Cube
Computational Systems Biology

Quantitative Methoden in der Molekularbiologie

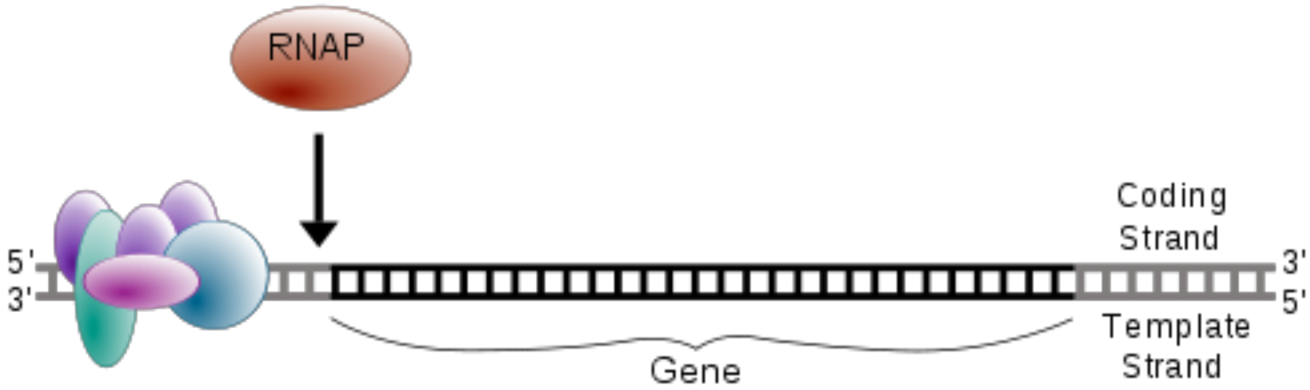
9. Quantification of nucleotide sequences (RT-PCR) and differential expression

WS 2015/16

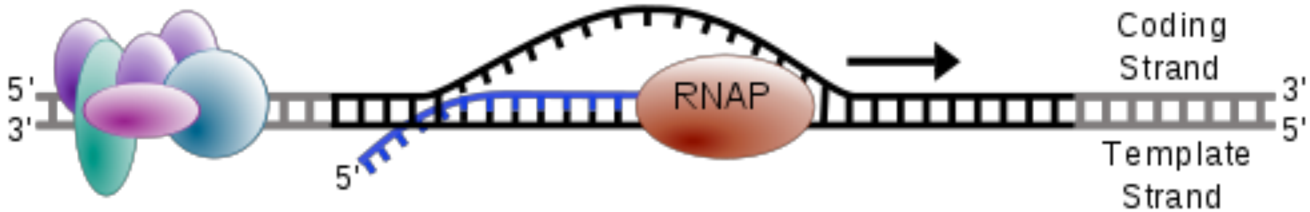
Outline

1. Differential expression: concept and definitions
2. Differential expression: statistical testing

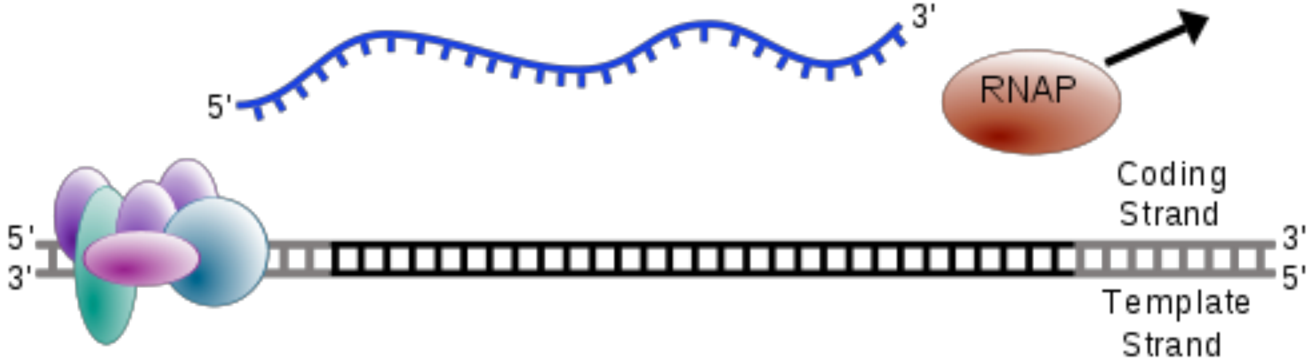
Gene expression (1)



