Statistical Analysis (1-way ANOVA)

Contents at a glance

I. Definition and Applications

One-way analysis of variance (ANOVA) tests allow you to determine if one given factor, such as drug treatment, has a significant effect on gene expression behavior across *any* of the groups under study. A significant p-value resulting from a 1-way ANOVA test would indicate that a gene is differentially expressed in at least one of the groups analyzed. If there are more than two groups being analyzed, however, the 1-way ANOVA does not specifically indicate which pair of groups exhibits statistical differences. Post Hoc tests can be applied in this specific situation to determine which specific pair/pairs are differentially expressed. This document will provide the necessary information for you to perform these analyses within GeneSpring.

II. Before Performing 1-way ANOVA – A Checklist

1. Do you have replicates for the experimental groups that you are about to compare? Statistical tests that compare one group to another, such as Student's t-test/ANOVA, need variance and means for each group. Without replicates, the variance for each group cannot be computed using standard methods. However, variance for experimental groups without replicates can be computed by applying the **GeneSpring Cross-Gene Error Model.** If no replicates are available, apply the **Error Model** based on **Deviation from 1** before proceeding. Please refer to the GeneSpring user manual, online tech notes, webinars, or cross-gene error model features sheet to learn more about the **Cross-Gene Error Model**.

2. Have you filtered out genes whose measurements are mostly unreliable?

3. Have you defined one parameter in the **Experiment Parameters** window indicating which sample belongs to which group?

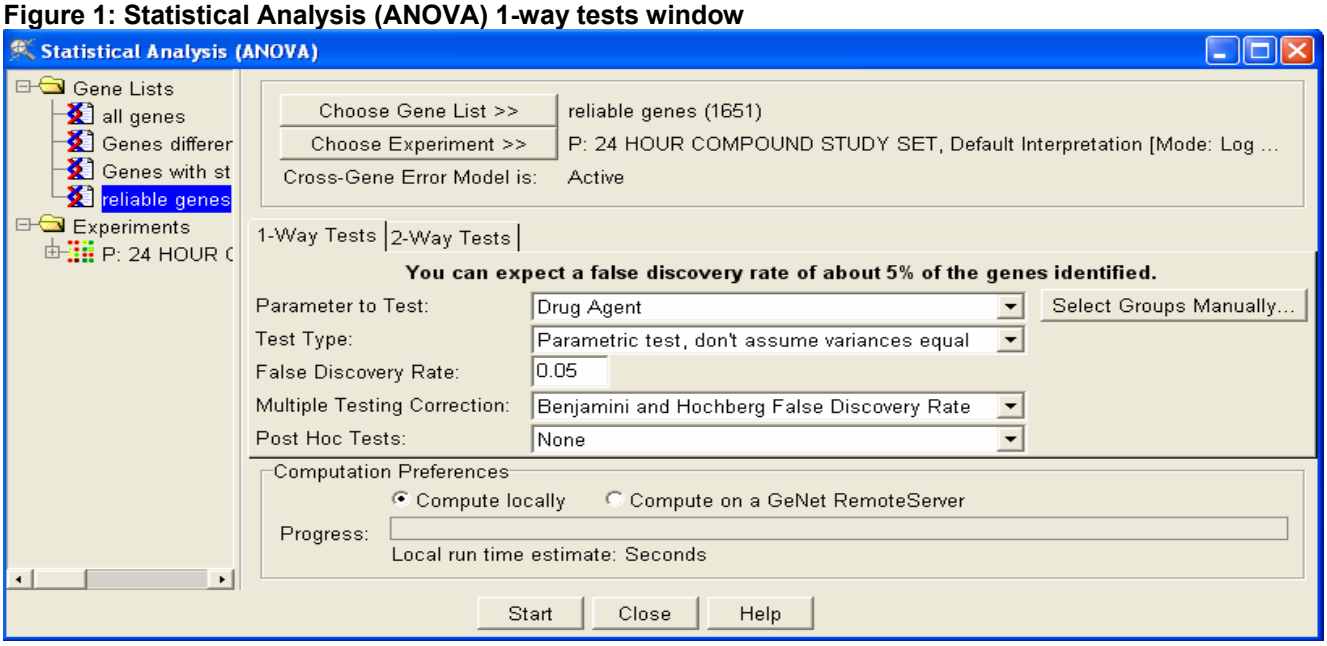
4. If you plan to use a parametric test, have you changed the analysis mode to **Log of Ratio** in the **Experiment Interpretation** window? Parametric tests assume that means of the populations under study are normally distributed (Gaussian distribution). Interpreting your data in log mode will make data more Normal/Gaussian than ratio mode.

