Microarray Data Analysis
Finding Differential Expressed Genes

國立臺灣大學 資訊所
Course: 生物資訊之統計與計算方法
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Outlines

- **Introduction: Three Demo Data Sets**
- **Hypothesis Testing**
  - ☐ **Parametric and Non-parametric Statistics**
- **Multiple Testing Corrections**
- **Post Hoc Tests**
- **Software: GeneSpring GX v7.3.1**

- **Bootstrap Analysis**
- **Permutation Test**

- **Significance Analysis of Microarrays (SAM)**
  - ☐ **Software: SAM**

- **Software: affylmGUI Demo**

Finding Differentially Expressed Genes

→ More than two samples

→ Two-sample (independent)

→ Paired-sample (dependent)

MA Table	exp01	exp02	exp03	exp04	exp05	exp•••	exp p
gene001	-0.48	-0.42	0.87	0.92	0.67		-0.35
gene002	-0.39	-0.58	1.08	1.21	0.52		-0.58

MA Table	exp01	exp02	exp03	exp04	exp05	exp•••	exp p	p-values	
gene001	-0.48	-0.42	0.87	0.92	0.67		-0.35	0.067	
gene002	-0.39	-0.58	1.08	1.21	0.52		-0.58	0.052	
gene003	0.87	0.25	-0.17	0.18	-0.13		-0.13	0.013	*
gene004	1.57	1.03	1.22	0.31	0.16		-1.02	0.016	*
gene005	-1.15	-0.86	1.21	1.62	1.12		-0.44	0.112	
gene006	0.04	-0.12	0.31	0.16	0.17		0.08	0.017	*
gene007	2.95	0.45	-0.40	-0.66	-0.59		-0.76	0.059	
gene008	-1.22	-0.74	1.34	1.50	0.63		-0.55	0.063	
gene009	-0.73	-1.06	-0.79	-0.02	0.16		0.03	0.516	
gene010	-0.58	-0.40	0.13	0.58	-0.09		-0.45	-0.009	*
gene011	-0.50	-0.42	0.66	1.05	0.68		0.01	0.068	
gene012	-0.86	-0.29	0.42	0.46	0.30		-0.63	0.030	*
gene013	-0.16	0.29	0.17	-0.28	-0.02		-0.04	0.002	*
gene014	-0.36	-0.03	-0.03	-0.08	-0.23		-0.21	0.423	
gene015	-0.72	-0.85	0.54	1.04	0.84		-0.64	0.084	
gene016	-0.78	-0.52	0.26	0.20	0.48		0.27	0.048	
gene017	0.60	-0.55	0.41	0.45	0.18		-1.02	0.018	*
gene018	-0.20	-0.67	0.13	0.10	0.38		0.05	0.538	
gene019	-2.29	-0.64	0.77	1.60	0.53		-0.38	0.053	
gene020	-1.46	-0.76	1.08	1.50	0.74		-0.70	0.074	
gene021	-0.57	0.42	1.03	1.35	0.64		-0.40	0.764	
gene022	-0.11	0.13	0.41	0.60	0.23		0.19	0.423	
gene•••									
gene n	-1.79	0.94	2.13	1.75	0.23		-0.66	0.723	

Microarray Data Matrix

gene001	-0.48	-0.42	0.87	0.92	0.67		-0.35

gene022	-0.11	0.13	0.41	0.60	0.23		0.19

Cy 5: treatment

Cy 3: control

■ Select a statistic which will rank the genes in order of evidence for differential expression, from strongest to weakest evidence.

(Primary Importance): only a limited number of genes can be followed up in a typical biological study.

■ Choose a critical-value for the ranking statistic above which any value is considered to be significant.

Example 1: Breast Cancer Dataset

cDNA microarrays

- Samples are taken from 20 breast cancer patients, before and after a 16 week course of doxorubicin chemotherapy, and analyzed using microarray. There are 9216 genes.

- **Paired data**: there are two measurements from each patient, one before treatment and one after treatment.

- These two measurements relate to one another, we are interested in the difference between the two measurements (the log ratio) to determine whether a gene has been up-regulated or down-regulated in breast cancer following that treatment.

log ratio

9216 x 20

Perou CM, et al, (2000), Molecular portraits of human breast tumours. Nature 406:747-752.
Stanford Microarray Database: http://genome-www.stanford.edu/breast_cancer/molecularportraits/

Example 2: Leukemia Dataset

- Bone marrow samples are taken from
  - 27 patients suffering from acute lymphoblastic leukemia (ALL，急性淋巴細胞白血病) and
  - 11 patients suffering from acute myeloid leukemia (AML，急性骨髓性白血病) and analyzed using Affymetrix arrays.
  - There are 7070 genes.

- **Unpaired data**: there are two groups of patients (ALL, AML).

- We wish to identify the genes that are up- or down-regulated in ALL relative to AML. (i.e., to see if a gene is differentially expressed between the two groups.)

MA Table	exp01	exp02	exp03	exp04	exp05	exp•••	exp	p
gene001	-0.48	-0.42	0.87	0.92	0.67			-0.35
gene002	-0.39	-0.58	1.08	1.21	0.52			-0.58
gene003	0.87	0.25	-0.17	0.18	-0.13			-0.13
gene004	1.57	1.03	1.22	0.31	0.16			-1.02
gene005	-1.15	-0.86	1.21	1.62	1.12			-0.44
gene006	0.04	-0.12	0.31	0.16	0.17			0.08
gene007	2.95	0.45	-0.40	-0.66	-0.59			-0.76
gene008	-1.22	-0.74	1.34	1.50	0.63			-0.55
gene009	-0.73	-1.06	-0.79	-0.02	0.16			0.03
gene010	-0.58	-0.40	0.13	0.58	-0.09			-0.45
gene011	-0.50	-0.42	0.66	1.05	0.68			0.01
gene012	-0.86	-0.29	0.42	0.46	0.30			-0.63
gene013	-0.16	0.29	0.17	-0.28	-0.02			-0.04
gene014	-0.36	-0.03	-0.03	-0.08	-0.23			-0.21
gene015	-0.72	-0.85	0.54	1.04	0.84			-0.64
gene016	-0.78	-0.52	0.26	0.20	0.48			0.27
gene017	0.60	-0.55	0.41	0.45	0.18			-1.02
gene018	-0.20	-0.67	0.13	0.10	0.38			0.05
gene019	-2.29	-0.64	0.77	1.60	0.53			-0.38
gene020	-1.46	-0.76	1.08	1.50	0.74			-0.70
gene021	-0.57	0.42	1.03	1.35	0.64			-0.40
gene022	-0.11	0.13	0.41	0.60	0.23			0.19
gene•••								
gene n	-1.79	0.94	2.13	1.75	0.23			-0.66

7070 x (27+11)

Golub, T.R et al. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531--537.
Cancer Genomics Program at Whitehead Institute for Genome Research
http://www.broad.mit.edu/cgi-bin/cancer/datasets.cgi
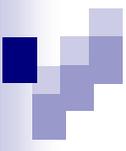
Example 3: Small Round Blue Cell Tumors (SRBCT) Dataset

cDNA microarrays

- There are four types of small round blue cell tumors of childhood:
  - Neuroblastoma (NB) (12),
  - Non-Hodgkin lymphoma (NHL) (8),
  - Rhabdomyosarcoma (RMS) (20) and
  - Ewing tumours (EWS) (23).
  - Sixty-three samples from these tumours have been hybridized to microarray.

- We want to identify genes that are differentially expressed in one or more of these four groups.

More on SRBCT:
http://www.thedoctorsdoctor.com/diseases/small_round_blue_cell_tumor.htm

Khan J, Wei J, Ringner M, Saal L, Ladanyi M, Westermann F, Berthold F, Schwab M, Antonescu C, Peterson C and Meltzer P. Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. Nature Medicine 2001, 7:673-679
Stanford Microarray Database

Calculate the expression ratio in control and experimental cases and to rank order the genes. Chose a threshold, for example at least 2-fold up or down regulation, and selected those genes whose average differential expression is greater than that threshold.

Problems: it is an arbitrary threshold.

- ☐ In some experiments, no genes (or few gene) will meet this criterion.
- ☐ In other experiments, thousands of genes regulated.

$$\frac{cS1=200}{cS2=100} = 2$$

BG=100
S1=300

BG=100
S2=200

- ☐ s2 close to BG, the difference could represent noise.
- ☐ It is more credible that a gene is regulated 2-fold with 10000, 5000 units)

- ☐ The average fold ratio does not take into account the extent to which the measurements of differential gene expression vary between the individuals being studied.

- ☐ The average fold ratio does not take into account the number of patients in the study, which statisticians refer to as the sample size.
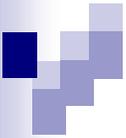
Define which genes are significantly regulated might be to choose 5% of genes that have the largest expression ratios.

Problems:

☐ It applies no measure of the extent to which a gene has a different mean expression level in the control and experimental groups.

☐ Possible that no genes in an experiment have statistically significantly different gene expression.

Hypothesis Testing

A *hypothesis test* is a procedure for determining if an assertion about a characteristic of a population is reasonable.

Example

someone says that the average price of a gallon of regular unleaded gas in Massachusetts is $2.5.

How would you decide whether this statement is true?

- □ find out what every gas station in the state was charging and how many gallons they were selling at that price.

- □ find out the price of gas at a small number of randomly chosen stations around the state and compare the average price to $2.5.

- Of course, the average price you get will probably not be exactly $2.5 due to variability in price from one station to the next.

Suppose your average price was $2.23. Is this three cent difference a result of chance variability, or is the original assertion incorrect?

A **hypothesis test** can provide an answer.

