

GeneMaths XT

Two color tutorial

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1. Import

1.1 Downloading the data

An example dataset will be used in order to explain the workflow of GeneMaths XT. This dataset is publicly available on the GEO website ('Gene Expression Omnibus').

1.1.1 Go to the GEO homepage: <http://www.ncbi.nlm.nih.gov/geo>, click in the box next to 'Query > GEO accession' and type **GDS742**.

1.1.2 Press <Go>.

1.1.3 Select **GSE1652** in the *GDS Summary* panel next to Series.

1.1.4 Scroll down the next page and select **SOFT formatted family file(s)**.

Download family	Format
SOFT formatted family file(s)	SOFT ?
MINiML formatted family file(s)	MINiML ?
Series Matrix File(s)	TXT ?

Figure 1-1. Download information.

1.1.5 On the next page select **GSE1652_family.soft.gz**.

1.1.6 Select <Save> and navigate to a path on your computer.

1.1.7 Press <Save> to save the file in the selected folder.

1.2 Importing the data in GeneMaths XT

1.2.1 Start GeneMaths XT by double clicking on the icon



on the desktop or from the task bar with **Start > Programs > Applied Maths > GeneMaths XT**.

1.2.2 Click <Next> in the welcome screen to begin the import of the data. If the welcome screen does not appear, choose **File > Import Wizard** in the *GeneMaths XT Main* window. The *Import Wizard* window pops up (see Figure 1-2).

1.2.3 Select the fourth option, **Import from other sources** and hit <Next>.

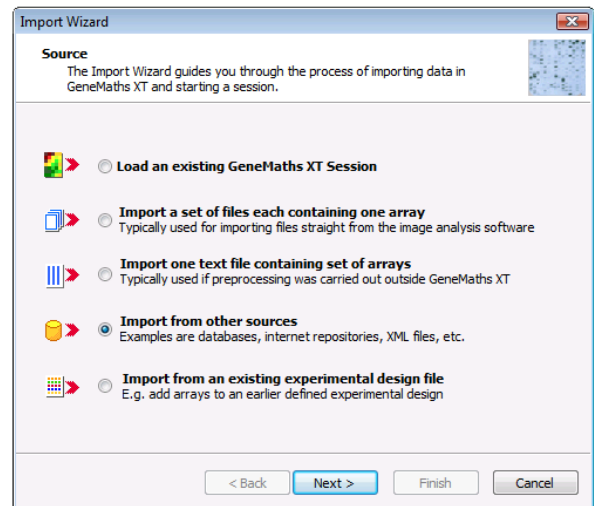


Figure 1-2. Import wizard: select data source.

1.2.4 Select **GEO's SOFT family** in the format list. A short description of the format is shown in the right panel (see Figure 1-3).

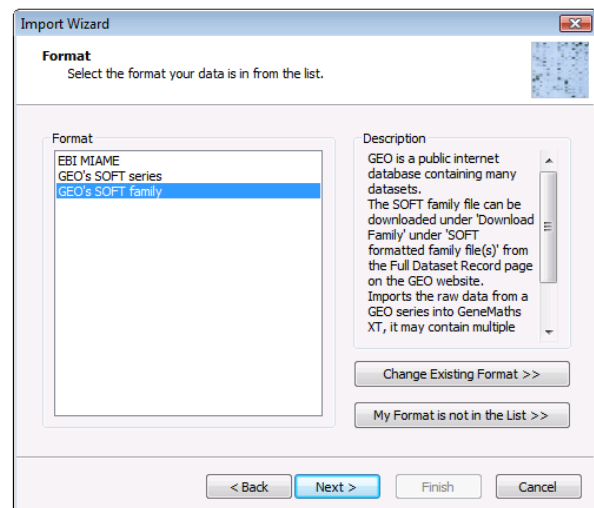


Figure 1-3. Import wizard: select format.

1.2.5 Click <Next>.

1.2.6 Browse for the stored file in the *File* panel. You can leave the top two panels empty (see Figure 1-4).

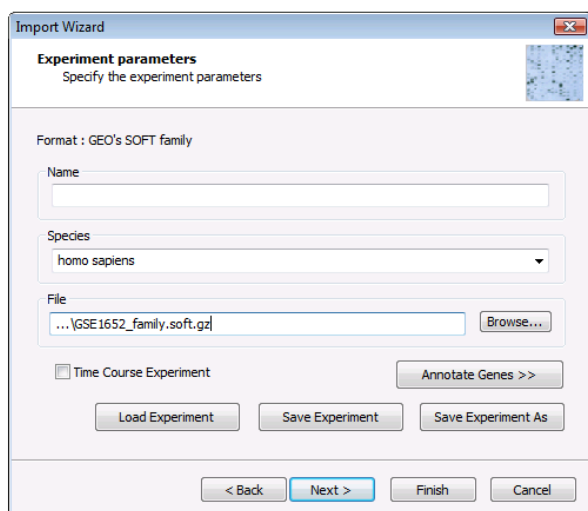


Figure 1-4. Start wizard: Input file.

1.2.7 Click <Next>.

1.2.8 Specify the name of the processed file e.g. GDS742.xps.

1.2.9 The *Calculation* dialog box pops up. The status of the import of the data is shown (see bottom of the box).

1.2.10 After the processing of the data (this may take a couple of minutes) GeneMaths XT will prompt to specify the contents of the columns in the *Define Format* dialog box (see Figure 1-5).

1.2.11 Select the second column, 'ID_REF' by clicking on it. The column is highlighted in pink. Specify the kind of data in this column by changing the settings in the *Column Information* panel. Select **Text** in the *Type* box and **Spot ID** in the *Text* box (see Figure 1-6).

1.2.12 Select the third column ('CH1_MEAN'). **Quantitation, Target, Foreground** and **Value** are automatically selected as the settings for the third column (see Figure 1-7).

GeneMaths XT automatically assigns the correct settings to columns 3-6 as specified in the dataset file. Only for column 2 and 7 the settings need to be changed.

1.2.13 Select the last column and fill out the correct settings (last row in Figure 1-8).

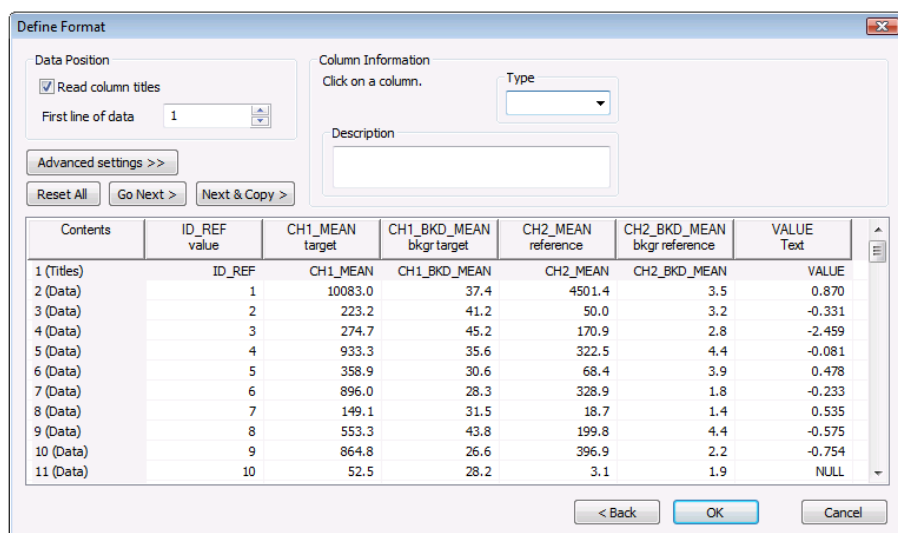


Figure 1-5. The *Define format* dialog box.

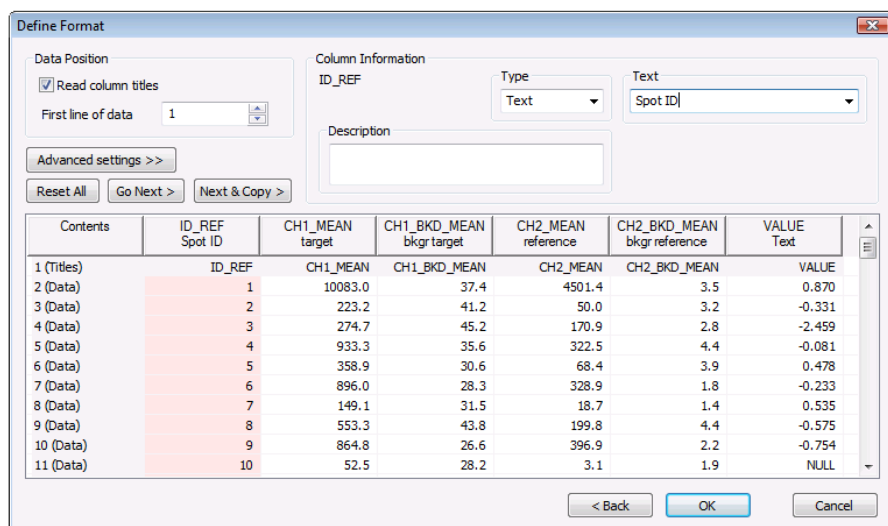


Figure 1-6. Settings for the second column.

