How to Use the Band Analysis Module in AlphaView® Software

It applies to AlphaView Version 3.0 and above.

Introduction

AlphaView software is a fast, quantitative and easy to use software tool that comes standard with FluorChem® and AlphaImager® imaging systems. In this technical note, we will walk through the steps in AlphaView software required to correctly perform spot densitometry on a sample. This powerful tool can be used for many different applications including quantification of bands from chemiluminescent blots and Ethidium Bromide gels.

Spot Densitometry Calculation

A precise spot densitometry calculation can be performed following these steps:

1. Acquire Image. When acquiring an image, be sure to select Saturation during the acquisition to ensure the bands of interest are not saturated, and therefore beyond the linear range of the CCD camera. Once the image is acquired, use the "Save" feature to save image for analysis.

Tip: *Never select "Save Modified" if you plan to perform spot densitometry on the image. Save Modified converts the image from 16 bit, to 8 bit, and the resulting data generated will not be as accurate.*

2. Select Analysis Tools and then Band Analysis. The Band Analysis module provides the image analysis tools needed to quickly extract quantitative data from images. The Band Analysis module is organized into 5 tabs: Region, Bkgrnd (Background), Std (Standard) Curve, Protocol and Report (Figure 1).

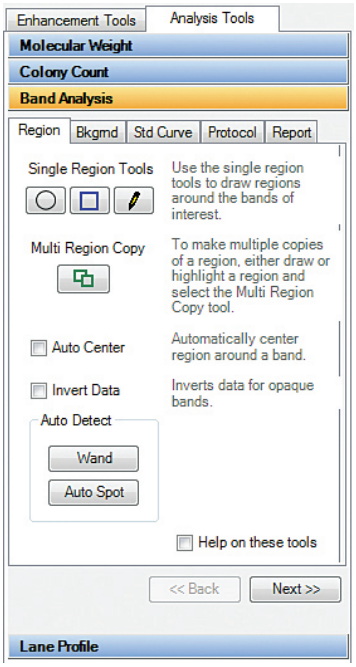

Figure 1. When an image is opened in AlphaView®, the Band Analysis Module will be active.

3. 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 Regions tab. 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 (Figure 2).

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.*

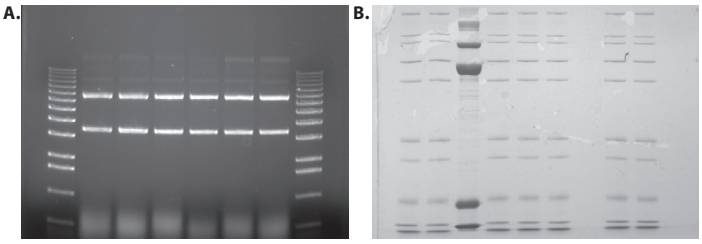

Figure 2. Selecting the Invert option when analyzing a sample. A. An ethidium bromide DNA gel. To analyze this sample, the user would not select the Invert option. B. A Coomassie stained SDS PAGE protein gel. To analyze this sample, the user would select Invert, so the software would recognize the dark bands as signal.

4. Draw Region(s) of Interest. 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, de-select the rectangle tool, highlight your drawn region of interest, and it can now be resized, repositioned, copied or deleted. An unlimited number of circular, rectangular or user-defined regions of interest can be designated on an image. Figure 3 demonstrates the proper technique for drawing a region of interest around a band.

Figure 3. Demonstration the proper technique for drawing a region of interest around an object. A. Region too large B. Region too close to band C. Recommended Region placement.

Tip: *Additional regions can be created easily using the Multi Region Copy tool (Figure 4) . Click on the "Multi Region Copy" button, the last created region will automatically be selected. To duplicate your next region of interest, place the pointer over the center of the band of interest and left-click the 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. Click on the Multi Copy Mode button or right click to exit the Multi Copy mode.

Figure 4. Multi Copy Region button.

Tip: *To delete a region, select the region(s), click on the <Delete> key.*

Once you have created one or more regions of interest, a data table will be created. Each drawn area of interest will correspond to a number on the Band Analysis Module Results dialog box (Figure 5). You can choose which columns to display in the data table by using the Show/ Hide Columns Feature which is found in the View Menu (Figures 6 and 7).

