Supporting Information

for

Transcription Inhibition by Platinum DNA Cross-links in Live Mammalian Cells

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MATERIALS AND METHODS

Materials and Methods. All chemical reagents were obtained from Sigma-Aldrich unless stated otherwise. Restriction enzymes, T4 polynucleotide kinase (T4 PNK), and T4 DNA ligase were purchased from New England Biolabs (NEB). Enzymatic reactions were carried out in reaction buffers provided by the supplier. Cisplatin and [Pt(*R*,*R*-dach)Cl_2] were synthesized as described.[1,2] Ethidium bromide (EtdBr) and nuclease S1 were obtained from Promega. Competent DH5α *E. coli* cells were purchased from Invitrogen. Oligonucleotides were obtained from Integrated DNA Technologies unless otherwise stated, and their mass spectra were recorded on a Bruker OmniFlex instrument. The analyte was mixed with a matrix containing 10 mg/mL of 2,4,6-trihydroxyacetophenone with 25 mM ammonium citrate in 50% MeCN solution and applied on the target using the dried droplet method. External calibration was performed using ABI Biosystems Calibration Mixture 2.

Vector Construction and Preparation. A mammalian vector expressing *Gaussia* luciferase, pCMV-GLuc (NEB), was deleted between 1501-3279 bp and 3698-3900 bp by the polymerase chain reaction (PCR) to remove BspQI restriction sites and the SV40 origin of

replication, yielding pGLuc.[3] pGLuc was treated sequentially with HindIII and BamHI to digest the vector between the *CMV* promoter and *GLuc* reporter genes. A synthetic insert designed for subsequent incorporation of the 1,2-d(G*pG*)-Pt DNA lesions (asterisks denote platination sites), prepared by annealing 5'-AGCTGGAAGAGCCAGAAGGTGGAAGAGC with 5'-GATCGCTCTTCCACCTTCTGGCTCTTCC to leave 5'-AGCT/GATC non-cohesive overhangs, was ligated to the HindIII/BamHI-digest of pGLuc. The ligation mixtures were transformed directly into DH5α cells on LB agar plates supplemented with 100 mg/L of ampicillin. Colonies were randomly picked and the plasmid was extracted (Qiagen miniprep kit). Incorporation of synthetic inserts was confirmed by restriction analysis and sequencing (MWG Operon, Huntsville, Alabama, USA) prior to large scale plasmid preparation. The vector designed to incorporate 1,3-d(G*pTpG*)-Pt lesions was prepared using 5'-AGCTGGAAGAGCACGAAGTGCGAAGAGC and 5'-GATCGCTCTTCGCA-CTTCGTGCTCTTCC with the same methodology. The modified vectors designed to incorporate 1,2-d(G*pG*)-Pt and 1,3-d(G*pTpG*)-Pt lesions were designated as pGLuc4temGG and pGLuc5temGTG, respectively.

Preparation of Platinated Insertion Strands. The 16-mer insertion strands required for oligonucleotide insertion into pGLuc4temGG and pGLuc5temGTG vectors, 5'-ACCTTCTGGCTCTTCC (GG-is) and 5'-CACTTCGTGCTCTTCC (GTG-is) respectively (Fig. S1), were synthesized using an Applied Biosystems 392 DNA/RNA synthesizer on 1 μmol scales by standard phosphoramidite protocols and purified with PolyPak II reverse-phase cartridges (Glen Research) in accord with the manufacturer's instructions. To prepare the 16-mer insertion strand with the 1,2-d(G*pG*)-[$Pt(NH_3)_2$] lesion (GG-is-PtA_2), desalted oligonucleotide (GG-is, 200 nmol) was allowed to react with *cis*-[$Pt(NH_3)_2Cl_2$] (300 nmol) in buffer (13.5 mL, 24 mM HEPES pH 6.8, 10 mM NaCl) for 24 h at 37 °C. The platinated oligonucleotide was purified by ion-exchange HPLC (IE-HPLC)

