Statistics of RNA-seq analysis

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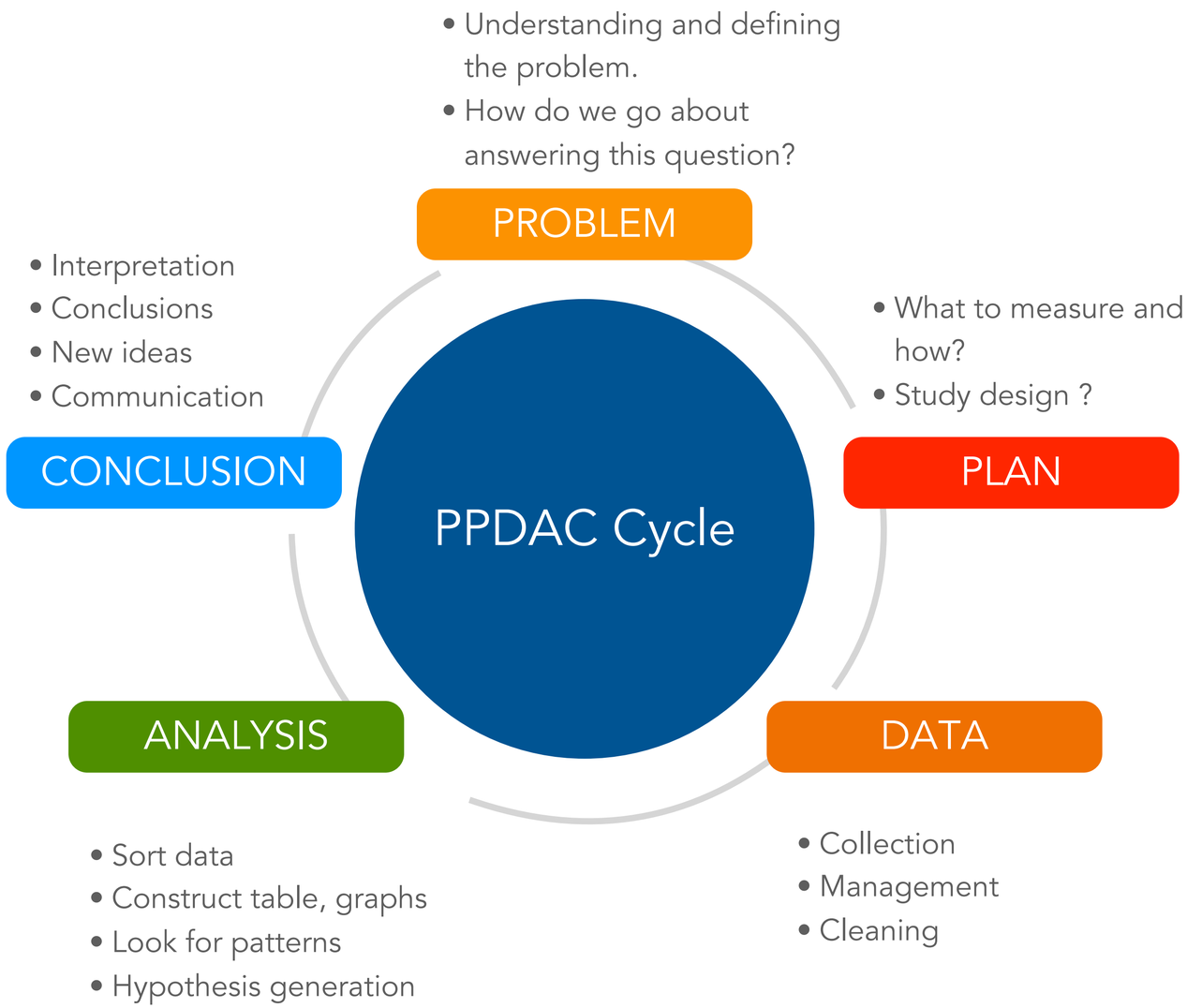
```
> dds <- DESeqDataSetFromMatrix(cnts, DataFrame(cond), ~ cond)
> dds <- DESeq(dds)
> results(dds)


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
```

Data literacy

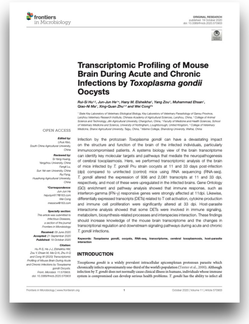
The ability to not only carry out statistical analysis on real-world problems, but also to understand and critique any conclusions drawn by others on the basis of statistics.

Statistics as an investigative process of problem-solving and decision-making



R. J. MacKay. R. W. Oldford. "Scientific Method, Statistical Method and the Speed of Light." Statist. Sci. 15 (3) 254 - 278, August 2000.

Statistics as an investigative process of problem-solving and decision-making



Transcriptomic Profiling of Mouse Brain During Acute and Chronic Infections by *Toxoplasma gondii* Oocysts

- IFN-γ response increases as infection progresses
- Calcium response pathways are downregulated

- Understanding and defining the problem.
- How do we go about answering this question?

PROBLEM

Toxoplasma gondii infection causes a host of severe neurological disorders. Our understanding of the molecular mechanisms associated with infection is incomplete.

We want to study the effect of Toxoplasma gondii infection (chronic and acute) in mouse brain

- What to measure and how?
- Study design ?

PLAN

- Total gene expression profile of the brain in infection versus no-infection
- A two-factor study with three biological replicates in each group with matched controls

PPDAC Cycle

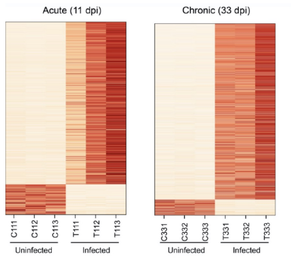
DATA

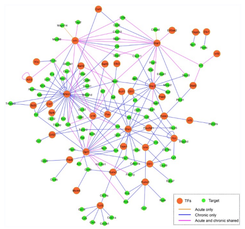
- Collection
- Management
- Cleaning

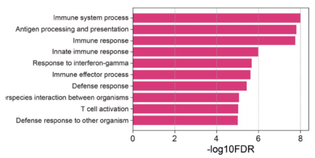
Profiling the total transcriptome with RNA-seq
Preprocessing and quality control

ANALYSIS

- Sort data
- Construct table, graphs
- Look for patterns
- Hypothesis generation







Hu et al. Profiling of Mouse Brain During Acute and Chronic Infections by Toxoplasma gondii Oocysts. Front. Microbiol. 2020

Outline

- Experimental Design
- General Statistical Concepts
- Statistical aspects specific to bulk RNA-seq analysis

Outline

- Experimental Design
- Statistical Concepts - Bite size statistics
- Statistical aspects of bulk RNA-seq analysis

Consequences of Poor Experimental Design

Inability to answer the questions we would like to answer

- Cost of experimentation.
- Limited & Precious material, esp. clinical samples.
- Immortalization of data sets in public databases and methods in the literature. Our bad science begets more bad science.
- Ethical concerns of experimentation: animals and clinical samples.

A Well-Designed Experiment

Should have

- Clear objectives
- Focus and simplicity
- Sufficient power
- Randomised comparisons

And be

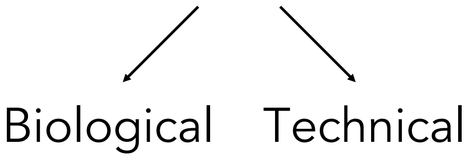
- Precise
- Unbiased
- Amenable to statistical analysis
- Reproducible

Experimental Factors

- Factors: aspects of experiment that change and influence the outcome of the experiment
  - e.g. time, weight, drug, gender, ethnicity, country, plate, cage etc.
- Variable type depends on type of measurement:
  - Categorical (nominal) , e.g. sex
  - Categorical with ordering (ordinal), e.g. tumour grade
  - Discrete, e.g. shoe size, number of cells
  - Continuous, e.g. body weight in kg, height in cm
- Independent and Dependent variables
  - Independent variable (IV): what you change
  - Dependent variable (DV): what changes due to IV
  - "If (independent variable), then (dependent variable)"

Sources of Variation

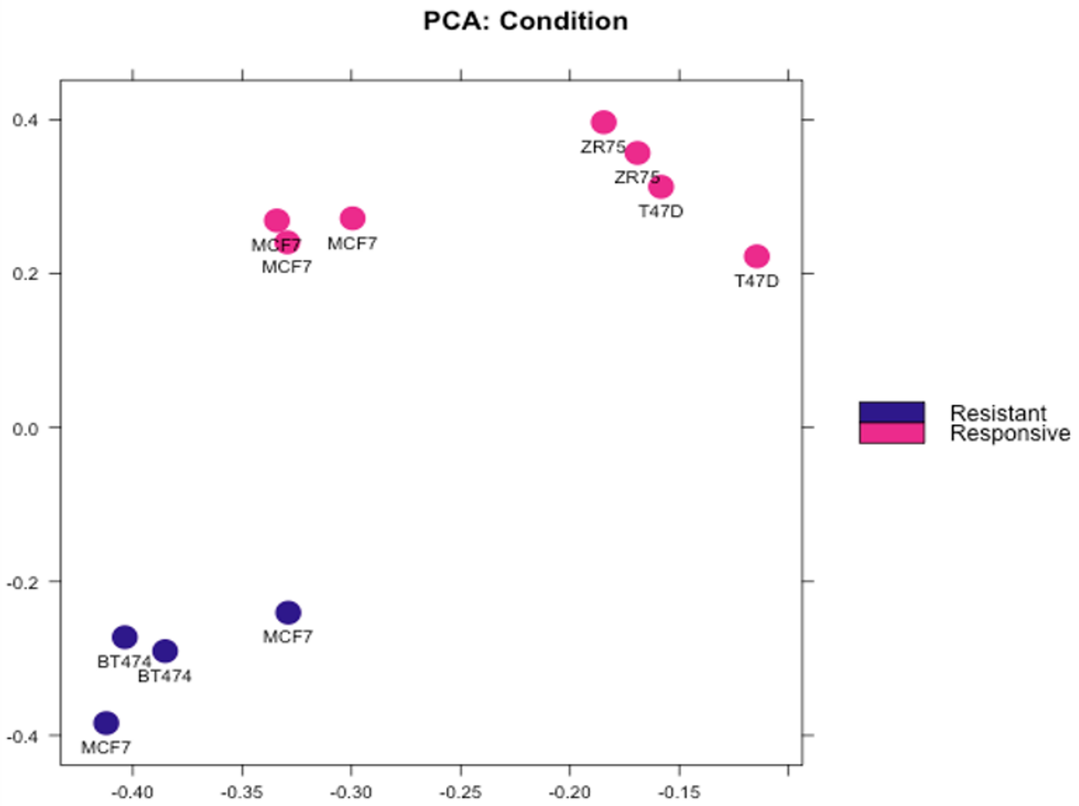
dependent variable = f (independent variable) + noise



