Selective enzymatic cleavage and labeling for sensitive capillary electrophoresis laser-induced fluorescence analysis of oxidized DNA bases

Cuiping Li, Hailin Wang*

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, 100085, China

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ABSTRACT

Oxidatively generated DNA damage is considered to be a significant contributing factor to cancer, aging, and age-related human diseases. It is important to detect oxidatively generated DNA damage to understand and clinically diagnosis diseases caused by oxidative damage. In this study, using selective enzymatic cleavage and quantum dot (QD) labeling, we developed a novel capillary electrophoresis-laser induced fluorescence method for the sensitive detection of oxidized DNA bases. First, oxidized DNA bases are recognized and removed by one DNA base excision repair glycosylase, leaving apurinic and apyrimidinic sites (AP sites) at the oxidized positions. The AP sites are further excised by the AP nicking activity of the chosen glycosylase, generating a nucleotide gap with 5'- and 3'- phosphate groups. After dephosphorylation with one alkaline phosphatase, a biotinylated ddNTP is introduced into the nucleotide space within the DNA strand by DNA polymerase I. The biotin-tagged DNA is further labeled with a QD-streptavidin conjugate via non-covalent interactions. The DNA-bound QD is well-separated from excess DNA-unbound QD by highly efficient capillary electrophoresis and is sensitively detected by online coupled laser-induced fluorescence analysis. Using this method, we can assess the trace levels of oxidized DNA bases induced by the Fenton reaction and UV irradiation. Interestingly, the use of the formamidopyrimidine glycosylase (FPG) protein and endonuclease VIII enables the detection of oxidized purine and pyrimidine bases, respectively. Using the synthesized standard DNA, the approach has low limits of detection of 1.1×10^{-19} mol in mass and 2.9 pM in concentration.

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1. Introduction

The oxidative damage of cellular DNA is linked to aging [1], cancer [2] and other age-related human diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [3,4]. The formation of oxidative lesions may result from numerous endogenous metabolic processes as well as from exogenous sources, such as ionizing radiation, UV radiation, redox-cycling drugs and carcinogenic compounds [5,6]. Therefore, there is urgent demand for rapid and sensitive detection methods for oxidatively generated DNA damage.

During the last few decades, a number of analytical methods have been developed to monitor oxidized DNA bases [7]. Among these methods, high performance liquid

chromatography-electrochemical detection and gas chromatography (GC)-mass spectrometry (MS) are two prominent contributions in the field [8–10]. In addition, ^{32}P -postlabeling assays [11], high-performance liquid chromatography tandem mass spectrometry [12], antibody-based immunoassay [13], photoelectrochemical-based detection [14,15] and single-cell gel electrophoresis [16] have been used to determine a variety of oxidative DNA lesions. These methods have their own advantages but limitations still exist. For example, the levels of 8-oxo-dG were overestimated by 10–50 times using GC-MS due to the introduction of artifacts resulting from the hydrolysis of DNA under acidic conditions and high temperatures during the derivatization procedure [17]. Immunological methods demanded high chemical selectivity and strong antigenicity of antibodies. Without these properties, nonspecific recognition of undamaged bases in DNA would affect the accuracy of the determination [18]. The available chromatographic methods and ^{32}P -postlabeling assays that are aimed at measuring an individual DNA base lesion require either

* Corresponding author. Tel.: +86 10 62849600; fax: +86 10 62849600.
E-mail address: hlwang@rcees.ac.cn (H. Wang).

chemical hydrolysis or enzymatic digestion of the oxidized DNA. In these pretreatment processes, additional oxidation of DNA could be introduced leading to artifacts.

Due to its highly efficient and rapid separation, high sensitivity and low sample consumption, capillary electrophoresis has been widely applied in various fields [19]. Capillary electrophoresis with UV or electrochemical detection has been used to analyze the oxidative damage of DNA. However, these assays exhibited low sensitivity [20]. In addition, capillary electrophoresis coupled to laser-induced fluorescence (CE-LIF) was developed to sensitively measure endogenous damage in mitochondrial and genomic DNA [21]. The method involved in the isolation and hydrolysis of genomic DNA or mtDNA, labeling of modified and unmodified nucleotides and micellar electrokinetic chromatography with laser-induced fluorescence detection. The labeling of excess unmodified nucleotides compromises the sensitivity of the CE-LIF detection of trace modified nucleotides.