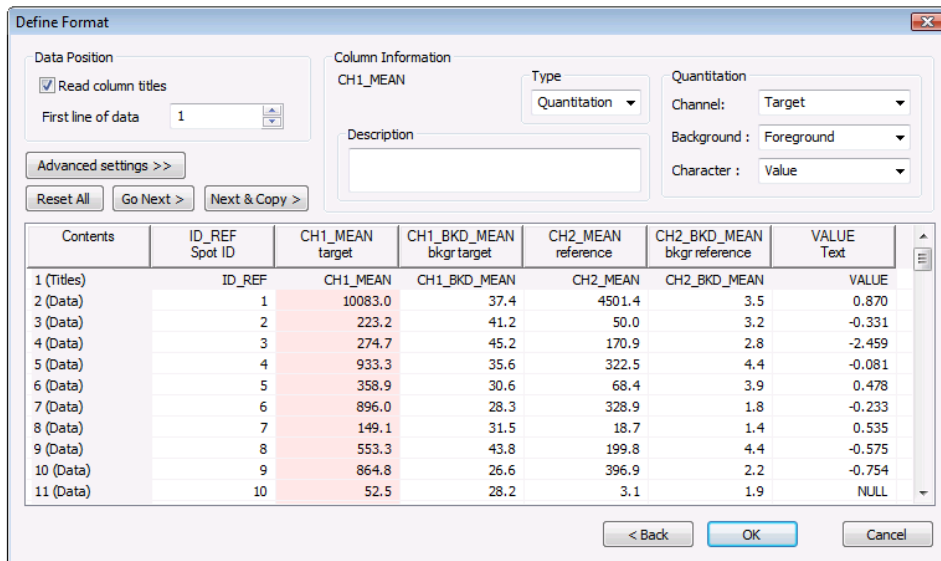


Figure 1-7. Settings for the third column.

Column		Type	Quantitation		
4	(CH1_BKG_MEAN)	Quantitation	Target	Background	Value
5	(CH2_MEAN)	Quantitation	Reference	Foreground	Value
6	(CH2_BKG_MEAN)	Quantitation	Reference	Background	Value
7	(VALUE)	Quantitation	Ratio	Foreground	Value

Figure 1-8. Settings for the different columns.

1.2.14 After specifying the correct column information for all data columns press <OK>.

1.2.15 The *Import mapping* dialog box pops up asking you to create a mapping for your data. This mapping tells GeneMaths XT which quantitations to use in the session.

1.2.16 Select **ID-REF** and hit ">". ID-REF is now placed in the right box (see Figure 1-9).

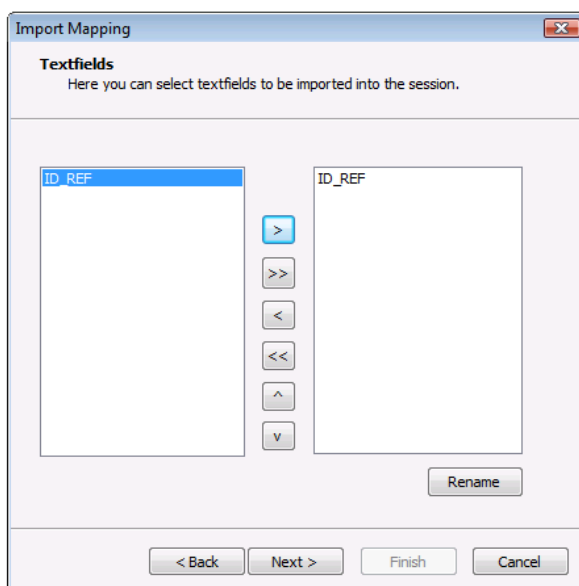


Figure 1-9. The *Import mapping* dialog box: step 1.

1.2.17 Click <Next>.


1.2.18 Mark the two checkboxes in the *Filter* panel of the next dialog box. Marking these boxes means we want to load both channels in a different layer and that we want to have the background for each channel in a different layer as well (see Figure 1-10).

1.2.19 In the *Quantitations* panel, use all four quantitations without an error value (see Figure 1-10), simply because there are no errors provided in the GDS file. Use the pull down menu to select the predefined signals.

1.2.20 In the next step of the import, select **VALUE** in the first column, select **No error** in the second column and hit ">" (see Figure 1-11). VALUE is now used as an extra quantitation.

1.2.21 Click <Next>.

GeneMaths XT will import the data in a new session. The *Main* window of GeneMaths XT appears as depicted in Figure 1-12. The session contains 5 layers (displayed in the top left panel), 3 row identifiers and 5 column identifiers (see Figure 1-14 and Figure 1-14).

NOTE: Do not forget to save your session on a regular basis by pressing .

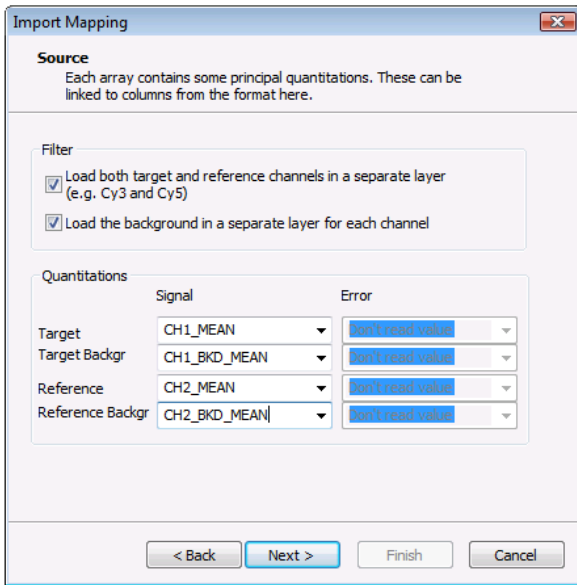


Figure 1-10. Import mapping: step 2.

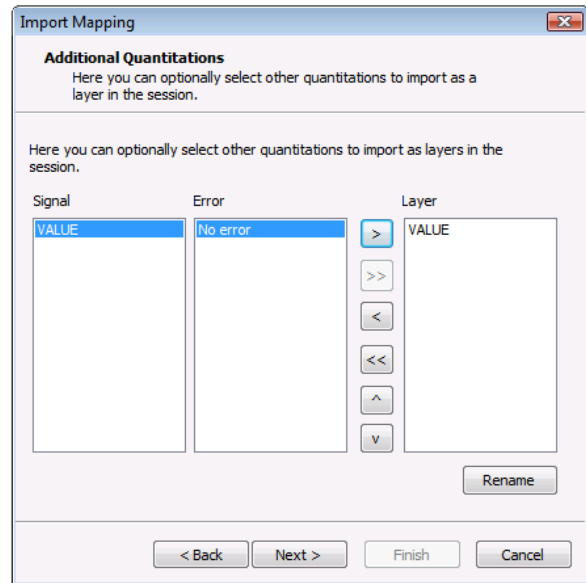


Figure 1-11. Import mapping: step 3.

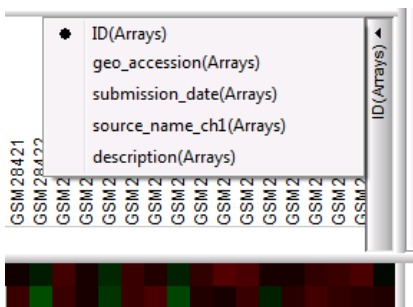


Figure 1-13. The column identifiers.

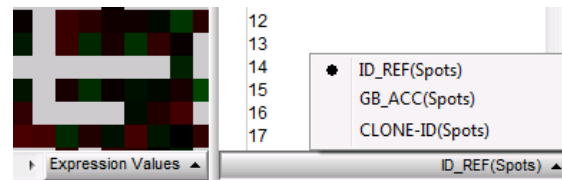


Figure 1-14. The row identifiers.

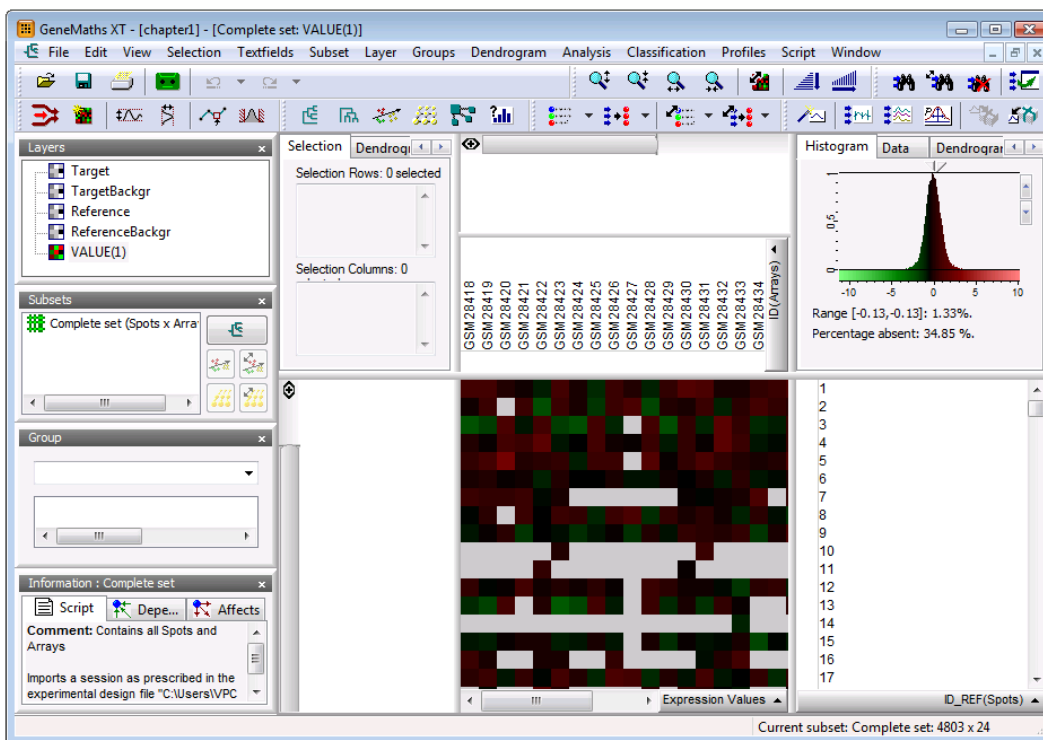


Figure 1-12. The Main window of GeneMaths XT after import of the data.

