Gene Expression Analysis of an Aging Study Using Illumina® Microarray Technology and Partek® Genomics Suite® 6.6

Overview

Using a complex dataset produced using Illumina Microarray (BeadChip) Technology, this tutorial compares the performance of different ANOVA models, and the impact on both genes lists and biological interpretation.

This tutorial will illustrate how to:

- Import Illumina data into Partek Genomics Suite
- Perform exploratory analysis using the PCA scatter plot
- Perform inferential statistics using ANOVA
- Generate lists of genes of interest
- Understand how different ANOVA models can influence statistical and biological results

Note: the workflow described below is enabled in Partek® Genomics Suite® software version 6.6. Please contact the Partek Licensing Team at licensing@partek.com to request this version or update the software release via *Help* > *Check for Updates* from the main command line. The screenshots shown below may vary across platforms and across different versions of Partek Genomics Suite software.

Description of the Data Set

The dataset used is based on 51 subjects run on the Illumina® Human Ref-8 BeadChip™ platform (Melov et al., 2007). Twenty-six of the subjects were categorized as “Young” with an age range of 18 to 28. The other 25 subjects were categorized as “Old” with an age range of 65 to 84. Skeletal muscle, a type of striated muscle tissue, was obtained via a biopsy from each of the subjects. Total RNA from the skeletal muscle cells was extracted, prepared, and run on BeadChips producing the data that is used with this tutorial. By using the appropriate statistical tools with this data set, it is possible to determine if there are any transcriptomic changes in the skeletal muscle tissue as people age.

Data and associated files for this tutorial can be downloaded by going to *Help* > *On-line Tutorials* from the Partek Genomics Suite software main menu. The data can also be downloaded directly from:
http://www.partek.com/Tutorials/microarray/Gene_Expression/Illumina/Illumina_Age_Data.zip

Importing Illumina® Files and the Data for this Tutorial

Data from Illumina's BeadStudio™ or GenomeStudio™ software package can be exported in a custom report file for seamless importation into Partek Genomics Suite. The BeadStudio™ and GenomeStudio™ plug-in required for the generation of the custom Partek report file, in addition to instructions for its installation, can be found here or by *Help > On-line Tutorials >Microarray > Tutorials > Gene Expression*.

Download the data to your local disk. For this tutorial, the data files are stored in the C:\Partek Training Data\ Illumina_Age_Data folder.

- Select **Gene Expression** from the Workflows panel on the right side of the tool bar in the Partek Genomics Suite main window (Figure 1)

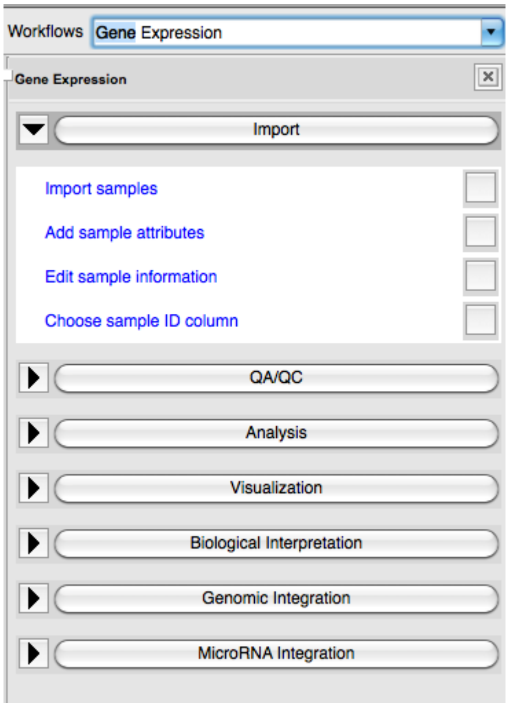
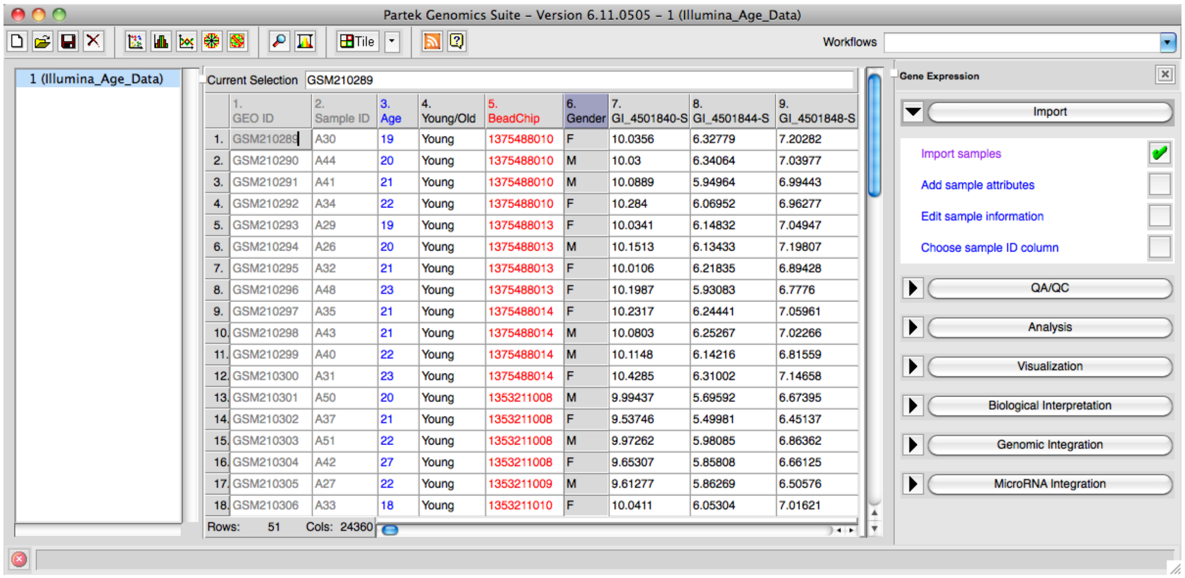


Figure 1: The Gene Expression workflow

- Select **Import samples** under the *Import* section of the workflow
- Select Load a project following Illumina GenomeStudio® export and **OK**
- Using the file chooser, browse to the C:\Partek Training Data\Illumina_Age_Data folder, select *Illumina_Age_Project.ppj* file and **Open**

After the *Illumina_Age_Project.ppj* file has finished importing, the project file will automatically display the data as spreadsheet *1 (Illumina_Age_Data*). You will see 51 rows representing 51 samples and 24,360 columns representing the genes in this spreadsheet as well as the sample attributes for each sample. Columns 1-6 describe the sample information, and the rest of the columns provide the probe intensities for the genes (Figure 2).



Current Selection GSM210289

	1. GEO ID	2. Sample ID	3. Age	4. Young/Old	5. BeadChip	6. Gender	7. GI_4501840-S	8. GI_4501844-S	9. GI_4501848-S
1.	GSM210289	A30	19	Young	1375488010	F	10.0356	6.32779	7.20282
2.	GSM210290	A44	20	Young	1375488010	M	10.03	6.34064	7.03977
3.	GSM210291	A41	21	Young	1375488010	M	10.0889	5.94964	6.99443
4.	GSM210292	A34	22	Young	1375488010	F	10.284	6.06952	6.96277
5.	GSM210293	A29	19	Young	1375488013	F	10.0341	6.14832	7.04947
6.	GSM210294	A26	20	Young	1375488013	M	10.1513	6.13433	7.19807
7.	GSM210295	A32	21	Young	1375488013	F	10.0106	6.21835	6.89428
8.	GSM210296	A48	23	Young	1375488013	F	10.1987	5.93083	6.7776
9.	GSM210297	A35	21	Young	1375488014	F	10.2317	6.24441	7.05961
10.	GSM210298	A43	21	Young	1375488014	M	10.0803	6.25267	7.02266
11.	GSM210299	A40	22	Young	1375488014	M	10.1148	6.14216	6.81559
12.	GSM210300	A31	23	Young	1375488014	F	10.4285	6.31002	7.14658
13.	GSM210301	A50	20	Young	1353211008	M	9.99437	5.69592	6.67395
14.	GSM210302	A37	21	Young	1353211008	F	9.53746	5.49981	6.45137
15.	GSM210303	A51	22	Young	1353211008	M	9.97262	5.98085	6.86362
16.	GSM210304	A42	27	Young	1353211008	F	9.65307	5.85808	6.66125
17.	GSM210305	A27	22	Young	1353211009	M	9.61277	5.86269	6.50576
18.	GSM210306	A33	18	Young	1353211010	F	10.0411	6.05304	7.01621

Rows: 51 Cols: 24360

Figure 2: Viewing the Analytical Spreadsheet with probes on columns and samples on rows

Note: For additional information about importing data please refer to **Chapter 4, Importing and Exporting Data,** in the Partek Genomics Suite User Manual (available from the Partek Genomics Suite software menu under *Help> User's Manual*). It is recommended that you go through **Chapter 6, The Pattern Visualization System,** before going through the next section of the tutorial. Finally, be aware that this tutorial covers only a specific type of analysis. For in-depth information covering all available analysis methods, see the Partek Genomics Suite User Manual.

Exploratory Data Analysis

Explore the data in spreadsheet *1 (Illumina_Age_Data)* by plotting a Principal Components Analysis (PCA) scatter plot of the data which is an excellent method for visualizing high-dimensional data.

