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Electrophoretic Mobility Shift Assay and Dimethyl Sulfate Footprinting for Characterization of G-Quadruplexes and G-Quadruplex-Protein Complexes

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Abstract

DNA G-quadruplexes are globular nucleic acid secondary structures which occur throughout the human genome under physiological conditions. There is accumulating evidence supporting G-quadruplex involvement in a number of important aspects of genome functions, including transcription, replication, and genomic stability, and that protein and enzyme recognition of G-quadruplexes may represent a key event to regulate physiological or pathological pathways. Two important techniques to study G-quadruplexes and their protein interactions are the electrophoretic mobility shift assay (EMSA) and dimethyl sulfate (DMS) footprinting assay. EMSA, one of the most sensitive and robust methods for studying the DNA-protein interactions, can be used to determine the binding parameters and relative affinities of a protein for the G-quadruplex. DMS footprinting is a powerful assay for the initial characterization of G-quadruplexes, which can be used to deduce the guanine bases involved in the formation of G-tetrads under physiological salt conditions. DMS footprinting can also reveal important information in G-quadruplex-protein complexes on protein contacts and regional changes in DNA G-quadruplex upon protein binding. In this paper, we will provide a detailed protocol for the EMSA and DMS footprinting assays for characterization of G-quadruplexes and G-quadruplex-protein complexes. Expected outcomes and references to extensions of the method will be further discussed.

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¹³Alternatively, covalent or non-covalent fluorophores [17, 18] and biotin [19, 38] labeled probes can be used.

¹⁴If working with RNA, it is essential to ensure the working environment is RNase-free. Wearing latex laboratory gloves (changed frequently) and using nuclease/RNase-free tips, reagents, and microfuge tubes are necessary common practices to prevent RNase contamination.

Keywords

Electrophoretic mobility shift assay (EMSA); Dimethyl sulfate (DMS) footprinting; DNA; G-quadruplex; Protein; Electrophoresis

1 Introduction

G-quadruplexes are formed in single-stranded guanine rich nucleic acid sequences and assembled by Hoogsteen hydrogen bonding of four guanines arranged within a planar tetrad and further stabilized by monovalent cations such as K^+ and Na^+ [1]. G-quadruplex formation has been observed in synthetic oligonucleotide sequences isolated from the human genome such as telomeres and gene promoter regions and more recently there has been significant number of advances in G-quadruplex detection that supports the existence of G-quadruplex structures in the genome of human cells significantly in gene regulatory regions and hot spots of genomically unstable regions of human chromosomes [2]. The association of G-quadruplexes to telomere biology, transcription regulation, and genomic instability undoubtedly suggest existence of proteins that modulate G-quadruplex conformation or serve as a platform for protein-protein interactions. The biological relevance of G-quadruplexes is linked to several G-quadruplex interacting proteins: shelterin complex proteins are known to function in telomere hemostasis [3]; and some proteins involve in G-quadruplex unfolding processes such as helicases [4] or G-quadruplex stabilization such as nucleolin [5]. In this paper, we will provide a detailed protocol for electrophoretic mobility shift assay (EMSA) and dimethyl sulfate (DMS) footprinting assay for investigating G-quadruplex and protein-G-quadruplex interactions. Expected outcomes and references to extensions of the method will be further discussed.

The established EMSA experiment has been applied to investigate many G-quadruplex-protein complex processes [6–10]. EMSA assay was originally described in 1981 by Fried and Crother [11] and Garner and Rezin [12] and became a widely used, robust method to elucidate crucial information for protein-nucleic acid interactions [6, 13, 14]. The underlying mechanism of the assay is that molecules in different size and charge show different electrophoretic mobilities when resolved on a native polyacrylamide or agarose gel [15]; the complex formed between nucleic acid and protein generally generate slower migrating species than free nucleic acids and DNA-protein complexes with lifetimes exceed the duration of the electrophoresis can be detected as distinct bands (Fig. 1). This technique poses several advantages. The assay is highly sensitive especially when using radioisotope labeled nucleic acids allowing the assay to be performed using nanomolar concentration of the protein and nucleic acids [16]. If high-sensitivity is not required, covalent or non-covalent fluorophores [17, 18] and biotin [19] labeled probes have also been successfully used and reported. Although the assay is often used for qualitative purposes, under appropriate conditions, apparent equilibrium constants for binding reactions can be obtained [20]. Another major advantage of the method is that the assay works well with both purified proteins as well as crude cell extracts [15]. Moreover, various size of nucleic acids and structures (single-stranded, double-stranded, quadruplex, triplex, hairpin, circular DNA) are

compatible and commonly used with the assay [16]. The DNA structure within the DNA-protein complex can be further characterized using DMS footprinting method.

DMS footprinting is a powerful biochemical method due to its ability to measure the relative reactivities of individual nucleotides within a DNA strand with DMS, which can provide essential information for structural characteristics of the DNA [21]. DMS is one of the oldest probes for footprinting, and it was first introduced for DNA mapping in 1977 [22], allowing detection of methylation by DMS at N7 of guanosine (N7-methylguanine) and N3 (N3-methyladenine) of adenine nucleotides [21, 22]. The glycosidic bond of methylated purine becomes unstable and breaks easily under alkali conditions at high temperatures (>90° C), which results in the cleavage of the sugar-phosphate backbone [22, 23] (Fig. 2). Because guanine bases are methylated fivefold faster than adenine bases, breakage at guanine residues results in darker bands than for adenine when ³²P end-labeled DNA fragments generated by DMS are resolved on a polyacrylamide gel [22, 24]. DMS footprinting has various applications. It can provide essential information for the existence of specific structural elements such as secondary structures. Moreover, it can be used to confirm high-resolution structures or to identify the regions that do not match predictions from the structure, which may suggest the region is dynamic or exists in multiple conformations [25–30]. DMS footprinting can also give significant information about complexes in the presence and absence of bound small molecule ligands and proteins, allowing identification of the regions of the structure that change upon ligand binding [31, 32].

