INCOGEN

# HIGH DENSITY FILTER READER

**Version 3**

# TUTORIAL

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# 1 INTRODUCTION

## 1.1 ABOUT THE HIGH DENSITY FILTER READER

Welcome to the High Density Filter Reader (HDFR). This program is designed to analyze images of high-density filters. Through a user-friendly, graphical interface, HDFR allows you to quickly and accurately identify clone locations on high-density membranes, quantify results for a single filter, and perform expression analyses between multiple filters. We welcome your feedback and comments regarding all aspects of this program.

## 1.2 ABOUT THIS TUTORIAL

This tutorial will lead you through a step-by-step analysis of a sample filter image. The role of the tutorial is to provide you with information about the main functions of the software and to assist you in analyzing your own filter images using the HDFR. For a detailed description of all functions of the HDFR and further information, please consult the **HDFR User's Manual**.

Sections preceded with "**Note:**" offer additional information that may not necessarily be related to the analysis of the sample image.

If you have already installed the HDFR on your computer, please skip to Section 3, **STARTING THE HDFR**, otherwise please continue to Section 2, **SETUP AND INSTALLATION**.

 

 

# 2 SET-UP AND INSTALLATION

## 2.1 MICROSOFT WINDOWS

To install the High Density Filter Reader on a computer running a Microsoft Windows operating system, place the HDFR Installation CD in your CD-ROM drive. If the *Autorun* feature is enabled, the installation will automatically start. If not, you can start the installation by opening **My Computer**, navigating to your CD-ROM drive, selecting the ***Windows*** directory and running ***Setup.exe***.

As you move through the installation, be sure to read each step. Please take the time to read the license agreement. If you have questions or problems with the license agreement, please do not continue the installation. Contact INCOGEN and we will help you in any way possible. Once you have installed the HDFR, you can review the license agreement by navigating to the directory where you installed the software and opening the file ***license.txt***.

Once you have accepted the license agreement, you may select the directory into which to install the HDFR (Figure 1a). The default destination directory is:

***C:\Program Files\INCOGEN\HDFR***.

If you would like to change the destination directory, press **Browse** and select a new location.

 

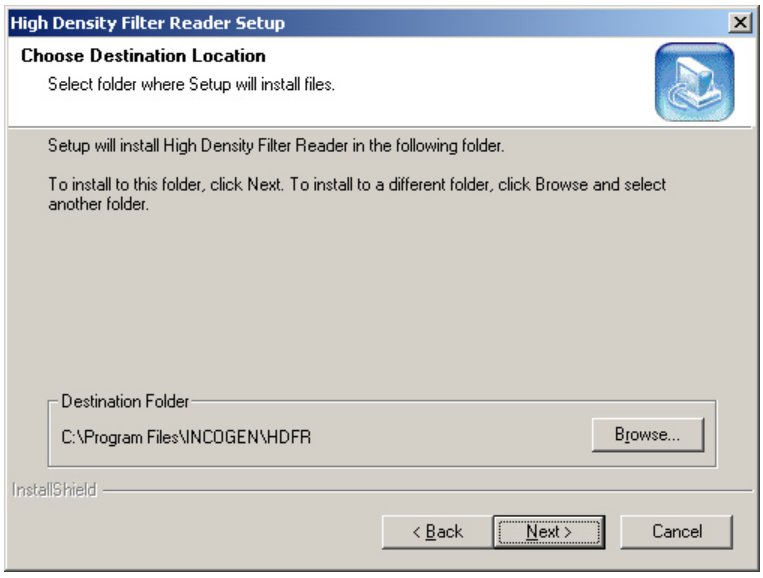


Figure 1a: Choosing a destination directory.

After you have selected a location for the software, you will be asked to specify what type of installation you would like to perform: ***Typical***, ***Compact*** or ***Custom***. In the typical installation, the files necessary for running the HDFR will be installed, as well as a set of sample files and an electronic copy of this tutorial. In the compact installation, only the files necessary for running the software will be installed. In the custom installation, you can choose which items you would like to install through a dialog like the one shown in Figure 1b below.

 

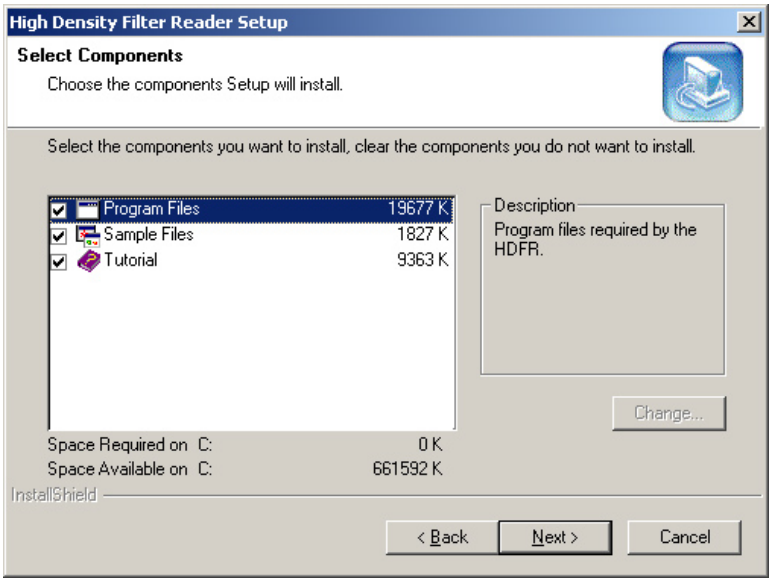


Figure 1b: Selecting the components to install.

Finally, the installation will prompt you for a location to place a shortcut. The default location is on the **Start** menu under **Programs** in a group called **INCOGEN**. You can change this location if you desire. Press **Next** to begin copying the files from the CD to your hard drive. You will see a progress meter as files are copied and your system is configured. When this completes, press **Finish**. The software is now ready to use.

 

## 2.2 UNIX - SOLARIS

To install the HDFR onto a computer running the Solaris operating system, please insert the HDFR CD into the CD-ROM drive. The CD should mount automatically and will typically appear in the directory: ***/cdrom/hdfr***. If the CD does not mount properly, please contact your local system administrator. If the problem persists, please contact HDFR Support at INCOGEN.

Once the CD is mounted, you should create a local HDFR source directory on your computer, for example: ***/usr/local/src/hdfr***. Then copy the file named ***install.tar*** from the ***solaris*** directory on the CD onto your local system. Once you've copied the file to your local system, use the *tar* command to un-tar the contents into your HDFR source directory. The following example outlines these tasks.

```
> mkdir /usr/local/src/hdfr
> cd /usr/local/src/hdfr
> cp /cdrom/hdfr/solaris/install.tar .
> tar -xvf install.tar
```

Before continuing the installation of the HDFR please verify that the appropriate patches have been installed on your computer. The patches are needed for the Java Runtime Environment (JRE) to run properly. Solaris 2.8 does not require any extra patches; however, versions 2.6 and 2.7 require patches as listed in the files ***README.i386*** (for PC-based architecture), or ***README.sparc*** (for Sparc-based architecture). The files are located in the directory ***x86_patches*** (PC) or ***sparc_patches*** (Sparc) inside the ***hdfr*** directory structure on your local system. Table 1 shows the names of the patches necessary for each operating system.

 

| Operating System | Patch File Name |
|---|---|
| Sparc Solaris 2.6 | ***1.2.2_05a_patches_sparc_5.6.tar*** |
| Sparc Solaris 2.7 | ***1.2.2_05a_patches_sparc_5.7.tar*** |
| x86 Solaris 2.6 | ***1.2.2_05a_patches_i386_5.6.tar*** |
| x86 Solaris 2.7 | ***1.2.2_05a_patches_i386_5.7.tar*** |

Table 1: A list of the Solaris patch names for various versions of the operating system.

The installation of these patches is covered in the ***README*** file in the appropriate directory. After the patches have been installed and the system has been rebooted, you will be ready to run the HDFR installer. The following steps outline this process.

1. Start X windows
2. Log in (or "su") as root.
3. Verify that your *DISPLAY ENV* variable is set up correctly. To check, run:
   ```
   > /usr/openwin/bin/xterm
   ```
   If a new xterm window opens up without problems, the display is set correctly. (The display will not be an issue if you log in as root at the system prompt and your current X windows session will be spawned by root, but may be a problem when you use the "su" command to become root during an already existing session.)
4. Change directory to the directory to which HDFR files were copied. For example:
   ```
   > cd /usr/local/src/hdfr
   ```
5. Run:
   ```
   > perl install.pl
   ```
   to start the installation script. The script prompts you to answer questions about the destination directory and the location of the source tar file for the HDFR. (The names shown in brackets contain the default answers. To accept the default for each question, simply hit the 'Enter' key.) The destination directory will contain the HDFR specific files and directories used when running the program. The source tar file refers to one of the ***hdfr.*.tar*** files, which are the

 

operating system specific files extracted from the initial ***install.tar*** file. The tar files in our example are contained in the directory ***/usr/local/src/hdfr***. Please submit an absolute path when specifying the destination directory and the tar file name. After the HDFR files have been installed you will be prompted to start the HDFR to finalize the installation. If the DISPLAY variable is not set correctly, you will see an error similar to: "*Exception in thread "main" java.lang.InternalError: Can't connect to X11 window server using ':0.0' as the value of the DISPLAY variable*". Please contact your local system administrator if you cannot change the display to your local computer.

6. After the HDFR has been run for the first time you will see a message stating that the installation is complete. You may now exit the HDFR and run it anytime as needed.

 

# 3 STARTING HDFR

## 3.1 MICROSOFT WINDOWS

If, during the installation, you chose the default location for placing the shortcut, you can start the HDFR by pressing **Start->Programs->INCOGEN->High Density Filter Reader** as shown in Figure 2. If you chose a different location for the shortcut, navigate to the appropriate folder to start the HDFR.

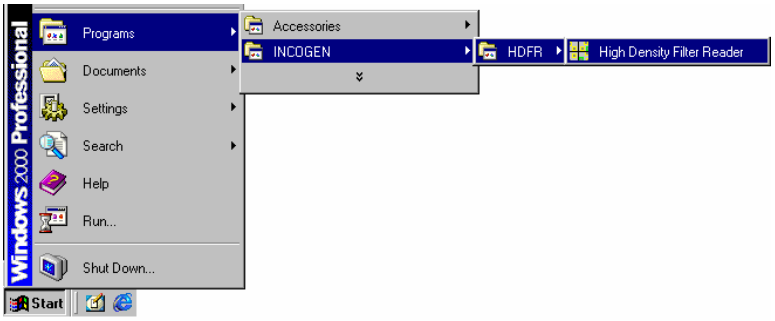


Figure 2: Starting the HDFR under Microsoft Windows.

## 3.2 UNIX - SOLARIS

The installation script described in Section 2.2 creates and places the ***runHDFR*** batch script in the directory into which the HDFR was installed. Execute this script to start the program, for example:

```
> /usr/local/hdfr/runHDFR
```

The program can be run by any user on the system, provided that the appropriate read and execute permissions are granted to the user.

