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Determination of the Binding Site-Size of the Protein-DNA Complex by Use of the Electrophoretic Mobility Shift Assay

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1. Introduction

In this chapter we describe how to perform an electrophoretic mobility shift assay (EMSA), also known as the band shift or gel retardation assay, to determine the binding site-size of the DNA binding protein using a series of DNA polymers. The binding site-size information of the DNA binding protein is a prerequisite for formulating any model of the proteins' function in DNA replication. EMSA is simple and quick, and if needed, the use of radioactive DNA make it highly sensitive and allow us to "see" the formation of distinct complexes of the DNA binding protein. The expectation of EMSA is that, once the length of the nucleotides is sufficient for the binding of protein, the protein-DNA complex remains intact and migrates as distinct band during gel electrophoresis. In addition, once the length of the nucleotides is sufficient for the binding of two or more proteins, the protein-DNA complexes migrate as distinct bands, usually referred to as a super shift: the higher the oligomeric protein-DNA complex, the lower the electrophoretic mobility. This technique can be used for both highly sequence-specific (e.g., transcription factor) (1,2) and non-specific proteins (e.g., single-stranded DNA binding protein) (3-7).

2. Some general considerations

1. Although EMSA was originally used in the detection of DNA-binding proteins in the crude cell extracts, it is thought that cell extracts contain many other DNA-binding proteins (and their regulatory proteins), and some small molecules (such as ATP and DNA metabolites), that are also able to interact with the DNA and the target protein; these molecules may cause some unpredictable effects on the reaction. Therefore, it is highly recommended to use purified protein(s) for EMSA.
2. When the studied protein has low DNA-binding activity, needs other loading factors, or binds to DNA in a strong sequence-specific way, a relatively high concentration of protein (in μM range) may be required to facilitate protein-DNA complex formation.
3. Protein binding to DNA usually needs to be in the "suitable" condition for optimal complex formation and stabilization. The conditions for the DNA binding process may

vary significantly in terms of pH, ionic strength, and necessary factors (such as metal ions or ATP).

4. When the protein-DNA interaction is highly cooperative, a particular care must be taken for determining its binding site-size by use of EMSA. If only one protein-DNA complex is visible when the DNA length is further increased (for example, to dT60 or more), it is likely inappropriate to determine its binding site-size using EMSA.

3. Experimental systems

1. Polyacrylamide gels. Differences in the size, aggregation state and pI of protein-DNA complexes will affect the choice of conditions used for EMSA. Generally, the lower percent of polyacrylamide gel will be considered for the bigger protein. Polyacrylamide gels, usually in 6-12% w/v, are made using an acrylamide to bisacrylamide weight ratio of 19:1 and TBE as the buffer in the gel and for electrophoresis.
2. Electrophoresis buffers. The process of electrophoretic separation may destabilize protein-DNA complexes, and thus the ionic strength, pH and composition of electrophoresis buffers should be adjusted to fit the need of the protein-DNA complex formation. If the binding condition for the formation of the protein-DNA complex is known, the electrophoresis buffer can be kept similar to the binding buffer. The most common buffer used in both polyacrylamide and agarose gels is TBE buffer. However, some metal-containing or metal-binding proteins should be analyzed in a buffer without EDTA. Sometimes the use of low ionic strength electrophoresis buffers at the same pH value can improve the stability of the protein-DNA complexes and the resolutions between bands; TE (10 mM Tris-HCl, 0.1 mM EDTA) or HE (10 mM HEPES, 0.1 mM EDTA) might be used in this case.
3. Sample buffers. Protein-DNA complexes are loaded on the polyacrylamide gel usually in the presence of 5-10% glycerol or sucrose. This can be included in the binding buffer or added before the protein-DNA complexes loading to the gel. Bromophenol blue and/or xylene cyanol (0.02% w/v) is usually used as an electrophoresis marker.
4. Visualization of gels. After electrophoresis, the gels are placed on filter paper, dried under vacuum at 80 °C, and then visualized by either autoradiography (exposed to X-ray film) or using a phosphorimager (exposed to the phosphor storage plate). Phosphorimaging is much more sensitive and the dynamic range is much greater than that of X-ray film. For phosphorimaging, the phosphor storage plate is scanned, and the data are digitized for quantitative analysis.