It is mandatory that you either have replicates or apply the cross-gene error model if no replicates are available, in order to perform 1-way ANOVA for groups under study. It is also recommended (though not mandatory) that your statistical analysis be performed on a set of reliable genes, instead of **all genes,** on the chips.

III. Overview of the Statistical Analysis (1-way ANOVA tests) window

1. Go to **Tools** toolbar and select **Statistical Analysis (ANOVA)**
3. In the resulting window, select the **1-Way Tests** tab

Figure 1: Statistical Analysis (ANOVA) 1-way tests window



- **Choose Gene List:** Select the gene list containing the set of genes you would like to analyze. Statistical tests will be performed only on genes in the selected gene list. Again, it is recommended that the **all genes** gene list should not be used. Instead, use a list of genes that has been filtered to remove genes with measurements mostly in the noise range or mostly flagged Absent.

- **Choose Experiment:** Choose the experiment and its proper interpretation to analyze. If you are using parametric tests, then your experiment interpretation should be in log-of-ratio mode.

- **Parameter to Test:** Select the parameter and the underlying groups to compare. In the example shown above, the parameter, 'Drug Agent' was selected to compare the effect of different drug agents on Sprague-Dawley rats. If you would like to compare only selected conditions for this parameter, open the **Select Groups Manually** window, and uncheck the conditions that you would like to ignore. Only groups that are checked will be analyzed.

- **Test Type:** Select the appropriate 1-way ANOVA test type. If you are using a parametric test, make sure your data has been log-transformed (by selecting log-of-ratio mode in experiment interpretation window).

- **False Discovery Rate:** Indicates the overall rate of false positive. The wording for this option, and its final effect on the number of false positives, changes according to the multiple testing correction selected in the option below.

- **Multiple Testing Correction:** This test option is not required for analysis, but it will allow you to keep the overall rate of false positive low.

- **Post Hoc Tests:** This test option is also not required for analysis, but selecting this option will allow you to determine which pair(s) among the groups under study have expression means that are statistically different.

IV. General background on 1-way ANOVA test

a. Null Hypothesis:

The hypothesis for each gene is that there is no difference in the mean gene expression intensities in the groups tested. In other words, the gene will have equal means across every group.

Example of a specific null hypothesis:

There is no difference in the mean gene expression intensities for the bcl-2 gene across all rat groups treated with different drug agents.

b. Number of genes analyzed:

All genes in the selected gene list will be analyzed. If there are 10,000 genes on your gene list (assuming you have all required measurements for each of the genes), then there are 10,000 separate analyses being performed and each gene will have a separate p-value.

c. Test Options:

Options	Specific test used (analyzing 2 groups)	Specific test used (analyzing more than 2 groups)
Parametric (variances equal)	Student's T-test	ANOVA
Parametric (variances not equal)	Welch t-test	Welch ANOVA
Parametric (use all available error estimate)	Welch t-test using error model variances	Welch ANOVA using error model variances
Nonparametric	Wilcoxon-Mann-Whitney test	Kruskal-Wallis test

d. Recommendations:

- The Welch test (variances not assumed equal) is recommended for most cases. This is set as the default.
- The parametric test, use all available error estimate, is similar to Welch test but has better variance estimates. To use this option, the **Cross-gene error model** needs to be activated in the **Experiment Interpretation** window.
- Student's t-test/ANOVA (variances assumed equal) should be used if very few replicates are available, or if some groups being analyzed do not have replicates.
- Nonparametric test makes the least assumptions about your data but should be used only when there are more than 5 replicates per group.

e. P-value

Indicates the probability of getting a mean difference between the groups as high as what is observed by chance. The lower the p-value, the more significant the difference between the groups.

