

# Electrophoretic mobility shift assay (EMSA) for detecting protein–nucleic acid interactions

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The gel electrophoresis mobility shift assay (EMSA) is used to detect protein complexes with nucleic acids. It is the core technology underlying a wide range of qualitative and quantitative analyses for the characterization of interacting systems. In the classical assay, solutions of protein and nucleic acid are combined and the resulting mixtures are subjected to electrophoresis under native conditions through polyacrylamide or agarose gel. After electrophoresis, the distribution of species containing nucleic acid is determined, usually by autoradiography of <sup>32</sup>P-labeled nucleic acid. In general, protein–nucleic acid complexes migrate more slowly than the corresponding free nucleic acid. In this protocol, we identify the most important factors that determine the stabilities and electrophoretic mobilities of complexes under assay conditions. A representative protocol is provided and commonly used variants are discussed. Expected outcomes are briefly described. References to extensions of the method and a troubleshooting guide are provided.

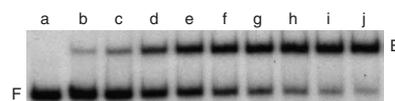
## INTRODUCTION

The electrophoresis mobility shift assay (EMSA) is a rapid and sensitive method to detect protein–nucleic acid interactions<sup>1–6</sup>. It is based on the observation that the electrophoretic mobility of a protein–nucleic acid complex is typically less than that of the free nucleic acid (Fig. 1). The current, widely-used assay differs little from that originally described by Fried and Crothers<sup>7</sup> and Garner and Revzin<sup>8</sup>; although precursors to the technique can be found in the earlier literature<sup>9–11</sup>. Mobility-shift assays are often used for qualitative purposes; although under appropriate conditions they can provide quantitative data for the determination of binding stoichiometries, affinities and kinetics<sup>3,6,12</sup>. Methods used in performing the assay differ for each purpose, and a large number of variants have been described in the literature (see Table 1).

## Advantages and limitations of EMSA

The mobility shift assay has a number of strengths. The basic technique is simple to perform, yet it is robust enough to accommodate a wide range of binding conditions (see Table 2 for representative ranges). Using radioisotope-labeled nucleic acids, the assay is highly sensitive, allowing the assays to be performed with small protein and nucleic acid concentrations (0.1 nM or less) and small (20 μl or less) sample volumes. When such high sensitivity is not needed, variants of the assay using fluorescence, chemiluminescence and immunohistochemical detection are also available<sup>13–17</sup>. A wide range of nucleic acid sizes (ranging from short oligonucleotides to several thousand nucleotides (nt) or base pairs (bp)<sup>18,19</sup>) and structures (single-stranded, duplex, triplex<sup>20</sup> and quadruplex<sup>21</sup> nucleic acids as well as small circular DNAs<sup>22</sup>) are compatible with the assay. Under favorable conditions, the distribution of proteins between several nucleic acid molecules can be monitored within a single solution<sup>18,23</sup>, as can the presence of complexes differing in protein stoichiometry and/or binding site distribution<sup>7,24</sup>. Proteins ranging in size from small oligopeptides to transcription complexes with  $M_r \geq 10^6$  can give useful mobility shifts<sup>25,26</sup> and the assay works well with both highly purified proteins and crude cell extracts<sup>27</sup>. These capabilities account in large part for the continuing popularity of the assay.

On the other hand, the EMSA is not without limitations. One theme of this protocol is the identification of potential problems and the suggestion of strategies that avoid or mitigate the most severe. The TROUBLESHOOTING section contains a guide for troubleshooting the most common problems that the authors have encountered. Perhaps the most important limitation is that samples are not at chemical equilibrium during the electrophoresis step. Rapid dissociation during electrophoresis can prevent detection of complexes, while even slow dissociation can result in underestimation of binding density. On the other hand, many complexes are significantly more stable in the gel than they are in free solution<sup>28–30</sup>; when this is the case, short electrophoresis times allow the resolution of patterns that closely approximate the distributions of species present in the samples at the start of electrophoresis. A second limitation is that the electrophoretic mobility of a protein–nucleic acid complex depends on many factors other than the size of the protein. Thus, an observed mobility shift does not provide a straightforward measure of the molecular weights or identities of proteins that are present in the complex<sup>12</sup>. The electrophoretic ‘supershift’ assay and assays that combine EMSA with western blotting or mass spectroscopy have been devised to allow identification of nucleic acid-associated proteins (summarized in Table 1), while a range of EMSA-based and non-EMSA methods



**Figure 1** | Titration of a 214-bp *lac* promoter DNA fragment with *Escherichia coli* CAP protein. Sample compositions are given in Table 3. The complex consists of a single molecule of CAP bound predominantly to the highest affinity CAP site in the *lac* promoter (CAP site 1<sup>49,66</sup>). Electrophoresis was carried out with a 10% wt/vol polyacrylamide (75:1 acrylamide:bisacrylamide) gel, cast and run in the Tris–acetate–EDTA buffer described in the protocol shown in Table 4. cAMP (final concentration 20 μM) was added to the electrophoresis running buffer. Pre-electrophoresis ensured that the cAMP was distributed throughout the gel before the samples were loaded. Band designations: F, free DNA; B, bound DNA (i.e., protein–DNA complex).

**TABLE 1** | Electrophoretic mobility shift assay (EMSA) variants.

| Method and reference(s)  | Purpose   |
|--|---|
| <b>1D methods</b>  |   |
| Time course <sup>7,39</sup>  | Measurement of association and/or dissociation kinetics   |
| Double-label assays <sup>31,32</sup>   | Determination of binding stoichiometry  |
| Continuous variation <sup>33,91</sup>  | Determination of binding stoichiometry  |
| Circular permutation <sup>92</sup>   | Detection of DNA bending  |
| Phased bends analysis <sup>79</sup>  | Determination of direction of a protein-induced bend with respect to a standard bend locus  |
| Binding partition analysis <sup>3,12,18,93,94</sup>  | Evaluation of binding cooperativity for nucleic acids with multiple protein binding sites; comparison of binding affinities of one protein for several target sequences, determination of the effect of one protein on binding affinity of a second |
| Reverse EMSA <sup>95</sup>   | Detection of nucleic acid binding, measurement of binding affinity. In this assay, the tracer species is the protein. In the classical EMSA, the nucleic acid is the tracer species   |
| Topoisomer EMSA <sup>22</sup>  | Detection of complexes formed with supercoiled DNA  |
| Cryogenic EMSA <sup>96</sup>   | Detection of labile complexes   |
| Antibody supershift <sup>84</sup>  | Identification of protein(s) carrying a specific epitope in mobility-shifted complex(es)  |
| Nucleosome shift <sup>97,98</sup>  | Detection of protein(s) that bind nucleosomes   |
| Heterogeneous subunit assay <sup>99</sup>  | Determination of whether binding activity is due to a protein monomer or a protein oligomer   |
| Binding site selection <sup>100</sup>  | Selection of oligonucleotides containing a specific protein binding site from an ensemble of partially randomized sequences, allowing subsequent amplification and sequencing   |
| Protein–protein <sup>101</sup>   | Detection of protein interactions in the absence of nucleic acids   |
| DNA–DNA and RNA–RNA <sup>102–104</sup>   | Detection of nucleic acid interactions in the absence of proteins   |
| Protein–oligosaccharide <sup>105</sup>   | Detection of protein binding to charged oligosaccharides  |
| <b>2D methods</b>  |   |
| EMSA followed by high temperature electrophoresis <sup>106</sup>                                     | Identification of DNA fragments containing specific binding sites for a given protein   |
| EMSA as an adjunct to footprinting <sup>37</sup>   | Reduction of signal from free nucleic acid in footprint pattern   |
| EMSA followed by electron microscopy <sup>107</sup>  | Characterization of conformation  |
| EMSA followed by SDS–polyacrylamide gel electrophoresis with western blot detection <sup>85,86</sup> | Identification of unknown binding proteins  |
| EMSA followed by MS <sup>87,88</sup>   | Identification of unknown binding proteins  |

