Comparative Antibody Titers Using A Microtiter Plate Assay Versus Dot Blotting on Nitrocellulose

William T. Gerthoffer, Ph.D.
Mariam Ba, M.S.

Department of Pharmacology
University of Nevada School of Medicine
Reno, NV 89503

Contact: William T. Gerthoffer, Ph.D.
Professor of Pharmacology
University of Nevada School of Medicine
1664 N. Virginia Street
Reno, NV 89557-0270
Phone: 775-784-4119
FAX: 775-784-1620
wtg@med.unr.edu

INTRODUCTION

Raising custom polyclonal antibodies in rabbits for research purposes requires that antibody titers be estimated for each serum sample collected. Relative titers of antibodies can be determined by several methods, the most common of which is an ELISA. One method is to bind the immunogen to a microtiter plate, react it with primary antibody and then detect the antigen-antibody complex using a tagged secondary antibody, which is often an alkaline phosphatase or horseradish peroxidase conjugate. The enzyme catalyzes a reaction resulting in a colored product that is detected with a UV/VIS microtiter plate reader. We wanted to determine if the enhanced sensitivity and linearity of fluorescence detection of the secondary antibody could also be used in a modified ELISA approach to determine antibody titers in serum. The availability of LI-COR® Odyssey® fluorescence scanners offers a convenient and sensitive alternative to a fluorescence plate reader for determining relative antibody titers in serum, ascites or in purified immunoglobulin preparations.

In this study, we used the Odyssey scanner to detect antigen-antibody complexes bound to a microtiter plate and antigen-antibody complexes bound to nitrocellulose. The aim was to compare the working range and the sensitivity of the two approaches in order to develop a standard protocol for assessing antibody titers in rabbit serum or in column fractions after affinity purification of antibody from serum. We find the ELISA approach where antigen is bound to the microtiter plate to be much less sensitive than a dot blot method where the antigen is bound to pure nitrocellulose.

METHODS

Antigen preparation

Recombinant human HSP27 and HSP22 were expressed in *E. coli* and purified by Ni-NTA affinity chromatography. Purity was assessed by SDS-PAGE, and protein content measured by the bicinchoninic acid method of Smith et al., 1985. Thirty to 100 ng of recombinant protein was used in the assays.

Modified ELISA method

Duplicate 100 µl samples of recombinant protein (0.3 or 1 µg/ml) in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 43 mM Na_2HP0_4 and 1.4 mM KH_2PO_4, pH 7.4) were incubated overnight in Immulon 1B 96-well microtiter plates (Thermo Electron Corp., 3355). The wells were washed four times with PBS. The wells were then blocked for one hour by adding 100 µl Odyssey blocking solution diluted to 0.5X in phosphate buffered saline. Primary antibody was diluted in 0.5X Odyssey Blocking Buffer and incubated for two hours at room temperature. Primary antibody was aspirated from the wells and the plates washed four times with PBS + 0.1% Tween 20. The appropriate secondary antibodies were diluted in 0.5X Odyssey blocking solution, added to the wells and incubated for 1 hr with rocking at room temperature. Secondary antibody was removed, and the wells washed four times with PBS + 0.1% Tween-20 and once with PBS. Goat-antimouse IgG-Alexa Fluor® 680 (Molecular Probes, A21058) was used to detect mouse anti-HSP27 IgG. Goat-antirabbit IgG-Alexa Fluor 680 (Molecular Probes, A-21109) was used to detect rabbit anti-HSP22 antibodies.

The Odyssey scanner includes a frame for holding microtiter plates and software to analyze fluorescence inten-sity of each well. The Immulon 1B microtiter plates were scanned in the 700 nm channel at a laser intensity of 5.0 (anti-HSP27) or 8.0 (anti-HSP22) and focus offset of 4 mm. We then used the Odyssey Application Software (version 1.2) to measure the fluorescent signal intensity in each well. The background fluorescence was corrected with a user defined area of interest, which was an empty well. That value was subtracted from each well. The corrected integrated intensity at 700 or 800 nm emission wavelength was determined for each well.