Band analysis results

Export View

Band	Sum	Area	Average
1	3,434,803	729	4,712
2	2,793,698	729	3,832
3	2,935,100	729	4,026
4	2,715,115	729	3,724
5	2,287,996	729	3,139
6	3,708,943	729	5,088
7	2,542,021	729	3,487
8	2,984,197	729	4,094
9	3,610,461	729	4,953
10	6,453,849	729	8,853
11	3,647,925	729	5,004
12	3,813,371	729	5,231
13	2,900,809	729	3,979
14	2,419,648	729	3,319

Figure 5. Band Analysis Results are displayed in a table format.

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.

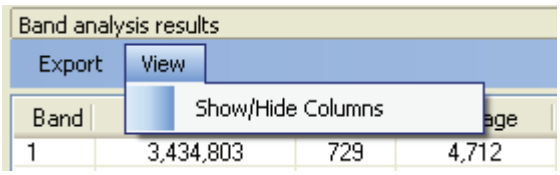

Figure 6. Show/Hide Columns Feature is found under the View Menu.

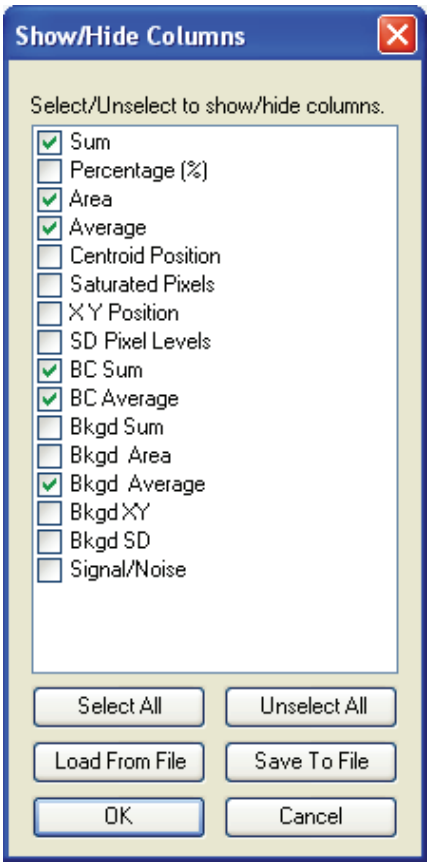

Figure 7. The Show/Hide Feature can be used to select which columns to display in the data table.

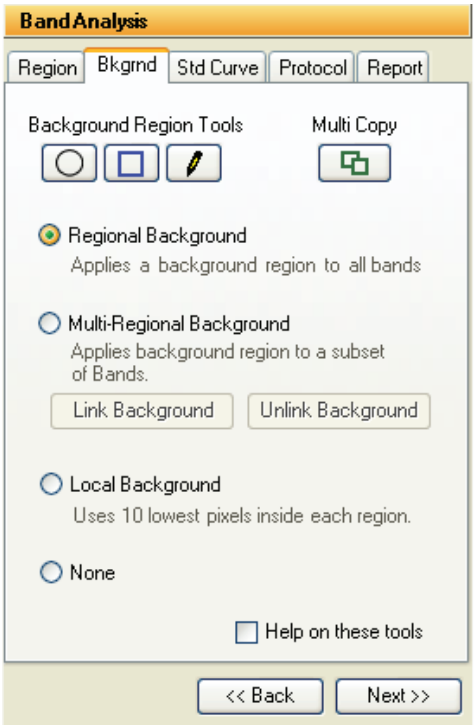

Figure 8. The Band Analysis Module showing the Background tab.

5. Background Correction. Using the Background tool tab, background 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 8) followed by the type of background correction you wish to apply.

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 the regions (Figure 9). The data table is automatically updated with Background Corrected (BC) values (Figure 10).

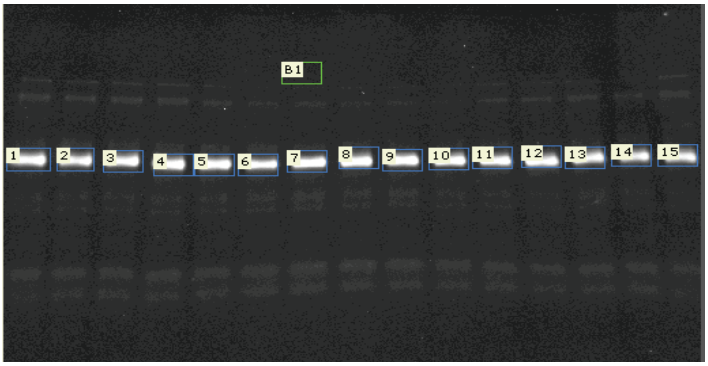

Figure 9. The background values from a single region are extracted and applied to all regions.

