

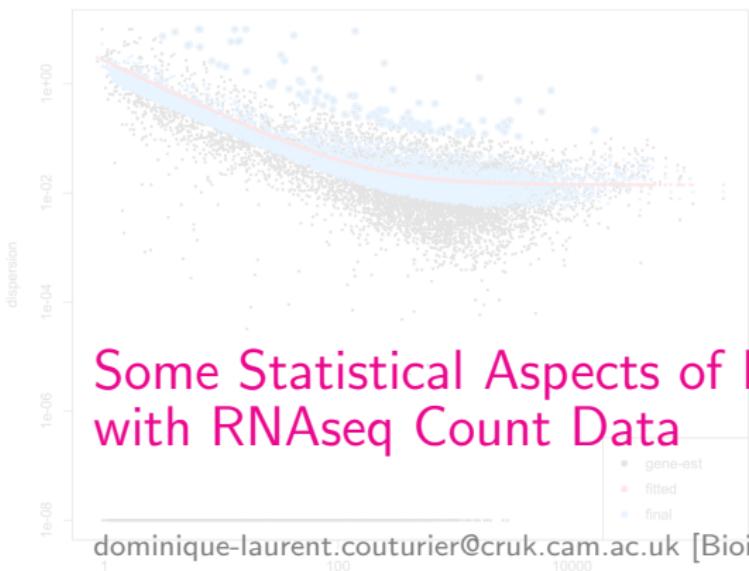


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Some Statistical Aspects of DE Analysis with RNAseq Count Data

(Source: O. Rueda, MRC-BSU; G. Marot, INRIA)

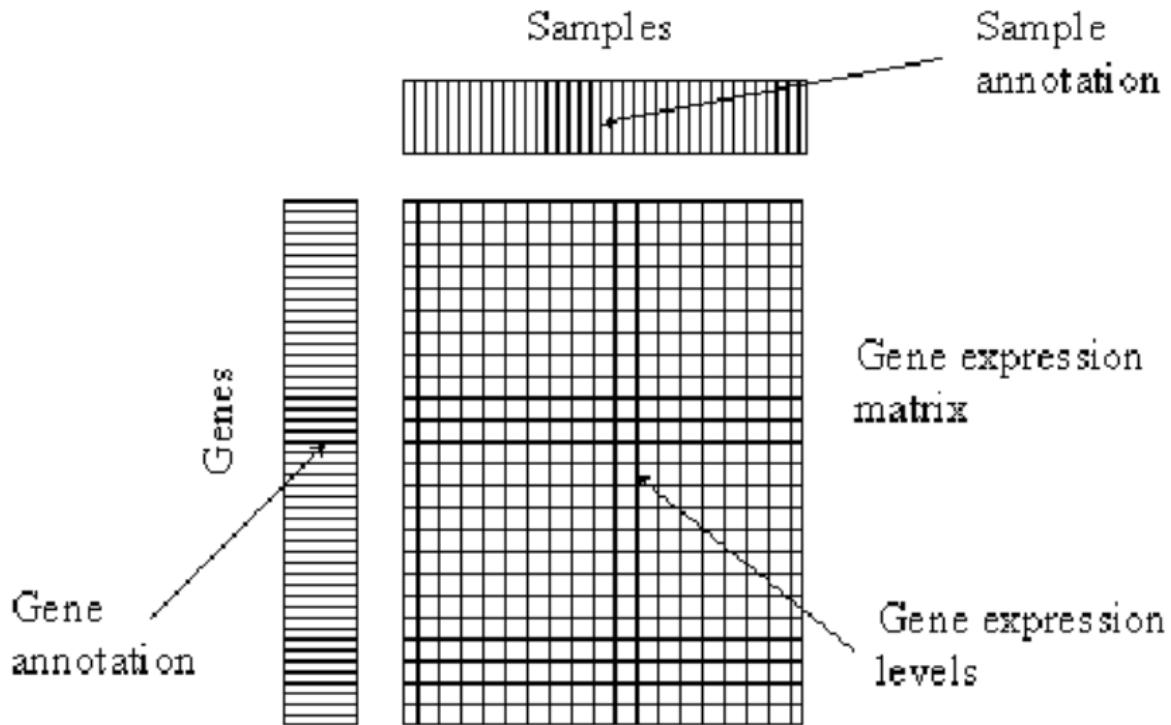
raw count for gene i , sample j

The mean is taken as "normalized counts" scaled by a normalization factor

$$K_{ij} \sim NB(s_{ij}q_{ij}, \alpha_i)$$

one dispersion per gene

Introduction



Introduction

```
> set.seed(777)
> cnts <- matrix(rnbinom(n=20000, mu=100, size=1/.25), ncol=20)
> cond <- factor(rep(1:2, each=10))

> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
> dds <- DESeq(dds)
> results(dds)

log2 fold change (MLE): cond 2 vs 1
Wald test p-value: cond 2 vs 1
DataFrame with 1000 rows and 6 columns
  baseMean log2FoldChange      lfcSE      stat     pvalue     padj
  <numeric>      <numeric>      <numeric>      <numeric>      <numeric>
1    97.3140     -0.682067    0.344525   -1.979730  0.0477339  0.745842
2   109.9860     -0.228819    0.450720   -0.507676  0.6116808  0.944354
3    98.8111      0.104291    0.462113    0.225683  0.8214483  0.978382
4   103.2615      0.306400    0.297682    1.029284  0.3033460  0.944354
5    97.9406      0.316338    0.357242    0.885501  0.3758864  0.944354
...
996   86.8057      0.0467703   0.287042    0.162939  0.8705668  0.980044
997  101.4437     -0.2070806   0.339886   -0.609264  0.5423495  0.944354
998   78.1356     -0.6372790   0.369515   -1.724637  0.0845930  0.824310
999   89.2920      0.7554725   0.306192    2.467314  0.0136131  0.614613
1000  103.5569     -0.0728875   0.348655   -0.209053  0.8344065  0.978382
```

Outline

► Part I: Quick recap

- ▷ Tests: Null and alternative hypotheses, Type I and type II errors, Power
- ▷ Experimental design & Sample size calculation.

► Part II: Modelling

- ▷ X design matrix,
- ▷ Linear regression,
- ▷ Negative binomial regression for counts.

