to waste.
 - d. Add the reaction mixture (275 μ L) 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 Eppendorf 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 Eppendorf tube in crushed dry ice for 2-3 min with the cap opened.
10. Dry the sample in a Speed-vac concentrator for approximately 2 h at 37 °C.
11. The resulting **dC***-containing oligodeoxynucleotide **2** can be used immediately or stored dry at -20 °C for up to 16 h.
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).

BASIC PROTOCOL 2

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** (Figures 2 and 3).

Materials: 0.1 mM Oligodeoxynucleotide **3** (Integrated DNA Technologies) in water

Water (HPLC grade, Sigma-Aldrich)

Uracil-DNA glycosylase reaction buffer 10x (UDG buffer, New England Biolabs)

Uracil-DNA glycosylase enzyme (UDG enzyme, New England Biolabs)

Phenol-chloroform-isoamyl alcohol 25:24:1 (BioUltra for molecular biology, Sigma-Aldrich)

3 M Sodium acetate, pH 5.2 (ACS reagent grade, Sigma-Aldrich)

Absolute ethanol (200 proof, Decon Labs)

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 Eppendorf tube

Vortex-mixer (Fisher Vortex Genie 2)

Thermostat-controlled oven-incubator set at 37 °C

Tabletop centrifuge (5424, Eppendorf)

Crushed dry ice

Tabletop 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. The oligodeoxynucleotide **3** is 5'-³²P-labeled 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 Eppendorf tube add 20 µL of water, 4 µL of UDG buffer, 12 µL of oligodeoxynucleotide **3** (100-200K cpm), and 4 µL of UDG enzyme (20 units/mL final) for a total reaction volume of 40 µL.
4. Mix the reaction using a vortex-mixer for 10 s.
5. Incubate at 37 °C for 1 h.
6. Add 40 µL of phenol:chloroform:isoamyl alcohol and vortex for 10 s.
7. Centrifuge at $21130 \times g$ for 3 min (Tabletop microcentrifuge).
8. Transfer the aqueous (top) layer to a new 1.5-mL Eppendorf tube. Proceed to ethanol precipitation and wash (steps 9-17) (Sambrook et al., 1989).
9. Add 4 µL of a 3 M stock solution of sodium acetate, pH 5.2.
10. Add 200 µL (5 volumes) of cold ethanol and vortex for a few seconds.
11. Place the 1.5 mL Eppendorf tube in crushed dry ice for 55 min.
12. Centrifuge at $17000 \times g$ at 4 °C for 45 min (tabletop centrifuge).
13. Remove the supernatant.

14. Add 90 μL of 8:2 EtOH:water to the Eppendorf tube and vortex for a few seconds.
15. Centrifuge at $17000 \times g$ at 4°C for 30 min (tabletop centrifuge).
16. Remove the supernatant.
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, 2009; Gates et al., 2004).
18. Resuspend the Ap-containing oligodeoxynucleotide **4** in 40 μL of water (approximately 2.5 μM)

Preparation of the cross-linked 5'- ^{32}P -labeled oligodeoxynucleotide duplex **5**

19. To a 1.5-mL Eppendorf tube add 4.5 μL of 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 30-50K cpm)
20. Mix the solution on a vortex-mixer for 20 s.
21. Incubate in a heating block at 50°C for 5 min.
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 h).
23. The **Ap-dC*** cross-link is formed during this incubation typically in yields of 88-95%. 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 (Figure 5).

It is important that the incubation time does not exceed 5 h 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 at -20°C for 1 d. The cross-linked DNA also can be ethanol precipitated and stored dry at -20°C for up to 3 d. Radiolytic degradation of the DNA can occur if the 5'- ^{32}P -labeled material is stored for long periods of time.