Quantum dot (QD) has unique optical properties, such as high fluorescence quantum yields and photostabilities against bleaching and is promising optical labels for sensitive analysis [22]. Our group has reported the ultrasensitive detection of BPDE-DNA adducts by taking advantage of QD and CE-LIF [23].

In this study, a sensitive method for the detection of oxidized DNA bases using selective enzymatic cleavage and labeling of the damaged positions in combination with CE-LIF analysis is reported for the first time. The selective cleavage of damaged bases can be achieved by a base excision repair (BER) glycosylase. For example, formamidopyrimidine glycosylase (FPG), which is used as a BER glycosylase, can recognize and remove a wide range of oxidized purines from the correspondingly damaged DNA [24]. The generation of a nucleotide gap caused by the action of the BER glycosylase can be further supplemented with a biotin-ddNTP. In combination with a QD-streptavidin conjugate, a DNA-biotin-streptavidin-QD complex can be produced. By measuring the QD-labeled DNA complex, CE-LIF analysis can be used to assess the level of oxidized DNA bases.

2. Materials and methods

2.1. Chemicals and materials

Escherichia coli, formamidopyrimidine glycosylase (FPG), endonuclease VIII and DNA polymerase I were purchased from New England BioLabs (Ipswich, MA). FastAP thermosensitive alkaline phosphatase was obtained from Fermentas (Thermo Scientific, Pittsburgh, PA). Calf thymus DNA, FeCl₂ and H₂O₂ were obtained from Sangon (Shanghai, China). Biotin-11-ddATP, biotin-11-ddGTP, biotin-11-ddCTP and biotin-11-ddUTP were obtained from PerkinElmer (Wellesley, MA). The Qdot 625 streptavidin conjugate and bovine serum albumin (BSA) were obtained from Invitrogen (Carlsbad, CA). All of the other solvents and chemicals were of analytical grade and obtained from the Beijing Chemical Reagents Company (Beijing, China) or Amresco (Solon, OH). All of the solutions were prepared using ultrapure water from a Purelab Ultra Bioscience water purification system with a 0.22 µm filter (ELGA, U.K.). An uncoated fused-silica capillary (50 µm i.d., 365 µm o.d.) was purchased from the Yongnian Optical Fiber Factory (Hebei, China).

2.2. Instrumentation

Capillary electrophoresis and laser-induced fluorescence (CE-LIF) analysis was carried out using a laboratory-built CE-LIF system, which has been previously described [25,26]. A helium-neon laser beam (375 nm, 30 mW, Melles Griot, Irvine, CA) was used for the

Fig. 1. Scheme for FPG DNA repair enzyme coupled with CE-LIF for detection of oxidized DNA bases.

QD excitation, and the emitted fluorescence was detected by a photomultiplier tube (PMT, model R1477, Hamamatsu Photonics, Japan) at 625 nm. During sample preparation, the temperature was controlled using a thermal cycler (Bio-Rad). A UV cross-linker was employed for UV irradiation of DNA (Beijing BINTA Instrument Technology Co., Beijing, China).

2.3. Oxidative DNA damage induced by the Fenton reaction

A dried calf thymus DNA pellet was dissolved in ultrapure water. The concentration of DNA was determined using a NanoDrop 2000 by measuring the absorbance at 260 nm. First, 10 µg of calf thymus DNA was incubated with 20 µM FeCl₂ and 100 µM H₂O₂ in a 2× Tris-acetate buffer (TA, 20 mM Tris and 13 mM acetic acid, pH 7.8) for 4 h at 37 °C in the dark with a final volume of 200 µl. Immediately thereafter, 20 µl (one-tenth of the total volume) of sodium acetate (3 M, pH 5.2) and 500 µl (2.5 times of the total volume) of cold 100% ethanol were added, and the temperature was maintained at −20 °C for 10 min. Finally, DNA was precipitated by centrifugation at 12,000 rpm for 10 min. The DNA pellet was washed three times with cold 70% ethanol. The DNA was dried in air and redissolved in 50 µl of 2× TA buffer. The concentration of DNA was measured using a NanoDrop 2000.