► Part III: Multiplicity correction

- ▷ Familywise error rate (FWER)
- ▷ False discovery rate (FDR)

mean of normalized counts

count for gene i, sample j

The mean is taken as "normalized counts" scaled by a normalization factor

$$K_{ij} \sim NB(s_{ij}q_{ij}, \alpha_i)$$

one dispersion per gene

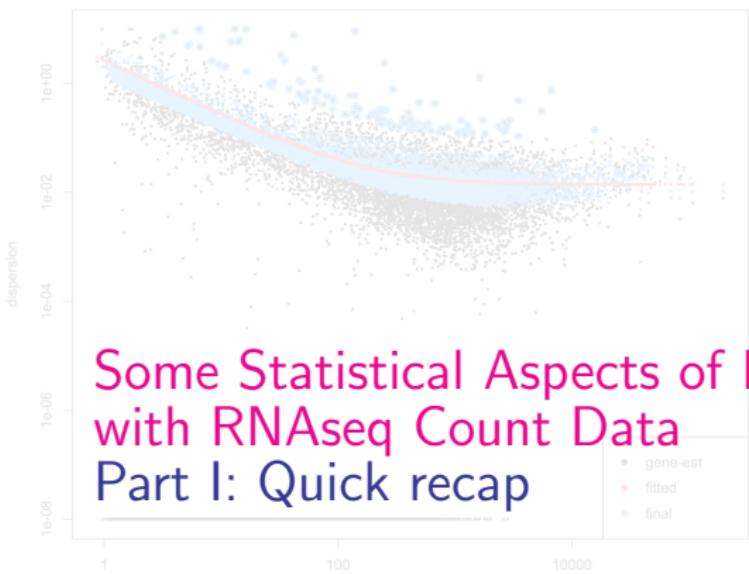


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Some Statistical Aspects of DE Analysis with RNAseq Count Data Part I: Quick recap

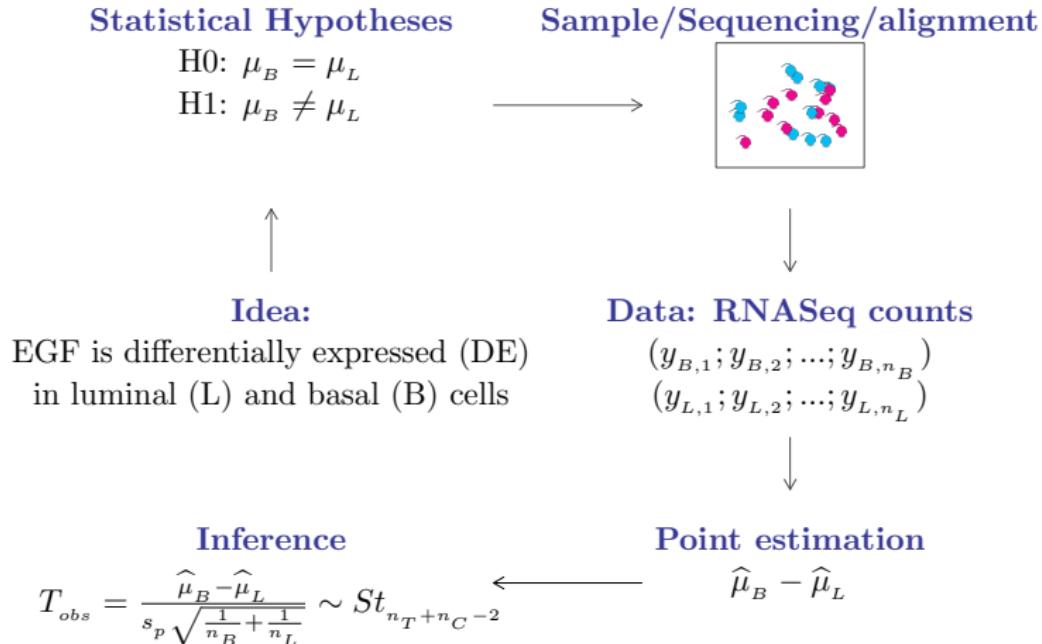
dominique-laurent.couturier@cruk.cam.ac.uk [Bioinformatics core]

The mean is taken as "normalized
counts scaled by a normalization
factor"

$$K_{ij} \sim NB(s_{ij}q_{ij}, \alpha_i)$$

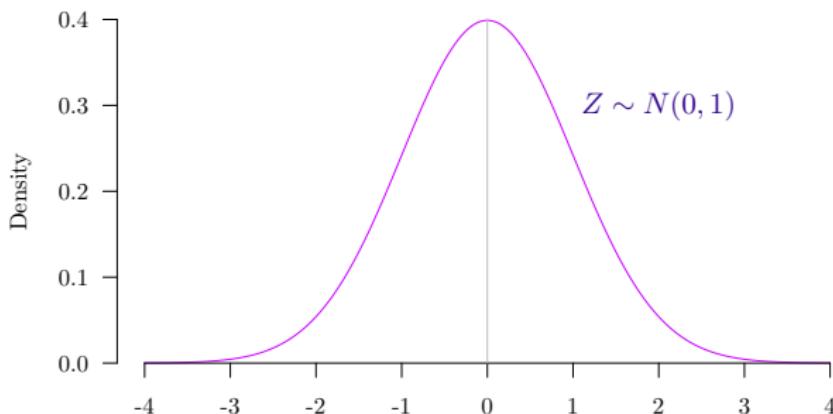
one dispersion per gene

Grand Picture of Statistics



Statistical tests

Assess how likely the observed test statistics is compared to the test statistics distribution under H_0 :



P-value for a two-sided test:

$$p\text{-value} = 2 \min [P(Z \leq Z_{obs} | H_0), P(Z \geq Z_{obs} | H_0)]$$

i.e. the probability of getting a test statistic as extreme or more extreme than the calculated test statistic if H_0 is true

Statistical tests

4 possible outcomes

Conclude:

- ▶ if $p\text{-value} > \alpha \rightarrow$ do not reject H_0 .
- ▶ if $p\text{-value} < \alpha \rightarrow$ reject H_0 in favour of H_1 .

		Test Outcome	
		H_0 not rejected	H_1 accepted
Unknown Truth	H_0 true	$1 - \alpha$ [TN]	α [FP]
	H_1 true	β [FN]	$1 - \beta$ [TP]

where

- ▶ α is the type I error, the probability of rejecting H_0 when H_0 is correct,
- ▶ β is the type II error, the probability of not rejecting H_0 when H_1 is correct.

Warnings

- ▶ 'absence of evidence is not evidence of absence',
- ▶ design may help minimising FP and FN (ie, maximising TN and TP).