DMS footprinting experiments are particularly useful and routinely used for characterization of G-quadruplexes and elucidation of DNA structure within DNA-protein complex. G-quadruplexes are formed by the association of the four guanine bases through Hoogsteen hydrogen bonding, which is stabilized by monovalent cations such as K⁺ and Na⁺ [1, 33–35]. In contrast to guanine residues in single- and double-stranded DNA, a guanine N7 in a G-tetrad is involved in Hoogsteen hydrogen binding and is thereby protected from methylation in the DMS footprinting experiment and subsequent cleavage under alkali conditions, thus enabling the identification of guanines involved in G-tetrad formation (Fig. 2) [23, 25, 29, 34, 36]. In addition to initial characterization of the G-quadruplexes, DMS footprinting technique can also be used to investigate G-quadruplex-protein interactions. Further protections can be expected from direct contacts between protein and G-quadruplex as well as from local changes within the G-quadruplex induced by protein binding [6–10].

2 Materials

Prepare all the solutions using filtered Milli-Q water. Unless stated otherwise, the reagents should be molecular biology grade or higher.

2.1 Denaturing-PAGE

1. TBE electrophoresis buffer (5×): 0.45 M Tris-HCl (pH 8.0), 0.45 M boric acid, 10 mM EDTA.
2. Urea (Thermo Fisher Scientific, cat. no. BP169–212).
3. 40% Acrylamide/Bis Solution 29:1 (Bio-Rad, cat. no. 1610146) (*see* Note 1).

4. Ammonium persulfate (APS) (Bio-Rad, cat. no. 1610700) (*see* Note 2).
5. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (Fisher Scientific, cat. no. 15524010) (*see* Note 3).
6. Alkaline gel loading dye (1×): 80% (by volume) formamide, 10 mM NaOH, 0.005% Bromophenol Blue (w/v).
7. Formamide (99.5%) (Thermo Fisher Scientific, cat. no. BP-228100).
8. Bromophenol Blue (Sigma Chemicals, cat. no. B5525).
9. Sodium Hydroxide (White Pellets) (Thermo Fisher Scientific, cat. no. BP350–212) (*see* Note 4).

2.2 Ethanol Precipitation of DNA

1. Sodium Acetate (pH = 7.3 M) (Sigma Aldrich, cat. no. S2404).
2. Ethanol, Absolute (200 Proof) (Fisher Scientific, cat. no. BP2818500).
3. Corning™ Costar™ Centrifugal Devices: Spin-X™ 0.45 µm CA (Thermo Fisher Scientific, cat. no. 07200388).

2.3 Preparation of ³²P-End-Labeled Oligonucleotides

1. 10× Phosphonucleotide Kinase (PNK) buffer (700 mM Tris–HCl pH = 7.6, 100 mM MgCl₂, 50 mM DTT) (New England Biolabs, cat. no. B0201S).
2. Adenosine 5' γ-³²P-ATP 5'-gamma ³²P Triphosphate ([γ-³²P] ATP) Triethylammonium salt—3000 Ci/mmol, 10 mCi/mL, EasyTide (PerkinElmer, cat. no. BLU502A250UC) (*see* Note 5).
3. T4 Polynucleotide Kinase (PNK), 10,000 U/mL (New England Biolabs, cat. no. M0201S).
4. Micro Bio-Spin™ P–6 Gel Columns, Tris Buffer (Bio-Rad, cat. no. 7326201).
5. UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, cat. no. 10977015).

2.4 Protein-DNA Complex Preparation

1. Binding Buffer: 20 mM Tris–HCl, pH = 7.6, 200 mM KCl.

¹-Acrylamide and bis-acrylamide are neurotoxic. Wear personal protective equipment to avoid exposure to hazards. Store refrigerated at 4 °C and protect from light.

²-Ammonium persulfate acts as a catalyst for the copolymerization of acrylamide and bis-acrylamide gels. The powder should be stored in a desiccator at room temperature. It decays slowly in solution, so it is better to prepare fresh solution the day it is used.

³-TEMED is an essential catalyst for polyacrylamide gel polymerization. It should be stored tightly sealed at 4 °C.

⁴-Special care is required to prepare a solution of sodium hydroxide. Solid pellets should be handled carefully. The solution must be prepared in a ventilated fume hood to provide containment. Dissolution of NaOH pellets in water is highly exothermic; solutions of sodium hydroxide should be prepared by slowly adding the sodium hydroxide pellets to water while avoiding excess heat accumulation. An ice bath can be used to chill the solution after each addition.

⁵-Exposure to γ-radiation and secondary X-rays from ³²P is hazardous. Safe handling of this isotope is essential. Exercise maximum precautions and shielding to prevent exposure to or spilling of ³²P. Radioactive waste from the columns should be checked using a Geiger counter on the radiation records. Radioactive material waste should be properly disposed.

