

H2: Differential Expression with Next Generation Sequencing Platforms

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Statistical Genomics: Master of Science in Bioinformatics

Outline

1 Intro

- Technology
- Data
- Normalization

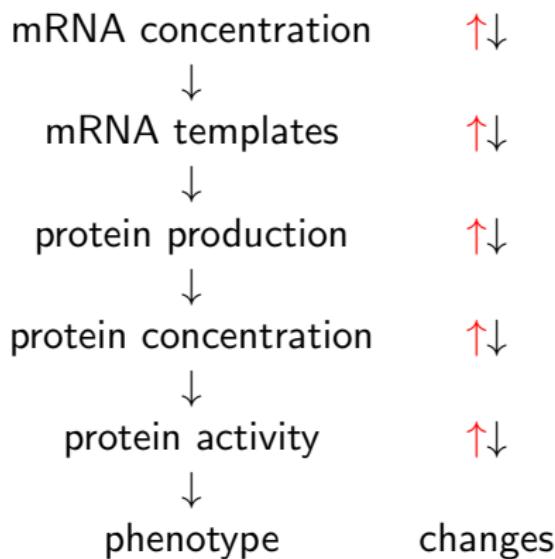
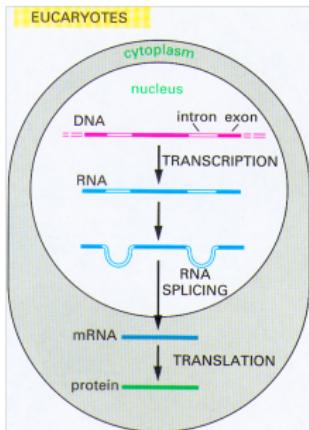
2 Statistical Model

- Poisson
- GLM
- Normalization
- Overdispersion

3 Statistical inference

Introduction

Central Dogma:



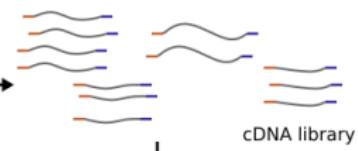
Sample of interest



Extract total RNA
and enrich targets



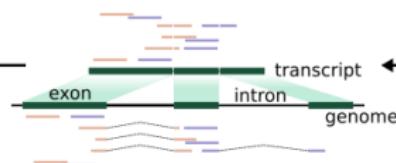
Fragment, reverse transcribe
ligate adapters, amplify



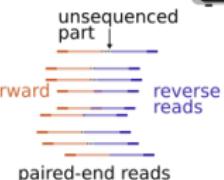
Data analysis

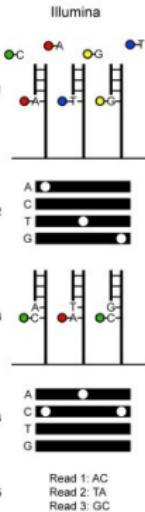
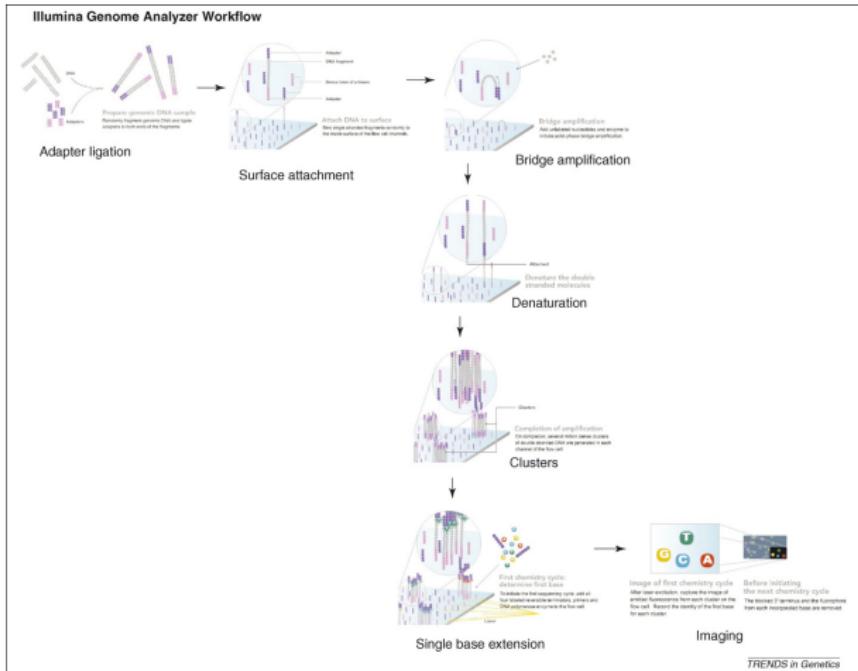
- differential expression
- variant calling
- annotation
- novel transcript discovery
- RNA editing
- ...

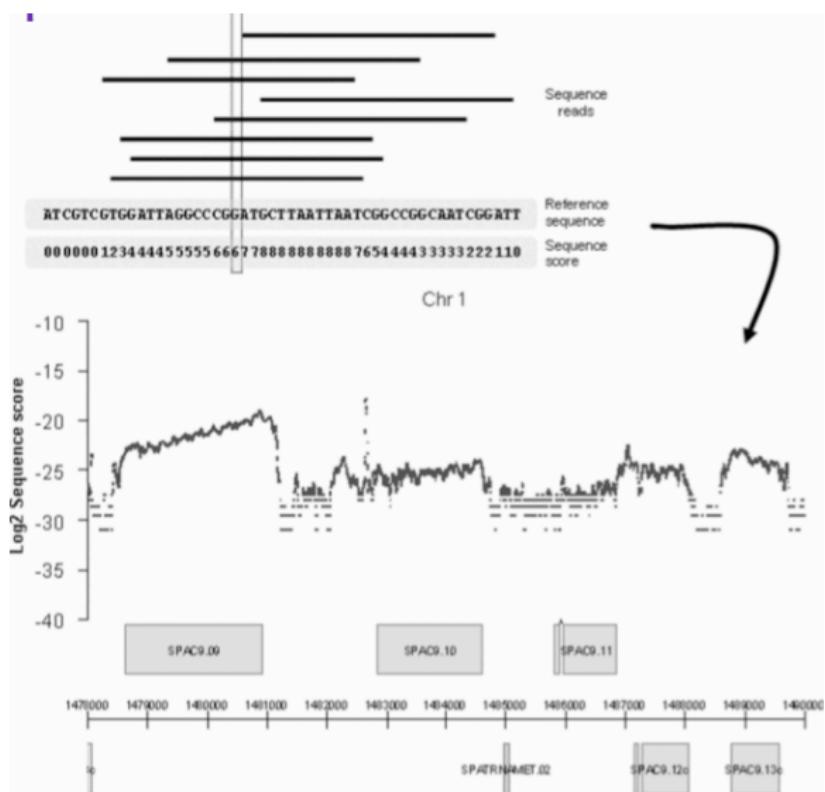
Transcriptome/genome mapping



Sequencing







Potential Problems

read counts



mapping, lane, flow cell, run bias

cDNA library



RNA extraction, rRNA, DNA conversion,...

mRNA levels



post transcriptional regulation, translation speed

protein levels



post translational regulation, modification, activity regulation...

phenotype

Potential Problems

read counts



mapping, lane, flow cell, run bias

cDNA library



RNA extraction, rRNA, DNA conversion,...

mRNA levels



post transcriptional regulation, translation speed

protein levels



post translational regulation, modification, activity regulation...

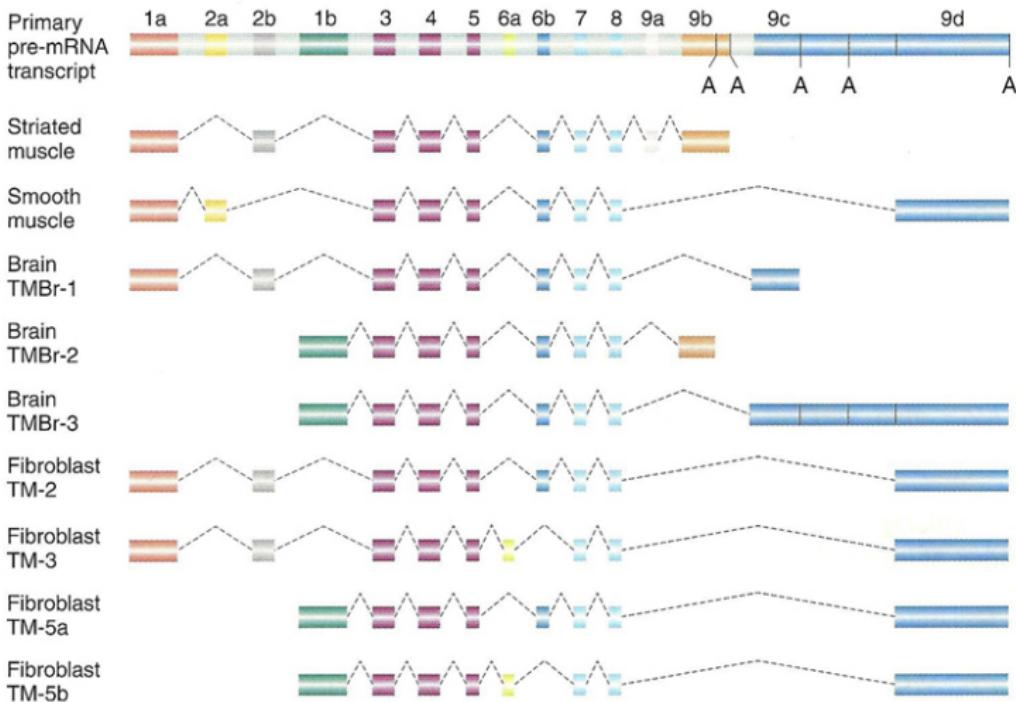
phenotype

- Number of reads depends on many factors
- expression level, total number of reads per library, transcript length etc.
- Here we focus on differences at gene level: transcripts have the same length.
- Systematic differences in read counts → systematic differences in mRNA levels



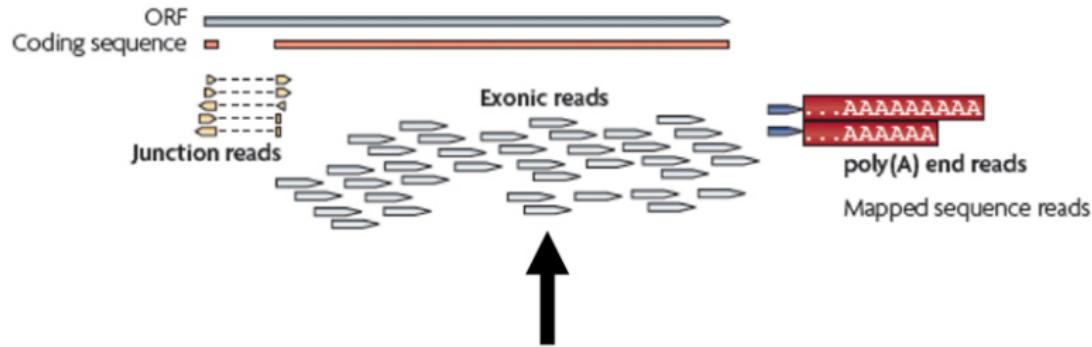
Alternative Splicing

Alternative splicing in tropomyosin



Alternative Splicing

Data from RNA-seq

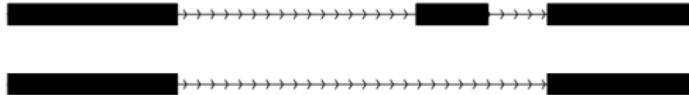
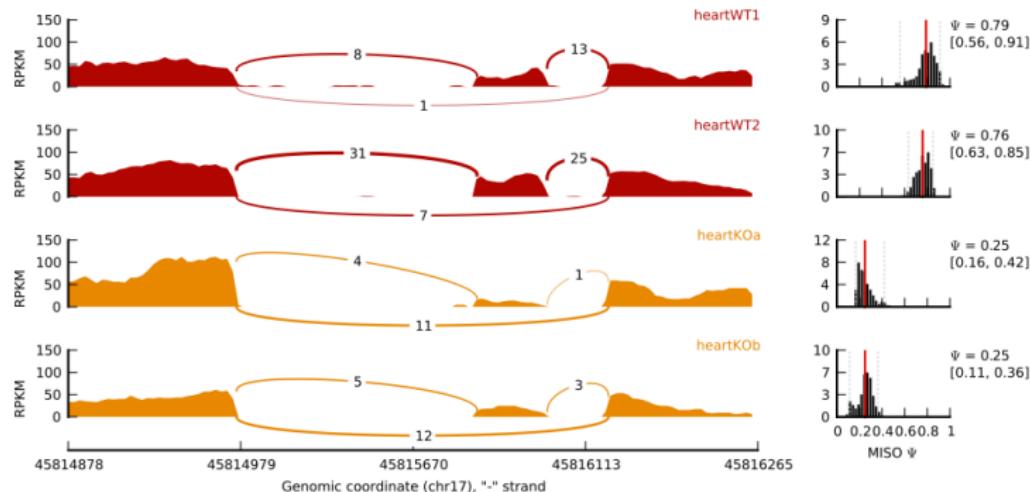


