Differential expression analysis for sequencing count data

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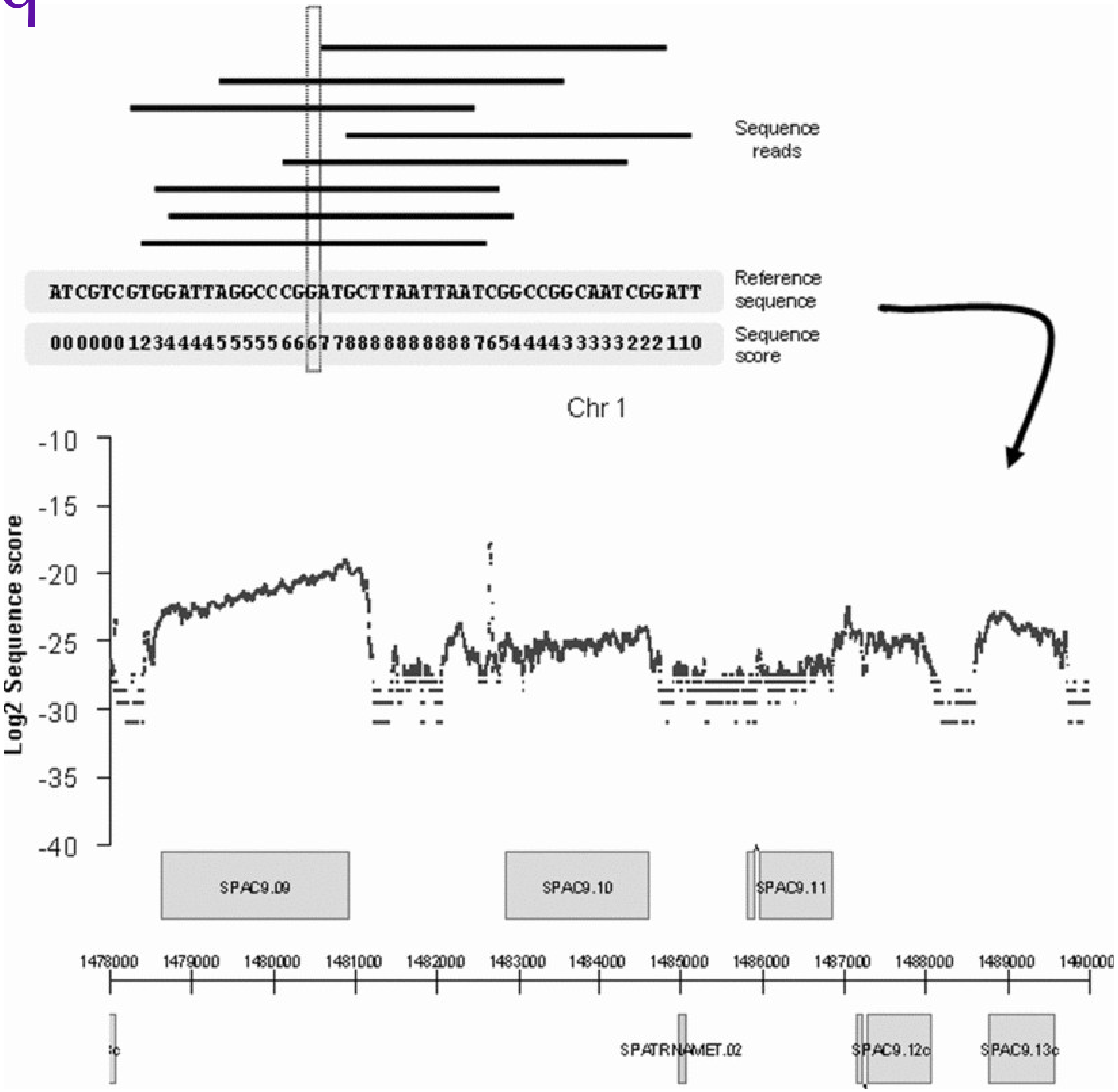
RNA-Seq


Sequence reads
ATCGTCGTGGATTAGGCCCGGATGCTTAATTAATCGGCCGGCAATCGGATT
Reference sequence
000000123444455555666778888888888765444433333222110
Sequence score
Chr 1
Log2 Sequence score
-10
-15
-20
-25
-30
-35
-40
SPAC9.09
SPAC9.10
SPAC9.11
1478000
1479000
1480000
1481000
1482000
1483000
1484000
1485000
1486000
1487000
1488000
1489000
1490000
SPATRNAMET.02
SPAC9.12c
SPAC9.13c

Count data in HTS

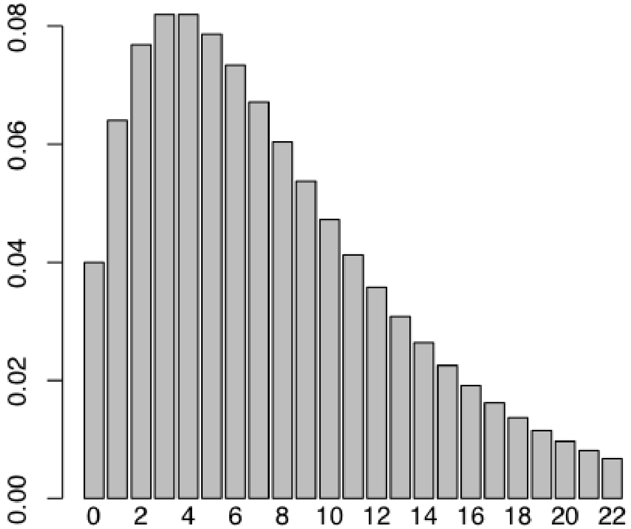
- RNA-Seq
- Tag-Seq

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

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Bioconductor packages

Bioconductor packages for testing for differential signal in sequencing count data:

- Based on negative-binomial distribution:
  - edgeR (Robinson, Mcarthy, Smyth)
  - DESeq (Anders, Huber)
  - BaySeq (Hardcastle, Kelly)

- Based on Poisson distribution:
  - DEGSeq (Wang et al.)