V. Multiple Testing Corrections (MTC):

When testing a set of genes for statistical significance across various groups, some of the genes may be falsely considered as statistically significant. If 10,000 genes are tested for differential expression between groups, with a significance p-value cutoff of 0.05, then the expected level of genes to be identified as significant by chance alone, even if there is no true differential expression, is 500 genes:

10,000 x 0.05 = 500 genes

Possible false positives = (# of genes) (p-value cutoff)

The purpose of a multiple testing correction is to keep the overall error rate/false positives to less than the user-specified p-value cutoff, even if thousands of genes are being analyzed.

a. Options

Test Type	Type of Error control	Genes identified by chance after MTC
Bonferroni	Family-wise error rate	If testing 10,000 genes with p-cutoff equals 0.05, then expects **0.05** genes to be significant by chance
Bonferroni step-down (Holm)		Same as above
Westfall and Young Permutation		Same as above
Benjamini and Hochberg	False Discovery Rate	If testing 10,000 genes with p-cutoff equals 0.05, then possible genes identified by chance is **5%** of genes that **passed** restriction (considered statistically significant)

b. Recommendations:

The recommended correction for multiple testing is Benjamini and Hochberg False Discovery Rate procedure. This procedure is the least stringent of all the methods mentioned above, but it provides a good balance between discovery of statistically significant differences in gene expression and protection against false positives (Type I error).

The stringency of MTC procedures mentioned increases as the number of genes being tested (genes on selected gene list) increases. The following example illustrates this situation:

If:

- number of genes on gene list = 10,000
- p-value cutoff = 0.05
- p-value for Gene A **without** MTC equals 0.000006

If the Bonferroni multiple testing correction was applied to this analysis, then the p- value for Gene A **with** MTC equals 0.06:

P–value with MTC = 10,000 x 0.000006

It is therefore recommended that you perform statistical analysis on a list of genes that have been filtered for unreliable genes since the multiple testing corrections are directly affected by the number of genes on your gene list.

For a more comprehensive discussion on multiple testing, see the Multiple Testing Corrections Features Sheet, refer to the user manual, or attend our Statistics workshop.

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VI. Post Hoc Tests:

1-way ANOVA determines whether a gene is differentially expressed in any of the conditions tested. However, it does not indicate which specific group pair(s) are the ones where statistical differences occur. Post Hoc Test can be used in conjunction with ANOVA to determine which specific group pair(s) are statistically different from each other.

a. Options:

Test Name	How it works
Tukey	All means for each condition are ranked in order of magnitude; group with lowest mean gets a ranking of 1. The pairwise differences between means, starting with the largest mean compared to the smallest mean, are tabulated between each group pair and divided by the standard error. This value, *q,* is compared to a Studentized range critical value. If *q* is larger than the critical value, then the expression between that group pair is considered to be statistically different.
Student-Newman-Keuls (SNK) test:	This test is similar to the Tukey test, except with regard to how the critical value is determined. All *q's* in Tukey's test are compared to the same critical value determined for that experiment; whereas all *q's* determined from SNK test are compared to a different critical value. This makes the SNK test slightly less conservative than the Tukey test.

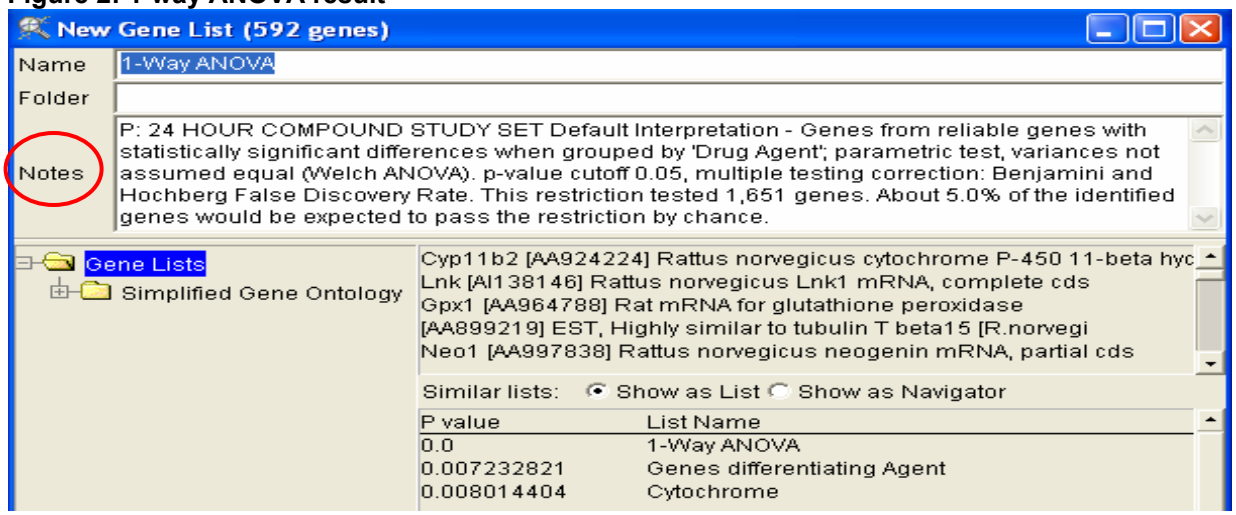
**** There are nonparametric and parametric versions of Tukey and Student-Newman-Keuls test. GeneSpring will apply the correct option based on whether a parametric or nonparametric ANOVA test was chosen.**

