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Electrophoretic Mobility Shift Assays

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Abstract

Experimental demonstration of regulatory protein interactions with the sequences upstream of potential target genes is an important element in gene expression studies. These experiments termed electrophoretic mobility shift assays (EMSAs) provide valuable insight into the mechanism of action of transcription factors. EMSAs combined with downstream applications such as transcriptional analysis help uncover precisely how regulatory proteins control target gene expression. This chapter comprises a guideline for expression and purification of recombinant transcription factor proteins followed by a detailed protocol for EMSAs.

Keywords: Electrophoretic mobility shift assay, Transcription regulator, Promoter, Gene regulation

1 Introduction

Transcription factors are regulatory proteins that bind to specific DNA sequence motifs upstream of genes in order to modulate their expression. In molecular biology EMSAs, also called gel retardation assays, are employed to ascertain if a protein of interest or several proteins bind to a specific DNA fragment. The method involves investigating whether a labeled DNA probe can interact specifically with a purified recombinant protein under in vitro conditions. A specific interaction between the DNA fragment and recombinant protein will generate a complex with a higher molecular weight than the labeled DNA fragment alone. Electrophoretic migration of a protein–DNA complex through a polyacrylamide gel will be slower or "retarded" compared to migration of DNA fragment alone. In order to determine if the protein–DNA complexes are specific, an excess of unlabeled specific DNA is added to the reaction, which competes with the labeled DNA fragment resulting in reduced levels of interaction between the labeled DNA fragment and the protein. In contrast, the addition of excess nonspecific DNA will have no effect on the specific interaction between a protein and a labeled DNA fragment. If the protein–DNA complex is nonspecific, addition of excess unlabeled specific or nonspecific DNA will reduce the interaction of the complex (Fig. 1).

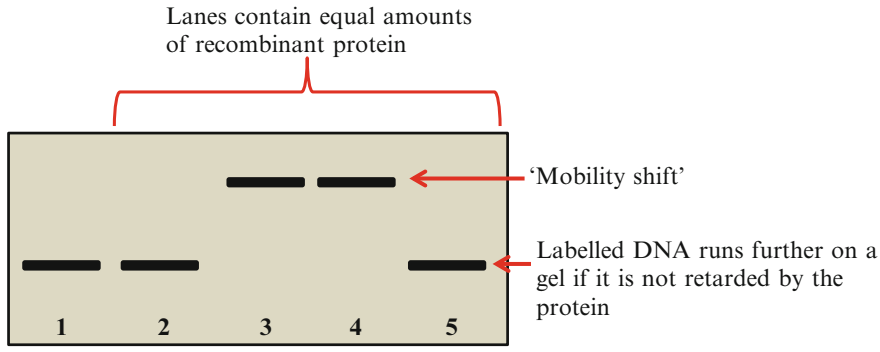

Fig. 1 Schematic representing an EMSA. Only labeled DNA probes are visible in the EMSA. They run slower if they are bound by the recombinant protein. *Lane 1* is a negative control containing a labeled DNA probe of interest (specific DNA) and no protein. *Lane 2* contains a labeled nonspecific DNA probe predicted not to interact to the protein of interest. *Lane 3* contains a labeled specific DNA probe. *Lane 4* contains the labeled specific DNA probe and 10× nonspecific unlabeled competitor DNA. *Lane 5* contains a labeled specific DNA probe and 10× specific unlabeled competitor DNA. *Lanes 2–5* contain the same amount of recombinant protein. An excess of unlabeled competitor DNA disrupts the gel shift only if it is specific

2 Materials

2.1 Protein Purification and Western Blot

1. Overnight expression autoinduction media: EMDmillipore
2. Lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0
3. Lysozyme (50 mg/ml) stock
4. 2× SDS reducing sample buffer: 100 mM Tris–HCl (pH 6.8), 4 % (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade), 0.2 % (w/v) bromophenol blue, 20 % (v/v) glycerol, 200 mM DTT (dithiothreitol). Store the SDS gel-loading buffer without DTT at room temperature. Add DTT from a 1 M stock just before the buffer is used. 200 mM β-mercaptoethanol can be used instead of DTT.
5. 4–12 % Bis-Tris pre-cast mini gel, 10 well, 1.5 mm thick
6. Immobilon P PVDF membrane 0.45 μm
7. 100 % methanol
8. Denionized water (dH_2O)
9. NuPAGE MES SDS Running Buffer (20×): Invitrogen or similar buffer
10. NuPAGE 20× Transfer Buffer: Invitrogen or similar buffer
11. Mini cell electrophoresis chamber
12. Transfer Module
13. His tag mouse monoclonal antibody-

