Cell Biosciences™

Technical Note 13

How to Use Band Analysis Module in AlphaView™ Q Software

Introduction

AlphaView Q software is fast, quantitative, and easy to use, providing the image and data analysis tools needed to analyze multicolor fluorescent Western blots acquired using the FluorChem® Q. In this technical note, we will walk through the steps in AlphaView Q software required to use the band analysis module.

Image Analysis Using the Band Analysis Module

The Band Analysis module provides the image analysis tools needed to quickly extract quantitative data from composite multichannel and single channel images. The tools within the Band Analysis module are designed to work directly on the composite multichannel image, without the need to analyze each channel separately. The Band Analysis module is organized into 5 tabs: Region, Bkgrnd (Background), Control, Std (Standard) Curve and Protocol (Figure 1).

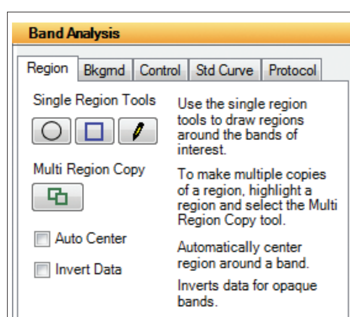

Figure 1. The Band Analysis Module showing the Region tab.

1. Determine if you need to select the Invert option. If your sample has dark bands on a lighter background (i.e. autorads, colorimetric samples), you must select the Invert box in the Region tab of the Band Analysis Module. This will set the analysis software to recognize high black levels as the signal of interest. If the sample is fluorescent, or has white bands on a dark background (i.e. Chemiluminescent Westerns), do not check the Invert box. The software defaults to recognizing high white levels as the signal of interest.

Tip: To determine if your sample has dark bands on a lighter background, make sure the REVERSE option is not selected in the Contrast Adjustment tool area. While this changes the visual appearance of the image, it does not perform the same function as the Invert checkbox.

2. Designation of bands. Beginning with the Region tab, regions of interest are designated with one region for each band of interest. To create your first region, select the blue rectangle tool (or circle or custom shape tool) located under the heading "Single Region Tools." Using the mouse pointer, make a box around your first band of interest. Once a region of interest is drawn, it can be resized, repositioned, copied or deleted.

Tip: For best results, create a box that is slightly larger than the band (Figure 2). Additional regions can be created easily using the Multi Region Copy tool. With the first region highlighted, select the green button under the heading "Multi Region Copy." To create your next region of interest, place the pointer over the center of the next band of interest and select that band. A second box should now appear on the screen around the second band. Repeat this procedure to create regions around all bands of interest (Figure 3).

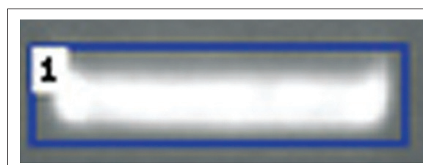

Figure 2. Demonstration of the proper technique for drawing a region of interest around an object.

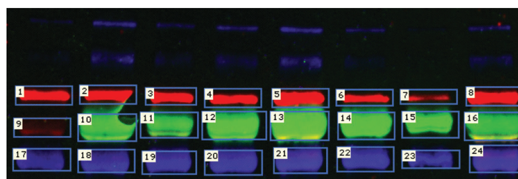

Figure 3. Composite display of a three channel image with 24 regions created using the Multi Region Copy tool. The 24 regions were created by first making a single region using the rectangle tool. Then, with this single region highlighted, the Multi Region Copy tool was selected and the additional 23 regions placed on the image by using the mouse pointer. After copying, the size of the regions were adjusted to fit each individual band.

Tip: If you select the "Auto Center" option, new regions will be automatically centered over each band.

Once you have created one or more regions of interest, a data table will be created (Figure 4). Each region of interest will show values for the red, blue and green channels.

