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A general method for quantifying sequence effects on nucleobase oxidation in DNA

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Abstract
Oxidative damage to DNA has long been associated with aging and disease, with guanine serving as the primary target for oxidation owing to its low ionization potential. Emerging evidence points to a critical role for sequence context as a determinant of the guanine ionization potential and the associated chemical reactivity of the guanine, as well as the spectrum of damage products that arise from oxidation. Recent studies also suggest that the generally accepted model of oxidation hotspots in runs of guanine bases may not hold for biologically relevant oxidants. One of the primary methods used to address these important problems of sequence context utilize gel electrophoresis to identify the location and quantity of base damage arising in model oligonucleotides. However, this approach has limited study to those agents that produce few strand breaks arising from deoxyribose oxidation, while ionizing radiation, Fenton chemistry and other biologically relevant oxidants produce sizeable proportions of both base and sugar damage. To this end, we have developed a universal method to quantify sequence context effects on nucleobase damage without interference by strand breaks from deoxyribose oxidation.

Keywords
Oxidative DNA damage; guanine oxidation; sequence selectivity; sequence context; nitrosoperoxycarbonate; γ-radiation; hydroxyl radical; strand breaks; exonuclease III

1. Introduction
DNA damage resulting from oxidative stress has been strongly associated with cancer, chronic degenerative diseases and aging (reviewed in refs. 1,2). While both the nucleobase and deoxyribose moieties of DNA are targets for oxidation, recent interest in charge transfer and sequence context effects on the location and quantity of damage have focused attention on the bases, with particular attention paid to guanine due to its low ionization potential (3) and the myriad products arising from its primary and secondary oxidation (4). Sequence context has been shown to play a significant role in modulating the ionization potential of guanine in duplex DNA and, hence, the reactivity of guanine with oxidizing agents. For example, it has been demonstrated that many one-electron oxidants, such as anthraquinones (5), rhodium complexes (6) and riboflavin-mediated photooxidation, selectively damage guanine when the base is located adjacent to other guanines (e.g., GG, GGG). This reactivity has been rationalized on the basis of the low ionization potential conferred to guanine in these sequence contexts and the migration of cationic holes to these sites from guanine radical cations located in sequence contexts conferring higher ionization potentials (7).
the other hand, we have recently demonstrated that nitrosoperoxycarbonate, an oxidant formed by reactive oxygen species during chronic inflammation, is selective for oxidizing guanines with the highest ionization potentials (8), while hydroxyl radical generated by Fe$^{+2}$-EDTA and γ-radiation are equally reactive with guanines irrespective of sequence context (Margolin et al., manuscript in preparation). We have, therefore, shown that sequence selectivity of guanine oxidation in double-stranded DNA is not only a function of sequence context, as has been previously thought, but also depends on the oxidant identity and its interactions with the DNA. Determination of sequence effects in nucleobase oxidation by various agents can thus provide valuable information on their mechanism of damage induction in DNA and on the relationship between reactivity and the potential to cause mutations.

The most widely employed approach to studying sequence context effects on DNA damage involves gel electrophoretic analysis of damage in model oligodeoxynucleotides exposed to oxidizing agents. Base damage in the oligos is converted to strand breaks by treating the DNA with either hot piperidine or with DNA repair enzymes such as E. coli formamidopyrimidine DNA glycosylase (Fpg) for oxidized purines and E. coli endonuclease IV (Nth) for oxidized pyrimidines (10). The strand breaks are then localized on sequencing gels and quantified by autoradiography or phosphorimager analysis. The problem inherent with this approach is that it is limited to oxidizing agents that produce only base damage, since oxidation of deoxyribose results in the formation of direct strand breaks and easily hydrolysable abasic sites that create a background of strand breaks that interfere with quantification of base-derived strand breaks. Such is the case with the biologically relevant oxidizing agent such as ionizing radiation, peroxynitrite, and Fenton chemistry arising with iron and copper (11).

