

Ultra-Rapid and Ultra-Sensitive Detection of Proteins in Chemiluminescent Western Blotting

David P. Chimento¹, Angela M. Zaorski¹, Lee Hedges², Carl A. Ascoli¹

¹Rockland Immunochemicals, Inc., PO Box 326, Gilbertsville, PA 19525,

²UVP, LLC, 2066 W. 11th Street, Upland, CA 91786.

Correspondence to CAA, Laboratory Director: ascoli@rockland-inc.com, 1-800-656-7625



The UVP BioSpectrum® Imaging System for high sensitivity detection of proteins in conjunction with the FemtoMax™ chemiluminescent-HRP substrate provides for extremely fast and sensitive detection of proteins by Western blot. We demonstrate the detection of 6 different commonly used epitope tags using Rockland Immunochemicals's epitope tag specific antibodies and a new 12-epitope tag control marker. The combination of high quality primary and secondary antibodies coupled with the new FemtoMax™ HRP substrate and the BioSpectrum® Imaging System allows the detection of femtogram amounts of target proteins with excellent sensitivity and specificity.

INTRODUCTION

Cell biologist, biochemists, and proteome scientists study proteins at varying levels within cells, with frequent low to very low expression levels often proving to be problematic when detecting by Western blot. Thus the two main issues for detection are specificity and sensitivity. Heterologous expression systems (mammalian, yeast, insect, *E. coli*) are sometimes used to enhance protein research [1,2]. These expression systems utilize molecular biology to construct expression vectors by cloning proteins of interest with a unique epitope tag fused onto one end. Many different epitope tag choices are commercially available to researchers [3,4,5,6,7]. This process can enhance both expression and detection of the protein of interest in cell lysates. Film and CCD cameras are now commonly used for data acquisition, although CCD-based cameras are considered the most robust technology [8]. Chemiluminescence produced by enzymatic reaction increases the sensitivity of a Western blot [9], and the high signal output allows for rapid collection of multiple exposures. FemtoMax™ is a high-burst luminol based chemiluminescent reagent that is superior at low level protein detection. Its high-level output (relative light units) allows for great sensitivity with very low background. The BioSpectrum® Imaging System is designed with a choice of high resolution and deeply cooled cameras* and provides automated pre-set or user-defined PC controls for gel imaging and analysis. By combining FemtoMax™ chemiluminescence with the BioSpectrum® Imaging System we report detection of proteins to mid-femtogram levels. To address the need for detection of heterologously expressed proteins, Rockland Immunochemicals now offers a user friendly and universal epitope-tag control sample for most commonly used epitope tags. This reagent is a tandem multi-epitope tag marker, allowing researchers a single universal loading control for most common epitope sequences for monitoring expression of almost any protein construct.

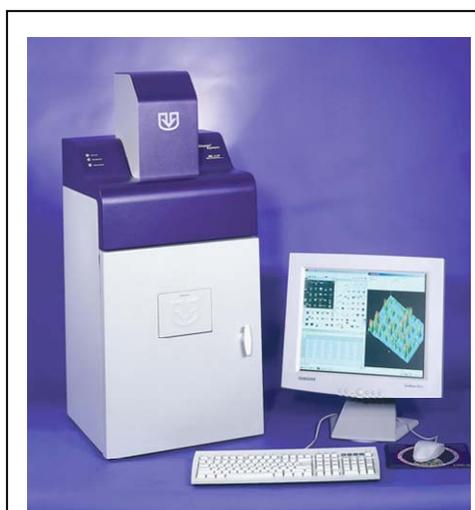


Figure 1. UVP BioSpectrum® system for data collection and analysis of chemiluminescent, bioluminescent, fluorescent and colorimetric imaging.

