Odyssey®

Infrared Imaging System

Odyssey Infrared EMSA Kit Instruction Manual

Revised June, 2006. The most recent version of this protocol, with color figures, is posted at http://biosupport.licor.com/support

LI-COR®

Biosciences

Doc# 984-08540

Contents

I. Kit Contents

Tube	Reagent	Volume	Storage
1	10 X Binding Buffer, 100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5	500 µl	-20 °C
2	25 mM DTT, 2.5% Tween®-20	500 µl	-20 °C
3	Poly (dI·dC), 1 µg/µl in 10 mM Tris, 1 mM EDTA; pH 7.5	125 µl	-20 °C
4	Sheared Salmon Sperm DNA, 0.5 µg/µl in 10 mM Tris, 1 mM EDTA; pH 7.5	125 µl	-20 °C
5	50% Glycerol	500 µl	-20 °C
6	1% NP-40	500 µl	-20 °C
7	1 M KCl	500 µl	-20 °C
8	100 mM $MgCl_2$	500 µl	-20 °C
9	200 mM EDTA, pH 8.0	500 µl	-20 °C
10	10 X Orange Loading Dye	500 µl	-20 °C

II. Storage

Items are shipped on dry ice. Upon receipt items should be stored as indicated above.

III. Introduction

Gel shift assays or electrophoretic mobility shift assays (EMSA) provide a simple method to study DNA-protein interactions. This assay is based on the principle that a DNA-protein complex will have different mobility during electrophoresis than non-bound DNA. These shifts can be visualized on a native acrylamide gel using labeled DNA to form the DNA-protein binding complex. To date, protocols require labeling DNA by radioisotope (1), digoxygenin (2), or biotin (3). The Odyssey® Infrared EMSA Kit coupled with IRDye® 700 EMSA oligonucleotides is an excellent alternative method to radioisotopic and chemiluminescent detection

methods for EMSA analysis and visualization (4,5). Using IRDye® EMSA reagents, assays can be completed in less than two hours with no gel transfer or film exposure. The gel doesn't even have to be removed from the glass plates for imaging.

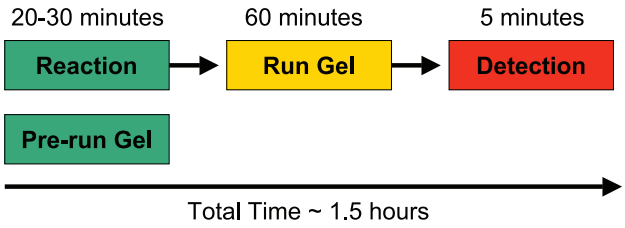

Figure 1. Infrared EMSA timeline with IRDye® 700 EMSA oligonucleotides.

