Preparation of a phosphotyrosinylated protein standard for 2D gel western blotting

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Introduction

- This talk is about an ongoing cancer research project at Kendrick Labs that utilizes carrier-ampholine 2D gel electrophoresis, our specialty. (CA-2D)
- EGFR (Epidermal growth factor receptor) is a protein well known to drive lung cancer. Two new EGFR inhibitors have recently gone through clinical trials. Both drugs are only effective on a subset of lung cancer patients. (1-2)
- A companion diagnostic test is needed to show which cancer patients would respond to the new drugs. Our goal is to help pharma companies develop such a test.

References

1. Thatcher, N. et. al. **(2015)** Necitumumab plus gemcitabine and cisplatin versus gemcitabine and cisplatin alone as first-line therapy in patients with stage IV squamous non-small-cell lung cancer (SQUIRE): an open-label, randomised, controlled phase 3 trial, ***Lancet Oncol*** *16, 763-774.*

2. Soria, J. C. et. al. **(2015)** Afatinib versus erlotinib as second-line treatment of patients with advanced squamous cell carcinoma of the lung (LUX-Lung 8): an open-label randomised controlled phase 3 trial, ***Lancet Oncol*** *16, 897-907.*

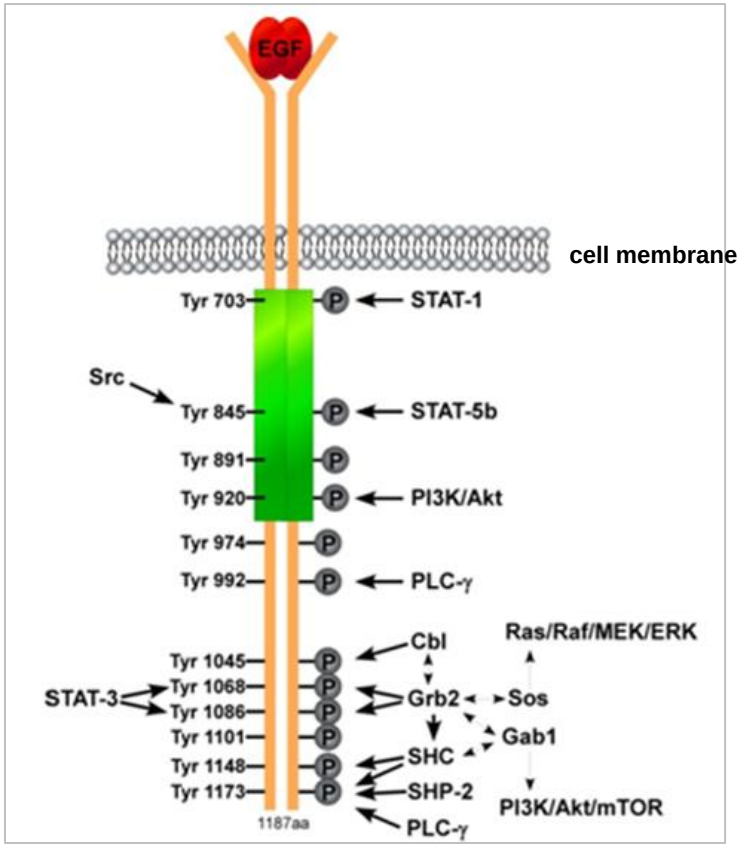
170 kDa EGFR is expressed in many cells but remains an unphosphorylated monomer until ligand appears

❖ **EGF** triggers receptor dimerization, which brings the inner chains together, triggering trans-tyrosine phosphorylation via kinase domains.

❖ The 12 phosphotyrosines (pTyr) become binding sites for mitogenic proteins that trigger cell division.

❖ Unphosphorylated EGFR is inactive.
It is easily detectable but does not correlate with EGFR inhibitor success in cancer patients.

❖We are developing a method to detect **pTyr-**EGFR, the active form.



