3000788 Intro to Comp Molec Biol

Lecture 10: RNA sequencing data processing

September 15, 2022

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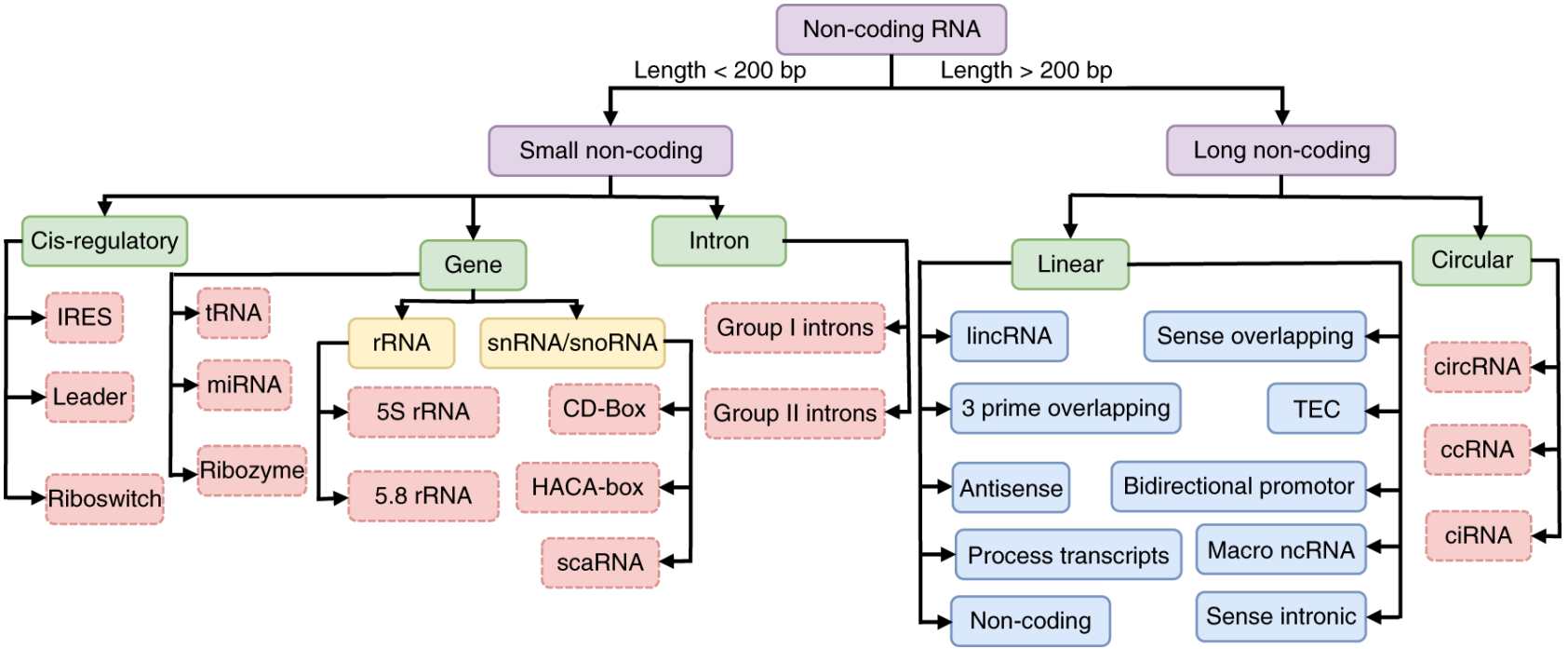
- Research Affairs
- Center of Excellence in Computational Molecular Biology (CMB)
- Center for Artificial Intelligence in Medicine (CU-AIM)

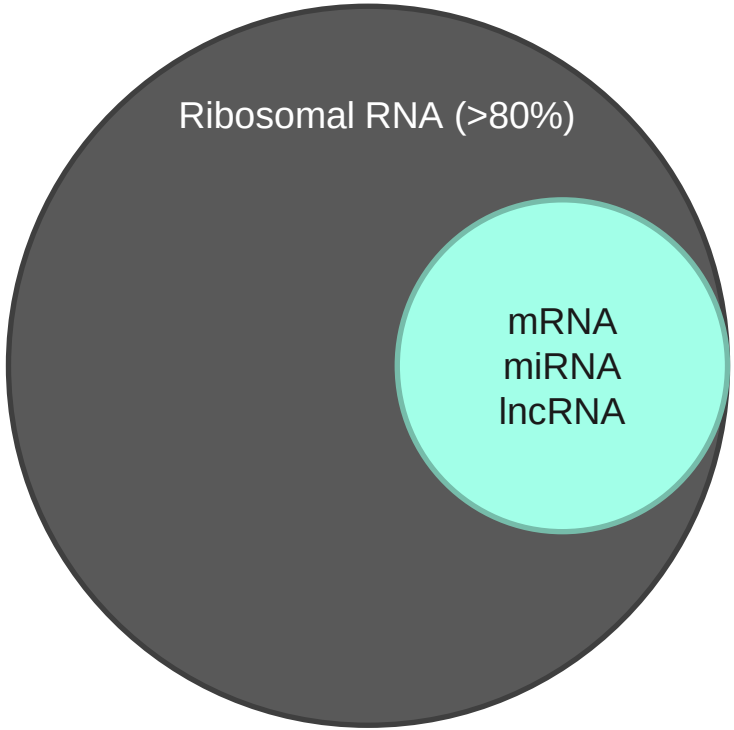
RNA-seq techniques

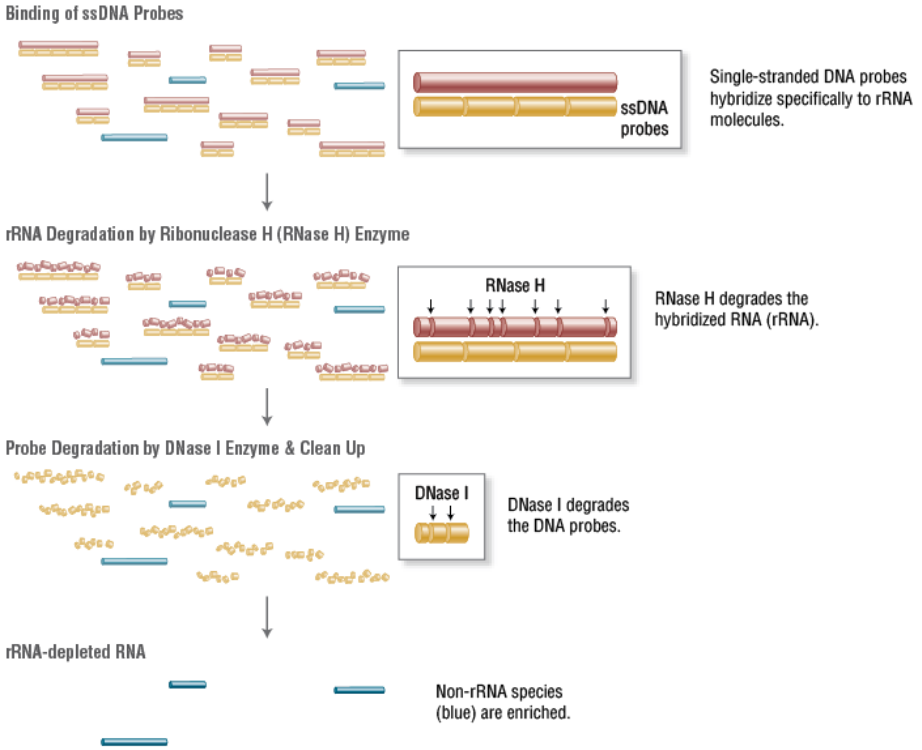
Non-coding RNAs



Amin, N. et al. Nature Machine Intelligence 1:246-256 (2019)

