Supporting Information: **Alkylating Probes for G-Quadruplex Structure and Evaluation of the Properties of the Alkylated G-Quadruplex DNA**

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Contents

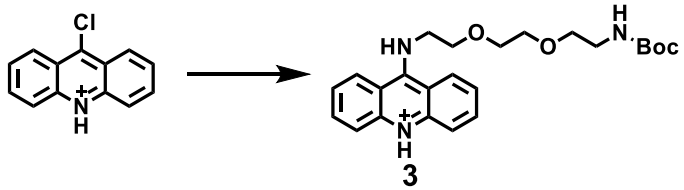
General

All air sensitive or water sensitive reactions were carried out under argon atmosphere using a high-vacuum line, dry equipment, and dry solvents.

^{1}H (400 MHz) NMR spectra were recorded on a Bruker 400 spectrometer. ^{13}C (125 MHz) NMR spectra were recorded on a Bruker AVANCE III 500 spectrometer. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectra were recorded on a Bruker AVANCE III 600 spectrometer. Chemical shifts of ^{1}H NMR were reported in ppm using deuterated solvents as internal standard ($CDCl_3$, δ 7.26; MeOD-d_4, δ 3.31). Multiplicity was described as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Chemical shifts of ^{13}C NMR were reported in ppm using deuterated solvents as internal standard ($CDCl_3$, δ 77.16; MeOD-d_4, δ 49.00). High resolution electrospray mass analysis was performed on a Bruker MicrOTOFQ II. MALDI-TOF mass analysis was performed on a Bruker autoflex speed mass spectrometer. Thin layer chromatography was performed on Silica gel 60 F254 (Merck-Millipore). Column chromatography was performed with Silica gel 60N [40-50 μm or 100-210 μm; KANTO CHEMICAL CO. INC. (Tokyo, Japan)]. Reverse phased high performance liquid chromatography was performed on JASCO HPLC system [PU-2089 as the pump, UV-2075 as the UV-detector, FP-2025 as the fluorescence detector, and CO-2065 as the column oven] purchased by JASCO Corporation (Tokyo, Japan) and COSMOSIL ODS columns [5C18-AR-II (4.6 or 1 S1
mm) for organic compound or 5C18-MS-II (4.6 or 10 x 250 mm) for oligonucleotide] purchased by Nacalai tesque (Kyoto, Japan). Ultraviolet-visible (UV-Vis) spectra were recorded on a BECKMAN

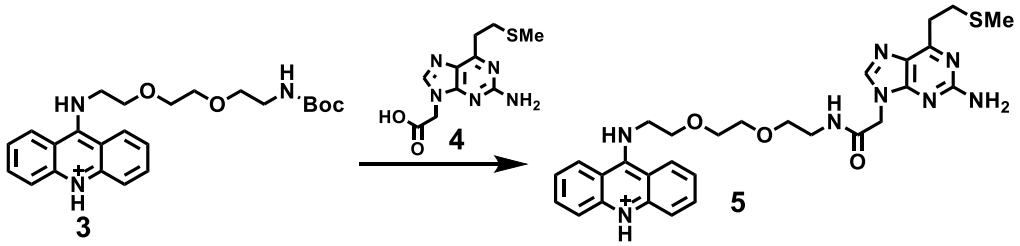
COULTER DU800 using 1 cm path length micro cell. Fluorescence spectra were recorded on a JASCO FP-5200 spectrometer using 1 cm path length quartz cell. pH measurement was performed on Seven Easy pH meter (Mettler Toledo), using ORION 8220BNWP (Thermo Fisher Scientific) as the electrode. Densitometric analysis of the gel was carried out on 20% denaturing polyacrylamide gel plates, and visualized and quantified by FLA-5100 Fluor Imager (Fujifilm Co., Tokyo, Japan). Reagents and organic solvents were purchased by Wako Pure Chemical Industries (Osaka, Japan), Tokyo Chemical Industry (Tokyo, Japan), Sigma-Aldrich, Nacalai tesque (Kyoto, Japan), and Junsei Chemical (Tokyo, Japan). DNAs were purchased by Japan Bio Services Co., LTD. (Saitama, Japan). dNTPs were purchased by Takara Bio (Shiga, Japan). Klenow fragment was purchased by New England Biolabs.

Synthesis of *tert*-butyl (2-(2-(2-(acridin-9-ylamino)ethoxy)ethoxy)ethyl)carbamate (3)



9-Chloroacridine (215 mg, 1.01 mmol) and *tert*-butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl) carbamate (303 mg, 1.22 mmol) were dissolved in phenol (*ca.* 1 g) and stirred at 100 ^{o}C. After stirring for 4 h, the reaction mixture was cooled to room temperature and poured into 1 N aq. NaOH (10 mL). The solution was extracted with CH_2Cl_2 (30 mL x 2), washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated. The crude sample was purified by column chromatography ($CHCl_3$/MeOH = 19/1, 9/1, 3/1) to give the desired product (**3**) (191.9 mg, 45%) as a yellow solid. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.18 (2H, d, J = 8.8 Hz), 8.11 (2H, d, J = 8.8 Hz), 7.66-7.70 (2H, m), 7.39 (2H, dd, J = 7.2, 7.6 Hz), 5.00 (1H, br-s), 3.99 (2H, dd, J = 4.4, 4.8Hz), 3.68 (4H, br), 3.58-3.62 (2H, m), 3.49 (2H, br), 3.36 (2H, d, J = 4.8 Hz), 1.39 (9H, s) ; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 155.84, 151.77, 148.42, 130.05, 128.55, 123.17, 122.96, 117.25, 79.11, 77.26, 70.17, 70.12, 70.04, 49.86, 40.21, 28.23; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{24}H_{31}N_3O_4$, 426.2387; found 426.2396