***Schematic representation of the EGF receptor bound to its ligand and depicting intracellular phosphorylation sites. F*rom the Alain Charest Lab (Tufts University) website**

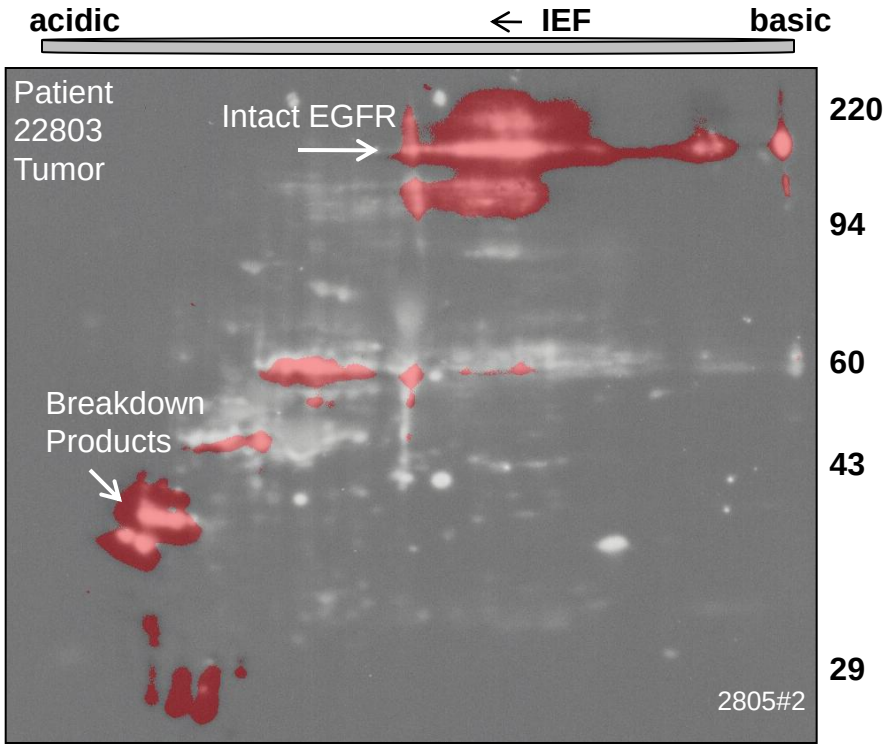
Previously, we identified active EGFR in lung cancer samples via CA-2D western blotting.

CA-2DE
1st Dimension: IEF in tube gel, SDS compatible
2nd Dimension: SDS PAGE in slab gel

Human lung tumor sample was homogenized in SDS buffer, run on a 2D gel, and the proteins transferred to PVDF.

Western blot overlay

- A pTyr antibody (white) was used for the first WB, then stripped off.
- An EGFR antibody (red) was used for a second WB.
- The images were overlaid to detect pTyr-EGFR.
- This signal is seen in 2/12 human squamous cell carcinoma samples



2D western blot overlay image.

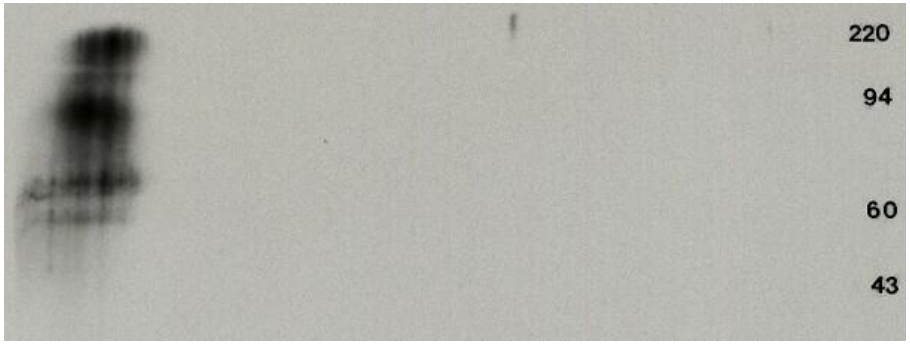
Problem:

- ❖ Results are western blot pictures
- ❖ In order to be useful for large sets, pictorial results *must* be quantified and presented as a number.