can be used for evaluation of binding stoichiometries<sup>31–34</sup>. A third limitation is that the electrophoretic mobility of a complex provides little direct information about the location of the nucleic acid sequences that are occupied by protein. This information is available from nuclease and chemical footprinting assays that can be performed independently of EMSA or in concert with it<sup>35–38</sup>. Finally, the time resolution of the current assay is defined by the interval required for manual solution handling. This limits kinetics studies to processes with relaxation times significantly larger than approximately 1 min required to mix reaction components and for electrophoretic migration into the gel matrix<sup>39</sup>. Strategies designed to improve the time resolution of the technique are in development (M.G.F., unpublished results).

**Alternatives to EMSA**

Many techniques are available for the detection and characterization of protein–nucleic acid complexes and most have advantages and disadvantages that differ from those of the EMSA. The most widely used alternative assays are nitrocellulose filter-binding<sup>40,41</sup> and footprinting<sup>35,36,42</sup>.

Filter binding is simple to perform and the manipulations are rapid enough to allow kinetic studies as well as equilibrium measurements<sup>43,44</sup>. Under favorable conditions, the assay can be highly sensitive and with the exception of the scintillation counter, the required equipment is inexpensive. Like the EMSA, filter-binding is a nonequilibrium technique. As a result, quantitative

analyses require careful evaluation of filter-retention efficiency<sup>44,45</sup>. The assay is not limited by the salt concentration of the sample and it can accommodate very large nucleic acids (e.g., the phage  $\lambda$  genome (48,502 bp<sup>46</sup>))<sup>47</sup>. In contrast, the electrophoretic process limits the salt concentrations of EMSA samples to 300 mM or less (1:1 salt) and to DNAs of 5,000 bp or less<sup>18,48,49</sup> (note that these limits are approximate and may vary with sample and gel compositions). The presence of more than one binding protein complicates filter-binding analysis, since retention of labeled nucleic acid is detected, and not the identity of bound proteins or the proportion of binding activity attributable to each. In addition, conditions that give efficient filter-retention of 1:1 protein–nucleic acid complexes do not result in significantly increased retention of higher stoichiometry complexes<sup>44</sup>, hence the classical assay is poorly suited for distinguishing complexes containing one protein from those containing more than one. Finally, under some solution conditions, single-stranded nucleic acids are retained by nitrocellulose filters<sup>50</sup>, resulting in a background of retained radioactivity that can mask the binding signal. Thus, for applications involving more than one nucleic acid-binding protein, the detection of more than one protein–nucleic acid complex in a mixture and/or binding to single-stranded nucleic acids, mobility shift assays offer clear advantages.

Footprinting assays<sup>35,51</sup> exploit the observation<sup>42</sup> that a protein bound to a specific nucleic acid sequence will interfere with the chemical or enzymatic modification of that sequence. A large



**TABLE 2** | Representative ranges of conditions for electrophoretic mobility shift assay (EMSA) using polyacrylamide gels.

| Quantity  | Range   | Comment  | Reference                         |
|---|---|--|-----------------------------------|
| Temperature ( <i>T</i> )  | $0\text{ }^{\circ}\text{C} \leq T \leq 60\text{ }^{\circ}\text{C}$  | Temperature range applies to electrophoresis as well as sample equilibration. The observed range was limited by the incubator used, not the assay. This range applies to electrophoresis using polyacrylamide gels only. As a result of changes in agarose gel properties with temperature our tests have not exceeded 37 °C   | M.G.F., unpublished result        |
| Pressure ( <i>P</i> )   | $\text{Ambient} \leq P \leq 500\text{ bar}$   | Sample and gel incubated in pressure chamber, electrophoresis performed under pressure   | 108                               |
| pH  | $4.0 \leq \text{pH} \leq 9.5$   | Care was taken to match conductivity of sample and electrophoresis buffer  | M.G.F., unpublished result        |
| Monovalent salt ( $M^+X^-$ )  | $1\text{ mM} \leq [M^+X^-] \leq 300\text{ mM}$  | Care was taken to match conductivity of sample and electrophoresis buffer. High-salt experiments were run in cold room to limit heating of gel. Buffer was circulated between cathodic and anodic reservoirs during electrophoresis*   | 7, 109, 110, 49                   |
| Oligovalent ions  | [Calcium acetate] $\leq 20\text{ mM}$<br>[Magnesium acetate] $\leq 20\text{ mM}$<br>[Spermidine chloride] $\leq 10\text{ mM}$<br>[Potassium phosphate] $\leq 300\text{ mM}$<br>[Sodium borate] $\leq 200\text{ mM}$<br>[Potassium sulfate] $\leq 300\text{ mM}$ | Approximate limits, obtained for each salt separately in studies of the CAP- and <i>lac</i> repressor-DNA interactions. Care was taken to match conductivity of sample and electrophoresis buffer. High-salt experiments were run in cold room to limit heating of gel. Limits reflect the stability of these protein-DNA complexes, not the resolution of the electrophoretic process | 109<br>M.G.F., unpublished result |
| Zwitterions (Z) (e.g., Gly, Pro, betaine)                                 | $[Z] \leq 2\text{ M}$   | Solutes used in samples for osmotic stress experiments   | 65, 66, 111, 112                  |
| Neutral solutes (N) (e.g., glycerol, ethylene glycol, sucrose)            | $[N] \leq 2\text{ M}$   | Sometimes added to samples to aid loading of gels. Used in samples for osmotic stress experiments. Use in gel buffer can stabilize some complexes during electrophoresis   | 65, 66, 113, 111–114              |
| Neutral polymers (e.g., polyethylene glycol 8,000, linear polyacrylamide) | $[\text{polymer}] \leq 10\% \text{ wt/vol}$   | Limited by difficulty in loading gels with viscous samples. Electrophoretic resolution was unaffected, even at the highest neutral polymer concentrations  | 29, 66                            |
| Reducing agents   | [DTT] $\leq 10\text{ mM}$<br>[2-mercaptoethanol] $\leq 10\text{ mM}$<br>[thioglycolic acid] $\leq 5\text{ mM}$  | DTT and 2-mercaptoethanol were added to samples only. Due to its negative charge, thioglycolate can be included in the gel running buffer and will migrate into the gel to ensure the presence of a reducing environment within the gel matrix   | 33<br>M.G.F., unpublished result  |

Unless indicated, conditions refer to sample equilibration prior to electrophoresis.

