

# *2D Gel Phosphoprotein Western blotting*

Nancy Kendrick, Jon Johansen and Matt Hoelter

Kendrick Labs Inc

[www.kendricklabs.com](http://www.kendricklabs.com)

# Talk Outline

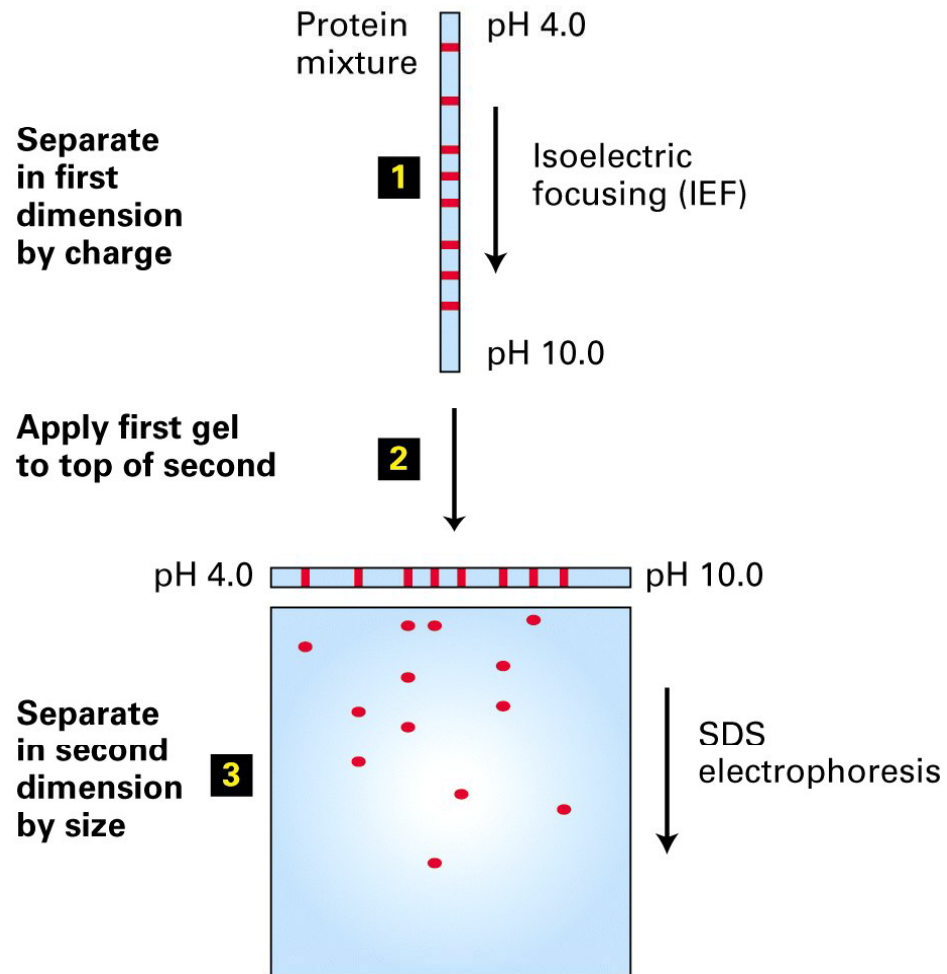
- ❖ Kendrick Labs, 2DE
- ❖ Sensitivity of Western blotting, usefulness for phosphoproteins
- ❖ P-Tyrosine Western blotting
- ❖ P-Serine & P-Threonine Western blotting



## Kendrick Labs Inc, Madison, WI Incorporated in 1987

- ❖ Service lab specializing in 2DE of protein samples from academia and industry. We outsource the final MS, mostly to Columbia University.
- ❖ 8 people, ~2500 2D gels per year.
- ❖ Samples are diverse including IPs, cultured cells treated with cancer drugs and mice KOs. We're constantly trying to improve our system.

# 2-Dimensional Electrophoresis



← IEF

Whole cell lysates give complex patterns, ~1000 spots

Comparing patterns takes considerable time.



Pancreatic Islet tissue. (Shown with permission of Dr. Mark Gray-Keller, University of Wisconsin)



How do we increase sensitivity?  
Use antibodies.

Antibodies give an enormous increase in sensitivity, they are **10-100X more sensitive than silver staining**. Silver staining is 10-40X more sensitive than Coomassie staining.

