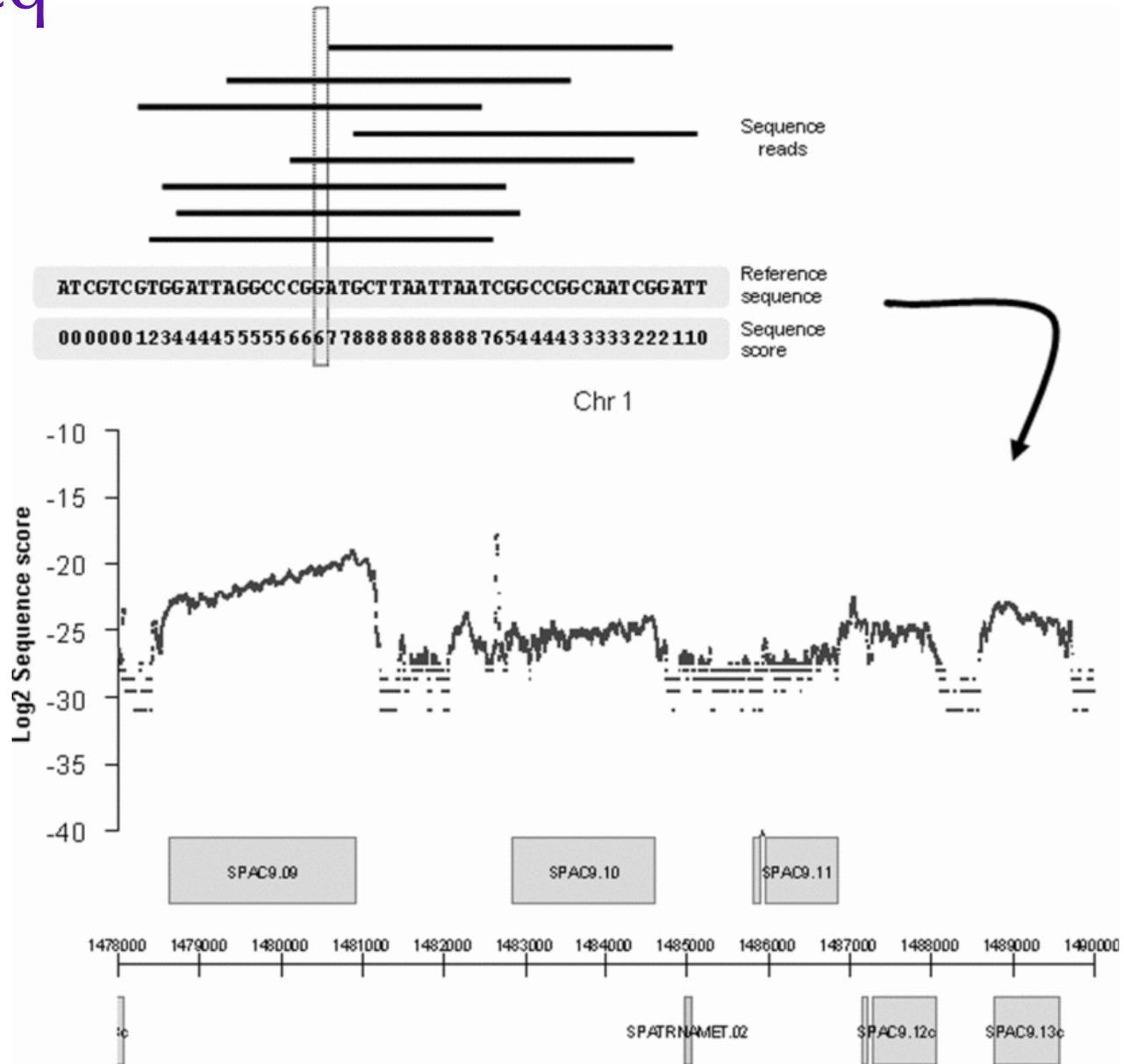


Differential expression analysis for sequencing count data

Simon Anders

RNA-Seq



Count data in HTS

- RNA-Seq
- Tag-Seq

Gene	GLiNS1	G144	G166	G179	CB541	CB660
13CDNA73	4	0	6	1	0	5
A2BP1	19	18	20	7	1	8
A2M	2724	2209	13	49	193	548
A4GALT	0	0	48	0	0	0
AAAS	57	29	224	49	202	92
AACS	1904	1294	5073	5365	3737	3511
AADACL1	3	13	239	683	158	40
[...]						

- ChIP-Seq
- Bar-Seq
- ...

Challenges with count data from HTS

discrete, positive, skewed

→ no (log-)normal model

small numbers of replicates

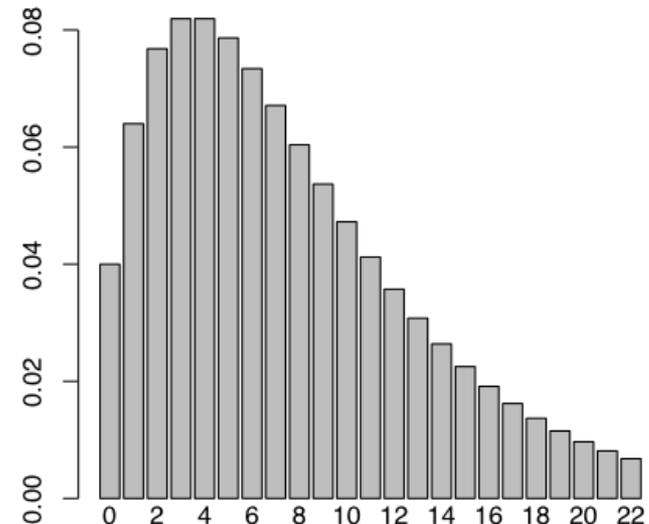
→ no rank based or permutation methods

sequencing depth (coverage) varies between samples

→ "normalisation"