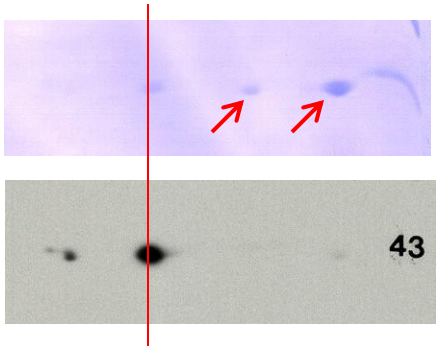
An internal standard is needed – a pTyr-protein that can be added to every WB sample before loading.

- It'd be a control for blot transfers, antibody reactivity and film exposures.
- EGFR density could be normalized to an internal standard and expressed as a number.

First we tried to buy a pTyr-protein:



Try 1: pTyr-BSA from Sigma-Aldrich at 1 ug load gives a very diffuse anti-pTyr WB pattern. Pattern isn't acceptable.



CB-stained PVDF shows 3 charge isomers

Anti-pTyr WB film shows 1 major pTyr isomer

Try 2: pTyr-JNK1 from University of Dundee at 1 ug load is mostly unphosphorylated, maybe 10%.
Too expensive, can't be loading a ug…

We decided to make our own pTyr-protein standard based on earlier work*

*http://www.kendricklabs.com/PP_NK_AES-10_32P-ProteinStds.pdf

We purchased a recombinant receptor tyrosine kinase (RTK) protein fragment from ProQinase, Alk48

- Anaplastic lymphoma kinase (ALK) is a well-studied RTK with a pTyr activation mechanism similar to EGFR.
- Alk48 is a piece of the cytosolic chain containing the kinase domain.
- Has 13 tyrosines that potentially can be phosphorylated.

The tyrosine phosphorylation reaction was carried out in vitro as per ProQinase instructions

- ALK48 protein was added to Standard Assay Buffer + ATP.
- The reaction was allowed to proceed 60 min at 30^{o} C,

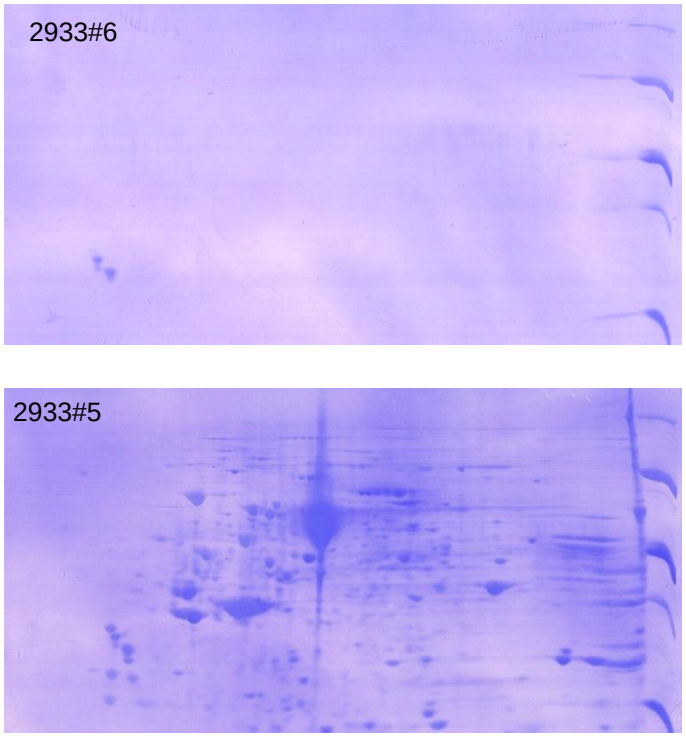
stopped with H_3P0_4, diluted with SDS buffer and aliquots run on CA-2D gels.

pTyr western blotting was used to check results.