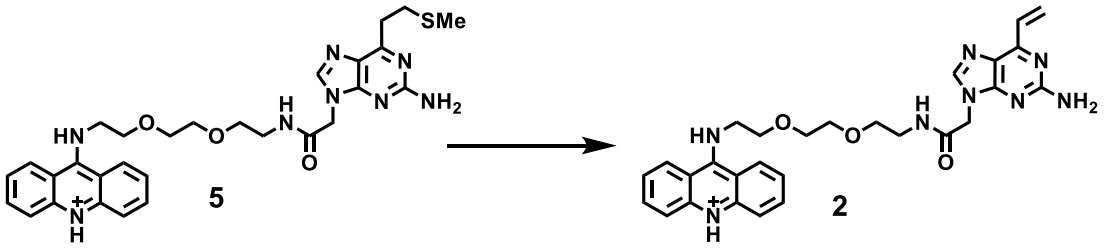
Synthesis of Acridine-amine-AVP precursor (5)



To a solution of **3** (42 mg, 98.7 μmol) in CH_2Cl_2 (0.1 mL), was added trifluoroacetic acid (0.4 mL) and stirred at room temperature for 2 h. The volatile was concentrated and co-evaporated with acetonitrile (5 mL x 3) under reduced pressure to afford yellow amorphous solid. This residue was dissolved in dry DMF (1.0 mL), and the mixture of HBTU (45 mg, 0.12 mmol), HOBt (16 mg, 0.12

mmol), DIPEA (175 μL, 1.00 mmol), and AVP coupling unit (**4** 32 mg, 0.12 mmol) was slowly added and stirred at room temperature. After 3 h, the mixture was concentrated under reduced pressure to afford yellow solid. This residue was purified by column chromatography ($CHCl_3$/MeOH = 30/1 to 15/1) to give yellow solid (21 mg). This product was further purified by reverse phased HPLC with C-18 column (Nacalai tesque: COSMOSIL 5C_{18}-AR-II, 10 x 250 mm) by a liner gradient of 0-30%/30min acetonitrile in 0.1% TFA buffer at a flow rate of 4 mL/min at 40.0 ^{o}C, and monitored by UV detection at λ = 254 nm and fluorescence detection (λ_{ex} = 266 nm, λ_{em} = 450 nm), to afford the desired product (22 mg, 32% as a mono TFA salt) as a pale yellow solid. The concentration of Acridine-amine-AVP precursor (**5**) was determined with quantitative NMR using maleic acid as an internal standard. (ε_{260} = 80,690 $M^{-1}\cdot cm^{-1}$, ε_{265} = 92,980 $M^{-1}\cdot cm^{-1}$)
^{1}H NMR (MeOD-d_4, 600 MHz) δ 8.53 (2H, d, J = 7.8 Hz), 8.15 (1H, s), 7.95 (2H, dt, J = 1.2, 8.4 Hz) 7.79 (2H, dd, J = 0.6, 8.4 Hz), 7.54 (2H, dt, J = 1.2, 8.4 Hz), 4.82 (2H, s), 4.35 (2H, dd, J = 4.8, 5.4 Hz), 3.99 (2H, dd, J = 4.8, 5.4 Hz), 3.71-3.72 (2H, m), 3.63-3.64 (2H, m), 3.52 (2H, t, J = 5.4 Hz), 3.35 (2H, t, J = 5.4 Hz), 3.24 (2H, t, J = 7.2 Hz), 2.92 (2H, t, J = 7.2 Hz), 2.13 (3H, s); ^{13}C NMR (MeOD-d_4, 150 MHz) δ 168.5, 162.9, 162.6, 160.3, 158.9, 158.0, 156.4, 147.4, 136.5, 126.4, 125.0, 119.5, 71.6, 71.3, 70.5, 69.9, 50.5, 49.6, 46.1, 40.4, 32.8, 32.0, 15.2; HRMS-ESI (m/z): $[M+H]^+$ calcd for $C_{29}H_{34}N_8O_3S$, 575.2547; found 575.2550

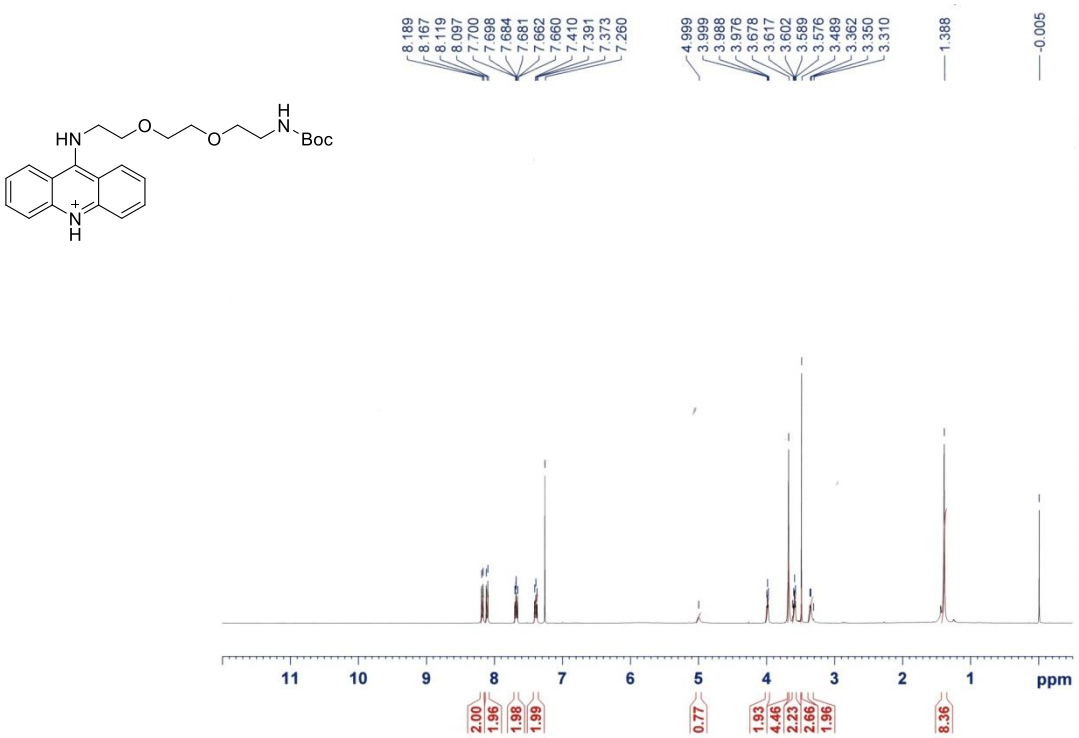
Synthesis of Acridine-amine-AVP (2)



