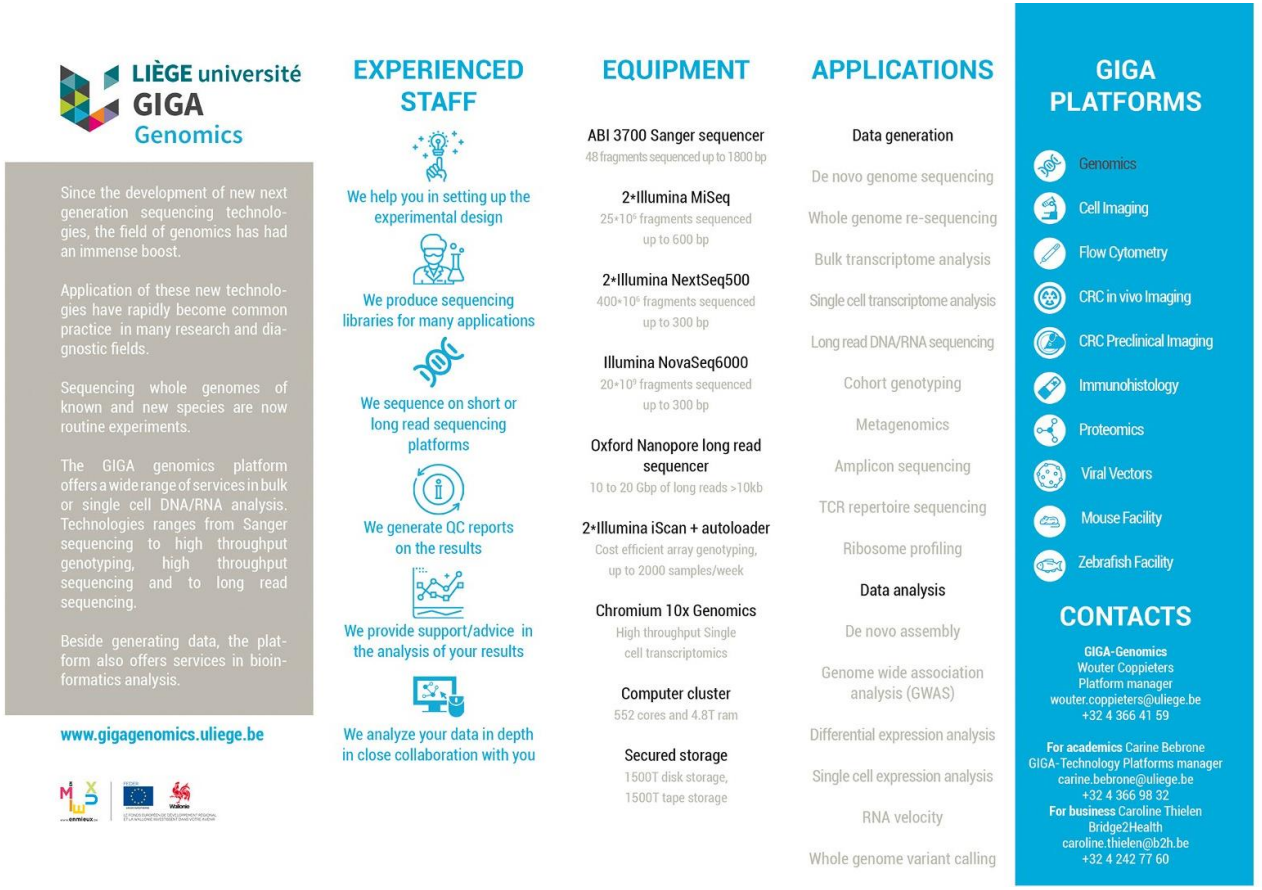
Lecture 9: RNA seq analysis in practice

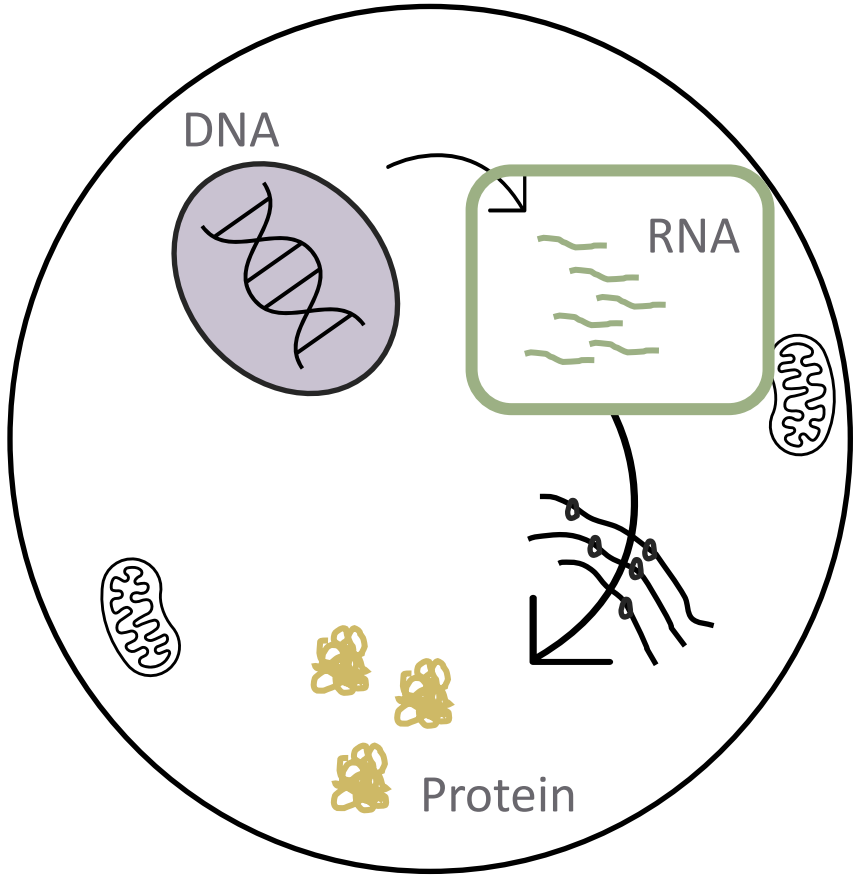
ARNAUD LAVERGNE
GIGA-Genomics
Bioinformatic team

GIGA - GENOMICS

- Platform
  - Sequencing services
    - Biological materials
    - DNA/RNA
    - Libraries
    - Sequencers
    - Data
  - High Throughput Sequencing (HTS) / Next Generation Sequencing (NGS)
  - Bioinformatic team
    - Data analysis
    - And more ...

