# Statistical analysis of RNA-seq

## Normalisation and differential expression

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# Aims of normalisation

Normalisation aims to ensure our expression estimates are:

- **comparable across features** (genes, isoforms, etc)
- **comparable across libraries** (different samples)
- **on a human-friendly scale** (interpretable magnitude)

Necessary for valid inference about DE

- between transcripts within samples
- between samples belonging to different biological conditions

# Basic Poisson model

Number of reads from gene $g$ in library $i$ can be captured by a Poisson model (Marioni et al. 2008):

$$\begin{aligned} r_{ig} &\sim Poisson(k_{ig}\mu_{ig}), \\ \implies \mathbb{E}(r_{ig}) &= k_{ig}\mu_{ig} \end{aligned}$$

where $\mu_{ig}$ is the concentration of RNA in the library and $k_{ig}$ is a normalisation constant.

$$\boxed{\hat{\mu}_{ig} = \frac{r_{ig}}{k_{ig}}}$$

# RPKM normalisation

Normalisation is a procedure for setting $k_{ig}$ such that the estimates of $\mu_{ig}$ are comparable between genes and across libraries.

$$\hat{\mu}_{ig} = \frac{r_{ig}}{k_{ig}}$$

The number of reads $r_{ig}$ is roughly proportional to

- the length of the gene, $l_g$
- the total number of reads in the library, $N_i$

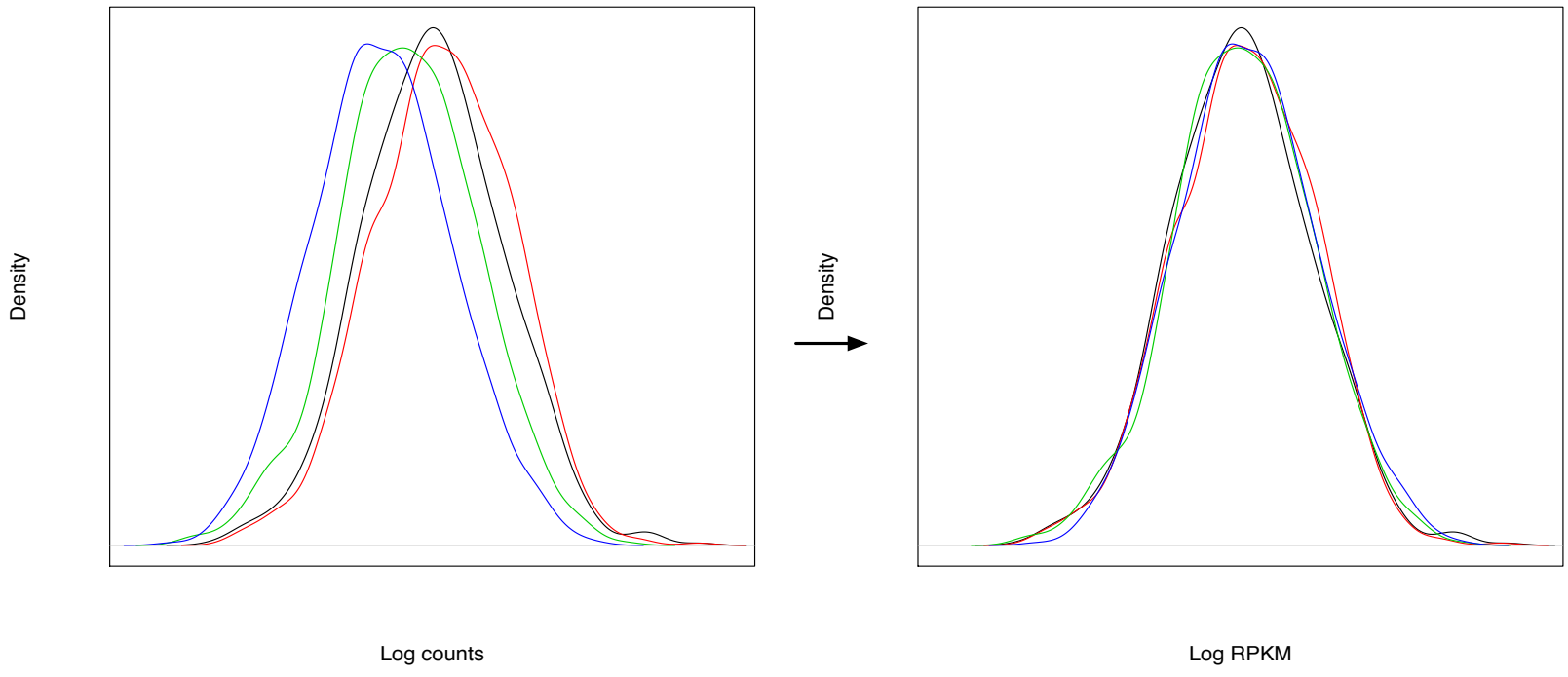
Thus it is natural to include them in the normalisation constant.

