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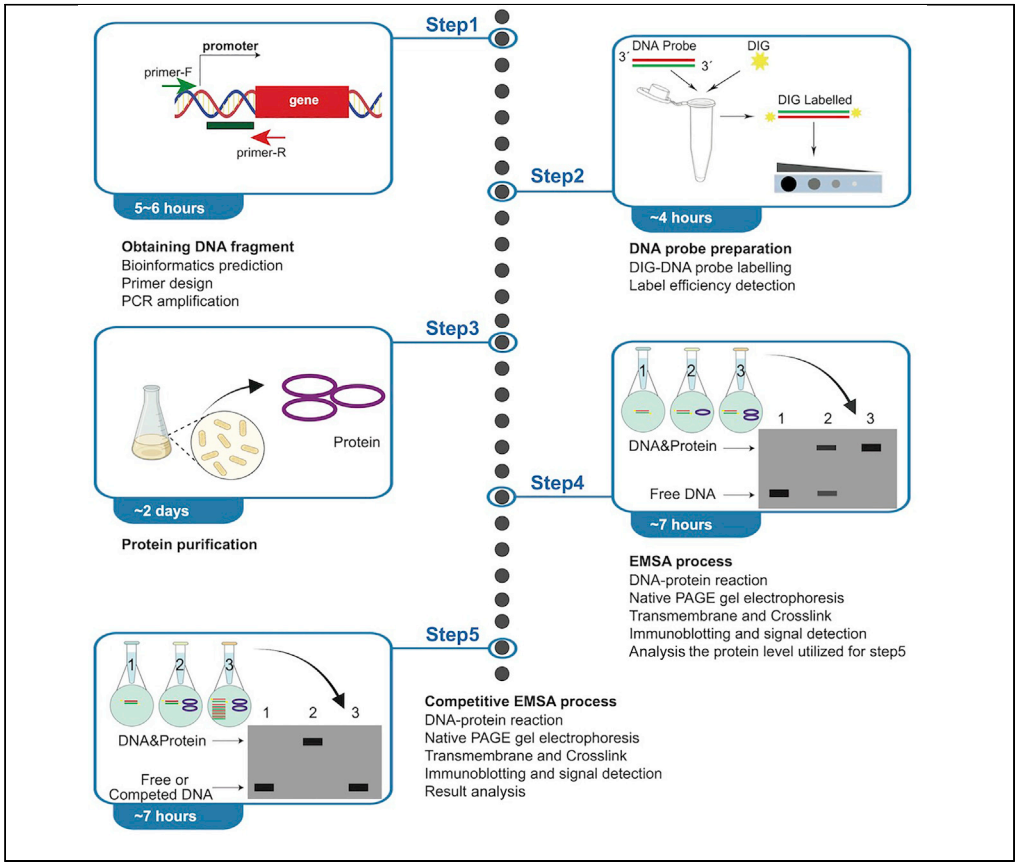
Protocol to detect nucleotide-protein interaction *in vitro* using a non-radioactive competitive electrophoretic mobility shift assay



Electrophoretic mobility shift assay (EMSA) is a classical and popular approach for DNA/RNA protein-binding affinity detection *in vitro*. This protocol describes a competitive EMSA assay using digoxigenin (DIG)-labeled probe, which solves the safety issues and limitations attributed to the short lifespan of the ^{32}P-radiolabeled DNA probe. We detail steps for DNA probe preparation, protein-DNA mixture coincubation, EMSA, and competitive EMSA process. We optimize the standard DIG-ddUTP-labeling EMSA protocol to high sensitivity with reproducible results.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A robust protocol for *in vitro* detection of protein-DNA interactions

A streamlined EMSA approach using non-radioactive digoxigenin-labeled probes

Determination of protein-DNA binding specificity by competition with unlabeled probes

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Protocol to detect nucleotide-protein interaction *in vitro* using a non-radioactive competitive electrophoretic mobility shift assay

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SUMMARY

Electrophoretic mobility shift assay (EMSA) is a classical and popular approach for DNA/RNA protein-binding affinity detection *in vitro*. This protocol describes a competitive EMSA assay using digoxigenin (DIG)-labeled probe, which solves the safety issues and limitations attributed to the short lifespan of the ^{32}P-radiolabeled DNA probe. We detail steps for DNA probe preparation, protein-DNA mixture coincubation, EMSA, and competitive EMSA process. We optimize the standard DIG-ddUTP-labeling EMSA protocol to high sensitivity with reproducible results.

For complete details on the use and execution of this protocol, please refer to Feng et al. (2022).

BEFORE YOU BEGIN

This protocol describes a reliable and detailed approach for the detection of DNA and protein complexes *in vitro* through competitive electrophoretic mobility shift assay (EMSA). A number of non-radioactive methods are available currently, such as the direct fluorescence staining of proteins and DNA in the PAGE shifting gels with fluorescent dye SYBR (Jing et al., 2003), immunodetection of biotin (Ludwig et al., 1995) or digoxigenin (DIG)-conjugated protein-DNA complex (Qi et al., 2006), as well as EMSA with infrared fluorescence-labeled DNA (Hsieh et al., 2016). The method employing DIG-ddUTP labeled probes is much more popular because it yields convincing results on confirmation analysis of the protein and its DNA binding capabilities *in vitro*. This protocol describes the details that help make the method reproducible and robust. We used *Escherichia coli* expressed protein AirA and the DNA fragment from gene *ler* promoter region, which has been demonstrated with strong binding affinity in enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 EDL933 (Feng et al., 2022) as examples to illustrate the different steps.

While the experimental materials described in this protocol used *E. coli* protein and DNAs, we have also used this protocol successfully in *Salmonella typhimurium*. Moreover, the protocol is applicable and reproducible for eukaryotic proteins and DNAs detection.

All buffers in the tables (buffers section) need to be prepared in advance and stored at the indicated temperature. These reagents that should be freshly prepared were well noted in each step correspondingly.

Reagents and gel preparation

Timing: 1 day

1. Reagents preparation.
   a. Prepare and store stock solutions as indicated in the manual.
   b. Freshly prepare working solutions on the day of polyacrylamide gel electrophoresis (PAGE) gel running as indicated at the below tables.

   Note: All the stock solutions except for commercially-sourced reagents should be filter-sterilized by passaging through a 0.22 μm filter.

2. Fresh native-PAGE gel preparation.

   Note: At least two slides of native-PAGE gels are needed for each EMSA procedure described in this protocol. Normally, 6% gel in 10 well, 1.0 mm glass gel systems are required.

