ModFit LT 3.0 and higher Training

Student Workbook

Part A of this workbook should be completed prior to the self-guided or on-site training.

Revision: 5/7/2001

Contents

Getting Started

If you wish, you can follow this introductory section using the files located on the Training CD. You can open them directly off the CD but it is better if you copy the Training folder to the ModFit LT folder on your hard disk. If you prefer not to load the files or do not have the CD, you can refer to the accompanying graphics. We also provide a set of accompanying video AVI files that can be viewed in lieu of this written tutorial.

In this first tutorial, we will frequently refer to two basic ModFit LT operations, “Open File” and “Auto Analysis”.

To open a file:

- Click on the **File** toolbar button.
- Navigate to your main hard disk and locate the folder of interest.
- Click on this folder and a list of files should be displayed on the left side of the dialog box.
- If the files do not appear, check the file filter and change it to “all files”.
- Select the file you want to open and click on the **Open** button.

All of the files we will need for part A are located in the **Generated Data Files** folder. The chart below shows the location of this folder.

Hard Disk (Top)
- ModFit LT
  - Training
    - Configuration Files
    - Data
      - Aneuploid Files
        - Reports
      - Diploid Files
        - Reports
      - Multiploid-Hypodiploid Files
        - Reports
      - Other Files
        - **Generated Data Files**
        - WithStds
      - Proficiency Test Files
        - ExamAnalysis
        - ReferenceReports
        - Reports
      - Tetraploid Files
        - Reports
    - Handouts

To perform an **Auto Analysis**, click on the **Auto** toolbar button. No other action is required.

Part A – What is a DNA Histogram? – Prerequisite tutorial

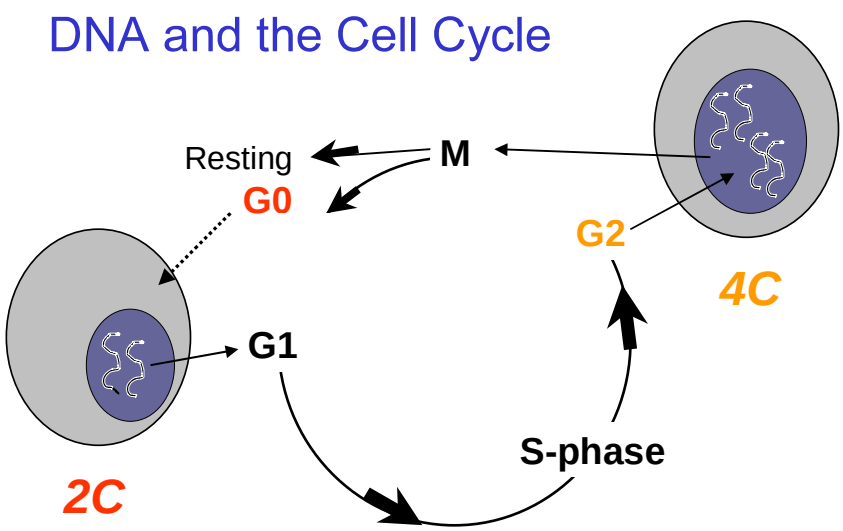


Figure 1

Study guide questions:

- *What is meant by 2C?*
- *How does the DNA content of cells in S-phase compare with cells in G0G1 and cell in G2M?*
- *Can we measure individual chromosomes?*

This diagram (see Figure 1) represents the eukaryotic cell cycle, highlighting the major cycle divisions. A resting cell in G0 ("gap zero") has the normal DNA content of chromosomes, called 2C because it has 2 complements of DNA. Sometimes induced by cytokines, cells will enter G1 and after some time interval will start to duplicate their DNA content. As soon as DNA replication begins, the cells enter into the synthesis phase, or S-phase. Cells in S-phase increase their DNA content as they progress through this stage. At the end of S-phase, cells enter G2 and later enter the stage of mitotic division, M, which produces two identical daughter cells, each with 2C DNA content.

Why do we measure total nuclear DNA? The use of DNA dyes such as propidium iodide (PI) provides a relatively easy means to determine how many cells are in each major stage of the cell cycle. In general, increased S-phase suggests an active cycling population. Note that this is not measuring individual chromosomes, but total DNA content only.

DNA Measurement

- DNA content + preparation + instrument noise

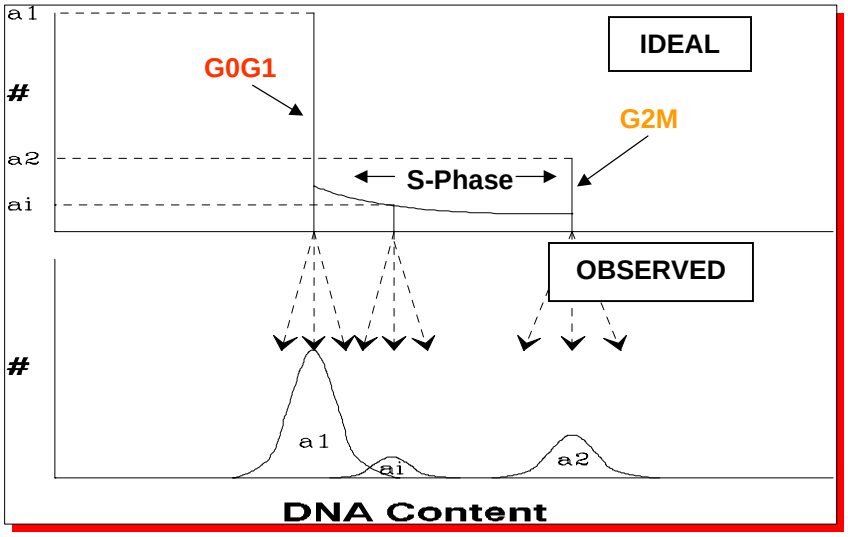


Figure 2 *Verity Software House, Inc.*

Study guide questions:

- *What is the expected relationship of the G2M and G0G1 peak positions?*
- *Why is a G0G1 peak shaped the way it is?*
- *What type of model component is typically used to model S-phase?*
- *What is the expected range of the debris distribution?*
- *Where would you expect to find aggregates?*

If we could make ideal measurements of the DNA content of cells in various stages of the cell cycle, we would see something like the upper panel in Figure 2. The number of observations is represented on the y-axis, and DNA content by the x-axis. We call such a distribution an ideal one-parameter DNA histogram, and it serves as a basis for our modeling analysis.

In practice, however, the measurements have some intrinsic error in them, influenced by biological factors such as sample source; electronic factors such as light source, detector and amplifier noise; and physical factors such as instrument alignment and optical filters. These factors broaden each point in the ideal histogram, as shown in the lower panel to form the DNA histogram we typically observe.

Now let's look at a representative simple DNA diploid histogram (di, or two-ploid).

We should note that the prefix "DNA" is used with diploid and as we'll see later, aneuploid, to distinguish the cytometric definition from its cytogenetic counterpart, (Cytometry 5, 445-446 (1984)). Instead of referring to a histogram as diploid we say DNA diploid.

❑ In ModFit LT, open the file DNA00.FCS.

In this simple example (see Figure 3), only the G0G1, S-phase and G2M compartments are shown; there are no debris or aggregate events. The small black triangles below the x-axis indicate the most probable peaks in the histogram.

Note, these files were created with a program that generates artificial histograms. This program allows us to specify percentages and location for each population. We use these generated histograms as "truth" for evaluating ModFit LT's models.

Let's do an automatic analysis; click on the Auto toolbar button on the toolbar. In Figure 4, the G0G1 and G2M peaks are modeled as Gaussian distributions, and the S-phase is estimated by a broadened rectangle. The lower graphic is an expanded view of the y-axis, useful for visualizing small distributions like the S-phase and aggregates. Note also that the G2M peak position is about twice the G0G1 peak position, as we would expect from the cell cycle characteristics reviewed earlier. For simple DNA histograms like this example, we expect the calculated S-phase (7.67%) to be very close to the actual S-phase (7.69%).

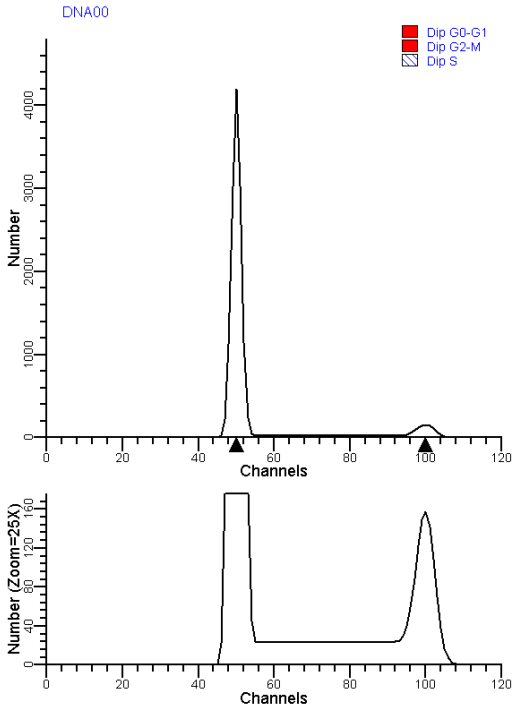


Figure 3

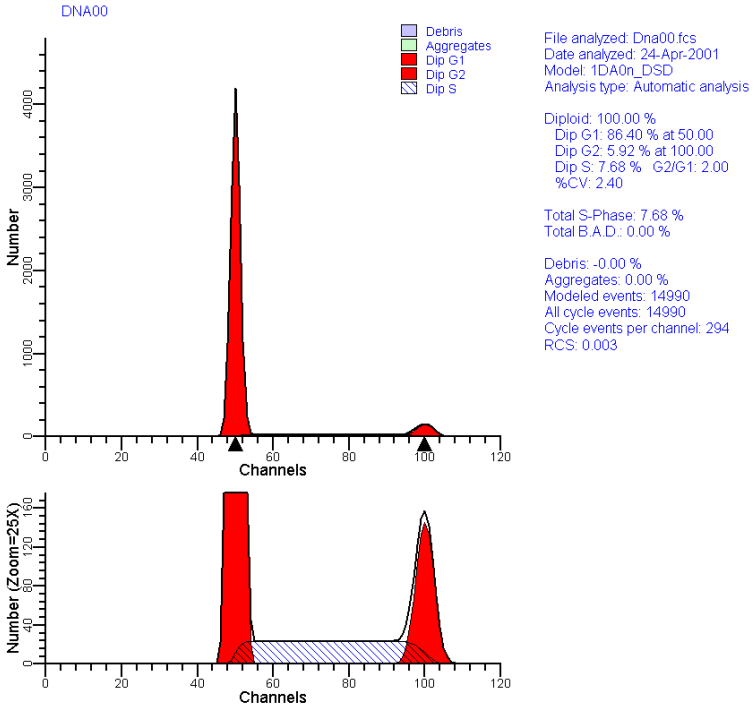


Figure 4

Let's add a little complexity to the histogram.

- Open the file DNA01.fcs

Notice the debris events downscale of the G0G1 peak, (see Figure 5).

An "event" in a listmode file contains a set of measurements that arises from a particle passing through the flow-cell that satisfies the acquisition trigger threshold setting.

Although it is more obvious below the G0G1 peak, keep in mind that the debris distribution underlies the entire histogram. Debris events are typically fragmented cells and/or nuclei produced during the sample preparation and staining, and may also arise from necrotic cells present in the original sample.

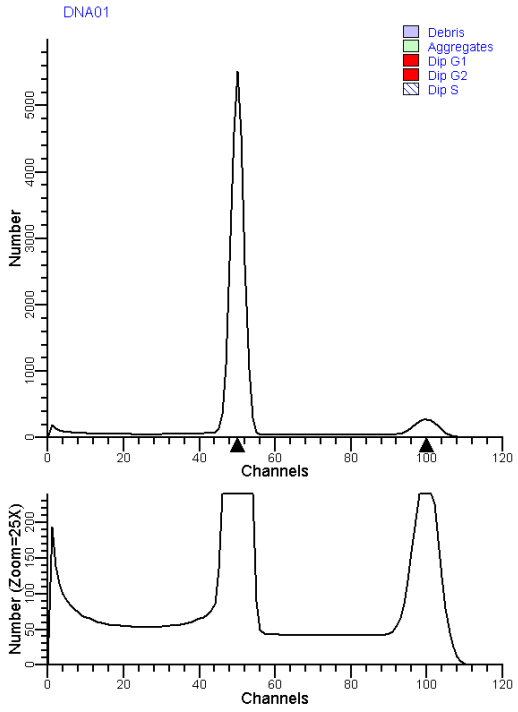


Figure 5

- Click the **Auto** toolbar button to see how the debris is modeled.

Even with the debris events underlying the G0G1, G2M and S-phase, the calculated S-phase (7.17%) is very close to the actual S-phase (7.05%).

Note: Debris modeling works best when the threshold or discriminator is as low as possible as represented in this example.

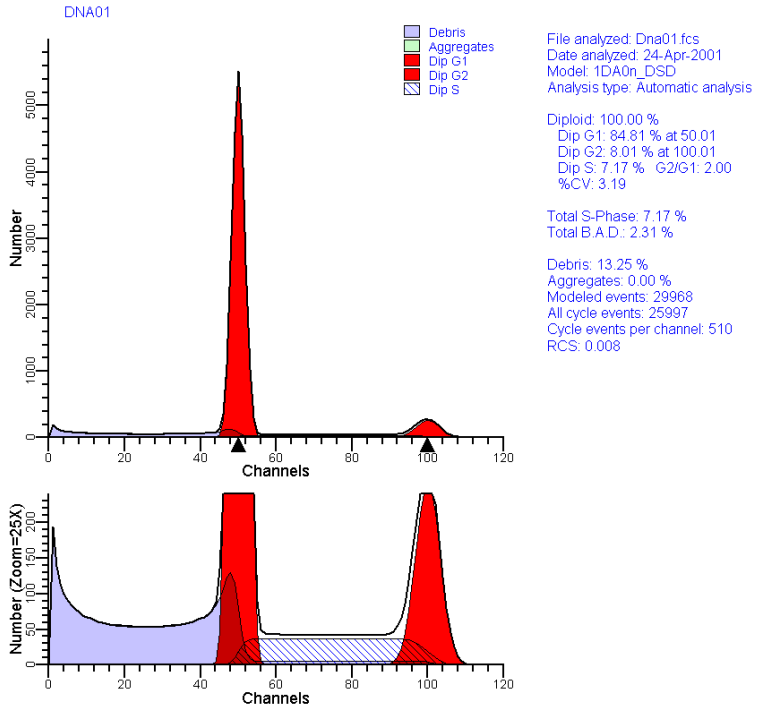


Figure 6

Now let's add the last component that we will need to consider, aggregates. Note in Figure 7 the additional peaks upscale in the histogram. These additional peaks represent aggregates between all events that make up the DNA histogram, the most notable being the doublet and triplet aggregates of the G0G1 peak. The amount and distribution of the aggregate forms are dependent on the sample source and preparation and may be highly variable. Be aware that an aggregate can include interactions between nuclei or between debris and nuclei.

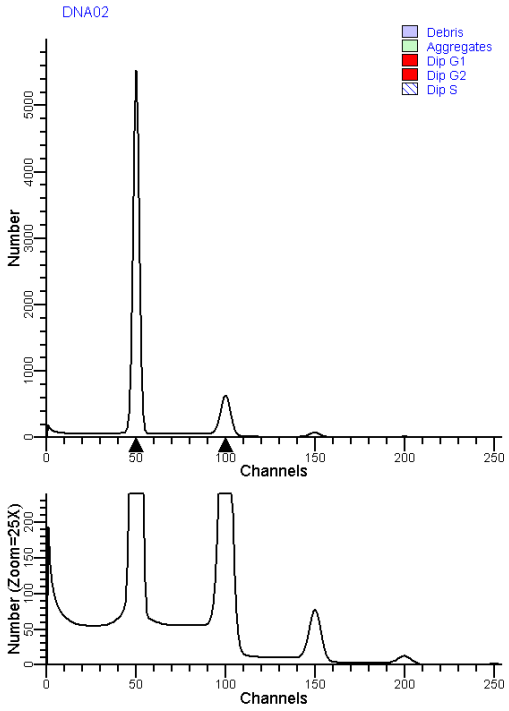


Figure 7

- Click the **Auto** toolbar button.

The aggregates are now modeled along with the other types of events. Notice in Figure 8 that the aggregate distribution extends under the G2M and G0G1 peaks and throughout the S-phase region. Even with aggregate events in the histogram, it is still possible to get good correlation between the calculated S-phase (6.64%) and the actual S-phase (7.05%).

You have seen a representation of the various model components in a typical DNA histogram: Gaussian, broadened rectangle, debris and aggregate distributions. Now, we're ready to look at even more complex data.

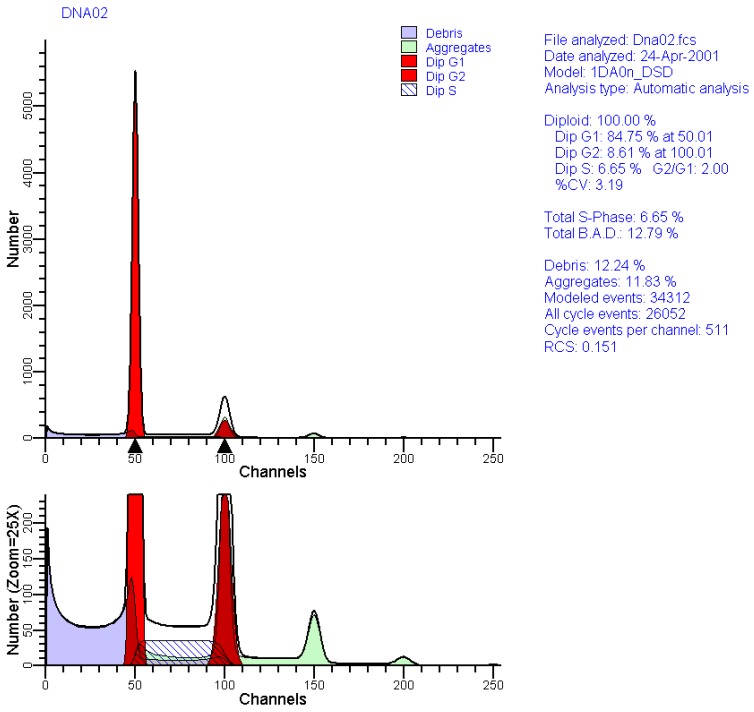


Figure 8

Study guide questions:
- What is a DNA aneuploid population?
- What is a DNA Index (DI)?
- Can you model a separate DNA aneuploid S-phase?
- How should you handle overlapping S-phase distributions?
- What is a DNA tetraploid population?
- Does the model chosen say anything about the biology of the sample?

- ❑ Open the file DNA03.FCS.

We have added a second cell cycle (see Figure 9), one that is DNA aneuploid. Although it is a more complex distribution, similar modeling concepts apply. Make sure you recognized the DNA diploid G0G1, DNA diploid G2M, DNA aneuploid G0G1, DNA aneuploid G2M, debris and aggregates.

"Aneuploid" comes from: an, not euploid, meaning not an even multiple of chromosomes.

The DNA index, DI, is defined as the position of the DNA aneuploid G0G1 peak divided by the position of the DNA diploid G0G1 peak. In this example the DI is 1.44.

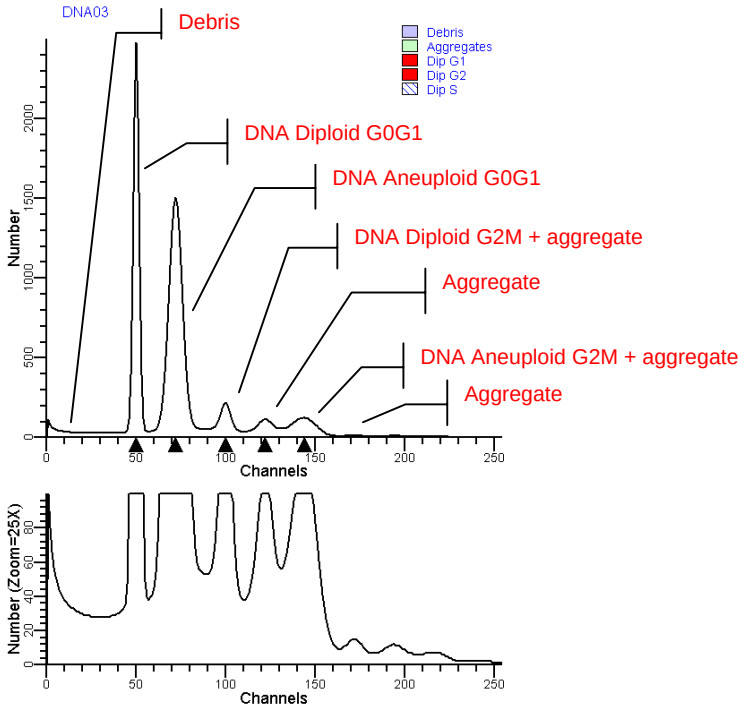


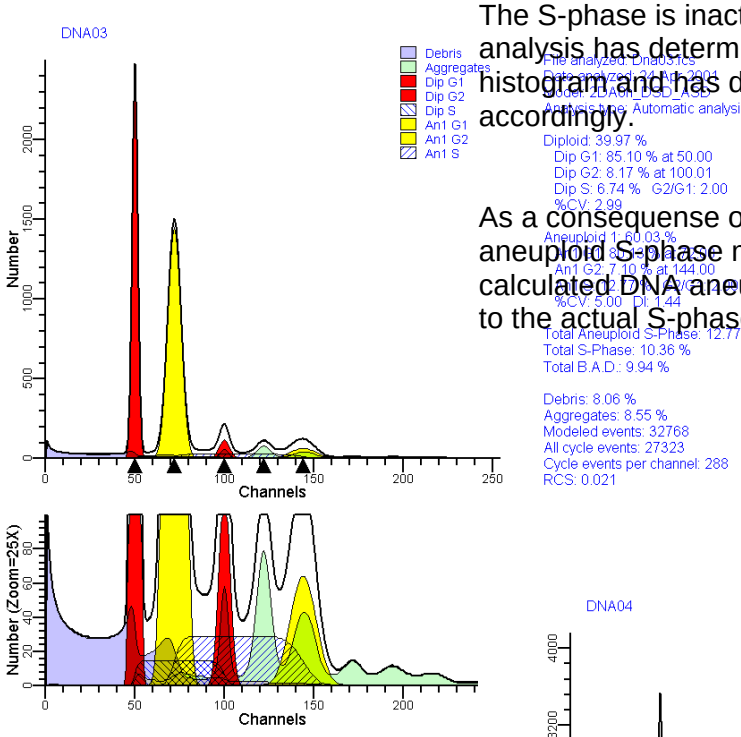
Figure 9

- ❑ Do an automatic analysis.

- ❑ Identify the various modeling components, including the two S-phase compartments (see Figure 10).

Even with the added complexity of the second population, the calculated DNA Diploid S-phase (6.71%) is very close to the actual value (6.91%). More importantly, the calculated DNA Aneuploid S-phase (14.51%) is also very close to the actual value (14.92%).

Figure 10



The S-phase is inactivated because ModFit LT's automatic analysis has determined that the "1.3 DI rule" applies to this histogram and has disabled the DNA diploid S-phase accordingly.

As a consequense of disabling the diploid S-phase, the aneuploid S-phase may be slightly elevated. In this case the calculated DNA aneuploid S-phase (15.22%) is still quite close to the actual S-phase (15.00%).

Figure 12

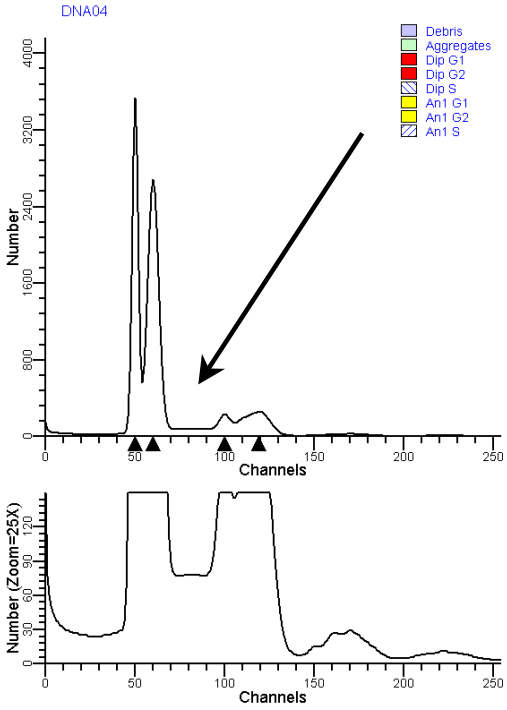


Figure 11

❑ Open file DNA04.fcs, an example of a DNA hyperaneuploid population.

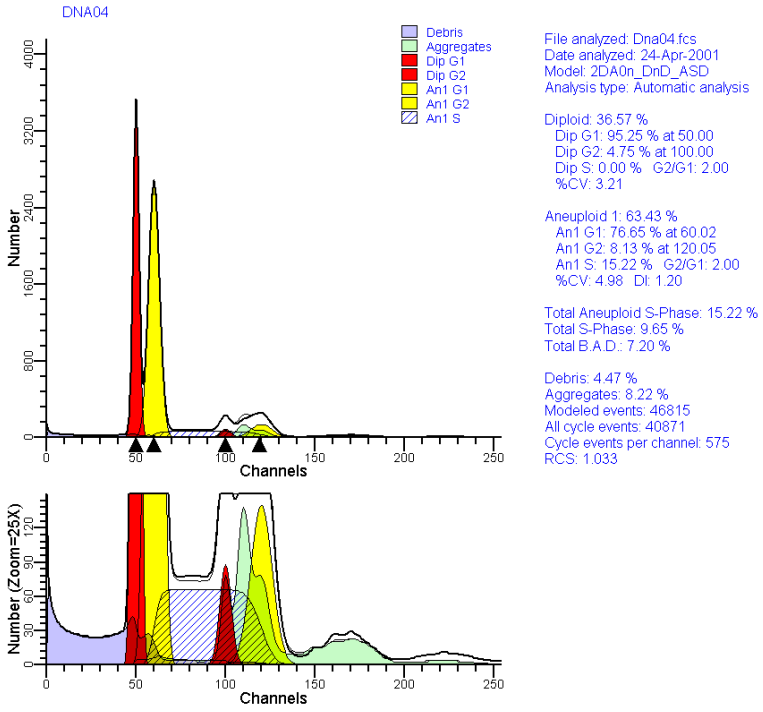
Notice (see Figure 11) that the two S-phase components overlap.

As a general rule, when two components of similar shape overlap significantly, the component of lesser significance should be turned off. **(Rule: II.B.1.a.1)**

As a result of extensive statistical validation studies, Verity Software House has established a rule that we use to determine whether to try to model overlapping S-phase components. It says, "If the DI is between 0.7-1.0 or 1.0-1.3 disable the diploid S-phase and make DNA diploid and DNA aneuploid G2M's dependent." **(Rule: II.B.1.d.2)**

❑ Do an automatic analysis of this file.

Notice (see Figure 12) that the DNA aneuploid DI is 1.20 and the DNA diploid S-phase is reported as zero.



Last, we'll introduce a special case of aneuploidy, that of a DNA tetraploid population.

❑ Open file DNA05.fcs (see Figure 13).

A DNA tetraploid population's G0G1 heavily overlaps the DNA diploid G2M. Another rule, as we saw earlier with the hyperdiploid example, states that overlapping components of similar shape should not be modeled. **(Rule: II.B.1.a.1)** Since the DNA aneuploid population is always of analytical importance, we typically disable the diploid G2M model component for DNA tetraploid histograms.

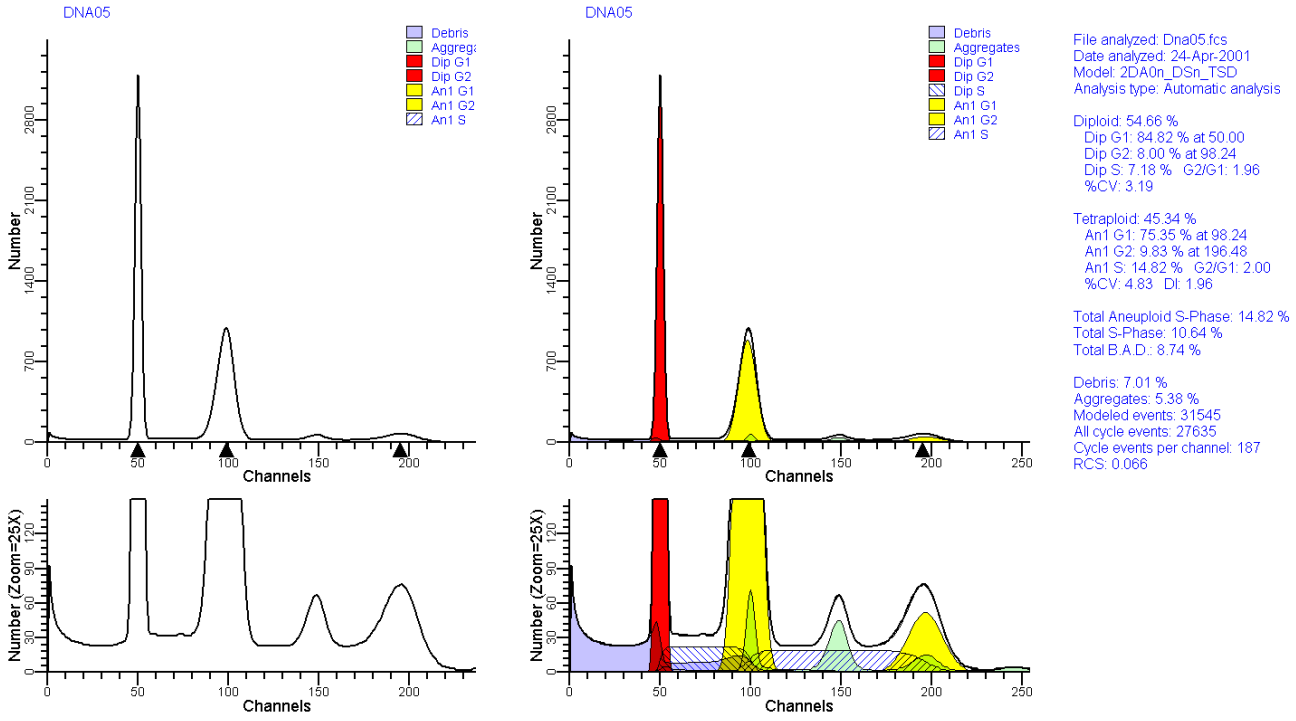
Figure 13

❑ Do an automatic analysis (see Figure 14).

As we discussed above, the DNA diploid G2M is disabled for DNA tetraploid histograms. Note that there is good agreement between the calculated DNA Aneuploid S-phase (14.52%) and the actual (14.87%).

ModFit LT has a special feature to adjust for this known source of error, which we will discuss in the next session.

Figure 14



In this example, the DNA aneuploid population is nearly half of the cycling populations present, so recognizing a DNA tetraploid histogram is not too difficult. There are times, though, when the decision to use a DNA tetraploid model for a proportionally smaller aneuploid population is not as well-defined. Later you will see an example of this when we go through the section on DNA tetraploid data files, and you will use the DNA Analysis Rules to guide your decision process. At this point, we hope you are familiar with typical DNA histogram patterns and how automatic analysis determines the underlying components.

You have learned how a DNA histogram relates to the cell cycle and you have seen that there are many types of events in a DNA histogram. The events in peaks such as G0G1 and G2M are modeled with Gaussian model components, S-phase events are modeled with a broadened rectangle, and debris and aggregates by their respective model components. We need to model the histogram because many of these model components are heavily overlapped.

You have seen that DNA histograms may contain more than one cycling population, with the second cycle called the DNA aneuploid population. You have also seen a special case of DNA aneuploidy, that of a DNA tetraploid population, and have learned some considerations that apply to the analysis of DNA tetraploid histograms. With this introduction in mind, you are now ready for some real data files and to look more closely at DNA analysis.

Study guide questions:

- ***What is meant by 2C?*** Cells that have the normal 2-complement set of chromosomes.

- ***How does the DNA content of cells in S-phase compare with cells in G0G1 and cells in G2M?*** Variable between G0G1 and G2M.

- ***Can we measure individual chromosomes?*** No, only total DNA present in the nucleus.

- ***What is the expected relationship of the G2M and G0G1 peak positions?*** The G2M position is usually about twice that of the G0G1 position.

- ***Why is a G0G1 peak shaped the way it is?*** Sample preparation, instrument and biological factors all contribute to broadening the observed DNA content into roughly a Gaussian distribution.

- ***What type of model component is typically used to model S-phase?*** A single rectangle, a conservative approach, is usually used for typical DNA histograms.

- ***What is the expected range of the debris distribution?*** Debris underlies the entire DNA histogram. The continuity of debris is the main reason why we do not recommend any gating strategy to "remove" debris.

- ***Where would you expect to find aggregates?*** The major aggregate forms will be found at two times (doublets) and three times (triplets) the G0G1 peak position, but other aggregate forms will be distributed throughout the histogram.

- ***What is a DNA aneuploid population?*** A cell cycle that is distinct from the DNA diploid population is called DNA aneuploid. Populations whose G0G1 is below the DNA diploid are called DNA hypodiploid, and those above are called DNA hyperdiploid.

- ***What is a DNA Index (DI)?*** DI is the mean position of the aneuploid G0G1 peak divided by the mean position of the diploid G0G1 peak.

- ***Can you model a separate aneuploid S-phase?*** In most circumstances, yes.

- ***How should you handle overlapping S-phase distributions?*** In general, DNA hyperdiploids with DI's of 1.3 or less and DNA hypodiploids with DI's of 0.7 or greater should have the DNA diploid S-phase compartment turned off.

- ***What is a DNA tetraploid population?*** A true DNA tetraploid population is one whose G0G1 DNA content is 4C; its G2M will be at 8C and an S-phase between the G0G1 and G2M.

- ***Does the model chosen say anything about the biology of the sample?*** Not necessarily. The model is chosen only to match a set of observations about the DNA histogram. For example, a histogram with one cycling population usually uses a "diploid' model, whether or not the G0G1 is truly a 2C DNA content. A DNA tetraploid model may be used for a DNA aneuploid population whose G0G1 significantly overlaps the diploid G2M position.