Determine mRNA abundance

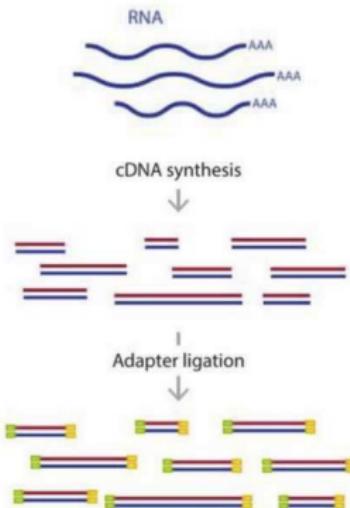
Infer relative level of expression

Alternative Splicing

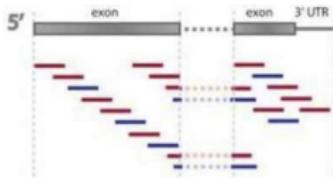
chr17:45816186:45816265:-@chr17:45815912:45815950:-@chr17:45814875:45814965:-



Single end vs paired end



Single-end sequencing



Paired-end sequencing

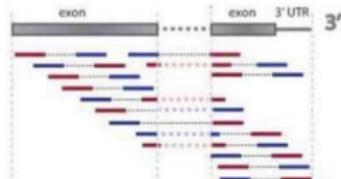
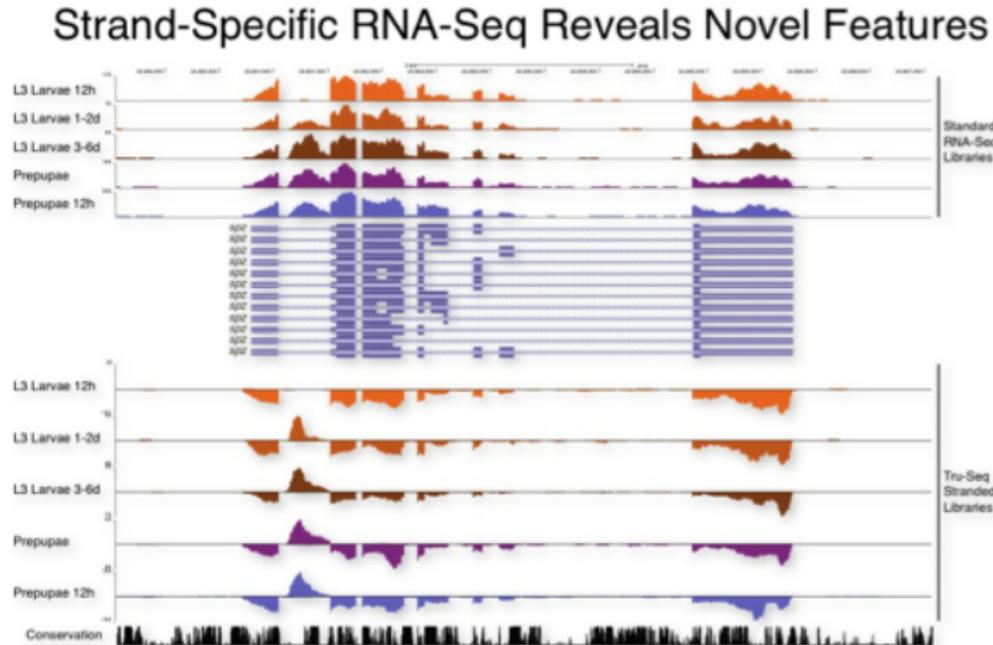


Image adapted from Zhereznova, et al., PLoS Genet. 2013 June; 9(6): e1003594.

Naive vs Strand specific

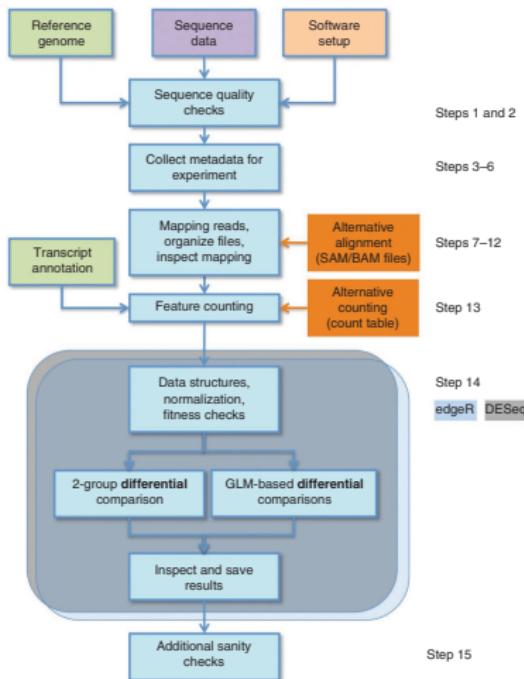


Steps in a RNA-seq Experiment

- ① Experimental design
- ② Experiment
- ③ Sampling
- ④ Library Prep
- ⑤ Sequencing/basecalling
- ⑥ Quality assessment of Reads
- ⑦ Read alignment to reference genome
- ⑧ Quality assessment of alignment
- ⑨ Summarization: read counts per feature (gene, exon, ...)
- ⑩ Gene Prioritization: Analysis of differential expression
- ⑪ Downstream Analysis



RNA-seq Data Analysis work flow



Anders et al. (2013) Nature Protocols.



Basecalling

Most researchers use standard base caller:

Illumina → Cassava → fastq files

http://en.wikipedia.org/wiki/FASTQ_format

Raw base quality QC

Quality score: $Q = -10 \log_{10} p$

fastQC: http:

//www.bioinformatics.babraham.ac.uk/projects/fastqc/

The screenshot shows the FastQC Report interface. At the top right, there's a banner for 'NEW OS X 10.9 MAMAK' with the text 'New design. Better apps. More ways your Mac works with iOS.' and a 'GET NOW' button. Below the banner, the main content area has a title 'FastQC Report' with a magnifying glass icon.

Summary

- ✓ Basic Statistics
- ✗ Per base sequence quality
- ✗ Per tile sequence quality
- ✓ Per sequence quality scores
- ! Per base sequence content
- ! Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ! Sequence Duplication Levels
- ! Overrepresented sequences
- ✓ Adapter Content
- ! Kmer Content

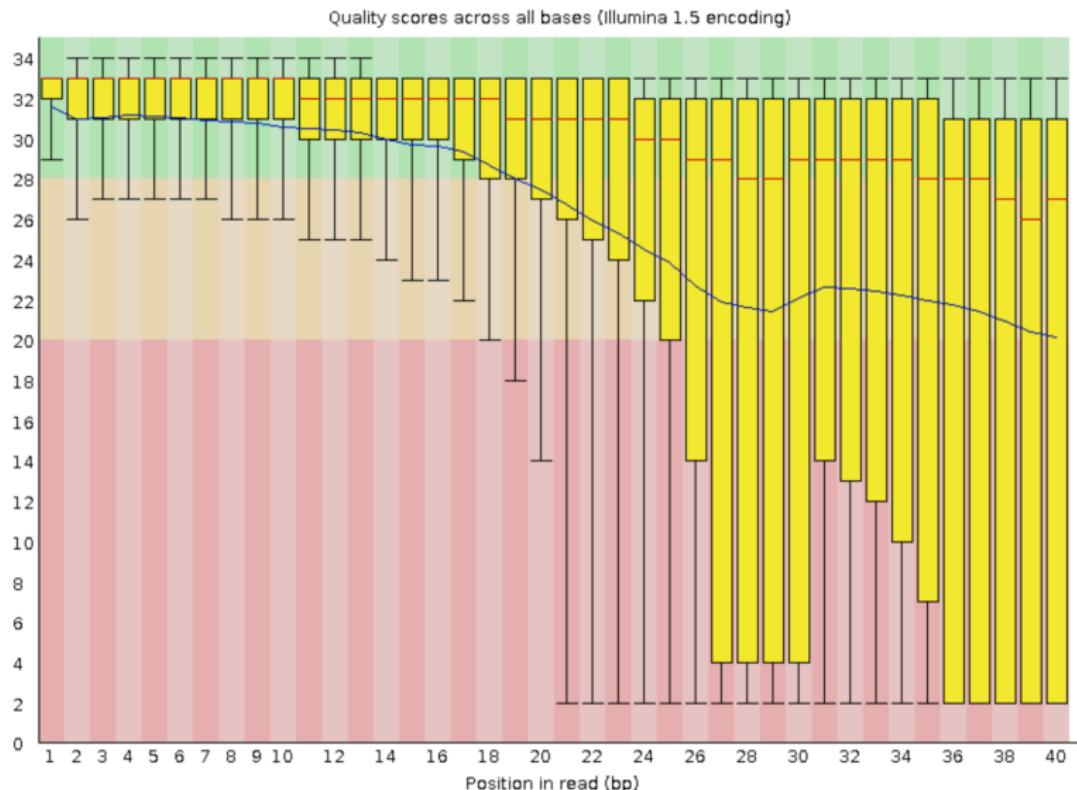
Basic Statistics

Measure	Value
Filename	bad_sequence.txt
File type	Conventional base calls
Encoding	Illumina 1.5
Total Sequences	395288
Sequences flagged as poor quality	0
Sequence length	40
%GC	47

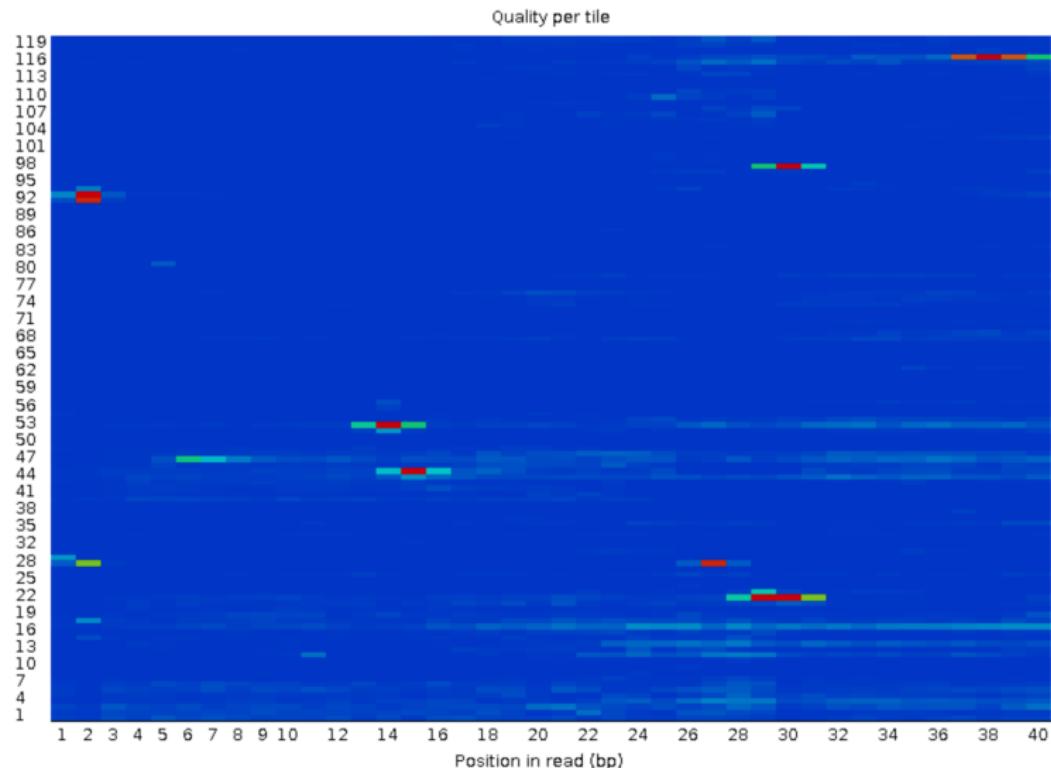
Per base sequence quality

Quality scores across all bases (illumina 1.5 encoding)

