@CSoneson

RNA-seq differential expression analysis

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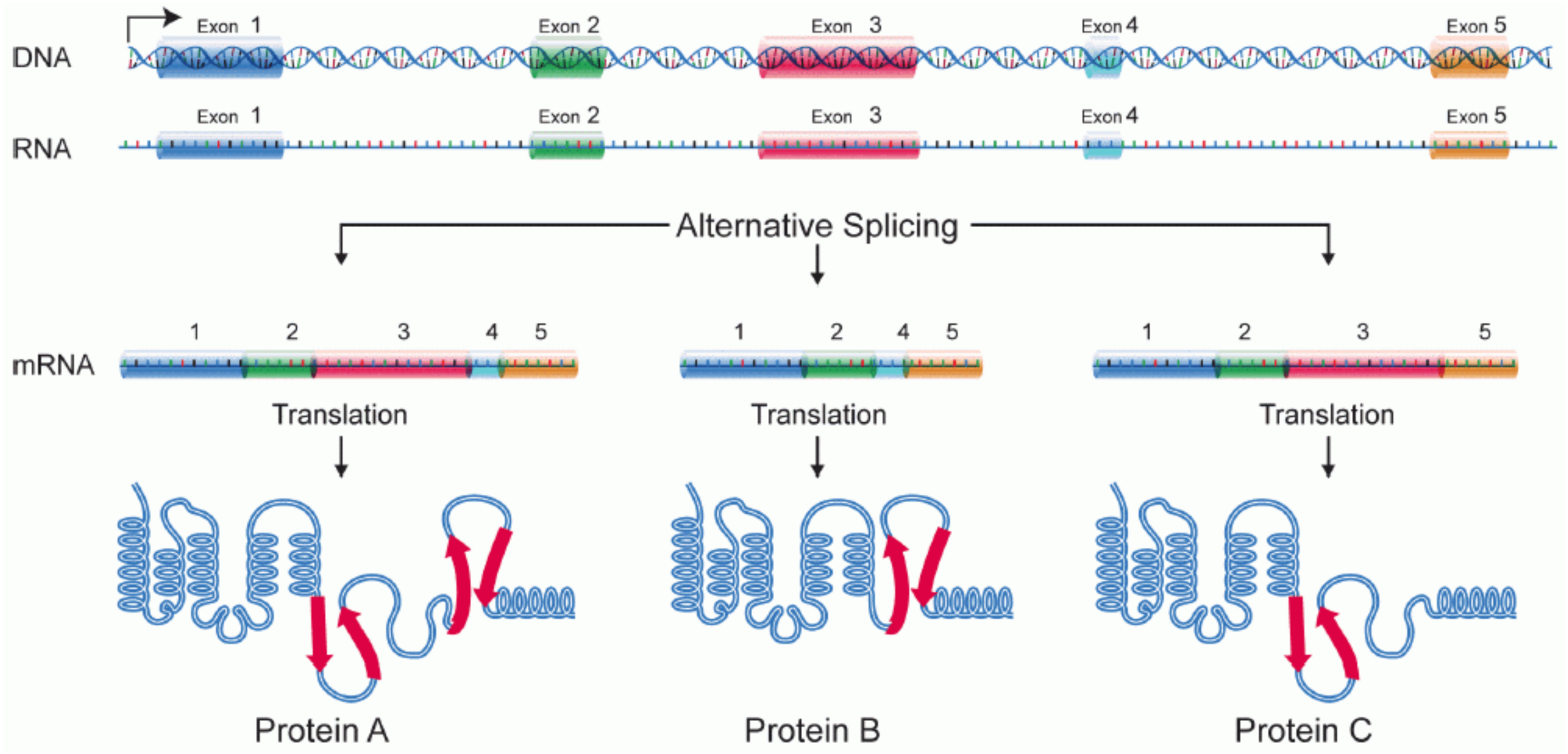
DIFFERENTIAL ANALYSIS


DNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
RNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Alternative Splicing
mRNA
1 2 3 4 5
1 2 4 5
1 2 3 5
Translation
Translation
Translation
Protein A
Protein B
Protein C

Wikipedia

Differential analysis types for RNA-seq

- Does the total output of a gene change between conditions? **DGE**
- Does the expression of individual transcripts change? **DTE**
- Does *any* isoform of a given gene change? **DTE+G**
- Does the isoform composition for a given gene change? **DTU/DIU/DEU**
- (Does *anything* change? GDE*)

- need *different* abundance quantification of transcriptomic features (genes, transcripts, exons)

*https://liorpachter.wordpress.com/2018/02/15/gde%C2%B2-dge%C2%B2-dtu%C2%B2-dte%E2%82%81%C2%B2-dte%E2%82%82%C2%B2/

Differential expression analysis

- Input: expression/abundance matrix
  (features x samples) + grouping/sample annotation

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516	SRR1039517	SRR1039520	SRR1039521
ENSG00000000003	693	451	887	416	1148	1069	774	581
ENSG00000000005	0	0	0	0	0	0	0	0
ENSG00000000419	466	515	623	364	590	794	419	510
ENSG00000000457	326	274	372	223	356	450	308	297
ENSG00000000460	91	75	61	48	110	95	100	82
ENSG00000000938	0	0	2	0	1	0	0	0

- Output: result table (one line per feature)

	logFC	logCPM	LR	PValue	FDR
ENSG00000109906	-5.882117	4.120149	924.1622	5.486794e-203	3.493826e-198
ENSG00000165995	-3.236681	4.603028	576.1025	2.641667e-127	8.410672e-123
ENSG00000189221	-3.316900	6.718559	562.9594	1.909251e-124	4.052512e-120
ENSG00000120129	-2.952536	7.255438	506.3838	3.881506e-112	6.179067e-108
ENSG00000196136	-3.225084	6.911908	463.2175	9.587512e-103	1.221008e-98
ENSG00000101347	-3.759902	9.290645	449.9697	7.323427e-100	7.772231e-96
ENSG00000211445	-3.755609	9.102440	433.4656	2.861624e-96	2.603138e-92
ENSG00000162692	3.616656	4.551120	402.0266	1.994189e-89	1.587300e-85
ENSG00000171819	-5.705289	3.474697	389.3431	1.150502e-86	8.140055e-83
ENSG00000152583	-4.364255	5.491013	376.1995	8.363745e-84	5.325782e-80

Differential expression analysis - input

	SRR1039508	SRR1039509	SRR1039512	SRR1039513	SRR1039516	SRR1039517	SRR1039520	SRR1039521
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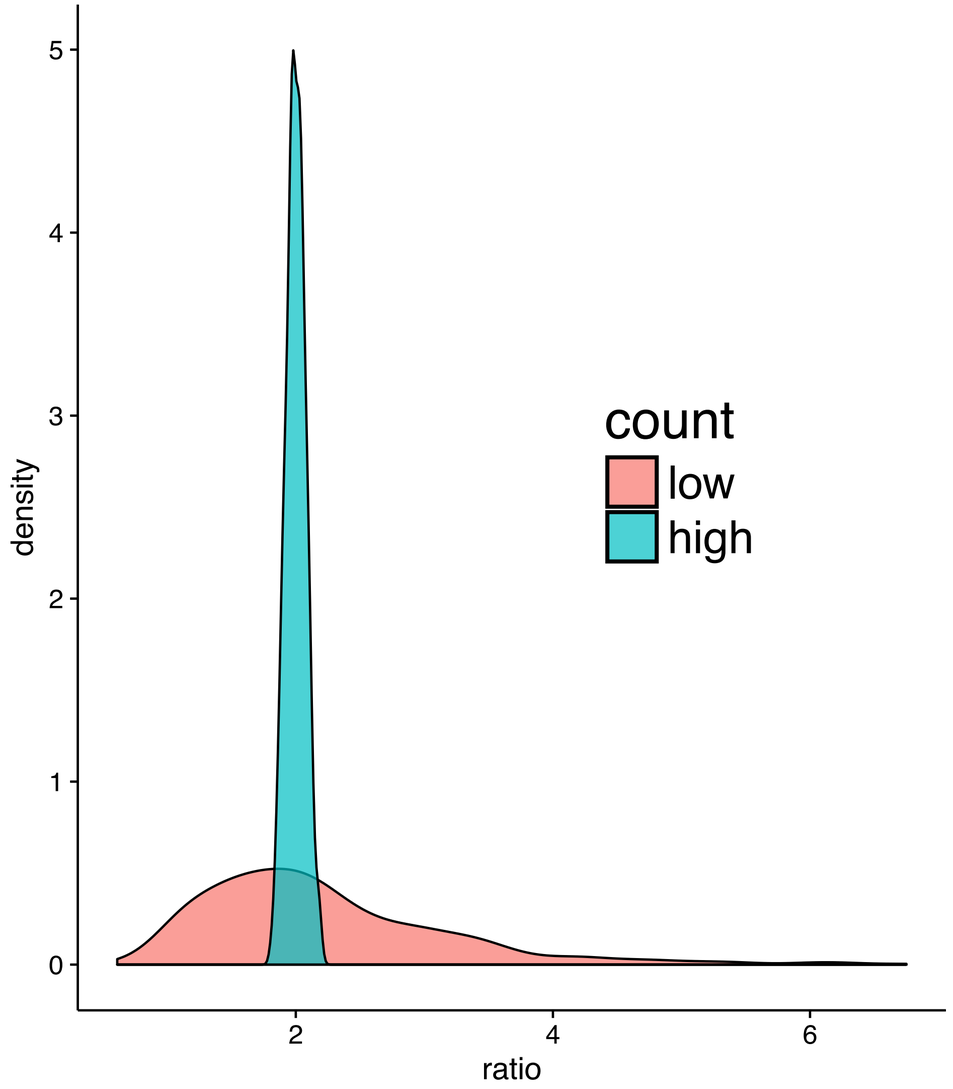
ENSG00000000938	0	0	2	0	1	0	0	0

- *Most* RNA-seq methods (e.g., edgeR, DESeq2, voom) need *raw counts* (or equivalent) as input
- **Don't** provide these methods with (e.g.) RPKMs, FPKMs, TPMs, CPMs, log-transformed counts, normalized counts, …
- Read documentation carefully!

Why not only relative abundances?



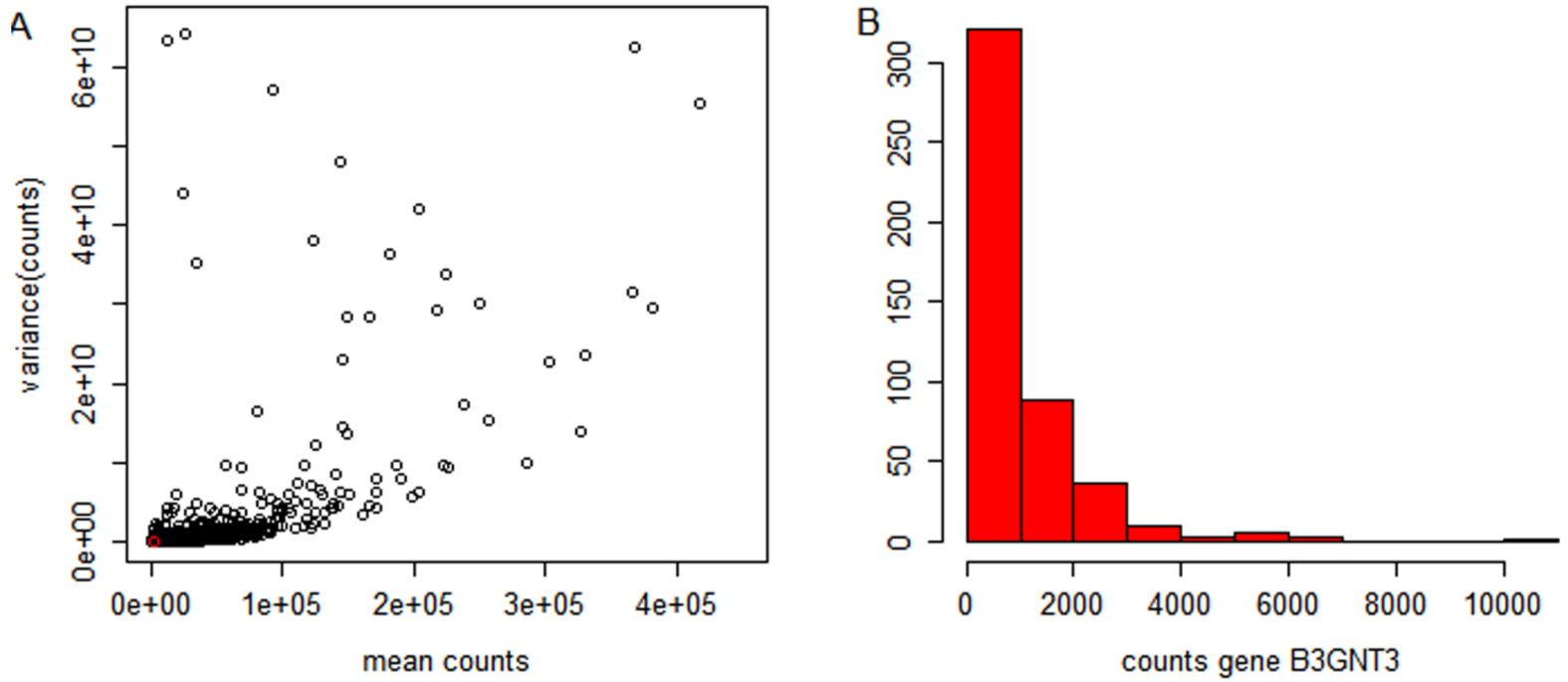
- Ex: ratio between two Poisson distributed variables
- Low count:
  mean = 20 vs mean = 10
- High count:
  mean = 2000 vs mean = 1000

