In-gel Immunochemical Detection of Proteins that Transfer Poorly to Membranes

Michael J. Theisen and Mark L Chiu
Department of Structural Biology
100 Abbott Park Road, Abbott Laboratories
Abbott Park, IL 60064-6098

The immunoblot, or "Western blot", is one of the most common and sensitive techniques for specific detection of proteins. The success of immunoblotting depends on efficient transfer of the protein of interest from a polyacrylamide gel to a blotting membrane. Samples containing large or hydrophobic proteins may not meet this criterion. Here we demonstrate an "in-gel" method for immunochemical detection of proteins, which obviates the need for a transfer step. When fluorescent secondary antibodies are detected with a LI-COR Odyssey® scanner, nanogram quantities of protein can be specifically detected by the in-gel protocol. A linear fluorescence response is seen over three orders of magnitude in protein concentration.

INTRODUCTION

One of the most sensitive and specific means of detecting and identifying proteins separated by electrophoresis is the immunoblot (10). The success of the technique depends on efficient transfer of proteins, under high voltage, from the gel to a nitrocellulose, nylon or polyvinylidene difluoride (PVDF) membrane. Unfortunately, not all protein samples are amenable to such a transfer, particularly those that have high molecular weights or post-translational modifications, or are hydrophobic (5). Conversely, small proteins may migrate to such an extent that they pass through the blotting membrane and are lost; this problem can be reduced by judicious choice of membrane material. Thus, it would be desirable to detect proteins of interest in-gel, immediately following electrophoresis, without the necessity of transferring to a membrane. While such protocols have long been known, they are rarely employed due to their laboriousness and low sensitivity (1,3,7,9).

Recently, several systems for in-gel immunostaining have become commercially available. The UnBlot™ kit (Pierce, Rockford, Illinois) allows in-gel detection of proteins by chemiluminescence. The latest development is the Odyssey® system (LI-COR Biosciences, Lincoln, Nebraska), which allows detection of proteins in the gel by fluorescence imaging. Conjugation of secondary antibodies to dyes with excitation and emission maxima at high wavelengths reduces background from intrinsic protein fluorescence. In this report, we describe the application of the Odyssey system to detection of a sample containing cytochrome P450 3A4 (CYP3A4). It is shown that poor transfer of this sample to the membrane makes detection difficult by standard immunoblotting techniques, while the in-gel immunoblot method specifically and sensitively identifies CYP3A4.

MATERIALS AND METHODS

N-terminally modified human CYP3A4 (4), expressed in *Escherichia coli* and purified to approximately 50% homogeneity, was a gift from the laboratory of Stephen G. Sligar (University of Illinois at Urbana-Champaign). Concentration of CYP3A4 was determined by the method of Omura and Sato (8). SDS-PAGE was carried out on a 4-20% polyacrylamide gradient gel with the Criterion™ system (Bio-Rad, Hercules, Califor-

nia), utilizing Tris/glycine/SDS running buffer (6). In the first experiment, the effectiveness of standard immunoblotting was compared to "in-gel" immunoblotting. For each CYP3A4 lane, approximately 3 µg of CYP3A4 material was diluted in sample buffer containing 5% v/v 2-mercaptoethanol, prior to loading onto the gel (6). SeeBlue® Plus2 molecular weight markers (Invitrogen, San Diego, California) were loaded in adjacent lanes. After electrophoresis, the gel was cut in half. One half was stained "in-gel" according to the protocol provided by LI-COR (http://biosupport.licor.com/support/docs/In-Gel-Western.pdf), as follows. The gel was first fixed in 50% v/v isopropanol / 5% v/v acetic acid, washed with ultrapure water and incubated overnight with rabbit anti-CYP3A4 primary antibody (Chemicon, Temecula, California), diluted 1:2000 in Odyssey Blocking Buffer (LI-COR) with 0.1% v/v Tween®-20. After thorough washing with PBS / 0.1% v/v Tween-20, the gel was incubated with IRDye™800-labeled goat anti-rabbit secondary antibody (Rockland Immunochemicals, Gilbertsville, Pennsylvania), diluted 1:2000 in Odyssey Blocking Buffer with 0.1% v/v Tween-20. The other half of the gel was transferred to a PVDF membrane (Pierce) using a Mini Trans-Blot® apparatus (Bio-Rad). The transfer buffer (60 mM Tris, 40 mM CAPS, 15% v/v methanol, pH 9.6) was stirred constantly and cooled by an insert chilled to –20°C. Transfer was carried out at 350 mA constant current, approximately 100 V, for two hours (2). The membrane was removed from the apparatus, blocked with LI-COR Odyssey Blocking Buffer containing 0.1% v/v Tween-20, and probed with antibodies as described above. Proteins that did not transfer from the gel were visualized by staining with Coomassie® Brilliant Blue R250 (Bio-Rad). All stained samples were scanned with the LI-COR Odyssey. The sample was placed on the glass surface of the scanner and irradiated by solid-state diode lasers exciting at 680 and 780 nm. The instrument focused and filtered the emitted fluorescent light for measurement by avalanche photodiodes.