2. Glycerol (Molecular Biology) (Thermo Fisher Scientific, cat. no. BP229–1).
3. Bovine Serum Albumin (BSA) (Thermo Fisher Scientific, cat.no. BP9704–100).
4. Dithiothreitol (DTT) (GoldBio, cat. no. DTT50).

2.5 Native-PAGE Preparation

1. TBE Electrophoresis Buffer (5×): 0.45 M Tris–HCl (pH 8.0), 0.45 M boric acid, 10 mM EDTA. Filter using a 0.22 µm filter. Store at 25° C.
2. 40% Acrylamide/Bis Solution 29:1 (Bio-Rad, cat. no. 1610146) (*see* Note 1).
3. Ammonium Persulfate (APS) (Bio-Rad, cat. no. 1610700) (*see* Note 2).
4. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) (Fisher Scientific, cat. no. 15524010) (*see* Note 3).
5. DNA loading buffer (10×): 50% glycerol by volume, 0.005% bromophenol blue (w/v). Store at –20 °C.
6. XAR-2Film(Individually Wrapped)(VWR,cat.no.IB1651579).
7. Plastic food wrap.

2.6 Methylation and Cleavage Reaction

1. 50 µg/mL calf thymus DNA (Thermo Fisher Scientific, cat. no. 15633019).
2. Dimethyl Sulfate solution (Sigma-Aldrich D186309).
3. Ethanol, Absolute (200 Proof) (Fisher Scientific, cat. no. BP2818500).
4. 2-Mercaptoethanol (Sigma-Aldrich, cat. no. M6250).
5. Glycerol (Molecular Biology) (Thermo Fisher Scientific, cat.no. BP229–1).
6. Piperidine, ReagentPlus, 99% (Sigma-Aldrich, cat. no. 104094).
7. Whatman® cellulose chromatography papers (Sigma-Aldrich, cat. no. Z270822).

3 Method

3.1 Preparation of ³²P-End-Labeled G-Quadruplex Samples

3.1.1 Purification of a Desired Full-Length Oligonucleotide Using a Denaturing PAGE

1. Prepare a gel cassette for a 20 cm × 16 cm × 1.6 mm gel and make 60 mL of denaturing 12% polyacrylamide gel solution by mixing 12 mL TBE buffer (5×), 18 mL of 40% acrylamide/bis-acrylamide (29:1), and 30 g urea, then adding water to 60 mL (*see* Note 6).

⁶.Clean glass plates (gel casting apparatus) using ethanol/methanol and wipe dry. The plates should be clamped together most of the way. Before adding APS and TEMED to the solution, mix the solution thoroughly and allow it to settle for a minute or so. Add APS and TEMED right before casting the gel, mix the solution very gently by swirling, avoiding any air bubbles, and carefully pour the gel solution between the plates. Avoid bubble formation in the gel while pouring. Gentle tapping on the plate with a fist can help direct the flow of the gel solution to avoid trapping bubbles.

2. After adding 350 μ L freshly prepared 10% APS and 20 μ L TEMED to the urea gel solution, fill the gel cassette with this solution using a propipetter and promptly insert the 1.6 mm comb. Let the gel polymerize, about 30 min to 1 h (see Note 6).
3. After the gel is polymerized, remove the comb gently and wash the wells using 1 $\times$ TBE buffer using a Pasteur pipette.
4. Place the gel cassette into the electrophoresis apparatus (Labrepc Model V15–17); clamp it down firmly. Pour 1 $\times$ TBE buffer into the top and bottom chambers (see Note 7).
5. Pre-run the gel at 200 constant voltage for 20–30 min.
6. Combine the DNA sample with alkaline gel loading dye and heat the sample at 95 $^{\circ}$ C for 5 min prior to loading into wells (see Note 8).
7. While samples are heating, rinse out the wells using a Pasteur pipette to make sure to get rid of the urea completely otherwise the urea will block the sample from entering the gel expeditiously. Load the samples into the gel.
8. Run the gel for about 2.5 h until bromophenol blue dye has migrated about half way down at a constant 200 V.
9. Pry the glass plates apart from the gel carefully so that the gel is still attached to one plate.
10. Place the plastic-wrapped gel over a UV fluorescent silica coated TLC plate and illuminate the gel from above with a shortwave UV illuminator (see Note 9).
11. Cut the desired DNA bands out with a razor blade. Chop the gel for a band into pieces, transferring them to a 1.5- or 2 mL microfuge tube. Crush the gel into very small piece, almost like a powder using a pipette tip, then add sufficient elution buffer containing 50 mM Sodium Acetate with 2 mM EDTA (pH 7) to cover the gel pieces. Rotate the tubes for overnight at 4 $^{\circ}$ C. Next day, remove the gel pieces using Corning Costar Centrifuge filters by following the manufacturer's instructions. For a second round of recovery, add more elution buffer into the gel, rotate another 2 h, and filter again, combining the filtrates.

⁷ Make sure to cover the bottom end of the plate with 1 TBE buffer and add 1 $\times$ buffer to the top of the gel apparatus until it covers the top of the gel.

