

Detect differentially expressed genes with ANOVA

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Analysis of variance (ANOVA) is a very powerful technique for identifying differentially expressed genes in a multi-factor experiment. In this data set, ANOVA will be used to generate a list of genes that are significantly differentially regulated by each treatment.

Adding factors and interactions

When setting up the ANOVA, the primary factors of interest, *Treatment* and *Time*, should be included. We will also include the interaction between *Treatment* and *Time*, *Treatment * Time*, because we are interested in whether different treatments behave differently over time. From our exploratory analysis using PCA, we also know that *Batch* is a major source of variation and needs to be included. Including *Batch* as a random factor will allow us to account for the batch effect.

- Select **Detect differentially expressed genes** from the *Analysis* section of the *Gene Expression* workflow
- Select **Treatment**, **Time**, and **Batch** in the *Experimental Factor(s)* panel
- Select **Add Factor >** to move the selections to the *ANOVA Factor(s)* panel
- Select both **Treatment** and **Time** in the *Experimental Factor(s)* panel by holding **Ctrl** on the keyboard while selecting each
- Select **Add Interaction >** to add the **Treatment * Time** interaction to the *ANOVA Factor(s)* panel (Figure 1)
- Do not select **OK** or **Apply**. We still need to add linear contrasts to the ANOVA model

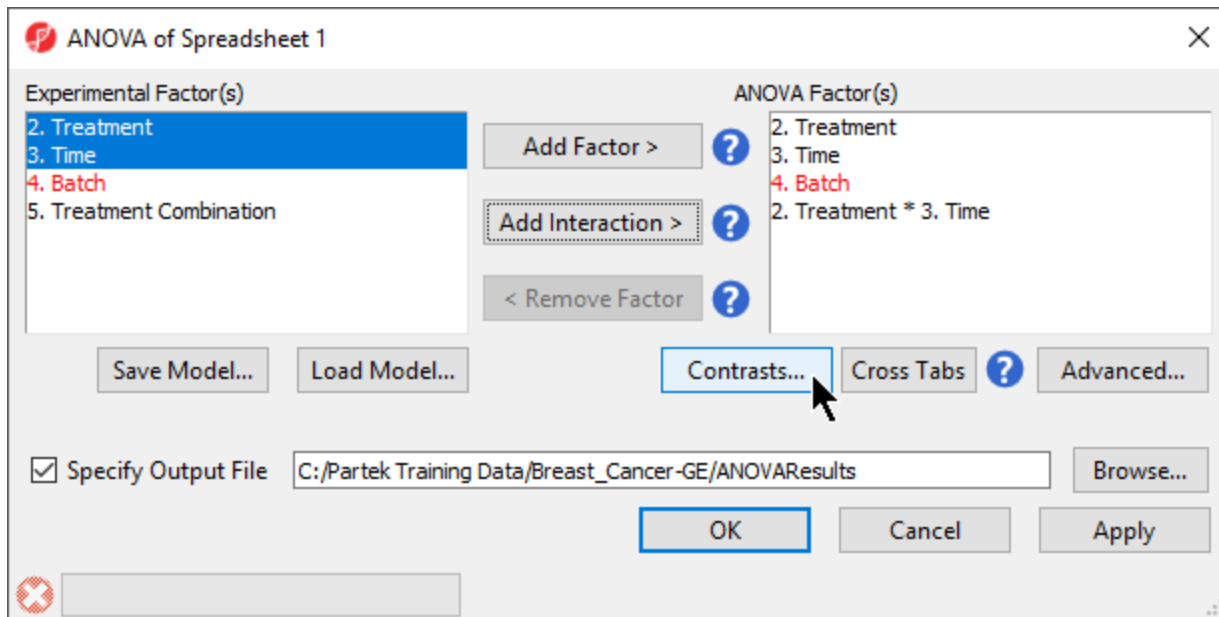


Figure 7. Adding factors and interactions to the ANOVA

Adding linear contrasts

ANOVA will output a p-value and F ratio for each factor or interaction; to get the fold-change and ratio between the different levels of a factor or interaction, linear contrasts, or comparisons, must be added.

- Select **Contrasts...** in the ANOVA dialog (Figure 1)
- Select **Yes** for *Data is already log transformed?*
- Select **Treatment * Time** from the *Select Factor/Interaction* drop-down menu

We will add contrasts comparing each of the three treatment groups to the control group.

- Select **E2 * 8** and **E2 * 48** from the *Candidate Level(s)* panel
- Select **Add Contrast Level >** to move them to the top panel (*Group 1*) on the right-hand side

The *Group 1* panel will be renamed after the contents of the panel. We can specify a name for the group.

