Electronic Supplementary Material (ESI) for ChemComm.

A gene-targeted polymerase-mediated strategy to identify *O*^{6}-methylguanine damage

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Experimental

1.1 Phosphoramidite synthesis: Benzi phosphoramidite was synthesized as previously described.[1]

1.2 Oligonucleotide synthesis: Modified DNA was synthesized by solid-phase DNA synthesis on a Mermade 4 DNA synthesizer (Bioautomation Corporation) in trityl-off mode. Universal Q SynBase™ CPG support columns were used as well as natural nucleotide phosphoramidites from Link Technologies Ltd. (Lanarkshire, Scotland). *O*^{6}-MeG phosphoramidite was purchased from Glen Research (Sterling, VA, USA). Oligonucleotide deprotection was carried out by treatment with 30% aqueous ammonium hydroxide at 55 ^{o}C for 10 hours, except in the case for the *O*^{6}-MeG DNA where a 1:1 solution of aqueous ammonium hydroxide and *N*-methylamine was used. The oligonucleotides were dried under reduced pressure, re-suspended in DNAse/RNase free water and filtered with 0.45 µM nylon filters (Merck Millipore) before HPLC purification. The oligonucleotide was purified by reverse phase HPLC using an Agilent eclipse XDB C-18 5 µM 4.6 x 150 mm column. The mobile phase consisted of 50 mM triethylammonium acetate (TEAA) and acetonitrile (ACN). The flow rate was set to 1.0 mL/min with a mobile phase starting at from 0-10% ACN over 18 min, increasing to 50% ACN from 18.1-20 min, and equilibrated from 20.1-25 min at 10% ACN. Fractions corresponding to the desired oligonucleotides were collected, lyophilized and re-suspended in DNAse/RNase free water. The Benzi Primer 1 and Benzi Primer 2 DNA (DNA sequences see Table 1) were purified by polyacrylamide gel electrophoresis. The DNA concentrations were measured by UV absorbance on a Varian Cary 100 Bio UV/Vis spectrophotometer.

1.3 Primer extension analysis: The Benzi Primer 1 (Table 1) DNA was 5'-end labelled using ^{32}P-ATP (Perkin Elmer) and T4 polynucleotide kinase (Promega Corp, Madison, WI, USA). The labelled Benzi DNA was annealed to complementary templates containing G or *O*^{6}-MeG, Codon-12 G and Codon-12 *O*^{6}-MeG in Table 1, respectively by heating the duplexes at 95 ^{o}C for 5 min and slow cooling overnight. KlenTaq M747K (a gift from Andreas Marx, University of Konstanz, Germany) primer extension reactions (10 µl) contained 20 nM enzyme, 10 nM DNA, 10 µM of each dNTP, 50 mM Tris HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% Tween 20. The reactions were incubated at either 55 or 72 ^{o}C for 10 min. For Dpo4-mediated reactions (10 µl), 20 nM enzyme (Trevigen), 10 nM DNA, 10 µM of each dNTP, 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT, 100 µg/ml bovine serum albumin, 5% glycerol was used and reactions were incubated at 37 ^{o}C for 30 min. For KlenTaq 1 (DNA Polymerase Technologies, St. Louis, MO, USA) reactions (10 µl), 25 units enzyme, 10 nM DNA, 10 µM of each dNTP, 250 mM Tris-HCl (pH 7.9), 15 mM $MgCl_2$, 0.13% Brij 58, 40 mM $(NH_4)_2SO_4$ was used and reactions were incubated at 55 ^{o}C for 10 min. Reactions were terminated by adding 10 µl of stop buffer (95% formamide, 20 mM EDTA and 0.5% xylene cyanol and bromophenol blue). Reaction mixtures (4 µl) were loaded onto 15% polyacrylamide/7 M urea gels and extension products were visualized from a phosphorimaging screen with a phosphorimager (BioRad, Hercules, CA, USA).

1.4 Linear amplification

Reaction mixtures (25 µl) contained 20 nM of KlenTaq (myPols), 1x KTQ buffer (50 mM Tris HCl (pH 9.1), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.1% Tween 20), 250 µM of each dNTP, 200 nM of the Benzi primer 2, and 2 nM of 27mer template (Fig. 3). Reactions with increasing ratio of *O*^{6}-MeG/G (Fig. 4) contained 2 nM of G 27mer template and x nM of *O*^{6}-MeG 27met template (x = 0; 0.5; 1; 1.5; 2 nM).