\* **CAUTION** Electrolysis of chloride salt solutions generates chlorine gas. Where the chance of chlorine generation exists, electrophoresis should be performed in a well-ventilated area and preferably a fume hood.

number of chemical and enzymatic agents are available for this purpose. In the classical assay, a radioisotope label is located at one end of one strand of the nucleic acid target. Following modification and any steps needed to cleave the nucleic acid at modification sites, fragments are resolved on a denaturing polyacrylamide gel. The resulting ladder of bands is visualized by autoradiography and gaps in the array indicate sites of protection by the protein(s) in the test mixture. Comparison of the protection pattern with sequencing reaction products can allow identification of protected sequences with single-nucleotide resolution. In addition to protection, the appearance of sites that are hypersensitive to modification can provide evidence of conformational change in the target nucleic acid<sup>42</sup>. Because most other methods provide little direct information about the identities of protein binding sites, this method remains the ‘gold standard’ for the identification of nucleic acid

sequences within or near binding sites. Since sequences of several hundred residues can be resolved on a typical sequencing gel, the technique is well suited for the analysis of the binding of several protein molecules to a single nucleic acid (a strength that footprinting shares with EMSA). In addition, the footprint signal can be obtained under conditions of binding equilibrium for the protein of interest. This is an important advantage over nonequilibrium assays such as EMSA. Variants of the footprinting assay optimized for quantitative detection of binding have been described<sup>35,52</sup>, and time-resolved methods have been developed that allow the analysis of binding kinetics as well as equilibria<sup>53,54</sup>.

Footprinting assays require simultaneous optimization of binding by the protein(s) of interest and the nucleic acid modification reaction(s) needed to produce the footprint signal. Thus, they are somewhat more difficult to perform than EMSA or filter binding



assays. In addition, because the radioisotope label is distributed over many nucleic acid fragments, the detection of binding by footprinting is less sensitive than the detection of binding using EMSA. In addition, incomplete binding results in a footprint pattern that contains contributions from both free and bound nucleic acids. This makes the protected regions indistinct, because the pattern of free nucleic acid is visible within the protected footprint. A popular solution to this problem is to use EMSA to separate complexes from free nucleic acids after the footprinting modification reaction. Following this step with denaturing gel electrophoresis allows one to independently visualize the fragment patterns of free and bound nucleic acid populations<sup>37,38</sup>. Finally, some proteins that bind nucleic acids without sequence specificity may not produce distinct footprints. For such systems, EMSA and filter binding assays provide binding signals that are easier to interpret than those available from footprinting.

### Strategic considerations that are relevant to EMSA

No single set of binding and electrophoresis conditions works well for all molecular systems. However, several variables can be optimized for the study of a particular interaction, including design of the nucleic acid target, binding reaction conditions and electrophoresis conditions, as discussed in the following text.

**Selection of nucleic acid target.** Short nucleic acids are easily synthesized and inexpensive to purchase. The small number of nonspecific protein binding sites in a small DNA or RNA can be advantageous when the binding protein has low sequence-specificity<sup>12</sup>. In addition, electrophoretic resolution of complexes from free nucleic acid is highest with small nucleic acids and this makes short electrophoresis times possible. On the other hand, all binding sites on a short nucleic acid are close to the molecular ends. This can result in aberrant binding due to structural and electrostatic end-effects<sup>55–57</sup>. Longer templates avoid these limitations but contain more nonspecific binding sites, migrate more slowly (requiring longer electrophoresis times) and generally give a smaller mobility-shift on protein binding<sup>6</sup>.

In the classical EMSA, the electrophoretic mobility of the nucleic acid is monitored. Nucleic acids can be labeled with radioisotopes<sup>58</sup>, covalent or noncovalent fluorophores<sup>13,59</sup> or biotin<sup>60,61</sup>. These labels can be detected by autoradiography, fluorescence imaging, chemiluminescent imaging and/or chromophore deposition, respectively. Alternatively, unlabeled nucleic acid can be used in the binding and electrophoresis steps and detected by postelectrophoretic staining with chromophores or fluorophores that bind nucleic acids<sup>7,8,14</sup>. Labeling the nucleic acid at the 5′- or 3′-end with [<sup>32</sup>P]phosphate is a widely used approach, as it is inexpensive, offers great sensitivity (10<sup>–18</sup> mol or less of 5′-ends can be detected on a routine basis<sup>7</sup>) and does not introduce artificial structures that might influence binding. Thus, when radioisotope use is permissible, we consider <sup>32</sup>P-labeling the method of choice for making nucleic acids detectable in the EMSA. When radioisotope use is not possible, covalent labeling of nucleic acid with a fluorophore allows both qualitative and quantitative EMSA applications<sup>62</sup>, although with lower sensitivity than is possible with radioisotope-labeled nucleic acids.

**Binding conditions.** Protein–nucleic acid interactions are sensitive to mono- and divalent salt concentrations<sup>63</sup> and pH<sup>64</sup>.

Although a typical Tris-based sample buffer is used in the protocol detailed here, good results have been obtained with many different buffers, including HEPES, MOPS (3-(*N*-morpholino)propanesulfonic acid), Bis-Tris (1,3-bis(tris(hydroxymethyl)methylamino)propane), Gly and phosphate. We favor buffers that approximate physiological salt concentrations and pH and provide any needed cofactors at appropriate concentrations. However, as long as the conductivity of the sample is not excessive, electrophoresis can be carried out with a wide range of sample compositions. This allows the concentration of binding buffer components to be adjusted to optimize complex formation.

**Additives.** Small neutral solutes such as glycerol or sucrose are often used to stabilize labile proteins. Such solutes can also enhance the stabilities of protein–nucleic acid interactions<sup>65,66</sup> and can be valuable additions to binding reaction mixtures. They are generally effective when the solute concentration is less than 2 M, but less is known about their effects at higher concentrations, where high viscosity and surface tension complicate solution handling. Addition of modest concentrations (0.1 mg ml<sup>–1</sup> or less) of a carrier protein (e.g., BSA) to the binding reaction can minimize nonspecific losses of binding proteins during solution handling. Similarly, nonionic detergents are sometimes helpful in maximizing protein solubility<sup>2</sup>. The useful concentrations of detergents vary with the identities of the detergent and the molecular system under study. Protease, nuclease and phosphatase inhibitors can be useful additives, especially when the protein sample is a partially-fractionated cell or nuclear extract<sup>27</sup>. Mixtures of inhibitors are commercially available and should be used according to the supplier's instructions. Finally, some systems require specific cofactors for correct function. Examples include cAMP for the *Escherichia coli* CAP (cAMP receptor) protein<sup>67</sup>, ATP for recombinases such as *E. coli* RecA or human Rad51 (refs. 68,69) and polyamines for some eukaryotic transcription factors<sup>70</sup>. Where necessary, small-molecule additives that stabilize complexes can be included in binding and gel buffers, to stabilize complexes during electrophoresis<sup>3,7</sup>.

