Electrophoretic Mobility Shift Assays using Infrared-Fluorescent DNA Probes

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ABSTRACT

Protein-DNA binding interactions are critical in several biological processes, especially the regulation of gene expression at the level of transcription initiation. An important technique for studying these interactions is the electrophoretic mobility shift assay (EMSA), whereby protein-DNA complexes are resolved on the basis of their mass:charge ratio using native polyacrylamide gel electrophoresis (nPAGE). Here we describe EMSA using PCR-generated, near infrared-fluorescent DNA probes, and IR fluorescence imaging to qualitatively and quantitatively study the interaction of transcriptional regulatory proteins from thermophilic organisms with different DNAs. Direct imaging of IR fluorophore-labeled DNA probes is advantageous because it provides high sensitivity (subnanomolar) without the need for intermediate staining steps or costly and problematic radiolabeled probes, thereby providing a more affordable and sensitive option to image protein-DNA on polyacrylamide gels by techniques such as EMSA.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Protocol development supported by grants from the NIH (1R15GM104833-01) and NSF (MCB-1714778).

MATERIALS

NAME	CATALOG #	VENDOR
Taq DNA Polymerase with Standard Taq Buffer - 2,000 units	M0273L	New England Biolabs

SAFETY WARNINGS

BEFORE STARTING

1. For the PCR synthesis of IR fluorophore-labeled, double-stranded DNA probes, a suitable 5′-end labeled primer needs to be obtained first. We routinely obtain 5′ IRDye® 700 or 800-labeled oligodeoxyribonucleotides from Integrated DNA Technologies (www.idtdna.com), 250 nmole or larger synthesis, HPLC purified. Such is paired with an unlabeled primer in a standard PCR reaction to yield a singly 5′ end-labeled, double-stranded DNA probe suitable for studying protein-DNA interactions by EMSA.
2. Reagent stocks needed are the same as those required for DNA analysis by native PAGE and IR fluorescence imaging. See https://doi.org/10.17504/protocols.io.mcyc2xw for details.

PCR synthesis of IR fluorophore-labeled DNA

1 The DNA probes used in our laboratory to study thermophile protein-DNA interactions are derived from the selection template DNA ST2R24, which was used in the identification of their preferred DNA-binding sites by the combinatorial approach REPSA. Sequence details may be found in the reference https://doi.org/10.1371/journal.pone.0159408. Such result in DNA probes of 60 bp or greater in length. While this protocol may also be used with shorter (20 to 30 bp) chemically synthesized DNA probes, the longer PCR-synthesized probes are more cost-effective when they use a common primer set. Also, we find that the longer DNA probes may be used at lower (subnanomolar) concentrations in binding reactions compared to their shorter counterparts. This may be due to the highly hydrophobic nature of the IRDye® labels, which become more significant on shorter length DNAs. A more detailed protocol describing the design and PCR synthesis of modular IR fluorescent DNA probes, which may be used in EMSA, may be found at https://doi.org/10.17504/protocols.io.wfjfbkn.

2 Following manufacturer's guidelines (https://doi.org/10.17504/protocols.io.ch7t9m), assemble a 25 µL PCR reaction containing: 1× NEB Standard Taq Reaction Buffer, 200 µM dNTPs, 100 nM each 5′ IRDye® 700 end-labeled primer IRD7_ST2R and unlabeled primer ST2L, 2 ng

ST2-derived template DNA, and 0.625 units *Taq* DNA polymerase in a 0.2 mL thin-wall PCR tube on ice. Mix thoroughly by repeated pipetting.

3 Program thermal cycler (Bio-Rad C1000 Touch™) for 20 cycles (PCR_20). For our ST2-derived DNAs, a run consists of the following steps: with lid heated to 105 °C, (1) 95 °C, 2:00 min, (2) 95 °C, 0:30 min, (3) 54 °C, 0.30 min, (4) 68 °C, 1:00 min, (5) Go to Step 2, 19×, (6) 68 °C, 2:00 min, and (7) 4 °C, ∞. Typically, a PCR_20 run takes approximately 60 min to complete.

4 After completion, PCR products can be quantitated directly using a Qubit™dsDNA HS Assay Kit and Quibit™ fluorometer (https://doi.org/10.17504/protocols.io.k5pcy5n). Similarly, PCR products may be qualitatively analyzed by native PAGE and IR fluorescence imaging (https://doi.org/10.17504/protocols.io.mcyc2xw).

