RNA-seq解析パイプライン：Transcript-based

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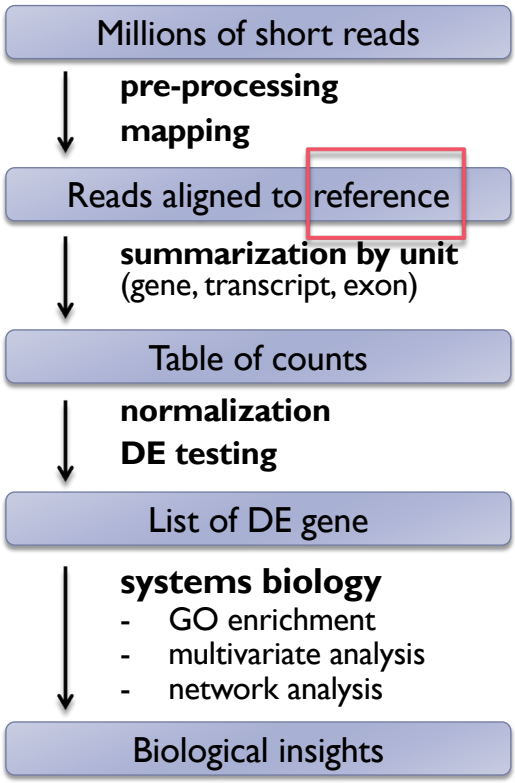
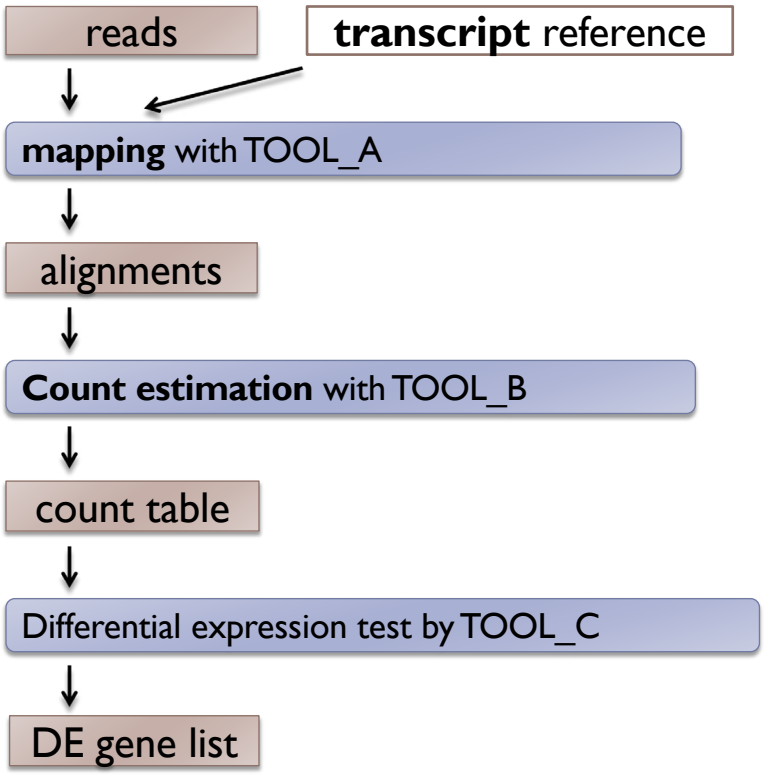
Two Basic Pipelines

- Choice of reference
  - **Genome** – standard for genome-known species
  - **Transcript** – the only way for genome-unknown species
    -- can be used for genome-known species