14. Chemiluminescent Kit, anti-mouse
15. Ni-NTA Agarose: Invitrogen
16. Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0
17. Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0
18. Bradford Assay reagents
19. *E. coli* strain carrying expression plasmid

2.2 Purification of a Biotinylated DNA Probe

1. 10 % TBE precast gel
2. TE buffer: 10 mM Tris pH 8.0, 1 mM EDTA
3. Ethidium bromide
4. 70 % Ethanol
5. 3 M sodium acetate
6. Spectrophotometer capable of quantifying DNA

2.3 EMSA

1. Binding buffer: 100 mM Tris pH 7.5, 500 mM KCl, 10 mM DTT, 5 % glycerol, 5 mM $MgCl_2$, 0.2 μg poly-(dI-dC)
2. Precast 6 % DNA Retardation Gels
3. Biodyne-B nylon membrane: Pall Corporation, AGB
4. Chemiluminescent Electrophoretic Mobility Shift Assay Kit: (we prefer the LightShift kit from Thermo Scientific).

3 Methods

3.1 Tag Selection for Recombinant Protein

Tags can interfere with protein function so it is important to consider their size, application, and other properties before deciding which tag to fuse to your protein of interest. Table 1 summarizes the applications and size of the following tags GST, His, HA, myc, FLAG, MBP. Small tags such as HA, His, FLAG, and myc are ~1 kDa in size and are therefore less likely to interfere with protein function. These tags can be added to either the N- or C-terminal of the protein (Fig. 2) (*see* **Note 1**). Another advantage of choosing a small tag is that the tag sequence can be added to your cloning primer to facilitate amplification of a tagged version of your gene of interest. The PCR product containing the tagged-gene can then be subcloned into an expression vector of your choice rather than purchasing a recombinant expression system required when using the larger MBP or GST tags. However, some proteins, such as AraC-type proteins are insoluble and notoriously difficult to purify. Addition of an MBP tag can facilitate the purification of such insoluble proteins [1].

Table 1
Tags commonly added to N- or C- terminal proteins in order to facilitate downstream applications such as protein purification, pull-down assays, western blots

Name	Sequence	MW (kDa)	Notes on application	Notes on construction
FLAG	DYKDDDDK	1	Pull down assays Very good for western blot	Can add sequence to primer
His (polyhistidine)	HHHHHH	1	Western blot, pull down ssays Very good for purification	Can add sequence to primer
MBP (maltose binding protein)		42	Good tag for purification of typically insoluble proteins	Need a plasmid system
GST (Glutathione *S*-transferase)		26	Pull down assays, protein purification, western blot	Need a plasmid system
HA (human influenza hemagglutinin)	YPYDVPDYA	1	Western blot, purification, pull down assays	Can add sequence to primer
Myc	EQKLISEEDL	1.2	Western blot, protein purification	Can add sequence to primer

Sequence information is shown for small tags

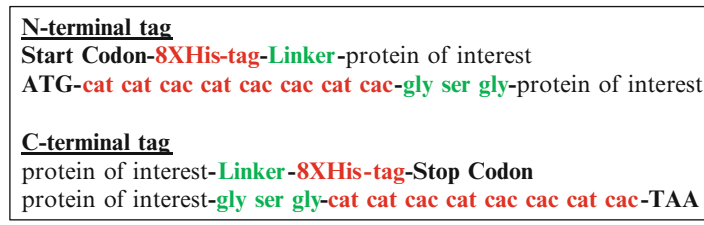

Fig. 2 Schematic representing the orientation 8×Histidine tags (DNA sequence in *red*) and linker (amino acid sequence in *green*) on the N- and C- terminal of a gene of interest. If the tag is added to the N-terminal of the gene, the start codon must be included in front of the tag, followed by a linker and the sequence of the gene (ensuring the codons are in frame). Similarly, when a tag is added to the C-terminal of a gene, the gene codons must run in frame with the linker, followed by the tag and then the stop codon

The His-tag will be described in this chapter because the His purification system is relatively straightforward and available from multiple commercial suppliers. When designing oligonucleotide primers to amplify a gene of interest, a linker can be added between the gene itself and the His-tag. For example, linkers encoding gly-gly-gly or gly-ser-gly can be used (Fig. 2). This helps to reduce the

possibility of the tag affecting the function of the recombinant protein (*see* **Note 2**). For best results, the tagged gene should be cloned into an inducible expression vector, for example under the control of an IPTG-inducible promoter and transformed into an cloning *E. coli* strain such as DH5α [2] (*see* **Note 3**).