Protein-DNA binding reaction

5 Depending on the variables being investigated, it may be necessary to make a serial dilution of thermophile transcriptional regulatory proteins (tTRPs). Routinely, 10-fold serial dilutions of tTRPs are made in a buffer (BLI100) containing 20 mM Tris-Cl [pH 7.8], 100 mM NaCl, and 0.05% Tween 20 nonionic detergent. This can efficiently be done using a 72-well Nunc™ MicroWell™ MiniTray, whose wells can hold up to 12 µL each. The tray is kept on ice when preparing dilutions to ensure maximal protein stability and to minimize evaporative losses. Note: tray may be reused, so long as previously used wells are marked.

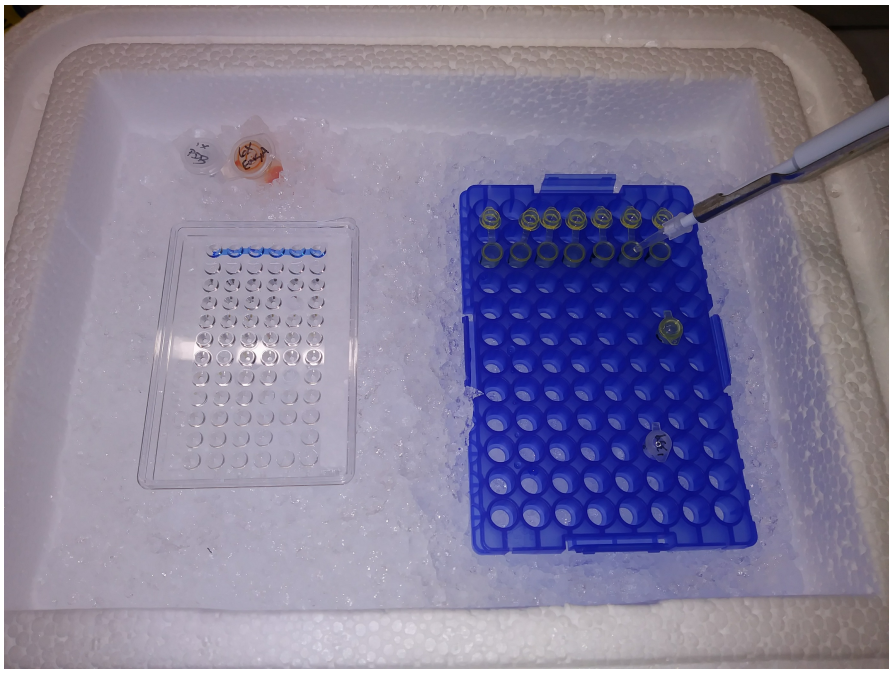

Figure 1. Ice bath set up for serially diluting DNA-binding proteins and assembling EMSA binding reactions. Nunc™ MiniTray™ is shown on left.

6 Protein-DNA binding reactions are assembled in 0.2 mL PCR tubes kept on ice. 4 µL of DNA probe (1.25 nM) in BLI100 is aliquoted to each tube. Afterward, 1 µL of a tTRP dilution is added and mixed by repeated pipetting. Tubes are then incubated in a mini dry bath or thermal cycler at the desired temperature for the durations chosen. We routinely incubate tTRPs for 20 min at 55 °C to effect equilibrium binding. Binding reactions may be terminated by transferring tubes to a reduced temperature (either room temperature or ice).

EMSA analysis of protein-DNA complexes

7 Thermophile TRP-DNA complexes are resolved at room temperature by native PAGE. Typically these are performed using hand cast 9.5%:0.5% acrylamide:bisacrylamide vertical minigels (10 cm) containing moderate ionic strength buffer (½× TBE). A description of their preparation and use may be found at https://doi.org/10.17504/protocols.io.mcyc2xw. Note: for certain tTRP-DNA complexes it is necessary to use a lower percentage polyacrylamide gel (6% to 8%) to observe appreciable complex entry into the gel. Such is not problematic as these gels are IR fluorescence imaged directly while remaining between plates and do not have to be physically manipulated.

