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An HPLC–tandem mass spectrometry method for simultaneous detection of alkylated base excision repair products

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ABSTRACT

DNA glycosylases excise a broad spectrum of alkylated, oxidized, and deaminated nucleobases from DNA as the initial step in base excision repair. Substrate specificity and base excision activity are typically characterized by monitoring the release of modified nucleobases either from a genomic DNA substrate that has been treated with a modifying agent or from a synthetic oligonucleotide containing a defined lesion of interest. Detection of nucleobases from genomic DNA has traditionally involved HPLC separation and scintillation detection of radiolabeled nucleobases, which in the case of alkylation adducts can be laborious and costly. Here, we describe a mass spectrometry method to simultaneously detect and quantify multiple alkylpurine adducts released from genomic DNA that has been treated with *N*-methyl-*N*-nitrosourea (MNU). We illustrate the utility of this method by monitoring the excision of *N*3-methyladenine (3mA) and *N*7-methylguanine (7mG) by a panel of previously characterized prokaryotic and eukaryotic alkylpurine DNA glycosylases, enabling a comparison of substrate specificity and enzyme activity by various methods. Detailed protocols for these methods, along with preparation of genomic and oligonucleotide alkyl-DNA substrates, are also described.

1. Introduction

Alkylation DNA damage is produced by a large number of environmental toxins, endogenous methyl donors, and chemotherapeutic agents [1], and thus the mechanisms of alkyl-DNA toxicity and mutagenicity are of great interest. Much of our understanding of the generation and enzymatic repair of DNA alkylation comes from the use of laboratory alkylating agents, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), *N*-methylnitrosourea (MNU), methylmethanesulfonate (MMS), and dimethylsulfate (DMS), which methylate DNA to produce *N*3- and *N*7-methylpurines (3mA, 3mG, 7mA, 7mG), O^6-methylguanine (O^6mG), *N*1-methyladenine (1mA) and to a lesser extent *N*3-methylcytosine (3mC), O^2-methylcytosine, and O^4-methylthymine (Fig. 1A) [2]. O^6mG is demethylated by alkyltransfereases Ada/MGMT, 1mA and 3mC are repaired by oxidative demethylases AlkB/ALKBH, and the remaining *N*3- and *N*7-methylpurines and O^2- and O^4-methylpyrimidines are repaired by various DNA glycosylases [3].

DNA glycosylases maintain genome integrity by initiating base excision repair (BER) of the large number of aberrant nucleobases that arise from deamination, oxidation, and alkylation [4]. These enzymes are specialized for particular types of damage, and all catalyze the hydrolysis of the *N*-glycosidic bond to generate a free nucleobase and an apurinic/apyrimidinic (AP) site (Fig. 1B). Some (bifunctional) DNA glycosylases contain a nicking (AP lyase) activity in addition to the base excision activity [5]. The resulting AP site is processed by AP endonuclease, DNA polymerase, and DNA ligase activities to restore undamaged DNA as part of the BER pathway

Abbreviations: 3mA, *N*3-methyladenine; 7mG, *N*7-methylguanine; 1mA, *N*1-methyladenine; εA, 1,N^6-ethenoadenine; MAG, methyladenine DNA glycosylase; TAG, *E. coli* 3-methyladenine DNA glycosylase I; AlkA, *E. coli* 3-methyladenine DNA glycosylase II; AAG, human alkyladenine DNA glycosylase; FAM, 6-carboxyfluorescein; MNU, *N*-methyl-*N*-nitrosourea; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MMS, methylmethanesulfonate; DMS, dimethylsulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; MRM, multiple reaction monitoring; CID, collision induced dissociation; ESI, electrospray ionization; ESI(+), positive ion mode electrospray ionization.

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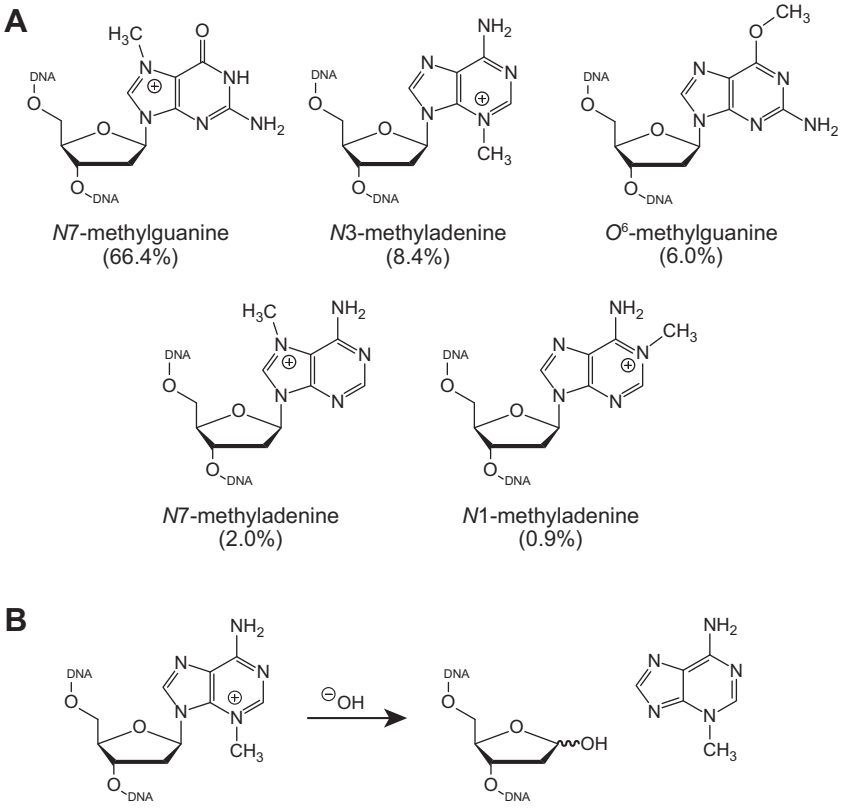

Fig. 1. Alkylpurine structures and the base excision reaction. (A) Structures of the major methylated purines produced by laboratory alkylating agents. Percentages refer to the relative amounts of methylpurines produced by MNU treatment [2]. (B) Schematic of the DNA glycosylase reaction.

[6]. DNA glycosylase activity was first demonstrated by Tomas Lindahl with the enzymatic release of free uracil from single- and double-stranded DNA [7]. Shortly thereafter, other bacterial and human DNA glycosylases were discovered that excise 3mA [8–14], as well as hypoxanthine [15,16] and a variety of oxidized bases (e.g., thymine glycol, 8-oxoguanine) [17,18], from DNA.

There are two predominant assays to monitor base excision activity of DNA glycosylases (Fig. 2). The more common method involves liberation of a single, defined lesion that has been chemically or enzymatically incorporated into an oligonucleotide [19–28] (Fig. 2A). AP sites generated by the glycosylase are nicked by alkali treatment or, in the case of the bifunctional enzymes, by intrinsic lyase activity, and the substrate and product fragments separated electrophoretically to quantify the ratio of substrate/product. Fragments have also been analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry [29]. The oligonucleotide-based glycosylase assay enables precise characterization of binding and catalysis of a specific lesion under well-defined conditions and nucleotide sequence contexts, and takes advantage of automated chemical DNA synthesis technology and availability of a large number of commercially available phosphoramidite precursors. This assay has therefore become a powerful method to characterize stable lesions, such as 8-oxoguanine, uracil, 5-methylcytosine, and 1,N^6-ethenoadenine [19,21,26,30,31]. However, some modifications, most notably *N*3- and *N*7-substituted alkylpurines, are prone to spontaneous depurination or ring opening under the conditions required for oligonucleotide synthesis and purification [32,33]. In addition, the oligonucleotide assay is not amenable to high-throughput analysis of multiple lesions.

Another common method involves excision of nucleobases from genomic DNA that has been pre-treated with an oxidizing or alkylating agent, followed by ethanol precipitation of the DNA and high performance liquid chromatography (HPLC) separation of the soluble (nucleobase) fraction [13,34–39] (Fig. 2B). This substrate has the advantage of allowing multiple types of alkylated bases to be evaluated and has been useful for characterizing lesions not amenable to chemical synthesis, such as the relatively labile 3mA [38,40]. The glycosylase activities and substrate preferences of the alkylpurine DNA glycosylases were initially characterized by scintillation detection of [^{3}H]-methylbases liberated from genomic DNA that had been pre-treated with a radiolabeled laboratory methylating agent such as *N*-[^{3}H]-methyl-*N*-nitrosourea (MNU) [13,34–37]. However, these reagents have become costly and pose an environmental hazard.

Tandem mass spectrometry (MS/MS) circumvents the need for radioactive reagents while enabling highly selective and sensitive quantitation of nucleobases, nucleosides, and nucleotides [41–44]. Mass spectrometry approaches have been used to identify modified nucleoside metabolites from rat liver [45], establish the spectrum of lesions produced in genomic DNA by oxidizing [46,47] and alkylating agents [48–51], and study oxidative damage repair [29,47,52–54]. To our knowledge, HPLC–MS/MS techniques capable of simultaneously detecting alkylated DNA adducts present in DNA [44,48,50,51] have not been utilized to assay the excision activity of DNA glycosylases.

The method described here involves reverse-phase HPLC separation of nucleobase products, followed by positive ion electrospray ionization [ESI(+)] mass spectrometric detection in multiple reaction monitoring (MRM) mode (Fig. 2B). We show that the four common methylpurines formed by reaction of DNA with MNU (7mG, 3mA, O^6mG, and 1mA) can be fully resolved based on their chromatographic retention times and MRM transitions. We illustrate the utility of the method by comparing the substrate specificities of five alkylpurine DNA glycosylases for 3mA and 7mG