Reactions were performed on a T3000 Thermocycler (Biometra). An initial denaturation step at 95 °C for 2 min was performed, followed by 35 cycles of 95 °C for 30 s, 40 °C for 30 s and 55 °C for 30 s, followed by a final step of elongation at 55°C for 3 min. For imaging, 10 µl of each reaction were added to 10 µl of 2% SDS in formamide, boiled for 10 min at 100 °C, then immediately placed on ice, and loaded on a home-made 15% acrylamide / 7 M urea gel, which was previously equilibrated for 30 min with 1x TBE (100 mM Tris base, 100 mM boric acid, 2 mM EDTA). Gels were run at 300 V, at rt for 30 min, and at 4 °C for further 2-2.5 h. Gels were imaged on a ChemiDoc MP Imaging System (BioRad), after 10 min incubation in 1x SYBR gold (Invitrogen) in TBE.

1.5 Molecular modelling studies: Structures were computed with the Molecular Operating Environment software (Chemical Computing Group). Crystal structure of WT KlenTaq with incoming ddCTP opposite template G (G1) was used (PDB ID: 3RTV). [2] PBD file was loaded as Molecular Assembly. The ternary complex of crystal structure KlenTaq:template:primer was prepped by Quick Prep option. Base pair before G1 in the crystal structure was G:C (G0:C0, template:primer). For modelling studies, C0 was replaced by Benzi by using the software Molecular Builder; G0 was kept as G for modelling studies on unmodified template, or replaced by O^6-MeG for modelling studies on O^6-MeG template. After bases replacement, for energy minimizations, the potential energy of the protein was fixed at distance 4.5 to 9 Å from Benzi-base pairs, and tethered at distance >9 Å, by applying Amber 10 ETH force field, with 0.1 gradient. Visualization was performed in the PyMol software (Schrodinger) and images were rendered with ray 2400.

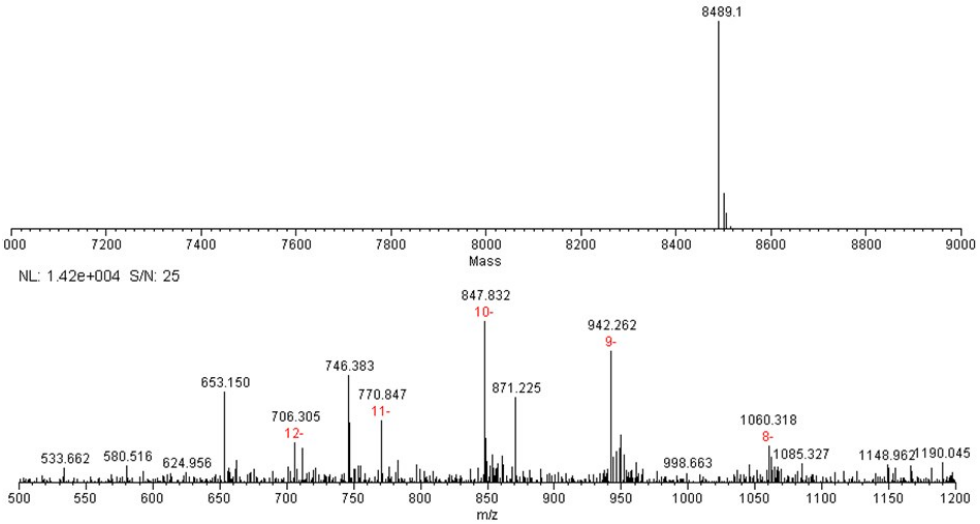

Figure S1. Mass spectra of codon-12 O^6-MeG DNA; sequence: 5'- GTA GTT GGA GCT GXT GGC GTA GGC AAG-3' (X = O^6-MeG, parent mass 8489)