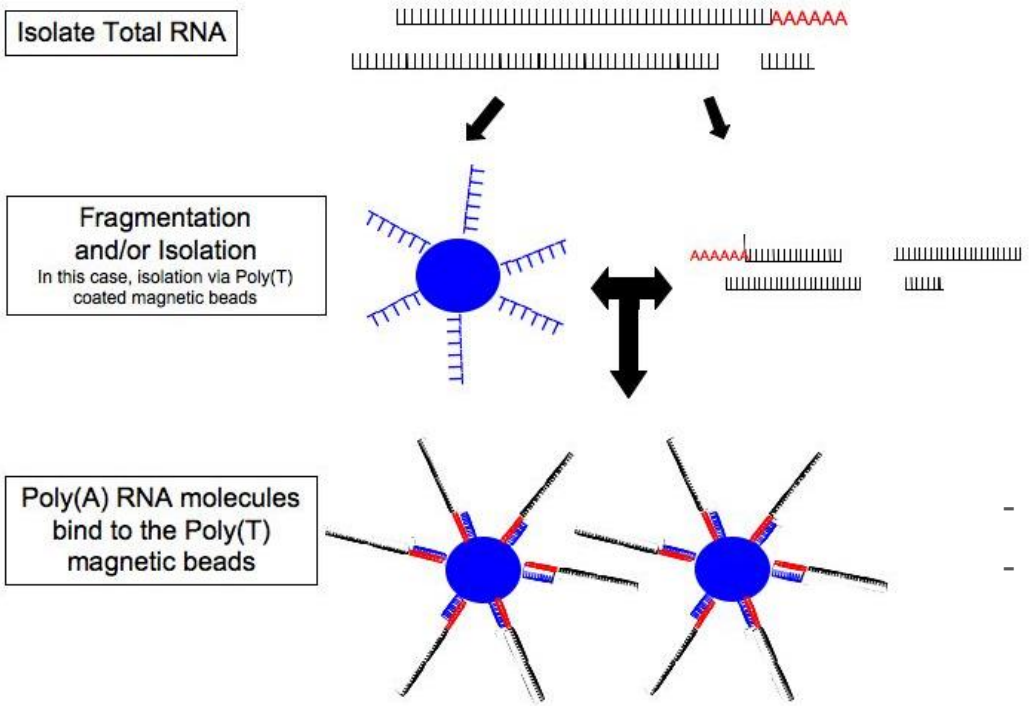
Total RNA sequencing

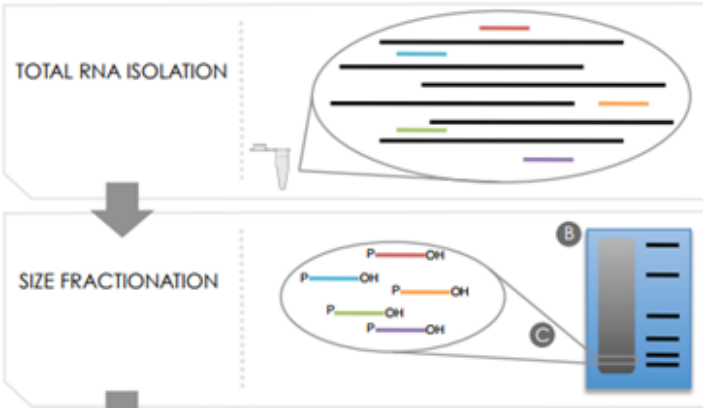




Source: New England BioLabs

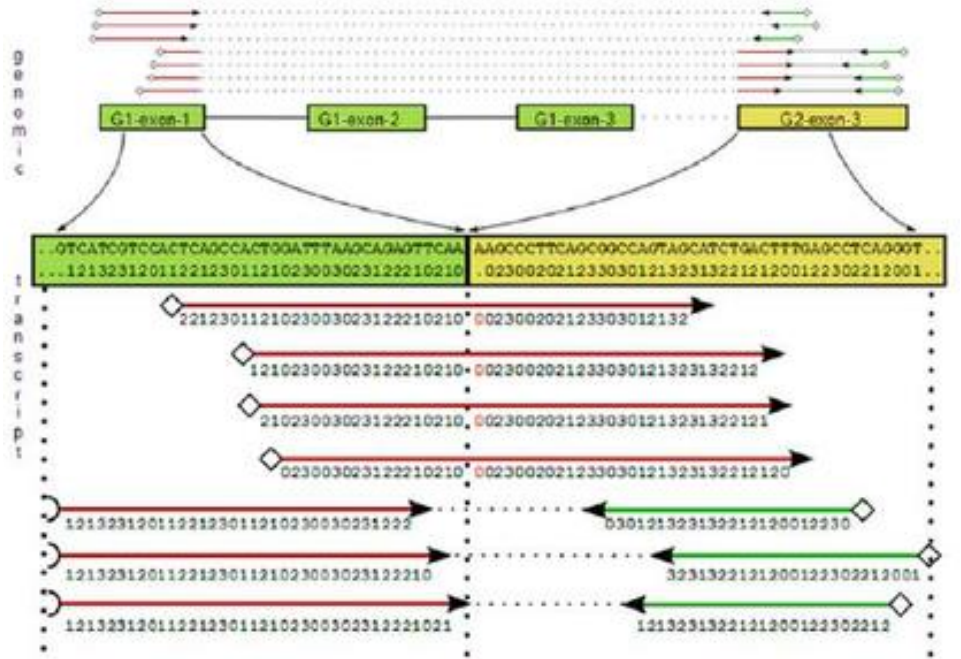
mRNA and miRNA sequencing





- Selection by polyT probe
- Size fractionation

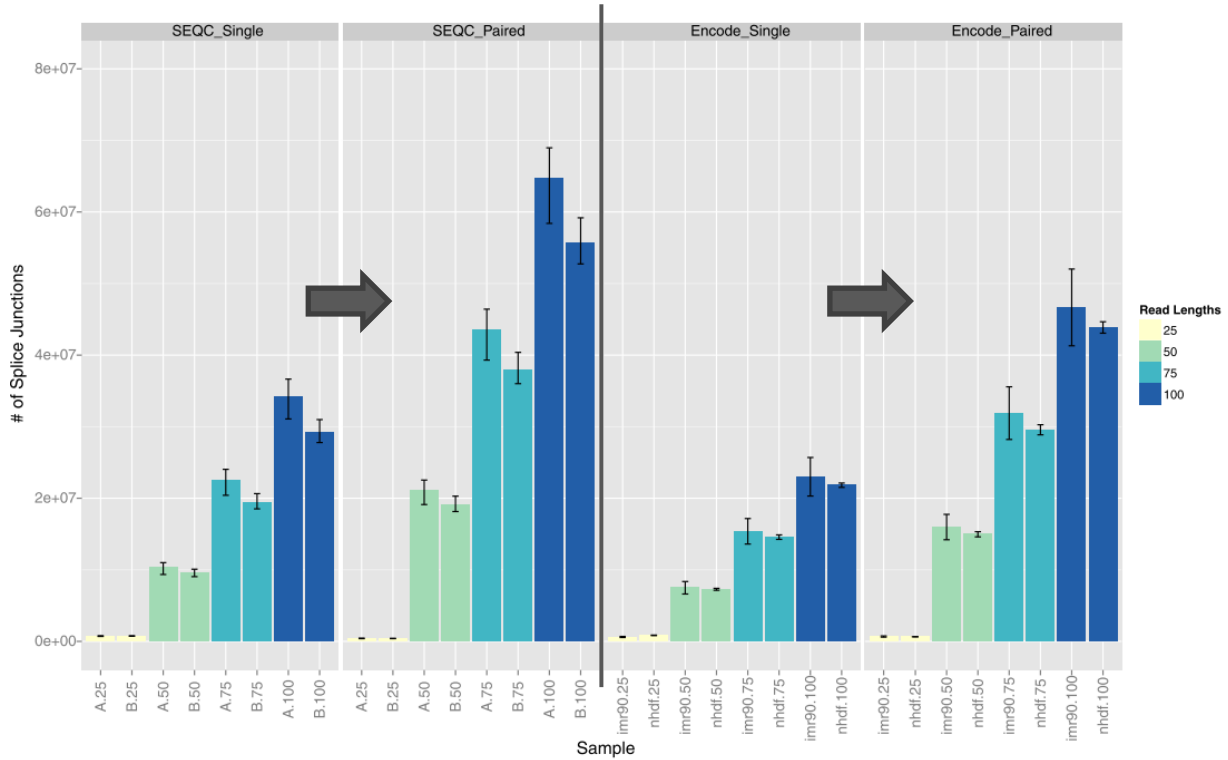
Transcript isoform detection



- **Single-end sequencing**: The read must span the exon junction
- **Paired-end sequencing**: As long as the forward and reverse reads came from different exons

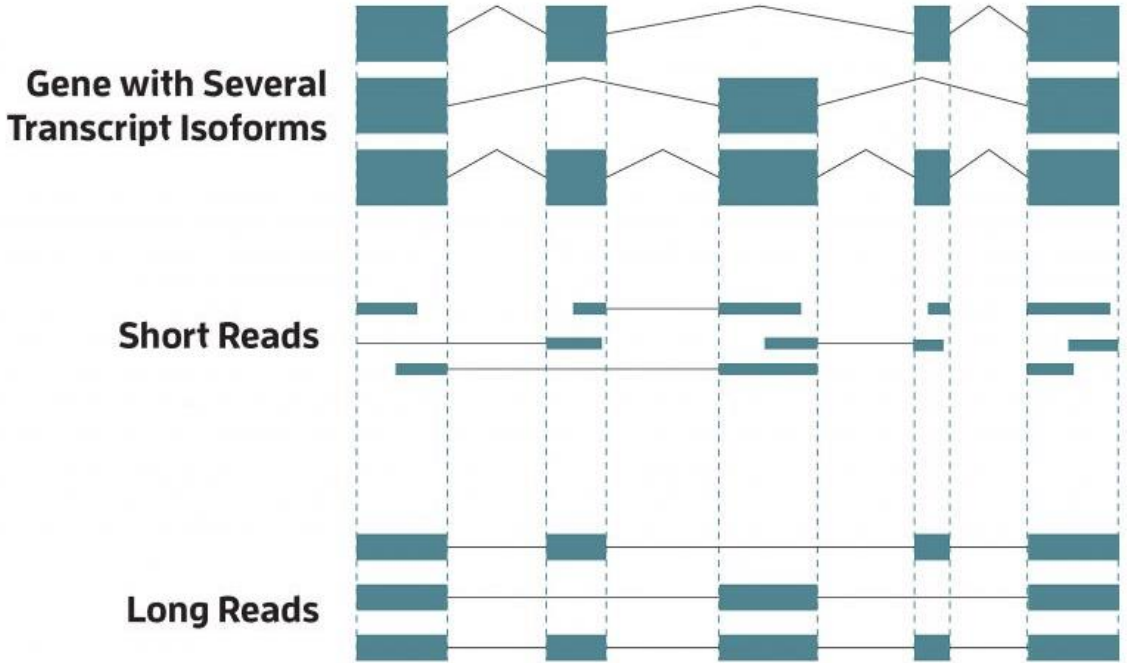
Sakarya, O. et al. PLoS Comp Biol 8:e1002464 (2012)

Impact of paired-end sequencing and read length



Chhangawala *et al.* Genome Biology 16:131 (2015)

Full-length transcript sequencing



https://www.genengnews.com/resources/tutorial/full-length-transcript-sequencing-no-assembly-required/

Choosing RNA-seq technique

- miRNA or mRNA or total-RNA

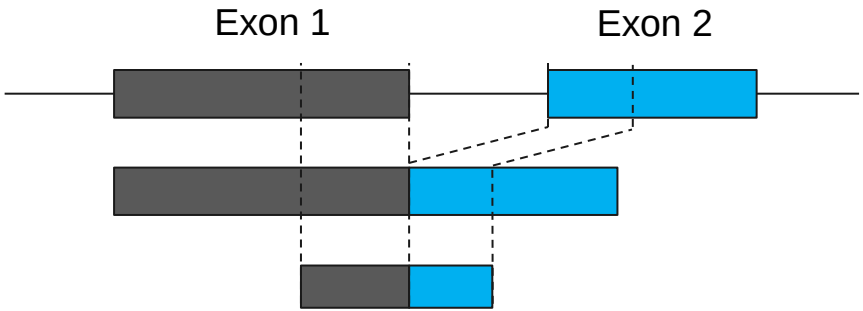
- Single-end or paired-end
  - Single-end is ok for gene-level quantification
  - Paired-end is needed to distinguish isoforms

- Illumina or 3rd generation sequencer
  - Long-read data is helpful for genes with complex isoforms and for detecting novel isoforms

RNA-seq analysis

Three primary pipelines

- Reference-free
  - Novel species, rely on *de novo* assembly
- Reference transcriptome
  - Fast, cannot discover new isoform
  - Ungapped, *k*-mer-based alignment
- Reference genome
  - Slow, but can detect new isoforms
  - Gapped alignment