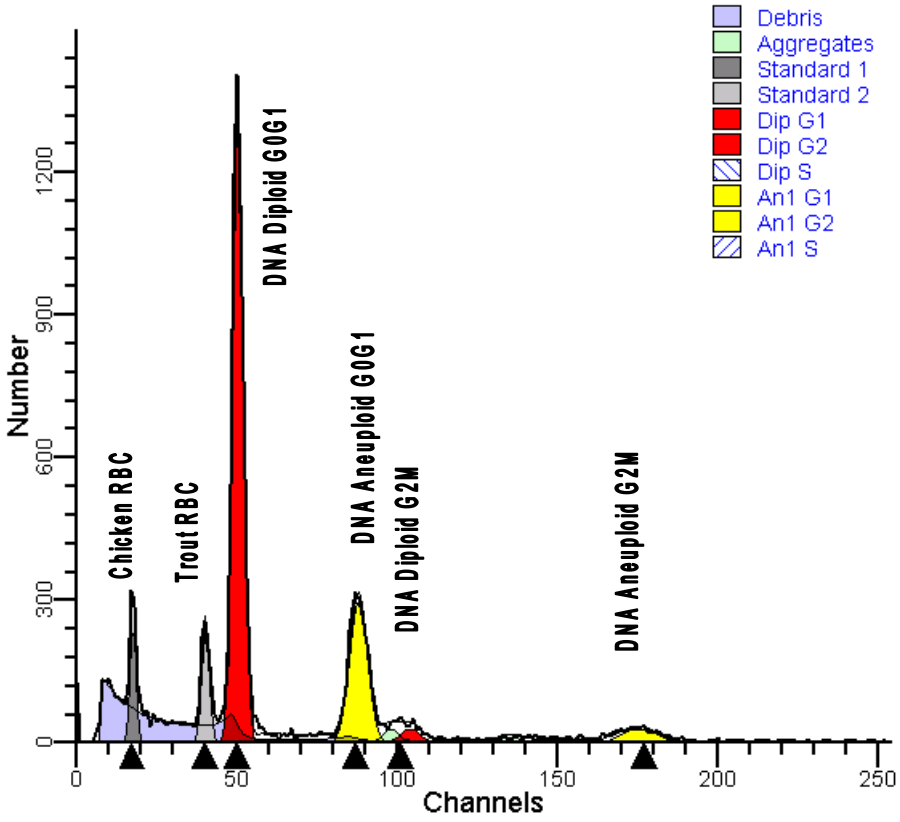
Part B – Introduction / Training overview – 5 minutes

This training is based on the DNA Histogram Analysis Rules found in Appendix 1 and has a goal of maximizing your interaction with the program during the training process. The rules are largely the result of an extensive review of thousands of data files with known clinical outcome by investigators from the US and Europe. (Bagwell CB, Baldetorp B, Clark GM, Spyratos F, et. al. Optimizing Flow Cytometeric DNA Ploidy and S-phase Fraction as Independent Prognostic Markers for Node-Negative Breast Cancer Specimens. Cytometry 2001 (June): Vol 46, #3).

This rule set is designed to greatly reduce the variability of results obtained by different operators who may otherwise follow different paths at key analysis decision points. Examples of such decision points include making a G2M dependent on G0G1 or allowing it to float; allowing a component to be modeled or disabled; and deciding if a given file is to use a DNA aneuploid or a DNA tetraploid model.

The example files used in this training are from a reference laboratory data set. To ensure that the program's **Auto Analysis** functions match the training files, we will load three program configuration files in part E: the **Auto Analysis** settings file, StdAutoSettings.aas; the peak finder settings file, StdPeakFinder.pf and the configuration settings file, StdConfiguration.mcf.

The histogram below shows a typical modeled histogram containing two standards, a DNA diploid and aneuploid population.

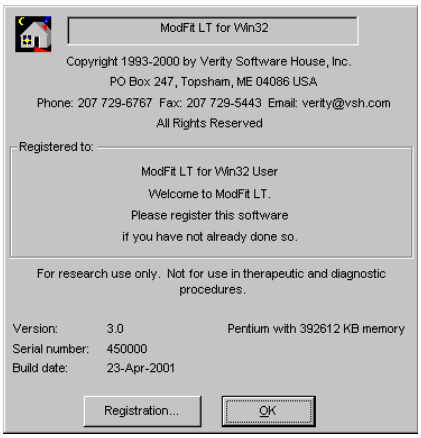


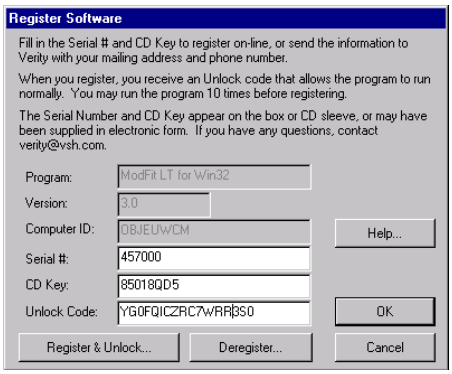
Part C – ModFit LT overview – 5 minutes

- Open ModFit LT 3.0 or above on your computer.

After the program is loaded, the **Program Banner** dialog box is displayed (see left figure below). The banner page contains phone, fax and email information for Verity Software House as well as program information such as the version, serial number and build date. It also contains two buttons. The **Registration** button will take you to the **Register Software** dialog box (see right figure below). The **OK** button will close the **Program Banner** dialog and allow you to start using the program.

*If the program is not registered, the **Register Software** dialog will be displayed when you start the program. See "Registering your new software" section in the ModFit LT User Guide to properly register the software.*

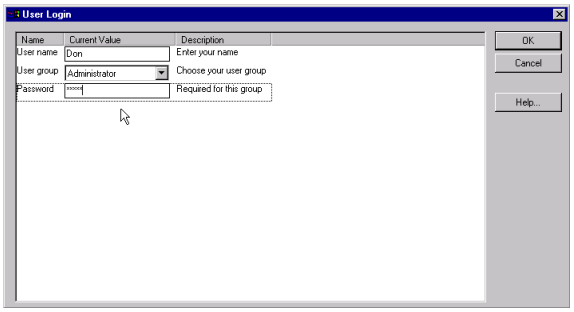




- Click the **Banner** dialog box's **OK** button.

The user login dialog is now displayed. It contains a **User Group** drop-down listbox containing the options, "Standard User", "Advanced User" and "Administrator". Each of these user levels has different associated "permissions" and controls access to certain program functions. A more detailed description of these **User Groups** categories and their permission functions can be found in the online Help and in the manual.

To configure ModFit LT, you must login as an "Administrator". If you are not an "Administrator" for ModFit LT, ask your administrator to perform the configuration steps in part E.



- Enter your first name, select Advanced User and type "Epsilon" for the password.

You will see only asterisks as you type each letter of the password.

- Click on the **OK** button.

The user login dialog box will disappear and you will now see the default application window.

- Click on the File toolbar button. 

Files can be opened using either the **File** button or the **File** menu. Reports can only be opened from the **File** menu.

- Navigate to the “Generated Data Files” folder.

This folder is the same one used in the first section. To show the subfolders inside a folder, double-click on the folder icon. This operation will expand the folders contents by one level. You can also double-click on the parent folder again to hide the lower levels.

- Select the data file “DNA00.FCS” from within the “Generated Data Files” folder and click on the **Open** button.

When you click on the **Open** button, the program will load and display a histogram in its “report-based” window. Notice that the un-modeled histogram is placed onto the report page in a default location and can be moved or resized as desired.

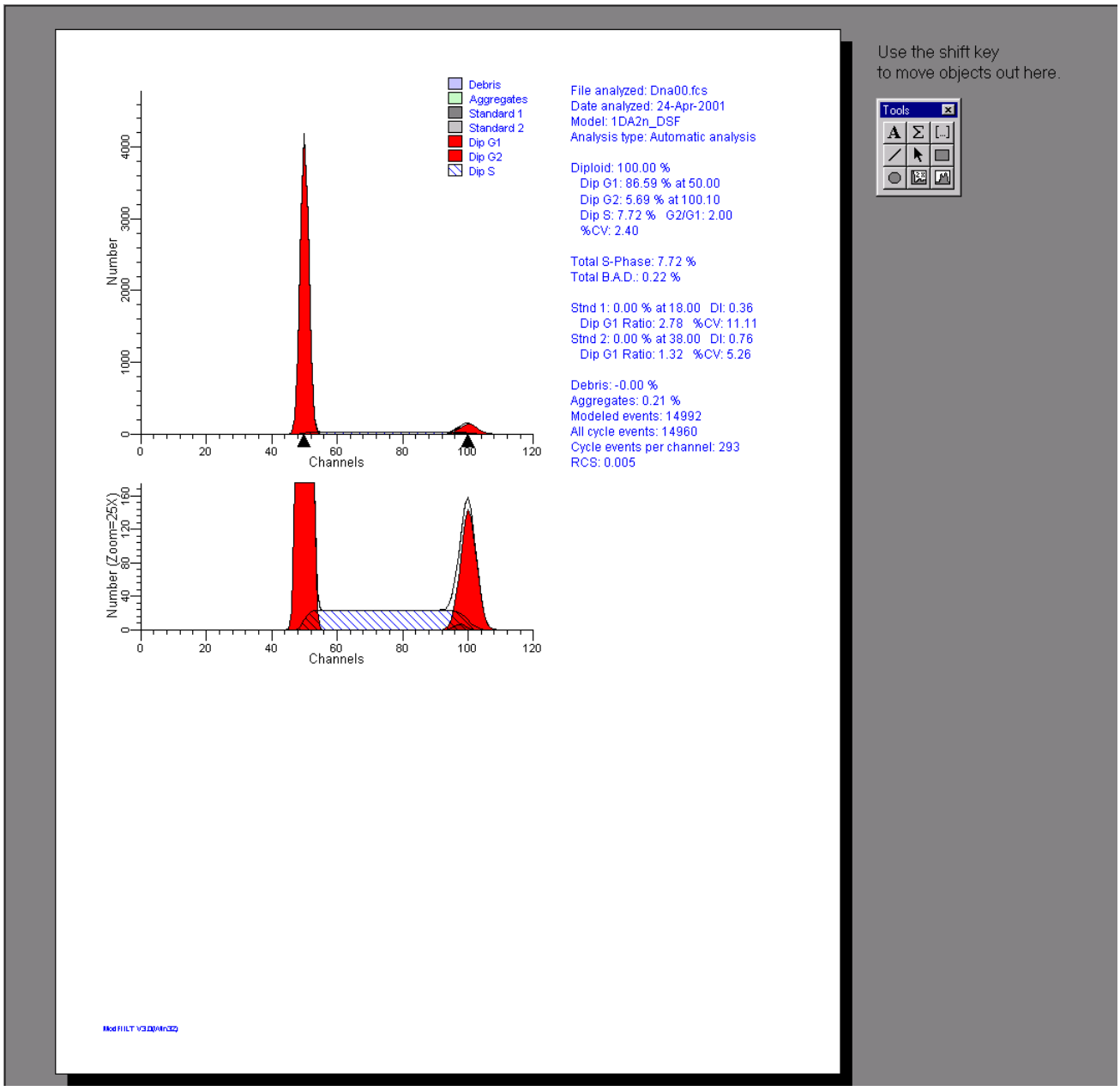
Once the cell cycle analysis is performed, the program will place a default set of results on the report page as well. Other graphics and result elements can be added to make this report a truly customized document. As we will see in the following tutorials, the format can be saved and used as a template for future analysis sessions.

Now that we have the program open and some data to work with, there are a couple of important program areas that need to be identified. Let’s start at the top of the screen. Here you will see a set of menu items (**File**, **Edit**, **View**, **Analysis**, **Tools**, **Help**). Underneath the menus are a line of buttons called “toolbar” buttons. These buttons provide a fast and easy way to access common program options.

Note that the buttons are arranged from left to right in the general sequence used for a typical analysis.

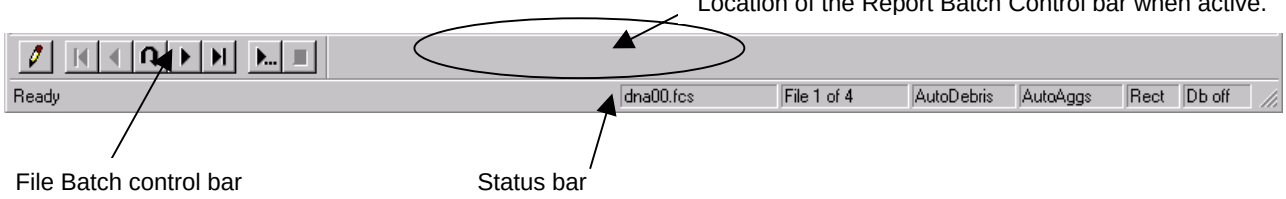
Below the buttons is the application window, containing the report page.

Note, you may move report objects off the report page by holding down on the Shift key while dragging the object off the page.



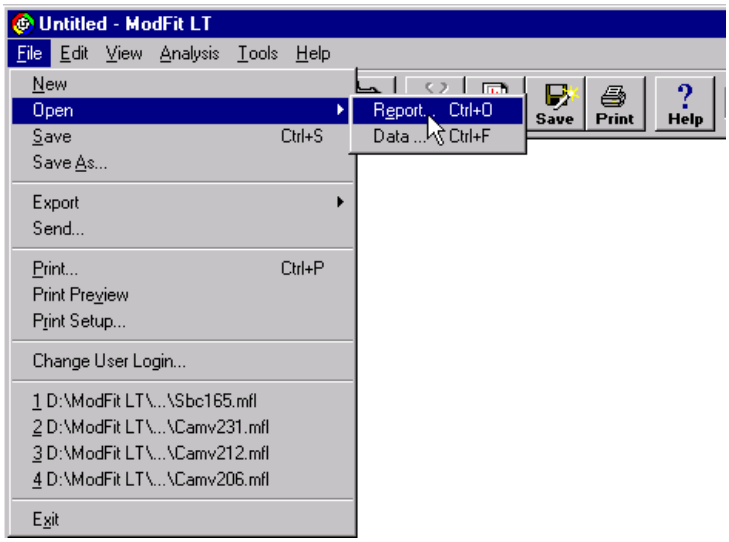
File Batch (see below) and **Report Batch** control bars are displayed below the application window when multiple files or reports are selected. Just below the batch control bar area is the **Status** bar. The **Status** bar contains information such as region position values when moving or adjusting a region; **AutoLinearity** status, name of the currently loaded file, batch item counter, flags for when **AutoDebris** and/or **AutoAggregates** are active, the type of S-phase shape used and the **Database** active status.

Note, when multiple reports are selected, the Report Batch control bar will be displayed in the area to the right of the File Batch control area.

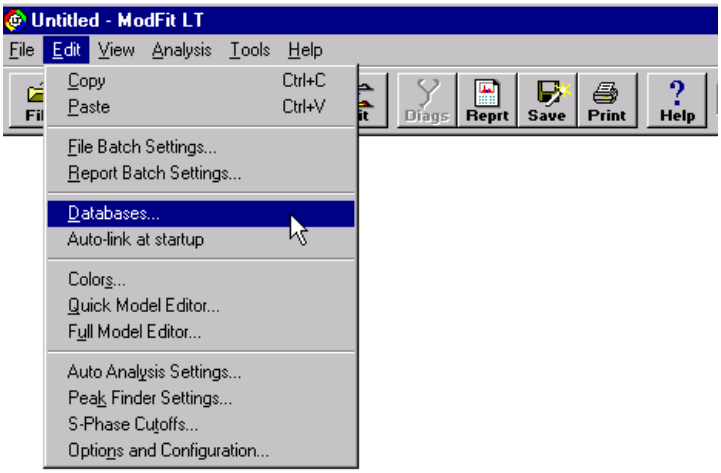


Under the **File** menu, you will find the menu item, "Open...**"**. When this option is selected, two additional sub-menu items appear, "Report…" and "Data…".

*The "Open->Report" menu is the only place you can open reports but "Open->Data" performs the same function as clicking on the **File** toolbar button.*



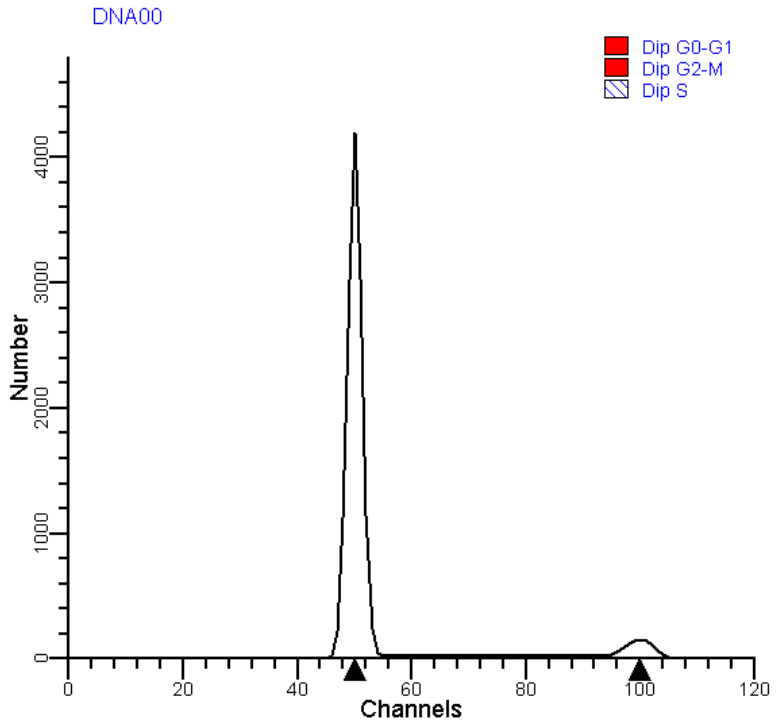
The **Edit** menu contains a number of other useful commands such as "Database...", "Quick Model Editor...", "Full Model Editor...", and "Options and Configuration...". You will have need of all these editing options in the later tutorials.



- Leave this data file open and let's move on to the next tutorial section.

Part D – AutoAnalysis: how it works. – 15 minutes

What are the black triangles under the DNA histogram graph (see figure below)?



A black triangle indicates a statistically significant peak that also satisfies a set of user-defined filters. The properties that control the sensitivity of finding "real" peaks are located in the **PeakFinder Settings** dialog box (**Edit** menu). Automatically identifying "real" peaks is quite important for the proper functioning of the **Auto Analysis** system. In the next tutorial, we will further examine these settings to better understand how they can optimize **Auto Analysis** for your specific type of data files.

- Click the **Auto** toolbar button. 

When you click on the **Auto** button, the program evaluates all the peaks found by the **Peak Finder** clustering system and then methodically determines a most probable DNA Ploidy pattern that is compatible with all the peaks identified. For example, if the algorithm finds a peak at channel 50, it then will examine the region around channel 100 for a second peak. If one is found, the **Auto Analysis** system further evaluates this peak to determine if it might be a G0G1 of a DNA aneuploid population or the G2M of the first peak. In this manner, the program incrementally builds a mathematical model that should best analyze the data. For this system to work well with your data, the **Peak Finder** and **Auto Analysis** settings need to be optimal for your laboratory. Normally, the default settings will do an adequate job, but a little effort on your part to tailor the system will have solid long-term benefits.

> *The **Auto Analysis** system can and will make mistakes and it is your responsibility to detect and fix these mistakes when they occur.*

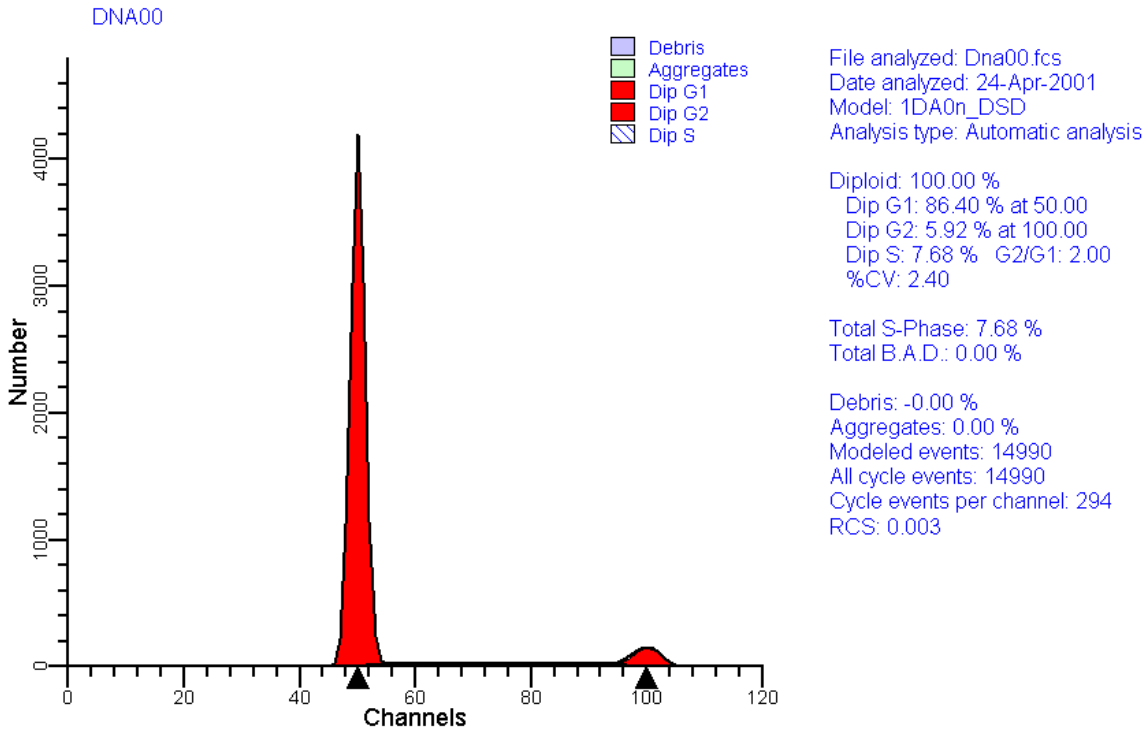
After creating the model, the program automatically determines the range positions and estimates for each model component. Starting with these initial estimates, the program then tries to minimize the differences between the model and the observed data by changing each model parameter according to a

method first published by Marquardt. The general idea behind modeling is that when the mathematical model's parameters are finally adjusted to minimize their difference to the observed data, they are assumed to represent the underlying biology represented by the histogram.

The figure below shows the histogram after **Auto Analysis**. Each model component is assigned a color, which aids in the understanding of the final fit. For example, both the G1 and G2 of the DNA diploid part of the model are colored "red". The smooth bold line that appears above each of these colored model components is the model's best-fit line (not well seen in this example). It should run through the data as close as possible. The analysis result that quantifies how well the model fits the data is the RCS or Reduced Chi-Square value. RCS values of less than five generally represent valid fits of the data. In this particular example, since the data has no statistical noise, the RCS is extremely low, 0.003. You will never see a RCS this low for typical DNA histogram data.

- ❑ Familiarize yourself with the layout of the model fit and the equation results.

We will go into a lot of detail on how to interpret this information in the later tutorials, G-K.



Part E – Peak Finder, Auto Analysis and Configuration Settings.

The files contained in the subsequent tutorials are from a few European laboratories that have a defined staining and acquisition protocol. For example, all the files have two internal standards, chicken and trout RBC's. During acquisition, the operators tried to align these standards to defined target channels. The preparative protocols used fresh or frozen tissue, which generally results in lower %CV's than paraffin embedded samples.

All these factors contribute to a particular type of DNA histogram that can be optimally analyzed by a specific set of **Peak Finder**, **Auto Analysis** and **Configuration** settings. In this tutorial, we will make sure your ModFit LT program has the benefit of these settings and we'll show you what you should do to optimize them for your data.

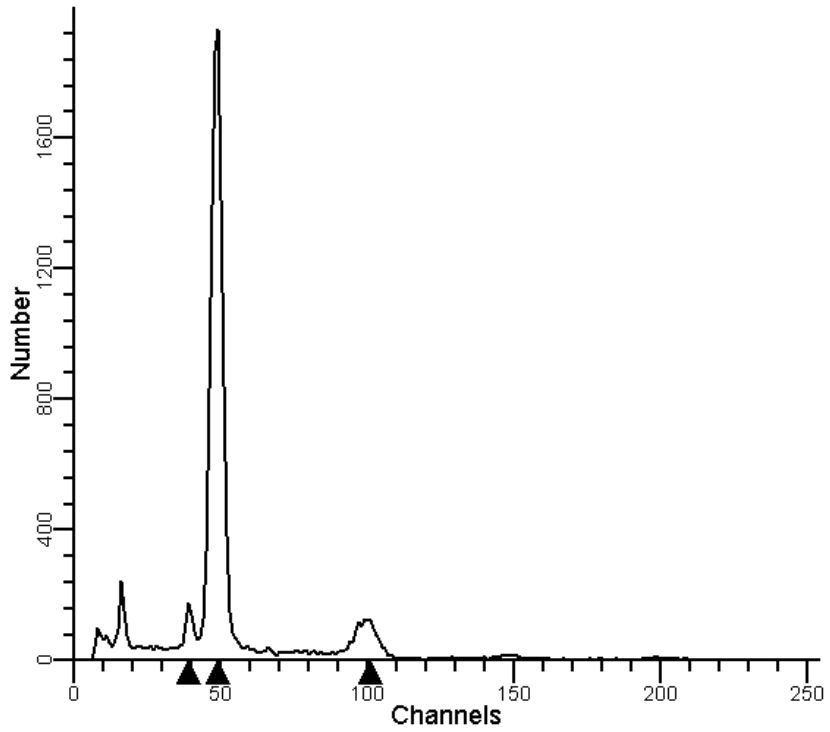
Let's load in one of these tutorial files to better examine the effects of these settings.

- Navigate to the "Diploid Files" and load "DipExample2".

  File location: "Training \ Data\ Diploid Files"

- Choose FL2-A as the parameter to use and then click **OK**.

  Do not click on the Auto toolbar button!



The first two peaks are the chicken and trout RCS's (see figure above). Depending on your current settings, the first peak may not have a black triangle associated with it.

One of the reasons standards are used in a histogram is to help detect DNA hypodiploids. A reference standard is needed to unambiguously determine the position of the DNA diploid

population, particularly when dealing with DNA hypodiploids and DNA hyperdiploids. You will see examples of these special histograms later when you work with DNA aneuploid files.

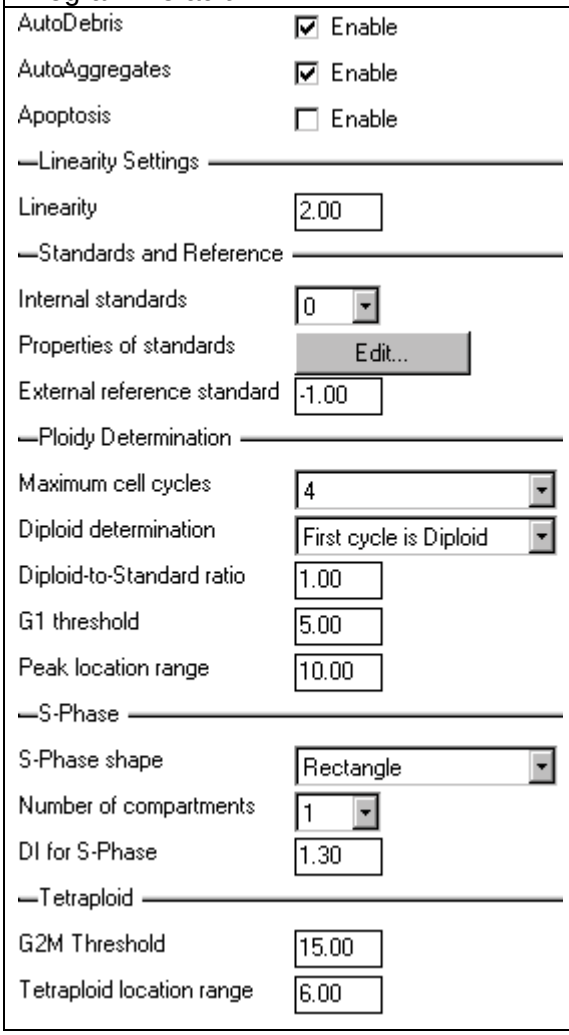
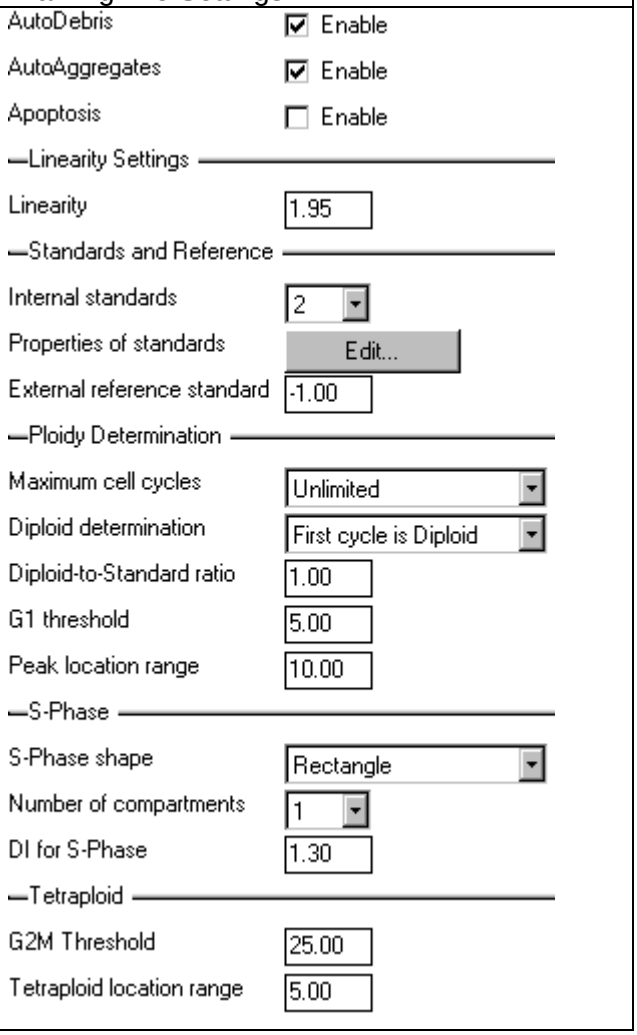
Loading Auto Analysis Settings

We will now load in all the settings that should make the analysis of this data a lot easier.

- From the **Edit** menu, choose "Auto Analysis Settings...".
- Scroll down to and click on the **Open…** button.
- Navigate to the "Configuration Files" folder (Training\Configuration Files\)
- Select the file *StdAutoSettings.aas* and click on the **Open** button.

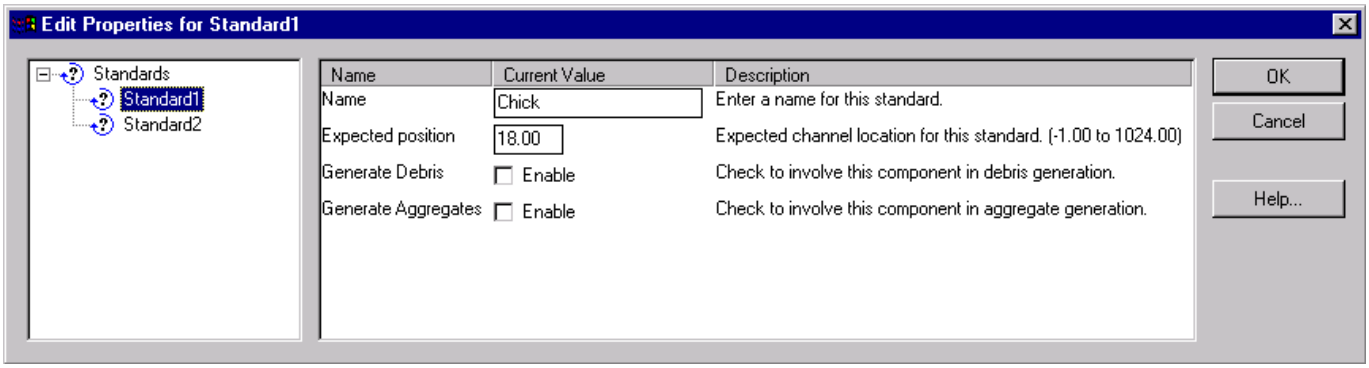
If you do not see the file, set the file filter to "show all files".

The two figures below show the differences between "Default" and "Training" file settings.

Program Default	Training File Settings
	

- Click on the **Edit**… button for the **Properties of standards** control.
- Click on the “Standard1” entry.

This dialog allows you to name the standard and to define its expected position in the histogram. The Standard2 object contains similar information for the second standard in this data set.



- Click on the “Standard2” entry to see how it has been set.

On a 256-channel histogram, the position of standard1 should be at channel 18 and the position of standard2 should be at channel 38. You can calculate the ratio of the position of either standard to the position of the DNA Diploid G0G1 population. This ratio can be used to help **Auto Analysis** determine the ploidy of the samples.

- Click on the **OK** Button to close the **Edit Properties for Standards** dialog box.
- In the **Edit Properties for Auto Analysis Settings** dialog box, scroll down to the **Ploidy Determination** section.

The **Ploidy Determination** option is a very powerful tool that can be a used to locate DNA hypodiploid populations and DNA aneuploid populations showing only one cycle.

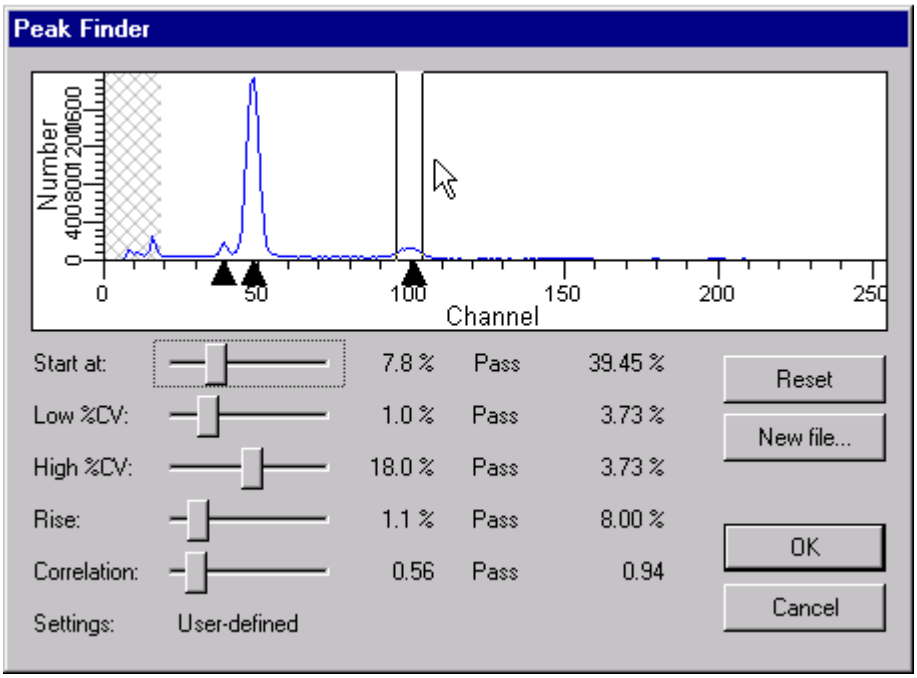
Note that the ploidy determination has been set for “First Cycle is Diploid”. This option has been intentionally left this way to make some points later on. If you had standards in your samples, you could set this option to use one of the standards to determine the expected location of the Diploid G0G1.

- Click the **OK** button to close the **Edit Properties for Auto Analysis Settings** dialog. These settings will be discussed later during the analysis and review of the data files.
- Click on the **Yes** button to save these settings as program defaults.

Loading Peak Finder Settings

- From the **Edit** menu, choose “Peak Finder Settings…”.
- Click on the **Show** button.

A new window (see figure below) will display the currently loaded histogram and the current **Peak Finder** settings.



- Slowly move your mouse pointer over the histogram.

The program will display two lines surrounding statistically significant peaks. In the lower part of the window, you will see a list of the **Peak Finder** filters; slide bars to adjust these options, the current **Peak Finder** settings, a column that indicates whether the currently selected peak passes each criteria and the peak’s actual value for that criteria or filter.