If $k_{ig} = 10^{-9} N_i l_g$, the units of $\hat{\mu}_{ig}$ are Reads Per Kilobase per Million mapped reads (RPKM) (Mortazavi et al. 2008).

This is the most elementary form of normalisation.

# RPKM normalisation

- RPKM works well for technical and some biological replicates
- $\mu_{ig} \simeq \mu_{jg}$ for all libraries $i$ and $j$
- RPKM units obtained by scaling of counts by $N_i^{-1}$

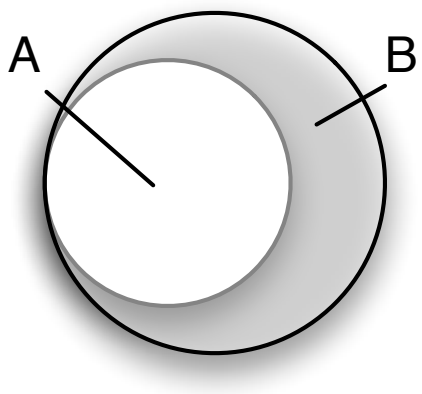


# Sample to sample normalisation

- Between different biological samples, homogeneity assumption does not hold
- Why is this a problem?

**Number of reads is limited**

E.g. counts from very highly expressed genes leave less real estate available for counts from lowly expressed genes




- Suppose you have two RNA populations A and B sequenced at same depth
- A and B are identical except half of genes in B are unexpressed in A
- Only $\sim$ half of reads from B come from shared gene set
- Estimates for shared genes differ by factor of $\sim 2$!

Robinson and Oslack 2010

# Poisson approximation to Binomial

- Total RNA output, $\sum_g \mu_{ig} l_g$, inversely affects read counts $r_{ig}$ (for fixed $\mu_{ig}$)
- RPKM normalisation assumes implicitly that total RNA output (unknown) is the same for all libraries:

$$r_{ig} \sim \textit{Binomial}\left(N_i, \frac{\mu_{ig} l_g}{\sum_g \mu_{ig} l_g}\right)$$

$$\sim \textit{Poisson}\left(N_i \frac{\mu_{ig} l_g}{\sum_g \mu_{ig} l_g}\right) \text{ as } N \to \infty$$

$$\implies \mathbb{E}(r_{ig}) = N_i \frac{\mu_{ig} l_g}{\sum_g \mu_{ig} l_g}$$

- RPKM assumption: $\forall i, \sum_g \mu_{ig} l_g = 10^9$ (so $\hat{\mu}_{ig} = \frac{r_{ig}}{10^{-9} N_i l_g}$)
- Better assumption: output between samples for a *core set only* of genes $G$ is similar: $\sum_{g \in G} \mu_{ig} l_g = \sum_{g \in G} \mu_{jg} l_g$

# TMM normalisation

The naive MLE is proportional to the normalised counts:

$$\hat{\mu}_{jg} = \frac{r_{jg}}{k_{jg}} = \frac{1}{10^{-9} l_g} \frac{r_{jg}}{N_j}$$

If $\sum_{g \in G} \hat{\mu}_{ig} l_g \neq \sum_{g \in G} \hat{\mu}_{jg} l_g$, the MLEs for *all* genes need to be adjusted.

Calculate scaling factor for sample $j$ relative to reference sample $i$:

$$\sum_{g \in G} \frac{r_{ig}}{N_i} \simeq S^{(i,j)} \sum_{g \in G} \frac{r_{jg}}{N_j}.$$

Adjust the MLEs for sample $j$ for *all* genes:

$$\hat{\mu}_{jg} = \frac{r_{jg}}{k_{jg}} = \frac{r_{jg}}{10^{-9} N_j l_g} \cdot S^{(i,j)}.$$

Robinson and Oslack 2010

# TMM normalisation

How to choose the subset $G$ used to calculate $S^{(i,j)}$?