2D gels are good for looking at post-translational modifications, especially phosphorylation.

...Match made in heaven.

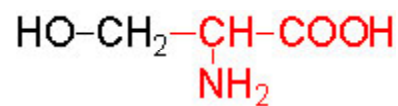


*The human genome has  
518 protein kinases*

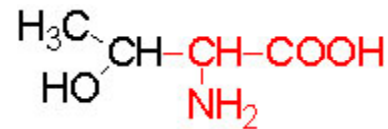
Phosphorylated proteins mediate:

- ❖ cell division (cancer)
- ❖ cellular differentiation
- ❖ hormonal signal transduction

*Three amino acids are phosphorylated by kinases in eukaryotes.*

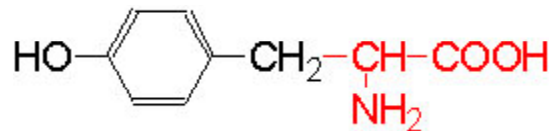


**serine**



**threonine**

Together  
~80% of total




**tyrosine**

~20% of total



# Western blot protocol

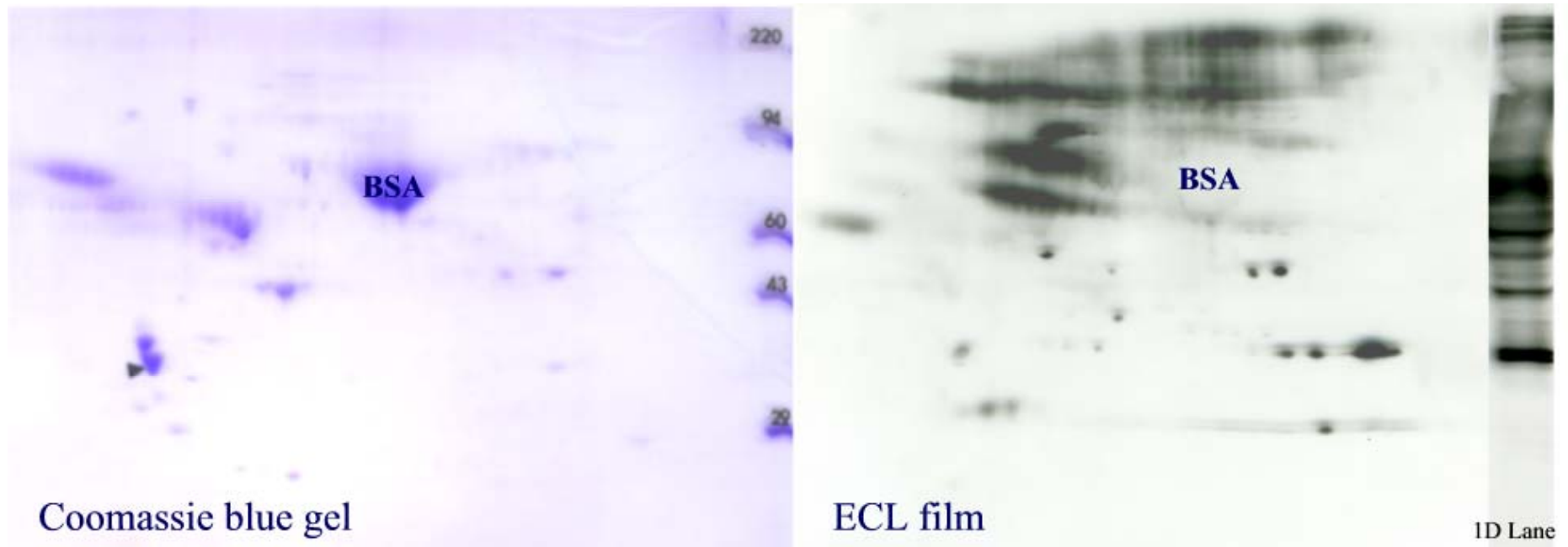
1. Perform 1D or 2DE; transfer the proteins to PVDF
2. Block in 5% BSA
3. Incubate with **anti-Phosphoprotein ab** overnight.
4. Wash 3X
5. Incubate the PVDF in 1:2000 secondary ab (goat anti-mouse HRP conjugated\*) for 2 hr
6. Wash 3X
7. Use GE Healthcare enhanced chemiluminescence (ECL or ECL-Advance) to detect signal



