INSTITUTO GULBENKIAN DE CIÊNCIA

The Gulbenkian Training Programme in Bioinformatics

NDARC16 - NGS Data Analysis, RNAseq, ChIPseq

Statistical Models for sequencing data: *from Experimental Design to Generalized Linear Models*

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Slides by ***Oscar M. Rueda*** *(CRUK-CI, U. Cambridge) et al*

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Outline

- Experimental Design
- Design and Contrast matrices
- Generalized linear models
- Models for counting data

To consult the statistician after an experiment is finished is often merely to ask him to conduct a post mortem examination. He can perhaps say what the experiment died of.

Sir Ronald Fisher (1890-1962)

[evolutionary biologist, geneticist and statistician]

An approximate answer to the right problem is worth a good deal more than an exact answer to an approximate problem.

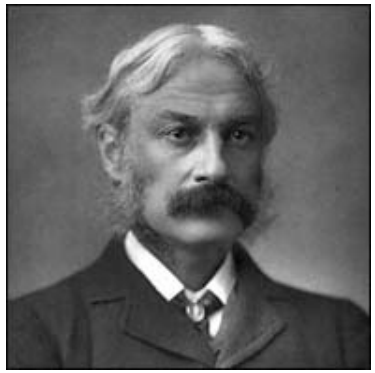
John Tukey (1915-2000)

[Statistician]

An unsophisticated forecaster uses statistics as a drunken man uses lamp-posts: for support rather than for illumination.

Andrew Lang (1844-1912)

[Poet, novelist and literary critic]



Experimental Design

Design of an experiment

- Select biological questions of interest
- Identify an appropriate measure to answer that question
- Select additional variables or factors that can have an influence in the result of the experiment
- Select a sample size and the sample units
- Assign samples to lanes/flow cells

Principles of Statistical Design of Experiments

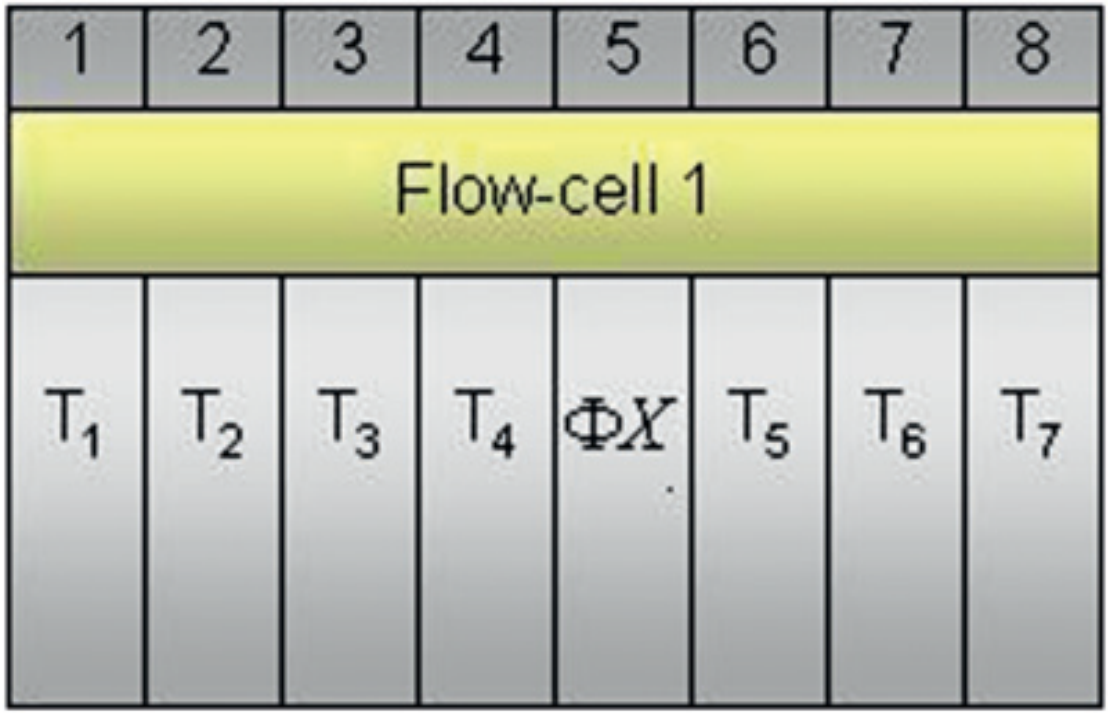
- R. A. Fisher:
  - Replication
  - Blocking
  - Randomization
- Used in microarray studies from the beginning
  (e.g. allocation of samples to slides)
- Bar coding makes easy to adapt them to NGS

Sampling hierarchy

There are three levels of sampling:

- Subject Sampling
- RNA sampling
- Fragment sampling.

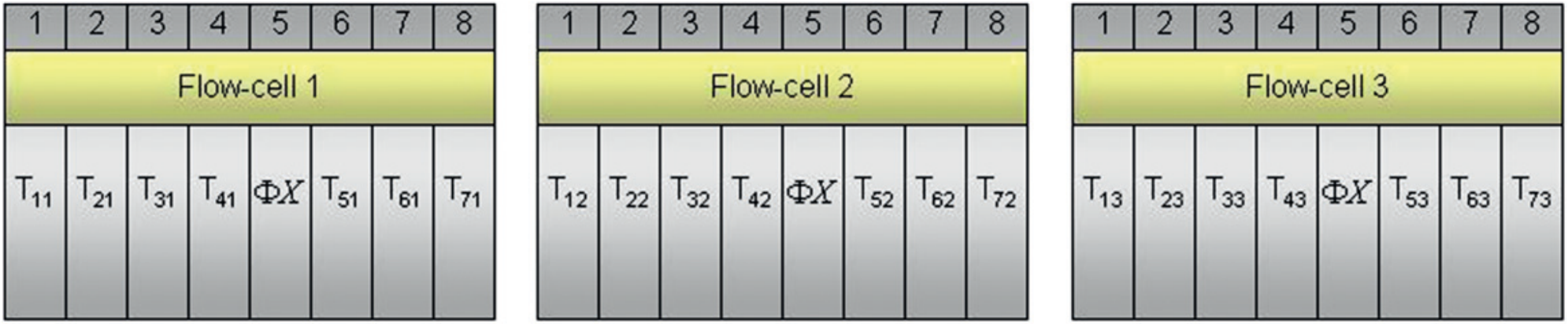
Unreplicated Data



Inferences for RNA and fragment-level can be obtained through Fisher's test. But they don't reflect biological variability.

Auer and Doerge. Genetics 185:405-416(2010)

Replicated Data



Inferences for treatment effect using generalized linear models (more on this later).

Is this a good design? We should randomize within block!

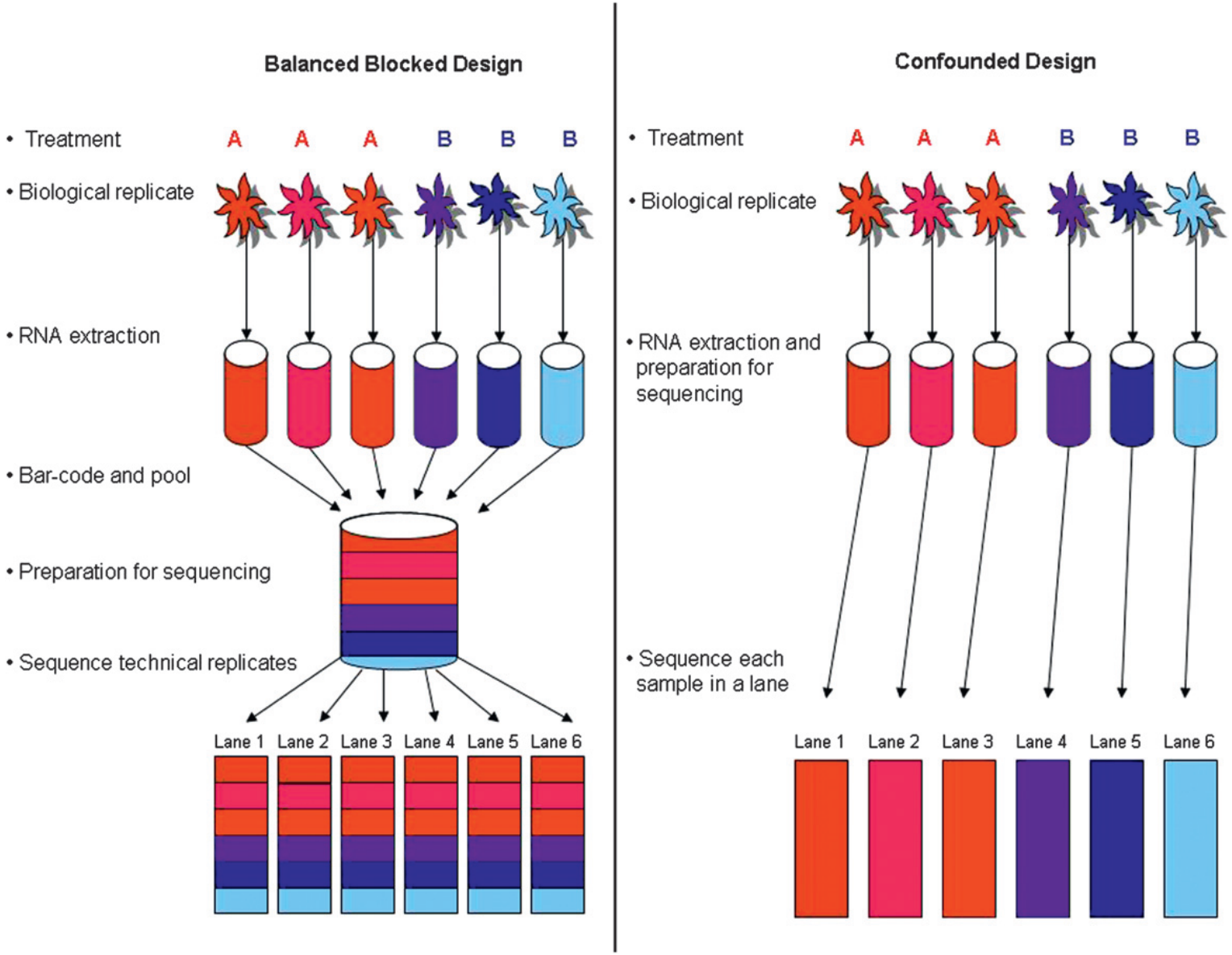
Balanced Block Designs

- Avoids confounding effects:
  - **Lane effects**: any errors from sample input into the flow cell until data output

    (e.g. systematically bad sequencing cycles, errors in base calling)
  - **Batch effects**: any errors from random RNA fragmentation until input into the flow cell

    (e.g. PCR amplification, reverse transcription artifacts)
  - Other effects non related to treatment.

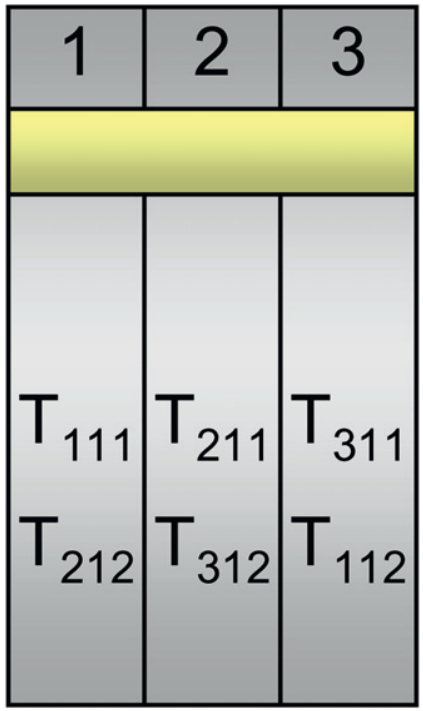
Balanced blocks by multiplexing



Auer and Doerge. Genetics 185:405-416(2010)

Balanced incomplete block design and blocking without multiplexing

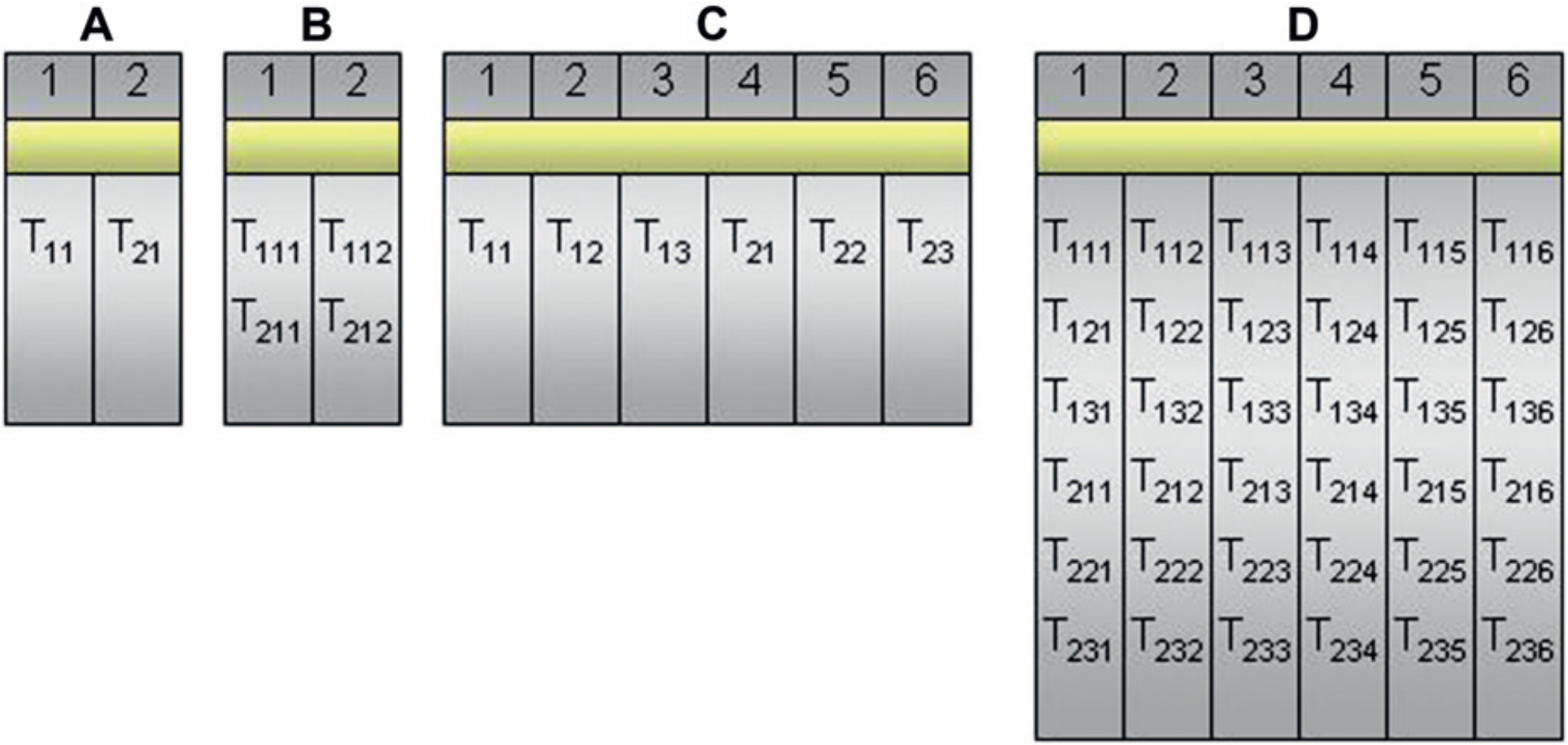
- Usually there are restrictions with the number of treatments, replicates, unique bar codes and available lanes for sequencing