2. Annotation

2.1 Row annotation

The annotation is automatically with the import of the file. The spots however, do not yet contain the GO ID as an identifier.

Several steps are needed in order to get the GO IDs for the genes. We will start from the GenBank accession numbers (GB_acc is one of the 3 row information fields, see Figure 1-14) and convert them to Unigene, then from Unigene to Entrez and then finally from Entrez to GO IDs. This is to show that there is a logical connection between all of these.

Please note that this long procedure is only needed when using custom arrays. For Affymetrix arrays we can use a direct import of annotations (see One Color Tutorial and GSEA Tutorial).

2.1.1 Select *Textfields > Annotations > Genbank to Unigene*. Select **Rattus norvegicus** as the organism and use **GB_ACC** as the GenBank ID (see Figure 2-1). Click **<OK>**.

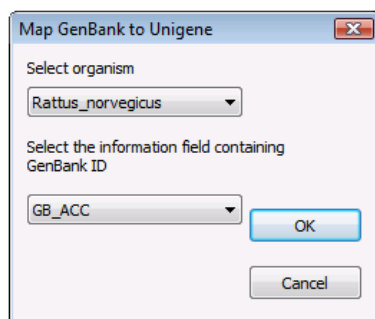


Figure 2-1. Conversion of GenBank accession numbers to Unigene.

Please be patient while GeneMaths XT is importing. When finished, you will have an extra row identifier named **Unigene Cluster ID** (see Figure 2-2).

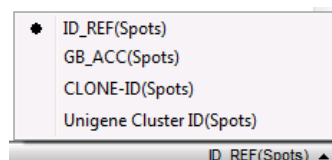


Figure 2-2. Unigene Cluster ID.

2.1.2 Select *Textfields > Annotations > Entrez Gene*. Choose **Entrez Gene To Unigene** and press **<OK>** (see Figure 2-3).

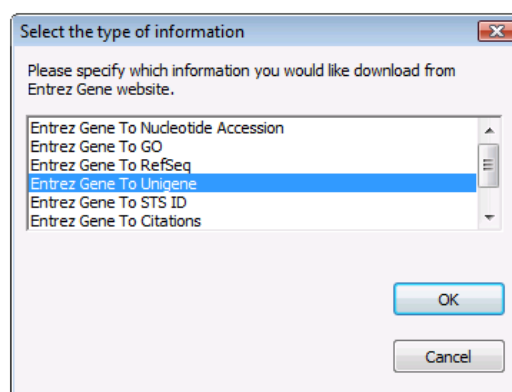


Figure 2-3. Get Entrez information.

2.1.3 In the next window, select the **Unigene Cluster ID** (left), the **UniGene Cluster** (right) and **GeneID** in the bottom panel (see Figure 2-4).

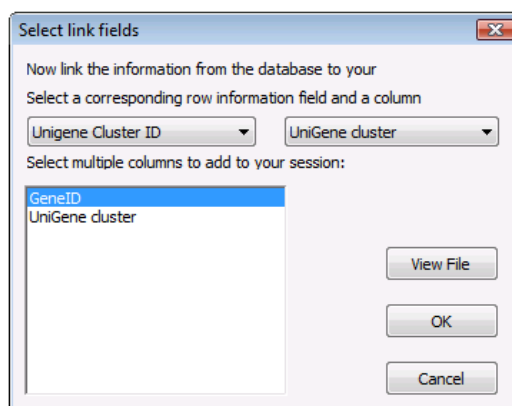


Figure 2-4. Linking the information.

With these settings the Unigene Cluster IDs are linked to the UniGene Cluster information in the external annotation file. **GeneID** is added to the row information fields (see Figure 2-5).

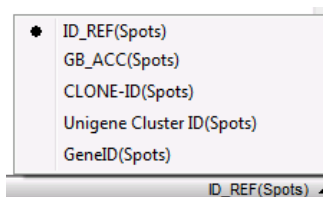


Figure 2-5. Gene ID in the list of row identifiers.

2.1.4 Select once more *Textfields > Annotations > Entrez Gene*, but this time select **Entrez Gene To GO** and press <OK>.

2.1.5 Fill out the next dialog with the settings shown in Figure 2-6 and press <OK>.

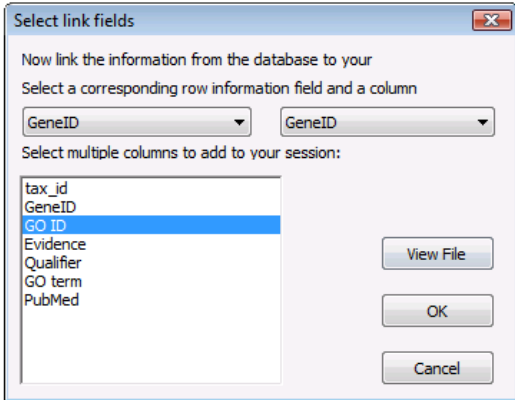


Figure 2-6. Adding the GO ID in the final step.

2.1.6 GO ID is added to the list of identifiers. Select GO ID from the list. The available GO ID(s) for each row entry is/are shown (see Figure 2-7).

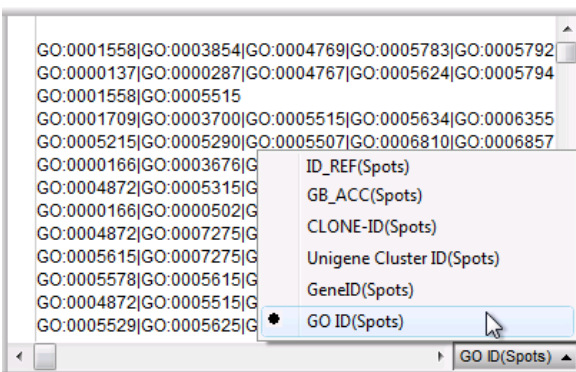


Figure 2-7. GO ID in the list of identifiers .

2.2 Column annotation

Four column identifiers are present after import of the data. In a next step, we are going to split the information present in the **description** information field (see Figure 2-8).

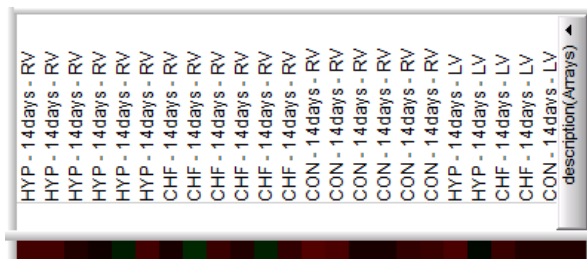


Figure 2-8. The description column identifier.

2.2.1 Select *Textfields > Split*. Fill out the dialog box as shown in Figure 2-9. Press <OK>.

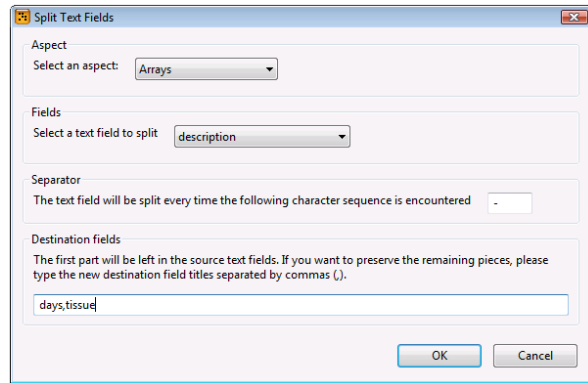


Figure 2-9. Split textfields.

Two column identifiers (days and tissue) are added to the list of column identifiers (see Figure 2-10).

2.2.2 Select the **tissue** identifier from the list. The information in the *Column names* panel is updated (see Figure 2-10).

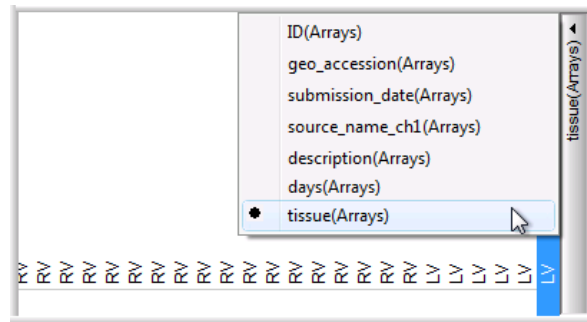


Figure 2-10. The column identifiers.

As a last step, we will rename the column identifier **description** to **disease state**.

2.2.3 Select *Textfields > Rename* and fill out the dialog box as shown in Figure 2-11. Press <OK>.

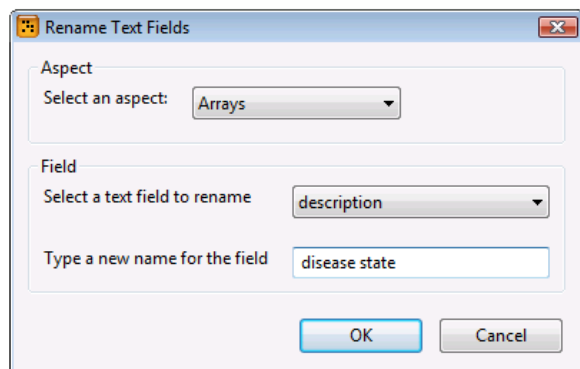


Figure 2-11. Rename a textfield.

3. Groupings

With the statistics we want to perform later on in mind, we need to define groupings, each containing a set of particular groups. The groupings will then later be the input for the statistical tools and visualizations.

3.1 Row groups

In the first step, we want to make row groups from the GO IDs.

