VIAGENE BIOTECH INC
IMPROVING LIFE SCIENCE

CoolShift-BTd, a General EMSA Kit Using Biotin-DNA-Probes

User's Manual

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Viagene EMSA kits are intended for research purpose only!

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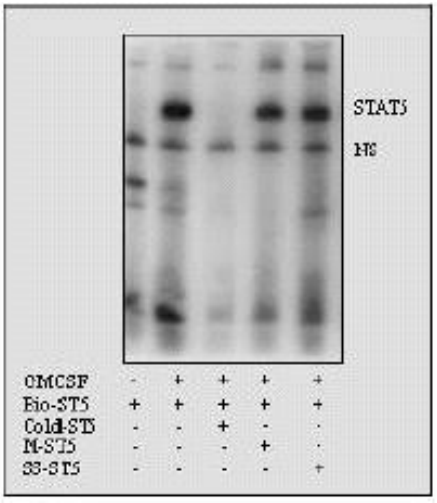
TABLE OF CONTENTS

A. Introduction

The Electrophoretic Mobility Shift Assay (EMSA) is a powerful tool for evaluating DNA-protein or RNA-protein interactions, which often referred to as gel shift or gel retardation. The assay is based on the principle that when subjected to electrophoresis, free DNA/RNA probes will migrate differently from a DNA/protein or RNA/protein complexes. The activated transcription factors (TF) that bind with DNA or RNA in nucleus can be detected with EMSA.

Viagene's non-radioactive EMSA kits are based on high sensitivity of the chemiluminescent technology. They are different from those on the market in the design of the probes and optimizing procedures. Comparing with radioactive systems, these non-radioactive EMSA Kits offer the sensitivity and speed of radioactive assays without the hazards, waste and probe instability problems. We tested each of these kits intensively and set up the optimum condition for each transcriptional factor. All these kits are ready to use and guaranteed to work.



The protocol is very easy to use. The standard kits come with all necessary components for performing 100 DNA/protein binding reactions and assays.

Viagene's non-radioactive EMSA kits can be stored for one year without loss of activity when the components are stored at recommended temperature.

B. Kit components (stored as indicated on labels)

1. The General EMSA Kit includes follows:

- 10X Binding Buffer A, B, N or G (4 °C) 1 vial
- Poly [dI: dC] (-20 °C) 1 vial
- Streptavidin-HRP (4 °C) 1 vial
- 6X Loading Buffer (4 °C) 1 vial
- 2X Blocking Buffer (4 °C) 1 bottle
- 5X Washing Buffer (4 °C) 2 bottle
- 2X Equilibration Solution (4 °C) 1 bottle
- ECL substrate A (Lightgen®-CL*, Sol. A, 4 °C) 1 bottle
- ECL substrate B (Lightgen®-CL*, Sol. B, 4 °C) 1 bottle
- 1% NP-40 (-20 °C) 1 vial
- 1M KCl (-20 °C) 1 vial
- 100mM $MgCl_2$ (-20 °C) 1 vial
- 100nM EDTA (-20 °C) 1 vial
- 50% Glyerol (-20 °C) 1 vial
- Operation Manual (-20 °C) 1 set

* Lightgen®-CL is for imaging with x-ray films. For imaging with imagers, Lightgen®-HL is needed.

2. EMSA-Controls (Option):

- Nuclear extracts with activated specific TF (-80 °C) 1 Vial*
- Nuclear extracts without activated TF (-80 °C) 1 Vial*

***** Since many customers have already had positive and/or negative controls, the complete kits from Viagene Biotech DO NOT include nuclear extracts (controls). However, a variety of positive/negative controls for a specific TF can be purchased, respectively. These controls will be shipped with dry ice.

C. Additional materials required

- Mini-polyacrylamide gel electrophoresis apparatus, and related chemicals and buffers.
- Electrophoretic transfer apparatus and buffers.
- X-ray films or a CCD imager (e.g. Viagene's Cool Imager™, Catalog# IMGR002).
- Sample storage apparatus such as refrigerators and ultra-low freezers.
- Orbital Shaker, vials and tubes.
- UV Crosslink apparatus or vacuum oven.
- Biotin-labeled probes, competitive and mutant probes.
- Positive-charged Nylon binding membrane.

D. Binding Reaction

1. Binding Reaction for Standard EMSA:

10X binding buffer	1.5 µl
Poly [dI: dC]	1.0 µl
Nuclear extracts	X µl
dH_2O	X µl
Total	15.0 µl

Mix well and let it sit at room temperature (R/T) for 20 minutes.

