

Dual Detection of Total Protein Profiles and Specific Proteins on Low Autofluorescence Poly(vinylidene difluoride) (PVDF) Membranes by a Combined Chemiluminescence and Fluorescence Approach Using a Xenon-Arc Lamp-Based CCD Camera Imaging System



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1 Introduction

Immunoblotting procedures typically require that replicate gels be run, one being stained with silver stain and the other electroblotted and probed using a chemiluminescent, fluorescent or colorimetric detection technique. The chief disadvantage to this approach is that the alignment of the gel and electroblot is difficult, as they usually differ somewhat in size due to swelling or shrinking of the gel during the transfer and staining process. As a result, procedures have been developed for the serial detection of total protein profiles directly on electroblots using reversible dyes and subsequent reprobing for specific antigens by standard immunodetection methods.

Amido black 10B dye, Coomassie® Brilliant Blue R-250 dye, Ponceau S dye and SYPRO® Ruby protein blot stain can be used to visualize total protein profiles, followed by detection of specific protein antigens with substrates of alkaline phosphatase or horseradish peroxidase. Unfortunately though, these stains are washed away during the immunoblotting component of the experiment. Thus, the aforementioned staining methods do not permit simultaneous detection of total protein and a specific target protein. Simultaneous detection of an individual protein and the total protein profile is particularly advantageous for analysis of two-dimensional electroblot patterns, as a single or very few landmarks are available on the final electroblot for registration with the total protein pattern.

In this study, a new dichromatic detection system for electroblotting is described. This new system allows the detection of total protein profiles using AquaBlue™ dye, a permanent fluorescent stain forming covalent bonds with primary amines of proteins (N-terminal and lysine residues), and detection of a specific protein using an alkaline phosphatase-conjugated antibody and the chemiluminescence substrate CDP-Star® substrate with Nitro-Block-II™ enhancer.

2 Materials and Methods

1D SDS-PAGE and Transfer. Proteins were separated on 12% SDS mini-gels and electroblotted to low fluorescence Immobilon-FL PVDF membranes using standard procedures.

2D Gel Electrophoresis and Transfer. Protein separation and transfer to a low fluorescence Immobilon-FL membrane were performed by Kendrick Labs, Inc. (Madison, WI) according to the method of O'Farrell (O'Farrell, P.H. J. Biol. Chem. 250: 4007-4021, 1975). Isoelectric focusing conditions were: 2% pH 3.5-10; size separation conditions: 10% acrylamide slab gel.

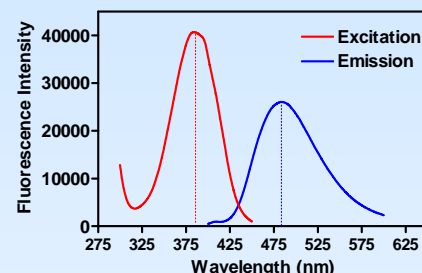
Protein Profile Staining with AquaBlue™ dye. Dried membranes were wetted in methanol, rinsed with water and equilibrated for 20 min in 5-mM sodium borate. Membranes were then stained for 10 min with the AquaBlue dye dissolved in 5 mM sodium borate, and washed in sodium borate for 20 min.

Immunodetection of TNF-α with CDP-Star® substrate with Nitro-Block-II™ Enhancer. Membranes stained with the AquaBlue dye were wetted in methanol and rinsed with water. Non-specific binding sites were blocked for one h in blocking buffer made of 5% w/w Blocking Reagent (PerkinElmer LAS, Inc.), 100 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20. After blocking, the blot was incubated 1 h with a rabbit polyclonal anti-TNF-α antibody, (Abcam plc, Cambridge, UK) diluted 1/10000 in blocking buffer. The blot was then reacted for 1 h with a goat anti-rabbit IgG alkaline phosphatase conjugate anti-rabbit secondary antibody (PerkinElmer LAS, Inc.), diluted 1/5000 in blocking buffer. After washing, the blot was immersed in CDP-Star substrate with Nitro-Block-II enhancer, and incubated for a minimum of 30 min, and up to 24 h, prior to imaging.