- For pair of libraries $(i, j)$ determine log fold change of normalised counts

$$M_g^{(i,j)} = \log \frac{r_{ig}}{N_i} - \log \frac{r_{jg}}{N_j}.$$

- and the mean of the log normalised counts

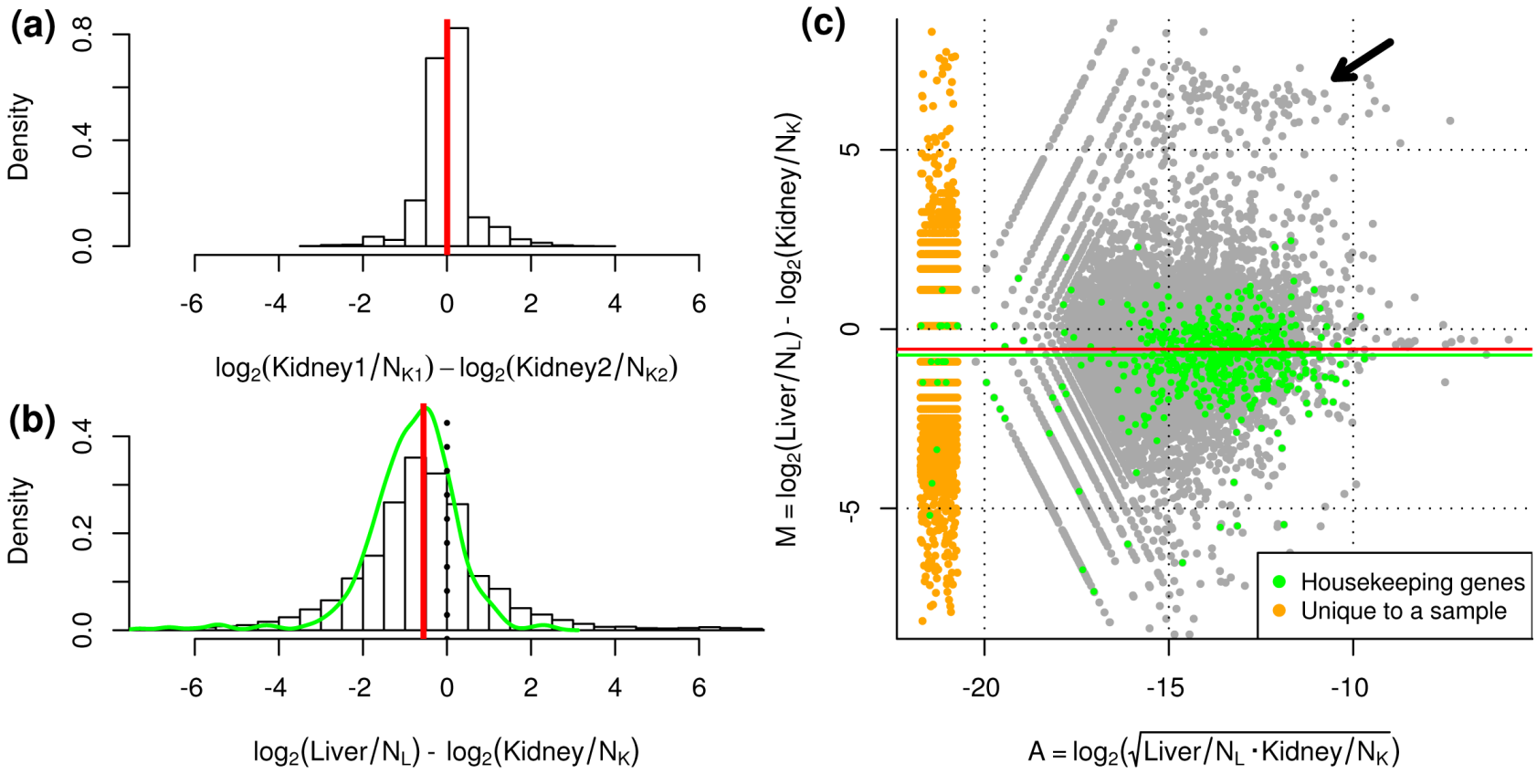
$$A_g^{(i,j)} = \frac{1}{2}\left[\log \frac{r_{ig}}{N_i} + \log \frac{r_{jg}}{N_j}\right].$$

- Set $G$ to genes remaining after trimming upper and lower $x\%$ of the $\{A_g\}$ and $\{M_g\}$. I.e. genes in $G$ have unexceptional values of $A_g^{(i,j)}$ and $M_g^{(i,j)}$

Robinson and Oslack 2010

# TMM normalisation (with edgeR)

- Compute summary of $\{M_g^{(i,j)}\}$ for genes in $G$ (weighted mean)
- Let $S^{(i,j)}$ be the exponential of this summary
- Adjust $\hat{\mu}_{jg}$ by a factor of $S^{(i,j)}$ for all genes $g$




Robinson and Oslack 2010

# Median log deviation normalisation (with DESeq)

An alternative normalisation provided in DESeq package

- For each gene $g$ in sample $i$, calculate deviation of $\log r_{ig}$ from the mean $\log r_{ig}$ over all libraries: $d_{ig} = \log r_{ig} - \frac{1}{I} \sum_i \log r_{ig}$.
- Calculate median over all genes: $\log S^{(i)} = \text{median}_i(d_{ig})$
- Adjust $\hat{\mu}_{ig}$ by a factor of $S^{(i)}$ for all genes $g$

edgeR and DESeq are both robust across genes (weighted mean of core set vs. median of all genes)

Call $\tilde{N}_i = \frac{N_i}{S_i}$ the "adjusted library size".

