Electronic Supplementary Information

Integration of Single-Molecule Detection with Magnetic Separation for Multiplexed Detection of DNA Glycosylases †

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EXPERIMENTAL SECTION

Materials. All oligonucleotides (Table 1) were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). Magnesium chloride ($MgCl_2$), ethylenediaminetetraacetic acid (EDTA), trizma hydrochloride (Tris-HCl, pH 8.0), sodium chloride (NaCl), immunoglobulin G (IgG) and human serum albumin (HSA) were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). Human 8-oxoguanine-DNA glycosylase 1 (hOGG1), 10× NEB buffer 2 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DL-Dithiothreitol (DTT), pH 7.9), 10 mg/mL bovine serum albumin (BSA), uracil DNA glycosylase (UDG), 10× UDG reaction buffer (200 mM Tris-HCl, 10 mM EDTA, 10 mM DTT, pH 8.0), endonuclease IV, 10× NEB buffer 3 (1M NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9), human alkyladenine DNA glycosylase (hAAG),

and T4 pyrimidine dimer glycosylase (T4-PDG) were obtained from New England Biolabs (Beverly, MA, USA). Thymine DNA Glycosylase (TDG) was bought from R&D System (Minneapolis, MN, USA). The streptavidin-coated magnetic beads (Dynabeads®M-280 Streptavidin, Dynal) were obtained from Invitrogen (California, CA, USA). Nuclear extract kit was brought from Active Motif (Carlsbad, CA, U.S.A.). All other reagents were of analytical grade and used without further purification. Ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, USA).

Table 1. Sequences of the Oligonucleotides[α]

note	sequence (5′-3′)
assistant probe 1	GTA CGT AGG TCG ATT CTC AGT ACT GCA CGT GAC AAT CT
assistant probe 2	CTG AGA ATC GAC CTA CGT ACT GCC UUA CUU AGC UTC CA
trigger 1 (15 nt)	ATT GTC ACG TGC AGT
trigger 1 (16 nt)	GAT TGT CAC GTG CAG T
trigger 1 (17 nt)	AGA TTG TCA CGT GCA GT
trigger 1 (18 nt)	AGA TTG TCA CGT GCAGTA
trigger 2 (15 nt)	GAA GCT AAG TAA GGC
trigger 2 (16 nt)	GGA AGC TA A GTA AGG C
trigger 2 (17 nt)	TGG AAG CTA AGT AAG GC
trigger 2 (18 nt)	TGG AAG CTA AGT AAG GCA
signal probe 1	biotin -ACT GCA CGX GAC AAT CT- AF488
signal probe 2	biotin -TGC CTT ACX TAG CTT CCA- Cy5

[α]In the assistant probe1, the underlined G indicates the damaged guanine (8-oxoG) modification, and the underlined U base indicates the damaged uracil deoxyribonucleotide modification. In the signal probe, the “X” indicates the abasic site mimic.

Preparation of DNA Stock Solutions. The 10 μM assistant probe 1, 10 μM assistant probe 2, 10 μM trigger probe 1 and 10 μM trigger probe 2 were incubated in a buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl , and 1 mM EDTA at 95 °C for 5 min, followed by slowly cooling to room temperature to form the double-stranded DNA (dsDNA) substrates. The obtained DNA stock solutions were stored at -20 °C for further use.

Conjugation of Signal Probes with the Streptavidin-Coated Magnetic Beads. The coupling of signal probes to magnetic beads (MBs) was carried out according to the protocol of Invitrogen Corporation. The 50 μL of streptavidin-coated MBs solution (10 mg/mL) was transferred into a 200 μL of centrifuge vial and washed twice with 1× B&W buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1 M NaCl). The supernatant was removed by magnetic separation and the MBs were re-suspended in 2× B&W buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl) to a final concentration of 5 μg/μL. Then 50 μL of 1 μM biotinylated signal probe 1 and 50 μL of 1 μM biotinylated signal probe 2 were mixed with 100 μL of 5 μg/μL MBs solution and incubated in the dark for 10 min on a roller mixer at room temperature. The mixture was then washed three times by magnetic separation using 1× B&W buffer to remove the uncoupled signal probes, and the remaining signal probe-MB conjugates were resuspended in 25 μL of TE buffer.