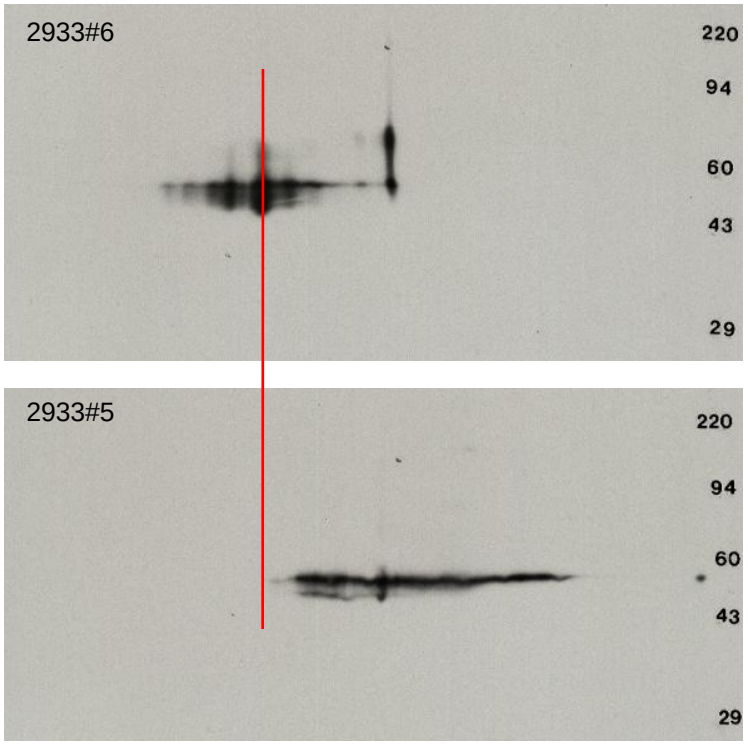
pAlk48 - pTyr Western Blot Results

100 ng pAlk48 only

100 ng pAlk48 + 200 µg human lung tumor



Coomassie stained PVDF shows total protein



Western blot obtained with anti-pTyr ab shows pAlk48 *changed position* in the presence of tumor sample.

Conclusions:

1. The tyrosine kinase reaction worked!
2. The variable, streaky pattern suggested incomplete phosphorylation.

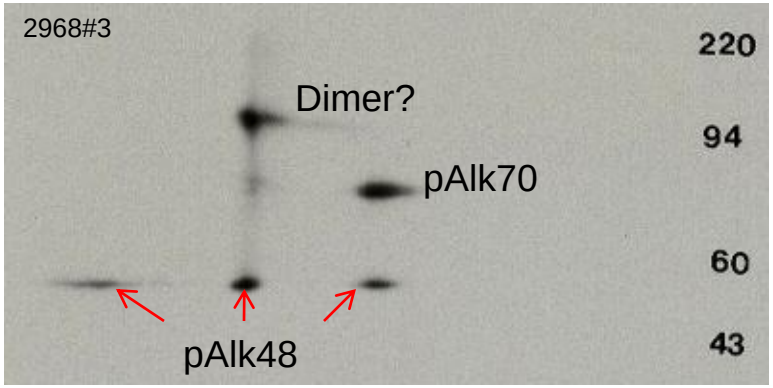
Looks like the kinase Rx is not going to completion. How can we force phosphorylation of all (most) tyrosines to 100%?

A ProQinase expert said:

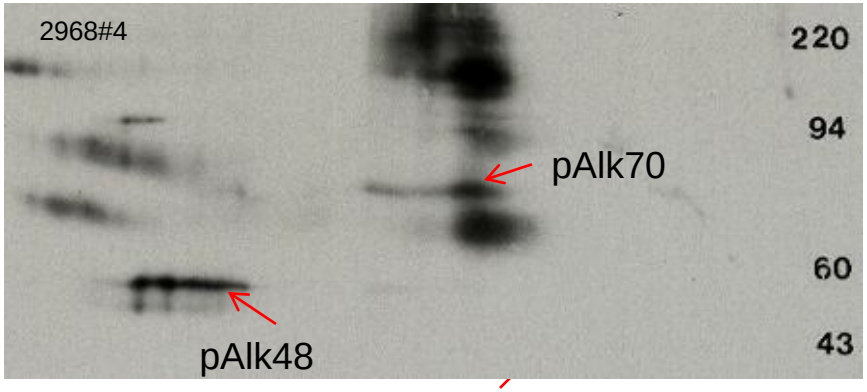
- Try omitting $MnCl_2$ which is known to decrease Vmax
- Vary $MgCl_2$ to maximize the kinase reaction