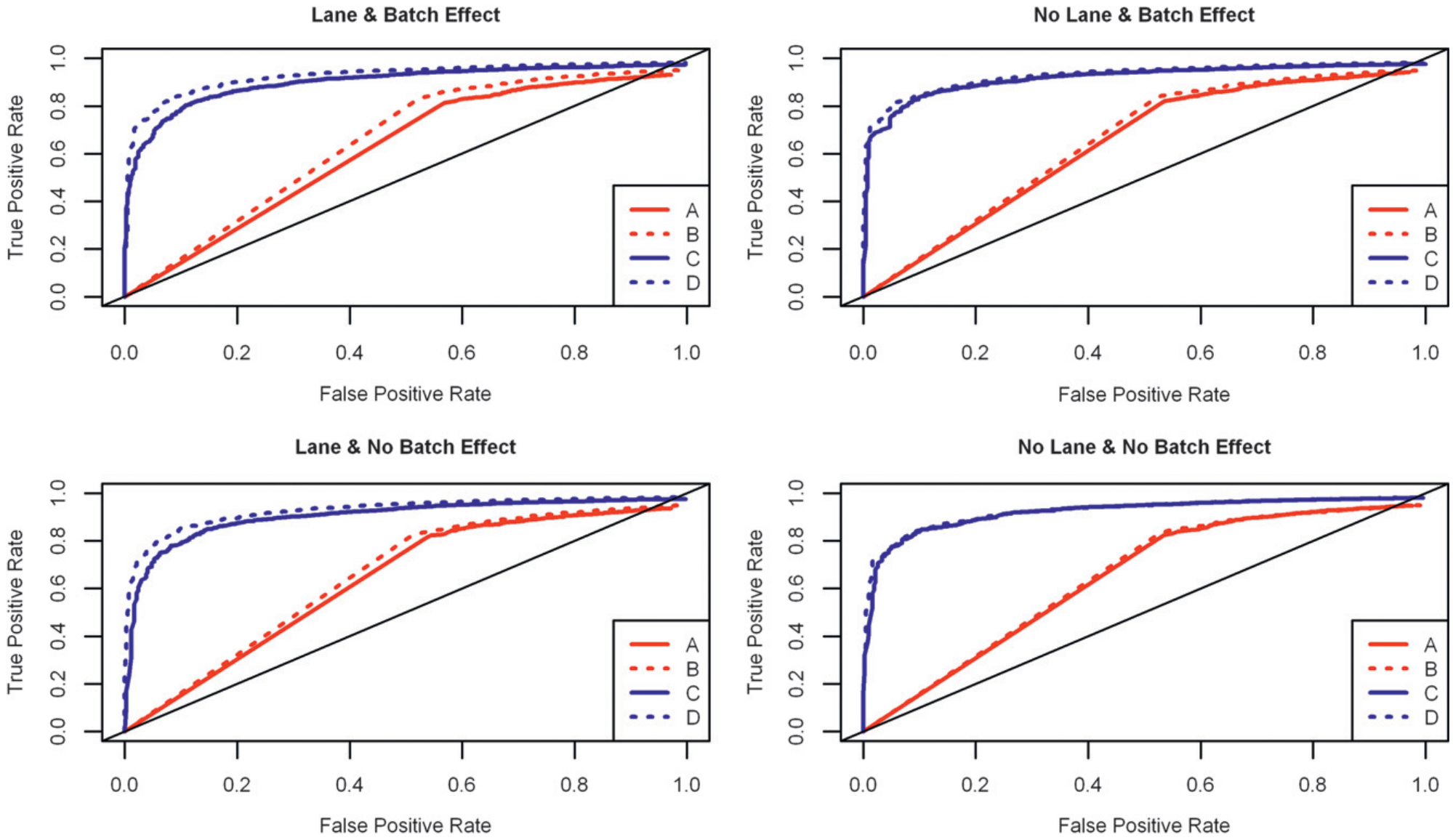
- 3 treatments (T_1, T_2, T_3)
- 1 subject per treatment (T_{11}, T_{21}, T_{31})
- 2 technical replicates (T_{111}, T_{112}, T_{211}, T_{212}, T_{311}, T_{312})

Auer and Doerge. Genetics 185:405-416(2010)

Simulations



Results



Auer and Doerge. Genetics 185:405-416(2010)

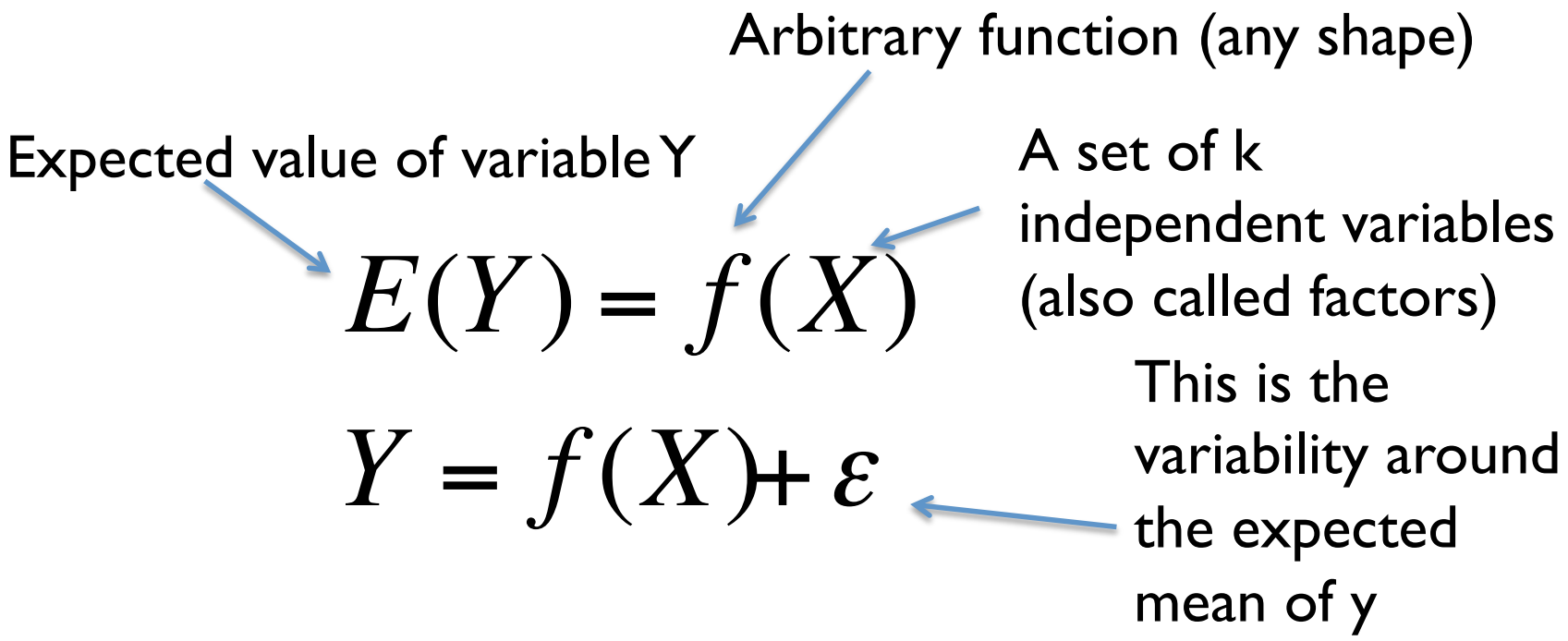
Benefits of a proper design

- NGS is benefited with design principles
- Technical replicates can not replace biological replicates
- It is possible to avoid multiplexing with enough biological replicates and sequencing lanes
- The advantages of multiplexing are bigger than the disadvantages (cost, loss of sequencing depth, bar-code bias…)

Design and contrast matrices

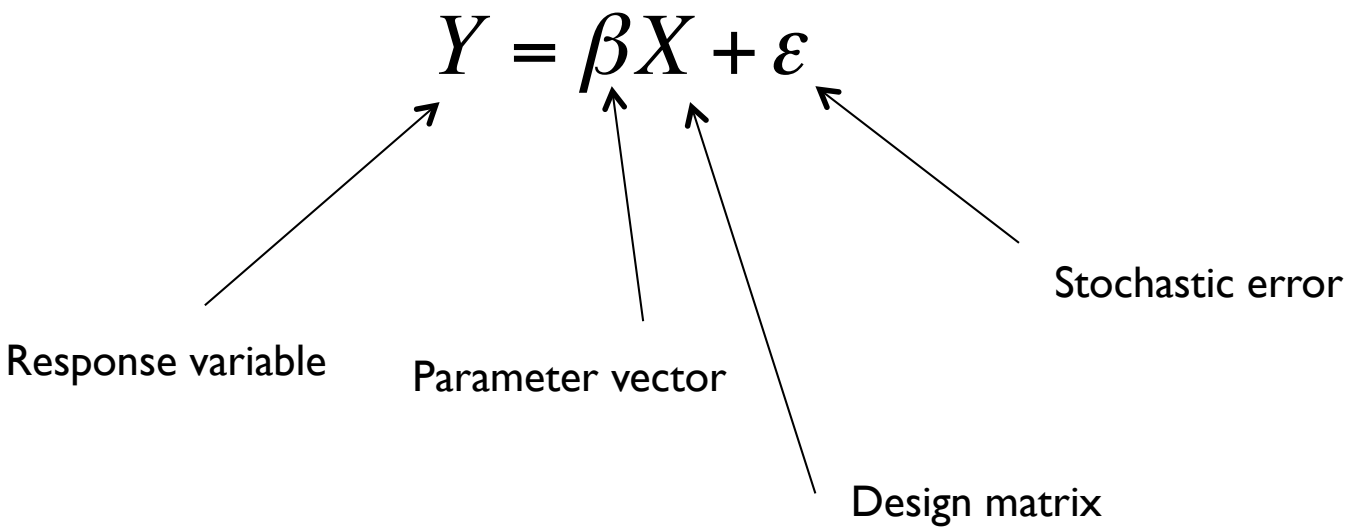
Statistical models

– We want to model the expected result of an outcome (dependent variable) under given values of other variables (independent variables)



Design matrix

- Represents the independent variables that have an influence in the response variable, but also the way we have coded the information and the design of the experiment.
- For now, let's restrict to models



Types of designs considered

- Models with 1 factor
  - Models with two treatments
  - Models with several treatments
- Models with 2 factors
  - Interactions
- Paired designs
- Models with categorical and continuous factors
- TimeCourse Experiments
- Multifactorial models

Strategy

- Define our set of samples
- Define the factors, type of factors (continuous, categorical), number of levels…
- Define the set of parameters: the effects we want to estimate
- Build the design matrix, that relates the information that each sample contains about the parameters
- Estimate the parameters of the model: testing
- Further estimation (and testing): contrast matrices

Models with 1 factor, 2 levels

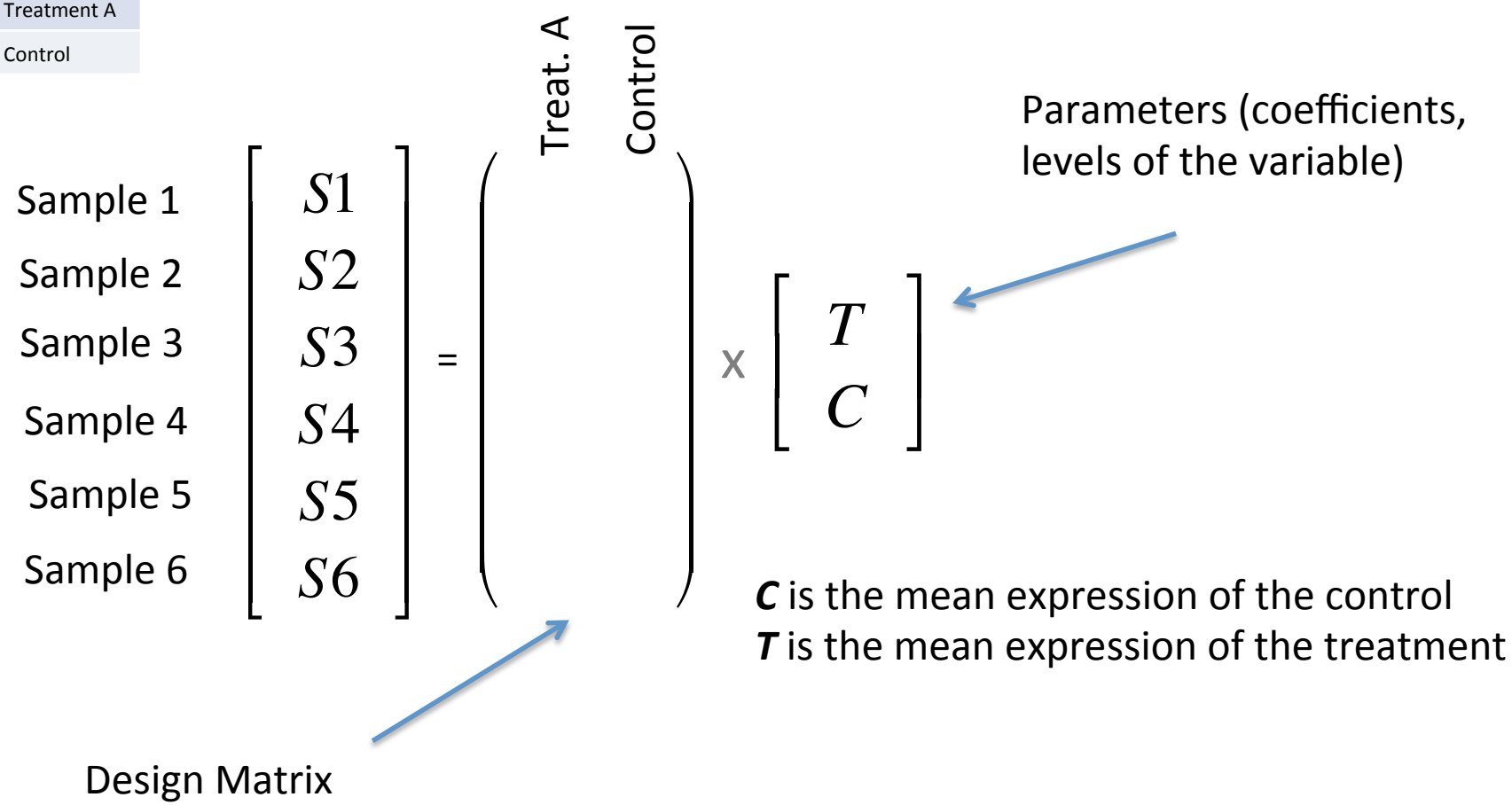
Sample	Treatment
Sample1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control

Number of samples: 6
Number of factors: 1
Treatment - number of levels: 2

Possible parameters (what differences are important)?
- Effect of Treatment A
- Effect of Control

Design matrix (models w/ 1 factor, 2 levels)

