The Use of Infrared Fluorescent Technology for the Analysis of Signal Transduction and Protein-Nucleic Acid Interactions

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Abstract

Cellular signaling processes are regulated by a number of molecular interactions. These include the binding of proteins to key regulatory sites on DNA, as well as phosphorylation of protein receptors, kinases and transcription factors. The analysis of these processes currently relies on the use of radioisotopic or chemiluminescent-based methods, neither of which gives optimal results.

We have developed methods employing two-color infrared fluorescent technology for the analysis of signal transduction events, as well as electrophoretic mobility shift assays (EMSA) for monitoring protein-DNA binding. Phosphorylated events of IFNg receptor, ERK, and Stat3 were studied with lysates of human cultured cells that were either unstimulated or specifically stimulated. Two different primary antibodies, mouse IgG specific for the phosphorylated epitope, and rabbit IgG against one of the proteins regardless of its phosphorylation status, were detected using fluorescent-labeled secondary antibodies coupled to either Alexa Fluor® 680 or IRDye™ 800. In all cases, phosphorylated and total amounts of these proteins could be visualized and quantitated simultaneously without the need for stripping and reprobing.

In a separate series of experiments, we demonstrated the ability of the Odyssey™ Infrared Imaging System to perform two-color in-gel Western blots. The in-gel procedure was able to detect as little as 10 pg of transferrin.

Finally, we developed a two-color system for analyzing protein-nucleic acid interactions by EMSA. Two different T7 RNA polymerase binding sites differing by a single nucleotide were labeled with either IRDye 700 or IRDye 800 and simultaneously combined with varying amounts of T7 RNA polymerase. Competitive protein binding could be monitored quantitatively following scanning of the gel on the Odyssey Infrared Imager.

The two-color capability of the Odyssey Imaging system facilitates the analysis of multiple signal transduction events and the analysis of competitive protein-nucleic acid interactions. Furthermore, two-color in-gel Westerns offer the ability to precisely analyze large, poorly transferred proteins with excellent sensitivity.

Methodology

Infrared dyes

Two near-infrared dyes were developed at LI-COR for nucleic acid and protein labeling. IRDye 700 has a λ_{max} of 680 nm and a $\lambda_{emission}$ of 715 nm and was used for nucleic acid labeling. IRDye 800 has a λ_{max} of 780 nm and a $\lambda_{emission}$ of 815 nm and was used for both protein and nucleic acid labeling.

Antibodies and Proteins

Rabbit anti-transferrin was purchased from DAKO, Inc. (Carpenteria, CA). Anti-pERK and anti-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-mouse and goat anti-rabbit antibodies were purchased from Rockland Immunochemicals (Boyertown, PA) and were labeled with IRDye 800 at LI-COR using standard methods (19). Alexa Fluor® 680 goat anti-rabbit and Alexa Fluor® 680 goat anti-mouse antibodies were obtained from Molecular Probes, Inc. (Eugene, OR). The In-Gel Western Chemiluminescent Detection Kit was obtained from Pierce Chemicals (Rockford, IL). T7 RNA polymerase (T7 RNAP) was obtained from Roche Molecular Biochemicals (Nutley, NJ).

Western blotting

Protein samples were loaded onto precast SDS-PAGE gels obtained from either Novex (Carlsbad, CA) or BioRad (Hercules, CA). Proteins were electrophoretically transferred to either nitrocellulose or PVDF membranes. After transfer of protein, the membranes were blocked with Odyssey Blocking Buffer (LI-COR). Both primary antibodies were incubated simultaneously with the blot, followed by washing. The blot was then incubated simultaneously with both secondary antibodies. Blots were scanned on the Odyssey Imager.

Detection of Phosphorylated and Non-phosphorylated Proteins

Lysates of unstimulated or EGF-stimulated A431 cells were obtained from Upstate Biotechnology (Lake Placid, NY). Phosphorylated and non-phosphorylated forms of ERK protein were detected simultaneously with general and phospho-antibodies.

