Electrophoretic Mobility Shift Assay (EMSA) Using IRDye® Oligonucleotides

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Abstract

This is a sample Mobility Shift Protocol (NFκB). Each oligo labeled with IRDye 700 provided by LI-COR® Biosciences for EMSA reactions will have an optimized protocol to measure the protein-DNA interaction. See the specific EMSA oligo pack insert for more information. As an example, the NFκB protein-DNA interaction will be described in this protocol.

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.

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Guidelines

I. 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 (1) radioisotope, (2) digoxygenin, or (3) biotin.The Odyssey® Family of Imagers (LI-COR® Biosciences) offers a quick and easily-adapted alternative method to radioisotopic and chemiluminescent detection methods for EMSA analysis and visualization.

A DNA oligonucleotide end-labeled with LI-COR IRDye is a good substrate for protein binding. LI-COR offers pre-annealed oligonucleotides specific to eight unique binding proteins. DNA detection using IRDye reagents is linear within a 50-fold dilution range, from 9.1 fmol to 0.18 fmol. Additional benefits include no hazardous radioisotope, no gel transfer to membrane or gel drying, no chemiluminescent substrate reagents, and no film exposure. Following electrophoresis, the gel can be imaged while remain- ing in the glass plates. If necessary, the gel can be placed back in the electrophoresis unit

and run longer.

Existing mobility shift assay protocols can be easily transformed into infrared assays by replacing the existing DNA oligonucleotides with oligonucleotides end-labeled with IRDye reagents.The binding conditions and electrophoresis conditions will remain the same as with any other EMSA detection method.

II. General Methodology

EMSA Oligonucleotides Labeled with IRDye 700

	Part Number
IRDye 700 p53 Consensus Oligonucleotide	829-07921
IRDye 700 STAT3 Consensus Oligonucleotide	829-07922
IRDye 700 CREB Consensus Oligonucleotide	829-07923
IRDye 700 NFκB Consensus Oligonucleotide	829-07924
IRDye 700 AP-1 Consensus Oligonucleotide	829-07925
IRDye 700 Sp-1 Consensus Oligonucleotide	829-07926
IRDye 700 HIF-1 Consensus Oligonucleotide	829-07929
EMSA Buffer Kit for the Odyssey	829-07910

Labeling DNA Fragments with IRDye Infrared Dyes

To obtain DNA fragments end-labeled with IRDye infrared dyes, oligos labeled with IRDye infrared dyes are used. It is critical that the DNA fragment is end-labeled rather than having dye incorporated into the DNA, which interferes with the formation of the DNA-protein complex.

Oligonucleotides are manufactured in single strand form; therefore, both forward and reverse DNA

oligo-nucleotides must be purchased. 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 and then leaving the oligonucleotides in the heat block and turning it off to slowly cool to room temperature.

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

III. Mobility Shift Sample Protocol (NFκB)

Each oligo labeled with IRDye 700 provided by LI-COR® Biosciences for EMSA reactions will have an optimized protocol to measure the protein-DNA interaction. See the specific EMSA oligo pack insert for more information. As an example, the NFκB protein-DNA interaction will be described in this protocol.

See 'STEPS' for protocol.

Figure 1. IRDye 700 NFκB oligonucleotides were separated on a native polyacrylamide gel (4-12% TBE, Invitrogen EC62352BOX) and imaged on the Odyssey® Infrared Imaging System.
Lane 1) no nuclear extract;
Lanes 2 and 5) 10 µg Raji nuclear extract;
Lanes 3 and 6) 5 µg Raji nuclear extract;
Lanes 4 and 7) 2.5 µg Raji nuclear extract.

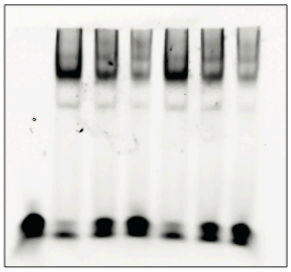
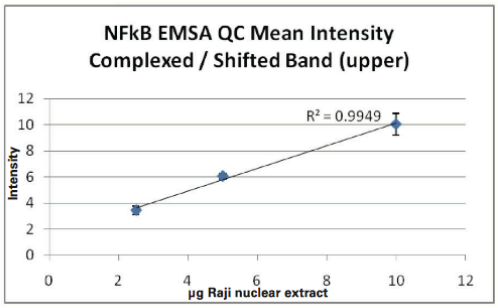


Figure 2. The uppermost shifted band in Lanes 2-7 of Figure 1 was analyzed to determine the level of NFkB binding to the IRDye 700 NFκB oligonucleotides.



