FluorChem FC2/HD2

USER GUIDE

Cell Biosciences™

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CELL BIOSCIENCES, INC.

FluorChem HD2 and FC2 User Guide

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FluorChem HD2 and FC2 User Guide

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Chapter 1:

Introduction and Setup

Chapter Overview

- Introduction to the FluorChem Systems and AlphaView Software
- About This Manual
- Customer Service and Technical Support
- FluorChem System Setup

Introduction to the FluorChem Systems and AlphaView Software

The FluorChem Systems are designed to meet the requirements of the most demanding chemiluminescence assays and also provide the most versatile fluorescence imaging system available today. These systems integrate the latest developments in camera and lens technology for imaging chemiluminescent and fluorescent samples with a wide dynamic range and fast image acquisition speeds.

The system is controlled by easy-to-use and intuitive AlphaView® software which performs image analysis and archiving and can also prepare images for desktop publishing.

AlphaView software includes image optimization tools that adjust contrast automatically or manually. Converting the image from positive to negative, using digital filters, applying a false color map, or other such techniques can be used to clarify hard-to-see portions of the image. Notes, labels, arrows, lines, and other drawing tools can be recorded directly onto the image using AlphaView software's annotation functions. Annotations are superimposed on the image upon hard copy printing and can either be saved as a template file or as part of the image. All AlphaView features are accessible via convenient on-screen buttons and menus in an intuitive, mouse-controlled interface.

AlphaView software also includes a broad array of analysis tools, including molecular weight calculation, R_f determination, lane profile, band analysis, quantitative PCR, microtiter plate reading, object distance measuring, gel scoring, and automatic colony counting.

FluorChem systems include all the necessary hardware and software for image capture, enhancement, and analysis. AlphaView software does not require a dedicated computer system and can operate simultaneously with other office-related software, such as word processing programs, spreadsheets, and desktop publishing software.

Images can be printed using a 256-level gray scale thermal printer or any printer with a Windows® driver. The low-cost, high-quality prints are ideal for lab notebook records or publication.

The "AlphaView System Quick Guide" on page 42 provides a condensed instruction sheet that acts as a quick reference guide for users already familiar with the software. Keeping copies of the appropriate instruction sheet(s) near the system for easy reference is highly recommended.

Mouse Functions

The system comes packaged with a two-button mouse. The left button activates functions and makes selections when using the software. In some cases, the right mouse button can recall or reactivate the function that was most recently assigned to the left mouse button.

Starting AlphaView Software

To start AlphaView software, double-click the AlphaView software icon on the Windows desktop.

About This Manual

This manual uses different fonts to indicate certain conditions:

- **Bold** text indicates the name of a button, a menu, or a function found in a menu.
- `Courier` text indicates an entry that is typed.
- Letters or words found between < > refer to keys on the keyboard.
- *NOTE* indicates key points and useful hints.
- **CAUTION** and **!WARNING!** statements indicate actions that may either harm the system or user, or affect the data quality.
- Icons or buttons to be pushed are placed next to their respective descriptions in the text.

Customer Service and Technical Support

The Cell Biosciences staff is available to respond to any questions or comments about the software. For questions, new software feature ideas, or general feedback, use the following contact methods:

Telephone
(408) 510-5500
(888) 607-9692 (toll free)

Fax
(408) 510-5599

E-mail
support@cellbiosciences.com

Web
www.cellbiosciences.com

Address
Cell Biosciences, Inc.
3040 Oakmead Village Drive
Santa Clara, CA 95051
USA

FluorChem System Setup

System Components

The FluorChem systems include the following:

- High-performance, high resolution, CCD camera
- Manual f/2.8 28-70mm zoom lens or other optional lens
- Computer with keyboard, mouse, and monitor (optional)
- Windows operating system (pre-installed)
- AlphaView image processing and analysis software (pre-installed and calibrated with computer and hardware system)
- MultiImage® II FC light cabinet containing a UV transilluminator, white light fold-down transilluminator and epi white lights with interference filter
- Fast lens (optional)
- Epi-illuminating UV lights (optional 254nm or 365nm)
- Printer (optional)
- ChromaLight (optional)

Upon receiving the system, it is critical to check the enclosed packing list to verify that all components are properly included.

System Placement

As with all electrical instruments, the FluorChem systems should be located on a table or bench top that is dry and stable, and away from water, solvents, or corrosive materials. In addition, the system should be placed away from interfering electrical signals and magnetic fields. If possible, a dedicated electrical outlet should be used to eliminate electrical interference from other laboratory instrumentation.

Cable Connections

The cable connectors and their respective mating ports are keyed or unique for each connection to eliminate potential wrong mating. The connections are illustrated and described later in this chapter.

!WARNING!

Make sure that the power is OFF and all power cords are disconnected while connecting the cables and setting up the system.

Hardware Installation

All software, peripheral drivers, and operating systems come factory-installed on computers purchased directly from Cell Biosciences. All components must be mated only to their correct ports during system installation.

Power Strip/Surge Protector Setup

Turn the power strip/surge protector power switch off. Plug the power strip/surge protector into a wall outlet (preferably a dedicated circuit) and turn the power on.

CAUTION

Do not plug the DE-500/DE-500 FC MultiImage II light cabinet into the same power strip as the FluorChem system. Using a separate circuit is highly recommended. The cabinet must be turned on after the computer's operating system has launched for the software to function properly.

Computer, Monitor, Mouse, and Keyboard Setup

The computer must have the monitor, mouse, and keyboard connected in the correct ports (see Figure 1-1). A standard three-prong power cable should be plugged into the back of the computer and the power strip.

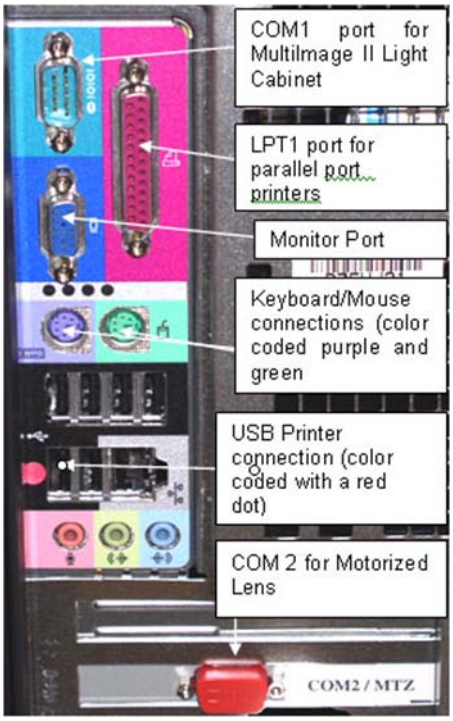


***Figure 1-1:** Computer, Monitor, Mouse, and Keyboard Setup*

Connect the monitor's video cable to the monitor port on the back of the computer. If after-market video cards were preinstalled into your computer system, connect the monitor's video cable to the video card connector. Connect the monitor's power cable to the power strip.

The mouse and keyboard connectors are color-coded and icon identified. Attach the mouse and keyboard cables to the connectors on the back of the computer by matching the colors. Some systems have USB mouse and keyboards.

The computer is now ready to be turned on. Turn the computer on by pushing the power button on the front of the unit.

Cabinet Installation Instructions

***Figure 1-2:** MultiImage II Light Cabinet (DE-500)—Pictured with the cabinet top (DE-500FC)*

Cabinet Setup

When you remove the light cabinet from its shipping carton, it is already partially assembled. The camera mounting assembly is packed separately in the same container. The UV transilluminator and cabinet top are both packed in separate boxes. Make sure you have received all the hardware before discarding the shipping carton.

1. The largest box will be the DE500 cabinet. This box will include camera bracket, camera bracket gasket, RS-232 cable, magnetic pad and pen.
2. Set the entire cabinet assembly on a level, flat surface. (There are indentations to the bottom of the cabinet, please use them for lifting and placement of the unit. The cabinet weighs 80 lbs with the UV transilluminator please take appropriate precautions in lifting and moving the light cabinet.) The footprint dimensions for the cabinet is 20" wide X 14" deep.
3. Open door and remove shipping foam from above and below fold down white light table.

NOTE: The camera bracket, gasket, RS-232 and magnetic pad/pen will be located in and around the shipping foam. Fold up white light table.

NOTE: Be sure to remove the inserts from the white sidelight bulbs.

4. Slide out UV tray. Unpack UV transilluminator and mount onto sliding tray (be sure to align UV transilluminator rubber feet into sliding tray open positions). Inside the cabinet, locate the power cord taped with RED tape. Uncoil and insert into the power socket for the UV transilluminator. (Additionally: if installing ChromaLight™, use the BLUE taped power cord for the transilluminator.)
5. Plug the light cabinet into the surge protector, turn on the cabinet, and test each of the switches on the front of the cabinet to ensure that all connections were made properly.

NOTE: Do not plug a transilluminator or the MultiImage II light cabinet into the same power strip as the computer; use a different circuit whenever possible.

6. AlphaView provides cabinet controls through the software. An RS-232 cable that is supplied with the cabinet is required for this purpose. Locate the RS-232 cable insert it into the corresponding socket, next to the power connect, and power switch on the rear of the DE500 (see Figure 1-3).

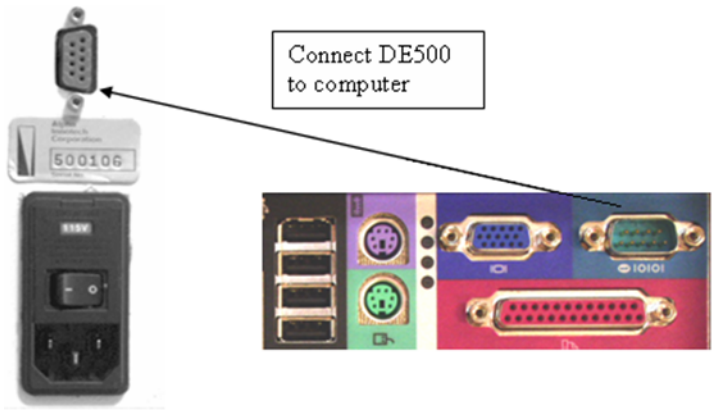


***Figure 1-3:** Cabinet Setup*

The DE-500 and DE-500FC MultiImage Light cabinets connect to the back of the computer via an RS-232 cable.

!WARNING!

If equipment is used in a manner not specified by the manufacturer, the protection provided by the equipment may be impaired.

CAUTION

The power supply cord is used as the main disconnect device. Ensure that the socket-outlet is located/installed near the equipment and is easily accessible.

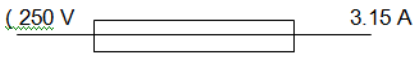
Symbol	Description
	Ultraviolet Light
	Risk of Electric Shock
	Hazard—Take Appropriate Precautions
	Earth (Ground) Terminal
	Fuse Symbol

***Table 1-1:** System Symbols*

!WARNING!

Please use protective equipment when using UV light. UV protective goggles, face shields, long sleeve lab coats can be obtained from major scientific distribution catalogs (e.g. Fisher Scientific).

Power Ratings:

DE 500FC MultiImage II cabinet is rated at:

250V 50/60 Hz 3A

Fuse: 250V 3.15A

Camera Components and Installation

Motorized Lens (Optional)

Figure 1-4 illustrates the different camera components for the motorized zoom lens (optional) for the FluorChem HD2.

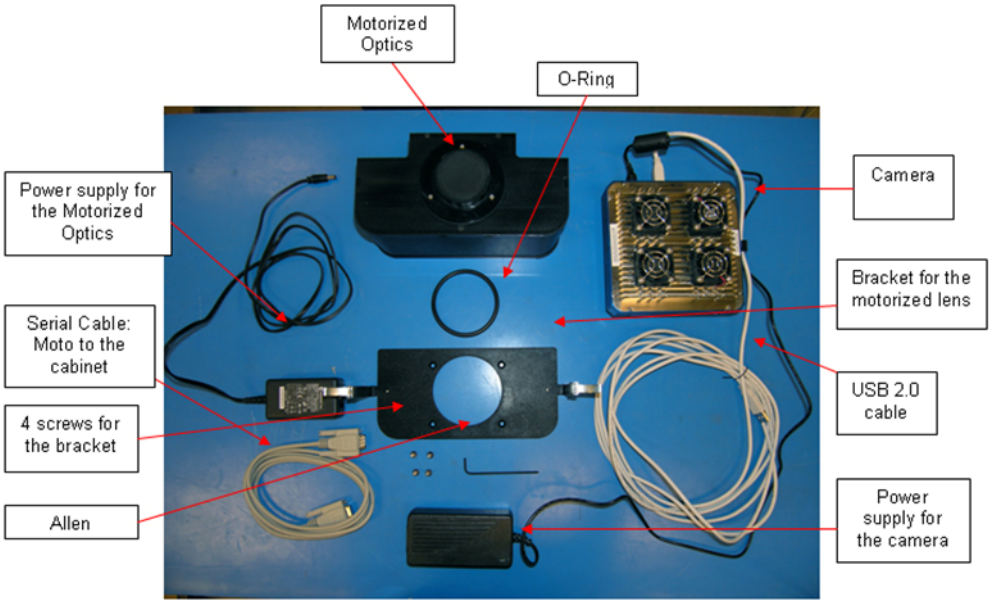


Figure 1-4: Camera Components

The following steps detail the installation and setup process:

1. Use an Allen wrench to attach the motorized lens bracket to the top of the DE-500 cabinet with the four screws provided.

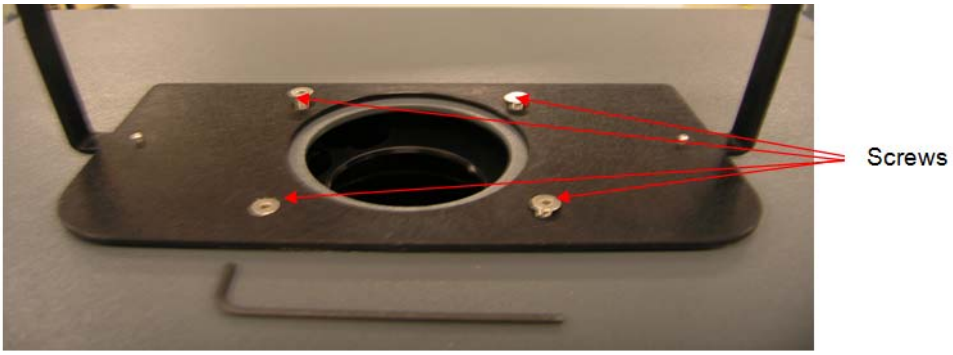


Figure 1-5: Camera Installation—Step 1

2. Place the o-ring in the middle of the bracket.

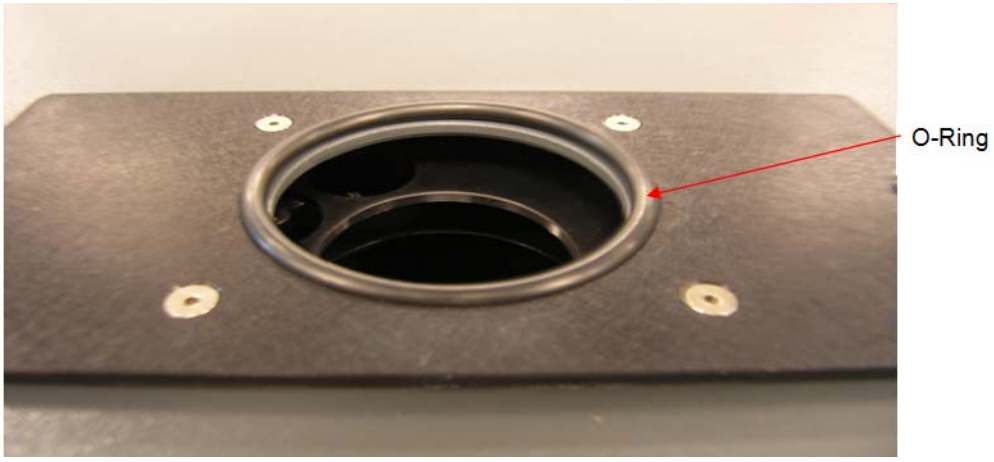


Figure 1-6: Camera Installation—Step 2

3. Place the motorized optics onto the bracket, carefully aligning it on top of the two protruding studs. The motorized optics and should fit perfectly. Once on top, use the two fasteners to secure the motorized optics.

***Figure 1-7:** Camera Installation—Step 3*

4. Place and secure the camera on top of motorized optics.

***Figure 1-8:** Camera Installation—Step 4*

CAUTION

Install two ¼"-20 x 1/4" button head screws on the camera sides to prevent light leaks. Do not use screws longer then 3/8" or the camera will be damaged.

1. Connect the camera cable and the camera power supply connectors to the back side of the camera.

Figure 1-9: Camera Setup—Step 1

2. Connect the power cable standard three-prong end to the power strip.

Figure 1-10: Camera Setup—Step 2

3. Complete the installation by connecting the camera cable to the USB port.

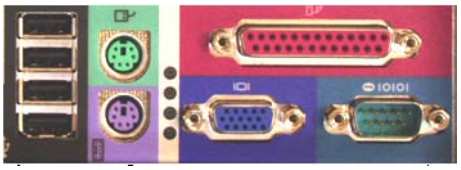


***Figure 1-11:** Camera Setup—Step 3*

Connecting the Printer

The Mitsubishi P95DW (UB) printer connections are color-coded for convenience. Plug the USB cable into the back of the printer and into the proper USB connector on the back of the computer. Plug in the standard three-prong power cable to the back of the printer and to the power strip, then turn the power on.

NOTE: The printer may be connected to any USB port on the computer; however, the driver may need to be manually re-installed depending on which USB port is used.

The Mitsubishi CP700 and CP770 printers connect via the on-board parallel (LPT1) port. Plug in the standard three-prong power cable on the back of the printer and into the power strip, then turn the power on. Set the printer up for color or black and white printing following the directions in the owner's manual supplied with the printer.

Power Up Sequence

Once all connections are made between computer, cabinet, camera, printer and monitor power can be applied in the following sequence:

1. Monitor
2. Computer
3. DE500 cabinet
4. Camera power supply—the camera will begin to cool to -10 °C.
5. Printer

After the cabinet filter wheel has finished homing, launch AlphaView software to begin deep cooling of the camera (minimum cooling period is 30 minutes).

Starting the FluorChem System with AlphaView Software

To launch AlphaView software, double-click the AlphaView icon on the Windows desktop.

System Information

To display system information, click Help > About. This option accesses a pop-up box (see Figure 1-12).

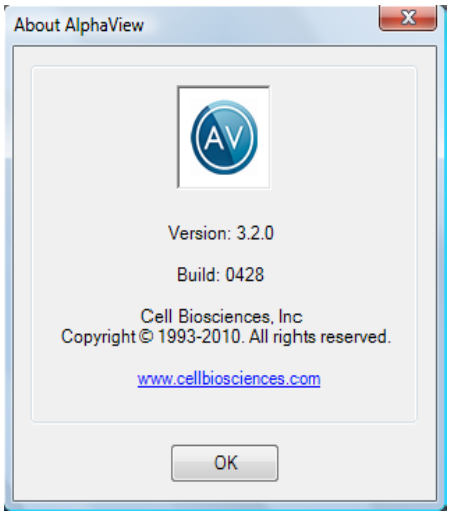


***Figure 1-12:** System Information*

This box shows the software version number. Use the information specific to your instrument and software when calling Cell Biosciences for technical support and software upgrades.

To close the box, click OK.

Chapter 2:

Getting Started—Basic Imaging Functions

Chapter Overview

- Basic Imaging Functions
- Automatic Image Capture (AIC) Software–Optional
- AlphaView System Quick Guide
- Contrast Adjustments
- Imaging Tools

Basic Imaging Functions

Power up the system computer and click on the AlphaView icon on the desktop to open AlphaView software.

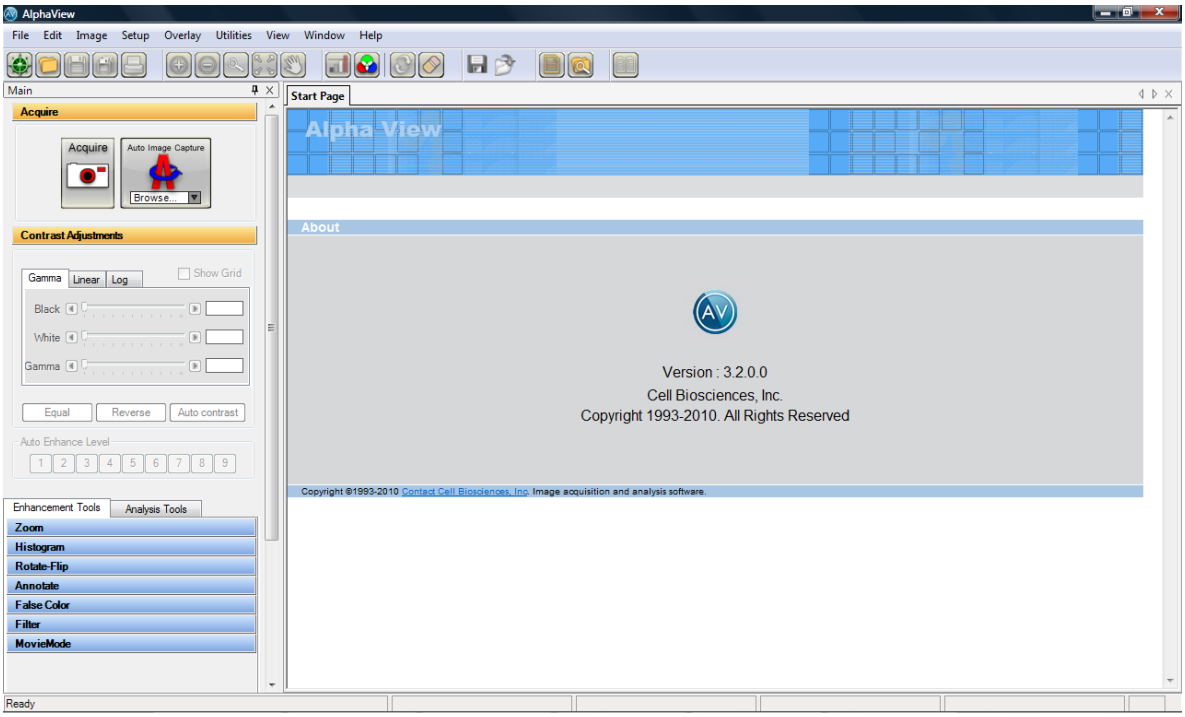


***Figure 2-1:** AlphaView Screen—Displaying the Image Area and Display Controls*

AlphaView software has four main control areas:

- **Acquire** for image acquisition
- **Contrast Adjustments** for image adjustment
- **Enhancement Tools** for image enhancement
- **Analysis Tools** for image analysis

Acquiring an Image Using the Navigator

Clicking the Navigator toolbar icon will guide the user through commonly used applications; in this example procedure, the "Ethidium Bromide" application is demonstrated.

***Figure 2-2:** AlphaNavigator—Selecting the Yes Option*

Selecting the "Yes, …" option displays the following dialog box.

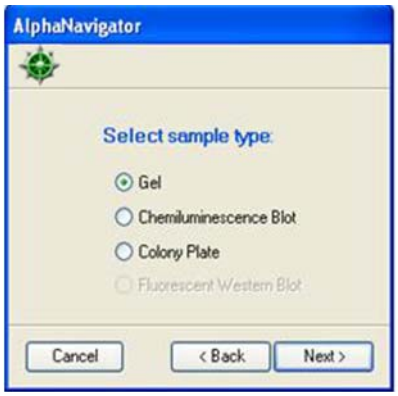
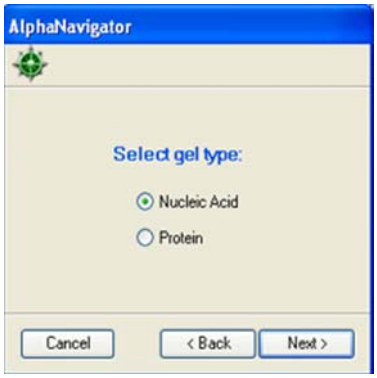


***Figure 2-3:** Selecting the Sample Type*

NOTE: The supported applications depend on the product.

Selecting Gel displays the following screen:



Selecting Nucleic Acid displays the following screen:

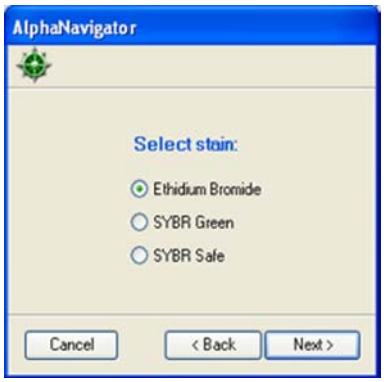


***Figure 2-4:** Selecting the Ethidium Bromide Stain Option*

Choosing Ethidium Bromide will select the proper light source and filter needed. The acquisition screen in the preview mode is displayed with the Auto Expose option selected.

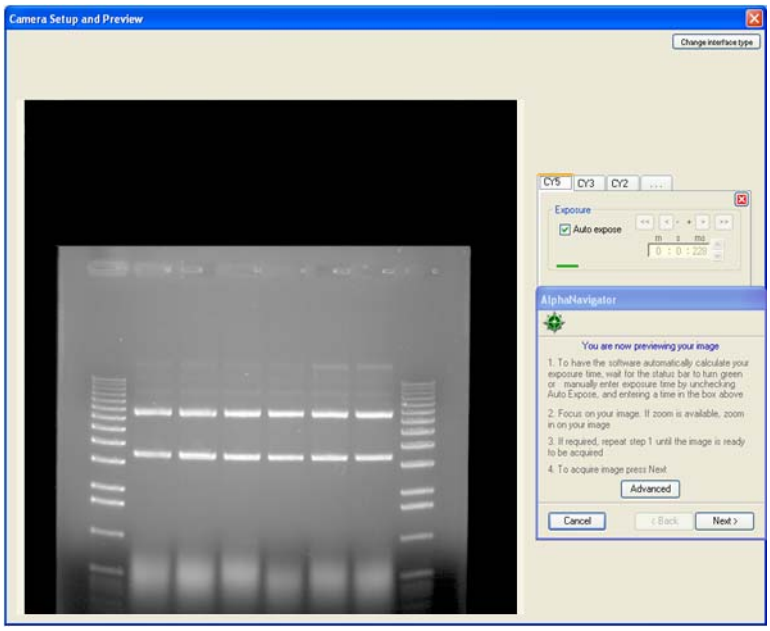


Figure 2-5: *Previewing Your Image*

AlphaNavigator displays navigation notes. Click on the Advanced button to access more acquisition controls, or click Next to acquire. If the Advanced button is selected:

***Figure 2-6:** Advanced Mode—Accessing Acquisition Controls*

The user can modify any control, hit Next to acquire, or Back to return to the preview mode.

If Next is selected the following screens are displayed:

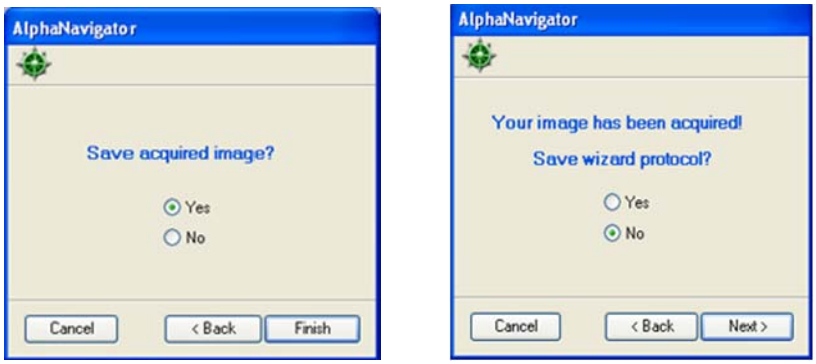


***Figure 2-7:** Image Acquisition Confirmation Prompts*

Selecting the Yes option and clicking Next will give a save protocol dialog box. The protocol will be added to the list of existing protocols.

Selecting the No option and clicking Next will generate the save image dialog box:

Selecting the Yes option and the clicking the Finish button will generate the save image dialog box and exit the Navigator. Selecting the No option, will close the Navigator.

The next time the Navigator is run, the user can run the saved protocol:

***Figure 2-8:** Running the Saved Protocol*

Selecting the No option, the following screen is generated with the last saved protocol selected.

***Figure 2-9:** Selecting the Saved Protocol*

The user can browse for other saved protocols by clicking on the down arrow. A list of existing protocols will be displayed:

Figure 2-10: *Selecting "My Eth. Boromide" Saved Protocol*

Selecting the saved protocol will go to the preview screen for the user to verify or acquire the setting.

Acquiring an Image Using the Acquire Button

In the Tool Bar, click the Camera Acquire icon, which launches the Camera Setup and Preview window; see Figure 2-11.

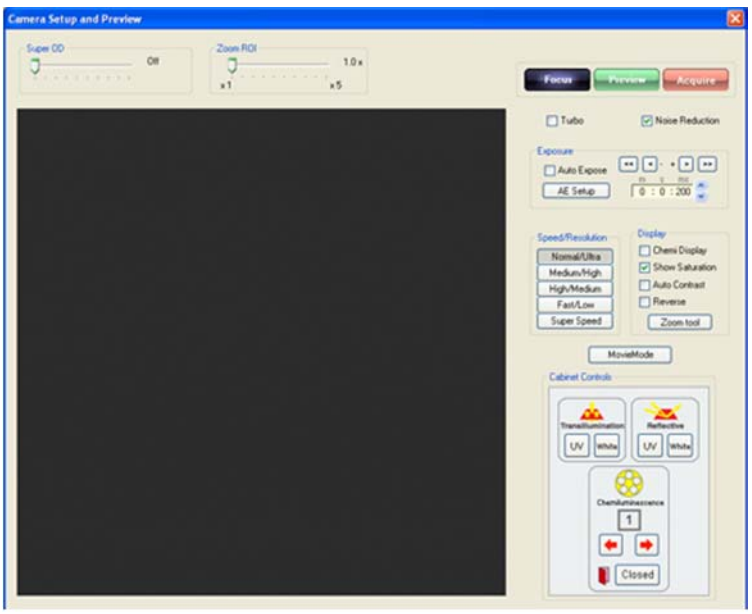


Figure 2-11: *Camera Setup and Preview Window*

This window provides camera exposure control, sensitivity/resolution control, lighting/filter wheel position control, contrast display options, and cabinet door status. The active imaging channels are laid out in a tabbed architecture. One tab for each channel that contains the complete acquisition configuration for that channel.

In addition, the system can enter Movie Mode by clicking the MovieMode button; see *"Movie Mode,"* starting on page 34.

Focus Control Mode

This window opens in Focus control mode (blue button). Focus control mode provides near-real time readout for easy sample positioning and optics adjustments. While in focus, select the lighting, filter, and binning options appropriate for your sample. You can then focus on your sample, select an aperture setting, and focus on your image.

NOTE: While in Focus control mode, we recommend clicking the Auto Expose check box to allow the software determine the optimal length of time for your image acquisition.

Expose Preview

Clicking the green Expose Preview box allows you to preview what your final image would look like, while still allowing you to make small adjustments to the options you select in focus.

Acquire Image

Select Acquire Image to capture your final image. You will then be able to save, print or analyze your image.

Auto Expose

The AlphaView features an Auto Expose function for ease of use. The Camera Setup and Preview window includes a check box to activate the Auto Expose function, as well as a button labeled Auto Expose Setup to configure the function.

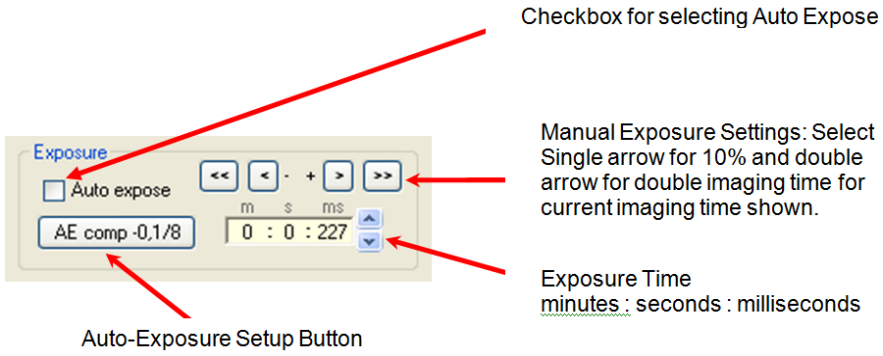


***Figure 2-12:** Auto Expose*

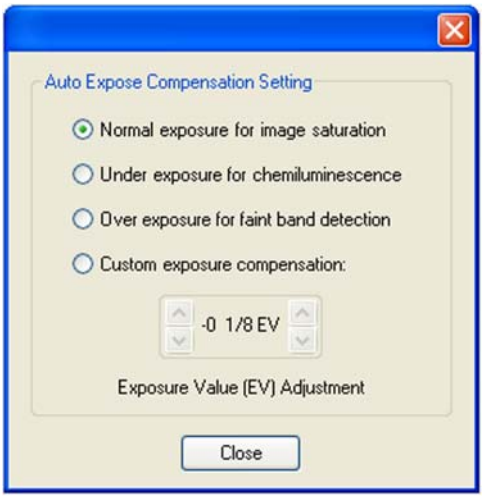


***Figure 2-13:** Auto Expose Compensation Setting*

Clicking the Auto Expose Setup button launches the Auto Exposure Compensation Setting window. This window offers four options:

- Normal exposure for image saturation. This setting is ideal for normal Colorimetric and Fluorescent imaging.
- Under exposure for chemiluminescence
- Over exposure for faint band detection.
- Custom exposure compensation. This setting allows the user to define the Exposure Value (EV). The left set of up/down arrow buttons change the EV value by whole unit intervals while the right set of up/down arrow buttons change the EV value by 1/8-unit intervals.

Auto Exposure works in both Preview and Acquire Image modes. The AlphaView software calculates the correct exposure time for the current situation. In Expose Preview mode, a status bar appears above the Auto Expose interface. In Acquire Image mode, a status window featuring a colored Auto Expose status bar for image acquisition appears. The status bar indicates the current status of the calculation. The status bar changes from red to yellow to blue to green to indicate progress. When it reaches green, the AlphaView software has calculated the correct exposure time. For exposure time calculations with saturated pixels in the image area, the status bar changes to pink to indicate the issue.

In Acquire Image mode, a green status bar indicates that the image is acquired. After achieving a green status, the new image loads and the Camera Setup and Preview window closes. In Expose Preview mode, the system continues repeated expose using the calculated exposure time until a different mode is selected.

Switching from Expose Preview mode to Acquire Image mode does not reset the calculation. If the system changes from Expose Preview mode to Acquire Image mode after calculating the correct exposure time in Expose Preview mode, images are acquired using the last calculated exposure time. Similarly, if a different sensitivity (binning) mode is selected after finishing the exposure calculation, the calculation is quickly converted and no recalculation is required.

The Exposure Value (EV) unit is the same as used in film photography (also called a "stop"). +1 EV will double the exposure time while –1 EV with halve the exposure time. The general exposure time using EV is found by the following formula:

$$(EVCompensatedTime) = Time * 2^{EV}$$

Where *Time* is the typical exposure time for the sample under test to reach full exposure as determined by the auto exposure tool.

Setting Exposure Time Without Auto Expose

Once the sample is positioned and the lens is properly focused, perform the following steps:

1. Close the MultiImage light cabinet door and check to ensure that the appropriate illumination source is turned on. In addition, check to ensure that the cabinet door indicator in the Cabinet Control software interface indicates Closed.
2. Uncheck the Auto Expose option.
3. Click on the green Expose Preview button and select the desired exposure time in to configure the system for the desired image intensity quality. Individual adjustments for milliseconds, seconds, minutes, and hours are available. The following list describes ideal exposure settings for different applications:
    - For most white-light applications, using a 50 ms exposure is sufficient. Final aperture adjustments can be made to optimize the image quality.
    - For UV fluorescence applications, using an exposure range between 8 ms and 4 seconds is sufficient. The aperture should be completely open when performing any adjustments. Selection Show Saturation is recommended for these applications.

- For low-light applications, such as chemiluminescence, a longer exposure time may be appropriate. In these cases, push the red Acquire Image button to directly acquire the image upon setting the desired exposure time. If a good exposure time is unknown, an alternative method is to utilize the Auto Expose feature configured to super sensitivity. Auto Expose image generation – with signal intensity showing—generally completes in less than three minutes. After Auto Expose image generation, a different sensitivity (binning) mode can be selected to generate a better resolution image. For further images, the software automatically calculates the new exposure time.

NOTE: When the system switches to Expose Preview mode, the image may flash or change brightness due to camera photon collection from the image over a longer period of time prior to sending the image to the computer's display readout.

Light Source and Filter Controls

The mechanical controls on the cabinet and the AlphaView software's virtual cabinet control interface (labeled as Cabinet Controls) are linked and provide near-real time synchronization and updating of light source and filter selection. Selecting a desired option via the software interface produces the same result as using the physical controls on the cabinet.

NOTE: A slight delay occurs upon pressing the button until the light source is fully activated.

Standard lighting choices include:

- Transillumination White: Protein gels, autorads, film, plates, and flasks
- Transillumination UV: Fluorescent gels such EtBr, SYPRO Red, SYBR Safe, etc.
- Reflective White: Colorimetric blots and membranes
- Reflective UV (optional): SYBR green, TLC plates, and Chemifluorescence
- ChromaLight (optional): GFP, Fluorescein, and SYBR green

Filters selection is linked to appropriate sample visualization.

Filter options include:

- Filter Position #1: Empty Position for Chemiluminescence detection
- Filter Position #2: Orange filter (595/55 nm) for Ethidium Bromide, colorimetric stains, film, SYPRO Orange
- Filter Position #3: Optional green filter (537/40 nm) for SYBR Green, SYBR Safe and Fluorescein
- Filter Position #4: Optional red filter (620/40 nm) for SYPRO Ruby, SYPRO Red and Deep Purple
- Filter Position #5: Optional blue filter (460/40 nm) for Hoeschst Blue

NOTE: If exposure time is less than 100 ms, yet signal is strong, close the aperture by stops.

Turbo Modes and Speed/Resolution Settings

The AlphaView System offers different speed and resolution settings to allow image acquisition across a variety of needs. Tweaking resolution and speed settings allow image acquisition to occur as quickly as possible, with the highest resolution possible, or somewhere in between. Available acquisition modes include 5 binning modes and 2 Turbo modes.

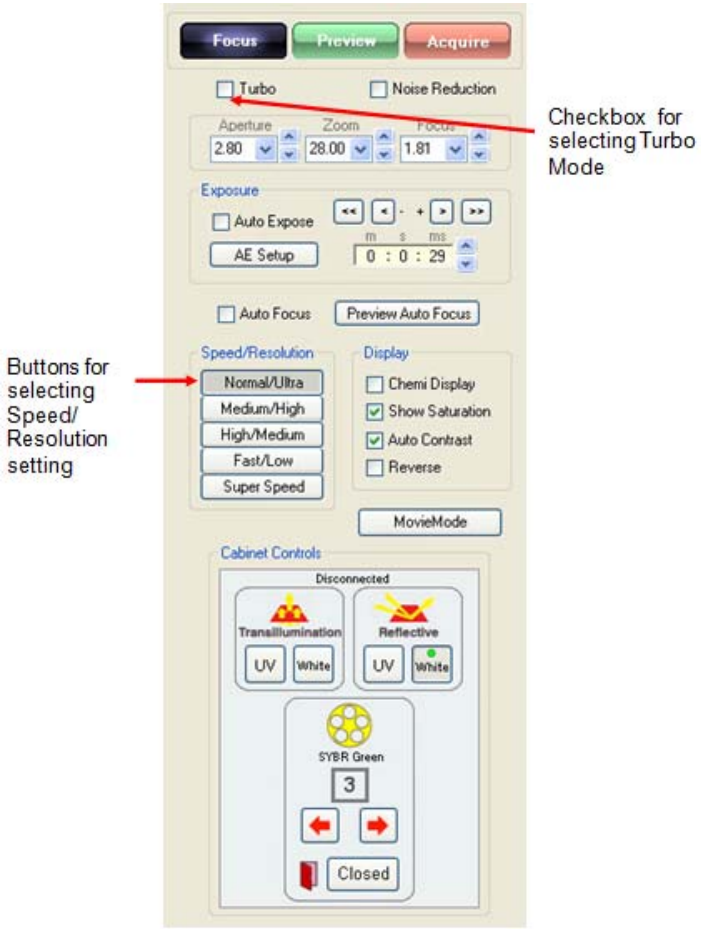


***Figure 2-14:** Turbo Modes and Speed/Resolution Settings*

The camera modes are selected by the Turbo Mode check box. When the Turbo Mode checkbox is unchecked the camera operates in Normal mode. Normal mode is useful for those imaging applications that require wide dynamic and quantification of low signal intensities. When the Turbo mode checkbox is checked the camera operates in Turbo Mode. Turbo Mode is used for the applications that require fast image acquisition to quickly visualize samples even those with low signal intensities.

Both Normal and Turbo mode can be used with each of five binning modes and Auto Expose functions to optimize image acquisition speed and for fluorescence and chemiluminescence applications.

Speed/Resolution Settings

Normal/Ultra Mode

Recommended for Fluorescence and Colorimetric Imaging, this mode captures the image using the CCD sensors full 2048x2048 pixel resolution to generate highest quality, vibrant images that are ready for quantitative analysis and publication.

Medium/High Mode

Recommended for Chemiluminescence, this mode captures images with a 2x2 pixel bin to decrease the required exposure time while maintaining a high resolution (1024x1024) image. This mode decreases exposure times from full resolution images by approximately a factor of four.

High/Medium Mode

Recommended for Chemiluminescence, this mode captures images with a 3x3 pixel bin (683x683 pixels) with decreased image acquisition time and medium resolution. This mode decreases exposure times from full resolution images by approximately a factor of nine

Fast/Low Mode

Recommended for Chemiluminescence, this mode captures images with a 4x4 pixel bin (512x 512 pixels) Similar to Super Speed this mode decrease exposure time but with a trade off in resolution. This mode decreases exposure times from full resolution images by approximately a factor of sixteen

Super Speed Mode

Recommended for Chemiluminescence, this mode captures the image performing an 8x8 pixel bin (256 x 256 pixels). This mode significantly decreases the exposure time required for acquiring a long chemiluminescent exposure. This mode's resolution is set to 256x256 pixels and is not recommended for precise quantitative analysis or publication. Super Speed mode is ideal for Expose Preview functionality when using Auto Expose to determine the chemiluminescent blot signal level. It is also useful for rapid visualization of signals for a qualitative assessment of the sample.

Gray Scale Optimization for Saturation and Contrast Displays

Figure 2-15 illustrates the check boxes available for modifying the displayed image.

***Figure 2-15:** Modifying the Displayed Image*

Any images set for analysis must not be over-exposed (too light) or under-exposed (too dark) for maximum clarity. Using the Show Saturation option (located below the camera control functions) displays the image areas assigned to each end of the gray scale spectrum. After inspecting the image's saturation, the imaging controls can be adjusted accordingly to optimize the image.

The Show Saturation option allows the user to access the Saturation Palette during image acquisition. The Saturation Palette is a modified gray scale palette in which green replaces black (gray level 0), and red replaces white (gray level 255, 4,095, or 65,535). With this palette, the image's over- and under-exposed areas appear as green or red, while areas within the linear range of the CCD chip appear in gray scale.

During image acquisition, it is critical to note the image's regions that appear red or green. The exposure time and the camera aperture can be adjusted to minimize the amount of red and green in the image area. Eliminating red and green in the actual sample area is especially critical for images that will be quantified. The saturation view can be turned off by unchecking Show Saturation once the red and green areas have been minimized and/or eliminated

The other three selections (Chemi Display, Auto Contrast, and Reverse) act as visualization tools designed to enhance contrast and provide flexibility image viewing. The Auto Contrast option displays the image with automatic black, white, and gamma adjustments according to the image histogram information (black/white levels). Reverse shows the sample as a negative by switching the black and white values. The Chemi Display option utilizes both auto contrast and reverse functions, as well as providing gamma adjustments, and is intended for use with chemiluminescent samples.

NOTE: These options are also available in the Movie Mode.

NOTE: These Contrast Display options are only visualization tools and do not change any image data acquired by the CCD camera.

Zoom ROI Tool

Zoom ROI is a tool designed for use with fast or fixed focal length lenses. These lenses typically have lower F-stop numbers than zoom lenses, but do not have zoom capabilities. Fast lenses are most commonly used to decrease exposure times in chemiluminescence applications. However, they can also be used for other applications, such as fluorescent gels. Since fixed focal length lenses do not have zoom rings, adjusting an acquired image's region of interest is possible using the AlphaView software interface.