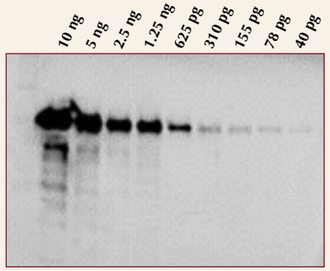
Two-Color In-Gel Western

Mixtures of λDE3 cell extract and purified Transferrin (Tf) were loaded onto 10% precast SDS-Page gels (Novex, Carlsbad CA), followed by electrophoresis at 10 V/cm at room temperature for 1 hour and 40 minutes. Gels were fixed for 15 minutes in 50% isopropanol at room temperature. Both primary antibodies were added simultaneously to the gel. After 1 hour of incubation, the gel was washed and then incubated with both secondary antibodies for 1 hour. The gel was then washed and scanned with the Odyssey Infrared Imager (LI-COR).

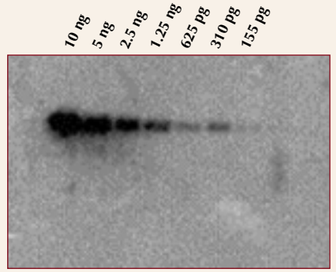
In-Gel Western

Serial dilutions of purified Tf were electrophoresed and gels were fixed as described above. Primary antibody used was rabbit-anti-Tf. Secondary antibody used for chemiluminescent detection was HRP-conjugated goat anti-rabbit, detected with the Pierce UnBlot chemiluminescent substrate by exposure to film. The secondary antibody used for detection on the Odyssey Infrared Imager (LI-COR) was Alexa Fluor® 680-labeled goat anti-rabbit.

Odyssey In-Gel Western

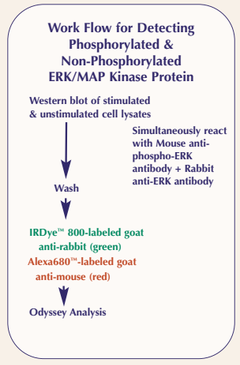


Chemiluminescent In-Gel Western

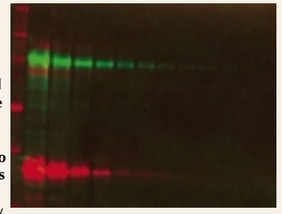


Beginning with 10 ng/lane, two-fold serial dilutions of purified human Transferrin were subjected to electrophoresis, followed by detection using an in-gel Western Protocol. The primary antibody for both gels was a 1:250 dilution of rabbit anti-transferrin. The secondary antibody for the Odyssey gel was a 1:1500 dilution of Alexa Fluor® 680-labeled goat anti-rabbit. The secondary antibody for the chemiluminescent gel was HRP-labeled goat anti-rabbit at a 1:500 dilution. The chemiluminescent gel was processed using the Pierce In-Gel Western Kit (Pierce Biochemicals, Inc.).

Work Flow for Detecting Phosphorylated & Non-Phosphorylated ERK/MAP Kinase Protein

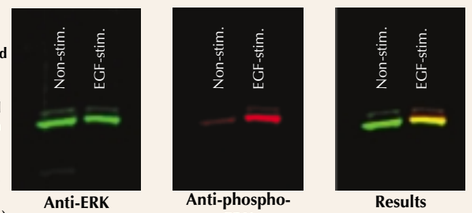


Two-Color In-Gel Western



A series of two-fold dilutions of a mixture of the T7-Tag extract from λDE3 lysogen and purified Transferrin (Tf) were loaded onto 10% SDS-polyacrylamide gels and subjected to electrophoresis. Gels were fixed and then probed with primary rabbit antibody against Tf and with primary mouse antibody against the T7-Tag. The secondary antibodies used were Alexa Fluor® 680 goat anti-mouse and IRDye 800 goat anti-rabbit. The two-color signal was detected on the Odyssey Infrared Imaging system (LI-COR).