3.2 Confirming Expression of Recombinant Protein in E. coli

It is important to confirm the nucleotide sequence of the recombinant expression construct to ensure that the gene and its tag will be expressed in frame before transformation into a strain of *E. coli* suitable for protein expression, such as BL21(DE3) (*see* **Note 4**) [3].

Once the expression strain has been selected, a western blot can be performed to verify the expression level, size, and quality of the recombinant protein. A western blot can also provide insight into whether the recombinant protein is present in the soluble fraction under various growth and induction conditions (*see* **Note 5**). Many types of expression systems are commercially available; an IPTG-inducible promoter will be used as an example here.

3.3 Pilot Expression Protocol: Preparation of Cell Extracts for Western Blot

1. Inoculate 10 ml overnight expression autoinduction media containing selection antibiotic with a single colony of the *E. coli* strain harboring either the IPTG-inducible recombinant expression plasmid or the empty plasmid.
2. Incubate overnight (approximately 16 h) at 37 °C with good aeration.
3. Pellet cells by centrifugation at 8800 × *g* for 10 min.
4. Decant supernatant and resuspend cell pellet in 1 ml lysis buffer containing 1 mg/ml lysozyme and incubate on ice for 20 min with gentle agitation.
5. Sonicate cells in an ice bath for 2 min (15 s bursts with 30 s intervals). Samples must be kept cold at all times. It is also important to ensure that no frothing occurs during sonication, which may result in protein denaturation.
6. Separate the soluble and insoluble fractions of the cell extracts by centrifugation at 16,000 × *g* for 30 min at 4 °C.
7. Aliquot the soluble fraction into a clean labeled tube on ice and resuspend the insoluble fraction in 1 ml lysis buffer. Remaining particulate insoluble material is likely not protein.
8. Add 30 µl aliquots of the soluble or insoluble fractions to 30 µl 2× SDS reducing sample buffer in a PCR tube and heat to 95 °C for 5 min.
9. Centrifuge the samples at 16,000 × *g* for 2 min to pellet cell debris.
10. Load the supernatant (10–30 µl aliquots) on a 4–12 % Bis-Tris acrylamide gel and separate by electrophoresis at 180 V for approximately 45 min.

11. During electrophoresis, prepare the PVDF membrane and filter paper (*see* **Note 6**).
12. Soak the sponges and filter paper in 1× transfer buffer until required.
13. Submerge the membrane in 100 % methanol for 10 s (*see* **Note 7**) and then transfer into dH_2O using a plastic forceps (*see* **Note 8**).
14. Transfer the separated proteins in the polyacrylamide gel to the PVDF membrane at 30 V for 1 h using an electroblotting apparatus.
15. Immuno-detection is performed using commercial antibodies specific for the tag fused to the expressed protein. For example, an anti-polyhistidine antibody (or antibody against an alternative tag fused to the protein of interest) is used as a primary antibody. His-tag antibodies can nonspecifically react with *E. coli* proteins containing histidine residues and it is therefore important to include a control *E. coli* strain carrying only the empty expression vector to distinguish between specific and nonspecific bands on the Western blot (*see* **Note 9**).
16. Confirm that the recombinant protein is in the soluble fraction. If it is in the soluble fraction, then further optimization is not needed before purification. If the recombinant protein is only detectable in the insoluble fraction, the conditions need to be optimized to increase solubility (*see* **Note 10**).

3.4 Purification of Recombinant Protein

When the protein expression conditions have been optimized, culture volumes can be scaled up before proceeding to the protein purification procedure.

1. Dilute the soluble recombinant His-tagged protein 1/50 in cold lysis buffer. For example, 1 ml of soluble protein is added to 50 ml cold lysis buffer and stored on ice.
2. Add 1 ml of Ni-NTA agarose (slurry) into the soluble protein and allow to rotate slowly on a shaker at 4 °C for 1 h or overnight. This allows the His-tagged recombinant protein to bind to the nickel in the agarose.
3. Prepare the column by passing 1 column volume of wash buffer through the column.
4. Cap the column and add the protein-slurry to the column.
5. Incubate for at least 15 min until the agarose settles to the bottom of the column.
6. Uncap the column and allow to flow through, some of which can be retained for analysis by Western blot.
7. Wash the column resin with 5 column volumes of wash buffer at a speed of 1 ml/min using a peristaltic pump.