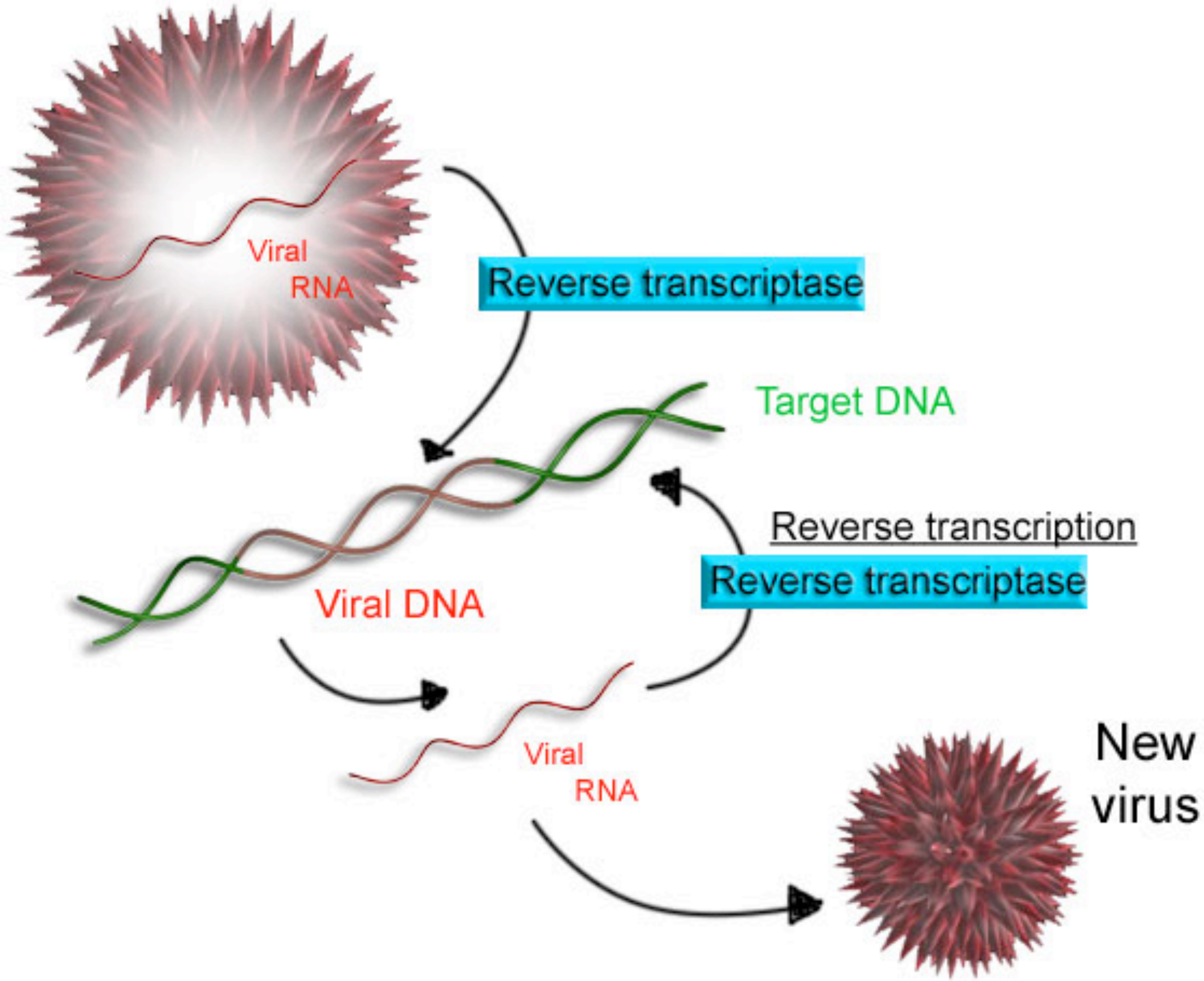
Gene expression (2)



