2D Gel PhosphoTyrosine Western blotting

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Talk Outline

- Kendrick Labs, 2DE
- Importance of focusing on protein subsets
- P-Tyrosine Western blotting
- Immunoprecipitation experiments
- Reciprocal Affinity Depletion (RAD)

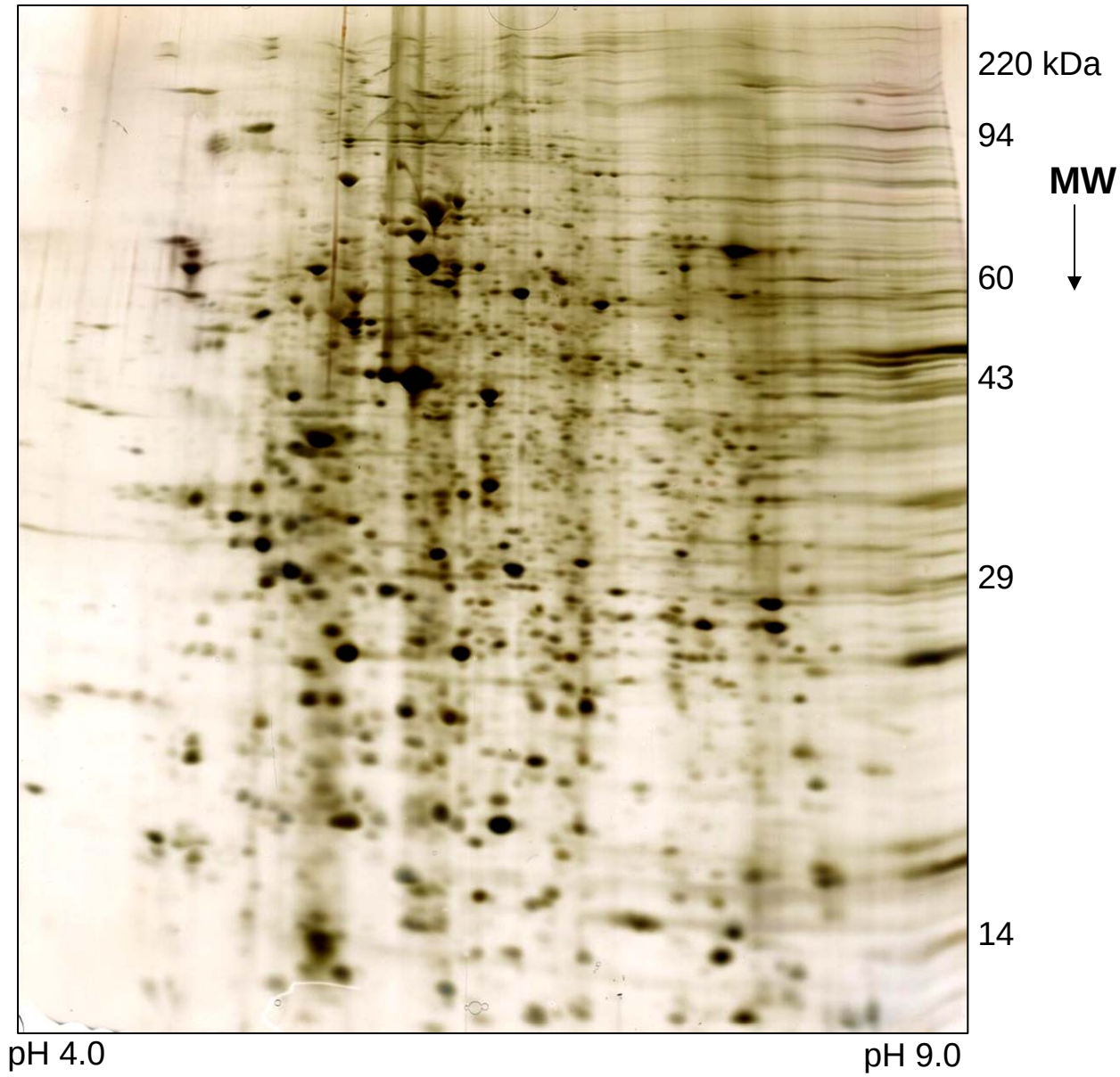
Kendrick Labs Inc, Madison, WI …since 1987

- Service lab specializing in 2DE of protein samples from academia and industry. Mass spectrometry (MS) is outsourced to university core labs.