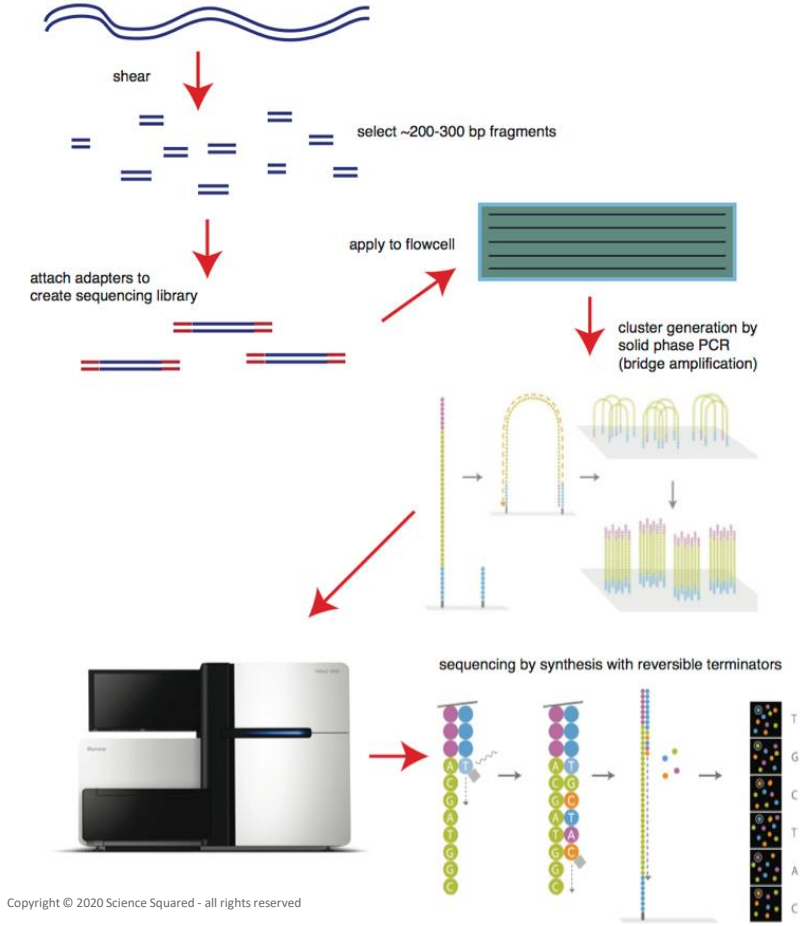


TRANSCRIPTOMICS



- Transcriptome
  - Gene expression

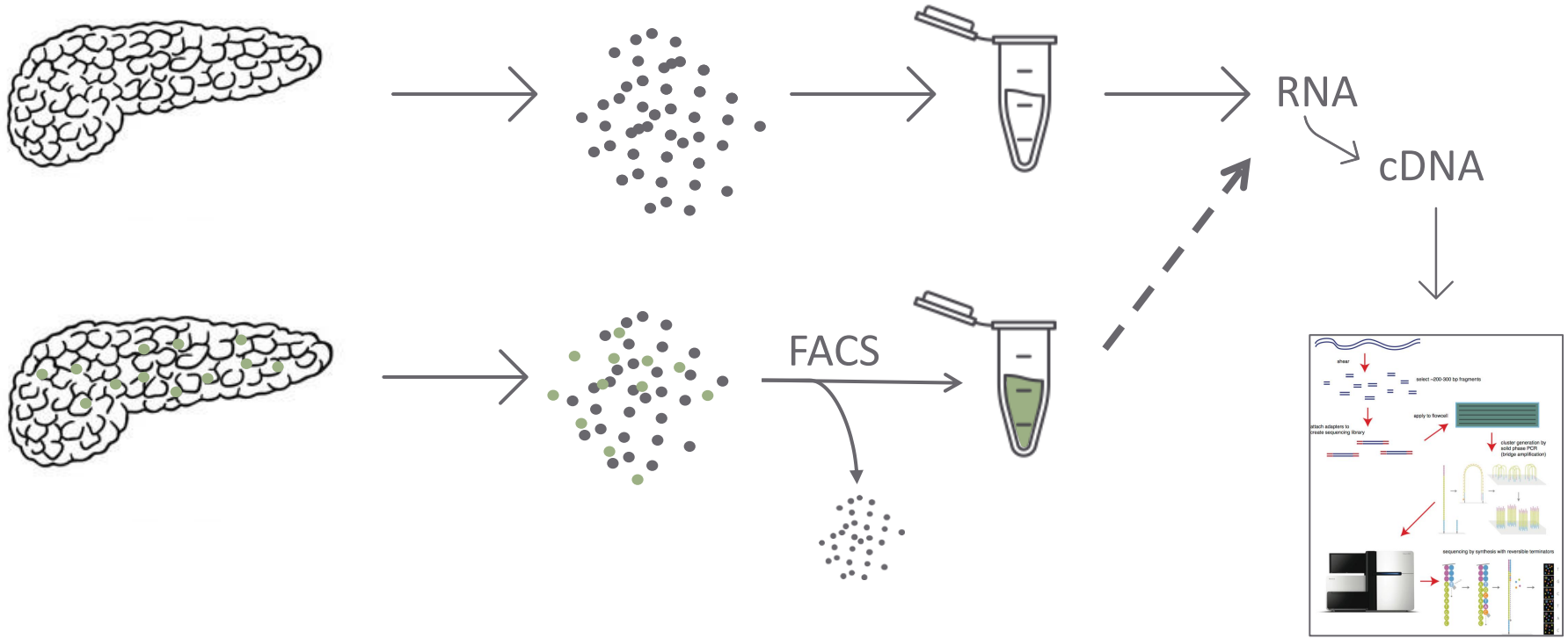
TRANSCRIPTOMICS



- Transcriptome
  - Gene expression
- High-Throughput Sequencing (HTS)
  - Next-generation sequencing (NGS)
  - Massively parallel sequencing
  - Millions of nucleotidic fragments
  - Genome-wide

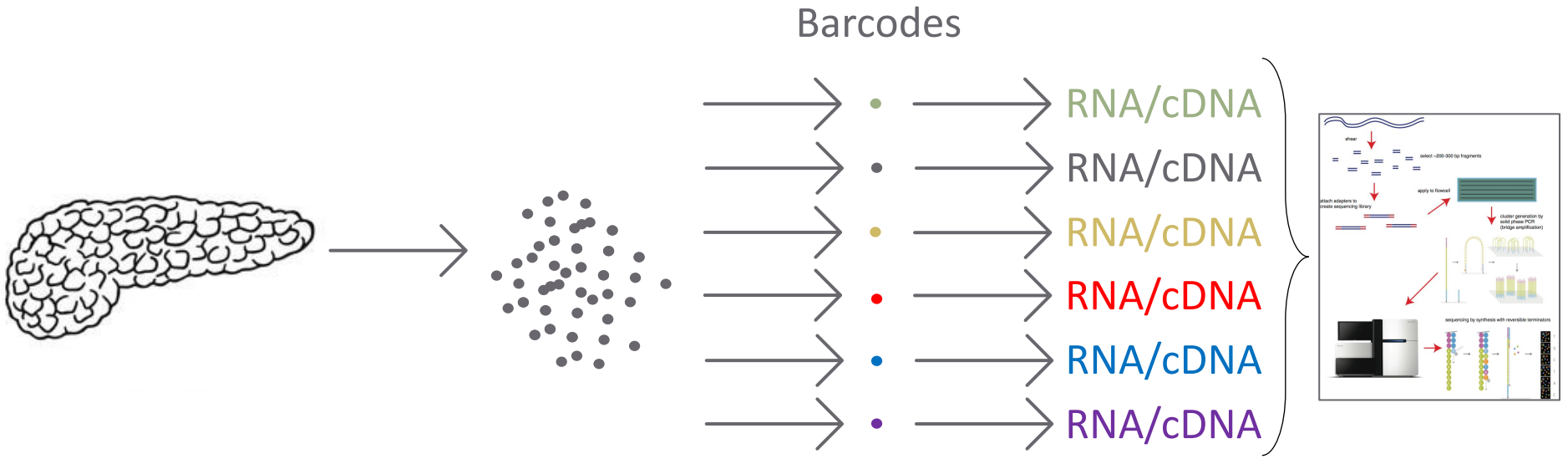
TRANSCRIPTOMICS

- RNA-Seq



TRANSCRIPTOMICS

- scRNA-Seq



SEQUENCERS

MiSeq
540 Mb -15 Gb
4 – 56 hours

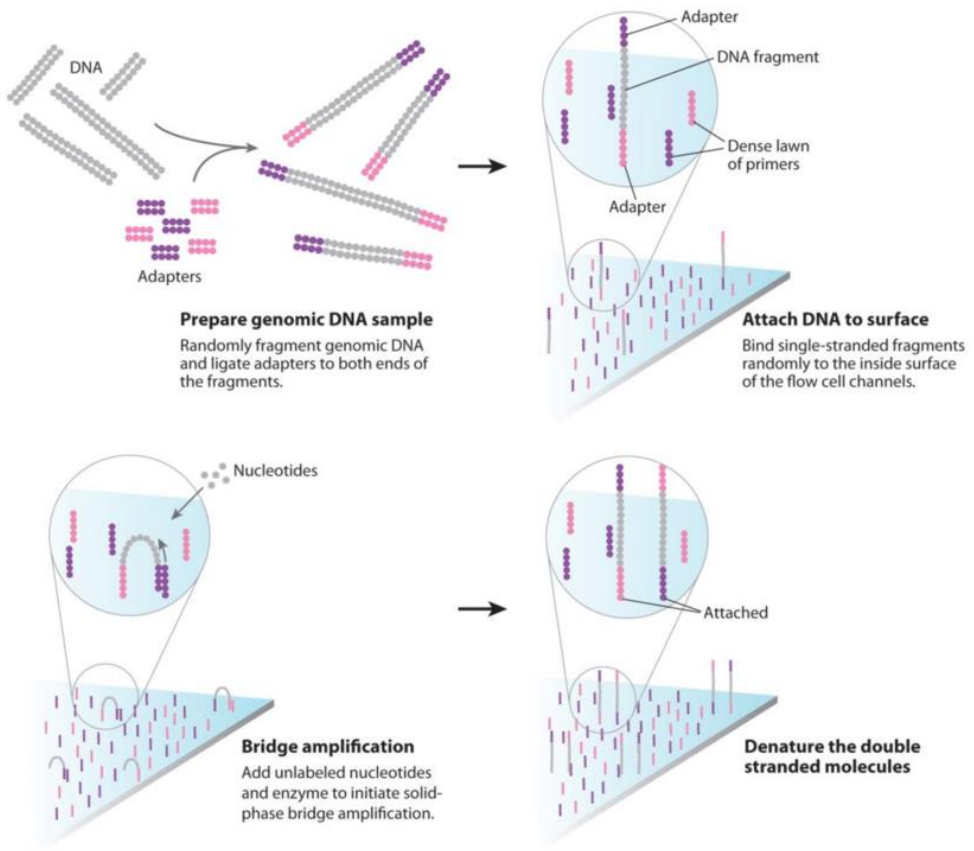
HiSeq
105 Gb - 1,5 Tb
1 – 3,5 days

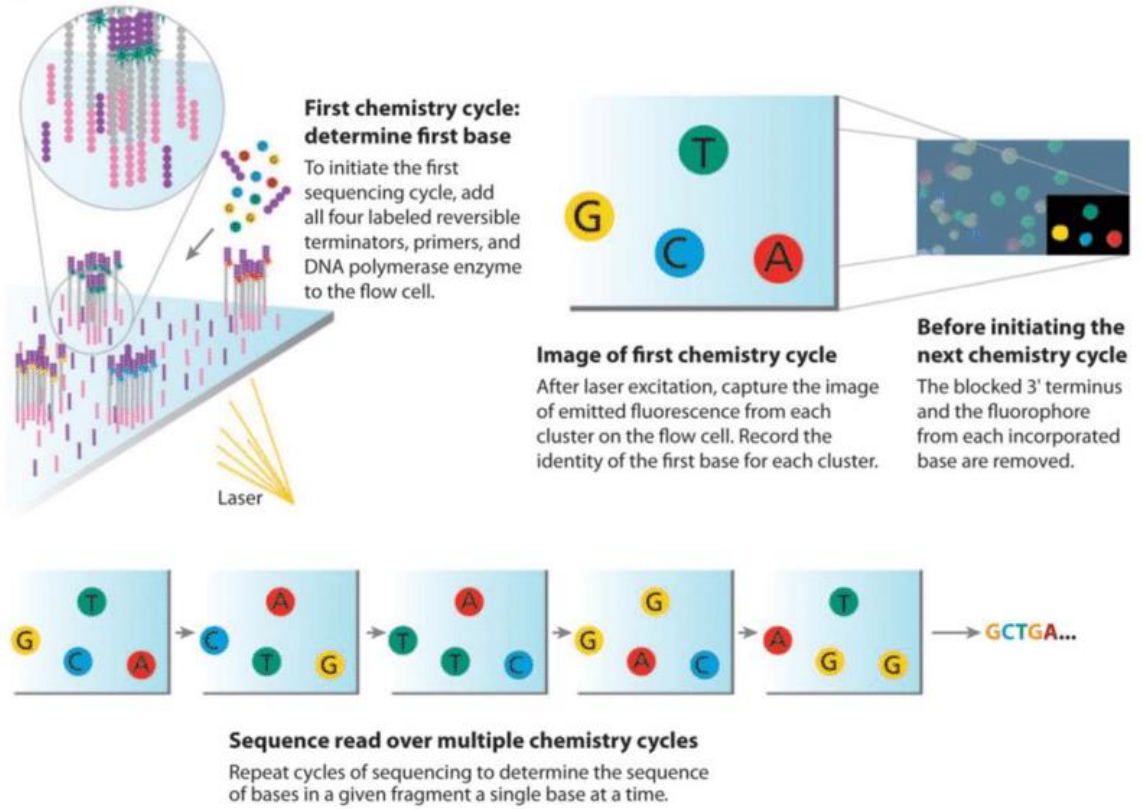
NextSeq
16,25 Gb - 120 Gb
11 – 29 hours

NovaSeq
65 Gb – 3 Tb
13 – 44 hours

Adapted from Illumina

SEQUENCING (HTS/NGS)