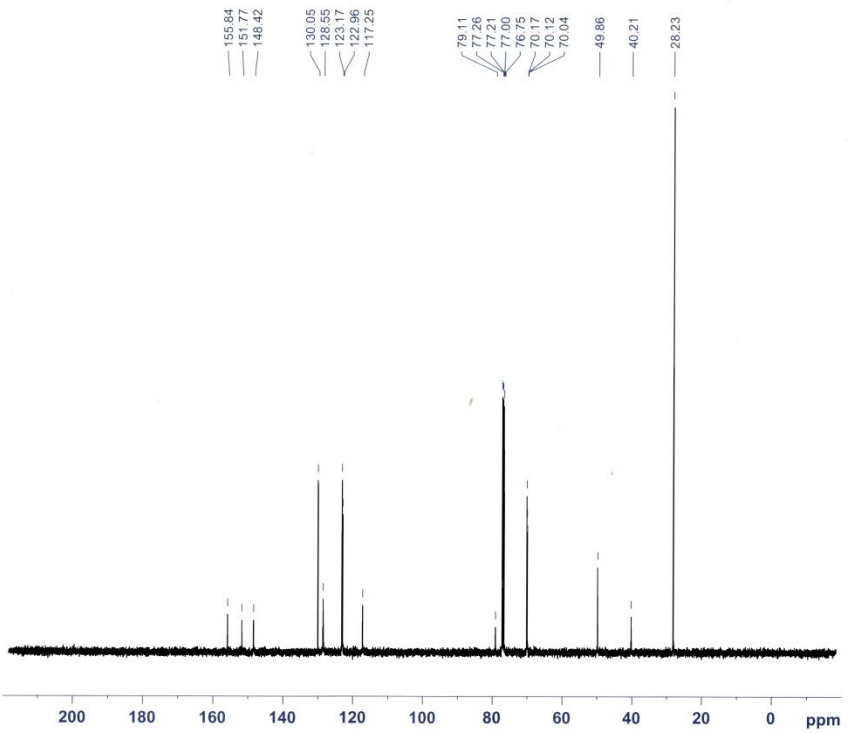
To a solution of precursors (**5** : 500 μM), was added 25 mM aq. MMPP (0.6 equiv.) dissolved in water and the mixture was incubated at 37 ^{o}C for 10 min to oxidize the sulfide group. To this mixture, was added either 1 M aq. KOH (50 equiv.) for Buffer A and Buffer C, or 1 M aq. NaOH (50 equiv.) for Buffer B. After incubated at 37 ^{o}C for 1 h, pH was neutralized by adding 1 M or 0.1 M aq. HCl. Progression of each reaction was monitored with reverse phased HPLC with C-18 column (Nacalai tesque: COSMOSIL 5C_{18}-AR-II, 4.6 x 250 mm) by a liner gradient of 0-30%/30min acetonitrile in 0.1% TFA buffer at a flow rate of 1 mL/min at 40.0 ^{o}C, and monitored by UV detection at λ = 254 nm and the structure was characterized by ^{1}H NMR and ESI mass spectrum measurement.
^{1}H NMR (MeOD-d_4, 600 MHz) δ 8.51 (2H, d, J = 8.4 Hz), 8.03 (1H, s), 7.95 (2H, dt, J = 1.2, 8.4 Hz), 7.77 (2H, d, J = 8.4 Hz), 7.54 (2H, dt, J = 1.2, 8.4 Hz), 6.93 (1H, dd, J = 10.8, 17.4 Hz), 6.84 (1H, d, J = 17.4 Hz), 6.02 (1H, d, J = 10.8 Hz), 4.78 (2H, s), 4.34 (2H, t, J = 5.4 Hz), 3.99 (2H, t, J = 5.4 Hz), 3.73-3.70 (2H, m), 3.66-3.62 (2H, m), 3.54-3.51 (2H, m), 3.39-3.33 (2H, m).
HRMS-ESI (m/z): $[M+2H]^{2+}$ calcd for $C_{28}H_{30}N_8O_3$, 264.1293; found 264.1308

tert-butyl (2-(2-(2-(acridin-9-ylamino)ethoxy)ethoxy)ethyl)carbamate (**3**)

^{1}H NMR ($CDCl_3$, 400 MHz)

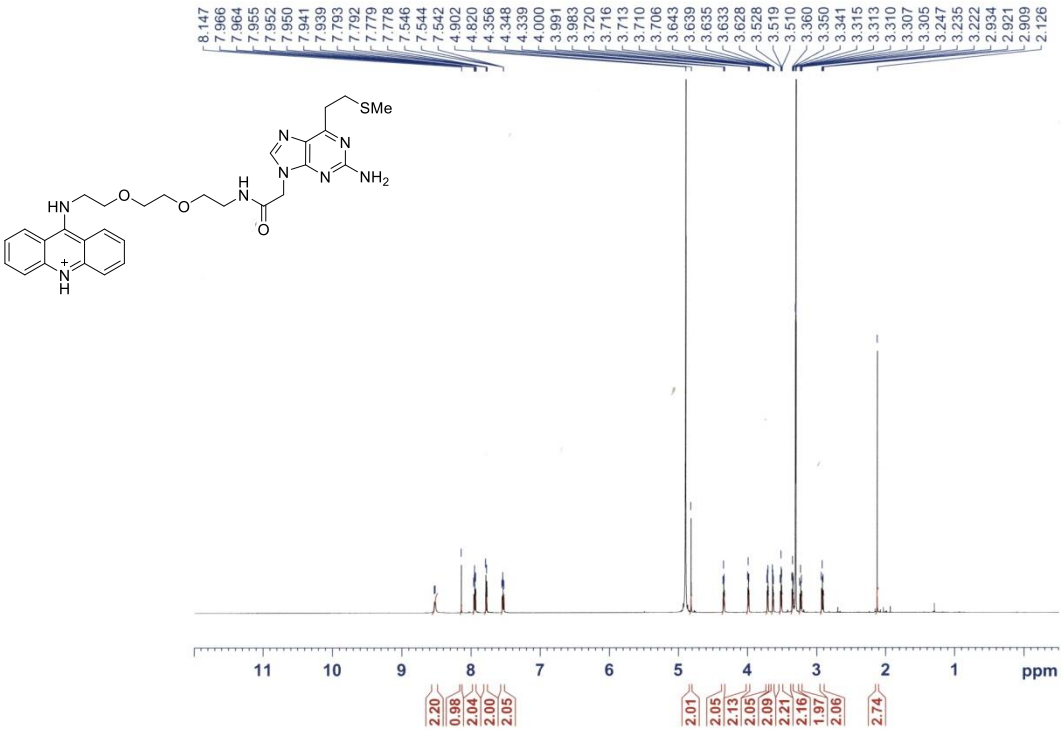


^{13}C NMR ($CDCl_3$, 125 MHz)

