Electrophoretic mobility shift assay (EMSA) by using biotins to detect protein-DNA interactions

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Introduction

Electrophoretic mobility shift assay (EMSA) is based on the simple rationale that proteins of differing size, molecular weight, and charge will have different electrophoretic mobilities in a non-denaturing gel matrix. In the case of a DNA–protein complex, the presence of a given DNA-binding protein will cause the DNA to migrate in a characteristic manner, usually more slowly than the free DNA, and will thus cause a change or shift in the DNA mobility visible upon detection (Figure 1). The EMSA protocols in our lab are based on the LightShift Chemiluminescent EMSA system (Thermo scientific) that perform gel shift assay without radioisotopes or digoxigenin. The system not only has high sensitivity that surpasses radioactive and digoxigenin method, but also is compatible with previously established binding conditions for popular DNA-protein interactions.

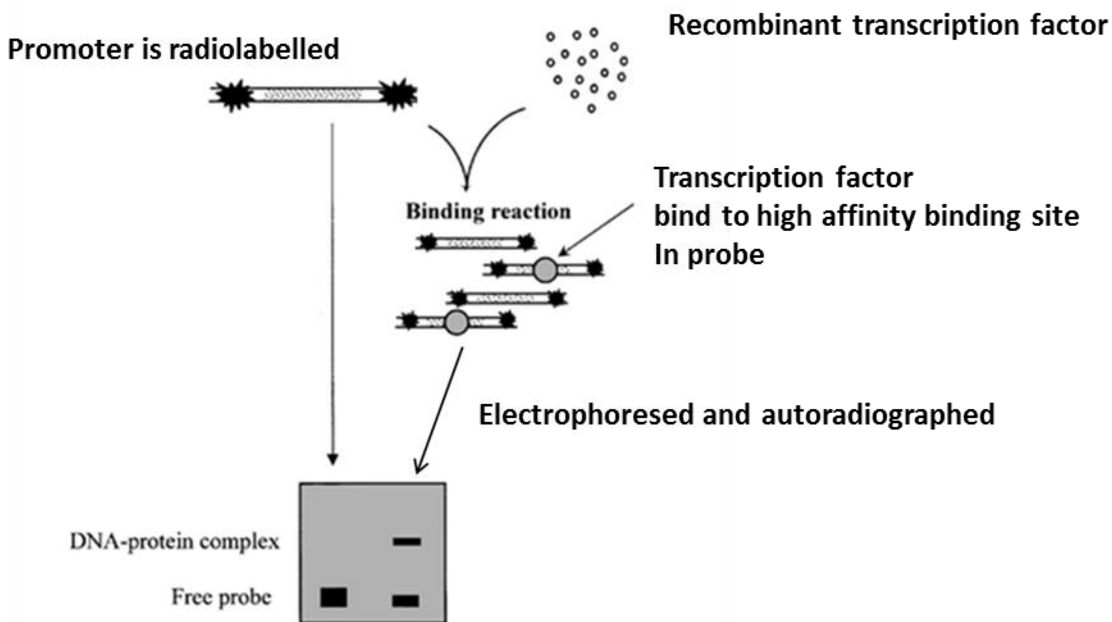


Figure 1. The principle of Electrophoretic mobility shift assay (EMSA) detecting protein-DNA interaction

Materials and Equipments

Materials:

Biotin–EBNA Control DNA, 50µL, 10fmol/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

The 60 bp biotin end-labeled duplex contains the following binding site:

5' BIOTIN-…TAGCATATGCTA…-3'

3'-…ATCGTATACGAT…-BIOTIN 5'

Unlabeled EBNA DNA, 50µL, 2pmol/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

The ~25 bp duplex contains the following binding site:

5'-…TAGCATATGCTA…-3'

3'-…ATCGTATACGAT…-5'