Terminology

- The *null hypothesis:*
  - H**0**: $\mu = 2.5$. (the average price of a gallon of gas is $2.5)
- The *alternative hypothesis:*
  - H**1**: $\mu > 2.5$. (gas prices were actually higher)
  - H**1**: $\mu < 2.5$.
  - H**1**: $\mu \neq 2.5$.
- The *significance level (alpha)*
  - Alpha is related to the degree of certainty you require in order to reject the null hypothesis in favor of the alternative.
  - Decide in advance to reject the null hypothesis if the probability of observing your sampled result is less than the significance level.
  - Alpha = 0.05: the probability of incorrectly rejecting the null hypothesis when it is actually true is 5%.
  - If you need more protection from this error, then choose a lower value of alpha .

Example

H_0: No differential expressed.

H_0: There is no difference in the mean gene expression in the group tested.

H_0: The gene will have equal means across every group.

H_0: $\mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 \, (\ldots = \mu_n)$
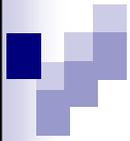
- *p* is the probability of observing your data under the assumption that the null hypothesis is true.
- *p* is the probability that you will be in error if you reject the null hypothesis.
- *p* represents the probability of **false positives** (Reject H_0 | H_0 true).

p=0.03 indicates that you would have only a 3% chance of drawing the sample being tested if the null hypothesis was actually true.

Decision Rule
- Reject H_0 if *P* is less than alpha.
- $P < 0.05$ commonly used. (Reject **H_0,** the test is significant)
- The lower the p-value, the more significant the difference between the groups.

P is *not* the probability that the null hypothesis is true!

$$Power = 1 - \beta.$$

Type I Error (alpha): calling genes as differentially expressed when they are NOT
Type II Error: NOT calling genes as differentially expressed when they ARE

Hypothesis Testing		Truth	
		H₀	H₁
Decision	Reject H₀	Type I Error (alpha) (false positive)	Right Decision (true positive)
	Don't Reject H₀	Right Decision	Type II Error (beta)

Hypothesis Testing
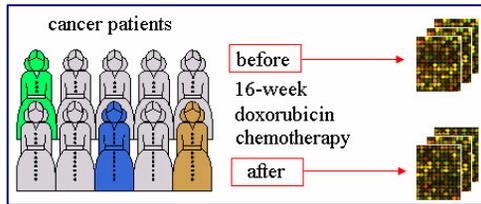
Dependent samples
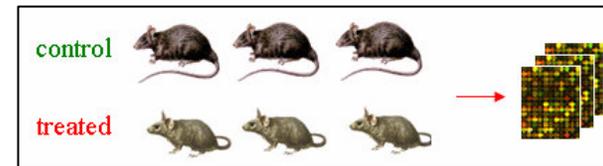
Independent samples

Comparison	Two Groups		More than two Groups
Hypothesis Testing	**Paired data**	**Unpaired data**	**Complex data**
Parametric (variance equal)	One sample t-test	Two-sample t-test	One-Way Analysis of Variance (ANOVA)
Parametric (variance not equal)	Welch t-test		Welch ANOVA
Non-Parametric (無母數檢定)	Wilcoxon Signed-Rank Test	Wilcoxon Rank-Sum Test (Mann-Whitney U Test)	Kruskal-Wallis Test

1. Determine the null and alternative hypothesis, using mathematical expressions if applicable.

2. Select a significance level (alpha).

3. Take a random sample from the population of interest.

4. Calculate a test statistic from the sample that provides information about the null hypothesis.

5. Decision

Hypothesis Testing:

two-sided z-test & p-value

H_0: $\mu = 35$ null hypothesis

H_1: $\mu \neq 35$ alternative hypothesis ($\mu > 35$; $\mu < 35$)
one-sided

α signifcant level: $= 0.05$

test statistic $z = \dfrac{\bar{X} - \mu}{\sigma/\sqrt{n}}$

Reject H₀ if $|z| > z_{0.05}$

$H_0 : \mu = m$

$H_1 : \mu \neq m$

$\alpha = P_{H_0}(|Z| > z_{\alpha/2})$

Sample Data: $= 33.6$
test statistic: $z = -2.47$

$(1 - \alpha)100\%$ Confidence Interval:

$P(z_{\alpha/2} < Z < z_{1-\alpha/2}) = 1 - \alpha$

p-value $= P_{H_0}(|Z| > z_0)$, $z_0 = \frac{\bar{X} - m}{\sigma/\sqrt{n}}$

The Classical Approach

z = -1.96   z = 1.96

z = -2.47   z = 0
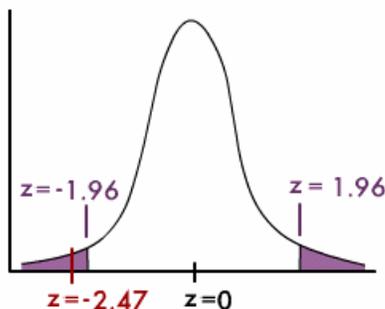
Conclusion: since the z value of the test statistic (-2.47) is less than the critical value of z= -1.96, we reject the null hypothesis.

The P-Value Approach

$\alpha = 0.025$ $\alpha = 0.025$
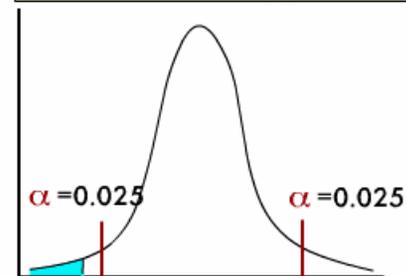
P -value = 0.0068 times 2 (for a 2-sided test) = 0.0136

Conclusion: since the P -value of 0.0136 is less than the significance level of α=0.05, we reject the null hypothesis.

If A Result is Statistically Significant

There are two possible explanations:

- The populations are identical, so there really is no difference.
  - □ By chance, you obtained larger values in one group and smaller values in the other.

  - □ Finding a statistically significant result when the populations are identical is called making a Type I error (false positives).

  - □ If you define statistically significant to mean "$P<0.05$", then you'll make a Type I error in 5% of experiments where there really is no difference.

OR

- The populations really are different, so your conclusion is correct.
  - □ The difference may be large enough to be scientifically interesting.

  - □ Or it may be tiny and trivial.

- The null hypothesis is that there is no biological effect.
    - □ For a gene in Breast Cancer Dataset, it would be that this gene is not differentially expressed following doxorubicin chemotherapy.
    - □ For a gene in Leukemia Dataset, it would be that this gene is not differentially expressed between ALL and AML patients.

- If the null hypothesis were true, then the variability in the data does not represent the biological effect under study, but instead results from difference between individuals or measurement error.

- The smaller the p-value, the less likely it is that the observed data have occurred by chance, and the more significant the result.

- $p=0.01$ would mean there is a 1% chance of observing at least this level of differential gene expression by random chance.

- We then select differentially expressed genes not on the basis of their fold ratio, but on the basis of their p-value.

H_0: no differential expressed.
- ■ The test is significant
    = Reject H_0
- ■ False Positive
    = (Reject H_0 | H_0 true)
    = concluding that a gene is differentially expressed when in fact it is not.

- ■ A p-value=0.05 indicates that you would have only a 5% chance of drawing the sample being tested if the null hypothesis was actually true.

- ■ The p-value is the smallest level of significance at which a null hypothesis may be rejected

- Question: What if I do a t-test on a pair of samples and fail to reject the null hypothesis--does this mean that there is no significant difference?
- Answer: Maybe yes, maybe no.

- For two-sample t-test, power is the probability of rejecting the hypothesis that the means are equal when they are in fact not equal. P(RH0 | not H0) Power is one minus the probability of Type-II error.

- The power of the test depends upon the sample size, the magnitudes of the variances, the alpha level, and the actual difference between the two population means.
- Usually you would only consider the power of a test when you failed to reject the null hypothesis.

- High power is desirable (0.7 to 1.0). High power means that there is a high probability of rejecting the null hypothesis when the null hypothesis is false.

- This is a critical measure of precision in hypothesis testing and needs to be considered with care.

The One-Sample t-test compares the mean score of a sample to a known value. Usually, the known value is a population mean.
Assumption: the variable is normally distributed.

One sample t-test

$H_0 : \mu = \mu_0$
$H_1 : \mu \neq \mu_0$ (two-tailed).
μ: population mean.
α: significant level (e.g., 0.05).
Test Statistic:

$$T = \frac{\bar{X} - \mu}{S/\sqrt{n}}, \quad t_0 = \frac{\bar{X} - \mu_0}{S/\sqrt{n}}$$

$\bar{X}$: sample mean.

S: sample standard deviation.

n: number of observations in the sample.

- Reject H_0 if $|t_0| > t_{\alpha/2,n-1}$.
- Power $= 1 - \beta$.
- $(1 - \alpha)100\%$ Confidence Interval for μ:
  $\bar{X} - t_{\alpha/2}S/\sqrt{n} \leq \mu < \bar{X} + t_{\alpha/2}S/\sqrt{n}$
- $p\text{-}value = P_{H_0}(|\mathbf{T}| > t_0), \mathbf{T} \sim t_{n-1}$.

Question
- whether a gene is differentially expressed for a condition with respect to baseline expression?
- H_0: μ =0 (log ratio)

MA Table	exp01	exp02	exp03	exp04	exp05	exp•••	exp P
gene001	-0.48	-0.42	0.87	0.92	0.67		-0.35
gene002	-0.39	-0.58	1.08	1.21	0.52		-0.58
gene003	0.87	0.25	-0.17	0.18	-0.13		-0.13

df = 28

t = -1.70 (.05)   t = +1.70 (.05)

t = -2.05 (.025)   t = +2.05 (.025)

t_0 = +1.9
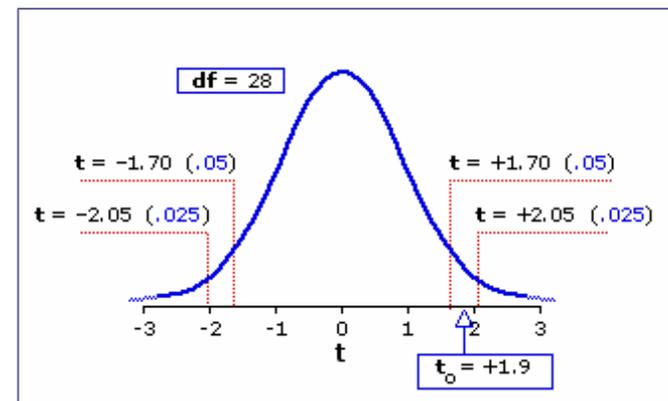
Paired Sample t-test

$H_0 : \mu_d = \mu_0$

$H_1 : \mu_d \neq \mu_0$ (two-tailed).