In a second experiment, the sensitivity limit of in-gel immunodetection was estimated by subjecting varying amounts of CYP3A4 to electrophoresis and immunostaining, as described above. For comparison, this gel was subsequently stained with Coomassie Brilliant Blue R250, and the detection limit of that method was visually estimated.

RESULTS AND DISCUSSION

Nearly complete transfer of the pre-stained molecular weight standards from the gel to the PVDF membrane was confirmed visually (Figure 1A). The standards were also visible by Odyssey scanning due to excitation of and subsequent fluorescence from the covalently conjugated dyes. Only a small amount of a molecular weight standard was retained, as seen on the Coomassie-stained gel (Figure 1B). In contrast, proteins from the CYP3A4 sample appeared as intense bands on the post-transfer, Coomassie-stained gel (Figure 1B), while only a few bands were present on the PVDF membrane (Figure 1A), indicating low transfer efficiency. On the "in-gel immunoblot" (Figure 1C), the band corresponding to CYP3A4 (confirmed by mass spectroscopy of peptide fragments, data not shown) was labeled clearly and specifically by anti-CYP3A4 antibody. The primary antibody incubation time can be shortened to 1 hr with similar sensitivity. Only a small area of nonspecific staining was

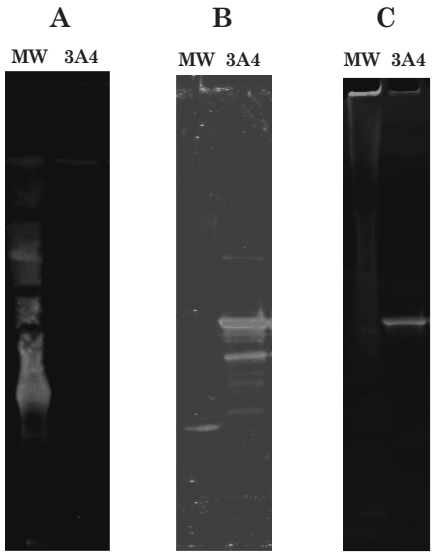

Figure 1. Comparison of immunoblot and in-gel detection. Lanes are labelled: MW=molecular weight standards; 3A4=CYP3A4. (A) PVDF membrane after electrophoretic transfer, probed with fluorescent secondary antibody. (B) The gel after electrophoretic transfer, stained with Coomassie Blue. (C) In-gel immunoblot, probed with fluorescent secondary antibody. Fluorescence was detected with LI-COR Odyssey.

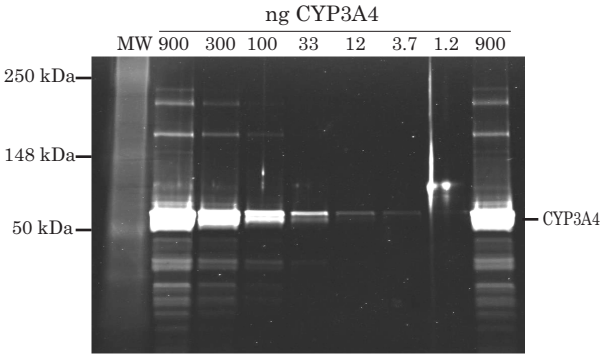

Figure 2. Sensitivity of the in-gel method for detection of CYP3A4. Lane 1 contains molecular weight standards. Lanes 2 - 13 contain 900, 300, 100, 33, 11, 3.7, 1.2 and 900 ng CYP3A4, respectively. The gel was probed with primary antibodies (anti-CYP3A4) at 1:2000 dilution, and subsequently with IRDye™800-labeled secondary antibody at 1:2000 dilution.

