



CoolShift-IRr, a General EMSA Kit Using IR Fluo-RNA Probe

User's Manual

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Viagene EMSA kits are intended for research purpose only!

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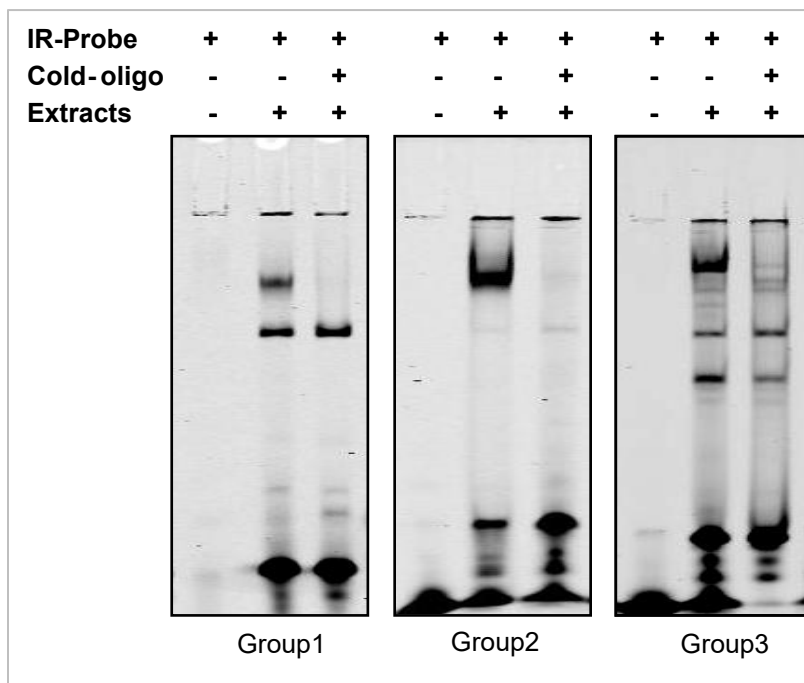
A. Introduction

The Electrophoretic Mobility Shift Assay (EMSA) is a powerful tool for evaluating RNA-protein or DNA-protein interactions, which often referred to as gel shift or gel retardation. With the “standard” radioactive EMSA techniques using P³²-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 day working.

With Viagene's non-radioactive EMSA kits using infrared (IR) fluorophore-labeled probes (IR-EMSA), an EMSA assay can be completed in ~2 hours. The IR-EMSA is not only a rapid way to perform EMSA, but the operation is also much easier than that of other detection methods, becoming the easiest and fastest way to detect the activation of transcription factors and other RNA/DNA-binding proteins.

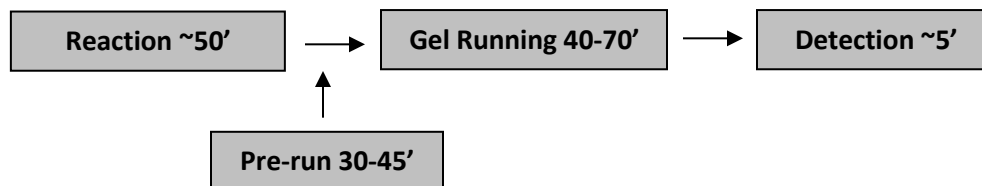
The principle of IR-EMSA is easy to understand: IR-EMSA is based on the use of probes labeled with infrared fluorophore, which are much smaller chemicals than that of RNA/DNA-protein complexes and move fast in non-denatured polyacrylamide gels, whereas the much larger RNA/DNA-protein complexes would migrate more slowly and would localize at a higher position

in the gel. The location of fluorescent-probes can be detected by imagers or scanners (see the sample picture below).



The non-radioactive infrared 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 when the components are stored at recommended condition and temperature.

