# Optimization of 2D Gel Transblotting for Host Cell Protein Analysis

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# Talk Outline

- Biologic drugs, recombinant proteins
- Problem of HCP contamination
- Using 2D gel WB to characterize antibodies against HCP.
- Work on optimizing transblotting
- Conclusions
- Collaborators

# Many cutting-edge drugs are biologics

- **What is a Biologic?**

  Biological products are those derived from natural sources. They can be composed of sugars, proteins, nucleic acids, or complex combinations, or may be living cells and tissues. Examples: flu vaccine, artificial skin, gene therapy, and especially **recombinant therapeutic proteins**. The latter are derived from cultured cells or bacteria.

- **How do biologics differ from conventional drugs?**

  **C**onventional drugs have known structures and may be chemically synthesized. In contrast, biologics are complex and not easily characterized. They tend to be heat sensitive and susceptible to microbial contamination.

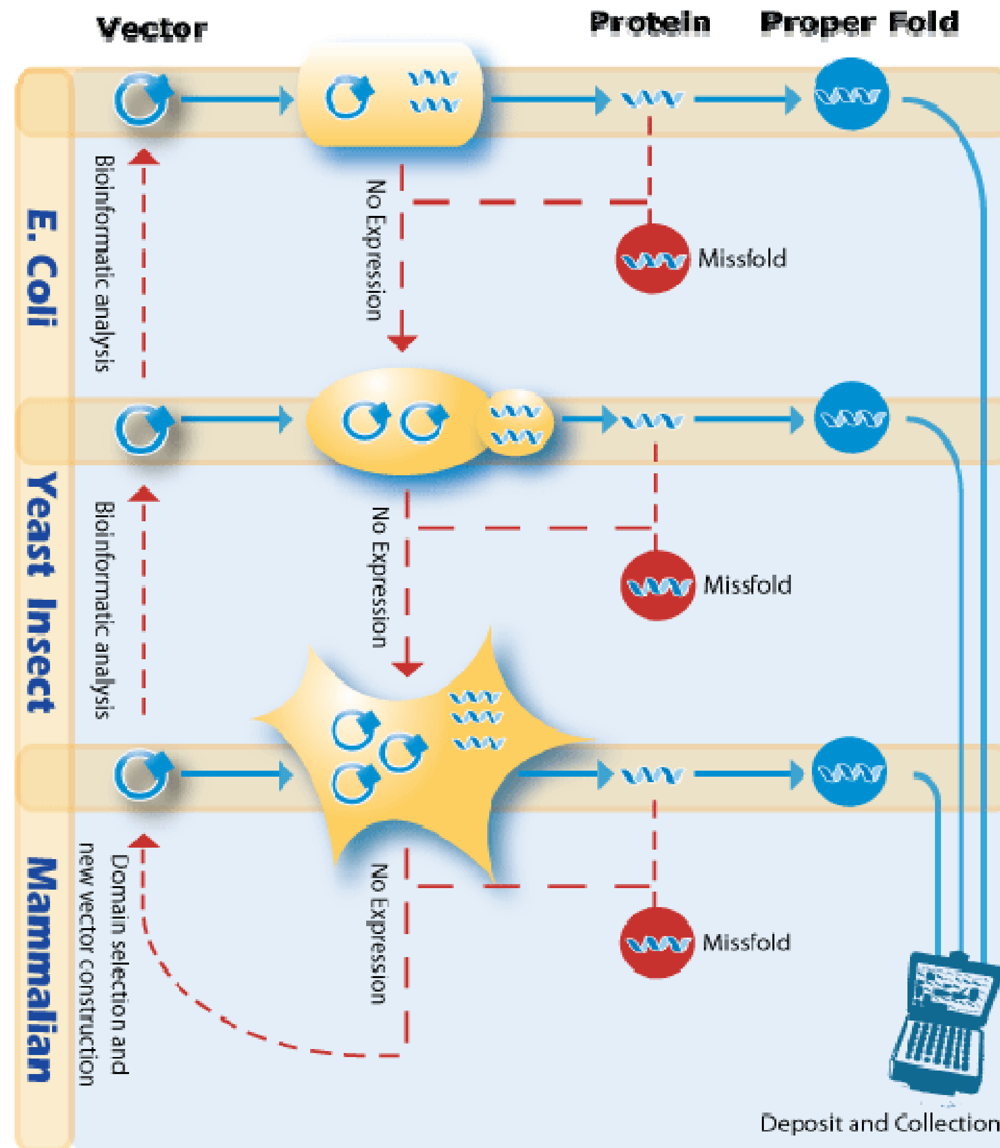
# Classes of Approved Recombinant-Protein Drugs with Examples (~100 total so far)

