Western Blot Protein Detection and Quantitation Based on Europium Labeled Proteins using a Plate Reader

Vanitha Thulasiraman[1], Jia-Ren Lin[2], Cathy Olsen[1], Rainer Muehlbacher[3], Brian Quast[1], Michael Katzlinger[3], Josef Atzler[3], Karlene A. Cimprich [2], Evan F. Cromwell[1]

[1]Molecular Devices LLC, Sunnyvale, California, USA; [2]Department of Chemical & Systems Biology, Stanford University, Stanford, California, USA; [3]Molecular Devices GmbH, Salzburg, Austria

Abstract

Protein detection is an important task for pharmaceutical and clinical research today and Western Blots (WB) are one of the most common methods employed for this purpose. Various techniques are used to detect proteins on WB membranes including fluorescence, silver staining, and chemi-luminescence. However, each technique has its limitations and there is a continuing need to improve quantitation, accuracy, and dynamic range of WBs. Here we report a novel system for WB membrane protein analysis that is incorporated into a SpectraMax® i3 or Paradigm Multi-Mode Microplate Readers. Membranes are incubated with Europium-chelate labeled secondary antibodies or streptavidin that bind specifically to the protein of interest. Europium (Eu) has a long fluorescence lifetime, on the order of 1 msec, and detection is done in time resolved fluorescence (TRF) mode, which significantly reduces background from auto-fluorescence or other sources of short lifetime emissions. The membranes are placed into a plate reader system where they are scanned with a TRF cartridge that has been optimized for WB scanning. The method does not involve enzyme detection, and the Eu-chelates are resistant to photo-bleaching so the signal remains stable for long periods of time (weeks to months). This allows repeat reading of membranes and potential for comparison of band intensities to known standards for more accurate quantitation. The TRF detection employs photon counting, hence the theoretical dynamic range is > 10^5. In practice, dynamic range is limited by saturation of binding sites on high-abundance bands and non-specific binding to background membrane. There is also no camera blooming, as can occur with chemi-luminescence or fluorescence detection, thus the system provides sharp bands and excellent image quality. The new ScanLater™ Western Blot Detection System is a simple, sensitive, and stable platform that provides excellent WB capability in a multi-mode plate reader extending the uses of this essential laboratory instrument.

Assay Principle

The ScanLater Western Blot Detection System simplifies the Western Blot protocol. The secondary antibody is labeled with an Eu-chelate and the blot is read on a plate reader. No substrates are needed and the blot can be scanned immediately after washing. Europium has a long fluorescence lifetime, on the order of 1 msec, and detection is done in time resolved fluorescence (TRF) mode which significantly reduces background from autofluorescence or other sources of short lifetime emissions.

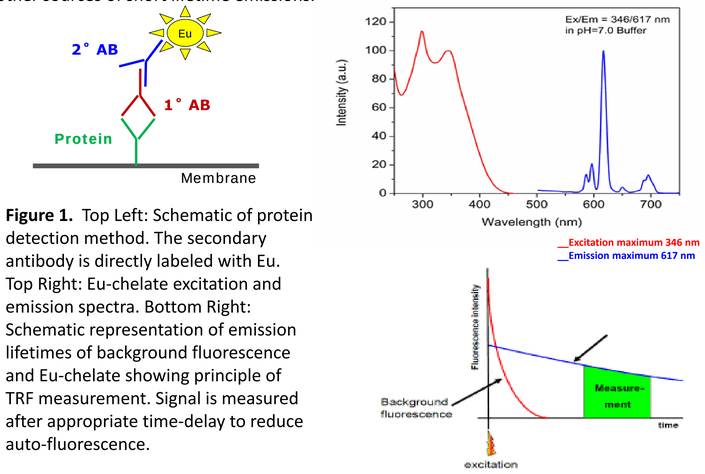


Figure 1. Top Left: Schematic of protein detection method. The secondary antibody is directly labeled with Eu. Top Right: Eu-chelate excitation and emission spectra. Bottom Right: Schematic representation of emission lifetimes of background fluorescence and Eu-chelate showing principle of TRF measurement. Signal is measured after appropriate time-delay to reduce auto-fluorescence.

Materials and Methods

Materials

- ScanLater Western Blot Assay Kit (10X Washing Buffer, 5X Blocking Buffer, Eu-Labeled Anti-Mouse IgG, Eu-Labeled Anti-Rabbit IgG, Eu-Labeled Streptavidin) were purchased from Molecular Devices, LLC.
- Western Blot membranes were scanned using a SpectraMax Paradigm or SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices).
- GST, rabbit biotinylated anti-GST, mouse anti-GST, rabbit anti-transferrin, and transferrin were purchased from Abcam. Chemiluminiscent substrate and HRP conjugate secondary antibody from Millipore.
- TGX 4-20% gels, dual stain protein standards, running and sample buffer, transfer buffer, and Trans Blot Turbo were purchased from Bio-Rad Laboratories. Immobilon FL membranes were purchased from Millipore.