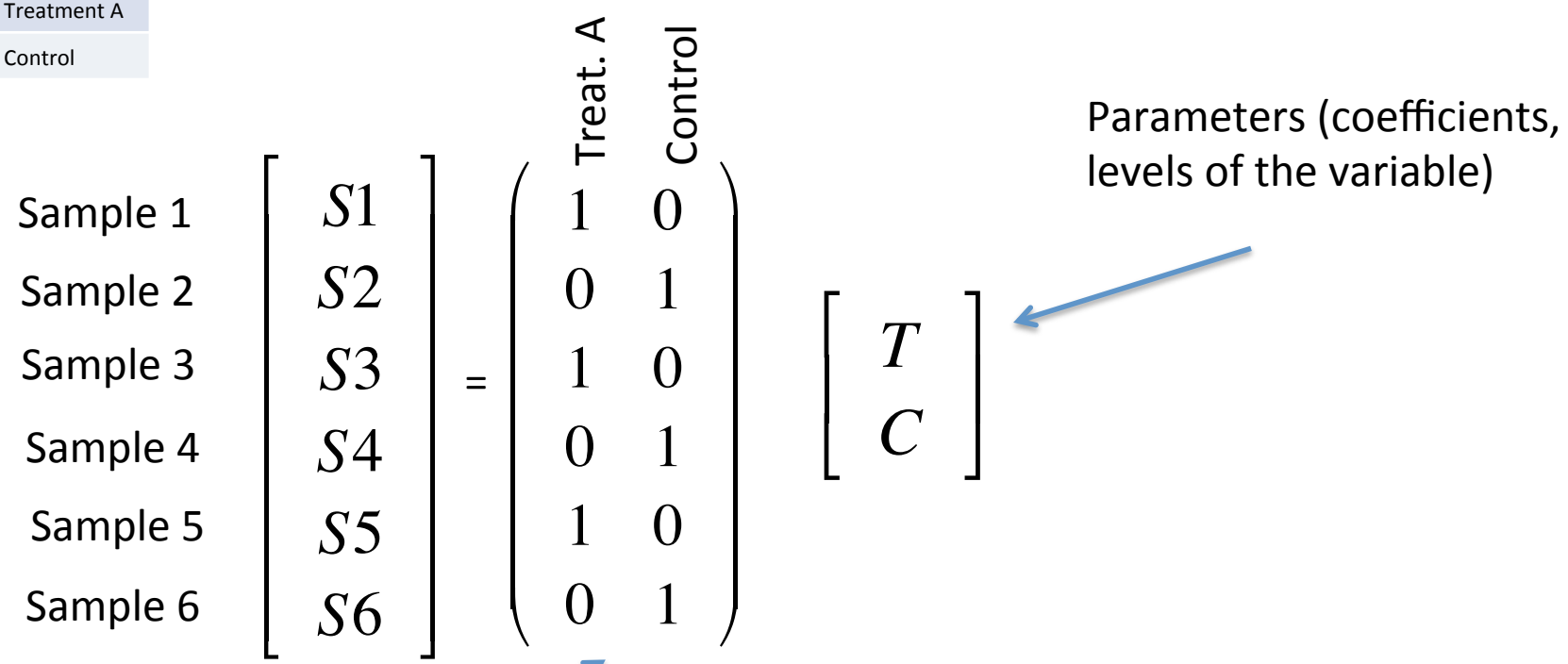
Sample	Treatment
Sample1	Treatment A
Sample 2	Control
Sample 3	Treatment A
Sample 4	Control
Sample 5	Treatment A
Sample 6	Control



Equivalent to a t-test

Design matrix (models w/ 1 factor, 2 levels)

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



Parameters (coefficients, levels of the variable)

Design Matrix

Equivalent to a t-test

Intercept

Different parameterization: using **intercept**

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

Let's now consider this parameterization:

C = *Baseline expression*

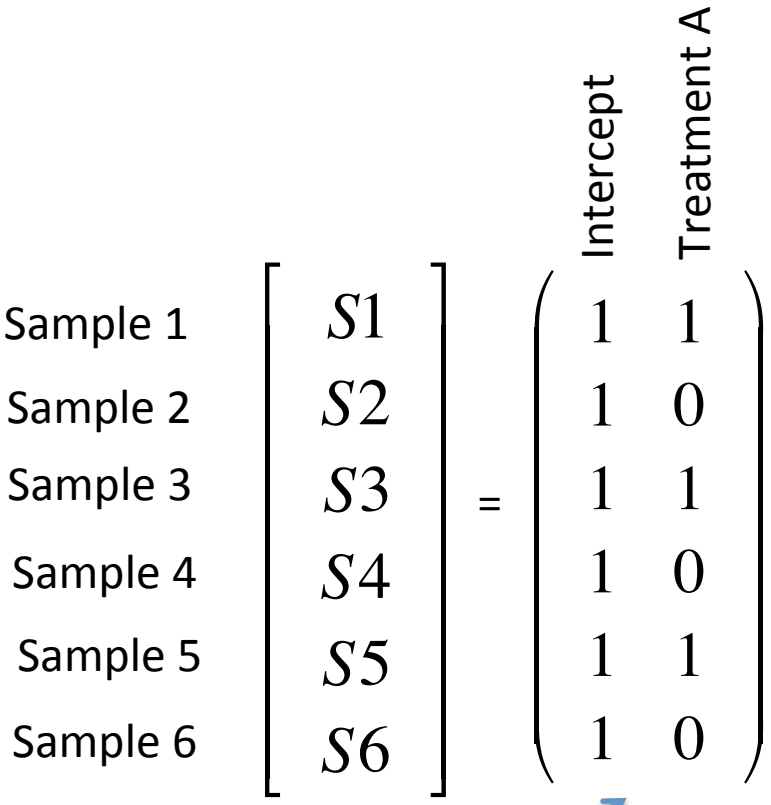
T_A = *Baseline expression* + *effect of treatment*

So the set of parameters are:

C = Control (*mean expression* of the control)

a = T_A – Control (*mean change in expression* under treatment)

Intercept



Parameters (coefficients, levels of the variable)

Design Matrix

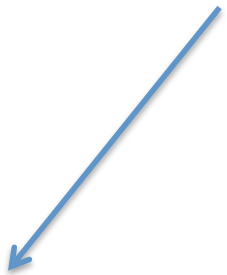
Intercept measures the baseline expression.

a measures now the differential expression between Treatment A and Control.

Contrast matrices

Are the two parameterizations equivalent?

$$\begin{bmatrix} 1 & -1 \end{bmatrix} \begin{bmatrix} \hat{T} \\ \hat{C} \end{bmatrix} = \widehat{T - C}$$



Contrast matrix

Contrast matrices allow us to estimate (and test) linear combinations of our coefficients.

Models with 1 factor, more than 2 levels

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

ANOVA models

Number of samples: 6
Number of factors: 1
Treatment - number of levels: 3

Possible parameters (what differences are important)?
- Effect of Treatment A
- Effect of Treatment B
- Effect of Control
- Differences between treatments?

Design matrix for ANOVA models

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

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} \end{pmatrix} \begin{bmatrix} T_A \\ T_B \\ C \end{bmatrix}$$

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ b \end{bmatrix}$$

Design matrix for ANOVA models

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

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \begin{bmatrix} T_A \\ T_B \\ C \end{bmatrix}$$

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ b \end{bmatrix}$$

Control = Baseline

T_A = Baseline + a

T_B = Baseline + b

Baseline levels

The model with intercept always take one level as a baseline:

The baseline is treatment A, the coefficients are comparisons against it!

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 0 & 1 \end{pmatrix} \begin{bmatrix} \beta_0 \\ b \\ c \end{bmatrix}$$

By default, R uses the first level as baseline

Exercise

Build contrast matrices for all pairwise comparisons for this design:

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \begin{bmatrix} T_A \\ T_B \\ C \end{bmatrix} \qquad \begin{pmatrix} \quad \end{pmatrix} \begin{bmatrix} \hat{T}_A \\ \hat{T}_B \\ \hat{C} \end{bmatrix}$$

Exercise

Build contrast matrices for all pairwise comparisons for this design:

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \\ 1 & 0 & 0 \\ 0 & 1 & 0 \\ 0 & 0 & 1 \end{pmatrix} \begin{bmatrix} T_A \\ T_B \\ C \end{bmatrix}$$

$$\begin{pmatrix} 1 & 0 & -1 \\ 0 & 1 & -1 \\ 1 & -1 & 0 \end{pmatrix} \begin{bmatrix} \hat{T}_A \\ \hat{T}_B \\ \hat{C} \end{bmatrix}$$

Exercise

Build contrast matrices for all pairwise comparisons for this design:

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 1 & 1 \\ 1 & 0 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ b \end{bmatrix} \qquad \begin{pmatrix} \quad \end{pmatrix} \begin{bmatrix} \hat{\beta}_0 \\ \hat{a} \\ \hat{b} \end{bmatrix}$$

Exercise

Build contrast matrices for all pairwise comparisons for this design:

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 1 & 1 \\ 1 & 0 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ b \end{bmatrix}$$

$$\begin{pmatrix} 0 & 1 & 0 \\ 0 & 0 & 1 \\ 0 & 1 & -1 \end{pmatrix} \begin{bmatrix} \hat{\beta}_0 \\ \hat{a} \\ \hat{b} \end{bmatrix}$$

Models with 2 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

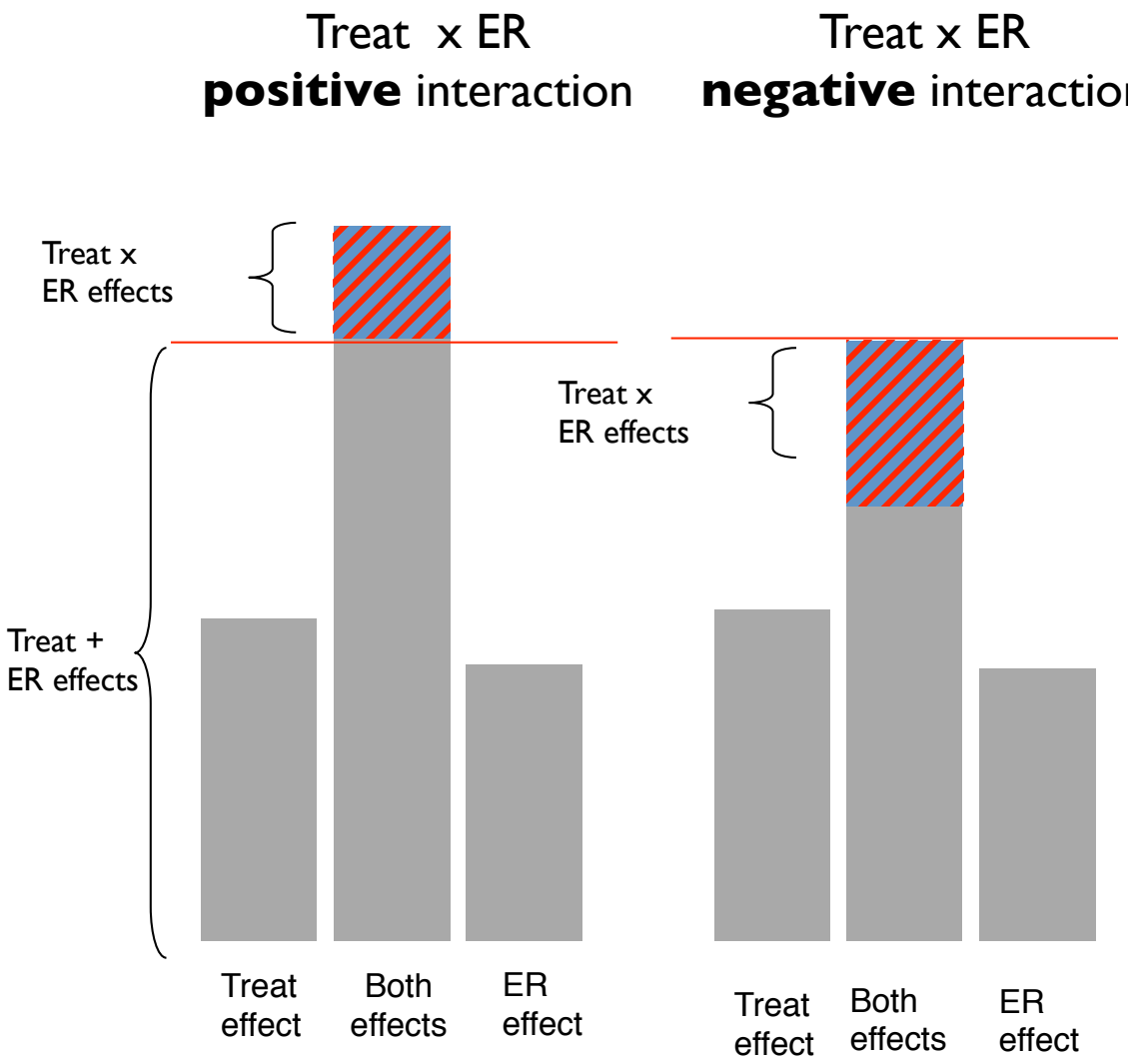
Number of factors: 2

Treatment - number of levels: 2

ER - number of levels: 2

Understanding Interactions

	No Treat	Treat A
ER -	S6, S8	S5, S7
ER +	S2, S4	S1, S3



Adapted from Natalie Thorne, Nuno L. Barbosa Morais

Models with 2 factors and no interaction

Model with no interaction: only ***main effects***

Number of coefficients (parameters):

Intercept + (♯levels Treat -1) + (♯levels ER -1) = 3

If we remove the intercept, the additional parameter comes from the missing level in one of the variables but, in models with more than 1 factor, it is a good idea to keep the intercept.

Models with 2 factors (no interaction)

R code:

```
>design.matrix <- model.matrix(~Treatment+ER)
```

(model with intercept)

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \\ S7 \\ S8 \end{bmatrix} = \begin{pmatrix} \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ er+ \end{bmatrix}$$

In R, the baseline for each variable is the first level.