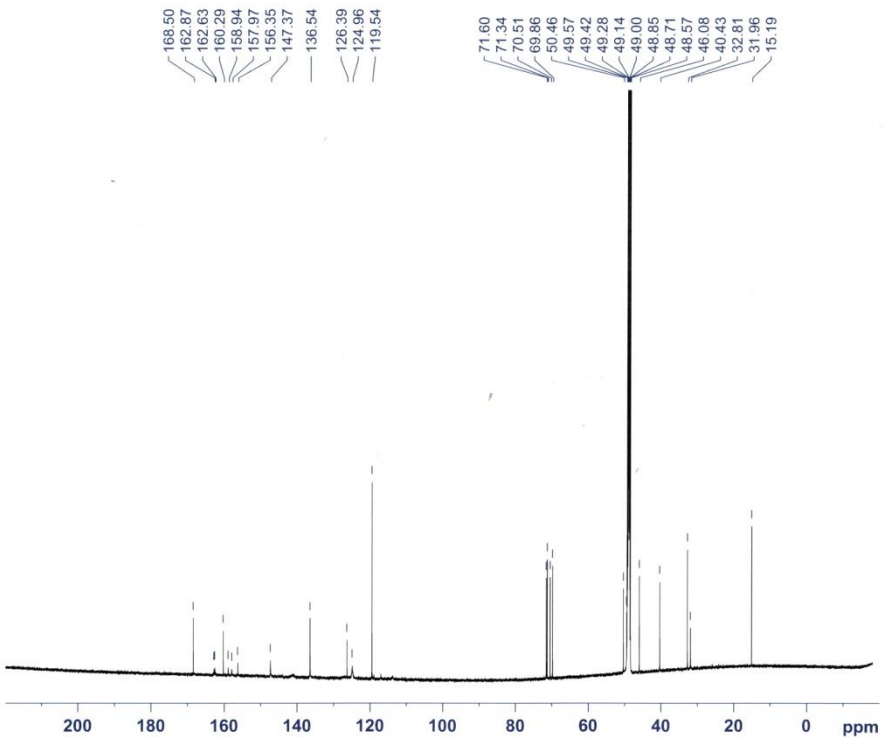


Acridine-amine-AVP precursor (**5**)

^{1}H NMR (MeOD-d_4, 600 MHz)

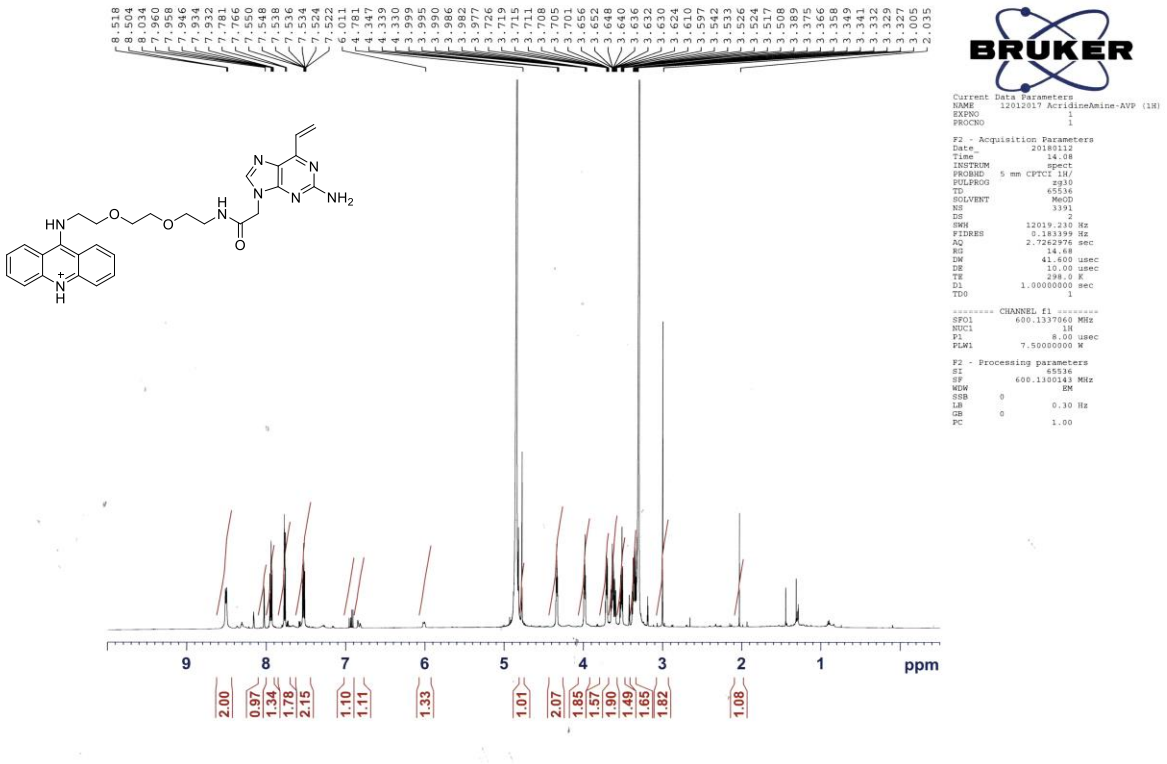


^{13}C NMR (MeOD-d_4, 150 MHz)



Acridine-amine-AVP (**2**)

^{1}H NMR (MeOD-d_4, 600 MHz)



General procedure of alkylating reaction with G-quadruplex DNA

(1) Annealing of G-4 DNA and measurement of CD spectra

5'-fluorescein (FAM) labeled human telomere DNA (50 μM) was diluted in following 1x buffers.