Band Analysis Result - All Channels

Output View Display Style

Band	Blue Sum	Blue Average	Green Sum	Green Average	Red Sum	Red Average	Area
1	39,847,749	27,033	10,819,374	7,340	11,454,450	7,770	1,474
2	41,277,295	28,003	13,420,159	9,104	12,846,043	8,715	1,474
3	34,320,745	28,600	10,218,848	8,515	11,090,631	9,242	1,200
4	42,972,403	29,153	13,606,053	9,230	10,923,027	7,410	1,474
5	43,442,567	29,472	17,565,845	11,917	16,478,874	11,179	1,474
6	35,890,636	28,193	12,419,997	9,756	7,985,309	6,272	1,273
7	31,369,895	27,541	10,176,448	8,934	5,198,763	4,564	1,139
8	39,983,967	27,126	13,413,078	9,099	15,801,727	10,720	1,474
9	41,678,796	28,275	11,632,507	7,891	6,484,442	4,399	1,474
10	66,094,694	29,934	60,395,704	27,353	9,428,750	4,270	2,208
11	60,911,122	29,858	44,952,925	22,035	8,523,328	4,178	2,040
12	76,066,561	30,329	72,405,544	28,869	9,544,186	3,805	2,508
13	72,714,662	30,995	90,344,204	38,509	11,063,866	4,716	2,346
14	64,859,223	30,251	75,977,032	35,437	7,683,330	3,583	2,144
15	58,681,000	28,252	56,955,050	27,421	6,466,819	3,113	2,077
16	67,841,955	29,368	58,626,471	25,379	11,178,814	4,839	2,310
17	74,787,202	42,300	16,116,985	9,115	5,802,611	3,282	1,768
18	82,739,508	45,737	19,003,444	10,504	6,326,062	3,496	1,809
19	81,504,511	47,166	17,459,787	10,104	5,900,375	3,414	1,728
20	94,886,317	48,116	21,416,018	10,860	6,449,015	3,270	1,972
21	94,526,090	50,013	22,800,643	12,063	7,204,910	3,812	1,890
22	92,265,137	47,485	21,770,715	11,204	5,976,989	3,076	1,943
23	63,540,288	44,496	14,513,689	10,163	4,128,488	2,891	1,428
24	100,676,276	48,402	22,753,076	10,938	8,311,075	3,995	2,080

Figure 4. Data table for the 24 regions created in Figure 3. Note that each region has Blue, Green and Red channel data values associated with each underlying channel.

The values in the Data Table are in units of pixel gray levels, each with a value between 0 and 65535 (16 bit depth) and are proportional to the light intensity on that pixel during the exposure time of the image.

The **Area** is the number of pixels in a region.

The **Sum** is the total value of all the pixel gray levels in a region. The Sum can be interpreted as the total signal from a region and best represents the total protein (or DNA) signal in bands.

The **Average** is the Sum divided by the Area. This is the average signal level from a region and best represents the average protein (or DNA) signal in the region of interest. The average gives the user the intensity value from the band without taking into account the area. This measurement is useful as it allows the user to directly compare two bands that are different sizes.

Tip: Each of the three channels may be examined separately by displaying a single channel in the Contrast Adjustments window and then adjusting the display style to display a single channel and selecting columns to display using the Show/Hide feature (Figure 5-6). If your sample has two colors in the image, you can use the show/hide features column to only show the values that relate to your image.

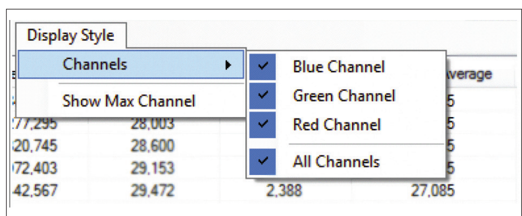

Figure 5. Display Style with All Channels selected. Any one, two or all three channels may be selected.

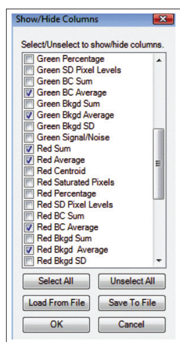

Figure 6. The Show/Hide Feature can be used to select which columns to display in the data table.

3. Background Correction. Using the Background tool tab, background fluorescence signal (which can arise from fluorescence detection chemistry, the sample matrix or the quality of the sample itself) can be removed. There are three background correction methods: Regional, Multi-Regional and Local.

