Quantification of 2D Gel Western Blot Images from Human Tumor Samples

Nancy Kendrick, Matt Hoelter, Andrew Koll and Jon Johansen

Kendrick Labs, Inc, Madison, WI
www.kendricklabs.com

Introduction

Kendrick Labs, Inc is a contract research organization; our clients are mostly scientists in pharmaceutical companies and academia. The goal of this project is to develop a test that will be useful for cancer researchers studying signaling pathways.

Previously, we have shown that *CA-2DE, in combination with **ECL western blotting can detect and identify low abundance tyrosine kinase (TK) proteins.

However, these results consist of western blot pictures. In order to be useful, the results must be *quantified and presented in a table as numbers*. This talk is about our preliminary quantification work.

- Note: Many TKs are difficult to dissolve. Only one buffer works well: ***SDS!
  Our carrier ampholine 2D system is compatible with SDS.
- SDS strongly interferes with mass spectrometry and IPG strip 2D; most core labs avoid it. They use SDS-free buffers and centrifuge out “cellular debris” containing important proteins. Kendrick Labs offers a unique method for visualizing TKs.

**CA-2DE: carrier ampholine 2D electrophoresis, **ECL: enhanced chemiluminescence, ***SDS: sodium dodecyl sulfate*

Receptor tyrosine kinase mechanism

- RTKs are large trans-membrane proteins. Ligand binding triggers dimerization, leading to trans-phosphorylation of tyrosines on the cytoplasmic chains (red circles with P)
- Then, c*ytoplasmic proteins* with affinity for specific phosphotyrosines relocate to the membrane, become activated, and trigger cascades of cell growth reactions.
- For more information see: Biology of Cancer, 2nd Ed. by Robert Weinberg, especially the EGFR movie on the CD.

Unphosphorylated RTKs are present in many tissues.

If no tyrosine phosphorylation, then no RTK activity!

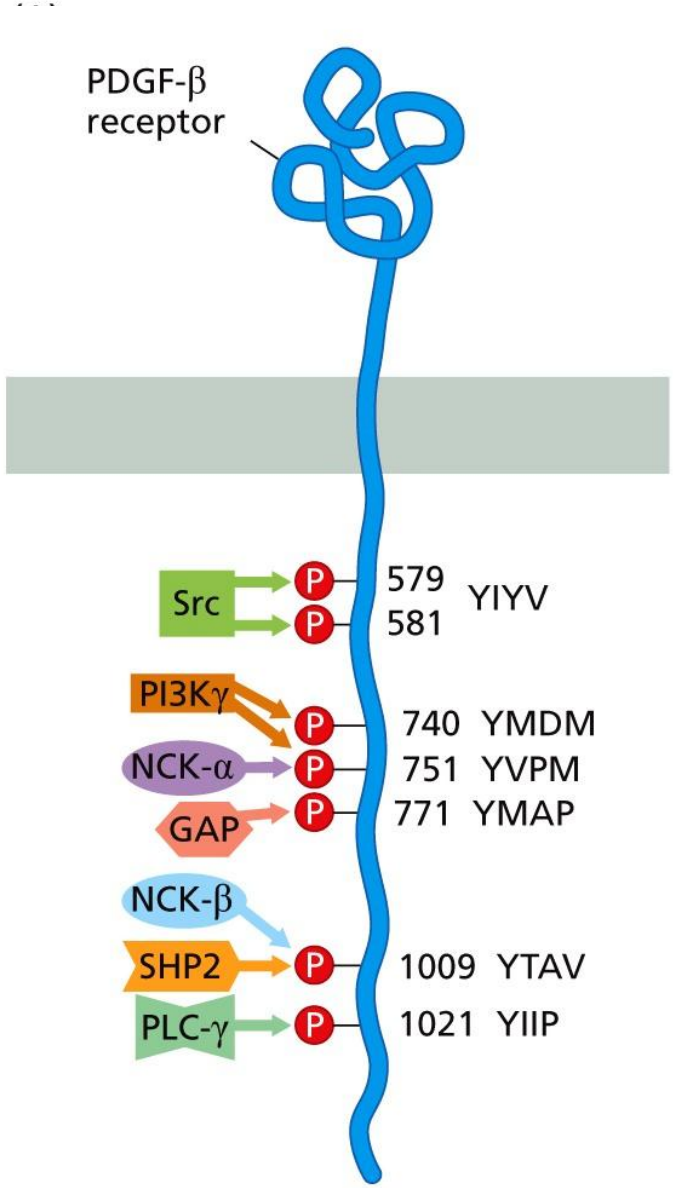


Figure 6.9 The Biology of Cancer (© Garland Science 2014)

Pharma companies have already developed inhibitors for several TKs involved in lung cancer. It's a hot research topic.