- Move the slide bars to change the criteria and see the peak filtering effect.

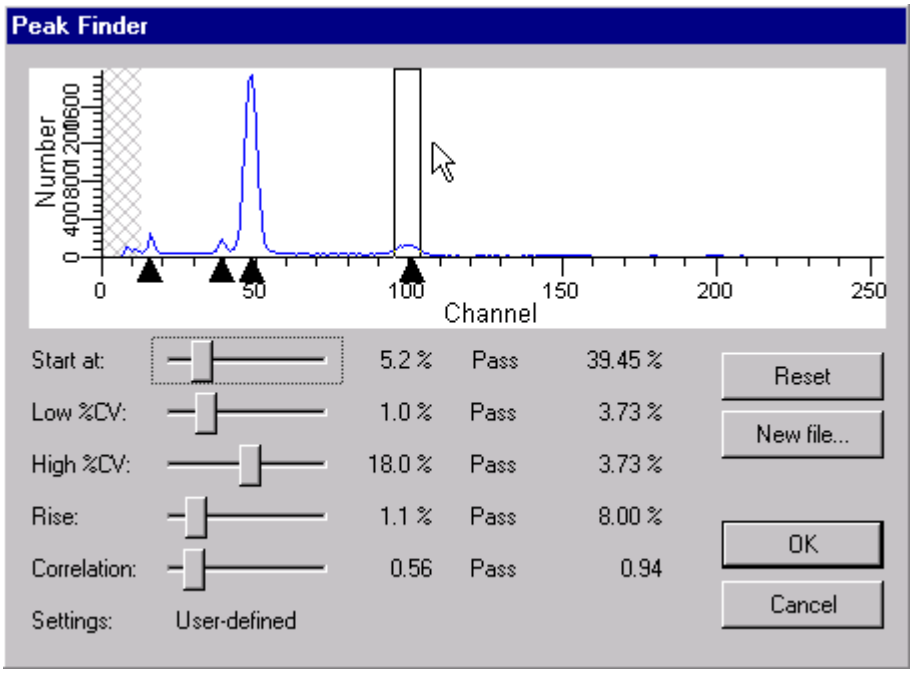
Don’t worry about changing these settings because you are about to reset them all in a moment. This technique is an easy way to find out why the program didn’t find a peak that you believe is there or is finding peaks that you don’t believe are peaks.

- Click on the **OK** button to close the **Peak Finder** dialog box.
- Scroll down to and click on the **Open…** button.
- Navigate to the Configuration Files folder (Training\Configuration Files\)
- Select the file *StdPeakFinder.pf* and click on the **Open** button.

If you do not see the file, set the file filter to “show all files”.

- Click on the **Show** button again to see the effect of these new **Peak Finder** values.

You should now see five black triangles indicating that the program has found four peaks.



*Note that you can restore the default **Peak Finder** values at anytime by clicking the **Reset** button in this dialog.*

The two figures below show the difference between the default values and the values set for the training files.

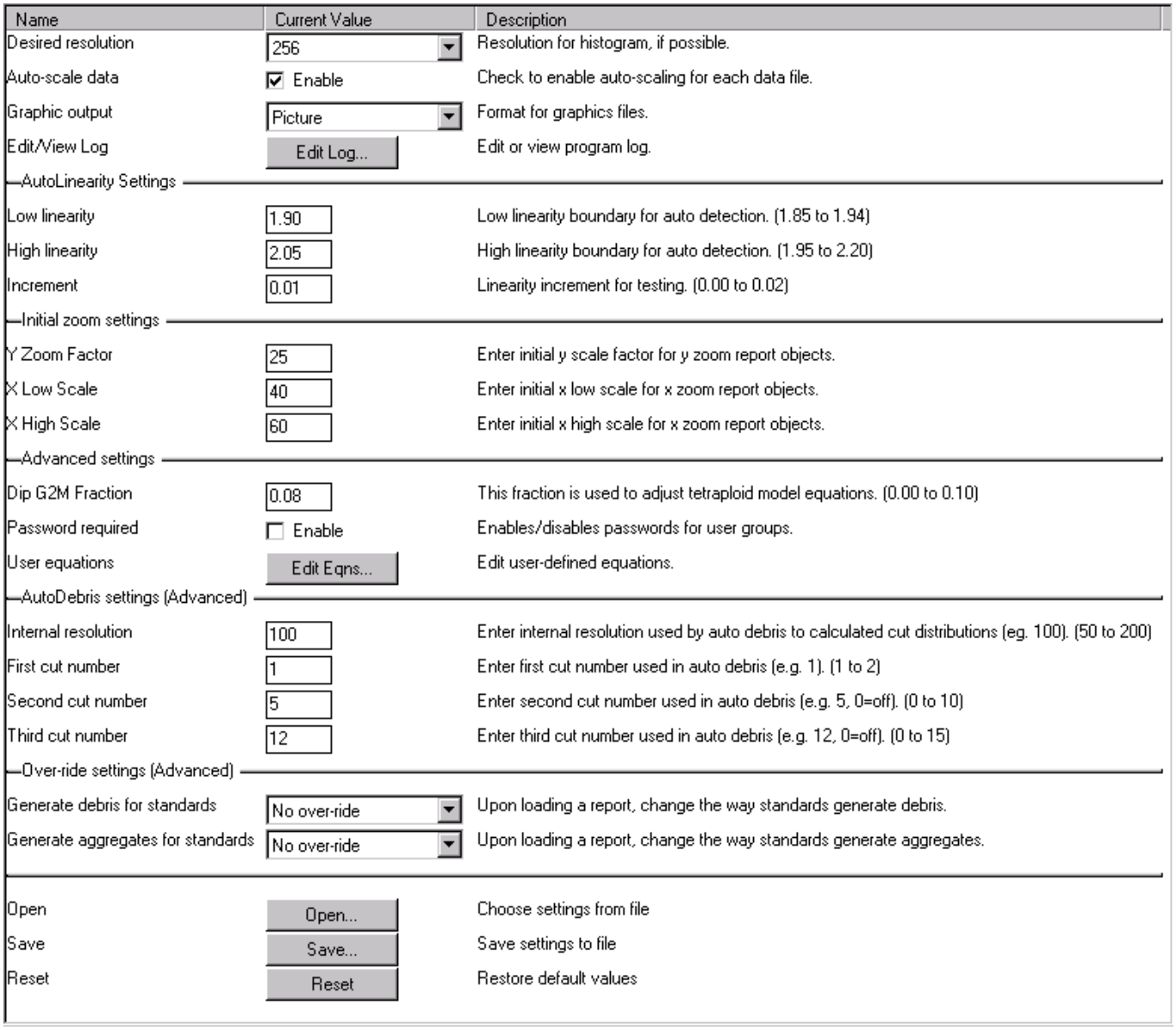
Program Default	Training file settings
Start at 10.00 Low %CV 1.00 High %CV 10.00 Rise 3.00 Correlation 0.65	Start at 5.20 Low %CV 1.00 High %CV 18.00 Rise 1.10 Correlation 0.56

- Click **OK** to close the **Edit Properties for Peak Finder Settings** dialog.
- Click the **Yes** button to save these settings as program defaults.

Options and Configuration

- From the **Edit** menu, choose “Options and Configuration…”.

The **Edit Properties for Options and Configuration** dialog box (see figure below) contains other configuration items. You are encouraged to review the manual or on-line help for a full description of these options.



Name	Current Value	Description
Desired resolution	256	Resolution for histogram, if possible.
Auto-scale data	☑ Enable	Check to enable auto-scaling for each data file.
Graphic output	Picture	Format for graphics files.
Edit/View Log	Edit Log...	Edit or view program log.
AutoLinearity Settings		
Low linearity	1.90	Low linearity boundary for auto detection. (1.85 to 1.94)
High linearity	2.05	High linearity boundary for auto detection. (1.95 to 2.20)
Increment	0.01	Linearity increment for testing. (0.00 to 0.02)
Initial zoom settings		
Y Zoom Factor	25	Enter initial y scale factor for y zoom report objects.
X Low Scale	40	Enter initial x low scale for x zoom report objects.
X High Scale	60	Enter initial x high scale for x zoom report objects.
Advanced settings		
Dip G2M Fraction	0.08	This fraction is used to adjust tetraploid model equations. (0.00 to 0.10)
Password required	☐ Enable	Enables/disables passwords for user groups.
User equations	Edit Eqns...	Edit user-defined equations.
AutoDebris settings (Advanced)		
Internal resolution	100	Enter internal resolution used by auto debris to calculated cut distributions (eg. 100). (50 to 200)
First cut number	1	Enter first cut number used in auto debris (e.g. 1). (1 to 2)
Second cut number	5	Enter second cut number used in auto debris (e.g. 5, 0=off). (0 to 10)
Third cut number	12	Enter third cut number used in auto debris (e.g. 12, 0=off). (0 to 15)
Over-ride settings (Advanced)		
Generate debris for standards	No over-ride	Upon loading a report, change the way standards generate debris.
Generate aggregates for standards	No over-ride	Upon loading a report, change the way standards generate aggregates.
Open	Open...	Choose settings from file
Save	Save...	Save settings to file
Reset	Reset	Restore default values

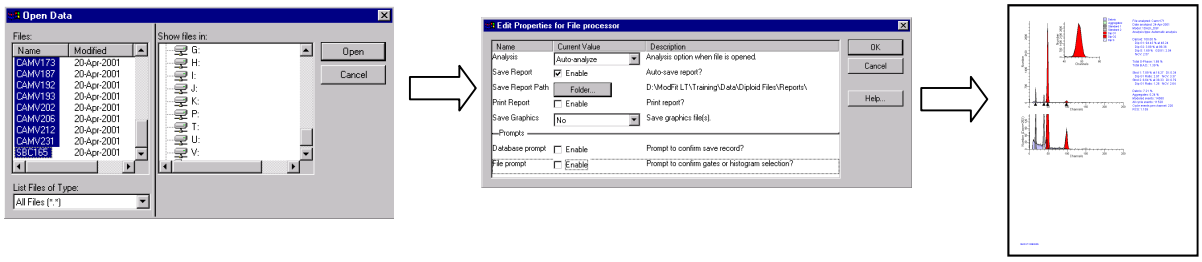
- Scroll down to and click on the **Open…** button.
- Navigate to the Configuration Files folder (Training\Configuration Files\)
- Select the file *StdConfiguration.mcf* and click on the **Open** button.
- If you do not see the file, set the file filter to “show all files”.

You are now ready to start analyzing files. The first part of the next section will discuss the general strategy you will use for each set of files. You will then go through each data set using this strategy.

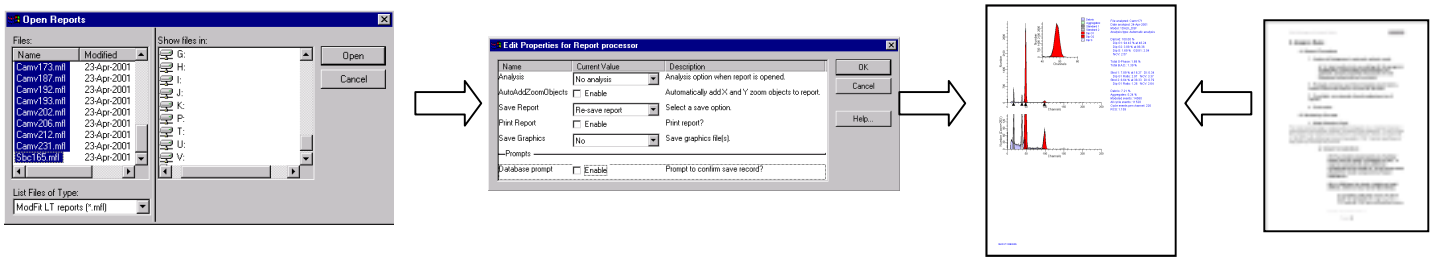
Part F – File Analysis and Review General Strategy – 5 minutes

The recommended file analysis strategy is a five-phase process.

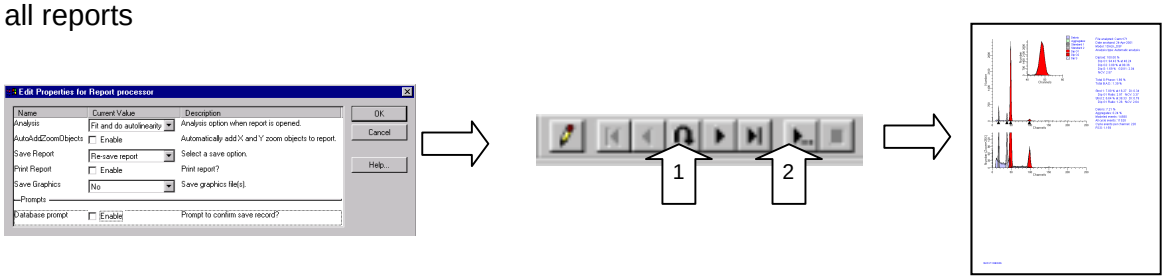
Phase 1 – **Auto Analysis** and report saving. Load the DNA report template and analyze all files using automatic analysis in batch mode, automatically saving reports.



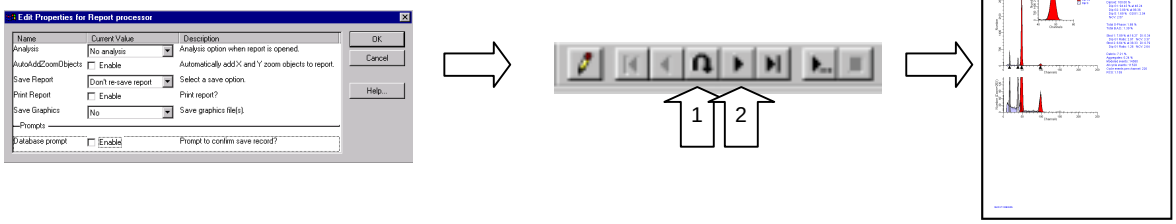
Phase 2 – Review of **Auto Analysis** reports. Load and review each stored report. The reviewing process is guided by a set of DNA analysis rules (see appendix 1). Each reviewed report is automatically re-saved. Of all the phases, Phase 2 requires the most attention.



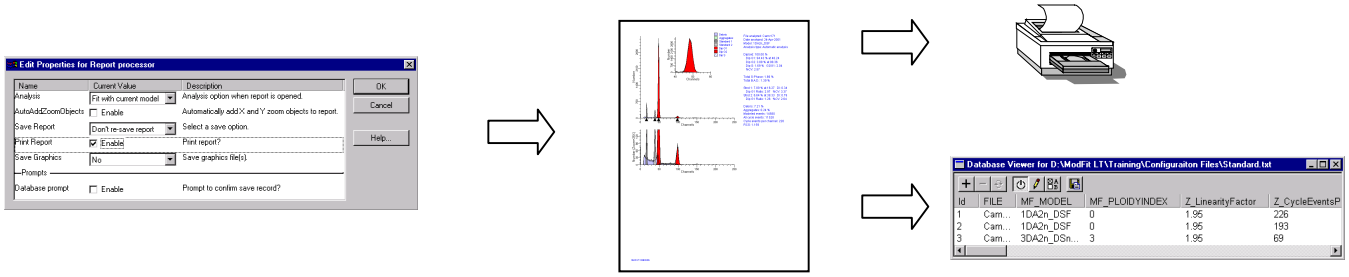
Phase 3 - Automatic Linearity Factor Detection. Using **Batch Report** processing, run **AutoLinearity** on all reports



Phase 4 - Final review. Final review of all reports making sure **AutoLinearity** did not create any problems.



Phase 5. Automatic report printing and/or database generation.



You will use the first four phases for each set of tutorial data files. This approach to DNA analysis is designed to efficiently and reproducibly analyze large numbers of histograms.

Note: The files in this training set are arranged into DNA diploid, DNA tetraploid, DNA aneuploid, and DNA multiploid / hypodiploids for training purposes only. This separation is neither required nor expected for routine analysis.

Part G - DNA diploid files – 40 minutes

DNA diploid histograms have one cell cycle and are reasonably easy to analyze. In the next few sections, we will use the four-phase approach to DNA analysis (see Part F for overview). The DNA histogram examples in this section have been carefully chosen to demonstrate some important aspects of the analysis, setting the stage for the more complex analyses that follow. As much as possible, we will introduce program features and cell cycle analysis strategies as we analyze the example files. In order to streamline these training tutorials, we will try to refrain from needless repetition. However, on a periodic basis, we will review the covered material.

Phase 1 – Perform Auto Analysis and generate report files.

We will first load our DNA report template, select the DNA diploid files, properly configure the **File Batch** control and then process all the files using **Auto Analysis**.

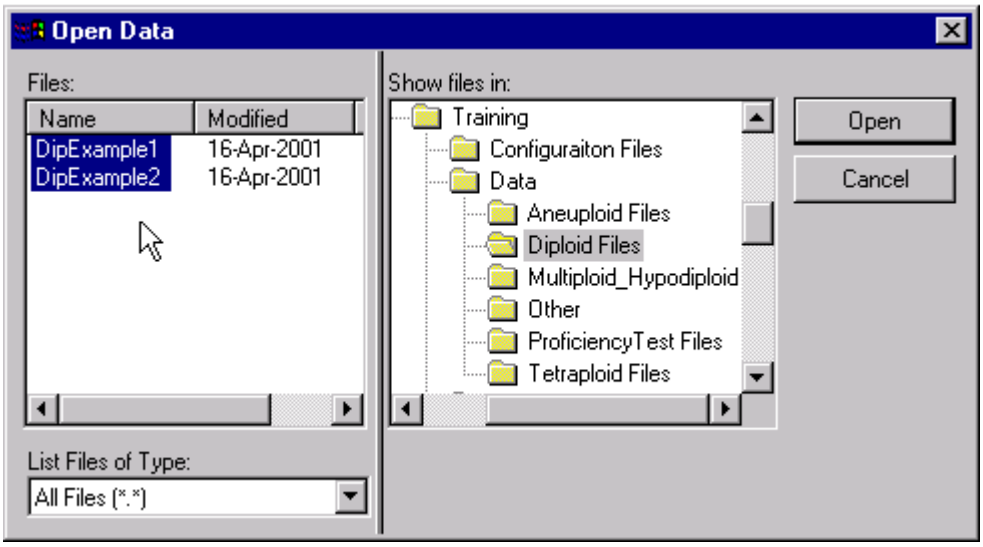
- Click on the **File** menu and choose **Open Report...**.
- Navigate to the folder under “Data”, select the file, “DNAReportTemplate.mfl” then click on **Open**.

Loading the DNA report template automatically configures all the report graphics. In these tutorials, we will be introducing you to the benefits of X and Y zoomed graphic objects. These report objects are automatically loaded with the report template. The report template is a convenient and efficient method for preparing the system for cell cycle analysis. After you have finished these tutorials and feel comfortable with DNA analysis, you may consider further tailoring the report for your own laboratory needs (see Report Design in the ModFit LT User Guide).

- Click on the **File** button on the toolbar (first toolbar button on left).
- Navigate to the “Diploid Files” folder.

Remember to click on the “Diploid Files” folder in the tree control shown on the right-side of the dialog. The files contained in this folder will be displayed in the listbox on the left-side of the dialog. If the file names do not appear, change the file filter to “all files”.

- Multiple-select all of the diploid example files (see figure below).

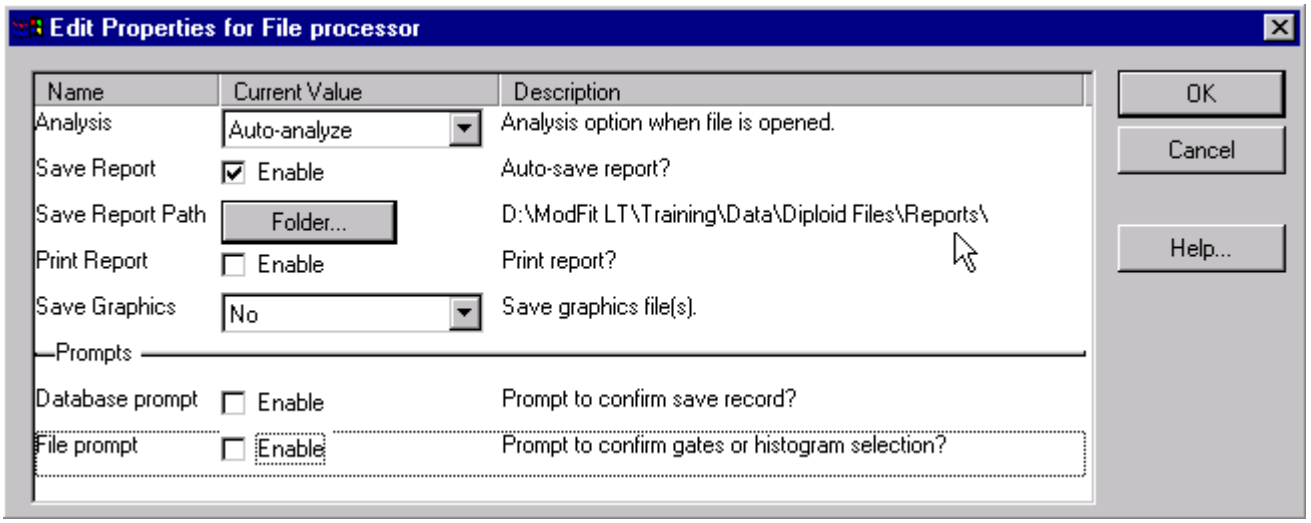


Selecting multiple files can be done a couple of ways. One of the easiest is to select the first file, press and hold the Shift key on the keyboard, and then click on the last file.

- Click the **Open** button.
- Select “FL2-A” as the analysis parameter.

When ModFit LT detects that the file contains multiple histograms, it automatically displays a dialog box with a list of parameters associated with each stored histogram. DNA cell-cycle analysis is usually done with the red fluorescence integrated or area signal.
If the data file were in a listmode format, the program would follow this dialog with further gating options.

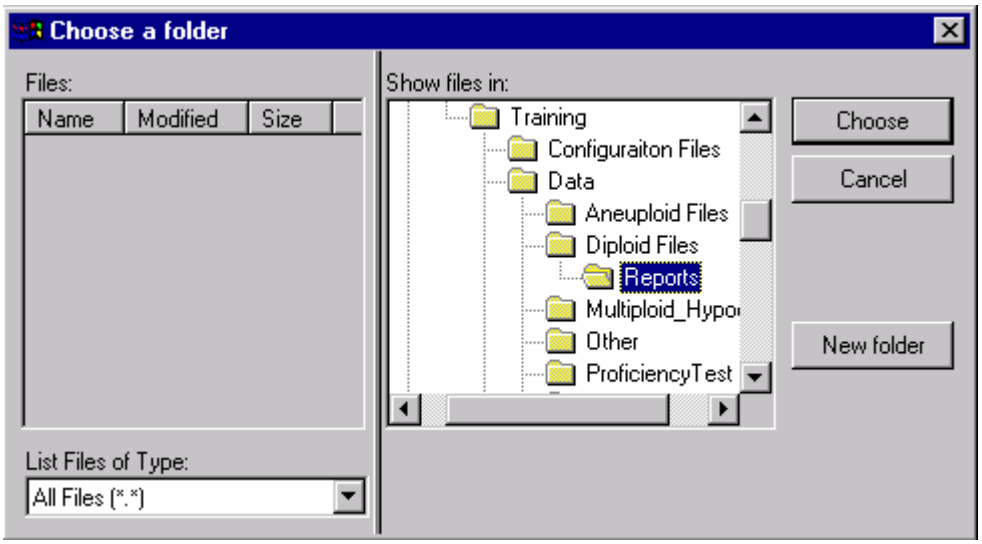
- Click **OK** to close the **Choose Histogram** dialog box.
- In the **Edit** menu, choose “**File Batch Settings**…”



*Another way to edit the **File Batch Settings** is to click on the **File Batch** control’s “pencil” icon.*

In this step, you are going to configure the **File Batch** processor to perform an **Auto Analysis** and save the report for each file.

- From the drop-down **Analysis** listbox, select “Auto-analyze” (see graphic above).
- Check the **Save Report** option (see graphic above).
- Select the **Save Report Path Folder…** button and navigate to the desired report folder location. For the DNA diploid files, we want to store the reports in the “Reports” folder under the “Diploid Files” folder (see graphic below).



Saving your reports in a different folder each day makes the process of selecting the group of folders for Phases 2 through 5 much easier. File management and archival are also easier if all the folders are in a single folder or directory.

- Select **Open** to store this report pathway and to close the **Choose a folder** dialog box.
- Uncheck the **Database** and **File prompt** checkboxes.

*By unchecking the **File prompt** checkbox, the program will no longer show you a **Choose Histogram** dialog for stacked histogram file formats or the listmode dialogs, for each file loaded. The assumption being made by the program is that all the subsequent files have the same type of data structure. If your laboratory is processing files from separate flow cytometers, this assumption may not be valid and it would be better to analyze these files separately.*

- Select **OK** to close the **Edit Properties for file processor** dialog.

We are now ready to analyze all the selected diploid files. In this tutorial section, we are only going to analyze two files, but normally there would be considerably more files, justifying all our preparative work. Also, keep in mind that we are segmenting these analyses into diploid, tetraploid, aneuploid, etc for teaching purposes only. When DNA histograms are typically analyzed, no such segmentation is necessary or desirable.

- In the **File Batch** control, click the **Redo** button to process the first file (see graphic below).

- Now select the **Auto-advance** button to analyze the remaining files (see graphic below).

*We have now analyzed all the files originally selected and stored their associated reports into the appropriate folder. In the next phase we will look at each of these stored reports, determine whether **Auto Analysis** made any mistakes, and then take the appropriate actions.*

*We need to reset these **File Batch** properties in order that the program doesn't prematurely start analyzing and saving reports when we start the next tutorial section.*

- Choose either **File Batch Settings** from the **Edit** menu or click on the "pencil" edit button on the **File Batch** control.
- Select "No analysis" from the **Analysis** drop-down listbox.
- Uncheck the **Save Report** option.
- Recheck the **File prompt** option.
- Click on **OK**.

This completes Phase 1.

Phase 2 – Initial Review

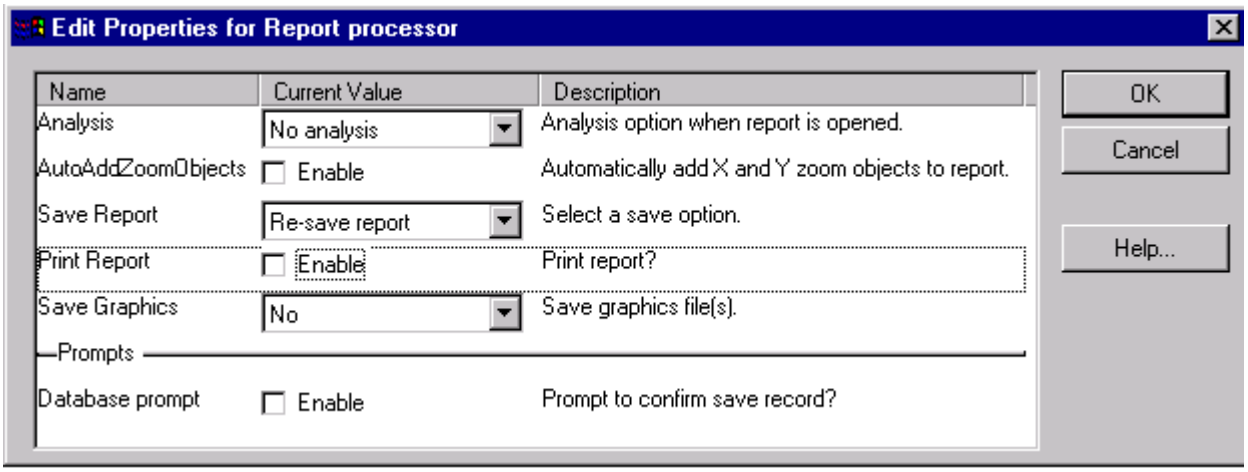
It is important to review each model fit to catch any mistakes the automatic analysis might have made. Adopt a reviewing style that can reliably detect not only major mistakes, but subtle ones as well. In Phase 1, we created two reports in the "Reports" folder under "Diploid Files". Now in Phase 2, we will inspect and possibly correct each of these reports. Phase 2 is really the hard part of cell cycle analysis and therefore will receive appropriate emphasis in these training tutorials.

We will select all the reports just created in Phase 1 and then appropriately configure the **Report Batch Processor**. We then will load each report and discuss the analysis in detail.

- From the **File** menu, choose **Open** -> **Report**...
- Navigate to the "Diploid Files\Reports" folder and multiple-select all of the reports.

*If you don't see any saved reports in this folder, you are either not in the right folder or you didn't configure the **File Batch** control properly in Phase 1.*

- Select **Open**.
- Choose **Report Batch Settings**… from the **Edit** menu or click on the **Report Batch** control's edit button.



*When the program detects that two or more reports were selected, it displays the **Report Batch** control and hides the **File Batch** control. Both of these controls look alike, but you can easily verify which one is on the desktop by putting the cursor over the control's edit button. The balloon help will let you know which one is present. In addition, the default position of the **Report Batch** control is to the right of the **File Batch** control.*

- Select "No analysis" in the **Analysis** drop-down listbox.

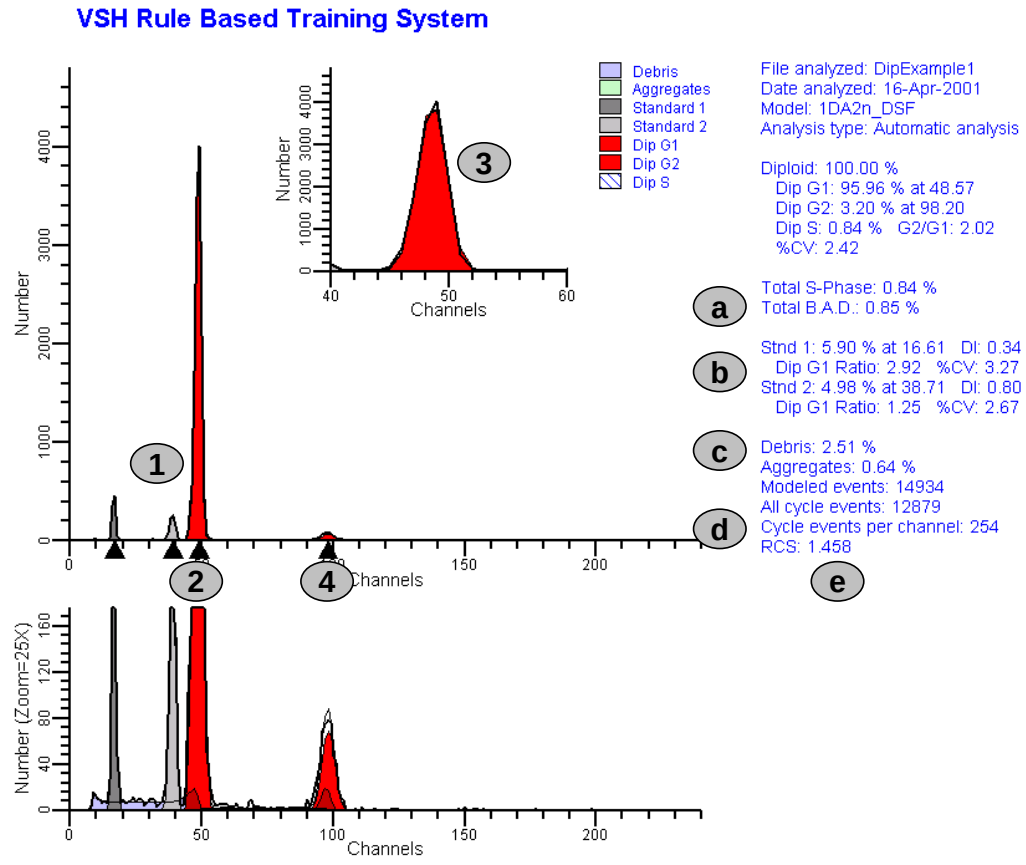
We have already done the initial analysis in Phase 1. In Phase 2 we are only evaluating and potentially fixing the stored reports.

- Choose "Re-save Reports" from the **Save Reports** drop-down listbox.

We need to "re-save" to make sure that any changes will be stored. It is possible to manually save the reports, but activating this option eliminates the possibility of forgetting to save the report after making changes.

The other option, "Auto-name and re-save", is normally not necessary. A special report-naming algorithm is used to convert the file name to a report name. If old reports need to be renamed using this algorithm, the "Auto-name and re-save" option can be used instead.

- Leave the rest of the options in their default states as shown in the figure above.
- Click **OK** to save the settings and close the dialog.
- Let's now examine the first report.



We have annotated the above report with numbers and letters. These labels represent the approximate position of your focus and eye position when evaluating the report. The model fit graphics is scanned in a left-to-right direction and the equations, top-to-bottom. We will begin these tutorials with just a few observations, but as we proceed, the number of these potential focus spots will increase. As we discuss these observations, any relevant analysis rule will be described. A copy of the complete rule set is in Appendix 1 of these training tutorials. To keep these tutorials short and to the point, we will only discuss some of the more pertinent focal spots for the current histogram. We'll occasionally add a spot with the label "?" for review purposes. When you see these labels, try to remember the previously presented important observations points. If you can't remember, go back and review.

1. The histograms in this tutorial have two internal standards, chicken and trout erythrocytes. These standards are useful for histogram alignment as well as detecting DNA hypodiploid ploidy patterns. We highly recommend their routine use for fresh/frozen types of preparations. When you look at these standards, evaluate their quality of fit, position and height. If the model didn't fit

the standard well, you might have to move or resize the standard's associated range. The rule regarding ranges is, II.B.2 "Do not change a range setting unless it is absolutely necessary to do so". In an earlier tutorial, you were instructed to load a previously stored Auto Analysis configuration file. The ***Auto Analysis Settings*** *contain important information on the internal standards, including their expected locations.*

Beneath each major peak in the DNA histogram should appear a black triangle. This triangle represents a statistically significant peak that satisfies additional filters (see Part D). Occasionally the peak finding algorithm will miss one of the standards because its concentration was too low or the debris was too high. This type of error is important to detect since it can dramatically affect what type of model ***Auto Analysis*** *selected. If the program misses one of the standards and the standard is visible, reposition the associated ranges about their appropriate peaks and click on the* ***Fit*** *button. If the standard is not visible (no black triangle), go to the* ***Quick Model Editor*** *in the* ***Edit*** *menu and appropriately change the number of standards; then click on the* ***Fit*** *button.*

The standards should be in consistent absolute position. If they're not, either the histogram was not acquired properly or there may be an additional DNA hypodiploid peak overlapping one of the standards. If the final concentration of standards is made consistent, the relative heights of the standards to the other peaks provides additional information about the final cellular concentration. If a mistake was made in concentration, many times it is evident by this type of comparison. If one of the standards, normally the trout, is much higher than the other standard, the presence of a DNA hypodiploid population should be suspected.

- The standards for this histogram have an appropriate fit, position and relative height.

2. To make an appropriate DNA ploidy determination for the histogram, the position of the DNA diploid G1 peak must be identified. The default color scheme for ModFit LT gives the DNA diploid G1 and G2 peaks a color of red, making their identification obvious. The rule regarding the position of the DNA diploid G1 is, I.F. "The position of the diploid G0G1 peak should always be placed in about the same channel. For 256 channel histograms the recommended location is channel 50."