- **Hormones:** Insulin (Humulin, the first in 1983), human growth hormone (Humatrope),
- **Cytokines:** Interferon alfa (Roferon-A), interleukin (Proleukin)
- **Clotting factors**: Factor VII (NovoSeven), factor VIII (Kogenate)
- **Monoclonal antibodies:** anti-VEGF (Avastin), anti-EGFR (Erbitux)
- **Vaccine products** Hepatitis B surface antigen (Recombivax HB), *B. burgdorferi* outer surface protein A (LYMErix)
- **Enzymes:** Glucocerebrosidase (Cerezyme), DNase (Pulmozyme)
- **Novel synthetic proteins**: Fusion protein of interleukin-2 and diphtheria toxin (Ontak)
- **Novel conjugates**: Covalently attached metal chelators: (Zevalin); Covalently attached chemotherapeutics: Mylotarg

Taken from: Dudzinski, D. & Kesselheim, A., *NEJM* **358**: 832, 2008

Recombinant Protein Synthesis Diagram taken from the Genway Biotech website: www.genwaybio.com

Host cells can be *E. coli*, yeast, insect, and mammalian cultured cells, especially CHO.

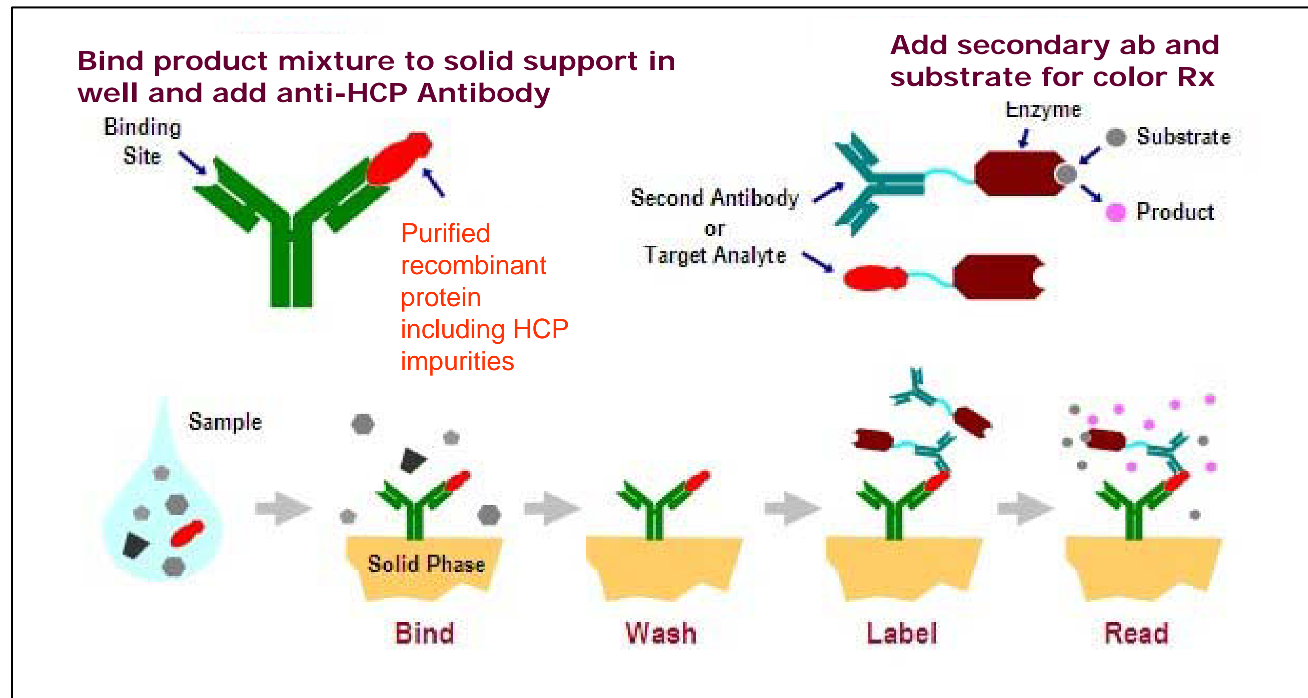


## 2D gel electrophoresis is useful at several stages of production.

- 2DE is used to characterize charge isoforms of recombinant proteins.
- 2D WB with an anti-HCP ab during production steps allows identification of contaminants by MS, *e.g.* (1)
- 2D WB may be used to characterize the anti-HCP antibodies used for Elisa quantification.