	No Treat	Treat A
ER -	S6, S8	S5, S7
ER +	S2, S4	S1, S3

Models with 2 factors (no interaction)

R code:

```
>design.matrix <- model.matrix(~Treatment+ER)
```

(model with intercept)

$$\begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ S5 \\ S6 \\ S7 \\ S8 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 1 \\ 1 & 0 & 1 \\ 1 & 1 & 1 \\ 1 & 0 & 1 \\ 1 & 1 & 0 \\ 1 & 0 & 0 \\ 1 & 1 & 0 \\ 1 & 0 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ er+ \end{bmatrix}$$

	No Treat	Treat A
ER -	S6, S8	S5, S7
ER +	S2, S4	S1, S3

Models with 2 factors and interaction

Model with interaction: ***main effects + interaction***

Number of coefficients (parameters):

Intercept + (♯levels Treat -1) + (♯levels ER -1)
+ ((♯levels Treat -1) * (♯levels ER -1)) = 4

Models with 2 factors (interaction)

R code:

```
>design.matrix <- model.matrix(~Treatment*ER)
```

(model with intercept)

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ er+ \\ a.er+ \end{bmatrix} \end{pmatrix}$$

"Extra effect" of Treatment A on ER+ samples

	No Treat	Treat A
ER -	S6, S8	S5, S7
ER +	S2, S4	S1, S3

Models with 2 factors (interaction)

R code:

```
>design.matrix <- model.matrix(~Treatment*ER)
```

(model with intercept)

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 1 & 1 \\ 1 & 0 & 1 & 0 \\ 1 & 1 & 1 & 1 \\ 1 & 0 & 1 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 0 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ a \\ er+ \\ a.er+ \end{bmatrix}$$

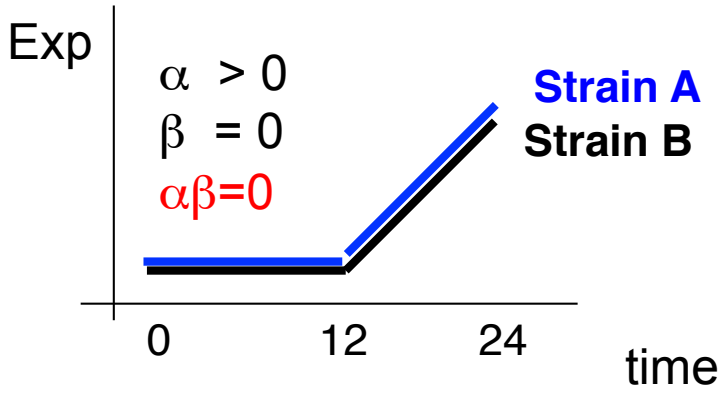
"Extra effect" of Treatment A on ER+ samples

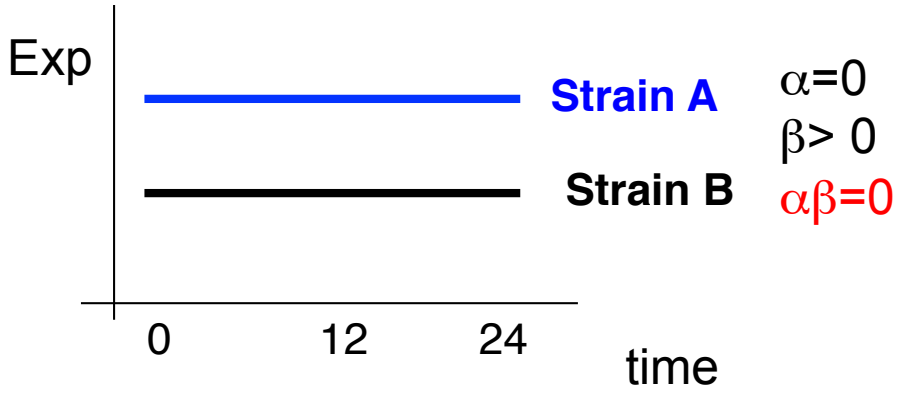
	No Treat	Treat A
ER -	S6, S8	S5, S7
ER +	S2, S4	S1, S3

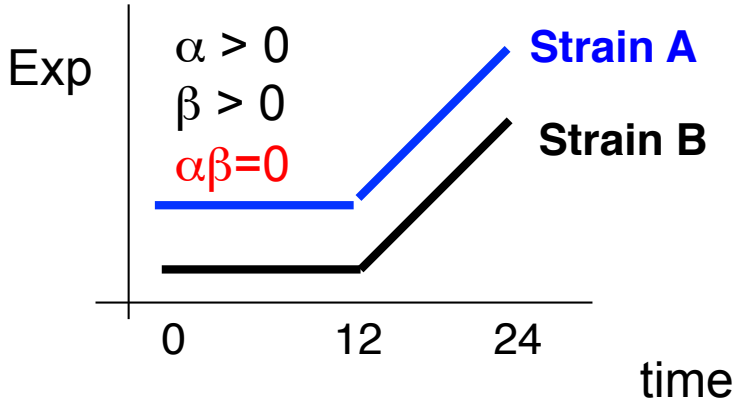
2 by 3 factorial experiment

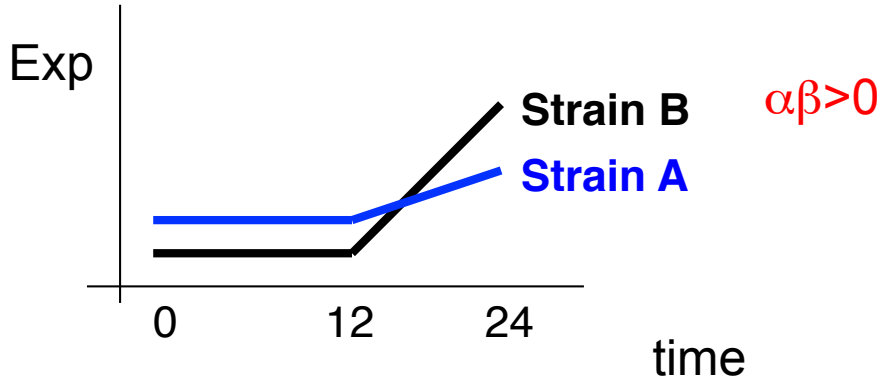
- Identify DE genes that have different time profiles between different mutants.

  α = time effect, β = strains, $\alpha\beta$ = interaction effect









Paired Designs

Sample	Type
Sample 1	Tumour
Sample 2	Matched Normal
Sample 3	Tumour
Sample 4	Matched Normal
Sample 5	Tumour
Sample 6	Matched Normal
Sample 7	Tumour
Sample 8	Matched Normal

Number of samples: 8
Number of factors: 1
Type - number of levels: 2

Sample	Type
Sample 1	Tumour
Sample 1	Matched Normal
Sample 2	Tumour
Sample 2	Matched Normal
Sample 3	Tumour
Sample 3	Matched Normal
Sample 4	Tumour
Sample 4	Matched Normal

Number of samples: 4
Number of factors: 2
Sample - number of levels: 4
Type - number of levels: 2

Design matrix for Paired experiments

We can gain precision in our estimates with a paired design because individual variability is removed when we compare the effect of the treatment within the same sample

R code:

```
>design.matrix <- model.matrix(~-1 +Type)
>design.matrix <- model.matrix(~-1 +Sample+Type)
```

(unpaired; model without intercept)

(paired; model without intercept)

Sample	Type
Sample 1	Tumour
Sample 1	Matched Normal
Sample 2	Tumour
Sample 2	Matched Normal
Sample 3	Tumour
Sample 3	Matched Normal
Sample 4	Tumour
Sample 4	Matched Normal

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 & 0 & 1 \\ 1 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 1 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 0 & 1 & 0 & 1 \\ 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 1 \\ 0 & 0 & 0 & 1 & 0 \end{pmatrix} \begin{bmatrix} S1 \\ S2 \\ S3 \\ S4 \\ t \end{bmatrix}$$

These effects only reflect biological differences not related to tumour/normal effect.

Analysis of covariance (models with categorical and continuous variables)

Sample	ER	Dose
Sample 1	+	37
Sample 2	-	52
Sample 3	+	65
Sample 4	-	89
Sample 5	+	24
Sample 6	-	19
Sample 7	+	54
Sample 8	-	67

Number of samples: 8

Number of factors: 2

ER - number of levels: 2

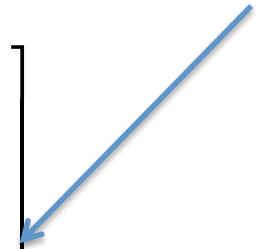
Dose: **Continuous**

Analysis of covariance (models with categorical and continuous variables)

R code:

```
>design.matrix <- model.matrix(~ ER + dose)
```

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 37 \\ 1 & 0 & 52 \\ 1 & 1 & 65 \\ 1 & 0 & 89 \\ 1 & 1 & 24 \\ 1 & 0 & 19 \\ 1 & 1 & 54 \\ 1 & 0 & 67 \end{pmatrix} \begin{bmatrix} \beta_0 \\ er + \\ d \end{bmatrix}$$



If we consider the effect of dose ***linear*** we use 1 coefficient (degree of freedom). We can also model it as non-linear (using splines, for example).

Sample	ER	Dose
Sample 1	+	37
Sample 2	-	52
Sample 3	+	65
Sample 4	-	89
Sample 5	+	24
Sample 6	-	19
Sample 7	+	54
Sample 8	-	67

Analysis of covariance (models with categorical and continuous variables)

Interaction: ***Is it the effect of dose equal in ER + and ER -?***

R code:

```
>design.matrix <- model.matrix(~ ER*dose)
```

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 1 & 37 & 37 \\ 1 & 0 & 52 & 0 \\ 1 & 1 & 65 & 65 \\ 1 & 0 & 89 & 0 \\ 1 & 1 & 24 & 24 \\ 1 & 0 & 19 & 0 \\ 1 & 1 & 54 & 54 \\ 1 & 0 & 67 & 0 \end{pmatrix} \begin{bmatrix} \beta_0 \\ er+ \\ d \\ er+.d \end{bmatrix}$$

If the interaction is significant, the effect on the dose is different depending on the levels of ER.

Sample	ER	Dose
Sample 1	+	37
Sample 2	-	52
Sample 3	+	65
Sample 4	-	89
Sample 5	+	24
Sample 6	-	19
Sample 7	+	54
Sample 8	-	67

Time Course experiments

Sample	Time
Sample 1	0h
Sample 1	1h
Sample 1	4h
Sample 1	16h
Sample 2	0h
Sample 2	1h
Sample 2	4h
Sample 2	16h

Main question: how does expression change over time?

If we model time as categorical, we don't make assumptions about its effect, but we use too many degrees of freedom

If we model time as continuous, we use less degrees of freedom but we have to make assumptions about the type of effect

Intermediate solution: ***splines***

Number of samples: 2

Number of factors: 2

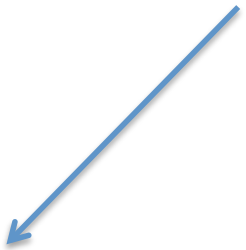
- Sample - number of levels: 2
- Time: Continuous or categorical?

Time Course experiments: no assumptions

R code:

```
>design.matrix <- model.matrix(~Sample + factor(Time))
```

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 & 0 & 0 \\ 1 & 0 & 1 & 0 & 0 \\ 1 & 0 & 0 & 1 & 0 \\ 1 & 0 & 0 & 0 & 1 \\ 0 & 1 & 0 & 0 & 0 \\ 0 & 1 & 1 & 0 & 0 \\ 0 & 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 0 & 1 \end{pmatrix} \begin{bmatrix} S_1 \\ S_2 \\ T_1 \\ T_4 \\ T_{16} \end{bmatrix}$$



We can use contrasts to test differences at time points.

Sample	Time
Sample 1	0h
Sample 1	1h
Sample 1	4h
Sample 1	16h
Sample 2	0h
Sample 2	1h
Sample 2	4h
Sample 2	16h

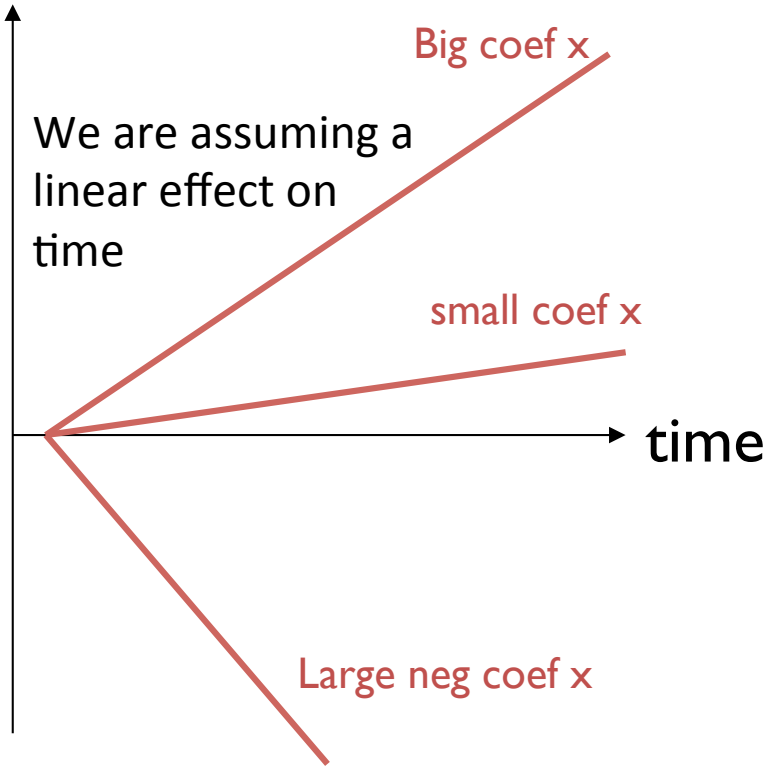
Time Course experiments

R code:

```
>design.matrix <- model.matrix(~Sample + Time)
```

Sample	Time
Sample 1	0h
Sample 1	1h
Sample 1	4h
Sample 1	16h
Sample 2	0h
Sample 2	1h
Sample 2	4h
Sample 2	16h

$$\begin{bmatrix} Y_1 \\ Y_2 \\ Y_3 \\ Y_4 \\ Y_5 \\ Y_6 \\ Y_7 \\ Y_8 \end{bmatrix} = \begin{pmatrix} 1 & 0 & 0 \\ 1 & 0 & 1 \\ 1 & 0 & 4 \\ 1 & 0 & 16 \\ 0 & 1 & 0 \\ 0 & 1 & 1 \\ 0 & 1 & 4 \\ 0 & 1 & 16 \end{pmatrix} \begin{bmatrix} S_1 \\ S_2 \\ X \end{bmatrix}$$



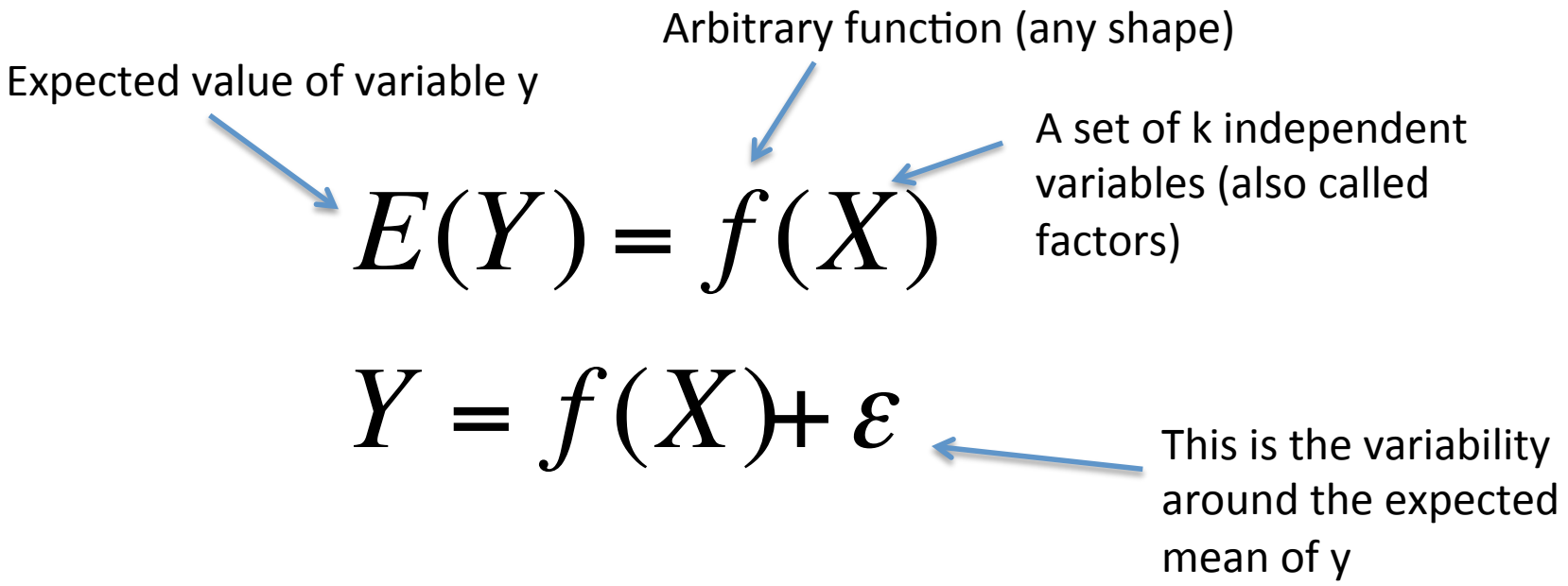
Intermediate models are possible: **splines**

