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1. RNA-Seq
   - Counting
   - Normalization
   - Practical Session

RNA-Seq: Expression value estimation

RNA-Seq

Genome

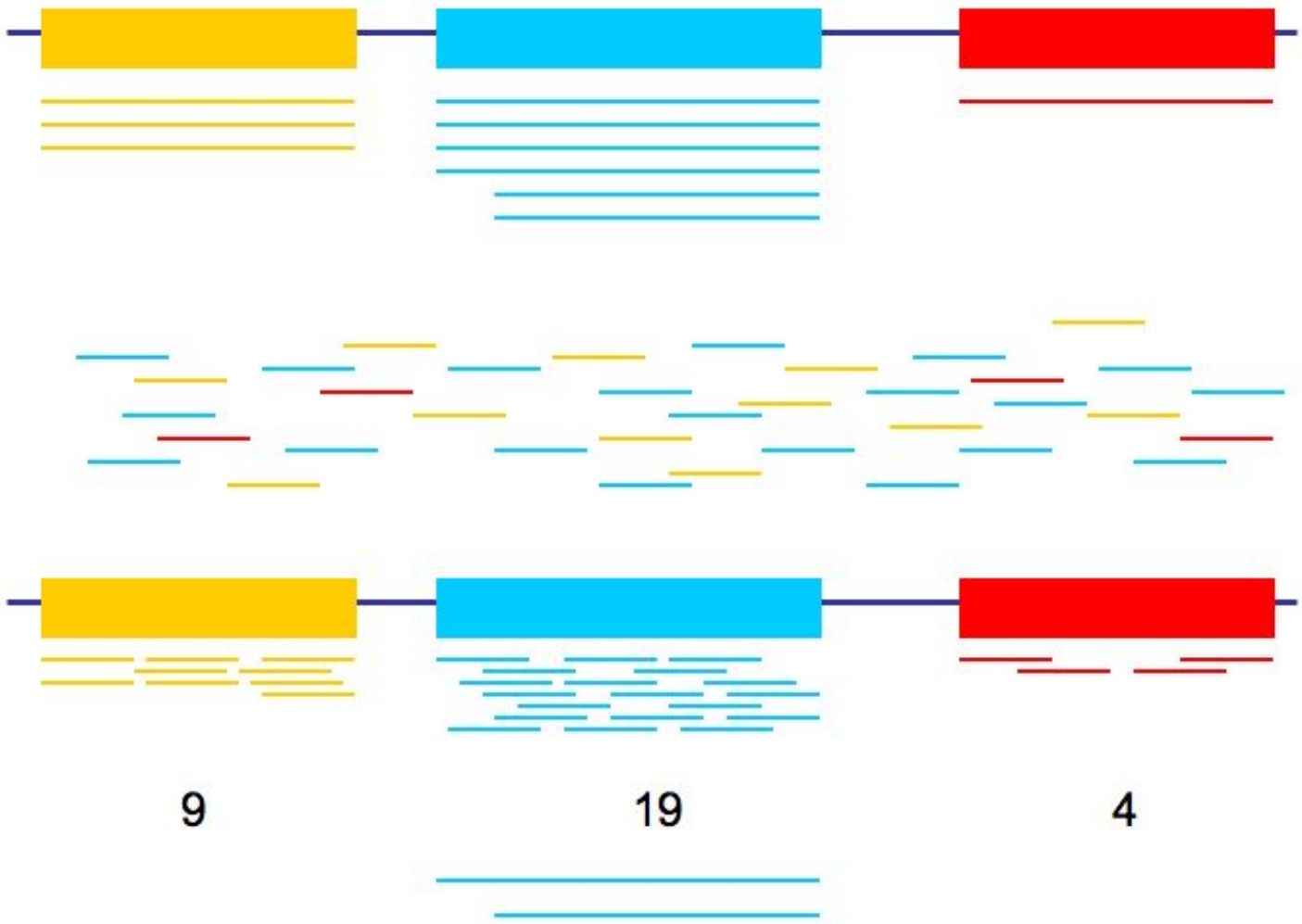
mRNA

Reads

Mapping

Expression count

Altern. Splicing



RNA-Seq: From reads to expression values

- RNA-Seq experiments result in large numbers of reads
- Reads are mapped against a reference genome
- We are interested in the expression strength of each gene.
- We could just count the reads for each gene.