To begin, select the Background tab (Figure 7) followed by the type of background correction you wish to apply.

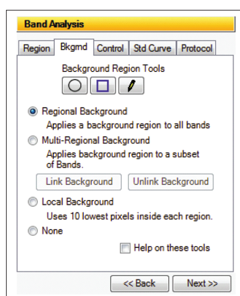

Figure 7. The Band Analysis Module showing the Background tab.

Regional Background. When the Regional Background function is active, a single background region will be applied to all object regions. To use this option, place a background region in an area of the image representative of the background level for all channels (Figure 8). The data table is automatically updated with Background Corrected (BC) values (Figure 9).

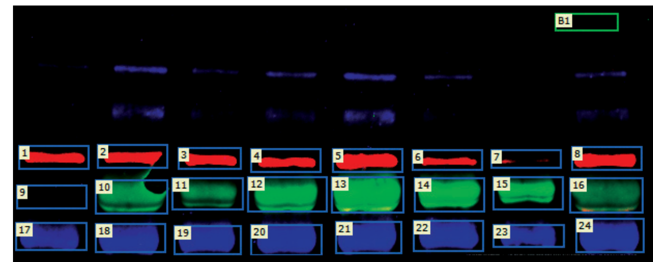

Figure 8. Composite display of a three channel image with regional background applied. The background values from a single region are extracted and applied to each of the three channels respectively.

Band Analysis Result - All Channels

Output View Display Style

Band	Blue BC Average	Green BC Average	Red BC Average
1	1,873	189	5,279
2	2,843	1,953	6,224
3	3,440	1,364	6,751
4	3,993	2,079	4,919
5	4,312	4,766	8,688
6	3,033	2,605	3,781
7	2,381	1,783	2,073
8	1,966	1,948	8,229
9	3,115	740	1,908
10	4,774	20,202	1,779
11	4,698	14,884	1,687
12	5,169	21,718	1,314
13	5,835	31,358	2,225
14	5,091	28,286	1,092
15	3,092	20,270	622
16	4,208	18,228	2,348
17	17,140	1,964	791
18	20,577	3,353	1,005
19	22,006	2,953	923
20	22,956	3,709	779
21	24,853	4,912	1,321
22	22,325	4,053	585
23	19,336	3,012	400
24	23,242	3,787	1,504

Figure 9. The data table showing the respective Background Corrected (BC) Average values for each channel after regional background correction.

Tip: More than one background region can be used and the average pixel level of the background region is applied. This feature is useful if the background of the sample is varied, making it difficult to create just one region that best represents the background.

Multi-Regional Background. Using this option, a background region can be applied to a subset of object regions. A second background region can then be applied to a second subset of object regions. Each subset of object regions is corrected by the linked background region to account for differences in background level across an image.

You will need to use the Link Background tool in conjunction with the Multi-Regional Background to link a background region to a subset of object regions. First place a background region in an area of the image representative of the background specific to the subset of object regions. Draw a rectangle by left clicking on the image and dragging to include the desired subset of bands and a single background region to be linked. Alternatively, you can use Ctrl + left click to select multiple regions and the specific background region. Finally, select the Link Background button. The data table is automatically updated with Background corrected values.

Repeat to link a second background region to a second subset of bands. Note that the colors of the linked regions are updated to indicate linkage.

Local Background. This option applies the average pixel level of the 10 lowest pixels in a band region to calculate the background. This option is useful if the image has regions with different background levels, since the background values will be unique and localized for each object. When using this option, it is important that the regions be slightly larger than the bands (see Figure 2). The data table is automatically updated with Background corrected values once this option is selected.

For a single color analysis, background correction could be the final step in your analysis. However, if your sample includes loading controls or a positive control, these factors can easily be included in your analysis by using the following features of the Band Analysis Module.

4. Loading Control Normalization. Once the regions of interest have been defined and background correction has been performed, loading control normalization takes place by choosing the Control tab (Figure 10). Loading control normalization is used to normalize experimental bands to the corresponding loading controls to adjust the data for variations in the amount of sample loaded in each lane.