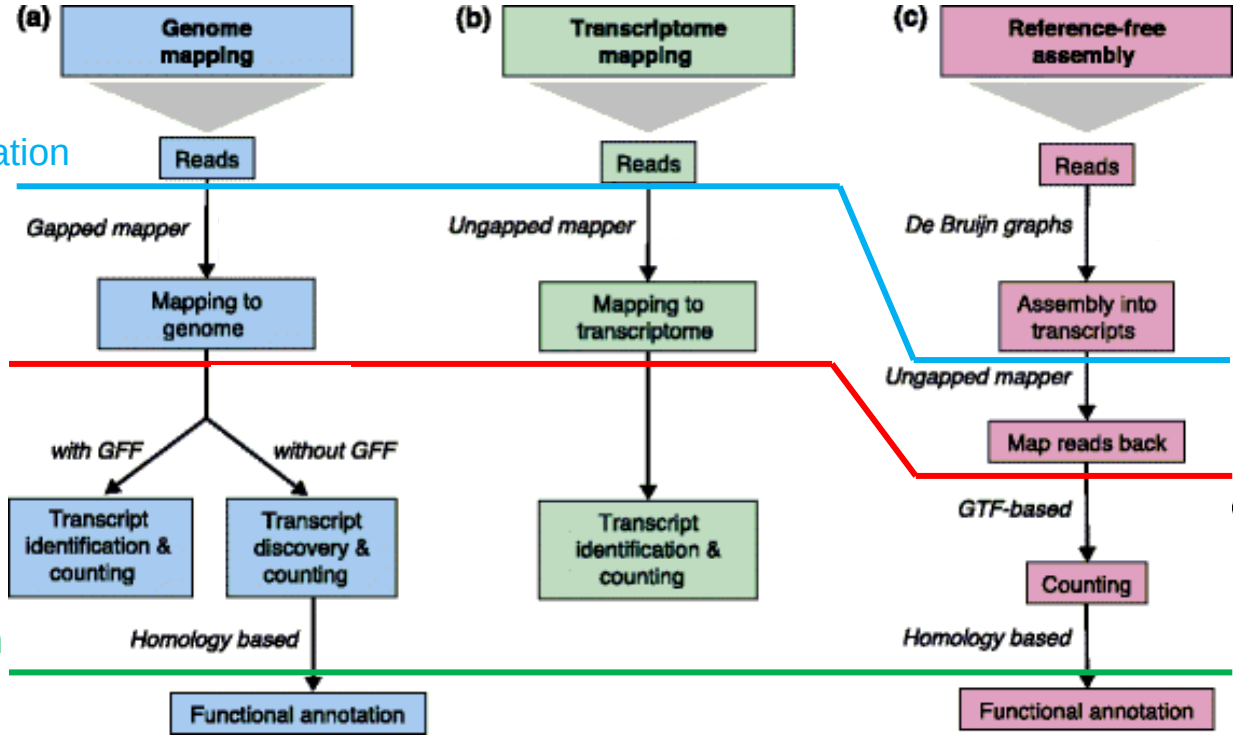


Pipeline overview

Reference preparation

Alignment

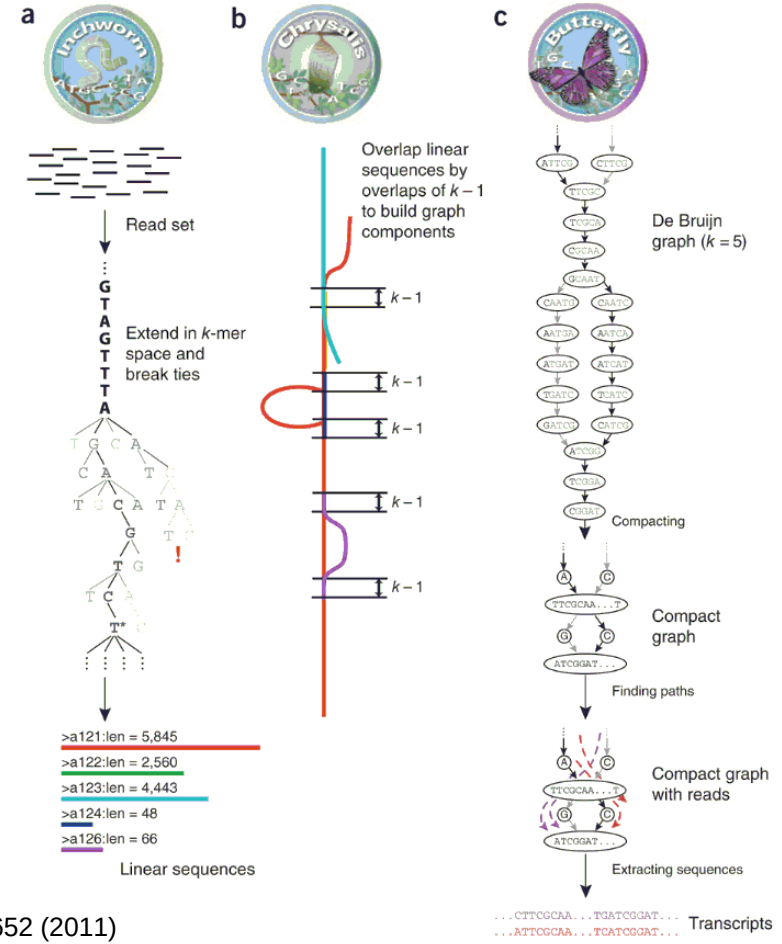
Quantification



Conesa *et al.* Genome Biology 17:13 (2016)

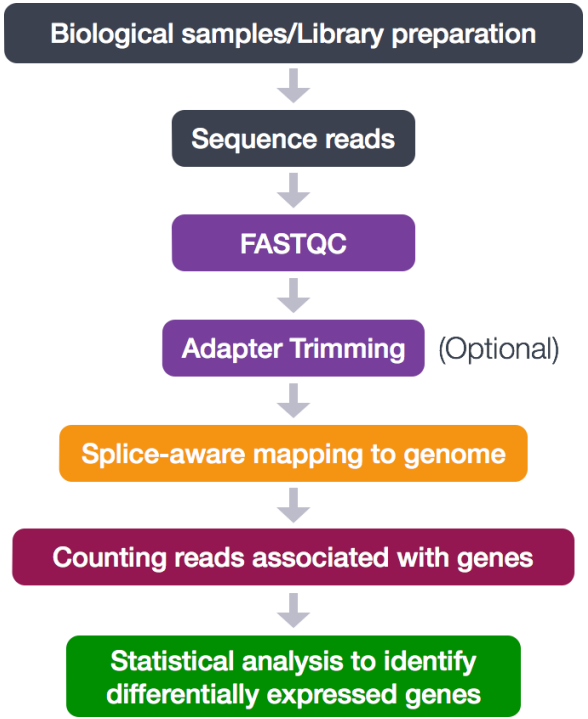
De novo transcript assembly

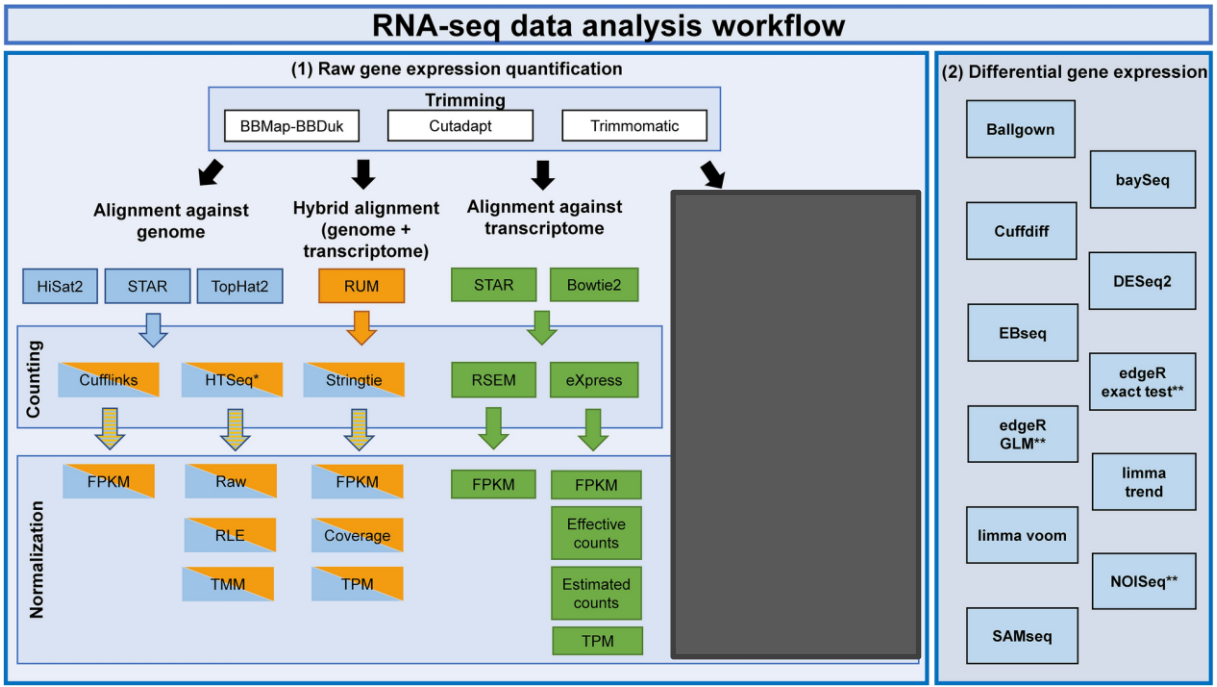
- Similar to genome assembly
  - Connect overlapping reads into contigs
- Handle the existence of isoforms
  - Cluster contigs with shared sequences
  - Generate all possible isoforms
- Used as database for downstream analysis



Grabherr *et al.* Nat Biotech 29:644-652 (2011)

Alignment-based pipelines

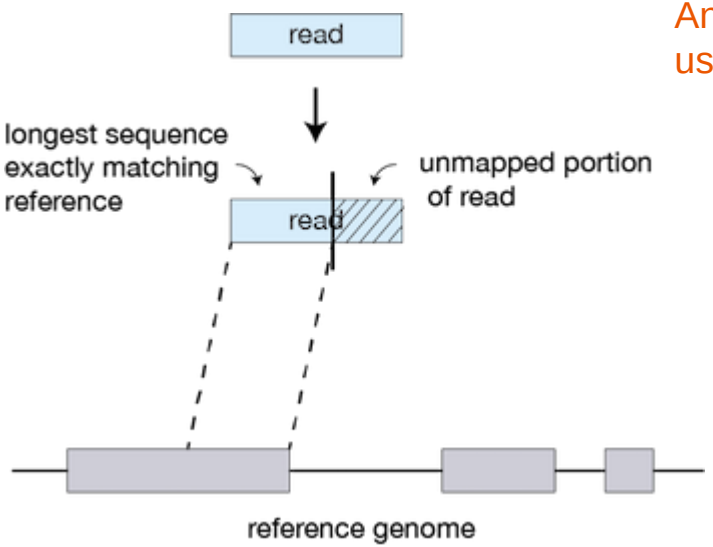




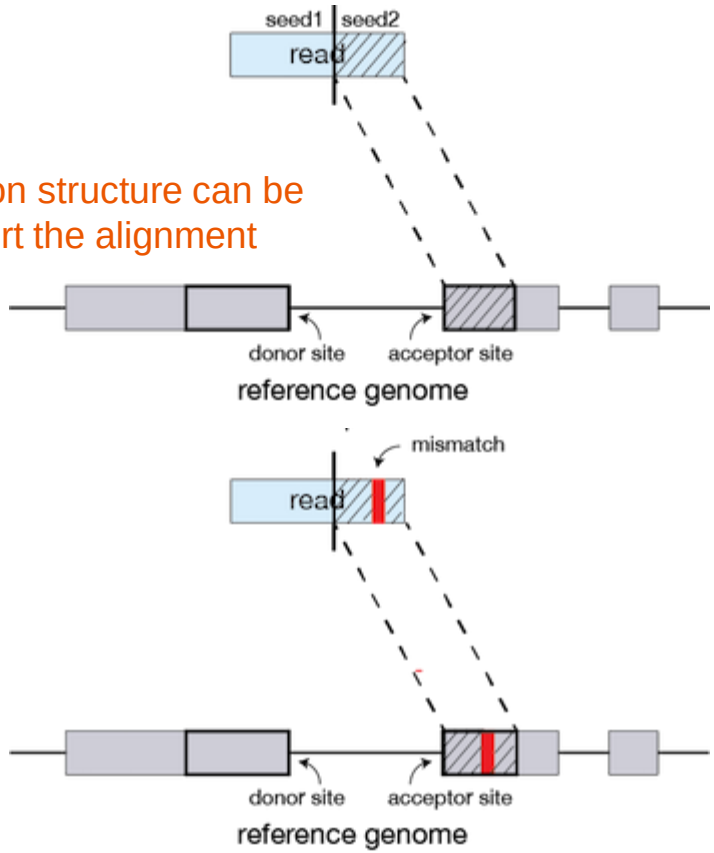
https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html

Corchete, L.A. *et al.* Sci Rep 10:19737 (2020)

Gapped alignment



Annotated exon structure can be used to support the alignment



https://hbctraining.github.io/Intro-to-rnaseq-hpc-O2/lessons/03_alignment.html

GTF/GFF genome annotation format

Sample GTF output from Ensembl data dump:

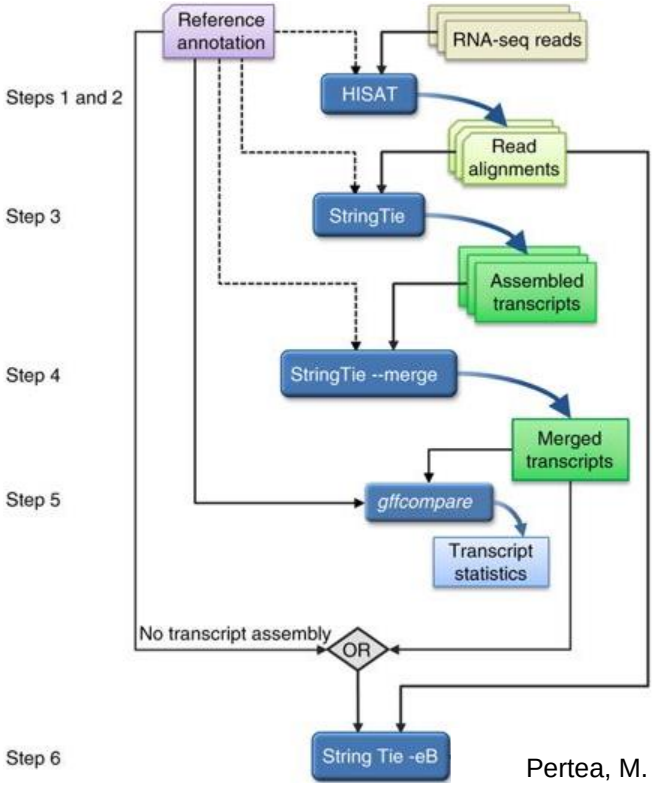
```
1 transcribed_unprocessed_pseudogene  gene        11869 14409 . + . gene_id "ENSG00000223972"; gene_name "DDX11L1"; gene_source "havana";
1 processed_transcript                transcript  11869 14409 . + . gene_id "ENSG00000223972"; transcript_id "ENST00000456328"; gene_name
```

Sample GFF output from Ensembl export:

```
X       Ensembl Repeat  2419108 2419128 42      .       .       hid=trf; hstart=1; hend=21
X       Ensembl Repeat  2419108 2419410 2502    -       .       hid=AluSx; hstart=1; hend=303
X       Ensembl Repeat  2419108 2419128 0       .       .       hid=dust; hstart=2419108; hend=2419128
X       Ensembl Pred.trans.     2416676 2418760 450.19  -       2       genscan=GENSCAN00000019335
X       Ensembl Variation       2413425 2413425 .       +       .
X       Ensembl Variation       2413805 2413805 .       +       .
```

- Tab-separated text file
- Chromosome ID, object name, base pair positions, strand, and other annotation details

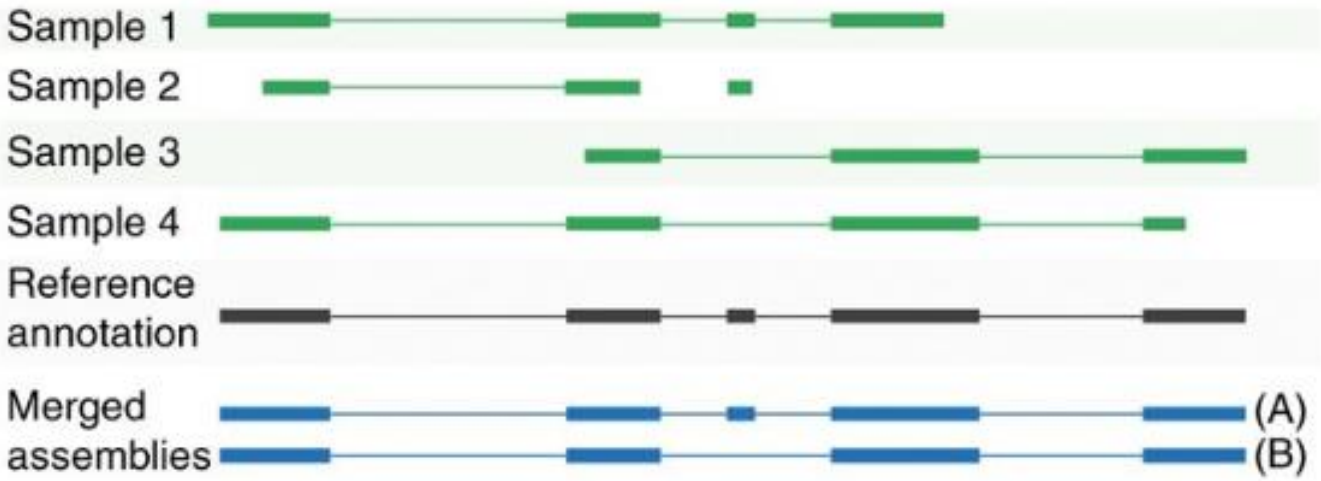
Multi-step post-alignment processing



- Initial alignment
- Assemble potential novel isoforms
- Merge isoforms across samples
- Re-quantify isoform abundances using the merged database of isoforms

Pertea, M. et al. Nature Protocols 11:1650-1667 (2016)

Importance of merging isoforms



Pertea, M. et al. Nature Protocols 11:1650-1667 (2016)

- Rare isoform may be missing in some samples
- Reads can get misinterpret if the correct isoform is not in the reference

GTF with abundance annotation

```
chr1    StringTie       transcript      36534   36849   1000    .       .       gene_id "STRG.1"; transcript_id "STRG.1.1"; cov "19.614035"; FPKM "6.688056"; TPM "10.944590";
chr1    StringTie       transcript      35245   36073   1000    -       .       gene_id "STRG.2"; transcript_id "STRG.2.1"; reference_id "ENST00000461467.1"; ref_gene_id "ENSG00000237613.2";
                                                                                ref_gene_name "FAM138A"; cov "0.327684"; FPKM "0.111735"; TPM "0.182847";
chr1    StringTie       transcript      52473   53312   1000    +       .       gene_id "STRG.3"; transcript_id "STRG.3.1"; reference_id "ENST00000606857.1"; ref_gene_id "ENSG00000268020.3";
                                                                                ref_gene_name "OR4G4P"; cov "0.119048"; FPKM "0.040593"; TPM "0.066429";
chr1    StringTie       transcript      137682  137965  1000    -       .       gene_id "STRG.4"; transcript_id "STRG.4.1"; reference_id "ENST00000595919.1"; ref_gene_id "ENSG00000269981.1";
                                                                                ref_gene_name "RP11-34P13.16"; cov "0.000000"; FPKM "0.000000"; TPM "0.000000";
chr1    StringTie       transcript      139283  139642  1000    .       .       gene_id "STRG.5"; transcript_id "STRG.5.1"; cov "3.111111"; FPKM "1.060837"; TPM "1.735993";
```

- Different tool outputs transcript abundance in different format

- GTF can accommodate abundance annotation in the last columns
  - Coverage (cov) = fraction of transcript length with mapped read
  - FPKM = Fragment per kilobase of exon per million reads mapped
  - TPM = Transcript per million

Units for transcript abundance

$$\text{FPKM} = \frac{\text{Read Count}}{\frac{\text{Transcript Length}}{1{,}000} \times \frac{\text{Total Read Count}}{1{,}000{,}000}}$$

Long transcript generates more fragments and more read counts

Experiment with higher sequencing depth generates more read counts

Similar to percentage (but per million)

$$\text{TPM} = \frac{\text{FPKM}}{\sum \text{FPKM}} \times 1{,}000{,}000$$

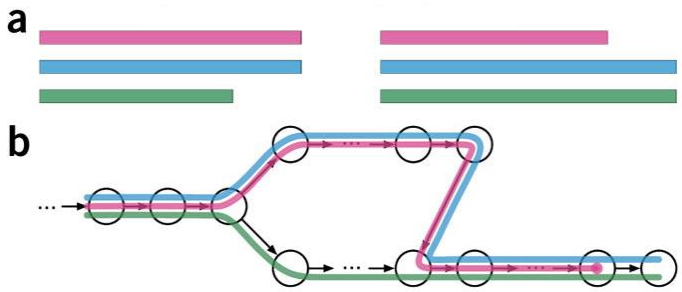
- Read count (number of mapped reads)
- FPKM = Fragment per kilobase of exon per million reads mapped
- TPM = Transcript per million

Alignment-based pipeline summary

- Initial alignment to reference genome (with annotated gene structure)
  - STAR / HISAT2
- [Optional]
  - Identify novel isoforms
  - Merge isoforms across samples
- Quantify transcript abundances
  - Read count / FPKM / TPM
  - StringTie2 / htseq-count

k-mer pseudoalignment

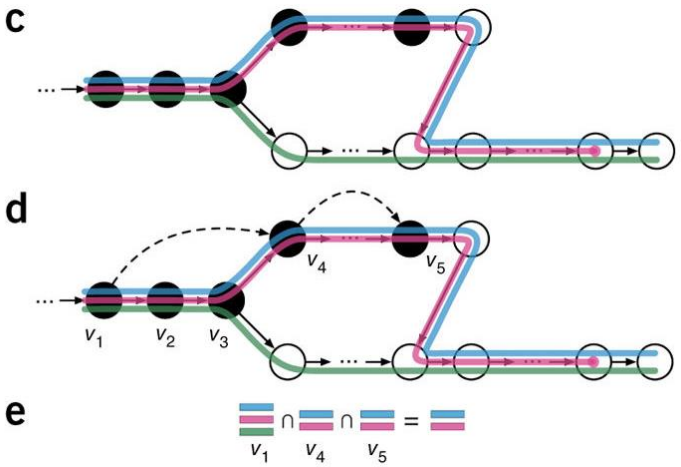
k-mer database for transcriptome



Bray *et al.* Nat Biotech 34:525-527 (2016)

- Create de Bruijn graph with *k*-mer as nodes
- Map node to transcripts with that *k*-mer
- Contig = a path on de Bruijn graph that mapped to the same transcript

