Measurement of Antigen by Enhanced Chemiluminescent Western Blot

BioTechniques 22:454-458 (March 1997)

Enhanced chemiluminescence has been used widely as a rapid and sensitive detection technique for proteins of interest. Here we report that the signal detected by enhanced chemiluminescence-based Western blot analysis is proportional to the amount of antigen loaded on the gel; however, the correlation coefficient differs with each antigen/antibody pair. We provide a simple and accurate protocol to quantify and estimate proteins in cell culture and cell-free systems. This protocol may be useful in other studies involving the manipulation of gene expression in vitro and in vivo.

The chemiluminescence in this protocol is generated by the oxidation of luminol. Horseradish peroxidase catalyzes the oxidation in the presence of an oxidant, hydrogen peroxide, to produce a luminol radical. This radical then forms an endoperoxide that decomposes to yield an electronically excited 3-amino-phenolate dianion, and the dianion emits light on return to its ground state (7). Since horseradish peroxidase is conjugated to the secondary antibody in commercially available Western blot detection kits, we reasoned that the intensity of chemiluminescence should be proportional to the number of secondary antibody molecules and therefore to the amount of antigen retained on the filter. Furthermore, in enhanced chemiluminescence-based Western blot detection kits, light emission is recorded by exposure to X-ray film (4). We concluded that signals could be quantified with densitometry since the exposure time is equal for each sample on the filter.

A Western blotting procedure with enhanced chemiluminescence detection was used to measure the relative amounts of two RNA-binding proteins in nuclear extract from Drosophila Schneider 2 cells. The Protein on Ecdysone Puffs (PEP) protein is a Drosophila heterogeneous nuclear ribonucleoprotein (hnRNP) and is recognized by the monoclonal antibody (MAb) Y1D2 (1). The SNF/D25 protein is a component of Drosophila U1 and U2 snRNP particles and is recognized by the MAb 4G3 (5). For our experiments, nuclear extract is prepared from cultured Schneider 2 cells by the protocol of Dignam et al. (3) and stored at -80°C. The total protein concentration in nuclear extract is determined by the protein microassay (Bio-Rad, Hercules, CA, USA) (2) with bovine serum albumin as a protein standard. Equal volumes of nuclear extract and 2× sodium dodecyl sulfate (SDS) gel loading buffer (6) are mixed and then boiled in a water bath for 10 min to denature the polypeptides. Variable amounts of the denatured mixture are loaded in alternating lanes on a 15% SDS-polyacrylamide gel (6), along with a molecular weight marker (Kaleidescope Precision Standard; Bio-Rad). Following fractionation by electrophoresis, the polypeptides are transferred electrophoretically from the gel to a nitrocellulose membrane (NitroBind™; Micron Separations Inc. [MSI], Westboro, MA, USA) (6). After the transfer, the membrane is incubated in TPBS [0.05% (vol/vol) Tween® 20 in phosphate-buffered saline (PBS) buffer] on a rocking platform for 30 min; this and all subsequent incubations are performed at room temperature. The membrane is incubated with blocking solution (5% skim milk powder in 10 mL TPBS) for 1 h on a rocking platform. One hundred microliters of hybridoma cell supernatant containing MAb Y1D2 are then added to the blocking solution and incubated further for 5–6 h. The membrane is washed with three changes of TPBS over 30 min, transferred into 10 mL blocking solution containing 2 µL horseradish peroxidase-labeled antibody specific for mouse IgG+IgM (H+L) (Kirkegaard & Perry Laboratories; Gaithersburg, MD, USA). The membrane is incubated in this solution for 2 h. The membrane is then washed as described above and immersed into substrate for 1 min. Substrate is prepared by mixing equal volumes of chemiluminescent substrate A and B from LumiGlo™ Substrate kit (Kirkegaard & Perry). This solution is stable for up to 1 h at room temperature and does not need to be protected from light (4). The membrane is removed from substrate without excess liquid and laid flat between two sheets of plastic wrap. The surface of the membrane to which the protein was applied is exposed to the X-ray film (Kodak X-OMAT™ AR5; Eastman Kodak, Rochester, NY, USA) for 10–20 s and developed in an X-ray film autodispenser (Figure 1). This film is saved to measure the PEP protein signal. The probing steps described above are repeated with hybridoma cell supernatant containing MAb 4G3, and another film (Figure 1) is obtained to measure the SNF/D25 protein signal. The films are scanned on a 9195A ScanJet™ Plus Scanner (Hewlett-Packard, Palo Alto, CA, USA) set for “high OD” (high optical density), “medium range” and “calibrated scanning”. The PEP and SNF/D25 band signals are analyzed with the 2D Gel Program in the MicroScan 1000 Gel Analyzer (Technology

![Figure 1. Enhanced chemiluminescence Western blot of Drosophila RNA-binding proteins.](image-url)
Resource, Nashville, TN, USA), using the trace background parameter in the program.  