Raw base quality QC

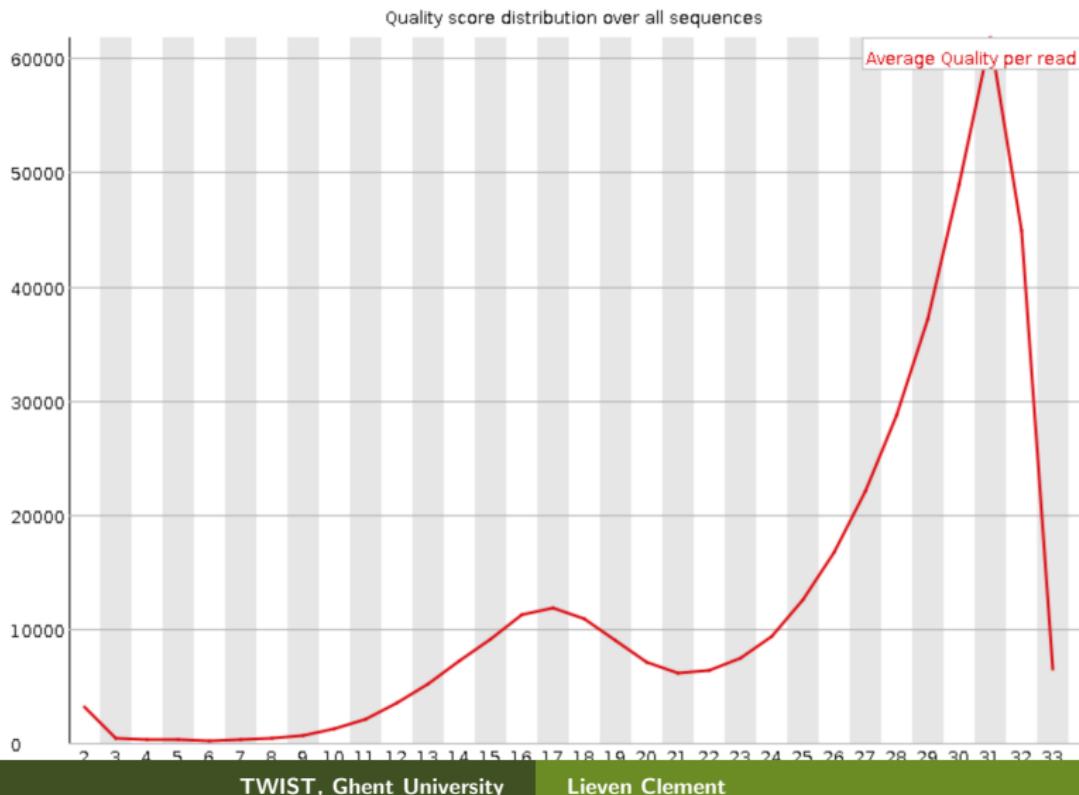


Raw base quality QC



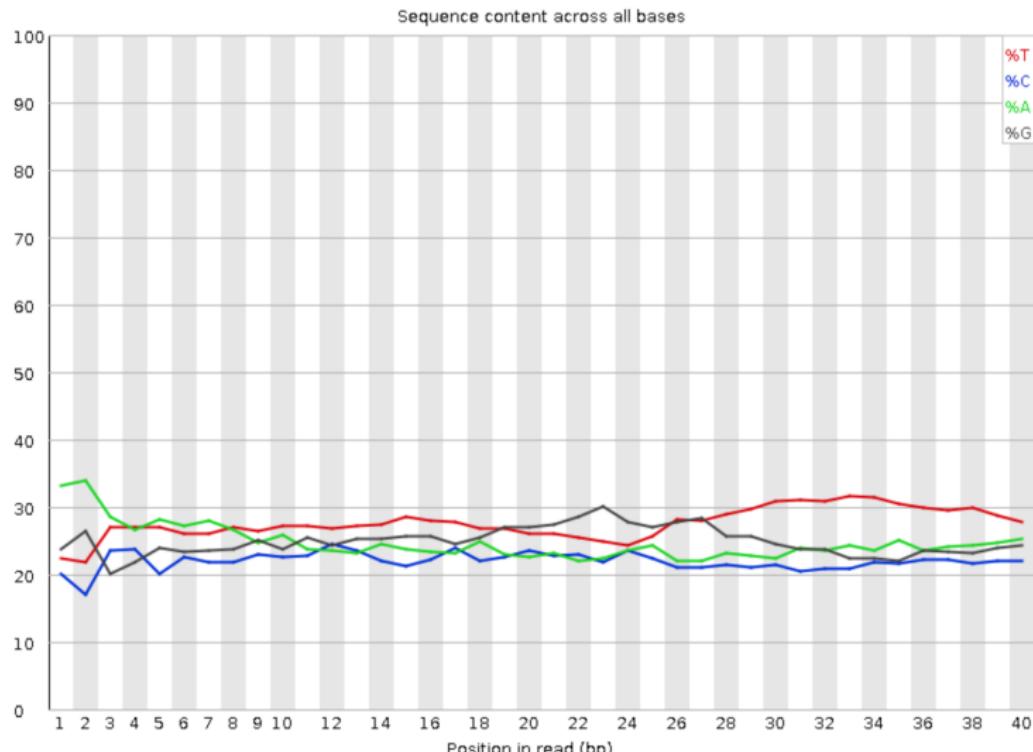
Raw base quality QC

✓ Per sequence quality scores



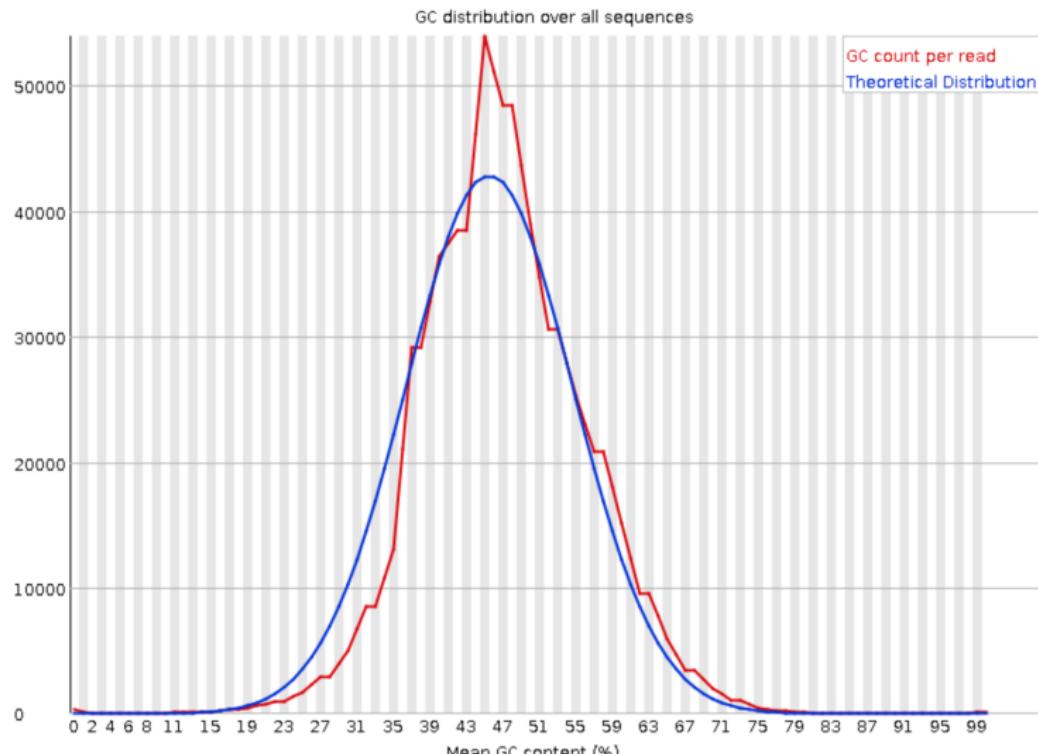
Raw base quality QC

⚠ Per base sequence content



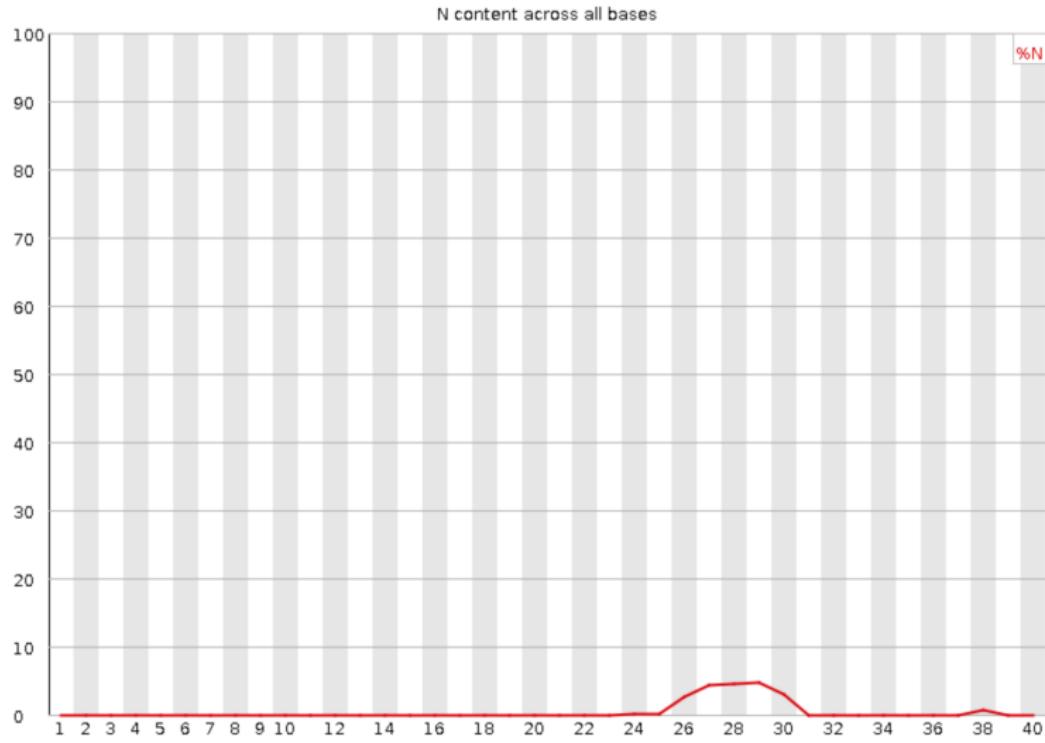
Raw base quality QC

⚠️ Per sequence GC content



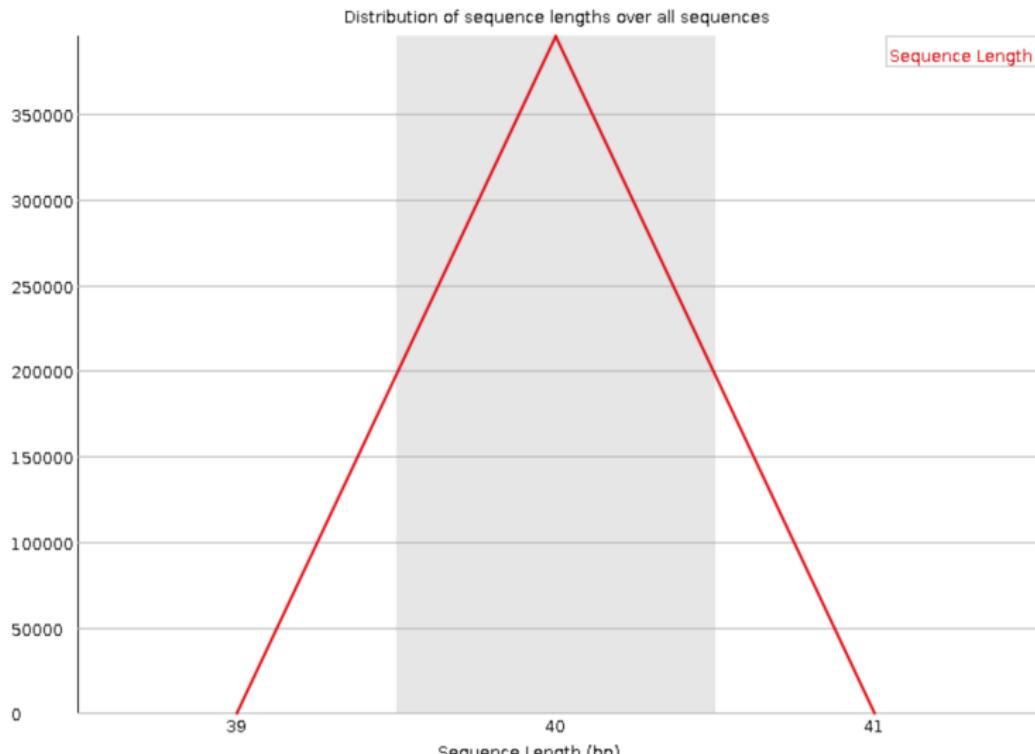
Raw base quality QC

Per base N content



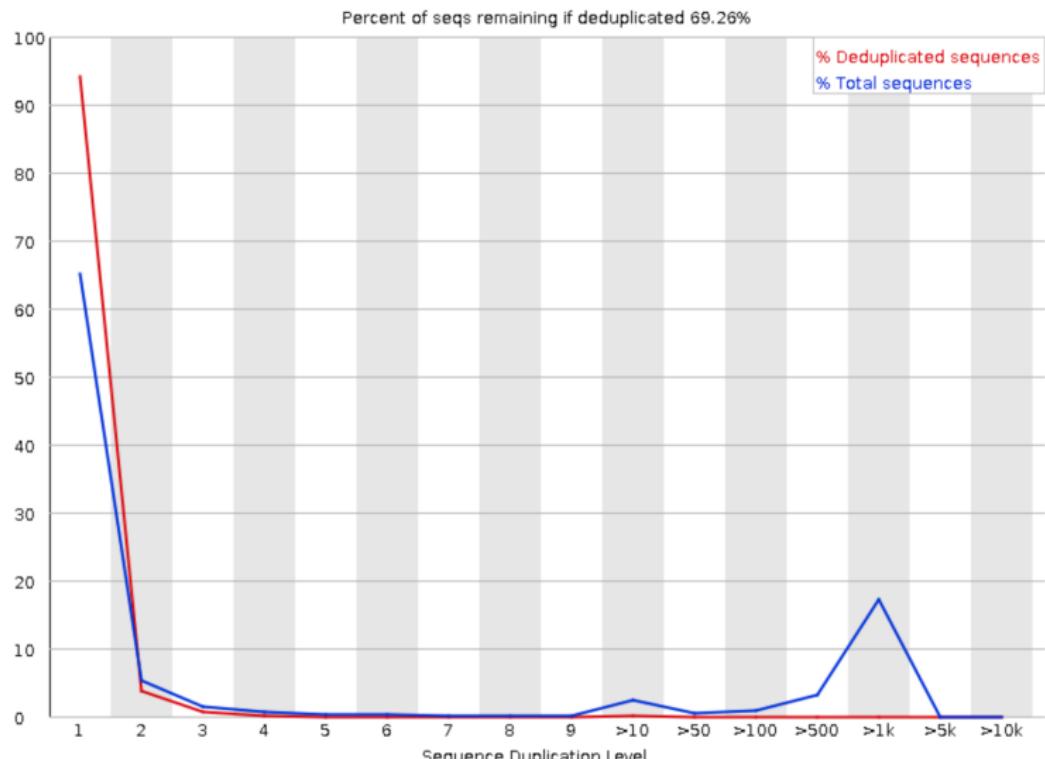
Raw base quality QC

Sequence Length Distribution



Raw base quality QC

⚠ Sequence Duplication Levels



Raw base quality QC

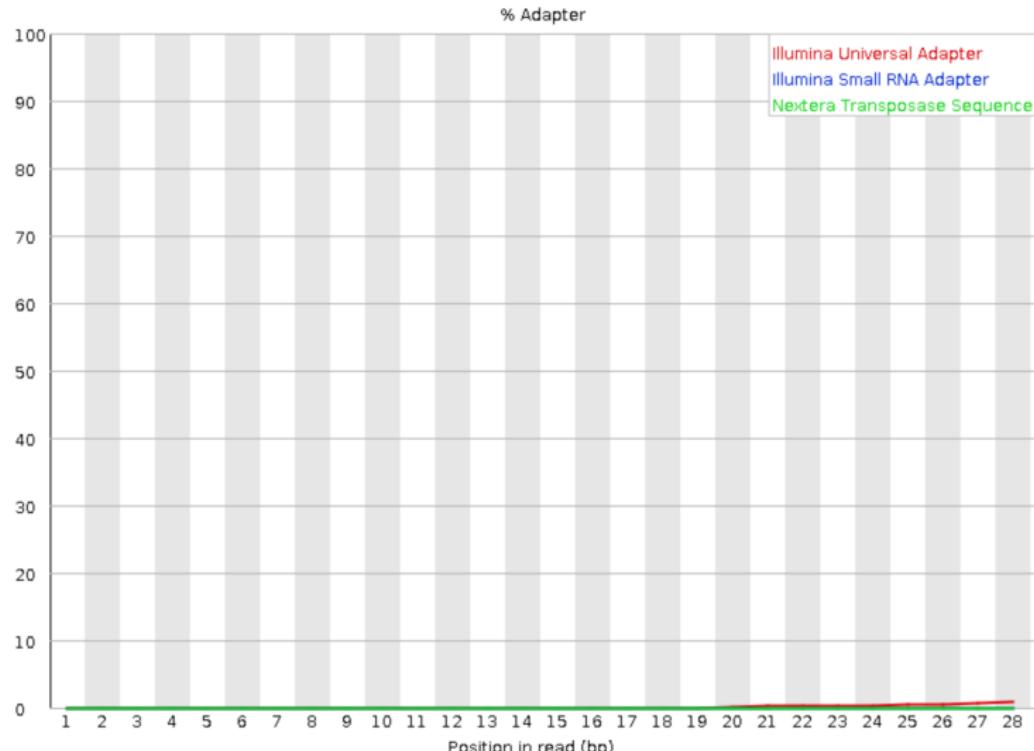
⚠ Overrepresented sequences

Sequence	Count	Percentage	Possible Source
AGAGTTTATCGCTTCCATGACGCAGAAGTTAACACTTC	2065	0.5224039181558763	No Hit
GATTGGCGTATCCAACCTGCAGAGTTTATCGCTTCCATG	2047	0.5178502762542754	No Hit
ATTGGCGTATCCAACCTGCAGAGTTTATCGCTTCCATGA	2014	0.5095019327680071	No Hit
CGATAAAAATGATTGGCGTATCCAACCTGCAGAGTTTAT	1913	0.4839509420979134	No Hit
GTATCCAACCTGCAGAGTTTATCGCTTCCATGACGCAGA	1879	0.47534961850600066	No Hit
AAAAATGATTGGCGTATCCAACCTGCAGAGTTTATCGCT	1846	0.4670012750197325	No Hit
TGATTGGCGTATCCAACCTGCAGAGTTTATCGCTTCCAT	1841	0.46573637449150995	No Hit
AACCTGCAGAGTTTATCGCTTCCATGACGCAGAACTAA	1836	0.46447147396328753	No Hit
GATAAAAATGATTGGCGTATCCAACCTGCAGAGTTTATC	1831	0.4632065734350651	No Hit
AAATGATTGGCGTATCCAACCTGCAGAGTTTATCGCTTC	1779	0.45005160794155147	No Hit
ATGATTGGCGTATCCAACCTGCAGAGTTTATCGCTTCA	1779	0.45005160794155147	No Hit
AATGATTGGCGTATCCAACCTGCAGAGTTTATCGCTTCC	1760	0.4452449859343061	No Hit