When the sample's region of interest is smaller than the acquisition window's and the system is not equipped with a zoom lens, the zoom ROI tool provides an ideal workaround. The Zoom ROI tool allows for software-based zooming without having to switch lenses. To use the Zoom ROI tool, focus on the image, then position the Zoom ROI slider until the region of interest occupies the preview window's entire image area. Use the Expose Preview and Acquire Image buttons to configure and acquire the final image. The Zoom ROI tool eliminates the need for cropping an image down to a desired size post-acquisition.

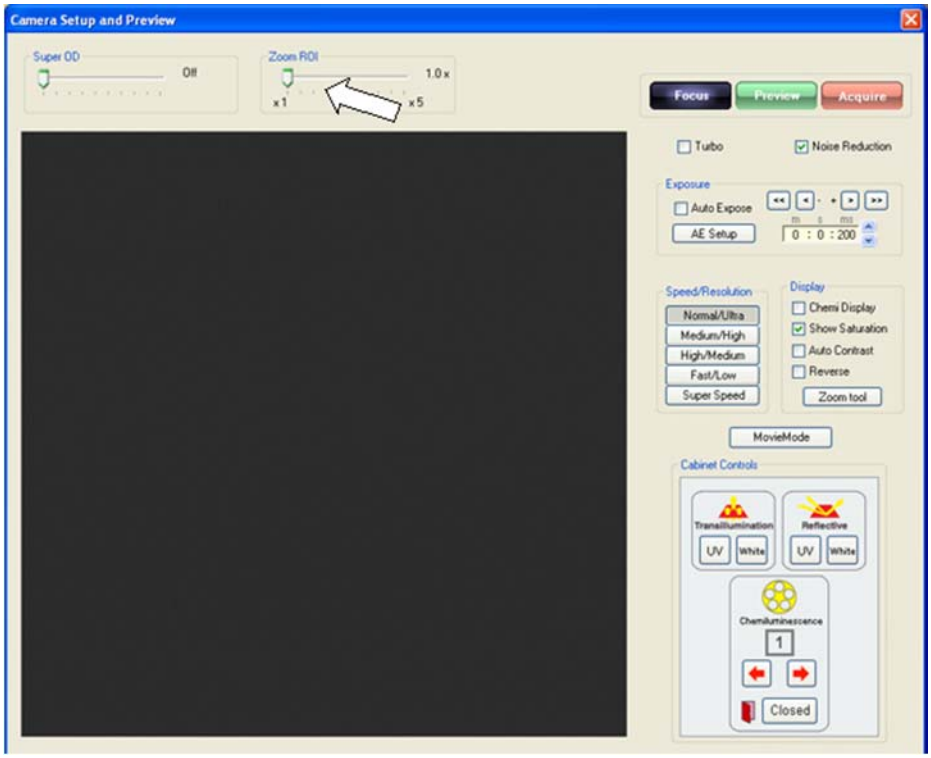


***Figure 2-16:** Zoom ROI Tool*

Saving Images

Once a satisfactory image has been captured, it should be saved as an original file for safe archiving. Click File > Save As or click the Save or Save As icons.

Super-OD Acquisition Feature

Super-OD™ is a process that delivers full dynamic range performance for digital imaging applications. Super-OD acquisition accomplishes this by extending the range of different light (or brightness) levels from the sample that can be viewed as a single image beyond that of a standard image. Super-OD acquisition can produce an image that represents more than 100-fold extension of the brightness range.

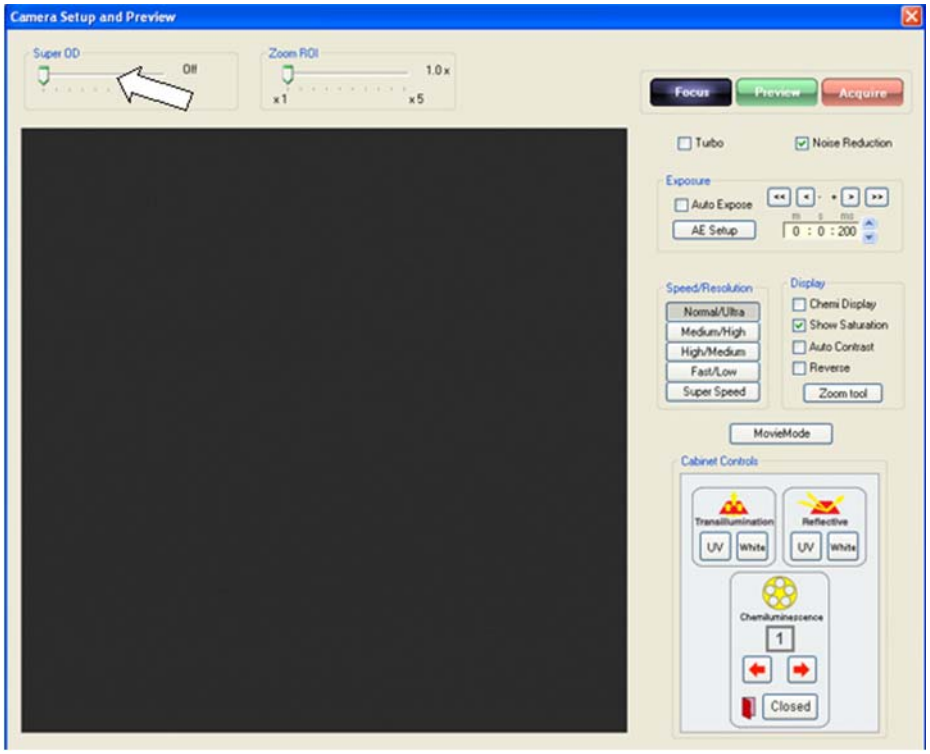


***Figure 2-17:** Super-OD Acquisition Feature Window*

The AlphaView acquisition window contains a Super-OD slider bar located above the image window display.

Acquiring in Super-OD Acquisition Mode

The user may acquire a Super-OD image with a factor from 2 to 500. Choose the Super-OD factor to use by sliding the Super-OD slider bar to the right. Super-OD acquisition is activated whenever the Super-OD slider is in any position other than "Off". As the slider bar is moved to the right the "Super-OD Factor" will increase and be displayed.

***Figure 2-18:** Super OD Slider*

In addition to the Super-OD Factor, the software also shows a time estimate for the total acquisition time. This time estimate is displayed as "Time x 'value'" where 'Time' is the initial exposure time and the 'value' is the multiplier to achieve the chosen Super-OD Factor. In the example above, the Factor is set to 8. The total acquisition time will be 544 times the initial exposure time. If the initial exposure time is 10 milliseconds, the total acquisition time for this acquisition will be 10 milliseconds x 544 = 5440 milliseconds or 5.44 seconds.

Initial Exposure Time for Super-OD Acquisition

Whenever Super-OD is activated the acquisition interface disables the exposure time input fields and switches to Auto Exposure mode. The initial exposure time used for Super-OD is determined by a proprietary Auto Exposure algorithm. Clicking the Expose Preview button in Super-OD mode will calculate and display the initial exposure time. Clicking the Acquire button in Super-OD mode will start the Super-OD acquisition process which first determines the initial exposure value and then performs the Super-OD acquisition process automatically.

Acquiring Super-OD Images

Slide the Super-OD slider bar to the desired Super-OD factor. Click the Acquire button. When the process is complete the resultant Super-OD image will be displayed in the AlphaView software main window.

Movie Mode

If kinetic, multiplex, color, or chemiluminescence experiments require the system to automatically capture several images at preset exposure times, preset time delay between images, preset lighting sources, or preset filter choices, then the AlphaView Movie Mode should be used. Movie Mode is accessible in the Camera Setup and Preview window. To launch the setup screen, click the Movie Mode button in the Camera Setup and Preview window. Movie Mode can also be accessed from Movie Mode in the ToolBox Enhancement Tools tab.

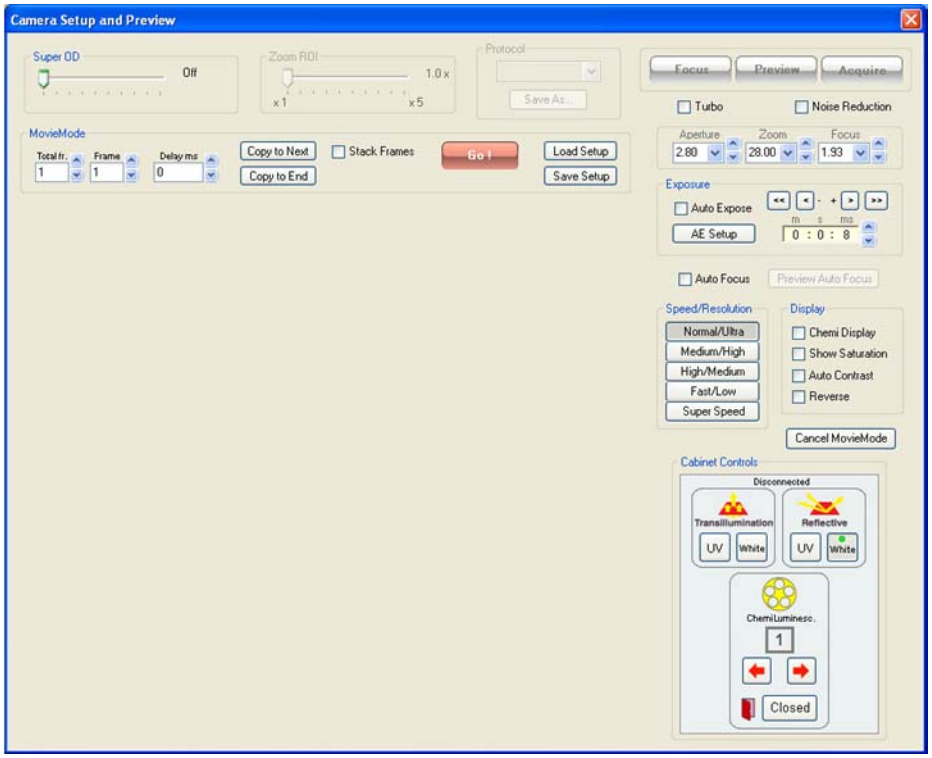


***Figure 2-19:** Movie Mode Setup*

The Total Frames field sets the total number of individual frames in the movie. Each movie can capture maximum of 50 frames (images) and a minimum of one frame.

The Frame field selects the active frame currently being used for condition customization. For example, if the movie uses three images, selecting Frame 1 allows for lighting and filter setup of the movie's first frame; selecting Frame 2 allows for setup of the movie's second frame. All frames can be adjusted manually using this technique, or specific lighting conditions can be copied to the next frame using the Copy To Next button. Copy To Next copies all settings from the previous frame to the current frame. The Copy to End button nscopies current frame setting to the entire frame set.

For chemiluminescence imaging, all lighting is typically shut off and the filter wheel is positioned for the nschemiluminescence position across all frames. In these situations, the exposure time is the only variable that changes from frame to frame. The Copy To Next button is a useful tool that optimizes setup time.

The Delay field configures a predetermined delay between captured imaged for kinetic experiments. The default Delay is set for the shortest possible delay (19 ms), but can be configured up to 50 minutes between images.

Once the movie setup is properly configured, click the Go button to begin the movie process. The system begins processing image acquisitions for each frame. When completed, the movie setup box disappears and the Toolbox window automatically configures to the Movie tools. This allows for movie playback, saving, loading, or new movie recording.

The movie display buttons offer the following functionality:

REC	Launches the Camera Setup and Preview window configured to Movie Mode.
PLAY	Displays the movie in a continuous loop.
STOP	Stops the movie at the current frame display.
PAUSE	Pauses movie playback at a user defined image.
REW (rewind)	Rewinds the movie to the first image.
REV (reverse)	Plays the movie in a continuous loop in reverse.
FWD (forward)	Forwards the movie to the last image.
DEL (delete)	Deletes the movie on the display. *NOTE: This function does not delete movies saved to the hard drive.*
LOAD	Loads a previously saved movie. *NOTE: This function does not load individual images previously captured in normal capture mode.*
SAVE	Saves a movie of images.

Saving An Individual Image from a Movie

After you load, play, and stop a movie at the desired image, it is possible to save the individual image seen on the screen. Use the SAVE AS button located on the tool bar to save the image in the desired location and file format on the local or network drives.

Saving a Partially Acquired Movie

It is possible to stop the movie in the middle of acquiring frames, the user can exit the movie mode to save the acquired frames only.

***Figure 2-20:** Saving a Partially Acquired Movie*

Option 1: Go to continue movie.

Option 2: Close Window to view and/or save acquired frames.

Save/Load Setup Acquisition

Individual movie parameters can be saved for future use. The Save Setup and Load Setup buttons save and load all Movie Mode setup parameters. This data is saved in files using the MVF format.

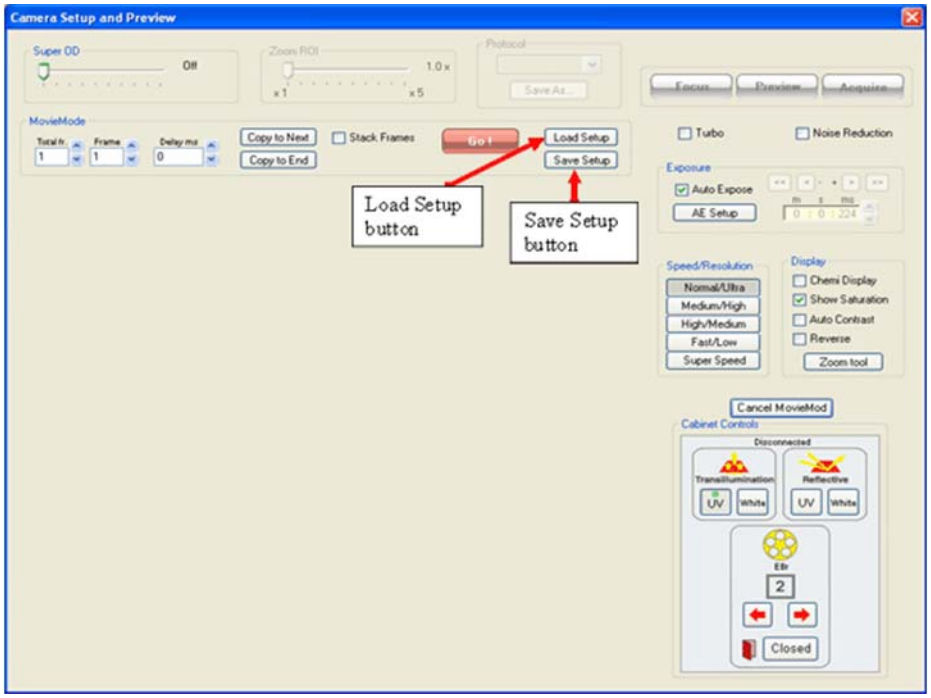


***Figure 2-21:** Movie Mode: Load/Save Setup*

Frame Stacking

The Movie Mode configuration of the Camera Setup and Preview window provides an option for stacking frames. If this selection is used during image acquisition, Stack Frames uses previous exposure information to sequentially add images to one another. Normal Sequence does not perform this addition.

NOTE: Stacking frames increases the noise level in acquired images.

Sample Case

Capture 5 frames at 1-5 sec exposure for total time exposure time of 15 sec

Display after summation of following frames:

Frame 1 = Image (1 sec exp time)

Frame 2 = Frame1 + Image (2 sec exp time)

Frame 3 = Frame2 + Image (3 sec exp time)

Frame 4 = Frame3 + Image (4 sec exp time)

Frame 5 = Frame4 + Image (5 sec exp time)

Automatic Image Capture (AIC) Software–Optional

For molecular biology researchers engaged in high throughput and particularly high volume work involving repetitive assays, Automatic Image Capture software is available as an option. This system automatically adjusts lens focus, filter settings (for fluorescent imaging), light settings and exposure for perfect images. All of this with just one click--making the acquisition of images faster and more convenient than standard imaging software.

NOTE: AIC requires motorized zoom optics.

Designing a User Protocol

To utilize the single click feature of Automatic Image Capture, a protocol specific to your sample must be defined and saved. Click the Acquire button to reach the Camera Setup and Preview Window.

All acquisition parameters that can be set by the user are available in the Camera Setup and Preview window and can be saved as a user protocol. Below is an example of the design of a protocol for imaging DNA gels stained with Ethidium Bromide.

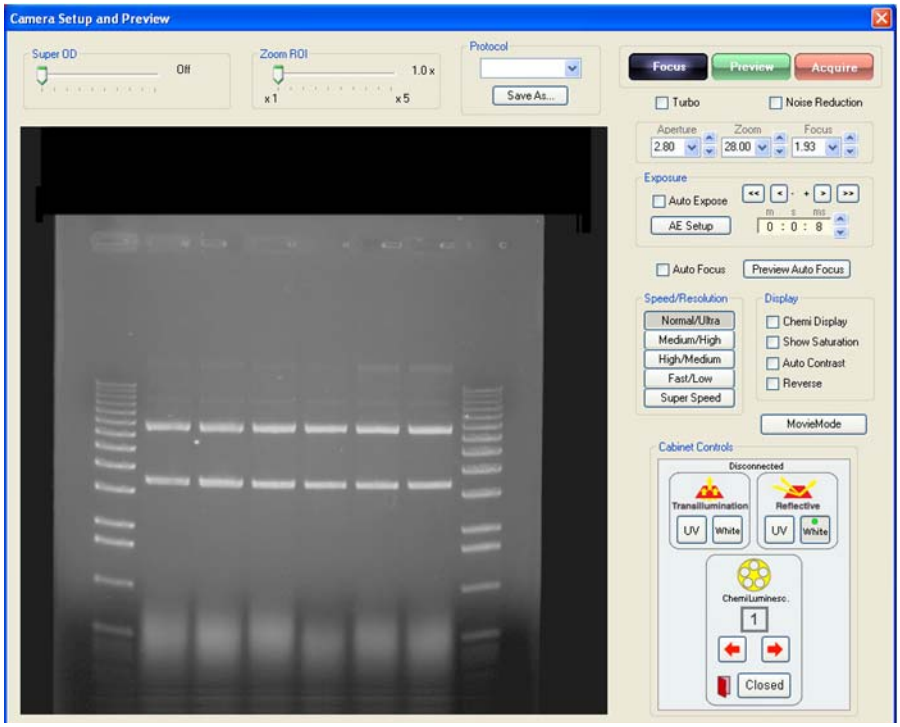


***Figure 2-22:** Example of the Protocol Design for Imaging DNA Gels Stained with Ethidium Bromide*

Next, click the Load/Save Protocol button to store your customized protocol.

The Save/Load Acquisition Protocol dialog box appears, allowing to you save and name your protocol.

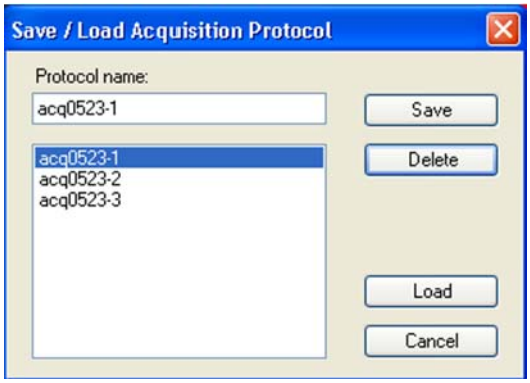


Figure 2-23: *Save/Load Acquisition Protocol*

This dialog box is also useful for loading a previously saved protocol to make small modifications, or delete existing protocols.

All settings and selections visible and accessed in the Camera Setup and Preview window are saved in this protocol. These settings include SuperOD, ZoomROI, lens settings (aperture, zoom and focus), exposure time (including auto exposure setup settings), sensitivity selection, display settings, cabinet lights and filter wheel position.

Auto Image Capture (AIC)

User-customized protocols can be recalled for single click image capture using the AlphaView software's Auto Image Capture feature. First, load the protocol of interest by clicking on the drop down menu from the Auto Image Capture icon.

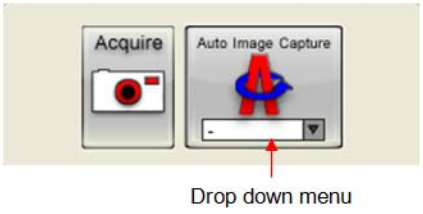


Figure 2-24: *Auto Image Capture (AIC)*

Then, once the protocol is selected, click the Auto Image Capture button to begin the acquisition process. Once the AIC process is initiated, a pop-up window, labeled Acquiring protocol, is displayed to indicate the status of the acquisition process. This progress window shows the current operation status and has some animations to indicate progress. The status messages towards the bottom of the window displays the specific operation and relevant parameters used during the process.

The LIGHTS section displays different simulated LEDs repeating left to right to indicate the status of the light source and filter selection as dictated by the protocol. The CAMERA section displays the current status of the image as the target is being focused as dictated by the protocol. The ACQUISITION section displays the current status of the exposure time as dictated by the protocol.

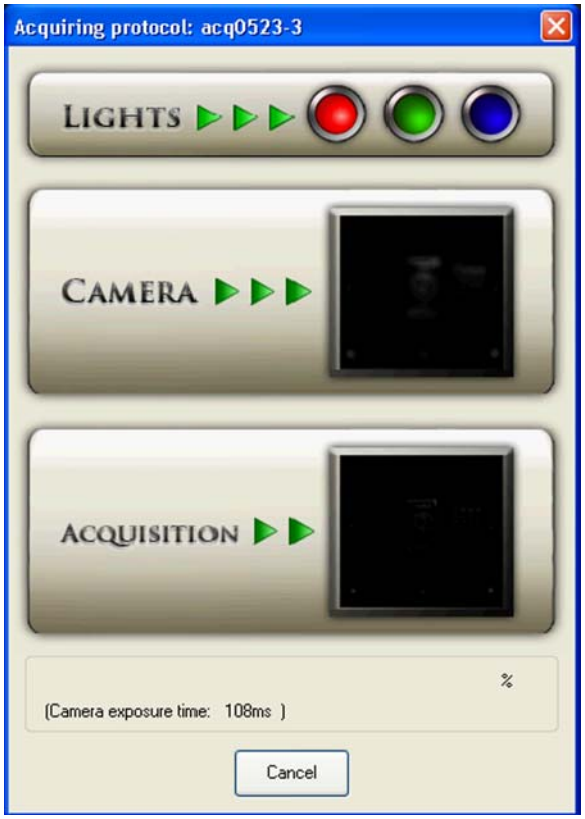


***Figure 2-25:** AIC Status Window*

NOTE: The images displayed in the Acquiring Protocol windows are animations to display status and do not reflect upon the image being acquired.

AlphaView System Quick Guide

NOTE: This is intended as a quick reference guide for acquisition. For more detailed information on the individual features, refer to their respective feature sections.

1. Power on the system:
   a. Turn on the computer, monitor, and optional printer.
   b. After the computer has finished loading the Windows desktop, turn on the power to the cabinet.
   c. The AlphaView software is activated by double clicking on the AlphaView icon.
2. Positioning and focusing on the sample:
   a. In the Tool Bar, select the Acquire icon to activate the image acquisition software features.
   b. In the Camera Setup & Preview window, click the Focus button.
   c. Open the door to the cabinet and position your sample on the preferred illumination source. Fluorescence samples that require epi or transillumination of UV energy should be placed on the purple UV filter glass. For colorimetric samples such as protein gels, films, or blots, use the fold-down white light table for your sample. For chemiluminescence use the fold-down white light table, or the adjustable tray if used in conjunction with the fast lens.
   d. Open the aperture on the camera lens all the way open to the smallest number by either pressing the up and down arrow keys, entering in a value and pressing the tab key. or selecting the preset parameters from the drop-down menu.
   e. With the door still open to allow light to enter into the cabinet, use the monitor's real-time readout display to position and focus your sample in the middle of the preview image.
   f. Focus on the object by either pressing the up and down arrow keys, entering in a value and pressing the tab key or selecting the preset parameters from the drop-down menu. For fluorescence and chemiluminescence, the door to the cabinet can be used to adjust the amount of light that enters into the cabinet if there is too much light to obtain a good focus setting. Do not adjust the aperture to do this. For colorimetric samples, it is necessary to decrease the aperture to acquire the image.

NOTE: Business cards and other pieces of paper with small text often are the easiest objects to obtain optimal focus settings with.

   g. Adjust the zoom setting by either pressing the up and down arrow keys, entering in a value and pressing the tab key, or selecting the preset parameters from the drop-down menu so that the area of interest on the sample takes up the entire image size on the screen.

3. Capturing a bright sample like fluorescently labeled gels, colorimetric samples and film:
   a. Close the cabinet door.
   b. Choose the appropriate optical filter for your sample type:

      Position #1 for colorimetric gels and film (no filter)

      Position #2 for Ethidium Bromide gel sand some colorimetric gels

      Positions #3-5 for other fluorescently labeled gels (optional filters)
   c. Turn on the illumination source (UV or white light) using the touch panel or virtual software controls.
   d. Select the green Expose Preview button.
   e. Check Show Saturation.
   f. Check Auto Expose.
   g. Select the Normal/Ultra resolution setting.
   h. Once the image in expose preview does not contain any saturation (red false color palette for white bands, green for dark bands) click Acquire Image. The exposure bar will turn green when this is complete. If the exposure bar is pink in color, saturation is still present in the image. For extremely bright images, particularly in white light applications, it may be necessary to reduce the aperture setting until the saturation is removed from the image.
4. Capturing a low light image (like chemiluminescence)
   a. Close the cabinet door (there should not be any lights on in the cabinet).
   b. Choose Position #1 in the filter wheel for no filter.
   c. Select the green Expose Preview button.
   d. Check Show Saturation.
   e. Check Chemi Display.
   f. Check Auto Expose.
   g. Select the Super Speed resolution setting.
   h. Wait for the exposure bar to indicate that the proper exposure time has been found and that there is no saturation in the image - the exposure bar will turn green when this is complete. If the exposure bar is pink in color, saturation is still present in the image.
   i. Select Medium/High or Normal/Ultra resolution and uncheck Auto Expose.
   j. Click Acquire Image.

NOTE: The exposure time will vary depending on which resolution setting is selected in step 4.h. If the exposure time calculated by the auto expose setting is too long, it is possible to use the Fast/Low or High/Medium setting instead. Alternatively, if the exposure time is short enough, Normal/High may be selected for a full resolution (4 million pixels) chemiluminescent image.

5. Save the original image:
   a. Select Save Image in the File menu or click on the SAVE or SAVE AS icon in the Tool Bar.
   b. Enter a file name and select the directory to which it should be saved (the directory path should be less than 100 characters).
   c. Specify the file format (TIF, BMP, PCX, MAC, color TGA).
   d. Click OK to save the file.
6. Enhance the display (optional):
   a. Adjust the black, white and gamma levels by moving the slider bars at the right of the image in the Contrast Adjust window, or select Auto Contrast.
   b. Apply the digital filters found in the Tool Box under the Enhancement and Filters (to stop a filter, hit any key on the keyboard; to reverse the effects of a filter, click Undo).
   c. Add text, boxes, arrows, etc. to the image using the annotation tools in the Tool Box under Enhancement and Annotate.
7. Print the image using the PRINT button in the Tool Bar or the pull-down File menu option.
8. Analyze the sample using the analysis features in Tool Box (ANALYSIS for quantitative analysis).

Contrast Adjustments

The Contrast Adjustments window allows for the best visualization possible of a sample utilizing the black, white, and gamma adjustments, as well as, image reverse and auto contrast.

The image on the screen is made up of picture elements (pixels) in an array. Each pixel is assigned a brightness (or a gray scale value) level between black and white. A very bright image has most of its pixels registering high gray level values and conversely, a very dark image has most pixels registering low gray level values (approaching zero).

The distribution of these gray values to the image is determined by the Contrast Adjustment controls. These controls regulate the Black level, White level, and Gamma setting (brightness linearity), allowing adjustment of the display to obtain the best image possible.

NOTE: These enhancement features modify the image display on the monitor only, and do not change the original quantitative data.

AlphaView software can also import RGB color images. The AlphaView Software automatically detects this process and the Contrast Adjustment tools are configured for color image adjustments.

NOTE: An image can be enhanced using these tools and then saved as a modified file for publications. However, to preserve the original image information, it is recommended that the file be saved as a different file name when using the save modified.

Using the Contrast Adjustments Tools for Grayscale Images

There are three sliding scales found in the image control area to the right of the image. Below each scale is a box displaying a number that corresponds to the position of the slider. By adjusting these sliding scales, the image display can be optimized.

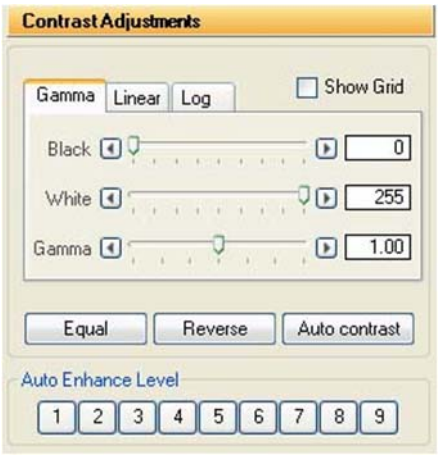


***Figure 2-26:** Contrast Adjustments Settings*

Figure 2-26 displays the imaging display tools: Black level, White level, Gamma setting with B/W/G, Linear, Log, and Equalize options.

To adjust any of these settings, place the cursor on the slider. Click and hold down the left mouse button while dragging the slider to a new setting. As the slider is moved along the scale, the image display is updated, along with the change in numeric value. The arrows above and below the scale bars can also be clicked to change the settings in single unit increments, or, the user may type in a specific unit.

Black Level Adjustment

The number beneath the Black Level scale corresponds to a gray level. There can be 256, 4095, or 65,536 possible gray levels depending on the system type. For the example below, an 8 bit image will be used with 256 total gray scale values. When the Black slider is at the very top of the scale, the number is 0. As the slider is moved downwards along the scale, the number increases and the image becomes progressively darker. This is because all pixels at the specified gray level and lower are shown on the screen as black pixels. If the slider is set to 0, all the pixels whose gray levels are at 0 are shown as black. If the setting is then changed to 60, all the pixels between 0 and 60 are shown as black and the image appears darker.

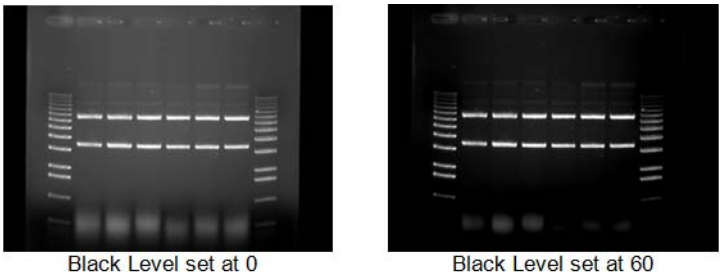


***Figure 2-27:** Black Level Adjustment Example*

White Level Adjustment

The number beneath the White Level scale also corresponds to a gray scale value. When the slider is at the very bottom of the scale, this number is 255. As the slider is moved upwards along the scale, the number decreases and the image becomes progressively lighter. This is because all pixels at the specified gray level value and above are shown on the screen as white pixels. For example, if the slider is set to 150, all the pixels between 150 and 255 are shown as white and the image appears lighter.

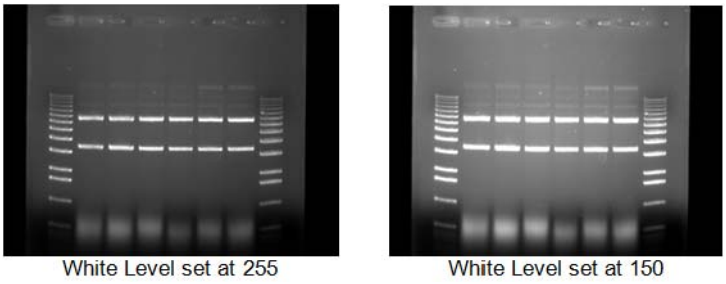


***Figure 2-28:** White Level Adjustment Example*

Gamma Setting Adjustment

Changing the Gamma setting affects the image brightness by adjusting the linearity of the image on the screen and printouts, but does not affect quantitative data.

The camera sees objects linearly while the human eye does not. When the Gamma setting is set to a value of 1, the image is displayed as the camera sees it. This, however, is different from what the human eye detects. By adjusting the Gamma setting, the user can make the image on the screen correspond to what is seen when he/she looks directly at the object. We recommend a Gamma setting of 0.55 for best visual representation.

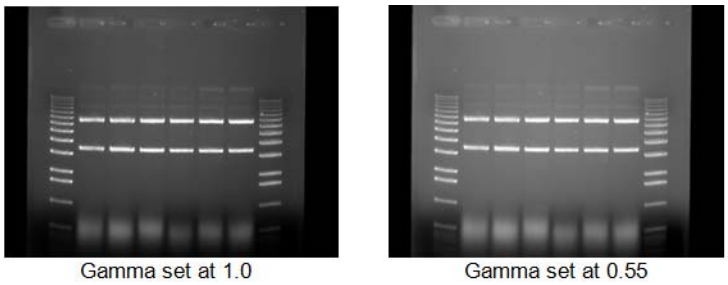


***Figure 2-29:** Gamma Setting Adjustment Example*

The Auto Contrast Selection Auto contrast

The Auto Contrast feature will automatically scale the black and white values of an image to more tightly fit the gray scale intensity profiles (histogram). This selection will use different black and white values for different images depending upon their unique histograms. A more dramatic visual change will take place for low light level images (such as chemiluminescence) where smaller portions of the histogram are used. This selection can be turned on or off and will adjust differently for each image.

The Reverse Button Reverse

The Reverse button inverts the gray levels of the displayed image, converting a positive image to negative, or vice versa. For instance, an image with black bands on a white background is converted into an image with white bands on a black background by simply clicking the Reverse button. Clicking the button a second time returns the image to its original form.

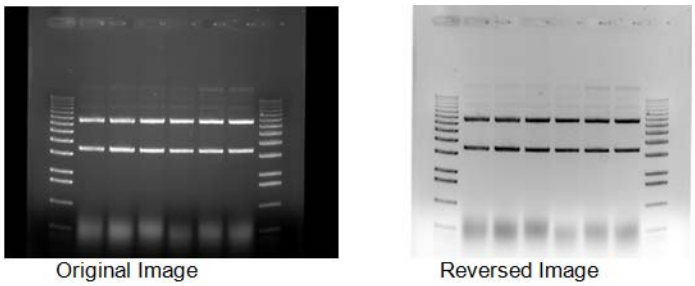
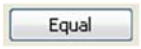


***Figure 2-30:** Original Image vs. Reversed Image*

NOTE: Reversing an image changes the way it is displayed on the screen, but does not change the quantitative data. For example, the bands in the above gel have the same density, regardless of whether the gel is displayed as white bands on a black background or black bands on a light background. For information on reversing pixel values, see Chapter 5, "Image Analysis Tools".

The Equal Button 

The Equal button flattens the image to show all pixel values in the original image. Equal automatically adjusts the image display for maximum contrast which is beneficial for faint band detection.

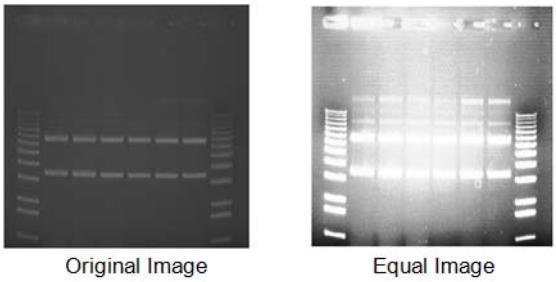


***Figure 2-31:** Original and Equal Image*

Making Linear, Log, or Equal Adjustments

Figure 2-32 shows an original image of film using default BWG settings.

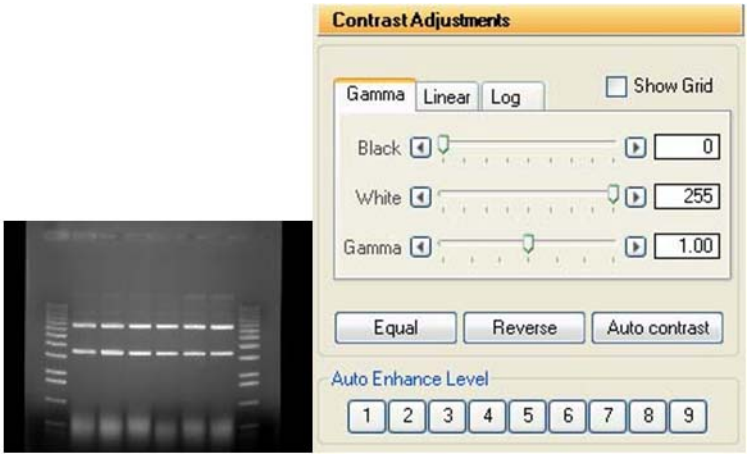


***Figure 2-32:** Original Image of Film with Default BWG Settings*

Figure 2-33 shows the image of film with linear Contrast Adjustments selected. The Linear tab provides minimum and maximum adjustment tools from 0 to 100%. Linear stretches the grayscale range of the displayed image to the system dynamic range of 0-65,535 grayscales.

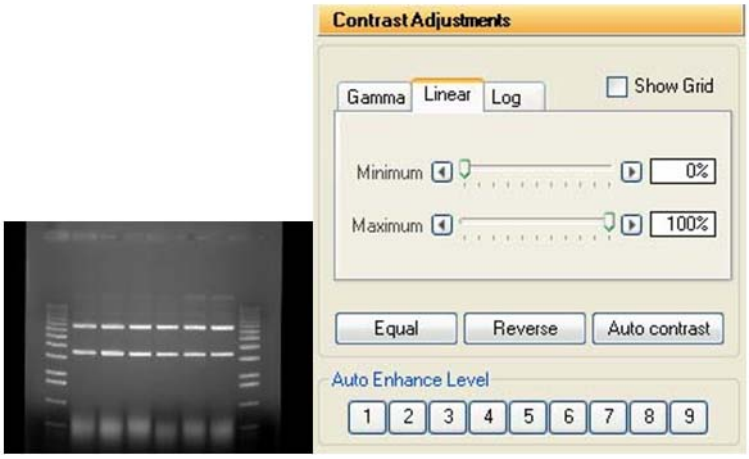


***Figure 2-33:** Contrast Adjustments—Linear Tab Settings*

Figure 2-34 shows the image of film with log the Contrast Adjustments selected the log provides minimum and maximum adjustment tools from 0 to 100%. The Log performs a logarithmic adjustment to the grayscale range of the displayed image.

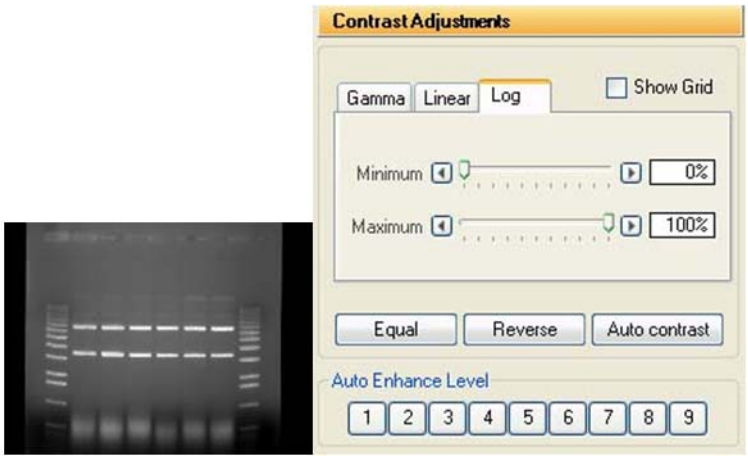


***Figure 2-34:** Contrast Adjustments—Log Tab*

Figure 2-35 shows the image of film with equal Contrast Adjustments selected. The Equal option automatically adjusts the image display for maximum contrast which is beneficial for faint band detection.

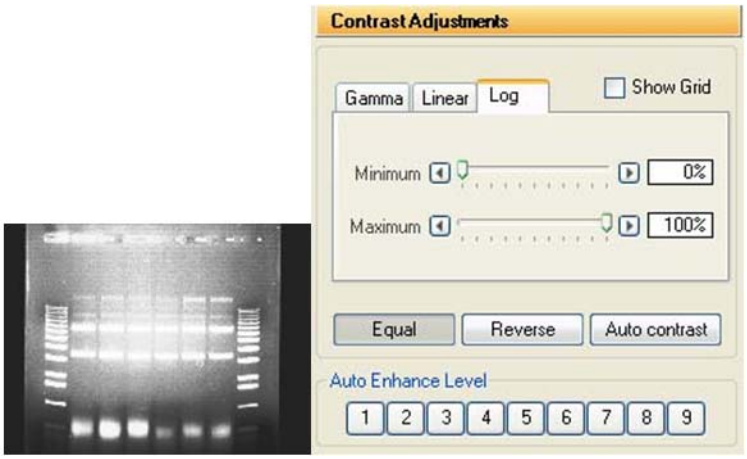


***Figure 2-35:** Contrast Adjustments—Log Tab*

Automatic Enhancement

Figure 2-36: Automatic Enhance Levels

This function is ideal for new or inexperienced users of the system since it offers 9 levels of automatic image enhancement of the black, white, and gamma levels simultaneously. For an inexperienced user, in can be difficult to adjust each black, white, and gamma buttons to their respective optimal positions. By clicking on one of the nine Auto Enhance Level buttons, the image is optimized according to a unique level. Button 1 will make the image 'darker'. Each increasing button click will 'lighten' up the image until button 9 is pressed which will make the image the 'lightest' possible.

To undo any Auto Enhance Levels, just press on the Reset button on the main interface.

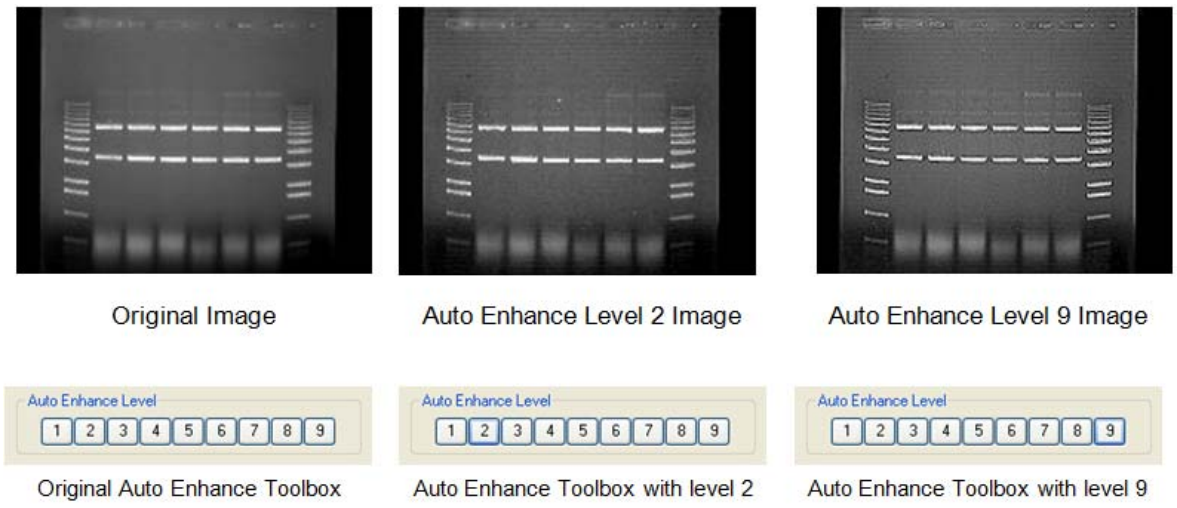


Figure 2-37: Image Levels and Enhance Tool Examples

Imaging Tools

Tool Bar

The Tool Bar window provides intuitive icons for the most common functions in AlphaView.

***Figure 2-38:** AlphaView Tool Bar*

Icon	Description
	The Navigator icon starts the AlphaNavigator wizard.
	The Open icon functions identically to the File Open function in the upper menu bar. This function is used to open previously saved images. Detailed instructions are available in Chapter 3, *"Drop-Down Menus"*.
	The Save and Save All icons function identically to the File > Save and File > Save All functions in the upper menu bar. This function is used to save captured images to the desired storage medium.
	Once an image is displayed, it can be printed on the default printer by clicking the Print icon in the Tool Bar window display. Most printers can be configured through the Windows operating system to be the default printer. Refer to your Windows operating manual for more information on installing a default printer.
	The Zoom Out and Zoom In icons provide easy zooming ability while you are active in image enhancement or analysis functions providing increased versatility. Detailed instructions are available in Chapter 4, *"Image Enhancement Tools"* as this function is also available in the Tool Box, Enhancement Tools.
	Assists the user to zoom in on a selected area, the average pixel is displayed.
	The Fit in Screen icon adjusts zoom factor to fit image in available screen area.
	The Image Drag icon is useful for to pan with a zoomed image. To activate this function, click the icon and move the mouse cursor to the image. The cursor will have changed to a small hand. Click the left mouse button and drag to move the image. When you are done, you can click the Image Drag icon again to deactivate it. *NOTE: Image Drag is only active when the image is zoomed in beyond 1X (greater than 100%). The icon is grayed out in other zoom modes.*

Icon	Description
	The Saturation icon allows for a quick image display of saturation. Completely saturation black regions (gray scale 0) will turn green and saturated white regions (i.e. gray scale 255, 4095, 65,535) will turn red. This is a useful tool to check for linearity of an image before analysis occurs. Saturation is a feature that is most important during the acquisition stages and is thoroughly detailed in the acquisition features of the system manuals.
	The Image Overlay icon creates an RGB image from three grayscale images. Select the images for the Red, Green and Blue channels in the selection window
	Clicking Reset returns the image to the system defaults as specified in the active default file. This is detailed later in Chapter 3, *"Drop-Down Menus"*.
	Clear removes any overlays currently displayed on the image. This function can be useful if annotations or other displays obscure parts of the image.
	Saves the analysis.
	Loads an analysis.
	The Notepad icon opens up a dialog box to allow the user to quickly track experimental conditions, comments, and any other details to be saved as an electronic copy for future reference. Detailed instructions are available in Chapter 3, *"Drop-Down Menus"* as this Notepad function is duplicated in the Utilities function in the upper header bar.
	The Open File Explorer opens windows file browser
	The Compare Image icon allows comparing two or more images in compare view.