One of the benefits of using the Odyssey® Infrared Imaging System for EMSA analysis is that it provides an easy method for quantification. However, there are issues to consider when using the Odyssey Imager 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 renders 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 tight band.This makes it more difficult to assign an intensity value to bands.

Optimization

Binding Reaction

A universal binding condition that applies to every protein-DNA interaction cannot be recommended, since binding conditions are specific for each protein-DNA interaction.Thus, the user should establish binding reaction conditions for each protein-DNA pair. Binding buffer should be the same for this method as with any other mobility shift detection method used.

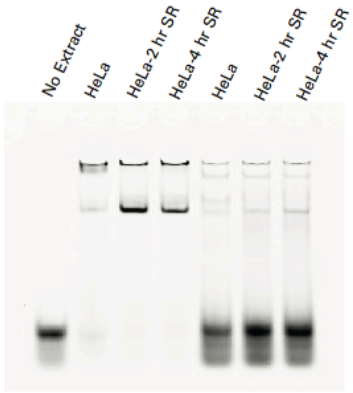
After the addition of DNA to the protein-buffer mix, reactions are incubated to allow protein to bind to DNA. Time required for binding is the same as when radioactively-labeled DNA fragments are used; a typical incu- bation condition is 20-30 minutes at room temperature. Since IRDye reagents are sensitive to light, it is best to keep binding reactions in darkness during incubation periods (e.g., put tubes into a drawer or simply cover the tube rack with aluminum foil). After the incubation period, native loading dye is added to the binding reaction.

NOTE: In some cases, it was observed that DNA control reactions (no protein) have lower signal than reactions containing protein.This may be due to lower stability of the dye in certain buffer conditions.The addition of 5 mM DTT and 0.5%Tween 20 to all reactions reduces this phenomenon.

IMPORTANT: It is critical not to use any blue loading dye (e.g., bromophenol blue), as this will be visible on the Odyssey® image. Use 10X Orange loading dye instead (LI-COR®, P/N 927-10100).

Figure 3. AP-1 EMSA using IRDye 700 end-labeled oligonucleotide duplex.
It is common to use unlabeled DNA duplex to determine binding specificity. Excess unlabeled DNA is added to the binding reaction; therefore, it competes with the labeled DNA for binding sites. If competition eliminates labeled DNA binding, no shift is observed (see last three lanes in gel), indicating that the binding reaction is specific.

Competition reactions contained 100-fold molar excess of wild-type oligonucleotide duplex. Nuclear extracts of HeLa, HeLa 2-hour serum response, and HeLa 4-hour serum response, were used to visualize an increase in AP-1 binding as a result of the serum response treatment to the HeLa cells.



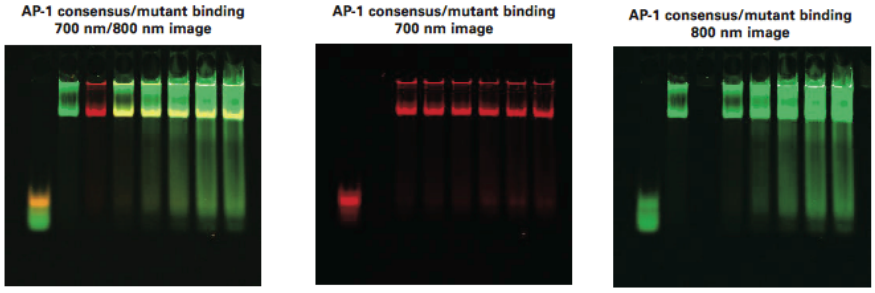


Figure 4. AP-1 EMSA using 2.5 µg HeLa 4-hour serum response nuclear extract to demonstrate binding specificity of AP-1 consensus DNA duplex. Binding specificity determination using Odyssey® two-color imaging.

Competition using mutant DNA duplexes is another common method to determine binding specificity. A mutant DNA sequence is used to compete with the wild-type binding sequence. Specific binding is observed when mutant DNA (unlabeled) does not reduce the binding of labeled wild-type DNA.Two-color analysis of mutant vs. wild-type binding is done using the Odyssey Infrared Imaging System.The wild-type oligos are labeled with IRDye 700 phosphoramidite and mutant oligos with IRDye 800 phosphoramidite. In the figure above, the mutant non-specific binding is very intense (800 nm image); however, there is no decrease in wild-type binding (700 nm image).