Band analysis results

Export View

Band	Sum	Area	Average	BC Sum	BC Average	Bkgd Average
1	3,434,803	729	4,712	1,451,923	1,992	2,769
2	2,793,698	729	3,832	932,561	1,279	2,558
3	2,935,100	729	4,026	866,927	1,189	2,875
4	2,715,115	729	3,724	717,654	984	2,757
5	2,287,996	729	3,139	332,817	457	2,712
6	3,708,943	729	5,088	1,828,852	2,509	2,667
7	3,322,808	729	4,558	1,269,944	1,742	2,951
8	3,031,364	729	4,158	975,583	1,338	15,382
9	3,610,461	729	4,953	1,413,255	1,939	3,215
10	6,453,849	729	8,853	4,299,654	5,898	3,099
11	3,647,925	729	5,004	1,674,522	2,297	9,011
12	3,813,371	729	5,231	1,756,132	2,409	2,909
13	2,900,809	729	3,979	927,405	1,272	15,354
14	2,419,648	729	3,319	436,768	599	2,736

Figure 10. The data table showing the Background Corrected (BC) 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. Background regions can also be copied from the data region (for exact size and shape). To copy a data region, select a data region, click on the "Multi Region Copy" of the background tab and then click on the area to use for 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 3). The data table is automatically updated with Background corrected values once this option is selected.

6. Standard Curve. The tab 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 11). 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.

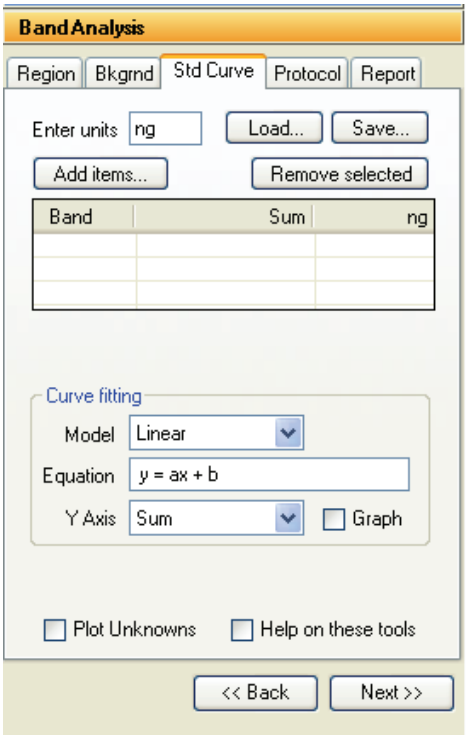

Figure 11. The Band Analysis Module showing the Standard Curve tab.

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 12). 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 13). You can change the value shown on the Y axis of the curve using the "Y axis" drop down menu located under "Curve fitting.

Figure 12. Standard Curve Calculation. In this example regions 1-9 were selected and the known concentration of each band was entered.

Tip: *The AlphaView software will automatically calculate points for unknown regions. As the values for the standard bands are 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*

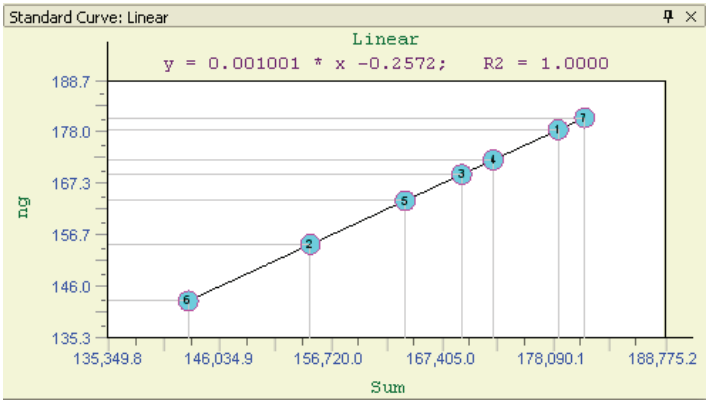

Figure 13. Standard Curve.

unknown objects on the image are displayed on the standard curve based on their 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 14). 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 15).*