[K^+ buffer : 100 mM KCl, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM K_2EDTA]

[Na^+ buffer: 100 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4 (pH 7.0), 1 mM Na_2EDTA]

To perform the thermal annealing of G-quadruplex, the DNA solution was heated at 90 ^oC for 10 min and cooled from 90 ^oC to 20 ^oC at a rate of 0.5 ^oC/min by using thermal cycler.

CD spectra using 5.0 μM FAM leveled G-4 DNA (annealed in the above-mentioned conditions) were recorded on a J-720WI (JASCO Co., Hachioji, Japan) equipped with a Peltier temperature controller. The CD spectra were recorded by using a micro quartz cell with 1 cm path length, from 500 to 200 nm at 0.2 nm intervals, at 2 nm band width, and 4 sec time per point and the data was averaged over eight scans.

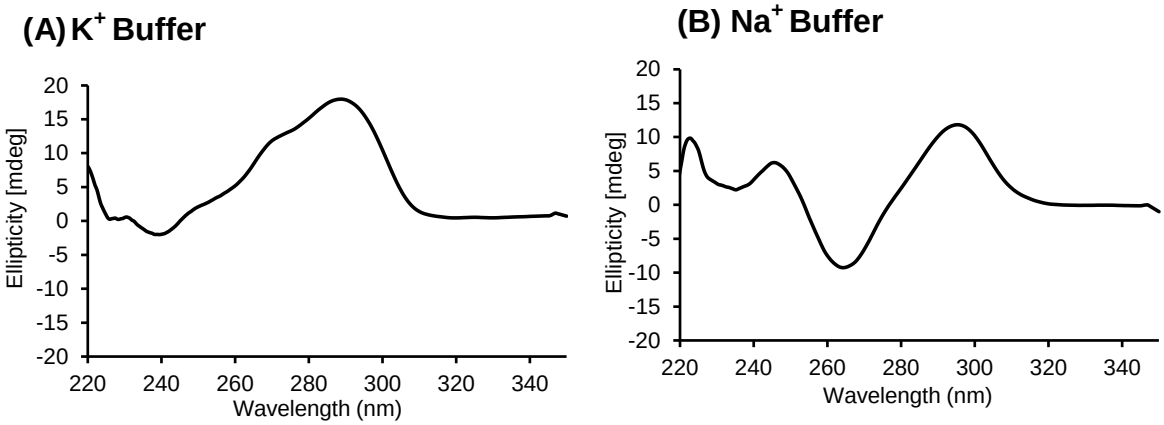


Fig. S1 CD spectra of non-alkylated G-4 DNA (A) K^+ buffer; (B) Na^+ buffer

(2) **Alkylating reaction with G-quadruplex DNA**

The alkylation reaction was performed with the target DNA [G-quadruplex DNA, ss-DNA or full-match ds-DNA (5.0 μM)] and alkylation probes (10-100 μM) in 1x buffers at 37 ^oC. After incubation, a part of the reaction mixture was collected and quenched by addition of 3 equiv. of loading dye (95% formamide, 50 mM EDTA pH 8.0, 0.05% BPB). The collected reaction mixture was analyzed by denaturing 16% polyacrylamide gel electrophoresis containing 20% formamide and 5.6 M urea, run at 400 V x 2 h 30 min in 1x TBE buffer. The fluorescence labelled DNAs were visualized and quantified with FLA-5100 (Fujifilm Co., Tokyo, Japan). The slowly migrating bands were cleaved from the gel and extracted by shaking in a buffer of 10 mM Tris-HCl (pH 8.0). The extract was filtered, freeze-dried, treated with Zip-tip (Merck-Millipore), and thereafter MALDI-TOF mass spectroscopy was measured.

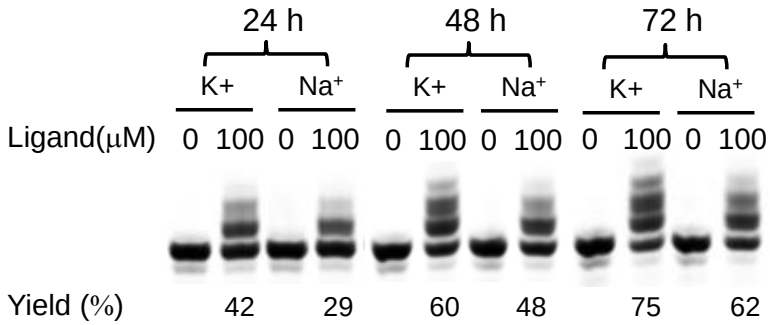


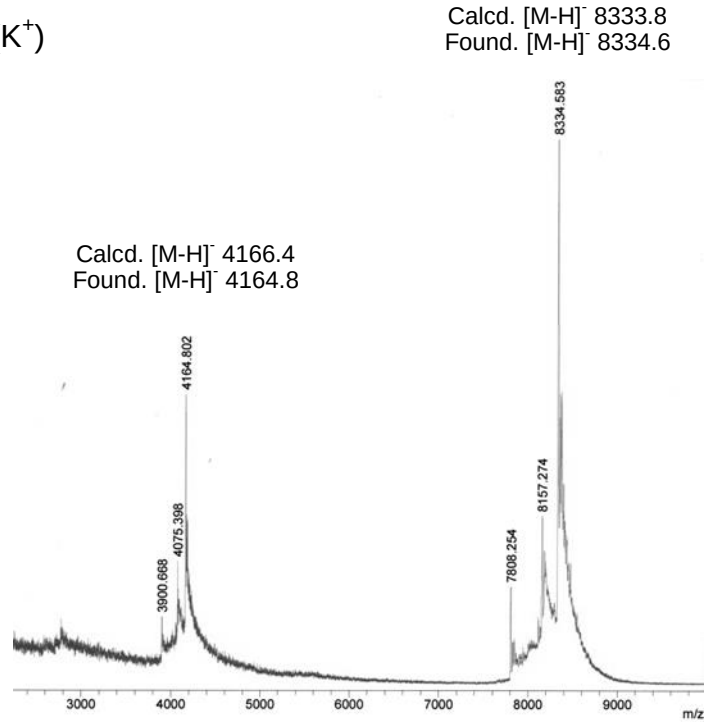
Fig. S2 Denaturing gel electrophoresis of the alkylation products using **2** (20 eq. to G-4 DNA

Potassium buffer (K^+): 100 mM KCl, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.0), 1 mM K_2EDTA, Sodium buffer (Na^+): 100 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4(pH 7.0), 1 mM Na_2EDTA. Acridine derivative (**2:** 0, 100 μM) was incubated with target G-4 (5 μM) at 37°C in potassium or sodium buffer.