Challenges for RNA-seq data analysis

- Choice of statistical distribution
- Normalization between samples
- Few samples -> difficult to estimate parameters (e.g., variance)

MODELING COUNTS

Characteristics of RNA-seq data

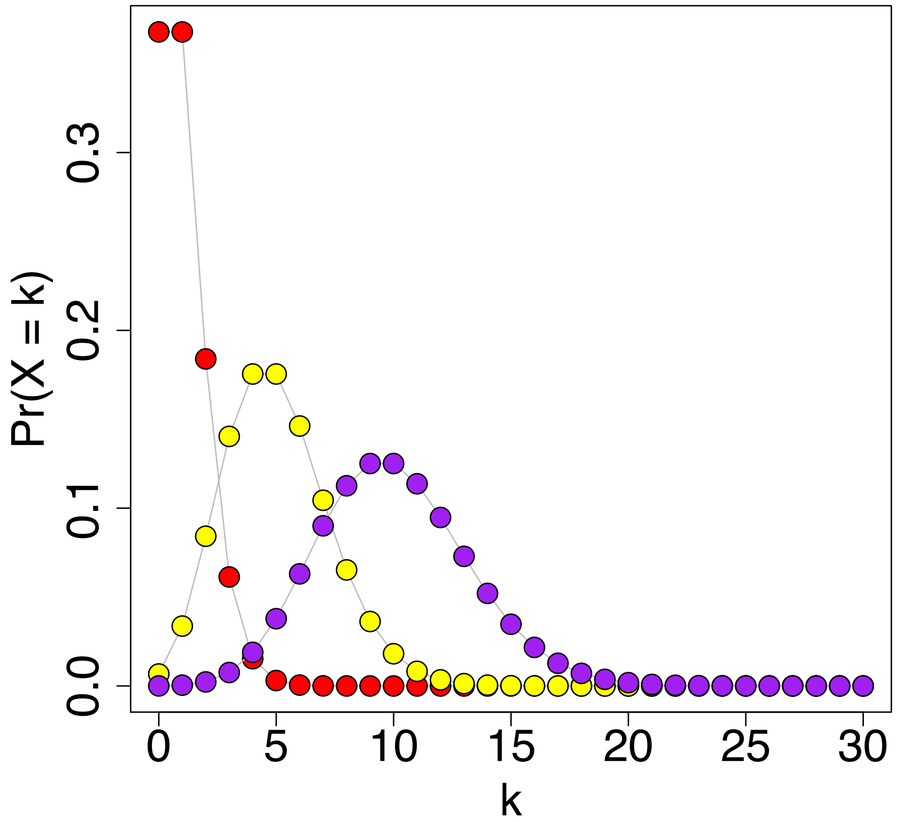


- Variance depends on the mean count
- Counts are non-negative and often highly skewed

Zwiener et al., PLoS One 2014

Modeling counts - the Poisson distribution

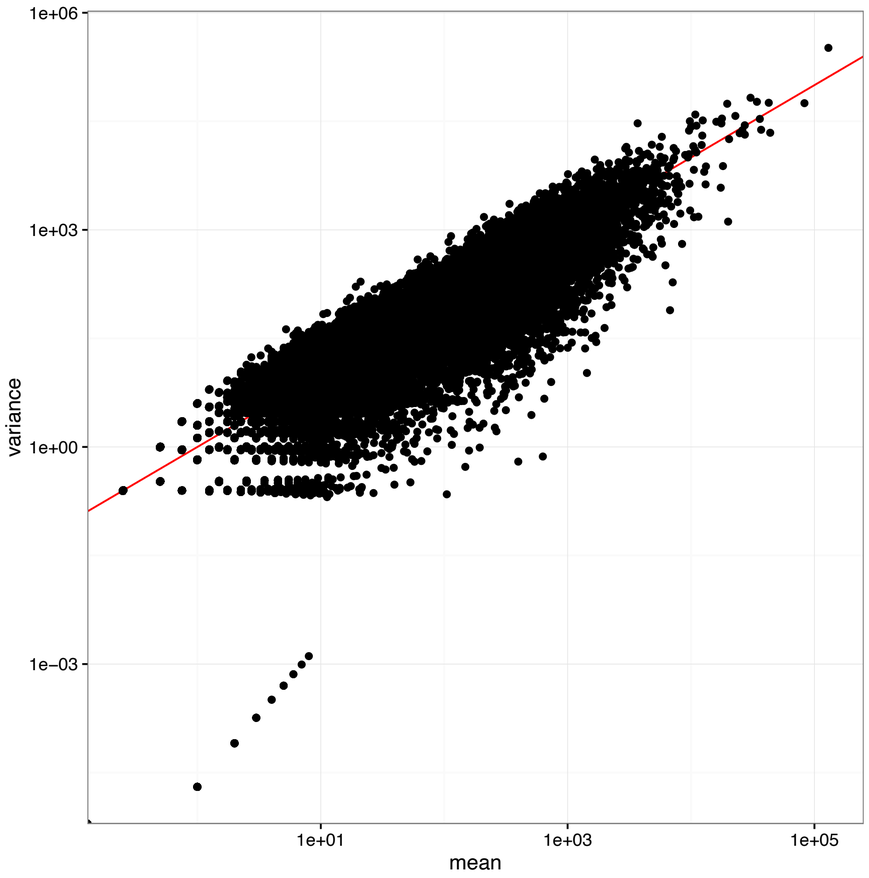
$$P(X = k) = \frac{\lambda^k e^{-\lambda}}{k!}$$



Modeling counts

Poisson distribution

- Quantifies sampling variability
- $var(X) = \mu$
- Represents technical replicates well (mRNA proportions are identical across samples)



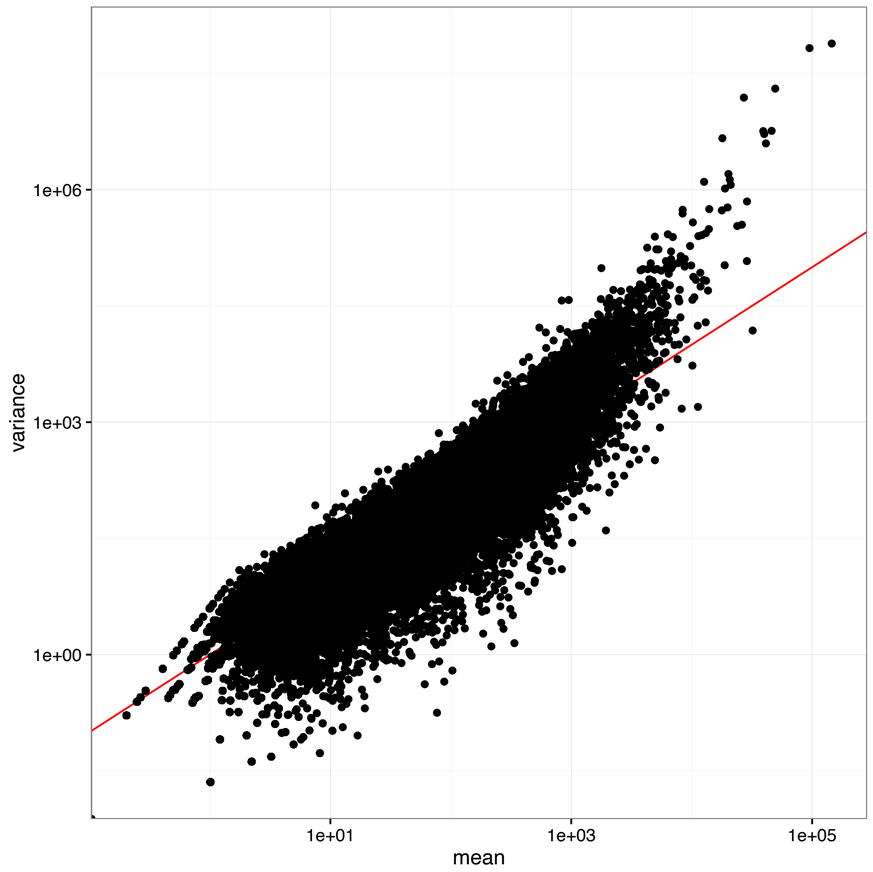
Example from SEQC data, same sample sequenced across multiple lanes

Modeling counts

Poisson distribution

- Does not fully capture variability across replicates (where mRNA proportions are not identical)