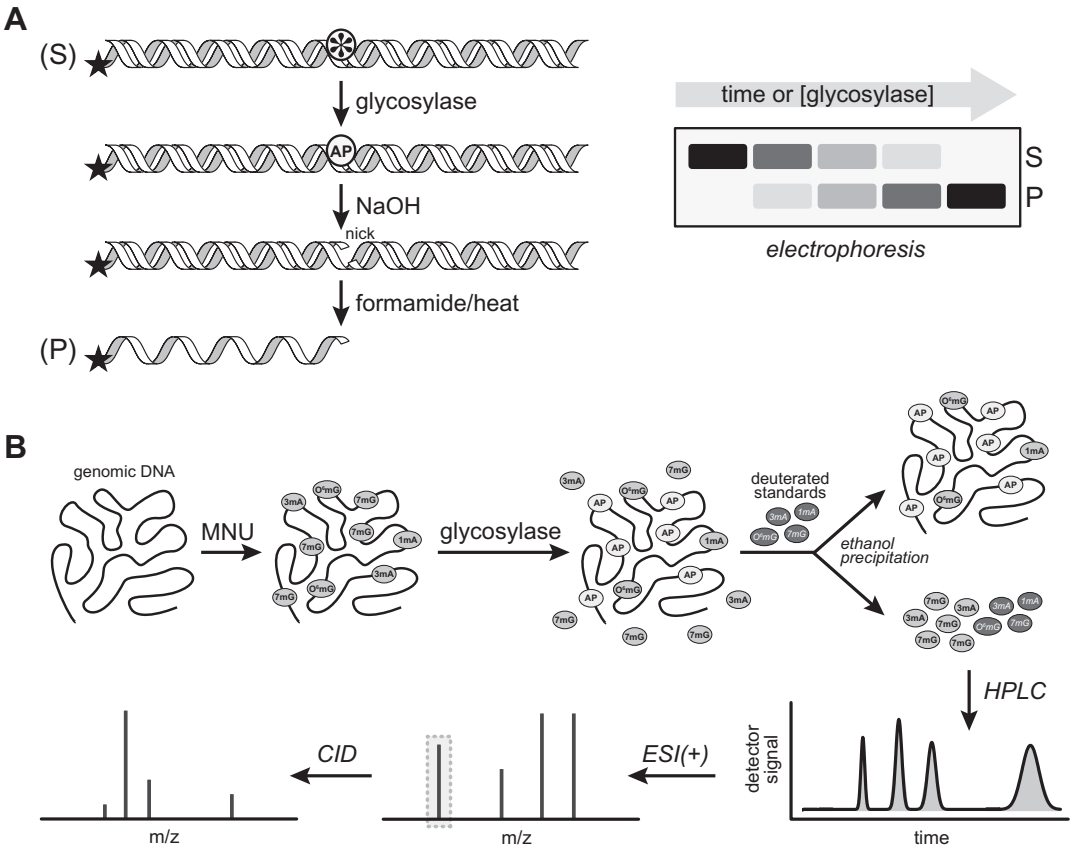

Fig. 2. Two methods for monitoring DNA glycosylase base excision activity. (A) Oligonucleotide assay. A ^{32}P- or fluorescently labeled (star) oligonucleotide containing a modified nucleobase (asterisk) is incubated with a DNA glycosylase to generate an AP site. At various time points, aliquots of the reaction are mixed with NaOH and heated to nick any AP sites. The substrate (S) and product (P) bands are separated by denaturing polyacrylamide gel electrophoresis, shown schematically to the right. (B) Genomic DNA assay. Genomic DNA is treated with a methylating agent (e.g., *N*-methyl-*N*-nitrosourea, MNU) to produce a spectrum of methylated nucleobases (gray ovals), some of which are excised from the DNA backbone by DNA glycosylases. The glycosylase reaction is quenched and spiked with deuterated internal standards (dark gray ovals) before the genomic DNA is ethanol precipitated and the soluble fraction containing free methylbases is subjected to HPLC–MS/MS. Ions were generated in positive ion electrospray ionization [ESI(+)] mode. MS/MS detection was based on multiple reaction monitoring of highly selective collision induced dissociation (CID) transitions with a triple quadrupole mass spectrometer.

and characterizing the kinetics of base excision for the DNA glycosylase AlkD. We also present representative data for the commonly used oligonucleotide base excision assay, along with detailed protocols for preparation and use of the different methylated DNA substrates used in the various glycosylase assays.

2. Materials and methods

Methods for chemical synthesis of deuterated nucleobase standards d_3-1mA, d_3-7mG, and d_3-O^{6}mG (Fig. S1) and detailed, step-by-step protocols for glycosylase substrate preparation and base excision assays are provided in the Supplemental Information.

2.1. Base excision assay using methylated genomic DNA and HPLC–MS/MS detection

2.1.1. Preparation of a methylated genomic DNA substrate

Methylated genomic DNA substrate was prepared by incubating 1 mg phenol:chloroform-purified calf thymus DNA and 1 μmol MNU in 0.3 M sodium cacodylate/0.1 M sodium perchlorate (pH 8.3) in the dark for 8 h at room temperature. The DNA was ethanol precipitated and the fibers washed with cold ethanol, resuspended in 1 ml Tris–EDTA pH 8.0 (TE) buffer, and dialyzed against additional TE buffer to remove any spontaneously hydrolyzed methyl adducts. Final yield was 0.8 mg DNA as determined by UV absorbance.

2.1.2. Excision of methylated bases from genomic DNA

In a 50 μl reaction, 10 μg of the methylated genomic DNA substrate (step 2.1.1) was incubated with either 5 N HCl for 1 h at 37 °C (to determine the upper limit of methylated bases present) or with 0–20 μM enzyme at 37 °C for varying amounts of time. The time course of HCl depurination shown in Table S1 was performed using 0.5 N HCl at 70 °C for 0.5–6 h. Enzymatic reactions contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 100 mM KCl, 10 mM dithiothreitol (DTT), 2 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 mg/ml bovine serum albumin (BSA). Reactions were terminated by addition of 50 μl stop buffer (0.5 mg/ml salmon DNA, 1 mg/ml BSA, 1 M NaCl) containing 10 μM of each deuterated nucleobase standard (d_3-1mA, d_3-3mA, d_3-7mG, d_3-O^{6}mG), followed by ethanol precipitation of the DNA. The supernatant was evaporated to dryness and the residue reconstituted in 50 μl ultrapure water and transferred to a 200-μl silanized autosampler vial equipped with a Teflon-lined bonded rubber septum in preparation for HPLC–MS/MS analysis.

2.1.3. Detection of excised bases by HPLC–MS/MS

2.1.3.1. Instrumentation. Sample analyses were carried out using a Waters Acquity UPLC system (Milford, MA), made up of a binary solvent manager, a refrigerated sample manager, and a heated column manager. MS/MS detection was performed using a ThermoElectron TSQ Quantum Ultra triple-stage quadrupole mass spectrometer (San Jose, CA) equipped with an *Ion Max* source hous-

ing, an ESI probe, and a 50 μm interior diameter stainless steel capillary.

2.1.3.2. Preparation of calibration standards. Calibration of the HPLC–MS/MS response was performed with standard solutions of 1mA, 3mA, 7mG, and O^6mG over a concentration range of 50–4000 nM for each compound. A stock solution of 1mA was prepared at 1 mM in 10 mM ammonium acetate. A stock solution of 3mA was prepared at 1 mM in water. Stock solutions of O^6mG and 7mG were prepared at 800 and 39 μM, respectively, in methanol. In order to account for matrix effects, stock analyte solutions were diluted in 50 μl mock glycosylase reactions containing 5 μM AlkD D113N, 50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/ml BSA. After incubating at 37 °C for 1 h, 50 μl stop buffer containing 10 μM of each deuterated standard was added and samples were processed as described above (step 2.1.2) in preparation for HPLC–MS/MS analysis. Stock solutions of d_3-1mA, d_3-3mA, and d_3-O^6mG were prepared at 100 μM in water. A stock solution of d_3-7mG was prepared at 100 μM in methanol.

Extraction recovery was determined by preparing two sets of 50 μl mock glycosylase reactions: the first containing 1 μM of each methylpurine and the second lacking analyte. After incubating at 37 °C for 1 h, 50 μl stop buffer containing 10 μM of each deuterated standard was added and samples were processed as described above (step 2.1.2) in preparation for HPLC–MS/MS analysis. Mock glycosylase samples previously lacking analyte were spiked with 1 μM of each methylpurine after resuspension in water.

2.1.3.3. HPLC. A Symmetry Shield RP18 column [2.1 × 150 mm, 3.5 μm particle size, Waters] equipped with an Acquity UPLC in-line stainless steel filter unit (0.2 μm, Waters) was used for all chromatographic separations. The column and autosampler tray temperatures were 25 and 10 °C, respectively. Mobile phases were made up of 0.2% perfluoropentanoic acid in (A) H_2O and in (B) MeOH:H_2O (95:5). Gradient conditions were as follows: 0–1 min, B = 0%; 1–8 min, B = 0–25% (curve 8); 8–9 min, B = 25–100% (curve 8); 9–10 min, B = 100–0% (curve 8);and 10–16 min, B = 0%. The flow rate was maintained at 0.4 ml/min. A software-controlled divert valve was used to transfer eluent to waste from 0 to 3 min and from 11 to 16 min. The sample injection volume was 10 μl. The autosampler injection valve and the sample injection needle were flushed and washed sequentially with 1 ml mobile phase B and 1 ml mobile phase A before each injection.

2.1.3.4. MS/MS, data acquisition, and processing. The mass spectrometer was operated in positive ion mode and quantitation was based on multiple reaction monitoring (MRM) of methylated adenines and guanines. Product ion mass spectra were acquired in the 30–200 m/z range, and each methylated base was readily identified by its characteristic precursor ion and major product ion. Full scan MS in positive ion electrospray mode (Supplemental Figs. S2–S5) gave a major protonated molecular ion with m/z 150 $[M+H]^+$ for each adenine analogue, m/z 166 $[M+H]^+$ for each guanine analogue, m/z 153 $[M+H]^+$ for d_3-1mA and d_3-3mA, and m/z 169 $[M+H]^+$ for d_3-7mG and d_3-O^6mG. Collision induced dissociation (CID) of m/z 150 of protonated 1mA and 3mA gave product ions with m/z 109 $[M+H-41]^+$ and 123 $[M+H-27]^+$, respectively. CID of m/z 166 of protonated 7mG and O^6mG gave product ions with m/z 124 $[M+H-42]^+$ and 134 $[M+H-32]^+$, respectively. CID of m/z 153 of protonated d_3-1mA and d_3-3mA gave a product ion with m/z 109 $[M+H-44]^+$ and 82 $[M+H-27]^+$, respectively. CID of m/z 169 of protonated d_3-7mG and d_3-O^6mG gave product ions with m/z 127 $[M+H-42]^+$ and 70 $[M+H-32]^+$, respectively. The mass transitions (precursor to product) monitored were 150 → 109 for 1mA, 150 → 123 for 3mA, 166 → 124 for 7mG, 166 → 134 for O^6mG,153 → 109 for d_3-1mA, 153 → 82 for d_3-3mA, 169 → 127 for d_3-7mG, and 169 → 70 for d_3-O^6mG. Although 1mA, 3mA, 7mG, and O^6mG do not have unique m/z transitions, chromatographic separation (Fig. S6) allowed for accurate quantitation. Data collection parameters were as follows: spray voltage, 4500 V; capillary temperature, 300 °C; sheath gas, 30 psi; and collision energies, varied. Data acquisition and quantitative spectral analysis were performed using Thermo-Finnigan Xcalibur version 2.0.7 and Thermo-Finnigan LCQuan version 2.5.5, respectively. Standard curves were prepared by plotting observed peak area ratios (analyte peak area/internal standard peak area) against corresponding known quantities of 7mG, 3mA, O^6mG, and 1mA (Fig. S7). Analyte amounts in experimental samples were determined by comparing observed peak area ratios to the standard curves.