“Tyrosine Kinases as Targets for Cancer Therapy” D. Krause and R. Van Etten, NEJM, 353: 172, 2005

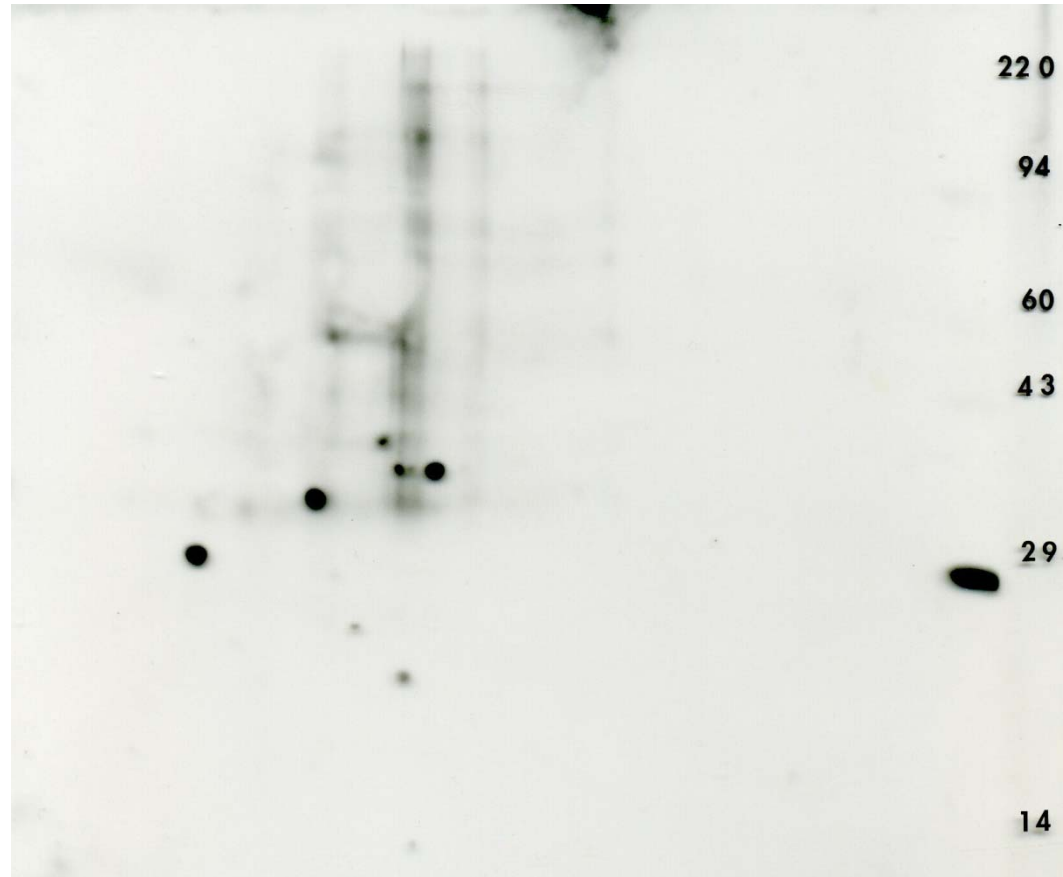
- ❖ “All TKs are under tight control so that *nonproliferating cells have very low levels of tyrosyl phosphorylated proteins.*”
- ❖ *Many oncogenes are TKs mutated to be always “on”*  
*so the phosphorylated substrates should be visible on 2D gels.*

# 2D Western blotting




25 micrograms of standard (RSV transformed 3T3 cells) from Exalpa. 1:1000 PY20 antibody dilution, 30 sec ECL exposure.