- Select the **Principal components analysis (PCA)** step in *QA/QC* section of the *Workflows* dialog. The *Scatter Plot* viewer with the PCA plot will appear as shown in Figure 3

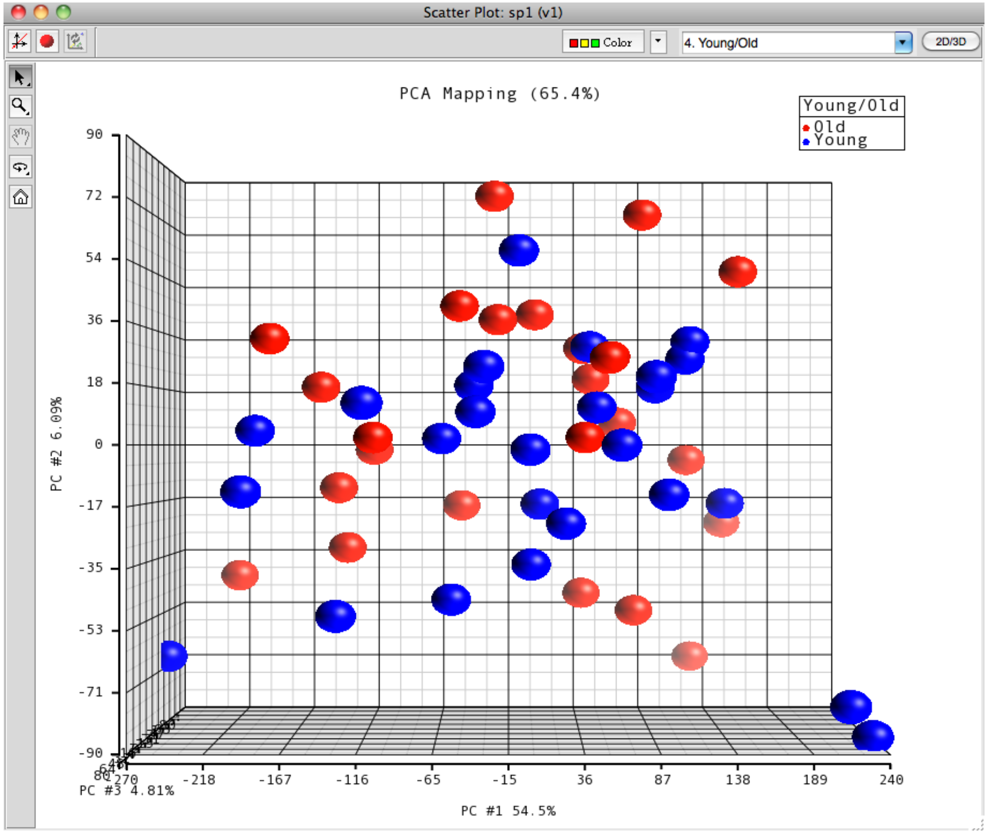


Figure 3 Viewing a PCA scatter plot of the Illumina® age data

In the scatter plot, each dot represents one sample and corresponds to a row on the *Illumina_Age_Data* spreadsheet. Select any point in the scatter plot, and the corresponding row will be highlighted in the spreadsheet. The color of the dot represents the categorized age of the sample: red represents the *Old* patients and blue represents the *Young* patients. Points that are close together in the plot have similar expression intensities across the transcriptome assayed, and points that are far apart in the plot have dissimilar expression intensities.

The PCA plot can be rotated in 3-dimensions.

- Select the *Rotate Mode* option (⟲) and drag the left mouse button to rotate the plot to examine the grouping pattern or outliers of the data on the first 3 principal components (PCs)
- Alternatively, you may press and hold the mouse wheel while moving the mouse to rotate the plot

On PC #3, a loose segregation between the *Old* and *Young* samples is apparent (Figure 4).

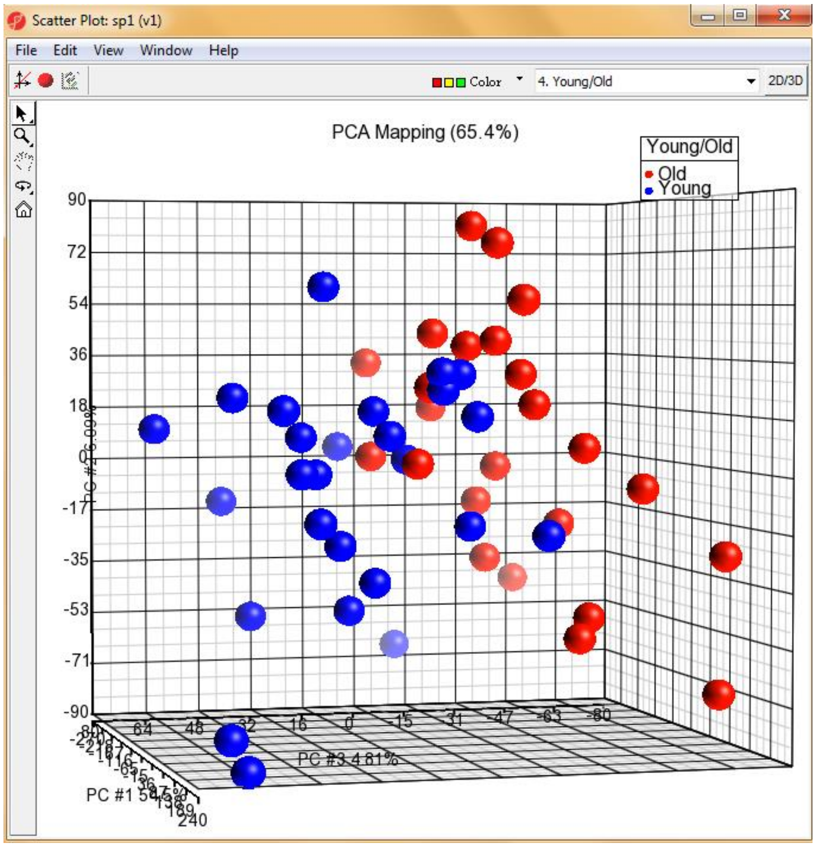


Figure 4: Viewing a PCA scatter plot of the Illumina Age data to detect grouping patterns in the data. From this angle, Old samples cluster on the right and Young samples are more on the left side

- Within the *Scatter Plot* viewer, select the **Plot Properties** icon (●) (or **Edit > Plot Properties**)
- In the *Style* tab, *Color* the points by 5. **BeadChip**
- Select **OK**

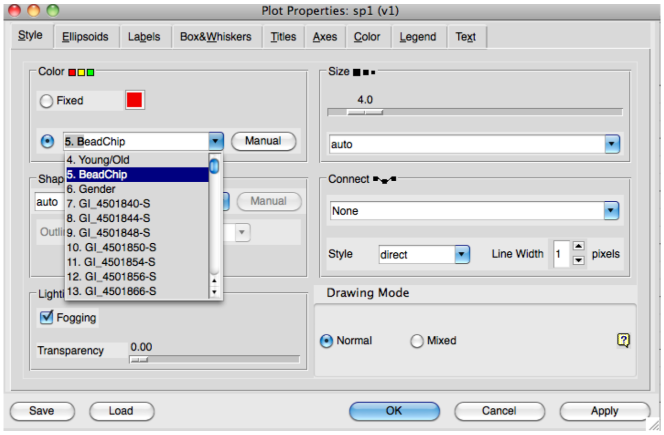


Figure 5: Plot Properties dialog to change how the PCA scatter plot is displayed

The dots are now colored based upon the *BeadChip*™ used for each sample (Figure 6). Notice that the samples group together based upon the *BeadChip* on which they were analyzed; this is an example of a technical batch effect or variability that is not due to the biology but to sample processing. Importantly, because we have now identified this batch effect using exploratory data analysis, the effect can be accounted for by including *BeadChip* as one of the factors in the ANOVA model. This allows the technical noise of the BeadChip to be minimized, thus allowing for better detection of the biological variation in the experiment.

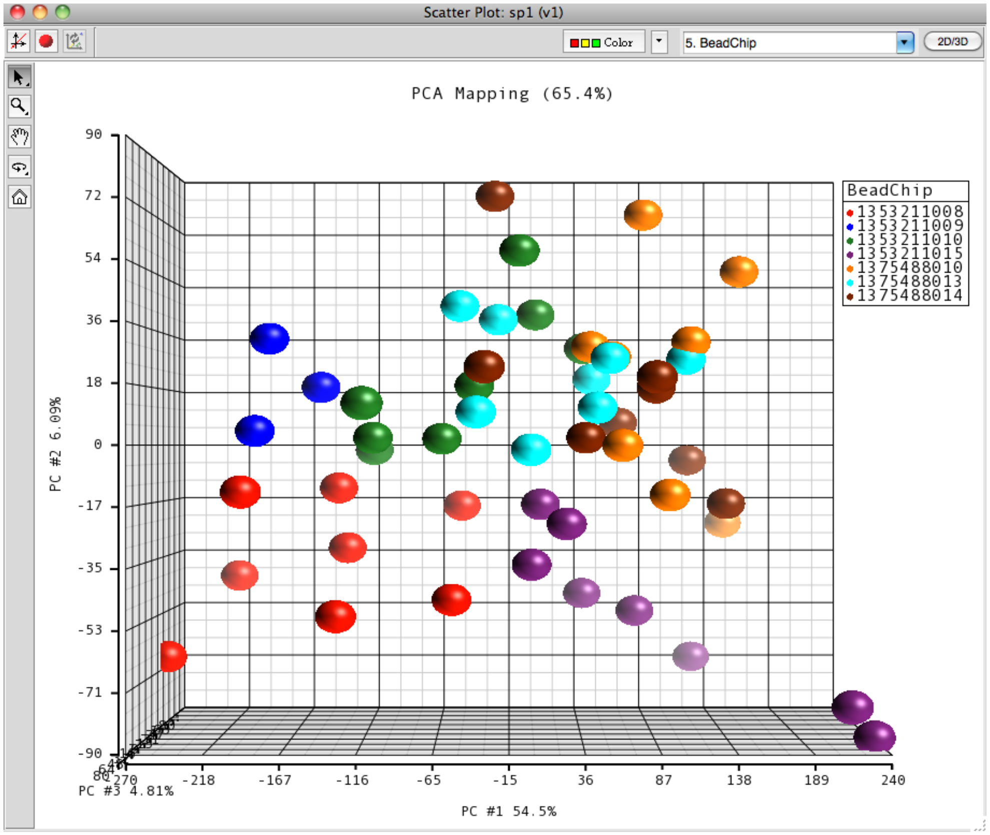


Figure 6: Viewing a PCA scatter plot of the Illumina Age data, colored by BeadChip

- Return to the Plot Properties dialog box (●) and configure the plot: *Size* the points by column **4. Young/Old**
- Select **Apply**

Changing the factor by which the dots in the PCA are colored can also be performed from within the PCA graph instead of going into the *Plot Properties* dialog. To do so, select the drop-down menus in the upper-right hand side of the PCA graph to select the factor the samples will be colored by. The factors for size, shape, and connect lines may also be selected from the same drop-down menu by first selecting the style (Figure 7).