2.2. Base excision assay using a defined oligonucleotide substrate

2.2.1. Preparation of oligonucleotide substrates

2.2.1.1. Chemical synthesis of stable lesion-containing oligonucleotides. Oligonucleotides containing stable lesions [1,N^6-ethenoadenine (εA), 1mA] were chemically synthesized by Integrated DNA Technologies or Midland Certified using phosphoramidite precursors (Glen Research). 25mer oligonucleotide [d(GACCACTACACCXATTCCTTACAAC)] containing a centrally located lesion (X) was either [^{32}P]-labeled or synthesized with 6-carboxyfluorescein (FAM) at the 5′ end and annealed to 2–3-fold molar excess of complementary oligonucleotide [d(GTTGTAAGGAATTGGTGTAGTGGTC)] to produce a 5 μM ^{32}P-DNA or a 50 μM FAM-DNA stock. [^{32}P]-oligos were subsequently purified by phenol:chloroform extraction and a G-25 spin column.

2.2.1.2. Enzymatic synthesis of 7mG-containing oligonucleotides. 7mG was enzymatically incorporated into DNA duplexes based on a method previously described by Asaeda et al. [30]. A 5′-FAM labeled oligonucleotide primer [d(GACCACTACACC)] was annealed to 3-fold excess of complementary oligonucleotide [d(GTTGTAAGGAATCGGTGTAGTGGTC)], which contained only one cytosine (underlined) in the single-stranded region of the template. Primer-template was extended using DNA polymerase I Klenow fragment (New England Biolabs) and a 4-fold excess of 2′-deoxy-7-methylguanosine 5′-triphosphate (d7mGTP, Sigma–Aldrich) over dCTP, dTTP, and dATP. Extention reactions (20 μl) were carried out at room temperature for 5 min and contained 10 μM primer-template, 400 μM d7mGTP, 100 μM d(C/T/A)TP, 1 U Klenow fragment, and polymerase buffer [66 mM Tris–HCl (pH 7.6), 6.6 mM NaCl, and 1.5 mM β-mercaptoethanol]. Reactions were quenched with 5 mM EDTA and the DNA was purified by phenol:chloroform extraction and exchanged into TE buffer using a G-25 spin column.

2.2.2. Excision of 7mG from an oligonucleotide substrate

Working stocks of FAM-labeled 7mG-oligonucleotides (step 2.2.1.2) were diluted to 500 nM in AAG activity buffer [50 mM sodium acetate (pH 6.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA]. Glycosylase reactions (80 μl) contained 5 μM Δ79AAG, 100 nM FAM-DNA, and AAG activity buffer, and were incubated at 37 °C. At various times, 8 μl aliquots were added to 2 μl 1 M sodium hydroxide and heated at 70 °C for 2 min. Samples were denatured by addition of 10 μl formamide/dye loading buffer [80% (w/v) formamide, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, and 10 mM EDTA pH 8.0] and heating at 70 °C for 5 min prior to running on a 20% acrylamide/8 M urea sequencing gel. Gels were run at 40 W for 45 min in 0.5X TBE buffer (45 mM Tris, 45 mM borate, and 1 mM EDTA pH 8.0) and imaged on a Typhoon Trio variable mode imager (GE Healthcare) in fluorescence mode using a 532-nm green laser and 526-nm emission filter.

2.3. Protein purification

Human AAG catalytic domain, which lacks 79 residues from the amino terminus (Δ79AAG) [55], *Saccharomyces cerevisiae* MAG [26], *Salmonella typhi* TAG wild-type and E38A mutant [40], and *Bacillus cereus* AlkD wild-type and D113N mutant [27] were purified as described previously.

The AlkC gene was cloned from *B. cereus* genomic DNA (ATCC 14579) into a modified pET-19b expression vector (Novagen) that contains a Rhinovirus 3C (PreScission protease) cleavable N-terminal His_{10}-Tag. The vector was transformed into *E. coli* Rosetta cells and overexpressed overnight at 16 °C upon addition of 0.1 mM IPTG. Cells were harvested in 50 mM Tris–HCl (pH 8.5), 500 mM NaCl, and 10% glycerol and lysed with an Avestin Emulsifer C3 homogenizer operating at ~20,000 psi. His_{10}-AlkC protein was purified using Ni–NTA (Qiagen) affinity chromatography. Following cleavage of the His_{10} tag, AlkC was further purified by heparin affinity and gel filtration chromatography to >99% homogeneity. Protein was concentrated to 2.4 mg/ml and stored in 20 mM Tris–HCl (pH 8.5), 200 mM NaCl, 10% glycerol, 2 mM DTT, and 0.1 mM EDTA.

3. Results

We set out to design a mass spectrometry method to simultaneously detect the various methylated nucleobases liberated from genomic DNA as a result of DNA glycosylase activity. Treatment of double-stranded DNA with MNU produces several methylated nucleobases (Fig. 1), with 7mG comprising 66% of the total methylated DNA, 3mA 8%, O^6mG 6%, 7mA 2%, 1mA 1%, and the other possible methylbases each comprising <1% (totaling ~4%). The remaining 12% methylated DNA products are phosphodiester backbone modifications [2]. We incubated MNU-treated calf thymus DNA with either mild acid for complete depurination or alkylpurine DNA glycosylases for more selective methylbase excision. The released methylpurines were separated and quantified by reversed-phase HPLC and ESI(+) MS/MS in MRM mode to obtain the highest possible selectivity (Fig. 2B). Nucleobases were quantified relative to internal deuterated standards that were added prior to chromatographic separation. We found that addition of an ion-pairing agent, perfluoropentanoic acid, to the mobile phase increased the retention times of the methylated bases and improved the HPLC separation. Direct infusion of methylated nucleobase standards allowed for tuning of the ESI source and optimization of MRM parameters.

3.1. Analytical figures of merit

We first determined response calibration parameters, analyte percent recovery, and lower limits of quantitation using mock glycosylase reactions containing an inactive AlkD D113N mutant [27,56] and known amounts of analytes, but lacking calf thymus DNA. The limits of detection and lower limits of quantitation were 500 fmol for all four bases measured. Signal responses for all methylpurines were linear over 0.5–40 pmol (injected amounts, 50–4000 nM) with all correlation coefficients (R^2) over 0.99 (Fig. S7). Loss of analytes during sample processing was minimal as extraction recovery values were 100 ± 7% for 1mA, 95 ± 6% for 3mA, 96 ± 6% for 7mG, and 101 ± 10% for O^6mG.

We next quantitated the total amount of each methylbase present in the MNU-treated DNA by acid depurination. Two concentrations of substrate were tested, and 1mA, 3mA, 7mG, and O^6mG products were measured over four different times from 0.5 to 6 h (Table S1). Depurination of all four bases from 3 μg DNA was complete after 30 min. However, using 10 μg DNA, 3mA depurination was complete after 30 min, but 3 h was needed for maximum 1mA and 7mG hydrolysis. Interestingly, the amounts of O^6mG decreased over time, suggesting that the O^6mG nucleobase was being degraded from acid treatment, either before or after excision from DNA. In support of this, O^6mG could not be detected from DNA treated with 5 N HCl. The relative amounts of total nucleobases detected from 3 and 10 μg DNA were 23:5:3:1 and 47:12:4:1 (7mG:3mA:O^6mG:1mA), respectively. Thus, on average the adenine adducts were consistent with previously reported values of MNU-treated DNA (66:8:6:1) while the guanine adducts were slightly underestimated [2]. When acid depurination of 10 μg DNA was performed with 5 N HCl at 37 °C for 1 h, the ratio of 7mG:3mA was 7.5:1 (Table S2), nearly identical to that previously reported, whereas 1mA levels were slightly higher than expected (3.3:1 3mA:1mA) [2].

3.2. Substrate selection by alkylpurine DNA glycosylases

We tested the utility of the HPLC–MS/MS method by measuring 3mA and 7mG excised from MNU-treated genomic DNA by several well-characterized DNA glycosylases from various organisms (Fig. 3A, Table S2). Eukaryotic and prokaryotic alkyl-DNA glycosylases are known to span a wide range of substrate specificities [4,57]. Human AAG/MPG [16,58], *S. cerevisiae* MAG [59,60], and *E. coli* AlkA [14,32] excise a broad range of alkylated and deaminated bases [26,37,61–63], while *B. cereus* AlkC and AlkD have intermediate specificities for cationic lesions, including 3mA and 7mG [27,38,56,64], and *E. coli* TAG [12] is highly specific for 3mA [39,40]. As negative controls, we tested an AlkD D113N mutant, which reduces 7mG activity 100-fold relative to wild-type AlkD, to levels indistinguishable from spontaneous 7mG depurination, as well as TAG E38A, which reduces 3mA activity 300-fold with respect to wild-type TAG [27,40].

As expected, after 1 h at 37 °C, all wild-type enzymes released the maximal or near-maximal amount of 3mA (Fig. 3A, Table S2). The TAG E38A and AlkD D113N mutants showed significantly reduced 3mA activity, similar to that previously reported for TAG E38A [40] and consistent with the observed catalytic impairment of AlkD D113N [27,56,64]. AAG, MAG, and AlkD showed robust 7mG activity, while TAG and AlkC displayed relatively weak removal of 7mG, again as expected. Consistent with previous results from oligonucleotide substrates [27,64], the AlkD D113N mutant exhibited greatly decreased 7mG activity. Minimal amounts of 3mA and 7mG were detected in a no-enzyme control, with ⩽6% nucleobases released compared to acid depurination.