⁸ DMS footprinting oligonucleotides can be designed with the addition of T7 bases at both ends of the sequence to increase footprinting resolution at the ends and to have an extended DNA environment for the G-quadruplex forming sequence. However, it is essential to confirm that the T flanking ends do not influence the G-quadruplex structure. One can easily check the quality of the G-quadruplex prior to experiment by performing simple 1D NMR experiment and observing the chemical shift region between 10.5–12 ppm, which is characteristic for the imino protons of guanines involved in the G-tetrad and connected with Hoogsteen hydrogen bonding [1]. Purified and desalted custom-DNA/RNA oligonucleotide probes (20–80 bp) can be directly purchased from commercial suppliers. We routinely synthesize and purify DNA oligonucleotides in our laboratory using β -cyanoethylphosphoramidite solid phase chemistry (Applied Biosystems Expedite 8909) as described previously [37]. In brief, the synthesized oligonucleotides are eluted from the column with a 50%:50% mixture of 40% methylamine:ammonia, heated for 10 min at 65 $^{\circ}$ C, purified on reverse-phase Micropure II columns (BioSearch Technologies) and subjected to sequential dialysis through 10 mM NaOH, water, 150 mM NaCl, and water before lyophilization.

⁹ It is important to note that the UV radiation used in transilluminators is harmful to both the skin and eyes. Use appropriate PPE for the hazard: UV face shield, goggles. Never view the UV lamp directly. Keep exposure time to a minimum because it can damage DNA. One can mark the locations of the DNA bands on the plastic wrap by using UV light exposure and the silica plate and then turning off the UV to cut the bands to decrease the exposure time.

12. Add 4 volumes of absolute (200-proof) ethanol to the eluted sample. Mix well and store at -20°C overnight or at -80°C for ~ 2 h. Centrifuge the sample for ~ 20 – 30 min at $12,000 \times g$ at 4°C . Carefully remove the ethanol, and gently rinse the recovered DNA pellet once with $250\ \mu\text{L}$ of ice-cold 75% ethanol. Remove the ethanol and allow the samples to sit open at room temperature to air-dry (*see* Note 10).
13. Resuspend the DNA in Milli-Q water and determine the concentration spectrophotometrically (*see* Note 11).

3.1.2 Preparation of ^{32}P -End-Labeled Oligonucleotides

1. To end-label the DNA, mix $2\ \mu\text{L}$ $10\ \mu\text{M}$ oligonucleotide, $2\ \mu\text{L}$ $10\times$ phosphonucleotide kinase buffer, $3\ \mu\text{L}$ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, $12\ \mu\text{L}$ Milli-Q water and $1\ \mu\text{L}$ PNK (phosphonucleotide kinase) (*see* Notes 5, 12–15).
2. Mix samples gently, spin down the tube contents, and incubate at 37°C for 30 – 40 min.
3. Run each sample through a Micro Bio-Spin column to remove any unreacted $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ based on the recommendations from the manufacturer; after the column was centrifuged at $1000 \times g$ for 2 min to remove the buffer from the column, the complete reaction mixture should then be loaded to the center of the column and eluted by centrifugation at $1000 \times g$ for 4 min (*see* Notes 16 and 17).

3.1.3 G-Quadruplex Folding of ^{32}P -Labeled Oligonucleotides

1. Set up the G-quadruplex folding reactions for a final target concentration of 50 nM for each oligonucleotide in 20 mM Tris-HCl ($\text{pH} = 7.4$) buffer, with and without 140 mM KCl (the DNA prepared in the absence of KCl will serve as a control) (*see* Note 18).
2. Anneal the DNA by incubating the samples at $>90^{\circ}\text{C}$ for 5 min, then cool down slowly to room temperature in the heating block (*see* Notes 17 and 19).

¹⁰ Remove the ethanol while avoiding contact with pellet so that the maximum salt possible is removed.

¹¹ Quantification of the DNA oligonucleotides can easily be performed by UV/Vis spectroscopy at 260 nm using their calculated extinction coefficients.

¹² 5'-end radiolabeling of nucleic acid probes are commonly used and provides high level of sensitivity. T4 Polynucleotide kinase (PNK) catalyzes the transfer of the gamma phosphate (^{32}P) of ATP to the 5'-hydroxyl terminus of DNA or RNA. Ammonium ions are strong inhibitors of the PNK enzymes, thus G-quadruplex forming nucleic acids should not be dissolved in or precipitated in ammonia-containing buffers before PNK treatment. We have also observed low labeling efficiency in our experiments if the nucleic acid is dissolved in the phosphate buffer before PNK treatment.

¹⁵ For a lower quantity of labeled DNA, all materials can be scaled down proportionately.

¹⁶ All recovered samples should be well over $500,000$ cpm if fresh ^{32}P -ATP is used.

¹⁷ Radiolabeled nucleic acid samples can be kept at 4°C for 2 weeks in a radioactive-storage refrigerator. Based on our experience, storage for longer than 2 weeks leads to degradation of nucleic acids.

¹⁸ The cation type influences G-quadruplex formation. For instance, G-quadruplexes formed in human telomeres are structurally polymorphic, with two equilibrating hybrid-type structures in K^{+} solution [39, 40] and a basket-type structure in Na^{+} solution [41]. Thus, cation in the G-quadruplex folding buffer and binding buffer should be correctly determined to study intended G-quadruplex system.

¹⁹ Most of the time, annealing of nucleic acids assist inducing appropriate G-quadruplex formation. Typically, the samples are heated to 90°C and slowly cool down to room temperature on heating block. If the samples are intended to be annealed, to prevent phosphodiester backbone cleavage, alkaline conditions in buffer should be avoided. Whether annealing is required or not can be determined empirically and quality can be checked by 1D NMR experiment.