Anders and Huber 2010

# Normalisation between genes

- So far we have looked at library-level scaling to make the expression of a given gene comparable across libraries
- In other words, we have been seeking to account for factors affecting all genes in a library similarly
- Are there factors affecting different genes differently?
- Recall normalisation equation:

$$\boxed{\hat{\mu}_{ig} = \frac{r_{ig}}{k_{ig}}}$$

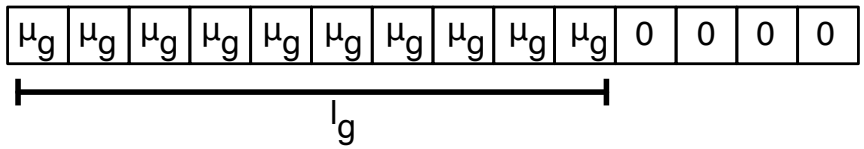
Consider the decomposition of $k_{ig} = kk_i k_g$

- $k$: global scaling to get more convenient units. E.g. $10^{-9}$.
- $k_i$: library-specific normalisation factors. E.g. $\tilde{N}_i = N_i / S^{(i)}$
- $k_g$: gene-specific normalisation factors. E.g. $l_g$

# Normalisation between genes

Where does the $l_g$ factor come from anyway?

Underlying assumption: constant Poisson rate across bases.




$$r_{igp} \sim Pois(kk_i \mu_g)$$

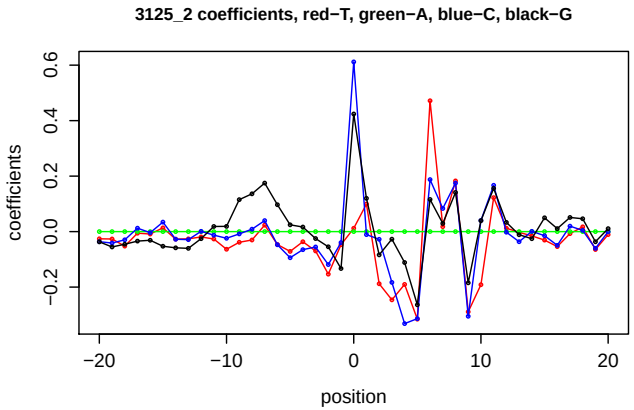
$$r_{ig} = \sum_{p=1}^{l_g} r_{igp}$$

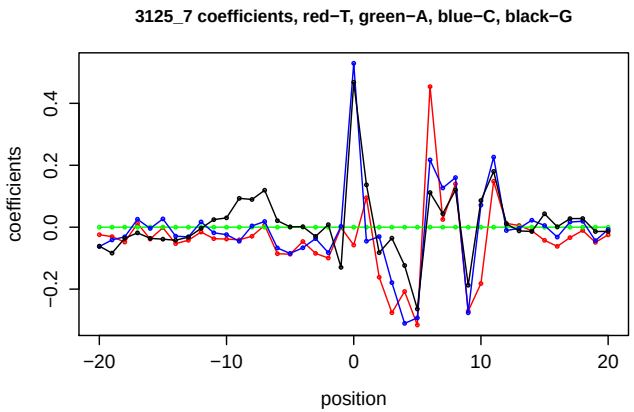
$$r_{ig} \sim Pois(kk_i \sum_{p=1}^{l_g} \mu_g)$$

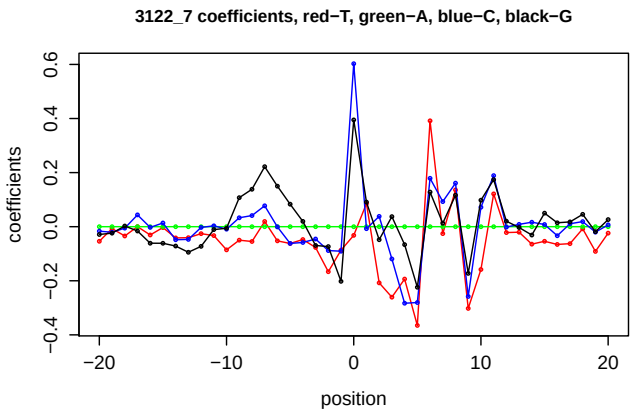
$$\sim Pois(kk_i l_g \mu_g)$$

$$\sim Pois(10^{-9} \tilde{N}_i l_g \mu_{ig})$$

# Normalisation between genes

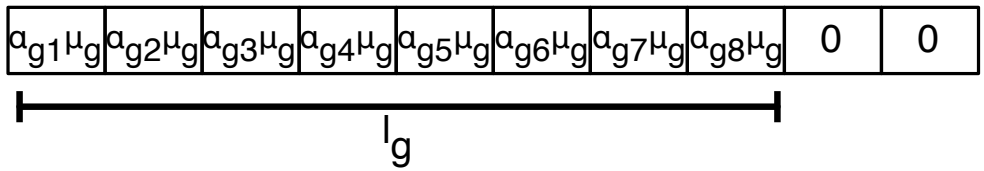



There are in fact local sequence-specific biases (Li et al. 2010, Hansen et al. 2010) (non-random amplification?).