- The position of the DNA diploid G1 peak is appropriate for this example.

3. The X-Zoomed graphic should be centered about the DNA diploid G1 peak. DNA near hyperdiploid peaks will be most evident in this graph. The analysis rule to follow regarding this type of ploidy pattern is, II.B.1.d "Choose a near-diploid model if the two G0G1 peaks can be clearly distinguished and the resulting fit seems appropriate".

- In this case, there is no split peak or other evidence of another DNA aneuploid population.

4. After the DNA diploid G1 peak has been identified and there is no evidence of a near hyperdiploid population, the associated G2 population should be inspected. If the peak finding algorithm has marked this peak as a significant peak that also satisfies your peak criteria (presence of black triangle), it will let the program independently find the G2 position (floating). Otherwise, it will calculate the G2 population's position based on the G1 position (dependent) and the ***Linearity Factor****. Normally you won't need to be aware of this logic, but occasionally it may be necessary to change the G2 position's dependency (see the next example file).*

The G2 standard deviation is always calculated from the G1's standard deviation.

- The G2 position and quality of fit is appropriate.

a. Total BAD (Background Aggregates and Debris) is defined as the fraction of the histogram from the lowest G1 to the highest G2 that is either debris or aggregates. Use the %Total BAD estimate as a histogram acceptance criteria for S-phase reliability. Recommended %Total BAD value is <=30%.

- The %Total BAD estimate for this histogram is quite low and therefore acceptable.

*b. If the model has internal standards, there will be a few additional equations added to the list, providing useful statistics. The absolute position, DI and Dip G1 ratio are important characteristics to examine, especially if the program is configured to determine its DNA ploidy based on one of the standards (see **Auto Analysis Settings**). If the histogram is shifted either to higher or lower intensity levels, the DI and Dip G1 ratio can reassure you that the identification of the standards was correct.*

- The standard's statistics are all within reasonable ranges.

c. The %Debris and %Aggregates are related to %Total BAD and are particularly useful when the %Total Bad is elevated. They provide important information targeting a particular problem in sample preparation. For example, if the %Aggregates estimate is quite high, it may be that you should consider vortexing your samples more vigorously.

- Both %Debris and %Aggregates are quite low and acceptable.

d. The "All cycle events" and the average "Cycle events per channel" are very important histogram quality measurements. Many times not enough counts are accumulated for optimal cell cycle analysis. Simulation studies indicate that an average "Cycle events per channel" of 100 or more is necessary for accurate S-phase estimation. In some cases, over 20,000 events are necessary to meet that requirement.

- The "All cycle events" and "Cycle events per channel" are quite acceptable.

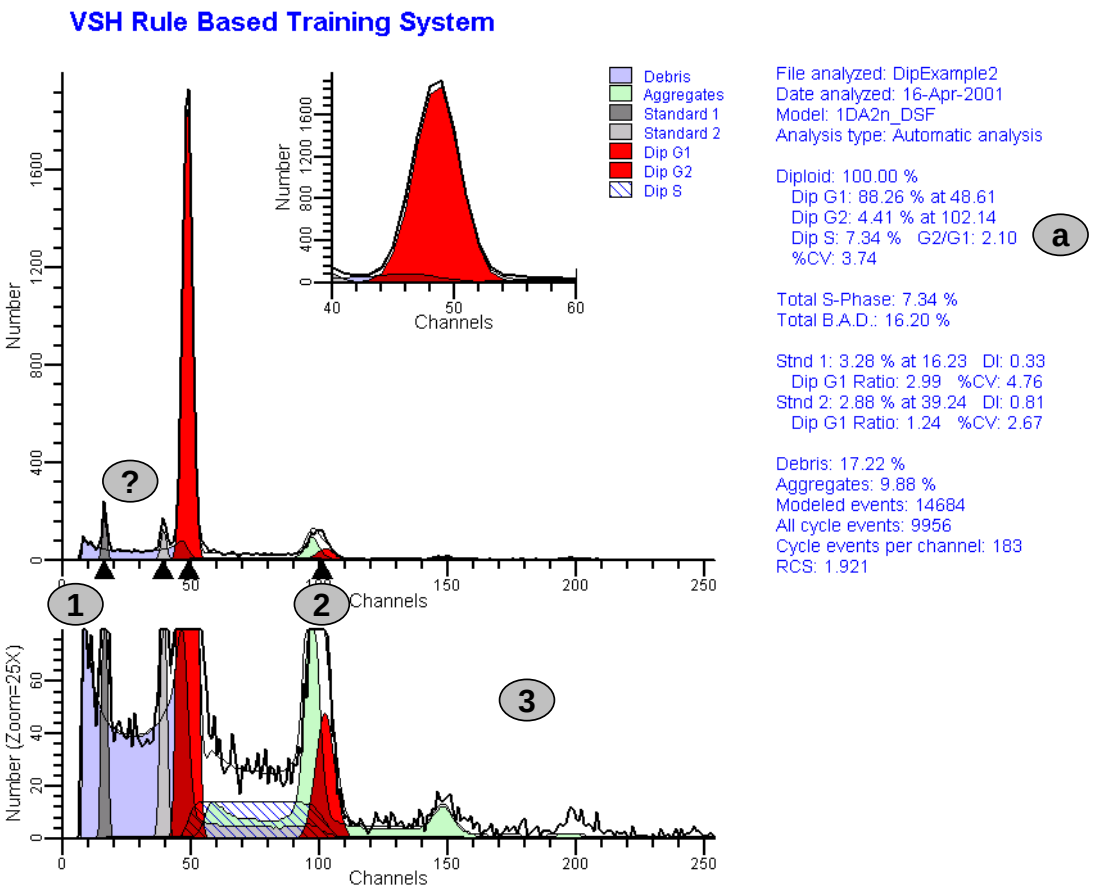
e. RCS (Reduced Chi-Square) is a measurement of the model fit quality. RCS values of greater than five may indicate that there is a population that is not being fit adequately. Elevated RCS values are generally associated with 1) inappropriate starting location of the debris model component, 2) non-Gaussian G1 peaks, 3) missing model components for aneuploid peaks and/or 4) non-linear amplifiers. If you see a high RCS value, try to find the reason and correct it if you can.

- The RCS value is within reasonable limits for this example.

We have spent a lot of time describing these points of focus for this histogram, but haven't done much to correct anything. The **Auto Analysis** algorithm should be correct about 90% of the time. The approach we are trying to teach is targeted at reliably finding those 10% failures and then correcting them in a reproducible manner.

- Click the **Next Batch** item button on the **Report Batch** control (see figure below).

The system will automatically save the current report and then load the next one.



1. Always check to make sure the debris model component starts at the appropriate beginning of the debris. Some flow cytometry discriminators accumulate a few events in these low channels, which can cause the debris range detection system to fail. This leakage normally occurs if the discriminator is set on a parameter other than the DNA content parameter. If the debris component doesn't start at the correct channel, it can affect the overall debris fit, which can ultimately affect all the other estimates. The debris range rule is II.B.2.a, "The beginning of the debris range should correspond to the channel with the highest debris counts." If the debris model component doesn't start at the correct position, move the debris range to the appropriate position.

The discriminator setting should be as low as possible. The very early debris channels (2 through 5) contain rich information concerning the underlying shape of the debris and therefore should not be eliminated by excessive discriminator thresholding (Rule:I.C, "Events should be discriminated on the DNA fluorescence parameter only. The discriminator level should be as low as possible without creating a debris peak that is greater than the highest G0G1 peak.").

- The debris component starts in the appropriate location for this example.

?. Try to remember the points previously discussed about internal standards (see preceding report).

2. If there is an identified peak at the DNA diploid G2 location and there are a significant number of aggregates, the fitted G2 position may end up being inappropriate. Normally the aggregate G1 doublets should align with the G2 position. If they don't align, the fit can be improved by using the Quick Model Editor to force the G2 to be dependent on the G1 (Rule: II.B.a.2, "If a G2M peak is clearly visible and well-defined, allow its mean to be fitted (float) unless there is another overlapping population.").

a. If there is a question on whether the G2's position is correct, examine the G2/G1 ratio. Normally the ratio will be between 1.95 and 2.0.

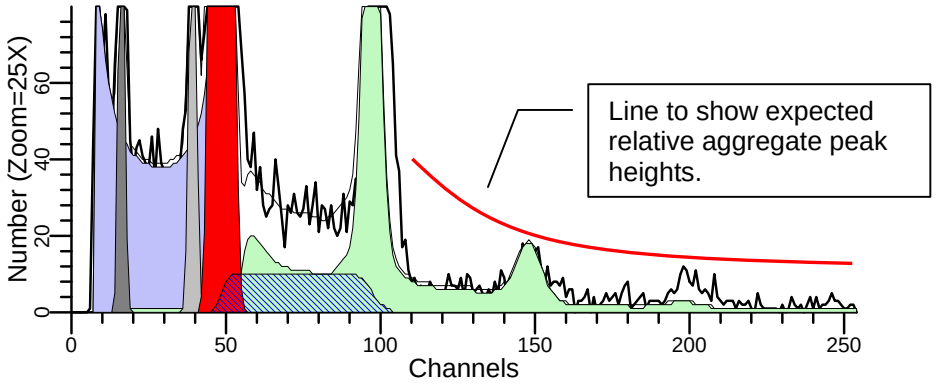
- The G2 is not aligned with the doublets and its G2/G1 ratio is high, 2.10. To correct this subtle problem follow the steps below:

- Choose the **Edit** menu and select "Quick Model Editor…".
- Uncheck the "Cycle1 G2M Visible" option.
- Click on **OK**.
- Click on the **Fit** button.

  - The cell cycle fit still isn't perfect, but it should improve when we find the optimal **Linearity Factor** in Phase 3.

3. Examine the 6C and 8C locations of the histogram. The Y-Zoomed Graphic is essential for visually inspecting the histogram for the presence of triplet(6C) and quadruplet (8C) aggregate peaks. As will be discussed in the next tutorial, the relative heights of these two peaks are important for the detection of low levels of DNA tetraploid cells.

Take a moment to appreciate the decreasing frequency of higher order aggregate peaks (see graph below).



Appreciating this decreasing pattern of higher order aggregates is an important step in developing your skill for detecting low levels of DNA tetraploid populations (next tutorial).

- There is no evidence of another aneuploid population. The quality of the aggregate fit will probably improve in Phase 3.

- Press the **Redo** button on the **Batch Report** control to force a re-saving of the report.

Normally, the system will automatically detect that the last report needs re-saving, but it is prudent to force the re-save operation to be sure.

This concludes Phase-2.

Phase 3 – Automatic Linearity Factor Detection

Both the dependent G2 positions and the aggregate model component are highly dependent on the **Linearity Factor**, defined as the ratio between the DNA diploid G2 and G1 positions. Since S-phase estimates are dependent on G2 as well as aggregates, it's important to determine the **Linearity Factor** in a reproducible manner. The **AutoLinearity** feature in **ModFit LT** will find the **Linearity Factor** that minimizes the RCS over a specified range. The default range is 1.90 to 2.05. The AutoLinearity algorithm looks at every **Linearity Factor** in the defined range at increments of 0.01 units, storing the **Linearity Factor** with the lowest RCS. Unfortunately, this process is computationally intensive and consequently should be done in the **Batch Report** processor.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu (see graph below).

- Select "Fit and do autolinearity" from the **Analysis** drop-down listbox.
- Leave the "Re-save" **Save Report** option selected.
- Select **OK**.
- Click on the "First Batch Item" button on the **Report Batch** control (see graph below).

*The program will immediately start analyzing the first report looking for the optimum **Linearity Factor**. In the lower-left corner of the status bar, you can monitor its progress.*

- Select the "Auto Advance" button on the **Report Batch** control to analyze the rest of the reports.

End of Phase 3.

Phase 4 –Final Review

Since Phase 3 can significantly change the quality of the cell cycle fits, it's important to perform a final review on all the reports. We will again use the **Report Batch** control to help us perform this task efficiently.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu
- Select "No analysis" in the **Analysis** drop-down listbox.
- Select "Don't re-save report" from the **Save Report** drop-down listbox.

*You don't really need to turn off the automatic saving of reports options, but since most of the reports are going to be acceptable, you might consider manually saving just those reports that need further adjusting. To manually save a report use the **Save** tool button.*

- Click on **OK**.
- Select the "First Batch Item" button on the Report Batch control.
- Inspect the first report using the method described in Phase 2.

*The first report should look just like the one in Phase 2 since not much changed with the new optimum **Linearity Factor**.*

- Select the "Next Batch Item" button on the **Report Batch** control.

*Notice that the fit of the aggregate component is much better with the optimized **Linearity Factor** and note that the RCS is much lower as well.*

This is the end section G: DNA Diploid Tutorial.

Part H - DNA tetraploid files – 15 minutes

In this section of the tutorial, you will analyze DNA tetraploid files following the four-phase analysis procedure. DNA tetraploid files can be very difficult to distinguish from DNA diploid histograms if the aneuploid fraction is very low. We will present a technique that should make this decision relatively easy and reliable. There are also a few specific modeling issues regarding DNA tetraploid models that need further explanation.

Note: The basic instructions for analysis are repeated to assist your learning each one. However, some of the notes and observations will be different.

Phase 1 – Perform Auto Analysis and generate report files.

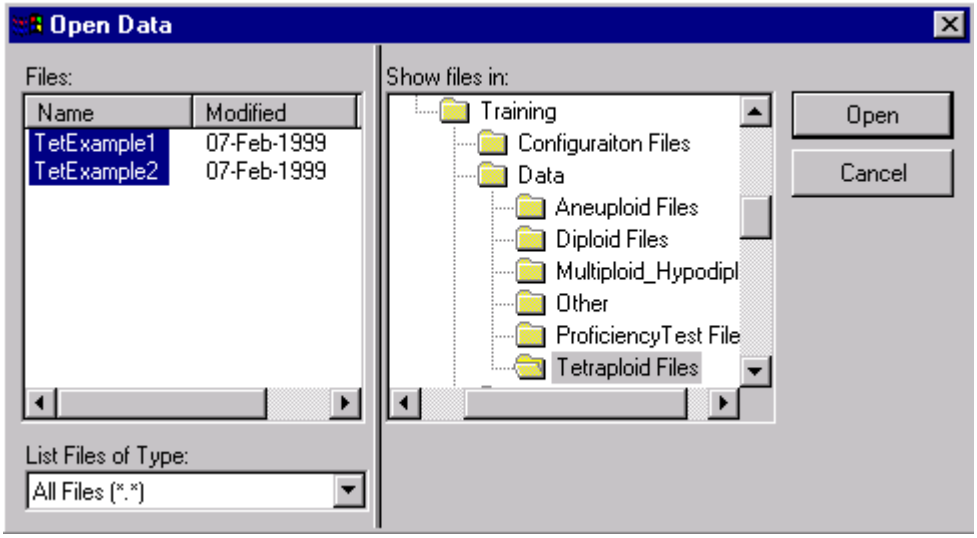
In this step, you are going to select all of the files for analysis, configure the **File Batch processor** to perform an **Auto Analysis**, and save a report for each analyzed data file.

If you already have a report on the desktop that has the X and Y zoom graphics, you don't need to load in the report template. If for some reason you have closed ModFit LT between this section and the last one, you will need to open the report template as you did in the Part G – DNA diploid files tutorial.

- Click on the **File** toolbar button.
- Navigate to the "Tetraploid Files" folder.

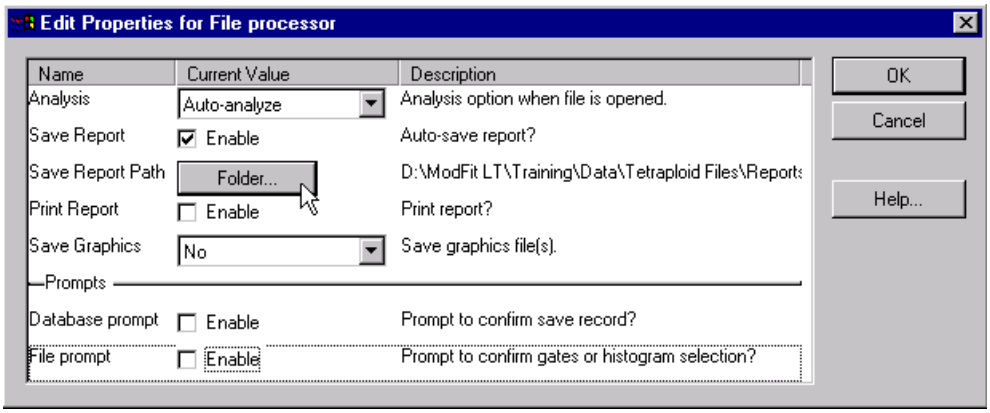
The data files are under folders specific for each part of the tutorial. The folder structure is shown on page A-1 of the manual.

- Multiple-select the data files listed in the Files window (see graphic below).



- Click the **Open** button.
- Select "FL2-A" as the analysis parameter.
- Click **OK** to close the **Choose Histogram** dialog box.
- In the **Edit** menu, choose "**File Batch Settings…**"

- In the **Edit Properties for File processor** dialog, choose “Auto-analyze” and enable **Save Report** checkbox as show in the figure below.



- Click on the “Folder…” button and navigate to the desired report folder location. For the DNA tetraploid files, this will be the “Reports” folder under the “Tetraploid Files” folder (see graphic above).

- Click the “**Open…**” button in the **Choose a Folder** dialog box to store this report pathway and to close the dialog box.

- In the **Edit properties for File Processor** dialog, be sure that the “Database prompt” and “File prompt” checkboxes are un-checked (see graphic above).

- Click **OK**.

- Click the **Redo** button on the **File Batch** control to process the first file.

- Click the **Auto-advance** button to analyze the remaining files.

We now need to reset the **File Batch** control’s properties.

- Choose either **File Batch Settings** from the **Edit** menu or click on the “pencil” edit button on the **File Batch** control.

- Select “No analysis” from the **Analysis** drop-down listbox.

- Uncheck the **Save Report** option.

- Recheck the **File Prompt** option

- Click on **OK**.

This completes Phase 1.

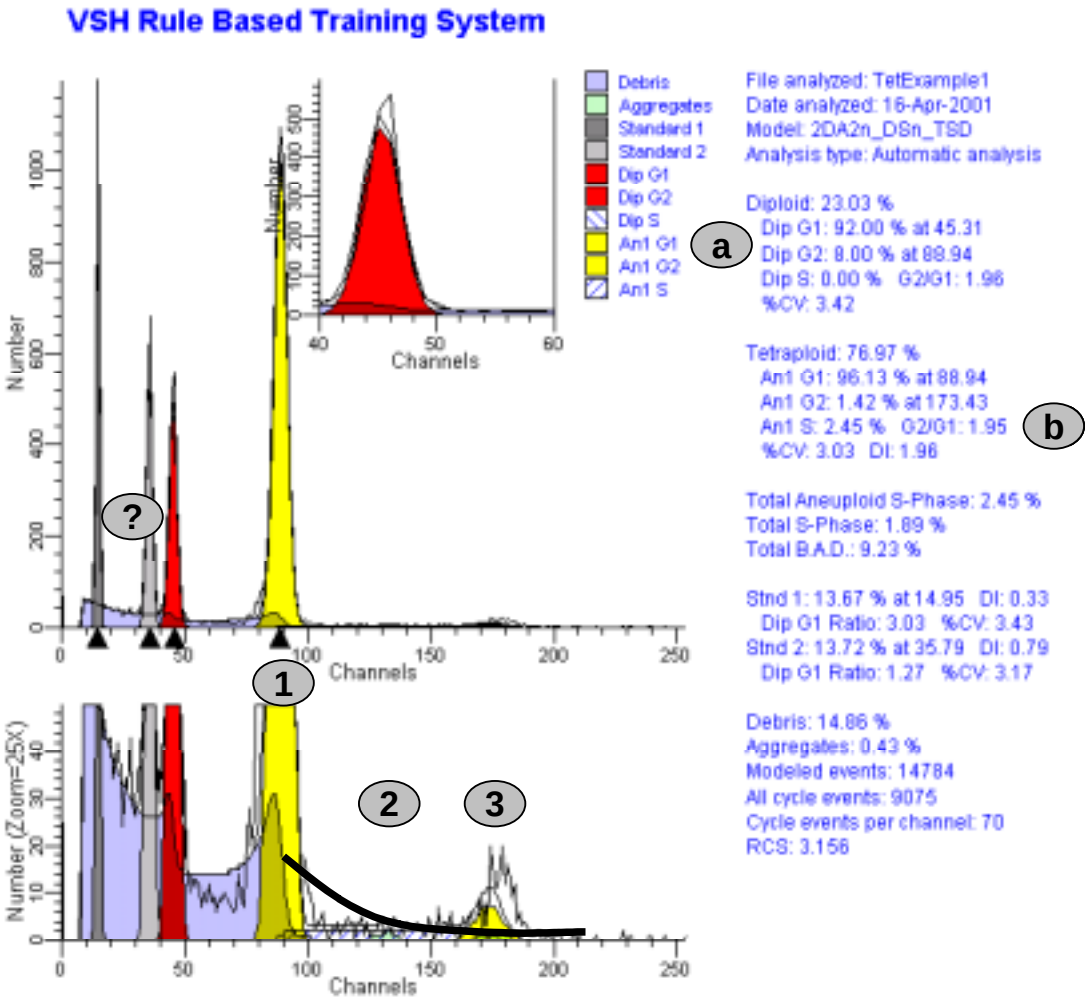
Phase 2 – Initial Review

Let's now review all these reports.

- From the **File** menu, choose **Open** -> **Report**...

Notice that the program took you to the folder where you had previously chosen to save the reports. If it didn't navigate to the "Tetraploid Files\Reports folder.

- Multiple-select all the reports.
- Click the **Open** button.
- Choose **Report Batch Settings**… from the **Edit** menu or click on the **Report Batch** control's edit button.
- Select "No analysis" in the **Analysis** drop-down listbox.
- AutoAddZoomObjects should be disabled.
- Choose "Re-save Reports" from the **Save Reports** drop-down listbox.
- Leave the rest of the options in their default states.
- Click **OK** to save the settings and close the dialog.
- Let's now examine the first DNA tetraploid report.



?. Try to remember all the points about the start of the debris and internal standards as well as the expected location of the DNA diploid G1 peak.

1. A large population at 4C (channel 90) is suggestive of a DNA tetraploid population. Further evidence to support this contention is the absence of a DNA diploid G2 peak. The diploid G2 is disabled for DNA tetraploid models because of the general rule:II.B.1.a.1, "If two model components are of similar shape and are highly overlapped (>75%), it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance." In this case, the DNA diploid G2 overlaps the DNA tetraploid G1 and is of lesser importance and therefore is automatically disabled for DNA tetraploid models.

- The DNA tetraploid model is appropriate for this histogram.

2,3. The 6C aggregation peak (2) is quite small compared to the larger DNA tetraploid G2 peak (3). This example is an obvious DNA tetraploid histogram because of the large size of the 4C peak. As we will see in the next report, the identification of DNA tetraploids becomes more difficult as the aneuploid fraction decreases. A good rule to employ regarding DNA tetraploids is Rule: II.B.1.b.2, "(Select a tetraploid model if) there is another peak at 8C that cannot be explained as an aggregate. a. Consider the 8C peak to be an aggregate if it is less than the 6C peak." Visualizing the slowly decreasing frequency of higher order aggregate peaks (see line in above graph) can help to detect these DNA tetraploids.

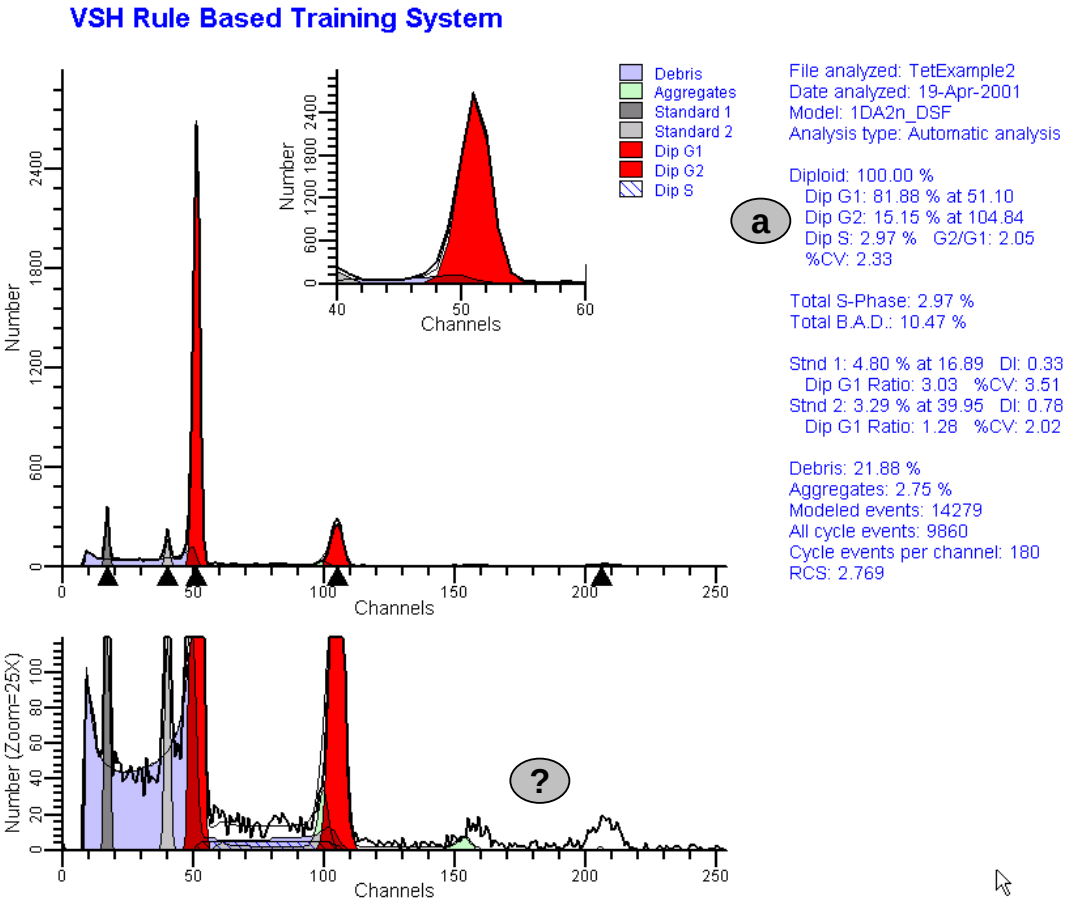
*Also, note that because the peak at (3) did not satisfy the **Peak Finder**'s criteria or filters, the DNA tetraploid G2 position is dependent on G1. Although the fit is not good right now, it is likely to improve in Phase 3.*

*a. If the DNA diploid G2 is disabled, why is there a 8% for its estimate? Because the DNA tetraploid model does not have a diploid G2, the tetraploid G1 is slightly larger than it should be because it is also capturing DNA diploid G2 counts. This effect causes the DNA tetraploid S-phase to be slightly underestimated. To compensate for low bias, the **Edit->Options and Configuration…** dialog has the setting, "Dip G2M fraction". As you might expect, this setting is currently 0.08 or 8%. For DNA tetraploid models, all affected equations are adjusted appropriately with this factor.*

*b. When a G2 is dependent, the G2/G1 ratio becomes equivalent to the **Linearity Factor**, which is currently 1.95.*

- **Auto Analysis** has appropriately selected a DNA tetraploid model for this histogram.

- Click the **Next Batch** item button on the **Report Batch** control.



?. Try to recount the previous discussion on 6C and 8C peaks. Can the peak at 8C, channel 210, really be just an aggregate?

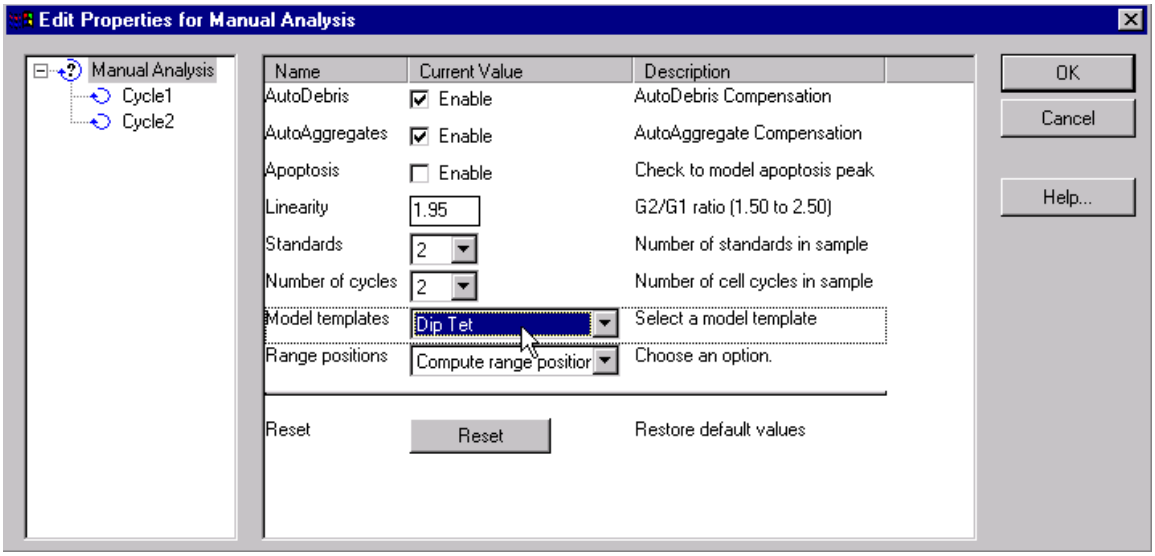
a. Also, note the rather large percentage for the DNA diploid G2. With solid tumors, very rarely do G2's exceed 10%. This elevated value is further evidence that the DNA diploid model selection was incorrect.

- The wrong model was selected in **Auto Analysis**. This histogram should be modeled with a DNA tetraploid model.

Let's now correct the problem.

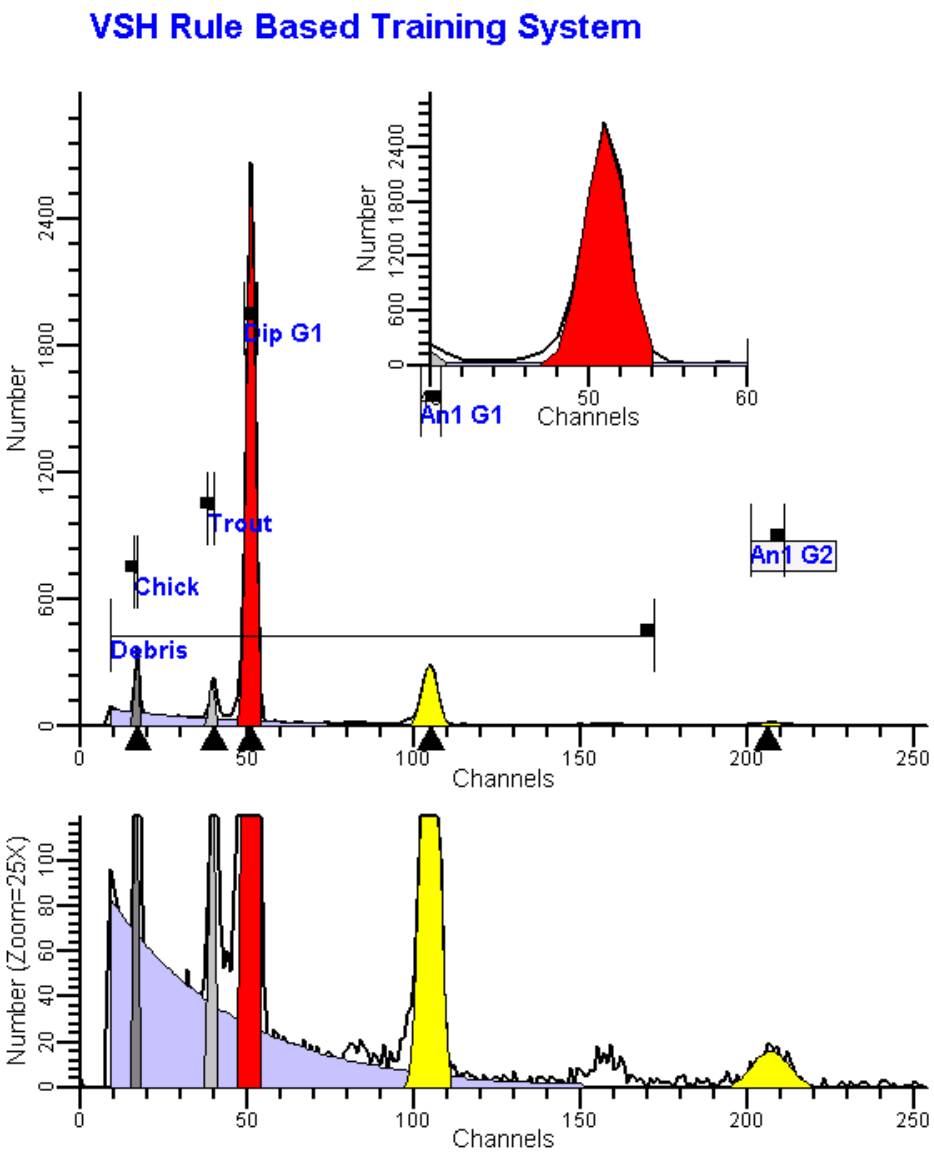
- Click on the **Mod** toolbar button (see graphic below).

- In the **Edit Properties for Manual Analysis** dialog box, change the "Number of cycles" value to "2" and select "Dip Tet" from the "Model template" drop-down listbox (see graphic below).



- Click on the **OK** button to close the dialog box and load the new model.

- Verify the position of the model ranges displayed on the report (see graphic below).



The only two ranges that need adjusting are the "An1 G1" and "An1 G2" ranges. They both need to be moved slightly to the right.

- Click on the **Fit** toolbar button.

The histogram should now be fitted with the DNA tetraploid model.

- Click on the **Save** toolbar button or the **Report Batch** control's Redo button to make sure this changed report is saved.

This concludes Phase 2.

Phase 3 – Automatic Linearity Factor Detection

Let's now find the optimum **Linearity Factor** all our stored reports.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu (see graph below).
- Select "Fit and do autolinearity" from the **Analysis** drop-down listbox.
- Leave the "Re-save" **Save Report** option selected.
- Select **OK**.
- Click on the "First Batch Item" button on the **Report Batch** control.
- When the first report is done processing, select the "Auto Advance" button on the **Report Batch** control to analyze the rest of the reports.

End of Phase 3.

Phase 4 –Final Review

Now for our final review…

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu
- Select "No analysis" in the **Analysis** drop-down listbox.
- Select "Don't re-save report" from the **Save Report** drop-down listbox.
- Click on **OK**.
- Select the "First Batch Item" button on the **Report Batch** control.
- Inspect the first report using the method described in Phase 2.

Notice that the DNA tetraploid G2 is now better positioned than it was before Phase 3.

- Select the "Next Batch Item" button on the **Report Batch** control.

Notice that the 6C aggregates are now better positioned than earlier and the RCS is much lower.

This is the end section H: DNA Tetraploid Tutorial.