μ_d: mean of population differences.

α: significant level (e.g., 0.05).

Test Statistic:

$$T_d = \frac{\bar{d} - \mu_d}{S_d/\sqrt{n}}, \quad t_d = \frac{\bar{d} - \mu_0}{S_d/\sqrt{n}}$$

$\bar{d}$: average of sample differences.

S_d: standard deviation of sample difference

n: number of pairs.

- Reject H_0 if $|t_d| > t_{\alpha/2, n-1}$.
- Power $= 1 - \beta$.
- $(1 - \alpha)100\%$ Confidence Interval for μ_d:
  $\bar{d} - t_{\alpha/2}S/\sqrt{n} \leq \mu_d < \bar{d} + t_{\alpha/2}S/\sqrt{n}$
- $p\text{-}value = P_{H_0}(|\mathbf{T}| > t_d), \mathbf{T} \sim t_{n-1}$.

Two Sample t-test (Unpaired)

$H_0 : \mu_x - \mu_y = \mu_0$

$H_0 : \mu_x - \mu_y \neq \mu_0$

α: significant level (e.g., 0.05).

Test Statistic:

$$t_0 = \frac{(\bar{X} - \bar{Y}) - \mu_0}{\sqrt{\frac{S_x^2}{n} + \frac{S_y^2}{m}}}$$

for homogeneous variances:
$df = n + m - 2$

for heterogeneous variances:
adjusted df

Reject H_0 if $|t_0| > t_{\alpha/2, df}$

Cancer type 1   Cancer type 2
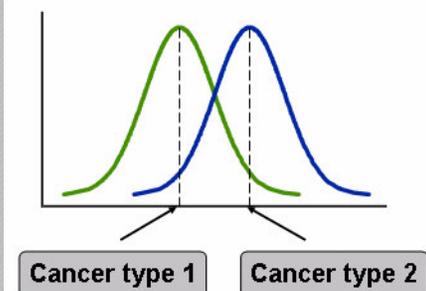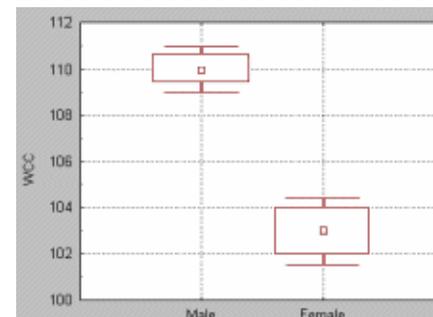
# Paired t-test
Applied to a gene From Breast Cancer Data

- The gene acetyl-Coenzyme A acetyltransferase 2 (ACAT2) is on the microarray used for the breast cancer data.

- We can use a paired t-test to determine whether or not the gene is differentially expressed following doxoruicin chemotherapy.

- The samples from before and after chemotherapy have been hybridized on separate arrays, with a reference sample in the other channel.
  - Normalize the data.
  - Because this is a reference sample experiment, we calculate the log ratio of the experimental sample relative to the reference sample for before and after treatment in each patient.
  - Calculate a single log ratio for each patient that represents the difference in gene expression due to treatment by subtracting the log ratio for the gene before treatment from the log ratio of the gene after treatment.
  - Perform the t-test. t=3.22 compare to t(19).
  - The p-value for a two-tailed one sample t-test is 0.0045, which is significant at a 1% confidence level.

- Conclude: this gene has been significantly down-regulated following chemotherapy at the 1% level.

Unpaired t-test

Applied to a Gene From Leukemia Dataset

- The gene metallothionein IB is on the Affymetrix array used for the leukemia data.
  - To identify whether or not this gene is differentially expressed between the AML and ALL patients.
  - To identify genes which are up- or down-regulation in AML relative to ALL.

- Steps
  - the data is log transformed.
  - t=-3.4177, p=0.0016

- Conclude that the expression of metallothionein IB is significantly higher in AML than in ALL at the 1% level.

Assumptions of t-test

- The distribution of the data being tested is normal.
  - □ For paired t-test, it is the distribution of the subtracted data that must be normal.
  - □ For unpaired t-test, the distribution of both data sets must be normal.
- **Plots**: Histogram, Density Plot, QQplot,…
- **Test for Normality**: Jarque-Bera test, Lilliefors test, Kolmogorov-Smirnov test.

- Homogeneous: the variances of the two population are equal.
- Test for equality of the two variances: Variance ratio F-test.

Note:

◆ If the two populations are symmetric, and if the variances are equal, then the *t* test may be used.

◆ If the two populations are symmetric, and the variances are not equal, then use the two-sample unequal variance t-test or Welch's *t* test.

One-Way ANOVA

Using Analysis of Variance, which can be considered to be a generalization of the *t*-test, when

- compare more than two groups (e.g., *drug 1*, *drug 2*, and *placebo*), or
- compare groups created by more than one independent variable while controlling for the separate influence of each of them (e.g., *Gender*, *type of Drug*, and *size of Dose*).

- For two group comparisons, ANOVA will give results identical to a *t*-test.
- One-way ANOVA compares groups using one parameter.

- We can test the following:
  - ☐ Are all the means from more than two populations equal?
  - ☐ Are all the means from more than two treatments on one population equal?
  (This is equivalent to asking whether the treatments have any overall effect.)

This comparison is performed for each gene.
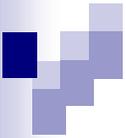
Assumptions

- The subjects are sampled randomly.
- The groups are independent.
- The population variances are homogenous.
- The population distribution is normal in shape.

As with t tests, violation of homogeneity is particularly a problem when we have quite different sample sizes.

Homogeneity of variance test

- Bartlett's test (1937)
- Levene's test (Levene 1960)
- O'Brien (1979)

Groups

	1	2	... j ...	k
	X_{11}	X_{12}	$\cdots\ X_{1j}\ \cdots$	X_{1k}
	X_{21}	X_{22}	$\cdots\ X_{2j}\ \cdots$	X_{2k}
			$\cdots$	
	X_{i1}	X_{i2}	$\cdots\ X_{ij}\ \cdots$	X_{ik}
	$\vdots$			$X_{n_k k}$
		$X_{n_2 2}$	$\cdots\ \ \vdots\ \cdots$	
	$X_{n_1 1}$		$X_{n_i j}$	

$$T_j = \sum_{i=1}^{n_j} X_{ij} \qquad \bar{X}_j = \frac{T_j}{n_j}$$

$$T = \sum_{j=1}^{k} T_j \qquad \bar{X} = \frac{T}{N}$$

$$S^2 = \sum_{j=1}^{k} \sum_{i=1}^{n_j} \frac{(X_{ij} - \bar{X})^2}{N-1}$$

$$H_0 : \mu_1 = \mu_2 = \cdots = \mu_k$$

$$X_{ij} = \mu_j + \epsilon_{ij} \qquad \begin{array}{l} i = 1, \cdots, n_j \\ j = 1, \cdots, k \end{array}$$
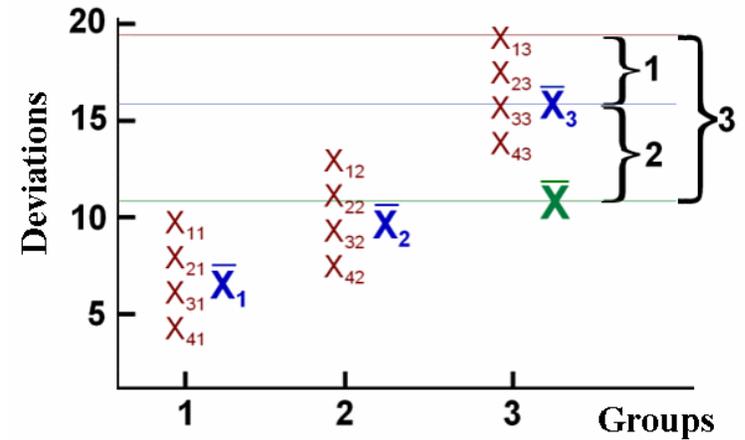
$$\epsilon_{ij} \sim N(0, \sigma^2)$$

$$(X_{ij} - \bar{X}) = (X_{ij} - \bar{X}_j) + (\bar{X}_j - \bar{X})$$

$$\sum_{j=1}^{k} \sum_{i=1}^{n_j} (X_{ij} - \bar{X})^2 = \sum_{j=1}^{k} \sum_{i=1}^{n_j} [(X_{ij} - \bar{X}_j) + (\bar{X}_j - \bar{X})]^2$$

$$\sum_{j=1}^{k} \sum_{i=1}^{n_j} (X_{ij} - \bar{X})^2 = \sum_{j=1}^{k} \sum_{i=1}^{n_j} (X_{ij} - \bar{X}_j)^2 + \sum_{j=1}^{k} \sum_{i=1}^{n_j} (\bar{X}_j - \bar{X})^2$$

$$SS_{Total} = SS_{Within} + SS_{Between}$$

$$F = \frac{MS_{Between}}{MS_{Within}}$$

Reject H_0, if $F_{obs} > F_{\{\alpha, k-1, N-k\}}$

ANOVA Table

Source	SS	df	MS	F	p
Between	SS_B	$p-1$	MS_B	MS_B/MS_W	< 0.05
Within	SS_W	$N-p$	MS_W		
Total	SS_T	$N-1$			

