

APPLICATION NOTE

Detection and quantitation of protein with ScanLater Western Blot Detection System

Introduction

Protein detection is important for pharmaceutical and clinical research today, and western blots are among the most common methods employed for this purpose. Various techniques are used to detect proteins on western blot membranes including fluorescence and chemiluminescence. However, each technique has its limitations, and there is a continuing need to improve quantitation, accuracy, and dynamic range. Here we report on a novel system for western blot membrane protein analysis that is incorporated into SpectraMax® i3 and SpectraMax® Paradigm® Multi-Mode Microplate Readers. We demonstrate extended dynamic range due to reduced background as well as stability of detection over time and number of reads. In addition, a comparison of Scanlater Western Blot System to a chemiluminescence method demonstrates improved sensitivity.

Assay principle

ScanLater® Western Blot System workflow follows standard gel loading and blotting methods up to the secondary antibody incubation step. Membranes are incubated with Europium-chelate labeled secondary antibodies or streptavidin that bind specifically to the primary antibody bound to the protein of interest (Figure 1).

Images are generated utilizing time resolved fluorescence (TRF) mode detection of Europium (Eu) which has a 1 ms fluorescence lifetime. This significantly reduces background from auto-fluorescence or other sources of short lifetime emissions. There is no camera blooming which is often seen with chemiluminescence or standard fluorescence detection; thus the system provides sharp bands and superior image quality.

The method does not involve enzyme detection, and the Eu-chelates are resistant to photo bleaching. Therefore, the signal remains stable for weeks to months. This stability enables repeat reading of membranes allowing more accurate quantitation. The new ScanLater™ Western Blot Detection System is a simple, sensitive, and stable platform that provides excellent protein analysis capability in a multi-mode plate reader.

Benefits

- Western blot protocol is unchanged except for incubation with secondary antibody
- No substrates required
- TRF detection using Europium-labeled secondary antibody reduces background while increasing dynamic range
- Digital photon counting provides unaltered TRF counts
- Europium resists photo bleaching and is stable for weeks, enabling reuse of blots

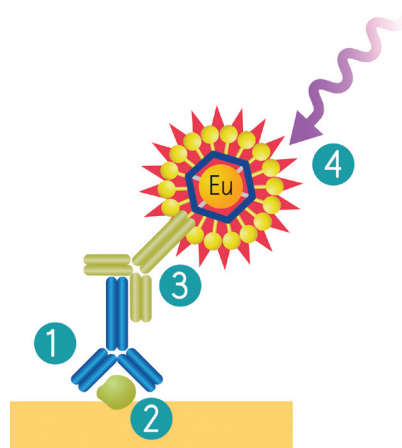


Figure 1. ScanLater Western Blot System workflow. Use existing primary antibody (1) for binding to protein of interest (2). Eu-labeled ScanLater Secondary Antibody (3) binds to primary antibody. Detection with ScanLater TRF Western Blot Detection Cartridge (4).

SoftMax Pro Software analysis of western blot data

All experiments were conducted using the ScanLater Western Blot Detection System for either the SpectraMax i3 or SpectraMax Paradigm Multi-Mode Microplate Reader. By using a microplate detection system, there are distinct advantages. First, digital photon counting provides TRF counts as raw data which are not altered. Images can be optimized and stored in SoftMax® Pro Software. Raw counts can also be directly exported by the software to an integrated Excel macro spreadsheet for analysis and quantification of protein. Alternatively, data can be directly exported to ImageJ for analysis.

Sensitivity and dynamic range

The sensitivity and dynamic range of the system were tested using glutathione S-transferase (GST). A three-fold serial dilution of GST in 1x running buffer was loaded on a 4-20% gradient gel and run for 30 minutes. Proteins were transferred to an Immobilon FL membrane and probed with biotin labeled rabbit anti-GST for 2 hours followed by incubation with ScanLater Eu-labeled streptavidin for 1 hour. The blot was washed, dried and scanned using SpectraMax Paradigm reader (Figure 2). The system demonstrated sub-picogram detection limit of GST with over 4 logs of positive response of the signal vs. amount of GST (Figure 3).