Assumption

Expression strength $\sim$ number of reads

This requires

- Expression strength $=$ number of transcripts
- Number of transcripts $\overset{?}{\sim}$ number of reads

... but it is not so simple.

RNA-Seq: From reads to expression values

Counting using HTSeq (Anders *et al.*, HTSeq – A Python framework to work with high-throughput sequencing data Bioinformatics 2014):

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Counting using HTSeq (Anders *et al.*, HTSeq – A Python framework to work with high-throughput sequencing data Bioinformatics 2014):

Given a file with aligned sequencing reads and a list of genomic features, the next step is to estimate the expression value of each feature.

$\Rightarrow$ first count how many reads map to each feature.

Feature: an interval (i.e., a range of positions) on a chromosome or a union of such intervals.

Example: a feature is a gene, and a gene is the union of all its exons.

RNA-Seq: From reads to expression values

Counting using HTSeq:

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  It then considers (in the union mode) the union of all sets $S(i)$. If a read overlaps more than one feature (e.g. two genes), then it is not counted. If the union is empty, the read is counted as no_feature'.

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- Outputs a table with counts for each feature as well as some statistics

Further common file formats

- Wiggle files are used to represent the coverage data (ie., continuous data
  Example:

```
variableStep chrom=chr2
300701 12.5
300702 12.5
300703 12.5
300704 12.5
300705 12.5
```

RNA-Seq: Sources of bias

Data from RNA-Seq experiments contains several sources of bias:

- Sample bias
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$\Rightarrow$ The number of reads per gene G is a combination of these factors:

$$\text{reads}(G) \sim v_{\text{sample}} \cdot N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

The goal of RNA-Seq is to find the true expression strength of each gene.

RNA-Seq: Normalizing data to reduce bias (I)

$$\text{reads}(G) \sim v_{\text{sample}} \cdot N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

The influence of v_{sample} is two-fold:

1. Smaller sample volumes result in fewer reads overall.

$\rightarrow$ we can treat v_{sample} as a part of N_{reads} during normalization.

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The influence of v_{sample} is two-fold:

1. Smaller sample volumes result in fewer reads overall.

$\rightarrow$ we can treat v_{sample} as a part of N_{reads} during normalization.

2. If sample volume is too small, low-expressed genes are not represented by any reads at all, and are missed.

$\rightarrow$ This problem can not be solved by data normalization. The sample volume has to be chosen correctly during wet-lab work!

RNA-Seq: Normalizing data to reduce bias (II)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

Since we know the N_{reads} for each sample, we can easily correct for differences between samples.

For two samples A, B:

$$\text{Expression}'(G_A) \sim \text{Expression}(G_A) : N_{\text{reads}}(A)$$

$$\text{Expression}'(G_B) \sim \text{Expression}(G_B) : N_{\text{reads}}(B)$$

And differential expression can then be computed as

$$\frac{\text{Expression}'(G_A)}{\text{Expression}'(G_B)}$$

RNA-Seq: Normalizing data to reduce bias (III)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

This is known as the RPM (reads per million) measure:

Reads per million — **RPM**

$$\text{rpm}(G) = \text{reads}(G) \cdot \frac{10^6}{N_{\text{reads}}}$$

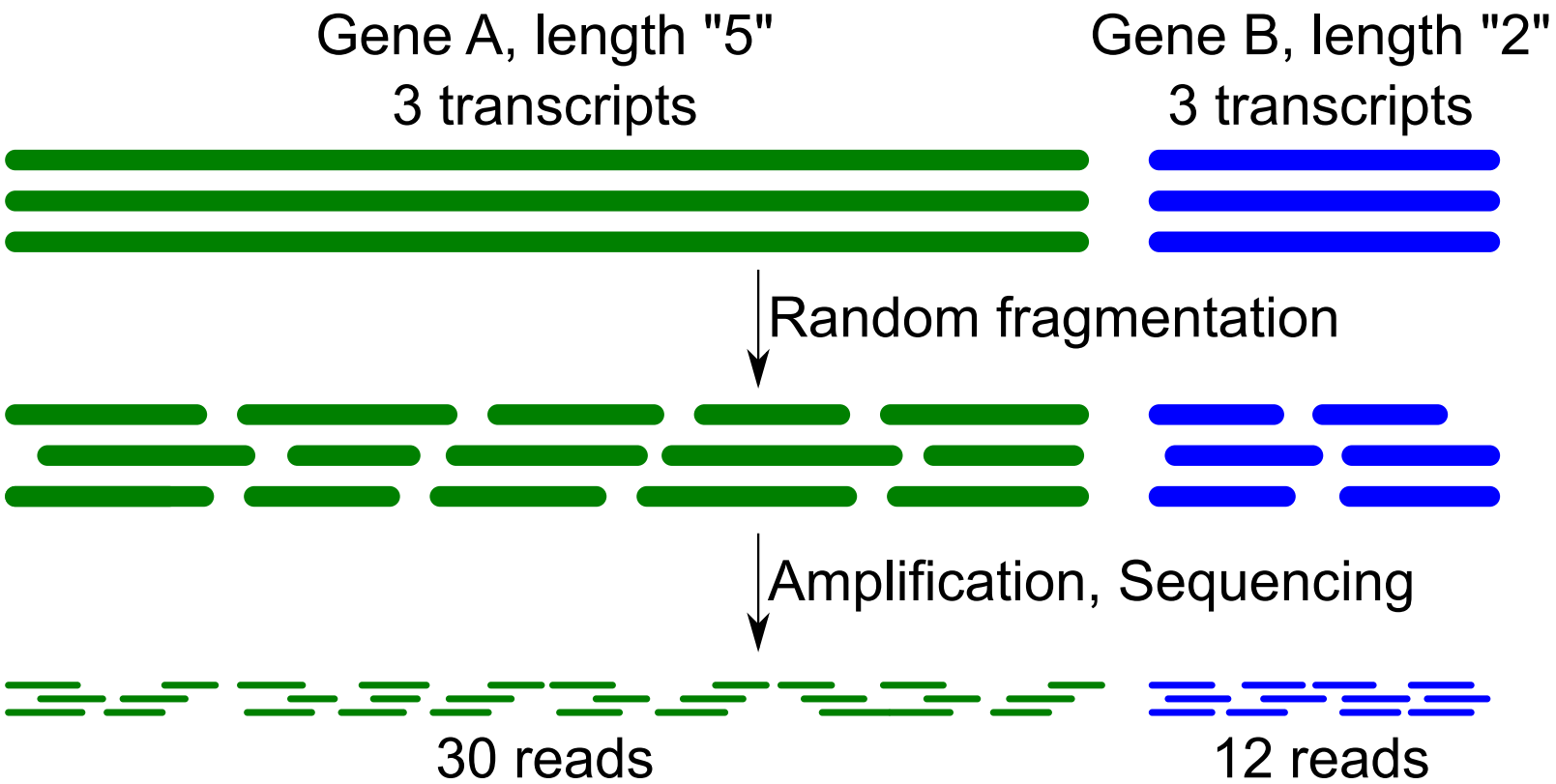
$\text{rpm}(G)$ is the expression of G relative to the total expression in the sample.

However, this is not without problems, as we will see in the next slide.

RNA-Seq: Normalizing data to reduce bias (IV)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

The influence of $\ell(G)$ is straight-forward:



RNA-Seq: Normalizing data to reduce bias (V)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

Since we know $\ell(G)$, we can correct for it. This leads to a new measure:

Reads per million per kilobase of exon model — **RPKM**

$$\text{rpkm}(G) = \text{reads}(G) \cdot \frac{10^6}{N_{\text{reads}}} \cdot \frac{1}{\ell(G)}$$

where $\ell(G)$ is the number of bases in all exons of G.

If a gene has several transcript variants (due to alternative splicing), each variant has its own (possibly different) rpkm value.

RNA-Seq: Normalizing data to reduce bias (VI)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot e_{\text{PCR}}(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

Sequence amplification by PCR is not unbiased.

Factors that influence amplification:

- GC rich sequences
- Sequences that form secondary structures
- ...

- We will treat PCR bias as part of technology-dependent bias.

RNA-Seq: Normalizing data to reduce bias (VII)

$$\text{reads}(G) \sim N_{\text{reads}} \cdot \ell(G) \cdot s_{\text{technology}}(G) \cdot \text{Expression}(G)$$

Different technologies have different biases.