- Set *Label* of the top panel to **E2**
- Select **Control * 0** from the *Candidate Level(s)* panel
- Select **Add Contrast Level >** to move it to the bottom panel (*Group 2*) on the right-hand side

- Set *Label* of the bottom panel to **Control**

The lower panel (*Group 2*) is considered the reference level. Because the data is log₂ transformed, the geometric mean will be used to calculate the fold change and mean ratio to place both on a linear scale instead of a log scale.

- Select **Add Contrast** (Figure 2)

Figure 8. Adding a contrast between E2 vs. Control at all time points.

To examine the time points of each treatment condition separately, we can select *Add Combinations* instead of *Add Contrast*. This adds every possible contrast for the levels in the *Group 1* and *Group 2* panels.

- Select **E2 * 8** and **E2 * 48** from the *Candidate Level(s)* panel
- Select **Add Contrast Level >** to move them to the top panel (*Group 1*) on the right-hand side
- Select **Control * 0** from the *Candidate Level(s)* panel
- Select **Add Contrast Level >** to move it to the bottom panel (*Group 2*) on the right-hand side
- Select **Add Combinations** to add contrasts for *E2 * 8 vs. Control * 0* and *E2 * 48 vs. Control * 0* (Figure 3)

Configure of Spreadsheet 1

Data is already log transformed? ☒ Yes ☐ No

Report comparisons as: ☒ Fold change ☐ Difference

Other Statistics: ☐ Estimate ☐ F ratio ☐ T statistic ☐ 95% CI for Fold change

Select Factor/Interaction: 2. Treatment * 3. Time

Candidate Level(s): Control * 0, E2 * 48, E2 * 8, E2+ICI * 48, E2+ICI * 8, E2+Ral * 48, E2+Ral * 8, E2+TOT * 48, E2+TOT * 8

Add Contrast Level > < Remove Contrast Level

Add Contrast < Remove Contrast Level

Add Contrast Add Combinations

| Contrast Name | Factor/Interaction | Status |
|-------------------------|------------------------|--------|
| E2 vs. Control | 2. Treatment * 3. Time | OK |
| E2 * 48 vs. Control * 0 | 2. Treatment * 3. Time | OK |
| E2 * 8 vs. Control * 0 | 2. Treatment * 3. Time | OK |

OK Cancel

Figure 9. Add Combinations creates contrasts for every combination of levels from the two group panels.

For this tutorial, we will not be considering the time points of each treatment condition individually. We can remove the *E2 * 8 vs. Control * 0* and *E2 * 48 vs. Control * 0* contrasts.

- Select **E2 * 8 vs. Control * 0** and **E2 * 48 vs. Control * 0** from the contrasts list
- Select **Delete**

We will now add contrasts for the other treatment conditions.

- Add contrasts for *E2+ICI vs. Control*, *E2+Ral vs. Control*, and *E2+TOT vs. Control* following the steps outlined for *E2 vs. Control*

There should now be four contrasts added to the contrasts panel (Figure 4).

Configure of Spreadsheet 1

Data is already log transformed? ☒ Yes ☐ No Base Report comparisons as: ☒ Fold change ☐ Difference

Other Statistics ☐ Estimate ☐ F ratio ☐ T statistic ☐ 95% CI for Fold change

Select Factor/Interaction:

Candidate Level(s)

- Control * 0
- E2 * 48
- E2 * 8
- E2+ICI * 48
- E2+ICI * 8
- E2+Ral * 48
- E2+Ral * 8
- E2+TOT * 48
- E2+TOT * 8

Add Contrast Level > < Remove Contrast Level

Add Contrast < Remove Contrast Level

| Contrast Name | Factor/Interaction | Status |
|--------------------|------------------------|--------|
| E2 vs. Control | 2. Treatment * 3. Time | OK |
| E2+ICI vs. Control | 2. Treatment * 3. Time | OK |
| E2+Ral vs. Control | 2. Treatment * 3. Time | OK |
| E2+TOT vs. Control | 2. Treatment * 3. Time | OK |

OK Cancel

Figure 10. Fully configured contrasts for the tutorial

- Select **OK** to add the contrasts to the ANOVA model

The *Contrasts...* button should now read *Contrasts Included* in the ANOVA dialog.