Detection of hOGG1 and UDG at the Single-Molecule Level. The enzyme reaction involves three consecutive steps. Firstly, the DNA glycosylases-induced excision reaction was performed in 10 μL of reaction mixture containing 0.4 μL of dsDNA substrates (10 μM), 1 μL of 10× NEB buffer 2, 0.1 μL of 100 μg/mL BSA, 1 μL of 10× UDG reaction buffer and different-concentration hOGG1 and UDG at 37 °C for 60 min. Secondly, 4 μL of magnetic bead-coupled signal probes, 2 μL of 10× NEB buffer 3, 5 U of endonuclease IV were added into the excision reaction system

with a total volume of 20 μL. The reactions were incubated at 37 °C in the dark for 60 min. Thirdly, the streptavidin-coated MBs were separated by magnetic separation for 3 min, and the supernatant solution was subject to single-molecule detection.

The images of single molecules were obtained by total internal reflection fluorescence microscopy (TIRF) (Nikon, Ti-E, Japan). After 2000-fold dilution, 15 μL of reaction product samples was used for TIRF imaging. The 488-nm and 640-nm lasers were used to excite the AF488 and Cy5, respectively. The photons were collected using an oil immersion 100× objective. The photons from the AF488 and Cy5 were collected by camera (Photometrics, Evolve 512). For data analysis, the AF488 counts and Cy5 counts from ten frames were counted by the image J software, with an imaging region of 600 × 600 pixels being counted in each frame.

Gel Electrophoresis. The DNA products were analyzed by a Bio-Rad ChemiDoc MP Imaging System. The products of dsDNA substrate stained with SYBR Gold were analyzed by 12% polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (9 mM boric acid, 9 mM Tris-HCl, 0.2 mM EDTA, pH 7.9) at 110 V constant voltage for 50 min at room temperature. The fluorescent DNA fragments of the enzyme reaction products were analyzed using an illumination source of Epi-green (460–490 nm excitation) and a 516–544 nm filter for AF488 fluorophore and an illumination source of Epi-red (625–650 nm excitation) and a 675–725 nm filter for Cy5 fluorophore.

Measurement of Fluorescence Spectra. The 20 μL of reaction products were diluted to a final volume of 60 μL with ultrapure water for the measurement of fluorescence spectra by a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan). The AF488 fluorescence was measured at the excitation wavelengths of 488 nm and the fluorescence intensity at 532 nm was used for

quantitative analysis of hOGG1. The Cy5 fluorescence was measured at the excitation wavelengths of 640 nm and the fluorescence intensity at 670 nm was used for quantitative analysis of UDG.

Inhibition Assay. Different-concentration $CdCl_2$ was incubated with 64 U/mL hOGG1 and 100 U/mL UDG at 37 °C for 15 min, followed by incubation with the enzyme reaction mixture containing 1× NEB buffer 2, 0.1 μL of BSA (100 μg/mL) and 0.4 μL of dsDNA substrates (10 μM) at 37°C for 60 min. Subsequently, 4 μL of MB-coupled signal probes, 2 μL of 10× NEB buffer 3, 5 U of endonuclease IV were added to the mixture and incubated at 37 °C for 60 min. Then the streptavidin-coated MBs were separated by magnetic separation for 3 min, and the supernatant solution was subjected to single-molecule detection. The relative activity of DNA glycosylases (*RA*) was measured according to eq. 1.

$$RA(\%) = \frac{N_i - N_0}{N_t - N_0} \times 100\% \quad (1)$$

where N_0 is the measured number of fluorescent molecules in the absence of DNA glycosylase, N_t is the measured number of fluorescent molecules in the presence of DNA glycosylase, and N_i is the measured number of fluorescent molecules in the presence of both DNA glycosylase and the inhibitor. The IC_{50} value was calculated from the curve of *RA* versus the $CdCl_2$ concentration.

