

Quantifications of Western Blots with ImageJ

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This protocol will allow you to relatively (no absolute values) quantify protein bands from western blot films. The quantification will reflect the relative amounts as a ratio of each protein band relative to the lane's loading control.

The same technique can be used for quantification of DNA or RNA from films.

ImageJ is a Java based (runs on all operating systems) freeware by Wayne Rasband from National Institute of Health (USA) and is available for download at: <http://rsb.info.nih.gov/ij/>

1 Scanning The Western Blot Film

It is best to use a transparency scanner (scanner with light source on both sides of the bed). With this kind of scanner when the minimum image area is selected the scanner minimizes the amount of background (Software dependant).

A regular scanner with a good resolution can also do the job.

Make high resolution scans from your films at 600 dpi in TIFF file format. Save this as your raw image, and store it somewhere safe (don't save anything over this original).

2 Prepare the Image

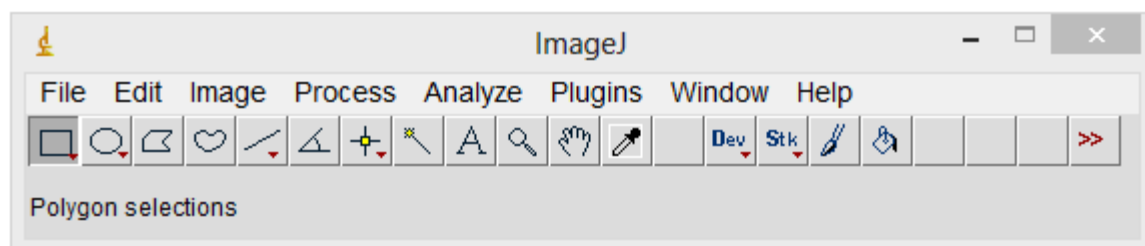
Make a copy of your original raw image and using Photoshop convert that into a JPEG file format, then change the picture mode to "Grayscale".

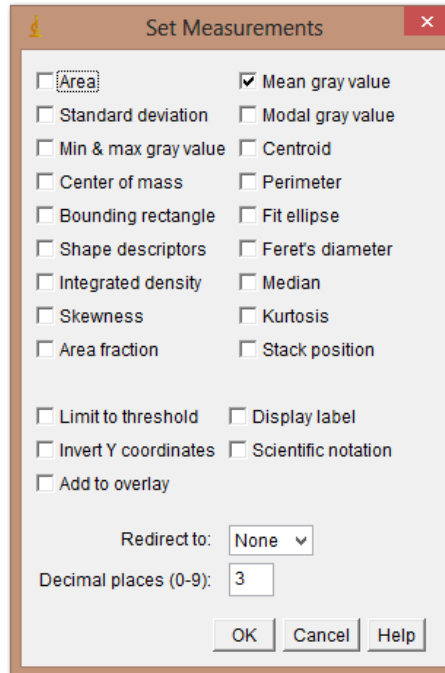
3 ImageJ

If you currently don't have ImageJ installed download it and install the program from the link at the top of the page 1.

3.1 Setting the Measurement Criteria

Under the "Analyze" menu select "Set Measurements". From the checkboxes have ONLY the "Grey Mean Value" checked.





3.2 Prepare the Image

Go to the “File” menu and open the JPEG file format for the film. Maximize the window and use “+” / “-” or “↑” / “↓” keyboard shortcuts to zoom. Use an appropriate zoom level. For spanning the image around in zoom mode select the “hand” tool from the ImageJ toolbox. (If you maximized the screen the toolbox may be hiding in the background).

3.3 Defining a Selection as Region of Interest (ROI)

If there are multiple proteins of interest on your film, for each row of protein band across the lanes you need to define a single region of interest.

Do this one row of protein at a time. Select the “rectangle” tool from ImageJ and draw a frame around the largest band of that row. You can drag around and resize the frame. Adjust it so that it covers the minimum area to contain the whole of the largest band of the row (sometimes a band may not be flat so you have to allow more area to include all of it).

Once you have the frame sized properly, click the “File” menu, “Save as”, “Selection” and save this frame with the protein name.

If other band rows (other protein) are present define and save a frame (ROI) for those as well. You need to do the same for the loading control bands as well.

3.4 Measurements

For each protein (row) to take measurements you will start at the first lane and use the same frame for all of the protein bands across the other lanes. It is very important that you use the same frame across a row. If you accidentally resize the frame or lose it you can go to “Open” under the “File” menu and reimport the same frame.

Place the frame on the first band. The frame should fit all the bands since you previously sized it according to the largest one. Centre the band inside the frame and use the “Ctrl”+“M” keyboard shortcut to record a measurement (“Command”+“M” on Mac or alternatively by clicking “Measure” under the “Analyze” menu). This will open the measurement window and display your data in order. Move the frame to the next lane and make measurement for that protein for all your samples (across the row).

When you finish a row export the data into a spreadsheet (e.g. Microsoft Excel) keeping track of which value belongs to which sample. Close the measurement window of ImageJ to clear the outputs.

With the same frame as the protein (row) you will now take a background measurement. To do so place the frame above or below every band in the row, in a place where there are no other bands or stains on film. Record measurements and export them into the spreadsheet.

Open the ROI of the other rows or loading controls and take the measurements in the same way for the bands and their backgrounds and export them into excel.

4 Spreadsheet Formulas & Calculations

Refer to the sample Excel sheet in Appendix A for disambiguation.

When you have all the data for the bands and their backgrounds along with the loading control bands and their backgrounds in your spreadsheet you need to write a few formulas to do the calculations.

- Invert the pixel density for all data (bands/controls + their backgrounds) in new columns. The inverted value is expressed as $255 - X$, where X is the value recorded by ImageJ. For example if the pixel density recorded by ImageJ is 234.5 then the inverted value should return 20.5.
- For the protein bands and loading controls, express the net value by deducting the inverted background from the inverted band value. For example, after doing the inversions if one band has a background of 3 and band value of 20.5 then the net value should return 17.5.
- When the net bands and loading controls are calculated as the final step, take a ratio of a net band value over the net loading control of that lane. For example if you had a net band value of 17.5 and a net loading control value of 4.5 for that protein band lane then the ratio is $17.5 / 4.5 = 3.88$.
- The final relative quantification values are the ratio of net band to net loading control. You can construct a bar (or other) graph to compare them.

Appendix A: Sample Spreadsheet

In the spreadsheet below, columns highlighted in orange have formulas behind them that calculate values based on the data from ImageJ in white columns.

Lane (Sample)	Protein X	(255 - Protein X) Inverted Protein X	Background	(255 - Protein X Background) Inverted Background	(Inverted Protein X - Inverted Background) Net Protein X
	Vector	222	33	238	17
Construct-1	195	60	235	20	40
Construct-2	200	55	237	18	37
Construct-3	180	75	238	17	58

Lane (Sample)	Loading Control	(255 - Loading Control) Inverted Load. Ctrl.	Load. Ctrl. Background	(255 - Load. Ctrl. Background) Inverted Load. Ctrl. Background	(Inverted Load. Ctrl. - Inverted Load. Ctrl. Background) Net Loading Control
	Vector	240	15	247	8
Construct-1	242	13	248	7	6
Construct-2	200	55	245	10	45
Construct-3	210	45	247	8	37

Lane (Sample)	(Net Protein X / Net Load. Ctrl.) Ratio
	Vector
Construct-1	6.666666667
Construct-2	0.822222222
Construct-3	1.567567568