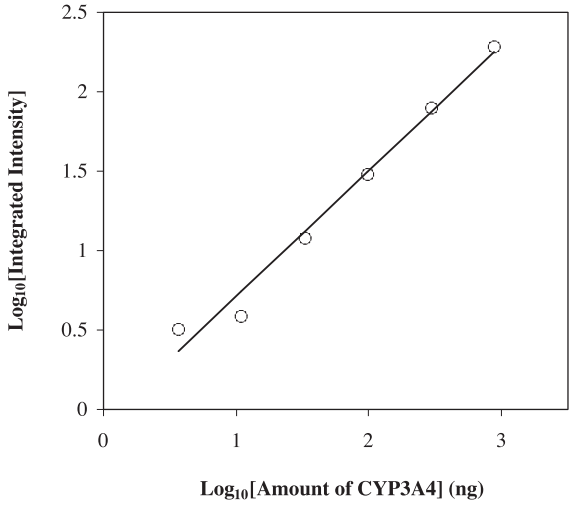

Figure 3. Linearity of fluorescence detection. The intensity of each CYP3A4 band (corresponding to 55 kDa molecular weight) was integrated with the LI-COR Odyssey software. The logarithm of CYP3A4 quantity is linear (R^2 = 0.98) with respect to the logarithm of the fluorescence intensity signal.

seen at the loading position of the molecular weight standards. The detection limit for CYP3A4 was approximately 3 ng (Figure 2). Over the range of 3 – 900 ng CYP3A4, the logarithm of integrated fluorescence intensity scaled linearly with the logarithm of the amount of CYP3A4 (Figure 3). The limit of detection using Coomassie staining was approximately 30 ng (data not shown), as estimated visually.

In-gel immunochemical detection of proteins offers several benefits over immunoblotting protocols. First, samples can be visualized whether or not they transfer to a membrane. Second, because there is no transfer or separate initial blocking step, the procedure is faster and simpler. For a poorly transferring protein of interest, it may be possible to further decrease background by first blotting contaminating proteins to a membrane, and then staining the proteins remaining in the gel. Background staining could also be reduced by optimizing the dilutions and incubation times for antibody binding.

REFERENCES

1. Adair, W.S., D. Jurivich and U.W. Goodenough. 1978. Localization of cellular antigens in sodium dodecyl sulfate-polyacrylamide gels. J Cell Biol *79*:281-285.
2. Burnette, W.N. 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem *112*:195-203.
3. Burridge, K. 1976. Changes in cellular glycoproteins after transformation: identification of specific glycoproteins and antigens in sodium dodecyl sulfate gels. Proc Natl Acad Sci U S A *73*:4457-4461.
4. Gillam, E.M., T. Baba, B.R. Kim, S. Ohmori and F.P. Guengerich. 1993. Expression of modified human cytochrome P450 3A4 in Escherichia coli and purification and reconstitution of the enzyme. Arch Biochem Biophys *305*:123-131.
5. Henderson, C.J. and C.R. Wolf. 1992. Immunodetection of Proteins by Western Blotting, pp. 221-233. *In* M. Manson (Ed.), Methods in Molecular Biology. The Humana Press, Totowa, NJ.
6. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*:680-685.
7. Olden, K. and K.M. Yamada. 1977. Direct detection of antigens in sodium dodecyl sulfate-polyacrylamide gels. Anal Biochem *78*:483-490.
8. Omura, T. and R. Sato. 1965. Isolation of Cytochromes P-45- and P-420. Methods in Enzymology *10*:556-561.
9. Rostas, J.A., P.T. Kelly and C.W. Cotman. 1977. The identification of membrane glycocomponents in polyacrylamide gels: a rapid method using 125I-labeled lectins. Anal Biochem *80*:366-372.
10. Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A *76*:4350-4354.

4308 Progressive Avenue • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH (Germany, Austria, Switzerland): +49 (0) 6172 17 17 771
LI-COR UK Ltd.: +44 (0) 1223 422104
www.licor.com

Doc# 979-07913