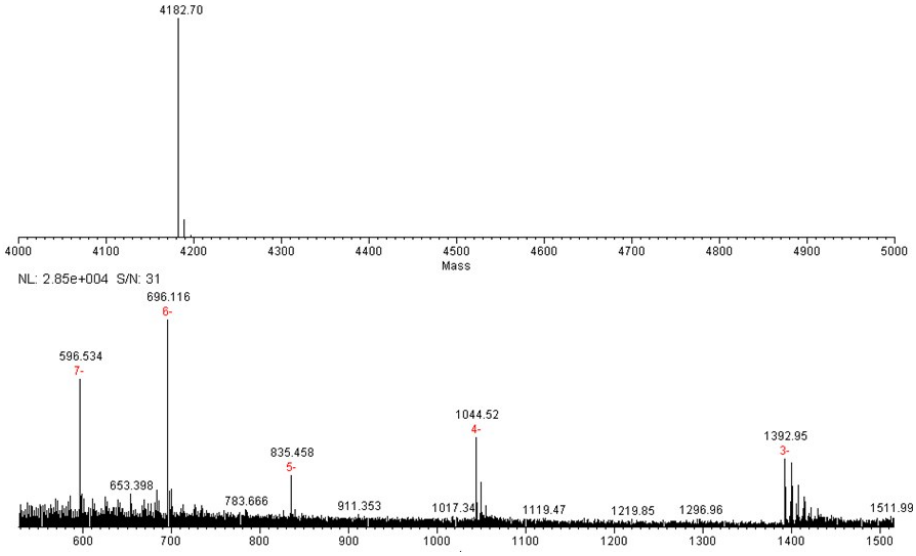

Figure S2. Mass spectra of codon-12 Benzi DNA; sequence: 5'- CTT GCC TAC GCC AP-3' (P = Benzi, parent mass 4182)

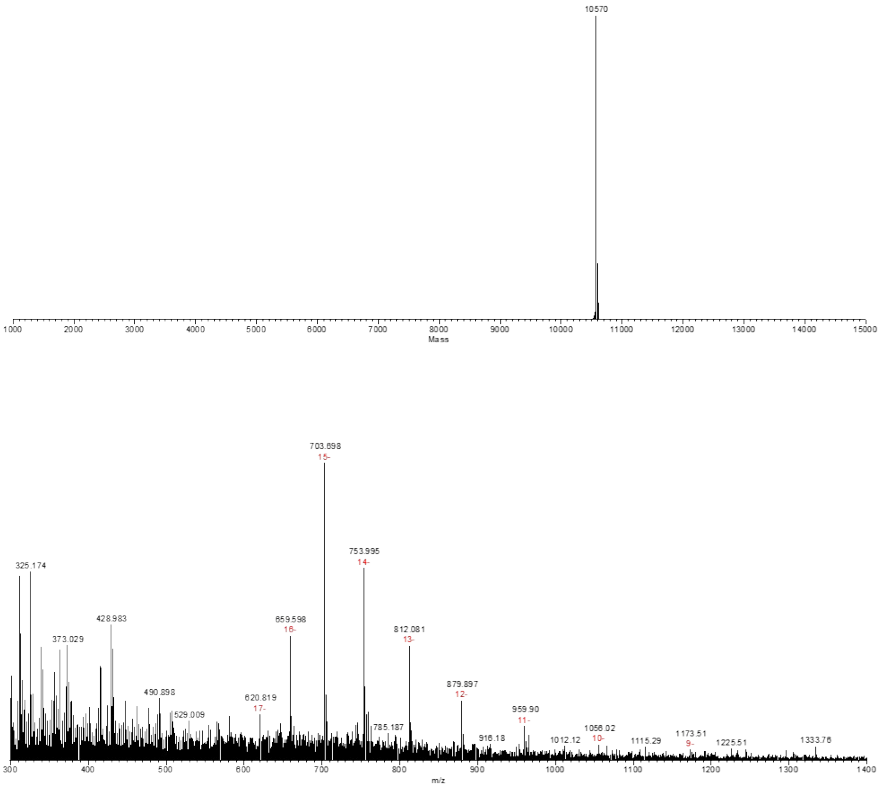

Figure S3. Mass spectra of Benzi-dT_{21} DNA; sequence: 5'- T_{21} CTT GCC TAC GCC AP-3' (P = Benzi, parent mass 10570)