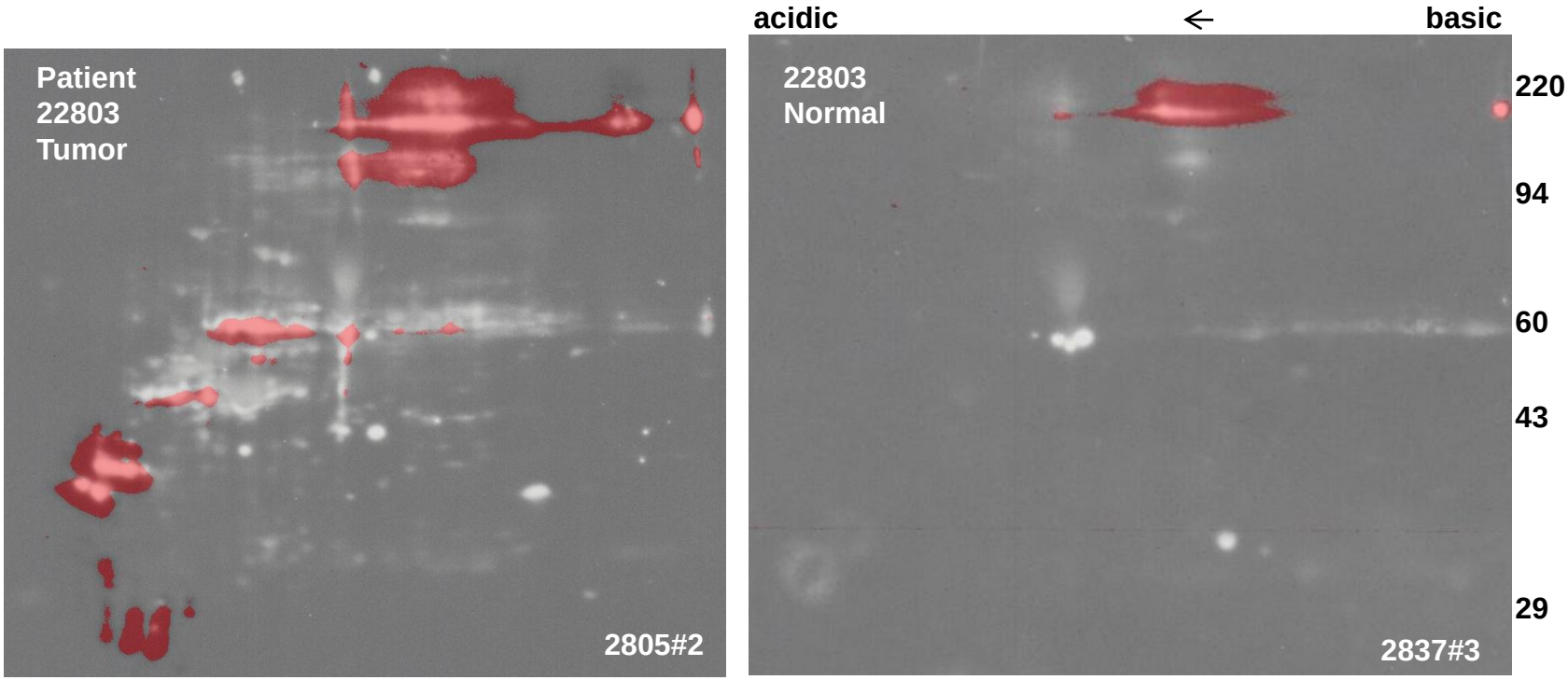
Tyrosine Kinase Protein	Abbrevation	Molecular weight	Inhibitor	Ref
Epidermal Growth Factor Receptor	EGFR	170,000	Several	[1,2]
Anaplastic Lymphoma Kinase	ALK	176,000	Crizotinib	[3]
Platelet Derived Growth Factor Receptor	PDGFR	175,000	Sorafenib	[4]
Hepatocyte Growth Factor Receptor	cMET, HGFR	160,000	Several	[5]
SRC (Cytoplasmic TK)	SRC	60,000	Dasatinib	[6]

Receptor tyrosine kinases tend to be around the same molecular weight and don't resolve on 1D gels. They're hard to measure. Genomic tests are only partially successful. A 2DE test that directly measures protein TK drivers should be useful to pharma companies.

References

1. Bronte, G., et al., Are erlotinib and gefitinib interchangeable, opposite or complementary for non-small cell lung cancer treatment? Critical reviews in oncology/hematology, 2014. 89(2): p. 300-13.
2. Roengvoraphoj, M., et al., Epidermal growth factor receptor tyrosine kinase inhibitors as initial therapy for non-small cell lung cancer: focus on epidermal growth factor receptor mutation testing and mutation-positive patients. Cancer treatment reviews, 2013. 39(8): p. 839-50.
3. Qian, H., et al., The efficacy and safety of crizotinib in the treatment of anaplastic lymphoma kinase-positive non-small cell lung cancer: a meta-analysis of clinical trials. BMC Cancer, 2014. 14: p. 683.
4. Bria, E., S. Pilotto, and G. Tortora, Sorafenib for lung cancer: is the "Battle" still open? Expert Opin Investig Drugs, 2012. 21(10): p. 1445-8.
5. Scagliotti, G.V., S. Novello, and J. von Pawel, The emerging role of MET/HGF inhibitors in oncology. Cancer Treat Rev, 2013. 39(7): p. 793-801.
6. Gold, K.A., et al., A Phase I/II Study Combining Erlotinib and Dasatinib for Non-Small Cell Lung Cancer. Oncologist, 2014. 19(10): p. 1040-1.

Previously, we have identified active EGFR* in lung cancer samples via western blotting overlays.



2D western blot overlays from tumor (left) and normal (right) tissue samples. EGFR (red) over pTyr (white) after stripping and reprobing same blot. *EGFR = epidermal growth factor receptor.

Results must be expressed as a number to compare many samples

Ultra high-sensitivity ECL western blotting is picky. Quantification is not trivial.

Enhanced Chemiuminescent (ECL) Western Blotting

First, proteins are transferred from a 2D gel to a paper-like membrane, Hybond ECL or PVDF.

