

GeneMaths XT

Loop design

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1. Import

1.1 Introduction

Mice that lack the aquaporin-1 gene (AQP-1) are unable to generate a hypertonic medullary interstitium in their kidneys. Mc. Reynolds et al.⁽¹⁾ investigated the gene expression profiles of wild-type mice and AQP-1 null mice.

In a first step total RNA was isolated from the medullas of 3 wild type mice and 3 aquaporin-1 null mice. Total RNA from each sample was amplified before labelling in the cDNA reaction. The hybridization scheme was based on a loop design: each sample from an individual mouse was hybridized to an array four times, generating four independent replicate measures of that RNA sample.

This example dataset will be used in order to explain the workflow of GeneMaths XT. This dataset is publicly available on the GEO website.

1.2 Downloading the data

1.2.1 Go to the GEO homepage: <http://www.ncbi.nlm.nih.gov/geo> ('Gene Expression Omnibus'). Click in the box next to 'Query > GEO accession' and type GSE2402.

1.2.2 Press <Go>.

1.2.3 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.

1.2.4 On the next page select **GSE2402_family.soft.gz**.

1.2.5 Select <Save> and navigate to the path on your computer.

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

(1) McReynolds et al., Renal medullary gene expression in aquaporin-1 null mice, *Am J Physiol Renal Physiol*, 288:315-321, 2005.

1.3 Importing the data in GeneMaths XT

1.3.1 Start GeneMaths XT by double clicking on the icon



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

1.3.2 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).

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

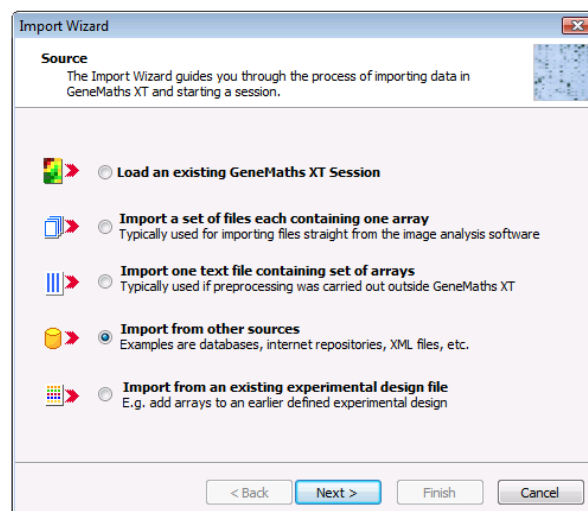


Figure 1-2. Import wizard: select data source.

1.3.4 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).

1.3.5 Click <Next>.

1.3.6 Browse for the file in the *File* panel. Leave the top two panels empty (see Figure 1-4).

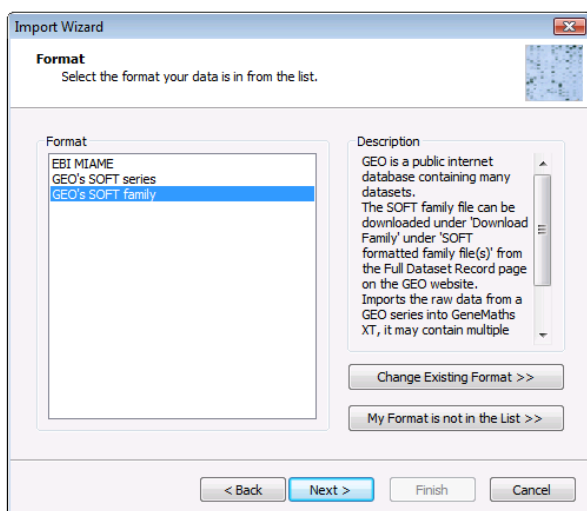


Figure 1-3. *Import wizard: select format.*

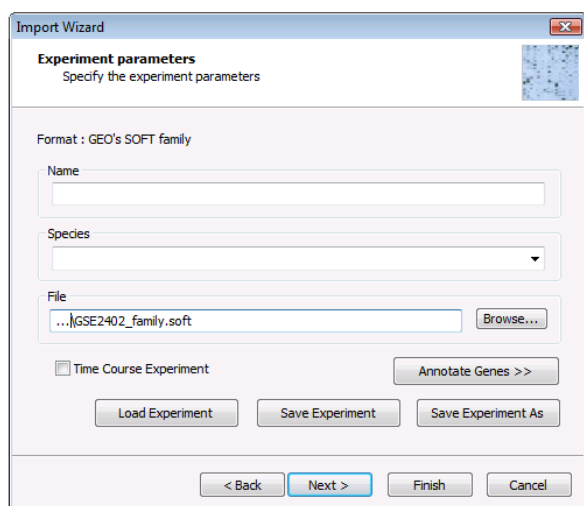


Figure 1-4. *Import Wizard: Input file.*

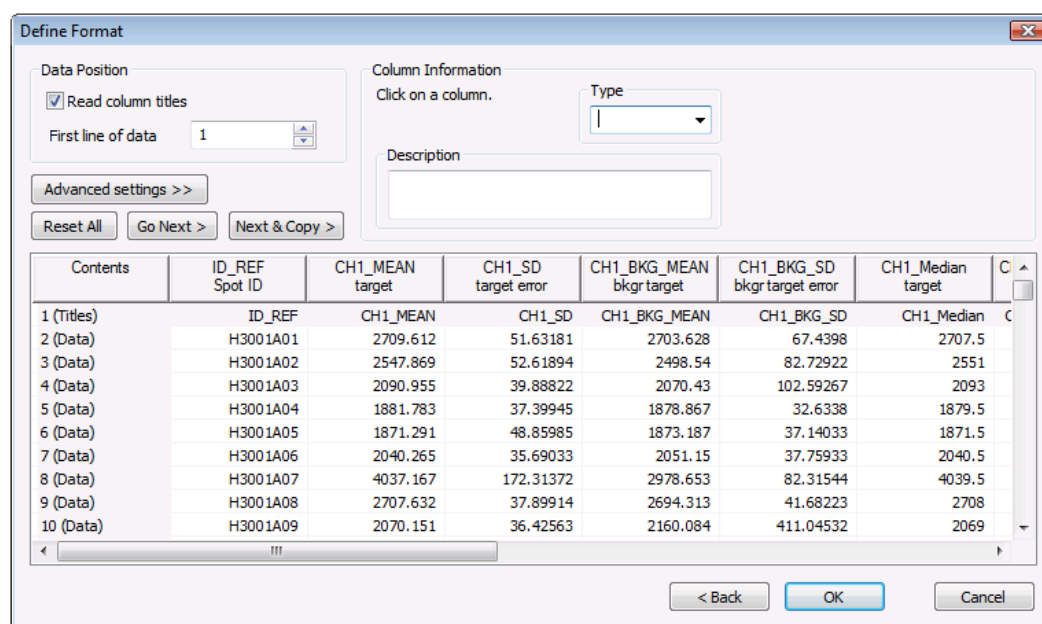


Figure 1-5. *The Define Format dialog box.*

1.3.7 Click *<Next>*.

1.3.8 Specify the name of the processed file e.g. GSE2402.xps.

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

1.3.10 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).

1.3.11 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 **SpotID** in the *Text* box (see Figure 1-6).

1.3.12 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 3-14 as specified in the file. Only for column 2 and 15 the settings need to be changed.

1.3.13 Select the last column and fill out the correct settings (last row in the list on the next page).

1.3.14 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.

1.3.15 Select **ID-REF** and hit *">"*. ID-REF is now placed in the right box (see Figure 1-8).

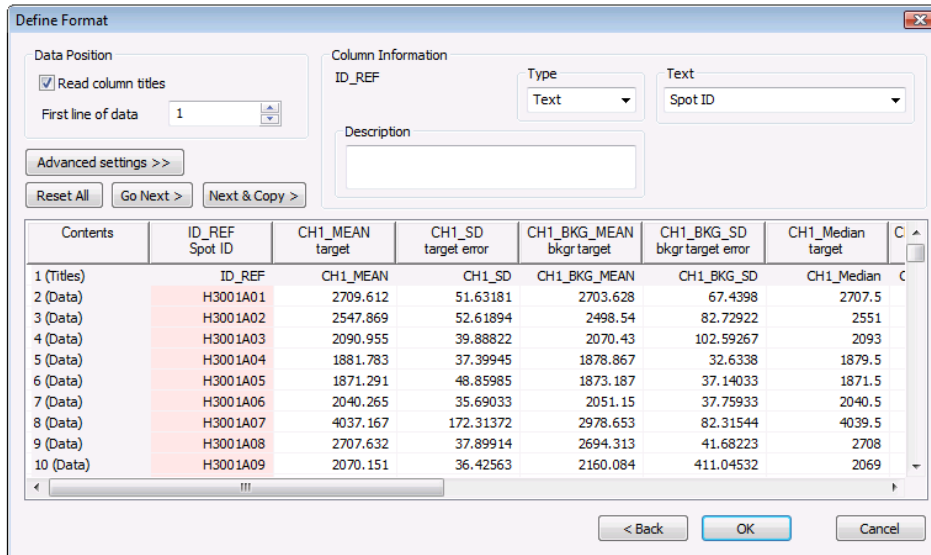


Figure 1-6. Settings for the second column.