3.3. Characterization of enzymatic activity by HPLC–MS/MS

We further investigated the utility of the mass spectrometry method by monitoring enzyme concentration dependence and kinetics of base excision from the genomic substrate. Using AlkD, we measured the release of 3mA and 7mG as a function of enzyme concentration (Fig. 3B). Each methylpurine showed a different concentration dependency. 3mA was completely excised with 0.5 μM AlkD, with an apparent dissociation constant ($K_{½}$) of 2.7×10^{-8} M, whereas at least 10-fold more enzyme (>5 μM) was required to completely remove 7mG ($K_{½} = 1.5 \times 10^{-6}$ M). These results are consistent with previously reported values using [^{3}H]-MNU-treated calf thymus DNA [38]. Additionally, the 7mG data are in good agreement with the $K_{½}$ determined using an oligonucleotide-based assay [64]. We next followed AlkD-catalyzed release of 3mA and 7mG over time (Fig. 3C). Under the conditions tested, AlkD completely excised 3mA within 24 min, with an observed second-order rate constant (k_{obs}) of 8.0×10^3 M^{-1} s^{-1}. AlkD-catalyzed excision of 7mG from genomic DNA was 36-fold slower ($k_{obs} = 2.2 \times 10^2$ M^{-1} s^{-1}) than that of 3mA, and about 5-fold slower than excision of

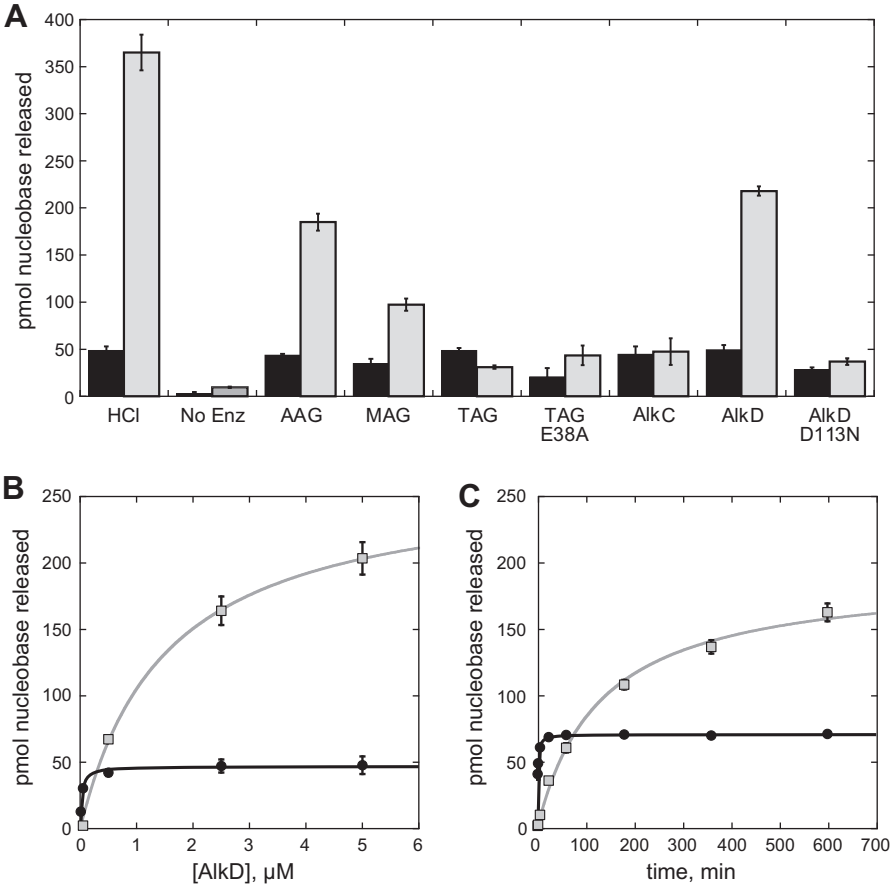

Fig. 3. Characterization of base excision from genomic DNA using HPLC–MS/MS. (A) Comparison of 3mA (black bars) and 7mG (gray bars) excision activities of several alkylpurine DNA glycosylases. Methylated genomic DNA was incubated with either 5 N HCl or 5 μM glycosylase for 1 h at 37 °C. Enzymes used: human Δ79AAG, *S. cerevisiae* MAG, *S. typhi* TAG wild-type and E38A mutant, *B. cereus* AlkC, and *B. cereus* AlkD wild-type and D113N mutant. The numbers used to generate this graph are provided in Supplemental Table S2. (B) Enzyme concentration dependence of 3mA (black circles) and 7mG (gray squares) excision by AlkD. Reactions containing 0.01–5 μM AlkD were incubated at 37 °C for 1 h. (C) Time dependence of 3mA (black circles) and 7mG (gray squares) excision by AlkD. Reactions containing 0.5 μM AlkD were incubated at 37 °C for 1 min to 10 h. Reaction mixtures were equilibrated at 37 °C for 3 min prior to the addition of enzyme.

7mG from an oligonucleotide substrate under comparable conditions [64].

3.4. Excision of 7mG from an oligonucleotide substrate

To illustrate the oligonucleotide-based base excision assay, we monitored base excision from a defined oligonucleotide substrate using denaturing polyacrylamide electrophoresis. As previously reported, it is possible to prepare a defined 7mG-containing oligonucleotide substrate that can be used for DNA glycosylase assays [26–28,30,55]. We enzymatically incorporated 7mG into the central position of a 25mer duplex and monitored release of the 7mG under single turnover conditions by the catalytic domain of human AAG (Δ79AAG) (Fig. 4). At time zero, about 28% of the total DNA was in the product form, consistent with the propensity of spontaneous depurination of 7mG (Fig. 4A) [64]. The k_{cat} for removal of 7mG by Δ79AAG was $(3.6 \pm 0.1) \times 10^{-4}\ s^{-1}$, about 5-fold slower than a previously reported value under similar conditions [28].

4. Discussion

Here, we describe biochemical methods to assay *in vitro* DNA glycosylase activities, focusing on the preparation of alkylpurine DNA substrates and a new mass spectrometry method we have developed to quantify multiple alkylpurine bases liberated from a genomic DNA substrate. Other HPLC–MS/MS methods have been developed to simultaneously detect alkylated DNA adducts present in DNA [44,48,50,51]. The method developed here expands on previous reports by examining the enzymatic excision of methylated adducts from DNA by alkylpurine DNA glycosylases. We show that this method is consistent with results from other DNA glycosylase assays, can be used to perform simultaneous kinetic characterization of base excision activity toward multiple nucleobase substrates, and has the potential to uncover new activities.

We measured 3mA and 7mG excision activities for several well-characterized DNA glycosylases and compared the results from previous work. This allowed us to compare MS/MS detection versus [^{3}H]-scintillation detection and kinetic parameters from genomic versus 7mG-containing oligonucleotide substrates. Firstly, the relative activities of AAG, MAG, TAG, AlkC, and AlkD for 3mA and 7mG are consistent with the literature [28,38,40,55,63–65]. Secondly, there is no significant difference between MS/MS and [^{3}H]-scintillation detection of 3mA and 7mG (as judged by TAG and AlkD activities, respectively) from genomic DNA [38,40]. Thirdly, the AlkD concentration-dependent release of both 3mA and 7mG from genomic DNA is comparable between [^{3}H]-scintillation and MS/MS modes of detection [38]. There was a modest (6-fold) difference in the kinetics of 7mG excised from genomic versus oligonucleotide DNA substrates [27,64], a difference most likely a result of the excess non-specific binding sites present in the

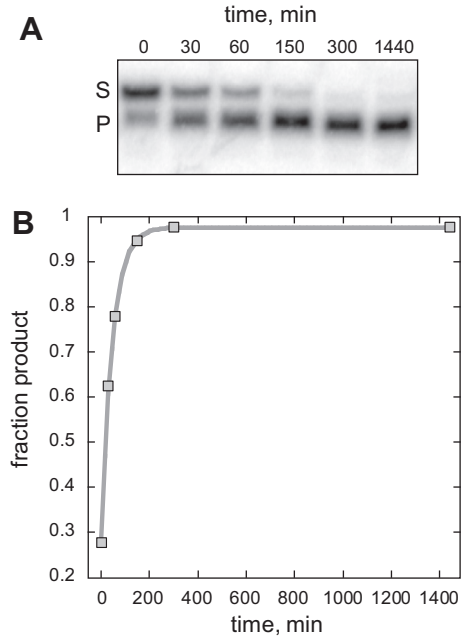

Fig. 4. Excision of 7mG from an oligonucleotide substrate by human Δ79AAG. Reactions contained 5 μM Δ79AAG, 100 nM FAM-DNA, 50 mM sodium acetate (pH 6.0), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/ml BSA. (A) Denaturing polyacrylamide gel showing 25mer 7mG-DNA (S) and AP-nicked 13mer product (P) at various reaction times. (B) Quantitation of the data in panel A.

genomic DNA substrate. Nonetheless, as previously shown for [^{3}H] detection [40,66], our results confirm that MS/MS detection is amenable to characterization of enzyme kinetics.

In addition to the ability to detect multiple nucleobase products, the use of genomic DNA provides the ability to probe less stable lesions that cannot be incorporated into oligonucleotides. Of interest is 3mA, a product of endogenous methyl donors (e.g., S-adenosylmethionine) [67,68] and alkylating agents used in chemotherapy [69,70]. Due to the short half-life of 3mA in DNA [71], it has not been possible to prepare purified oligonucleotides containing a single 3mA modification suitable for use in biochemical assays, although methylsulfonate derivatized lexitropsin (Me-Lex) peptides have been useful to study the biological effects of 3mA [72,73]. The data presented here represents the first kinetic characterization of 3mA release by AlkD, and reveals that AlkD excises 3mA at a rate one order of magnitude greater than 7mG, suggesting that 3mA may be the preferred substrate of AlkD. In addition, 3mA excision by AlkD is slightly faster ($k_{obs} = 8.0 \times 10^3\ M^{-1}\ s^{-1}$) than by 3mA glycosylases TAG ($k_{obs} = 3.9 \times 10^3\ M^{-1}\ s^{-1}$) and Mag-III ($k_{obs} = 6.4 \times 10^3\ M^{-1}\ s^{-1}$) [40,66].

We believe our method will be invaluable in providing detailed information about substrate specificities among closely related glycosylases. Simultaneous detection of nucleobase products will enable high-throughput structure–function analysis of substrate specificity, including evaluation of mutants that are predicted to alter specificity, since amino acid substitutions may differentially affect activity toward each lesion. Although neither 1mA nor O^{6}mG are typical glycosylase substrates, we included them in this analysis because they are produced at significant levels by various laboratory methylating agents. It is conceivable that newly discovered or mutant glycosylases could have activity toward these nucleobases, as previously observed for 1mA excision by *Archaeoglobus fulgidus* AlkA [25]. Finally, we note that detection of methylated bases by HPLC-MS/MS is not confinedto genomic DNA substrates. We are able to detect 1mA excised from a 25mer oligonucleotide by acid depurination (data not shown). Thus, this MS/MS method may be more generally applied to other DNA repair activities, such as 1mA conversion to adenine by ALKBH orthologs [74].

5. Conclusions

DNA glycosylase (base excision) activity can be monitored from oligonucleotides containing a single lesion or from genomic DNA containing a large number of different lesions. The oligonucleotide assay is more amenable to precise control of experimental parameters and single-turnover kinetics, while also being relatively inexpensive and easy to implement, but is limited to more stable lesions. Conversely, the genomic DNA assay allows simultaneous evaluation of enzymatic activity from DNA containing multiple types of potential substrates, including less stable lesions, but suffers from increased cost and difficulty of implementation. Use of radiolabeled methylating agents like [^{3}H]-MNU have become prohibitive, necessitating alternative detection methods of non-radiolabeled adducts. We demonstrate the use of a robust HPLC–MS/MS method for detecting DNA glycosylase nucleobase products, which will enable high-throughput analysis of substrate specificities and activities of alkylation specific DNA repair proteins. The required nucleobase standards are either commercially available (1mA, 3mA, 7mG, O^{6}mG, d_3-3mA, d_3-O^{6}mG) or straightforward to synthesize (d_3-1mA, d_3-7mG, d_3-O^{6}mG), and we believe our method should be accessible to most laboratories that have HPLC–MS/MS capabilities.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2013.07.020.