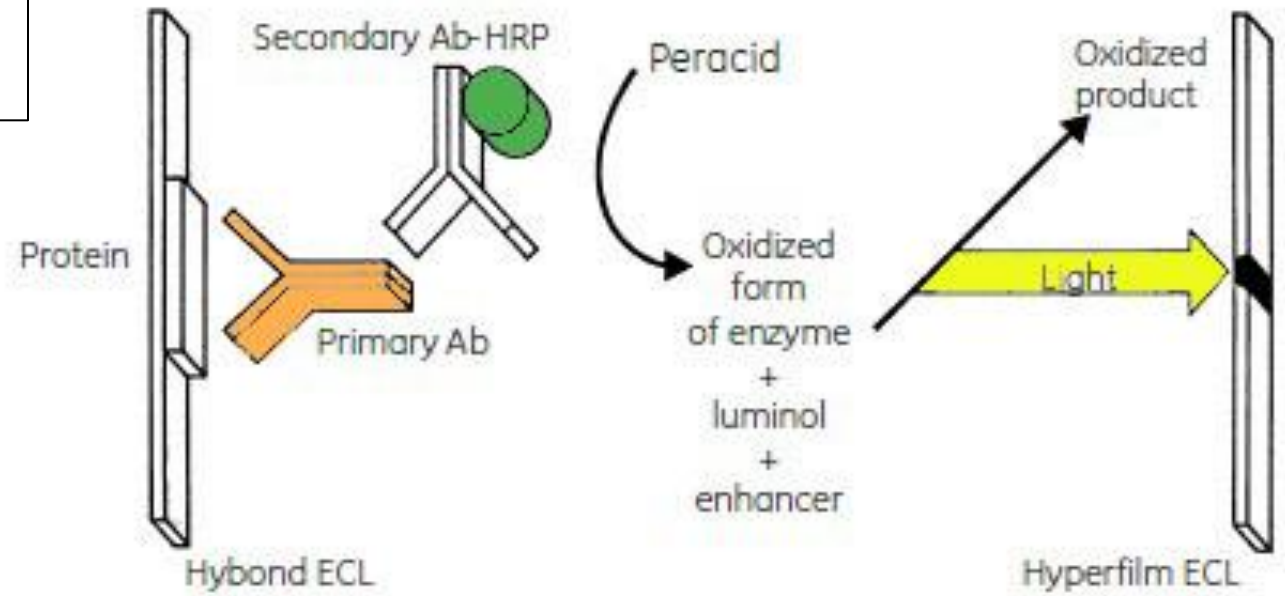


Figure 1. Principles of ECL Western blotting

Figue 1 is taken from Amersham ECL Western Blotting detection reagents and analysis system, Product Booklet Code RPN2106/8/9. (GE Healthcare) HRP = horse radish peroxidase.

The ECL light reaction peaks at about 15 min. The darkness of a film pattern depends on where you are in the curve.

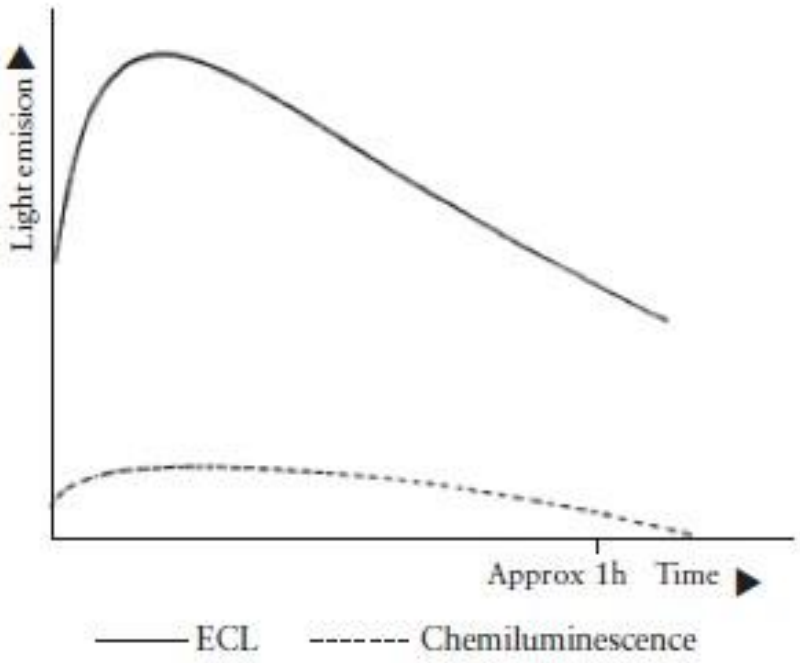
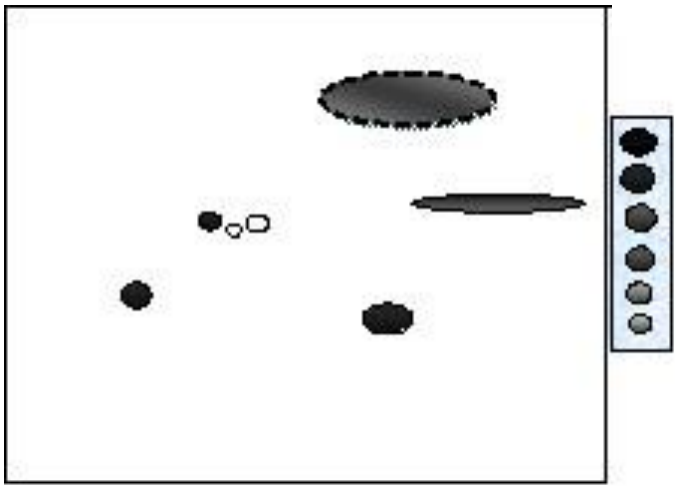


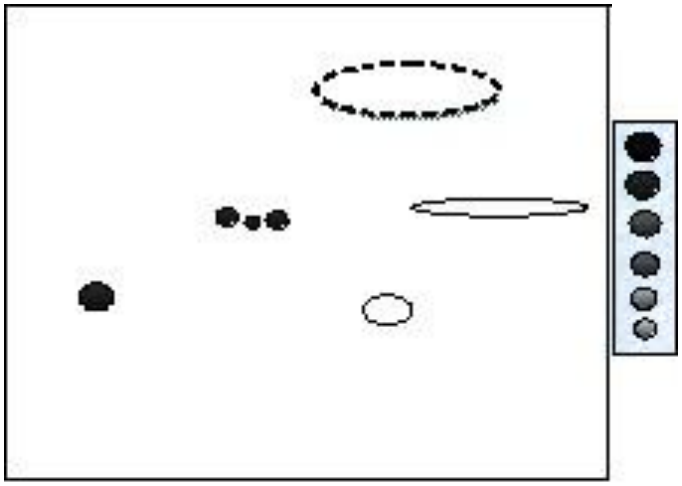
Figure 3. Graph of light emission versus time, showing the difference between chemiluminescence and ECL.

Taken from Amersham ECL Western Blotting detection reagents and analysis system, Product Booklet Code RPN2106/8/9. (GE Healthcare) ECL light peaks at about 15 minutes.

Results *within* a given ECL WB are quantitative. The problem is comparing *between* blots.



Tumor



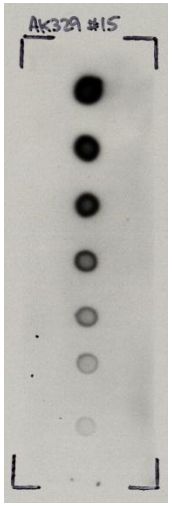
Normal

We decided to add a dot blot standard curve strip to every 2D western blot. If the plot showed linearity, results could be normalized relative to one of the dots.

Optimizing the dot blots wasn't trivial.