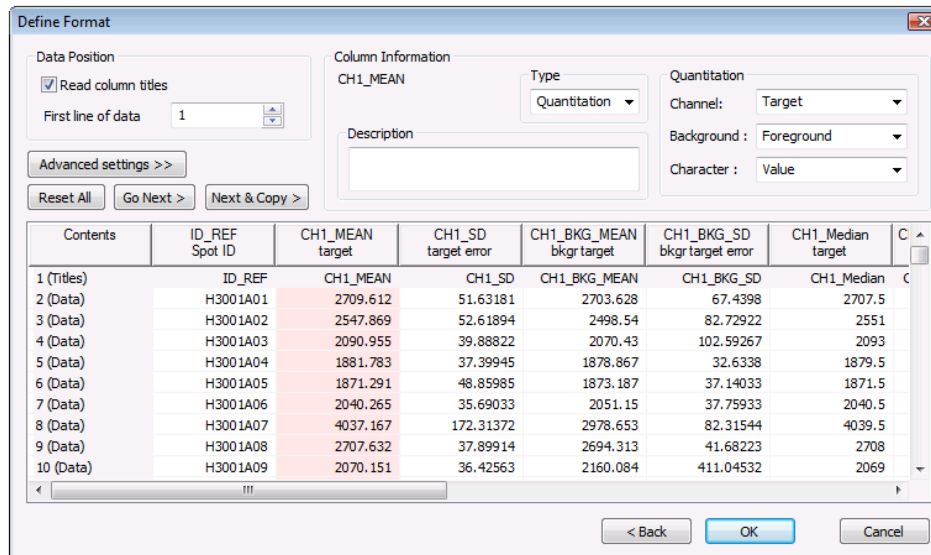


Figure 1-7. Settings for the third column.

Column	Type	Quantitation
4 (CH1_SD)	Quantitation	Target Foreground Error
5 (CH1_BKG_MEAN)	Quantitation	Target Background Value
6 (CH1_BKG_SD)	Quantitation	Target Background Error
7 (CH1_MEDIAN)	Quantitation	Target Foreground Value
8 (CH1_BKG_MEDIAN)	Quantitation	Target Background Value
9 (CH2_MEAN)	Quantitation	Reference Foreground Value
10 (CH2_SD)	Quantitation	Reference Foreground Error
11 (CH2_BKG_MEAN)	Quantitation	Reference Background Value
12 (CH2_BKG_SD)	Quantitation	Reference Background Error
13 (CH2_MEDIAN)	Quantitation	Reference Foreground Value
14 (CH2_BKG_MEDIAN)	Quantitation	Reference Background Value
15 (VALUE)	Quantitation	Ratio Foreground Value

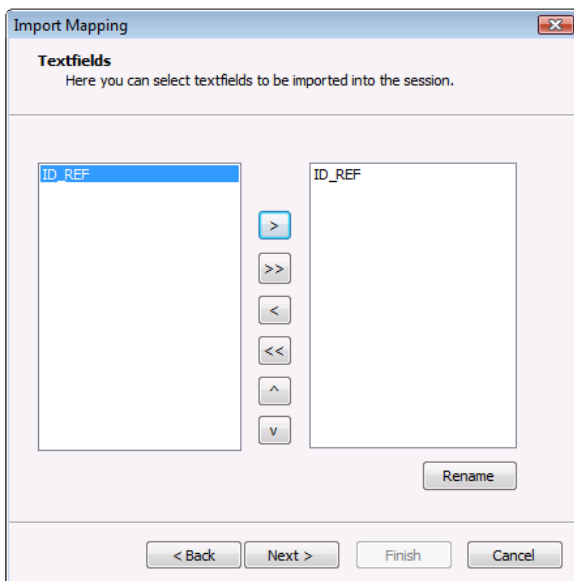


Figure 1-8. The *Import Mapping* dialog box: step 1.

1.3.16 Click <Next>.

1.3.17 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 1-9).

1.3.18 In the *Quantitations* panel, use the **MEDIAN** quantitations as depicted in Figure 1-9 and the standard deviations ('SD') as their error values. Use the pull down menu to select the predefined signals.

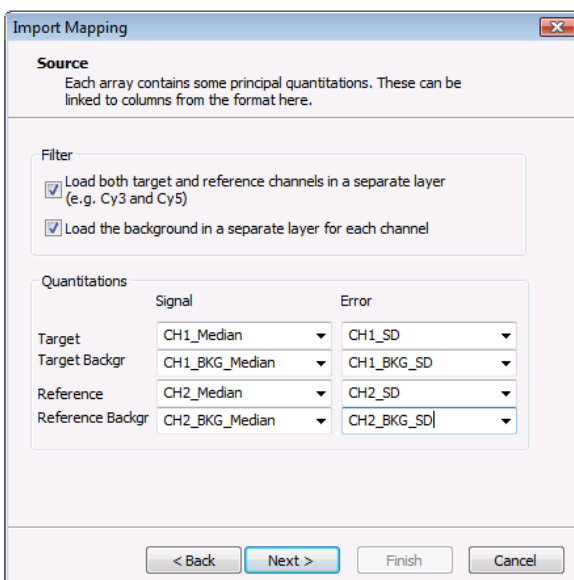


Figure 1-9. *Import Mapping*, step 2.

1.3.19 In the next step of the import, you can define an extra quantitation. In this exercise, we do not need an extra layer.

1.3.20 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 4 layers (displayed in the top left panel), 9 row identifiers and 4 column identifiers (Figure 1-10 and Figure 1-11).

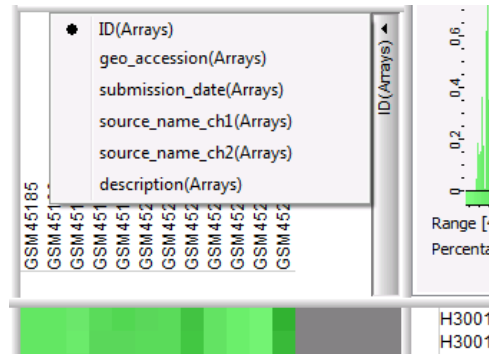


Figure 1-10. 6 column identifiers.

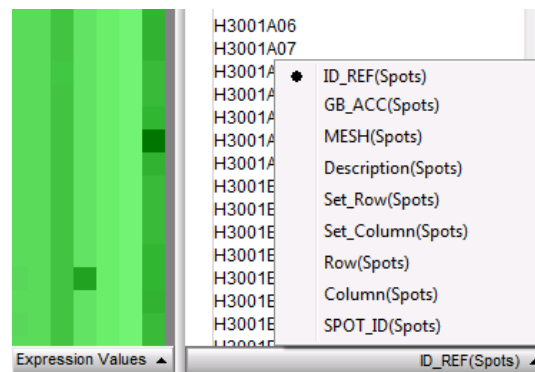



Figure 1-11. 9 row identifiers.

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

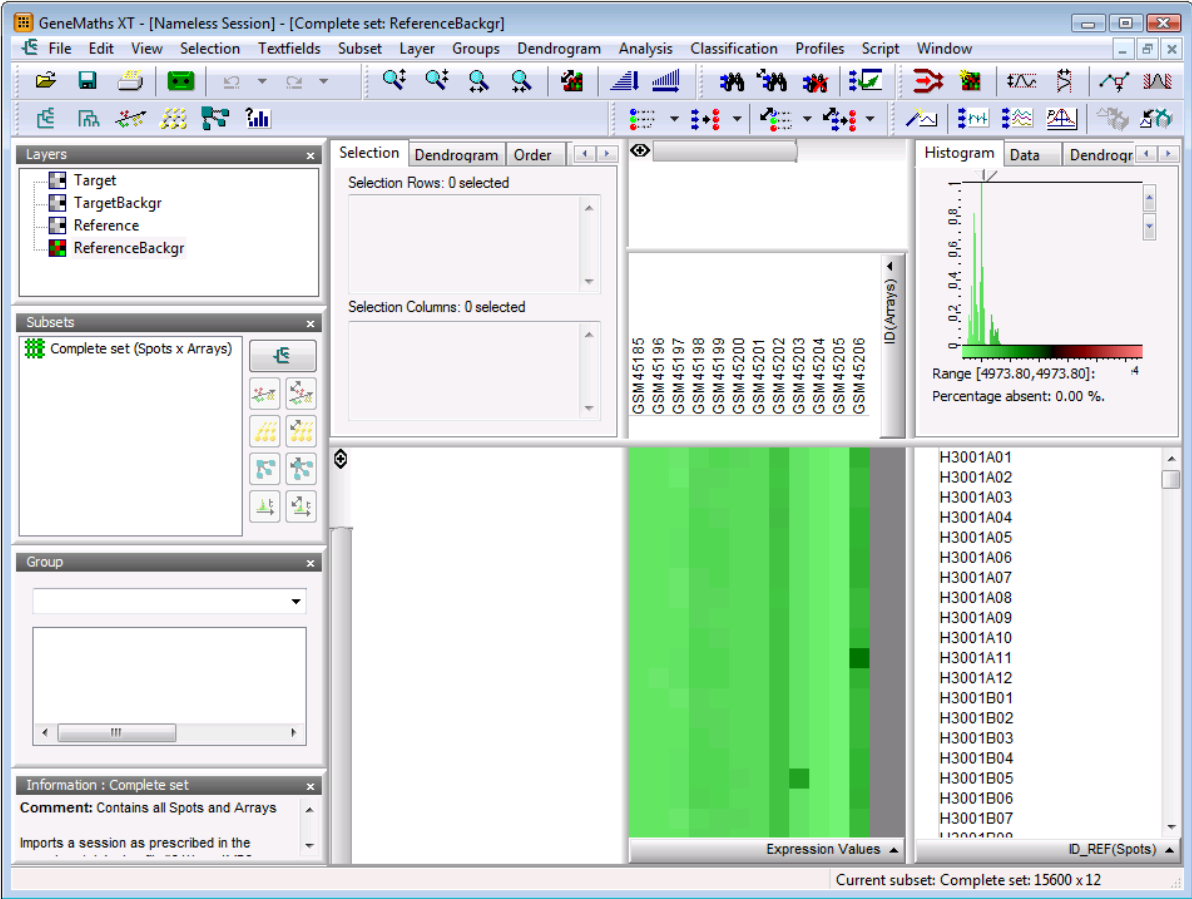


Figure 1-12. The Main window of GeneMaths XT after import of the data.

2. Annotation

The annotation is automatically done via the import of the file. A file called 'loopannot.txt' contains additional array information for this dataset and can be found on our website.

2.0.1 Select 'Download' in the menu bar of our home page (www.applied-maths.com) and select 'Manuals & Tutorials'. Scroll down the page and select 'GeneMaths XT Tutorials'. In the new window, click with your right mouse button on 'Array Annotations' next to 'Loop Design' and select 'Save Target As...'. Save the file as a text file in a folder on your computer.