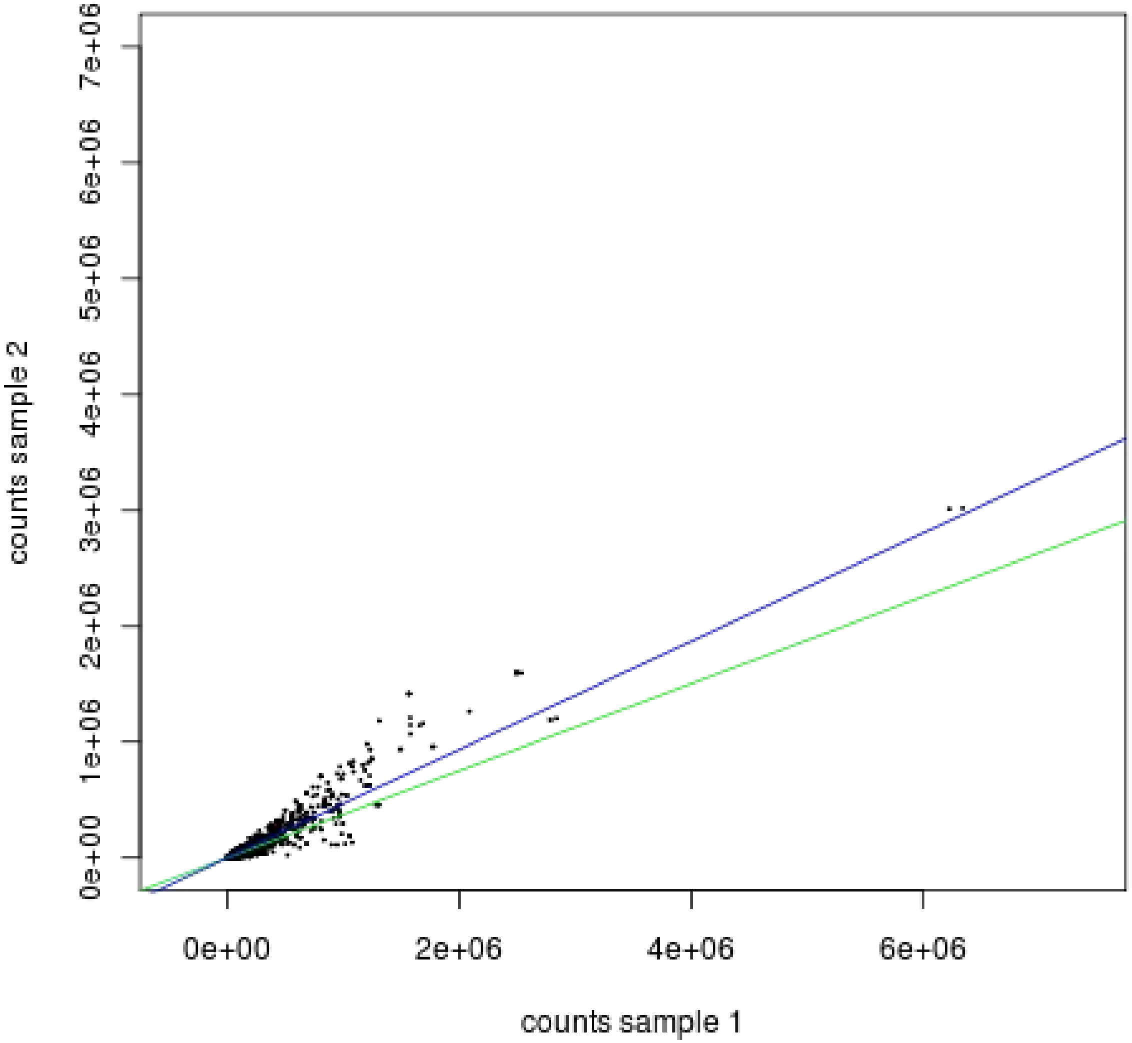
Normalisation for library size

- 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 or sample A to sample B.
- We use the median of all these ratios.

Normalisation for library size



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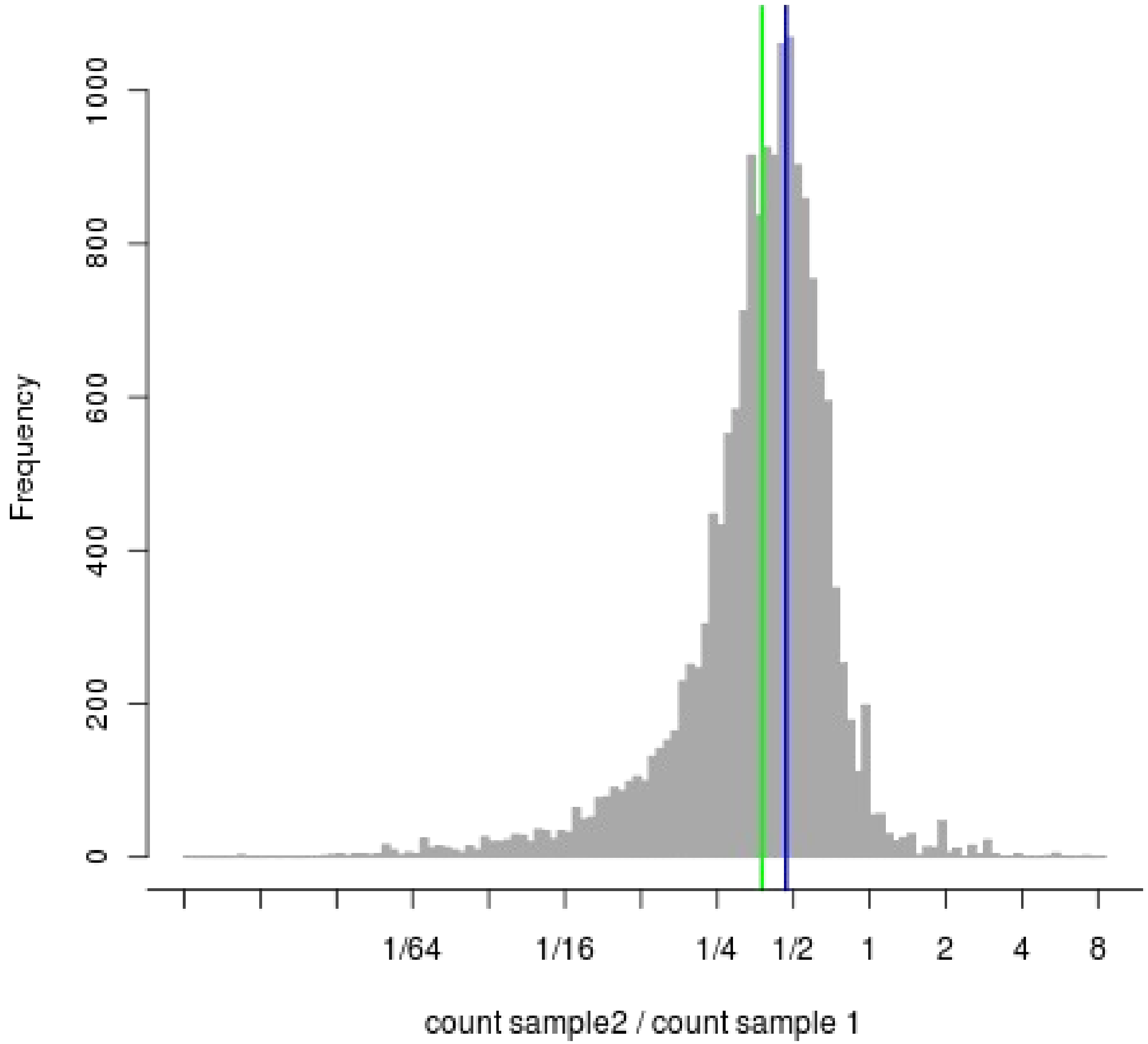
Normalisation for library size



