Quantitative western blot protocol
by Erik Andersen (Horvitz lab) January, 2006

I used this protocol to determine the amount of mono, di or trimethylation of histone H3 as compared to total histone H3 levels in *C. elegans* embryo protein extracts. The protocol for embryo protein extracts was adapted by Melissa Harrison in the Horvitz lab from protocols used by the van den Hueval, Meyer and Strome labs. I measured the amount of protein using a Comassie-based assay from BioRad (cat #: 500-0002). The Typhoon 9400 fluorescence imaging system from Amersham (now GE) was used to measure the fluorescence from each blot.

Running the SDS PAGE gels:

1. For all gels mentioned in this protocol, I used BioRad 18% Tris HCl SDS PAGE gels (cat #: 345-0024) for the Criterion gel system (cat #: 165-6001). These gels gave me the most reproducible results and were very easy to use. I ran all gels for 1.5 hours at 150 V, just long enough for the dye to run off. I loaded protein samples from each genotype in quadruplicate (for amounts see the Deterimine the linear range section).

Transfer of the protein from gel to nitrocellulose membrane:

1. I used Schleicher and Schuell PROTRAN 0.2 μm pore size nitrocellulose (item #: 10402480).
2. The transfer buffer is stored at room temperature in a 50X stock. I make the 1X transfer buffer fresh each time, but there is no reason to do it this way. The transfer buffer recipe following this protocol has been adapted for use with histones by the Kozarides lab.
3. I cut the lanes and foot off of the gel and while immersed in transfer buffer assembled the cassette.
4. All transfers were done in the BioRad Trans-Blot cell (cat #: 170-3939) for 2.5 hours at 400 mA.
5. After transferring, I put the blot in Ponceau S for 15 minutes. Rinsed briefly in distilled water, and then cut the blot with a razor blade to remove sections of the blot containing any proteins larger than 30 kD and smaller than 10 kD. Additionally, I cut off the ladder after marking the blot with a pencil. All of my secondary antibodies bound to pen ink and protein ladders.

Western blots:

1. The blots were blocked in PBST with 5% BSA for 30 minutes at room temperature with agitation. Blotto or 5% milk eliminated most signals detected by western blot.
2. The blots were incubated with primary antisera in PBST with 5% BSA for one hour at room temperature with agitation. See below for concentrations.
3. I rinsed the blots three times quickly and then did three five minutes washes at room temperature with agitation.
4. The secondary antibodies in PBST with 5% BSA were incubated with the blots in dark chambers for 30 minutes at room temperature. See below for concentrations.
5. The blots were washed the same as step 3. I found that extended washing at this step decreased detection of the fluorescent-conjugated secondary antisera.

6. The blots were removed from the dark chambers in a semi-dark room (just dim the lights) and put into plastic sheet protectors (WilsonJones Heavy-weight sheet protectors 21413) The plastic sheet protector was cut with a razor to slightly larger than the blot, and excess liquid was rolled out of the protector.
7. The blots in protective plastic were put in a dark chamber until the fluorescence was quantified using the Typhoon imaging system. The blots were never allowed to dry out.

Determine the linear range of the antisera:

For all western blots, I determined the linear range for each primary antibody using a constant amount of secondary antibody and the same scanning and data processing conditions to detect the fluorescence. I never could get any antibody to work within a linear range using HRP-conjugated secondary antibodies (perhaps for obvious reasons seeing as it is an enzymatic amplification of signal). I used Cy3 and Cy5 labeled secondary antibodies from Jackson Immunoresearch, which were kept at 4ºC until used (no longer than one month).

1. I loaded the gel with a linearly increasing amount of protein (as measured using the BioRad kit) 6.25, 12.5, 25, 50 μg. The linear range for almost all antisera fell within this range.
2. At least two different concentrations of the primary antibodies were tested for linear range. I started with 1:1000 and 1:5000.
3. The secondary antibody was kept constant at 1:500 for both Cy3 and Cy5-conjugated antisera.
4. I plotted the fluorescence values and analyzed the linear regression using Microsoft Excel.

Quantification of the amount of signal from western blot:

I used the Typhoon 9400 fluorescence imaging system from GE for all quantifications. The instructions below are specific to the program used by the Bell lab at MIT.

1. Double-click on the Typhoon Scanner Control icon on the desktop.
2. Put the blot into the Typhoon by lifting the lid and take note of the orientation of your gel and the space where it is sitting (by the alphanumeric designations on the sides of the scan plate).
3. Make the scan window the size of the blot to be scanned by dragging from bottom left to top right position while holding down the left mouse button.
4. Select Fluorescence under Acquisition mode and then click Setup.
5. Select the emission filter for Cy3 and Cy5.
6. Set the PMT setting. This setting has to be optimized. I would try Cy3 at 450 and Cy5 at 600.
7. Select the orientation that your blot was placed into the Typhoon under the Orientation option click box.
8. Click the Press Sample check box
9. Select the pixel size for quality of image and scan time. I use 200 microns.
10. Click Scan button.
11. Name the file. I include the date, what was scanned and the PMT settings.
12. The Typhoon will then scan the image.

13. When the scan is finished, double-click on the ImageQuant TL v2003.03 icon
14. Select Analysis Toolbox from the start-up menu.
15. Open the file you just scanned.
16. You should see a black-and-white image of the blot you just scanned.
17. Draw boxes using the area tool around your bands.
18. Subtract background using any method and empirically determine which works best.
19. Click on the fourth button from the left that looks like a table with the top row and left column colored grey.
20. Now, you should see your quantification on the right half of the screen. Name the boxes accordingly.
21. Under File, choose Export to Excel.
22. Analyze data using Excel. I calculate the mean signal and standard deviation for histone H3 levels and then for the modification mark. Using the levels of H3, I normalize the modification state signal and plot using a bar graph.

Recipes:

2X Sample buffer recipe

100 mM Tris pH 6.7
15% glycerol
2% β-mercaptoethanol

50X Phosphate transfer buffer recipe

Mix 500 mL of 1M Na_2HPO_4 and 425 mL of 1M NaH_2PO_4 to make 925 mL of 50X stock at pH 6.7.

For 500 mL of 1M Na_2HPO_4, mix 70.98 g of stock with MilliQ water.
For 500 mL of NaH_2PO_4, mix 60 g of stock with MilliQ water.

PBST

20 mM Tris pH 7.4
500 mM NaCl
0.05% Tween-20