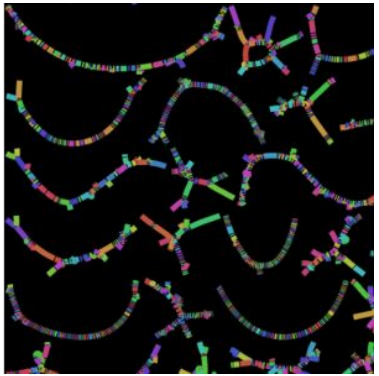
de-novo Transcriptome Assembly

Ecole EB3I n1 - 2025



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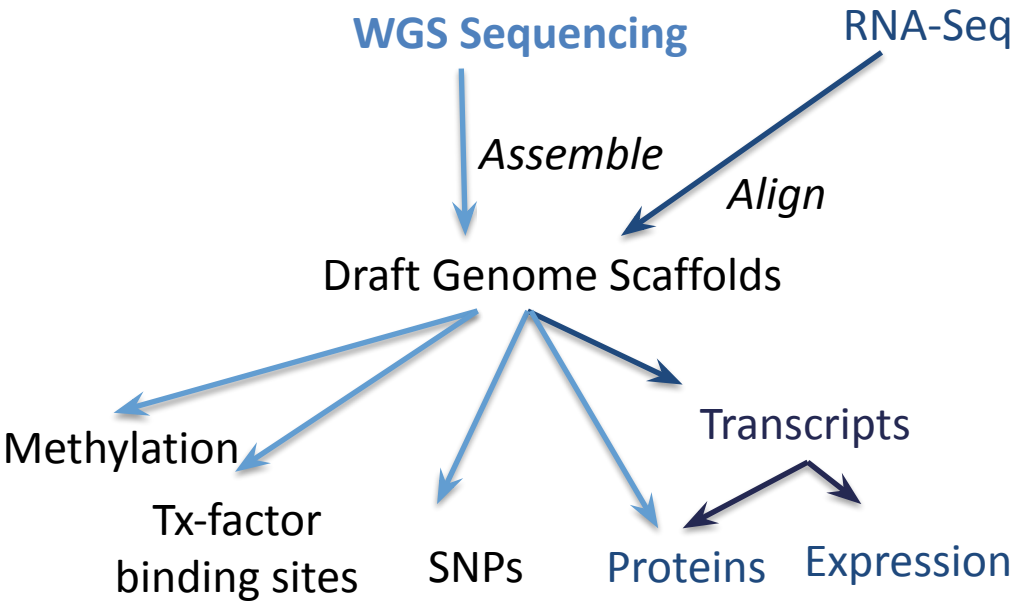
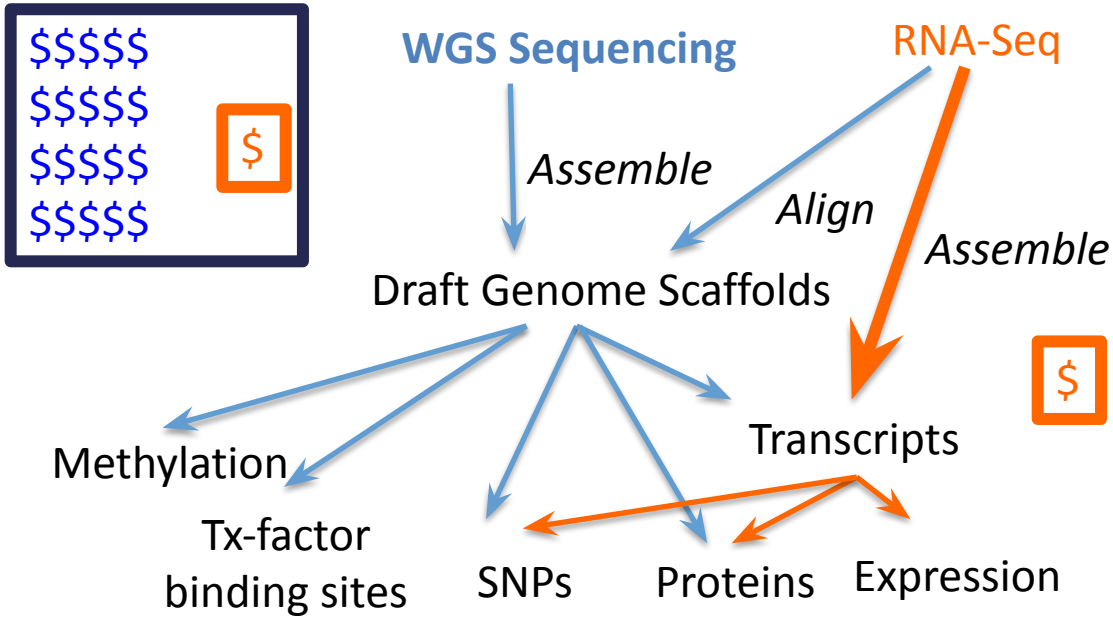
A Paradigm for Genomic Research



A **Maturing** Paradigm for Transcriptome Research



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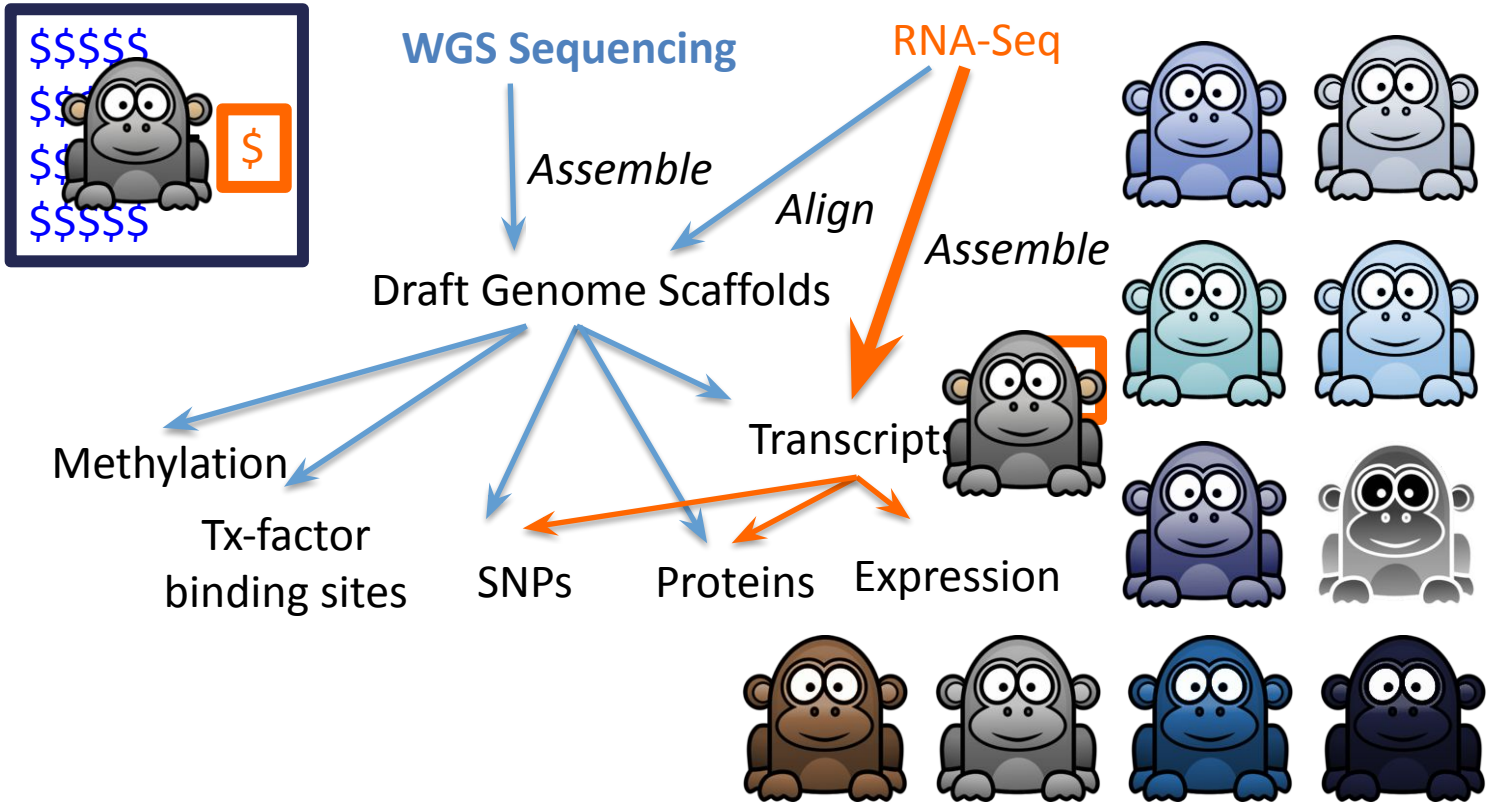
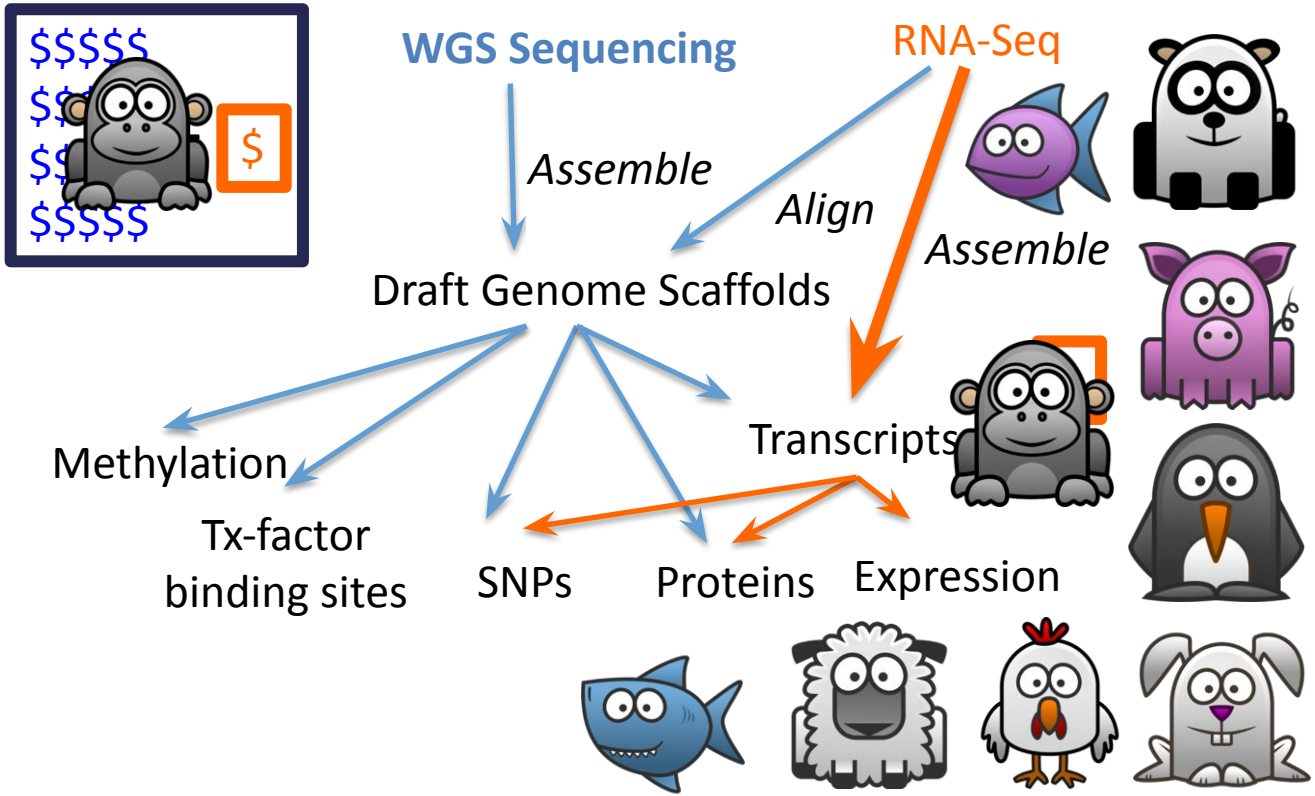
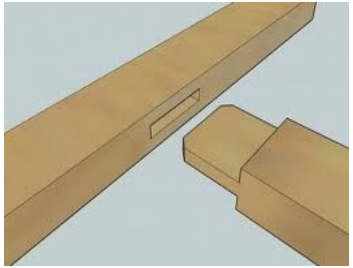
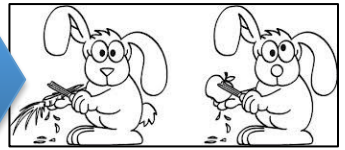