using a linear gradient of 25-40% B over 10 min, where solvent A contained 20 mM Tris·HCl pH 8.0, 20% acetonitrile and solvent B contained solvent A supplemented with 1 M NaCl (Fig. S2). The final yield was 66 nmol (33.0%). The 16-mer insertion strand with the 1,3-d(G*pTpG*)-[$Pt(NH_3)_2$] lesion (GTG-is-PtA_2) was prepared by treating GTG-is with *cis*-[$Pt(NH_3)_2Cl_2$] using the same reaction conditions and purified with IE-HPLC, with a final yield of 32 nmol (16%, Fig. S2). Preparations of the insertion strands containing the 1,2-d(G*pG*)-[Pt(dach)] lesion (GG-is-Pt(dach)) and 1,3-d(G*pTpG*)-[Pt(dach)] lesion (GTG-is-Pt(dach)) were accomplished with the same protocol except that [$Pt(dach)Cl_2$] (300 nmol) was used (Fig. S3). Final yields for GG-is-Pt(dach) and GTG-is-Pt(dach) were 48 nmol (24%) and 33.5 nmol (17%) respectively. The insertion strands were characterized by MALDI-TOF MS to determine their molecular mass and analyzed for nucleotide composition by enzymatic digestion, using a reported procedure and are summarized in Table S1.[4]

	1,2-d(G*pG*)-Pt lesion	**1,3-d(G*pTpG*)-Pt lesion**
Unplatinated	5′-ACCTTCTGGCTCTTCC GG-is	5′-CACTTCGTGCTCTTCC GTG-is
$[Pt(NH_3)_2]^{2+}$	5′-ACCTTCTGGCTCTTCC GG-is-PtA_2	5′-CACTTCGTGCTCTTCC GTG-is-PtA_2
$[Pt(dach)]^{2+}$	5′-ACCTTCTGGCTCTTCC GG-is-Pt(dach)	5′-CACTTCGTGCTCTTCC GG-is-Pt(dach)

Figure S1. Sequences of platinated and unplatinated insertion strands.