2.0.2 Select *Textfields > Import*.

2.0.3 Select the **Arrays** aspect and navigate to the annotation file in the *File* panel.

2.0.4 Use the **ID** field in the session and the **ID** column in the file to link the data (see Figure 2-1). Select

Ch1Type and **Ch2Type** while holding the SHIFT button. Press <OK>.

2.0.5 Click on the column identifier tab in the *Main* view. The new information fields are added to the list of identifiers (red rectangle).

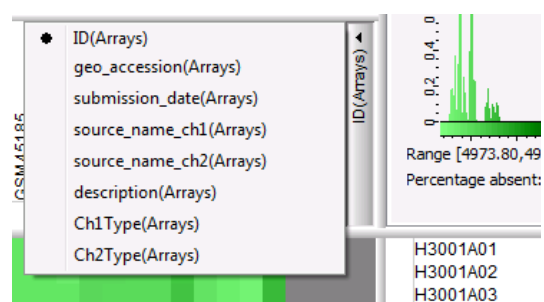


Figure 2-2. New column identifiers.

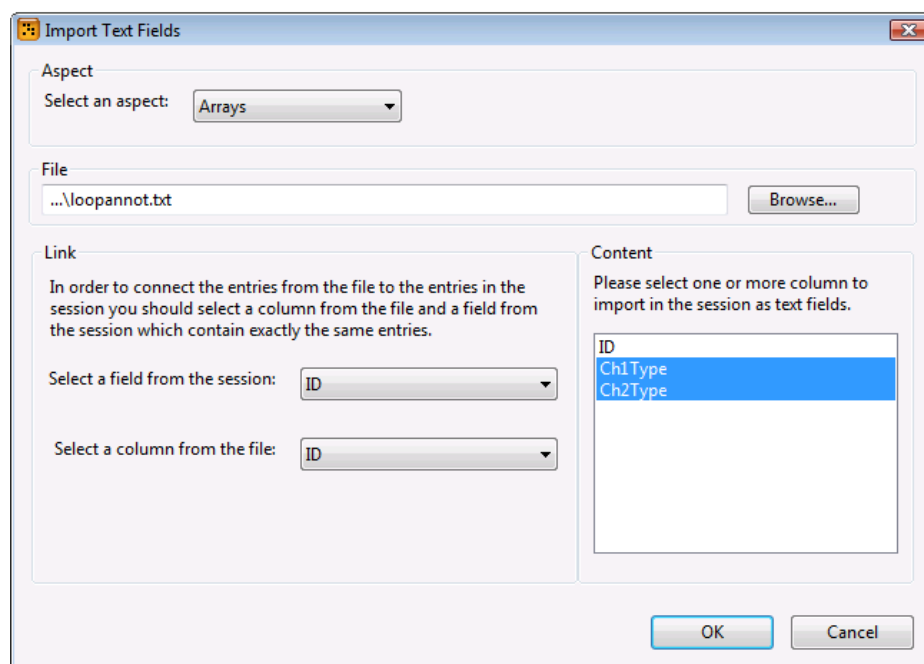


Figure 2-1. Adding new information fields to the list of column identifiers.

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.

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

3.0.2 In the next window, select **Ch1Type** from the *Name* pull down menu and click *<OK>* (see Figure 3-1).

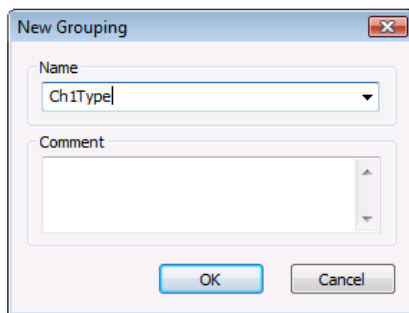


Figure 3-1. Grouping from Ch1Type.

3.0.3 Ch1Type is selected as the text field in the next window (see Figure 3-2). Uncheck ALL the limitations and click *<OK>*.

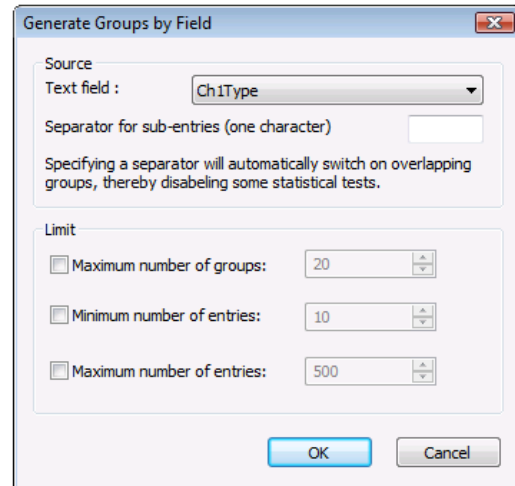


Figure 3-2. Creating groups based on Ch1Type.

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

3.0.5 Repeat the previous steps for **Ch2Type** (see Figure 3-4).

3.0.6 Press *<Exit>*.

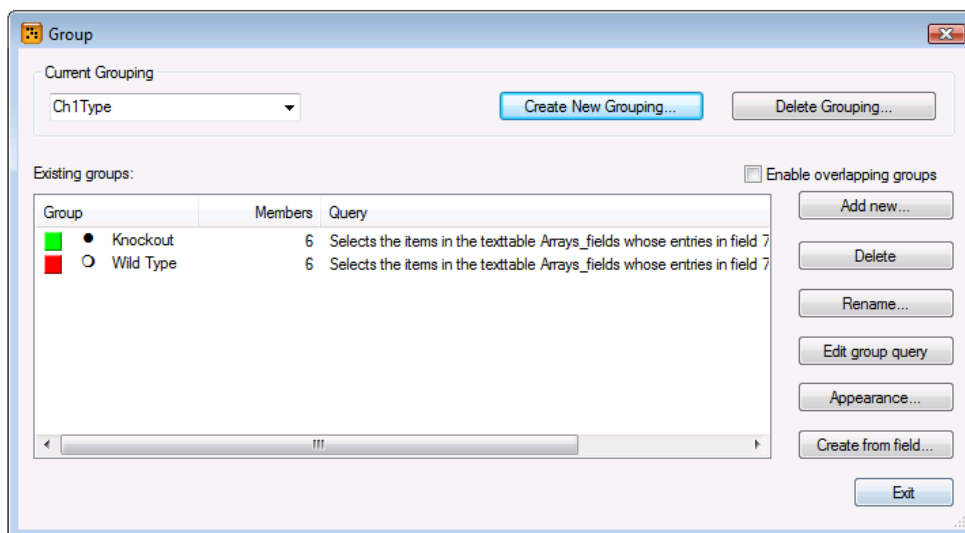


Figure 3-3. Groups based on Ch1Type.

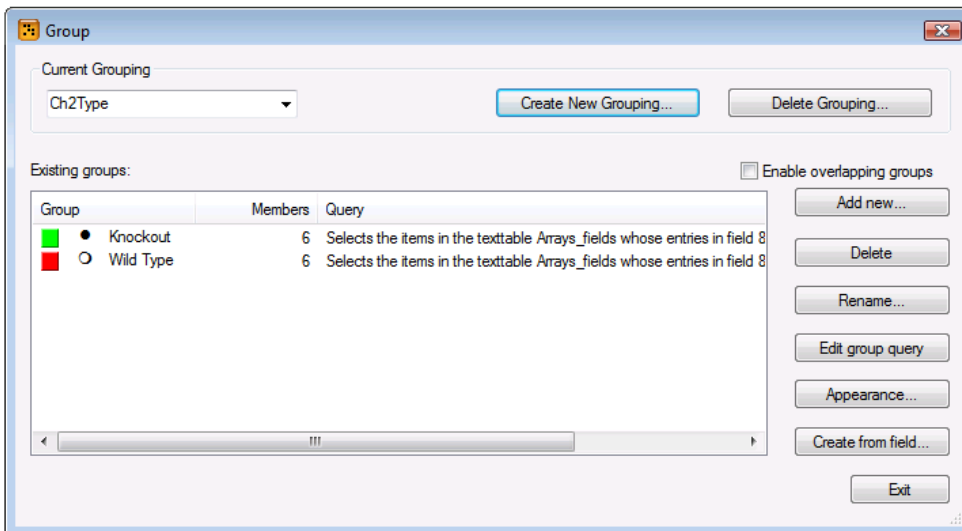


Figure 3-4. Groups based on Ch2Type.

4. Visualizing the loop design

GeneMaths XT offers a nice visualization tool to show the design of your arrays.

4.0.1 Select *View > Draw Design*.

4.0.2 Select the array fields containing the sample names for the Cy5 channel (red, target) and the Cy3 channel (green, reference); `Sample_source_name_ch1` and `Sample_source_name_ch2` respectively. Select the two groupings corresponding to each channel and press `<OK>` (see Figure 4-1).

In the next window, the set-up of the microarray is displayed: each sample (3 WT mice and 3 KO mice) is measured four times (two times labeled with a red dye and two times labeled with a green dye).

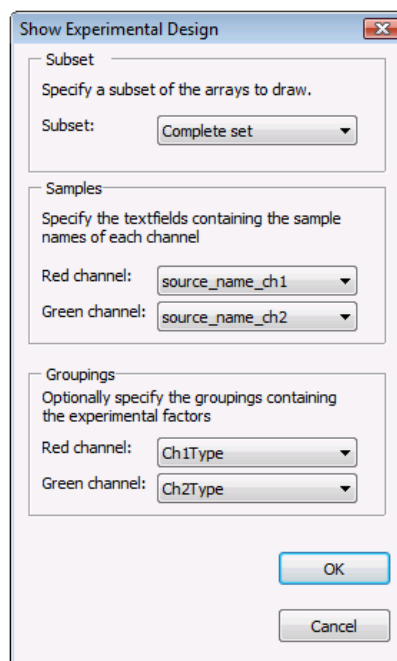


Figure 4-1. Settings for displaying the experimental design.