large dynamic range (0 ... 10^5) between genes

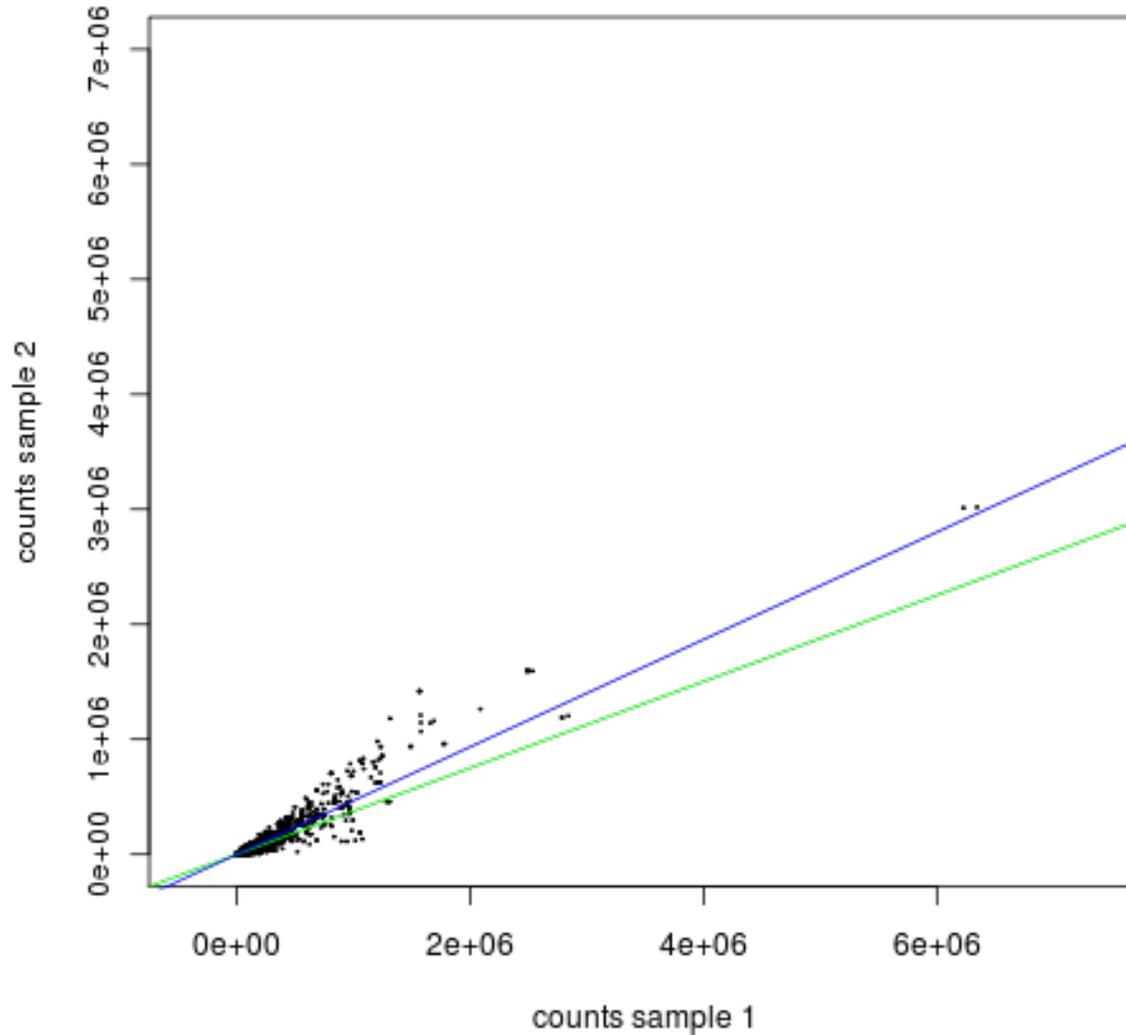
→ heteroskedasticity matters



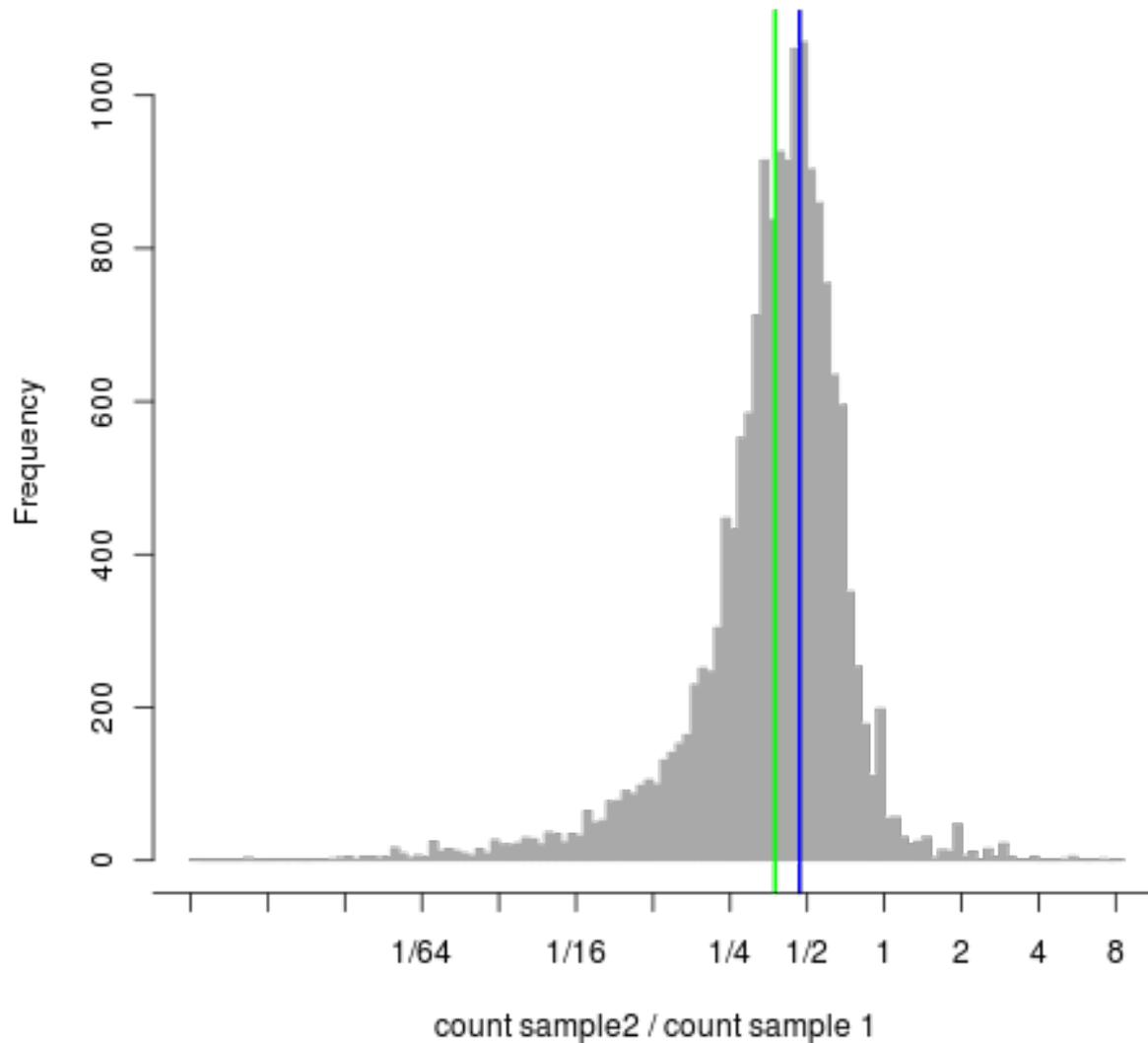
Normalisation for library size

- If sample A has been sampled deeper than sample B, we expect counts to be higher.
- Simply using the total number of reads per sample is not a good idea; genes that are strongly and differentially expressed may distort the ratio of total reads.
- By dividing, for each gene, the count from sample A by the count for sample B, we get one estimate per gene for the size ratio of sample A to sample B.
- We use the median of all these ratios.

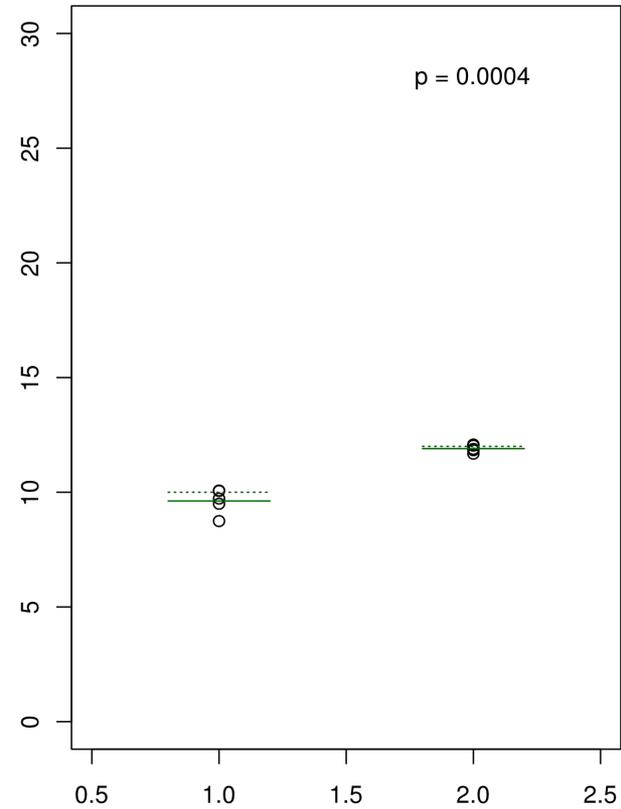
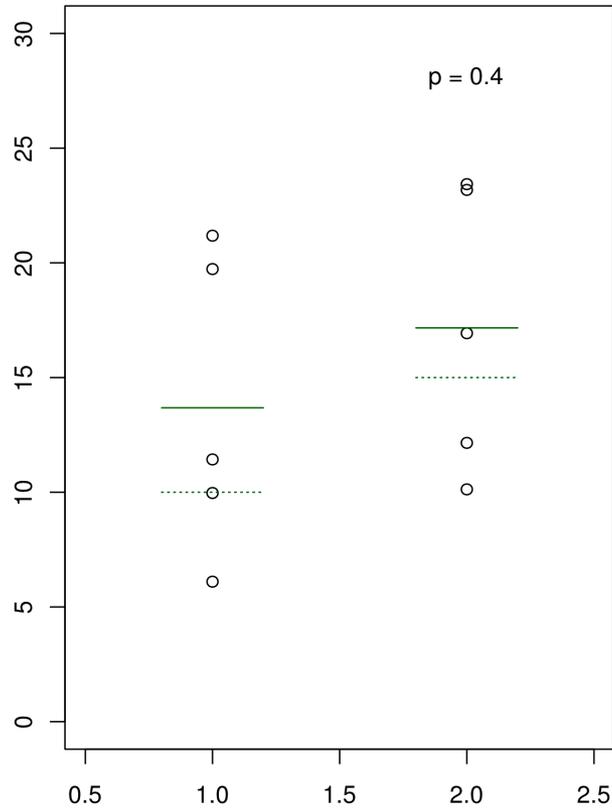
Normalisation for library size



