

Method Sheet 25

How to perform a two way ANOVA to analyse dose response data

Overview

This method sheet explains how to perform a two way ANOVA statistical test to analyse dose response data when there are two different compounds being tested in the same experiment. Using this type of ANOVA is necessary when analysing data from experiments where different concentrations of a control substance (e.g. DMSO) and a candidate inhibitor were analysed in the same experiment. A two way (or two factor) ANOVA allows us to address three separate questions. First, does the concentration of the treatment significantly affect the observed responses? Second, does the type of treatment significantly affect the observed responses? Third, does the compound behave differently than the vehicle at different concentrations (technically called an interaction)? We must address each of these questions because there is a chance that the vehicle alone (DMSO) could impact the bioassay readout, for example, by slowing the growth of cells at high concentrations.

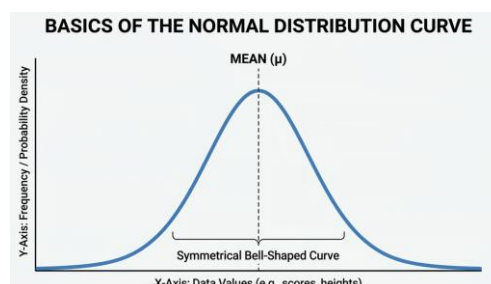
How ANOVA overcomes the problem of multiple testing

One of the key problems in statistics is the danger of discovering false positive “significant” results through multiple testing. This can occur when you complete multiple statistical tests at the same time. For example, if you compared 20 different drug treatments to a control using 20 individual t-tests, it is likely that at least one would give a false positive “significant” p-value, even if none of the drugs have any real activity, since the chances of obtaining a p-value of less than 0.05 is one in twenty just by chance alone.

ANOVA overcomes this by evaluating the variance of the entire dataset in a single step. Instead of many individual comparisons, it initially tests whether any differences exist between the group means. If this initial test is not significant (i.e. if the p-value for the interaction is greater than 0.05), you stop at that point, as it indicates there is no difference between the control substance and the test inhibitor. If this test is significant ($p < 0.05$), it tells you that at least some of the concentrations of the treatment are significantly different from the vehicle control. To find out which ones, and how significant these are, you must perform a post-hoc test - such as Tukey’s or Dunnett’s test. These post-hoc tests build on the earlier ANOVA analysis, automatically correcting for any impact of multiple testing, and tell you exactly which treatments and concentrations give significantly different results from the control condition and their p-values.

Testing for data normality in drug discovery data

ANOVAs are designed to analyse data that are **normally distributed**. That means, if you were to plot a curve showing how frequently each value appears in a large data set, it would appear as a bell shape curve. This curve is what we call the normal distribution (see image at right).



Although most forms of data from biological assays yield data that are normally distributed, we prefer not to assume that, and typically perform a statistical test, such as the **Shapiro-Wilk test**, to ensure the data are distributed in this way before choosing a statistical test. The problem with drug discovery data, and bioassay results generally, is that the number of independent experiments is typically small, perhaps three or four repeats of the same experiment (i.e. n=3 or 4).

This small number presents a difficulty for the Shapiro-Wilk test, as it can only work effectively with much larger 'n' values. A further problem common to drug discovery work is that a single screen can test hundreds or thousands of different compounds. Applying hundreds or thousands of normality tests would be both impractical and prone to type I errors. Therefore, when analysing screening data, we typically assume that the data are close enough to normal distribution for use in ANOVA on the basis of historical use of the assay and visual inspection to ensure there are no very large outliers within replicate measurements of the same treatments.

Statistical packages suitable for ANOVA testing

Microsoft Excel does not have the capability to perform ANOVA tests. Specialised statistical analysis software packages such as GraphPad PRISM and SPSS can be used to perform ANOVA, but require payment of a licence fee. Examples of software packages that are free to use and capable of performing ANOVA include R and Jamovi. As Jamovi uses a simple online web interface similar to commercial alternatives often used in industry, the method below explains how to perform a two way ANOVA using Jamovi.