PROTOCOL

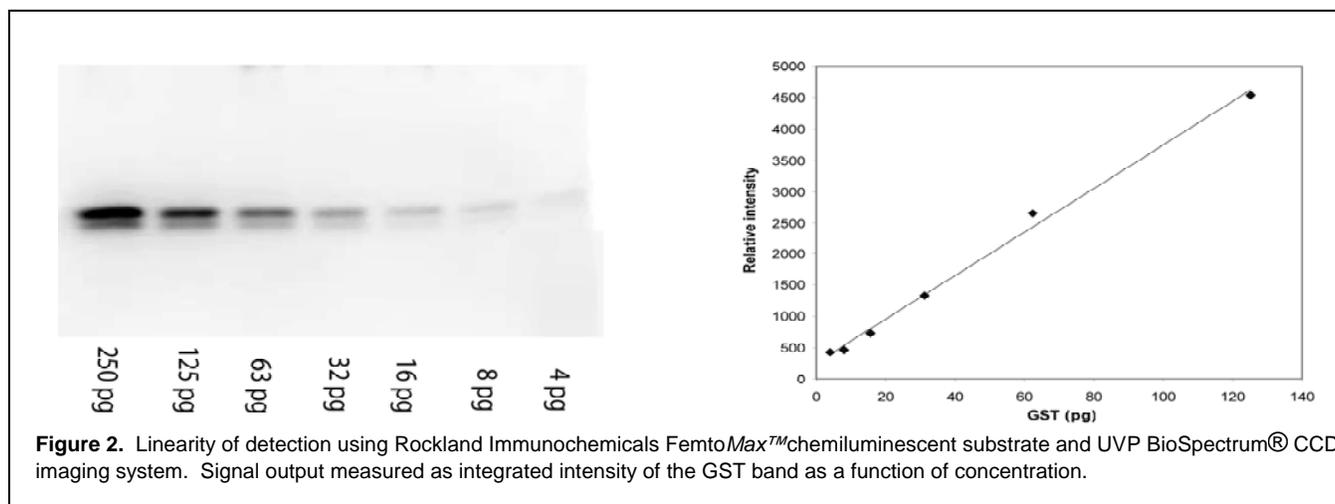
- Perform SDS-PAGE and Western blot transfer
- Block membrane and probe with epitope-tag antibodies
- Probe using HRP conjugated secondary antibodies
- Add TMBE substrate and perform data collection with BioSpectrum® Imaging System

METHODS

Level of detection assays: Recombinant GST (p/n 000-001-200) at 250 picograms was adjusted using a two-fold serial dilution to a final concentration of 1 pg and was electrophoresed on a 4-20% SDS-PAGE gel. The gel was blotted to nitrocellulose membrane and blocked for 1 h in 4 % w/v BSA (p/n BSA-30) in PBS. The GST blots were probed with mouse anti-GST (p/n 200-301-200) diluted to 1:500 by incubating in BLOTTO at 4 °C overnight. Detection was performed by incubating the blot in peroxidase conjugated goat anti-mouse IgG (p/n 610-103-121) diluted 1:10,000 in TBS with 1% BSA. In the second LOD assay, recombinant GST was run as stated above and probed similarly with mouse anti-GST, but using a biotin conjugated goat anti-mouse IgG (p/n 610-1602) followed by detection with peroxidase streptavidin (p/n S000-03) diluted to 1:20,000. **Epitope detection assay:** Epitope tag marker (p/n MB-301-0100) was diluted 4-fold into reducing loading buffer and 5 μ L were loaded onto a 4-20% SDS-PAGE gel. The gel was blotted to nitrocellulose and blocked for 2 h at 20 °C in 5% BLOTTO in TBS. Each blot was probed with one of the following anti-epitope antibodies: anti-GST (Mouse) (p/n 200-301-200), anti-Myc tag biotin conjugated (p/n 600-406-381), anti-HIS⁶ tag biotin conjugated (p/n 600-406-382), anti-FLAG (p/n 600-406-383), anti-HA (p/n 600-406-384) or anti-VSV-G (p/n 600-401-386) at a 1:1,000 dilution overnight at 4 °C. All secondary antibody or streptavidin peroxidase conjugates were incubated in TTBS + 1.0 % BSA and incubated for 1 h at 20°C. The anti-GST (mouse) was detected with peroxidase conjugated rabbit-anti-mouse (p/n 610-4302). The biotinylated anti-Myc and anti-HIS⁶ were detected with peroxidase conjugated streptavidin (p/n S000-03). Anti-FLAG, anti-HA and anti-VSV-G (rabbit) were all detected using goat anti-rabbit IgG (p/n 611-103-122). **Data collection:** The two-component FemtoMax™ reagent was mixed 1:1 and applied directly to the blot. Chemiluminescent data was collected using the UVP BioSpectrum® CCD Imaging System. Images were previewed with 5x5 binning with real time exposure compensation. Camera settings were manipulated in preview mode to optimize the exposure and determine the appropriate final exposure settings. Exposures of 30 sec up to 5 min at 2x2 binning were used for data collection. Data processing was performed with on-chip integration, and resultant images were displayed and analyzed in the VisionWorks® LS software package.