**Competing nucleic acid.** Often a protein sample will contain more than one nucleic acid binding activity. When secondary binding activities obscure the one of interest, the addition of unlabeled competing nucleic acid to the reaction mixture can reduce the binding of secondary proteins to the labeled target<sup>27</sup>. This strategy works when the protein of interest binds the target nucleic acid with greater affinity than it binds the competitor and when the secondary binding activities do not discriminate between competitor and target sequences. Since competing nucleic acids also reduce the amount of specific binding, even under favorable conditions, it is best to test a range of competitor concentrations to optimize discrimination of specific and nonspecific binding. Commonly used competitors include genomic DNAs, poly d(A–T) and poly d(I–C)<sup>5</sup>.

**Electrophoresis conditions.** The resolution of complexes depends on their stability during electrophoresis. Since many buffers are compatible with electrophoresis, the composition and concentration of gel and running buffer components can be adjusted to optimize the stability of complexes. The most popular buffers are variants of Tris–borate–EDTA<sup>71,72</sup>, Tris–acetate–EDTA<sup>73</sup> or Tris–Gly<sup>27</sup>. As previously mentioned, low molecular weight cofactors

and/or nonspecific stabilizers such as glycerol or ethylene glycol can be included in gel buffers and running buffers to enhance the stability of complexes. In some circumstances, it may be feasible to perform electrophoresis in the buffer that was used in the binding reaction<sup>3,6</sup>. This avoids subjecting the sample to a change of buffer conditions during gel loading and as reaction components migrate into the gel. This has the advantage of avoiding any perturbation to the molecular system that might be due to a change in buffer composition; it also eliminates the need to independently optimize binding and electrophoresis buffer conditions.

Although both polyacrylamide and agarose gels have been used for EMSA<sup>7,8,74</sup>, polyacrylamide gels offer better electrophoretic resolution for protein–DNA and protein–RNA complexes of  $M_r \leq 500,000$  (ref. 3). In addition, some complexes are significantly more stable in polyacrylamide matrices than in agarose gels or free solution<sup>7,29</sup>. While the mechanism of this effect is not completely understood, for a few molecular systems it has been shown that the dissociation rates of complexes decrease with increasing gel concentration<sup>28,30</sup>. On the other hand, the resolution of large com-

plexes and the duration of electrophoresis is much longer in high percentage gels. Thus, optimization of gel concentration can be a valuable precursor to the most critical EMSA applications. One effective optimization strategy is to start with a relatively low concentration gel (e.g., 5% wt/vol acrylamide) and to increase this concentration systematically until any improvement in complex stability is balanced by loss of electrophoretic resolution and/or the onset of impractically long electrophoresis times.

The following is a protocol for a representative mobility shift assay. The protein is purified *E. coli* cAMP receptor protein (CAP)<sup>49,67</sup> and the nucleic acid is a 214-bp restriction fragment from the *E. coli lac* promoter–operator region<sup>48</sup>. The binding buffer consists of 10 mM Tris (pH 7.5 at 20 °C), 100 mM KCl, 1 mM EDTA, 0.1 mM DTT, 20 μM cAMP, 5% vol/vol glycerol, 0.010 mg ml<sup>-1</sup> BSA and the electrophoresis was carried out in a 10% wt/vol polyacrylamide (75:1 acrylamide:bisacrylamide) gel, cast in 40 mM Tris–acetate, 2.5 mM EDTA (pH 7.8 at 20 °C) and run in 40 mM Tris–acetate, 2.5 mM EDTA (pH 7.8 at 20 °C), supplemented with 20 μM cAMP. The results of this assay are shown in **Figure 1**.

## MATERIALS

### REAGENTS

- TEMED\* (*N,N,N',N'*-tetramethylethylenediamine) (Sigma, cat. no. T9281)
  - ▲ **CRITICAL** Store tightly sealed at 4 °C. ▲ **CRITICAL** Unless stated otherwise, reagents should be 'molecular biology grade' or better.
  - ▲ **CRITICAL** Some materials and solutions described in the following text are necessary only if the investigator intends to cast his or her own polyacrylamide gels. These are indicated with an asterisk (\*). Precast gels available commercially are a convenient and viable alternative to the ones made in the laboratory, although their use reduces the investigator's ability to modify gel concentration, crosslinking or electrophoresis buffer compositions.
- Ammonium persulfate\* (Sigma, cat. no. A3678) ▲ **CRITICAL** Store in a desiccator at 4 °C.
- Acrylamide monomer\*, electrophoresis grade (Sigma, cat. no. A3553)
  - ▲ **CRITICAL** Store refrigerated at 4 °C. Protect from light.
- *N,N'*-methylene bisacrylamide\* (USB Corporation, cat. no. 75821)
  - ▲ **CRITICAL** Store refrigerated at 4 °C. Protect from light.
- Protein solution or cell extract containing nucleic acid binding activity
  - Note:* Purification methods vary according to the protein in question. Methods for preparing cell- and nuclear extracts that can be used for EMSA have been described elsewhere<sup>74–76</sup>. ▲ **CRITICAL** The preparation of protein solutions for assay can be time consuming. Since nucleic acid binding activities are often labile, samples should be prepared and stored under conditions that maximize the lifetime(s) of the active protein(s) and minimize the potential for undesirable modifications and proteolysis.
- <sup>32</sup>P-labeled target nucleic acid ! **CAUTION** Exposure to β-radiation and secondary X-radiation from <sup>32</sup>P is hazardous. Most research institutions specify procedures for the safe handling of this isotope, which should be followed stringently.
  - Note:* Methods are available for labeling DNA and RNA with <sup>32</sup>P at 5'-ends, 3'-ends, or internally<sup>72,77</sup>.
- 10× Tris–acetate–EDTA (TAE) electrophoresis buffer (see REAGENT SETUP)
  - Note:* If a commercially prepared gel is used, it is best to use an electrophoresis buffer that matches the one with which the gel was cast. Consult the product literature for information on the selection and preparation of electrophoresis buffers.
- 40% wt/vol acrylamide–bisacrylamide stock solution\* (see REAGENT SETUP)
- 10× Dye + glycerol stock solution (see REAGENT SETUP)
- 10× Binding buffer (see REAGENT SETUP)

### EQUIPMENT

- Electrophoresis power supply (250 V, 200 mA capacity recommended, 100 V, 25 mA capacity minimal). Most commercial power supplies sold for

SDS–polyacrylamide gel electrophoresis (PAGE) or DNA-sequencing applications are suitable

- Vertical electrophoresis apparatus. Most commercially available vertical gel electrophoresis systems are suitable. This equipment includes the glass plates, spacers, well-forming combs, clamps, and optionally, a gel-casting stand, needed to prepare and to run a polyacrylamide gel. While more elaborate equipment is available, the basic Studier 'gel box'<sup>78</sup> works well for most EMSA applications
- Side-arm flask and stopper, access to mild vacuum or water aspirator, to degas gel mixtures before polymerization
- Autoradiography film cassette and Kodak XAR-5 film (Kodak) or cassette and storage phosphor screen. Access to a darkroom equipped with film developer is needed for film autoradiography. Access to a phosphorimager instrument is needed for storage phosphor screen autoradiography
- Plastic food wrap. Types with relatively low 'cling' such as Saran Wrap are easier to work with than others
- Accurately calibrated air-displacement pipettors (e.g., Gilson Pipetman). Instruments that deliver 1–10 μl, 2–20 μl and 20–200 μl are needed to span the volume ranges given in the example protocol. Standard-taper tips are suitable for most solution handling, but narrow-taper gel-loading tips (e.g., Corning, cat. no. 4853) are necessary for the gel-loading step
- Plexiglass sheets (5-mm thick)

### REAGENT SETUP

**10× Binding buffer** 100 mM Tris (pH 7.5 at 20 °C), 10 mM EDTA, 1 M KCl, 1 mM DTT, 50% vol/vol glycerol, 0.10 mg ml<sup>-1</sup> BSA. As described in the INTRODUCTION, many variations can be used.

