Focal Points

Application Note FP-117

UVP BioImaging Systems

Chemiluminescent Immunoblot Detection with Enhanced Luminol HRP Substrates and Digital CCD Cameras

Introduction

Immunoblotting has emerged as a powerful and convenient procedure for the protein chemist engaged in detecting and quantifying specific proteins. The development of chemiluminescent detection has eliminated the dependence on radioisotopes while maintaining the sensitivity of radioisotope detection. Traditionally, detection was achieved on film, but the limited linear dynamic range of film and the high sensitivity afforded by chemiluminescent substrates has led to the increased use of charge coupled device (CCD) cameras. CCD sensor technology can quantify a two-fold increase in linear dynamic range over film. Coupled with powerful data analysis software, the CCD camera has significantly decreased the amount of time needed to detect, quantify and analyze immunoblot data. The researcher using CCD camera technology can completely eliminate costly film processing chemicals and hazardous waste. In addition, images captured with a CCD camera can be analyzed immediately using a software application and easily imported to documents for publication. Images generated on film must be scanned and then analyzed. This is time-consuming and resolvable image data is lost in this needless step.

Abstract

Chemiluminescent detection requires recognition of an analyte of interest by an enzyme-conjugated ligand. The ligand can be any molecular species that binds specifically and with high affinity to the analyte. Horseradish peroxidase (HRP) serves as the enzyme for many chemiluminescent substrates because it is inexpensive and has a relatively small molecular mass. BioWest™ Extended Duration Chemiluminescent Substrate (UVP) consists of a luminol/enhancer and stable peroxide buffer. When the two components are mixed in the presence of HRP, a chemical reaction occurs and light is emitted. BioWest Substrate is sensitive to mid-femtogram levels of the analyte being detected and light emission continues up to 24 hours. The high sensitivity and long light duration of BioWest Substrate are ideally suited for use with the CCD camera-based BioChemi™ System (UVP). The BioChemi System consists of a high-resolution, deeply cooled, scientific-grade, digital CCD camera; very sensitive optics; EpiChemi™ II Darkroom Enclosure; 3UV™ Transilluminator; computer; monitor; thermal printer; and LabWorks™ Image Acquisition and Analysis Software.

In this study, the BioChemi System was used to acquire images, analyze and compare BioWest and ECL™ Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, APB) in dot blot and Western blot applications. The dot blot was also compared to blots exposed to film but analyzed using the BioChemi System and LabWorks Software.

Materials and Methods

Dot Blot

The Easy-Titer® ELIFA Unit (Pierce) was assembled according to the manufacturer's instructions for dot blotting. Nitrocellulose membrane (Pierce) was cut to fit rows A-D of the Easy-Titer ELIFA Unit and Hybond™-P PVDF Membrane (APB) was cut to fit rows E-H. Before assembly, Hybond-P Membrane was wetted with methanol (Pierce) and equilibrated with several exchanges of Milli-Q® Water. Nitrocellulose membrane was wetted and equilibrated with Milli-Q Water simultaneously with the Hybond-P Membrane. HRP-conjugated streptavidin (SA-HRP, Pierce) was reconstituted with Milli-Q Water at a concentration of 1 µg/µl (stock). The stock solution was subsequently diluted to 1.25 pg/µl in BupH™ Modified Dulbecco's Phosphate Buffered Saline (DPBS, Pierce). Serial dilutions of SA-HRP (Figure 1) were prepared, loaded in triplicate into the wells of the Easy-Titer ELIFA Unit and filtered through each membrane for 5 minutes. Membranes were washed three times with 300 µl of DPBS per well. Substrate working solutions were prepared immediately before use. BioWest Substrate was prepared by mixing equal volumes of luminol/enhancer with stable peroxide. ECL Plus™ Substrate was prepared by mixing detection reagents A and B in a 40:1 ratio. BioWest Substrate was applied to the nitrocellulose membrane and ECL Plus Substrate was applied to the Hybond-P Membrane per the manufacturer's recommendations. Following incubation, excess substrate was drained and the membranes were placed in a plastic sheet protector for imaging on CL-XPosure Film (Pierce) and with the BioChemi System.