RESULTS

We assayed the sensitivity our reagents and hardware by performing a level of detection assay (LOD). GST was loaded in a two-fold serial dilution series, starting at 250 pg and ending with 1 pg of GST total protein load. A blot was performed as described above and the blot was probed with Rockland Immunochemicals Ms-anti-GST mAB, and data were collected on the BioSpectrum® Imaging System. **Figure 2** shows LOD data for this assay ranging from 250 pg down to 4 pg. The BioSpectrum® Imaging System utilized a Canon EF 50mm F1.2L lens. The large aperture of this lens made it ideal for collecting data in low-light conditions, a requirement for sensitive Western blot detection. Using the VisionWorks®LS software, we performed *area density analysis* on each band in the image, and calculated the signal level from each sample. Pixel intensities from the scanned membrane were plotted against the protein concentration and a linear regression fit was performed. The graph illustrates a wide linear response allowing detection of a large range of concentrations.



The very high affinity of the biotin-streptavidin interaction can be used to achieve the highest level of detection for biological assay systems. We tested the sensitivity of our experimental setup by probing the GST blot using biotin-streptavidin conjugated reagents. **Figure 3** shows Western blot data for detection of the GST control sample using a biotinylated primary antibody and a streptavidin-peroxidase conjugate. Using this detection system we were able to achieve mid-femtogram detection levels.

Accurate and sensitive results were also achieved for the detection of several unique epitope-tags. Data for epitope tag blots were collected by treating each blot with FemtoMax™ reagent and collecting chemiluminescent data on the UVP BioSpectrum® CCD imaging station.

Figure 4 shows data for 6 different antibodies specific for GST, FLAG, HA, Myc, VSV-G, or HIS⁶ epitope tags. The detection was robust and showed little cross reactivity with other non-specific proteins present in the lysate with low overall background. This clearly demonstrates the specificity of the antibodies and validates the 12-epitope tag marker as a very user friendly tool for the monitoring of heterologous protein expression. Because the optimum amino-terminal or carboxyl-terminal position of a hexahistidine (His⁶) affinity epitope is often empirically determined by the researcher, we assayed whether the anti-hexahistidine (HIS⁶) antibody is able to detect the HIS⁶ epitope in both C-terminus and N-terminus orientations. In addition we also tested the ability of our anti-MBP antibody to detect MBP from an *E.coli* lysate. The hexahistidine (HIS⁶) epitope fused to the amino terminus of GFP or fused at the carboxyl terminus β -galactosidase, were used as test proteins for the ability of our anti-His⁶ antibody to detect the each orientation of the His-affinity tag fusion proteins; N-terminal tagged His⁶-Sumo-GFP (40 kDa), and Carboxyl terminal tagged β -galactosidase -His⁶ (120 kDa). We again treated our completed western blot with FemtoMax™, and collected a 4x4 bin image for 30 s on the BioSpectrum® Imaging System. The data showed that the anti-His⁶ antibody (p/n 600-406-382) was able to detect 10 ng of His⁶ located at either the amino terminus of recombinant GFP or carboxyl terminus of recombinant β -galactosidase (**Figure 5**). The anti-MBP antibody was able to clearly detect less than 15 ng of MBP migrating at a molecular weight of 43 kDa (**Figure 5**). We observed that the recombinant proteins and the 12-tag marker are detected with equivalent sensitivity by our anti-epitope antibodies.