- Samples are diverse including IPs, cultured cells, and mice KOs. We're constantly trying to improve our system.

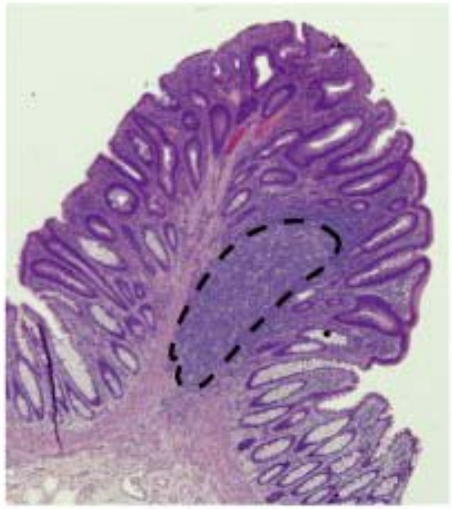
Whole cell lysates give complex patterns, ≥ 1000 protein spots

Cultured cells contain ~6000 proteins, differentiated cells probably fewer, ~4000-5000.



Acute Lymphoblastic Leukemia (ALL) cell line) Shown with permission of Dr. Terzah Horton, Baylor College Medicine.

Why focus on protein subsets? Animal tissues get very complicated.



small tubular adenoma (4×)

Example: Intestinal villus showing the beginning of colon cancer .

- Intestinal cells are differentiating as they migrate up the villi. The tumor cells are differentiating as well. It's a very complex system with thousands of proteins.
- 2D gel Western blotting is a sensitive way to focus in on protein subsets.

Figure from The Biology of Cancer by Robert Weinberg, 2006

Which Subsets? Phosphotyrosine-containing proteins.

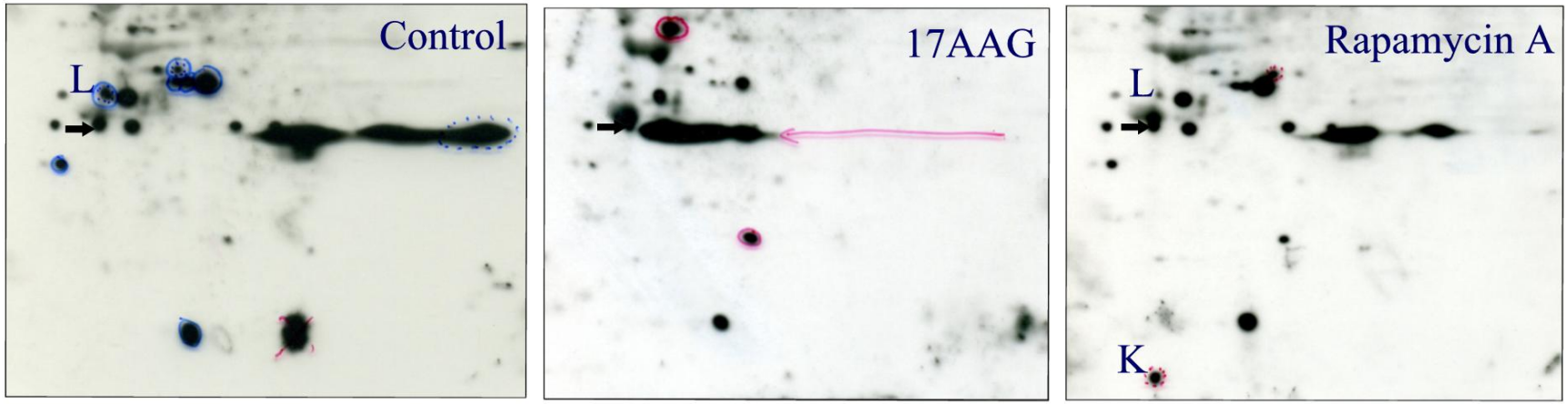
- Tyrosine kinases (TK) mediate cell growth and division by phosphorylating tyrosine residues in specific proteins. Their activity is low in normal tissue but often very high in malignancies.

