

Western Blot Image Analysis

1. Purpose

To measure the relative intensity of bands observed by western blotting using pixel information obtained using Kodak Molecular Imaging Software Version 4.0. To determine if there is a statistically significant difference in signal intensity between wildtype and mutant protein extracts for a western blot band of interest.

2. Procedure

2.1 Processing images with Kodak Molecular Imaging Software Version 4.0

Using the Kodak Molecular Imaging Software Version 4.0, open the required “.bip” file.

Select Manual ROIs. Select New ROI Set. Select ROI Rectangle tool and draw a rectangle around the object of interest. Copy the rectangle to create a series of comparable objects of interest on a single membrane, ensuring the area of the rectangle is identical for each object, contains the entire object in each case and does not overlap with other objects to be compared.

Use the ROI (Region of Interest) Analysis Data window to view the information about the selected ROI Set. For each membrane or series of comparable measurements, create a new ROI Set.

Export images as “.jpg” files and ROI analysis data as “.txt” files.

2.2 Statistical analysis with Microsoft Excel

To perform a t-test analysis of mean intensity measurements from Kodak Molecular Imaging Software import ROI analysis data to Microsoft Excel software from the exported “.txt” file.

Data points for 4 wildtype and 4 mutant samples are paired by spatial arrangement on gel and compared pairwise to minimise the impact of subtle background artifacts on image analysis.

The \log_{10} transformation of mean intensity values is calculated to convert the data distribution from a skewed distribution to a more symmetric distribution that better suits the “normality” assumptions of the subsequent t-test.

$$\log_{10}(\text{mean intensity}) = \text{LOG10}(\text{mean intensity})$$

Determine the ratio of the \log_{10} (mean intensity) values for each wildtype-mutant pair
 $\log_{10} \text{ ratio} = \text{LOG10}(\text{mutant mean intensity}) - \text{LOG10}(\text{wildtype mean intensity})$

Calculate the average of the \log_{10} ratios across all wildtype-mutant pairs

$$\text{AV} = \text{AVERAGE}(\text{LOG10 ratio1}:\text{LOG10 ratio4})$$

Calculate the standard deviation of the \log_{10} ratios across all wildtype-mutant pairs

$$SD=STDEV(\text{LOG}_{10} \text{ ratio1}:\text{LOG}_{10} \text{ ratio4})$$

Calculate the standard error of the mean of the \log_{10} ratios across all wildtype-mutant pairs (use this value for error bars when plotting average \log_{10} ratios)

$$SE=SD/(\text{SQRT}(n = 4 \text{ pairs of samples}))$$

Calculate the t-statistic for the \log_{10} ratios across all wildtype-mutant pairs

$$t\text{-statistic}=AV/SE$$

Calculate the p-value for the above t-statistic to determine the significance of the \log_{10} ratios across all wildtype-mutant pairs (Null hypothesis = there is no difference between the wildtype-mutant pairs, i.e. $AV=0$)

$$p\text{-value}=\text{TDIST}(\text{ABS}(t\text{-statistic}), n-1 \text{ pairs of samples} =3, 2 \text{ tail distribution})$$

Using a p-value significance cut-off of 0.05;

- p-value > 0.05, no significant difference between wildtype and mutant \log_{10} mean intensities.
- p-value <0.05, significant difference between wildtype and mutant \log_{10} mean intensities.

(Note: A paired t-test analysis will yield the same p-value as this t-test).