Example from SEQC data, replicates of the same RNA mix

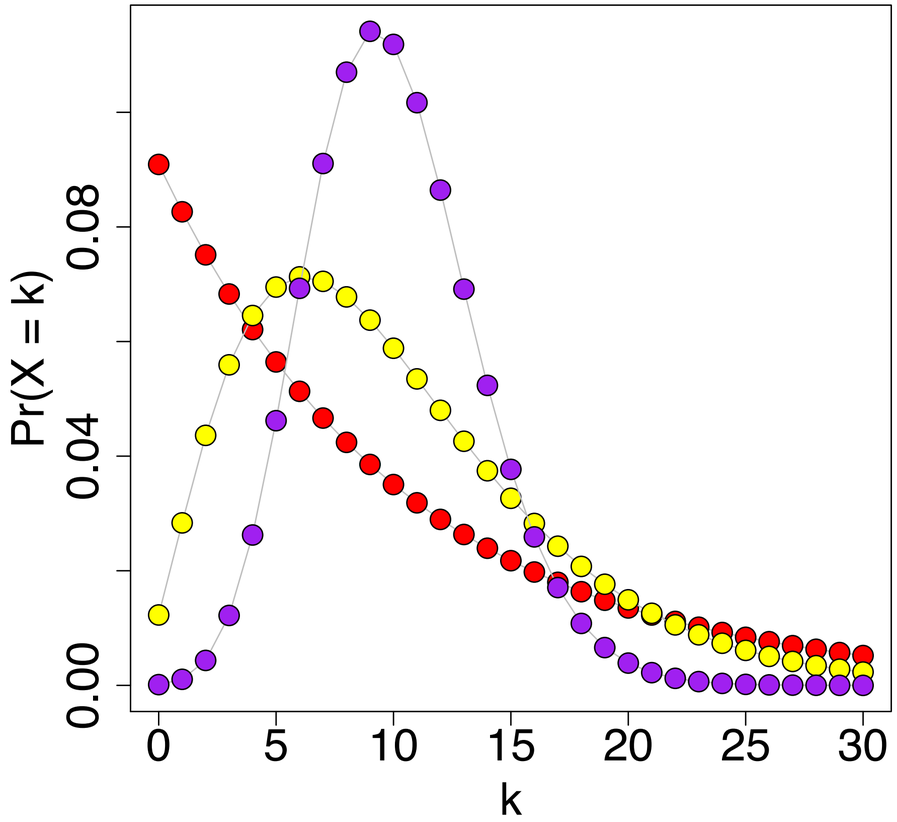


Modeling counts - the Negative Binomial distribution

$$P(X = k) = \binom{k + r - 1}{k} \cdot (1 - p)^r p^k$$

Generalizes the
Poisson distribution

One of several ways
of capturing
over-dispersion

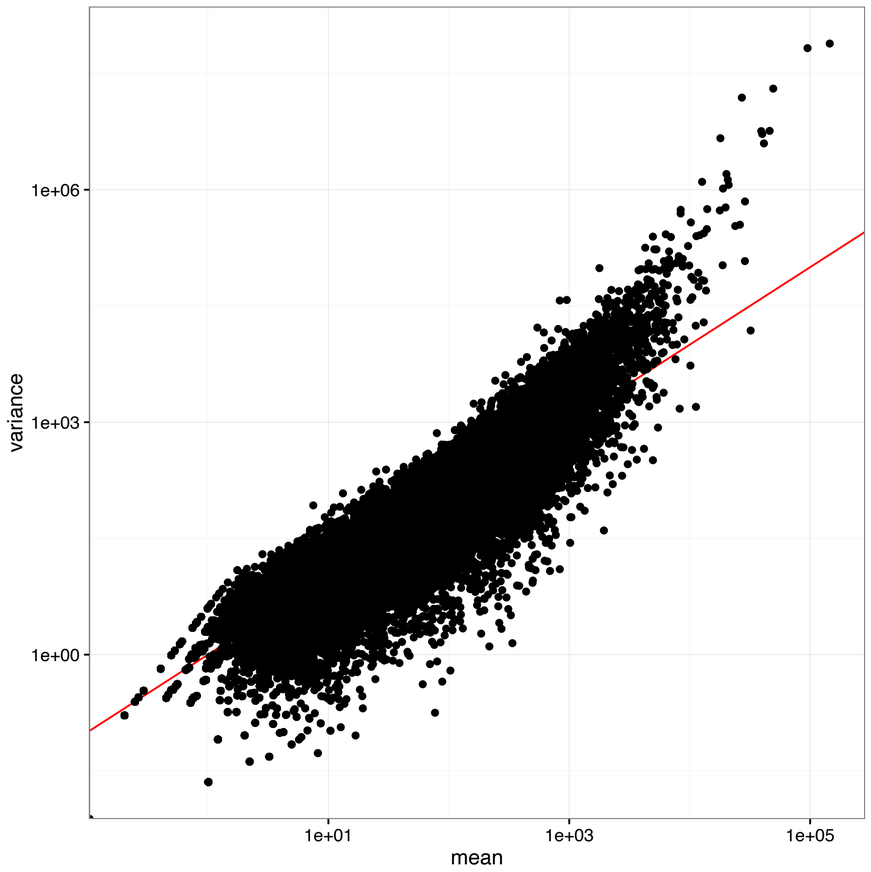


Modeling counts

Negative binomial distribution

- $var(X) = \mu + \theta\mu^2$
- θ = dispersion
- $\sqrt{\theta}$ = "biological coefficent of variation"
- Allows mRNA proportions to vary across samples (according to a gamma distribution)
- Captures variability across biological replicates better

Example from SEQC data, replicates of the same RNA mix



With count data…

- *linear* modeling (and thus t-tests, ANOVA, etc) is no longer suitable for inference

- Instead, we use *generalized linear models*

A very brief intro to GLMs

- A GLM consists of three parts:
  - A *distribution*, specifying the conditional distribution of the response Y given the predictor values
  - A *linear predictor*

$$\eta = \beta_0 + \beta_1 x_1 + \ldots + \beta_p x_p$$

  - A *link function* g, linking the conditional expected value of Y to η: $g(\mathrm{E}[Y|X]) = \eta$

The linear model is a GLM

- A GLM consists of three parts:
  - A *distribution*, specifying the conditional distribution of the response Y given the predictor values (Gaussian)
  - A *linear predictor*

$$\eta = \beta_0 + \beta_1 x_1 + \ldots + \beta_p x_p$$

  - A *link function* g, linking the conditional expected value of Y to η: $g(\mathrm{E}[Y|X]) = \eta$ (Identity function)

Other commonly used GLMs

- Logistic regression - binary response
  - Binomial distribution
  - logit link function
- Loglinear regression - count response
  - Poisson distribution
  - log link function

GLMs for RNA-seq

- Negative Binomial distribution
- Log link function
- Implemented e.g. in edgeR and DESeq2

GLMs vs transformation

- The link function in the GLM transforms the *mean*, not the observed values
- Thus, we can transform the systematic part without changing the assumptions on the random part
- By transforming the response (the observed values), we change also the random part (e.g., the association between mean and variance)

voom

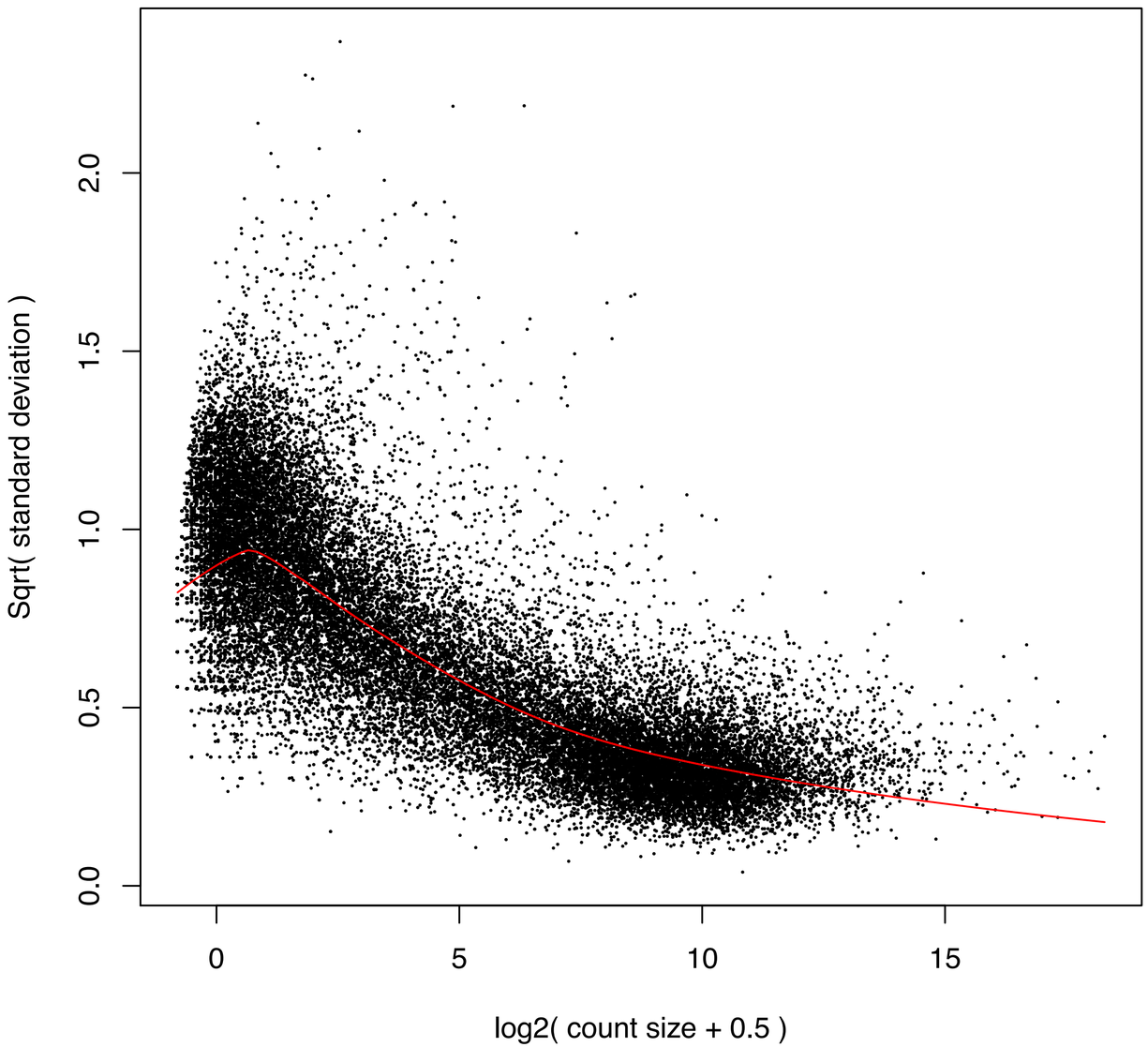
- Instead of modeling the counts, we can *transform* them to a suitable scale and model them with a normal distribution ("microarray-like").

- **voom** (part of the limma package) calculates logCPM values

$$y_{gi} = \log_2 \left(\frac{r_{gi} + 0.5}{R_i + 1.0} \times 10^6 \right)$$

- Transformed data is heteroskedastic (variance depends on mean) - use weighted least squares

voom - mean/variance relationship



BETWEEN-SAMPLE NORMALIZATION

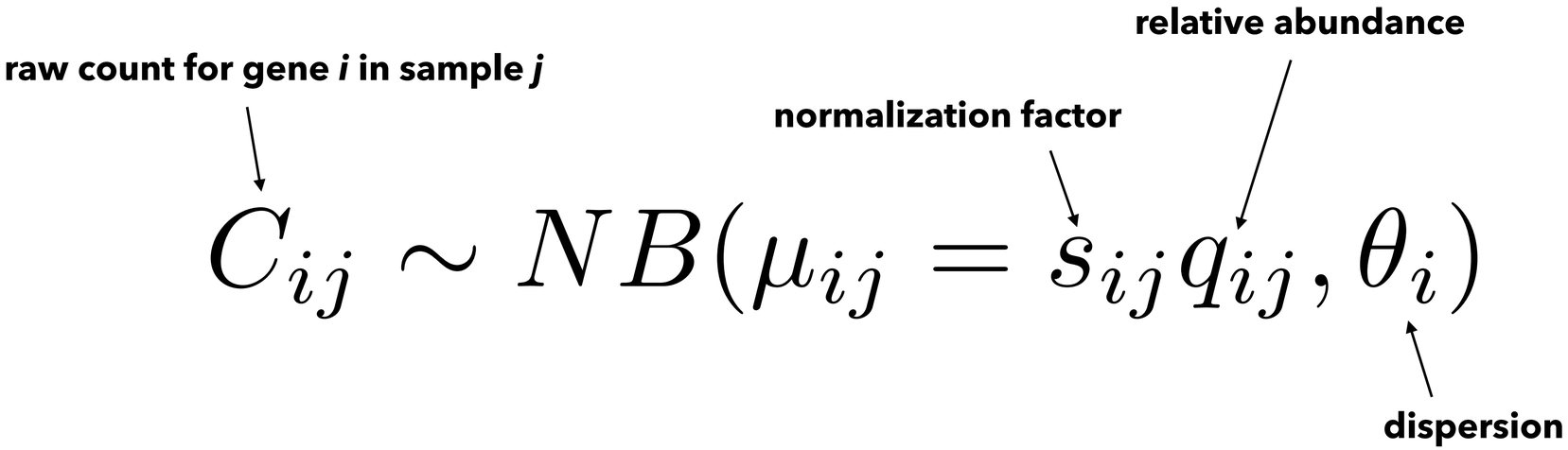
Normalization

Observed counts depend on:

- abundance
- gene length
- sequencing depth
- sequencing biases
- …

- “As-is”, not directly comparable across samples

Normalization



- s_{ij} is a normalization factor (or *offset*) in the model

- counts are not explicitly scaled

  - important exception: voom/limma (followed by explicit modeling of mean-variance association)

Simple example - offsets

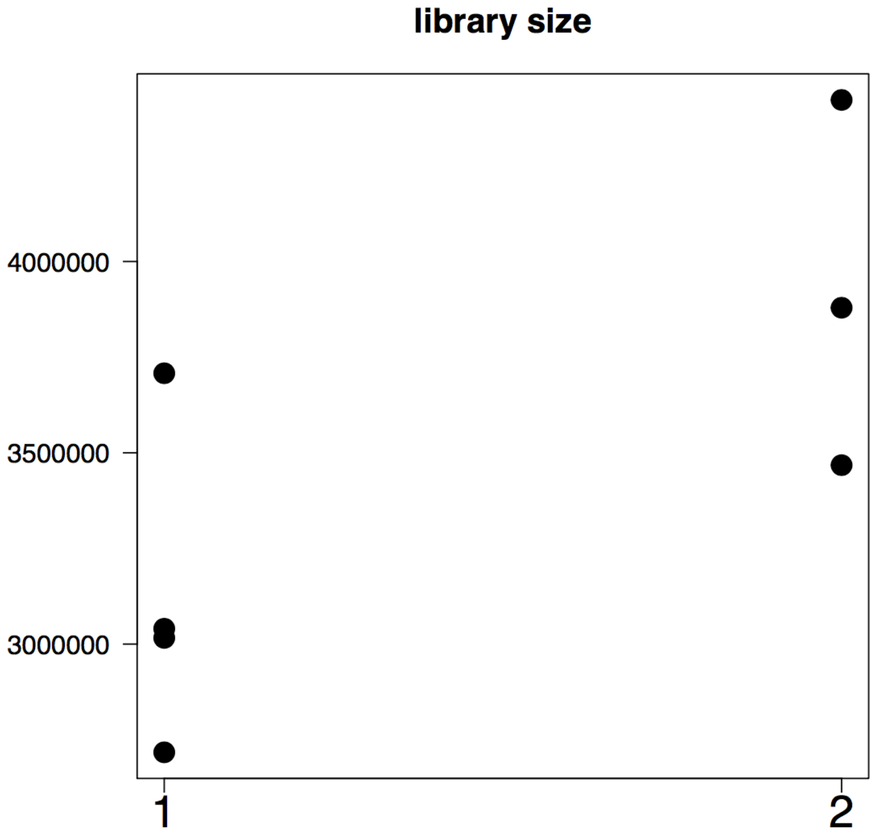
- Assume that we have RNA-seq reads for one gene. Is the gene differentially expressed?