**1. Hunter, A. et. al Separation of host cell proteins oppa and dppa from recombinant apolipoprotein in an industrial hic unit operation. *Biotechnol Prog,* 2009. *25(2*): p. 446-53.**

## To measure HCP contamination for the FDA: obtain a *antibody against HCP* and set up a quantitative Elisa assay



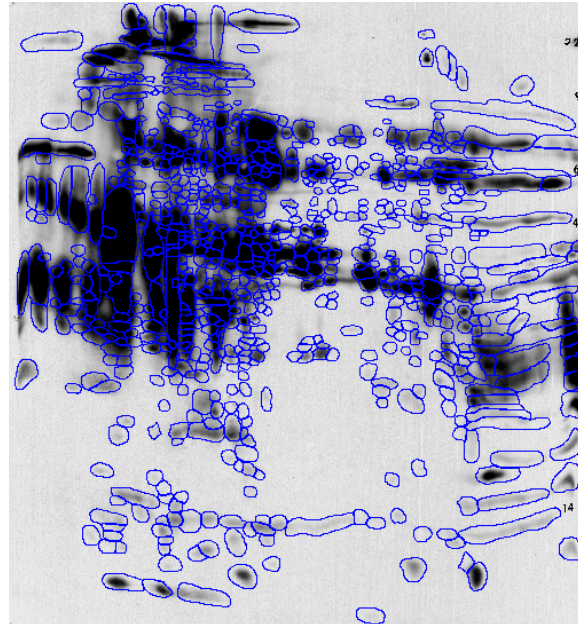

Elisa testing can be standardized and validated for GMP. However, it's only as good as the polyclonal antibody.

❖ Kendrick Labs is getting requests to characterize anti-HCP ab using 2D Western blotting. The number of spots on a 2D film is compared to the number on a silver-stained pattern from the same sample.

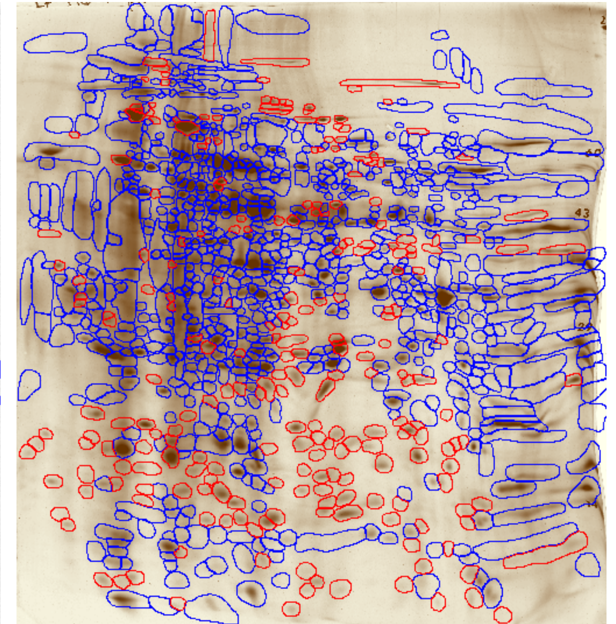
❖ If the Western shows a high percentage of the silver-stained proteins, it’s a good antibody.

# Example of HCP Antibody Analysis

Silver-stained 2D gel from HCP shows 1045 spots with Progenesis SS




Western blot with a anti-HCP antibody shows 798 spots



Results: Red shows spots present on silver but missing from WB (blue).

Final Result: 798/1045 spots = 76%. Not a perfect antibody, but not bad.

# Western blotting is difficult to standardize

- Protein recovery on the blot isn't known. Basic proteins are known to transfer differently than acidic ones.

