

Glycoprotein Detection with the Odyssey[®] Infrared Imaging System

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INTRODUCTION

Glycosylation is one of the most common and important events in post-translational modification, with over half of all proteins believed to be glycosylated.¹ Cellular glycoconjugates play important roles in many biological processes and have been implicated in cancer development, retrovirus infection and other diseases. Carbohydrate-binding proteins known as lectins bind to specific oligosaccharides, and can serve as markers to identify certain cell types or cellular components. Lectins have very high binding specificity and have been used to characterize and purify oligosaccharides.² This application note describes the use of lectins to detect glycoproteins in Western blot format using the Odyssey[®] Infrared Imaging System.

Concanavalin A (Con A) is a carbohydrate-binding protein that binds specifically to the most commonly occurring sugars: α -D-mannose, α -D-glucose and, with lower affinity, α -N-acetylglucosamines (GlcNAcs)^{3,4}. Commercially available biotinylated Con A and IRDye[®] 800CW-streptavidin (LI-COR, Part # 926-32230) were used to detect various glycoproteins by Western analysis. Infrared detection with biotin/IRDye-streptavidin and the Odyssey system is robust and sensitive with a detection limit of 4-8 ng, and eliminates the need for colorimetric and chemiluminescent reagents. Con A can also be covalently labeled with IRDye[®] 800CW (using one of LI-COR's IRDye[®] 800CW Protein Labeling Kits, Part # 928-38040 or 928-38044) and used to detect glycoproteins directly, with a detection limit of 8-16 ng on a Western blot. By adding an antibody against the protein of interest in combination with a lectin, two-color detection can be performed on cell lysates to detect the target protein and make inferences about glycosylation state. A com-

ination of lectins can also be used to dissect the carbohydrate composition of a target protein.

For more information about IRDye products used in this study, go to <http://www.licor.com/bio/reagents/irdyes.jsp>.

MATERIALS AND METHODS

Sensitivity of biotinylated Con A / IRDye 800CW-streptavidin and IRDye 800CW-Con A

Two-fold serial dilutions of α 2-macroglobulin, glucose oxidase and RNase B (Sigma Cat# M6159, 49178 and NEB Cat# P7817S) were mixed and separated on 4-12% polyacrylamide Tris-glycine gels (Novex/Invitrogen). Samples were electrophoretically transferred onto nitrocellulose membrane (Osmonics). For all blots in this study, blocking was carried out with Odyssey Blocking Buffer (LI-COR, Part # 927-40000) for 1 h at room temperature; washing was performed in PBST (PBS + 0.1% Tween[®]-20) for 4 x 5 min, followed by a 5 min rinse in PBS before imaging.

One blot was incubated with biotinylated Con A (Vector Laboratories, Cat# B1005) diluted 1:1000 in Odyssey Blocking Buffer + 0.2% Tween-20 for 3 h. The blot was washed, then incubated in a 1:10,000 dilution of IRDye 800CW-streptavidin (LI-COR, Part # 926-32230) for 1 h, then washed again. The blot was imaged with the Odyssey Infrared Imaging System.

As an alternative to biotin-streptavidin detection, Con A (concanavalin A; EY Laboratories, Inc., Cat. # L1104) was directly conjugated with amine-reactive IRDye 800CW (IRDye 800CW Protein Labeling Kit Part # 928-38040) and purified by dialysis. A dye/protein ratio of 1.7 was obtained. The resulting IRDye 800CW-Con A (1 mg/ml) was diluted 1:1000 and used to detect an identical blot. The blot was washed, then imaged with Odyssey.