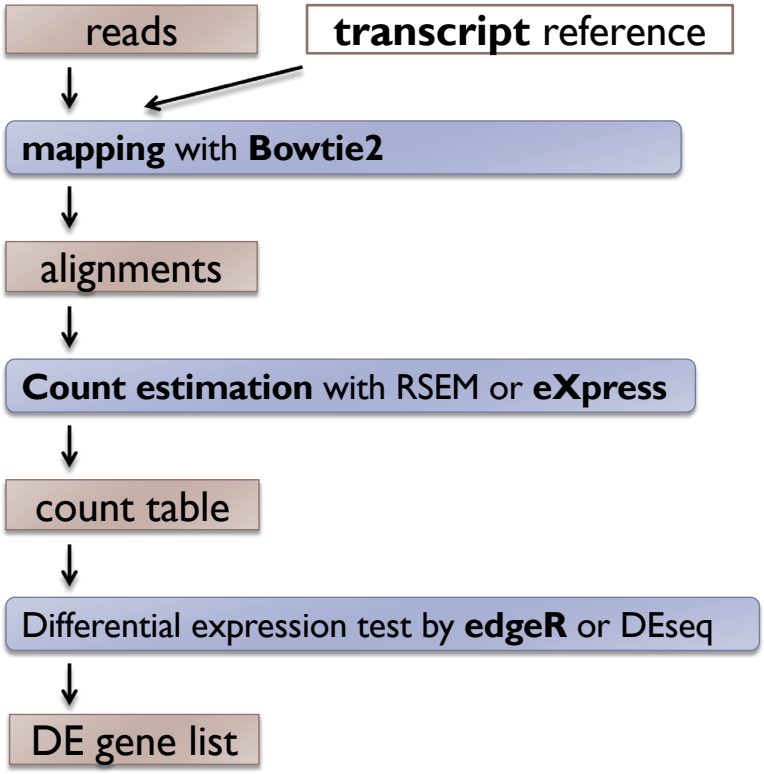


A transcript-based pipeline

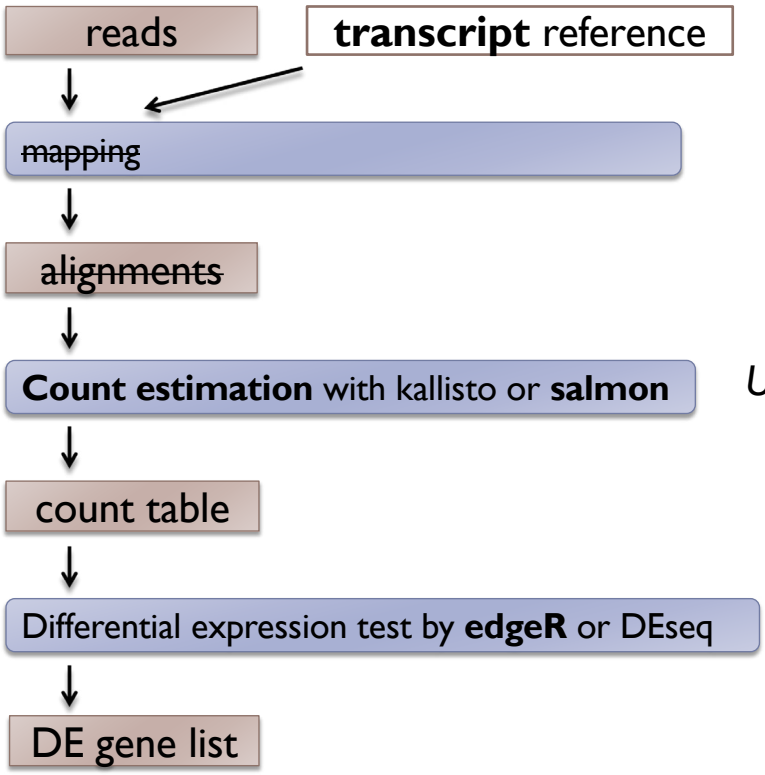


old

A transcript-based pipeline



A transcript-based pipeline (alignment-free method)



Ultrafast!

Alignment-free RNAseq quantification

- Software
  - Salmon
  - Kallisto
  - Sailfish
- Concept
  - Precise alignments are not required to assign reads to their origins.
  - => "psudo-alignment" using a de bruijn graph information (kallisto), a k-mer approach (Sailfish old ver.) or a "quasi-mapping" (Salmon)
- Benefit
  - Ultrafast
  - Computationally cheap
  - Accuracy: similar or better than mapping-based methods