- HRP-secondary antibody gave linear dot blot plots, but wasn't stable. Various conditions had to be worked out.
- Finally, the dot blots were standardized. All following results are from dot blots loaded with a mouse/rabbit IgG mixture.

ng/dot:
12
8
4
2
1
0.5
0.1



Amersham Hyperfilm (GE Healthcare)
versus
Kodak Biomax (Sigma)

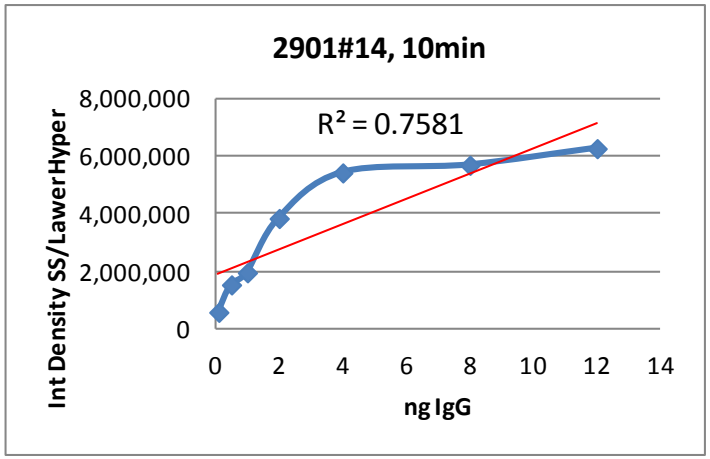
We use both. Which is better for quantification?

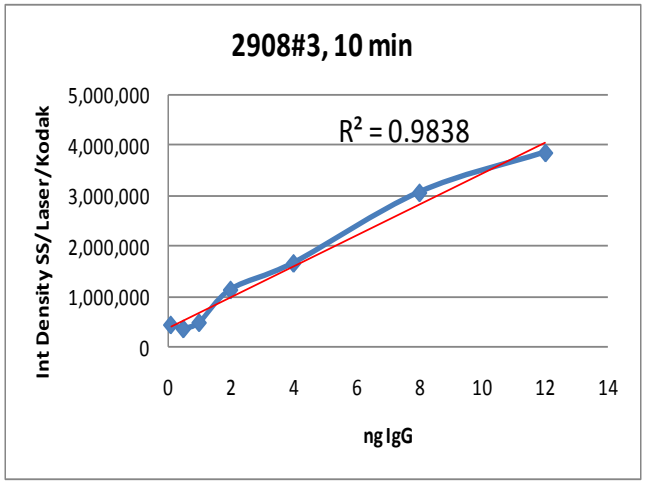
Test method

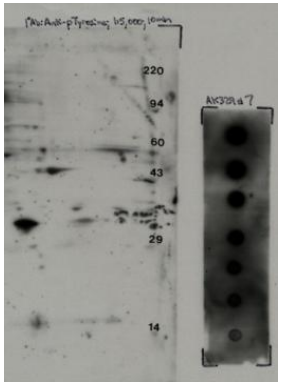
- Ran 4 pairs of Tumor/Normal samples in duplicate (8 2D gels x 2) Western blotted one set with Hyperfilm and the other with Kodak. Included identical dot blots on all.

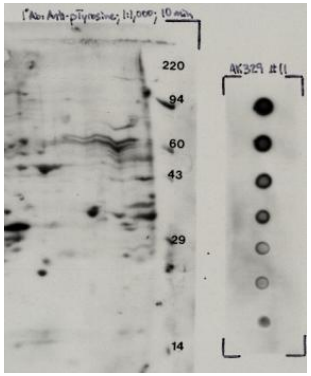
- Scanned films with our calibrated laser densitometer - linear from 0 – 3 OD.
- Analyze with SameSpots software (TotalLab, UK.)

Examples: Hyperfilm versus Kodak Biomax









Hyperfilm,10 min (2901#14)

Kodak, 10 min (2908#3)

Dot blot results:

Amersham ECL Hyperfilm			Kodak Biomax MR		
Sample	Gel ID, exposure time	Dot blot R^2	Sample	Gel ID, exposure time	Dot blot R^2
30417 T	Optimization		30417 T	2908#1, 3 min	0.9917
				2908#1, 10 min	0.9830
30417 N			30417 N	2908#2, 3 min	0.9925
				2908#2, 10 min	0.9356
30934 T			31026 T	2908#3, 3 min	0.9917
				2908#3, 10 min	0.9838
30934 N			31026 N	2908#4, 3 min	0.9689
				2908#4, 10 min	0.9856
31102 T	2901#12, 3 min	0.9618	31102 T	2908#5, 3 min	0.9163
	2901#12, 10 min	0.8695		2908#5, 10 min	0.9789
31102 N	2901#13, 3 min	0.9639	31102 N	2908#6, 3 min	0.9838
	2901#13, 10 min	0.9185		2908#6, 10 min	0.9739
31026 T	2901#14, 3 min	0.9488	22803 T	2908#7, 3 min	0.9977
	2901#14, 10 min	0.7581		2908#7, 10 min	0.9534
31026 N	2901#15, 3 min	0.9429	22803 N	2908#8, 3 min	0.9740
	2901#15, 10 min	0.6965		2908#8, 10 min	0.9677
Hyperfilm average R^2		**0.8825**	**Kodak average R^2**		**0.9737**