6% native-PAGE gel prepared for gel electrophoresis

Reagent	Final concentration	Amount
40% Acryl/Bis solution (37.5:1)	6% (v/v)	3 mL
5× TBE	1×	4 mL
10% Ammonium persulfate (AP)	0.1% (v/v)	200 μL
TEMED	0.1% (v/v)	20 μL
ddH_2O	N/A	12.78 mL
Total	**N/A**	**20 mL**

Note: PAGE gels are best prepared on the day of the EMSA experiment and allowed to polymerize for at least one hour.

⚠ CRITICAL: Acryl/Bis solution, AP and TEMED are toxic and users of this protocol should exercise caution while handling them. Gels should be made in a fume hood.

Recombinant protein AirA expression and purification

Timing: 2 days

This section is for the expression and purification of the recombinant protein AirA. This EMSA analysis protocol can be applied to proteins of different types: cytoplasmic or nuclear proteins, purified protein fragments or cellular expressed recombinant proteins, heterologous expressed proteins, as well as those *in vitro* synthesis protein fragments. For the purpose of this protocol, the AirA protein with an N-terminal 6×His-epitope was recombinantly expressed in and purified from the *E. coli* BL21 DE3 strain.

3. Express the AirA-His_6 protein from the vector pET28a in *E. coli* BL21 DE3 strain, and purify the protein through affinity chromatography.
   a. Measure the protein concentration.
      i. Use Pierce BCA Protein Assay Kit (Thermo Scientific, Cat# 23227) for the protein concentration measurement following the manufacturer's instructions (https://www.thermofisher.cn/document-connect/document-connect.html?url=https://assets.thermofisher.cn/TFS-Assets%2FLSG%2Fmanuals%2FMAN0011430_Pierce_BCA_Protein_Asy_UG.pdf).
      ii. Aliquot 50 μL of those proteins with concentrations greater than 100 nM in each 0.2 mL tube.
      iii. Store proteins at −80°C for further utilization.

Table 1. Set of primers used for amplification of DNA fragments

Primer name	Source	Primer sequence (5′-3′)
P_{ler}-Forward	GENEWIZ	TCCTGGGGATTCACTCGCTTG
P_{ler}-Reverse	GENEWIZ	TCATAATAAATAATCTCCGC
rpoS-Forward	GENEWIZ	CTTCCAGTGTTGCCGCT
rpoS-Reverse	GENEWIZ	CCCGTACTATTCGTTTGCC

b. Thaw the proteins on ice before performing downstream steps.

Note: Affinity chromatography is not the sole or best way for protein purification. Protein purification step is often experience-oriented, either way can be preferred depending on each laboratories' experience, such as ion exchange chromatography and gel exclusion chromatography, among others.

Note: High protein purity, concentration and activity are often key to the success and reproducibility of the experiment. If the starting material is crude cytoplasmic or nuclear proteins, a concentration step is normally required. Proteins can be concentrated with centrifugal filters (e.g., the Millipore Amicon Ultra ones).

⚠ CRITICAL: High protein purity without other interfering protein contamination is often the key for measuring its binding affinity to DNA. In order to rule out the interference of non-specific binding of protein and DNA, which would possibly lead to false positive or negative conclusions, protein quality should be ensured. In addition, adding heparin or other non-specific DNA such as poly [d(I-C)] into the EMSA reaction system is an effective method to prevent the non-specific binding of proteins to DNA (troubleshooting 1 and 2).

Amplification and purification of DNA fragments

Timing: 5 h

This section describes the steps of amplification and purification of DNA fragments. Typically, 300 bp to 500 bp DNA fragments located at upstream of the transcription start site of target genes are amplified for subsequent EMSA experiments.

4. Perform the PCR amplification of the promoter region of gene *ler* and the control gene *rpoS*.
   a. PCR amplify the DNA fragment [size=439 bp] from the promoter region of gene *ler* using Ex Taq (Takara) and primers P_{ler}-Forward and P_{ler}-Reverse as follows (Table 1) using below program indicated in the Table 2.
   b. A similar length of DNA fragment [size=384 bp] was selected and amplified for the control gene *rpoS*.

Note: The size of DNA fragment should be elaborately designed, preferably within 500 bp. DNA fragments longer than 500 bp could cause the target protein to bind to multiple sites on the detected DNA in subsequent reactions (troubleshooting 1).

Table 2. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	95°C	30 s	
Annealing	55°C	30 s	25–35 cycles
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

5. DNA quality detection and purification.
   a. Analyze the qualities of PCR products on a 1% agarose gel.
   b. Purify DNAs using QIAquick Gel Extraction Kit (QIAGEN, Cat# 28704) following the manufacturer's instructions (https://www.qiagen.com/cn/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-gel-extraction-kit/?catno=28704).

Note: The elution volume of purified DNA is typically 30 μL, and the final concentration should be greater than 100 ng/μL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Digoxigenin-AP, Fab fragments (1:10,000 dilution)	Roche	Cat# 11093274910
Bacterial and virus strains		
EHEC O157:H7 EDL933	ATCC	43895
BL21(DE3) Chemically Competent Cells	TransGen Biotech	Cat# CD601-02
Chemicals, peptides, and recombinant proteins		
Ex Taq DNA Polymerase	Takara	Cat# RR001A
DIG-ddUTP solution	Roche	Cat# 11363905910
Terminal Transferase, 400 U	Roche	Cat# 3333566001
Blocking Reagent	Roche	Cat# 11096176001
CSPD, ready-to-use	Roche	Cat# 11755633001
poly [d(I-C)]	Roche	Cat# 10108812001
AirA-His_6	This study	N/A
Critical commercial assays		
Pierce BCA Protein Assay Kit	Thermo Scientific	Cat# 23227
QIAquick Gel Extraction Kit	QIAGEN	Cat# 28704
Oligonucleotides		
P_{ler}-Forward: TCCTGGGGATTCACTCGCTTG	This study	N/A
P_{ler}-Reverse: TCATAATAAATAATCTCCGC	This study	N/A
rpoS-Forward: CTTCCAGTGTTGCCGCT	This study	N/A
rpoS-Reverse: CCCGTACTATTCGTTTGCC	This study	N/A
Recombinant DNA		
pET28a (+) plasmid encoding AirA of EHEC O157:H7	(Feng et al., 2022)	N/A
Other		
Nylon membrane	Roche	Cat# 11417240001
T series Multi-Block Thermal Cycler	LongGene	T20
Extra Thick Blot Paper	Bio-Rad	Cat# 1703955
Oven	Tianyu	DGG-10
Water Bath	OuNuo	SY-1-2
Mini-PROTEAN Tetra cell	Bio-Rad	1658004
Mini Trans-Blot module	Bio-Rad	1658036
Image system	GE	Amersham Imager 600
0.2 mL PCR tube	Axygen	PCR-02D-C
0.22 μm filter	Merck	Cat# CLS431212

MATERIALS AND EQUIPMENT

5× Labelling buffer

Reagent	Final concentration	Amount
Potassium cacodylate	1 M	0.214 g
Tris-HCl (1 M, pH 7.4)	0.125 M	125 μL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
Bovine serum albumin	0.125% (w/v)	1.25 mg
ddH_2O	N/A	~ 875 μL
Total	**N/A**	**1 mL**

Store the solution at −20°C after sterilization. Use it within one year.