8. Recap the column and add 500 µl of elution buffer to the resin and incubate for 5 min.
9. Uncap the column and collect the eluate from the column in a micro-centrifuge tube.
10. Repeat steps 8 and 9 *four* more times.
11. Collect purified elution fractions 1–5 in 500 µl aliquots and analyze on a 4–12 % Bis-Tris acrylamide gel. Determine the protein concentrations by Bradford Assay.
12. Protein samples can be stored at 4 °C for use within 1 week or in the longer term at −80 °C in 50 µl aliquots.

3.5 Preparation of a Biotinylated DNA Probe

A DNA probe can be labeled radioactively, fluorescently, or with biotin. Radioactively labeled probes are the most sensitive but are less frequently used due to the cumbersome safety requirements. Biotin-labeled oligonucleotide primers are routinely available from commercial suppliers and when used for amplification, the DNA fragments generate biotinylated DNA probes. The probe chosen can comprise only the upstream regulatory sequences for a given gene/operon or extended intergenic regions (*see* **Note 11**). Although the probe can be extracted and purified from an agarose gel, for best results purification should be performed from a non-denaturing 10 % polyacrylamide gel using the following technique.

1. Mix the probe PCR with 6× loading dye and load into a 10 % polyacrylamide gene TBE gel.
2. Perform electrophoresis at 100 V for 65 min in 1× TBE buffer.
3. Following electrophoresis, remove the gel and stain with ethidium bromide.
4. Visualize the probe very briefly under UV light and extract from the gel using a scalpel (*see* **Note 12**).
5. Cut a small hole in the bottom of a 1.5 ml micro-centrifuge tube and place in a 2 ml microcentrifuge tube. Then place the acrylamide "slice" in the 1.5 ml tube, which sits into the 2 ml microcentrifuge tube.
6. Centrifuge the tubes for 1 min at 16,000 × *g*. This macerates the gel "slice" through the hole as it passes into the 2 ml microcentrifuge tube for collection.
7. Mix 200 µl of TE buffer with the macerated acrylamide and incubate at 37 °C overnight and then centrifuge at 16,000 × *g* for 5 min.
8. Decant the supernatant into a clean micro-centrifuge tube.
9. Add another 200 µl of TE buffer to the acrylamide, vortex, centrifuge again at 16,000 × *g* for 2 min and collect the supernatant.

10. Pool both supernatant fractions and centrifuge at 16,000 × *g* for a further 2 min.
11. Harvest the supernatants, leaving the last 10 μl to avoid recovery of any residual acrylamide.
12. Precipitate the DNA with 1 ml 70 % EtOH containing 40 μl of 3 M sodium acetate and centrifuge at 16,000 × *g* for 10 min.
13. Discard the supernatant and wash the DNA pellet with 200 μl 70 % EtOH then centrifuge at 16,000 × *g* for 10 min.
14. Discard the supernatant again and allow the DNA pellet to air dry at room temperature for 10–15 min.
15. Resuspend the DNA pellet in 30 μl TE buffer and determine the DNA concentration using a spectrophotometer.

3.6 EMSA

To investigate if the recombinant protein binds to the DNA sequence of interest, a pilot reaction should be performed with the purified recombinant protein, purified biotinylated DNA probe, and binding buffer. If a shift is observed in this pilot experiment, the next step is to repeat the reaction with doubling dilutions of the purified protein in order to determine the minimum protein required to shift a known concentration of DNA (*see* **Note 13**).

The protocol for carrying out the EMSA is as follows:

1. Prepare a 20 μl binding reaction containing a minimum of 10 μl binding buffer, 2–10 ng biotinylated probe and the purified protein.
2. Incubate the reaction at room temperature for 20 min before loading directly onto a 6 % DNA Retardation Gel and electrophoresing at 100 V for 65 min. These gels contain 0.5× TBE which provides an environment of low-ionic strength to promote protein–DNA interaction (*see* **Notes 14** and **15**).
3. Transfer the DNA or DNA–protein complex onto a Biodyne-B nylon membrane (Pall Corporation, AGB) at 4 °C in prechilled 0.5 % TBE at 80 V for 60 min.
4. Cross-link the membrane under UV light for 10 min (*see* **Note 16**).
5. Detection of the bands representing labeled probes or interaction complexes can be performed using various commercial chemiluminescent imaging kits (such as the LightShift chemiluminescent electrophoretic mobility shift assay kit) and visualized using a chemiluminescent imager (*see* **Notes 17** and **18**).