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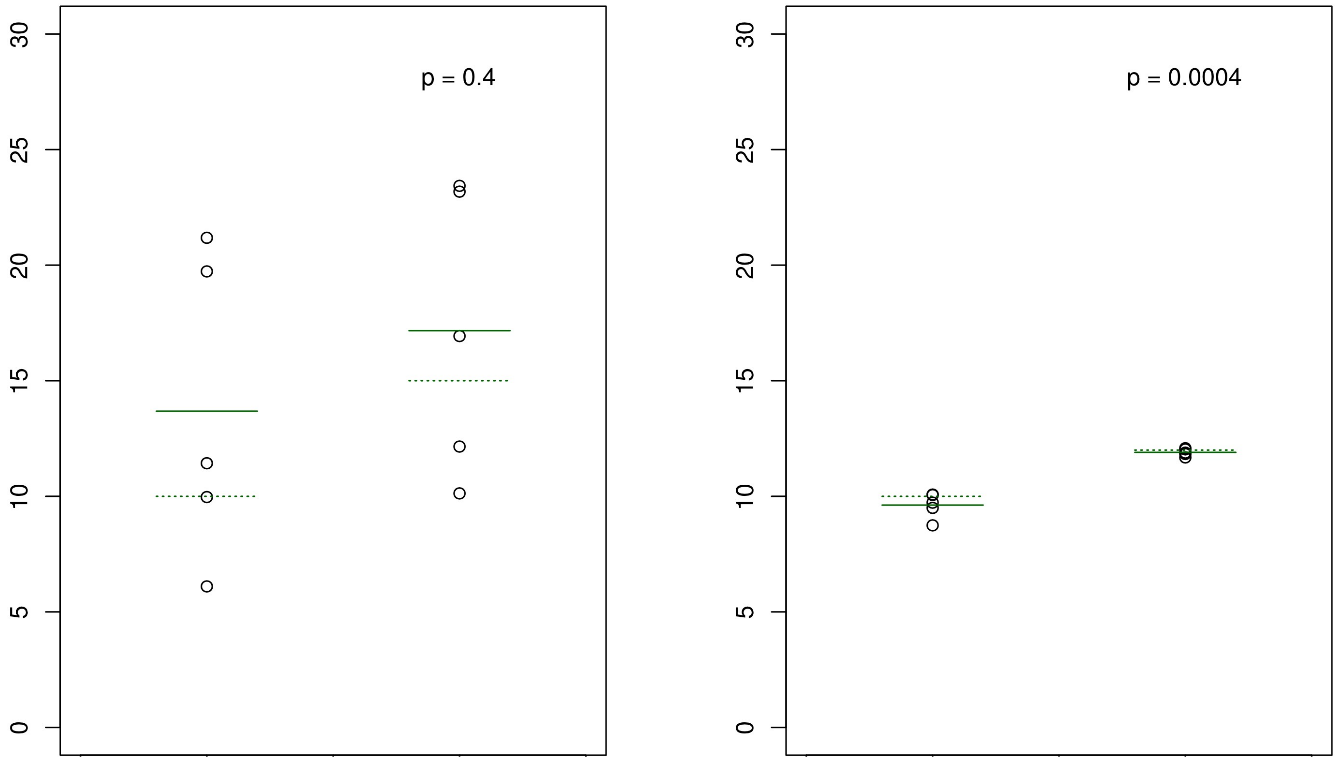
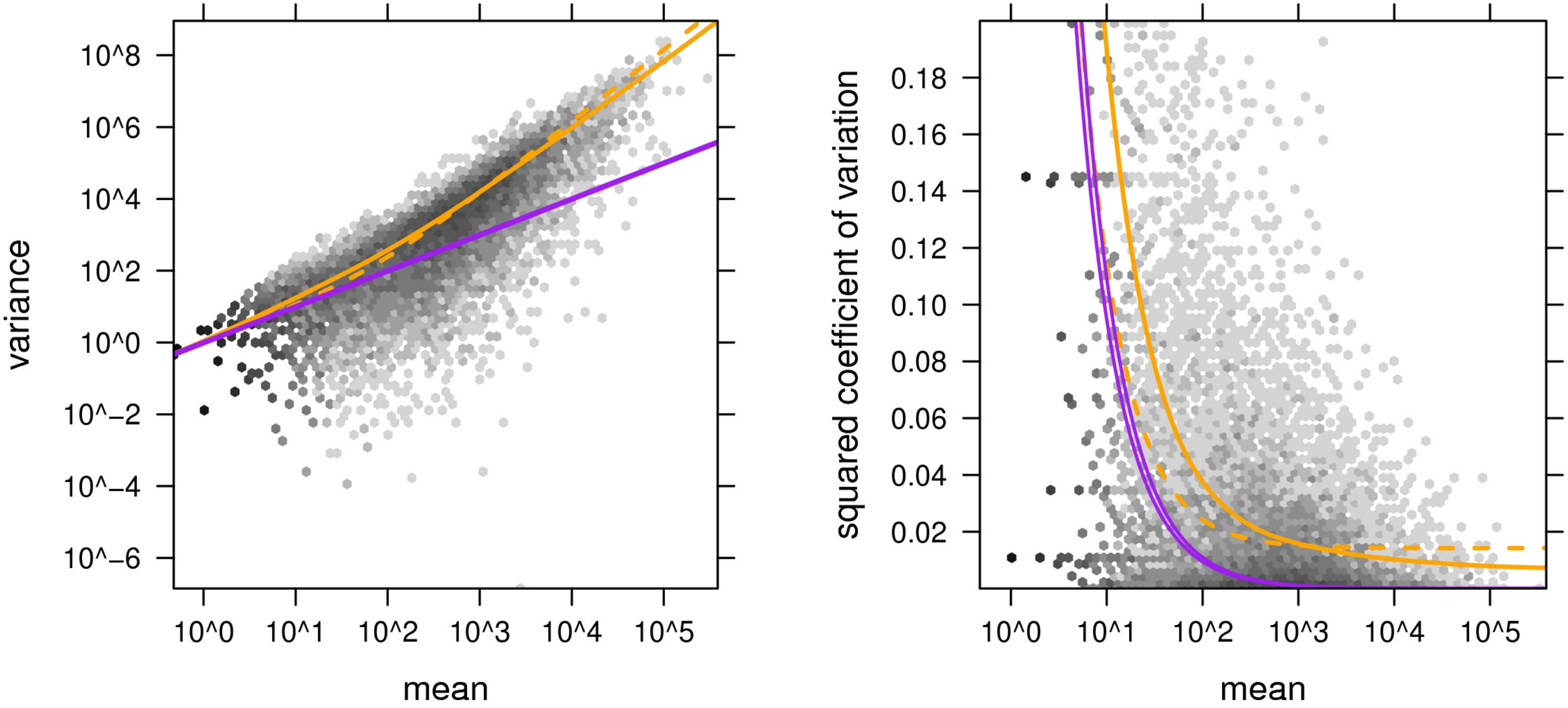
Effect size and significance



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	$v = \mu + f(\mu^2)$	—

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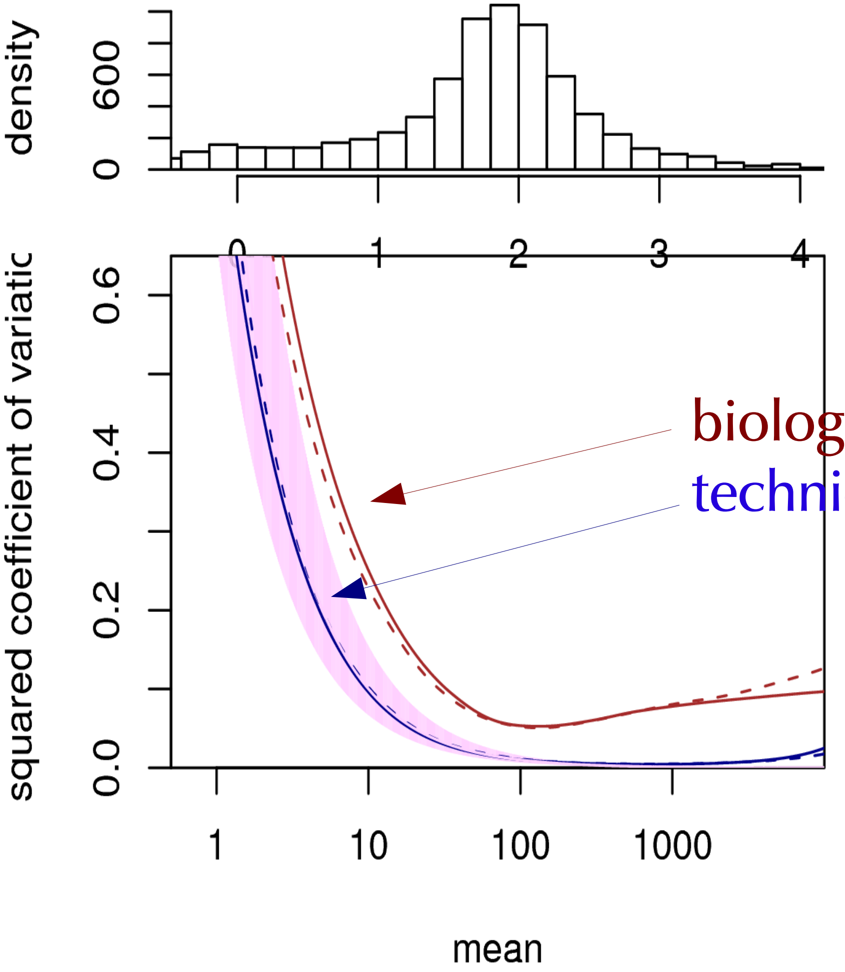
Technical and biological replicates



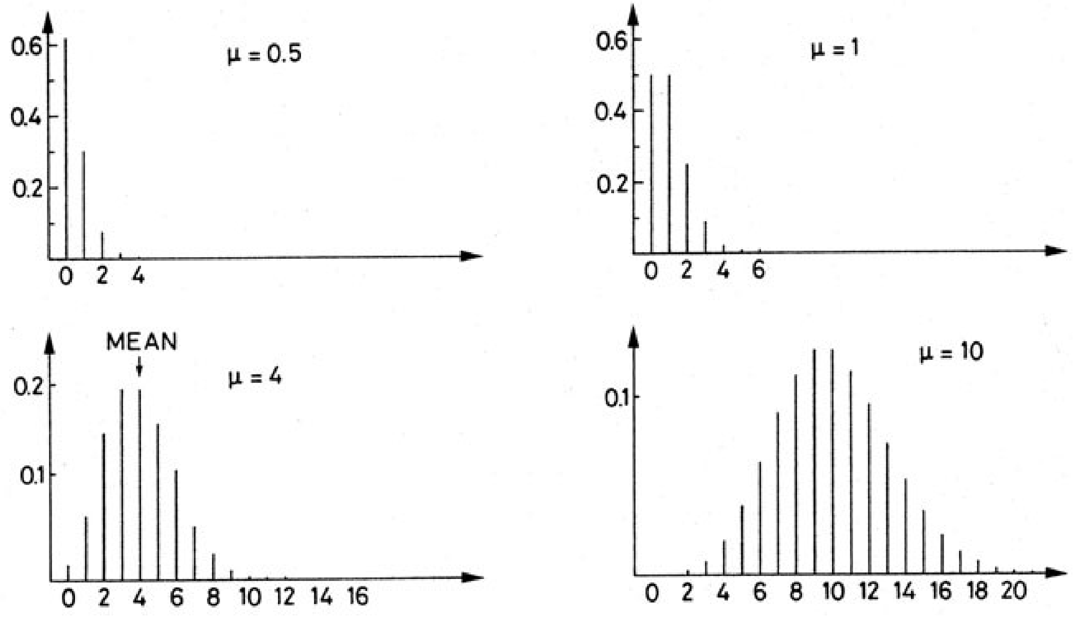
biological replicates

technical replicates

RNA-Seq of yeast [Nagalakshmi et al, 2008]

Poisson (I)

- The Poisson distribution turns up whenever things are counted
- Example: A short, light rain shower with *r* drops/m^2. What is the probability to find *k* drops on a paving stone of size 1 m^2?