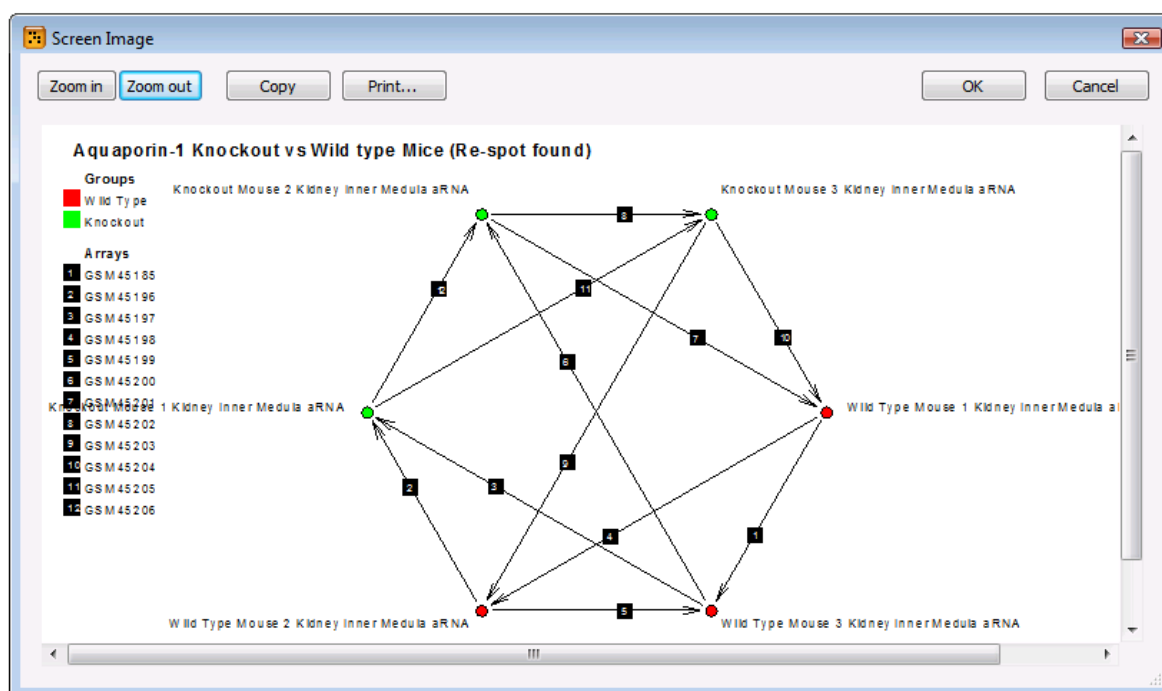


Figure 4-2. Graphical representation of the loop design.

5. Preprocessing

Prior to the analysis of the data, thorough preprocessing steps need to be performed. In these preprocessing steps, sources of variation from the measured expression values are removed.

5.1 Sources of variation

First we are going to look at the data and its major sources of variation by creating an ANOVA report. ANOVA models the measured expression level of each gene as a linear combination of the major sources of variation.

5.1.1 Select *Layer > Normalization > Anova Report*. Fill out the settings as depicted in Figure 5-1.

5.1.2 Press **<OK>**. The ANOVA report is displayed (see Figure 5-2).

The SS-value describes for each effect its contribution to the global variation in the experiment. From the obtained ANOVA table we can see that all effects contribute to the variation. In the preprocessing steps we are going to remove most sources of variation.

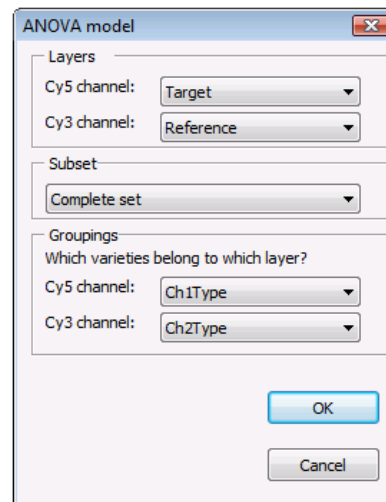


Figure 5-1. Settings for ANOVA.

5.2 Background correction

First we are going to correct the target and reference layer for non specific background noise.

5.2.1 Select *Layer > Data Preparing > Background Correction*. Select the target layers and use the subtract algorithm (see Figure 5-3). Store the corrected values in layer **Tar_BG_corr**.

5.2.2 Press **<OK>**.

Source	SS	df	MS
Array	2.26401e+011	11	2.05819e+010
Dye	7.29041e+011	1	7.29041e+011
Array*Dye	2.83209e+011	11	2.57463e+010
Gene	2.42757e+012	15599	1.55623e+008
Spot	9.35145e+011	171589	5.44991e+006
Variety*Gene	4.65402e+010	15599	2.98354e+006
Dye*Gene	9.89021e+010	15599	6.34028e+006
Residual	4.35017e+011	155990	2.78875e+006
Total	5.18182e+012	374399	

μ	=	The overall average signal
A	=	Array effect
D	=	Dye effect
AD	=	Array-dye effect
G	=	Gene effect
VG	=	Variety-gene effect
AG	=	Array-gene effect or spot effect
DG	=	Dye-gene effect
SS	=	Sums of Squares
df	=	Degrees of freedom
MS	=	Mean Squares (= SS/df)

Figure 5-2. ANOVA report.

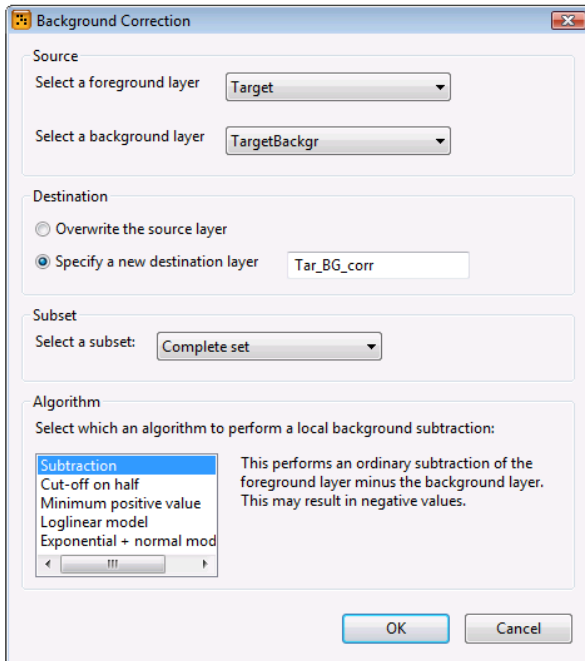


Figure 5-3. Background correction for the target layers.

5.2.3 Apply background correction to the reference layer. Select *Layer > Data Preparing > Background Correction* and use the settings as depicted in Figure 5-4 and store the corrected values in a new layer called **Ref_BG_corr**.

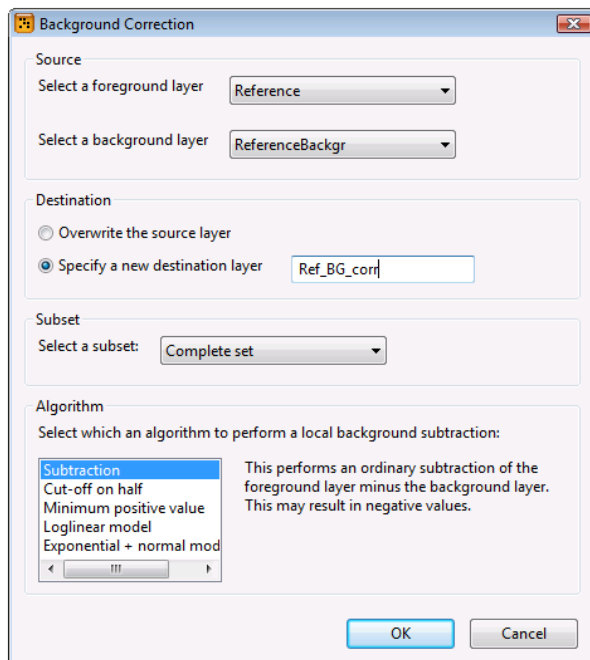


Figure 5-4. Background correction for the reference layers.

5.3 Variance stabilization

The variance stabilization method (vsn) is a method to preprocess DNA microarray intensity data. This transformation is equivalent to the natural logarithm in the high-intensity range and to a linear transformation in the low-intensity range. In an intermediate range, the 'arsinh' function interpolates smoothly between the two. An advantage of vsn-transformation over log-transformation is that vsn works also on values that are negative after background subtraction.

5.3.1 Call the variance stabilization R-routine (Bioconductor) with *Layer > Data Preparing > Variance Stabilization*.

5.3.2 Fill in the settings as shown in Figure 5-5 and press **<OK>**.

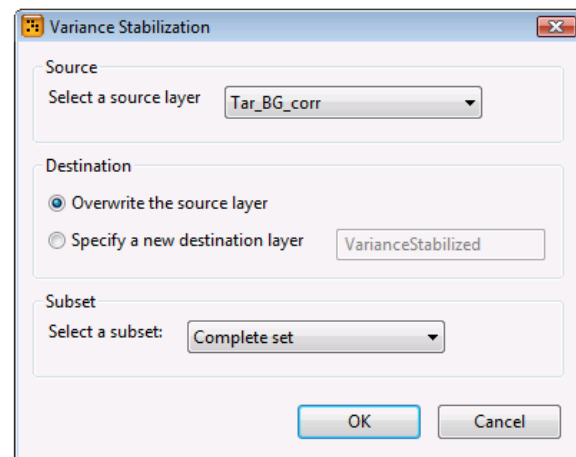


Figure 5-5. Variance stabilization.

5.3.3 Repeat the previous step for the **Ref_BG_corr** layer.

5.3.4 Select *Layer > Normalization > Anova Report*. Fill out the settings as depicted in Figure 5-6.

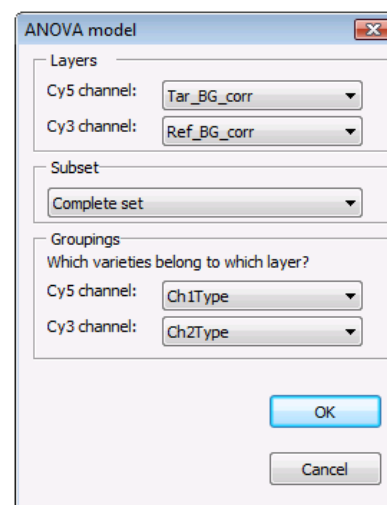


Figure 5-6. Settings for ANOVA.