3. Materials

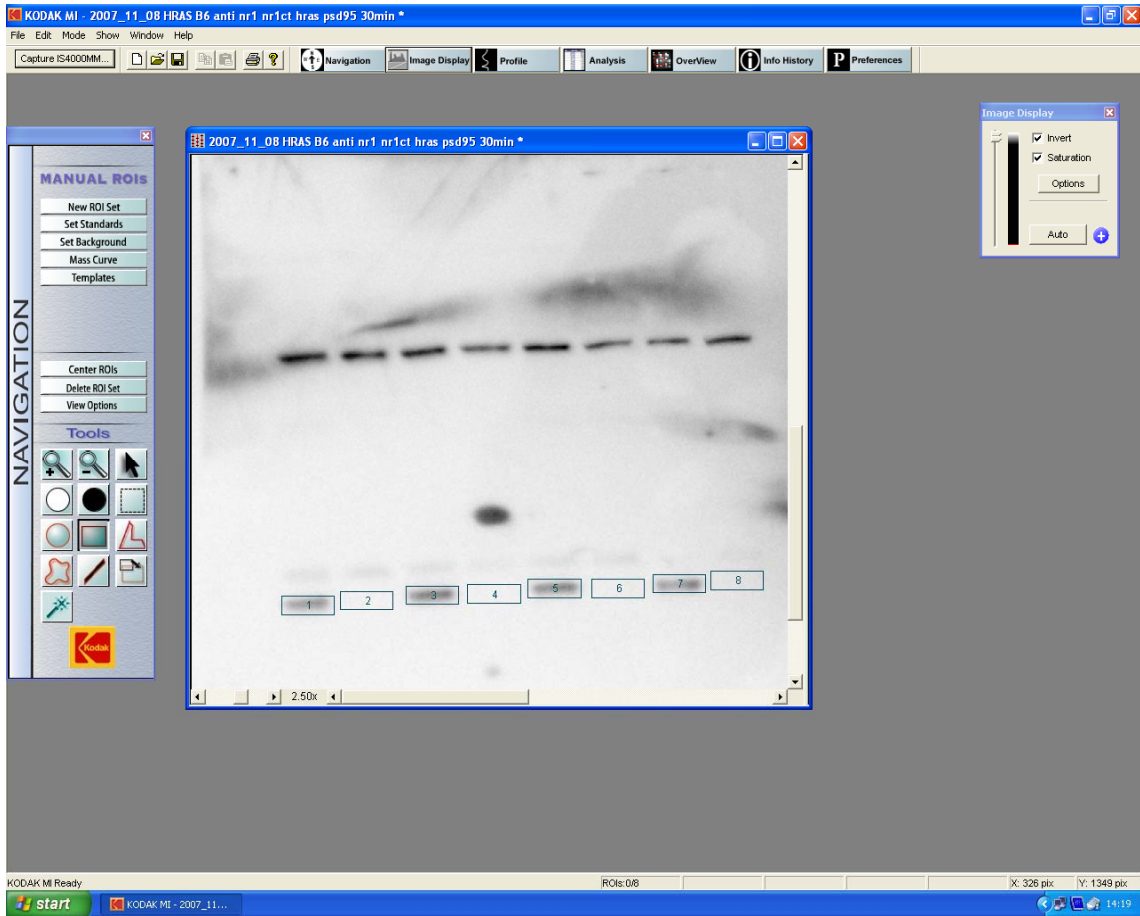
Kodak Molecular Imaging Software Version 4.0
Microsoft Excel software

4. Quality Control

Care must be taken to ensure that the ROI is an identical size and shape for each object to be compared.

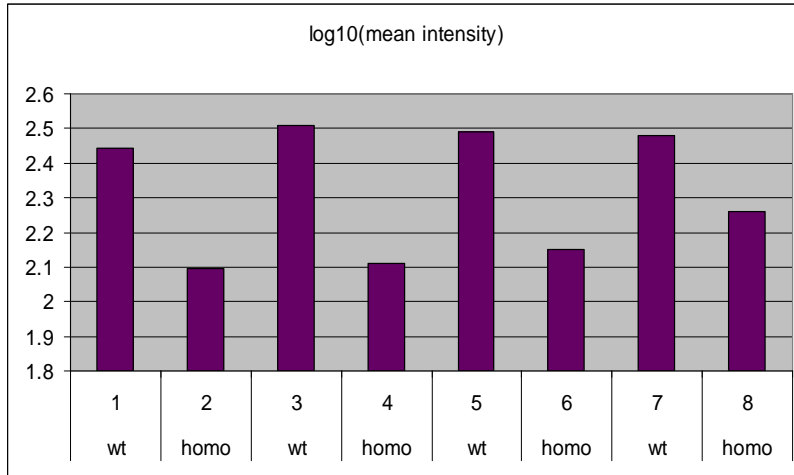
5. Example Data

5.1 KODAK MI software display



5.2 Region of interest data statistical manipulation with Microsoft Excel

Mouse line HRAS B6
Date captured 2007_11_08
Antibody details anti-HRAS Santa Cruz sc-29 1:500,
 anti-mouse HRP Upstate 12-349 1:15000
Exposure time 30 min