3.7 Determination of Protein–DNA Interaction Specificity Using Competitor DNA

To determine if the observed protein–DNA interaction is specific, competitor DNA is added to the reaction. Generally competitor DNA is unlabeled DNA that is either specific (i.e. identical to the labeled probe) or nonspecific (a DNA fragment that is not predicted to interact with the protein). Competitor DNA is added to

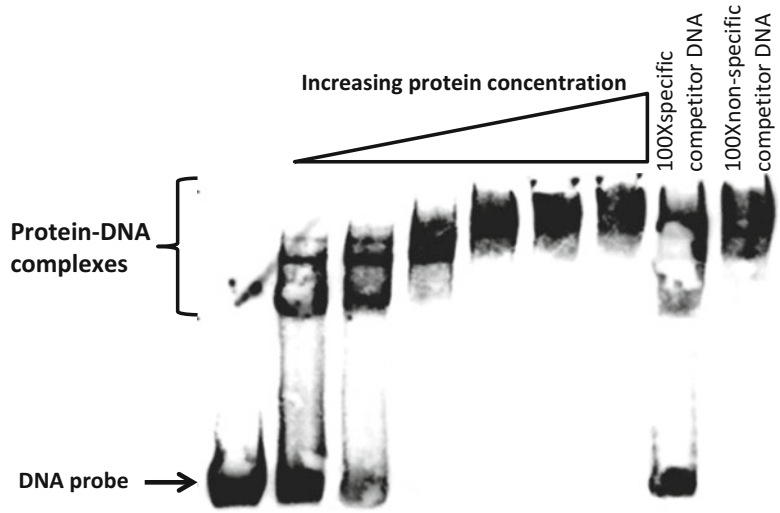

Fig. 3 A standard amount of biotinylated DNA probe is added to each well. *Lane 1* contains probe and no protein. *Lanes 2–7* include increasing amounts of recombinant protein. *Lane 8* and *9* also contain 100× specific and nonspecific competitor DNA respectively

the binding reaction at a final concentration of 1×, 10×, and 100× the concentration of the labeled probe. An excess of specific competitor DNA but not nonspecific competitor DNA should disrupt the interaction of a protein bound to its target probe (Fig. 3) [4].

4 Notes

1. N- or C-terminal tags can interfere with protein function. Therefore, it is recommended that both recombinant proteins (an N- terminal and a C-terminal version) are tested in EMSAs.
2. A minimum of five histidine residues are required for purification of tagged proteins, although this can be increased to eight residues to facilitate an improved Western blot signal using anti-polyhistidine antibodies.
3. This strain contains several mutations to aid the cloning procedure: *recA1* increases insert stability, *endA1* increases plasmid yield, *lacZΔM15* allows blue/white screening. Blue/white screening is a useful tool to identify candidate recombinant clones. β-galactosidase is an enzyme encoded by the *lacZ* gene. When X-gal, an analog of lactose, is added to the growth media, β-galactosidase breaks it down producing a blue 5-bromo-4-chloro-indoxyl pigment which is visible in the *E. coli* colony. However, strains carrying the *lacZΔM15* mutation are deficient in many N-terminal residues of *lacZ* gene [5]. Strains

carrying this mutation are unable to produce the blue break down product when X-gal is added to the media unless transformed with a plasmid expressing a complementing fragment of *lacZ* in a process called α-complementation [6]. A multiple cloning site within the *lacZ* fragment of vectors pUC19 or pBluescript facilitates blue/white screening [7]. During cloning, the *lacZ* fragment will be disrupted and will no longer complement the chromosomally encoded *lacZ* gene causing a white colony to form. Therefore, blue colonies express intact LacZα which breaks down X-gal and do not contain an insert. White colonies however, have an interrupted LacZα due to the presence of an insert. White colonies can be streaked on media containing selective antibiotic for further testing.

