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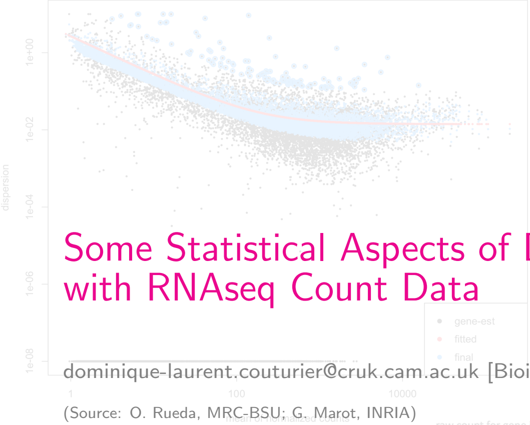


Some Statistical Aspects of DE Analysis with RNAseq Count Data

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

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

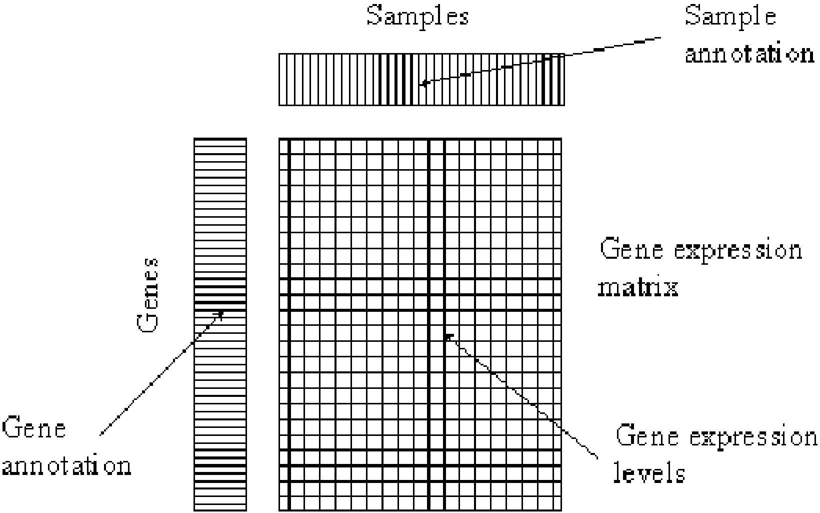
raw count for gene i, sample j

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

one dispersion per gene

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

Introduction

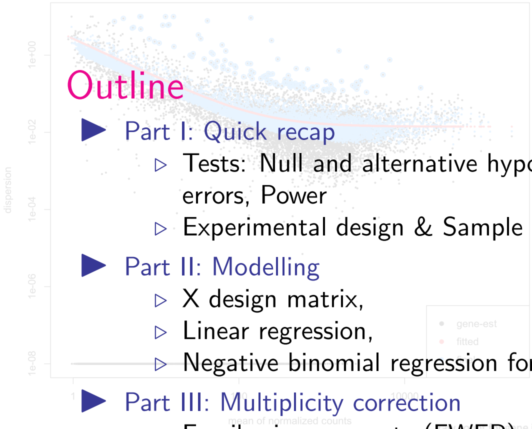


Introduction

```
> 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> <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)

raw count for gene i, sample j

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

one dispersion per gene

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

Some Statistical Aspects of DE Analysis with RNAseq Count Data

Part I: Quick recap

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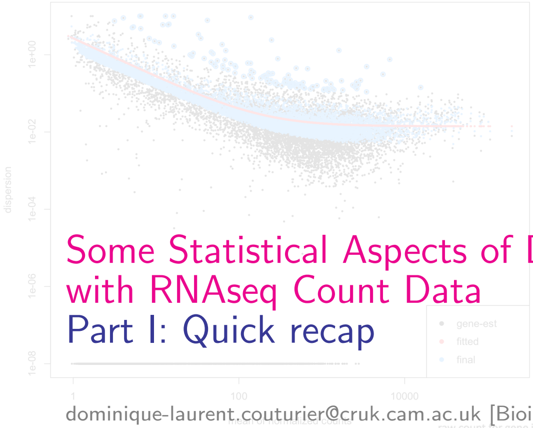


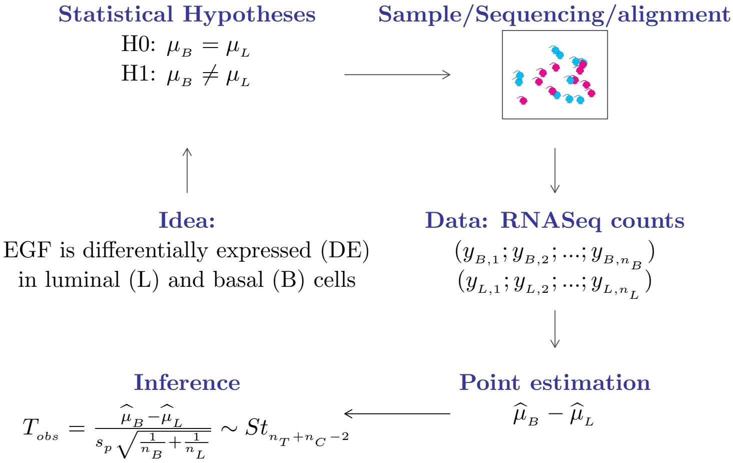
raw count for gene i, sample j

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

one dispersion per gene

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

Grand Picture of Statistics



Statistical Hypotheses

H0: $\mu_B = \mu_L$

H1: $\mu_B \neq \mu_L$

Sample/Sequencing/alignment

Data: RNASeq counts

$(y_{B,1}; y_{B,2}; ...; y_{B,n_B})$

$(y_{L,1}; y_{L,2}; ...; y_{L,n_L})$

Point estimation

$\widehat{\mu}_B - \widehat{\mu}_L$

Inference

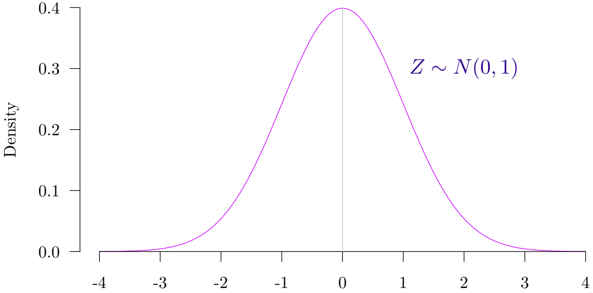
$$T_{obs} = \frac{\widehat{\mu}_B - \widehat{\mu}_L}{s_p\sqrt{\frac{1}{n_B}+\frac{1}{n_L}}} \sim St_{n_T+n_C-2}$$

Idea:

EGF is differentially expressed (DE) in luminal (L) and basal (B) cells

Statistical tests

Assess how likely the observed test statistics is compared to the test statistics distribution under H0:



P-value for a two-sided test:

p-value $= 2\ \text{min}\left[P(Z \leq Z_{obs}|\text{H0}), P(Z \geq Z_{obs}|\text{H0})\right]$

i.e. the probability of getting a test statistic as extreme or more extreme than the calculated test statistic if H0 is true

Statistical tests

4 possible outcomes

Conclude:

- if p-value $> \alpha \quad \rightarrow$ do not reject H0.
- if p-value $< \alpha \quad \rightarrow$ reject H0 in favour of H1.

		Test Outcome	
		H0 not rejected	H1 accepted
Unknown Truth	H0 true	$1 - \alpha$ [TN]	α [FP]
	H1 true	β [FN]	$1 - \beta$ [TP]

where

- α is the type I error, the probability of rejecting H0 when H0 is correct,
