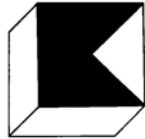


**Over:** Rethinking phosphorylation site identification by mass spectrometry



# Kendrick Labs Update

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## 2D gel Western blotting using anti-Ubiquitin antibody from Bethyl Labs

Using 2D gels to compare the top ~1200 proteins in cell lysates for differences is often fruitful, but the worry is that low abundance proteins are missed. One way to increase sensitivity by  $\geq 100$ -fold while reducing complexity is 2D gel Western blotting using antibodies against post-translational modifications. Over the past two years we've had good luck with phosphoserine, phosphothreonine, and phosphotyrosine WB. Recently, Matt Hoelter, Biochemist, has been optimizing anti-Ubiquitin and anti-SUMO WB with good success.

**anti-Ubiquitin WB:** The ubiquitin-proteasome system functions universally in eukaryotes to regulate protein levels by rapid breakdown (Figure 1.) Ubiquitin, an 8.5 kDa protein, is highly conserved between species; the human genome contains over 500 ubiquitin E3 ligases that target individual proteins. So this system is *important*. Polyubiquitylation, for example, is responsible for the fast turnover of P53 (20 min half-life). Mono-ubiquitylation and SUMOylation have vastly different consequences such as causing the protein to relocate within the cell.

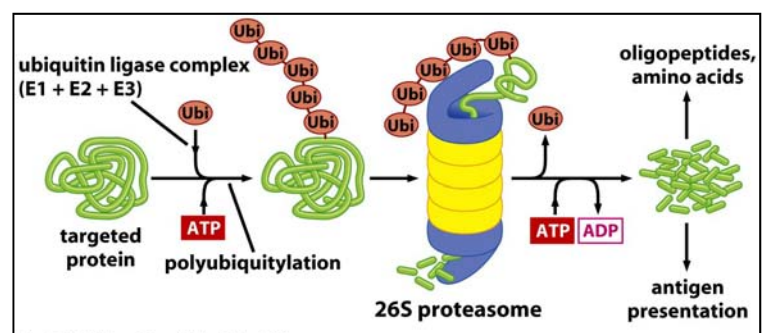


Figure 1. The ubiquitin-proteasome system. Taken from R Weinberg Biology of Cancer, Garland Science 2007, p242

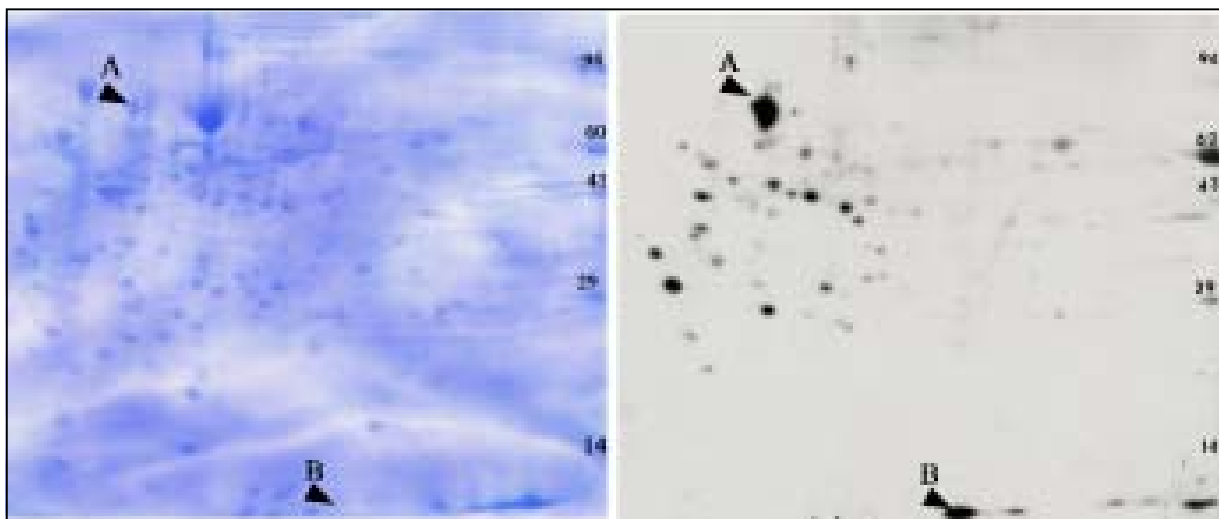


Figure 2. Left shows a Coomassie stained 2D PVDF blot from rat liver cytosol diluted with SDS/Urea buffer. Even though the background is splotchy, the 2D pattern is visible. We routinely stain blots and color-scan them to facilitate matching to a duplicate Coomassie gel for spot cutting for mass spectrometry. Right: ECL film obtained after overnight incubation of the left-hand blot with anti-ubiquitin antibody from Bethyl Labs diluted 1:2000. The film and blot are superimposable. The proteins lighting up on the film don't match abundant proteins in the Coomassie pattern (arrows) suggesting good specificity.