Biotin-probe	0.5 µl
Total	15.5 µl

Allow mixture to react at R/T for at least 20 minutes.

* The total 2-5 µg of nuclear proteins in the volume of 3 µl or less are required for non-radioactive EMSA, and the protein concentration of nuclear extracts should be 1 µg/µl or higher for best results.

2. Reaction for Testing Binding Condition:

10X binding buffer	1.5 µl
Poly [dI: dC]	1.0 µl

Optional reagent*	1 µl
Nuclear extracts	X µl
dH_2O	X µl
Total	15.0 µl

Mix well, and sit at R/T for 20 minutes.

* Optional reagent could be 1% NP-40 , 1M KCl, 100mM $MgCl_2$, 100nM EDTA, or 50% Glycerol . Testing the effect of each reagent needs to setup a separated reaction.

Biotin-probe	0.5 µl
Total	15.5 µl

3. Competition Reaction*:

10X binding buffer	1.5 µl
Poly [dI: dC]	1.0 µl
Nuclear extracts	X µl
Cold oligonucleotides or Mutant oligonucleotides	X µl (20-100 fold over that of biotin-probe)
dH_2O	X µl
	15 µl

Mix well, and sit at R/T for 20 minutes .

Biotin-probe	0.5 µl
Total	15.5 µl

Mix well, and allow mixture to react at R/T for at least 20 minutes.

* Usually, competitive EMSA is performed after positive DNA/protein complexes are detected by regular EMSA.

4. Supershift EMSA Reaction*:

10X Supershift buffer	1.5 µl
Poly [dI: dC]	1.0 µl
Nuclear extracts*	X µl
dH_2O	X µl
Total	15.0 µl

Mix well and sit at room temperature (R/T) for 20 minutes.

Biotin-probe	0.5 µl
Total	15.5 µl

Allow mixture to react at R/T for 20 minutes.

Supershift Antibody	1-4 µl
Total	15.5 µl + vol. of antibody

Allow mixture to react at R/T for at least 30 minutes.

* Usually, supershift EMSA is performed after positive DNA/protein complexes are detected by regular EMSA.

E. Gel preparation

1. Prepare and make 6.5% mini Gels:

10X TBE	1.0 ml
40% Acrylamide/Bis	2.8 ml
50% Glycerol	1.0 ml
dH_2O	15.2 ml
TEMED	20 µl
10% AP	350 µl
Total	20.0 ml

20 ml is enough to make 2 mini gels (90 X 70 X 1.5 mm）

2. Prepare pre-cooled 0.25X TBE:

10X TBE	30 ml
dH_2O	1170 ml
Total	1200 ml

3. Pre-running:

Pre-run the gel(s) for at least 30 min at 120V in cooled 0.25X TBE on ice, then, flush each well with 0.25X TBE before loading samples.

F. Electrophoresis:

1. Prepare samples：

Binding reaction from Section D.	15.0-20 µl
6X loading buffer	3.0-4 µl
Total	~18-24 µl

Mix well, sit at R/T for 3 minutes, and centrifuge for 3 minute at 14,000 rpm.

2. Load Samples：

Load all the supernatant (~18-24 µl) into gel wells.

3. Electrophoresis:

Run the gel on ice at 180V until bromophenol blue gets to the lower end of gels (~50-80 min).

G. Electro-transfer:

Electro-transfer can be performed by wet trans-blot or semi-dry trans-blot.

1. **Wet Trans-blot (Bio-Rad)**

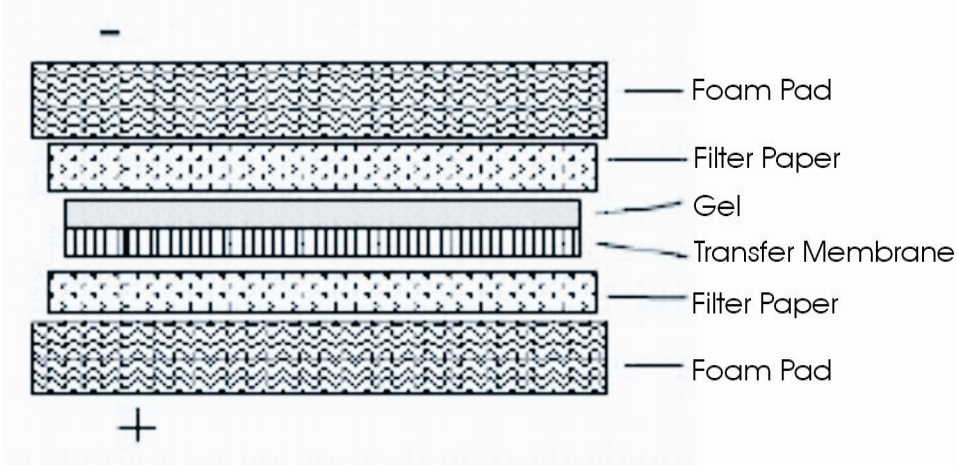
   (1) Prepare transfer buffer: 0.5X TBE 1200 ml.