Experimental design 1: Minimising biases

3 fundamental aspects of sounds experiments (Fisher 1935)

- ▶ Replication

Try to capture all sources of variability
(Biological versus technical variability)

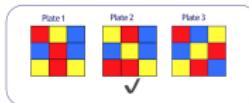
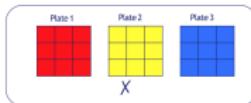
- ▶ Blocking

Try to remove technical biases/confounding
(Lane and batch effects)



- ▶ Randomisation

Try to remove confounding due to other factors



Experimental design 2: boosting power

Power- / Effect size- / Sample size- calculations

4 ingredients:

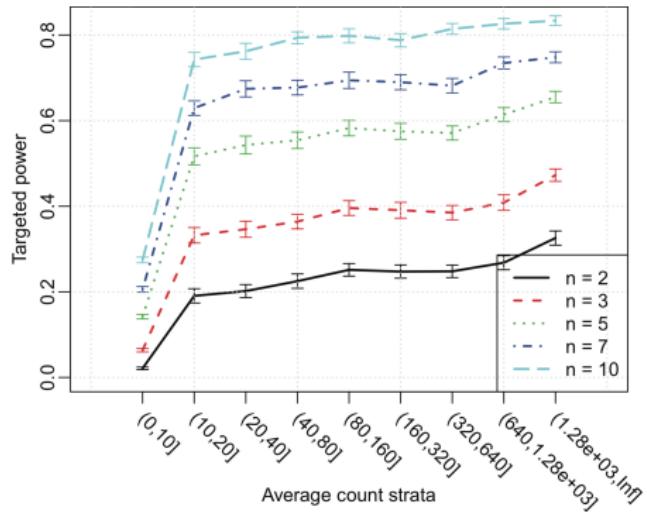
- ▶ $1 - \beta$, the power,
- ▶ δ , the effect size: function of μ_L and μ_B
(log fold change, standardised difference),
- ▶ n , the sample size (number of biological replicates),
- ▶ α , the type I error.
- ▷ ϕ , nuisance parameters
(variability, sequencing depth, multiplicity correction)

'Give me 3 of them, I will deduce the fourth':

- ▶ **Power calculation:** Aim is to define the probability ($1 - \beta$) to detect an effect size of interest (δ) at the α level with a sample size of n biological replicates.
- ▶ **Sample size calculation:** Aim is to define the sample size (n) allowing to detect an effect size of interest (δ) at the α level with a given probability ($1 - \beta$).

Experimental design 2: boosting power

Power- calculations in DE analyses



(Wu, Wang and Wu (2015))

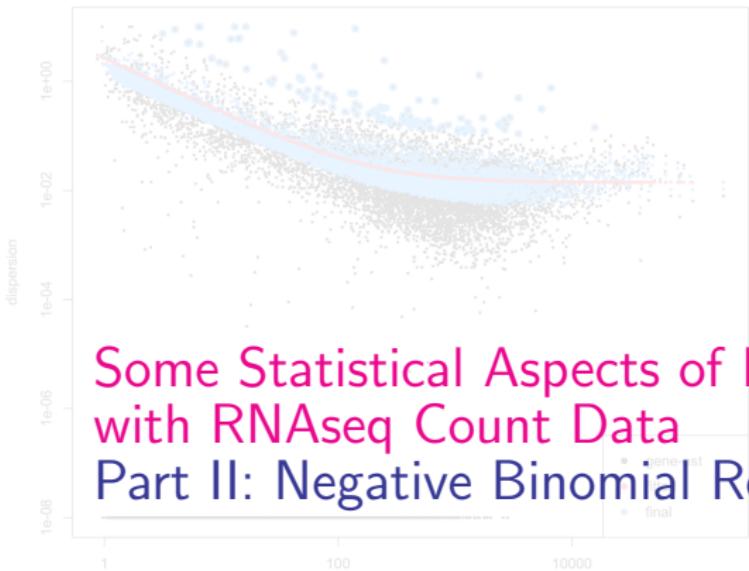


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Some Statistical Aspects of DE Analysis with RNAseq Count Data Part II: Negative Binomial Regression

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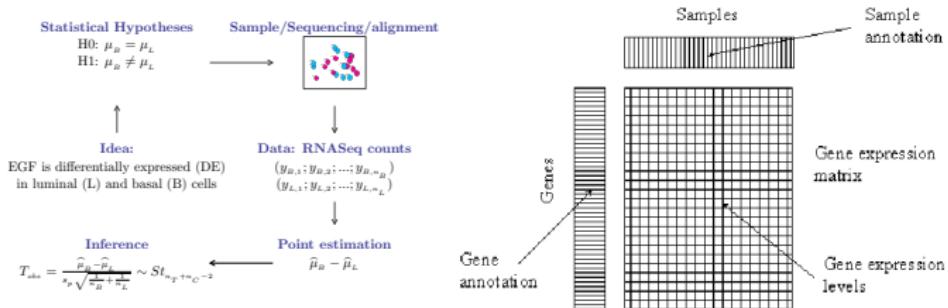
(Source: O. Rueda, MRC-BSU)

The mean is taken as "normalized count" divided by a normalization factor

$$K_{ij} \sim NB(s_{ij}q_{ij}, \alpha_i)$$

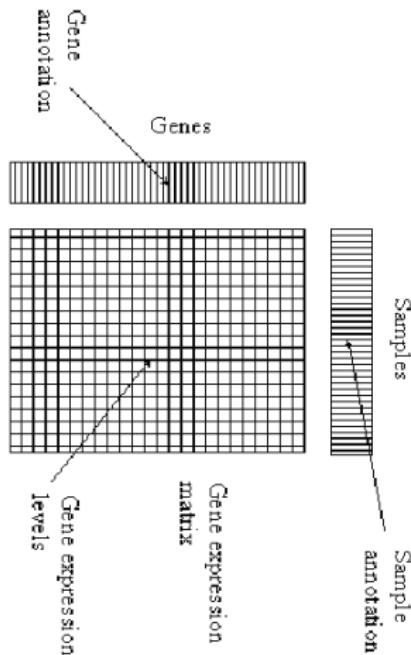
one dispersion per gene

Statistical modelling



Aim: Model the count data of each gene as a function of the conditions of interest (treatment, age, sex, batch, aso.)

