Agilent Feature Extractor Plug-In for GeneSpring GX

FE Plug-In Quick Start Guide

This document describes the new Feature Extractor Plug-In for GeneSpring GX.

What is the Feature Extractor Plug-In?

The plug-in enhances the importing of data files produced by Agilent Feature Extraction software. The plug-in allows you to import a different set of columns from a FE data file and a number of FEPARAM fields as Sample Attributes, and to convert Agilent flag information into the GeneSpring GX flag information.

The source code for the plug-in is available and is located in the source directory of the Programs folder in the GeneSpring data folder. It is released under the LGPL license.

What is in this Quick Start guide?

Agilent Technologies

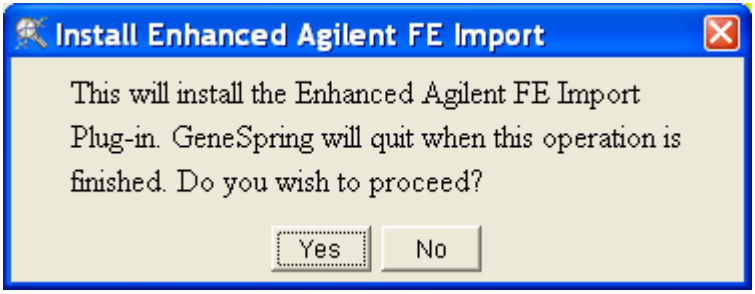
Installation of the Plug-In

The FE plug-in for GeneSpring GX can be installed easily by dragging and dropping the Installer ZIP file onto an open main window of GeneSpring GX. The file is a special GeneSpring ZIP file that allows the easy installation of the new functionality into GeneSpring GX.

1. Download the installer ZIP file from the Agilent Technologies Web site at

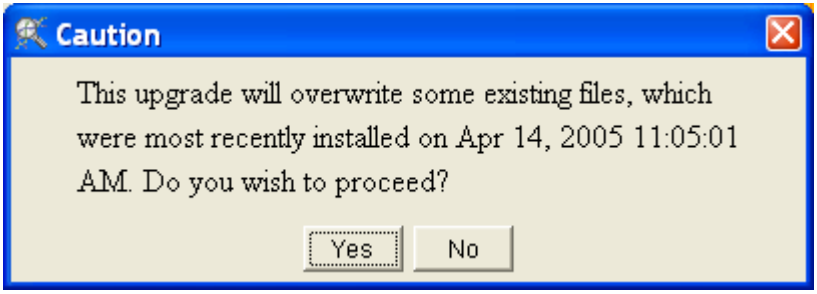
http://www.chem.agilent.com/scripts/generic.asp?|page-34734&indcol=Y&prodcol=Y

2. Save the ZIP file to your local hard drive.
3. **3**Start GeneSpring GX.
4. **4**Drag the ZIP file onto the main GeneSpring GX window.
5. A dialog box displays.



6. Press **OK** to continue or **Cancel** to cancel the installation process.

7 A **Caution** dialog box may display indicating that the installation will overwrite certain files. This may happen if previously the plug-in had been installed or a normalization scenario with the name **Agilent FE** was created. You can abort the installation by pressing **No** and determine if the files can be safely overwritten.



8 After the installation is complete, a new dialog box displays indicating that the installation was successful.

9 Press **OK** to continue.

10 GeneSpring GX closes automatically.

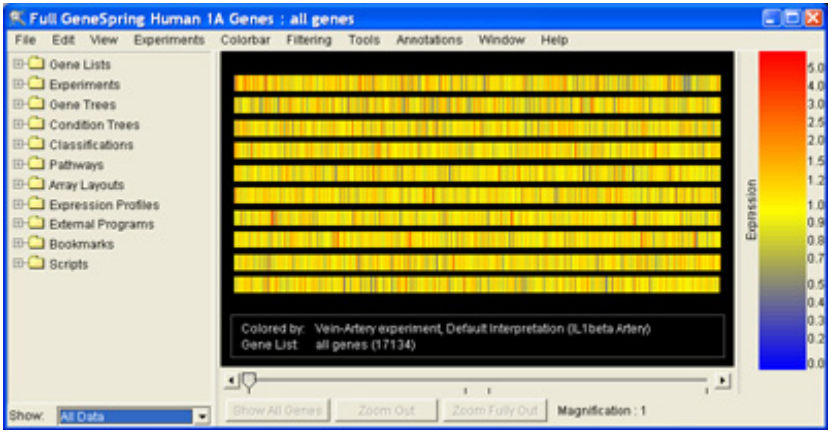
The plug-in is now installed correctly, and Agilent FE data can be loaded.

Loading data produced by Agilent FE

1 Start GeneSpring GX.

2 If you do not yet have the Agilent genome in GeneSpring GX, download the genome from the Agilent Web site by selecting **File**

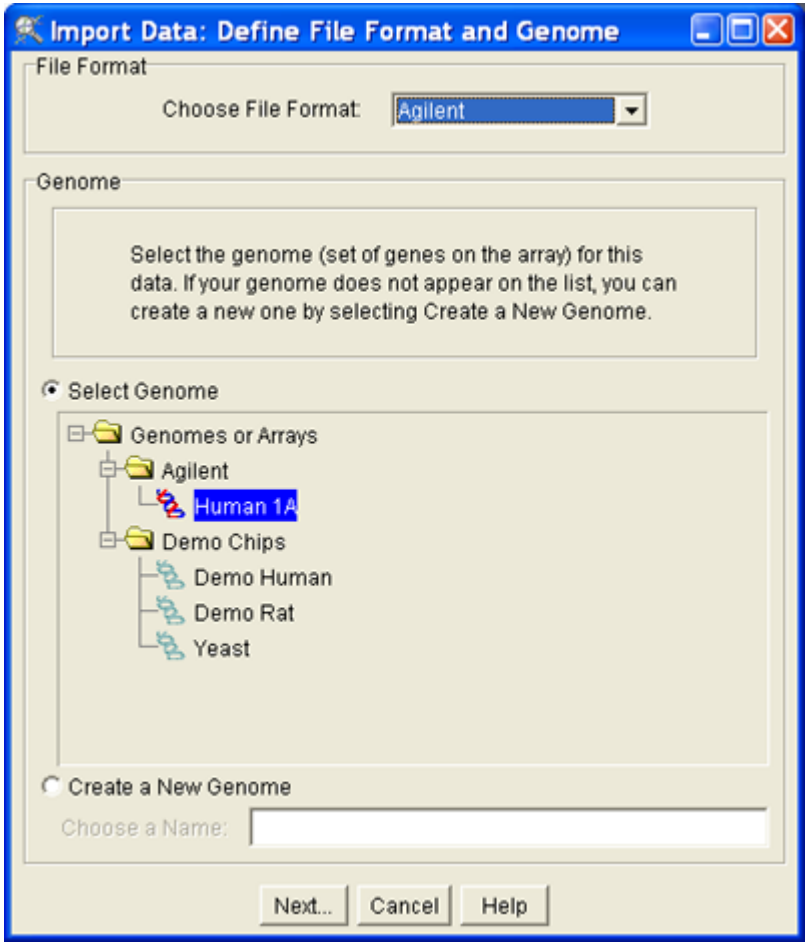
> Import Genome. See the User Manual for more information on importing or creating genomes in GeneSpring GX.