Detection of purified glycoproteins using biotinylated Con A/IRDye 800CW-streptavidin

Several purified proteins were digested with Peptide: N-Glycosidase F (PNGase F; New England BioLabs, Cat. # P0704S) which cleaves between the innermost GlcNAc and asparagine residues of N-linked glycoproteins. Undigested and digested proteins (20 µg each) were separated on 4-12% gels. One replicate gel was stained with Coomassie™ (Invitrogen SimplyBlue™ Protein Stain) to detect total protein; another was transferred to nitrocellulose. After blocking, blots were incubated for 1.5 h with 1:1000 biotinylated Con A in Odyssey Blocking Buffer + 0.2% Tween-20, washed, incubated with 1:10,000 IRDye 800CW-streptavidin for 1 h, and washed again. Blots and gels were imaged with Odyssey, using the 800 nm channel to image IRDye 800CW and the 700 nm channel to image Coomassie staining.

Detection of EGFR in A431 cell lysates using human anti-EGFR / AlexaFluor® 680 antibody and IRDye 800CW-Con A

Undigested and PNGase F-digested A431 cell lysates (25 µg each) were separated on replicate 4-12% gels. One gel was stained with Coomassie to confirm equal sample loading, and the other was transferred to nitrocellulose. After blocking, blots were incubated with 1:1000 mouse anti-human EGFR (Biosource, Cat. # AHR5062) in Odyssey Blocking Buffer + 0.2% Tween-20 overnight at 4 °C. Blots were washed, then incubated simultaneously with 1:2500 Alexa Fluor® 680 goat-anti-mouse (Molecular Probes Cat. # A21057) and 1:1000 IRDye 800CW-Con A for 1 h. After washing, blots were imaged with Odyssey in the 700 nm (total EGFR) and 800 nm (Con A) channels.

Characterization of EGFR in normal and cancer cell lysates using labeled lectins

The lectins Con A, WGA (wheat germ agglutinin; EY Laboratories, Inc., Cat. # L2101) and UEA-I (*Ulex europaeus*; EY Laboratories, Inc., Cat. # L-2201) were directly conjugated with IRDye 800CW using standard amine-directed labeling techniques, and purified by dialysis.

Normal epidermal and cancer epidermal (A431) lysates were separated on 4-12% gels, then stained with Coomassie or transferred to nitrocellulose. Blots were blocked, then incubated

with 1:1000 mouse anti-human EGFR as above. After washing, they were incubated with 1:2500 Alexa Fluor® 680 goat-anti-mouse and 1:1000 IRDye 800CW-Con A, IRDye 800CW-WGA, or IRDye 800CW-UEA I (all 1 mg/ml). After washing, blots were imaged with Odyssey in the 700 nm (total EGFR) and 800 nm (lectin) channels.

RESULTS AND DISCUSSION

Biotinylated Con A is a convenient reagent that facilitates straightforward detection of glycoproteins using IRDye 800CW-streptavidin to visualize Con A binding. This technique was used to detect several purified glycoproteins (Fig. 1A). As an alternative, Con A was conjugated with IRDye 800CW by a standard amine-directed labeling chemistry, generating a directly labeled lectin reagent that streamlines the detection process (Fig. 1B). Sensitivity of both detection methods was determined in Western blot format using three known glycoproteins: α2-macroglobulin from human plasma⁵ (180 kDa), glucose oxidase from *A. niger*⁶ (82 kDa), and RNase B from bovine plasma⁷ (17 kDa). The limits of detection with biotinylated Con A/IRDye 800CW-streptavidin and IRDye 800CW-Con A were 4-8 ng and 8-16 ng, respectively.

In Fig. 2A, a selection of purified proteins were examined in greater detail. Protein samples were treated with PNGase F, a glycosidase which removes all terminal and internal N-linked glycosylation. A shift in migration of the band in digested samples indicates that N-linked oligosaccharides were present in the sample. Band shifts were observed for α2-macroglobulin, glucose oxidase, and transferrin. Phosphorylase and BSA did not show a band shift, implying a lack of N-linked glycosylation. Data were consistent with published reports^{5, 6, 8, 9, 10}.