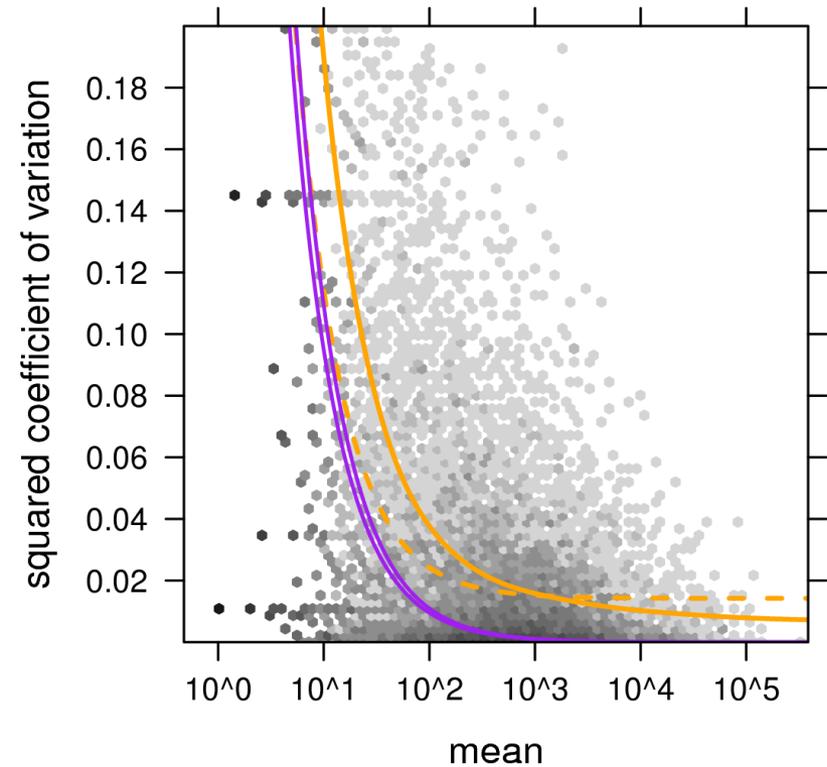
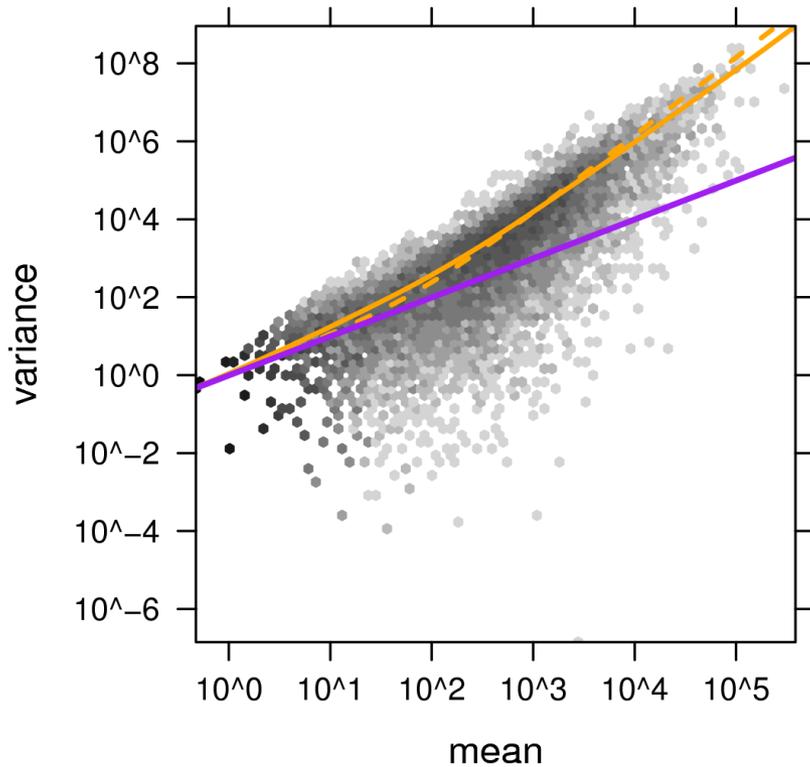
Normalisation for library size