3 Select **File > Import data**.

4 In the **Select One Data File** window, select an FE output file to import from the file-chooser dialog box provided.

5 The **Import Data: Define File Format and Genome** window displays.



6 GeneSpring GX tries to determine the data file's format is by examining the contents. If it is successful, it reports the name in the “Choose File Format:” drop-down box.

The drop-down menu may contain more than one data format, and it will always contain the “Custom” data format. GeneSpring GX can recognize many different data file formats, but it will

only report the names of data files that are consistent with the current data files. If the Agilent FE output file contains 2-color data, the format is called *Agilent*. If the file contains 1-color data, the format is called *Agilent FE (1 Color)*.

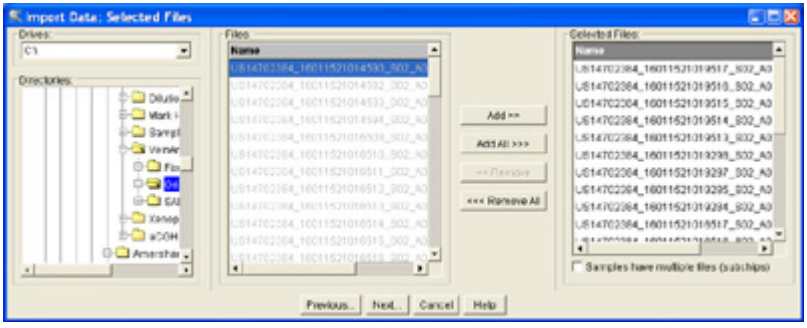
More than the two data formats, *Agilent* and *Custom*, may be available in the drop-down menu if you or the administrator have created a Recognized Custom format before. This Recognized Custom Format will most likely be chosen as the default.

*NOTE: To ensure that the FE plug-in will be used, be sure that the **Choose File Format** drop-down box is set to "Agilent" or "Agilent FE (1-Color)" as appropriate to the data type.*

7 Verify that the **Select Genome** field indicates the correct genome for loading the data files, and press **Next** to continue.

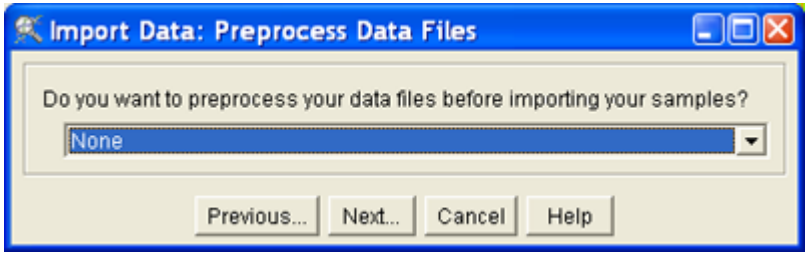
8 The **Import Data: Selected Files** dialog box displays to allow you to add more files to be imported.

You can now choose multiple files to import by navigating to the correct folder on the left. Select the files in the middle window and press **Add** to add the files to the list on the right. Press **Next** to continue.

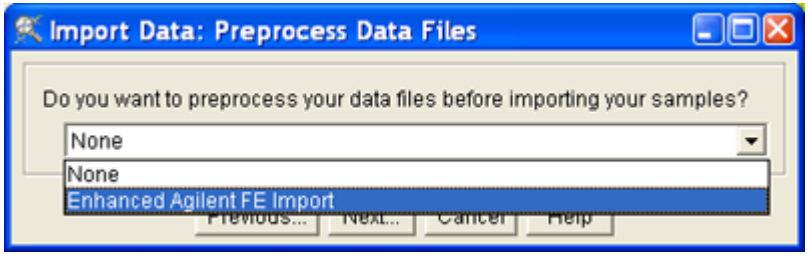


NOTE: If you want to attach image files to Samples, such as JPG images of the arrays, you must place the image files and data files in the same folder.

9 After you select all of the files that you want to load, the **Import Data: Preprocess Data Files** dialog box displays.



10 Select **Enhanced Agilent FE Import** from the drop-down menu, and click **Next**.



11 The plug-in imports the correct columns—for example, for 2-color data, rProcessedSignal is imported as SIGNAL and gProcessedSignal as CONTROL, and it also allows you to convert Agilent flags to GeneSpring flags.

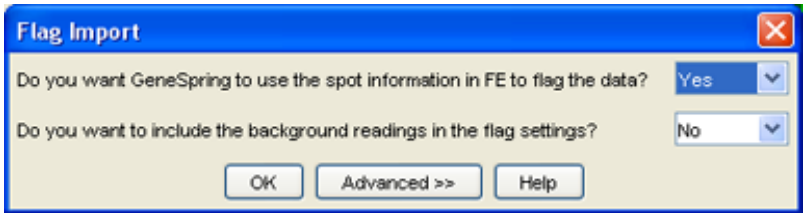
If you choose to use the Enhanced Agilent FE Import plug-in as directed in step 10, the Agilent FE flag information is converted to GeneSpring flag information.

The **Flag Import** dialog box displays with two questions. How you answer the questions determines *how* GeneSpring GX will interpret the FE flags:

- **No – No**: Ignore all of the FE flags.
- **Yes – Yes**: Import all of the FE flags.
- **Yes – No**: Import only FE flags that deal with spot information. This is the default.

- **No – Yes**: Import only FE flags that deal with background information.