A **Maturing** Paradigm for Transcriptome Research

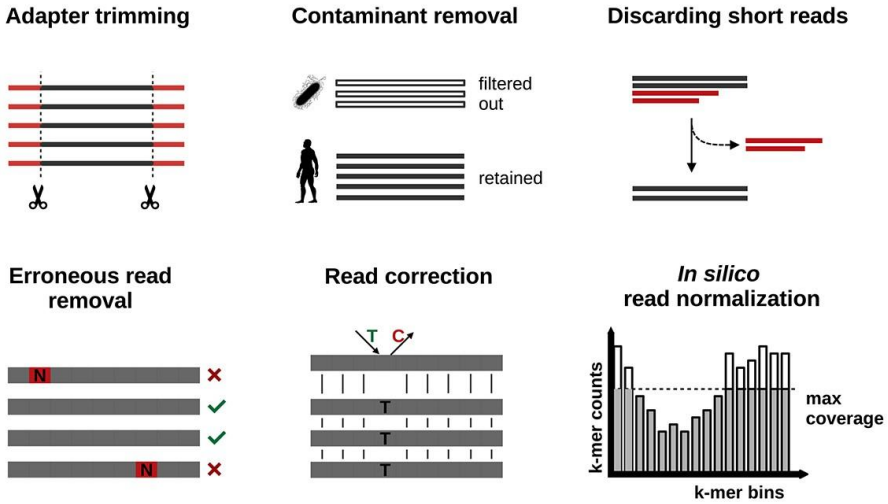


RNA Seq de novo analysis workflow





Data cleaning

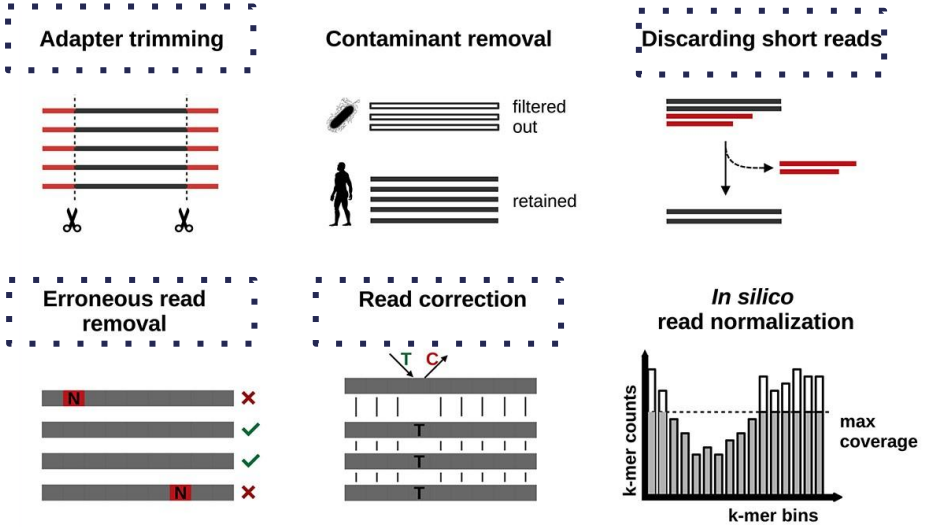


https://doi.org/10.1093/bib/bbab563

Bias should be corrected in reverse order of their generation

1. Sequencing biases (bad quality, unknowns)
2. Library preparation
   - Adaptors and primers sequences
   - Poly A/T tails
3. Biological sample (low complexity, rRNA, contaminants)

Data cleaning



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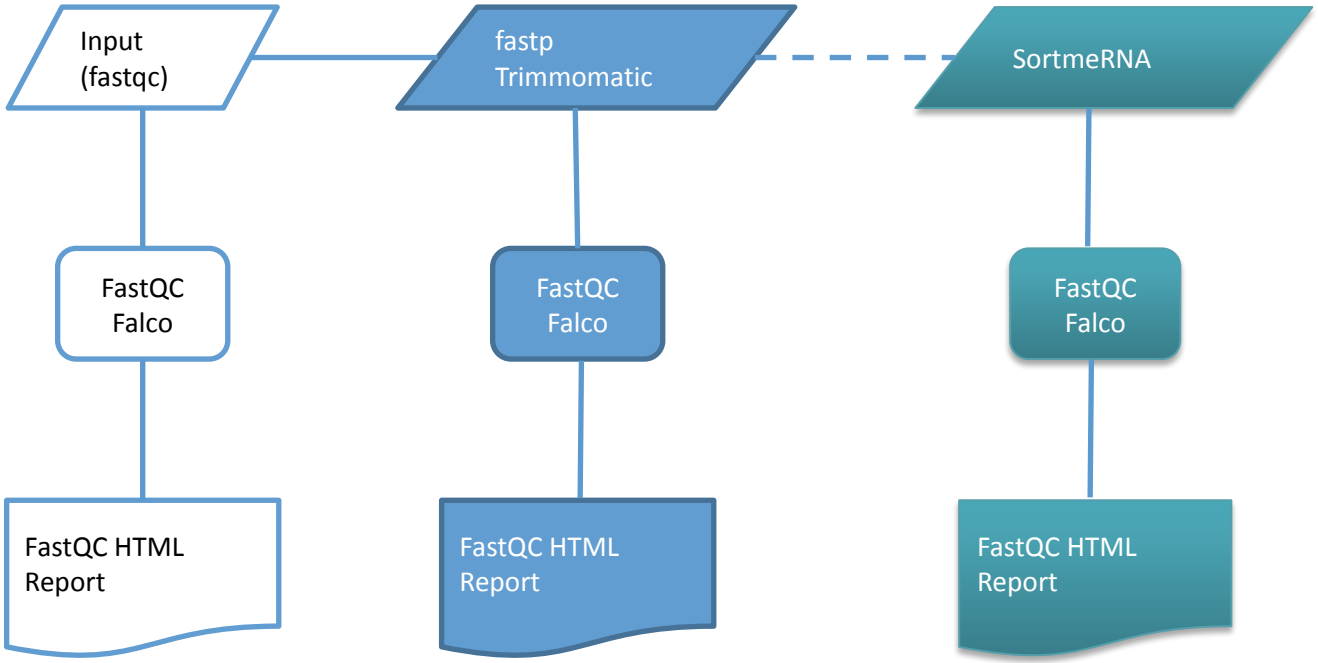

Alternative : Sickle, TrimGalore

Alternative : phyloFlash, BBMap

Contaminations

Euphausia superba (Uwe Kils. 2011)

Contaminations

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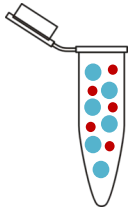
Contaminations

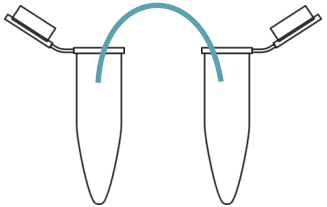


in-contamination

for ex. rRNA

third-party contamination

for ex. food - parasite



cross-contamination

for ex. experiment

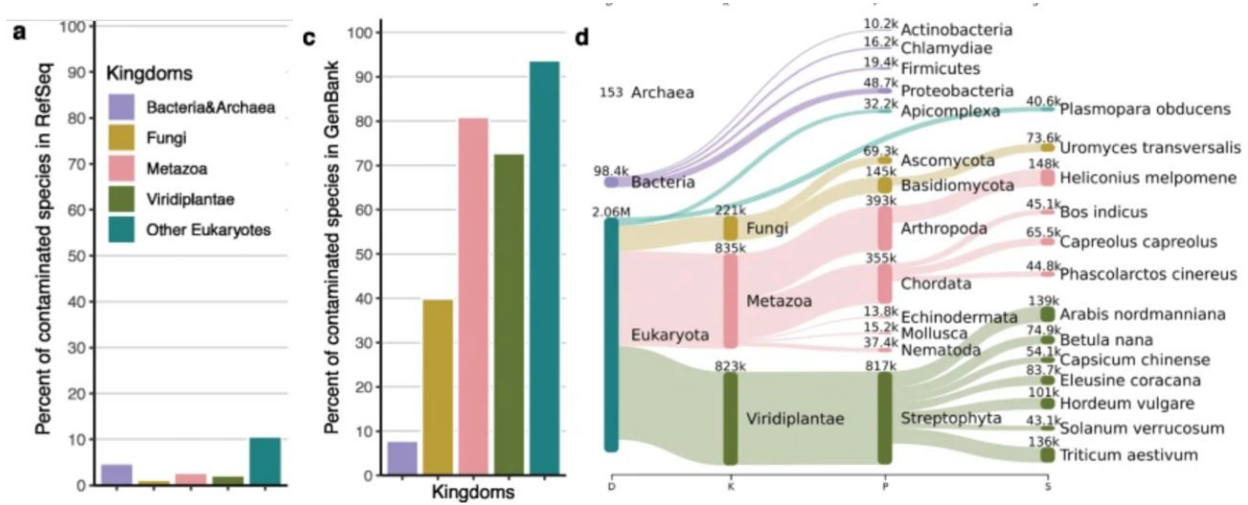
- Most of (all) Illumina sequencing dataset are somewhat contaminated
- Illumina sequencing is especially susceptible to contamination due to the coverage depth
- It seems inherent to the method
- “Index misassignment between multiplexed libraries is a known issue” (Illumina, Inc., 2018); it potentially can produce contaminations in the sequenced datasets

Thanks to Serafim Nenarokov

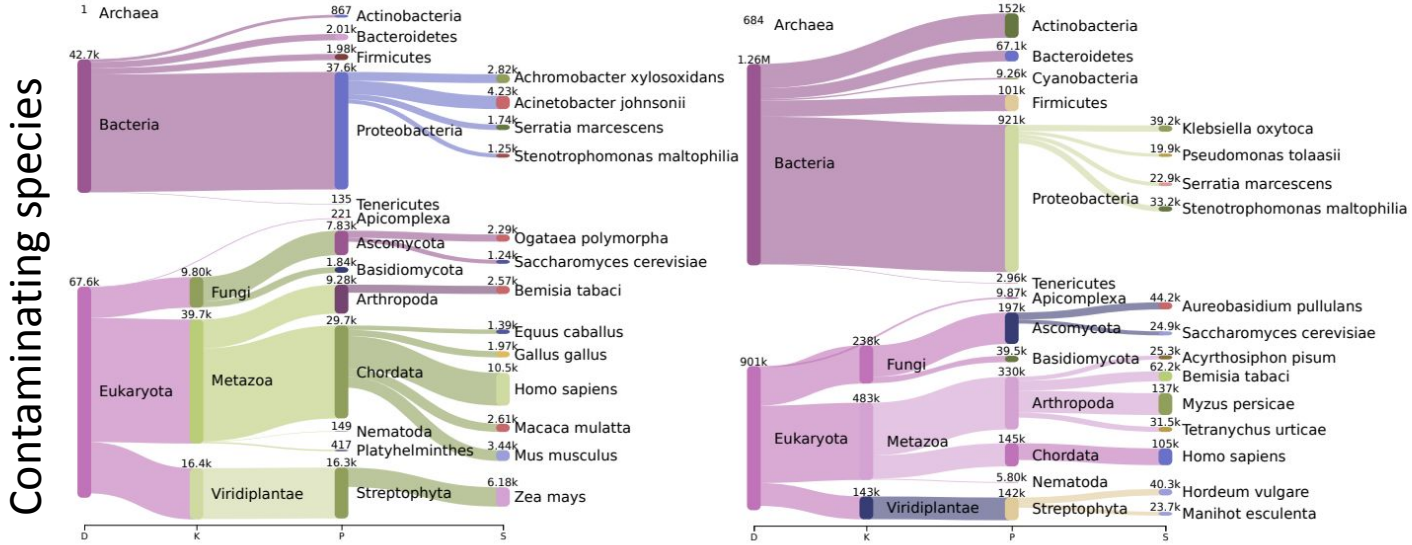
Contaminations

« Conterminator reported 114,035 and 2,161,746 contaminated sequences affecting 2767 and 6795 species in RefSeq and GenBank, respectively »

Contaminated sequences



Contaminations



Supplementary Figure 1: **Sanksey plot of most contaminating species in RefSeq and GenBank. left** Sanksey plot five kingdoms: Bacteria&Archaea, Fungi, Metazoa, Viridiplantae and other Eukaryotes. **right** Distribution of contaminating species in GenBank.

Steinegger, M., Salzberg, S.L. Terminating contamination: large-scale search identifies more than 2,000,000 contaminated entries in GenBank. *Genome Biol* **21,** 115 (2020). https://doi.org/10.1186/s13059-020-02023-1

rRNA contamination

One of the most common contamination

90-95% of total RNA correspond to rRNA

Hopefully it belongs to the sequenced organism but can also belongs to symbiont parasite or …. Aliens

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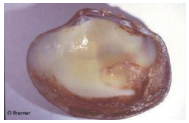

Ruditapes philippinaru

Vibrio tapetis

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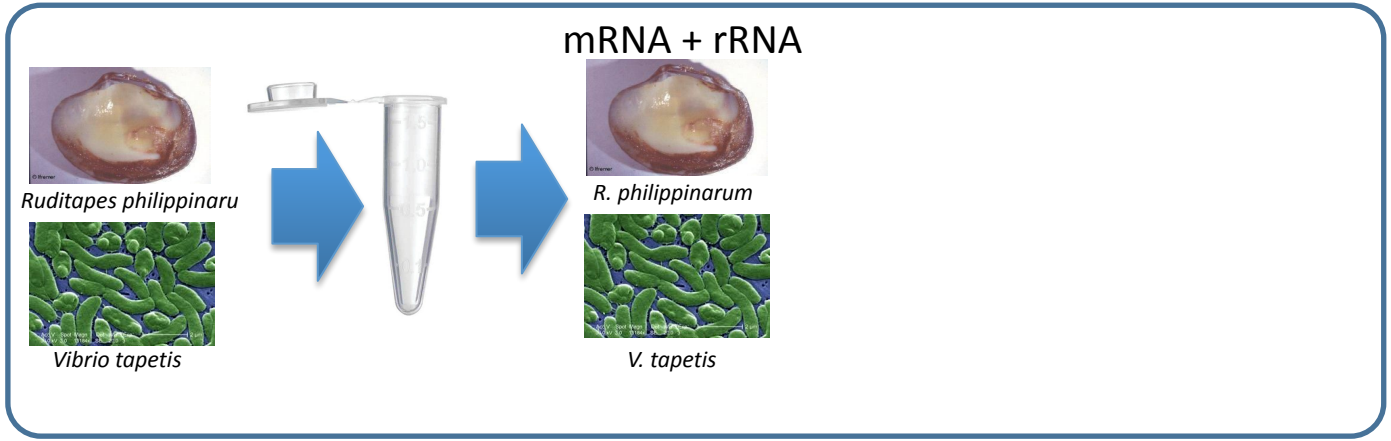

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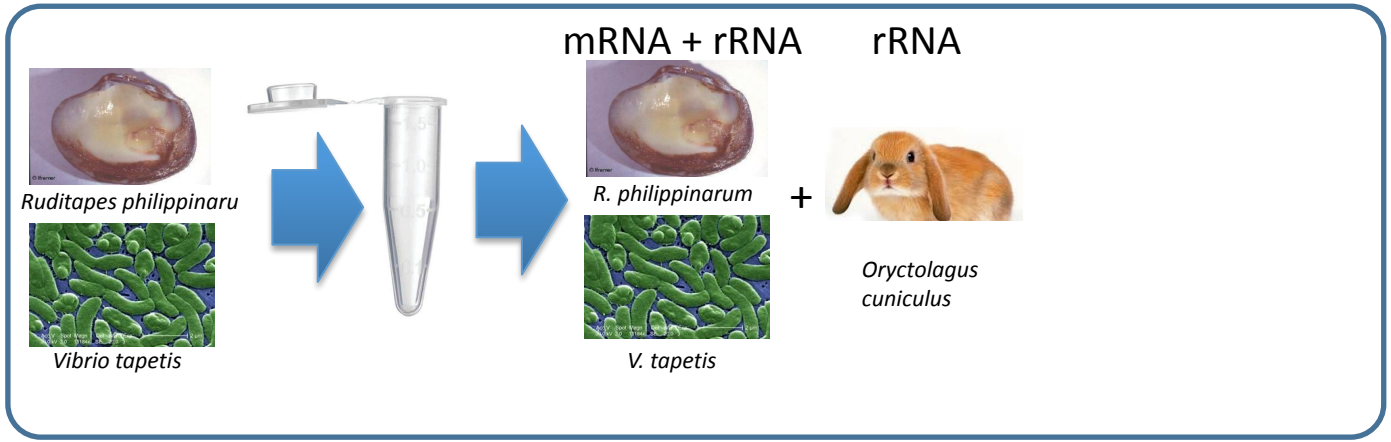