Welch ANOVA

Welch's F Test

- Use when the sample sizes are unequal.
- Use when the sample sizes are equal but small.

$$H_0 : \mu_1 = \mu_2 = \cdots = \mu_k$$

$$X_{ij} = \mu_j + \epsilon_{ij}$$

$$\epsilon_{ij} \sim N(0, \sigma_j^2)$$

$$i = 1, \cdots, n_j$$

$$j = 1, \cdots, k$$

$$s_j^2 = \frac{\sum_{i=1}^{n_j}(X_{ij} - \bar{X}_j)^2}{n_j - 1}$$

$$w_j = \frac{n_j}{s_j^2}$$

$$\bar{X}' = \frac{\sum_{j=1}^{k} w_j \bar{X}_j}{\sum_{j=1}^{k} w_j}$$

$$F' = \frac{\frac{\sum_{j=1}^{k} w_j(\bar{X}_j - \bar{X}')^2}{k-1}}{1 + \frac{2(k-2)}{k^2-1} \sum_{j=1}^{k}\left(\frac{1}{n_j-1}\right)\left(1 - \frac{w_j}{\sum_{j=1}^{k} w_j}\right)^2}$$

$$df' = \frac{k^2 - 1}{3 \sum_{j=1}^{k}\left(\frac{1}{n_j-1}\right)\left(1 - \frac{w_j}{\sum_{j=1}^{k} w_j}\right)^2}$$

Reject H_0, if $F'_{obs} > F_{\{\alpha, k-1, df'\}}$

B-statistic

Lonnstedt and Speed, Statistica Sinica 2002: parametric empirical Bayes approach.

- B-statistic is an estimate of the posterior log-odds that each gene is DE.
- B-statistic is equivalent for the purpose of ranking genes to the penalized t-statistic $t = \frac{\bar{M}}{\sqrt{(a+s^2)/n}}$, where a is estimated from the mean and standard deviation of the sample variances s^2.

$$M_{gj}|\mu_g, \sigma_g \sim N(\mu_g, \sigma_g^2)$$

$$B_g = \log \frac{P(\mu_g \neq 0|M_{gj})}{P(\mu_g = 0|M_{gj})}$$

Penalized t-statistic

Tusher et al (2001, PNAS, SAM)
Efron et al (2001, JASA)

$$t = \frac{\bar{M}}{(a+s)/\sqrt{n}}$$

Lonnstedt, I. and Speed, T.P. Replicated microarray data. *Statistica Sinica* , 12: 31-46, 2002

General Penalized t-statistic

(Lonnstedt et al 2001)

$$t = \frac{b}{s^* \times SE}$$

multiple regression model

Penalized two-sample t-statistic

$$t = \frac{\bar{M}_A - \bar{M}_B}{s^* \times \sqrt{1/n_A + 1/n_B}}, \quad \text{where } s^* = \sqrt{a + s^2}$$

Robust General Penalized t-statistic

Non-parametric Statistics

- Do not assume that the data is normally distributed.
- There are two good reasons to use non-parametric statistic.

  - *Microarray data is noisy:*
    - there are many sources of variability in a microarray experiment and outliers are frequent.
    - The distribution of intensities of many genes may not be normal.
    - Non-parametric methods are robust to outliers and noisy data.

  - *Microarray data analysis is high throughput:*
    - When analysising the many thousands of genes on a microarray, we would need to check the normality of every gene in order to ensure that t-test is appropriate.
    - Those genes with outliers or which were not normally distributed would then need a different analysis.
    - It makes more sense to apply a test that is distribution free and thus can be applied to all genes in a single pass.
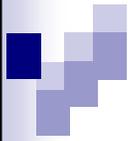
■ Given *n* pairs of data, the sign test tests the hypothesis that the median of the differences in the pairs is zero.

■ The test statistic is the number of positive differences.

■ If the null hypothesis is true, then the numbers of positive and negative differences should be approximately the same.

■ In fact, the number of positive differences will have a Binomial distribution with parameters *n* and *p*.

Pair	Before	After	Sign
1	89	73	+
2	83	77	+
3	80	58	+
4	72	77	−
5	77	70	+
6	74	62	+
7	69	67	+
8	65	68	−
9	60	44	+
10	55	50	+
11	54	46	+
12	50	38	+
13	42	47	−
14	48	40	+
15	44	43	+
16	38	29	+
17	36	25	+

The Sign Test:

when $n_1 = n_2 \leq 50$

$H_0 : P = Q = \frac{1}{2}$

$H_1 : P \neq Q \neq \frac{1}{2}$

$T = \#" +"$

At $\alpha = 0.01$, two-tailed test,

reject H_0 if $T \geq 14$ when $N = 17$.

(Binomial Probability)

$\#" +" = 14$

$\#" -" = 3$

The obtained T=14 is equal to the critical value, so we reject H_0.

■Null hypothesis: the population median from which both samples were drawn is the same.

■The sum of the ranks for the "positive" (up-regulated) values is calculated and compared against a precomputed table to a p-value.

■ Sorting the absolute values of the differences from smallest to largest.
■ Assigning ranks to the absolute values.
■ Find the sum of the ranks of the positive differences.

■ If the null hypothesis is true, the sum of the ranks of the positive differences should be about the same as the sum of the ranks of the negative differences.

Pair	Before	After	Diff.	Rank
1	89	73	16	15.5
2	83	77	6	7
3	80	58	22	17
4	72	77	−5	5
5	77	70	7	8
6	74	62	12	13.5
7	69	67	2	2
8	65	68	−3	3
9	60	44	16	15.5
10	55	50	5	5
11	54	46	8	9.5
12	50	38	12	13.5
13	42	47	−5	5
14	48	40	8	9.5
15	44	43	1	1
16	38	29	9	11
17	36	25	11	12

The Wilcoxon signed-rank Test:

$H_0 : \mu_1 = \mu_2$
$H_1 : \mu_1 \neq \mu_2$

$T = \min\{\sum_+ \text{Rank}, \sum_- \text{Rank}\}$

At $\alpha = 0.01$, two-tailed test,
    reject H_0 if $T \neq 23$ when $N = 17$.
(Table)

(The zero difference is ignored when assigning ranks. $N_{new} = N_{old} - \#\{ties\}$)

$T = \min\{\sum_+ \text{Rank} = 140, \sum_- \text{Rank} = 13\}$
    $= 13$

The obtained T=13 is less than the critical value 23, so we reject H_0.

Mann-Whitney Test

(Wilcoxon Rank-Sum Test, unpaired)

■The data from the two groups are combined and given ranks. (1 for the smallest, 2 for the second smallest,...)
■The ranks for the larger group are summed and that number is compared against a precomputed table to a p-value.

Group		Rank	
G_1	G_2	G_1	G_2
26	16	3	11
22	10	4	17
19	8	7.5	19
21	13	5.5	13.5
14	19	12	7.5
18	11	9	15.5
29	7	2	20
17	13	10	13.5
11	9	15.5	18
34	21	1	5.5
$n_1 = 10$	$n_2 = 10$	$R_1 = 69.5$	$R_2 = 104.5$

The Mann-Whitney U Test:

$$H_0 : F_1 = F_2$$
$$H_1 : F_1 \neq F_2$$

$$U = n_1 n_2 + \frac{n_1(n_1+1)}{2} - R_1$$
or
$$U' = n_1 n_2 + \frac{n_2(n_2+1)}{2} - R_2$$

$$R_i = \sum_i \text{Rank}$$

At $\alpha = 0.05$, two-tailed test for $n_1 = 10, n_2 = 10$, reject H_0 if $U \leq 23$ or $U' \geq 77$ (Table)

U: the number of times that a score from Group 1 is lower in rank than a score from Group 2.

$$U = 85.5, \quad U' = 14.5$$
The obtained $U = 85.5$ is less than the critical value 77, so we reject H_0.

Kruskal-Wallis Test

- The Kruskal Wallis test can be applied in the one factor ANOVA case. It is a non-parametric test for the situation where the ANOVA normality assumptions may not apply.

- Each of the *ni* should be at least 5 for the approximation to be valid.

$$H_0 : \mu_1 = \mu_2 = \cdots = \mu_k$$

$$H_1 : \mu_i \neq \mu_j \quad \text{for at least one set of } i \text{ and } j$$

$$W = \frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{R_j^2}{n_j} - 3(N+1)$$

$$W \sim \chi^2_{k-1} \text{ under } H_0$$

Reject H_0 if $W > CHIPPF(\alpha, k-1)$,
the chi-square percent point function

Groups

1	2	$\cdots$	j	$\cdots$	k
X_{11}	X_{12}	$\cdots$	X_{1j}	$\cdots$	X_{1k}
X_{21}	X_{22}	$\cdots$	X_{2j}	$\cdots$	X_{2k}
		$\cdots$			
X_{i1}	X_{i2}	$\cdots$	X_{ij}	$\cdots$	X_{ik}
$\vdots$					$X_{n_k k}$
	$X_{n_2 2}$	$\cdots$	$\vdots$	$\cdots$	
$X_{n_1 1}$			$X_{n_i j}$		

Rank Data

1	2	$\cdots$	j	$\cdots$	k
R_{11}	R_{12}	$\cdots$	R_{1j}	$\cdots$	R_{1k}
R_{21}	R_{22}	$\cdots$	R_{2j}	$\cdots$	R_{2k}
		$\cdots$			
R_{i1}	R_{i2}	$\cdots$	R_{ij}	$\cdots$	R_{ik}
$\vdots$					$R_{n_k k}$
	$R_{n_2 2}$	$\cdots$	$\vdots$	$\cdots$	
$R_{n_1 1}$			$R_{n_i j}$		

$$F(x) = P(X \leq x) = P(X \leq G(\alpha)) = \alpha$$

$$x = G(\alpha) = G(F(x))$$

The percent point function (ppf) is the inverse of the cumulative distribution function.