Compare View

Two or more images can be opened in compare view. To open compare view, click on the Compare View icon.

If only two images are opened in the tab view, images will directly open in compare view. If more than two images are opened in tab view then a dialog box pops up, through which image can be selected to open in compare view.

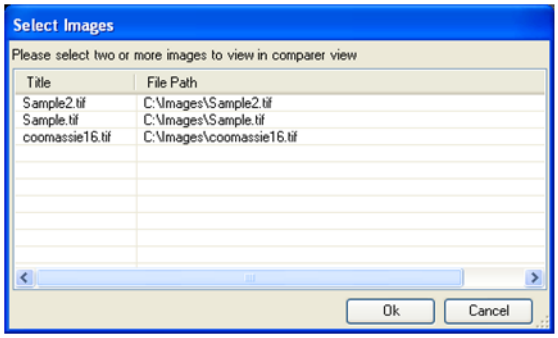


***Figure 2-39:** Selecting Images in Compare View*

If two images of same size are opened in compare view then synchronize checkbox, in status bar, will be available to use. By activating synchronize function scroll and zoom can be applied on both images together.

To switch back from compare view to tab view simply clicks on the Compare View icon.

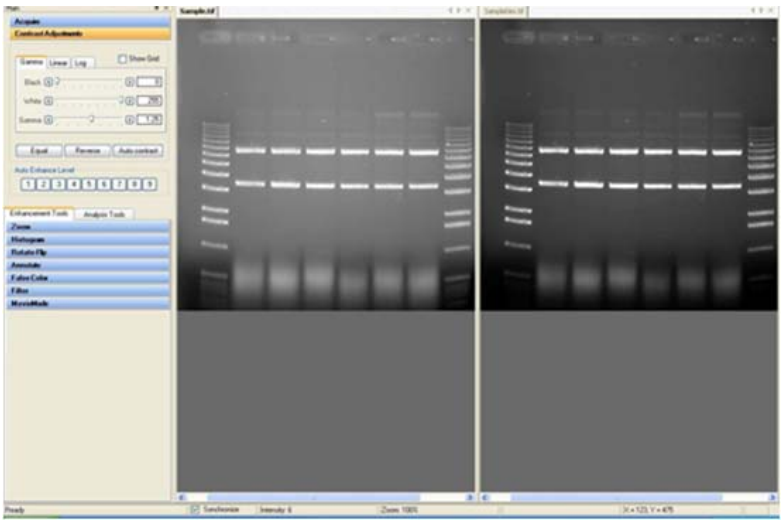


***Figure 2-40:** Switching Back from the Compare View*

Another way is by drag one image tab to see the docking options as shown in Figure 2-41.

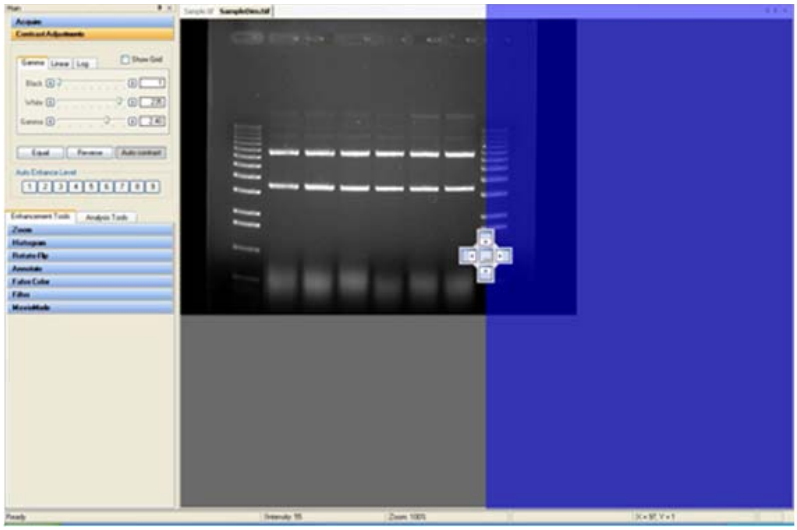


Figure 2-41: How to Enter the Compare View

After selecting appropriate place, when you drop the image tab, Compare view will be opened.

Figure 2-42: Compare View Result

Tool Box

The Tool Box window contains an intuitive interface for performing all image enhancement and analysis functions.

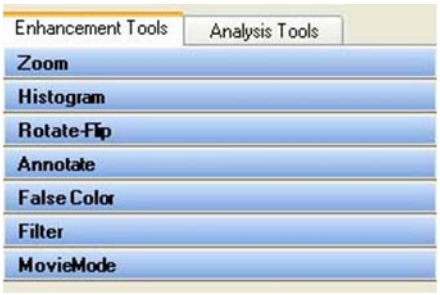


***Figure 2-43:** Tool Box*

The Enhancement Tools option contains the controls for enhancing and adjusting the image. This includes software filtering, false colors, zoom factors, and other unique features. The Analysis Tools contain the controls for quantitative analysis including gel smiling corrections, band matching, Lane Profile densitometry, multiplex band analysis, molecular weight calculations, colony counting, and arrays. Both the Enhancement Tools and the Analysis Tools are detailed in Chapter 4, *"Image Enhancement Tools"* and Chapter 5, *"Image Analysis Tools"*, respectively.

Status Bar

The Status Bar is located on the bottom of the monitor and provides a real time display of the mouse cursor x, y position, the image zoom factor, and the grayscale intensity at the mouse cursor x, y position.

***Figure 2-44:** Status Bar*

Chapter 3:

Drop-Down Menus

Chapter Overview

- Main Menu
- File Menu
- Edit Menu
- Image Menu
- Setup Menu
- Overlay Menu
- Utilities Menu
- View Menu
- Window Menu
- Help Menu

Main Menu

Across the top of the screen is a Windows menu bar containing several system operation functions. These include file saving and loading, edit, image, setup, overlay, file utilities, view, window and help functions.

***Figure 3-1:** AlphaView Drop-Down Menu Bar*

File Menu

Use this menu to save an image as a file, retrieve a previously saved image, select different printers, print an image to a parallel printer, overlay multiple images in RGB color channels, close an image, log-off of the system or exit the system.

***Figure 3-2:** File Pull-Down Menu*

File > Open Menu

This function opens an image, which has been previously saved as a TIF, GLP, BMP, PCX, TGA, PIC, JPG, PNG, FCZ or Macintosh® TIFF (MAC) file.

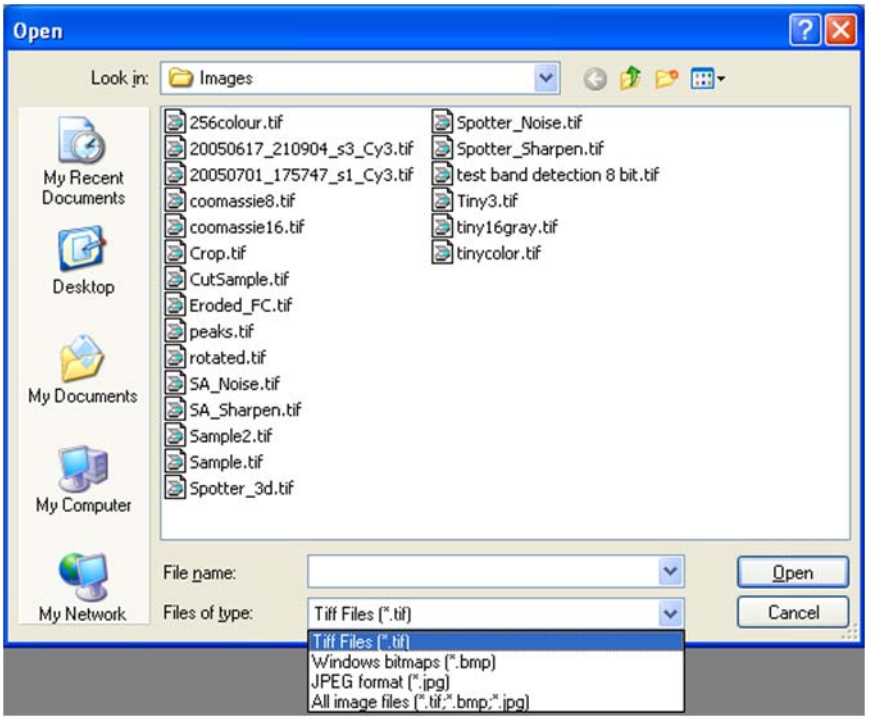


Figure 3-3: File > Open Dialog Box

Using the left mouse button, click on the name of the file to be loaded. That name is then highlighted in the list and appears in the text box below the File name prompt.

Alternate disk drives can be accessed using the "Look In" dialog box.

Once the file has been selected, click on the **Open** button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and the selected image appears in the image window on the screen.

To dismiss the dialog box without loading an image, click on the **Cancel** button.

File > Save/Load Analysis

You can use Save/Load analysis feature from file drop-down menu, to save all your work and then load it back for later use.

NOTE: The load analysis menu item can only be accessed when the user opens any the analysis tools. If the image opened has an analysis saved, the item will be activated.

Figure 3-4: Save/Load Analysis Feature

File > Close Menu

This function closes the image currently displayed on the screen.

File Save, Save As, Save Modified, and Save All Menus

Save allows original images to be saved in several different formats. **Save As** allows images that have previously been saved to be saved in a different location or as a different file type without affecting the original image. **Save Modified** saves the image as a 8-bit color image with annotations burned into the image. **Save All** saves all images in .tiff format only.

AlphaView has the ability to save files in several formats; see Figure 3-5.

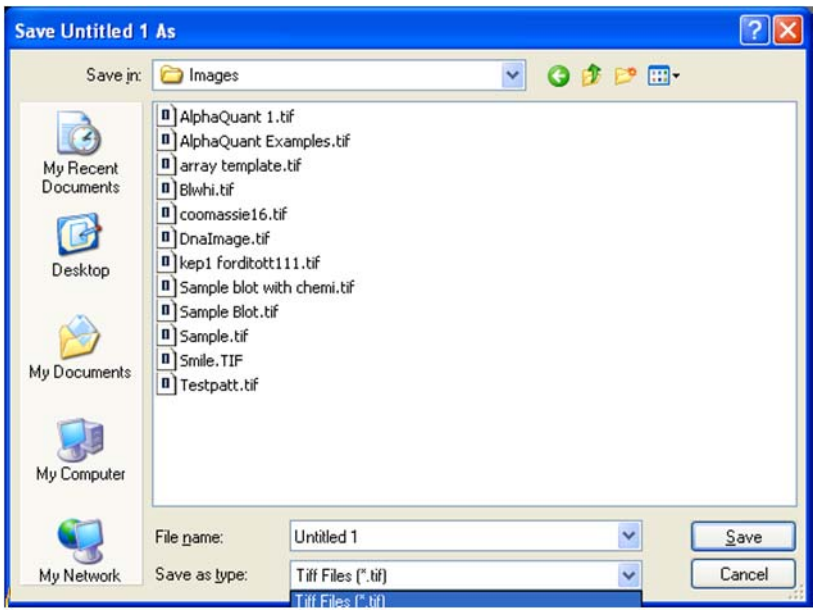


Figure 3-5: File > Save As Dialog Box

Enter a new file name in the text box adjacent to the File Name prompt. Next, choose a file type from the **Save As** type list.

AlphaView will automatically give the appropriate 3-character extension. AlphaView will also create a file with the same base name and a .STP extension. This setup file saves information specific to this file, such as Black Level, White Level, Gamma Setting and 1D-Multi template placement. If the file is accessed later, these settings will be recalled.

File Types

TIFF is the default file format for AlphaView files. TIFF is an acronym for "tagged image file format" and was developed as a flexible and machine-independent graphic file format. Saving as a TIFF file will allow users to double-click TIFF files from Windows Explorer and automatically launch the application on any machine that has AlphaView loaded on it. Please refer to "Preferences" on page 72 for information on how to customize the default file type.

Mac TIFF is the Apple Macintosh version of the TIFF file format. Mac TIFF files have the extension .MAC so they can be easily distinguished from Windows TIFF files. Most software can distinguish between Mac and Windows TIFF formats and can accept either. AlphaView offers the option of both formats in the event that only one of the two is acceptable.

GLP is a proprietary file format that allows changes to only be made in AlphaView programs. It will accept 8-bit and 16-bit images and cannot be opened in any other software program.

BMP, PCX, TGA, PIC, JPG, GLP are additional graphic file formats which may be useful when saving an image for desktop publishing. These file formats can be imported directly into many Macintosh® and PC programs. (For more information, see Appendix A, *"Opening AlphaView Files in Other Software Programs"*.)

NOTE: Do not use these formats to save images that will be analyzed later, since pixel data can be lost or altered when saving files in these formats.

NOTE: Not all of the file types listed above can be saved as a 16 bit file. The AlphaView software allows 8 bit Tiff images to be saved as BMP, JPG, or Tiff format. 16 bit Tiff images can only be saved as Tiff images. Some may require you to convert the image to an 8 bit file first.

Original versus Modified Files

An Original image file is one in which the data is saved in an unaltered form. This option should be selected if the image will be analyzed later. If the Black level, White level, or Gamma settings have been adjusted, the new values are saved but the pixel values are not altered. When this file is opened at a later time, AlphaView will display it with the values that were displayed when the image was saved, however, it is still possible to revert to the original raw image file by selecting Reset on the Tool Bar.

Annotation information cannot be saved with the Original image option. It can, however, be saved as an Overlay. Please refer to "Overlay" on page 66 for more information.

If the image was saved as an original file using an older system, some distortion may occur when viewing it in desktop publishing or word processing programs. If this occurs, save a copy of the image in the modified format before importing it into another software package.

An image that is saved as a modified file permanently retains the changes to the image's Black level, White level, and Gamma setting. Annotations and any filtering performed are also saved with the image, replacing original image information with the new information.

NOTE: If the image is saved as a Modified file it is converted to an 8-bit image.

Print

This function sends the image to the default printer specified in Print Setup.

Printer Setup

This function displays a dialog box in which the settings for the parallel printer are specified. When all the pertinent printing preferences have been specified, click on the OK button. If you purchased a printer with AlphaView, this will be preset from the factory.

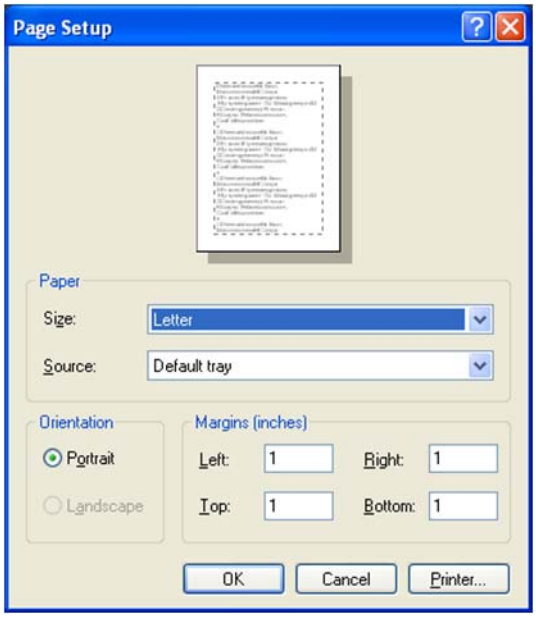


Figure 3-6: Page Setup Dialog Box

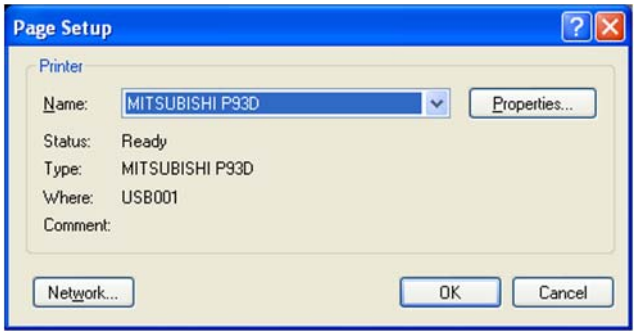


Figure 3-7: Page Setup—Printer Dialog Box

For more information on using the Print menu, see the Windows manual.

Exit Function

The Exit function closes AlphaView. To restart AlphaView from Windows, double-click on the AlphaView icon.

Edit Menu

The Edit menu provides the ability to copy, crop, reset and remove any annotations or filters that have been added to the original image.

***Figure 3-8:** Edit Pull-Down Menu*

Edit Activation

To activate the **Copy** and **Crop** functionality, place a check mark next to **Edit Activation**. This will turn the mouse cursor into a + sign that will allow you to highlight the region of interest for the image. After **Edit Activation** is highlighted, the desired area of interest is drawn using the mouse.

***Figure 3-9:** Ready to Crop or Copy*

Once this is completed, you can select either the **Copy** or **Crop** function in the **Edit** menu options.

Copy and Crop

Copy will copy the desired area of interest into the Windows Clipboard and allow you to paste into any desktop publishing package (i.e. Word, Excel, Adobe Photoshop, etc.).

Crop will display just the region of interest as the active window in the AlphaView interface.

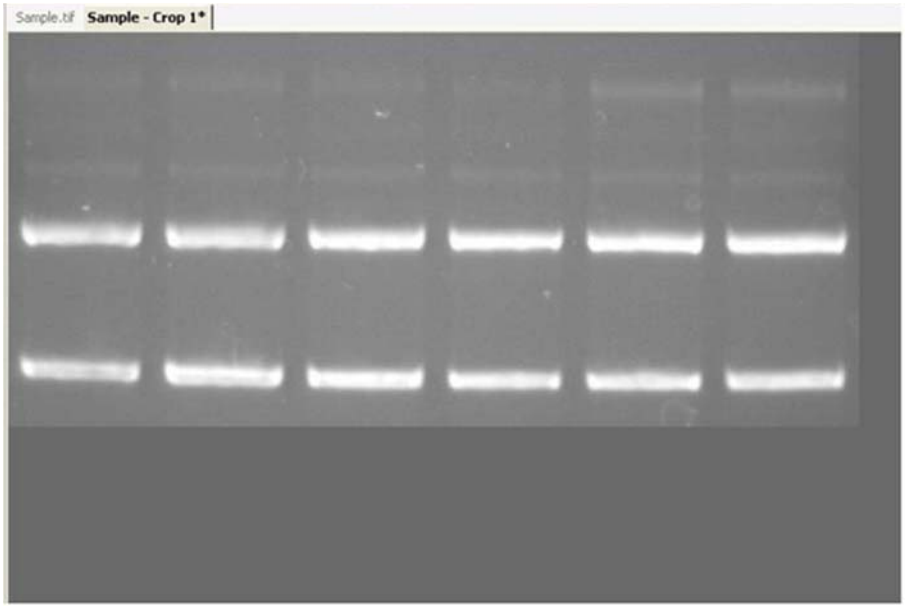


Figure 3-10: AlphaView Interface—Sample Crop 1

Reset and Clear Options

The Reset option configures the Black, White, and Gamma settings to default settings. The Clear option removes any annotations that are present on the image.

Image Menu

The Image menu provides the ability to perform a variety of image processing functions.

***Figure 3-11:** Image Pull-Down Menu*

Overlay

To superimpose images, use the **Overlay** function under the Image File menu. This function will display separate multiplexed images or a RGB color image as a compiled image with the appropriate color channel images added together. A simple way to acquire multiple images for this function is to use the Movie Mode function in image acquisition and acquire a series of identical images.

The Overlay Images option allows you to overlay up to three different images with three different color channels. You can select the **Browse** button for each color channel and select the appropriate images to be used for generating a color image. For example, if you have a saved grayscale images of an identical gel taken with a SYPRO red filter for the red stain and a SYBR green filter for the green stain, you can choose these images in the appropriate Red and Green Channels to generate a composite image with the red and green colors mapped onto the compiled image.

NOTE: The images must be the same bit depth and resolution for the software to overlay the images.

Equalize

The equalize option performs a duplicate function to the **Equal** option in the Contrast Adjustment Window. This is a useful function for detecting faint bands on a sample.

Arithmetic

The Arithmetic function is used to add, subtract, average and divide several images together to generate a compiled image.

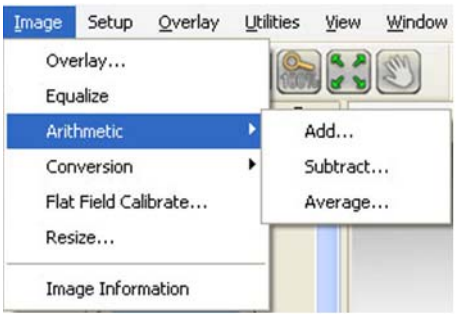


***Figure 3-12:** Image > Arithmetic Menu*

To average a set of images together open one of the images in the set and then select 'Average a Set…' under the Image pull-down menu. A prompt will appear allowing the user to select all of the images that for the set. It is possible to browse the directories looking on the network drives and removable media if necessary. Once all of the images have been selected click on the open button to finish the image set. The resulting image is an average of all of the images together. This is a useful function for extending the dynamic range on a set of similar images by allowing bright spots and faint spots to be seen on the same image.

The other functions are adding, subtracting and dividing images together. Adding together images is frequently used for colorimetric markers run together with chemiluminescent samples. Subtracting images is often used to remove noise from a sample by running dark images first and subtracting them out of the final image. The most common application for quotient is for those technical users who run their own flat field corrections. This can be done using the Flat Field Calibrate selection under the Image pull down menu which will be described in detail later in this section.

All three of these arithmetic functions are performed by opening the main image that will be adjusted. Next select the appropriate arithmetic function under the image pull down menu. Then select the image that is to be added, subtracted or divided from the original image and select open. The dialog box will disappear and the resultant image will appear.

NOTE: Images that have been arithmetically altered are ideal for publications and documentation, however, they are strongly not recommended for analysis as the pixel values have been adjusted.

Conversion

Because AlphaView can generate 16-bit files, the conversion option is useful when an image is to be imported into a program that only accepts 8-bit images. Choosing this option will convert a 16-bit image into an 8-bit image.

A 48-bit color image can be converted into a 16 bit grayscale image by simply averaging the RGB values for each pixel.

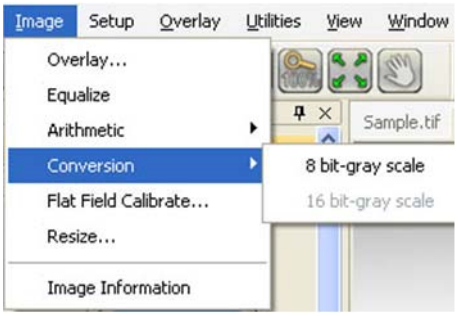


***Figure 3-13:** Image > Conversion Menu*

Flat Field Calibrate (Manual)

Flat Field Calibrate is a function that is used to 'flatten' the image so that the pixel data is even across the entire image area. This is a function that is useful for large gels and other applications that use the entire field of view for an image.

Creating flats can be art in itself; there are many documents on the internet that can help users interested in this arena to create the ideal flat for the application. However, some useful flats that have been created in the past involve very simple tools like a piece of 8.5 x 11 regular low quality copy paper (higher quality paper contains watermarks that will show up in the final image). It is essential that both the flat and the gel images be identical, including the aperture, zoom (if applicable) and focus settings on the lens.

Step-by-Step Manual Flat Field Calibration applied to an existing image:

1. Place the gel or other application in the cabinet or dark room.
2. Adjust the aperture, zoom (if applicable) and focus on the lens.
3. Use auto-expose set to the normal selection and acquire an image of the gel. (Alternatively, it is possible to select show saturation and then use expose preview and adjust the exposure time manually to just under saturation.)
4. Save the image of the gel.
5. Next remove the gel from the UV transilluminator or white light tray and clean and/or dry off the surface if necessary using glass cleaner.
6. Place the white piece of paper onto the appropriate surface. (For example, if the UV transilluminator was used, place the paper onto the UV transilluminator; if the white light tray was used, place the piece of paper onto the white light tray.)
7. Turn on the appropriate light source used (white light, UV transilluminator, epi lights, etc.).

8. Without changing anything on the lens acquire another image of the 'Flat' image following step 3 again.
9. Save the Flat image.
10. Open the original gel or other application image.
11. Select Flat Field Calibrate from the Image pull down menu.
12. Browse the directories for the 'Flat' image created.
13. Click on open. Make sure to save the flat field calibrated image for future use.

Resize

The Resize function is to resize an image to a specific dimension for use in graphical presentations. You have the option to 'Preserve aspect ratio' to avoid image dimensional distortion, or you can deactivate this function and configure the image resolution to the desired width and height dimensions.

Figure 3-14: *Resize Image Dialog Box*

NOTE: It is recommended that you DO NOT perform quantitative analysis on resized images.

Image Information

The Image Information function provides a dialog box with all detailed image properties. To remove this dialog box from the screen, click OK.

***Figure 3-15:** Image Information Dialog Box*

Setup Menu

This menu customizes the system settings by allowing users to save default parameter preferences and customize the software settings.

***Figure 3-16:** Setup Pull-Down Menu*

Print Info

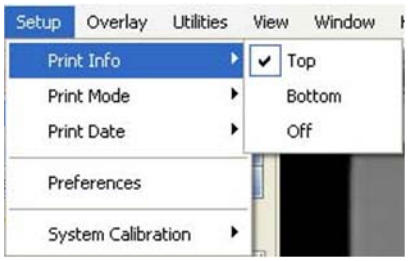


Figure 3-17: Setup > Print Info Menu

When printing an image, basic image information is included on the print. This includes the exposure time, the Black level, White level, and Gamma setting, the date and time the image file was generated, an image ID number, and the name of the file to which the image is stored.

To print this information at the top of the print, choose Top from this menu. To print at the bottom of the print, choose Bottom.

NOTE: Printing image information at the top or bottom of a print may obscure a small portion of the image. To print the image with no information on it, choose Off.

Print Mode

AlphaView software provides custom printing options.

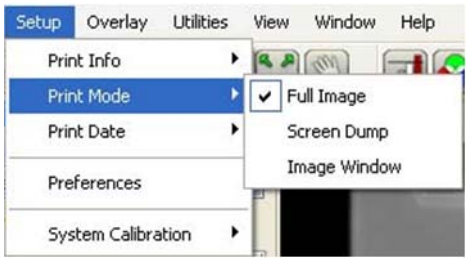


Figure 3-18: Setup > Print Mode Menu

Printing can be achieved in three different methods:

- Full Image—Prints the original image. Does not print zoomed images or images overlaid with data screens.

- Screen Dump—Prints the imaging area. Well suited for printing images overlaid with data screens and/or graphs, zoomed images, etc.
- Image Window—Prints the highlighted window.

Print Date

Under the Setup menu there is a selection labeled "Print Info". This allows the user to change the format in which the date is printed. The choices are MM/DD/YYYY and DD/MM/YYYY.

Preferences

In order to change the preferences of the system, you will need to find the administrator of the AlphaView software program to log in; the login is enforced only on data altering tool tabs (image acquire, cabinet settings, and auto enhancement).

***Figure 3-19:** User Login Dialog Box*

There are five tabs in the Preferences menu (the AlphaView Stand Alone software does not contain the Image Acquire or Cabinet Settings tabs):

- General—Configures prompts and file saving/opening formats.

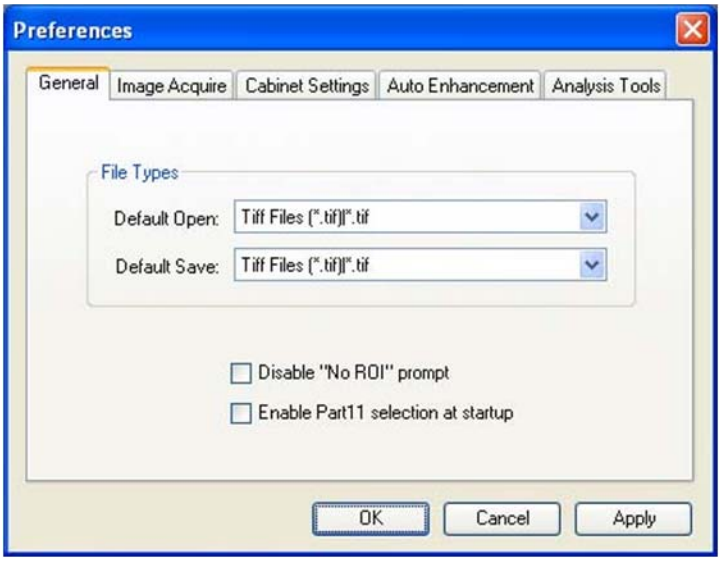


Figure 3-20: Preferences—General Tab

- Image Acquire—Inverts the image seen by the camera and adjusts the ROI values.

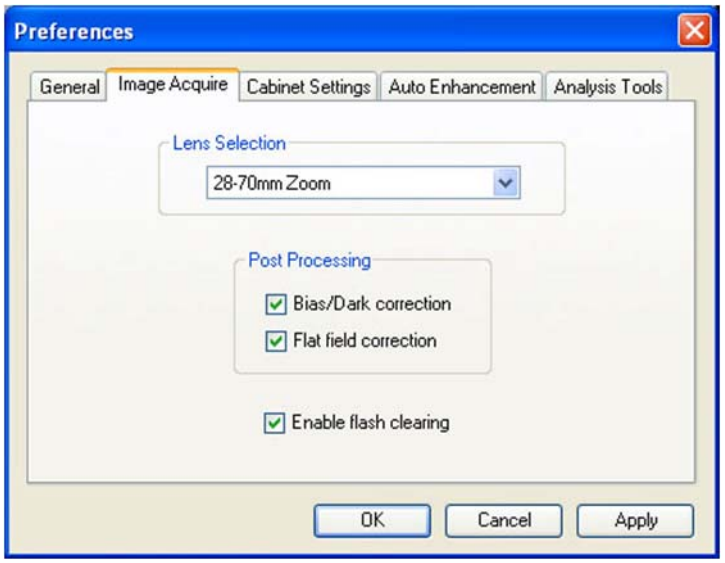


Figure 3-21: Preferences—Image Acquire Tab

NOTE: Cell Biosciences does not recommend that you make any changes to these settings without contacting technical support first. Some features on this tab are not currently implemented in the software.

- Cabinet Settings—Used for adjusting the port settings and customizing filter positions on the cabinet.

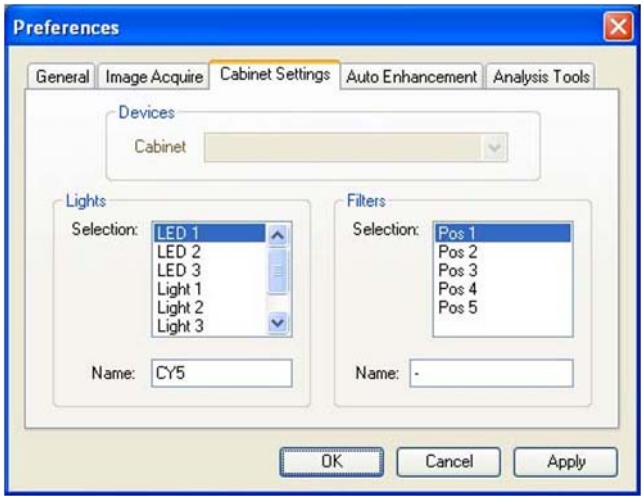


***Figure 3-22:** Preferences—Cabinet Settings Tab*

- Auto Enhancements—Used to customize the Auto Enhance Levels located in the Image Enhancement tool box.

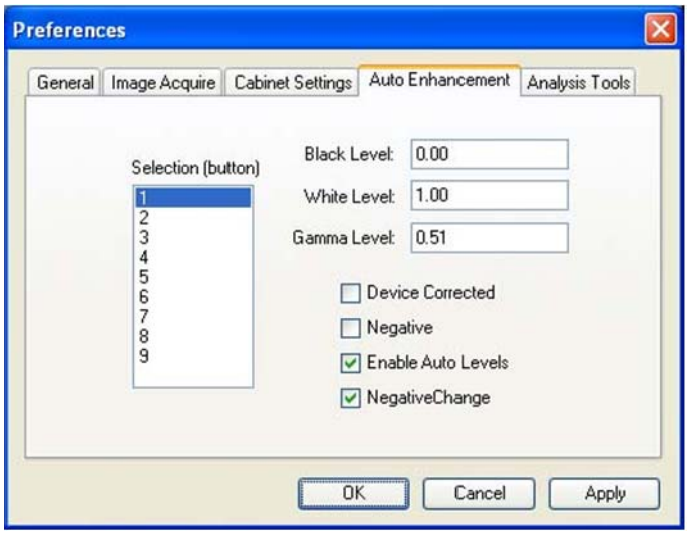


***Figure 3-23:** Preferences—Auto Enhancement Tab*

To make changes to the preferences, select your change by typing in a new value or select/de-select the appropriate box with a check mark and then select apply. Some settings may require that the software be re-started for the change to take effect.

- Analysis Tools—Used to show/hide additional image analysis tools. Any change in the settings will require software be re-started for the change to take effect.

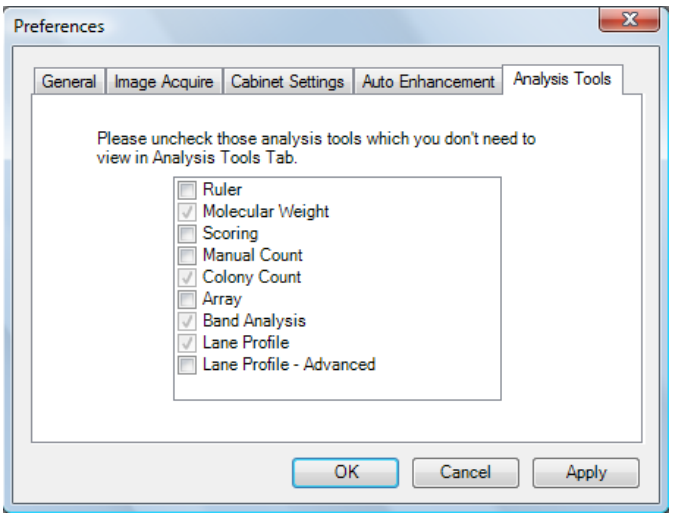


***Figure 3-24:** Preferences—Analysis Tools Tab*

System Calibration

In order to use system calibration, it will be required the administrator log in of the AlphaView software program.

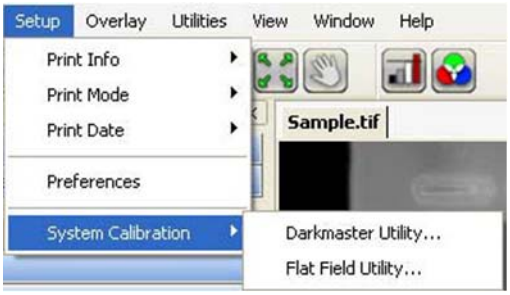


***Figure 3-25:** Setup—System Calibration Menu*

Darkmaster Utility—See Appendix D, "Bias and Dark Master Utility".

Flat Field Calibration (Auto)—See Appendix E, "Flat Field Calibration".

Overlay Menu

The Overlay menu provides a means of saving and retrieving annotation overlays. This is especially useful when a standard gel format is run repeatedly. Lane numbers, molecular weight marker sizes, and other pertinent information can be stored as an Overlay file and retrieved at a later date. This eliminates the need to re-enter the information each time a new image is captured.

***Figure 3-26:** Overlay Pull-Down Menu*

An overlay is any set of annotations (text, boxes, arrows, etc.) that have been drawn on the image. They can be saved as a group and opened later. If repetitive samples are being imaged, an overlay eliminates the need to re-enter the same information (such as lane numbers, standard sizes, etc.) continually.

Load Overlay

The Load Overlay function allows Overlay files to be retrieved and applied to the image currently displayed.

Opening an Overlay after an image has been captured places the annotations on top of the image. They can be stored as part of the image by saving the file as a modified file. Please refer to "File Save, Save As, Save Modified, and Save All Menus" on page 60 for more information.

Select the name of the file to be loaded. (If necessary, change the directory or drive.) The file name is then highlighted in the list and appears in the text box below the Filename prompt.

Once the file has been selected, click on the OK button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and the annotations in the selected file appear on the image.

To dismiss the dialog box without loading annotations, click on the CANCEL button.

Save Overlay

Once annotations have been made, select Overlay > Save Overlay.

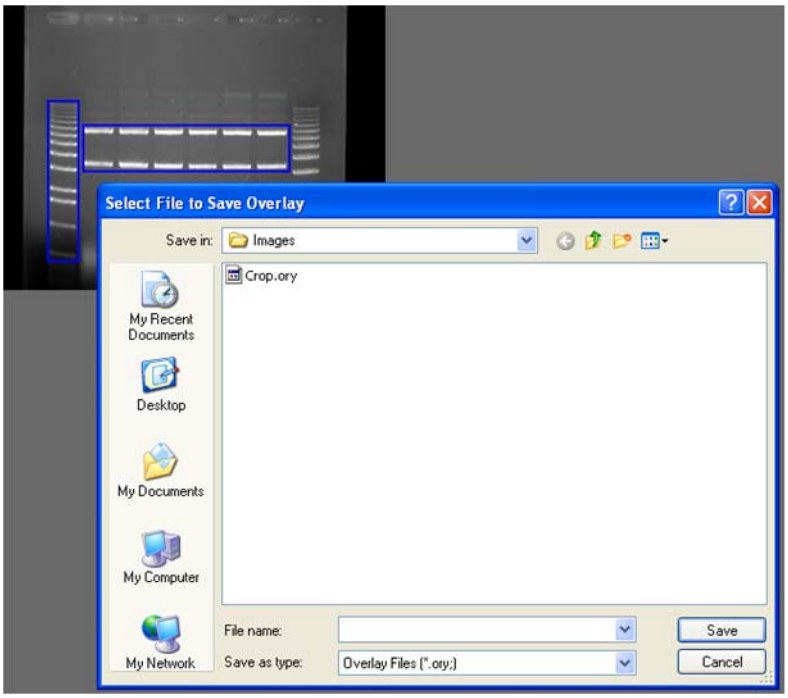


Figure 3-27: Save Overlay File Selection Dialog Box

Enter a new file name in the text box below the File Name prompt. AlphaView will automatically give the appropriate 3-character extension.

The current directory is the one in which the new overlay file will be saved. If necessary, change the directory or drive as described in Section 3.1.

Once a name has been entered and the appropriate directory has been accessed, click the SAVE button to save the overlay file.

Overlay Libraries

AlphaView contains a library of overlays that can be accessed through the Load Overlay function described above. This library of overlays is stored in the Image folder located in the AlphaView directory:

08WHITE.OVR / 08BLACK.OVR	8 lane labels in white/black
10WHITE.OVR / 10BLACK.OVR	10 lane labels in white/black
12WHITE.OVR / 12BLACK.OVR	12 lane labels in white/black
15WHITE.OVR / 15BLACK.OVR	15 lane labels in white/black
24WHITE.OVR / 24BLACK.OVR	24 lane labels in white/black
HINDIII.OVR	λHindIII label

The objects in these overlays can be repositioned, resized, re-colored, copied or deleted as needed.

Show Annotation

To displays or hides annotations in Analysis modules.

Utilities Menu

A number of functions are now handled by Windows programs. To access many of these programs while in AlphaView, open the Utilities menu and select the program of choice.

***Figure 3-28:** Utilities Pull-Down Menu*

Notepad

The Notepad is a blank screen that allows the user to make notes about the experiment and save them as an ASCII file. The Notepad is useful for saving any imaging comments or experimental conditions with the saved image for future reference.

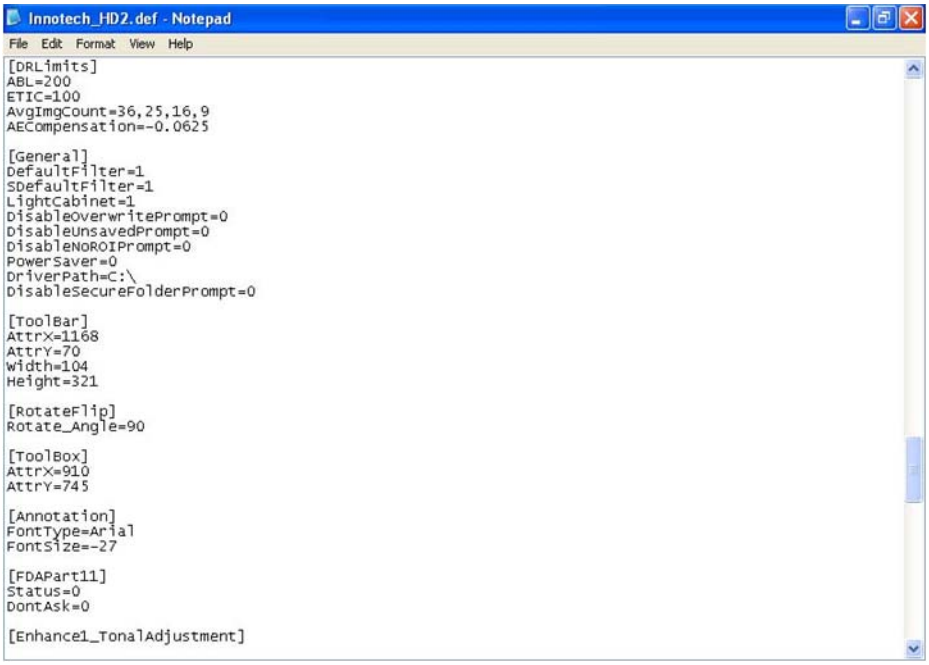


Figure 3-29: Notepad Display Window

Explorer

Windows Explorer allows access to files and other information saved on the local machine or the network, if applicable.

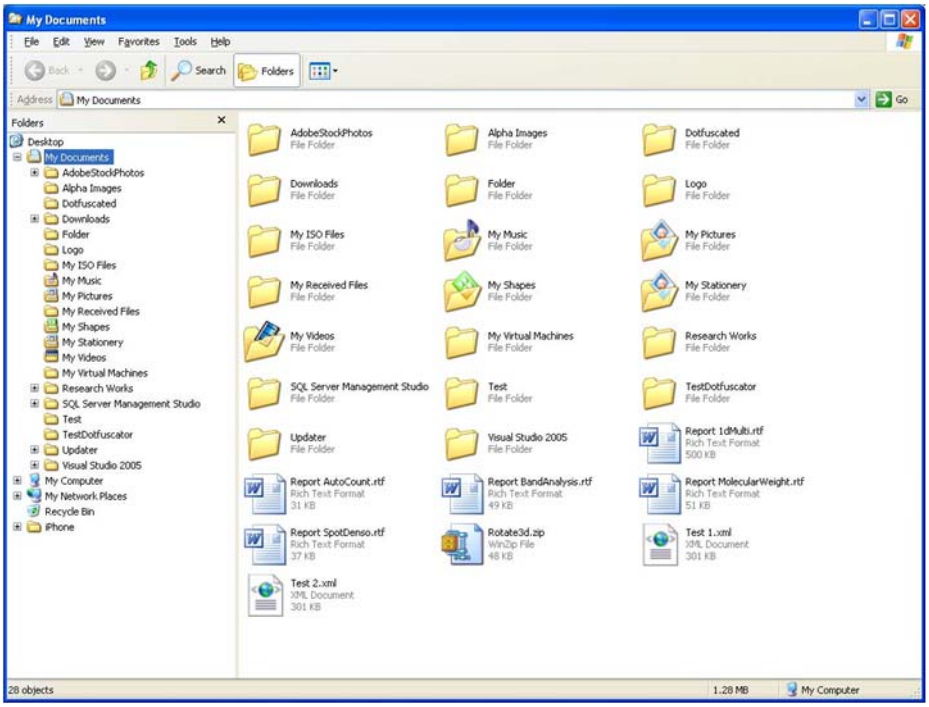


***Figure 3-30:** Windows Explorer Window*

View Menu

The View menu provides the ability to control the display of the on-screen control tools as well as provide image enhancement abilities.