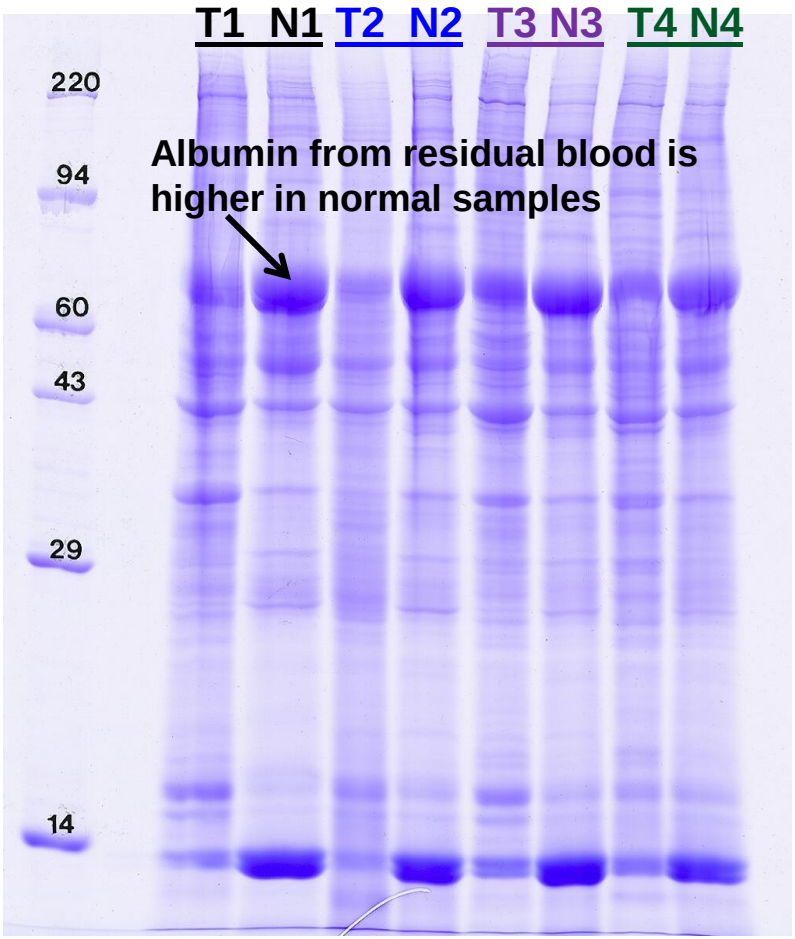
Conditional formatting:
$R^2 < 0.95$ = red

Kodak Biomax film is better for quantification. Reasonable criteria for acceptance: $R^2 \geq 0.95$ for dot blot.

Analysis of human lung tumor samples

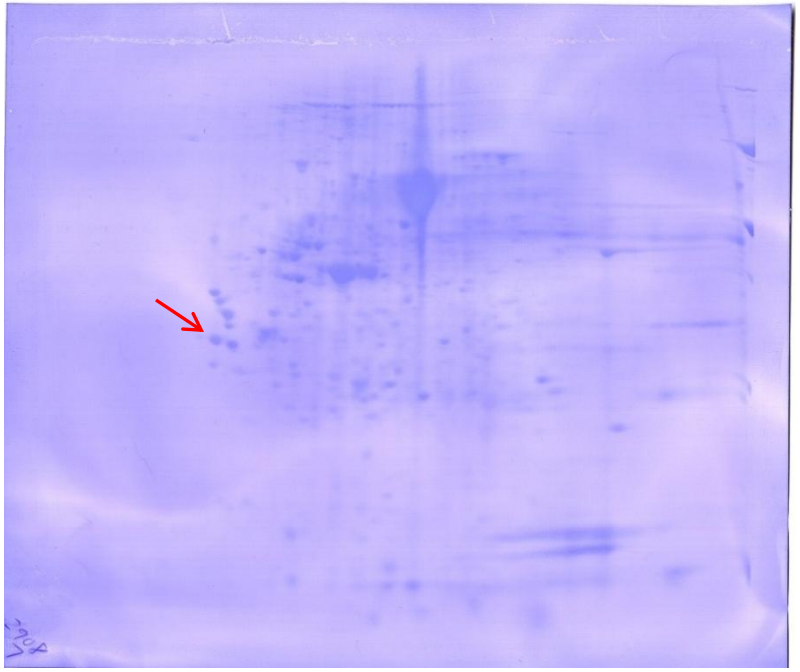
Consider:

1. Protein patterns from tumors are different than those from matched normal tissue. The latter is bloodier in this case.
2. We're trying to quantify *low-abundance TKs*. High abundance proteins will be ignored.

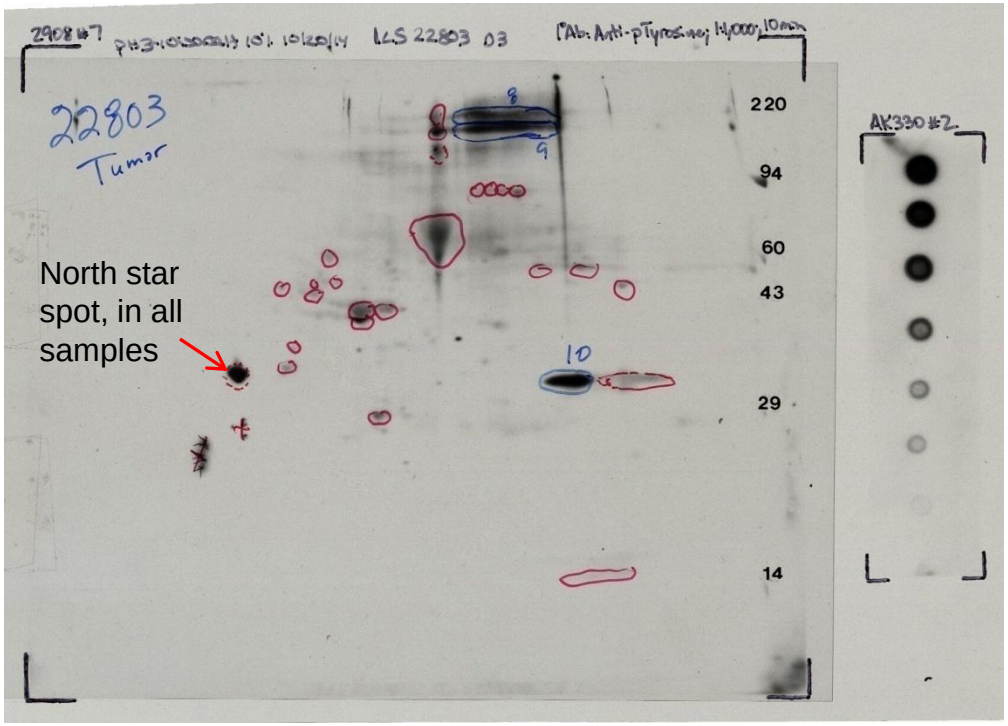


Four matched pairs of human squamous cell carcinoma. T = tumor, N = normal tissue.

High abundance proteins are visible on the Coomassie-stained PVDF blot



Coomassie-stained PVDF



WB film exactly matches Coomassie PVDF

1. Cover ECL film with transparency.
2. Align film with printed PVDF Coomassie image using corner marks.
3. Outline Coomassie-stained proteins in red on transparency.
4. Outline low abundance protein spots unique to film in blue (8, 9 & 10 above.)
5. Analyze low abundance proteins in all samples using SameSpots software.