- β is the type II error, the probability of not rejecting H0 when H1 is correct.

Warnings

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

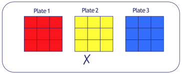
Experimental design 1: Minimising biases

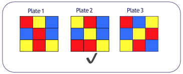
3 fundamental aspects of sounds experiments (Fisher 1935)

- 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:

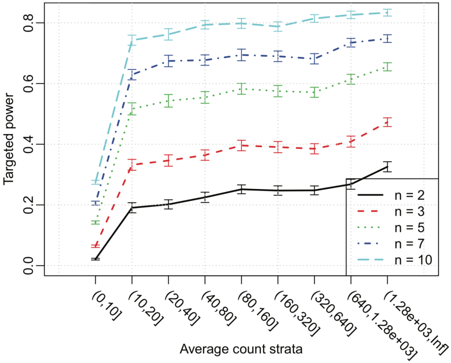
- $1-\beta$, the power,
- δ, the effect size: function of μ_L and μ_B (log fold change, standardised difference),
- n, the sample size (number of biological replicates),
- α, the type I error.
- ϕ, nuisance parameters (variability, sequencing depth, multiplicity correction)

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

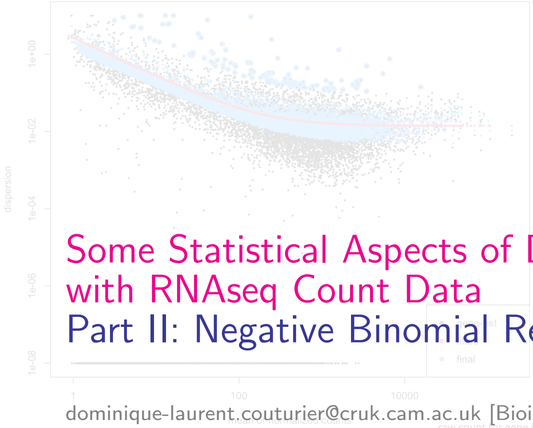
- Power calculation: Aim is to define the probability ($1-\beta$) to detect an effect size of interest (δ) at the α level with a sample size of n biological replicates.
- Sample size calculation: Aim is to define the sample size (n) allowing to detect an effect size of interest (δ) at the α level with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

Experimental design 2: boosting power

Power- calculations in DE analyses



(Wu, Wang and Wu (2015))



Some Statistical Aspects of DE Analysis with RNAseq Count Data

Part II: Negative Binomial Regression

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

(Source: O. Rueda, MRC-BSU)

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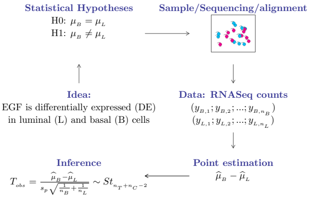
Statistical modelling



Statistical Hypotheses

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Point estimation

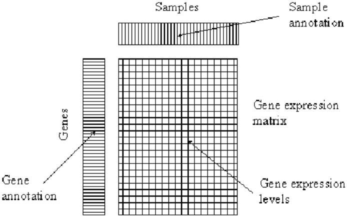
$\widehat{\mu}_B - \widehat{\mu}_L$

Inference

$T_{obs} = \frac{\widehat{\mu}_B - \widehat{\mu}_L}{s_p\sqrt{\frac{1}{n_B}+\frac{1}{n_L}}} \sim St_{n_T+n_C-2}$

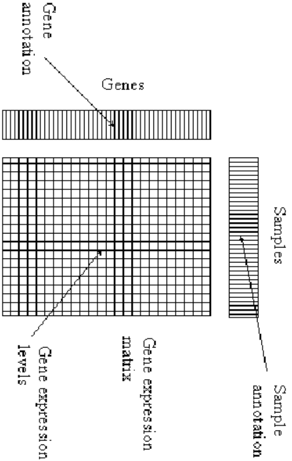
Idea:

EGF is differentially expressed (DE) in luminal (L) and basal (B) cells



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

Statistical modelling



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

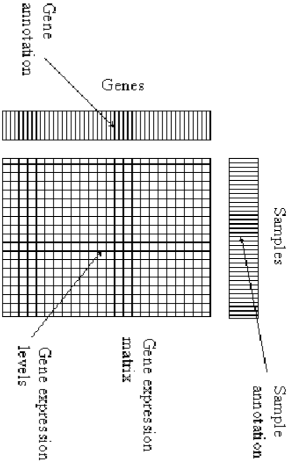
$$\mathsf{E}[\mathbf{y}] = f(\mathbf{X})$$

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,
- $\mathsf{E}[\mathbf{y}]$ 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



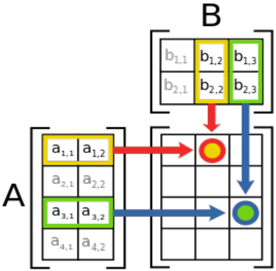
$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \epsilon$$

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

where

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