Genotype	Serial Number	ROI Type	Net Intensity	Sum Intensity	Mean Intensity	Interior Area (pixels)	log10 (mean intensity)	log10 ratio (mean intensity)
Wt	1	Rect	214490	561278	276.7643127	2028	2.442110089	
Homo	2	Rect	-933	252567	124.5399399	2028	2.095308652	-0.346801438
Wt	3	Rect	293576	654560	322.7613525	2028	2.508881527	
Homo	4	Rect	-1911	261729	129.0576935	2028	2.110783899	-0.398097628
Wt	5	Rect	270153	627081	309.2115479	2028	2.490255705	
Homo	6	Rect	-3356	286648	141.3451691	2028	2.15028097	-0.339974735
Wt	7	Rect	224733	610053	300.815094	2028	2.478299624	
Homo	8	Rect	-7054	368126	181.521698	2028	2.258928545	-0.219371079

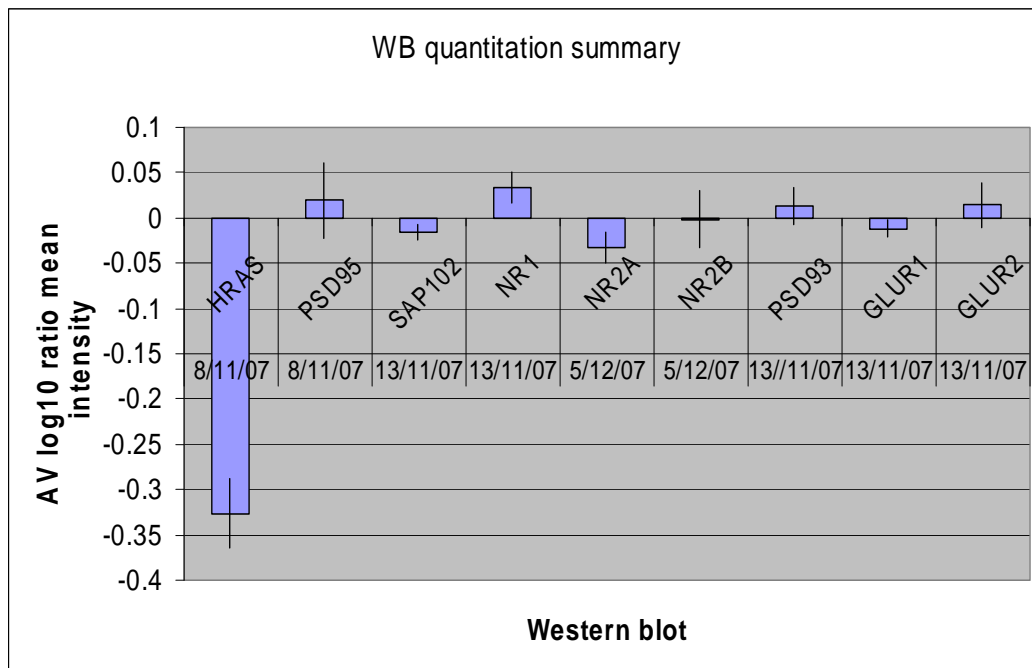
average log10ratio (mean intensity)	std dev log10ratio (mean intensity)	std error (n=4 pairs)	t-statistic (AV/SE)	p-value (df=n pairs-1=3, 2 tailed)
-0.32606122	0.075709473	0.037854736	-8.613485413	0.003290428