BASIC PROTOCOL 3

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'- ^{32}P -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 “10x” reaction buffer (UDG buffer, New England Biolabs)

Uracil-DNA glycosylase enzyme (UDG enzyme, New England Biolabs)

Phenol-chloroform-isoamyl alcohol 25:24:1 (BioUltra for molecular biology, Sigma-Aldrich)

3 M Sodium acetate, pH 5.2 (Sigma-Aldrich)

Absolute ethanol (200 proof, Decon Labs)

8:2 (v:v) Ethanol:water

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 Eppendorf tube

Vortex-mixer (Fisher Vortex Genie 2)

Thermostat-controlled oven-incubator set at 37 °C

Tabletop centrifuge (5424, Eppendorf)

Tabletop 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 site-containing unlabeled oligodeoxynucleotide **7**

1. In a 1.5 mL Eppendorf tube add 10 µL of oligodeoxynucleotide **6** (a 0.1 mM stock solution in water), 4 µL of UDG buffer, 21 µL of water, and 5 µL of UDG enzyme (25 units).
2. Mix using a vortex-mixer for 30 s.
3. Incubate at 37 °C for 1 h.
4. Add 40 µL of phenol:chloroform:isoamyl alcohol and vortex for a few seconds.
5. Centrifuge at $21130 \times g$ for 3 min (tabletop centrifuge).

6. Remove the aqueous layer and ethanol precipitate as described in Basic Protocol 2, steps 9-16).
7. Dry the **Ap**-containing oligodeoxynucleotide **7** in a Speed-vac concentrator for 2-3 min at room temperature (23 °C).

Preparation of the cross-linked duplex **8**

1. Add 10 µL of water to the freshly prepared **Ap**-containing oligodeoxynucleotide **7**.
2. Add to the Eppendorf 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.
3. Resuspend the **dC***-containing oligodeoxynucleotide **2** in 32.5 µL of water and vortex vigorously for 30 s.
4. Transfer the **dC***-containing oligodeoxynucleotide **2** to the Eppendorf tube containing the **Ap**-oligodeoxynucleotide.
5. Incubate in a thermostat-controlled heating block at 50 °C for 5 min.
6. 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 h).

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

BASIC PROTOCOL 4

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,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 1X (TBE buffer, BioReagent, for molecular biology, Sigma-Aldrich)

Formamide loading buffer (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)

Water (HPLC 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)

Glass gel plates (16 × 19.7 cm) with 2 mm thick spacers and comb

1.5, 2 mL Eppendorf tubes

100 mL beaker and magnetic stir bar

20 mL disposable syringe and needle

Electrophoresis power source (2060-FBS, E-C Apparatus Corporation)

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

Vortex-mixer (Fisher Vortex Genie 2)

Centrifuge (5424, Eppendorf)

Poly-Prep® Chromatography column (spin column, Bio-Rad Laboratories)

Clinical centrifuge (spin-bucket, IEC)

C-18 Sep-Pak® cartridges (Waters, 1 cc, 100 mg, cat #WAT023590)

Speed-vac Concentrator (SC 110, Savant)

Gel purification of cross-link 8

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 (20-25 μ L) to the stirred 100 mL beaker from step 1.
4. Add 200 μ L of aqueous ammonium persulfate to the stirred 100 mL beaker from step 2.
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 (1.5-2 h).
8. After polymerization is complete, remove the comb and bottom spacer slowly, and wash out the wells with a solution of 1X TBE using a 20-mL disposable syringe equipped with a disposable needle.
9. Mount the plate assembly onto the gel stand. Fill top and bottom wells of the gel stand with 1x TBE.
10. Before loading samples, electrophorese the gel for 30 min at 300 V.
11. Add 20 μ L of formamide loading buffer to the dry cross-link **8** sample.
12. Vortex and spin down for 10 s.
13. Wash the gel wells once again with 1 X TBE.
14. Load the sample onto the wells of the gel.
15. Rinse the sample tube with 10 μ L of formamide loading buffer.
16. Vortex and spin down for 10 sec.
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 12-14 cm from the well (approximately 6 h).
20. Remove the plate assembly from the stand and separate the plates carefully and remove the gel from both plates.
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 5-7 cm from the wells (Figure 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 Eppendorf tube.
24. Crush the gel slice with the tip of a glass rod and add 1 mL of elution buffer.
25. Shake the sample utilizing a vortex mixer for at least 1 h.
26. Centrifuge the 2 mL Eppendorf tube for 2 min at $21130 \times g$.
27. Transfer the liquid supernatant to an assembled spin-column with a 2 mL Eppendorf 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 2-3 min.
31. Evaporate to dryness via Speed-vac concentrator at room temperature (approximately 2-3 h).

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.