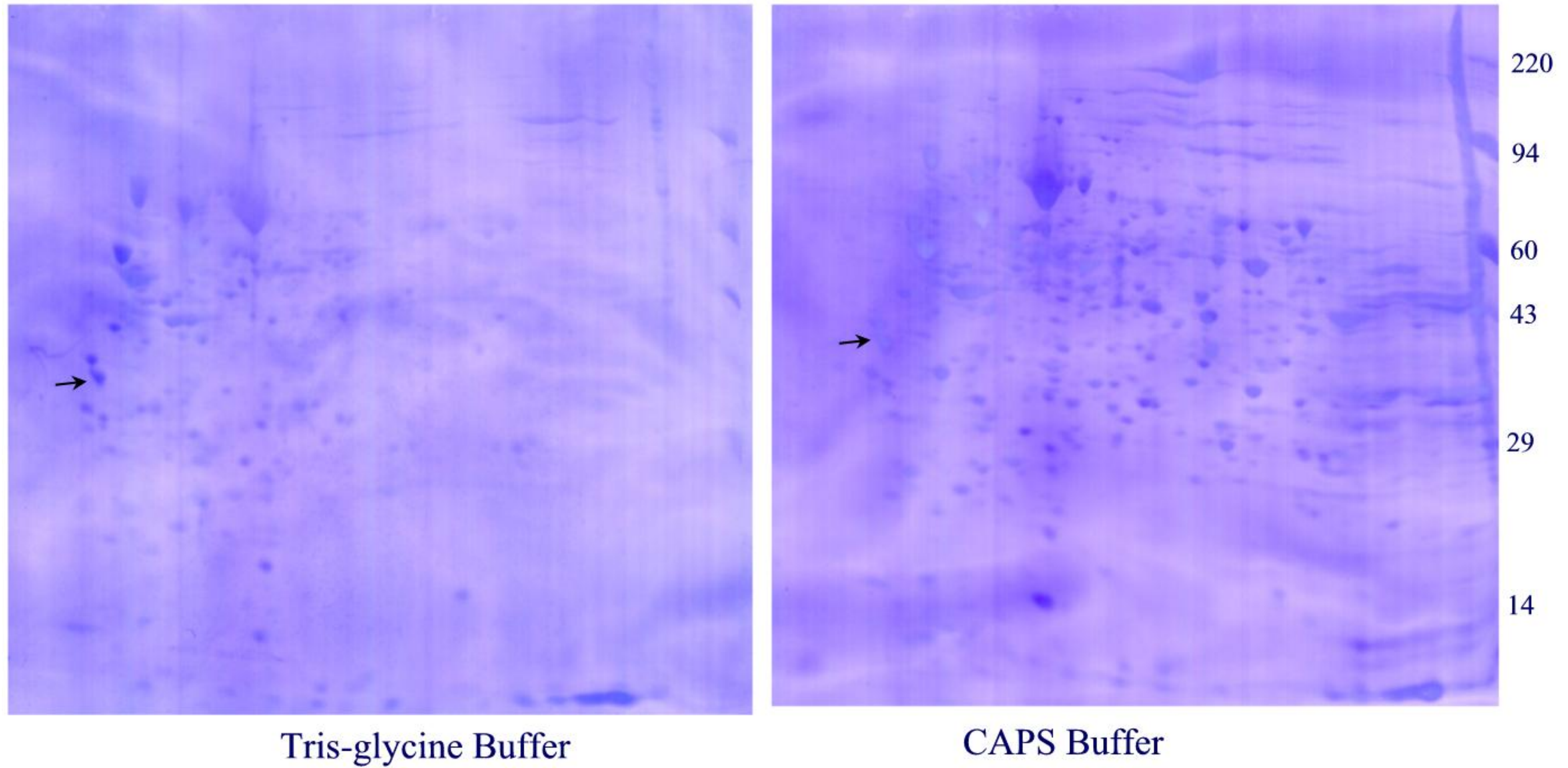
- Antibody specificity varies from animal to animal and species to species. (Host cell protein antibodies are polyclonal.)