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

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  - 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.

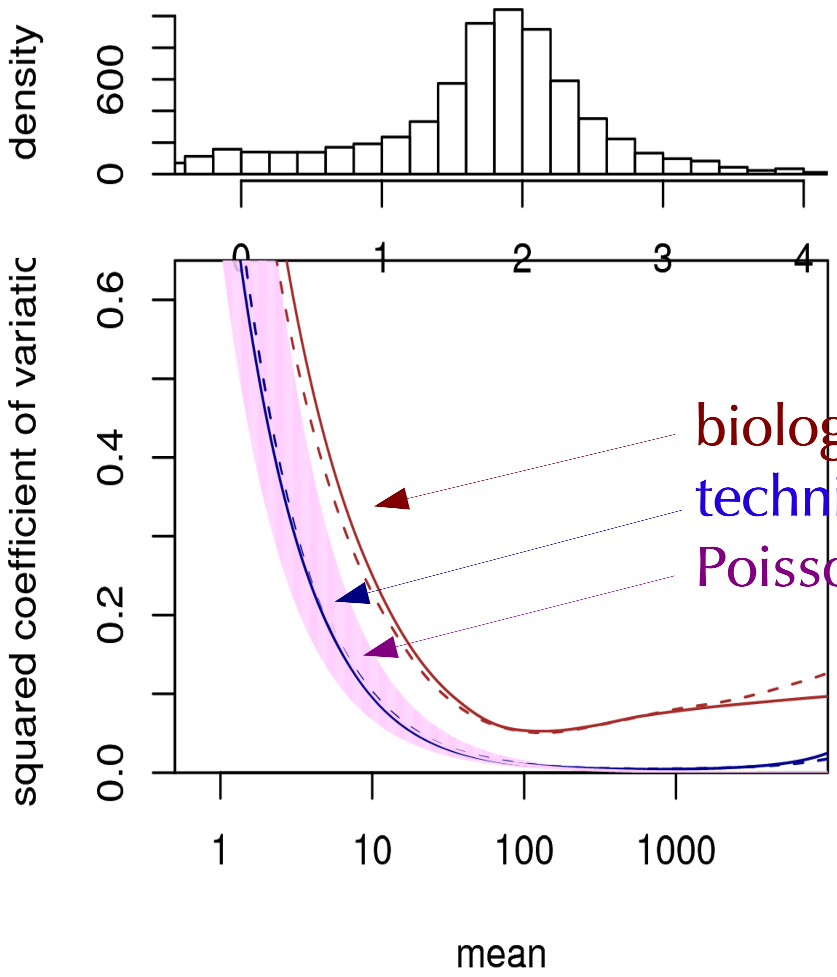
Technical and biological replicates

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



biological replicates

technical replicates

Poisson noise

RNA-Seq of yeast [Nagalakshmi et al, 2008]

Summary: Noise

We distinguish:

- Shot noise
  - unavoidable, appears even with perfect replication
  - dominant noise for weakly expressed genes

can be computed

- Technical noise
  - from sample preparation and sequencing
  - negligible (if all goes well)
- Biological noise
  - unaccounted-for differenced between samples
  - Dominant noise for strongly expressed genes

needs to be estimated from the data

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Which null hypothesis?

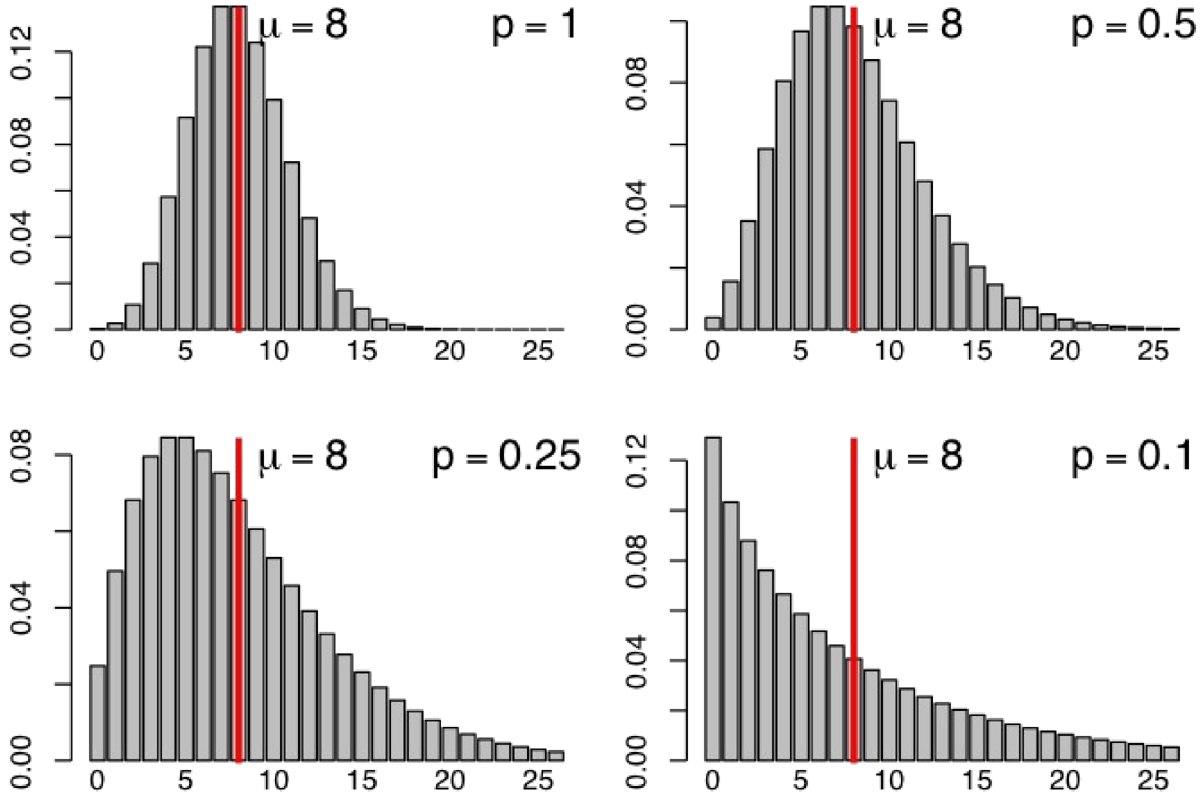
In a statistical test, we attempt to *reject* a null hypothesis.

Given two samples with different experimental conditions, which null hypothesis covers the question of biological interest?

- The concentration of transcripts from gene *i* is equal in the two samples.
  - → shot noise is all we need to know
- The difference of the concentrations is of a magnitude as is expected between replicate samples.
  - → Estimate of biological variability needed