3.2 EMSA Assay

3.2.1 Complex Preparation

1. Prepare binding buffer in ice containing 20 mM Tris-HCl (pH = 7.4), 200 mM KCl, 2 mM EDTA, 0.15 mg/mL BSA and 2 mM DTT (*see* Notes 20 and 21).
2. Prepare the protein in desired concentration (706 nM) using binding buffer (*see* Notes 22 and 23).
3. Take the 1 μ L 10 nM folded G-quadruplex sample in 1.5 mL microfuge tube in shielding box that is placed on ice, and after DNA sample chill on ice for 5–10 min, add the 8.5 μ L 706 nM protein solution prepared in binding buffer. Equilibrate the binding reaction mixture for an hour in shielding box placed on ice (Table 1, *see* Note 24).
4. Prepare 1 μ L 10 nM folded G-quadruplex sample in 1.5 mL microfuge tube and add the 8.5 μ L binding buffer solution. This sample will be used as a control (Table 1).
5. Add 2% glycerol to in binding reaction mixture prior to loading on the gel (*see* Note 25).

²⁰Special care and precautions are required when protein is mixed with the DNA. If addition results in any cloudiness, the binding reaction should be optimized in a way where protein stays in solution. Conditions that give complete binding of the DNA G-quadruplex to the protein can be established in EMSA experiments prior to performing DMS footprinting reactions. The binding buffer composition should be optimized for specific protein-DNA interactions and should be at physiological conditions for excellent buffering capacity as well as biological relevance. Various buffer conditions can be used in the binding reactions such as Tris-based, HEPES, Bis-Tris, and Phosphate [16]. Determining the correct pH of the binding and electrophoresis buffer is a common practice for manipulating the mobilities of the protein-DNA complexes [42]. Protein charge can significantly affect the resolution of the protein-DNA complex. Proteins which are neutral or positively charged display reduced mobility on the native gel and negatively charged proteins may have electrophoretic mobility comparable to free DNA. Mono- and divalent salt can be added in binding reaction to stabilize complexes [43]. Since the samples will be subjected to electrophoresis, the conductivity of the samples should not be excessive. Monovalent salt concentration should be in the range of $1 \text{ mM} < \text{M} + \text{X} < 300 \text{ mM}$ to match conductivity of the sample and electrophoresis [20]. Addition of carrier proteins such as BSA (Bovine Serum Albumin) [44, 45] and poly (dI-dC) [46] can be added to abolish any nonspecific interactions within the complex. Nonionic detergents in binding reactions were also reported to increase the solubility of the protein and stability of the complexes [47]. If the cell or nuclear extract is used instead of purified protein, then protease, nuclease and phosphatase inhibitors can be included into binding reaction [48]. DTT or 2-Mercaptoethanol (<10 mM) can be added in the samples if a reducing environment is necessary for the complex [45]. Any component added into binding reaction to improve protein-Gquadruplex should also be added in control reactions for accurate comparison.

²¹For free DNA samples, gels can be run at 200 V at room temperature. However, for protein-DNA complexes, temperature is an important factor and variable that requires careful attention and should be determined empirically. All the binding reaction components as well as electrophoresis buffer should be brought the reaction temperature before preparing the complex.

²²Unlike nucleic acids, protein preparation can be time consuming and challenging. Protein expression and purification methods may vary according to protein and have been described elsewhere [49–52]. We have prepared example protein used in our representative protocol with an N-terminal His6-tag using the pET-28a (+) vector (Novagen) in *E. coli* BL21 (DE3) cells (Promega) and purified using Ni^{2+} charged His-trap, Q-seph, and size exclusion columns. Special care should be taken to maintain nucleic-acid binding activity of the protein. Protein solutions should be prepared and stored under the conditions that maximize the storage time of the active protein. If the protein is stored as frozen aliquots, the aliquot should be thawed right before the complex preparation for the assay to decrease the amount of the time it sits at 4 °C and to obtain the maximum nucleic acid binding activity.

²³The optimal concentration necessary for the protein to interact with a constant amount of nucleic acids should be determined empirically [16].

²⁴The binding reaction must reach equilibrium for obtaining reproducible results. The equilibration time may vary depending on the interacting molecules and binding reaction conditions such as temperature and concentration of the substrate and ligand; it can be determined empirically by comparing mole-fractions of bound and unbound nucleic acid amount in a series of binding reactions incubated for different time periods and resolved on the same native gel.

²⁵Addition of neutral solutes such as glycerol, ethylene glycol <2 M into samples help loading the samples into wells of the gel [16]. Moreover, they may further stabilize some complexes during the electrophoresis. It should be noted that neutral solutes higher than 2 M concentration might increase viscosity and might make the solution handling harder.