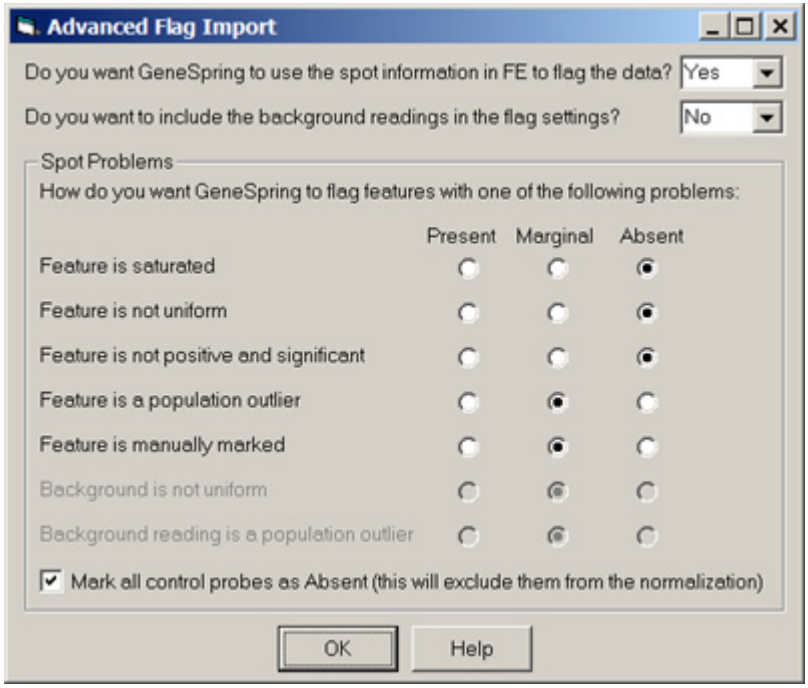
Click **Advanced...** if you want to import only specific FE flags. The **Advanced Flag Import** dialog box displays with a more granular set of choices. See step 12.



The three buttons at the bottom of the dialog box offer the options

- **OK** continues the import process by displaying the **Suggested Normalization** window and starting to read and convert the data files.
- **Advanced** displays the **Advanced Flag Import** dialog box where you can change the default conversion settings.
- **Help** displays the appropriate Help page (URL).

12 Click **Advanced...** to access the **Advanced Flag Import** dialog box where you can change the conversion settings.



You can respond **Yes** or **No** to the prompt, *Do you want GeneSpring to use the spot information in FE to flag the data?*

- If you respond Yes, the first five choices in the **Spot Problems** box are enabled along with the **Mark all control probes as Absent** check box.
- If you respond **No**, the entire **Spot Problems** box is disabled along with the *Do you want to include the background readings in the flag setting?* prompt. The new data file that the plug-in creates will contain a **GeneSpring Flags** column where everything is marked **P** for **Present**.

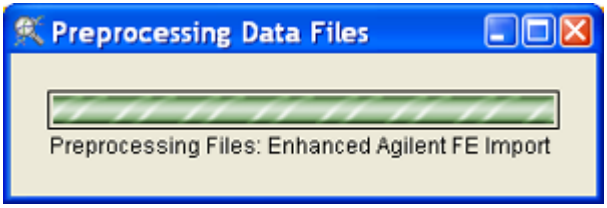
You can also respond **Yes** or **No** to the prompt *Do you want to include the background readings in the flag settings?*

- If you respond **No**, the last two choices in the **Spot Problems** box are disabled (**Background is not uniform** and **Background reading is a population outlier**) and both options are imported as **Present**.
- If you respond **Yes**, the last two choices are enabled and set to **Marginal**.

Alternatively, you can change the conversion setting for each FE flag value by selecting the appropriate radio buttons and pressing **OK** to begin importing.

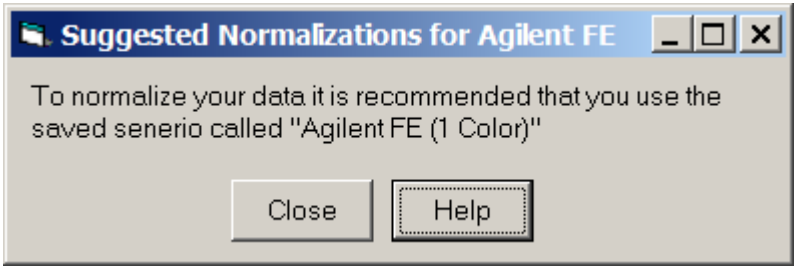
See appendix A for a detailed explanation of the flag logic and how the settings in the **Advanced Flag Import** dialog box influence the outcome.

13 A window displaying a progress bar displays indicating that the preprocessing is active.

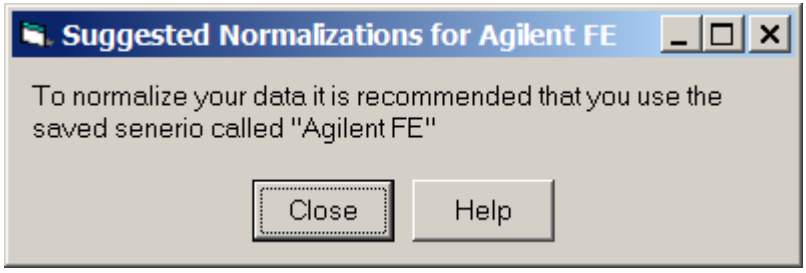


14 The **Suggested Normalizations for Agilent FE** dialog box displays automatically after you have completed the Flag Import settings. The version of this dialog box that displays depends on whether the plug-in recognized the file as being in 1-color or 2-color file format.