10× TBE	60 ml
dH_2O	1140 ml
Total	1200 ml

   (2) Presoak the membrane: in 0.5X TBE for at least 10 minutes.

   (3) Prepare wet trans-blot：

   Carefully remove one glass from the gel and mark the orientation of the gel. Cover the gel with one sheet of pre-wet Whatman paper (1mm). With the paper on the lower sider, gently separate the paper with gel away from glass plate. Sandwich the gel with presoaked binding-membrane and Whatman papers following the picture shown below.



   (4) Transfer: Transfer in 0.5× TBE at 390mA for 40 minutes.

2. **Semi-Dry Trans-blot (Bio-Rad)**

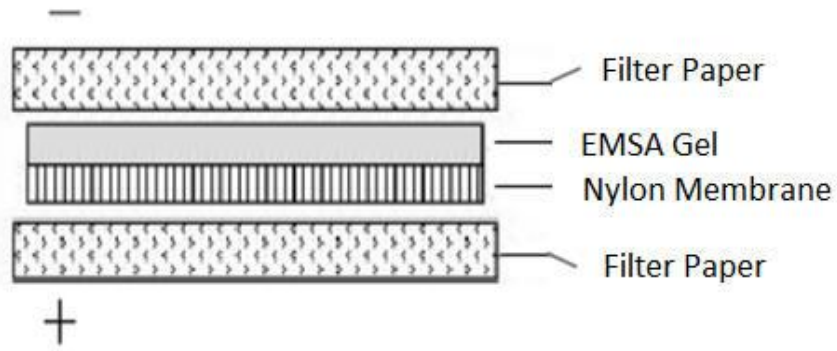
   (1) Prepare transfer buffer: 0.5X TBE 200 ml.

10× TBE	10 ml
dH_2O	190 ml
Total	200 ml

   (2) Presoak the membrane: in 0.5X TBE for at least 10 minutes.

   (3) Prepare Semi-dry trans-blot：

Carefully remove one glass from the gel and mark the orientation of the gel. Cover the gel with one sheet of pre-wet Whatman paper (1mm). With the paper on the lower sider, gently separate the paper with gel away from glass plate. Sandwich the gel with presoaked binding-membrane and Whatman papers following the picture shown below.



(4) Semi-dry electro-transfer: perform **t**ransfer in 0.5× TBE at constant 300mA for 20 minutes.

H. Immobilization of bound DNA

After the transfer, remove the nylon-membrane and rinse it in 0.5X TBE. Place the membrane in a UV linker to crosslink DNA by following the manufacturer's guidance. In our lab, the energy of 600-800mJ is applied for crosslinking DNA to nylon membrane in Stratagene Stratalinker 1800.

It has been reported that DNA can be immobilized on a nylon membrane in a vacuum oven at 80 ^{o}C for 2 hours, but we have not tried it in our lab.

I. Chemiluminescence Reaction:

1. **Prepare the reagent:**

   Gently warm the Blocking Buffer and Wash Buffer in a 37-50^{o}C water bath until all the precipitates are dissolved completely.

2. **Block the Binding-membrane:**

   (1) Prepare 1 X blocking buffer:

2 X Blocking buffer	7.5 ml
dH_2O	7.5 ml
Total	15 ml

   (2) Block the membrane with 15 ml of 1X blocking buffer for 15 minutes at R/T on a shaker.

3. **Streptavidin-HRP binding reaction:**

(1) Prepare streptavidin/HRP binding buffer:

2 X Blocking buffer	7.5 ml
dH_2O	7.5 ml
Streptavidin-HRP	15 µl
Total	15 ml

(2) Discard blocking buffer and incubate the membrane with 15 ml of HRP binding buffer for 15 minutes at R/T on a shaker.

4. Wash the membrane:

(1) Prepare 1 x Washing solution:

Wash buffer (stocking)	8 ml
dH_2O	32 ml
Total	40 ml

(2) Wash the membrane(s) 4 times with 10 ml of 1X washing solution, 5 minutes for each wash.