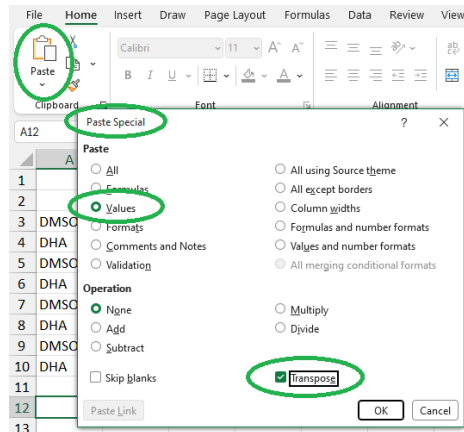
Arranging your data for analysis in Jamovi

- 1) The arrangement of data in your Excel file is slightly different from that required by Jamovi for analysis by ANOVA.
- 2) First, you must bring together the results from each of the three or four separate experiments into the same table.
- 3) List the concentrations of extract or compound used from the second cell of the top row, including the zero concentration.
- 4) Then insert headings in the first cell of the following rows for each of the two different treatments (e.g. DMSO and the compound dihydroartemisinin, DHA, in this example).
- 5) Then paste as values only (not cell references) the results from each of the different experiments - your table should now look something like this:

	A	B	C	D	E	F	G	H	I	J	K	L
1												
2		256	128	64	32	16	8	4	2	1	0	
3	DMSO	101%	104%	101%	100%	102%	95%	109%	103%	99%	100%	Exp 1
4	DHA	27%	30%	40%	44%	55%	69%	81%	86%	92%	100%	Exp 1
5	DMSO	111%	111%	111%	104%	108%	106%	117%	114%	102%	100%	Exp 2
6	DHA	33%	37%	45%	50%	57%	70%	85%	100%	97%	100%	Exp 2
7	DMSO	102%	103%	105%	109%	104%	103%	104%	98%	97%	100%	Exp 3
8	DHA	34%	37%	41%	46%	62%	65%	81%	90%	93%	100%	Exp 3
9	DMSO	102%	101%	107%	110%	100%	106%	99%	99%	91%	100%	Exp 4
10	DHA	29%	31%	35%	43%	55%	64%	82%	89%	89%	100%	Exp 4

- 6) Now we have to rotate this table by 90° so that the orientation of the data are compatible with the Jamovi interface.
- 7) Highlight and copy the whole table, including all of the concentrations.
- 8) Click on an empty cell below the selected table.

- 9) Click on 'Paste' then 'Paste Special' at the bottom of the menu options, and in the options box that appears, select both checkboxes for 'Values' and 'Transpose'



- 10) Your table of data values should now be rotated by 90°, as shown below:

12		DMSO	DHA	DMSO	DHA	DMSO	DHA	DMSO	DHA
13	256	101%	27%	111%	33%	102%	34%	102%	29%
14	128	104%	30%	111%	37%	103%	37%	101%	31%
15	64	101%	40%	111%	45%	105%	41%	107%	35%
16	32	100%	44%	104%	50%	109%	46%	110%	43%
17	16	102%	55%	108%	57%	104%	62%	100%	55%
18	8	95%	69%	106%	70%	103%	65%	106%	64%
19	4	109%	81%	117%	85%	104%	81%	99%	82%
20	2	103%	86%	114%	100%	98%	90%	99%	89%
21	1	99%	92%	102%	97%	97%	93%	91%	89%
22	0	100%	100%	100%	100%	100%	100%	100%	100%
23		Exp 1	Exp 1	Exp 2	Exp 2	Exp 3	Exp 3	Exp 4	Exp 4