Statistical modelling : Linear regression



(Wikipedia)

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

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

where

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

Matrix multiplication:
the element $\mathbf{C}_{i,j}$ (ith row, jth column of the matrix **C**) is obtained by

- multiplying **term-by-term** the entries of the ith row of **A** and the jth column of **B**,
- and summing these products.

Statistical modelling : Strategy

- Collect the information related to each sample for the predictors of interest,
- define $\boldsymbol{\beta}$, the sets of parameters we are interested in,
- build the $\mathbf{X}$ matrix that relates
  the sample information with the $\boldsymbol{\beta}$
  this step is automatically done in R by specifying the regression formula in the function lm() or DEseq2()
- estimate the $\boldsymbol{\beta}$ 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:
  $\boldsymbol{\pi} = \frac{e^{\mathbf{X}\boldsymbol{\beta}}}{1+e^{\mathbf{X}\boldsymbol{\beta}}}$,
- 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
Sample1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control

$$\begin{array}{l} \text{Sample 1} \\ \text{Sample 2} \\ \text{Sample 3} \\ \text{Sample 4} \\ \text{Sample 5} \\ \text{Sample 6} \end{array} \underset{y}{\begin{pmatrix} \; \\ \; \\ \; \\ \; \\ \; \\ \; \end{pmatrix}} = \underset{X}{\begin{array}{cc} \text{Control} & \text{Treat. A} \\ \multicolumn{2}{c}{\begin{pmatrix} \; & \; \\ \; & \; \\ \; & \; \\ \; & \; \\ \; & \; \\ \; & \; \end{pmatrix}} \end{array}} \overset{\beta}{\begin{bmatrix} \beta_1 \\ \beta_2 \end{bmatrix}}$$

β_1 = μ_C is the mean expression of the control

β_2 = μ_A is the mean expression of the treatment A group

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

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

$$\begin{array}{r} \text{Sample 1} \\ \text{Sample 2} \\ \text{Sample 3} \\ \text{Sample 4} \\ \text{Sample 5} \\ \text{Sample 6} \end{array} \underset{y}{\begin{pmatrix} \\ \\ \\ \\ \\ \\ \end{pmatrix}} = \underset{X}{\begin{array}{cc} \text{intercept} & \text{Shift bw C and A} \\ \multicolumn{2}{c}{\begin{pmatrix} & \\ & \\ & \\ & \\ & \\ & \end{pmatrix}} \end{array}} \quad \begin{array}{c} \beta \\ \begin{bmatrix} \beta_1 \\ \beta_2 \end{bmatrix} \end{array}$$

β_1 = μ_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

$$\underset{y}{\begin{bmatrix} \\ \\ \\ \\ \\ \\ \end{bmatrix}} = \underset{X}{\begin{pmatrix} \\ \\ \\ \\ \\ \\ \end{pmatrix}} \overset{\beta}{\begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix}}$$