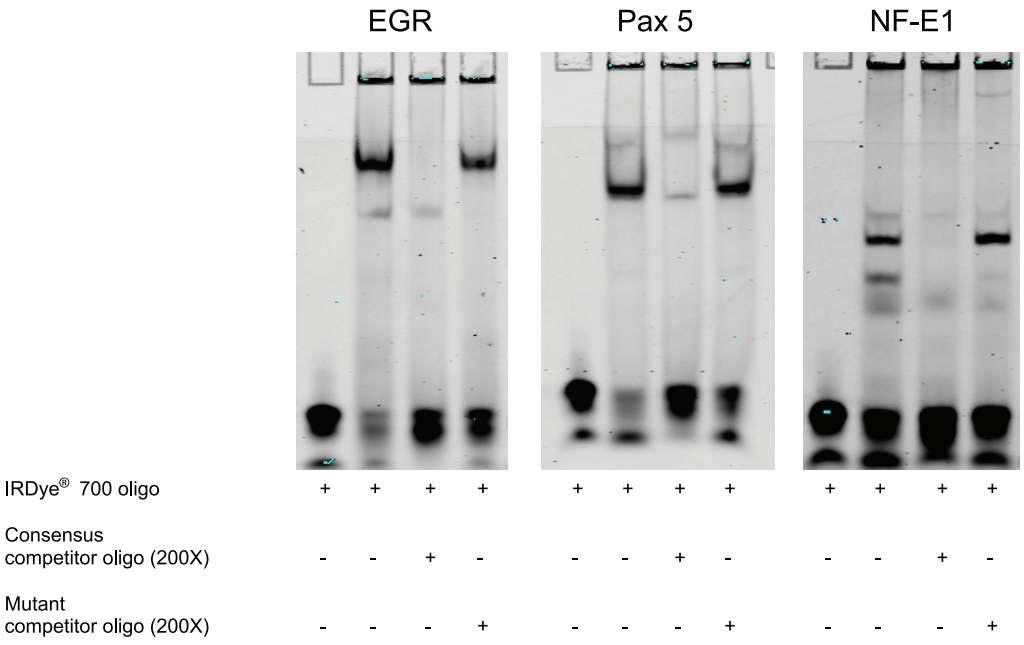

Figure 2. IRDye® 700 EMSA of 3 different transcription factor targets.

IV. Additional Materials Required

- IRDye® end-labeled DNA. IRDye® 700-labeled double-stranded DNA oligonucleotides are available from LI-COR for various transcription factors. See www.licor.com for the most up to date selection. Or see *IRDye® Labeling of DNA Fragments* in *Section V.* for further details on how to obtain IRDye® end-labeled oligonucleotides.

⚠ **Important:** DNA must be end-labeled. Internal labels (as generated by random priming) will inhibit DNA-Protein complex formation.

- Ultrapure nuclease-free water.
- Polyacrylamide gel (5% gel is a good starting point) in Tris-acetate, Tris-borate, or Tris-glycine-EDTA and corresponding electrophoresis buffer.
- Electrophoresis unit.
- Odyssey® Infrared Imaging System.

V. General Methodology for Infrared Electrophoretic Mobility Shift Assay (IR-EMSA)

IRDye Labeling of DNA Fragments

IRDye® 700-labeled double-stranded DNA oligonucleotides are available from LI-COR for various transcription factors (see www.licor.com for the most up to date selection). To obtain IRDye® end-labeled DNA fragments for other transcription factors of interest, IRDye® infrared dye-labeled oligos can be used. It is critical that the DNA fragment is end-labeled rather than having dye incorporated into the DNA. Dye incorporation into the DNA fragment interferes with the formation of the DNA-Protein complex.

Synthetic oligonucleotides 5′ end-labeled with IRDye® 700 or IRDye® 800 are available from LI-COR Biosciences (www.licor.com). Oligonucleotides are manufactured in single-strand form; therefore, both sense and antisense DNA oligonucleotides must be purchased.

⚠ **Important:** Both oligonucleotide sequences should be end-labeled with the same IRDye®. There is a significant decline (~70%) in signal intensity when using only one end-labeled oligonucleotide.

Once oligonucleotides are obtained, they need to be annealed to form a double-stranded DNA fragment. Oligonucleotides are annealed by placing the oligonucleotide set in a 100°C heat block for 5 minutes. Leave the oligonucleotides in the heat block and turn it off to slowly cool to room temperature.

Binding Reaction

A universal binding condition that applies to every protein-DNA interaction can not be recommended, since binding conditions are specific for each protein-DNA interaction. Thus, you should establish binding reaction conditions for each protein-DNA pair. The Infrared EMSA Kit provides all the reagents necessary to optimize an IR-EMSA binding reaction.

Binding Reaction Optimization

Optimized binding reactions for all infrared dye-labeled EMSA-ready oligonucleotides are provided in their pack inserts, along with a suggested positive control nuclear extract. All other IR-EMSA binding reactions will need further optimization. Optimization can be done by adding supplementary components to the binding buffer supplied in this kit. This can be done using the binding reaction setup in Table 1. Reactions should be set up and added in the order listed in Table 1.

Table 1. Example IR-EMSA binding reaction optimization experiment.