- Biological “noise”
  - Biological processes are inherently stochastic
  - Single cells, cell populations, individuals, organs, species….
  - Timepoints, cell cycle, synchronized vs. unsynchronized
- Technical noise
  - Reagents, antibodies, temperatures, pollution
  - Platforms, runs, operators
- Replication is required to capture variance

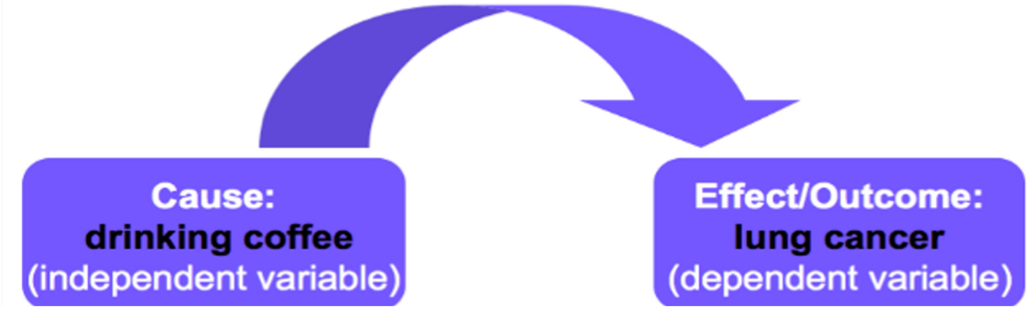
Types of Replication

- Biological replication:
  - In vivo:
    - Patients
    - Mice
  - In vitro:
    - Different cell lines
    - Re-growing cells (passages)
- Technical replication:
  - Experimental protocol
  - Measurement platform (i.e. sequencer)



Confounding Factors

- Also known as extraneous, hidden, lurking or masking factors, or the third variable or mediator variable.
- May mask an actual association or falsely demonstrate an apparent association between the independent & dependent variables.
- Hypothetical Example would be a study of coffee drinking and lung cancer.



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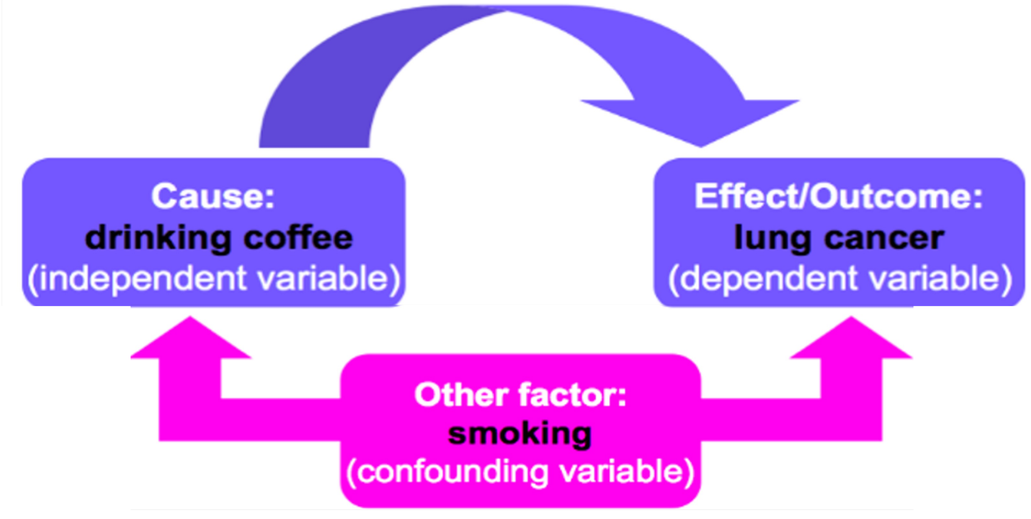
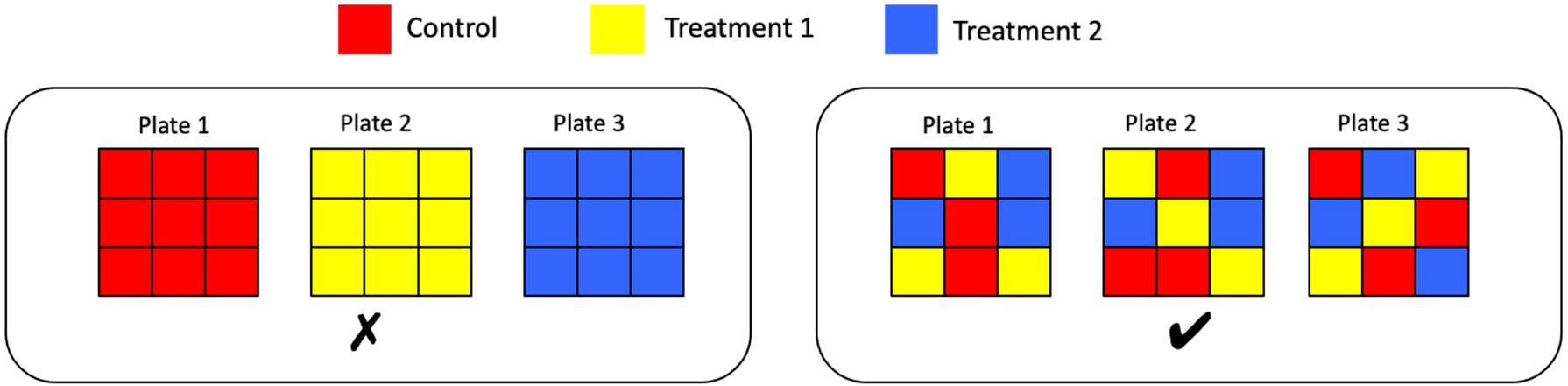


Solutions

- Write it all down!!!!!!!!
- Controlling technical effects:
  - Randomisation
    - Statistical analyses assume randomised comparisons
    - May not see issues caused by non-randomised comparisons
    - Make every decision random not arbitrary
    - Caveat: over-randomization can increase error
  - Blinding
    - Especially important where subjective measurements are taken
    - Potentially multiple degrees of blinding (eg. double-blinding)

Randomised Block Design

- Blocking is the arranging of experimental units in groups (blocks) that are similar to one another.

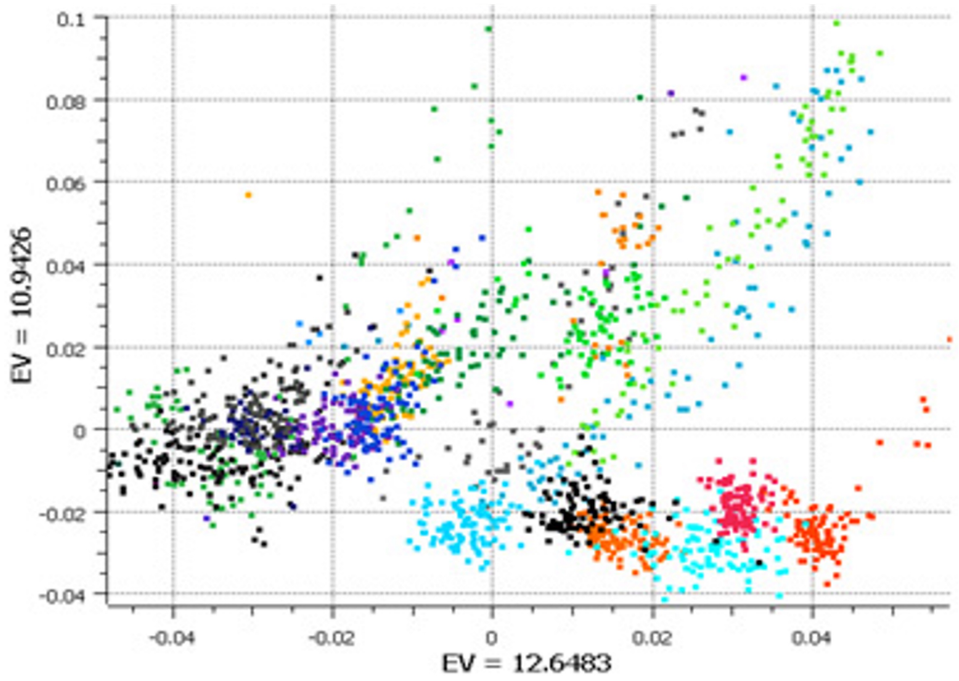


- Each plate contains spatially randomised equal proportions of:
  - Control
  - Treatment 1
  - Treatment 2
- controlling plate effects.