8 Before loading onto a polyacrylamide gel, 2 µL EMSA Loading Dye (20% glucose, 0.9% Orange G) is added to each tube and mixed thoroughly by repeated tapping. Reaction mixtures are consolidated by brief centrifugation in a minifuge. 6.5 µL from each tube is loaded sequentially into a corresponding gel well filled with ½× TBE running buffer. This can be achieved using standard 0.1 – 10 µL tips and a corresponding pipettor. Dispense slowly to optimize layering of the sample at the bottom of the well. Do not introduce air into the tip when aspirating a

sample, as this will interfere with dispensing.

9 After about 5 min, to allow all samples to settle in the gel wells fully, the cover is placed on the electrophoresis chamber. Electrophoresis is initiated at a reduced voltage (50 V, voltage limited) for 5 min, to allow optimal stacking of the protein-DNA complexes and their efficient entry into the gel. Electrophoresis is continued at a standard voltage (10 V/cm, 100 V) until the Orange G band approaches the bottom of the gel.

10 Once the run is completed, the tank cover is removed, running buffer from both chambers drained, and the gel removed from the electrode assembly. **Do not remove the gel from its glass plates** - IR fluorescence imaging can be performed directed on the gel while sandwiched. Briefly rinse gel/plates sandwich and blot dry before placing on the imager. Note: if you do not plan to image your gel immediately, it is best to store it at 4 ºC, to minimize band diffusion. We have obtained adequate images form gels stored several hours under these conditions.

IR fluorescence imaging and quantitation

11 To perform infrared (IR) fluorescence imaging of PAGE minigels, we use a LI-COR Odyssey imager and LI-COR Image Studio™ 5.2 software. A detailed protocol for IR fluorescence imaging may be found at https://doi.org/10.17504/protocols.io.mcyc2xw. The primary difference between this standard protocol and imaging EMSA gels is that Preview and Scan are imaged at far higher Intensity levels (7 or more), given the small quantity of IR-labeled DNA present in each binding reaction. Note: as quantitation of EMSA data can be quite important, care must be taken to ensure no saturated pixels (white) are present in any of the bands to be quantitated. Such yield an infinity value and interfere with the analysis. Rescan at a lower Intensity setting as needed.

12 Quantitation of IR fluorescent DNA in each band follows the steps detailed in protocol https://doi.org/10.17504/protocols.io.mcyc2xw. Note that a Manual Find may be needed to identify and quantify all bands desired. The goal is to identify and quantitate both the unbound DNA band and the protein-DNA complex band for each binding reaction. An example of an imaged and quantitated EMSA experiment is shown in Figure 1.

Figure 1. Quantitation of FadR binding to the Mutant 3 sequence. Shown are two-fold dilutions of FadR from 64 to 2 nM (Lanes 1- 6) and a DNA alone control (Lane 7). FadR-DNA complexes are on top and unbound DNA below. Quantitation values determined using a LICOR Odyssey imager are shown to the lower left of each band. Experimental details may be found at https://doi.org/10.1371/journal.pone.0184796.

Determining protein-DNA dissociation constants

13 It is possible to use quantitative EMSA data to determine the dissociation constant (K_D) for a protein-DNA complex. Values that need to be known are the total concentrations of DNA probe and protein present in the binding reaction. For each reaction, the imaging data provide the relative amounts of DNA present in each species: unbound DNA and protein-DNA complex, with the sum of these values constituting the total DNA recovered from the binding reaction. Thus, one can use a standard binding equilibrium equation:

$$K_D = [DNA_{free}] * [Protein_{free}] / [Protein{\cdot}DNA_{complex}]$$

where,

$$[DNA_{free}] = [DNA_{total}] * \text{fraction unbound}$$

$$[Protein{\cdot}DNA_{complex}] = [DNA_{total}] * \text{fraction protein-DNA complex}$$

$$[Protein_{free}] = [Protein_{total}] - [Protein{\cdot}DNA_{complex}]$$

Using the data from the above example, where the total DNA was 1 nM in each reaction, and choosing the protein concentration that yielded approximately a 50:50 distribution between unbound and protein-DNA complex species (Lane 3, 16 nM protein], we find [DNA_{free}] = 0.42 nM, [$Protein{\cdot}DNA_{complex}$] = 0.58 nM, and [$Protein_{free}$] = 15.4 nM. These values yield a calculated K_D = 11 nM. Note that the 50:50 distribution lane was chosen to make the K_D calculation given the strong signals present in both DNA species.