Published in final edited form as:

Biochem Biophys Res Commun. 2007 April 6; 355(2): 431–437.

DNA damage reduces *Taq* DNA polymerase fidelity and PCR amplification efficiency.

Jan A. Sikorsky^{a,*}, Donald A. Primerano^b, Terry W. Fenger^c, and James Denvir^b

^aLeadAmerica, Boca Raton, FL, 33432

^b Department of Biochemistry and Microbiology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25704

^c Division of Forensic Science, Marshall University, Huntington, WV, 25704

Abstract

DNA damage blocks DNA polymerase progression and increases miscoding. In this study we assessed the effects of specific lesions on *Taq* DNA polymerase fidelity and amplification efficiency. In the presence of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), *Taq* DNA polymerase inserted dCMP and to a lesser extent dAMP. 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodA) instructed the incorporation of dTMP and caused a pronounced n-1 deletion not observed in other systems. The presence of an abasic lesion led to dAMP incorporation and n-1 deletions. In addition, we introduce the Mean Modified Efficiency (MME) as a more precise method for determining PCR amplification efficiency of damaged templates. Using this method, we were able to quantify reductions in amplification efficiency of templates containing 8-oxodG (single or multiple), 8-oxodA, or abasic sites. Because the MME method can detect small reductions in amplification efficiency, it may be useful in comparing the extent of damage in environmentally degraded or archival DNA specimens.

INTRODUCTION

Chemical and radiation damage to DNA results in the formation of modified bases. Replication across these lesions by DNA polymerases results in the misincorporation of nucleotides and the establishment of mutations in daughter chromosomes [1–5]. The synthesis of oligonucleotides containing chemically defined bases permits the study of translesion synthesis and consequent misincorporation by a number of DNA polymerases [6–16]. 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) can assume either a *syn* or *anti*-orientation depending on its base pairing partner [6–9]. When paired with 2'-deoxycytosine, 8-oxodG takes on the *anti* conformation and forms a normal Watson-Crick base pair [8–9]. In its *syn* form, 8-oxodG can associate with 2'-deoxyadenosine by Hoogsteen pairing [6–7]. In 8-oxodG translesional syntheses, the ratio of dCMP to dAMP insertion depends on the class of DNA polymerase. DNA Polymerase I, the Klenow fragment, and polymerase β preferentially incorporate dCMP, while eukaryotic polymerases α and δ incorporate dAMP more frequently [10]. 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodA) resides in the *anti* orientation and causes only minor conformational changes to duplexed DNA [11]. On synthetic templates containing 8-oxodA, the Klenow fragment of *E. coli* DNA polymerase I and *Taq* DNA polymerase exclusively

*To whom correspondence should be addressed. Jan A. Sikorsky, Ph.D., 1515 South Federal Hwy, Suite 301, Boca Raton, FL 33432, Ph: 561.368.8085x218, Fx: 561.368.8151, jsikorsky@lead-america.org

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

incorporate dTMP in the presence of all four dNTPs [11–13]. When nucleotides are presented individually, these two enzymes insert dTMP and to a lesser extent dGMP which demonstrates that 8-oxodA has limited mutagenic potential [12]. The Klenow fragment and DNA polymerase α preferentially insert dAMP opposite both synthetic and natural abasic sites [14–16]. Abasic sites also promote sequence-dependent 1 and 2 bp deletions [14–16]. In addition to miscoding, misorientation of the 3' hydroxyl of mismatched bases at the primer terminus can stall polymerization by DNA polymerase I [1,10].

