Journal of Pharmacological and Toxicological Methods 63 (2011) 279–282

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Quantitative immunofluorescent blotting of the Multidrug Resistance-associated Protein 2 (MRP2)

Phillip M. Gerk *

Dept. of Pharmaceutics, Virginia Commonwealth University, MCV Campus, School of Pharmacy, Room 454F Smith Bldg, 410 N. 12th Street, PO Box 980533, Richmond, VA 23298–0533, United States

ARTICLE INFO

Article history:
Received 19 July 2010
Accepted 12 January 2011

Keywords:
ATP-binding cassette transporters
Immunofluorescence
MRP2/ABCC2
Placenta
Protein quantitation
Western blotting

ABSTRACT

Introduction: Quantitation of the expression levels of proteins involved in drug transport and disposition is needed to overcome limitations of film-based detection of chemiluminescent immunoblots. **Purpose:** The purpose was to describe and validate a quantitative immunofluorescent blotting method for detection of ATP-Binding Cassette Transporter Isoform C2/Multidrug Resistance-associated Protein 2 (ABCC2/MRP2). **Methods:** Western blotting was performed by electrophoresis of membrane vesicle protein isolated from *Sf*9 cells overexpressing MRP2 subsequently blotted with infrared labeled secondary antibody. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor; Lincoln, NE). The images were analyzed using the Odyssey Application Software to obtain the integrated intensities, followed by linear regression of the intensity data. **Results:** The limits of quantitation for the time-*in*sensitive technique described here were from 0.001 μg to 0.5 μg of total membrane protein, the coefficient of variation of the slope was 8.9%; r^2 values were 0.986 ± 0.012. The utility and sensitivity of this technique were demonstrated in quantitating expression of MRP2 in human placental tissue samples, in which MRP2 was present in low abundance. **Discussion:** The immunofluorescent blotting technique described provides sensitive, reproducible, and quantitative determinations of large, integral membrane proteins such as MRP2, all with potential long-term cost savings.

1. Introduction

Immunoblotting is a standard technique for the detection of numerous proteins. Most commonly, the technique requires polyacrylamide gel electrophoresis, transfer to a nitrocellulose membrane, and blotting with a primary antibody followed by a secondary antibody providing a means of detection such as horseradish peroxidase-mediated chemiluminescence, and finally exposure to film. Film is then commonly analyzed by densitometry using widely available programs such as ImageJ or NIH Image. However, these widely used methods are not conducive to quantitative comparisons due to time-sensitivity of the chemiluminescence and the narrow linear range of film. Additionally, it can be subjective in terms of film exposure times, and can be difficult to reproduce.

More recently, other methods avoiding the use of film have arisen. There are high-density imagers capable of directly capturing the chemiluminescence with a CCD camera. While these imagers have sophisticated capabilities for image analysis, they still require chemiluminescence. Additionally, makers of chemiluminescence kits have also improved their products to provide a more constant, longer-lasting signal. However, such improvements result in recurring expenses which can be significant.

In the past several years, a new approach has been marketed in which the secondary antibody is tagged with a fluorophore rather than conjugated with an enzyme. This approach frees the user from the time-sensitivity of chemiluminescence, while reducing long-term costs. Additionally, this approach can enable quantitative rather than semi-quantitative comparisons over a wide dynamic range. However, to date there are no available reports validating the quantitation of immunofluorescent blotting of ATP-Binding Cassette (ABC) transporters.

Furthermore, the current literature is deficient in papers applying and validating this technique for many commonly studied proteins. The time-*in*sensitive technique described here has a 500-fold linear range of quantitation, with absolute intra-gel and inter-gel variabilities below 15%. Although this technique has been applied to several soluble proteins (for example, estrogen receptor α (Long & Nephew, 2006) and the chemokine receptor CXCR2 (Baugher & Richmond, 2008)), it has rarely been applied to integral membrane proteins such as ABC transporters. Finally, we applied this technique to the quantitation of human Multidrug Resistance-associated Protein 2 (MRP2) in human placental villous tissue samples, where MRP2 is variably expressed.

* Tel.: +1 804 828 6321; fax: +1 804 828 8359.
E-mail address: pmgerk@vcu.edu.

1056-8719/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.vascn.2011.01.003

2. Materials and methods

*Sf*9 cells were grown in SFII-900 SFM (Invitrogen) in suspension culture (27 °C, 140 rpm) to ~3×10^6 cells/ml at >97% viability (as determined using trypan blue exclusion) and diluted to 1×10^6cells/ml before infection. Then 5×10^8 cells were infected in the presence of 5% fetal bovine serum using a high-titer recombinant baculovirus stock containing the full coding region of the human MRP2/ABCC2 (NM_000392) at a multiplicity of infection of six; 48 h later, sucrose-fractionated plasma membrane vesicles were isolated and stored as described (Ito, Suzuki, & Sugiyama, 2001; Gerk, Li, & Vore, 2004). Protein concentrations were determined as described (Lowry, Rosebrough, Farr, & Randall, 1951; Gerk et al., 2004).

Western blotting was performed by loading various amounts (0.001–1 μg) of membrane protein on 8% tris–glycine denaturing 10- or 15-well gels (Novex) and separating proteins by electrophoresis, then transferring to nitrocellulose. After blocking nonspecific binding with the Odyssey blocking buffer (Li-Cor Biosciences), mouse anti-MRP2 (M_2-III6, Alexis) subsequently, infrared labeled secondary antibodies goat anti-mouse IRDye 800 (Li-Cor Biosciences) was added to bind to the primary antibody. The bound complex was detected using the Odyssey Infrared Imaging System (Li-Cor; Lincoln, NE). The images were analyzed using the Odyssey Application Software, version 1.2 (Li-Cor) to obtain the integrated intensities. Numerical data were analyzed using GraphPad Prism 4.0, using unweighted non-linear regression ($y=a+bx$) to compare the best-fit model ($y=a+bx$ vs. $y=bx$) by an F-test.