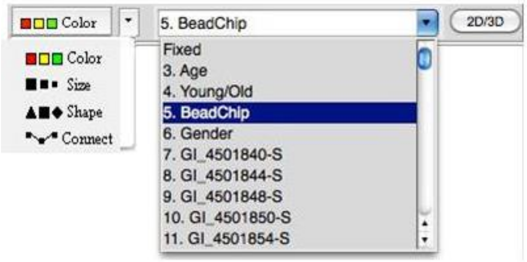


Figure 7: Displaying the pull-down menus for changing graphical styles in the PCA plot

Notice now that the data shows both the age of the samples from the different sized dots as well as the BeadChips used for each sample based on the color of the dots (Figure 8). Both *Old* (small dots) and *Young* (big dots) samples were run on the same BeadChips (colors) over the entire experiment. By noting that there are both small dots and large dots sharing the same colors, it can be inferred that the experiment design is balanced.

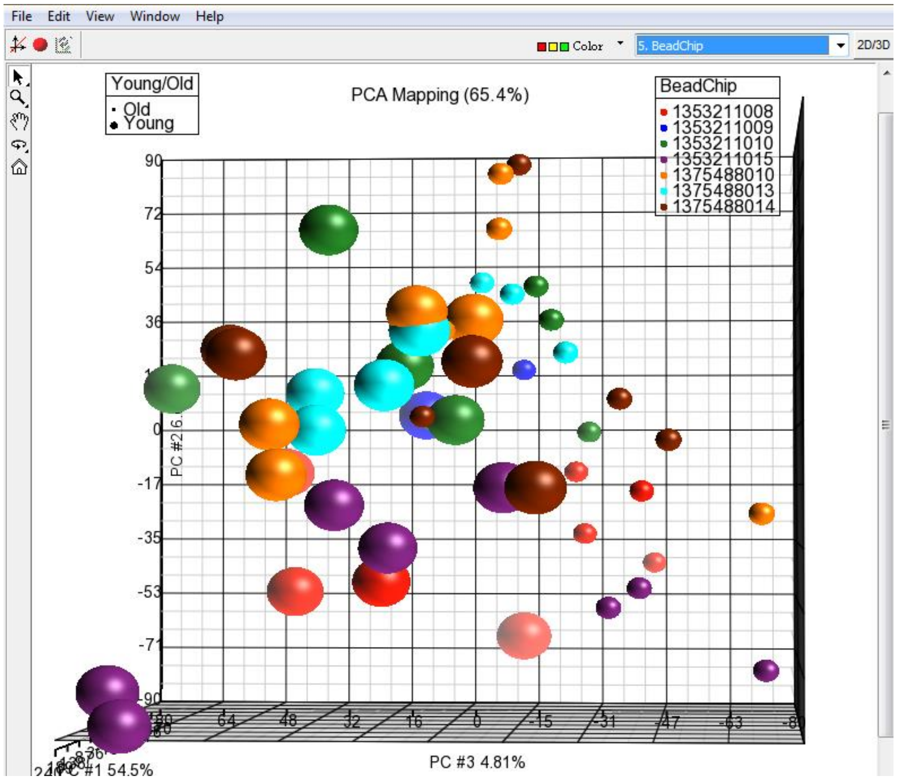
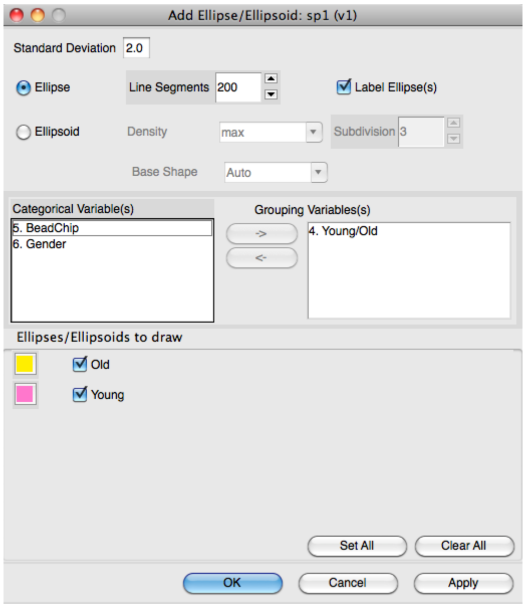


Figure 8: Visualizing the scatter plot with data layering: Color by BeadChip™ and Size by Age

Another way to visualize a cluster pattern is to overlay the data with ellipses. Follow these steps to include ellipses for the *Young/Old* groups.

- Select the **Ellipsoids** tab in the *Plot Properties* dialog
- Select the **Add Ellipse/Ellipsoid** button
- Select the **Ellipse** radio button
- Double click on **4. Young/Old** to move it to the *Grouping Variable(s)* panel
- Select **OK** (Figure 9)



Add Ellipse/Ellipsoid: sp1 (v1)

Standard Deviation 2.0

● Ellipse Line Segments 200 ☑ Label Ellipse(s)

○ Ellipsoid Density max Subdivision 3

Base Shape Auto

Categorical Variable(s): 5. BeadChip, 6. Gender

Grouping Variables(s): 4. Young/Old

Ellipses/Ellipsoids to draw

■ ☑ Old

■ ☑ Young

Figure 9: Adding ellipses to the Young/Old clusters

Now, rotate the plot to the PC#3 plane. You can see from the ellipses that the data is clustered by *Young* and Old *with* almost no overlap, signifying a difference in variation between the two groups (Figure 10).

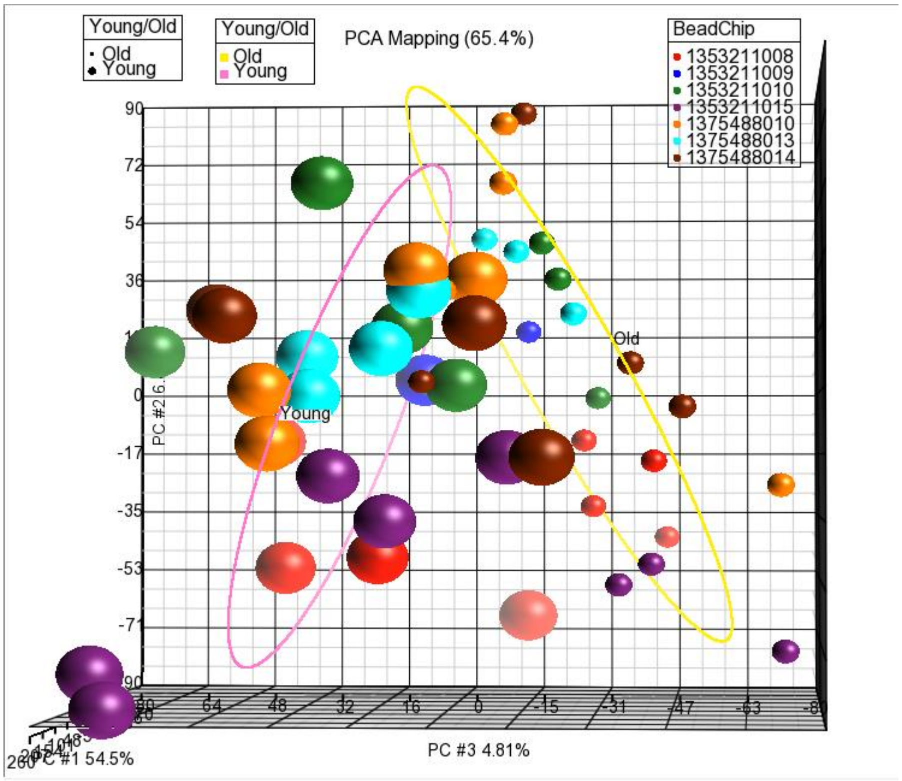


Figure 10: Viewing a scatter plot of data colored by BeadChip, sized by Young/Old, and grouped with Young/Old ellipses

PCA is an example of exploratory data analysis and is useful for identifying outliers and major effects in the data. From this scatter plot exercise, both age and BeadChip™ categorical factors demonstrate significant sources of variation in the data set based upon the grouping patterns.

The variation in *Gender* is not a significant source of variation as there is no distinct separation of the two groups in the scatter plot. Visualize this on your own using the same steps included used to visualize the other factors in the PCA plot.

For additional information on the PCA implementation, refer to **Chapter 11 Inferential Statistics** in the Partek Genomics Suite User Manual.

Identifying Differentially Expressed Genes using a one-way ANOVA

Analysis of variance (ANOVA) is a very powerful technique for identifying differentially expressed genes and can be used in either a simple or a complicated multi-factor experiment. One of the key attributes of the Partek® ANOVA is its applicability to a wide range of use cases. In this first example, conducting a one factor ANOVA on a continuous variable such as *Age* is the equivalent of linear regression analysis.

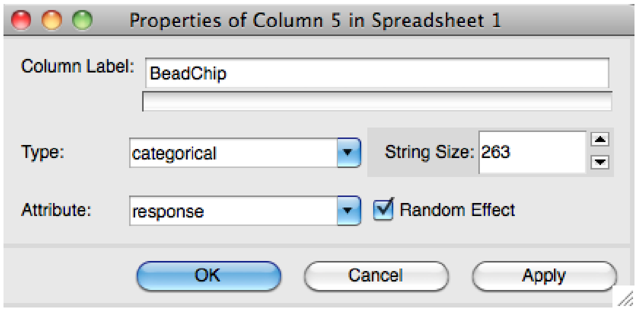
Random vs. Fixed Effects – Mixed Model ANOVA

Most factors in analysis of variance (ANOVA) are fixed effects or factors whose levels represent all the levels of interest. In this study, *Young/Old* is a fixed effect. If the levels of a factor only represent a random sample of all the levels of interest, the factor is a random effect. The 7 BeadChips™ and 51 subjects in this study represent only a random sample of the global subjects about which inferences are made, thus, they are random effects. The random effects (such as *BeadChip*) appear in red, while continuous variables (such as *Age*) appear blue on the spreadsheet and in the ANOVA dialog. How the effects are listed for each factor is important to any successful ANOVA analysis as designating the incorrect effects can lead to incorrect p-values. In general, a random effect designated as a fixed effect will produce an over-optimistic p-value, leading to a higher false discovery rate.