AAAATGATTGGCGTATCCAACCTGCAGAGTTTATCGCTT	1729	0.4374026026593269	No Hit
CGTATCCAACCTGCAGAGTTTATCGCTTCCATGACGCAG	1713	0.43335492096901496	No Hit
ATCCAACCTGCAGAGTTTATCGCTTCCATGACGCAGAAG	1708	0.43209002044079253	No Hit
CAGAGTTTATCGCTTCCATGACGCAGAAGTTAACACTT	1684	0.42601849790532476	No Hit
CAACCTGCAGAGTTTATCGCTTCCATGACGCAGAAGTTA	1668	0.4219708162150128	No Hit
TGCAGAGTTTATCGCTTCCATGACGCAGAAGTTAACACT	1668	0.4219708162150128	No Hit
TATCCAACCTGCAGAGTTTATCGCTTCCATGACGCAGAA	1630	0.4123575722005221	No Hit
GTCATGGAAGCGATAAAACTCTGCAGGTTGGATAACGCCA	1620	0.40982777114407726	No Hit
AACTCTCGCTCATGGAAGCGATAAAACTCTGCAGGTTGG	1616	0.4088158507214993	No Hit



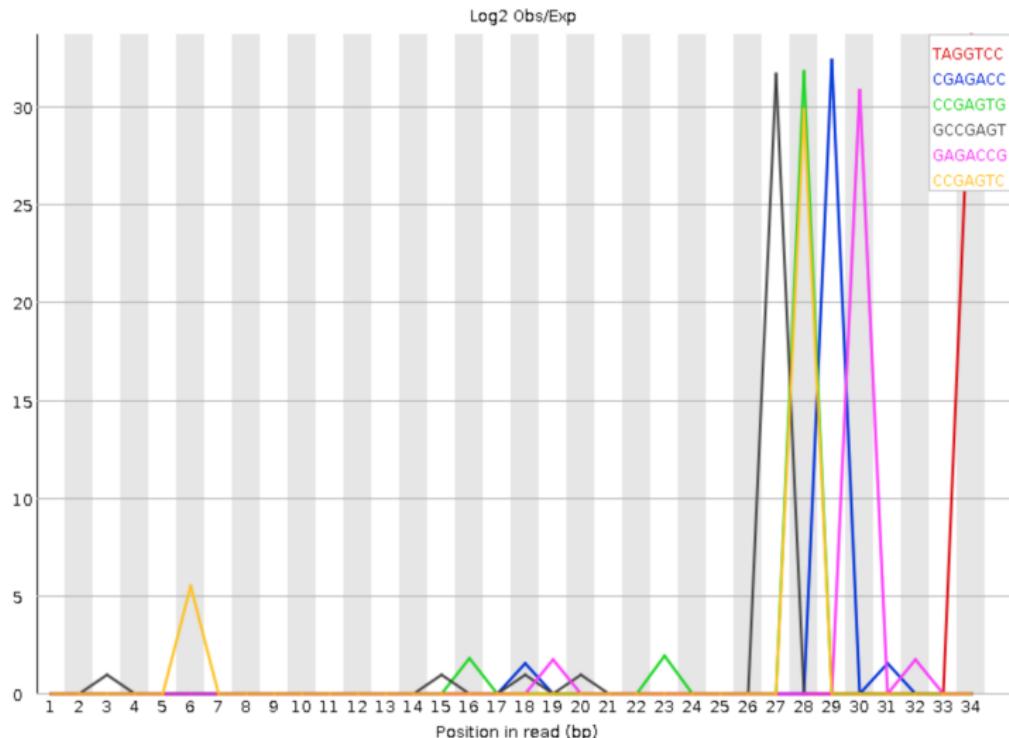
Raw base quality QC

Adapter Content



Raw base quality QC

⚠ Kmer Content



Preprocessing

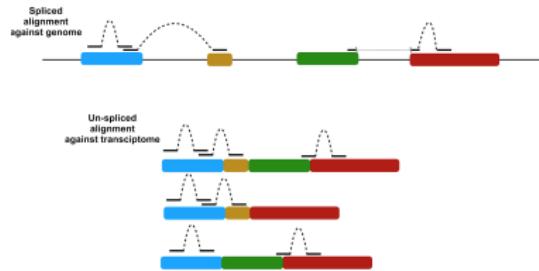
- Read Trimming
 - Adaptor sequence
 - Bar code
 - (deteriorating bases at the end of reads)
 - often already done by the sequencing provider.
 - remaining polyA tails
- Read filtering
 - low quality reads
 - PhiX reads (should be removed already by sequence provider)
 - in RNA-seq never remove duplicates because they can occur for highly expressed transcripts
- Perform fastQC again

Alignment

- DNA
 - bowtie2, BWA, ...
 - Needs: genomic reference sequence + cleaned reads
- RNAseq
 - Aligning to transcriptome: annotation-bias, you throw away data: very fast: Salmon and Kallisto.
 - Genome: problem Gaps
Star, tophat2, Rsubread, ...
 - Needs: genomic reference sequence + genomic annotation+ cleaned reads

http://wwwdev.ebi.ac.uk/fg/hts_mappers/

To the genome: gap aware!



