Non-Radioactive EMSA Kits with IR Fluo-Probes

User's Manual

VER. 03.16

03/2016

This manual is used for Viagene's complete EMSA kits with catalog number beginning with IRTF….

Viagene EMSA kits are intended for research purpose only!

Viagene Biotech Inc.

3802 Spectrum Blvd., Suite 124
Tampa, FL 33612

Tech-Support: 813.902.2209
Fax: 813.903.0649
Skype: viagene-biotech
E-mail: viagene@hotmail.com

TABLE OF CONTENTS

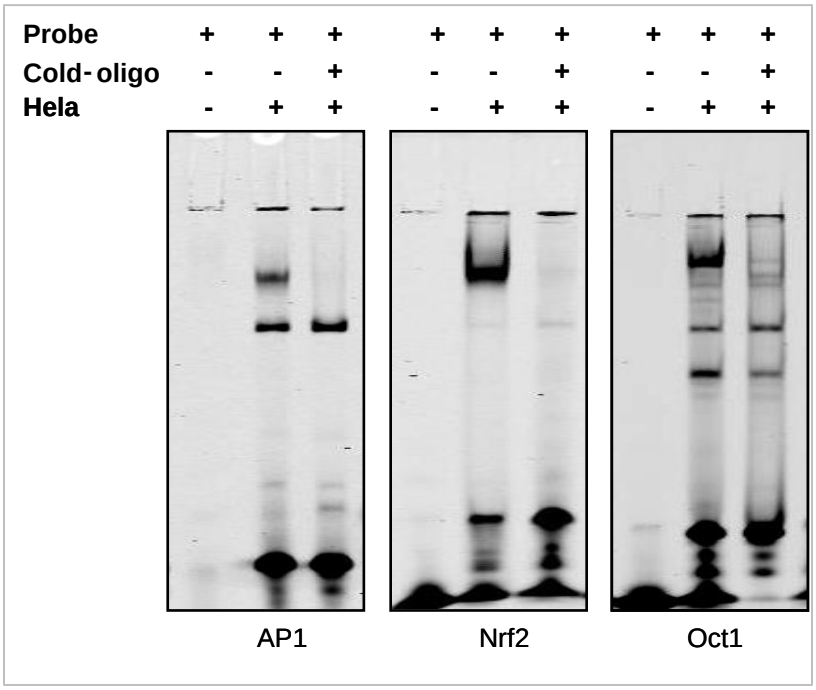
A. Introduction

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. 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 also is much easier than that of other detection methods, becoming the easiest and fastest way to detect the activation of transcription factors and other DNA/RNA-binding proteins.

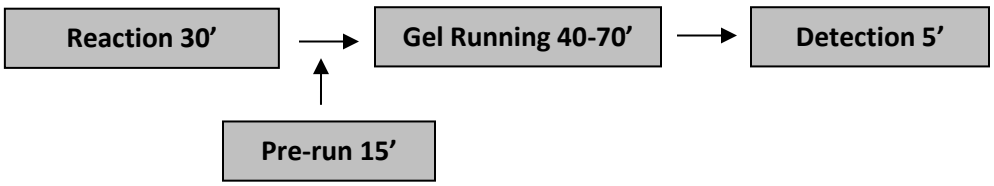
The principle of IR-EMSA is easy to understand: IR-EMSA is based on the use of probes labeled with infrared fluorophores, which are much smaller chemicals than that of probe/TF complexes and move fast in non-denatured polyacrylamide gels, whereas the much larger probe/TF complexes would migrate more 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 complete non-radioactive IR-EMSA kits come with IR-probes and all necessary components for performing 30/60 DNA/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 EMSA standard Kits include the follows:

- ❶ 10X Binding Buffer A, B, N or G (4 °C) 1 Vial
- ❷ Poly [dI: dC] (-20 °C) 1 Vial
- ❸ IR Fluorophore-Labeled Probe (-20 °C) 1 Vial
- ④Cold Oligonucleotide (-20 °C) Option
- ⑦Mutant Unlabeled Oligonucleotide (-20 °C) Option
- ❺ IR-EMSA enhancer (4 °C) 1 Vial
- ❻ 6X Loading Buffer (4 °C) 1 Vial
- ⑧ 10X Supershift Buffer (4 °C) Option
- ⑨ Supershift Antibody Option
- User's 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, 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

- Samples with activated transcriptional factors.
- Mini-polyacrylamide gel electrophoresis apparatus and related chemicals and buffers.
- Centrifuge and centrifuge tubes.
- Li-Cor Odyssey infrared scanners.
- Sample storage apparatus such as refrigerators and ultra-low freezers.
- Orbital Shaker, vials and tubes.

D. Binding Reaction

1. Binding Reaction:

10X binding buffer ❶	1.5 µl
Poly [dI: dC] ❷	1.0 µl
IR-EMSA enhancer❺	2.0 µl
Nuclear extracts*	X µl
dH_2O	X µl

Total	19.0 µl

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

IR Fluo-probe ❸	1 µl
Total	20 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.

2. Competition Reaction*:

10X binding buffer❶	1.5 µl
Poly [dI: dC] ❷	1.0 µl
IR-EMSA enhancer❺	2.0 µl
Nuclear extracts	X µl
Cold oligonucleotides ④	
or Mutant oligonucleotides ⑦	X µl (20-100 fold over that of IR Fluo-probe)
dH_2O	X µl
	19 µl

Mix well and sit at R/T for 20 minutes

IR Fluo-probe ❸	1.0 µl
Total	20 µl

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

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

3. Supershift EMSA Reaction*:

10X Supershift buffer ⑧	1.5 µl
Poly [dI: dC] ❷	1.0 µl
IR-EMSA enhancer❺	2.0 µl
Nuclear extracts*	X µl
dH_2O	X µl
Total	18.0 µl

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

IR Fluo-probe ❸	1 µl
Total	19 µl

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

Supershift Antibody ⑨	1-4 µl
Total	19 µl + vol. of antibody

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

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

E. Gel preparation

1. **Prepare and make 5% 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 30-60 minutes at 120V in cooled 0.25X TBE on ice, then, flush each well with 0.25% TBE before loading samples.

F. Electrophoresis:

1. **Prepare samples：**

Binding reaction from section D.	20-24 µl
6X loading buffer ❻	3 - 4 µl
Total	23-28 µl

Mix well, sit at R/T for 2-3 min and centrifuge for 5 minute at 14,000 rpm.

2. **Load samples：**

Load all the supernatant (23-28 µl/each) into gel wells.

3. **Electrophoresis:**

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

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 (2-2.5 mm) = one glass plate thickness of 1-1.5 mm + 1/2 of gel thickness (1-1.5 mm).
4. Set the scanning channel at 700nm and the intensity to 8.5.
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 probes used.	Use more IR Fluo- probes.
	Degraded DNA/RNA probes	The solutions should be DNase or RNase free.
	Incorrect operation of imager or scanner	Read and follow operation manuals
	Probes unable to bind to target proteins	Label DNA/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. Nuclear 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 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

Problems	Possible reasons	Solutions
		DNA/RNA-protein complexes.
	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/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 run-time
	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. 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. Hendrcikson, 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 24 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/Bisacrylamide solution.