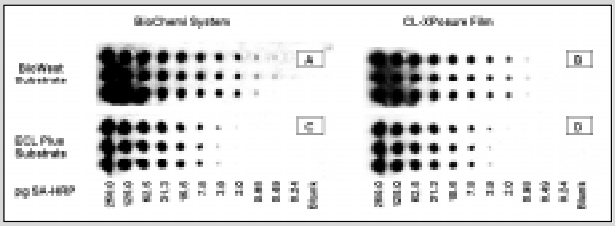


Figure 1. Dot blots for SA-HRP were detected using BioWest™ Substrate (Figures A and B) or ECL Plus™ Substrate (Figures C and D). Images were captured using the BioChemi™ System (Figures A and C) or CL-XPosure™ Film (Figures B and D). BioWest Substrate allows visual detection to 0.49 pg of SA-HRP when captured on the BioChemi™ System (Figure A). A two-minute capture on the BioChemi System generates an equivalent image to a two-minute exposure to CL-XPosure Film (compare Figure A to Figure B). ECL Plus Substrate only allows visual detection of 3.9 pg of SA-HRP when captured on the BioChemi System (Figure C). Again, this two-minute capture is equivalent to the same blot exposed to film (compare Figure C to Figure D). BioWest Substrate is eight-fold more sensitive that ECL Plus Substrate in this application (compare Figure A to Figure C and Figure B to Figure D).

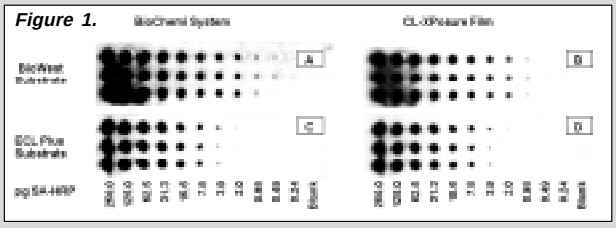
Western Blot

Recombinant mouse IL-2 (BD PharMingen) was serially diluted (Figure 3) in ImmunoPure® Lane Marker Reducing Sample Buffer (Pierce). Serial dilutions were boiled for 3 minutes and 10 µl of each sample was separated at 175 volts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10-20% Tris-Glycine gels (Invitrogen) using the XCELL II™ Mini-Cell Electrophoresis Unit (Invitrogen). Nitrocellulose and Hybond-P Membranes were wetted and equilibrated as described for the dot blot. Protein transfer to these membranes was performed for 2 hours at 200 mA in the Mini Trans-Blot® Cell (Bio-Rad). Membranes were blocked overnight with SuperBlock® Blocking Buffer in phosphate buffered saline (PBS) (Pierce) containing 0.05% Tween®-20 (Pierce). Anti-mouse IL-2 monoclonal antibody (BD PharMingen) was diluted to 2 µg/ml with SuperBlock® Blocking Buffer in PBS containing 0.05% Tween®-20. Each blot was agitated for 1 hour at RT with 12 ml of the diluted primary antibody. Blots were washed six times for 5 minutes each with BupH™ DPBS (Pierce) containing 0.05% Tween-20. HRP-Conjugated Goat Anti-Rat IgG (H+L) (Pierce) was reconstituted with Milli-Q® Water and diluted to 40 ng/ml with SuperBlock Blocking Buffer in PBS containing 0.05% Tween-20. Blots were agitated for 1 hour at RT with 12 ml of the diluted secondary antibody. Washing was repeated as described above. Substrates were prepared as described for the dot blots. Blots were imaged and analyzed on the BioChemi System.