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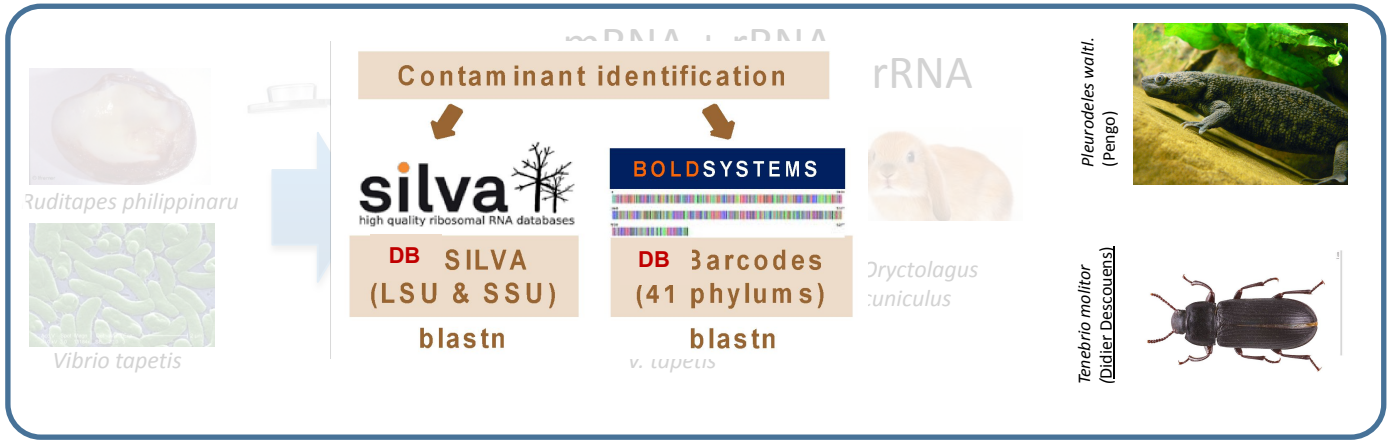


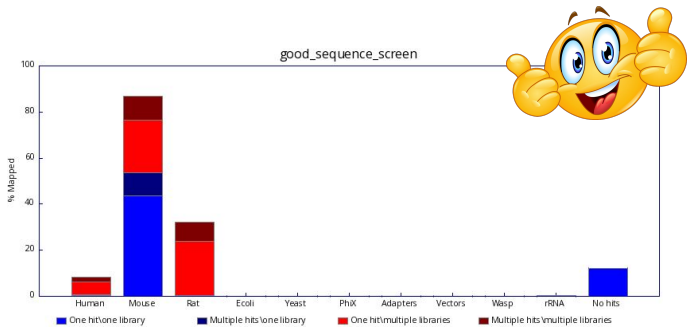
Detect third-party/cross contamination

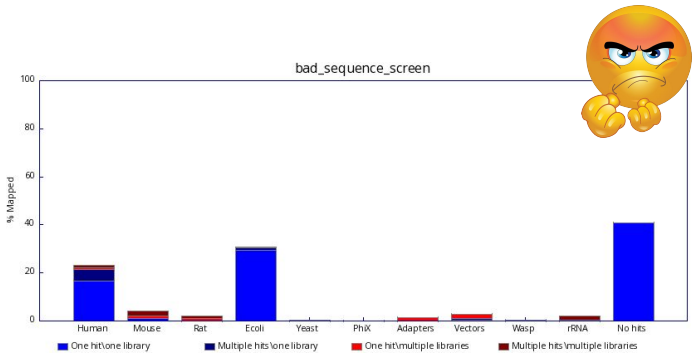
Reads that do not originate from the organism and/or RNA species of interest
f. ex. reads originating from an endosymbiont bacterium in an eukaryote organism of interest

Tools : short-read taxonomic classifiers

With a eukaryotic read dataset, **kraken2** could be used to exclude reads classified as bacterial, archaeal, fungal or from plants.
Alternative **Centrifuge** for microbial reads

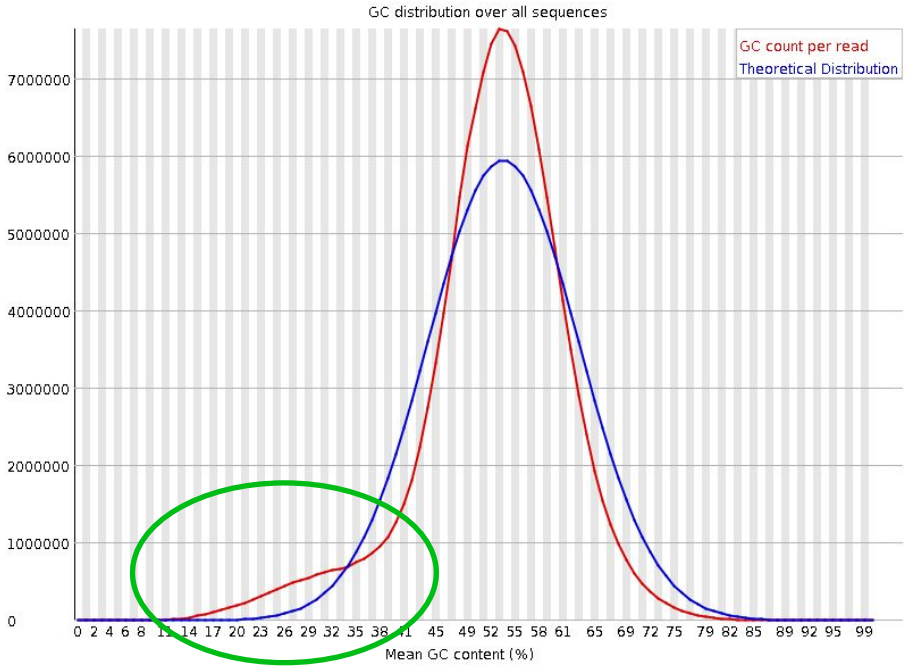
screen-only alternative **FastQ Screen**





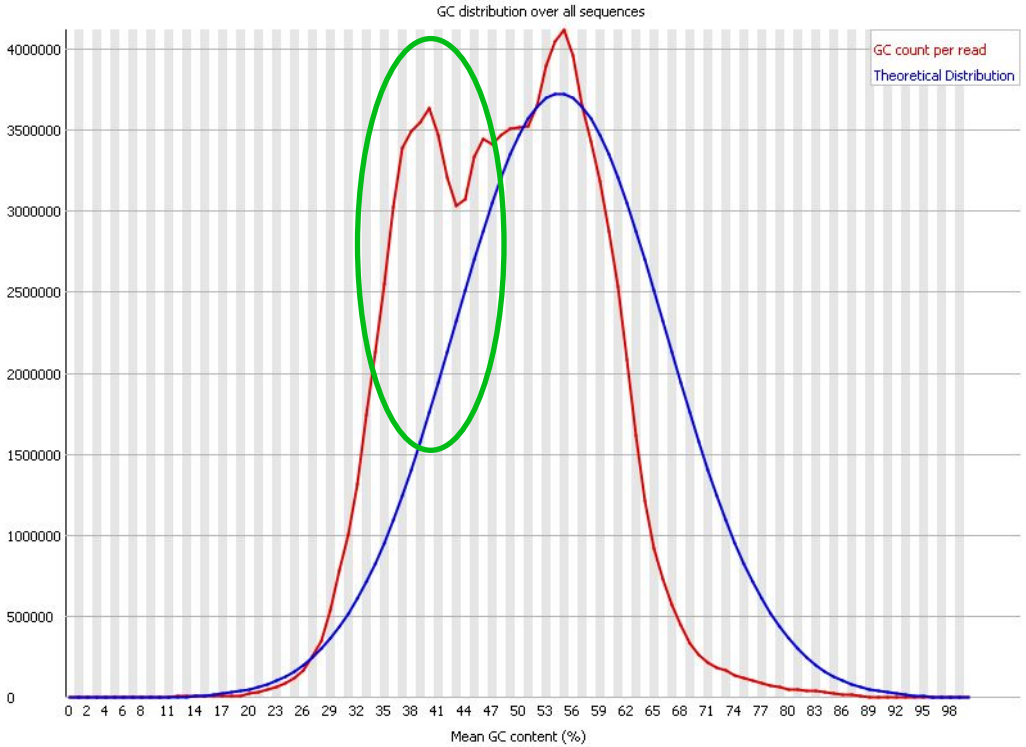
FastQC: Per sequence GC content

A contamination ?