# PVDF transblotting has been around for years

Basic Protocol

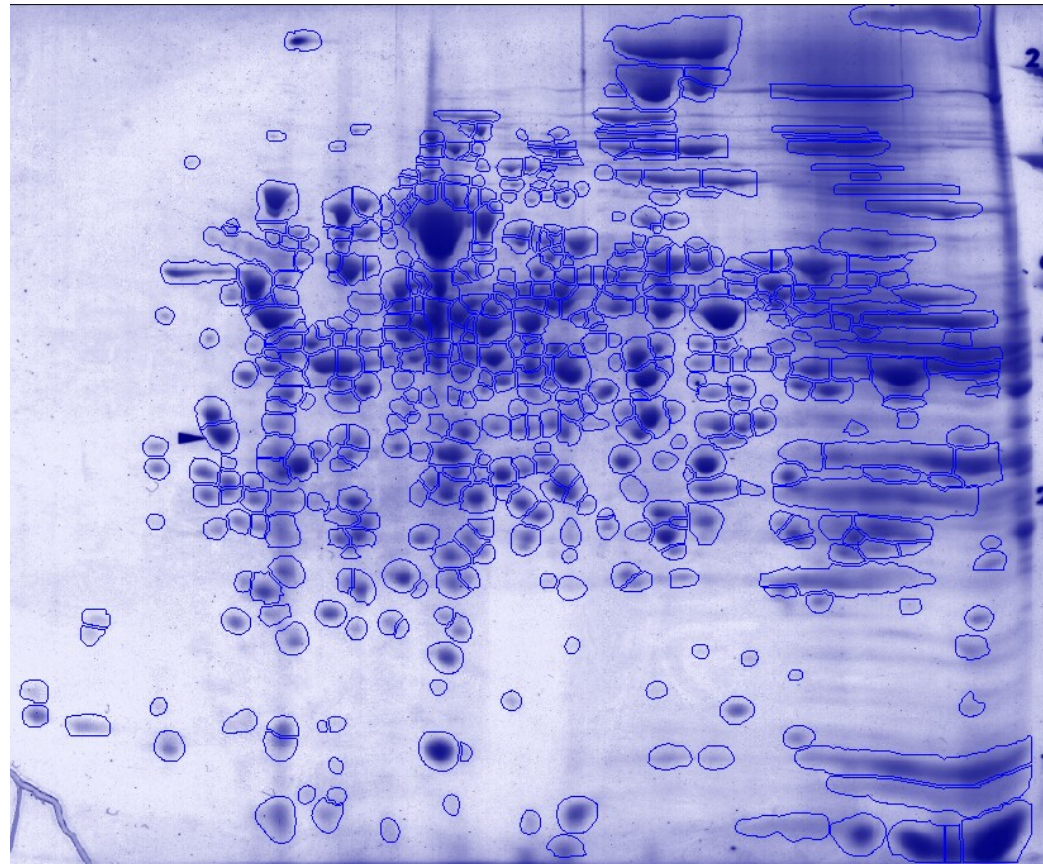
- Transfer the 2D gel proteins to a PVDF membrane using either Tris-glycine buffer, pH 8.8, *or* CAPS buffer, pH 11. (SOPs L-1610, L-1614 are similar).
- Stain with Coomassie and scan to record the 2D pattern.
- Rinse, block and incubate with a primary antibody overnight.
- Visualize the proteins with a secondary HRP-ab that induces ECL fluorescence; expose to x-ray film.