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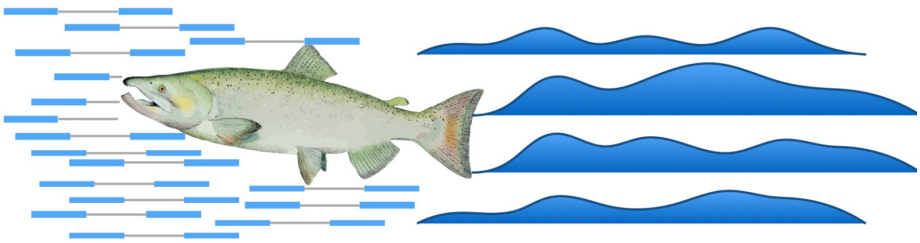


Salmon —*Don't count . . . quantify!*

- ultra-fast
- stable; sophisticated; well-documented
- Alevin for single-cell RNA-seq

Overview

Salmon is a tool for quantifying the expression of transcripts using RNA-seq data. Salmon uses new algorithms (specifically, coupling the concept of *quasi-mapping* with a two-phase inference procedure) to provide accurate expression estimates very quickly (i.e. *wicked-fast*) and while using little memory. Salmon performs its inference using an expressive and realistic model of RNA-seq data that takes into account experimental attributes and biases commonly observed in *real* RNA-seq data.

Citing Salmon

If you find Salmon useful, or have suggestions for improv
Salmon in your work, please cite the Salmon paper:

https://combine-lab.github.io/salmon/

Salmon

Salmon is a tool for quantifying the expression of transcripts using RNA-seq data. Salmon uses new algorithms to provide accurate expression estimates very quickly.

```
(example)

$salmon index …    # step 1. build index
$salmon quant …    # step 2. quantification
```

- Input
  - reference (fasta) and reads (fastq)
- Output
  - Count estimation table : **`quant.sf`**

Let's Try **Salmon**

ex401

Map 75-bp Illumina reads to a transcript reference and quantify the abundance.

Prepare reads and reference genome

Sequences for this exercise are stored in **`~/gitc/data/SS/`**.

```
IlluminaReads1.fq : Illumina reads in fastq format
minimouse_mRNA.fa : a set of transcript sequnences
```

Build index of reference sequence

```
$salmon index -t minimouse_mRNA.fa \
  -i minimouse_mRNA.fa.salmon.idx -k 31
```

Quantification

```
$salmon quant -i minimouse_mRNA.fa.salmon.idx \
  -l A -o salmon_out -r IlluminaReads1.fq
```