Mathematical models, based on exponential DNA amplification during the polymerase chain reaction (PCR), permit the use of real-time PCR data in template abundance calculations [17]. For example, in one application of the comparative threshold ($\Delta\Delta C_T$) method, target mRNA abundance is determined by comparing its observed threshold cycle (C_T) values to the C_T s from a characterized reference mRNA [18–19]. This method assumes reaction efficiencies from the target and reference genes are equivalent and close to one [19]. These mathematical models do not consider the effect of differences in template sequence and quality on amplification efficiency. Nogva and Rudi observed that the *Listeria monocytogenes* 23S rRNA gene amplifies less efficiently than its hlyA gene and that restriction enzyme digests of input genomic DNA improve the amplification efficiencies of both genes. They concluded that amplification efficiency during early PCR cycles is less than the efficiency during the exponential phase [20]. Work in our lab adds template quality as a factor that impacts early cycle reaction efficiency [21]. We found that while some base modifications in synthetic templates (e.g. single 8-oxodG) have no impact on *Taq* amplification efficiency, other lesions (e.g. thymidine dimer and 8-oxodA bases) dramatically reduce *Taq* efficiency. We also show that the Relative Threshold Cycle (RTC) method can quantify differences in amplification efficiency of templates with the latter lesions [21]. The RTC method has recently been used to show that sperm from infertile men contain more DNA damage than sperm DNA from fertile men [22].

Studies on the mutagenic impact of modified bases on *Taq* DNA polymerase are limited to 8-oxodA. To extend these studies, we determined the miscoding potential of 8-oxodG, an abasic site, as well as 8-oxodA in PCRs catalyzed by *Taq* polymerase. In addition, although 8-oxodG causes a transient inhibition of chain extension by the Klenow fragment [10], we did not observe a reduction in amplification efficiency on 8-oxodG templates using the RTC method [21]. Therefore we developed the Mean Modified Efficiency (MME) method as a more sensitive measure of changes in amplification efficiency. We report here that (1) *Taq* DNA polymerase fidelity is reduced when oxidized bases or abasic sites are present in the template, (2) both 8-oxodA and abasic sites induce deletion formation, and (3) the MME method can detect more subtle differences in amplification efficiency between normal templates and those with 8-oxodG bases.

MATERIALS AND METHODS

Synthesis and Amplification of Oligonucleotide Templates

Ninety base oligonucleotides containing either normal or modified bases (7,8-dihydro-8-oxo-2'-deoxyguanosine, 7,8-dihydro-8-oxo-2'-deoxyadenosine, or abasic site (tetrahydrofuran ring)) were synthesized on an Applied Biosystems (ABI, Foster City, CA) Model 394 DNA Synthesizer in the Marshall University Genomics Core Facility as described previously [21]. Primers were designed to amplify either the full-length 90-mer oligonucleotide or a 45 base region with no base modifications (the internal control region (ICR); Fig. 1).

Purification and Sequencing of Real-Time PCR products

PCR products were sized by agarose gel electrophoresis (data not shown) and purified using the Qiagen® QiaQuick PCR amplification kit [23]. To prevent chemical degradation post-purification, purified products were eluted in Qiagen Buffer EB (10mM Tris-Cl, pH 8.5) and stored at -20°C .

Purified PCR products were sequenced using ABI Big Dye Terminator chemistry with *Taq* FS polymerase [23]. Due to the shortness of templates (< 100 bp), modifications to the reference protocol were made. 1 l of purified PCR product was added to 9 l of a master-mix containing 1 l of ready reaction mix (including *Taq*, ddNTPs, dNTPs, MgCl_2 , and buffer), 0.5 l of a 10 M unidirectional sequencing primer (PCR primers [21]), and 7.5 l dH_2O . The reaction mixes were cycle sequenced using optimized sequencing parameters (30 cycles: 95°C for 30 s, 50°C for 20 s, 60°C for 4 min).

Sequencing product purification and analysis

Sequencing reactions were purified using Centri-Sep 100 spin columns (Princeton Separations, Princeton, NJ; [23]). Samples were denatured at 95°C for 4 min and loaded onto an Applied Biosystems 310 Genetic Analyzer for analysis. Purified reactions were processed using the Seq POP6 Rapid (1 ml) E module (Sequence Analysis software v3.3 (ABI)) and analyzed using the CE-1 base-calling algorithm. Computer analyzed base calls were manually edited for accuracy. Sequence comparisons were made using ABI Sequence Navigator v1.0.1 software or the SeqLab Analysis platform (Accelrys, San Diego, CA).