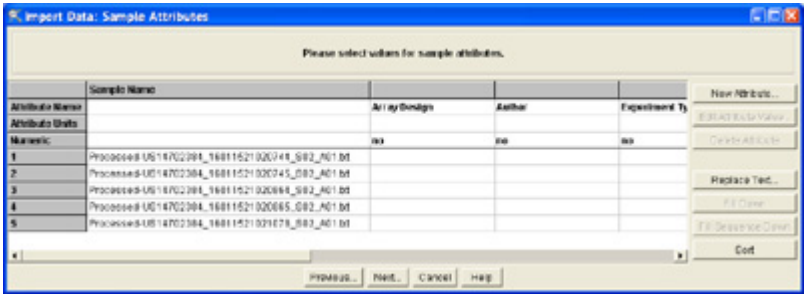
For 1-color data



For 2-color data

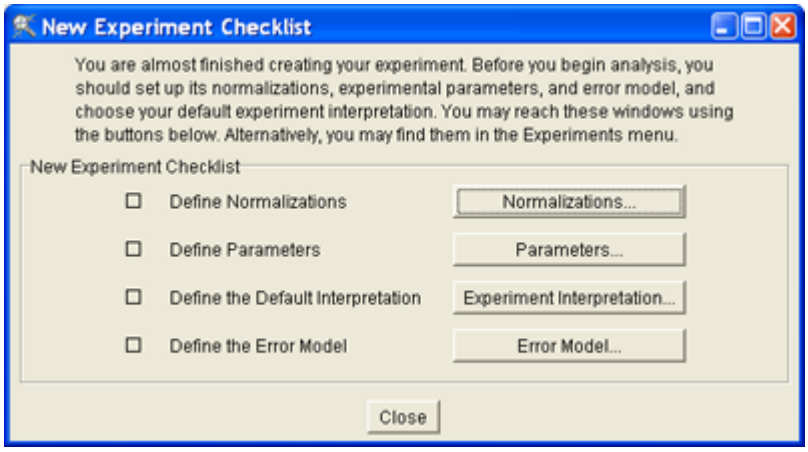


15 Here the import procedure is the same as the normal import procedure. Continue to import the samples.

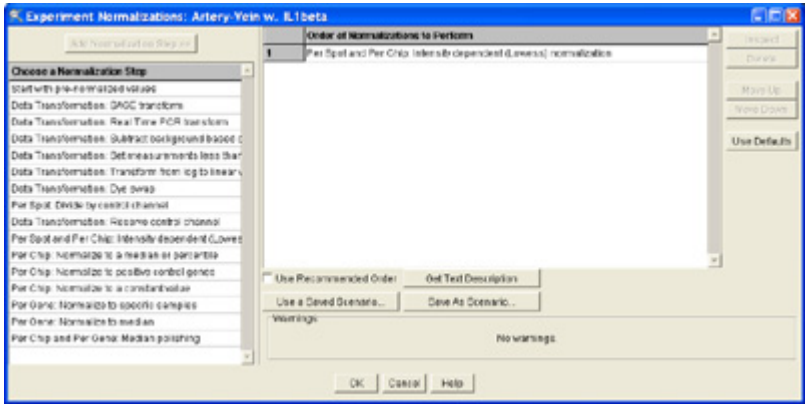


Agilent FE normalization scenarios

When all samples are loaded and used to build an experiment, we recommend that you apply the **Agilent FE** or **Agilent FE (1-Color)** scenario.



To apply the normalization scenario, click the **Normalizations button** in the **New Experiment Checklist** dialog box or click **Experiments > Experiment Normalization**.

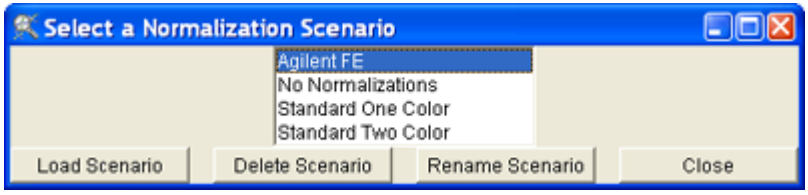


The default two-color data normalization scenario is the “Per Spot and Per Chip: Intensity dependent (Lowess) normalization”. Since Agilent Feature Extraction already applies Lowess normalization to the data, there is no need for a second Lowess normalization (technically, however, it would not be detrimental).

For Agilent FE 2-color data, a more appropriate normalization scenario is to simply apply a ratio calculation of the two channels, followed by a per-gene normalization to ensure all ratio’s for each gene are normalized to 1.

During installation of the plug-in, a **Saved Scenario** was installed to perform this normalization. To apply the normalization scenario:

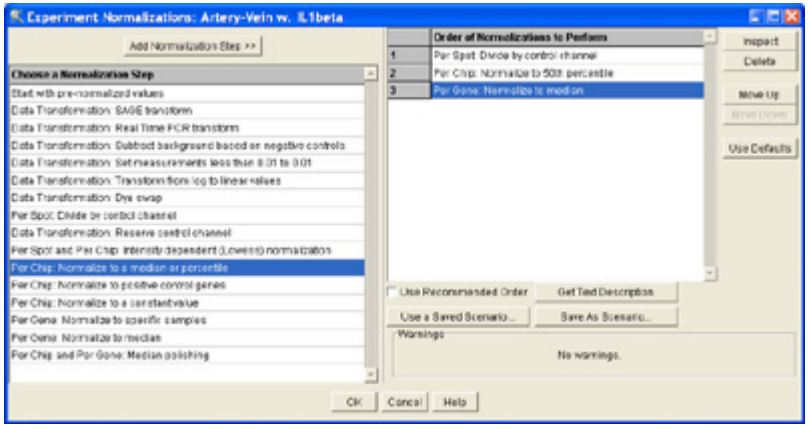
1 Select **Use a Saved Scenario...**



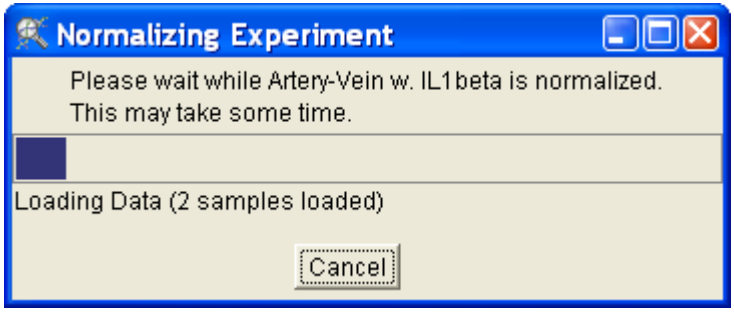
2 Select the **Agilent FE** or **Agilent FE (1-color)** scenario from the list and press **Load Scenario**.

See appendix B for details on the Agilent FE Normalization scenario.

3 The new normalization scenario is loaded and displayed in the Normalization window.



4 Press **OK** to apply the new normalization scenario to the experiment.



After the Agilent FE normalization scenario is applied, the data is ready to be analyzed in GeneSpring GX in the usual manner.

Appendix A – Flags

Agilent FE output data contain a large number of flags that can be used to indicate various problems with a feature on an array. Usually, this flag information can be easily used in GeneSpring GX after the data files are imported (using the “Filter on Data file” filter). However, there a number of features in GeneSpring GX that cannot use this flag information unless the information is first converted into special GeneSpring flags. The Agilent FE plug-in converts the complex set of FE flags into three-levels of GeneSpring flags: Absent (A), Marginal (M). or Present (P). The flag columns from Agilent FE output and their meaning are provided in table 1.