- Select **OK** to perform the ANOVA

ANOVA results spreadsheet

The result of the 3-way mixed model ANOVA is displayed in a new spreadsheet, *ANOVA-3way (ANOVAResults)* that is a child of the *Breast_Cancer.txt* spreadsheet. In *ANOVAResults*, each row represents a probe(set)/gene with the columns containing the results of the ANOVA (Figure 5).

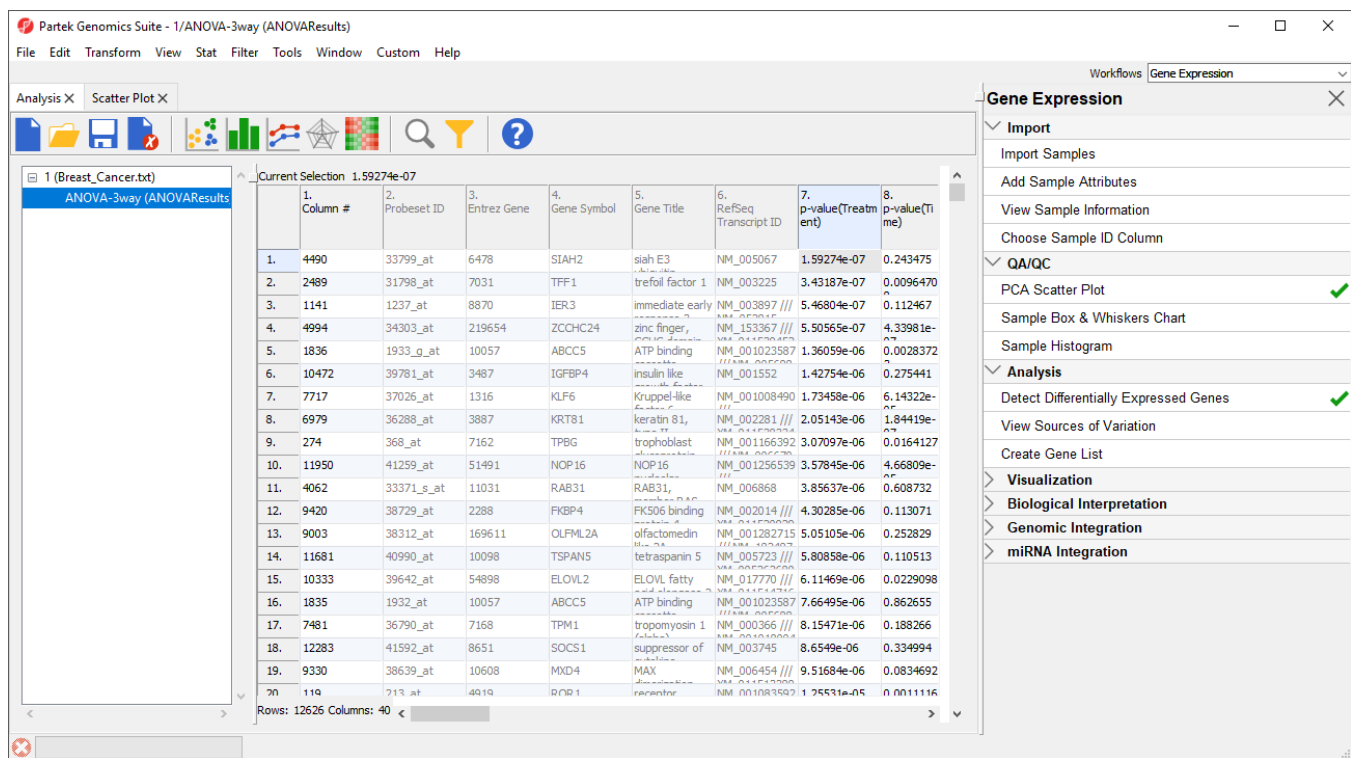


Figure 11. Viewing the ANOVA Results spreadsheet. Probe(sets)/genes are on rows and the ANOVA results are on columns.

By default, the rows are sorted in ascending order by the p-value of the first factor, which places the most significantly differentially expressed gene between different treatments at the top of the spreadsheet.

Each factor in the ANOVA adds p-value, F value, and SS value columns. F value is a ratio of signal to noise; high values indicate that the probe(set)/gene explains variation in the data set due to the factor. SS value is the sum of squares.

Each contrast in the ANOVA adds p-value, ratio, and fold-change columns. The p-value is calculated using log space. The ratio and fold change are calculated using linear space.

Viewing the sources of variation

Sources of variation captured in the ANOVA can be viewed for the entire data set or for individual probe(sets)/genes.

- Select **View Sources of Variation** from the *Analysis* section of the *Gene Expression* workflow

The Sources of Variation plot will open in a new tab (Figure 6).