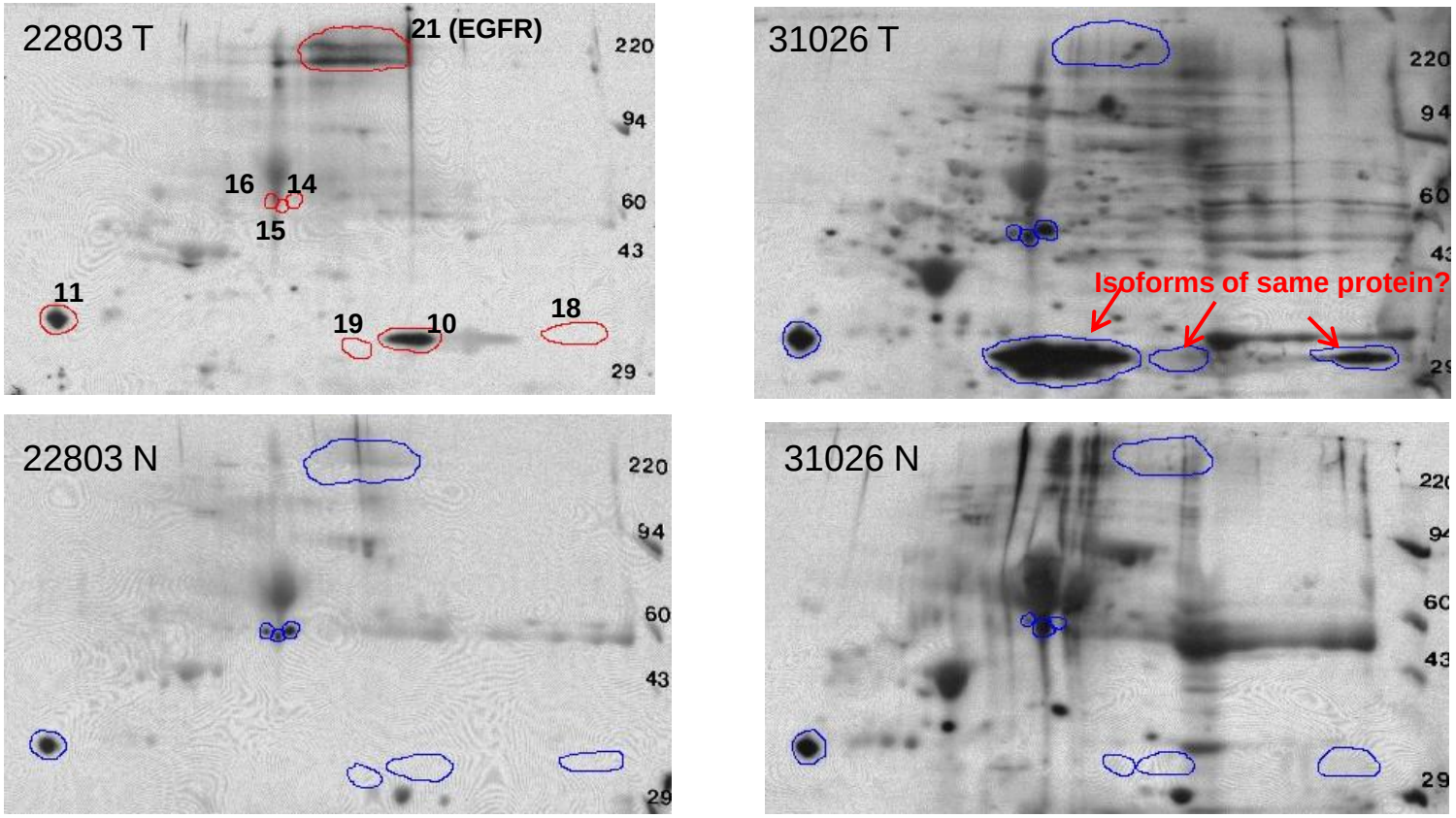
Quantitative Analysis using SameSpots software

Analysis Steps

1. Scan the films with a calibrated laser densitometer.
2. Align the 2D gel images on the computer screen.
3. Hand outline and match spots of interest.
4. Subtract background, normalize spot densities, create montages.
5. Export data to Excel.

TOTALLAB

Eight pTyr spots of interest were outlined in every image.



Examples of two matched pTyr western blots pairs: 22803 tumor (top left), 22803 normal (bottom left); 31026 tumor (top right), 31026 normal (bottom right.) Spot numbering is shown in the upper left image only. Spot 11 is the “North Star”. If abundant proteins are disallowed, only a few proteins differ between tumor and matched normal tissue. The EGFR 175 kDa protein is not present in the 31026 film, but a different protein at ~30 kDa is strongly lighting up.

Quantitative Results from SameSpots software.