This suggests a variable-rate model with weights $\alpha_{gp}$:

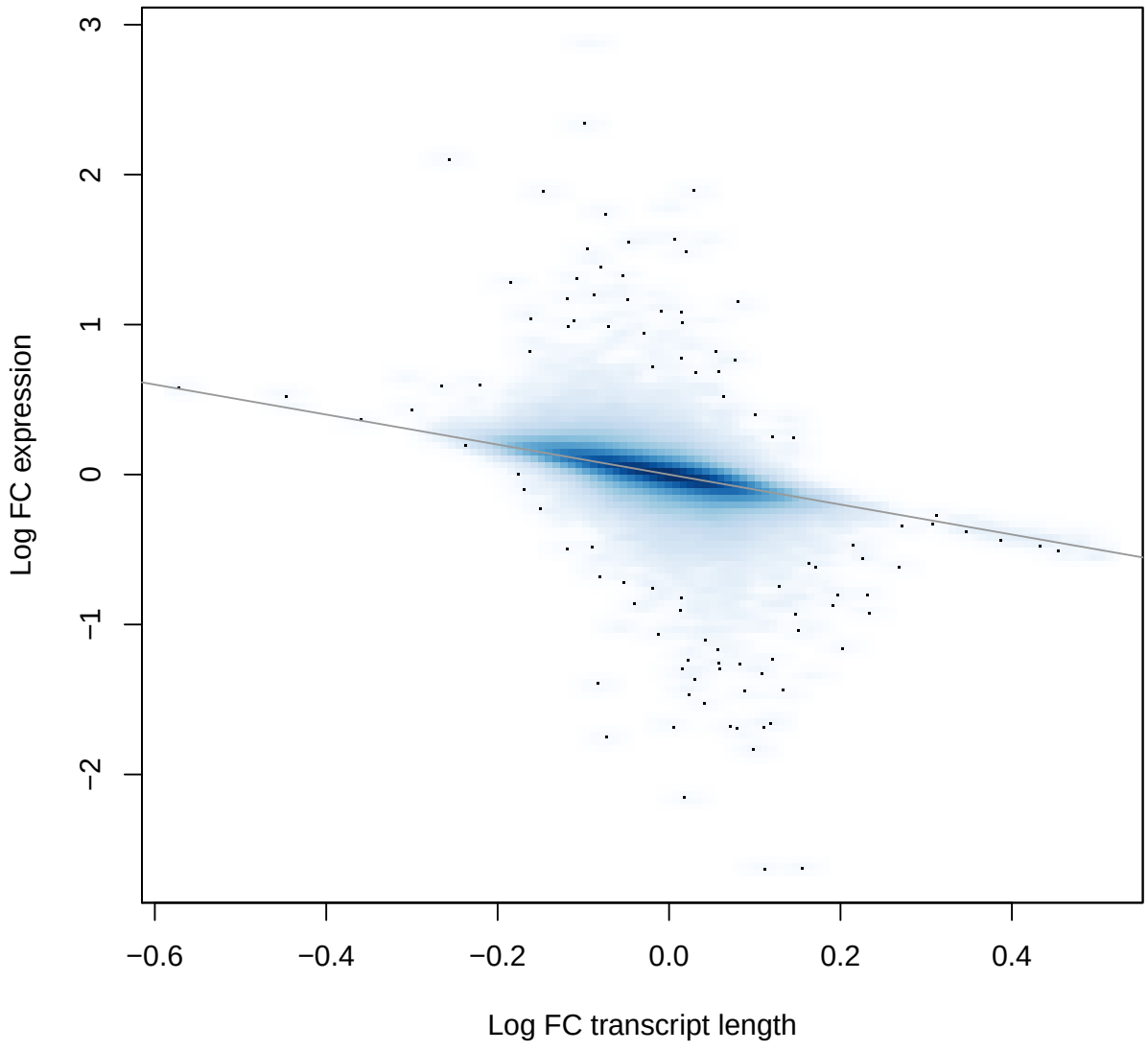



$$r_{ig} \sim Pois(kk_i \sum_{p=1}^{l_g} \alpha_{gp}\mu_{ig})$$

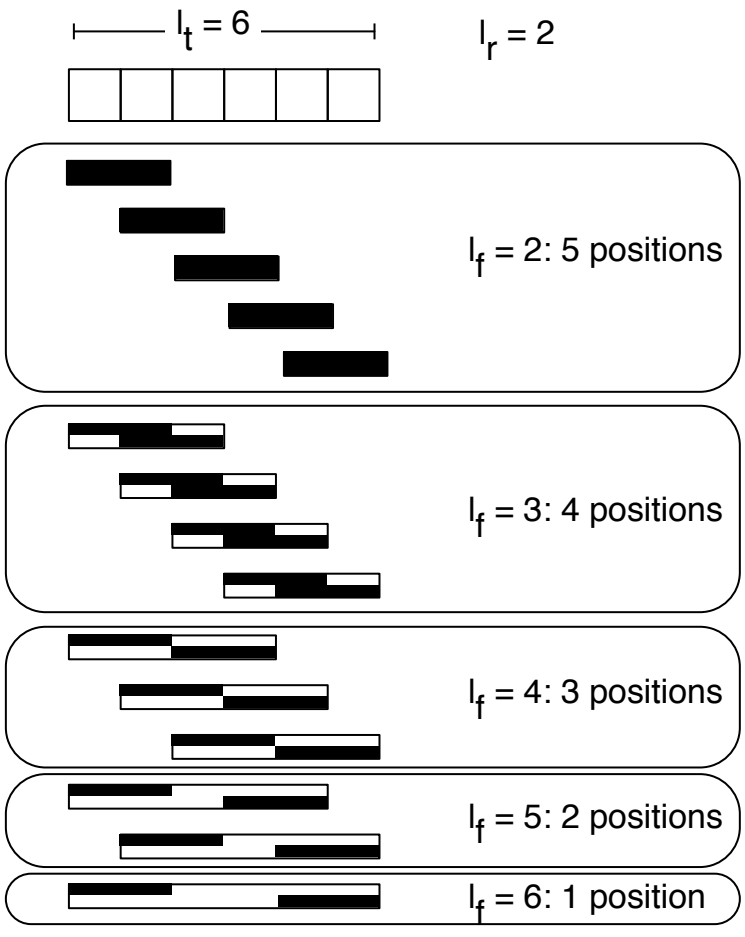
$$\sim Pois(kk_i \tilde{l}_g \mu_{ig})$$