Flag description	Column headers in Agilent FE output
Feature is saturated	gIsSaturated, rIsSaturated
Feature is not uniform	gIsFeatNonUnifOL, rIsFeatNonUnifOL
Feature is not positive and significant	gIsPosAndSignif, rIsPosAdSignif
Feature is a population outlier	gIsFeatPopnOL, rIsFeatPopnOL
Feature is manually marked	IsManualFlag
Background is not uniform	gIsBGNonUnifOL, rIsBGNonUnifOL
Background reading is population outlier	gIsBGPopnOL, rIsBGPopnOL
Is Control Probe	Control Type

Table 1 Flag columns in Agilent FE output. The *g* and *r* used as the first letter of the column header name indicate the Green (Cy3) and Red (Cy5) channels, respectively

Flag logic

To map the complex 14 binary flag columns (there are 4096 flag combinations) to the simple three-value GeneSpring flags (A, M, and P), a mapping that depends on the seriousness of the flags was applied. Certain flag values carry more weight that other flag values. The most serious flags are mapped as “Absent,” medium serious flags are mapped as “Marginal," and all other features are mapped as “Present.”

Flag values in the FE output are *independent* flags, that is, a feature can be saturated, but uniform and a background population outlier. GeneSpring GX will keep the Lowest GeneSpring flag value (low is A, next is M then followed by P).

For example, if a feature is not flagged as either saturated or non-uniform, but flagged as a population outlier and not manually marked or a control probe (and not making use of the Background mapping), it will have a "score" of P,P,M,P,P,P,P and will therefore receive the GeneSpring flag "M" or "Marginal" (table 2).

Flag description	Value in FE output	GeneSpring flag
Feature is saturated	0	Pass
Feature is not uniform	0	Pass
Feature is not positive and significant	1	Pass
Feature is a population outlier	1	Marginal
Feature is manually marked	0	Pass
Background is not uniform	0	Pass
Background reading is population outlier	0	Pass
Is Control Probe	0	Pass
Result		Marginal

Table 2 Feature is a population outlier and gets marked *Marginal* since this is the lowest of flag scores

A second example concerns a feature that is flagged as a saturated feature and as a population outlier (and all other flags are not set in FE output). It gets marked as "A" because the feature is saturated. Since this is the lowest possible flag value in GeneSpring GX, the entire feature is flagged as "A" or "Absent" (table 3).

Flag description	Value in FE output	GeneSpring flag
Feature is saturated	1	Absent
Feature is not uniform	0	Pass
Feature is not positive and significant	0	Absent
Feature is a population outlier	1	Marginal
Feature is manually marked	0	Pass
Background is not uniform	0	Pass
Background reading is population outlier	0	Pass
Is Control Probe	0	Pass
		Absent

Table 3 Feature is both saturated and a population outlier and gets flagged *Absent* since that is the lowest of the flag scores

If the background information is used to determine the flag values, those features that are marked as either having a non-uniform background or are background-population outliers, get marked as “M” or “Marginal” probes by default, or whatever is set in the dialog box (table 4).

Flag description	Value in FE output	GeneSpring flag
Feature is saturated	0	Pass
Feature is not uniform	0	Pass
Feature is not positive and significant	1	Pass
Feature is a population outlier	0	Pass
Feature is manually marked	0	Pass
Background is not uniform	0	Pass
Background reading is population outlier	1	Marginal
Is Control Probe	0	Pass
		Marginal

Table 4 Feature is a background population outlier and gets marked *Marginal* since this is the lowest of flag scores

Appendix B – Agilent File Format

Agilent FE transfers these fields to GeneSpring GX.

GeneSpring information	Agilent FE fields	
	For 2-color data	For 1-color data
Gene Name	ProbeName (If column is empty, use SystematicName. If that column is empty, use FeatureNum.)	Same
Signal	rProcessedSignal	gProcessedSignal
Reference (a.k.a. "Control" within GeneSpring)	gProcessedSignal	N/A
User Provided Signal Precision	LogRatioError is converted using the following calculation. For a given gene in the FE output, let: RPS = rProcessedSignal (imported as Signal) GPS = gProcessedSignal (imported as Control) LRE = logRatioError Then import the following: $signalprecision = GPS\sqrt{(e^{LRE^2} - 1)}$	gProcessedSigError
Description	Description	Same
GenBank ID	GenBank	Same
Flags	The preprocessor needs to translate the eight different flag settings.	Same
Sample Attributes	All of the FEPARAMS and STATS fields are loaded as Sample Attributes. The names of the Sample Attributes are the names provided on the FEPARAMS (or STATS) row. The values are the values displayed below the field name in the DATA row. The type in the TYPE row can be used to determine if a Sample Attribute is numeric or not. **1** FE type: integer or float → GeneSpring: numeric **2** FE type: text → GeneSpring: non-numeric	Same

Table 5 Interpreting the Agilent file format

Appendix C – Agilent Normalization Scenario for 1-Color Data

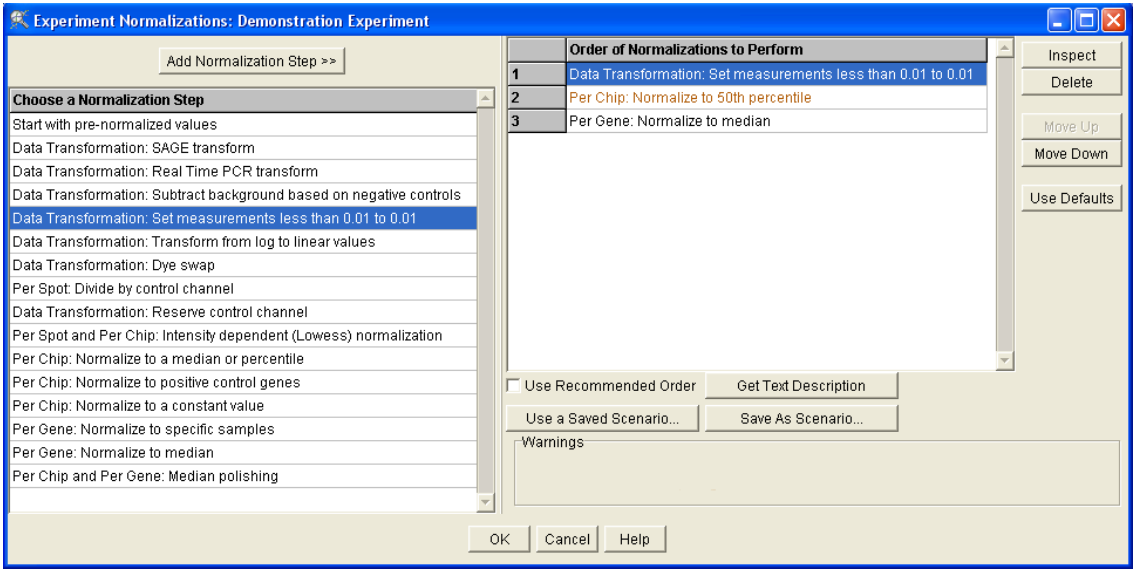
For optimal use of the expertise that is built into Agilent Feature Extraction software, Agilent recommends that you use a normalization scenario that is different than the default one-color scenario. This recommended normalization scenario makes use of the converted flag information that allows you to exclude the control and otherwise flagged probes.