	2908#05 31102-T 3 min	2908#05 31102-D 10 min	2908#06 31102-N 3 min	2908#06 31102-N 10 min	2908#07 22803-T 3 min	2908#07 22803-T 10 min	2908#08 22803-N 3 min	2908#08 22803-N 10 min	2908#01 30417-T 3 min	2908#01 30417-T 10 min	2908#02 30417-N 3 min	2908#02 30417-N 10 min	2908#03 31026-T 3 min	2908#03 31026-T 10 min	2908#04 31026-N2 3 min	2908#04 31026-N 10 min
ng IgG	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.	Norm. Vol.
12	1,914.2	702.2	833.7	383.3	991.5	421.8	669.7	321.7	921.6	496.8	720.7	339.9	602.8	341.3	854.6	530.5
8	711.7	361.0	440.7	264.8	654.0	330.6	335.0	189.3	194.5	165.3	516.7	321.6	404.0	268.9	432.5	328.9
4	257.0	195.9	265.0	190.8	322.8	233.0	172.0	140.0	232.2	213.0	216.6	176.8	165.0	156.3	256.7	225.3
2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	30.5	40.1	38.1	46.0	22.0	27.9	40.2	46.1	30.5	38.4	30.4	32.9	29.3	40.4	33.6	35.3
0.5	25.8	34.3	15.7	19.4	11.4	15.8	19.0	23.3	14.7	17.8	40.2	38.7	22.4	29.6	51.1	50.7
0.1	-	4.3	6.5	6.1	-	3.6	9.5	12.3	-	2.8	18.1	18.7	27.4	34.8	25.0	27.1
Spot #																
10	28.2	18.6	8.6	3.5	441.7	237.1	4.7	1.9	38.2	31.6	5.4	4.3	434.6	210.2	8.6	6.9
11	330.7	158.3	41.5	34.0	219.6	126.4	108.3	71.6	878.0	391.0	472.7	200.1	402.1	198.5	461.3	286.8
14	174.8	68.4	48.5	21.4	0.5	0.4	32.5	22.6	266.7	110.6	1.3	2.5	0.4	0.3	105.4	39.0
15	33.4	21.4	25.9	10.0	2.3	5.6	17.1	11.0	107.4	50.4	162.4	54.7	5.3	4.2	38.5	32.7
16	2.4	2.8	5.8	5.3	0.6	0.5	10.2	9.3	21.2	14.0	9.5	4.6	1.2	0.8	11.0	8.3
18	15.8	3.5	7.6	2.8	7.3	2.6	4.9	1.4	899.0	428.0	9.6	5.3	10.6	11.7	12.7	3.7
19	112.6	84.3	3.7	0.9	3.1	1.0	2.3	0.6	7,360.2	3,012.2	4.3	2.7	3.2	1.1	4.2	2.5
21	71.9	46.0	48.3	52.8	594.3	473.0	28.4	34.3	210.0	159.4	102.9	45.4	37.6	47.9	36.0	13.5

Table 1. Raw data from SameSpots software. Integrated density within a spot outline normalized as a percent of the 2 ng spot on the dot blot for that film.

Comparison of 3 & 10 min film exposures

		31102 T/N	**31102** T/N	**22803** T/N	**22803** T/N	**30417** T/N	**30417** T/N	**31026** T/N	**31026** T/N
Spot #	MW	3 min*	10 min	3 min	10 min	3 min	10 min*	3 min	10 min
21	175 kDa	1.5	0.9	**21**	**14**	2.0	3.5	1	4
14	60 kDa	3.6	3.2	0.01	0.02	211	44	0.004	0.01
15	60 kDa	1.3	2.1	0.1	0.5	0.7	0.9	0.14	0.13
16	60 kDa	0.4	0.5	0.1	0.1	2.2	3.0	0.11	0.10
18	30 kDa	2.1	1.3	1.5	1.9	94	81	0.8	3.2
10	30 kDa	3.3	5.4	93	125	7.1	7.4	51	30
19	30 k Da	30	90	1.3	1.7	1700	1129	0.8	0.4
11	32 kDa	8.0	4.7	2.0	1.8	1.9	2.0	0.9	0.7
		2908 #5* & 6		2908# 7&8		2908 #1&2*		2908 #3&4	

Table 2. Tumor/normal ratios for two film exposures. T/N ratio >10, red; < 0.1, green. Ideally, the two film exposures would give the same value. *R^2 values for dotblots < 0.95.

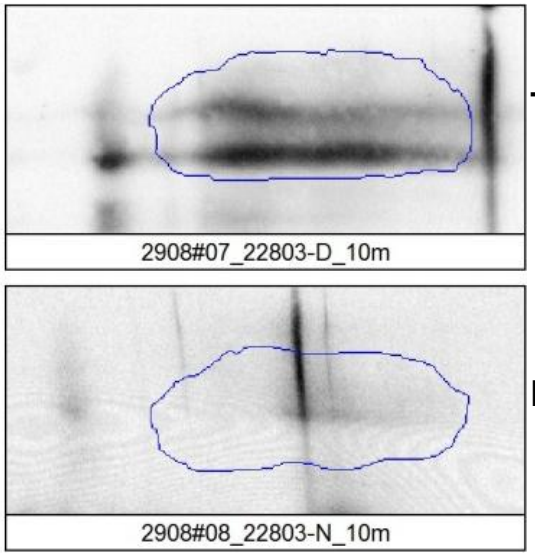
- There is fairly good agreement between 3 and 10 min exposures.
- Setting the red/green cutoff to detect $\geq$ 10-fold changes highlights the same spot regardless of film exposure.

Final Report: Does it reflect actual differences in films?

	pTyr-Protein Fold Increase: Tumor/Normal							
Patient #	S21, EGFR	S18, 30-A	S10, 30-B	S19, 30-C	S14, 60-X	S15, 60 -Y	S16, 60-Z	S11 32-marker
31102	1	2	4	60	3	2	0.5	6
22803	17	2	109	2	0.02	0.3	0.05	2
30417	3	87	7	1414	127	0.8	2.6	2
31026	2	2	41	1	0.01	0.1	0.1	1

Table 2. Fold change (Tumor/Normal ratios) for eight pTyr proteins in four samples. The 3 and 10 min exposure values were averaged. Red: ratio > 10; Green: ratios < 0.1.

