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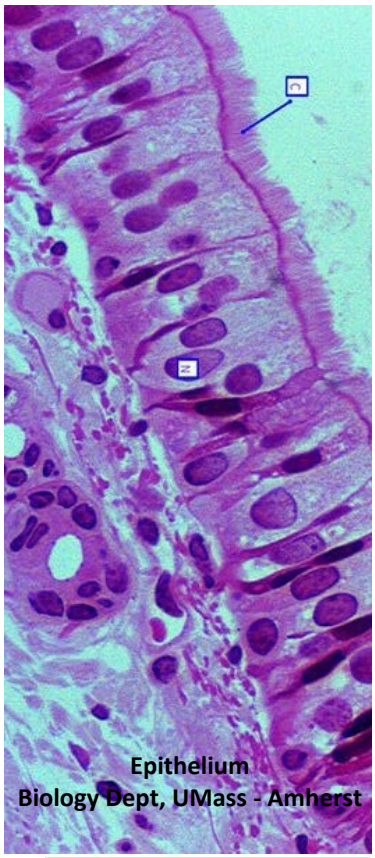
Kendrick Labs Update

Kendrick Labs, Inc. is a Proteomics Contract Research Organization specializing in two-dimensional gel electrophoresis (2DE).


Epithelium
Biology Dept, UMass - Amherst

In this first issue of a new series, we'll start with an overview of 2DE services currently performed at Kendrick Labs.

2DE is a biochemical method for separating complex mixtures of proteins into individual species. Proteins are first separated by charge using isoelectric focusing (IEF), then by size using SDS slab gel electrophoresis. The starburst pattern of protein spots is visualized on the final 2D slab gel by staining or Western blotting. Proteins of interest may be identified by mass spectrometry.

Two variations of 2DE are currently in use world-wide that differ in IEF protocol. The most common method by far, which we'll call IPG-2DE here, uses immobilized pH gradient strips for IEF. The classic method, carried out at Kendrick Labs, uses carrier ampholines polymerized in acryamide tube gels for IEF (CA-2DE).

IPG-2DE: In this method, the IEF pH gradient is immobilized on commercially available solid supports called IPG strips. For a review see Angelika Gorg et. al. [1]. Although the strips are easier to deal with than tube gels, they are incompatible with the detergent SDS, by far the best reagent for dissolving proteins [2]. Sample preparation for strips utilizes nonionic detergents and urea that poorly solubulize many proteins. Centrifugation to remove particulates before loading adds to variability. Loading by strip rehydration with sample is not quantitative [3-4]. To compensate, Differential In Gel Electrophoresis (DIGE, 5-6] is used in which samples are pre-labeled with Cy dyes and combined before IEF.

Inside this issue

CA-2DE, originally developed by O'Farrell [7], was refined at many facilities in the following years including Kendrick Labs [8]. In this method, acrylamide tube gels are polymerized with carrier ampholines that form a pH gradient when voltage is applied. During IEF the ampholines and proteins migrate to a steady-state position. Although the tube gels worked reasonably well, a few problems were apparent at the onset. Extruding tube gels and positioning them on slab gels was tricky. Tube gel elasticity caused minor variability. The early software for pattern matching was primitive so finding differences in large sets of complex 2D gels was tedious.

Today, these problems have been solved at Kendrick Labs. The CA-2DE method has been standardized through use of written SOPs. SameSpots software allows exact pattern alignment before matching; computerized analysis of many variable complex patterns has become straightforward. DIGE is not necessary. CA-2DE may be validated without it.

CA-2DE is compatible with SDS, the best reagent by far for solubilizing proteins (2).