- Illumina sequencing prefers longer reads

→ long genes with small changes appear more interesting than short genes with strong changes.
There is no good solution for this problem.
One could take a random region of e.g. 250 bp from each gene.

- GC bias: Expression of low-GC regions can appear twice as strong
Usually GC content of genes is not very different in one organism.

→ New third generation sequencing technologies eliminate the PCR step.

RNA-Seq: Normalization measures

So far we have discussed

- **RPM** – correcting for sample volume and sequencing depth
- **RPKM** – also correcting for transcript length

We mention a further measure, a variant of RPKM:

Fragments per kilobase exon model per million reads — **FPKM**

FPKM is used by *Cufflinks*. Built on a statistical model, it reflects the likelihood that one would observe the fragments in the experiment, given the proposed abundances on the transcripts.

Quantile normalization: Motivation

RPM, RPKM and FPKM normalize by the total number of reads, N_{reads}.

This is based on two assumptions:

The **percentage** of reads assigned to a gene G
reflects its expression strength.

and

A change in that percentage between two samples
reflects a change in expression strength.

But: N_{reads} is dominated by a small number of highly-expressed genes.

50% of all reads are due to 5% of the expressed genes
in a typical human sample.

Quantile normalization: Example 1

Consider two sequenced samples A and B.

- Both contain the same number of reads.
- Our simple organism has three genes: X, Y, and Z.
- Gene expression is unchanged except for one highly-expressed gene
- How is this reflected in the percentages?

	Reads			Percentages		
Gene	A	B	interpretation	A	B	interpretation
X	10	10	no change	10	5	**50% down**
Y	40	40	no change	40	20	**50% down**
Z	50	150	300% up	50	75	**50% up**
	100	200				

Quantile normalization: Example 2

Consider two sequenced samples A and B with the same number of reads.

- This organism has four genes: X, Y, and $\hat{X}$, $\hat{Y}$.
- $\hat{X}$ and $\hat{Y}$ are only active in sample B

	Reads			Percentages		
Gene	A	B	interpretation	A	B	interpretation
X	20	20	no change	20	10	**50% down**
Y	80	80	no change	80	40	**50% down**
$\hat{X}$	0	20	switched on	0	10	switched on
$\hat{Y}$	0	80	switched on	0	40	switched on
	100	200				

Quantile normalization: Conclusion

From the examples, we can draw the conclusion that...

> ...the number of reads expected to map to a gene is not only dependent on the **expression level** and **length of the gene**, but also the **composition of the RNA population** that is being sampled. Thus, if a large number of genes are unique to, or highly expressed in, one experimental condition, the sequencing 'real estate' available for the remaining genes in that sample is decreased.
>
> *Robinson & Oshlack, Genome Biology 2010, 11:R25*

Quantile normalization as proposed solution

The quantile normalization procedure by Bullard *et al.* works as follows:

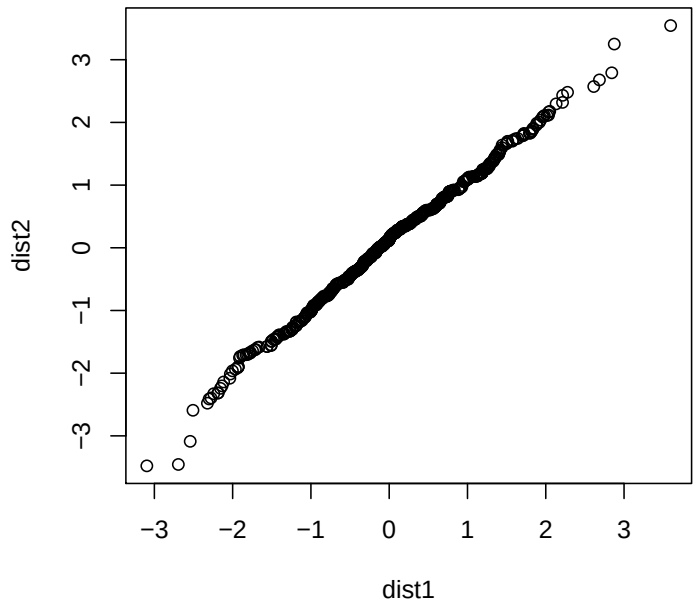
1. Count the reads for each gene
2. Ignore all genes with 0 reads
3. Compute the upper quartile, q_{75}
   q_{75} is the number of reads needed such that
   75% of the (nonzero) genes have less than q_{75} reads.
4. Divide all read counts by q_{75}.