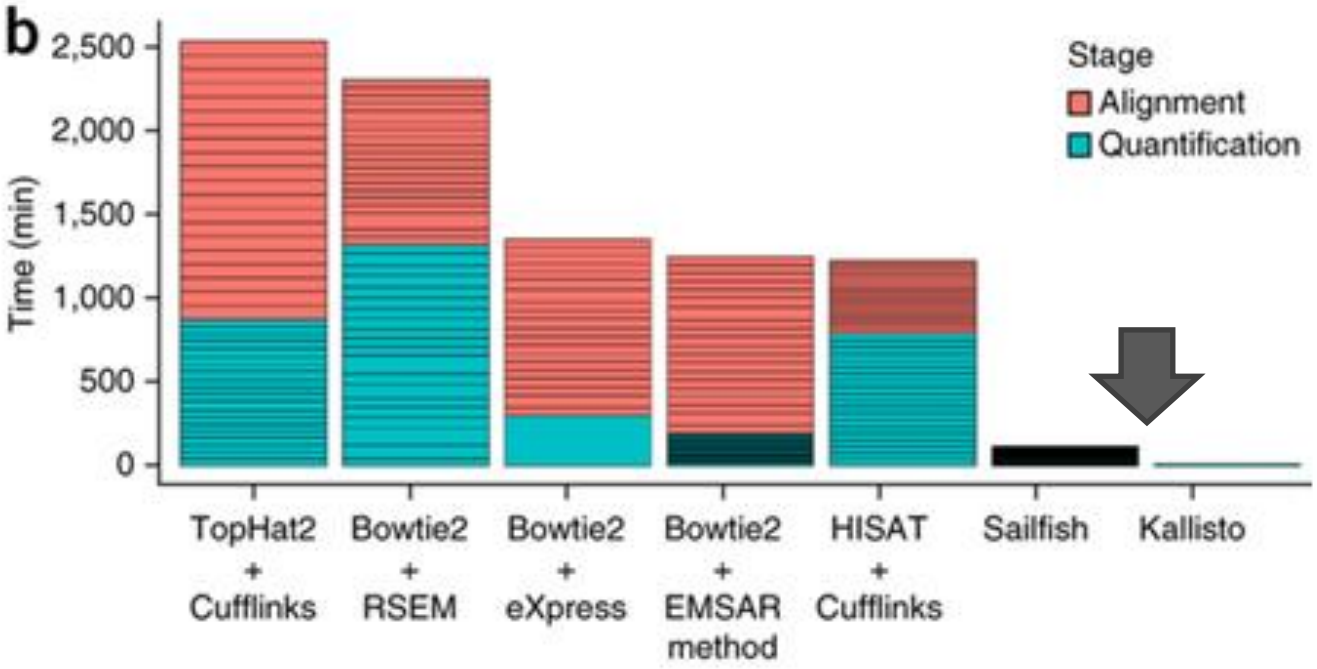
k-mer pseudoalignment



Bray *et al.* Nat Biotech 34:525-527 (2016)

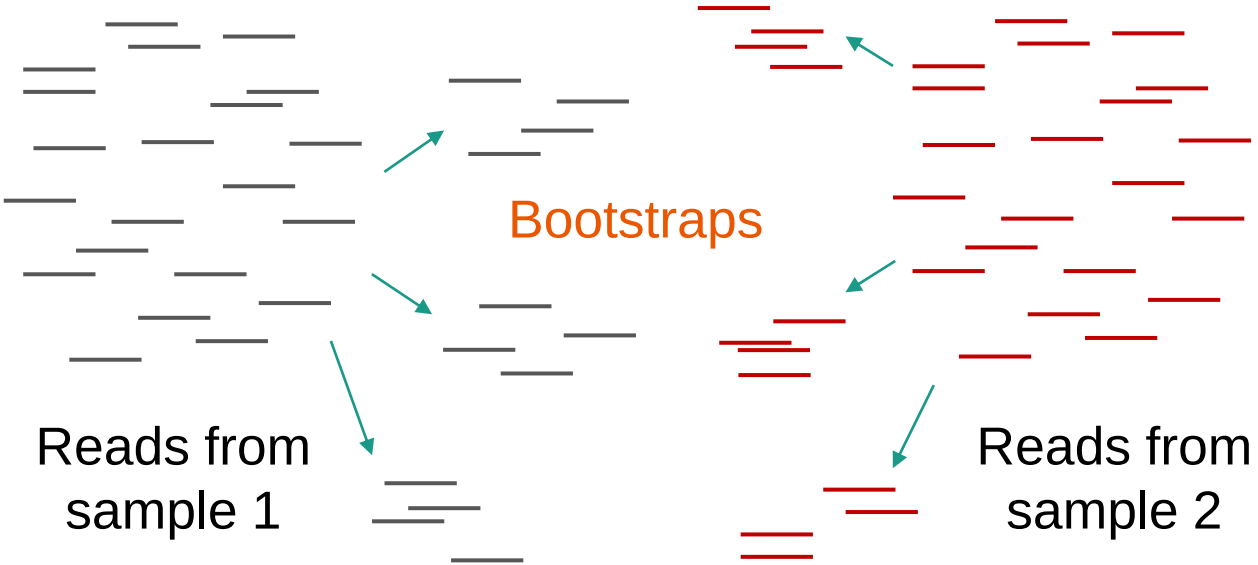
- For a new read, all of its *k*-mers are mapped against contigs
  - Ignore the ordering of *k*-mers on the read
- Report only contigs that are compatible with all *k*-mers
- Speed up by skipping uninformative *k*-mer
  - V_2 and V_3 regions
  - Only 2-4 *k*-mer lookups are enough

>100 fold speed up with pseudoalignment



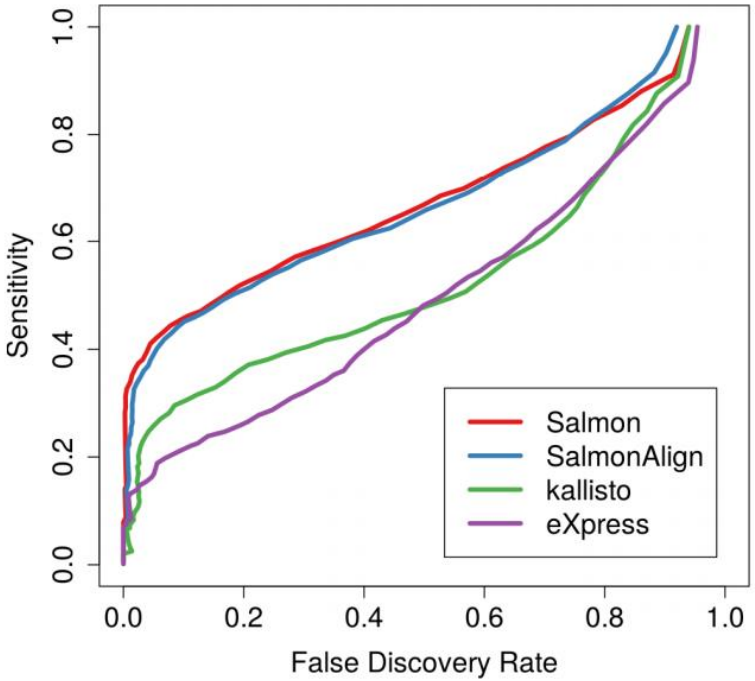
Bray *et al.* Nat Biotech 34:525-527 (2016)

Bootstrapping enabled by pseudoalignment



- Bootstrapping estimates technical variances

Salmon: improved *k*-mer alignment

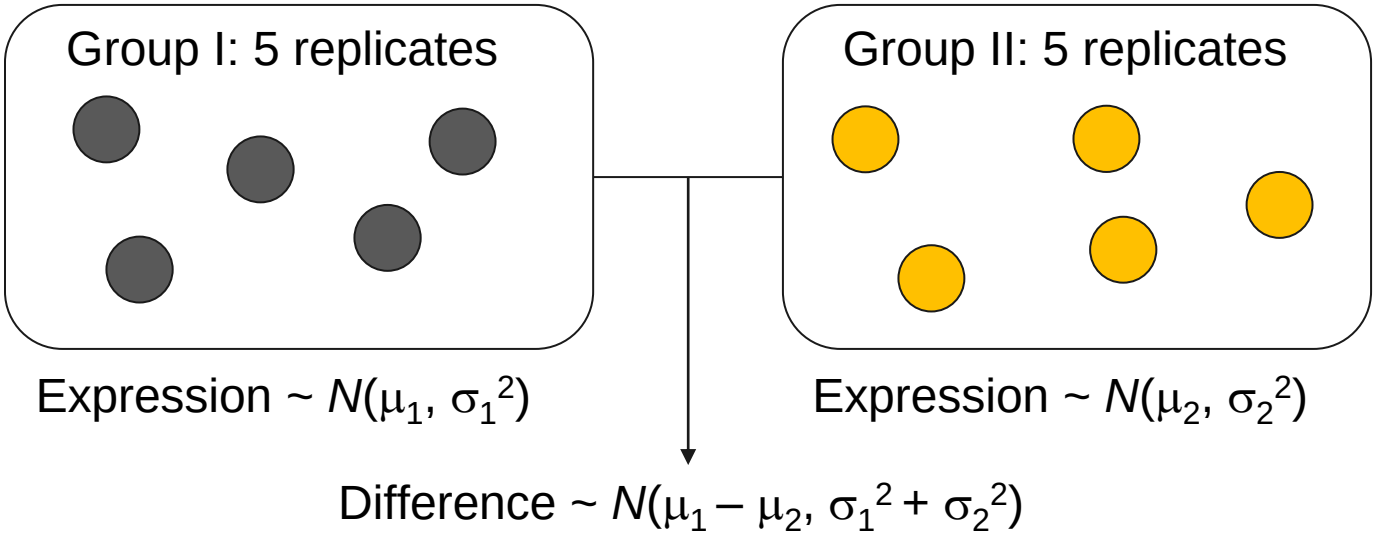


Patro *et al.* Nat Methods 14:417-419 (2017)

- Also track the location of *k*-mer on the input read → semi-alignment
- Correct quantification based on GC content and 3' / 5' amplification biases