3.2.2 Preparation of the Native PAGE

1. Clean the large and small gel plate pairs of 20cm × 16cm × 1.6mm gel cast, spacers and comb with methanol and 70% ethanol using Kimwipes to remove any debris that might interfere gel polymerization.
2. Prepare the polymerization mixture by mixing 6 mL TBE buffer (5×), 6 mL of 40% acrylamide/bisacrylamide (29:1) and adding water to 60 mL. The final concentration of acrylamide is 4% (*see* Notes 26 and 27).
3. Thoroughly mix 400 µL freshly made 10% APS and 25 µL TEMED right before you are ready to cast the gel (*see* Note 28).
4. Pour the polymerizing mixture into the glass plate slowly, avoiding bubbles (*see* Note 29).
5. Insert the 1.6 mm comb, 10 well comb gently and clamp it from top to plate. Let the gel polymerize, about 30 min to 1 h.
6. After the gel is polymerized remove the comb gently and wash the wells using 0.5× TBE buffer using a pasture pipette.
7. Place the gel plate into the electrophoresis apparatus; clamp it down firmly. Pour chilled 0.5× TBE buffer into the top and bottom chambers (*see* Notes 26 and 30).
8. Pre-run the gel at 20 V constant voltage for 20–30 min at 4 °C (*see* Notes 20 and 31).
9. Rinse out the wells using pasture pipette.
10. Load 5 µL samples into wells of the gel. Make sure to load only dye (same amount with your samples in other wells) into near end wells of the gel for even running (*see* Note 32).
11. Run the gel for 3 min at 150 V and followed by an hour at constant 80 V at 4 °C (*see* Note 33).

²⁶Composition of the gel and electrophoresis buffer should be adjusted empirically based on the molecular system. The most common EMSA buffers are Tris–Borate–EDTA, Tris–Acetate–EDTA, and Tris–Glycine. 5% glycerol might be included in electrophoresis buffer to increase complex stability. Moreover, thioglycolate (<5 mM) can be included into the gel running buffer if a reducing environment within the gel matrix is necessary for the complex [16].

²⁷The gel percentage should be optimized empirically. Resolution of the complexes can be increased by higher percentage of the gel. The complexes of DNA fragments at the range of 25–1500 bp length and proteins at the range of 10,000–500,000 Daltons can be resolved in polyacrylamide gels at 4–10%. 3–6% agarose gels can also be used depending on the size of the complexes [20]. One can start at low concentration of gel (3–4%) and gradually increase the gel concentration until the best resolution is obtained.

²⁸Mix the solution thoroughly before adding APS and TEMED, settle it down for approximately a minute. Add APS and TEMED right when one is ready to cast the gel, mix the solution very gently to avoid any air bubbles.

²⁹Avoid bubble formation during polymerization. Since bubbles don't conduct electrical current, they prevent continuous migration of the species in binding reaction during electrophoresis. If a bubble is formed, tap the plate gently where the bubble is formed to help the bubble migrate to the top or part of the plate, remove the bubbles using thin spacers, and add more polymerization mixture for filling the plate completely.

³⁰Make sure there is no bubble formation at the bottom part of plates. If any observed, they can be removed by slowly replacing the plates by tilting.

³¹The low voltage for pre-run at 4 °C is required to avoid gel heating and bringing the gel and electrophoresis components to the same temperature with the binding reaction components.

³²Loading smaller sample volumes into the wells of the gel result in a narrow starting zone and thus sharper bands.

³³Because lifetimes of protein-DNA complexes should be more than the time required by free DNA to leave the sample for detecting the binding, initial loading voltage can be critical and can be controlled with the application of the high voltage in the beginning of

12. End the electrophoresis and take out the spacers first, start prying from the side to remove the gel from the plates.
13. Put a sheet of Whatman 1 filter paper on top of the gel and try to lift the end closest to you with the gel on it.
14. Lay the paper, gel side up, on the radiation shield pad and cover the gel with plastic wrap. Cut off extra material (*see* Note 34).
15. Transfer the wrapped gel to the gel dryer (Bio-Rad, Model 583), sandwiching between two sheets of filter paper (*see* Note 35).
16. Make sure the water in the vacuum control reservoir is between min and max (closer to max) and start the vacuum. Check that the entire area is under vacuum by running fingers along the groove around the edges. Dry the gel for 1 h.
17. Take the dried gel out and put it in a Phosphorimager cassette. Expose overnight or over the weekend.
18. Detect the signals using a Storm PhosphorImager (Fig. 3).
19. Quantification can be obtained using Image Quant 5.1 software from Amersham Biosciences.

3.3 DMS Footprinting Assay

3.3.1 Complex Preparation

1. Mix the folded and unfolded G-quadruplex samples from step 2 of Subheading 3.1.3 with the protein in shielding box that is placed on ice (*see* Note 20). To ensure that the DNA binds quantitatively to the protein, the protein should be present at a concentration well above the K_d and should be in excess of the DNA.
2. Incubate the reaction mixture to allow complete complex formation between protein and G-quadruplex in shielding box placed on ice (*see* Note 24).
3. Include two control reactions without protein for both folded and unfolded G-quadruplex.

3.3.2 DMS Methylation and Native Gel Purification of Methylated DNAs and Protein-DNA Complexes

1. Treat the DNA and DNA-protein complex samples in the presence and absence of KCl to methylate the DNA by addition of DMS to 0.5% and 1 μ g Calf Thymus DNA, incubating the methylation mixture for 7 min at room temperature (*see* Note 36).

electrophoresis for couple minutes. Excessive heating by high voltage should be avoided. In addition, decreasing the volume load into the wells can effectively decrease the distance of free electrophoresis.

³⁴The wrapped gel should be free of air bubbles and wrinkles which would interfere with autoradiography.

³⁵Drying the gel is important to prevent the diffusion of the separated species on the gel; however, we have also obtained successful imaging when the wrapped gel is incubated in the cassette at 4 °C overnight without drying.

