Carrier-Ampholine 2D Electrophoresis is useful for analysis of Host Cell Protein Antibody Coverage

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Abstract

ELISA testing using anti-HCP (host cell protein) antibodies remains the work-horse for detecting host cell protein in recombinant drug substances. [1] ELISA antibodies (ab) are generated against numerous host cell proteins by different B cell lineages within an animal. Each antibody must be tested to ensure broad reactivity with many proteins. A narrow antibody might give sensitive, linear test results for a few strongly antigenic proteins, but miss the majority of HCP contaminants. Carrier ampholine 2D gel western blotting (CA-2D) in combination with sensitive silver staining may be used to determine ELISA antibody coverage. Since CA-2D is compatible with sodium dodecyl sulfate (SDS), sample preparation is straightforward. Reproducible patterns are obtained. We have developed a computer comparison protocol to match between complex western blot and silver-stain HCP patterns via an intermediate image from the Coomassie-stained PVDF membrane. SameSpots software from TotalLabs is used to compare patterns and tabulate results.

Introduction

Two-dimensional electrophoresis (2DE) separates protein mixtures first by charge using isoelectric focusing (IEF), then by size using SDS slab gel electrophoresis. The resulting starburst pattern of protein spots is visualized by staining or western blotting. *Two* 2DE methods are now in use which differ in IEF protocol.

1. IPG-2D is the most common 2D protocol. [2] In this method, the IEF pH gradient is immobilized on precast commercially available strips. The strips are easy to use, but incompatible with the detergent SDS, by far the best reagent for dissolving proteins They are also incompatible with salt concentrations above 10 mM. Sample preparation is often complex since urea and Triton-X only partially dissolve membrane proteins. Undissolved protein, dismissed as “cell debris”, is sometimes removed by centrifugation and discarded!

2. CA-2D using carrier ampholines in polyacrylamide tube gels, the classic method, is uncommon but compatible with SDS. [3-5] After loading, voltage is applied across the tube gels, causing the pH gradient to form, and sample proteins to migrate to a steady-state position. Initially, CA-2D had problems. Pouring tube gels was tricky. Tube gel elasticity caused pattern variability that 1990’s primitive software could not handle.

CA-2D problems are now solvable. The method may be standardized through use of written SOPs. New sophisticated software allows pattern alignment and analysis of multiple images. Evidence will be provided below that the method, in combination with western blotting and silver staining may be used to assess coverage of ELISA antibodies for host cell proteins.

Materials and Methods

E. coli cultured cells, strain K12 MG1665 was a gift from Scarab Genomics (Madison, WI). The anti-*E.coli* HCP antibody (AP117) was purchased from Cygnus Technologies (Southport, NC). PVDF (Immmobilon P, 0.45 µm) was purchased from Millipore (Billerica, MA). Ultrapure Acrylamide stock solution was purchased from National Diagnostics (Charlotte, NC). Isodalt Servalytes (pH 3-10) were purchased from

Serva (Heidelberg, Germany). All other electrophoresis reagents were purchased from approved vendors that were either ISO certified or had completed a Supplier Quality Questionnaire.

Sample Preparation: The E. coli pellet was lysed in SDS Boiling Buffer without reducing agents diluted 1:1 Osmotic Lysis Buffer containing nucleases, protease inhibitors, and phosphatase inhibitors. The sample was sonicated for 5 min, heated in a boiling water bath for 5 min, and treated with Omnicleave endonuclease (Epicentre, Madison, WI) to break down polynucleotides. A protein determination was performed using the BCA assay (Smith et.al. Anal. Biochem. 150:76-85, and Pierce Chemical Co., Rockford, IL) The sample was then diluted to 2.5 and 0.25 mg/ml in 1:1 SDS boiling buffer: Urea sample buffer before loading 25 µg (silver stain) and 250 µg (western blot). Buffer composition is provided on this link www.kendricklabs.com/preparation.htm

Two-dimensional electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (O'Farrell, P.H., J. Biol. Chem. 250: 4007-4021, 1975; Burgess-Cassler, A., Johansen, J., Santek, D., Ide J., and Kendrick N., Clin. Chem. 35: 2297, 1989) by Kendrick Labs, Inc. (Madison, WI). Isoelectric focusing was carried out in a glass tube of inner diameter 3.3 mm using 2.0% pH 3-10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 20,000 volt-hrs. One µg (PVDF) or 100 ng (silver) of an IEF internal standard, tropomyosin, was added to each sample. This protein migrates as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2; an arrow on the stained gels marks its position. The tube gel pH gradient plot for each set of Servalytes was determined with a surface pH electrode.