Effect size and significance



Variance depends strongly on the mean



Variance calculated from comparing two replicates

Poisson

$$v = \mu$$



Poisson + constant CV

$$v = \mu + \alpha \mu^2$$

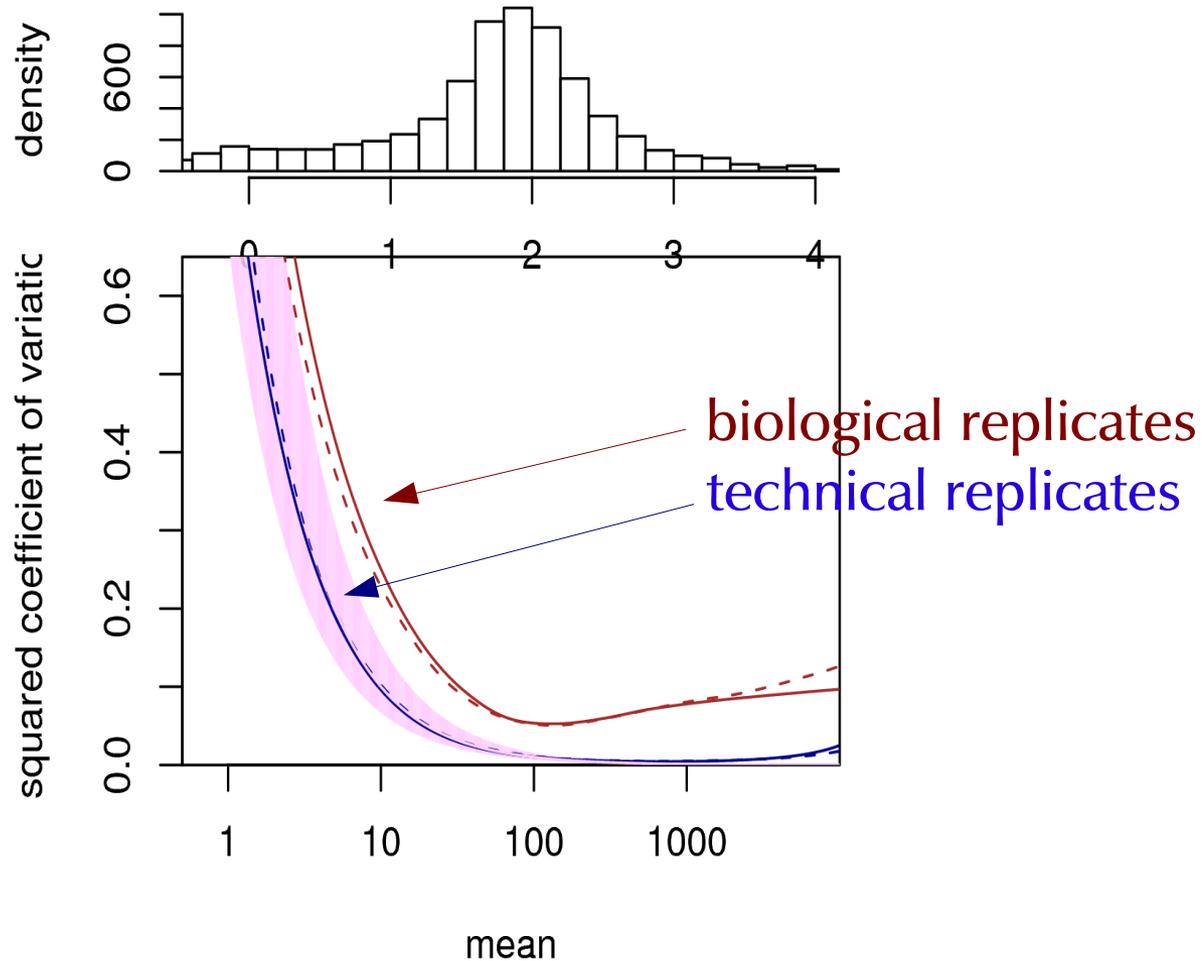


Poisson + local regression

$$v = \mu + f(\mu^2)$$

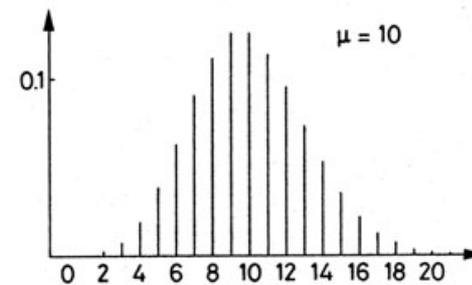
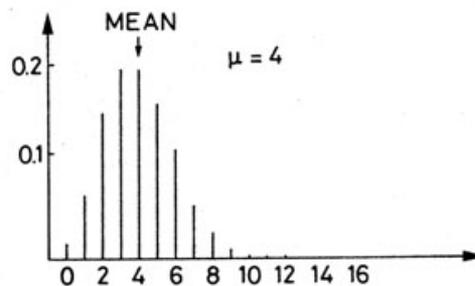
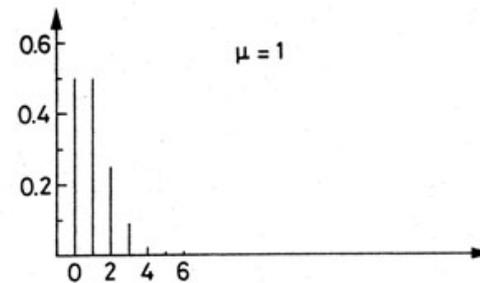
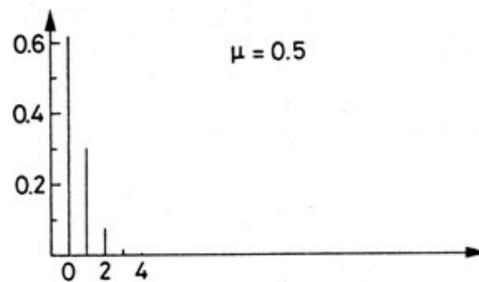


Technical and biological replicates



Poisson (I)

- The Poisson distribution turns up whenever things are counted
- Example: A short, light rain shower with r drops/m². What is the probability to find k drops on a paving stone of size 1 m²?



Poisson (II)

For Poisson-distributed data, the variance is equal to the mean.

Hence, no need to estimate the variance

according to several authors: Marioni et al. (2008), Wang et al. (2010), Bloom et al. (2009), Kasowski et al. (2010), Bullard et al. (2010)

- Really?

Is HTS count data Poisson-distributed?

To sort this out, we have to distinguish *two* sources of noise.

Shot noise

- Consider this situation:
 - Several flow cell lanes are filled with aliquots of the *same* prepared library.
 - The concentration of a certain transcript species is *exactly* the same in each lane.
 - We get the same total number of reads from each lane.
- For each lane, count how often you see a read from the transcript. Will the count all be the same?

Shot noise

- Consider this situation:
 - Several flow cell lanes are filled with aliquots of the *same* prepared library.
 - The concentration of a certain transcript species is *exactly* the same in each lane.
 - We get the same total number of reads from each lane.
- For each lane, count how often you see a read from the transcript. Will the count all be the same?
- Of course not. Even for equal concentration, the counts will vary. This *theoretically unavoidable* noise is called *shot noise*.

Shot noise

- Shot noise: The variance in counts that persists even if everything is exactly equal. (Same as the evenly falling rain on the paving stones.)
- Stochastics tells us that shot noise follows a *Poisson distribution*.
- The standard deviation of shot noise can be *calculated*: it is equal to the square root of the average count.

Sample noise

Now consider

- Several lanes contain samples from biological replicates.
- The concentration of a given transcript varies around a mean value with a certain standard deviation.
- This standard deviation cannot be calculated, it has to be *estimated* from the data.

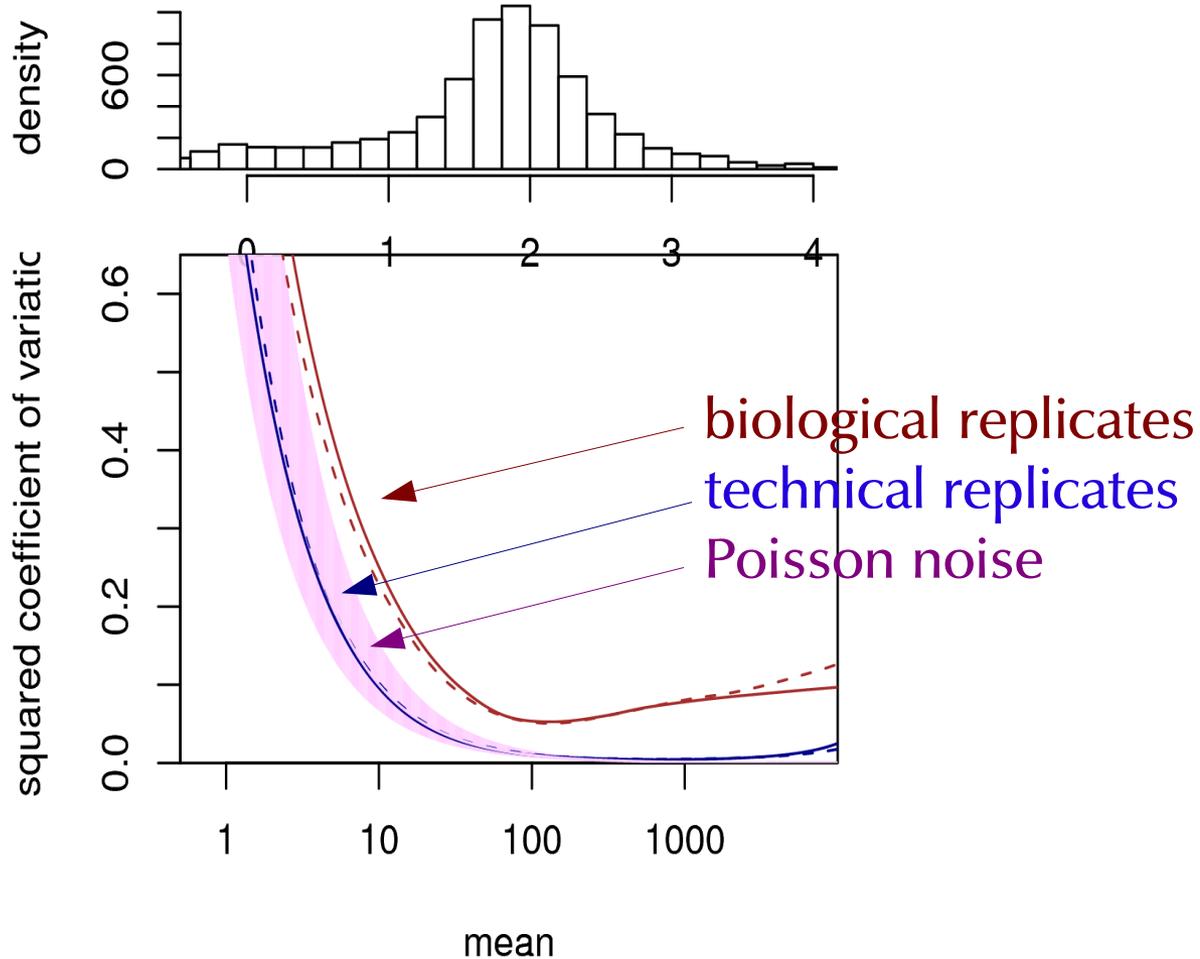
Technical and biological replicates

Nagalakshmi *et al.* (2008) have found that

- counts for the same gene from different *technical* replicates have a variance equal to the mean (Poisson).
- counts for the same gene from different *biological* replicates have a variance exceeding the mean (overdispersion).

Marioni *et al.* (2008) have looked confirmed the first fact (and confused everybody by ignoring the second fact).