Statistical modelling



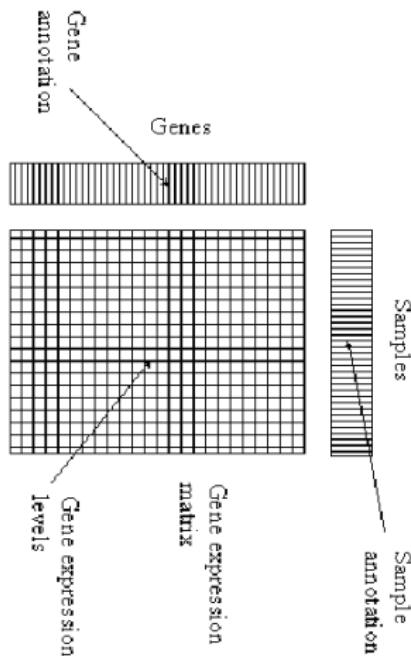
$$\mathbf{y} = f(\mathbf{X}) + \epsilon$$
$$E[\mathbf{y}] = f(\mathbf{X})$$

where

- ▶ \mathbf{y} denotes the $(n \times 1)$ vector of expression intensities of a given gene,
- ▶ \mathbf{X} denotes the $(n \times p)$ design/predictor matrix,
- ▶ ϵ denotes the $(n \times 1)$ stochastic error vector,
- ▶ $E[\mathbf{y}]$ denotes the expectation of \mathbf{y}

Express the count data vector of a given gene, \mathbf{y} , as a function f of characteristics of the samples (\mathbf{X} : age, treatment, aso) plus a stochastic error vector ϵ

Statistical modelling : Linear regression

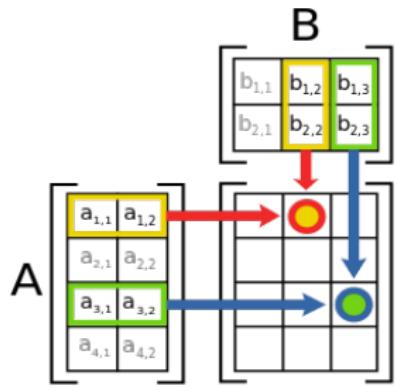


$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \boldsymbol{\epsilon}$$
$$E[\mathbf{y}] = \mathbf{X}\boldsymbol{\beta}$$

where

- ▶ \mathbf{y} denotes the $(n \times 1)$ vector of expression intensities of a given gene,
- ▶ \mathbf{X} denotes the $(n \times p)$ design/predictor matrix,
- ▶ $\boldsymbol{\beta}$ denotes the $(p \times 1)$ parameter vector,
- ▶ $\boldsymbol{\epsilon} \sim N(0, \sigma^2)$ denotes the $(n \times 1)$ stochastic error vector,
- ▶ $E[\mathbf{y}]$ denotes the expectation of \mathbf{y}

Statistical modelling : Linear regression



(Wikipedia)

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \epsilon$$

$$E[\mathbf{y}] = \mathbf{X}\boldsymbol{\beta}$$

where

- ▶ \mathbf{y} denotes the $(n \times 1)$ vector of expression intensities of a given gene,
- ▶ \mathbf{X} denotes the $(n \times p)$ design/predictor matrix,
- ▶ $\boldsymbol{\beta}$ denotes the $(p \times 1)$ parameter vector,
- ▶ $\epsilon \sim N(0, \sigma^2)$ denotes the $(n \times 1)$ stochastic error vector,
- ▶ $E[\mathbf{y}]$ denotes the expectation of \mathbf{y}

Matrix multiplication:

the element $C_{i,j}$ (i th row, j th column of the matrix \mathbf{C}) is obtained by

- ▶ multiplying **term-by-term** the entries of the i th row of \mathbf{A} and the j th column of \mathbf{B} ,
- ▶ and summing these products.

Statistical modelling : Strategy

- ▶ Collect the information related to each sample for the predictors of interest,
- ▶ define β , the sets of parameters we are interested in,
- ▶ build the X matrix that relates the sample information with the β
this step is automatically done in R by specifying the regression formula in the function `lm()` or `DEseq2()`
- ▶ estimate the β and use statistical inference to assess significance (p -values)
these two points are done by the function `lm()` or `DEseq2()`

Statistical modelling : $\mathbf{X}\boldsymbol{\beta}$ (For information)

- ▶ Linear regression:

$$E[\mathbf{y}] = \mathbf{X}\boldsymbol{\beta},$$

- ▶ Cox regression:

$$h(t) = h_0(t)e^{\mathbf{X}\boldsymbol{\beta}},$$

- ▶ Logistic regression:

$$\pi = \frac{e^{\mathbf{X}\boldsymbol{\beta}}}{1+e^{\mathbf{X}\boldsymbol{\beta}}},$$

- ▶ Mean expression levels for a given gene in DESeq2:

$$E[\mathbf{y}] = 2^{\mathbf{X}\boldsymbol{\beta}},$$

Statistical modelling : X contrast matrix

Contrast matrices for models with

- ▶ one factor / categorical predictor,
 - ▷ two experimental conditions (dichotomous predictor),
[t-test](#)
 - ▷ several experimental conditions,
[One-way ANOVA](#)
- ▶ two factors / categorical predictors,
 - ▷ without interaction,
 - ▷ with interaction,
[Two-way ANOVA](#)

Design matrix for models with a two-level factor

Sample	Treatment
Sample1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control

Number of samples: 6

Number of factors: 1 with 2 levels (Control and Treatment A)

Possible parameters (What differences are important)?

- Effect of Treatment A
- Effect of Control

Design matrix for models with a two-level factor: No intercept

Sample	Treatment
Sample 1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control

$$\begin{matrix} \beta \\ \beta_1 \\ \beta_2 \end{matrix}$$

Control Treat. A

$$\begin{matrix} \text{Sample 1} \\ \text{Sample 2} \\ \text{Sample 3} \\ \text{Sample 4} \\ \text{Sample 5} \\ \text{Sample 6} \end{matrix} \left(\begin{array}{c} \quad \\ \quad \\ \quad \\ \quad \\ \quad \\ \quad \end{array} \right) = \left(\begin{array}{c} \quad \\ \quad \\ \quad \\ \quad \\ \quad \\ \quad \end{array} \right)$$

y X

$\beta_1 = \mu_C$ is the mean expression of the control

$\beta_2 = \mu_A$ is the mean expression of the treatment A group

Design matrix for models with a two-level factor: With intercept

Sample	Treatment
Sample 1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control

$$\begin{array}{l} \text{Sample 1} \\ \text{Sample 2} \\ \text{Sample 3} \\ \text{Sample 4} \\ \text{Sample 5} \\ \text{Sample 6} \end{array} \left(\begin{array}{|c|} \hline y \\ \hline \end{array} \right) = \left(\begin{array}{|c|} \hline \text{Intercept} \\ \hline \text{Shift bw C and A} \\ \hline \beta \\ \hline \beta_1 \\ \hline \beta_2 \\ \hline \end{array} \right) \quad \begin{array}{|c|} \hline X \\ \hline \end{array}$$

$\beta_1 = \mu_C$ is the mean expression of the control
 β_2 is the shift in mean between the group A and the control group

Design matrices for models with a two-level factor:

R Code

Open the R Markdown Document 'StatsRNAseq_Couturier.Rmd'
and go to Section '[Contrast matrices / One 2-level factor](#)'

```
> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
```

Design matrix for models with a three-level factor

Sample	Treatment
Sample1	Treatment A
Sample 2	Treatment B
Sample 3	Control
Sample 4	Treatment A
Sample 5	Treatment B
Sample 6	Control

Number of samples: 6

Number of factors: 1 with 3 levels (Control, Treatment A, Treatment B)

Possible parameters (What differences are important)?