Cell Culture and Preparation of Cell Extracts. Human lung adenocarcinoma cell line (A549 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Gibco, U.S.A.) and 1% penicillin-streptomycin (Invitrogen, USA) at 37 °C in a humidified chamber with 5% CO_2. The cell lysates were prepared using the nuclear extract kit (ActiveMotif, Carlsbad, CA, USA) according to the manufacturer's protocol.

SUPPLEMENTARY RESULTS

Verification of the formation of dsDNA substrate and its stability. To verify the formation of dsDNA substrates and the stability of dsDNA substrates in the presence of Endo IV, we performed nondenaturating PAGE. In the presence of assistant probe 1, assistant probe 2, trigger 1 and trigger 2, a distinct band of 54 bp is observed (Fig. S1, lane 1), which migrates more slowly due to its large molecular weight than the bands of assistant probe 1/trigger 1 duplex (Fig. S1, lane 2) and assistant probe 2/ trigger 2 duplex (Fig. S1, lane 3). In addition, in the presence of Endo IV, the band size of dsDNA substrates containing assistant probe 1, assistant probe 2, trigger 1 and trigger 2 (Fig. S1, lane 4) is same to the band in the absence of Endo IV (Fig. S1, lane 1). These results clearly demonstrate that the dsDNA substrates containing assistant probe 1, assistant probe 2, trigger 1 and trigger 2 can be formed and its stability is not influenced by Endo IV.

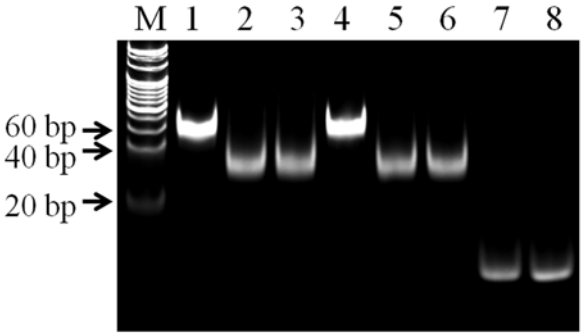

Fig. S1 PAGE analysis of the formation of dsDNA substrate (lanes 1–3) and its stability in the presence of 5 U of Endo IV (lanes 4–6) with SYBR Gold as the indicator. Lane M, DNA ladder marker; lane 1, 0.5 μM assistant probe 1 + 0.5 μM assistant probe 2 + 0.5 μM trigger 1 + 0.5 μM trigger 2; lane 2, 0.5 μM assistant probe 1 + 0.5 μM trigger 1; lane 3, 0.5 μM assistant probe 2 + 0.5 μM trigger 2; lane 4, 0.5 μM assistant probe 1 + 0.5 μM assistant probe 2 + 0.5 μM trigger 1 + 0.5 μM trigger 2 + 5 U of Endo IV; lane 5, 0.5 μM assistant probe 1 + 0.5 μM trigger 1 + 5 U of Endo IV; lane 6, 0.5 μM assistant probe 2+ 0.5 μM trigger 2 + 5 U of Endo IV; lane 7, 2.5 μM

trigger 1; lane 8, 2.5 μM trigger 2.