Multi factorial models

- We can fit models with many variables
- Sample size must be adequate to the number of factors
- Same rules for building the design matrix must be used:
  - There will be one column in design matrix for the intercept
  - Continuous variables with a linear effect will need one column in the design matrix
  - Categorical variable will need *#levels -1* columns
  - Interactions will need *(#levels -1) x (#levels -1)*
  - It is possible to include interactions of more than 2 variables, but the number of samples needed to accurately estimate those interactions is large

Statistical models

We want to model the expected result of an outcome (dependent variable) under given values of other variables (independent variables)



Linear models

– The observed value of Y is a linear combination of the effects of the independent variables

Arbitrary number of independent variables

$$E(Y) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_k X_k$$

Polynomials are valid

$$E(Y) = \beta_0 + \beta_1 X_1 + \beta_2 X_1^2 + \ldots + \beta_p X_1^p$$

$$E(Y) = \beta_0 + \beta_1 \log(X_1) + \beta_2 f(X_2) + \ldots + \beta_k X_k$$

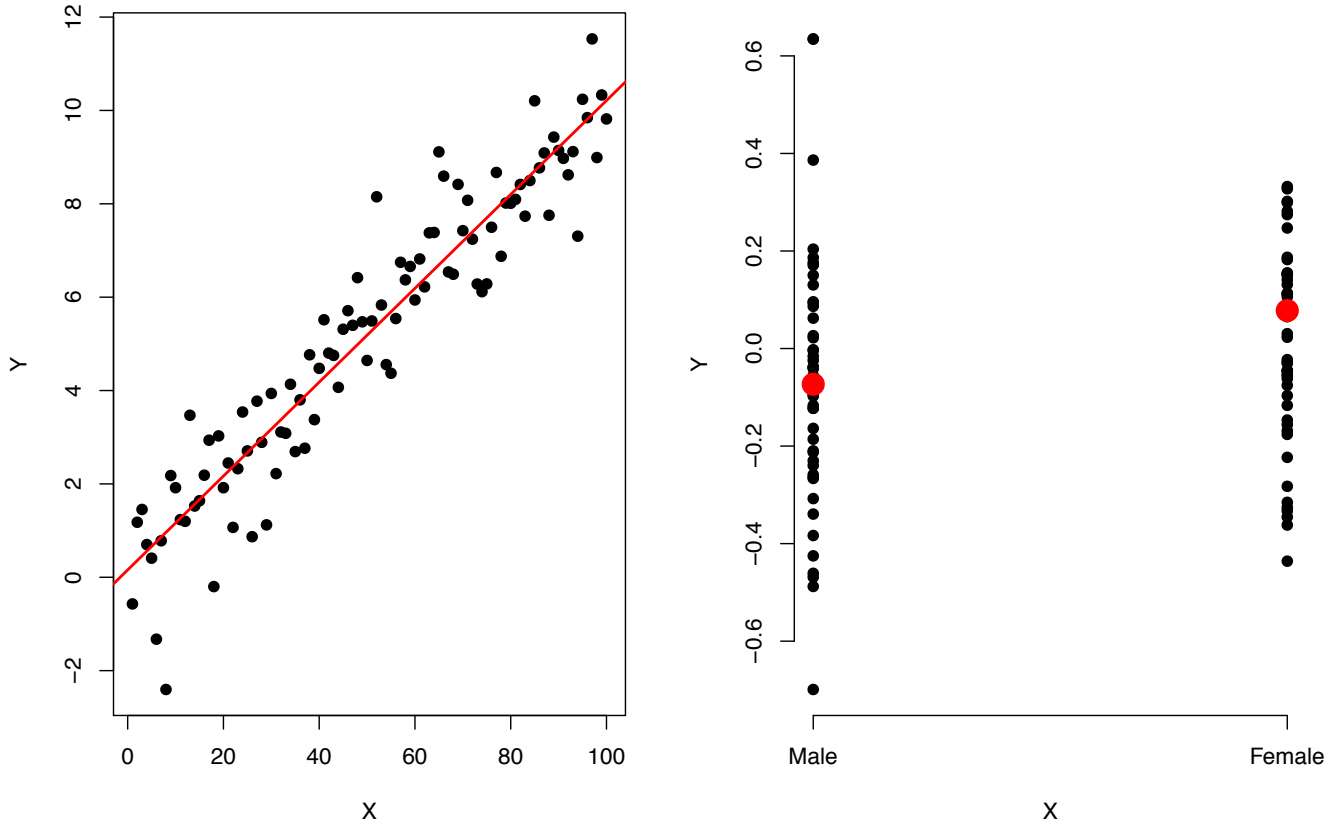
We can use functions of the variables if the effects are linear

Smooth functions: not exactly the same as the so-called ***additive models***

– If we include categorical variables the model is called **General Linear Model**

Model Estimation

We use **least squares estimation**



Given n observations (y_1,..y_n,$\boldsymbol{x}_1$,..$\boldsymbol{x}_n$) minimize the differences between the observed and the predicted values

Model Estimation

$$Y = \beta X + \varepsilon$$

β → Parameter of interest (effect of X on Y)

$\hat{\beta}$ → **Estimator** of the parameter of interest

$se(\hat{\beta})$ → **Standard Error** of the estimator of the parameter of interest

$$\hat{\beta} = (X^T X)^{-1} X^T Y$$

$$se(\hat{\beta}_i) = \sigma \sqrt{c_i}$$

where c_i is the i^{th} diagonal element of $\left(X^T X\right)^{-1}$

$\hat{y} = \hat{\beta} x$ → Fitted values (predicted by the model)

$e = y - \hat{y}$ → Residuals (observed errors)

Model Assumptions

In order to conduct statistical inferences on the parameters on the model, some assumptions must be made:

- The observations 1,..,n are independent
- Normality of the errors: $\varepsilon_i \sim N(0,\sigma^2)$
- Homoscedasticity: the variance is constant
- Linearity

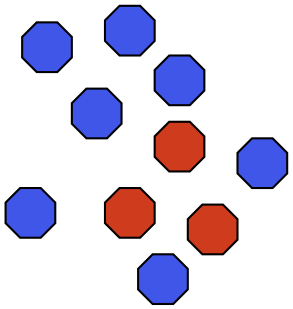
Hypothesis testing

- Everything starts with a biological question to test:
  - **What genes are differentially expressed under one treatment?**
  - **What genes are more commonly amplified in a class of tumours?**
  - **What promoters are methylated more frequently in cancer?**
- We must express this biological question in terms of a parameter in a model
- We then conduct an experiment, obtain data and estimate the parameter
- How do we take into account uncertainty in order to answer our question based on our estimate?

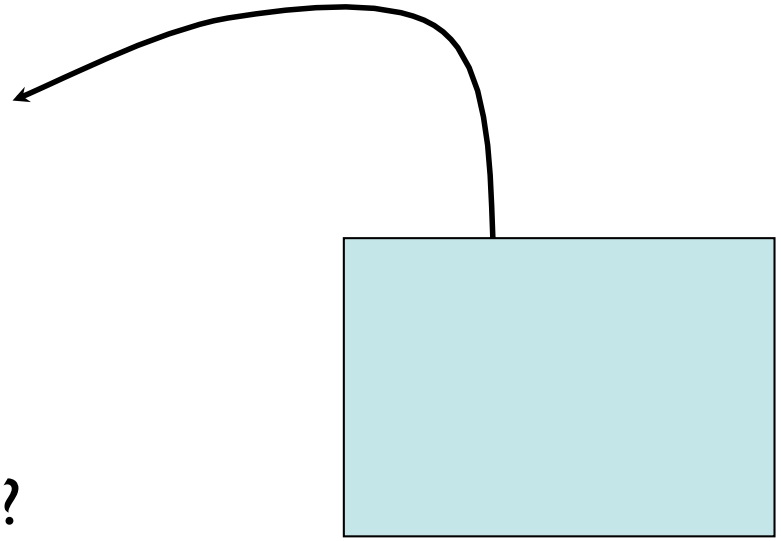
Sampling and testing

Discrete observations

#red = 3



Random sample of 10 balls from the box



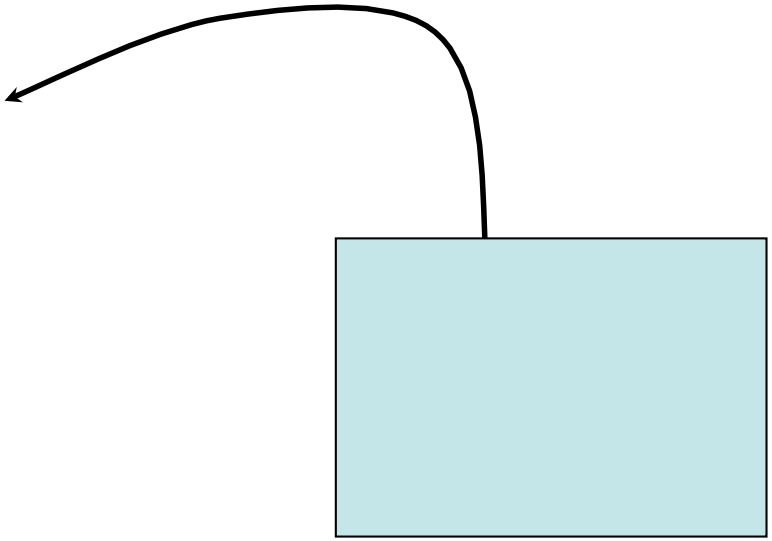
When do I think that I am not sampling from this box anymore?

How many reds could I expect to get <u>just by chance alone!</u>

10% red balls and
90% blue balls

Sample

Random sample of 10 balls from the box



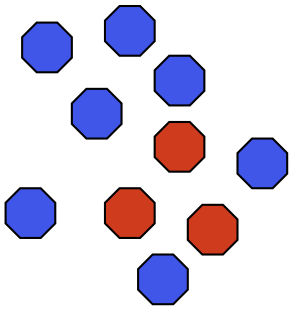
10% red balls and
90% blue balls

Null hypothesis

(about the population that is being sampled)

Discrete observations

#red = 3



Test statistic

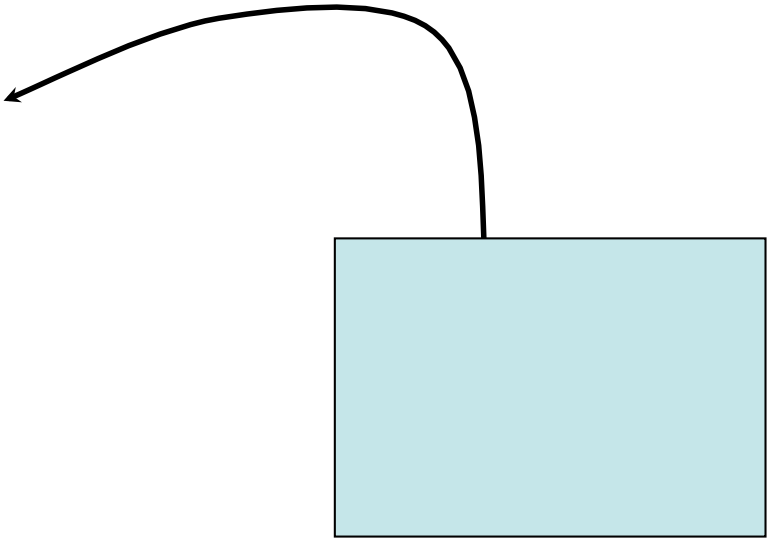
Rejection criteria (based on your observed sample, do you have evidence to reject the hypothesis that you sampled from the null population)

Sample

Continuous observations

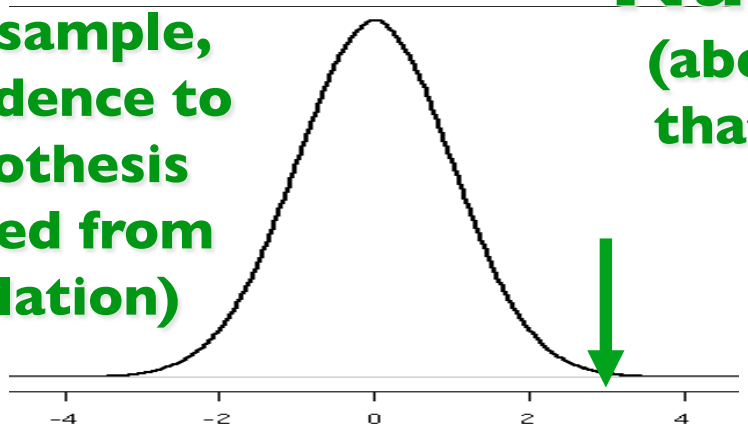
4, 2.3, 5.2, 4.7, 2.1, 3.5, ……..

mean = 3, sd = 0.6



Test statistic

Rejection criteria (based on your observed sample, do you have evidence to reject the hypothesis that you sampled from the null population)



Null hypothesis (about the population that is being sampled)

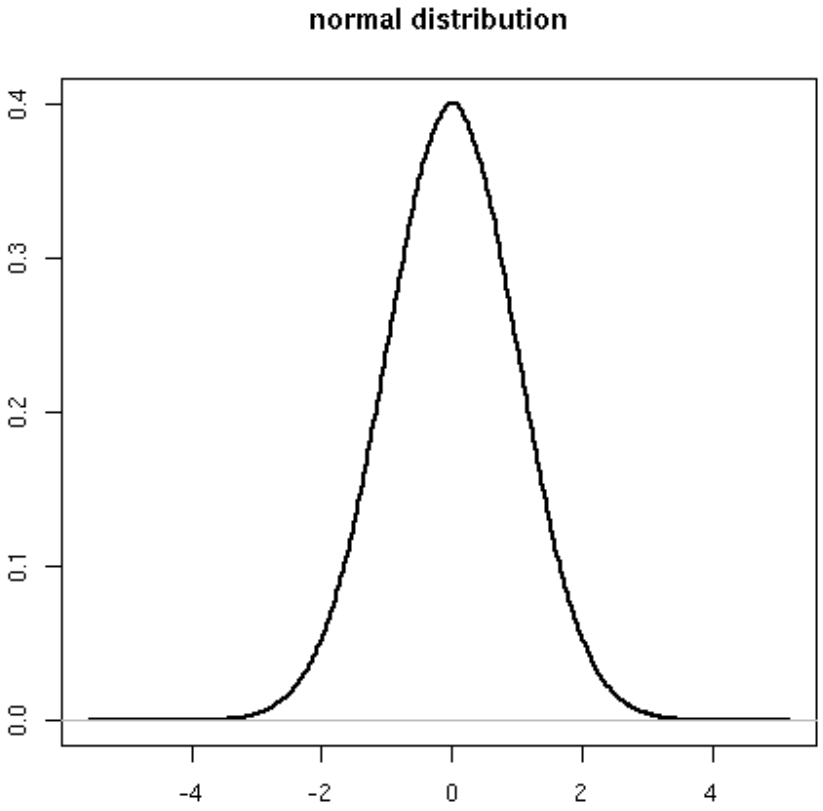
Lets formalise the test!