Isolation of Acridine-amine-AVP adducted DNA

The target G-quadruplex DNA (5.0 μM) and Acridine-amine-AVP (10 or 25 μM) were incubated in 1x buffers at 37 ^oC. After incubated for 72 h, excess ligand was filtered off by centrifugation using amicon-ultra (membrane for 3 kDa; Merck-Millipore). The crude mixture was purified by sequencing gel electrophoresis containing 20% acrylamide and 7 M urea, run at 900V x 11 h in 1x TBE buffer. The separated DNA bands were cleaved from the gel and extracted by shaking in a buffer of 10 mM Tris-HCl (pH 8.0) and 1 mM Na_2EDTA. The extract was filtered, freeze-dried, and purified by Sep-pak C-18 Cartridge (Waters Co.) and NAP-10 column (GE Healthcare). The purified DNA was treated with Zip-tip (Merck-Millipore), thereafter MALDI-TOF mass spectroscopy was measured. The concentration of Acridine-amine-AVP-adducted DNA was determined by UV absorption at 260 nm using the molar extinction coefficients: 338,150 M^{-1} cm^{-1} [Calculated as a mono alkylated DNA; 257,460 M^{-1} cm^{-1} (5'-fluorescein labeled human telomere DNA) and 80,690 $M^{-1}cm^{-1}$(**2**)]

(A) Potassium buffer (K^+)



(B) Sodium buffer (Na^+)

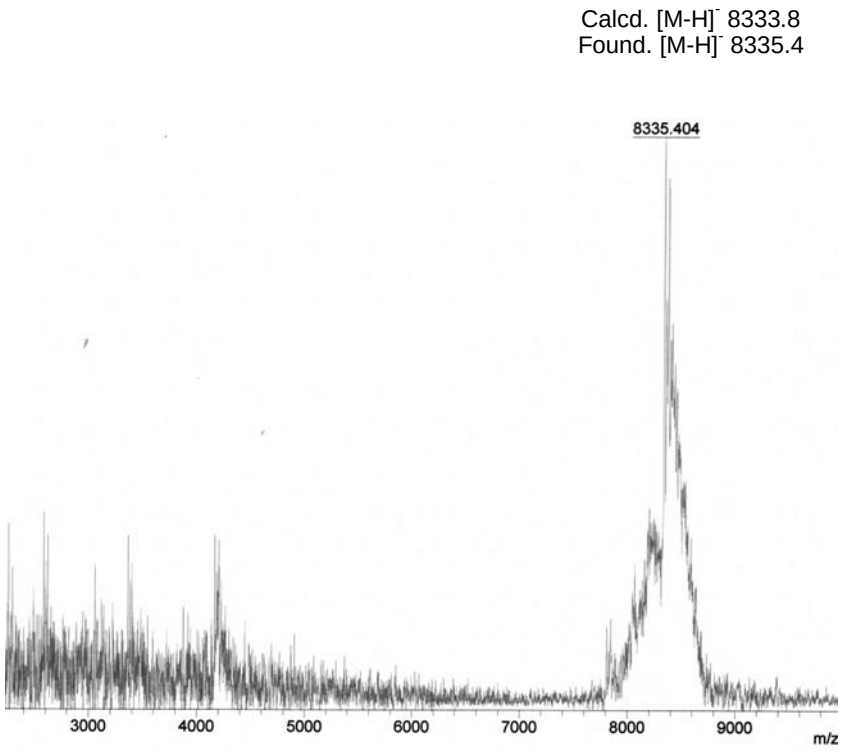


Fig. S3 MALDI-TOF mass spectra of alkylated G-4 DNA

(A) The adducts was formed in the K^+ buffer conditions and purified by gel.
(B) The adducts was formed in the Na^+ buffer conditions and purified by gel.