Epstein-Barr Nuclear Antigen (EBNA) Extract, 125µL, store at -20°C

Poly (dI•dC), 125µL, 1µg/µL in 10mM Tris, 1mM EDTA; pH 7.5, store at -20°C

50% Glycerol, 500µL, store at -20°C

1% NP-40, 500µL, store at -20°C

1 M KCl, 1mL, store at -20°C

100mM $MgCl_2$, 500µL, store at -20°C

200mM EDTA pH 8.0, 500µL, store at -20°C

5X Loading Buffer, 1mL, store at -20°C

Stabilized Streptavidin-Horseradish Peroxidase Conjugate, 1.5mL, store at 4°C

Chemiluminescent Substrate, stable for 6 months at room temperature or 1 year at 4°C

Luminol/Enhancer Solution, 80mL

Stable Peroxide Solution, 80mL

Blocking Buffer, 500mL, store at 4°C

4X Wash Buffer, 500mL, store at 4°C

Substrate Equilibration Buffer, 500mL, store at room temperature or 4°C

Positively charged nylon membrane

5X TBE (450mM Tris, 450mM boric acid, 10mM EDTA, pH 8.3)

Equipments:

X-ray film or CCD camera

UV lamp or crosslinking device equipped with 254nm bulbs or 312nm transilluminator

Electrophoresis apparatus

Electroblotter or capillary transfer apparatus

High-quality blotting paper

Circulating water bath

Plastic forceps

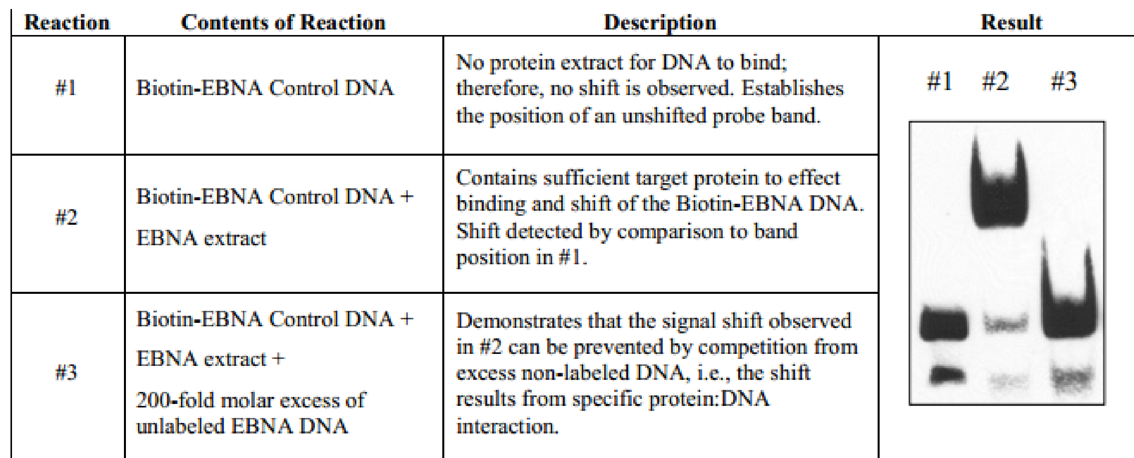
Polyacrylamide gel in 0.5X TBE

Procedures for electrophoretic mobility shift assays

A. Designs for EMSA experiments

1. Perform positive control reaction and understanding the EMSA experiments (Table 1)

Before we start experiment, the principle of EMSA should be showed. As in Table 1, line 1 is a negative control that running biotin-EBNA control DNA without any proteins. No protein in line1, no shift is observed. Therefore, it will show the position of an unshifted probe band. When biotin-EBNA control DNA is added with EBNA extract, the band showing DNA-protein complex appeared in line 2. Then when you add many fold excess of unlabeled EBNA DNA into the mix of biotin-EBNA control DNA and EBNA extract, the band showing DNA-protein complex will disappear because the unlabeled DNA successfully bind with protein by compete with labeled DNA. For every EMSA, this three binding experiments should be performed.



