

Analysis of Quantitative data One-Way + Two-Way ANOVA

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Comparison between more than 2 groups One factor = One predictor One-Way ANOVA

Signal-to-noise ratio



Signal
Noise= statistical significanceSignal
Noise= no statistical significanceNoise

SignalDifference between the meansNoiseVariability in the groups

= F ratio

One-Way Analysis of variance

Step 1: Omnibus test

• It tells us if there is a difference between the means but not which means are significantly different from which other ones.

Step 2: Post-hoc tests

• They tell us if there are differences between the means pairwise.



Source of variation	Sum of Squares	df	Mean Square	F	p-value
Between Groups	18.1	4	4.5	6.32	0.0002
Within Groups	51.8	73	0.71		
Total	69.9				



Continuous variable



5 differences: $\sum_{1}^{5} (mean_n - grand mean)^2$

Sum of squared errors Between the groups

Source of variation Sum of Squares		df	Mean Square	F	p-value
Between Groups	18.1				
Within Groups					
Total	69.9				

Continuous variable



Source of variation	Sum of Squares	df	Mean Squares	F	p-value
Between Groups	18.1				
Within Groups	51.8				
Total	69.9				



	Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Signal	Between Groups	18.1	k-1			
Noise	Within Groups	51.8	n-k			
	Total	69.9				

df: degree of freedom with df = n-1 n = number of values, k=number of groups Between groups: df = 4 (k-1) Within groups: df = 73 (n-k = n_1 -1 + ... + n_5 -1)



	Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Signal	Between Groups	18.1	4	4.5		
Noise	Within Groups	51.8	73	0.71		
	Total	69.9				

df: degree of freedom with df = n-1 18.2/4 = 4.5 51.8/73 = 0.71

Mean squares = **Sum of Squares / n-1 = Variance!**



Source of variation	Sum of Squares	df	Mean Squares	F ratio	p-value
Between Groups	18.1	4	4.5	6.34	0.0002
Within Groups	51.8	73	0.71		
Total	69.9				

Mean squares = Sum of Squares / n-1 = Variance

$$= ratio = \frac{Variance between the groups}{Variance within the groups (individual variability)} = \frac{4.5}{0.71} = 6.34$$

Comparison of more than 2 means

- Running multiple tests on the same data increases the **familywise error rate**.
- What is the familywise error rate?
 - The error rate across tests conducted on the same experimental data.
- One of the basic rules ('laws') of probability:
 - The Multiplicative Rule: The probability of the joint occurrence of 2 or more independent events is the product of the individual probabilities.

 $\mathsf{P}(\mathsf{A},\mathsf{B})=\mathsf{P}(\mathsf{A})\times\mathsf{P}(\mathsf{B})$

For example:

 $P(2 \text{ Heads}) = P(\text{head}) \times P(\text{head}) = 0.5 \times 0.5 = 0.25$

Familywise error rate

- <u>Example</u>: All pairwise comparisons between 3 groups A, B and C:
 A-B, A-C and B-C
- Probability of making the Type I Error: **5%**
 - The probability of not making the Type I Error is 95% (=1 0.05)
- Multiplicative Rule:
 - Overall probability of <u>no Type I errors</u> is: 0.95 * 0.95 * 0.95 = 0.857
- So the probability of making <u>at least one Type I Error</u> is 1-0.857 = 0.143 or 14.3%
 The probability has increased from E⁹/₂ to 14.3%
 - The probability has increased from 5% to 14.3%
- Comparisons between 5 groups instead of 3, the familywise error rate is 40% (=1-(0.95)ⁿ)