4. Determination of the binding site-size of the protein-DNA complex by use of the electrophoretic mobility shift assay: Single-stranded DNA binding protein (SSB)

Step 1. Use of a purified protein

As mentioned above, cell extracts contain many other proteins and some small molecules, able to interact with the target DNA and protein, which may cause some unpredictable effects on the DNA-binding reaction. Therefore, it is highly recommended to use purified protein(s) for EMSA. More and more proteins have become available in a pure form now, especially by using recombinant technology. The overexpression of DNA-binding proteins or their domains is essential for their purification, characterization and structure

determination. The bacterial host *E. coli* is the first choice of expression system; it is simple to use and inexpensive to culture. For example, the gene encoding the single-stranded DNA binding protein (SSB) from *Pseudomonas aeruginosa* PAO1 can be PCR-amplified from the genomic DNA (3), inserted into the pET21b vector, and the gene can be expressed in transformed *E. coli* cell by using the inducer isopropyl thiogalactoside (IPTG). *P. aeruginosa* SSB can be then purified from the soluble supernatant by Ni²⁺-affinity chromatography (HiTrap HP; GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Protein purity can be determined by Coomassie-stained SDS-PAGE.

Step 2. Preparation of protein-DNA complexes

Binding mode is not always the same for several DNA-binding proteins. For example, differences in the binding condition, such as the ionic strength, pH and some small molecules included in the reaction mixture, can affect the binding mode of SSB (8). These factors also influence the stability of the SSB-DNA complexes in the binding reaction. For determination of the binding site-size of SSB using EMSA, various lengths of ssDNA oligonucleotides or other series of ssDNA homopolymers will be needed; radiolabeling can be carried out with [³²P]ATP (6000 Ci/mmol; PerkinElmer Life Sciences) and T4 polynucleotide kinase (Promega, Madison, WI, USA). The SSB-DNA complexes will be formed with different protein concentrations, usually at range of 10⁻⁹-10⁻⁷ M. For *P. aeruginosa* PAO1 SSB, 0, 19, 37, 77, 155, 310, 630, 1250, 2500, and 5000 nM protein was used for the complexes formation, respectively. *P. aeruginosa* PAO1 SSB was incubated for 30 min at 25°C with 1.7 nM radioactive DNA substrates (dT15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, and 80) in a total volume of 10 µL in 20 mM Tris-HCl pH 8.0 and 100 mM NaCl. The composition of the binding buffer may be adjusted to fit the need of study or the nature of the protein of interest.

Step 3. Fractionation of the SSB-DNA complexes using EMSA

Polyacrylamide or agarose gels can be used to fractionate mixture of protein-DNA complexes prior to further analysis. For *P. aeruginosa* PAO1 SSB, the complexes were formed in a total volume of 10 µL (see above), and aliquots (5 µL) were removed from each reaction solution and added to 2 µL of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples can be resolved on a native 8% polyacrylamide gel at 4 °C in TBE buffer for 1 h at 100 V and visualized by autoradiography or phosphorimaging.