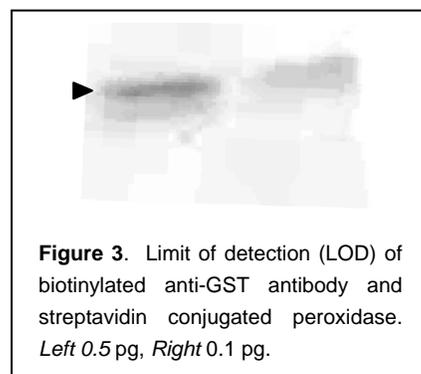


Figure 3. Limit of detection (LOD) of biotinylated anti-GST antibody and streptavidin conjugated peroxidase. Left 0.5 pg, Right 0.1 pg.

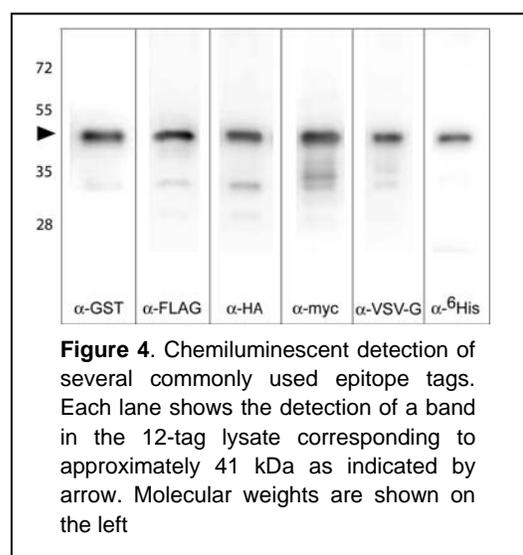


Figure 4. Chemiluminescent detection of several commonly used epitope tags. Each lane shows the detection of a band in the 12-tag lysate corresponding to approximately 41 kDa as indicated by arrow. Molecular weights are shown on the left

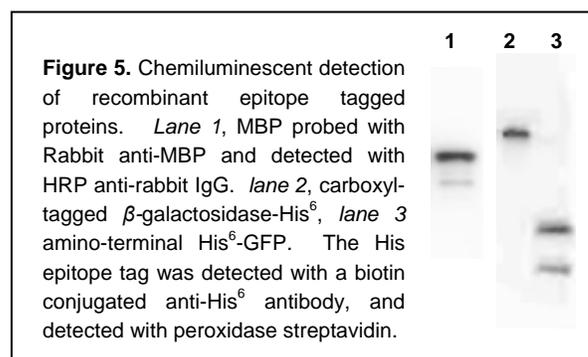


Figure 5. Chemiluminescent detection of recombinant epitope tagged proteins. Lane 1, MBP probed with Rabbit anti-MBP and detected with HRP anti-rabbit IgG. lane 2, carboxyl-tagged β -galactosidase-His⁶, lane 3 amino-terminal His⁶-GFP. The His epitope tag was detected with a biotin conjugated anti-His⁶ antibody, and detected with peroxidase streptavidin.

DISCUSSION

The data presented here illustrate the advantages of using advanced chemiluminescent reagents and imaging hardware for Western blot detection. An important advantage is the ability to collect images rapidly along with high sensitivity thereby increasing overall productivity. Using the VisionWorks® software we quickly and easily quantified each band in our Western blots. We demonstrated that the combination of UVP's BioSpectrum® Imaging System along with Rockland Immunochemicals antibody reagents and FemtoMax™ enhanced peroxidase chemiluminescence substrate allows for ultra-rapid (seconds) and ultra-sensitive (femtogram) data collection. The data here also highlights a new epitope tag control that contains 12 of the most commonly used epitope or affinity markers currently used by researchers. This versatile control, along with appropriate anti-epitope antibodies, allows researchers assay control for monitoring protein expression of most types of academic and commercial vectors with a single robust reagent for monitoring the specificity in their assays.