CDP-Star is a Registered Trademark of Applied Biosystems Group. Protected under one or more of U.S. Patents 5,326,882; 4,931,569; 5,582,980; 5,851,771; 5,538,847; 5,145,772; 4,978,614.

3 Fluorescence Characteristics of the AquaBlue Protein Stain

Once covalently bound to proteins, the AquaBlue dye fluoresces, with a peak of excitation at 383 nm, and a peak of emission at 484 nm. Spectra of excitation and emission of the coupled dye are illustrated below.



Protein profiles permanently stained with the AquaBlue dye can be imaged using a simple UV light box, or a xenon-arc lamp-based CCD imager such as the ProXPRESS™ 2D Proteomics Imaging System equipped with appropriate filters. Membranes can be imaged dry, before immunodetection, or wet, in the presence of the CDP-Star substrate.

Initial exposure settings recommended for AquaBlue Dye Fluorescent Signal Detection using the ProXPRESS 2D Proteomics Imaging System:

- Top illumination
- 100 msec, 100 μm resolution
- Excitation filter 390/70 nm (cat. 1442-508)
- Emission filter 480/30 nm (cat. 1442-537)

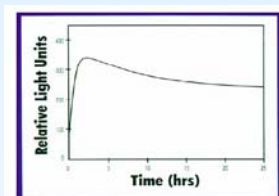
4 Chemiluminescence Detection using CDP-Star Substrate with Nitro-Block-II Enhancer

The CDP-Star reagent is a 1,2-dioxetane compound that is a substrate for alkaline phosphatase. After addition of the CDP-Star substrate to a blot, the dephosphorylation product accumulates rapidly. Its subsequent decay, which occurs at a constant rate, generates a light output. Maximum light emission occurs two hours after substrate addition to the membrane, with continuous light output for a minimum of 24 h.

Light emission from the CDP-Star reaction can be detected by a variety of methods, including exposing the membrane to a KODAK BioMax Light or XAR film, or detecting signal with a CCD-based imager, such as the ProXPRESS 2D Proteomics Imaging System.

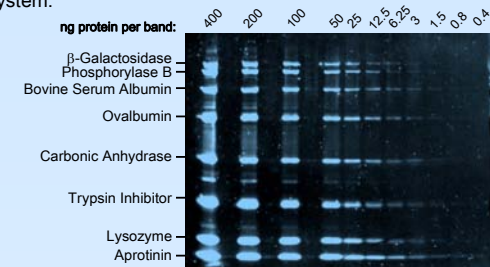
Initial exposure settings recommended for CDP-Star chemiluminescence detection using the ProXPRESS 2D Proteomics Imaging System:

- No illumination,
- Emission 0/0 (empty filter slot)
- 1-2 min exposure
- 100 μm resolution