- Effect of Treatment A
- Effect of Treatment B
- Effect of Control
- Differences between treatments?

Design matrix for models with a two-level factor: No intercept

Sample	Treatment
Sample1	Treatment A
Sample 2	Treatment B
Sample 3	Control
Sample 4	Treatment A
Sample 5	Treatment B
Sample 6	Control

$$\begin{bmatrix} \beta \\ \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix} = \begin{bmatrix} y \\ x \end{bmatrix}$$

$\beta_1 = \mu_C$ is the mean expression of the control

$\beta_2 = \mu_A$ is the mean expression of the treatment A group

$\beta_3 = \mu_B$ is the mean expression of the treatment B group

Design matrix for models with a two-level factor: With intercept

Sample	Treatment
Sample1	Treatment A
Sample 2	Treatment B
Sample 3	Control
Sample 4	Treatment A
Sample 5	Treatment B
Sample 6	Control

$$\begin{bmatrix} \beta \\ \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix} = \begin{bmatrix} y \\ x \end{bmatrix}$$

$\beta_1 = \mu_C$ is the mean expression of the control

β_2 is the shift in mean between the group A and the control group

β_3 is the shift in mean between the group B and the control group

Design matrices for models with a three-level factor:

R Code

Open the R Markdown Document 'StatsRNAseq_Couturier.Rmd'
and go to Section '[Contrast matrices / One 3-level factor](#)'

```
> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
```

Design matrix for models with two two-level factors

Sample	Treatment	ER status
Sample1	Treatment A	+
Sample 2	No Treatment	+
Sample 3	Treatment A	+
Sample 4	No Treatment	+
Sample 5	Treatment A	-
Sample 6	No Treatment	-
Sample 7	Treatment A	-
Sample 8	No Treatment	-

Number of samples: 8

Number of factors: 2 two-level factors

Design matrix for models with two two-level factors: No interaction

Sample	Treatment	ER status
Sample 1	Treatment A	+
Sample 2	No Treatment	+
Sample 3	Treatment A	+
Sample 4	No Treatment	+
Sample 5	Treatment A	-
Sample 6	No Treatment	-
Sample 7	Treatment A	-
Sample 8	No Treatment	-

$$\begin{bmatrix} & \\ & \end{bmatrix} = \begin{bmatrix} & \\ & \end{bmatrix}$$

y x

$\beta_1 = \mu_C$ is the mean expression of the control

β_2 is the shift in mean between the group A and the control group

β_3 is the shift in mean between the ER+ group and the control group

Design matrix for models with two two-level factors: With interaction

Sample	Treatment	ER status
Sample 1	Treatment A	+
Sample 2	No Treatment	+
Sample 3	Treatment A	+
Sample 4	No Treatment	+
Sample 5	Treatment A	-
Sample 6	No Treatment	-
Sample 7	Treatment A	-
Sample 8	No Treatment	-

$$\begin{bmatrix} & \\ & \end{bmatrix} = \begin{bmatrix} & \\ & \end{bmatrix}$$

y x

$$\begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \\ \beta_4 \end{bmatrix}$$

$\beta_1 = \mu_C$ is the mean expression of the control

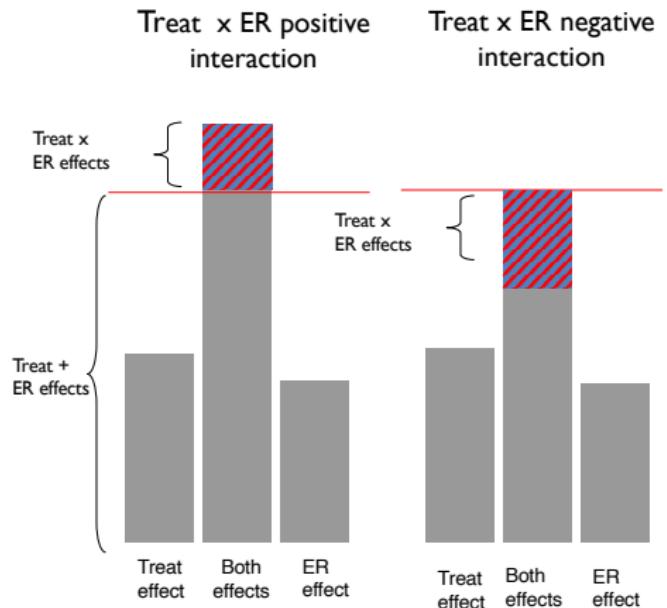
β_2 is the shift in mean between the group A and the control group

β_3 is the shift in mean between the ER+ group and the control group

β_4 is the additional shift in mean for patients of the ER+ and Treatment A groups

Design matrix for models with two two-level factors: With interaction

Sample	Treatment	ER status
Sample 1	Treatment A	+
Sample 2	No Treatment	+
Sample 3	Treatment A	+
Sample 4	No Treatment	+
Sample 5	Treatment A	-
Sample 6	No Treatment	-
Sample 7	Treatment A	-
Sample 8	No Treatment	-



Design matrices for models with two two-level factors:

R Code

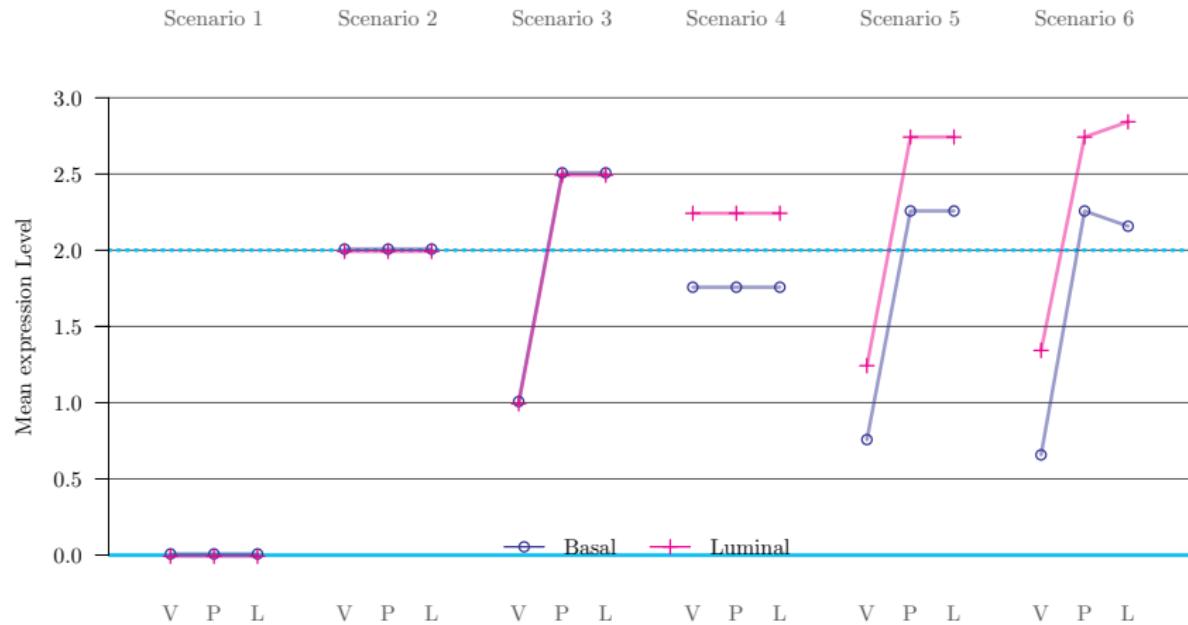
Open the R Markdown Document 'StatsRNAseq_Couturier.Rmd'
and go to Section '[Contrast matrices / Two 2-level factors](#)'

```
> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
```

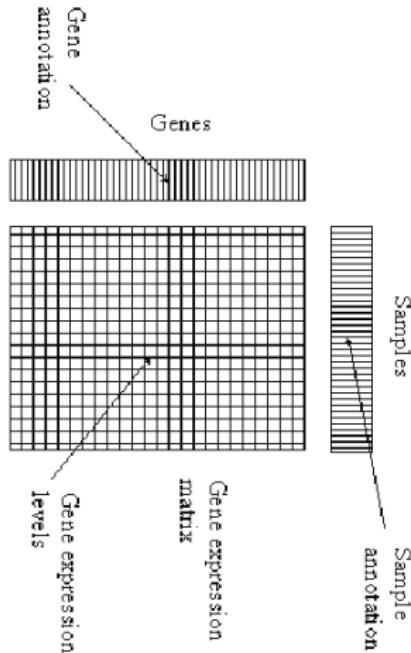
Models with 2 factors: possible scenarios

2 factors:

- ▶ cell type (2 levels): luminal versus basal
- ▶ mouse type (3 levels): virgin, pregnant, lactating



Negative binomial regression: Model



$$\mathbf{y} \sim \text{NB}(\mu, \phi)$$

$$E[\mathbf{y}] = \mu = s 2^{\mathbf{X}\beta}$$

where

- ▶ \mathbf{y} denotes the $(n \times 1)$ count vector of expression intensities of a given gene,
- ▶ \mathbf{X} denotes the $(n \times p)$ design/predictor matrix,
- ▶ β denotes the $(p \times 1)$ parameter vector,
- ▶ ϕ denotes the dispersion parameter,
- ▶ s denotes the scaling factor vector (library size),
- ▶ $E[\mathbf{y}] = \mu$ denotes the expectation of \mathbf{y}

Negative binomial regression: Probability mass function

$$\mathbf{y} \sim \text{NB}(\boldsymbol{\mu}, \phi)$$

$$f(\mathbf{y}|\boldsymbol{\mu}, \phi) = \frac{\Gamma(\mathbf{y} + \frac{1}{\phi})}{\Gamma(\frac{1}{\phi})\Gamma(\mathbf{y} + 1)} \left(\frac{\phi\boldsymbol{\mu}}{1 + \phi\boldsymbol{\mu}} \right)^{\mathbf{y}} \left(\frac{1}{1 + \phi\boldsymbol{\mu}} \right)^{\frac{1}{\phi}}$$

with expectation and variance given by

- ▶ $E[\mathbf{y}] = \boldsymbol{\mu} = s 2^{\mathbf{X}\boldsymbol{\beta}}$
- ▶ $\text{Var}[\mathbf{y}] = \boldsymbol{\mu}(1 + \phi\boldsymbol{\mu})$

Negative binomial regression: Log2 FC

```
log2 fold change (MLE): cond 2 vs 1
Wald test p-value: cond 2 vs 1
DataFrame with 1000 rows and 6 columns
  baseMean log2FoldChange    lfcSE      stat     pvalue     padj
  <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
1     97.3140      -0.682067  0.344525 -1.979730  0.0477339  0.745842
2    109.9860      -0.228819  0.450720 -0.507676  0.6116808  0.944354
...
999   89.2920      0.7554725  0.306192  2.467314  0.0136131  0.614613
1000 103.5569      -0.0728875  0.348655 -0.209053  0.8344065  0.978382
```

- ▶ $E[y|'cond 1'] = 2^{\hat{\beta}_1}$
- ▶ $E[y|'cond 2'] = 2^{\hat{\beta}_1 + \hat{\beta}_2} = 2^{\hat{\beta}_1} 2^{\hat{\beta}_2}$
 - ▷ If not DE, $\hat{\beta}_2 = 0$ so that $E[y|'cond 2'] = 2^{\hat{\beta}_1} 2^0 = 2^{\hat{\beta}_1}$,
 - ▷ If DE, $\beta_1 \neq 0$ so that $E[y|'cond 2'] = 2^{\hat{\beta}_1} 2^{\hat{\beta}_2}$
Interpretation: *Multiplicative change in observed gene expression level of* $2^{\hat{\beta}_2} = 2^{-0.682067} = 0.6232717$ *compared to the condition 1*