By analyzing the films, regression analyses were performed to establish the correlation between the protein signals on the films and the amount of total protein in nuclear extract loaded on the gel. The signals were plotted individually against the amount of total protein loaded on the gel (see Figure 2). The SNF/D25 signal (y) is related to the amount (x) of total protein in a lane by the linear equation \( y = 0.66263x + 0.233261, r^2 = 0.99056 \) (Figure 2, panel A). A different situation was found for the PEP protein. The linear equation for PEP is \( y = 0.73541x + 0.16703, r^2 = 0.95597 \) (Figure 2, panel B). However, a slightly better coefficient for the PEP signal was obtained using the logarithmic equation \( y = 12.37038\log(x)-3.219, r^2 = 0.98679 \) (Figure 2, panel C). Therefore, for each protein, the relationship between the amount of total protein and the antigen signal is specific and must be determined empirically. We speculate that the affinities of MAb Y1D2 and MAb 4G3 toward their correspondent proteins are different. 

Despite these differing correlations, the relative amounts of PEP and SNF/D25 proteins can be estimated with confidence. If the ratio of the two signals is plotted against the amount of total protein, a nearly linear graph \( r^2 = 0.91112 \) is still obtained when the total protein is within the range of 1.8–9.0 \( \mu \text{g} \), but above 9.0 \( \mu \text{g} \), the graph reaches a plateau. Since the graphs for PEP and SNF/D25 proteins can be considered parallel over this interval, either antigen provides an appropriate loading control for estimating the relative amounts of the other. 

This technique may be useful in other studies requiring quantification or an estimation of the antigen level. For example, in a transfection system, the expression level of the transfected gene can be determined by comparing the amount of its protein product to that of an endogenous protein. Or, the expression levels of downstream genes can be elucidated numerically and correlated to the expression level of the transfected gene, given the availability of appropriate antibodies. Moreover, we have found that the enhanced chemiluminescence Western blotting is sensitive to the level of PEP protein in single Drosophila larvae. Determining the useful range for comparison is the critical step for this kind of estimation and must be derived for each pair of antibodies. 

REFERENCES


4. Kirkegaard & Perry Laboratories. 1995. LumiGLO™ Substrate Kit for Chemiluminescent Detection of Horseradish Peroxidase-Reagents 2 × 120 mL Kit, Catalog No. 54-16-00, 6 × 120 mL Kit, Catalog No. 54-16-01 Manufacturer’s Instructions, Gaithersburg, MD. 


Benchmarks

Cold Spring Harbor, NY.


We thank Kenneth Sorensen and Carl Urbinati for comments on the manuscript. This work was supported by the Potts Foundation. Address correspondence to Sally A. Amero, Department of Molecular and Cellular Biochemistry, Loyola University Chicago, 2160 South First Avenue, Maywood, IL 60153, USA. Internet: samero@luc.edu

Received 29 May 1996; accepted 19 August 1996.

Donghui Huang and Sally A. Amero
Loyola University Chicago
Maywood, IL, USA

Reusing Hybridization Mixtures

BioTechniques 22:458-462 (March 1997)

Presently, I am screening plasmid libraries by colony blot hybridization at a stringency that was calculated to permit up to about 35% average mismatch between the probe and the target sequences (1). During the sequential rounds of hybridization required first to identify positive clones in high-density screening plates and then to select well-isolated positive single colonies on low-density plates, I use identical hybridization conditions. Therefore, considerable savings in time, materials and the generated amount of high-level radioactive waste would be possible if the hybridization solution, containing the $^{32}$P-labeled probe, could be saved and then reused in subsequent rounds.

The following statement appears in Sambrook et al. (Reference 3; page 1.103): “hybridization mixtures containing complementary strands of DNA should be discarded since there is no satisfactory way to denature the double-stranded DNA that forms during the first round of hybridization”. In an attempt to reduce the amount of probe needed to perform several consecutive rounds of hybridization, I have developed an effective way to reuse hybridization mixtures. The method maintains optimal signal strength (i.e., there is no detectable decrease in signal strength beyond that expected from radioisotope decay) and is convenient to perform. The method has allowed a single hybridization mixture to be reused five times over an 18-day period without compromising the quality of the results obtained. The conditions employed at each step are described below. DNA restriction fragments to be used as hybridization probes were recovered