Derivation of the MME Method

Exponential amplification of a DNA template is given by

$$x_n = (1 + E)^n x_0. \quad 1$$

where x_n is total DNA fluorescence at cycle n , and E is amplification efficiency [17]. In the MME assay, modified templates generate unmodified templates at one efficiency and these PCR-derived unmodified templates amplify at a different efficiency. To model this two-efficiency amplification, we derived the following:

$$E_m = \frac{E_u(T - M)}{M[(1 + E_u)^{C_T} - 1]}, \quad 2$$

where E_m is the efficiency of modified templates, E_u the efficiency of PCR-derived unmodified templates, T the threshold fluorescence, M the fluorescence of the input (modified) template, and C_T the cycle number at which threshold fluorescence is reached. (See Supplemental Materials for complete derivation.) In order to solve for E_m using equation 2, it was necessary to obtain values for C_T , T , E_u , and M . T was set at 1 and C_T was given as output from ABI Prism® 7000 SDS Software.

In amplifications with a single efficiency, E is related to the slope (m) in a plot of the logarithm of input template concentration against C_T values:

$$E = 10^{-(1/m)} - 1. \quad 3$$

In order to determine m , we prepared unmodified template at concentrations that ranged over six orders of magnitude and amplified the entire unmodified template. We plotted the C_T values for each PCR against the base 10 logarithm of the template concentration and determined the

slope (m) of the best fit line. This method was used in the determination of both E_U and E_{ICR} .

We used an indirect approach in the determination of M for the modified templates. This approach relied on the amplification of the ICR within each modified and unmodified template. The initial fluorescence value (x_0) for an input template can be calculated from T , C_T , and the ICR amplification efficiency (E_{ICR}) as follows:

$$x_0 = \frac{T}{(1 + E_{ICR})^{C_T}}. \quad 4$$

Since the same template stocks are used for both the 45-mer and 90-mer experiments, it is appropriate to substitute the input fluorescent values x_0 from ICR data as input measures for M .

Assigning Statistical Significance

In order to establish statistical significance of differences in MME calculations, 95% confidence intervals were computed for the MME of each template. Five replicate amplifications of both the ICR and full-length templates were performed at six input concentrations (see Table 1). For each of these five reaction pairs, equation 2 was applied and the mean calculated generating MME measurements for each concentration. The mean and standard deviation of these measurements was computed and used to calculate a 95% confidence interval for the measurement [24]. Differences between MMEs for two different templates are considered statistically significant if their respective confidence intervals do not overlap.

RESULTS

8-oxodG, 8-oxodA, and an abasic site reduced PCR amplification efficiency

We amplified ICR and full-length targets from equivalent amounts of unmodified templates and templates containing 8-oxodG, 8-oxodA, or abasic sites. The reduced amplification efficiencies of the full-length OxodG3A, OxodA1, and abasic templates are readily apparent from rightward shifts in full-length amplification curves (Fig. 2). We determined the unmodified template amplification efficiency (E_U), the ICR amplification efficiency (E_{ICR}), the initial template fluorescence (x_0), and the threshold cycle (C_T) from the ICR curves for each template and quantified the reduction in efficiency using the MME method (Table 1). When compared to the unmodified template (OxoControl), significant reductions in MME were observed for all five of the modified templates. The MMEs for OxodG1, OxodG2A, and OxodG3A templates were 0.509, 0.414, and 0.319, which represented reductions of 34.4%, 46.6%, and 95.9%, respectively (Table 1); all three of these MMEs were significantly different from the control template efficiency at $p < 0.05$. The OxodG1 and OxodG2A templates showed no significant difference in RTC efficiency [21]. For all other templates, calculated MMEs were comparable to RTC estimations [21]. We note that for a given template there is no correlation between modified efficiencies and input template concentration (Table 1).

