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Detection of Glu-Glu-Tagged Proteins in Mammalian Cell Culture Media by Dot Immunoblotting

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ABSTRACT

A dot immunoblotting technique has been developed to estimate the relative expression levels of tagged recombinant human proteins in mammalian cell culture media. Variations in sample denaturation, blocking agents and membrane composition and treatment were used to optimize the signal-to-noise ratio of the defined procedure. The method is rapid, with sensitivity extending to the low nanomolar range for a number of recombinant proteins. This technique should have general utility for antibody-based measurements of other tagged and non-tagged proteins in cell culture media or in biological fluids.

INTRODUCTION

Screening of mammalian cell culture supernatants for the expression of an exogenous protein is required at virtually all stages in the production of recombinant proteins. Optimization for transfection efficiency, selection of clones and product amplification depend on the rapid identification of cultures expressing the desired level of protein. Techniques that are commonly used to assess protein expression include separation of a protein mixture by SDS-PAGE, followed by staining with a variety of protein stains such as Coomassie Blue® or silver, SDS-PAGE-Western blotting or ELISAs using protein-specific antibodies (1,3). The relative advantages and disadvantages of each of these techniques depend on the speed and sensitivity required, the presence of interfering substances in the culture media and the availability of protein-specific antibodies.

As part of a strategy for the high-throughput production of many novel

recombinant proteins, it was necessary to develop a fast, sensitive method for measuring the relative levels of protein expression in mammalian cell cultures. Here, we report on the development of a dot immunoblotting procedure used to estimate the concentration of N- or C-terminal Glu-Glu-tagged proteins in cell culture media.

MATERIALS AND METHODS

Immobilon™-P, -NC and -PSQ transfer membranes were obtained from Millipore (Bedford, MA, USA). The Mini-fold® I dot blot system was purchased from Schleicher & Schuell (Keene, NH, USA). Murine antibodies to the Glu-Glu peptide, EYMPME, were obtained from Covance (Madison, WI, USA), and HRP-conjugated goat anti-mouse IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). West Dura Extended Duration® substrate and Super Signal Ultra® for ECL™ were obtained from Pierce Chemical (Rockford, IL, USA); Hyperfilm™ ECL and Hoeffler TE22 blotting apparatus were both purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Tris-Glycine 4%–20% polyacrylamide gradient gels and sample buffer were obtained from Novex (San Diego, CA, USA). Supported nitrocellulose paper (0.2 µm) was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Water was prepared by Milli-Q® filtration (Millipore).

Dot Immunoblotting of Mammalian Cell Culture Conditioned Media

A template of twofold serial dilutions of serum-free conditioned media from transfected BHK cells was prepared in a deep 96-well microplate by initially transferring 285 µL conditioned cell culture media into a well containing 15 µL 20× denaturing buffer [Buffer D, 200 mM Tris-HCL (pH 7.4), 1 M NaCl, 0.1 M β-mercaptoethanol and 0.08% Triton® X-100]. A standard curve, starting at 75 ng standard Glu-Glu tagged protein, was set up in the same manner in a separate row of wells

on each plate. All serial dilutions were prepared in a final concentration of 1× Buffer D. The plates were covered and incubated at 65°C for 10 min. The dot blot apparatus was assembled according to the manufacturer's instructions and the Immobilon-P transfer membrane was prewetted in 100% methanol for 15 s and rinsed in water for 2 min. No filter paper backing was used. When other transfer membranes were tested, they were pretreated according the manufacturer's instructions. The membrane was placed on the dot blot apparatus while still wet, and each well was loaded under vacuum with 100 µL from the appropriate well of the sample template.

After the media was vacuum-filtered through each well, the wells were rinsed twice with 100 µL Buffer W [10 mM Tris-HCl (pH 7.4) and 50 mM NaCl]. When the rinses were complete, the vacuum was turned off and the membrane was placed in 50 mL 1× Buffer D and incubated at 65°C for 10 min. Each membrane was then rinsed with water, placed between two sheets of Whatman® 3M filter paper and dried in a vacuum oven at 100°C for 30 min. After drying, the membrane was rinsed with methanol for 15 s and then with water for 2 min.