We have developed a method that obviates the background of deoxyribose oxidation-induced strand breaks. Using relatively inexpensive 3'-phosphorothioate-protected oligodeoxynucleotides, the background of strand breaks is removed from the analysis by digestion of the oxidized oligos with E. coli exonuclease III (ExoIII). Subsequent treatment with hot piperidine or DNA repair enzymes exposes the base damage as strand breaks that can be localized and quantified in sequencing gels. This approach provides a nearly universal method for defining the sequence context effects on oxidative damage to DNA.

2. Materials

2.1. Gel electrophoresis and purification of oligodeoxynucleotides

1. Oligodeoxynucleotides for analysis, as well as their complements, can be ordered from Integrated DNA Technologies (www.idt.com) or any company specializing in custom oligodeoxynucleotide synthesis. As an example, we and others have used the following model oligodeoxynucleotide for studies of sequence context effects on guanine oxidation: 5’-CGTACTCTTTGGTXGYTXGYTTCTTCTAT-3’ (7,8). This sequence contains consensus portions on both 5’ and 3’ ends, as well as a TGG sequence that is placed at the same position in all oligodeoxynucleotides and acts as a normalization standard. Damage at guanines in the variable sequences XGY (where X and Y are thymine, cytosine, guanine or adenine) is always normalized to the damage at the central guanine of the TGG sequence (See Notes 1 and 2).

2. For experiments with agents that induce high levels of direct strand breaks (i.e., deoxyribose oxidation), the oligodeoxynucleotides should contain three consecutive phosphorothioate linkages at their 3’ ends (also available from Integrated DNA Technologies): 5’-CGTACTCTTTGGTXGYTXGYTTCTTCTTC-
T-S-A-S-T-3’, where S is a phosphorothioate linkage. The complements to these oligodeoxynucleotides should also contain three phosphorothioate linkages at their 3’ ends (See Note 3).

3. TBE buffer (10×): 0.89 M Tris base, 0.89 M boric acid, 0.02 M EDTA (disodium salt), pH 8.3. Store at ambient temperature.

4. 40% acrylamide:bis-acrylamide (19:1) solution from American Bioanalytical. Store at 4 °C. Acrylamide is a neurotoxin in unpolymerized form and should be handled with care.

5. N,N,N,N’-Tetramethyl ethylenediamine (TEMED), from Sigma-Aldrich

6. Ammonium persulfate, 10% (w/v) aqueous solution, prepared directly before use.

7. Gel electrophoresis system: Model S2 Sequencing gel electrophoresis apparatus (Lab Repco).

8. Power Pac 3000 power supply with a temperature probe (Bio-Rad).

9. Elution buffer: 0.5 M ammonium acetate, 10 mM magnesium acetate

10. Ultrafree-MC Centrifugal Filter Devices (Millipore)

11. 3 M sodium acetate, pH 5.2; 100% ethanol; 70% ethanol.

2.2 Labelling of oligodeoxynucleotides

1. γ-[³²P]-ATP, 10 mCi/ml, 6000 Ci/mmol (Perkin Elmer)

2. T4 Polynucleotide kinase (PNK) and PNK reaction buffer (10×) (New England Biolabs)

3. Sephadex G-25 spin columns (Roche)

2.3 Damage reactions of oligodeoxynucleotides and preparation of samples for gel analysis

1. 2 M piperidine solution

2. E. coli Fpg glycosylase and 10× reaction buffer (New England Biolabs or Trevigen)

3. Glycogen (Roche)

4. Formamide gel loading buffer: 95% (v/v) formamide, 20 mM EDTA, pH 8.0, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol

2.3 Sequencing gel analysis and autoradiography

1. See Section 2.1

2. Imaging Screen K (Bio-Rad)

3. Phosphorimagter Storm 820 model (GE)

2.4 Image analysis

ImageQuant software (GE)

2.5 Removal of direct strand breaks from damaged oligodeoxynucleotides

1. E. coli Exo III and 10× exonuclease III reaction buffer (NEbuffer 1) (New England Biolabs)
2. Sephadex G-25 spin columns (Roche)

3. Methods

This method for studying the sequence effects on nucleobase oxidation uses small, synthetic 5'-32p-labeled, double-stranded oligodeoxynucleotides containing guanines in defined sequence contexts. After treatment with a damaging agent, a strand break is introduced at the sites of guanine oxidation by treatment with either hot piperidine or Fpg glycosylase (reviewed in ref. 10). The relative instability of most of the primary and secondary guanine oxidation products to treatment by either one or both of these agents ensures the complete conversion of most guanine oxidation products to strand breaks. The treated oligodeoxynucleotides are then resolved on a DNA sequencing gel and the strand breaks formed at each oxidized guanine are quantified using standard image analysis software.

