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

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This paper describes the UVP BioSpectrum® Imaging System for high sensitivity detection of proteins in conjunction with the FemtoMax™ chemiluminescent-HRP substrate. We also demonstrate the detection of 6 different commonly used epitope tags using Rockland's epitope tag specific antibodies and a new 12-epitope tag control marker. Rockland's 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 proteomic 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, and 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 lysate. For data acquisition, film and CCD cameras are now commonly used, though 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 uses a high resolution 4.0 megapixel CCD camera and provides automated pre-set or user-defined PC controls for gel imaging and analysis. By combining FemtoMax™ with the BioSpectrum® we report detection

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

of proteins to 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.

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

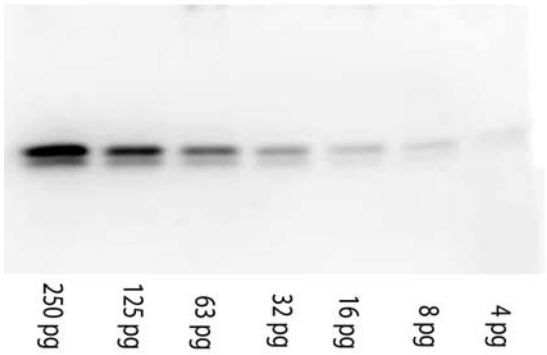
METHODS

Level of detection assays: Recombinant GST (p/n 000-001-200) was made at 250 picograms and a two-fold serial dilution to a final concentration of 1 pg was run on a 4-20% SDS-PAGE gel. . The gel was blotted as above. The membrane was blocked for one hour in 4 % w/v BSA (p/n BSA-30) in PBS. The GST blots were probed with Rockland 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 Rockland's 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*: Rockland epitope tag marker (p/n MB-301-0100) was diluted 4-fold into reducing loading buffer and 5 µL were loaded onto a 4-20% SDS-PAGE gel. The gel was blotted to nitrocellulose and blocked for 2 hours 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-HIS^6 tag biotin conjugated (p/n 600-406-382), FLAG (p/n 600-406-383), HA (p/n 600-406-384), VSV-G (p/n 600-401-386), at 1:1000 dilution overnight at 4 °C. All secondary antibody or streptavidin, peroxidase conjugates were incubated in TTBS + 1.0 % BSA and incubated for 1 hour at 20°C. The anti-GST (mouse) was detected with peroxidase conjugated rabbit-anti-mouse (p/n 610-4302). The anti-Myc and anti-HIS^6 were detected with peroxidase conjugated streptavidin (p/n S000-03). The anti-FLAG, anti-HA, 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 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 seconds up to 5 minutes at 2x2 binning were used for data collection. Data processing was performed with on-

chip integration, and resultant images were displayed and analyzed in 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's Ms-anti-GST mAB, and data were collected on the BioSpectrum. **Figure 1** shows LOD data for this assay ranging from 250 pg down to 4 pg. It is worth noting that the BioSpectrum configuration used 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 (**Figure 2**). The graph illustrates a wide linear response allowing detection of a large range of concentrations.



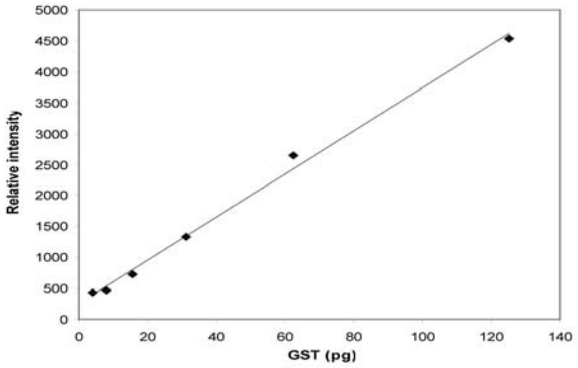


Figure 2. Linearity of detection using FemtoMax and BioSpectrum imaging system. Signal output measured as integrated intensity of the GST band as a function of concentration

The interaction of biotin-streptavidin is a very strong and 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 blot using biotin and streptavidin conjugated reagents. **Figure 3** shows Western blot data for detection of the GST control sample using a biotinylated primary antibody and a streptavidin conjugated secondary antibody. Using this detection system we were able to achieve femtogram LOD.

Figure 3. Biotin-Streptavdin interaction LOD. *Left 0.5* pg, *Right* 0.1 pg.

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 image station. **Figure 4** shows data for 6 different antibodies specific for GST, FLAG, HA, Myc, VSV-G, or HIS^6 epitope tags. The detection was robust and showed little cross reactivity with other non-specific proteins present in the lysate, and low overall background, clearly demonstrating the specificity of the antibodies and validating 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 (His^6) affinity epitope is often empirically determined by the researcher, we assayed whether the anti- hexahistidine (HIS^6) antibody is able to detect the HIS^6 epitope in both orientations. In addition we also tested the ability or our anti-MBP antibody to detect MBP from an *E.coli* lysate. The hexahistidine (HIS^6) 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-His^6 antibody to detect the each orientation of the His-affinity tag fusion proteins; N-terminal tagged His^6-Sumo-GFP (40 kDa), and Carboxyl terminal tagged *β*-galactosidase -His^6 (120 kDa). We again treated our completed western blot with FemtoMax™, and collected a 4x4 bin image for 30 seconds on the BioSpectrum. The data showed that the anti-His^6 antibody (p/n 600-406-382) was able to detect 10 ng of His^6 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. .

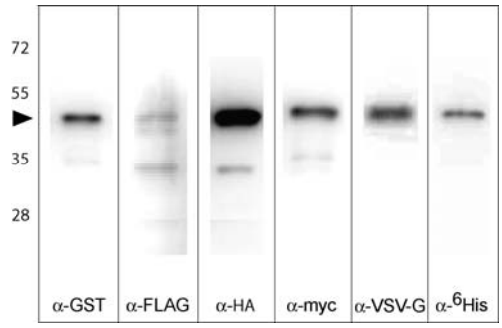


Figure 4. Chemiluminescent detection of several commonly used epitope tags. Each lane shows the detection of a band in the 12-tag lysate corresponding to approximately 41 kDa as indicated by arrow. Molecular weights are shown on the left.

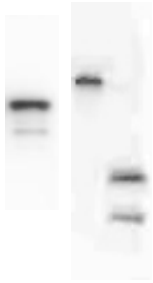


Figure 5 Chemiluminescent detection of recombinant epitope tagged proteins. *Lane 1*, MBP probed with Rabbit anti-MBP and detected with anti-rabbit IgG. *lane 2*, carboxyl-tagged *β*-galactosidase-His^6, *lane 3* amino-terminal His^6-GFP. The His epitope tag was detected with a biotin conjugated anti-His^6 antibody, and detected with peroxidase streptavidin.

DISCUSSION

The data presented here illustrate the advantages of using advanced chemiluminescent reagents and advanced 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™ along with Rockland antibody reagents and FemtoMax enhanced peroxidase 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 (BioChemi HR, LMS-26E)	UVP	82026-850
BioSpectrum 500 (BioChemi HR, FI-26X)	UVP	82026-852
BioSpectrum 500 (BioChemi HR, LM-26E)	UVP	82026-848

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