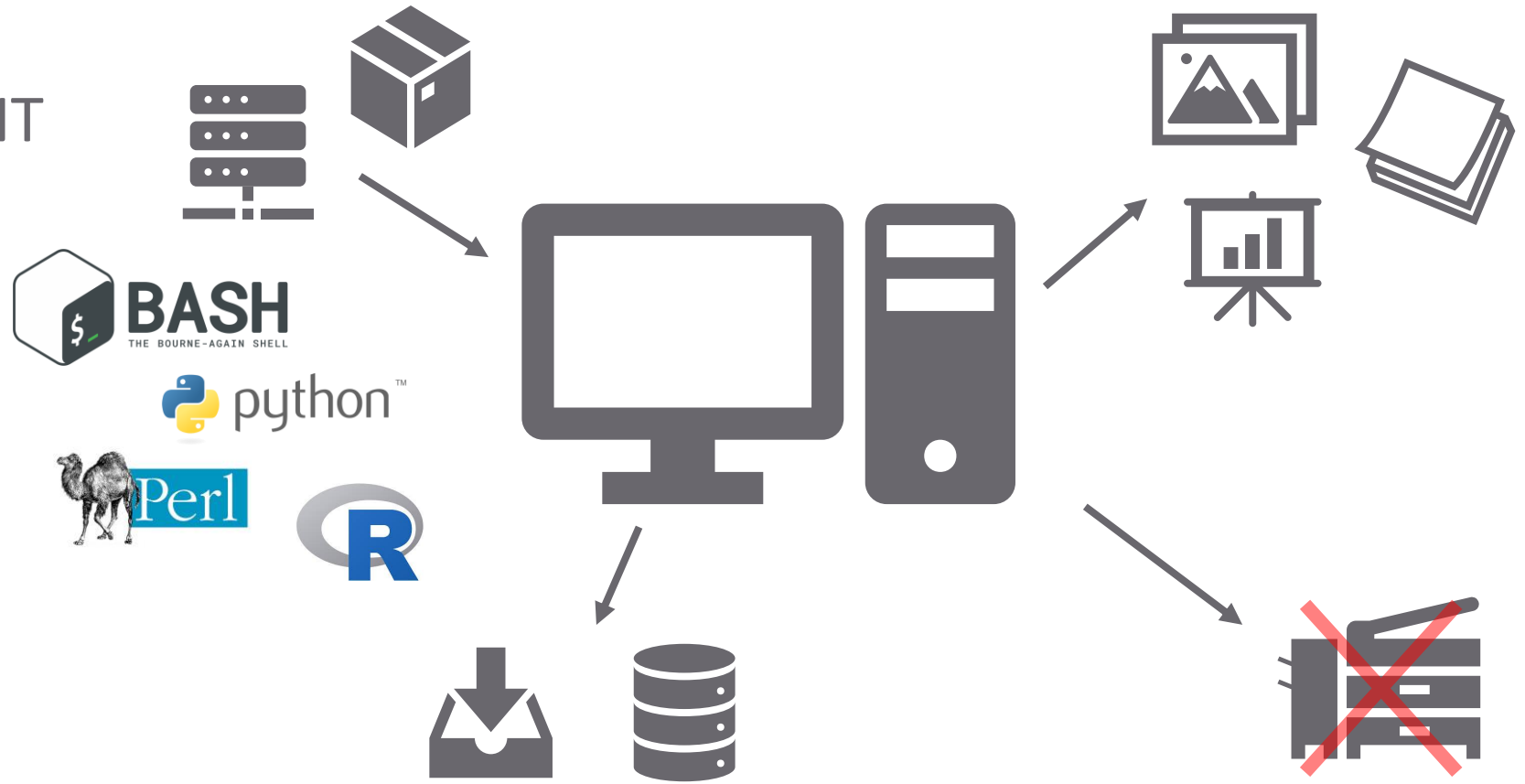




Adapted from Illumina

COMPUTING

- DATA MANAGEMENT
  - DATA PROCESSING
  - STORAGE
  - RESULTS
- REPRODUCTIBILITY
  - PIPELINES
  - CONTAINERS



DATA MANAGEMENT

LAPTOP
250Gb – 1Tb
4-8 Go RAM
4-8 CPUs

LAB COMPUTER
1 - 5 Tb
32 - 128 Go RAM
8-16 CPUs

CLUSTER/STORAGE
1.5 Pb
256 Go RAM
32 CPUs

DATA MANAGEMENT

- 1.2 Tb
- 12 Billions of reads
- 100-1000 samples
  - Multiple experiments
  - Unique combinations of indexes