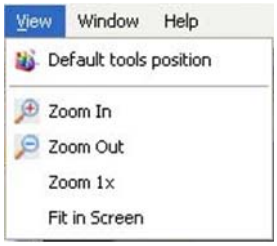


***Figure 3-31:** View Pull-Down Menu*

Default Tools Position

Four (4) main control windows exist within AlphaView: Tool Bar, Contrast Adjustment, Tool Box, and Status Bar:

Figure 3-32: Tool Bar

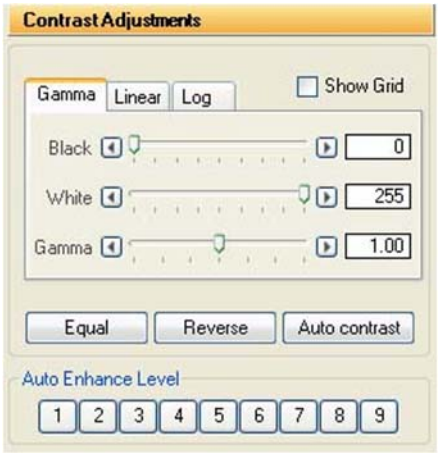


Figure 3-33: Contrast Adjustments Window

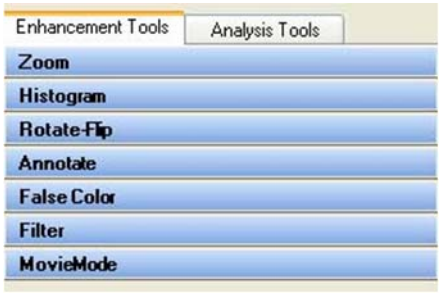


Figure 3-34: ToolBox Window

Figure 3-35: Status Bar

These control windows automatically open when AlphaView is launched for additional ease of use and to generate a common 'look and feel'. Since these items are 'floating' tools, you can click on Default Tools Position to move all tools to the default locations for more intuitive operation. Lastly, except for the status bar, it is possible to select and move any of the other windows to a custom location.

Zoom Functions

Additional options provide the ability to Zoom In and Zoom Out on the image, Zoom to 1X and to Fit to Screen.

NOTE: Zoom In and Zoom Out are duplicate functions for the Zoom In and Zoom Out icons in the ToolBar and the Zoom options in the Enhancement Tools.

Window Menu

The Window menu provides the ability to navigate from one image window to next image window. It also allows user to close all documents.

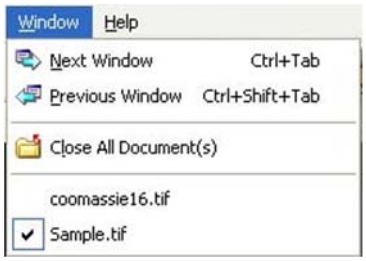


***Figure 3-36:** Window Pull-Down Menu*

Help Menu

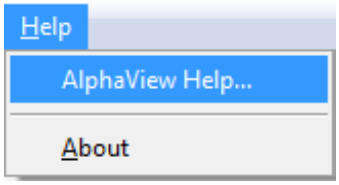


***Figure 3-37:** Help Pull-Down Menu*

Complete instructions on how to use the software along with detailed explanations of all features are available from the AlphaView Help option of the Help menu.

To display system information, select the About option in the Help menu. This button accesses a pop-up box (see Figure 3-38), which shows the system serial number and software version number. Use this information when contacting Cell Biosciences for technical support, software upgrades, etc.

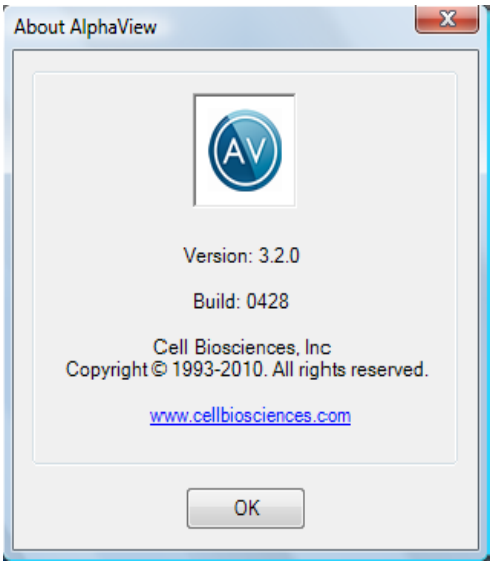


***Figure 3-38:** AlphaView—About Help Display*

To close the box, click on the OK button.

Chapter 4:

Image Enhancement Tools

Chapter Overview

- Tool Box Enhancement Tab
- Zoom
- Histogram
- Rotate-Flip
- Annotate
- False Color
- Filter
- Movie Mode

Tool Box Enhancement Tab

Image enhancement tools are contained within the Tool Box as indicated. This tool set allows the user to zoom the image, rotate-flip the image, show the image histogram, perform automatic image enhancement, annotate on the image, display false colors, apply software filters, and activate the Movie function.

NOTE: Many image enhancement tools do not function with multichannel images.

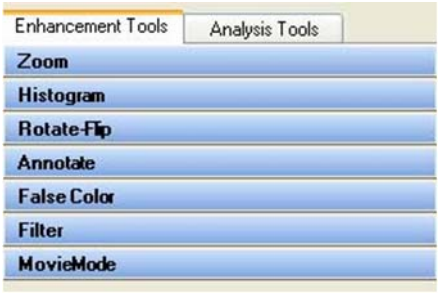


***Figure 4-1:** Tool Box*

Zoom

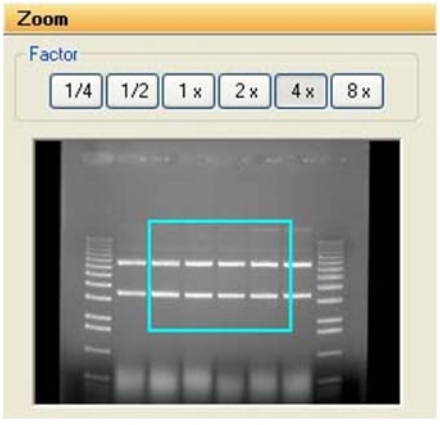


***Figure 4-2:** Zoom Tool*

The Zoom tool function magnifies an image, making details easier to see, and allows movement around the magnified image.

An image can be displayed ¼x, ½x, 1x, 2x, 4x, or 8x larger than the original display by clicking on the appropriate buttons. To return to the original magnification, click on the 1X button.

When an image is magnified, only part of it can be displayed on the screen at any one time. To see different parts of the magnified image, use the green Pan Control box. The outer box shows a thumbnail of the entire image while the inner green box represents the portion currently displayed on the screen.

To view different regions of a magnified image, move the cursor into the inner box. Click and hold down the left mouse button. The cursor changes to a hand; use it to drag the green box until the desired region of the image appears on the screen.

Alternatively, use the scroll bars in the image window to move the image up or down, and left or right.

On-screen Zoom tools are also available located on the main Tool Bar. This function duplicates the Zoom tool in Tool Box, and also allows for Image Drag to easily pan the image during any analysis functions located in "Zoom" on page 86 (in the Analysis Tools option).

Image Drag icon in ToolBar

Zoom Icons in ToolBar

Histogram

The histogram is a graphical display of the proportion of pixels assigned to each of the 4,095 gray levels.

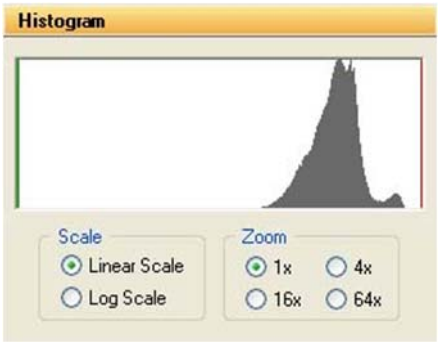


Figure 4-3: Histogram Display in the Tool Box

The image is made up of picture elements (pixels) having brightness levels ranging from black to white. A very bright image will have most of its pixels registering high gray levels and conversely, a very dark image will have most pixels with low gray levels (approaching zero).

The histogram is displayed in the lower left corner of the screen, below the image window. The horizontal axis represents the gray scale range: black at the left end and white at the right end, with levels of gray in between. The number of pixels registering a particular gray level determines the height of each bar along the axis.

A Coomassie blue-stained protein gel visualized with a white light box has a histogram reflecting mostly bright pixels:

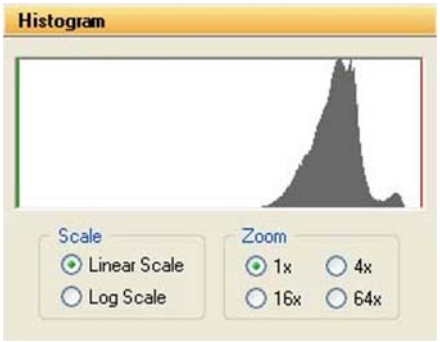


***Figure 4-4:** Histogram of a Typical Coomassie Gel*

Most of the pixels are found in the light portion of this histogram. The dark bands represent a small number of pixels and include a variety of gray values, and therefore do not show up as a single peak.

The histogram function is particularly useful to verify that an image spans the maximum range of gray levels. When an image is to be used for analysis, it is especially important that the gray level range be as large as possible. If an image does not include most of the gray levels, we recommend repeating the image capturing process.

Rotate-Flip

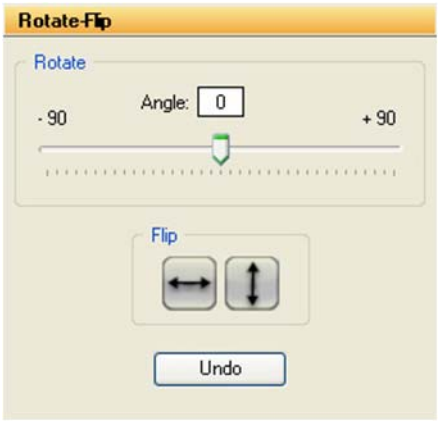


Figure 4-5: The Rotate-Flip Tool

The Rotate-Flip tool function rotates the image in a clockwise or counterclockwise direction by 1° increments up to a maximum of 90° in either direction. This is a useful tool if the image is not aligned properly during the capturing process.

To rotate an image, click and hold down on the center sliding bar with the left mouse button and move it left or right until the desired angle of rotation appears in the rotate box. Release the left mouse button and image will rotate to the desired angle. To undo a rotation, just click on the Undo button. Also, a Flip option allows for the image to be rotated 180° in a vertical or horizontal fashion.

The Reset button on the main software interface will also remove any rotations or image flips and return to the display to the original image.

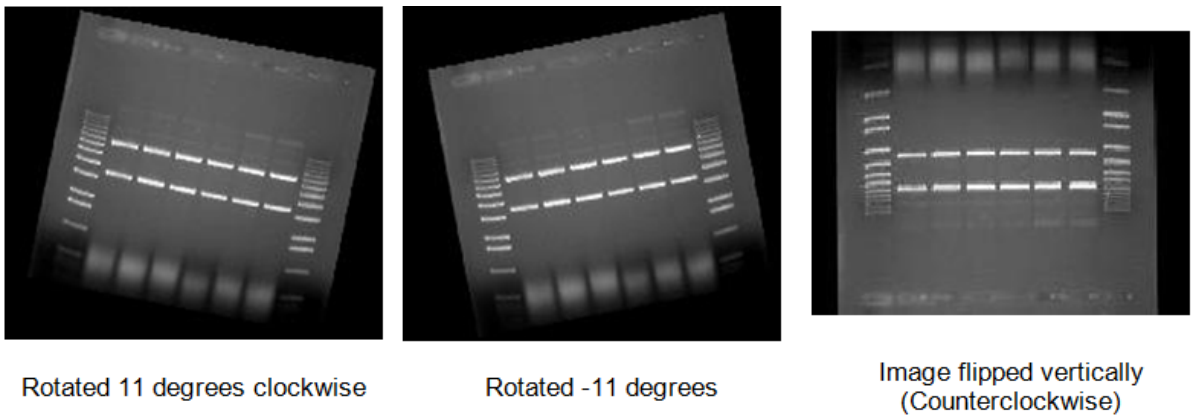


Figure 4-6: Rotate-Flip Tool Images

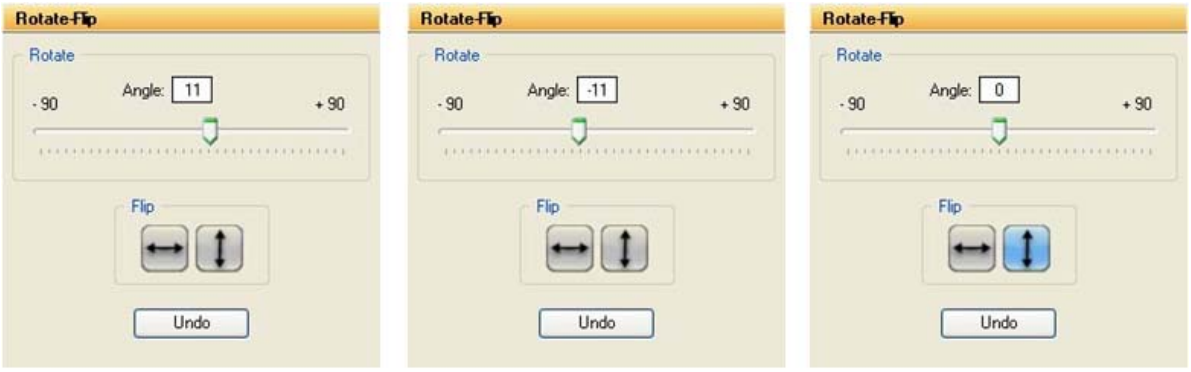


***Figure 4-7:** Rotate-Flip Tool Controls*

Annotate

The annotation tools, include a number of different options for adding text (including Greek symbols), drawing arrows and otherwise marking an image.

NOTE: These tools are for annotation only. For information on drawing objects for quantitation purposes, see Chapter 5, "Image Analysis Tools".

Figure 4-8: Annotate Toolbox

Object Attributes

Use the COLOR, PEN WIDTH, PEN STYLE, LINE ENDS, TEXT STYLE and/or TEXT ORIENT menus to specify object attributes. Attributes can be assigned to the cursor before drawing or typing. Alternatively, they can be assigned to an object while it is in "edit" mode (see the following subsections for more details).

Annotation Colors

Annotations can be displayed in a variety of colors. The color options are displayed by clicking the COLOR radio button. To select a color, simply click the cursor on the button labeled with the desired color. The color button appears depressed, indicating that it is selected. Any annotations subsequently entered will appear in that color. It should be noted, however, that annotations are printed in gray scale on the video printer. Further, when an image is saved as a modified image, the annotations are saved in gray-scale, not color.

Line Thickness

The PEN WIDTH option specifies the thickness of lines when using the freehand, lines, box and circle drawing tools. Click on the appropriate checkbox for the desired width. All annotations subsequently entered will appear at that width.

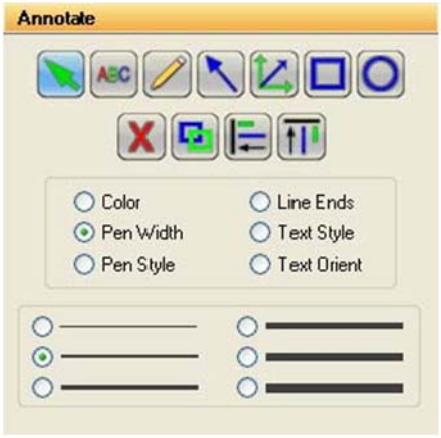


***Figure 4-9:** Pen Width Selection Tools*

Line Types

The PEN STYLE option specifies the style of lines when using the freehand, lines, box and circle drawing tools. Click on the appropriate checkbox for the desired style. All annotations subsequently entered will appear in that style.

NOTE: These pen styles only work with a thin line (see the "Line Thickness" section).

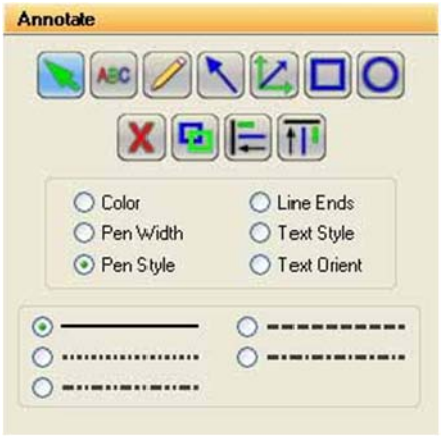


***Figure 4-10:** Pen Style Selection Tools*

Arrows and Straight Lines

The LINE ENDS option specifies the style of the ends of straight lines (no arrow, single arrow or double arrow). Click on the appropriate checkbox for the desired style.

NOTE: These line ends work with any line thickness.

***Figure 4-11:** Line Ends Selection Tools*

Text Background and Font

The TEXT STYLE option specifies the style of text. Click on the appropriate checkbox to show text with or without a background. An opaque background is useful if annotations will be made on an image that has wide variations in gray scale. By using an opaque background, text will not be "lost" in the background of the image.

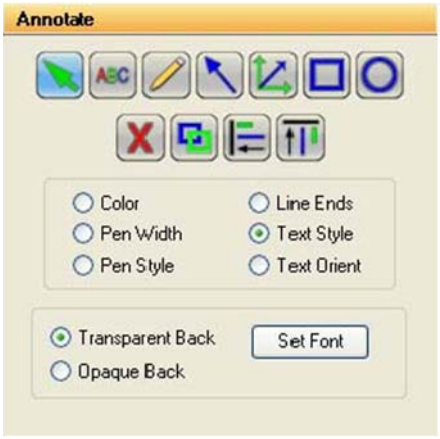


***Figure 4-12:** Text Style Selection Tools*

This is also the window in which specific font is chosen. When the Set Font button is depressed, a selection box appears, from which text style can be chosen.

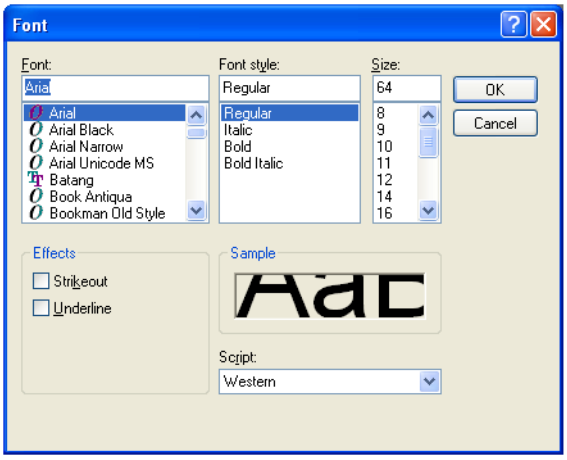


***Figure 4-13:** Font Selection Dialog Box*

NOTE: To choose Greek symbols (such as α, β, λ, π, *and* θ*) choose the Symbol font:*

a b c d e f g h i j k l m n o p q r s t u v w x y z

αβχδεφγηιφκλμνοπθρστυϖωξψζ

Text Orientation

In the TEXT ORIENT window, select whether text should be oriented vertically, horizontally, or at an angle (in 15° increments).

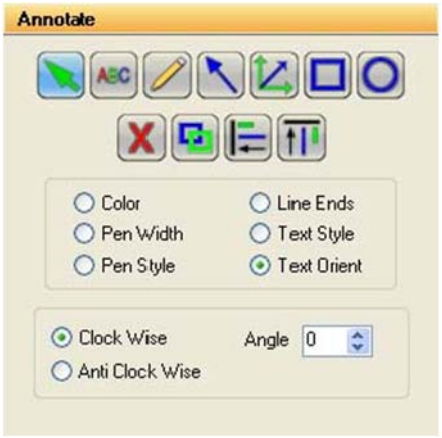


***Figure 4-14:** Text Orient Selection Tools*

NOTE: Only rotate fonts that are True Type (indicated by TT in front of the name); other fonts (such as Courier and Fixedsys) do not re-scale properly, giving unpredictable results.

Drawing Tools

Once the object attributes have been defined, click the cursor on any of the drawing tool buttons to assign the function associated with that button to the mouse. The cursor will change from an arrow to a cross, indicating that AlphaView is in "drawing" mode.

After selecting a drawing tool, move the cursor to the correct position on the image to begin drawing. Press and hold the left mouse button and move the mouse to the end point of the object to be drawn. Release the left button, and the object should appear.

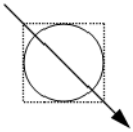
Boxes will appear at the corners of the new object and the cursor will revert to an arrow, indicating that AlphaView is now in "edit" mode. At this point, the object can be resized or repositioned. The color, pen thickness, line type, etc. can also be changed, simply by clicking on the desired choice (as described in *"Object Attributes,"* starting on page 91).

To draw another object, click the right mouse button to return to "draw" mode, or click on one of the drawing tool icons.

Icon	Description
	The selection tool, allows the user to select all drawing for further operations, such as vertical alignment.
	The button labeled with an "ABC" adds text to the image. Place the cursor at the location on the image where the left edge of the text should appear. Click the left mouse button and begin typing. To place another piece of text, click where it should be placed. Once all text is entered, click on the right mouse button. To edit text, double-click on it. An edit window will appear, in which changes can be made. To change fonts, see *"Text Background and Font,"* starting on page 93.
	The button labeled with a pencil icon allows the user to draw lines freehand. After clicking on the pencil, move the cursor to the correct position on the image to begin drawing. Press and hold the left mouse button. Using the mouse, move the cursor as if it were a pencil. When finished drawing, release the mouse button.
	The button labeled with a diagonal line and arrow, draws arrows and straight lines. After clicking on the button, move the cursor to the position on the image where the line should begin. Press and hold the left mouse button. Using the mouse, move the cursor to the other end point of the line then release the mouse button. The arrow can be adjusted by clicking on one of the boxes at the end (the other will serve as an anchor point) or by clicking in the middle to drag the entire arrow.
	The button labeled with angles and arrows on its side is a line drawing tool, very similar to the one described above. The significant difference is that this tool limits the angle that the line can be drawn to increments of 45°.
	The button labeled with a square draws a rectangle or square of any size on the image. After clicking on the button, move the cursor to the position that should correspond to one of the corners of the rectangle. Press and hold the left mouse button. Using the mouse, move the cursor to enlarge the rectangle. When it reaches the desired size, release the left mouse button.
	The button labeled with a circle draws a circle of any size. After clicking on the button, move the cursor to the position on the image where the circle should be started. Press and hold the left mouse button. Using the mouse, move the cursor to enlarge the circle. When the circle reaches the desired size, release the left mouse button.

***Table 4-1:** Drawing Tool Icon Descriptions*

Hint: to draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.



Sample Annotations

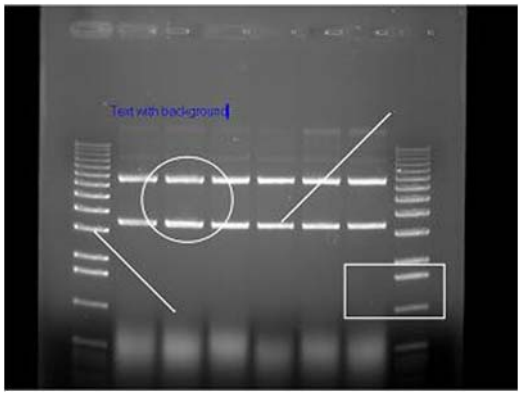


***Figure 4-15:** Sample Annotations*

Annotated image showing: freehand drawings, lines with various characteristics, circles, squares, text with various characteristics

Editing Tools

When the cursor is in "edit" mode, it can be clicked on an object to select it.

NOTE: The cursor can be toggled between "edit" and "drawing" modes by clicking the right mouse button.

When an object is selected, small square boxes appear at the corners. Selected objects can be resized, copied, deleted or moved:

- To resize an object, click on one of the gray boxes at the corners of its perimeter and drag the box until the object reaches the desired size.
- To copy an object, use the Copy tool.
- To delete an object, use the Cut tool or the Eraser.
- To move an object, click within its boundary and drag it into the desired location.

To select more than one object, outline them with the mouse; any objects that fall completely within the outline drawn will be selected.

NOTE: The entire object must be enclosed by the cursor's movement in order to be selected.

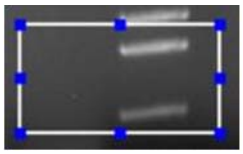


***Figure 4-16:** A Selected Object*

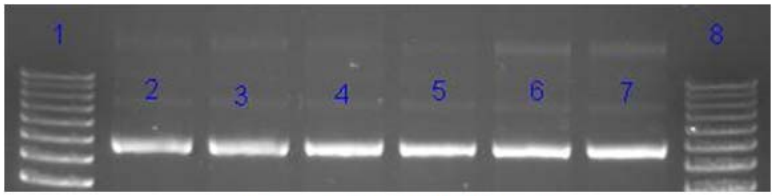
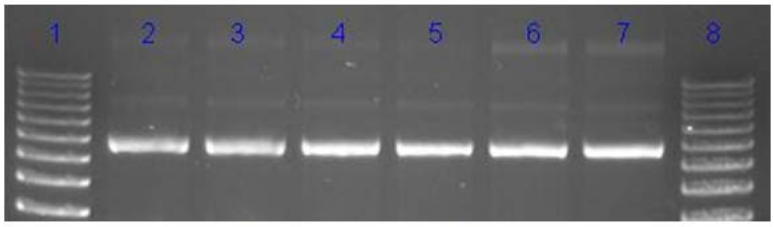
Icon	Description
	Once an object or group of objects has been selected, clicking on the Cut tool deletes it from the image.
	The Copy tool makes an exact copy of the selected object. The new object becomes the selected object, and can be repositioned by placing the cursor within the object's boundary and moving it to the desired location.
	The Horizontal Alignment tool aligns annotations in a straight horizontal line. This is especially useful for labeling lanes, etc. To use this tool, draw text on the image, select it, click the Horizontal Alignment tool, then deselect the text. The text will now be aligned in a straight line across the image.  Text Prior to Horizontal Alignment  Text After Horizontal Alignment
	The Vertical Alignment tool aligns annotations in a straight vertical line. This is especially useful for labeling markers, etc. To use this tool, draw text on the image, select it, click the Vertical Alignment tool, then deselect the text. The text will now be aligned in a straight line down the image.

***Table 4-2:** Editing Tools*

False Color

These tools consist of eleven pre-defined color palettes that can be applied to an image. To select a palette, simply click on one of the four buttons labeled **Gray Palette**, **High-Low**, **Next** or **Previous**.

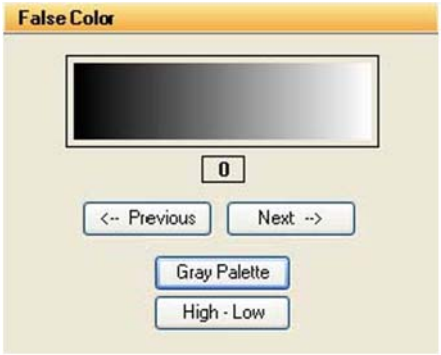


***Figure 4-17:** False Color Selection Box*

When a palette is selected, its range of colors is displayed to the left of the palette buttons and automatically applied to the image. To apply a different palette to the image, click **Next** or **Previous**.

NOTE: Changes in Black level, White level, and Gamma setting can alter the effect of each of the palettes, and can enhance the results produced.

Gray Scale (Palette 0)

This is the default or standard gray scale, consisting of different gray levels, ranging from black to white.

High–Low (Palette 1)

This is a modified gray scale palette in which black is replaced with green, and white is replaced with red. Over- and under-exposed areas of the image are thus shown as green or red, while areas within the linear range of the CCD chip are shown in gray scale. The Saturation Palette is especially useful during quantitation, as areas outside the linear range of the instrument do not give accurate quantitative information. This palette allows the user to avoid those areas during quantitative analysis.

This palette can also be accessed by clicking the Show Saturation checkbox in the Camera Setup and Preview function (accessible by clicking on the Camera icon in the Tool Bar window)

Other Palettes

These are color substitution palettes in which the gray levels are translated into different color ranges. These palettes can be useful to help distinguish features and highlight details on an image.

- Palette 2 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to red; saturated to blue, and values in between to green.
- Palette 3 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to blue; saturated to green, and values in between to red.
- Palette 4 maps the gray scale levels to a red/green/blue palette. Values of 0 are mapped to green; saturated to red, and values in between to blue.
- Palette 5 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to cyan; saturated to yellow, and values in between to magenta.
- Palette 6 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to yellow; saturated to magenta, and values in between to cyan.
- Palette 7 maps the gray scale levels to a cyan/magenta/yellow palette. Values of 0 are mapped to magenta; saturated to cyan, and values in between to yellow.
- Palette 8 maps the gray scale levels to shades of red palette. This palette may be useful when viewing red color images.
- Palette 9 maps the gray scale levels to a red palette. Values of 0 are mapped to dark red; saturated to white, and values in between to shades of red.
- Palette 10 maps the gray scale levels to shades of blue palette. This palette may be useful when viewing blue color images.
- Palette 11 maps the gray scale levels to a blue palette. Values of 0 are mapped to dark blue; saturated to white, and values in between to shades of blue. This palette may be useful when printing an image of a Coomassie-stained protein gel onto a color printer.
- Palette 12 maps the gray scale levels to shades of green palette. This palette may be useful when viewing green color images.
- Palette 13 maps the gray scale levels to a green palette. Values of 0 are mapped to dark green; saturated to white, and values in between to shades of green. This palette may be useful when printing an image of a SYBR® Green I-stained protein gel onto a color printer.
- Palette 14 maps the gray scale levels to an orange palette. Values of 0 are mapped to dark orange; saturated to white, and values in between to shades of orange. This palette may be useful when printing an image of an EtBr-stained gel onto a color printer.

Filter

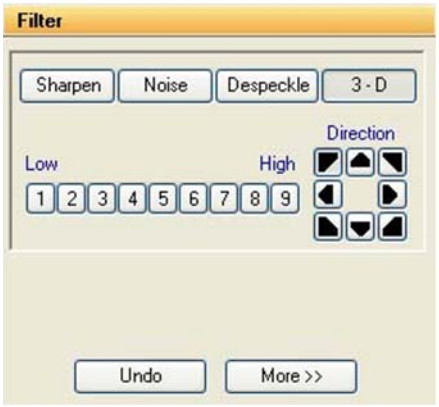


***Figure 4-18:** Filters Toolbox with 3-D (Contour) Selected*

AlphaView includes a variety of enhancement filters that can improve the appearance of an image. Some filters sharpen detail, others smooth and reduce random noise. Still others help visualize edges and separate closely spaced bands or objects. Depending upon the unique characteristics of an image, the results of each filtering operation vary. Assess the characteristics of the image and then select the filter designed to minimize its imperfections.

When an image is filtered, the original image information is replaced with the results of the filtering operation. As a result, the original image information is altered. To avoid losing the original image, save it as an original TIFF file before applying a filter.

When the FILTERS button is selected, a pop-up box appears. If the desired filter is not shown, choose MORE to display more filters. One or many filters can be applied to a single image.

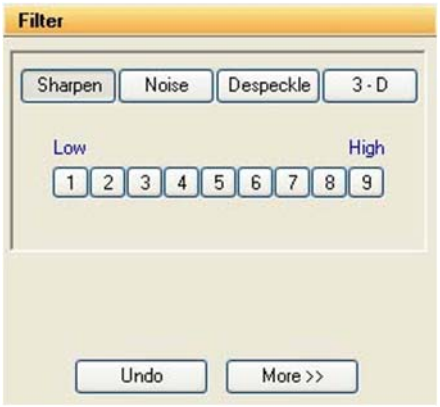


***Figure 4-19:** Filters Toolbox with Sharpen Highlighted*

Figure 4-20: Filters Toolbox with More Selected

General Information

To enhance an image, filters change the value assigned to each pixel. The new value assigned to a pixel is determined based on the values of the other pixels in its local vicinity (or "neighborhood"). The neighborhood is a two-dimensional matrix of pixel values, where each dimension has an odd number of elements. The "pixel of interest" is the one at the center of the neighborhood. This is the pixel whose old value is being replaced with a new one as the result of the filtering algorithm.

The pixels in a neighborhood provide information about the brightness trend. This information is important to the filtering process. The brightness trend is also referred to as the "spatial frequency." Images with high spatial frequency content contain large, closely spaced changes in pixel values. For example, on a black and white checkerboard, the smaller the squares, the higher the frequency content.

Images with low spatial frequency content (for example, images of clouds) contain large areas of slowly changing pixel values.

Most of the filter options available (with the exception of the Noise filters) use a weighted summation process to determine the value assigned to the pixel of interest. Each pixel in a 3x3 neighborhood is multiplied by a "convolution kernel" having the same dimensions. The resulting sum is assigned to the pixel of interest.

P1 P2 P3 P4 P5 P6 P7 P8 P9	X	K1 K2 K3 K4 K5 K6 K7 K8 K9	(K1xP1)+ (K2xP2)+ (K3xP3)+ (K4xP4)+ (K5xP5)+ (K6xP6)+ (K7xP7)+ (K8xP8)+ (K9xP9)
3x3 Pixel Neighborhood (P5 is being calculated)		Convolution kernel	**New Value for P5**

Each element of the convolution kernel is a weighting factor, also called a "convolution coefficient." The size and arrangement of these weighting factors determine the type of transformation the image will undergo. Changing a weighting factor influences the overall sum and, therefore, affects the value given to the pixel of interest.

Sharpening Filters

These filters can increase image sharpness and provide edge enhancement. However image noise may be enhanced as well. These filters accentuate the high-frequency details of an image while leaving the low-frequency content intact. High frequency portions of the image get brighter while low frequency portions become black.

Sharpen level 9 (high) has the largest effect on the image. Sharpen level 5 has an intermediate effect. Sharpen 1 (low) has the most subtle effect on the image. 9 different sharpening levels are available for optimization of the image.

Noise Filters

This filtering process uses the values of the pixels contained in the area surrounding a pixel to determine the new value given to the pixel of interest. The noise filter sorts the pixels in the neighborhood into ascending order and picks the middle or median pixel value as the new value for the pixel of interest. Three levels of noise reduction are available.

Despeckle Filters

The despeckle filter is a type of smoothing filter based on data rejection. Pixels in the neighborhood (usually the adjacent pixels) are used as a data set upon which the average and standard deviation of the set are calculated. If the pixel of interest (the center of the neighborhood) is different from the neighborhood average (either greater or lesser) by a threshold (a multiple of the standard deviation) it is replaced by the average value.

The effect of the filter is to "smooth" pixels that are much different from their neighbors. Artifacts such as hot pixels, cosmic rays etc. are commonly rejected by this type of filter. The filter strength is controlled by the threshold factor. For large factors very little data is rejected as only very large deviations are required for rejection, where as, low thresholds result in more smoothing. (For example, setting #1 results in outliers of 1 standard deviation greater or lesser than the neighborhood average to be corrected for, setting #2 results in outliers of 2.5 standard deviations to be corrected for and setting #3 results in outliers of 5 standard deviations to be corrected for.)

These filters are particularly effective in eliminating random noise contained in an image and produce less blurring than the noise filters.

3-D (Contour) Filters

These filters are particularly useful for visualizing faint bands. They produce a "3-D" effect, defining edges and making details easier to see.

3-D level 9 (high) has the largest effect on the image. 3-D level 5 has an intermediate effect 3-D level 1 (low) has the least effect on the image. Nine different 3-D levels are available for optimization of the image.

In addition, 3-D allows the user to control the direction of the 3-D shadowing effect. Once the level is refined, just click on a direction arrow to visualize the shadowing adjustment. The default shadow direction is in the lower right hand corner direction and 9 different directions are available.

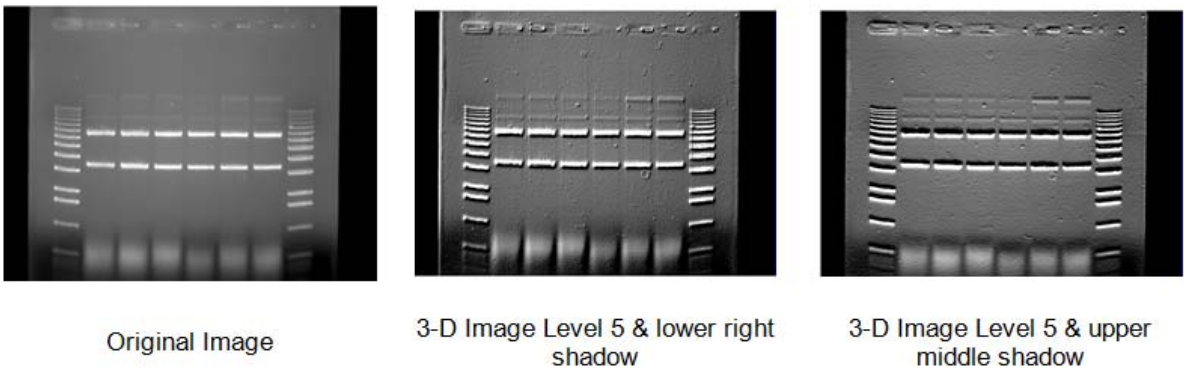


Figure 4-21: 3D Image Examples

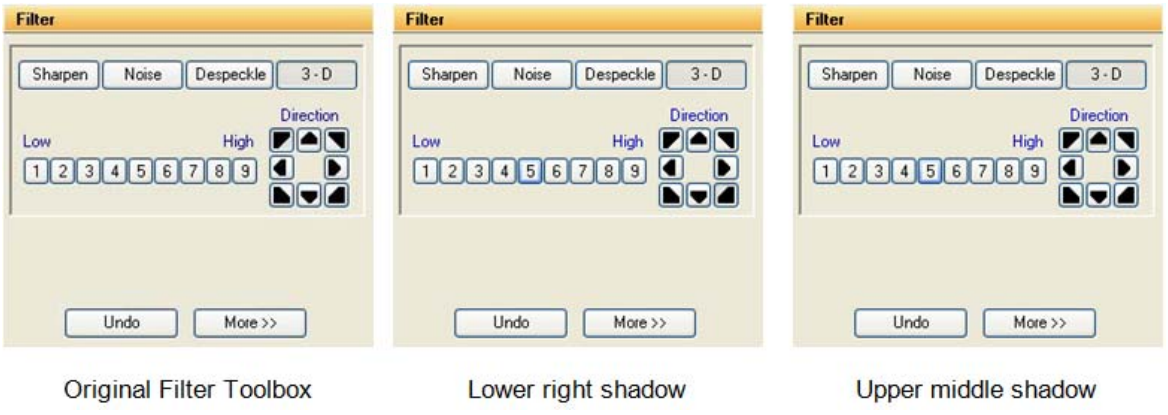


Figure 4-22: 3D Image Controls

Smoothing Filters

These filters are very useful for reducing the visual noise present in an image. When a smoothing filter is applied to an image, rapid changes in intensity are averaged out with the remaining pixels in the neighborhood, thereby decreasing the high frequency content. The visual result is a slight smoothing of the image because sharp pixel transitions are averaged with their surroundings.

Smooth High has the largest effect on the image. Smooth Med has an intermediate effect. Smooth Low has the most subtle effect on the image.

Edge Filters

These filters use Laplacian enhancement to highlight edges, regardless of direction. All edge-enhancement operations attenuate the low frequencies of the image. Regions of constant intensity or linearly increasing intensity become black as a result of these transformations, and regions of rapidly changing intensity values are highlighted.

NOTE: The White level may need to be adjusted after using the Edge filters in order to see the result of this filtering process.

Horizontal Edge Filter

This filter brightens horizontal edges. This can be useful in pinpointing bands on a gel. The horizontal edge filter (Horz. Edge) enhances image edges by shifting an image vertically by one pixel and then subtracting the shifted image from the original. In an area of constant pixel intensity, the subtraction yields black pixel values. At an edge, which is an area with large changes in intensity, the subtraction yields light-colored pixel values. The larger the difference in intensities, the lighter the resultant pixels.

NOTE: After applying the horizontal edge filter the entire image may appear black, and might require reducing the White level in order to better visualize the results.

Vertical Edge Filter

This filter (Vert. Edge) brightens vertical edges using the approach described for the horizontal edge filter (see above), except that the image is shifted horizontally before the shifted image is subtracted from the original. In this case, the vertical edges produce light-colored pixel values. As in the horizontal edge filter, it may be necessary to adjust the White level in order to better visualize the results of this filtering process.

Custom Filter

This function allows the use of customized filters. Using the weighting factors of the other filters as a frame of reference, experiment with new weighting factor values.

Undo Button

The upper right button is labeled **Undo**. This reverses the last filtering process applied to an image. To revert the image to its original state after multiple filters have been applied, press the **Reset** button on the main interface.

Examples of Filter Results

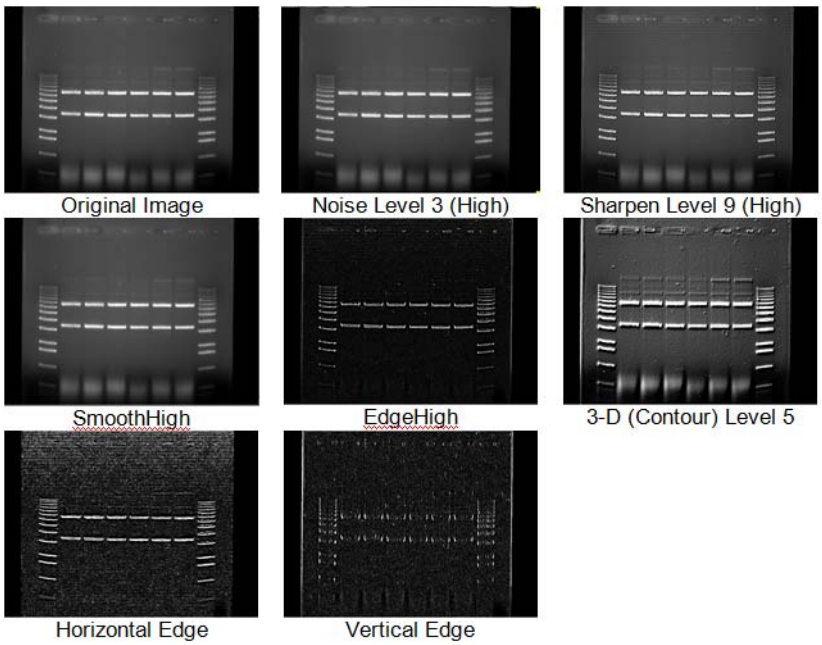


***Figure 4-23:** Examples of Filter Results*

Movie Mode

To open and play existing movies, click on the Movie Mode tab in the Enhancement Tools.

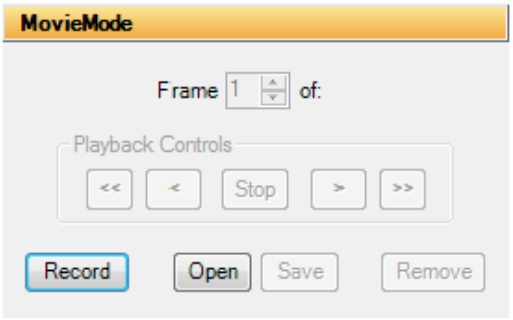


***Figure 4-24:** Movie Mode In the Enhancement Tools*

Click the **Open** button to open and play a previously recorded movie. To record a movie, click on the **Record** button. This will launch the Camera Setup and Preview window. Please refer to "Movie Mode" on page 34 for detailed instructions on recording movies.

Chapter 5:

Image Analysis Tools

Chapter Overview

- Tool Box Analysis Tools Tab
- Molecular Weight Determination
- Colony Count
- Band Analysis Tools
- Lane Profile
- Additional Analysis Tools

Tool Box Analysis Tools Tab

Default image analysis tools are contained within the Tool Box as indicated. This tool set allows the user to perform molecular weight determinations, automatic counting of colonies and cells, multiplex band analysis and lane profile densitometry.

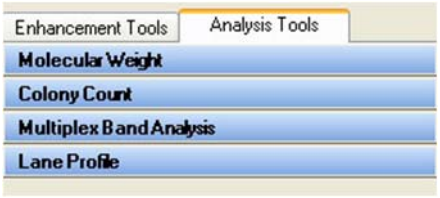


***Figure 5-1:** Default Analysis Tools in the ToolBox*

Molecular Weight Determination

The Molecular Weight button (in Tool Box, Analysis Tools) opens a set of tools for entering the values of known molecular weight markers and determining the molecular weights of unknown bands on the image. When the Molecular Weight button is selected, a function box appears in the area to the lower left of the screen, and a data box appears on the image. (If the data window obscures part of the image containing bands of interest, it can be moved.)

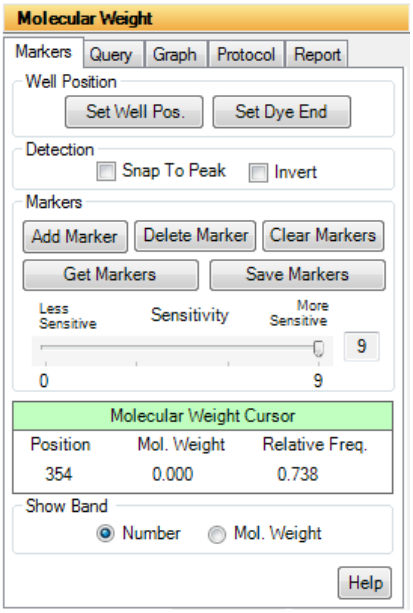


Figure 5-2: Molecular Weight Tools

Figure 5-3: Molecular Weight Data Box

At the top of the data box is a bar with two menus: Marker and Query. The area below the menu bar is divided into two sections. Molecular weight marker data is displayed in the upper section and the calculated or query data is displayed in the lower section.