**Degassing gel polymerization solutions** The composition of the gel polymerization solution is given in **Table 3**. We have found that polymerization is more efficient and the resulting gels more homogeneous if the solution is thoroughly degassed before the polymerization reaction is started. Our equipment for degassing gel solutions consists of a thick-walled side-arm flask with a volume at least 4× greater than the solution to be treated, a stopper for the flask, a magnetic stir bar and stir plate to agitate the solution and a vacuum hose connecting the side-arm flask to a mild vacuum source ('house' vacuum or a water aspirator are sufficient). The gel solution (*minus the TEMED*) is stirred at room temperature (20 °C ± 2 °C), under vacuum, until bubble formation stops. This typically requires 5–15 min. Polymerization should be started (by addition of TEMED) within 30 min of degassing treatment; if this is not possible, degas the solution (*minus the TEMED*) again before use. ! **CAUTION** Always interpose a trap between the side-arm flask and the vacuum source to prevent contamination of the vacuum source with acrylamide. ! **CAUTION** Always wear eye protection, especially when working with glassware under vacuum.

# PROTOCOL

**TABLE 3** | Composition of typical gel polymerization mixtures.

| Components: combine and perform steps in top-down order   | Volume        |                |                |
|---|---------------|----------------|----------------|
|   | 5% wt/vol gel | 10% wt/vol gel | 15% wt/vol gel |
| 10× Tris–acetate–EDTA electrophoresis buffer. 400 mM Tris, 25 mM EDTA, brought to pH 7.8 with acetic acid                             | 2.0 ml        | 2.0 ml         | 2.0 ml         |
| 40% wt/vol acrylamide–bisacrylamide stock solution  | 2.5 ml        | 5.0 ml         | 7.5 ml         |
| H <sub>2</sub> O  | 15.5 ml       | 13.0 ml        | 10.5 ml        |
| Ammonium persulfate   | 0.05 g        | 0.05 g         | 0.05 g         |
| Degas under mild vacuum 15 min with stirring. A stoppered side-arm flask connected to a water aspirator or 'house vacuum' works well* |               |                |                |
| Start polymerization by adding TEMED ( <i>N,N,N',N'</i> -tetra-methylethylenediamine)   | 12 μl         | 12 μl          | 12 μl          |

\* **CAUTION** Always interpose a trap between the side-arm flask and the vacuum source, to prevent accidental contamination of the vacuum source with acrylamide.

**TAE buffer** 400 mM Tris, 25 mM EDTA, brought to pH 7.8 with acetic acid. As described in the INTRODUCTION, many variations of electrophoresis gel and running buffers can be used.

**40% wt/vol Acrylamide–bisacrylamide stock solution** Acrylamide monomer, 39.5 g per 100 ml; *N,N'*-methylene bisacrylamide 0.5 g per 100 ml. Filter the resulting solution through a 0.45-μm filter. Store at 4 °C in a brown glass bottle. Alternatively, acrylamide–bisacrylamide stock solutions\* can be purchased from commercial sources (e.g., 40% wt/vol acrylamide–bisacrylamide solution for electrophoresis, 37.5:1 acrylamide:bisacrylamide (Sigma, cat. no. A7168)). Although this product is convenient, the higher acrylamide:bisacrylamide ratio

(approximately 75:1 wt/wt) of our formulation results in a gel with fewer crosslinks at a given acrylamide concentration than the 37.5:1 mixture. We believe that the 75:1 formulation gives better resolution of large complexes than do solutions containing greater proportions of bisacrylamide. **CAUTION** Acrylamide and bisacrylamide are neurotoxic. Weigh these reagents in a draught-free area. Wear a particle mask, gloves and eye protection when handling acrylamide powders. Never pipet solutions by mouth.

**10× Dye + glycerol stock solution** 10 mM Tris, 1 mM EDTA, 50% vol/vol glycerol, 0.001% wt/vol bromophenol blue, 0.001% wt/vol xylene cyanol FF.

## PROCEDURE

### Gel preparation ● TIMING Less than 3 h

1| Ensure that gel plates, spacers and comb are clean. Remove any fingerprints or other residue from glass plates with a swab saturated with methanol. Ensure that all components are dry before continuing. If using commercially available precast gels, skip Steps 1–3 and start the protocols at Step 4 (pre-electrophoresis).

2| Prepare the polymerization mixture. The amounts of reagents required for one gel with dimensions 0.08 × 12 × 18 cm<sup>3</sup> (nominal volume 17.8 ml), containing 5–15% wt/vol polyacrylamide, are shown in **Table 3**.

**CAUTION** Acrylamide and bisacrylamide are neurotoxins. Wear gloves and use eye protection when handling the polymerization solution. Never pipette solutions by mouth.

**CRITICAL STEP** Polymerization of thoroughly degassed solutions can be rapid. Viscosity can become too great to allow the solution to be poured or transferred by pipette within approximately 5 min. Therefore, do not start the polymerization reaction until ready to cast the gel.

3| Pour the polymerizing mixture into the glass plate assembly. Avoid bubble formation. Insert the well-forming comb immediately and allow the gel to polymerize for at least 2 h.

**CRITICAL STEP** Prevent bubble formation. Since bubbles do not conduct electrical current, they disrupt electrophoretic migration. Thus, it is important to minimize the number and size of bubbles trapped during polymerization. Pour the polymerizing mixture slowly to avoid bubble formation. Tilting the gel-plate assembly 45° from the vertical can minimize bubble formation during pouring. If bubbles are trapped in the solution, finish pouring, stand the assembly on end so that the plates are vertical and gently tap the assembly with a spatula to dislodge the bubbles. Once bubbles have floated to the top of the solution, they should be popped or removed before inserting the well-forming comb.

**PAUSE POINT** Polymerized gels can be stored at room temperature (20 °C ± 2 °C), with open edges sealed with plastic film, for several hours or up to 1 week at 4 °C and 100% humidity. We use a plastic box in a refrigerator for this purpose. A paper wick attached to the side of the box and saturated with water is sufficient to maintain the humidity.

### Pre-electrophoresis ● TIMING 0.5–1 h

4| Remove comb and bottom spacer (if used) from gel and mount the gel in the vertical electrophoresis apparatus.