- « Run »
- No storage on device

DATA PROCESSING

- DEMULTIPLEXING
  - Reads → Samples
  - ~ 20M / sample
  - « Fastq »
    - Identifier
    - Sequence
    - Separator
    - Quality score
      - Ascii +33

```
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CTTCTGGAGAGGAGTTCCTCTGATATGAATTAAGGTTTTCCCTTCTGTCATGACCAGAAGAAGGTTTTATCTGTGCCACACTACTTTTCATTTTCTGTTGCCAGTTGGTCCAATA
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+
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:FF,FF
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TTCAGCCTGGAANGCAGATACTCTCAATAATATTAAT
+
FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF:FFFFFFFFFFFFFFFF:FFFFFFF:FFFFF:FFFFFFFFFFFFFFFFFFFFFFFF:FFF,FFFFFFFFFFFF:FFF#FFFFF:FFFFFFFFFFFF
FFFFFF
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Symbol	ASCII Code	Q-Score	Symbol	ASCII Code	Q-Score
!	33	0	6	54	21
"	34	1	7	55	22
#	35	2	8	56	23
$	36	3	9	57	24
%	37	4	:	58	25
&	38	5	;	59	26
'	39	6	<	60	27
(	40	7	=	61	28
)	41	8	>	62	29
*	42	9	?	63	30
+	43	10	@	64	31
,	44	11	A	65	32
-	45	12	B	66	33
.	46	13	C	67	34
/	47	14	D	68	35
0	48	15	E	69	36
1	49	16	F	70	37
2	50	17	G	71	38
3	51	18	H	72	39
4	52	19	I	73	40
5	53	20			

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90,0000%
20	1 in 100	99,0000%
30	1 in 1000	99,9000%
40	1 in 10,000	99,9900%
50	1 in 100,000	99,9990%
60	1 in 1,000,000	99,9999%

RESEARCHERS

RESEARCHERS

WORKFLOW

Transcriptomics

« Gene Expression »

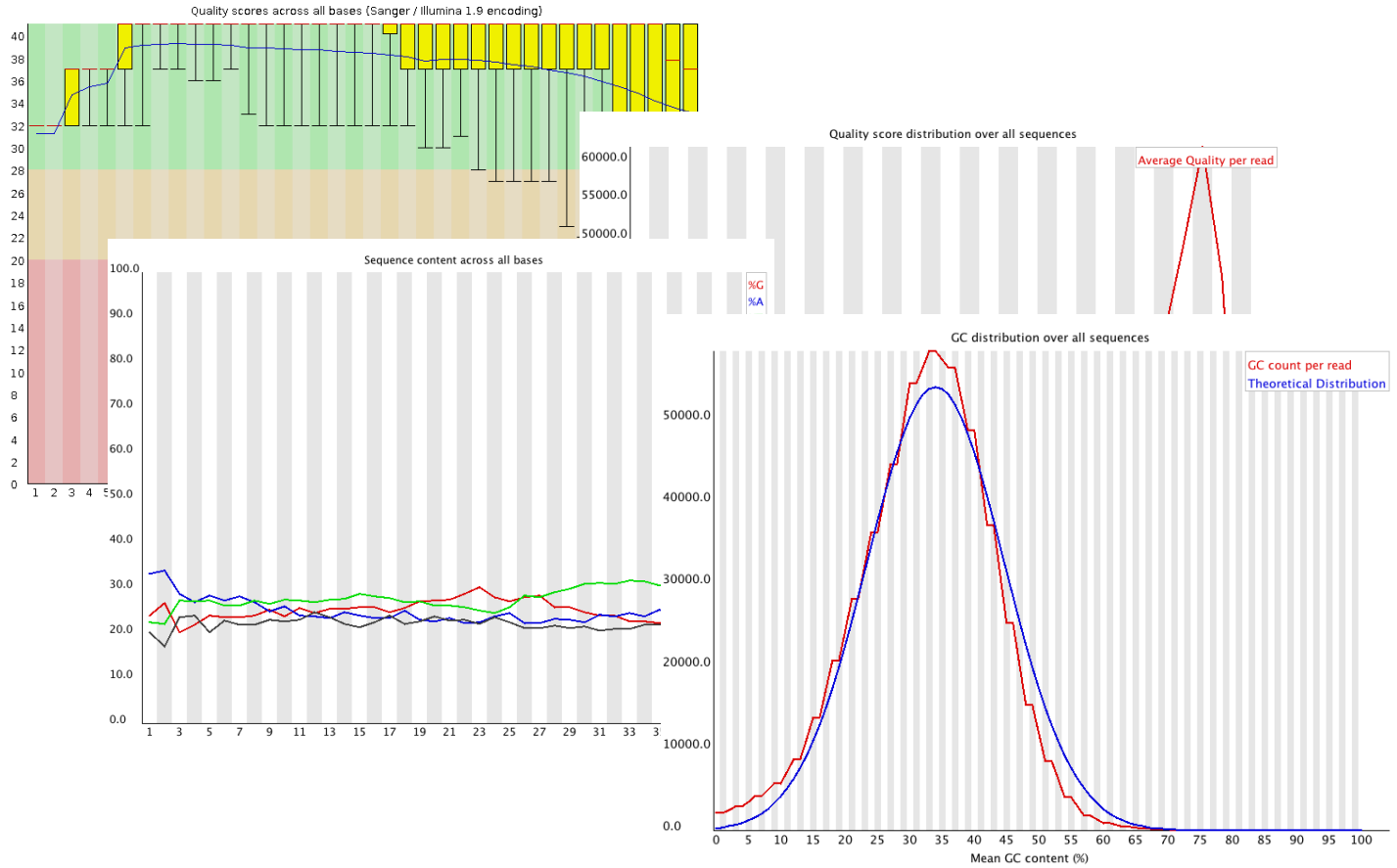
- QC Sequencing
- Mapping
- Quantification
- QC Mapping/Quantification

- Downstream Analysis
  - Clustering
  - Differential Expression

QC SEQUENCING

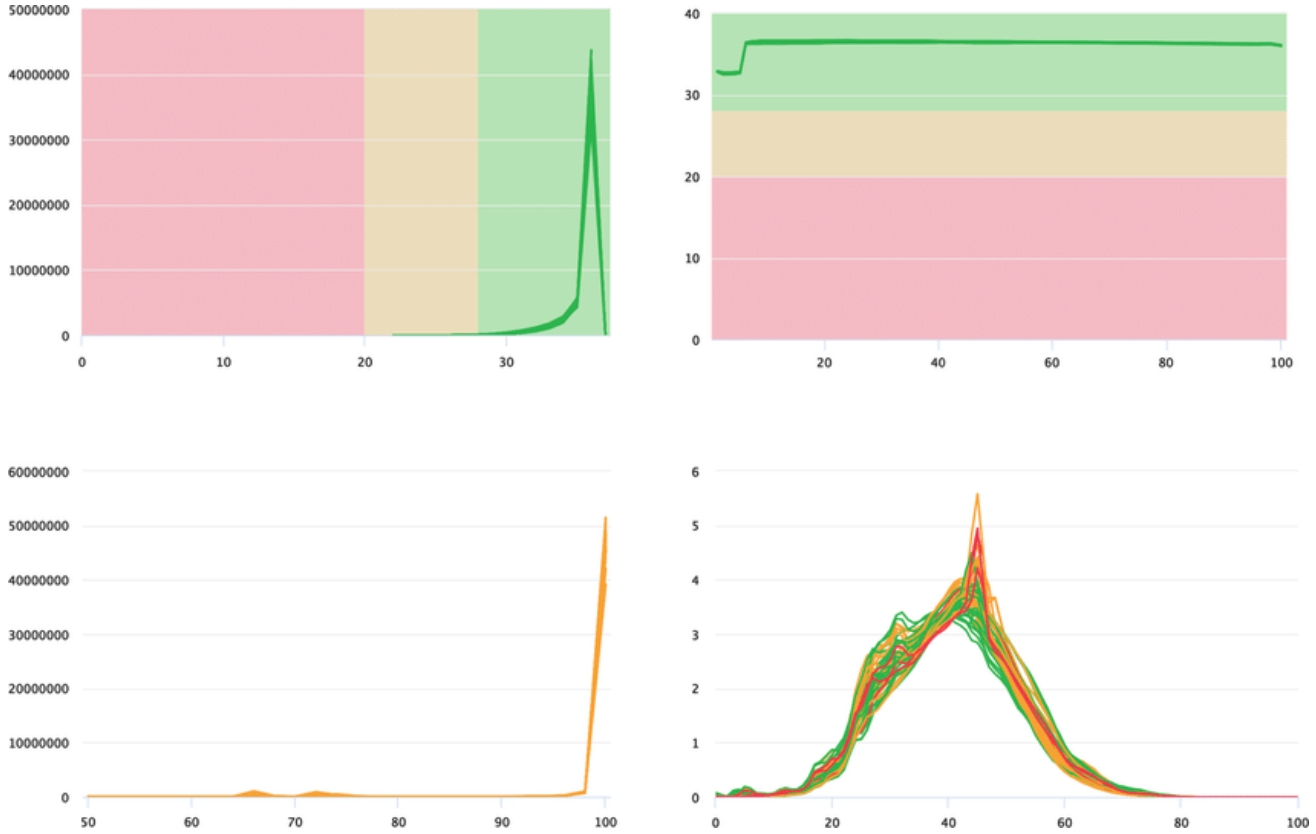
FastQC

- Number of reads
- Base calling quality
- Sequece quality
- GC content
- Sequence length
- Duplication levels
- Adapter content
- Overrepresented sequences
- …



QC SEQUENCING

MultiQC

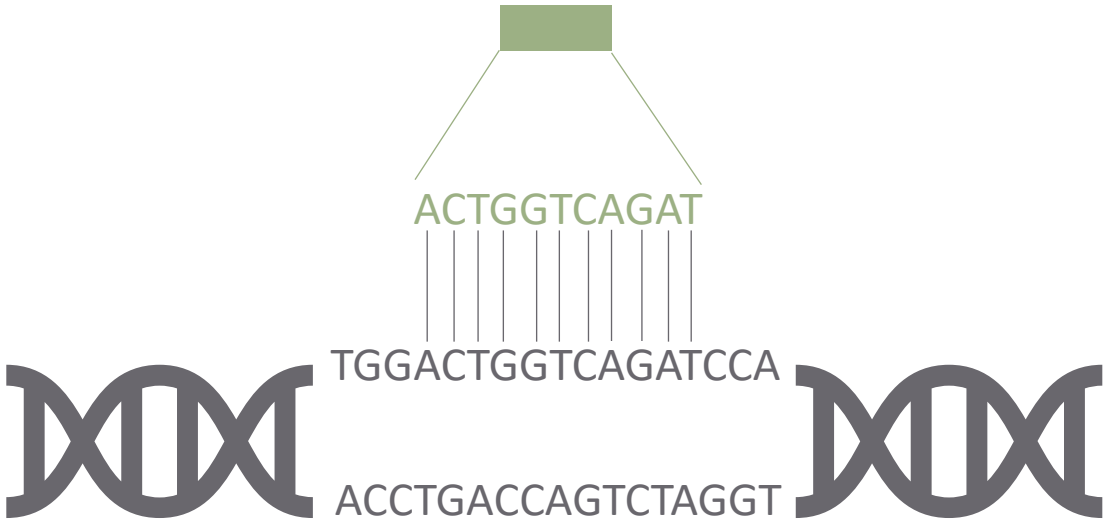


MAPPING

- Alignment
- Origin of reads

- Reference
  - Genome sequence
  - Gene set

- Database
  - Ensembl, UCSC, …



Ensembl

UCSC

REFERENCE

Genome (FASTA)

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CCCTAACCCCTAACCCTAACCCTAACCCTAACCCTAACCTAACCCTAACCCTAACCCTAAC
CCTAACCCTAACCCTAACCCTAACCCTAACCCCTAACCCTAACCCTAAACCCTAAACCCTA
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GGGCGGGGGGAGGGTGGCGCCGTGCACGCGCAGAAACTCACGTCACGGTGGCGCGG