Randomised Block Design

- Good design example: Alzheimer's study from GlaxoSmithKline

Plate effects by plate
Left PCA plot show large plate effects.
Each colour corresponds to a different plate



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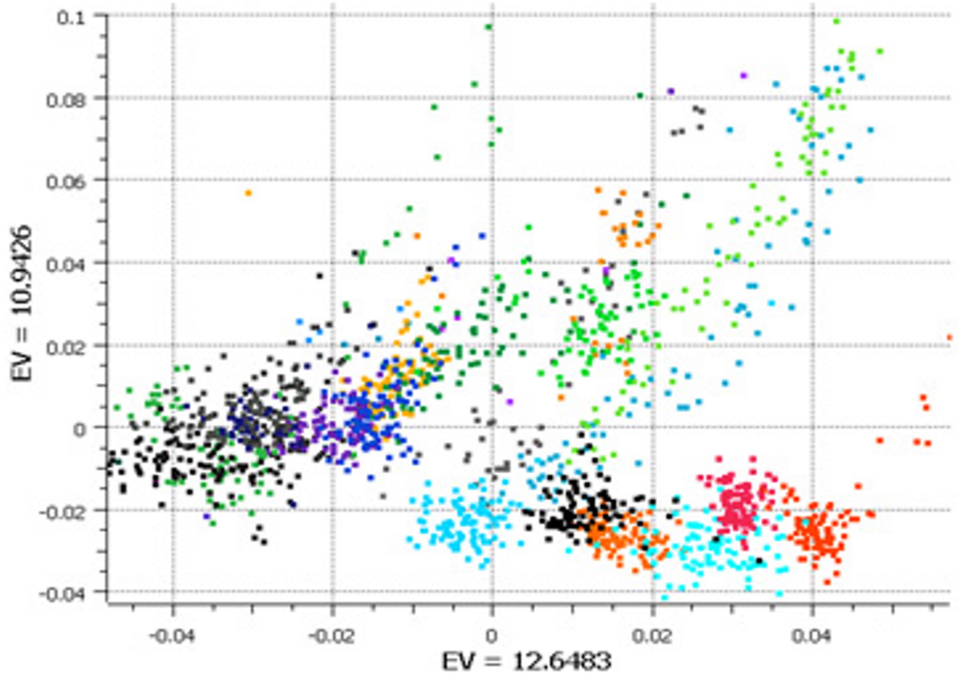
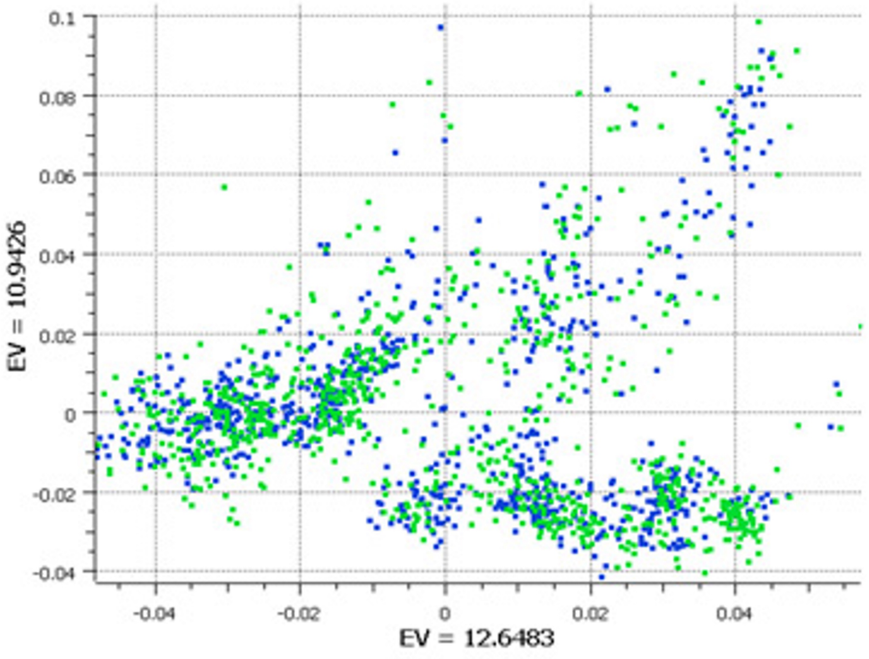


Plate effects by case/control
Right PCA plot shows each plate cluster contains equal proportions of cases (blue) and controls (green).



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Experimental Controls

- Ideal : Everything is identical across conditions except the variable you are testing
- Controlling errors
  - Type I: False Positives
    - Negative controls: should have minimal or no effect
  - Type II: False Negatives
    - Positive controls: known effect
- Technical controls
  - Detect/correct technical biases
  - Normalise measurements (quantification)

Examples of Experimental Controls

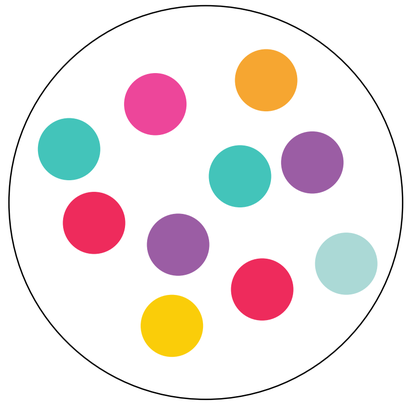
- Wild-type organism (knockouts)
- Inactive siRNA (silencing)
- Vehicle (treatments)
- Spike-ins (quantification/normalisation)
- “Gold standard” data points
- Multi-level controls
- e.g. contrast Vehicle/Input vs. Treatment/Input

Outline

- Experimental Design
- Statistical Concepts
- Statistical aspects of bulk RNA-seq analysis

Basics on inferential statistics and hypothesis testing

Population: the complete set of individuals that we are interested in



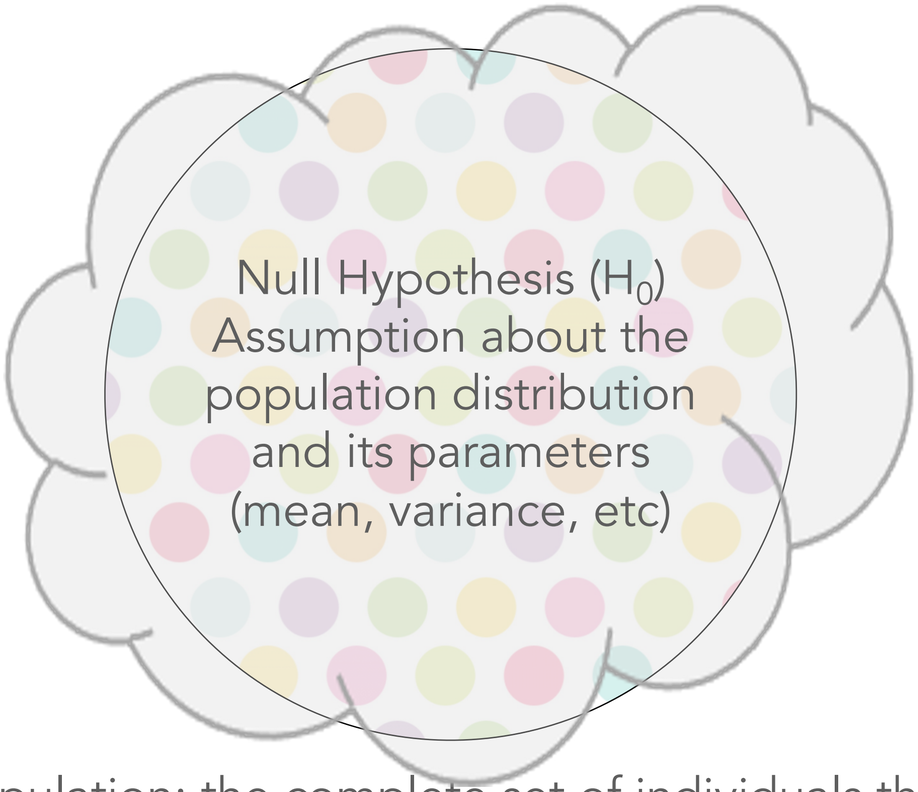
Sample: smaller set of individuals that is representative of the population

Variable: what we are interested in measuring

Basics on inferential statistics and hypothesis testing

Inference means two things:

1. Estimating population parameters
2. Testing hypothesis regarding the population distribution



inference

Population: the complete set of individuals that we are interested in

Sample: smaller set of individuals that is representative of the population

Variable: what we are interested in measuring

Slide content adapted from Matt Castle

Basics on inferential statistics and hypothesis testing

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A simple example

A neurologist is testing the effect of a drug on response time by injecting 100 rats with a unit dose of the drug subjecting each to neurological stimulus and recording its response time. The neurologist knows that the mean response time for rats not injected with the drug is 1.2 seconds. The mean of the 100 injected rats response times is 1.05 seconds with the sample standard deviation of 0.5 seconds. Do you think that the drug has an effect on response time ?

H_0: Drug has no effect on response time

H_1: Drug has an effect on response time

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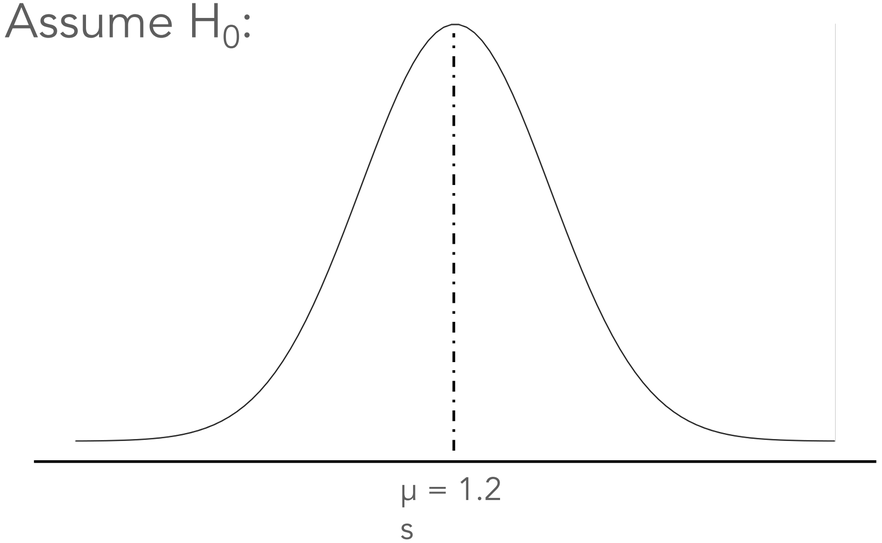
Assume H_0:



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H_0: $\mu = 1.2$ s

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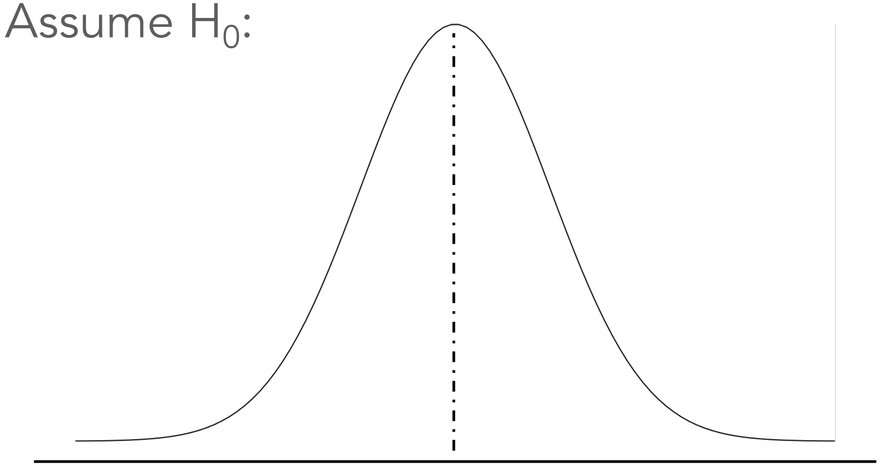
Calculate test statistic

$$t = \frac{m - \mu}{s/\sqrt{n}}$$

Assume H_0:



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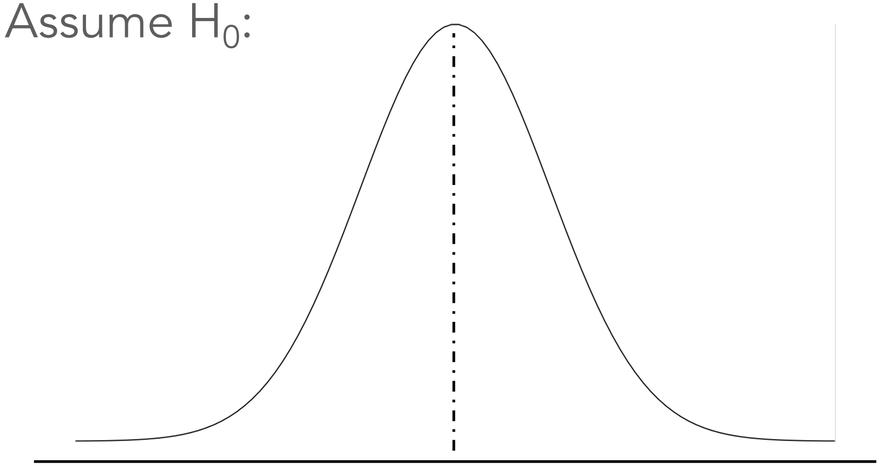
H_1: $\mu \neq 1.2$ s

Calculate test statistic

1.05 → m, 1.2 → μ, 0.5 → s, 100 → n

$$t = \frac{m - \mu}{s/\sqrt{n}}$$

Assume H_0:



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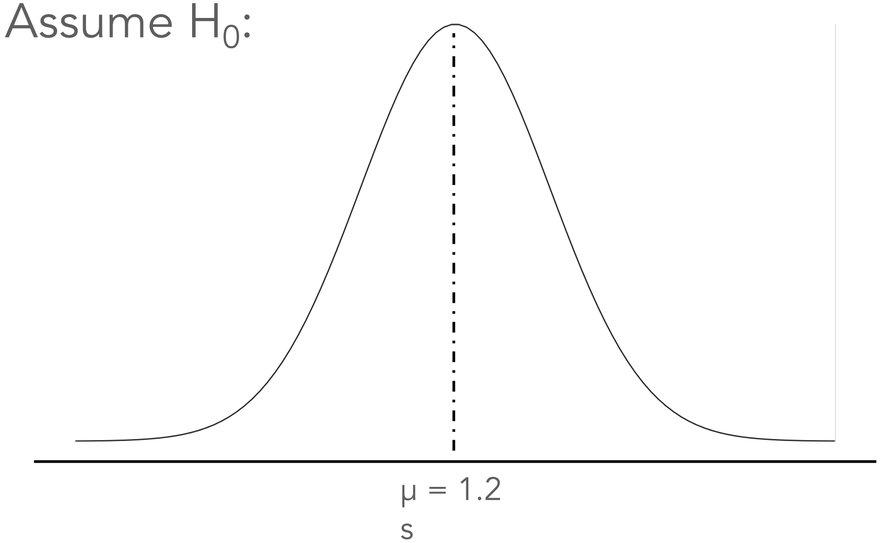
Calculate test statistic

$$t = \frac{m - \mu}{s/\sqrt{n}} = -3$$

Assume H_0:



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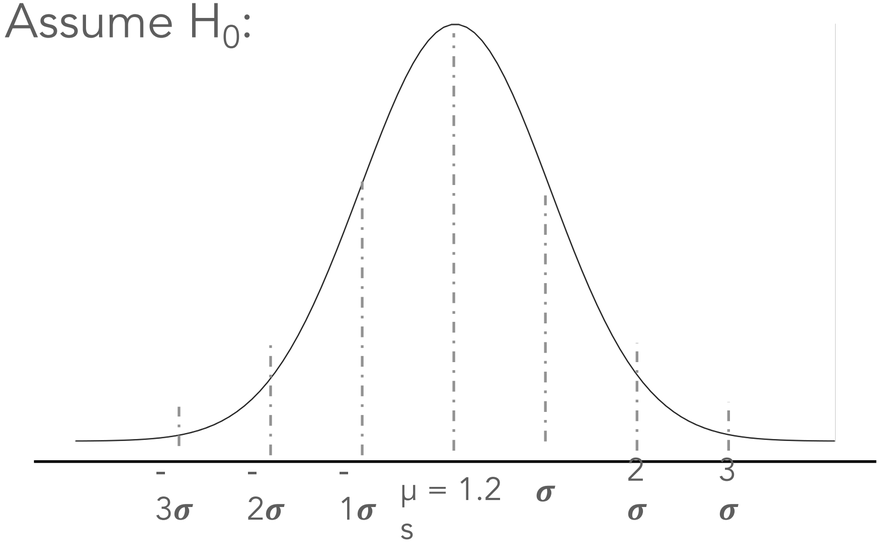

This means that the sample mean (1.05) is 3 standard deviations away from the mean

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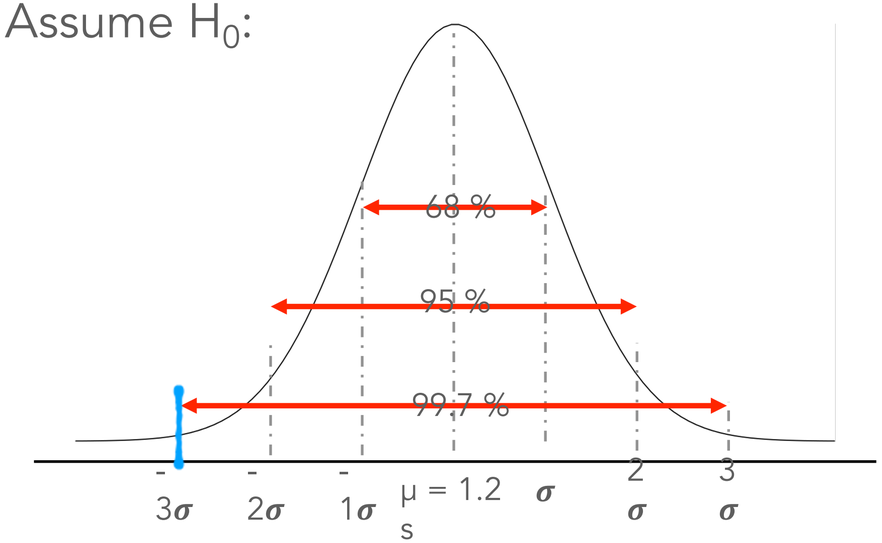

This means that the sample mean (1.05) is 3 standard deviations away from the mean

What is the probability of observing a test statistic as extreme as 1.05?

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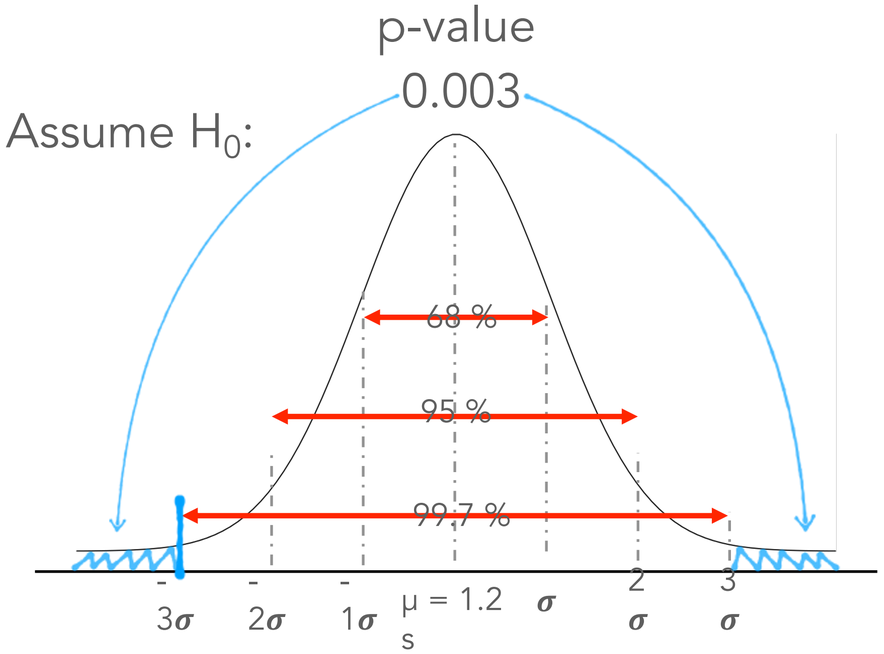

This means that the sample mean (1.05) is 3 standard deviations away from the mean

What is the probability of observing a test statistic as extreme as 1.05?