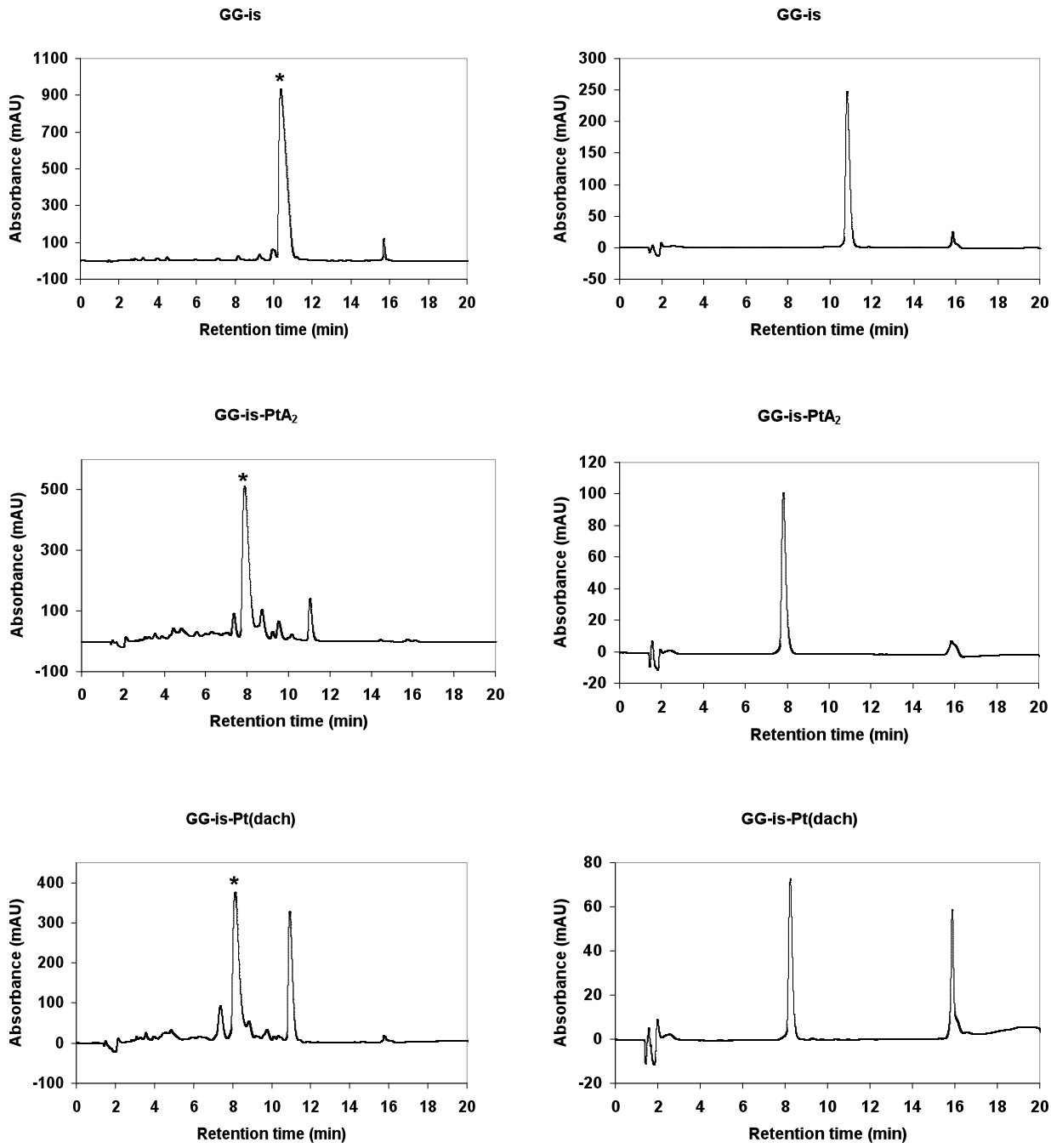

Figure S2. HPLC purification of 16-mer insertion strands for pGLuc4temGG; chromatogram of the preparative separation (left column) and analysis of purified oligonucleotides (right column). Asterisks mark the target peaks containing the desired products during preparative separation.