The rinsed membrane was then immediately placed in 100 mL Buffer B (Buffer W containing 0.05% Tween® 20 and 0.5% nonfat dry milk) and gently agitated on an orbital shaker for 30 min at room temperature. Each membrane was quickly rinsed with Buffer T (Buffer W containing 0.05% Tween 20); fresh Buffer B was added and each membrane was incubated with 0.1 µg anti Glu-Glu antibody/mL for 1 h at room temperature or overnight at 4°C. The antibody-containing solution was discarded, and each membrane was washed 3× for 10 min with Buffer T. After a quick rinse with Buffer W, the membranes were incubated with 0.1 mg/mL HRP-conjugated goat anti-mouse IgG in Buffer B for 1 h at room temperature with gentle shaking. Each membrane was then washed 3× for 10 min each with Buffer T and quickly rinsed sequentially with Buffer W and water. The membranes were then placed in a sheet protector and incubated with the ECL substrate according to the manufacturer's instructions and

placed on a sheet of Kodak XAR-5™ film (Eastman Kodak, Rochester, NY, USA) for development by autoradiography, or the membranes were placed directly in a Lumi Imager™ (Roche Molecular Biochemicals, Indianapolis, IN, USA) for quantitation.

SDS-PAGE and Western Blotting

Samples of BHK conditioned media were prepared for electrophoresis by mixing them with an equal volume of 2× Tris-glycine sample buffer containing 0.2 M dithiothreitol. All samples were heated at 100°C for 5 min immediately before loading the gels. Electrophoresis and transfer of proteins to nitrocellulose membranes were conducted according to the manufacturer's instructions. The transfer buffer used contained 25 mM Tris, 0.2 M glycine and 20% methanol. Following transfer at 500 mA for 1 h or 50 mA for 18 h,

the membranes were blocked for 10 min at room temperature with Western A buffer [50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.05% Igepal CA-630, 150 mM NaCl and 0.25% gelatin] containing 10% nonfat dry milk. The membranes were quickly rinsed with Western A buffer and incubated with 0.1 µg anti-Glu-Glu antibody/mL of Western A buffer containing 2.5% nonfat dry

milk. After gentle agitation for 1 h at room temperature or overnight at 4°C, the membranes were washed 3× with Western A buffer for 10 min each. Fresh Western A buffer containing 2.5% nonfat dry milk and 0.2 µg/mL HRP-conjugated goat antimouse IgG were added, and the membranes were incubated for 1 h at room temperature with gentle agitation. The membranes

Figure 1. Dot immunoblotting of BHK conditioned media for detection of recombinant Glu-Glu-tagged protein. Samples of BHK conditioned media were spiked at 111 nM with one of four purified Glu-Glu-tagged proteins (Lanes 1–4). Serial dilutions (Rows A–H) were then made into BHK conditioned media and the immunoblotting was carried out as described in the Materials and Methods section. The apparent molecular weights of the proteins were zp60 CEE, 7000 Da; z11 NEE, 18000 Da; zobNEE, 17000 Da; and zc8NEE, 8400 Da.

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were then washed 3× for 10 min each in Western A buffer, followed by a quick rinse in water. Next, the membranes were visualized with Super Signal Ultra according to the manufacturer's instructions and quantitated using the Lumi Imager as described before. Statistical analysis was performed using the software package Stata.

RESULTS AND DISCUSSION

Some preliminary experiments were undertaken to examine the effects of sample denaturation, membrane type and treatment and the effects of various blocking agents on the signal-to-noise ratio of the dot immunoblotting procedure. Each parameter was tested with a number ($n = 4-6$) of different purified Glu-Glu-tagged proteins that were spiked at 1–100 nM concentrations into samples of BHK conditioned media.