SDS compatibility with CA-2DE, first reported by the Andersons [9], was later optimized at Kendrick Labs [7]. Thus, samples like the epithelial tissue shown above can be homogenized in SDS buffer, heated to 100° C until the solution clarifies, then loaded *without centrifugation*. The resultant CA-2DE gels give quantitative results. Evidence is presented on the following pages that CA-2DE is:

- Compatible with SDS
- A quantitative method for analysis of most proteins
- Extremely sensitive when used with Western blotting

SDS Compatibility

CA-2DE is compatible with SDS because, although this detergent binds to proteins stoichiometrically, it comes off during IEF as shown in Figures 1-3.

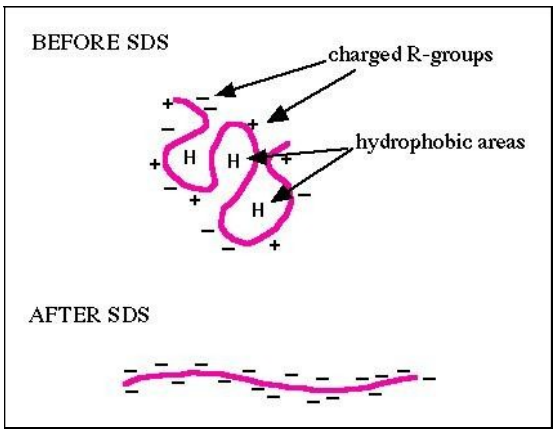


Figure 1. SDS binding mechanism. When proteins are heated in the presence of SDS and β-mercaptoethanol (BME), SDS binds to the peptide backbone and imparts a uniform charge/mass ratio. BME reduces disulfide bridges. All secondary and tertiary structure is lost.

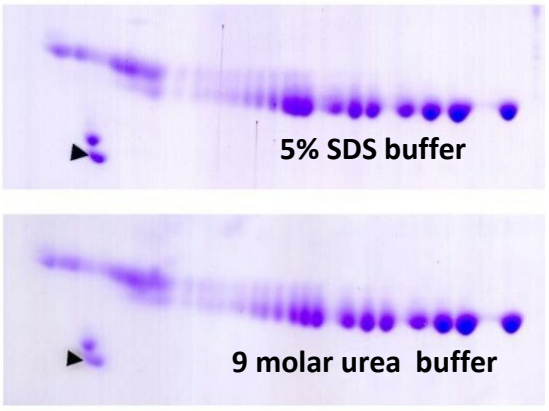


Figure 2. Example: Carbamylated Creatine Phosphokinase Standard dissolved in either SDS (top) or 9M urea (bottom) buffer before IEF. The arrowhead marks the lower isoform of tropomyosin, pI marker of MW 33 kDa and pI 5.2. SDS does not interfere with IEF of this protein.

How can negatively charged SDS be used to dissolve proteins prior to IEF where a single charge change is detectable? Leigh and Norman Anderson (10) showed that during CA-IEF SDS is stripped off proteins to make micelles with NP-40, a non-ionic detergent. The charged micelles migrate to the acid end of the tube gel where they form a bulb that is discarded.