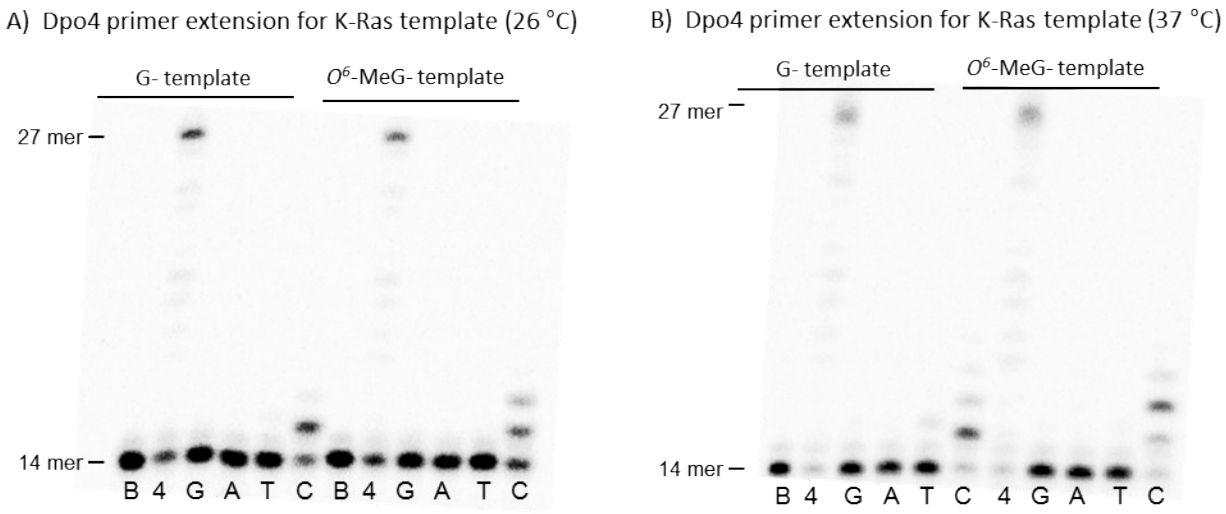

Figure S4. Dpo4-mediated elongation past Benzi:G and Benzi:O^6-MeG at (A) 26 °C (B) 37 °C.

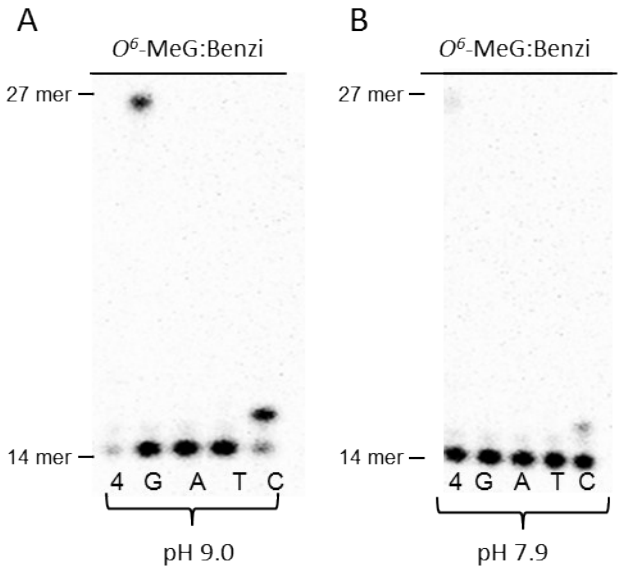

Figure S5. KlenTaq (2.5 units) elongation past Benzi:O^6-MeG at 55 °C (A) pH 9 and (B) and pH 7.9.

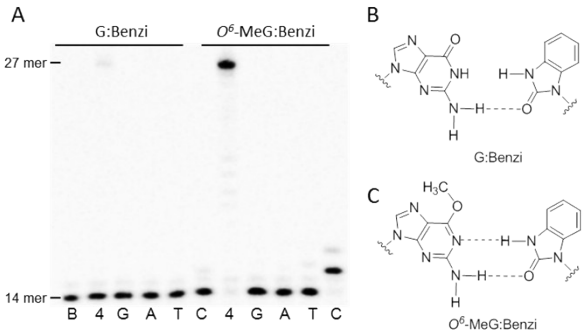

Figure S6. (A) KlenTaq M747K-mediated elongation past G:Benzi and *O*^{6}-MeG:Benzi primer template DNA at 55 °C for 10 min (B = 14mer blank, 4 = 4 canonical dNTPs, G = dGTP, A = dATP, T = dTTP, and C= dCTP) and (B) proposed hydrogen bonding between *O*^{6}-MeG and Benzi.

