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

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Introduction

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 chemiluminescence. 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® Multi-Mode Microplate Reader. Membranes are incubated with Europium-chelate-labeled secondary antibodies or streptavidin that bind specifically to primary antibody against the protein of interest. The membranes are placed into a plate reader system where they are scanned with a time-resolved fluorescence (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. There is also no camera blooming, as can occur with chemi-luminescence or fluorescence detection, thus the system gives sharp bands and excellent image quality. In this study we present results from evaluation of Eu-chelate labeled anti-rabbit, anti-rat, & anti-mouse secondary antibodies. Dynamic range and sensitivity was determined using a glutathione-S-transferase (GST) dilution series with Eu-labeled anti-rabbit antibody. Increases in expression levels of Hsp70 in heat shocked cells were analyzed using Eu-labeled anti-mouse IgG. Molecular weights of antigens were determined using a biotinylated protein ladder detected with Eu-streptavidin. The new SpectraMax Western Blot system is a simple, sensitive, and stable platform that provides excellent WB capability in a Microplate reader.

Assay Principle

The ScanLater 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. 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. Europium has a long fluorescence lifetime, on the order of 1 msec, and detection is done in TRF mode, which significantly reduces background from autofluorescence or other sources of short lifetime emissions.

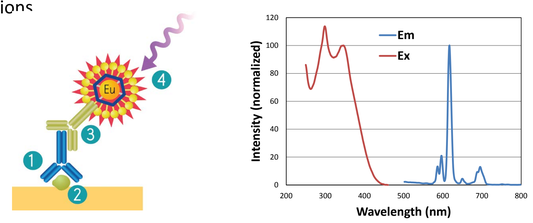

Figure 1. Left: ScanLater Western Blot System workflow: Use existing primary antibody (1) for binding to protein of interest (2). Eu-labeled ScanLater secondary antibody (3) binds to primary antibody. Detection with ScanLater TRF detection cartridge (4). Right: Eu-chelate excitation and emission spectra, with excitation maximum of 346nm and emission maximum of 617nm.

Materials & Methods

- ScanLater™ reagents (Protein Ladder, 10X Washing Buffer, 5X Blocking Buffer, Eu-Labeled Anti-Mouse IgG, Eu-Labeled Anti-Rabbit IgG, Eu-Labeled Anti-Rat IgG, Eu-Labeled Streptavidin) were purchased from Molecular Devices, LLC.
- 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.
- IL17, rat anti-IL17, mouse anti-hsp 70 were purchased from R&D systems
- 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, 5 min each.
- Secondary antibody at the dilution of 1:5000 in 1X blocking buffer were added to the blots and incubated for 60 min. Blots were washed 3 times in 1X wash buffer, 5 min each. A final rinse was done with water for 15 sec and then the blots were air-dried before scanning.
- Protein ladder was loaded at 4 ul to determine molecular weight of antigen (figure 2), and the pre-stained bands were used to monitor gel electrophoresis, transfer efficiency and for gel and blot alignment.
- The membranes were scanned in a SpectraMax Paradigm or SpectraMax i3 Multi-Mode Microplate reader using SoftMax® Pro software.

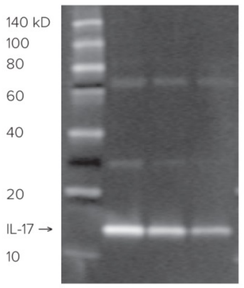

Figure 2. Estimation of molecular weight of IL-17 using the ScanLater Protein Ladder. Pre-stained bands appear at 18, 31 and 70 kd.

Sensitivity & Dynamic Range

The sensitivity and dynamic range of the system was tested using GST. A three-fold serial dilution series 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 2 hours followed by incubation with Eu-labeled streptavidin for 1 hour. The blot was washed, dried and scanned using a SpectraMax i3 or a SpectraMax Paradigm system. The system demonstrated sub-picogram detection limit of GST with over 4 logs of positive response of signal vs. amount of GST.

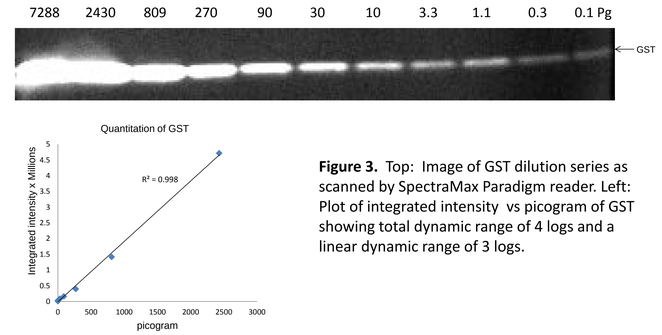

Figure 3. Top: Image of GST dilution series as scanned by SpectraMax Paradigm reader. Left: Plot of integrated intensity vs picogram of GST showing total dynamic range of 4 logs and a linear dynamic range of 3 logs.

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 GST 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-GST o/n at 4˚C followed by incubation with Eu-labeled streptavidin for 1 hr. Blots were washed, dried, and scanned on the same day and after 57 days using a SpectraMax i3 or SpectraMax Paradigm system.