2.1. Application

Reports of the expression of human MRP2 range from very low or undetectable in the human placenta (Pascolo et al., 2003; Evseenko, Paxton, & Keelan, 2006) to comparable to hepatic expression in term placentae (Meyer Zu Schwabedissen et al., 2005), while other reports do not provide quite enough information to make this comparison (St-Pierre et al., 2000; Azzaroli et al., 2007). Some reports demonstrate high interpatient variability in MRP2 expression (Meyer Zu Schwabedissen et al., 2005; Vaidya, Walsh, & Gerk, 2009). Therefore, the above methods were applied to detect and quantitate MRP2 protein in normal human placental homogenate. Human placentae from normal pregnancies were obtained under approval from the VCU Institutional Review Board; subjects gave written informed consent prior to enrollment. After removing the fetal and maternal plates, the soft villous tissue was excised, cleared of major blood vessels, finely minced, and thoroughly rinsed with normal saline. The tissue (200–300 mg wet tissue) was homogenized with a Polytron homogenizer at 16,000 rpm for 30 s. The samples were then centrifuged at 500 rcf at 4 °C for 10 min, and the supernatant was aliquoted and stored at −80 °C. After determining protein concentrations as described above, samples (25 μg protein) were analyzed by immunoblotting as above. Sample intensities were compared to a calibration curve on the same blot to determine the equivalent amount of MRP2 protein using MRP2-expressing membranes as a standard.

2.2. Protocol

1. Reagents and solutions

1.1. Tris–glycine SDS sample buffer (2X)
2 ml glycerol (#0854-1 L; Amresco, Solon, OH)
2.5 ml Trizma base 0.5 M (T-6066; Sigma-Aldrich, St. Louis, MO)
4 ml 10% sodium dodecyl sulfate (SDS) (L-4509; Sigma-Aldrich)
0.5 ml of 0.1% bromphenol blue (BP-114-25; Fisher, Pittsburgh, PA)
1 N hydrochloric acid (HX0603-75; EMD, Gibbstown, NJ); to adjust pH to 6.8
18 M Ω H_2O to adjust total volume to 10 ml

1.2. Running buffer: prepared 10X (dilute to 1X before use)
30.54 g Trizma base
144.13 g Glycine (G-8898; Sigma-Aldrich)
10 g SDS
1 N HCl to adjust pH to 8.3
18 M Ω H_2O to adjust total volume to 1000 ml

1.3. Transfer buffer: (pre-chilled to 4 °C)
1.45 g Trizma base
7.2 g glycine, add both to ~750 ml dH_2O
200 ml HPLC grade methanol (JT9093-3; VWR Scientific, West Chester, PA)
18 M Ω H_2O to adjust total volume to 1000 ml, let stir ~5–10 min for thorough mixing

1.4. Blocking buffer: Odyssey blocking buffer (927–40000; Li-Cor Biosciences, Lincoln, NE)

1.5. TBS binding buffer: (prepared 10X)
24.2 g Trizma base
80 g sodium chloride (S-7653; Sigma-Aldrich)
add both of the above to approximately 800 ml 18 M Ω water
adjust pH using 5 N HCl to pH 7.6 (about 20 ml)
18 M Ω H_2O to adjust total volume to 1000 ml

1.6. Other reagents
Tween 20 (20605-500 ml; USB Corp., Cleveland, OH)
10% w/v SDS solution (dissolve 1 g SDS in 8–9 ml 18 M.Ω H_2O, adjust volume to 10 ml)

1.7. Antibodies
Primary antibody: mouse anti-MRP2 (M_2-III6; Alexis Biochemicals, San Diego, CA)
Secondary antibody: goat anti-mouse Alexa Fluor 680 (827–06901; Li-Cor Biosciences)

2. Consumables
Novex 8% tris–glycine pre-cast 10-well gels (EC6015BOX; Invitrogen, Carlsbad, CA)
Benchmark prestained protein ladder (10748010; Invitrogen)
Whatman qualitative filter paper grade 1 (1001–917; GE Healthcare, Piscataway, NJ)
Nitrocellulose 0.2 μm Protran BA85 (10-402-594; Schleicher& Schuell, Keene, NH)

3. Apparatus
Pipette roller: break a 10 ml disposable polystyrene pipette to a length corresponding to 4 ml markings (i.e., break off pieces below 2 ml and above 6 ml; retain piece between 2 ml and 6 ml)
For running the gel: X-Cell Sure Lock Mini Cell (EI905; Invitrogen)
For transfer: BioRad transfer apparatus (Mini Trans-Blot Cell; Bio-Rad, Hercules, CA)
DC Power Supply (PS500X; Hoefer Scientific Instruments, San Francisco, CA)

4. Procedure

4.1. Prepare samples appropriately, in 1X sample buffer, to deliver an appropriate amount of protein per well, in a maximum of 20 μl. Routinely, it is better to plan on adding 20 μl to each well. Mix well, briefly centrifuge.

4.2. Heat denature at 37 °C for 30 min (note that high temperatures even for short times will destroy MRP2). Gently flick tubes, then briefly centrifuge again.Set voltage to 125 V to begin electrophoresis. Let the gel run about 45 min (1 gel) to an hour (2 gels), but typically not past the point at which the dye runs out the bottom of the gel.

4.3. Set up the BioRad transfer apparatus in an ice bucket, such that the transfer cell is covered by ice on all sides, place the BioRad cooling unit (ice-slab), and a stir bar.