4. This strain has mutations in the *lon* and *ompT* genes which encode proteases that can affect protein yield [8]. DE3 indicates that the host strain is a lysogen of λDE3 phage and encodes a chromosomal copy of T7 RNA polymerase. This polymerase gene is under control of the *lacUV5* promoter, facilitating IPTG-induced expression of recombinant genes cloned in pET vectors [9]. There are many commercial strains of *E. coli* harboring various genetic mutations and/or plasmids which can be tested for optimal expression of various recombinant proteins. For example, expression of recombinant proteins that contain rare codons not commonly expressed in *E. coli*, such as proline, can be achieved using strain Rosetta(DE3) pLysS (MerckMillipore). This is a BL21 derivative harboring a plasmid which supplies the tRNAs for rare *E. coli* codons AGG, AGA, AUA, CUA, CCC, and GGA. In addition, there are several *E. coli* strains specifically engineered for expressing toxic proteins [10].
5. Induction of the protein of interest, for example from an IPTG-inducible system generally necessitates optimization. The recombinant *E. coli* strain can be grown in standard media such as Luria-Bertani and inducer added as the cells enter exponential phase. However, this process can require significant optimization to determine the optimal time of induction, length of induction, and concentration of IPTG. It is best to avoid high concentrations of IPTG as this can be associated with production of recombinant protein mainly in the insoluble fraction. As an alternative to IPTG, a mixture of glucose and lactose can be added to buffered media and expression of the recombinant protein allowed to proceed overnight without the need to optimize induction times. In such cultures *E. coli* first utilizes the glucose which helps to repress the expression of recombinant protein. As the glucose is exhausted, the *E. coli* will begin to simultaneously utilize the lactose and express the recombinant protein. Media of this type can be prepared in the laboratory or purchased commercially, for example overnight

expression (OnEx) autoinduction media from EMDmillipore can prove to be very helpful for protein expression using pET plasmids. Cultures grown in overnight expression media should be grown with good aeration to ensure maximal yield of recombinant protein.

6. The PVDF membrane required will differ depending on the size of the recombinant protein. For example, Immobilon P membrane^{SQ} is best suited for proteins less than 10 kDa while the Immobilon P membrane is more suited to higher molecular weight proteins. Six sponges are required for the western blot. One sponge is used to measure the size of filter paper required. Two pieces of filter paper are cut slightly smaller than the sponge, and the PVDF membrane cut slightly smaller than the filter paper. The top left corner of the membrane is cut to indicate "lane 1."
7. The membrane is very sensitive to handling especially if it will be subsequently exposed to X-ray film. To avoid observing black marks and creases prior to X-ray film exposure, always use a plastic forceps in the top left corner of the membrane (above lane 1).
8. Following submersion of the membrane in methanol, the membrane is transferred to deionized H_2O. The membrane will rise to the top of the water dish and is at risk of drying out. To avoid this, vigorously shake the water dish from side to side until the membrane sinks to the bottom. The dish can then be placed on a shaker table for all remaining wash steps.
9. Low concentrations of imidazole present in the lysis buffer should reduce nonspecific reaction of antibodies with histidine residues in native *E. coli* proteins.
10. Reducing the growth temperature from 37 to 30 °C or even room temperature can increase the solubility of the recombinant protein
11. It is also possible to incorporate base pair changes into the DNA probe to investigate if certain sequences are crucial for protein binding.
12. UV light introduces mutations into DNA. If used to visualize ethidium bromide stained DNA, exposure to UV light must be very brief. There are alternatives to ethidium bromide that do not require UV visualization if required [11, 12].
13. Many factors affect the specificity and strength of the protein–DNA interaction, including the ionic strength and pH of the binding buffer; the presence or absence of divalent cations such as Zn^{2+} and Mg^{2+}, nonionic detergents and carrier proteins such as bovine serum albumin. These factors need to be considered and can be determined empirically by manipulating

the binding buffer. There may also be information available in the literature on the DNA-binding requirements of specific proteins or families of proteins with shared structural characteristics.

14. Bromophenol blue can interfere with the reaction and it is recommended that loading dye be avoided. Glycerol is generally a constituent of the binding reaction and can be used to load the binding reaction into the wells of polyacrylamide gels.
15. EMSAs must be electrophoresed under nondenaturing conditions. SDS will disrupt protein–DNA interactions so it is important to use a protein electrophoresis tank that is never exposed to SDS-containing gels.
16. Following cross-linking, the membrane can be stored safely in a cool, dry place until detection is performed.
17. For increased sensitivity, the membrane can be exposed to X-Ray film and developed in a dark room.
18. If a recombinant protein does not bind to a labeled DNA fragment under the above conditions, it does not necessarily mean that the protein does not interact with the DNA sequence. The temperature and time of the binding reaction can be optimized or it is also possible that the protein requires modification in vivo before it binds a segment of DNA. For example, EMSAs revealed that the phosphorylated MgrA transcriptional regulator from *S. aureus* binds to the *norB* promoter but not the *norA* promoter, whereas dephosphorylated MgrA bound to the *norA* promoter and not *norB* [13].

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