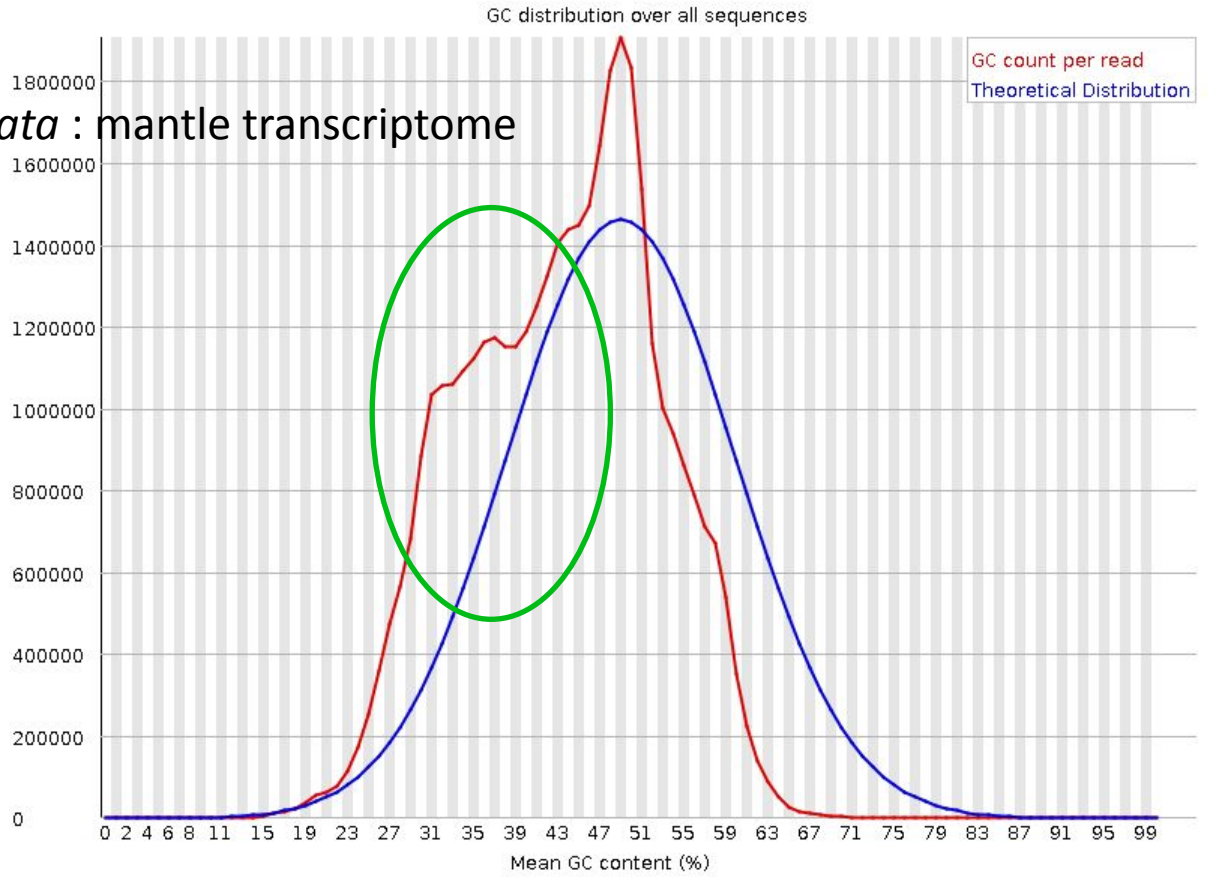
Can this be fixed ? Maybe…

FastQC: Per sequence GC content

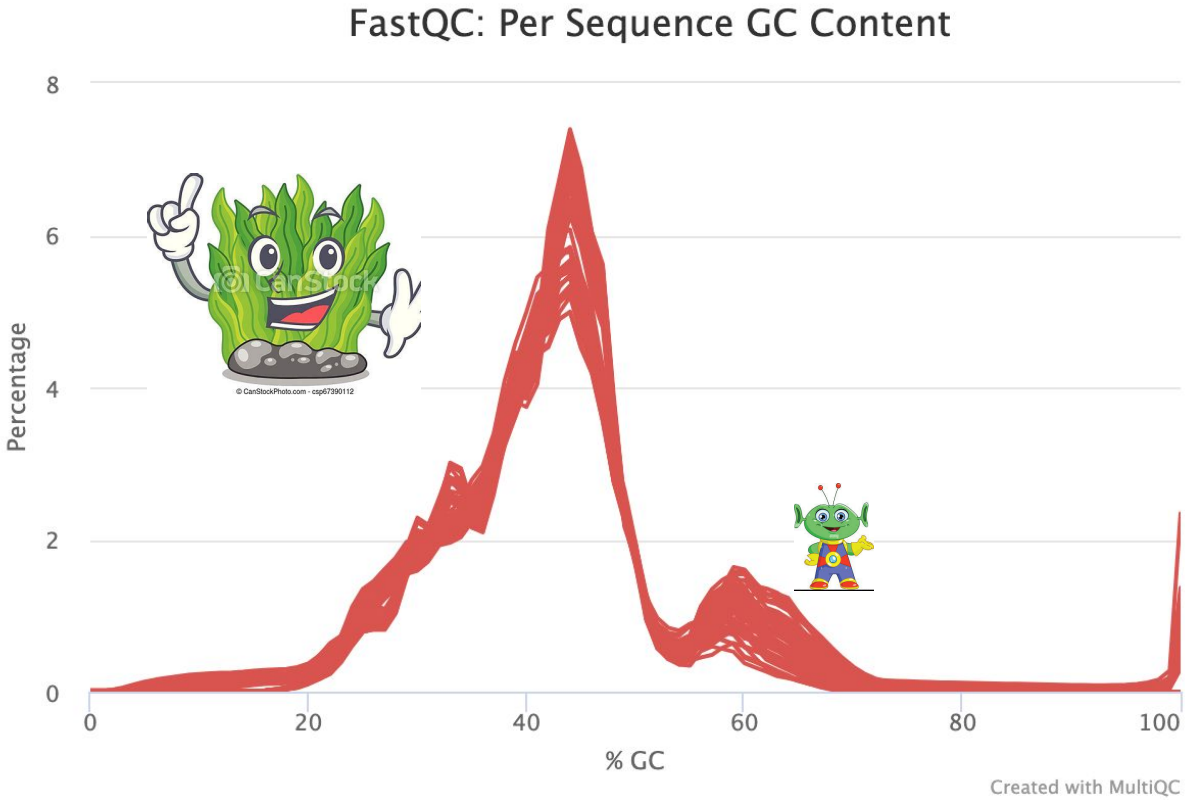


Third-party contamination : detection

Sabellaria alveolata : mantle transcriptome



Third-party contamination : detection



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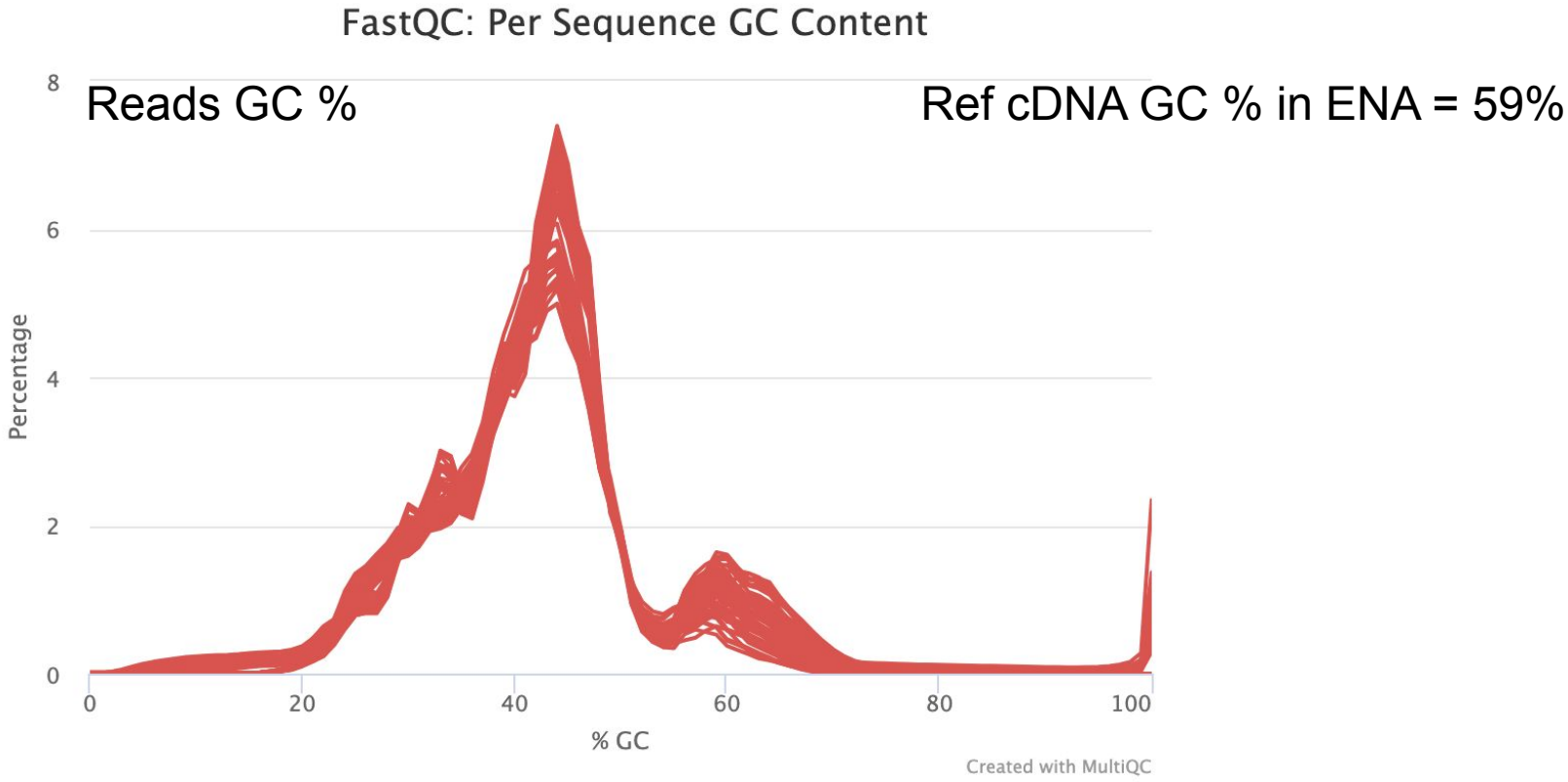

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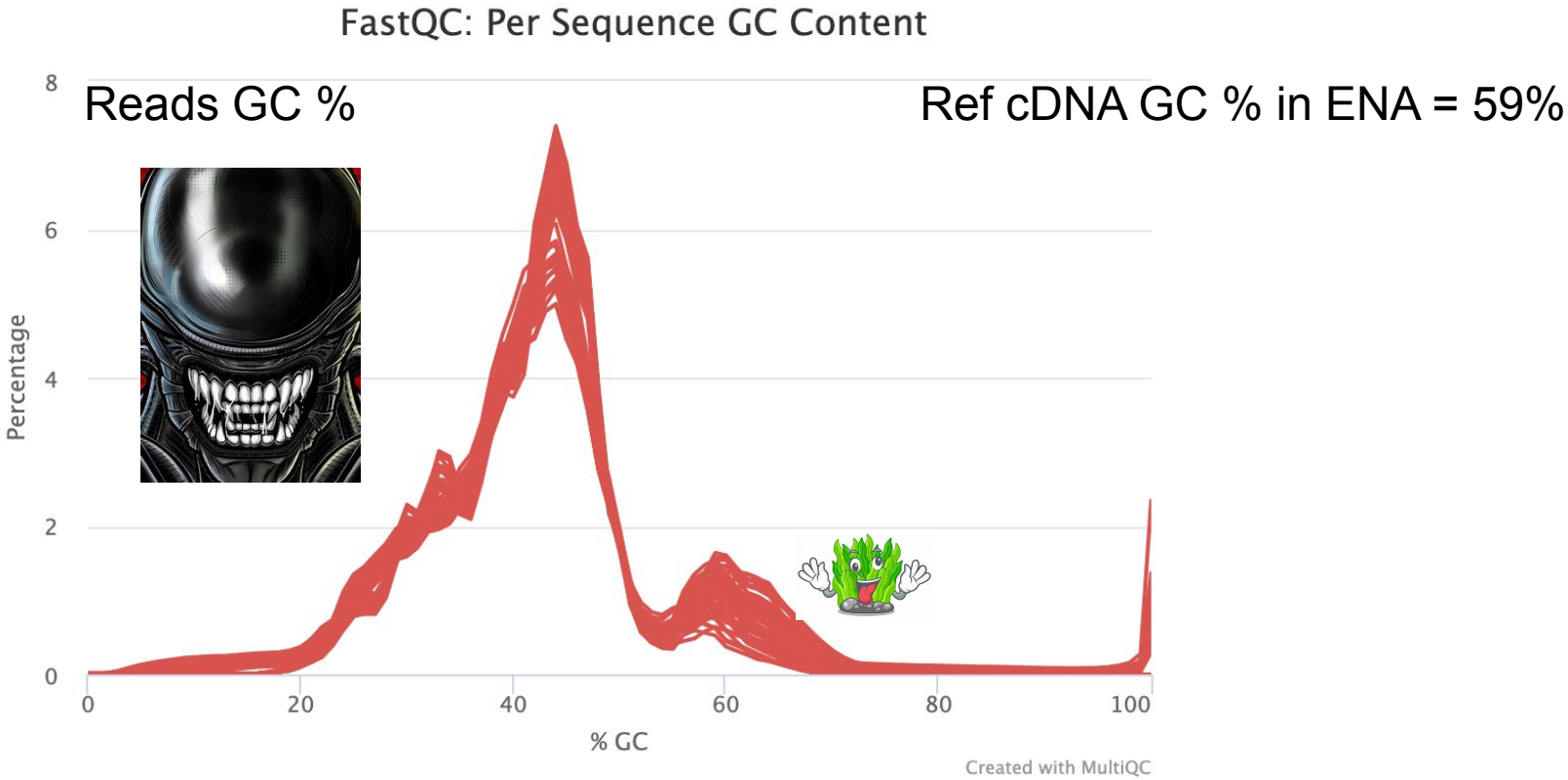


Removing rRNA contamination

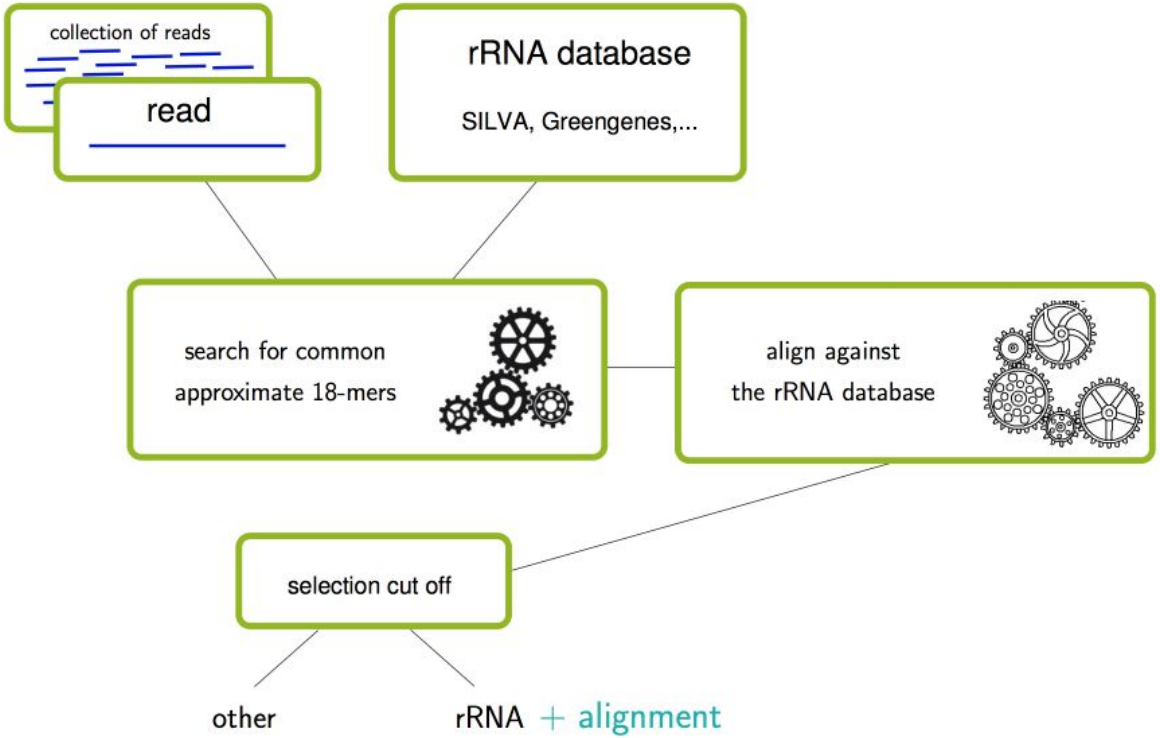
Prior to sequencing :

- Ribodepletion kits
- Selection polyA

After sequencing :

- Remove rRNA reads from raw reads (ex. SortMe RNA)
- Detect rRNA transcripts in the assembly (RNAmmer)

SortMeRNA



Selected for *de novo* assembly

https://github.com/sortmerna

Detect rRNA transcripts : RNAMMER

The program uses hidden Markov models trained on data from the 5S ribosomal RNA database and the European ribosomal RNA database project

Alternative Barrnap : *BAsic Rapid Ribosomal RNA Predictor*
https://github.com/tseemann/barrnap

```
# ------------------------------------------------------------------------------------------------------
##gff-version2##source-version RNAmmer-1.2##date 2009-11-16
##Type DNA
# seqname        source      feature  start     end       score    +/-      frame    attribute
# ------------------------------------------------------------------------------------------------------
AE000511         RNAmmer-1.2      rRNA     448462    448577    49.2     +        .        5s_rRNA
AE000511         RNAmmer-1.2      rRNA     1473564   1473679   49.2     -        .        5s_rRNA
AE000511         RNAmmer-1.2      rRNA     1045067   1045183   40.3     +        .        5s_rRNA
AE000511         RNAmmer-1.2      rRNA     445339    448223    3056.5   +        .        23s_rRNA
AE000511         RNAmmer-1.2      rRNA     1473918   1476803   3032.8   -        .        23s_rRNA
AE000511         RNAmmer-1.2      rRNA     1207586   1209074   1801.4   -        .        16s_rRNA
AE000511         RNAmmer-1.2      rRNA     1511140   1512627   1803.6   -        .        16s_rRNA
```