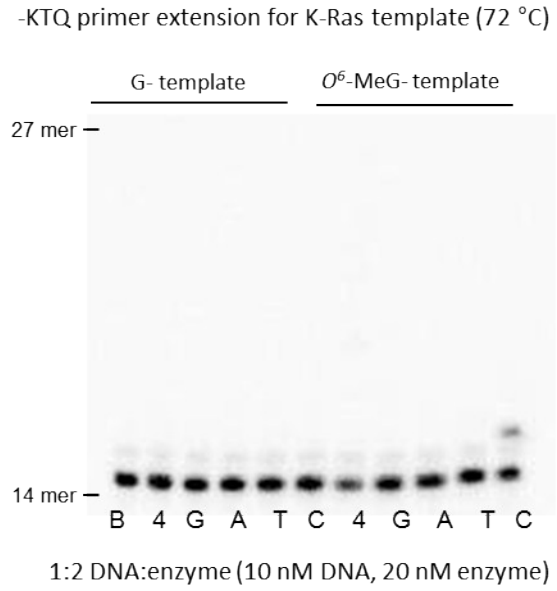

Figure S7. KlenTaq M747K elongation past Benzi:G and Benzi:*O*^{6}-MeG at 72 °C.

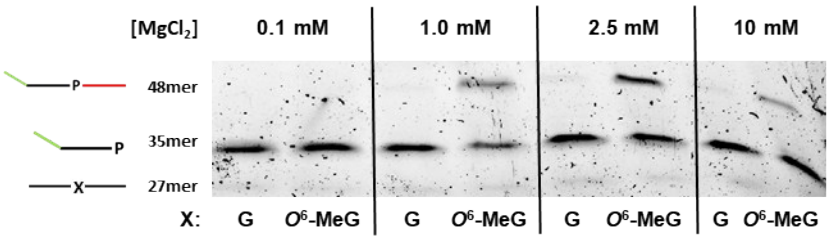

Figure S8. KlenTaq-mediated linear amplification with varying concentrations of $MgCl_2$. Reaction conditions are the same as described for the linear amplification, except for the varying concentrations of $MgCl_2$ of 0.1, 1.0, 2.5 and 10 mM $MgCl_2$. The lowest (0.1 mM) and highest concentrations of $MgCl_2$ (10 mM) show no extension and reduced extension, respectively, in comparison to 2.5 mM $MgCl_2$.

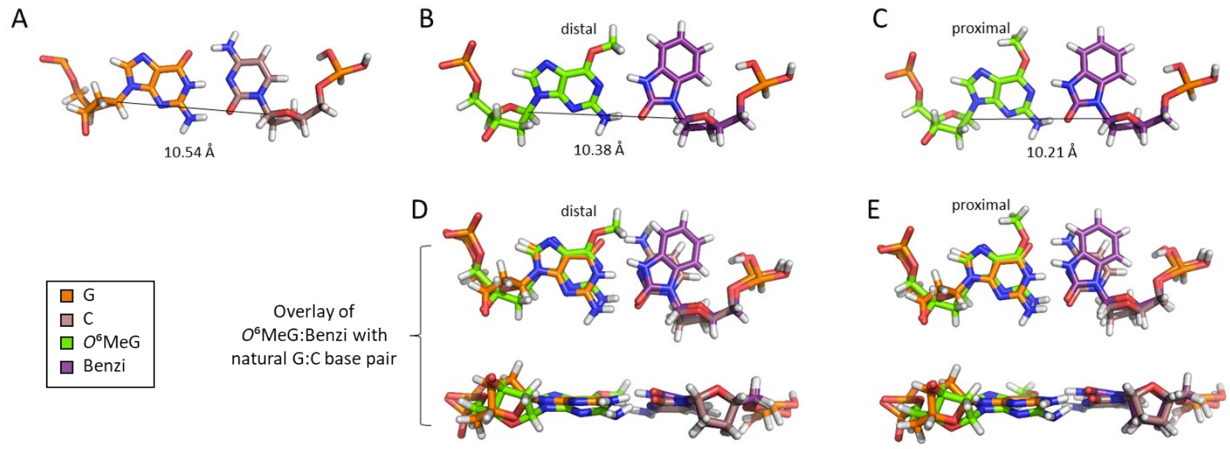

Figure S9. Modelled structures with wild type KlenTaq with C1'-C1' distances shown in Å for terminal template:primer base pairs (A) canonical G:C (B) *O*^{6}-MeG:Benzi with the methyl group in distal conformation and (C) *O*^{6}-MeG:Benzi with the methyl group in proximal conformation. In addition, overlay structures comprised of G:C and *O*^{6}-MeG:Benzi where determined for (D) the methyl in distal conformation and (E) the methyl in the proximal conformation.

References:

[1] H. L. Gahlon, S. J. Sturla, *Chem. Eur. J.* 2013, **19**, 11062-11067

[2] K. Betz, A. Nilforoushan, L. A. Wyss, K. Diederichs, S. J. Sturla and A. Marx, *Chem. Comm.* 2017, **53**, 12704-12707.