In Fig. 2B, proteins were transferred to nitrocellulose for Western analysis and probed with biotinylated Con A. After detection with IRDye 800CW-streptavidin, α2-macroglobulin and glucose oxidase showed strong bands in the undigested samples and the absence of corresponding bands in the digested samples, indicating that these proteins contain carbohydrate residues capable of binding Con A. Phosphorylase and BSA are not glycoproteins and were not detected by Con A. Interestingly, although a band shift

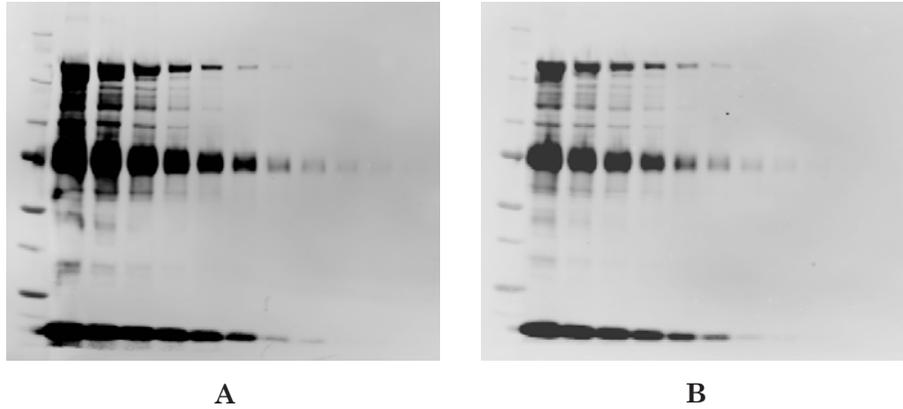


Fig. 1. Western blot of two-fold serial dilutions of α 2-macroglobulin, glucose oxidase and RNase B from 2 μ g to 244 pg. Limit of detection for glucose oxidase was 4 ng (lane 10) with biotinylated Con A / IRDye 800CW-streptavidin (panel A), and 8 ng (lane 9) with IRDye 800CW-Con A (panel B).

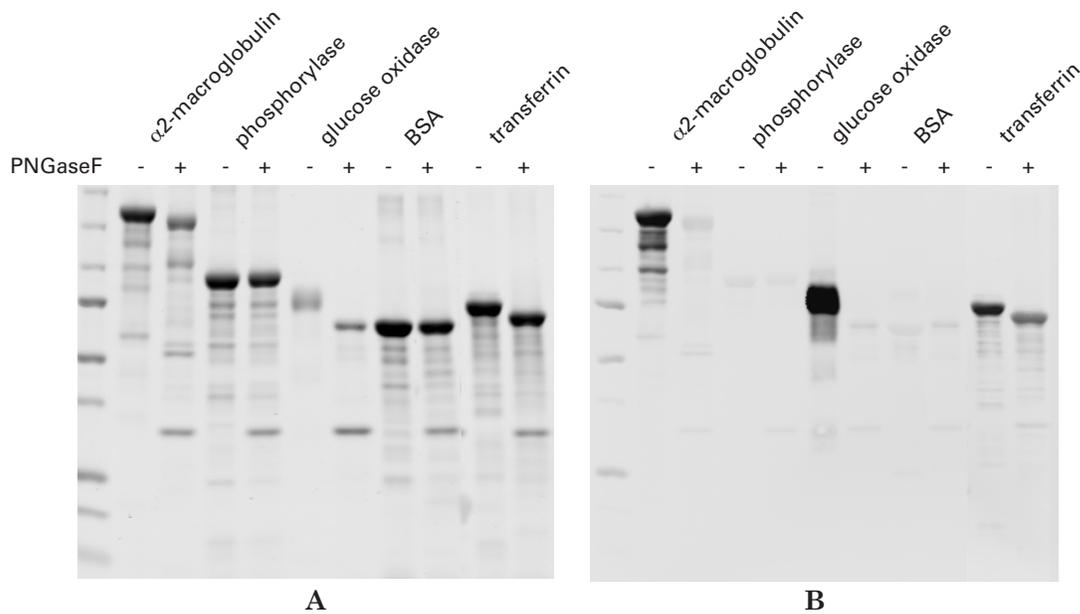


Fig. 2. A) Coomassie stain of untreated and PNGase F-treated proteins. B) Western blot showing undigested (-) and PNGase F-digested (+) proteins. Blot was probed with biotinylated Con A and detected with IRDye 800CW-streptavidin.