## PTyr WB example from client:



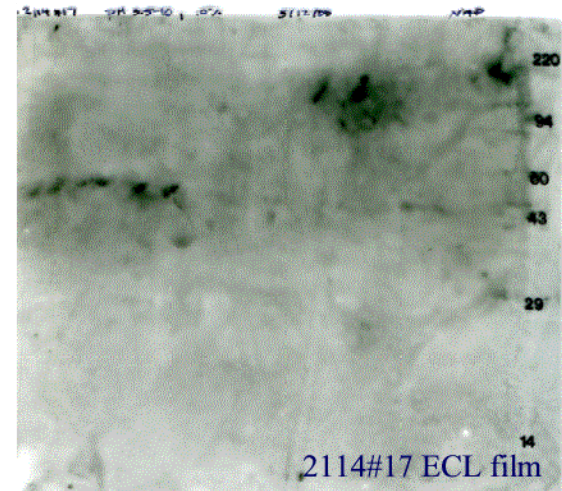
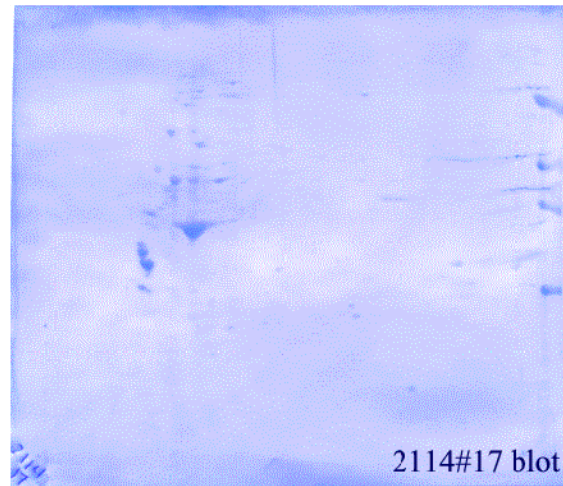
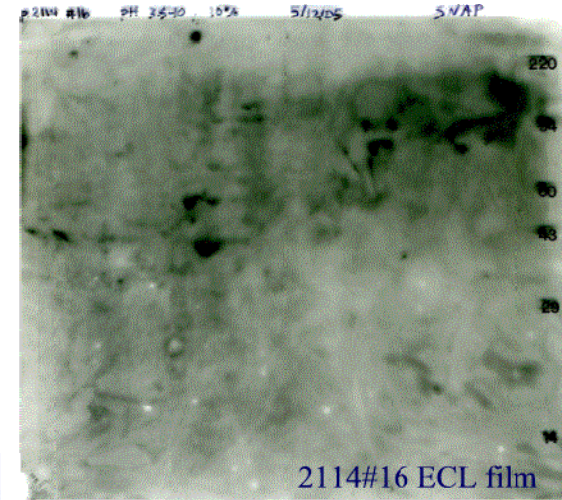
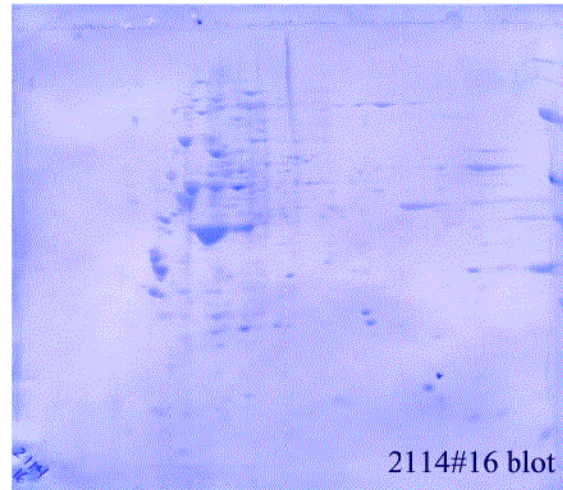
siRNA knockdown of mouse embryonic fibroblast  
(shown with permission from academic client).



We currently have more requests for P-Ser & P-Thr Western blotting than for P-Tyr

- Approximately 30% of cellular proteins are phosphorylated  
(Philip Cohen, TIBS 25, 596, 2000),

In the past they haven't worked well.