2.4. Sample preparation

2.4.1. FPG protein or endonuclease VIII treatment to remove the damaged nucleotides

4 μ g of calf thymus DNA with and without the Fenton reaction treatment was incubated with 8 U FPG in 1 $\times$ NEBuffer I (10 mM Bis Tris Propane-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.0, supplied by NEB) supplemented with 100 μ g/ml BSA for 90 min at 37 °C to remove the damaged nucleotides, leaving a nucleotide gap with 5' and 3' phosphate groups. The reaction was terminated by heating at 75 °C for 10 min.

Similarly, 5 U endonuclease VIII was also used to treat 4 μ g of calf thymus DNA pretreated or untreated by the Fenton reaction in 1 $\times$ endonuclease VIII reaction buffer (10 mM Tris-HCl, 75 mM NaCl, 1 mM EDTA, pH 8.0, supplied by NEB) for 90 min at 37 °C. The enzyme was inactivated by heat treatment at 75 °C for 10 min.

2.4.2. Dephosphorylation of nucleotide gap by alkaline phosphatase

After FPG protein or endonuclease VIII treatment, 4 U FastAP thermosensitive alkaline phosphatase was added to the sample solution supplemented with 2 mM MgCl₂ followed by incubation for 20 min at 37 °C in 1 $\times$ FastAP buffer (10 mM Tris-HCl, 5 mM MgCl₂, 100 mM KCl, 0.02% Triton X-100 and 100 μ g/ml BSA, pH 8.0) to release the 5' and 3' phosphate groups from the nucleotide

gap in the DNA. The enzyme was inactivated by heating at 75 °C for 10 min.

2.4.3. Biotin-ddNTP incorporation at the nucleotide gap by DNA polymerase I

After the FPG protein and alkaline phosphatase treatment, a nucleotide space with 3' and 5' hydroxy groups was generated. 2 U DNA polymerase I, biotin-11-ddATP, biotin-11-ddGTP, biotin-11-ddCTP, biotin-11-ddUTP (0.4 μ M each, final concentration) and 5 mM MgCl₂ were added to the solution, and a biotin-labeled ddNTP was introduced into the nucleotide space next to the 3'-OH end. The mixture was treated at 37 °C for 60 min, and the reaction was terminated by heating at 75 °C for 20 min.

Then, the biotin-labeled DNA sample was ethanol precipitated with 0.2 volume of 2.0 M NH₄Ac and 2.5 volume of cold 100% ethanol and washed twice with cold 70% ethanol to remove any enzyme and reaction buffer. The DNA pellet was dried and redissolved in 10–20 μ l of 2 $\times$ TA buffer. The concentration of biotin-labeled DNA was determined using the NanoDrop 2000.

2.4.4. QD-streptavidin conjugate binding with biotin-labeled DNA

3 nM QD-streptavidin conjugate was incubated with varying concentrations of biotin-tagged DNA in 2 $\times$ TA buffer with a final volume of 20 μ l at room temperature for 10 min to form the QD-DNA complex. Finally, the prepared samples were analyzed by capillary electrophoresis and laser-induced fluorescence.

Fig. 2. CE-LIF analysis of QD-labeled oxidative DNA damage induced by the Fenton reaction. Electrophoretograms from (a) to (c) corresponding to 0.1, 0.5 and 1 ng/ μ l biotinylated DNA, respectively. Peaks 1 and 2 correspond to the free QD-streptavidin conjugate and QD-streptavidin-biotin-DNA complex, respectively. The oxidized bases were cut by FPG.

2.5. Capillary electrophoresis and laser-induced fluorescence (CE-LIF) analysis

An uncoated fused-silica capillary that was 50 μm i.d. $\times$ 365 μm o.d. with a total length of 26 cm and an effective length of 20 cm from the inlet to the detection window was used. The sample was electrokinetically injected into the capillary by utilizing an injection voltage of 15 kV for 5 s and separated by a voltage of 20 kV. CE separation was carried out in 1 $\times$ Tris-glycine buffer (TG, 30 mM Tris and 160 mM glycine, pH 8.5). After each analysis, the capillary was washed using 0.02 M NaOH for 2 min, H₂O for 1 min and then 1 $\times$ TG separation buffer for 2 min to rebalance the capillary. Each sample was detected at least three times.