was observed for transferrin after digestion with PNGase F, digested transferrin was still detected by Con A. It is possible that the transferrin oligosaccharide detected by Con A is coupled via O-linkage and was therefore not digested by PNGase F. Transferrin is predicted to have an O-linkage in addition to N-linkage⁸. *It is impor-*

*tant to note that some lectins exhibit non-specific hydrophobic binding to nonglycosylated molecules. As shown here, specificity should always be confirmed using appropriate glycosidases.*²

It would be very useful if this technique could not only yield information about glycosylation state, but also confirm the identity of the glyco-

protein in a whole cell lysate using a specific antibody. To this end, epidermal growth factor receptor (EGFR), another glycoprotein¹¹, was analyzed with a two-color detection methodology using anti-EGFR primary antibody and Alexa Fluor[®] 680 goat-anti-mouse secondary antibody. IRDye 800CW-Con A was used to simultaneously detect glycosylation in the 800 nm channel. PNGase F was again employed to confirm specificity. Detection of EGFR protein is shown in red, and glycoprotein detection with Con A is shown in green (Fig. 3A). In the overlaid images, colocalization of red and green pixels results in yellow color, indicating that both anti-EGFR antibody and Con A bound to the EGFR band – evidence that EGFR is glycosylated. Removal of the carbohydrate residue by digestion with PNGase F resulted in a pronounced shift in mobility, thereby validating this conclusion. Coomassie staining (Fig. 3B) confirms equal sample loading. No purification or enrichment of EGFR was required to perform this analysis.

Alterations in glycosyl epitopes can play key roles in tumor progression. EGFR, a popular target for anticancer therapy¹², has been shown to have alterations in its glycosylation pattern in certain cancers¹¹. In Fig. 4, we used several IRDye 800CW labeled lectins (Con A, WGA and UEA-I) to differentiate EGFR glycosylation patterns between normal skin tissue versus cancer

skin tissue. The EGFR glycoprotein was detected as in Fig. 3, and oligosaccharide composition was simultaneously characterized using different IRDye 800CW-conjugated lectins. WGA preferentially binds to β -GlcNAcs and sialic acid^{14, 15}, whereas UEA-I binds to α -fucose^{16, 17}. As shown in Fig. 4 (B-D), EGFR bound to Con A and WGA, but not to UEA-I, implying that EGFR contains a combination of α -D-mannose, α -D-glucose, α -N-GlcNAcs, β -GlcNAcs, and/or sialic acid, but not α -fucose.

EGFR was only detected in the epidermal cancer tissue lysate and not in the normal epidermal tissue. The Coomassie stain of the normal skin lysate showed only a single dominant band that may correspond to keratin. Therefore, due to technical difficulties that may stem from the quality of the lysate, EGFR was not detected in normal skin lysate. Despite this issue, the data suggest that infrared-labeled lectins could be a useful tool for differentiation of normal and cancer cells based on glycosyl epitope expression.

It is important to note that although lectin blots offer useful information, they do not provide proof of a carbohydrate structure and are not a substitute for more specific and quantitative analytical approaches.¹ Lectin binding is dependent on many factors; the results, while suggestive of carbohydrate structure, are not definitive. Nevertheless, lectin detection with