β_1 = μ_C is the mean expression of the control

β_2 = μ_A is the mean expression of the treatment A group

β_3 = μ_B is the mean expression of the treatment A 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

$$\underset{y}{\begin{bmatrix} \\ \\ \\ \\ \\ \\ \end{bmatrix}} = \underset{X}{\begin{pmatrix} \\ \\ \\ \\ \\ \\ \end{pmatrix}} \overset{\beta}{\begin{bmatrix}\beta_1\\ \beta_2\\ \beta_3\end{bmatrix}}$$

$\beta_1 = \mu_C$ 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
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	-

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

$$\underset{y}{\begin{bmatrix} \\ \\ \\ \\ \end{bmatrix}} = \underset{X}{\begin{pmatrix} \\ \\ \\ \\ \end{pmatrix}}$$

β_1 = μ_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

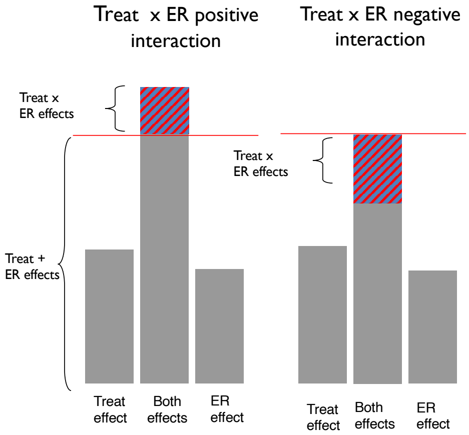
Sample	Treatment	ER status
Sample1	Treatment A	+
Sample 2	No Treatment	+
Sample 3	Treatment A	+
Sample 4	No Treatment	+
Sample 5	Treatment A	-
Sample 6	No Treatment	-
Sample 7	Treatment A	-
Sample 8	No Treatment	-

$$\underbrace{\begin{bmatrix} \\ \\ \\ \\ \\ \\ \\ \\ \end{bmatrix}}_{y} = \underbrace{\begin{pmatrix} \quad & \quad & \quad & \quad \\ \\ \\ \\ \\ \\ \\ \\ \end{pmatrix}}_{X} \begin{bmatrix} \beta_1 \\ \beta_2 \\ \beta_3 \\ \beta_4 \end{bmatrix}$$

β_1 = μ_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
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	-



Design matrices for models with two two-level factors: R Code

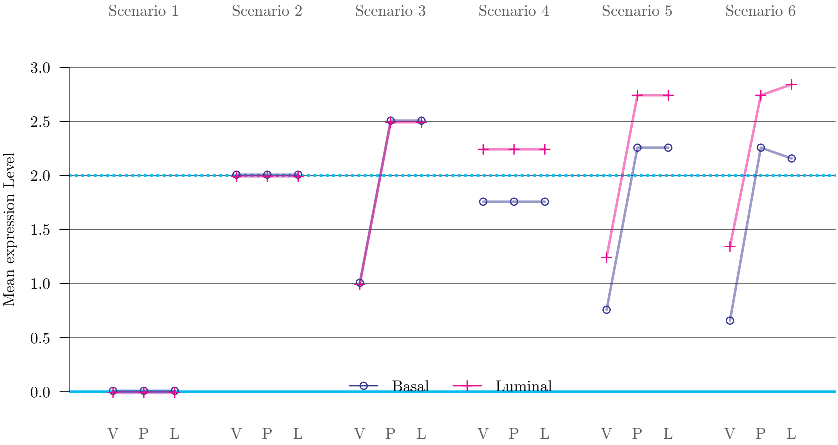
Open the R Markdown Document ‘StatsRNAseq_Couturier.Rmd’ and go to Section ‘Contrast matrices / Two 2-level factors’

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

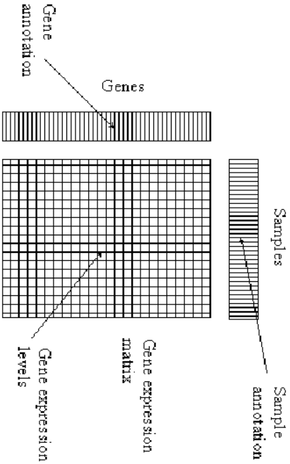
Models with 2 factors: possible scenarios

2 factors:

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



Negative binomial regression: Model



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

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

where

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

Negative binomial regression: Probability mass function

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

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

with expectation and variance given by

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

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
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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
```