Hypothesis testing

- Distribution from null population is normal, defined by a mean and variance
  - $N(\mu_0,\sigma_0)$
- Take a sample size n, observe
  - $N(\mu_1,\sigma_1)$
- Null hypothesis : random sample comes from population with $N(\mu_0,\sigma_0)$

- If $x=c(x_1,\ldots x_n) \in N(\mu_0,\sigma_0)$

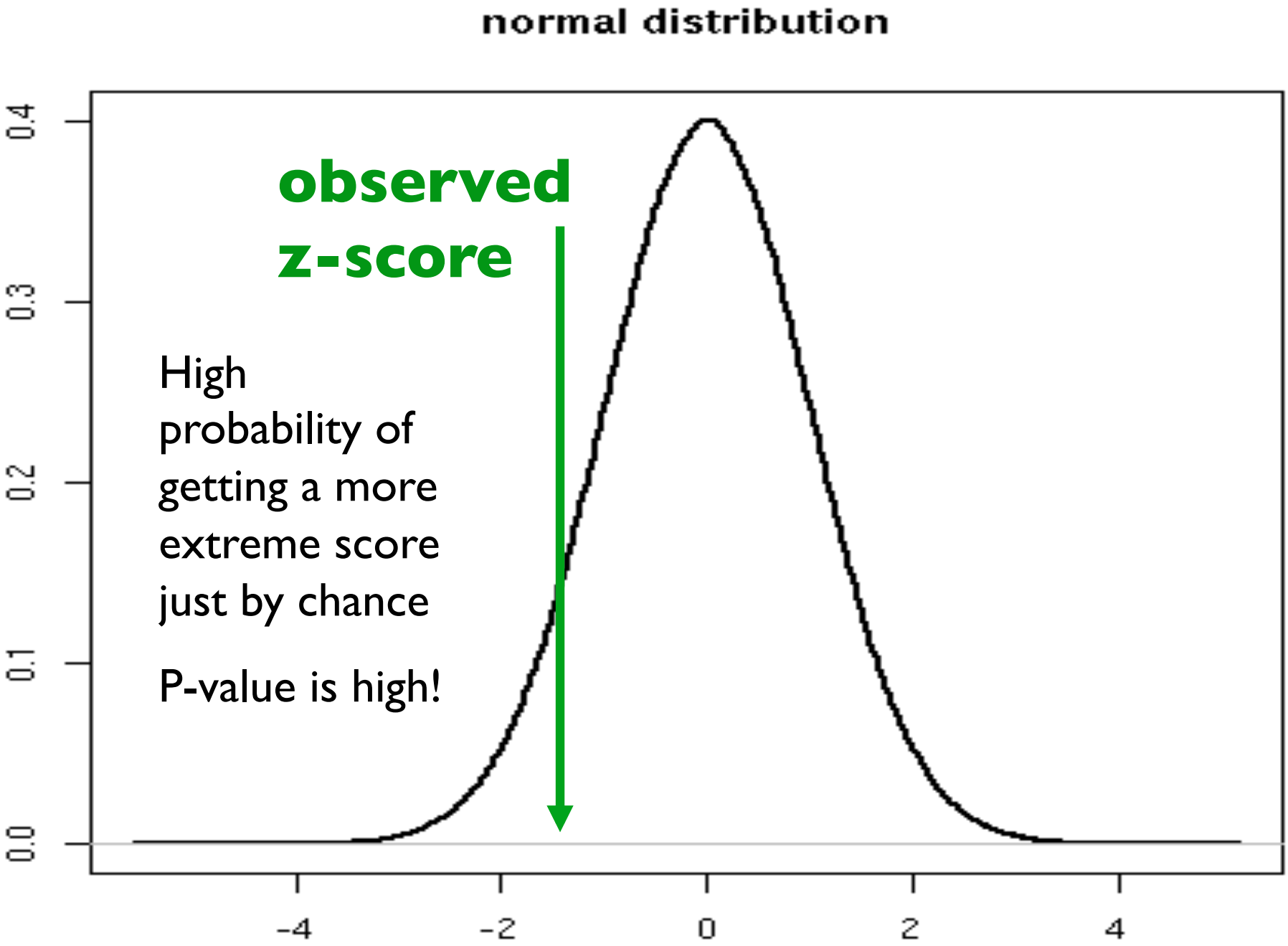
$$\frac{\text{mean}(x) - \mu_0}{\sigma_0/\sqrt{n}} \sim N(0, 1)$$

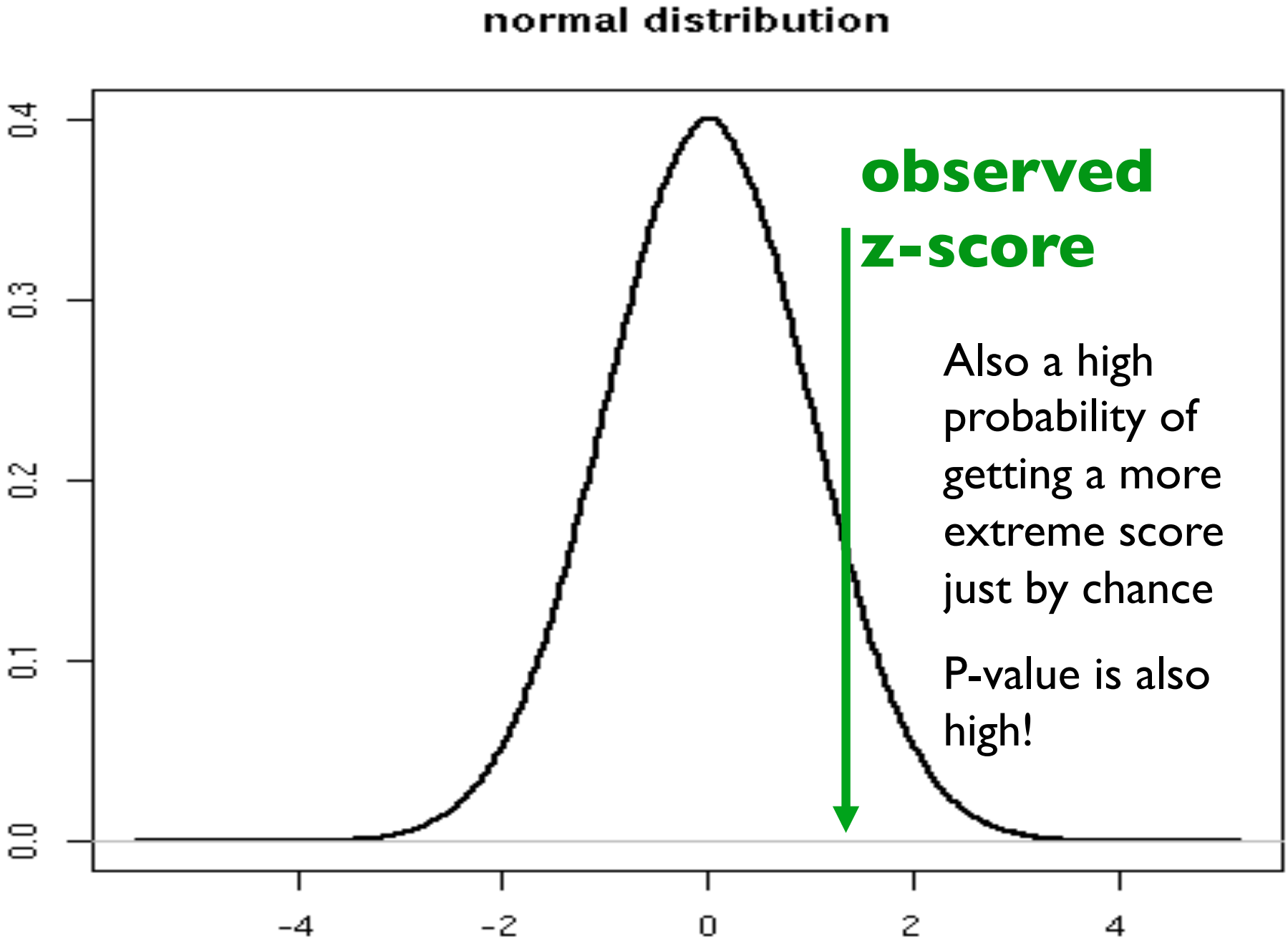


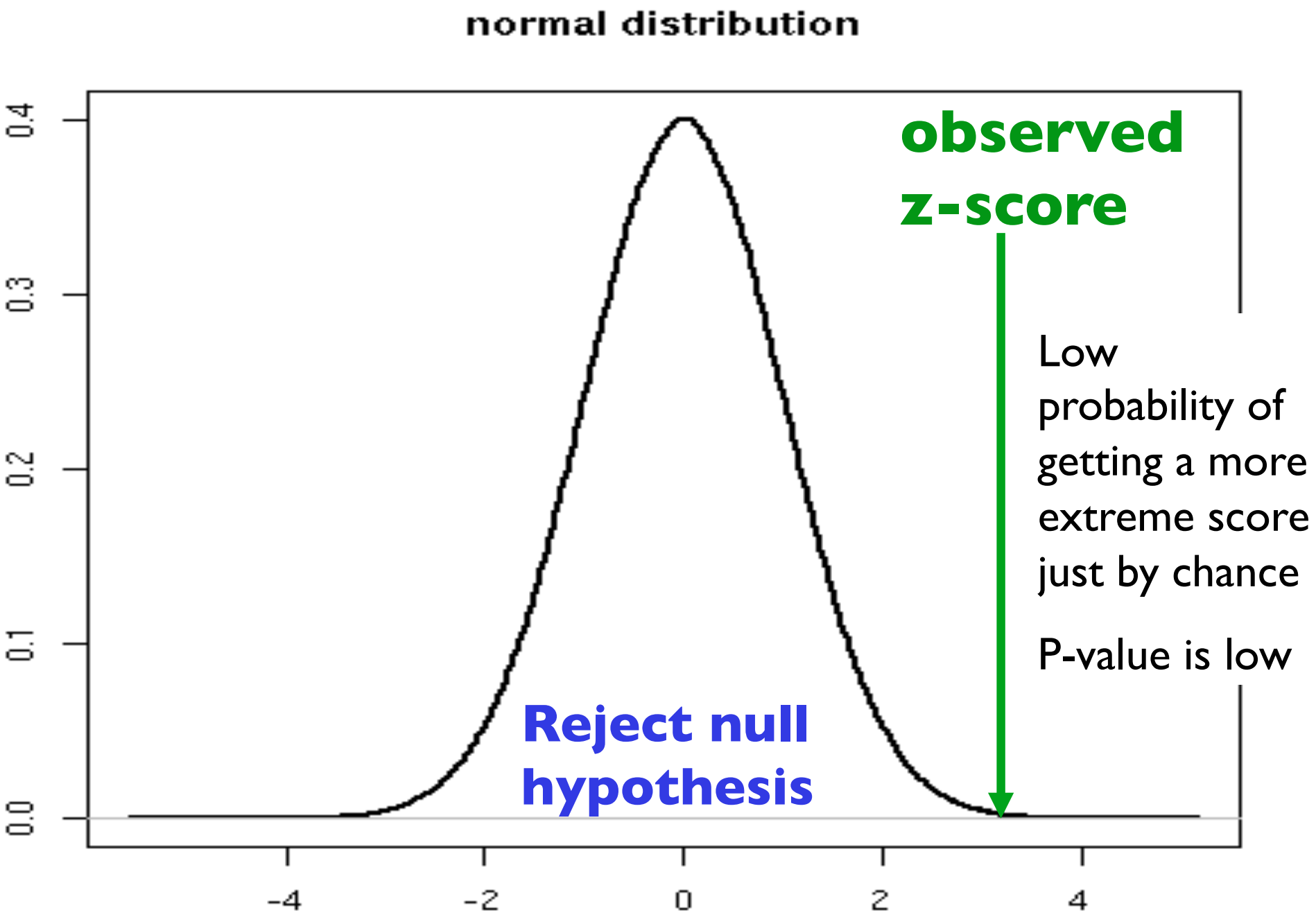
This standardised statistic is called the **z-statistic**

Most of the data are between +/- 3 sd's

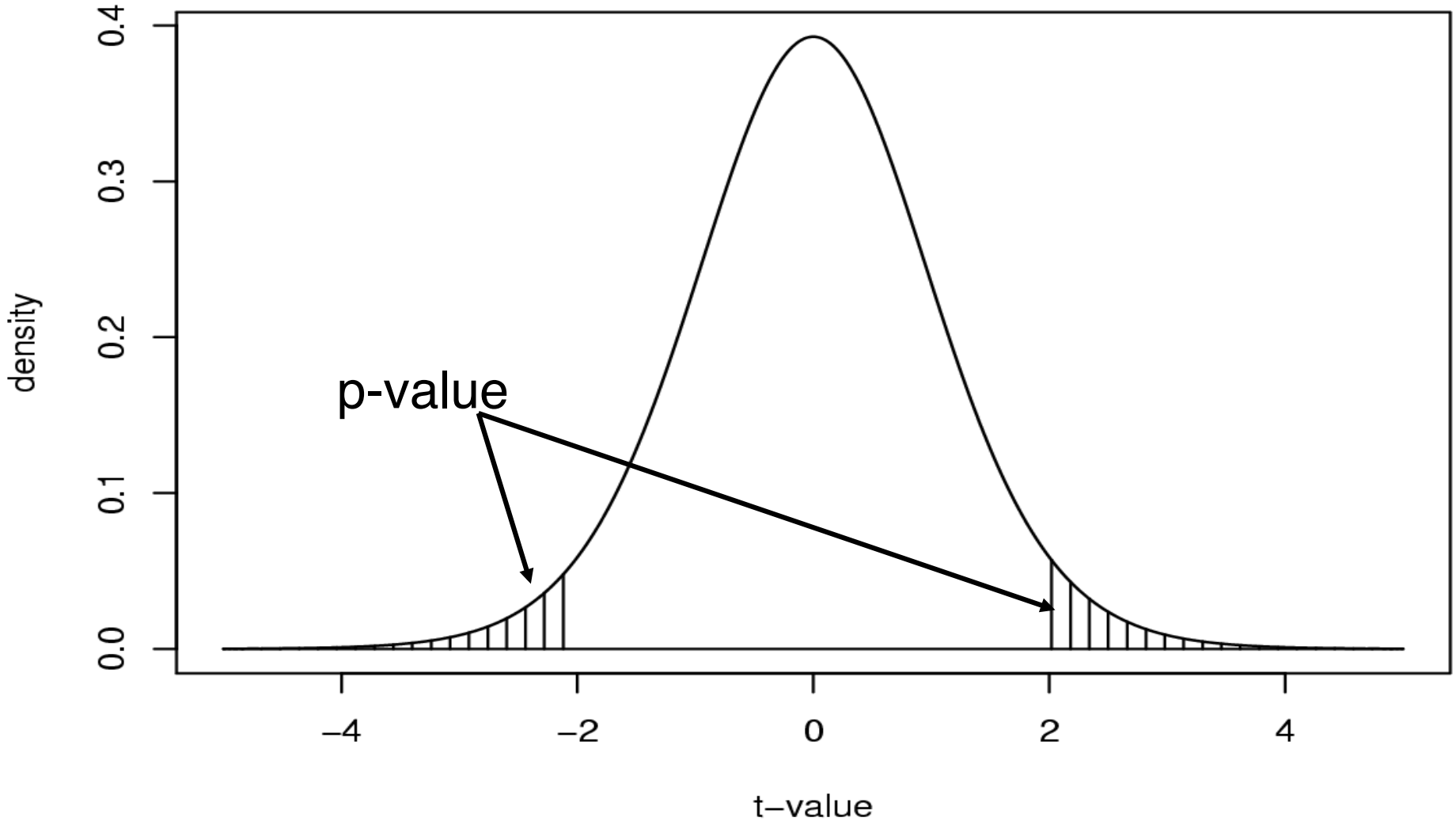
If you observed a z-score greater than +/- 3 this is evidence that you did not sample from the hypothesized distribution


normal distribution
observed
z-score
High
probability of
getting a more
extreme score
just by chance
P-value is high!
0.0
0.1
0.2
0.3
0.4
-4
-2
0
2
4


normal distribution
0.0
0.1
0.2
0.3
0.4
-4
-2
0
2
4
observed
z-score
Also a high probability of getting a more extreme score just by chance
P-value is also high!