Step 4. Determination of the binding site-size of protein-DNA complexes

After fractionating and phosphorimaging, complexed and free DNA bands can be visualized and quantified, and the binding site-size of DNA-protein complex can be determined. For example, various lengths of radioactive ssDNA oligonucleotides d(T5)n were complexed with SSB and with different protein concentrations. If the DNA space (length) is enough, the second or third studying protein will bind to the same complex where one protein of interest has been already pre-bound. Therefore, we can calculate the length of DNA required for the second joining protein (Figure 1). For example, SSB from *Klebsiella pneumoniae* binds to short ssDNAs (dT30-50) to form a complex in which a single protein is bound to the ssDNA (Figure 2), and two proteins could bind to dT55-75 (Figure 3). The presence of an extra 5 nt in dT55, as compared with dT50, provides enough interaction space for the binding of a second SSB protein, which occupies around 27 nt ssDNA (55/2=27.5). Furthermore, three SSB proteins could bind to dT80-85 (Figure 4), which occupies around 26 nt ssDNA per SSB protein (80/3=26.7). Thus, combining together

the EMSA results with the number of the SSB-DNA (dT30–85) complexes, it can be found that the binding-site size of SSB from *Klebsiella pneumoniae* is 27 ± 1 nt, as determined using EMSA under this binding condition (Figure 5).

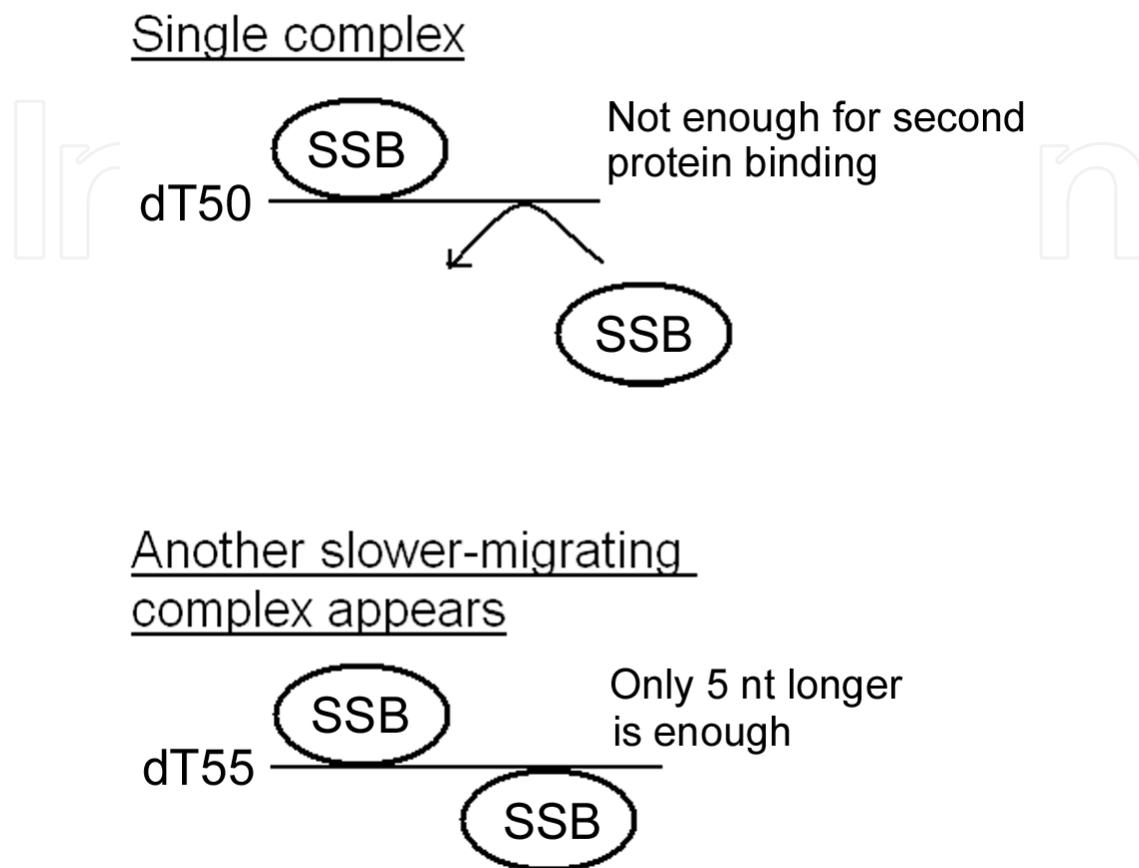


Fig. 1. Schematic model for the supershift of the SSB binding to the ssDNA. If the DNA provides enough interaction space for the binding of a second protein, we can observe another slower-migrating complex from EMSA when the protein concentration is increased.