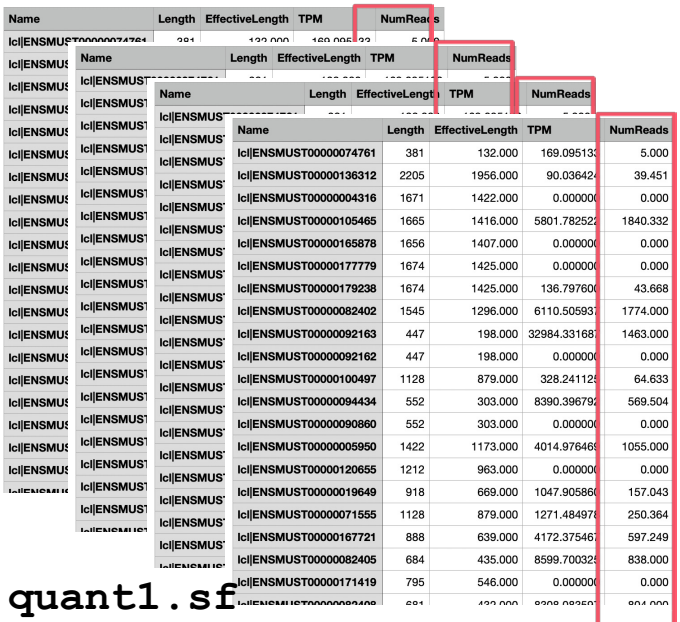
Salmon outputs

NumReads => edgeR

quant.sf

Name	Length	EffectiveLength	TPM	NumReads
lcl|ENSMUST00000074761	381	132.000	169.095133	5.000
lcl|ENSMUST00000136312	2205	1956.000	90.036424	39.451
lcl|ENSMUST00000004316	1671	1422.000	0.000000	0.000
lcl|ENSMUST00000105465	1665	1416.000	5801.782522	1840.332
lcl|ENSMUST00000165878	1656	1407.000	0.000000	0.000
lcl|ENSMUST00000177779	1674	1425.000	0.000000	0.000
lcl|ENSMUST00000179238	1674	1425.000	136.797600	43.668
lcl|ENSMUST00000082402	1545	1296.000	6110.505937	1774.000
lcl|ENSMUST00000092163	447	198.000	32984.331687	1463.000
lcl|ENSMUST00000092162	447	198.000	0.000000	0.000
lcl|ENSMUST00000100497	1128	879.000	328.241125	64.633
lcl|ENSMUST00000094434	552	303.000	8390.396792	569.504
lcl|ENSMUST00000090860	552	303.000	0.000000	0.000
lcl|ENSMUST00000005950	1422	1173.000	4014.976469	1055.000
lcl|ENSMUST00000120655	1212	963.000	0.000000	0.000
lcl|ENSMUST00000019649	918	669.000	1047.905860	157.043
lcl|ENSMUST00000071555	1128	879.000	1271.484978	250.364
lcl|ENSMUST00000167721	888	639.000	4172.375467	597.249
lcl|ENSMUST00000082405	684	435.000	8599.700325	838.000
lcl|ENSMUST00000171419	795	546.000	0.000000	0.000

Salmon to edgeR

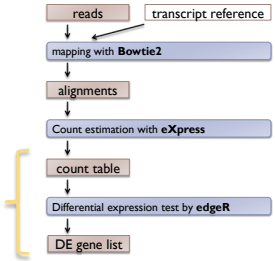


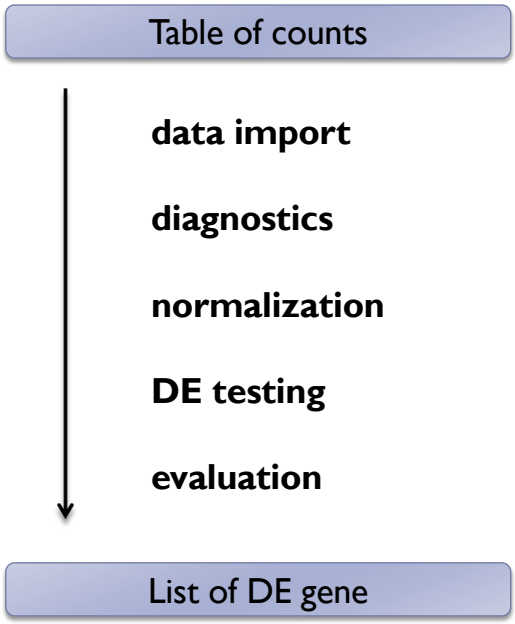
quant1.sf
quant2.sf
quant3.sf
quant4.sf

count matrix

Name	Lib-1|NumReads	Lib-2|NumReads	Lib-3|NumReads	Lib-4|NumReads
lcl|ENSMUST00000074761	5.000	27.957	15.037	230.000
lcl|ENSMUST00000136312	39.451	674.000	61.696	42.809
lcl|ENSMUST00000004316	0.000	528.689	0.000	156.235
lcl|ENSMUST00000105465	1840.332	521.304	111.396	0.000
lcl|ENSMUST00000165878	0.000	148.549	0.000	218.000
lcl|ENSMUST00000177779	0.000	470.496	348.508	215.000
lcl|ENSMUST00000179238	43.668	0.000	0.000	29.956
lcl|ENSMUST00000082402	1774.000	195.495	1.534	192.288
lcl|ENSMUST00000092163	1463.000	499.000	260.604	159.365
lcl|ENSMUST00000092162	0.000	454.000	104.191	198.000
lcl|ENSMUST00000100497	64.633	447.000	0.000	0.000
lcl|ENSMUST00000094434	569.504	0.000	0.000	159.985
lcl|ENSMUST00000090860	0.000	410.000	1.608	0.000
lcl|ENSMUST00000005950	1055.000	332.068	135.311	1.002
lcl|ENSMUST00000120655	0.000	266.106	239.000	192.000
lcl|ENSMUST00000019649	157.043	406.000	0.000	6.017
lcl|ENSMUST00000071555	250.364	0.000	264.000	0.000
lcl|ENSMUST00000167721	597.249	4.998	89.000	135.272

```
$salmon quantmerge …
```





edgeR

- A Bioconductor package for differential expression analysis of digital gene expression data
- **Model**: An over dispersed Poisson model, negative binomial (NB) model, is used
- **Normalization**: TMM method (trimmed mean of M values) to deal with composition effects
- **DE test**: exact test and generalized linear models (GLM)

edgeR (classic)