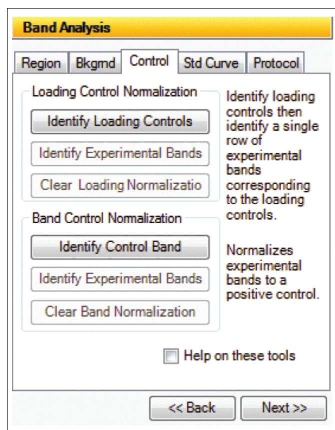

Figure 10. The Band Analysis Module showing the Control Tab.

To perform loading control normalization, you will need to first identify the row of loading controls and then identify a row of experimental bands. A second row of experimental bands may then be identified by repeating this process (Figure 11).

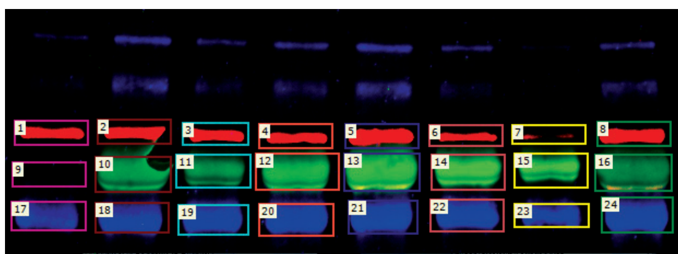

Figure 11. Loading Control Normalization. In this example, the Blue channel regions 17-23 were identified as the loading controls. The Red channel regions 1-8 were identified as one set of experimental proteins. The Green channel regions 9-16 were identified as a second set of experimental proteins. Note that region 9 was created even though no green signal level is present so that there are equal numbers of protein bands for loading control normalization.

Identify Loading Controls. Select the "Identify Loading Controls" button and select the channel (red, green or blue) for the loading controls. Identify each member of the row of loading control bands using the mouse pointer. You may either left click on each of the regions individually or draw a rectangle to select a single row of regions. Deselect the "Identify Loading Controls" button when done or right click.

Identify Experimental Bands. Select the "Identify Experimental Bands" button and select the channel for each row of experimental bands. Identify each member of a single row of experimental bands using the mouse pointer. Just like with the loading controls, you can either left click on each of the regions individually or draw a rectangle to select a single row of regions. The "Identify Experimental Bands" button will automatically deselect when the number of bands identified in the row reaches the number of loading control bands. Experimental bands and their corresponding loading control will now be outlined in the same color.

Band Analysis Result - All Channels

Output View Display Style

Band	Blue BC Average	Green BC Average	Red BC Average	LCN Average
1	1,873	189	5,279	6,639
2	2,843	1,953	6,224	6,520
3	3,440	1,364	6,751	6,612
4	3,993	2,079	4,919	4,619
5	4,312	4,766	8,688	7,535
6	3,033	2,605	3,781	3,650
7	2,381	1,783	2,073	2,311
8	1,966	1,948	8,229	7,631
9	3,115	740	1,908	931
10	4,774	20,202	1,779	21,162
11	4,698	14,884	1,687	14,579
12	5,169	21,718	1,314	20,392
13	5,835	31,358	2,225	27,196
14	5,091	28,286	1,092	27,310
15	3,092	20,270	622	22,596
16	4,208	18,228	2,348	16,904
17	17,140	1,964	791	-
18	20,577	3,353	1,005	-
19	22,006	2,953	923	-
20	22,956	3,709	779	-
21	24,853	4,912	1,321	-
22	22,325	4,053	585	-
23	19,336	3,012	400	-
24	23,242	3,787	1,504	-

Figure 12. Loading Control Normalization Data Table.

The Loading Control Normalized Average (LCN Average) then taking into account the loading controls and allows you to directly compare the intensities across all lanes, as values normalized for differences in total signal per lane from the calculation of the specified loading control.

5. Band Control Normalization.

The Band Control Normalization tool can be used to normalize a row of experimental bands to a positive control. First, select the channel and identify the region for the positive control and then identify the regions for the experimental bands (Figure 13). The control band and the corresponding experimental bands must be in the same channel. Multiple sets of positive controls and experimental bands may be identified and each set may be in a distinct channel.