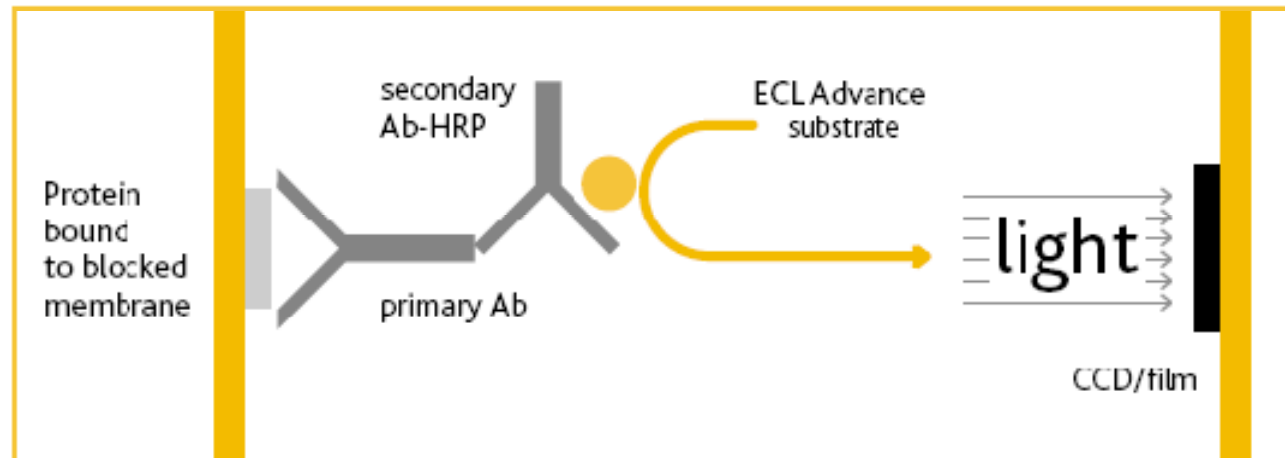


**Anti-P-Serine and P-Threonine antibodies combined**

Typical results with ECL for 1:200 ab dilution.

# ECL Advance from GE Healthcare

Principle of ECL Advance chemiluminescent detection



GE web site says ECL Advance sensitivity is at pg level, much greater than ECL, so primary ab concentrations may be reduced.

# We chose Qiagen antibodies:

- **PhosphoSerine ab Q5 (IgG & IgM)** for detection of phosphorylated serine residues, **irrespective of surrounding amino acids**
- **PhosphoThreonine ab Q7 (IgG)** for detection of phosphorylated threonine residues, **irrespective of surrounding amino acids**

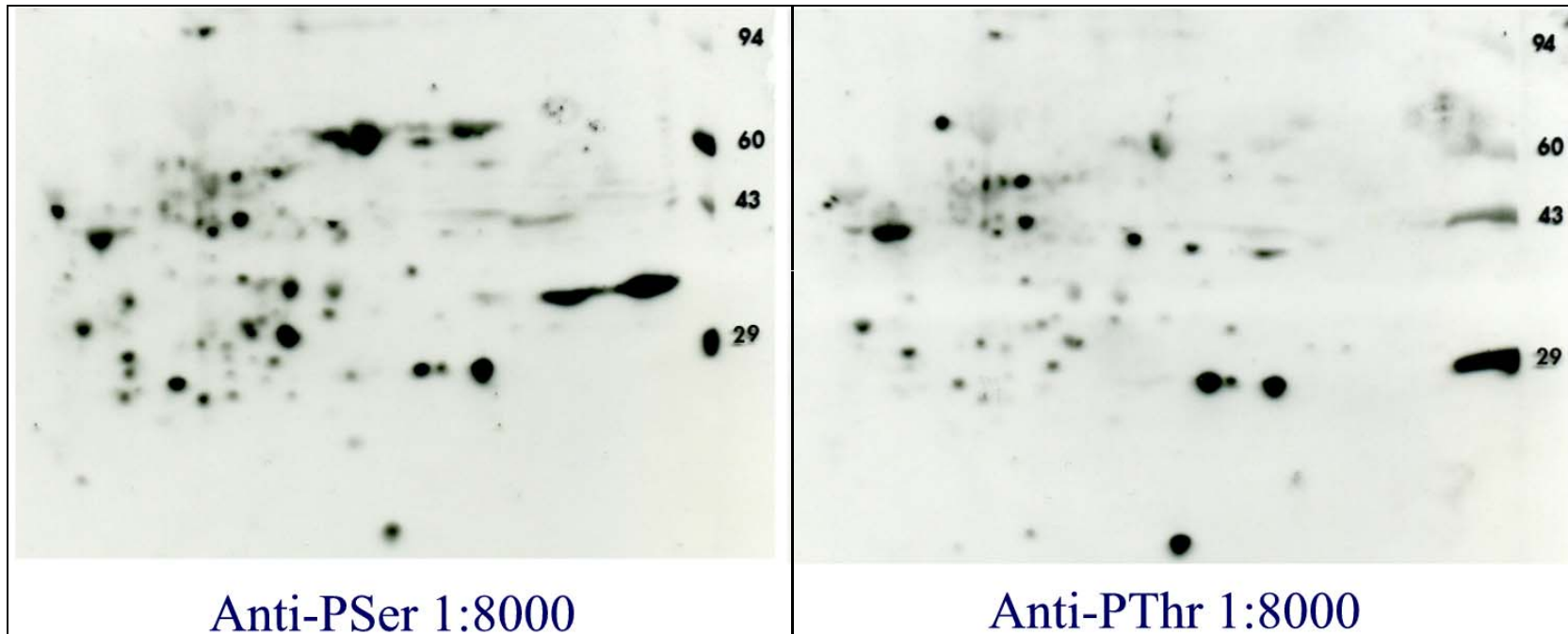
Each is \$340 for 100  $\mu$ l; suggested dilution is 1:200, enough for 1 2D gel.