We did this for 2 proteins: Alk48 (E. coli) and Alk70 (insect sf9 cells). Checked results by 1D, looked good, then mixed the 48 and 70 kDa Alks for 2D pTyr WB

2D pTyr WB of combined pAlk48 + pAlk70



20 ng (pAlk48 + pAlk70)



20 ng (pAlk48 + pAlk70) + transformed 3T3 cells (positive pTyr control)

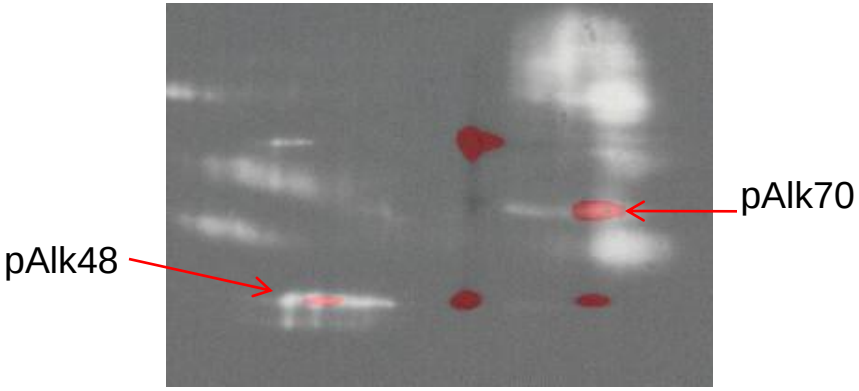
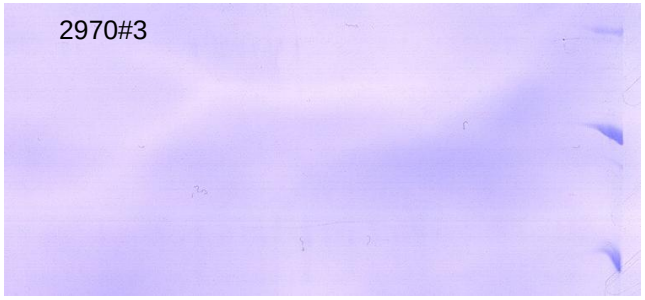


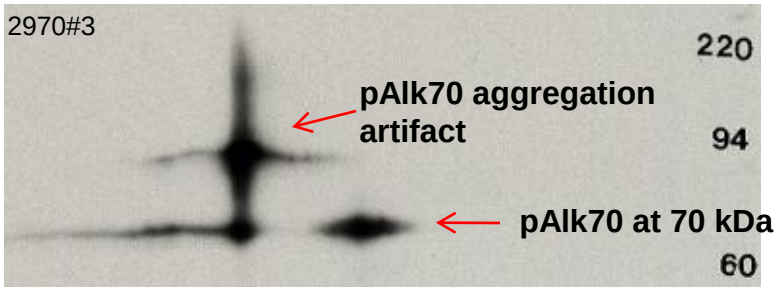
Image overlay

? Maybe the dimerization has something to do with mixing the 2 pAlks?
No, problems occurred for pAlk70 alone too.