2.6. UV irradiation of DNA

Calf thymus DNA was irradiated on ice with a 254 nm germicidal UV lamp (a series of five 8 W tubes) for 0.5 h, 1 h and 2 h at a UV ray source-to-sample distance of 10 cm. The unirradiated DNA was used as the control. Following exposure to UV irradiation, the DNA was further treated as described in the sample preparation and analyzed by CE-LIF.

2.7. Preparation of biotinylated DNA as the standard

A 285 bp dsDNA with a biotinylated 5'-end on one strand was prepared via a PCR strategy. Then, the PCR products were separated by 2% agarose gel electrophoresis and purified from the gel according to the manufacturer instructions provided for the gel kit (ISOLATE II PCR and Gel Kit, BiOLINE). The varying concentrations of biotin-labeled DNA were incubated with 3 nM QD-streptavidin conjugate in 2 $\times$ TA buffer for 10 min at room temperature prior to CE-LIF. The unlabeled 285 bp dsDNA was used as the control.

3. Results and discussion

3.1. Method design

Oxidatively generated DNA damage can be recognized and excised by specific DNA glycosylases and converted to apurinic or apyrimidinic sites (AP sites). Based on this biological feature, several DNA repair enzymes have been used for the detection of oxidative purines and pyrimidines [27,28]. In this study, we exploited two types of DNA glycosylases (i.e., FPG and endonuclease VIII) to make a nick at the oxidized base of calf thymus DNA. Then, DNA polymerase I was employed to selectively label the nicked positions. Finally, the selectively labeled DNA was rapidly analyzed using CE-LIF.

To illustrate the approach in detail, FPG protein was used as an example (Fig. 1). The FPG protein has been widely used for the detection of various oxidative purine damage, such as 8-oxoguanine, fapy-guanine, methy-fapy-guanine, 8-oxoadenine and fapy-adenine [27,29]. The FPG protein as an N-glycosylase recognizes the oxidized bases and excises them from the N-glycosidic bonds in double-stranded DNA to generate AP sites. Due to the AP-lyase activity of the FPG protein, 3'- and 5'-phosphodiester bonds at the same AP sites are cleaved, and one nucleotide gap forms for each oxidized base. Meanwhile, both 5' and 3' phosphate groups are present [30]. To fill the gap with a nucleotide using DNA polymerase, the 3'-phosphate group should be replaced with a 3'-hydroxyl group. Therefore, FastAP thermosensitive alkaline phosphatase was employed to catalyze the release of the 5'- and 3'- phosphate groups from DNA to form 5'- and 3'- hydroxyl groups. Subsequently, a biotinylated ddNTP was introduced into the nucleotide gap through the incorporation of a biotin-11-ddNTP by DNA polymerase I. Then, the biotinylated DNA was further

Fig. 3. CE-LIF analysis of oxidative DNA damage caused by UV irradiation. (A) Electrophoretograms from CE-LIF analysis of oxidative DNA damage induced by UV irradiation for 0.5 h, 1 h, 2 h. Peaks 1 and 2 correspond to the QD-streptavidin conjugate and QD-DNA complex, respectively. (B) The plot of the QD-DNA complex as a function of the UV irradiation time. Error bars stand for the standard deviation of three repetitive measurements. The oxidized bases were cut by FPG. 5 ng/ μL biotinylated DNA was used in CE-LIF analysis.

labeled by QD via noncovalent interactions of biotin and streptavidin from DNA and QD, respectively. Using these sequential steps, a QD-streptavidin-biotin-DNA complex with high fluorescence intensity was generated. Finally, CE-LIF was employed to separate and detect the QD-labeled DNA from free QD. Using this method, the fluorescence intensity of QD-labeled DNA is expected to be proportional to the quantity of oxidized bases and indicative of the oxidative damage to DNA.

3.2. Detection of oxidatively generated DNA damage induced by the Fenton reaction

First, the oxidative damage to DNA caused by the classic Fenton reaction was investigated. The metal ions (e.g., iron (II) and Cu (II)) can react with hydrogen peroxide to yield highly reactive hydroxyl radicals, and this reaction is referred to as the Fenton reaction [31]. The hydroxyl radicals can cause various DNA damage including chain breakage, abasic sites and base oxidation [32].