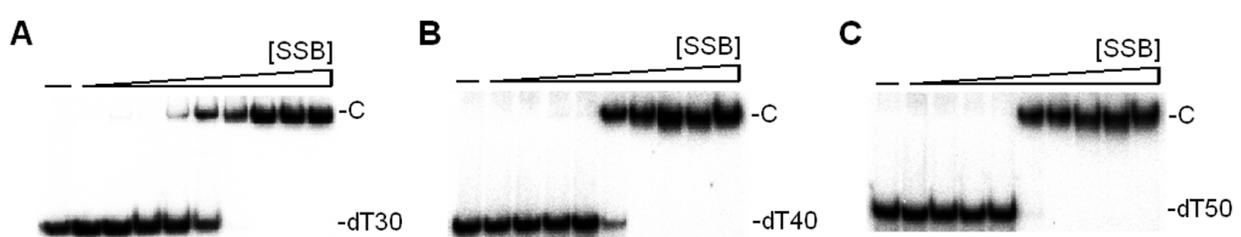


Fig. 2. Binding of *Klebsiella pneumoniae* SSB to dT30–50. The reaction solutions contained DNA substrate dT30, dT40, or dT50 and the SSB (0–5.0 μ M). SSB (0, 19, 37, 77, 155, 310, 630, 1250, 2500, and 5000 nM) was incubated for 30 min at 25 °C with 1.7 nM of (A) dT30, (B) dT40, or (C) dT50 in a total volume of 10 μ L in 20 mM HEPES pH 7.0 and 500 mM NaCl. Aliquots (5 μ L) were removed from each reaction solution and added to 2 μ L of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples were resolved on a native 8% polyacrylamide gel at 4 °C in TBE buffer (89 mM Tris borate and 1 mM EDTA) for 1 h at 100 V and visualized by autoradiography. This SSB binding to dT30–50 forms a single complex.