After equilibration for 10 min in buffer "O" (10% glycerol, 50mM dithiothreitol, 2.3% SDS and 0.0625 M tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (1.0 mm thick). SDS slab gel electrophoresis was carried out for about 5 hrs at 25 mA/gel. The following proteins (Sigma Chemical Co., St. Louis, MO; Millipore, Billerica, MA) were used as molecular weight standards: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000) and lysozyme (14,000). These standards appear as bands at the basic edge of the silver-stained (Oakley, B.R. et. al. *Anal. Biochem. 105:*361-363, 1980) 10% acrylamide slab gels. The gels were dried between sheets of cellophane paper with the acid edge to the left.

Western Blotting: After slab gel electrophoresis, the 2D gel for blotting was placed in transfer buffer (10 mM CAPS, pH 11.0, 10% methanol) and transblotted onto PVDF membranes overnight at 225 mA and approximately 100 volts/ two gels. The blot was stained with Coomassie Brilliant Blue R-250, desktop scanned, and then blocked for two hours in 5% Carnation Nonfat Dry Milk (NFDM) in Tween-20 tris buffer saline (TTBS), and rinsed in TTBS. The blot was then incubated in primary antibody (Goat anti-*E. coli* [Cygnus Cat. # AP117 and Lot # 99] diluted to 0.5 µg/ml in 2% NFDM TTBS) overnight and rinsed 3 x 10 minutes in TTBS. The blot was then placed in secondary antibody (Anti-Goat IgG-HRP [Sigma Cat. # A5420 and Lot # 090M4822] 1:10,000 diluted in 2% NFDM) for two hours, rinsed in TTBS as above, treated with ECL, and exposed to x-ray film (1 minute, 3 minute, and 10 minute exposures). Using X-ray film provides a higher throughput than a chemiluminescent imager when sets of blots are analyzed.

Computerized Comparisons: Western blot films (1 minute and 3 minute exposures) and duplicate silver-stained gels were obtained from the sample and scanned with a laser densitometer (Model PDSI, Molecular Dynamics Inc, Sunnyvale, CA). The scanner was checked for linearity prior to scanning with an NIST-calibrated Neutral Density Filter Set (Melles Griot, Irvine, CA). The images were analyzed using Progenesis Same Spots software (version 4.5, 2011) and Progenesis PG240 software (version 2006, TotalLab, Newcastle upon Tyne, United Kingdom). The general method of computerized analysis for these pairs included image warping in conjunction with detailed manual checking. The lighter exposure of the western blot films was used to aid in spot matching for the overexposed areas of the darker exposure. Spots detected with the antibody were added to the master spot set even if not detectable by silver staining.

RESULTS

SDS Compatibility: *How can negatively charged SDS be used to dissolve proteins prior to IEF where a single charge change is detectable?* SDS denatures proteins and binds to the peptide backbone as shown schematically in Figure 1. Leigh and Norman Anderson showed that during isoelectric focusing in tube gels, SDS is stripped off proteins to make micelles with IGEPAL a non-ionic detergent (previously NP-40). The charged micelles migrate to the acid end of the tube gel where they form a bulb that is discarded. [3] We cannot be certain that SDS is stripped off all proteins all of the time. However, samples loaded with 2.5% SDS and up to 100 mM NaCl give quite reproducible patterns.

Further evidence for SDS compatibility is provided in Figure 2 which shows a protein standard, carbamylated creatine phosphokinase (CPK), that has been deliberately boiled with urea to produce multiple charge isoforms. Carb-CPK dissolved in SDS buffer gives essentially the same 2D pattern as that dissolved in urea buffer, confirming that the SDS has been stripped off.

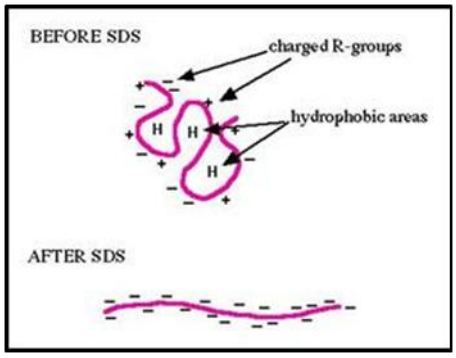

Figure 1. SDS binding mechanism. When proteins are heated in the presence of SDS and β-mercaptoethanol (BME), SDS binds to the peptide backbone and imparts a uniform charge/mass ratio. BME reduces disulfide bridges. All secondary and tertiary structure is lost.