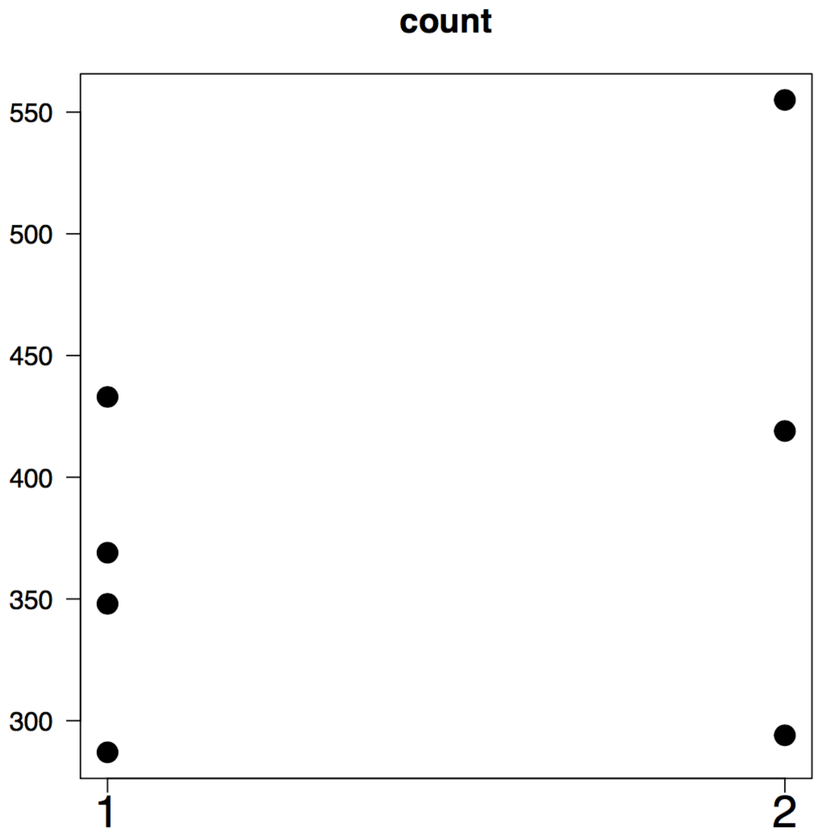
```
count.data <- data.frame(counts = c(369, 287, 348, 433, 555, 294, 419),
                         cond = c("1", "1", "1", "1", "2", "2", "2"))
glm.pois <- glm(counts ~ cond, family = poisson, data = count.data)
coefficients(summary(glm.pois))

##             Estimate Std. Error z value  Pr(>|z|)
## (Intercept)   5.8840    0.02638 223.050 0.000e+00
## cond2         0.1626    0.03853   4.219 2.451e-05
```

Simple example - offsets

- Relate counts to library sizes





Simple example - offsets

- Incorporate library size as offset

```
count.data$lib.size <- c(3040296, 2717092, 3016179, 3707895,
                         4422272, 3467730, 3879114)
glm.pois <- glm(counts ~ cond + offset(log(lib.size)), family = poisson,
                data = count.data)
coefficients(summary(glm.pois))

##             Estimate Std. Error  z value Pr(>|z|)
## (Intercept) -9.06944    0.02638 -343.802  0.00000
## cond2       -0.06635    0.03853   -1.722  0.08506
```

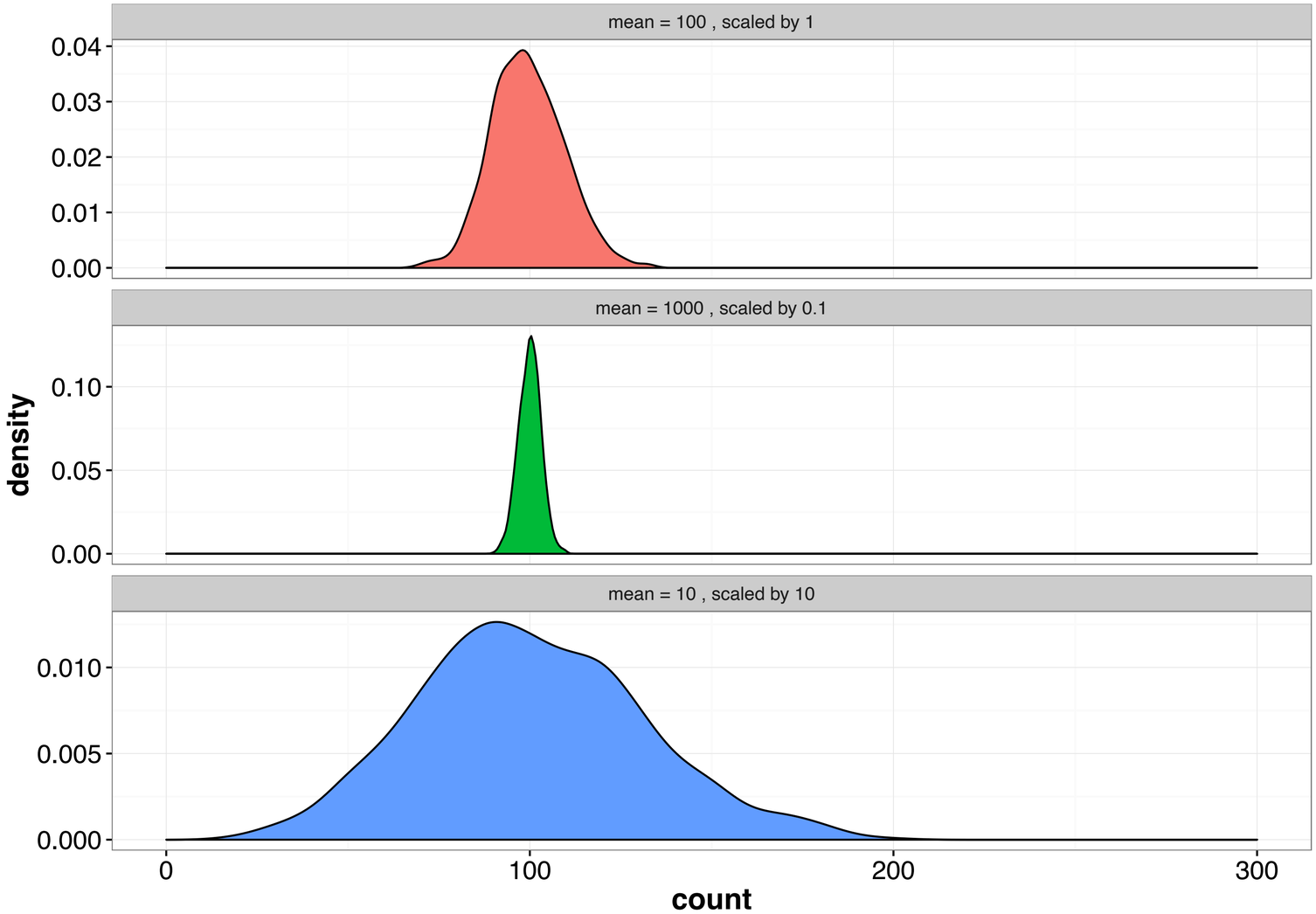
Why offset rather than scaling?

Poisson distributed variables with different means, scaled to have mean = 100

Raw count
mean = 100

Raw count
mean = 1000,
scaled by 0.1

Raw count
mean = 10,
scaled by 10



How to calculate normalization factors?

Attempt 1: **total count** (library size)

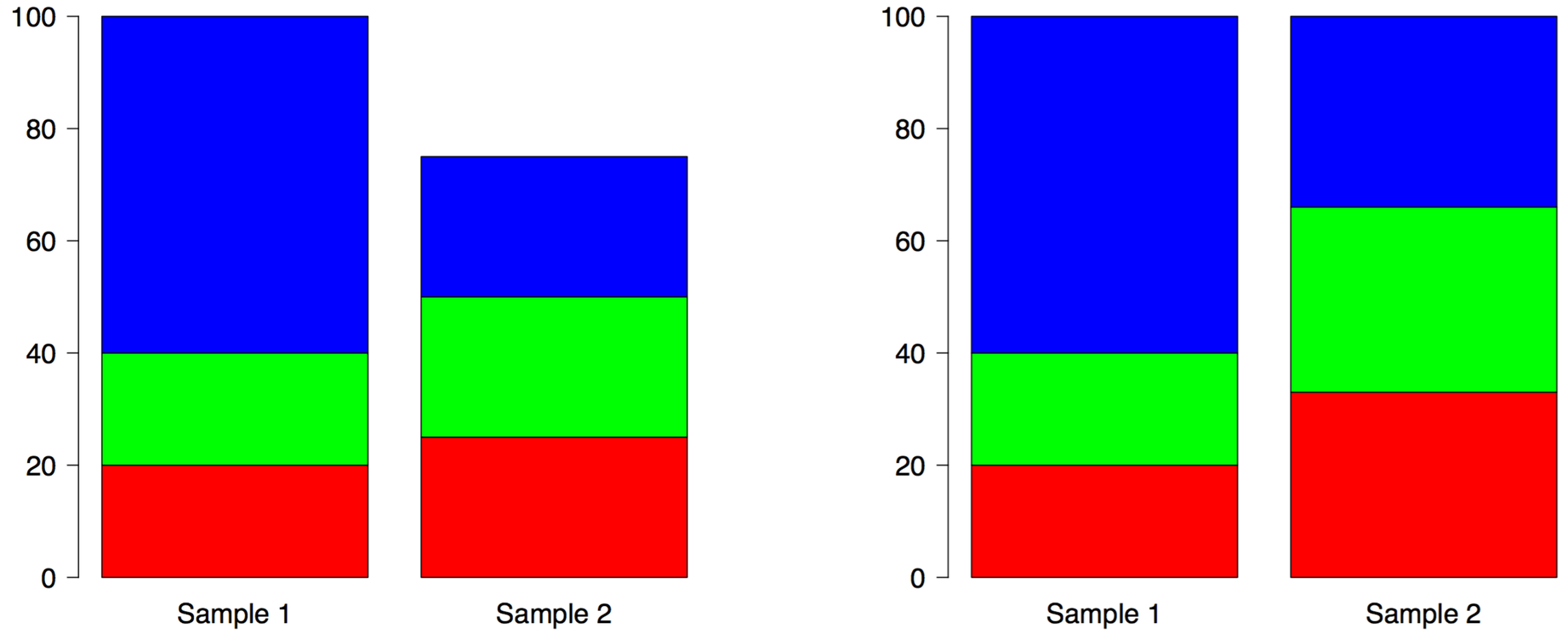
- Define a reference sample (one of the observed samples or a “pseudo-sample”) - gives a “target library size”
- Normalization factor for sample *j* is defined by

$$\frac{\text{total count in sample } j}{\text{total count in reference sample}}$$