The recommended Agilent FE(1-Color) normalization involves three steps:

The Agilent FE (1 Color) normalization scenario has three steps:

1. Data transformation: Set measurements from < 0.0 to 0.0
2. Per chip: Normalize to the median or percentile
3. Per gene: Normalize to median

Each step is discussed in detail.

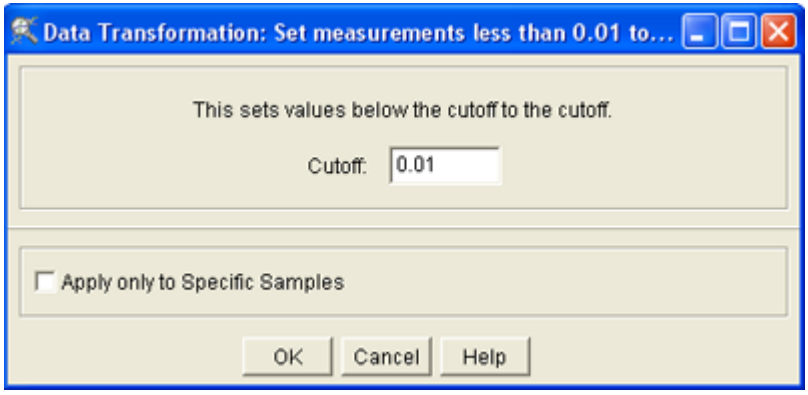


Data transformation: Set measurements from < 0.01 to 0.01

The data transforming normalization involves applying a mathematical modification to a variables values. During normalization, GeneSpring GX recalculates the data values and uses them in subsequent analyses.

The Set Measurements option sets any measurements less than a specified cutoff value to the cutoff value. By default, this value is 0.01.

- For Agilent FE (1 Color) data, set the cutoff value to the default by typing **Cutoff: 0.01** in **Cutoff** text box and click **OK**.



By default, this cutoff value is set to 10, but for this example scenario we have lowered this value to 0.01 to allow lower values.

Per chip: Normalize to the median or a percentile

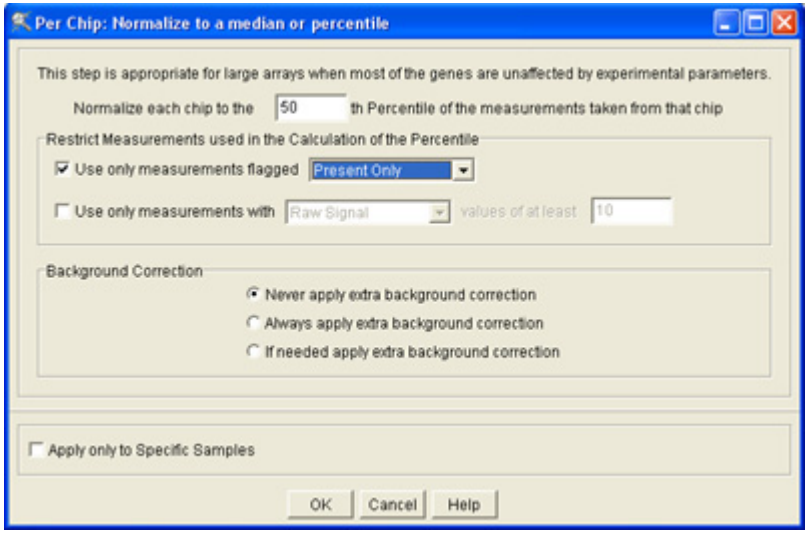
Per-chip normalizations control the intensity of chip-wide variations. These variations may result from inconsistent washing, inconsistent sample preparation, or other microarray production or microfluidics imperfections.

The Normalize to a Median or Percentile option lets you divide all of the measurements on each chip by a specified percentile value. By default, this value is 50.0%.

- For Agilent FE (1 Color) data, type 50.0% in the percentile text box if the default value is not already displayed.

- Check the **Use only measurements flagged** box and set the drop-down menu to **Present Only**
- Click the **Never apply extra background correction** button

Click **OK** when done.



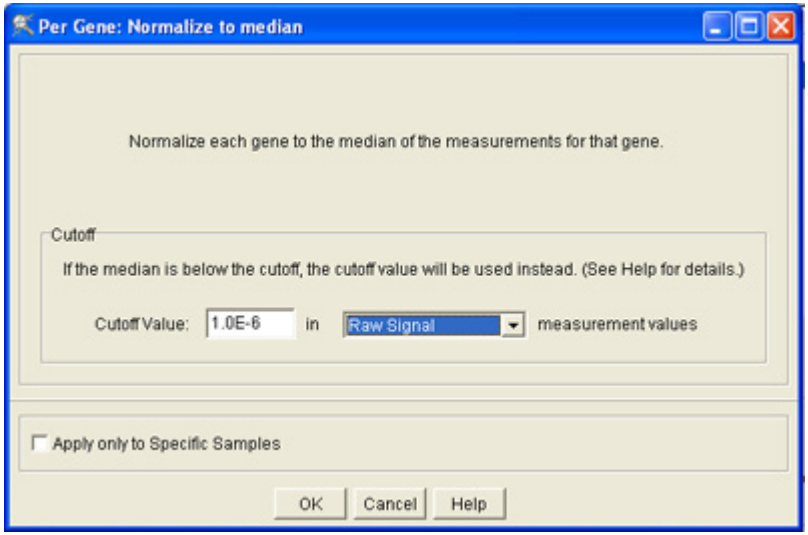
Identical chips from different samples may show different intensities due to external factors. You can offset this by asuming that all genes were functioning normally when sampled, and that their intensities are normal for what they were doing. By calling that normal state 1 for every chip in the experiment, you can normalize these variations in gene intesities.