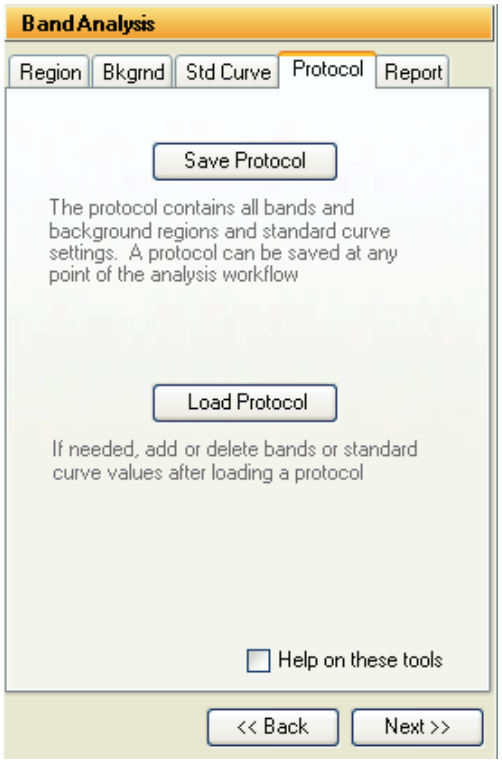

Figure 14. The Band Analysis Module showing the Protocol tab.

Loading an Existing Protocol. To use an existing protocol on a new image, open the image in AlphaView. Go to the Protocol tab in the Band Analysis module and click on Load Protocol. This will load all band and background regions created, along with the standard curve settings used.

Loading a Saved Analysis. A saved analysis can be loaded only on the original image. To use a saved analysis, open the original image in AlphaView. Under the File drop down menu, go to Analysis and click on Load Analysis. This will load all band and background regions created, along with the standard curve settings used.

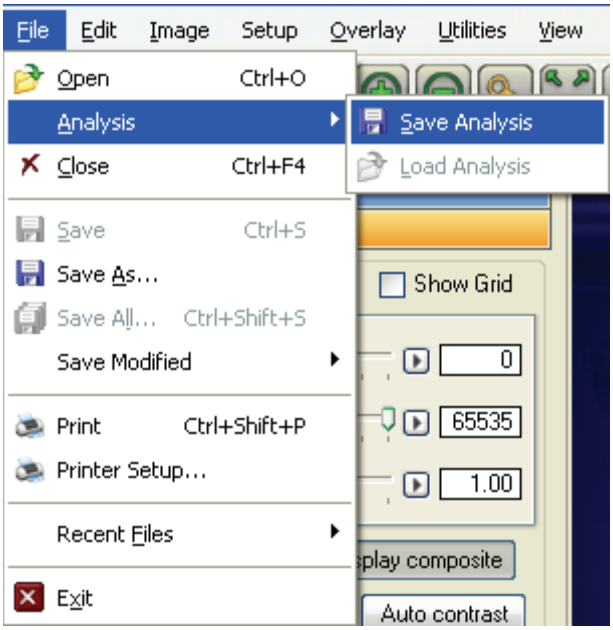

Figure 15. The File drop down menu with Save Analysis selected.

8. Generating a Report. The Report tab is used for creating a report for archiving and saving your image and analysis in a single document (Figure 16). If you have an existing template you have previously saved, you can select it from the existing templates pull down menu. Select which image you want on your report and the size of the image you want (1/4 is the default size). Next, select the type of data you would like in your report (data sheet, graph and/or acquisition information). You

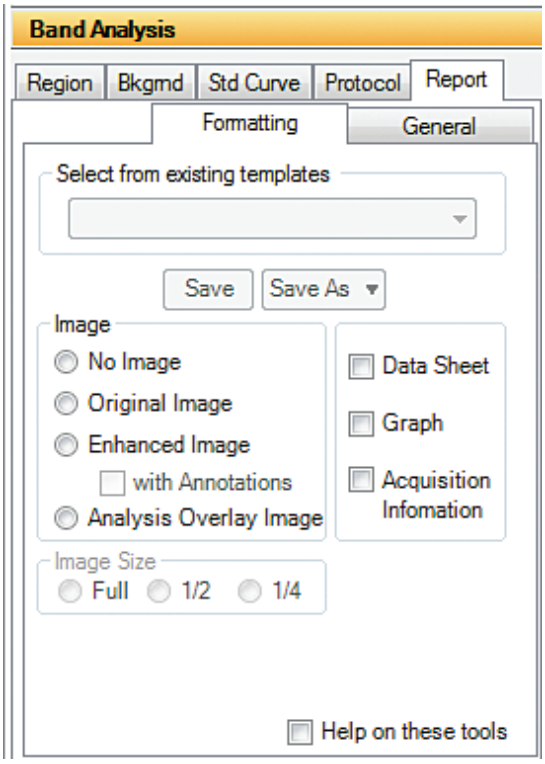

Figure 16. The Band Analysis Module showing the Report tab.

can use the Save and Save As buttons to create a report template at this time. If you have finished entering your formatting options, click on the General tab. Enter the report name, user name and any notes you would like to include in your report. Click the Generate Report button to display the report (Figure 17). You can then save the report as either an html or pdf document or send the report directly to your printer.