The negative-binomial distribution

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



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

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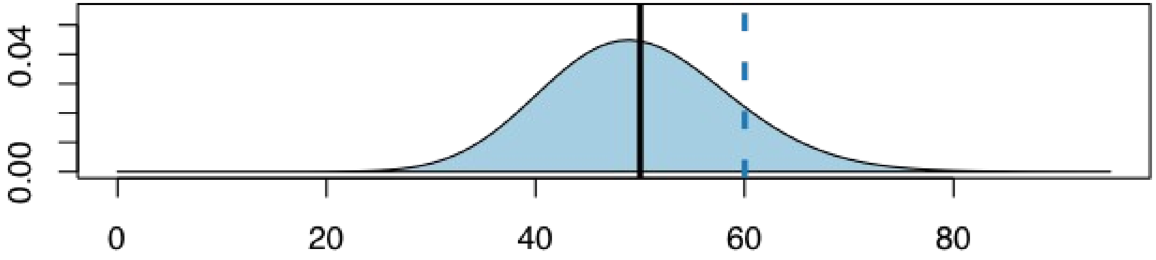
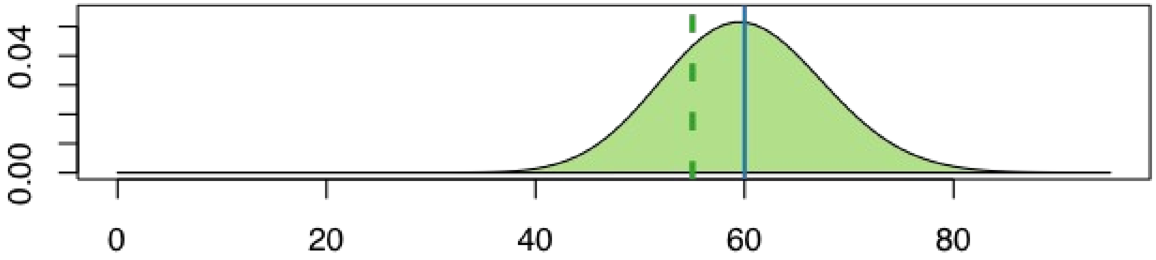
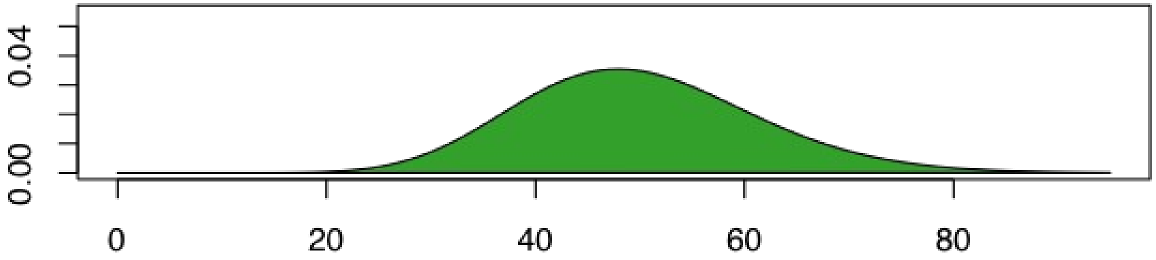
The NB distribution from a hierarchical model



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:

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

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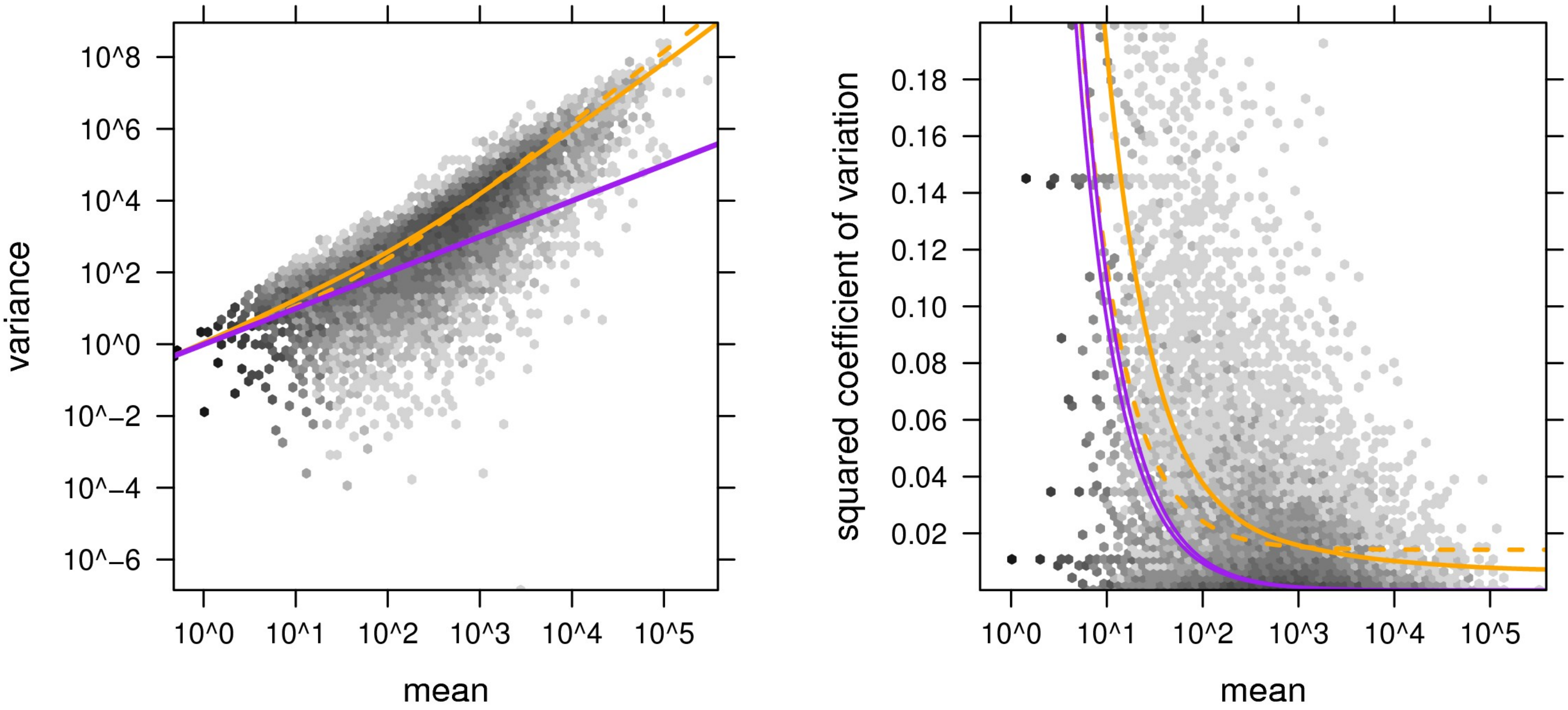
Model fitting

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

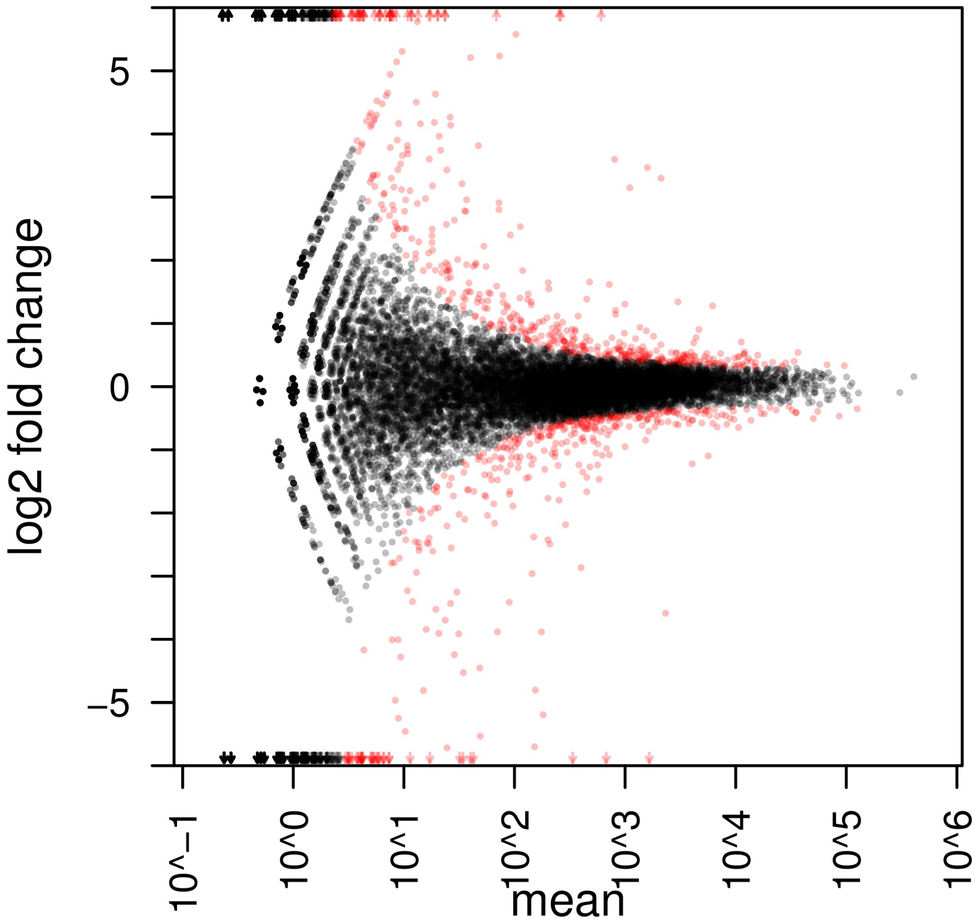


Testing for differential expression

- For each of two conditions, add the count from all replicates, and consider these sums $\mathrm{K}_{i\mathrm{A}}$ and $K_{i\mathrm{B}}$ 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 $k_{i\mathrm{A}}+\mathrm{k}_{i\mathrm{A}}$, 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]