These normalizations are only made on genes considered normal and flagged Present. Genes flagged Marginal or Absent are ignored to avoid skewing the data.

Per gene: Normalize to the median

The last step in normalizing Agilent FE (1 Color) data involves normalizing the expression value for each gene across all arrays.

- For Agilent FE (1 Color) data, set the cutoff value to 0.01 in **Raw Signal** data measurement values, and click **OK**.



There are two options that can be set in this normalization step: (1) a cutoff value can be set to ensure that values lower than the cutoff will be replaced with the cutoff value and (2) the cutoff value can be applied to either the normalized value or the Raw signal value.

The recommended normalization step has a cutoff set for the Raw signal value of $1.0E^{-6}$. This, together with the cutoff value for the Control value of "$1.0E^{-6}$," ensures that all normalized values are sensible.

Normalization completed

When you have completed the three-steps for normalizing Agilent FE (1 Color) data, the following will have occurred:

- Values below 0.01 were set to 0.01.
- Each measurement was divided by the 50.0th percentile of all measurements in that sample.
- Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10 then each measurement for that gene was divided by 10 if the numerator was above 10, otherwise the measurement was thrown out.

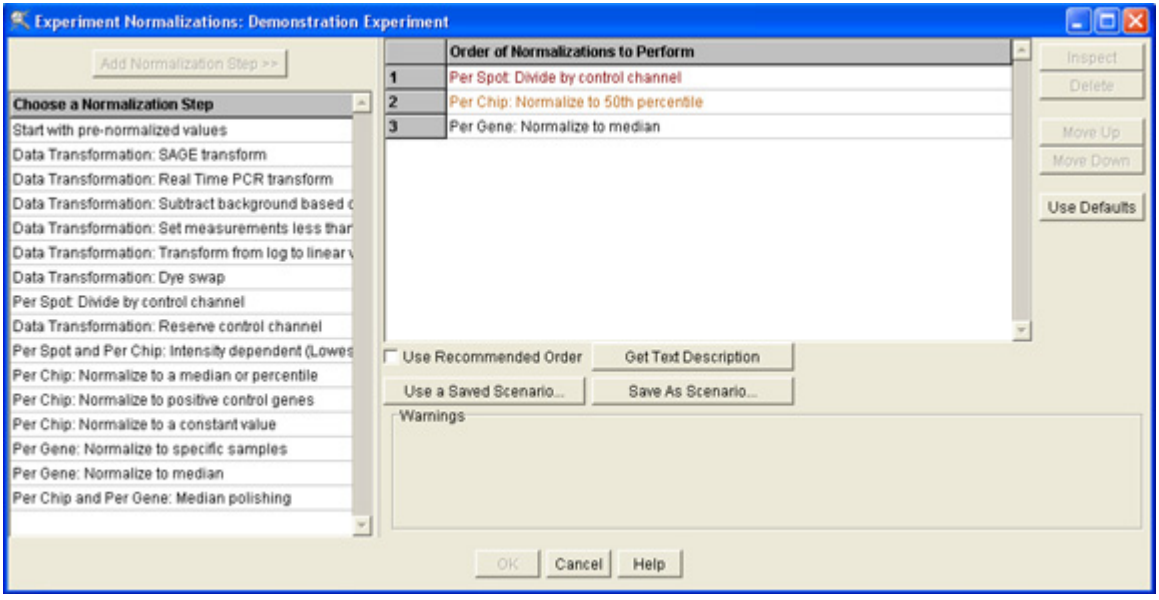
Appendix D – Agilent Normalization Scenario for 2-Color Data

For optimal use of the expertise that is built into Agilent Feature Extraction software, Agilent recommends that you use a normalization scenario that is different than the default two-color scenario usually used. This recommended normalization scenario also makes use of the converted flag information that allows you to exclude the control and otherwise flagged probes.

The recommended normalization involves three steps:

1. Per spot: Divide by the control channel
2. Per chip: Normalize to the 50th percentile
3. Per gene: Normalize to the median

Each step will be discussed in detail.

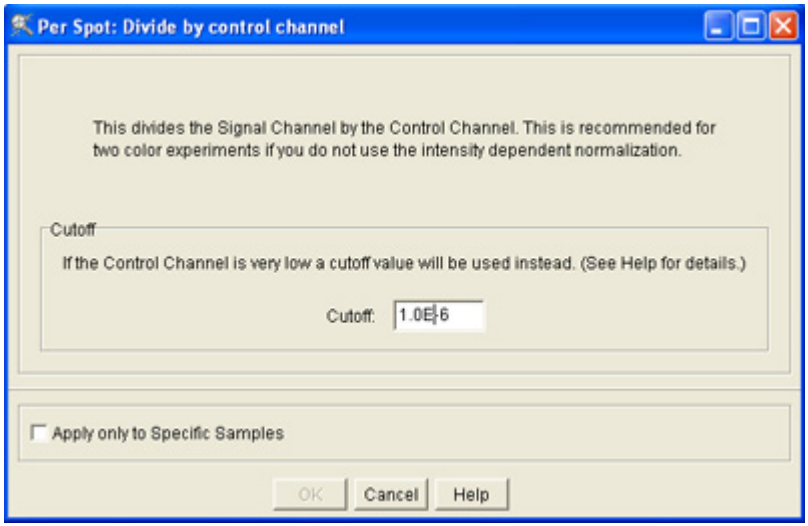


Per spot: Divide by control channel

GeneSpring GX extracts two columns from the Agilent FE output file that represent the red and green channel signal values, after processing in Agilent Feature Extraction. These two channels,

called ***rProcessedSignal*** (for the red or Cy5 channel data) and ***gProcessedSignal*** (for the green or Cy3 channel data) are loaded respectively as the *Signal* and *Control* channels in GeneSpring GX.

Any normalization scenario in GeneSpring GX involves the ratio between the Signal and Control channels. The first step in the normalization scenario is to create this ratio with the normalization step "Per Spot: Divide by control channel."