Entering Known Molecular Weights for Markers

An unlimited number of molecular weight standards can be defined, either all in one lane, or in multiple lanes. To input markers, open the Marker menu and select Add Marker.

NOTE: To enter standards from previously-saved files, see "Applying a Set of Saved Markers" on page 115.

Notice that the cursor flashes and has a short, horizontal line associated with it. Move the cursor to the lane containing the molecular weight standards. Beginning at the top of the lane, position the cursor so the horizontal line is aligned with the band.

If the **Snap to Peak** function has been activated, the cursor jumps to the highest pixel value in the band as the cursor approaches it. If bands are so tightly spaced that the line is not coinciding with the band, turn off **Snap to Peak** and place the marker on the band manually.

When the horizontal line is positioned correctly, click the left mouse button. A dialog box appears requesting the band's molecular weight. Using the numeric keypad in the dialog box (or the numbers on the keyboard), enter the known molecular weight. Alternatively, enter the value using the keyboard.

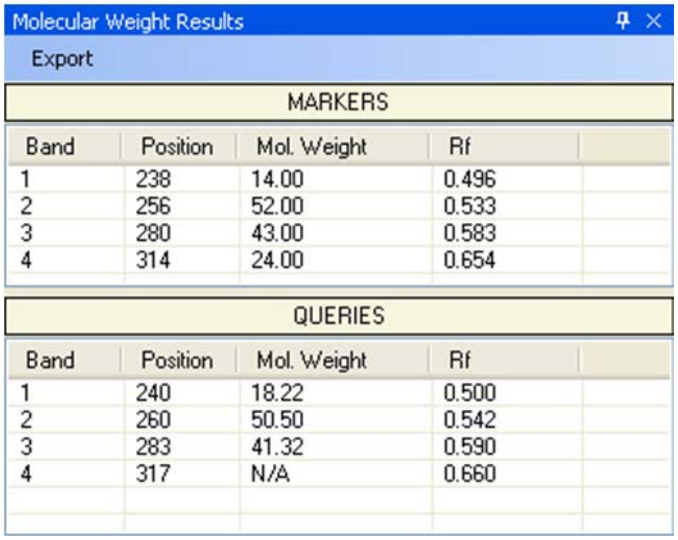
After entering the molecular weight, press either the OK button in the dialog box (or the <Enter> key on the keyboard). The dialog box disappears, the number of the band appears on the image, and the band's data is added to the Markers section of the data box. The horizontal red line remains, indicating that the cursor is ready to select the next band as described above.

After entering the value for the last marker, click the Add Markers Complete button instead of OK. This will deactivate the value-entering function and will return the cursor to its normal mode. (To reactivate the value-entering function, select Add Marker from the menu again, or click the right mouse button.)

The Molecular Weight Data Box

The data box is located near the top of the image. Initially, no values are displayed in the data box. When a molecular weight value is assigned to a band, the following information is displayed in the data box:

- Band numbers are assigned beginning with the first band, continuing in the order selected.
- The position corresponds to the band's location along the y-axis, ranging from 0 to 1030.
- The value displayed under the Molecular Weight. heading is the molecular weight value entered for a known and or the value calculated for an unknown band.
- The R_f value for each band is also displayed. Unless otherwise specified, AlphaView assumes the origin (R_f value = 0.00) is located at the top of the screen, and dye front (R_f value = 1.00) is at the bottom of the screen. Using these points as a frame of reference, AlphaView calculates R_f values for the intermediate bands.


Molecular Weight Results

Export

MARKERS

Band	Position	Mol. Weight	Rf
1	238	14.00	0.496
2	256	52.00	0.533
3	280	43.00	0.583
4	314	24.00	0.654

QUERIES

Band	Position	Mol. Weight	Rf
1	240	18.22	0.500
2	260	50.50	0.542
3	283	41.32	0.590
4	317	N/A	0.660

***Figure 5-4:** Molecular Weight Marker and Query Data*

If the data box obscures part of the image, it can be resized or moved using Windows® functions, or it can be hidden using the Hide Data checkbox.

Repositioning and Deleting Markers

Marker band indicators can be repositioned simply by positioning the cursor, clicking, and dragging the line to the desired location.

To delete a molecular weight marker, point the cursor at the appropriate marker band and click the left mouse button. This highlights the band in question by putting a red box around it. (Alternatively, clicking in the data table highlights the marker's information and selects the band on the image.)

Click on the Delete Marker function in the Marker menu. The marker is deleted, as is the band's data in the marker data table. Markers entered after the deleted bands are renumbered on the image and the data table.

To delete all the markers and start over, select the Clear Markers function in the Marker menu.

Determining Molecular Weights of Unknown Bands

After marker values are entered, the molecular weight of any unknown band can be determined.

Manually Selecting Bands

To indicate unknown bands manually, select the Add Band function from the Query menu. Just as in Add Marker above, a line will appear attached to the cursor. Point the cursor at the band of interest and click the mouse button. The molecular weight of the band is automatically calculated and displayed in the Queries section of the data box.

To select a second band, click the right mouse button and the cursor line will reappear. Repeat this procedure for all bands for which molecular weights is to be determined.

NOTE: The molecular weight markers appear in blue while the queries appear in green.

The molecular weight of a band is calculated based on the graph of the known marker bands.

NOTE: If a query band lies outside of the markers, it will be extrapolated in "Least Squares Fit" mode or given a value of "N/A" in "Point-to-Point" mode. For more information, see "The Graph Tool" on page 118.

Automatic Band Finding

AlphaView includes an algorithm for automatically finding bands in query lanes. To use this function, select Auto Query from the Query menu. A yellow vertical line will appear attached to the cursor. Position the line over the lane of interest and click the left mouse button. The bands in the lanes will be selected automatically, and their data will appear in the Data box.

Repositioning and Deleting Bands

Bands can be repositioned simply by positioning the cursor, clicking, and dragging the band to the desired location.

To delete an unknown band indicator, point the cursor at the band and click the left mouse button. The band in question is highlighted. (Alternatively, clicking in the data table highlights the band's information and selects it on the image.)

Click on the Delete Band function in the Query menu. The band will be deleted, as will the band's data in the query data table. Bands entered after the one deleted are renumbered on the image and the data table.

To delete all the queried bands, select the Clear Queries function in the Query menu.

Using the Molecular Weight Standards Library

AlphaView contains a library of DNA and protein molecular weight standards. Additional sets of markers can also be added to this library for later access. For a complete list of pre-loaded files and the band sizes in each, see Appendix B, *"AlphaView Molecular Weight Library Files"*.

Applying a Set of Saved Markers

From the Marker menu in the data box, select Get Markers from File. A dialog box appears.

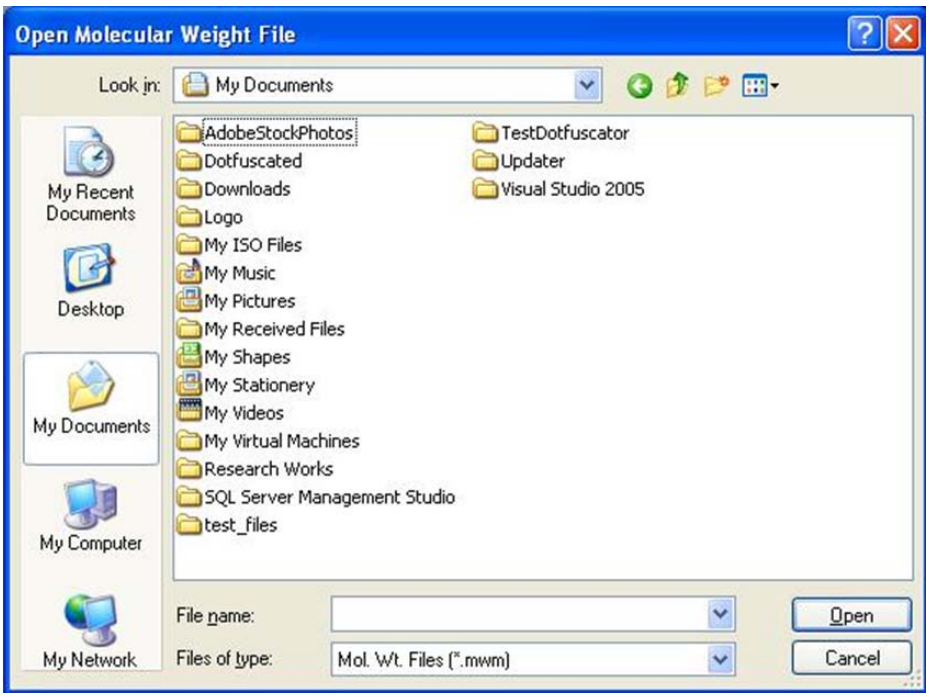


***Figure 5-5:** Get Markers from the Open Molecular Weight File Dialog Box*

Using the left mouse button, click on the name of the file to be loaded. That name is then highlighted in the list and appears in the text box below the File Name: prompt.

The current directory path is shown beneath the Folders heading. Below this is a graphical depiction of the path, and the sub-directories of the current directory. If the file of interest is in a different directory than the one open, double-click on the appropriate folder icon.

Once the file has been selected, click on the OK button to load the file. (Alternatively, double-click on the file name.) The dialog box disappears and a new dialog box appears.

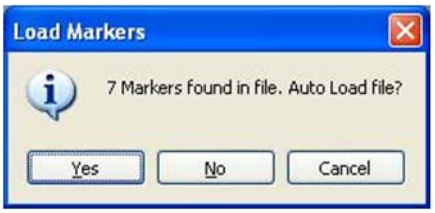


***Figure 5-6:** Load Markers Auto Load Dialog Box*

This box gives the file name selected, as well as the number of markers contained within the file. It also give the user the option of auto-loading the file.

- If Yes is selected, a vertical line will appear attached to the cursor. Place the vertical line in the marker lane on the gel, and click. AlphaView will automatically search for the markers in the lane. In the above example, the brightest 7 bands in the lane would be selected, and the values for Boe II's 100 bp DNA marker ladder would be assigned to the bands.
- If No is selected, AlphaView will display a cursor with a red line attached. Text in the upper right-hand corner of the image window will tell the user how many markers have been entered, and the value of the next marker to be entered.

***Figure 5-7:** Manual Load Dialog*

To begin entering markers, move the cursor to the top band in the lane and align the horizontal line with the band. Click the left mouse button. Notice that the position for that band is added to the markers data table.

Move the cursor to the next band, click the left mouse button again to assign the next value in the series. Repeat this process until the values of all the marker bands are assigned.

To quit this function before all markers have been placed, click the right mouse button.

Adding a Set of Molecular Weight Markers to the Library

If the same set of molecular weight markers will be run repeatedly, there is no need to enter values on each new image. Instead, save the molecular weight values in a file and apply them to the marker lane of any subsequent images.

To save a set of molecular weight standards, click on Write Markers to File in the Marker menu after the marker values have been entered.

Following Windows conventions (255 characters or less), enter a new file name in the text box below the File Name: prompt. AlphaView will automatically give the appropriate 3-character extension.

The current directory path is shown beneath the Directories heading. Below this is a graphical depiction of the path, and the sub-directories of the current directory. If the file is to be saved in a different directory than the one open, double-click on the appropriate folder icon.

Alternate disk drives can be accessed using the Drives heading.

Once a name has been entered and the appropriate directory has been accessed, click OK. All of the marker values that are currently in the Data window will be saved to this new file. To access them in the future, follow the steps in "Applying a Set of Saved Markers" on page 115.

Special Functions

Snap to Peak

This feature makes it easier to place the cursor on a band. If the **Snap to Peak** function has been activated, the cursor jumps to the highest pixel value in the band as the cursor approaches it.

If bands are so tightly spaced that the line is not coinciding with the band, turn off **Snap to Peak** and place the marker on the band manually.

If the cursor is jumping to areas between bands instead of the bands themselves, check to see that the **Bands Darker than Background checkbox** is properly set.

Calculating R_f Values

To obtain accurate R_f values, specify the location of the wells (i.e., origin) and the dye front using the functions Set Well Pos./Start and Set Dye Front/End both found in the Marker menu.

To designate the location of the wells, point the cursor at Set Well Pos./Start. Position the cursor so it is pointing anywhere along the wells and click the left mouse button. A horizontal bar appears, defining the location of the origin. By clicking and dragging, the bar can be moved for better positioning.

Repeat this procedure using Set Dye Front/End to indicate the location of the dye front.

Once the origin and dye front have been defined, they are used to calculate the R_f values of any bands that are added. If dye fronts are not assigned, AlphaView uses the top and bottom of the image as the boundaries for calculating R_f values.

The Molecular Weight Cursor Box

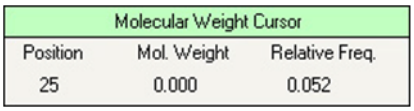


***Figure 5-8:** Molecular Weight Cursor Box*

The **Molecular Weight Cursor box**, found in the Molecular Weight toolbox, reports the location on the y-axis, the molecular weight and the R_f value of the current position of the cursor. These data are updated as the cursor is moved. This information can be helpful for positioning the cursor in a precise location, or for a quick estimate of a band's size.

The Graph Tool

The Graph function is found in the lower left corner of the Molecular Weight tools box.

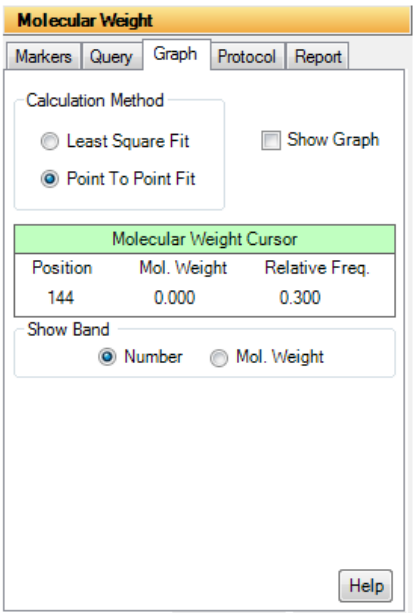


***Figure 5-9:** Molecular Weight Tools*

Clicking in the Graph box displays the semi-log graph of the molecular weight data that is used to calculate the molecular weights of unknown bands. (To remove the graph window from the display, click on the Graph checkbox a second time.) The x-axis corresponds to the band's vertical location on the screen, and the y-axis is a log representation of molecular weight. By clicking on the appropriate button, the data can be toggled between a least squares fit and a point-to-point fit.

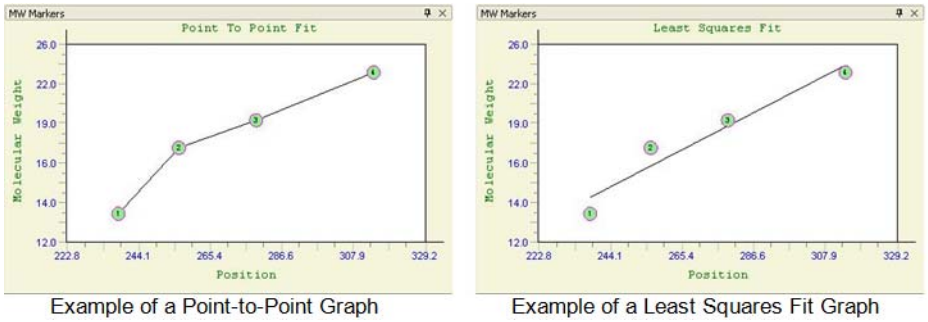


***Figure 5-10:** Example of Point-to-Point and Least Squares Fit Graph*

The graph is useful to verify that the molecular weight data is entered correctly, and can help determine the most linear region of the molecular weight standards. Further, if a query band is clicked, its position will be shown on the graph by dashed lines (in Figure 5-10).

Colony Count

The Colony Count tools in the Tool Box, Analysis Tools make it easy to count the number of objects on an image, such as colonies on a Petri dish or viral plaques.

Click on the Colony Count. The following sets of tools will be displayed in the lower left of the screen.

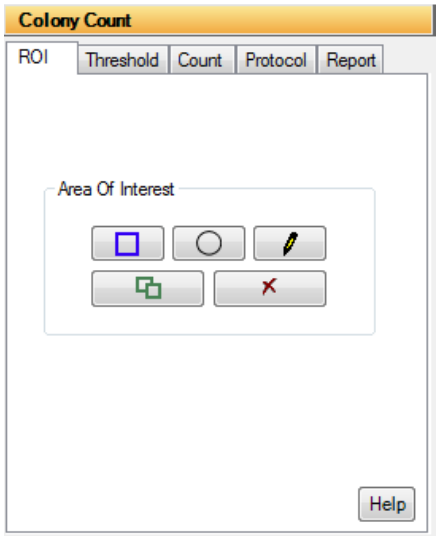


***Figure 5-11:** Colony Count Tools*

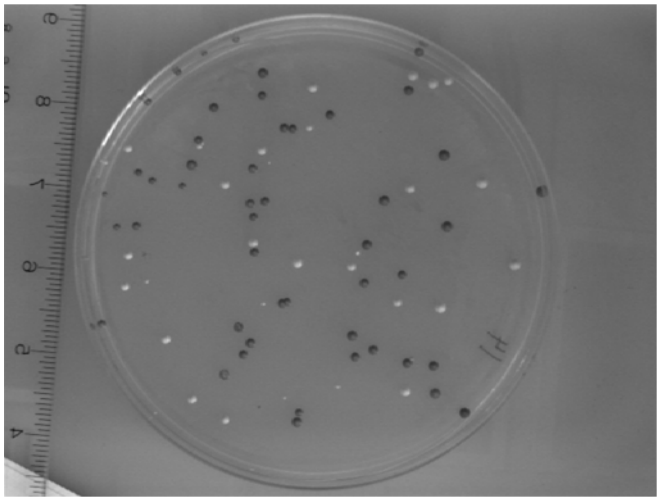


***Figure 5-12:** Sample with Two Types of Objects*

When two types of objects are counted, the objects are highlighted in different colors. They can also be marked on the image using a "+" or an "X".

Follow these three steps to perform an automatic colony count:

1. Define and Position Area(s) of Interest

There are three drawing tools with which areas of interest can be designated. Click on the desired AOI (Area of Interest) button in the toolbox to define areas on the image containing the objects to be counted.

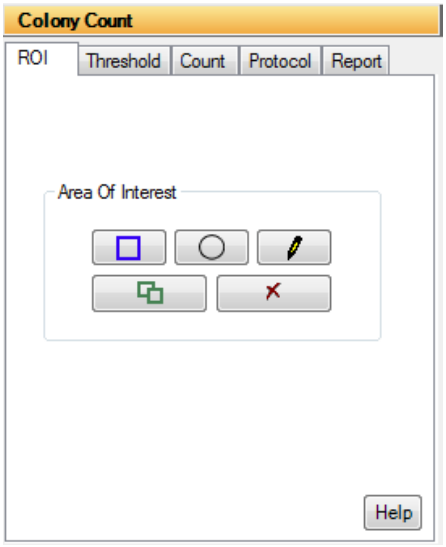
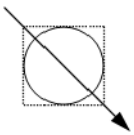


***Figure 5-13:** Colony Count Tools*

Circles: This is the most appropriate choice for counting colonies on a Petri dish. After clicking on the button in the toolbox labeled with a circle, move the cursor into the image area to the edge of the Petri dish. Click the left mouse button and move the cursor to open the circle. When the circle corresponds to the perimeter of the Petri dish, release the mouse. The circle has "handles" around it, and can be moved or re-sized at this point.

Hint: To draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.



Rectangles: After clicking on the button in the toolbox labeled with a square, move to one of the four corners of the region to be enclosed. Click the left mouse button and move the cursor to the corner diagonally opposite.

NOTE: The box that opens as the cursor moves. Release the left mouse button when the box has reached the desired size. The rectangle has "handles" around it, and can be moved or re-sized at this point.

Freehand Drawings: To draw a freehand object, select the freehand icon; the mouse will change to a pencil. Draw an object of the desired size and shape. When the mouse is released, the start and end points of the object will be connected.

Manipulating Areas of Interest: Areas of interest can be moved, copied and deleted. To perform any of these functions, an area must first be selected by clicking on it or dragging the cursor around it. Its color changes to gray, indicating that it is active. More than one area of interest can be selected at any given time.

To move an area of interest click on the selected area. While holding down the left mouse button, drag the area to the desired location.

To delete an area of interest click on the cut button in the toolbox (designated by a pair of scissors). The selected area of interest is removed and any other areas of interest are renumbered accordingly.

To copy a selected area of interest, click on the copy button (found next to the cut button). A second copy of the area of interest appears overlapping the first one. Click and drag the copy to the desired location.

2. Set the Density Thresholds

The objects counted by the automatic counting routine are based on the difference in the gray levels of the objects as compared to the background (e.g., the colonies on the petri dish are either darker or lighter than the surrounding area). The density thresholds define the gray levels that are recognized by the counting routine.

To set the density threshold, click and drag the sliders. As the sliders are moved, the regions of the image that fall within the gray scale range are highlighted in the image area. Adjust the slider positions until the objects to be counted are highlighted. The numbers next to the Min and Max settings change to reflect the positions of the sliders. These numbers represent the minimum and maximum gray scale values that are currently being detected. Any object whose gray level falls between these two numbers will be counted.

If there are two sets of objects to count, click on the second colony count button (next to the Count button) and adjust the density threshold so the second set of objects are highlighted.

3. Count the Objects

When the density threshold settings and size settings are satisfactory, click on the Count button. If two types of objects are to be counted, click the second colony count button and click Count again. The counts are shown for each area of interest and the totals are given in an colony count display window on the image.

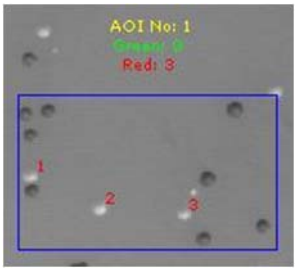


Figure 5-14: Colony Count Sample Results for an AOI

Editing Tools

After the objects have been automatically counted, AlphaView will automatically open the Edit Tools functions. If editing is necessary, these tools manually override decisions made by the automatic counter.

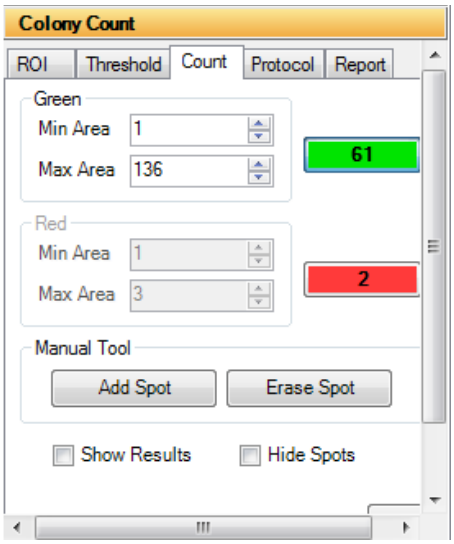


Figure 5-15: Colony Count Editing Tools

Displaying Spots

Spots are displayed as red (or green) numbers on the image. These can be changed to symbols ("+" or "x") by holding the <shift> key and clicking the right mouse button. Repeat to return to numbers.

Spots can be hidden from view by checking the Hide Spots checkbox.

Specify the Size Range of the Objects

If the image has objects of different sizes but only those in a certain size range are of interest, use the Area Controls.

Specify the size range by adjusting the minimum and maximum diameter settings. The numbers below the Min. Area and Max. Area headings indicate the minimum and maximum diameter settings in pixels. Any object whose diameter falls between these two numbers will be counted.

As these number are changed, objects will be added to (or deleted from) the display, and the Count windows will be updated.

Using the Add Spot Tool

Occasionally, the automatic counting function may fail to count an object on the image. This could occur for a variety of reasons, including inappropriate Density and Size Threshold parameter settings.

Objects can be added to the total count numbers using the Add Spot button. Click on the appropriate count box (red or green). Next, click on the Add Spot button. Point the cursor at the object to be added and click the left mouse button. The object will be highlighted and the count will automatically increase.

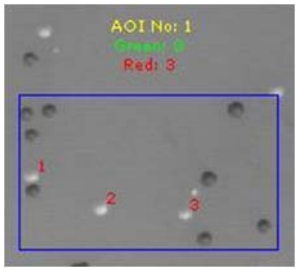


***Figure 5-16:** Results of Colony Count After Manual Addition of Three Spots*

To add a second object, click on the right mouse button to re-enter "add" mode, then click on the desired object with the left mouse button.

Using the Erase Spot Tool

This function removes any extra spots that were counted either automatically or manually. For example, if a portion of an AOI has high background or other noise, a number of objects may erroneously be included in the count. Draw a box around the object or set of objects. Objects will be displayed with a white background to indicate that they are selected. Clicking Erase Spot deletes all selected objects at once and automatically reduces the total count information.

Spot Count Data

When AlphaView opens, the Colony Count data box is hidden. To display spot count data, click the checkbox to deselect Hide Data. The following data window will appear:

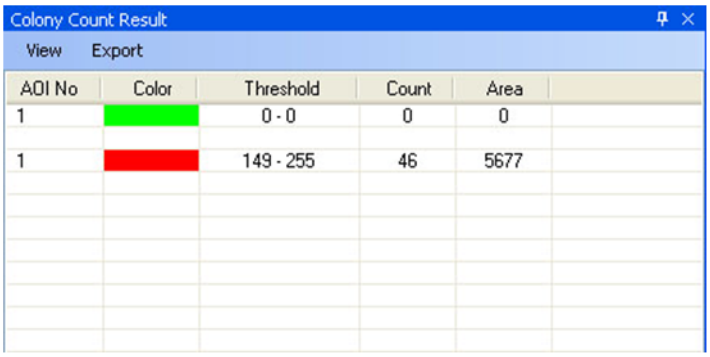


Figure 5-17: Colony Count Data Window Showing AOI Summary Data

This window shows the summary data for the Area(s) of interest (AZ). This includes the threshold values that were set for each count type, the number of objects found for each count type, and the total pixels in all of the spots.

To see specific details on each spot, click View > Spot Details. Figure 5-18 displays an example of what displays:

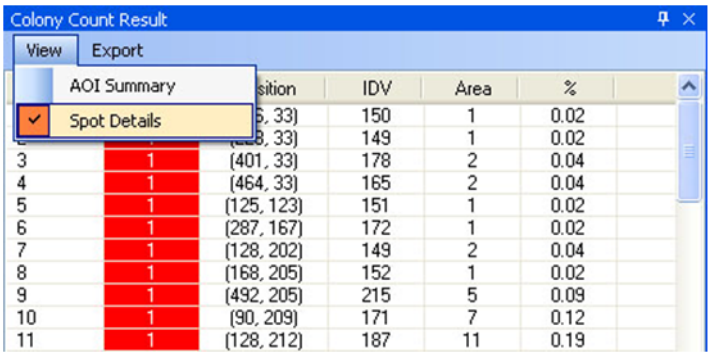


Figure 5-18: Selecting the Colony Count Data Window to Show Individual Spot Details

The data table gives the individual spot number, the AOI in which it was counted, its coordinates, the integrated density value (IDV) for each spot, the area (number of pixels) for each spot, and the % contribution to the total.

NOTE: Manually-added spots show areas of 1 pixel.

Band Analysis Tools

In the ToolBox, Analysis Tools, a tab labeled Band Analysis opens a set of tools with which the density of bands, spots or other objects can be measured. A two dimensional area of interest (or Object) is created and the density is obtained through the corresponding pixel intensity values designated as IDV or Integrated Density Value.

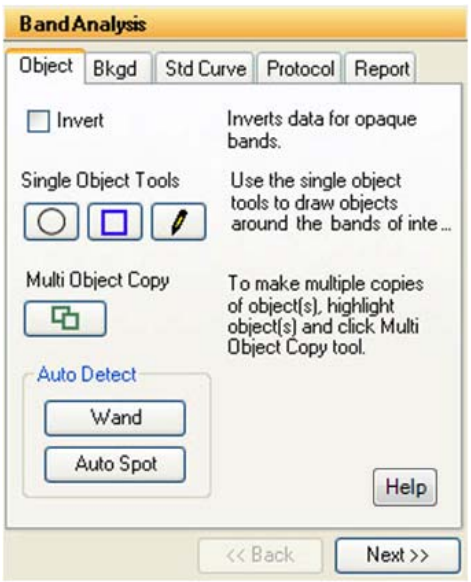


***Figure 5-19:** Band Analysis Object Tab*

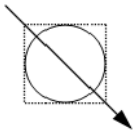
Creating an Object Area of Interest

Object Boxes:

An area of interest can be created in several ways. The user can manually draw an area of interest through the three OBJECT buttons. Objects can be enclosed with a box (rectangle), ellipse, or freehand drawing. Select the one that most closely corresponds to the shape of the objects on the image.

To draw a rectangle or ellipse, click on the button labeled with a box (or a circle) beneath the OBJECT header in the toolbox. The cursor automatically changes to a "+" when it is in the image area, indicating that AlphaView is in "drawing" mode. Move the "+" to the corner of the object to be measured, click the left mouse button, move the cursor, and release the mouse button when the box (or circle) surrounds the object.

Hint: To draw a perfect circle around a portion of an image, first visualize a square surrounding the area of interest. Position the mouse in the upper left hand corner of the square. Click and drag the mouse down across the area of interest at a 45° angle until the circle encloses the area of interest.



To draw a freehand object, select the freehand icon; the mouse will change to a pencil. Draw an object of the desired size and shape. When the mouse is released, the start and end points of the object will be connected.

Once the mouse is released, the cursor changes to an arrow (indicating that AlphaView is in "edit" mode), and the object is shown in gray with handles around it. The object can now be resized using the handles or repositioned by clicking within the boundaries and dragging to a new location.

To draw another object, return AlphaView to "drawing" mode by clicking the right mouse button.

If a box or ellipse is large and includes significant background area, the corresponding density value may be large. The higher the background, the greater its contribution to the total of pixel values in that object. Therefore, objects should be drawn so that they fully enclose areas of interest, but do not include an excessive number of background pixels or pixels from neighboring regions:

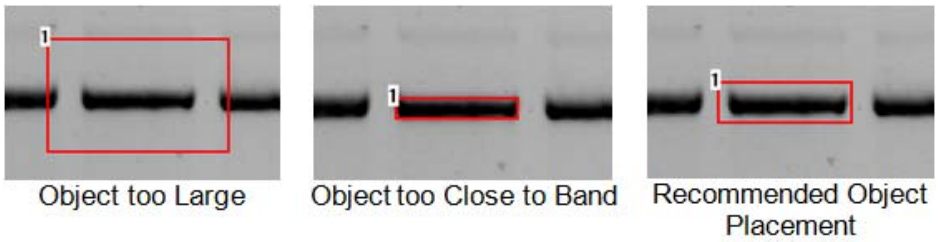


***Figure 5-20:** Drawing Objects Examples*

Hint: Keeping the data window open can reduce the speed at which objects are drawn and manipulated on the screen. Therefore, it may be preferable to hide the data window until all objects and backgrounds are drawn and in place.

Hint: Accurate Local Background Correction method requires the object box to contain a few pixel values that are true representations of the background level. If the object is too small and the band fills the object box then local background correction will result in significant errors.

Magic Wand and AutoSpot

These two selections are automated detection features designed to recognize sample in an image and draw Object boxes around it. They are found under the Auto Detect heading in the main Band Analysis. Tab. Once an object is created the dimensions can be altered and the background subtracted similar to manually drawn objects.

***Figure 5-21:** Magic Wand and AutoSpot Tools*

Magic Wand

Selecting Magic Wand will activate a wand like symbol and the following data table:

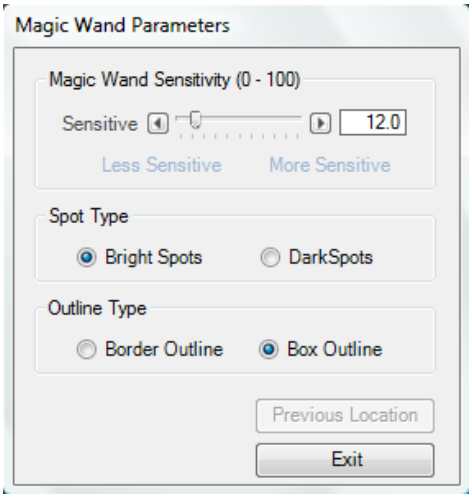


***Figure 5-22:** Magic Wand Parameter Window*

The tip of the wand should be centrally positioned over the sample spot on the image and then simply click the mouse. The software will use either an "edge detection" algorithm to trace an area of interest around the spot or draw a box around the sample. A right mouse click will reactivate the wand icon for another selection.

Magic Wand Sensitivity

The Magic Wand Sensitivity slide bar will alter the parameter for how much of the spot will be detected. The slide bar ranges from 0 to 100. The smaller the number the less of the spot the software will define. The greater the sensitivity number the more of the object the software will find.

HINT: Selecting a brighter pixel within the band (or spot) will have magic wand draw a tighter area of interest. Correspondingly, a less intense pixel will draw a larger perimeter around the selected spot.

Spot Type

Under the Spot Type heading the Bright Spots selection should be checked if the image contains bright spots on a dark background (e.g. ethidium bromide stained fluorescent gels and chemiluminescent blots). The Dark Spots selection should be made when the sample contains dark sample on a light background (example: film, Coomassie blue protein gels). The software should automatically determine this selection, but some manual intervention may be necessary. Remember as in all portions of AlphaView software if the image has been reversed (negative image) using the Reverse selection on the Contrast Adjustment window the Dark Spot selection should not be made. Reverse is a visual alteration only and does not affect the original image capture data. (e.g. a chemiluminescent blot is captured using the Reverse mode in order for it to appear as film, the correct selection is still Bright Spots).

Outline Type

Border Outline will use the edge detection methods to determine the spot area. Box Outline is the default setting and will draw a box shape around the sample usually including a significant portion of background. User preference and image particulars will determine which outline type is best.

Previous Location

Previous Location will automatically select the spot on the image last selected for magic wand and apply any changes made for sensitivity. This feature is designed to quickly evaluate how the different sensitivity numbers will affect the drawn area using the same selected point in a spot. The X,Y coordinates are given for the previous spot as well.

Exit

Exit will remove the Magic Wand Parameter window.

Auto Spot

Auto Spot is a feature designed to find multiple spots within a region of interest. It is accessed through AutoSpot under Auto Detect in the Multiplex Band Analysis tab. Once it is selected the following tab will appear and the cursor will be ready to draw an area of interest.

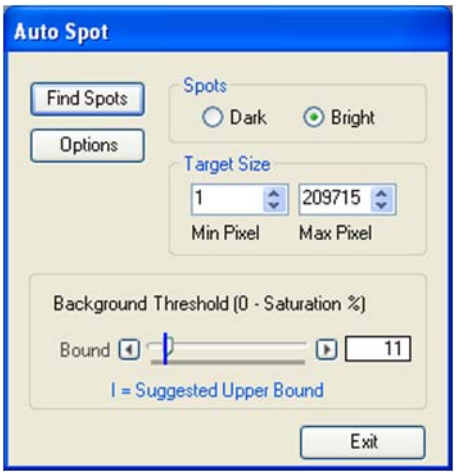


***Figure 5-23:** Auto Spot*

Area of Interest

After selecting AutoSpot an area should be drawn on the image by clicking and dragging the mouse. The smaller and more defined the Area of Interest is, the better the resulting data. The area can be manipulated using the manipulation tabs at the edge of the red box.

Options

The Options selection allows either a box outline to be drawn around the spots or a border outline. The box outline is the default setting and will include more of a background in the drawn area than the border outline. User preference and image particulars will determine which outline type is best for sensitivity can also be adjusted by sliding the bars for normal spots (<98% saturation) and for saturated spots (>98% saturation).

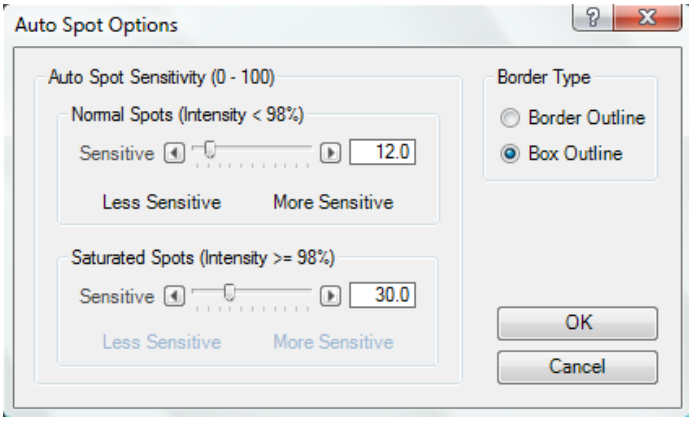


Figure 5-24: Auto Spot Options

Bright Spot vs Dark Spot

The Bright Spots selection should be checked if the image contains bright spots on a dark background (e.g. ethidium bromide stained fluorescent gels and chemiluminescent blots). The Dark Spots selection should be made when the sample contains dark sample on a light background (film, commassie blue protein gels etc.). The software should automatically determine this selection, but the user may have to override it if the software incorrectly evaluates an image. Remember as in all portions of AlphaView software if the image has been reversed (negative image) using the Reverse selection on the Contrast Adjustment window the Dark Spot selection should not be made. Reverse is a visual alteration only and does not effect the original image capture data. (EG if a chemiluminescent blot is captured using the Reverse mode in order for it to appear as film, the correct selection is still Bright Spots).

Find Spots

The Find Spot button is selected once the correct area of interest is drawn and the Bright Spot/Dark Spot selection is correctly made. A green outline will be drawn around the detected spots.

Background Threshold

The background threshold slide bar will adjust the criteria used for finding spots. It is on a scale of 0 to 100% saturation. The sliding bar maps the dynamic range of the camera. By calculating the fringe pixels in the image or ROI, the initial threshold value and suggested lower (or upper) bound value are calculated and shown on the sliding bar. The user can always override the threshold value by moving the sliding bar or typing in the counter box.

Target Size

The user can input the minimum and maximum area in pixels of the spots or bands of the desired targets. The default values are 1 pixel minimum and no limit for maximum pixels.

Get Data

Get Data is selected once a satisfactory spot outline is achieved. This will convert all drawn objects into standard Spot Densitometry objects with associated density numbers.

Manipulating Objects

Selecting Objects

To select an object, click on it with the mouse. "Handles" will appear at its corners.

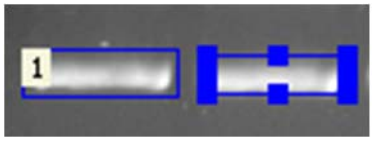


***Figure 5-25:** Non-Selected and Selected Objects*

To select a second object instead of the first, click on the desired object.

To select more than one object at a time, drag the mouse around all of the objects of interest.

NOTE: An object must be completely surrounded by the mouse operation in order to be selected.

To select more than one object where it is not feasible to drag the mouse around objects, hold the <shift> key and click on each object to be selected.

To de-select an object, click the left mouse button outside of the selected object. The handles disappear, indicating that the object is no longer selected.

Copying Objects

To draw boxes or ellipses enclosing the same number of data points, use the Copy function.

First, draw or select an object of the desired shape and size as described above. Next, click on the Copy button in the toolbox. A duplicate of the selected object will appear on the screen. This object can then be moved to the desired position.

To make multiple copies of an object, continue to click on the Copy button until the desired number of copies is displayed.

Moving Objects

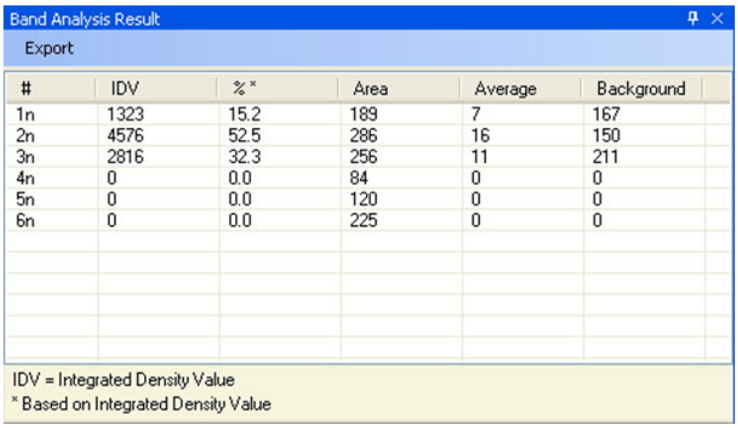
An object can be moved to fine-tune its position simply by clicking on it, holding down the left mouse button and dragging it to the desired location.

Deleting Objects

To remove an object or set of objects, select the object(s). Select the Delete button on keyboard. All the selected objects are removed from the image window and their associated data is cleared from the data window.

Band Analysis Measurements

As objects are drawn, their density data is automatically calculated and displayed in a data window. Any time an object is drawn or detected, the data in the window are updated. If the Band Analysis Results window obscures a region of interest, resize and/or move it following Windows conventions.


Band Analysis Result

Export

#	IDV	%*	Area	Average	Background
1n	1323	15.2	189	7	167
2n	4576	52.5	286	16	150
3n	2816	32.3	256	11	211
4n	0	0.0	84	0	0
5n	0	0.0	120	0	0
6n	0	0.0	225	0	0

IDV = Integrated Density Value
* Based on Integrated Density Value

***Figure 5-26:** Example of a Band Analysis Data Window*

Data Definitions

Please refer to Appendix C, *"Data Description Tables"* for a complete listing and description of the various quantities.

is the number assigned to each object on the image in the order in which they were drawn. This object number is also shown in the corner of the object.

IDV is the sum of all the pixel values after background correction:

$$IDV = \Sigma(\text{each pixel value - BACK})$$

% is the percentage that each box, ellipse, or freehand drawing contributes to the total density measured thus far, taking background correction into consideration. The sum of the values in this column will be 100.

Area is the size (in pixels) of the region enclosed by the box, ellipse, or freehand drawing.

Average is the average value (after background correction) of the pixels enclosed.

$$AVG = IDV \div AREA$$

Background is the background value that will be subtracted from all the pixels in the object. (See "Background Tab–Calculating Background Values" on page 135 for more information.)

Inverting Data

The **Invert** function reverses the gray scale assignments so that 0 corresponds to white and 4,095 corresponds to black. This control is found on the **Region tab**.

If the image has dark bands and light background, then **Invert** should be selected by placing an "X" in its box. If the image has light bands and dark background, the **Invert** option should not be activated.

NOTE: This function does not alter the appearance of the image (unlike the Reverse button described in Chapter 2, "Getting Started—Basic Imaging Functions").

Background Tab–Calculating Background Values

Background subtraction is an important part of image densitometry analysis. There are three background subtraction options in the Band Analysis tools.

$$IDV = \Sigma(\text{each pixel value} - BACK)$$

***Figure 5-27:** Background Tab*

No Background

If no background calculation method has been selected, the background value in the data window (BACK) is reported as zero (0). Therefore, the object's integrated density value will be equal to the sum of all the pixel values within the box, ellipse, or freehand drawing. Recommendation: No Background or Manual Background is suggested for AutoSpot, Magic Wand (only the border outlines though) or any object where little if any background is included in the object area of interest.

Automatic Background

When the Auto Background function is active (an "X" appears in the checkbox), AlphaView determines the average of the 10 lowest pixel values in each individual object and assigns that value to BACK. This option is useful if the image has regions with different background levels, because the background values (BACK) will be unique for each object.

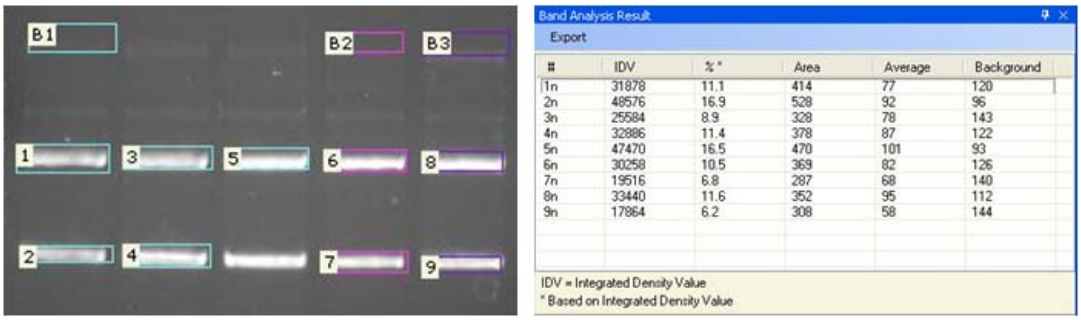
NOTE: If Auto Background is active, it will override the manual background option (described below).