- $E[\mathbf{y}|\text{‘cond 1}'] = 2^{\widehat{\beta}_1}$
- $E[\mathbf{y}|\text{‘cond 2}'] = 2^{\widehat{\beta}_1+\widehat{\beta}_2} = 2^{\widehat{\beta}_1}2^{\widehat{\beta}_2}$
  - If not DE, $\beta_2 = 0$ so that $E[\mathbf{y}|\text{‘cond 2}'] = 2^{\widehat{\beta}_1}2^0 = 2^{\widehat{\beta}_1}$,
  - If DE, $\beta_2 \neq 0$ so that $E[\mathbf{y}|\text{‘cond 2}'] = 2^{\widehat{\beta}_1}2^{\widehat{\beta}_2}$
    Interpretation: *Multiplicative change in observed gene expression level of* $2^{\widehat{\beta}_2} = 2^{-0.682067} = 0.6232717$ *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 Z-test to assess if a Log2 FC is significantly different from 0:

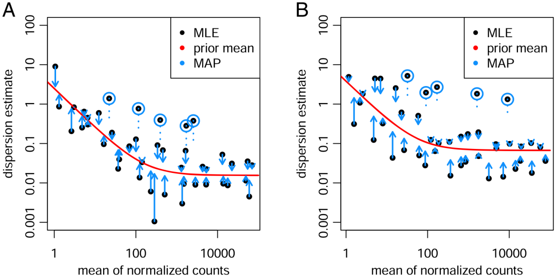
- **H0:** $\beta_2 = 0$ versus **H1:** $\beta_2 \neq 0$
- Z-statistic $= \frac{\widehat{\beta}_2}{\widehat{\sigma}_{\widehat{\beta}_2}} = \frac{-0.682067}{0.344525} = -1.979730$
- P-value with $Z \sim N(0,1)$ under **H0** is given by

```
> 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
  - $\widehat{\boldsymbol{\beta}}$, the estimate of the parameter vector,
  - $\widehat{\phi}$, 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].

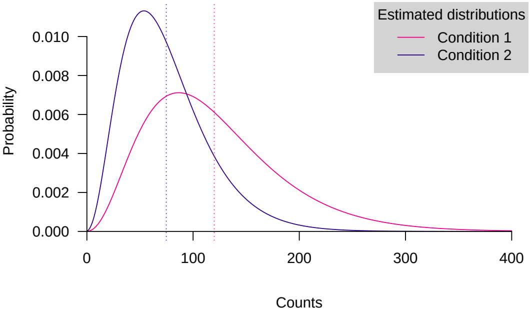


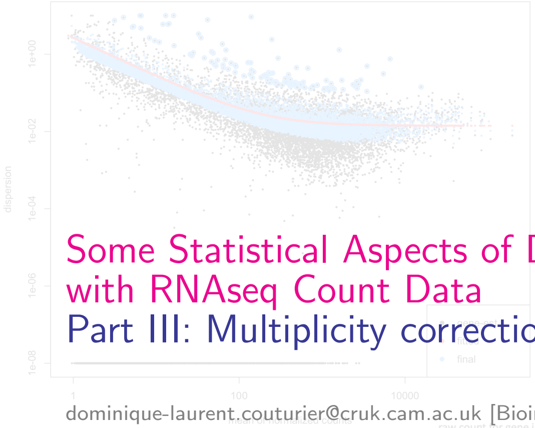
(Love et al (2015))

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





Some Statistical Aspects of DE Analysis with RNAseq Count Data

Part III: Multiplicity correction

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

(Source: G. Marot, INRIA)

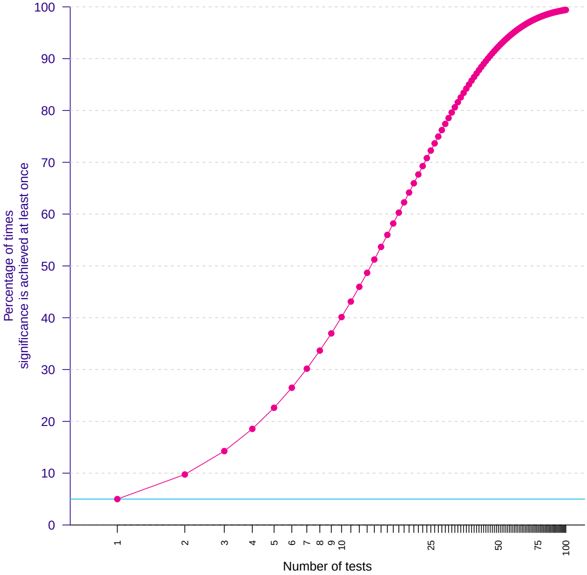
raw count for gene i, sample j

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

one dispersion per gene

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

Multiplicity correction: Familywise error rate



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.

The False Discovery Rate (FDR)

Idea : Do not control the error rate but the proportion of error
$\Rightarrow$ 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)
```