$$\sim Pois(10^{-9}\tilde{N}_i \tilde{l}_g \mu_{ig})$$

# Accounting for sequencing biases with mseq



# Normalisation between genes (adjust for insert size distro)




$$\tilde{l_t} = \sum_{l_f=l_r}^{l_t} p(l_f|l_t)(l_t - l_f + 1)$$

(assuming each position equally likely)

$$\tilde{l_t} = \sum_{l_f=l_r}^{l_t} p(l_f|l_t) \sum_{p=1}^{l_t-l_f+1} \alpha(p,t,l_f)$$

(weight $\alpha(p,t,l_f)$ for fragments of length $l_f$ at position $p$, transcript $t$)

If pre-selection fragments roughly uniform up to $l_t$ within insert size distribution, then $p(l_f|l_t) \simeq p(l_f)$

Glaus et al 2012

# Differential expression

We have obtained library and gene specific normalisation factors to make counts/concentration estimates as comparable as possible.

This allows us to:

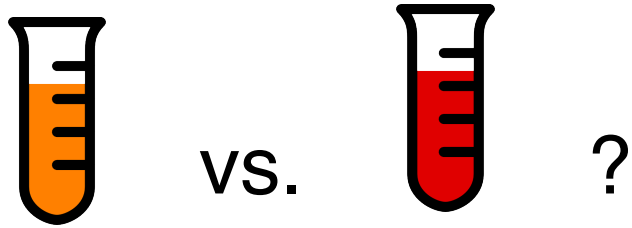
- obtain reasonably unbiased log fold changes between two groups of samples
- obtain $p$-values under the null hypothesis of no differential expression

Recall hypothesis testing:

- define a function of the data, $T$ (the *test statistic*)
- derive distribution of $T$ under the null (e.g. no DE)
- define critical regions of $T$
- compute observed value $t$ from actual data
- reject null if $t$ is in a critical region

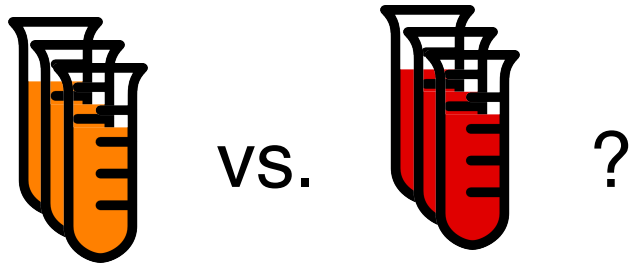
# Differential expression

Option 1. $H_0 : \mu_{1g} = \mu_{2g}$




Option 2.

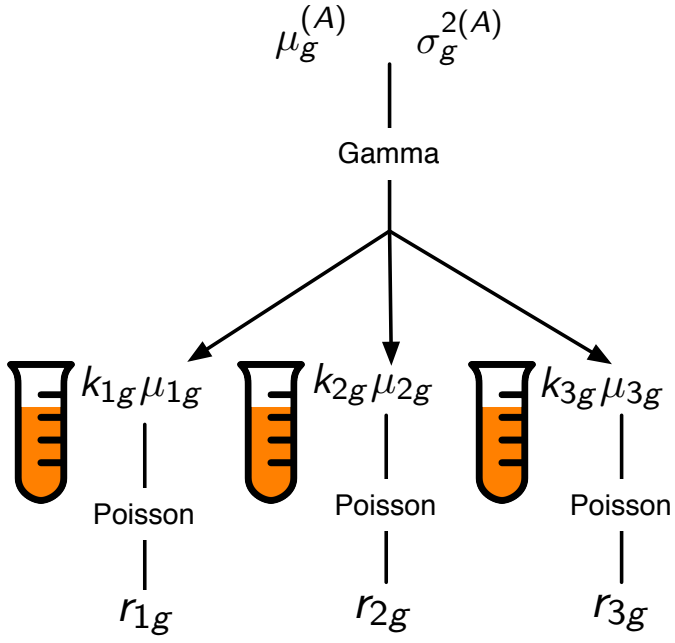
$H_0 : \mu_{1g} = \mu_{2g} = \mu_{3g} = \mu_g^{(A)} = \mu_{4g} = \mu_{5g} = \mu_{6g} = \mu_g^{(B)}$




Both options are inadequate!