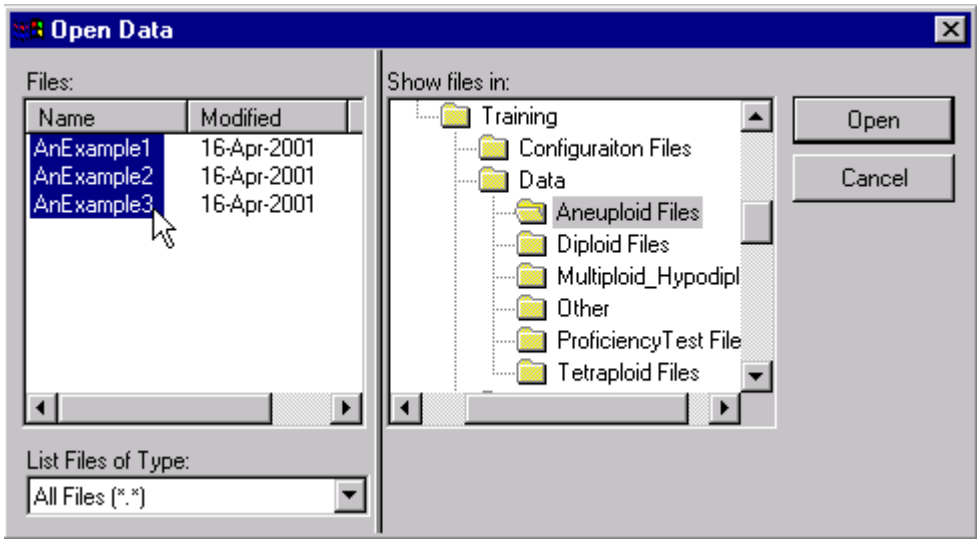
Part I - DNA aneuploid files – 40 minutes

DNA aneuploid histograms are slightly more difficult to interpret than DNA diploids because they have an additional cell cycle, creating more complexity to histogram interpretation and analysis. However, the four-phase approach to this type of analysis is still the method of choice.

Phase 1 – Perform Auto Analysis and generate report files.

In a manner similar to the preceding sections, we will first load our DNA report template (if necessary), select the DNA aneuploid files, properly configure the **File Batch** control and then process all the files using **Auto Analysis**.

- Click on the **File** menu and choose **Open Report...**.
- Navigate to folder under “Data”, select the file, “DNAReportTemplate.mfl” then click on **Open**.
- Click on the **File** toolbar button (first tool button on left).
- Navigate to the “Aneuploid Files” folder.
- Multiple-select all of the aneuploid example files (see figure below).



Multiple selection can be done in several ways. In the previous sections, we used a “block selection” technique. Another technique called the “marquis box” method is sometimes more efficient. Place the cursor in the white space just to the right of the first file name. While holding the left (PC only) mouse button down, drag the mouse pointer diagonally across the files of interest. You will see a marquis box with dotted lines over the encompassed selected file names.

- Click the **Open** button.
- Select “FL2-A” as the analysis parameter.
- Click **OK** to close the **Choose Histogram** dialog box.

As in earlier tutorials, we will configure the **File Batch** control to automatically analyze all the files and save the reports.

- From the **Edit** menu, choose "**File Batch Settings**…" or select the **File Batch** control's "pencil" button.
- From the drop-down **Analysis** listbox, select "Auto-analyze".
- Check the **Save Report** option.
- Select the **Save Report Path Folder…** button and navigate to the desired report folder location. For the DNA aneuploid files, we want to store the reports in the "Reports" folder under the "Aneuploid Files" folder.
- Select **Open** to store this report pathway and to close the **Choose a folder** dialog box.
- Uncheck the **Database** and **File prompt** checkboxes.
- Select **OK** to close the **Edit Properties for file processor** dialog.

Now we are able to automatically process the files.

- In the **File Batch** control, click the **Redo** button to process the first file.
- Now select the **File Batch** control's **Auto-advance** button to analyze the remaining files.

All the files have now been analyzed and the reports saved.

We now need to reset these **File Batch** properties in order that the program doesn't prematurely start analyzing and saving reports when we start the next tutorial section.

- Choose either **File Batch Settings** from the **Edit** menu or click on the "pencil" edit button on the **File Batch** control.
- Select "No analysis" from the **Analysis** drop-down listbox.
- Uncheck the **Save Report** option.
- Click on **OK**.

This completes Phase 1.

Phase 2 – Initial Review

As we have seen in the previous sections, it is possible for the automatic analysis algorithms to make mistakes. The number of mistakes will potentially increase as the complexity of the DNA histogram increases. When you notice that there are two or more additional cell cycles, be more alert to possible analysis problems and carefully employ the left-to-right method of evaluating the fit.

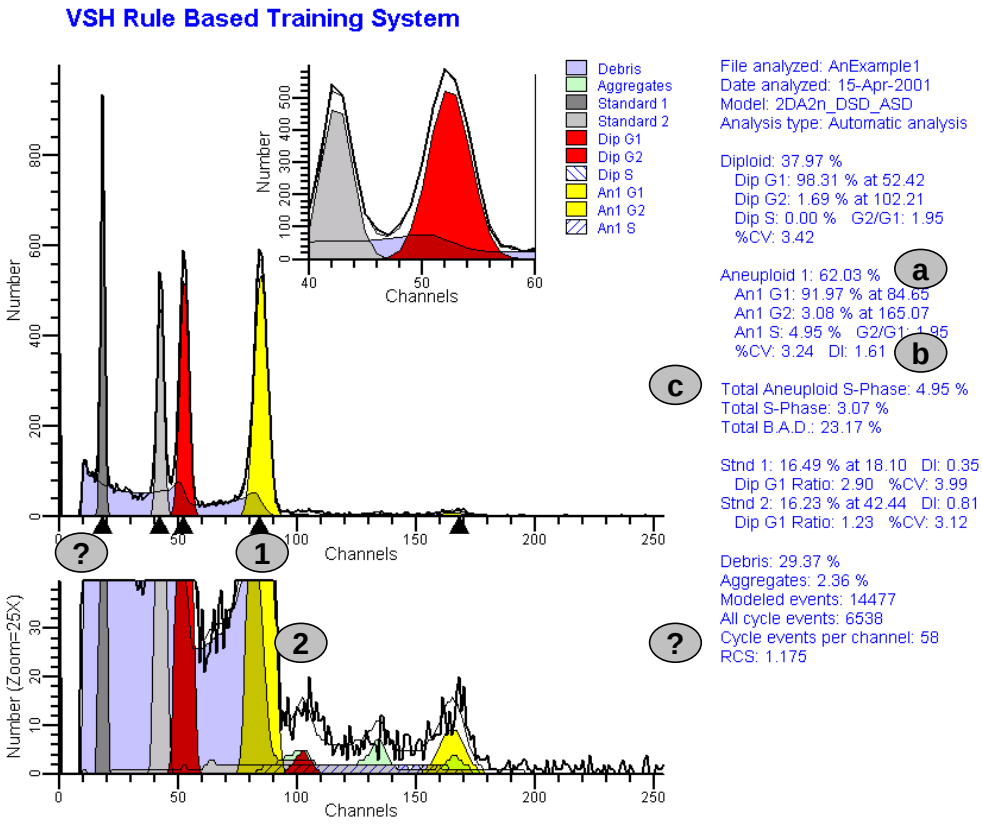
Let's load in the reports.

- From the **File** menu, choose **Open** -> **Report**...
- Navigate (if necessary) to the "Aneuploid Files\Reports" folder and multiple-select all of the reports.

*If you don't see any saved reports in this folder, you are either not in the right folder or you didn't configure the **File Batch** control properly in Phase 1.*

- Select **Open**.
- Choose **Report Batch Settings**… from the **Edit** menu or click on the **Report Batch** control's "pencil" icon.
- Choose 'No analysis' from the **Analysis** drop-down listbox.
- Choose "Re-save report" from the **Save Report** drop-down listbox.
- Leave the rest of the options in their default states.
- Click **OK** to save the settings and close the dialog.
- Let's now examine our first DNA aneuploid report.

In these tutorials, we are only identifying a few new focus spots for each presented report. We occasionally will ask you via the "?" spot to review previously discussed material. However, when you evaluate the report, you need to consider everything we have covered, whether it is marked or not. This type of rigorous approach will generally detect most of the subtle problems that lead to variable results.



?. Try to recall the points made about examining the debris fit.

1. More than half of the DNA aneuploid populations are obvious when they occur. The default color for the first aneuploid G1 and G2 populations is yellow.

- An obvious single aneuploid population is present and **Auto Analysis** chose the correct model.

2. Always try to identify the DNA diploid G2 population. The question you need to ask yourself is whether the DNA diploid G2 is being fit appropriately or not. If it is not, you might need to change the model to a DNA tetraploid model (Rule:II.B.1.b.3, "Choose a tetraploid model over an aneuploid model if the diploid G2M overlaps too significantly with an aneuploid G1." and Rule:II.B.1.b.3.b, "Inappropriate fits of the diploid G2M are usually associated with a zero or very high calculated percentage or a location that results in a G2/G1 ratio outside the expected range.").

- The DNA diploid G2 population is modeled well. Since this G2 is dependent on the G1, the quality of the fit will improve in Phase 3 when a more optimal **Linearity Factor** is determined.

a. On the average, the aneuploid fraction (AF) will be between 40-60%.

- The Aneuploid Fraction (AF) is 62%.

b. Always check the DNA Index of the DNA aneuploid population to make sure it corresponds to the fitted graphics.

- The DI is 1.6.

c. The total aneuploid S-phase is the key analysis result regarding DNA aneuploid S-phases. If there are multiple DNA aneuploid populations, this value will be the weighted average of all the aneuploid S-phases.

The total S-phase result is only included for backward compatibility. It is not recommended to report this value since it is highly influenced by AF because it is a weighted average that includes the DNA diploid S-phase. Because the variability of AF approaches a high %CV of 20, the prognostic strength of this variable is unreliable.

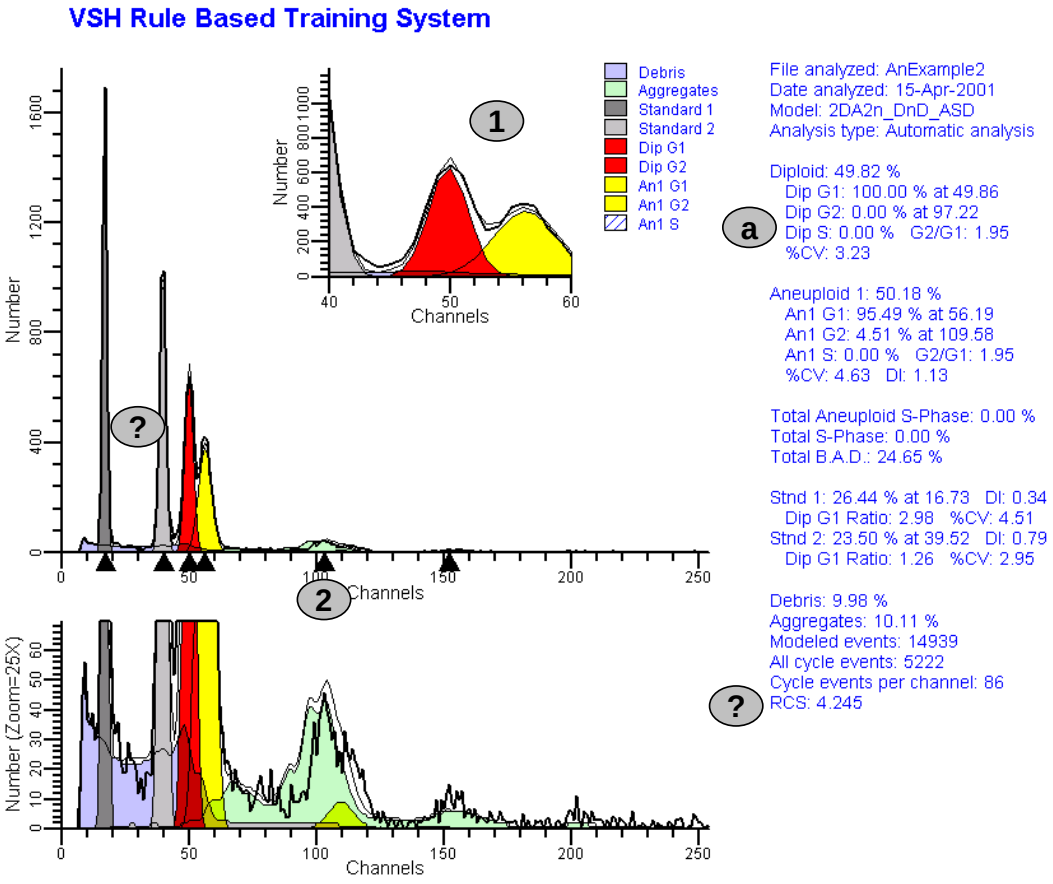
- The Total Aneuploid S-phase is 4.9%.

?. Try to remember the previous discussion about "Cycle events per channel". Does this histogram have an optimal number of events?

You can still quantify S-phase with sub-optimal numbers of counts but the uncertainty of the estimate will be higher.

Auto Analysis seems to have done a reasonable job with this histogram. Let's look at the others.

- Click the **Next Batch** item button on the **Report Batch** control.



?. Try to remember our previous discussion about internal standards. If the internal standards' concentrations are the same as in the previous files, what can be deduced about the cellular concentration?

1. There are a number of modeling ramifications regarding DNA near hyperdiploid histograms. Since the DNA diploid and aneuploid S-phase populations overlap so significantly, the DNA diploid S-phase is automatically inactivated when ***Auto Analysis*** *detects that the DI is less than or equal to 1.3 (Rule:II.1.a, "If two model components are of similar shape and are highly overlapped (>75%), it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance." and Rule: II.1.d.2, "If the DI is between 0.7-1.0 or 1.0-1.3 disable the diploid S-phase and make both G2's dependent").*

An easy way to inspect and change many of these activation and dependency model attributes is by the ***Quick Model Editor*** *under the* ***Edit*** *menu.*

- A DNA near hyperdiploid G1 population is present. **Auto Analysis** picked the correct model.

2. Because of the above stated Rule:II.1.d.2, both the DNA diploid and aneuploid G2 peaks are dependent on their respective G1 peaks. Since both G2's are dependent, they may not yet fit the data properly because the optimal ***Linearity Factor*** *has not yet been calculated.*

- The DNA diploid and aneuploid G2's are not fitted well but that situation should improve in Phase 3.

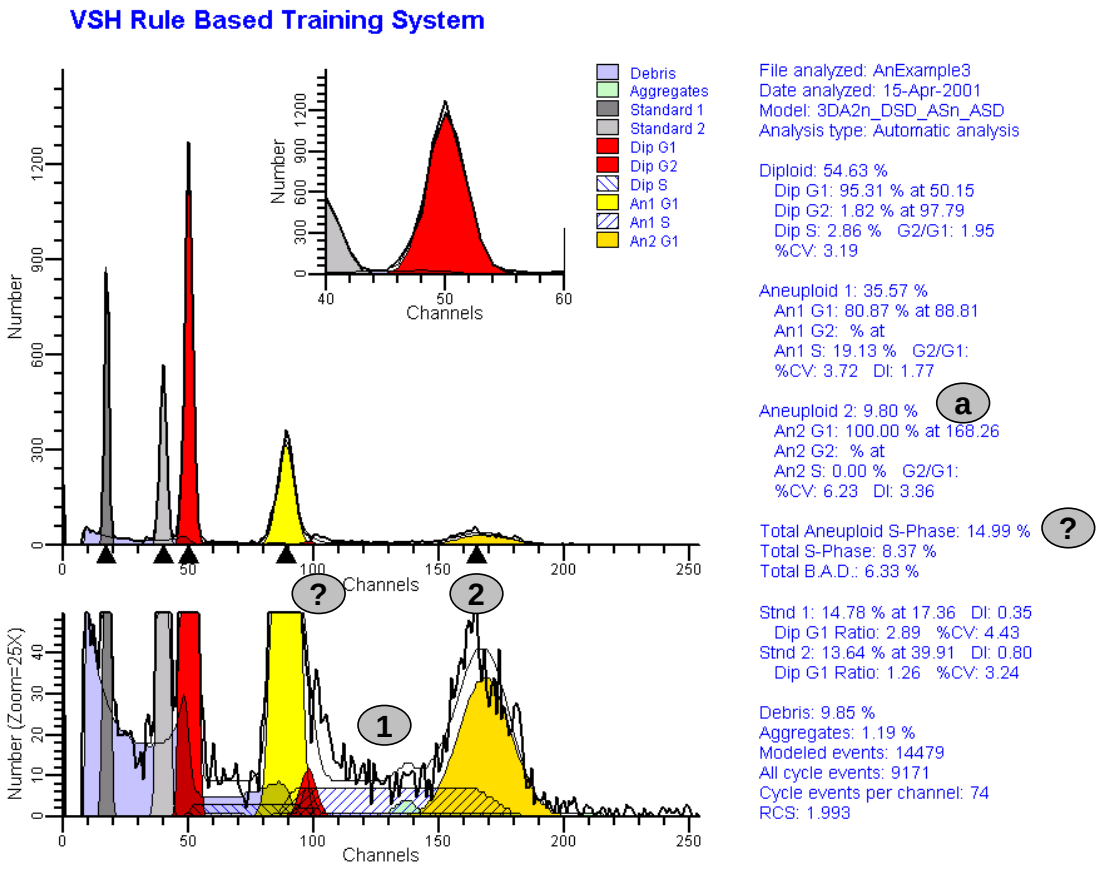
If you run into a situation where you want to make sure you check something in the final review phase, a useful technique is to use the annotation reporting objects to call attention to that aspect of the report. After the final review, the annotation can easily be removed.

a. Because we are using a near diploid model, the DNA diploid S-phase is inactivated and appears as zero in the evaluated equation list.

- The DNA diploid S-phase is zero as expected.

We have found nothing to change for this report. Let's move on to the final report for this tutorial section.

- Click the **Next Batch** item button on the **Report Batch** control.



?. Try to recall the discussion about the DNA diploid G2. Should we be worried that it is not perfectly positioned? Why?

1. We always use a convoluted (broaded) single rectangle for the S-phase model component when analyzing solid tumor DNA histograms (Rule:II.B.a.3, "Always model S-phase as a single broadened rectangle."). Broadened rectangles are advantageous since they are not as sensitive to the presence of small G1 shoulders or other S-phase "imperfections" as some of the other proposed S-phase components (broadened trapezoids, polynomials and multiple compartments).

*2. A second DNA aneuploid population is assigned, by default, a slightly darker yellow. In this example, the change in color should be the initial tip-off that a second aneuploid population has been detected by the **Auto Analysis** procedure. Why did the system make this choice? We feel that it is important to know how **Auto Analysis** decides when a peak represents a valid G1 population. This knowledge can be used to control and tailor the program's operating characteristics to efficiently and reliably analyze DNA histograms. Once the technical reasons for this decision are presented, we'll discuss what you should do about this particular situation.*

*The relevant configuration settings in **Auto Analysis** are the "G1 threshold" and the "Tetraploid G2M threshold". In order for the system to assign a peak as a valid G1 peak, its estimated area must equal or exceed the "G1 threshold". In this case, the "G1 threshold" is set to 5%, which is certainly less than the peak at channel 170. Since this peak is also a possible G2 for the first aneuploid population, **Auto Analysis** must determine whether it is too large to be a valid G2. The important setting for this determination is the "Tetraploid G2M threshold". This particular histogram's peak at channel 170 is estimated to exceed the current G2M threshold setting and therefore **Auto Analysis** has assigned it to be the G1 of another aneuploid population.*

*Did **Auto Analysis** make the right choice? If you look carefully at this peak, it doesn't look like a typical G2. It's large and it appears split. It may indeed represent a second aneuploid population, but the problem is that because it is so far upscale, the program cannot model its S-phase and G2. The rules that pertain to this situation are, Rule:II.B.1.c.1, "Only choose this model (Aneuploid) if the potential aneuploid's G0G1 cannot be explained as an aggregate or some other part of another cycle (e.g. G2M)." and Rule:II.B.1.c.2, "and there are adequate channels to model the entire cycle".*

If there are not enough channels to model the S-phase and G2, then the results will be in error and it is better to ignore these results than to try to include them.

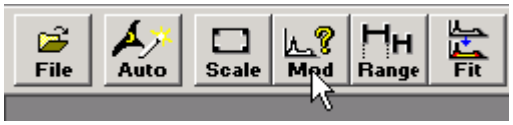
- The correct model should be one aneuploid population. We need to adjust the model.

We'll correct the model in just a moment. Let's finish up our focus areas.

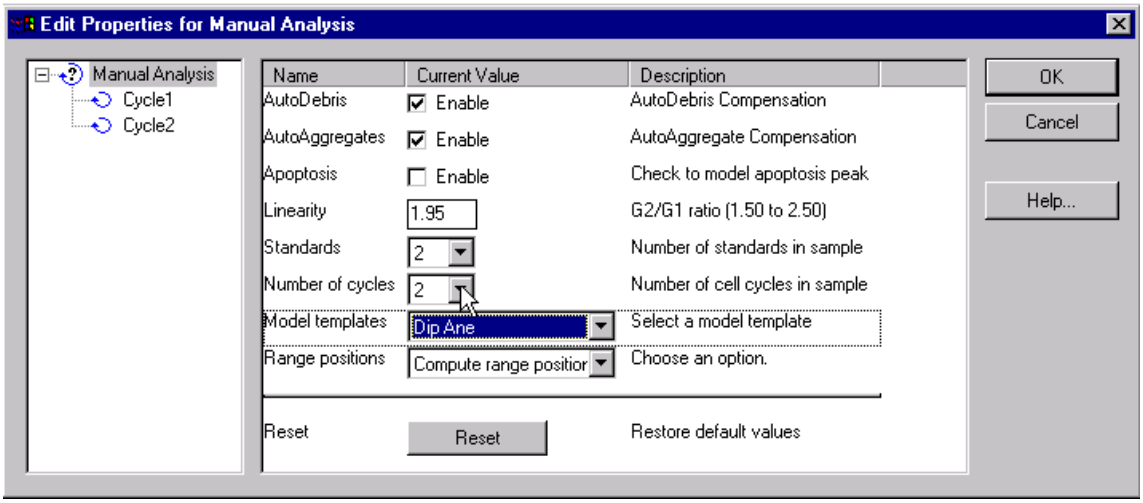
a. The second way to identify the second aneuploid population is by inspection of the model equation lists.

?. Try to recall the definition of Total Aneuploid S-phase. Why is this value different from the Aneuploid 1 S-phase?

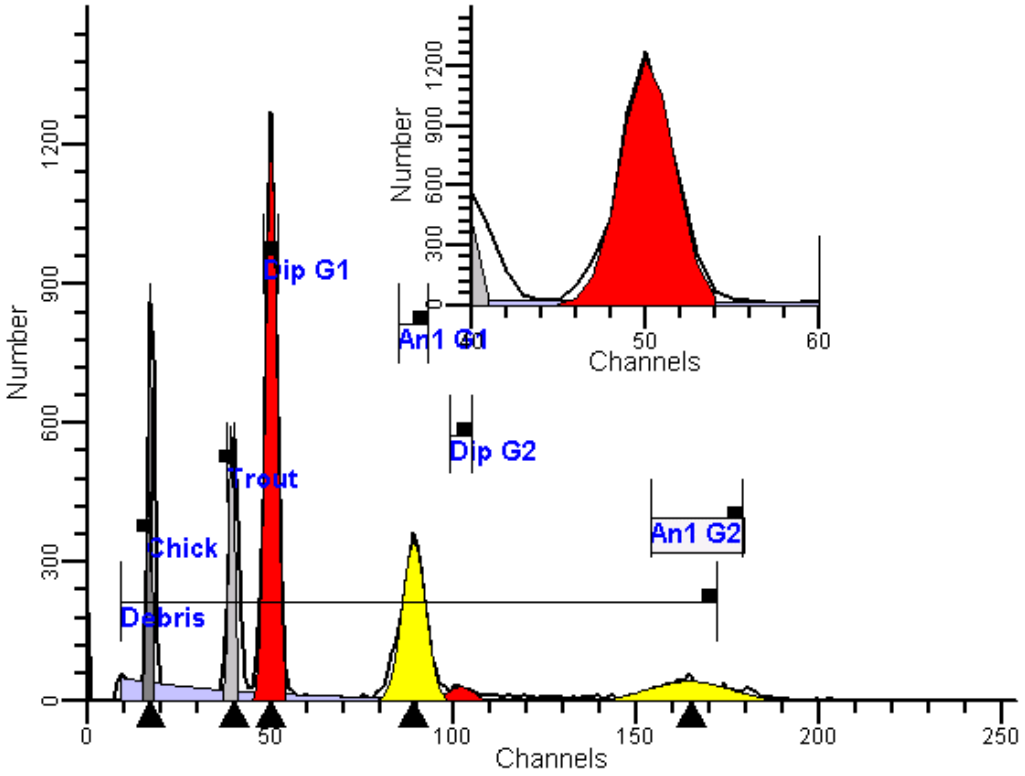
- Click on the **Choose Model** toolbar button (see graphic below).



- Choose "2" from the "Number of cycles" drop-down listbox (see graphic below).



- If necessary, choose "Dip Ane" from the "Model templates" drop-down listbox.
- Click **OK**.
- Make sure the ranges are as shown below:



The only two ranges necessary to adjust was the Dip G2 (moved a little to the right) and the An1 G2 (widen to accommodate the entire peak). Remember the range moving rule, "Do not change a range setting unless it is absolutely necessary to do so."

- Click on the **Fit** toolbar button.

If you did the steps properly, there should now only be one aneuploid population.

- Click on the **Redo Report Batch** control button or click on the **Save** toolbar button to make absolutely sure this modified report has been saved.

This concludes Phase-2.

Phase 3 – Automatic Linearity Factor Detection

Let's now find the optimum **Linearity Factor** all our stored reports.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu (see graph below).
- Select "Fit and do autolinearity" from the **Analysis** drop-down listbox.
- Leave the "Re-save" **Save Report** option selected.
- Select **OK**.
- Click on the "First Batch Item" button on the **Report Batch** control.
- When the first report is done processing, select the "Auto Advance" button on the **Report Batch** control to analyze the rest of the reports.

End of Phase 3.

Phase 4 –Final Review

Now for our final review…

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu
- Select "No analysis" in the **Analysis** drop-down listbox.
- Select "Don't re-save report" from the **Save Report** drop-down listbox.
- Click on **OK**.
- Select the "First Batch Item" button on the **Report Batch** control.
- Inspect the first report using the method described in Phase 2.

Notice that the DNA diploid G2 is now better positioned than it was before Phase 3.

- Select the "Next Batch Item" button on the **Report Batch** control.

Notice that the aggregates are now better positioned than earlier and the RCS is much lower.

- Select the "Next Batch Item" button on the **Report Batch** control.

Notice that the DNA diploid G2 is better positioned and our change from two aneuploid populations to one is present

This is the end section I: DNA Aneuploid Tutorial.

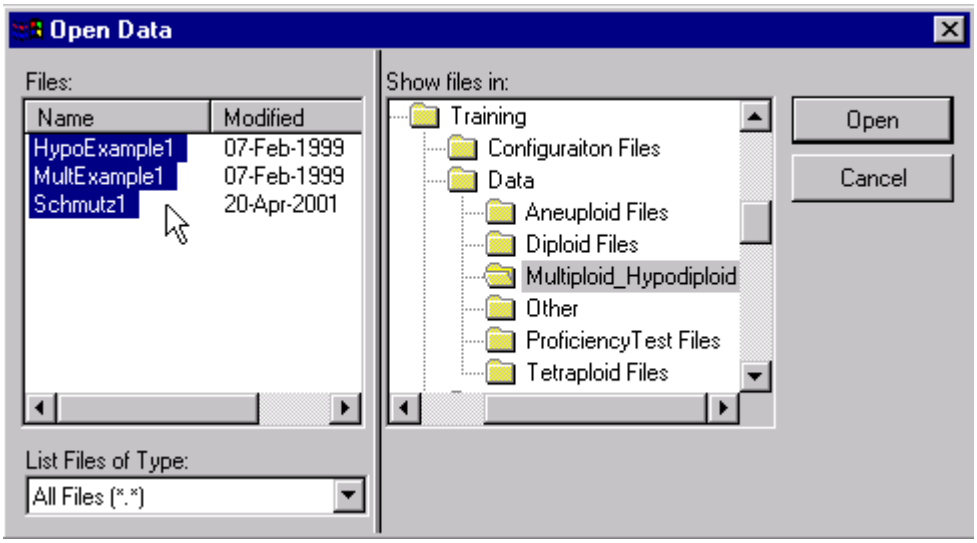
Part J – Multiploid / Hypodiploid Files – 15 minutes

We will again use the four-phase approach to analyze these more difficult DNA histograms.

Phase 1 – Perform Auto Analysis and generate report files.

In a manner similar to the preceding sections, we will first load our DNA report template (if necessary), select the DNA aneuploid files, properly configure the **File Batch** control and then process all the files using **Auto Analysis**.

- Click on the **File** menu and choose **Open Report...**.
- Navigate to folder under “Data”, select the file, “DNAReportTemplate.mfl” then click on **Open**.
- Click on the **File** toolbar button (first tool button on left).
- Navigate to the “Multiploid_Hypodiploid Files” folder (see figure below).
- Multiple-select all of the example files (see figure below).



- Click the **Open** button.
- Select “FL2-A” as the analysis parameter.
- Click **OK** to close the **Choose Histogram** dialog box.

As in earlier tutorials, we will configure the **File Batch** control to automatically analyze all the files and save the reports.

- From the **Edit** menu, choose "**File Batch Settings**…" or select the **File Batch** control's "pencil" button.
- From the drop-down **Analysis** listbox, select "Auto-analyze".
- Check the **Save Report** option.
- Select the **Save Report Path Folder…** button and navigate to the desired report folder location. For the DNA multiploid and hypodiploid files, we want to store the reports in the "Reports" folder under the "Multiploid_hypodiploid Files" folder.
- Select **Open** to store this report pathway and to close the **Choose a folder** dialog box.
- Uncheck the **Database** and **File prompt** checkboxes.
- Select **OK** to close the **Edit Properties for file processor** dialog.

Now we are ready to automatically process the files.

- In the **File Batch** control, click the **Redo** button to process the first file.
- Now select the **File Batch** control's **Auto-advance** button to analyze the remaining files.

All the files have now been analyzed and the reports saved.

We now need to reset these **File Batch** properties in order that the program doesn't prematurely start analyzing and saving reports when we start the next tutorial section.

- Choose either **File Batch Settings** from the **Edit** menu or click on the "pencil" edit button on the **File Batch** control.
- Select "No analysis" from the **Analysis** drop-down listbox.
- Check the "File prompt" checkbox.
- Uncheck the **Save Report** option.
- Click on **OK**.

This completes Phase 1.

Phase 2 - Initial Review

Let's load in these reports and see what **Auto Analysis** did.

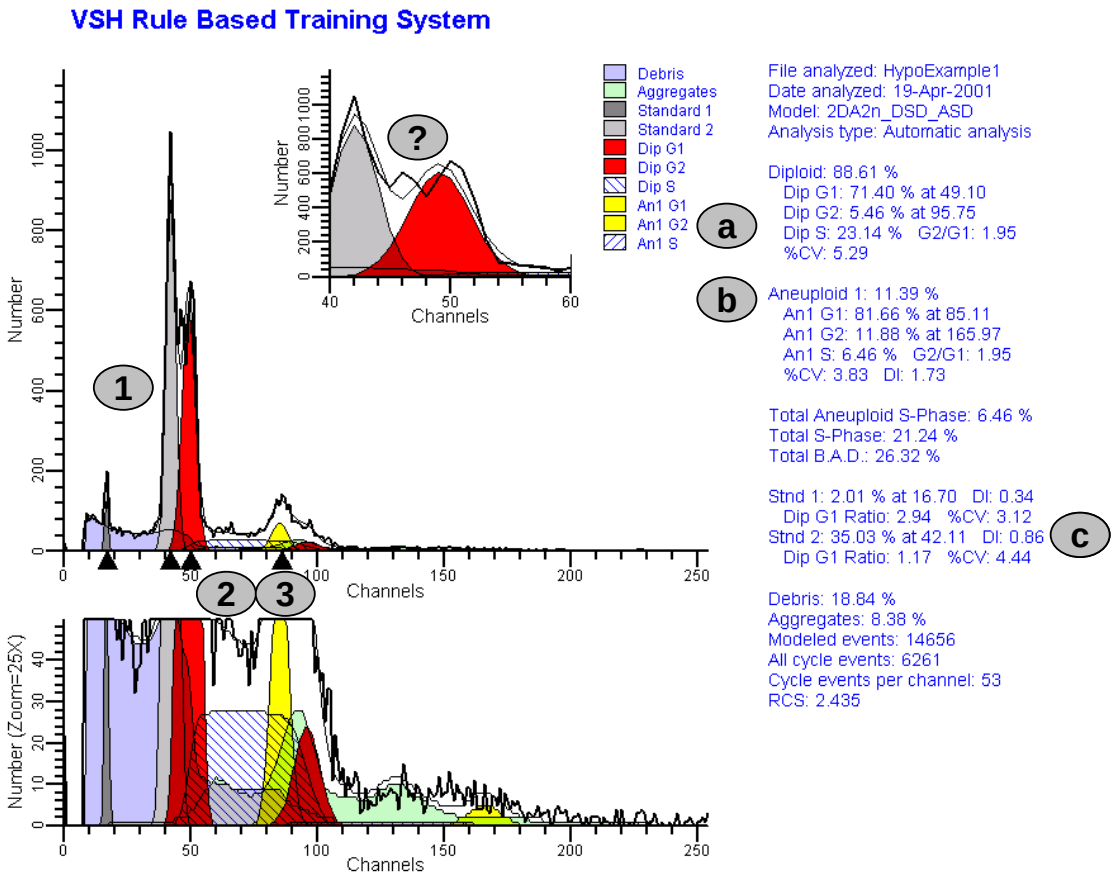
- Click on **Report** menu and choose **Open** -> **Report…**

Notice that the program took you to the folder where you had previously chosen to save the reports.

- Multiple-select all the listed reports.
- Click the **Open** button.

Configure the **Report Batch** control to efficiently evaluate these reports.

- Choose **Report Batch Settings**… from the **Edit** menu or click on the **Report Batch** control's "pencil" icon.
- Choose 'No analysis' from the **Analysis** drop-down listbox.
- Choose "Re-save report" from the **Save Report** drop-down listbox.
- Leave the rest of the options in their default states.
- Click **OK** to save the settings and close the dialog.
- Let's examine our first report.



?. Try to remember when to use the X-Zoomed graphic.

1. Notice that the second standard, trout erythrocytes, seems elevated compared to the first standard.

2 and a. Notice that the DNA Diploid S-phase is elevated.

3. There is also an elevated peak at roughly twice the position of the peak identified at (1).

c. Finally, note that the results associated with the second standard model component shows a DI of 0.86 when it normally ranges between 0.78 and 0.80.

All these observations support the conclusion that this histogram has the relatively rare DNA hypodiploid DNA ploidy pattern (expected frequency ~2% for breast cancer).