Dot blot method

To facilitate even distribution and uniform spot size of the antigen solution, pure nitrocellulose membrane was gently pressed onto the rings embossed on the underside of a microtiter plate lid. This leaves a faint circular mark of about 32 mm^2 area. Antigen diluted in PBS was spotted in duplicate 2 µl samples onto 10 x 50 mm strips of nitrocellulose and allowed to adsorb completely (about 3-5 min). The membrane strips were placed in 35 mm culture dishes and were blocked by incubating in 0.5X Odyssey blocking solution for 1 hr at room temperature. Primary antibody was diluted in 0.5X Odyssey blocking buffer and incubated with shaking for 1 hour at room temperature. Membranes were washed four times for five minutes with PBS + 0.1% Tween-20. Membranes were then incubated 1 hr at room temperature in Goat-antirabbit Alexa Fluor 680 secondary antibody diluted 1:50,000 to 0.02 µg/ml in 0.5X Odyssey blocking solution. Secondary antibody was removed, and the membranes washed four times for 5 min with PBS + 0.1% Tween-20 and once with PBS. Membranes were scanned using the "Membrane" preset, 169 µm resolution, medium quality, 5.0 (anti-HSP27) or 8.0 (anti-HSP22) laser intensity, 700 nm emission wavelength (Odyssey Application Software version 1.2). Integrated intensity of each dot was corrected for background fluorescence using the median setting and a 3 pixel border of a circular object. Integrated intensities of data from both the ELISA method and the dot blot method were exported to spreadsheet software and to GraphPad Prism version 4.0 (GraphPad Software, San Diego USA) for curve fitting and graphing.

RESULTS AND DISCUSSION

***Anti-HSP27 IgG*.**

To establish the feasibility of using the modified ELISA method to titer antibodies, we initially used a purified anti-HSP27 monoclonal antibody from Stressgen Biotechnologies Corp. (SPA-803) and purified recombinant human HSP27. Recombinant protein was prepared by expressing the human HSP27 in *E. coli* and isolating a pure fraction from bacterial lysate by ammonium sulfate precipitation and DEAE chromatography as previously described (Larsen et al., 1997). Ten to 100 ng of protein were used to coat 96 well ELISA plates. A series of anti-HSP27 dilutions was prepared ranging from 0.1 to 2 µg/ml. The primary antibody was incubated 2 hr at room temperature with rocking. Alexa Fluor 680 labeled goat anti-mouse IgG was diluted 1:1,000 to 2 µg/ml and incubated for 1 hr. After four washes with PBS, fluorescence at 700 nm was measured with the Odyssey scanner and integrated intensity of each well calculated. Figure 1A shows the signal intensity as a function of primary antibody concentration. We compared three different antigen levels on the plates -10, 30 and 100 ng/well. The signal intensity varied as function of antigen levels, but saturated

rather quickly as a function of primary antibody concentration. These results suggest the ELISA method will detect differences in the amount of antigen-antibody complex on the plate, but the signal saturates within a narrow range of antibody concentrations.

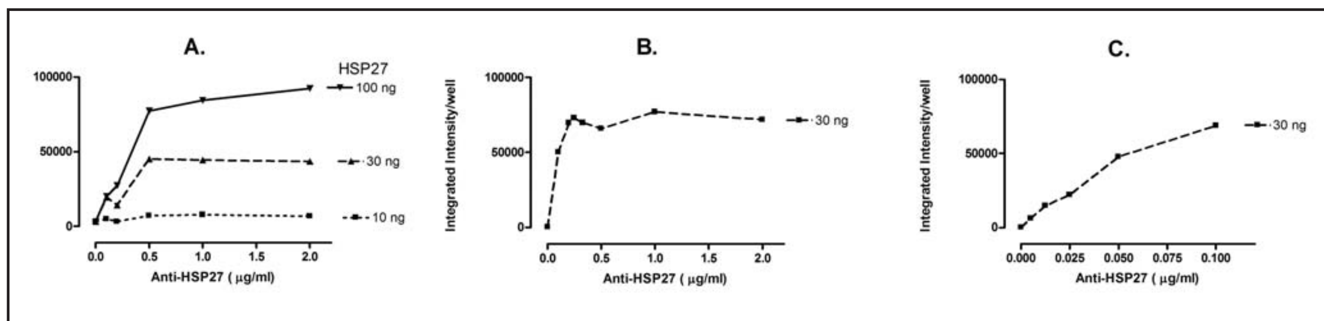

Figure 1. Antibody titering by modified ELISA (A.) vs. dot blot method (B. and C.)

We then compared the signal from the ELISA method (Fig. 1A.) to an established "dot blot" technique (Figure 1B.) in which 2 µl samples of antigen are spotted by hand onto strips of pure nitrocellulose. The same primary antibody concentrations were used for detection of the spots, but the secondary antibody was diluted to 0.04 µg/ml in the dot blot method instead of 2 µg/ml used in the ELISA method. Figure 1B shows that the signal intensity at the same levels of antigen is higher and saturates rapidly at primary antibody concentrations less than 0.2 µg/ml. To define the linear range of the dot blot assay more accurately, we repeated the experiment using much lower primary antibody dilutions (0.005 - 0.1 µg/ml) and 0.04 µg/ml secondary antibody (Figure 1C.). The integrated intensity of the dots was a linear function of antibody concentration at 30 ng antigen loaded on the blots. These results show the sensitivity and linearity of detecting HSP27 antigen is superior when using a nitrocellulose as the binding substrate when compared to 96 well ELISA microtiter plates. Both primary and secondary antibodies can be used at significantly lower concentrations in the dot blot assay, which would realize significant cost savings. However, the dot blot assay is somewhat more sensitive to the skill of the technician preparing the dot blots.

