VIAGENE BIOTECH INC
IMPROVING LIFE SCIENCE

CoolShift-IRd, a General EMSA Kit Using IR Fluo-DNA Probe

User's Manual

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Viagene EMSA kits are intended for research purpose only!

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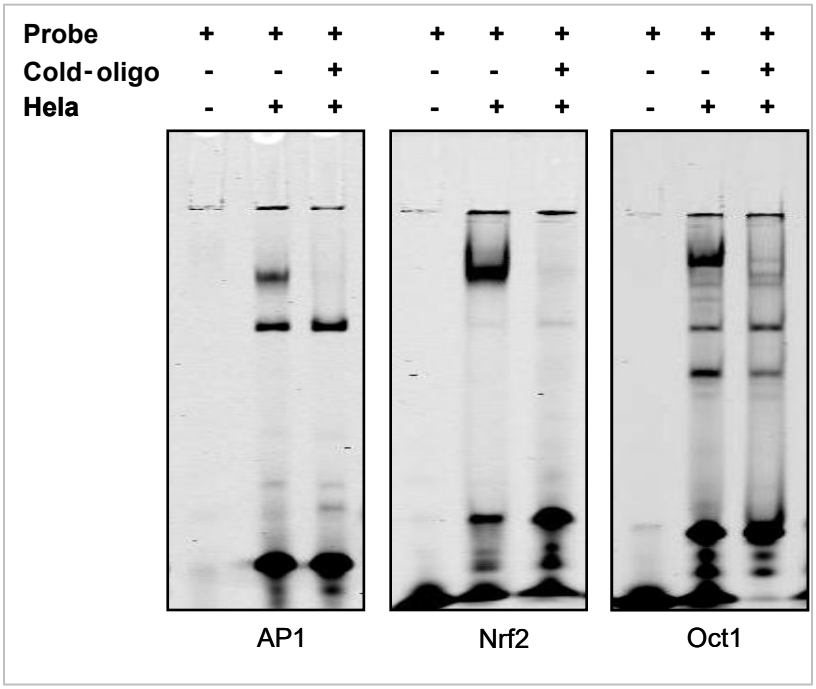
A. Introduction

The Electrophoretic Mobility Shift Assay (EMSA) is a powerful tool for evaluating DNA/RNA-protein interactions, which 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 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 (TF) and other DNA/RNA-binding proteins.

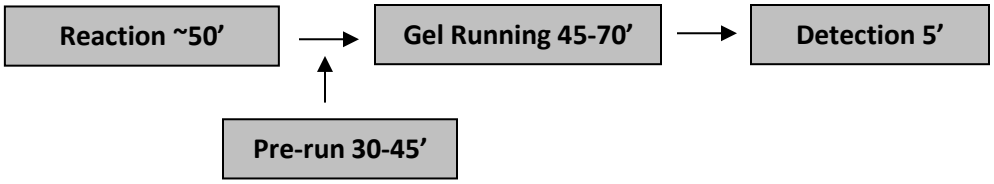
The principle of IR-EMSA is easy to understand: IR-EMSA is based on the use of probes labeled with infrared fluorophore, which are much smaller chemicals than that of DNA/RNA-protein complexes and move fast in non-denatured polyacrylamide gels, whereas the much larger DNA/RNA-protein complexes would 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 general infrared DNA-EMSA kit comes with all necessary components for performing 100 DNA-protein binding reactions and assays. The kit 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 EMSA Kit includes follows:

- 10X Binding Buffer A, B, N or G (4°C or -20°C) 1 vial
- Poly [dI: dC] (-20°C) 1 vial
- 6X Loading Buffer (4°C or -20°C) 1 vial
- 1% NP-40 (-20°C) 1 vial
- 100mM DTT (-20°C) 1 vial
- 1M KCl (-20°C) 1 vial
- 100mM $MgCl_2$ (-20°C) 1 vial
- 100nM EDTA (-20°C) 1 vial
- 50% Glycerol (-20°C) 1 vial
- Operation Manual 1 set

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, positive/negative controls for a specific TF 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 activated transcriptional factors.
- Centrifuge and centrifuge tubes.
- Li-Cor Odyssey infrared scanners.
- Sample storage apparatus such as refrigerators and ultra-low freezers.

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	14.0 µl

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

IR Fluo-probe	1.0 µl
Total	15 µl

Allow mixture to react at R/T for 20-30 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. If severe aggregates are observed in gel wells, 1 µl of 2% Tween-20 may be added in the binding reaction.

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	14.0 µl

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

* Optional reagent could be 1% NP-40, 100mM DTT, 1M KCl, 100mM $MgCl_2$, 100nM EDTA, or 50% Glycerol. For testing effects of optional reagents on DNA-protein interaction, the reaction needs to be setup separately for each reagent.

IR Fluo-probe	1.0 µl
Total	15.0 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 IR Fluo-probe)
dH_2O	X µl
	14 µl

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

IR Fluo-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 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	14.0 µl

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

IR Fluo-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 DNA-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
ddH_2O	1170 ml
Total	1200 ml

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 µl
6X loading buffer ❻	3 - 4 µl
Total	13-28 µl

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 µl/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 dry 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.

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 DNA probes	The solutions should be DNase free.
	Incorrect operation of imager or scanner	Read and follow operation manuals
	Probes unable to bind to target proteins	Label DNA with large molecules may prevent probes from binding to target proteins.

Problems	Possible reasons	Solutions
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. Nuclear extraction at low temperature. Store extracts at -80 or liquid nitrogen. Perform Western blot to check target protein.
	Not enough Proteins	Protein concentration should be 1-3 μg/μl. Total 2-5μg protein is used for EMSA.
	Too much poly[dI:dC] used	Too much Poly[dI:dC] would also reduce specific DNA-protein interaction.
	High volume of samples used.	Nuclear proteins are extracted by buffer with high salts. High volume of samples increases salt concentration which would reduce or prevent formation of DNA-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 activity is suspected, treat extracts and buffers with diethyl pyrocarbonate. Exclude divalent cations wherever possible.

Problems	Possible reasons	Solutions
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:

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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. Follow the laboratory regulation when handling Acrylamide/Bisacrylamide solution.