5| Fill the upper and lower buffer reservoirs with electrophoresis running buffer. For gels obtained with the polymerization protocol given earlier, the appropriate solution is 1× concentrated TAE buffer. Use a Pasteur pipette to rinse the submerged surfaces

of the sample wells with buffer, to ensure that no debris is present. A Pasteur pipette with a 90° bend in the narrow end can be useful for displacing air bubbles trapped at the bottom of the gel.

**▲ CRITICAL STEP** Charged reagents that are incompatible with the gel polymerization reaction can be moved into the gel by electrophoresis. An appropriate concentration of the reagent of interest is mixed with the running buffer prior to pre-electrophoresis. An example of this is the inclusion of cAMP in the electrophoresis buffer of the assay shown in **Figure 1**.

**6|** Place approximately 5 μl of 1× dyes + glycerol solution in each well of the gel and conduct pre-electrophoresis at approximately 10 V cm<sup>-1</sup> of gel length. Reduce this voltage if gel heating is evident.

**▲ CRITICAL STEP** The dye migration pattern tests whether the gel density and conductivity are homogeneous and reveals any defect in the shape of the sample well. Discard any gel that does not give narrow, well-resolved dye bands at this stage.

**Sample preparation and equilibration ● TIMING 1–1.5 h**

**7|** Prepare samples and equilibrate for 30 min at 20 °C ± 1 °C. This can be carried out, in part, while the gel is undergoing pre-electrophoresis. A titration protocol is normally used to determine the optimal concentration of protein required to interact with a constant amount of nucleic acid. Sample variation is minimized by preparing a premix of components that are present in constant concentrations and distributing a constant volume of this solution to individual sample tubes. All nonprotein components should be mixed and brought to the reaction temperature before adding the protein. This ensures that the protein encounters all solution components at close to their final concentrations. As an example, a typical titration protocol used for the study of the CAP–DNA interaction is given in **Table 4**. Note that cAMP is a special additive needed for sequence-specific DNA-binding activity in this molecular system.

**▲ CRITICAL STEP** Equilibration time will depend on the identities of the interacting molecules and on many of the reaction conditions (examples include but are not limited to temperature, protein, and nucleic acid concentrations and salt concentration). Failure to attain equilibrium may give irreproducible results. A minimal control for the attainment of equilibrium is to divide each sample in an assay into two aliquots and to incubate one subset for 30 min and the other subset for 60 min (or more). Analyze the mixtures using identical electrophoresis conditions. Equal mole-fractions of bound and free nucleic acids in corresponding samples are evidence of close approach to binding equilibrium.

**Electrophoresis ● TIMING 0.5–5 h depending on the electrophoretic resolution required**

**8|** At the end of the equilibration period, rinse the wells of the gel and load the samples. In the experiment shown in **Figure 1** (reaction compositions given in **Table 4**), the entire sample (30 μl) was loaded. However, smaller sample volumes can be used (a practical lower limit is imposed by the need to cover the bottom surface of the well with a uniform layer of sample solution; for typical gels, this can be as small as 5 μl). Small sample volumes are advantageous because they result in narrow starting zones at the start of electrophoresis and, accordingly, sharper bands at the end of electrophoresis. Gel loading is conveniently done using an air-displacement pipettor equipped with narrow-taper ‘gel-loading’ tips (see EQUIPMENT). Many sample compositions are dense enough to form a layer cleanly at the bottom of the sample wells. For those that are not, inclusion of glycerol to a final concentration of 2–5% vol/vol will facilitate loading. (Many other dense solutes work equally well for this purpose.) Place the loaded tip within 1 mm of the gel surface and slowly expel the sample. Avoid expelling an air bubble after the sample, since the turbulence caused by the escaping bubble will disrupt the narrow zone of the undisturbed sample.

**9|** Load tracking dye in flanking, unused wells as a marker, and perform electrophoresis at 10 V cm<sup>-1</sup>. Reduce the voltage if the gel becomes warm during electrophoresis. To minimize complex dissociation, the electrophoresis interval should be as short

**TABLE 4 |** Composition of binding reactions for the assay shown in **Figure 1**.

| Component  | Sample               |      |      |      |      |      |      |     |      |      |
|--|----------------------|------|------|------|------|------|------|-----|------|------|
|  | a                    | b    | c    | d    | e    | f    | g    | h   | i    | j    |
|  | <b>Volumes in μl</b> |      |      |      |      |      |      |     |      |      |
| 10× Binding buffer: 100 mM Tris (pH 7.5 at 20 °C), 10 mM EDTA, 1 M KCl, 1 mM DTT, 50% vol/vol glycerol, 0.10 mg ml <sup>-1</sup> BSA                     | 3                    | 3    | 3    | 3    | 3    | 3    | 3    | 3   | 3    | 3    |
| <sup>32</sup> P DNA: 214 bp <i>lac</i> promoter–operator fragment <sup>48</sup> , 1.25 × 10 <sup>-8</sup> M, approximately 4,000 c.p.m. μl <sup>-1</sup> | 1.5                  | 1.5  | 1.5  | 1.5  | 1.5  | 1.5  | 1.5  | 1.5 | 1.5  | 1.5  |
| 200 μM cAMP  | 3                    | 3    | 3    | 3    | 3    | 3    | 3    | 3   | 3    | 3    |
| H <sub>2</sub> O   | 22.5                 | 21.5 | 20.5 | 18.5 | 16.5 | 14.5 | 12.5 | 7.5 | 20.5 | 18.5 |
| DNA-binding protein: <i>Escherichia coli</i> CAP, 1.4 × 10 <sup>-8</sup> M   | 0                    | 1    | 2    | 4    | 6    | 8    | 10   | 15  |      |      |
| <i>E. coli</i> CAP, 1.4 × 10 <sup>-7</sup> M   |                      |      |      |      |      |      |      |     | 2    | 4    |



## PROTOCOL

as possible, consistent with resolution of the species of interest. Note that some molecular systems are not perturbed by the addition of tracking dyes to the samples. For these systems, it is convenient to add dyes directly to the samples, before loading the gel.

### Detection of electrophoretic bands ● TIMING 30 min to several days, depending on the radioactivity of the sample

**10|** At the end of electrophoresis, remove the gel and plate assembly from the electrophoresis device and dry it thoroughly with paper towels.

▲ **CRITICAL STEP** The gel and plate assembly must be dry before separating the plates. Residual buffer trapped between the plates can promote vacuum formation as the plates are pried apart and this can distort the gel. In addition, transfer of free buffer to the gel surface can result in elution of radioactive nucleic acid from the gel during subsequent steps. This 'free' radioisotope is a nuisance to autoradiography and a potential safety hazard.

**11|** Gently separate the plates, leaving the gel adherent to one of the plates. Wrap the gel and plate in a single layer of plastic food wrap. The wrapped surface of the gel should be free of air bubbles and wrinkles, since these will interfere with autoradiography. For short (24 h or less) autoradiographic exposures, this preparation is sufficient. For longer exposures, drying the gel on a paper or membrane support or freezing it at  $-80\text{ }^{\circ}\text{C}$  (see following text) will minimize band broadening due to diffusion.