Negative binomial regression: Significance

```
log2 fold change (MLE): cond 2 vs 1
Wald test p-value: cond 2 vs 1
DataFrame with 1000 rows and 6 columns
  baseMean log2FoldChange    lfcSE      stat     pvalue     padj
  <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
1     97.3140     -0.682067  0.344525 -1.979730 0.0477339  0.745842
2    109.9860     -0.228819  0.450720 -0.507676 0.6116808  0.944354
...
999   89.2920      0.7554725  0.306192  2.467314 0.0136131  0.614613
1000 103.5569     -0.0728875  0.348655 -0.209053 0.8344065  0.978382
```

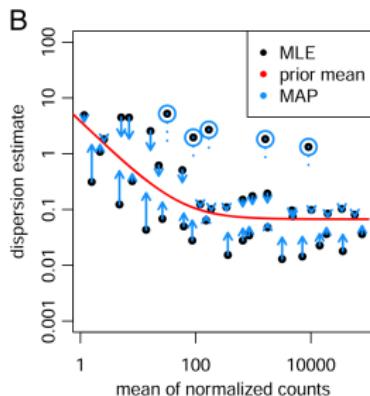
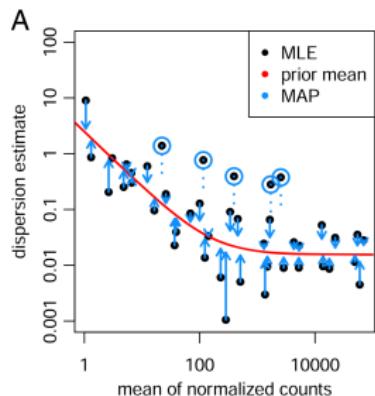
Wald T-test to assess if a Log2 FC is significantly different from 0:

- ▶ **H0:** $\beta_2 = 0$ versus **H1:** $\beta_2 \neq 0$
- ▶ Z-statistic = $\frac{\hat{\beta}_2}{\hat{\sigma}_{\hat{\beta}_2}} = \frac{-0.682067}{0.344525} = -1.979730$
- ▶ P-value with $Z \sim N(0, 1)$ under **H0** is given by
 - > `2*(1-pnorm(abs(-1.979730)))`

```
[1] 0.04773388
```

Negative binomial regression: Assumed Distribution

- ▶ The **assumed distribution of counts per condition for a given gene** depends on
 - ▷ $\hat{\beta}$, the estimate of the parameter vector,
 - ▷ ϕ , the estimate of the dispersion parameter for that gene.
- ▶ There are **3 ways to estimate ϕ in DESeq2:**
 - ▷ **gene-wise** dispersion estimates via ML (black dots) [not efficient],
 - ▷ **smooth curve** (red line) [strong assumption],
 - ▷ Bayesian **combination of both** [mid-way optimal solution].

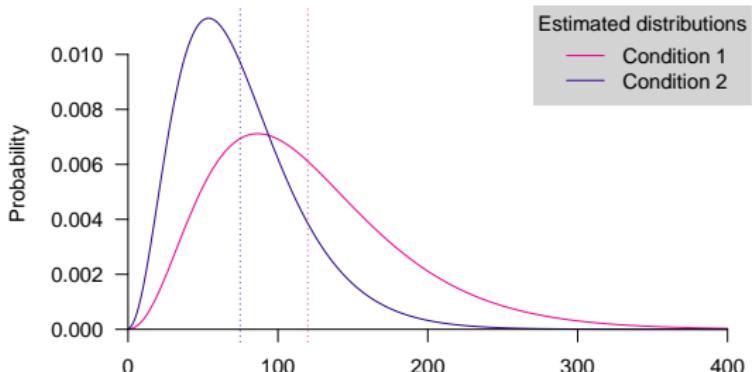


(Love et al (2015))

Negative binomial regression: Assumed Distribution

```
-> mcols(dds)[,c("Intercept","cond_2_vs_1","dispGeneEst","dispFit","dispersion")]
DataFrame with 1000 rows and 5 columns
  Intercept cond_2_vs_1 dispGeneEst dispFit dispersion
  <numeric>   <numeric>    <numeric> <numeric>   <numeric>
1     6.90565 -0.682067  0.294082  0.234624  0.274708
2     6.89102 -0.228819  0.479231  0.230525  0.479231
...
999    6.05380  0.7554725  0.206644  0.229562  0.213730
1000   6.73029 -0.0728875  0.304930  0.235483  0.282745
```

- ▶ For gene 1 and condition 1, we have
 $y \sim NB(\hat{\mu} = 2^{6.90565} = 119.8969, \hat{\phi} = 0.274708)$
- ▶ For gene 1 and condition 2, we have
 $y \sim NB(\hat{\mu} = 2^{6.90565} 2^{-0.682067} = 74.72831, \hat{\phi} = 0.274708)$





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Some Statistical Aspects of DE Analysis with RNAseq Count Data Part III: Multiplicity correction

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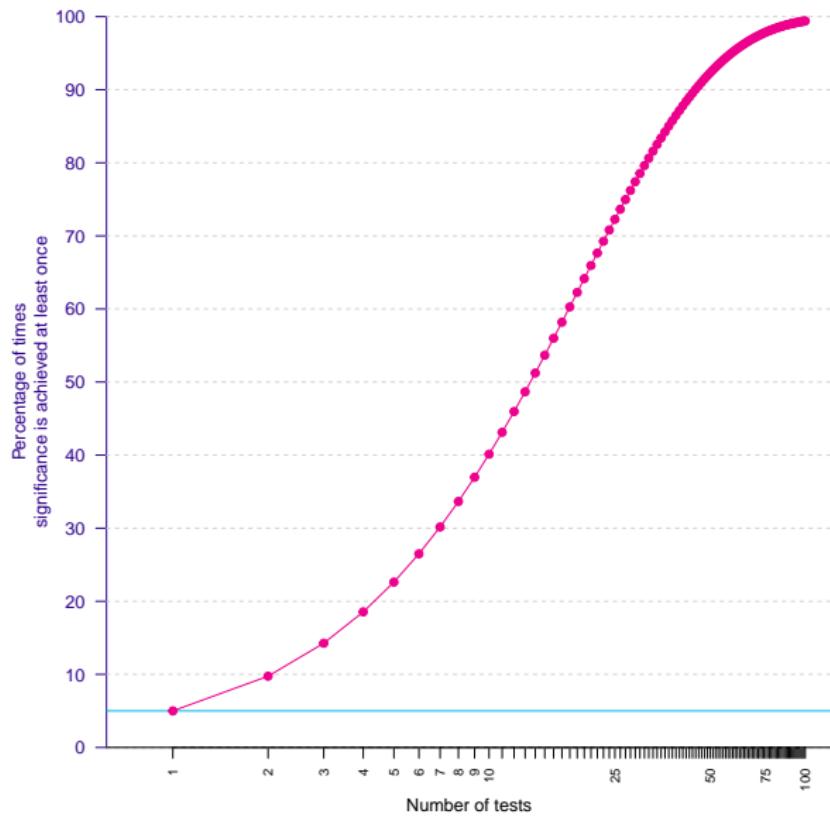
(Source: G. Marot, INRIA)