In this study, the FPG protein was used to generate nicks for the oxidized bases. The sample and control consisted of calf thymus DNA that was treated or untreated by the Fenton reaction, respectively. After the treatment of a series of enzymes as described in the Section 2, biotinylated DNA with different final concentrations (i.e., 0.1, 0.5, and 1 ng/ μ l) was combined with the QD-streptavidin conjugate. As described in the Section 3.1, oxidatively damaged DNA can be labeled with QD due to the introduction of biotinylated-ddNTP by the sequential action of FPG, alkaline phosphatase and DNA polymerase I. DNA without damaged bases cannot be recognized and excised by FPG and further untagged by QD. After the CE-LIF analysis, a strong fluorescence signal corresponding to QD-labeled DNA (Peak 2) was observed for the Fenton reaction-treated calf thymus DNA (Fig. 2). In contrast, only a weak signal corresponding to QD-labeled DNA was observed for the untreated calf thymus DNA. The existence of the QD-DNA complex in the control was most likely due to the calf thymus DNA containing oxidative DNA damage and AP sites prior to treatment with the Fenton reaction. When no QD-streptavidin conjugate was used for labeling, no QD-DNA complex was observed. The fluorescence intensity of the QD-labeled DNA increased as the amount of oxidative DNA damage increased (Fig. 2).

In addition to the QD-DNA complex (peak 2), the unbound QD-streptavidin conjugate (peak 1) was also present in the reaction solution. The QD-DNA complex must be separated from the free QD-streptavidin conjugate. Agarose gel electrophoresis analysis indicated that the QD-streptavidin conjugate migrated toward the

anode (data not shown), suggesting that the QD-streptavidin conjugate was modified by a slight negative charge. Due to the excess negative charge of DNA, the charge-mass ratio of the QD-DNA complex was higher than that of the QD-streptavidin conjugate. The migration of the QD-DNA complex depends on the electroosmotic flow (EOF) and electrophoretic mobility of QD-DNA, which moves in the opposite direction of the EOF. In contrast, the movement of the QD-streptavidin conjugate was primarily driven by the EOF. Therefore, the QD-streptavidin conjugate migrates faster than the QD-DNA complex. Due to this mobility difference, the QD-streptavidin conjugate and QD-DNA complex were well separated in the CE analysis (Fig. 2).

3.3. CE-LIF analysis of oxidative DNA damage induced by UV irradiation

Ultraviolet (UV) irradiation from sunlight is a major factor in the formation of skin cancer [33]. UV light can be absorbed by DNA, which leads to the formation of thymine dimers. In addition, UV light induces the formation of reactive oxygen species (ROS), which results in oxidative DNA lesions [34]. Due to the low oxidation electric potential, guanine is a major target of the photoreaction. The most prominent guanosine oxidation product is 8-oxo-7, 8-dihydroguanine (8-oxoGua). Because 8-oxoGua is also the substrate of the FPG protein [35], the DNA damage caused by UV irradiation was investigated using our approach.

Fig. 4. Endonuclease VIII cut-based CE-LIF analysis of oxidative pyrimidine lesions induced by the Fenton reaction. Electrophoretograms from (a) to (c) were obtained from 0.5, 1 and 5 ng/ μ l of biotin-labeled DNA, respectively. Peaks 1 and 2 correspond to the QD-streptavidin conjugate and QD-DNA complex, respectively.

DNA was irradiated by 254 nm UV light, which is very close to the maximum absorption of DNA ($\lambda = 260$ nm). Under the chosen condition, calf thymus DNA was irradiated with UV light for 0.5 h, 1 h and 2 h. After the treatment of a series of enzymes, 5 ng/ μ l irradiated DNA was combined with the QD-streptavidin conjugate, and the QD-DNA complex was determined by CE-LIF analysis. Based on the CE-LIF analysis of QD-labeled DNA, 0.5 h irradiation stimulates the most significant damage response (Fig. 3). As the irradiation time increased, the peak area of QD-labeled DNA remained the same. After UV irradiation was increased to 2 h, the peak area decreased significantly. The reduction in the damage signal may be due to complete DNA fragmentation resulting from long irradiation. Therefore, this method can be used to determine the oxidative DNA damage generated by UV irradiation.

