

Notes & Tips

## A nonisotopic assay for unambiguous assignment of DNA glycosylase functionality

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Received 27 February 2007

Available online 10 April 2007

DNA glycosylase assays still commonly involve the use of radioactively end-labeled DNA substrates, although in recent years a few nonradioactive methods have been described [1–3]. All reported assays, however, have in common that they are strand cleavage assays. In contrast to DNA glycosylases with inherent strand-scission activity (i.e., DNA glycosylases/AP-lyases), monofunctional DNA glycosylases do not cleave the DNA backbone but leave behind an abasic or apurinic/aprimidinic (AP)<sup>1</sup> site in DNA [4]. Thus, for the analysis of monofunctional DNA glycosylase products with those assays, a posttreatment of samples is necessary to induce strand scission adjacent to the abasic site. The lack of current DNA glycosylase assays to concurrently detect abasic DNA products and AP-lyase products has likely been a contributing factor in controversies on the classification of some DNA glycosylases such as the adenine glycosylase MutY [5–7]. Indeed, it has been suggested that conflicting glycosylase assay results may have been caused by artifacts arising from different handling of DNA samples (heat or base treatment) and/or high temperature of gels during denaturing PAGE, taking into account the inherent lability of abasic sites [6,7].<sup>2</sup> Another limitation of current DNA glycosylase assays is that DNA substrates are restricted to relatively short DNA duplexes (typically ≤30 bp). The use of longer DNA substrates may allow, for instance, investigations aimed at delineating the mecha-

nisms by which repair enzymes find their specific target sites in DNA, analogous to corresponding studies involving other proteins such as restriction endonucleases [9].

Herein, a nonisotopic DNA glycosylase assay that overcomes both limitations is presented. The assay is performed on DNA substrates nearly 200 bp in length and monitored by subjecting untreated DNA glycosylase products to native, urea-enhanced PAGE. The method is based on reports in which gel electrophoresis under these conditions has been employed for efficient separation of intact DNA fragments (100–300 bp) from those containing a single nick [10,11]. The observed retardation in gel electrophoretic mobility of nicked DNA has been attributed to loss of stacking at the nicked site, which induces a kink in the DNA molecules [11]. In this study it was hypothesized that a similar effect may be observed for DNA fragments containing a single AP site, considering the available data in the literature which suggest that duplex DNA is somewhat kinked at the abasic site and/or contorts more easily relative to regular DNA [12].

To prepare appropriate DNA substrates, nicking endonucleases (NEases) were utilized as tools together with a DNA ligase (Fig. 1). The initial DNA duplex fragments containing recognition sequences for NEases in close vicinity were prepared from four overlapping oligonucleotides by assembly PCR and are sufficiently long to be visualized in a gel after a standard staining procedure without requiring radiolabeling. Treatment of the intact DNA duplexes with the corresponding NEases led to clean and full conversion to gapped DNA products (supplementary Fig. 1A). The addition of a mismatch-forming oligonucleotide and T4 DNA ligase to a purified gapped DNA sample resulted subsequently in the quantitative formation of an intact duplex DNA fragment with a single mismatch (see supplementary Fig. 1B).

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<sup>1</sup> Abbreviations used: AP, apurinic/aprimidinic site; dsDNA, double-stranded DNA; NEases, nicking endonucleases; UDG, uracil-DNA glycosylase.

<sup>2</sup> The classification of DNA glycosylases by another commonly employed assay, which is based on monitoring the absence or presence of reduced Schiff base intermediates (borohydride trapping assay) [8], may also yield discordant results, as was the case for MutY [6].