# Observation: Coomassie blue-stained CAPS blots look better than tris-glycine blots



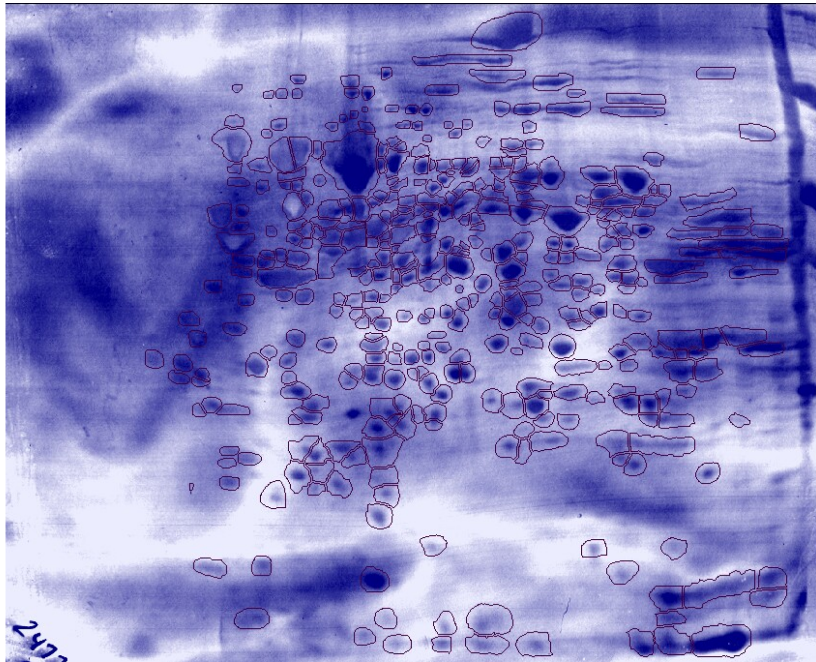

Rat liver cytosol test sample was equally loaded on both 2D gels

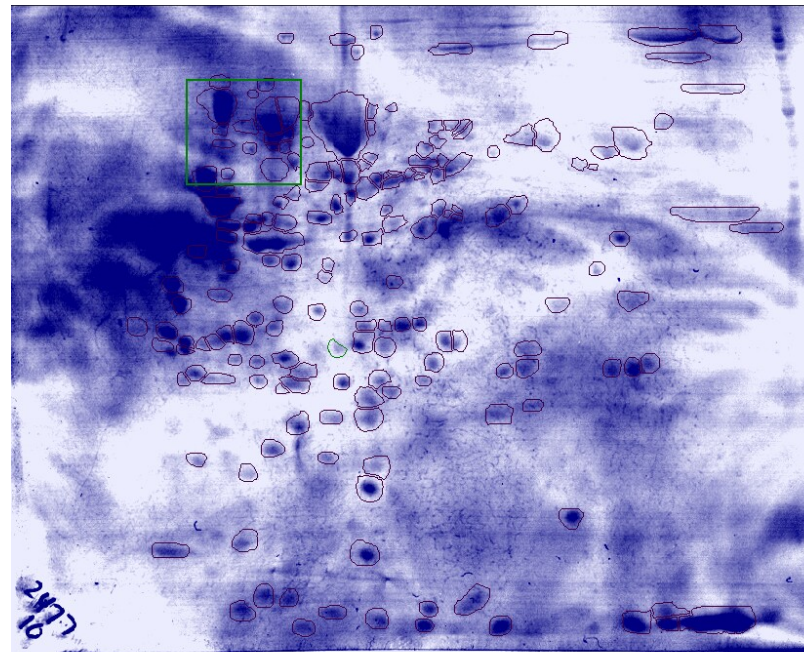
# We counted spots to check differences



A Coomassie blue-stained 2D gel analyzed with Progenesis SS showed 482 polypeptide spots.

# Similar analysis of CAPS and Tris-glycine stained blots showed:





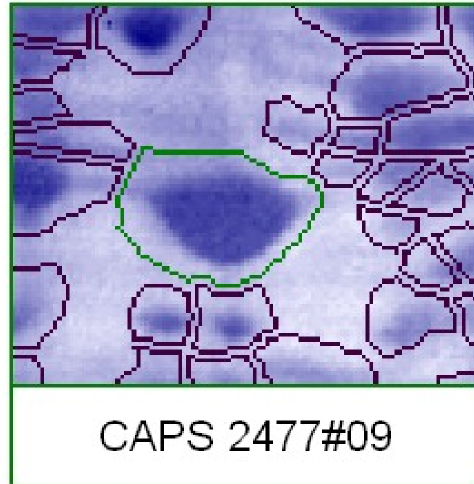
**281 spots for CAPS (58%)**

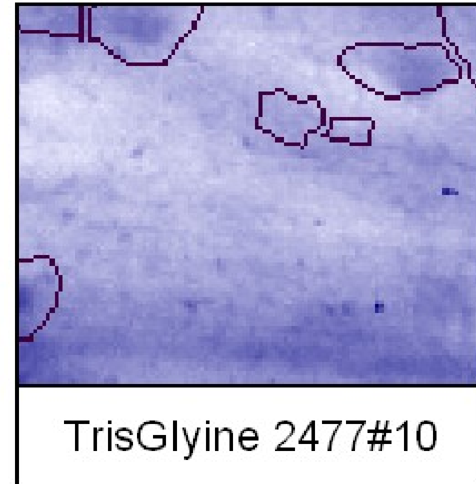
**178 spots for Tris-glycine (37%)**

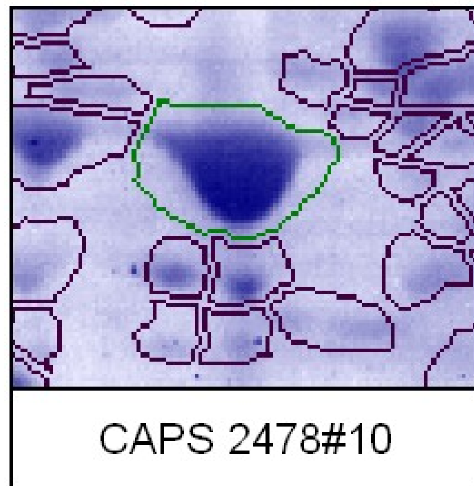
**Much better recovery?**

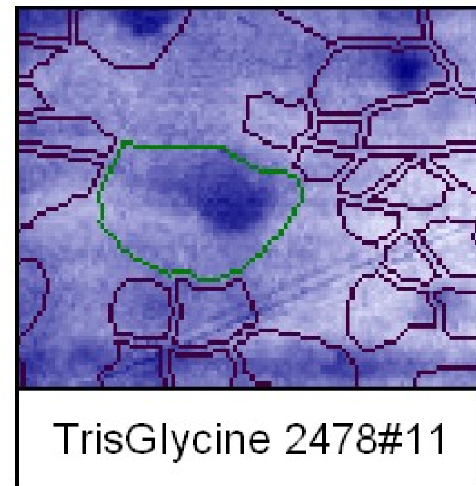
# But it is real or a staining problem?