³⁶10% DMS should be freshly made in 50% ethanol as a stock solution. For a no-DMS control, samples can be treated with only 50% ethanol instead of 0.5% (final) DMS. Mix each sample with the pipet as the reagents are added, marking the start of each tube's reaction. After all reaction tubes are started, do a spin-down centrifugation of the tubes and set them back in the rack. From the start of

2. Include total of four control reactions without DMS for both folded and unfolded G-quadruplex samples with protein and without protein (*see* Note 36, Fig. 4c).
3. Add 0.07% β -mercaptoethanol and 2% glycerol to experimental samples as well as non-DMS control samples (*see* Note 37).
4. Prepare a gel cassette for a 20 cm $\times$ 16 cm $\times$ 1.6 mm gel, and make 60 mL of 8% nondenaturing polyacrylamide gel solution by mixing 12 mL TBE buffer (5 $\times$), 12 mL of 40% acrylamide/ bisacrylamide (19:1), and 36 mL water (*see* Notes 6, 26, and 27).
5. After adding 350 μ L freshly prepared 10% APS and 20 μ L TEMED to the gel solution and pouring the gel, insert the 1.6 mm comb. Let the gel polymerize, about 30 min to 1 h (*see* Notes 6 and 29).
6. Remove the comb and rinse out the wells with 1 $\times$ TBE buffer.
7. Place the gel cassette into the electrophoresis apparatus. Pour 1 $\times$ TBE buffer into the top and bottom chambers (*see* Notes 7 and 30).
8. Load the samples onto the gel and run at 200 V for DNA samples and at 100 V for protein-DNA complexes. The gel should take 2–3 h (*see* Notes 21, 33, and 38).
9. Place plastic wrap over the gel after removing the back plate. Use about 4 min of exposure time for the Kodak XAR-2 film (*see* Note 39).
10. Visualize the DNA and complex location of DNA bands within the gel via autoradiography.
11. Cut the desired band out from the gel using razor blade and transfer each sample into a 1.5-mL microfuge tube. Follow **steps 11** and **12** in Subheading 3.1 for recovery of the DNA (*see* Note 40).
12. Dissolve the pellet in 50 μ L 10% piperidine. Heat the samples at 90 $^{\circ}$ C for 16 min in 10 mM Tris-HCl pH = 7.6 buffer (*see* Notes 41 and 42) to cleave the methylated DNA.

DMS addition, time 7 min for each of these samples, precisely. The methylation time might vary depending on the system used and the best incubation time should be determined empirically.

³⁷It is important that the DMS modification reaction be quenched effectively at the desired time. In this experiment β -mercaptoethanol is found to be sufficient to quench the DMS reaction. Addition of neutral solutes such as glycerol, ethylene glycol <2 M into samples help load the samples into wells of the gel [16]. Moreover, they may further stabilize some complexes during the electrophoresis. It should be noted the neutral solutes higher than 2 M concentration might increase viscosity and might make the solution handling harder.

³⁸If there are wells that are open at the sides of the gel, load neutral loading dye in these wells to prevent “drag” on the samples so the bands will run evenly. Center wells do not require this care.

³⁹The radioactivity levels can be quantified using a Geiger counter or scintillation counter. If the samples were not about 100,000 cpm when loaded, the incubation can be up to 10–15 min. Ensure that the gel and Kodak film are aligned very well and stay stable during the incubation time.

⁴⁰Check the pellet on the Geiger counter as the ethanol is removed to make sure the radioactivity is primarily staying in the tube with the pellet.

⁴¹The piperidine cleavage will only start when samples are heated.

⁴²Don't use heat when evaporating the piperidine or water washes because it may damage the DNA samples.

13. Spin samples down and then transfer them to the Speedvac for an hour for complete drying (*see* Note 42).
14. Add 50 μ L water to each sample pellet with vigorous vortexing, then return the samples to the Speedvac for 30–60 min to dry them completely again. Repeat the wash with 50 μ L water resuspension for each sample, vortexing the samples very well, and drying them again for 30–90 min until completely dry.

3.3.3 Separation of Cleavage Products on Denaturing PAGE

1. Set up a gel cassette for a 30 cm $\times$ 30 cm $\times$ 0.4 mm gel and prepare 60 mL of denaturing 16% polyacrylamide gel solution by mixing 12 mL TBE buffer (5 $\times$), 24 mL of 40% acrylamide/bis-acrylamide (29:1), and 30 g urea, then adding water to 60 mL total volume (*see* Notes 6 and 43).
2. After adding 350 μ L 10% APS and 20 μ L TEMED slowly to the gel solution, to minimize the formation of bubbles, fill a gel cassette with this solution using a propipetter and promptly insert the 0.4 mm comb (*see* Note 6).
3. Allow the gel to polymerize for 30 min–1 h.
4. Pour 1 $\times$ TBE buffer in lower and upper chambers of electrophoresis apparatus (*see* Note 7).
5. Remove the comb and rinse out the wells with 1 TBE buffer (*see* Note 44).
6. Pre-run the gel at 1600 V, 45 mA, 300 W for 30 min. With the Owl Separations apparatus S4S, the temperature should go to about 40–50 $^{\circ}$ C.
7. Rinse out the wells using a Pasteur pipette to remove the urea completely.
8. Resuspend piperidine-treated DNA sample with alkaline gel loading dye for a final concentration of 30,000 cpm/10 μ L. Heat the samples at 95 $^{\circ}$ C for 5 min and place them directly on ice prior to loading into wells.
9. Load 10,000 cpm/3 μ L sample into wells and run the gel at 1800 V with a maximal current of 35 mA (*see* Note 45).
10. After the desired resolution is obtained, end the electrophoresis run. Working as quickly as possible, remove the spacers first, then pry from the sides to break the gel from the plates.
11. Immediately, before the gel has time to cool, put a sheet of Whatman[®] cellulose chromatography paper on top of the gel and, moving quickly, roll the paper back at a corner, encouraging the edge of the gel to follow the paper. When enough of the gel has been lifted off the plate onto the paper, the remainder of the gel can

⁴³. In this step, it is very important to clean off gel plates with methanol and ethanol several times to remove any debris that might interfere with gel polymerization or promote bubble formation.