Taq DNA polymerase fidelity is influenced by lesions on DNA template

To determine if 8-oxodG, 8-oxodA, and abasic lesions in oligonucleotide templates have miscoding potential, real-time PCR products from normal and modified oligonucleotide amplifications were directly sequenced (Figs. 3A–E). The forward PCR primer (Fig. 1) was used to sequence PCR products. Since this primer binds to the 3' end of the complement of the input oligonucleotide, any sequence readout must be derived from strands complementary

to the input oligonucleotide and therefore should convey information on nucleotides incorporated across from the lesion. The sequence of the OxoControl PCR product matched its expected sequence (Fig. 3A). PCR products from the three templates with 8-oxodG lesions gave rise to a mixture of readouts at the positions of base modifications. The majority of 8-oxodG amplicons showed the expected Watson-Crick incorporation of dCMP at the lesion site (s), while a smaller fraction contained dAMP which creates a stable transversion (G=C to T=A; Figs. 3B, 3C, and data not shown). Amplicons derived from the 8-oxodA and abasic templates each revealed two different sequences. For the 8-oxodA template, the predominant sequence contained dTMP opposite the lesion site (Fig. 3D). In addition, some amplicons exhibited a one base deletion which originated at 8-oxodA position and caused a frameshift in the sequence readout. This n-1 sequence was inferred from subpeaks at positions 28, 39, 47, 49, 54, and 57 (down arrows in Fig. 3D); no comparable subpeaks were observed in PCR products derived from the unmodified or 8-oxodG templates. The abasic site instructed the incorporation of dAMP in the majority of products. In addition, a less abundant n-1 deletion originating at the abasic site was apparent (down arrows in Fig. 3E).

DISCUSSION

We have previously shown that DNA lesions perturb PCR amplification to different degrees and that the reduced amplification efficiency of modified templates results in a delay during the initial cycles of amplification which in turn translates into an increase in the threshold cycle C_T [21]. Under the assumption that amplification efficiencies of the ICR and full-length productions are identical and interchangeable between templates, the RTC method detects large differences in amplification efficiencies of templates containing 8-oxodA, TT Dimer, or abasic lesions. However, the RTC method does not detect differences between the control template and templates with a single 8-oxodG or two 8-oxodGs separated by 13 bases [21]. The MME method refines the RTC method for quantifying aggregate damage to DNA templates by utilizing exact measures of the unmodified template amplification efficiencies (E_U) (equation 2) following concepts developed by Pfaffl and coworkers [25]. Using the MME method, differences in amplification efficiency were observed between OxoControl and OxodG1 templates and between OxoControl and OxodG2A templates (both comparisons are statistically significant at $p < 0.05$; Table 1). These findings indicate that while the RTC formula is appropriate for the comparison of templates where large differences in amplification rates exist, the more refined MME formula is suitable for the comparison of templates with smaller reductions in efficiency.

Bypass of 8-oxodA, 8-oxodG, and abasic lesions by several DNA polymerases has been extensively characterized [4,10–12,15,26]. In addition to impacting the rates of nucleotide insertion and extension, these modified bases can pair with normal bases and increase the rate of mutagenesis. We studied the mutagenic potential of 8-oxodA, 8-oxodG, and abasic sites by sequencing PCR products derived from templates with these modified bases. In translesional syntheses catalyzed by the Klenow fragment or DNA polymerase α , abasic lesions (either deoxyribose or tetrahydrofuran) preferentially instruct the insertion of dAMP [15,26]. In addition, 1 and 2-bp deletions have been detected opposite abasic sites where the frequency of deletion formation is dependent on the sequence context at the site of modification [15,26]. In our experimental system we also found PCR products with n-1 deletions in addition to the dAMP insertion and observed a reduction in amplification efficiency. Shibutani and coworkers proposed that abasic sites could cause deletions by a template re-alignment in which the dAMP inserted opposite the abasic site pairs with the template base 5' to the lesion (where T is the preferred base) and allows for polymerase catalyzed extension [15,27].