Reaction	Contents of Reaction	Description	Result
#1	Biotin-EBNA Control DNA	No protein extract for DNA to bind; therefore, no shift is observed. Establishes the position of an unshifted probe band.	#1 #2 #3
#2	Biotin-EBNA Control DNA + EBNA extract	Contains sufficient target protein to effect binding and shift of the Biotin-EBNA DNA. Shift detected by comparison to band position in #1.	
#3	Biotin-EBNA Control DNA + EBNA extract + 200-fold molar excess of unlabeled EBNA DNA	Demonstrates that the signal shift observed in #2 can be prevented by competition from excess non-labeled DNA, i.e., the shift results from specific protein:DNA interaction.	

2. Pipeline for EMSA experiments.

EMSA experiments usually contain several steps including gel preparation, binding reaction, electrophoreses binding reaction, transfer binding reaction into nylon membrane, crosslink transferred DNA to membrane, and detection (Figure 2).

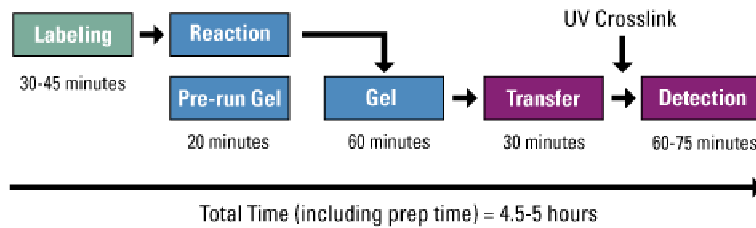


Figure 2. The pipeline of EMSA by using the LightShift Chemiluminescent EMSA system

B. Prepare and Pre-Run Gel

1. Prepare a native polyacrylamide gel in 0.5X TBE or use a pre-cast DNA retardation gel. The appropriate polyacrylamide percent depends on the size of the target DNA and the binding protein. Most systems use a 4 -6% polyacrylamide gel in 0.5X TBE.
2. Place the gel in the electrophoresis unit, and clamp it to obtain a seal. Fill the inner chamber with 0.5X TBE to a height several millimeters above the top of the wells. Fill the outside of the tank with 0.5X TBE to just above the bottom of the wells, which reduces heat during electrophoresis. Flush wells and pre-electrophorese the gel for 30-60 minutes. Apply 100V for an $8 \times 8 \times 0.1$cm gel.
3. Proceed to Section C while gel is pre-electophoresing

C. Prepare and Perform Binding Reactions

Notes:

• Include controls in the assay to ensure the system is working properly (see Procedure, Section A).

• Do not vortex the Control DNA or the EBNA extract.

1. Thaw all binding reaction components, EBNA Control System components and Test System samples, and place them on ice. Do not thaw the EBNA Extract until immediately before use. Thaw the EBNA Extract at room temperature. DO NOT heat the EBNA Extract, which includes thawing in your hand.
2. Prepare complete sets of 20 binding reactions for the Control EBNA System and/or the Test System according to Procedure Section A, Tables 2 and 3; add the reagents in the order listed in the tables. Do not vortex tubes at any time during this procedure.
3. Incubate binding reactions at room temperature for 20 minutes.
4. Add 5µL of 5X Loading Buffer to each 20µL binding reaction, pipetting up and down several times to mix. DO NOT vortex or mix vigorously

D. Electrophorese Binding Reactions

1. Switch off current to the electrophoresis gel.
2. Flush the wells and then load 20µL of each sample onto the polyacrylamide gel.

3. Switch on current (set to 100V for 8 × 8 × 0.1cm gel) and electrophorese samples until the bromophenol blue dye has migrated approximately 2/3 to 3/4 down the length of the gel. The free biotin-EBNA Control DNA duplex migrates just behind the bromophenol blue in a 6% polyacrylamide gel.