STAR: Spliced Transcripts Alignment to a Reference

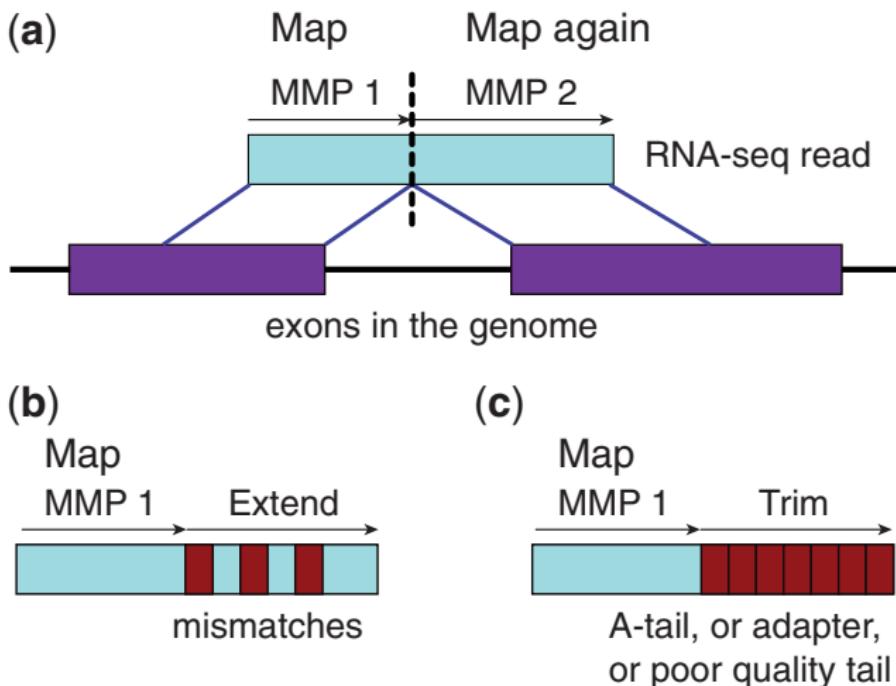
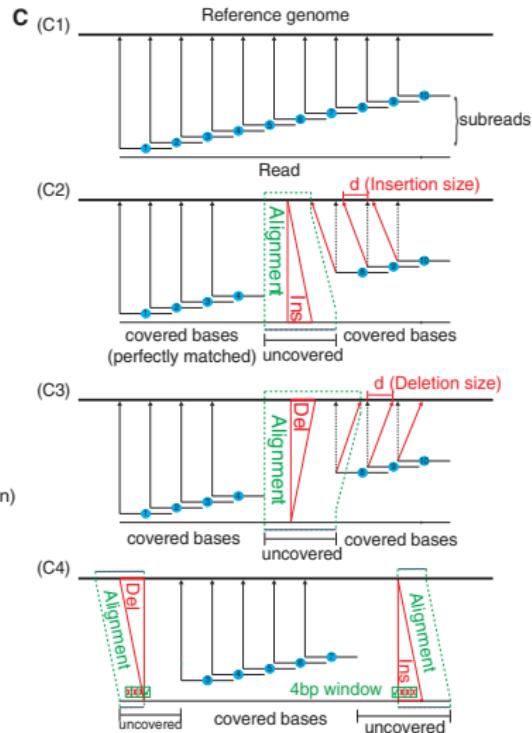
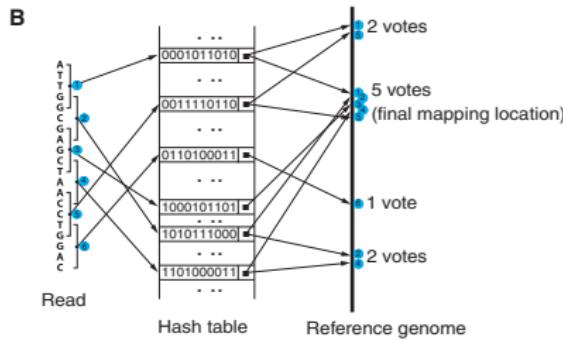
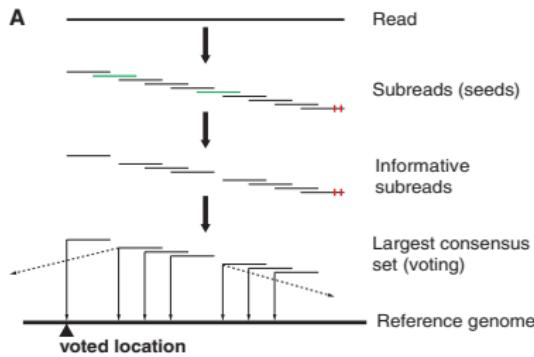


Fig. 1. Schematic representation of the Maximum Mappable Prefix search in the STAR algorithm for detecting (a) splice junctions, (b) mis-

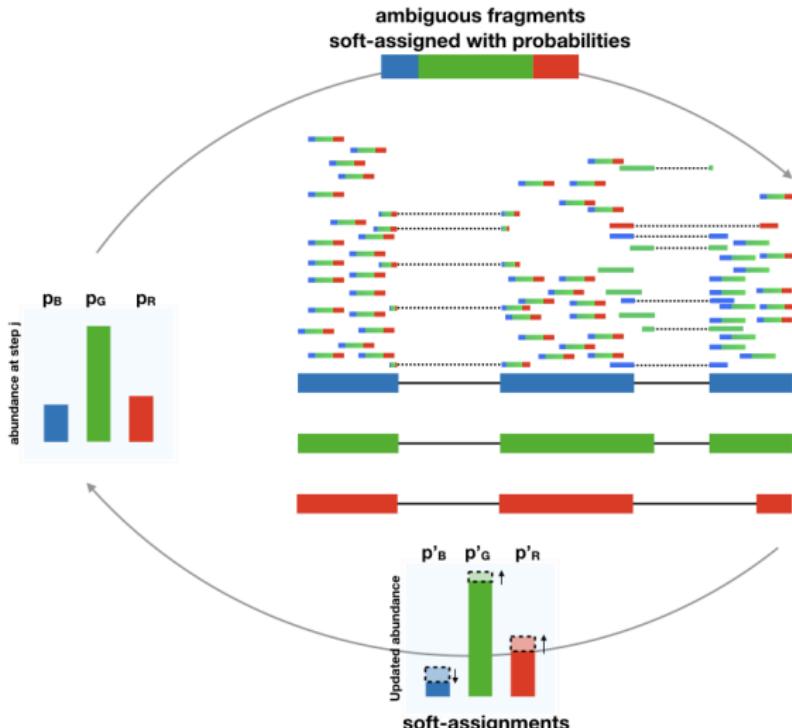


Rsubread: integration read alignment into R



et al. (2013) Nucleic Acids Research, 41(10):e108, 2013

Salmon: fast and bias-aware quantification of transcript expression (Mapping to the transcriptome)



Post alignment QC

- fastQC
 - Coverage plots
 - Removal of biological contamination if not of interest mRNA
(only small fraction of RNA pool):
rRNA, ncRNA, mitochondrial RNA
- Normally removed with kits prior to sequencing

Summarization upon mapping to the genome

- Most applications summarize reads based upon known annotation: bias
- Generate counts for genes, transcript or exons
- Count read instead of nt
- Count each read only ones
- Discard reads that
 - do not map uniquely
 - overlap with several genes
 - with a bad quality score

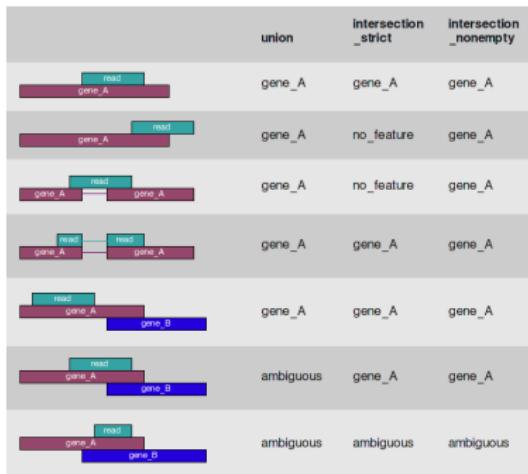


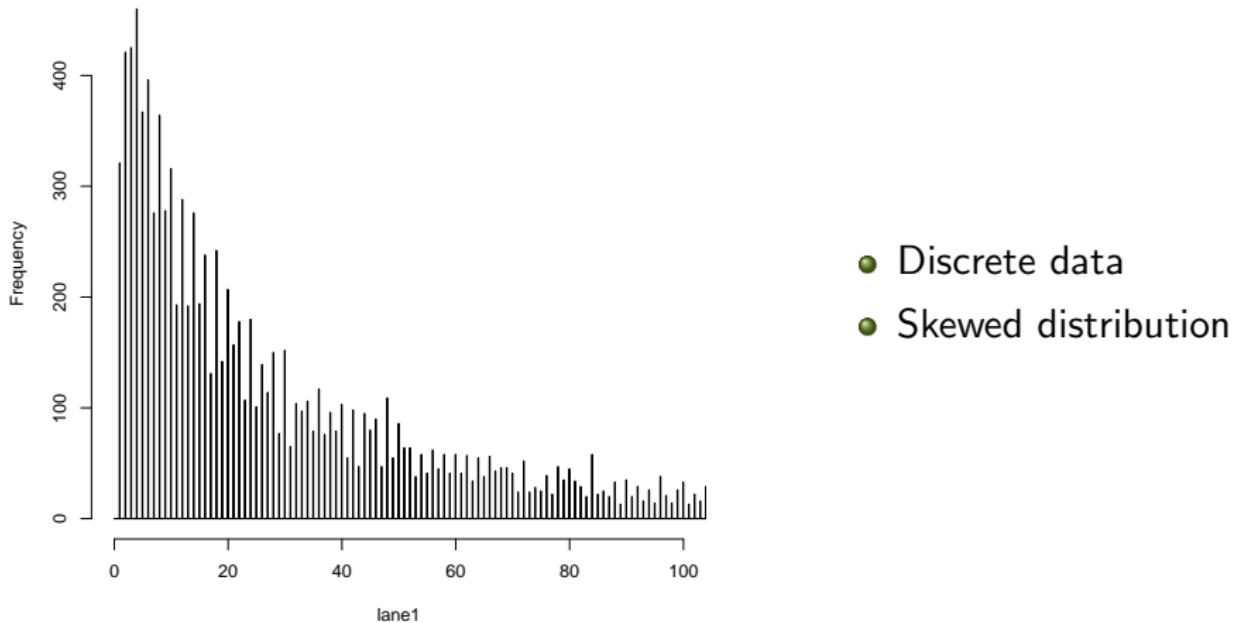
Figure 4.1: Overlap modes; Image from the HTSeq package developed by Simon Anders.

Summarization upon mapping to the transcriptome

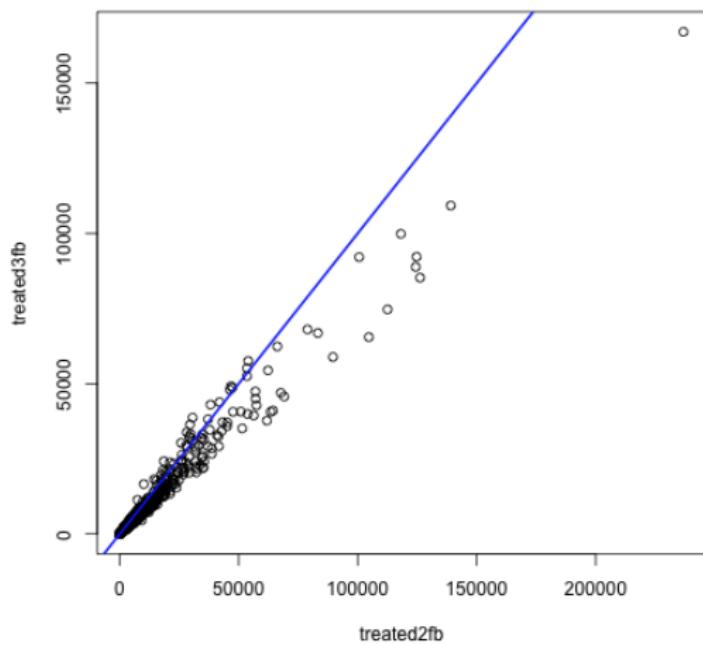
- Results in counts at the transcript level.
- Sum of transcript level counts to obtain gene-level count
- Account for potential difference in transcript usage between samples : via average transcript length (see normalisation, why?)
- Has been shown to be more accurate: e.g. Soneson et al. (2015). F1000Research, 4. doi: 10.12688/f1000research.7563.1

Classical Approach: Gene level data

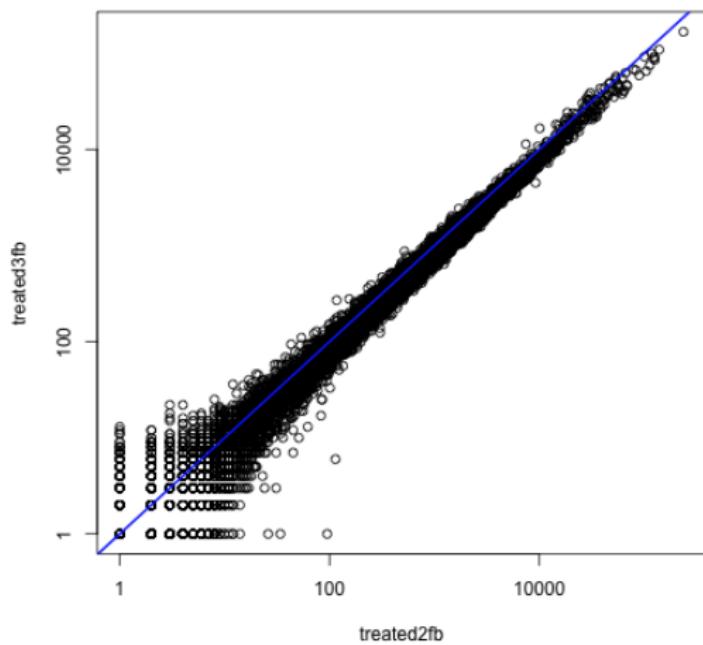
- gene x sample matrix
- Differential expression well studied by statisticians
- Count data:
 - many zeroes
 - very large range
 - biological variability?