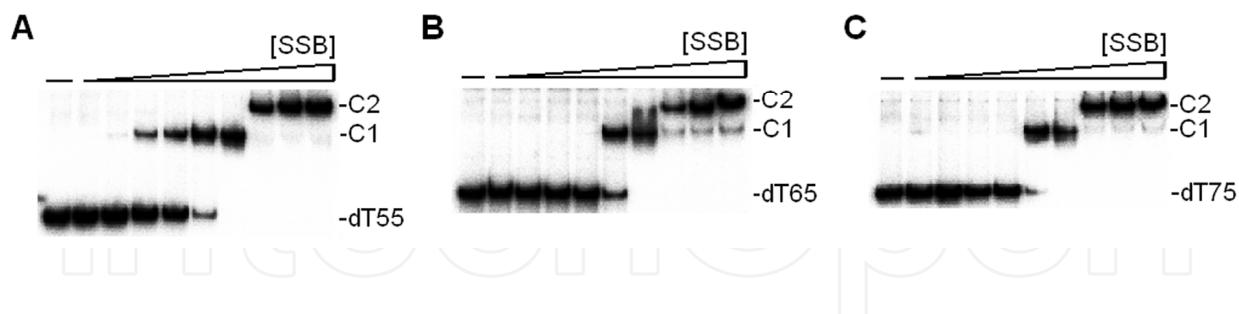


Fig. 3. Binding of *Klebsiella pneumoniae* SSB to dT55–75. The reaction solutions contained DNA substrate dT55, dT65, or dT75 and the SSB (0–5.0 μ M). SSB (0, 19, 37, 77, 155, 310, 630, 1250, 2500, and 5000 nM) was incubated for 30 min at 25 $^{\circ}$ C with 1.7 nM of (A) dT55, (B) dT65, or (C) dT75 in a total volume of 10 μ L in 20 mM HEPES pH 7.0 and 500 mM NaCl. Aliquots (5 μ L) were removed from each reaction solution and added to 2 μ L of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples were resolved on a native 8% polyacrylamide gel at 4 $^{\circ}$ C in TBE buffer (89 mM Tris borate and 1 mM EDTA) for 1 h at 100 V and visualized by autoradiography. This SSB binding to dT55–75 forms two different complexes.

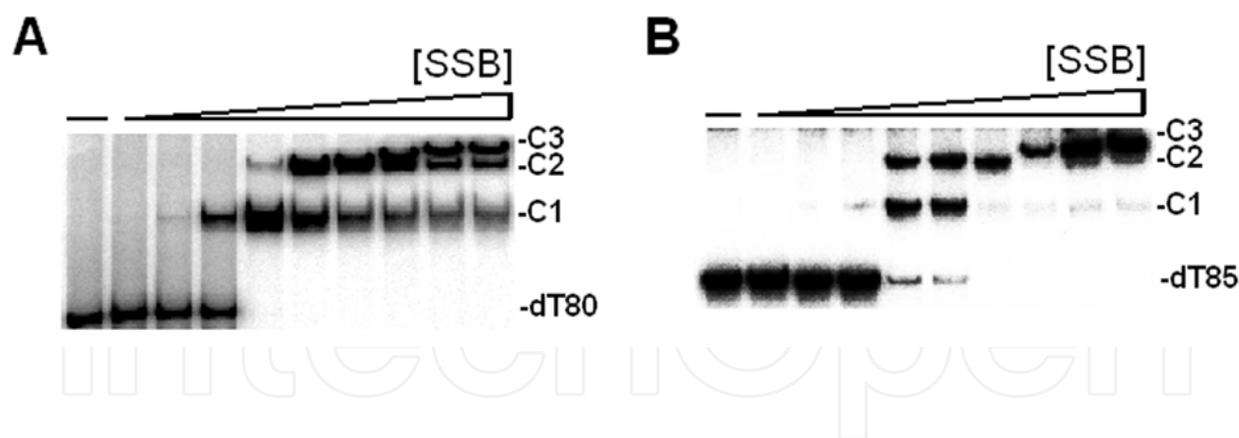


Fig. 4. Binding of *Klebsiella pneumoniae* SSB to dT80–85. The reaction solutions contained DNA substrate dT80 or dT85 and the SSB (0–5.0 μ M). SSB (0, 19, 37, 77, 155, 310, 630, 1250, 2500, and 5000 nM) was incubated for 30 min at 25 $^{\circ}$ C with 1.7 nM of (A) dT80 or (B) dT85 in a total volume of 10 μ L in 20 mM HEPES pH 7.0 and 500 mM NaCl. Aliquots (5 μ L) were removed from each reaction solution and added to 2 μ L of gel-loading solution (0.25% bromophenol blue and 40% sucrose). The resulting samples were resolved on a native 8% polyacrylamide gel at 4 $^{\circ}$ C in TBE buffer (89 mM Tris borate and 1 mM EDTA) for 1 h at 100 V and visualized by autoradiography. This SSB binding to either dT80 or dT85 forms three different complexes.