$\mu_{ig}$ is the RNA concentration parameter for library $i$, which *varies* across biological replicates.

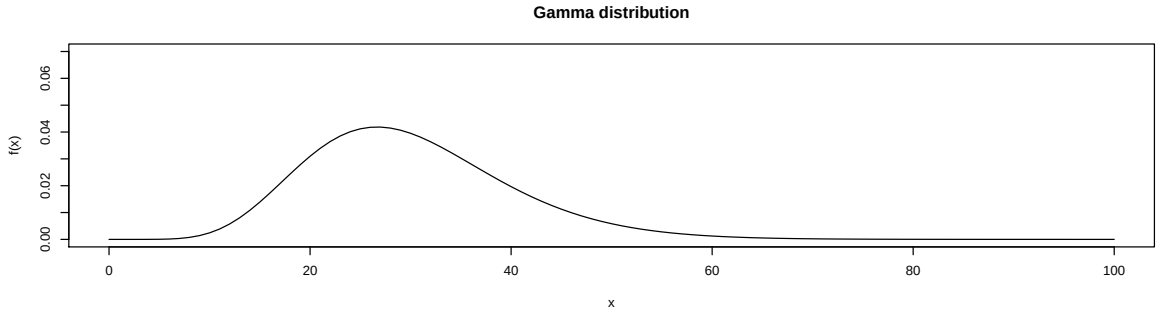
# Negative binomial distribution



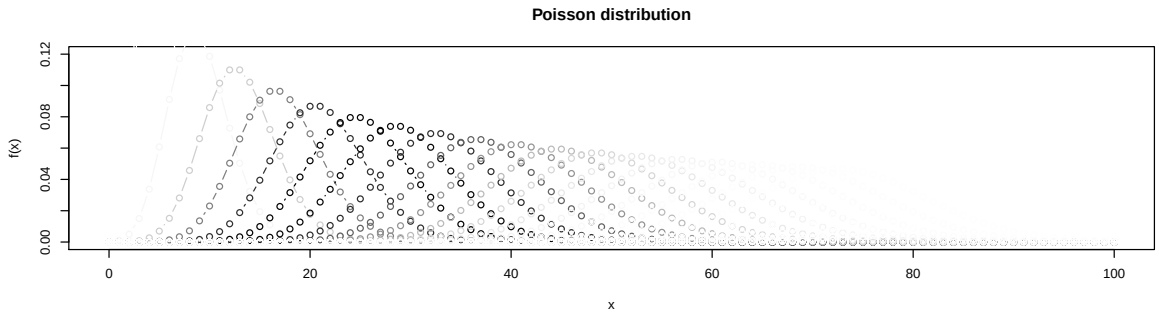

If the rate parameter of the Poisson distribution is not fixed, but varies according to a Gamma distribution, then the counts follow a **negative binomial distribution**.

Unlike the Poisson, the variance is greater than the mean.

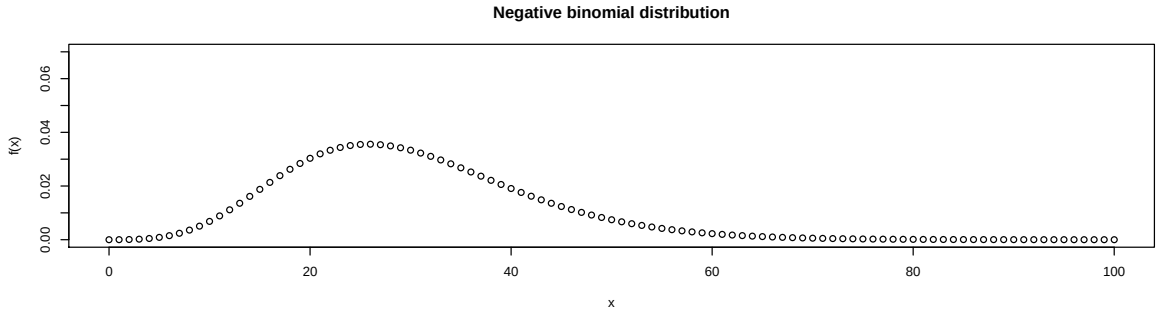
# Negative binomial distribution




Biological variance




Poisson noise




Read count variance

$$\text{Mean(NB)} = \text{mean(Gamma)} = \mathbb{E}(\text{mean(Poisson)})$$
$$\text{Var(NB)} = \text{var(Gamma)} + \mathbb{E}(\text{var(Poisson)})$$

# Negative binomial distribution

Number of reads from gene $g$ in library $i$ of condition $c$ can be captured by a negative binomial model:

$$r_{cig} = NB(k_{ig}\mu_{cg}, s_{cg})$$

where $\mu_{cg}$ and $s_{cg}$ are, respectively, the mean and dispersion for reads from gene $g$ in condition $c$.