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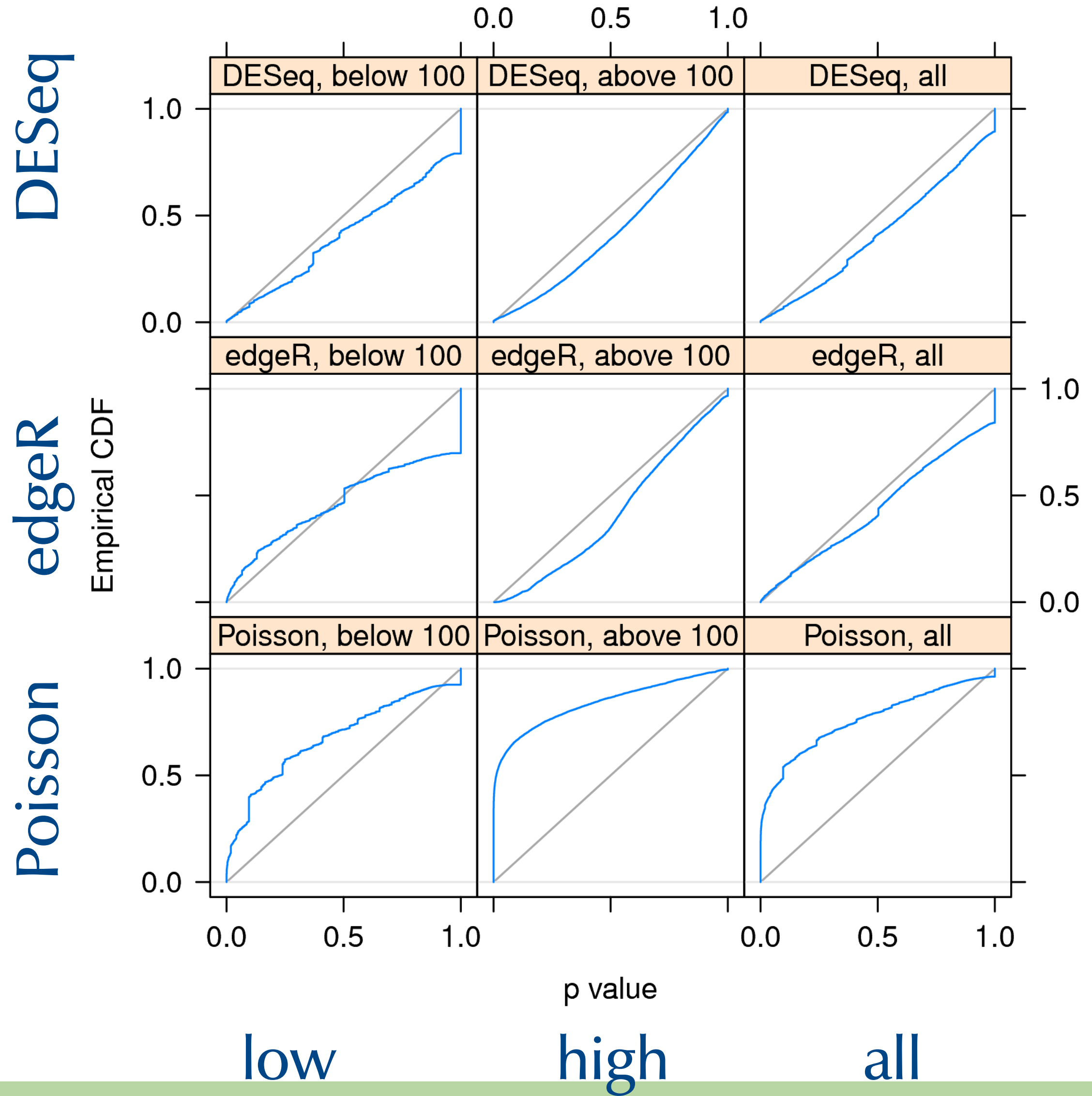
Type-I error control



Comparison of replicates:

no differential expression,

expect uniform *p* values

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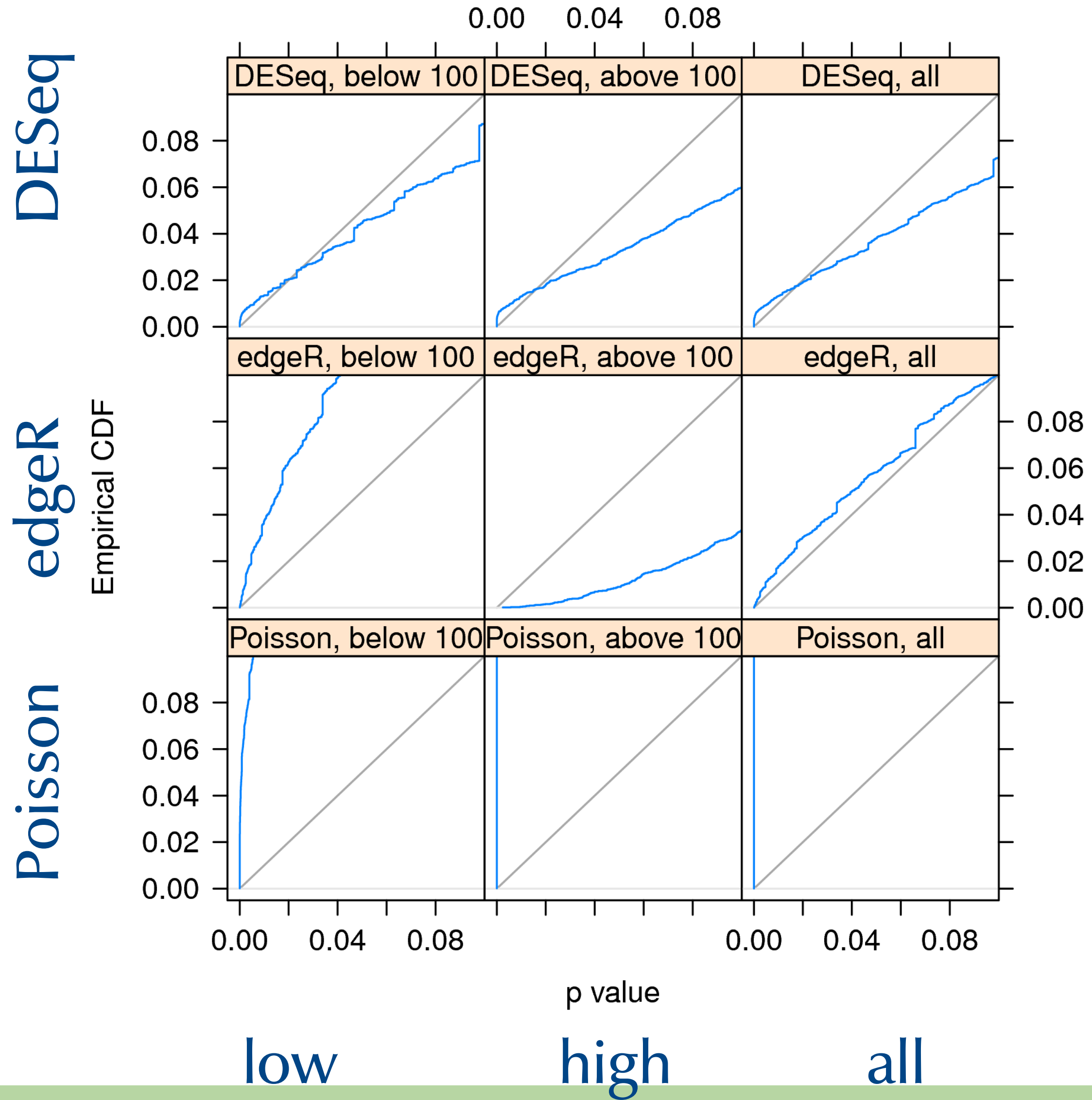
Type-I error control: Zoom