3.1.1 Select *Groups > Edit Row Groups* and click on *<Create New Grouping>*.

3.1.2 In the next window, select **GO ID** from the *Name*-pull down menu and click *<OK>* (see Figure 3-1).

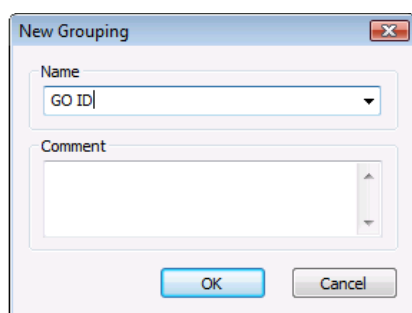


Figure 3-1. Grouping from the GO IDs.

3.1.3 GO ID is selected as the text field in the next window (see Figure 3-2). As you can see in the gene identifier list (see Figure 2-7), the same gene can contain multiple GO IDs, separated by a “|” (e.g. GO:0006955|GO:0005529|GO:0007166). Use a “|” (a

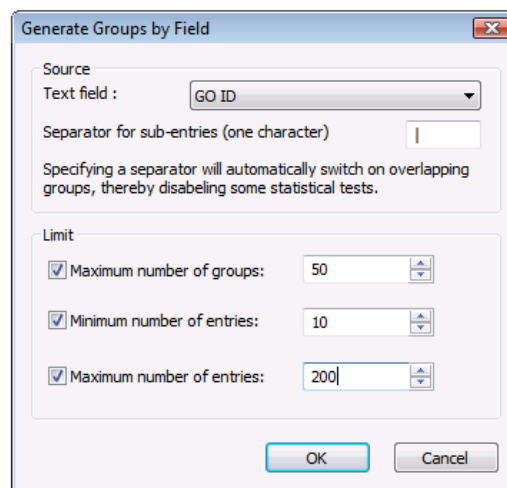


Figure 3-2. Creating groups based on the GO ID.

pipe) for the delimiter. This will split the multiple IDs for a certain row entry.

3.1.4 Specify the limitations as shown in Figure 3-2. The filtering is useful because we do not want small groups, this would make them useless in further analysis. Click *<OK>*.

3.1.5 The groups based on the settings are shown in the next window (see Figure 3-3).

3.1.6 Press *<Exit>*.

GeneMaths XT offers the possibility to link a grouping to a website. We will link the GO ID grouping to the GO-website. Later on we can use this link when using statistics reports.

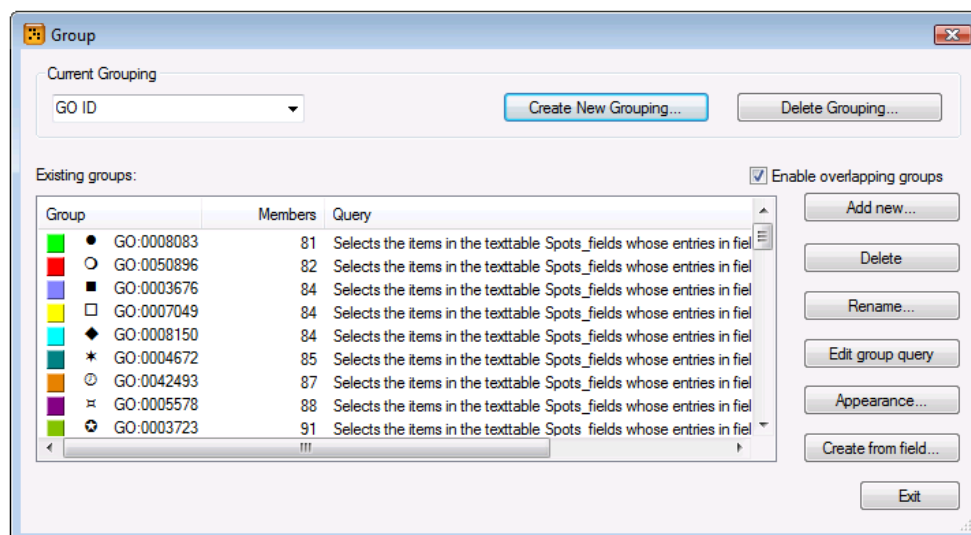


Figure 3-3. Groups based on GO IDs .

3.1.7 Select *Groups* > *Row Group Link*.

3.1.8 In the next dialog box, select **GO ID** and 'http://www.godatabase.org/cgi-bin/amigo/go.cgi?action=replace_tree&query=###'. Click **<OK>**.

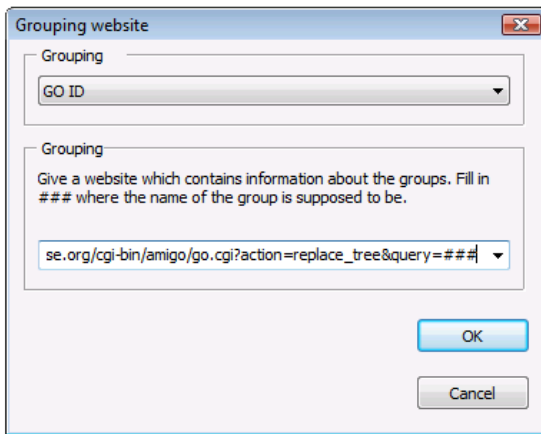


Figure 3-4. Create a group link.

3.2 Column groups

In the next step, we want to make groups from the column information fields **tissue** and **disease state** (see section 2.2).

3.2.1 Select *Groups* > *Edit Column Groups* and click on **<Create New Grouping>**.

3.2.2 In the next window, select **tissue** from the *Name*-pull down menu and click **<OK>** (see Figure 3-5).

3.2.3 Tissue is selected as the text field in the next window (see Figure 3-6). Uncheck ALL the limitations and click **<OK>**.

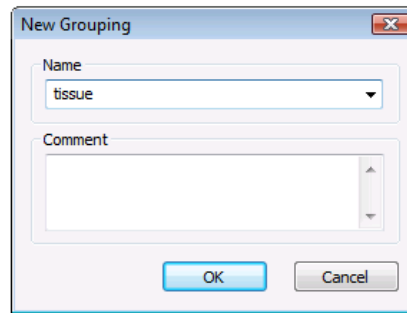


Figure 3-5. Grouping from tissue.

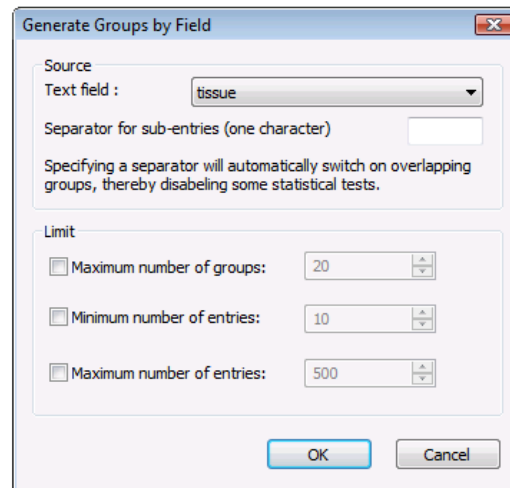


Figure 3-6. Creating groups based on the tissue.

3.2.4 The groups based on the settings are shown in the next window (see Figure 3-7).

RV = right ventricle

LV= left ventricle

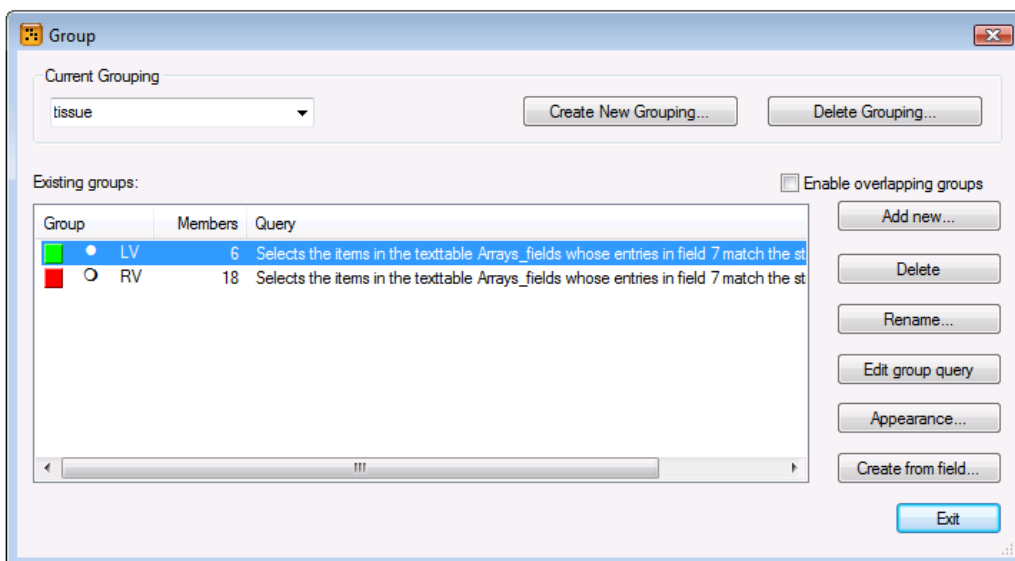


Figure 3-7. Groups based on the tissue.