In silico normalization of reads

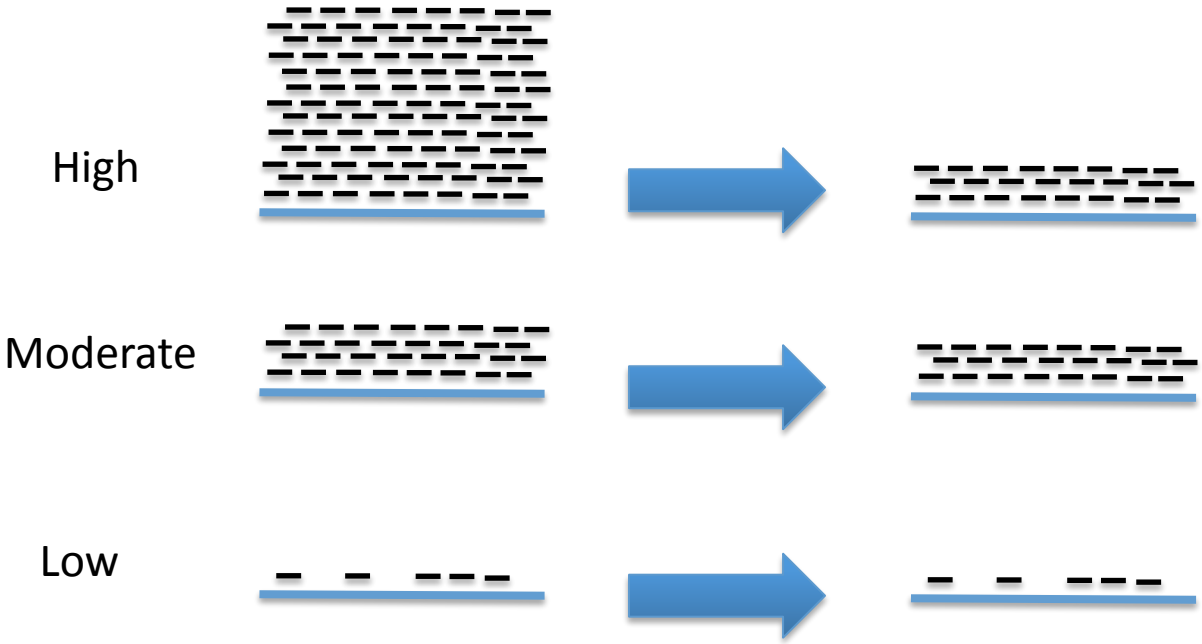
- By definition RNAseq display a wide range of expressions
  Very low expressed -> Very highly expressed transcripts

- The information given by reads from high expression transcripts is redundant, and very high coverage also brings more sequencing errors

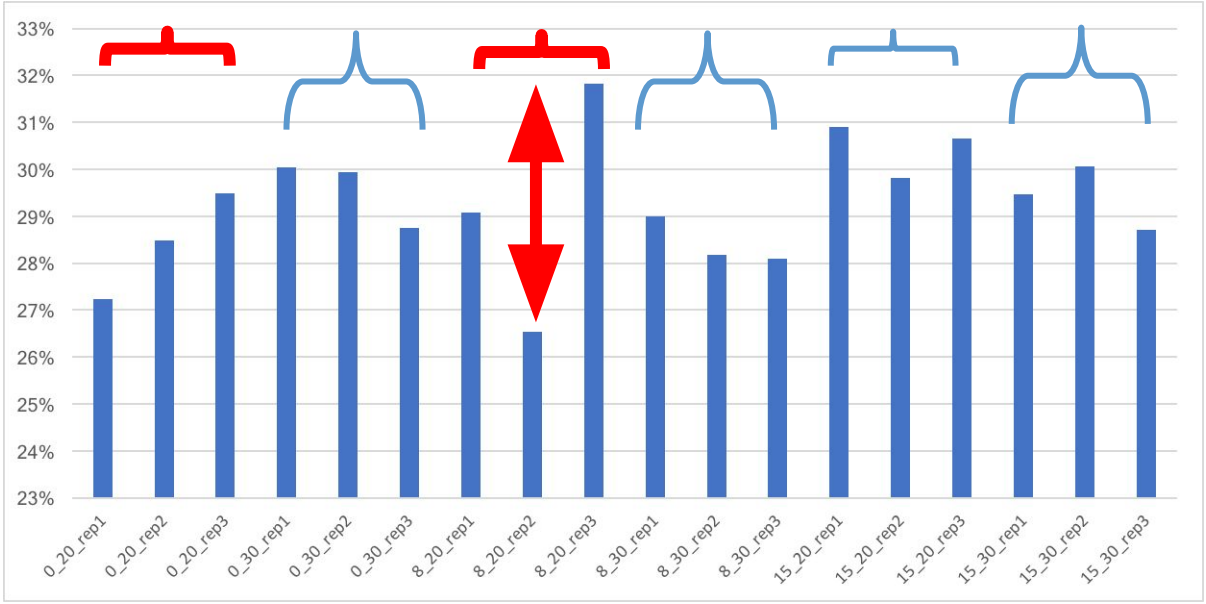
- De-novo assemblers do not benefit from coverage increase beyond a certain point (> 200 millions reads) , and fewer data means quicker assemblies

How to decrease coverage of highly expressed transcripts without decreasing that of low expressed transcripts ?

In silico normalization of reads



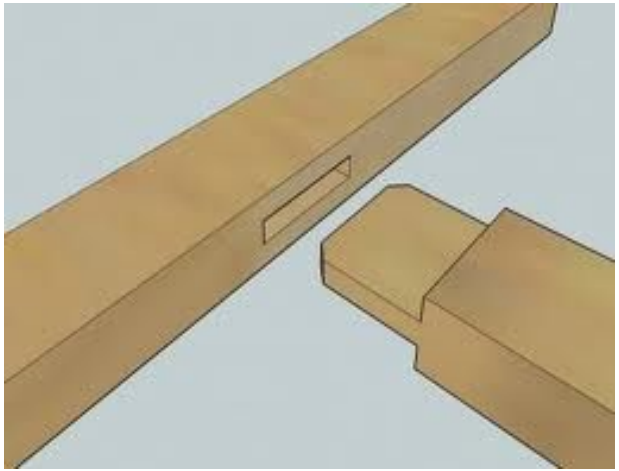
Normalisation prior to assembly : replicats congruence



Reads *in silico* normalisation

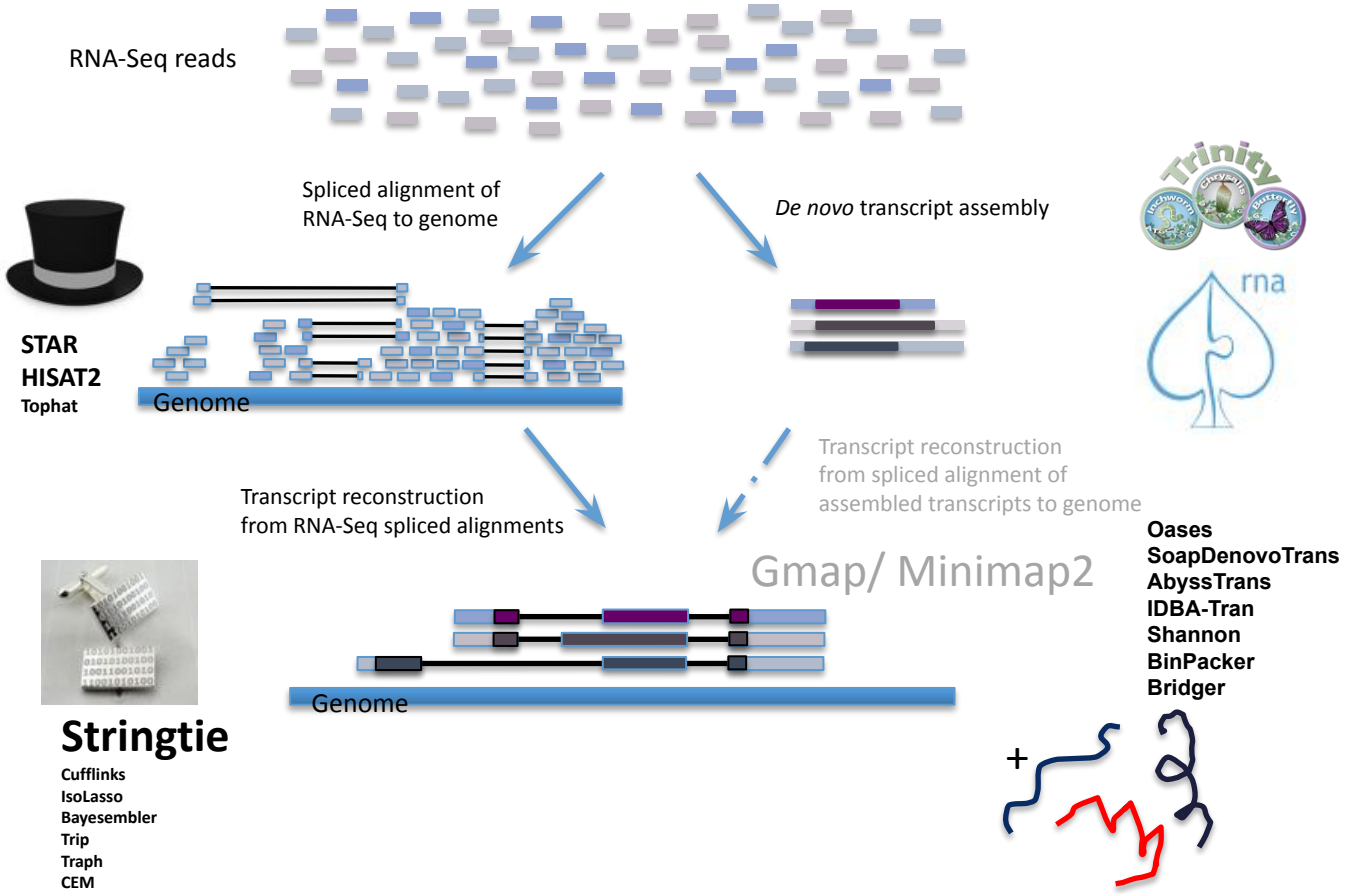
Tools that can perform *in silico* read normalization

- `Trinity` assembler also offers in-built *in silico* normalization
- `khmer` – https://github.com/dib-lab/khmer (using the diginorm algorithm)
- `Bignorm` – https://git.informatik.uni-kiel.de/axw/Bignorm
- `NeatFreq` – https://github.com/bioh4x/NeatFreq
- `ORNA` – https://github.com/SchulzLab/ORNA



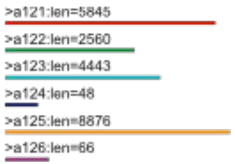
TRANSCRIPTOME ASSEMBLY STRATEGIES

Contemporary strategies for transcript reconstruction from RNA-Seq



Trinity – How it works:

RNA-Seq reads → **Linear contigs** → **de-Bruijn graphs** → **Transcripts + Isoforms**



...CTTCGCAA...TGATCGGAT...
...ATTCGCAA...TCATCGGAT...

Running a typical Trinity job requires ~1 hour and ~1G RAM per ~1 million PE reads.

Thousands of disjoint graphs

Results

Result: linear sequences grouped in *components*, *contigs* and sequences

```
>TRINITY_DN889_c0_g1_i1 len=259 path=[473:0-258] [-1, 473, -2]
GAACAATGTCTACACTGTCTTCAACTTGGATGACAAGGAACTTTCATTGGCTCAAGCTAA
CTACAATTCATCTCTGAAACCAGATATTGAAGAAATCAAGGATACTGTCCCTAGCGCTGT
GCTGGCTCCACAATACTACAACACATTCTCAGCTGACCCAACTGCCACTGCAGTCACTGG
TAACATCTTTGCACCAGAGGCCACTATGTCCATGGCTGCTCCAGCTAATGCTTCTAGAAA
CTCTTCATTAAACTCTCCT

>TRINITY_DN810_c0_g1_i2 len=226 path=[407:0-225] [-1, 407, -2]
GATGATATCAACAATGAGACTTGTGAACCAGGTGAAGAAAACTCTTTCTTTGTATGCGAC
CTAGGTGAAATTGAAAGATTGTACGCTAACTGGTGGAAAGAACTACCAAGAGTTCAGCCA
TTTTACGCTGTCAAGTGTAACCCAGATTTGAAGATAATAAGAAAATTGGCTGACCTCGGA
```

TRINITY_DNW|cX_gY_iZ (until release 2.0 cX_gY_iZ previously **compX_cY_seqZ**

TRINITY_DNW|cX defines the graphical component generated by Chrysalis (from clustering inchworm contigs).
Butterfly might tease subgraphs apart from each other within a single component, based on the read support data . This gives rise to subgraphs (**gY**).: trinity genes
Each subgraph then gives rise to path sequences (iZ). : trinity isoforms
(path) list of vertices in the compacted graph that represent the final transcript sequence and the range within the given assembled sequence that those nodes correspond to.

rnaSPAdes usage and results

Derived from SPAdes is a versatile toolkit designed for assembly and analysis of sequencing data. SPAdes allows to assemble genomes, metagenomes, transcriptomes, viral genomes etc.

Output

- `transcripts.fasta` – default assembly
- `hard_filtered_transcripts.fasta` – includes only long and reliable transcripts with rather high expression.
- `soft_filtered_transcripts.fasta` – includes short and low-expressed transcipts, likely to contain junk sequences.

