VIAGENE BIOTECH INC
IMPROVING LIFE SCIENCE

CoolShift-BTr, a General EMSA Kit Using Biotin-RNA-Probes

User's Manual

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Viagene EMSA kits are intended for research purpose only!

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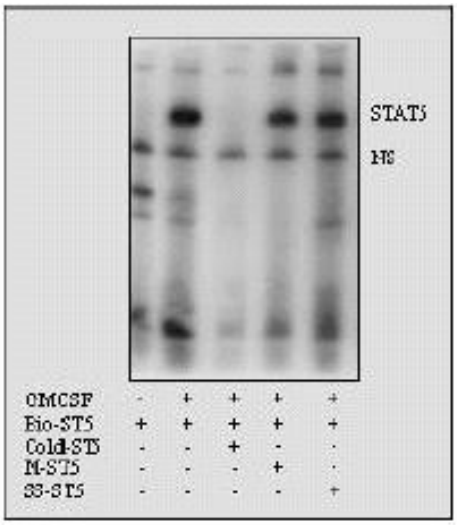
A. Introduction

The Electrophoretic Mobility Shift Assay (EMSA) is a powerful tool for evaluating RNA-protein or DNA-protein interactions, which is often referred to as gel shift or gel retardation. With the "standard" radioactive EMSA techniques using P^{32}-labeled oligonucleotides, x-ray film and film developers, the results can only be obtained after laborious procedures and 2-3 days of film exposure time, working with radioactive materials. Even for non-radioactive EMSA using oligonucleotide probes labeled with DIG, the experimental result can only be obtained after 2 days of work. With Viagene's non-radioactive EMSA kits using biotin-labeled probes (Biotin-EMSA) and high sensitivity of the chemiluminescent technology, a biotin-EMSA assay can be completed in ~6 hours.

The principle of non-radioactive RNA EMSA is easy to understand: This RNA EMSA kit is based on the use of probes labeled with biotin, which are much smaller chemicals than that of RNA-protein complexes and move fast in non-denatured polyacrylamide gels, whereas the much larger RNA-protein complexes would migrate more slowly and localize at a higher position in the gel. The location of biotin-probes can be detected by x-ray films, an imager or scanner (see the sample picture on the right).



The non-radioactive Biotin-labeled RNA-EMSA kits come with all necessary components for performing 100 RNA/protein binding reactions and assays. The kits can be stored for one year without loss of activity if the components are stored under recommended conditions and temperatures.

B. Kit components (stored as indicated on labels)

1. The General EMSA Kit includes follows:

- 10X RNA EMSA Binding Buffer (4°C or -20°C) 1 vial
- tRNA (-20°C) 1 vial
- RNA EMSA Enhancer (-20°C) 1 vial
- RNase Inhibitors (-20°C) 1 vial
- 6X Loading Buffer (4°C or -20°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. RNA EMSA Controls (Option):

- Extracts with specific RNA-binding proteins (-80°C) 1 Vial*
- Extracts without RNA-biding protein (-80°C) 1 Vial*

***** Since many customers have already had positive and/or negative controls, the complete kits from Viagene Biotech DO NOT include control extracts. However, the positive/negative controls can be purchased, separately. Please contact us for the availability.

C. Additional materials required

- Mini-polyacrylamide gel electrophoresis apparatus, and related chemicals and buffers.
- Sample storage apparatus such as refrigerators and ultra-low freezers.
- Orbital Shakers, vials and tubes.
- Biotin-labeled RNA probe (biotin-RNA-probe), competitive and mutant probe.
- Antibody(s) for Supershift EMSA.
- Samples with RNA-binding proteins.
- Centrifuge and centrifuge tubes.
- Li-Cor Odyssey infrared scanner.
- Sample storage apparatus such as refrigerators and ultra-low freezers.

D. Binding Reaction

1. Binding Reaction for Standard EMSA:

10X RNA EMSA binding buffer	1.5 µl
tRNA	1.0 µl
RNase Inhibitor	1.0 µl
Cell extracts	X µl
DEPC dH_2O	X µl
Total	14.0 µl

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

Biotin-RNA-probe	1.0 µl
Total	15 µl

Allow mixture to react at R/T for 20-30 minutes.

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

2. Reaction for Testing Binding Condition:

10X RNA EMSA binding buffer	1.5 µl
tRNA	1.0 µl
RNase Inhibitor	1.0 µl
Optional reagents*	1 µl
Cell extracts	X µl
DEPC dH_2O	X µl
Total	14.0 µl

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

* Optional reagent could be 1M KCl, 100mM $MgCl_2$, 200nM EDTA, 100mM DTT, 50% Glycerol or 1% NP-40. Testing the effect of each reagent needs to setup a reaction, respectively.