Parametric Tests

- Assume that the data follows a certain distribution (normal distribution).
- Assuming equal variances and Unequal variances.
- More powerful.
- Not appropriate for data with outliers.

t-test	Non-parametric
Easy	Easy
Powerful	Robust
Widely Implemented	widely implemented
Not appropriate for data with outliers	Less powerful

Note:

Because of the loss of power, classical non-parametric statistics have not become popular for use with microarray data, and instead bootstrap methods trend to be preferred.

Non-Parametric Tests

When certain assumptions about the underlying population are questionable (e.g. normality).

- Does not assume normal distribution
- No variance assumption

- Ranks the order of raw/normalized data across conditions for analyses
- Not affected by interpretation mode (GeneSpring)

- Decrease effects of outliers (Robust)
- Not recommended if there is less than 5 replicates per group
- Needs a high number of replicates
- Less powerful

Bootstrap Analysis
Robust
Powerful
Requires use of specialist packages or programming.
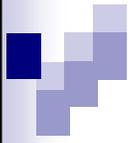
Volcano Plot

The Y variate is typically a probability (in which case a -log10 transform is used) or less commonly a p-value.

The X variate is usually a measure of differential expression such as a log-ratio.
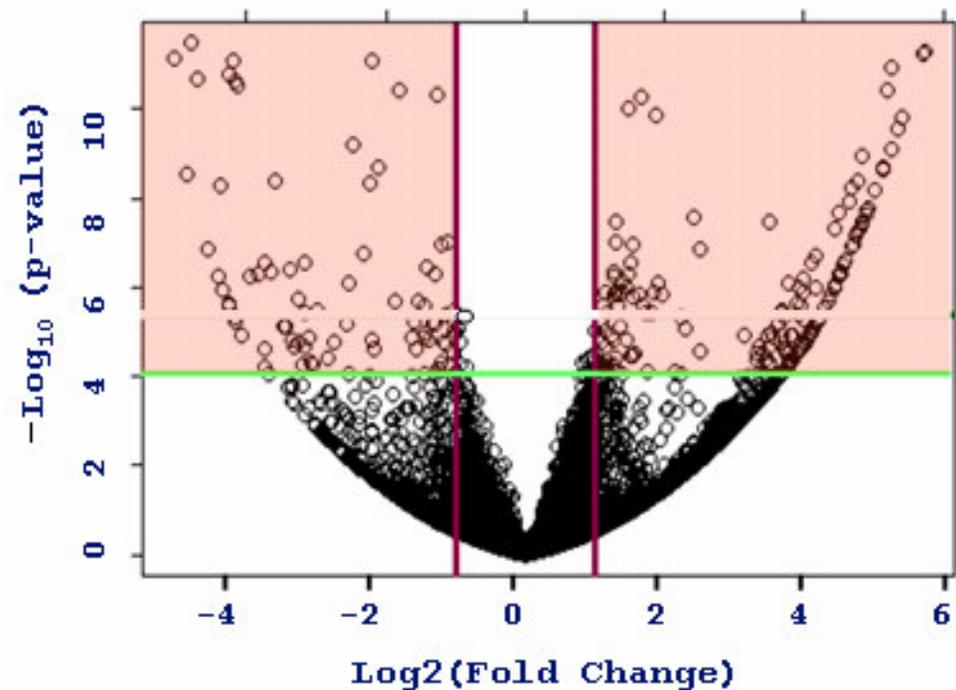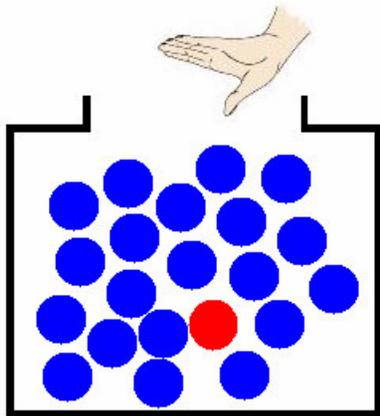
Imagine a box with 20 marbles: 19 are blue and 1 is red.
What are the odds of randomly sampling the red marble by chance?
It is 1 out of 20.

Now let's say that you get to sample a single marble (and put it back into the box) 20 times.
Have a much higher chance to sample the red marble.
This is exactly what happens when testing several thousand genes at the same time:

Imagine that the red marble is a false positive gene: the chance that false positives are going to be sampled is higher the more genes you apply a statistical test on.

Multiplicity of Testing

X: false positive gene

$P(X>=1)$

$= 1-P(X=0)$

$= 1- 0.95^n$

Number of genes tested (N)	False positives incidence	Probability of calling 1 or more false positives by chance ($100(1-0.95^N)$)
1	1/20	5%
2	1/10	10%
20	1	64%
100	5	99.4%
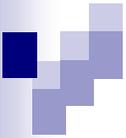
Multiplicity of Testing

- There is a serious consequence of performing statistical tests on many genes in parallel, which is known as multiplicity of p-values.

- Take a large supply of reference sample, label it with Cy3 and Cy5: no genes are differentially expressed: all measured differences in expression are experimental error.
  - By the very definition of a p-value, each gene would have a 1% chance of having a p-value of less than 0.01, and thus be significant at the 1% level.
  - Because there are 10000 genes on this imaginary microarray, we would expect to find 100 significant genes at this level.
  - Similarly, we would expect to find 10 genes with a p-value less than 0.001, and 1 gene with p-value less than 0.0001
  - The p-value is the probability that a gene's expression level are different between the two groups due to chance.

Question:

1. How do we know that the genes that appear to be differentially expressed are truly differentially expressed and are not just artifact introduced because we are analyzing a large number of genes?

2. Is this gene truly differentially expressed, or could it be a false positive results?

■ Multiple testing correction adjusts the p-value for each gene to keep the overall error rate (or false positive rate) to less than or equal to the user-specified p-value cutoff or error rate individual.

Multiple Testing

	# Reject H_0	# not Reject H_0	
# true H_{0j}	V	U	m_0
# true H_{1j}	S	T	m_1
	R	m - R	m

V : false positives = Type I errors

T : false negatives = Type II errors

Type One Errors Rates

$$PCER = \frac{E[V]}{m}$$

$$PFER = E[V]$$

$$FWER = P(V \geq 1)$$

$$FDR = E[\frac{V}{R}] \quad \text{if } R > 0$$

Power = Reject the false null hypothesis

$$\text{Any-pair Power} = P(S \geq 1)$$

$$\text{Per-pair Power} = \frac{E[S]}{m_1}$$

$$\text{All-pair Power} = P(S = m_1)$$

Multiple Testing Corrections

Test Type	Type of Error control	Genes identified by chance after correction
Bonferroni Bonferroni Step-down Westfall and Young permutation	Family-wise error rate	If error rate equals 0.05, expects **0.05** genes to be significant by chance
Benjamini and Hochberg	False Discovery Rate	If error rate equals 0.05, **5%** of genes considered statistically significant (that pass the restriction after correction) will be identified by chance (false positives).

most stringent

More false negatives

More false positives

least stringent

- The more stringent a multiple testing correction, the less false positive genes are allowed.
- The trade-off of a stringent multiple testing correction is that the rate of *false negatives* (genes that are called non-significant when they are) is very high.
- FWER is the overall probability of false positive in all tests.
  - Very conservative
  - False positives not tolerated
- False discovery error rate allows a percentage of called genes to be false positives.
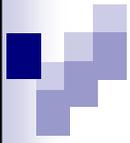
(1) Bonferroni

- The p-value of each gene is multiplied by the number of genes in the gene list.

- If the corrected p-value is still below the error rate, the gene will be significant:
  - Corrected p-value= p-value * n <0.05.
  - If testing 1000 genes at a time, the highest accepted individual un-corrected p-value is 0.00005, making the correction very stringent.

- With a Family-wise error rate of 0.05 (i.e., the probability of at least one error in the family), the expected number of false positives will be 0.05.

- This correction is very similar to the Bonferroni, but a little less stringent.
- The p-value of each gene is ranked from the smallest to the largest.
  - The ith p-value is multiplied by the number of genes present in the gene list

    Corrected P-value= p-value * (n – i + 1) < 0.05
- if the end value is less than 0.05, the gene is significant.
- It follows that sequence until no gene is found to be significant.

Example:

Let n=1000, error rate=0.05

Gene name	p-value before correction	Rank	Correction	Is gene significant after correction?
A	0.00002	1	0.00002 * 1000=0.02	0.02<0.05 ➡ Yes
B	0.00004	2	0.00004*999=0.039	0.039<0.05 ➡ Yes
C	0.00009	3	0.00009*998=0.0898	0.0898>0.05 ➡ No

(3) Westfall and Young Permutation

- Both Bonferroni and Holm methods are called single-step procedures, where each p-value is corrected independently.
- The Westfall and Young permutation method takes advantage of the *dependence structure* between genes, by permuting all the genes at the same time.

- The Westfall and Young permutation follows a step-down procedure similar to the Holm method, combined with a bootstrapping method to compute the p-value distribution.
- Because of the permutations, the method is very slow.
- The Westfall and Young permutation method has a similar Family-wise error rate as the Bonferroni and Holm corrections.

- P-values are calculated for each gene based on the original data set and ranked.
- The permutation method creates a pseudo-data set by dividing the data into artificial treatment and control groups.
- P-values for all genes are computed on the pseudo-data set.
- The successive minima of the new p-values are retained and compared to the original ones.
- This process is repeated a large number of times, and the proportion of resampled data sets where the minimum pseudo-p-value is less than the original p-value is the adjusted p-value.

