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Method Article

Preparation of DNA interstrand cross-link repair intermediates induced by abasic sites



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A B S T R A C T

DNA interstrand cross-links (ICLs) are extremely deleterious DNA lesions, which can block different DNA transactions. A major step in ICL repair involves strand cleavage activities flanking the cross-linking site, also known as unhooking. The cleavage generates a single-stranded DNA remnant attached to the unbroken strand, often referred to as the unhooked ICL repair intermediates. The unhooked ICLs are substrates for specialized DNA polymerases, leading to the eventual restoration of the duplex DNA structure. Although these repair events have been outlined, the understanding of molecular details of the repair pathways has been hindered by the difficulty of preparing structurally defined ICL repair intermediates. Here, we present a straightforward method to prepare model ICL repair intermediates derived from a ubiquitous type of endogenous DNA modification, abasic (AP) sites. AP-derived ICLs have emerged as an important type of endogenous ICLs. We developed the method based on commercially available materials without the requirement of synthetic chemistry expertise. The method is expected to be accessible to any interested labs in the DNA repair community.

- The method exploits the alkaline lability of ribonucleotides and uses designer oligonucleotides to create ICL repair intermediates with varying lengths of the unhooked strand.
- Strand cleavage at ribonucleotides is achieved using NaOH, which avoids the potential for incomplete digestion during enzymatic workup due to specific substrate structures.
- The method is grounded on the high cross-linking yield between an AP lesion and a nucleotide analog, 2-aminopurine, via reductive amination, developed by Gates and colleagues.

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A R T I C L E I N F O

Method name: Preparation of DNA interstrand cross-link repair intermediates induced by abasic sites

Keywords: DNA interstrand cross-links, DNA repair, DNA damage, DNA lesion, DNA adduct

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Abbreviations: 2AmPr, 2-aminopurine; AP sites, abasic sites; dU, deoxyuridine; FAM, 6-fluoresceine; MES, 2-(N-morpholino)ethanesulfonic acid; ODN, oligodeoxynucleotide; UDG, uracil-DNA glycosylase.

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SPECIFICATIONS TABLE

Subject Area;	Biochemistry, Genetics and Molecular Biology
More specific subject area;	<i>DNA repair</i>
Method name;	<i>Preparation of DNA interstrand cross-link repair intermediates induced by abasic sites</i>
Name and reference of original method;	<i>Jin Tang, Feng Tang, Linlin Zhao, Facile Preparation of Model DNA Interstrand Cross-Link Repair Intermediates Using Ribonucleotide-Containing DNA, DNA Repair, 2022, DOI: https://doi.org/10.1016/j.dnarep.2022.103286</i>
Resource availability;	<i>All materials are commercialized.</i>

Background

The concept of using chimeric RNA/DNA oligonucleotides followed by the digestion of ribonucleotides to construct psoralen-induced ICL repair intermediates was first explored by Couvé et al. [1]. Later, Semlow et al. also used a similar strategy to adjust the length of unhooked intermediates to assess the repair capability of NEIL3 glycosylase [2]. Although these early studies have laid the conceptual groundwork, the methods have not been carefully evaluated for semi-preparative scale synthesis and for additional ICL substrates. In addition, both methods rely on RNase, and the efficiency and yield of enzymatic digestion-based methods are often limited by the cross-linking chemistry, the spacing of rNMP residues, and steric hindrance. Here, we seek to develop a chemical-based method based on the alkaline lability of rNMPs to generate strand cleavage at rNMPs sites. The resulting cleavage sites provide convenient access to the ICL substrates harboring different lengths of the unhooked strand.

Method details

Step 1: Generation of an oligodeoxynucleotide (ODN) containing an abasic (AP) site

- Materials and apparatus

 1. Chemicals were from Sigma Aldrich or Fisher Scientific and were of the highest grade available.
 2. Uracil-DNA glycosylase (UDG) and RNase HII were from New England Biolabs.
 3. DNA sequencing gel equipment, Bio-Rad Mini-PROTEAN gel casting and electrophoresis equipment set, and Milli-Q water
 4. ODNs containing modified nucleotides were purified by high-performance liquid chromatography (HPLC) and were from Integrated DNA Technologies. The sequences of DNA substrates described in this method are as follows:

A 30-nt oligodeoxynucleotide containing a deoxyuridine was used as a precursor for AP site-containing substrates (hereinafter referred to as T1).