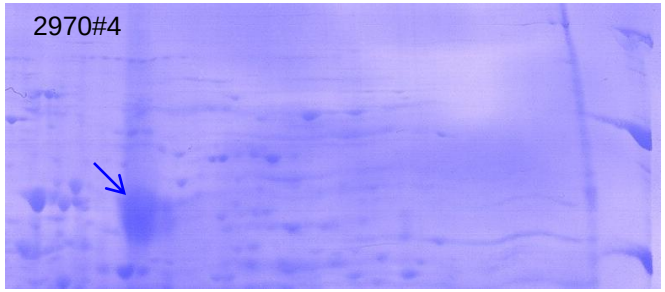
pAlk70 alone shows an aggregation artifact; pAlk70 in the presence of human tumor sample streaks.

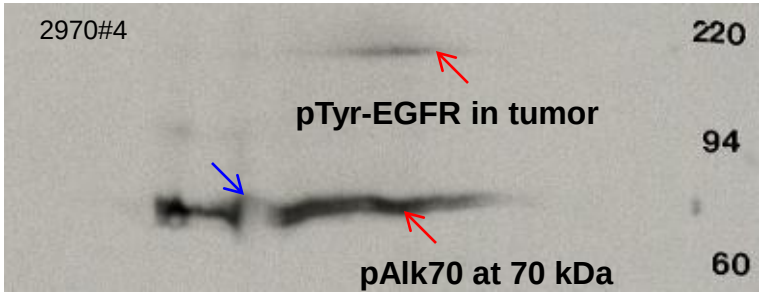
10 ng pAlk70 alone





10 ng pAlk70 + 200 µg tumor sample



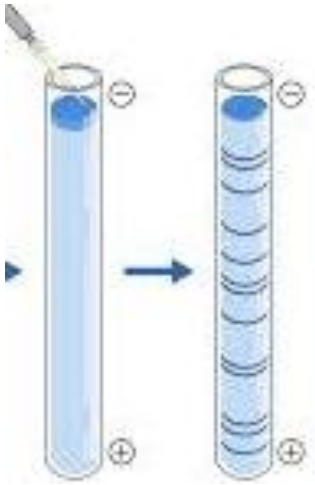


Coomassie stained PVDF shows total protein

Anti-pTyr WB film

This human tumor sample, 22803, showed a strong pTyr-EGFR signal in 2011 (see slide 4) that declined after 3 year storage at -80^{o} C. We think that pAlk70 streaking is caused by dimerization during IEF with unphosphorylated EGFR and other RTKs in the tumor.

Hypothesis on what's happening



IEF in tube gel

Known: As isoelectric focusing (IEF) progresses, negatively charged SDS is stripped off proteins to join micelles of NP-40, a nonionic detergent. The micelles migrate to the acidic end of the tube gel and form a bulb that is discarded.

Hypothesis: In the absence of SDS, the pAlk proteins renature during IEF, then dimerize with themselves or other RTK chains. IEF of the dimerized pAlk is impaired; it streaks. When SDS is added back prior to 2nd dimension, the pAlk proteins dissociate (mostly) and migrate normally on the slab gel, but at displaced IEF positions.

Dimerization is the problem, not incomplete phosphorylation.

Conclusions, future direction

1. The pAlk48 and pAlk70 have potential for being excellent internal standards, *if we can figure out a way to stop dimerization during IEF.*
2. Next, we'll try iodoacetylation of the pAlks to prevent dimerization. There are numerous cysteine and lysine targets spread throughout the Alk fragments.

Acknowledgements

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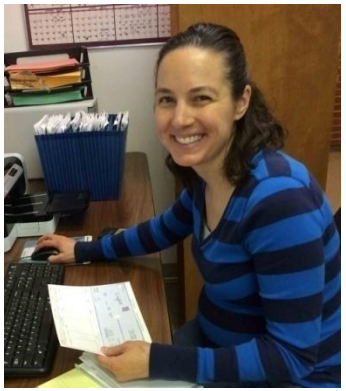
Matt Hoelter
Western Blot Manager
AES Executive Director

Andrew Koll
Biochemist



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Thanks to Dr. Daniel Muller for comments and advice
ProQinase Group Leader
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RTK mechanism, inspiration

The Biology of Cancer
by Robert Weinberg
Publisher: Garland Science
Second Edition 2013