Maybe the tris-glycine blots don’t stain well.


CAPS 2477#09


TrisGlyine 2477#10


CAPS 2478#10


TrisGlycine 2478#11

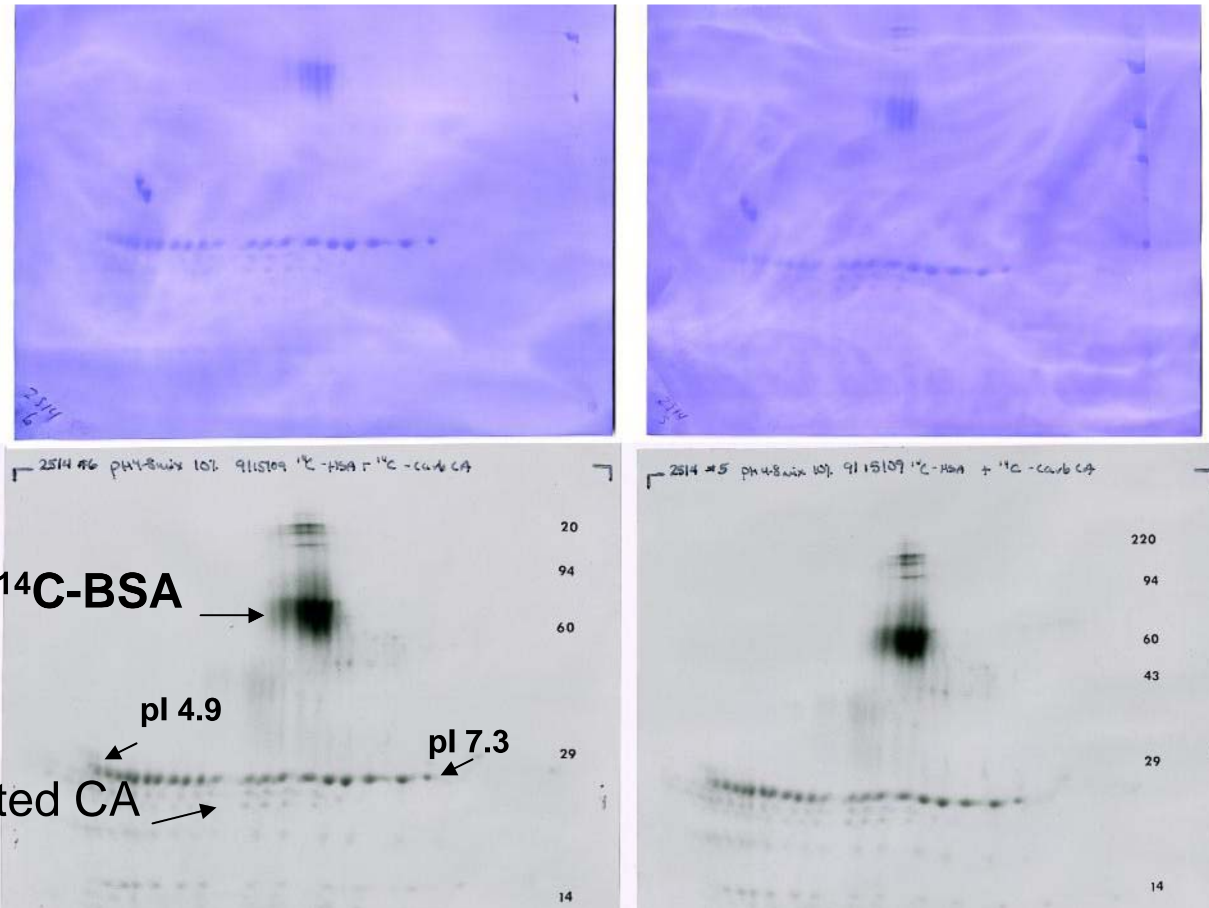
# We tried using $^{14}C$-labeled proteins to compare recoveries on the blots

1. Run 2D gels with $^{14}C$-BSA and $^{14}C$-carbamylated CA (pI marker) that had been previous synthesized and stored at -80$^{o}$ C for 20 years (1,2).
2. Transblot duplicate gels to PVDF using CAPS and Tris-glycine
3. Expose to x-ray film and quantify spots.