Comparison of replicates:

no differential expression,

expect uniform *p* values

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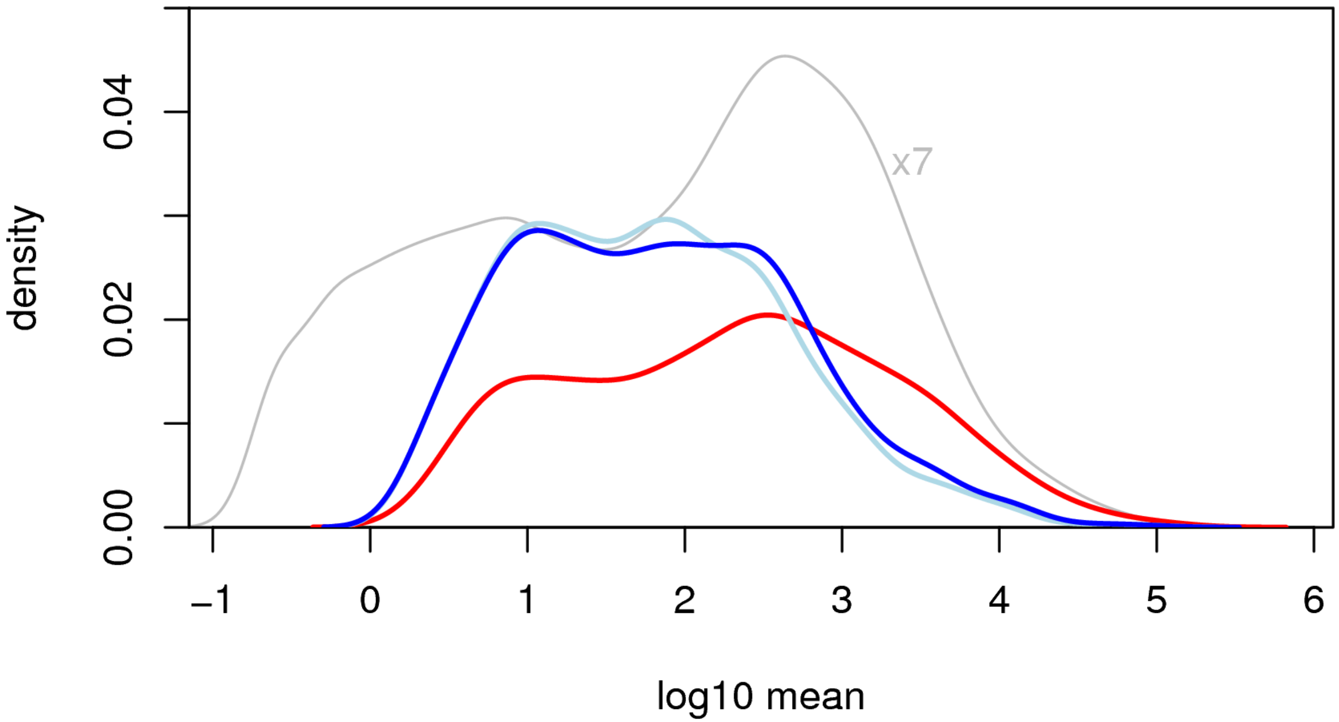
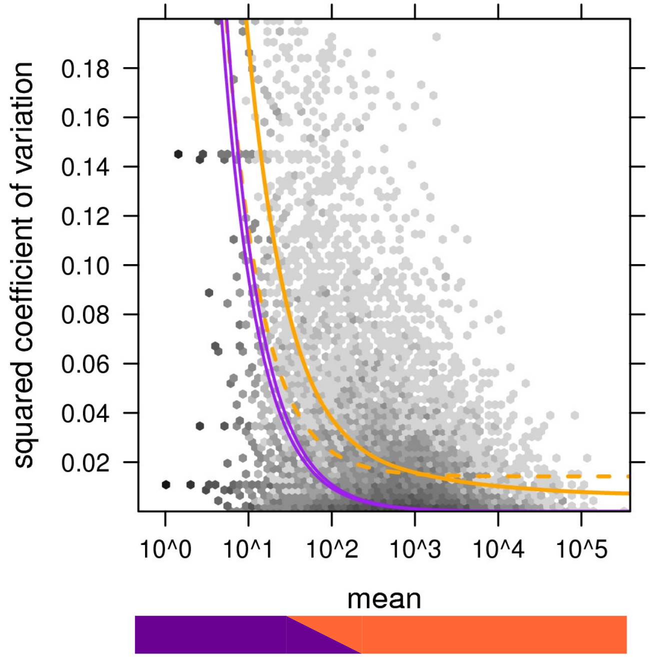
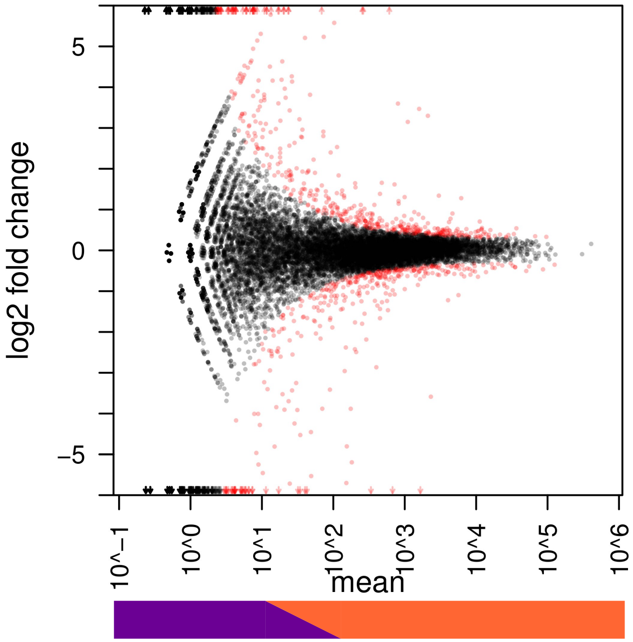
Distribution of hits along the dynamic range



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

Two noise ranges





	dominating noise	*How to improve power?*
🟪	shot noise (Poisson)	deeper sampling
🟧	biological noise	more biological replicates

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Working without replicates

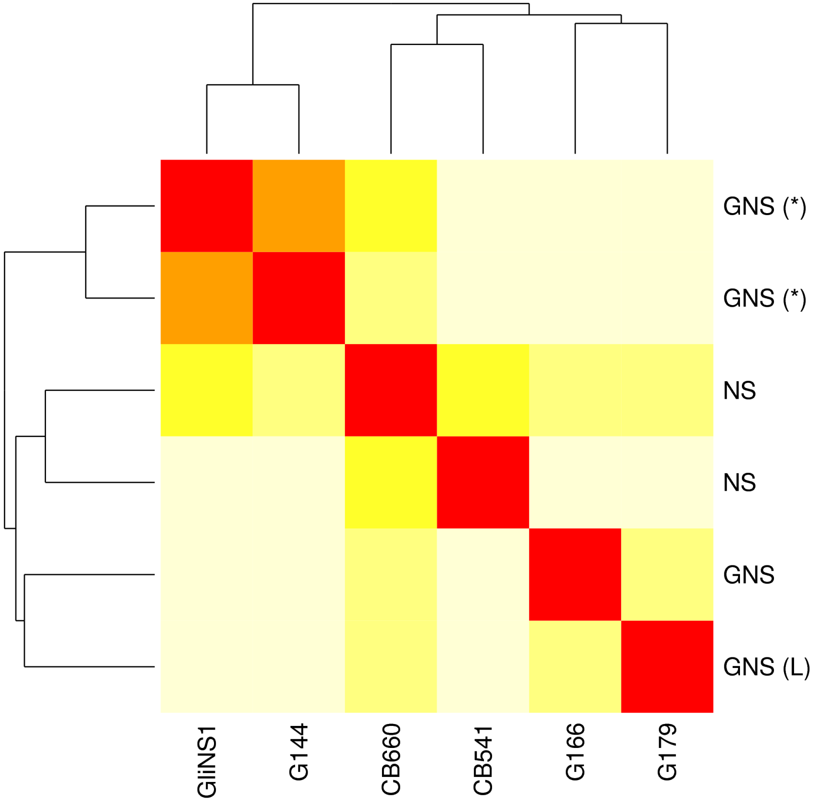
One can infer the variance from a comparison of different conditions.

- 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 homoskledastic.



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

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

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Further use cases

Similar count data appears in

- comparative ChiP-Seq
- barcode sequencing
- …

and can be analysed with *DESeq* as well.

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.

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Coming soon: GLMs for DESeq

- Until now, DESeq only supported simple comparisons.
- There are many use cases requiring more general models.
- Implementation of generalized linear models (GLMs) for DESeq now ready for use.

The model

$$K_{ij} \sim NB(\mu_{ij}, \alpha_i)$$

$$\text{with} \qquad \log \frac{\mu_{ij}}{s_j} = \eta_{ij} = \beta_{i0} + \sum_l \beta_{il} x_{jl}$$

K_{ij}	read count for gene i in sample j
s_j	size factor for sample j
α_i	dispersion for gene i
μ_{ij}	expectation for gene i in sample j
η_{ij}	linear predictor for gene i in sample j
x_{jl}	l-th for predictor sample j (indicator or quantitative)
β_{il}	l-th regression coefficient for gene i

The model

- This is a generalized linear model:
  - Link function: log link, with sample-dependent size factors
  - Family: negative binomial with known dispersion

- The dispersion is estimated from the fitted variance-mean relation, reading off at the sample average:

$$\tilde{\alpha}_i = v(\hat{q}_i)/\hat{q}_i^2 \qquad \text{with} \qquad \hat{q}_i = \sum_j \frac{k_{ij}}{s_j}$$

- The negative binomial is in the exponential family if the dispersion is given.