5× Binding buffer

Reagent	Final concentration	Amount
HEPES	100 mM	1 g
EDTA (0.5 M, pH 8.0)	5 mM	100 μL
$(NH_4)_2SO_4$	50 mM	66.07 mg
DTT (0.05 M)	5 mM	1 mL
Tween 20	1% (v/v)	100 μL
KCl (3 M)	150 mM	500 μL
ddH_2O	N/A	~ 9.3 mL
Total	**N/A**	**10 mL**

Store the solution at 4°C after sterilization. Use it within six months.

5× TBE buffer

Reagent	Final concentration	Amount
Tris	445 mM	53.91 g
Boric acid	445 mM	27.51 g
EDTA (0.5 M, pH 8.0)	10 mM	20 mL
ddH_2O	N/A	~ 980 mL
Total	**N/A**	**1 L**

Store the solution at 25°C after sterilization. Use it within six months.

0.5× TBE buffer for native-PAGE gel running & membrane transfer buffer

Reagent	Final concentration	Amount
5× TBE buffer	0.5×	100 mL
ddH_2O	N/A	900 mL
Total	**N/A**	**1 L**

Store the solution at 25°C after sterilization. Use it within six months.

5× Loading buffer, with bromophenol blue

Reagent	Final concentration	Amount
5× TBE buffer	0.25×	50 μL
Glycerol	34% (v/v)	340 μL
Bromophenol blue	0.2% (w/v)	2 mg
ddH_2O	N/A	610 μL
Total	**N/A**	**1 mL**

Store the solution at −20°C. Use it within one year.

Maleic acid buffer, pH 7.5

Reagent	Final concentration	Amount
Maleic acid (1 M)	0.1 M	100 mL
NaCl (3 M)	0.15 M	50 mL
ddH_2O	N/A	850 mL
Total	**N/A**	**1 L**

Store the solution at 25°C after sterilization. Use it within six months.

Note: Adjust pH to 7.5 using NaOH.

10× Blocking buffer		
Reagent	Final concentration	Amount
Blocking reagent	10% (w/v)	10 g
Maleic acid buffer	N/A	100 mL
Total	**N/A**	**100 mL**
The dissolved buffer can be stored at 4°C for one week.		

Note: The Blocking reagent is difficult to dissolve. The reagent should be taken out from −20°C in advance and put in 65°C water bath with constant stirring for approximately one hour to dissolve, and to ensure that no precipitate in the solution.

1× Blocking buffer		
Reagent	Final concentration	Amount
10× Blocking buffer	1×	20 mL
Maleic acid buffer	N/A	180 mL
Total	**N/A**	**200 mL**
Freshly prepare the 1× Blocking buffer, do not store it.		

Washing buffer		
Reagent	Final concentration	Amount
Tween 20	0.3% (v/v)	3 mL
Maleic acid buffer	N/A	1 L
Total	**N/A**	**1 L**
Store the solution at 25°C after sterilization. Use it within six months.		

Detection buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M, pH 9.5)	0.1 M	100 mL
NaCl (3 M)	0.1 M	33.3 mL
ddH_2O	N/A	866.7 mL
Total	**N/A**	**1 L**
Store the solution at 25°C after sterilization. Use it within six months.		

STEP-BY-STEP METHOD DETAILS

DIG-label DNA and label efficiency detection

Timing: 4 h

This step describes how to label DNA probe with DIG and perform the label efficiency detection. During this step, the DIG-ddUTP solution and Terminal Transferase were utilized for labeling the 3′ end of DNA. Oligonucleotides, including either RNA and single- or double-stranded DNA can be labeled with high efficiency (Green and Sambrook, 2022; Hocquellet et al., 1997). Depending on the probe labeling efficiency, this step can be repeated as needed and probes can be pooled together for further utilization. The DIG-labeled probe with a high detection efficiency is paramount to obtaining optimal and reproducible results.

1. DIG-labeling of the target DNA fragment.
   a. Aliquot 200 ng of the *ler* promoter DNA fragments in a 0.2 mL PCR tube and add PCR-grade water to the reaction to a total volume of 10 μL.
   b. Prepare the following reaction buffer (Table 3) on ice and gently mix the buffer with a pipette.
   c. Add 10 μL of the above reaction buffer to step (a) tube with target DNA fragment, mix gently by pipetting.
   d. Incubate the reaction tube in a PCR machine as indicated in the below program (Table 4). Set the lid temperature at 95°C.

   Note: The temperature and incubation time is critical for the reaction. To ensure accuracy, pre-set the PCR machine to 37°C before starting the DIG label reaction program.

   Optional: After the DIG label reaction is complete, it is optimal to set the denaturation step to 95°C for 5 min, followed by slow cooling at 25°C for DNA annealing. This step can ensure the thorough dissociation of the left Terminal Transferase with the DIG-labeled DNA inside the reaction, thus eliminating the non-specific binding band of Terminal Transferase in the following step (Tokunaga and Stegeman, 2014).

   e. Add 2 μL 0.2 M EDTA (pH 8.0) to above reaction tube to stop the labeling reaction.
   f. Add 3 μL of PCR-graded water to the reaction to a total volume of 25 μL. The final concentration for DIG-labeled DNA is 8 ng/μL.
   g. Repeat the DNA labeling reaction and process as described in steps a-f for the negative control gene *rpoS*.

   Note: Different negative control genes can be selected according to the type of target protein. Herein, the conserved housekeeping gene *rpoS* coding for a subunit of RNA polymerase was selected (Dong and Schellhorn, 2009).