Taq DNA polymerase incorporated dUMP opposite 8-oxodA in the majority of PCR products. (Our PCRs contained dUTP in place of dTTP.) This is consistent with the preferential

incorporation of dTMP opposite 8-oxodA observed for the Klenow fragment, *Taq* polymerase, and eukaryotic polymerases α and β [11–13]. However, in other less abundant amplicons derived from the OxodA1 template, we observed a one base deletion immediately after the modified base. Since this apparent base skipping is not observed in the OxoControl or 8-oxodG templates, it can not be the result of simple primer repositioning. This effect of 8-oxodA has not been observed in other experimental systems where deletions could readily be detected [11,13,28]. Several differences in the PCR components may explain this unexpected result. (1) The insertion kinetics of dUMP opposite 8-oxodA have not been characterized and it is possible that the observed decrease in MME and n-1 deletion formation in 8-oxodA-templated reactions were at least initially the result of stalling after dUMP incorporation. (2) Our template sequence is derived from the human mitochondrial genome and has a run of four G's immediately 5' to the 8-oxodA site [29]. Although this proposed mechanism is not dependent on the insertion of dAMP as described in the Shibutani model, a stretch of G:C base pairs might stabilize misaligned extension products and thereby favor deletion formation.

Nucleotide insertion in 8-oxodG templates is dependent on the type of DNA polymerase. Eukaryotic DNA polymerases α and δ favor insertion of dAMP while native DNA polymerase I and the Klenow fragment incorporate dCMP and to a lesser extent dAMP opposite 8-oxodG. With the latter two enzymes there is a transient inhibition of extension 3' to the modified base [10]. We observed a preferential incorporation of dCMP over dAMP at 8-oxodG sites by *Taq* DNA polymerase and a reduction in MME which are consistent with the prokaryotic DNA polymerase findings.

PCR products of varying length are created during initial stages of the PCR and these products only influence reaction kinetics during the first few cycles [20]. Our work implies that template quality also impacts PCR efficiency only during these stages [21]. Once sufficient bypass products form, normal exponential amplification proceeds. Without taking template quality into consideration, quantification of damaged DNA by real-time PCR could underestimate the amount of input template or overestimate amplifiable template. In addition, workers studying low copy DNA having incurred environmental damage (e.g. ancient DNA) should interpret DNA sequence data with increased caution given the likelihood of lesion induced mutagenesis during the PCR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This project was supported by NIH Grants P20 RR-16477 and P20 RR-020180 and NIH Grant 2001-RC-CX-K002. Points of view in this document are those of the author(s) and do not necessarily represent the official position or views of the NIH, the NCRR, or the U.S. Department of Justice. The authors would like to thank the Department of Veterans Affairs for use of equipment and laboratory space.

References

1. Steitz T. DNA polymerases: structural diversity and common mechanisms. *J Biol Chem* 1999;274:17395–17398. [PubMed: 10364165]
2. Wang D, Kreutzer D, Essigmann J. Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutat Res* 1998;400:99–115. [PubMed: 9685598]
3. Marnett L. Oxyradicals and DNA damage. *Carcinogenesis* 2000;21:361–370. [PubMed: 10688856]
4. Wallace SS. Biological consequences of free radical-damaged DNA bases. *Free Radic Biol Med* 2002;33:1–14. [PubMed: 12086677]
5. Cooke M, Evans M, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* 2003;17:1195–1214. [PubMed: 12832285]

6. Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* 1986;83:4908–4912. [PubMed: 2941760]
7. McGregor D, Lang M. Carbon tetrachloride: genetic effects and other modes of action. *Mutat Res* 1996;366:181–195. [PubMed: 9033667]
8. Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury. I. Free radical biochemistry. *J Toxicol Clin Toxicol* 1993;31:139–171. [PubMed: 8433411]
9. Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury. II. Are free radicals the cause of toxin-induced liver injury? *J Toxicol Clin Toxicol* 1993;31:173–196. [PubMed: 8433412]
10. Shibutani S, Takeshita M, Grollman A. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* 1991;349:431–434. [PubMed: 1992344]
11. Guschlbauer W, Duplaa AM, Guy A, Teoule R, Fazakerley GV. Structure and in vitro replication of DNA templates containing 7, 8-dihydro-8-oxoadenine. *Nucleic Acids Res* 1991;19:1753–1758. [PubMed: 1851559]
12. Shibutani S, Bodepudi V, Johnson F, Grollman A. Translesional synthesis on DNA templates containing 8-oxo-7, 8-dihydrodeoxyadenosine. *Biochemistry* 1993;32:4615–4621. [PubMed: 8485138]
13. Kamiya H, Miura H, Murata-Kamiya N, Ishikawa H, Sakaguchi T, Inoue H, Sasaki T, Masutani C, Hanaoka F, Nishimura S, Ohtsuka E. 8-Hydroxyadenine (7, 8-dihydro-8-oxoadenine) induces misincorporation in in vitro DNA synthesis and mutations in NIH 3T3 cells. *Nucleic Acids Res* 1995;23:2893–2899. [PubMed: 7659512]
14. Takeshita M, Chang C, Johnson F, Will S, Grollman A. Oligodeoxynucleotides containing synthetic abasic sites. *J Biol Chem* 1987;262:10171–10179. [PubMed: 2440861]
15. Shibutani S, Takeshita M, Grollman A. Translesional synthesis on DNA templates containing a single abasic site. *J Biol Chem* 1997;272:13916–13922. [PubMed: 9153253]
16. Asagoshi K, Terato H, Ohyama Y, Ide H. Effects of guanine-derived formamidopyrimidine on DNA replication. *J Biol Chem* 2002;277:14589–14597. [PubMed: 11839760]
17. Rasmussen, R. Quantification on the LightCycler instrument. In: Meuer, S.; Wittwer, C.; Nakagawara, K., editors. *Rapid Cycle Real-time PCR: Methods and Applications*. Springer Press; Heidelberg: 2001. p. 21–34.
18. Livak K. ABI Prism 7700 Sequence Detection System, User Bulletin 2. PE Applied Biosystems. 1997
19. Livak K, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$. *Method, Methods* 2001;25:402–408.
20. Nogva HK, Rudi K. Potential influence of the first PCR cycles in real-time comparative gene quantifications. *Biotech* 2004;37:246–253.
21. Sikorsky JA, Primerano DA, Fenger TW, Denvir J. Effect of DNA Damage on PCR Amplification Efficiency with the Relative Threshold Cycle Method. *Biochem Biophys Res Commun* 2004;323:823–830. [PubMed: 15381074]
22. San Gabriel M, Zhang X, Zini A. Estimation of human sperm gene-specific deoxyribonucleic acid damage by real-time polymerase chain reaction analysis. *Fertil Steril* 2006;85:797–799. [PubMed: 16500368]
23. Withrow AG, Sikorsky J, Downs JC, Fenger T. Extraction and analysis of human nuclear and mitochondrial DNA from electron beam irradiated envelopes. *J Forensic Sci* 2003;48:1302–1308. [PubMed: 14640275]
24. Remington, R.; Schork, M. *Statistics with Applications to the Biological and Health Sciences*. Prentice Hall Inc; Englewood Cliffs, NJ: 1970.
25. Pfaffl M. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45. [PubMed: 11328886]
26. Strauss BS BS. The 'A rule' of mutagen specificity: a consequence of DNA polymerase bypass of non-instructional lesions? *Bioessays* 1991;13:79–84. [PubMed: 2029269]
27. Shibutani S, Grollman AP. On the mechanism of frameshift (deletion) mutagenesis in vitro. *J Biol Chem* 1993;268:11703–11710. [PubMed: 8505300]

28. Tan X, Grollman AP, Shibutani S. Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2'-deoxyadenosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA lesions in mammalian cells. *Carcinogenesis* 1999;20:2287–2292. [PubMed: 10590221]
29. Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJ, Staden R, Young IG. Sequence and organization of the human mitochondrial genome. *Nature* 1981;290:457–465. [PubMed: 7219534]

Fig 1. Model of oligonucleotide amplification

A) Amplification of full-length oligonucleotides which differ in the number and type of modified bases (indicated by *). B) The internal control region (ICR) contains no modified bases and therefore amplifies with the same efficiency in each template.