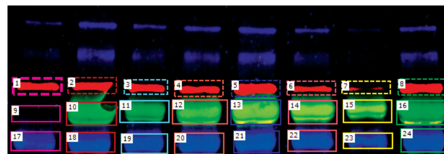

Figure 13. Band Control Normalization. In this example, region 1 in the red channel was selected as a control band and regions 2-8 were selected as the associated experimental bands. Note that the region outlines are dashed to indicate the corresponding control and experimental bands.

Identify Positive Control. Select the "Identify Control Band" button and select the desired channel (red, green or blue). Identify the positive control band using the mouse pointer.

Identify Experimental Bands. Select the "Identify Experimental Bands" button. Identify each of the experimental bands to be normalized to this positive control band. Just like with the loading controls, you can either left click on each of the regions individually or draw a rectangle to select a single row of regions. Deselect the "Identify Experimental Bands" button or right click when done.

Once a positive control is selected, the software will immediately display the fold change represented by the band of interest in each lane, relative to the positive control. In the example shown above, the samples have been normalized using Loading Control Normalization and thus the fold-change results are not influenced by variance in sample loading (Figure 14).

Band Analysis Result - All Channels

Output View Display Style

Band	Red BC Average	LCN Average	PCN Average	Fold Change
1	5,279	6,639	100.00	±1.00
2	6,224	6,520	98.21	-1.02
3	6,751	6,612	99.61	-1.00
4	4,919	4,619	69.57	-1.44
5	8,688	7,535	113.50	+1.14
6	3,781	3,650	54.99	-1.82
7	2,073	2,311	34.81	-2.87
8	8,229	7,631	114.96	+1.15
9	1,908	931	-	-
10	1,779	21,162	-	-
11	1,687	14,579	-	-
12	1,314	20,392	-	-
13	2,225	27,196	-	-
14	1,092	27,310	-	-
15	622	22,596	-	-
16	2,348	16,904	-	-
17	791	-	-	-
18	1,005	-	-	-
19	923	-	-	-
20	779	-	-	-
21	1,321	-	-	-
22	585	-	-	-
23	400	-	-	-
24	1,504	-	-	-

Figure 14. Band Control Normalization Data Table showing the Positive Control Normalization (PCN) Average and Fold Change of regions 1-8.

6. Standard Curve. The button in the Band Analysis toolbox labeled Std Curve opens a set of tools that create a calibration curve for applications such as quantitative PCR and Western Blot band quantitation (Figure 15). The calibration curve functions allow quantitation of the bands based on a set of standards. A minimum of two standard bands must be specified, but the accuracy of the calibration curve increases as the number of standard bands and their range of values increases. The standard curve feature can only be performed on single channel images. To create a standard curve for a multicolor image, you can extract a three color image into its individual channels and then proceed with calculating a standard curve.

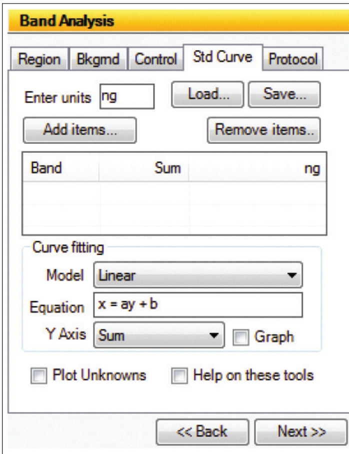

Figure 15. The Band Analysis Module showing the Standard Curve tab.

Specifying Units. Once the regions are defined, click on the Standard Curve tab. Enter the units in which the results should be reported (e.g. ng, ul, pg, %) in the "Enter units" box.

Designating the Standard Bands. Select "Add Items" and click on the first region using the mouse pointer (Figure 16). Continue to select regions until all objects used for standard curve calculation are listed. For each band whose value is known input the concentration in the last column. The band number changes from white to green, indicating that it is now a standard. Once a second value has been entered, a curve is displayed (Figure 17).