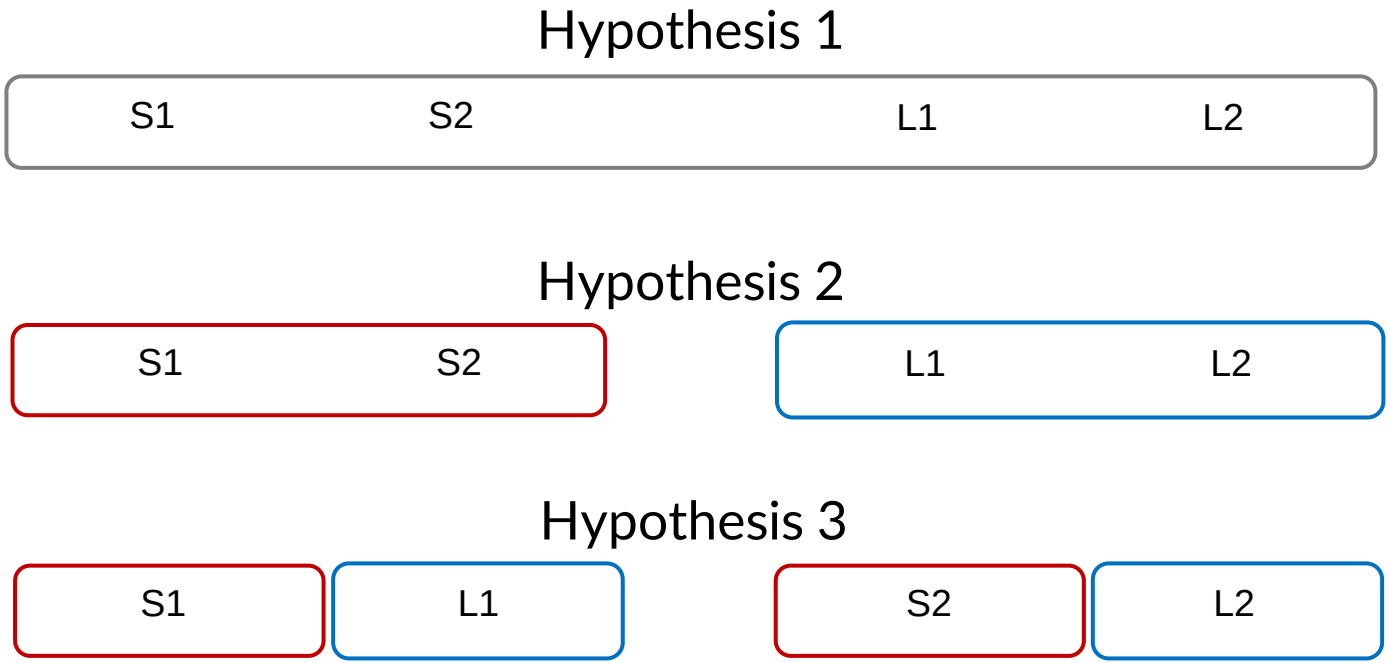
Differential expression analysis

DE for microarray & nanostring

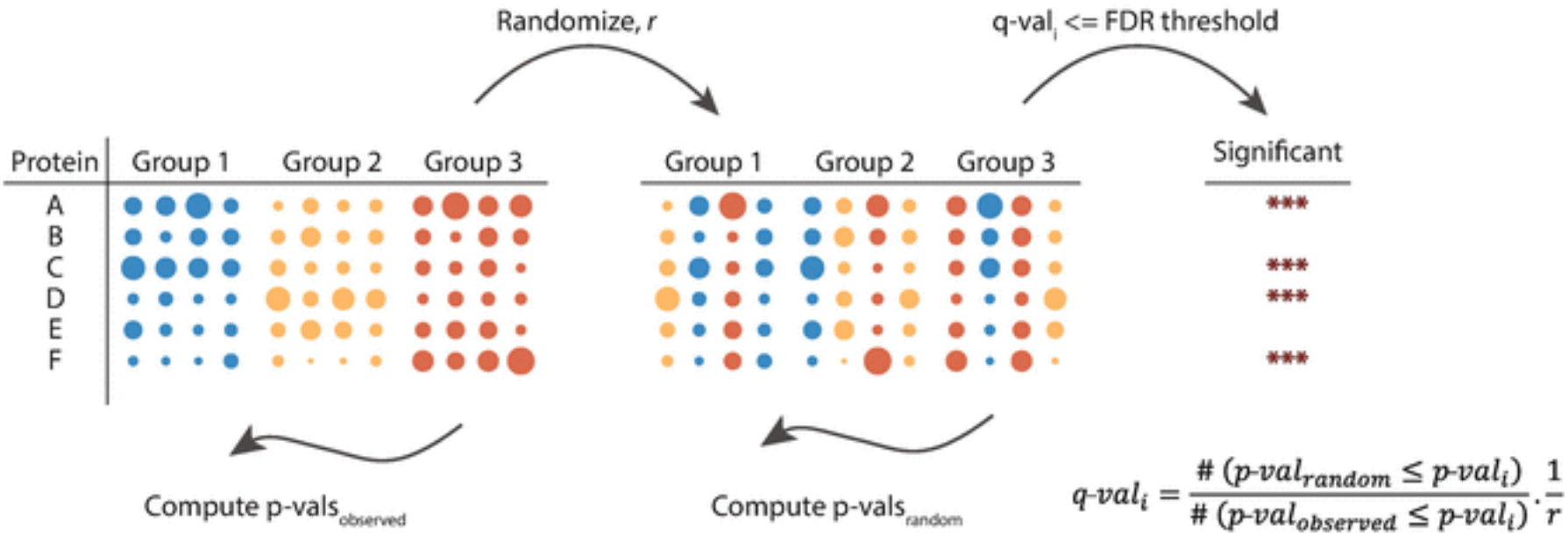


- Simple *t*-test for normally distributed abundance data

DE as nested model testing / likelihood ratio test



DE as permutation test

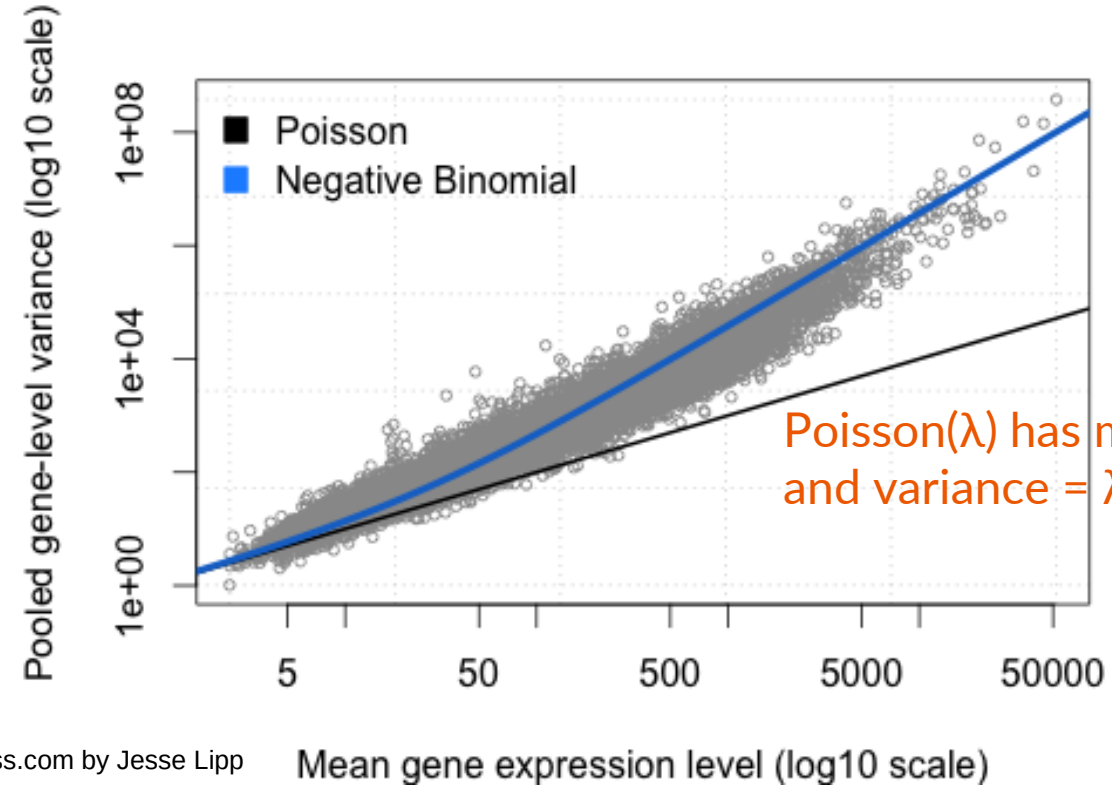


Tyanova and Cox. Cancer Systems Biology pp 133-148. (2018)

- Permuting sample labels = remove condition-specificity

DESeq2 model for read count

The distribution of RNA-seq read count



Poisson(λ) has mean = λ
and variance = λ

Image from bioramble.wordpress.com by Jesse Lipp

Negative binomial model

- NB(r, p) = the number of failures that we will see in a series of Bernoulli trials with probability of success p until we obtain r successes
  - X O O X X X O O X O O = 5 failures until 6 successes

- $\mathrm{P_{NB}}(k; r, p) = \binom{k+r-1}{k}(1-p)^k p^r$
  - k + r – 1 locations to place k failures (the last location must be success)

- Mean = $\frac{pr}{(1-p)}$
- Variance = $\frac{pr}{(1-p)^2} = \frac{pr}{(1-p)}\frac{1}{(1-p)} = \frac{pr}{(1-p)}\left(1+\frac{p}{1-p}\right)$

$$= \frac{pr}{(1-p)} + \frac{p^2 r}{(1-p)^2} = \frac{pr}{(1-p)} + \left(\frac{pr}{1-p}\right)^2 \frac{1}{r}$$

Another view of negative binomial model

- $\mathrm{P}_{\mathrm{NB}}(k; r, p) = \int_0^\infty \mathrm{P}_{\mathrm{Poisson}(\lambda)}(k) \cdot \mathrm{P}_{\mathrm{Gamma}\left(r, \frac{1-p}{p}\right)}(\lambda)\, d\lambda$
- Negative binomial distribution is a continuous mixture of Poisson distribution, with mixing weights Gamma-distributed
  - Same as Gamma site-specific mutation rates

- Bulk gene expression is an average over many cells
- Mixture of read counts from multiple cells, each following Poisson(λ)

DESeq2 model of gene expression

- Read count $K_{i,j} \sim NB(\mu_{i,j}, \sigma_{i,j}^2)$, for gene *i* from sample *j*
  - $\mu_{i,j}$ = sample effects x sequencing effects
  - $\sigma_{i,j}^2 = \mu_{i,j}$ + gene-specific effects x $\mu_{i,j}^2$

- Sample effects
  - Control / Treatment
  - Confounding factors: age, time after treatment, etc.
  - Log FC = $\sum_r x_{j,r}\beta_{i,r}$ where $x_{j,r}$ are design parameters for sample *j* and β_{ir} are the effect sizes
    - Linear effect model

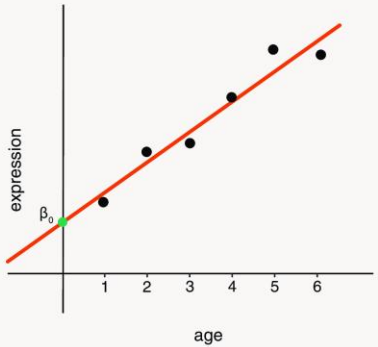
Linear effect models

Covariates: quantitative measurements (e.g. age)

Factors: categorical variables (e.g. genotype)

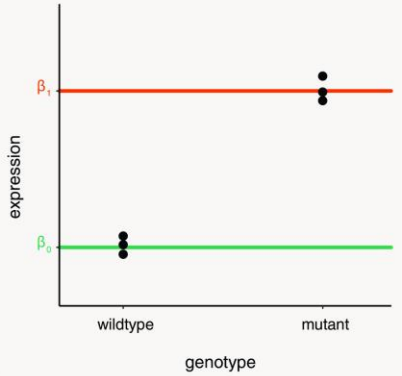
Regression model

$expression = \beta_0 + \beta_1 age$



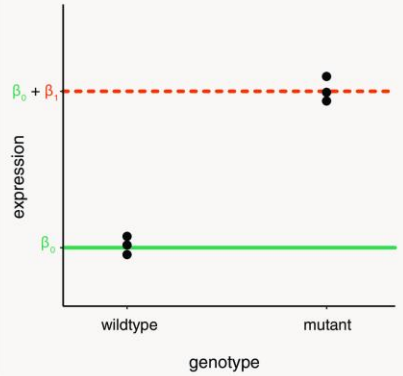
Means model

$expression = \beta_1 wildtype + \beta_2 mutant$



Mean-reference model

$expression = \beta_1 + \beta_2 mutant$



Law, C.E. et al. F100Res 9:1444 (2020)

Legend

- Original data points

Expected gene expression *(based on model)*

Expected gene expression *(of non-reference levels in mean-reference model)*

Linear model for multiple effects

Model

$$E(y) = 1.03 + 1.09x_1 + 1.97x_2 + 0.82x_1x_2$$

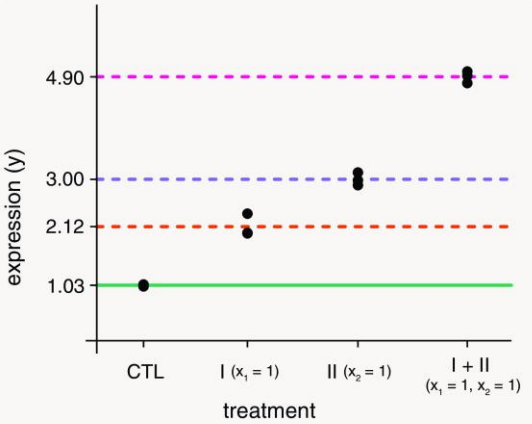
$E(y) = 1.03$	$= 1.03$	*(for control)*
$E(y) = 1.03 + 1.09$	$= 2.12$	*(for treatment I)*
$E(y) = 1.03 + 1.97$	$= 3.00$	*(for treatment II)*
$E(y) = 1.03 + 1.09 + 1.97 + 0.82$	$= 4.90$	*(for treatments I & II)*