- **Several good antibodies are available for phosphotyrosine.**

Western blotting procedure

1. Transfer all the proteins to a membrane called PVDF
2. Incubate with an antibody (PY20) that selectively binds to phosphotyrosine groups on proteins
3. Visualize the proteins with a secondary ab carrying an HRP group that activates ECL, which fluoresces

P-Tyr WB example from a client:



Anti-PhosphoTyrosine Western Blot Results for HOS 24 hr Samples

Cultured human osteosarcoma cells WB, shown with permission of Dr. Yair Gazett, University of Texas.

We couldn’t identify the proteins - not enough material for MS.

Why not IP the P-Tyr proteins from large amounts of SM with the PY20 ab bound to agarose resin?

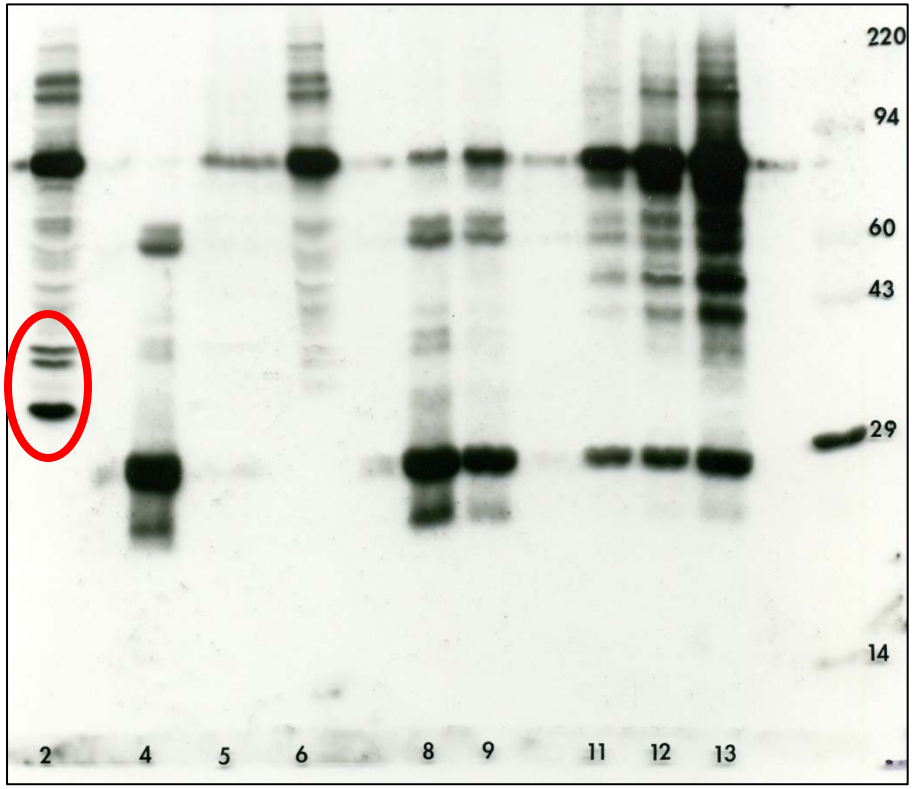
- From the same denatured sample in SDS buffer used for 2D WB so that we can find the changing proteins again.

IPing from complex samples in SDS buffer has several variables:

- Ethanol precipitation or dilution to <0.1% to remove SDS
- Exalpha or Waxman buffer for IP
- Overnight or 2 hr IP