Group

Permutation

Group
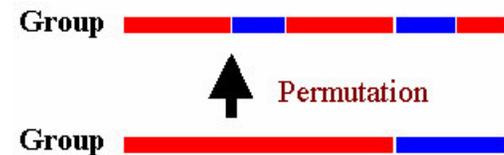
MA Table	exp01	exp02	exp03	exp04	exp05	exp...	exp	n	p-values
gene001	-0.48	-0.42	0.87	0.92	0.67		-0.35		0.067
gene002	-0.39	-0.58	1.08	1.21	0.52		-0.58		0.052
gene003	0.87	0.25	-0.17	0.18	-0.13		-0.13		0.013 *
gene004	1.57	1.03	1.22	0.31	0.16		-1.02		0.016 *
gene005	-1.15	-0.86	1.21	1.62	1.12		-0.44		0.112
gene006	0.04	-0.12	0.31	0.16	0.17		0.08		0.017 *

$$\text{corrected p-value:} \approx \frac{\#\{p^* < 0.05\}}{n!}$$

(4) Benjamini and Hochberg FDR

- This correction is the least stringent of all 4 options, and therefore tolerates more false positives.
- There will be also less false negative genes.
- The correction becomes more stringent as the p-value decreases, similarly as the Bonferroni Step-down correction.
- This method provides a good alternative to Family-wise error rate methods.
- The error rate is a proportion of the number of called genes.
- FDR: Overall proportion of false positives relative to the total number of genes declared significant.

Corrected P-value= p-value * $(n / R_i) < 0.05$

Let n=1000, error rate=0.05

Gene name	p-value (from largest to smallest)	Rank	Correction	Is gene significant after correction?
A	0.1	1000	No correction	0.1 > 0.05 ➡ No
B	0.06	999	1000/999*0.06 = 0.06006	0.06006 > 0.05 ➡ No
C	0.04	998...	1000/998*0.04 = 0.04008	0.04008 < 0.05 ➡ Yes
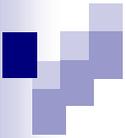
Recommendations

- The default multiple testing correction in GeneSpring is the Benjamini and Hochberg False Discovery Rate.

- It is the least stringent of all corrections and provides a good balance between discovery of statistically significant genes and limitation of false positive occurrences.

- The Bonferroni correction is the most stringent test of all, but offers the most conservative approach to control for false positives.

- The Westfall and Young Permutation is the only correction accounting for genes coregulation. However, it is very slow and is also very conservative.

- As multiple testing corrections depend on the number of tests performed, or number of genes tested, it is recommended to select a prefiltered gene list.

If There Are No Results with MTC

- increase p-cutoff value
- increase number of replicates
- use less stringent or no MTC
- add cross-validation experiments

Post Hoc Tests

Applicable when comparing more than 2 groups.

One-way ANOVA model

$$\text{H0: } \mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5 (\ldots = \mu_n)$$

If H0 is rejected for a **gene**, there is still no information about where differences are observed.

How does one determine which specific differences are significant?

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.

assuming
equal sample sizes and
homogeneity of variance

Group	A	B	C	D
Mean	2	3	7	8

alpha $= 0.01$
$n = 5$
$df = 16$

$$\sqrt{\frac{MSE}{n}} = \sqrt{\frac{.5}{5}} = 0.316$$

$$q = \frac{\overline{X}_i - \overline{X}_j}{\sqrt{\frac{MSE}{n}}}$$

- Parametric and non-parametric
- Unequal sample sizes
- Variance assumption

"r" is the number of means spanned by a given comparison.

r, df, alpha $\longrightarrow$ studentized range statistic q

1. $r = 4$, $q_{.01} = 5.19$

A vs D: $q = \dfrac{8-2}{0.316} = 18.99$, $p < 0.01$

2. $r = 3$, $q_{.01} = 4.79$

  a.   A vs C: $q = \dfrac{7-2}{0.316} = 15.82$, $p < 0.01$

  b.   B vs D: $q = \dfrac{8-3}{0.316} = 15.82$, $p < 0.01$

3. $r = 2$, $q_{.01} = 4.13$

  a.   A vs B: $q = \dfrac{3-2}{.316} = 3.16$, $p > 0.01$

  b.   B vs C: $q = \dfrac{7-3}{.316} = 12.66$, $p < 0.01$

  c.   C vs D: $q = \dfrac{8-7}{.316} = 3.16$, $p > 0.01$

Honestly Significant Difference (HSD)

$$HSD = q\sqrt{\frac{MS_{Within}}{n}} \qquad \frac{M_1 - M_2}{\sqrt{MS_w\left(\frac{1}{n}\right)}}$$

Tukey's HSD Post-hoc test is applied in exactly the same way that the Student-Newman-Keuls is, with the exception that r is set at k for all comparisons.

- (k vs 1, k vs 2,.., k vs k-1) (k-1 vs 1, k-1 vs 2,…, k-1 vs k-2) …(…2 vs 1)

$$r = k, \; df, \text{alpha} \longrightarrow \text{studentized range statistic } q$$

- All alpha's in Tukey's test are compared to the same critical value.
- All alpha's in SKN test are compared to a different critical value.
- This test is more conservative (less powerful) than the Student-Newman-Keuls.

- Bootstrap Sample/data
  - with replacement: different individuals in the bootstrap data could have the same value from the real data.
  - without replacement: each of the real values is only used once in the bootstrap data.

- Bootstrap analysis are more appropriate for microarray analysis than either t-test or classical non-parametric tests.
  - don't require that the data are normally distributed.
  - robust to noise and experimental artifacts.

- Under the null hypothesis; there is no difference in gene expression between the two groups. If that were the case, then any of the measurements in the data could have been observed in any of the individuals.
  - ex: any of the AML patients could have had any of the 38 measurements associated with both the AML and ALL patients.

- The bootstrap works by constructing a large number of random data sets by resampling from the original data, in which each individual is randomly allocated one of the measurements from the data, which could be from either of the groups.

■ The bootstrap data sets look like the real data, in that they have similar values, but are biologically nonsense because the values have been randomized.

■ Aim: the aim of the test is to compare some property of the real data with a distribution of the same property in random data sets.

Bootstrap Analysis

Ex: two-sample t-test

Bootstrap Samples

Original Data

t

$t^{(1)}$

$t^{(2)}$

$t^{(B)}$

$t = 0.3.1596$

$B = 1000000$

Histogram of Bootstrap t statistics

$$\sum_{i=1}^{B} I(|t^{(i)}| > t) = 9750$$

bootstrap p-value < 0.001
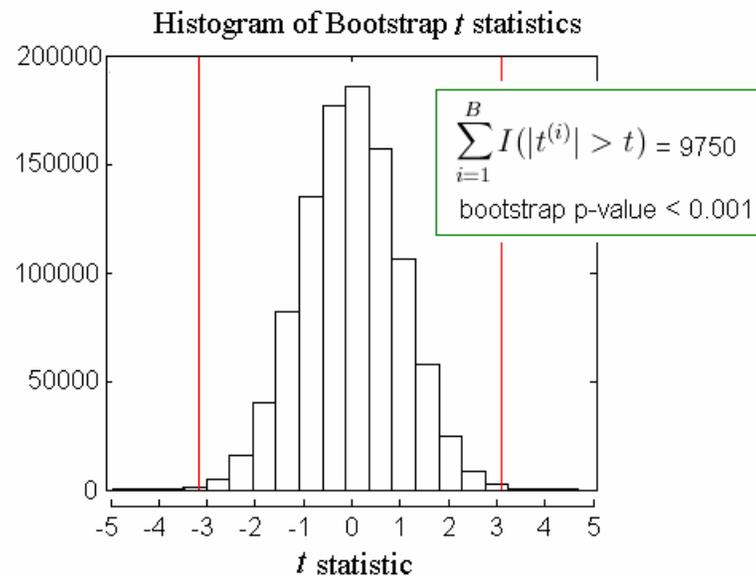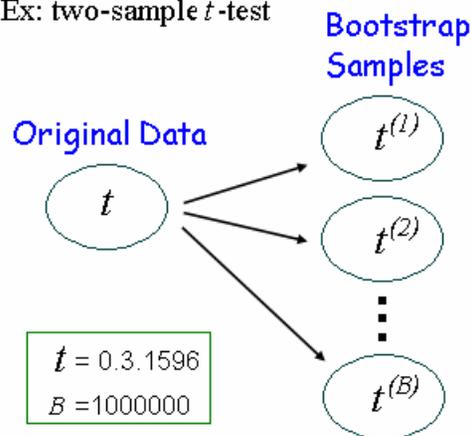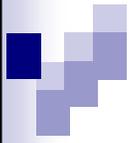
t statistic

Steps of Bootstrap Analysis

1. We generate an empirical distribution using the t-statistics calculated from the randomized bootstrap data.

2. The t-statistic from the real data is compared with the distribution of t-statistics from the bootstrap data.

3. We calculate an empirical p-value by computing the proportion of bootstrap statistics that have a more extreme value than the t-statistic from the real data.

   □ if the real t-statistic is in the belly of the distribution, then it is indistinguishable from t-statistics generated from randomized data.

   □ if the statistic from the real data is towards the edge of the bootstrap distribution, then it is unlikely that the experimental result can have arisen by chance, and we would conclude that the gene is significantly differentially expressed.

Permutation Test

- The permutation test is a test where the null-hypothesis allows to reduce the inference to a randomization problem.
- The process of randomizations makes it possible to ascribe a probability distribution to the difference in the outcome possible under H0.

- The outcome data are analyzed many times (once for each acceptable assignment that could have been possible under H0) and then compared with the observed result, without dependence on additional distributional or model-based assumptions.