$$\text{p-value} = 2\ \min[P(t \le t_{obs} | H_0), P(t \ge t_{obs} | H_0)]$$

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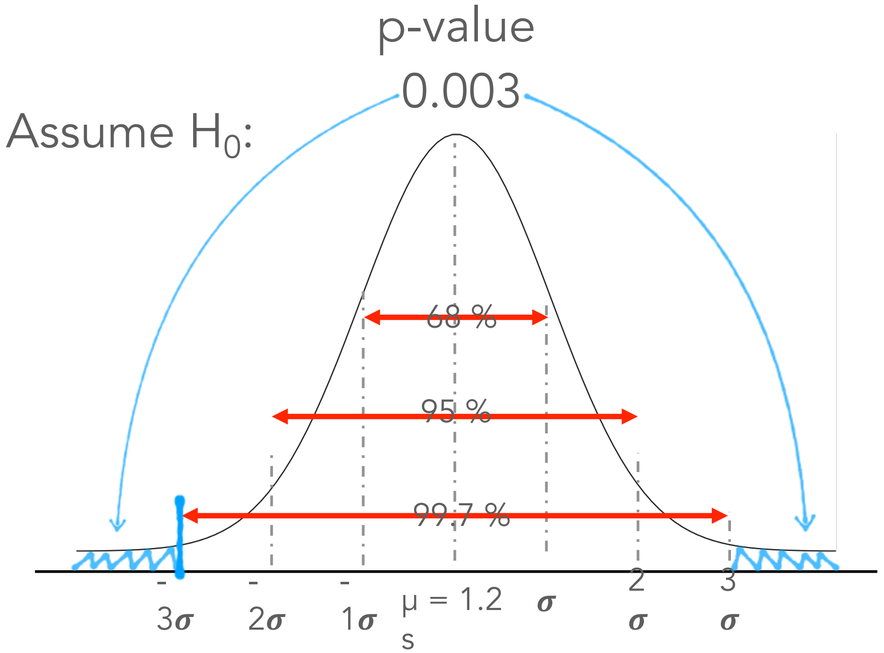

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Calculate test statistic

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

We reject the null hypothesis!

Example adapted from Sal Khan

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Constructed the null and alternative hypothesis about the population

H_0: μ = 1.2 s

H_1: μ ≠ 1.2 s

Calculate test statistic

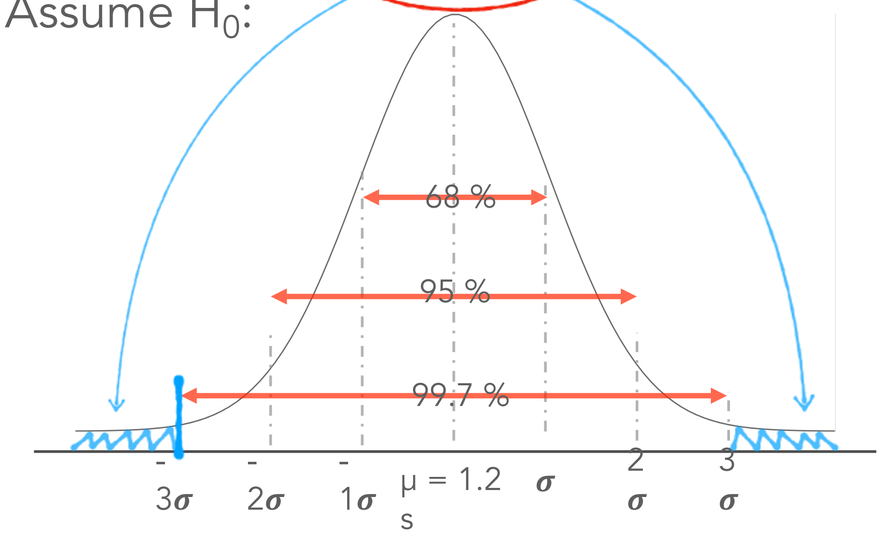
$$t = \frac{m - \mu}{s/\sqrt{n}} = -3$$

Calculated test statistic

Obtained p-value

p-value 0.003

Assume H_0:



This means that the sample mean (1.05) is 3 standard deviations away from the mean

What is the probability of observing a test statistic as extreme as 1.05?

$$\text{p-value} = 2\ \min[P(t \le t_{obs} | H_0), P(t \ge t_{obs} | H_0)]$$

Reached a conclusion

We reject the null hypothesis!

Example adapted from Sal Khan

Key Concepts - Hypothesis Testing

- All statistical tests are based on assumptions!
- All statistics can be wrong
- Statistical tests are probabilistic in nature
- There is always a chance that the result is wrong (even when all assumptions met perfectly):
  - Either significant result when no difference (Type I),
  - Or insignificant results when there is an actual difference (Type II)

Type I and Type II Errors

- All hypothesis tests involve making a decision:

Is this result significant or not?

- This decision can be wrong in two ways:

Type I error or False positive

This is when you reject the null hypothesis when it is true

"You're pregnant !"

Type II error or False negative

This is when you fail to reject the null hypothesis when it isn't true

"You're not pregnant"

Slide content adapted from Matt Castle

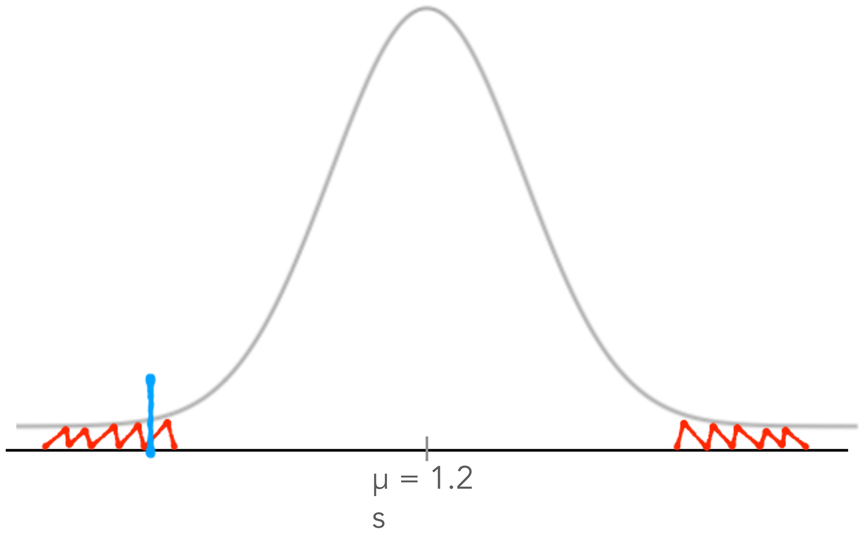
Type I and Type II Errors

H_0: $\mu = 1.2$ s

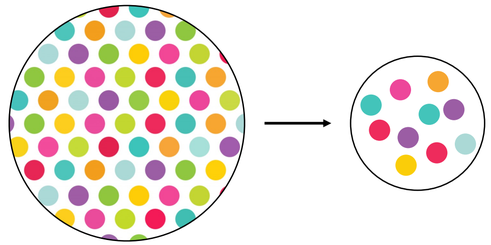
H_1: $\mu \neq 1.2$ s

if p-value > α → do not reject H_0
if p-value < α → reject H_0 in favour of H_1

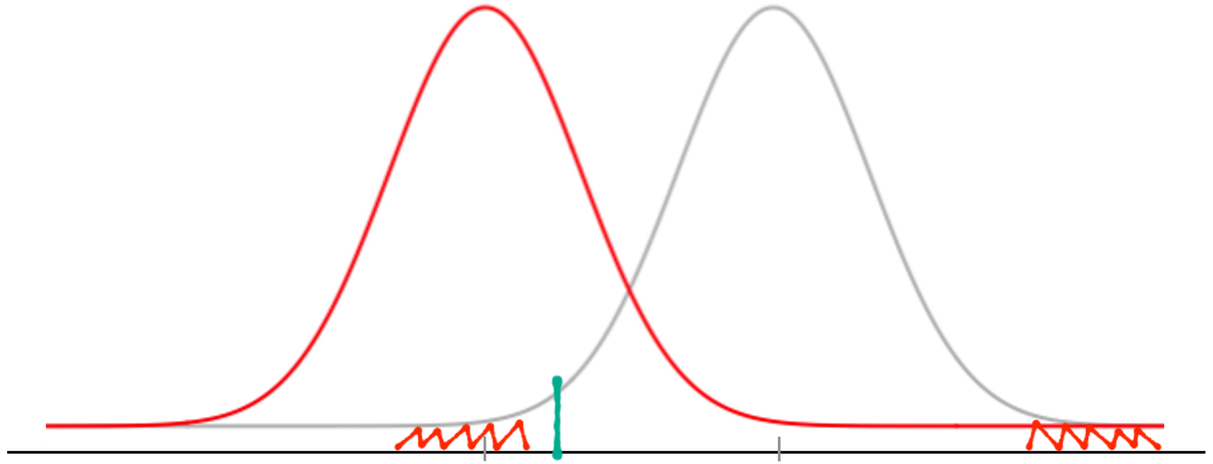
α=0.05 → the type I error, the probability of rejecting
H_0 when H_0 is correct



Suppose H_1 true:



Depending on your sampling, you might fail to reject H_0



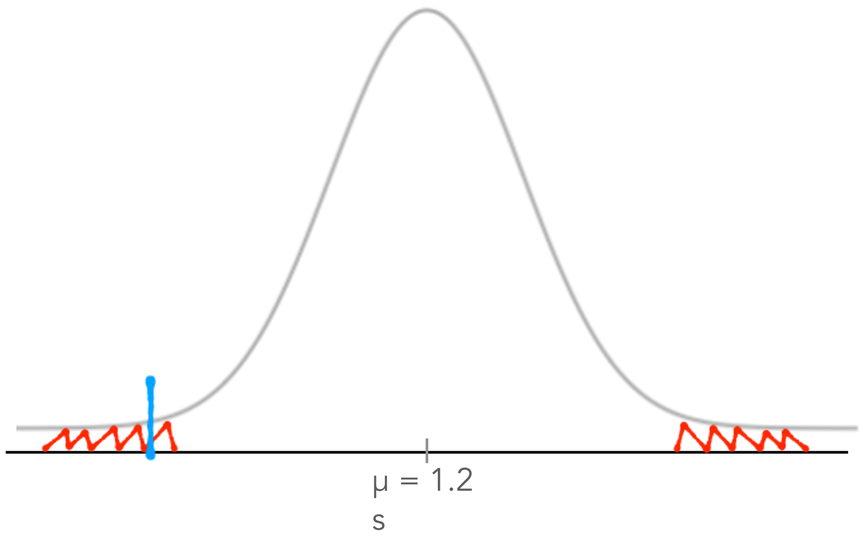
Type I and Type II Errors

H_0: $\mu = 1.2$ s

H_1: $\mu \neq 1.2$ s

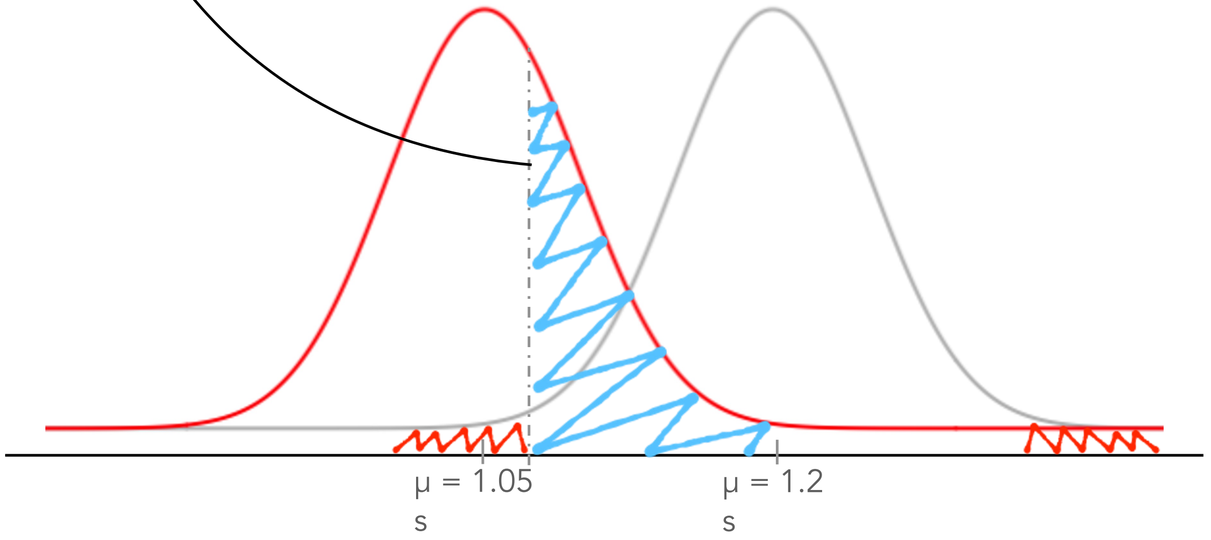
if p-value > α → do not reject H_0
if p-value < α → reject H_0 in favour of H_1

α=0.05 → the type I error, the probability of rejecting
H_0 when H_0 is correct



Suppose H_1 true:

β → the type II error, the probability of not rejecting
H_0 when H_1 is correct



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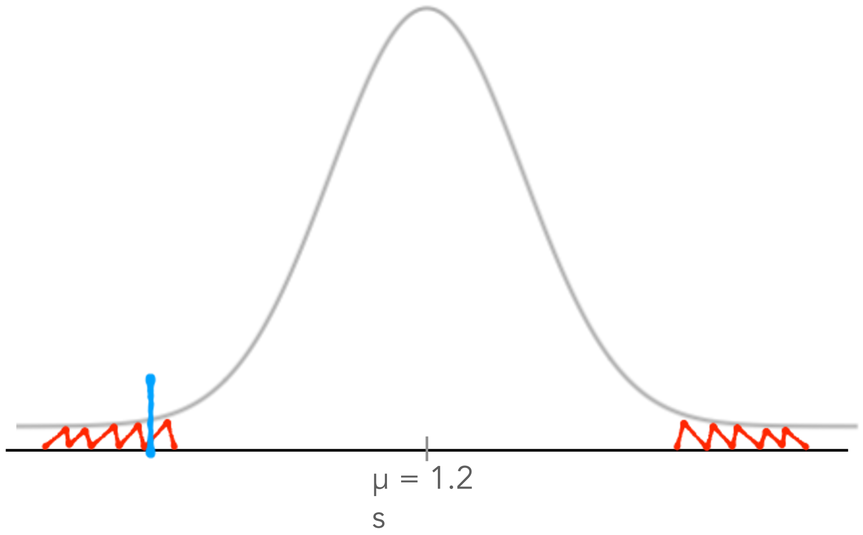
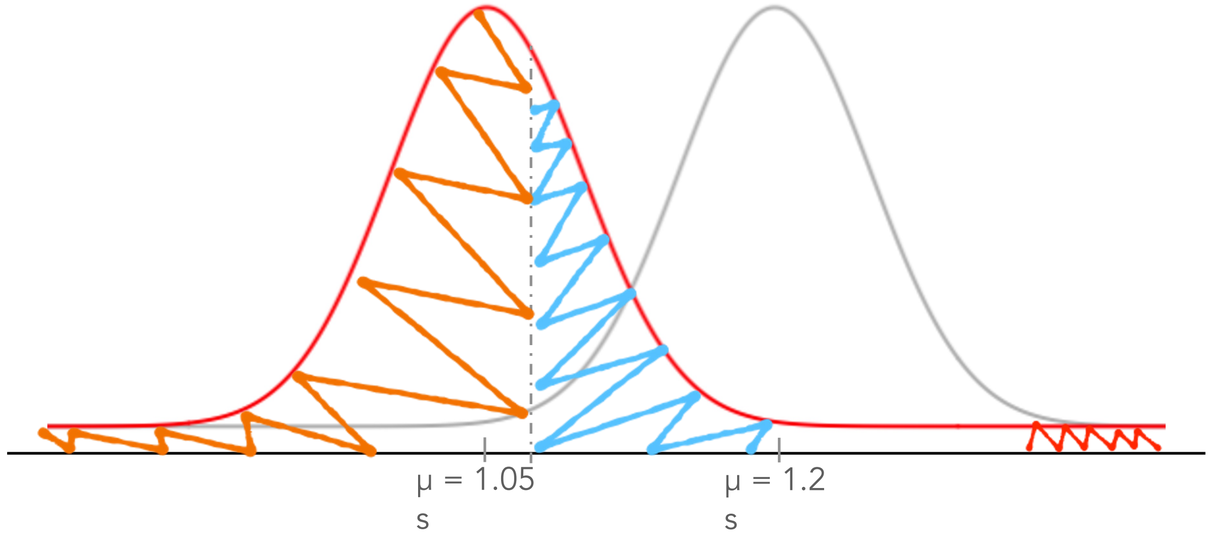


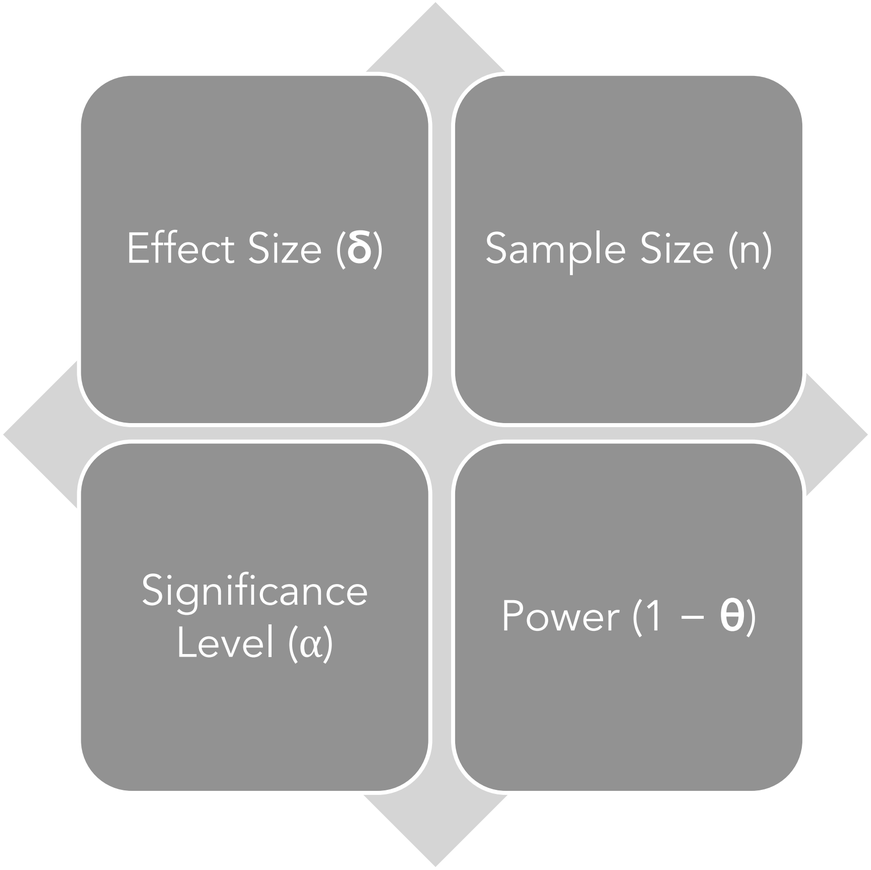
Suppose H_1 true:

θ → the type II error, the probability of not rejecting
H_0 when H_1 is correct

1- **θ** → Power is the probability that we actually detect an effect that exists