The influence of RNA composition

- Observed counts are relative

- High counts for some genes are “compensated” by low counts for other genes

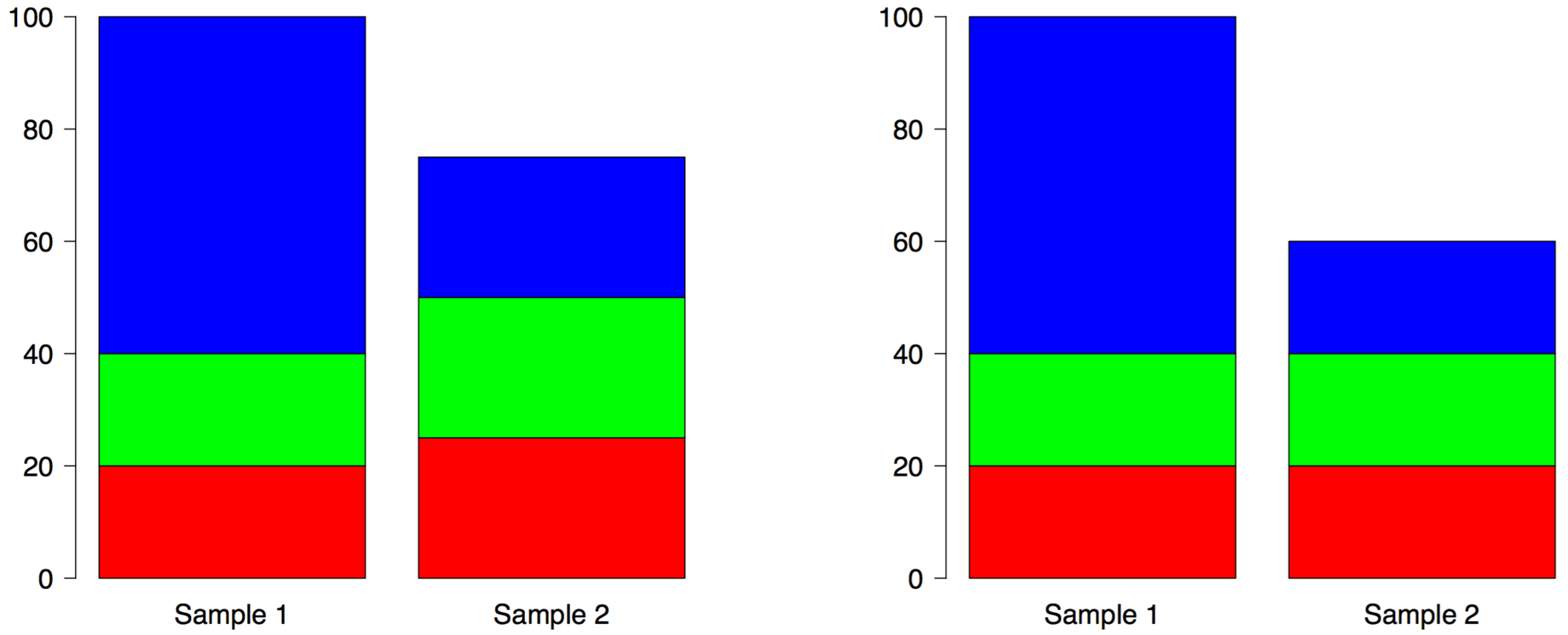


How to calculate normalization factors?

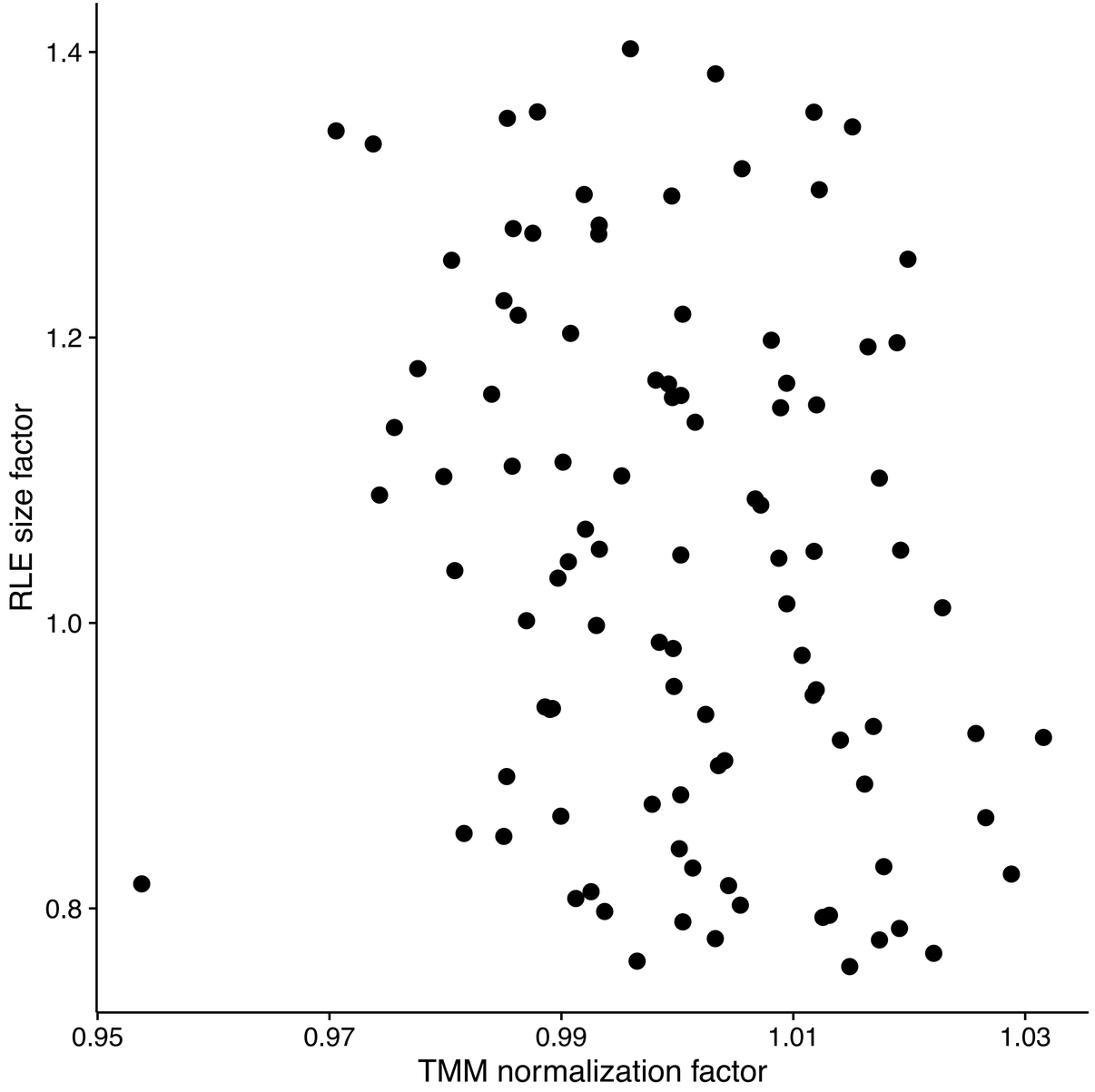
- Attempt 2: total count (library size) * compensation for differences in composition
- Idea: use only non-differentially expressed genes to compute the normalization factor
- Implemented by both edgeR (TMM) and DESeq2 (median count ratio)
- Both these methods assume that most genes are not differentially expressed

How to calculate normalization factors?

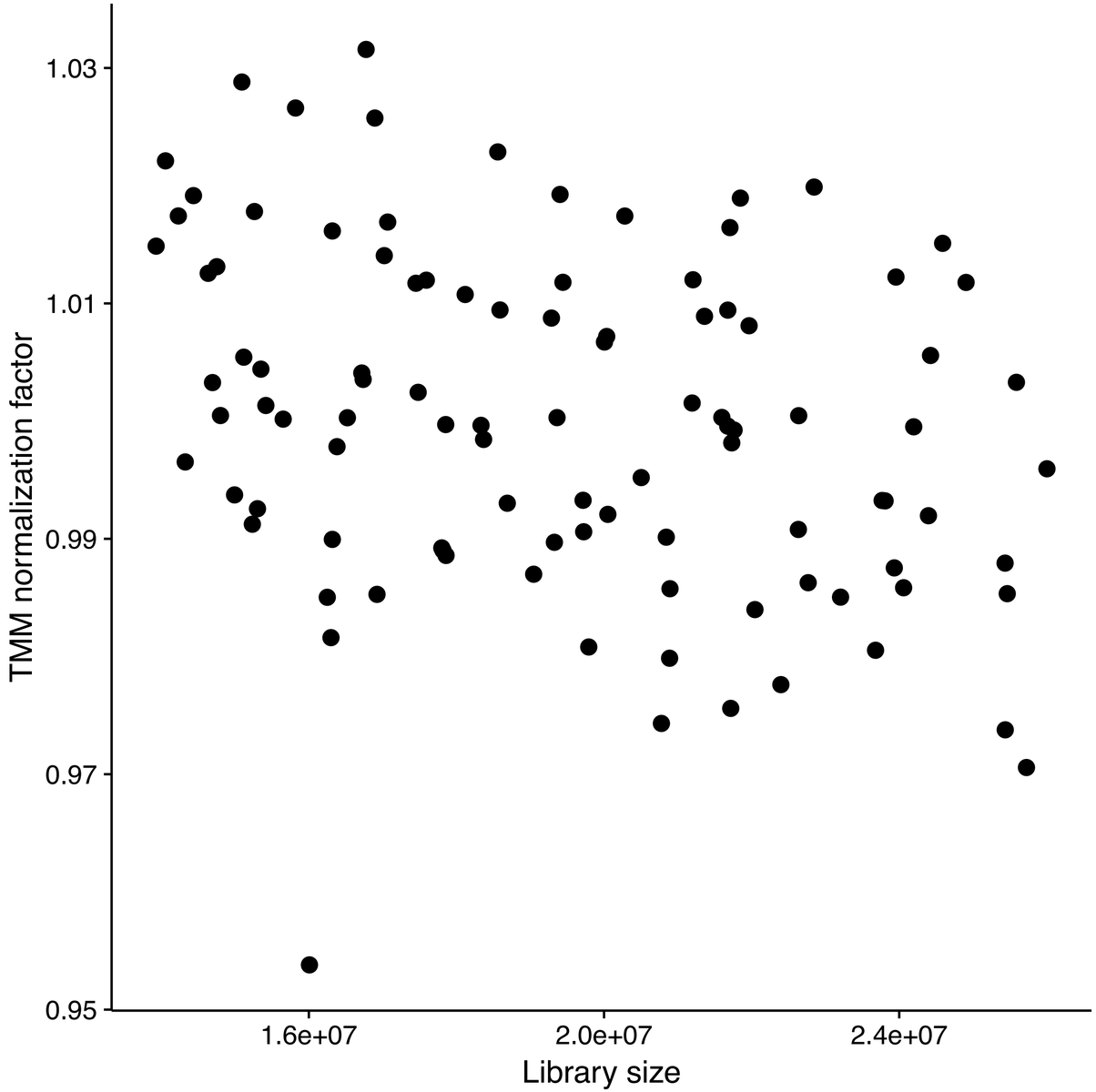
- Attempt 2: total count (library size) * compensation for differences in composition