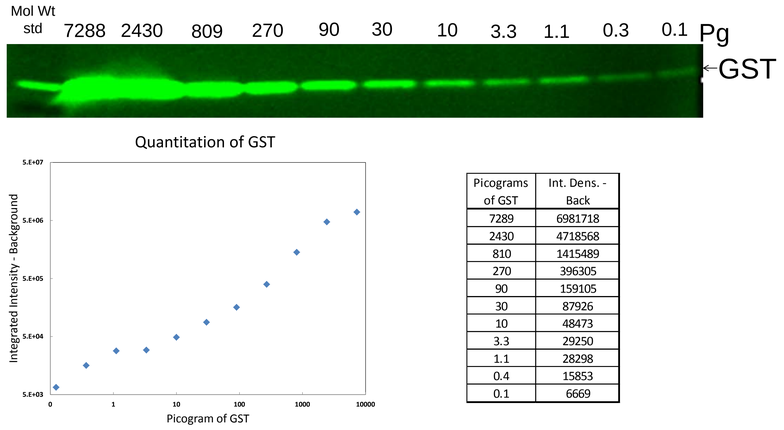
Methods

- Proteins were resolved in TGX 4-20% gel at 200V for 30 min and electrotransferred onto PVDF membrane using the Trans Blot Turbo for 7 min. Blots were rinsed in TBST for 30 sec and then blocked in 1X blocking buffer for 1 hour.
- Primary antibody was added directly into the blocking buffer at the appropriate dilution and incubated for either 2 hr at room temperature (rt) or over night (o/n) in a cold room (4˚C). Blots were washed 3 times in 1X wash buffer for 5 minutes each time.
- Secondary antibody at the dilution of 1:5000 in 1X blocking buffer were added to the blots and incubated for 60 minutes. Blots were washed 3 times in 1X wash buffer for 5 minutes each time. A final rinse was done with water for 15 seconds and then the blots were air-dried before scanning.
- The membranes were scanned in a SpectraMax Paradigm or SpectraMax i3 reader using SoftMax® Pro software.

SpectraMax i3 Microplate Reader + MiniMax™ Imaging Cytometer

Sensitivity & Dynamic Range

The sensitivity and dynamic range of the system was tested using glutathione S-transferase (GST). A three-fold serial dilution of GST in 1x Sample buffer was loaded on a 4-20% gradient gel run for 30 min. Proteins were transferred to an Immobilon FL membrane and probed with biotin labeled rabbit anti-GST for 2hr followed by incubation with Eu-labeled streptavidin for 1 hr. The blot was washed, dried and scanned using SpectraMax Paradigm Multimode Plate Reader. The system demonstrated sub-picogram detection limit of GST with over 4 logs of positive response of signal vs. amount of GST.

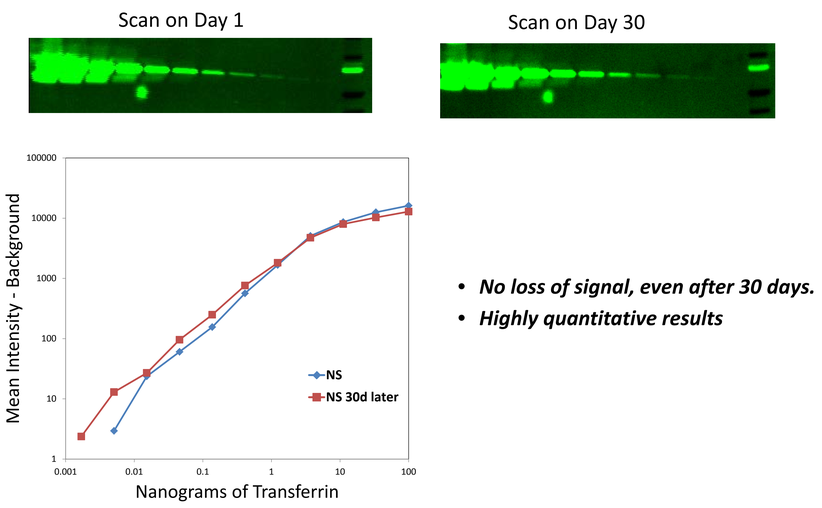


Picograms of GST	Int. Dens. - Back
7289	6981718
2430	4718568
810	1415489
270	396305
90	159105
30	87926
10	48473
3.3	29250
1.1	28298
0.4	15853
0.1	6669

Figure 2. Top: Image of GST dilution series as scanned by SpectraMax Paradigm Multimode Detection Platform. (Note: False-color scale is used to show large dynamic range). Bottom: Integrated intensities from individual bands showing total dynamic range of 4 logs and a linear dynamic range of 3 logs. Images were processed using ImageJ software.