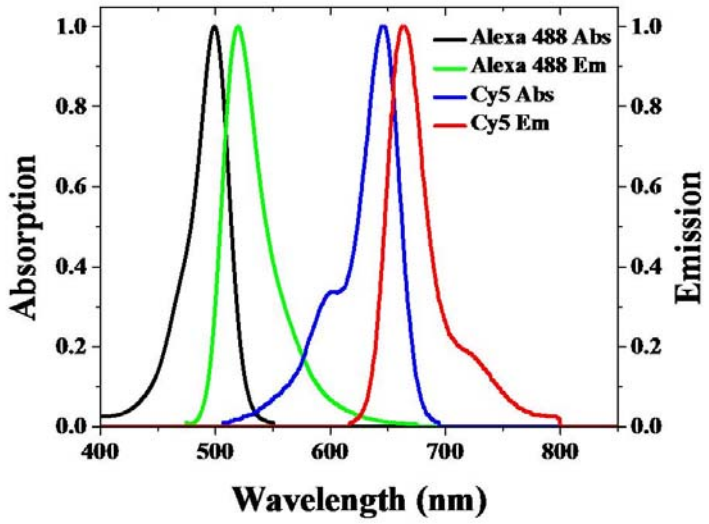

Fig. S2 The normalized absorption and emission spectra of AF 488 and Cy5. Black line, absorption spectrum of AF488; green line, emission spectrum of AF488; blue line, absorption spectrum of Cy5; red line, emission spectrum of Cy5.

Verification of the stability of trigger probe/signal probe in the presence and absence of Endo IV. To verify the stability of trigger probe/signal probe in the presence and absence of Endo IV, we performed nondenaturating PAGE with direct excitation of AF488 and Cy5 (Fig. S3). Distinct bands from trigger probe 1/signal probe 1 duplex (Fig. S3) and trigger probe 2/signal probe 2 duplex (Fig. S3, lane 3) are observed in the absence of Endo IV. A new distinct band of 8 nt resulting from the cleaved trigger probe 1/AF488-labeled signal probe 1 duplex (Fig.S3, lane 2) and a new distinct band of 9 nt resulting from the cleaved trigger probe 2/Cy5-labeled signal probe 2 duplex (Fig.S3, lane 4) are observed in the presence of Endo IV. These results suggest that the trigger probe /signal probe duplex is stable in the absence of Endo IV and the signal probe in trigger probe/signal probe duplex can be cleaved by Endo IV, resulting in the release of

fluorophore and trigger probe in solution.

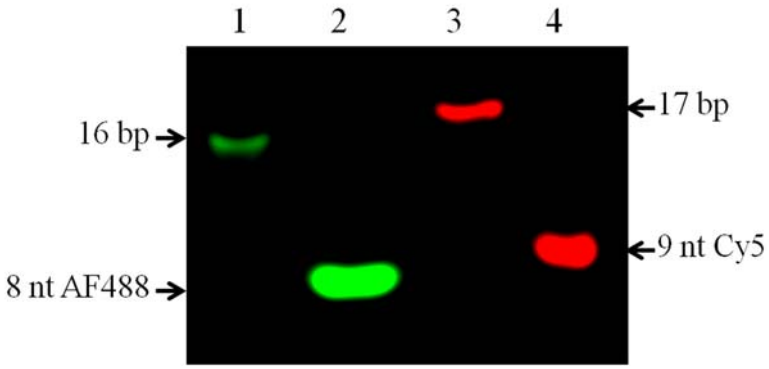

Fig. S3 PAGE analysis of the stability of trigger probe/signal probe in the presence (lane 2 and lane 4) and absence (lane 1 and lane 3) of Endo IV (5U) with direct excitation of AF488 and Cy5. Lane 1, 0.5 μM trigger 1 + 0.5 μM AF488-labeled signal probe 1; lane 2, 0.5 μM trigger 1 + 0.5 μM AF488-labeled signal probe 1 + 5 U of Endo IV; lane 3, 0.5 μM trigger 2 + 0.5 μM Cy5-labeled signal probe 2; Lane 4, 0.5 μM trigger 2 + 0.5 μM Cy5-labeled signal probe 2 + 5 U of Endo IV.