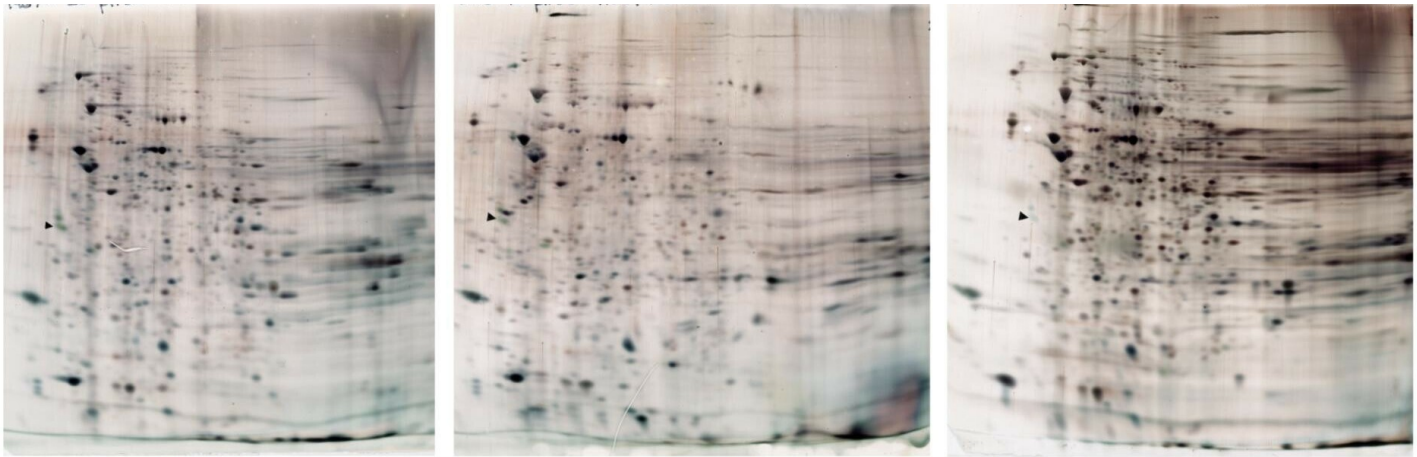


Figure 3. Rat liver microsomes prepared 3 ways. For this figure, microsomes (pellet obtained after homogenization, low speed spin and then 100,000 x g spin) were purchased and dissolved in either SDS buffer with heating (left) in urea buffer (middle), or SDS buffer with heating followed by dilution with urea buffer (right). Standard format (13 x15 cm) gels are shown loaded with 50 ug protein and silver stained. IEF was carried out with pH 3.5-10 ampholines. Microsomes prepared with SDS *and* urea, show a more complex pattern than the other two. *TIP: Once sample preparation is optimized, don't change it.* Changing sample preparation may alter the pattern because of hidden salts and lipids that affect the pH gradient.

Quantitative Method

CA-2DE with Coomassie staining is quantitative for most proteins as shown below and on the next page

Experimental Procedure

- Rat liver cytosol was diluted with buffer containing 2.5% SDS + 4.5 M urea before running on large format 2D gels in triplicate at loads of 200, 400 and 600 µg. Kendrick Labs standard operating procedures were used for all steps. The gels were Coomassie blue-stained and scanned with a laser densitometer calibrated to be linear over 0-3 OD. Sixty polypeptide spots were quantified with Progenesis software from Nonlinear Dynamics. See Figures 4 and 5 below for images, Table 1 for results.

- In reality, spot volume is not used for 2D gel quantification. Measurements are always normalized to correct for gel-to-gel staining differences by using spot percentage. **Spot % = (spot volume-bkg/all spot volumes -bkg combined) ×100**. A summary of Results and Conclusions are provided on the next page.