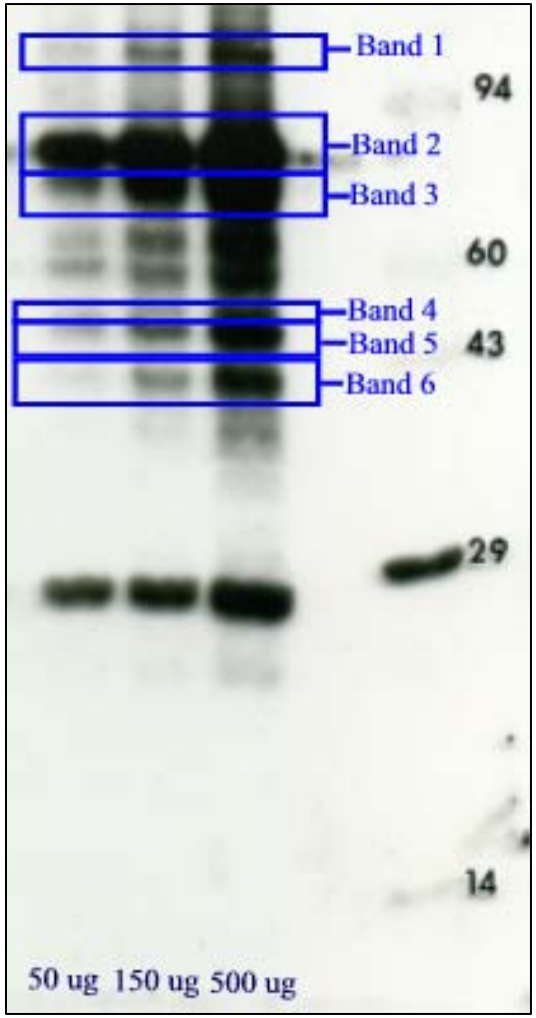
P-Tyr IP: comparison of EtOH ppt versus dilution to remove SDS

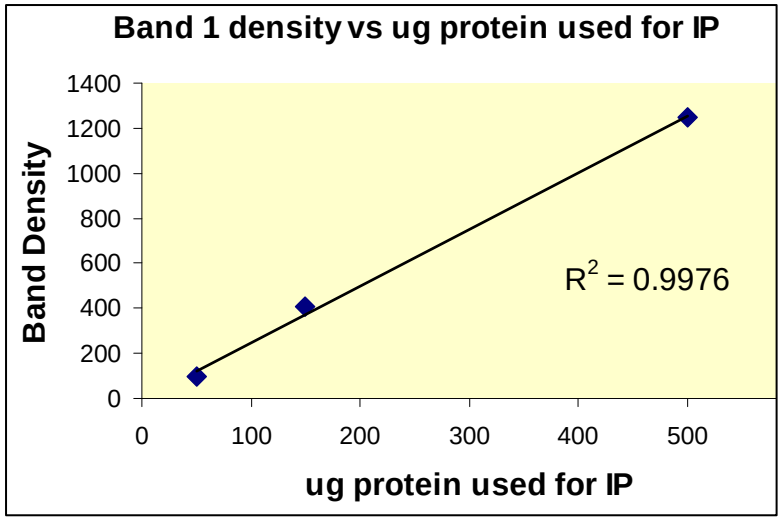
Lane	Sample
2	50 µg lysate, no IP
4	beads only
6	EtOH insoluble pellet
8-9	50 µg EtOH ppt, IP ON
11	50 µg diluted out, IP ON
12	150 µg diluted out, IP ON
13	500 µg diluted out, IP ON

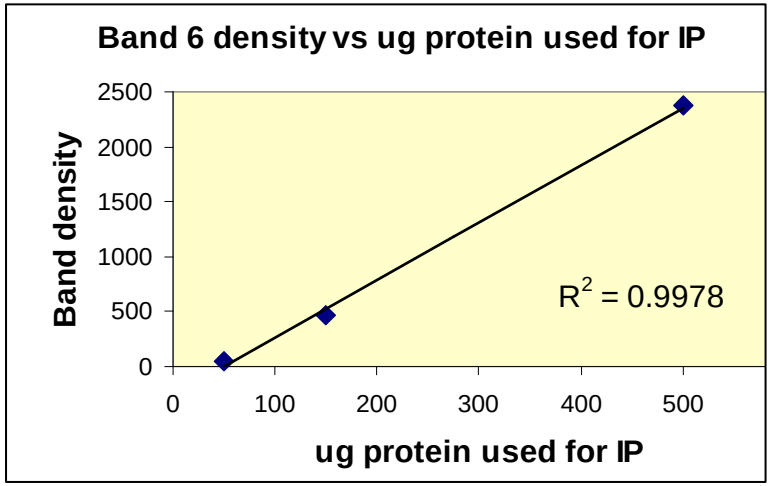


Conclusion: diluting out the SDS works best but 3 bands are missing.