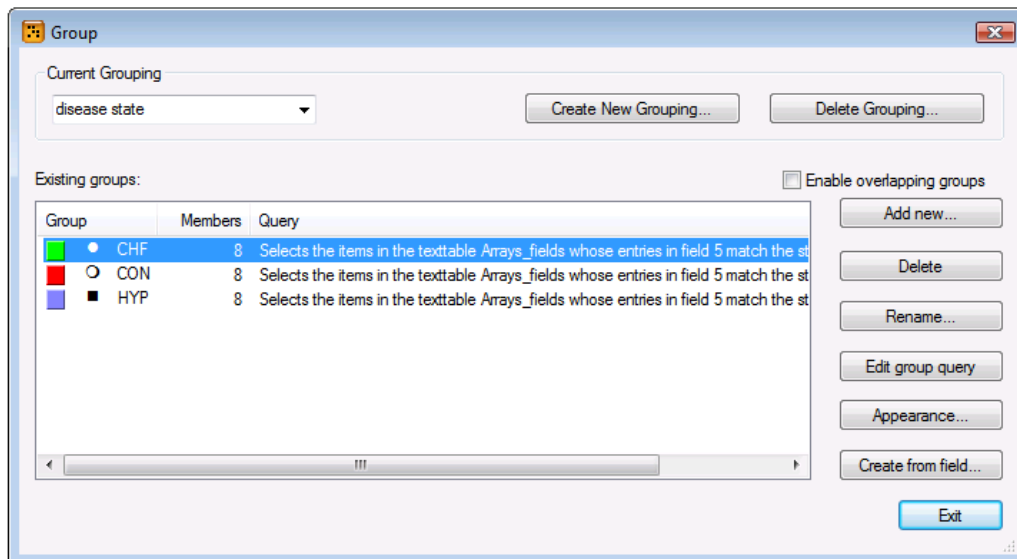


Figure 3-8. Groups based on the disease states.

3.2.5 Repeat steps 3.2.1 until 3.2.3 for **disease state** (see 3-8).

CHF = decompensated hypertrophy

CON = control

HYP = compensated hypertrophy

3.2.6 Press <Exit>.

4. Preprocessing

4.1 The preprocessing diagram

In order to perform data analysis on this dataset, we first need to preprocess our data.

4.1.1 In GeneMaths XT, select *Layer > Preprocessing diagram*.

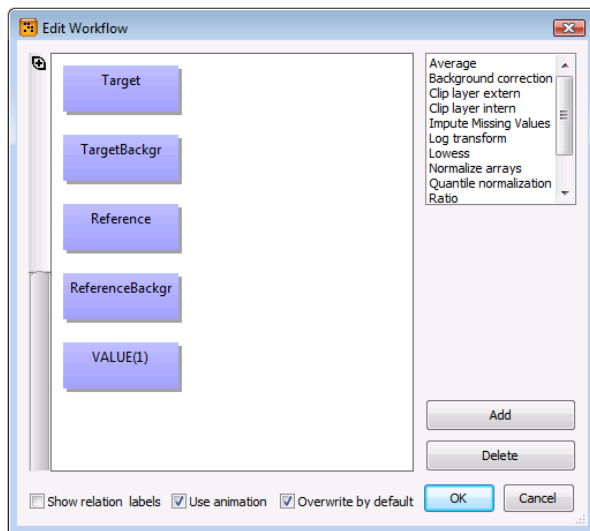


Figure 4-1. Preprocessing window.

In the *Preprocessing* window, all layers present in the session are displayed. On the right side of the window, the preprocessing tools are listed.

4.1.2 Uncheck *Overwrite by default*.

4.2 Background correction

4.2.1 In the *Preprocessing* window, select the **Target** and **TargetBackgr** layer. To select both layers hold the CTRL key.

4.2.2 Select **Background correction** from the list tools.

A description of this tool is displayed below the list of preprocessing tools.

4.2.3 Press **<Add>**. Double clicking on the tool in the list does the same.

4.2.4 Leave the settings in the next window unaltered and press **<OK>**.

4.2.5 In the next window, store the background corrected values in a new layer called **Tar_BG_corr**.

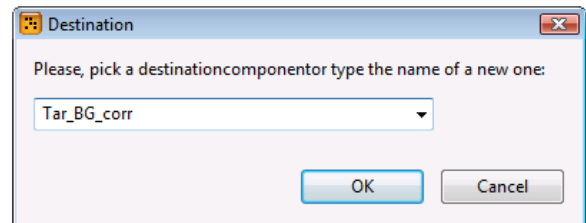


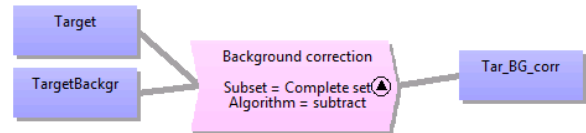
Figure 4-2. Define a new destination layer.

*NOTE: Make sure you have unchecked the option **Overwrite by default**.*

4.2.6 Press **<OK>**.

4.2.7 The result of the background subtraction is stored in the **Tar_BG_corr** layer.

4.2.8 Click on the arrow in the Background correction box. Two parameters are displayed.



4.2.9 To change the parameters, double click in the pink box.

4.2.10 Check **Exponential + normal model** and press **<OK>**.

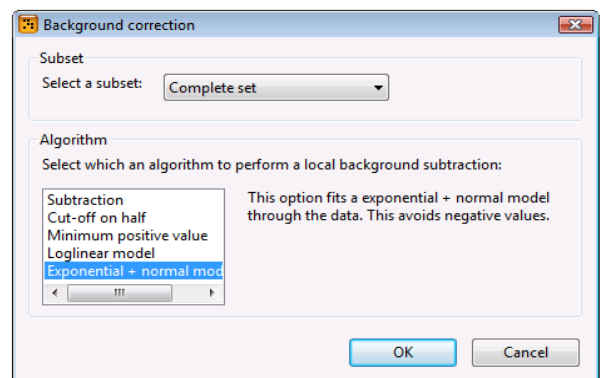


Figure 4-3. Background correction settings.

4.2.11 Repeat step 4.2.1 - 4.2.10 for the **Reference** and **ReferenceBackgr** layer. Store the result in a new layer called **Ref_BG_corr** and make sure the **Exponential +**

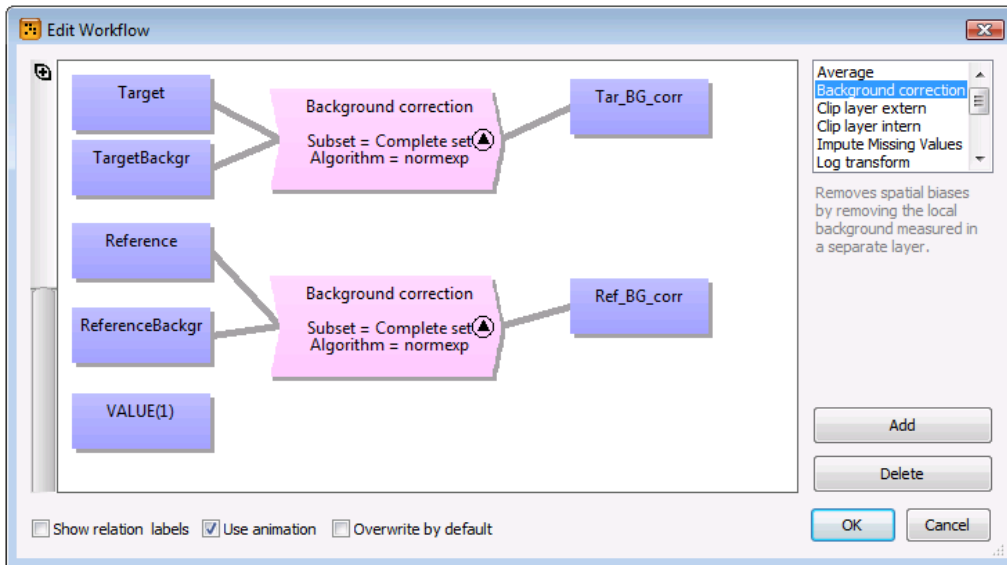


Figure 4-4. The *Preprocessing* window: Background correction.

normal model is selected from the list of available algorithms.

The *Preprocessing* window should now look like Figure 4-4.

4.3 Log transformation

In the next step we are going to make new layers that are the logarithm of the newly created background subtracted layers (Tar_BG_corr and Ref_BG_corr). The log transformed layers will have a normal distribution. This is a prerequisite to perform several statistical tests on your data.

*NOTE: Make sure you have unchecked the option **Overwrite by default** in the *Preprocessing* window.*

4.3.1 Select the layer **Tar_BG_corr** in the *Preprocessing* window.

4.3.2 Select **Log Transform** from the list of preprocessing tools and press **<Add>**. Double clicking on the name does the same.

4.3.3 Store the result of the log transformation in a new layer called **LogTar**.

4.3.4 Press **<OK>**.

4.3.5 Repeat the previous step for the **Ref_BG_corr** layer. Store the values in a layer called **LogRef** (see Figure 4-5).

4.3.6 Press **<OK>**.

A *Calculation* dialog box pops. After calculating the preprocessing steps defined in the *Preprocessing* window, the new layers are added to the list of layers in the *Layers* window (see Figure 4-6).

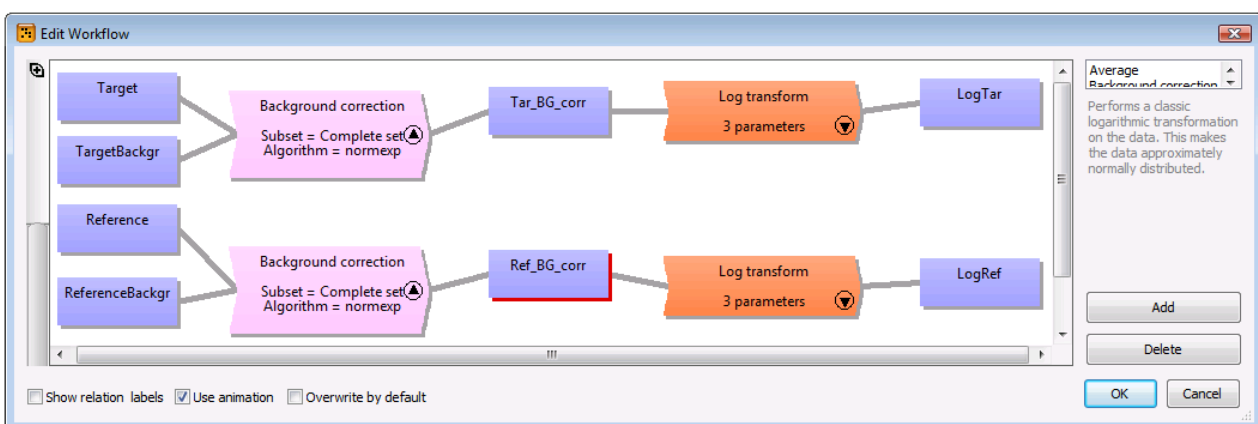


Figure 4-5. The *Preprocessing* window: Log Transformation.