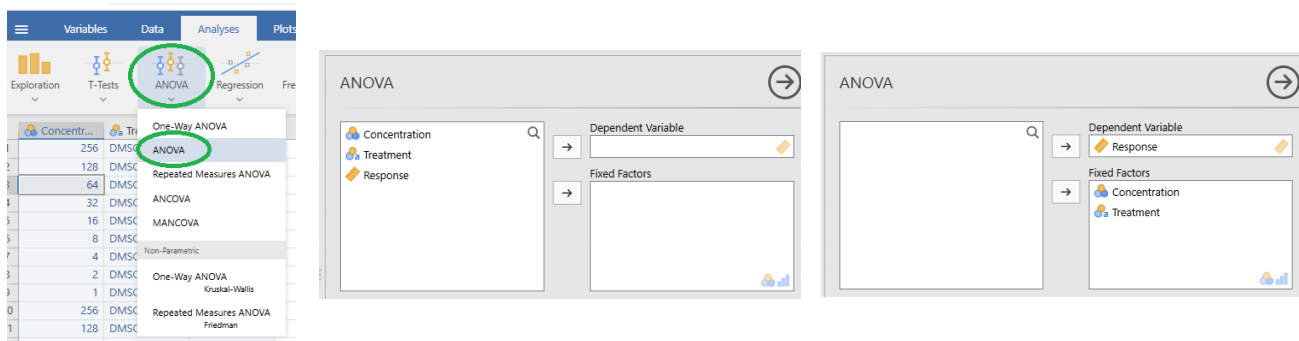
- 11) Delete the bottom row listing the experiment numbers shown in this example.
- 12) Prepare a new table of three columns below this with three headings, 'Concentration', 'Treatment' and 'Response'
- 13) Select all the concentration values from the first column of the previous table, then paste them 8 times (or 6 if only 3 experiments) without any empty gaps so there is a single column of 8 repeats of the same set of concentration values, starting just below the heading 'Concentration'
- 14) Just below the 'Treatment' heading, insert the name of the first treatment (should be DMSO) into every cell in the upper half of the second column.
- 15) Then, in all cells of the second half of the same column, insert the name of the second treatment (in this example, DHA), again without any gaps.
- 16) Highlight then drag (or cut and paste) each column of 10 result values for DMSO and stack them below each other without any gaps into the first half of the third column just below the 'Response' heading.
- 17) Do the same for each column of 10 result values for the DHA treatment, making sure they match with the correct treatment names in the second column.
- 18) You should now have three columns - the first contains the concentrations, the second contains the names of the treatments, and the third all of the results, without any gaps.
- 19) Now highlight all of the values in the response column, and convert them back from percentages into numerical values by formatting the cells as numbers with at least 3 decimal places.
- 20) It should now look something like the table shown at right:

25	Concentra	Treatmen	Response
26	256	DMSO	1.011
27	128	DMSO	1.042
28	64	DMSO	1.007
29	32	DMSO	0.998
30	16	DMSO	1.020
31	8	DMSO	0.946
32	4	DMSO	1.085
33	2	DMSO	1.029
34	1	DMSO	0.992
35	0	DMSO	1.000
36	256	DMSO	1.105
37	128	DMSO	1.110
38	64	DMSO	1.115

Using Jamovi for two way ANOVA

- 1) Jamovi is a free, open-source software application for statistical analysis, available at the following website: <https://www.jamovi.org/>
- 2) If you are able to download Jamovi to your own PC, it will run faster and be easier to use, but there is also a free to access cloud version available from the same website.
- 3) To use the cloud option, click on 'Jamovi Cloud' then 'Start' then choose a convenient log in method (e.g. with a Google account).
- 4) If you receive a message indicating that the server is busy, it is because too many users are accessing the free service presently, try again a little while later.
- 5) Highlight and copy your Excel data WITHOUT the headings.
- 6) Paste them from cell A1 of the Jamovi web sheet.
- 7) Double click on the heading letter 'A' of the first column and type 'Concentration' in the uppermost text box that appears.
- 8) Do the same for column 'B' and rename it with 'Treatment'
- 9) Do the same for column 'C' and rename it with 'Response', then click the up arrow in a circle to close the edit box.
- 10) Your data should now look something like this in Jamovi:
- 11) Click on the large 'ANOVA' button in the top ribbon, then select the 'ANOVA' option.
- 12) A new dialogue box appears as shown below, use the mouse to click on the term 'Response' and drag it across to move it into the 'Dependent variable' box.
- 13) Move 'Concentration' and 'Treatment' into the 'Fixed factors' box, as shown below:

	Concentr...	Treatment	Response
1	256	DMSO	1.011
2	128	DMSO	1.042
3	64	DMSO	1.007
4	32	DMSO	0.998
5	16	DMSO	1.020
6	8	DMSO	0.946
7	4	DMSO	1.085
8	2	DMSO	1.029
9	1	DMSO	0.992
10	0	DMSO	1.000
11	256	DMSO	1.105
12	128	DMSO	1.110