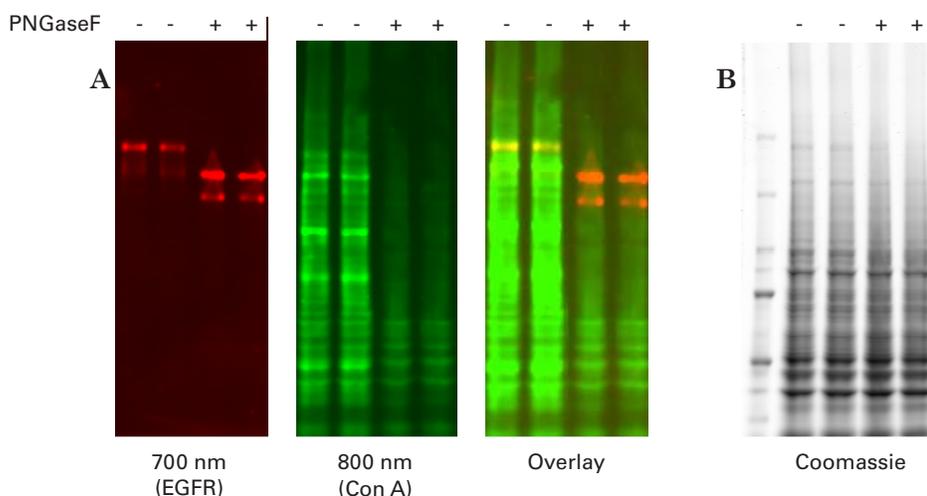


Fig. 3. A) Each panel shows duplicate lanes of A431 cell lysate, untreated or digested with PNGase F. EGFR protein was detected in the 700 nm channel (red), glycoproteins were detected in the 800 nm with dye-conjugated Con A (green), and the overlay panel shows a composite image. Yellow band (overlap of red and green) indicates glycosylated EGFR. The shift in mobility of EGFR following PNGase F treatment indicates that N-linked sugars were removed by the glycosidase, providing further confirmation. B) Coomassie stain of a replicate gel, confirming equal loading of samples.

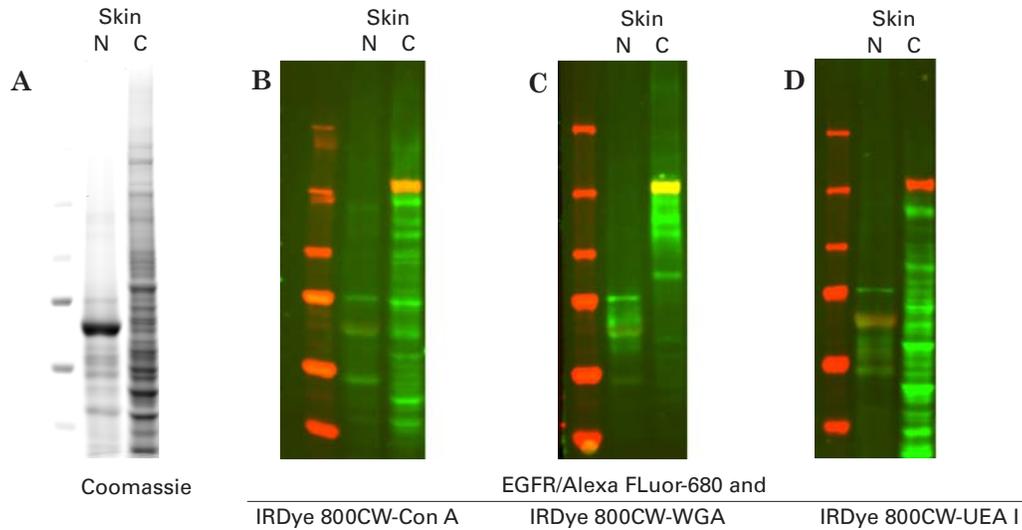


Fig. 4. A) Lysates of normal (N) and cancer (C) tissues from human epidermis. Gel was stained with Coomassie. B-D) Western blots showing two-color detection of EGFR using mouse anti-human EGFR antibody/Alexa Fluor[®] 680 goat-anti-mouse antibody (red), and IRDye 800CW (green) directly conjugated to Con A (panel B), WGA (panel C) and UEA-I (panel D).

the Odyssey system offers useful advantages, including variety, ease of use, and the ability to perform analysis with only a modest amount of target protein. Biotinylated lectins and IRDye 800CW streptavidin are a ready-to-use solution, and the ability to generate directly-labeled lectins provides an opportunity to further simplify detection.

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