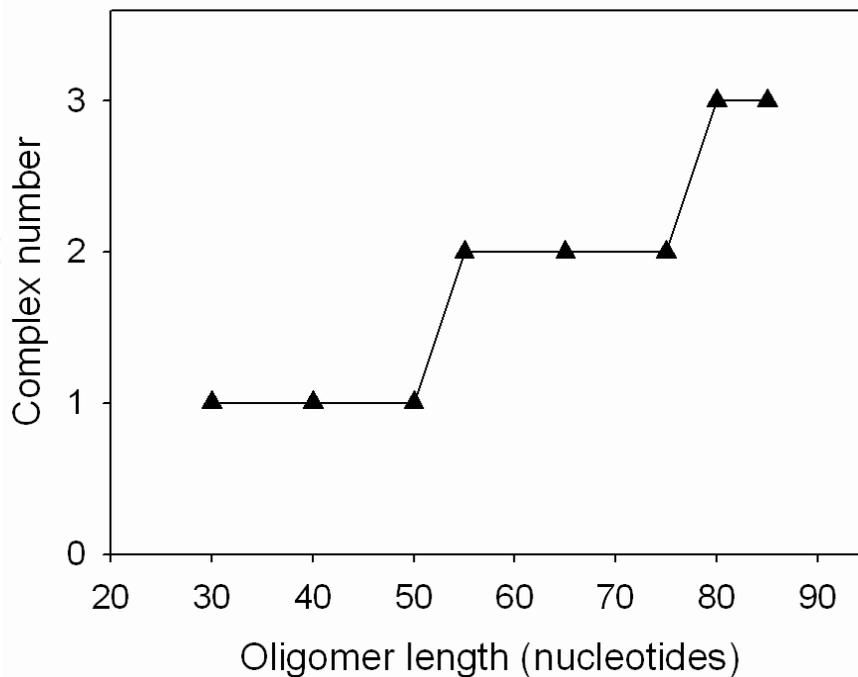


Fig. 5. Complex number of *Klebsiella pneumoniae* SSB as a function of the length of the ssDNA determined using EMSA. The binding site-size of other DNA-binding protein may also be determined using a similar protocol.

Step 5. Estimation of the apparent dissociation constants

In addition to the binding site-size, EMSA can also be used to estimate the apparent dissociation constants for the protein-DNA complexes when the protein concentration is known accurately and the used DNA is at low concentrations. If the DNA concentration is much lower than the apparent dissociation constant, its value will be equal to the protein concentration needed to have 50% of protein-DNA complex formed. The radio-intensity of free DNA and each protein-DNA complex, with increasing amounts of the protein, can be scanned and quantified by using phosphorimaging. For example, using *Salmonella enterica* serovar Typhimurium LT2 SSB (4), the apparent dissociation constant for the SSB-ssDNA complex 1 (K_{d1}) was estimated from the protein concentration that binds 50% of the input DNA; the apparent dissociation constant for the SSB-ssDNA complex 2 (K_{d2}) was estimated from the protein concentration that forms 50% of the complex 2, and K_{dn} could be also calculated in a similar manner if multiple complexes are formed.

Step 6. Analysis of oligomeric state by gel filtration chromatography

We have previously described how to determine the binding-site size of DNA-binding protein like SSB. Information about the oligomeric state of protein of interest may also be important for analysis of its binding mode. Assuming that the protein has a shape and partial specific volume similar to the standard proteins, the native molecular mass of the protein can be estimated and compared with its predicted mass in monomer from the amino acid sequence. For example, the native molecular mass for *S. enterica* serovar Typhimurium LT2 SSB is approximately 4.1 times the molecular mass of a *StSSB* monomer, and thus *S. enterica* serovar Typhimurium LT2 SSB in solution is a tetramer.

5. Conclusion

Development of more targeted drugs or antibiotics against many pathogenic bacteria has been hampered due to the lack of knowledge of DNA replication and the overall molecular biology of the organisms. For example, *K. pneumoniae* used here is a ubiquitous opportunistic pathogen that colonizes at the mucosal surfaces in humans and causes severe diseases; many clinical strains of *K. pneumoniae* are highly resistant to antibiotics. Since SSB is required for DNA replication, blocking the activity of SSB would be detrimental to bacterial survival. A characteristic of any protein-nucleic acid complex is its "site size", i.e. the average number of nucleotide residues occluded by the bound protein. Here we describe how to determine the binding-site size of DNA-binding protein using EMSA in different binding conditions. The information obtained through the use of this methodology may be very helpful in studying the binding mode(s) of DNA-binding protein. However, a particular care must be taken when the DNA-binding protein of interest binds to DNA with high cooperativity, as like PriB. If only one protein-DNA complex is visible when the length of the DNA is further increased (for example, to dT60 or more), it is likely inappropriate to determine its binding site-size using EMSA.