How can I decide if my effect is random or fixed? A simple way to tell if a factor is random or fixed: if you were to repeat the experiment would the same levels of each factor be used again?

- Young/Old – **Yes**, the same age groups would be used again - a **fixed** effect
- Gender – **Yes**, the same genders would be used again - a **fixed** effect
- BeadChip - **No**, the samples would be ran on different BeadChips - a **random** effect

You can specify which factors are random and which are fixed when you import your data, or after importing, by right-clicking on the column corresponding to a categorical variable, selecting **Properties,** and checking **Random effect (**Figure 11**)**. By doing that, the ANOVA will deal with the factors appropriately.


Properties of Column 5 in Spreadsheet 1

Column Label: BeadChip

Type: categorical

String Size: 263

Attribute: response

☑ Random Effect

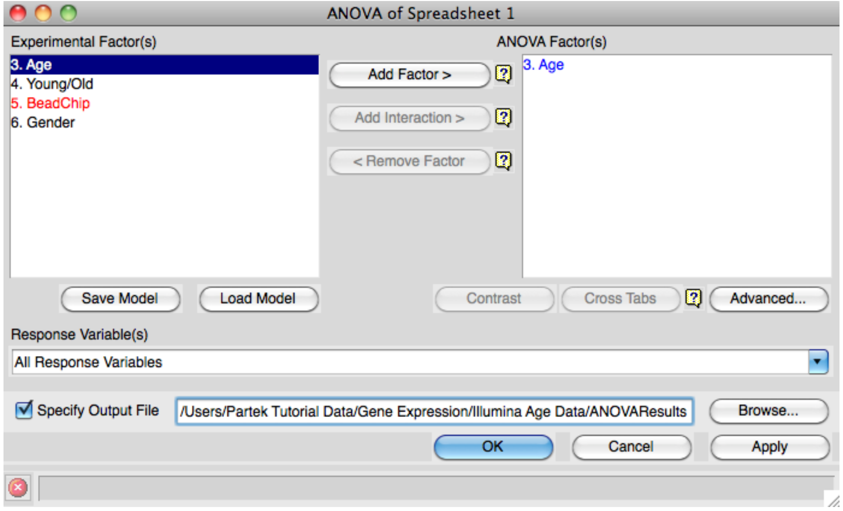
Figure 11: Configuring the Properties dialog box to display the random effect check-box

The rest of this tutorial will discuss how different ANOVA models can affect the statistical and biological results of the data explored thus far, specifically by comparing a one-way ANOVA model and a multi-factor ANOVA model.

Setting up a One-Way ANOVA Model

To set up the one-way ANOVA with (numerical) *Age* as the factor, follow these steps:

- To invoke the ANOVA dialog, select **Detect differentially expressed genes** in the *Analysis* section of the workflow
- In the *Experimental Factor(s)* panel, select **3. Age**.
- Use the **Add Factor >** button to move the selection to the *ANOVA Factor(s)* panel (Figure 12)
- Select **OK** to perform the analysis
- You may be warned that you have not specified contrasts (fold-change) and asked if you would like to specify them now. Select **No**


ANOVA of Spreadsheet 1

Experimental Factor(s)
3. Age
4. Young/Old
5. BeadChip
6. Gender

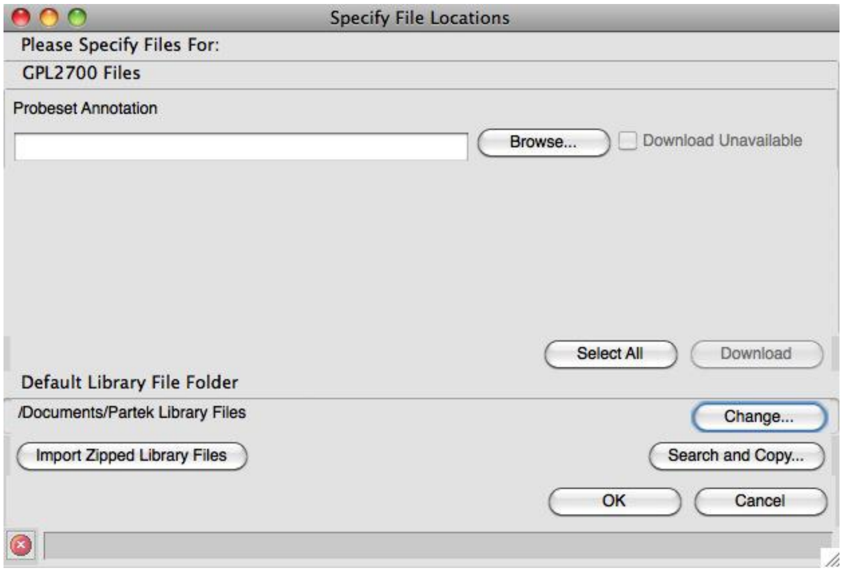
ANOVA Factor(s)
3. Age

Response Variable(s): All Response Variables

☑ Specify Output File: /Users/Partek Tutorial Data/Gene Expression/Illumina Age Data/ANOVAResults

Figure 12: Configuring the ANOVA model to include Age

You may be asked to specify the location of the probeset annotation file (Figure 13) which is located in the same folder as the original project file (named *GPL2700.annotation.txt*).



Specify File Locations

Please Specify Files For:

GPL2700 Files

Probeset Annotation

Default Library File Folder

/Documents/Partek Library Files

Figure 13: Specifying the location of the annotation file

The ANOVA result will be displayed in a child spreadsheet called *Regression-1way (ANOVAResults)*. In the child results spreadsheet, each row represents a gene, and the columns represent the statistical results of that gene along with corresponding gene annotations. By default, the genes are sorted in ascending order by the p-value of the first factor, *Age*. Hence, the most statistically significantly differentially expressed gene based on *Age* is at the top of the spreadsheet. Linear regression can be represented as either a correlation (column 9 of the result sheet) or as a p-value representing the strength of the correlation (column 6): the stronger the correlation between *Age* and a gene's expression, the lower the p-value.

In some cases, a continuous numerical factor such as *Age* may be correlated to all of the response variables. With spreadsheet *1 (Illumina_Age_Data)* is active, follow these steps to examine these correlations:

- Select Stat > Correlate > Many to One…
- Set *Correlate* to All Response Variables
- Set *With* to **3. Age**
- Set *Measure* to Pearson (Linear) Correlation
- Select **OK**

A new spreadsheet is created as a child of the intensity spreadsheet called *Correlation:corr*. Once again, each row represents a gene, and the columns represent the statistical results. The list is sorted in ascending order by the first p-value column in the spreadsheet which is *p-value(correlation).*

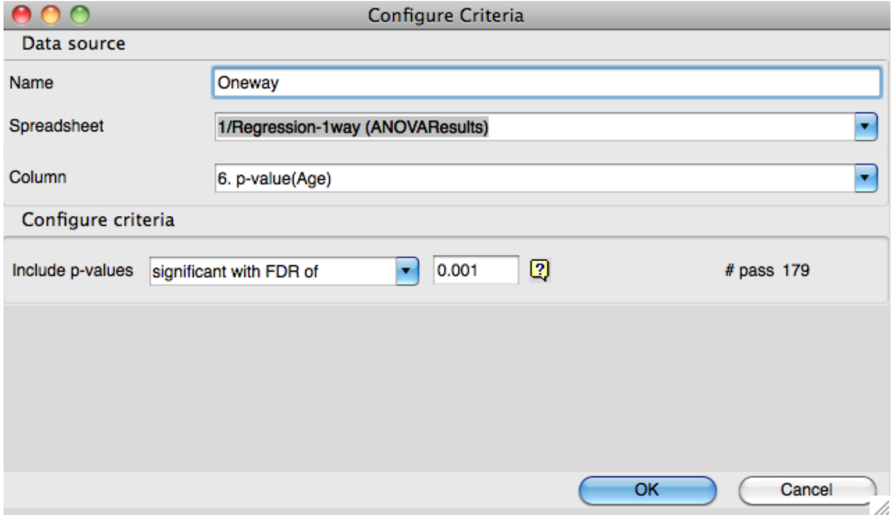
Notice that the statistical results of the ANOVA-based analysis and the results of the correlation-based analysis are identical. That is, the gene order and p-values are the same between the two spreadsheets *Correlation:corr* and *Regression-1way(ANOVAResults).* Additionally, the *r* value output in the correlation analysis is equivalent to the

PartialCorr(Age) output in the ANOVA analysis. This demonstrates that using ANOVA to analyze a continuous variable is equivalent to performing a linear correlation analysis.

Create Gene List

Now that statistical results from the microarray experiment have been obtained, the results of the 24,354 genes can be used to create a new spreadsheet of just those genes that pass criteria. This will make analyzing the data more streamlined by focusing on just those genes with the highest degree of differential expression. In Partek Genomics Suite software, the *List Manager* can be used to specify numerous criteria to use in the generation of the list of genes of interest. The following section will illustrate how to use the *List Manager* to create a gene list.