Matrix

```
> model.matrix(~treat1 * treat2)
```

	(Intercept)	treat1YES	treat2YES	treat1YES:treat2YES
1	1	0	0	0
2	1	0	0	0
3	1	0	0	0
4	1	1	0	0
5	1	1	0	0
6	1	1	0	0
7	1	0	1	0
8	1	0	1	0
9	1	0	1	0
10	1	1	1	1
11	1	1	1	1
12	1	1	1	1

Plot



Law, C.E. et al. F100Res 9:1444 (2020)

DESeq2 model of gene expression

- Read count $K_{i,j} \sim NB(\mu_{i,j}, \sigma_{i,j}^2)$, for gene *i* from sample *j*
  - $\mu_{i,j}$ = sample effects x sequencing effects
  - $\sigma_{i,j}^2 = \mu_{i,j}$ + gene-specific effects x $\mu_{i,j}^2$

- Sequencing effects
  - Sequencing depth (sample-specific)
  - GC content (gene-specific)
  - Gene length (gene-specific)

DESeq2 model of gene expression

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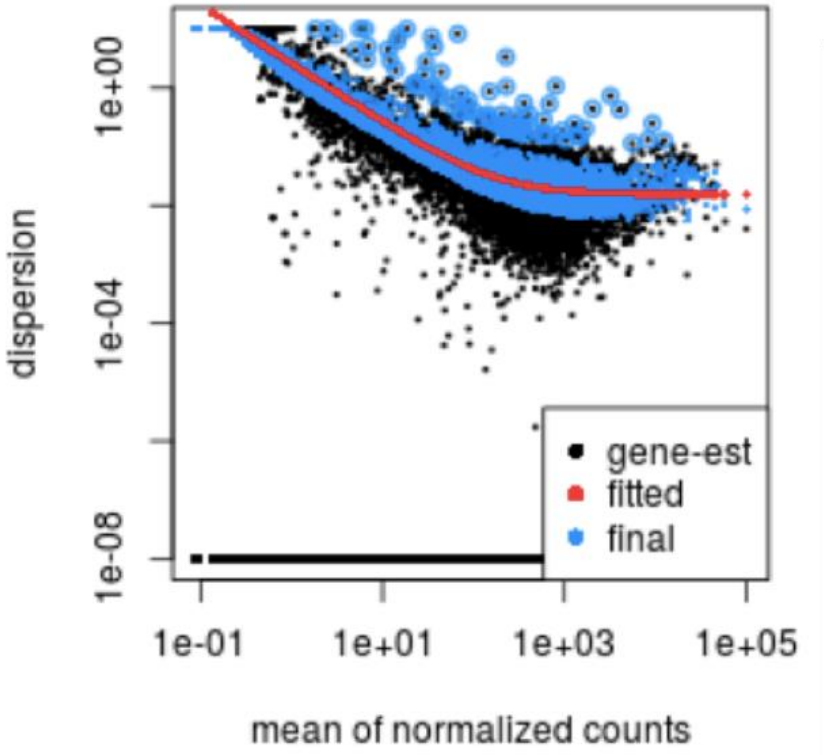
- Gene-specific effects on variance
  - **Assumption**: Genes with similar expression should have similar variances
  - Regression of gene-specific effects versus $\mu_{i,j}$
  - Also called dispersion

Two-step Bayesian approach for dispersion fitting



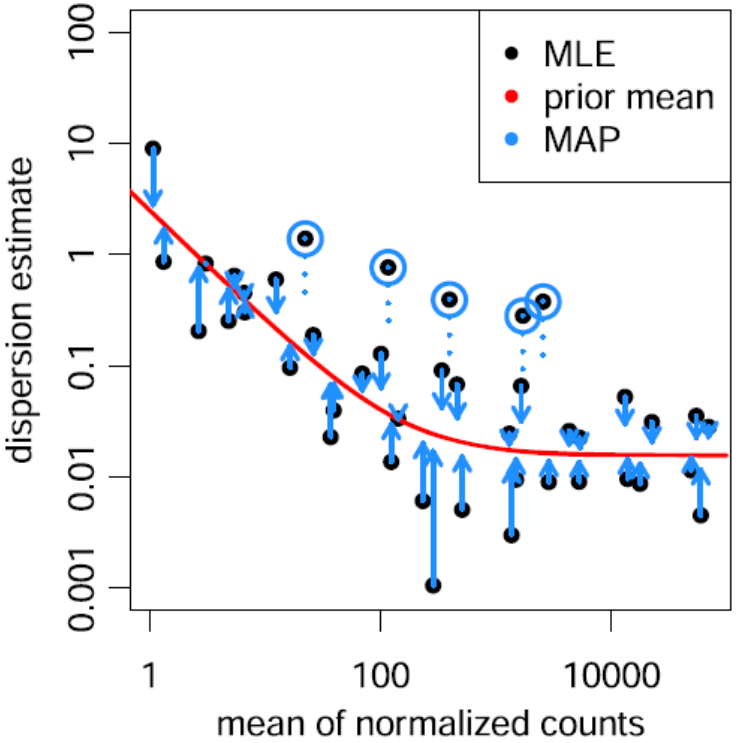
- Dispersion = $\frac{\sigma_{i,j}^2 - \mu_{i,j}}{\mu_{i,j}^2} = \left(\frac{\sigma_{i,j}}{\mu_{i,j}}\right)^2 - \frac{1}{\mu_{i,j}}$
- For genes with high expression level,

  $\text{Log}(\text{Dispersion}) \approx 2 \cdot \text{Log}\left(\frac{\sigma_{i,j}}{\mu_{i,j}}\right)$

- Fit trend using local regression
  - Similar to moving average

Love, Huber, and Anders. Genome Biol. 15:550 (2014)

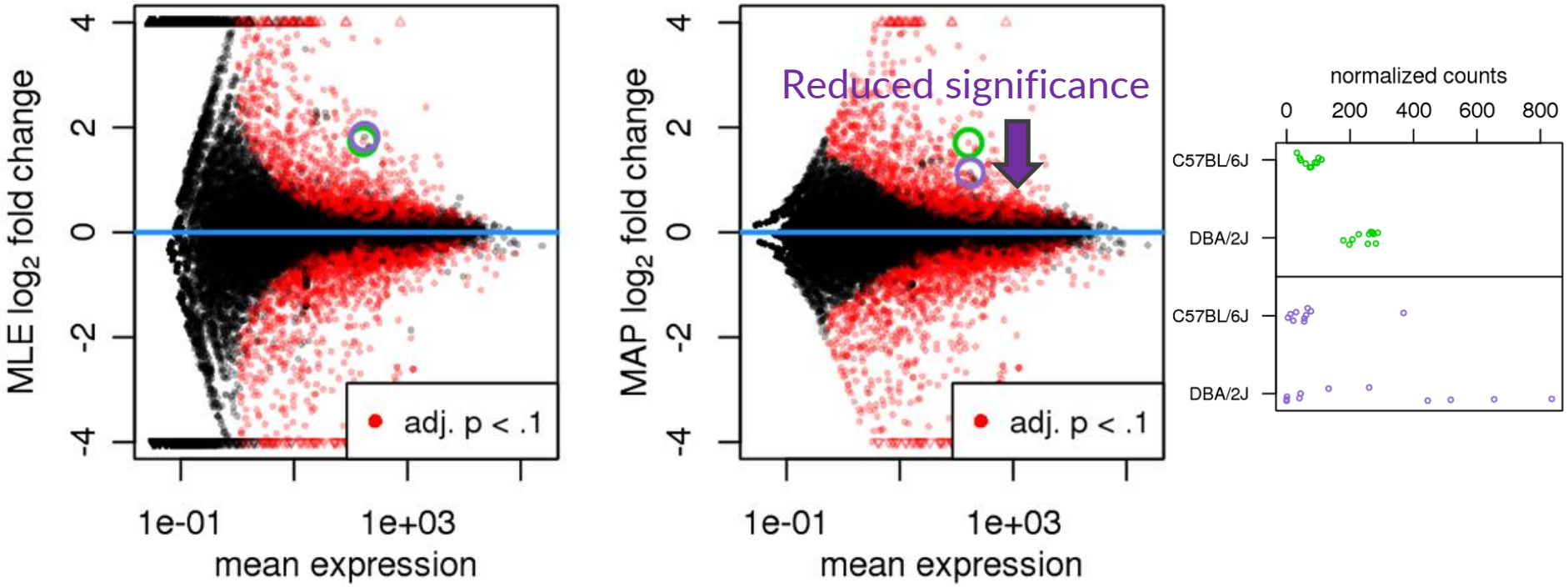
Two-step Bayesian approach for dispersion fitting



- Estimate of dispersion is noisy if there are few samples
- MLE = direct estimate
- MAP = **Bayesian** update using the fitted trend as prior
- Genes with very high dispersions may reflect true biological variations

Love, Huber, and Anders. Genome Biol. 15:550 (2014)

Impact of two-step Bayesian update



Love, Huber, and Anders. Genome Biol. 15:550 (2014)

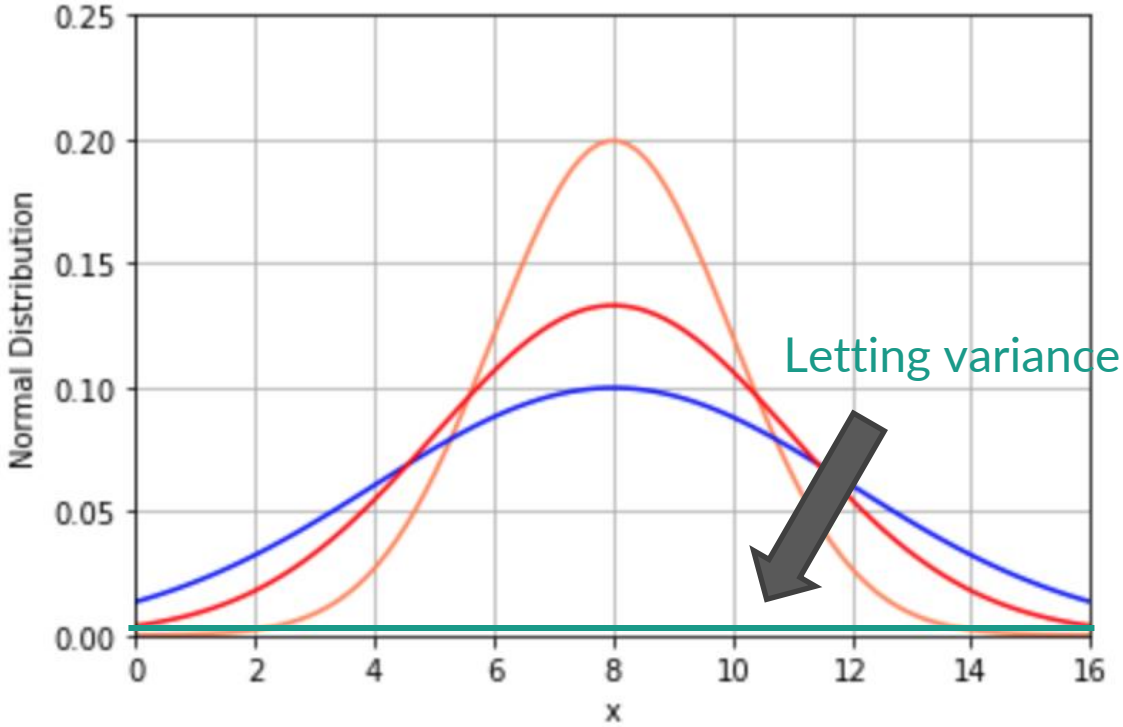
Caveats of two-step Bayesian update

- The used prior models are typically conservative
  - Dispersions follow the average fitted trend
  - Sample effect $\beta_{i,r}$ follows Normal(0, σ_r^2)