The variance has two components:

$$\sigma^2_{cg} = k_{ig}\mu_{cg} + k^2_{ig}\mu^2_{cg}s_{cg}$$

Poisson noise Overdispersion

- Notice there are now **two parameters to estimate**
- How do we obtain precise estimates of the dispersion if we have a small number of libraries per condition?

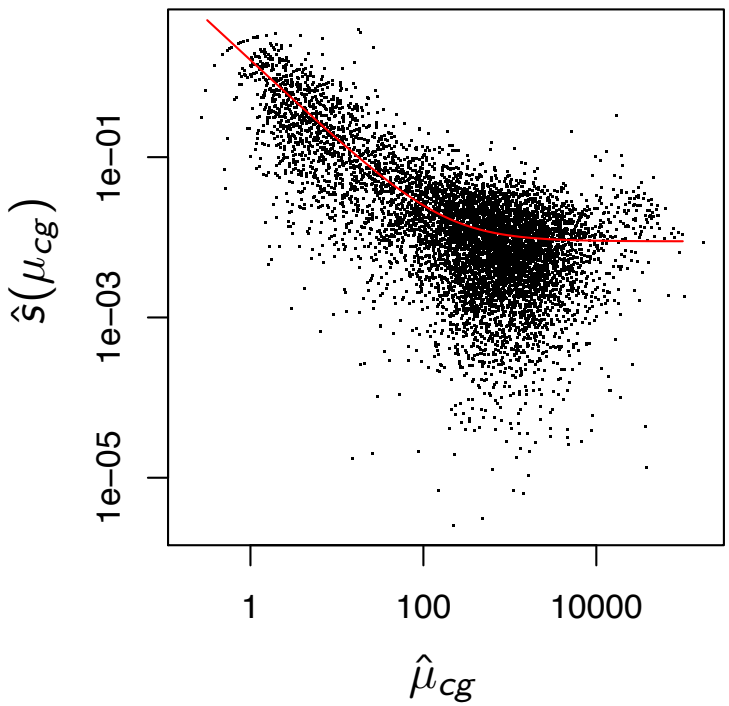
# DESeq

How do we estimate the variance robustly?

Assumption: **dispersion is a smooth function of the mean**.

$$\sigma^2_{cg} = k_{ig}\mu_{cg} + k^2_{ig}\mu^2_{cg}s(\mu_{cg})$$

Poisson noise Overdispersion




Use fitted values (or values above the line) instead of raw estimates.

This is a form of pooling (sharing of information across genes) to stabilise the estimates.

Anders and Huber 2010

# Differential expression with DESeq

Back to hypothesis testing...

$$r_{cig} = NB(k_{ig}\mu_{cg}, s_{cg})$$

$H_0 : \mu_{1g} = \mu_{2g}$.

Perform a negative binomial exact test.

**How extreme is the partitioning of counts between the two conditions under the null?**

# Differential expression with DESeq

Let the observed condition-specific counts be $q^*_{cg} = \sum_i r_{cig}$.

The probability of the data under the null is $P^* = P(q^*_{1g}, q^*_{2g} | \hat{\mu}_g, \hat{\sigma}^2_g)$.

Obtain gene-wise exact $p$-values:

$$p_g = \frac{\sum_{q_{1g}, q_{2g} : P(q_{1g}, q_{2g} | \hat{\mu}_g, \hat{\sigma}^2_g) < P^* \wedge q_{1g} + q_{2g} = q^*_{1g} + q^*_{2g}} P(q_{1g}, q_{2g} | \hat{\mu}_g, \hat{\sigma}^2_g)}{\sum_{q_{1g}, q_{2g} : q_{1g} + q_{2g} = q^*_{1g} + q^*_{2g}} P(q_{1g}, q_{2g} | \hat{\mu}_g, \hat{\sigma}^2_g)},$$

where $\hat{\mu}_g$ and $\hat{\sigma}^2_g$ are estimates for the mean and variance under the null.

Anders and Huber 2010

# Differential isoform expression

- At the gene level, counts are often observed (however beware of isoform switching)
- At other levels (isoforms, haplo-isoforms) counts almost always have to be estimated (e.g. with MMSEQ) because reads map to multiple overlapping transcripts
- Count-based methods such as DESeq can be used to obtain differential isoform expression results by using estimated counts instead of observed counts
- A more powerful approach is to take into account posterior uncertainty in expression estimates (MMDIFF; Turro et al 2014)

# Concluding remarks

- Variation in total RNA output per sample leads to biases in expression estimates (limited real estate)
- Variation in sequence composition of genes leads to biases (non-random hexamer priming)
- Normalisation seeks to correct for these biases
- Biological and Poisson variability can be modelled with a negative binomial distribution
- Variance of negative binomial hard to estimate gene-by-gene (best to share information acrosss genes)
- Negative binomial exact test produces $p$-values under the null of no differential expression