For analysis of guanine oxidation by an agent that produces significant amounts of deoxyribose oxidation, a protocol modification is introduced that allows the removal of the background of direct strand breaks. This is accomplished preparing double-stranded oligodeoxynucleotides that contain exonuclease-resistant phosphorothioate linkages at their 3’ ends and treating these oligodeoxynucleotides with ExoIII after the damage reaction is complete (See Note 3). Phosphorothioate linkages protect the 3’ ends of the parent oligodeoxynucleotides and oligodeoxynucleotides containing only base lesions from digestion by ExoIII (12). Oligodeoxynucleotides containing strand breaks now have exposed 3’ ends that are substrates for the enzyme. ExoIII recognizes substrates with 3’-hydroxyl, 3’-phosphate and 3’-phosphoglycolate termini (13), as well as substrates containing abasic sites that are cleaved endonucleolytically (14) and thus removed from base damage analysis. After the ExoIII reaction is complete, the only [32P]-labeled oligodeoxynucleotides remaining in solution are the parent molecules or those containing damaged bases that can be revealed as strand breaks following reaction with hot piperidine or Fpg treatment and gel electrophoresis (see Figure 1). Our control experiments have shown that ExoIII treatment does not alter the sequence selectivity of guanine oxidation observed in sequence damage experiments (i.e., the presence or absence of ExoIII does not affect the quantity and location of base damage, as shown in Figure 2 for the guanine-specific oxidant, nitrosoperoxycarbonate).

The first part of this section describes the steps necessary to determine sequence selectivity of guanine oxidation by agents that selectively oxidize guanines in duplex DNA. The second part describes the modifications of the method that provide for removal of the background of direct strand breaks induced by agents capable of significant deoxyribose oxidation. When working with a new damaging agent it is necessary to measure the amount of direct strand breaks that it causes, in order to determine if the ExoIII treatment described in the second part of the method should be used. This can often be accomplished with a single dose-response experiment with and without hot piperidine treatment. The same dose-response experiment should be conducted to determine a dose of the damaging agent that will be used in all subsequent experiments. A dose that is typically chosen should be high enough to induce statistically significant damage at every guanine of interest, as measured by a paired Student’s T-test. However, it should be low enough to damage less than 30% of the parent oligodeoxynucleotide. According to a Poisson distribution, this low level of damage ensures that each oligodeoxynucleotide sustains an average of one or fewer damage reactions. If these single-hit conditions are violated, sequencing gel quantification of DNA damage becomes impossible due to the inability to quantify a second damage event.
3.1 Purification of oligodeoxynucleotides by gel electrophoresis

All synthetic oligodeoxynucleotides should be purified before use in sequence damage experiments to remove failure sequences and damaged molecules. Gel electrophoresis is the most efficient and reliable method for oligodeoxynucleotide purification (See Note 4). Due to the high frequency of nucleobase damage occurring in synthesis, all oligodeoxynucleotides should be treated with hot piperidine prior to purification (See Note 5).

3.1.1 Treatment of oligodeoxynucleotides by hot piperidine—All oligodeoxynucleotides are dissolved in TE buffer (10 mM Tris, 0.5 mM EDTA, pH 8.0) to a final concentration of 100–500 pmol/µl. An equal volume of 2 M piperidine solution in distilled and deionized water is added and the oligodeoxynucleotides are incubated at 90 °C for 20 min. Following drying under vacuum (e.g., Speedvac), the samples are again dissolved in TE containing bromophenol blue dye and 20–25% glycerol to a DNA concentration of 100–500 pmol/µl.