 

 

# 4 LOADING AN IMAGE

The HDFR recognizes images of filters in the following formats: TIFF, GIF, JPEG (JPG). To open a new image, you can select **File->Open Image** from the Main Menu (Figure 3a), or click on the **Image** *FastFlow* button (Figure 3b).

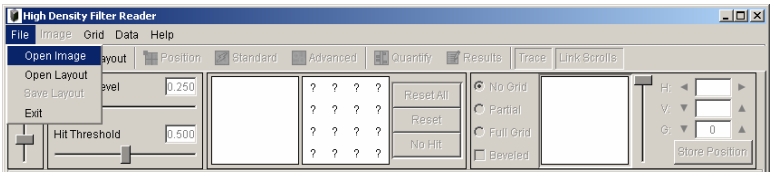


Figure 3a: Opening an image by using the **File** menu.

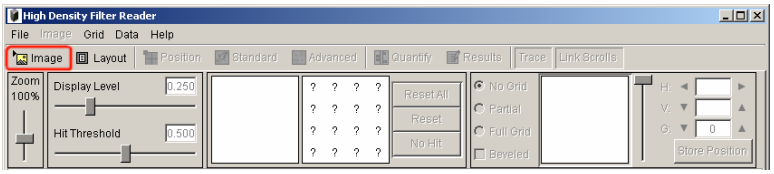


Figure 3b: Opening an image by using the *FastFlow* button.

Select the directory into which you installed the HDFR. (For example, the default install directory for the Windows operating system is:

***C:\PROGRAM FILES\INCOGEN\HDFR2***.)

That directory contains a sub-directory named ***samples*** which contains three sample image files:

1. ***sample1-4x4a.jpg***
2. ***sample2-4x4b.jpg***
3. ***sample3-4x4b.tif***

 

Double-click on the file called ***sample3-4x4b.tif*** to select it for analysis (Figure 4). If you are running Microsoft Windows, it is possible that you do not see the extensions (.jpg, .tif) of the sample images because you enabled the "Hide file extensions" feature.

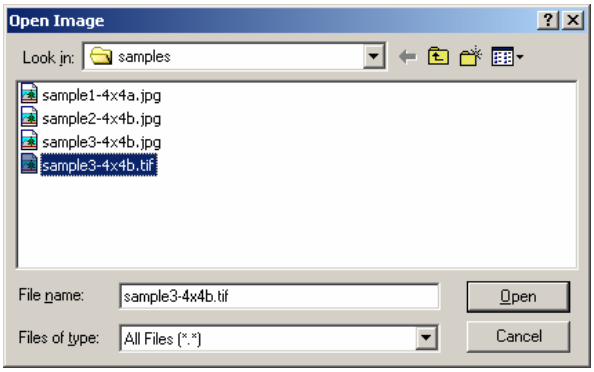


Figure 4: The file chooser window with the image file ***sample3-4x4b.tif*** selected for analysis.

After the image file has been selected and loaded into the HDFR, it will appear inside of the image panel (Figure 5).

 

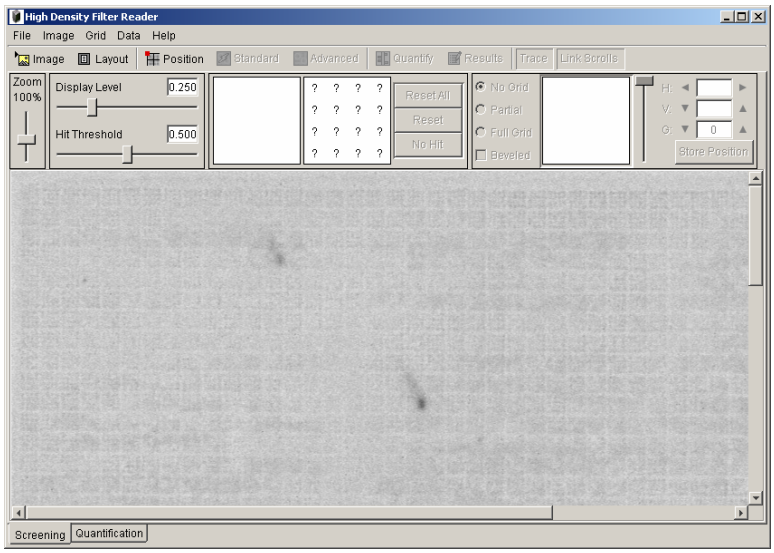


Figure 5: The sample image ***sample3-4x4b.tif*** opened inside of the HDFR.

At this time, you have successfully loaded a filter image into the HDFR. You can now proceed to the next step, which allows you to select a filter layout that corresponds to your filter.

 

 

# 5 LOADING A FILTER LAYOUT

The next step in the analysis process is the selection of the appropriate filter layout. You can either use and modify an existing layout, or you can design a layout from scratch. A sample layout has been installed with the HDFR. To load it, select **File->Open Layout** from the Main Menu (Figure 6) or press the **Layout** *FastFlow* button.

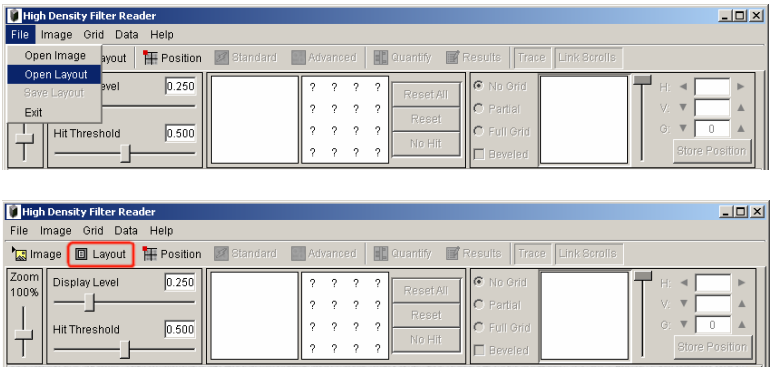


Figure 6: Importing a layout file by using the **File** menu.

Again, using the system file chooser, select the ***samples*** sub-directory inside of the directory into which the HDFR was installed and double-click on the file called ***SampleLayout.hlf*** (Figure 7).

 

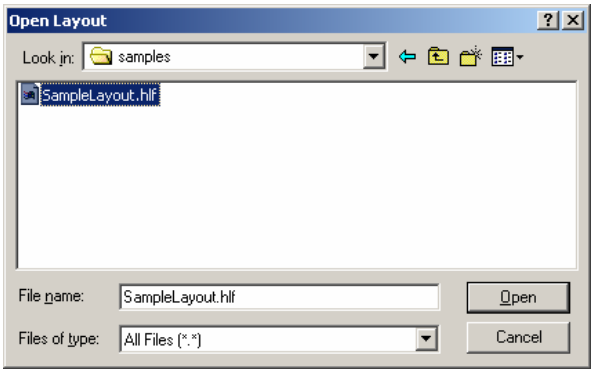


Figure 7: Choosing the layout file ***SampleLayout.hlf*** (***hlf*** = **H***DFR* **L***ayout* **F***ile*).

Once the layout file has been loaded, the following pop-up window will appear:

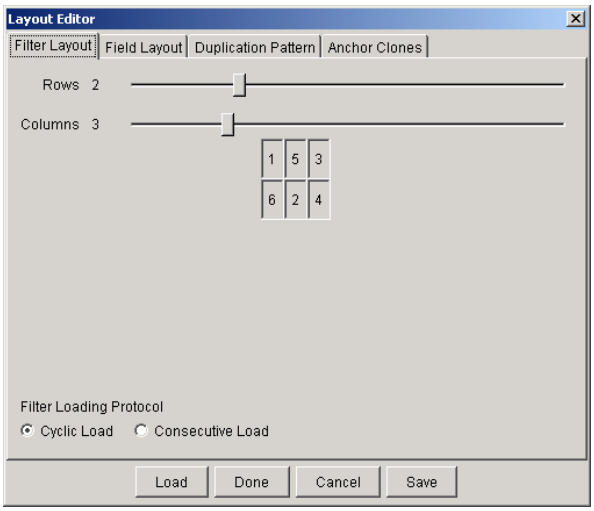


Figure 8: The layout dialog box (**Filter Layout** tab selected).

 

The layout window allows you to choose and modify the following layout parameters:

1. **Filter Layout**
2. **Field Layout**
3. **Duplication Pattern**
4. **Anchor Spots**

Select the **Filter Layout** tab (it will already be selected by default) to bring the filter layout to the foreground. Using the horizontal slider bars, you may select the number of rows and columns corresponding to the number of fields on the filter. The example image contains a 2x3 layout. Use the upper slider to adjust the number of rows to 2 and the lower slider to adjust the number of columns to 3. Once you have selected the correct number of fields, you may click on the small images of the fields to select their order. The first field on which you click becomes field #1, the second becomes field #2, etc. If you have mistakenly assigned a wrong number to a field, click on it again to erase the current number and select the correct field. Use the field numbering scheme shown in Figure 8 for the image you are currently analyzing.

For information on filter loading protocols, see Appendix A.

Once the correct filter layout has been selected, click on the **Field Layout** tab to bring it to the foreground. This window allows you to choose between a 96 and 384 well plate and choose the location of the A1 well. Use the buttons to adjust your set-up to correspond to Figure 9.

 

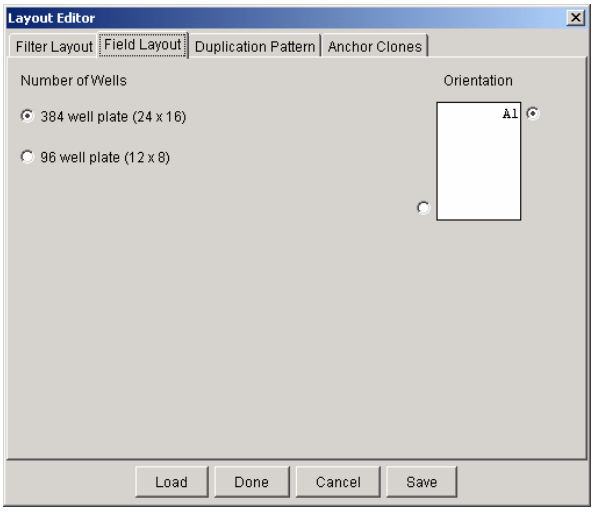


Figure 9: The **Field Layout** dialog box.

After selecting the correct field layout, proceed to the **Duplication Pattern** window by clicking on the corresponding tab. Use this window to select the appropriate gridding pattern for the filter image. It is crucial to select the correct pattern. The algorithm will not recognize any hits correctly if the selected pattern does not correspond to the gridding pattern used on the filter. The clones on the filter in the example image ***sample3-4x4b*** were spotted in the pattern shown below:


Figure 10: The 4x4 duplication pattern used in the production of the filter ***sample3-4x4b***.

 

The duplication pattern for this image has been saved with the description name **4x4 b**. Select the name using the slider bar as shown in Figure 11.