Verification of the stability of single-stranded signal probes in the presence of Endo IV. To verify the stability of single-stranded signal probes in the presence of Endo IV but without trigger probes, we performed nondenaturating PAGE with direct excitation of AF488 and Cy5 (Fig. S 4). A new distinct band of 8 nt resulting from the cleaved trigger probe 1/AF488-labeled signal probe 1 duplex (Fig. S4, lane 1) and a new distinct band of 9 nt resulting from the trigger probe 2/Cy5-labeled signal probe 2 duplex (Fig. S4, lane 2) are observed in the presence of Endo IV. In the presence of single-stranded signal probe 1 and single-stranded signal probe 2 alone, a distinct band of 16 nt equivalent to the AF488-labeled signal probe 1 (Fig. S4, lane 3) and a distinct band of 17 nt equivalent to the Cy5-labeled signal probe 2 (Fig. S4, lane 4) are observed, respectively,

even in the presence of Endo IV. These results demonstrate that the intact AP site in single-stranded signal probe 1 and single-stranded signal probe 2 alone cannot be cleaved by Endo IV.

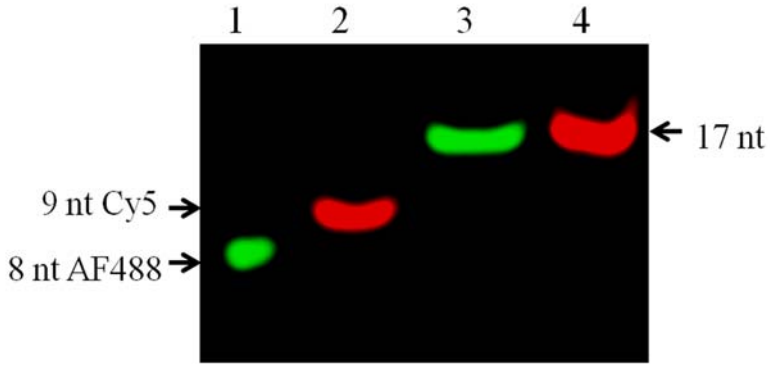

Fig. S4. PAGE analysis of the stability of single-stranded signal probes in the presence of Endo IV (5U) with direct excitation of AF488 and Cy5. Lane 1, 0.5 μM trigger 1 + 0.5 μM AF488-labeled signal probe 1 + 5 U of Endo IV; lane 2, 0.5 μM trigger 2 + 0.5 μM Cy5-labeled signal probe 2 + 5 U of Endo IV; lane 3, 0.5 μM 0.5 μM AF488-labeled signal probe 1 + 5 U of Endo IV; lane 4, 0.5 μM Cy5-labeled signal probe 2 + 5 U of Endo IV.

Verification of the Proposed Method. We used fluorescence measurement to investigate the feasibility of the proposed method for hOGG1 and UDG assays (Fig. S5). A distinct AF488 fluorescence signal with a characteristic emission peak of 532 nm is observed in the presence of hOGG1 (Fig. S5A, green line), but no significant AF488 signal is observed in the absence of hOGG1 (Fig. S5A, black line). Similarly, a distinct Cy5 fluorescence signal with a characteristic emission peak of 670 nm is observed in the presence of UDG (Fig. S5B, red line), but no significant Cy5 fluorescence signal is observed in the absence of UDG (Fig. S5B, blue line). When both hOGG1 and UDG are present, AF488 and Cy5 fluorescence signals are observed

simultaneously (Fig. S5C)