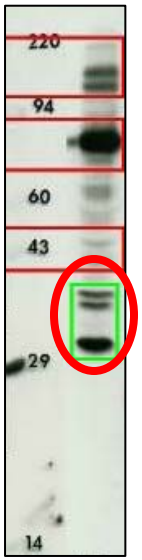
Overnight IPs are quantitative



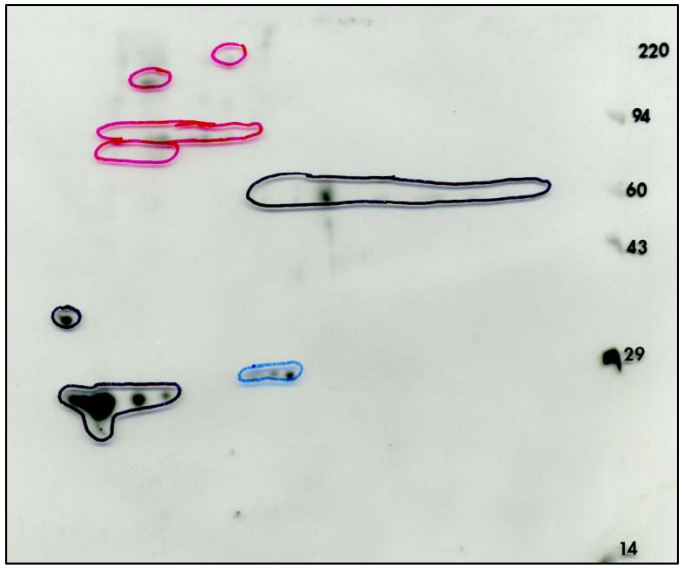




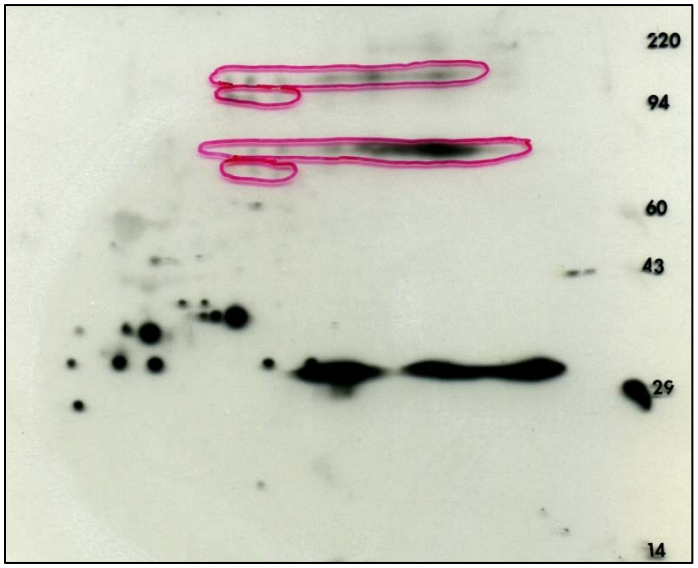
IP for 2D gel analysis didn't work



1D, No IP



IP from 500 µg HOS cell lysate



200 µg HOS cell lysate, no IP

Letting this go for a while because:

a. Too many variables for the moment, too expensive in time and supplies

b. Rumor has it that the PY20 works better for P-Tyr on *native* proteins. So maybe this antibody will never bring down the above proteins between 30 and 40 kDa.

Another approach….

RAD: Reciprocal Affinity Depletion

invented by
Dr. David Huang
GeneTel Labs
Madison, WI

Reciprocal **A**ffinity **D**epletion (RAD) Method to find proteomic differences between tissues

Normal mouse homogenate

Immunize chickens

Purify chicken antibodies against normal mouse proteins

Make an affinity column

Pass **diseased mouse homogenate** through anti-normal column

Proteins unique to diseased tissue pass through in the eluent. High abundance proteins stay on the column.