normal distribution
0.0
0.1
0.2
0.3
0.4
-4
-2
0
2
4
observed
z-score
Low
probability of
getting a more
extreme score
just by chance
P-value is low
Reject null
hypothesis

The p-values for two-sided tests

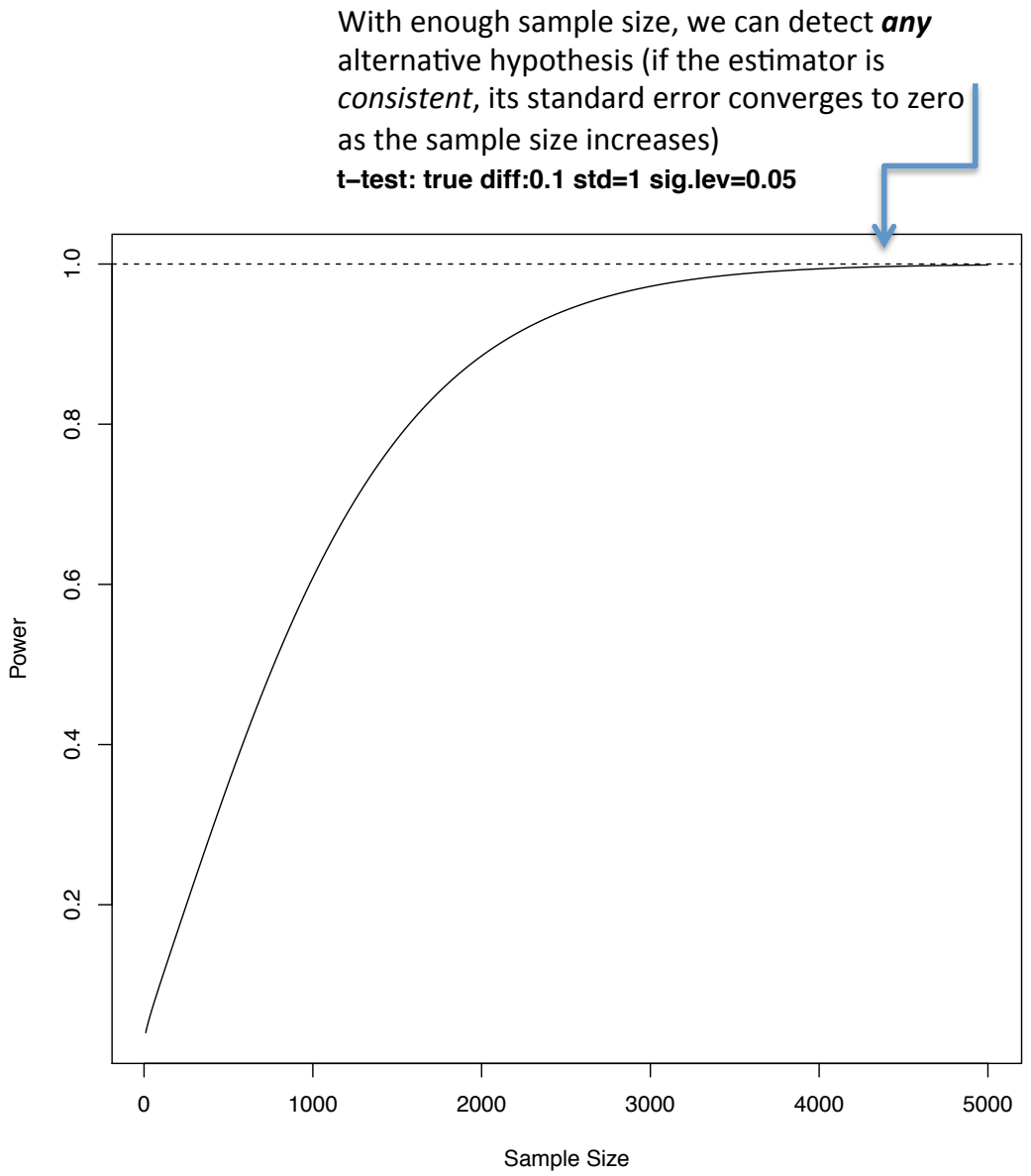


Type I and Type II errors

- **Type I** error: probability of rejecting the null hypothesis when it is true. Usually, it is the significance level of the test. It is denoted as **α**.
- **Type II** error: probability of not rejecting the null hypothesis when it is false. It is denoted as **β**.
- Decreasing one type of error increases the other, so in practice we fix the type I error and choose the test that minimizes type II error.

The power of a test

- The power of a test is the probability of rejecting the null hypothesis at a given significance level when a specific alternative is true
- For a given significance level and a given alternative hypothesis in a given test, the power is a function of the sample size
- What is the difference between statistical significance and biological significance?



Multiple testing problem

- In High throughput experiments we are fitting one model for each gene/exon/sequence of interest, and therefore performing thousands of tests

  E.g.: $p < 0.05$ means $< 1/20$ chance of rejecting H_0 when it should be accepted. So what happens if we do 1000+ tests?

- Type I error is not equal to the significance level of each test
- Multiple test corrections try to fix this problem (Bonferroni, FDR,…)

Controlling the number of errors

N = number of hypotheses tested
R = number of rejected hypotheses
$\mathbf{n_0}$ = number of true hypotheses

	Null Hypothesis True	Alternative Hypothesis True	Total
Not Significant (don't reject)	# True Negative	# False Negative (Type II error)	N- # Rejections
Significant (Reject)	# False positive (Type I error)	# True positive	R = # Total Rejections
Total	n_0	$N-n_0$	N

Bonferroni Correction

If the tests are independent:

$P_{(\text{at least one false positive} \mid \text{all null hypothesis are true})} =$

$P_{(\text{at least one p-value} < \alpha \mid \text{all null hypothesis are true})} = \mathbf{1 - (1-\alpha)^N}$

Usually, we set a threshold at α/N.

Bonferroni correction:

reject each hypothesis at **α/N** level

It is a very conservative method!

False Discovery Rate (FDR)

N = number of hypotheses tested
R = number of rejected hypotheses
$\mathbf{n_0}$ = number of true hypotheses

	Null Hypothesis True	Alternative Hypothesis True	Total
Not Significant (don't reject)	# True Negative	# False Negative (Type II error)	N - # Rejections
Significant (Reject)	V= # False positive (Type I error)	# True positive	R= # Total Rejections
Total	n_0	$N-n_0$	N

Family Wise Error Rate: **FWER** = $P(V \geq 1)$
False Discovery Rate: **FDR** = $E(V/R \mid R>0)\, P(R>0)$

FDR aims to control the set of false positives among the rejected null hypotheses.

Benjamini & Hochberg (BH) step-up method to control FDR

Benjamini & Hochberg proposed the idea of controlling FDR, and used a step-wise method for controlling it.

Step 1: compare **largest** p-value to the specified significance level α:
If $p_m^{ord} > \alpha$ then don't reject corresponding null hypothesis

Step 2: compare second largest p-value to a modified threshold:
If $p_{m-1}^{ord} > \alpha * (m-1)/m$ then don't reject corresponding null hypothesis

Step 3:
If $p_{m-2}^{ord} > \alpha * (m-2)/m$ then don't reject corresponding null hypothesis

. . .

Stop when a p-value is lower than the modified threshold:

All other null hypotheses (with smaller p-values) are rejected.

Slide by Alex Lewin, Ernest Turro, Paul O'Reilly

Adjusted p-values for BH FDR

The final threshold on p-values below which all null hypotheses are rejected is $\alpha j^*/m$ where j^* is the index of the largest such p-value.

BH:
compare p_i to $\alpha j^*/m$ $\longleftrightarrow$ compare mp_i/j^* to α

Can define 'adjusted p-values' as mp_i/j^*

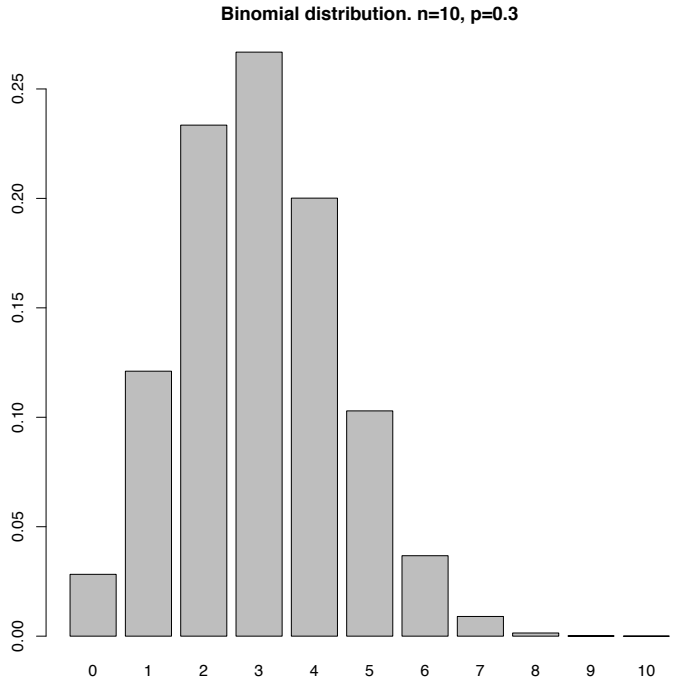
But these 'adjusted p-values' tell you the level of FDR which is being controlled (as opposed to the FWER in the Bonferroni and Holm cases).

Models for counting data

Binomial Distribution

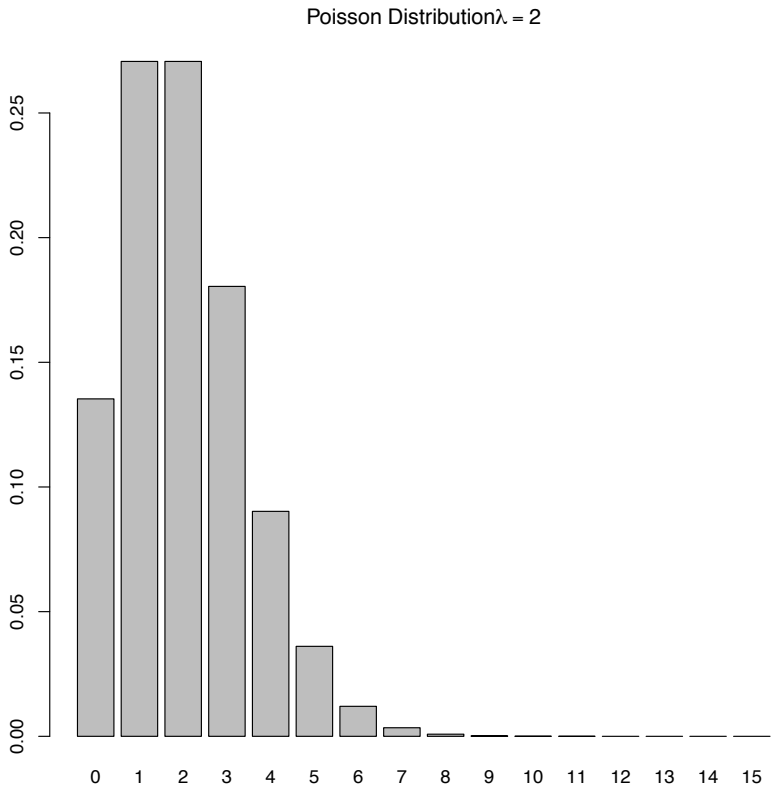
- It is the distribution of the number of events in a series of *n* independent *Bernoulli* experiments, each with a probability of success *p*.
- Y can take integer

  values from 0 to n
- E(Y)=np
- Var(Y)= np(1-p)



Poisson Distribution

- Let Y ~ B(n,p). If *n* is large and *p* is small then *Y* can be approximated by a Poisson Distribution (*Law of rare events*)
- Y ~ P(λ)
- E(Y)=λ
- Var(Y)=λ

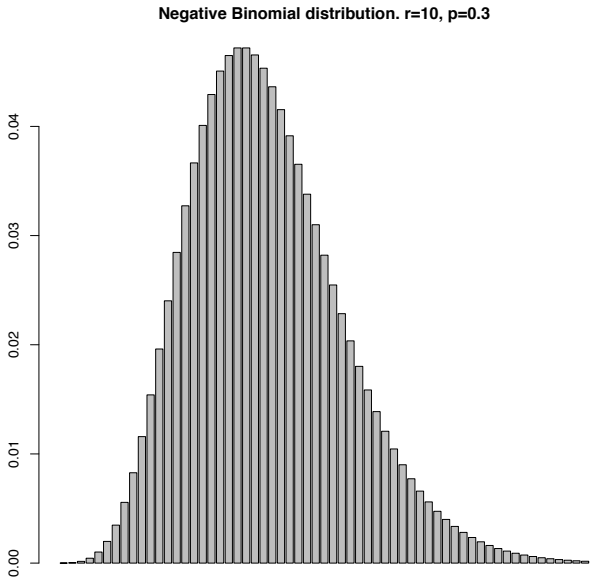


Negative Binomial Distribution

- Let Y ~ NB(r,p)
- Represents the number of successes in a Bernoulli experiment until *r* failures occur
- The distribution of a continuous mixture of Poisson distributions where λ follows a Gamma distribution
- It can be seen as a overdispersed Poisson distribution