Technical and biological replicates



Summary: Noise

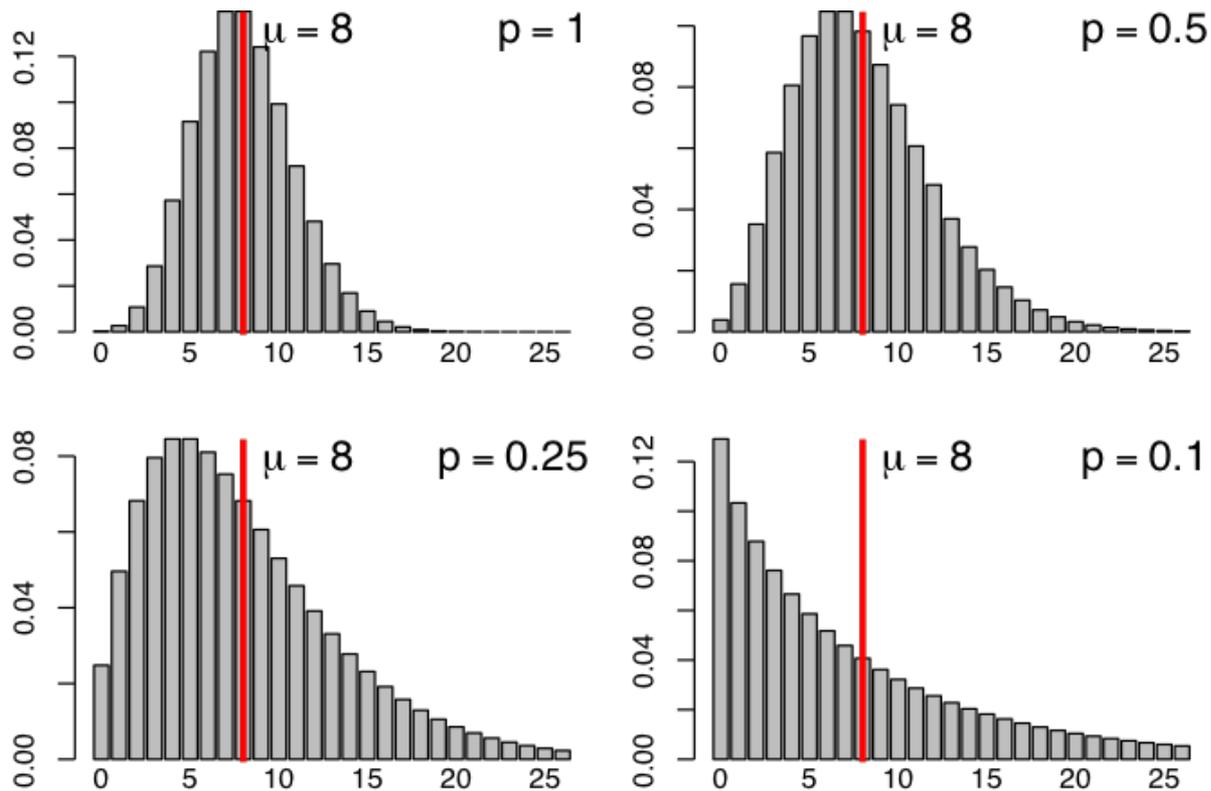
We distinguish:

- Shot noise
 - unavoidable, appears even with perfect replication
 - dominant noise for weakly expressed genes
- Technical noise
 - from sample preparation and sequencing
 - negligible (if all goes well)
- Biological noise
 - unaccounted-for differences between samples
 - Dominant noise for strongly expressed genes

can be computed
needs to be estimated from the data

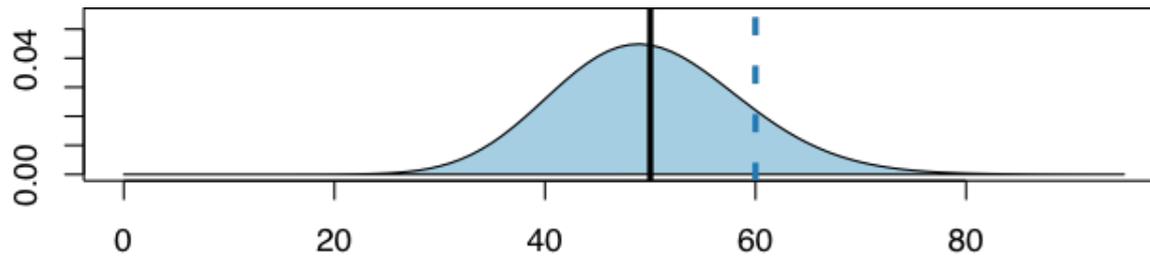
The negative-binomial distribution

A commonly used generalization of the Poisson distribution with *two* parameters

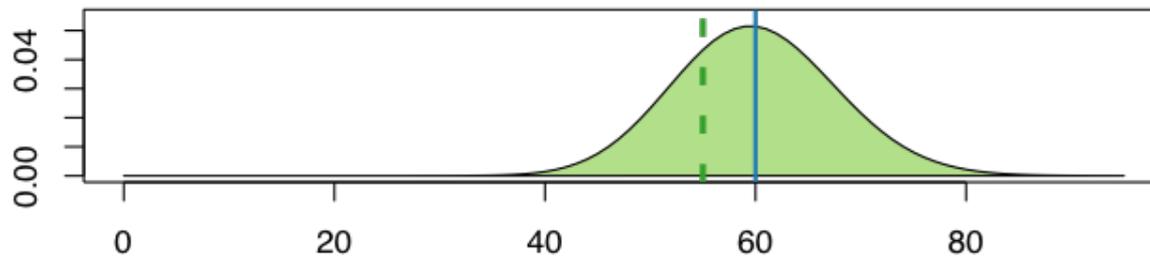


$$\Pr(Y = k) = \binom{k + r - 1}{r - 1} p^r (1 - p)^k \quad \text{for } k = 0, 1, 2, \dots$$

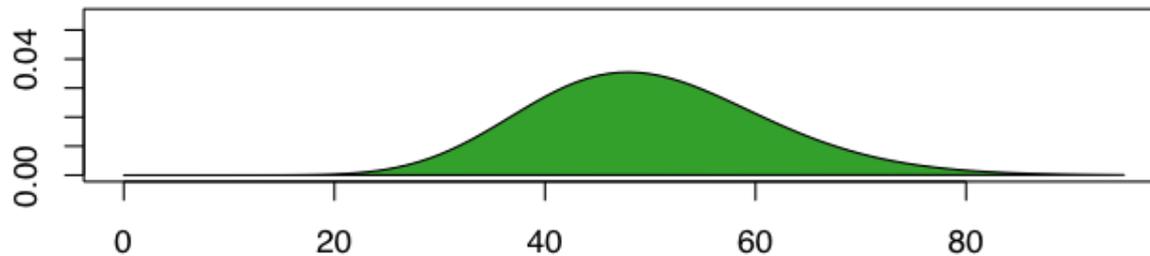
The NB distribution from a hierarchical model



Biological sample
with mean μ and
variance v



Poisson distribution
with mean q and
variance q .



Negative binomial
with mean μ and
variance $q+v$.

Testing: Null hypothesis

Model:

The count for a given gene in sample j come from negative binomial distributions with the mean $s_j \mu_\rho$ and variance $s_j \mu_\rho + s_j^2 v(\mu_\rho)$.

s_j relative size of library j
 μ_ρ mean value for condition ρ
 $v(\mu_\rho)$ fitted variance for mean μ_ρ

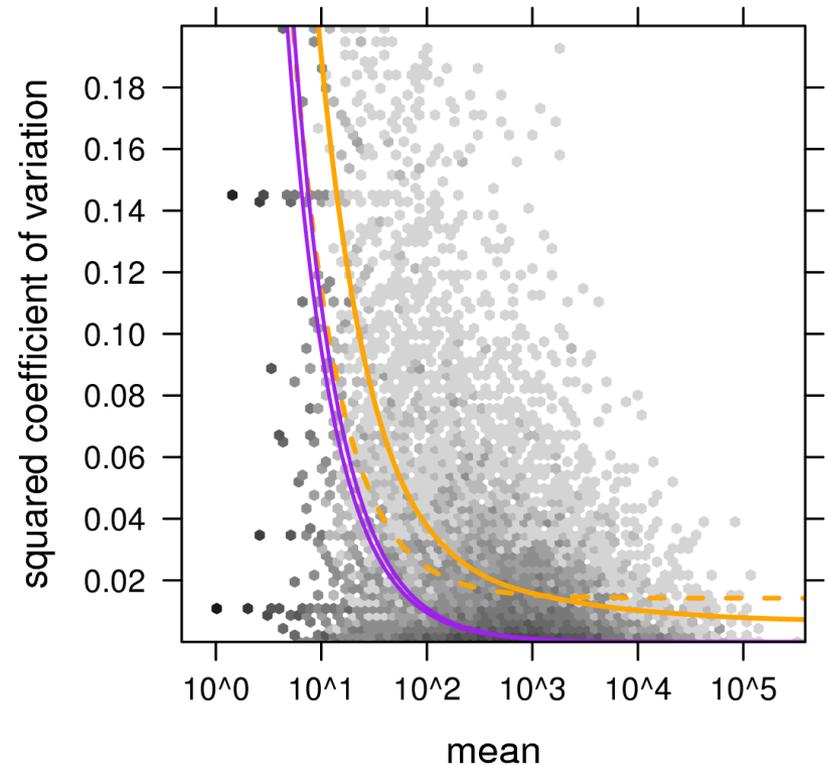
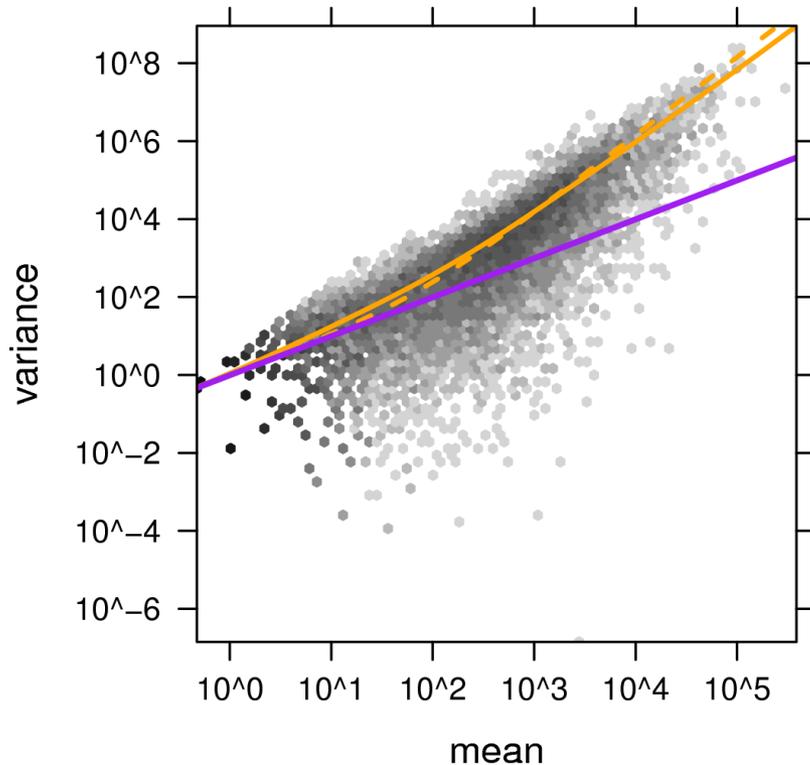
Null hypothesis:

The experimental condition r has no influence on the expression of the gene under consideration:

$$\mu_{\rho_1} = \mu_{\rho_2}$$

Model fitting

- Estimate the variance from replicates
- Fit a line to get the variance-mean dependence $v(\mu)$
(local regression for a gamma-family generalized linear model, extra math needed to handle differing library sizes)

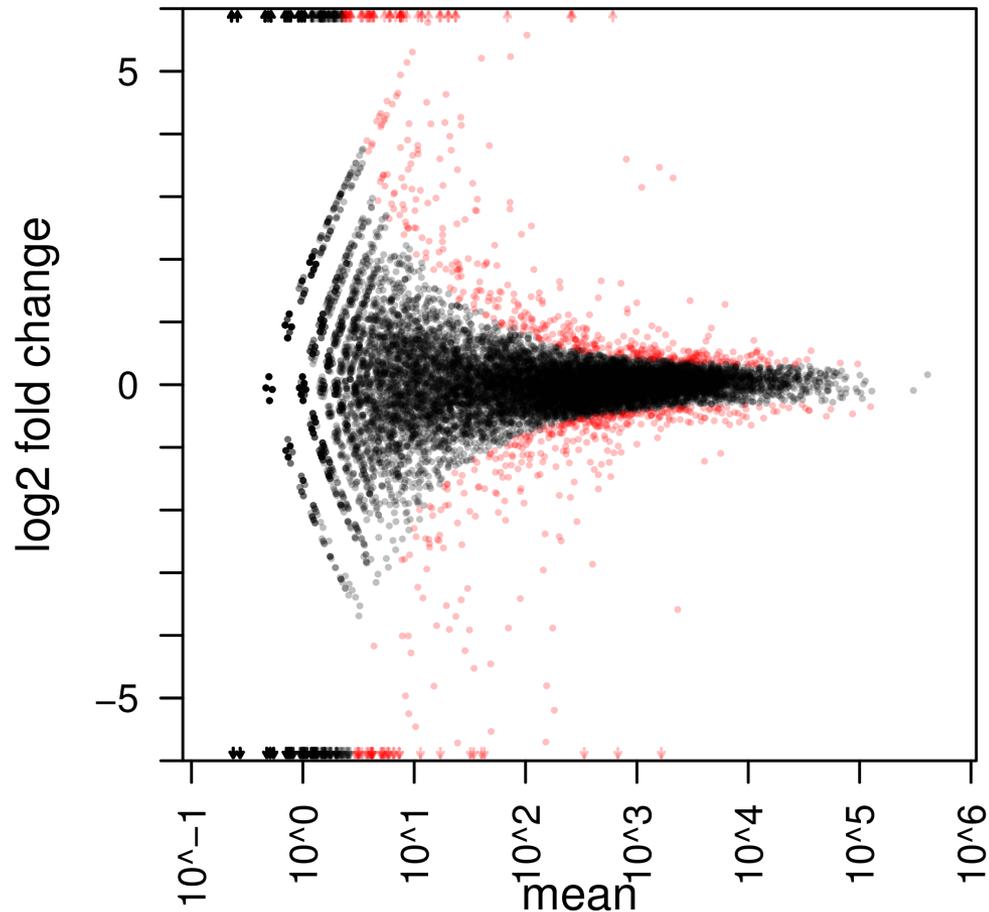


Testing for differential expression

- For each of two conditions, add the count from all replicates, and consider these sums K_{iA} and K_{iB} as NB-distributed with moments as estimated and fitted.
- Then, we calculate the probability of observing the actual sums or more extreme ones, conditioned on the sum being $k_{iA} + k_{iA'}$, to get a p value.

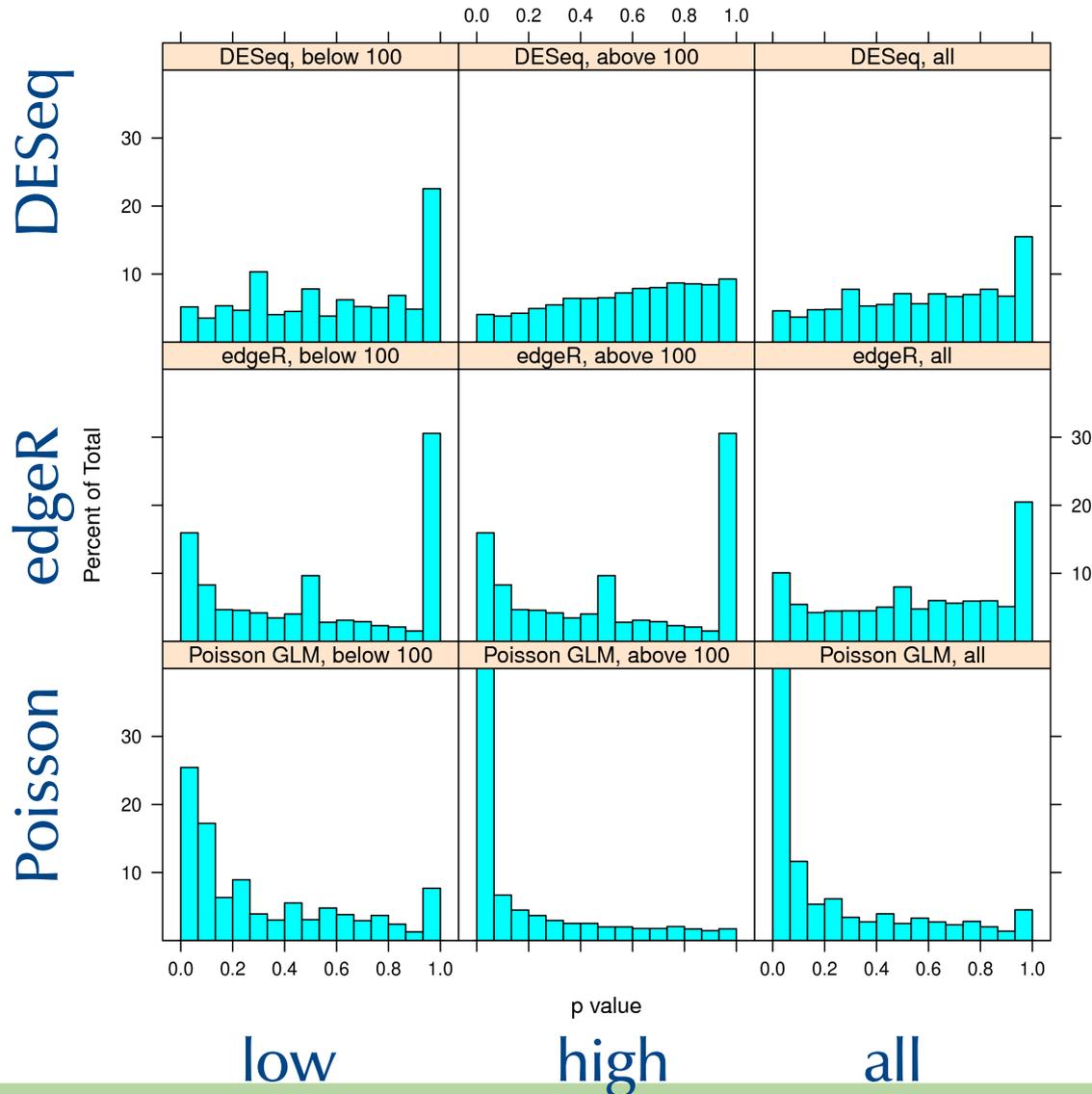
(similar to the test used in Robinson and Smyth's *edgeR*)