Reciprocal Affinity Depletion

Diseased mouse homogenate

Immunize chickens

Purify chicken antibodies against diseased mouse proteins

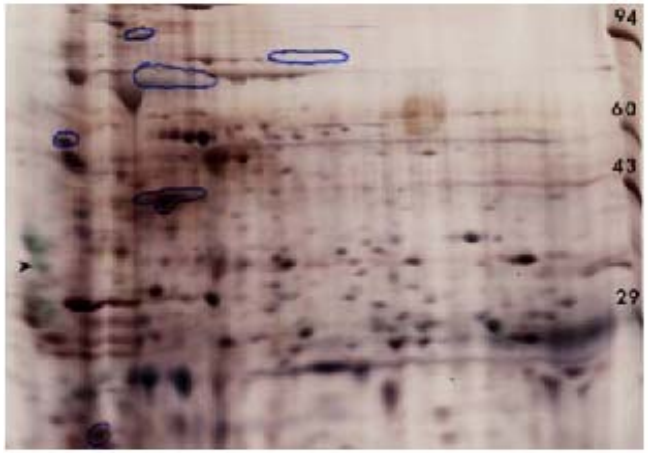
Make an affinity column

Pass **normal mouse homogenate** through anti-diseased column

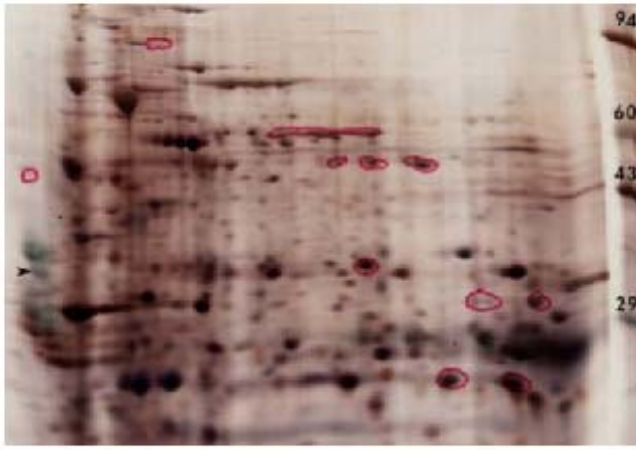
Proteins unique to normal tissue pass through in the eluent. High abundance proteins stay on the column.

The eluents are greatly enriched in proteins differing between the tissues. Comparison by 2D gel electrophoresis to find differences becomes straightforward as does subsequent identification of differing protein spots by mass spectrometry.