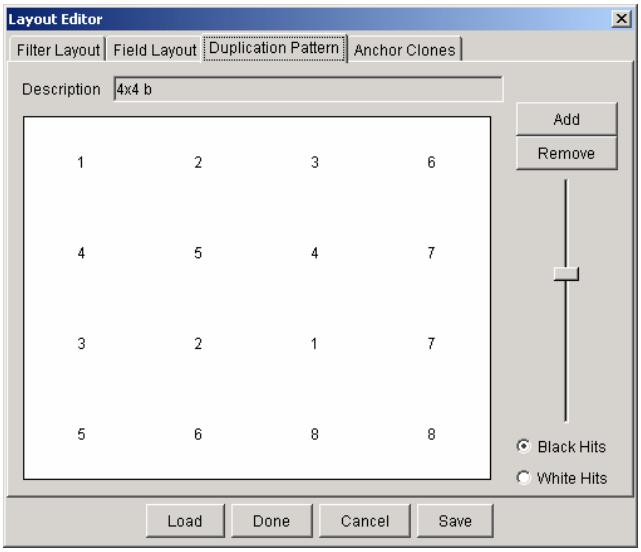


Figure 11: Selecting the appropriate duplication pattern for the filter image ***sample3-4x4b***.

Using the "**Black Hits / White Hits**" radio button, you can select whether the image of the filter contains dark hits on a light background or vice versa.

**Note:** You can also define your own gridding pattern for use with the HDFR. To do so, click on the **Add** button to bring up the pattern definition window. Pattern densities ranging from 3x3 to 8x8 can be chosen with the slider. After selecting the appropriate density, you can fill in the pattern by clicking on the fields. Use of this interface is similar to the numbering of fields in the **Filter Layout** window. (The pattern field index increments with every other click for use with a duplicate gridding pattern.) For more information, please refer to the **HDFR User's Manual**.

 

Anchor spots are not used in this example. For a description of the Anchor Spots tab and related functionality, see Appendix B.

Once you have completed the steps described in this section, click on the **Done** button to proceed to the analysis steps. You may also save the layout for future use by clicking on the **Save** button before clicking the **Done** button.

**Note:** When the HDFR is executed, it looks for a default layout file called ***default.hlf*** located in the ***HDFR*** root directory. If that file is found, the layout saved in that file is used for the analysis of all filter images unless another layout file is loaded. This is particularly useful if you analyze many filters that use the same layout. To take advantage of this feature, you should save your layout file under the name ***default.hlf*** in the ***HDFR*** root directory.

 

# 6 ANALYSIS

## 6.1 GRID PLACEMENT

After the correct layout has been loaded into the HDFR, a grid will appear superimposed on the filter image and you will see the pattern you have chosen in the pattern window (Figure 12a).

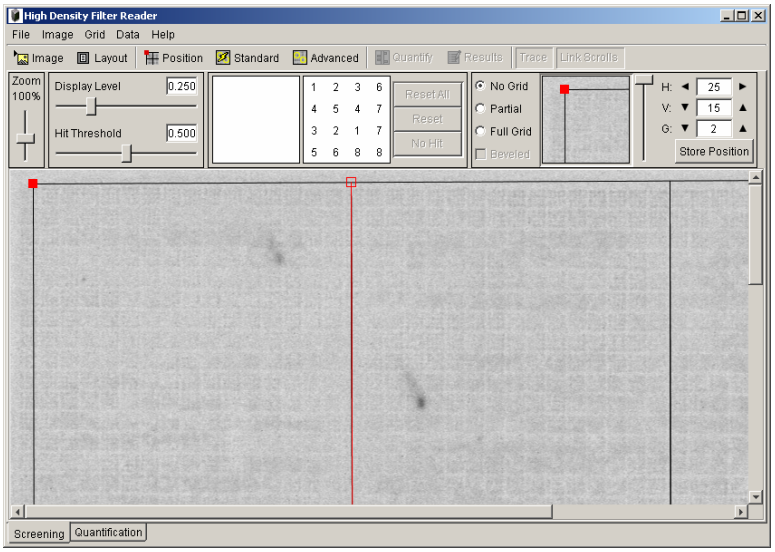


Figure 12a: The grid is superimposed onto the filter image ***sample3-4x4b***.

You may choose between different views by selecting the **Full Grid** (Figure 12b) or the **Partial Grid** (Figure 12c) options located just right of center at the top of the HDFR window. **No Grid** . The **Beveled** option offers a raised perspective (by alternating the grid cell colors) of the grid on the filter. This visual aid for placing the grid is particularly useful when the gap between the cells is set to zero.

 

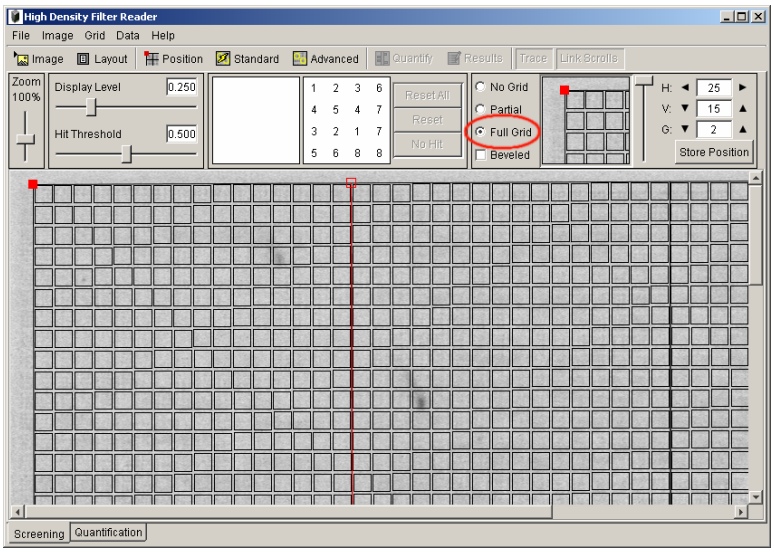


Figure 12b: The **Full Grid** option.

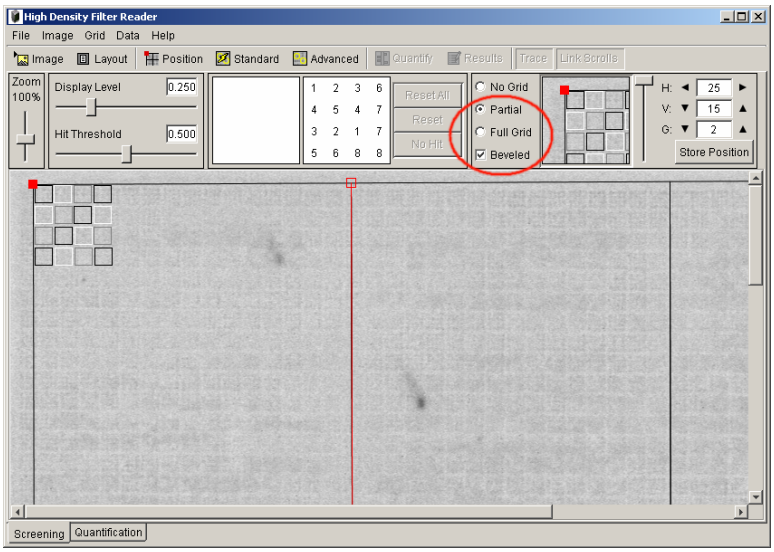


Figure 12c: The **Partial Grid** option with the **Beveled** option enabled.

 

The grid can be moved into the correct position by using the control points (squares at the corner points of the grid and between some fields). The control points can be moved with the mouse, the arrow keys, by entering numerical values for the coordinates into text fields at the top right, or by using the arrow icons next to the field coordinates. Each control point can be selected by clicking on it, or, once a control point is selected, by hitting the spacebar to move to the next control point. The spacebar cycles through the control point on the outside corners before selecting the inside ones. The active control point will automatically come into view in the main image panel. It will also be visible in the small grid alignment window to the left of the control points adjustment controls.

Select the outside control points (a filled-in square represents an active and movable state) and position them such that their x and y coordinates correspond to the coordinates shown in Figures 13a-d.

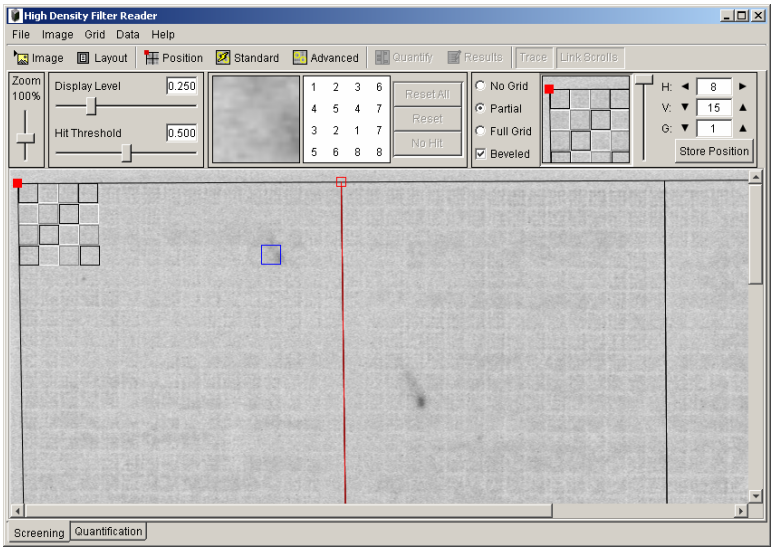


Figure 13a: The coordinates for the upper left corner are (8,15).

 

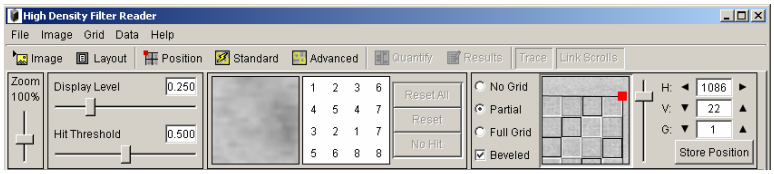


Figure 13b: The coordinates for the upper right corner are (1086, 22).

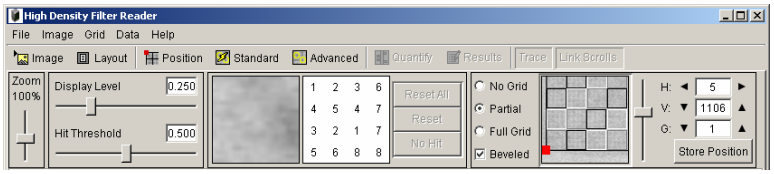


Figure 13c: The coordinates for the lower left corner are (5, 1106).

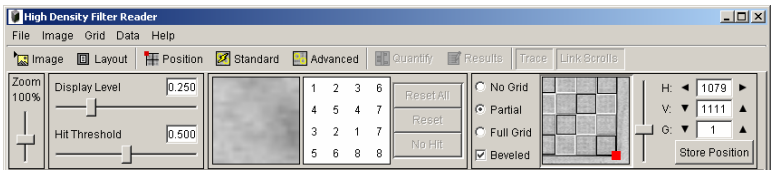


Figure 13d: The coordinates for the lower right corner are (1079, 1111).