2. Label efficiency detection for DIG-labeled DNA.
   a. Make ten-fold serial dilutions of the above-labeled DNA using PCR-graded water in 0.2 mL PCR tubes.
      i. Mark the tubes with DNA concentrations from high to low as No. 1–4.
      ii. Mark the control tube without DNA probe as No. 5 according to Table 5.
   b. Cut a small piece of nylon membrane (1 cm × 5 cm) and orderly spot 1 μL of samples from tubes No. 1–5 onto the membrane.
   c. Put the nylon membrane into a 120°C oven for 15 min with an Extra Thick Blot Paper at the bottom to perform the crosslinking step, during which step the nucleic acid can be fixed to the membrane.

   Note: Nylon membrane is a positively charged membrane with a pore diameter of 0.45 μm, which is widely applicable in Southern, Northern, and spotting blot. A strong detection but low background signal can be achieved through color or chemiluminescence detection in DIG-labeled probe hybridization process. Therefore, the nylon membrane is pivotal in signal capture for obtaining optimal results during the protocol processing.

   Note: It is better to prepare and spot probes with serial dilutions of DNA with concentrations ranging from high to low, and cut a triangle mark at the upward right side of the nylon membrane to mark the side of the membrane. Make sure the membrane with probes is always right-side up and take care not to invert or rotate the membrane.

   Note: Turn on the oven to 120°C in advance; be equipped with proper gloves to prevent accidental injury during the experimental procedure.

Optimal: UV crosslinking can also be performed for nucleic acid fixing to the membrane with a following-up air-dry step for approximately 30 min.

Pause point: It is possible to pause at this point, and the baked membrane can be stored at 4°C for 1 week between two pieces of Blot paper (3 mm).

d. Wash the membrane.
   i. Take the membrane out from the oven carefully and put it in an appropriate plastic container (2 cm × 8 cm).
   ii. Soak the membrane properly in 10 mL Washing buffer and incubate it at 25°C with horizontal shaking for 2 min at 60 rpm.
e. Block the membrane.
   i. Pour out the Washing buffer carefully, take care not to disturb the membrane inside.
   ii. Add 10 mL 1× Blocking buffer and incubate it at 25°C with horizontal shaking for 30 min at 60 rpm.

Optimal: As an alternative, several other commercial Blocking solutions are also available. Alternatively, a manually prepared Blocking buffer containing 1× Maleic acid buffer with 100 μg/mL herring sperm DNA and 3% bovine serum albumin (BSA) can also be used.

f. Equilibrate the membrane with Anti-DIG antibody.
   i. Pour out the Blocking buffer, take care not to disturb the membrane inside.
   ii. Add 10 mL Anti-DIG antibody solution (Dilute Anti-DIG-AP antibody 1:10,000 in 1× Blocking buffer) and incubate it at 25°C with horizontal shaking for 30 min at 60 rpm.
g. Pour out the Anti-DIG antibody solution, take care not to disturb the membrane inside. Add 10 mL Washing buffer and incubate it at 25°C with horizontal shaking for 15 min at 60 rpm.
h. Repeat the above washing step (g) once.
i. Equilibrate the membrane with Detection buffer.
   i. Pour out the Washing buffer, take care not to disturb the membrane inside.
   ii. Add 10 mL Detection buffer and equilibrate the membrane at 25°C with horizontal shaking for 2–5 min at 60 rpm.
j. Incubate the right-side membrane with DNA probe with 0.5 mL CSPD for 5 min at 25°C. Take caution that the membrane should not be exposed to light during this step.
k. Chemiluminescent exposure of the membrane in Amersham Imager 600 with elongated times from 10 s till 5 min for both the *ler* promoter and *rpoS* probes.

Optional: The tray of Amersham Imager 600 is optional to cover with a proper sized plastic wrap for membrane incubation with CSPD, which can save the amount of CSPD and hence maximize its work efficiency before performing immunochemistry exposure.

l. Save each scanning image and compare the labeling efficiency of target and control DNA probes.

Note: Make sure the nylon membrane with probes is maintained right-side up during washing, equilibrating, and detection.

Note: The labeled DNA probes can be stored at 4°C for short time (within 1 month). The probes can be stored at −20°C long-term.

⚠ CRITICAL: Probe labeling success is achieved if clear signals can be detected for the 0.008 ng DIG-labeled DNA dot within 5 min of exposure. The labeled probe with the lowest detection signal in 0.08 ng DNA dot can also be utilized albeit with lower efficiency, but higher amounts of probes should be used in the following steps.

Table 3. Mixture for DIG-label reaction

Reagent	Final concentration	Amount
5× Labelling buffer	2×	4 μL
$CoCl_2$ solution (25 mM)	10 mM	4 μL
DIG-ddUTP solution (1 mM)	0.1 mM	1 μL
Terminal Transferase (400 U/μL)	40 U/μL	1 μL
Total	N/A	10 μL

However, other situations indicated the labeling DNA was not sufficient, and it was necessary to check the experimental steps and re-conduct the DIG labeling process (troubleshooting 3).

Pause point: It is possible to pause at this point.

Coincubation and reaction of the DNA and target protein mixture

Timing: 1 h

This step describes how to perform the DNA and target protein mixture reaction. If the detected protein has binding affinity to the tested nucleic acid, protein-nucleic acid complexes are expected to occur after coincubation under appropriate time and conditions. The larger of the molecular weight of the complex, the slower the migration on polyacrylamide gel electrophoresis compared with the probes without bounded protein. Based on this theory, a retarded phenomenon on the gel displaying a "shifted" band at the front of the membrane in comparison to the mobility of the free probe indicates that there is a binding interaction activity between the target protein and the DNA probe (Figure 1) (Holden and Tacon, 2011).

3. Prepare the reaction mixture for AirA protein and *ler* promoter or *rpoS* DNA fragment.
   a. Prepare the AirA protein and *ler* promoter DNA fragment mixture reactions in 0.2 mL tubes on ice as indicated in Table 6.
   b. Mix well gently with a pipette and quickly spin down the reagents in each tube.
   c. Repeat the steps a-b for the *rpoS* DNA fragment.

Note: The concentration of the AirA protein should be no less than 100 nM. Low protein concentration can result in a lower detection signal after immunoblotting, and simply increasing the amounts of protein may cause samples to spill from the PAGE gel wells.

⚠ CRITICAL: The ingredient of the Binding buffer in this part is not fixed, since different target proteins may require different components for proper binding reactions, which need to be determined or validated by certain other experiments. Cofactors, such as magnesium ions, iron ions, calcium ions and biotin, are often components needed for the binding reactions (troubleshooting 2).