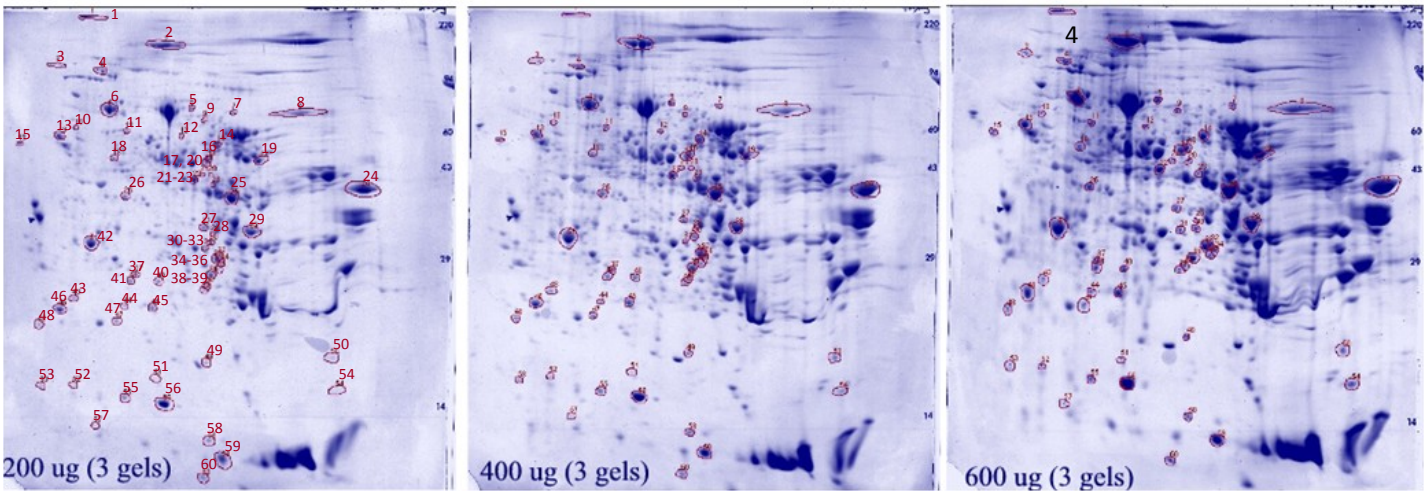


Figure 4. 2D gel patterns obtained from rat liver cytosol diluted with buffer containing 2.5% SDS + 4.5 M urea. Sixty polypeptide spots, outlined in red, were quantitatively analyzed using Progenesis Discovery software. Spot numbers are enlarged on the left panel. Results are shown in Table 1 on the next page. A close-up of spot outlining and linearity plot is shown below for spot 8. The arrow indicates the lower spot of an internal standard added to every sample, tropomyosin, pI 5.3 and MW 33 kDa.

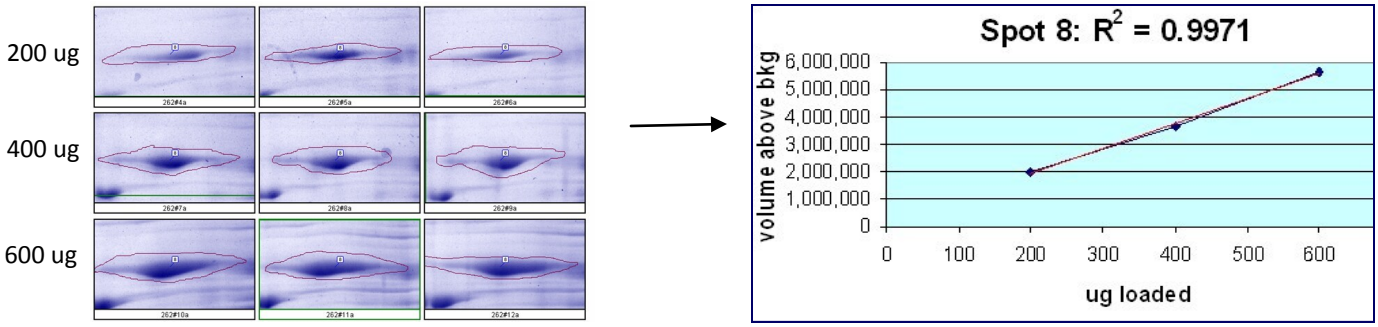


Figure 5. Quantitative analysis of protein spot 8, molecular weight 76,100, pI 8.1. Spot outlining is shown on the left; a plot of spot 8 volume versus protein load is shown on the right.