- 14) This tells Jamovi that the dependent variable (the response we are measuring) is present in the column with heading 'Response', and that there are two factors to analyse separately in this ANOVA: Concentration and Treatment.
- 15) As soon as you have done this, a table showing the results of the ANOVA appears, which should look something like this:

ANOVA

ANOVA - Response

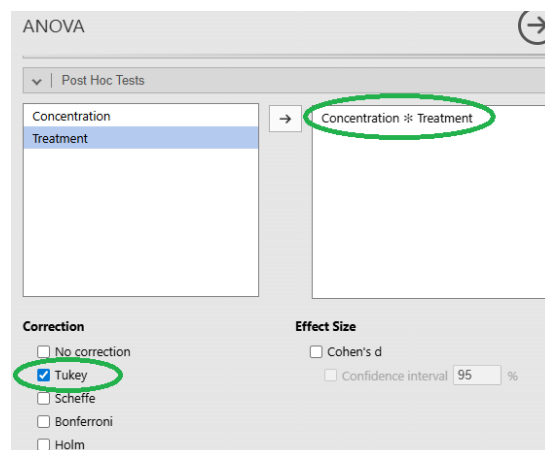
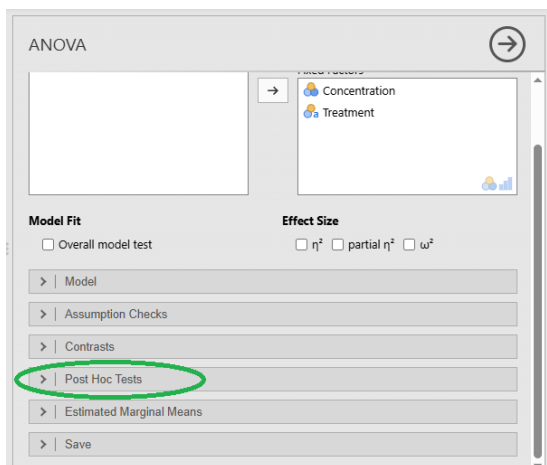
	Sum of Squares	df	Mean Square	F	p
Concentration	1.084	9	0.12049	62.2	< .001
Treatment	3.098	1	3.09803	1599.2	< .001
Concentration * Treatment	1.413	9	0.15704	81.1	< .001
Residuals	0.116	60	0.00194		

Interpreting the data from the initial ANOVA test

- 1) We have performed the ANOVA to answer three questions: (i) Does the concentration of the treatment significantly affect the observed responses? (ii) Does the type of treatment significantly affect the observed responses? (iii) Does the compound behave differently than the vehicle at different concentrations?
- 2) In the example shown above, the right-most column of the table shows three different p-values that give us answers to these three questions.
- 3) We typically set the threshold for significance as being any p-value that is less than 0.05, which means we can be 95% sure that the difference seen is real and not just arising from chance.
- 4) If the p-value for the third test is greater than 0.05, we would stop here and perform no further analysis, since that would indicate that the test substance does not have any inhibitory properties greater than the vehicle alone.
- 5) In this example, however, all three p-values are below the threshold, so we now want to ask an additional question, which concentrations of the compound of interest give a significant reduction in the response?
- 6) To answer this, we now have to extend the analysis with a post-hoc test

Using Tukey's post-hoc test in Jamovi

- 1) In the ANOVA control pane, scroll down and click on 'Post hoc tests'
- 2) Move the 'Concentration x Treatment' heading (technically called the interaction) into the right hand box to tell Jamovi that this is the main test we are interested in.
- 3) Select the check box for 'Tukey' to tell Jamovi which type of post-test to use.