Thus, each contig name has

- individual unique id (`NODE_XX`)
- length (`length_XXXX`)
- kmer coverage (`cov_XXXX`) : (is always lower than the read (per-base) coverage)

Distinct isoforms will have different unique ids, and may have different coverage and length as well. The only thing they share is the gene id (given after _g). For example:

```
NODE_217_length_5488_cov_28.472321_g133_i0
NODE_260_length_5302_cov_30.767137_g133_i1
```

Trinity – rnaSPAdes : Pro - Cons

Trinity

+ old (large community of users. Brian Hass)
+ Very good documentation
+ Complete: many features.
+ Ability to assemble long and complex transcripts,
+ management of multiple isoforms.
- Slower and more fragmented assemblies
- Development stopped (2024)

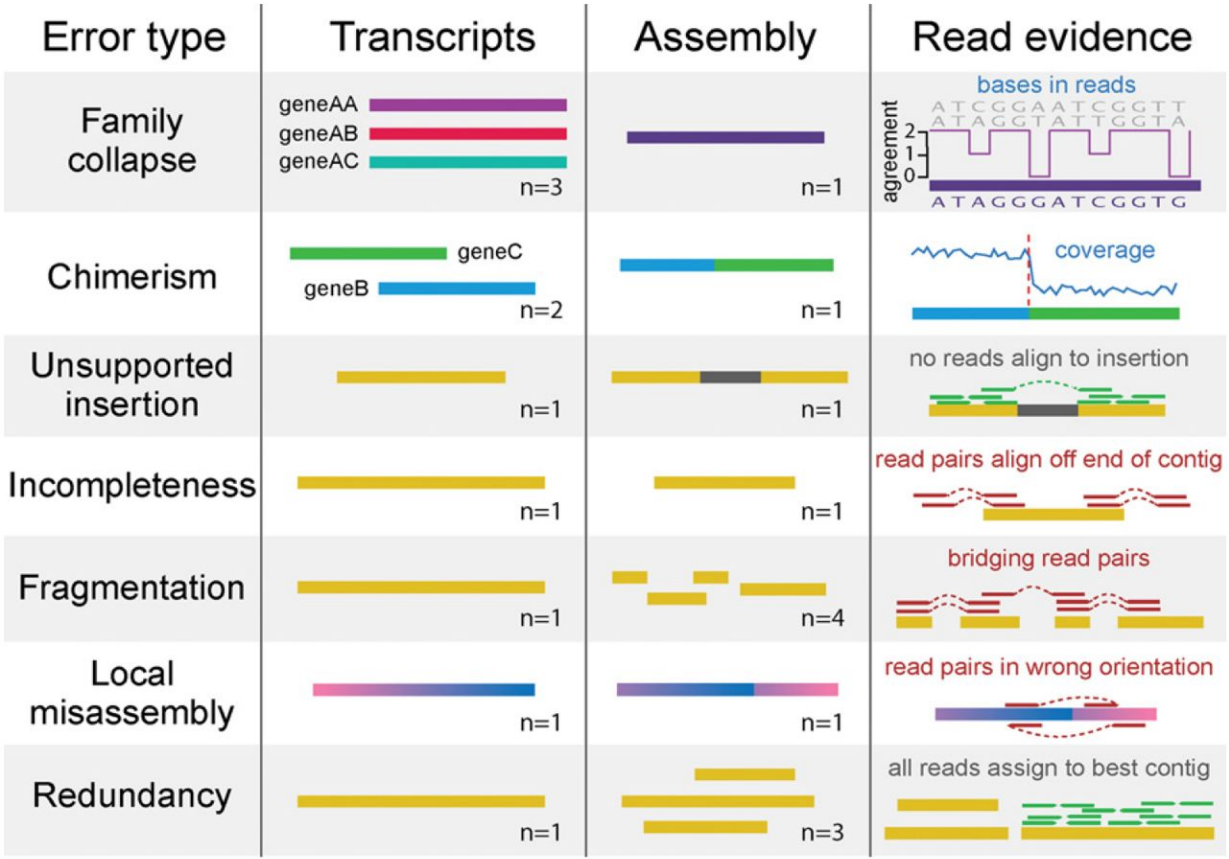
rnaSPAdes

+ speed
+ precision
+ especially for data with poor coverage or heterogeneous samples.
+ wider phylogenetic range
+ include long read support
- Less rare isoforms

Transcriptome assembly

ASSEMBLY QUALITY ASSESSMENT AND CLEANNING

De novo Transcriptome Assembly is Prone to Certain Types of Errors



Smith-Unna et al. Genome Research, 2016

Assembly quality assessment

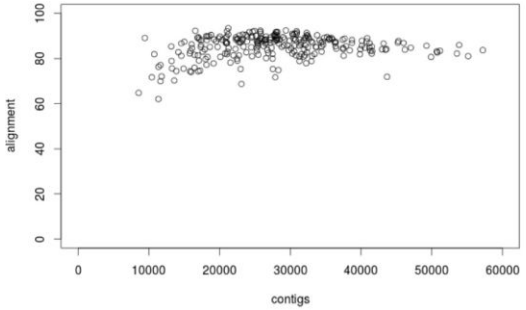
- Generating general Assembly metrics
- Comparing the assembled sequences to the reads used to generate them (reference-free)
- Comparing the assembled sequences
  - to catalogue of orthologous genes
  - to conserved gene domains
  - to transcriptomes or genomes of closely related species.

Metrics

- The number of contigs in the assembly
- The size of the smallest contig
- The size of the largest contig
- The number of bases included in the assembly
- The mean length of the contigs
- The number of contigs <200 bases
- The number of contigs >1,000 bases
- The number of contigs >10,000 bases
- The number of contigs that had an open reading frame
- The mean % of the contig covered by the ORF
- NX (e.G. N50): the largest contig size at which at least X% of bases are contained in contigs at least this length
- % Of bases that are G or C
- GC skew

Realignment metrics

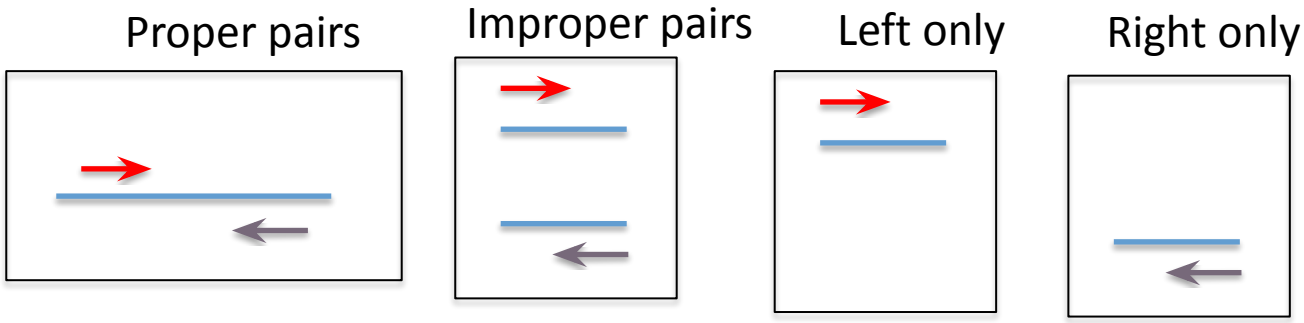
The realignment rate gives how much of the initial information is inside the contigs.



A typical ‘good’ assembly has ~80 % reads mapping to the assembly and ~80% are **properly paired**.

Given read pair: → ←

Possible mapping contexts in the Trinity assembly are reported:



Assembly evaluation : read remapping

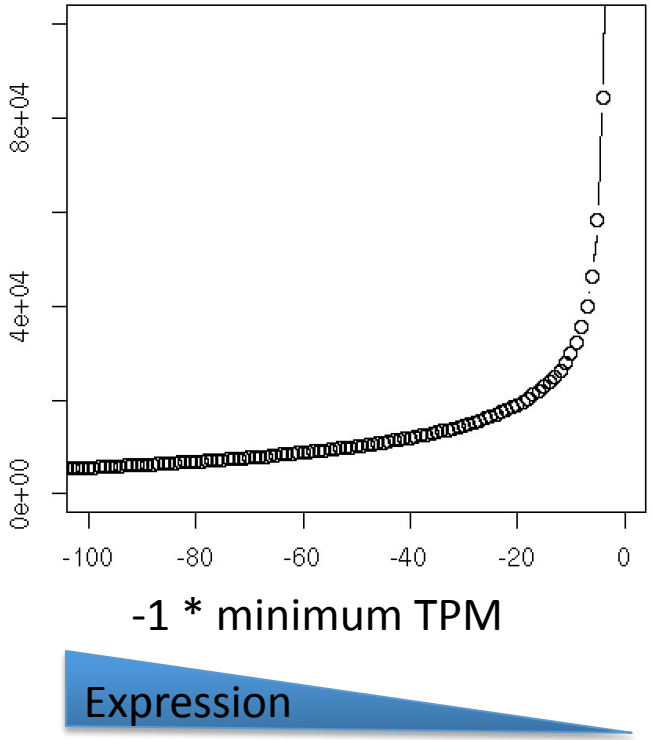
Quality Assessment: Calculate the coverage and depth of each contig to identify transcripts that are well or poorly supported by the raw data.
Expression quantification: Estimate the level of expression (f.e. TPM) of each transcript.
Correction and Refinement: Identify regions of low coverage, sequence variations, or potential errors in the assembly.

Tools :
Unspliced (fast) aligners: *Bowtie2* or *BWA* are fast and efficient for aligning short sequences to reference sequences.
K-mers-based quantifiers: *Kallisto* Or *Salmon* do not perform traditional base-by-base alignment, but use k-mer indexing (or near-alignments) approaches of reads on transcripts for fast and highly accurate quantification.

Alternative to N50 ?

Often, most assembled transcripts are *very* lowly expressed
(How many 'transcripts & genes' are there really?)

Cumulative nb of Transcripts ordered by decreasing expression level



1.4 million Trinity transcripts

N50 ~ 500 bases

24k transcripts

Correspond to 90% of expression

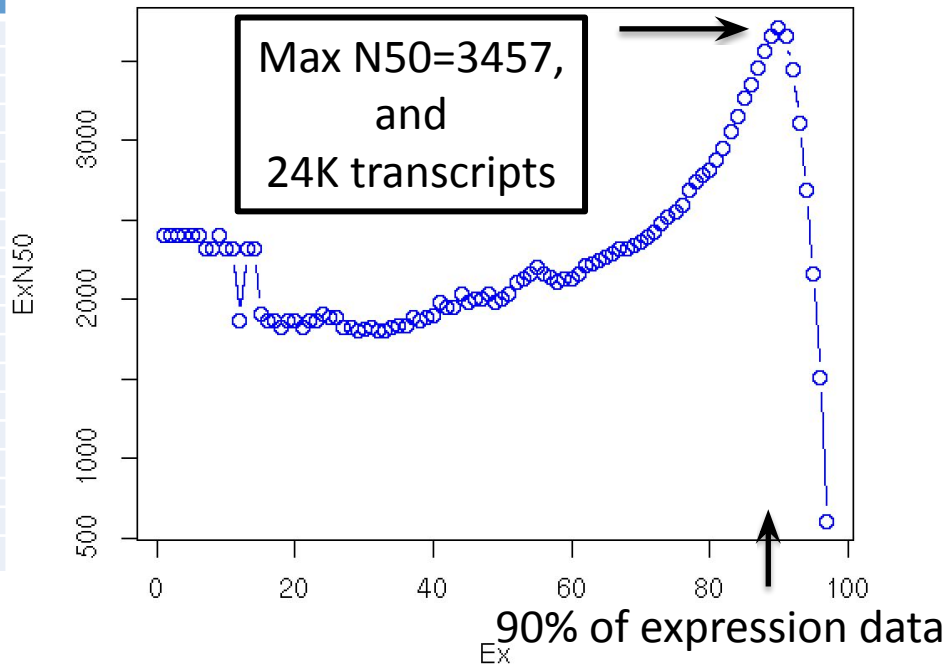
* Salamander transcriptome

Alternative to N50 : ExN50 – E90N50

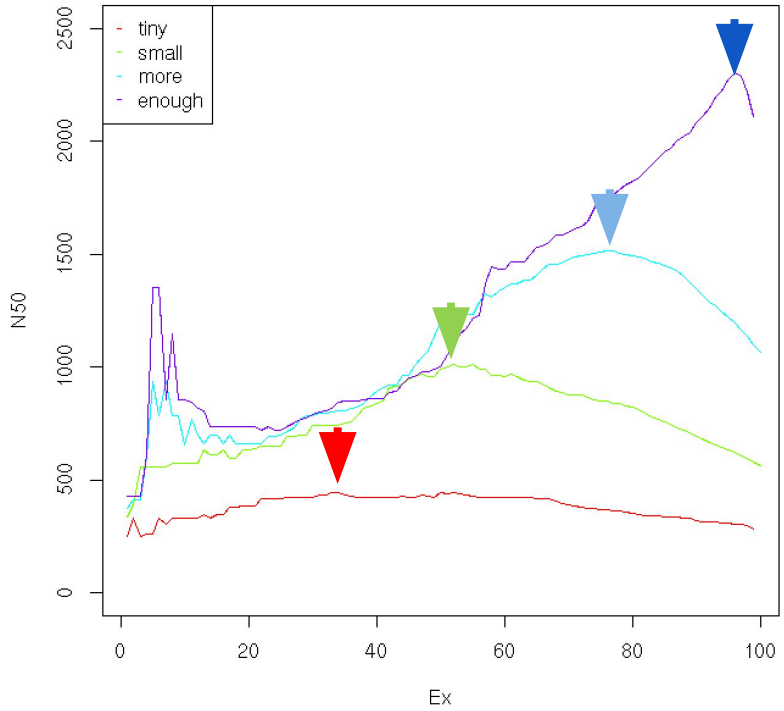
Compute N50 Based on the Top-most Highly Expressed Transcripts (ExN50)

- Sort contigs by expression value, descendingly.
- Compute N50 given minimum % total expression data thresholds => ExN50

#E	min_expr	E-N50	num_transcripts
E2	89129.251	2397	1
E3	89129.251	2397	2
E5	66030.692	2397	3
E6	66030.692	2397	4
E8	66030.692	2397	5
...	.....	.....	....
E86	9.187	3056	12309
E87	7.044	3149	14261
E88	6.136	3261	16646
E89	4.538	3351	19635
E90	**3.939**	**3457**	**23471**
E91	3.077	3560	28583
E92	2.208	3655	35832
E93	1.287	3706	47061
...	.....	.....	....
E97	0.235	2683	275376
E98	0.164	2163	428285
E99	0.128	1512	668589
E100	0	606	1554055



ExN50 Profiles for Different Trinity Assemblies Using Different Read Depths



Millions of Reads

Thousands of Reads

Note shift in ExN50 profiles as you assemble more and more reads.