5. Equilibrate the membrane:

(1) Prepare 1 X Equilibration buffer:

2 X Equilibration buffer	6 ml
dH_2O	6 ml
Total	12 ml

(2) Equilibrate the membrane after washing, with 12 ml of 1X Equilibration buffer at R/T for 5 minutes on shaker.

6. Chemiluminescent detection:

(1) Prepare the Chemiluminescent substrate solution:

Lightgene CL (for X-Ray films):

Solution A	1.2 ml
Solution B	1.2 ml
Total	2.4 ml

Lightgene HL (for imagers):

Solution A	1.2 ml
Solution B	1.2 ml
Total	2.4 ml

2-2.4 ml is enough to cover a mini-gel membrane (~9 x 7 cm).

(2) Add 2.0-2.4 ml of chemiluminescent substrates onto the membrane.

(3) Depending on the detection technique, proceed with one of the following procedures.

J. Chemiluminescence imaging*:

1. Detecting with Chemiluminescence Imager:

An imager for capturing chemiluminescent picture should have very high sensitivity, which should have the capacity to compute thousands of pictures in minutes. Cool Imager™ by Viagene (Catalog IMGR002) is well designed for detecting chemiluminescent images of Western immunoblotting, DNA/RNA hybridization or non-radioactive EMSA. In the manipulation of the Cool Imager™, you do not need to remove the chemiluminescent substrate from the membrane for imaging. The pictures will appear on the screen in 2-5 minutes. Please refer to the operation manual of Cool Imager™ for chemiluminescent imaging.

2. Detecting with exposure and develop X-ray film:

When using X-ray film to capture a chemiluminescent image of blots, one must have a darkroom, film developer, filming chemicals and X-film, etc. After the chemiluminescent reaction (usually 1-2 minutes), discard the chemiluminescent substrate and wrap the blots with a piece of transparent paper or membrane. Place a sheet of X-ray film on the surface of the transparent paper. Exposure time may vary depending on the strength of chemiluminescent signaling, and several X-ray films with different exposure time may need to attain the desired pictures. Develop the films according to the manufacturer's instructions of the X-ray film developer.

*The general EMSA kit from Viagene Biotech includes enough amounts of materials for performing 100 EMSA reaction/assays. However, if the user needs to repeat adding chemiluminescence substrates onto the same membrane to get images with different exposures, it would be necessary to 1) purchase extra amounts of substrates from Viagene Biotech, or 2) use other brands of HRP-related chemiluminescence substrate using for Western blotting or immune-detection.

The standard ECL EMSA kits include chemiluminescence substrates (Lightgen CL) for imaging pictures with X-films. For imaging pictures with digital imagers, the kits with higher sensitivity of substrates (Lightgen HL) are needed. When ordering, please indicate that x-ray film or an imager is used for imaging EMSA pictures.

K. Troubleshooting:

Problem	Cause	Recommendation
Weak or no imaging	Poor transfer. Exposure time is too short. Not enough biotin-probe is used. The image is on the other side of the blot. The Extracted proteins degraded	Use electro-blotting for best results. Increase time of exposure Increase probe used Revert the blot and try again Try using protease inhibitors
High background	Membrane is dry. Particulates in blocking or wash buffer Exposure time is too long	Keep membrane moist during detection. Gently warm until no particulate remain. Shorten exposure time
No shift observed.	Not enough extract. Quality of extract is not good or the extract degraded	Use more extract. Try using protease inhibitors or high quality extract.
The image is too dark.	Over-exposed The chemiluminescent is too strong. Too much biotin probe is used.	Shorten the exposure time. Dilute the chemiluminescent substrate for 1-5 multiple. Use higher dilution of biotin-probe

For more troubleshooting and detail discussion of EMSA problems, please see webpage: http://www.viagene.com/supports/EMSA_Forum/ECL_EMSA_Q&A.htm

L. References:

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8. Liu R.Y. et al. (1999). Tumor necrosis factor-alpha-induced proliferation of human Mo7e leukemic cells occurs via activation of nuclear factor kappaB transcription factor. J Biol. Chem. 274:13877-85.

LI. Notes:

1. Upon receipt, check the package and the kit components immediately. If problems arise, please contact Viagene Biotech within **72 hours**.
2. Before opening vials, spin down the components in the vials.
3. The kit can be stored for 12 months at the condition indicated on the labels.
4. When kits are stored at low temperature, white precipitates may be observed. Warm up the bottles in a water bath to dissolve the precipitate before use.
5. Follow this instruction strictly to obtain the best results.
6. Follow the laboratory regulation when handling Acrylamide/Bis solution.