- Select the **Regression-1way(ANOVAResults)** spreadsheet
- To invoke the *List Manager*, select **Create gene list** in the *Analysis* section of workflow. The *List Manager* dialog box will open.
- Select the **Advanced** tab. To create criteria for the gene list, select the **Specify New Criteria** button to open the *Configure Criteria* dialog box
- *Name* the criteria meaningfully, like **Oneway** in this example
- For *Spreadsheet*, ensure that the **Regression-1 way (ANOVAResults)** is selected as this is the spreadsheet that should be used to create the new gene list
- Ensure that **6. p-value(Age)** is specified for *Column*
- In the *Configure criteria* panel, *include p-values* **significant with FDR** of **0.001** as shown in (Figure 14)



Configure Criteria

Data source

Name: Oneway

Spreadsheet: 1/Regression-1way (ANOVAResults)

Column: 6. p-value(Age)

Configure criteria

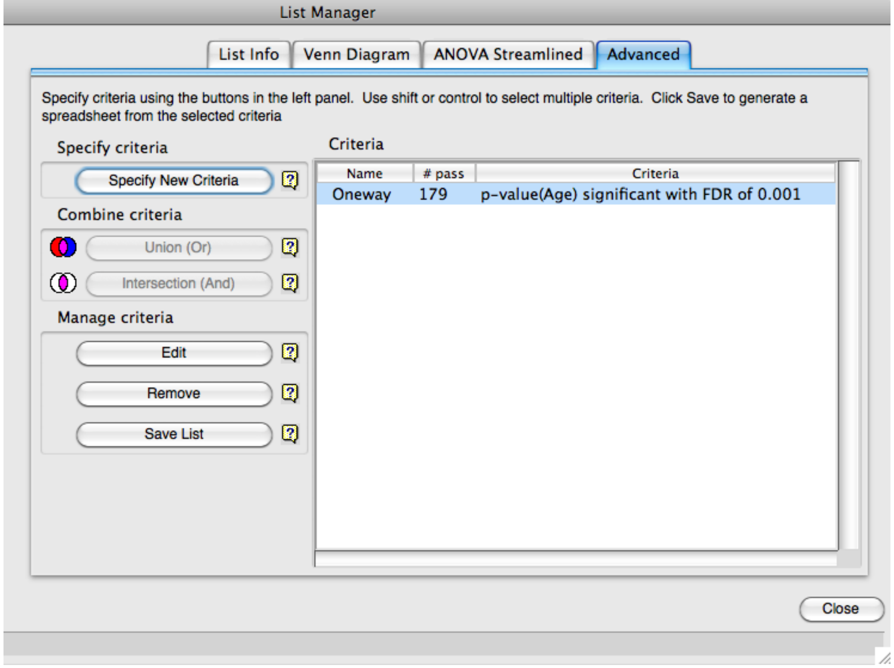
Include p-values: significant with FDR of 0.001

pass 179

Figure 14: Create a gene list from the one-way ANOVA

In the *Configure criteria* section of the dialog box, different criteria may be selected for *Include p-values*, and the numerical cut off may be narrowed or widened to create the gene list. There is additional information regarding the criteria options available in the help bubble, which can be viewed by left-clicking on the yellow bubble with a question mark (?). The number of genes that pass the cut off criteria will be shown next to the *# pass* field. In this example, 179 genes pass the criteria of FDR <= 0.001.

- Select **OK** if you have not already done so. The will then be listed in the *Criteria* panel of the *List Manager* dialog (Figure 15)
- Select **Close**



Specify criteria using the buttons in the left panel. Use shift or control to select multiple criteria. Click Save to generate a spreadsheet from the selected criteria

Name	# pass	Criteria
Oneway	179	p-value(Age) significant with FDR of 0.001

Figure 15: Viewing list in the List Manager

To save the list into Partek Genomics Suite software:

- Select **Save List** in the *List Manager* dialog box
- A new dialog box appears with checkboxes for all lists created. Select those you wish to save and select **OK**

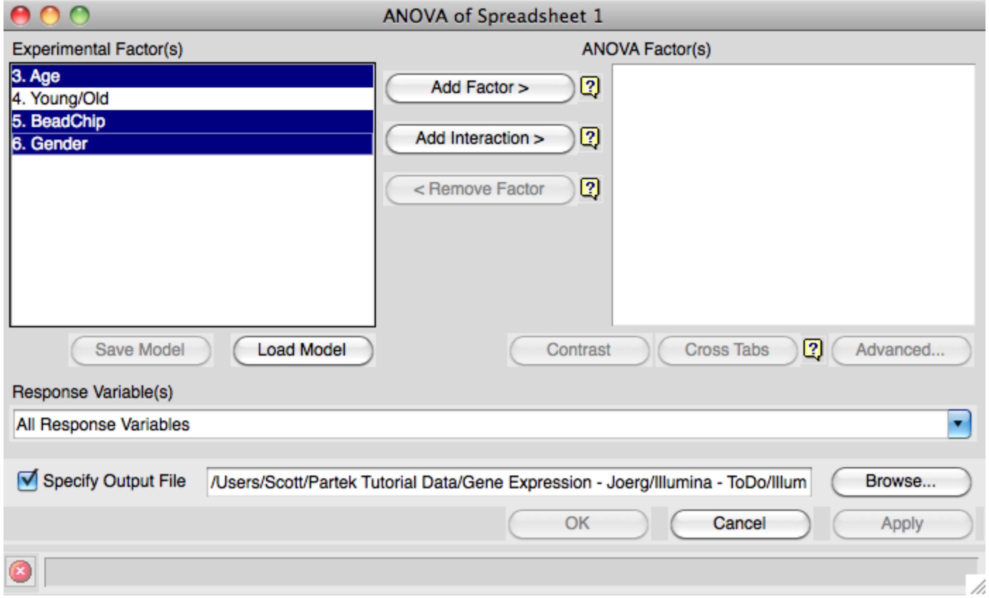
The spreadsheet *Oneway (Oneway.txt)* has been created and added to the spreadsheet list in Partek Genomics Suite. The spreadsheet *Oneway.txt* includes the 179 genes that passed the FDR 0.001 criteria and with all of the statistical and annotation information from the *Regression-1way (ANOVAResults)* spreadsheet.

Identifying Differentially Expressed Genes using a Two-Way ANOVA

By adding additional factors, the ANOVA model might better fit the data if more factors accurately describe the variables that affect the response variables (gene expression). Thus, by incorporating the same variables into the ANOVA that are affecting the microarray data, the technical effects can be accounted for, and the biologically relevant effects become more apparent. Additionally, secondary factors such as gender (which may not be of primary interest in the experiment) provide additional information that could be of interest to a researcher. From the PCA diagram, BeadChip™ was found to be a source of variation, thus inclusion of BeadChip in the ANOVA may make the model fit the data better. The inclusion of BeadChip minimizes or removes the technical artifacts, allowing for the variation attributable to *Age* to be more accurate. Likewise, variations due to *Gender* may also improve the model. One factor that may not be obvious is the inclusion of the interaction between *Age* and *Gender*. The interaction of *Age* and *Gender* will model changes between these two factors. This interaction tests the assumption that muscle tissue between a men and women age the same way by identifying genes where the expression is altered by age, but the age effect is different between the two genders. Omitting the *Age*Gender* interaction assumes that muscle tissue in men and a women age identically. Biologically speaking, this could be incorrect, and so the ANOVA model without the interaction term would increase the noise in our model, thus decreasing the sensitivity of the statistics for *Age*.

Set up the multi-factor ANOVA model to include *Age*, *Gender*, *BeadChip*™, and the *Age*Gender* interaction.

- Select the intensity spreadsheet *1 (Illumina_Age_Data)*
- Invoke the ANOVA dialog by selecting **Detect differentially expressed genes** in the *Analysis* section of the workflow
- In the *Experimental Factor(s)* panel of the ANOVA dialog box, select **3. Age, 5. BeadChip,** and **6. Gender** by pressing <Ctrl> and left clicking each factor (Figure 16)
- Use the **Add Factor >** button to move the selections to the *ANOVA Factor(s)* panel



ANOVA of Spreadsheet 1

Experimental Factor(s)

3. Age
4. Young/Old
5. BeadChip
6. Gender

ANOVA Factor(s)

Response Variable(s)

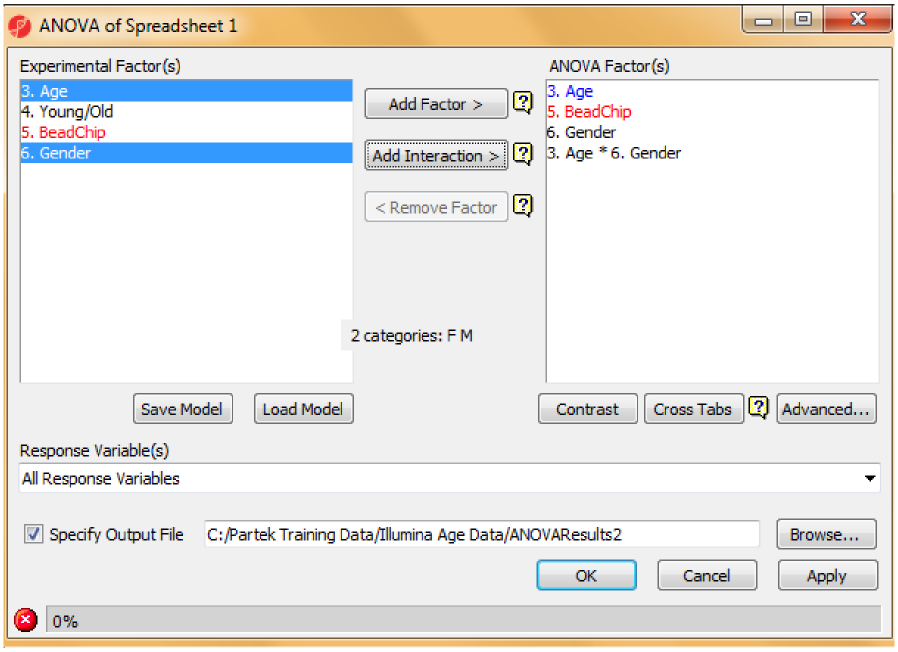
All Response Variables

☑ Specify Output File /Users/Scott/Partek Tutorial Data/Gene Expression - Joerg/Illumina - ToDo/Illum

Figure 16: Configuring the ANOVA model to include Age, BeadChip, and Gender as factors in the model

- To specify the interaction, select both *Age* and *Gender* in the *Experimental Factor(s)* panel
- Select the **Add Interaction >** button to add the **3. Age*6. Gender** interaction in the *ANOVA Factor(s)* panel for the finalized ANOVA model

- In the **Specify Output File** field, add a **2** to the end of the name so that the results from the new ANOVA do not overwrite the previous ANOVA results. Use Figure 17 to ensure that the dialog box is set up correctly



ANOVA of Spreadsheet 1

Experimental Factor(s)