Monitoring Tyrosine Phosphorylation Of ERK1 and ERK2 by Two-Color Western Blotting

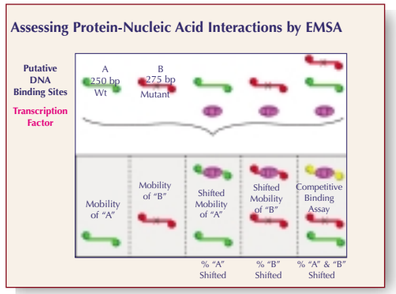


Anti-ERK | Anti-phospho-ERK | Results Overlayed

ERK1 and ERK2 (p44/42 MAP Kinases) were detected on a Western blot of non-stimulated or EGF-stimulated A431 cell lysates. Total ERK and phosphorylated ERK were detected by simultaneous reaction with rabbit anti-ERK and mouse phospho-ERK primary antibodies followed by simultaneous reaction with IRDye 800-labeled goat anti-rabbit (green) and Alexa Fluor® 680-labeled goat anti-mouse (red) secondary antibodies. The yellow color in the composite image indicates the presence of both ERK and phosphorylated ERK.

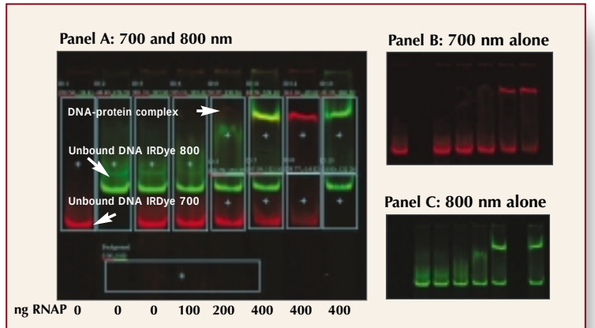
Electrophoretic Mobility Shift Assay

Double end-labeled DNA fragments containing the T7 promoter were synthesized in the PCR reaction using infrared dye (IR dye) labeled primers. The IRDye 700 M13 forward and reverse primers were used to synthesize a 250 bp fragment containing wild type (wt) promoter. The IRDye 800-labeled M13 primers were used to generate a 275 bp fragment with T7 promoter carrying a point mutation. The pSPORT1 plasmid (Roche Molecular Biochemicals) was used as a template. In a typical binding reaction, 3 ng of labeled DNA fragment(s) were mixed with different amounts of T7 RNAP in the presence of binding buffer (40 mM Tris-HCl pH8; 6mM $MgCl_2$; 10 mM DTT; 2 mM spermidine; 0.5% Tween-20; 5mM DTT).



Schematic illustration of the principles of the model electrophoretic mobility shift assay for T7 RNA polymerase binding.

In addition, control reactions containing only buffer and DNA fragments (each fragment separately or both together) were also assembled. The reactions were incubated at room temperature for 10 minutes, after which an equal volume of 2X native loading dye (20% glycerol, 0.01% bromophenol blue, 50 mM Tris-HCL pH 8.3; 10 mM EDTA; 30 mM Na-acetate) was added. Complexes were resolved on a 5% native polyacrylamide gel. Electrophoresis was performed at room temperature at about 10 V/cm in electrophoresis TAE buffer (50 mM Tris-HCL pH 8.3; 10 mM EDTA; 30 mM Na-acetate). Imaging and quantification of DNA fragments and protein-DNA complexes were done by the Odyssey Infrared Imaging System at 700 and 800 nm wavelengths.



Binding of T7 RNAP to two DNA fragments containing the T7 promoter sequence, labeled with either IRDye 700 (red) or IRDye 800 (green). Panel A: Image of the gel when scanned at 700 and 800 nm wavelengths simultaneously. Yellow indicates the presence of both dyes in a pixel. Panels B and C show the same gel at a single wavelength, 700 or 800, respectively.

Summary

- Using two-color detection, signal transduction pathways may be simultaneously analyzed for the phosphorylation status of cell receptors and transcription factors.
- IR yields superior performance for in-gel Western protein detection.
- Two-color capability enables direct comparisons of competitive factors affecting protein-nucleic binding.

Application Note #543
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