Sample Denaturation

Although most Glu-Glu-tagged proteins we analyzed could be adequately detected without prior reduction and denaturation, the dot blot signal intensity for most of these proteins was increased twofold to threefold by prior incubation at 65°C for 10 min in the presence of 5 mM β -mercaptoethanol. Inclusion of 0.002%–0.004% SDS or Triton X-100 in the denaturation buffer produced a further two- to threefold increase in signal intensity. These data are consistent with the notion that the access of the anti-tag antibody to the Glu-Glu tag is restricted in the native state of these recombinant proteins. On the other hand, concentrations of SDS above 0.004% and of Triton X-100 above 0.016% completely prevented detection by immunoblotting, which suggests that protein binding to the membranes was inhibited or the Glu-Glu tag was rendered

inaccessible to antibody under these conditions. Since the useful concentration range with Triton X-100 was somewhat broader than with SDS, we have used the former detergent for the remainder of our experiments.

Membrane Composition and Treatment

Compared to Immobilon-NC or -PSQ membranes, the Immobilon-P membranes that were prepared as described earlier produced the highest signal-to-noise ratio. The most consistent results were obtained with prewetted membranes assembled in the dot blot apparatus without paper backing. Of the three membranes tested, Immobilon-PSQ appeared to have the highest level of nonspecific background staining. The dot blot signal intensity for most of the proteins analyzed was also increased by a post-blotting incubation

Figure 2. Comparison of dot immunoblotting with SDS-PAGE Western blot analysis for estimating the levels of Glu-Glu-tagged protein in BHK conditioned media. Duplicate samples of conditioned media from 51 different BHK clones representing six different recombinant Glu-Glu-tagged proteins (4–12 different clones for each protein) were taken for analysis by dot or Western blotting as described. Each point represents a concentration determined from serial dilution of the culture medium from a single clone of BHK cells.

at 65°C for 10 min in the presence of 0.004% Triton X-100 and 5 mM β -mercaptoethanol. No further increase was observed by increasing the temperature to 100°C. Compared to drying the membrane at room temperature or drying at room temperature in a vacuum dessicator, a further 40%–50% increase in signal intensity was observed by drying the membranes at 100°C for 30 min in a vacuum oven.

Blocking Agents

A number of agents are reported to be effective in reducing nonspecific binding of antibodies to membranes in immunoblotting experiments (1–3). When compared to blocking in 0.5% nonfat dried milk, blocking with 1% PVP-40 was inconsistent with a high level of background antibody staining that is often observed. In contrast, nonfat dried milk at 0.5%, 2.5% or 10%

(wt/vol) produced dot blots that were consistently interpretable, with low background staining. Because suitable results were obtained with 0.5% nonfat dried milk, this amount was selected as our standard blocking concentration.

Sensitivity and Comparison with Detection by Western Blotting

The sensitivity of the dot blot procedure was assessed using a dilution series (0.9–111 nM) of four purified Glu-Glu-tagged proteins in BHK conditioned media. These proteins covered a range of sizes from 7–18 kDa. Each of the four proteins tested could be reliably detected down to the low nanomolar level (Figure 1). To evaluate how the dot blot procedure compared with SDS-PAGE and Western blotting for detection of recombinant proteins in cell culture media, duplicate samples of conditioned media containing one of

six different Glu-Glu-tagged recombinant proteins (representing 51 different BHK clones) were taken for analysis (Figure 2). The relative concentrations of Glu-Glu-tagged proteins in the conditioned media measured by the two procedures was highly correlated ($r = 0.834$, $P < 0.0001$).

In summary, the outlined procedure provides a rapid, sensitive method for measuring the levels of recombinant Glu-Glu-tagged protein secreted into the culture media of mammalian cells. We expect this procedure will be useful for antibody-based measurement of other tagged and non-tagged recombinant proteins in cell culture media or biological fluids.

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