Differential expression



RNA-Seq data: overexpression of two different genes in flies [data: Furlong group]

Type-I error control

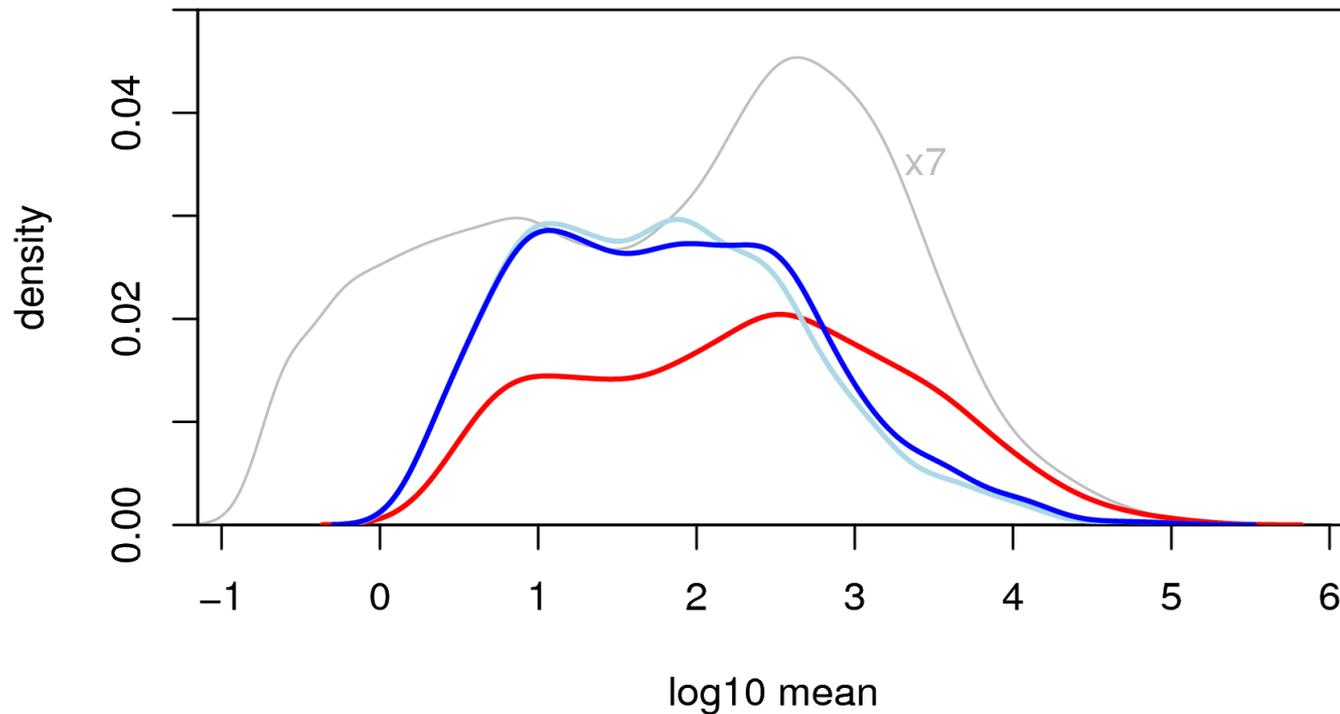


Comparison of replicates:

no differential expression,

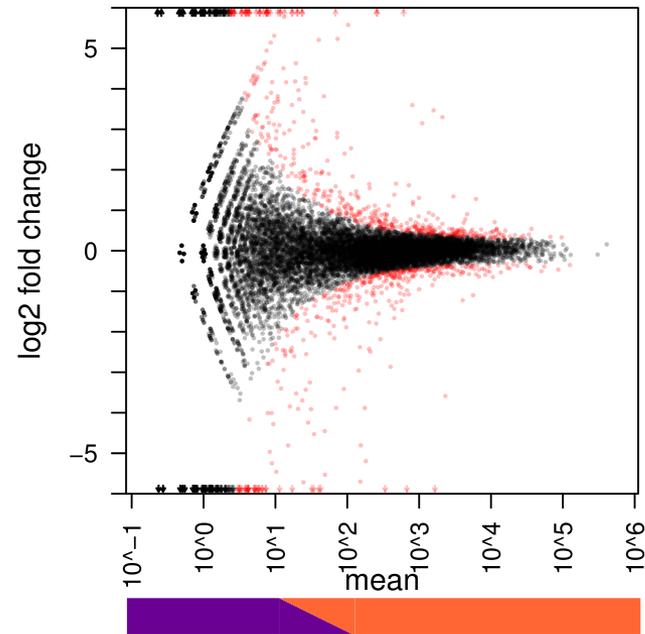
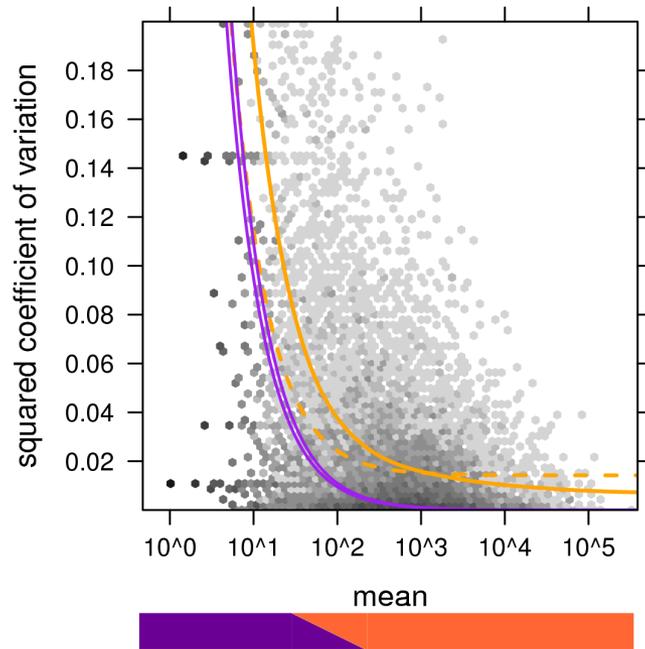
expect uniform p values

Distribution of hits along the dynamic range



- all genes
- differentially expressed according to DESeq
- differentially expressed according to edgeR

Two noise ranges



dominating noise



shot noise (Poisson)



biological noise

How to improve power?

deeper sampling

more biological replicates

Alternative splicing

- So far, we counted reads in *genes*.
- To study alternative splicing, reads have to be assigned to *transcripts*.
- This introduces ambiguity, which adds uncertainty.
- Current tools (e.g., *cufflinks*) allow to quantify this uncertainty.
- However: To assess the significance of differences to isoform ratios between conditions, the assignment uncertainty has to be combined with the noise estimates.
- This is not yet possible with existing tools.

Working without replicates

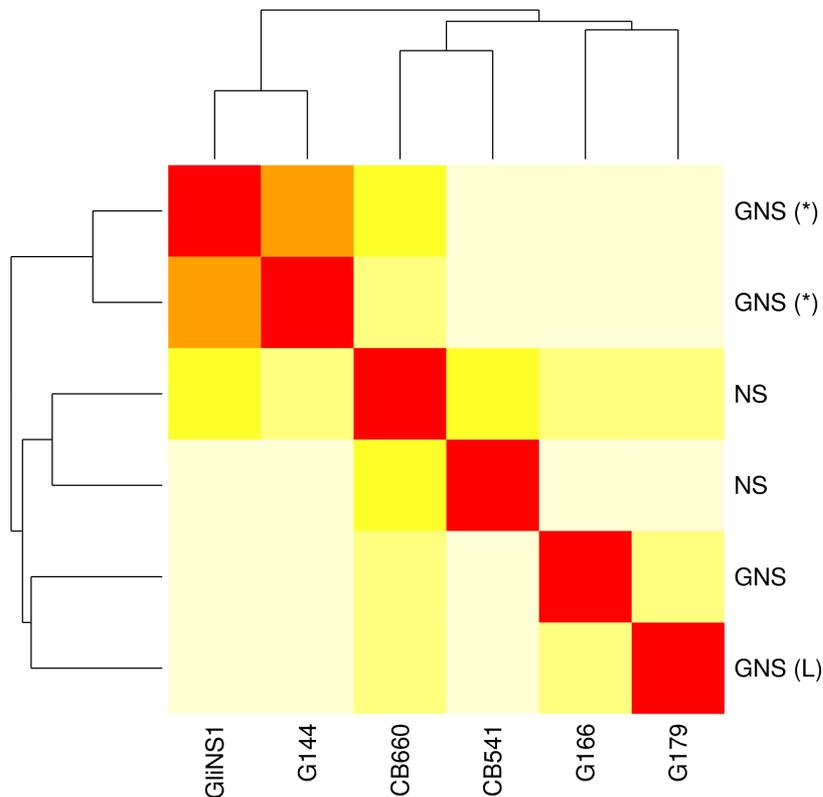
One can infer the variance from a comparison of different conditions.