⁴⁴. Very gently pull the comb forward a little and pour buffer behind the comb. Use a syringe to bump bubbles out of the bottom area and behind the plates as well. Make sure there are no bubbles between the plates at the bottom. Flex the comb back and forward with respect to the gel plate as you wiggle the comb out. This step is important and prevents the wells from deforming on the bottoms or sides since a vacuum is created as the comb is removed.

⁴⁵. Loading smaller sample volumes into the wells of the gel result in a narrow starting zone thus sharper bands.

be removed from the plate by rolling the paper carefully the rest of the way away from the plate, avoiding tears.

12. Cover the gel with plastic wrap. Cut off extra material and dry the gel using a gel dryer at 80 °C for an hour with a vacuum, conditions provided by a gel dryer (*see* Note 35).
13. Place the dried gel in a PhosphorImager cassette. Expose to a PhosphorImager screen overnight or over the weekend.
14. Detect the signals using a Storm PhosphorImager.

3.3.4 Analysis and Anticipated Results—In order to draw accurate conclusions from the results, all the controls should be included in the assay and all the reactions should be treated identically in the presence and absence of protein/KCl/DMS. Discrete bands should be observed for the fragments of unfolded G-quadruplex resulted from digestion at each guanine positions (Fig. 4c, lane 3). The top band of the gel representing the full product can be measured as a reference point (Fig. 4c). The reference point in the absence of DMS should be more intense from the reactions including DMS. When DNA is folded, a guanine N7 in a G-tetrad is involved in Hoogsteen hydrogen binding and is protected from methylation and subsequent cleavage (Fig. 4c, lane 7). Hence, it is anticipated that the reference point will be denser in folded reaction compared to unfolded reaction. Similarly, protein binding will result in additional contacts and lead to protection of close-by residues from DMS modifications compared to the unbound control reaction (Fig. 4c, lanes 7 and 8). It should be also noted that folding and/or protein binding might result in over-cleaved products because formation of additional contacts might cause some regions of the DNA to be more exposed to solution or constrains structure at a certain orientation that is more favorable to DMS reaction. In footprinting assays the analysis is mostly done qualitatively. However, ImageQuant software enables manual boxing of individual bands and provides side-by-side analysis of the bands of treated and untreated samples. The data can be normalized to increase accuracy of the data interpretation. Normalization can be done by two methods: by dividing the intensity of each band to the average of all of the measured bands in a given lane or dividing the intensity of each band to the reference point.

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Fig. 1. The principle of EMSA. The binding of protein to the DNA or RNA would increase their size and cause slow mobility of complexes in the native polyacrylamide gel

Fig. 2.

The proposed mechanism for DMS methylation and subsequent cleavage reaction

Fig. 3.

The As1411 G-quadruplex binding to human nucleolin protein. Samples contained 1×10^{-9} M DNA (5'-GGTGGTGGTGGTTGTGGTGGTGGTGG-3'); free ^{32}P -labeled As1411 G-quadruplex (**a**) and As1411G4/Nucleolin complex (**b**) resolved on a 4% w/v polyacrylamide gel cast and run in $0.5\times$ TBE buffer. The binding buffer contained 20 mM Tris (pH 7.6 at 4 °C), 200 mM KCl, 2 mM dithiothreitol, 0.15 mg/mL bovine serum albumin

Fig. 4.

(a) General schematic diagram of a DMS footprinting experiment for G-quadruplexes and protein-G-quadruplex complexes. DNA bases in the given sequence are color coded; Red = Guanine, Blue = Thymine, Green = Adenine. (b) Separation of species on a native PAGE gel. The binding of protein to the DNA would increase their size and cause slow mobility of complexes in the native polyacrylamide gel (c) Representative data for DMS chemical footprinting of G-quadruplex sequence in the presence of 140 mM K⁺ (lane 7), 0 mM K⁺ (lane 3) and in the presence of protein and 140 mM K⁺ (lane 8), in the presence of protein and 0 mM K⁺ (lane 4). Lanes 1, 2, 5, and 6 serve as control experiments in the absence of DMS

Table 1

Composition of binding reactions for the assay shown in Fig. 3

Components	a Volume (μ L)	b Volume (μ L)
10 nM 32 P DNAG4 in 20 mM Tris-HCl pH = 7.6 100 mM KCl	1	1
706 nM Protein in 20 mM Tris-HCl (pH = 7.4), 200 mM KCl, 2 mM EDTA, 0.15 mg/mL BSA and 2 mM DTT	–	8.5
Binding Buffer: 20 mM Tris-HCl (pH = 7.4), 200 mM KCl, 2 mM EDTA, 0.15 mg/mL BSA and 2 mM DTT	8.5	–
40% Glycerol	0.5	0.5
V_{total}	10	10