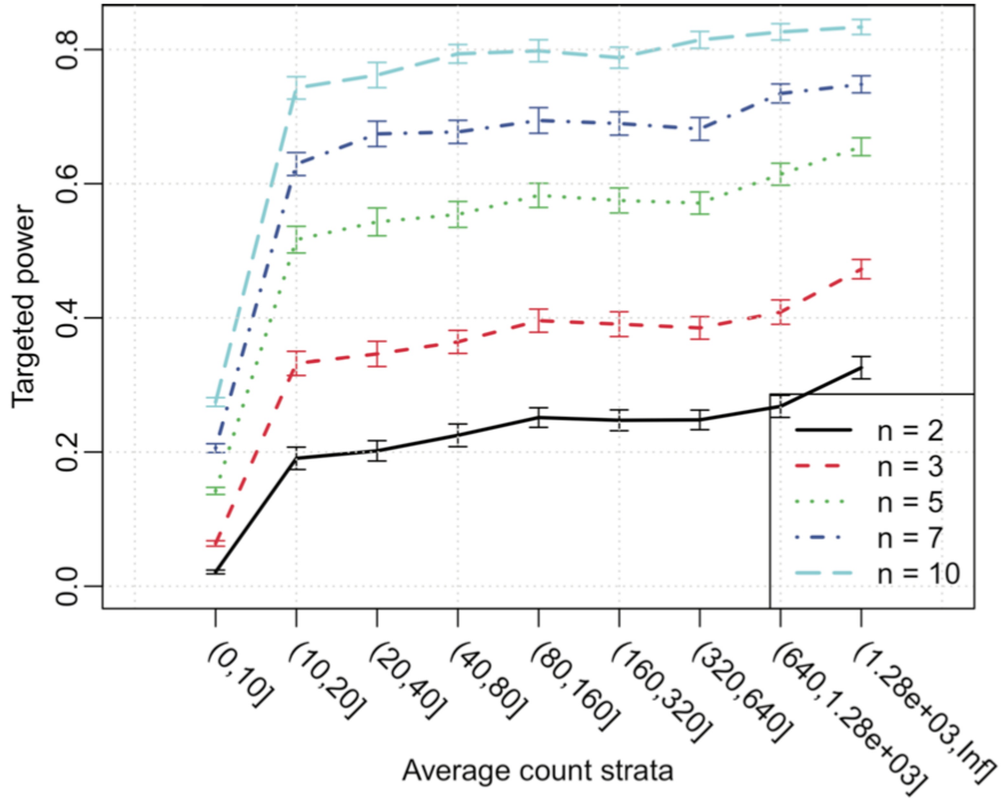


Power Analysis



- The four concepts are linked
- If we know three, we can work out the forth
- Power calculation: Aim is to define the probability (1-**θ**) 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 – **θ**).

Power Analysis in Differential Expression Analysis



(Wu, Wang and Wu (2015))

Outline

- Experimental Design
- Statistical Concepts - Bite size statistics
- Statistical aspects of bulk RNA-seq analysis

Statistical Aspects of Differential Expression Analysis

Linear Modeling

Model the expression of each gene as linear combination of explanatory factors (eg. treatment, age, sex, etc.)

$$Y \sim Normal(\mu, \sigma)$$

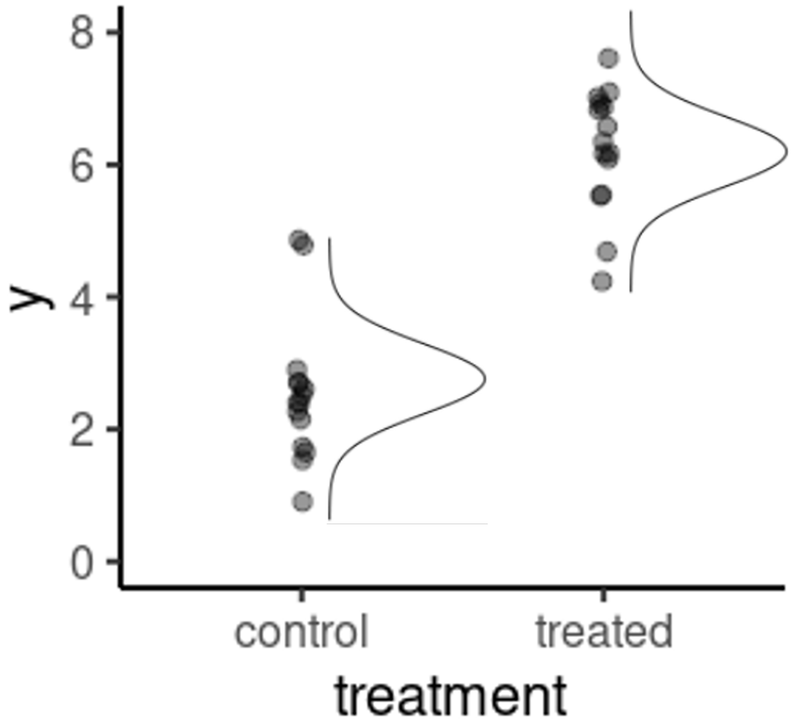
$$\mu = \beta_0 + \beta_1 * treatment + \beta_2 * age + \ldots$$

y - expression of the gene

$\boldsymbol{\beta_i}$ - parameters we want to estimate from the data

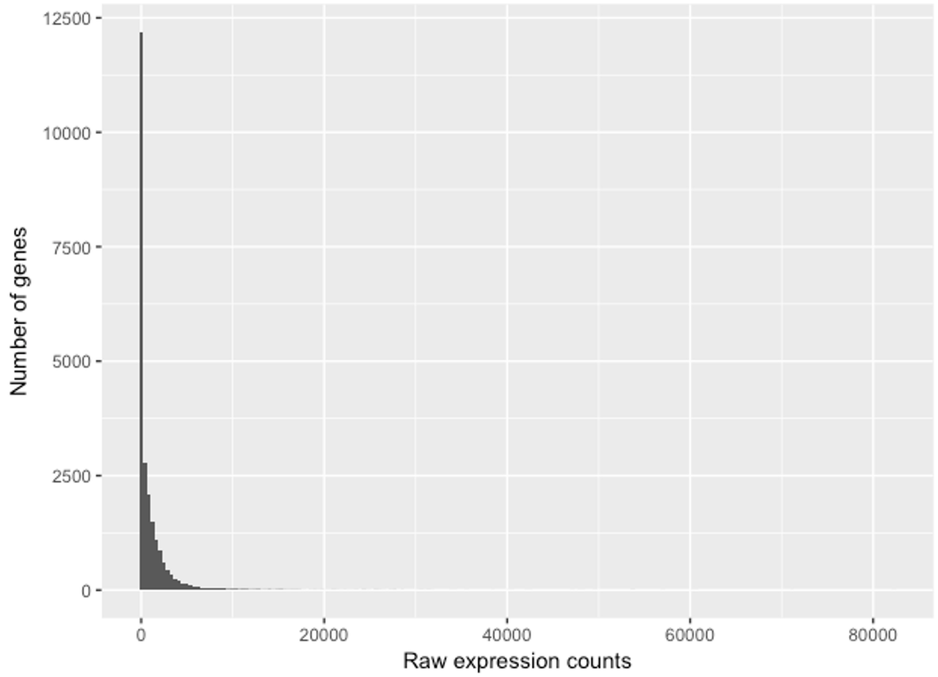
$\boldsymbol{\beta_0}$ - the “intercept” (the value of expression when all other parameters are set at a reference level)

σ - the standard deviation (uncertainty) of our model (also estimated from the data)



Statistical Aspects of Differential Expression Analysis

Characteristics of RNA-seq data



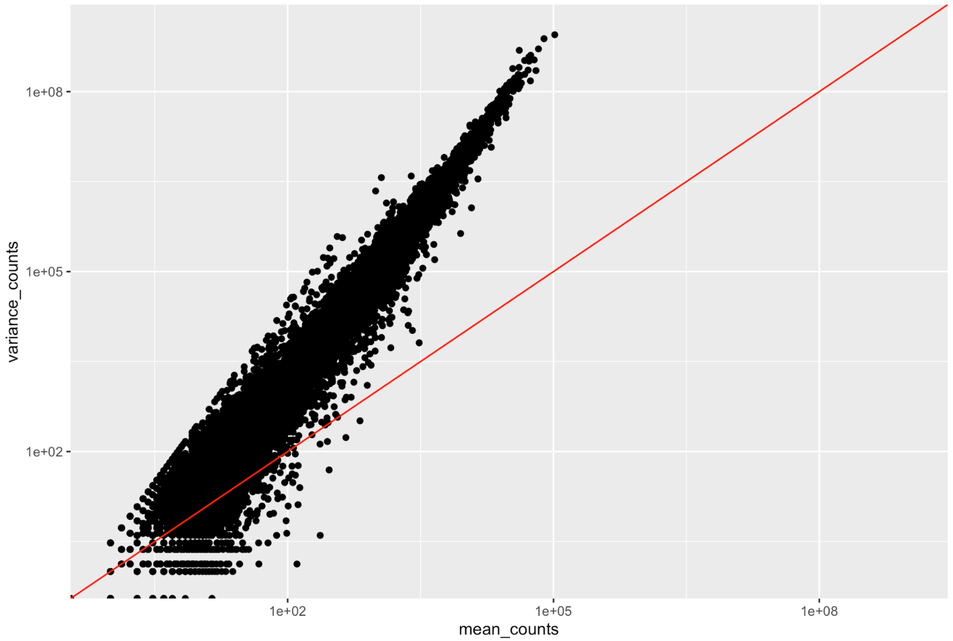
This plot illustrates some common features of RNA-seq count data:

- a low number of counts associated with a large proportion of genes
- a long right tail due to the lack of any upper limit for expression
- large dynamic range

Looking at the shape of the histogram, we see that it is not normally distributed.

Statistical Aspects of Differential Expression Analysis

Characteristics of RNA-seq data



To assess the properties of the data we are working with, we can look at the mean-variance relationship.

For the genes with high mean expression, the variance across replicates tends to be greater than the mean (scatter is above the red line).

Essentially, the Negative Binomial is a good approximation for data where the mean < variance, as is the case with RNA-Seq count data.