Lane 1	Free IRDye 700 AP-1 consensus oligonucleotide and IRDye 800 AP-1 mutant oligonucleotide with no nuclear extract;
Lane 2	Nuclear extract with 0:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 3	Nuclear extract with 1:0 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 4	Nuclear extract with 1:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 5	Nuclear extract with 1:2 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;

Lane 6	Nuclear extract with 1:3 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 7	Nuclear extract with 1:4 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 8	Nuclear extract with 1:5 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;

References

1. Wolf, S.S., Hopley, J.G., and Schweizer, M. (1994) The Application of 33P-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 Research, **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 Research, **23**, 3792-3793.

Materials

IRDye Consensus Oligonucleotide See guidelines by LI-COR

Odyssey EMSA Buffer Kit 829-07910 by LI-COR

10X Orange Loading Dye 927-10100 by Licor

Protocol

Gel Preparation

Step 1.

Native pre-cast polyacrylamide gels such as 5%TBE (BioRad) or 4-12%TBE (Invitrogen) are recommended. Alternatively, the recipe below can be used to prepare a 4% native gel.

NOTES

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NOTE: The protein shift detected on each gel type (i.e., 5% vs 4-12%) will be unique.

Gel Preparation

Step 2.

Prepare 4% native polyacrylamide gel containing 50 mMTris, pH 7.5; 0.38 M glycine; and 2 mM EDTA:

For 40 ml mix:

- 5 mL 40% polyacrylamide stock (Polyacrylamide-BIS ratio = 29:1)
- 2 mL 1 MTris, pH 7.5
- 7.6 mL 1 M Glycine
- 160 µL 0.5 M EDTA
- 26 mL H2O
- 200 µL 10% APS
- 30 µL TEMED

Gel Preparation

Step 3.

Pour the gel between glass plates and wait about 1-2 hours to polymerize.

DURATION
02:00:00

Oligo Preparation

Step 4.

Dilute oligos in 1XTE for final concentration of 20 pmol/µL.

NOTES
Margaret Dentlinger 10 May 2017

EMSA oligonucleotides from LI-COR Biosciences are pre-annealed.

Oligo Preparation

Step 5.

Place 5 µL of forward IRDye 700 oligo into a new tube and add 5 µL of reverse IRDye 700 oligo.

Oligo Preparation

Step 6.

Anneal oligos by placing the oligo set in a 100° C heat block for 3 minutes. Leave the oligos in the heat block and turn it off to slowly cool to room temperature.

DURATION
00:03:00

Oligo Preparation

Step 7.

Dilute annealed oligos 1 µL in 199 µL water. This is your working DNA stock. Oligos can be stored at -20° C for up to a year if protected from light.

Binding Reaction

Step 8.

For NFκB IRDye 700 oligonucleotide, the following binding reaction is a good starting point.

Reaction	µL
10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5)	2
Poly(dI•dC) 1 µg/µL in 10 mM Tris, 1 mM EDTA; pH 7.5	1
25 mM DTT/2.5%Tween®-20	2
Water	13
IRDye 700 NFκB	1
Raji nuclear extract (Positive control) (5 µg/µL)	1
TOTAL	**20**

Binding Reaction

Step 9.

After the addition of the DNA to the protein-buffer mix, reactions are incubated to allow protein binding to DNA. A typical incubation condition is 20-30 minutes at room temperature.

DURATION
00:30:00

NOTES
Margaret Dentlinger 14 Dec 2016

Since IRDye 700 infrared dye is sensitive to light, it is best to keep binding reactions in the dark during incubation periods (e.g., put tubes into a drawer or cover the tube rack with aluminum foil).

Electrophoresis

Step 10.

Add 1 µL of 10X Orange loading dye (LI-COR®, P/N 927-10100), mix, and load on a gel.

Electrophoresis

Step 11.

Run the gel at 10 V/cm for about 30 minutes in non-denaturing buffer (i.e., 1X TGE or TBE buffer).

DURATION
00:30:00

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

Imaging

Step 12.

Gels can be imaged either inside the glass plates or removed from the glass plate. When removing gel from the glass plates, take care not to deform or tear the gel. Scan the gel. Please refer to your

manual for specific information on your model of imager.