3. Age
4. Young/Old
5. BeadChip
6. Gender

ANOVA Factor(s)

3. Age
5. BeadChip
6. Gender
3. Age * 6. Gender

2 categories: F M

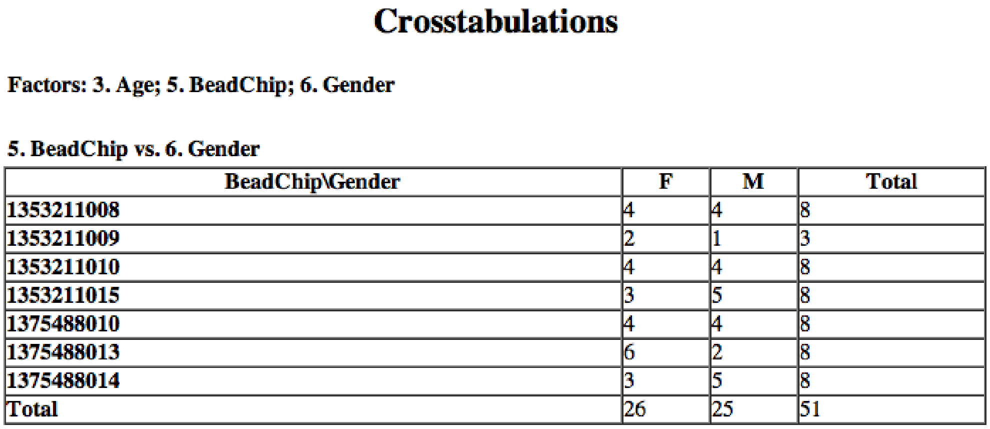
Response Variable(s): All Response Variables

☑ Specify Output File: C:/Partek Training Data/Illumina Age Data/ANOVAResults2

0%

Figure 17: The finalized ANOVA dialog box for the multi-factor ANOVA model

- Before the ANOVA is run, the distribution of the samples can be observed (Figure 18) by selecting **Cross Tabs**



Crosstabulations

Factors: 3. Age; 5. BeadChip; 6. Gender

5. BeadChip vs. 6. Gender

BeadChip\Gender	F	M	Total
1353211008	4	4	8
1353211009	2	1	3
1353211010	4	4	8
1353211015	3	5	8
1375488010	4	4	8
1375488013	6	2	8
1375488014	3	5	8
Total	26	25	51

Figure 18: Viewing the Crosstabulation table that describes the distribution of the samples

The cross-tabulation table describes the sample distribution based upon the categorical factors included in the ANOVA model. Notice that *BeadChip™* and *Gender* are the only categorical factors as *Age* is a constant variable and *Age*Gender* is an interaction. Hence, when the ANOVA is run, this is considered a two-way ANOVA.

The samples are evenly distributed between males and females on *BeadChips* 1353211008, 1353211010, and 1375488010 as there are 4 males and 4 female samples on each of these chips. The rest of the *BeadChips* are imbalanced with respect to males and females. While perfectly balanced data is preferred for analysis, Partek Genomics Suite software can easily handle imbalances by taking the weighted averages of the samples. If zeros appear in the cross-tabulation table, there is an absence of samples for that factor; Partek Genomics Suite will attempt to estimate the variation for a factor with missing samples in a category in order to continue the analysis. However, if the imbalance is too great, the ANOVA result table will display “?” instead of p-values which means analysis could not be performed because of the overwhelming imbalance. If this occurs, it is best to consult a statistician to determine if a different ANOVA model should be used.

- Close the *crosstabulations* viewer and select **OK** in the ANOVA dialog to conduct the statistical analysis
- Select **No** in the dialogue box warning you that no contrast had been specified

The result *spreadsheet ANCOVA-2way (ANOVAResults2)* is created. Just as with the previous ANOVA spreadsheet, the *ANCOVA-2way (ANOVAResults2)* is a child of the intensity (*Illumina_Age_Data)* spreadsheet. Again, each row represents a gene, and the columns represent the gene annotations and the statistical calculations for that gene. The genes are sorted in ascending order by *p-value*(*Age*). The most noticeable difference in the new spreadsheet is that p-value columns are now included for *Age*, *BeadChip*, *Gender*, and the *Age*Gender* interaction, instead of just one p-value for *Age* provided previously in the *Regression-1way (ANOVAResults)* spreadsheet.

Viewing the Sources of Variation

The *sources of variation* plot is a graphical way to assess the relative contribution of each factor to the variation in the response variables (gene expression intensity values) across the whole array.

- **Select Plot sources of variation** from the *Analysis* section of the workflow with the *ANCOVA-2way (ANOVA Results2)* spreadsheet active
- Select the **Bar Chart (Signal To Noise)** tab and select **OK**. The *Sources of Variation* plot is shown in Figure 19

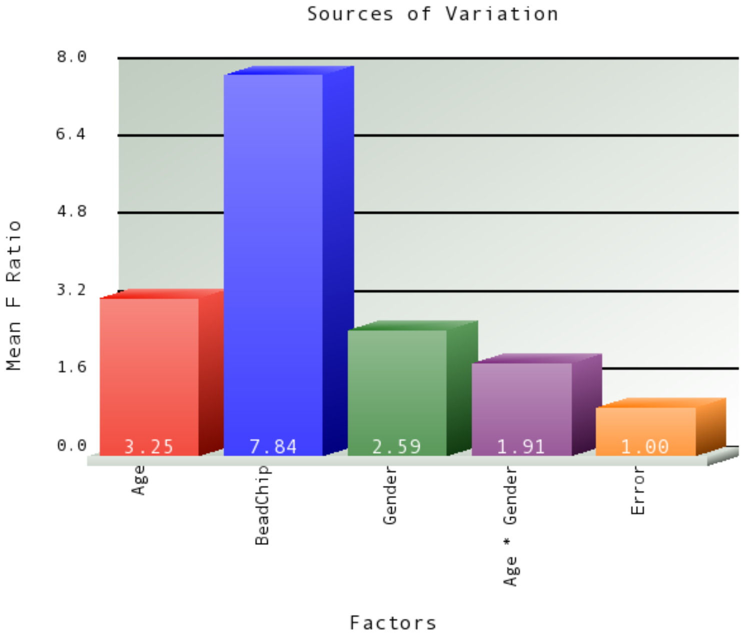


Figure 19: Viewing the Sources of Variation plot

This plot presents the average effect sizes across all the genes for each of the factors. All the factors in the ANOVA model are displayed on the X-axis along with a bar representing noise (*Error*) or the variability in the data not explained by the other factors. The Y-axis represents the *Mean F-ratio* (signal-to-noise) of all the genes. Compare each signal bar to the error bar; if the bar is higher than the error bar, the factor has a larger effect size than the effect size of background noise. If a bar is lower than the background noise, it means that the factor has less of an effect size than the effect size of the background noise.

In Figure 19, the biological and technical factors all show a larger average effect size than the background noise. In general, each factor or interaction in the ANOVA mode that has an average effect size larger than the background effect size should be included because that factor improves the statistical results generated in the ANOVA. If a factor or interaction with a significant effect size is not included, the variation that would otherwise be attributed to that factor is considered as noise in the ANOVA model. Increasing noise in the ANOVA model will decrease the p-values of some genes, hence it is important to include *Age*, *Gender*, *BeadChip*, and the *Age*Gender* interaction in this model.

Also notice that the tallest *Mean F ratio* bar is attributed to *BeadChip*. By including *BeadChip* in the ANOVA model, you have already performed a batch correction.

Below are the *Sources of Variation* graphs for both the multi-way and one-way ANOVAs (Figure 20). The *Age* effect size is 50% higher in the multi-way graph than in the one-way graph because the additional factors decrease the amount of noise in the ANOVA model, boosting the *Age* effect size relative to the noise (*Error*).