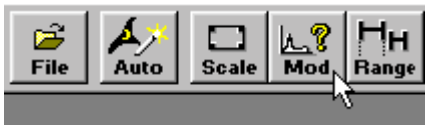
There are a number of modeling ramifications to DNA hypodiploid histograms. In this particular example, since the DNA hypodiploid G1 peak overlaps the trout standard, we must deactivate this standard (Rule:II.B.a.1, "If two model components are of similar shape and are highly overlapped (>75%), it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance.").

This same rule applies to the overlapping DNA hypodiploid and diploid S-phases. Since the DNA diploid S-phase is of lesser importance, it must be deactivated.

The difficult part of this histogram interpretation is whether the additional aneuploid population is really just part of the DNA hypodiploid cycle. We need to use another one of our rules to help us with this decision (Rule:II.B.1.c.1, "Only choose this model (aneuploid) if the potential aneuploid's G0G1 cannot be explained as an aggregate or some other part of another cycle (e.g. G2M)."). Since this additional peak could be the DNA hypodiploid's G2, we initially reject the ***Auto Analysis*** *decision to add a DNA aneuploid population to the model.*

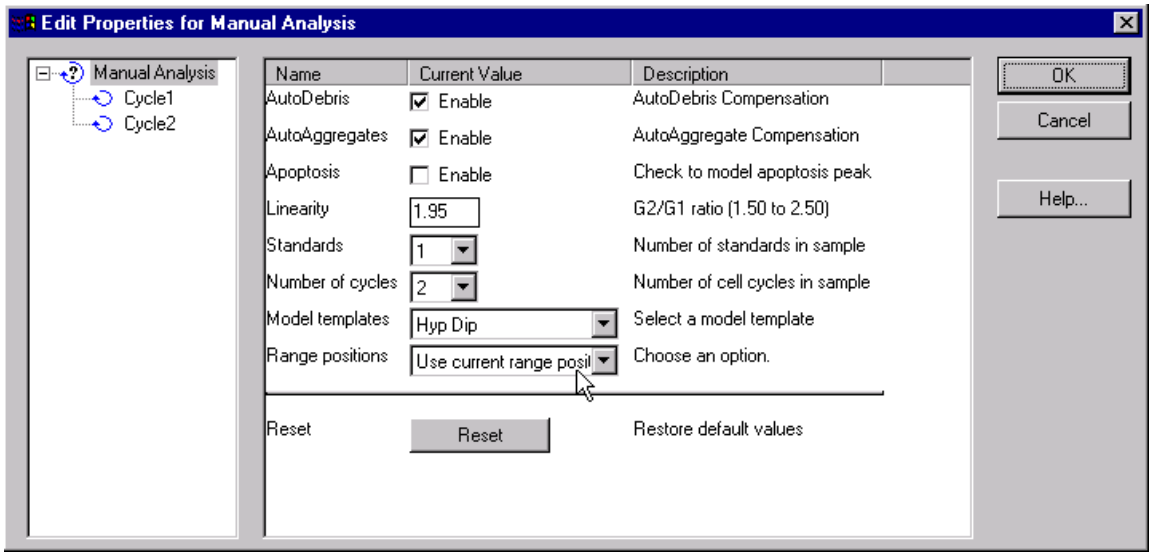
Let's pick a better model.

- Click on the **Mod** toolbar button.

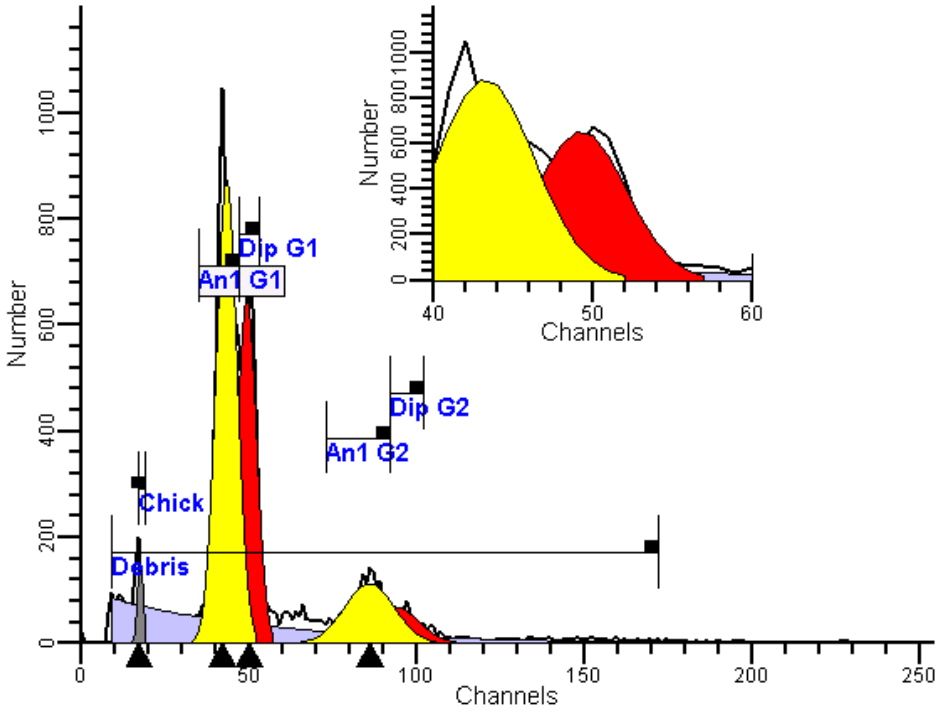


- In the **Edit Properties for Manual Analysis** dialog box, change the number of standards to "1" (see graphic below).
- From the **Model template** drop-down listbox choose "Hyp Dip" (see graphic below).
- Select "Use current range positions" from the **Range positions** drop-down listbox (see graphic below).

If you leave this option set to "Compute range positions", the program will move all of the ranges. Since ***Auto Analysis*** *calculated most of the populations correctly, it is best to use the "Use current range positions" option in order to minimize the number of ranges to move or resize.*



- Click on **OK**.
- Only adjust the “An1 G1” and “An1 G2” ranges (see graphic below).

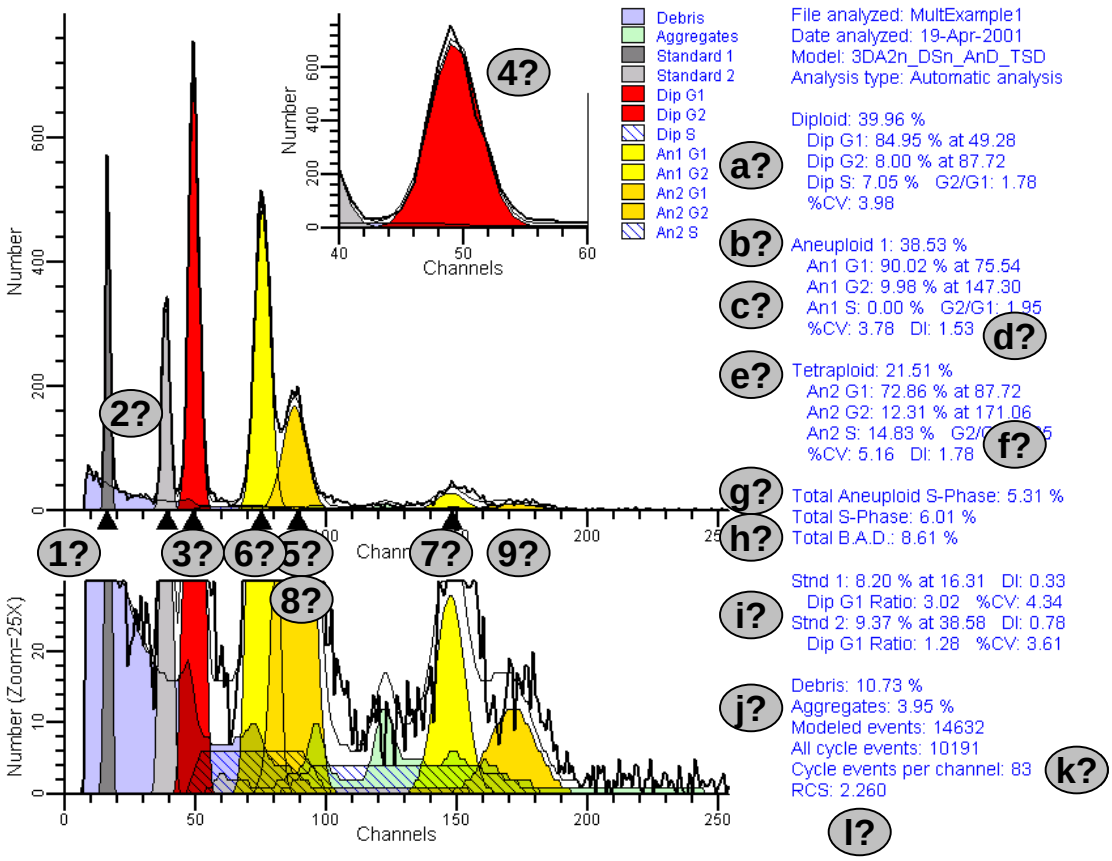


- Click on the **Fit** toolbar button.

The DNA hypodiploid G2 and the aggregates still don’t fit well, but that situation will probably improve in Phase 3.

- Click on the **Report Batch** control’s **Next Batch Item** button to load the next report.

VSH Rule Based Training System



Although this histogram is quite complex, it is easily interpretable and most of the rules we have introduced thus far, will apply. In many ways, this histogram summarizes practically all that you have learned; therefore, we will use it as a final summary. If you don't feel comfortable with any of the following questions regarding this histogram, go back and review them in the appropriate earlier tutorials.

1?. Start of the debris component. Is it correct?

2?. What can be said about the internal standards in this sample?

3?. Why is this peak assigned to be a DNA diploid G1? What is a quick way of knowing whether a G1 or G2 model component is part of the DNA diploid or aneuploid population?

4?. Is there any evidence of a near diploid population?

5?. Can you see the DNA diploid G2? What does the absence of a DNA diploid G2 peak relate to the type of model that is used for peak (5)?

6?, b?, d?. What are the %AF and DI for this DNA aneuploid G1? What are the definitions for %AF and DI?

7?. Is this peak a reasonable G2 for the first DNA aneuploid G1? How do you know it belongs to the first DNA aneuploid G1?

8?, e?, f?. What are the %AF and DI for the second DNA aneuploid G1?

c?. Why is this S-phase zero? What rule is being used? Has the rule been applied to the appropriate DNA aneuploid population?

9?. Is this peak a reasonable G2 for the second DNA aneuploid G1?

a?. Why is the DNA diploid G2, 8%?

g?. How is the "Total Aneuploid S-phase" calculated?

h?. Does this sample have an acceptable level of %BAD? What is %BAD?

I?. Are the internal standards in their appropriate locations? What can be said about the cellular concentration assuming the internal standards are always added to a constant final concentration?

j?. If you were going to try to improve your method of preparation, what would you work on most, minimizing debris or aggregates?

k?. Have an adequate number of events been accumulated? How do you know? What's optimal?

l?. Is the quality of the fit reasonable? When would you suspect that some part of the histogram is not being fit properly?

If you feel comfortable answering the above questions, then you have learned what we intended to teach. If you don't, go back and review the earlier tutorials.

*The important observations that relate to our task in Phase 2 are questions: 6, 8 and c. These two aneuploid populations have S-phases that are heavily overlapped and therefore one of the S-phases needs to be inactivated. The question to answer is whether **Auto Analysis** picked the correct population to inactivate. The more dominant aneuploid population is the first one according to our estimates of their respective aneuploid fractions; therefore, the second DNA aneuploid S-phase should be deactivated, not the first.*

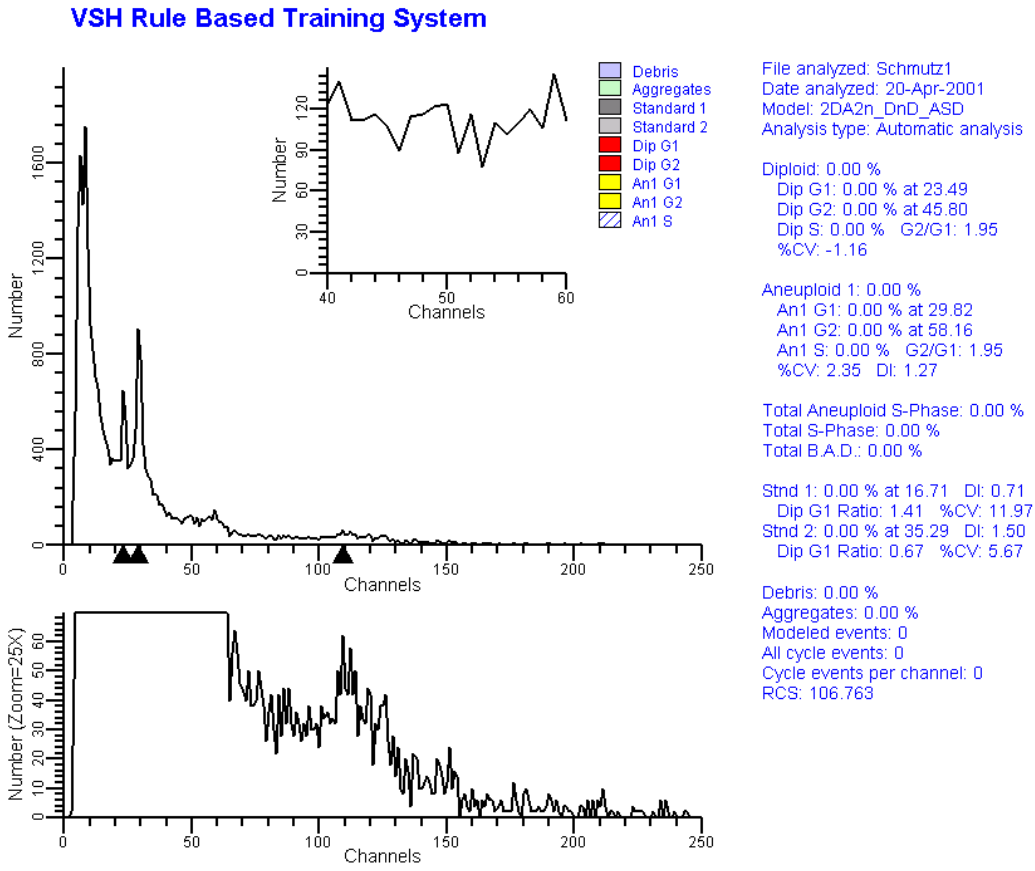
In the big scheme of things, this error is rather minor. However, our intent in this tutorial is to teach a method of analyzing DNA histograms that results in different operators obtaining the same answer by allowing the rules to guide us during key decision points. Let's now correct this relatively minor mistake.

- The second DNA aneuploid population's S-phase should be deactivated and the first, activated.

- Choose "Quick Model Editor…" from the **Edit** menu.
- Scroll down until you see "Cycle2 S-phase" and activate its checkbox.
- Inactivate "Cycle3 S-phase".
- Select **OK**.
- Click on the **Fit** toolbar button.
- Click on the **Save** toolbar button.

Let's now look at the last report.

- Click on the **Report Batch** control's **Next Batch Item** button to load the next report.



It's very important to recognize this type of histogram when it crosses your desk. Occasionally, a DNA histogram will be of such quality that it should not be subjected to further examination. This kind of histograms is a waste of your time and if reported, can undermine clinicians' confidence in this technology and can conceivable harm the patient. The old adage, "Garbage in, garbage out", is particularly relevant for this type of histogram. You can ask for another sample, but do not try to be heroic and make something out of nothing.

This concludes Phase 2.

Phase 3 – Automatic Linearity Factor Detection

Let's now find the optimum **Linearity Factor** all our stored reports.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu (see graph below).
- Select "Fit and do autolinearity" from the **Analysis** drop-down listbox.
- Leave the "Re-save" **Save Report** option selected.
- Select **OK**.
- Click on the "First Batch Item" button on the **Report Batch** control.
- When the first report is done processing, select the "Auto Advance" button on the **Report Batch** control to analyze the rest of the reports.

End of Phase 3.

Phase 4 –Final Review

Now for our final review…

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu
- Select "No analysis" in the **Analysis** drop-down listbox.
- Select "Don't re-save report" from the **Save Report** drop-down listbox.
- Click on **OK**.
- Select the "First Batch Item" button on the **Report Batch** control.
- Inspect the first report using the method described in Phase 2.

The DNA hypodiploid model fit should look reasonable.

- Select the "Next Batch Item" button on the **Report Batch** control.

Notice that the second DNA aneuploid population has zero percent S-phase now.

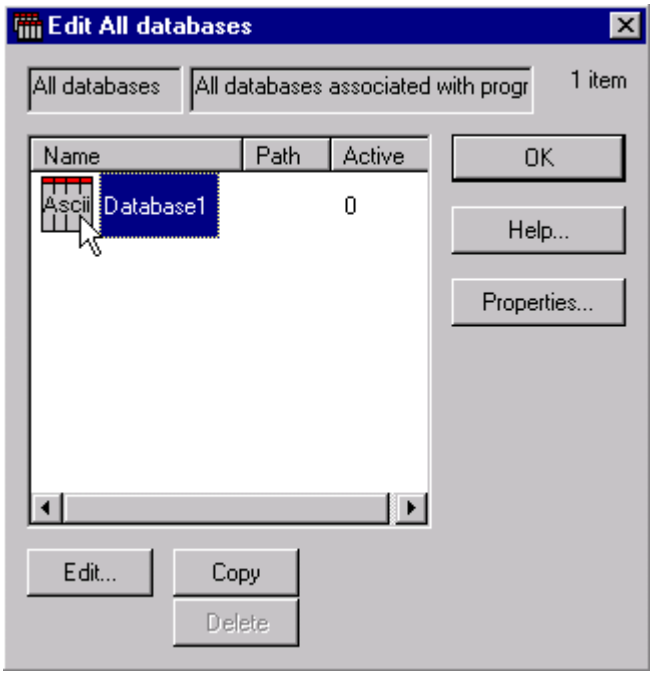
*It is very rare that the **Linearity Factor** detection system will create problems and necessitate a modeling change, but it can happen. Thus, you are obligated to recheck every report for this final time.*

This is the end section J: DNA Multiploid_Hypodiploid Tutorial.

Part K – Phase 5 - Databasing and Printing Reports – 15 minutes

Now that all your reports have been analyzed by the four-phase method, you can automatically reprocess these reports in Phase 5 and generate a complete database of the results and a printout of the final reports. This tutorial will attempt to show you exactly what you need to know to do these important tasks.

- From the **Edit** menu, choose "Databases…"
- Select "Database1" in the listbox (see graphic below).

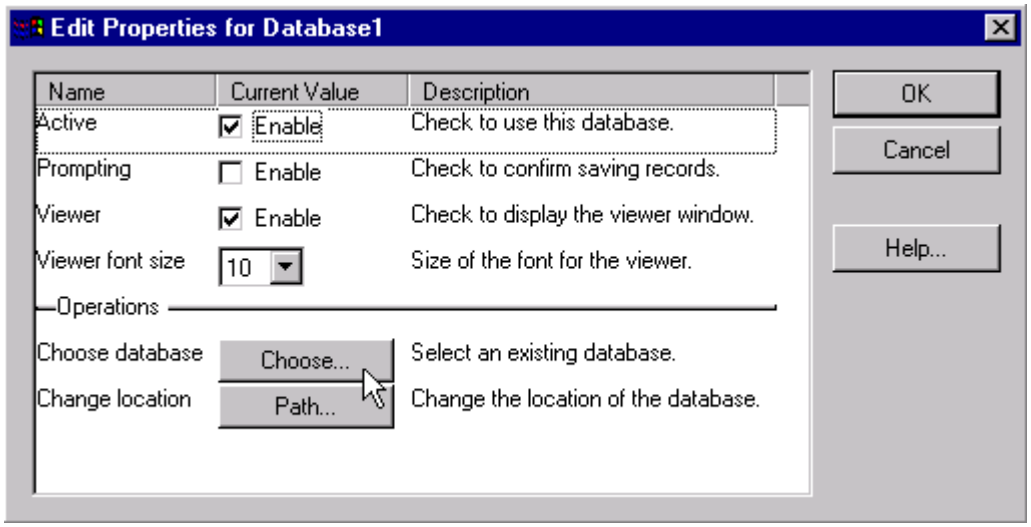


The program supports multiple simultaneous databases. By default, there is just one database, "Database1", available.

If the current database item is "Standard" then this part of the tutorial has been already done by someone else. Just follow the steps anyway.

We are going to use a previously designed database to import all the database fields.

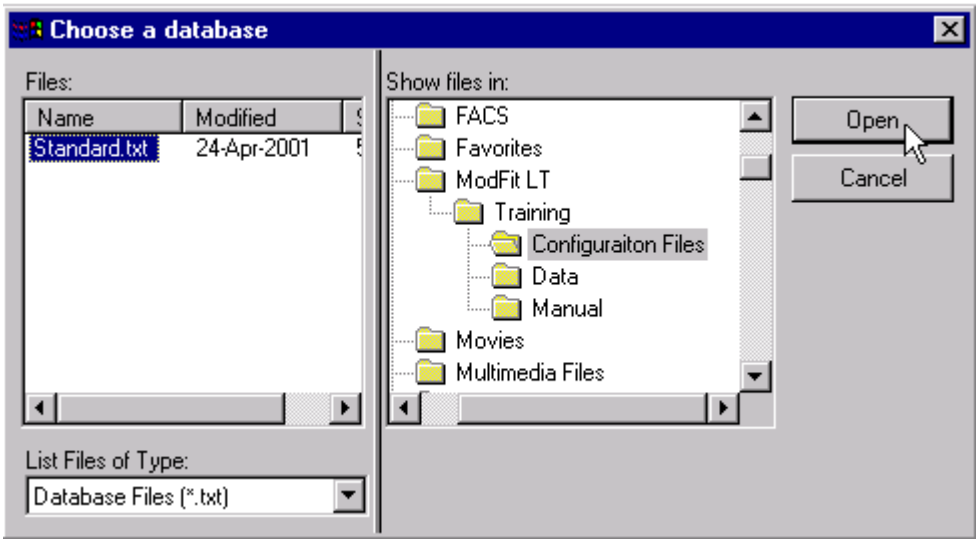
- Click on **Properties…**.



- Check and uncheck the options as shown above.

*The **Active** checkbox will activate the database for the current modeling session. We don't want the database system to prompt us to save a record and the **Viewer** allows us to inspect the records of the database.*

- Click on the **Choose** button (see graphic above).
- Navigate to the "Standard.txt" file in "Training\Configuration" folder (see graphic below).



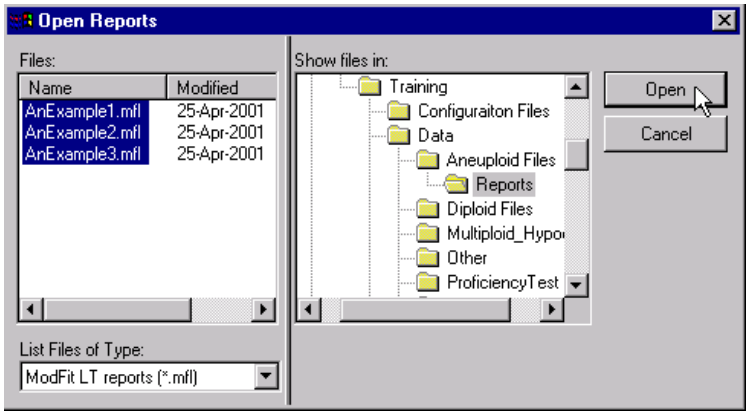
- Click on **Open**.

The viewer will appear and the database will now be named, "Standard".

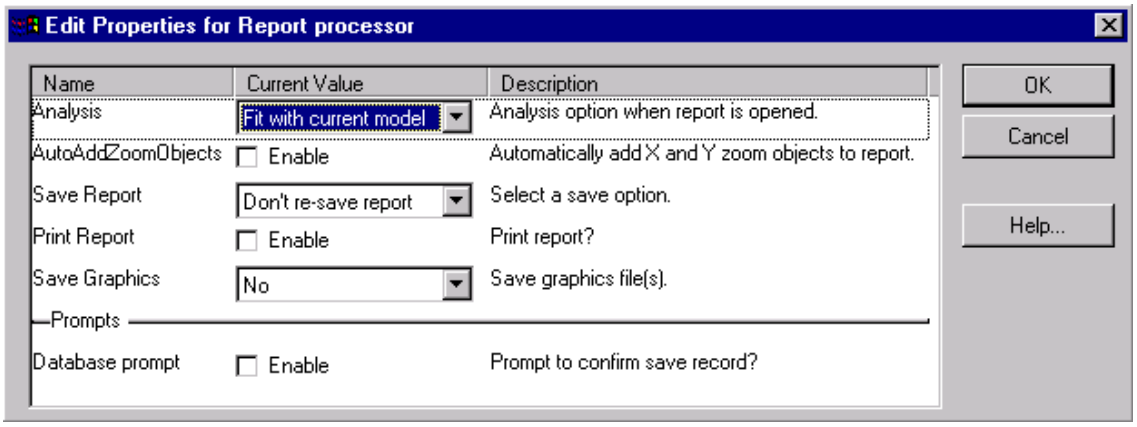
- Click on **OK**.
- Use the horizontal scroll bar on the **Viewer** to inspect all the database fields that will be databased.

We are going to analyze just a few reports to demonstrate how this last phase works.

- Click on the **File** menu, choose **Open->Reports**.
- Navigate to the “Training\Data\Aneuploid Files” folder (see graphic below).



- Multiple-select the aneuploid example files.
- Click on **Open**.
- Either choose “Report Batch Settings…” from the **Edit** menu or click on the **Report Batch** control’s “pencil” button.



- Select “Fit with current model” from the **Analysis** drop-down listbox.

*This selection is the important one. You are telling the **Report Batch** control to fit the data with the current model when it loads in the report. If the database system is active, all the results that match the database fields will be copied to the database and will appear as a new record in the **Viewer**.*

- If you have a printer connected to your computer, enable the **Print Report** checkbox.
- Click on **OK**.
- Click on the **Report Batch** control’s **Redo** button.

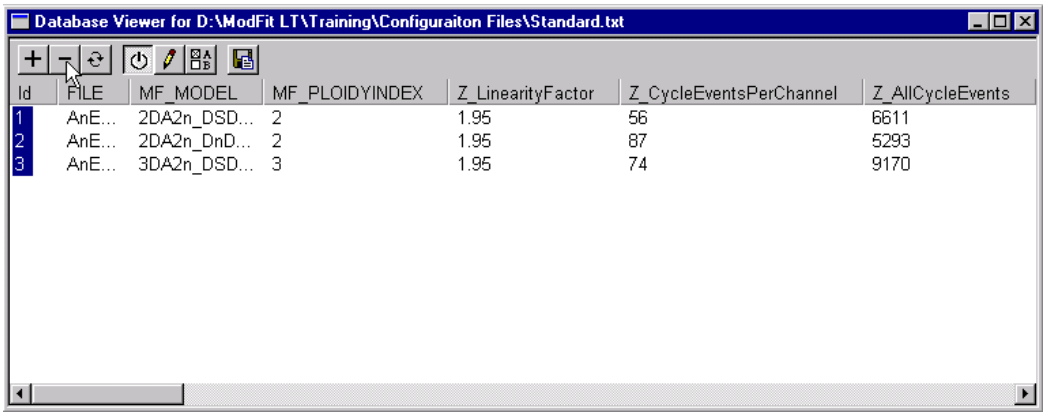
Notice that the first record is stored in the database. Use the horizontal scrollbar of to examine all the stored database fields.

- Click on the **Report Batch** control’s **Auto Advance** button.

All the rest of the reports will be databased. Keep in mind that you can multiple-select hundreds of reports for databasing and reporting.

We're going to delete the records just stored so that these tutorials can be re-used without changing the instructions.

- Click on the first column for Id "1".
- Hold down the Shift key and click on the first column for Id "3" (see graphic below).



- Click on the **Delete** record button (-).
- Click **Yes** when asked whether you really want to delete the records.

Let's go back and turn off the databasing until we are ready to use it.

- Choose "Databases…" from the **Edit** menu.
- Click on the "Standard" database item to select it.
- Click on the **Properties…** button.
- Uncheck the **Active** and **Viewer** entries.
- Click on **OK**.

Go take a look at the reports if you printed them.

This is the end of section K: Phase 5 - Databasing and Printing Reports.

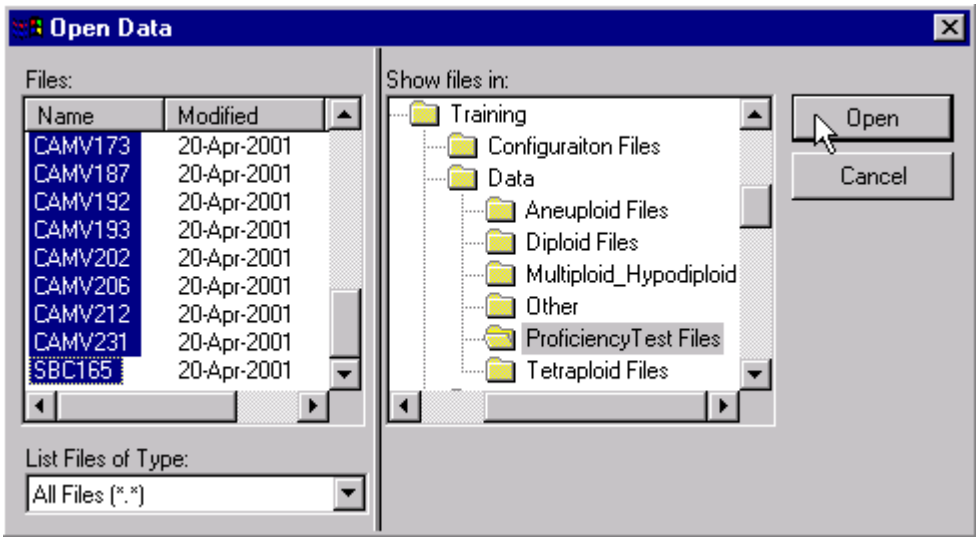
Part L – Proficiency Testing – 50+ minutes

It's now time to test your knowledge of performing cell cycle analysis on DNA histograms. We have gathered some easy, not-so-easy and hard histograms for each DNA ploidy type. In this section, we will be asking you to use the four-phase approach to analyze all these files. After your final review, you will configure the system to store all your analysis results to a particular database. That file will be imported into a specially designed Excel worksheet to evaluate your cell cycle analysis skills. Take your time and try to remember the left-to-right technique in evaluating the reports in Phase 2. Remember, use the rules in Appendix 1 to guide you through this process. Good luck!

Phase 1 – Perform Auto Analysis and generate report files.

In a manner similar to the preceding sections, you will first load our DNA report template (if necessary), select all the proficiency files, properly configure the **File Batch** control and then process all the files using **Auto Analysis**.

- Click on the **File** menu and choose **Open Report...**.
- Navigate to folder under "Data", select the file, "DNAReportTemplate.mfl" then click on **Open**.
- Click on the **File** toolbar button (first tool button on left).
- Navigate to the "Proficiency Test Files" folder.
- Multiple-select all files (see figure below).



- Click the **Open** button.

If the first file happens to have multiple "stacked" histograms, you may be prompted to enter the analysis parameter. Choose "FL2-A" when prompted.

As in earlier tutorials, we will configure the **File Batch** control to automatically analyze all the files and save the reports.

- From the **Edit** menu, choose "**File Batch Settings**…" or select the **File Batch** control's "pencil" button.
- From the drop-down **Analysis** listbox, select "Auto-analyze".
- Check the **Save Report** option.
- Select the **Save Report Path Folder…** button and navigate to the desired report folder location. For these files, we want to store the reports in the "Reports" folder under the "Proficiency Test Files" folder.
- Select **Open** to store this report pathway and to close the **Choose a folder** dialog box.
- Uncheck the **Database** and **File prompt** checkboxes.
- Select **OK** to close the **Edit Properties for file processor** dialog.

Now we are ready to automatically process the files.

- In the **File Batch** control, click the **Redo** button to process the first file.
- Now select the **File Batch** control's **Auto-advance** button to analyze the remaining files.

After some processing, all the files will be **Auto Analyzed** and the reports saved.

- Click on the **Save** toolbar button to make sure the last report is saved.

We now need to reset these **File Batch** properties in order that the program doesn't prematurely start analyzing and saving reports if you start another tutorial section when your done.

- Choose either **File Batch Settings** from the **Edit** menu or click on the "pencil" edit button on the **File Batch** control.
- Select "No analysis" from the **Analysis** drop-down listbox.
- Uncheck the **Save Report** option.
- Check the **File prompt** checkbox.
- Click on **OK**.

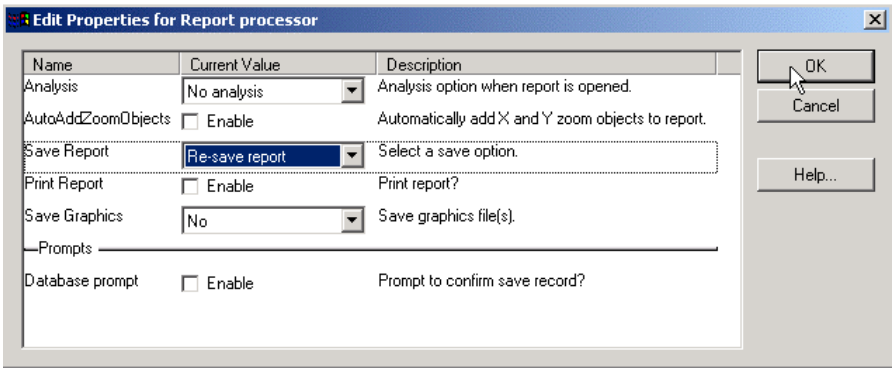
This completes Phase 1.

Phase 2 – Initial Review

Use the left-to-right method and carefully review each report.

Let's load in all the reports.

- From the **File** menu, choose **Open** -> **Report**...
- Navigate (if necessary) to the "Proficiency Test Files\Reports" folder and multiple-select all of the reports.
- Select **Open**.
- Choose **Report Batch Settings**… from the **Edit** menu or click on the **Report Batch** control's "pencil" icon.
- Choose 'No analysis' from the **Analysis** drop-down listbox.
- Choose "Re-save report" from the **Save Report** drop-down listbox.
- Leave the rest of the options in their default states (see graphic below).



- Click **OK** to save the settings and close the dialog.

Review the first report. Make any changes that are necessary. Once you're satisfied, click on the **Report Batch** control's **Next Batch Index** button to go the next report. Since you configured the **Report Batch** processor to save the reports, any changes you have made will be automatically saved when you move to the next report. Continue in this manner until you have processed all the reports.

End of Phase 2

Phase 3 – Automatic Linearity Factor Detection

Let's now find the optimum **Linearity Factor** all your stored reports.

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu (see graph below).
- Select "Fit and do autolinearity" from the **Analysis** drop-down listbox.
- Leave the "Re-save" **Save Report** option selected.
- Select **OK**.
- Click on the "First Batch Item" button on the **Report Batch** control.
- When the first report is done processing, select the **Auto Advance** button on the **Report Batch** control to analyze the rest of the reports.
- Click on the **Save** toolbar button to make sure the last report is saved.

End of Phase 3.