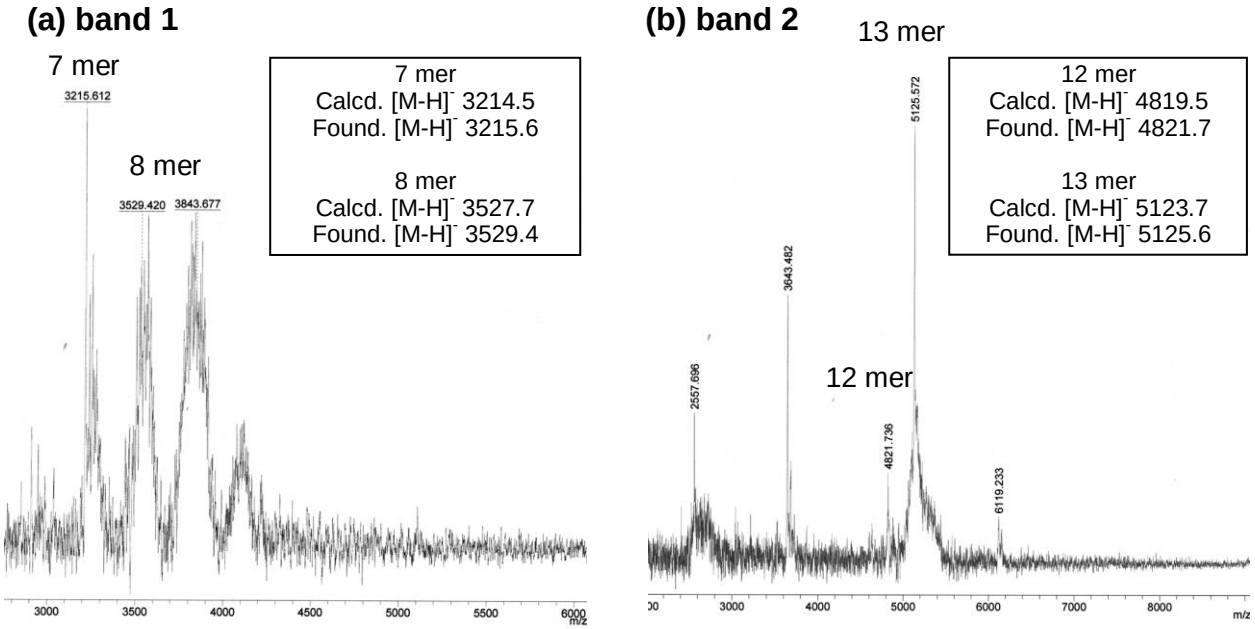
Measurement of CD melting spectra

1.5 μM of DNA (Acridine-amine-AVP adducted DNA, Non-alkylated DNA, or Non-alkylated DNA including 5 equiv. of 9-aminoacridine as a reversibly binding ligand) in buffers was thermally annealed by heating at 90 ^{o}C for 10 min and cooling to 25 ^{o}C at a rate of 0.5 ^{o}C/min using thermal cycler. CD spectra were recorded on a J-720WI (JASCO Co., Hachioji, Japan) equipped with a Peltier temperature controller. The CD spectra were recorded by using a micro quartz cell with 1 cm path length, from 500 to 200 nm at 0.2 nm intervals, at 2 nm band width, and 4 sec time per point and the data was averaged over two scans. For the measurement of melting temperature, CD spectra were recorded from 20 ^{o}C to 90 ^{o}C at an interval of 2 ^{o}C, with temperature increase at a rate of 1 ^{o}C/min.

Enzymatic hydrolysis of alkylated or non-alkylated DNA

The estimation of alkylated nucleobase in the target quadruplex DNA was performed by enzymatic hydrolysis reaction. The purified alkylated DNA (1 μM), *t*RNA (0.5 μg/μL), Snake Venom Phosphodiesterase I (5 x 10^{-3} U/μL), and Bacterial Alkaline Phosphatase (5 x 10^{-3} U/μL) were incubated in a 1x buffer for Bacterial Alkaline Phosphatase [50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$] at 37 ^{o}C for 30 min. The partial digestion of non-alkylated DNA (1 μM) was performed with Snake Venom Phosphodiesterase (5 x 10^{-5} U/μL) and Alkaline Phosphatase (5 x 10^{-5} U/μL) in 1x buffer at 37 ^{o}C for 30 min. After incubation, the enzyme reaction was quenched by adding the same volume of formamide containing 10 mM Na_2EDTA. The products were analyzed and purified by denaturing 16% polyacrylamide gel electrophoresis containing 20% formamide and 5.6 M urea, run at 400 V x 150 min in 1x TBE buffer. The novel bands were cleaved from the gel and extracted by shaking in a buffer of 10 mM Tris-HCl (pH 8.0) and 1 mM Na_2EDTA. The extract was filtered, freeze-dried, and purified by NAP-10 column (GE Healthcare). The purified DNA was treated with Zip-tip (Merck-Millipore), thereafter MALDI-TOF mass spectroscopy was measured.

(A) Potassium Buffer



(B) Sodium Buffer

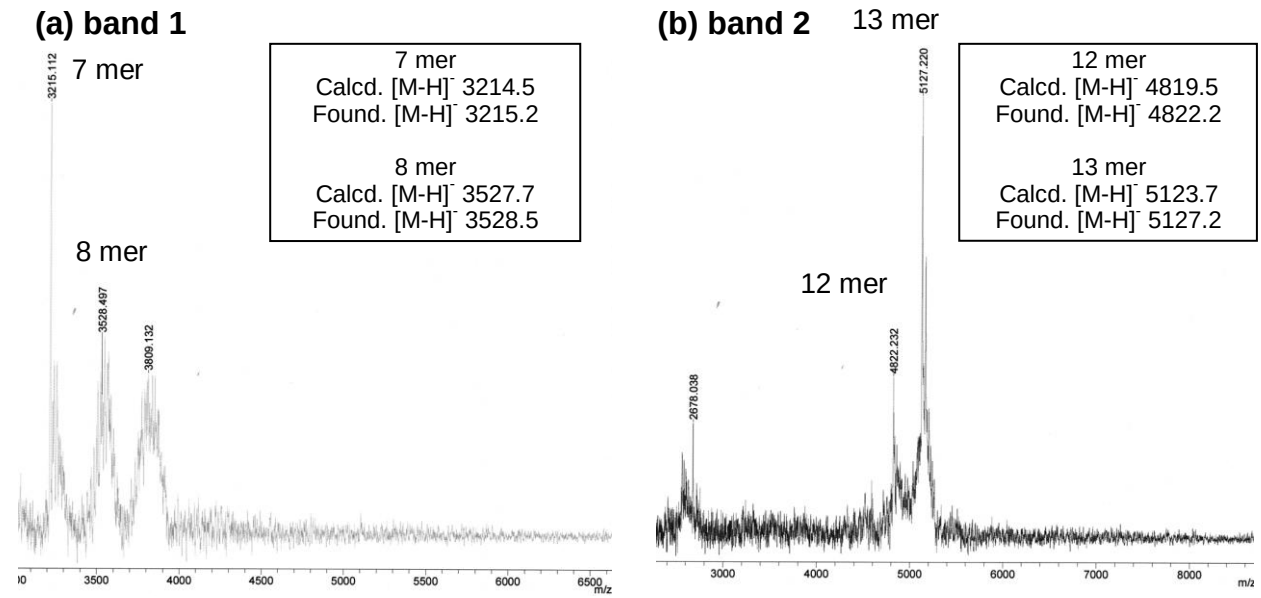


Fig. S4 MALDI-TOF mass spectra of the digested fragments

(A) The adducts was formed in K^+ buffer , purified and digested by 3'-exonuclease.
(B) The adducts was formed in Na^+ buffer , purified and digested by 3'-exonuclease.