Anti-HSP22 IgG

The purpose of the study was to establish a rapid method suitable for comparing titers of custom antibodies raised in rabbits. We compared the ELISA method to the dot blot method using recombinant human HSP22 as the antigen. Rabbit anti-human HSP22 antibodies were raised against a synthetic peptide corresponding to a C-terminal peptide (NELPQDSQEVTCT) from the human HSP22 sequence. The peptide was synthesized, conjugated to Keyhole limpet hemocyanin, and antiserum produced by Sigma Genosys Corp. Thirty and 100 ng of recombinant HSP22 were bound to microtiter plates or spotted onto nitrocellulose strips as described above. Antibodies were affinity purified from the third and fifth bleeding of one rabbit (number 218) taken 2 weeks apart. The titers of anti-HSP22 were then compared using the ELISA method (Figures 2A. and 2B.) and the dot blot method (Figures 2C. and 2D.). The solid lines show integrated intensity of the wells or dots containing 100 ng HSP22, and the dotted lines show integrated intensity when 30 ng of the antigen protein was used. Both ELISA plates and dot blots were scanned at laser intensity of 8.0 to enhance fluorescence intensity. Both methods detected the anticipated increase in antibody titers between bleeds 3 and 5 (compare Figs. 2A. and 2B. vs Figs. 2C. and 2D.). However, the dot blot method produced about a six-fold higher signal intensity when using the same antibody concentrations compared to the ELISA methods (cf. Figs. 2A .and 2C.). Note the difference in the Y axis scales. Although the ELISA approach can be used to assess relative antibody levels in different serum samples, it is less sensitive and requires considerably more secondary antibody than the dot blot method.

We have shown that a modified ELISA method using direct detection of fluorescent secondary antibody might be suitable for assessing antibody titers over a 10-fold range of dilutions. The sensitivity of the microtiter plate method is substantially less than that of a simpler dot blot technique, which uses far less antigen and secondary antibody. However, if antigen and secondary antibody reagents are not limiting, and throughput is paramount, the microtiter plate method might lend itself better to automated processing in a high throughput environment. The useful working range of primary antibody dilutions is somewhat better using the dot blot method – 50 fold vs. 10 fold for the ELISA method when using anti-HSP27 antibody. There are several possible reasons for the difference between the methods. The protein bound to nitrocellulose may be available to the antibody in more optimum configuration, or binding of purified protein to nitrocellulose may be more efficient compared to binding to plastic microtiter plates. The latter effect would result in an apparent decrease in detection threshold.

A. 218-3 ELISA

B. 218-5 ELISA

Images corresponding to ELISA data in Figs. 2A. and 2B.

Images corresponding to Dot Blot data in Figs. 2C. and 2D.

C. 218-3 Dot Blot

D. 218-5 Dot Blot

Figure 2. Comparison of rabbit anti-human HSP22 antibody titers in sequential bleeds using ELISA vs. dot blotting methods.

In conclusion, we have found titering antibody preparations can be done rapidly and accurately using direct fluorescent detection of secondary antibody in either a modified ELISA method or a classical dot blot method. The dot blot method is more economical, more sensitive and has superior working range of antibody dilutions. The dot blot method is probably best for a small lab environment where modest throughput is required but there is limited budget for reagents.

LITERATURE CITED

1. Larsen, J. K., Yamboliev, I. A., Weber, L. A. and Gerthoffer, W. T. Phosphorylation of the 27-kDa heat shock protein via p38 MAP kinase and MAPKAP kinase in smooth muscle. *Am J Physiol* 273: L930-L940, 1997.

2. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal Biochem* 150: 76-85, 1985.

LI-COR and Odyssey are registered trademarks of LI-COR, Inc. All other trademarks belong to their respective owners.

4308 Progressive Ave. • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
North America: 800-645-4267 • International: 402-467-0700 • FAX: 402-467-0819
LI-COR GmbH*: +49 (0) 6172 17 17 771 • LI-COR UK Ltd.: +44 (0) 1223 422104
www.licor.com
* Serving Austria, Czech Republic, Germany, Hungary, Slovakia, and Switzerland