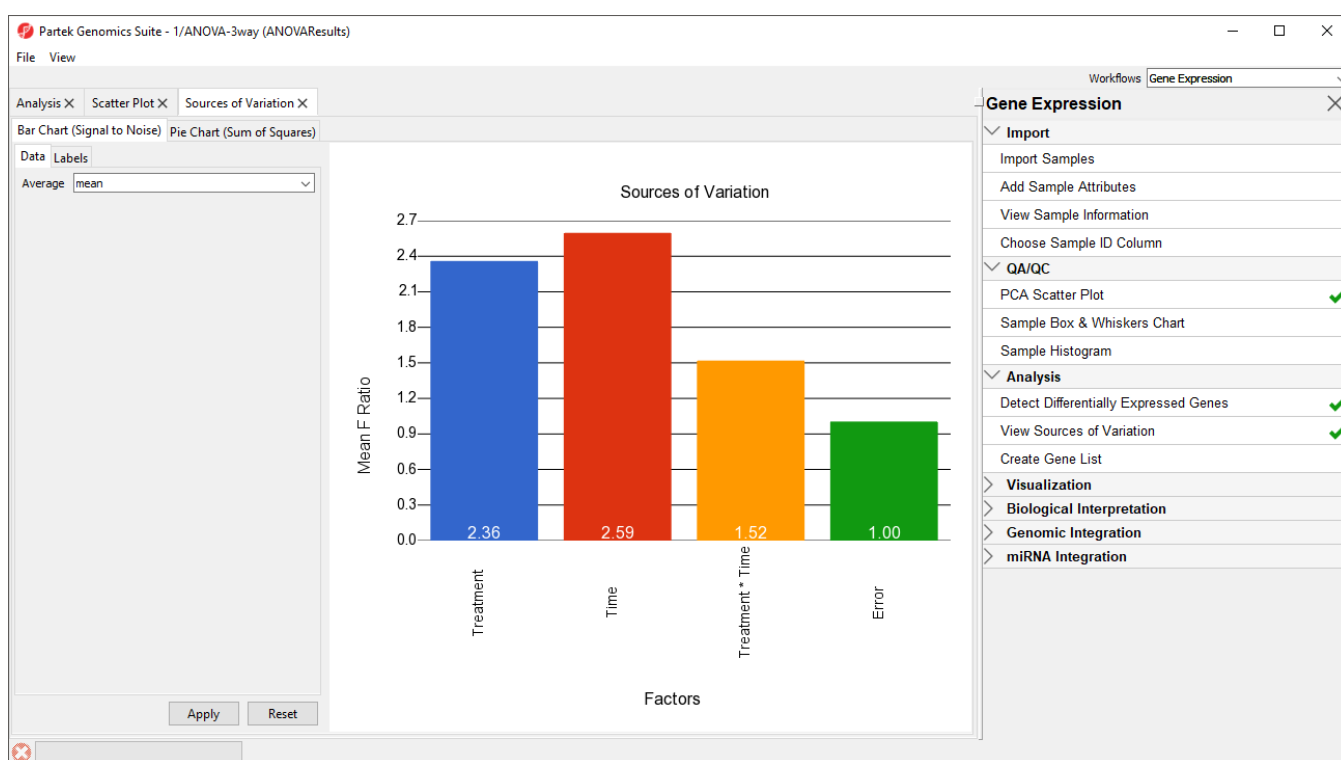


Figure 12. Viewing the sources of variation plot. Non-random factors are included when ANOVA is run using the default REML modle.

This plot presents the signal to noise ratio across all probe(set)/genes for each of the non-random factors and interactions in the ANOVA model. The y-axis represents the average mean square or F ratio, the ANOVA measure of variance, for all the probesets. Each bar is a factor and random error is also included. If the factor has a greater mean F ratio than Error, the factor contributed significant variation to the data set.

Note that *Batch* is not included as a factor. This is because *Batch* is a random factor and accounted for by the ANOVA model.

The sources of variation for each probe(set)/gene can be viewed individually.

- Right-click on a row header in the *ANOVAResults* spreadsheet
- Select **Sources of Variation** from the pop-up menu

The plot will open in a new tab. For additional plots that can be invoked from the ANOVA results spreadsheet, see the [Visualizations](#) user guide.

« [Exploring the data set with PCA](#) [Removing batch effects](#) »

Additional Assistance

If you need additional assistance, please visit [our support page](#) to submit a help ticket or find phone numbers for regional support.



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