3.1.2 Preparative gel electrophoresis

1. These instructions assume the use of the S2 gel sequencing apparatus from Gibco/BRL, sold by Lab Repco. It is essential that the glass plates are cleaned in 1 M NaOH followed by thorough rinsing with deionized water and wiping with a small amount of acetone or ethyl acetate to dissolve residual contaminants. Sigmacote is applied to the internal surface of one of the plates before assembly to promote sticking of the gel to only one plate. Large binding clips can be used to hold the gasket in place.

2. Prepare 20% acrylamide, 8.3 M urea gel in 10× TBE by mixing 130 g of urea with 26 ml of 10× TBE buffer, 130 ml of 40% 19:1 acrylamide:bis-acrylamide solution and water to a total volume of 260 ml. Gently heat the mixture with stirring to dissolve all of urea, followed by filtration through a membrane with 0.45 µm pores to remove any particulate. To this solution, add 600 µl of 10% ammonium persulfate solution and 60 µl of TEMED. Immediately load ~180 ml of this mixture into the assembled plates using a 60 ml syringe to prepare a 1.2 ml thick, 20% acrylamide gel in 1×TBE; tap to remove any bubbles that form between the plates. Insert a comb with 10 mm x 5 mm teeth and secure it with binding clips. The gel should polymerize in ~30 min.

3. Pre-run the gel with 1×TBE running buffer at 70 w until the gel reaches about 39 °C (use any type of surface temperature probe).

4. Before the samples are loaded on the gel, the wells should be extensively washed with 1×TBE running buffer using a syringe with a small gauge needle to remove particulate and unpolymerized acrylamide.

5. Load no more than 40 nmol (400 µg) of oligodeoxynucleotide into each lane and run the gel for ~6 hr with 1×TBE running buffer at ~70 w to produce a temperature of ~45 °C.

3.1.3. Elution of oligodeoxynucleotides from the gel

1. Separate the glass plates and wrap the plate attached to the gel in Saran wrap or other thin, clear plastic wrap. Visualize oligodeoxynucleotides in the gel by placing the plate on a white sheet of paper in a darkroom and illuminating it from above with a short-ray UV lamp. Mark the DNA bands on the Saran wrap with a black,
fine tipped marker, then use a razor blade to cut out only the upper half of each band; the lower half may contain poorly resolved failure sequences.

2. Place the gel slices into 1.5 ml tubes and macerate the pieces by repeated rolling of a pipette tip along the tube wall. Add elution buffer to the top of the tube and leave the tubes vortexing overnight at the low setting in a 4 °C cold room.

3. Remove the gel particulate using Ultrafree-MC centrifugal filter devices (Millipore). Distribute the filtrate to 1.5 ml tubes in 300 µl aliquots. To each tube add 100 µl of 3 M sodium acetate solution and 1 ml of 100% ethanol. Incubate the tubes at −80 °C for 1 hr and pellet the precipitated DNA at 16,000×g for 30 min at 4 °C. Carefully wash the DNA pellets several times with 70% cold ethanol and air-dry them. Dissolve the dried oligodeoxynucleotides in the buffer that will be used to carry out damage reactions.

3.2 Preparation of [\(^{32}\)P]-labeled, duplex oligodeoxynucleotides

1. Oligodeoxynucleotides with sequences of interest are 5'-end labeled with \(^{32}\)P by transferring a phosphate group from \(\gamma\)-\(^{32}\)P-ATP to the oligodeoxynucleotide using T4 polynucleotide kinase (PNK). In a single tube, mix approximately 100 pmol of oligodeoxynucleotide, 20 units of T4 PNK, 5 µl of 10× T4 PNK buffer, 5 µl of \(\gamma\)-\(^{32}\)P-ATP and deionized water to a final volume of 50 µl. Incubate at 37 °C for 1 hr. Remove unreacted ATP by passing the reaction mixture over a G-25 Sephadex column that has been washed with the buffer in which damage reactions will be carried out (See Note 6).

2. For annealing of complementary oligodeoxynucleotides, add to the mixture approximately 200 pmol of complementary strand. Incubate the mixture in a heating block at 90–95 °C for about 1 min, then turn the heating block off and allow it to cool to 37 °C (this takes ~90 min). The resulting solution will contain ~100 pmol of 5'-\(^{32}\)P] labeled, double-stranded oligodeoxynucleotide, as well as ~100 pmol of unlabeled, single-stranded complementary oligodeoxynucleotide.