Kit Component	Reaction							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Ultra Pure water	14 µl	13 µl	12 µl	12 µl	12 µl	12 µl	12 µl	12 µl
10 X Binding Buffer (Tube 1)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
25mM DTT/2.5% Tween-20 (Tube 2)	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
1 µg/µl Poly (dI·dC) (Tube 3)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Optional Components								
0.5 µg/µl Salmon Sperm DNA (Tube 4)	-	-	1 µl	-	-	-	-	-
50% Glycerol (Tube 5)	-	-	-	1 µl	-	-	-	-
1% NP-40 (Tube 6)	-	-	-	-	1 µl	-	-	-
1 M KCl (Tube 7)	-	-	-	-	-	1 µl	-	-
100 mM MgCl2 (Tube 8)	-	-	-	-	-	-	1 µl	-
200 mM EDTA (Tube 9)	-	-	-	-	-	-	-	1 µl
IRDye™ end labeled oligo (50nM)	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
Protein Extract (5 µg/ µl)	-	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl
TOTAL VOLUME	*20 µl*	*20 µl*	*20 µl*	*20 µl*	*20 µl*	*20 µl*	*20 µl*	*20 µl*

NOTE: In some cases, DNA control reactions (no protein) were observed to have lower signal than reactions containing protein. This may be due to lower stability of the dye in certain buffer conditions. The addition of DTT and Tween-20 to all reactions reduces this phenomenon.

Reactions are incubated to allow protein binding to DNA. Time required for binding is the same as when radioactively-labeled DNA fragments are used; a typical incubation condition is 20-30 minutes at room temperature. Since IRDyes® are sensitive to light, it is best to keep binding reactions in the dark during incubation periods (e.g. put tubes into a drawer or simply cover the tube rack with aluminum foil). After the incubation period, 2 µl of 10X Orange Loading Dye (Tube 10) should be added to the 20 µl EMSA reaction.

⚠ **Important:** It is critical not to use any blue loading dye (e.g. bromophenol blue), as this will be visible on the Odyssey image.

Electrophoresis

Separation of protein-DNA complexes is usually performed by loading binding reactions onto native polyacrylamide gels. Percentage of the gel depends on the protein size and DNA fragment, but a 5% gel is a good starting point. Electrophoresis is usually performed at 10 V/cm at room temperature or at 4 °C in Tris-acetate, Tris-borate, or Tris-glycine-EDTA gel and buffer.

NOTE: For best results, perform electrophoresis in dark (simply put a cardboard box over the electrophoresis apparatus).

Imaging

There is no need to remove the gel from the glass plates. This makes gel handling easier and allows running the gel further, if needed, after scanning is completed. Possible deformations or tearing of the gel while separating plates are also eliminated.

⚠ **Important:** It is important to set the Odyssey® focus offset to the center of the gel. For example, if the bottom glass plate is 1 mm thick and the gel 1 mm thick, set the focus offset to 1.5 mm.

Quantification

One of the benefits of using Odyssey for EMSA analysis is that it provides an easy method for quantification. However, there are issues to consider when using Odyssey to quantify EMSA results. The primary issue is that the free DNA fragment has much less signal than the DNA fragment when bound to a protein, making quantification of the unbound DNA inaccurate. The addition of DTT/Tween-20 to the binding reaction stabilizes the dye and reduces this phenomenon.

In addition, it is unrealistic to perform quantification analyses under the assumption that the free DNA band in the control, containing DNA only (no extract), should equal the sum of the signals of the free and bound DNA in the samples where the protein-DNA binding reaction occurs. Using end-labeled oligonucleotide duplexes as the DNA source and nuclear extract as a protein source render this assumption impractical, due to the non-specific binding that occurs from using a nuclear extract. Oligonucleotides can also complicate quantification because the free oligonucleotides form a smear rather than a nice tight band. This makes it more difficult to assign an intensity value to the bands.

⚠ **Important:** When quantifying Odyssey® generated EMSA data, great care must be taken in experimental design and analysis.

