



ABSTRACT

We describe and highlight a variety of applications for glycoprotein detection using a single near-infrared detection system. Near-infrared wavelengths provide low background from biological materials, buffer components, and standard membranes used in Western and lectin blotting applications. Our system is optimized for two-color near-infrared detection, offering flexibility and multiplexing capabilities for glycoprotein analysis. We highlight applications for glycoprotein analysis such as glycoprotein gel staining, O-GlcNAc specific antibody detection, glycoprofiling using direct or indirect lectin detection, enzymatic glycoprotein labeling, and lectin arrays. The Odyssey[®] Infrared Imaging System and IRDye[®] labeled conjugates (antibodies, streptavidin and lectins) provide a single optimized solution for detecting a variety of glycoprotein interactions.

INTRODUCTION

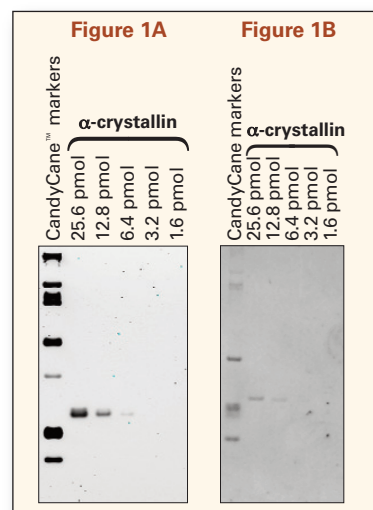
Glycoprotein analysis is a growing area of research that requires sensitive and quantitative applications. We have adapted several one-color, visible glycoprotein applications to near-infrared applications that are detected at 700 nm and 800 nm. Detection at near-infrared wavelengths eliminates fluorescence from biological compounds and buffer components while reducing background for membrane and array based applications. The use of lectins to characterize glycoprotein structures provides a powerful tool to decipher complex structures found in carbohydrates. Lectins are proteins found in plants, viruses, microorganisms and animals that have unique specificities for carbohydrate moieties. Profiling of glycoproteins is shown using lectins labeled with IRDye or biotinylated lectins detected with IRDye labeled streptavidin conjugates. Endo- and exoglycosidases are also used to characterize glycoprotein structures. PNGase F, an amidase, removes the entire carbohydrate structure of N-linked glycans cleaving between the innermost GlcNAc and the asparagine residue. Neuraminidase is a sialidase, which removes α -2-3, α -2-6, and α -2-8 linked N-acetyl neuraminic acid residues. Near-IR detection of multiple lectins after glycoprotein digestion is shown in a single experiment using the Odyssey Infrared Imager.

METHODS & RESULTS

All membranes were blocked with Odyssey Blocking Buffer (LI-COR) unless otherwise stated. All antibody and lectin incubations were performed in Odyssey Blocking Buffer including 0.2% Tween[®]-20. Washes contained 1x PBS + 0.2% Tween-20. Lectin blots processed in the MPX[™] contained 0.1% SDS in washes and antibody incubations to reduce background.

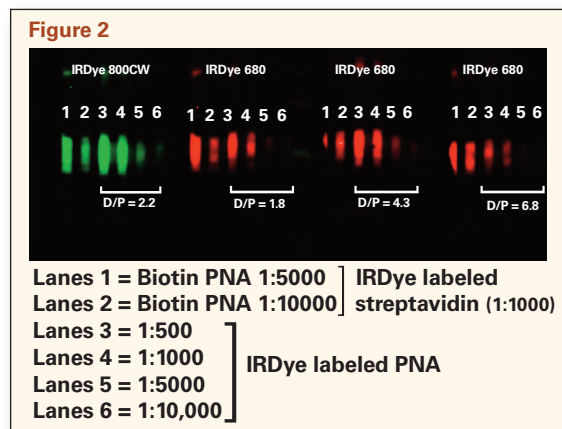
Total Glycoprotein Stain and Western Blot Detection of O-Linked Glycoproteins

Dilutions of α -crystallin were resolved by SDS-PAGE. Figure 1A shows the gel stained with Krypton Glycoprotein Stain (ThermoScientific), and imaged on the Odyssey at 700 nm. Figure 1B shows Western blot detection with mouse anti-O-GlcNAc antibody (Covance) and incubated with IRDye 800CW goat anti-mouse secondary antibody (1:5000) prior to imaging on Odyssey.