Statistical Aspects of Differential Expression Analysis

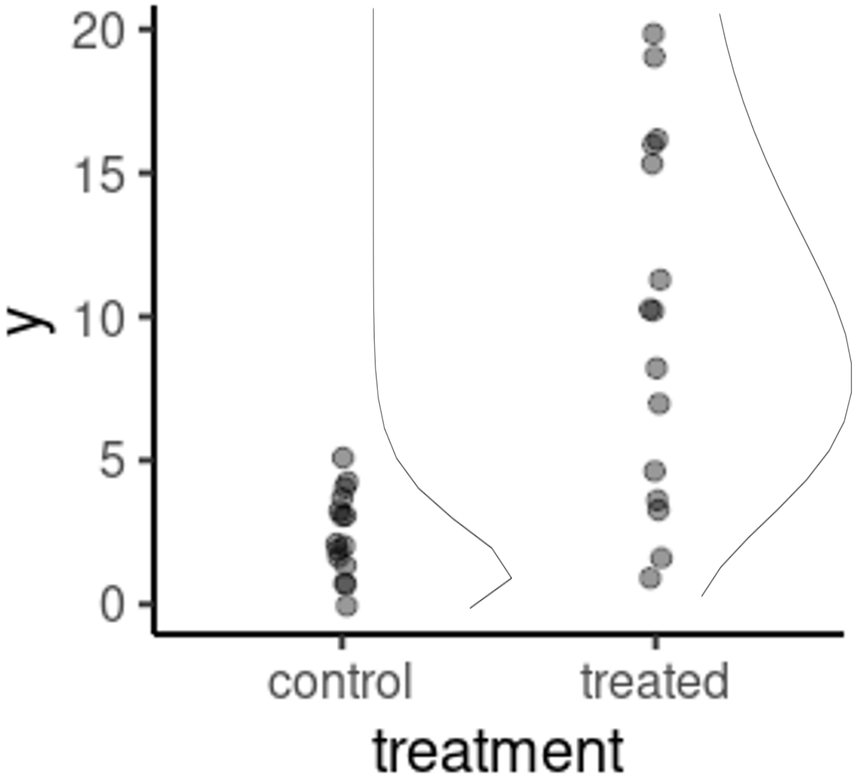
Linear Modeling

Model the expression of each gene as linear combination of explanatory factors (eg. treatment, age, sex, etc.)

$$Counts \sim NB(\mu, \phi)$$

$$\mu = sq$$

$$log2(q) = \beta_0 + \beta_1 * treatment + \beta_2 * age + \ldots$$



counts - expression of the gene

$\boldsymbol{\beta_i}$ - parameters we want to estimate from the data

$\boldsymbol{\beta_0}$ - the “intercept” (the value of expression when all other parameters are set at a reference level)

ϕ - the “dispersion” (uncertainty) of our model (also estimated from the data)

s - scaling factor (sequencing depth and transcript composition)

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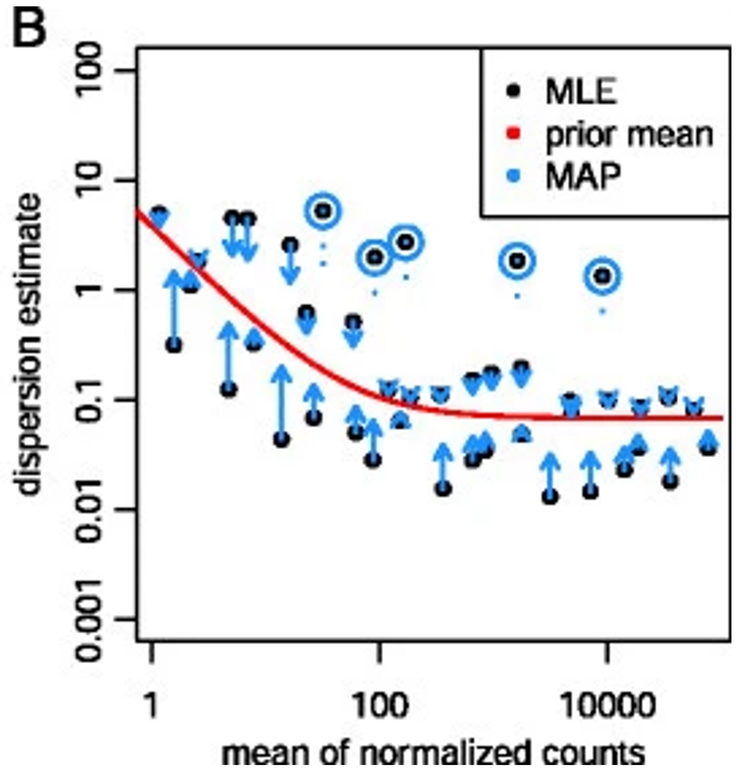

Statistical Aspects of Differential Expression Analysis

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Coefficients are estimated for each sample group along with their standard error.

The coefficients are the estimates for the **log2 fold-changes**, and will be used as input for hypothesis testing.

Statistical Aspects of Differential Expression Analysis

Linear Modeling

$$Counts \sim NB(\mu, \phi)$$

$$\mu = sq$$

$$log2(q) = \beta_0 + \beta_1 * treatment + \beta_2 * age + \dots$$

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s - scaling factor (sequencing depth and transcript composition)

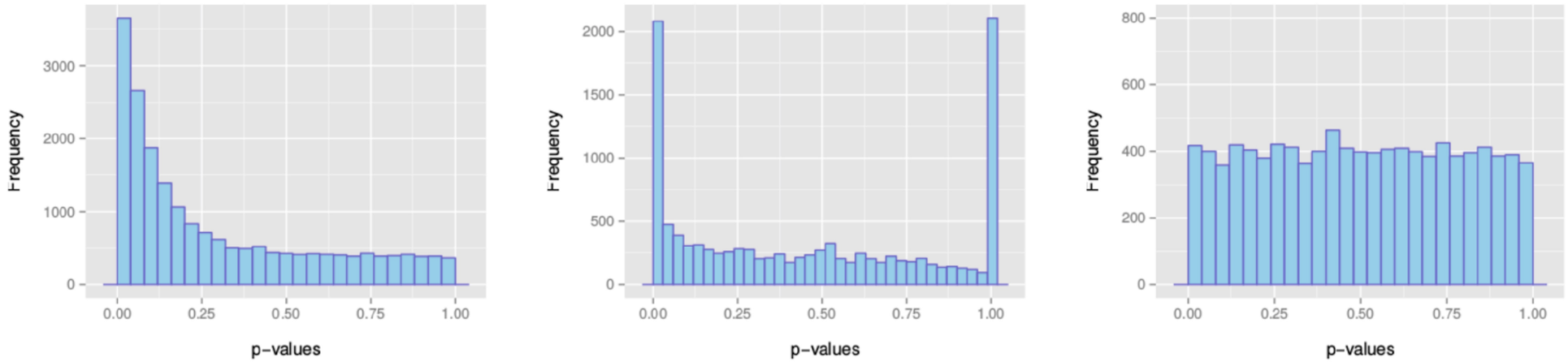
Summary:

- Use **negative binomial linear regression** to model gene expression in RNA-seq
- Calculate **size factors** for each sample to account for differences in sequencing depth and transcript composition between samples
- Estimate **dispersion** for each gene by “borrowing” information across genes for more precise estimates when sample sizes are small (as is typical in RNA-seq experiments)
- Estimate model **coefficients** which are used to define test hypothesis **($\boldsymbol{\beta_i}$ = 0)**

Description of DESeq2 model: Love, Huber & Anders (2014)

P-value Histograms

Examples of expected overall distribution



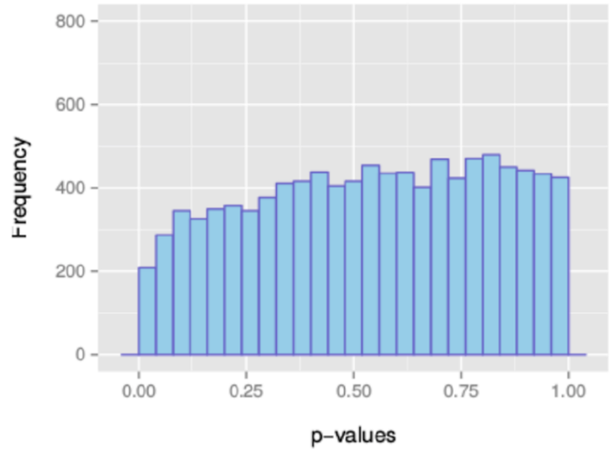
(a) : the most desirable shape

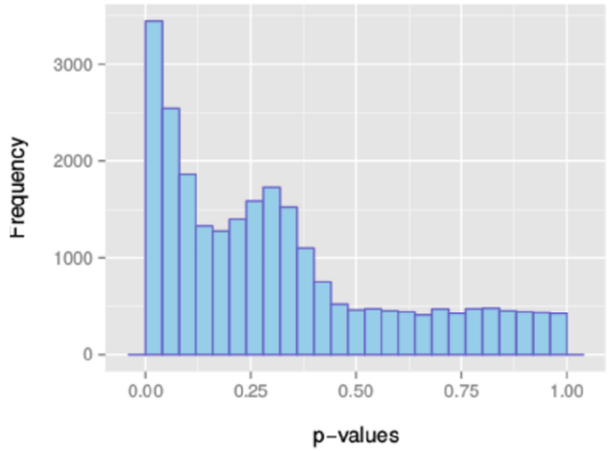
(b) : very low counts genes usually have large p-values

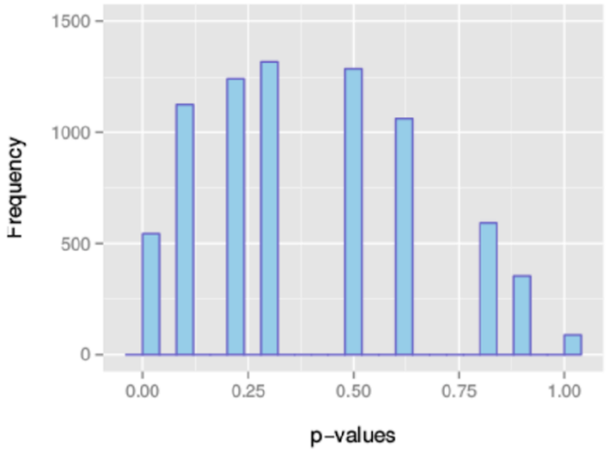
(c) : do not expect positive tests after correction

P-value Histograms

Examples of unexpected 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

Multiplicity Correction

- A gene with a significance cut-off of **α** = 0.05, means there is a 5% chance it is a false positive.
- If we test for 20,000 genes for differential expression at **α** = 0.05, we would expect to find 1,000 genes by chance
- If we found 3000 genes to be differentially expressed total, roughly one third of our genes are false positives!
- The more genes we test, the more we inflate the false positive rate. This is the multiple testing problem.

Multiplicity Correction

- Bonferroni: The adjusted p-value is calculated by: α^{k} (k = total number of tests). This is a very conservative approach
- FDR/Benjamini-Hochberg: Benjamini and Hochberg (1995) defined the concept of FDR and created an algorithm to control the expected FDR below a specified level given a list of independent p-values.

Conclusions

- Assumptions assumptions assumptions