5.3 Region of interest data analysis summary with Microsoft Excel

Western blot summary statistics

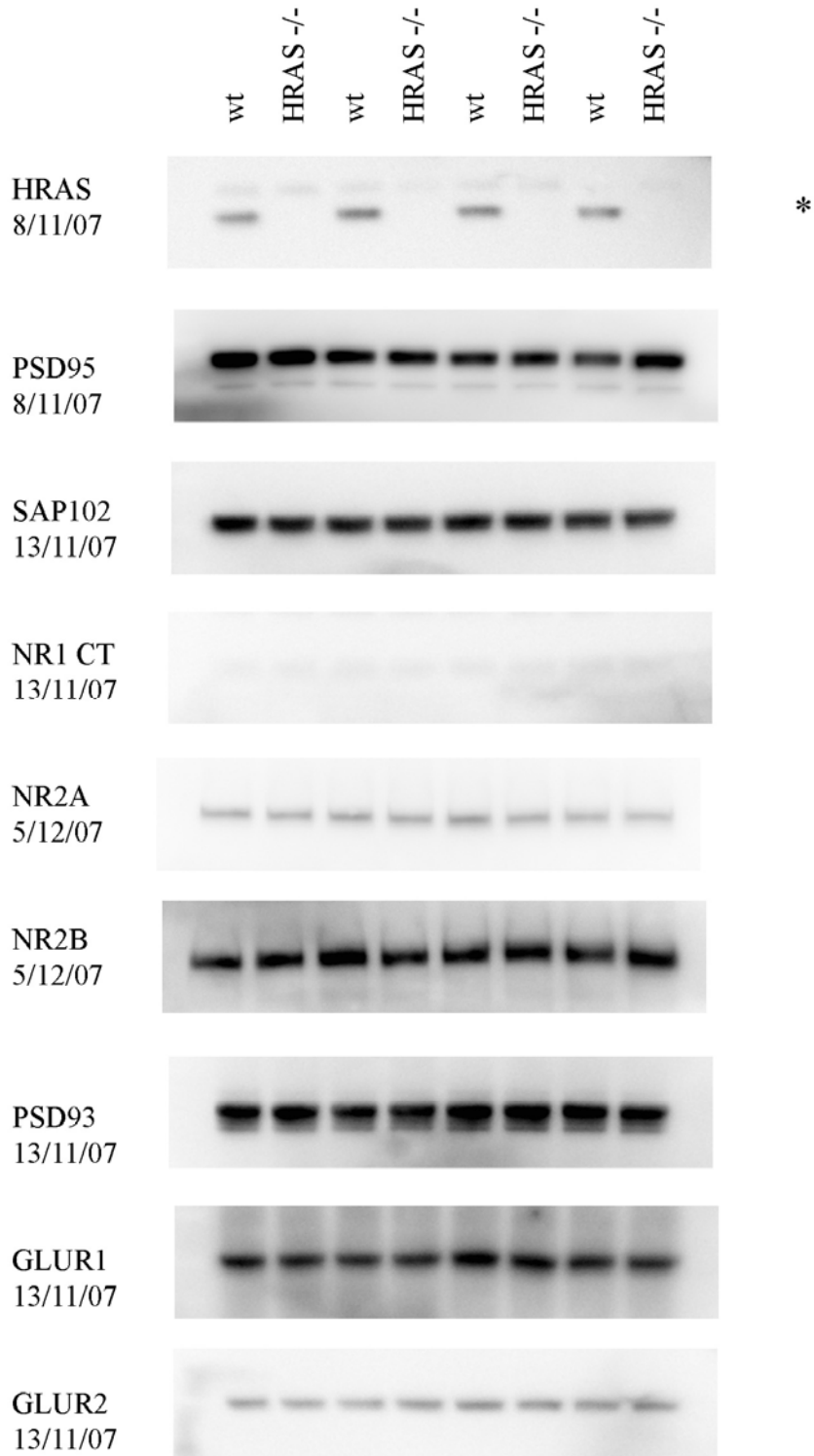
Mouse line: HRAS B6
Samples: 4wt:4homo
Genotype confirmed: Yes

Date	Western blot	AV log ₁₀ ratio mean intensity	SEM	p-value	Comment
8/11/07	HRAS	-0.32606122	0.037854736	0.003290428	decrease
8/11/07	PSD95	0.019552849	0.041276885	0.668053463	
13/11/07	SAP102	-0.015057137	0.008720041	0.182671389	
13/11/07	NR1	0.033912049	0.017249864	0.144016688	
5/12/07	NR2A	-0.032720131	0.017189103	0.153096722	
5/12/07	NR2B	-0.001577817	0.031889841	0.963648888	
13//11/07	PSD93	0.013422313	0.020981412	0.567854784	
13/11/07	GLUR1	-0.011838689	0.009197676	0.28836345	
13/11/07	GLUR2	0.01414052	0.024428392	0.60328522	



5.4 Western blot summary image

HRAS B6 western blot screening



6. Supporting Information

7. Document History

This document was created by Rachel T. Uren on 22 February 2008.