# Preliminary 1D Results - RLH

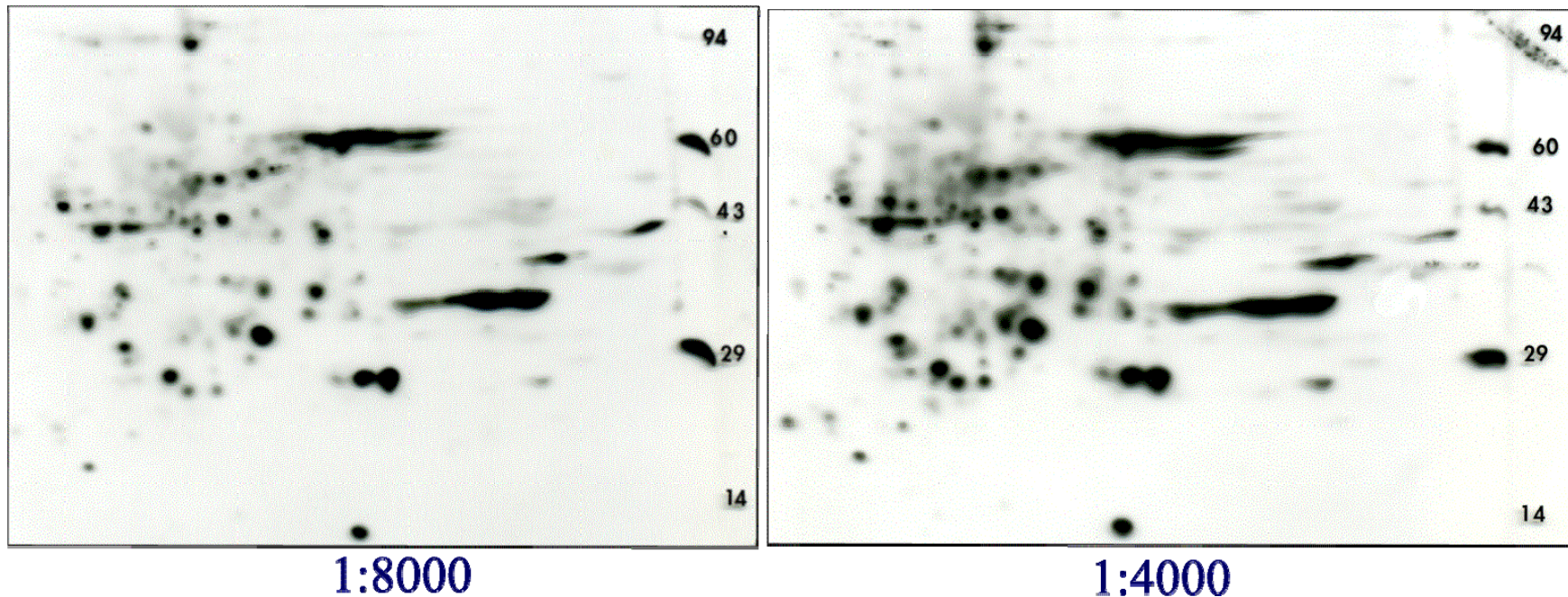
- 1. The Qiagen antibodies give nice 1D banding pattern at 1:8000 dilution with little nonspecific secondary binding.** The ECL Advance is greatly increasing sensitivity as hoped.
- 2. Experiments to show specificity were unsuccessful.** Free phospho-amino acids didn't inhibit. Alkaline phosphatase didn't have an effect. However, nonspecific binding would be expected to be the same between control and test samples.
- 3. We decided to proceed to 2D Western blotting.**