Bullard et al. Bioinformatics 2010, 11:94

Visualizing quantile normalized data

After quantile normalisation one can visualise the distribution of the expression values in each sample using either

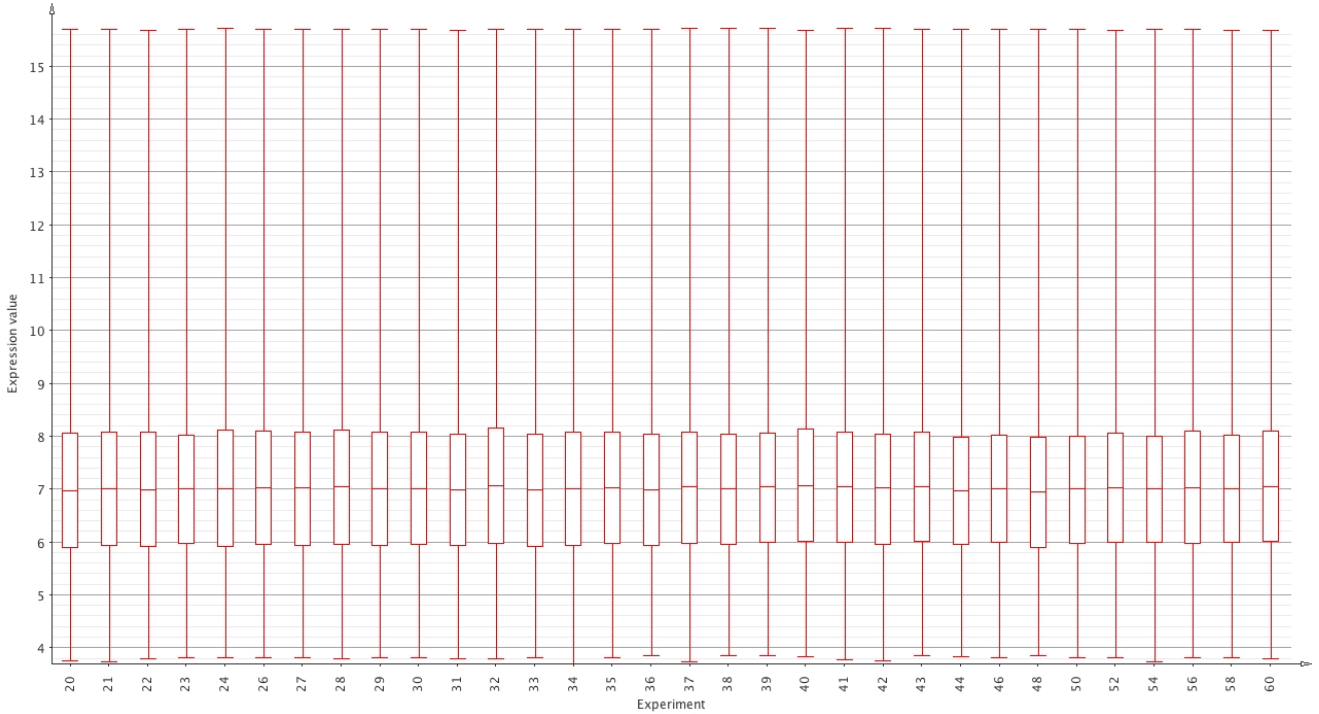
- A qq-plot: it plots the quantiles of two distributions as a scatter plot against each other. Two very similar distributions have almost a scatter along the diagonal:



Visualizing quantile normalized data

After quantile normalisation one can visualise the distribution of the expression values in each sample using either

- A boxplot: shows for each experiment the box plot of the expression values. Quantile normalised data should have very similar looking box plots.



Practical Session

Questions?

Practical Session

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Now off to the fifth practical session

Learning Objectives of Practical Session

- Run HTSeq-count
- Load .bam files after RNA-seq mapping with STAR into Mayday SeaSight
- Learn how to normalise count data in Mayday