**Note:** To help accurately place the grid, you should click on a cell containing a positive hybridization event several cells away from the active corner. The cell will appear outlined in blue and a zoomed-in view of that cell will appear to the left of the pattern window. You may now use the zoomed-in view to position the grid until the hybridization spots are in the correct position. You should use the mouse to move the grid to an approximate position and then use the arrow keys or the up/down/left/right buttons next to the field coordinates to accurately position the grid.

 

Once the four outside corners have been positioned correctly, you may use the squares on the inside perimeter of the grid to adjust the distance between the fields. Specify the (x,y) coordinates for the two inside squares as shown in Figures 13e and 13f.

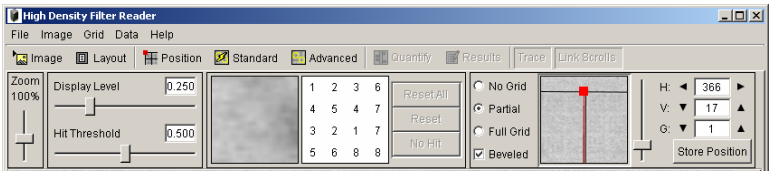


Figure 13e: The coordinates for the control point which determines the horizontal spacing between fields are (366, 17).

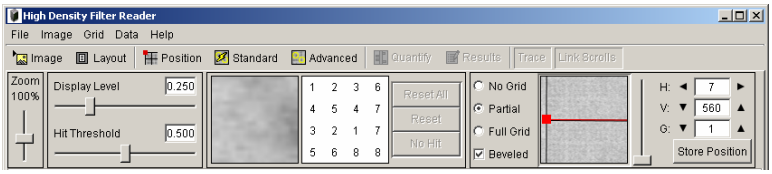


Figure 13f: The coordinates for the control point which determines the vertical spacing between fields are (7, 560).

To specify the correct gapping between cells, adjust the **Gap** controls (marked by **G**). The value of the **Gap** should be set to 1 for this example.

After the grid has been placed correctly over the filter image, click on the **Standard** (standard analysis) *FastFlow* button (Figure 14) to analyze the filter. The analysis of the filter generally takes a few seconds, depending on the speed of your computer.

 

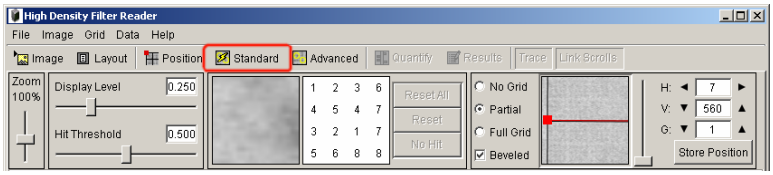


Figure 14: Starting an analysis using the Standard (analysis) FastFlow button.

## 6.2 DATA VISUALIZATION AND MANIPULATION

The HDFR offers users an intuitive and user-friendly way to browse through and manipulate the data once the filter image has been analyzed. In the following sections some of those features are explained as they are applied to the example image. For a detailed description of all features, please refer to the **HDFR User's Manual**.

### 6.2.1 Overview

After the analysis of the filter is completed, the data will be presented to you in two ways. The analysis results are displayed in a spreadsheet format on the left side of the image (Figure 15a) and hybridization events (hits) are also circled on the image itself. You may use the **Trace Hits** option to toggle the display of identified hybridization events that fall above the **Display Level** (see Section 6.2.2). The spreadsheet data consists of four columns. The first column (**ID**) represents the pattern number. The second column (**Value**) displays the confidence level of the call. The third column (**Clone**) displays the clone name obtained from the cell address, pattern, and library index. The fourth column is used to mark user input with a star. The value in the second column is used to determine whether the particular call constitutes a hit and/or whether it is displayed.

To view the numerical results for a particular cell, you can simply click on the cell of interest. The cell selected in Figure 15a is in

 

field 5. Find this cell on your image and click on it. This action has four effects: 1) The cell becomes highlighted in blue, 2) a zoomed in view of the cell appears in the zoom window, 3) the pattern for the cell appears in the pattern window, and 4) the spreadsheet display automatically focuses on the rows corresponding to the cell and highlights them. You can also obtain focus of a certain cell by clicking on any of the columns in the spreadsheet. For further descriptions on navigating between cells, please refer to Section 6.2.4.

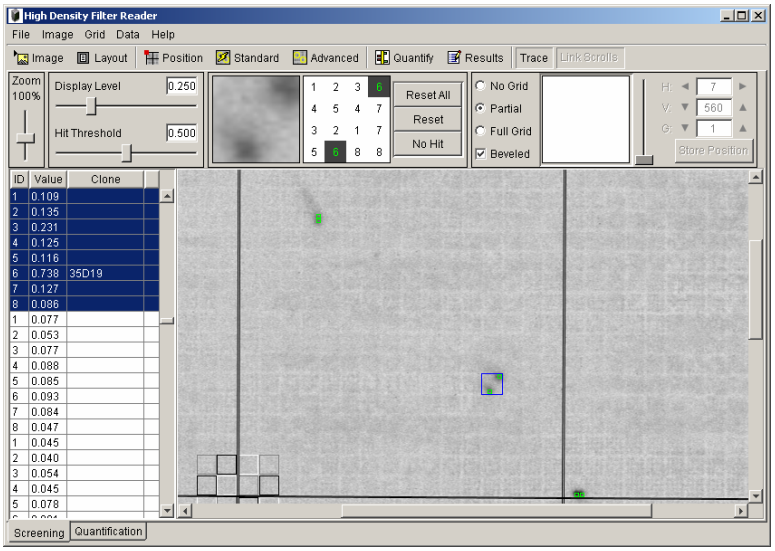


Figure 15a: The results of the analysis are displayed in a spreadsheet and represented on the image by circles drawn around the hybridization events. The outlined cell and pattern correspond to the clone location 35D19.

**Note:** If the filter contains many irregularities, users have the option to execute the "advanced analysis" which is more time consuming, but is also more sensitive than the standard analysis which is executed by default. To perform the advanced analysis, press the **Advanced** (analysis) *FastFlow* button. For a detailed description of both analysis algorithms, please refer to the **HDFR User's Manual**.

 

### 6.2.2 Hit Threshold and Display Level

The two horizontal sliders named **Hit Threshold** and **Display Level** are used to control visualization of the data. Only hybridization events with values greater than the current **Hit Threshold** slider value are considered true "hits" and are displayed in green. Values that fall below the **Hit Threshold**, but are above the **Display Level** slider value are considered potential candidates for a hit and are displayed in yellow. Values that fall below the Display Level are not displayed. In Figure 15a, the **Hit Threshold** was set to 0.500 and the **Display Level** was set to 0.233. The "6" pattern in the highlighted field was assigned a confidence value of 0.738. It therefore falls above the **Hit Threshold** and is displayed in green. That pattern and cell location correspond to the clone address 35D19. Figure 15b focuses on the cell and pattern corresponding to the clone 37L20. The "7" pattern was assigned a confidence value of 0.361, which falls below the **Hit Threshold**, but above the **Display Level** and is therefore drawn in yellow to signal a potential hit.

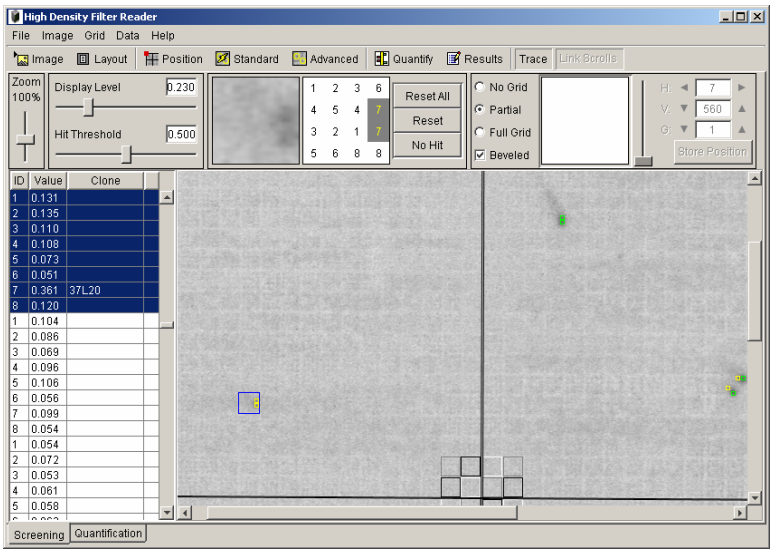


Figure 15b: The results of the analysis are displayed in a spreadsheet format and represented on the image by circles drawn around the

 

hybridization events. The outlined cell and pattern correspond to the clone location 37L20.

**Note:** The fastest method to set the correct threshold for a whole filter (which means that all of the "true" hits as deemed by the user are drawn in green) is to choose the weakest hybridization signal that corresponds to a "true" hit and to set the **Hit Threshold** just below it.

### 6.2.3 Zooming

There are three ways of taking advantage of the zooming feature. The entire image can be magnified by using the Zoom slider. Also, as described in Section 6.2.1, by left-clicking on a cell, the cell appears magnified in the small zoom window to left of the pattern window. In addition, local regions of the image can be magnified by clicking and holding the right mouse button on any part of the filter image. A magnification of the region around the mouse pointer will appear and will be repainted dynamically when the pointer is moved until the right mouse button is released (Figure 16).

 

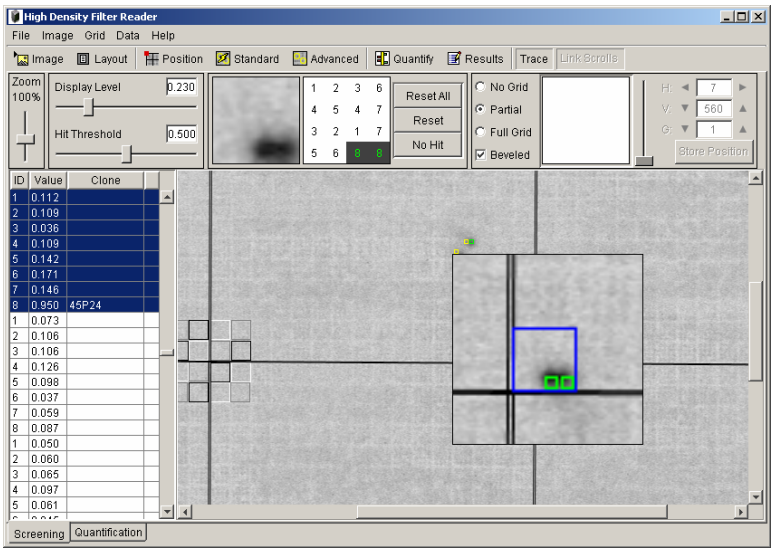


Figure 16: An example of dynamically magnifying a region of the filter by using the right mouse button.