Spot 21, EGFR
175 kDa
Patient 22803



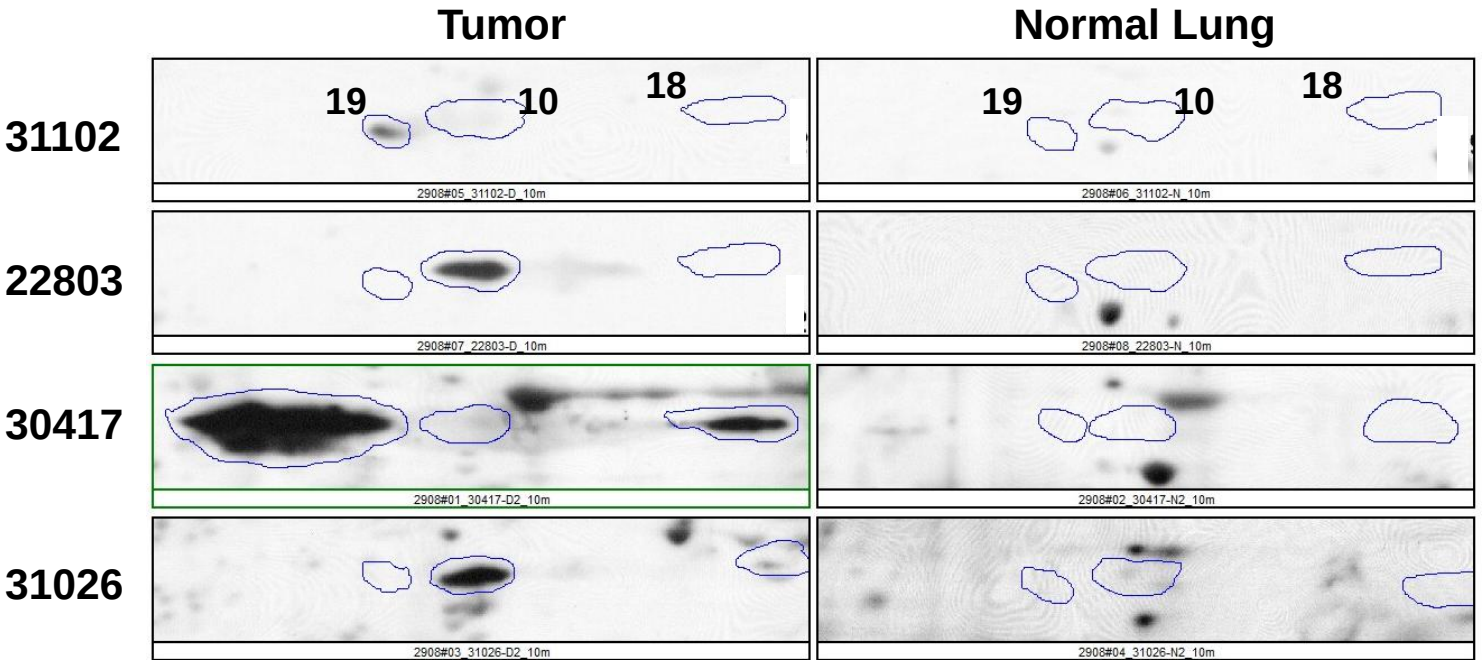
Tumor

Normal

Spots 19, 10, & 18

	pTyr-Protein Fold Increase: Tumor/Normal							
Patient #	S21, EGFR	S19, 30-C	S10, 30-B	S18, 30-A	S14, 60-X	S15, 60 -Y	S16, 60-Z	S11 32-marker
31102	1	60	4	2	3	2	0.5	6
22803	17	2	109	2	0.02	0.3	0.05	2
30417	3	1414	7	87	127	0.8	2.6	2
31026	2	1	41	2	0.01	0.1	0.1	1

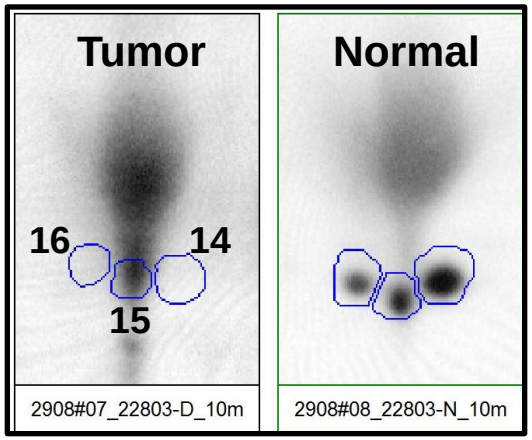
Table 2. Fold change (Tumor/Normal ratios) for eight pTyr protein spots in four samples. Red: T/N ratio > 10; Green: T/N ratio < 0.1.



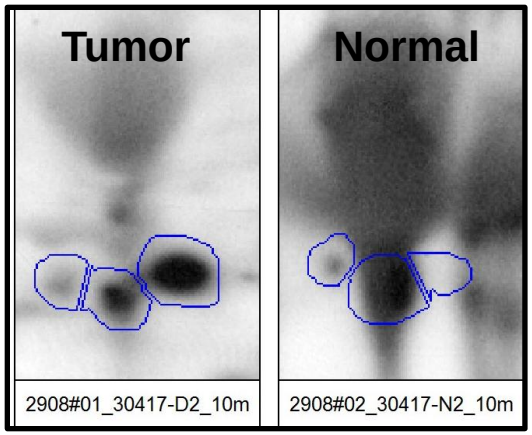
Spots 14 &16 are much darker in normal tissue for 2 patients.

	pTyr-Protein Fold Increase: Tumor/Normal							
Patient #	S21, EGFR	S19, 30-C	S10, 30-B	S18, 30-A	S14, 60-X	S15, 60 -Y	S16, 60-Z	S11 32-marker
31102	1	60	4	2	3	2	0.5	6
22803	17	2	109	2	0.02	0.3	0.05	2
30417	3	1414	7	87	127	0.8	2.6	2
31026	2	1	41	2	0.01	0.1	0.1	1

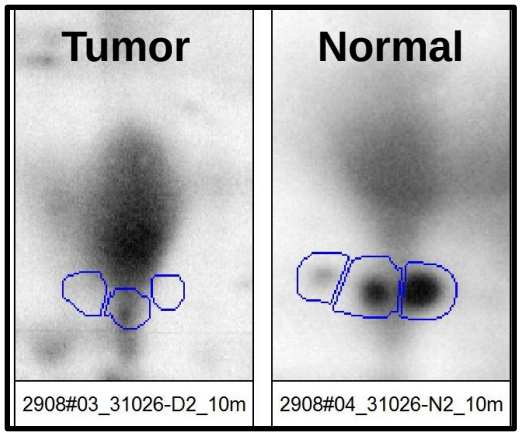
Table 2. Fold change (Tumor/Normal ratios) for eight pTyr proteins in four samples.
Red: T/N ratio > 10; Green: T/N ratios < 0.1.



22803



30417



31026

Conclusions:

1. Numerical results presented in the final table show good agreement with differences observed visually on the ECL films. This preliminary work suggests that protein differences in 2D gel western blots can be expressed numerically.

2. The dot blot standard curves provide criteria for quantitative comparisons.

Future work:

1. Validate the method to determine within-day and between-day variability.

Acknowledgements

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Jon Johansen
Lab Manager

Matt Hoelter
Western Blot Manager

Andrew Koll, Biochemist

TOTALLAB

Thanks to the 2D software developers at TotalLab
http://www.totallab.com

RTK mechanism, inspiration

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