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Gene Set (GTF)

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#!genome-version GRCh38
#!genome-date 2013-12
#!genome-build-accession NCBI:GCA_000001405.27
#!genebuild-last-updated 2019-03
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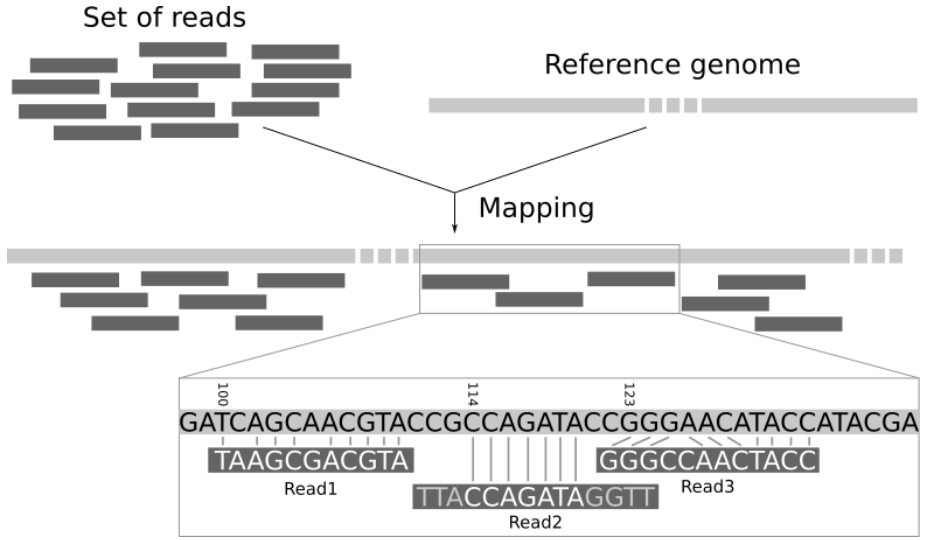
MAPPING

- Homo Sapiens
  - Genome
  - Gene Set
- Softwares
  - STAR
  - HISAT
  - …
- High RAM/CPUs



MAPPING

- Genome Indexing
  - Quick queries
- High RAM/CPUs

MAPPING

- SAM/BAM files
  - FLAG - Information
  - RNAME - Chromosome
  - POS – Location of 1st base
  - MAPQ – Quality score
  - CIGAR - Operations

Flag	Description
1	read is mapped
2	read is mapped as part of a pair
4	read is unmapped
8	mate is unmapped
16	read reverse strand
32	mate reverse strand
64	first in pair
128	second in pair
256	not primary alignment
512	read fails platform/vendor quality checks
1024	read is PCR or optical duplicate

Paired-End

A00801:76:HGJCYDSXY:4:1544:20401:36699 **99** **1** **3112677** **255** **150M** **=** **3112770** **244**
CTAGGAGATAGTAGGGATTGGGAAGCAACTACTGAAAGGTCTGTGTCTTCTTTGTGGATGATAAAATATTCTGGAATTATATTGTATGCTAGGCGCACAATCTTGTGACCATAGTACAGATATTCAACAGATAAATTTTGTGTGCTATGA
F:FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF:FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF:FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF:FFFFFFFFFF:FFFFF
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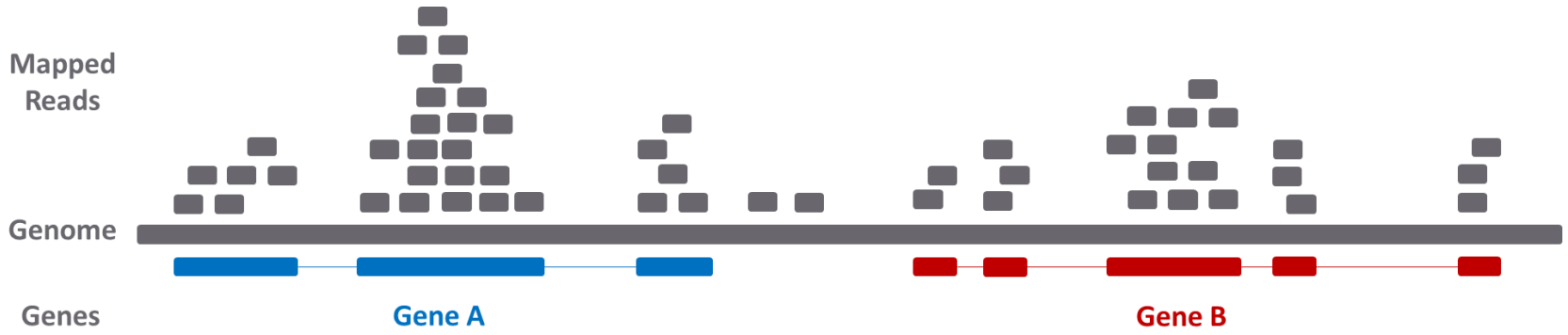
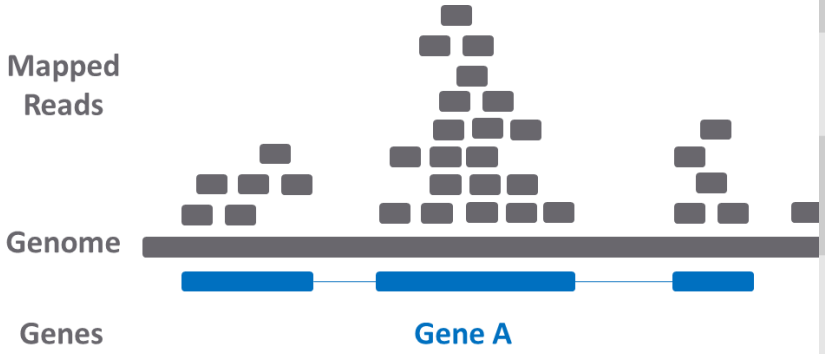
QUANTIFICATION (RNA)

- Gene Expression



QUANTIFICATION (RNA)

- Gene Expression



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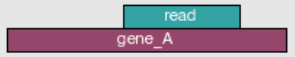
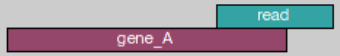
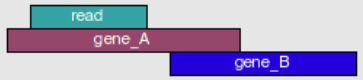
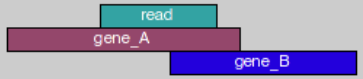
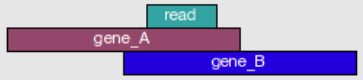
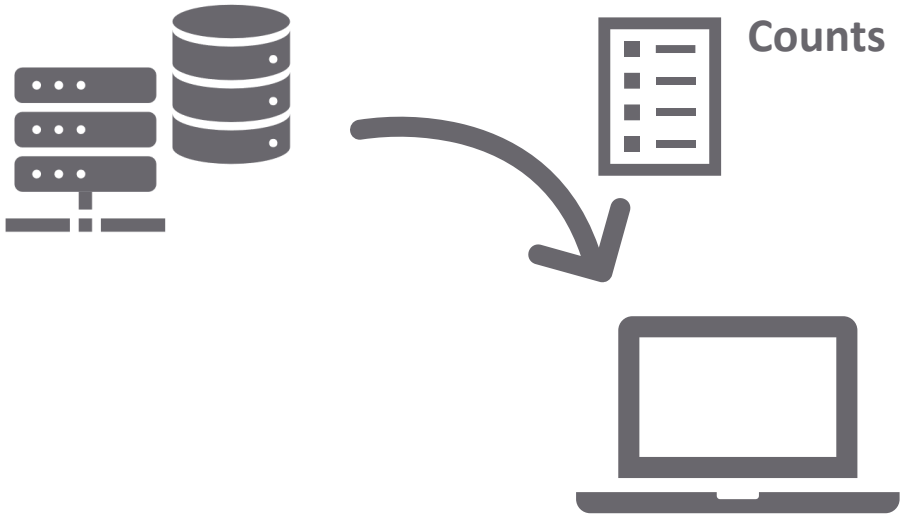
	gene_A	gene_A	gene_A
	gene_A	no_feature	gene_A
	gene_A	no_feature	gene_A
	gene_A	gene_A	gene_A
	gene_A	gene_A	gene_A
	ambiguous	gene_A	gene_A
	ambiguous	ambiguous	ambiguous

QUANTIFICATION (RNA)

- Gene Expression
- « Count matrix »
- Major output



Each column is a sample

Each row is a gene

GENE ID	KD.2	KD.3	OE.1	OE.2	OE.3	IR.1	IR.2	IR.3