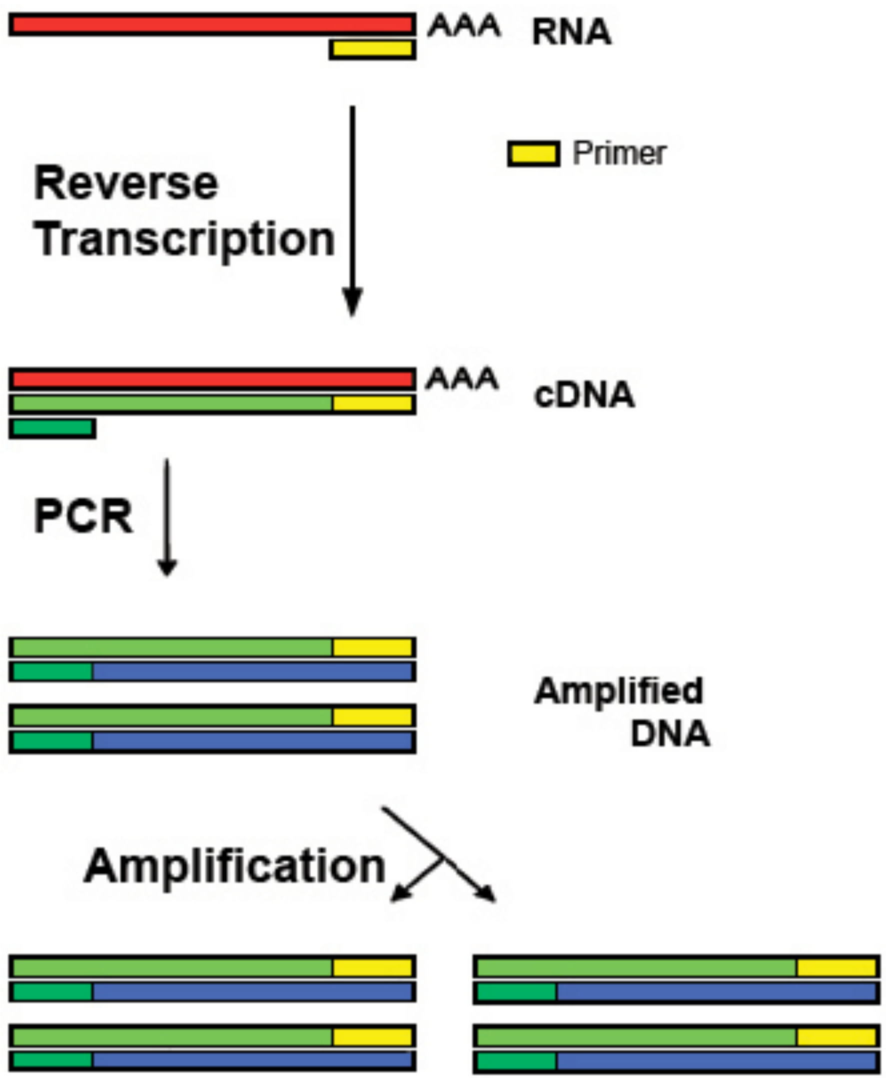
Gene expression (3)



Reverse transcription



Amplification of mRNA



Normalization for differences

Endogenous controls:

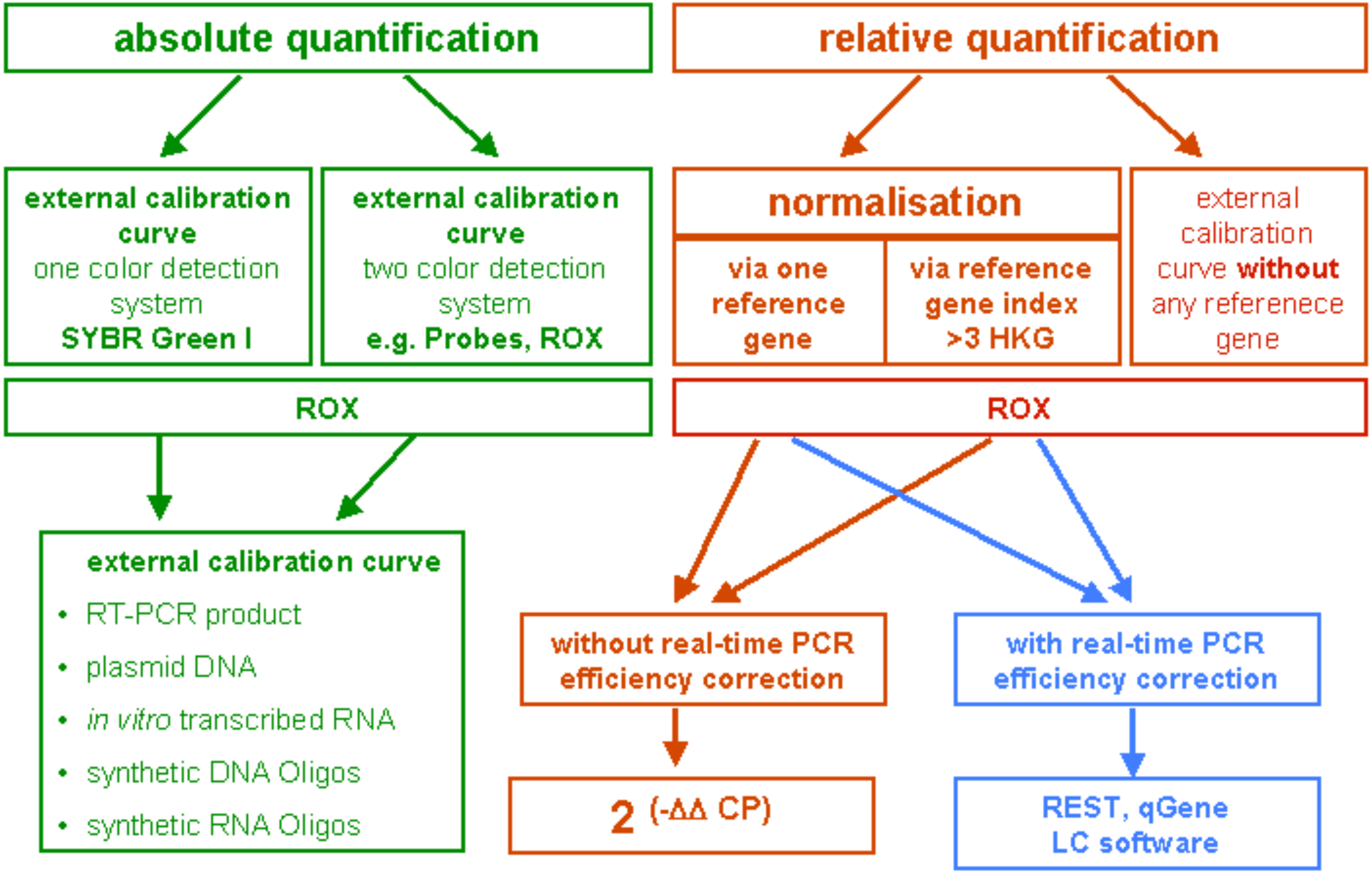
- Expressed at constant background level
- Not affected by condition(s) under investigation
- Housekeeping genes, e.g.:
  - RNaseP
  - rRNA
  - β-Actin

Quantification of target and control

- Separate assays (same machine)

- Multiplex reactions in same assay using differently labeled probes

Quantification strategies



M.W. Pfaffl Biospectrum 2004

Differential expression

Sample A:
- Target gene
- Control gene

Sample B:
- Target gene
- Control gene

Relative quantification: $\Delta\Delta C_T$

$\Delta C_T = C_{T,Target} - C_{T,Control}$

$\Delta C_{T,A} = C_{T,Target,A} - C_{T,Control,A}$

$\Delta C_{T,B} = C_{T,Target,B} - C_{T,Control,B}$

$\Delta\Delta C_T = \Delta C_{T,B} - \Delta C_{T,A}$

Relative Fold change $= (1+E)^{-\Delta\Delta C_T}$

$\Delta\Delta C_T$ Amplication efficiency

Requirements for endogenous controls:

- Should not be affected by treatment under investigation
- Must amplify with an efficiency equal to that of the target gene

Assessing efficiencies:

- Measuring ΔC_T values across dilutions (regression plot of ΔC_T vs. $\log_{10}$(concentration))
- General criterion:
  $\Delta\Delta C_T$ method valid if $|\text{slope}| < 0.1$

Outline

1. Differential expression: concept and definitions

2. Differential expression: statistical testing

Testing for differential expression

- Null hypothesis H_0:
  Results in our experiment are the consequences of pure chance! Experimental treatment has no effect!