- 4) A new table of results will appear on the right hand side of the screen.
- 5) This table contains far more information than you need for your dissertation, so you will have to focus only on those results that matter the most.
- 6) Scroll to the right-most column of the table, which should contain the **p-values** you are looking for with a column heading of P_{Tukey} (as shown in the left image below):

Post Hoc Tests

Post Hoc Comparisons - Concentration * Treatment

Comparison				Mean Difference	SE	df	t	P-value	
Concentration	Treatment	Concentration	Treatment						
256	DMSO	-	256	DHA	0.73225	0.0311	60.0	23.52799	<.001
-	-	-	128	DMSO	-0.00775	0.0311	60.0	-0.24902	1.000
-	-	-	128	DHA	0.70400	0.0311	60.0	22.62029	<.001
-	-	-	64	DMSO	-0.02050	0.0311	60.0	-0.65869	1.000
-	-	-	64	DHA	0.63875	0.0311	60.0	20.52373	<.001
-	-	-	32	DMSO	-0.01500	0.0311	60.0	-0.48197	1.000
-	-	-	32	DHA	0.58300	0.0311	60.0	18.73242	<.001
-	-	-	16	DMSO	0.00500	0.0311	60.0	0.16066	1.000
-	-	-	16	DHA	0.46900	0.0311	60.0	15.06948	<.001
-	-	-	8	DMSO	0.01625	0.0311	60.0	0.52213	1.000
-	-	-	8	DHA	0.37175	0.0311	60.0	11.94473	<.001
-	-	-	4	DMSO	-0.03000	0.0311	60.0	-0.96393	1.000
-	-	-	4	DHA	0.21925	0.0311	60.0	7.04474	<.001
-	-	-	2	DMSO	0.00475	0.0311	60.0	0.15262	1.000

Concentration	Treatment	Concentration	Treatment	Mean Difference	SE	df	t	P-value	
256	DMSO	-	256	DHA	0.73225	0.0311	60.0	23.52799	<.001
-	-	-	128	DMSO	-0.00775	0.0311	60.0	-0.24902	1.000
-	-	-	128	DHA	0.70400	0.0311	60.0	22.62029	<.001
-	-	-	64	DMSO	-0.02050	0.0311	60.0	-0.65869	1.000
-	-	-	64	DHA	0.63875	0.0311	60.0	20.52373	<.001
-	-	-	32	DMSO	-0.01500	0.0311	60.0	-0.48197	1.000
-	-	-	32	DHA	0.58300	0.0311	60.0	18.73242	<.001
-	-	-	16	DMSO	0.00500	0.0311	60.0	0.16066	1.000
-	-	-	16	DHA	0.46900	0.0311	60.0	15.06948	<.001
-	-	-	8	DMSO	0.01625	0.0311	60.0	0.52213	1.000
-	-	-	8	DHA	0.37175	0.0311	60.0	11.94473	<.001
-	-	-	4	DMSO	-0.03000	0.0311	60.0	-0.96393	1.000
-	-	-	4	DHA	0.21925	0.0311	60.0	7.04474	<.001
-	-	-	2	DMSO	0.00475	0.0311	60.0	0.15262	1.000
-	-	-	2	DHA	0.12750	0.0311	60.0	4.09671	.016
-	-	-	1	DMSO	0.07150	0.0311	60.0	2.30737	.728
-	-	-	1	DHA	0.11450	0.0311	60.0	3.67501	.054
0	DMSO	-	0	DMSO	-0.01100	0.0311	60.0	-0.35373	.728
-	-	-	0	DHA	0.04100	0.0311	60.0	1.31737	.998

- 7) Almost all of the p-values in the table will be of no value to your dissertation.
- 8) Instead, you must focus on the comparison between the zero concentration of DMSO, which is the negative control, and each of the different concentrations of DMSO and compound or extract of interest.
- 9) You find the appropriate p-value by looking first at columns 1 and 2 of the table, which give the ID of the first comparator.
- 10) Then look down the list in the third and fourth columns in the table to find the ID of the second comparator.
- 11) In the example shown above, we are comparing 0 μ M DMSO (the control) with 256 μ M DMSO, and the result of the test is a p-value of 0.998 (non-significant).
- 12) You should now scroll down this table and take a note of every p-value that compares the 0 μ M DMSO control condition to every other treatment.
- 13) You will use these p-values in your dissertation to put the stars on your dose response data charts.

Notes

- A much better post-hoc test to use in this type of analysis is Dunnett's post-hoc test, since this compares every treatment against the control, and ignores all the other possible comparisons, resulting in a much simpler and easier to read table of p-values.
- As Jamovi is unable to perform a Dunnett's test, we perform a Tukey test instead, but if you are able to perform ANOVA in another software package, you should choose the Dunnett's post-hoc test option.

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