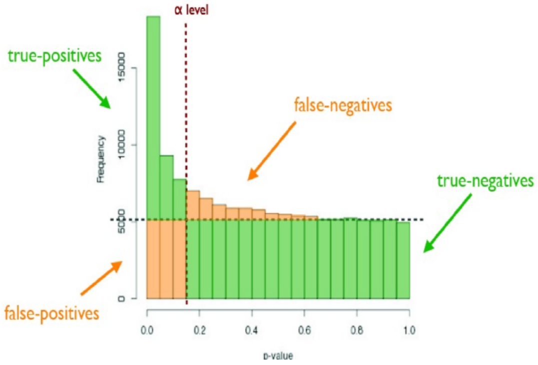
```
log2 fold change (MLE): cond 2 vs 1
Wald test p-value: cond 2 vs 1
DataFrame with 1000 rows and 6 columns
      baseMean log2FoldChange     lfcSE      stat    pvalue      padj
     <numeric>      <numeric> <numeric> <numeric> <numeric> <numeric>
1      97.3140      -0.682067  0.344525 -1.979730 0.0477339  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

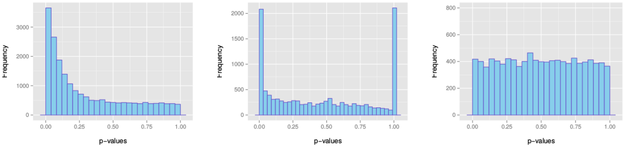
Standard assumption for p-value distribution



Source : M. Guedj, Pharnext

p-values histograms for diagnosis

Examples of expected overall distribution



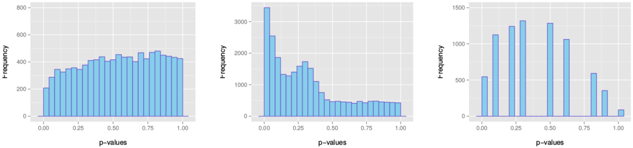
(a) : the most desirable shape

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

(c) : do not expect positive tests after correction

p-values histograms for diagnosis

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

```
> 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>
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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
```