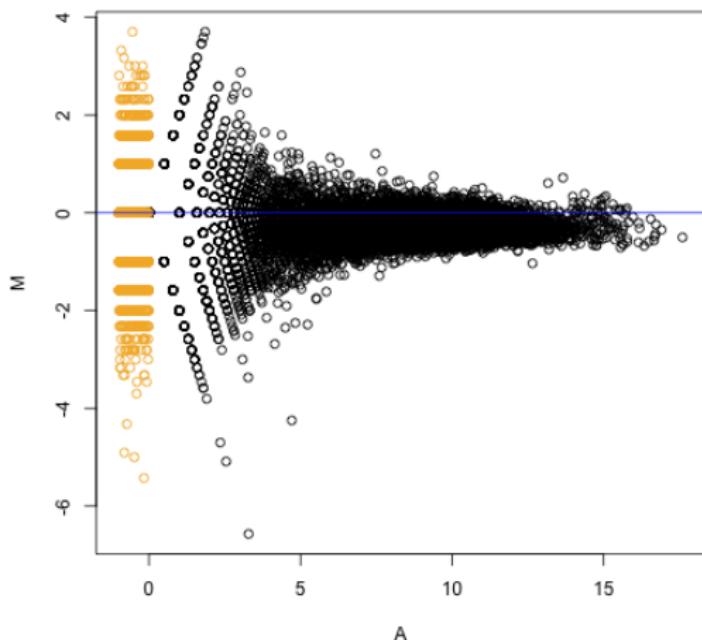
Normalization



Normalization



Normalization



- $M = \log_2(Y_2) - \log_2(Y_1)$
- $A = \frac{[\log_2(Y_2) + \log_2(Y_1)]}{2}$

Normalization

- Sequencing depth

	group	lib.size	norm.factors
treated2fb	treated	15620018.00	1.00
treated3fb	treated	12733865.00	1.00
untreated3fb	untreated	10283129.00	1.00
untreated4fb	untreated	11653031.00	1.00

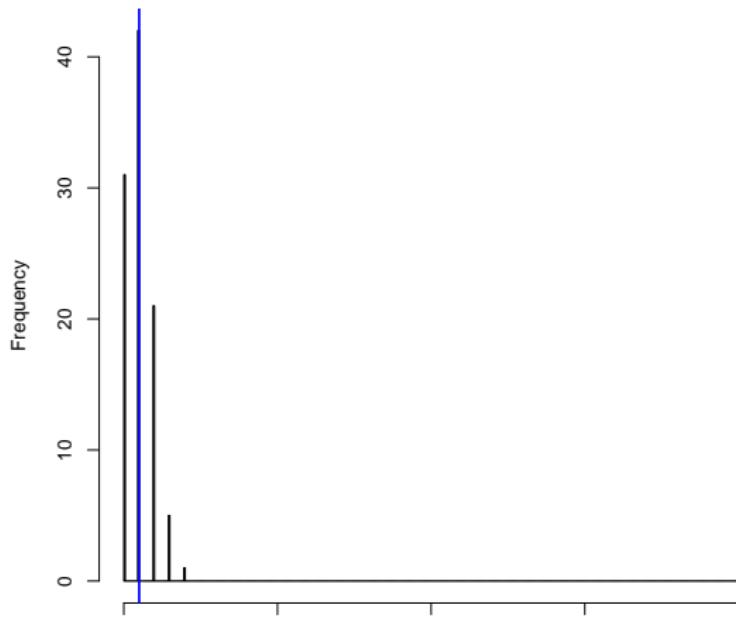
- use lib.size to normalize?
- Convert reads in counts per million

Modeling Counts

- Marioni (2008) Genome Research showed that technical replicates are $Poisson(\mu)$
- Properties: $\mu=\text{mean}=\text{variance}$

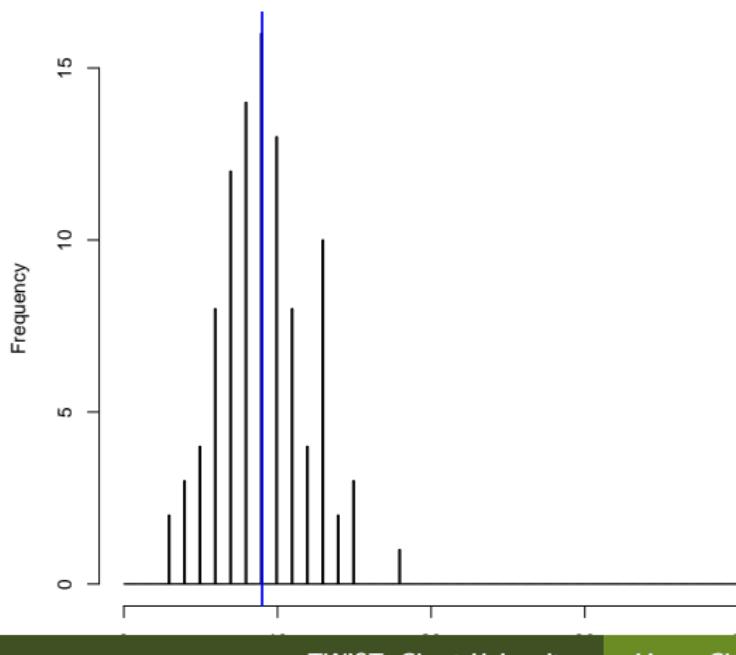
Modeling Counts

mu = 1



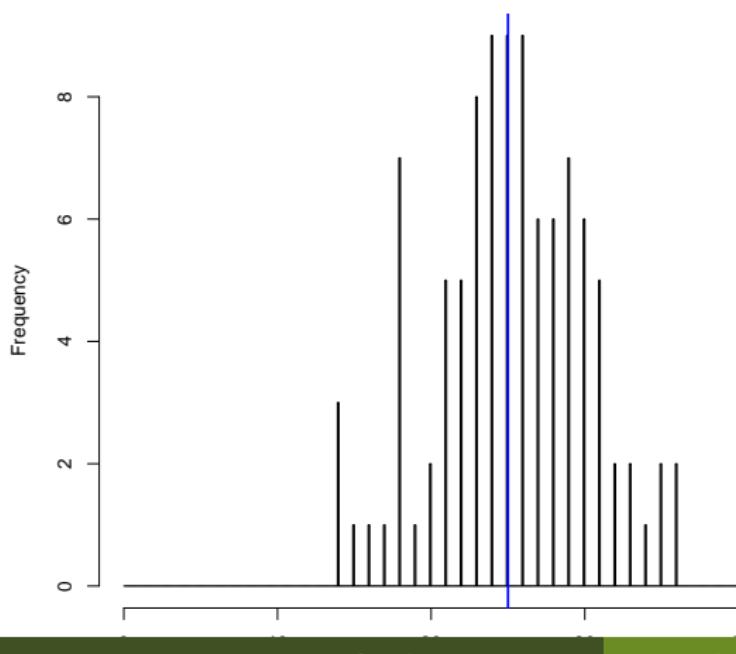
Modeling Counts

mu = 9



Modeling Counts

$\mu = 25$



Modeling Counts

- The Poisson distribution is commonly used $\text{Poisson}(\mu)$
- Properties: $\mu = \text{mean} = \text{variance}$
- Relative error decreases with increasing mean
- CV = standard deviation / mean = $\sqrt{\mu}/\mu = 1/\sqrt{\mu}$

Mean	CV
1	1
9	1/3
25	1/5
100	1/10

Generalized linear model for seq data

$$\begin{cases} y_{ig} & \sim \text{Poisson}(\mu_{ig}) \\ \log(\mu_{ig}) & = \eta_{ig} \\ \eta_{ig} & = \sum_{k=1}^N x_{ik} \beta_{gk} \end{cases}$$

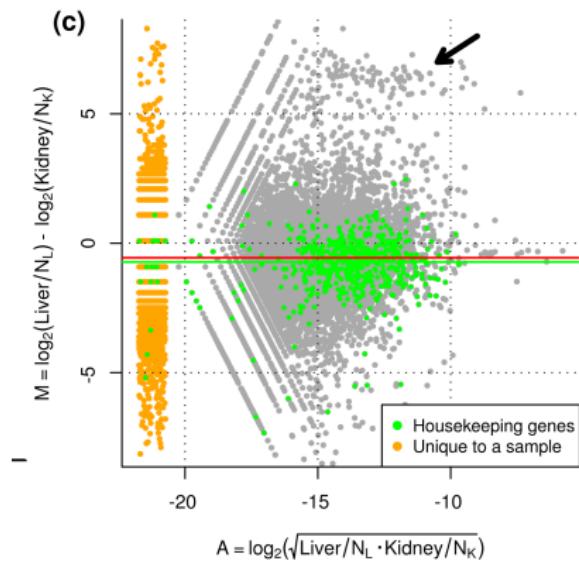
- y_{gi} : count for gene g of subject i
- x_{ik} : predictor variable k evaluated for subject i
- η : linear predictor
- β_{gk} : effect for predictor variable k and gene g

GLM with normalization

$$\left\{ \begin{array}{l} y_{ig} \sim \text{Poisson}(\mu_{ig}) \\ \mu_{ig} = \lambda_{ig} S_{ig} \\ \log(\mu_{ig}) = \eta_{ig} \\ \eta_{ig} = \sum_{k=1}^N x_{ik} \beta_{gk} + \log S_{ig} \end{array} \right.$$

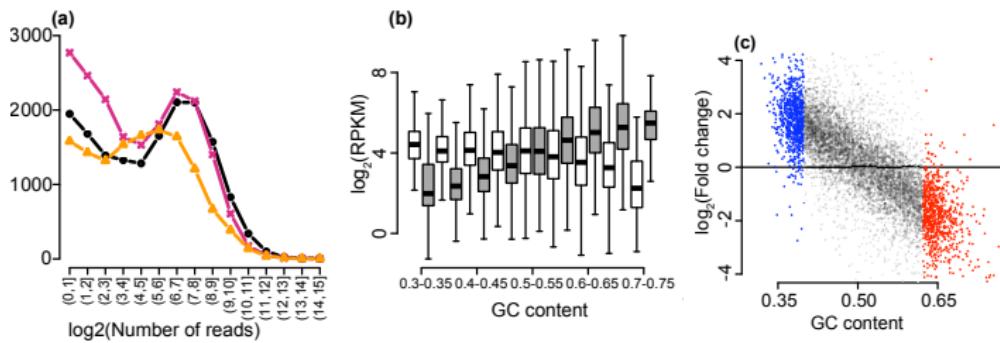
- y_{gi} : count for gene g of subject i
- x_{ik} : predictor variable k evaluated for subject i
- η : linear predictor
- β_{gk} : effect for predictor variable k and gene g
- S_{ig} : effective library size for gene g of subject i

Normalization with lib.size??



Robinson and Oshlack (2010). Genome Biology.





Hansen, Irizarry and Wu (2012). Biostatistics.

Normalization: S_{ij}

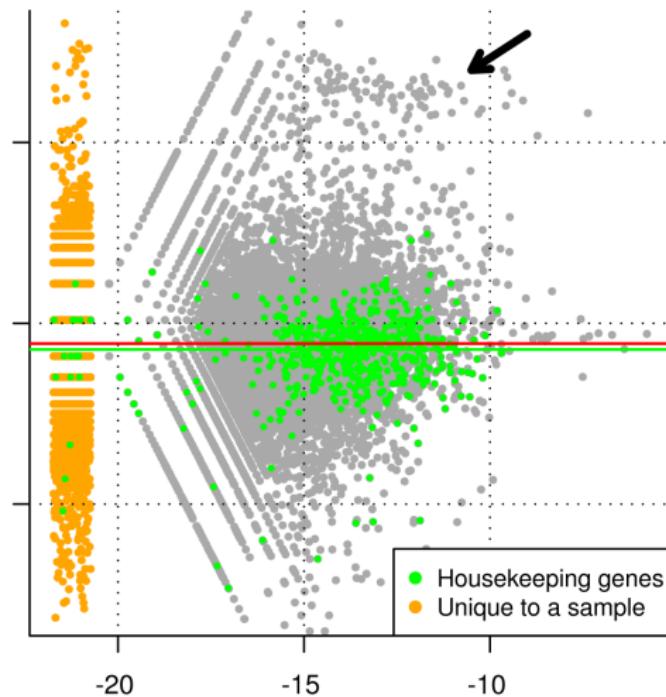
- Upper quartile Bullard et al. (2010) BMC Bioinformatics.
- scale normalization: edgeR package, Robinson and Oshlack (2010) Genome biology
- Geometric mean: DESeq, Anders and Huber (2010) Genome Biology
- Gene specific normalization: S_{gij}
 - GC content
 - gene length
 - cqn package, Hansen, Irizarry and Wu (2012) Biostatistics.