3.3 Damage reactions and sample preparation for sequencing gel analysis (see Note 6)

1. When working with a new damaging agent, a dose-response study should be performed with several different concentrations of the damaging agent to determine: a) the optimal dose of the damaging agent to be used in sequence-specific damaging reactions; and b) the level of direct strand breaks produced by the damaging agent. In cases where an agent produces high levels of direct strand breaks (>10% of the total strand breaks revealed after hot piperidine or Fpg treatment) that can interfere with the quantification of damage induced at guanines, ExoIII should be used (see Section 4).

2. Each damage reaction is performed in triplicate, with triplicate control samples to which only the vehicle is added. Note that, except for the absence of the damaging agent, the control tubes are treated exactly the same way as the damage reactions. Proceed with the damage reactions according to the protocol. Each reaction should contain 5–15 µl of labeled oligodeoxynucleotide for sufficiently strong signal.

3. After the damage reaction is complete, the damaging agent is removed by passing each sample over a G-25 spin column (See Note 7).

4. Sites of base damage are now converted to strand breaks either by treatment with hot piperidine or Fpg (10):
a. For hot piperidine treatment, aliquot a defined volume of the damaged oligodeoxynucleotide into a separate tube and add equal volume of 2 M piperidine solution. Incubate the tubes at 90 °C for 20 min, then dry the reaction under vacuum (e.g., Speedvac; see Note 9). Add 5 µl of formamide gel loading buffer to each tube.

b. For Fpg treatment, add the appropriate volume of 10× Fpg buffer and 1–3 units of Fpg to a defined volume of damaged oligodeoxynucleotide (see Note 10). Incubate at 37 °C for 1 hr. To precipitate the DNA after the reaction, add 3 M NaOAc (pH 5.2) equal to one-half of the reaction volume, 1 µl of glycogen, and 3.5 volumes of 100% ethanol. Incubate at −80 °C for 1 hr, then pellet the DNA by centrifugation at 16,000×g for 30 min at 4 °C. Air-dry the pellets and dissolve them in 5 µl of formamide gel-loading buffer.

3.4 Separation of damage reaction products on DNA sequencing gel and autoradiography

1. Prepare 0.4 mm thick sequencing gels containing 8.3 M urea and 20% acrylamide sequencing gel in 1×TBE as described in steps 1 and 2 of section 3.1.2, using the 0.4 mm spacers. The sequencing gel is only 0.4 mm thick (compared to 1.2 mm thick preparative gel), so prepare only one-half of the total gel solution volume described in step 2 of section 3.1.2 (i.e., 130 ml).

2. Pre-run the gel at 70 w in 1× TBE until temperature reaches ~39 °C.

3. Wash the wells of the gel with 1×TBE as described in step 4 of section 3.1.2

4. Load 3 µl of each sample in each well and run the gel at ~70 w to achieve a temperature of ~45 °C for 3 hr (see Note 11)

5. Separate the plates, taking care that the gel adheres to only one of the plates. Wrap the plate with the gel with Saran wrap. Position the glass plate with the gel facing upward and place the Imaging screen on top of the gel. Expose for 2–12 hr as needed to provide a strong exposure when damage bands are weak (see Note 12).

6. Scan the Imaging plate according to manufacturers directions to obtain a digital image of the gel. An example of a typical result is shown in Figure 2A.

3.5 Quantification of damage at each guanine using phosphorimager software

1. Locate the position of each guanine of interest within each lane, using sequencing standards as needed (see Note 11).

2. Following user directions for the software, determine the amount of radioactivity corresponding to each band as a percentage of total radioactivity in the entire lane (including the band corresponding to intact, parent oligodeoxynucleotide).

3. Calculate the average percentage of total radioactivity present in each of the three bands (derived from the TGG and two XGY sites) for the three control and three oxidant treated samples.

4. Subtract the average values for the controls from the average values for the oxidized samples to obtain the net signal due to guanine oxidation (See Note 13).