# Qiagen Anti-PSer vs Anti-PThr abs



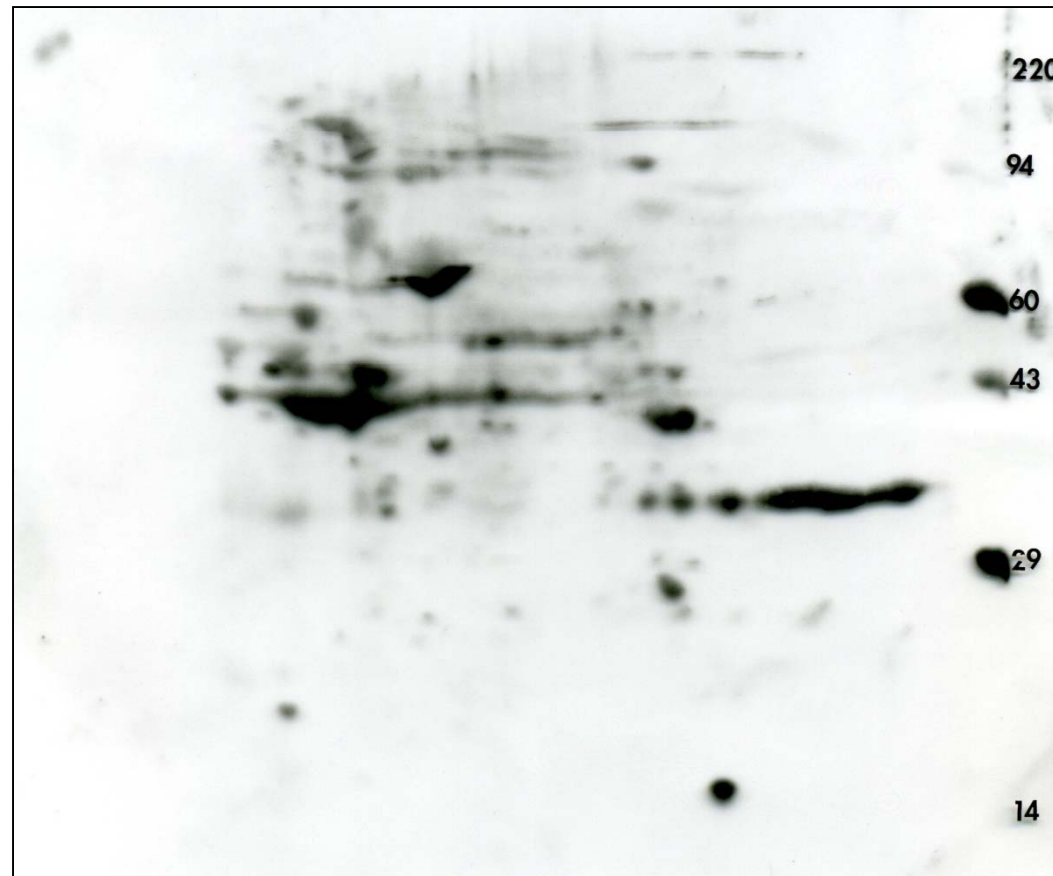
200  $\mu$ g of RLH loaded for two 2D gels run identically. Western blotting was identical except for the primary ab. Central areas of both ECL Advance films expanded. A few proteins are common to both, but the patterns do not match.

# Anti-PSer + anti-PThr combined:



The abs were combined before the dilutions. 1:4000 clearly gives a darker yet clean pattern. (Secondary ab was not changed.) This is what we're currently using for clients. Our combined cost for PSer/PThr abs + ECL Advance is \$65/blot, not too bad.

# Example from client



PSer/PThr pattern of embryonic mouse heart KO (shown with permission of academic investigator).



## *Summary*

- ❖ 2DE is reproducible and quantitative, but the stains aren't very sensitive.
- ❖ Focusing on phosphoprotein Western blotting is a way to increase sensitivity.
- ❖ The PY-20 ab against P-tyrosine is sensitive, reasonably priced and works well for 2DE WB.
- ❖ ECL-Advance is a major break-through for P-Ser and P-Thr Western blotting.