COMMENTARY

Background Information

Interstrand DNA-DNA cross-links are often studied as critical, medically-relevant DNA lesions generated by anticancer chemotherapeutic agents such as *cis*-platin, carboplatin, bendamustine, or chlorambucil (Cheson and Leoni, 2011; Johnson et al., 2014; Povirk and Shuker, 1994; Rajski and Williams, 1998; Schärer, 2005; Zhu et al., 2013). In addition, there is evidence that some as-yet-unidentified endogenous DNA cross-link(s) may drive human aging (Bergstrahl and Sekelsky, 2007; Niedernhofer et al., 2005). 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 (Chen and Schuster, 2013; Rajendran et al., 2011).

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; Kozekov et al., 2010; Povirk and Shuker, 1994). Furthermore, only a small number (1-3%) of the initial alkylation events go forward to generate interstrand cross-links (Kozekov et al., 2010; Povirk and Shuker, 1994; Rink et al., 1993). 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; Carrette et al., 2013; Gao and Orgel, 1999; Gruppi et al., 2014; Haque et al., 2014; Harwood et al., 2000; Hentshel et al., 2012; Hong et al., 2006; Manoharan et al., 1999; Mukherjee et al., 2014; Nakatani et al., 2002; Nishimoto et al., 2013; Noll et al., 2001; O'Flaherty et al., 2013; Pujari et al., 2014; Schärer, 2005; Tomás-Gamasa et al., 2014; Ye et al., 2013), 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 oligodeoxynucleotides. The Protocol for post-synthetic modification of the oligodeoxynucleotides 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 oligodeoxynucleotides (Gao and Orgel, 1999; Lindahl et al., 1977; Negishi et al., 1987; Varshney and van de Sande, 1991) to access one of the original forms of “click chemistry”, hydrazone formation (Kool et al., 2013; Wang and Canary, 2012), 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 oligodeoxynucleotides 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 (Catalano et al., 2015; Dutta et al., 2007; Johnson et al., 2013; Price et al., 2015; Price et al., 2014; Yang et al., 2015).

Critical Parameters

In the preparation of the dC*-containing oligodeoxynucleotide, 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 oligodeoxynucleotides 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 oligodeoxynucleotide 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 oligodeoxynucleotide duplexes of various lengths. However, the melting temperature of the oligodeoxynucleotides 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 (Sági et al., 2001; Vesnaver et al., 1989). 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 at 4 °C for 4 d to obtain 40% yield in a 12 base pair duplex. The location of the dU residue in the oligodeoxynucleotide also can affect yield of Ap generation by UDG. dU residues near the end of oligodeoxynucleotides 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 one to three days. In approximately 5-6 h, the dC*-containing oligodeoxynucleotide can be obtained and the dried sample stored at -20 °C for 16 h (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 h of set-up time and 5 h of incubation. Gel purification of the cross-linked duplex requires approximately 8 h. 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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Figure 1.
Conversion of 2'-deoxycytidine (dC) to ⁴N-amino-2'-deoxycytidine (dC*).

Figure 2.
Conversion of 2'-deoxyuridine (dU) to an abasic site (Ap).

Figure 3.
Formation and possible structures of the Ap-dC* interstrand cross-link.

1 5' -GGTGTGAGGGAGTGAGTGACATTGGAGAGTGTGTT
2 5' -GGTGTGAGGGAGTGAGTGAC*ATTGGAGAGTGTGTT
3 5' ³²P-AACACACTCTCCAA^{dU}ATCACTCACTCCCTCACACC
4 5' ³²P-AACACACTCTCCAA^{Ap}ATCACTCACTCCCTCACACC
5 5' ³²P-AACACACTCTCCAA^{Ap} A ATCACTCACTCCCTCACACC
3' -TTGTGTGAGAGGTT A ^{C*}TAGTGAGTGAGGGAGTGTGG
6 5' -AACACACTCTCCAA^{dU}ATCACTCACTCCCTCACACC
7 5' -AACACACTCTCCAA^{Ap}ATCACTCACTCCCTCACACC
8 5' -AACACACTCTCCAA^{Ap} A ATCACTCACTCCCTCACACC
3' -TTGTGTGAGAGGTT A ^{C*}TAGTGAGTGAGGGAGTGTGG

Figure 4.
Sequences used in these Protocols.

Figure 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 h. 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 ^{32}P -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**.

Figure 6.

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 h. 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**.