**12|** *Autoradiography.* Working in a suitable darkroom (for film autoradiography) or in a laboratory with dim light (for phosphor screen autoradiography), place film or phosphor screen in an exposure cassette. Place the wrapped gel and plate assembly in the cassette, with gel-side toward film or screen. Close the cassette. Expose film or screen at  $4\text{ }^{\circ}\text{C}$  for intervals up to 24 h, and at  $-80\text{ }^{\circ}\text{C}$  for longer intervals.

▲ **CRITICAL STEP** Flexible cardboard or plastic film cassettes accommodate glass gel plates better than the traditional rigid metal cassette, but they do not absorb beta-radiation emitted by the gel. If cassettes are stacked during the exposure interval, separating them with plexiglass sheets (5-mm thick) will minimize cross-exposure fogging of adjacent films or phosphor screens.

**13|** At the end of the exposure period, develop the latent image on phosphor screen or film, according to manufacturer's instructions. Example gel pictures are shown in **Figures 1–4**.

### ? TROUBLESHOOTING

#### ● TIMING

Steps 1–3, gel preparation: less than 3 h

Steps 4–6, pre-electrophoresis: 0.5–1 h

Step 7, sample preparation and equilibration: 1–1.5 h. This step can be initiated during pre-electrophoresis (Steps 4–6)

Step 8, electrophoresis: 0.5–5 h. The time required will vary depending on the experiment

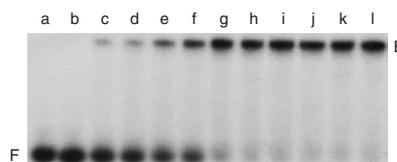
Steps 9–13, detection of electrophoretic bands: typically 30 min to several days. Autoradiographic exposure time will depend on concentration of nucleic acid and its specific radioactivity

### ? TROUBLESHOOTING

The 'pilot conditions' previously described work well for many binding proteins. However, if binding is not detected, the protocol may require modification. Troubleshooting advice can be found in **Table 5**.

**TABLE 5 |** Troubleshooting table.

| Problem                                | Possible reasons               | Solutions  |
|--|--------------------------------|--|
| No bands visible after electrophoresis | Low nucleic acid concentration | Verify nucleic acid concentration. Repeat experiment with appropriate concentration of nucleic acid  |
|  | Inefficient labeling           | Check reaction components for concentration and enzyme activity  |
|  | Nucleic acid degradation       | If sample is radioactive, verify that nucleic acid is intact by running a sequencing gel. If nuclease activity is suspected, treat buffers with diethyl pyrocarbonate. Exclude divalent cations wherever possible. Commercially available RNase inhibitors are useful to protect RNA substrates. Commercially-available phosphatase inhibitors can prevent substrate dephosphorylation |



**Figure 2 |** Titration of a 16-residue single-stranded DNA with human AGT protein. All samples contained  $8.7 \times 10^{-7}\text{ M}$  DNA; samples b–l contained in addition 0.57, 1.14, 1.72, 2.29, 2.86, 3.44, 4.01, 4.58, 5.16, 5.73 and  $6.60 \times 10^{-6}\text{ M}$  AGT protein, respectively. The binding buffer contained 10 mM Tris (pH 7.6 at  $20\text{ }^{\circ}\text{C}$ ), 50 mM KCl, 1 mM DTT,  $10\text{ }\mu\text{g ml}^{-1}$  BSA. Samples were resolved on a 10% wt/vol polyacrylamide gel cast and run in 40 mM Tris-Acetate, 2 mM EDTA, 50 mM KCl. Band designations: F, free DNA; B, protein-DNA complex. Continuous variation electrophoretic mobility shift assay (EMSA) and analytical ultracentrifugation analyses support the conclusion that the complex has a 4:1 stoichiometry<sup>19,33</sup>.

**TABLE 5** | Troubleshooting table (continued).

| Problem   | Possible reasons   | Solutions  |
|---|--|--|
| All bands are smeared or streaked   | Uneven gel polymerization  | Use fresh gel components. Degas thoroughly before polymerization. If polymerization interfered with casting gel, reduce <i>N,N,N',N'</i> -tetramethylethylenediamine (TEMED) concentration. If gel requires greater than 1 h to polymerize, increase ammonium persulfate concentration   |
|   | Excessive gel heating  | Check concentrations of gel and running buffer. If they are correct, reduce voltage during electrophoresis   |
|   | Sample conductivity too high   | Reduce salt concentration in nucleic acid or sample buffer   |
| Free nucleic acid migrates normally. Nucleic acid mobility unchanged in presence of protein   | Low protein concentration  | Verify protein concentration. Use larger volumes of protein stock or more concentrated stock in preparing samples  |
|   | Protein is inactive  | Run SDS–polyacrylamide gel electrophoresis to rule out degradation. If the binding protein is an enzyme, test for activity. Test higher concentrations of protein to detect residual binding activity. A new, more active preparation of protein may be necessary  |
|   | Protein is negatively charged and comigrates with nucleic acid   | Check migration of protein alone under EMSA conditions. If protein comigrates with nucleic acid, reduce pH of binding and electrophoresis buffers <sup>64</sup>  |
| Free nucleic acid migrates normally. No nucleic acid detectable in samples containing protein | Nucleic acid degradation   | Verify that nucleic acid is intact. If nuclease activity is suspected, treat glassware and buffers with diethyl pyrocarbonate. Exclude divalent cations wherever possible. Use commercial RNase and phosphatase inhibitors   |
|   | Binding occurred but complexes dissociated during electrophoresis. Liberated nucleic acid is too diffuse to detect | Minimize gel running times; use more concentrated gel; include stabilizing solutes in gel buffer; reduce salt concentration in binding and electrophoresis buffers to increase electrostatic stabilization; lower electrophoresis temperature (run gel in cold room); reduce or eliminate competing nucleic acid (this can be added back with care once a useful binding signal is obtained) |
| Free nucleic acid migrates normally. Complex bands smeared or streaky                         | Binding occurred but complexes dissociated in gel during electrophoresis   | Minimize gel running times; use more concentrated gel; include stabilizing solutes in gel buffer; reduce salt concentration in binding and electrophoresis buffers to increase electrostatic stabilization; lower electrophoresis temperature (run gel in cold room); reduce or eliminate competing nucleic acid   |
|   | Binding occurred but complexes dissociated in the well prior to electrophoresis                                    | Minimize interval that sample is in well before electrophoresis. Complexes may be destabilized by component(s) of running buffer. If so, they may be more stable in gel and running buffers that more closely resemble the composition of binding buffer   |
|   | Binding occurred but complexes dissociated in the well during electrophoresis                                      | To minimize gel 'dead time,' use smallest sample volume possible; conduct electrophoresis at high voltage (approximately 50 V cm <sup>-1</sup> ) until samples enter the gel, then reduce to approximately 10 V cm <sup>-1</sup>   |
|   | Binding occurred but samples containing protein are too salty  | Reduce salt concentration in protein stock and/or in binding buffer  |
| Free band is sharp, complex band(s) are broad and indistinct                                  | Heterogeneous protein  | Multiple species may be due to post-translational modification or to partial degradation without loss of binding activity  |
| Complex and free bands are broad and indistinct   | Sample zone is too large (measured from top of sample to bottom of well) at the start of electrophoresis           | Reduce sample volume. Increase density of sample (e.g., increase glycerol concentration) to facilitate gel loading. Minimize time between loading and electrophoresis  |
|   | Electrophoresis period too long  | Reduce run-time  |
|   | Nucleic acid degradation   | Verify that nucleic acid is intact. If nuclease activity is suspected, treat extracts and buffers with diethyl pyrocarbonate. Exclude  |