- The variance will be overestimated, maybe drastically.
- The power is smaller, maybe much smaller.

Still, this is the best one can do without replicates.

Variance-stabilizing transformation

The estimated variance-mean dependence allows to derive a transformation that renders the count data approximately homoskedastic.



This is useful, e.g., as input for the `dist` function.

[Tag-Seq of neural stem cell tissue cultures, Bertone Group]

Further use cases

Similar count data appears in

- comparative ChiP-Seq
- barcode sequencing
- ...

and can be analysed with *DESeq* as well.

Conclusions

- Proper estimation of variance between *biological* replicates is vital. Using Poisson variance is incorrect.
- Estimating variance-mean dependence with local regression works well for this purpose.
- The negative-binomial model allows for a powerful test for differential expression
- Preprint on *Nature Precedings*:
“Differential expression analysis for sequence count data”
- Software (*DESeq*) available from Bioconductor and EMBL web site.

Google for
DESeq



- Co-author: Wolfgang Huber
- Funding: European Union (Marie Curie Research and Training Network “Chromatin Plasticity”) and EMBL

Advertisement

HTSeq

A Python package to process
and analyse HTS data

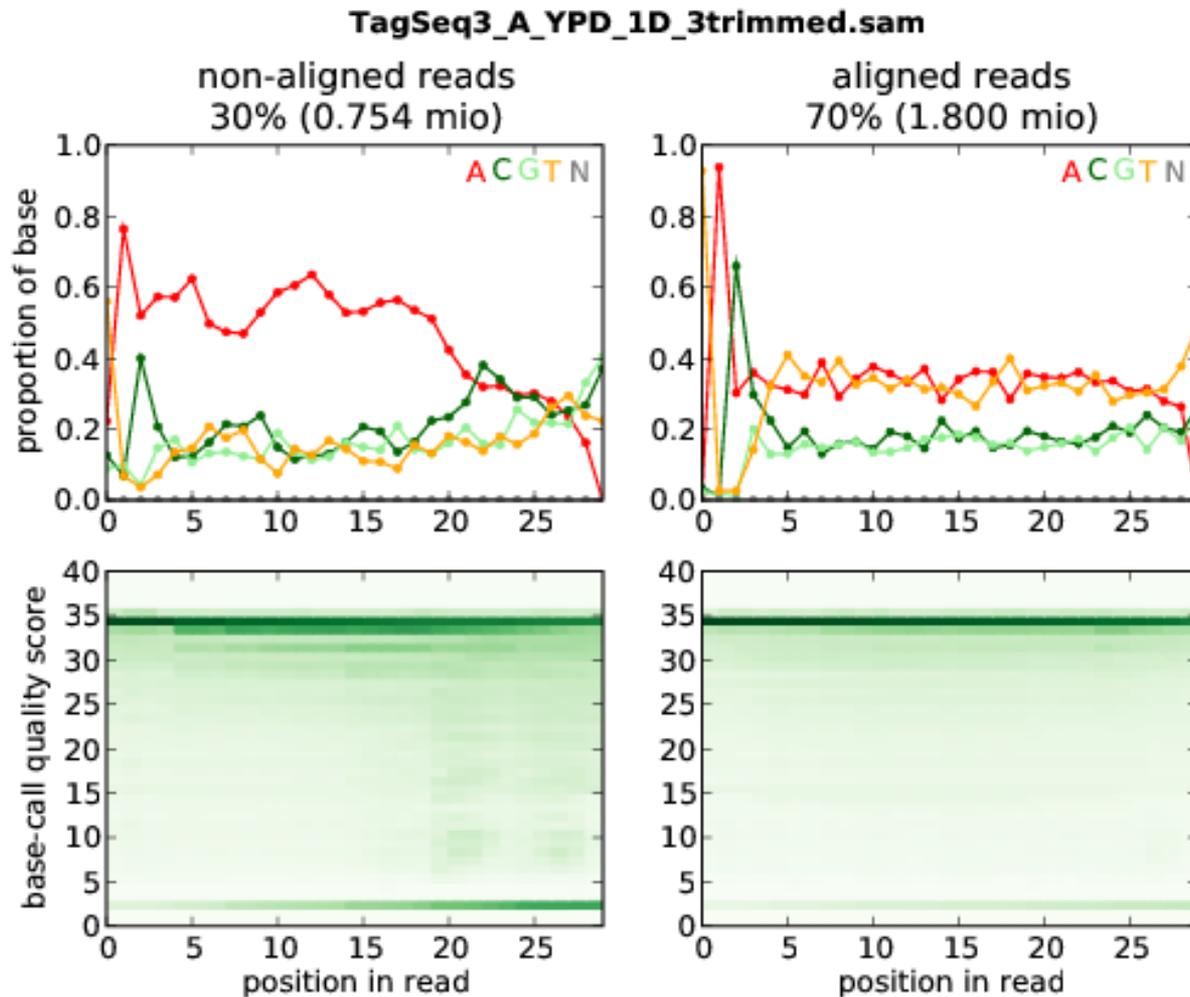
HTSeq: Features

- A framework to process and analyse high-throughput sequencing data with Python
- Simple but powerful interface
- Functionality to read, statistically analyse, transform sequences, reads, alignment
- Convenient handling of position-specific data such as coverage vectors, or gene and exon positions
- Well documented, with examples for common use cases.
- In-house support

HTSeq: Typical use cases

- Analyse base composition and quality scores for quality assessment of a read
- Trim of adapters in snRNA-Seq
- Calculate coverage vectors for ChIP-Seq
- Assign reads to genes to get count data from RNA-Seq (incl. handling of spliced reads, overlapping genes, ambiguous maps, etc.)
- Split reads according to multiplex tags
- etc.

Quality assessment with HTSeq



HTSeq: Availability

- HTSeq is available from <http://www-huber.embl.de/users/anders/HTSeq>
- Testers wanted

Negative-binomial model (I)

- Suppose, we have m replicates of a given condition, and obtain counts for n genes.
- The concentration of gene i in replicate j is a random variable Q_{ij} , which is i.i.d. for $j=1, \dots, m$ with mean q_{i0} and variance σ_i^2 .
- Let K_{ij} be the count value for gene i in replicate j . Its expectation value is $s_j \mu_i$ with size factor s_j .
- Given $Q_{ij} = q_{ij}$, the sequencing is a Poisson process and hence: $K_{ij} \sim \text{Pois}(s_j q_{ij})$.

Negative-binomial model (II)

- If Q_{ij} has mean μ_i and variance σ_i^2 , what is the marginal (“mixing”) distribution of $K_{ij} \sim \text{Pois}(s_j q_{ij})$?
- If one assumes Q_{ij} to be gamma-distributed, the answer is:
- K_{ij} follows a negative binomial (NB) distribution with mean $s_j q_{i0}$ and variance $s_j q_{i0} + s_j \sigma_i^2$.

Model fitting

- Estimate relative library sizes s_j .
- Within a set of replicates, calculate for each gene sample mean and sample variance of k_{ij}/s_j .
- To get an unbiased estimate of σ_i^2 , subtract an “average shot-noise” of $\frac{\hat{q}_i}{m} \sum_j \frac{1}{\hat{s}_j}$.
- Fit a line through the graph of mean and variance estimates (with a gamma-family local regression).

Model:

K_{ij} follows a negative binomial (NB) distribution with mean $s_j q_{i0}$ and variance $s_j q_{i0} + s_j \sigma_i^2$.

Diagnostic plot for variance fit

Residuals ECDF plot for condition 'A'