5.3.5 Press **<OK>**. The ANOVA report is displayed.

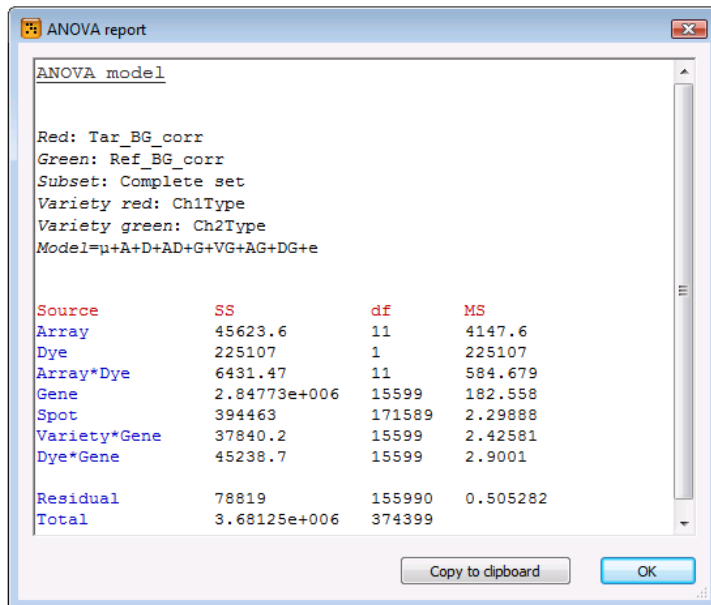


Figure 5-7. ANOVA report.

From the obtained ANOVA table (Figure 5-7) we can see that the contribution of all of the effects has dropped compared to our first ANOVA report (Figure 5-2). The Gene effect has the highest impact. The Spot effect (= spots of a gene may contain a different amount of cDNA on the different arrays) has the second highest impact followed by the Array effect.

5.4 Normalization

A. Analyzing the data

5.4.1 Select **Profiles > Plot Wizard**. Select **Columns** as orientation and because we want different layers to plot against each other, select **One column, different layers** and click **<Next>**.

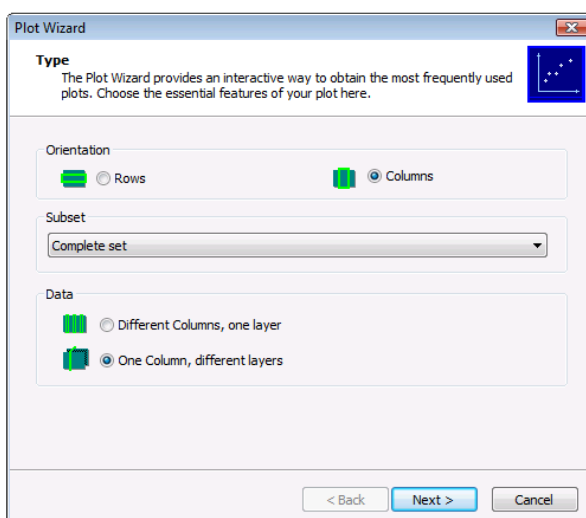


Figure 5-8. Plot Wizard: step 1.

5.4.2 In the next window select **Entries**. Select all entries and click **<Next>** to move to the next step.

NOTE: To select all entries select the first entry scroll down the list and select the last entry while holding the CTRL button.

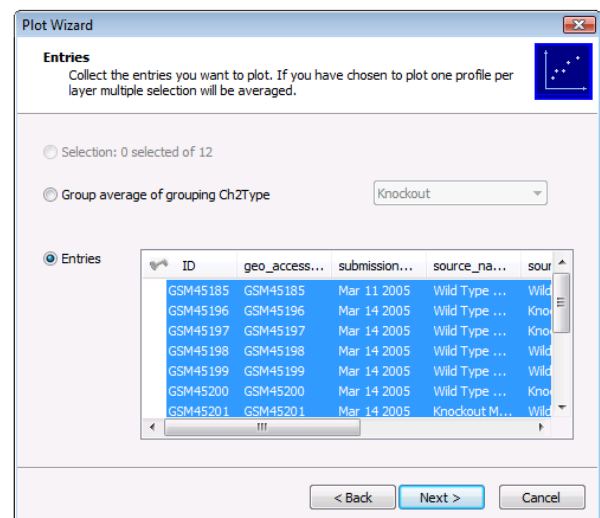


Figure 5-9. Plot Wizard: step 2.

5.4.3 In the third window, select the two transformed layers **Tar_BG_corr** and **Ref_BG_corr**. Click **>** and click **<Next>**.

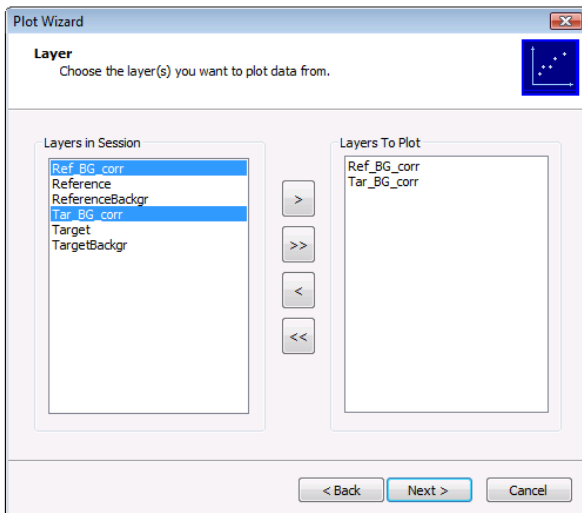


Figure 5-10. Plot Wizard: step 3.

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

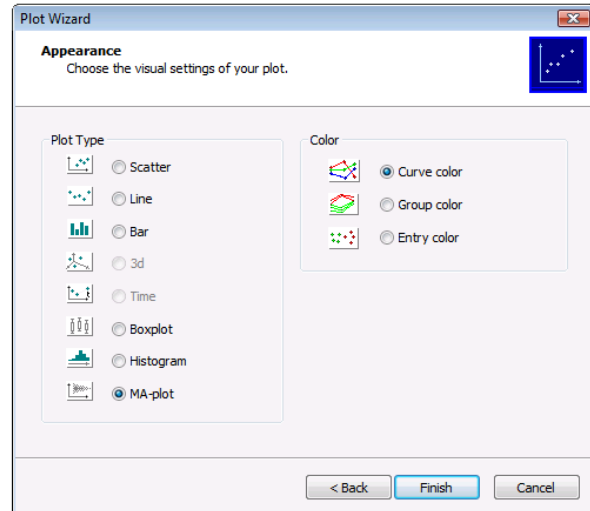


Figure 5-11. Plot Wizard: final step.

The plot appears in GeneMaths XT. We need to set the X and Y axes correctly for the plot.

5.4.5 Select the **Ref_BG_corr** layer in the *Profiles* panel. Click right and choose *Set as y-axis* (see Figure 5-12).

5.4.6 Select the **Tar_BG_corr** layer and choose *Set as x-axis*.

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

5.4.8 Select *Profiles > Selected Profile > Lowess Plot*. Press **<OK>** in the next window. The plot should now look like the one in Figure 5-13.

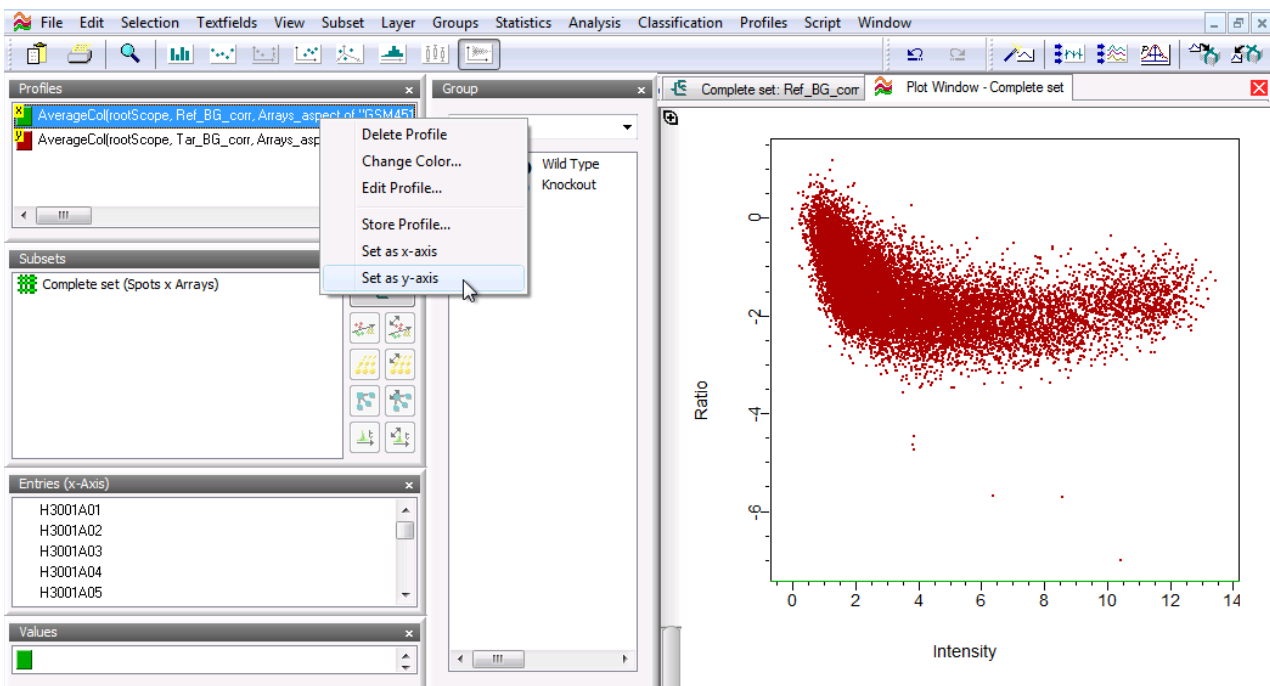
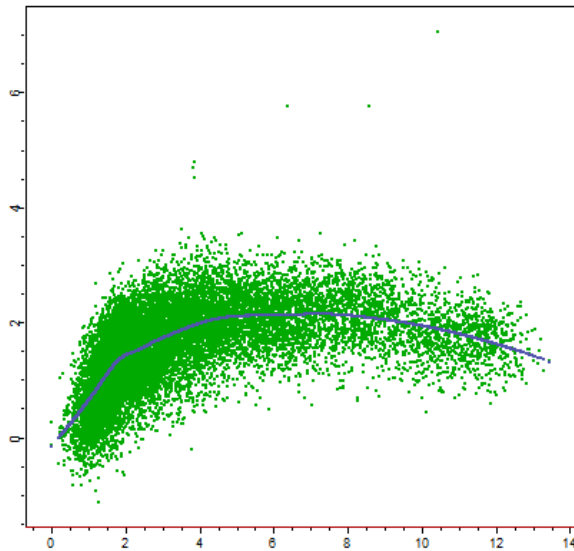


Figure 5-12. MA-plot.



rageCol(rootScope, Tar_BG_corr, Arrays_aspect of "GSM45185", Arrays_aspect of "GSM...