### 6.2.4 Navigation Shortcuts

In addition to clicking on a particular cell of interest to obtain focus for it, the HDFR provides you with several shortcuts to jump between cells. This allows you to quickly navigate through cells of interest on the filter. After a cell has been selected and a blue outline is drawn around it, arrow keys in combination with control keys can be used to move the focus represented by the blue rectangle. The arrow keys move the cursor left, right, up, or down. Movement on the edges of the grid is wrapped around. The space bar has the same effect as the right arrow key. The 'Alt', 'Ctrl', and 'Shift' keys may be used in combination with the left and right arrow keys to move to the next or previous "hit" or "miss". ("Next" refers to cells to the right or down, and "previous" refers to cells to the left, or up.) The 'Alt' key indicates you wish to move to a hit. The 'Ctrl' key indicates you wish to move to a miss. The 'Shift' key, in addition to the 'Ctrl' and 'Alt' keys, means that you wish hit and miss to be determined by the **Hit**

 

**Threshold** instead of the **Display Level**. Table 2 gives a listing of the shortcuts and their functions.

| Action | Result |
|---|---|
| Alt + right | Move to the next cell that contains a hybridization event with value above the **Display Level**, or was user-defined. (yellow, blue or green) |
| Alt + left | Move to the previous cell that contains a hybridization event with value above the **Display Level**, or was user-defined. (yellow, blue or green) |
| Ctrl + right | Move to the next cell that does not contain a hybridization event. (below **Display Level**) |
| Ctrl + left | Move to the previous cell that does not contain a hybridization event. (below **Display Level**) |
| Shift + Alt + right | Move to the next cell that contains a hybridization event with value above the **Hit Threshold**, or was user-defined. (blue or green) |
| Shift + Alt + left | Move to the previous cell that contains a hybridization event with value above the **Hit Threshold**, or was user-defined. (blue or green) |
| Shift + Ctrl + right | Move to the next cell that contains a candidate hybridization event that falls below the **Hit Threshold**, but above the **Display Level**. (yellow) |
| Shift + Ctrl + left | Move to the previous cell that contains a candidate hybridization event that falls below the **Hit Threshold**, but above the **Display Level**. (yellow) |

Table 2: A listing of the navigation shortcuts and their functions.

### 6.2.5 Local Operations and Re-analysis

Sometimes it may be advantageous to keep results for specific areas of the filter as they are and to re-analyze the remainder of the filter. This may be particularly useful when the filter contains varying background levels or is especially difficult to analyze. You can use the 'Insert' and 'Delete' keys in combination with the 'Shift' key to mark or delete hits as "committed" and adjust the thresholds or re-analyze the rest of the filter without affecting the marked cells. Hits or misses that

 

were marked as "committed" based on user input are distinguished by an asterisk next to the clone entry in the spreadsheet view and the outlines of the hits are drawn in blue. Table 3 lists the keys and their functions.

| Action | Result |
|---|---|
| Insert | Mark all hybridization events in the current cell as "committed". (Green and yellow are marked as hits.) |
| Delete | Delete all hybridization events in the current cell. |
| Shift+Insert | Mark hybridization events above the **Hit Threshold** (green) as "committed" and delete hybridization events below the **Hit Threshold** in the current cell. |
| Shift+Delete | Reset calls in current cell to original values. |

Table 3: The shortcut keys and functions for marking calls in individual cells as "committed" for further re-analysis of filter.

### 6.2.6 User Calls

An additional option for the user to manipulate the results is to change the call to a different pattern than the pattern obtained by the algorithm. As an example, select the clone 37L20 by either clicking on the filter image, or the corresponding row in the spreadsheet (Figure 17). By clicking on it, a zoomed-in view of the filter and the corresponding hit appears in the upper part of the HDFR.

 

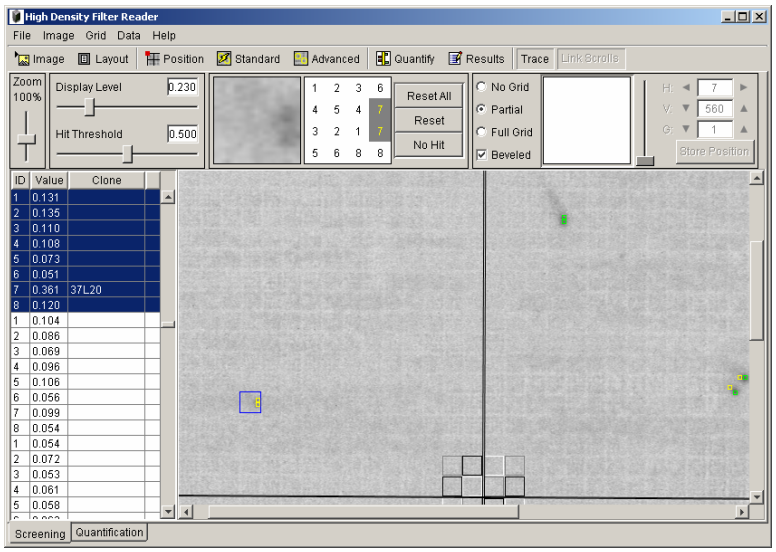


Figure 17: Selecting a detailed view of the clone 37L20.

Let us assume that the hit was really a "5" rather than a "7". To change it, first click on one of the "7"s in the small pattern window and then click on one of the "5"s. Changing a hit has the following four consequences (Figure 18):

1. The hit pattern changes from a "7" to a "5" pattern in the pattern window.
2. The small circles in the filter image turn blue to indicate user input.
3. The clone entry corresponding to the "7" pattern in the spreadsheet disappears and the row is marked with an asterisk in the last column to indicate user input.
4. A clone entry corresponding to pattern "5" is added in the row corresponding to that pattern and the row is marked with an asterisk to indicate user input.

 

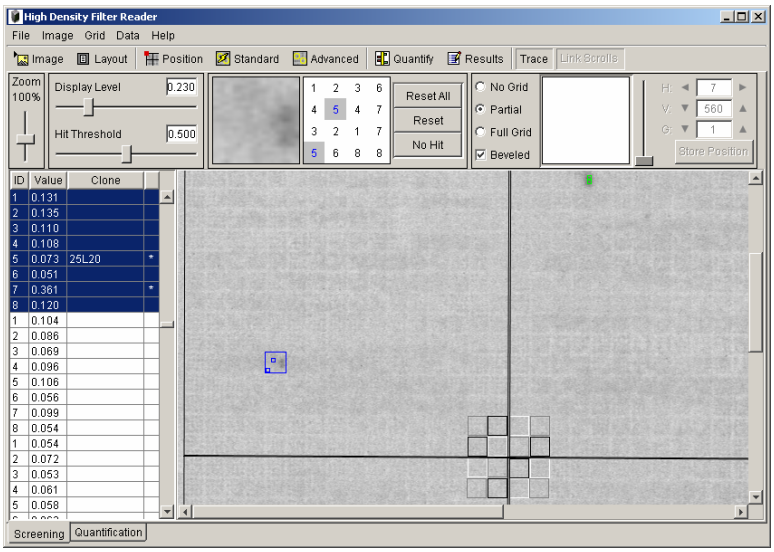


Figure 18: User-initiated change in the hybridization pattern.

By clicking on the **Reset** button (Figure 19) the user input can be reset for the highlighted cell only. (This operation is equivalent to the 'Shift+Delete" shortcut.) To reset all user-initiated changes for the entire filter, click on the **Reset All** button.

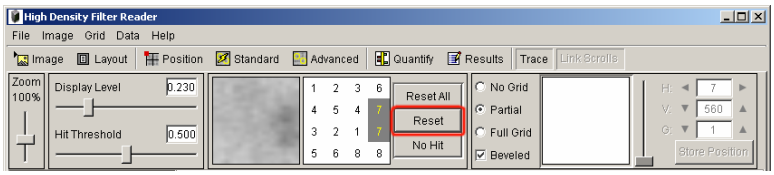


Figure 19: Resetting the results for a single cell.

 

## 6.3 QUANTIFICATION OF HYBRIDIZATION EVENTS

You can quantify the level of hybridization events, by selecting **Quantify Results** from the **Data** menu (Figure 20).

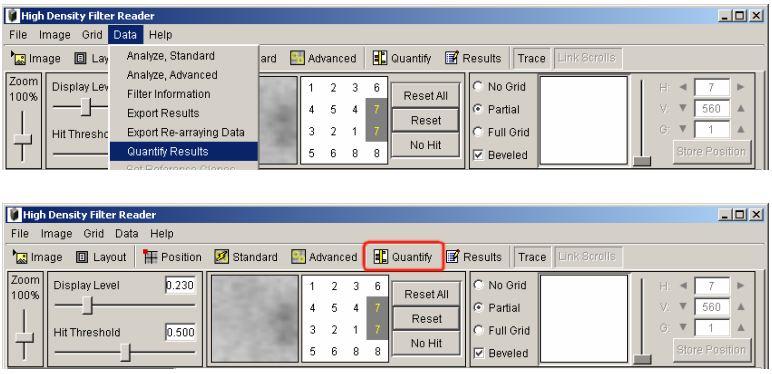


Figure 20: Quantifying the hybridization events by selecting **Quantify Results** from the **Data** menu.

After a few seconds, during which the quantification analysis takes place, you will notice that HDFR has switched to the **Quantification** mode. You can move between the previous, clone address identification screen, and the quantification screen by clicking on the **Screening** / **Quantification** tabs in the lower left corner (Figure 21).

 

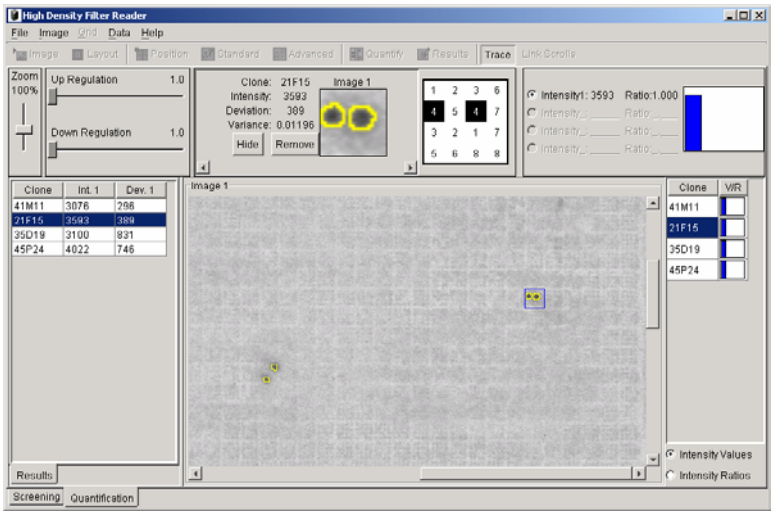


Figure 21: The **Quantification** screen. The cell containing the clone 21F15 is selected.

The quantification mode contains several new or different features not found in the clone address identification (**Screening**) mode. The following paragraphs describe those features.