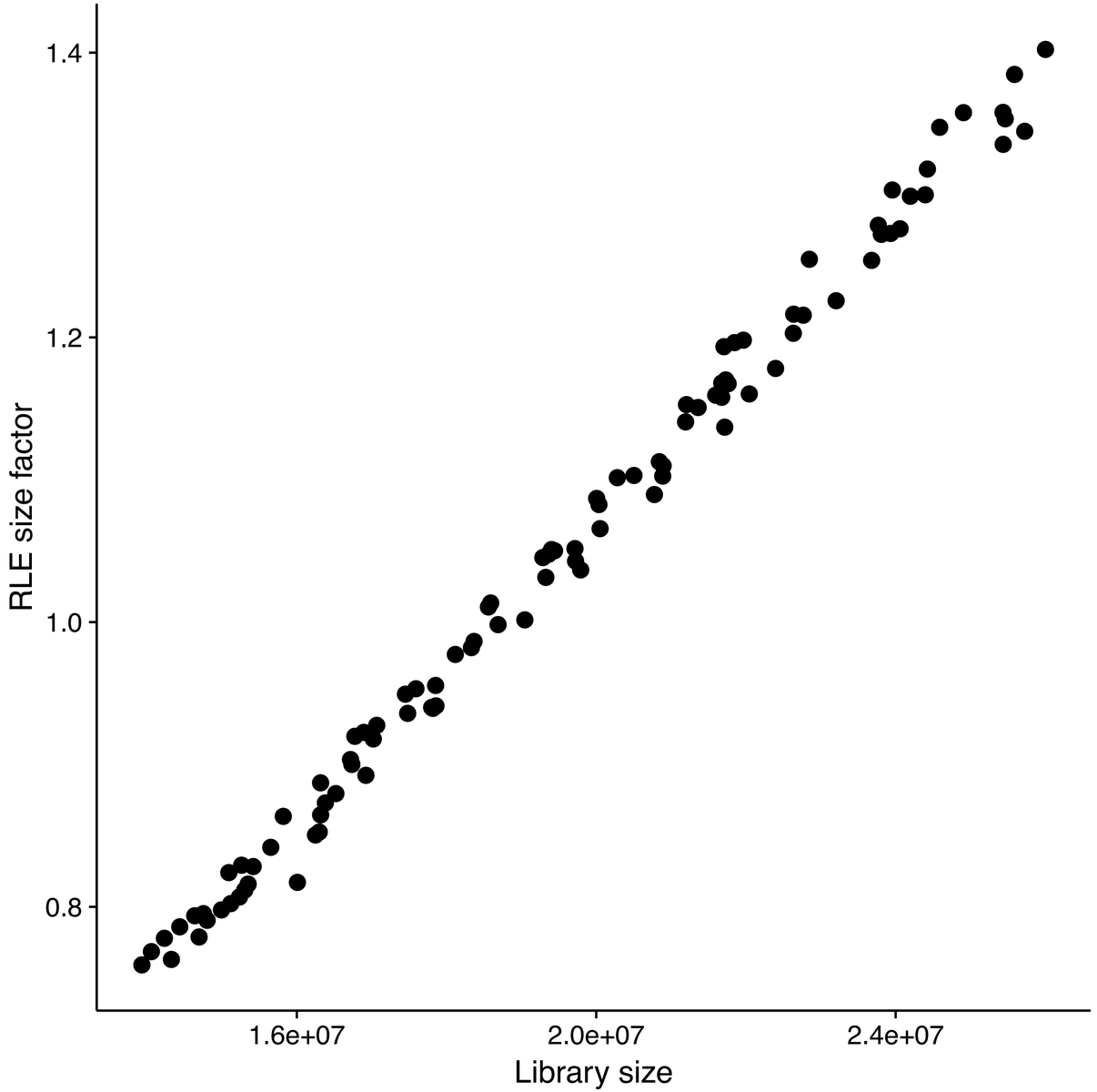
“Normalization factors” (edgeR) vs “size factors” (DESeq2)



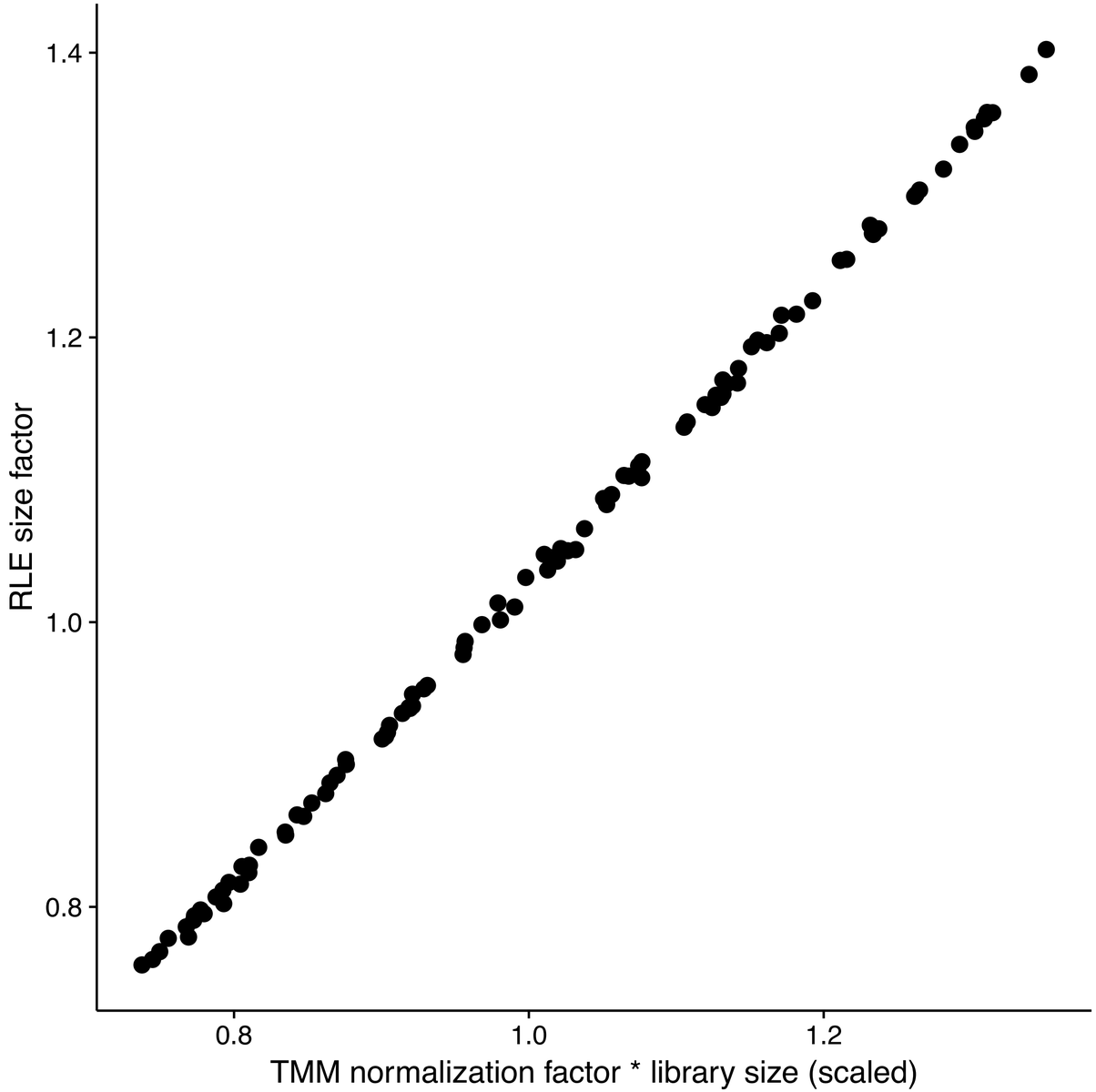
“Normalization factors” (edgeR) vs library size



“Size factors” (DESeq2) vs library size



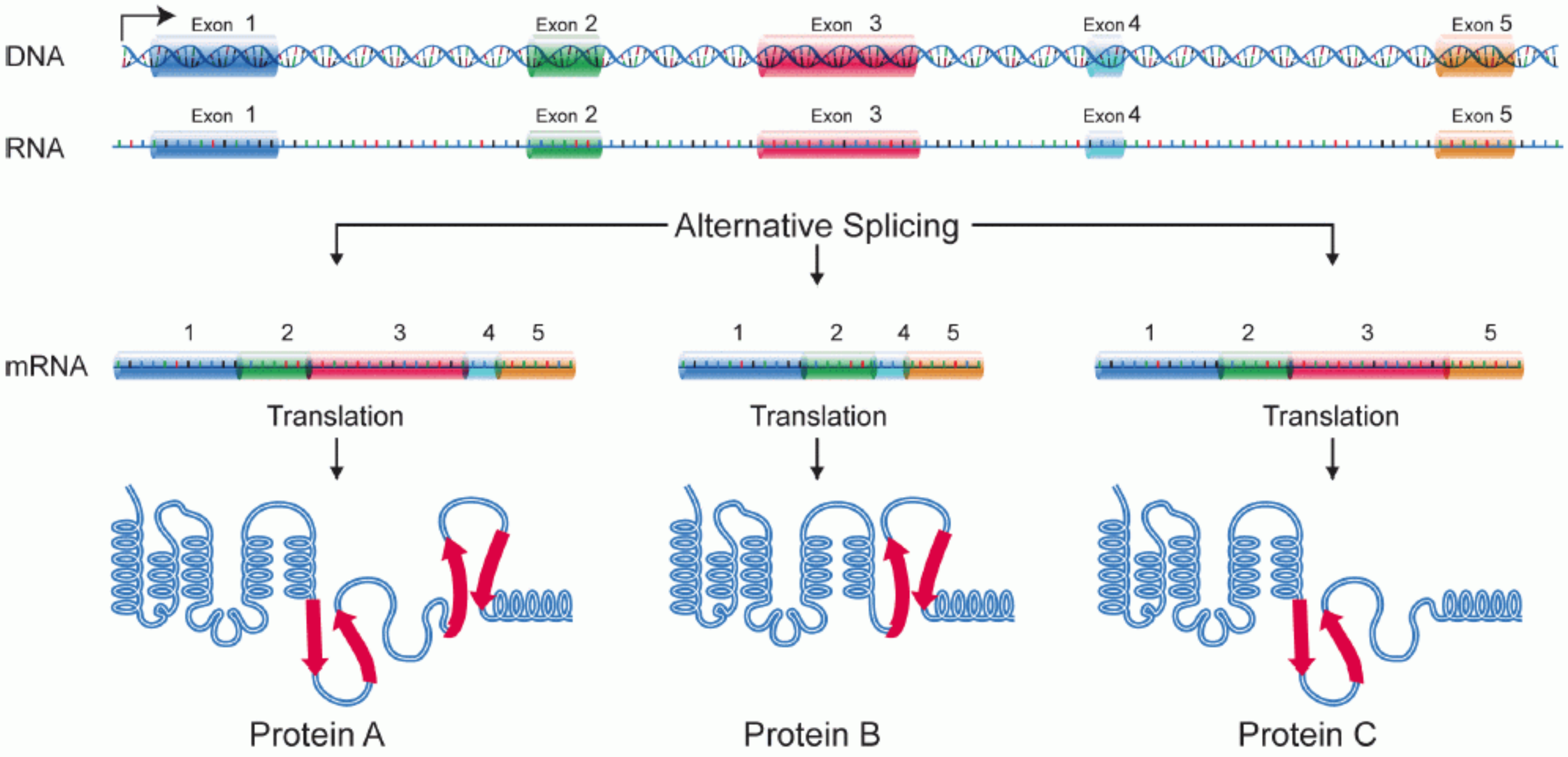
“Effective library sizes” (edgeR) vs “size factors” (DESeq2)



Other types of normalization

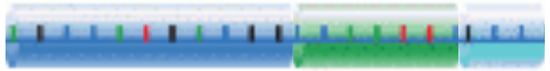
- Normalization factors can be computed based on a pre-determined subset of features that we “know” shouldn’t change between samples (spike-ins, house-keeping genes).
  - Need to assume that these features behave similarly to the endogenous genes.
  - May be required in targeted sequencing experiments or other settings where the assumption that “most genes don’t change” is not realistic.