DESeq with GLMs: Applications

- Factorial designs with interactions
- Paired samples
- Regression of expression on genotype, etc.
- Alternative splicing
    - Count for each exon, then fit a model for each gene, and test the interaction between exon and experimental condition.
- Methylation (HELP)
- ChIP-Seq: ChIP vs input crossed with condition

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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 Preecedings*:
  "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

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: $K_{ij} \sim \mathrm{Pois}(\, s_j q_{ij} \,)$.

Negative-binomial model (II)

- If Q_{ij} has mean μ_i and variance σ_i^2, what is the the marginal (“mixing”) distribution of $K_{ij} \sim \text{Pois}(\, s_j \text{q}_{ij}\,)$?
- 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 $s_j q_{i0} + s_j \sigma_i^2$.

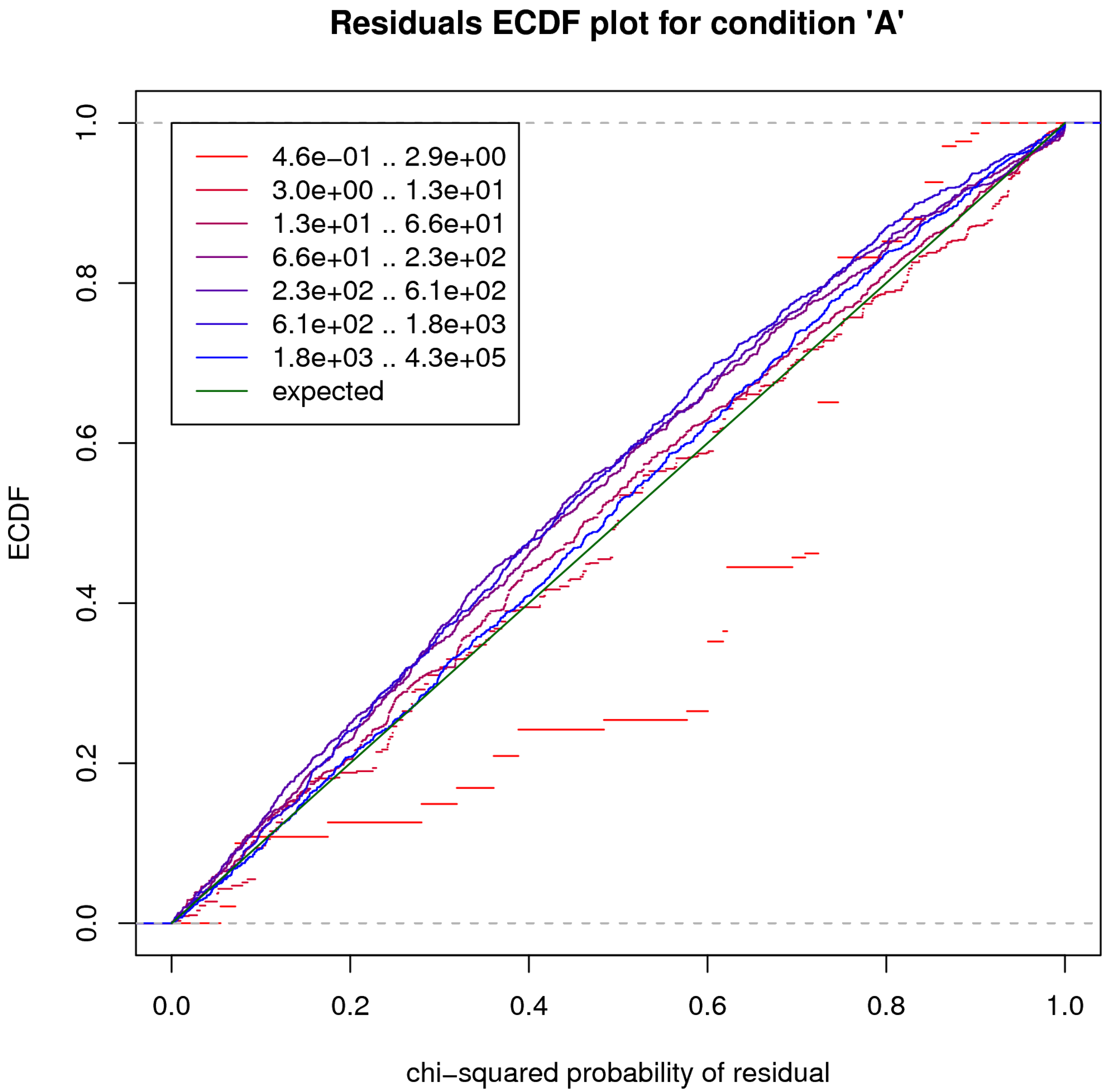
Model fitting

- 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 $\frac{\hat{q}_i}{m} \sum \frac{1}{\hat{s}_j}$
- 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



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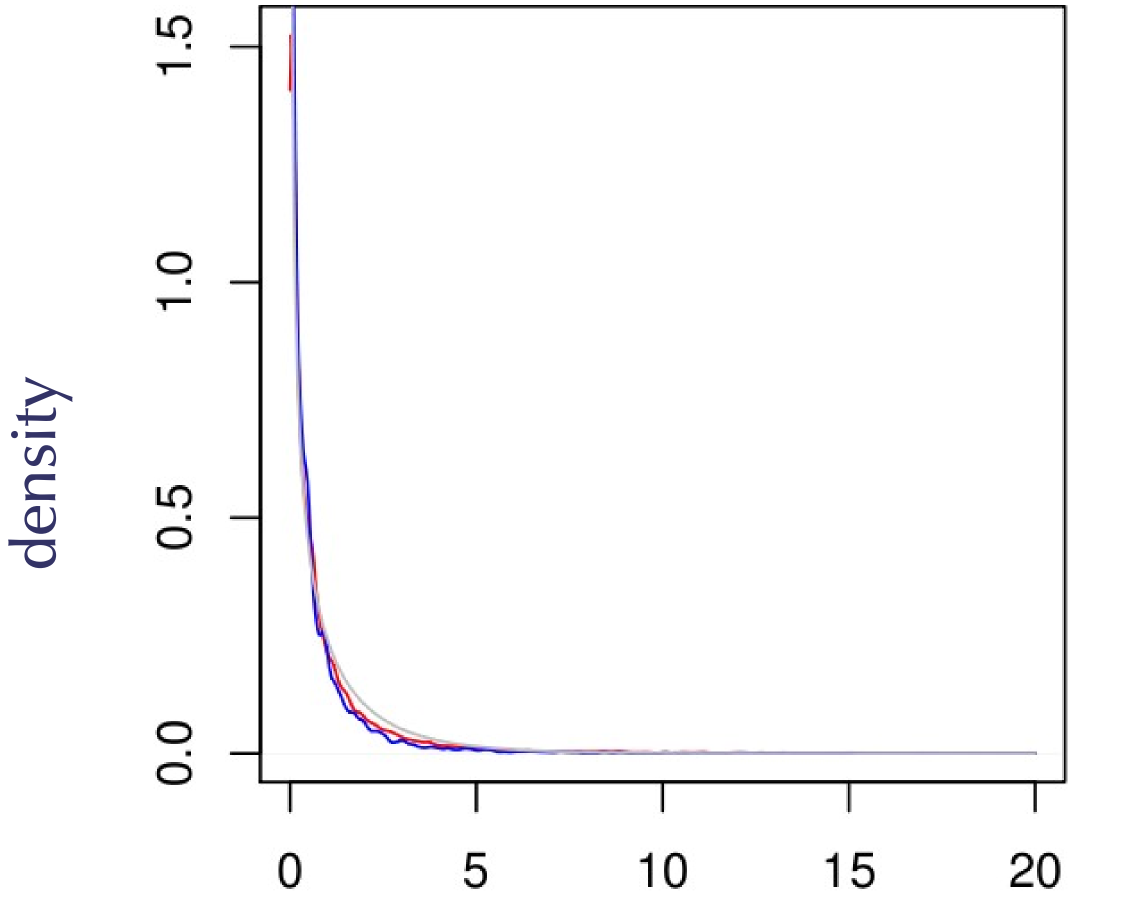
Variance residuals distribution



per-gene sample variance / fitted variance

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