⚠ CRITICAL: pH is another critical part of the Binding buffer, and care should be taken to match the conductivity of the samples and electrophoresis buffer (Altschuler et al., 2013; Hellman and Fried, 2007). Here, pH 7.6 was used for detecting AirA. However,

Table 4. Program for DIG label reaction

Steps	Temperature	Time	Cycles
1	37°C	5 min	1
Hold	4°C	Forever	

Table 5. Serial dilution of DIG-labeled DNA probe

Tube	Oligo (μL)	From tube No.	ddH_2O (μL)	Dilution	Final concentration (ng/μL)
1	20	Original	0	n/a	8
2	2	1	18	1: 10	0.8
3	2	2	18	1: 100	0.08
4	2	3	18	1: 1,000	0.008
5	0	N/A	20	N/A	0

the pH of Binding buffer should be adjusted and optimized accordingly according to the properties of the target proteins (troubleshooting 2).

d. Perform the reaction program in a PCR machine set with the below program (Table 7).

Note: Here, the incubation program is set at 30°C for 20 min for protein-DNA reaction. However, the reaction program can be variable based on the chemical properties of different proteins. A wide range of binding temperatures have been described (Hellman and Fried, 2007). For example, the optimal reaction temperature for the catabolite activator protein (CAP) from *E. coli* is typically 37°C (Garner and Revzin, 1981), while the reaction temperature for the CtrA protein from *Ehrlichia chaffeensis* cells is low (e.g., 2°C–25°C) (Cheng et al., 2011). Therefore, the reaction temperature and time need to be adjusted and optimized based on the characteristics of target proteins accordingly (troubleshooting 2).

e. Take out the tubes and put them on ice immediately after the reaction program was finished.
f. Add 5 μL of 5× Loading buffer to each reaction tube, mix well by pipetting and briefly spin down the tubes.

Note: Any denaturing agent contamination in the Loading buffer should be avoided, otherwise the protein-DNA complex may be disturbed and result in uncertain experimental conclusions.

Native-PAGE gel electrophoresis

Timing: 1.5 h

This step describes the native-PAGE gel electrophoresis process.

4. Native-PAGE gel electrophoresis.

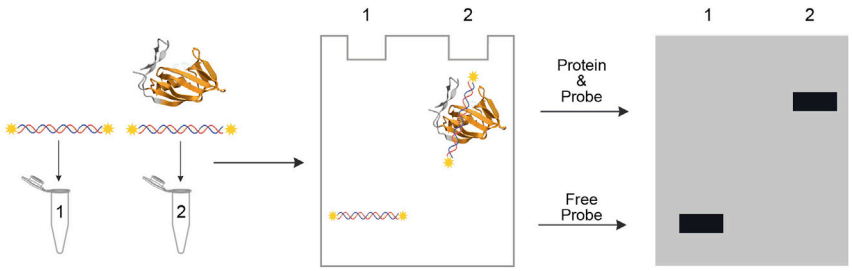

Figure 1. A figure illustrated the schematic diagram of the EMSA method
Target protein and DIG-labeled DNA probe are co-incubated under appropriate conditions for protein-DNA reaction (left panel). The reaction mixtures without (1) or with (2) target proteins are loaded into a native PAGE gel, and immunoblotted to detect protein-DNA interactions (middle and right panel). A retarded gel shifting band is expected if the target protein has binding activity to the tested DNA probe.

Table 6. Reaction mixture for the binding of protein-DNA complex

Reagent	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
5× Binding buffer (μL)	4	4	4	4	4	4
DIG-labeled DNA (20 nM) (μL)	1	1	1	1	1	1
AirA protein (150 nM) (μL)	0	2	4	6	8	10
ddH_2O (μL)	15	13	11	9	7	5
Total (μL)	20	20	20	20	20	20

Note: Normally the acrylamide concentration within 6%–8% for PAGE gel is appropriate for the majority of experimental procedures. The acrylamide concentration can be optimized accordingly if the detected protein-DNA complex size is special.

a. Pre-run the 6% native PAGE gel with 0.5× TBE running buffer at 80 V for 30 min using a constant voltage power supply.

Note: 0.5× TBE buffer is normally utilized for native PAGE gel electrophoresis. However, for some proteins like GSK-3β (Chen et al., 2016), the ion concentrations in 0.5× TBE buffer is too high to maintain the stability of formed protein-DNA complexes. In such cases, lower ion concentration (e.g., 0.25× TBE) is needed to meet the requirement of specific proteins.

⚠ CRITICAL: It is conventional (and desirable) to conduct the pre-running step for polyacrylamide gels to clear the ions that would increase conductivity, which will hence, generate too much heat for the gel during running.

Optional: To make the sample well easier for visualization, 2 μL 5× Loading buffer can be preloaded in each well before loading samples.

b. Load 20 μL of reaction samples in each well in order according to the protein concentration from low to high (i.e., from left to right is 0, 15, 30, 45, 60, and 75 nM) with a pipette.
c. Run the native PAGE gel in above pre-run buffer at 120 V for 60 min using a constant voltage power supply at 4°C cold room or on ice.

Note: The gel running process can be normally performed at 25°C. However, to maximize the stability of the protein-DNA complex, it is better to be performed at a low temperature (i.e., 4°C).

Note: The gel running time can be variable according to the experimental DNA size, and the bromophenol blue inside the Loading buffer can be used as an indicator of gel running time (i.e., until the dye reaches the bottom of the gel) (troubleshooting 4).

Membrane transfer and crosslinking

Timing: 2 h

This step describes the membrane transfer and crosslinking process. Herein, the wet transfer method with a "sandwich" structure was selected for membrane transfer. Based on our experience,

Table 7. Reaction program for the binding of protein-DNA complex

Steps	Temperature	Time	Cycles
1	30°C	20 min	1
Hold	4°C	Forever	

Set the lid temperature at 95°C.

high protein and nucleotide transfer efficiency can be achieved using wet transfer method, since both the PAGE gel with DNA and protein, and the membrane can be thoroughly soaked with the transfer buffer during the process. However, for the semi-dry transfer method, the transfer occurred between the two electrode plates, and a high operational experience was required. Therefore, the membrane transfer efficiency of proteins and DNA, and their retention on the membrane are often difficult to control during semi-dry transfer, leading to a fluctuant result and difficulty for experimental results interpretation. As a result, the wet transfer method is recommended and utilized here.

5. Membrane transfer.
   a. Pry open the glass plate carefully and remove these excess gels from the sample wells after the PAGE gel electrophoresis.