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Supplemental Information

An HPLC-tandem mass spectrometry method for simultaneous detection of alkylated base excision repair products

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Supplemental Methods

Chemicals and reagents

Calf thymus DNA, salmon testis DNA, *N*-methyl-*N*-nitrosourea, phenol:chloroform:isoamyl alcohol (pH 8.0), sodium cacodylate, sodium perchlorate, sodium HEPES, Trizma base, potassium chloride, sodium chloride, ammonium acetate (NH_4OAc), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), *N*7-methyladenine (7mA), *O*^{6}-methylguanine (O^{6}mG), *N*7-methylguanine (7mG), 6-amino-2-chloropurine, methanol-d_3, iodomethane-d_3, ethanol, and perfluoropentanoic acid were purchased from Sigma-Aldrich Chemicals (Saint Louis, MO). 2′-deoxyguanosine monohydrate and 2′-deoxyadenosine monohydrate were from ChemGenes Corp. (Wilmington, MA). *N*3-methyladenine (3mA) was purchased from EMD Biosciences (Darmstadt, Germany). Hydrochloric acid and *N*1-methyladenine (1mA) were purchased from Thermo Fisher Scientific (Waltham, MA). HPLC-grade methanol and water were from J.T. Baker (Phillisburg, NJ). *N*3-methyl-d_3-adenine (d_3-3mA) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). *N*1-methyl-d_3-adenine (d_3-1mA), *N*7-methyl-d_3-guanine (d_3-7mG), and *O*^{6}-methyl-d_3-guanine (d_3-O^{6}mG) were chemically synthesized by the Vanderbilt Institute of Chemical Biology Synthesis Core. d_3-O^{6}mG is also available from Cambridge Isotope Laboratories, Inc.

Buffers

Cacodylate/Perchlorate Buffer: 300 mM sodium cacodylate/100 mM $NaClO_4$ (pH 8.3) [Dissolve 48 g sodium cacodylate in 100 ml 1 M sodium perchlorate. Adjust the pH to 8.3 with 2 N NaOH and increase volume to 1 L with ddH_2O.]

TE Buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA

5X Glycosylase Reaction Buffer: 250 mM HEPES (pH 7.5), 500 mM KCl, 50 mM DTT, 10 mM EDTA, 0.5 mg/ml BSA

2X Glycosylase Stop Buffer: 0.5 mg/ml salmon DNA, 1 mg/ml BSA, 1 M NaCl

10X Annealing Buffer: 100 mM MES (pH 6.5), 400 mM NaCl

10X Polymerase Buffer: 660 mM Tris-HCl (pH 7.6), 66 mM $MgCl_2$, 15 mM β-mercaptoethanol

Formamide Loading Buffer: 80% (w/v) formamide, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, 10 mM EDTA (pH 8.0)

10X TBE Buffer: 890 mM Tris, 890 mM borate, 20 mM EDTA (pH 8.0) [Add 108 g Tris base, 55 g boric acid, and 40 ml 0.5 M EDTA (pH 8.0). Adjust the volume to 1 L with ddH_2O.]

Synthesis of d_3-1mA

Iodomethane-d_3 (150 µl, 2.4 mmol) was added dropwise to a stirred solution of deoxyadenosine monohydrate (150 mg, 0.6 mmol) in dry, degassed dimethylacetamide (2 ml) at room temperature. The reaction mixture was stirred at 28°C for 24 h. Dry acetone was added, and the precipitate (*N*1-methyl-d_3-2′-deoxyadenosine iodide) was filtered, dried (212 mg, 90%), dissolved in water (10 ml), and heated at 95°C for 2 h. After cooling, concentrated ammonia was added to pH 8 and the precipitate was separated by filtration to give pure d_3-1mA (80 mg, 50%). ^{1}H-NMR (DMSO-d_6): δ = 8.12 (s, 1H), 7.81 (s, 1H). LC-ESI: *m/z* = 153.

Synthesis of d_3-7mG

Iodomethane-d_3 (162 µl, 2.60 mmol) was added dropwise to a stirred solution of deoxyguanosine monohydrate (70 mg, 0.260 mmol) in dry, degassed dimethylsulfoxide (2 ml) at room temperature. After 45 min, excess iodomethane-d_3 was removed *in vacuo* with a rotary evaporator and 3 M hydrochloric acid (3 ml) was added. The reaction mixture was heated at 80°C for 1 h. After cooling, the reaction mixture was neutralized with 1 M sodium hydroxide. The precipitate was separated by filtration and washed with water to give pure d_3-7mG (30 mg, 68%). ^{1}H-NMR (DMSO-d_6): δ = 11.5 (br s, 1H), 7.03 (s, 1H), 6.03 (s, 2H). LC-ESI: *m/z* = 169.

Synthesis of d_3-O^{6}mG

2-amino-6-chloropurine (100 mg, 0.59 mmol) was dissolved in dry methanol-d_3 (1 ml), and sodium methoxide-d_3 (65 mg, 1.2 mmol, prepared from methanol-d_3 and sodium) was added to the solution at room temperature. The reaction mixture was heated at 80°C for 48 h. The solvent was removed by evaporation, and the resulting residue was purified by flash chromatography [methylene chloride:methanol (85:15)] to give pure d_3-O^{6}mG as a white power (84 mg, 85%). ^{1}H-NMR (DMSO-d_6): δ = 12.44 (br s, 1H), 7.82 (s, 1H), 6.15 (s, 2H). LC-ESI: *m/z* = 169.

Supplemental Tables

Table S1. Quantitation of adducts released by acid hydrolysis					
DNA (µg)	Time (h)	Adduct (pmol)			
		1mA	3mA	7mG	O^6mG
3.0 [0.3X]	0.5	3.76 ± 0.14	21.2 ± 1.2	86.8 ± 0.5	9.40 ± 0.37
	1	3.86 ± 0.22	18.8 ± 1.9	87.6 ± 4.3	8.93 ± 0.72
	3	3.69 ± 0.22	19.0 ± 0.6	89.1 ± 4.1	5.71 ± 0.51
	6	3.53 ± 0.07	18.9 ± 0.7	83.4 ± 1.7	3.54 ± 0.38
10.0 [1X]	0.5	5.14 ± 0.66	74.3 ± 7.6	187 ± 13	25.8 ± 1.1
	1	5.82 ± 0.55	82.4 ± 5.3	244 ± 26	30.4 ± 4.2
	3	7.44 ± 0.59	74.2 ± 4.6	292 ± 20	23.9 ± 1.4
	6	7.59 ± 0.24	71.1 ± 4.5	306 ± 3	12.5 ± 0.6
Reactions were performed at 70°C for the indicated times and contained 0.5 N HCl, the indicated quantities of MNU-treated calf thymus DNA, 50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/ml BSA.					

Table S2. Quantitation of 3mA and 7mG adducts released by DNA glycosylases				
Enzyme	3mA (pmol)		7mG (pmol)	
	Absolute	Relative to HCl	Absolute	Relative to HCl
(5N HCl)	48.7 ± 4.3	1.00	365 ± 19	1.00
No enzyme	3.10 ± 1.57	0.06	9.67 ± 0.70	0.03
AAG	43.6 ± 1.6	0.90	185 ± 9	0.51
MAG	34.9 ± 4.9	0.72	97.2 ± 6.5	0.27
TAG	48.7 ± 2.7	1.00	31.2 ± 1.7	0.09
TAG E38A	20.7 ± 9.3	0.43	43.5 ± 10.4	0.12
AlkC	44.7 ± 8.3	0.92	47.5 ± 14.2	0.13
AlkD	49.5 ± 5.0	1.01	218 ± 5	0.60
AlkD D113N	28.4 ± 2.2	0.58	36.9 ± 3.5	0.10
Reactions were performed at 37°C for 1 hour and contained 5 µM enzyme, 10 µg MNU-treated calf thymus DNA, 50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/ml BSA. Treatment with 5 N HCl also generated 14.5 pmol 1mA. O^6mG was not detected.				

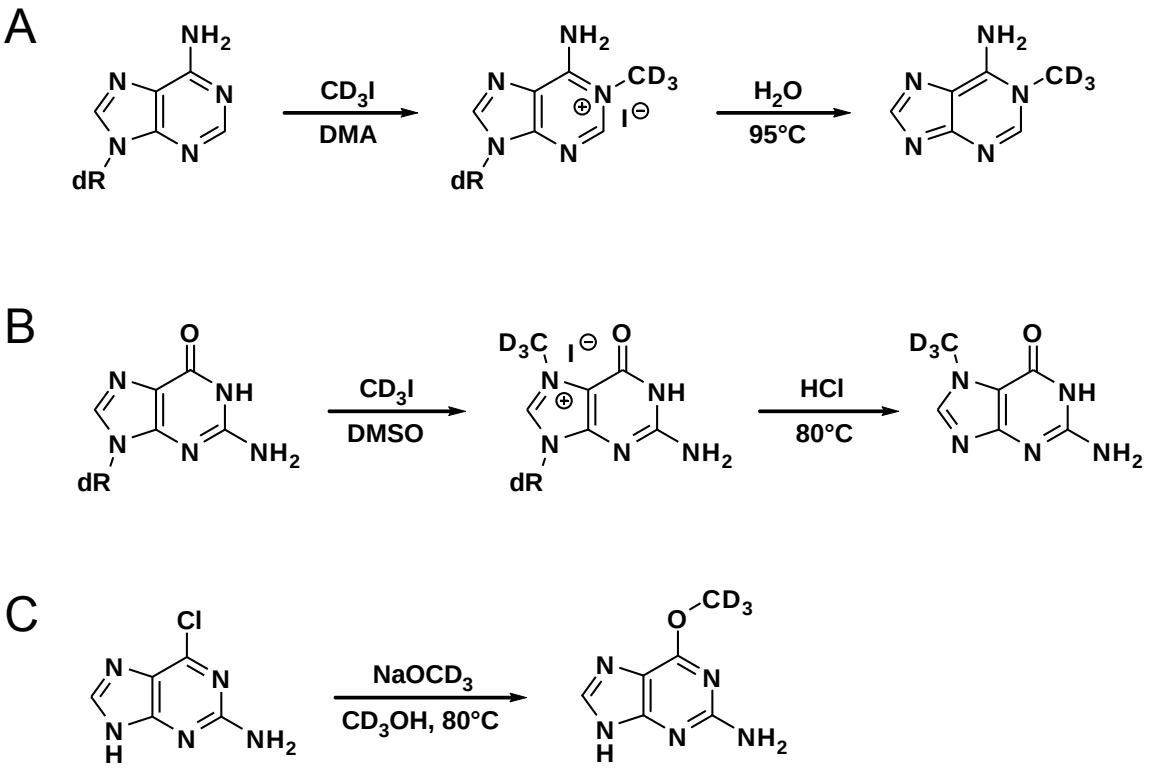

Figure S1. Synthesis of deuterated nucleobase standards. (A) d_3-1mA, (B) d_3-7mG, and (C) d_3-O^{6}mG.