- input: **count data** (not RPKM or TPM)
- output: gene table with DE significance statistics (FDR)

```
(example)

$ R
> library(edgeR)                        #load edgeR library
> dat <- read.delim("count_data.txt", …) #import count table to R
> group <- c(rep("M", 3), rep("H", 3))   #assign groups
> D <- DGEList(dat, group=group)         #import data to edgeR
> D <- calcNormFactors(D)                #normalization (TMM)
> D <- estimateCommonDisp(D)             #estimate common dispersion
> D <- estimateTagwiseDisp(D)            #estimate tagwise dispersion
> de <- exactTest(D, pair=c("M", "H")) #DE test
> topTags(de)
Comparison of groups: H-M
            logConc    logFC      P.Value          FDR
AT5G48430 -15.36821 6.255498 9.919041e-12 2.600872e-07
AT5G31702 -15.88641 5.662522 3.637593e-10 4.083773e-06
AT3G55150 -17.01537 5.870635 4.672331e-10 4.083773e-06
…
```

Advanced

edgeR (GLM)

- input: **count data** (not RPKM or TPM)
- output: gene table with DE significance statistics (FDR)

```
(example)

$ R
> library(edgeR)                        #load edgeR library
> dat <- read.delim("count_data.txt", …) #import count table to R

> treat <- factor(c("M", "M", "M", "H", "H", "H"))
> treat <- relevel(treat, ref="M")
> design <- model.matrix(~treat)
> rownames(design) <- colnames(y)

> D <- DGEList(dat, group=treat)         #import data to edgeR
> D <- calcNormFactors(D, method="TMM")   #normalization (TMM)
> D <- estimateDisp(D, design)           #estimate dispersion
> fit <- glmFit(D, design)               #fitting to model
> lrt <- glmLRTt(D, coef=2))             #DE test
> topTags(lrt)
> …
```

Let's try edgeR

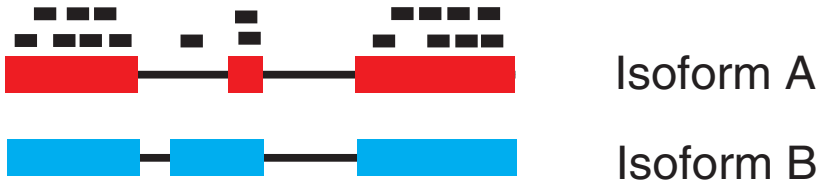
- edgeR classic
  - ex402: Differential expression analysis with edgeR (pairwise)
- edgeR linear model [advanced]
  - ex403-1: Differential expression analysis with edgeR (GLM)
  - ex403-2: Differential expression analysis with edgeR (GLM; considering batch effect)

Advanced

Multi-mapping issue in estimating abundance

- Source of repeats caused multi-mapping issues in RNA-seq analysis
  - Isoforms
  - Very similar paralogs
  - Transposable elements

  } cannot align reads uniquely
- Mapping ambiguity should be taken into consideration.



- Solution
  - Ignore, split equally, EM algorithm, read coverage-based
  - RSEM, Slamon, Kallisto: EM

Advanced

Table 1
Computational strategies and methods that handle multi-mapped reads.