Results

Dot Blot

A SA-HRP dot blot was detected on the BioChemi System and CL-XPosure Film using BioWest Substrate (Figures 1A and 1B). To illustrate the sensitivity of the BioChemi - BioWest Systems combination, parallel dot blots were performed and detected using ECL Plus Substrate (Figures 1C and 1D), a competitor's high-sensitivity, long-duration substrate. All exposures were for two minutes. BioWest Substrate allowed for the detection of as little as 0.49 pg of SA-HRP using either the BioChemi System or CL-XPosure™ Film for image capture (Figures 1A and 1B). ECL Plus Substrate detected only 3.9 pg by the BioChemi System or CL-XPosure Film (Figures 1C and 1D), which is an eight-fold decrease in sensitivity compared to the BioWest Substrate. The BioChemi-BioWest Systems combination was capable of generating equivalent images to film in the same time exposure (compare Figure 1A to Figure 1B).



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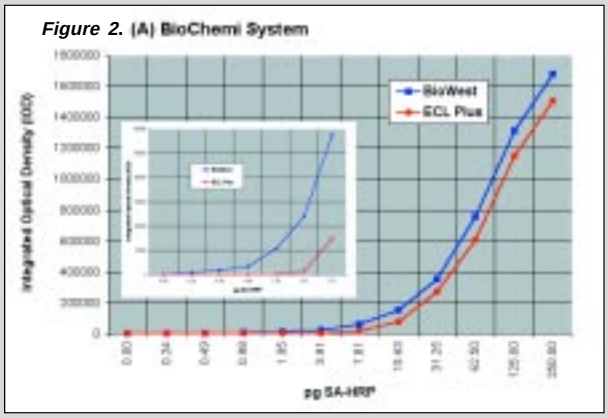
The SA-HRP dot blots from Figures 1A and 1C were quantified and analyzed directly using Labworks 4.0 Image and Analysis Software (UVP). Dot blots in Figure 1B and 1D were scanned on the Model GS-700 Imaging Densitometer (Bio-Rad) and analyzed using Labworks 4.0 software. Light emission for BioWest Substrate is more intense than light emission for ECL Plus Substrate at every concentration of SA-HRP assayed with the BioChemi System (Figure 2A).



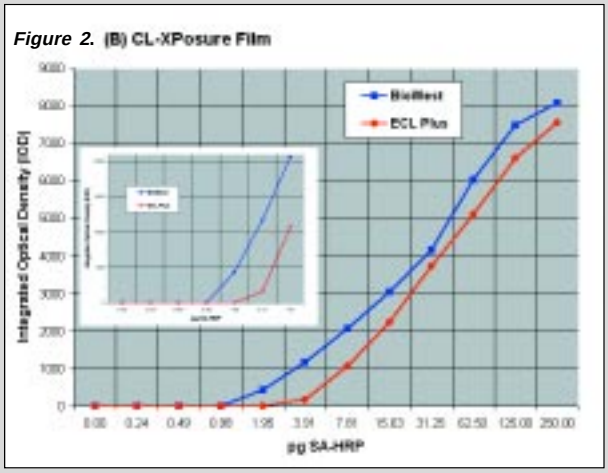


Figure 2. SA-HRP dot blots were detected using BioWest Substrate and ECL Plus Substrate. Images were captured using the BioChemi System (Figure A) or CL-XPosure Film (Figure B). Data captured by the BioChemi System was quantified directly using Labworks 4.0 Software. CL-XPosure Film Data was scanned on the Model GS-700 Imaging Densitometer then imported and quantified using Labworks 4.0 Software. Insets for each figure represent an expanded view of the lower range of detection.

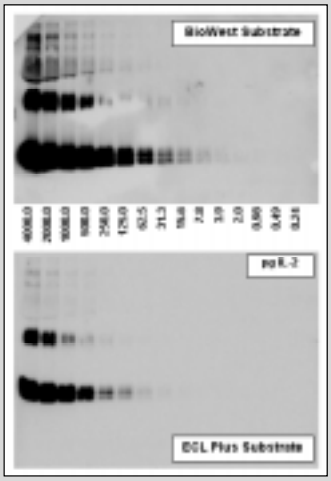
The inset of Figure 2A represents an expansion of the lower detectability limit (LDL) for each substrate. This graphical representation of the images in Figure 1A and Figure 1C confirms a six- to eight-fold increase in sensitivity on this dot blot when using BioWest Substrate vs. ECL Plus Substrate (Figure 2A inset). The data generated on the BioChemi System is similar to that obtained on CL-XPosure Film (Figure 2A and Figure 2B). However, the LDL on CL-XPosure Film is

not as sensitive as it is on the BioChemi System (Figure 2B inset), illustrating the improved dynamic range possible using the BioChemi System.