1/2-SBSRNA4	57	41	64	55	38	45	31	39
A1BG	71	40	100	81	41	77	58	40
A1BG-AS1	256	177	220	189	107	213	172	126
A1CF	0	1	1	0	0	0	0	0
A2LD1	146	81	138	125	52	91	80	50
A2M	10	9	2	5	2	9	8	4
A2ML1	3	2	6	5	2	2	1	0
A2MP1	0	0	2	1	3	0	2	1
A4GALT	56	37	107	118	65	49	52	37
A4GNT	0	0	0	0	1	0	0	0
AA06	0	0	0	0	0	0	0	0
AAA1	0	0	1	0	0	0	0	0
AAAS	2288	1363	1753	1727	835	1672	1389	1121
AACS	1586	923	951	967	484	938	771	635
AACSP1	1	1	3	0	1	1	1	3
AADAC	0	0	0	0	0	0	0	0
AADACL2	0	0	0	0	0	0	0	0
AADACL3	0	0	0	0	0	0	0	0
AADACL4	0	0	1	1	0	0	0	0
AADAT	856	539	593	576	359	567	521	416
AAGAB	4648	2550	2648	2356	1481	3265	2790	2118
AAK1	2310	1384	1869	1602	980	1675	1614	1108
AAMP	5198	3081	3179	3137	1721	4061	3304	2623
AANAT	7	7	12	12	4	6	2	7
AARS	5570	3323	4782	4580	2473	3953	3339	2666

QC MAPPING/QUANTIFICATION

MultiQC

General Statistics

Copy table | Configure Columns | Plot | Showing 8/8 rows and 8/10 columns.

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned	% Trimmed	% Dups	% GC	M Seqs
SRR3192396	67.5%	71.9	93.7%	97.8	4.0%	78.9%	51%	104.4
SRR3192397	66.6%	63.0	94.7%	87.1	3.5%	77.2%	49%	92.0
SRR3192398	50.9%	36.5	88.2%	58.7	5.0%	55.3%	47%	66.6
SRR3192399	52.3%	42.3	88.2%	65.6	5.0%	57.4%	47%	74.3
SRR3192400	70.3%	63.4	77.3%	73.4	7.2%	74.1%	45%	94.9
SRR3192401	71.2%	63.8	76.4%	72.8	6.3%	76.3%	45%	95.2
SRR3192657	73.1%	67.1	91.2%	85.0	3.1%	82.2%	51%	93.1
SRR3192658	71.2%	66.9	89.7%	87.1	3.4%	82.3%	52%	97.1

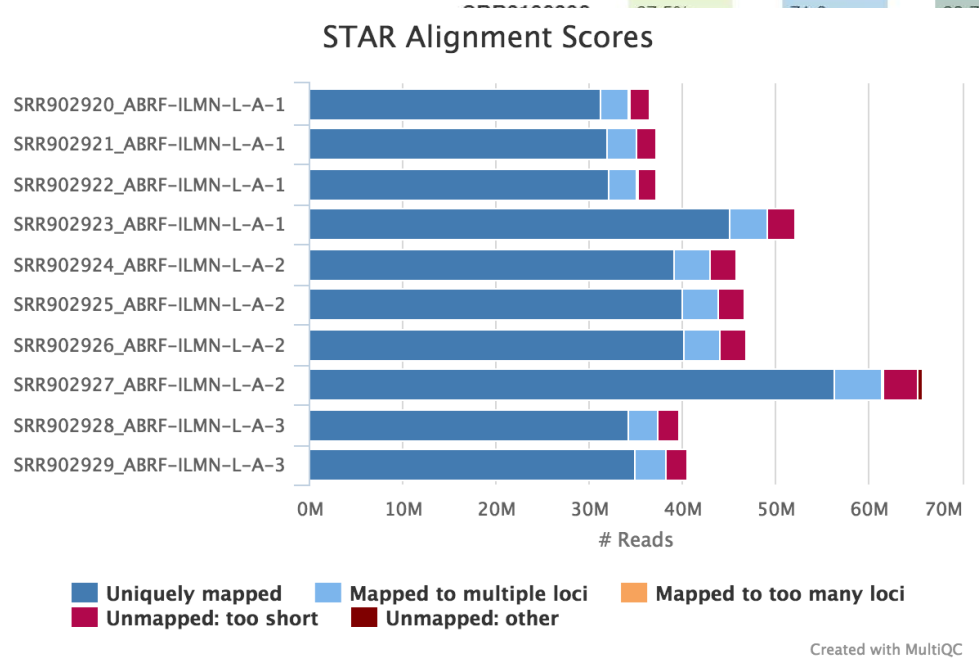
QC MAPPING/QUANTIFICATION

MultiQC

General Statistics

Copy table | Configure Columns | Plot | Showing 8/8 rows and 8/10 columns.

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned	% Trimmed	% Dups	% GC	M Seqs
			%	97.8	4.0%	78.9%	51%	104.4
			%	87.1	3.5%	77.2%	49%	92.0
			%	58.7	5.0%	55.3%	47%	66.6
			%	65.6	5.0%	57.4%	47%	74.3
			%	73.4	7.2%	74.1%	45%	94.9
			%	72.8	6.3%	76.3%	45%	95.2
			%	85.0	3.1%	82.2%	51%	93.1
			%	87.1	3.4%	82.3%	52%	97.1



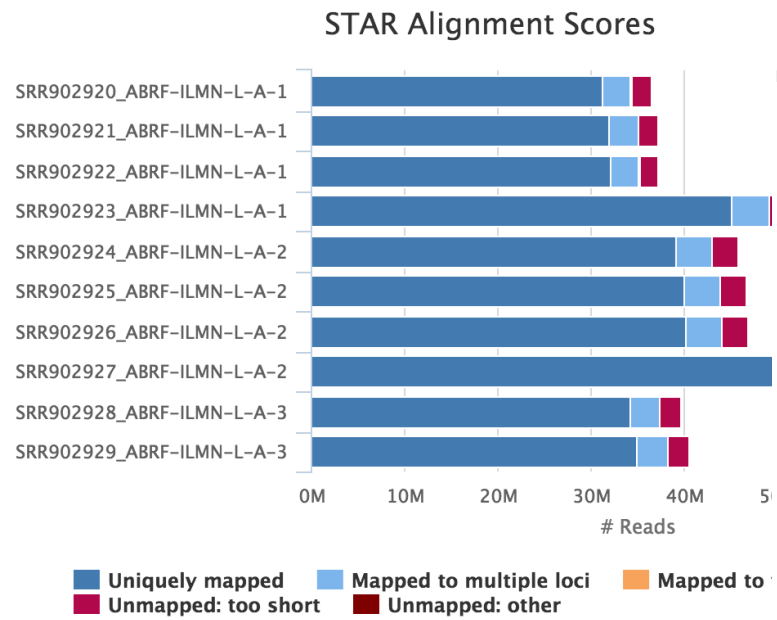
QC MAPPING/QUANTIFICATION

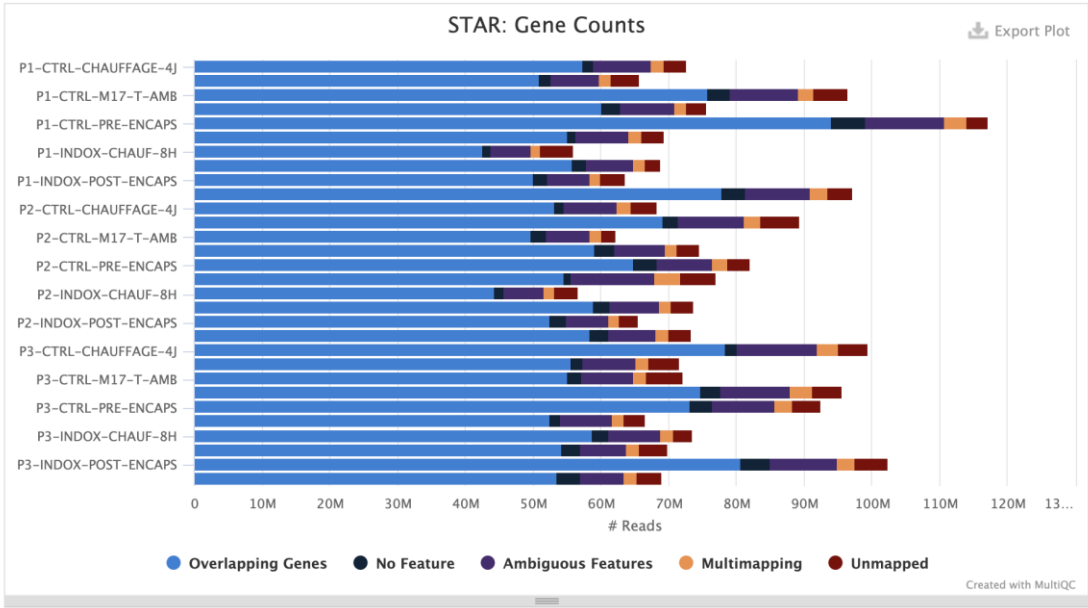
MultiQC

General Statistics

Copy table | Configure Columns | Plot | Showing 8/8 rows and 8/10 columns.

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned	% Trimmed	% Dups	% GC	M Seqs
			%	97.8	4.0%	78.9%	51%	104.4
			%	87.1	3.5%	77.2%	49%	92.0

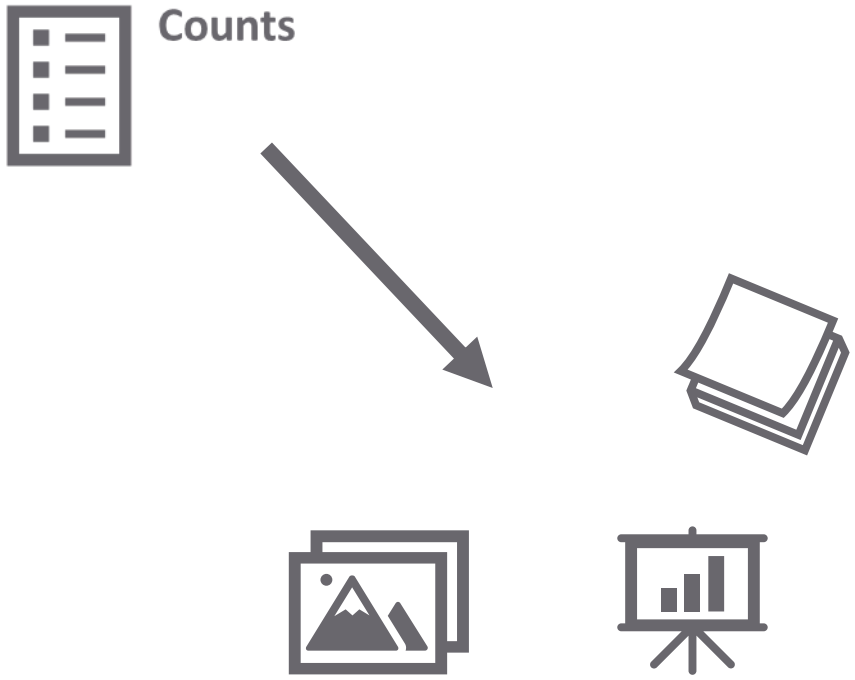