VII. Interpreting the Results

a. Results from 1-way ANOVA without Post Hoc test applied

Figure 2 below shows an example of a 1-way ANOVA result without a Post Hoc test applied. The **Notes** section indicates what setting was used for this analysis and the percentage of genes that could have been identified by chance. The genes in this gene list were found to have measurements considered statistically different across at least one group-pair. You cannot tell which exact group was differentially expressed from this analysis.

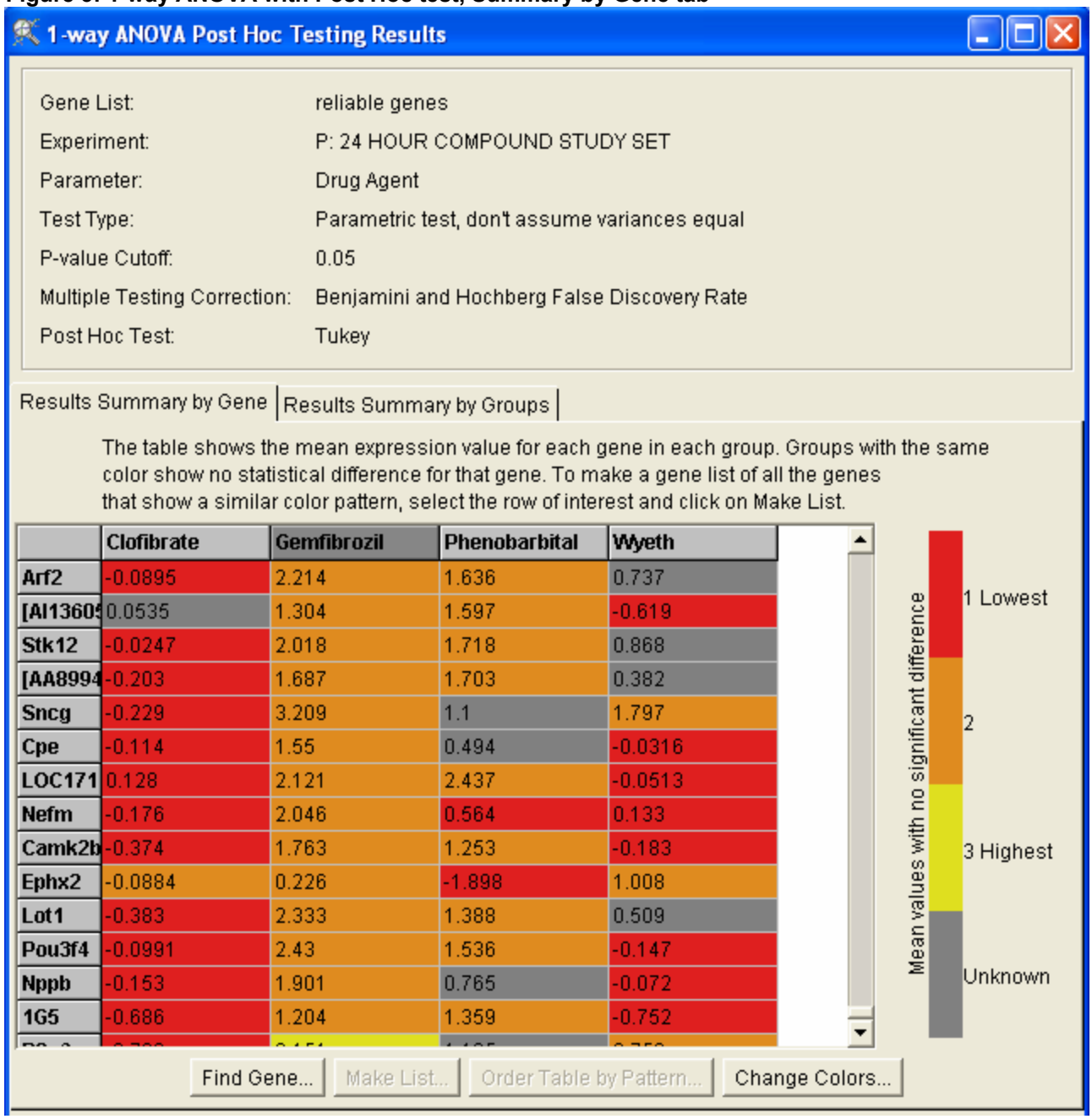
Figure 2: 1-way ANOVA result



b. Results of 1-way ANOVA with Post Hoc test applied

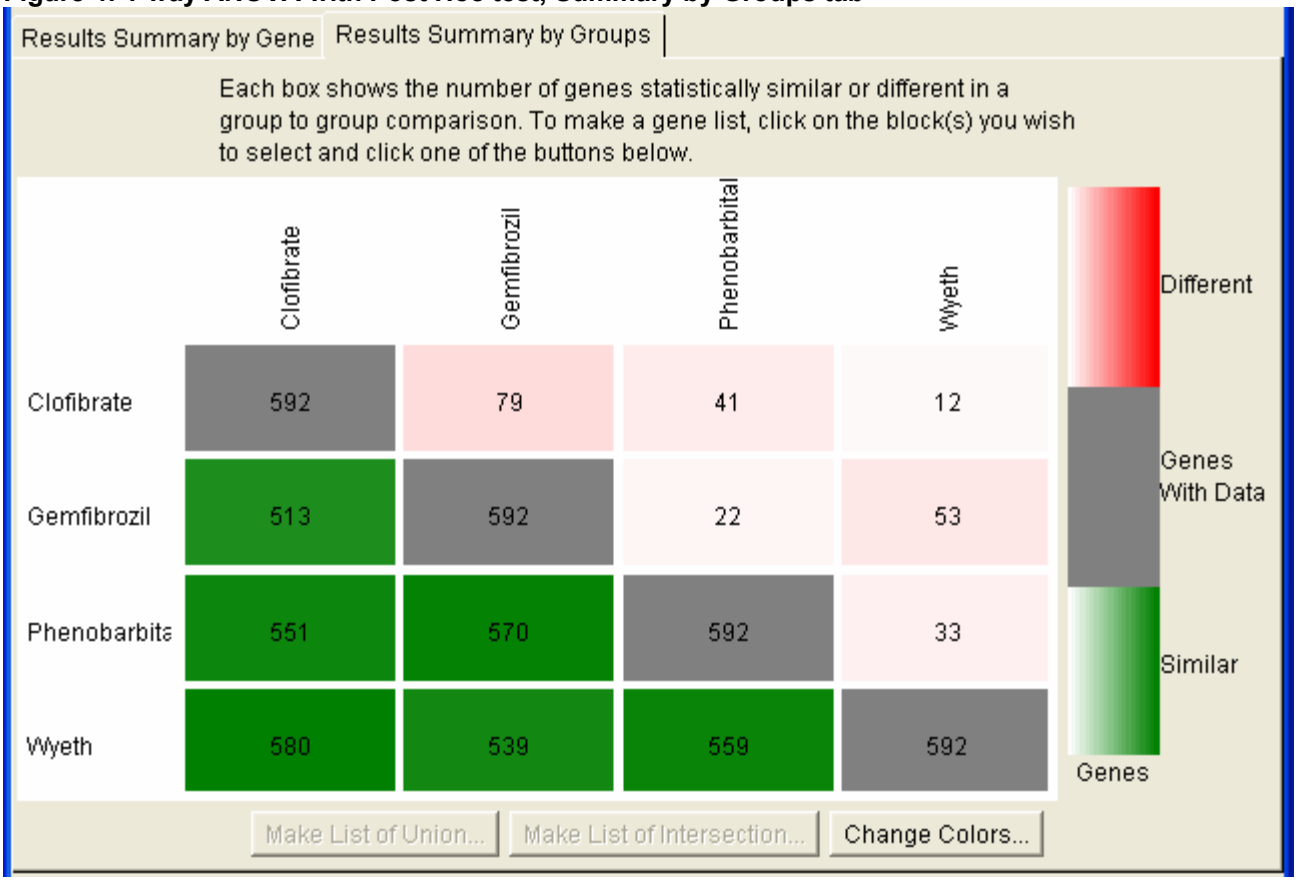
1-way ANOVA with Post Hoc test applied will return the window shown in Figure 2, and also the windows shown in Figures 3 and 4 below.