Infrared EMSA timeline



B. Kit components (stored as indicated on labels)

1. The General RNA 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
▪ 6X Loading Buffer (4°C or -20°C)	1 vial
▪ 100mM DTT (-20°C)	1 vial
▪ 1M KCl (-20°C)	1 vial
▪ 100mM MgCl ₂ (-20°C)	1 vial
▪ 200mM EDTA (-20°C)	1 vial
▪ 50% Glycerol (-20°C)	1 vial
▪ DEPC dH ₂ O (4°C or -20°C)	1 vial
▪ Operation Manual	1 set

2. RNA EMSA Controls (Option):

▪ Extracts with specific RNA-binding proteins (-80°C)	1 Vial*
▪ Extracts without RNA-binding 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.
- IR dye-labeled probe (IR Fluo-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 μ l
Cell extracts	X μ l
<u>DEPC dH₂O</u>	<u>X μl</u>
Total	14.0 μ l

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

<u>IR Fluo-RNA-probe</u>	<u>1.0 μl</u>
Total	15 μ l

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

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

2. Reaction for Testing Binding Condition:

10X RNA EMSA binding buffer	1.5 μ l
tRNA	1.0 μ l
RNase Inhibitor	1.0 μ l
Optional reagents*	1 μ l
Cell extracts	X μ l
<u>DEPC dH₂O</u>	<u>X μl</u>
Total	14.0 μ l

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

* Optional reagent could be 1M KCl, 100mM MgCl₂, 200nM EDTA, 100mM DTT, 50% Glycerol or 1% NP-40. Testing the effect of each reagent needs to setup a reaction, respectively.

<u>IR Fluo-probe</u>	<u>1.0 μl</u>
Total	15.0 μ l

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

3. Competition Reaction*:

10X binding buffer	1.5 μ l
tRNA	1.0 μ l
RNase Inhibitor	1.0 μ l
Cell extracts	X μ l
Cold oligonucleotides or Mutant oligonucleotides	X μ l (20-100 fold over that of IR Fluo-probe)
<u>DEPC dH₂O</u>	<u>X μl</u>
	14 μ l

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

<u>IR Fluo-probe</u>	<u>1.0 μl</u>
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Total	15.0 μ l
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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 μ l
tRNA	1.0 μ l
RNase Inhibitor	1.0 μ l
Cell extracts*	X μ l
<u>DEPC dH₂O</u>	<u>X μl</u>
Total	14.0 μ l

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

<u>IR Fluo-probe</u>	<u>1.0 μl</u>
Total	15.0 μ l

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

<u>Supershift Antibody</u>	<u>1-4 μl</u>
Total	15 μ l + 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 ₂ O	15.5 ml
TEMED	20 μ l
<u>10% AP</u>	<u>350 μl</u>
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
<u>dH₂O</u>	<u>1170 ml</u>

Total	1200 ml
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3. Pre-running:

Pre-run the gel(s) for 30-45 minutes 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.	15-19 μ l
<u>6X Loading buffer</u>	<u>3 - 4 μl</u>
Total	18-23 μ l

Mix well, sit at R/T for 2-3 minutes, and centrifuge for 2-3 minute at 14,000 rpm.

2. Load Samples:

Load all the supernatant (18-23 μ l/each) into gel wells.

3. Electrophoresis:

Run the gel on ice at 180V until bromophenol blue gets to the lower end of gels (~45-70 min).

G. Fluorescence Detection:

1. Remove the glass cassette(s) with gel from electrophoresis unit and clean the glass plate(s) with lint-free wipes.
2. Place the cassette with gel directly on the Odyssey scan bed.
3. Set focus offset of Odyssey
Value of focus = (thickness of 2 glass plates + thickness of gel) x 50%.
4. Set the scanning channel at 700nm and the intensity to 9.
5. Start scanning following the operation instruction of fluorescence detectors or imagers.