   Note: Make sure the target protein did not run off the gel during the procedure.

   b. Assemble a "sandwich" structure for the wet transfer procedure.
      i. Pre-wet the nylon membrane with proper size (i.e., same as the glass plate) into transfer buffer for 5 min.
      ii. Put two pieces of filter paper (Blot paper, 3 mm) and sponges into a proper plastic box containing enough transfer buffers.
      iii. Assemble the membrane transfer clip as below order: negative charged transfer clip side > sponge > filter paper > PAGE gel > nylon membrane > filter paper > sponge > positive charged transfer clip side.
      iv. Put the membrane transfer clip inside the membrane transfer tank with the orientation that the gel surface is connected to the negative electrode, and the nylon membrane side is connected to the positive electrode (Figure 2).

   Note: Air bubbles can cause incomplete transfer. Avoid bubbles generation during the "sandwich" assembly operation process, a glass rod is often used to dislodge the bubbles.

   Note: Make sure the operator is wearing gloves, and try not to touch the membrane since either protein or oil on one's hands can affect the membrane transfer efficiency and contaminate the membrane.

   c. Perform the membrane transfer at 4°C cold room with an ice bag on one side of the transfer tank, setting the transfer program at 80 V, 200 mA for 60 min.

   Note: Prevention of too much heat generation during the membrane transfer process. The transfer program is optional according to experimental times, and a program with 200 mA–400 mA at 30–60 min is normally selected. The membrane transfer time depends on the DNA fragment size; the longer the DNA lengths, the longer transfer duration is often needed (troubleshooting 2 and 3).

6. Put the transferred membrane on the center of a Thick Blot Paper with proper size (14 × 16 cm) flatly.
7. Transfer the whole paper with membrane into a 120°C oven, baking for 15 min to fix the nucleic acid on the membrane.

   Note: Take care about the direction of the transferred membrane, the side in contact with the PAGE gel contains the exposed DNA. The direction of the membrane would directly affect the signal strength after immunochemistry exposure, since the right-side of the membrane with exposed DNA is conducive to the reaction of DIG-labeled fragments with antibodies.

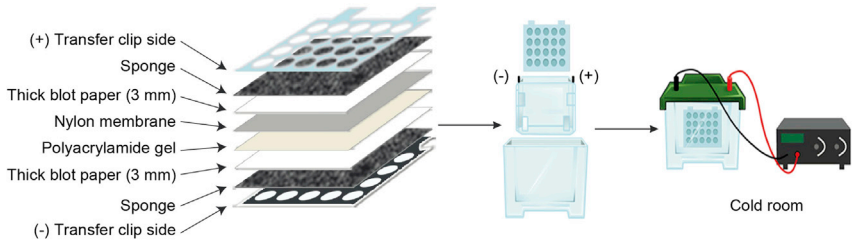

Figure 2. A representative figure displaying the wet-transfer procedure

Pause point: It is possible to pause at this point. The transferred membrane can be carefully stored at 4°C for 1 week between two sheets of Blot paper (3 mm).

Immunoblotting and chemiluminescent detection

Timing: 2.5 h

This step describes how to perform immunoblotting and chemiluminescent detection.

8. Immunoblotting.
   a. Put the membrane into a suitable sized plastic container (10 cm × 15 cm), add 30 mL of Washing buffer and soak the membrane thoroughly, incubating at 25°C with horizontal shaking for 2 min at 60 rpm.
   b. Pour out the Washing buffer carefully, take care not to disturb the membrane inside. Add 50 mL 1× Blocking buffer and incubate it at 25°C with horizontal shaking for 30 min at 60 rpm (troubleshooting 5 and 6).
   c. Pour out the Blocking buffer carefully, take care not to disturb the membrane inside. Add 20 mL Anti-DIG antibody solution (Dilute Anti-DIG-AP antibody 1:10,000 in 1× Blocking buffer) and incubate it at 25°C with horizontal shaking for 30 min at 60 rpm (troubleshooting 5 and 6).
   d. Pour out the Anti-DIG antibody solution carefully, take care not to disturb the membrane inside. Add 50 mL Washing buffer and incubate it at 25°C with horizontal shaking for 15 min at 60 rpm.
   e. Repeat the above washing step (d) once.
   f. Equilibrate the membrane in 30 mL detection buffer at 25°C with horizontal shaking for 2–5 min at 60 rpm.
9. Chemiluminescent detection.
   a. Cover the tray of Amersham Imager 600 with a proper sized plastic wrap. Put the right-side of the membrane onto the tray and incubate it with 1 mL CSPD for 5 min at 25°C.

   Note: Take caution that this process should be protected from light and the membrane should be maintained damp.

   b. Perform chemiluminescent detection by auto-exposure using Amersham Imager 600. Further adjust the exposure program manually according to the autoexposure time till to 5 min (troubleshooting 3 and 5).

   Note: Pay attention to the direction of the membrane. The right-side membrane with DIG-DNA fragment should be always upward during the immunoblotting and chemiluminescent detection process.

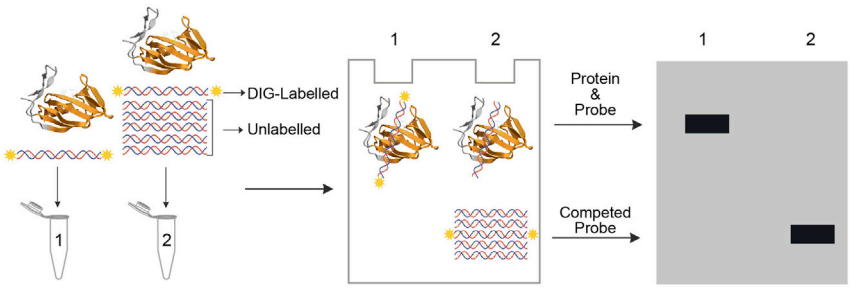

Figure 3. A figure illustrated the schematic diagram of the competitive EMSA assay

Target protein, DIG-labeled DNA probe and 50–100 fold more unlabeled DNA probe are co-incubated at specific conditions for reaction (left panel). The reaction mixtures without (1) or with (2) unlabeled DNA probe are loaded into a native PAGE gel, and immunoblotted to detect competitive protein-DNA interaction (middle and right panel). Those unlabeled DNA probe effectively compete for the binding of the target protein to the DIG-labeled DNA probe, and the retarded band is expected to disappear if the target protein has specific binding activity to the tested DNA probe.

Note: If the signal is too weak, it can be resolved by extending the CSPD incubation time or chemiluminescent exposure time to 10 min.