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. (Figures 4 and 5).

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 and run for 30 minutes. Proteins were transferred to Immobilon FL and probed with rabbit anti-transferrin for 2 hours, followed by probing with Eu-labeled anti-rabbit IgG for 1 hour. Blots were washed, dried and scanned seven times in a row using a SpectraMax Paradigm reader (Figure 6).

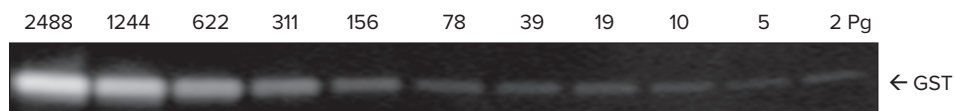


Figure 2. Image of GST dilution series as scanned by SpectraMax Paradigm reader.

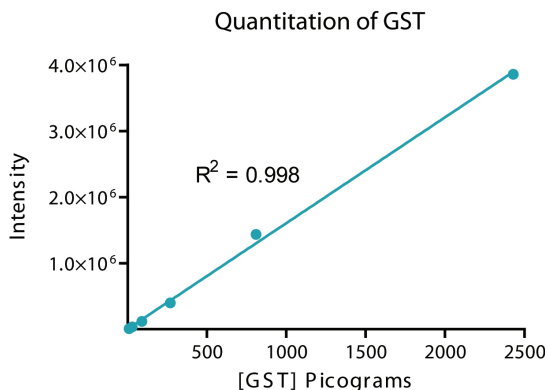


Figure 3. Integrated intensities from individual bands shown in Figure 2 analyzed with the SoftMax Pro integrated Excel macro showing total dynamic range of 4 logs and a linear dynamic range of 3 logs.

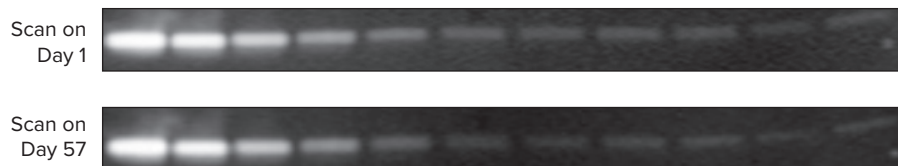


Figure 4. Eu-label signal stability and resistance to photo bleaching.

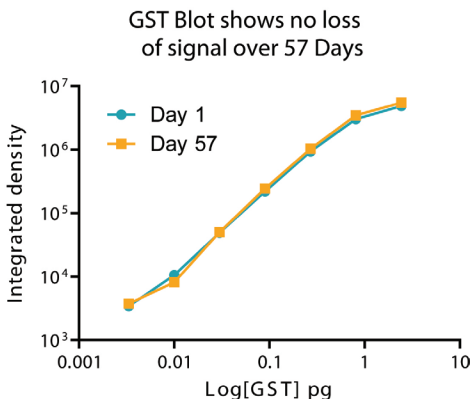


Figure 5. Plot of mean intensity vs. picograms of GST illustrates signal stability after 57 days.

Comparison to chemiluminescence western blot

HEK293T cells were treated with 0, 50, and 100 ppm of methyl methanesulfonate (MMS), a carcinogen that causes DNA damage. The extracts (80 µg) were run on a gel, and blots were probed with mouse anti-Rad18 followed by either ScanLater Eu-labeled anti-mouse antibody or HRP labeled anti-mouse antibody for the chemiluminescent assay (Immobilon Western Chemiluminescent HRP Substrate, Millipore cat. no. WBKLS 0500).

Rad18 is a protein that plays an essential role in DNA damage repair. It is present in two forms, ubiquitinated and non-ubiquitinated. Shown in Figure 7, exposure to MMS decreases the amount of ubiquitinated Rad18 and increases the amount of non-ubiquitinated Rad18. Quantitation of the ScanLater blot was done using SoftMax Pro Software to capture the signal and export directly to an Excel macro for analysis. An AlphaInnotech chemiluminescent imager was used to capture signal from the chemiluminescent blot. Quantitative analysis of the ScanLater blot shown in Figure 8 shows an increase in Rad18 protein and decrease in ubiquitinated Rad18 corresponding to increasing levels of MMS. This correlation was less clear using the chemiluminescent method.