H. Troubleshooting:

Problems	Possible reasons	Solutions
No shifted bands & no free probes are observed	Poor labeling of probes.	Check IR Fluo- labeling efficiency.
	No enough amount of IR probe used.	Use more IR Fluo- probes.
	Degraded RNA probes	The solutions should be RNase free.
	Incorrect operation of imager or scanner	Read and follow operation manuals

Problems	Possible reasons	Solutions
	Probes unable to bind to target proteins	Label RNA with large molecules may prevent probes from binding to target proteins.
All bands are smeared or streaked	Uneven gel polymerization	Use fresh gel components. Degas before polymerization. If polymerization interfered with casting gel, reduce TEMED concentration. If gel requires greater than 1 h to polymerize, increase ammonium persulfate concentration
	Excessive gel heating	Check concentrations of gel and running buffer. If they are correct, reduce voltage during electrophoresis
	Sample conductivity too high	Reduce salt concentration in nucleic acid or sample buffer
Only free probe bands can be observed	Proteins degraded	Use high quality extraction kits with protease inhibitor. Cell extraction at low temperature. Store extracts at -80 or liquid nitrogen. Use RNase inhibitor with reaction if probe is RNA. Perform Western blot to check target protein.
	Not enough Proteins	Protein concentration should be 1-3 $\mu\text{g}/\mu\text{l}$. Total 2-5 μg protein is used for EMSA.
	Too much tRNA used	Too much tRNA would also reduce specific RNA-protein complexes.
	High volume of samples used.	Cellular proteins are extracted by buffer with high salts. High volume of samples increases salt concentration which would reduce or prevent formation of RNA-protein complexes.
	No target proteins in the sample.	Express target protein by an external gene or change a cell line with target protein
	Target proteins are not activated	Treat cells with proper cell factors or other stimulating factors
Free band is sharp, complex band(s) are broad and indistinct	Heterogeneous protein	Multiple species may be due to post-translational modification or to partial degradation without loss of binding activity
Complex and free bands are broad and indistinct	Sample zone is too large (measured from top of sample to bottom of well) at the start of electrophoresis	Reduce sample volume. Increase density of sample (e.g., increase glycerol concentration) to facilitate gel loading. Minimize time between loading and electrophoresis
	Electrophoresis too long	Reduce the time of electrophoresis.
	Nucleic acid degradation	Verify that nucleic acid is intact. If nuclease

Problems	Possible reasons	Solutions
		activity is suspected, treat extracts and buffers with diethyl pyrocarbonate. Exclude divalent cations wherever possible. Use RNase and phosphatase inhibitors
Nucleic acid stuck in well, no free species visible	Protein/nucleic acid ratio is too high	Reduce the concentration of protein or increase the concentration of unlabeled nonspecific competitor
	Protein is aggregated	Change binding conditions to improve protein solubility. Possible modifications: add solutes that stabilize folded (compact) forms of proteins (e.g., glycerol); keep protein stocks and binding reactions at ice temperature; avoid freeze-thaw cycles with protein stocks; include non-ionic detergents in protein storage buffer and/or binding buffer
	Free nucleic acid and complexes are too large for gel system	Try lower percentage polyacrylamide or reduce the acrylamide/bisacrylamide ratio. Test agarose gel as alternative to polyacrylamide

For more troubleshooting and detail discussion of EMSA problems, please see webpage:

http://www.viagene.com/supports/EMSA_Forum/IR_EMSA_Q&A.htm

I. References:

1. Crothers, D.M. (1998) *Nature* 325:464-5.
2. Garner, M.M. & Revzin, A. (1986) *Trends in Biochemical Sciences* 11:395-6.
3. Hendrickson, W. (1985) *Bio Techniques* 3:198–207.
4. Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) *Nucleic Acids Research* 11:1475–1489.
5. Bannister, A. and Kouzarides, T. (1992). Basic peptides enhance protein-DNA interaction in vitro. *Nucl. Acids Res.* 20:3523.
6. Kironmai, K.M., et al. (1998). DNA-binding activities of Hop1 protein, a synaptonemal complex component from *Saccharomyces cerevisiae*. *Mol. Cell Biol.* 18:1424-35.
7. Liu R.Y. et al (1999). Constitutive activation of the JAK2/STAT5 signal transduction pathway correlates with growth factor independence of megakaryocytic leukemic cell lines. *Blood.* 93:2369-79.
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.

J. 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. Follow this instruction strictly to obtain the best results.
5. For a successful RNA EMSA, maintaining a RNase free environment is essential.
6. Follow the laboratory regulation when handling Acrylamide/Bisacrylamide solution.