Figure 5-13. MA-plot with the correct axes.

The MA-plot (see Figure 5-13) shows that at a certain average level of intensities, the ratio M approximates a certain constant level. However, some deviation in 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.

B. Lowess normalization

5.4.9 Select *Layer > Normalization > Lowess*. Select the layers with the transformed intensities (Figure 5-14).

5.4.10 Click <OK> and keep the default settings in the next dialog.

5.4.11 Click <OK>.

5.4.12 Select the first profile in the *Profiles* panel. Click right and choose *Edit Profile*. Press <OK>.

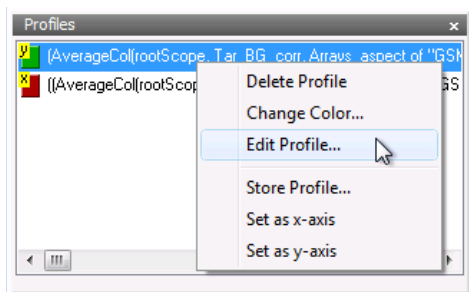


Figure 5-15. *Edit Profile*.

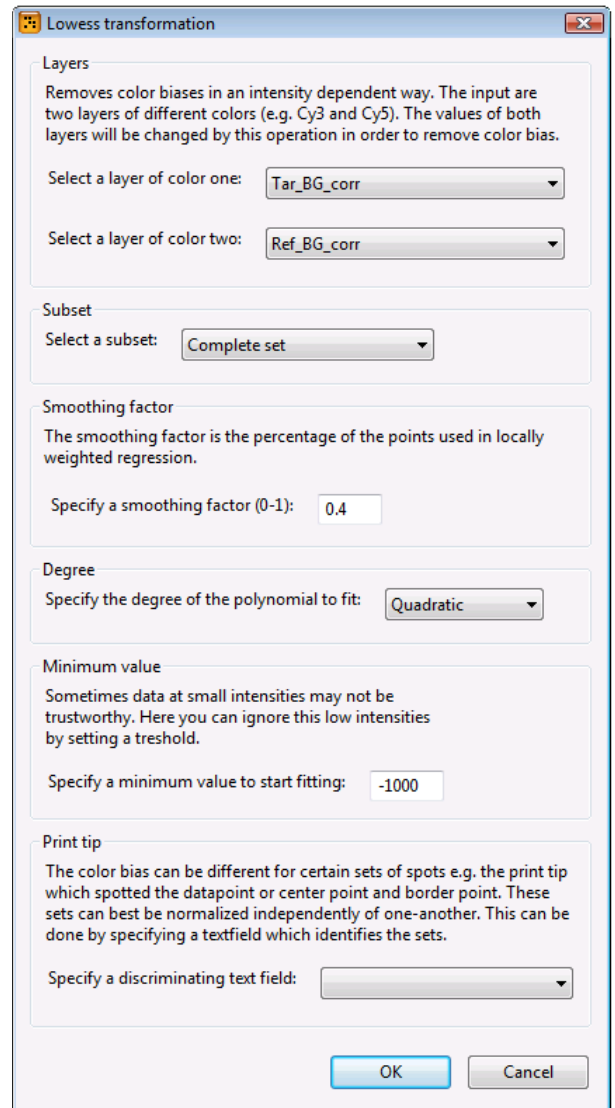


Figure 5-14. Lowess Normalization settings.

5.4.13 Repeat the previous step for the other profile in the *Profile* panel. The plot should now look like Figure 5-16. There is a compensation for the intensity dependent effect. Lowess thus allows for both normalization and linearization of the data.

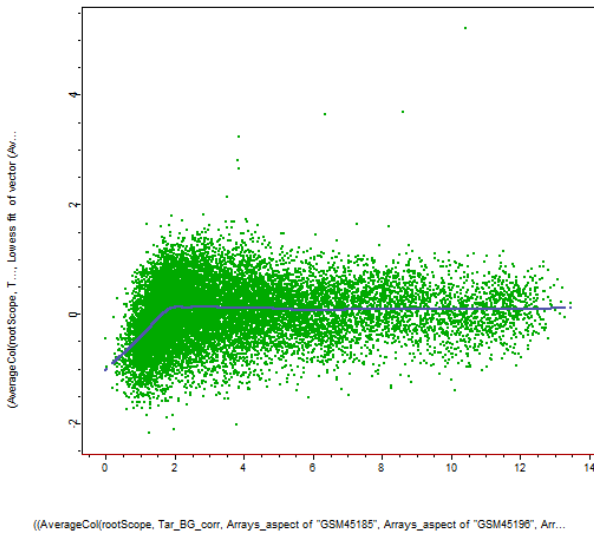
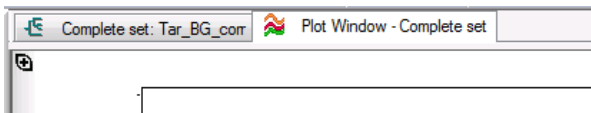


Figure 5-16. The MA-plot after Lowess normalization.

5.4.14 Go back to the *Main* window by clicking on the *Complete set* tab (see screenshot below).



5.4.15 Select *Layer > Normalization > Anova report*. Fill out the settings as depicted in Figure 5-17.

5.4.16 Press **<OK>**. The ANOVA report is displayed (Figure 5-18).

From the obtained ANOVA table (Figure 5-18) we can see that the effects of Dye and Array-dye have dropped substantially compared to Figure 5-7. By normalizing the genes we have compensated for the dye effect and subsequently also for the cross effect of array and dye.

ANOVA model

Red: Tar_BG_corr
Green: Ref_BG_corr
Subset: Complete set
Variety red: Ch1Type
Variety green: Ch2Type
Model= $\mu+A+D+AD+G+VG+AG+DG+e$

Source	SS	df	MS
Array	45623.2	11	4147.56
Dye	0.169905	1	0.169905
Array*Dye	1.13749	11	0.103409
Gene	2.84773e+006	15599	182.558
Spot	394463	171589	2.29888
Variety*Gene	34257.6	15599	2.19614
Dye*Gene	26621	15599	1.70658
Residual	73317.3	155990	0.470012
Total	3.42201e+006	374399	

Copy to clipboard OK

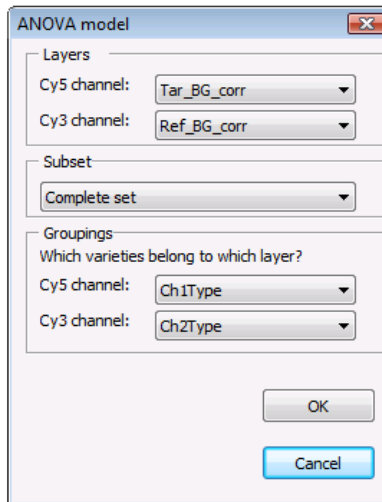


Figure 5-17. Settings for ANOVA.

C. Array normalization

In the next step we are going to normalize our two layers.

5.4.17 Select *Layer > Normalization > Arrays*.

5.4.18 Select the **Tar_BG_corr** layer and store the normalized array in a new layer called **Normalized_Tar**. Do not change the rest of the settings and press **<OK>** (see Figure 5-19).

5.4.19 Repeat the previous step for the **Ref_BG_corr** layer and store the layer as **Normalized_Ref**.

The histograms of the new layers **Normalized_Tar** and **Normalized_Ref** are now centered around zero.

Figure 5-18. ANOVA report.

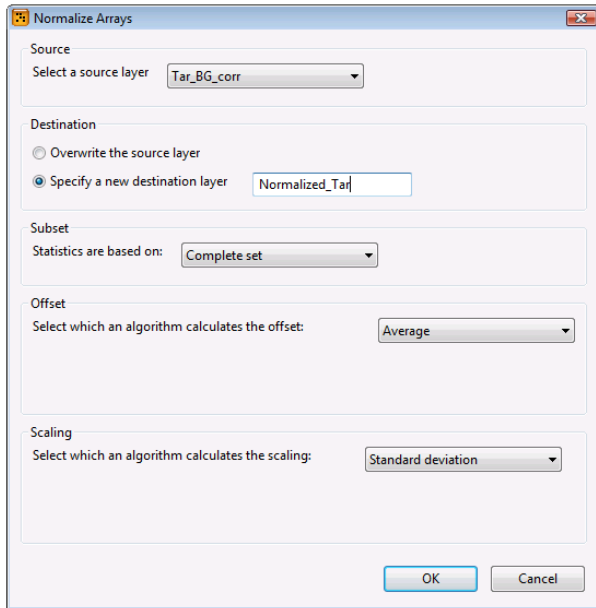


Figure 5-19. Settings for the normalization of the arrays.