Conclusion

Using ScanLater western blot technology, users can follow the workflow that is optimized for their application. Secondary antibody labeled with Eu enables low background detection in TRF mode on a SpectraMax i3 or SpectraMax Paradigm Multi-Mode Microplate Reader. Images are stored in SoftMax Pro Software which provides integrated export to a custom Excel macro or ImageJ for analysis.

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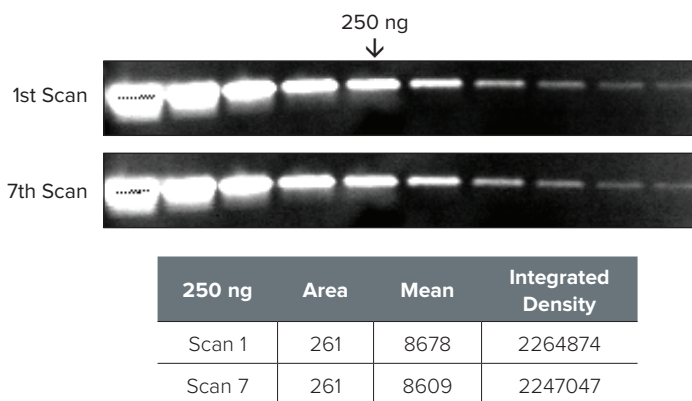


Figure 6. Signal is very stable and reproducible even after scanning multiple times.

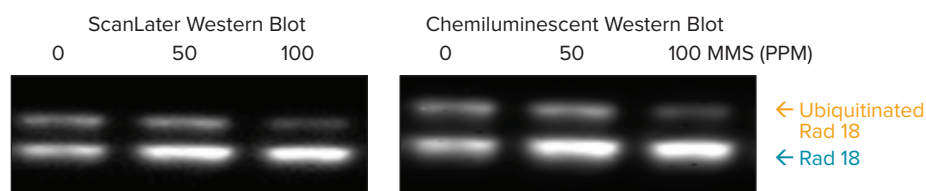


Figure 7. Comparison of detection of endogenous and ubiquitinated forms of Rad18, using ScanLater Western Blot and Chemiluminescent Systems.

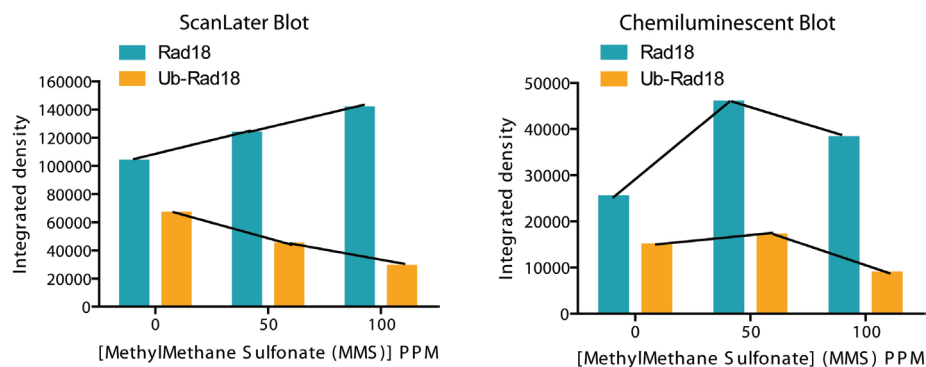


Figure 8. Scan later clearly detects a quantitative increase in the amount of unmodified Rad18 and decrease in the amount of ubiquitinated Rad18 with increase in the level of MMS. The data shows that the ScanLater TRF Western Blot is as sensitive as and more precise than the chemiluminescent blot.

ScanLater Western Blot Detection System provides quantitation of protein with a dynamic range of 4 logs and a linear range of 3 logs. In addition, the signal is stable over time and over multiple

scans. ScanLater Western Blot Detection System is a simple, sensitive, and stable platform that provides excellent protein analysis capability in a multi-mode microplate reader.