T1: 5'-FAM/AGA CTG CAT /dU/AC GAA GCG AAT CCT TCC CCC

FAM, 6-fluoresceine; dU, deoxyuridine

Two 17-nt oligomers containing a 2-aminopurine and two flanking ribonucleotides were used for cross-linking and preparation of ICL repair intermediates with two DNA remnants of different sizes (hereinafter referred to as R1 and R2).

R1: 5'-CGC TTC rGTA /2AmPr/TrG CAG TC

R2: 5'-CGC TrUC GTA /2AmPr/TG CrAG TC rN denotes ribonucleotides; 2AmPr, 2-aminopurine

- Buffers and solutions

 1. UDG reaction buffer (5X): 500 mM HEPES pH 7.7, 5 mM dithiothreitol, 5 mM EDTA (ethylenediaminetetraacetic acid disodium salt)
 2. 1 M NaOH
 3. 1 M NaBH₄
 4. 5X TBE buffer: 445 mM Tris, 445 mM boric acid, 10 mM EDTA
 5. Milli-Q water

- Procedure

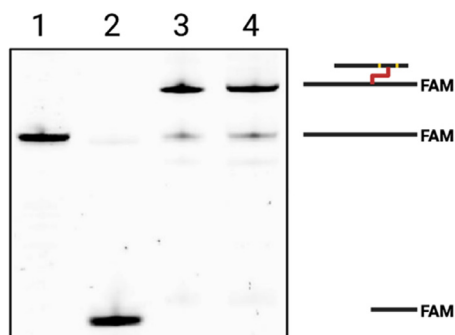


Fig. 1. Representative denaturing PAGE analysis of the cross-linking reaction. Lane 1, T1. Lane 2, reaction products of AP-containing T1 after the NaOH cleavage reaction. Lane 3, cross-linking reaction products of AP-containing T1 with R1. Lane 4, cross-linking reaction products of AP-containing T1 with R2. Samples in lanes 3 and 4 contained 5-fold more materials relative to those in lanes 1 and 2. The gel image was adapted from the Fig. 1d of Ref [4].

1. Dissolve commercial ODNs in water to a final concentration of approximately 400 μM .
2. To a total of 3 nmol T1, add 8 μL 5X UDG reaction buffer and 6 μL 5,000 unit/ μL UDG. Dilute to a total volume of 40 μL with water. Allow the UDG digestion for 6 hours at 37°C.
3. After the reaction, the formation of AP sites is confirmed by alkaline-mediated cleavage at AP sites followed by denaturing polyacrylamide gel electrophoresis (PAGE) analysis.
4. To an aliquot of 0.5 μL reaction mixture, add 2.5 μL water to dilute it 6-fold. The resulting DNA concentration is 12.5 μM .
5. Take 1 μL diluted reaction mixture, add 3.7 μL 1 M NaOH and 7.8 μL water, incubate at 65°C for 1 hour followed by neutralization with HCl. This reaction cleaves the AP-containing T1 at AP sites and generates single-strand breaks.
6. Take 1 μL diluted reaction mixture, add 1 μL 1 M NaBH₄, 10.5 μL water and incubate on ice for 30 min. NaBH₄ quenches the reaction by reducing any unreacted AP sites.
7. Analyze the reaction products using a 7 × 10 cm 18% denaturing PAGE. Recipe of the gel solution: 18% acrylamide/bis-acrylamide (19:1), 7 M urea, 1X TBE solution.
8. Relative to T1 (Fig. 1, lane 1), a faster-migrating band in the NaOH-treated sample, corresponding to an ODN product from single-strand break, confirms the formation of AP sites (Fig. 1, lane 2). The yield of the UDG reaction can be calculated by dividing the band intensity of the faster migrating band by the sum of band intensities from the same lane. The yield of AP sites is over 95% using the reaction recipe described above. If a lower yield is observed, consider extending the UDG digestion time or adding more UDG.

Step 2: Purification of the AP-containing T1 from the UDG reaction mixture

- Materials

1. Bio-Spin® Columns with Bio-Gel® P-6 (catalog No. 7326227)
2. Benchtop centrifuge
3. Phenol/chloroform mix: UltraPure™ phenol:chloroform:isoamyl alcohol (25:24:1, v/v; catalog No. 15593031)
4. Thermo Scientific Savant SpeedVac DNA130 vacuum concentrator system
5. Thermo Scientific NanoDrop 1000 spectrophotometer

- Procedure

1. Add 40 μL phenol/chloroform solution to the UDG reaction mixture. Vortex for 20 s. Centrifuge at 16,000 g for 5 min. Remove the bottom layer (phenol/chloroform), add another 40 μL phenol/chloroform solution to the remaining solution. Vortex and centrifuge again.
2. Transfer the upper layer solution to a clean centrifuge tube.
3. Remove phenol and salt using a Bio-Rad P-6 column. Replace the buffer in the Bio-Rad P-6 column with water (follow the protocol from manufacture), and load the upper layer solution

to a buffer exchanged P-6 column. Centrifuge at 1,000 g for 4 minutes. Now the AP-containing T1 is in water. Measure the concentration with a NanoDrop spectrophotometer. Calculate the concentration based on the Beer-Lambert law.