Fig 2. Amplification of oligonucleotides containing modified bases in real-time

Unmodified and modified oligonucleotides were amplified using primers targeted for either the ICR (—+) or the full-length oligonucleotide (—x). Delta Rn is the baseline subtracted PCR product fluorescence normalized to an internal dye (ROX). A rightward shift in amplification profiles indicates a reduction in amplification efficiency relative to the ICR. All PCR profiles show data points taken from cycles 1 through 40. Each amplification profile represents the average of 5 separate experiments.

Fig 3. Sequence analysis of PCR products from 90mer oligonucleotides

PCR products from OxoControl (panel A), OxodG1 (panel B), OxodG2A (panel C), OxodA1 (panel D), or abasic (panel E) templates were subjected to cycle sequencing. The positions of modified bases are designated (**M**) in the sequence header. dAMP incorporation opposite 8-oxodG bases is marked with an asterisk (*). N-1 sequence deletions derived from 8-oxodA and abasic templates are indicated by solid down arrows. dTMP incorporation in the abasic template is marked by a number sign (#).

Table 1
Mean modified efficiencies of oligonucleotide templates^a

	Input Template	Mean $C_{T(90mer)}$	x_0^b	Modified Efficiency (Em), ^{cd}	Mean Modified Efficiency (MME)	MME S.D.
OxoControl	100fmole	4.16	6.48E-02	0.925	0.776	0.0792
	10fmole	7.17	1.09E-02	0.760		
	1fmole	11.0	8.65E-04	0.760		
	100amole	14.5	9.10E-05	0.720		
	10amole	17.8	1.07E-05	0.704		
OxodG1	1amole	21.2	9.68E-07	0.789	0.509	0.113
	100fmole	4.32	9.57E-02	0.536		
	10fmole	6.81	1.65E-02	0.618		
	1fmole	11.1	1.38E-03	0.432		
	100amole	15.0	1.40E-04	0.328		
OxodG2A	10amole	17.3	1.60E-05	0.622	0.414	0.140
	1amole	21.1	1.54E-06	0.518		
	100fmole	4.83	8.97E-02	0.418		
	10fmole	7.26	1.31E-02	0.613		
	1fmole	11.1	1.36E-03	0.475		
OxodG3A	100amole	14.5	1.49E-04	0.463	0.0319	0.00600
	10amole	19.2	1.49E-05	0.223		
	1amole	22.5	1.31E-06	0.290		
	100fmole	9.19	4.40E-02	0.043		
	10fmole	12.4	7.80E-03	0.030		
OxodA1	1fmole	15.8	7.91E-04	0.029	0.1009	0.0245
	100amole	19.3	7.60E-05	0.028		
	10amole	22.8	7.58E-06	0.027		
	1amole	25.7	7.97E-07	0.035		
	100fmole	7.65	4.10E-02	0.128		
Abasic	10fmole	10.2	7.75E-03	0.127	0.0134	0.00250
	1fmole	13.6	9.18E-04	0.104		
	100amole	16.9	1.01E-04	0.101		
	10amole	20.7	1.07E-05	0.0768		
	1amole	24.0	1.27E-06	0.0692		
	100fmole	10.4	4.86E-02	0.0179		
	10fmole	13.6	8.49E-03	0.0129		
	1fmole	17.1	9.71E-04	0.0108		
	100amole	20.6	8.71E-05	0.0117		
	10amole	23.9	9.02E-06	0.0127		
	1amole	27.2	8.81E-07	0.0143		

^aThreshold (T) equals 1 for all calculations.

^bInitial fluorescence (x_0) was calculated using equation 4.

^c E_{U} s were determined using equation 3; equaling 0.936 (OxoControl), 0.943 (OxodG1), 0.922 (OxodG2A), 0.963 (OxodG3A), 0.968 (OxodA1), and 0.946 (Abasic), respectively.

^dMMEs were derived using equation 2.