Quantitative Method continued:

			Spot Volume				Spot %						Spot Volume				Spot %	
Spot #	pI	MW kDa	CV (n=3) 200 ug	CV (n=3) 400 ug	CV (n=3) 600 ug	linearity R^2	Ave Spot %	CV (n=9) Spot %	Spot #	pI	MW kDa	CV (n=3) 200 ug	CV (n=3) 400 ug	CV (n=3) 600 ug	linearity R^2	Ave Spot %	CV (n=9) Spot%
1	~5.7	300	16%	9%	7%	0.9902	1.66	12	31	6.9	34.3	13%	18%	6%	0.9983	0.47	18
2	6.3	176	24%	21%	9%	0.9985	7.74	16	32	7.2	32.8	13%	20%	26%	0.9961	1.05	15
3	5.4	131	8%	10%	13%	0.9737	0.32	13	33	7.1	32.6	9%	4%	7%	0.9765	1.44	11
4	5.7	118	2%	7%	10%	0.7858	1.24	20	34	7.2	31.9	7%	5%	18%	0.9999	0.69	9
5	6.7	79.9	11%	8%	9%	0.9977	0.77	12	35	7.2	31.0	3%	12%	12%	0.9942	0.46	37
6	5.8	78.4	4%	8%	12%	0.9889	6.14	9	36	7.0	30.5	4%	5%	6%	0.9977	1.63	7
7	7.4	78.3	6%	8%	15%	0.9981	0.39	14	37	6.1	29.5	5%	31%	9%	0.9722	0.64	16
8	8.1	76.0	9%	2%	2%	0.9971	5.01	4	38	6.9	29.4	10%	3%	11%	0.9810	0.59	10
9	6.9	74.0	18%	14%	16%	0.9837	0.26	16	39	6.9	28.8	10%	8%	8%	0.9998	0.36	22
10	5.5	67.9	4%	10%	4%	0.9822	0.16	8	40	6.3	28.7	5%	6%	12%	0.9990	0.66	5
11	6.0	65.5	4%	13%	11%	0.9940	0.38	8	41	6.0	28.7	7%	18%	7%	0.9934	0.75	14
12	6.5	64.0	7%	16%	13%	0.9982	0.25	15	42	5.7	33.8	2%	1%	6%	0.9970	5.87	6
13	5.4	60.8	3%	1%	7%	0.9902	2.04	4	43	5.6	27.0	9%	8%	2%	0.9994	0.50	10
14	7.1	60.3	2%	14%	5%	0.9852	1.70	10	44	6.0	26.2	19%	1%	7%	0.9950	0.38	26
15	4.9	58.4	8%	7%	5%	0.9999	0.41	14	45	6.2	26.2	5%	1%	16%	0.9909	1.38	17
16	7.0	54.1	11%	8%	11%	0.9836	0.95	16	46	5.4	25.7	3%	2%	5%	0.9972	2.44	5
17	6.9	52.8	8%	17%	5%	0.9778	1.14	24	47	5.9	24.8	7%	7%	7%	0.9992	0.67	9
18	5.9	52.6	5%	9%	9%	0.9689	0.52	11	48	5.3	24.4	2%	3%	14%	0.9982	0.57	12
19	7.6	52.0	6%	6%	3%	0.9794	2.66	8	49	6.9	22.0	6%	7%	18%	0.9960	0.52	16
20	7.0	48.2	37%	10%	13%	0.9582	0.13	34	50	8.9	21.2	12%	10%	7%	0.9916	0.85	24
21	6.8	47.7	17%	6%	7%	0.9986	0.17	17	51	6.3	19.5	12%	5%	8%	0.9994	0.16	28
22	6.7	44.4	7%	8%	9%	0.9905	1.75	15	52	5.6	18.6	17%	2%	10%	0.9994	0.28	22
23	7.1	43.6	36%	27%	8%	0.9706	0.32	24	53	5.3	18.3	20%	11%	15%	0.9943	0.40	15
24	9.4	41.0	10%	4%	9%	0.9887	13.51	8	54	9.0	17.9	9%	8%	5%	0.9665	0.88	60
25	7.4	40.9	8%	3%	8%	0.9999	7.06	4	55	6.0	17.5	18%	6%	20%	0.9999	0.81	12
26	6.0	40.5	5%	6%	14%	0.9940	0.96	8	56	6.3	17.0	7%	0%	5%	0.9999	5.83	7
27	6.8	37.6	10%	17%	4%	0.9999	0.71	13	57	5.7	14.8	62%	4%	24%	0.9999	0.18	36
28	7.1	36.3	32%	9%	16%	0.9837	0.08	23	58	6.9	14.5	2%	1%	15%	0.9975	0.72	6
29	7.5	35.9	1%	13%	16%	0.9850	4.96	12	59	7.2	10.1	3%	2%	6%	0.9972	3.95	13
30	7.1	35.0	1%	1%	7%	0.9921	0.70	8	60	6.7	4.1	13%	5%	18%	0.9846	0.90	13
									All Spots: Ave			11%	8%	10%	0.9874		15