4. Dilute the AP-containing T1 to a concentration of 45 μ M with Milli-Q water or reduce the volume using a SpeedVac vacuum concentrator (without drying).

Step 3: Preparation of DNA interstrand cross-links between AP-T1 and R1 (or R2)

- Materials

1. Bio-Rad C1000 Touch Thermal Cycler
2. DNA sequencing gel apparatus, Bio-Rad Mini-PROTEAN gel casting and electrophoresis equipment set

- Buffers and solutions

1. MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (10X): 300 mM MES pH 6.5, 1 M NaCl, 10 mM EDTA
2. M NaOAc pH 5.4
3. M NaCNBH₃
4. TBE buffer (5X): 445 mM Tris, 445 mM boric acid, 10 mM EDTA

- Procedure

1. Anneal an AP-containing T1 with R1 (or R2). To a solution of 26.7 μ L 45 μ M AP-containing T1 (1.2 nmol), add 1.44 nmol R1 (or R2), 4 μ L 10X MES buffer, and water to a total volume of 40 μ L. Mix the solution. Incubate the solution following a temperature gradient: 75°C for 5 min, 75 to 40°C at 1°C/min, 40°C for 10 min, down to 4°C.
2. Check the annealing efficiency using a 7 \times 10 cm 18% native PAGE (recipe for gel solution: 18% acrylamide/bis-acrylamide (19:1), 0.35X TBE). Optimize the annealing procedure if the annealing efficiency is lower than 95% (see Note 1 for details).
3. Cross-linking between an AP lesion and a nucleotide analog, 2-aminopurine, via reductive amination. To the solution containing annealed AP-T1/R1, add 2.5 M NaCNBH₃ and 1.5 M NaOAc pH 5.4 to final conditions of 250 mM NaCNBH₃ and 750 mM NaOAc. Incubate the solution at 37°C for 12-14 hr.
4. Check the cross-linking efficiency using a 7 \times 10 cm 18% denaturing PAGE. Fluorescein signals decrease after the overnight incubation, likely due to the change of pH. Approximately 5-fold more cross-linked reaction products are needed to produce similar fluorescent signals relative to T1. The cross-linked products migrate slower than T1 (Fig. 1, lanes 3 and 4). The cross-linking reaction yield is calculated by dividing the intensity of the slower migrating band by the sum of the intensities from the same lane. Usually, the cross-linking yield is greater than 90% [3].

Step 4: Preparation of the unhooked ICLs

- Materials

1. A dry bath incubator with a heated lid
2. Corning® Costar® Spin-X® centrifuge tube filters (catalog No. CLS8162)
3. 3M Empore™ extraction disk cartridges (C18) (catalog No. 56221-502)
4. Thermo Scientific Savant SpeedVac DNA130 vacuum concentrator system
5. DNA sequencing gel equipment, Bio-Rad Mini-PROTEAN gel casting and electrophoresis equipment set
6. Thermo Scientific NanoDrop 1000 spectrophotometer
7. shrimp alkaline phosphatase (New England Biolabs, catalog No. M0371S)

- Buffers and solutions

1. 5 M NaOH
2. 1 M Tris pH 8.0
3. 5% ACN (acetonitrile, HPLC grade)/H₂O
4. 95% ACN/H₂O

- Procedure

1. To a solution of 100 μ L with 10 μ M AP-TA/R1 (or R2) ICLs, add 4.17 μ L 5 M NaOH, incubate at 55°C for 1 hour (set the dry bath temperature to 55°C and lid temperature to 95°C).