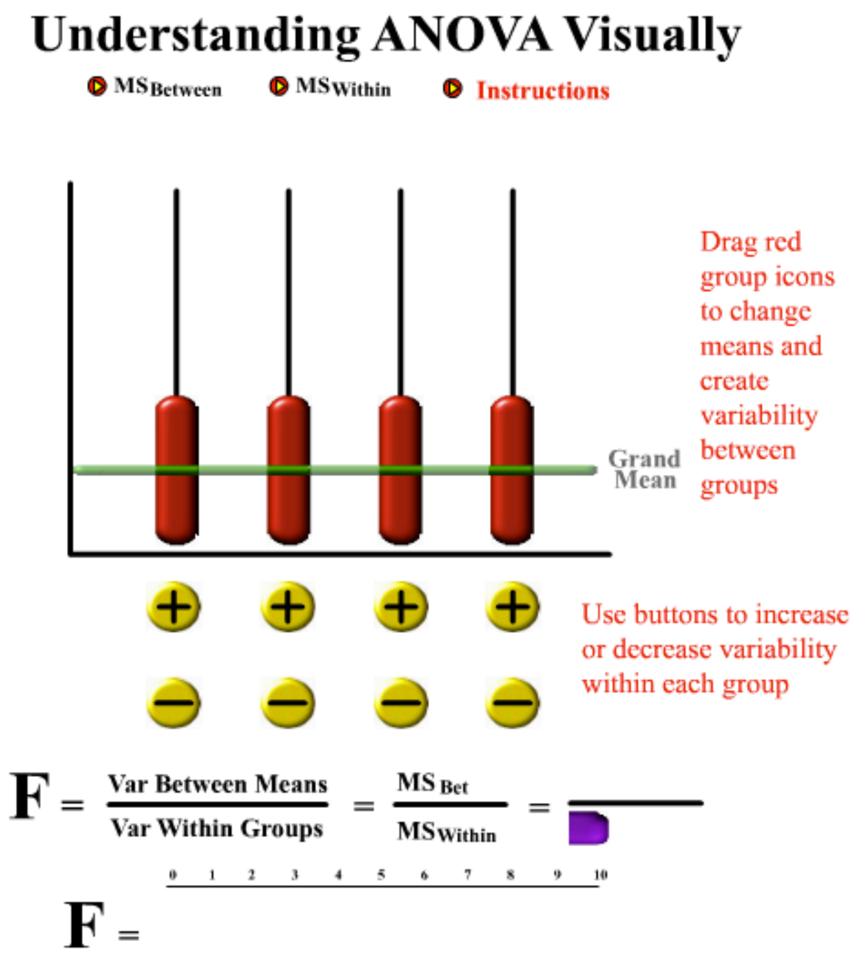
- Is H_0 true or false?

- Statistical testing!

Test concept

- If H0 is true, then the average result values (C_T, ΔC_T, $\Delta\Delta C_T$) should be the same.
- Comparing means from two groups (Samples A,B): t-Test
- Comparing means from more groups (Samples A,B, …): ANOVA (ANalysis Of VAriance)
- One different factor in Samples A,B, …:
  - One-Way ANOVA
- Multiple different factors in Samples A,B,…:
  - Multi-Way ANOVA