Phase 4 –Final Review

Now for your final review…

- Click on the **Report Batch** control's "pencil" button or choose **Report Batch Settings** from the **Edit** menu
- Select "No analysis" in the **Analysis** drop-down listbox.
- Select "Don't re-save report" from the **Save Report** drop-down listbox.
- Click on **OK**.
- Select the "First Batch Item" button on the **Report Batch** control.
- Inspect all the reports by using the **Report Batch** control's **Next Batch Item** button.

*If you need to make changes to the reports at this point, you need to completely reanalyze the file, including the **AutoLinearity** analysis. Don't forget to save the report with the **Save** button when you are done. Do not use **Auto Analysis** since it will automatically load a new model and reset all your changes.*

*To perform the **AutoLinearity** analysis on a single report, use the following procedure.*

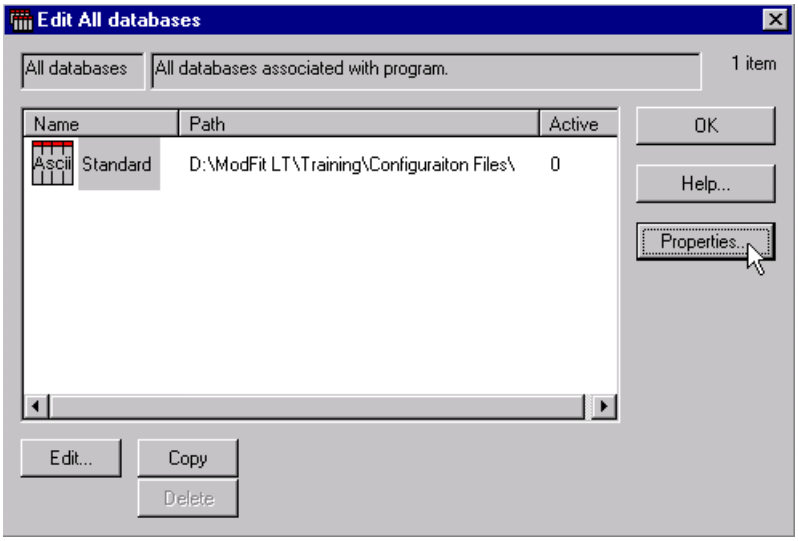
*Choose "Quick Model Editor…" from the **Edit** menu.*
*Check the **AutoLinearity** checkbox.*
*Click on **OK**.*
*Click on the **Fit** toolbar button.*

End of Phase 4.

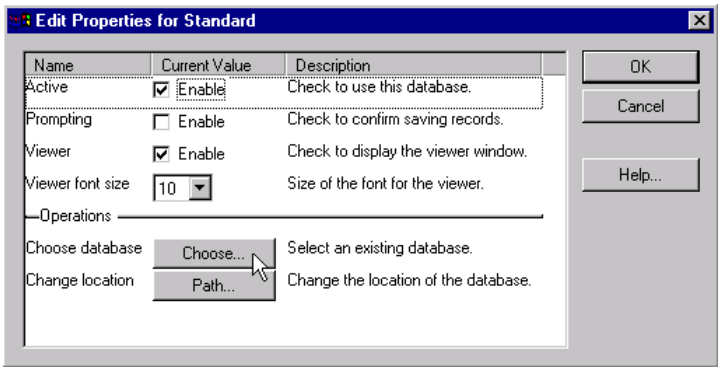
Phase 5 – Databasing and Printing Reports

Now that all the reports have been reviewed, we will database the results and compare them with the "Reference" analyses. It's very important that you have exactly the right number of reports. For this Proficiency Exam, you should have 29 reports.

- From the **Edit** menu, choose "Databases…"
- Select "Standard" in the listbox (see graphic below).



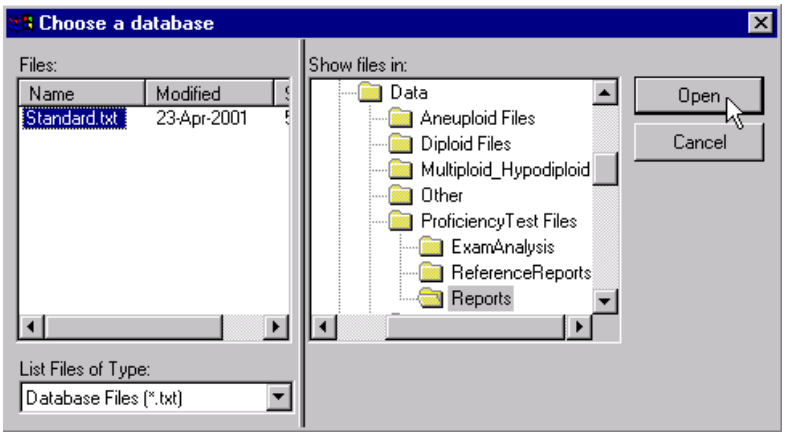
- Click on **Properties…**.



- Check and uncheck the options as shown above.

*The **Active** checkbox will activate the database for the current modeling session. We don't want the database system to prompt us to save a record and the **Viewer** allows us to inspect the records of the database as they are created.*

- Click on the **Choose** button (see graphic above).
- Navigate to the "Standard.txt" file in "Training\Data\Proficiency Test Files\Reports" folder (see graphic below).



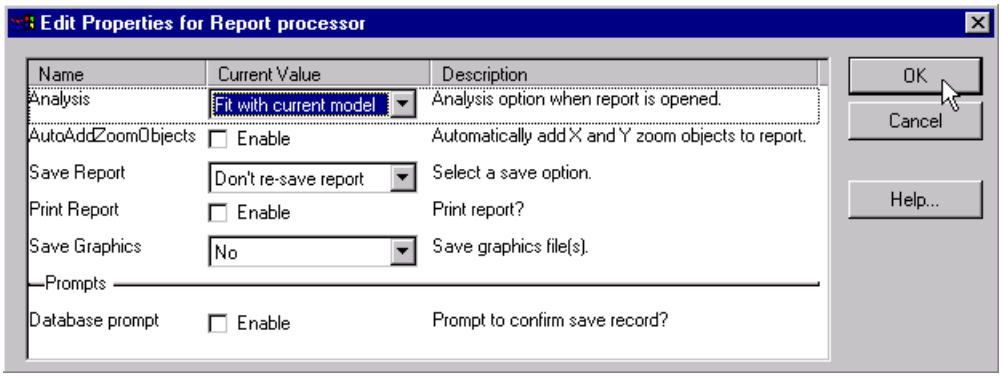
- Click on **Open**.

*The **Viewer** will appear and the database will be named, "Standard".*

- Click on **OK** to close the **Edit Properties for Standard** dialog.
- Click on Ok to close the **Edit All Databases** dialog.

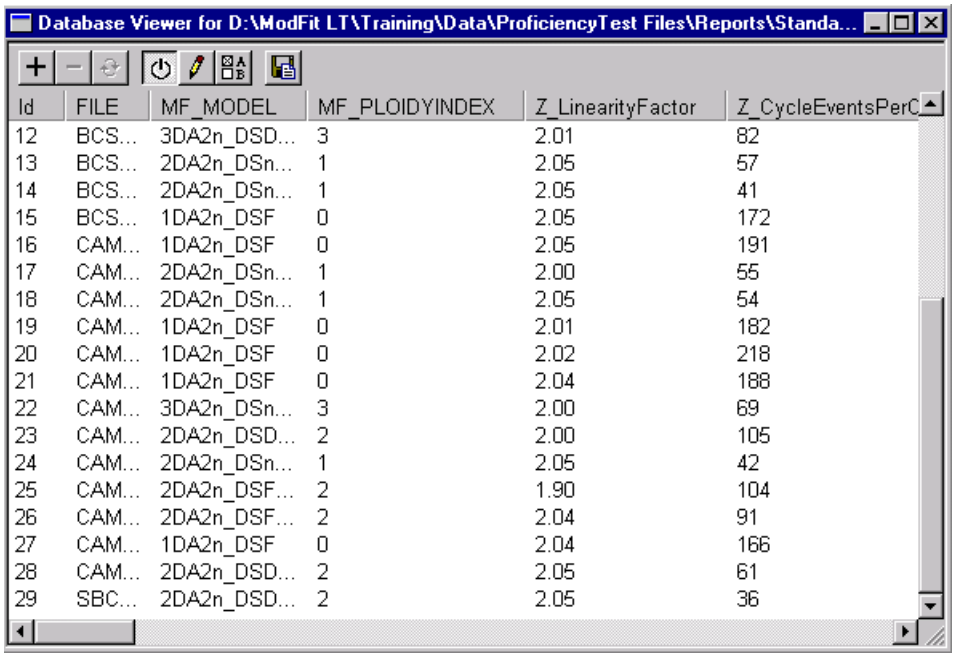
*If there are records in the **Viewer**, delete them all using the same procedure as discussed in the previous tutorial.*

- Either choose "Report Batch Settings…" from the **Edit** menu or click on the **Report Batch** control's "pencil" button.



- Select "Fit with current model" from the **Analysis** drop-down listbox.
- Click on **OK**.
- Click on the **Report Batch** control's **Go to the first batch item** button.
- Click on the **Report Batch** control's **Auto Advance** button.

When you are done, your **Viewer** should look like the graphic below:



Id	FILE	MF_MODEL	MF_PLOIDYINDEX	Z_LinearityFactor	Z_CycleEventsPerC
12	BCS...	3DA2n_DSD...	3	2.01	82
13	BCS...	2DA2n_DSn...	1	2.05	57
14	BCS...	2DA2n_DSn...	1	2.05	41
15	BCS...	1DA2n_DSF	0	2.05	172
16	CAM...	1DA2n_DSF	0	2.05	191
17	CAM...	2DA2n_DSn...	1	2.00	55
18	CAM...	2DA2n_DSn...	1	2.05	54
19	CAM...	1DA2n_DSF	0	2.01	182
20	CAM...	1DA2n_DSF	0	2.02	218
21	CAM...	1DA2n_DSF	0	2.04	188
22	CAM...	3DA2n_DSn...	3	2.00	69
23	CAM...	2DA2n_DSD...	2	2.00	105
24	CAM...	2DA2n_DSn...	1	2.05	42
25	CAM...	2DA2n_DSF...	2	1.90	104
26	CAM...	2DA2n_DSF...	2	2.04	91
27	CAM...	1DA2n_DSF	0	2.04	166
28	CAM...	2DA2n_DSD...	2	2.05	61
29	SBC...	2DA2n_DSD...	2	2.05	36

As mentioned earlier, it is very important that you have exactly 29 records in this database (see graphic above, last line).

We are now done databasing and should turn the **Database** system off.

- Click on “Databases...” under the **Edit** menu.
- Select the “Standard” database item.
- Click on the **Properties...** button.
- Uncheck the **Active** and **Viewer** checkboxes.
- Click on **OK**.
- Click on **OK** again.

Importing into Excel

We are ready to import this database into our specially designed Excel 2000 Workbook.

- With Windows Explorer (normally found in “Start\Programs\Accessories”), navigate to “Training\Data\Proficiency Test Files\ExamAnalysis” folder.

  If you are running these tutorials from the Macintosh and you have Excel 2000 for the Mac, use Finder to navigate to the Excel workbook file.

- Double-click on the “ProficiencyTestResults.xls” file.
- Click on the “Your Data” tab at the bottom of the workbook.
- If there are any cells containing data, select and delete them.

- Click on **Open...** under Excel's **File** menu.
- Choose "Text Files..." from the **File Type** drop-down listbox.
- In the **Look in** tree-list control, navigate to "Proficiency Test Files\Reports".
- Select "Standard.txt" and click on **Open**.
- When the **Text Import Wizard** appears, click on the **Finish** button.
- Select all the rows and columns in the Standard.txt worksheet.

For Windows users this operation is conveniently done by pressing the Ctrl – Shift – End keys all at the same time.

- Select **Copy** from the **Edit** menu.
- Click on the title bar of the **Proficiency Test Results** workbook to activate it.
- Click on the upper-left cell to set the focus.
- Select **Paste** from the **Edit** menu.

If all went according to plan, you should have the first 30 rows filled with data.

- Go back to the "Standard.txt" workbook and close it.
- If prompted to save contents of the clipboard, choose the **No** button.

Once you are done importing data into Excel from ModFit LT, it is a good idea to close this workbook. If you leave it open in Excel, then ModFit LT will not be able to work with it, which can eventually result in an error.

- Click on the "Diploid Analyses" worksheet tab.

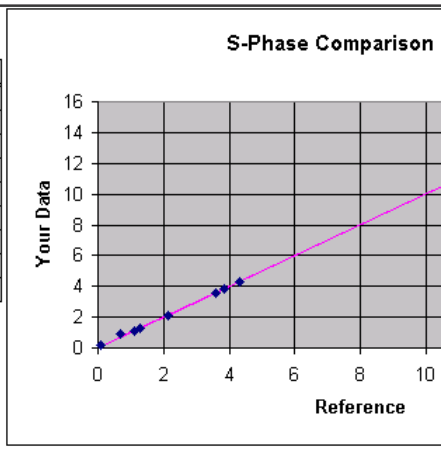
Diploid Analyses

Rows: 4,7,12,16,17,20,21,22,28

Files	Reference	Your Data	Abs S Diff	Ploidy Code
BCS057	0.08	0.2	0.12	0
BCS071	3.85	3.85	0	0
BCS145	0.7	0.9	0.2	0
BCS184	3.61	3.61	0	0
CAMV151	2.14	2.14	0	0
CAMV168	4.32	4.32	0	0
CAMV171	1.29	1.29	0	0
CAMV173	13.61	13.61	0	0
CAMV212	1.12	1.12	0	0

S-Phase Analysis

Average:	0.036
Corr:	1.000



Ploidy Classification Analysis

	Diploid	Tetraploid	Aneuploid	Multiploid	Hypodiploid	Total
Number	9	0	0	0	0	9
Percent	100.0%	0.0%	0.0%	0.0%	0.0%	100.0%

The table in the upper-left of the worksheet should receive most of your attention. The first column shows the names of the DNA diploid files. The next two columns are the comparison between the "Reference" and "Your Data" S-phases. If there is a difference of greater than 0.1%,

*the cell will be shaded in red. If any of these cells are red, review the **Part G – DNA Diploid files** tutorial and re-examine your relevant reports. If you still can't figure out why they are different, look at the corresponding "Reference" report (ProficiencyTestFiles\ReferenceReports). Try to only review the "Reference" reports as a last resort.*

Underneath this table is another table that calculates the average absolute difference and the correlation coefficient between the "Reference" and "Your Data" S-phases. If these values are shaded in red then the mistakes made were probably more than just minor mistakes and you need to eventually redo this proficiency exam.

*The last table, "Ploidy Reclassification Analysis", shows a breakdown of the analyzed files. In the case of the DNA diploid files, the first column should show 100% of the files categorized as DNA diploids. If you don't have the correct DNA ploidy for the histogram, then the S-phase will not be accurate or reproducible. This table may provide clues as to what type of DNA ploidy you are confusing with the DNA diploids. For example, if a few files were misclassified as DNA tetraploids, you should go back to the **Part H - DNA Tetraploid files** tutorial and review it completely.*

The graph on the right plots the "Reference" vs. "Your Data" S-phases. This graph provides an overall qualitative comparison between these two sets of S-phases.

Once you have finished the "Diploid Analyses", click on the "Tetraploid Analyses" worksheet tab. The format of this worksheet is the same as we have just discussed except that it analyzes your proficiency with DNA tetraploid histograms.

Continue this analysis with "Aneuploid Analyses" and "Multiploid Analyses".

We at Verity hope you have found these DNA histogram analysis tutorials useful. We are convinced that if users are adequately trained, they can efficiently analyze DNA histograms and obtain consistent and relevant DNA Ploidy and S-phase estimates.

This is the end of section L: Proficiency Testing.

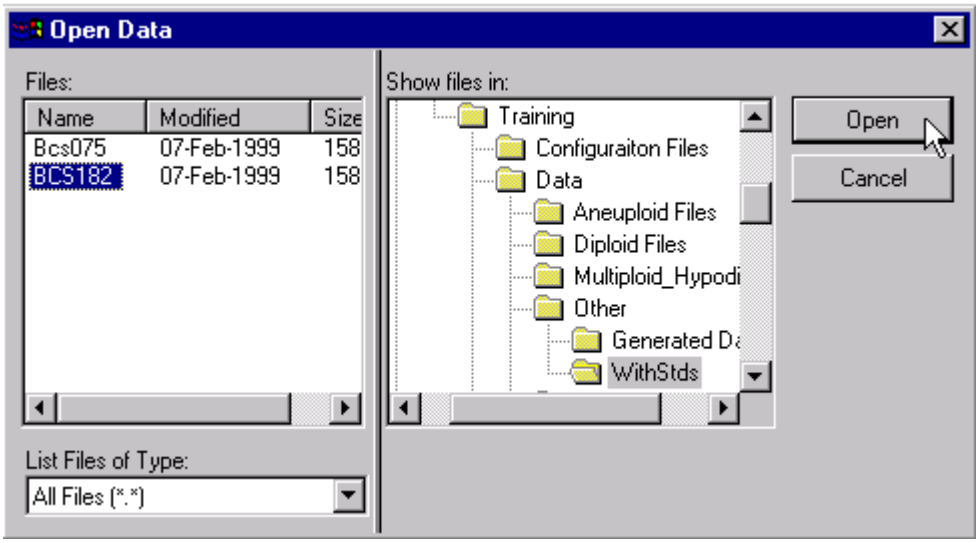
Part M- Advanced Modeling Techniques:

Occasionally, the standard set of models provided with ModFit LT cannot handle a particular analysis problem. In these situations, it is necessary to be able to edit some low level aspects of the DNA histogram model. In this section, we will introduce how to do these kinds of edits with a histogram that cannot be modeled accurately with the standard models.

Phase 1 – Perform Auto Analysis and generate report files.

In a manner similar to the preceding sections, we will first load our DNA report template (if necessary), select the problem file and then process the file using **Auto Analysis**.

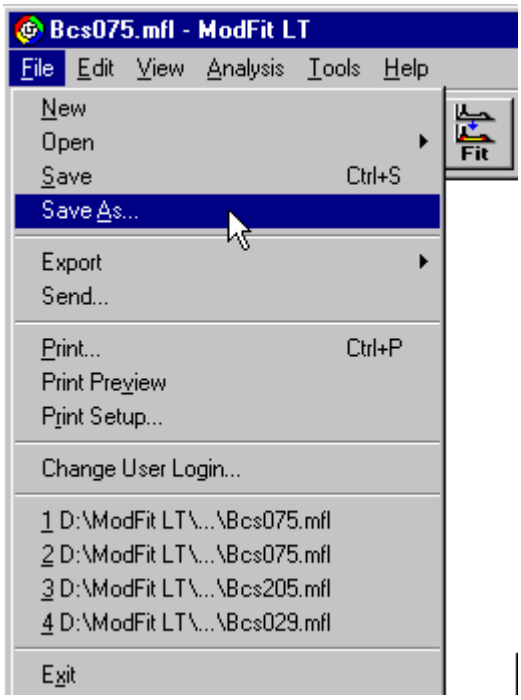
- Click on the **File** menu and choose **Open Report...**.
- Navigate to the folder under “Data”, select the file “DNAReportTemplate.mfl” and click on **Open**.
- Click on the **File** toolbar button (first tool button on left).
- Navigate to the “WithStds” folder (see figure below).
- Select file “BCS182" (see figure below).



- Click the **Open** button.
- Select “FL2-A” as the analysis parameter.
- Click **OK** to close the **Choose Histogram** dialog box.

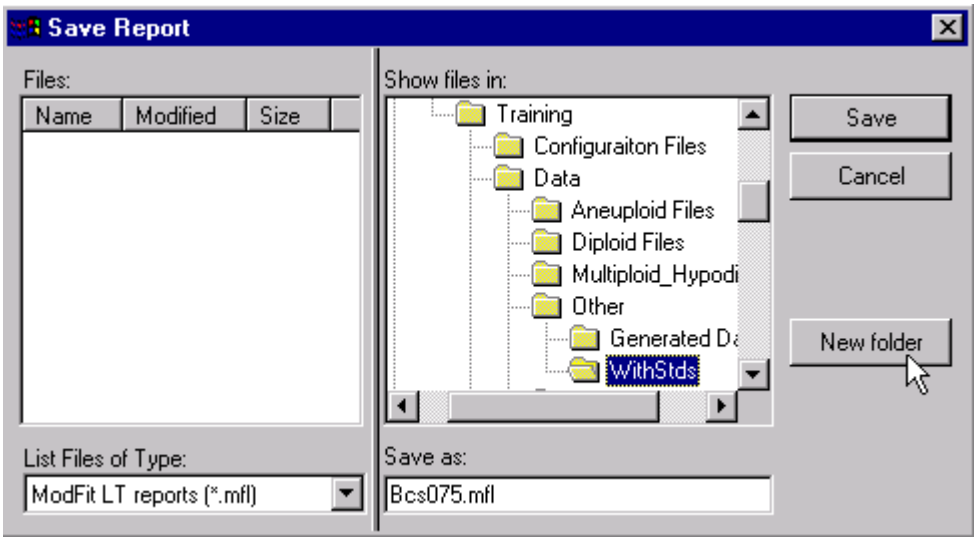
*Since we only chose one file, the **File Batch** processor is not loaded and will not be used.*

- Click on the **Auto** button to initiate **Auto Analysis**.
- Click on the **File** menu and select the "Save As…" menu

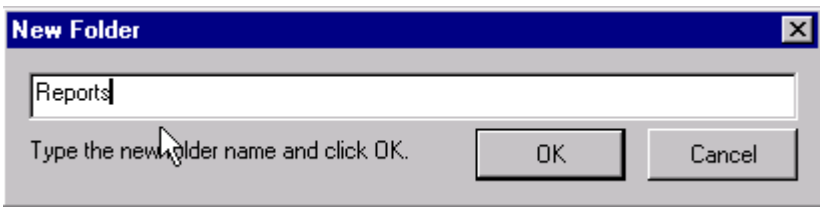


*The steps below assume that there currently is no **Reports** folder under the WithStds folder. If there is a folder there already, you don't need to do the next three steps.*

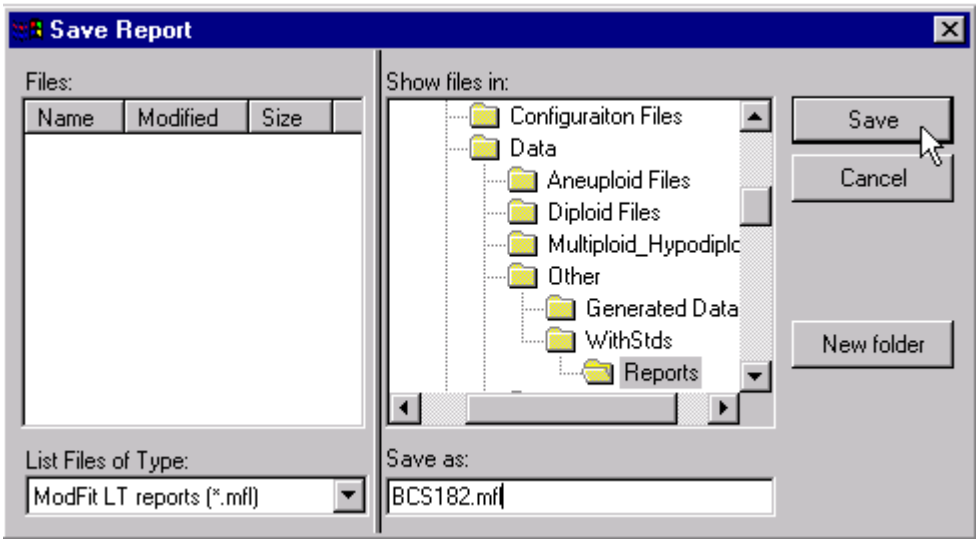
- Navigate to the "WithStds" folder and click on the **New folder** button.



- Type "Reports" into the edit line for the name of the new folder.



- Click on **OK**.
- Select the new “Reports” folder, then type the file name “BCS205.MFL into the “Save as:” edit box.



- Click on **Save**.
- The **Auto Analysis** report for file BSC182.fcs has now been save to the folder we just created.

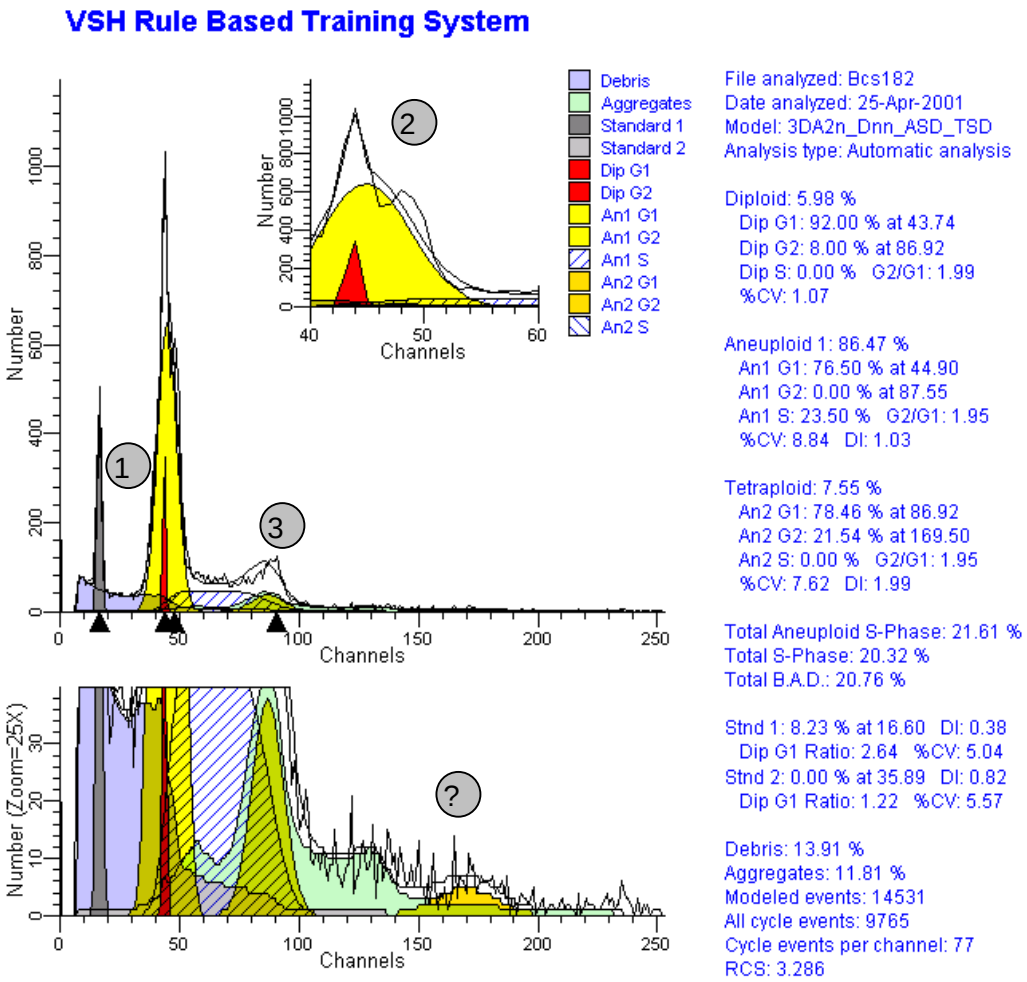
*This process of manually saving a report file using the **Save As** option sets the location where subsequent reports will be saved.*

This completes Phase 1.

Phase 2 - Initial Review

Since we are working with a single file, we do not have to reload the report to do the Phase 2 review.

- Let's examine the report.



1,2. Notice that the second standard, trout erythrocytes, is not visible and that the DNA diploid and aneuploid G1 populations are trying to fit the same area. Whenever you see two model components of similar shape trying to fit data in the same area of the histogram, a mental red flag should appear. As we have discussed before, superimposed model components cannot be expected to perform reliably in this situation. The rule II.B.1.a.1 states, "If two model components are of similar shape and are highly overlapped (>75%), it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance." As you will see in a moment, we are not only going to disable a model component of lesser importance but we will also add a model constraint to make it work the way we want.

*3,?. **Auto Analysis** has classified the peak near channel 85 as the G1 of a third cycle. Take a careful look at the Y-zoomed graph below the histogram and decide whether you agree with this decision or not. Try to back your decision up with one of the analysis rules. Which is the appropriate rule to use?*

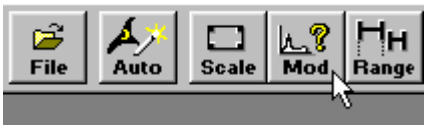
- The second standard must be turned off and the first G1 modeled as a DNA hypodiploid. Because the "near diploid" rules also apply, the S-phase of the lesser significant population (diploid population) needs to be turned off and the G2 populations made dependent on their corresponding G1 populations.

- Since the 8C peak is not greater than the 6C peak, we have no evidence of a third cell cycle and we should therefore change the model accordingly.

If you missed the second observation and conclusion, review the DNA tetraploid tutorial.

Let's start by selecting a better model that solves some of the identified problems associated with this analysis.

- Click on the **Mod** toolbar button.

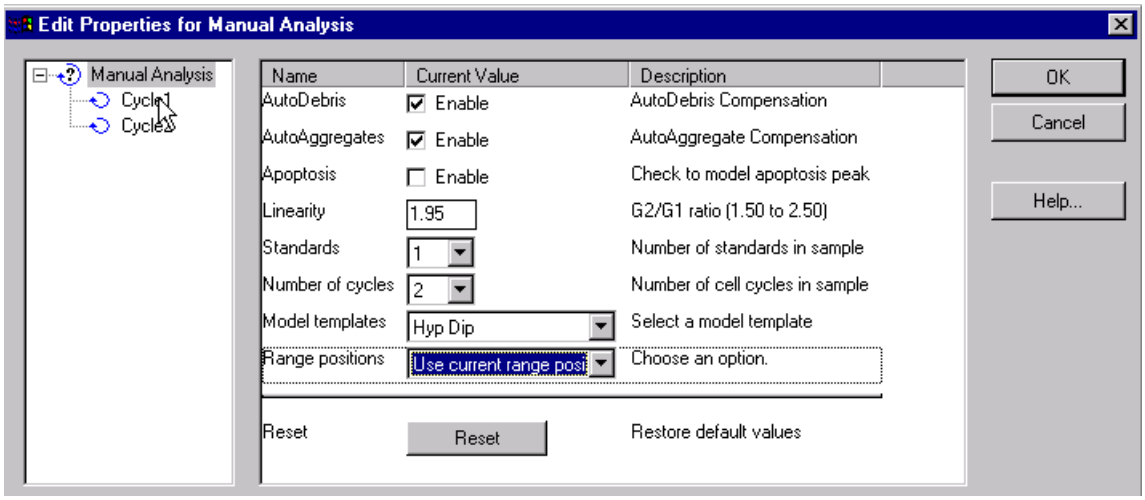


- In the **Edit Properties for Manual Analysis** dialog box, change the number of standards to "1" (see graphic below).

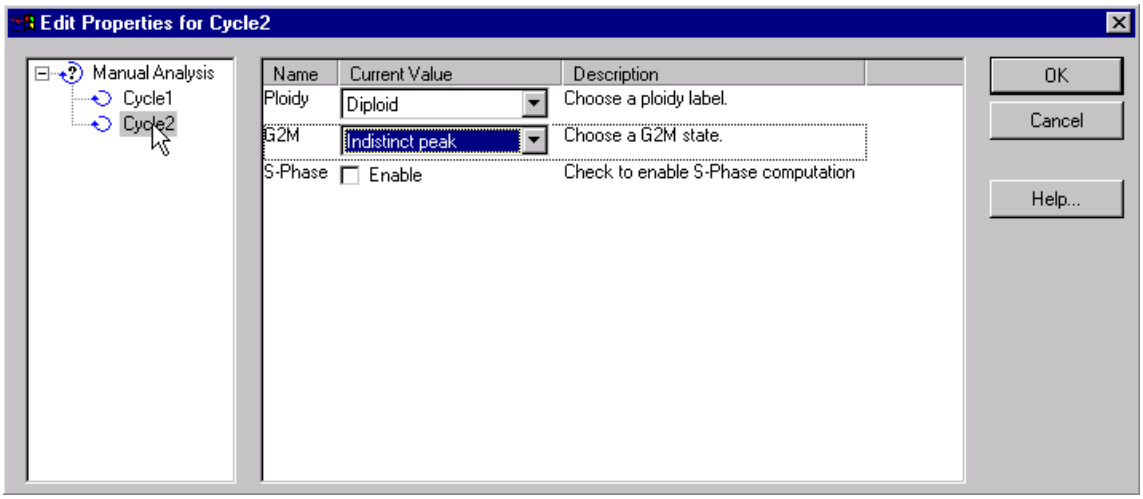
- From the **Model template** drop-down list box, choose "Hyp Dip" (see graphic below).

- Select "Use current range positions" from the **Range positions** drop-down list box (see graphic below).

*If you leave this option set to "Compute range positions", the program will move all of the ranges. Since **Auto Analysis** calculated most of the populations correctly, it is best to use the "Use current range positions" option in order to minimize the number of ranges to move or resize.*



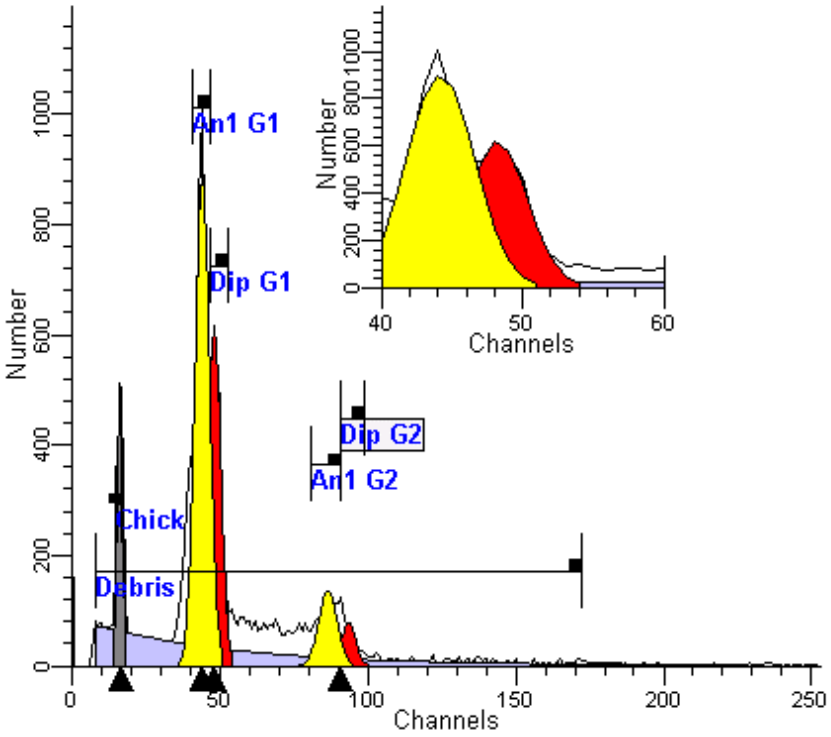
- Click on the "Cycle2" object under Manual Analysis (left panel).



- Select “Indistinct peak” from the **G2M** dropdown listbox (see figure above).