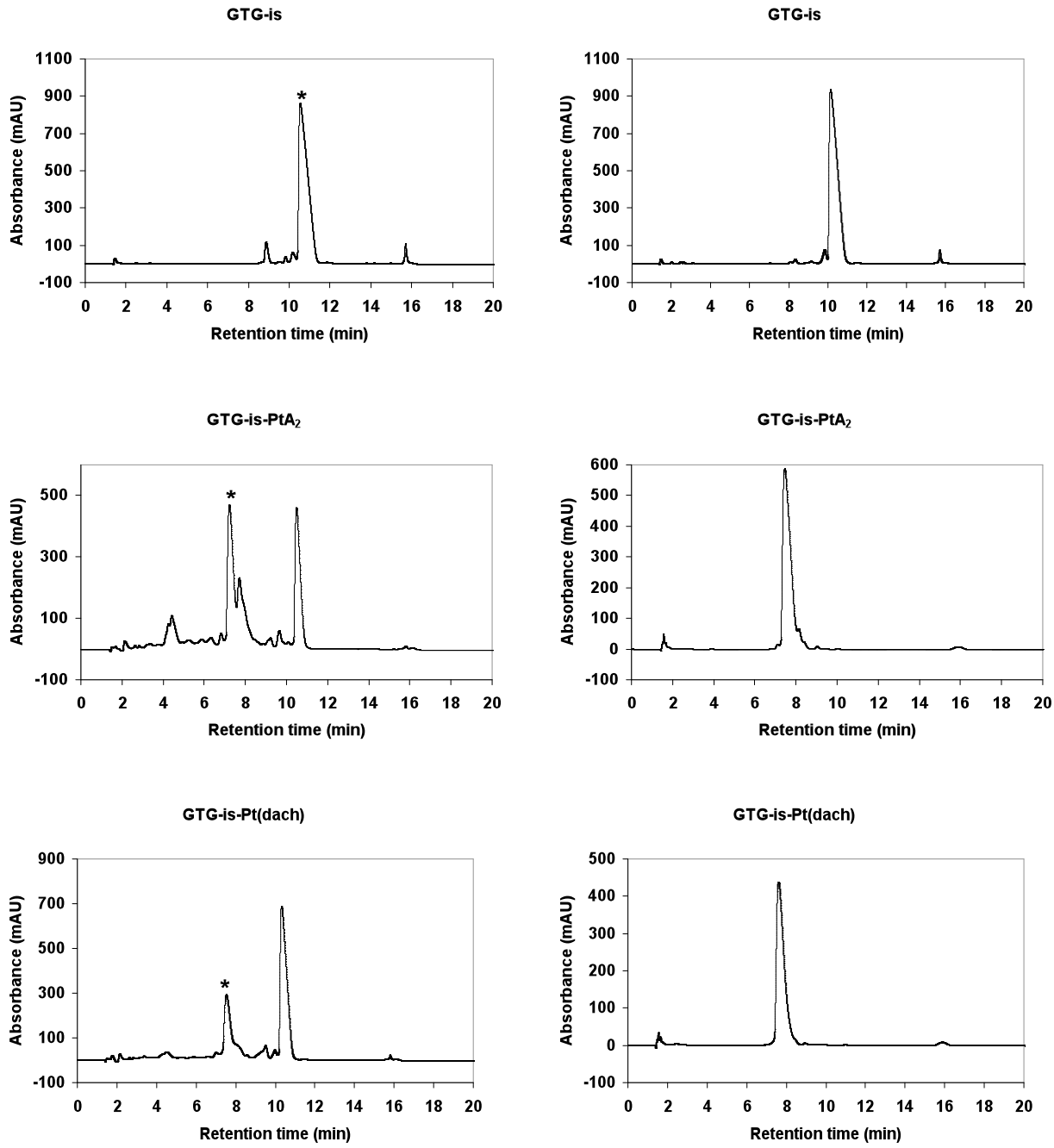

Figure S3. HPLC purification of 16-mer insertion strands for pGLuc5temGTG; chromatogram of the preparative separation (left column) and analysis of purified oligonucleotides (right column). Asterisks mark the target peaks containing the desired products during preparative separation.

Table S1. Characterization of the insertion strand by nucleotide composition analysis[a] and MALDI-TOF MS.

insertion strand	Nucleotide composition analysis								MALDI-TOF MS (m/z)	
	dC		dG		T		dA			
	obs'd	calc'd	obs'd	calc'd	obs'd	calc'd	obs'd	calc'd	obs'd	calc'd
GG-is	7.2	7	1.8	2	6.1	6	0.9	1	4760	4758
GG-is-PtA_2	7.1	7	0.1	0	5.9	6	0.9	1	4988	4987
GG-is-Pt(dach)	7.1	7	0	0	6.0	6	0.9	1	5068	5065
GTG-is	7.2	7	1.9	2	6.1	6	0.8	1	4761	4758
GTG-is-PtA_2	7.1	7	0.1	0	6.0	6	0.8	1	4987	4987
GTG-is-Pt(dach)	7.1	7	0	0	6.0	6	0.9	1	5065	5065

[a]The insertion strand (500 pmol) was digested with nuclease S1 (10 U) for 12 h at 37 °C followed by calf intestinal phosphatase (10 U) treatment for 4 h at 37° C. Analyses of digests were carried out using reverse-phase HPLC with a Supelcosil LC-18-S column; the gradient used was 5-15% B over 30 min, where solvent A contained 10 mM NaOAc and solvent B contained 100% methanol.

Preparation of Complementary Strands. The 24-mer insertion strands required for gapping of pGLuc4temGG and pGLuc5temGTG vectors, 5'-TTTTGGAAGAG-CCAGAAGGTTTTT (GG-cs) and 5'-TTTTGGAAGAGCAC-GAAGTGTTTT (GTG-cs) respectively, were synthesized by using an Applied Biosystems 392 DNA/RNA synthesizer on 1 μmol scales by standard phosphoramidite protocols. The oligonucleotides were purified by isopropanol precipitation.

Preparation of Site-Specifically Gapped Plasmid. The gapped plasmid was prepared by tandem nicking with Nt.BspQI and removal of the intervening nicked strand by trapping with its complementary strand in large molar excess (Fig. S4). A 200 μg portion of the plasmid was incubated with Nt.BspQI (200 U) at 50 °C for 2 h. The enzyme was deactivated by heating the reaction mixture at 80 °C for 20 min and removed by phenol/chloroform/isoamyl alcohol (25:24:1) treatment. A single-stranded gap was formed by trapping the nicked strand with its complementary strand. The nicked plasmid solution was concentrated with a diafiltration centrifugal device (Pall, MWCO 30 KDa), supplemented with synthetic cDNA

(1:1000 molar ratio) in annealing buffer (10 mM Tris·HCl pH 8.0, 2 mM $MgCl_2$, 0.4 M NaCl), and heated at 80 °C for 5 min then cooled at 4 °C for 5 min for 10 cycles. The gapped plasmid was purified by repeated washing (6 times) through a diafiltration centrifugal device (Millipore, MWCO 30 KDa) and quantitated by using UV spectroscopy.