5.4.20 Select *Layer > Normalization > Anova report*. Fill out the settings as depicted in Figure 5-20.

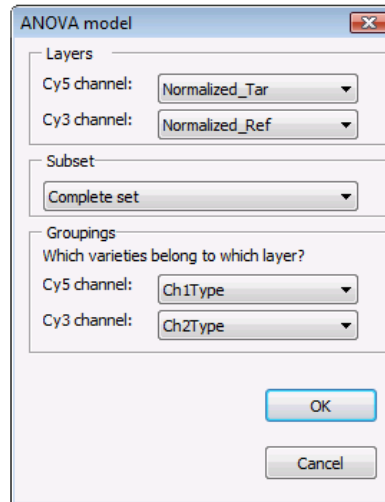


Figure 5-20. Settings for ANOVA.

5.4.21 Press **<OK>**. The ANOVA report is displayed (Figure 5-21).

From the obtained ANOVA table (Figure 5-21) we can see that the Array, the Dye and Array-dye effects have dropped significantly compared to the previous ANOVA report (Figure 5-18). With these dye and array effects reduced to the minimum, we can start a comparison knowing that **the major source of variation will be the expression level of the different genes.**

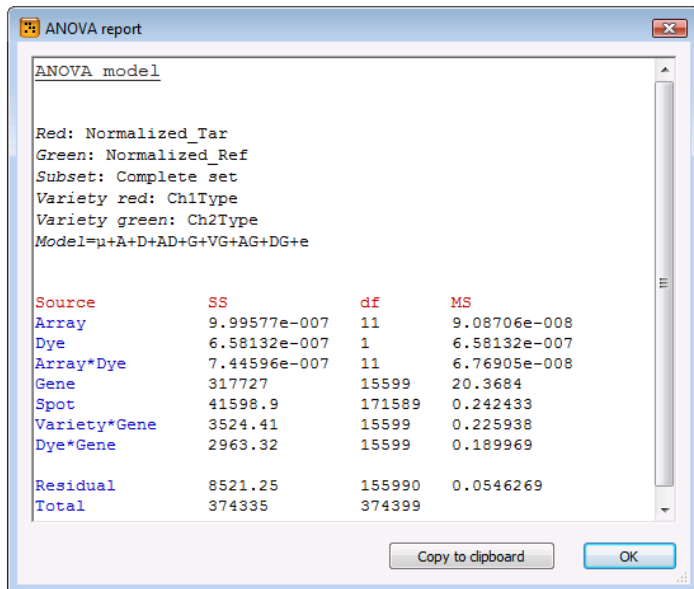


Figure 5-21. ANOVA report.

6. Statistics & Analysis

Now we have a starting point for our statistical analysis. We are going to perform a LIMMA-test to find differentially expressed genes between the wild type and the knockout mice.

6.0.1 Select *Profiles* > *Statistics Wizard*. Leave the orientation on Rows and press <Next> (see Figure 6-1).

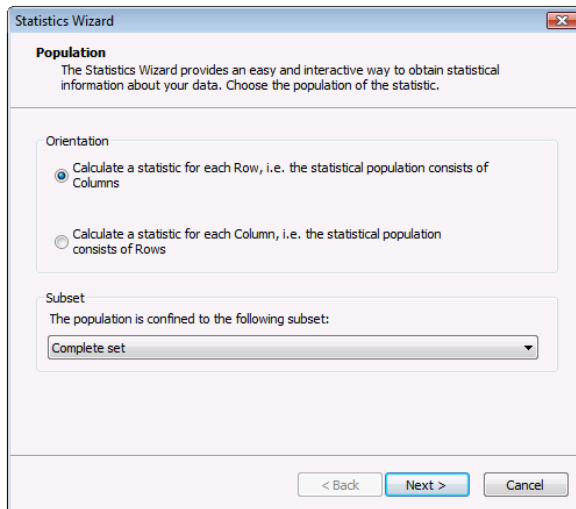


Figure 6-1. *Statistics Wizard*, step 1.

6.0.2 Select **General LIMMA** from the submenu 'Independent test (two groups)'.
LIMMA will tests whether the mean of the two groupings 'Wild type' and 'Knockout' in the two-color design are equal or not.

LIMMA will tests whether the mean of the two groupings 'Wild type' and 'Knockout' in the two-color design are equal or not.

6.0.3 Click <Next> (see Figure 6-2).

6.0.4 In the next window, make sure that **Normalized_Ref** is selected and the two different groups in the Ch2Type-grouping. Select **p-value** as output and click <Next> (see Figure 6-3).

6.0.5 In the next window, select **Normalized_Tar** in the *Layer* panel, **source_name_ch2** (cy3) and **source_name_ch1** (cy5) in the *Samples* panel and **Ch1Type** as grouping for the transformed target layer (Figure 6-4).

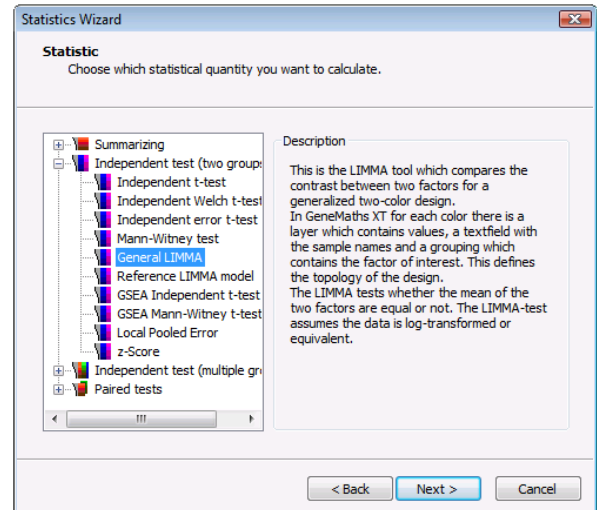


Figure 6-2. *Statistics Wizard*, step 2: selecting the test.

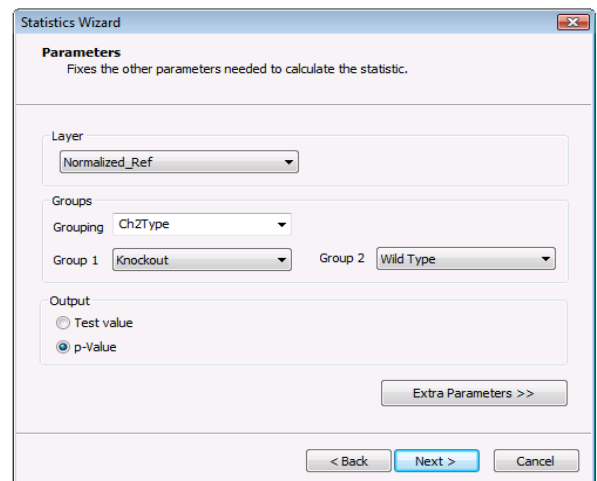


Figure 6-3. *Statistics Wizard*, step 3.

6.0.6 Do not use a correction for multiple testing in the fourth step.

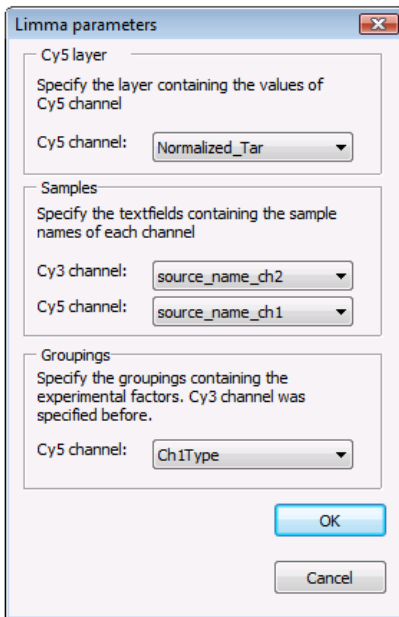


Figure 6-4. Statistics Wizard, step 4.

6.0.7 Click <Finish>.

6.0.8 Select the newly created profile in the *Profiles* tab. Click right on the profile name (see Figure 6-5) and select *Sort From Profile*.

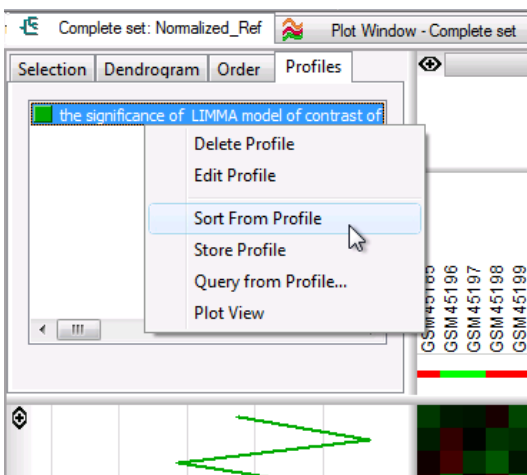


Figure 6-5. Sort From Profile.

6.0.9 Click right on the *Profile* panel and select *Show as Numbers* (see Figure 6-6).

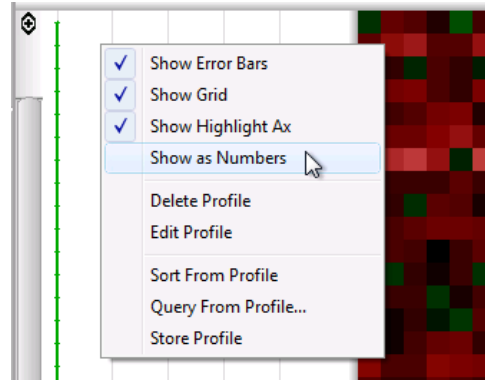


Figure 6-6. Show as Numbers.

In the *Profile* panel, the p-values for the genes are shown. These p-values give an indication if the mean of the two groupings (Wild type and Knockout) are the same. Row entries having a p-value close to 0 indicates that these genes are differentially expressed between the Wild type and Knockout mice. In a next step we are going to select those genes with a p-value smaller than 0.001.