We're ready to try the Bethyl Labs antibody with experimental samples! Submit your samples now and get anti-ubiquitin WB for \$300/package including 2D gel, transblotting, immuno-staining with Bethyl Labs anti-ubiquitin ab, duplicate Coomassie-stained gel for MS and electronic photos of blot, gel and ECL film. As always, we'll do fast, free repeats if something goes awry. Protein identification by mass spectrometry would be extra.

## Rethinking phosphorylation site identification by mass spectrometry (MS)

A few years ago our lab group spent considerable time optimizing 2D Western blotting for phosphoproteins (P-Tyr, P-Ser and P-Thr) as a tool to identify novel kinase substrates. See [www.kendricklabs.com](http://www.kendricklabs.com) for more information. Subsequently, clients have had good success with the Western blotting (WB) and also with protein identification of corresponding spots cut from Coomassie blue stained duplicate 2D gels. However, taking it one step further to identify phosphorylation sites by MS has failed in almost every instance. Note that we send samples out for MS. From 2005 thru 2007 we worked with 3 separate core facilities that all gave disappointing results. Our 2008-9 collaborator, Dr. John Leszyk at the University of Massachusetts Core Facility, has successfully identified phosphorylation sites for four projects in 2008. But he's having difficulty with *our* phosphorylated proteins.

*Why is this not working?* We turned to the literature for answers. New method papers imply that identification of phosphorylation sites is straightforward using direct MS. Matthias Mann's group studied the "phosphoproteome" of mouse melanoma tumors (1). They homogenized tumor tissue, digested with trypsin and Lys-C, performed phosphopeptide enrichment using SCX chromatography /TiO<sub>2</sub> beads and finally LC-MS. Results: A total of 4443 proteins were identified along with 5600 phosphorylation sites on 2250 proteins. Schreiber et al in a recent review of quantitative phosphoproteomics (2) states "Improved isotope labeling and phosphopeptide enrichment strategies in conjunction with more powerful mass spectrometers and advanced data analysis have been integrated in highly efficient phosphoproteomics workflows, which are capable of monitoring up to several thousand site-specific phosphorylation events within one large-scale analysis." But we're not sure how this qualitative general approach can be applied to real-life problems. There are no sample comparisons, for example. The "phosphoproteome" is known to vary with cell type and stimuli. So we looked for *specific cases* of phosphosite identification.

We used the Highwire search engine (<http://highwire.stanford.edu/cgi/search>) to search for papers specifically describing MS analysis of phosphorylation sites. For the short period January 2009, 156 papers were found containing the phrase "phosphorylation site". Interestingly, many of these studies did not involve MS but rather used immunoprecipitation (IP), WB, and various molecular biology techniques (especially transfection with mutant alleles) to study phosphorylation (eg 3-5). The papers that did use MS analyzed dark gel bands from IPs. For example, Wegierski et al identified PTyr sites by transfecting flag-tagged TRPV4 gene into 2 cell lines, purifying the recombinant protein by IP before doing MS from a Coomassie-stained SDS gel band (6). The Burlingame Lab in UCSF analyzed p53 phosphorylation sites after IP out of cell extracts (10<sup>8</sup> cells) followed by SDS PAGE with Coomassie staining (7). Going back a couple of years, Lee et al found phosphorylation sites on cytochrome oxidase subunit I by starting with COX protein extracted from mitochondria from 250 grams of cow heart and resolved by SDS PAGE & Coomassie (8).

**Conclusions:** We have greatly underestimated the amount of material that's needed to determine amino acid phosphorylation sites. Enough protein to give a *dark* Coomassie blue stained 2D spot, 1-5 ug protein in the phosphorylated isoform, is required. The protein of interest must be known and the sequence in the databases. One way to obtain enough material is by generating flag-tagged recombinant protein and purifying by immunoprecipitation with a commercially available anti-flag affinity resin. Dr. Leszyk agrees and notes that the problem with WB is that it's capable of detecting protein amounts far below the limit of detection of the mass spectrometer.

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