Familywise error rate

- <u>Solution</u> to the increase of familywise error rate: correction for multiple comparisons
 Post-hoc tests
- Many different ways to correct for multiple comparisons:
 - Different statisticians have designed corrections addressing different issues
 - e.g. unbalanced design, heterogeneity of variance, liberal vs conservative
- However, they all have **one thing in common**:
 - the more tests, the higher the familywise error rate: the more stringent the correction
- Tukey, Bonferroni, Sidak, Benjamini-Hochberg ...
 - Two ways to address the multiple testing problem
 - Familywise Error Rate (FWER) vs. False Discovery Rate (FDR)

Multiple testing problem

- **<u>FWER</u>**: **Bonferroni**: $\alpha_{adjust} = 0.05/n$ comparisons e.g. 3 comparisons: 0.05/3=0.016
 - Problem: very conservative leading to loss of power (lots of false negative)
 - 10 comparisons: threshold for significance: 0.05/10: 0.005
 - − Pairwise comparisons across 20.000 genes ⊗
- <u>FDR</u>: Benjamini-Hochberg: the procedure controls the expected proportion of "discoveries" (significant tests) that are false (false positive).
 - Less stringent control of Type I Error than FWER procedures which control the probability of <u>at least</u> <u>one</u> Type I Error
 - <u>More power</u> at the cost of increased numbers of Type I Errors.
- Difference between FWER and FDR:
 - a p-value of 0.05 implies that 5% of all tests will result in false positives.
 - a FDR adjusted p-value (or q-value) of 0.05 implies that 5% of significant tests will result in false positives.

One-Way Analysis of variance

Step 1: Omnibus test

• It tells us if there is (or not) a difference between the means but not which means are significantly different from which other ones.

Step 2: Post-hoc tests

- They tell us if there are (or not) differences between the means pairwise.
- A correction for multiple comparisons will be applied on the p-values.
- These post hoc tests should only be used when the ANOVA finds a significant effect.

Exercise: One-way ANOVA

protein expression.xlsx

- <u>Question</u>: is there a difference in protein expression between the 5 cell lines?
- 1 Plot the data
- 2 Check the assumptions for parametric test



Parametric tests assumptions

1	Test for normal distribution					
2	Anderson-Darling test					
3	A2*	0.3797	0.3141	1.166	1.439	0.2011
4	P value	0.3446	0.5029	0.0035	0.0007	0.8590
5	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
6	P value summary	ns	ns	**	***	ns
7						
8	D'Agostino & Pearson test					
9	K2	0.1236	0.7508	9.375	22.59	1.280
10	P value	0.9401	0.6870	0.0092	<0.0001	0.5274
11	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
12	P value summary	ns	ns	**	****	ns
13						
14	Shapiro-Wilk test					
15	W	0.9295	0.9535	0.8197	0.7531	0.9671
16	P value	0.3752	0.6888	0.0029	0.0004	0.7411
17	Passed normality test (alpha=0.05)?	Yes	Yes	No	No	Yes
18	P value summary	ns	ns	**	***	ns
19						
20	Kolmogorov-Smirnov test					
21	KS distance	0.1485	0.1704	0.1980	0.2058	0.1035
22	P value	>0.1000	>0.1000	0.0603	0.0424	>0.1000
23	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	No	Yes
24	P value summary	ns	ns	ns	*	ns
25						
26	Number of values	12	12	18	18	18
0.7						

Normal QQ plot





Parametric tests assumptions

1	Test for normal distribution					
2	Anderson-Darling test					
3	A2*	0.7849	0.3412	0.2086	0.1524	0.4727
4	P value	0.0295	0.4303	0.8386	0.9495	0.2138
5	Passed normality test (alpha=0.05)?	No	Yes	Yes	Yes	Yes
6	P value summary	*	ns	ns	ns	ns
7						
8	D'Agostino & Pearson test					
9	K2	2.037	0.6827	0.5884	0.8869	2.902
10	P value	0.3611	0.7108	0.7451	0.6418	0.2344
11	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
12	P value summary	ns	ns	ns	ns	ns
13						
14	Shapiro-Wilk test					
15	W	0.8553	0.9458	0.9657	0.9868	0.9313
16	P value	0.0427	0.5773	0.7142	0.9935	0.2050
17	Passed normality test (alpha=0.05)?	No	Yes	Yes	Yes	Yes
18	P value summary	*	ns	ns	ns	ns
19						
20	Kolmogorov-Smirnov test					
21	KS distance	0.2278	0.2049	0.1373	0.1016	0.1646
22	P value	0.0857	>0.1000	>0.1000	>0.1000	>0.1000
23	Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes
24	P value summary	ns	ns	ns	ns	ns
25						
26	Number of values	12	12	18	18	18
27						