**References:**

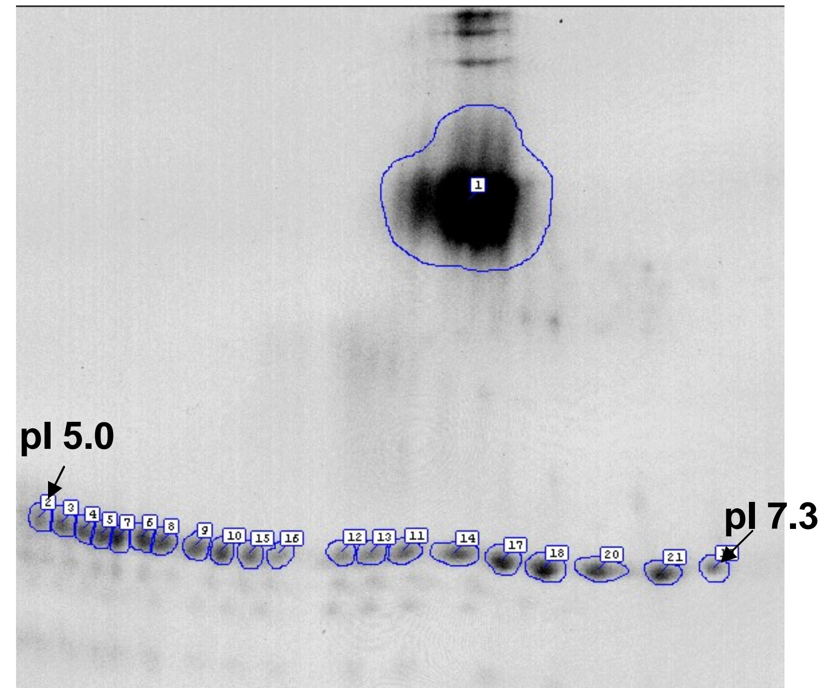
1. **Jentoft, N, Dearborn DG. Protein labeling by reductive alkylation. Methods Enzymol, 1983: 570-79.**
2. **Burgess-Cassler, A, Johansen, J, Santek, D, Ide, J & Kendrick N, Computerized quantitative analysis of CB-stained serum proteins separated by 2DE. Clin Chem 35: 2297-2304, 1987.**

# A pH 4-8 IEF gradient had to be used to resolve the $^{14}$C-carbamylated CA



# Spot Quantification shows no difference in recovery on CAPS vs TG blots

| Carb-CA Spot pI | TrisGlycine Spot Volume | CAPS spot Volume | Ratio Spot Volume Caps/TrisGly |
|---|---|---|---|
| 4.99 | 245 | 176.9 | 0.7 |
| 5.08 | 310 | 235.0 | 0.8 |
| 5.14 | 238 | 215.3 | 0.9 |
| 5.2 | 285 | 215.0 | 0.8 |
| 5.27 | 336 | 269.5 | 0.8 |
| 5.37 | 293 | 200.0 | 0.7 |
| 5.46 | 313 | 267.9 | 0.9 |
| 5.54 | 311 | 252.4 | 0.8 |
| 5.63 | 280 | 249.1 | 0.9 |
| 5.7 | 313 | 251.0 | 0.8 |
| 5.86 | 218 | 155.0 | 0.7 |
| 6.01 | 266 | 194.0 | 0.7 |
| 6.11 | 435 | 361.3 | 0.8 |
| 6.27 | 273 | 226.2 | 0.8 |
| 6.45 | 192 | 141.4 | 0.7 |
| 6.61 | 453 | 383.9 | 0.8 |
| 6.75 | 531 | 411.5 | 0.8 |
| 6.97 | 224 | 125.1 | 0.6 |
| 7.11 | 464 | 418.0 | 0.9 |
| 7.3 | 389 | 335.0 | 0.9 |
| | | Average | 0.8 |



## *Conclusions*

- CAPS transfer buffer allows better visualization of proteins on Coomassie stained PVDF blots.
- However, protein *recoveries* on the blot with CAPS *are* not necessarily higher. Recovery probably varies between protein species and has to be checked.
- 2D WB is a useful tool for scientists evaluating recombinant proteins.

# Collaborators:

Jon Johansen, Lab Manager

Matt Hoelter, Biochemist, AES Executive Director