Making use of the transcript abundances



Wikipedia

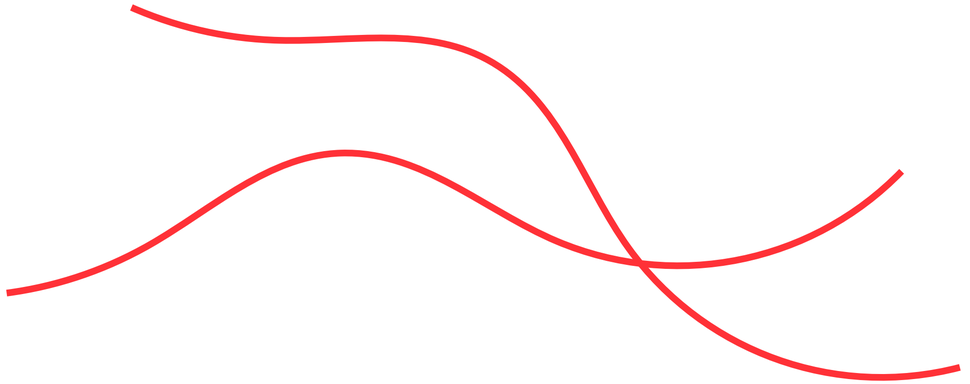
Impact of differential isoform usage on gene-level counts

T1  length = **L**

T2  length = **2L**

sample 1

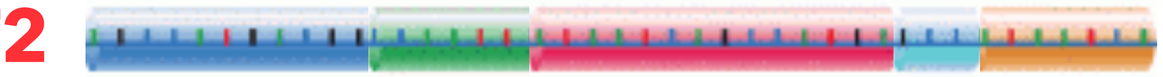
sample 2



Impact of differential isoform usage on gene-level counts

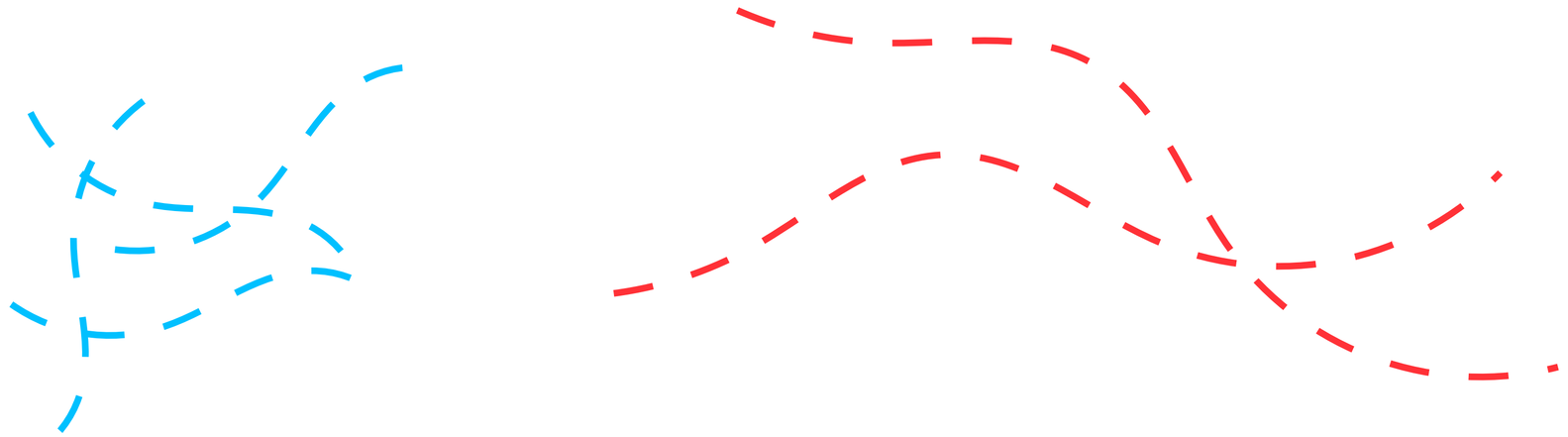
T1 length = **L**

T2 length = **2L**

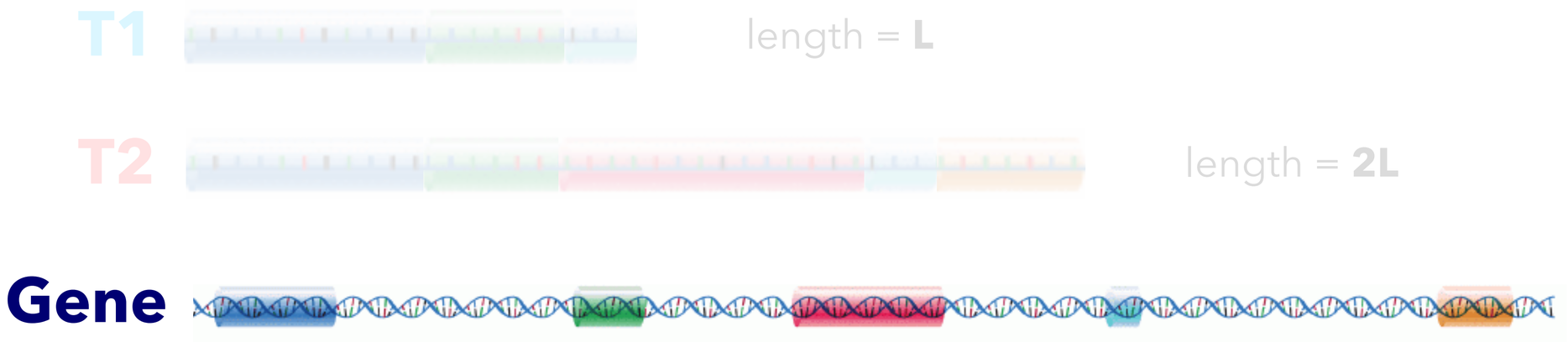


sample 1

sample 2

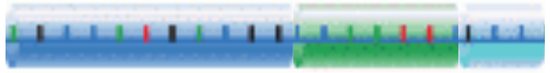


Impact of differential isoform usage on gene-level counts



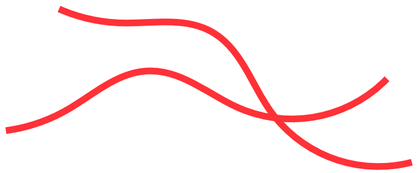
Gene	S1	S2
Count	150	150

Average transcript lengths

T1  length = **L**

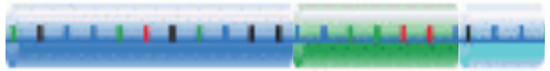
T2  length = **2L**

$$ATL_{g1} = 1 \cdot L + 0 \cdot 2L = L$$

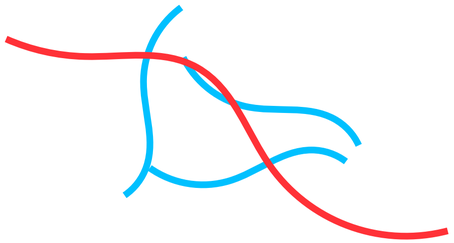


$$ATL_{g2} = 0 \cdot L + 1 \cdot 2L = 2L$$

Average transcript lengths

T1  length = **L**

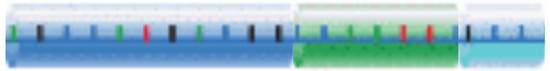
T2  length = **2L**



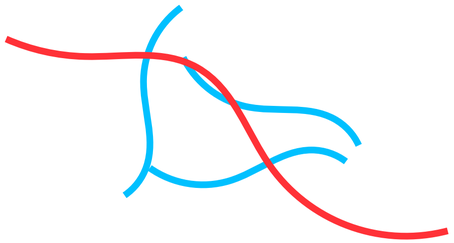
$$ATL_{g1} = 0.75 \cdot L + 0.25 \cdot 2L = 1.25L$$

$$ATL_{g2} = 0.5 \cdot L + 0.5 \cdot 2L = 1.5L$$

Average transcript lengths

T1  length = **L**

T2  length = **2L**

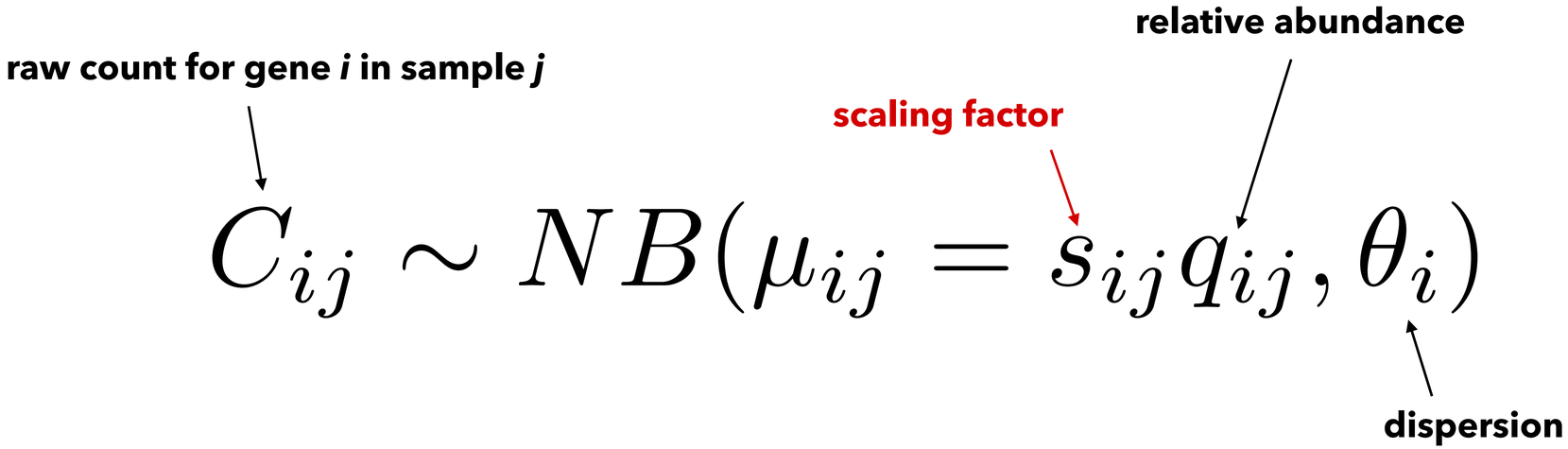


$$ATL_{g1} = 0.75 \cdot L + 0.25 \cdot 2L = 1.25L$$

$$ATL_{g2} = 0.5 \cdot L + 0.5 \cdot 2L = 1.5L$$

weights obtained from transcript TPM estimates

Offsets (“scaling factors”)



- Extend scaling factor for given sample and gene to include the average length of the transcripts from the gene that are present in the sample

Offsets ("average transcript lengths")

- Similar to correction factors for library size, but sample- **and** gene-specific
- Transcript abundance levels (TPMs) can be obtained from (e.g.) Salmon or kallisto
- Average transcript length for gene *g* in sample *s*:

$$ATL_{gs} = \sum_{i \in g} \theta_{is} \bar{\ell}_{is}, \qquad \sum_{i \in g} \theta_{is} = 1$$

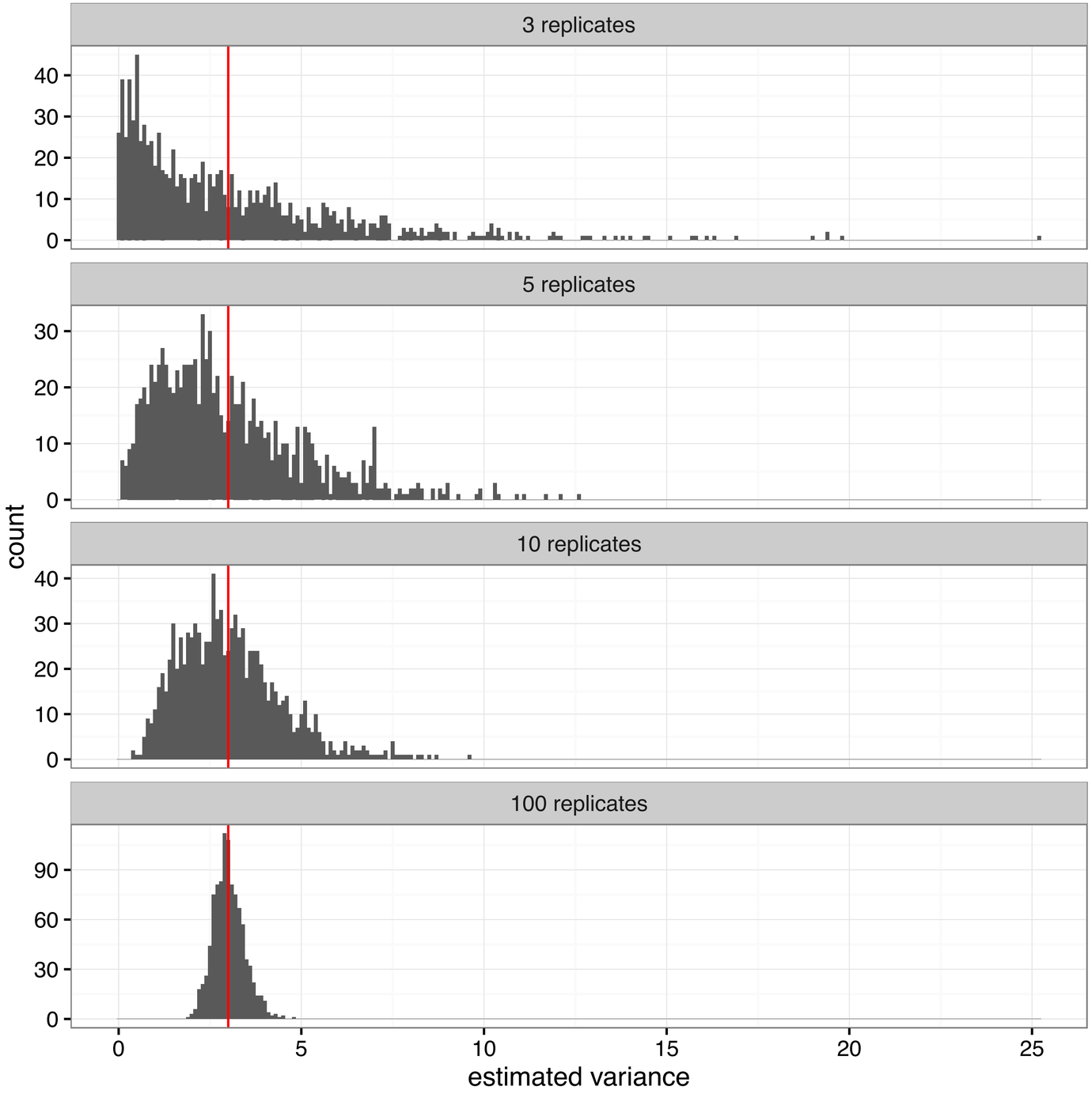
$$\bar{\ell}_{is} = \text{effective length of isoform } i \text{ (in sample } s)$$
$$\theta_{is} = \text{relative abundance of isoform } i \text{ in sample } s$$