Manual Background

Using this option, different region(s) of the image representative of different background densities can be specified. Further, the user can specify exactly which objects of interest should correspond to which background objects.

To manually specify the background, use the buttons beneath the Background heading in the Band Analysis toolbox to draw a box (or ellipse or freehand drawing) around an area representative of background density. If the background varies across an image, draw several background objects in order to represent each of the various background levels.

Next, specify the object(s) from which each background density should be subtracted. To associate a background with an object(s), select the object(s) and the background as described in "Selecting Objects" on page 132. When all are selected, click on the Link Bckgrd button in the Band Analysis toolbox. All the objects will assume the color of the background object, indicating that they are associated with each other.



#	IDV	% *	Area	Average	Background
1n	31878	11.1	414	77	120
2n	48576	16.9	528	92	96
3n	25584	8.9	328	78	143
4n	32886	11.4	378	87	122
5n	47470	16.5	470	101	93
6n	30258	10.5	369	82	126
7n	19516	6.8	287	68	140
8n	33440	11.6	352	95	112
9n	17864	6.2	308	58	144

***Figure 5-28:** Using Manual Background to Assign Different Background Values to Different Objects: Objects 1-5 are linked to B1; 6-7 to B2; 8-9 to B3*

In the Band Analysis data window, these objects now show a non-zero background density value in the BACK column.

To disassociate an object from its background, select the object and click on the Unlink Bckgrd button. The object reverts to its original color, indicating that it is no longer associated with any background box or ellipse. The object's value in the data window changes and its entry in the BACK column returns to zero.

Note: If Auto Background is active, manual background settings will be overridden.

Mass Standard Calibration Curves for Quantitative PCR

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. The calibration curve functions allow quantization of the bands on a gel based on a set of standards. A minimum of two standard bands must be input, but the accuracy of the calibration curve increases as the number of standard bands and their range of values increase.

Defining the Bands as Objects

Before generating a standard curve, define the bands using the object drawing tools in the Band Analysis toolbox. As described in "Selecting Objects" on page 132, the size of an object can influence the integrated density value. Therefore, we recommend using the same sized box, ellipse or freehand drawing when defining a standard curve. (For instructions, see "Copying Objects" on page 133.)

Specifying the Quantization Units

Once the objects are defined, click on the Std Curve button. The Standard Curve Toolbox will appear in which the results should be reported (e.g. ng, μL, pg, %) in the Enter units box

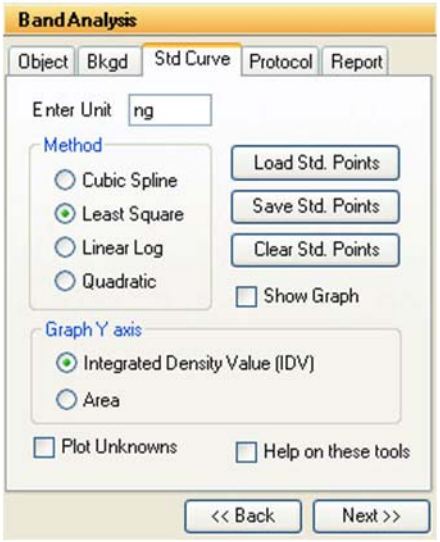


***Figure 5-29:** Standard Curve Tab*

Designating the Standard Bands

Click on one of the bands whose value is known. In the dialog box displayed, enter the value for the band, using either the keyboard or the numeric keypad in the dialog box. When the entry is correct click on the OK button. The band number changes from white to green, indicating that it is now a standard.

To enter the next value, click on another band. The dialog box will open again and will show an estimated value (based on the object's IDV value and on the standard value already entered). Click OK to accept this value, or enter a value.

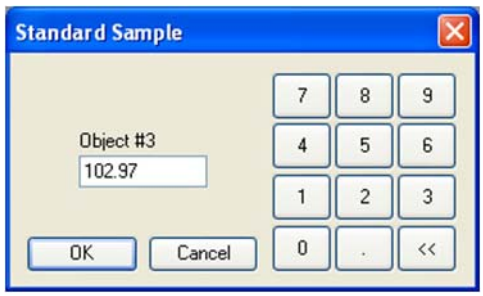


***Figure 5-30:** Entering the Value for a Standard Band*

Once the second value has been entered, a curve is displayed. 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.

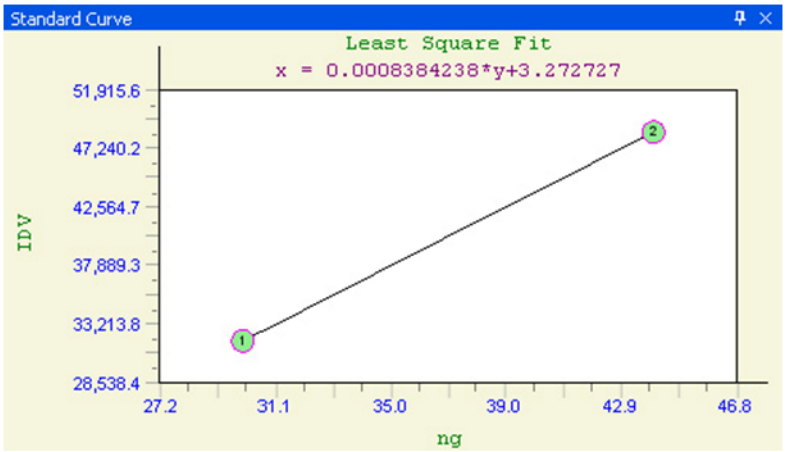


Figure 5-31: Standard Curve

Enter values for each band whose amount is known. As more standard points are added, the calculated values of the unknown points may change.

To remove a band from the set of standards, click on it again. The band number changes from green back to white, indicating that it is no longer a standard. To de-select all the standard points at once, click on the Clear Std Pts button in the toolbox.

To change the value of a standard band, click on it twice. In the dialog box, the estimated value for that band is displayed. Accept this value by clicking OK, or enter a new value.

Displaying the Curve

If the box displaying the standard curve obscures a part of the image, it can be resized using Windows operating system functions. It can be hidden from view by clicking the Hide Curve checkbox in the Standard Curve toolbox.

Quantization Values of Unknown Bands

As the values for the standard bands have been entered, the quantitation values of the unknown bands are automatically calculated. To see the calculated values of the unknowns, click on the Exit button in the standard curve toolbox. This returns to the Band Analysis tools and data box.

Note: The graph is based on a Smoothed Cubic Spline fit. To use other curve-fitting methods, exports the data table as an ASCII file and open it in any statistical analysis package.

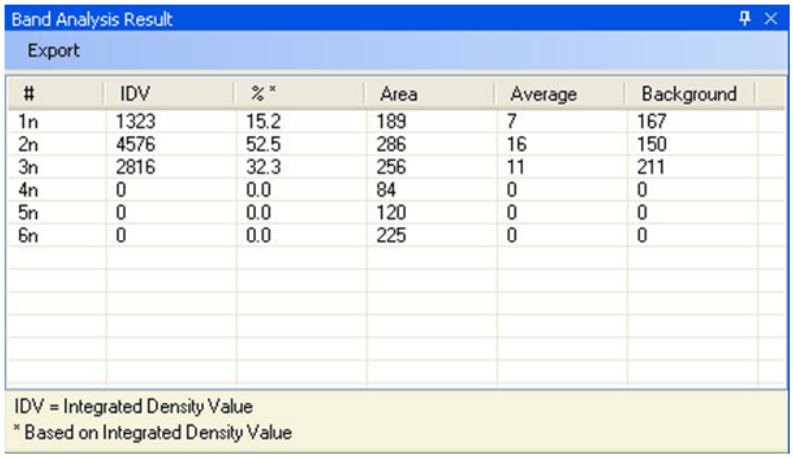


***Figure 5-32:** Band Analysis Data Box*

The third column in the data box contains values for the standards (entered by the user), and the values for the unknown bands (calculated based on the curve). The heading of this column reflects the units entered as in "Specifying the Quantization Units" on page 137

An "n" next to the object number indicates that the objects are unknowns (or non-standards) whose value has been interpolated. An "a" means that the object lies outside of the curve, and its value has been approximated through extrapolation. Standards have an "s" next to the object number, and are highlighted in green to differentiate them from unknown bands.

Lane Profile

The **Lane Profile** option in the **Analysis Tools** tab of the **ToolBox** accesses a set of densitometry and molecular weight determination tools with which bands on a gel can be automatically analyzed in a lane format.

When **Lane Profile** is selected, the **Lanes tab** is displayed with the following functions:

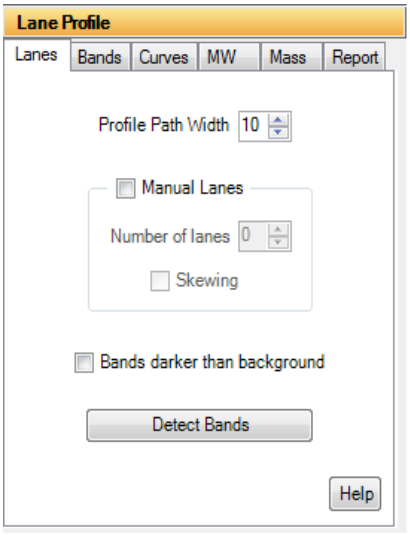


Figure 5-33: Lane Profile Lanes Tab

Profile Path Width

The **Profile Path Width** is the area from which the pixel density is measured. The default setting is 10. To adjust the setting, click on the up or down arrows or enter a value and hit the enter button on the keyboard.

The density values for Lane Profile graphs are determined by the average pixel density between the blue lines on the template.

A **Profile Path Width** that is too narrow may not include enough information and can result in a noisy graph. By contrast, a **Profile Path Width** that is too wide can incorporate background pixels that will reduce the pixel average and dilute the actual data.

Auto Lane Detection Using an Area of Interest

AlphaView can automatically detect and analyze the lanes and bands in the area of interest specified by the user. To draw an area of interest, click and hold the mouse and draw a box around the sample area of interest. Image artifacts (well position marks, etc.) should not be included in the box as AlphaView may record them as bands. After an area of interest is drawn, click on the **Detect Bands** button in the **Lanes tab**. AlphaView will automatically detect the lanes and analyze the bands in the area of interest.

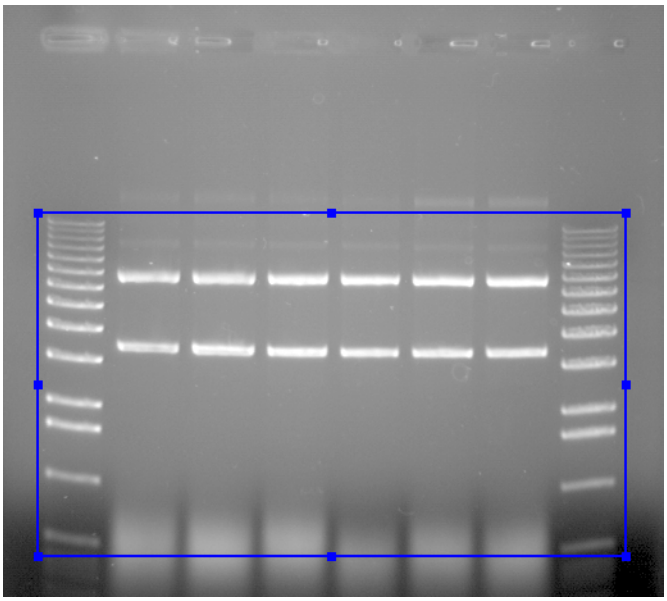


Figure 5-34: *Area of Interest Drawn*

Manual Lane Detection

Select the **Manual Lanes** checkbox to manually detect the lanes on the image. Once selected, the Auto Grid template will appear on the image.

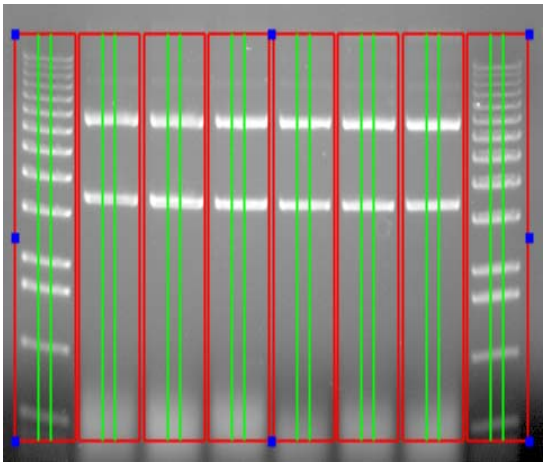


Figure 5-35: *Lane Profile Auto Grid Template*

Setting Up the Lane Profile Template

Red lines in the Auto Grid template indicate borders of adjacent lanes. Green lines define the **Profile Path Width**.

Manual Lanes Box

Select the **Number of Lanes** up to a maximum of 24 lanes. When the number of lanes is adjusted, the template displayed on the image is updated accordingly.

If the lanes on the gel are horizontally oriented (sideways) the image can be rotated. To change the orientation of the image to coincide with the template, click on the **Enhancement Tools** tab in the **Toolbox** and select the **Rotate-Flip** option. Click and drag the rotation bar +/- 90 degrees until the desired orientation is reached.

After specifying the correct number of lanes and changing the image orientation, adjust the outside borders of the template until they frame the lanes to be scanned. To do this, first click on the template. Next, select any of the blue boxes along the outside border of the template and drag it until the desired size is reached. Adjust the template until the border of the template frames the lanes to be scanned and the red lines lie between each of the lanes.

If the lanes of the gel are not perfectly straight, click the **Skewing** checkbox in the **Manual Lanes** box. The template will display four blue boxes, one at each corner of the template. Click and drag the blue boxes as needed until the template is positioned properly over the skewed lanes.

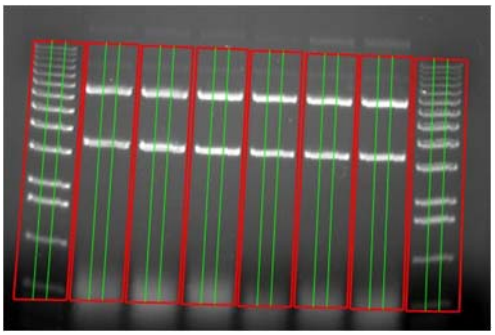


***Figure 5-36:** Skewed Lane Profile Template Properly Aligned on a Gel*

Profile Path Width

Adjust the Profile Path Width value as needed. The path width should not exceed the band edges and cannot extend beyond the red lane borders. As this value is changed, the template displayed on the image updates accordingly.

Bands Darker than Background Option

Select the **Bands darker than background** checkbox if the image being analyzed has dark bands/signal on a white background which are usually colorimetric gels. The **Bands darker than background** option is only necessary when the actual sample has signals that are visible by the naked eye, for example Coomassie Blue stained gels, Silver stained gels, and pieces of autorad film.

Image Analysis–Detect Bands

Select the **Detect Bands** button to automatically measure and calculate the densities of each lane. To have AlphaView detect all bands in the image automatically select **Detect Bands** immediately after opening an image. Otherwise, select **Detect Bands** after drawing an area of interest or after the **Manual Lane** settings and template have been adjusted.

Viewing Results

Once the **Detect Bands** button is selected, AlphaView automatically analyzes the image, detects the peaks for each lane, integrates the area under each peak, displays results and moves to the **Bands tab**.

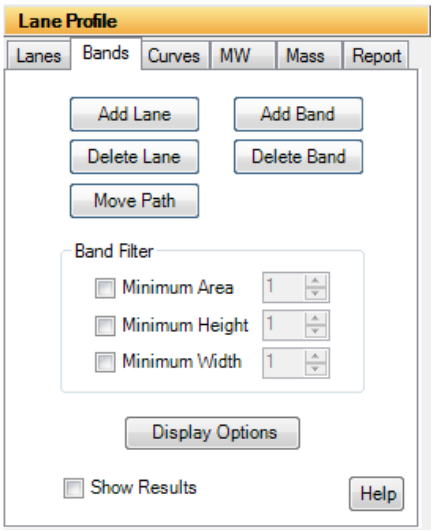


***Figure 5-37:** Lane Profile Bands Tab*

Peak integration adjustments that can be made manually will be described in detail later in this section. The Analyzed Image and the Lane Profile Results windows are displayed.

Analyzed Image

Individual bands will be identified by number on the analyzed image. When a lane is selected in the image its borders will turn pink. The corresponding lane data will be displayed in the Lane Profile Results windows.

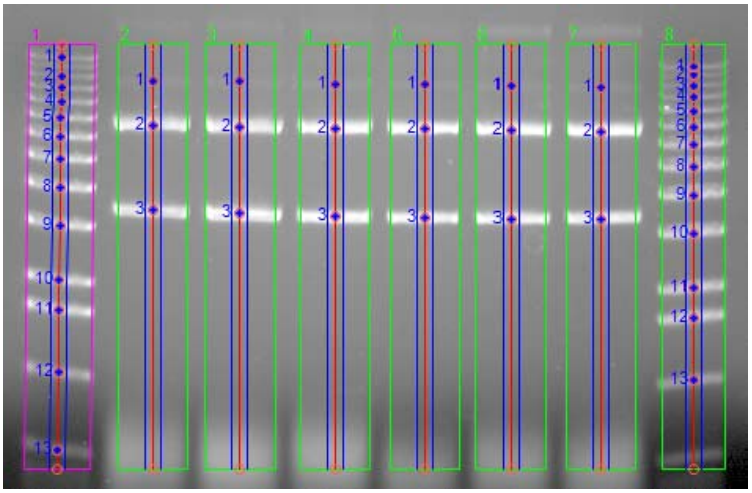


***Figure 5-38:** Analyzed Image*

Lane Profile Results Windows

The following results windows will also be displayed after the image is analyzed. These windows can be resized or docked by the user if desired:

- Data Table (middle). The Data Table displays peak information for each lane in the image.
- Lane Graphs (lower left). This area displays a graph for each lane.
- Active Graph (lower right). The active graph displays peak numbers and integration data for the lane selected.

To view data and the Active Graph for a lane, select a lane either from Analyzed Image or from the Lane Graphs. To view peak data for a specific band, select the band in the Analyzed Image or the peak from the Active Graph. The corresponding data will be highlighted in the Data Table. AlphaView uses the following color-coding to indicate what data is being displayed:

- The lane graph that is currently selected will be grey.
- The data for the currently selected lane will be highlighted in yellow in the Data Table.
- The data for the currently selected band or peak will be highlighted in dark yellow in the Data Table, and the peak fill in the Active Graph will also be dark yellow.

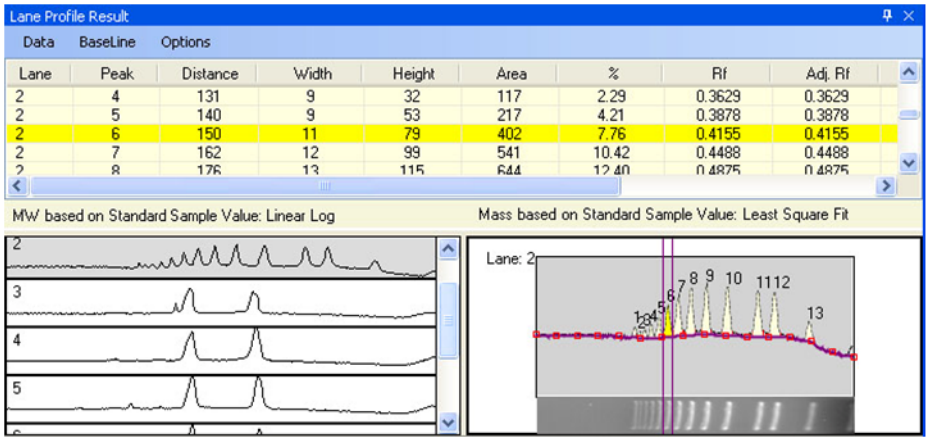


Figure 5-39: Results showing Color Coding in Data Table (top), Lane Graph (lower left) and Active Graph (lower right)

Lane Graph Display

Graphs representing all Analyzed Image lanes are shown in this area. After band detection, Lane 1 will be selected by default and its corresponding peak data will also be displayed in the Data Table and Active Graph. To view data for a different lane simply click on its lane graph.

When the image contains many lanes, there may be too many graphs in the Lane Graph display to see reasonable detail. To reduce this view to only four lane graphs, select the **Options** menu directly above the Data Table and deselect **Show All Lanes**.

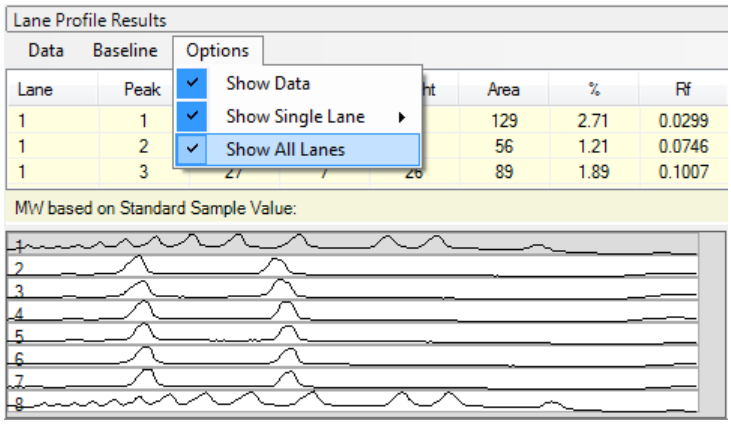


Figure 5-40: Deselecting Show All Lanes

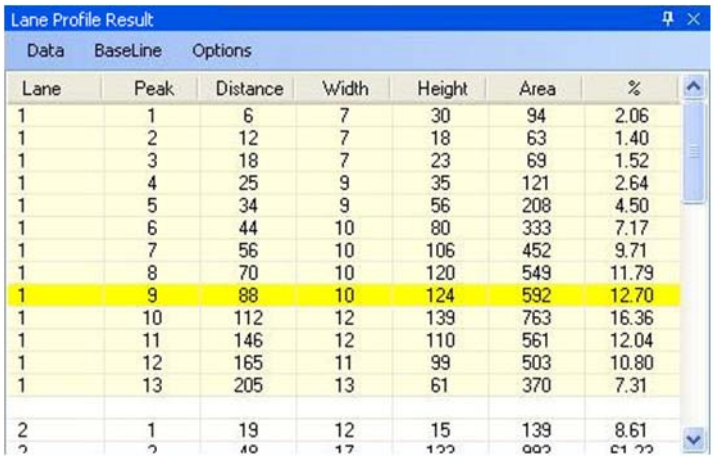
Active Graph

The Active Graph is shown in the lower right. The x-axis represents the distance (in pixels) from the top of the area of interest drawn or the Auto Grid template used. The y-axis represents the average pixel intensity across the width of the scan.

NOTE: Active Graphs are shown without axes in order to show as much detail as possible in a small window. To show the graph's axes, choose ***Show Axes*** *from the* ***Show Single Lanes*** *option in the* ***Options*** *menu located above the Data Table.*

Data Table

Once a peak is defined, its integration data and associated information are displayed in the Data Table. The data table is updated automatically when peaks are added or deleted, when peak boundaries are redefined, or if the background value is reset.


Lane Profile Result

Data BaseLine Options

Lane	Peak	Distance	Width	Height	Area	%
1	1	6	7	30	94	2.06
1	2	12	7	18	63	1.40
1	3	18	7	23	69	1.52
1	4	25	9	35	121	2.64
1	5	34	9	56	208	4.50
1	6	44	10	80	333	7.17
1	7	56	10	106	452	9.71
1	8	70	10	120	549	11.79
1	9	88	10	124	592	12.70
1	10	112	12	139	763	16.36
1	11	146	12	110	561	12.04
1	12	165	11	99	503	10.80
1	13	205	13	61	370	7.31
2	1	19	12	15	139	8.61

Figure 5-41: *Example Data Table*

The Data Table contains the following columns of data:

- **LANE** indicates the lane number where the peak is located.
- **PEAK** is the number assigned to each peak on the graph.
- **DISTANCE** is the migration distance (in pixels) for each peak as measured from the top of the region used for data analysis.
- **WIDTH** and **HEIGHT** are the calculated peak width and peak height.
- **AREA** is the integrated pixel intensity for the area under each peak and can be used to represent band intensity.

- **%** is the percentage each peak contributes to the total density. The sum of this column will be 100% for each lane.

Lane Profile Results Menu

The drop down selections at the top of the Data Table (**Data**, **Baseline** and **Options**) allow for the user to perform editing, visualization and outputting tasks pertaining to the Active Graph window.

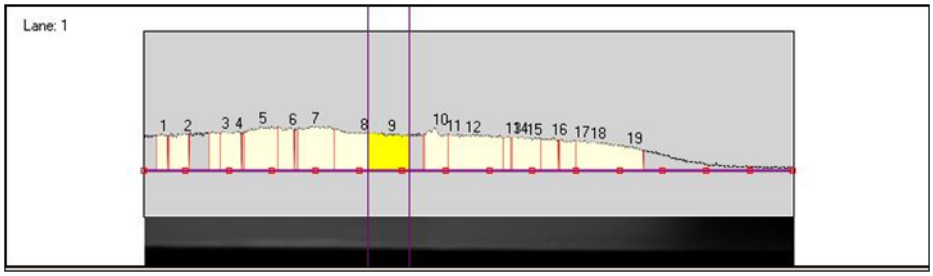


***Figure 5-42:** Visualization of the Active Graph*

Data

The **Data** drop down menu allows the user to superimpose different lanes onto the Active Window using the **Overlay Control** selection. For detailed instructions, see "Overlaying Graphs" on page 153. **Print** will allow the user to print any or all of the profile and data table windows. **Clipboard** will allow the user to send any or all of the profile and data table windows to the clipboard for pasting into different programs (e.g. Excel).

Baseline

Baseline manipulation and background subtraction can be performed using this menu. For detailed instructions, see "Adjusting the Baseline" on page 150.

NOTE: Background subtraction can have a dramatic effect on how many peaks are recognized as bands. Try changing the background subtraction to optimize band recognition.

Options

Selecting **Show Data** will display the Data Table. Selecting **Show Single Lane** will display the Acitve Graph. Selecting **Show All Lanes** will display the Lane Graphs. From the **Show Single Lane** menu option, **Background Color** allows the user to select a variety of different background colors for the Active Graph. **Show Axes** allows the user to display the axes on the graph. **Show Image Strip** will display a strip of the lane being currently analyzed.

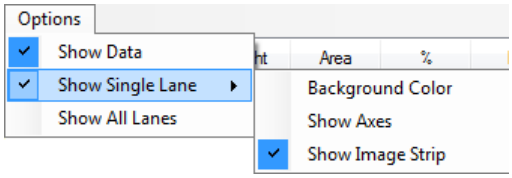


Figure 5-43: Options > Show Single Lane Menu

Editing Peaks

Editing Peak Boundaries

The boundaries of existing peaks can be readjusted to make the peaks wider or narrower. To adjust either peak boundary, click on the peak to select it. Next, point the cursor at the vertical boundary line, hold down the left mouse button and drag the line to the desired location. When it is in place, release the mouse button.

The data table is automatically updated to reflect any adjustments. Both the peak and the data in the data table will be blue to indicate that this is a user-defined (vs. automatically detected) peak.

Visualizing Peak Placements

To determine exactly where a peak is located relative to the original image, it is often helpful to see the graph and the gel image in the same orientation. The Show Strip function (found in the Options menu) displays the area of the gel that has been defined as a lane across the length of the x-axis.

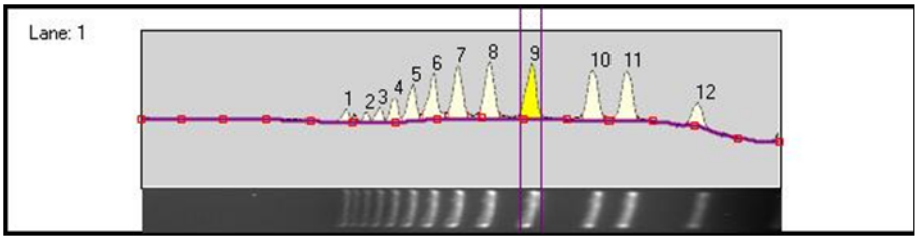


Figure 5-44: Graph With Show Strip Selected

NOTE: If Show Strip fails to show a portion of the image, check that the appropriate driver for the VGA card is selected.

Adjusting the Baseline

As a default, the baseline value is set to Auto Base. As the baseline is adjusted, the values for all peaks are updated to reflect the new baseline.

All of the following functions are found in the **Baseline menu** under **Background Subtraction**:

Auto Base

This method breaks the red line defining the left boundary of the lane into sixteen regions, and outputs sixteen points which reflect the average backgrounds across those sixteen regions. The user can manipulate the 16 points by clicking and dragging. The user may also designate more points than the 16 and manipulate those in the same fashion.

Intra-Lane

This method uses the lower of the two values at the edges of the lane along its travel as the background. If the section of the image is used is wider than the bands, then this is a very effective way of removing co-migrating material from your measurements. This method should only be used if the bands are completely enclosed. This is because when the edges of your lane area intersect any of your bands, band material will be considered as background, severely affecting your results.

Rubber Band

This method can be thought of as stretching a rubber band underneath the lane profile. This option does not work well if the values at the ends of the intensity profile are lower than the rest of the intensity pixels. We do not recommend this method for images with poorly separated bands.

Minimum Profile

This method takes the lowest value on the profile of each lane as the background for that lane. We do not recommend this method for lanes where the beginning or end of the lane is the lowest point as this is dependent on where the lane boundary was placed when it was defined. This can be hard to repeat between analysis of the same gel.

Valley to Valley

This method requires that you have performed band detection first. The background is taken as the line between the edges of the bands in the lane. You enter a value (see "Set Parameters" on page 151) of maximum slope in the accompanying entry box to avoid situations where the edge between overlapping bands, which is not at the background intensity, causes the background to climb too high.

Rolling Disc

This method requires you to enter a parameter for the size of the disc (see "Set Parameters" on page 151) in the entry box. This option calculates the background as if a disc, with the radius you have entered, were rolling underneath the lane profile. The larger the radius of the disc, the less the background rises with the profile.

Set Parameters

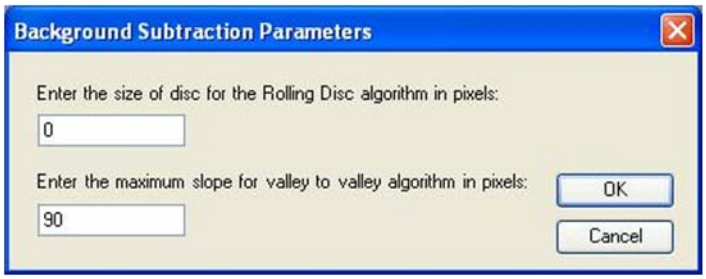


***Figure 5-45:** Background Subtraction Parameters Dialog Box*

This dialog box is used to change values for Rolling Disc and Valley to Valley background methods.

Base Lock

The Base Lock function will designate the active graph baseline for all of the lanes in the data table. To de-select this option click on the Base Lock a second time. The check mark will disappear.

Horizontal Base

Activating the Horizontal Base function will move the baseline to the 0 position (no background). The baseline can be moved vertically by clicking the baseline and dragging it upwards.

Reset Base

Activating the Reset Base function will move the baseline to the 0 position (no background). Unlike Horizontal Base the baseline cannot be moved.

Base Subtract

This selection will subtract the current baseline from the intensity value line. Un-check Base Subtract to deactivate this feature.

Interpreting Lane Profile Data

AlphaView provides a variety of tools to help interpret Lane Profile scan data.

V. Line

Typically, the peaks on a graph correspond to the bands in a lane. The **V. Line** tool located in the Lane Graph Display can help determine if the peaks on two different graphs represent bands at the same molecular weight position (i.e. the same distance from the top of the gel).

To compare peaks on different graphs, roll the mouse over the Lane Graphs. A vertical line which can be scrolled across the graphs will appear. The intensity value at the point where the **V. Line** crosses the graph will be displayed.

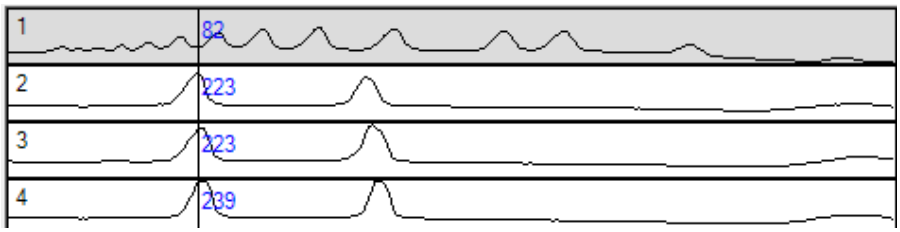


***Figure 5-46:** V.Line Tool Intensity Display*

Alternatively, the **V. Line** can also display hash marks which is a useful tool for comparing peak heights. To toggle between displaying intensity values and hash marks when using the V. Line, click the right mouse button.

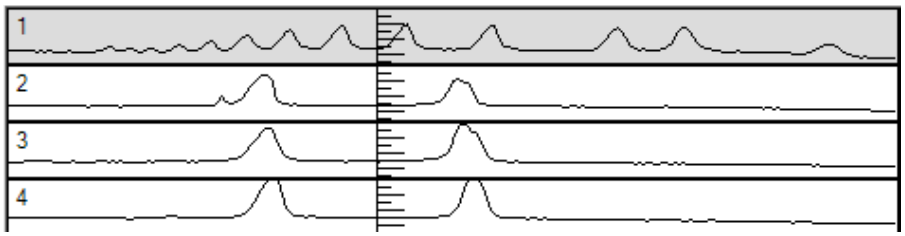


***Figure 5-47:** V.Line Tool Hash Marks Display*

NOTE: The hash marks are most useful when only four graphs are shown so that enough detail is displayed. To change the Lane Graph display, select the **Options** *menu which is directly above the Data Table and deselect* **Show All Lanes**.

Overlaying Graphs

When comparing the bands in one lane with those in another it is often convenient to display the graphs so that one is superimposed over another. After clicking on a graph so that it is enlarged in the image area, select the Overlay Control function (from the Data menu) to display the Lane Overlay Control dialog box. In this dialog box, specify the lane(s) whose graph(s) should be superimposed and the color and line pattern in which they should be displayed.

- The first column in the dialog box contains the numbers of the graphs which can be superimposed.
- The second column reports whether or not the graph of a particular lane is currently superimposed. These ON/OFF settings toggle back and forth by clicking on them.
- The colored bar in the third column indicates the color in which a graph will be displayed when it is superimposed. To select a color option, click on the color bar until the desired color is shown.
- The last column in the dialog box specifies the pattern of the line when the graph is superimposed. To select a pattern, click on the current pattern until the desired pattern is shown.

It is especially important to choose different patterns if the graphs will be printed, as different colors cannot be distinguished using a gray scale printer.

When the selections have been made, click on the DONE button to dismiss the dialog box and superimpose the selected graph(s). (If no graphs have been selected, the dialog box is simply dismissed.)

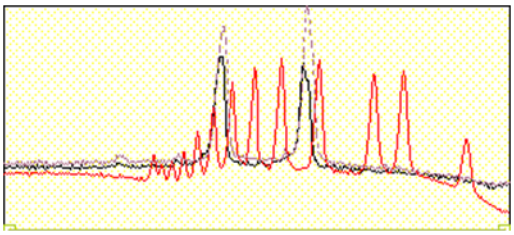


***Figure 5-48:** Three Graphs Overlaid*

To turn all graph superimposing off, select the **Deselect All** button at the bottom of the dialog box.

Bands Tab Overview

Several lane editing and viewing options appear in the **Bands tab**. Each will be explained in detail in this section.

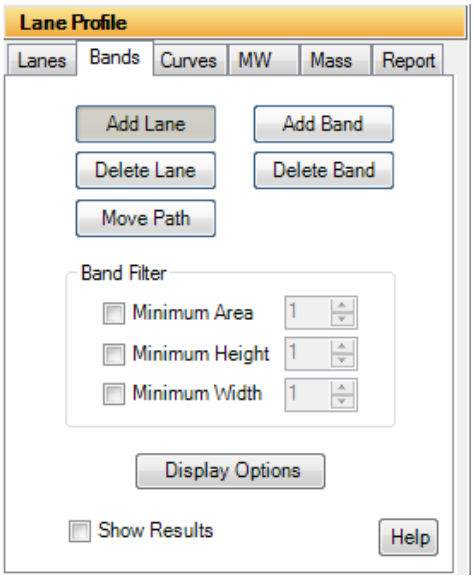


Figure 5-49: *Lane Editing Features*

Add Lane

This option allows an additional lane to be placed on the image. To add a lane, select the **Add Lane** button and place the mouse on the image. A plus sign cursor with a new lane grid will display on the image where lanes can be added. Roll the mouse over the image to move the cursor to the desired position. When the desired position is reached, click the mouse to drop the lane on the image. To add more lanes, move the cursor to another position on the image and click to add the next new lane. Continue until all new lanes are added. Click the **Add Lane** button again to exit the editing mode. The Data Table will readjust accordingly, adding the lane and calculating results for the bands within the lane.

Delete Lane

This option allows lanes to be deleted. To delete a lane, select the **Delete Lane** button and place the mouse on the image. A cursor will display on the image. Place the cursor anywhere on the lane to be deleted, then click the mouse to delete the lane. Continue until all desired lanes are deleted. Click the **Delete Lane** button again to exit the editing mode. To delete lanes when not in editing mode, simply click on the lane and select the Delete key on the keyboard. The Data Table will readjust to remove the deleted lane data.

Move Path

This feature allows for the horizontal manipulation of the center line in each of the found bands. To do this, click on the **Move Path** button. To move the path over a band, position the cursor over the band centroid, then click and drag to move the path. To move a lane path, position the cursor over an area in a lane without bands, then click and drag to move the lane path. To skew an entire lane path, click on the round end point of

the path at the top or bottom of the lane, then click and drag the lane to skew it to the desired position. Click the **Move Path** button again to exit the editing mode. The integrated area of each band will be updated in the Data Table as the curve is refigured to match the pixels surrounding the centroid mark.

Add Band

This option allows bands to be added. To add a band, select the **Add Band** button and place the mouse on the image. A plus sign cursor will display on the image. Place the cursor on the band of interest, then click the mouse to add the band. Continue until all new bands are added. Click the **Add Band** button again to exit the editing mode. The Data Table will readjust accordingly, and results for the new bands will be displayed.

Delete Bands

This option allows bands to be deleted. To delete a band, select the **Delete Band** button and place the mouse on the image. A cursor will display on the image. Place the cursor on the band of interest, then click the mouse to delete it. Continue until all bands of interest are deleted. Click the **Delete Band** button again to exit the editing mode. To delete bands when not in editing mode, simply click on the band and select the Delete key on the keyboard. The Data Table will readjust accordingly, and results for the deleted bands will be removed.

Band Filter - Adjusting Peak Detection Parameters

The parameters in the **Band Filter** box on the **Bands tab** allow adjustment of the automatic peak finder parameters.

***Figure 5-50:** Band Filter Parameters*

There are three attributes that determine whether or not the automatic peak finder recognizes a region of the graph as a peak: **Minimum Area**, **Minimum Height** and **Minimum Width**. To apply minimum peak find attributes to the analyzed image, select the appropriate checkbox. Either enter a value or click on the arrow(s) to adjust the setting. If a value is entered manually, hit the Enter key on the keyboard to apply the changes. If a minimum peak find attribute will not be used, deselect its checkbox.

As each setting is adjusted, the automatic peak finder rescans the graph and defines the peaks based on the new settings. A peak is recognized only if it meets the criteria defined in the minimum peak attribute settings.

*NOTE: Using the **Minimum Width** control setting is the quickest way to regulate the number of peaks detected by the automatic peak finder.*

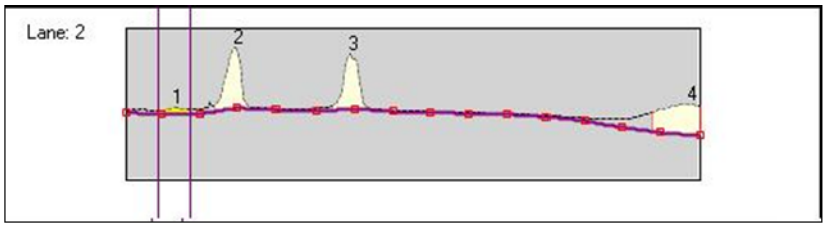


***Figure 5-51:** Minimum Width is 10*

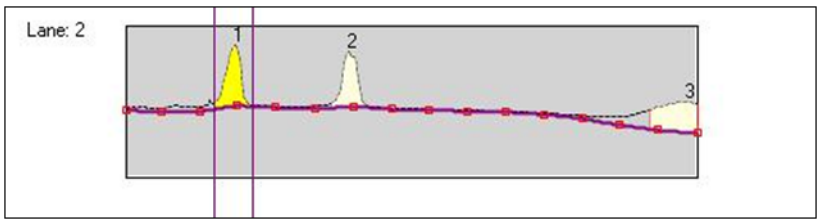


***Figure 5-52:** Minimum Width is 17*

Display Options

These features allow the user to change the visual display of the lanes and bands on the analyzed image. To access the dialog box select the **Display Options** button from the **Bands tab**.

- The Lane Property box provides the following options:
  - Show Center Line: displays the vertical line which runs through the center of each of the bands.
  - Show Lane Boundary: displays the perimeter of each of the lanes found in the gel (selected by default).
  - Show Profile Boundary: displays the profile path width in each of the lanes (selected by default).
  - Names: the selected radio button will display the lane name by number (Numerical) or by letter (Character).
- Under Band Representation, the Cross selection designates bands with a cross symbol. The Box selection will display a two-dimensional box around each of the bands.
- Show Band Properties turns the band property display on/off. The Band Property value to be displayed is selected under Band Property.

- The Band Name Property box allows the user to select what value to display as the bands property. The choices are Position (from top to bottom) in Numerals, Position (from top to bottom) in Characters, Relative Front, Adjusted Relative Front, Molecular Weight Standard or Molecular Weight Calculated.

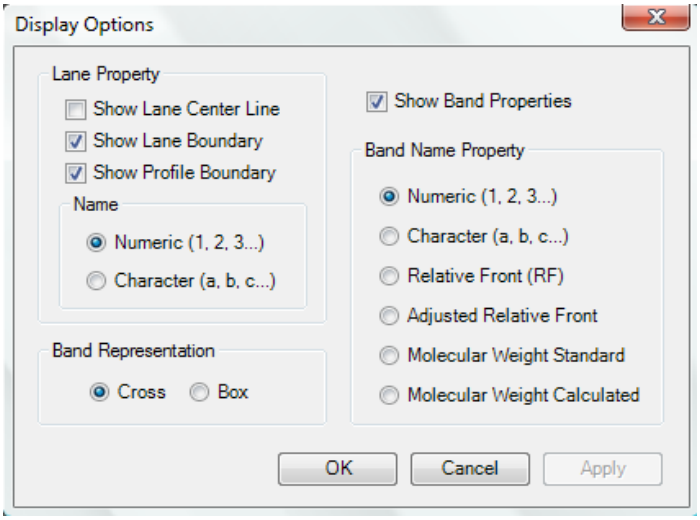


Figure 5-53: Display Options

Gel Smiling Correction

Options on the **Curves tab** allows the user to adjust for gel smiling or curvature. Using these tools will increase the accuracy of molecular weight calculations and automatic band matching. After gel smiling adjustments have been applied, a new column of data will be generated with an Adjusted R_f. The R_f values reported are relative values from 0 to 1 where 0 is the origin and 1 is the lane front without correction for gel smiling as commonly occurs in gel electrophoresis. The Adjusted R_f is the corrected value.

Suggestions

The software calculates Adjusted R_f based on the band's position relative to the nearest gel smiling curve. When the band lies between two curves with different curvatures, a weighting is applied where greater weight is given to the nearest gel smiling curve. For the most accurate calculations, enough curves should be added to properly mimic the curvature of the sample. We also suggest placing at least one curve above the top band and below the bottom band in a lane. If these curves are added, the software will use the lane origin and front as the first and last curves which are assumed to be straight.

Applying Gel Smiling Curves

After identifying and editing any lanes or bands, access the gel smiling tools by clicking on the **Curves tab**.

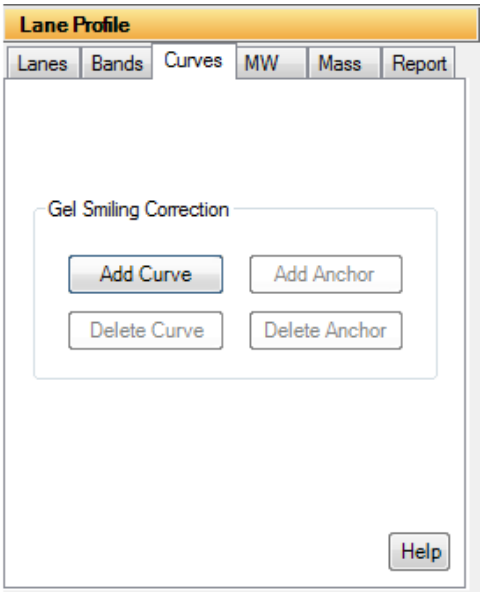


***Figure 5-54:** Curves Tab*

Add Curve

Select the **Add Curve** button and then click on the desired area of the image to add a curve line. The curve line will have three anchor points: one will be placed at the position of the mouse click on the image and the other two will be on either end of the image boundary. Click the **Add Curve** button again to exit the editing mode.