Table 1. Spot Volume and Spot % results obtained from 60 polypeptide spots on 9 gels. Spot outlines are shown in Fig. 4. Spots with high error (CV for spot percentage > 20 or R^2 values from the plots < 0.95) are highlighted with colors. **Orange** indicates a spot splitting problem. In that case, isoforms of the same or adjacent proteins are merging at higher loads; small shifts in the splitting line induce relatively high error into the measurement. Note that for computer comparisons, when a protein has several charge isoforms that are unchanging by eye between samples on different gels, our Analysts outline them as one protein rather than splitting them to maximize spot count. **Green** indicates that the spot was very faint on the lowest load gels. The fainter the spot, the higher the error. Pink indicates a protein aggregation problem. One protein spot, number 4, shows about the same density at 400 and 600 µg. This protein streaks in the horizontal direction at higher loads causing spot density to be outside the outline. Note: streaky proteins on the basic end of the gel usually give a quantitative response when the whole streak is outlined.

Summary

- Average CV* for Spot volume = 10% (n=180 values, 3 spots each)
- Average CV for Spot percentage = 15% (n=60 values, 9 spots each)
- Average R^2 value for "spot volume vs µg loaded" plots = 0.9874 (n=60 plots)

Conclusions

- The CA-2DE system resolves protein mixtures reproducibly and quantitatively for samples prepared with 2.5% SDS.
- The method's reproducibility and linearity would be expected to hold for lower abundance proteins even though they can't be detected with Coomassie blue staining.

*CV = coefficient of variation = standard deviation times 100/mean

Western blotting

CA-2DE Western blotting has high sensitivity and specificity.

Antibodies are soluble proteins produced by B-cells of the immune system that bind tightly to antigenic determinants on other proteins. Protein dissociation constants for antibody-antigen binding range from 10^{-8} to 10^{-12} where the smaller the number the higher the affinity [10]. The extraordinarily tight binding of antibodies to an antigenic determinant (epitope) is quite useful.

Commercially produced antibodies have added a whole dimension to biomedical research. They are custom-made by many companies for use in protein detection tests including immunohistochemistry, protein arrays, ELISA assays and Western blotting. In the latter method, proteins in a sample are resolved by 1D or 2D electrophoresis and then transferred to a PVDF membrane at Kendrick Labs. After transblotting, the PVDF is stained with Coomassie blue and scanned to record the general 2D pattern. The stain washes off during subsequent incubation with blocking agent and primary antibody; it does not interfere. Finally the membrane is incubated with a secondary antibody that illuminates in the presence of a chemical called ECL followed by exposure to x-ray film. (Film development is faster and more efficient than chemiluminescent scanning when Western blots are run in large sets.) The film patterns are superimposable with the *image* of the stained blot. Matching from the film to the blot image and from there to a stained duplicate gel for spot cutting allows straightforward protein identification by mass spectrometry.

CA-2DE Western blotting has many purposes. For example, it is useful for tracking protein purification via immunoprecipitation, for finding proteins with post-translational modifications such as lysine acetylation, and for determining if low abundance proteins are present in a tissue. High affinity antibody binding gives high sensitivity for the antigen.