⚠ CRITICAL: With the protein amount increasing, a gradually increased detection signal for DNA and protein binding would be detected (Figure 5). According to the results, we can see that when 60 nM (i.e., 8 μL) of the target protein is added, DNA and protein are completely combined to form a protein-DNA complex. Therefore, this protein concentration will be selected for further competitive gel electrophoresis migration experiment, which is carried out to address the specificity of the protein-DNA binding in the settled experimental conditions.

Optimal: The detected binding signal of target protein to DNA may be due to non-specific binding. Therefore, to rule out this possibility, poly [d(I-C)] or poly [d(A-T)] are often used as non-specific competitors for EMSA (Buratowski and Chodosh, 2001; Ferraz et al., 2021). Herein, we also performed the exact same procedure as steps 3–9 above, except that 1 μg poly [d(I-C)] (Roche, Cat# 10108812001) was added to the above protein-DNA reaction mixtures listed in Table 6.

Competitive EMSA process

Timing: 7 h

This section describes the competitive EMSA process. A competitive EMSA can be used to evaluate the specificity of protein binding activity to DNA, using the same sequence but unlabeled DNA fragment. Excessive unlabeled DNA fragments could compete with the labeled probes for protein binding. With the concentration increase of the unlabeled DNA fragments, the binding of DIG-labeled DNA probes to proteins would decrease. Therefore, in the competition experiment design, the utilized concentration of the unlabeled DNA in experiment can reach to 50–100 times more than the labeled probe. Those unlabeled DNA fragments effectively compete for the binding of the target protein to the DIG-labeled DNA fragments, and the retarded band is expected to weaken or even disappear if the target protein has specific binding activity to the tested DNA probe. (Figure 3).

10. Prepare the competitive EMSA reaction buffers on ice as indicated in Table 8. Mix well gently by pipette and spin down with a short time.

Note: The reaction system and conditions of the competitive EMSA process should be exactly same as the reactions in the previous EMSA process, except for the changes in DNA

Table 8. Reaction mixture for the competitive binding of protein-DNA complex

Reagent	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
5× Binding buffer (μL)	4	4	4	4	4	4
DIG-labeled DNA (20 nM) (μL)	1	1	1	1	1	1
Unlabeled DNA (1,000 nM) (μL)	0	0	0	0.5	1	2
AirA protein (150 nM) (μL)	0	4	8	8	8	8
ddH_2O (μL)	15	11	7	6.5	6	5
Total (μL)	20	20	20	20	20	20

composition and content, so as to eliminate the interference factors of the experiment and ensure the accuracy of experimental results interpretation.

Note: The reaction performed for the *ler* promoter and negative control *rpoS* should be same.

11. Perform the protein-DNA reaction program as indicated in above step 3d and Table 7.
12. Perform the steps 3e–9 for protein-DNA binding signal detection.

EXPECTED OUTCOMES

A series of signal dots on the nylon membrane can be seen in the probe label efficiency detection, for both *ler* promoter and *rpoS*. The DIG signal gradually weakens along with the increase of the dilution series. As a result, the DIG signal can be clearly detected when the amount of labeled DNA was 0.008 ng (Figure 4, lane 4), indicating that the labeling reaction was successful, which can fulfill the experimental requirements.

With the concentrations of AirA protein increasing, slow migrating bands are observed for the DIG-labeled *ler* promoter through the gel migration detection and analysis, indicating that AirA protein binds to the *ler* promoter to form a protein-DNA complex. When the AirA amount is 60 nM, all the DNAs are completely bound by the protein (Figure 5, left panel). However, in the negative control group, the increased amount of the protein AirA does not affect the *rpoS* migration level (Figure 5, right panel). Further, the EMSA results are not affected by non-specific DNA competitor poly [d(I-C)] (Figure 6), ruling out the possibility of non-specific binding of AirA protein to the *ler* promoter. Collectively, these preliminary results indicate that AirA has a binding activity to the *ler* promoter.

A gradually decreased level of DIG-labeled *ler* promoter bound to AirA can be detected along with the addition of specific competitor molecules (i.e., the unlabeled *ler* promoter). In the competitive analysis section. The DIG-labeled *ler* promoter is completely competed down when the added DNA

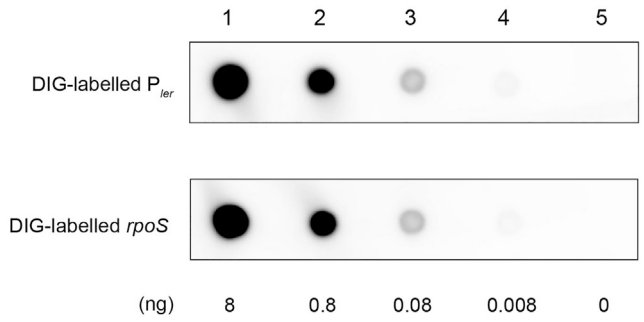

Figure 4. DNA labeling efficiency detection

The DIG labeling efficiency for the promoter region of *ler* (top panel) and *rpoS* fragment (bottom panel) were detected and indicated, respectively. The immunoblot displayed here was captured after 20 s of exposure. The content of each probe was shown at the bottom of each lane.

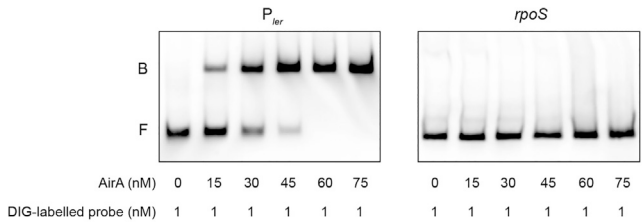

Figure 5. The EMSA detection for the target protein AirA with *ler promoter and* the negative control *rpoS*

The reactions of different concentrations of protein AirA with the promoter region of gene *ler* (left panel) and control gene *rpoS* (right panel) were loaded, and figures were displayed after immunoblotting, respectively. In each lane, the DNA probe level is confined (i.e., 1 nM), together with a gradually increased AirA concentration, accordingly. B and F represent the bound and free DNA fragments, respectively. The concentrations of AirA and DIG-labeled probes were indicated at the bottom of each blot. The blots displayed here were captured after 15 s of exposure.

competitor level is 100 nM (Figure 7, left panel). However, in the negative control group, the increased amount of unlabeled *rpoS* has no effect on the migration of DIG-labeled *rpoS* (Figure 7, right panel). These results indicate that the binding of the protein AirA to *ler* promoter is specific.