Primer extension assay

First, for the alkylation of G4-template, the mixture (20 μL) of G4-template (5 μM) and AVP probe **2** (100 μM) in sodium or potassium buffer (pH 7.0) was incubated at 37 ^{o}C for 3 days. Then, the diluted solution (1 μM G4-template, 5 μL) was added to a solution (5 μL) of the primer DNA (0.34 μM) in NE buffer 2 (100 mM NaCl, 20mM Tris-HCl, 20 mM $MgCl_2$, 2 mM DTT, pH 7.9, New England Biolabs), heated at 93 ^{o}C and gradually cooled to room temperature for the annealing. To the solution were added dNTP (0.8 μL, final 0.2 mM) and the Klenow Fragment (exo-) (0.2 μL, final 0.1 U/μL, New England Biolabs), then the mixture was incubated at 37 ºC for 5 min. The reaction mixture was quenched by a loading buffer (80% formamide, 10 mM EDTA, 10 μL), then cooled to 0^{o}C. Electrophoresis was performed on a 14% denaturing polyacrylamide gel containing 30% formamide with 1×TBE and 5.3 M urea at 300 V and 40 ^{o}C for 70 min.

The replication reaction using longer DNA template containing G-4 structure

1) Preparation of Klenow fragments

The expression and purification of Klenow fragment DNA polymerase was performed as previously reported with a slight modification. Briefly, *E.coli* EG2523 (New England Bio Labs) was transformed with the plasmid encoding Klenow fragment fused at its N-terminus with maltose binding protein. The cells were cultured at 37 °C in LB medium to an A_{600} of around 0.5, followed by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside and further culture for 3 hours. The cultured cells were harvested and lysed by sonication. After centrifugation, the soluble fraction was loaded on the column packed with amylose resin (New England BioLabs) equilibrated with 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA and 1 mM DTT. Klenow fragment as eluted by the addition solution containing maltose (20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 10 mM maltose). The eluate was dialyzed against 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and 2 mM $CaCl_2$. The 3.5 mL of solution containing Klenow fragment was mixed with 700 μL of 50 μg/mL the Factor Xa protease (New England BioLabs) and incubated at 25 °C overnight. After treatment, Klenow fragment was loaded on a Hitrap Heparin column (GE Healthcare) equilibrated with 10 mM phosphate buffer (pH 7.0), 100 mM NaCl and 1 mM DTT, and purified with the linear gradient of NaCl. The fractions containing Klenow fragment were collected and concentrated by Amicon Ultra-15 (Merck) followed by purification through Hiload Superdex 75 (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1 mM EDTA and 1 mM DTT. The fractions containing Klenow fragment were collected, concentrated and dialyzed against 50 mM Tris-HCl (pH 7.2), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 50% glycerol. The purified Klenow fragment was kept under -30 °C until use. The concentration was determined by UV absorbance at 280 nm using the molar extinction coefficient of 58,790.

2) **The replication reaction**

Ten micro molar primer and alkylated or non-alkylated longer G4 template (Table S1) were annealed in the buffer used in the replication reaction: 40 mM Tris-HCl (pH 8.0), 150 mM KCl, 8 mM $MgCl_2$, and 2 mM spermidine. Reaction mixture contained 1 μM Klenow Fragment and 1.5 μM longer G4

template in a buffer containing 250 μM dNTPs, 150 mM KCl, 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, and 2 mM spermidine. After preparation of the solution, the mixtures were incubated at 37 °C. The reaction was stopped by the addition of the loading buffer. Products were separated by on 10% polyacrylamide gels containing 7 M urea at 70 °C for 1 hour at 200 V in 1×TBE. The gel images were captured using a Fujifilm FLA-5100 fluorescent imager.

Table S1. Sequences of the DNA templates used in this study

Sequence name	Sequence (5' to 3')[a]
Primer[b]	[FAM]-GAT CAC TAA TAC GAC TCA CTA TAG GAG ATG ACA CGA ACT AGG CTC GGT GCT CTC-3'
longer G4 template	GCCGTTTCGTAGTA**GGGTTAGGGTTAGGGTTAGGG**CAGAGA*GAGCACC GAGCCTAGTTCGTGTCATCTCCTATAGTGAGTCGTATTAGTGATC*

[a] G-quadruplex-forming, primer binding sites are shown in bold and italic respectively.

[b] "[FAM]" indicates the modification introduced by 5'-fluorescein phosphoramidite.

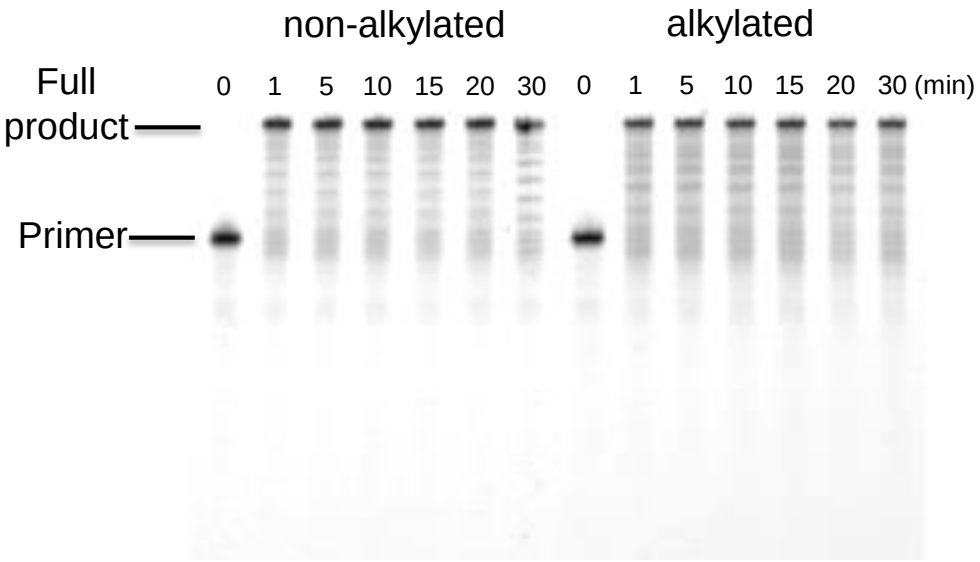


Fig. S5 Denaturing gel electrophoresis of products of replication using non-alkylated and alkylated G-4 structure in the absence of K^+

Reaction mixture contained 1 μM Klenow Fragment DNA polymerase, 1.5 μM longer G4 template and primer (10 μM) in a buffer containing 250 μM dNTPs, 40 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, and 2 mM spermidine