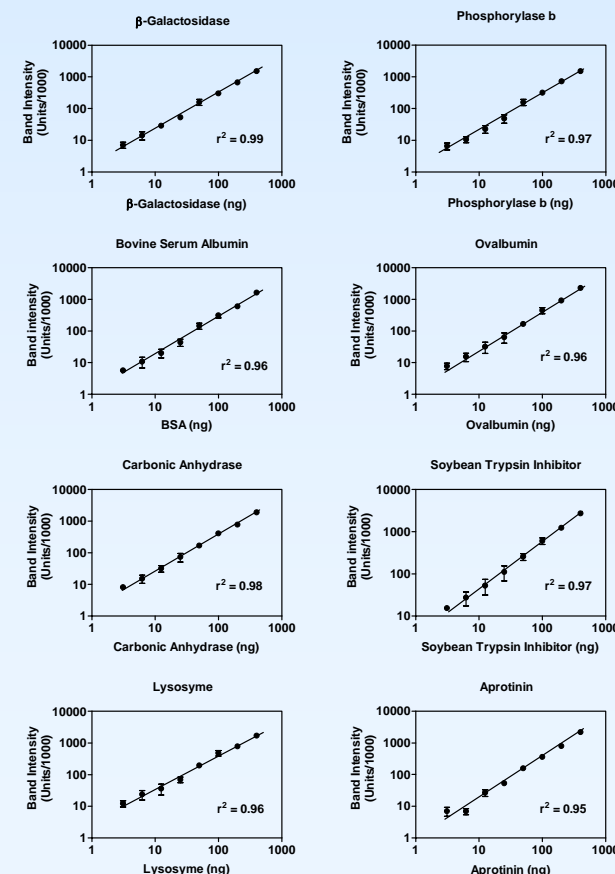


5 Detection of Protein Profiles from PVDF Membranes Stained with AquaBlue dye

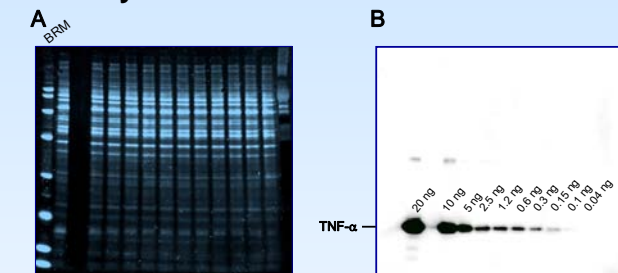
Broad Range Markers (BRM; Bio-Rad cat. 16-0317) contain about equal amounts of nine protein standards. In order to determine the sensitivity and linearity of the AquaBlue Protein Stain, two-fold serial dilutions of Broad Range Markers were separated on a 12% SDS mini-gel and electroblotted to an Immobilon-FL membrane. After staining with the AquaBlue Protein stain, fluorescence was detected with the ProXPRESS 2D Proteomics Imaging System.



Above: AquaBlue dye allows detecting as little as 1.5 ng protein for each of the bands present in the Broad Range Markers. Below: Protein standards from triplicate gels were quantified using the Phoretix 1D software (Nonlinear Dynamics Ltd). Signal is linear from 3 to 400 ng of protein.

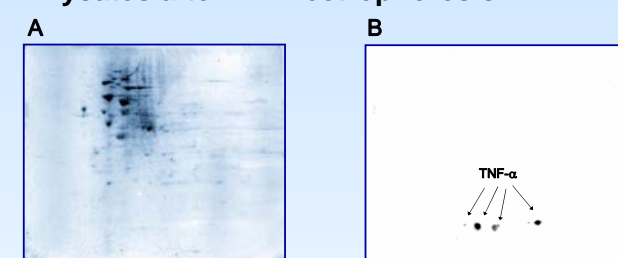


6 Dichromatic Western Blot Detection of Tumor Necrosis Factor spiked into CHO Cell Lysates after 1D SDS-PAGE



CHO clear cell lysates (1 μg) were spiked with serial dilutions of recombinant human TNF-α, run on a 12% SDS mini-gel and electroblotted to Immobilon-FL membrane. A) Detection of the total protein profiles with the AquaBlue Protein Stain. B) Immunodetection of spiked TNF-α using a rabbit polyclonal antibody anti-TNF-α and a secondary antibody conjugated to alkaline phosphatase (AP). Signal was revealed using the CDP-Star substrate with Nitro-Block-II enhancer. BRM: Broad range markers

7 Dichromatic Western Blot Detection of Tumor Necrosis Factor in CHO Cell Lysates after 2D Electrophoresis



CHO clear cell lysate (100 μg) was spiked with 50 ng of recombinant human TNF-α, run on 2D gels as described in Materials and Methods and electroblotted to Immobilon-FL membrane. A) Detection of the total protein profiles with the AquaBlue Protein Stain. B) Immunodetection of spiked TNF-α using a rabbit polyclonal antibody anti-TNF-α and a secondary antibody conjugated to alkaline phosphatase (AP). Signal was revealed using the CDP-Star substrate with Nitro-Block-II enhancer.

8 Conclusions

We have developed a new dichromatic detection system allowing the simultaneous imaging from a low fluorescence PVDF membrane of total protein profiles and of a specific protein detected by immunoblotting.

- The AquaBlue fluorescent Protein Stain is permanent, but does not interfere with subsequent immunoblotting.
- The AquaBlue Protein Stain detects as little as 1.5 ng of a protein band. Signal is linear up to at least 400 ng per band.
- The CDP-Star chemiluminescence substrate is highly sensitive (detects less than 0.1 ng of protein per band) and is ideal for long or multiple exposures, without any loss in signal.