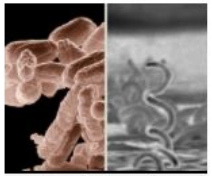
* Candida transcriptome

BUSCO analysis

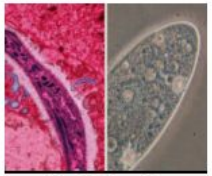
BUSCO(http://busco.ezlab.org/)
Assessing genome assembly and annotation completeness with Benchmarking Universal Single-Copy Orthologs

BUSCO

Datasets *(Beta versions, updated sets and additional lineages coming soon)*


Bacteria sets


Eukaryota sets


Protists sets


Metazoa sets

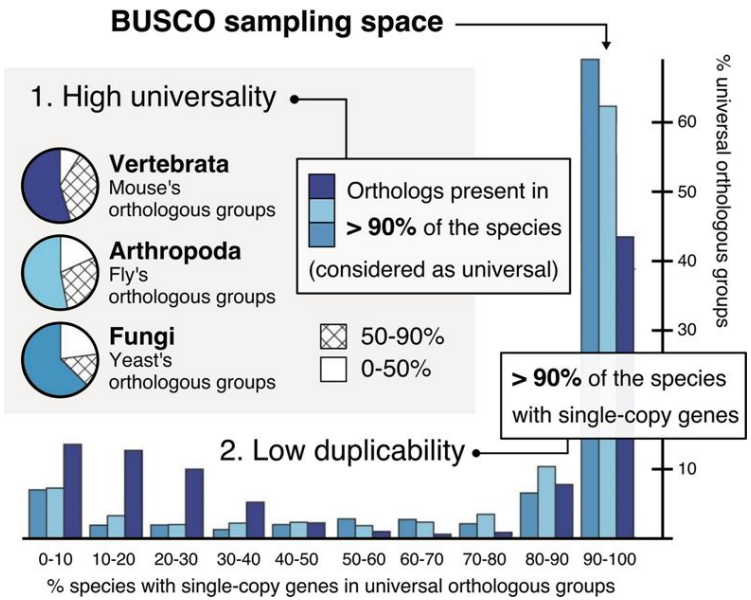

Fungi sets


Plants set

Arthropods: Vertebrates: Fungi: Bacteria:

Metazoans: & & Eukaryotes: & & &

BUSCO analysis



193 lineages !!

acidobacteria_odb10.2024-01-08.tar.gz
aconoidasida_odb10.2024-01-08.tar.gz
actinobacteria_class_odb10.2024-01-08.tar.gz
actinobacteria_phylum_odb10.2024-01-08.tar.gz
actinopterygii_odb10.2024-01-08.tar.gz
agaricales_odb10.2024-01-08.tar.gz
agaricomycetes_odb10.2024-01-08.tar.gz
alphabaculovirus_odb10.2024-01-08.tar.gz
alphaherpesvirinae_odb10.2024-01-08.tar.gz
alphaproteobacteria_odb10.2024-01-08.tar.gz
alteromonadales_odb10.2024-01-08.tar.gz
alveolata_odb10.2024-01-08.tar.gz
apicomplexa_odb10.2024-01-08.tar.gz
aquificae_odb10.2024-01-08.tar.gz
arachnida_odb10.2024-01-08.tar.gz
archaea_odb10.2024-01-08.tar.gz
arthropoda_odb10.2024-01-08.tar.gz
ascomycota_odb10.2024-01-08.tar.gz
aves_odb10.2024-01-08.tar.gz
aviadenovirus_odb10.2024-01-08.tar.gz
bacillales_odb10.2024-01-08.tar.gz
bacilli_odb10.2024-01-08.tar.gz
bacteria_odb10.2024-01-08.tar.gz
bacteroidales_odb10.2024-01-08.tar.gz
bacteroidetes-chlorobi_group_odb10.2024-01-08.tar.gz

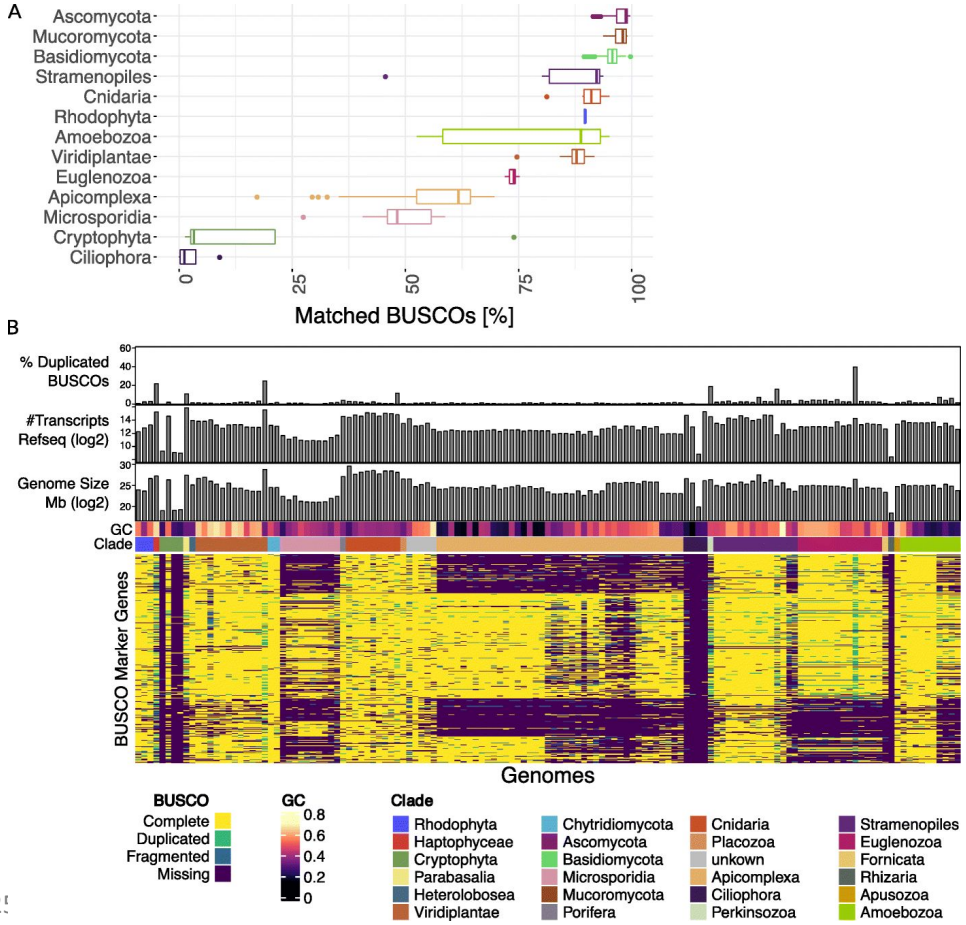
…….

BUSCO Results

```
# BUSCO version is: 5.3.2
# The lineage dataset is: eukaryota_odb10 (Creation date: 2020-09-10, number of genomes: 70,
number of BUSCOs: 255)
# BUSCO was run in mode: transcriptome

        ***** Results: *****

        C:97.2%[S:57.6%,D:39.6%],F:2.4%,M:0.4%,n:255
        248     Complete BUSCOs (C)
        147     Complete and single-copy BUSCOs (S)
        101     Complete and duplicated BUSCOs (D)
        6       Fragmented BUSCOs (F)
        1       Missing BUSCOs (M)
        255     Total BUSCO groups searched


# BUSCO version is: 5.3.2
# The lineage dataset is: arthropoda_odb10 (Creation date: 2020-09-10, number of genomes: 90,
number of BUSCOs: 1013)
# BUSCO was run in mode: transcriptome

        ***** Results: *****

        C:96.9%[S:56.8%,D:40.1%],F:1.8%,M:1.3%,n:1013
        981     Complete BUSCOs (C)
        575     Complete and single-copy BUSCOs (S)
        406     Complete and duplicated BUSCOs (D)
        18      Fragmented BUSCOs (F)
        14      Missing BUSCOs (M)
        1013    Total BUSCO groups searched
```

BUSCO v6.0.0 *is the current stable version!*

BUSCO limitation

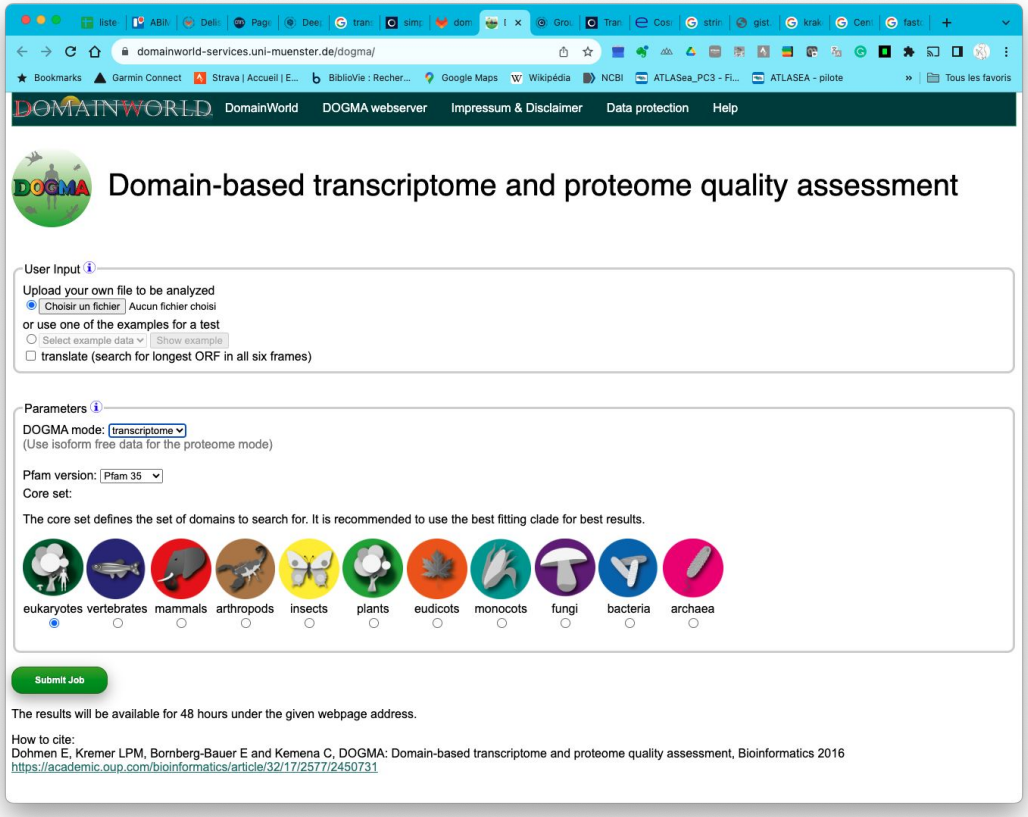


https://github.com/Finn-Lab/EukCC/

Saary, P., Mitchell, A.L. & Finn, R.D. Estimating the quality of eukaryotic genomes recovered from metagenomic analysis with EukCC. *Genome Biol* **21,** 244 (2020). https://doi.org/10.1186/s13059-020-02155-4

BUSCO Alternative

Instead of scoring on the basis of conserved genes (BUSCO) , completeness is assessed on the basis of conserved protein domains (DOGMA).



https://domainworld.uni-muenster.de/

Transcriptome thinning / redondancy reduction

de novo assembled contigs include transcriptional artifacts,

- pre-mRNA and ncRNA in addition to the protein-coding transcripts
- alternative splicing which manifests as transcript isoforms.

It may not always be necessary to retain all such sequences

Exclude transcripts that can be considered as being lowly expressed on the basis of abundance metrics such as TPM (e.g. TPM <1.00)

Clustering tool with combination of sequence identity and sequence coverage thresholds

- CD-HIT and MMSeqs2
  - the longest sequence in each cluster or the sequence with the most commonality
  - the longest isoform is not necessarily the most expressed (and vice versa).

Clustering + shared read support
Corset,Grouper or Compacta

Creation of SuperTranscripts : stitches all unique exons from the isoforms into a single, linear sequence.

New de novo transcriptome assemblers

- IDBA-Tran (Peng et al., Bioinf., 2014)
- IDBA-MTP (Peng et al., RECOMB 2014)
- SOAPdenovo-Trans (Xie et al., Bioinf., 2014)
- Fu et al., ICCABS, 2014
- StringTie (Pertea et al., Nat. Biotech., 2015)
- Bermuda (Tang et al., ACM, 2015)
- Bridger (Chang et al., Gen. Biol. 2015)
- BinPacker (Liu et al. PLOS Comp Biol, 2016)
- FRAMA (Bens M et al., BMC Genomics 2016)
- rnaSPAdes (Bushmanova et al., *GigaScience 2019)*
- ….
- Cstone (Linheiro and Archer, PLOS Comp Biol, 2021)

BinPacker - https://github.com/macmanes-lab/BINPACKER

Bridger - https://github.com/fmaguire/Bridger_Assembler

inGAP-CDG - https://sourceforge.net/projects/ingap-cdg/

DTA-SiST - https://github.com/jzbio/DTA-SiST

IDBA-tran - https://github.com/loneknightpy/idba

IsoTree - https://github.com/david-cortes/isotree

Oases - https://github.com/dzerbino/oases

RNA-Bloom - https://github.com/bcgsc/RNA-Bloom

rnaSPAdes - https://github.com/ablab/spades

SOAPdenovo-Trans - https://github.com/aquaskyline/SOAPdenovo-Trans

Trans-ABySS - https://github.com/bcgsc/transabyss

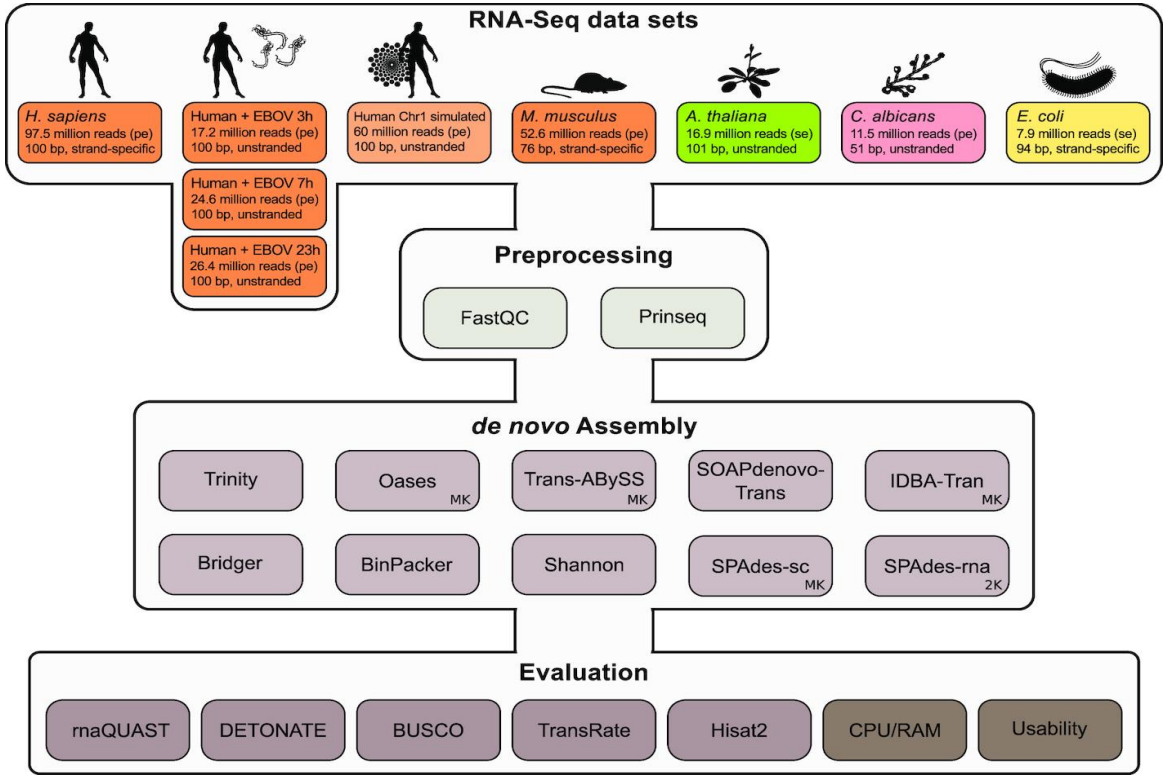
TransLig - https://sourceforge.net/projects/transcriptomeassembly/