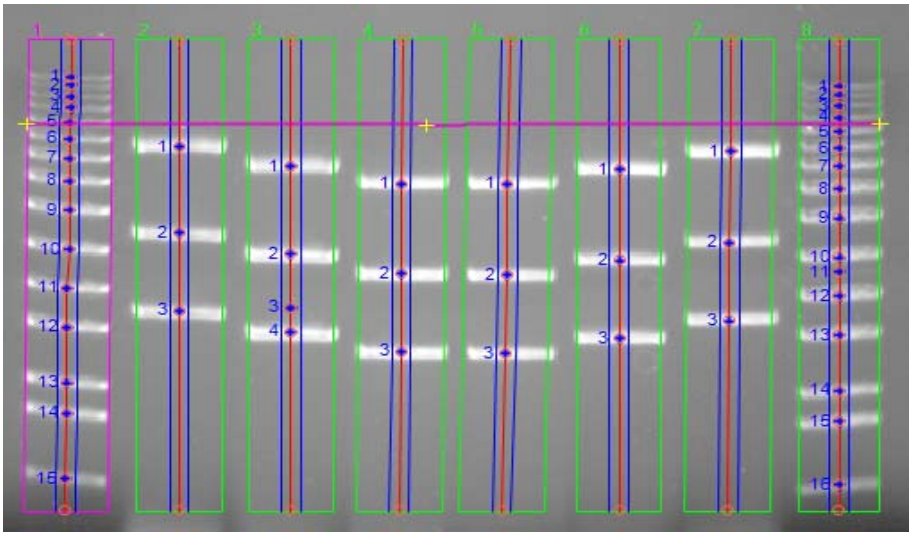


Figure 5-55: Curve Line Added to the Image

To create the curve, click on any of the three yellow plus sign (+) anchors on the curve line and drag it to the desired position. When an anchor has been selected, it will change from a plus sign to a square.

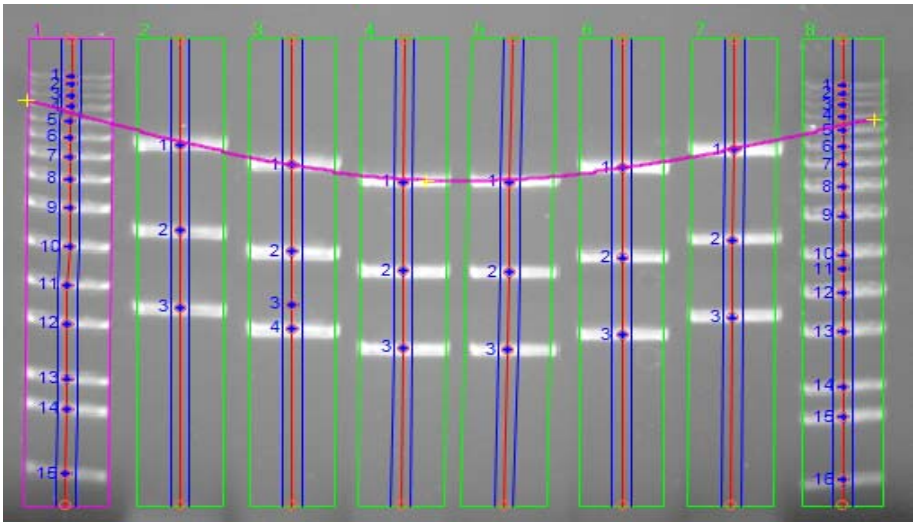


Figure 5-56: Curve After Using Anchors to Adjust the Line

Once a curve has been added, the Adj. R_f values will update in the Data Table, and the following other Gel Smiling options are enabled:

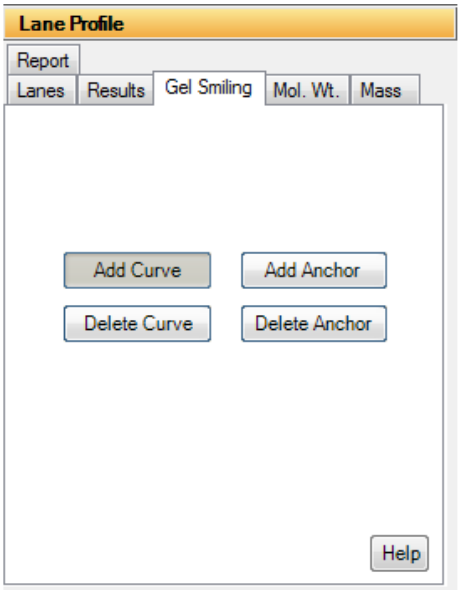


***Figure 5-57:** Curve Options*

Adding and Deleting Anchors

Anchor points can be added by selecting the **Add Anchor** button. Roll the mouse to the area of the curve where the new anchor should be placed. The cursor will change to a plus sign in areas where an anchor can be added. Click on the curve to add the anchor. Click the **Add Anchor** button again to exit the editing mode.

Additionally, anchor points can be deleted by selecting the **Delete Anchor** button. Click once on an existing anchor to delete it. Click the **Delete Anchor** button again to exit the editing mode. To delete anchors when not in editing mode, simply click on the anchor and select the Delete key on the keyboard.

Delete Curve

A curve can be deleted by selecting the **Delete Curve** button. Click on an existing curve to delete it. Click the **Delete Curve** button again to exit the editing mode. To delete curves when not in editing mode, simply click on the curve and select the Delete key on the keyboard.

Additional Gel Smiling Curves

Add enough curves to properly mimic the curvature of the sample. The more curves applied, the more accurate calculations will be for molecular weight and band matching. After the initial curve is applied the software will attempt to mimic the curvature of the surrounding curves when new curves are applied. This results in less editing of the additional curves.

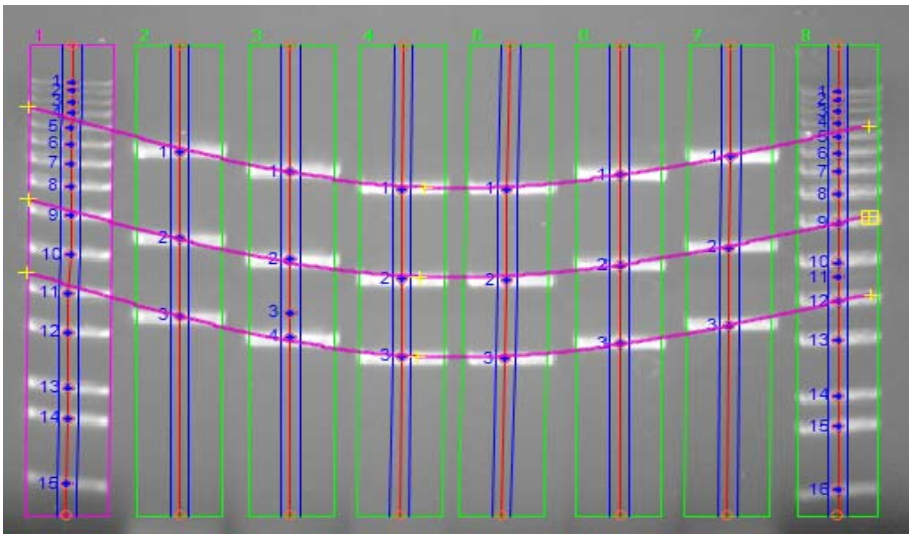


Figure 5-58: Adding Gel Smiling Curves

Molecular Weight Tab

AlphaView supports molecular weight calculations based on molecular weight standards. Click on the **MW tab** to enter standard values.

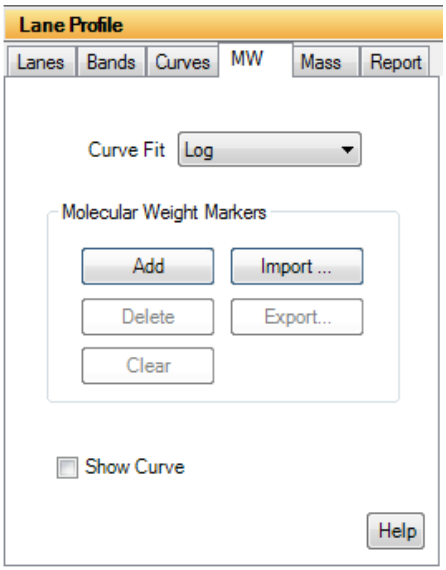


Figure 5-59: MW Tab

Curve Fit

From the **Curve Fit** drop-down menu, select the molecular weight standards curve fitting option. The fit options are: log, linear, point to point fit, cubic spline and quadratic.

Import

To apply an existing set of molecular weight standard values select the **Import** button and select the desired *.mwm file.

NOTE: A set of preconfigured molecular weight standards files (.mwm files) are installed with AlphaView software.*

The molecular weight standards file can either be auto loaded or manually loaded.

Auto Load requires the user to select a lane to apply the molecular weight standard file. After the *.mwm file is selected, select YES to **Auto Load**. Roll the mouse over the image, the cursor will be a yellow line. When the yellow line is over the desired standard lane, click on the lane. AlphaView will automatically identify each band in the lane and associate it with a molecular weight standard in the imported file.

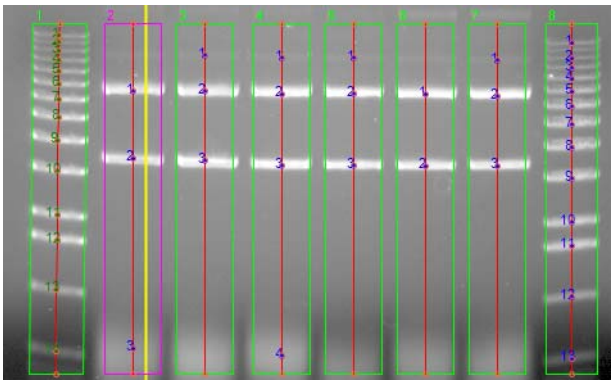


***Figure 5-60:** Molecular Weight Auto Load*

Manual load requires the individual bands in a lane to be correlated manually with the standards in the imported file. After the *.mwm file is selected, select NO for **Manual Load**. Move the cursor to the first band of interest in a lane and click. Text at the top of the image will indicate how many molecular weight standards are yet to be identified with a band selection, and what the molecular weight is. Continue to click on bands to identify molecular weight standards until all have been identified.

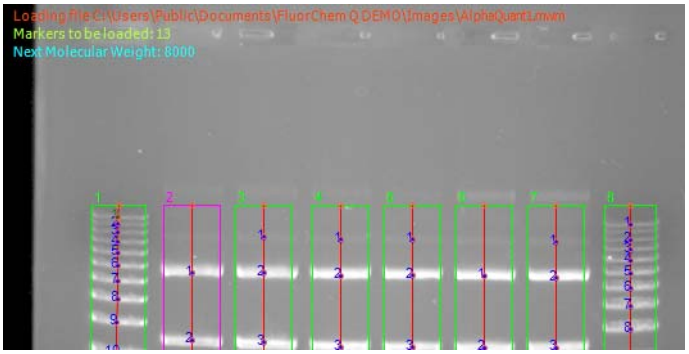


Figure 5-61: Molecular Weight Manual Load

Add Molecular Weight Marker

To identify a band as a molecular weight marker, select the **Add** button. Click on the first band in the known standard lane of the image. Enter the marker weight in the dialog box, then click OK.

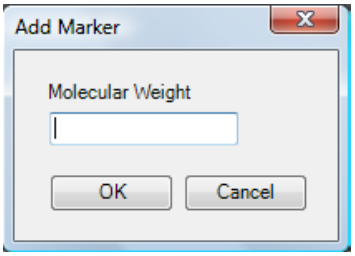


Figure 5-62: Add Molecular Weight Dialog Box

Continue adding markers until all bands are identified. Click the **Add** button again to exit the editing mode.

Delete Molecular Weight Marker

To delete a molecular weight marker, select the **Delete** button. Move the cursor to the band in the image and click to delete it. Click the **Delete** button again to exit the editing mode.

Clear Molecular Weight Markers

To clear all molecular weight markers in a lane, select the **Clear** button. Roll the mouse over the image. The cursor will be a yellow line. When the yellow line is over the desired lane, click on the lane. Once this is done, all molecular weight marker information in the lane will be removed. Click the **Clear** button again to exit the editing mode.

Molecular Weight Standards Results Display

After molecular weight standards have been identified, they will be color coded green in the Data Table. The curve fit used to calculate results will also be displayed below the Data Table.

Lane	Peak	Distance	Width	Height	Area	%	Rf	Adj. Rf	MW Std
1	1 s	35	8	28	104	2.07	0.1045	0.1045	10,000.00
1	2 s	40	7	15	61	1.23	0.1194	0.1194	8,000.00
1	3 s	47	7	20	62	1.25	0.1403	0.1403	6,000.00

MW based on Standard Sample Value: Quadratic

***Figure 5-63:** Molecular Weight Standards Results Display*

The calculated molecular weight for each band in the sample will now be displayed in the **Calc MW** column in the Data Table:

Lane	Peak	Distance	Width	Height	Area	%	Rf	Adj. Rf	MW Std	Calc MW
2	1	52	17	149	1142	45.62	0.1905	0.1905		3,401.29
2	2	105	19	128	995	39.75	0.3846	0.3846		1,542.73
2	3	253	31	15	369	14.62	0.9267	0.9267		169.63

***Figure 5-64:** Calculated Sample Molecular Weight*

Export

The set of molecular weight standards used for analysis can be saved for future use. To do this, click the **Export** button. A save dialog box will appear. Enter the name of the file and change the directory location if desired. If there are molecular weight standards in more than one lane, a lane must first be selected. To do this, roll the mouse over the image and move the yellow line cursor to the lane that contains the markers of interest. Click on the lane and save the file.

Show Molecular Weight Standard Curve

Select the **Show Curve** checkbox to display the molecular weight standards curve as calculated by the software. When this box is checked, the plot will be displayed in the lower right hand corner of the software window.

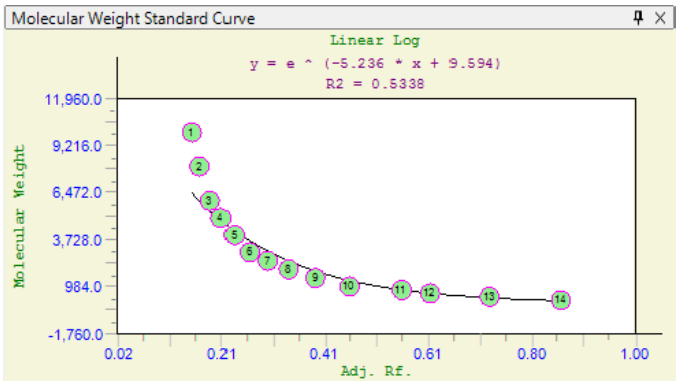


***Figure 5-65:** Molecular Weight Standard Curve*

Mass Standards

AlphaView supports mass calculations based on mass standards. Click on the **Mass** tab to enter individual mass standards or apply the total mass for a molecular ladder.

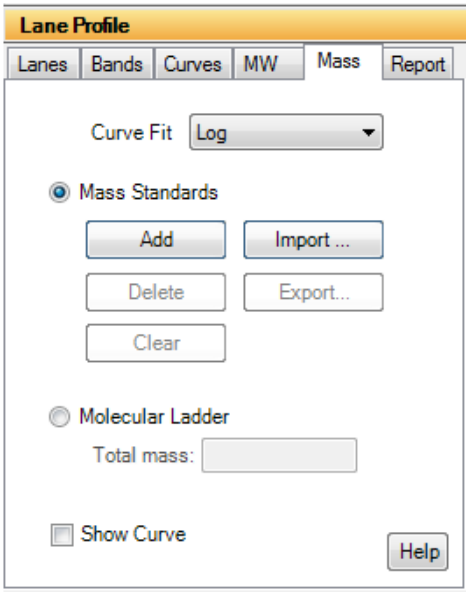


***Figure 5-66:** Mass Standards Tab*

Curve Fit

From the **Curve Fit** drop-down menu, select the mass standards curve fitting option. The fit options are: log, linear, point to point fit, cubic spline and quadratic. Curve fits must be specified regardless if the Mass Standard or Molecular Ladder option is selected.

Using Mass Standards

To use mass standards, select the **Mass Standards** radio button on the **Mass** tab. The following options become active.

Import

To apply an existing set of mass standards, select **Import** then select the desired *.msm file.

NOTE: A set of preconfigured mass standard standards files (.msm files) are installed with AlphaView software.*

The mass standards file can either be auto loaded or manually loaded.

Auto Load requires the user to select a lane to apply the mass standard file to a lane. After the *.msm file is selected, select YES to **Auto Load**. Roll the mouse over the image, the cursor will be a yellow line. When the yellow line is over the desired standard lane, click on the lane. AlphaView will automatically identify each band in the lane and associate it with a mass standard in the imported file.

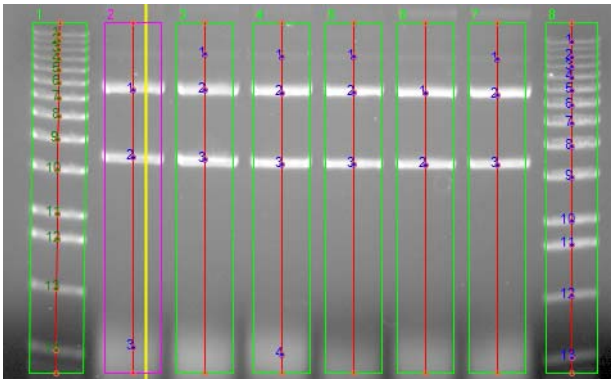


Figure 5-67: Mass Standard Auto Load

Manual load requires the individual bands in a lane to be correlated manually with the standards in the imported file. After the *.msm file is selected, select NO for **Manual Load**. Move the cursor to the first band of interest in a lane and click. Text at the top of the image will indicate how many mass standards are yet to be identified with a band selection, and what the mass is. Continue to click on bands to identify mass standards until all have been identified.

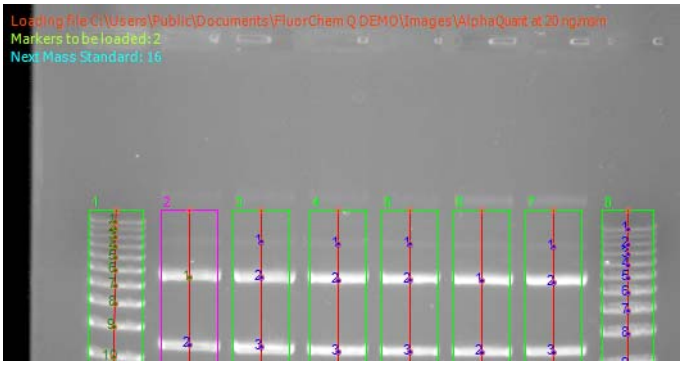


Figure 5-68: Mass Standard Manual Load

Add Mass Standard Marker

To identify a band as a mass standard, select the **Add** button. Click on the first band in the desired lane of the image. Enter the mass in the dialog box, then click OK.

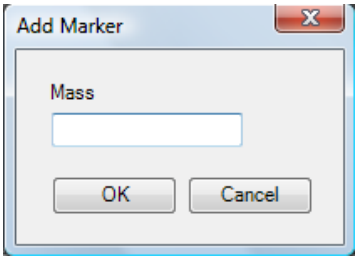


***Figure 5-69:** Add Mass Standard Dialog Box*

Continue adding standards until all bands are identified. Click the **Add** button again to exit the editing mode.

Delete Mass Standard Marker

To delete a mass standard, select the **Delete** button. Move the cursor to the band in the image and click to delete it. Click the **Delete** button again to exit the editing mode.

Clear Mass Standard Markers

To clear all mass standards in the image, select the **Clear** button. This will delete all markers in the image.

Export

The set of mass standards used for analysis can be saved for future use. To do this, click the **Export** button. A save dialog box will appear. Enter the name of the file and change the directory location if desired. If there are mass standards in more than one lane, a lane must first be selected. To do this, roll the mouse over the image and move the yellow line cursor to the lane that contains the markers of interest. Click on the lane and save the file.

Using a Molecular Ladder

To use a molecular ladder, select the **Molecular Ladder** radio button on the **Mass** tab. Enter the total mass that was loaded into the molecular weight standard lane. Next, roll the mouse over the image, the cursor will be a yellow line. When the yellow line is over the desired ladder lane, click on the lane.

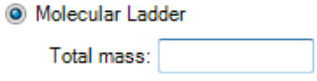
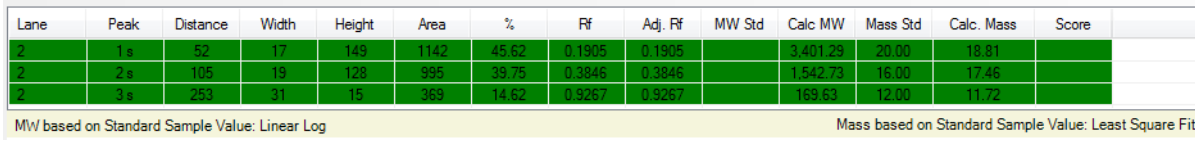


***Figure 5-70:** Molecular Ladder Total Mass Entry*

Mass Standards Results Display

After mass standards have been identified, they will be color coded green in the Data Table. The curve fit used to calculate results will also be displayed below the Data Table.



Lane	Peak	Distance	Width	Height	Area	%	Rf	Adj. Rf	MW Std	Calc MW	Mass Std	Calc. Mass	Score
2	1 s	52	17	149	1142	45.62	0.1905	0.1905		3,401.29	20.00	18.81	
2	2 s	105	19	128	995	39.75	0.3846	0.3846		1,542.73	16.00	17.46	
2	3 s	253	31	15	369	14.62	0.9267	0.9267		169.63	12.00	11.72	

MW based on Standard Sample Value: Linear Log Mass based on Standard Sample Value: Least Square Fit

***Figure 5-71:** Mass Standards Results Display*

The calculated mass for each band in the sample will now be displayed in the **Calc. Mass** column in the Data Table:

Lane	Peak	Distance	Width	Height	Area	%	Rf	Adj. Rf	MW Std	Calc MW	Mass Std	Calc. Mass
3	1	24	13	10	72	2.59	0.0879	0.0879		5,164.61		9.00
3	2	52	18	154	1297	45.83	0.1905	0.1905		3,401.29		20.24
3	3	106	23	174	1464	51.58	0.3883	0.3883		1,519.89		21.77

***Figure 5-72:** Calculated Sample Mass*

Show Mass Standards Curve

Select the **Show Curve** checkbox to display the mass standards curve as calculated by the software. When this box is checked, the plot will be displayed in the lower right hand corner of the software window.

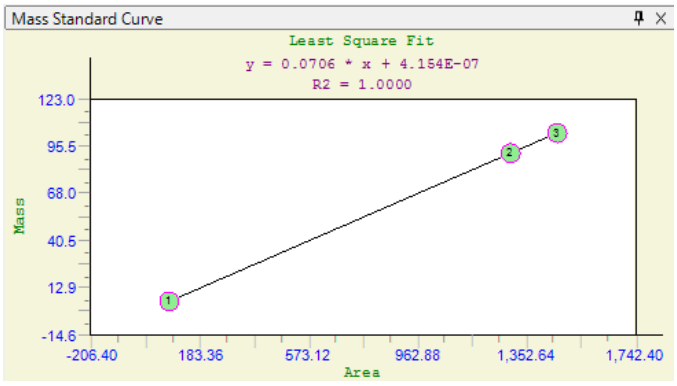


***Figure 5-73:** Mass Standard Curve*

Advanced Lane Profile Options

Lane Profile also includes two advanced options: **Band Scoring** and **Band Matching**. To view these options, they must be selected in **Preferences**. To do this, select **Setup** and **Preferences** from the **Main Menu.** Go to the **Analysis Tools tab** and select the **Lane Profile - Advanced** checkbox and click **Apply**.

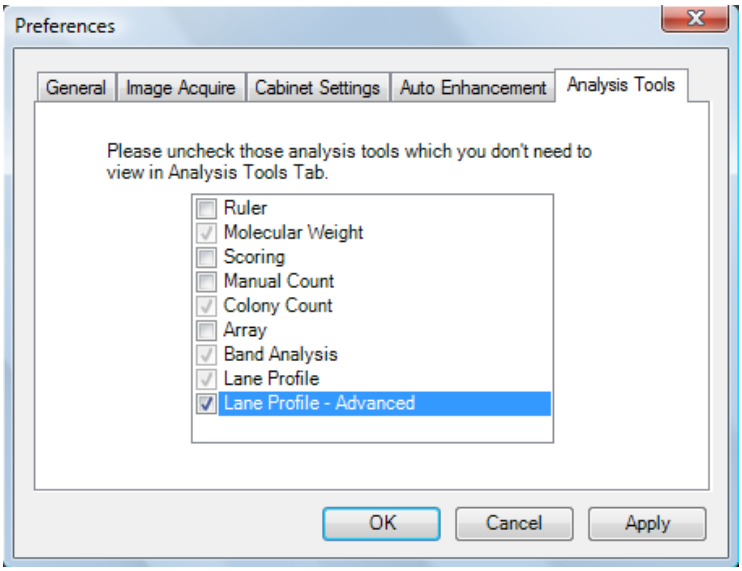


***Figure 5-74:** Preferences Analysis Tools Tab*

Lane Profile will now display two new tabs for each of the advanced options.

Band Scoring Tab

To perform band scoring, select the **Scoring** tab.

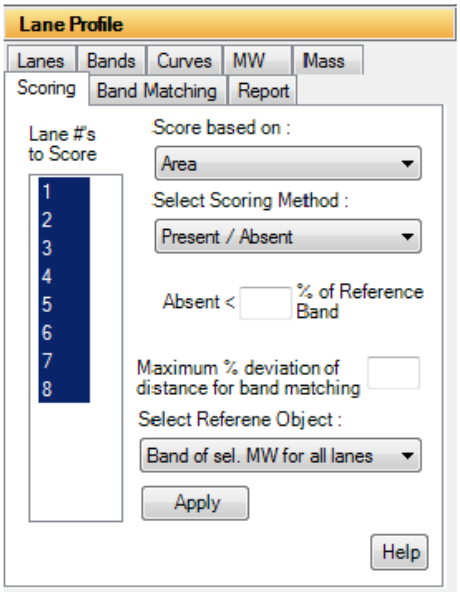


Figure 5-75: Band Scoring Tab

Lane #'s to score: Choose the lanes to score by highlighting the lanes to be included.

Score based on: Select the parameter to base the scoring on. The choices are Area, Mass, and Height. The Area and Height are calculated automatically in Lane Profile. In order to use the Mass as the scoring parameter the user must calculate the Mass of every band by applying a Mass Standard. This can be done separately or in conjunction with MW calculations.

Select Scoring Method: Select the scoring method of interest. The choices are: Present/Absent; High/Medium/Low/Absent; % of control; and Quantity.

- Present/Absent: this method is used as a qualitative measurement for review. The researcher is interested in whether or not a lane has a specific band of interest based on some criteria (area, mass, or height).
- Additional Criteria: When choosing Present/Absent as the scoring method the user must enter a value for determining the presence or absence. The value is a percent. When the value of an unknown band falls below that percentage value it is classified as Absent, when it exceeds the value it is classified as Present.
- High/Medium/Low/Absent: This method is used to classify bands into groups based on Area, Mass, or Height. The researcher will determine break points for each classification (high, medium, low, and absent). The software will place each band into one of these classifications depending on the user entered criteria.

- Additional Criteria: When choosing the High/Medium/Low/Absent method the user must enter in values to use as break points for each of these classifications. The values entered are percentages of the reference band based on the parameter chosen in the "score based on" field.

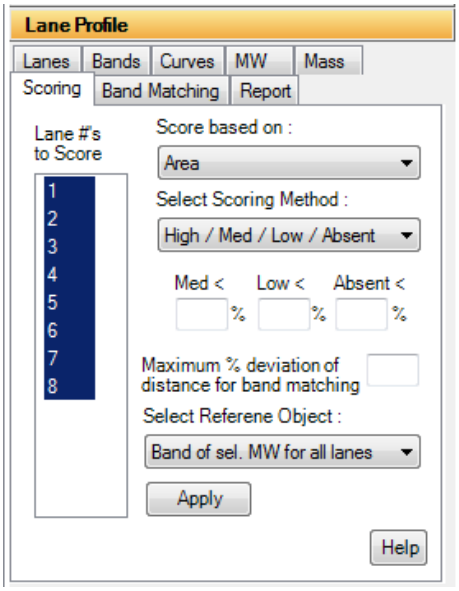


***Figure 5-76:** Band Scoring - Select Scoring Method*

Band Matching, Similarity Matrix, and Dendrograms

AlphaView provides features for matching bands between lanes. The resultant data can then be used to generate similarity matrices and/or dendrograms. Band matching features are accessible under the Analysis menu within Lane Profile's Auto Lane feature.

Prerequisites for Band Matching

Prior to performing band matching, a number of steps must be followed:

1. Lanes and Bands must be detected and edited through the Auto Lane feature.
2. If Molecular Weights are to be calculated, molecular weight markers must be loaded prior to band matching. For more information, please refer to "Molecular Weight Tab" on page 161.
3. (Optional) A new Gel Smiling tool has been added to AlphaView. This tool allows the user to adjust the software's calculations for M.W., Adj. R_f, and Band Matching by drawing the shape of the gel smile or curvature. Applying the Gel Smiling tool will aid in the accuracy of automatically matching bands and will result in less user edits afterwards. Refer to the section on Gel Smiling tool for further instructions.

Band Matching

Band Matching dialog window is accessible through the Analysis menu on the Auto Lane's Profile and Data window. Select Band Matching from the Analysis menu.

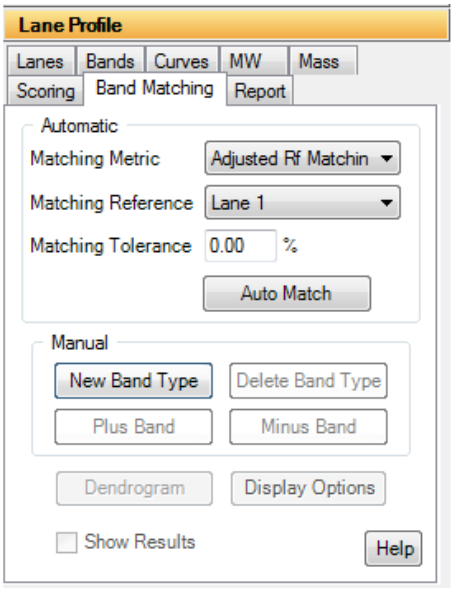


***Figure 5-77:** Band Matching Tab*

Functionality is provided for both Automatic and Manual band matching. Each will be explained in detail.

NOTE: These are not exclusive. After Automatic Band Matching has been performed the user is able to use the Manual matching features to edit or correct the automatic findings.

Automatic Band Matching

Matching Metric

Select either Adjusted R_f Matching or Generic R_f Matching as the matching metric.

- **Adjusted R_f Matching**: When Adjusted R_f Matching is selected the software will match bands based on the data values in the Adjusted R_f column of the Profile and Data window. These values result from the gel smile/curvature rather than the absolute vertical R_f value. Adjusted R_f is calculated based on the addition of M.W. markers and/or the R_f Curve lines which serve to correct the Generic R_f values for gel smiling.

- **Generic R_f Matching**: When Generic R_f Matching is selected the software will match bands based on the data values in the R_f column of the Profile and Data window. These values are the relative R_f calculated vertically where the origin of the lane is set to zero and the lane front is set to 1. Gel smiling or curvature is not adjusted for in this selection.

Matching Reference

Select a lane to serve as the matching reference. It is advised that you choose a lane that either contains most of the bands of interest or spans the range of bands of interest while also containing many of the bands of interest. This will reduce the amount of effort required for post match edits.

Matching Tolerance

Enter a value to be used as the matching tolerance. The tolerance is converted into the amount of displacement two bands may have and still be matched by the software. The larger the tolerance value the larger the range the software will use for matching. The valid range is 0-10%. A value of 1-5% typically works best. A lower percentage works well for tightly spaced bands.

Auto Match

After selecting a matching metric, reference, and tolerance click the Auto Match button for the software to perform auto matching. When matching has been completed the Display Results and Generate Dendrogram buttons will become active. Refer to the appropriate sections below for a description of Similarity Results and Dendrogram creation.

Editing Match Results

Following Automatic band matching the user may need to manually edit or correct some matches. The tools under Manual Matching can be used for this purpose. Refer to the section below on Manual Matching for a description of each tool.

Manual Band Matching

Manual band matching tools are useful for quickly matching lanes with only a few bands or for manual edits and corrections to Automatic Band Matching results.

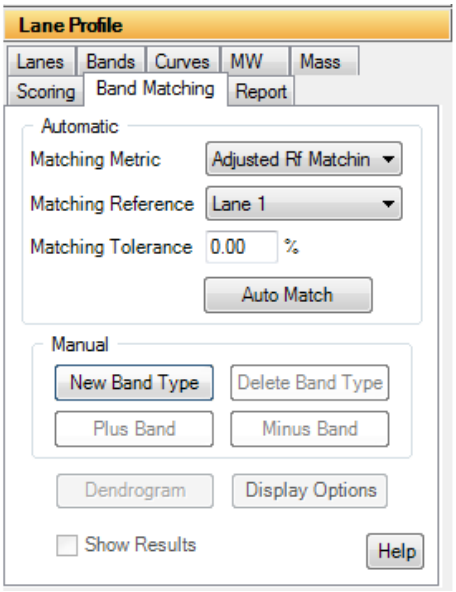


***Figure 5-78:** Band Matching Tab*

New Band Type

Select the New Band Type tool by clicking the New Band Type button. A Band Type is a band with a unique R_f. or Adj. R_f. Value. Each band within a lane will be part of a band type and no two bands within a lane can be of the same band type.

After selecting the New Band Type tool, click on a band to draw a band type line. Repeat this process of selecting the tool and clicking a band until a band for each unique band type has been selected.

Deleting a Band Type

To delete a band type, select the Delete Band Type button and click on a band type line.

Adding Bands to a Band Type

Select the Plus Band tool by clicking the Plus Band button. Click on a band type line to select it, the line will be highlighted in yellow. Now click on a band that belongs to this band type. Repeat this process for all bands belonging to this band type. Then repeat the process for each band type until all bands have been identified as belonging to a band type.

Removing Bands from a Band Type

Select the Minus Band tool by clicking on the Minus Band button. Click on a band type line to select it, the line will be highlighted in yellow. Now click on the band that you would like to remove from the selected band type.

Undo/Redo

The Undo and Redo buttons will undo and redo the last action performed respectively.

Band Matching Results—Display Results

After bands have been matched a table is generated with the matching results. Click the Display Results button to view the matching results.

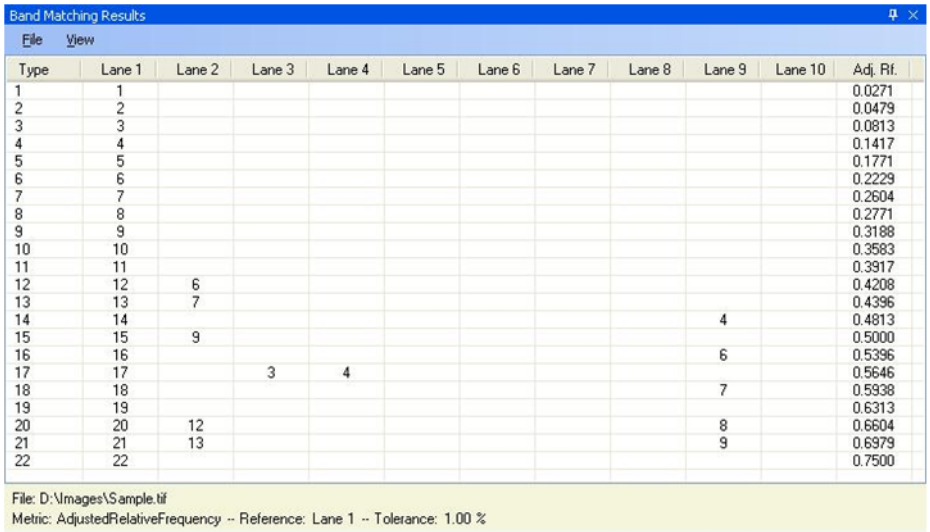


***Figure 5-79:** Band Matching Results Window Displaying the Similarity Matrix*

Viewing Result Tables

The match results can be viewed in one of four configurations. Select the configuration desired under the View menu. In each view Band Type is identified in the left most column. Band numbers appear in their R_f Position within each lane. Lane numbers are identified in the first row. The file location, matching metric, matching reference, and matching tolerance are displayed below the tables.

- **Mixed Table**—Displays both matched and unmatched bands in one table.
- **Separate Tables**—Displays matched and unmatched bands in separate tables.
- **Matched Table**—Displays only the results of matched bands.
- **Unmatched Table**—Displays only the results of unmatched bands.

Exporting and Printing Band Matching Results

Print and Export are selectable under the File menu. Select Print to send the report to a local or network printer. Select Export to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Dendrogram and Similarity Matrix Generation

After bands have been matched dendrograms and similarity matrices can be generated from the data. Click the Generate Dendrograms button to view these.

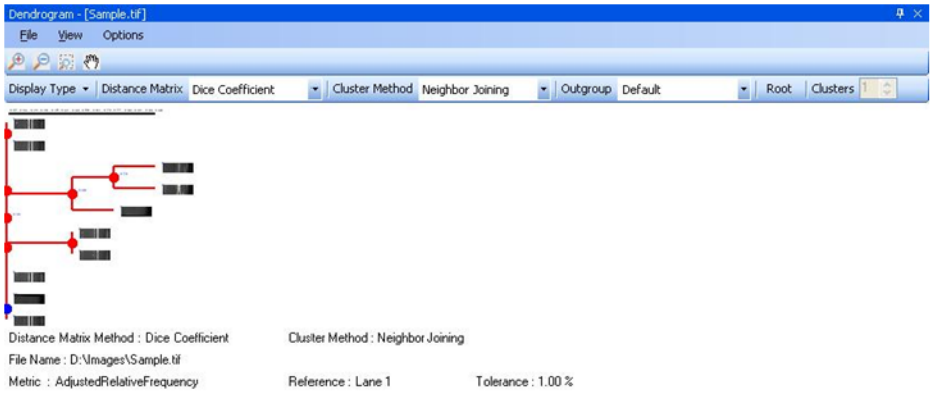


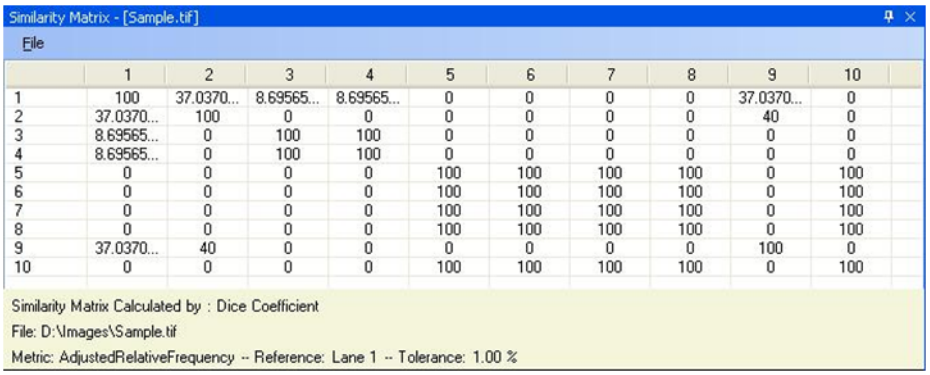
***Figure 5-80:** Dendrogram Window*

Dendrogram window where Dendrograms are generated and Similarity Matrices are accessed.

Similarity Matrix

Select a method from the Distance Matrix pull down for calculating similarity. The choices are Dice Coefficient, Jaccard Coefficient, Pearson Coefficient, and Frequency Similarity. These are standard statistical algorithms, references can be found in most statistical text books.

Select the Display Matrix option from the Options to display the Similarity Matrix.



	1	2	3	4	5	6	7	8	9	10
1	100	37.0370...	8.69565...	8.69565...	0	0	0	0	37.0370...	0
2	37.0370...	100	0	0	0	0	0	0	40	0
3	8.69565...	0	100	100	0	0	0	0	0	0
4	8.69565...	0	100	100	0	0	0	0	0	0
5	0	0	0	0	100	100	100	100	0	100
6	0	0	0	0	100	100	100	100	0	100
7	0	0	0	0	100	100	100	100	0	100
8	0	0	0	0	100	100	100	100	0	100
9	37.0370...	40	0	0	0	0	0	0	100	0
10	0	0	0	0	100	100	100	100	0	100

Figure 5-81: *Similarity Matrix*

The similarity matrix is a graphical display of the similarity between lanes.

Exporting and Printing the Similarity Matrix

Print and Export are selectable under the File menu. Select Print to send the report to a local or network printer. Select Export to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Dendrograms

The dendrogram window is accessed by clicking the Generate Dendrograms button in the Band Matching dialog.

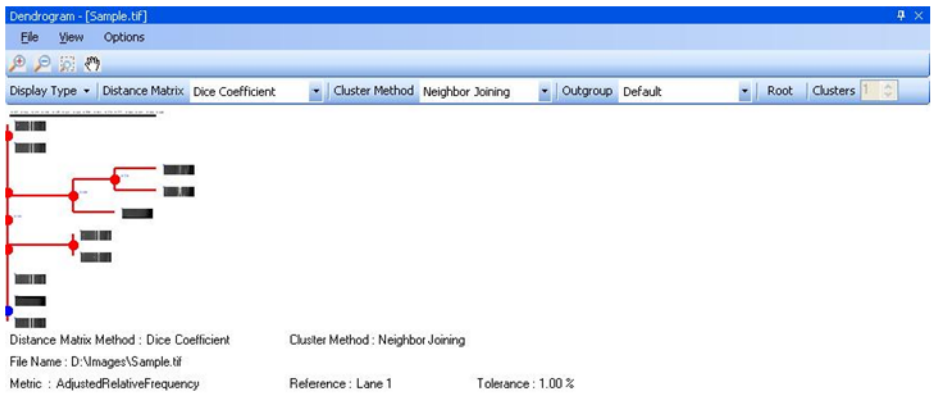


Figure 5-82: *Dendrograms*

Distance Matrix

Select a method from the Distance Matrix pull down for calculating similarity. The choices are Dice Coefficient, Jaccard Coefficient, Pearson Coefficient, and Frequency Similarity. These are standard statistical algorithms, references can be found in most statistical text books.

Cluster Method

Select a method for calculating the clustering from the Cluster Method pull down. The choices are Neighbor Joining, UPGMA, WPGMA, Single Linkage, Complete Linkage, Ward, Median, and Centroid. These are standard statistical algorithms, references can be found in most statistical text books.

Clusters

Select the number of clusters you would like displayed in the dendrogram. The values are 1 to maximum number of lanes in the image. This option is disabled when Neighbor Joining is chosen for the cluster method since the method determines the number of clusters.

OutGroup

When Neighbor Joining is chosen for the cluster method the OutGroup option is enabled allowing the user the ability to set the number of Out Groups to display.

Exporting and Printing the Dendrogram

Print and Export are selectable under the File menu. Select Print to send the report to a local or network printer. Select Export to export the results to the clipboard or to file. When exporting to a file there are two file types that can be saved, standard text file (*.txt) or a comma separated value file (*.csv). When exporting to Excel, use the *.csv file type to skip the Excel import wizard or use export to clipboard followed by Paste in Excel.

Display Type

Under the View menu the user may select to view the dendrogram in the Phylogram or Cladogram format.

Collapse/Expand

The Collapse/Expand tool allows the user to collapse or expand the dendrogram by clicking on a red intersection marker.

Zoom In/Out Tools

The zoom in and out tools allow zoom control over the dendrogram window.

Zoom with Selection Tool

The zoom with selection tool allows the user to zoom an area of interest within the dendrogram defined by mouse.

Grab Tool

The grab tool allows the user to navigate the entire dendrogram in a zoomed display by clicking and dragging to window.

Common Features

Default analysis tool has three common features namely, Protocol, Report, and Export Results.

Protocol

***Figure 5-83:** Protocol Tab*

The **Protocol** tab is used to save and load protocols for use on replicate blots.

Saving a protocol differs 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: An analysis may be saved at anytime by using the file drop down menu.

***Figure 5-84:** Saving an Analysis*

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.

Report

Using Report Tab, user can generate reports based on analysis data. Reports offers different options to customize the report, which can be saved on file as an Html Format (*.html) or Rich Text Format (*.rtf). User can also take print of report.