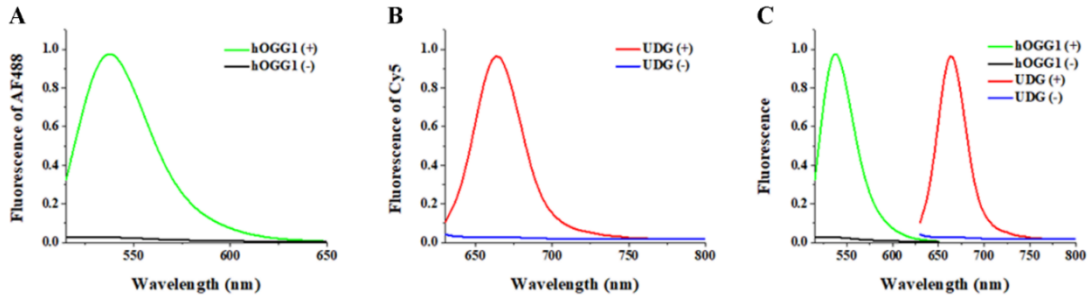

Fig. S5 (A) Fluorescence emission spectra of AF488 in the presence (green line) and absence (black line) of hOGG1, respectively. (B) Fluorescence emission spectra of Cy5 in the presence (red line) and absence (blue line) of UDG, respectively. (C) Fluorescence emission spectra of AF488 and Cy5 in the presence and absence of hOGG1 and UDG, respectively. The hOGG1 concentration is 32 U/mL, and the UDG concentration is 50 U/mL.

Optimization of Experimental Conditions. To achieve the best performance of simultaneous detection of UDG and hOGG1, we optimized the experimental conditions including the length of trigger probes, the concentration of signal probes, and the Endo IV reaction time. When the trigger probe is too short, the resultant dsDNA substrate may be unstable at the reaction temperature. When the trigger probe is too long, it is hard to dissociate from the resultant dsDNA substrate in response to the treatment of hOGG1 and UDG. Thus, the length of trigger probe should be optimized. We designed four trigger probes 1 for hOGG1 and four trigger probes 2 for UDG with the base length varying from 15 to 18 nt. As shown in Fig. S6A, the F/F_0 value increases with the increasing length of trigger probe from 15 to 17 nt, and reaches the highest value at the length of 17 nt for both trigger probe 1 and trigger probe 2 (F and F_0 are the fluorescence intensity in the presence and absence of DNA glycosylase, respectively). Thus, the trigger probe with the length

of 17 nt is used in the subsequent research.

We further optimized the amount of Endo IV and its reaction time, respectively. As shown in Fig. S6B, the F/F_0 value enhances with the increasing amount of Endo IV, and reaches the maximum value at the amount of 5 U probably due to the exhaustion of signal probes (F and F_0 are the fluorescence intensity in the presence and absence of DNA glycosylase, respectively). Thus, 5 U of Endo IV is used in the subsequent research. As shown in Fig. S6C, the F/F_0 value improves with the reaction time, and reaches a plateau at the reaction time of 60 min due to either the exhaustion of signal probes or the inactivation of Endo IV (F and F_0 are the fluorescence intensity in the presence and absence of DNA glycosylase, respectively). Thus, 60 min of Endo IV reaction time is used in the subsequent experiments.

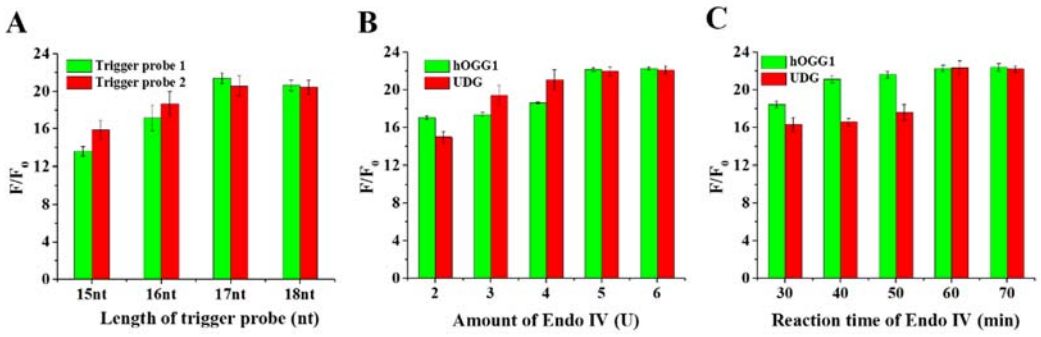

Fig. S6 (A) Effect of the length of trigger probes on the fluorescence intensity of hOGG1 (green column) and UDG (red column). (B) Effect of Endo IV amount on the fluorescence intensity of hOGG1 (green column) and UDG (red column). (C) Effect of Endo IV reaction time on the fluorescence intensity of hOGG1 (green column) and UDG (red column). F and F_0 are the fluorescence intensity in the presence and absence of hOGG1 and UDG, respectively. The concentration of hOGG1 is 32 U/mL, and the concentration of UDG is 50 U/mL. Error bars show the standard deviation of three experiments.