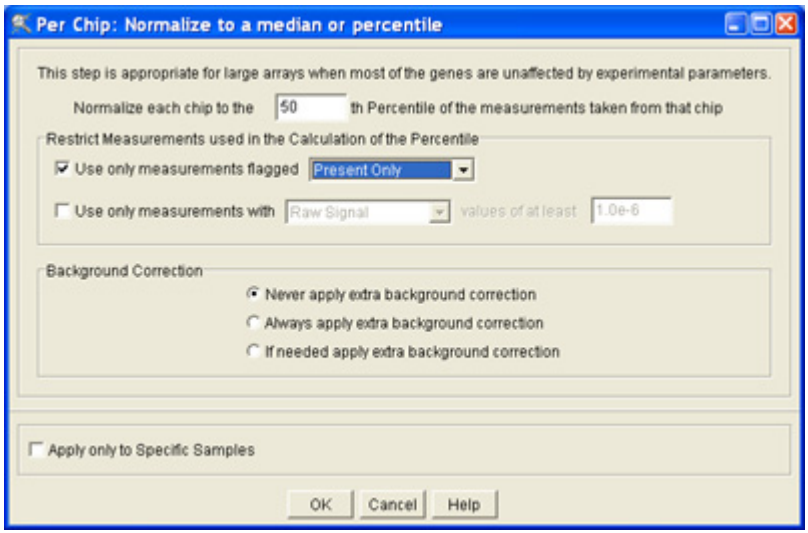
The Control Channel cutoff value is the only option that you can set for this normalization step. If the Control Channel value is below the cutoff value, the cutoff value is used for the control channel to ensure that sensible ratios are calculated.

By default, this cutoff value is set to 10, but for this example scenario we have lowered this value to $1.0E^{-6}$ to allow lower values for the control channel.

Per chip: Normalize to 50th percentile

The second normalization step normalizes the expression value to the median of the expression values on the array. This ensures that the expression values for each array can be compared across chips by centering each of the distributions for each array around 1, making comparison across arrays possible.

When you determine the median value for the distribution of signal values, you can exclude from the calculation each probe that has been flagged as being unreliable. This ensures that the median calculation and normalization is not skewed by outliers or control probes. It also ensures that normalized values are sensible.



The recommended scenario only considers probes that are marked with a “Present” or P GeneSpring flag. This is indicated by the Normalization-step option “Use only measurements flagged Present Only.”

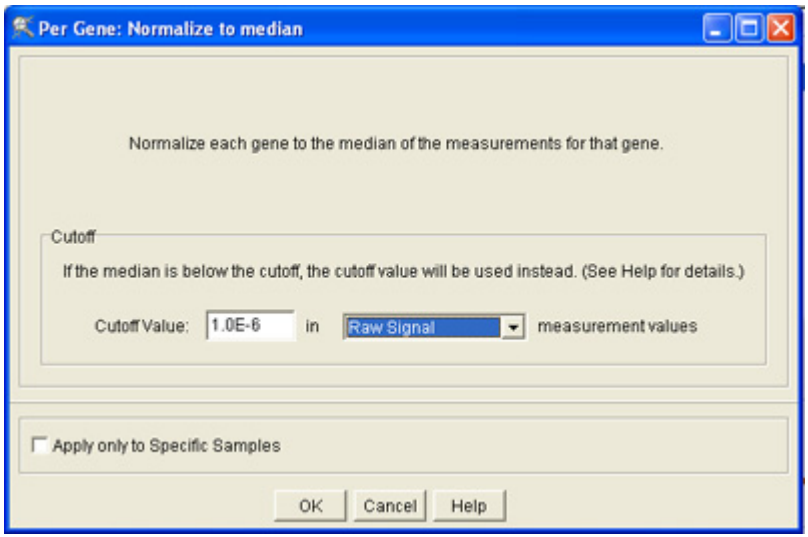
Preprocessing the data using the Agilent FE plug-in's default settings results in the *exclusion* of probes that are control probes, saturated, feature population outliers, non-uniform features, or manually flagged.

To include the features that are only flagged to be marginal, the option can be changed by selecting **Present or Marginal** from the drop-down list.

NOTE: *The features are only excluded in calculating the median value. The features are not excluded from the experiment and continue to be available for review or analysis. The excluded features will also have the same normalization applied to when compared to the non-excluded probes.*

Per gene: Normalize to median

The last normalization step normalizes the expression value for each gene across all the arrays around 1. This allows you to compare the genes across all the conditions or arrays, regardless of the actual normalized expression value (ratio).

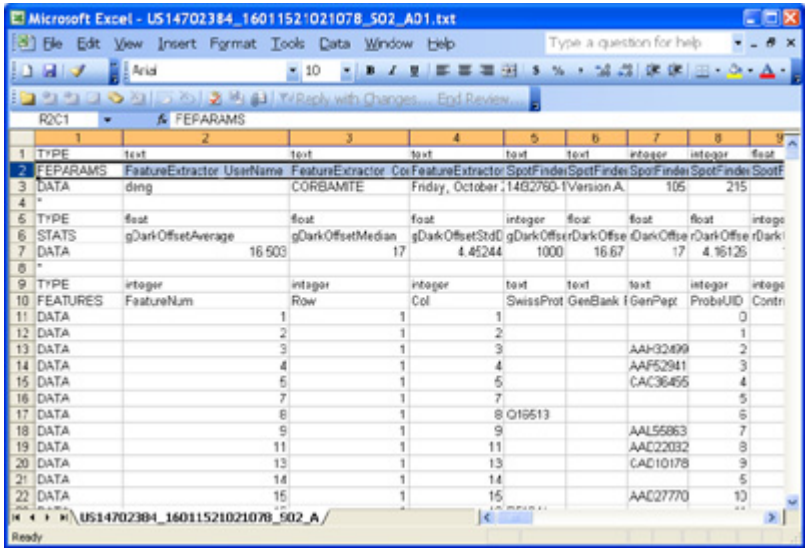


There are two options that can be set in this normalization step: (1) a cutoff value can be set to ensure that values lower than the cutoff will be replaced with the cutoff value and (2) the cutoff value can be applied to either the normalized value or the Raw signal value.

The recommended normalization step has a cutoff set for the Raw signal value of $1.0E^{-6}$. This, together with the cutoff value for the Control value of $1.0E^{-6}$, ensures that all normalized values are sensible.

Appendix E – Extracted Sample Attributes

The Agilent FE plug-in extracts a number of fields from the Feature Extraction output file and stores them as Sample Attributes in GeneSpring GX. The fields that are extracted from the FE output file are located in line 2 of the FE output file marked “FEPARAMS” and in line 6 marked “STATS.”



All items from the FEPARAMS are extracted as Sample Attributes. The names of the Sample Attributes are the same as the name of the FEPARAMS or STATS fields..

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In this book

This book contains brief instructions to help you get started with your Agilent Feature Extractor Plug-In for GeneSpring GX.

The information includes instruction on

- Installing the Feature Extraction Plug-In
- Loading and normalizing data produced by Feature Extraction Software
- Identifying flags related to specific types of problems

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