LIMITATIONS

The procedure described here is for *in vitro* detection or confirmation of the binding activity of a certain protein and associated DNA fragment. To detect the specific binding site of the target protein on DNA, other experiment(s) is required, such as the DNase I foot-printing assay. Also, this protocol has size restrictions (within 500 bp) for the DIG-labeled DNA fragment. The current protocol should be tested and specific steps should be optimized when working in longer DNA fragments. In addition, the Binding buffer mentioned in this experimental method is not suitable for all proteins, and other experiment(s) are required for the composition determination of the Binding buffer according to the characteristics of the specific protein.

TROUBLESHOOTING

Problem 1

Multiple migration bands are appeared (before you begin step 3; before you begin step 4).

Potential solution

Degradation of the target protein can cause multiple bands. Avoid repeated freezing and thawing of the purified protein, and thaw aliquoted proteins slowly on ice.

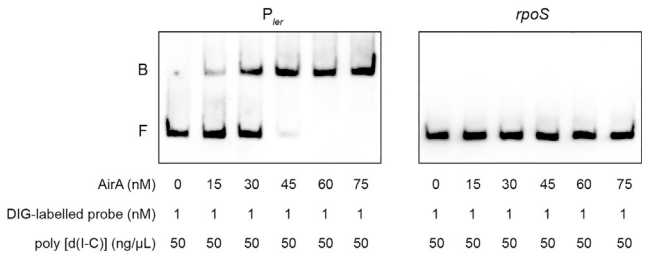

Figure 6. The binding of target protein AirA to *ler* promoter is not affected by non-specific DNA competitor poly [d(I-C)]

Poly [d(I-C)] was added in the same protein-DNA reactions as described in Figure 5. A native PAGE gel running and immunoblotting were further performed and the immunoblot figures were displayed. In each lane, the DNA probe level (*ler* promoter or *rpoS*) (i.e., 1 nM) and poly [d(I-C)] (i.e., 50 ng/μL) are confined, together with a gradually increased AirA concentration, accordingly. B and F represent the bound and free DNA fragments, respectively. The concentrations of protein AirA, DIG-labeled probes and poly [d(I-C)] were indicated at the bottom of each blot. The blots displayed here were captured after 15 s of exposure.

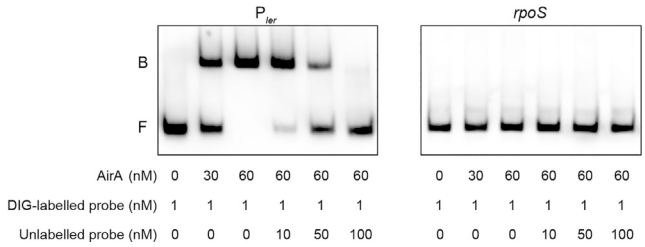

Figure 7. The competition EMSA result for the protein AirA with *ler* promoter and the negative control *rpoS*

Selected concentrations of protein AirA (i.e., 0, 30 and 60 nM) with 1 nM DIG-labeled DNA probe and a range of unlabeled DNA concentrations (i.e., 0, 10, 50, 100× times more) in each lane were loaded accordingly, for both target gene *ler* (left panel) and the control gene *rpoS* (right panel). B represents the AirA bound DNA fragments and F represents the free DNA fragments. The concentrations of protein AirA, DIG-labeled probes and unlabeled probes were indicated at the bottom of each blot, respectively. The blots displayed here were captured after 15 s of exposure.

The truth that multiple binding sites are located at the DNA fragment can also cause the multiple bands. The DNA fragment with fewer predicted binding sites must be elaborately designed according to the prediction result of bioinformatics analysis.

Problem 2

No migration band is appeared (before you begin step 3; Major step 3; Major step 5).

Potential solution

Protein amount is insufficient in the protein-DNA reaction or the purified protein does not maintain 100% activity. High protein activity must be carefully confirmed and maintained in the protein operation step (i.e., Epitope tags recombination may affect the natural property of the protein or the protein activity may be lost in the purification steps); Add five-times more proteins according to the theoretical value of protein-DNA binding.

Membrane transfer is not sufficient. Extend the membrane transfer time accordingly.

Improper binding condition or Binding buffer in the protein-DNA reaction. Some necessary components are needed for the binding reaction. For some proteins, the formation of protein-DNA complexes depends on other additional factors, such as the acetyl phosphate (Liu et al., 2020), zinc ions (Choi et al., 2017), and biotin (Yang et al., 2015). The current protocol can also theoretically be used to detect the interaction of RNA polymerase in association with sigma factor with its cognate promoters. However, both CAP and cyclic AMP are required for this interaction in *E. coli* (Garner and Revzin, 1981), therefore the experimental conditions should be adjusted accordingly.

Problem 3

No detected signal or the signal is too weak (Major step 2; Major step 5; Major step 9).

Potential solution

DNA labeling efficiency is low, that is, the 0.08 ng DNA spot showed no detected signal in the probe labeling efficiency detection step. Improve the purity (uniqueness) and concentration of the PCR amplified DNA fragment and re-perform the DNA probe labeling step.

Incomplete membrane transfer. Use wet transfer method and extend the transfer time or increase the transfer voltage.

Short chemiluminescent exposure time, short incubation time with CSPD or insufficient CSPD amount. Increase the amount and incubation time of the CSPD with membrane or prolong the exposure time.

Problem 4
The protein-DNA complex is unstable (Major step 4).

Potential solution
High ion concentrations in the electrophoresis gel system damages the complex stability. Reduce the ion level of the electrophoresis buffer from 0.5× TBE to 0.25× TBE. Overheat is generated. Perform the binding reaction of DNA and protein at a low temperature and run the electrophoresis on ice.

Problem 5
The background signal of the whole membrane is too high (Major step 8; Major step 9).

Potential solution
Non-thorough blocking or washing of the membrane. Increase the Blocking buffer amounts or prolong the washing time to ensure that the membrane is fully immersed in the indicated buffers in all steps.

The membrane is overdried during the detection step. Cover the membrane with a cling film during the CSPD incubation, and keep damp during the exposure process.

Problem 6
The membrane displays a kind of spotty background (Major step 8).

Potential solution
Precipitates inside some solutions. Double-check and mix well all the buffers before using, and make sure there are no precipitates visible inside all buffers.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bin Yang, yangbin@nankai.edu.cn.

Materials availability
This study did not generate any unique materials.

Data and code availability
All data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS
F.W., T.Y., W.Y., P.W., and Y.L. worked together to optimize the protocol condition; F.W., T.Y., and W.Y. conducted the methods in this article. F.W., T.Y., W.Y., and B.Y. wrote the manuscript. The work was supervised by B.Y.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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