VI. Mobility Shift Experiment Example using AP-1 IRDye 700 Infrared Dye Labeled Oligonucleotides (LI-COR, Part # 829-07925)

Prepare and Pre-Run Gel

1.	Use a pre-cast 5% TBE native acrylamide gel or prepare a native acrylamide gel in 0.5X TBE.
2.	Pre-electrophorese gel in 0.5X TBE for 30 minutes at 70 V (for a 9 x 7 cm gel) or 10 V per cm gel.
3.	While gel is pre-running, proceed to *Binding Reaction* steps.

Binding Reaction

1.	Reactions should be set up and added in the order listed:

Kit Component	Reaction			
	1	**2**	**3**	**4**
Ultra Pure Water	12 µl	12 µl	9 µl	9 µl
10 X Binding Buffer (Tube 1)	2 µl	2 µl	2 µl	2 µl
25mM DTT/2.5% Tween-20 (Tube 2)	2 µl	2 µl	2 µl	2 µl
1 µg/µl Poly (dI·dC) (Tube 3)	1 µl	1 µl	1 µl	1 µl
1% NP-40 (Tube 6)	1 µl	1 µl	1 µl	1 µl
100 mM $MgCl_2$ (Tube 8)	1 µl	1 µl	1 µl	1 µl
200X Consensus Competitor Oligo	0 µl	0 µl	1 µl	0 µl
200X Mutant Competitor Oligo	0 µl	0 µl	0 µl	1 µl
AP-1 IRDye® End-labeled Oligo (Part # 829-07925)	1 µl	1 µl	1 µl	1 µl
HeLa 4hr Serum Response Nuclear Extract (2.5 µg/µl)	-	2 µl	2 µl	2 µl
TOTAL VOLUME	*20 µl*	*20 µl*	*20 µl*	*20 µl*

2.	Gently mix tube by tapping the side of the tubes.
3.	Briefly centrifuge sample into the bottom of the tube.
4.	Place tubes in box or drawer protected from light and incubate for 20 minutes at room temperature.
5.	Add 2 µl of 10 X Orange Loading Dye (Tube 10) to each reaction tube gently pipette to mix.

Electrophoresis of Binding Reaction

1.	Turn off pre-running gel current.
2.	Flush gel wells with 50 µl of electrophoresis buffer.
3.	Load 20 µl of each sample reaction into the wells of the gel.
4.	Turn on current to 70 V (or 10 V per cm of gel)
5.	Cover gel box with dark box.
6.	Electrophorese for 1 hour or until orange dye migrates to the bottom of the gel.

Detection

1.	Remove gel from electrophoresis unit and dry off glass plates with a lint free wipes.
2.	Place gel in glass plates directly on the Odyssey scan bed.

3.	Set focus offset of Odyssey (2.5 mm) = glass plate thickness (2 mm) + 1/2 of gel thickness (0.5 mm). Set Scan Intensity of the 700 nm channel equal to 8.
4.	Start scan.

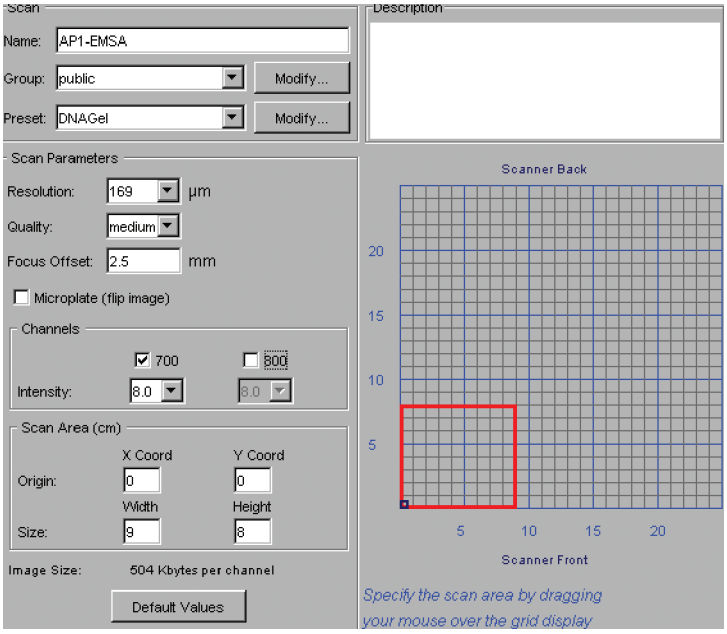

Figure 3. Scan parameters shown in Odyssey Scanner Console window.