Western Blots

A Western blot of IL-2 was detected with BioWest Substrate and ECL Plus Substrate using the BioChemi System (Figures 3A and 3B). The images in Figures 3A and 3B were acquired together, but split here for illustrative purposes. Visual inspection of the blots in Figure 3A and 3B show that IL-2 is detectable to 0.98 pg using BioWest Substrate. This is a 16-fold increase in sensitivity over ECL Plus Substrate, which only allows visible detection to 15.6 pg. The images generated on the BioChemi System were analyzed and quantified using LabWorks 4.0 software to confirm the visual results. Figure 4 graphically depicts a three-fold or greater increase in intensity of light emitted with BioWest Substrate compared with ECL Plus Substrate at all concentrations of IL-2 assayed. BioWest Substrate exhibits sensitivity to less than 1 pg of IL-2, while ECL Plus Substrate generates no signal above background in the plotted range (Figure 4 inset).

Figure 3. Western blots for IL-2 were detected using BioWest™ Substrate (Figure A) and ECL Plus™ Substrate (Figure B). Images were captured using the BioChemi™ System. BioWest™ Substrate allows visual detection to 0.98 pg of IL-2 while ECL Plus™ Substrate only allows visual detection to 15.6 pg. BioWest™ Substrate is 16-fold more sensitive than ECL Plus™ Substrate in this application.



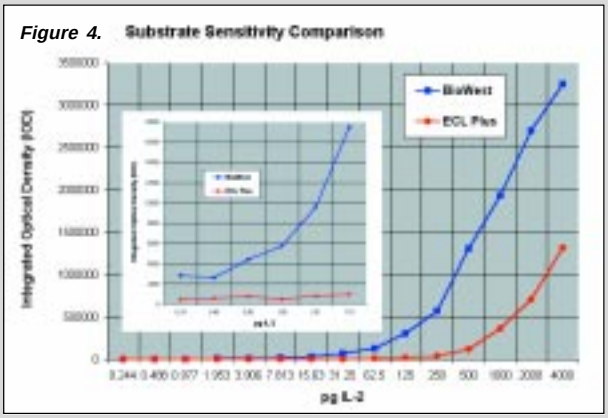


Figure 4. IL-2 Western blots were detected using BioWest™ Substrate and ECL Plus™ Substrate. Images were captured on the BioChemi™ System and quantified using LabWorks™ 4.0 Software. The inset represents an expanded view of the lower range of detection.

Discussion

Quantified comparisons of blots confirmed an eight-fold increase in sensitivity of BioWest Substrate over ECL Plus Substrate in a dot blot application and a 16-fold increase in sensitivity in a Western blot application. This sensitivity is best documented on a deeply cooled, high-resolution CCD-based camera system like the BioChemi System, as the increased dynamic range allows detection over a greater concentration range of antigen. The sensitivity of the BioChemi System allowed detection of blots in less time than exposing blots to film and with twice the linear dynamic range of scanned images from film. Instantaneous digitization of the image enabled immediate enhancement for publication and/or subsequent quantitative analysis. This permanently archived our experimental data for safe storage and saved valuable time preparing this research for publication.

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UVP.com

UVP, Inc. • 2066 W.11th Street, Upland, CA 91786 • (800) 452-6788
(909) 946-3197 • Fax: (909) 946-3597 • E-Mail: uvp@uvp.com

Ultra-Violet Products Ltd. • Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge CB4 1TG UK
+44(0)1223-420022 • Fax: +44(0)1223-420561 • E-Mail: uvp@uvp.co.uk