Reports also allows user to save report settings/configuration as template or can modify existing templates.

Formatting

Formatting tab allows user to format reports before saving or sending it to printer.

User can create new report or select existing report template to create a report using the drop down list. If no report template is defined/saved, it will be disabled.

Choose image that will be part of report with the image size. 1/4 is the default image size.

Also choose different information (Data sheet, Graph, Acquisition information) that will be part of report.

Report settings/configuration can be saved using Save button, in case of new or Save as in case of modifying an existing report template.

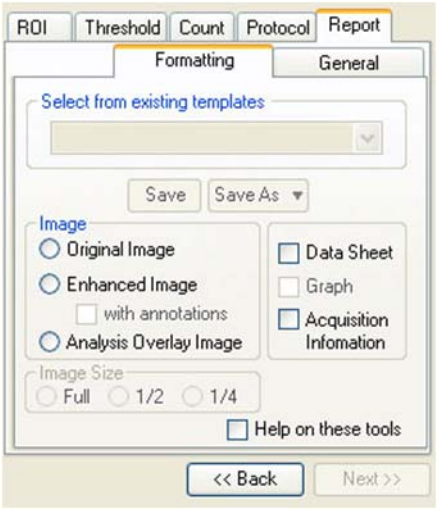


***Figure 5-85:** Report – Formatting Tab*

General

General tab allows user to give report a title, enter analysis notes / comments or give user name.

Clicking Generate Report will create a report based on report settings/configuration.

***Figure 5-86:** Report – General Tab*

Export Results

Once the analysis has completed then there is option to export the result. Either the results can print directly to a printer, or export the results as ASCII data for direct importation into Excel or other spreadsheet programs

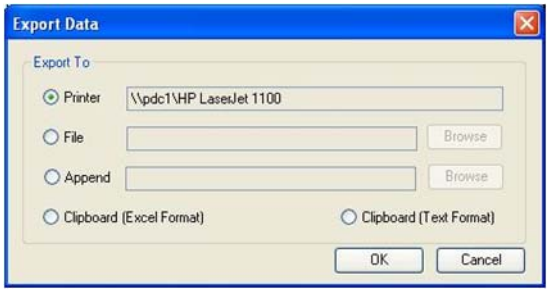


***Figure 5-87:** Common Export Dialog Box*

Sending Data to a Printer

To send the data directly to the Video Printer or Default Printer, click on the Export button and click in the circle next to appropriate printer.

Sending Data to a File

If you would like to take the data from the system and import it into another computer, the data can be saved to a diskette or network which will allow you to open the data file on a separate workstation connected to the network.

Click on the Export button and then on the File option. Specify the path and file name to send the data file. The data is saved as an ASCII file and can be imported into most spreadsheet programs. ASCII is a very common file format output option for numerical data.

Appending Data to a Same File

Export dialog also allows you to append data to an existing exported file.

NOTE: This feature is applicable to AutoCount and Array analysis modules.

Sending Data to a Spreadsheet Program

To send the data results to Excel or other spreadsheet programs, click on the Export button, click on the clipboard option, then click on OK. If you have Excel or another spreadsheet program loaded on the system and running in the background, you can simply press the ALT and TAB keys simultaneously to move into the spreadsheet program. You can then import the data directly to the desired spreadsheet from clipboard.

NOTE: The spreadsheet program must be installed on the computer in order to export the data to that program.

When the appropriate export source has been selected, click on the OK button to send the data.

Exporting Quantitative Data Lane Profile

The exporting of Lane Profile is similar to what has been defined above with some extra feature.

The Lane Profile has following window to export data.

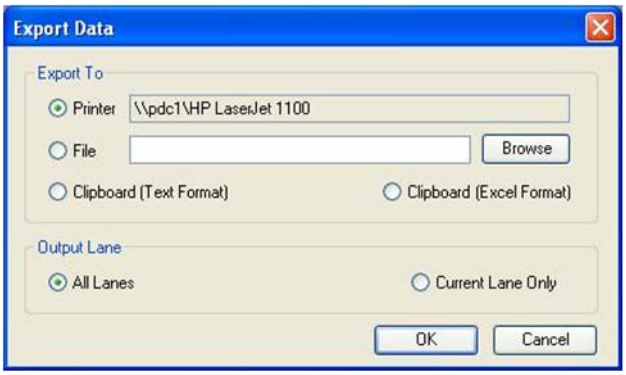


***Figure 5-88:** Lane Profile Export Dialog Box*

Output Lane

This option allows user to export what type of data to be exported. Selecting All Lanes will export all lanes data or selecting Current Lane will only export current lane data.

To send the data, click on the OK button.

Additional Analysis Tools

***Figure 5-89:** Additional Analysis Tools in the ToolBox*

Additional image analysis tools can be activated to show up under the tool box as shown here. To activate these tools:

1. Go to Setup> Preferences > Analysis tools tab.
2. Select tools to show.

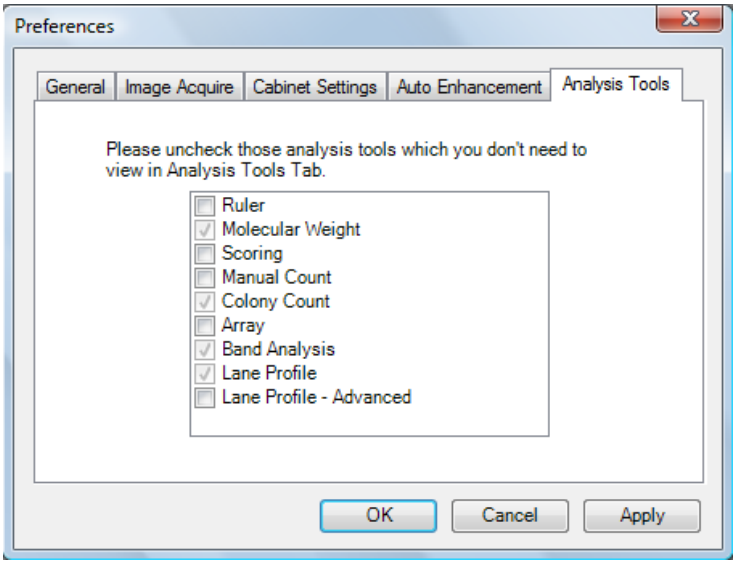


Figure 5-90: Preferences—Analysis Tools Tab

This tool set allows the user to perform ruler measurements, gel scoring, manual counting of colonies and cells, and perform high density array analysis.

The Ruler Function

The Ruler function allows the user to create a scale based on a standard and to measure distances from a set origin to a chosen location.

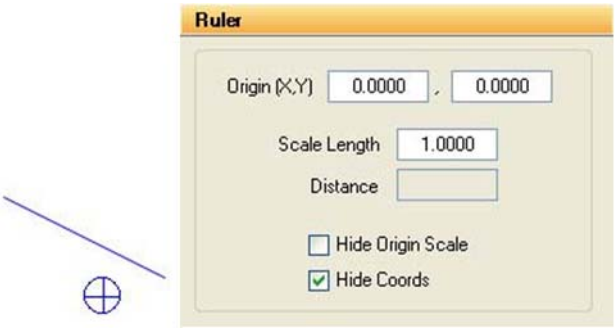


Figure 5-91: The Ruler Tools and Ruler Toolbox

Using the Ruler Function

To access the Ruler function, click on **ToolBox**, **Analysis Tools** and then on **Ruler**. A line with a box at each end and a circle with a cross in it will appear on the screen.

Before any measurements can be obtained, a standard scale must be established. The scale that is created will serve as the standard for all measurements. Therefore, it is advisable to draw the scale line according to some standard measurement within the image (such as a ruler).

Using the mouse, click and drag one end of the line and then the other end into position. Once the line is in position, click in the **Scale Length box** and type the length of the standard. Press <enter> to save this value.

Distance is measured from the origin (designated by the circle with a cross in it) to the mouse location. Place the origin marker appropriately, and then move the mouse. The distance will be constantly updated in the **Distance box**.

To hide the scale and origin, click on the **Hide Origin Scale checkbox**.

Cartesian Coordinates

The **Ruler** function can also be used to create a Cartesian coordinate system. The origin icon has default values (0.000, 0.000). These can be changed by clicking on the **Origin box**, typing in new values, and clicking <enter>.

Once the origin is placed on the screen in its desired location, the relative coordinates of the mouse at any location are shown at the right-hand side of the screen. In the example above, the cursor is 0.747 inches to the right of the origin and 1.071 inches below. To hide the coordinates, click on the **Hide Coordinates checkbox**.

The Scoring Function

The Scoring function is designed for gene expression applications to check for presence or absence of specific samples. The scoring function can be used for a quick manual identification of several different sample types. The software allows for up to three different scores on a gel, blot, or microtiter plate.

To score an image, click on the Scoring button in Toolbox 4. Define the appropriate number of rows and columns of your image by using the appropriate controls. You can also define the amount of spacing between your rows and columns to compensate for any variations between lanes or wells.

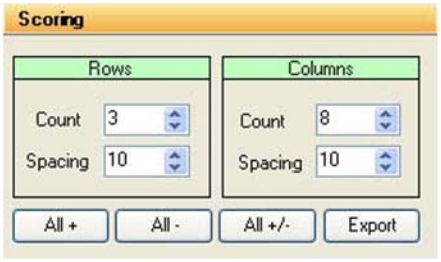


Figure 5-92: Scoring Controls

Once you have selected the number of rows and columns to be displayed, you may adjust the template to fit your image. Use the left button on the mouse to click, hold, and drag the outside borders of the template so they frame the lanes or wells to be scored. Clicking, holding, and dragging on or within any border moves the entire template. Clicking on the blue corners surrounding the template resizes it.

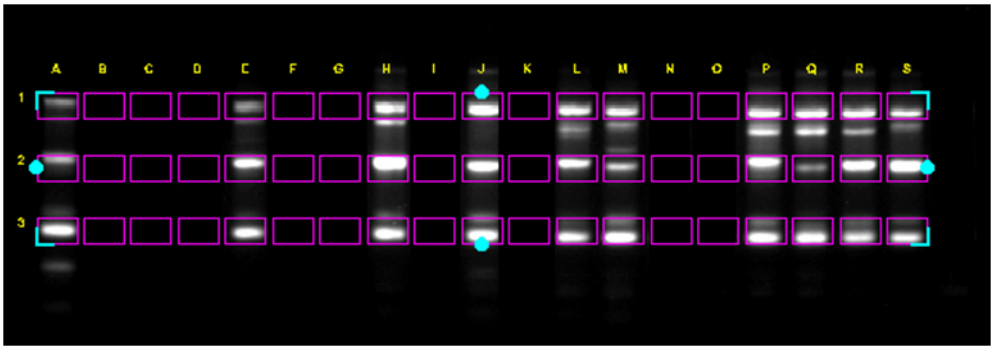


Figure 5-93: Scoring Image with Template Positioned

Scoring the Sample

Three different scoring options are available: score with a positive, negative, or positive / negative. The software will display an "X" if you have a positive sample or three "–" signs arranged in a vertical fashion if you score with a negative. Nothing will appear if you score with a positive/negative. You may use the right and left mouse buttons to determine different scores. The right mouse button will score the sample with an "X". The left mouse button will score the sample with three "-" signs vertically arranged.

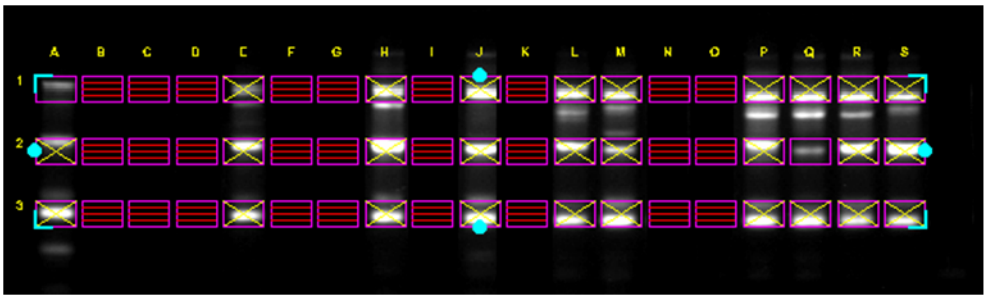


Figure 5-94: *Scoring for positives (+), negatives (-), and positive / negative (nothing)*

For faster scoring, you may look at the entire image and visually determine whether the samples are primarily positive, negative, or positive / negative. By clicking on the All +, All -, or All +/- boxes, all of the samples on the image will have either an "X", three "-", or nothing depending on the button you clicked.

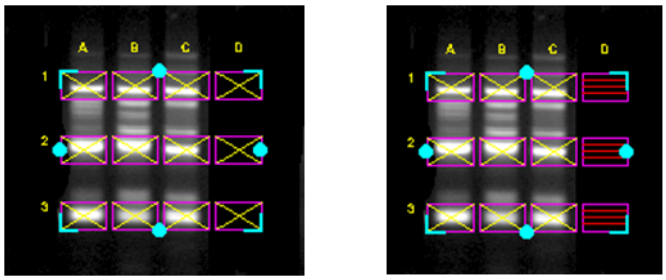


Figure 5-95: *Image after All+ button pressed vs. Image after All+ button Pressed and Individual Sample Scoring*

Figure 5-96: *Output and Scoring Options*

Manual Count

Click on **Manual Count**. The following sets of tools will be displayed in the lower left of the screen. Some samples are extremely difficult to count because of their shape, size, or lack of intensity variation from the background. To accurately count these samples, we have developed the manual counting feature within our software. To manually count the objects in an image, click on **Manual Count** in **Tool Box**, **Analysis Tools**. You may use either a green x or a red +. If you are only counting one type of sample, you may simply use the x which is the default to begin counting in the software.

Figure 5-97: Manual Count Tools

Placing Markers to Count

Point the cursor at an object to be counted and click the left mouse button. An "x" is placed over the object, ensuring that the same object will not be mistakenly counted twice. In the toolbox, the number in the **Count** box automatically increases by 1. Click on all of the objects of this type or color to be counted.

To count objects of another type or color (such as white vs. blue colonies in a β-gal assay), use right mouse button to click on each object as above.

Figure 5-98: Section of an Image After Manual Counting and Corresponding Data

Erasing and Hiding Count Markers

To erase a marker that has been manually placed, click on the marker on the image. It will disappear and the count will be reduced by one.

The Sensitivity indicator determines how close the cursor must be to a marker in order to delete it. Higher numbers allow the user to click further away from the marker to delete it; however, if Sensitivity is set too high, it will be impossible to place markers on spots that are close together.

Hiding Your Count Markers from the Screen

The software will record all counts as you continue clicking on your objects. If the counting becomes confusing, you may click on the **Hide** button to hide off of the x's and +'s that were used to count your objects on the screen. If you wish to make a print of your image hiding the count, make sure that the **Hide** button has been pressed to high your count markers. You can then go to **Tool Box**, **Enhancement Tools**, **Annotate**, and label the image with the appropriate. Click on the **Print** button to obtain a hardcopy.

Erasing the Count Markers and Data

Once you have finished counting your objects and have obtained the data you need, you may clear the count by clicking on the **Zero** button. This will erase all of the count markers from the screen and zero the count data to allow you to begin counting again with the same image or another image that you have acquired.

Analyzing Arrays

The **Array** analysis tools (found in the **ToolBox**, **Analysis Tools**) make it easy to measure the relative gray levels of objects in a uniformly spaced array, such as microtiter plate wells or dot blots.

These tools allow spot number, orientation and size to be specified. For microtiter plates, two options are available: measuring the density within a circle (well), or measuring a central spot and a surrounding torus (halo) separately.

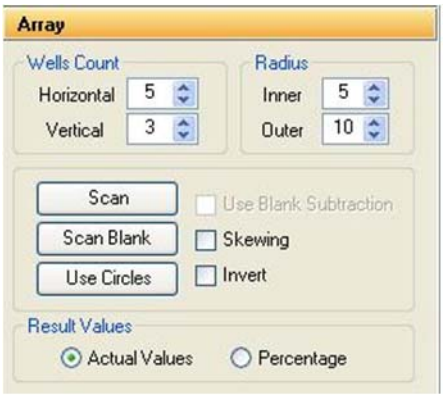


***Figure 5-99:** ARRAY Toolbox*

Once these parameters have been set, a template can be saved in a default file and recalled at a later time.

Setting Up an Array Template

To access the **Array** tools, open **ToolBox**, **Analysis Tools** and click on the **Array** button. A template is displayed on the image, depicting the layout of the objects to be measured. Up to 10,000 objects in a 100x100 array can be measured. (The default is an array of 96 circles, arranged in 8 rows of 12.)

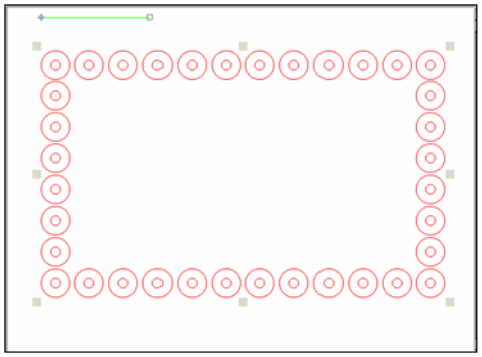


***Figure 5-100:** ARRAY Template*

The tool box work area displays a number of buttons and controls for specifying the number of objects, along with their orientation and sizes.

Analyze Arrays with Circles or Squares

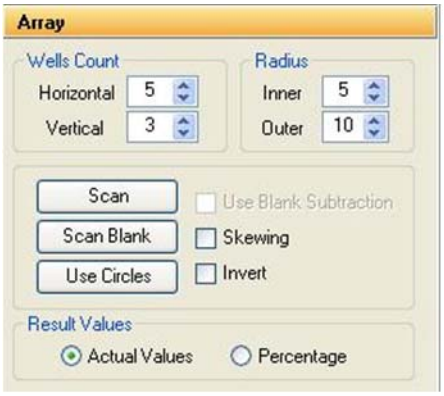


***Figure 5-101:** Array Dialog Box*

The controls labeled Hori. Wells and Ver. Wells let the number of horizontal and vertical objects be set (to a maximum of 100 and 100, respectively). The controls labeled Center and Outer let the size of the center and outer circles be set. Click on an arrow to increase or decrease the number of objects per row or column, or the size of the objects. As the number and size are adjusted, the template displayed over the image is updated to reflect the changes.

Aligning the Template

Once the number of objects has been set, adjust the placement of the template.

If the image of the sample is not parallel with the template, the template can be realigned by adjusting the green line so it is parallel to the top edge of the sample. Place the cursor on the box at either end of the line. Hold the left mouse button down and drag the line until it is parallel to the image.

Next, adjust the placement of the template so the objects coincide with the areas of interest on the image. Move the cursor to any of the square "handles" on the outside of the template. Hold down the left mouse button and drag the handles until the template is properly positioned.

If the image is not perfectly rectangular, the template can be skewed by clicking the Skewing checkbox.

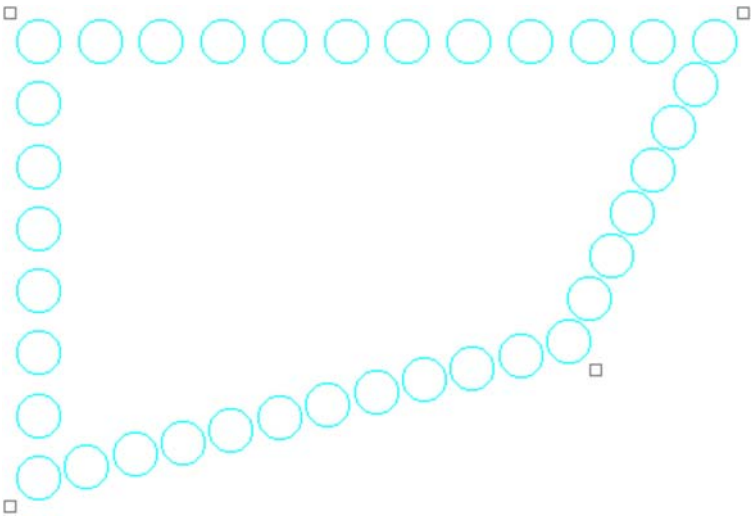


***Figure 5-102:** A Skewed Template*

Specifying the Areas to Be Measured

For microtiter plate analysis, the toolbox controls labeled Center and Outer specify the size of the inner circle and outer torus scanned to measure each well. The number in the center of each of these controls indicates the diameter of the circles. Click on an arrow to increase or decrease the diameters. As the number is adjusted, the objects on the template grow larger or smaller, reflecting the changes.

When specifying the size of the area to be measured, exclude shadows or other imaging artifacts. Depending upon the lighting conditions when the image was captured, there may be a crescent-shaped shadow obscuring part of the bottom of the wells, especially those near the edge of the microtiter plate. We recommend decreasing the size of the sampling area so that these regions are not included.

To measure the total density within each object, reduce the Center value to zero (0) and adjust the Outer value until the objects on the screen are slightly smaller than the areas of interest on the image. Reposition the corner objects using the mouse until the objects are properly aligned over the objects.

Measuring Density

Once the objects are sized and positioned correctly, click on the **Scan** button in the tool box. The density values will be calculated and displayed under each object.

The values are the average pixel values within the object, adjusted to a scale of 0 to 100. (0 corresponding to black pixel values, and 100 corresponding to white pixel values.)

If both a center and an outer circle have been specified, two values are displayed. The upper value corresponds to the inner circle and the lower value corresponds to the torus.

The Invert Box

The **Invert** function reverses the relative density assignments so that 0 corresponds to white and 100 corresponds to black. If the image has dark areas of interest on a light background, then **Invert** should be selected by placing an "X" in its box. (If the image has light areas of interest on a dark background, the **Invert** option should not be activated.) Unlike the **Reverse** option described in "The Reverse Button" on page 47, this function does not alter the appearance of the image.

Removing Background Using the Scan Blank Function

Since a microtiter plate is not completely transparent, it contributes to the density measured when samples are scanned. A non-uniform light source also affects the density measured. To ensure that the density measured is attributable only to the sample in the wells, scan a "blank" before scanning samples.

Load an image of a clean, unused microtiter plate and align the objects as described above. Once the array has been set up, click on the Scan Blank button. The average pixel gray value for each object (and torus) is measured and set to zero (0).

Now, when samples are scanned, the gray value of the blank is subtracted from that of the sample before it is scaled. This ensures that the density reported only reflects the sample in each well.

When Scan Blank has been selected, the background values obtained apply to all subsequent scans. To indicate that a background is being applied, the Scan Blank button changes to read Clear Blank. To turn off this background subtraction function, simply click on Clear Blank.

Lane Profile - Advanced

When the **Lane Profile - Advanced** checkbox is selected, **Band Scoring** and **Band Matching** tabs are added to the **Lane Profile** options in the **Analysis Tools tab** of the **Toolbox**. For complete details, please see "Advanced Lane Profile Options" on page 170.

Common Export Result Feature

Once the analysis has completed then there is option to export the result. Either the results can print directly to a printer, or export the results as ASCII data for direct importation into Excel or other spreadsheet programs

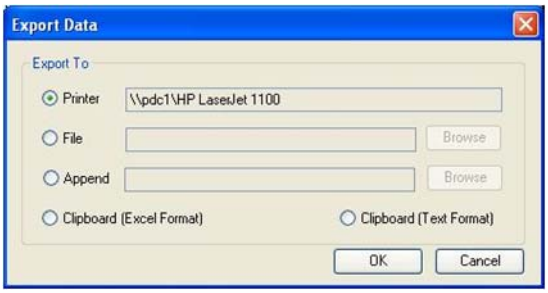


***Figure 5-103:** Common Export Dialog Box*

Sending Data to a Printer

To send the data directly to the Video Printer or Default Printer, click on the Export button and click in the circle next to appropriate printer.

Sending Data to a File

If you would like to take the data from the system and import it into another computer, the data can be saved to a diskette or network which will allow you to open the data file on a separate workstation connected to the network.

Click on the Export button and then on the File option. Specify the path and file name to send the data file. The data is saved as an ASCII file and can be imported into most spreadsheet programs. ASCII is a very common file format output option for numerical data.

Appending Data to a Same File

Export dialog also allows you to append data to an existing exported file.

NOTE: This feature is applicable to AutoCount and Array analysis modules.

Sending Data to a Spreadsheet Program

To send the data results to Excel or other spreadsheet programs, click on the Export button, click on the clipboard option, then click on OK. If you have Excel or another spreadsheet program loaded on the system and running in the background, you can simply press the ALT and TAB keys simultaneously to move into the spreadsheet program. You can then import the data directly to the desired spreadsheet from clipboard.

NOTE: The spreadsheet program must be installed on the computer in order to export the data to that program.

When the appropriate export source has been selected, click on the OK button to send the data.

Appendix A:

Opening AlphaView Files in Other Software Programs

Appendix Overview

- Viewing AlphaView Images
- Using ResEdit

Viewing AlphaView Images

Cell Biosciences generated files have been tested in the software packages below. For successful imports, the command line is given.

Programs for the Macintosh® operating system were tested on a PowerMac® 8100/100AV. Results may vary for different software versions and/or hardware configurations.

		.TIF	.BMP	.GIF	.MAC	.PCX
Adobe Photoshop	2.51 LE (Mac)	Open	no	Open	Open As/TIFF	no
Adobe Photoshop	3.0 (Mac)	no	Open	Open		
Canvas	3.5 (Mac)	use ResEdit*	no	no	use ResEdit*	no
Microsoft Word	6.0 (Mac)	Insert Picture	Insert Picture	no	Insert Picture	no
Microsoft Word	6.0a (Win)	Insert Picture	Insert Picture	no	Insert Picture	Insert Picture
Microsoft Excel	5.0 (Mac)	no	no	no	no	no
Microsoft Excel	5.0c (Win)	Insert Picture	Insert Picture	no	Insert Picture	Insert Picture
NIH Image	1.59 (Mac)	Open	no	no	Open	no

***Table A-1:** Viewing AlphaView Images - Programs and File Type Requirements. *For instructions on using ResEdit™, see next page.*

Additional packages, such as Claris Works and PowerPoint have also been tested. For these systems, it is necessary to save the file with a ".TIFF" extension in order for them to recognize the file as a TIF format.

If you have a specific software package that is not listed here, contact Cell Biosciences. This PC-formatted disk contains a gel image saved as five of the file types that AlphaView can generate. Try opening each of these files in your software package to determine compatibility. We recommend that you start with the .TIF file, as this is the default Cell Biosciences file format.

Using ResEdit

1. Save image in IS-1000/500 as a TIFF or MACTIFF.
2. Obtain a copy of the free ware ResEdit by downloading from Apple Computers through the Internet.
3. Open ResEdit.

   An animated startup display will show up and continue until you click on the mouse or any key.

A dialog box will appear.

4. Open the TIFF image.

   Another dialog box will appear asking if you want to add a resource fork.

5. Click OK.
6. Click File > Get Info for This File.
7. In the File Info window, change the Type to TIFF (instead of TEXT), and the Creator to DAD2 (instead of DOSA). (Must be typed in all CAPS as shown here).
8. Close the window and save changes.
9. Quit ResEdit.

After this procedure, the icon will change to a TIFF icon and the file may be opened in Canvas.

Appendix B:

AlphaView Molecular Weight Library Files

Appendix Overview

- List of Standards

List of Standards

A library including the following DNA, RNA and protein molecular weight standards has been incorporated into AlphaView.

Values of size standards are given in base pairs.

***Table B-1:** AlphaQuant Standards*

AlphaQuant 1	AlphaQuant 2	AlphaQuant 3	AlphaQuant 4	AlphaQuant 5	AlphaQuant 6	AlphaQuant 7
10000	2000	5000	1000	500	2000	5000
8000	1800	4500	800	400	1000	3000
6000	1600	4000	700	300	500	2000
5000	1400	3500	600	250	250	1000
4000	1200	3000	500	200	100	500
3000	1000	2500	400	175		
2500	800	2000	300	150		
2000	700	1500	200	125		
1500	600	1000	100	100		
1000	500	700		75		
800	400	500		50		
600	300			25		
400	200					
200	100					
	50					

Table B-2: DNA Sizing Standards

DNA Size Standards (in bp)								
HINDIII	PHIX174	BRL10BP	BRL50BP	BRL100BP	PRO100BP	BRL123BP	BRL1KB	BRLHIMW
23130	1353	200	500	1500	1500	4182	12216	48502
9416	1078	190	450	1400	1000	4059	11198	38416
6557	872	180	400	1300	900	3936	10180	33498
4361	603	170	350	1200	800	3813	9162	29942
2322	310	160	300	1100	700	3690	8144	24776
2027	281	150	250	1000	600	3567	7126	22621
564	271	140	200	900	500	3444	6108	19399
125	234	130	150	800	400	3321	5090	17057
	194	120	100	700	300	3198	4072	15004
	118	110	50	600	200	3075	3054	12220
	72	100		500	100	2952	2036	10086
		90		400		2829	1636	8612
		80		300		2706	1018	8271
		70		200		2583	517	
		60		100		2460	396	
		50				2337	344	
		40				2214	298	
		30				2091	220	
		20				1968	201	
		10				1845	154	
						1722	134	
						1599	75	
						1476		
						1353		
						1230		

DNA Size Standards (in bp)								
HINDIII	PHIX174	BRL10BP	BRL50BP	BRL100BP	PRO100BP	BRL123BP	BRL1KB	BRLHIMW
						1107		
						984		
						861		
						738		
						615		
						492		
						369		
						246		
						123		

Table B-3: *RNA and Protein Standards*

RNA Size Standards		Protein Markers (in kD)						
BRLRNA1	BRLRNA2	BRLPROT1	BRLPROT2	BRLPROT3	BRL10KD	NOVEXM12	SIG-MAHMW	SIG-MAPMW
1770	9490	87.0	200.0	43.0	200	200.0	205	190
1520	7460	60.0	97.4	29.0	120	116.3	116	108
1280	4400	53.0	68.0	18.4	110	97.4	97	89
780	2370	46.0	43.0	14.3	100	66.3	84	77
530	1350	40.0	29.0	6.2	90	55.4	66	61
400	240	34.0	18.4	3.0	80	36.5	55	41
280		29.0	14.3		70	31.0	45	36
155		27.0			60	21.5	36	
		21.0			50	14.4		
		15.0			40	6.0		
		6.5			30	3.5		
					20	2.5		
					10			

Appendix C:

Data Description Tables

Appendix Overview

- Single Channel Columns

Single Channel Columns

Region Tab	
Band	Band Identifying Number
Sum	Sum of all pixel gray levels in Band
Percentage (%)	Percentage Sum =(Sum/ Total of all Band Sums)*100
Area	Area of Band Region in pixels
Average	Average pixel level of Band = (Sum/Area)
X Y Position	XY position of Band Region centroid
SD Pixel Levels	Standard Deviation of pixel gray levels in Band
Background Tab	
BC Sum	Background Corrected Sum = (BC Average *Area)
BC Average	Background Corrected Average = (Average – Bkgd Average)
Note	Subtracting the Bkgd Average from the Average accounts for differences in area
Bkgd Sum	Sum of all pixel gray levels in Background Region
Bkgd Area	Area of Background Region
Bkgd Average	Average pixel level of Background region = (Bkgd Sum /Bkgd area)
Bkgd XY	X Y position of Background region
Bkgd SD	Standard deviation of pixel gray levels in background region
Signal/Noise	Signal to Noise ratio of the band region = (Average– Bkgd Average) /Bkgd SD
Control Tab	
LCN Sum	Loading Control Normalized Sum = (LCN Average* Area)
LCN Average	Loading Control Normalized Average = (BC Average of Experimental Band/BC Average of corresponding Loading control) * mean of all loading controls.

NOTE: If a background correction has not been applied, Average is used.

LC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Loading Control, (e.g. Red1/ Green 12)
PCN Sum	Positive Control Normalized Sum=(NCN Average *Area)
PCN Average	Positive Control Normalized Average = (LCN Average of experimental band / LCN Average of control band) *100.
Note	If loading controls have not been applied, BC Average is used. If a background correction has not been applied, Average is used.
PC Regions	Specifies the channel and region number used for each Experimental Band and corresponding Band Control, (e.g. Red1/ Red 6)
Fold Change	Fold Change of experimental band relative to control

*NOTE: If LCN Average experimental band >LCN Average control band then fold change = LCN average experimental/LCN average control. If LCN Average of the experimental band < LCN Average of control then fold change = (-1) * 1/(LCN average experimental / LCN average.*

Std Curve Tab	
"ng"	Values of Band region in Std curve in units as defined

Appendix D:

Bias and Dark Master Utility

Appendix Overview

- Bias and Darkmaster File Generation

Bias and Darkmaster File Generation

The system is shipped with a set of darkmaster and bias files that meet the requirements of most commonly encountered imaging applications (1, 4, and 8 mins). Some applications, however, such as extremely low-level signal detection with lengthy exposure times may benefit from generating specific darkmaster files matched to the exposure time of the application for optimum performance. The AlphaView software includes a utility for generating bias and darkmaster files.

NOTE: A darkmaster for a given time need only be constructed once. Subsequent imaging applications will then apply the optimum darkmaster from the set of all darkmasters in the system.

CCD images exhibit a non-zero black level (bias) and temperature/time dependant levels (primarily thermally generated electrons called dark current) which are typically quite stable and can be corrected. Once there levels are characterized they can be removed from the image under consideration. This process is uniformly referred to as image calibration (along with flat field correction) to produce an image that contains only signals from the object under test and some residual noise from the original error sources.

The minimum requirements for accurate determination of bias and dark current levels is thermal stability and light tightness. Any fluctuations in temperature will alter the bias and dark current levels. Any light leaks will result in an additional source for errors that will not be correctly removed from images under consideration.

Bias and darkmaster files are created by collecting a set of 16 images that are combined in such a way to reduce the noise levels and reject spurious signals (such as cosmic rays, etc). A set of such files for darkmasters are created at different exposure times to create a library of darkmaster files. The files are logged into the program folder and used for all subsequent imaging sessions.

When an object is imaged, the exposure time of the image is used to select the darkmaster with a similar exposure time and the darkmaster is scaled such that the dark current levels match that of the image under consideration. The bias and scaled darkmaster files are then subtracted from the image under consideration which results in an image with no bias (offset or black level errors) and elimination of dark current signals. As with any calibration method there are still noise artifacts that remain in the image after correction. At the time of acquisition an image contains the actual bias level plus bias level noise (also called read noise) in addition there is the dark current signal and its noise signals. The bias and darkmaster calibrations remove the bias and dark current signals but the noise from those signal remains (other techniques must be employed to address the residual noise-like frame averaging).

The best darkmaster images will have a similar exposure time to that which is typically used. The utility allows building darkmaster files of any exposure time (in 1 minute increments) so a darkmaster with a time that matches the typical exposure time can be constructed. It is best to have the darkmaster exposure time be greater than the time of interest. For example, if 4.5 minutes is a typical time then a 5 minute darkmaster would be best. Shorter or much longer times will still work, but optimal dark current correction occurs when using darkmaster exposure times greater than the test image exposure time (but no too much longer-round up to the next highest minute).

To use the utility to construct bias and darkmaster files, perform the following steps:

1. Allow camera to reach temperature and stabilize (at least 30 minutes after power on is recommended).
2. Put a cap on the lens mounting plate to block all light from the camera head. A lens cap will leak light. Creating darkmaster file with the camera and lens mounted to a cabinet is not recommended. If the camera cannot be dismounted then close the aperture and position the most opaque filter under the lens and turn off the cabinet before proceeding.
3. Access the utility from the application menus; see Figure D-1.

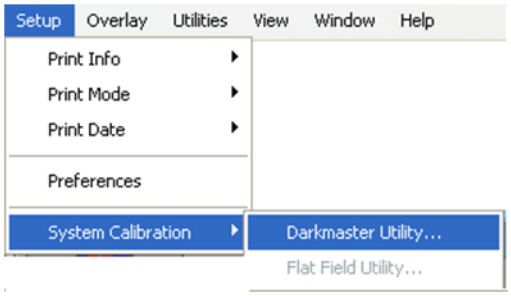


***Figure D-1:** Setup Menu Options*

4. Log in to the utility (call tech support for the password).

***Figure D-2:** User Login*

5. Enter the times for darkmaster files creation separated by commas.

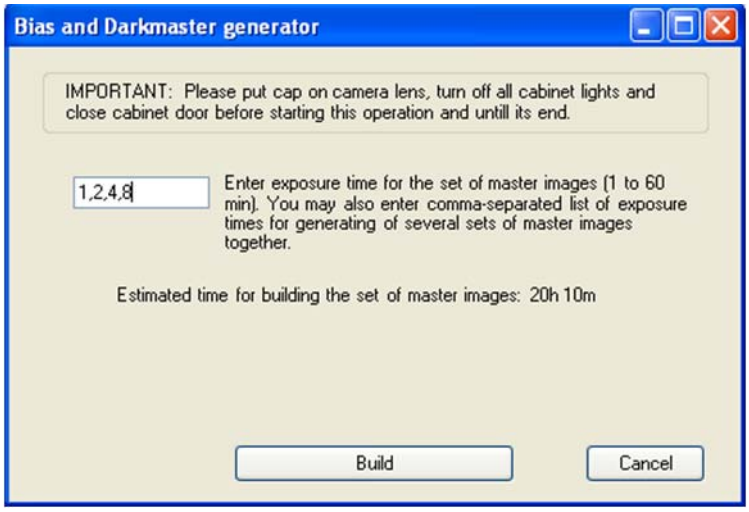


***Figure D-3:** Bias and Darkmaster Generator*

If bias files do not exist, they are created automatically. If only bias files are desired, enter a time of 0 minutes. Notice that the times to create the calibration images are quite long so please plan accordingly.

The estimated time of completion is given.

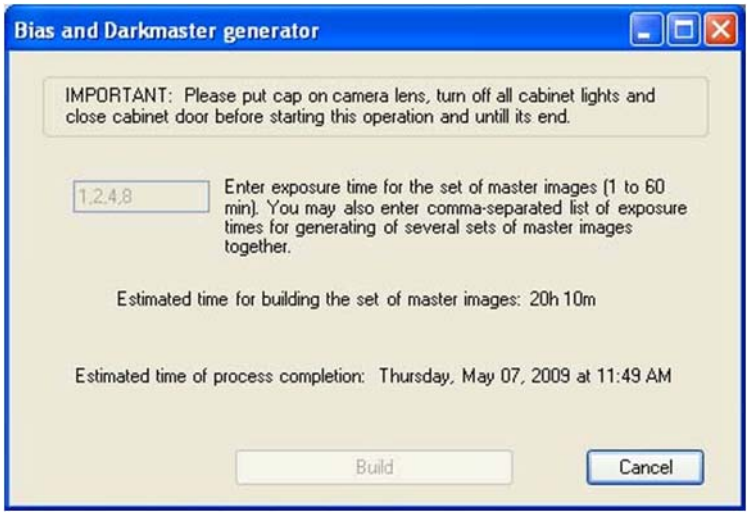


***Figure D-4:** Bias and Darkmaster Generator—Time Set*

A final notice is given when the process is complete.

Appendix E:

Flat Field Calibration

Appendix Overview

- Introduction
- Calibration Procedure

Introduction

The flat field calibration procedure is performed at installation or at the factory. Flat field files are provided for the most commonly encountered imaging situations. The purpose of the flat field calibration is to remove significant illumination and detection path non-uniformities from acquired images. Properly calibrated fluorescent images will be uniform for accurate quantification of signals levels within 5% at any position in the image.

A flat field calibration is only valid for images acquired under identical imaging conditions specific to that flat field file (not including exposure time or binning factor). A specific flat field file calibrates both the illumination intensity field and the image formation optics (lens and filter) and sample position within the cabinet. Consequently any change to either the illumination field or the imaging optics will degrade the calibration. Examples of such changes are positioning the sample at a different level within the cabinet, changing lens focus and aperture settings, replacing filters, or repositioning the camera and lens bracket.

The flat field calibration is applied automatically as part of the image acquisition process when a matching flat field is present in the application directory (C:\Program Files\name of system). If a matching flat field file is not present, then a flat field calibration is not applied. Also the flat field calibration may be disabled in the camera setup and preview window during acquisition.

The flat field image naming convention follows the format "FlatNxM.tif" where N is an index that lists the excitation light source and M is an index that lists the emission filter wheel position.

Calibration Procedure

Flat field calibration is appropriately utilized when no changes are made between flat field calibration and the imaging of biological samples.

The appropriately named flat field file will be placed in the application directory and subsequently applied to image acquisition at those illuminations and filter settings. The flat utility applies a smoothing function to the acquired image to remove irregularities due to mottling or fibers in the paper.

1. Place a uniform target such a piece of white laser printer paper at the sample location that will be used for fluorescent imaging.

***Figure E-1:** Flat Field Target Inside System*

2. Focus on the surface of the flat target (paper target shown below).

NOTE: It is important to set lens focus and aperture settings to those to be used for image acquisition.

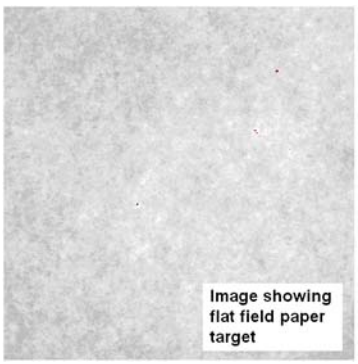


***Figure E-2:** Flat Field Target Image Example*

3. Acquire a grayscale image of the target with the desired illumination and filter settings. Use the top speed resolution setting possible (Normal/Ultra) and Zoom ROI setting = 1. The image should be generally bright with pixel gray levels between 50% and 90% of saturation (intensity values = 30,000 - 58,000). A few saturated (red) pixels are allowed.

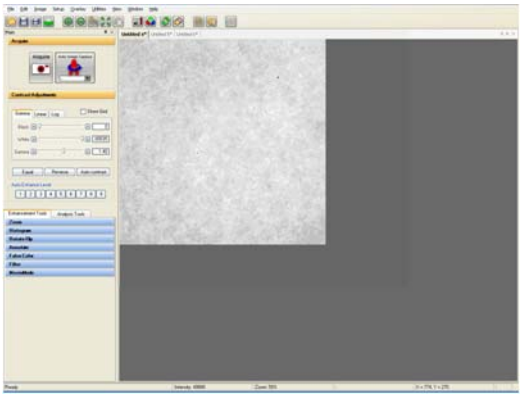


***Figure E-3:** AlphaView Screen Shot Example*

4. Select Flat Field Utility from the Setup > System Calibration menu.

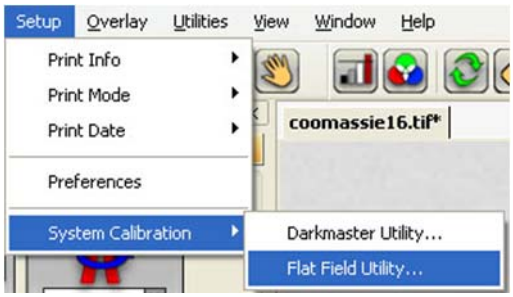


***Figure E-4:** Flat Field Menu Path*

5. Complete the login screen security entry. The default is provided below:

   User name: master

   Password: master

***Figure E-5:** Security Login Screen*

6. Image selection screen—review images created for flat field calibration. Images shows should match images acquired for flat field calibration.

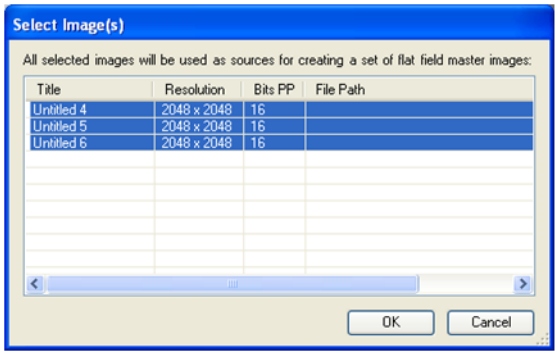


***Figure E-6:** Flat Field File Image Selection*

7. Select OK (or follow instructions).

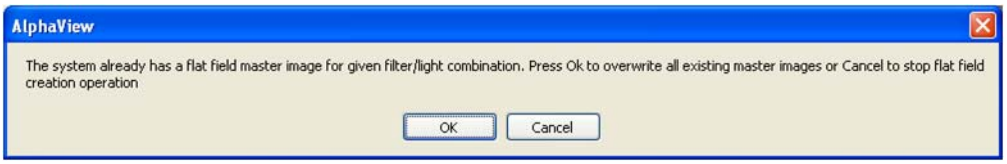


***Figure E-7:** Confirmation of System Containing Flat Field Master Image*

8. Select OK. Flat field calibration is now complete

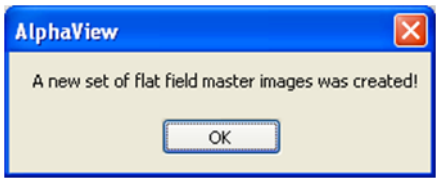


***Figure E-8:** Confirmation of New Set of Flat Field Master Images Created*

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