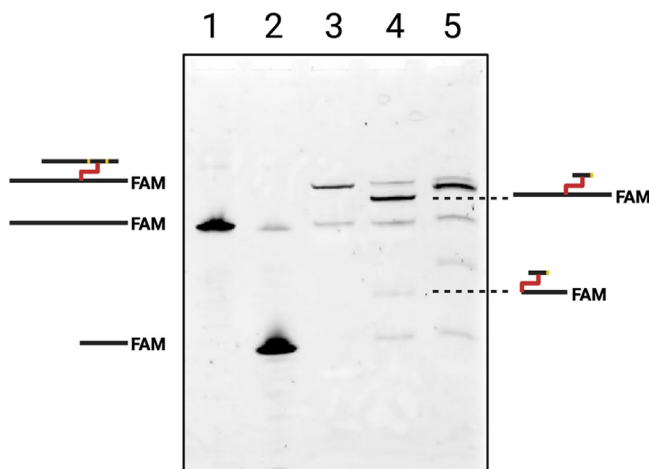


Fig. 2. Representative 7×10 cm denaturing PAGE analysis for monitoring the yield of unhooked ICLs. Lane 1, T1. Lane 2, AP-containing T1 after NaOH treatment. Lane 3, cross-linking reaction products of AP-containing T1 with R1. Lane 4, the sample from lane 3 treated with NaOH (ICL1 crude products). Lane 5, the sample from lane 4 of Fig. 1 treated with NaOH (ICL2 crude products).

2. Monitor the reaction yield using a 7×10 cm 18% denaturing PAGE (Fig. 2). Optimization of the yield of unhooked ICLs by altering the concentration of NaOH, incubation time, and temperature may be needed for different ICL substrates. With the ODNs used in this procedure, the highest yield of ICLs was observed using 0.2 M NaOH at 55°C for 60 min. The resulting unhooked ICLs with a remnant of R1 or R2 are referred to as ICL1 and ICL2, respectively.
3. Separate the reaction products using a 30×38 cm 0.75 mm 18% denaturing PAGE.
4. Cut the desired gel bands containing ICL1 (or ICL2) using a clean razor blade. Freeze the gel band in a -20°C freezer. Crush the gel with a glass rod.
5. Extract the unhooked ICLs with 1 mL 10 mM Tris pH 8.0 at 4°C overnight. Remove the gel particles with a Spin-X centrifuge tube filter by centrifuging at 16,000 g for 5 min. The unhooked ICLs are in Tris and other components in the PAGE media.
6. Clean ICL1 (or ICL2) by solid-phase extraction. Pre-condition the C18 cartridge by washing the cartridge with 2 mL 5% ACN/ H_2O , 2 mL 95% ACN/ H_2O , and 2 mL 5% ACN/ H_2O . Load the sample onto the C18 cartridge, wash the cartridge with 1 mL 5% ACN/ H_2O , and elute ICL1 (or ICL2) using 500 μL 95% ACN/ H_2O . Remove the organic solvent using a SpeedVac vacuum concentrator. Reconstitute the sample with 20 μL Milli-Q water, and measure the concentration using a NanoDrop spectrophotometer.
7. Check the purity of the unhooked ICLs with 30×38 cm 0.4 mm 18% denaturing PAGE (Fig. 3).

Notes:

1. AP-containing ODNs are heat labile, and a lower annealing temperature is desired to avoid the formation of DNA strand breaks. The annealing procedure is optimized in terms of the denaturing temperature, denaturing time, temperature gradient, annealing temperature, and buffer composition and pH. The annealing yield is monitored by the native polyacrylamide gel electrophoresis.
2. ICLs generated using this procedure contain a mixture of phosphate-containing and dephosphorylated 3'-end of the "unhooked" strand (remnant of R1 or R2), as evidenced by mass spectrometry and PAGE analyses.¹ If a homogeneous unhooked ICL substrate is desired, an additional step to remove the phosphate group using alkaline phosphatase can be included before PAGE purification. 1 unit of shrimp alkaline phosphatase completes the removal of a phosphate group from 1 pmol DNA in 30 min at 37°C.