5. Normalize the two signals for guanines in the XGY sequence contexts by dividing their signal values by that for the TGG normalization sequence. An example of a typical result showing the dependence of guanine oxidation by nitrosoperoxycarbonate as a function of guanine ionization potential is shown in Figure 2B.
3.6 Removing a background of direct strand breaks

1. This section describes modifications to the procedure described in section 3.2, in which the background of direct strand breaks produced by some oxidizing agents is removed prior to analysis of guanine oxidation. This is accomplished using oligodeoxynucleotides containing phosphorothioate linkages at their 3’ ends and ExoIII to remove oligodeoxynucleotide fragments that contain 3’ ends as a result of deoxyribose oxidation. Hot piperidine or Fpg treatment is subsequently used to introduce strand breaks at the sites of guanine oxidation.

2. Follow steps 3.1.1 through 3.2.2, part 3 (See Note 14).

3. ExoIII treatment is achieved by adding an appropriate volume of 10× NEbuffer1 buffer and 1–5 units of ExoIII to a defined volume of damaged oligodeoxynucleotide. Incubate the tubes at 37 °C for 1 hr (shorter incubation times may be sufficient). De-salt each reaction by passing it over a G-25 spin column (see Note 7).

4. To each of the G-25 eluents, add an equal volume of 2 M piperidine solution and incubate at 90 °C for 20 min. Dry completely under vacuum (e.g., Speedvac; see Note 15) and add 5 µl of formamide gel loading buffer to each tube.

5. Proceed with steps described in sections 3.2.3 (see Note 16) and 3.2.4. An example of the typical result of using ExoIII to remove the background of direct strand breaks is shown in Figure 3A. Relative reactivities of guanines in different sequence contexts with a hydroxyl radical formed by (Fe-EDTA)2− treatment is shown in Figure 3B.

4. Notes

1. It is imperative to have a common normalization sequence in each oligodeoxynucleotide to act as an internal control, as lane-to-lane variations in signal intensity make rigorous quantification impossible without the internal control.

2. It is possible to design longer oligodeoxynucleotides that contain more than two sequence contexts in addition to the internal control. Care should be taken to avoid using shorter oligodeoxynucleotides with low melting temperatures, as they may undergo partial denaturation during incubations at 37 °C.

3. The presence of three phosphorothioate linkages completely inhibits the 3’-to-5’ exonuclease activity of the ExoIII used to remove direct strand breaks induced by the oxidation agents.

4. The oligodeoxynucleotides are received in solid form and contain truncated (i.e., failure) sequences that may interfere with quantification of damage on sequencing gels. It is, therefore, imperative to purify full-length oligodeoxynucleotides away from the truncated sequences. Gel electrophoresis is the most efficient and effective method for oligodeoxynucleotide purification.

5. Synthetic oligodeoxynucleotides also contain detectable amounts of oxidized nucleobases, most notably guanine. This background of base damage may pose serious problems for the analyses by reducing the dynamic range for quantification of guanine oxidation. To remove this background of base oxidation, the oligodeoxynucleotides can be treated with hot piperidine prior to their purification by gel electrophoresis. If using oligodeoxynucleotides containing various modifications, such as biotynilation, unnatural bases or phosphorothioate linkages,
it is often advisable to keep in mind that these modifications may be affected by the hot piperidine treatment.

6. Usually, washing the column four times with 300 µl of buffer is sufficient for complete buffer exchange.

7. The spin column can be washed with deionized water (2x300 µl).

8. It is preferable to perform the [32P]-labeling of the oligodeoxynucleotides and the damage reactions on the same day to minimize [32P]-induced base damage (i.e., radiolytic DNA damage) during storage.

9. Since piperidine is a volatile and toxic chemical, incubations should be carried out in an appropriate fume hood in tubes with screw-top caps.

10. Careful attention should be paid to a manufacturer’s definition of unit values and concentrations for all commercial enzyme preparations. This is particularly important for Fpg, since unit definitions and concentrations often differ for the various manufacturers.