- Perform a permutation test (general):
  1. Analyze the problem, choice of null-hypothesis
  2. Choice of test statistic **T**
  3. Calculate the value of the test statistic for the observed data: **tobs**
  4. Apply the randomization principle and look at all possible permutations, this gives the distribution of the test statistic **T** under H0.
  5. Calculation of p-value:

$$p = P(T \geq t_{obs} \mid H_0) \approx \frac{\#\{t^* \geq t_{obs}\}}{\# \text{ permutations}}$$

Ref: Mansmann, U. (2002), Practical microarray analysis: resampling and the Bootstraap.Heidelberg.

Coexpression of genes

Data

H_0: Gene 1 and Gene 2 are not correlated.

Test statistic T:

Pearson (or Spearman) correlation coefficient, calculate t_{obs}

Gene1	Gene2
g_1^1	g_1^2
⋮	⋮
g_n^1	g_n^2

Randomization: Under H_0 it is possible to permute the values observed for Gene 2. There are n! possibilities.

p-value: $p = P(T \geq t_{obs} \mid H_0) \approx \dfrac{\#\{ T^* \geq t_{obs} \}}{n!}$

$g_{(1)}^1$	$g_{(1)}^2$
⋮	⋮
$g_{(n)}^1$	$g_{(n)}^2$

Random Permutation for group labels

Gene 1	Gene 2	Group		Group
1.4482	1.0709	1		2
0.4850	0.9324	1		1
1.1331	1.2379	1		4
		⋮		⋮
0.8015	0.6765	2		1
		⋮		⋮
1.3726	1.2373	3		4
		⋮		⋮
1.1030	1.735	4		2
0.5148	1.0015	4		3

The permutation test allows determining the statistical significance of the score for every gene.

Images from
http://www.siliconegenetics.com

Agilent Technologies

Analysis Guides
http://www.chem.agilent.com/scripts/generic.asp?lpage=34708&indcol=Y&prodcol=Y

GeneSpring Tutorials
http://www.chem.agilent.com/Scripts/Generic.ASP?lPage=34743&indcol=Y&prodcol=Y

**Chapter 13
GeneSpring Manual 7.2**

response

$$y_j$$

$j = 1, 2, \ldots n$ samples

$i = 1$
2
$\vdots$
p
genes

data
x_{ij}

Tusher, Tibshirani and Chu (2001), "Significance analysis of microarrays applied to the ionizing radiation response". PNAS 2001 98: 5116-5121.

$$d_i = \frac{r_i}{s_i + s_0}$$

Two class, unpaired data

$$y_j = 1 \text{ or } 2$$

$$r_i = \bar{x}_{i2} - \bar{x}_{i1}$$

s_i standard deviation

s_0 exchangeability factor

d_i
d_1
d_2
$\vdots$
d_p

order stat.

$d_{(p)}$
$\vdots$
$d_{(2)}$
$d_{(1)}$

permutations of the response values y_j

$$\begin{matrix} d_{(p)}^{*b} \\ \vdots \\ d_{(2)}^{*b} \\ d_{(1)}^{*b} \end{matrix} \quad b = 1, 2, \ldots B$$

$$\bar{d}_{(i)} = (1/B) \sum_b d_{(i)}^{*b}$$

expected order statistics

$$\begin{matrix} \bar{d}_{(p)} \\ \vdots \\ \bar{d}_{(2)} \\ \bar{d}_{(1)} \end{matrix}$$

$$d_{(i)} = \bar{d}_{(i)}$$

$$d_{(i)} - \bar{d}_{(i)} > \Delta$$
significant positive

$$\bar{d}_{(i)} - d_{(i)} > \Delta$$
significant negative

upper cut-point $\text{cut}_{up}(\Delta)$

lower cut-point $\text{cut}_{low}(\Delta)$

$$\begin{bmatrix} d_{(p)} \\ \vdots \\ d_{(2)} \\ d_{(1)} \end{bmatrix}$$

vs

$$\begin{bmatrix} \bar{d}_{(p)} \\ \vdots \\ \bar{d}_{(2)} \\ \bar{d}_{(1)} \end{bmatrix}$$
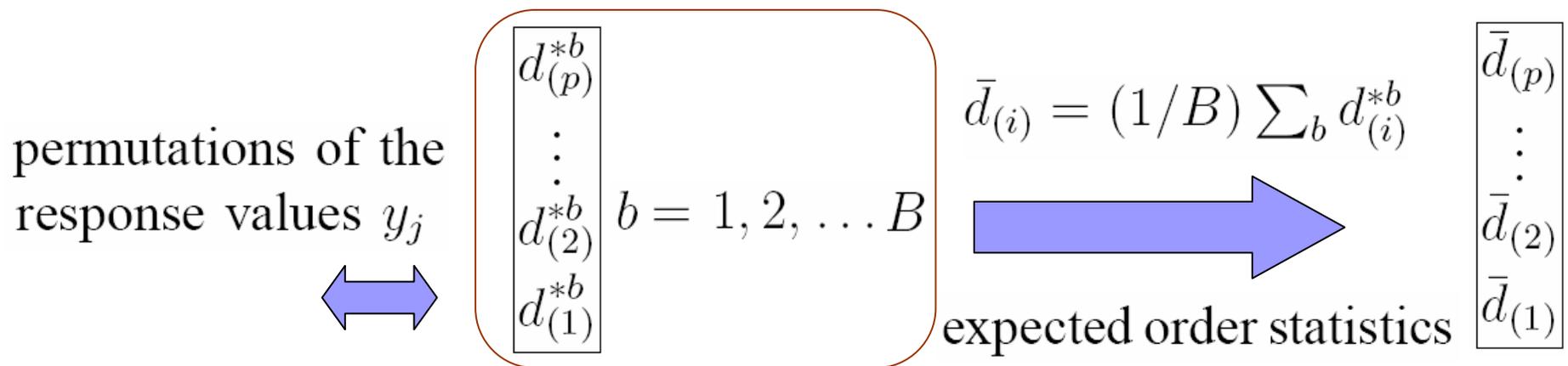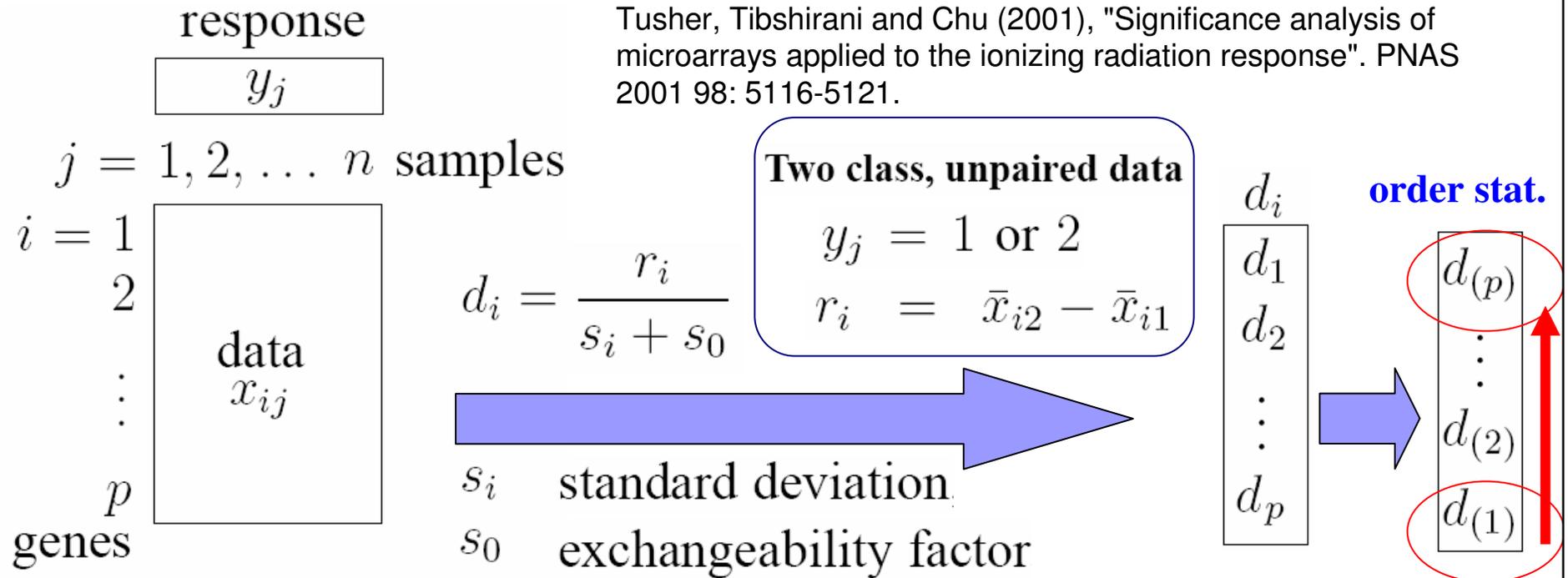
number of falsely called genes
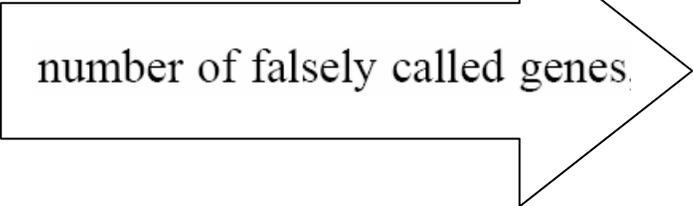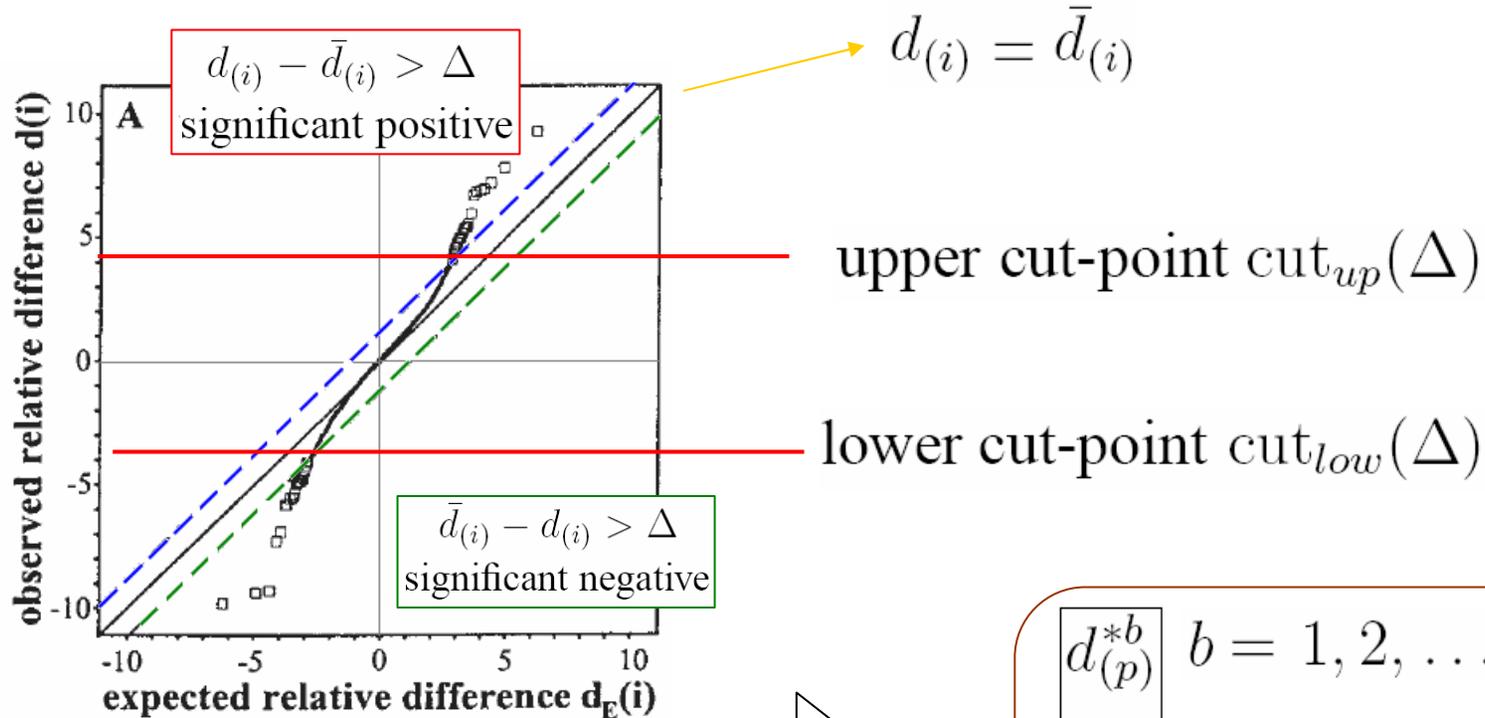
$$\begin{bmatrix} d_{(p)}^{*b} \\ \vdots \\ \vdots \\ d_{(2)}^{*b} \\ d_{(1)}^{*b} \end{bmatrix} \quad b = 1, 2, \ldots B$$