PARAMETER ESTIMATION

Example:

estimate variance of normally distributed variable

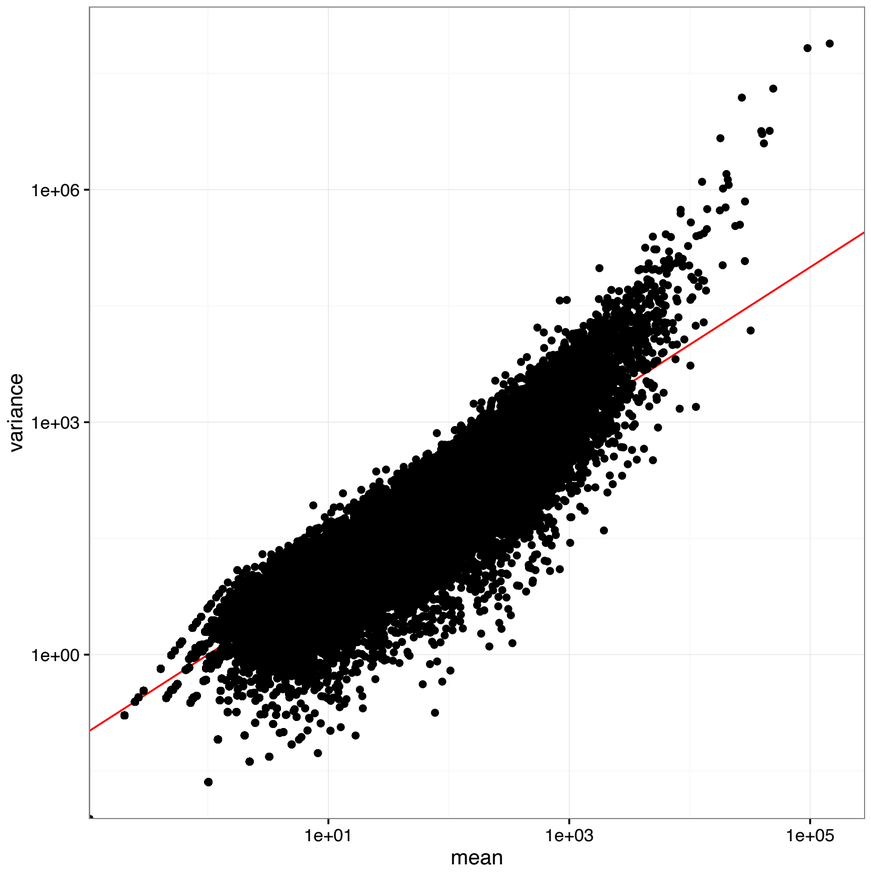
True value = 3



Modeling counts

- **Negative binomial distribution**
  - $var(X) = \mu + \theta\mu^2$
  - θ = dispersion
  - $\sqrt{\theta}$ = "biological coefficent of variation"
  - Allows mRNA proportions to vary across samples (according to a gamma distribution)
  - Captures variability across biological replicates better

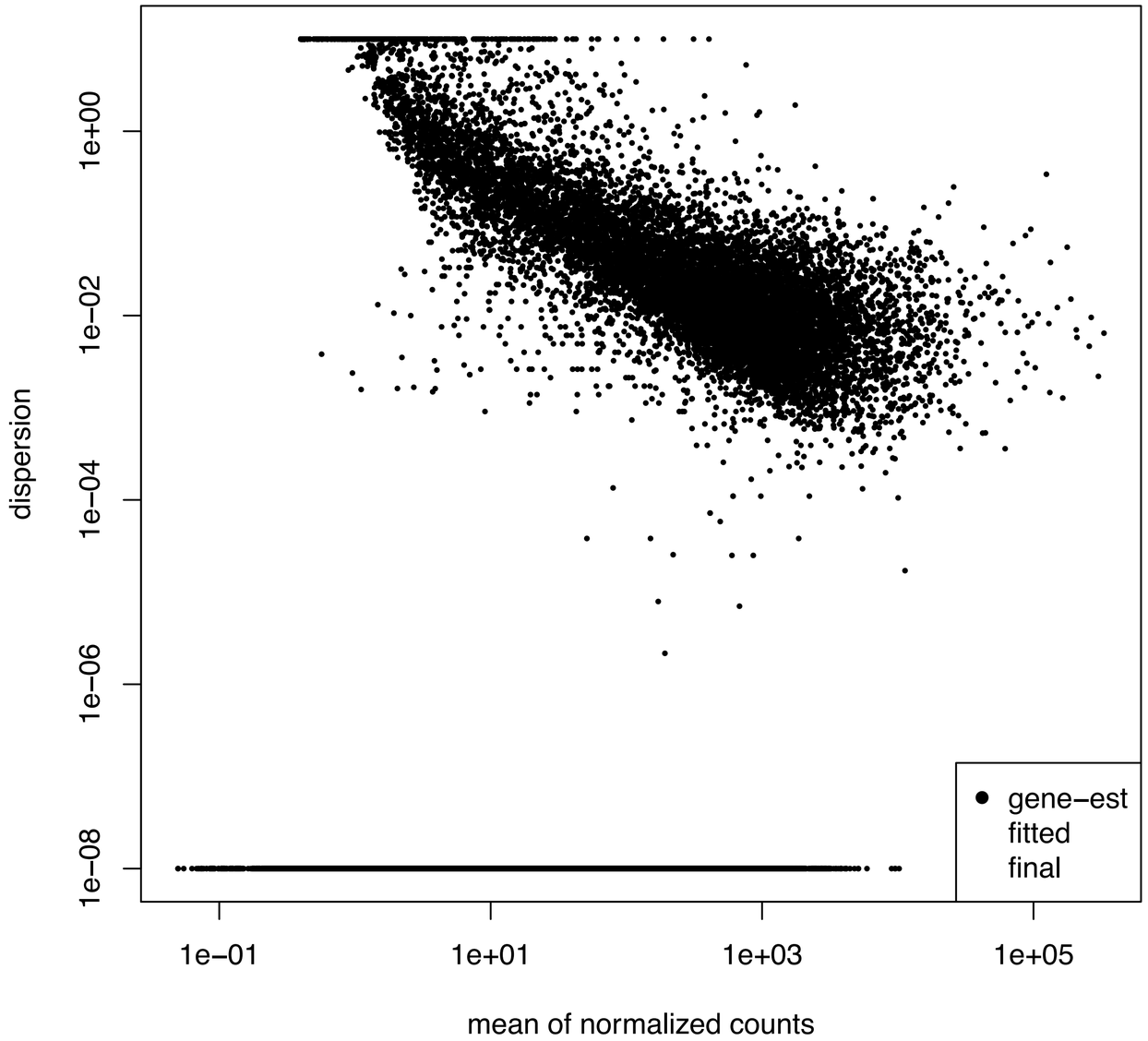
Example from SEQC data, replicates of the same RNA mix



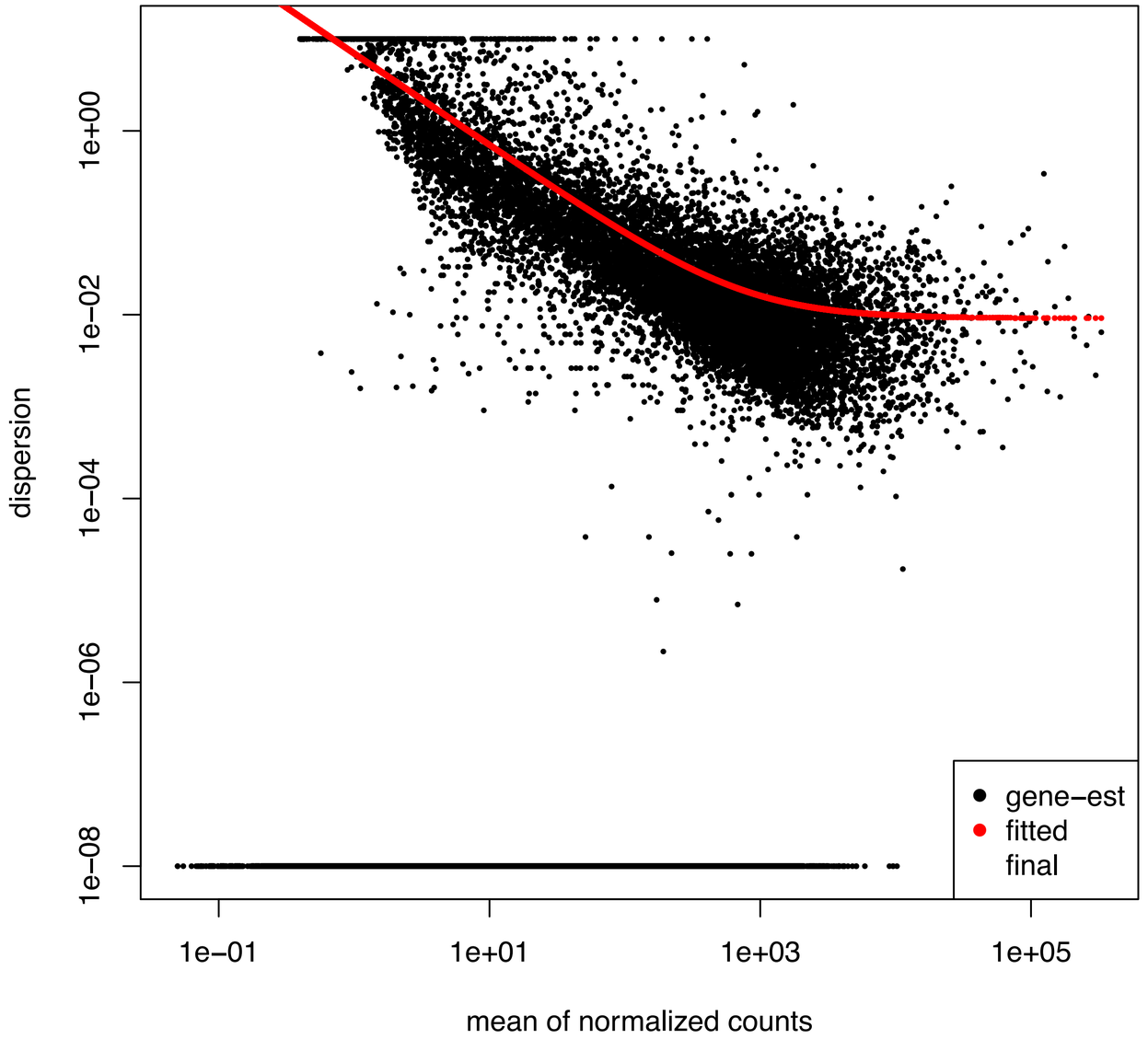
Shrinkage dispersion estimation

- Take advantage of the large number of genes
- Shrink the gene-wise estimates towards a center value defined by the observed distribution of dispersions across
  - all genes (“common” dispersion estimate)
  - genes with similar expression (“trended” dispersion estimate)

Shrinkage dispersion estimation



Shrinkage dispersion estimation



Shrinkage dispersion estimation