6.0.10 Click right on the profile name in the *Profiles* tab (see Figure 6-5) and select *Query from Profile*.

6.0.11 Set the threshold to < 0.001 and press <OK>.

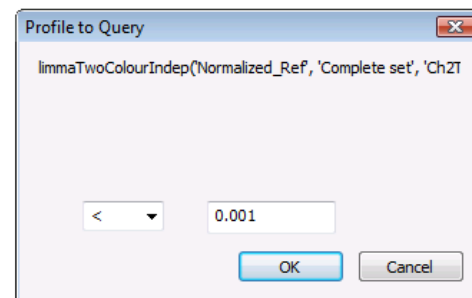
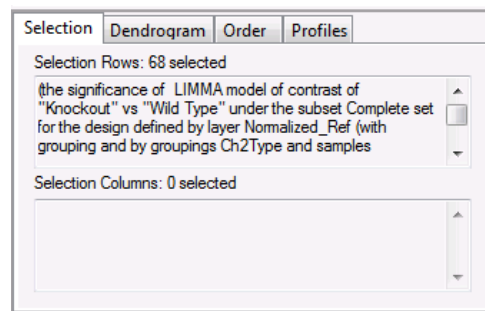


Figure 6-7. Setting a criterion for the test statistic.

6.0.12 Select the *Selection* tab in the *Profile* panel. A short description is given of the selected rows. 85 rows (= genes) with a p-value smaller than 0.001 are selected (blue arrow in the *Main Window*).



Next we are going to store the selection in a new subset and look at the descriptions of the differentially expressed genes.

6.0.13 Select *Subset > Selection to Subset* and give the new subset the name **Differentially expressed**. Press **<OK>**.

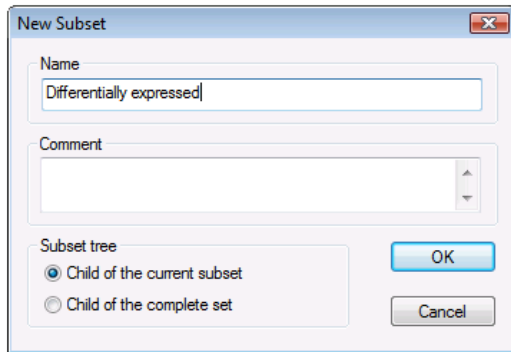
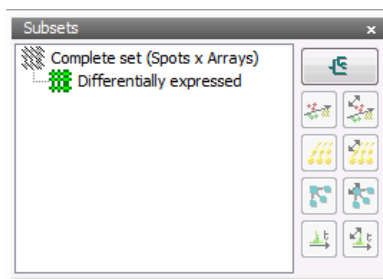



Figure 6-8. Create a new subset.



Next we are going to look which genes of the Differentially expressed subset are downregulated and which ones are upregulated.

6.0.14 Click on the down arrow next to the array groupings button  and select **Ch1Type**. Ch1Type is now set as the current grouping.

6.0.15 Select *Profiles > Plot Wizard*. Select **Columns** for the orientation, **Differentially expressed** as subset and **Different Columns, one layer** in the *Data* panel. Click **<Next>**.

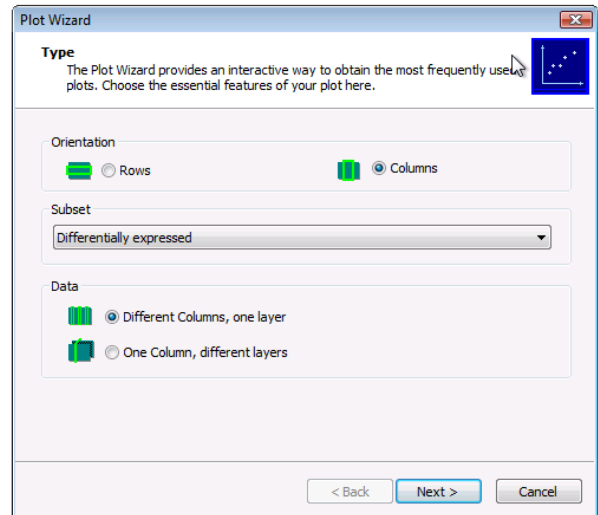


Figure 6-9. Plot Wizard: step 1.

6.0.16 In the next window select **Group average of grouping Ch1Type** and press **<Next>** to move to the next step.

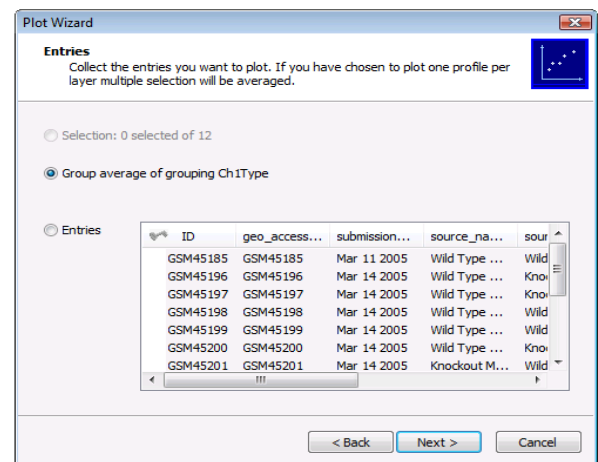


Figure 6-10. Plot Wizard: step 2.

6.0.17 In the third window, select the **Normalized_Tar** layer and click **<Next>**.

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

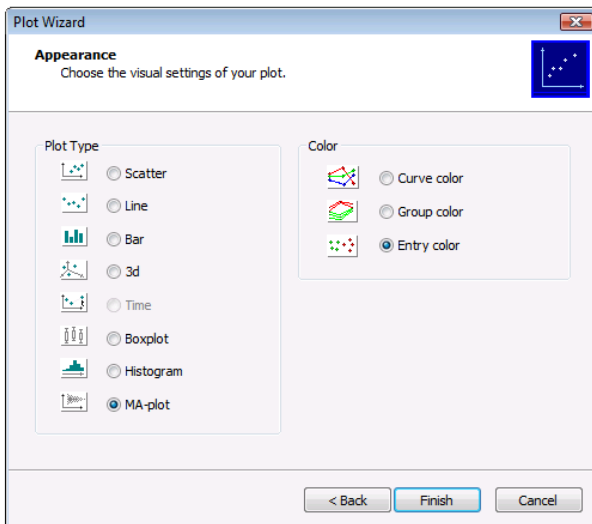
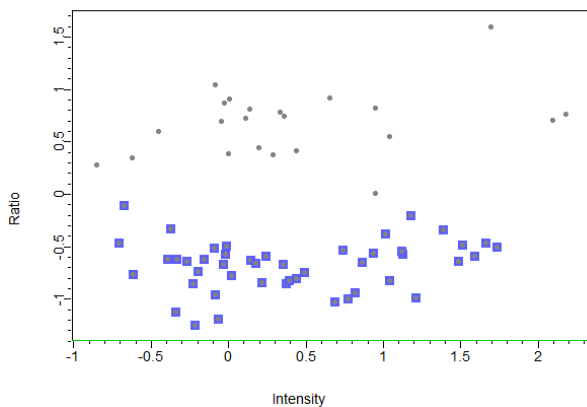


Figure 6-11. Plot Wizard: final step.

6.0.19 Make sure that no entries are selected by pressing F4.

6.0.20 Select all entries below the zero of the Y-axis by pressing the SHIFT button and dragging the left mouse button over the screen.



6.0.21 Select *Groups > From Row Selection* and click *<Add new>*.

6.0.22 In the new window, enter a name for the group, e.g. **Downregulated** and press *<OK>*.

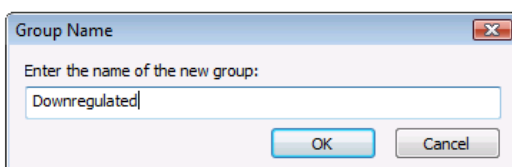


Figure 6-12. Enter a name for the new group.

6.0.23 Press *<Yes>* to confirm the assignment of the new group and press *<Exit>*.

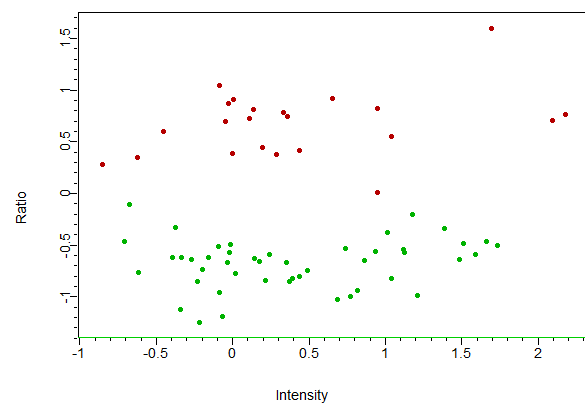
6.0.24 Make sure that no entries are selected by pressing F4.

6.0.25 Select all entries above the zero of the Y-axis.

6.0.26 Select *Groups > Edit Row Groups* and click *<Add new>*.

6.0.27 In the new window, enter a name for the group, e.g. **Upregulated** and press *<OK>*. Press *<Yes>* and press *<Exit>* to close the window.

6.0.28 Make sure that no entries are selected by pressing F4. The plot should now look like the one in the next screenshot:



6.0.29 Select several entries in the plot by holding the CTRL button and clicking with your left mouse button on the entries.

6.0.30 Click on the 'Differentially expressed: Normalized_Tar' tab to go back to the *Main* window.

6.0.31 The selected entries are marked with a blue arrow in the *Row entries* panel. The color codes (upregulated versus downregulated) are also displayed.

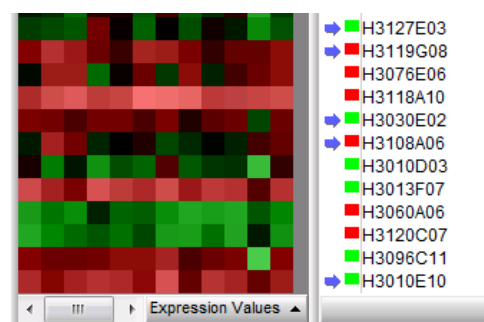


Figure 6-13. Selected entries.

6.0.32 In order to find the up- and downregulated genes in the **Normalized_Ref** layer, repeat steps 6.0.14-6.0.30 with the Ch2Type set as grouping and the Normalized_Ref as layer.