# Phosphorylated protein detection is more efficient by fluorescent Western blot

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## Introduction

The detection of phosphorylated proteins usually requires showing both nonphosphorylated and phosphorylated species. For traditional chemiluminescent methods, the negligible size difference between the phosphorylated and nonphosphorylated protein of interest requires probing with a phospho-specific antibody, then stripping, and then re-probing with the protein-specific antibody. Along with potential negative effects on data quality, the process of stripping and re-probing can take a considerable amount of time and reagents.

The drawbacks from the traditional chemiluminescent method of detection can be overcome by using fluorescent secondary antibodies. Fluorescent secondary antibodies bearing two different fluorophores (with non-overlapping spectra) allow for multiplexing; this means both phosphorylated and non-phosphorylated species can be detected simultaneously.[1]

The ability to multiplex not only makes IR detection the faster way to visualize phosphorylated and non-phosphorylated versions of a protein of interest, but it also makes quantitation more accurate. Stripping a blot is not always even or completely effective; moreover, the process of stripping can sometimes take off some of the original protein.[2] Quantitation of proteins before and after stripping is less accurate than if no stripping were involved. Thus, a protocol that visualizes modified and unmodified proteins at the same time offers improved accuracy as well as convenience of speed (see Table 1 for comparison).

## Methods

### Multiplexing protocol: Probing for two proteins of the same weight

Untreated and IFNα-treated HeLa lysates were loaded on an SDS polyacrylamide gel and resolved by electrophoresis. The proteins were transferred to a PVDF membrane using Azure Transfer Buffer. The membrane was split and one membrane was probed using a chemiluminescent detection protocol while the other membrane was probed using a protocol with fluorescent antibodies as detailed below.

### Chemiluminescent detection

After transfer, the first blot was probed for phospho-STAT1. First the membrane was blocked for 30 minutes before incubation with 4 µg rabbit anti-phospho-STAT1 for 1 hour. The membrane was then rinsed twice with 25 mL Azure Fluorescent Wash Buffer (AFWB) followed by three 5 minute washes with AFWB. Next, it was incubated with 3 µg anti-rabbit-HRP for 1 hour, followed by a wash as before with AFWB. After washing, the blot was incubated with 10 mL of the chemiluminescent substrate Radiance for 2 minutes. The blot was imaged directly by the Azure cSeries.

| Time Investment for Western Blots | | |
|---|---|---|
| **Protocol steps** | **Chemi** Time (min) | **Fluorescent** Time (min) |
| Blocking | 30 | 10 |
| Primary incubation | 60 | 60 |
| Wash | 15 | 15 |
| Secondary incubation | 60 | 60 |
| Wash | 15 | 15 |
| Add substrate | 2 | – |
| PBS rinse or Water rinse | – | 5 |
| | 5 | – |
| Stripping | 5 | |
| Wash | 15 | |
| Blocking again | 30 | |
| Primary incubation | 60 | |
| Wash | 15 | |
| Secondary incubation | 60 | |
| Wash | 15 | |
| Add substrate | 2 | |
| **TOTAL TIME** | **6 hrs 29 min** | **2 hr 45 min** |

Table 1. Time required for each western blot protocol.

After imaging the blot, the probed membrane was stripped: first, the membrane was rinsed in high purity water for 5 minutes; second, the membrane was placed in Azure HRP Stripping Buffer for 5 minutes. Following stripping, the membrane was washed with 25 mL AFWB, 2 times fast and then 3 times for 5 minutes each.

To probe for STAT1, the membrane was blocked again for 30 minutes and then incubated with 4 µg mouse anti-STAT1 for 1 hour. The membrane was then rinsed twice with 25 mL AFWB followed by three 5 minute washes with AFWB. Next it was incubated with 3 µg anti-rabbit-HRP for 1 hour, followed by a wash with AFWB in a similar manner as before. After washing, the blot was incubated with 10 mL Radiance for 2 minutes. The blot was imaged again by the Azure cSeries.

### Using fluorescent antibodies and Infrared (IR) detection

After transfer, the membrane was blocked for 10 minutes at room temperature. To probe for the proteins of interest, the membrane was incubated with 4 µg rabbit anti-phospho-STAT1 and 4 µg mouse anti-STAT1 for 1 hr. The membrane was then rinsed twice with 25 mL Azure IR Fluorescent Wash Buffer (AIWB) followed by three 5 minutes washes with AIWB. Following the wash, the membrane was incubated with 4 µg of anti-rabbit-800 and 4 µg anti-mouse-700 for 1 hour. The probed membrane was washed with AIWB in the same manner as before. After the AIWB wash, the blot was rinsed with 25 mL of PBS for 5 minutes before imaging. The blot was imaged with the Azure cSeries, using the filters IR-800 (green) and IR-700 (red).