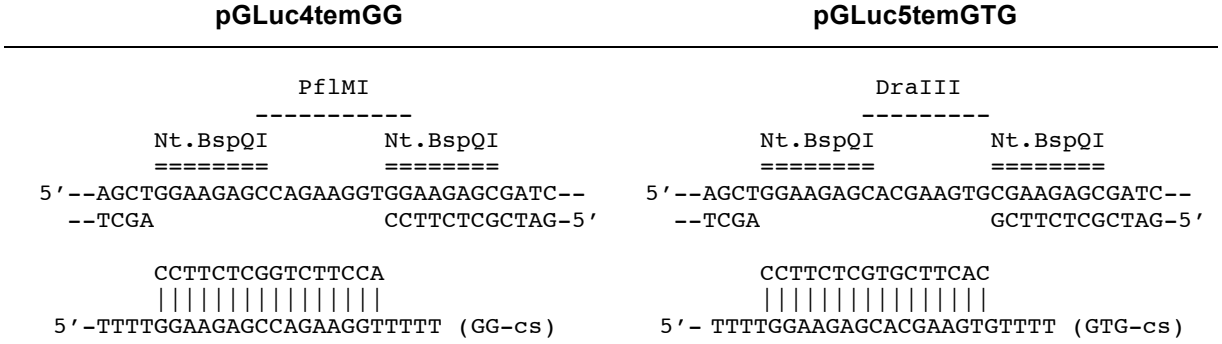

Figure S4. DNA sequence at the gapping site of pGLuc4temGG (left) and pGLuc5temGTG (right) with nicked strand annealed to its complementary strand.

Incorporation of insertion strands into gapped plasmid. The insertion strands (1.2 nmol) were phosphorylated by T4 PNK (600 U) at 37 °C for 1 h. The phosphorylated insertion strands were treated with phenol/chloroform/isoamyl alcohol (25:24:1), purified by ethanol precipitation, and dried in vacuo. The gapped plasmid (40-70 μg) was added at 1:10 to 1:100 molar ratios (vs. the insertion strands) and the mixture was annealed in a thermocycler (80 °C for 10 min followed by cooling to 4 °C at -1 °C/min). The reaction was supplemented with T4 DNA ligase (80 U) in buffer (50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) and incubated at 16 °C for 12 h. The mixture was separated by using preparative agarose gel electrophoresis (0.8-1.0% w/v) containing 0.5 μg/mL EtdBr. The DNA bands were revealed using a handheld UV lamp and the band with higher electrophoretic mobility, corresponding to covalently closed circular product, was excised. Plasmid extraction from the gel was carried out using a commercial kit (Promega). The platinated plasmids were purified further by treatment with PflMI (15 U) or DraIII (15 U) at 37 °C for 30 min followed by heat deactivation at 65 °C for 20 min and preparative agarose

gel electrophoresis (0.8-1.0% w/v). The DNA bands were revealed with a handheld UV lamp and the band with higher electrophoretic mobility, corresponding to covalently closed circular product, was excised. Gel extraction was carried out as described above (Qiagen) and the eluted plasmids were dialyzed against TE buffer. Quantitation of the platinated and non-platinated plasmid was carried out using the picogreen assay (Molecular Probes). Overall yields range between 5-15%.

Restriction Analysis on Ligated Platinated/Non-platinated Plasmids. pGLuc4temGG plasmid (100 ng) was incubated with PflMI (2 U) at 37 °C for 15 min and the enzyme was inactivated by heating at 75 °C for 15 min. The plasmids were analyzed using agarose gel electrophoresis (0.8-1.0% w/v) containing 0.5 μg/mL EtdBr. Pt-DNA lesions were chemically removed by treating the platinated plasmid (100 ng) with 0.2 M NaCN at 55 °C for 10 h, followed by dialysis against water for 4 h, prior to restriction enzyme treatment. pGLuc5temGTG (100 ng) was treated with DraIII (2 U) in the same manner. Control lanes containing untreated plasmids were added for comparison. The gels were imaged using the BioRad Fluor-S MultiImager.