Figure 3: 1-way ANOVA with Post Hoc test, Summary by Gene tab



This window lists all the genes considered differentially expressed by statistical criteria. Groups with the highest color differential have the most significant difference. Groups with the same color show no statistical difference for that gene. A group colored grey is considered to be unknown because the significance of its mean difference cannot be determined with confidence from the test used.

Figure 4: 1-way ANOVA with Post Hoc test, Summary by Groups tab



This window indicates the total number of genes that are statistically differentially expressed between the groups being compared in the matrix. Greater color saturation indicates greater difference (or similarity). Total number of genes analyzed is shown in the box colored grey. Gene list can be generated from each, or combination of the boxes, by highlighting the appropriate boxes and selecting **Make List of Union** or **Make List of Intersection**.

VIII. Viewing P-values Generated

The associated p-value for the genes on this gene list could be viewed in GeneSpring using the following methods:

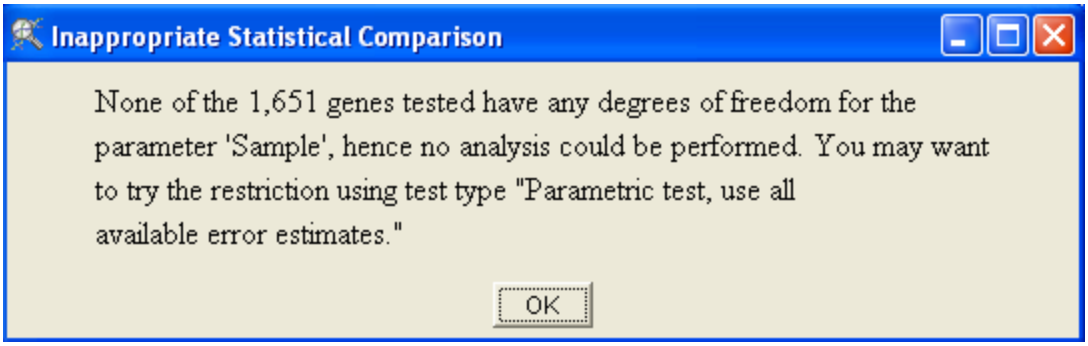
1. **Gene List Inspector:** Double-click on the selected gene list to open up the Gene List Inspector window. P values are shown under the **P-value** columns.

2. **Ordered List:** Select the gene list and go to **View** ⇒ **Ordered List**. Genes are displayed according to p-values: smallest p-values are on the left-hand side, highest p-values are on the right-hand side.

3. **Export out of GeneSpring:** Highlight the gene list and go to **Edit** ⇒ **Copy** ⇒ **Copy Annotated Gene List** and select to export out **Gene List Associated Values**.

IX. Most frequently asked questions and answers

Q. Why do I get an error message saying I have no degrees of freedom (such as the message shown below)?



A. This error message indicates that there are no replicates in the groups being compared. The degree of freedom is a mathematical way of representing the number of replicates/samples. Zero degrees of freedom indicates there are no replicates, and thus a 1-way ANOVA **CANNOT** be performed. If no replicates are available, but you would still like to perform a statistical analysis, then the **Cross-Gene Error Model** needs to be activated and the "Parametric test, use all available error estimate" must be used.

If you **do** have replicates but get this error message, then check your parameter to ascertain that it was set up correctly to indicate which samples are considered replicates. GeneSpring will not automatically know which samples are replicates unless specified correctly in the **Experiment Parameter** window and selected in the **Parameter to Test** field.

Q. Why do I get zero genes passing the restriction when I perform statistical analysis?

A. There can be several explanations for this observation:

i. Analysis criteria might be too stringent (low p-value cut-off and conservative multiple testing correction)
ii. Not enough replicates in each group resulting in insufficient power to detect real differences between groups under study
iii. Biologically, there may not be differential gene expression.

X. References

Zar, J. (1999) Biostatistical Analysis. (4th ed.) Upper Saddle River, NJ, Prentice Hall.