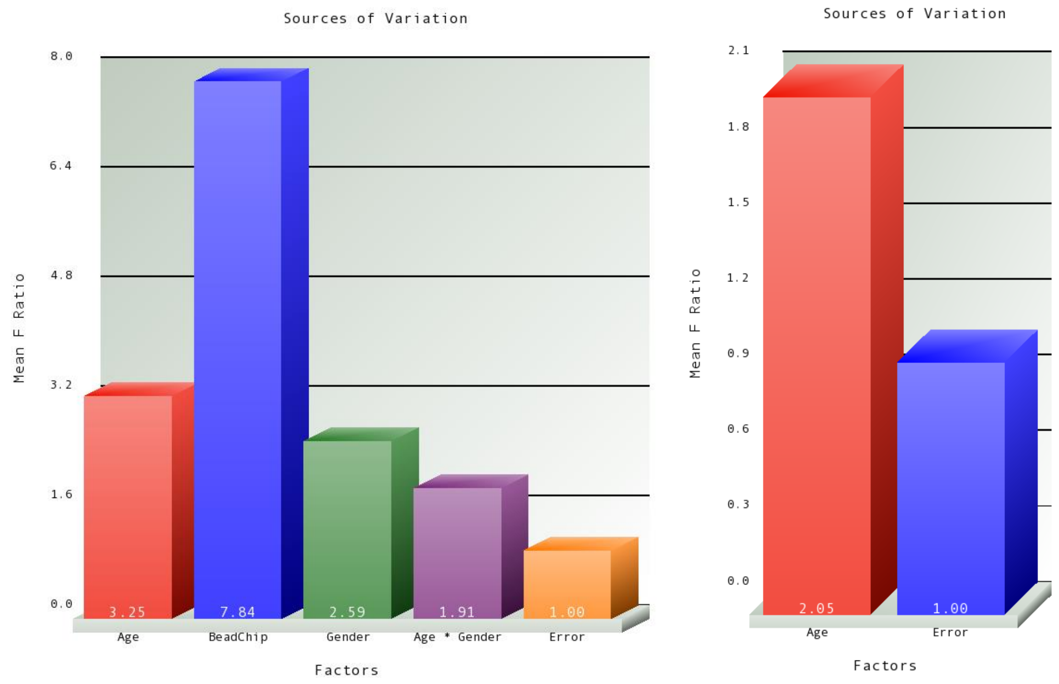


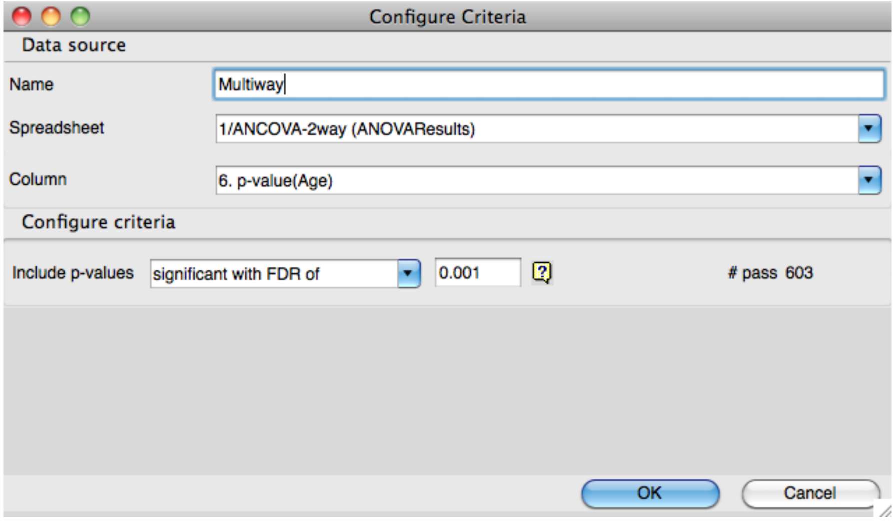
Figure 20: Viewing the Plot Sources of Variation for the multi-way (left) and one-way ANOVA (right)

To view the source of variation plot for a single gene, right click on a row header corresponding to the gene in either of the ANOVA spreadsheets and select **Sources of Variation** from the pop-up menu.

Create Gene List for the multi-factor ANOVA

In order to compare the differences between the results of the two ANOVAs, generate another gene list from the *ANCOVA-2way (ANOVAResults2)* spreadsheet.

- Select **Create Gene List** in the workflow. In the *List Manager* dialog, select the **Advanced** tab. Select the **Specify New Criteria** button to open the *Configure Criteria* dialog box \
- For *Name*, specify **Multiway**
- Ensure that the **ANCOVA-2way (ANOVAResults2)** *Spreadsheet* is selected as this is the spreadsheet that will be used to create the new gene list
- For *Column*, select **6. p-value(Age)**
- In the *Configure criteria* portion, *Include p-values* **significant with FDR** of **0.001** as shown in Figure 21
- Click **OK** to add the gene list criteria to the List Manager dialog box as shown in Figure 22



Configure Criteria

Data source

Name: Multiway

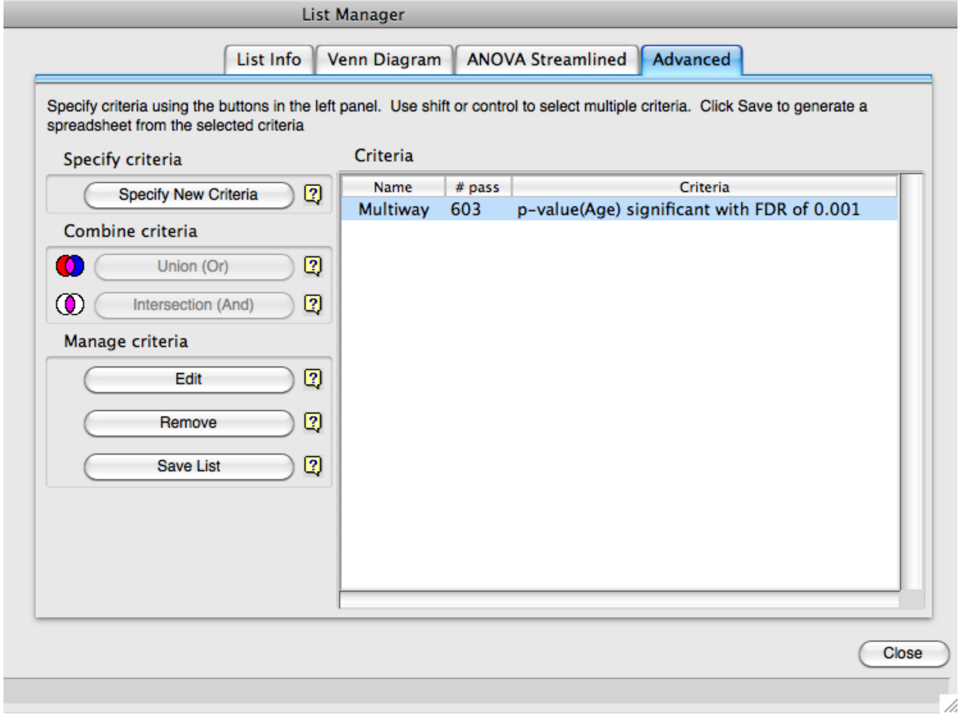
Spreadsheet: 1/ANCOVA-2way (ANOVAResults)

Column: 6. p-value(Age)

Configure criteria

Include p-values: significant with FDR of 0.001 # pass 603

Figure 21: Create a gene list from the multi-way ANOVA



List Manager

Specify criteria using the buttons in the left panel. Use shift or control to select multiple criteria. Click Save to generate a spreadsheet from the selected criteria

Criteria

Name	# pass	Criteria
Multiway	603	p-value(Age) significant with FDR of 0.001

Figure 22: Viewing the newly created list in the List Manager

To save the list to the spreadsheet navigator within Partek Genomics Suite, follow these steps:

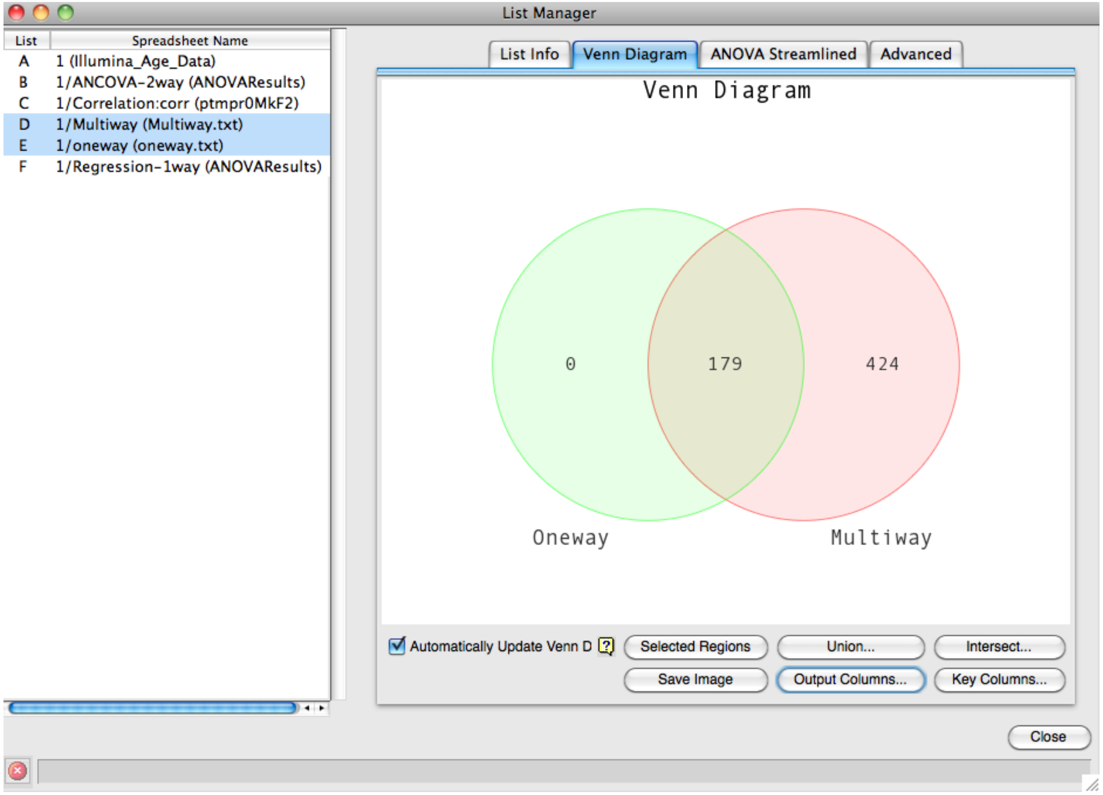
- Click **Save List** in the *List Manager* dialog box
- A *List Creator* dialog box listing the spreadsheet criteria that were created. Check **Multiway.** Select **OK** and **Close**

The spreadsheet *Multiway.txt* has been created and added to Partek Genomics Suite (spreadsheet navigator and within *List Manager*). The spreadsheet *Multiway.txt* includes the 603 genes that passed the FDR 0.001 criteria along with all of the statistical and annotation information from the *ANCOVA-2way (ANOVAResults2)* spreadsheet.

Comparing the Gene Lists

Next, compare the two gene lists created with a Venn diagram to see if genes from the two ANOVA tests overlap with each other.

- Select **Create gene list** in the *Analysis* section of the workflow
- Highlight both **Oneway.txt** and **Multiway.txt** lists in the *List Manager* dialog
- Select the **Venn Diagram** tab (Figure 23)
- Double-click on any part of the white background surrounding the Venn diagram to invoke the *Configure Venn Diagram* dialog. Here you can change fonts and give titles to the regions of the diagram. For instance, the *List Names* were changed to **Oneway** and **Multiway**



List Manager

List	Spreadsheet Name
A	1 (Illumina_Age_Data)
B	1/ANCOVA-2way (ANOVAResults)
C	1/Correlation:corr (ptmpr0MkF2)
D	1/Multiway (Multiway.txt)
E	1/oneway (oneway.txt)
F	1/Regression-1way (ANOVAResults)

Figure 23: Viewing the Venn diagram that shows the overlap of genes between the two one-way ANOVA and the two-way ANCOVA gene lists

The *Oneway.txt* list completely overlaps with the *Multiway.txt* list, so those same genes were identified in both lists. Notice that the *Multiway* list is more than three times larger than the *Oneway* list and recall that the same criteria including error rates were used to generate both gene lists. Because of the additional factors included in the two-way ANCOVA model, the variation described by those additional factors allows a more precise calculation of the importance of the *Age* factor, resulting in more genes with lower p-values that passed the gene list creation criteria. By accounting for as many meaningful sources of variation in the experiment as possible and minimizing the technical *BeadChip* batch effect (simply by including it in the ANOVA model), more genes are detected as being significantly differentially expressed due to *Age*.