**Magnification window**

Click on the clone 21F15 either in the table or on the cell containing the clone on the filter, as shown in Figure 21. The magnification window now contains a traced outline view of the hit within the selected cell. There are several numbers next to the magnification screen: the **Clone** name; the **Intensity** of the hit; the **Deviation**, or difference between the intensities of each of the colonies within the duplicate hit; and the **Variance** for all pixels in the cell. Pressing on the **Hide** button temporarily hides the filter image from view (this becomes useful in Section 6.4, **Expression Analysis Between Filters**) while pressing the **Remove** button permanently removes the filter from the current analysis. (The image is not removed from the hard disk; it can be reloaded and re-analyzed.) When the filter image is hidden, the **Hide** button label is changed to **Show**. Pressing **Show** restores the view of the image.

 

**Tabular Data View**

The data view in the left panel summarizes the clone names (**Clone**), intensity values (**Value**) and deviations (**Dev**) for all hits.

**Bar Chart**

The bar chart located in the upper right corner gives a visual representation of the intensity of the selected hit. It is particularly useful when comparing hits from multiple filters. This functionality, as well as the numbers and radio buttons next to the bar chart, will be described in detail in the next section.

**Threshold Sliders**

The function of the threshold sliders will be explained in the next section.

## 6.4 EXPRESSION ANALYSIS BETWEEN MULTIPLE FILTERS

Let us now analyze a second filter and perform a quantification of the hits on the filter. To do so, press on the **Screening** tab in the lower left corner. Pressing on the tab will return you to the clone address identification mode (Figure 22).

 

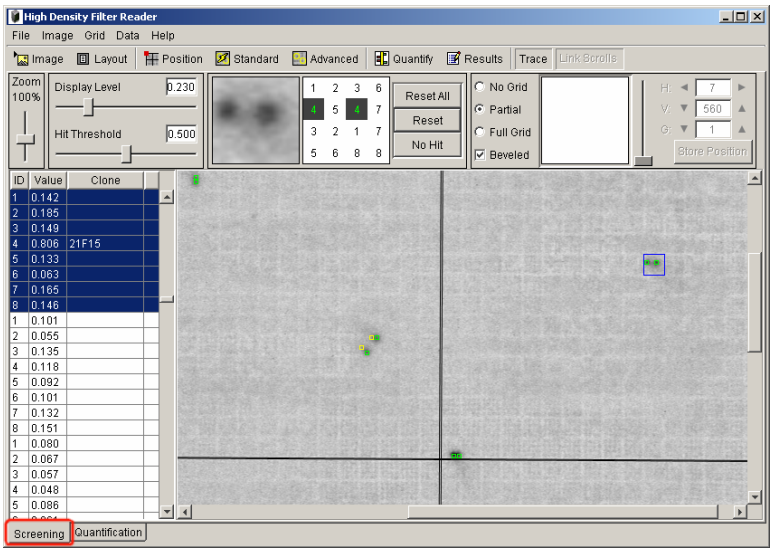


Figure 22: Returning to the clone address identification screen.

To load another image, click on the **Image** *FastFlow* button, or open the **File** menu and select **Open Image**. (If you encounter problems during the next several steps, please refer to Section 4, **Loading an Image**.) You will be prompted whether you wish to discard the clone address identification results for the current image (Figure 23).

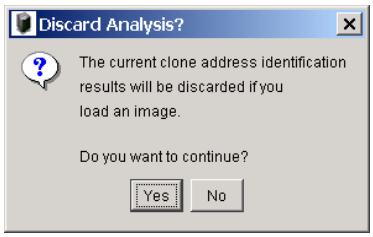


Figure 23: The user is prompted whether to discard existing results before loading a new image.

 

Select **Yes** and load the image named ***sample2-4x4b.jpg*** using the file chooser.

Activate the grid by clicking on the **Position Grid** *FastFlow* button and place the grid control points in the positions shown in the table below and set the inter-cell gap to 1 by using the gap adjustment controls.

| **Control Point Position** | **Coordinates (x, y)** |
|---|---|
| Upper left | (14,11) |
| Upper right | (1100, 11) |
| Lower left | (15, 1093) |
| Lower right | (1098, 1090) |
| Horizontal spacing | (375, 11) |
| Vertical spacing | (15, 552) |

Table 4: Grid control point positions for filter image ***sample2-4x4b.jpg.***

Perform the standard analysis by clicking on the **Std. Analysis** button. The HDFR screen should look similar to Figure 24.

 

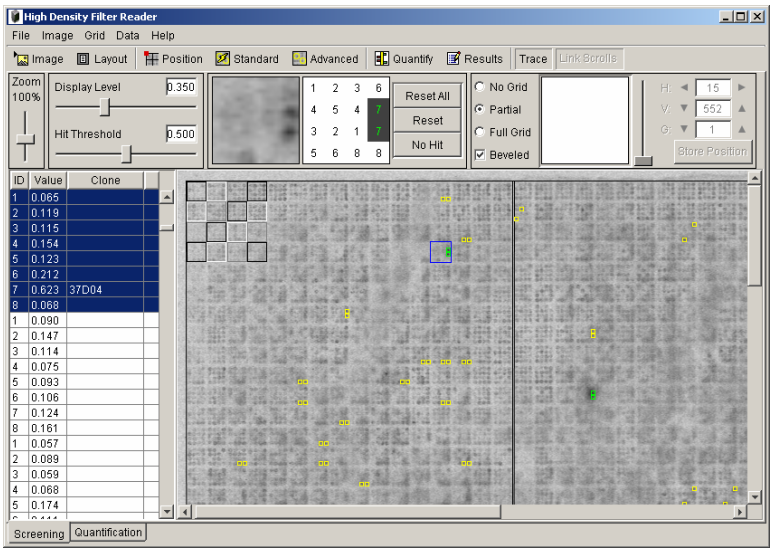


Figure 24: Clone address identification for filter image ***sample2-4x4b.jpg.***

Set the **Hit Threshold** and **Display Level** sliders to 0.530 to eliminate the false negatives. The screen should now look similar to Figure 25.

 

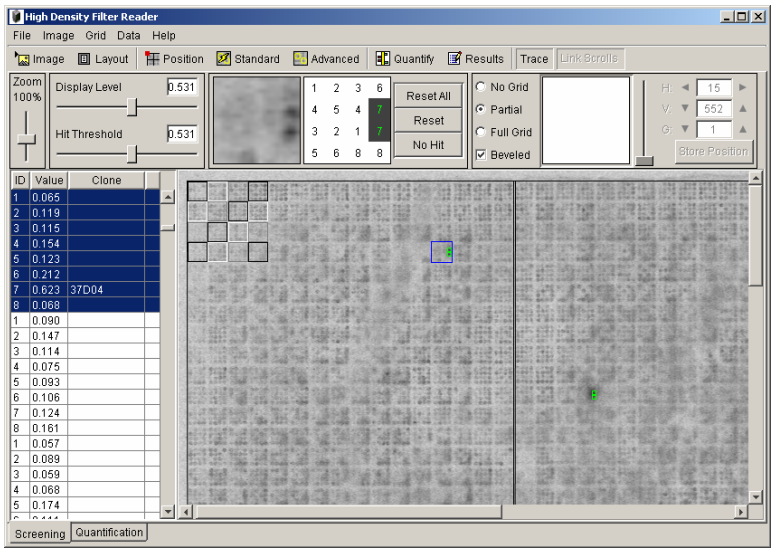


Figure 25: Clone address identification for filter image ***sample2-4x4b.jpg.***

Next, select **Quantify Results** from the **Data** menu to perform a quantification analysis on this filter image. You will be prompted to provide reference clones to normalize the two filters. Using reference clones to normalize filter images when comparing two or more filters is necessary to compensate for variations in intensities due to different exposure times or other factors that may lead to differences in clone intensity values that are not based on differences in expression levels. Select the clone 41M11 as the reference clone by highlighting it in the left panel and clicking the **Add** button (Figure 26). Then click **OK**.

 

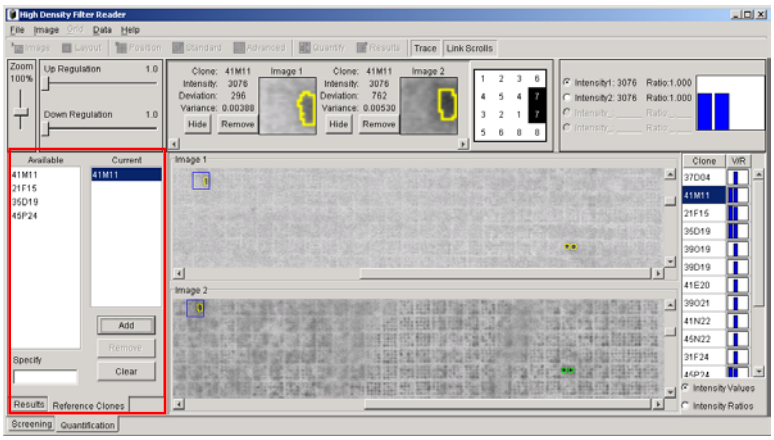


Figure 26: Selecting reference clones to normalize the intensity of filters used for expression analysis.

**Note:** if you don't know the name of the reference clone, you can locate the clone on the filter and click on it to highlight it in the list. Then simply press **Add** to designate it as a reference clone. If the clone of interest is not the only clone in the cell, the clone with the lowest duplication pattern number will be highlighted.

You will see the quantification screen with a split-panel view displaying both filter images. Beneath the reference clones panel, click on the Results tab to view the numeric comparisons of the clones on the two filters. Click on the clone 21F15 in the table to see it highlighted on both of the filter images (Figure 27).

 

Figure 27: Comparing expression levels between two filters using filter image 1 (***sample3-4x4b.tif***) as the control.

You will notice that there are now two magnification windows displaying the highlighted cell from each filter. Clones on the filter that was chosen as the control are all highlighted in yellow. On the sample filter, clones outlined in green signify a lower normalized intensity value than the control (down-regulation) and clones outlined in red signify a higher normalized intensity value than the control (up-regulation). The bar chart in the upper right corner provides a visual representation of the relative expression levels. The numbers next to the bar chart give the intensities as well as the ratios of the expression levels. By clicking on the radio buttons next to the bar chart, the filter image that is used as the control can be switched. The first filter was chosen as the control by default. To select the second filter as the control, simply click on the radio button next to **Value 2**. You will notice that all clones on the image of the second filter (***sample2-4x4b.jpg***) will become outlined in yellow (control) and that the clones on the image of the first filter (***sample3-4x4b.tif***) will become outlined in red or green, depending on whether they were up- or down-regulated (Figure 28).

 


Figure 28: Comparing expression levels between two filters using filter image 2 (***sample2-4x4b.jpg***) as the control.

**Note:** When a cell contains multiple hits, values for the first pattern that corresponds to a hit are shown by default. To obtain values for a different hit pattern within the same cell, click on the corresponding pattern in the duplication pattern window, the cell magnification window, or select it from the table.

**Threshold Sliders**

Using the sliders to the left of the magnification windows, you can adjust the thresholds for drawing the clones in green or red. If, for example, you only wish to see clones that were up- or down-regulated by a factor of two or greater outlined in red and green, you can slide both sliders to the 2.0 position. Only clones that were up- or down-regulated by a factor of two or greater will be outlined in red or green. All clones with up- or down-regulation factors greater than 2.0 will be drawn in yellow (Figure 29).