One-Way ANOVA in Prism 8

nalyze Data Built-in analysis	×		Parameters: One-Way ANOVA (and Nonp.	arametric or Mixed)	
Which analysis?	Analyze which data sets?	Parameters: One-Way ANOVA (and Nonparametric or Mixed)	Experimental Design Repeated Measures	Multiple Comparisons Options Residuals	
Transform, Normalize Transform Transform concentrations (X) Normalize Prune rows Remove baseline and column math Transpose X and Y Eraction of total	A:Mussels B:Pellets C:Flakes Parameters: One-Way ANOVA (and Nonparametric or N Experimental Design Repeated Measures Multiple Com;	Experimental Design Repeated Measures Multiple Comparisons Options R Followup tests None. Compare the mean of each column with the mean of every other column. Compare the mean of each column with the mean of a control column. Control column: Column A : Mussels ✓ ✓	 Correct for multiple comparisons using Test: Tukey (recommended) Correct for multiple comparisons by co Test: Two-stage step-up method of Don't correct for multiple comparisons Test: Fisher's LSD test 	g statistical hypothesis testing, Recommended.	
	Experimental design No matching or pairing Each row represents matched, or repeated measure Group A Group B Group Data Set-A Data Set-B Data Set Y Y Y Assume Gaussian distribution of residuals? No Mark Han MOVA	 Compare the means of preselected pairs of columns. Selected pairs: Select Test for linear trend between column mean and left-to-right column order. Which test? Use choices on the Options tab to choose the test, and to set the defaults for future ANOVAs. 	Multiple comparisons options Swap direction of comparisons (A-B) v Report multiplicity adjusted P value for Each P value is adjusted to account for Family-wise significance and confidence le Graphing Graph confidence intervals. Graph ranks (nonparametric). Graph differences (repeated measure Additional results	VS. (B-A). or each comparison. or multiple comparisons. evel: Parameters: One-Way ANOVA (and Nonparametric or Mixed) Experimental Design Repeated Measures Multiple Comparisons Option What graphs to create? Measidual plot Correct model? Equal variance?	is Residuals
Grouped analyses Contingency table analyses Survival analyses	(a) Yes, Use ANOVA. (b) No. Use nonparametric test. Assume equal SDs? (c) Yes. Use ordinary ANOVA test. (c) No. Use Brown-Forsythe and Welch ANOVA tests.		Descriptive statistics for each data se Report comparison of models using A Report goodness of fit. Output Show this many significant digits (for eve P value style: GP: 0.1234 (ns), 0.0332 Make options on this tab be the default	Predicted Y	725
	Based on your choices (on all tabs), Prism will perform: - Ordinary one-way ANOVA. Learn	Learn Cancel	ОК	Actual residual Actual residual Diagnostics for residuals Are residuals dustered or heteroscedastic? Brown-Forsythe and Barlett's tests. Are the residuals Gaussian? Normality tests of Anderson-Darling, D'Agostino, Shapiro-Wilk and Ko Make options on this tab be the default for future One-Way ANOVAs.	Imogorov-Smirno

Have a go!