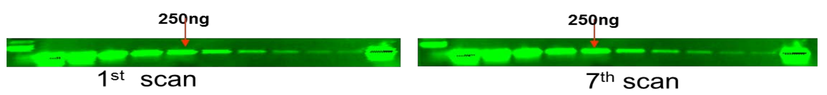
Signal Stability

An exceptional feature of the Eu-labels is signal stability and resistance to photo-bleaching. Blots can be scanned later as the signal is stable for months. To show long-term stability, a three-fold serial dilution of transferrin 1x Sample buffer was loaded on a 4-20% gradient gel run for 30 min. Proteins were transferred to an Immobilon FL membrane and probed with rabbit anti-transferrin o/n at 4˚C followed by incubation with Eu-labeled anti-rabbit IgG for 1 hr. Blots were washed, dried, and scanned on the same day and after a month using a SpectraMax Paradigm.



- ***No loss of signal, even after 30 days.***
- ***Highly quantitative results***

In addition, blots can be scanned multiple times without loss of signal. Serial two-fold dilution of transferrin in 1x Sample buffer was loaded on a 4-20% gradient gel run for 30 min. Proteins were transferred to Immobilon FL and probed with rabbit anti-transferrin for 2 hrs, followed by probing with Eu-labelled anti-rabbit IgG for 1 hr. Blots were washed, dried and scanned multiple times using a SpectraMax Paradigm.



250ng	Area	Mean	IntDen
Scan 1	261	8678	2264874
Scan 7	261	8609	2247047

Signal is very stable & reproducible even after scanning multiple times.

Applications

- ***Detect very low amounts of endogenous ubiquitinated form of Rad-18, involved in post-replication repair of UV-damaged DNA using Scan later western blot***

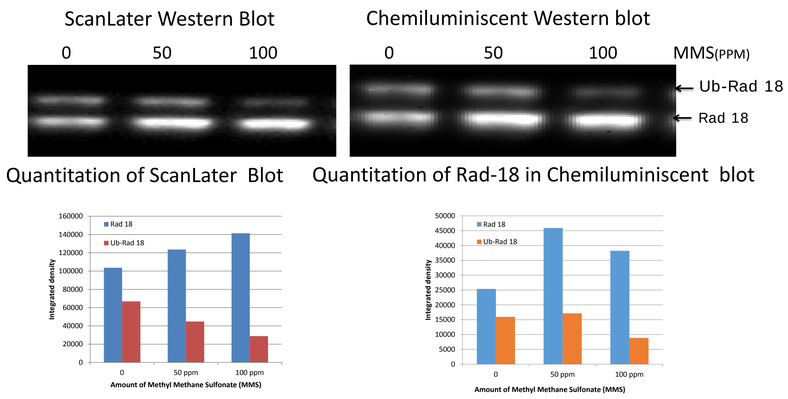


Figure 5. Detection of ubiquitinated Rad-18 in HEK 293 cells on treatment with different concentration (0, 50, 100 ppm) of carcinogen MMS, an alkylating agent. Comparable result using Scan later vs. Chemiluminiscence western blot (Stanford University).

- ***Detect very low amounts of endogenous phosphorylated form of pChk1, involved in post-replication repair of UV & MMS-damaged DNA using Scan later western blot.***

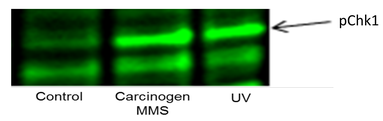


Figure 6. Detection of phosphorylated form of pChk1 in HEK 293 cells treated with carcinogen MMS and UV radiation (Stanford University).

- ***Detect trace amounts of endogenous phosphorylated form of JNK1, involved in cell signalling in stimulated and inhibitor treated cells using Scan later western blot.***

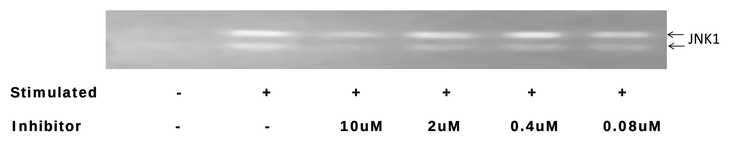


Figure 7. Detection of phosphorylated form of JNK1 in NHLF (Natural human lung fibroblast) cells treated with stimulants and inhibitory drugs (result from alpha site customer).

Summary

- Sensitivity: Detection of low amounts of endogenous phosphorylated and ubiquitinated protein in human cells.
- Reduced background: Europium has a long fluorescence lifetime, on the order of 1 msec, and detection is done in Time Resolved Fluorescence (TRF) mode, which significantly reduces background from auto-fluorescence or other sources of short lifetime emissions.
- Save time: No time-consuming ECL optimization.
- Reduce costs: Eliminate the need for laborious and expensive X-ray films and developer.
- Ready to go: Provides excellent WB capability to SpectraMax® i3 or SpectraMax Paradigm Multi-Mode Microplate Readers, extending uses of this essential laboratory instrument.

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