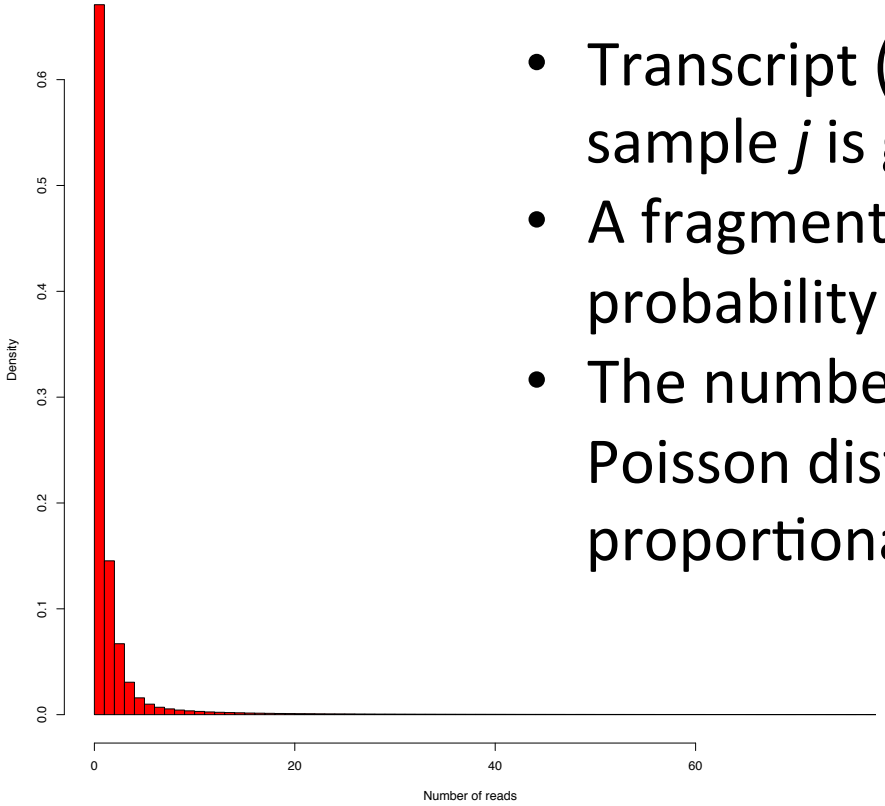
$$p = \frac{\mu}{\sigma^2}$$ → Overdispersion parameter

$$r = \frac{\mu^2}{\sigma^2 - \mu}$$ → Location parameter



Sequencing data

Gene	Sample 1	Sample 2
ERBB2	0	45
MYC	14	23
ESR1	56	2

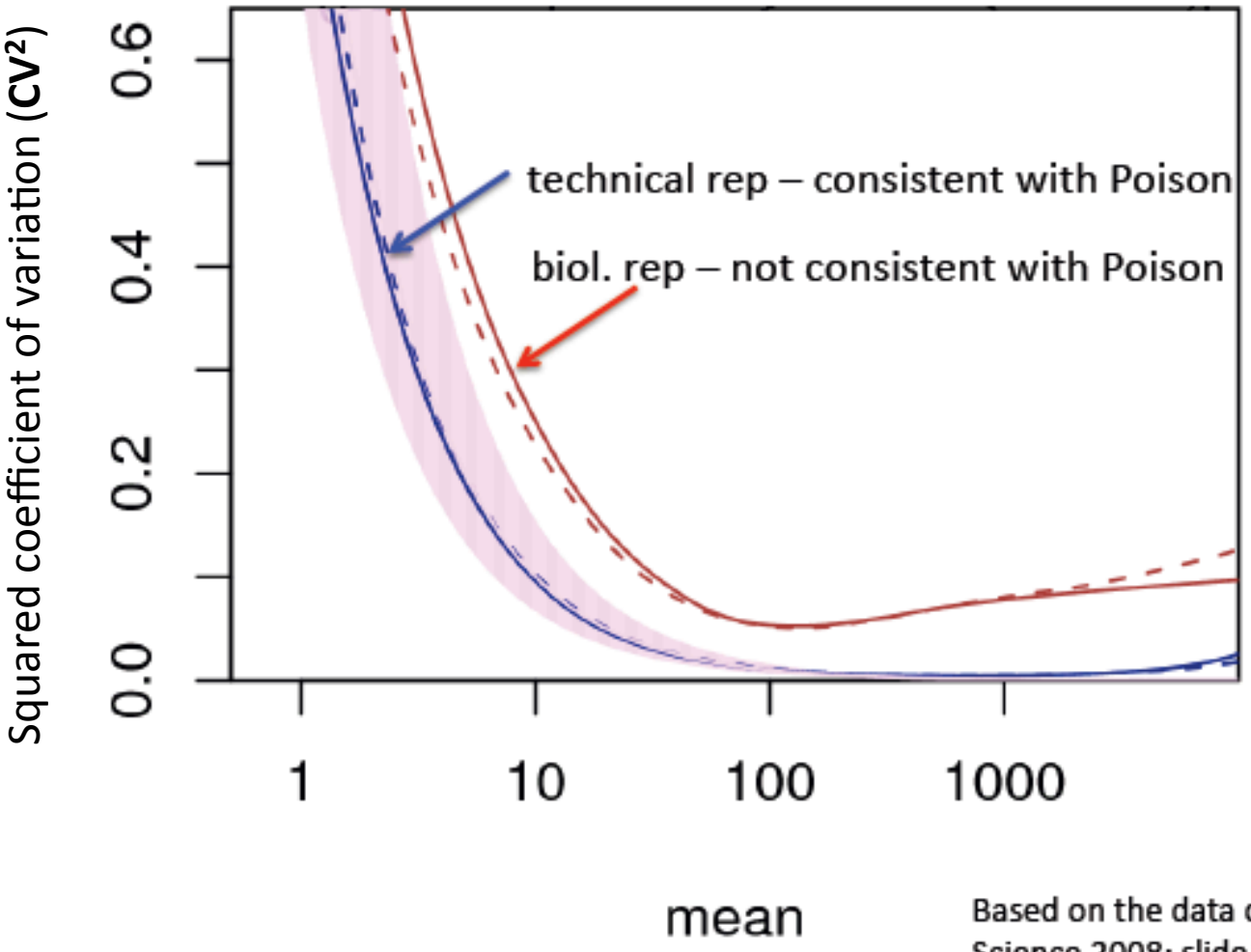


- Transcript (or sequence, or methylation) *i* in sample *j* is generated at a rate λ_{ij}
- A fragment attaches to the flow cell with a probability of p_{ij} (small)
- The number of observed tags y_{ij} follows a Poisson distribution with a rate that is proportional to $\lambda_{ij}p_{ij.}$

The variance in a Poisson distribution is equal to the mean

Adapted from slides by Benilton Carvalho, Tom Hardcastle

Extra variability



Based on the data of Nagalakshmi et al. Science 2008; slide adapted from Huber;

Adapted from slides by Benilton Carvalho

Negative binomial model for seq. data

- For subject j, on transcript i:

$$Y_{ij}|\lambda_{ij} \sim P(\lambda_{ij})$$

- Different subjects have different rates, which we can model through:

$$\lambda_{ij} \sim \Gamma(\alpha, \beta)$$

- This hierarchy changes the distribution of Y:

$$Y_{ij} \sim \mathrm{NB}\left(\alpha, \frac{1}{1+\beta}\right)$$

Adapted from slides by Benilton Carvalho

Estimating Overdispersion with edgeR

edgeR (Robinson, McCarthy, Chen and Smyth):

- Total CV^2=Technical CV^2 + Biological CV^2

Decreases with sequencing depth

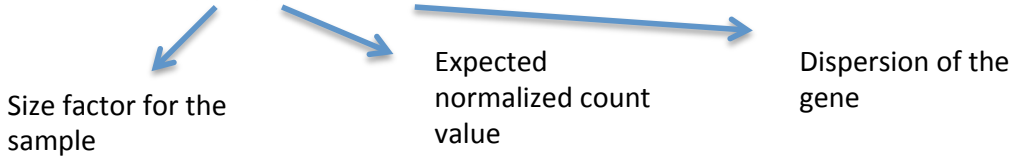
Variability in gene abundance between replicates

- Borrows information from all genes to estimate BCV
  - Common dispersion for all tags
  - Empirical Bayes to shrink each dispersion to the common dispersion

Estimating Overdispersion with DESeq

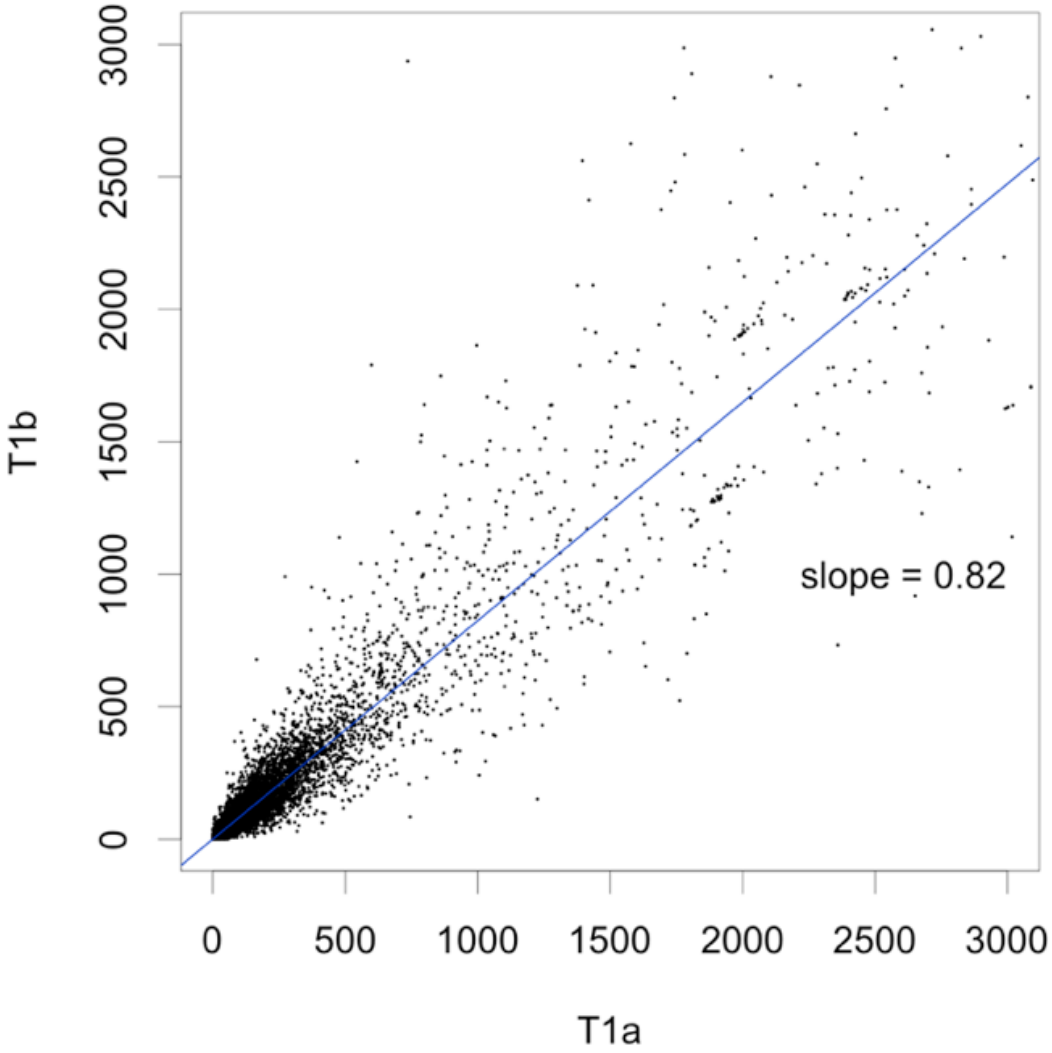
DESeq (Anders, Huber):

- $\text{Var} = s\mu + \alpha s^2 \mu^2$



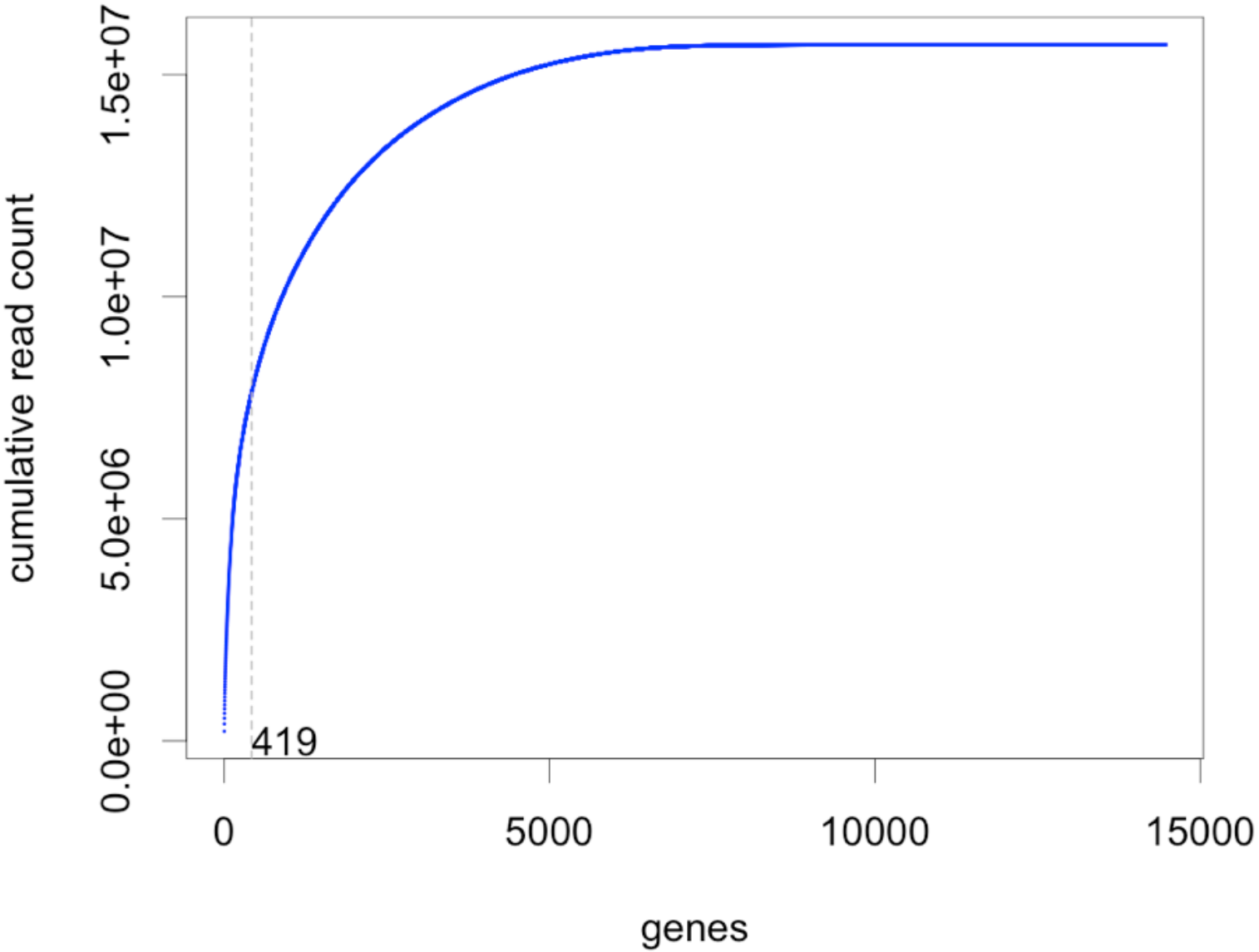
- `estimateDispersions()`
  1. Dispersion value for each gene
  2. Fits a curve through the estimates
  3. Each gene gets an estimate between (1) and (2)

Reproducibility



Slide by Wolfgang Huber

A few genes get most of the reads



Slide by Wolfgang Huber

Effective library sizes

- Also called normalization (although the counts are not changed!!!)
- We must estimate the effective library size of each sample, so our counts are comparable between genes and samples
- Gene lengths?
- This library sizes are included in the model as an ***offset*** (a parameter with a fixed value)

$$\log \mu_{ij} = s_j + \sum_k \beta_{ik} x_{kj}$$

Estimating library size with edgeR

edgeR (Robinson, McCarthy, Chen and Smyth):

- Adjust for sequencing depth and RNA composition (total RNA output)
- Choose a set of genes with the same RNA composition between samples (with the log fold change of normalised counts) after trimming
- Use the total reads of that set as the estimate

Estimating library size with DESeq

DESeq (Anders, Huber):

- Adjust for sequencing depth and RNA composition (total RNA output)
- Compute the ratio between the log counts in each gene and each sample and the log mean for that gene on all samples
- The median on all genes is the estimated library size

References

- Anders and Huber. Genome Biology, 2010; 11:R106
- Auer and Doerge. Genetics 2010, 185:405-416
- Harrell. Regression Modeling Strategies
- Robles et al. BMC Genomics 2012, 13:484
- Venables and Ripley. Modern Applied Statistics with S