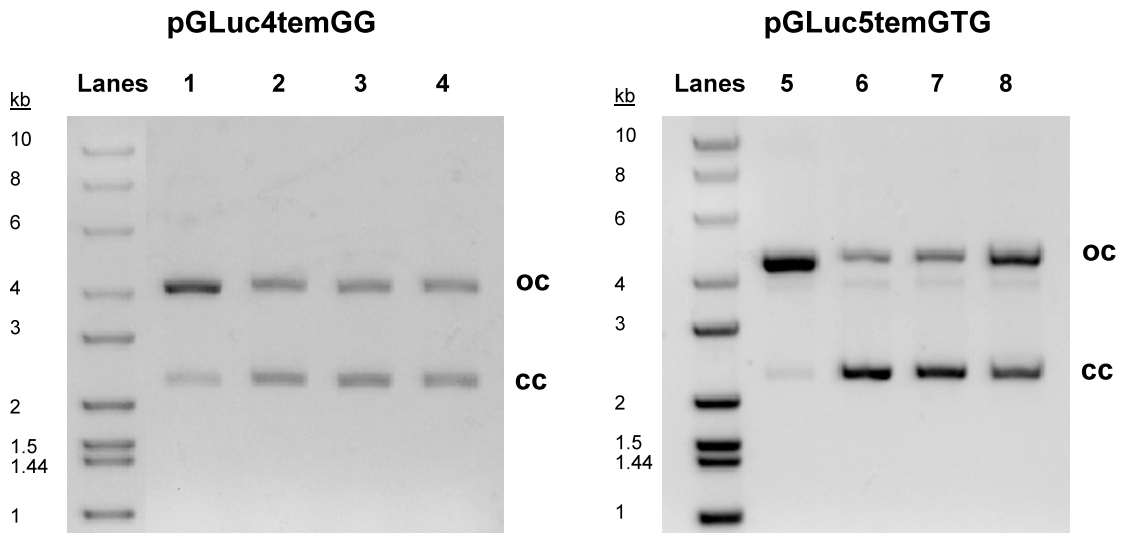

Figure S5. Ligation experiment revealing gapped pGLuc4temGG (left) and pGLuc5temGTG (right) in the presence of different insertion strands treated with T4 DNA ligase at 16 ºC for 12 h; lane 1: gapped pGLuc4temGG alone, lane 2: plasmid + GG-is, lane 3: plasmid + GG-is-PtA_2, lane 4: plasmid + GG-is-Pt(dach); lane 5: gapped pGLuc5temGTG alone, lane 6: plasmid + GTG-is, lane 7: plasmid + GTG-is-PtA_2, lane 8: plasmid + GTG-is-Pt(dach).

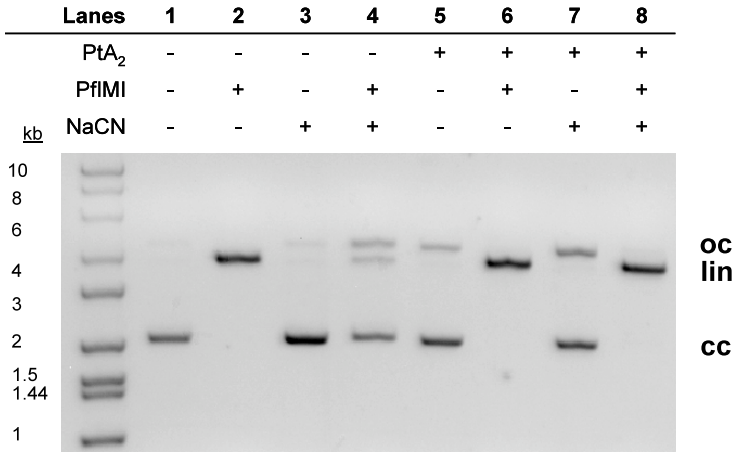

Figure S6. Restriction analysis of platinated/non-platinated plasmids with PflMI before/after cyanide treatment; pGLuc4temGG+is (lanes 1-4) and pGLuc4temGG+is-PtA_2 (lanes 5-8).

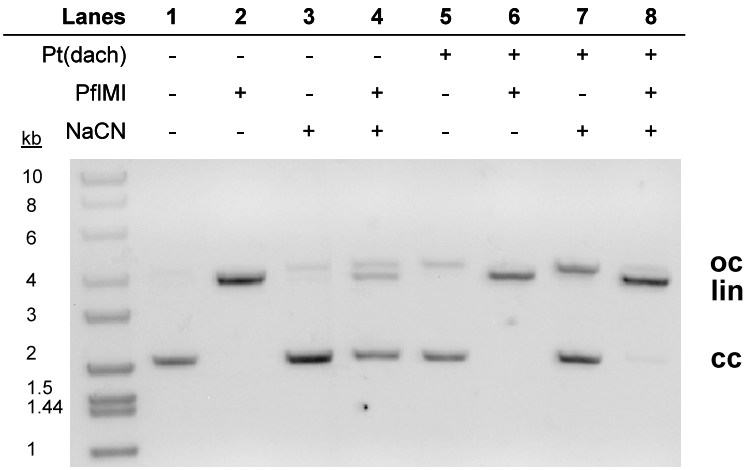

Figure S7. Restriction analysis of platinated/non-platinated plasmids with PflMI before/after cyanide treatment; pGLuc4temGG+is (lanes 1-4) and pGLuc4temGG+is-Pt(dach) (lanes 5-8).