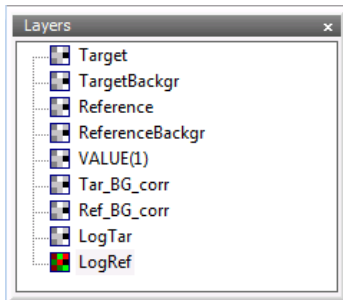


Figure 4-6. The *Layers* window with the new layers.

4.4 Normalization

First we are going to take a look at the data as it is now.

4.4.1 Select *Profiles* > *Plot Wizard*. Select **Columns** as the orientation. Because we want different layers to plot against each other (LogTar vs. LogRef) select **One column, different layers** and click <Next>.

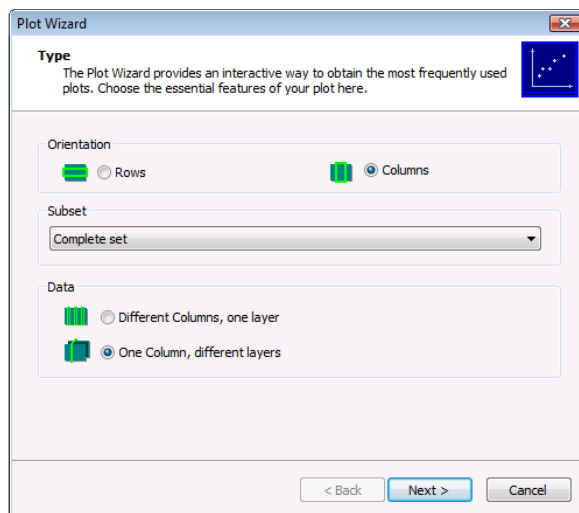


Figure 4-7. *Plot Wizard*: step 1.

4.4.2 In the next window select **Entries**. Select all entries and click <Next> to move to the next step (see Figure 4-8).

4.4.3 In the third window, select the two log transformed layers **LogRef** and **LogTar**. Click ">" and click <Next> (see Figure 4-9).

4.4.4 In the final window, select **MA-plot** in the *Plot type* panel and **Curve color** in the *Color* panel (see Figure 4-10). Click <Finish>. ($M = \log_2(\text{Red Intensity}/\text{Green Intensity})$ and $A = \sqrt{(\text{Red Intensity} \times \text{Green Intensity})}$).

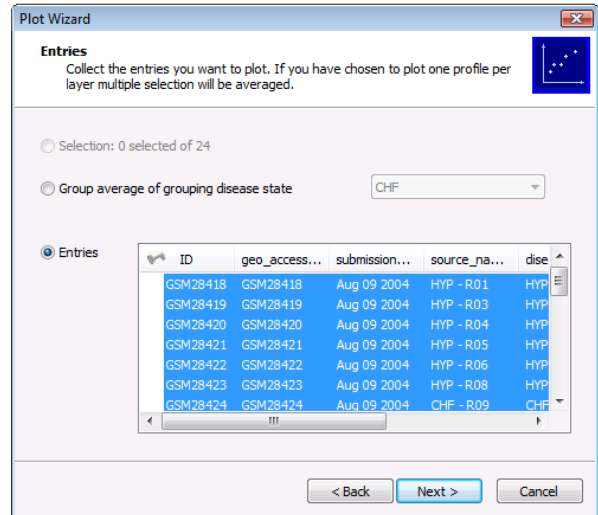


Figure 4-8. *Plot Wizard*: step 2.

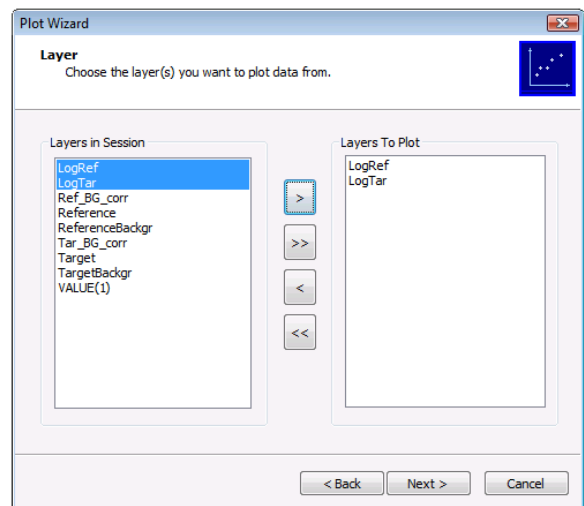


Figure 4-9. *Plot Wizard*: step 3.

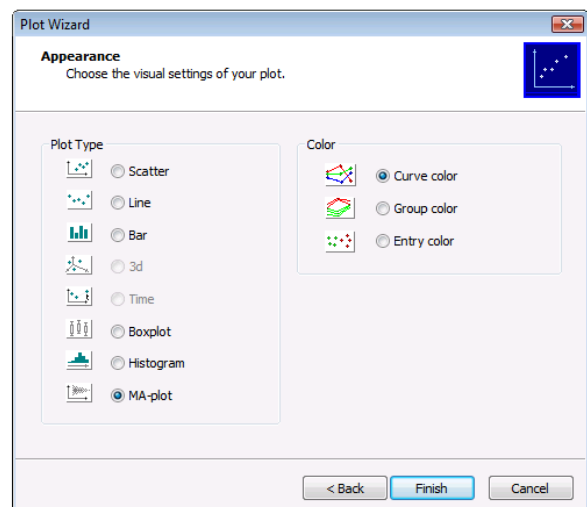


Figure 4-10. *Plot Wizard*: final step.

The plot appears in GeneMaths XT (see Figure 4-11). We need to set the X and Y axes correctly for the plot.

4.4.5 Select the profile of the **LogRef** layer in the *Profiles* panel (see left panel in Figure 4-11). Click right and choose *Set as y-axis*.

4.4.6 Select the profile of the **LogTar** layer and choose *Set as x-axis*.

4.4.7 Select *Profiles > Selected Profile > XY to RI*.

4.4.8 Select *Profiles > Selected Profile > Lowess Plot*. Press <OK>.

The plot should now look like the one in Figure 4-12.

The MA-plot (see Figure 4-12) shows that at a certain average level of intensities, the ratio M approximates a certain constant level. However, when A (= intensity) is below a certain threshold the ratio deviates from this constant level. This means that the detection levels for underexpression differ between the two channels. Long story short, the extreme deviation of the plot is caused by a difference in sensitivity between the two channels. A solution for this problem is to use an 'intensity dependent normalization'. We will use the Lowess normalization to compensate for these intensity dependent effects.

4.4.9 Select *Layer > Preprocessing diagram*.

4.4.10 In the *Preprocessing* window, select the **LogTar** and **LogRef** layers. To select both layers hold the CTRL key.

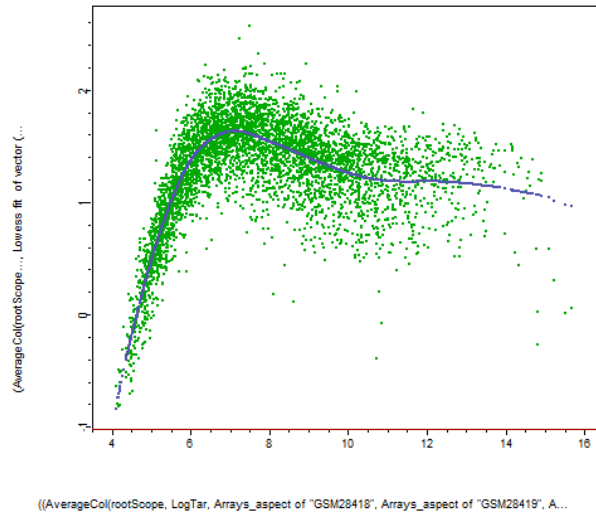


Figure 4-12. MA-plot with the corrected axes.

4.4.11 Select **Lowess** from the list of preprocessing tools and press <Add>. Double clicking on the name does the same.

The result of the Lowess normalization is stored in the **LogTar** and **LogRef** layers (see Figure 4-13).