Visual ANOVA

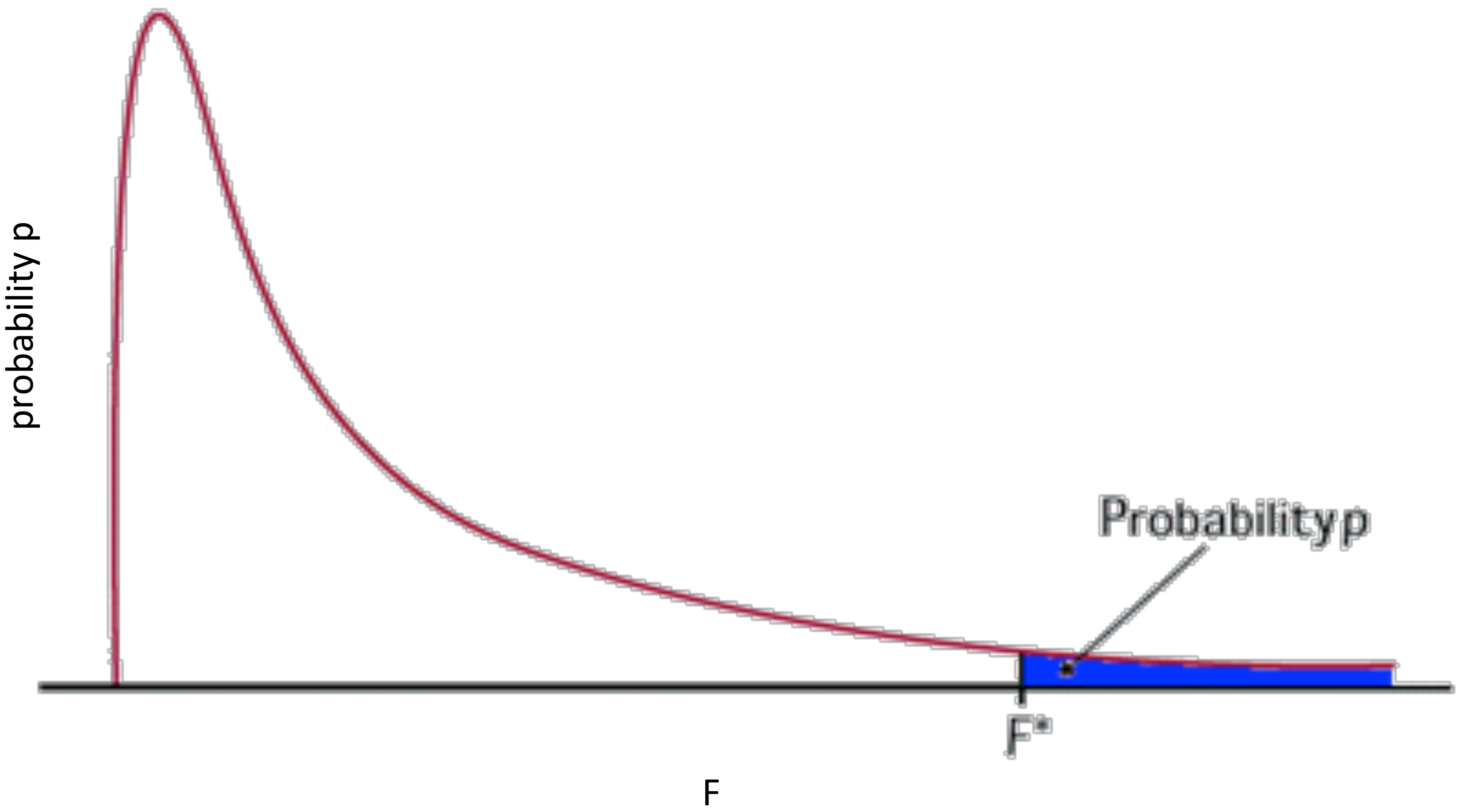


http://web.utah.edu/stat/introstats/anovaflash.html

One-Way ANOVA

- Number of groups: m
- Number of members per group: n
- Means within groups: M_a, M_b, M_c, …
- Mean of means: $M=(M_a + M_b + M_c + \ldots)/m$
- Variance within groups: $s^2{}_a$, $s^2{}_b$, $s^2{}_c$, …
- Average variance within all groups: s^2
- $s^{*2}=((M_a-M)^2+(M_b-M)^2+(M_c-M)^2+\ldots)/(m-1)$
- $F=n*s^{*2}/s^2$

F distribution



F distribution tables

- Critical F values for given level of significance
- Numerator $v_1 = df_n = m-1$
- Denominator $v_2 = df_d = m(n-1)$
- E.g.: http://stattrek.com/online-calculator/f-distribution.aspx

Expressing variation in results

Normally distributed values:

standard error $= \frac{s}{\sqrt{n}}$ (s: standard deviation; n: sample size)

margin of error $= Z * \frac{s}{\sqrt{n}}$ (Z: confidence level, e.g. 2.58 for 99%)

Error propagation:

- In addition and subtraction of quantities: new margin of error is square root of the sum of each margin of error squared.
- In multiplication and division of quantities: new margin of error is square root of the sum of each error divided by its quantity squared

$$\sqrt{moe_1^2 + moe_2^2 + \dots}$$

$$\sqrt{\left(\frac{moe_1}{quant_1}\right)^2 + \left(\frac{moe_2}{quant_2}\right)^2 + \dots}$$