Analysis of variance: results

QQ plot

												1			
1	Table Analyzed	Transform of Prote	in expression				Resid	ual plot		Homosceda	sticity plot	<u>ब</u> 0.5			*
2	Data sets analyzed	A-E					1.0		0.8			sidu		Color.	
3							0.5-		- 0.6−	•	•	ě .			
4	ANOVA summary								idua	ŝ					
5	F	8.127							······ දි. 0.4-	8.		dic			
6	P value	<0.0001					-0.5-		역 적 0.2-			ے۔ <u>5</u>	959.5		
7	P value summary	****											6		
8	Significant diff. among means (P < 0.05)?	Yes	Homoger	neity	v of variance	e	-1.0	0.0 0.2	0.0	-0.2 0.	0 0.2		-0.5 ().0 0.f	5
9	R square	0.3081					Pred	icted Y		Predic	ted Y		Actual	residual	
10								-	Ano Writeaura						
11	Brown-Forsythe test					9	Ordinary one-way ANOVA								
12	F (DFn, DFd)	0.3031 (4, 73)					Multiple comparisons								
13	P value	0.4222													
14	P value summary	ns				2	Number of families	1							
15	Are SDs significantly different (P < 0.05)?	No				3	Alpha	0.05							
16						4		0.00							
17	Bartlett's test					5	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value			
18	Bartlett's statistic (corrected)	5.829				6	A vs. B	0.2505	-0.07808 to 0.5790	No	ns	0.2177	A-B		
19	P value	0.2123	F=0.6727	/0.08	278=8.13	7	A vs. C	0.07521	-0.2247 to 0.3751	No	ns	0.9555	A-C		
20	P value summary	ns				8	A vs. D	-0.3053	-0.6052 to -0.005359	Yes	*	0.0440	A-D		
21	Are SDs significantly different (P < 0.05)?	No				9	A vs. E	-0.1331	-0.4330 to 0.1669	No	ns	0.7275	A-E		
~						10	B vs. C	-0.1/53	-0.4/52 to 0.124/	No	ns	0.4807	B-C		
23	ANOVA table	SS		DF	MS	12	B vs. E	-0.3835	-0.6834 to -0.08360	Yes	**	0.0055	B-E		
24	Treatment (between columns)	2.691		4	0.6727	13	C vs. D	-0.3805	-0.6487 to -0.1122	Yes	**	0.0015	C-D		
25	Residual (within columns)	6.043		73	0.08278	14	C vs. E	-0.2083	-0.4765 to 0.05998	No	ns	0.2021	C-E		
26	Total	8,734		77		15	D vs. E	0.1722	-0.09604 to 0.4405	No	ns	0.3839	D-E		
5						16						\square			
68	Normality of Residuals					17	Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
29	Test name	Statistics		P value	Passed normality test (alu	10	Avs. B	-0.03123	-0.2817	0.2505	0.1175	12	12	3.016	73
30	Anderson-Darling (A2*)	0 4188		0.3201	Yes	20	Avs. C Avs. D	-0.03123	0.2740	-0.3053	0.1072	12	18	4 026	73
31	D'Agostino-Pearson omnibus (K2)	0 1697		0.9187	Yes	21	A vs. E	-0.03123	0.1018	-0.1331	0.1072	12	18	1.755	73
32	Shapiro-Wilk (W)	0.9863		0.5717	Ves	22	B vs. C	-0.2817	-0.1064	-0.1753	0.1072	12	18	2.311	73
33	Kolmogorov-Smirnov (distance)	0.07889		0 1000	Yes	23	B vs. D	-0.2817	0.2740	-0.5557	0.1072	12	18	7.330	73
33	(distance)	0.01000		0.1000	100	24	B vs. E	-0.2817	0.1018	-0.3835	0.1072	12	18	5.058	73
a	a	-				25	C vs. D	-0.1064	0.2740	-0.3805	0.09590	18	18	5.611	73
	T					26	C vs. E	-0.1064	0.1018	-0.2083	0.09590	18	18	3.071	73
	INC	Finality				21	U VS. E	0.2740	0.1018	0.1722	0.09590	18	18	2.540	73
						20									

Ordinary one-way ANOVA ANOVA results

Analysis of variance: results



Exercise: Repeated measures ANOVA neutrophils.xlsx



- A researcher is looking at the difference between 4 cell groups. He has run the experiment 5 times. Within each experiment, he has neutrophils from a WT (control), a KO, a KO+Treatment 1 and a KO+Treatment2.
- **Question**: Is there a difference between KO with/without treatment and WT?