4.4.12 Press <OK>.

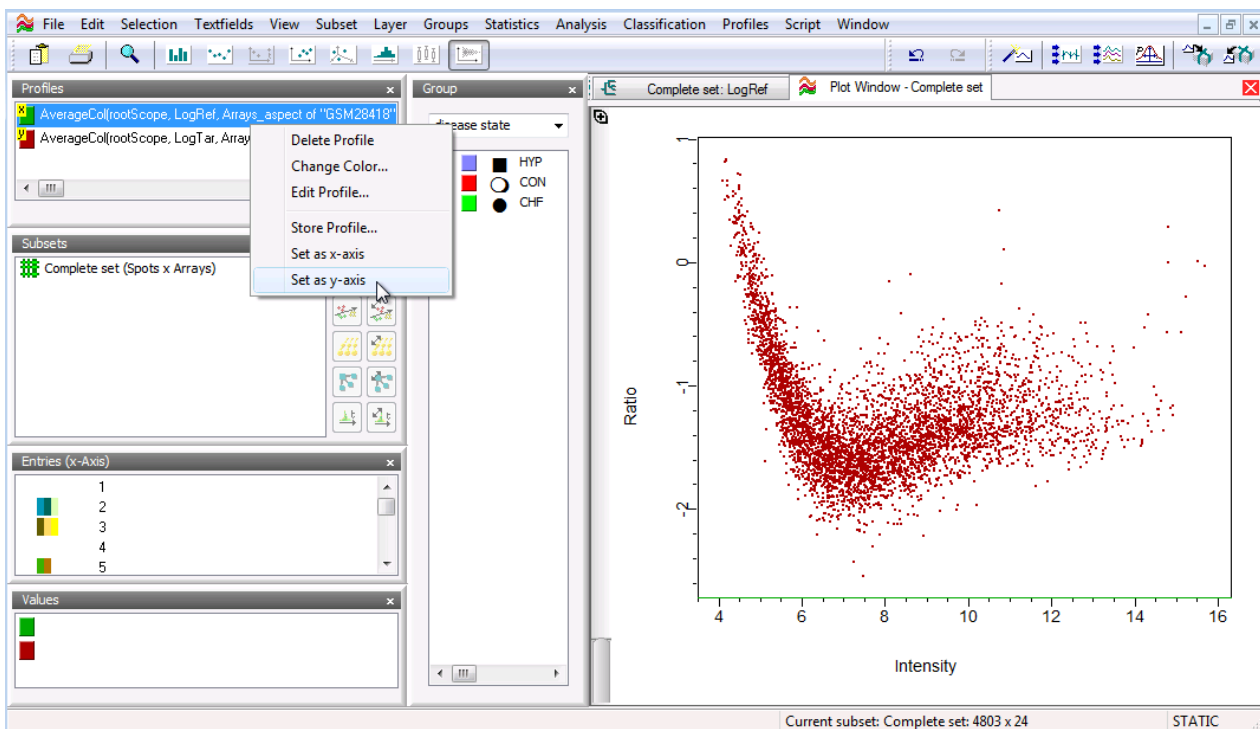


Figure 4-11. MA-plot.



Figure 4-13. The *Preprocessing* window: **Lowess**.

4.4.13 Select the first profile in the *Profiles* panel. Click right and choose *Edit Profile* (see Figure 4-14). Click <OK>.

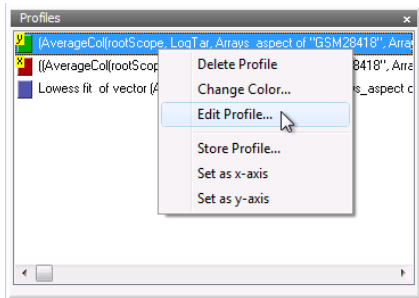


Figure 4-14. The *Profiles* panel.

4.4.14 Repeat the previous step for the other two profiles in the *Profile* panel. The plot should now look like Figure 4-15.

There is a clear compensation for the intensity dependent effect. Lowess thus allows for both normalization and linearization of the data.

4.5 Ratio

We are going to subtract LogTar and LogRef from each other in order to calculate the ratio of the layers.

4.5.1 Select *Layer > Preprocessing diagram*.

4.5.2 In the *Preprocessing* window, select the **LogTar** and **LogRef** layers. To select both layers hold the CTRL key.

4.5.3 Select **Ratio** from the list of preprocessing tools and press <Add>. Double clicking on the name does the same.

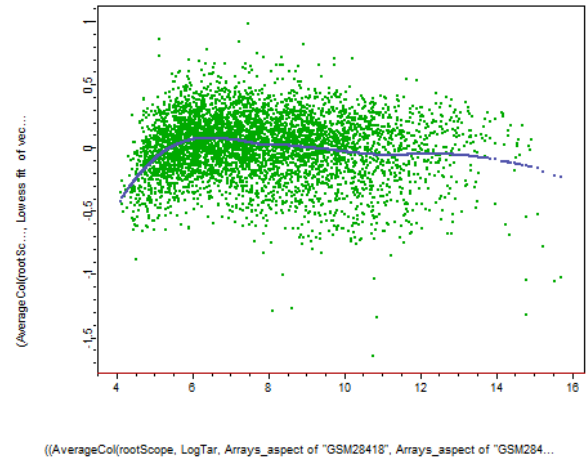


Figure 4-15. The **MA-plot** after **Lowess normalization**.

4.5.4 Store the values in a new layer called **Ratio**. Press <OK>.

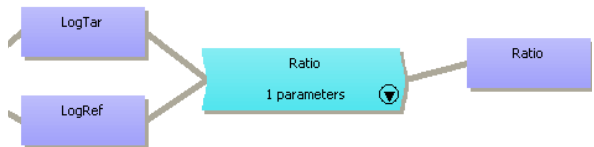


Figure 4-16. The *Preprocessing* window: **ratio**.

4.5.5 Press <OK>.

The **Ratio** layer is created and is added to the list of layers in the *Layers* window.


The new layer **Ratio** is a good starting point for statistical analysis. The histogram in the *Main* window shows a Gaussian distribution, centered around zero.

5. Statistics & Analysis

In the last step we will perform an ANOVA test on the **control (CON)** and **decompensated hypertrophy (CHF)** arrays of the **right ventricle (RV)**.

In a first step we are going to make a subset, containing all arrays of the **right ventricle**.

5.0.1 Click on the down arrow next to the column

groupings button  and select **tissue** from the drop-down menu. The tissue is now set as grouping.

5.0.2 Select **tissue** from the list of column identifiers.

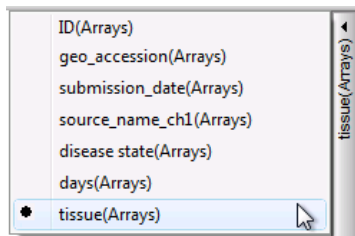


Figure 5-1. List of column identifiers.

5.0.3 In the *Group* window, select the **tissue** grouping from the list of defined groupings (see Figure 5-2).

All groups belonging to the tissue grouping are listed in the *Group* window (see Figure 5-2).

5.0.4 CTRL click on the square next to **RV** (see Figure 5-2)..

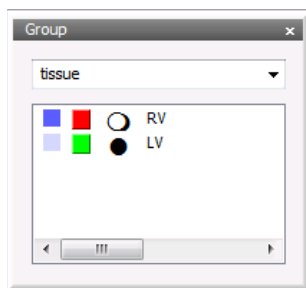


Figure 5-2. The *Group* window.

All arrays belonging to the RV (=right ventricle) group are selected in the *Column* panel (see Figure 5-3).

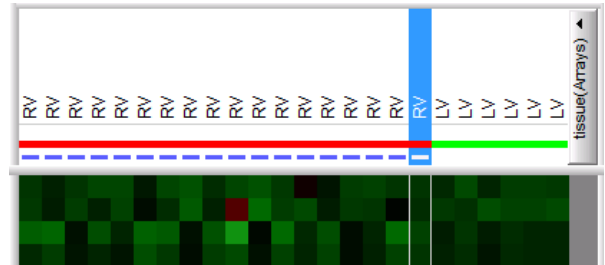


Figure 5-3. Selected arrays.

5.0.5 Select *Subset > Selection to Subset* and name the subset **Right ventricle** (see Figure 5-4). Press <OK>.

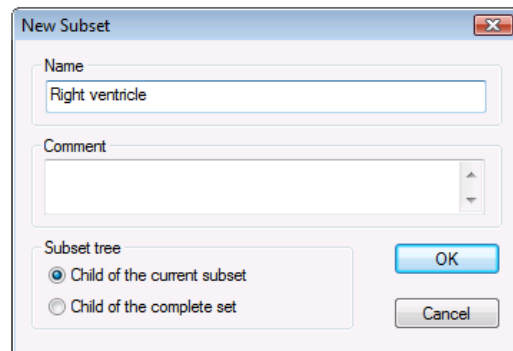


Figure 5-4. Creating a subset of the complete set.

5.0.6 In the *Main* window, the **Right ventricle** subset is stored as a subset of the Complete set.

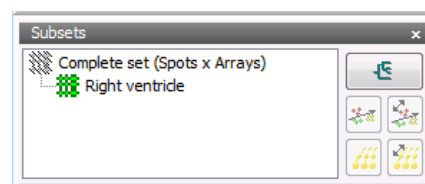



Figure 5-5. The *Subsets* window.

5.0.7 Clear the selection by pressing F4.

Next we are going to select the arrays belonging to the **decompensated hypertrophy** and **control** disease state.

5.0.8 First make sure that the **Right ventricle** subset is selected in the *Main* window.

5.0.9 Click on the down arrow next to the array

groupings button  and select **disease state** from the drop down menu. The **disease state** is now set as grouping.

5.0.10 Select **disease state** from the list of column identifiers (see Figure 5-1).

5.0.11 In the *Group* window, select the **disease state** grouping from the list of defined groupings (see Figure 5-6).

All groups belonging to the disease state grouping are listed in the *Group* window (see Figure 5-6).

5.0.12 CTRL click on the square next to **HYP** and **CHF** (see Figure 5-6)..

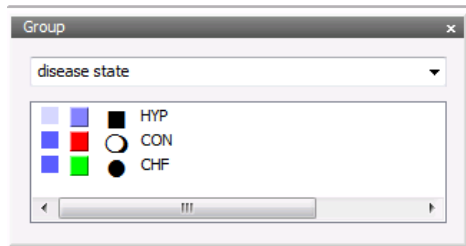


Figure 5-6. The *Group* window.

All arrays belonging to the CON (= control) and CHF (decompensated hypertrophy) groups are selected in the *Column* panel (see Figure 5-7).