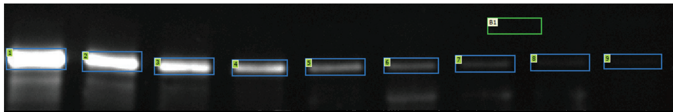

Figure 16. Standard Curve Calculation. In this example regions 1-9 were selected and the known concentration of each band was entered.

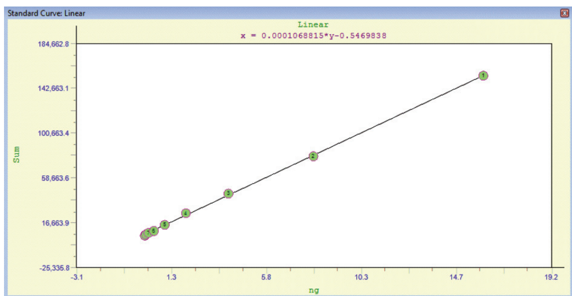

Figure 17. Standard Curve.

Tip: The AlphaView Q software will automatically calculate points for unknown regions. As the values for the standard bands have been entered, the values of the unknown bands are automatically calculated. The calculated values of the unknowns are automatically updated in the spreadsheet. The points corresponding to the standard bands are labeled in yellow. Points for the unknown objects on the image are displayed on the standard curve based on their integrated density values. These are labeled in white. Enter values for each band whose amount is known. As more standard points are added, the calculated values of the unknown points may change.

7. Saving Protocol and Analysis Settings. The Protocol tab is used to save and load protocols for use on replicate blots (Figure 18). A protocol or analysis contains all band and background regions created with the loading controls, band controls and standard curve settings used. A protocol or analysis may be saved at any point in the analysis workflow.

Tip: Saving a protocol is different from saving an analysis in that protocols may be used on images other than the original image while a saved analysis is available for loading only on the original image. Note that an analysis may be saved at anytime by using the File drop down menu (Figure 19).

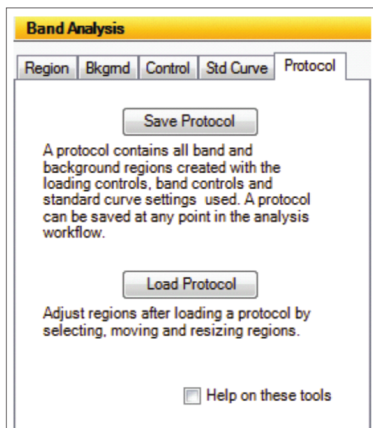

Figure 18. The Band Analysis Module showing the Protocol tab.

Figure 19. The File drop down menu with Save Analysis selected.

8. Exporting Data. The Output tool provides a way to export the data to a printer, to the clipboard, or to an ASCII file (Figure 20). This ASCII file can then be imported into a spreadsheet for further analysis and/or graphing.

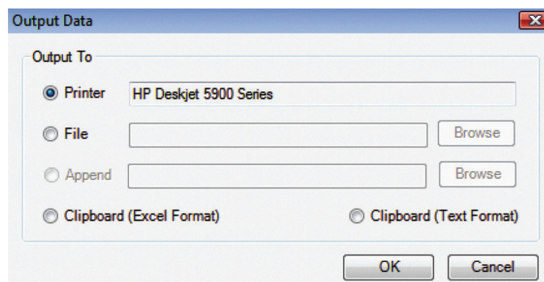

Figure 20. Output Data Selection Box.

To export data, click on the appropriate box to specify where the data should be sent. To create a file, enter a name for it in the text box. To send the data, click on the OK button.

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AlphaView Q is an easy-to-use software tool for both image acquisition and analysis of multicolor fluorescent Western blots and Chemiluminescent Western blots. AlphaView Q is specifically designed to meet the requirements for multichannel image analysis in molecular biology labs by providing tools in the software for loading control normalization. AlphaView Q comes standard with the FluorChem Q, a Western blot imaging system for fluorescent and chemiluminescent blots.

For more information, visit us at: http://www.cellbiosciences.com

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