Answer: There is a significant difference from WT for the first and third groups.

Comparison between more than 2 groups Two factors = Two predictors Two-Way ANOVA

Two-way Analysis of Variance (Factorial ANOVA)

Source of variation	Sum of	Df	Mean Square	F	p-value
	Squares				
Variable A (Between Groups)	2.665	4	0.6663	8.42	<0.0001
Within Groups (Residual)	5.775	73	0.0791		
Total	8.44	77			

Source of variation	Sum of Squares	Df	Mean Square	F	p-value
Variable A * Variable B	1978	2	989.1	F (2, 42) = 11.91	P < 0.0001
Variable B (Between groups)	3332	2	1666	F (2, 42) = 20.07	P < 0.0001
Variable A (Between groups)	168.8	1	168.8	F (1, 42) = 2.032	P = 0.1614
Residuals	3488	42	83.04		



- Interaction plots: Examples
 - Fake dataset:
 - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)

Genotype	Condition	Value
Genotype 1	Condition 1	74.8
Genotype 1	Condition 1	65
Genotype 1	Condition 1	74.8
Genotype 1	Condition 2	75.2
Genotype 1	Condition 2	75
Genotype 1	Condition 2	75.2
Genotype 2	Condition 1	87.8
Genotype 2	Condition 1	65
Genotype 2	Condition 1	74.8
Genotype 2	Condition 2	88.2
Genotype 2	Condition 2	75
Genotype 2	Condition 2	75.2

- Interaction plots: Examples
 - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)



Single Effect

- Interaction plots: Examples
 - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)

Zero or Both Effect



- Interaction plots: Examples
 - <u>2 factors</u>: **Genotype** (2 levels) and **Condition** (2 levels)

Interaction



Alcohol	None		21	Pints	4 Pints		
Gender	Female	Male	Female	Male	Female	Male	
	65	50	70	55	45	30	
	70	55	65	65	60	30	
	60	80	60	70	85	30	
	60	65	70	55	65	55	
	60	70	65	55	70	35	
	55	75	60	60	70	20	
	60	75	60	50	80	45	
	55	65	50	50	60	40	

Example: goggles.xlsx

- The 'beer-goggle' effect
 - The term refers to finding people more attractive after you've had a few beers. Drinking beer provides a warm, friendly sensation, lowers your inhibitions, and helps you relax.
- <u>Study</u>: effects of alcohol on mate selection in night-clubs.
- Pool of independent judges scored the levels of attractiveness of the person that the participant was chatting up at the end of the evening.
- **Question**: is subjective perception of physical attractiveness affected by alcohol consumption?
 - Attractiveness on a scale from 0 to 100

None 2 Pints 4 Pints

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1978	2	989.1	F (2, 42) = 11.91	< 0.0001
Alcohol Consumption	3332	2	1666	F (2, 42) = 20.07	< 0.0001
Gender	168.8	1	168.8	F (1, 42) = 2.032	0.1614
Residual	3488	42	83.04		

<u>With significant interaction</u> (real data)

Without significant interaction (fake data)

ANOVA table	SS	DF M	S F (DFn, DFd)	P value
Interaction	7.292	2 3.64	6 F (2, 42) = 0.06872	0.9337
Alcohol Consumption	5026	2 251	3 F (2, 42) = 47.37	< 0.0001
Gender	438.0	1 438.	0 F (1, 42) = 8.257	0.0063
Residual	2228	42 53.0	5	