Biotin-RNA-probe	1.0 µl
Total	15.0 µl

Mix well and allow mixture to react at R/T for 20-30 minutes.

3. Competition Reaction*:

10X binding buffer	1.5 µl
tRNA	1.0 µl
RNase Inhibitor	1.0 µl
Cell extracts	X µl
Cold oligonucleotides	
or Mutant oligonucleotides	X µl (20-100 fold over that of biotin-RNA-probe)
DEPC dH_2O	X µl
	14 µl

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

Biotin-RNA-probe	1.0 µl
Total	15.0 µl

Mix well and allow mixture to react at R/T for 20-30 minutes.

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

4. Supershift EMSA Reaction*:

10X Supershift buffer	1.5 µl
tRNA	1.0 µl
RNase Inhibitor	1.0 µl
Cell extracts*	X µl
DEPC dH_2O	X µl
Total	14.0 µl

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

Biotin-RNA-probe	1.0 µl
Total	15.0 µl

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

Supershift Antibody	1-4 µl
Total	15 µl + vol. of antibody

Allow mixture to react at R/T for 30-60 minutes.

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

E. Gel preparation

1. Prepare and make 5.0% mini gels:

10X TBE	1.0 ml
40% Acrylamide/Bisacrylamide	2.55 ml
50% Glycerol	1.0 ml
dH_2O	15.5 ml
TEMED	20 µl
10% AP	350 µl
Total	20.42 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 ❻	3.0-4 µl
Total	~18-24 µl

Mix well, sit at R/T for 3 minutes, and centrifuge for 3 minute at 140,000 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)

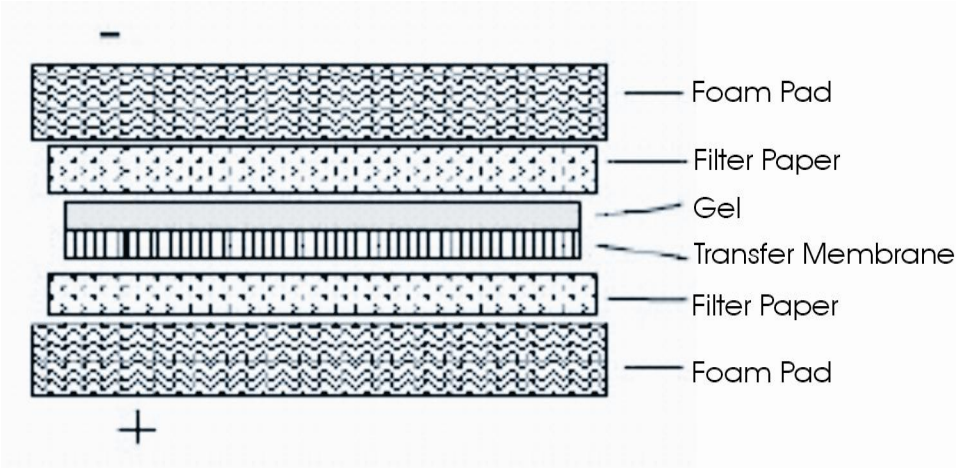
(1) Prepare transfer buffer: 0.5X TBE 1200 ml.

10× TBE	60 ml
ddH_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 side, 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)

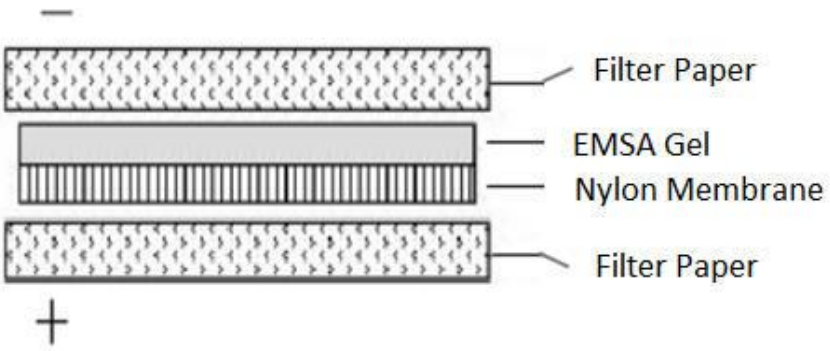
(1) Prepare transfer buffer: 0.5X TBE 200 ml.

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 RNA

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

It has been reported that RNA 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-HRP❺	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	0.6 ml
Solution B	0.6 ml
dH_2O	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 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, RNA/DNA 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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M. 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. Follow the laboratory regulation when handing Acrylamide/Bis solution.
6. Follow the laboratory regulation when handling Acrylamide/Bis solution.