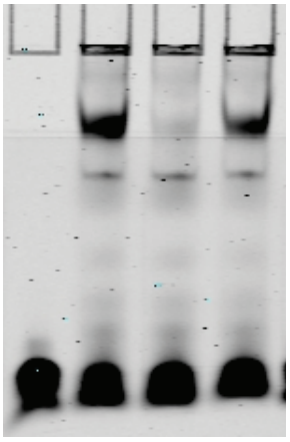

Figure 4. AP-1 IRDye® 700 EMSA.

AP-1IRDye™700 oligo	+	+	+	+
Consensus competitor oligo (200X)	-	-	+	-
Mutant competitor oligo (200X)	-	-	-	+

VII. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
Weak or no signal.	Did not add DTT/ Tween®-20 to binding reaction.	Add 1 µl of 25 mM DTT/ 2.5% Tween-20 to binding reaction.
	Not enough IRDye® labeled DNA used.	Increase amount of IRDye® labeled DNA added to the reaction.
	Target DNA degraded.	Verify integrity of DNA.
	Imaged in the wrong channel of the Odyssey.	When using IRDye® 700-labeled DNA turn on the 700 nm laser.
No shift bands detected or weak signal.	**Auto Sensitivity** selected in Odyssey Software.	Change **Sensitivity** setting to manual and adjust **Sensitivity** manually.
	Scanned gel with **Intensity** too low.	Increase **Intensity** parameter in Odyssey Software to 8 and scan again.
	Incorrect **Focus Offset**.	Adjust the **Focus Offset** in Odyssey software to equal the thickness of the glass plate plus half the thickness of the gel and scan again.
	DNA/Protein complex disrupted due to heat or vortexing.	Run gel with cooled buffer.
		Do not vortex binding reaction.
	Not enough extract.	Add more extract to reaction.
	Degraded extract.	Minimize freeze thaw cycles.
		Use protease inhibitors.
	System not fully optimized.	Use additives in the kit to determine their effects on binding efficiency.
Spots or speckling.	Contamination on glass surfaces.	Clean glass gel plates and the Odyssey scanning surface with isopropanol.

VIII. References

1. Wolf, S.S., Hopley, J.G., and Schweizer, M. (1994) The application of ^{33}P-labeling in the Electrophoretic mobility shift assay. *Biotechniques* 16, 590-592.
2. Suske, G., Gross, B., and Beato, M. (1989) Non-radioactive method to visualize specific DNA-protein interactions in the band shift assay. *Nucleic Acids Res.* 17, 4405.

3. Ludwig, L.B., Hughes, B.J., and Schwartz, S.A. (1995) Biotinylated probes in the electrophoretic mobility shift assay to examine specific dsDNA, ssDNA or RNA-protein interactions. *Nucleic Acids Res.* 23, 3792-3793.

4. Li, Y., Ahmed, F., Ali, S., Philip, P.A., Kucuk, O., and Sarkar, F.H. (2005) Inactivation of nuclear factor κB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. *Cancer Res.* 65:6934-6942.

5. Geddie, M.L., O'Loughlin, T.L., Woods, K.K., and Matsumura, I. (2005) Rational design of p53, and intrinsically unstructured protein, for the fabrication of novel molecular sensors. *J. Biol. Chem.* (M508149200).

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH (Germany, Austria, Switzerland, Czech Republic, Hugary, Slovakia): +49 (0) 6172 17 17 771
LI-COR UK Ltd.: +44 (0) 1223 422104
www.licor.com