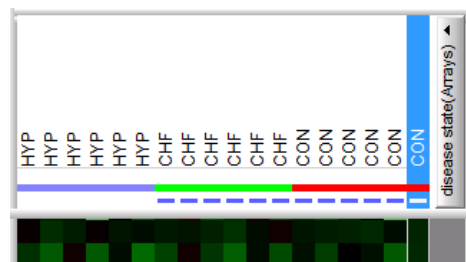


Figure 5-7. Selected arrays.

5.0.13 Select *Subset > Selection to Subset*, name the subset **Decompensated vs. Control** and enable *Child of the current subset*. Press <OK>.

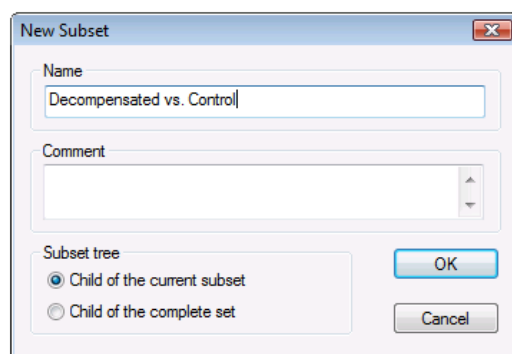
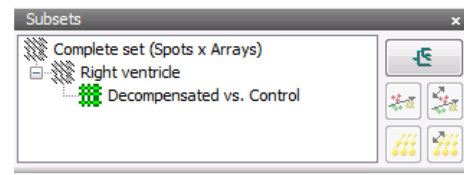


Figure 5-8. Creating a subset.

The *Subsets* window should now look like this:



5.0.14 Select *Profiles > Statistics Wizard*. Leave the orientation on **Row** set **Decompensated vs. Control** as subset and press <Next>.

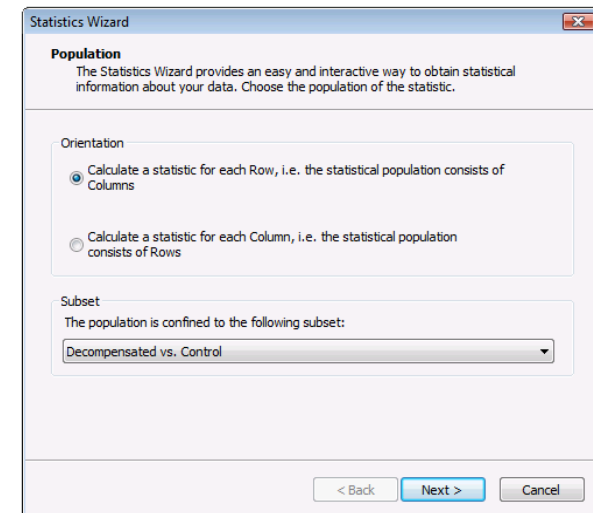


Figure 5-9. *Statistics Wizard*, step 1.

5.0.15 Select **ANOVA** test (under 'Independent test (multiple groups)') from the list and click <Next> (see Figure 5-10).

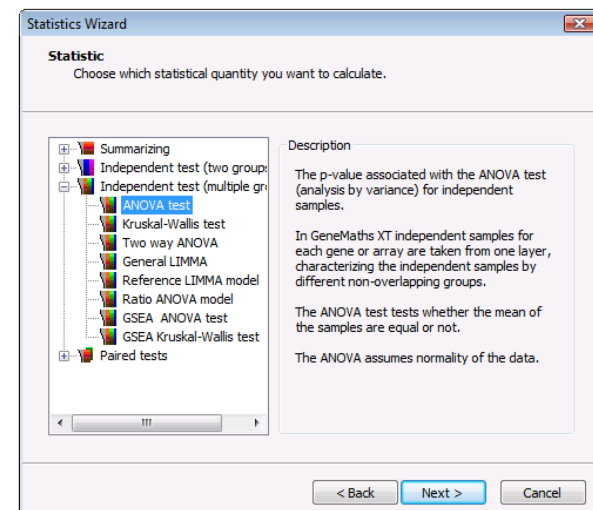


Figure 5-10. *Statistics Wizard*, step 2.

5.0.16 In the next window, make sure that **Ratio** is selected and the **disease state** in the *Groups* panel. Select **p-value** as output and click <Next> (see Figure 5-11).

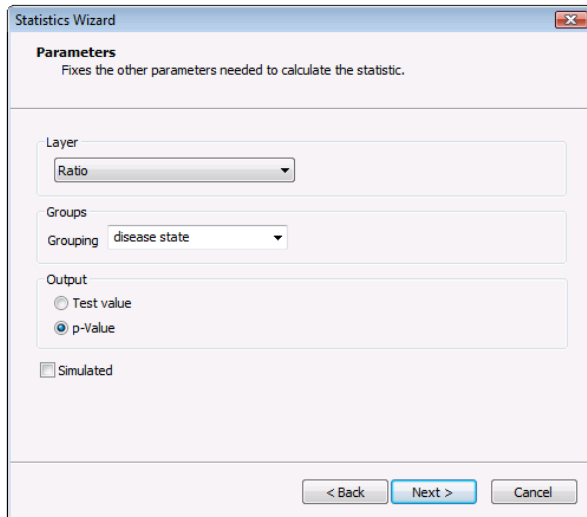


Figure 5-11. Statistics Wizard, step 3.

5.0.17 In the last window, choose the *Benjamini & Hochberg procedure* to correct for multiple testing and press *<Finish>* (see Figure 5-12).

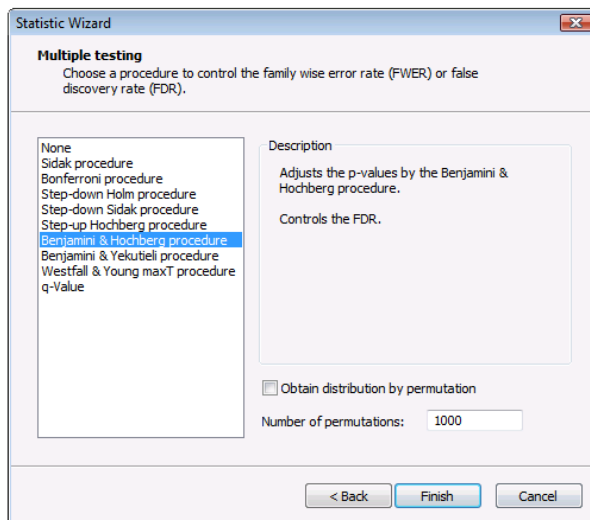


Figure 5-12. Statistics Wizard, step 4.

5.0.18 Click on the newly created profile in the *Profiles* tab. Right-click on the profile name and select *Sort From Profile* (see Figure 5-13).

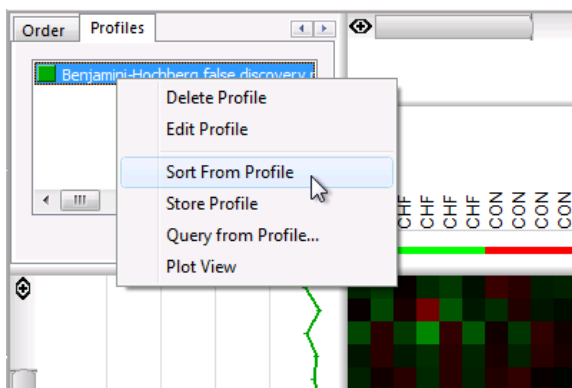


Figure 5-13. Sort From Profile.

5.0.19 Right click in the *Profile* panel (see Figure 5-14) and select *Show as Numbers*.

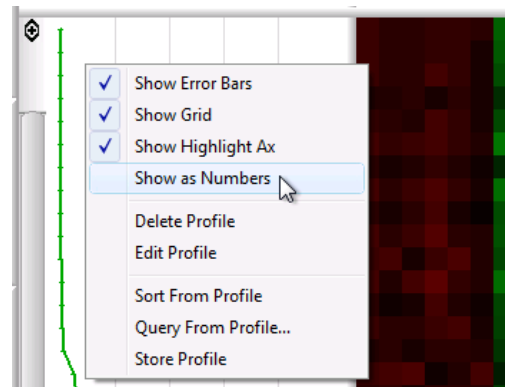


Figure 5-14. Show as Numbers.

In the *Profile* panel, the p-values for the row entries are shown. These p-values give an indication if the entries are significantly differentially expressed between the two groups or not. The lower the p-values the more differentially expressed.

5.0.20 Right-click in the *Profile* panel (see Figure 5-14) and select *Query From Profile*.

5.0.21 Set the threshold of the p-values to '*< 0.05*' and press *<OK>*.

All entries having a p-value of less than 0.05 are selected (see Figure 5-15)

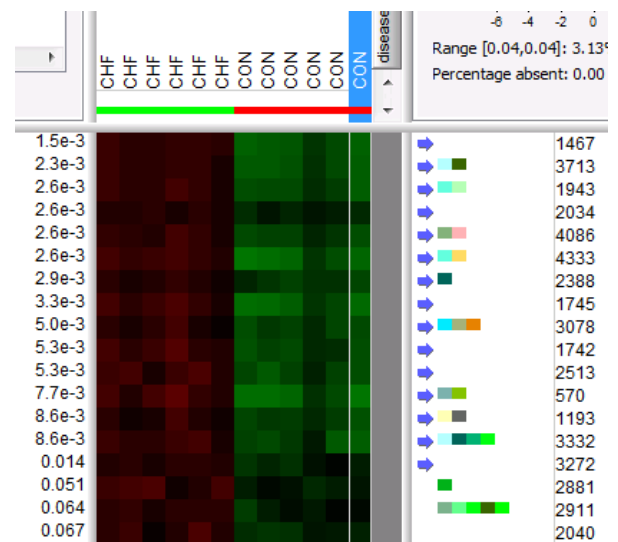


Figure 5-15. Selected row entries.