Besides having an impact on the size of the gene lists, using an ANOVA model that provides a better fit to the data also impacts the biological interpretation of the data. Below is a side-by-side comparison of the *GO Enrichment* tool results from both gene lists (Figure 24).

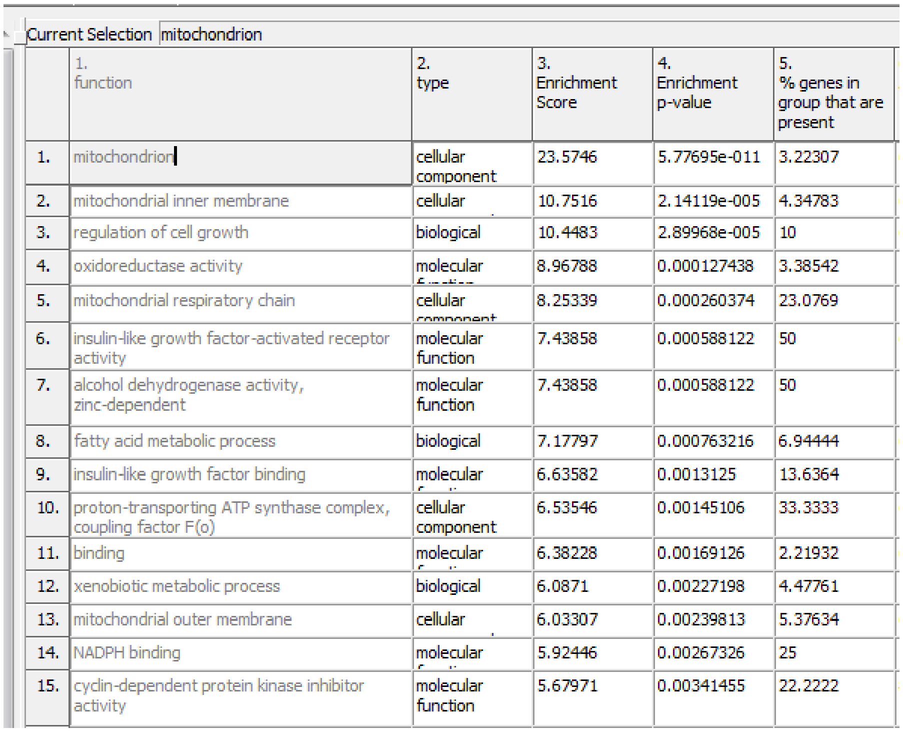
The top list is the one-way ANOVA *GO Enrichment* results, and the list on the bottom is from the multi-way GO *Enrichment* results. The top 15 functional groups showing gene enrichment are listed in both spreadsheets. Notice that the enrichment scores, which are an indicator for over-representation of genes in a functional group, are higher in the multi-way ANOVA than in the one-way ANOVA and that the p-values are smaller in the *Multiway ANOVA* results. Also, the top 15 functional groups in the multi-way relate to mitochondrial functional groups more so than in the one-way.

Hence, the ANOVA model that is selected for analysis affects not only the statistical analysis but can impact the ultimate biological interpretation of the data.

Note: In order to run *GO Enrichment*, certain properties may need to be associated with the spreadsheets.

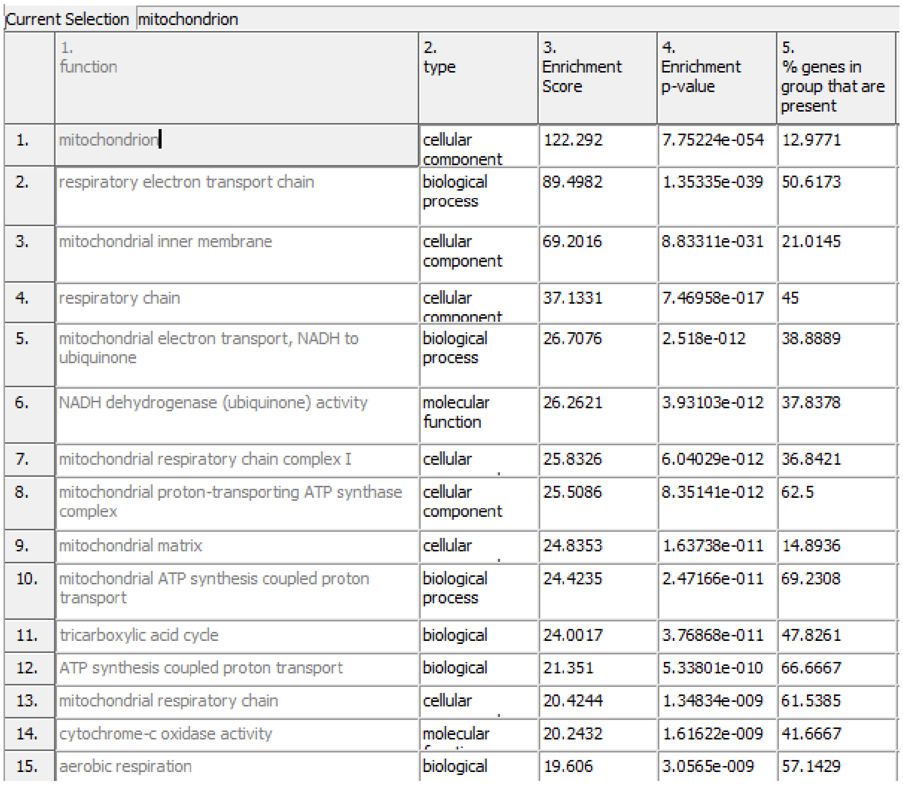
- Right-click on the *1 (Illumina_Age_Data)* spreadsheet and select **Properties**
- Select **Set Name Column** and select the **Gene symbol** radio button
- Select **OK** and **OK** again. This has now identified which field from the annotation file contains the gene symbol (needed for the background genes on the array)
- Right-click on the gene-list spreadsheet and select **Properties**
- In the *Properties of Spreadsheet*, select **Gene Symbol Column** from the *Add Property* pull-down menu
- Select **Add**
- Select **4. Gene Symbol** for *Gene Symbol Column* and select **OK**

To learn more about the GO Enrichment tool in software Genomics Suite, please read the GO Enrichment User Guide available from *Help > On-line Tutorials > User Guides*.



Current Selection mitochondrion

	1. function	2. type	3. Enrichment Score	4. Enrichment p-value	5. % genes in group that are present
1.	mitochondrion	cellular component	23.5746	5.77695e-011	3.22307
2.	mitochondrial inner membrane	cellular	10.7516	2.14119e-005	4.34783
3.	regulation of cell growth	biological	10.4483	2.89968e-005	10
4.	oxidoreductase activity	molecular	8.96788	0.000127438	3.38542
5.	mitochondrial respiratory chain	cellular component	8.25339	0.000260374	23.0769
6.	insulin-like growth factor-activated receptor activity	molecular function	7.43858	0.000588122	50
7.	alcohol dehydrogenase activity, zinc-dependent	molecular function	7.43858	0.000588122	50
8.	fatty acid metabolic process	biological	7.17797	0.000763216	6.94444
9.	insulin-like growth factor binding	molecular	6.63582	0.0013125	13.6364
10.	proton-transporting ATP synthase complex, coupling factor F(o)	cellular component	6.53546	0.00145106	33.3333
11.	binding	molecular	6.38228	0.00169126	2.21932
12.	xenobiotic metabolic process	biological	6.0871	0.00227198	4.47761
13.	mitochondrial outer membrane	cellular	6.03307	0.00239813	5.37634
14.	NADPH binding	molecular	5.92446	0.00267326	25
15.	cyclin-dependent protein kinase inhibitor activity	molecular function	5.67971	0.00341455	22.2222



Current Selection mitochondrion

	1. function	2. type	3. Enrichment Score	4. Enrichment p-value	5. % genes in group that are present
1.	mitochondrion	cellular component	122.292	7.75224e-054	12.9771
2.	respiratory electron transport chain	biological process	89.4982	1.35335e-039	50.6173
3.	mitochondrial inner membrane	cellular component	69.2016	8.83311e-031	21.0145
4.	respiratory chain	cellular component	37.1331	7.46958e-017	45
5.	mitochondrial electron transport, NADH to ubiquinone	biological process	26.7076	2.518e-012	38.8889
6.	NADH dehydrogenase (ubiquinone) activity	molecular function	26.2621	3.93103e-012	37.8378
7.	mitochondrial respiratory chain complex I	cellular	25.8326	6.04029e-012	36.8421
8.	mitochondrial proton-transporting ATP synthase complex	cellular component	25.5086	8.35141e-012	62.5
9.	mitochondrial matrix	cellular	24.8353	1.63738e-011	14.8936
10.	mitochondrial ATP synthesis coupled proton transport	biological process	24.4235	2.47166e-011	69.2308
11.	tricarboxylic acid cycle	biological	24.0017	3.76868e-011	47.8261
12.	ATP synthesis coupled proton transport	biological	21.351	5.33801e-010	66.6667
13.	mitochondrial respiratory chain	cellular	20.4244	1.34834e-009	61.5385
14.	cytochrome-c oxidase activity	molecular	20.2432	1.61622e-009	41.6667
15.	aerobic respiration	biological	19.606	3.0565e-009	57.1429

Figure 24: Viewing the GO Enrichment results from the one-way ANOVA (top) and the multi-way ANOVA (bottom)

End of Tutorial

This is the end of the tutorial. If you need additional assistance with this data set, contact the Partek Technical Support staff at +1-314-878-2329 or email us at support@partek.com.

References

Melov S., Tarnopolsky M.A., Beckman K., Felkey K., Hubbard A. Resistance exercise reverses aging in human skeletal muscle. PLoS One 2007;2(5): e465.

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