E. Electrophoretic Transfer of Binding Reactions to Nylon Membrane

1. Soak nylon membrane in 0.5X TBE for at least 10 minutes.
2. Sandwich the gel, nylon membrane and blotting paper in a clean electrophoretic transfer unit according the manufacturer's instructions. Use 0.5X TBE cooled to ~10ºC with a circulating water bath. Use very clean forceps and powder-free gloves, and handle the membrane only at the corners.

Note: Use clean transfer sponges. Avoid using sponges that have been used in Western blots.
3. Transfer at 380mA (~100V) for 30 minutes. Typical transfer times are 30-60 minutes at 380mA using a standard tank transfer apparatus for mini gels (8 × 8 × 0.1cm).
4. When the transfer is complete, place the membrane with the bromophenol blue side up on a dry paper towel. (There should be no dye remaining in the gel.) Allow buffer on the membrane surface to absorb into the membrane. This will only take a minute. Do not let the membrane dry. Immediately proceed to Section F.

F. Crosslink Transferred DNA to Membrane

Crosslink at 120mJ/cm2 using a commercial UV-light crosslinking instrument equipped with 254nm bulbs (45-60 second exposure using the auto crosslink function).

After the membrane is crosslinked, proceed directly to Section G. Alternatively, the membrane may be stored dry at room temperature for several days.
Notes: Do not allow the membrane to get wet again until ready to proceed with Section G.

G. Detect Biotin-labeled DNA by Chemiluminescence

The recommended volumes are for an 8 × 10cm membrane. If larger gels are used, adjust volumes in Section G accordingly. Perform all blocking and detection incubations in clean trays or in plastic weigh boats on an orbital shaker.
1. Gently warm the Blocking Buffer and the 4X Wash Buffer to 37-50°C in a water bath until all particulate is dissolved. These buffers may be used between room temperature and 50°C as long as all particulate remains in solution. The Substrate Equilibration Buffer may be used between 4°C and room temperature.
2. To block the membrane add 20mL of Blocking Buffer and incubate for 15 minutes with gentle shaking.
3. Prepare conjugate/blocking buffer solution by adding 66.7µL Stabilized Streptavidin-Horseradish Peroxidase Conjugate to 20mL Blocking Buffer (1:300 dilution).

Note: This conjugate/blocking buffer solution has been optimized for the Nucleic Acid Detection Module and should not be modified.

4. Decant blocking buffer from the membrane and replace it with the conjugate/blocking solution. Incubate membrane in the conjugate/blocking buffer solution for 15 minutes with gentle shaking.
5. Prepare 1X wash solution by adding 40mL of 4X Wash Buffer to 120mL of ultrapure water.
6. Transfer membrane to a new container and rinse it briefly with 20mL of 1X wash solution.
7. Wash membrane four times for 5 minutes each in 20mL of 1X wash solution with gentle shaking.
8. Transfer membrane to a new container and add 30mL of Substrate Equilibration Buffer. Incubate membrane for 5 minutes with gentle shaking.
9. Prepare Substrate Working Solution by adding 6mL Luminol/Enhancer Solution to 6mL Stable Peroxide Solution.

Note: Exposure to the sun or any intense light can harm the Working Solution. Keep the Working Solution in an amber bottle and avoid prolonged exposure to intense light. Short-term exposure to typical laboratory lighting will not harm the Working Solution.

10. Remove membrane from the Substrate Equilibration Buffer, carefully blotting an edge of the membrane on a paper towel to remove excess buffer. Place membrane in a clean container or onto a clean sheet of plastic wrap placed on a flat surface.
11. Pour the Substrate Working Solution onto the membrane so that it completely covers the surface. Alternatively, the membrane may be placed DNA side down onto a puddle of the Working Solution. Incubate membrane in the substrate solution for 5 minutes without shaking.
12. Remove membrane from the Working Solution and blot an edge of the membrane on a paper towel for 2-5 seconds to remove excess buffer. Do not allow the membrane to become dry.
13. Wrap the moist membrane in plastic wrap, avoiding bubbles and wrinkles.