TABLE 5 | Troubleshooting table (continued).

| Problem   | Possible reasons   | Solutions   |
|---|--|---|
| Nucleic acid stuck in well, no free species visible | Protein/nucleic acid ratio is too high                       | Reduce the concentration of protein or increase the concentration of unlabeled nonspecific competitor   |
|   | Protein is aggregated  | Change binding conditions to improve protein solubility. Possible modifications: add solutes that stabilize folded (compact) forms of proteins (e.g., glycerol); keep protein stocks and binding reactions at ice temperature; avoid freeze–thaw cycles with protein stocks; include non-ionic detergents in protein storage buffer and/or binding buffer |
|   | Free nucleic acid and complexes are too large for gel system | Try lower percentage polyacrylamide or reduce the acrylamide/bisacrylamide ratio. Test agarose gel as alternative to polyacrylamide   |

ANTICIPATED RESULTS

In a typical experiment, protein complexes migrate more slowly than free nucleic acid (Fig. 1). The mobility decrement that is observed depends on many factors including the sizes of protein and nucleic acid, the number of protein molecules bound, protein charge, and for rod-like DNAs, whether the DNA is bent on complex formation<sup>3,79</sup>. Current versions of the EMSA provide little direct information about the location(s) of the binding site(s) that are occupied in protein–nucleic acid complexes. However, footprinting assays<sup>36,42,80</sup> carried out in parallel with EMSA provide an efficient means of identifying occupied sequences.

Although the CAP–DNA complex shown in Figure 1 has a 1:1 stoichiometry, in other systems the first complex to form may have a different protein:nucleic acid ratio. For example, the first detectable complex to form in the titration of a 16 nt single-stranded DNA with the human AGT DNA-repair protein has a 4:1 stoichiometry<sup>33</sup> (Fig. 2). A range of EMSA methods are available for the determination of stoichiometries<sup>3,6,12</sup> and investigators are also encouraged to consider non-EMSA methods such as analytical ultracentrifugation<sup>81–83</sup>.

Occasionally, several complexes of differing stoichiometry will form in a single binding reaction. Under favorable conditions, these can be resolved, resulting in a ‘ladder’ of bands in which each stoichiometric step is represented (Fig. 3). When more than one binding protein is present in the reaction mixture, complexes may form containing individual proteins or any possible combination of proteins. An example of such an assay with purified *E. coli* CAP and *lac* repressor proteins is shown in Figure 4. Partition analysis methods<sup>7,12,18</sup> are available for the characterization of multiple equilibria of the kinds shown in Figures 3 and 4.

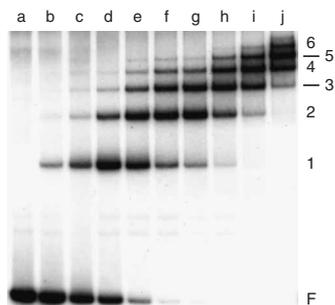


Figure 3 | Titration of a 203-bp *lac* promoter DNA with *Escherichia coli* lactose repressor. All samples contained  $1.91 \times 10^{-9}$  M DNA; samples b–j contained 0.08, 0.16, 0.32, 0.48, 0.61, 0.83, 0.99, 1.32,  $1.66 \times 10^{-8}$  M repressor protein, respectively. The binding buffer contained 10 mM Tris (pH 8 at 20 °C), 1 mM EDTA, 50 mM KCl, 100  $\mu$ g ml<sup>-1</sup> BSA, 5% vol/vol glycerol. Samples were resolved on a 5% wt/vol polyacrylamide gel cast and run at room temperature (20 °C  $\pm$  2 °C) in 45 mM Tris–borate (pH 7.8), 2.5 mM EDTA. Electrophoretic species are designated F, free DNA or numbered (1–6) to indicate the repressor: DNA ratio of the corresponding complex<sup>7</sup>. Under these conditions, repressor binds nonspecific sites as well as its specific operator sequences.

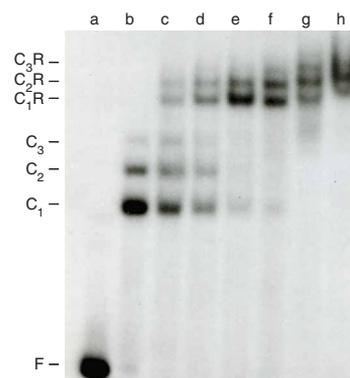


Figure 4 | Titration of 1:1, 2:1 and 3:1 CAP–*lac* promoter complexes with *lac* repressor. All samples contained a 214-bp *Escherichia coli lac* promoter–operator DNA<sup>90</sup> ( $3.7 \times 10^{-10}$  M). Samples b–h contained CAP protein ( $7.1 \times 10^{-9}$  M). Samples c–h contained *lac* repressor at 0.7, 1.5, 2.2, 2.9, 3.6 and  $7.3 \times 10^{-9}$  M, respectively. The binding buffer was 10 mM Tris (pH 8.0 at 20 °C), 1 mM EDTA, 50 mM KCl, 20  $\mu$ M cAMP. Electrophoresis was carried out at room temperature in a 5% wt/vol polyacrylamide gel run in 45 mM Tris–borate (pH 8.0), 2 mM EDTA, 20  $\mu$ M cAMP. Symbols: F, free DNA; C<sub>1</sub>, C<sub>2</sub> and C<sub>3</sub>, complexes with 1, 2 and 3 CAP dimers bound per DNA molecule, respectively; C<sub>1</sub>R, C<sub>2</sub>R and C<sub>3</sub>R, complexes with one repressor tetramer and 1, 2 and 3 CAP dimers bound per DNA molecule, respectively.

When the source of binding activity is an unpurified or partially purified cell extract, the identification of the protein(s) present in a resolved complex is often the most important challenge. Because cell extracts contain large numbers of nucleic acid-binding proteins, it is risky to assume that the dominant binding activity associated with a nucleic acid is the one of interest to the investigator. The classical approach to this problem has been the electrophoretic supershift assay, in which an antibody against a candidate protein reduces the gel-mobility of the protein–nucleic acid complex, producing a secondary mobility shift<sup>84</sup>. More recently, 2D strategies in which EMSA is paired with SDS-PAGE with western blot detection<sup>85,86</sup>, or paired with mass spectrometry<sup>87,88</sup> have started to bring the power of proteomics to bear on this problem. A detailed protocol describing EMSA paired with SDS-PAGE and mass spectrometry for protein identification has recently been published<sup>89</sup>.

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