3.4. Measurement of oxidative pyrimidine damage using endonuclease VIII

Another N-glycosylase (i.e., endonuclease VIII) was also investigated. Similar to the FPG protein, endonuclease VIII also possesses both DNA N-glycosylase activity and abasic site nicking activity. Notably, endonuclease VIII can be employed to detect oxidative

pyrimidine lesions including 5, 6-dihydroxythymine, 6-hydroxy-5, 6-dihydrothymine, thymine glycol, uracil glycol, 5-hydroxy-5-methylhydantoin, and methyltartronylurea [29,36].

The experimental procedure is the same as that used for the FPG protein. Calf thymus DNA that was treated and untreated by the Fenton reaction was defined as the sample and control, respectively. 0.5, 1 and 5 ng/ μ l biotinylated DNA were used to bind with the QD-streptavidin conjugate followed by CE-LIF analysis. Peaks 1 and 2 correspond to the QD-streptavidin conjugate and QD-DNA complex, respectively. As shown in Fig. 4, the signal of QD-labeled DNA in the treated DNA (sample) is much higher than that of the untreated DNA (control). In addition, the fluorescence intensity of the QD-DNA complex significantly increased as the amount of biotinylated DNA in the treated DNA increased. Therefore, endonuclease VIII combined with CE-LIF analysis can be employed to determine oxidized pyrimidine bases.

3.5. CE-LIF analysis of the sensitivity and linearity of the method

The 285 bp dsDNA with a biotin-tag at the 5'-end of only one strand was used as the standard. The biotinylated DNA with varying concentrations was combined with the QD-streptavidin conjugate.

Fig. 5. CE-LIF analysis of 285 bp dsDNA with biotin-tag at the 5'-end of one strand. (A) Electrophoretograms from CE-LIF analysis of biotinylated 285 bp dsDNA. The standard was varied from 0 (QD) to 3.7×10^{-17} mol DNA unlabeled with biotin was the control. (B) The linear plot of the QD-streptavidin-biotin-DNA complex as a function of the concentration of biotinylated DNA. The error bars correspond to the standard deviation of three repetitive measurements.

In comparison to calf thymus DNA (approximately 3800 bp), the shorter standard dsDNA (285 bp) after binding to the QD-streptavidin conjugate should exhibit a shorter migration shift from the unbound QD-streptavidin conjugate. To achieve satisfactory separation, the total length of the capillary was increased to 40 cm with an effective length of 34 cm. As shown in Fig. 5A, the peaks corresponding to the QD-labeled DNA are well separated from the unbound QD-streptavidin conjugate. As shown in Fig. 5B, the peak area of the QD-labeled DNA was proportional to the concentration of the standard DNA in a range of 2.5×10^{-11} – 1×10^{-9} mol/l. The correlation equation is $F = 6.91C - 0.51$ with a correlation coefficient of $R^2 = 0.997$ ($n = 3$). The dynamic range spans two orders of magnitude. The lower limits of detection are approximately 2.9×10^{-12} mol/l in concentration and 1.1×10^{-19} mol in mass based on an estimate from the signal to noise ratio (S/N) = 3.

4. Conclusions

In this study, we developed a novel method for the detection of oxidatively generated DNA damage using capillary electrophoresis laser-induced fluorescence combined with DNA repair enzymes. The method has low limits of detection (i.e., 1.1×10^{-19} mol in mass and 2.9 pM in concentration). In comparison to commonly used HPLC-ECD (high performance liquid chromatography-electrochemical detection), this method exhibits a lower limit of detection and does not require time-consuming DNA digestion. The developed method can be applied to detect oxidatively generated DNA damage caused by the Fenton reaction and UV irradiation.

Capillary electrophoresis is widely applied to bioanalysis due to its highly efficient and rapid separation ability, high sensitivity and low sample consumption. Appropriate DNA repair enzymes can be utilized to recognize and remove specific oxidative DNA damage. Therefore, by coupling CE-LIF with various DNA repair enzymes, the levels of different base damage can be rapidly assessed. As demonstrated in this study, the FPG protein can be employed to assess oxidized purine bases, and endonuclease VIII can be utilized to estimate oxidized pyrimidine bases. Potentially, to estimate the total amount of oxidative pyrimidine damage and purine damage due to chemical or ionizing radiation, the FPG (purine oxidative damage) and endonuclease VIII (pyrimidine oxidative damage) proteins can be used simultaneously to treat the damaged DNA.

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