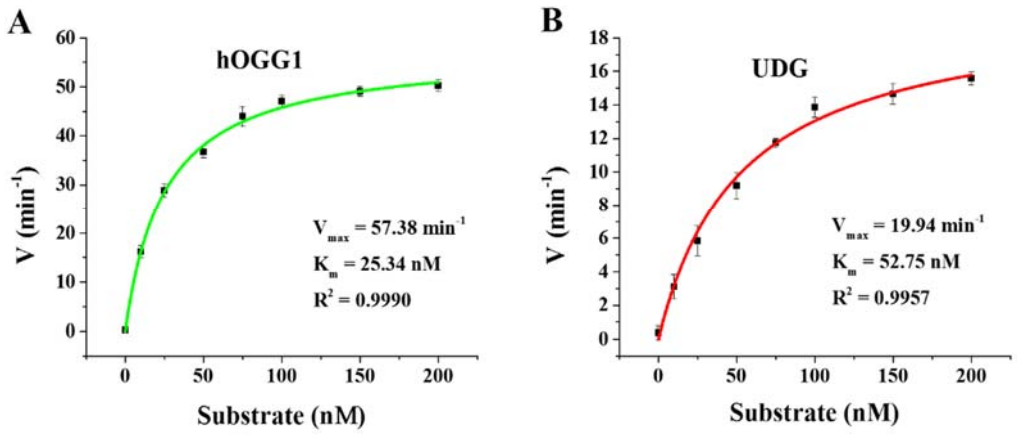

Fig. S7 Variance of initial velocity with the concentration of DNA substrates in response to hOGG1 (A) and UDG (B), respectively. The green/red curve shows fitting. Error bars show the standard deviation of three experiments.

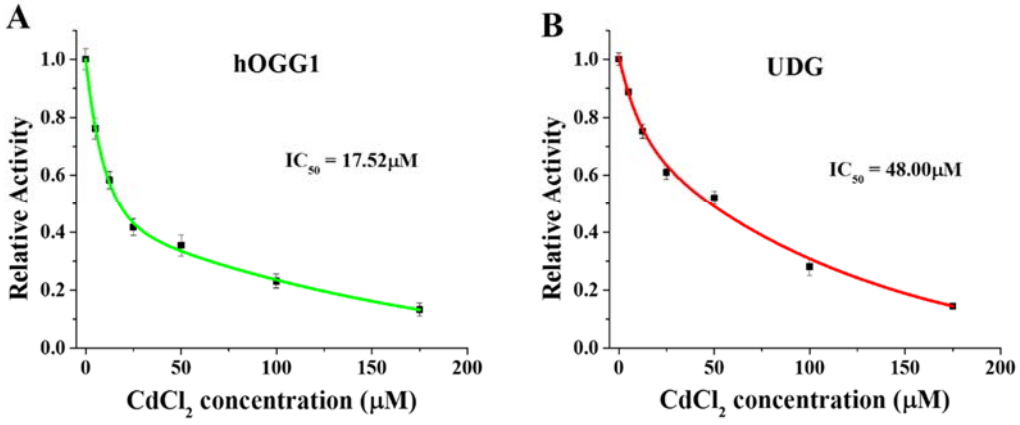

Fig. S8 (A) Variance of the relative activity of hOGG1 (A) and UDG (B) in response to different-concentration $CdCl_2$. The green/red curve shows fitting. The hOGG1 concentration is 64 U/mL, and the UDG concentration is 100 U/mL. Error bars show the standard deviations of three experiments.