Trinity - https://github.com/trinityrnaseq/trinityrnaseq

Assemblers comparison

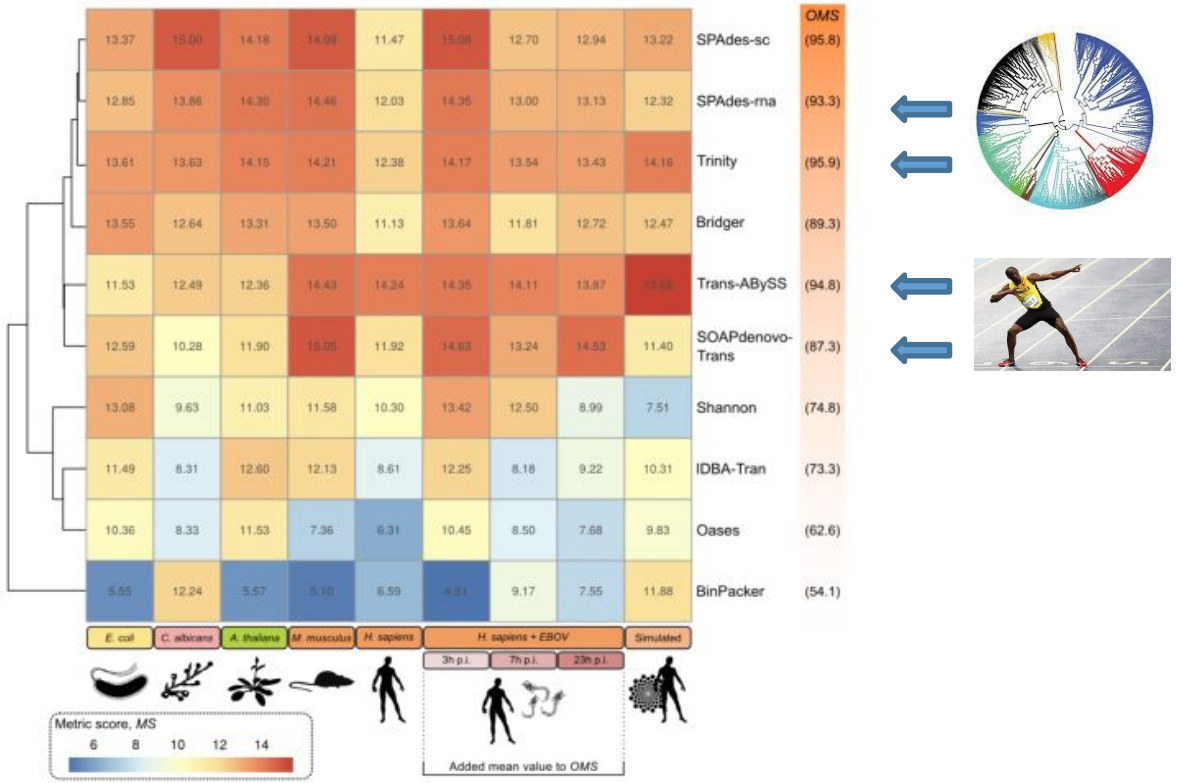
- Qiong-Yi Zhao et al., Optimizing de novo transcriptome assembly from short-read RNA-Seq data: a comparative study. BMC Bioinformatics 2011, 12(Suppl 14):S2
- Clarke, K., Yang, Y., Marsh, R., Xie, L., & Zhang, K. K. (2013). Comparative analysis of de novo transcriptome assembly. Science China Life Sciences, 56(2), 156–162. doi:10.1007/s11427-013-4444-x
- (Vijay et al., 2013) Challenges and strategies in transcriptome assembly and differential gene expression quantification. A comprehensive in silico assessment of RNA-seq experiments. Molecular ecology. PMID: 22998089
- (Haas et al., 2013) De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature protocols. PMID: 23845962
- (Lu et al., 2013) Comparative study of de novo assembly and genome-guided assembly strategies for transcriptome reconstruction based on RNA-Seq. Sci China Life Sci.
- Chen, G., Yin, K., Wang, C., & Shi, T. (n.d.). De novo transcriptome assembly of RNA-Seq reads with different strategies. Science China Life Sciences, 54(12), 1129–1133. doi:10.1007/s11427-011-4256-9
- (He et al., 2015) Optimal assembly strategies of transcriptome related to ploidies of eukaryotic organisms. BMC genomics. DOI: 10.1186/s12864-014-1192-7
- S. B. Rana, F. J. Zadlock IV, Z. Zhang, W. R. Murphy, and C. S. Bentivegna, “Comparison of De Novo Transcriptome Assemblers and k-mer Strategies Using the Killifish, Fundulus heteroclitus,” *PLoS ONE*, vol. 11, no. 4, p. e0153104, Apr. 2016.
- (Wang and Gribskov, 2016) Comprehensive evaluation of de novo transcriptome assembly programs and their effects on differential gene expression analysis. Bioinformatics. PMID: 27694201
- M. Hölzer and M. Marz, “De novo transcriptome assembly: A comprehensive cross-species comparison of short-read RNA-Seq assemblers,” Gigascience, vol. 8, no. 5, pp. 57–16, May 2019.
- Sadat-Hosseini et al. (2020) Combining independent *de novo* assemblies to optimize leaf transcriptome of Persian walnut. PLoS ONE 15(4): e0232005. https://doi.org/10.1371/journal.pone.0232005
- Jackson DJ, Cerveau N, Posnien N..De novo assembly of transcriptomes and differential gene expression analysis using short-read data from emerging model organisms - a brief guide. Front Zool. 2024 Jun 20;21(1):17. doi: 10.1186/s12983-024-00538-y.

Assemblers comparison



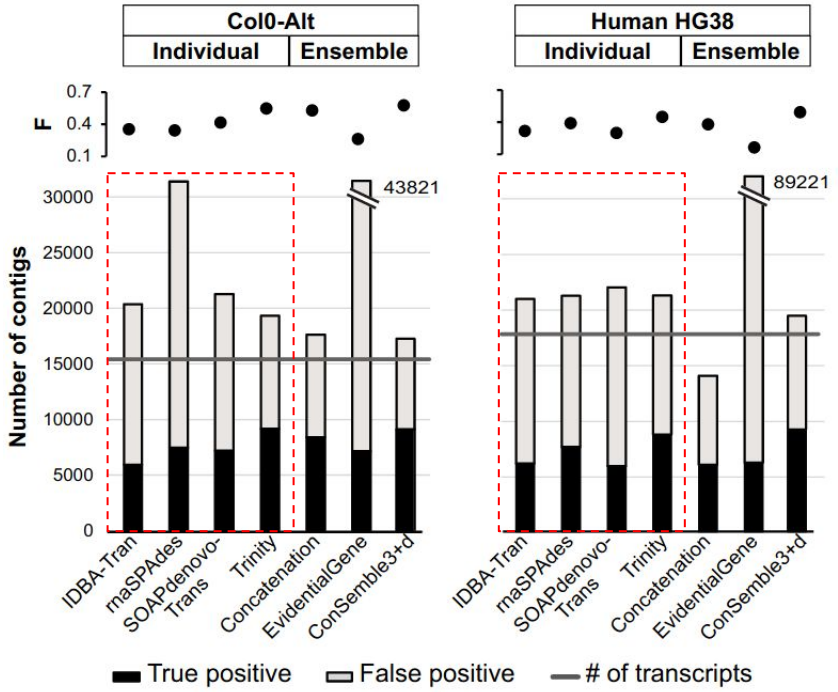
GigaScience, Volume 8, Issue 5, May 2019, giz039, https://doi.org/10.1093/gigascience/giz039

Assemblers comparison



GigaScience, Volume 8, Issue 5, May 2019, giz039, https://doi.org/10.1093/gigascience/giz039

Individual assemblers are not perfect …



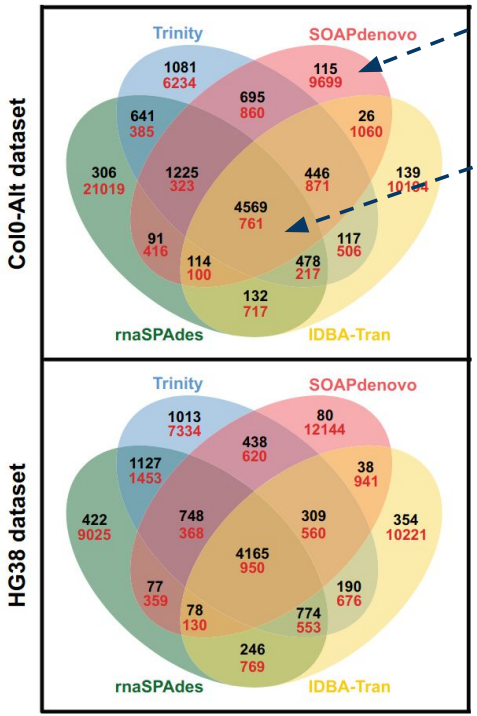
- Incomplete assemblies (missing transcripts)
- False positives (chimeric or incorrectly assembled sequences) => over-estimation
- Results vary depending on the parameters and sequencing depth

The **F-score** measures the overall quality of an assembly by combining precision and recall

Voshall, Adam, Sairam Behera, Xiangjun Li, et al. « A Consensus-Based Ensemble Approach to Improve Transcriptome Assembly ». BMC Bioinformatics 22, nº 1 (2021): 513. https://doi.org/10.1186/s12859-021-04434-8.

Integrative approach by consensus

Principle: combine several assemblies and retain only the transcripts present in several methods.



115 correct contigs
9699 incorrect contigs

4569 correct contigs
761 incorrect contigs

Advantages:

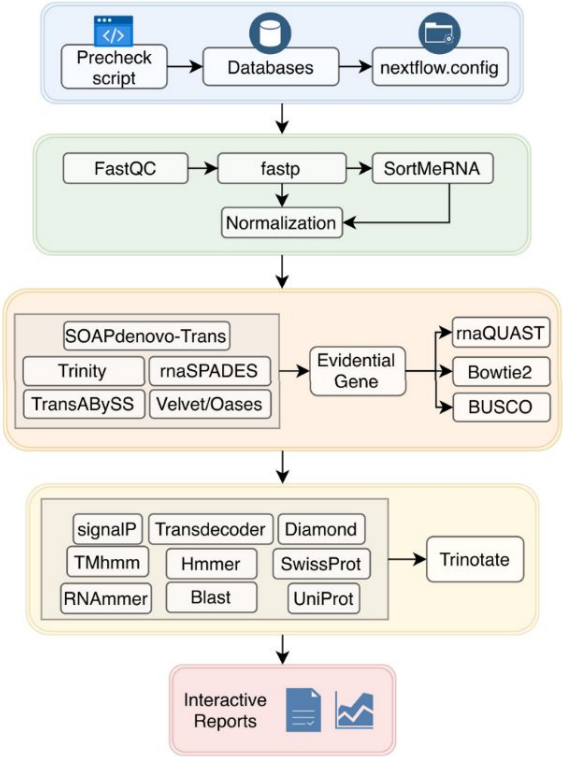
- Reduced errors
- Improved accuracy (fewer false positives)
- More robust transcriptome, especially for non-model organisms

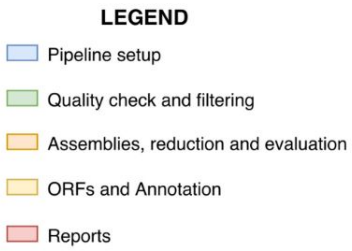
Integration does not seek to maximize quantity, but to optimize quality

Voshall, Adam, Sairam Behera, Xiangjun Li, et al. « A Consensus-Based Ensemble Approach to Improve Transcriptome Assembly ». BMC Bioinformatics 22, nº 1 (2021): 513. https://doi.org/10.1186/s12859-021-04434-8.

Integrative approach by consensus

TransPi—a comprehensive TRanscriptome ANalysiS PIpeline for de novo transcriptome assembly





DRAP, **EvidentialGene**, Concatenation, ConSemble, Pincho

use a combination of clustering and classification methods to generate a nonredundant consensus assembly from different assemblers

Rivera-Vicéns, Ramón E., Catalina A. Garcia-Escudero, Nicola Conci, Michael Eitel, et Gert Wörheide. « TransPi—a Comprehensive TRanscriptome ANalysiS PIpeline for de Novo Transcriptome Assembly ». Molecular Ecology Resources 22, nº 5 (2022): 2070-86. https://doi.org/10.1111/1755-0998.13593.