fall above $\text{cut}_{up}(\Delta)$
or
fall below $\text{cut}_{low}(\Delta)$

$$\text{False Discovery Rate (FDR)} = \frac{[\text{median (or 90th percentile) of the number of falsely called genes}]}{[\text{the number of genes called significant}]}$$

The q-value of a gene is the false discovery rate for the gene list that includes that gene and all genes that are more significant. It is computed by finding the smallest value of $\hat{\Delta}$ for which the gene is called significant, and then is the FDR corresponding to $\hat{\Delta}$.

| Response type	Coding
Quantitative	Real number eg 27.4 or -45.34
Two class (unpaired)	Integer 1, 2
Multiclass	Integer 1, 2, 3, ...
Paired	Integer -1, 1, -2, 2, etc. eg - means Before treatment, + means after treatment -1 is paired with 1, -2 is paired with 2, etc.
Survival data	(Time, status) pair like (50,1) or (120,0) First number is survival time, second is status (1=died, 0=censored)
One class	Integer, every entry equal to 1
Time course, two class (unpaired)	(1 or 2)Time(t)[Start or End]
Time course, two class (paired)	(-1 or 1 or -2 or 2 etc)Time(t)[Start or End]
Time course, one class	1Time(t)[Start or End]
Pattern discovery	eigengenek, where k is one of 1,2,... number of arrays

Table 1: Response Formats

Software: SAM

SAM assigns a score to each gene in a microarray experiment based upon its change in gene expression relative to the standard deviation of repeated measurements.

- *False discovery rate*: is the percent of genes that are expected to be identified by chance.

- *q-value*: the lowest false discovery rate at which a gene is described as significantly regulated.

- *Output plot*: the number of observed genes versus the expected number. This visualizes the outlier genes that are most dramatically regulated.

SAM: Significance Analysis of Microarrays
http://www-stat.stanford.edu/~tibs/SAM/
Gene Set Enrichment Analysis
http://www.broad.mit.edu/gsea/

Tusher VG, Tibshirani R, Chu G.(2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci* 98(9):5116-21.

Software: Limma, LimmaGUI, affylmGUI

LimmaGUI - Untitled

File Spot Types RNA Targets Layout Normalization Linear Model Evaluate TopTable Plot Help

LimmaGUI

Welcome to LimmaGUI, a package for Linear Modelling of Microarray Data

Data Set Name

Untitled
- R and G
- Background Correction
- Spot Quality Weighting
- M and A
- Layout
- Parameterizations

PARAMETERIZATIONS

M A Scatter Plot for 811

gene
80C
NegativeControl
Pro25G

Limma: Linear Models for Microarray Data

http://bioinf.wehi.edu.au/limma/

LimmaGUI: a menu driven interface of Limma

http://bioinf.wehi.edu.au/limmaGUI

- Smyth, G. K. (2005). Limma: linear models for microarray data. In: Bioinformatics and Computational Biology Solutions using R and Bioconductor, R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, W. Huber (eds.), Springer, New York, Chapter 23. (To be published in 2005)
- Smyth, G. K. (2004). Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology 3, No. 1, Article 3.

Tilapia - Statistical Microarray Analysis using LimmaGUI - Microsoft Internet Explorer

檔案(F) 編輯(E) 檢視(V) 我的最愛(A) 工具(T) 說明(H)

上一頁 搜尋 我的最愛 媒體

Tilapia - Statistical Microarray Analysis using LimmaGUI
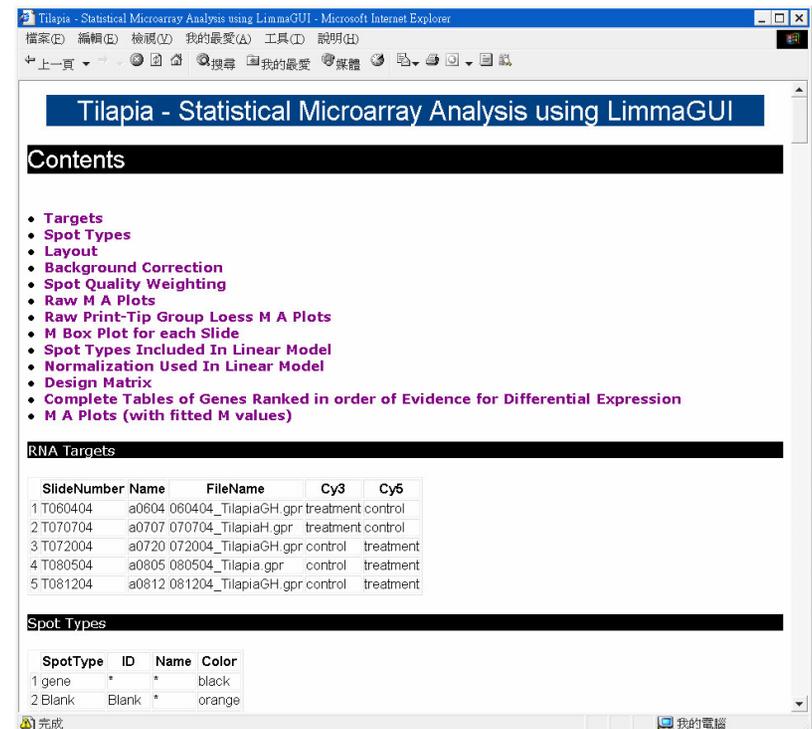
Contents

- Targets
- Spot Types
- Layout
- Background Correction
- Spot Quality Weighting
- Raw M A Plots
- Raw Print-Tip Group Loess M A Plots
- M Box Plot for each Slide
- Spot Types Included In Linear Model
- Normalization Used In Linear Model
- Design Matrix
- Complete Tables of Genes Ranked in order of Evidence for Differential Expression
- M A Plots (with fitted M values)

RNA Targets

	SlideNumber	Name	FileName	Cy3	Cy5
1	T060404	a0604	060404_TilapiaGH.gpr	treatment	control
2	T070704	a0707	070704_TilapiaH.gpr	treatment	control
3	T072004	a0720	072004_TilapiaGH.gpr	control	treatment
4	T080504	a0805	080504_Tilapia.gpr	control	treatment
5	T081204	a0812	081204_TilapiaGH.gpr	control	treatment

Spot Types

	SpotType	ID	Name	Color
1	gene	*	*	black
2	Blank	Blank	*	orange

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Other Related Issues

**Reference for Finding
Differential Expressed Genes**

http://idv.sinica.edu.tw/hmwu/SMDA/DifferentialExpressedGene/index.htm

- Clustering and Classification
- Analysis of Replicates Arrays
- Time Series Samples
- Experimental Design
- …

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http://idv.sinica.edu.tw/hmwu

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