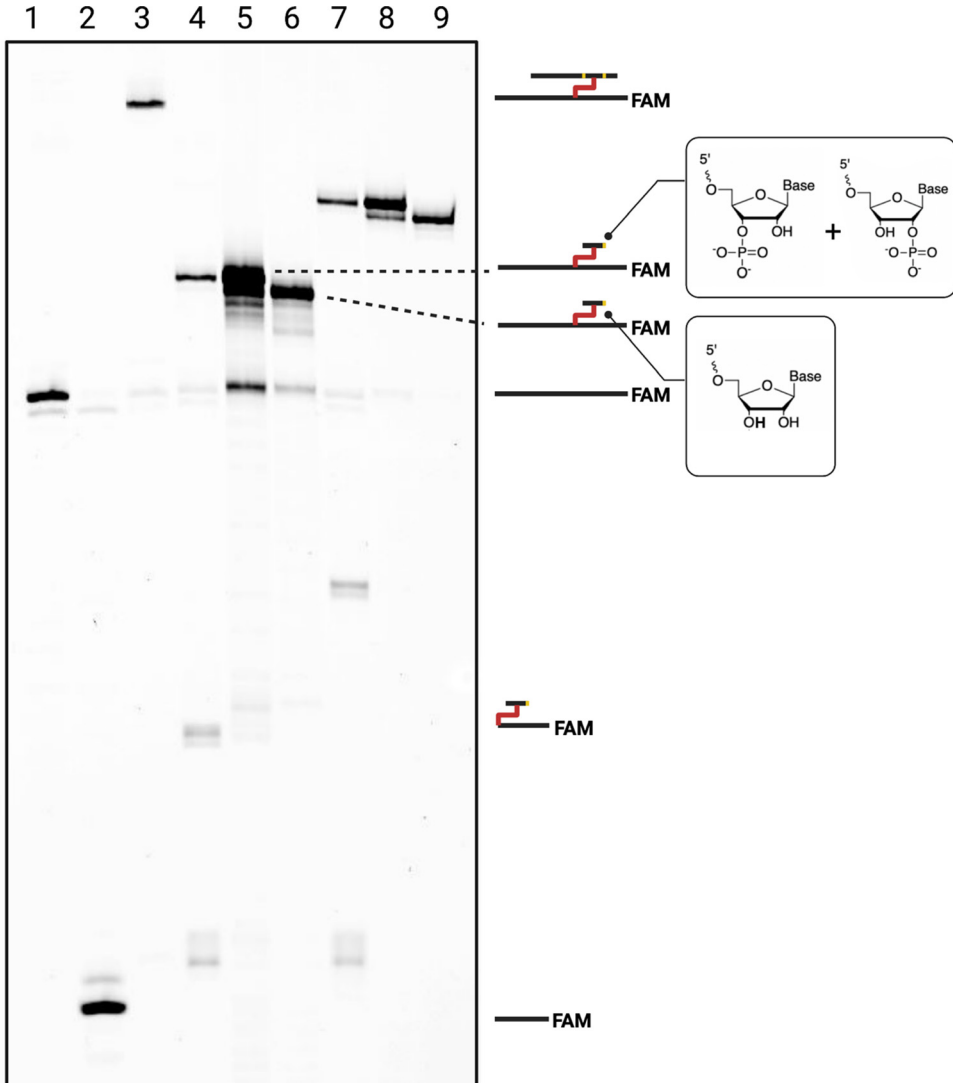


Fig. 3. Representative denaturing PAGE analysis of the ICLs after purification and alkaline phosphatase treatment. Samples in lanes 1 to 4 are the same as those in Fig. 2. Lane 5, ICL1 after gel purification (95% purity based on the observed intensities). The major source of impurity may be the dissociation of the two cross-linked strands as evidenced by the presence of starting materials in lane 5. Lane 6, the sample from lane 5 treated with alkaline phosphatase. The experiment facilitates the assignment of multiple product bands in lane 5. Lane 7, the same as lane 5 in Fig. 2. Lane 8, the gel-purified ICL2 (99% purity based on the observed intensities). Lane 9 is the sample from lane 8 treated with alkaline phosphatase.

- Although ODNs and oligoribonucleotides bearing a 5'-phosphate generally migrate faster than those with 5'-OH, the 3' terminal chemistry affects the migration differently. For oligoribonucleotides, those with a 3'-OH terminus migrate slower than the phosphorylated counterpart [5]. This explains the usual migration pattern observed in the chimeric products (ICL1 and ICL2), with phosphorylated products migrating slower than their hydroxylated counterparts (lanes 5, 6, 8, 9 in Fig. 3).

Method validation

The molecular weight of the ICL substrates can be verified by mass spectrometry. However, substrates with more than 30 nt tend to have poor ionization efficiencies. The signal can be enhanced by injecting more materials onto the LC-MS system. For example, 30 pmol of ICL1 was loaded onto an Agilent Zorbax SB-C18 column (0.5 × 250 mm, 5 μm in particle size) connected to a Thermo TLQ mass spectrometer. Solvent A: water, and solvent B: methanol with 400 mM hexafluoroisopropanol. Samples were analyzed at 8 uL/min with a 50-minute gradient starting at 5% B and increasing to 20% B in 5 min, then to 45% B in 45 min. Example mass spectrometric data are shown in Fig S3 of Ref [4]. The presence of the cross-linked remnant can also be verified using primer extension assays. ICLs block DNA replication and result in products corresponding to polymerase stalling at or prior to the cross-linked nucleotide. An example is shown in Fig 3 of Ref [4].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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