 

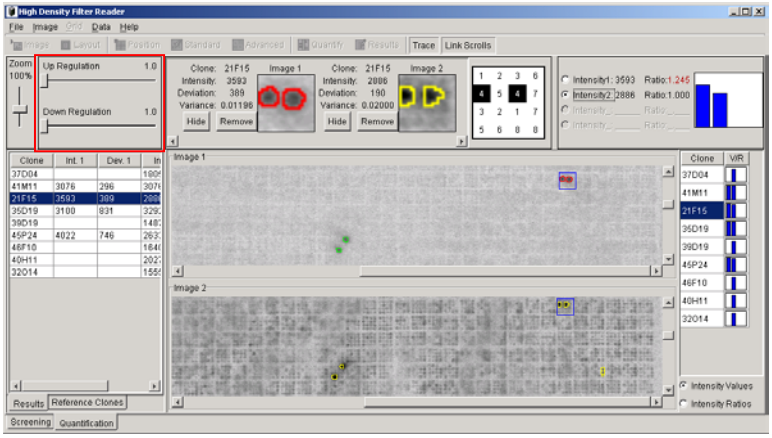


Figure 29: The **Threshold** slider can be used to only view the green/red color-coding for clones above and below a certain expression ratio.

**Re-normalizaton**

In the described example, all intensity values were normalized with filter image 1 as the base. The filters can be re-normalized with filter image 2 as the base by selecting **Normalize** from the **Data** menu and selecting filter image 2. You will be prompted to enter the reference clones again.

Up to four filter images can be viewed simultaneously in the quantification screen by repeating the steps outlined in this section. If a fifth filter image is loaded for quantification, the first image is removed.

 

 

# 7 DATA EXPORT

**NOTE: The data export functions have been disabled in the demo version of HDFR.**

## 7.1 STANDARD – CLONE ADDRESS IDs

Once you are satisfied with the screening results as they appear on the filter, you may press the **Results** *FastFlow* button to export the data (Figure 30). Be sure you are in screening mode; otherwise, you will export quantification results (see Section 7.2).

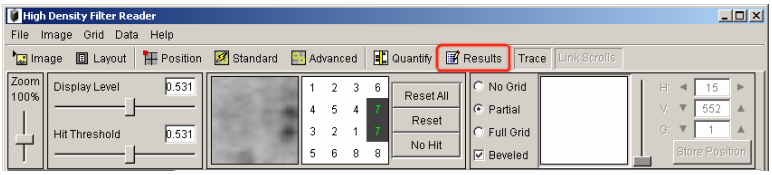


Figure 30: Exporting clone address identification results by clicking on the **Results** *FastFlow* button.

The following window will appear (Figure 31):

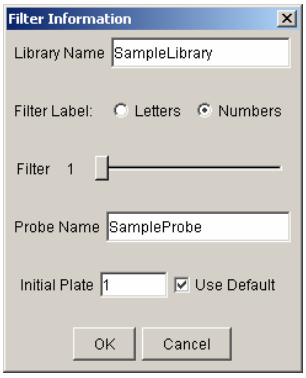


Figure 31: The filter information dialog box.

 

This window allows you to enter the name of the library, the filter labeling scheme, the filter number, and the probe name. For this example, call the library *SampleLibrary*, select **Numbers** as the filter-labeling scheme, choose the number of the filter to be **1,** and call the probe name *SampleProbe*. Click **OK** to proceed to the next window (Figure 32).

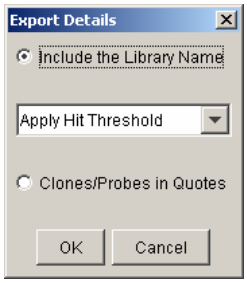


Figure 32: Dialog box to choose various data export options.

This window allows you to choose the output format of the data. The first option, when checked, includes the library name as the first line of the results file. The second option allows you to choose whether to export only clones that were marked by the user (blue) and clones that fall above the **Hit Threshold** (green), or whether to also include clones that fall below the **Hit Threshold** but above the **Display Level** (yellow). The third option puts quotation marks around the clones and probes to facilitate import into MS-Excel and other spreadsheet programs; otherwise, a clone name such as 37E24 may be interpreted as a number in scientific notation: $37*10^{24}$.

**Note**: When importing an HDFR results file into MS-Excel, be sure to set the *column data format* of the first column to text.

Clicking the **OK** button allows you to proceed to the file chooser screen (Figure 33). This screen allows you to choose the directory and the name of the file to which you would like to save the results. Select the ***samples*** directory and choose the file name ***sampleResults.txt***.

 

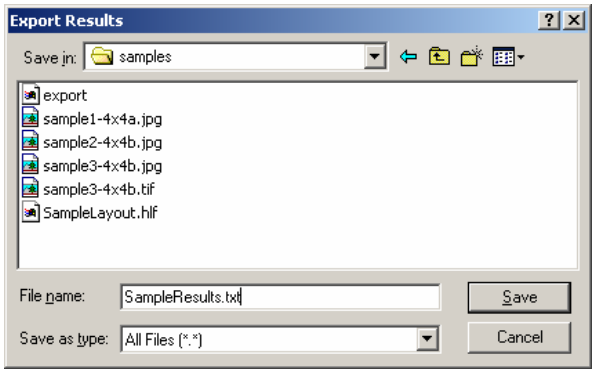


Figure 33: The file chooser for data output.

Once the data has been saved, you may view and manipulate it using any text editor. Figure 34 shows an example of viewing the data in the Windows operating system using MS Notepad.

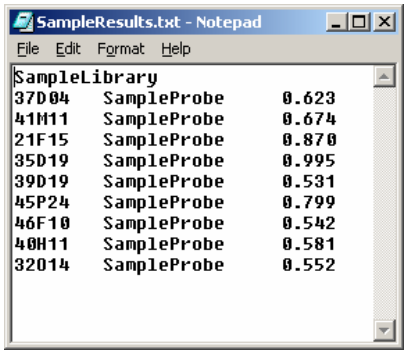


Figure 34: Viewing the exported data in MS Notepad.

 

## 7.2 STANDARD – QUANTIFICATION DATA

To export quantification data, press the **Results** *FastFlow* button (Figure 35) when you are in quantification mode.

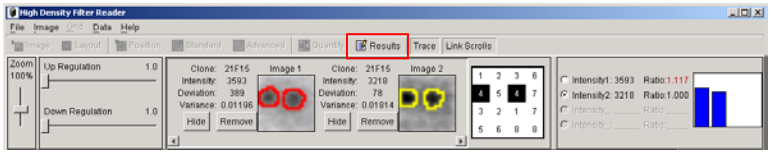


Figure 35: Exporting quantification results by clicking on the **Results** *FastFlow* button.

The following window will appear (Figure 36):

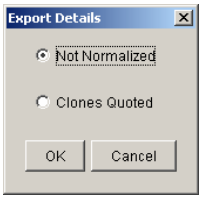


Figure 36: The quantification data export options.

By default, the exported quantification values will be those that have been normalized using the reference clones. To export the raw values before normalization, select the "Not Normalized" option. The "Clones Quoted" option is identical to the corresponding option for exporting clone address identification data.

After saving the data, you can view it using any text editor. The quantification data is exported in tab-delimited format. The first column gives the clone addresses. The next pair of columns gives the intensity values and the standard deviations for filter image 1, the subsequent pair of columns gives the intensity values and the standard deviations for filter image 2, etc. (Figure 37)

 

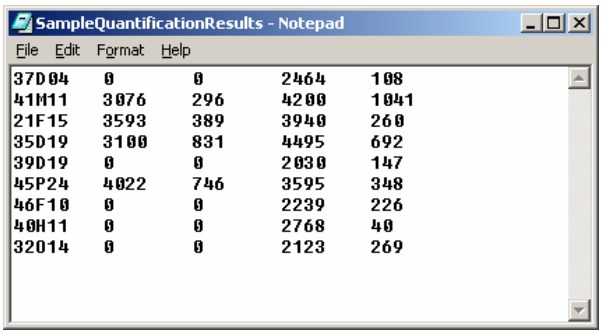


Figure 37: Quantification results for two filter images viewed in MS Notepad. The first column gives the clone addresses, the second and third column give the intensity values and the standard deviations for filter image 1, respectively, the fourth and fifth column give the intensity values and the standard deviations for filter image 2, respectively.

## 7.3 RE-ARRAYING

In addition to exporting the data in the previous format, it can be exported directly for re-arraying using the Genetix Q-bot. To export the data for re-arraying, select **Data->Export Re-arraying Data** from the toolbar, as shown in Figure 38.

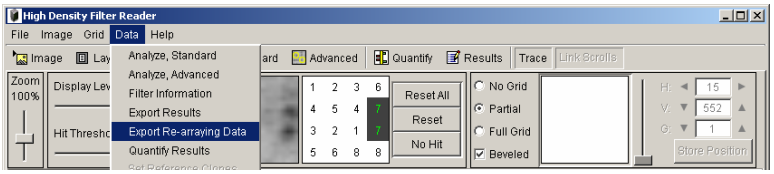


Figure 38: Using the Main Menu to select the **Export Re-arraying Data** option.

The following window (Figure 39) allows you to enter comments and/or bar codes for the plates containing the clones spotted

 

onto the filter. You can use the slider bar to choose the number of the plate for which you would like to enter information. This window also allows you to decide whether to apply the **Display Level** or the **Hit Threshold** to the exported clones. The **Hits / Misses** pull-down menu allows you to decide whether to export all clones with positive hybridization events, or all clones that were "missed" by the probe.

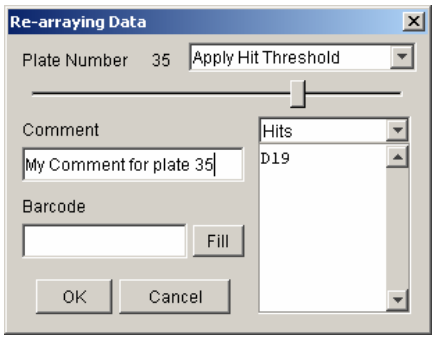


Figure 39: Adding comments, bar codes and choosing parameters for the re-arraying file.

After clicking **OK**, you can select the location and name of the file to which you would like to save the data. Once the data has been saved, you can view and further manipulate it, or use it directly with the Genetix Q-bot. Below is an example of the output file viewed in MS Notepad (Figure 40).

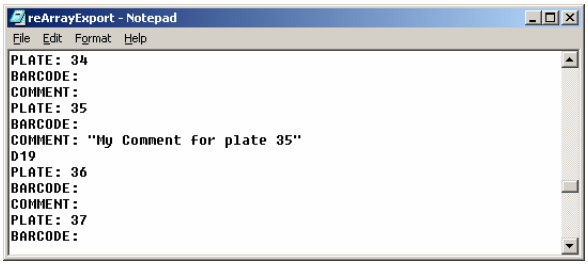


Figure 40: An example re-arraying file.