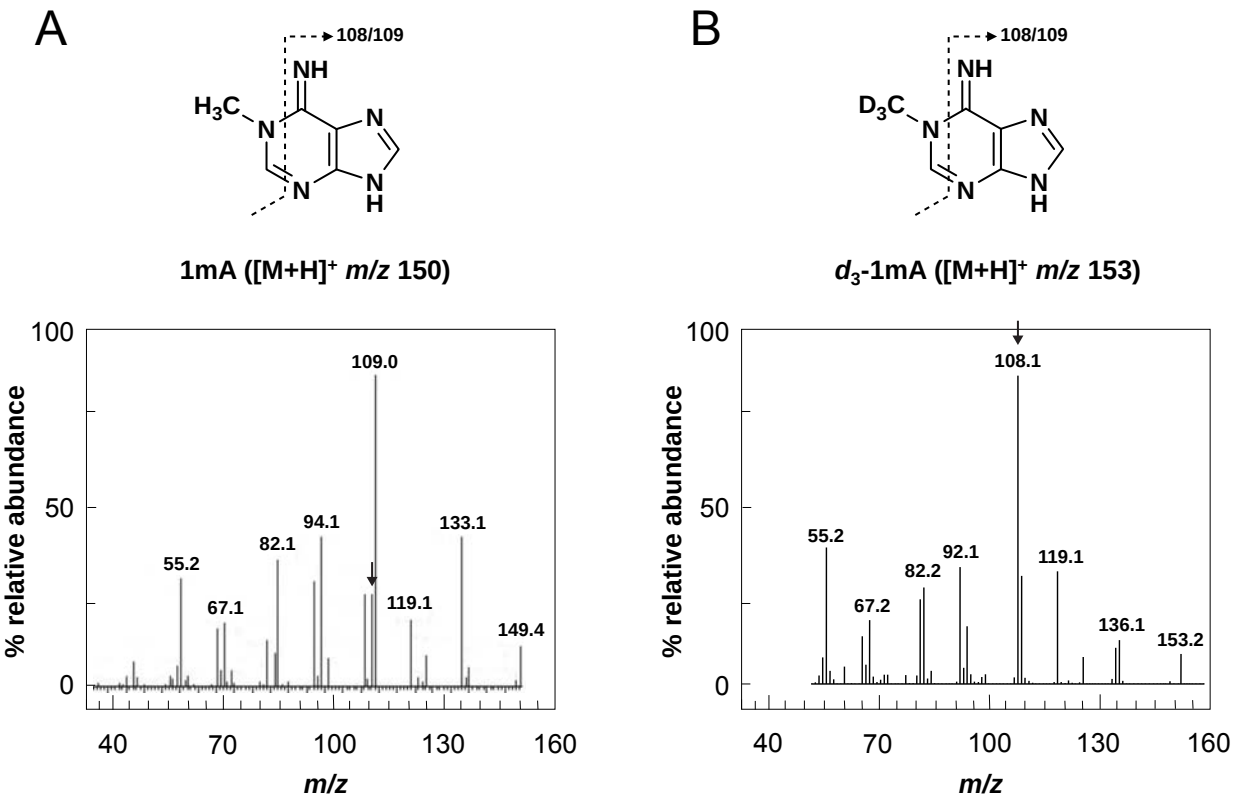

Figure S2. MS/MS-ESI(+) product ion spectra of $[M + H]^+$ ions of (A) 1mA and (B) d_3-1mA. The CID fragments used for quantitation are indicated in the spectra with arrows. The dashed lines in the chemical structures indicate the proposed sites of dissociation.

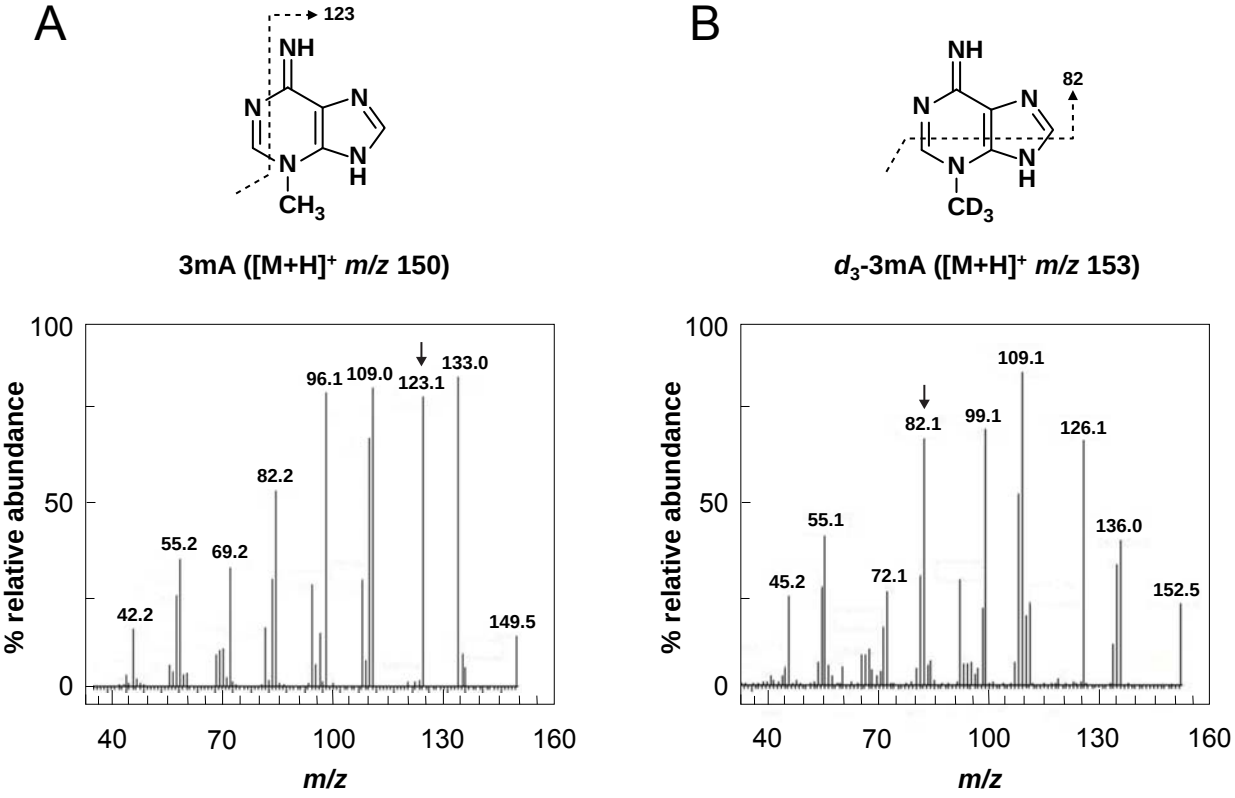

Figure S3. MS/MS-ESI(+) product ion spectra of $[M + H]^+$ ions of (A) 3mA and (B) d_3-3mA. The CID fragments used for quantitation are indicated in the spectra with arrows. The dashed lines in the chemical structures indicate the proposed sites of dissociation.

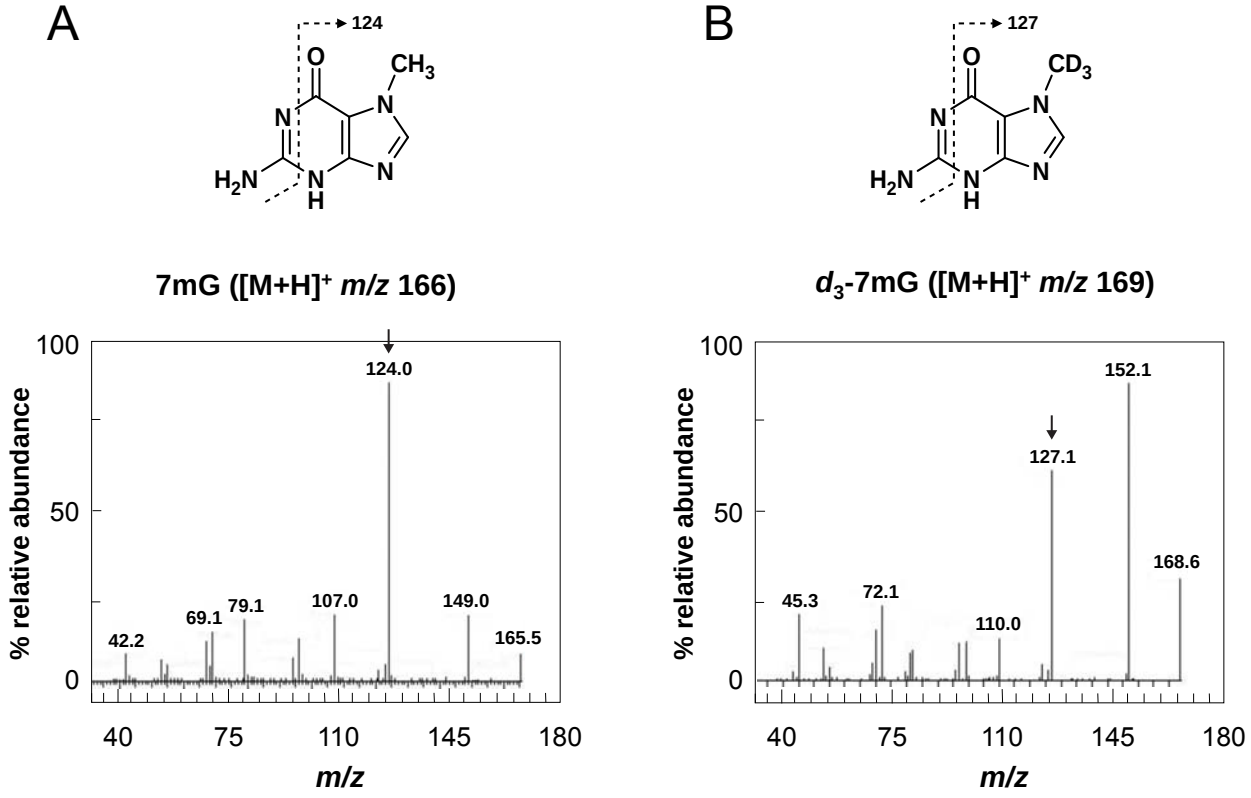

Figure S4. MS/MS-ESI(+) product ion spectra of $[M + H]^+$ ions of (A) 7mG and (B) d_3-7mG. The CID fragments used for quantitation are indicated in the spectra with arrows. The dashed lines in the chemical structures indicate the proposed sites of dissociation.