Tool	Quantification level	Input	Strandedness can be specified	Count type	Strategy	Paired end	Confidence level	Focus
HTSeq-count	Gene	BAM	Y	Counts	Ignore	Y	N	Long RNA
STAR geneCounts	Gene	Fastq	Y	Counts	Ignore	Y	N	Long RNA
Cufflinks	Transcript	BAM	Y	RPKM	Split equally, Rescue	Y	N	Long RNA
featureCounts	Gene	BAM	Y	Counts	Ignore, count all, split equally	Y	N	Long RNA
CoCo	Gene	BAM	Y	Counts, CPM, TPM	Rescue	Y	N	Small RNA Long RNA
ERANGE	Transcript	BAM	N	RPKM	Rescue	Y	N	Long RNA
EMASE	Transcript	BAM	N	Counts, TPM	EM	Y	N	Long RNA
IsoEM2	Both	SAM	Y	FPKM, TPM	EM	Y	Confidence intervals	Long RNA
Kallisto	Transcript	Fastq	Y	TPM	EM	Y	Bootstrap values	Long RNA
RSEM	Both	Fastq, BAM	Y	Counts, TPM, FPKM	EM	Y	95% credibility intervals	Long RNA
Salmon	Transcript	Fastq	Y	Counts, TPM	EM	Y	Bootstrap values	Long RNA
MMR	N/A	BAM	Y	N/A	Read coverage	Y	N/A	Long RNA
MuMRescueLite	Genomic loci	Custom format	N	Counts	Read coverage	N	N	Short sequence tags
Rcount	Gene	BAM	Y	Counts	Read coverage	N	N	Long RNA
ShortStack	Gene	Fastq, BAM	N	Counts, RPM	Read coverage	N	N	Small RNA
mmquant	Gene	BAM	Y	Counts	Gene Clustering	Y	N	Small RNA Long RNA
SeqCluster	Gene	BAM	N	Counts	Gene clustering	N	N	Small RNA
Fuzzy method	Gene	Custom format	N	Fuzzy counts	Fuzzy sets	N	Fuzzy counts	Small RNA Long RNA
geneQC	Gene	SAM	Y	NA	ML	Y	Mapping uncertainty level	Small RNA Long RNA

(Deschamps-Francoeur et al., 2020)

Advanced

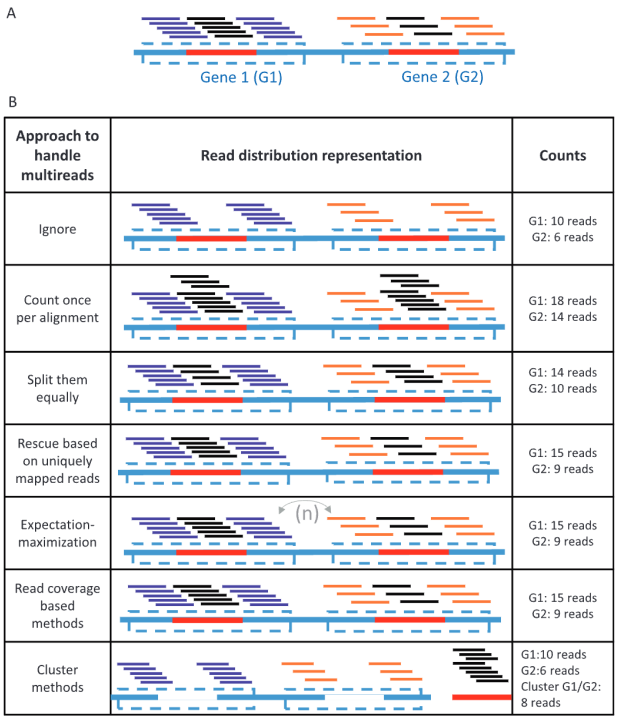


Fig. 3. Strategies to deal with multi-mapped reads. (A) Example of two genes sharing a duplicated sequence and the distribution of RNA-seq reads originating from them. The two genes are represented by boxes outlined by dashed lines and their common sequence is illutrated by a red line. The reads are represented by lines above the genes, purple for reads that are unique to Gene 1, orange for reads that are unique to Gene 2 and black for reads that are common to genes 1 and 2. (B) General classes to handle multi-mapped reads include ignoring them, counting them once per alignment, splitting them equally between the alignments, rescuing the reads based on uniquely mapped reads of the gene, expectation–maximization approaches, rescuing methods based on read coverage in flanking regions and clustering methods that group together genes/transcripts with shared sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Deschamps-Francoeur et al., 2020)

落ち穂拾い

- 一般化線形モデル in RNA-seq
  - 前回GITC NGS解析入門＞統計学入門 p63～
  - https://github.com/nibb-unix/gitc202402-unix/blob/main/textbook/3_statistics.pdf
- False Discovery Rate (FDR)
  - 前回GITC NGS解析入門＞統計学入門 p38 ~
  - https://github.com/nibb-unix/gitc202402-unix/blob/main/textbook/3_statistics.pdf