Normalization EdgeR

(c)

$$M = \log_2(\text{Liver}/N_L) - \log_2(\text{Kidney}/N_K)$$

$$A = \log_2(\sqrt{\text{Liver}/N_L \cdot \text{Kidney}/N_K})$$



TMM normalization details

A trimmed mean is the average after removing the upper and lower x% of the data. The TMM procedure is doubly trimmed, by log-fold-changes M_{gk}^r (sample k relative to sample r for gene g) and by absolute intensity (A_g). By default, we trim the M_g values by 30% and the A_g values by 5%, but these settings can be tailored to a given experiment. The software also allows the user to set a lower bound on the A value, for instances such as the Cloonan *et al.* dataset (Figure S1 in Additional file 1). After trimming, we take a weighted mean of M_g , with weights as the inverse of the approximate asymptotic variances (calculated using the delta method [24]). Specifically, the normalization factor for sample k using reference sample r is calculated as:

$$\log_2(TMM_k^{(r)}) = \frac{\sum_{g \in G^*} w_{gk}^r M_{gk}^r}{\sum_{g \in G^*} w_{gk}^r} \text{ where } M_{gk}^r = \frac{\log_2\left(\frac{Y_{gk}}{N_k}\right)}{\log_2\left(\frac{Y_{gr}}{N_r}\right)}$$

$$Y_{gk}, Y_{gr} > 0.$$

The cases where $Y_{gk} = 0$ or $Y_{gr} = 0$ are trimmed in advance of this calculation since log-fold-changes cannot be calculated; G^* represents the set of genes with valid M_g and A_g values and not trimmed, using the percentages above. It should be clear that $TMM_r^{(r)} = 1$.

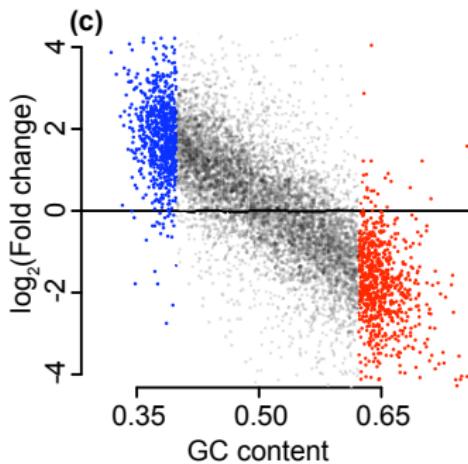
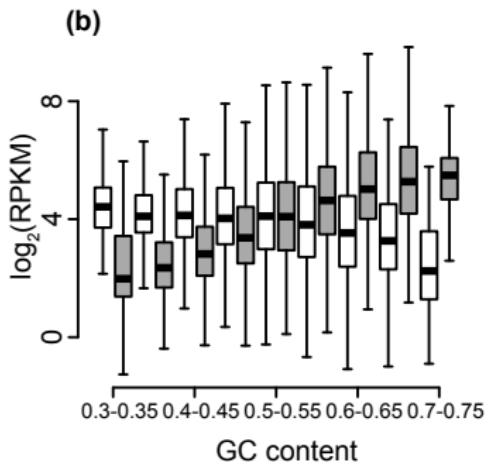
As Figure 2a indicates, the variances of the M values at higher total count are lower. Within a library, the vector of counts is multinomial distributed and any individual gene is binomial distributed with a given library size and proportion. Using the delta method, one can calculate an approximate variance for the M_g , as is commonly done with log relative risk, and the inverse of these is used to weight the average.

We compared the weighted with the unweighted trimmed mean as well as an alternative robust estimator (robust linear model) over a range of simulation parameters, as shown in Figure S4 in Additional file 1.



Normalization cqn

(Hansen, Irizarry and Wu (2012). Biostatistics)



Normalization cqn

(Hansen, Irizarry and Wu (2012). Biostatistics)

We present a normalization algorithm motivated by a statistical model that accounts for both the need to correct systematic biases and the need to adjust for distributional distortions. We denote the log gene expression level for gene g at sample i with $\theta_{g,i}$, which we consider a random variable. For most g , $\theta_{g,i}$ are independent and identically distributed across i . We assume that the marginal distribution of the $\theta_{g,i}$ is the same for all samples i , and denote it by G . Note that this variability accounts for the difference in gene expression across different genes. The p covariates thought to cause systematic errors are denoted with $\mathbf{X}_g = (X_{g,1}, \dots, X_{g,p})'$. Examples of covariates considered here are GC-content, gene length, and gene mappability defined as the percentage of uniquely mapping subreads of a gene. To model the observed counts $Y_{g,i}$ for gene g in sample i we write:

$$Y_{g,i} | \mu_{g,i} \sim \text{Poisson}(\mu_{g,i})$$

with

$$\mu_{g,i} = \exp \left\{ h_i(\theta_{g,i}) + \sum_{j=1}^p f_{i,j}(X_{g,j}) \right\}$$

with $f_{i,j}(\bar{X}_j) = 0 \forall j$ for identifiability. Here, the h_i s are non-decreasing functions that account for the fact that count distributions are distorted in non-linear ways across the different samples (Figure 2(a)). The $f_{i,j}$ s account for sample dependent systematic biases. Data exploration suggested

Normalization cqn

(Hansen, Irizarry and Wu (2012). Biostatistics)

For any given i , the distribution of $h_i(\theta_{g,i})$ is unspecified and Figure 2(b) shows that values can range from $-\infty$ to 8. First we observe that when $\mu_{g,i}$ is large, $\log(Y_{g,i}) | \mu_{g,i}$ is approximately normal with mean $\log(\mu_{g,i})$ and variance $1/\mu_{g,i}$. The small variance implies that for large $\mu_{g,i}$

$$\log(Y_{g,i}) | \mu_{g,i} \approx \log(\mu_{g,i}) = h_i(\theta_{g,i}) + \sum_{j=1}^p f_{i,j}(X_{g,j}),$$

showing that for a fixed i and large $\mu_{g,i}$, the distribution of $\log(Y_{g,i})$ is equal to $h_i(\theta_{g,i})$ except for a location shift given by $\sum_{j=1}^p f_{i,j}(X_{g,j})$. Even though the shape of $h_i(G)$ is left unspecified, the quantiles of $\log(Y_{g,i})$ shift by $\sum_{j=1}^p f_{i,j}(X_{g,j})$. We therefore use quantile regression to estimate the $f_{i,j}$ s. To assure the large $\mu_{g,i}$ assumption is satisfied, instead of fixing the quantile choice, we use median regression on a subset of genes with average counts beyond a lower bound.

To estimate the h_i s we take advantage of the fact that

$$E \left\{ \log(Y_{g,i}) - \sum_{j=1}^p f_{i,j}(X_{g,j}) \right\} = h_i(\theta_{g,i})$$

and that the distribution of $\theta_{g,i}$ does not depend on i , to use subset quantile normalization (Wu and Aryee, 2010).

The specifics of our algorithm are as follows:

1. Select a subset of genes with $\bar{Y}_{g..} > 50$. Then for each i , use median regression on $\log(Y_{g,i})$ to estimate the parameters that define the splines $f_{i,j}$ and determine $\hat{f}_{i,j}$.

Normalization cqn

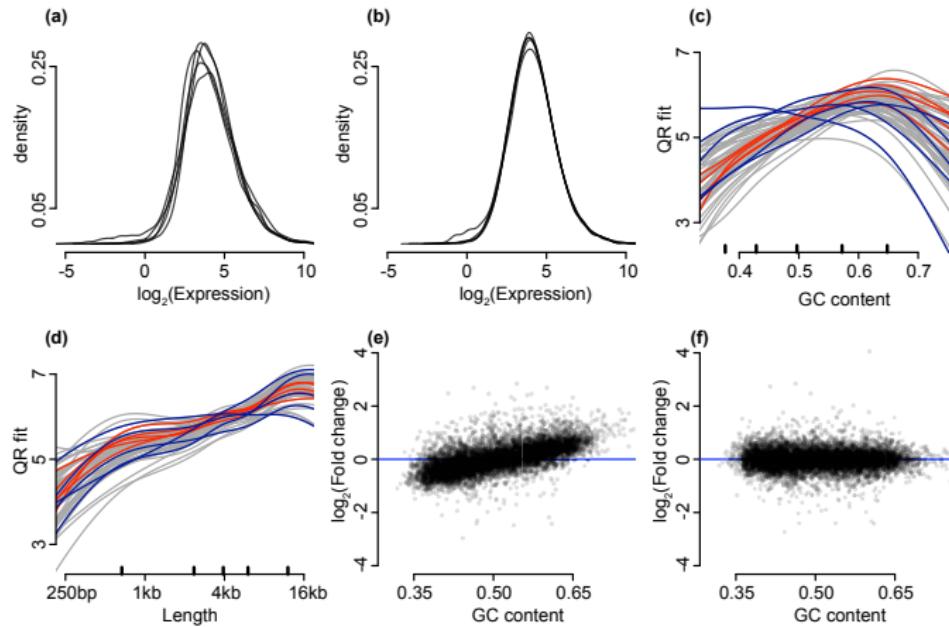
(Hansen, Irizarry and Wu (2012). Biostatistics)

2. For each i , apply quantile normalization to $\log(Y_{g,i}) - \sum_{j=1}^p \hat{f}_{i,j}(X_{g,j})$ to obtain \hat{h}_i^{-1} .
3. For each gene g on each sample i , define a *normalization offset* as $\exp[\log(Y_{g,i}) - \hat{h}^{-1}\{\log(Y_{g,i}) - \hat{f}_{i,j}(X_{g,j})\}]$.

The algorithm returns an offset rather than normalized data for two reasons. First, for interpretability we want to preserve the data as counts, i.e. integer numbers. Due to the large sampling error, small counts should be treated with caution thus users of the algorithm benefit from access to these original counts. Second, the most widely used methodology for identifying differentially expressed genes from RNA-seq data model the counts in a way that sampling error from counting process (such as Poisson) and variation in gene expression (θ) are taken into account (Robinson and others, 2010; Anders and Huber, 2010). Providing an offset allows direct application of these existing methods which take counts as input and can be easily adapted to adjust for offsets.

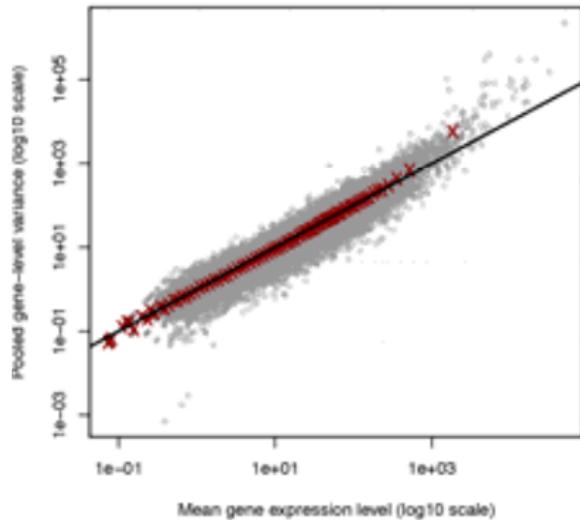
Normalization cqn

(Hansen, Irizarry and Wu (2012). Biostatistics)

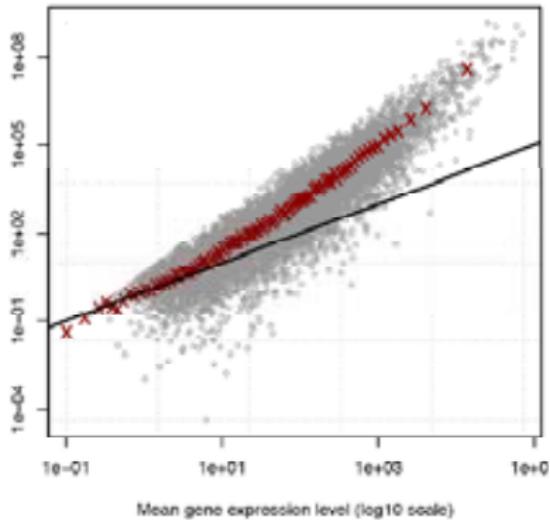


Mean Variance relationship

Technical replicates



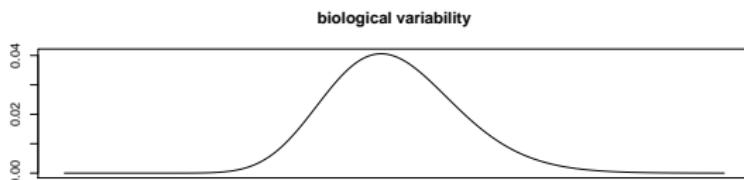
Biological replicates



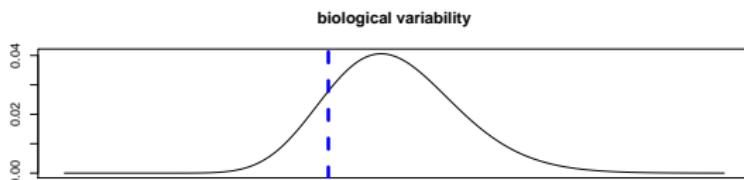
Mean variance relationship

	Seq technology	true expression
total variability	= technical variability + biological variability	
$\text{Var}[y_{gi}]$	$= \mu_{gi} + \phi_g * \mu_{gi}^2$	
Total CV ²	= Technical CV ² + biological CV ²	
Total CV ²	$= \frac{1}{\mu_{gi}}$ + ϕ_g	