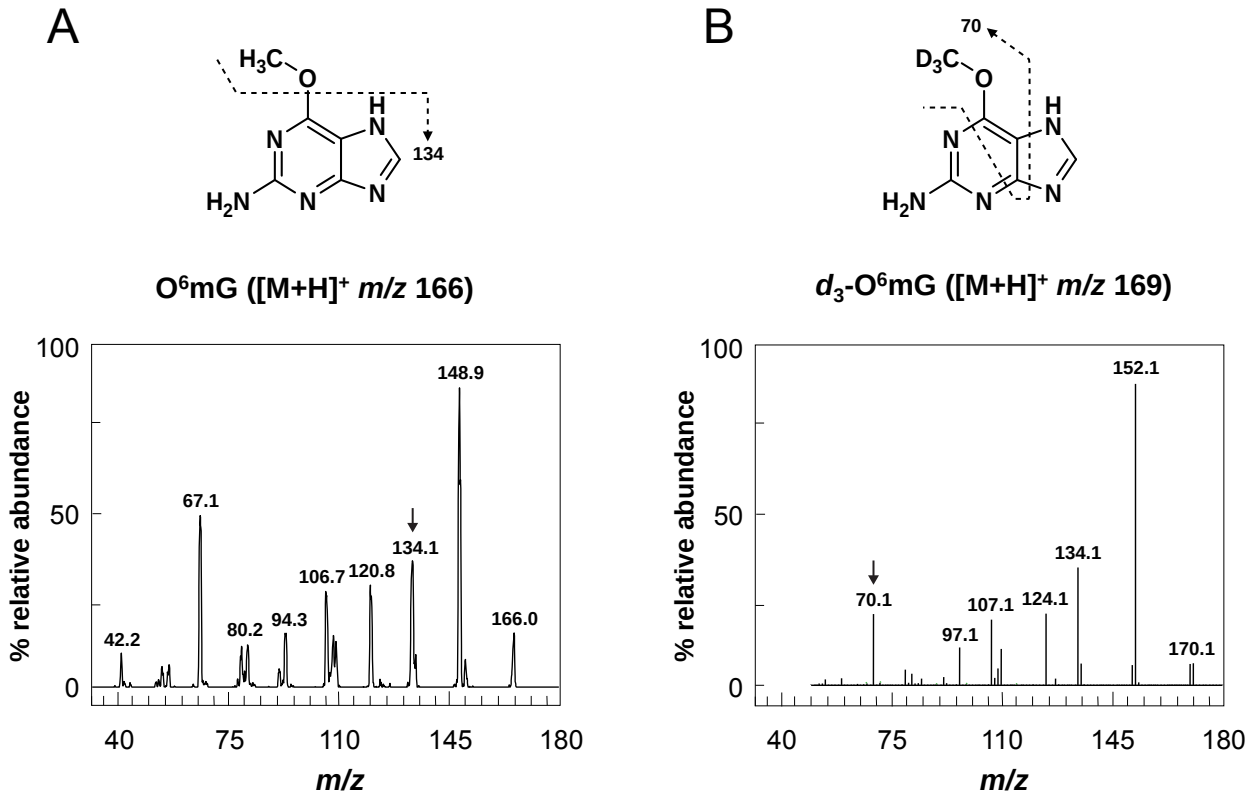

Figure S5. MS/MS-ESI(+) product ion spectra of $[M + H]^+$ ions of (A) O^6mG and (B) d_3-O^6mG. The CID fragments used for quantitation are indicated in the spectra with arrows. The dashed lines in the chemical structures indicate the proposed sites of dissociation.

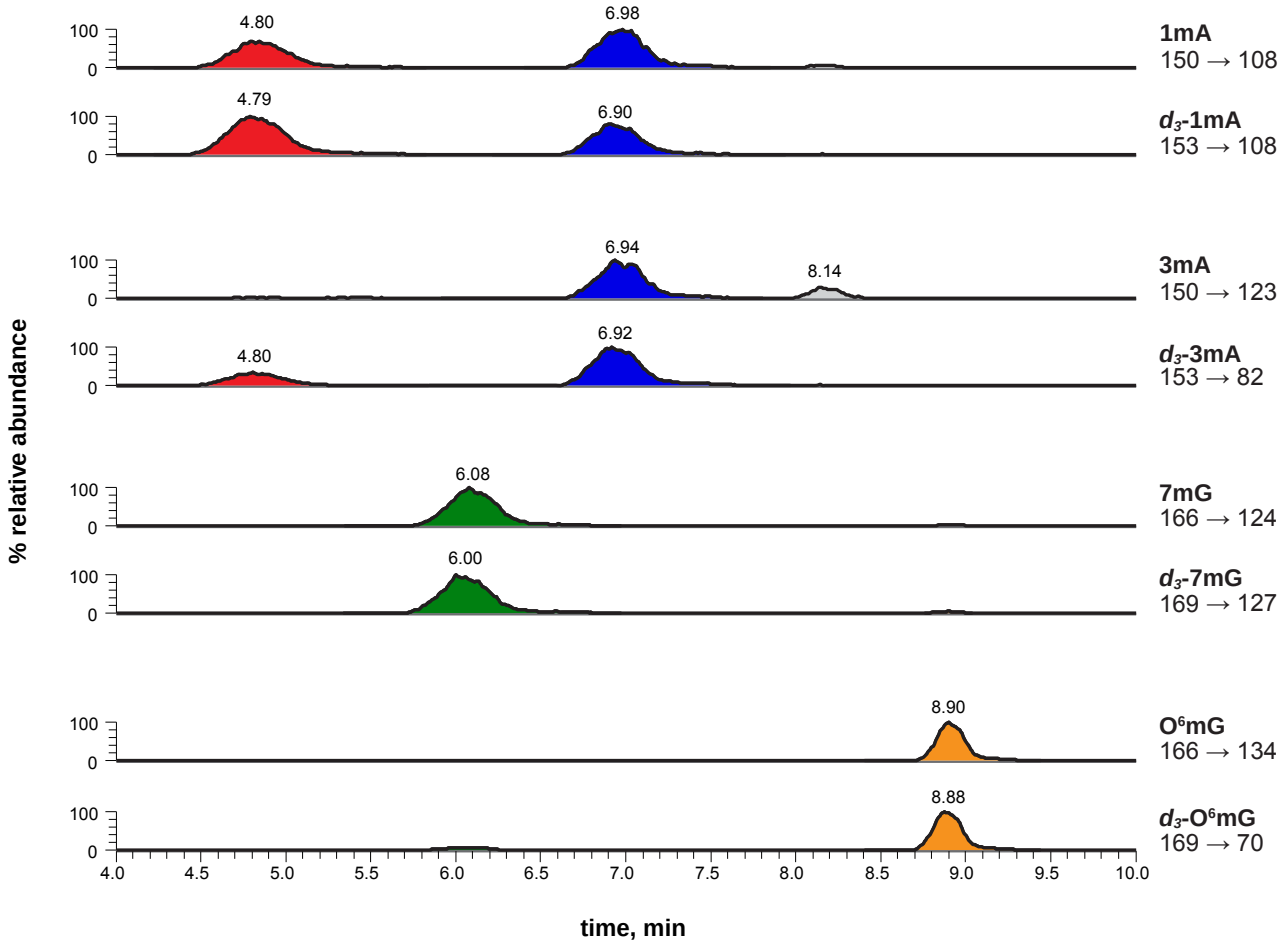

Figure S6. Representative HPLC-MS/MS chromatograms of unlabeled (H_3-Me-) and deuterium labeled (d_3-Me-) methylated nucleobases. Target compounds and their MRM transitions are indicated to the right of the chromatograms. Retention times are indicated above each peak. Due to the high polarity of the guanine and adenine analogues, an ion-pairing agent (perfluoropentanoic acid) was necessary to improve retention and resolution. Addditionally, a shielded reverse phase analytical column was used to minimize strong analyte-silanol interactions (peak tailing). Red, 1mA and d_3-1mA; blue, 3mA and d_3-3mA; green, 7mG and d_3-7mG; orange, O^6mG and d_3-O^6mG; gray, unidentified contaminant.

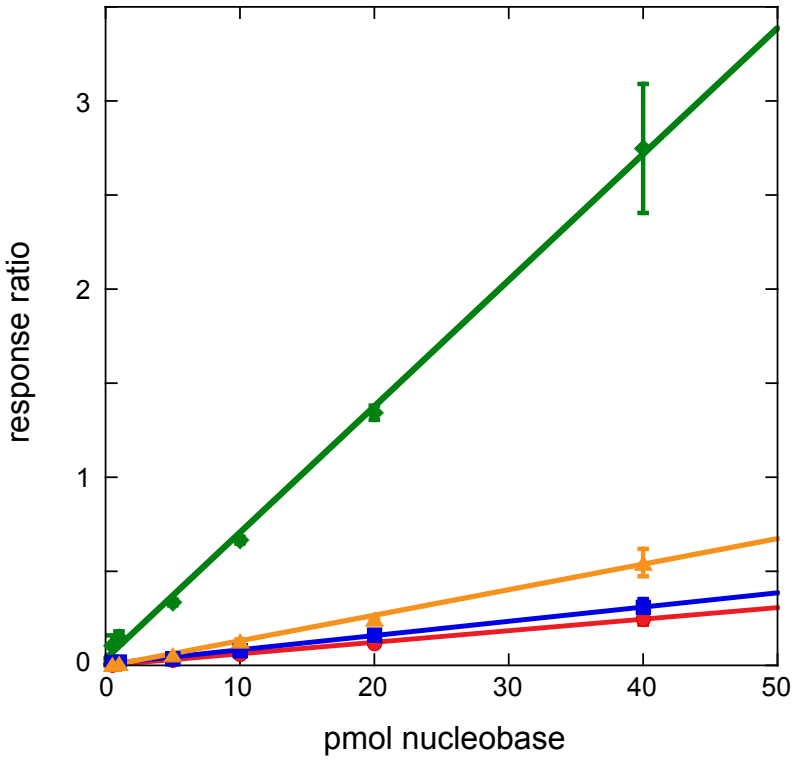

Figure S7. Representative calibration curves. The response ratio is the ratio of peak areas of undeuterated/deuterated methylated nucleobase. Calibration curves for all analytes were linear over a range of 0.5-40 pmol with all correlation coefficients (R^2) over 0.99. Red, 1mA and d_3-1mA; blue, 3mA and d_3-3mA; green, 7mG and d_3-7mG; orange, O^6mG and d_3-O^6mG.

Detailed Protocols

2.1.1. Preparation of methylated genomic DNA substrate

(1) Dissolve 1 mg calf-thymus DNA (Sigma-Aldrich D4522) in 1 ml TE buffer.

(2) Add 1 ml phenol:chloroform:isoamyl alcohol (25:24:1) (pH 8). Gently mix and let stand on ice for 10 min. Centrifuge at 4°C and 5,000 x *g* for 5 min. Recover top (aqueous) layer. Repeat until top layer is no longer cloudy.