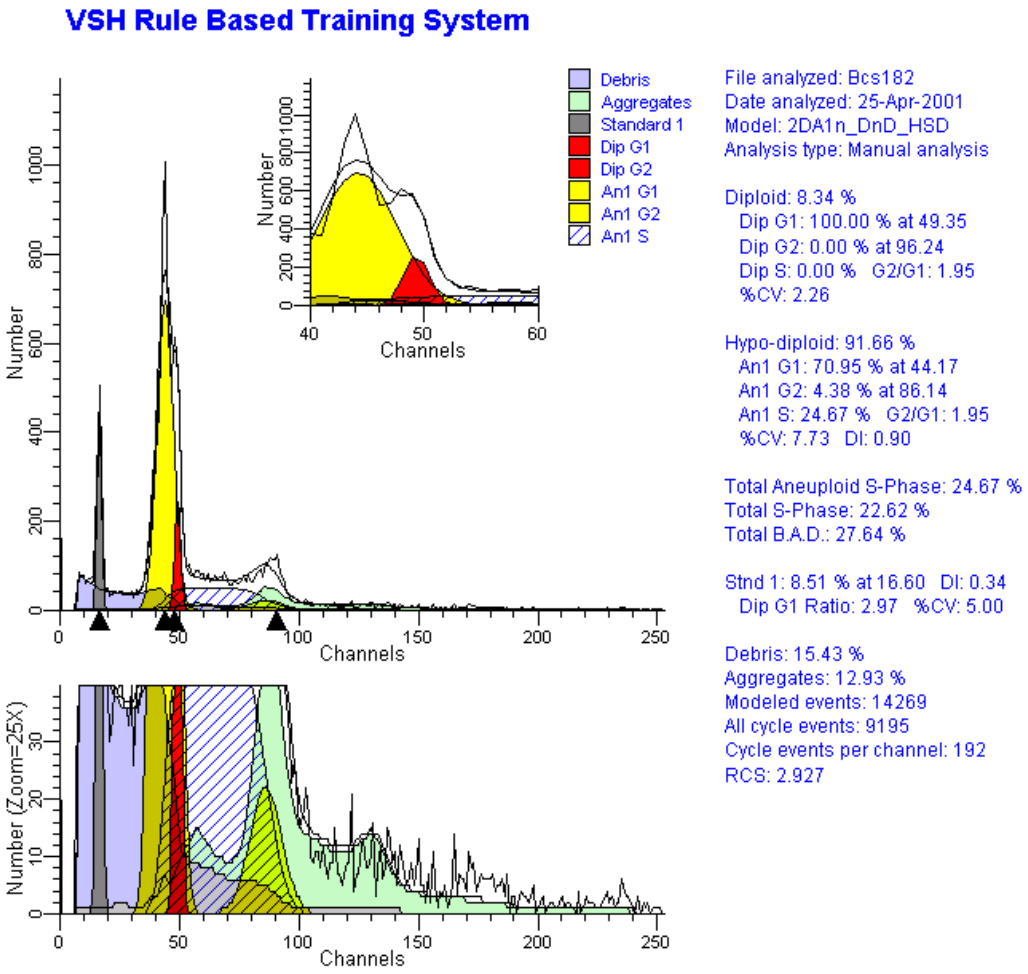
When “Indistinct peak” is chosen, the model calculates the G2M position from the G1 position by multiplying its mean position by the current linearity factor. An “Indistinct peak” option adds rigidity and stability to the affected G2M model component.

- The **S-phase** option should be unchecked.
- Click on **OK**.
- You will need to adjust the “Dip G1”, “Dip G2”, “An1 G1” and “An1 G2” ranges (see graphic below).



- Click on the **Fit** toolbar button.

Note that your analysis may not be the same as the one shown below. In a moment will make the system a lot more stable.

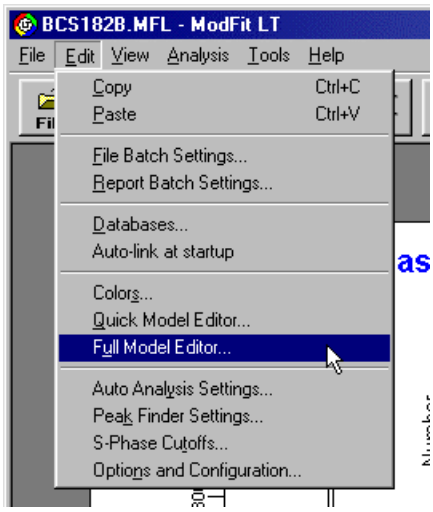


As we mentioned earlier, when two model components overlap heavily, it may be necessary to add more constraints to the model to give the fit some stability. In this case, the CV of the DNA hypodiploid population is much larger than the DNA diploid population. In order to make the model work as we want, we need to force the standard deviations of these two model components to be the same.

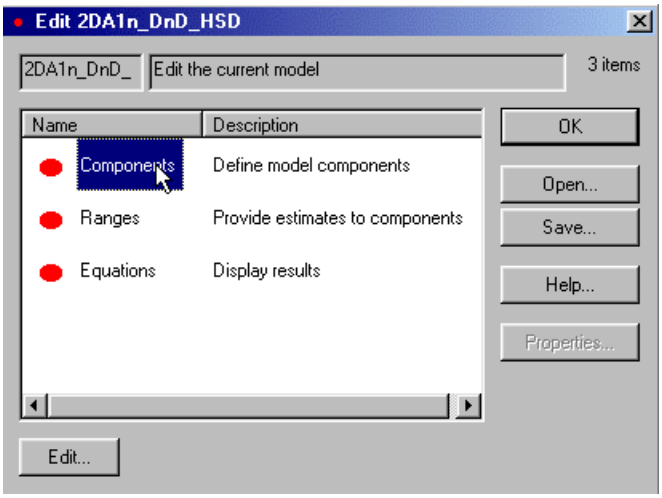
This case is very rare but it does serve as an excellent demonstration of how these DNA models can fail when populations overlap too much.

- Make standard deviation of the diploid population dependent on the standard deviation of the aneuploid by a factor of 1.

- Choose “Full Model Editor…” from the **Edit** menu (see graphic below).

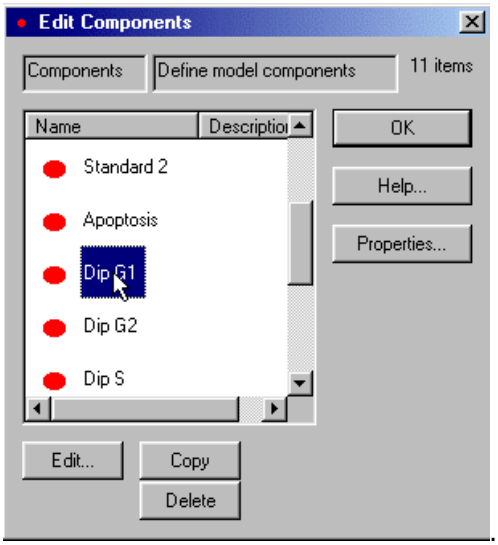


- Double click on the "Components" object in the Edit 2SA1n_DnD_HSD dialog box..

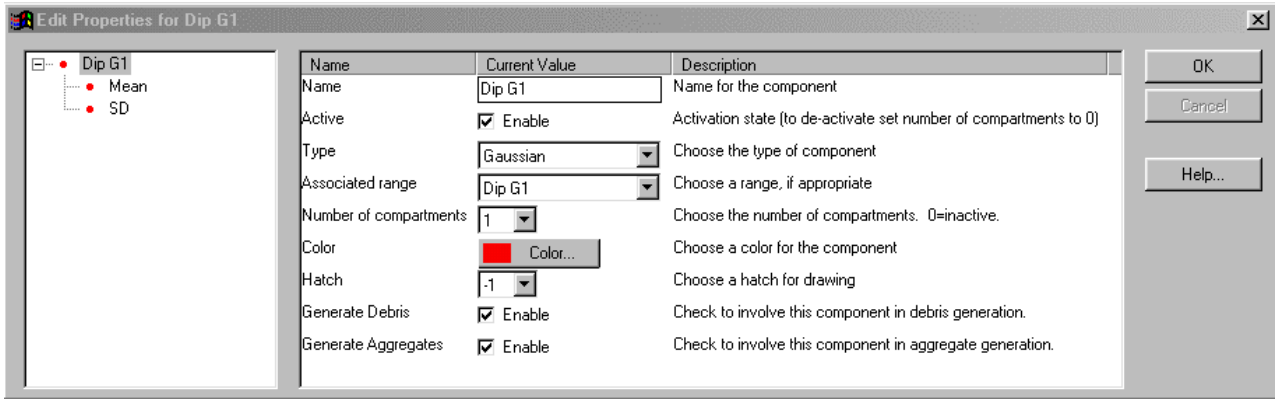


This dialog box lists three groups of model objects, “Components”, “Ranges” and “Equations”. The “Component” group contains the individual objects and details that make up the mathematical model (rectangle, gaussian, debris, and aggregate components). The “Range” group contains the list of ranges and how they are associated with the components. The “Equations” group contains the equations used to calculate the results shown on the report.

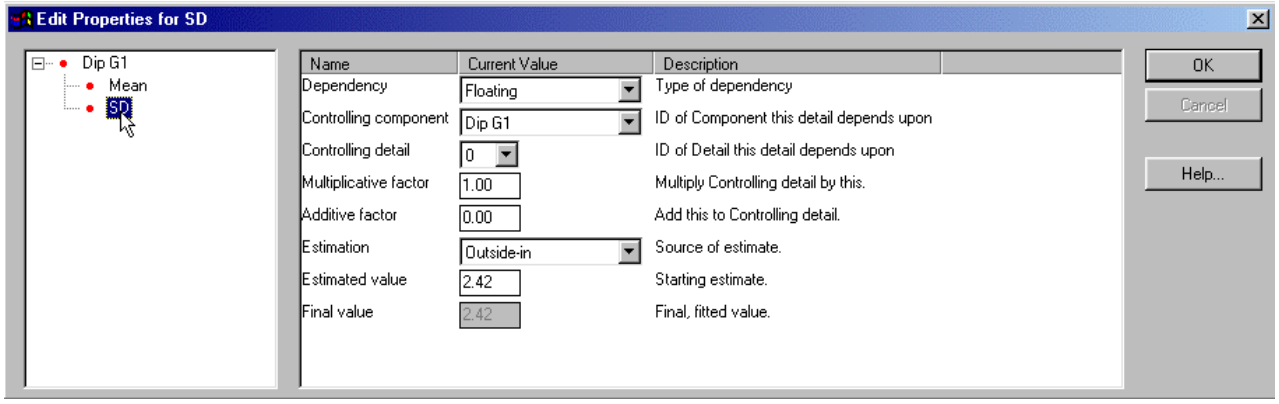
- Double Click on the "Dip G1" (see graphic below)



The **Edit Properties for Dip G1** dialog box will be displayed (see graphic below). This dialog allows you to edit all of the attributes for the “Dip G1” model component.



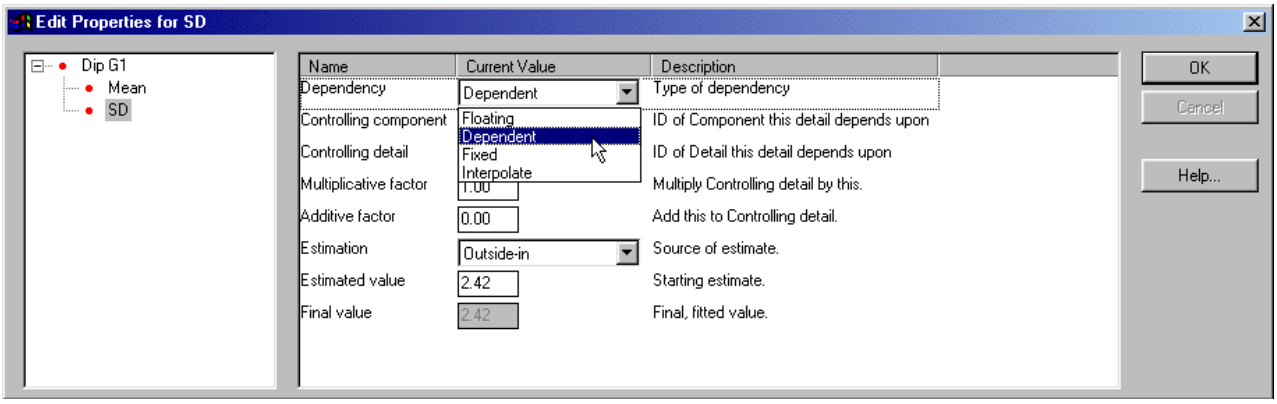
- Click on the "SD" object in the left window of the **Edit Properties for Dip G1** dialog box (see below).



The panel on the right side (see figure below) shows the details associated with the calculation of the “Dip G1” standard deviation. Note that the "Dependency" listbox is set as "Floating" which means that

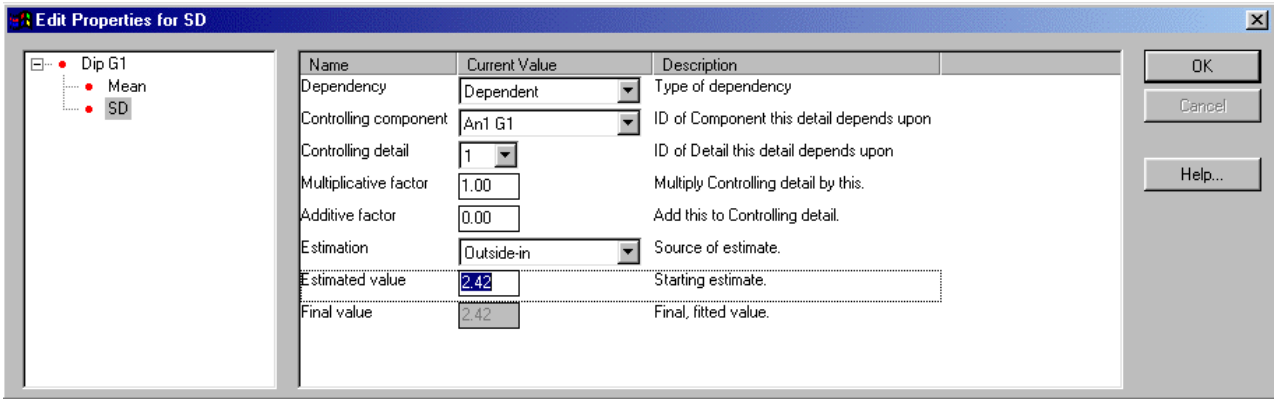
the program is free to adjust this value to optimize the fit. When the "Dependency" detail is "Floating", most of the other values in the dialog are not used by the program.

- Select "Dependent" in the "Dependency" dropdown list (see graphic below).



- Change the "Controlling detail" component from "Dip G1" to "An1 G1" (see graphic below)

- Check that the other values are set as they are in the graphic below.



By setting these properties of the "Dip G1 SD" up in this manner, we are telling the model to make the DNA diploid standard deviation dependent on the DNA hypodiploid's standard deviation by a factor of one. The end result will be that the widths of these two components will be the same.

Take a moment to appreciate the flexibility implicit in this design. You can identify relationships between any model components. Specialized models can be constructed and saved for future use.

For typical DNA analysis, don't overuse this powerful ability to control the models. Since these low level edits cannot be standardized, they should only be used in the few cases where the models just will not work properly.

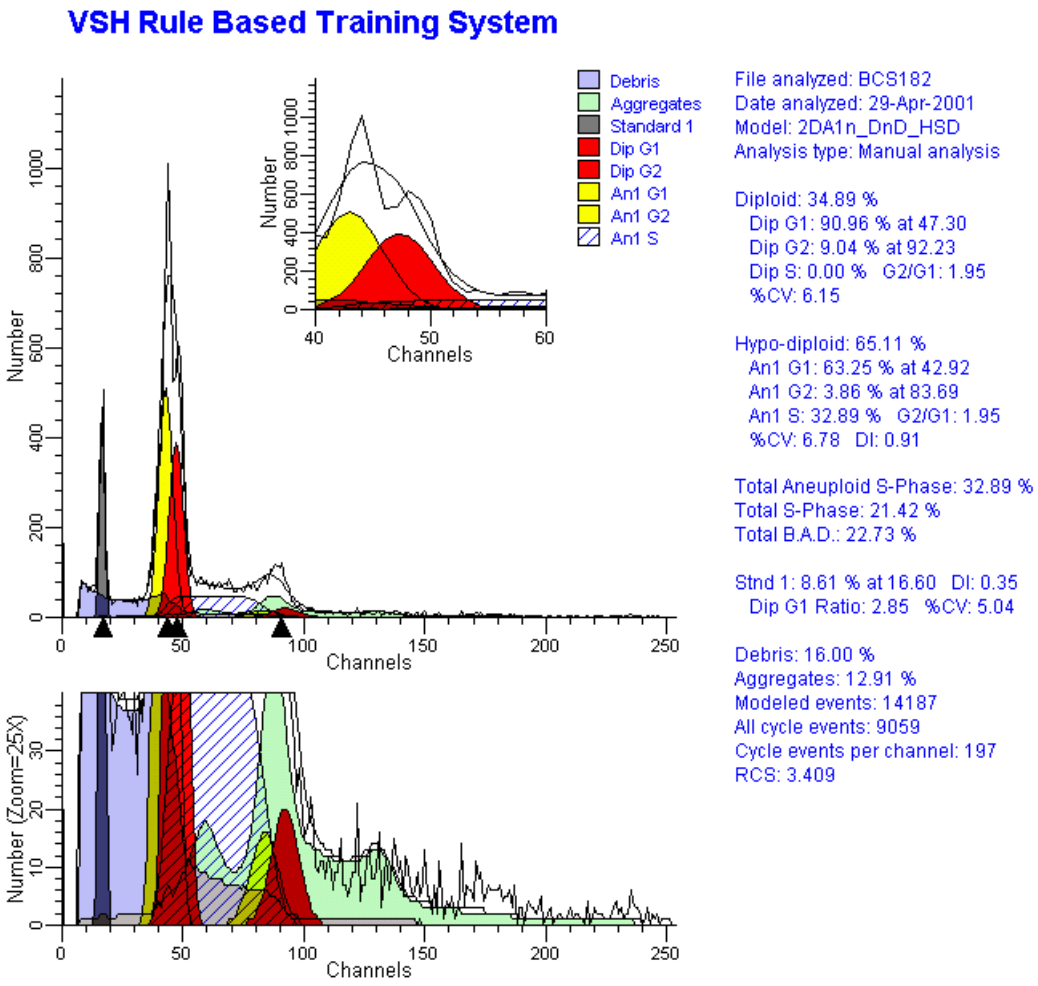
- Click on **OK** for the **Edit Properties for Dip G1** dialog box.

- Click on **OK** for **Edit Components** dialog box.

- Click on **OK** for **Edit 2DA1n_DnD_HSD** dialog box.

- Click on the **Fit** button.

- The graph below shows the new analysis. This analysis should now show a reasonable fit for both the DNA hypodiploid G1 and G2 populations (yellow elements) and the DNA diploid G1 and G2 populations (red elements).



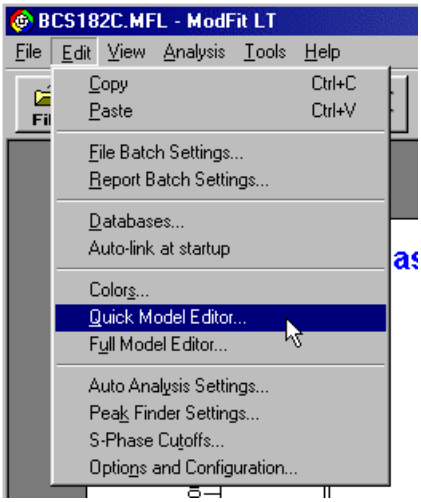
- Click on the **Save** button

This concludes Phase 2.

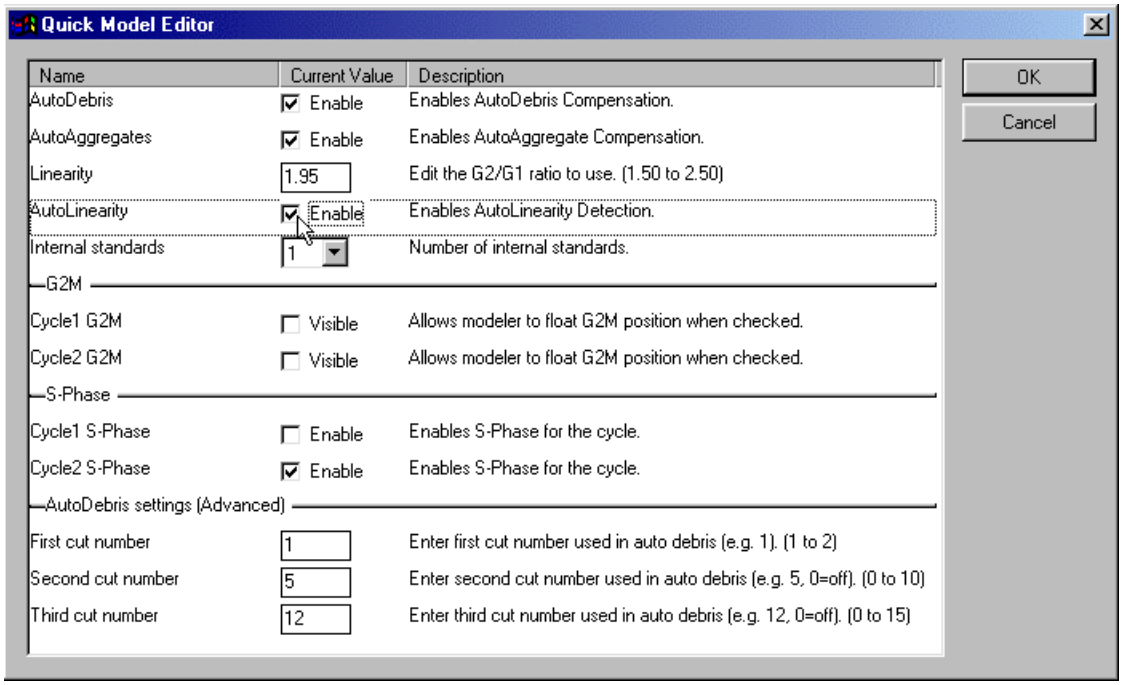
Phase 3 – Automatic Linearity Factor Detection

Let's now find the optimum **Linearity Factor**.

- Select "Quick Model Editor" from the **Edit** menu.

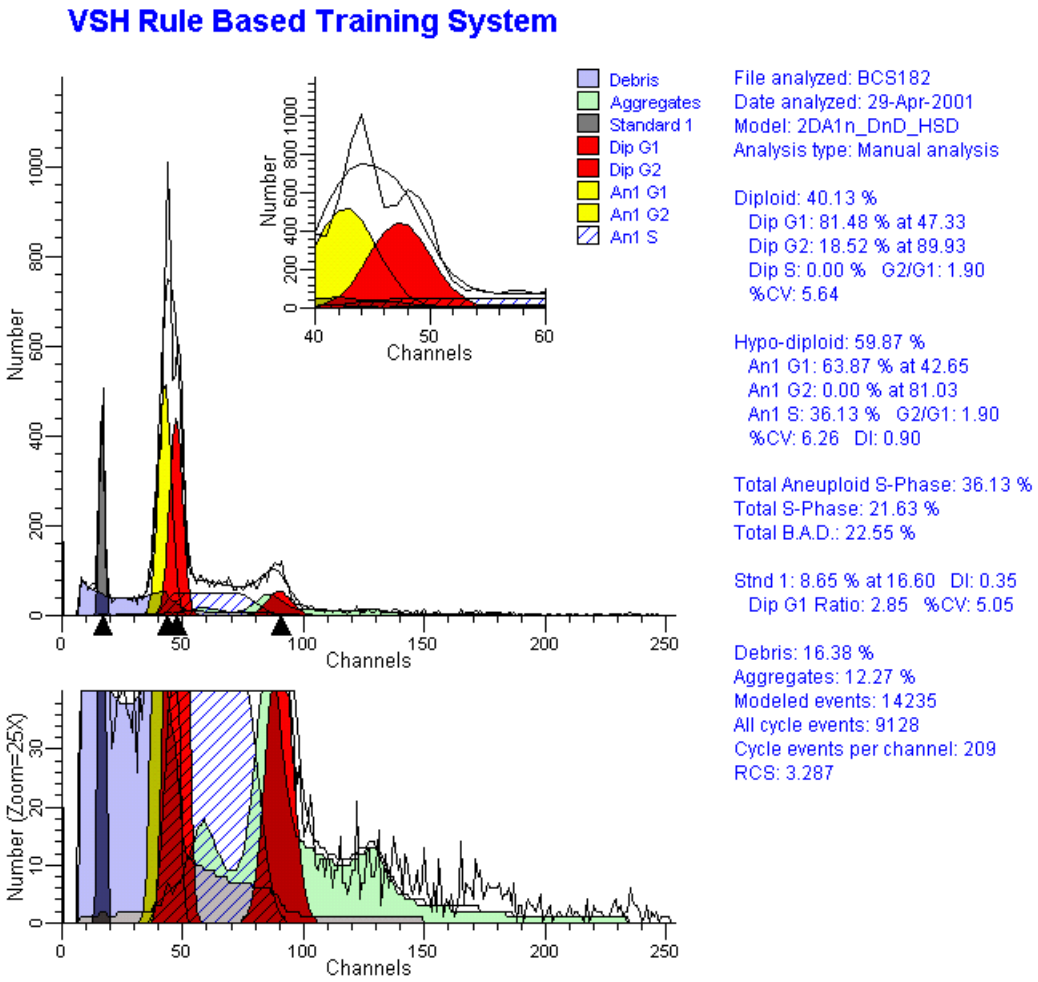


- Enable the **AutoLinearity** option (see graphic below).



- Select **OK**

- Click on the **Fit** button.
- Click on the **Save** button.



End of Phase 3.

Phase 4 –Final Review

Now for our final review…

- Inspect the first report to make sure the new **Linearity Factor** did not negatively affect the fit.

The DNA hypodiploid model fit should look reasonable. Notice that the DNA diploid population has zero percent S-phase.

You might now want to print this report and or add the analysis results to a database.

This is the end section M - Advanced Modeling Techniques Tutorial.

Rules for Obtaining High Quality DNA Histograms and Optimizing Correlation of S-phase Estimates Between Operators

Note: This document may change over time as additional data is obtained from participating laboratories.

Rules for Obtaining High Quality DNA Histograms and Optimizing Correlation of S-phase Estimates Between Operators

I. Acquisition Rules

A. Parameter

The DNA fluorescence parameter should be either a linear integral or area type of signal. It is important to maintain a consistent gain setting. See further under point 1.*F*

B. Linearity

The linearity of the DNA fluorescence amplifier should be tested. Non-linear amplifiers should not be used for DNA analysis.

C. Discriminator

Events should be discriminated on the DNA fluorescence parameter only (e.g. red fluorescence for Propidium Iodide). The discriminator level should be as low as possible without creating a debris peak that is greater than the highest G0G1 peak.

D. Gating

Gating should not be performed during acquisition. Gating is only recommended for multiparameter DNA histograms such as cytokeratin vs DNA or BrdUrd vs DNA. Gating on light scatter is not recommended due to the heterogeneity of the distribution. Signal processing gating, signal peak height vs signal peak area, to eliminate aggregates is only recommended if the aggregates are clearly and completely separated from singlet particles which is usually only true for experimental tissue culture cell lines.

E. Number of Events and Resolution

Simulation studies indicate that for accurate S-phase estimates, there should be an average of approximately 100 events per channel between the lowest G1 and highest G2 of the histogram when the resolution is 256 channels. If a histogram has its diploid G0G1 on channel 50 and the last G2 of an aneuploid population is at 200, there should be at least 15,000 events between channels 50 and 200.

F. Location of diploid G0G1

The position of the diploid G0G1 peak should always be placed in about the same channel. For 256 channel histograms the recommended location is channel 50. For 1024 channel histograms (not normally recommended because it requires collection of 400% more events for same histogram at 256 channels), the location is channel 200.

G. Changing Gains

1. Normally adjust the gain to center the DNA diploid peak on a particular channel (e.g. 50).

2. When a hyper-tetraploid population is observed during acquisition, it is desirable to reduce the gain so that its G2M and some channels consisting of only background are on scale.

3. Note, after adjusting gain, acquisition must be restarted. Gain should be reset to the normal location when running the next sample.

G. Time or Chronology Parameter

If the instrument supports either time or chronology it is highly recommended to view a time vs. DNA parameter to detect any peak shifts during acquisition.

II. Analysis Rules

A. General Procedure

1. Analyze all histograms in automatic analysis mode.

a) For best results, try to use settings for the program's configuration, peak finding characteristics and automatic analysis properties that results in most histograms being analyzed accurately.

2. Evaluate and review each stored analysis report and re-analyze if the model used is incorrect for the data.

3. If available, run automatic linearity adjustment on all reports.

4. Final review.

B. Reviewing Process

1. Model Selection Check

The most important step in analyzing DNA histograms in a consistent manner is checking the correct ploidy model for a particular DNA histogram. In some cases this process may require several analyses to achieve the correct and optimal fit, i.e. the RCS value should be as low as possible (< 3.0). Use the rules below to help guide you through this process.

a) General Considerations

(1) If two model components are of similar shape and are highly overlapped (>75%), it may be necessary to add additional constraints to the model or, in the worse case, disable the model component of lesser importance.

(2) If a G2M peak is clearly visible and well-defined, allow its mean to be fitted (float) unless there is another overlapping population.

(a) If a floating G2M yields a G2/G1 ratio that is outside the expected linearity range (e.g. <1.8 or >2.2), make the G2M's mean and standard deviation dependent on G1 by the appropriate linearity factor (e.g. 1.95).

(3) Always model S-phase as a single, broadened rectangle.

(4) When choosing between two very similar models, select the one that gives consistent results with slightly different range settings.

(a) An example of this rule might be when trying to use an aneuploid model with a near-tetraploid type of histogram. If the aneuploid model only works with very specific range settings, choose the tetraploid model instead.

b) Tetraploid Model Selection

(1) Select a tetraploid model if the DI is close to the expected diploid G2/G1 ratio

(a) Use +/- 0.15 ratio units as a guide but note that if the diploid G2 is not modeled properly, a tetraploid model may be necessary even though it falls outside of the above range.

(2) There is another peak at (8C) that cannot be explained as an aggregate.

(a) Consider the 8C peak to be an aggregate if it is less than the 6C peak.

(3) Choose a tetraploid model over an aneuploid model if the diploid G2M overlaps too significantly with an aneuploid G1.

(a) The diploid G2M will generally only model properly if there is a clearly distinguished peak at its expected location.

(b) Inappropriate fits of the diploid G2M are usually associated with a zero or very high calculated percentage or a location that results in a G2/G1 ratio outside the expected range.

c) Aneuploid Model Selection

(1) Only choose this model if the potential aneuploid's G0G1 cannot be explained as an aggregate or some other part of another cycle (e.g. G2M).

(2) and there are adequate channels to model the entire cycle.

d) Near diploid Model Selection

(1) Choose the near diploid model if the two G0G1 peaks can be clearly distinguished and the resulting fit seems appropriate.

(2) If the DI is between 0.7-1.0 or 1.0-1.3 disable the diploid S-phase and make both G2's dependent.

(3) For very near-diploids, it may be necessary to force the standard deviations of the two G0G1 model components to be equal to yield an appropriate fit.

e) Hypo-diploid Model Selection

(1) Only select a hypo-diploid model when there are standards or normal controls that accurately determine the expected diploid G0G1 position.

(2) If the hypo-diploid G0G1 overlaps one of the standards, disable the standard model component and re-model.

(3) Near-diploid rules apply

2. Range Positions Check

The most common reason for uncorrelated results between two fits using the same model is inattention to range positions. Do not change a range setting unless it is absolutely necessary to do so.

a) Debris Range

(1) The beginning of the debris range should correspond to the channel with the highest debris counts (see Figure 1, Range: Debris for examples of correct and incorrect placements).

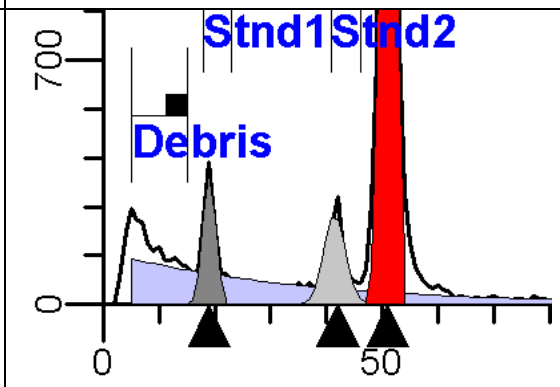
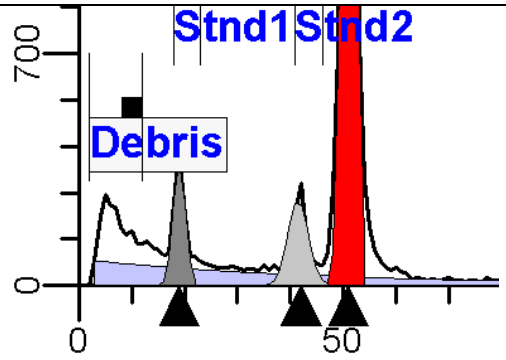
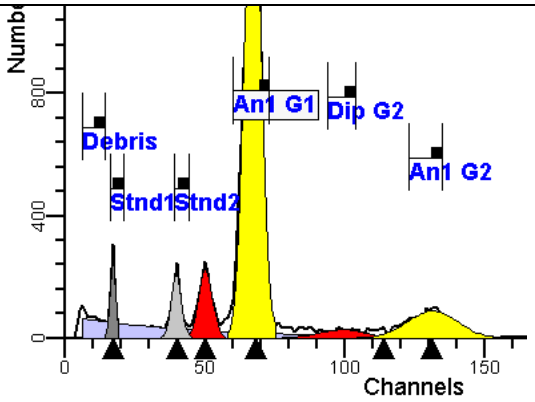
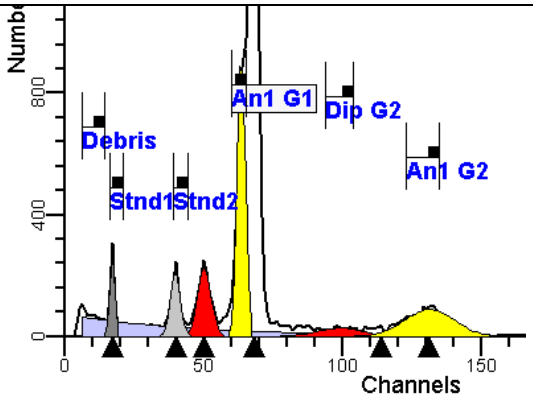
b) Peak Ranges

(1) Center range about the peak and make sure estimates appropriately fit the data (see Figure 1, Range: Peak G0G1 and Range: Peak G2M examples of correct and incorrect placements).

(2) Exceptions to centering the range are for near-diploid and near-tetraploid G0G1 peaks. These ranges need to be displaced to yield reasonable estimates for the underlying peaks.

III.Figures (400x300 gifs)

Figure 1

Rule Description	Correct	Incorrect #1
Range: Debris		
Range: AN1 G1		
Range: AN1 G2