- Implicitly favor the null hypothesis of no differential expression

- What if the goal of the experiment is to show that two treatments provide the same difference?
  - Bayesian update in DESeq2 can be disabled
  - Uninformative prior: Normal(0, ∞)

Uninformative prior



Letting variance → infinity

https://stats.stackexchange.com/questions/453784/variance-in-normal-distribution

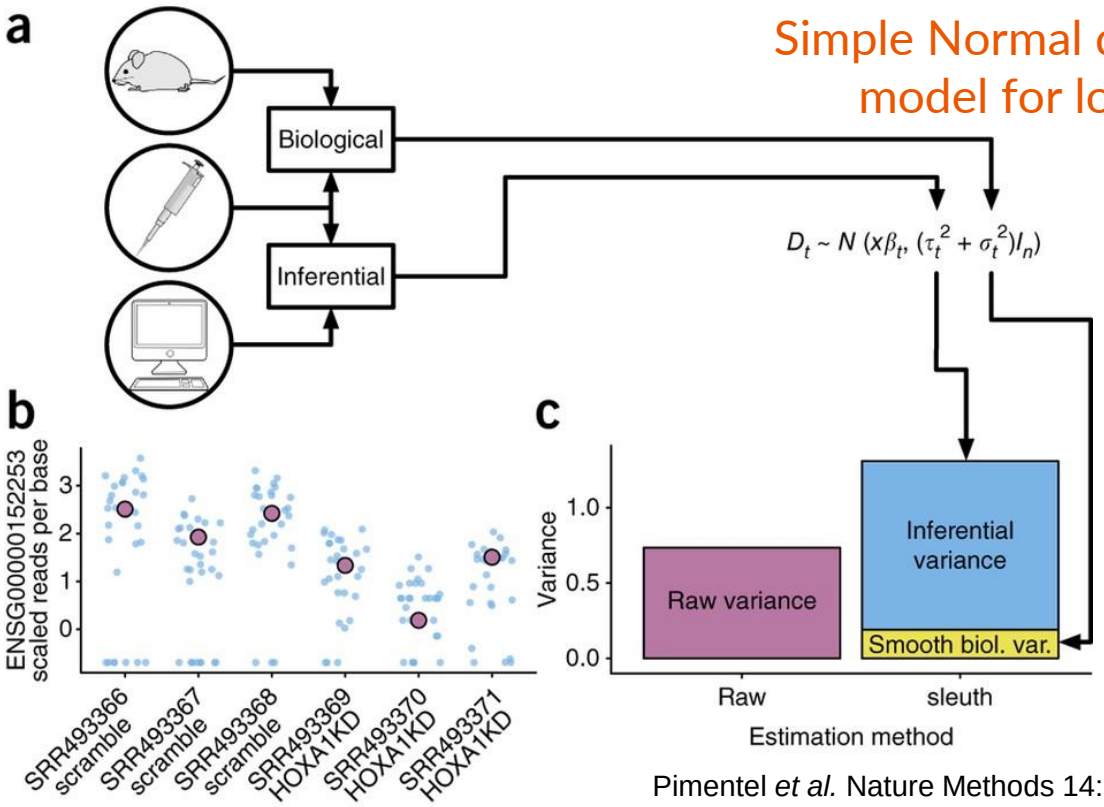
DE as a test of effect size

- Sample effects
  - Log FC = $\sum_r x_{j,r}\beta_{i,r}$ where $x_{j,r}$ are design parameters for sample j and β_{ir} are the effect sizes
- Wald test for each $\beta_{i,r}$: $\frac{\beta_{ir}}{\mathrm{SE}(\beta_{ir})} \sim$ Standard Normal

sleuth model for TPM

Making use of bootstrap to estimate variance

Technical variance estimates from bootstrapping



Simple Normal distribution model for log TPM

Pimentel *et al.* Nature Methods 14:687-690 (2017)

Normal distribution model for log TPM

- True expression: $y_{t,i} = x_i^T \beta_t + \varepsilon_{t,i}$ for sample *i* and transcript *t*
- Observed expression: $D_{t,i} = y_{t,i} + \zeta_{t,i}$

- Noises are normally distributed: $\varepsilon_{t,i} \sim N(0, \sigma_t^2)$ and $\zeta_{t,i} \sim N(0, \tau_t^2)$
  - Transcript-specific

- Full model: $D_t \sim N(x^T \beta_t, (\sigma_t^2 + \tau_t^2) I_n)$

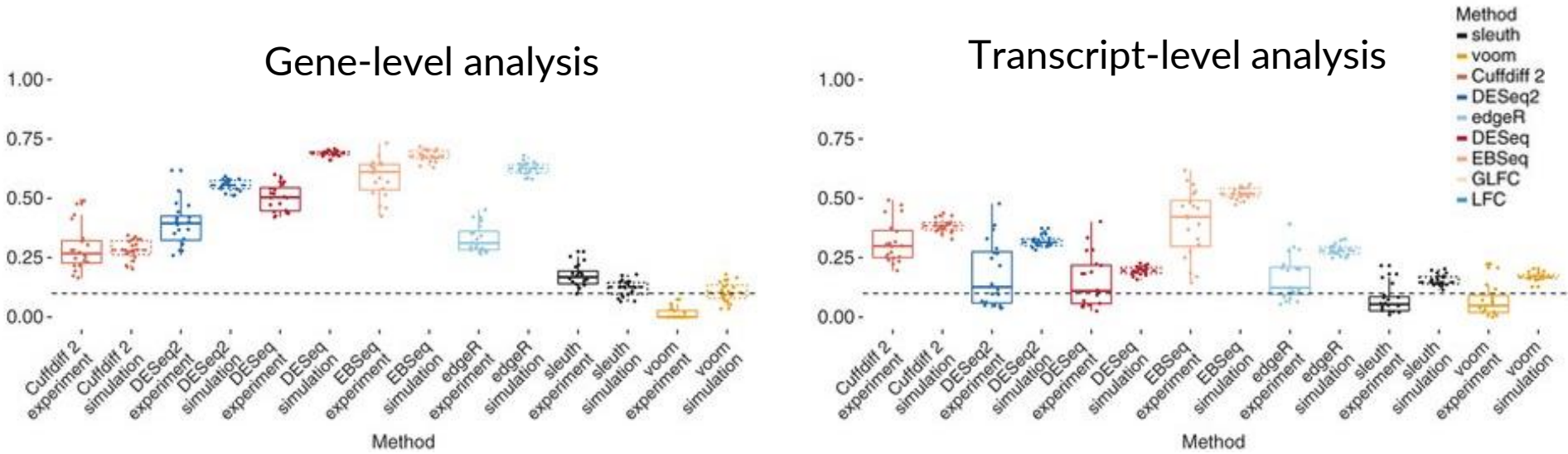
DE for sleuth

- Full model: $D_t \sim N(x^T \beta_t, (\sigma_t^2 + \tau_t^2) I_n)$

- Know from τ_t^2 bootstrapping
- Estimate from σ_t^2 data

- Fit β_t under various design matrices x (hypotheses)
- Compare likelihood ratios

Variance estimates improve accuracy of DE



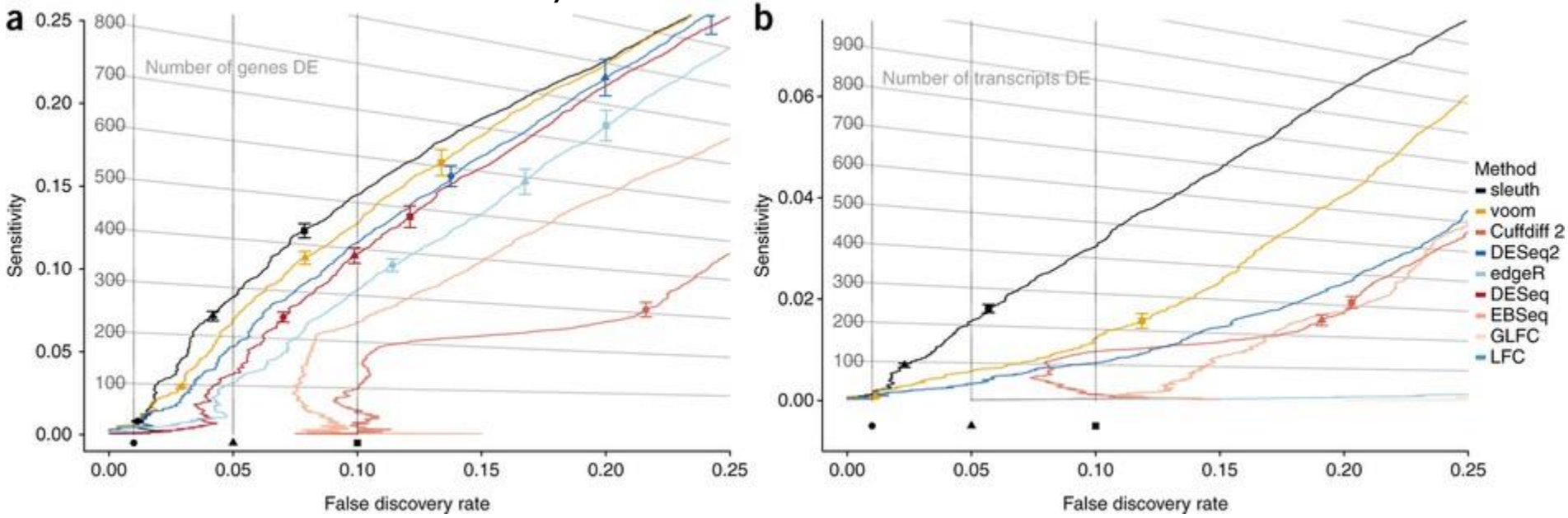
Pimentel *et al.* Nature Methods 14:687-690 (2017)

- All approaches were set to control False Discovery Rate at 10%
- Only **sleuth** and **voom** achieved the target

Variance estimates improve sensitivity of DE

Gene-level analysis

Transcript-level analysis



Pimentel *et al.* Nature Methods 14:687-690 (2017)

Differential expression summary

- DE can be formulated in multiple ways but depend heavily on the model of gene expression distribution

- Read count model using Negative Binomial distribution
  - Bayesian update to improve the estimate of variance
  - Tied to genome-based pipeline: STAR

- Log TPM model using Normal distribution
  - Estimate technical variance directly using bootstrapping
  - Tied to transcriptome-based pipeline with *k*-mer pseudoalignment
  - kallisto / salmon

Any question?

- See you next week on September 20th 9-10:30am