DOWNSTREAM ANALYSIS

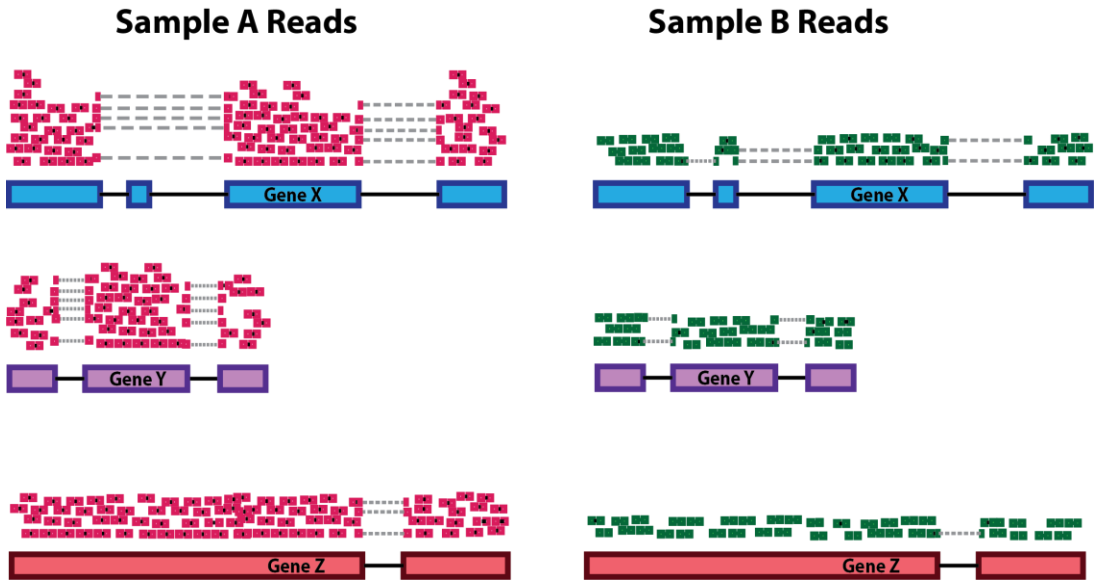
- Experimental Design

- R
  - DESeq2
  - EdgeR
  - Voom
  - ROTS
  - Limma
  - …



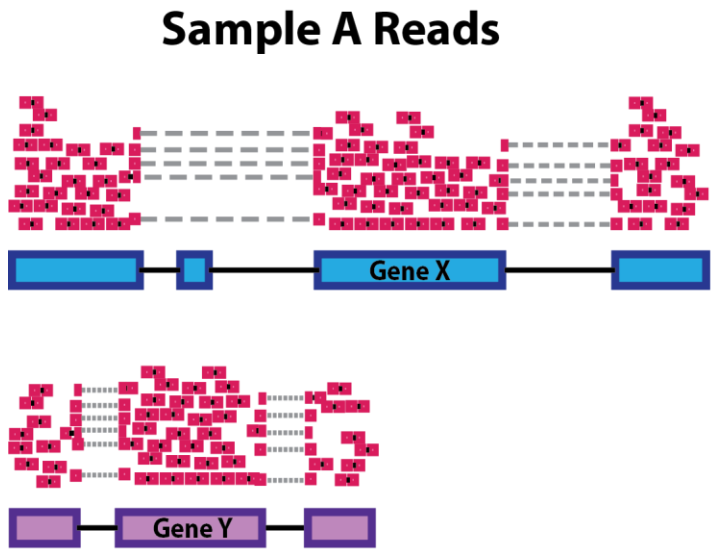
NORMALIZATION

- Scaling raw count values
  - Comparisons between genes and/or samples
- Factors to consider:
  - Sequencing depth



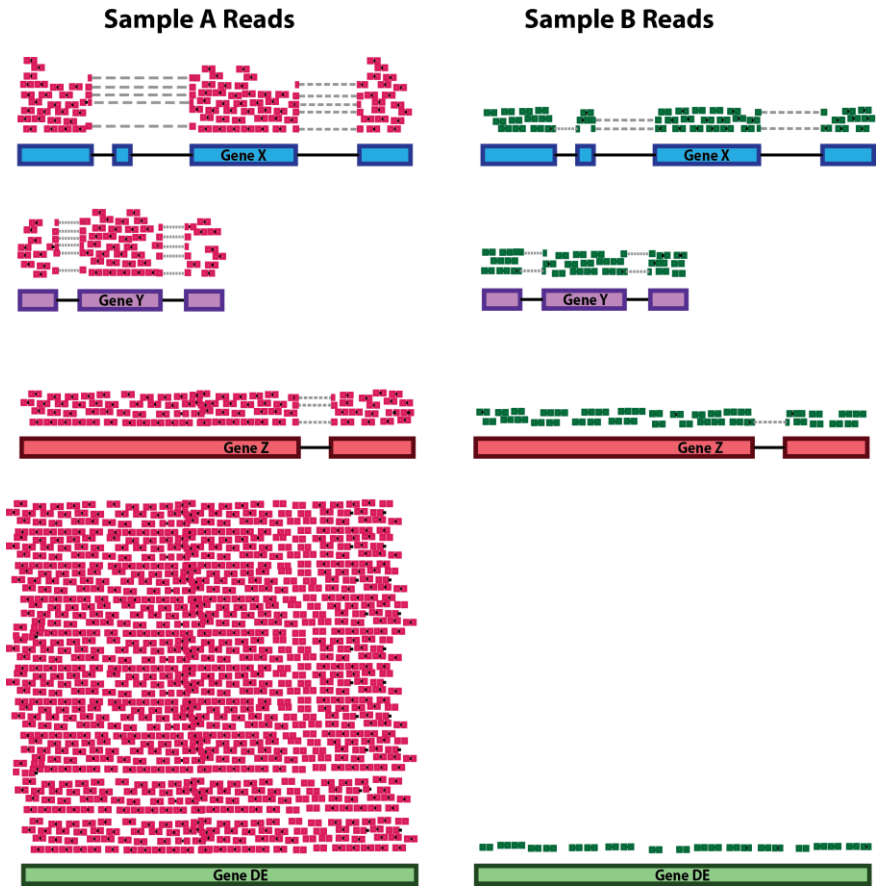
NORMALIZATION

- Scaling raw count values
  - Comparisons between genes and/or samples
- Factors to consider:
  - Sequencing depth
  - Gene length



NORMALIZATION

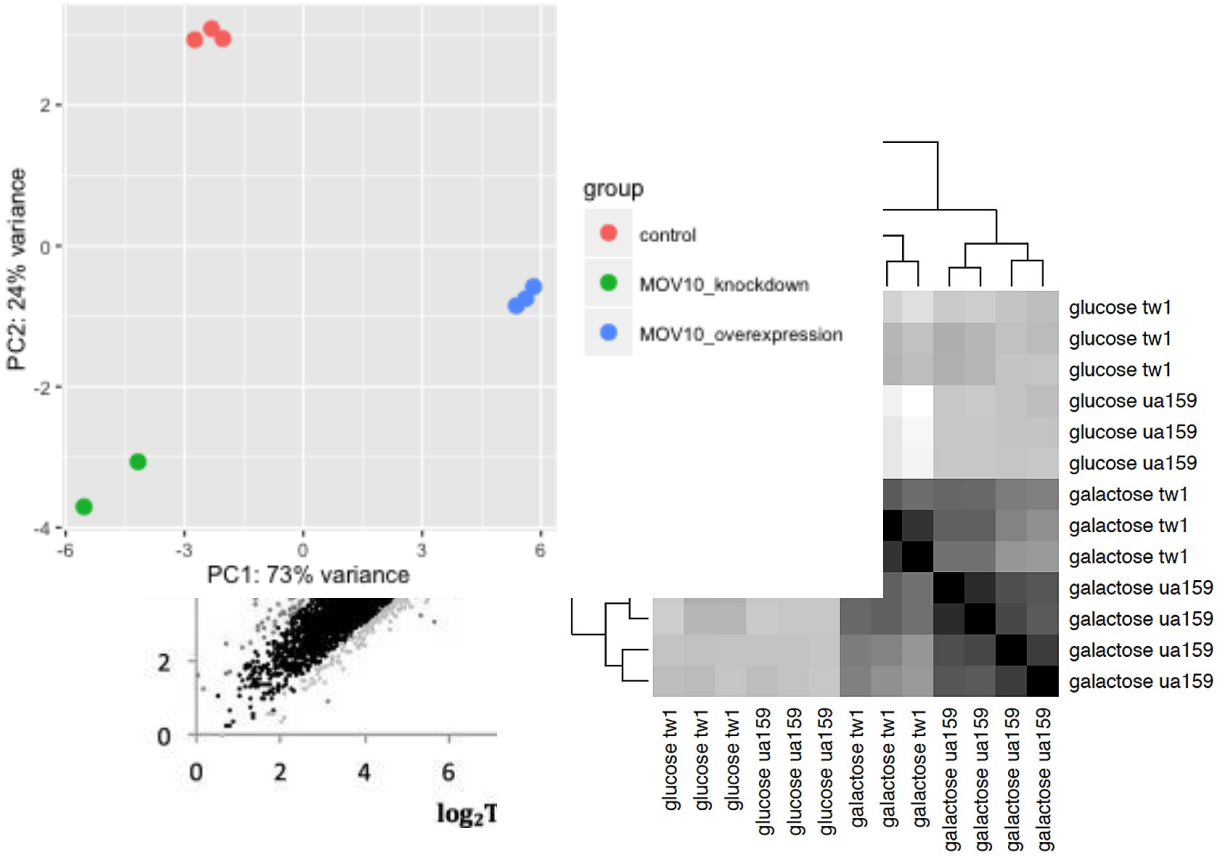
- Scaling raw count values
  - Comparisons between genes and/or samples
- Factors to consider:
  - Sequencing depth
  - Gene length
  - RNA composition
- Essential for DE analysis



Normalization method	Description	Accounted factors	Recommendations for use
CPM (counts per million)	Counts scaled by total number of reads	Sequencing depth	Gene count comparisons between replicates of the same samplegroup; **NOT for within sample comparisons or DE analysis**
TPM (transcripts per kilobase million)	Counts per length of transcript (kb) per million reads mapped	Sequencing depth and gene length	Gene count comparisons within A sample or between samples of the same sample group; **NOT for DE analysis**
RPKM/FPKM (reads/fragments per kilobase of exon per million reads/fragments mapped)	Counts per million reads mapped per length of transcript (kb)	Sequencing depth and gene length	Gene count comparisons between genes within A sample; **NOT for between sample comparisons or DE analysis**
Deseq2's **median of ratios**	Counts divided by sample-specific size factors determined by median ratio of gene counts relative to geometric mean per gene	Sequencing depth and RNA composition	Gene count comparisons between samples and for **DE analysis**; **NOT for within sample comparisons**
Edger's **trimmed mean of M values (TMM)**	Uses A weighted trimmed mean of the log expression ratios between samples	Sequencing depth, RNA composition, and gene length	Gene count comparisons between and within samples and for **DE analysis**

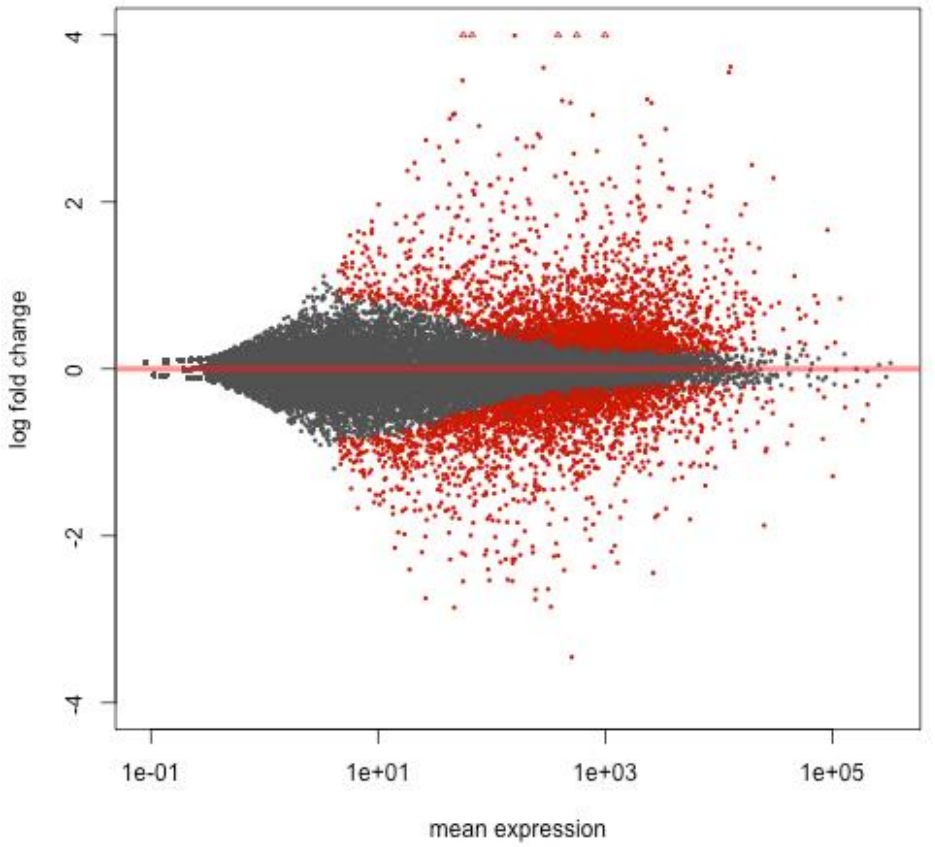
DOWNSTREAM ANALYSIS

- Experimental Design
- Clustering
  - Sample correlation
  - Hierarchical clustering
  - Principal Component Analysis



DOWNSTREAM ANALYSIS