Glycoprofiling with Lectins

Glycoprotein analysis can be performed by lectin blotting using IRDye labeled lectins or IRDye labeled streptavidin and detected on Odyssey at 700 nm and 800 nm. Reactions containing fetuin (Sigma) digested with neuraminidase (New England Biolabs) were loaded on 10% SmartGels (LI-COR) using preparative well combs (Figure 2). The gel was transferred to a nitrocellulose membrane, blocked, and placed in the Multiplexer[™] (MPX) Blotting System (LI-COR). Each channel was incubated with biotinylated PNA, followed by IRDye streptavidin or IRDye labeled PNA. Non-digested fetuin showed no signal for the PNA lectin (data not shown).

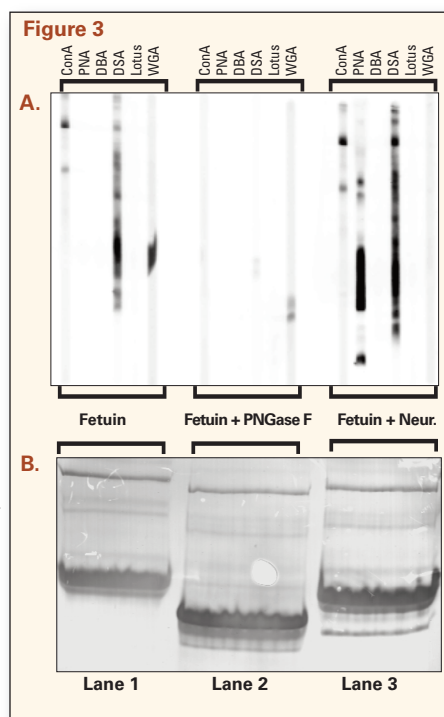


Fetuin digested with either PNGase F, neuraminidase (Sigma), or untreated, was resolved on 10% SmartGels and transferred to a nitrocellulose membrane (Figure 3A). The blocked membrane was placed into the MPX unit and each channel was probed with a specific biotinylated lectin (Associates of Cape Cod and Vector Labs). MPX channels were washed as described, the blot removed from the MPX and incubated with IRDye 800CW Streptavidin (1:10,000).

Total Protein Stain

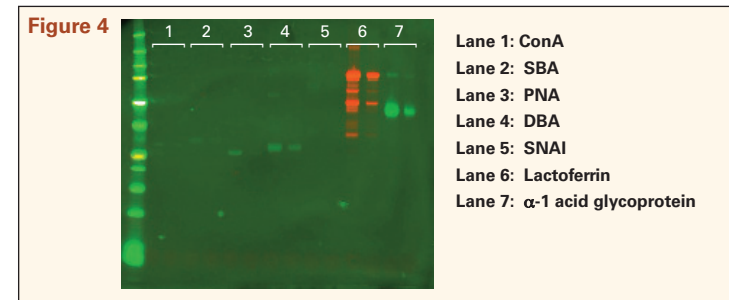
Membrane staining is done post-lectin blotting to confirm presence of protein and enzyme activity. Membranes were stained with Simply Blue Safe Stain (Invitrogen) for 30 minutes. Destaining solution (40% methanol and 10% acetic acid) was added to the membrane for a maximum of 5 minutes prior to imaging at 700 nm (Figure 3B).

Fetuin incubated with PNGase F (Lane 2) shows a large shift to lower molecular weight compared to the untreated sample (Lane 1). Fetuin incubated with neuraminidase (Lane 3) shows a smaller shift in molecular weight.



Lectin Arrays

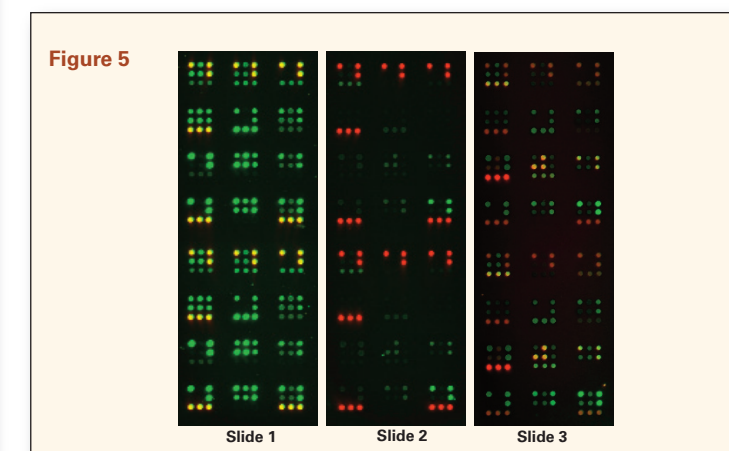
Antibody verification was done by Western blot for five biotinylated lectins with rabbit anti-lactoferrin (Jackson ImmunoResearch) or chicken anti- α -1 Acid Glycoprotein (Affinity BioReagent) primary antibodies (Figure 4). Blocked membranes were incubated with IRDye 800CW donkey anti-chicken and IRDye 680 goat anti-rabbit secondary antibodies. The Western blot shows α -1 acid glycoprotein antibody cross-reacts with all lectins except SNA I. The lactoferrin antibody did not cross-react with any of the lectins and is the best choice for use on the GlycoArray (Qiagen) slides.