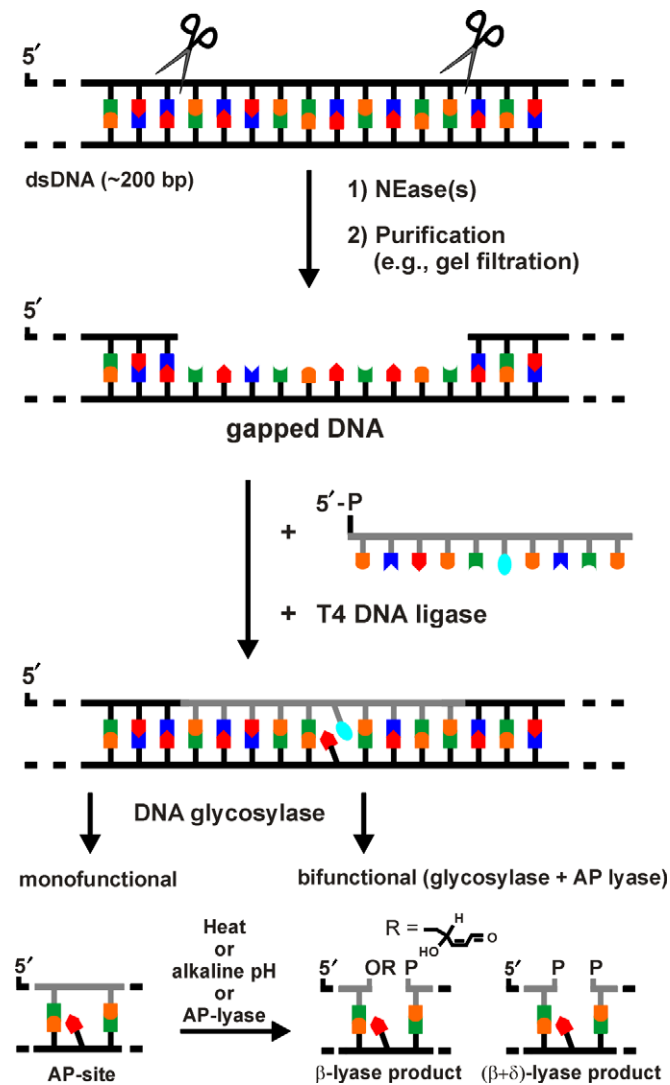


Fig. 1. Outline of the experimental procedure. Single-stranded breaks or nicks are generated in close vicinity on dsDNA by nicking endonucleases (NEases), leading to gapped DNA upon removal of cleaved DNA oligomers. Subsequently, a 5'-phosphorylated, mismatch-forming oligonucleotide is ligated into gapped DNA. The resulting intact DNA fragment bearing a single mismatch is afterward treated with a DNA glycosylase, and samples are analyzed by native, urea-enhanced PAGE.

Because uracil-DNA glycosylases (UDGs) are well known to be monofunctional glycosylases [5,12], DNA substrates with single mismatches containing uracil were initially generated. For all five uracil-containing substrates investigated, which differ in the DNA sequence context surrounding the mismatch and/or mismatch identity (see supplementary Fig. 2A), the obtained DNA products at incubation with *Escherichia coli* UDG exhibited a markedly lower electrophoretic mobility than the corresponding singly mismatched DNA duplex in urea-enhanced PAGE (Fig. 2A and supplementary Figs. 2B and 2C, lanes 1–3). The smallest but at all times detectable shift between the bands for the DNA fragments containing a mismatch or an abasic site was observed for the 9-x/G pair (x=U or abasic site), while a visibly larger shift was observed for the identical pair at a different location (6-x/G; see supplementary Fig. 2B). The observed differences in gel retardation are likely caused by different conformations at the abasic site, which depend on the nature of the base opposite the abasic site and the immediate flanking bases [12].

To simulate a bifunctional DNA glycosylase assay, the DNA fragments with an abasic site obtained from the release of free uracil from the uracil-containing substrates were treated by either Endonuclease III or by Endonuclease VIII, both of which have different AP lyase activities. As expected, the resulting AP lyase products displayed a significantly reduced electrophoretic mobility compared to the DNA fragments with an abasic site (Fig. 2A, lanes 4 and 5). These data clearly demonstrate that DNA duplex fragments containing a single mismatch and their corresponding mono- and bifunctional DNA glycosylation products can be separated and detected by urea-enhanced PAGE.

To demonstrate that the assay can be employed for the straightforward assignment of DNA glycosylase functionality, the method was applied on MutY using DNA duplexes containing either a single 8-oxoG/A or a single G/A mismatch. As shown in Fig. 2B, at incubation of the DNA duplex with the 8-oxoG/A mismatch with MutY, the major product (88–92%) possessed a slightly, but distinguishably lower gel mobility compared to the substrate, while the minor product exhibited a large gel shift. These