Analyze Data			×					
	Parameters: Two-Way ANOVA	(or Mixed Model)			×			
Built-in analysis 🗸 🗸	RM Design RM Analysis Fac	ctor names Multiple (Comparisons Options	Residuals				
Which analysis?	Data arrangement					~]	
Transform, Normalize	Table format:	Parameters: Iwo-Wa	iy ANOVA (or Mixed M					
Transform	Grouped	RM Design RM Ana	lysis Factor names M	ultiple Compariso	ons Options Residuals			
Transform concentrations (X)	A:Y	Data arrangement						
Normalize	1 Title	Table format	Group A	Group	Group C	13		
Prune rows	2 Title	Grouped	Title	Title	Title	1 3		
Remove baseline and column math	3 Title		A:Y1 A:Y2	B:Y1	B:Y2 C:Y1 C:Y2			Parameters: Two-Way ANOVA (or Mixed Model)
Transpose X and Y	4 Title	1 Title				3		
Fraction of total	Matching by which facto	2 Title				3		RM Design RM Analysis Factor names Multiple Comparisons Options Residuals
XY analyses	Each column represents	3 Title			Parameters: Two-Way AN	OVA (or Mi	ixed Model)	Multiple comparisons test
 Column analyses 	Each row represents a	4 Title	hand	www			Multiple Companiones In the Land	Test: Sidak (more power, recommended)
Grouped analyses	Assume sphericity (equi	Factor names			RM Design RM Analysis Factor names Multiple Comparisons Options Residua			O Correct for multiple comparisons by controlling the Ealse Discovery Rate.
Two-way ANOVA (or mixed model)	Assume sphericity (equa	Name the factor th	nat defines the columns:	Gender	What kind of comparis	son?		Test: Two-stage step-up method of Benjamini, Krieger and Yekutieli (recommended) 💦 🗸
Three-way ANOVA (or mixed model)	Wee Ne erregelisser-Gr	Name the factor th	int definites the <u>c</u> olumns	Alcohol	Compare each cell me	an with the c	other cell mean in that row	Opon't correct for multiple comparisons. Each comparison stands alone.
Row means with SD or SEM	Tes. No correction.	Name the factor th	hat defines the <u>r</u> ows:	Alconol	Group A Group B			Te <u>s</u> t: Fisher's LSD test
Multiple t tests - one per row		Name of matched g	<u>s</u> et (i.e. person or block)	Subject		Data Set-A	A Data Set-B	Multiple comparisons options
E Contingency table analyses					A:Y1	A	A:Y2 B:Y1 B:Y2	Swap direction of comparisons (A-B) vs. (B-A).
Survival analyses					1	Mean	Mean	Each P value is adjusted to account for multiple comparisons.
Parts of whole analyses					2	(Mean)+	Mean {	Family-wise significance and confidence level: 0.05 (95% confidence interval)
Multiple variable analyses	Based on your choices (on				3	Man	Maan	Graphing options
• Nested analyses	- Ordinary two-way And				3	mean	- Indan	Graph confidence intervals.
Generate curve								Additional results
🗄 Simulate data					How many compariso	ns?		Narrative <u>r</u> esults.
					Compare each colur	nn mean wit	h every other column mean.	Show cell/row/column/grand means.
					Compare each colur	nn mean wit	h the control column mean.	Report goodness of fit.
					Control column:	roup A : Fer	nale ~	Show this many significant disits (for avarything avcent B values)
	Help			Learn				P value style: GP: 0.1254 (hs), 0.0552 (*), 0.0021 (**), 0.0 V
					Which toot?			Make options on this tab be the default for future Two-Way ANOVAs.
					Use choices on the Ont	ions tab to c	hoose the test, and to set the defaults for	
					future ANOVAs.			Learn Cancel OK
							I	
Have a cal							Learn Cancel	ОК
паче а до!							Canter	