Glycoprotein interactions with lectins can be profiled on the Odyssey in an array format using the GlycoArray slides (Qiagen). The slides were processed using the manufacturer's buffers and protocols adapted with near-IR reagents for detection on the Odyssey. Four detection modes are available:

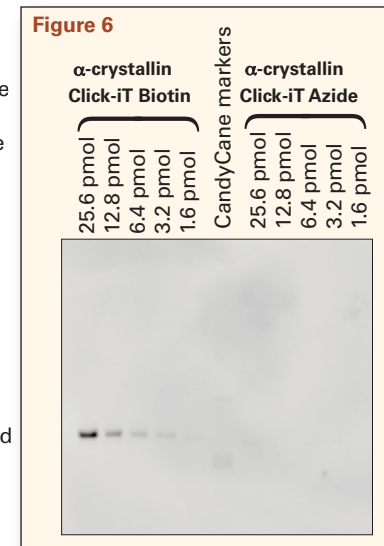
- ❖ Directly labeled glycoprotein
- ❖ Primary labeled antibody detection
- ❖ Secondary labeled antibody detection
- ❖ Labeled streptavidin detection

The software provided with the array gives a histogram and interpretation table which is most powerful for comparative analysis between samples. Glycostructure categories are identified from the software. Slide 1 was incubated with purified lactoferrin (Sigma), 200 nM, and slide 2 was the negative control incubated with water (Figure 5). Both slides were then incubated with rabbit anti-lactoferrin primary antibody followed by IRDye 800CW goat anti-rabbit secondary antibody. The slides were scanned at 21 μ m on Odyssey at 700 nm and 800 nm. Slide 3 shows two-color detection of both lactoferrin (4 nM) by IRDye secondary antibody and IRDye 700DX α -1 acid glycoprotein (200 nM).



Enzymatic Detection of O-linked Glycoproteins

Detection of the O-linked glycoprotein, α -crystallin, was done by an enzymatic method that first modifies the O-GlcNAc to an azide (Click-iT[™] O-GlcNAc Enzymatic Labeling System, Invitrogen). The azide modified protein is further reacted with biotin-alkyne and a copper catalyst to produce a biotinylated glycoprotein (Click-iT Biotin Protein Analysis Detection Kit, Invitrogen). The azide modified and biotinylated α -crystallin products were resolved by SDS-PAGE and transferred to a PVDF membrane (Figure 6). The membrane was blocked, then incubated with IRDye 800CW Streptavidin (1:10,000) for 1 hour prior to imaging on Odyssey.



CONCLUSIONS

- Odyssey Infrared Imaging System was used to highlight eight applications with near-infrared reagents for glycoprotein detection, including total protein and glycoprotein staining, O-GlcNAc Western blotting and enzymatic labeling, lectin blotting and lectin arrays.
- As little as 6.4 pmoles of the O-linked glycoprotein, α -crystallin, could be detected with Krypton Glycoprotein Stain and an O-GlcNAc specific antibody using near-infrared detection.
- MPX provides a single platform for optimization of lectin dilution, incubation time, and wash conditions in a two-color format.
 - Neuraminidase treated fetuin exposes terminal structures of sialylated glycoproteins and PNA is detected by direct labeling with IRDye or IRDye labeled streptavidin.
 - Fetuin digested with PNAase F shows loss of nearly all lectin signal. Only lectins binding to O-linked glycostructures on fetuin would be detected.
- Glycoprotein digestions can be monitored by total protein staining of membranes and imaging on the Odyssey.
- Two-color lectin arrays can be performed at 50-fold lower protein concentrations than recommended.
- Sensitivity of 1.6 pmoles of α -crystallin was achieved when combined with near-infrared detection using enzymatic detection.