## Results and Conclusions

In this note, two corresponding western blot membranes were subjected to either chemiluminescent or IR detection for analysis of phosphorylated STAT1. HeLa cells were either treated with IFNα to induce phosphorylation of STAT1 or left untreated, and then protein lysates were harvested and resolved by gel electrophoresis followed by transfer to a membrane for western blotting. Each blot was probed for STAT1 and phospho-STAT1 using the same primary antibodies, but secondary antibodies that were either HRP-conjugated (Figure 1) or fluorescently labeled (Figure 2) were used depending on the detection method.

The comparisons presented here aim to demonstrate the improved convenience and accuracy of fluorescence detection as compared to traditional chemiluminescent methods. The main differences between methods include secondary antibodies used and the number of steps involved. The extra steps in chemiluminescent detection methods include stripping the membrane, which leaves the possibility for uneven removal of antibodies and possible loss of target proteins. Additionally, chemiluminescent detection required approximately 6.5 hours of time to attain data of both unmodified and phosphorylated STAT1. With fluorescent secondary antibodies, STAT1 and phospho-STAT1 could be imaged simultaneously with different IR filters in the digital imager in less than 3 hours (see Table 1). Both protocols can provide quality results, so it's important to choose the protocol that best fits your needs.[1,2]

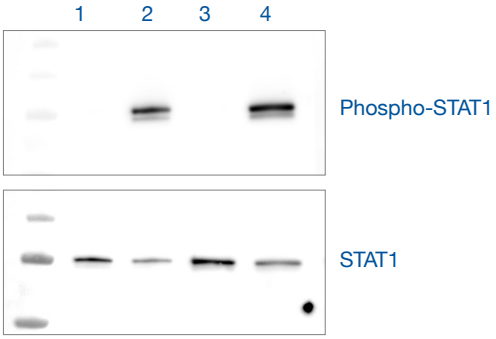


**Figure 1. Chemiluminescent western blot of STAT1 and phospho-STAT1.** The blot was first probed with anti-phospho-STAT1 and imaged. After stripping, the blot was re-probed with anti-STAT1 and imaged. Lanes: Ladder, 1) 10 µg untreated HeLa lysate, 2) 10 µg IFNα-treated, 3) 20 µg of untreated, 4) 20 µg IFNα-treated.

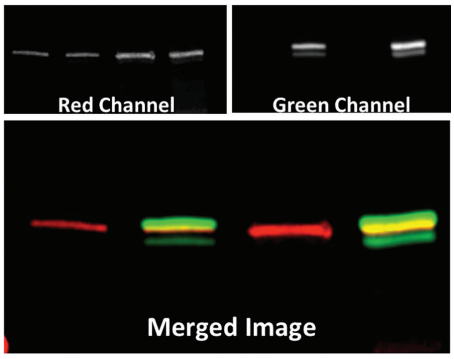


**Figure 2. Fluorescent western blot of STAT1 and phospho-STAT1.** The blot was probed with anti-phospho-STAT1 and anti-STAT1 followed by fluorescent secondary antibodies, and then imaged on Azure cSeries. Top right is the green channel, using IR-800; top left is the image of the red channel, using IR-700. Bottom image is both channels merged. Lanes are the same as in Figure 1.

## References


1. Gingrich, J.C., Davis, D.R., Nguyen, Q. Multiplex Detection and Quantitation of Proteins on Western Blots Using Fluorescent Probes. *BioTechniques.* 2000. 29:636-642
2. MacPhee, D.J. Methodological Considerations for Improving Western Blot Analysis. *Journal of Pharmacological and Toxicological Methods.* 2010. 61: 171-177.


| Products and Reagents | Product Number |
|---|---|
| Low Fluorescence PVDF Membrane | AC2105 |
| Azure Transfer Buffer | AC2127 |
| Azure Fluorescent Wash Buffer | AC2113 |
| Azure IR Fluorescent Wash Buffer | AC2145 |
| Goat-Anti Rabbit HRP Secondary Antibody | AC2114 |
| Goat-Anti Mouse HRP Secondary Antibody | AC2115 |
| AzureSpectra 700 Goat-anti-mouse Secondary Antibody | AC2129 |
| AzureSpectra 800 Goat-anti-rabbit Secondary Antibody | AC2134 |
| Radiance Chemiluminescent Substrate | AC2101 |
| Azure HRP Stripping Buffer | AC2154 |

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