(3) Add NaCl to 1 M final concentration [1 ml DNA/TE + 250 µl 5 M NaCl], followed by 3 volumes of cold 100% ethanol [1.25 ml DNA/TE/NaCl + 4 ml ethanol]. Centrifuge at 4°C and 4,000 x *g* for 5 min. Remove supernatant and wash pellet with 1 ml cold 70% ethanol twice. Let pellet dry completely.

(4) Resuspend pellet in 500 µl ice-cold cacodylate/perchlorate buffer (0.3 M sodium cacodylate/0.1 M $NaClO_4$ pH 8.3), and dialyze overnight against 1 L cacodylate/perchlorate buffer at 4°C.

(5) Prepare a 10 mM stock of MNU (Sigma-Aldrich N1517) in water. Add 1 µmol MNU to the DNA solution [100 µl 10mM MNU + 0.5 ml DNA]. Incubate in the dark 8 h at room temperature.

(6) Transfer reaction to glass beaker. Add NaCl to a final concentration of 1 M [150 µl 5 M NaCl + 0.6 ml DNA/MNU]. Slowly add 2.5 ml cold 100% ethanol.

(7) Wind DNA fibers onto sealed Pasteur pipet. Wash fibers with 1-2 ml cold 100% ethanol. Very gently resuspend fibers in 1 ml TE buffer in a glass tube and let stand on ice 15 min or until homogenous. Dialyze overnight against 2 L TE buffer at 4°C. Dialyze 3 times against 600 ml TE at 4°C for 3 h.

(8) Determine DNA concentration by UV absorption at 260 nm.

2.1.2. Excision of methylated bases from genomic DNA substrate

(1) Prepare 5X stock of glycosylase buffer and 2X stop buffer. Prepare 100 μM stock solutions of deuterated nucleobase standards in water (d_3-1mA, d_3-3mA, d_3-O^6mG) or methanol (d_3-7mG). Prepare a working solution containing 10 μM internal d_3-methylbase standards by 10-fold dilution of the stock solutions into stop buffer.

(2) In a 50 μl reaction, add 0.2 mg/ml methylated genomic DNA, 5 μM enzyme, and 1X glycosylase buffer according to the table below. Incubate at 37°C for 1 h. (Alternatively, periodically remove 50 μl aliquots from a 500 μl reaction to monitor the reaction time course.)

	[Final]	[Stock]	Volume
DNA Glycosylase	5 μM	50 μM	5 μl
Genomic DNA	0.2 mg/ml	1 mg/ml	10 μl
Glycosylase Reaction Buffer	1X	5X	10 μl
Water			25 μl
			50 μl

(3) Quench reaction with 50 μl 2X stop buffer spiked with deuterated nucleobase standards.

(4) Add 300 μl cold 100% ethanol. Incubate at -20°C for 20 min. Centrifuge at 4°C and 20,000 x *g* for 15 min.

(5) Remove supernatant and evaporate to dryness using either a rotary evaporator or a heating block at 60°C under a steady stream of nitrogen gas.

(6) Resuspend pellet in 50 μl water and transfer to a 200 μl silanized autosampler vial equipped with a Teflon-lined bonded rubber septum in preparation for HPLC-MS/MS analysis.

2.2.1. Preparation of oligonucleotide substrates

2.2.1.1a. Chemical synthesis—fluorescence detection of DNA

(1) Synthesize lesion-containing oligonucleotide [d(GACCACTACACCXATTCCTTACAAC)] with 6-carboxyfluorescein (FAM) at either the 5' or the 3' end and unlabeled complementary oligonucleotide [d(GTTGTAAGGAATTGGTGTAGTGGTC)].

(2) Anneal 100 μM FAM-lesion DNA to 200 μM complementary DNA in the presence of 1X annealing buffer (10 mM MES pH 6.5, 40 mM NaCl) in a screw cap microcentrifuge tube. The annealing reaction can be carried out using a thermocycler or by placing the tube in a 200 ml water bath at 80°C and letting it cool to room temperature. The formulation below results in 50 μM duplex DNA after annealing:

	[Final]	[Stock]	Volume
FAM-Lesion Strand	100 μM	500 μM	10 μl
Complementary Strand	200 μM	500 μM	15 μl
Annealing Buffer	1X	10X	5 μl
Water			25 μl
			50 μl

2.2.1.1b. Chemical synthesis—^{32}P detection of DNA

(1) Synthesize lesion-containing oligonucleotide [d(GACCACTACACCXATTCCTTACAAC)] and unlabeled complementary oligonucleotide [d(GTTGTAAGGAATTGGTGTAGTGGTC)].

(2) Label 50 µM lesion strand with 1 µM γ[P^{32}]-ATP, 40 µM cold ATP, and 10 U polynucleotide kinase (PNK). Incubate at 37°C for 25 min. Quench reaction by heating at 70°C for 10 min.

	[Final]	[Stock]	Volume
γ[^{32}P]-ATP	1 µM	1.67 µM	6 µl
Lesion-DNA	50 µM	500 µM	1 µl
PNK	1 U/µl	10 U/µl	1 µl
PNK Buffer	1X	10X	1 µl
ATP (cold)	40 µM	400 µM	1 µl
			10 µl

(3) Anneal 5 µM [^{32}P]-lesion DNA to 15 µM complementary DNA in the presence of 1X annealing buffer (10 mM MES pH 6.5, 40 mM NaCl) in a screw cap microcentrifuge tube by placing the tube in a 200 ml water bath at 80°C and letting it cool to room temperature. The formulation below results in 5 µM duplex DNA after annealing:

	[Final]	[Stock]	Volume
[^{32}P]-Lesion Strand	5 µM	50 µM	5 µl
Complementary Strand	15 µM	500 µM	1.5 µl
Annealing Buffer	1X	10X	5 µl
Water			38.5 µl
			50 µl

(4) Add 50 µl phenol:chloroform:isoamyl alcohol (25:24:1) (pH 8). Mix thoroughly and spin at 25°C and 700 x *g* for 1 min to separate layers. Remove top layer and add to G-25 spin column (GE Healthcare) that has been pre-equilibrated with TE buffer. Spin G-25 column at 25°C and 700 x *g* for 1 min.

2.2.1.2. Enzymatic synthesis of 7mG-containing oligonucleotides

(1) Anneal primer/template. Mix 30 μM 5'-FAM or [^{32}P]-labeled primer DNA (d(GACCACTACACC)), 100 μM reverse template DNA [d(GTTGTAAGGAATCGGTGTAGTGGTC)], and 1X annealing buffer (10 mM MES pH 6.5, 40 mM NaCl) in a screw cap microcentrifuge tube. Anneal using a themocycler or a 200-ml 80°C water bath allowed to cool to room temperature.

	[Final]	[Stock]	Volume
FAM-DNA Primer	30 μM	50 μM	10 μl
Template DNA	100 μM	500 μM	3.3 μl
Annealing Buffer	1X	10X	1.7 μl
Water			1.7 μl
			16.7 μl

(2) Extend primer/template. In a 20 μl reaction, add 10 μM labeled primer/template, 5 U DNA pol I Klenow Fragment, 400 μM d7mGTP, 100 μM d(ATC)TP, and 1X DNA pol buffer (66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 1.5 mM β-mercaptoethanol). Incubate at room temperature for 45 min.

	[Final]	[Stock]	Volume
Primer/Template	10 μM	30 μM	6.7 μl
d7mGTP	400 μM	4 mM	2 μl
d(A/T/C)TP	100 μM	1 mM	2 μl
Klenow	0.25 U/μl	5 U/μl	1 μl
DNA pol Buffer	1X	10X	2 μl
Water			6.3 μl
			20 μl

(3) Quench extension reaction by adding 20 μl 10 mM EDTA.

(4) Add 40 μl phenol:chloroform:isoamyl alcohol (25:24:1) (pH 8). Mix thoroughly, spin at 25°C and 700 x *g* for 1 min to separate layers. Remove top layer and add to G-25 spin column (GE Healthcare) that has been pre-equilibrated with TE buffer. Spin G-25 column at 25°C and 700 x *g* for 1 min. Maximum recovery is 40 μl 5 μM labeled double-stranded 7mG-DNA.

(5) Duplexes containing 7mG mispairs can be created by re-annealing the 7mG duplexes in the presence of 100-fold excess complementary strand containing T, G, or A opposite the 7mG. Single-stranded 7mG-DNA is obtained by re-annealing the 7mG duplexes in the presence of 100-fold excess unlabeled 7mG strand with G in place of 7mG.

2.2.2. Excision of lesions from oligonucleotide substrates

(1) Dilute oligonucleotide substrate to 500 nM in 1X glycosylase reaction buffer (50 mM HEPES pH 7.5, 100 mM KCl, 10 mM DTT, 2 mM EDTA, and 0.1 mg/ml BSA).
(2) In an 80 µl reaction, add 5 µM enzyme, 100 nM FAM-DNA, and 1X glycosylase reaction buffer. Incubate at 37°C.

	[Final]	[Stock]	Volume
DNA Glycosylase	5 µM	50 µM	2 µl
FAM-DNA	100 nM	500 nM	4 µl
Glycosylase Reaction Buffer	1X	5X	4 µl
Water			10 µl
			20 µl

(3) For each time point, remove an 8 µl aliquot and add 2 µl 1 M NaOH. Heat at 70°C for 2 min.
(4) Add 10 µl formamide loading buffer (80% (w/v) formamide, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol, 10 mM EDTA pH 8.0) and heat at 70°C for 5 min. Store samples on ice.
(5) Pour a 20% acrylamide/8M urea sequencing gel. Add 48 g urea, 50 ml 40% 19:1 acrylamide:bisacrylamide solution (Calbiochem Omnipur), and 10 ml 10X TBE buffer, and adjust the volume to 100 ml with ddH_2O. Add 200 µl 10% ammonium persulfate, mix, and then add 30 µl TEMED (BioRad Laboratories) to catalyze polymerization.
(6) Pre-run the gel at 40 W in 0.5X TBE (45 mM Tris, 45 mM borate, 1 mM EDTA pH 8.0). Load 8 µl glycosylase reaction and run gel at 40 W for 1 h or until dyes are separated by ½ inch.
(7) Separate glass plates carefully so that gel remains on one plate and cover with plastic wrap.
    a. FAM-DNA: Place gel-side down onto phosphorimager and image using fluorescence mode under a 532-nm laser and a 526-nm emission filter.
    b. [^{32}P]-DNA: Place phosphor screen onto gel for ~2 hours prior to imaging and image using storage phosphor acquisition mode under a 633-nm red laser and a 390-nm bandpass filter.
(8) Quantify band intensities using ImageQuant (GE Healthcare) or ImageJ (http://rsb.info.nih.gov/ij/).