 

# 8 SUMMARY

By following the example in this tutorial you should have obtained a working knowledge of all essential functions of the HDFR. This information should allow you to use the program to analyze images of your own high-density filters. If you have any additional questions regarding the HDFR, or would like to comment on the program, please do not hesitate to contact INCOGEN by phone at 800-286-6599 or email hdfr@incogen.com. We hope you will enjoy using the HDFR and will find it helpful in your research. We look forward to your feedback!

 

 

# 9 APPENDIX A – ANCHOR SPOTS

Some laboratory protocols provide for the inclusion of anchor spots to aid in the placement of the grid for analysis. Anchor spots can be extraordinarily helpful in this way. However, it may not be desirable to have those spots included in the analysis or results. HDFR provides an easy mechanism for specifying the locations of the anchor spots, and will not include them in the analysis. When defined, the anchor spots will be traced in red and signified in the results table by the capital letter 'A' as shown in the figure below.

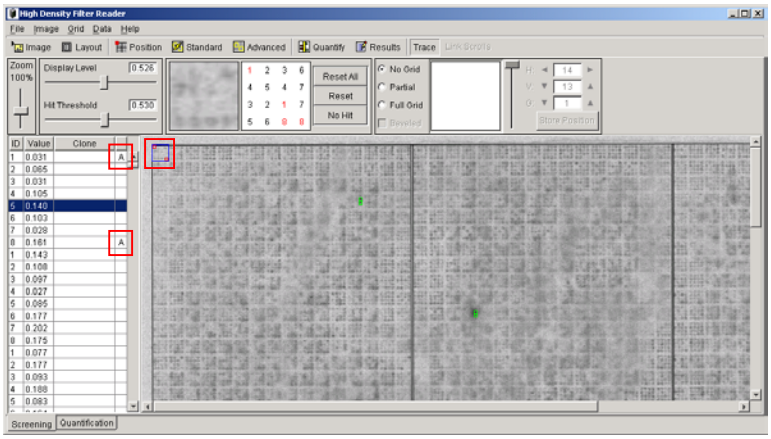


Figure A1: Anchor spots are outlined in red on the image and represented by the letter 'A' in the table.

Anchor spots are saved in the layout and are therefore defined through the Layout Editor (Section 5). The rightmost tab on the Layout Editor is for defining anchor spots, as shown in Figure A2 below.

 

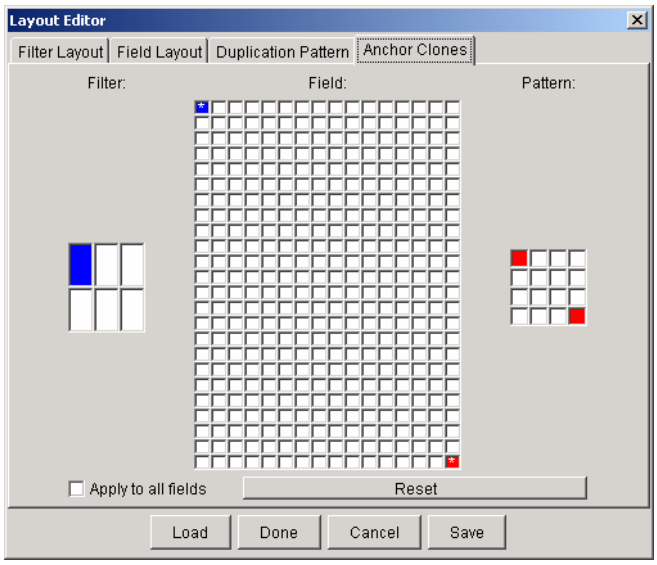


Figure A2: The anchor spots tab of the Layout Editor. On the left is the filter grid; in the middle, the field grid; on the right, the cell grid.

On the left is a small representation of the filter, with the dimensions specified on the Filter Layout tab of the Layout Editor. The top left field is selected by default. This field is represented in the middle by a large grid of small boxes; it has either 96 or 384 cells depending on the selection on the Field Layout tab. The top left cell is selected by default. This cell is represented on the right by a small grid of small boxes; this grid has the same dimensions as the duplication pattern selected on the Duplication Pattern tab.

Note: Changing the dimensions of the filter, field, or duplication pattern on the other tabs will cause any selected anchor spots to become invalid.

To set the top left spot of the top left cell of the top left field on the filter as an anchor spot, the user would click on the top left field in the filter representation on the left, then click on the top left cell in the field representation in the middle, then click on the top left box in the cell representation on the right. The box in the cell grid will turn red,

 

showing that it has been selected as an anchor. The cell in the field grid will display an asterisk ("*"), showing that it contains an anchor spot.

To remove that spot from the set of anchor spots, the user must only click on it again in the cell grid. The box in the cell grid will revert to white. If no other boxes in the cell were selected as anchor spots, the asterisk will be removed from the cell in the field grid.

To remove all spots from a particular cell, the user can click on the cell of interest in the field grid. The asterisk will be removed and all boxes in the cell grid will revert to white.

If all fields contain an anchor spot in the same position of the same cell, the user can select "Apply to all fields" *before* selecting the anchor spot. With this option selected, all choosing and removing of anchor spots will apply to all fields on the filter, not just the current field.

An individual field can be completely cleared of anchor spots by pressing **Reset** when the field is selected in the filter grid and "Apply to all fields" is unchecked. All fields can be reset by pressing **Reset** when "Apply to all fields" is selected.

Anchor spots will be saved in the layout whenever the layout is saved and reloaded the next time the layout is opened. Refer to Section 5 for more information about saving and opening layouts.

 

 

# 10 APPENDIX B – FILTER LOADING PROTOCOL

Two popular ways of gridding samples onto a filter are supported by HDFR. The default method is *cyclic* and the second method is *consecutive*. The choice of loading protocol impacts the names assigned to clones during analysis.

The example in this tutorial uses a filter with six fields, designated on the filter as shown in figures B1 and B2 below, and a 4x4 duplication pattern. Using this layout, the filter can accommodate 48 plates.

In cyclic loading, each field in order is gridded with samples from an individual plate in each cycle. In the first cycle for this example filter, all pattern 1 positions on field 1 are loaded with samples from plate 1, pattern 1 positions on field 2 with samples from plate 2, and so forth. In the second cycle, all pattern 2 positions on field 1 are loaded with samples from plate 7, pattern 2 positions on field 2 with samples from plate 8, and so forth. Using cyclic loading, the configuration of the example filter is shown below. Each field lists the plates from which its samples would be taken and the corresponding patterns the samples would fill.

 

| ***Field 4*** | ***Field 1*** | ***Field 6*** |
|---|---|---|
| pattern 1 => plate 4 | pattern 1 => plate 1 | pattern 1 => plate 6 |
| pattern 2 => plate 10 | pattern 2 => plate 7 | pattern 2 => plate 12 |
| pattern 3 => plate 16 | pattern 3 => plate 13 | pattern 3 => plate 18 |
| pattern 4 => plate 22 | pattern 4 => plate 19 | pattern 4 => plate 24 |
| pattern 5 => plate 28 | pattern 5 => plate 25 | pattern 5 => plate 30 |
| pattern 6 => plate 34 | pattern 6 => plate 31 | pattern 6 => plate 36 |
| pattern 7 => plate 40 | pattern 7 => plate 37 | pattern 7 => plate 42 |
| pattern 8 => plate 46 | pattern 8 => plate 43 | pattern 8 => plate 48 |
| ***Field 2*** | ***Field 5*** | ***Field 3*** |
| pattern 1 => plate 2 | pattern 1 => plate 5 | pattern 1 => plate 3 |
| pattern 2 => plate 8 | pattern 2 => plate 11 | pattern 2 => plate 9 |
| pattern 3 => plate 14 | pattern 3 => plate 17 | pattern 3 => plate 15 |
| pattern 4 => plate 20 | pattern 4 => plate 23 | pattern 4 => plate 21 |
| pattern 5 => plate 26 | pattern 5 => plate 29 | pattern 5 => plate 27 |
| pattern 6 => plate 32 | pattern 6 => plate 35 | pattern 6 => plate 33 |
| pattern 7 => plate 38 | pattern 7 => plate 41 | pattern 7 => plate 39 |
| pattern 8 => plate 44 | pattern 8 => plate 47 | pattern 8 => plate 45 |

Figure B1: An example of a six-field filter with a 4x4 duplication pattern gridded using the cyclic loading method.

To select cyclic loading as the method for a filter, simply choose "Cyclic Loading" under Filter Loading Protocol on the Filter tab of the Layout Editor. Please see Section 5 for a description of the Layout Editor. The loading protocol selection will be saved as part of the layout.

In consecutive loading, each field is gridded with samples until it is completely filled before loading of the next field commences. For consecutive loading of the example filter, field 1 would be loaded as follows: all pattern 1 samples will be taken from plate 1, pattern 2 samples from plate 2, pattern 3 samples from plate 3, and so forth. Field 2 for this example will fill patterns 1-8 with samples from plates 9-16, respectively; field 3 samples will come from plates 17-24. The example filter would be filled as shown below using the consecutive loading protocol.

 

| ***Field 4*** | ***Field 1*** | ***Field 6*** |
|---|---|---|
| pattern 1 => plate 25 | pattern 1 => plate 1 | pattern 1 => plate 41 |
| pattern 2 => plate 26 | pattern 2 => plate 2 | pattern 2 => plate 42 |
| pattern 3 => plate 27 | pattern 3 => plate 3 | pattern 3 => plate 43 |
| pattern 4 => plate 28 | pattern 4 => plate 4 | pattern 4 => plate 44 |
| pattern 5 => plate 29 | pattern 5 => plate 5 | pattern 5 => plate 45 |
| pattern 6 => plate 30 | pattern 6 => plate 6 | pattern 6 => plate 46 |
| pattern 7 => plate 31 | pattern 7 => plate 7 | pattern 7 => plate 47 |
| pattern 8 => plate 32 | pattern 8 => plate 8 | pattern 8 => plate 48 |
| ***Field 2*** | ***Field 5*** | ***Field 3*** |
| pattern 1 => plate 9 | pattern 1 => plate 33 | pattern 1 => plate 17 |
| pattern 2 => plate 10 | pattern 2 => plate 34 | pattern 2 => plate 18 |
| pattern 3 => plate 11 | pattern 3 => plate 35 | pattern 3 => plate 19 |
| pattern 4 => plate 12 | pattern 4 => plate 36 | pattern 4 => plate 20 |
| pattern 5 => plate 13 | pattern 5 => plate 37 | pattern 5 => plate 21 |
| pattern 6 => plate 14 | pattern 6 => plate 38 | pattern 6 => plate 22 |
| pattern 7 => plate 15 | pattern 7 => plate 39 | pattern 7 => plate 23 |
| pattern 8 => plate 16 | pattern 8 => plate 40 | pattern 8 => plate 24 |

Figure B2: An example six-field filter with a 4x4 duplication pattern gridded using the consecutive loading method.

To select consecutive loading as the method for a filter, simply choose “Consecutive Loading” under Filter Loading Protocol on the Filter tab of the Layout Editor. Please see Section 5 for a description of the Layout Editor. The loading protocol selection will be saved as part of the layout.

 