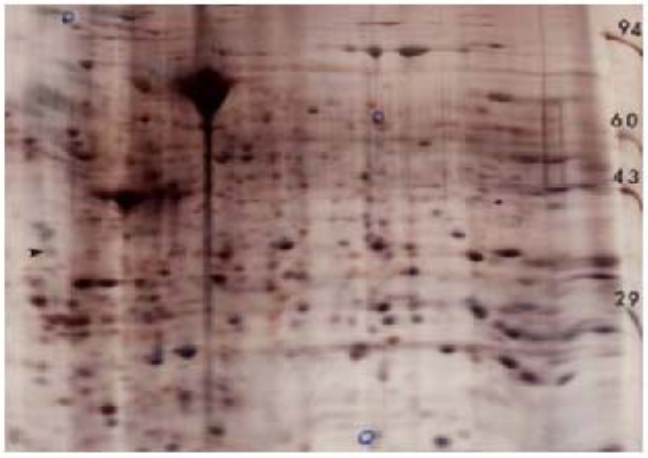
First RAD try with lung homogenate



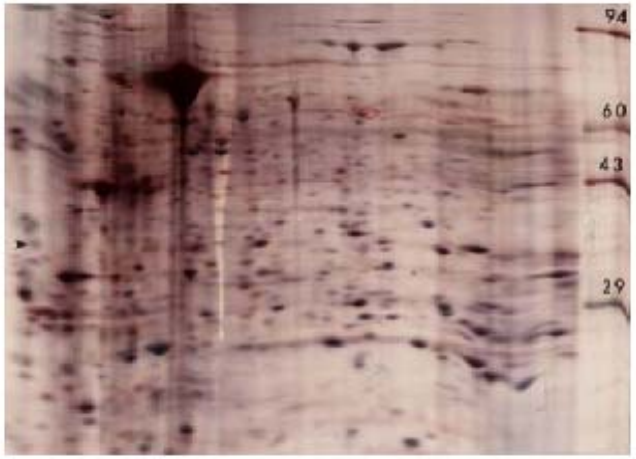
RAD Control Tissue Homogenate



RAD Diseased Tissue Homogenate



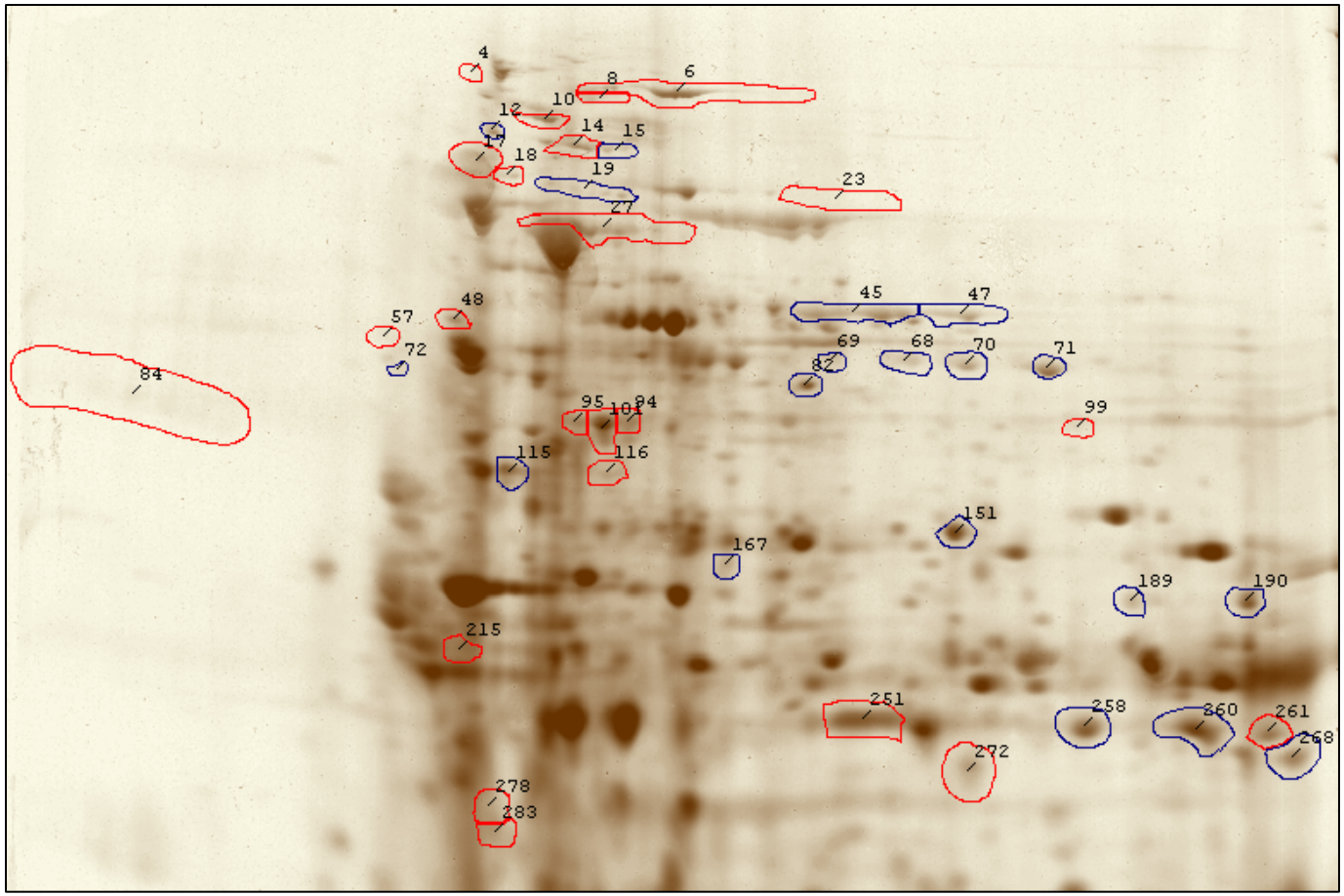
Control Tissue Homogenate before RAD



Diseased Tissue Homogenate before RAD

2D gel patterns from RAD and original samples from mouse lung homogenates (with permission). The client has requested anonymity and nondisclosure of details pending publication.

Computerized comparison showed 41 proteins changing between the samples



New Plan is to combine RAD with P-Tyr Western blotting

- RAD affinity columns take 3 months to generate but are reusable.
- Fresh tissue could be used for the depletions for phosphoprotein studies.
- From the effluent we could run duplicate 2D gels, one for P-Tyr WB comparisons and one for MS.

Collaborators:

Jon Johansen
Lab Manager

Matt Hoelter
Biochemist