11. Though the position of each guanine within the oligodeoxynucleotides is known and can usually be determined from the relative migration of the DNA fragments, the identity of the cleavage sites can be verified using Maxam-Gilbert sequencing standards (15) or [32P]-labeled synthetic oligodeoxynucleotides with lengths corresponding to the oligodeoxynucleotides arising from guanine oxidations at the three sites.

12. The time needed for an appropriate exposure of the phosphorimager plate depends on the strength of the signal to be quantified and on the condition of the phosphorimaging plate.

13. The statistical significance of differences between control and oxidized samples can be determined using a paired Student’s T-test.

14. Depending on the proportion of deoxyribose oxidation-induced strand breaks caused by an oxidant, the radioactive signal for base oxidation will be diminished following ExoIII digestion. This may necessitate starting with larger amounts of [32P]-labeled oligodeoxynucleotides to produce base damage signals sufficient for quantification. Typically, we use two-fold more of the radioactive oligodeoxynucleotides when analyzing hydroxyl radical-induced guanine oxidation, as compared to the starting amounts in the experiments with agents producing a higher proportion of base damage (e.g., nitrosoperoxycarbonate).

15. The ExoIII must be de-activate and/or completely removed before converting guanine base damage to strand breaks with Fpg, since the newly exposed 3'-ends will be susceptible to digestion by residual ExoIII. Hot piperidine treatment denatures and inactivates ExoIII, so no additional steps are necessary in the procedure.

16. ExoIII-digested samples will contain very short, [32P]-labeled oligodeoxynucleotide fragments that will migrate faster than the fragments arising from guanine oxidation and thus will not interfere with the intended analysis. However, the short fragments may run off the gel into the running buffer, so care should be taken in handling and discarding the contaminated running buffer after the experiment. In addition, the gel should be wrapped with at least two layers of Saran or similar plastic wrap to avoid contaminating the phosphorimaging screen with radioactivity from the short fragments that readily elute from the gel during exposure or from residual contaminated running buffer.
References


Figure 1.
Schematic representation of the ExoIII method for removing direct strand breaks from analyses of sequence context effects on base damage. (A) Oligodeoxynucleotides contain a $[^{32}\text{P}]$ label at their 5' ends and three consecutive phosphorothioate linkages at their 3' ends. (B) Treatment with an agent that oxidizes deoxyribose produces direct strand breaks that possess unprotected 3' ends. (C) ExoIII recognizes unprotected 3' ends and hydrolyzes the oligodeoxynucleotide in a 3'-to-5' direction, releasing the 5' $[^{32}\text{P}]$ label. (D) After gel filtration chromatography, the oligodeoxynucleotide that contained the direct strand break has lost its 5' label and is not be detected during subsequent electrophoresis and autoradiography.
Figure 2.
(A) A typical autoradiogram of a sequencing gel obtained in an experiment with nitrosoperoxycarbonate, a selective oxidant of guanines (8). An oligonucleotide with the sequence 5’-CGTACTCTTTGGTAGATAGCTTCTCTAT-3’ was damaged with 0 and 2 mM nitrosoperoxycarbonate and treated with hot piperidine to convert base lesions to strand breaks. The resulting damage products were separated on a sequencing gel. (B) Plot of the relative amounts of piperidine-sensitive guanine lesions in different sequence contexts produced by nitrosoperoxycarbonate as a function of sequence-specific guanine ionization potential (7,8).
Figure 3.

(A) Illustration of the ExoIII method for removing direct strand breaks. The oligodeoxynucleotides employed in Figure 2 were damaged with Fe$^{+2}$-EDTA, an oxidant that produces a high proportion of deoxyribose oxidation and thus direct strand breaks. These direct breaks were removed by treatment with Exo III, which leaves the intact oligodeoxynucleotides containing guanine base lesions ready for analysis by hot piperidine cleavage. The faster and slower migrating bands in the doublets apparent in lane 2 represent 3'-phosphoglycolate-ended and 3'-phosphate-ended DNA fragments, respectively. Both of these “direct strand break” products are completely removed by ExoIII, as is apparent in lane 4. (B) Plot of the relative amounts of piperidine-sensitive guanine lesions in different ionization potentials.
sequence contexts produced by Fe$^{2+}$-EDTA as a function of sequence-specific guanine ionization potential (7).