The mean is taken as "normalized count" divided by a normalization factor

$$K_{ij} \sim NB(s_{ij}q_{ij}, \alpha_i)$$

one dispersion per gene

Multiplicity correction: Familywise error rate



Multiplicity correction

Experimental design

Exploration

Normalization

Differential analysis

Multiple testing

The Family Wise Error Rate (FWER)

Definition

Probability of having at least one Type I error (false positive), of declaring DE at least one non DE gene.

$$FWER = \mathbb{P}(FP \leq 1)$$

The Bonferroni procedure

Either each test is realized at $\alpha = \alpha^*/G$ level

or use of adjusted pvalue $pBonf_i = \min(1, p_i * G)$ and $FWER \leq \alpha^*$.

For $G = 2000$, $\leq \alpha^* = 0.05$, $\alpha = 2.510^{-5}$.

Easy but conservative and not powerful.

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The False Discovery Rate (FDR)

Idea : Do not control the error rate but the proportion of error
⇒ less conservative than control of the FWER.

Definition

The false discovery rate of [Benjamini and Hochberg, 1995] is the expected proportion of Type I errors among the rejected hypotheses

$$\text{FDR} = \mathbb{E}(FP/P) \text{ if } P > 0 \text{ and } 0 \text{ if } P = 0$$

Prop

$$\text{FDR} \leq \text{FWER}$$

Multiplicity correction

```
> set.seed(777)
> cnts <- matrix(rnbinom(n=20000, mu=100, size=1/.25), ncol=20)
> cond <- factor(rep(1:2, each=10))

> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
> dds <- DESeq(dds)
> results(dds)

log2 fold change (MLE): cond 2 vs 1
Wald test p-value: cond 2 vs 1
DataFrame with 1000 rows and 6 columns
  baseMean log2FoldChange    lfcSE      stat     pvalue     padj
  <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
1     97.3140     -0.682067  0.344525 -1.979730  0.0477339  0.745842
2    109.9860     -0.228819  0.450720 -0.507676  0.6116808  0.944354
3     98.8111      0.104291  0.462113  0.225683  0.8214483  0.978382
4    103.2615      0.306400  0.297682  1.029284  0.3033460  0.944354
5     97.9406      0.316338  0.357242  0.885501  0.3758864  0.944354
...
996    86.8057      0.0467703  0.287042  0.162939  0.8705668  0.980044
997   101.4437     -0.2070806  0.339886 -0.609264  0.5423495  0.944354
998    78.1356     -0.6372790  0.369515 -1.724637  0.0845930  0.824310
999    89.2920      0.7554725  0.306192  2.467314  0.0136131  0.614613
1000   103.5569     -0.0728875  0.348655 -0.209053  0.8344065  0.978382

> p.adjust(results(dds)[,"pvalue"],method="BH")[c(1:5,996:1000)]
[1] 0.7458417 0.9443538 0.9783822 0.9443538 0.9443538 0.9800445 0.9443538 0.8243099
[9] 0.6146133 0.9783822
```

Multiplicity correction

Experimental design

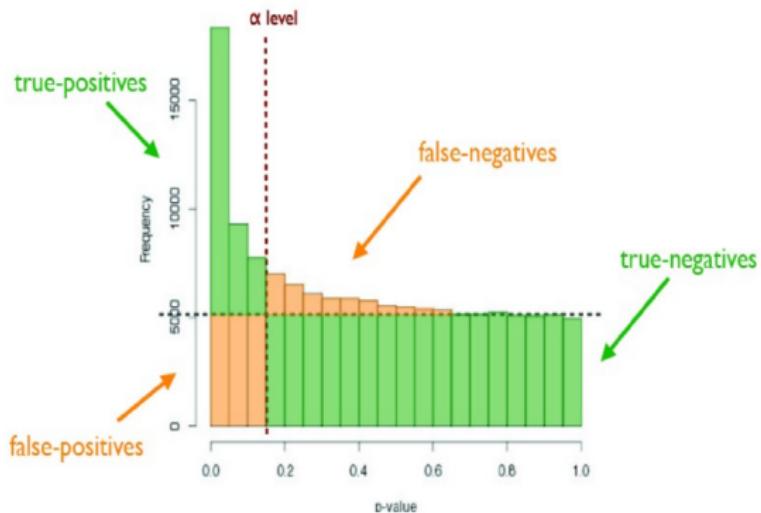
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Standard assumption for p-value distribution



Source : M. Guedj, Pharnext

Multiplicity correction

Experimental design

Exploration

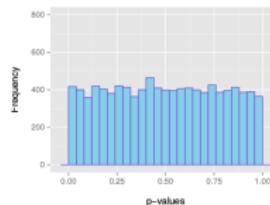
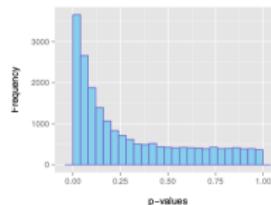
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p-values histograms for diagnosis

Examples of expected overall distribution



(a) : the most desirable shape

(b) : very low counts genes usually have large p-values

(c) : do not expect positive tests after correction

Multiplicity correction

Experimental design

Exploration

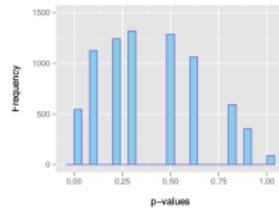
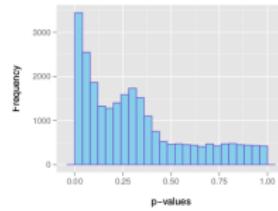
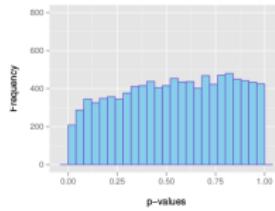
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Examples of not expected overall distribution



- (a) : indicates a batch effect (confounding hidden variables)
- (b) : the test statistics may be inappropriate (due to strong correlation structure for instance)
- (c) : discrete distribution of p-values : unexpected

CONCLUSION

```
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