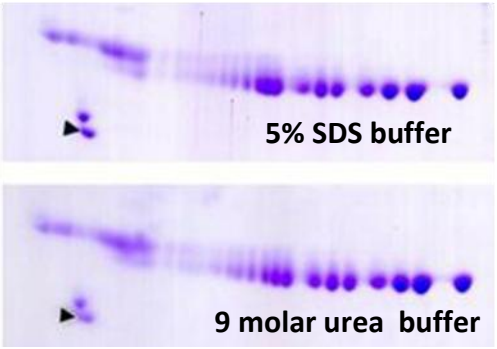

Figure 2. Example: Carbamylated CPK Standard dissolved in either SDS (top) or 9M urea (bottom) buffer shows the same 2D pattern. The arrow marks the lower isoform of tropomyosin, pI marker of MW 33 kDa and pI 5.2.

Our website provides eight additional CA-2D gel examples showing SDS compatibility. www.kendricklabs.com/membranes&vesicles.htm The examples are rat liver cytosol, *E. coli* whole cell preparation, CHO outer membrane, rat liver microsomes, Fusobacteria outer membrane, red blood cell ghosts, mouse brain synaptosomes and hepatocyte inner membrane.

A list of over 280 publications that cite Kendrick Labs, Inc or show are gels is also provided on our website www.kendricklabs.com/References-clients.htm Samples prepared in SDS were run on CA-2D gels for virtually all of these references.

Western-Silver Alignment.

Overview: Total proteins in the HCP sample are visualized by silver staining 2D gels. Proteins reacting with the antibody are visualized by western blotting (WB). The two patterns are matched to determine antibody coverage (percent of total protein spots detected by the antibody.) Matching corresponding spots on the two patterns is accomplished with SameSpots software from TotalLab. The process is made possible by Coomassie blue (CB) staining the PVDF membrane before WB.

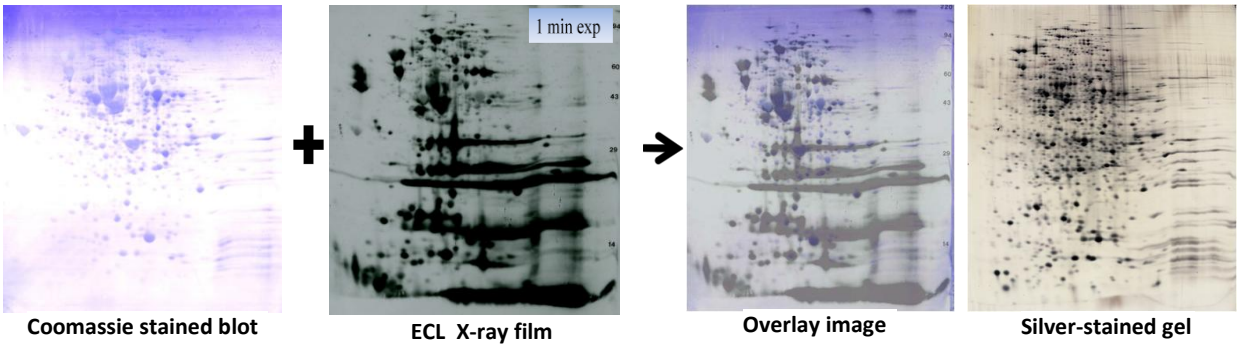

Figure 3. Alignment images for *E. coli* HCP. Duplicate 2D gels are loaded with 25 µg HCP lysate for silver staining. A third gel is loaded with 250 µg and transferred to PVDF for western blotting. Before WB, the PVDF membrane is stained with Coomassie blue (CB) to create a Rosetta stone image that matches both silver and film. After scanning, the PVDF is destained, and incubated with the Cygnus anti-HCP antibody. Multiple ECL film exposures are generated to maximize sensitivity. Over-laying the WB film image over the CB PVDF image allows exact matching.

Matched spots become warp vector sites for SameSpots software. Analysts carefully align images, propagate spot outlines to all, collect data, and create final reports.

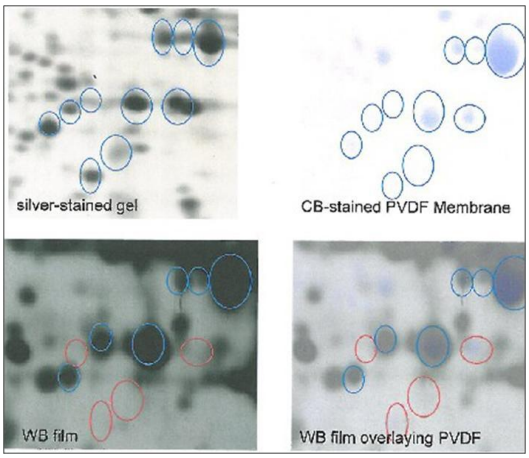

Figure 4. Magnified view of silver, CB PVDF, film and overlay. Top: Matches between silver & blot. Bottom: Matches between film and blot. Spots detected on the WB film are outlined in blue. Spots detected by silver stain but not by antibody are outlined in red.