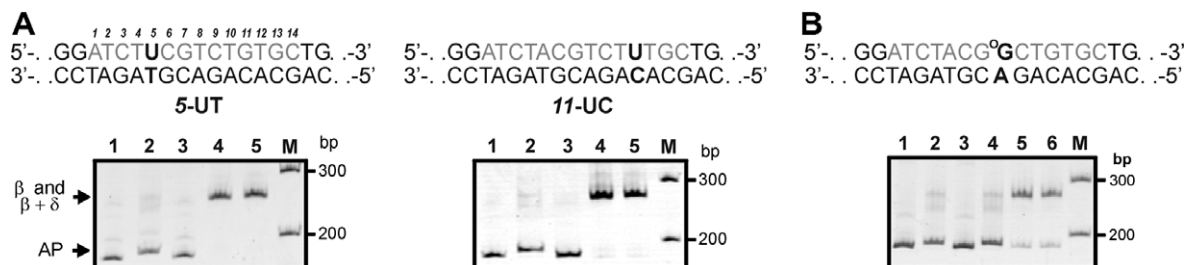


Fig. 2. Urea-enhanced PAGE of simple glycosylase (AP) and glycosylase/lyase ( $\beta$  and  $\beta+\delta$ ) products. Partial sequences of DNA substrates are shown above each electrophoregram. Italicized numbers correspond to base locations of oligonucleotides (gray) ligated into gapped dsDNA. (A) Analysis of dsDNA substrates with a 5-UT or 11-UC mismatch before (lanes 1 and 3) and after incubation with UDG (lanes 2), with UDG and *Endo*III (lanes 4), or with UDG and *Endo*VIII (lanes 5). (B) Analysis of the dsDNA substrate carrying a single 8-oxoG/A mismatch after incubation for 1 h at 37 °C with wild-type MutY (lanes 2 and 4) or mutant MutY S120K (lanes 5 and 6). Enzyme concentrations were 0.2  $\mu$ M (lanes 2 and 5) or 0.6  $\mu$ M (lanes 4 and 6), respectively. Lanes 1 and 3 are samples in the absence of enzyme. Lanes M correspond to a 100-bp DNA marker (Promega). All DNA samples were analyzed on 7.5% polyacrylamide gels containing 4 M urea, which were run for 3–4 h at 12.5 V/cm (ambient temperature) in 1x TBE buffer.

data provide clear evidence that MutY is mainly a simple DNA glycosylase and has at most a weak associated lyase activity, in agreement with some prior studies [6]. Although the gel shift of the simple glycosylation product is small, it could be reproducibly detected in every experiment. Similar data were obtained for the DNA duplex with the G/A mismatch (not shown). Incubation of either DNA substrate with the mutant MutY S120K led to products with considerably decreased gel electrophoretic mobility (see Fig. 2, lanes 5 and 6), consistent with the finding that this mutant is a bifunctional enzyme [13].

In summary, a new method that allows for the unequivocal determination of DNA glycosylase functionality is introduced. Moreover, this method may be used, in a strand cleavage assay format, for quantitative analyses of DNA glycosylase activity on long dsDNA substrates.

### Acknowledgments

The author thanks Dr. Sheila S. David for support and a gift of wild-type and mutant S120K MutY samples.

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2007.04.006](https://doi.org/10.1016/j.ab.2007.04.006).

### References

- [1] M.V. Garcia-Ortiz, R.R. Ariza, T. Roldan-Arjona, A chemiluminescent method for the detection of DNA glycosylase/lyase activity, *Anal. Biochem.* 298 (2001) 127–129.
- [2] A. Maksimenko, A.A. Ishchenko, G. Sanz, J. Laval, R.H. Elder, M.K. Saparbaev, A molecular beacon assay for measuring base excision repair activities, *Biochem. Biophys. Res. Commun.* 319 (2004) 240–246.
- [3] S. Madhusudan, F. Smart, P. Shrimpton, J.L. Parsons, L. Gardiner, S. Houlbrook, D.C. Talbot, T. Hammonds, P.A. Freemont, M.J. Sternberg, G.L. Dianov, I.D. Hickson, Isolation of a small molecule inhibitor of DNA base excision repair, *Nucleic Acids Res.* 33 (2005) 4711–4724.
- [4] A.K. McCullough, A. Sanchez, M.L. Dodson, P. Marapaka, J.S. Taylor, R.S. Lloyd, The reaction mechanism of DNA glycosylase/AP lyases at abasic sites, *Biochemistry* 40 (2001) 561–568.
- [5] H.E. Krokan, R. Standal, G. Slupphaug, DNA glycosylases in the base excision repair of DNA, *Biochem. J.* 325 (1997) 1–16.
- [6] S.D. Williams, S.S. David, Evidence that MutY is a monofunctional glycosylase capable of forming a covalent Schiff base intermediate with substrate DNA, *Nucleic Acids Res.* 26 (1998) 5123–5133.
- [7] P.M. Wright, J. Yu, J. Cillo, A.L. Lu, The active site of the *Escherichia coli* MutY DNA adenine glycosylase, *J. Biol. Chem.* 274 (1999) 29011–29018.
- [8] B. Sun, K.A. Latham, M.L. Dodson, R.S. Lloyd, Studies on the catalytic mechanism of five DNA glycosylases. Probing for enzyme-DNA imino intermediates, *J. Biol. Chem.* 270 (1995) 19501–19508.
- [9] D.M. Gowers, G.G. Wilson, S.E. Halford, Measurement of the contributions of 1D and 3D pathways to the translocation of a protein along DNA, *Proc. Natl. Acad. Sci. USA* 102 (2005) 15883–15888.
- [10] H. Kuhn, E. Protozanova, V.V. Demidov, Monitoring of single nicks in duplex DNA by gel electrophoretic mobility-shift assay, *Electrophoresis* 23 (2002) 2384–2387.
- [11] E. Protozanova, P. Yakovchuk, M.D. Frank-Kamenetskii, Stacked-unstacked equilibrium at the nick site of DNA, *J. Mol. Biol.* 342 (2004) 775–785.
- [12] J. Lhomme, J.F. Constant, M. Demeunynck, Abasic DNA structure, reactivity, and recognition, *Biopolymers* 52 (1999) 65–83.
- [13] S.D. Williams, S.S. David, A single engineered point mutation in the adenine glycosylase MutY confers bifunctional glycosylase/AP lyase activity, *Biochemistry* 39 (2000) 10098–10109.