RELATED PRODUCTS

Equipment and Reagents	Vendor	Catalog
12-epitope tag marker	Rockland Immunochemicals	MB-301-0100
Anti-GST (Mouse)	Rockland Immunochemicals	200-301-200
Anti-FLAG	Rockland Immunochemicals	600-406-383
Anti-HA	Rockland Immunochemicals	600-406-384
Peroxidase streptavidin	Rockland Immunochemicals	S000-03
BLOTTO Immunoanalytical Grade (Non-Fat Dry Milk)	Rockland Immunochemicals	B501-0500
FemtoMax™ Super Sensitive Chemiluminescent HRP Substrate	Rockland Immunochemicals	FEMTOMAX-110
FemtoMax™ Chemiluminescent Western Blotting Kit for Rabbit	Rockland Immunochemicals	KCA003
* BioSpectrum 500 Imaging System with BioChemi 500 Camera (4 mpx, -28°C cooling absolute and regulated), 50mm f1.2 lens, Motorized Lift, 2UV transilluminator and VisionWorksLS Software	UVP	97-0362-01
BioSpectrum 600 Imaging System with OptiChemi 600 Camera (3.2 mpx, -60°C cooling from ambient), 50mm f1.2 lens, Motorized Lift, 2UV transilluminator and VisionWorksLS Software	UVP	97-0402-01
BioSpectrum 800 Imaging System with MegaCam 600 Camera (8.3 mpx, -50°C cooling), 50mm f1.2 lens, Motorized Lift, 2UV Transilluminator and VisionWorksLS Software	UVP	97-0492-01

REFERENCES

1. Kimple ME, Sondak J. Overview of affinity tags for protein purification. *Curr. Protoc. Protein Sci.* unit 9.9, (2004)
2. Chen S, Gray D, Ma J, Subramanian S. Production of Recombinant Proteins in Mammalian Cells, *Curr. Protoc. Protein Sci.* unit 5.10, (2001).
3. Riggs, P.D. Expression and Purification of Maltose-Binding Protein Fusions. *Molecular Biotechnology*, v15 pp 51-53 (1990).
4. Pati, U.K. (1992) Novel vectors for expression of cDNA encoding epitope-tagged proteins in mammalian cells. *Gene* 114:285–288.
5. Maru, Y. et al. (1996) The Dimerization Property of Glutathione S-Transferase Partially Reactivates Bcr-Abl Lacking the Oligomerization Domain *J. Biol. Chem.* 271,15353-15357.
6. Sung MK, Ha CW, Huh WK. A vector system for efficient and economical switching of C-terminal epitope tags in Saccharomyces cerevisiae. *Yeast* 2008, (4):301-11.
7. Korf U, Kohl T, van der Zandt H, Zahn R, Schleege S, Ueberle B, Wandschneider S, Bechtel S, Schnölzer M, Otteleben H, Wiemann S, Poustka A.. Large-scale protein expression for proteome research. *Proteomics*. (14):3571-80.
8. Lamarcq L, Lorimier P, Negoescu A, Labat-Moleur F, Durrant I, Brambilla E. Comparison of seven bio- and chemiluminescent reagents for in situ detection of antigens and nucleic acids. *J. of Biolum. Chemilum.* Volume 10 Issue 4, Pages 247 - 256.
9. Akhavan-Tafti H, Schaap AP, Arghavani Z, DeSilva R, Eickholt RA, Handley RS, Schoenfelner BA, Sugioka K, Sugioka Y. CCD camera imaging for the chemiluminescent detection of enzymes using new ultrasensitive reagents. *J Biolumin Chemilumin.* 1994 9:155-64.

For more information on products or [CUSTOM SERVICES](#), please visit our web site at www.rockland-inc.com.

BioSpectrum® and VisionWorks® are registered trademarks of UVP, LLC.

This document is copyrighted. All rights are reserved. This document may not, in whole or part, be copied, photocopied, reproduced, translated, or reduced to any electronic medium or machine-readable form without prior consent, in writing, from Rockland Immunochemicals, Inc. ©10/01/2008, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA. All rights reserved.

Rev.0 (10/01/2008)