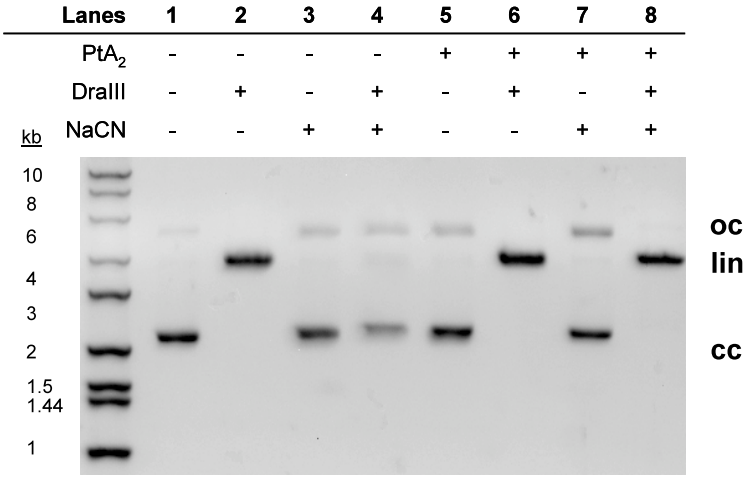

Figure S8. Restriction analysis of platinated/non-platinated plasmids with DraIII before/after cyanide treatment; pGLuc5temGTG+is (lanes 1-4) and pGLuc5temGTG+is-PtA_2 (lanes 5-8).

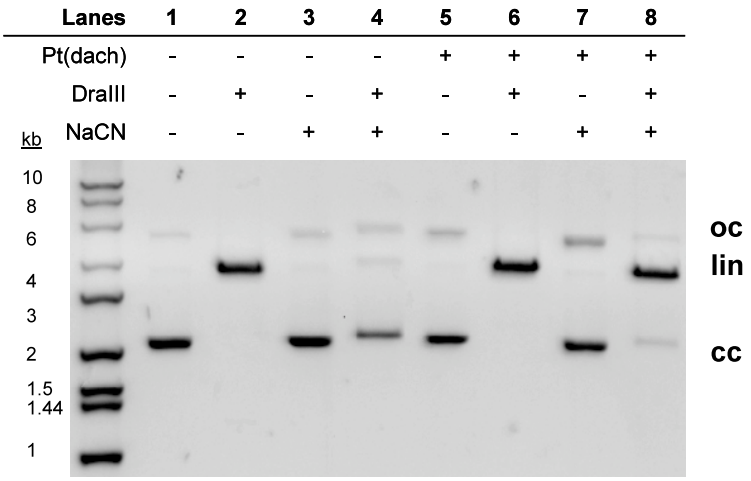

Figure S9. Restriction analysis of platinated/non-platinated plasmids with DraIII before/after cyanide treatment; pGLuc5temGTG+is (lanes 1-4) and pGLuc5temGTG+is-Pt(dach) (lanes 5-8).

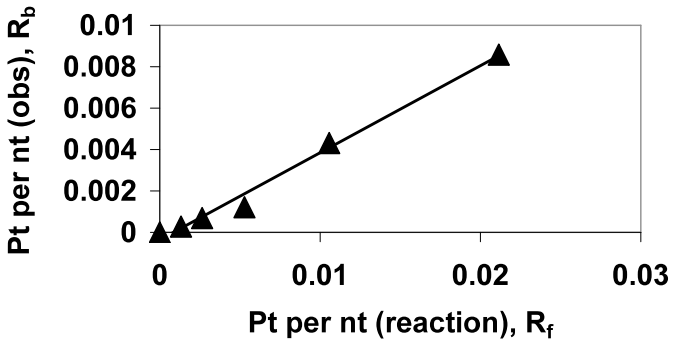

Figure S10. Platination of pGLuc after treatment with cisplatin (0.5, 1, 2, 4, 8 μM) for 16 h at 25 °C in buffer (24 mM HEPES pH 6.0, 10 mM NaCl).

REFERENCES

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