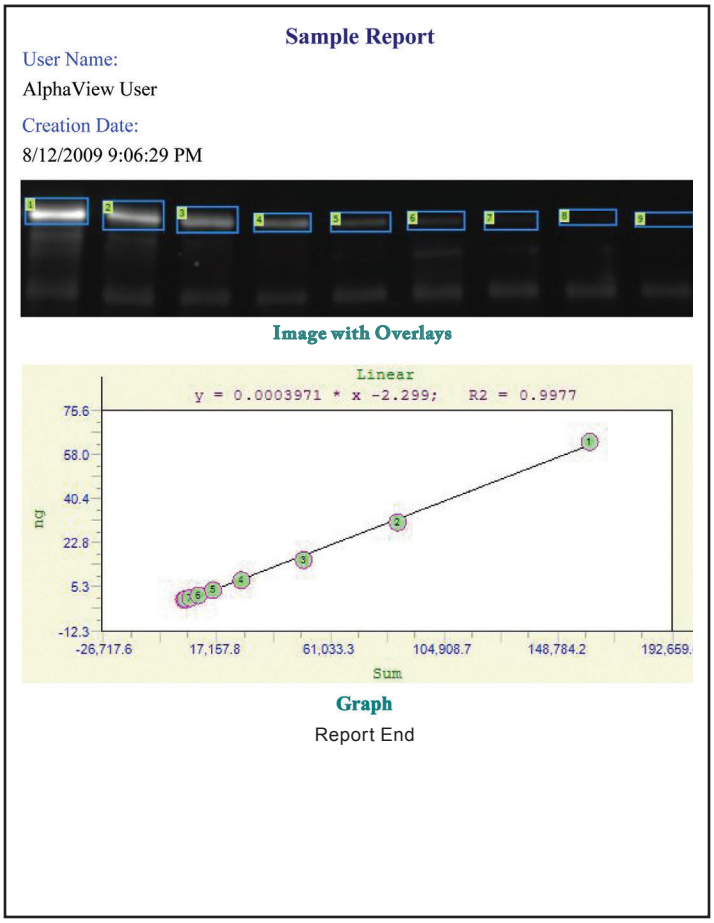

Figure 17. Sample of a report generated from the Band Analysis Module.

9. Exporting Data. The Export tool provides a way to export the data to a printer, to the clipboard, or to an ASCII file (Figure 18). This ASCII file can then be imported into a spreadsheet for further analysis and/or graphing.

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.

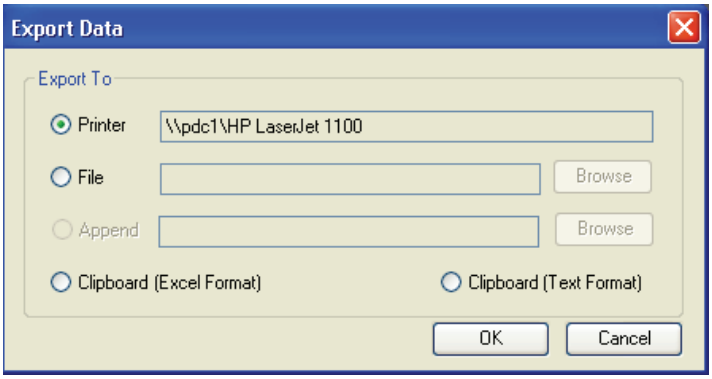

Figure 18. Output Data Selection Box.

AlphaView is an easy-to-use software tool for both image acquisition and analysis that allows scientists to rapidly generate results and images for publications and presentations. AlphaView is designed to meet the requirements for image analysis in molecular biology labs, including analysis tools for determining band concentrations and molecular weight, colony counting and determining macroarray intensity values. AlphaView is compatible with our FluorChem and AlphaImager family of imagers.

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