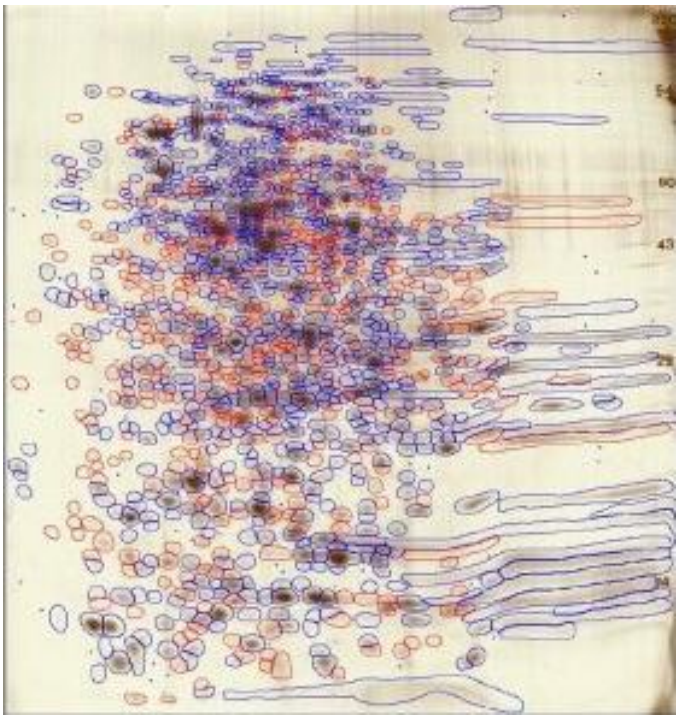

Figure 5. Final Result: 993/1400 total spots are detected by the antibody = 71% coverage. Blue outlines show spots present on both silver and film; red shows spots only in silver. Small blue dots show spots detected only by western blotting and added to the total.

Reproducibility

Assay reproducibility: In two instances *E. coli* HCP antibody analyses were performed in triplicate on the same sample i.e. 1 run/day on 3 different days, with each run analyzed by a different 2D Analyst. The CV (SD/Mean*100, n = 3) for % coverage was 8.1% in one case and 5.1% in the other.

Discussion

Genome sequencing technology has advanced at incredible speed over the past ten years, to a point that sequencing of human genomes is now available for ~$1000 each. [6] This fosters an expectation that advances in protein analysis too will soon be fast and automatable. Yet protein technology has not caught up. What's going on?

There are only four chemically distinct nucleotides making up DNA strands: adenine, thymine, cytosine and guanine. Human DNA is constant in all cells and easy to amplify. These facts make DNA analysis straightforward and automatable. In contrast, there are ~20 chemically distinct amino acids making up each of the > 20,000 proteins corresponding to protein coding genes. Alternative splicing adds an unknown number of protein variants to the mix. [7] Furthermore, post-translational modifications (PTM) of proteins such as glycosylation, phosphorylation, and acetylation complicate analyses. PTMs are widespread in mammals, transient in nature, and very important; they cannot be ignored. [8] Furthermore, proteins exist in cells as dynamic complex mixtures. Before analyses can be automated, it is important to have a gold standard showing what is actually there.

Proteins vary greatly in solubility characteristics depending on amino acid sequence. One substance, SDS, has emerged as the best reagent by far for dissolving proteins. [9] Methods which are incompatible with SDS, including IPG-2D and in some cases, mass spectrometry, are at a disadvantage when analyzing complex mixtures because a subset of proteins will be missed. In our opinion, no single method exists that suffices to give gold standard analysis of complex protein mixtures. Rather, using 2 or more orthogonal methods to verify conclusions is the path to take. CA-2D is one such method to add to the arsenal of SDS PAGE, IPG-2D, mass spectrometry and high affinity antibodies (ELISA assay, western blot).

Conclusions: CA-2D in combination with western blotting is a useful way to assess antibody reactivity with complex HCP protein samples. SDS compatibility is an jadvantage because sample preparation is simplified. This method is orthogonal to mass spectrometry in that it detects whole proteins instead of peptides. Although time-consuming and labor intensive, CA-2D remains a powerful method to resolve protein mixtures.

References

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