Receptor Tyrosine Kinase Western blotting: The example shown below is a CA-2DE Western blot using an antibody against phosphotyrosine in combination with CA-2DE of a whole cell lysate of a human lung cancer sample and matched control. A glycosylated ~200 kDa protein is lighting up in the cancer sample, at about the molecular weight of a receptor tyrosine kinase. No signal was seen from normal tissue taken from the same lung. CA-2DE Western blotting of the cancer sample with an antibody against epidermal growth factor receptor (EGFR) showed a strong signal that co-migrated with the phosphotyrosine signal suggesting the protein was EGFR (data not shown). However, three attempts to confirm the identity by spot cutting and mass spectrometry (LC/MS/MS) have failed. In most cases proteins may be identified from spots cut from Kendrick Labs 2D gels. However, for the case below, phosphotyrosine Western blotting gives a clear signal that is much more sensitive than mass spectrometry.

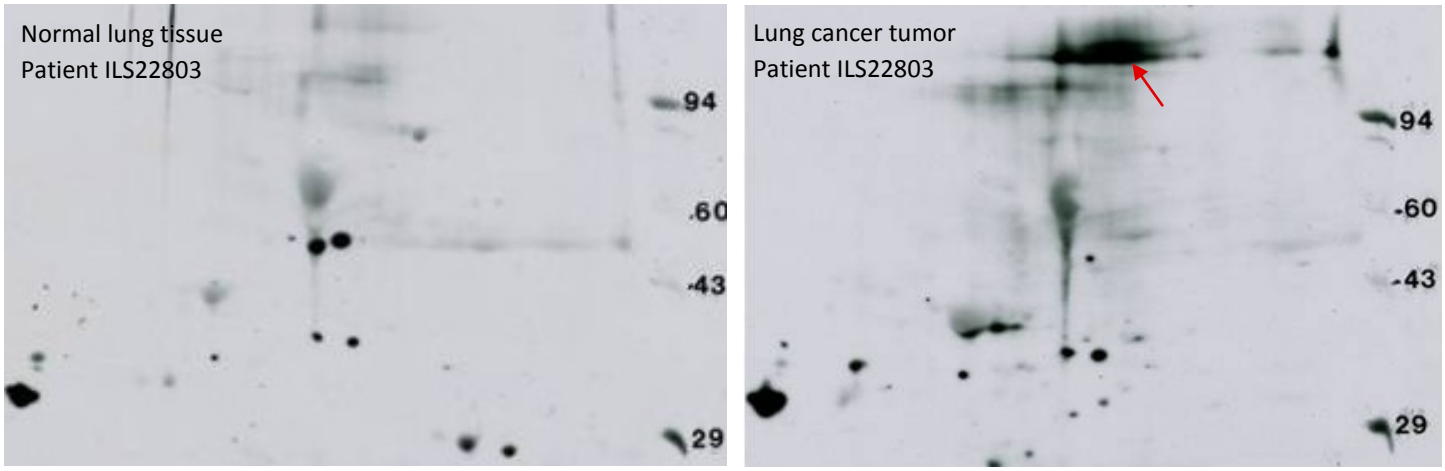


Figure 6. These films obtained with the PY20 phosphotyrosine antibody demonstrate the remarkable sensitivity of CA-2D Western blotting. Right: Phosphotyrosine Western blot of *whole cell lysate* from a human lung tumor sample purchased from a tissue bank. The arrow marks a putative receptor tyrosine kinase, probably Epidermal Growth Factor Receptor, known to be a driver of lung cancer. Right: corresponding pTyr WB from a matched normal lung tissue from the same patient. Conditions: standard format 2D gel (13 x 15 cm), 200 ug protein load, PY20 phosphotyrosine antibody with overnight incubation. Binding to the heavily loaded MW markers on the right allows matching between lots. These proteins (phosphorylase A, 94 kDa, and carbonic anhydrase, 29 kDa) are sold by CalBiochem as phosphotyrosine MW markers. The protein marked by the red arrow co-migrated with the protein highlighted by EGFR Western blotting.

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