6. Acknowledgement

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7. Experimental protocol

Determination of the binding site-size of SSB (or other DNA-binding protein)

Equipment and reagents

- 30% acrylamide/bis- acrylamide stock solution
- Binding buffer: 20 mM Tris-HCl, pH 8.0, 100 mM NaCl. The composition of the binding buffer may be adjusted to fit the particular study or the nature of the studied protein
- Various lengths of ssDNA homopolymers. The duplex DNA can be used for other DNA-binding protein
- [$\gamma^{32}\text{P}$]ATP solution at 10mCi/mL, specific activity 3000 Ci/mmol
- T4 polynucleotide kinase
- Purified SSB protein
- Loading buffer: 0.25% bromophenol blue and 40% sucrose
- TBE buffer (89 mM Tris borate and 1 mM EDTA)
- Vertical electrophoresis system and a power pack

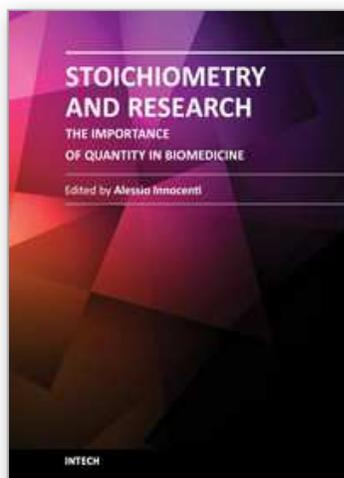
Experimental method

1. Phosphorylate the ssDNA homopolymers of varying length in 10 μL of T4 polynucleotide kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , 5 mM dithiothreitol) supplemented with 1 μL of [$\gamma^{32}\text{P}$]ATP and 1 μL of T4 polynucleotide kinase. Incubate at 37 $^\circ\text{C}$ for 1 h.
2. Dilute radiolabeled DNA substrates to suitable concentration (for example, 1–10 nM; for determination of the apparent dissociation constant) using binding buffer.

3. Incubate radiolabeled DNA substrates with different concentrations of protein, respectively, for 30 min at 25 °C, in a total volume of 10 μ L in binding buffer.
4. Remove aliquots (5 μ L) from each reaction solution and add it to 2 μ L of loading buffer and load on a native 8% polyacrylamide gel at 4°C in TBE buffer for 1–2 h at 100 V, depending on the length of DNA.
5. Dry the gel under vacuum and autoradiograph.
6. Summarize the number of DNA-protein complexes formation for each length of DNA used.
7. Evaluate DNA length required for two and three complexes formation.
8. Calculate the length (the binding site-size) needed for one protein binding.

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The aim of this book is to provide an overview of the importance of stoichiometry in the biomedical field. It proposes a collection of selected research articles and reviews which provide up-to-date information related to stoichiometry at various levels. The first section deals with host-guest chemistry, focusing on selected calixarenes, cyclodextrins and crown ethers derivatives. In the second and third sections the book presents some issues concerning stoichiometry of metal complexes and lipids and polymers architecture. The fourth section aims to clarify the role of stoichiometry in the determination of protein interactions, while in the fifth section some selected experimental techniques applied to specific systems are introduced. The last section of the book is an attempt at showing some interesting connections between biomedicine and the environment, introducing the concept of biological stoichiometry. On this basis, the present volume would definitely be an ideal source of scientific information to researchers and scientists involved in biomedicine, biochemistry and other areas involving stoichiometry evaluation.

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