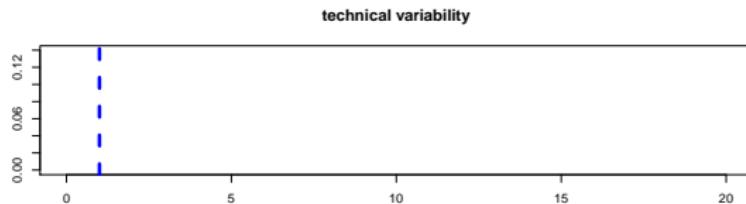
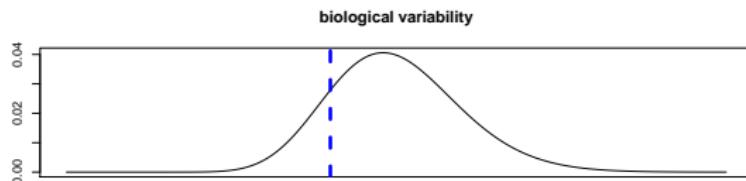
Sources of variability in a sequencing experiment



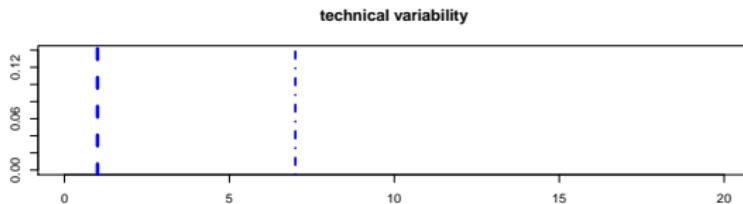
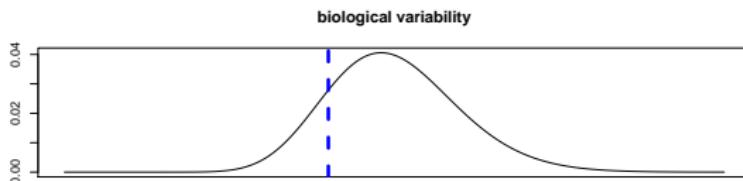
Sources of variability in a sequencing experiment



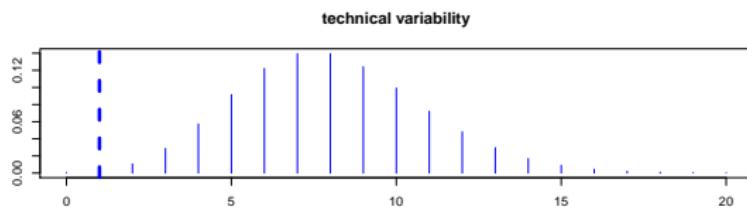
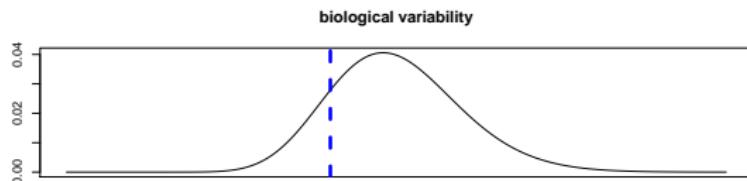
Sources of variability in a sequencing experiment



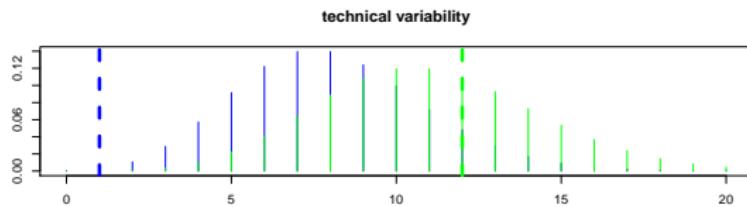
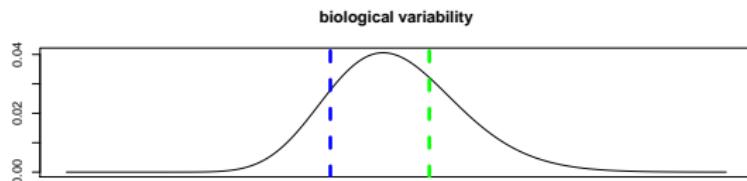
Sources of variability in a sequencing experiment



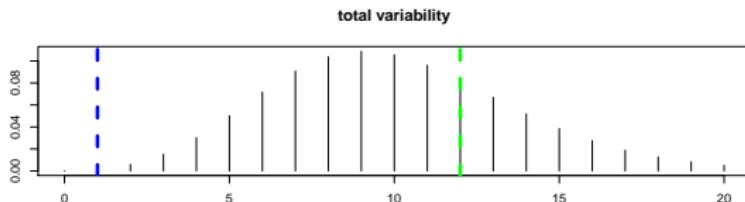
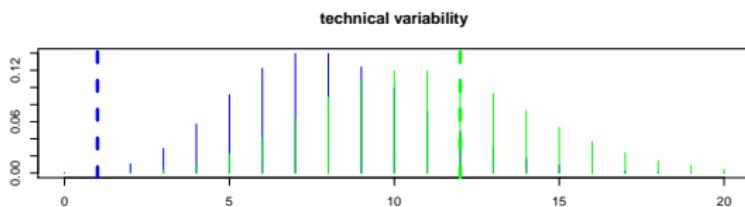
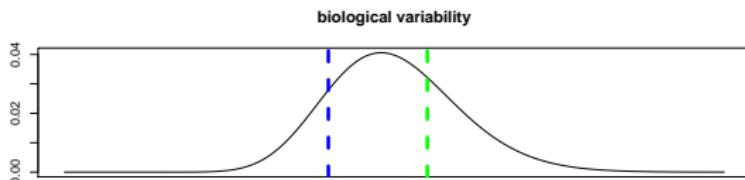
Sources of variability in a sequencing experiment



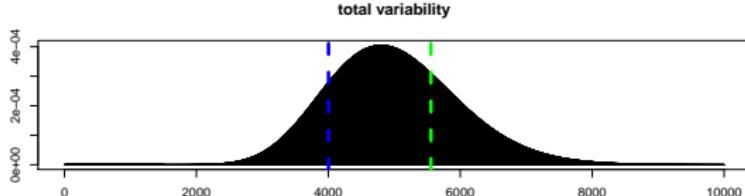
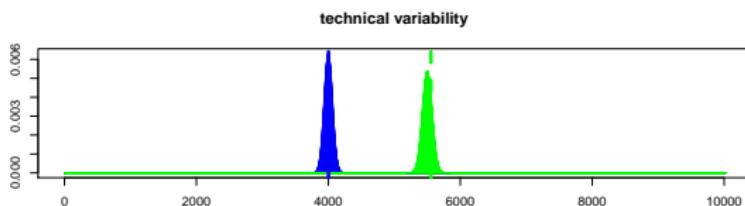
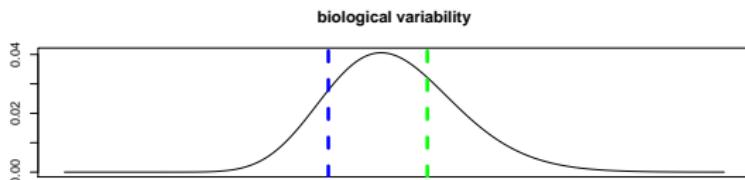
Sources of variability in a sequencing experiment



Sources of variability in a sequencing experiment



Sources of variability in a sequencing experiment



Improved model for RNA-seq data

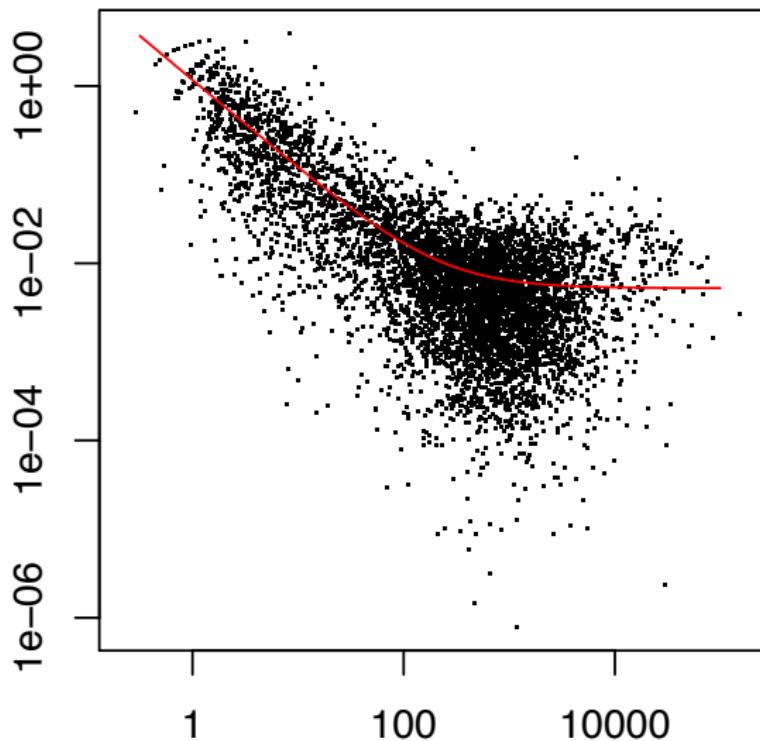
$$\left\{ \begin{array}{lcl} y_{ig} & \sim & \text{NB}(\mu_{ig}, \phi_{ig}) \\ \mu_{ig} & = & \lambda_{ig} S_{ig} \\ \log(\mu_{ig}) & = & \eta_{ig} \\ \eta_{ig} & = & \sum_{k=1}^N x_{ik} \beta_{gk} + \log S_{ig} \end{array} \right.$$

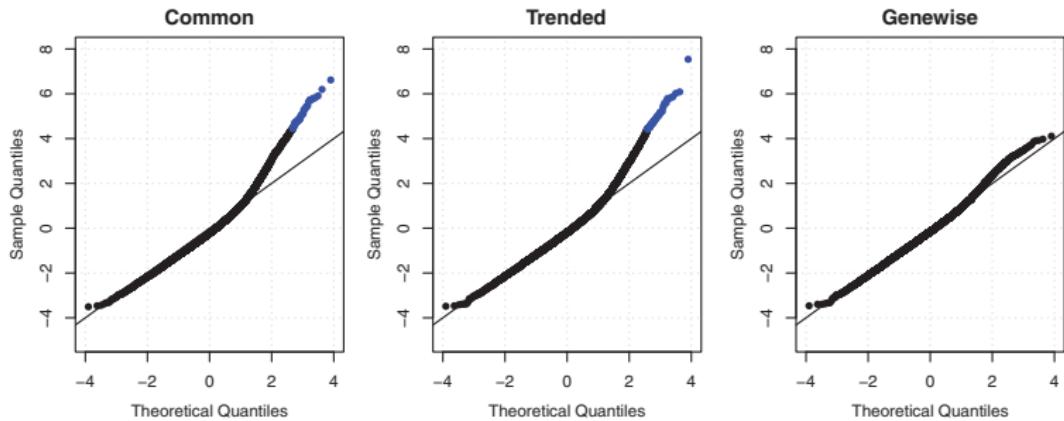
- y_{gi} : count for gene g of subject i
- x_{ik} : predictor variable k evaluated for subject i
- β_{gk} : effect for predictor variable k and gene g
- S_{ig} : effective library size for gene g of subject i



Estimating overdispersion

- For every single gene: not enough data
- Common dispersion for all genes
- Trended dispersion
- Gene wise, EB shrinkage to a common (trended) dispersion:
Borrow strength across genes (McCarthy & Smyth (2012).
Nucleic Acid Research)





McCarthy et al. (2012) NAR

Hypothesis testing

- Asymptotic statistical tests exist to test if the coefficients of the GLM are different from zero
- Implemented in edgeR
- Again we have to correct for multiple testing !!!