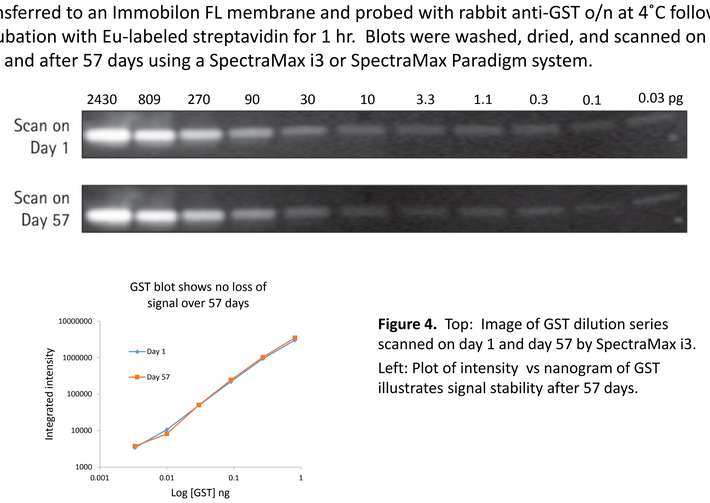

Figure 4. Top: Image of GST dilution series scanned on day 1 and day 57 by SpectraMax i3. Left: Plot of intensity vs nanogram of GST illustrates signal stability after 57 days.

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 hr followed by probing with Eu-labelled anti-rabbit IgG for 1 hr. Blots were washed, dried and scanned multiple times using a SpectraMax i3 or SpectraMax Paradigm system.

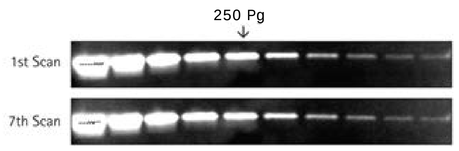

Figure 5. Signal from same blot run multiple times using ScanLater system. Images of 1st and 7th scans are shown. Results from 250pg band show <1% change in signal intensity.

250pg	Area	Mean	Integrated Density
Scan 1	261	8678	2264874
Scan 7	261	8609	2247047

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

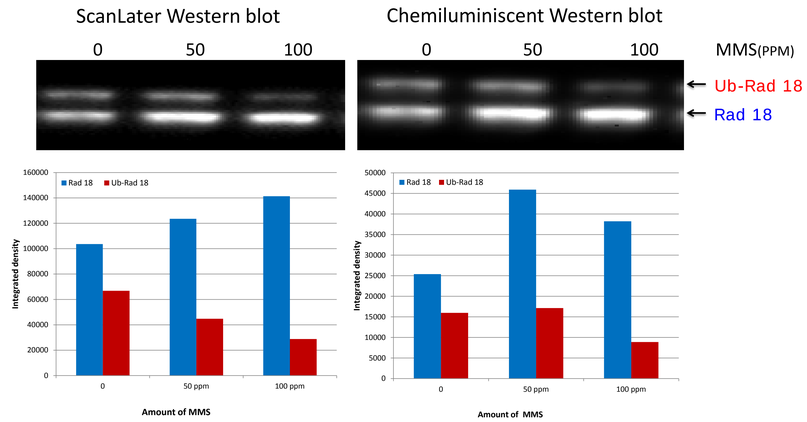

Figure 6. Detection of ubiquitinated Rad-18 in HEK 293 cells after treatment with different concentration (0, 50, 100 ppm) of carcinogen methyl methane Sulfonate (MMS), an alkylating agent. ScanLater western blot is as sensitive as and more precise than the Chemiluminiscence western blot . Data courtesy of the J-R Rin and K Cimprich, Stanford University.

Increase in Hsp 70 quantified when CHO & HeLa cells are exposed to heat shock.

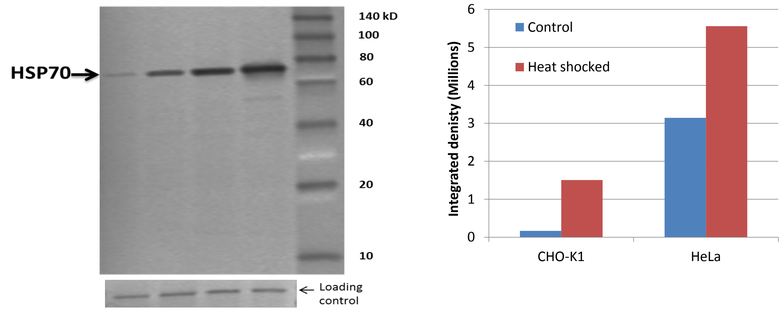

Figure 7. Left: Western blot shows an increase in Hsp 70 that occurs when cells are exposed to temperatures that are higher than normal. Right: ScanLater quantitation of signal from hsp70 bands.

Summary

- ***Quantitative:*** ScanLater Western blot detection system provides quantitation of protein with a dynamic range of 4 logs and a linear dynamic range of 3 logs.
- ***Reduce 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.
- ***Stable labeling of blots:*** Allows reanalysis of blots over extended periods of time.
- ***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 simple, sensitive, & stable platform for excellent protein analysis in a SpectraMax® Multi-Mode Microplate Reader

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