- Experimental Design
- Clustering
  - Sample correlation
  - Euclidian distance
  - Principal Component Analysis
- Differential Expression Analysis
  - Pairwise comparisons
  - MA plot



DOWNSTREAM ANALYSIS

- Experimental Design
- Clustering
  - Sample correlation
  - Euclidian distance
  - Principal Component Analysis
- Differential Expression Analysis
  - Pairwise comparisons
  - MA plot
  - DE genes

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
PAX5	1531,91362	6,1280938	0.14505696	28,091789	1,23E-173	1,59E-169
SOX9	348,04912	3,5537120	0.15748166	20,475861	3,53E-93	2,27E-89
PDX1	830,75570	-1,8973788	0.12438094	-15,203018	3,38E-52	1,45E-48
ISL1	655,25202	-1,9729198	0.13344796	-14,715372	5,14E-49	1,65E-45
ARX	526,74210	2,2554297	0.15754888	14,227414	6,19E-46	1,59E-42

Summary

out of 21769 with nonzero total read count
adjusted p-value < 0.1
LFC > 0 (up) : 985, 4.5%
LFC < 0 (down) : 929, 4.3%
outliers [1] : 0, 0%
low counts [2] : 8914, 41%
(mean count < 8)
[1] see 'cooksCutoff' argument of ?results
[2] see 'independentFiltering' argument of ?results

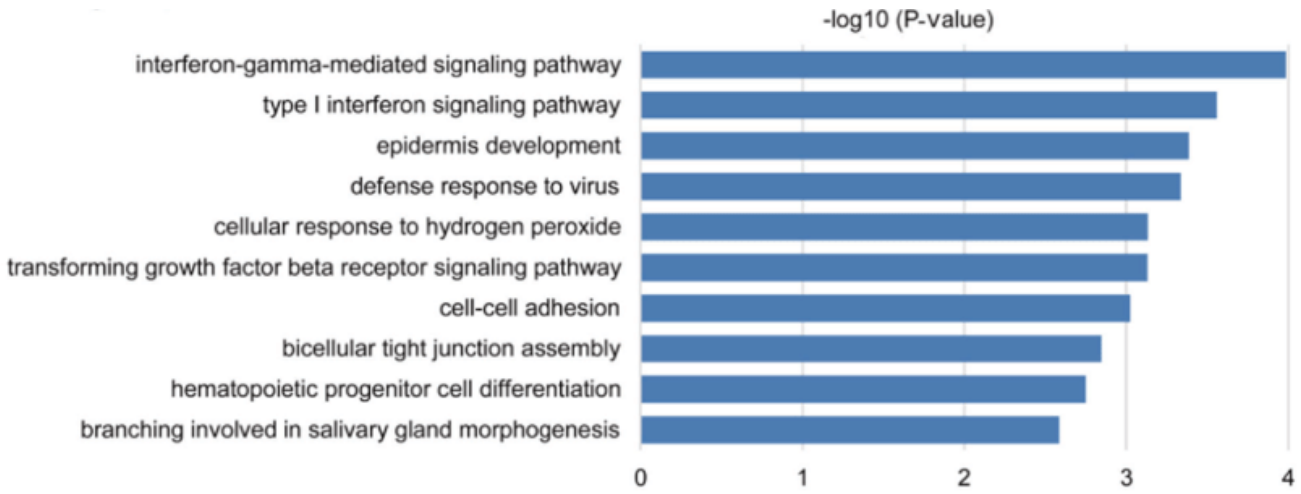
MULTIPLE TEST CORRECTION

- P-value
  - $p < 0,05$
  - 5% chance of false positive
  - One test per gene
  - 20.000 genes -> 1000 genes by chance

- P-value adjusted
  - **FDR/Benjamini-Hochberg:** (default in DESeq2) it ranks the genes by p-value, then multiply each ranked p-value by “total number of tests”/rank
  - Bonferroni: p-value * « total number of tests »
  - Q-value / Storey method: « The minimum FDR that can be attained when calling that feature significant »

DOWNSTREAM ANALYSIS

- Biological meaning
- Gene ontology / Gene Set Enrichment Analysis
  - GSEA
  - Enrichr
  - GOrilla
  - PANTHER
  - …



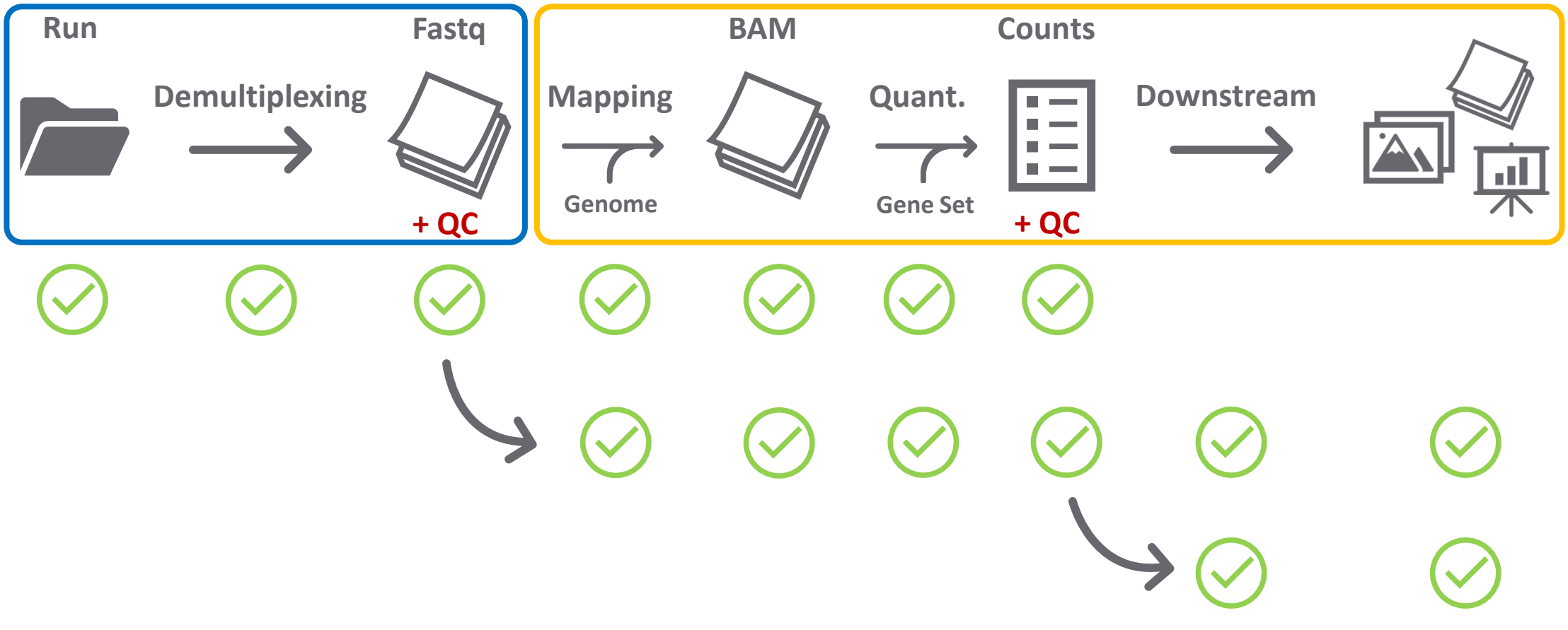
GSEA
Gene Set Enrichment Analysis

Enrichr

GORILLA
Gene Ontology enRIchment anaLysis and visuaLizAtion tool

PANTHER
Classification System

SUMMARY


Run
Fastq
Demultiplexing
+ QC
BAM
Counts
Mapping
Genome
Quant.
Gene Set
+ QC
Downstream

REPRODUCTIBILITY

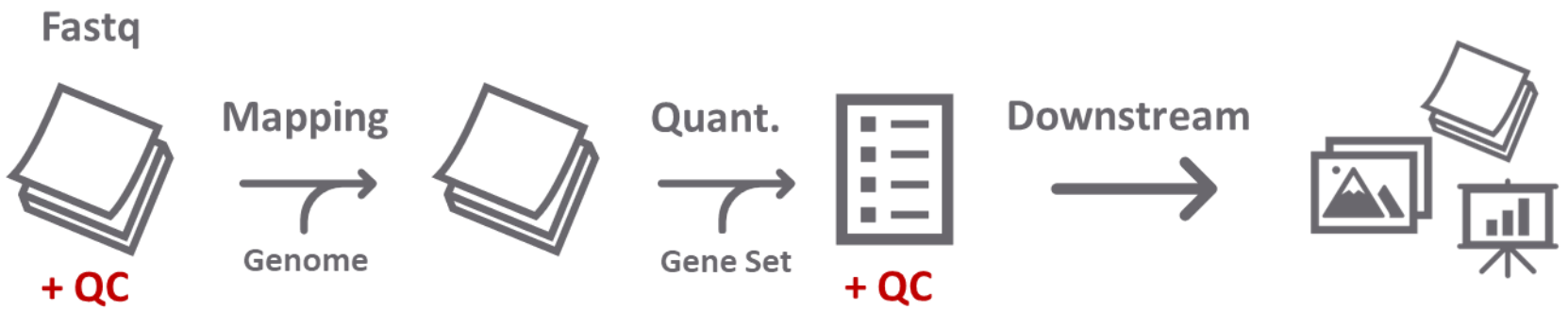
PIPELINES & CONTAINERS

REPRODUCTIBILITY

- Pipelines
  - Set of successive actions
    - Softwares
    - Parameters
    - References

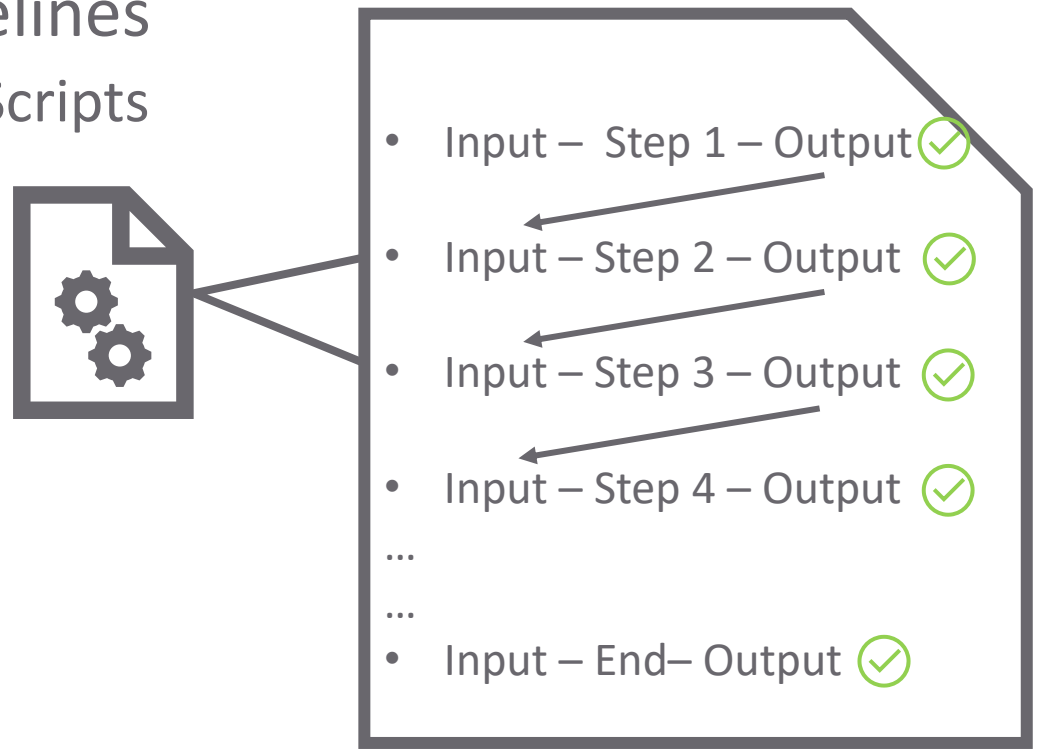
nextflow

Snakemake



REPRODUCTIBILITY

- Pipelines
  - Scripts

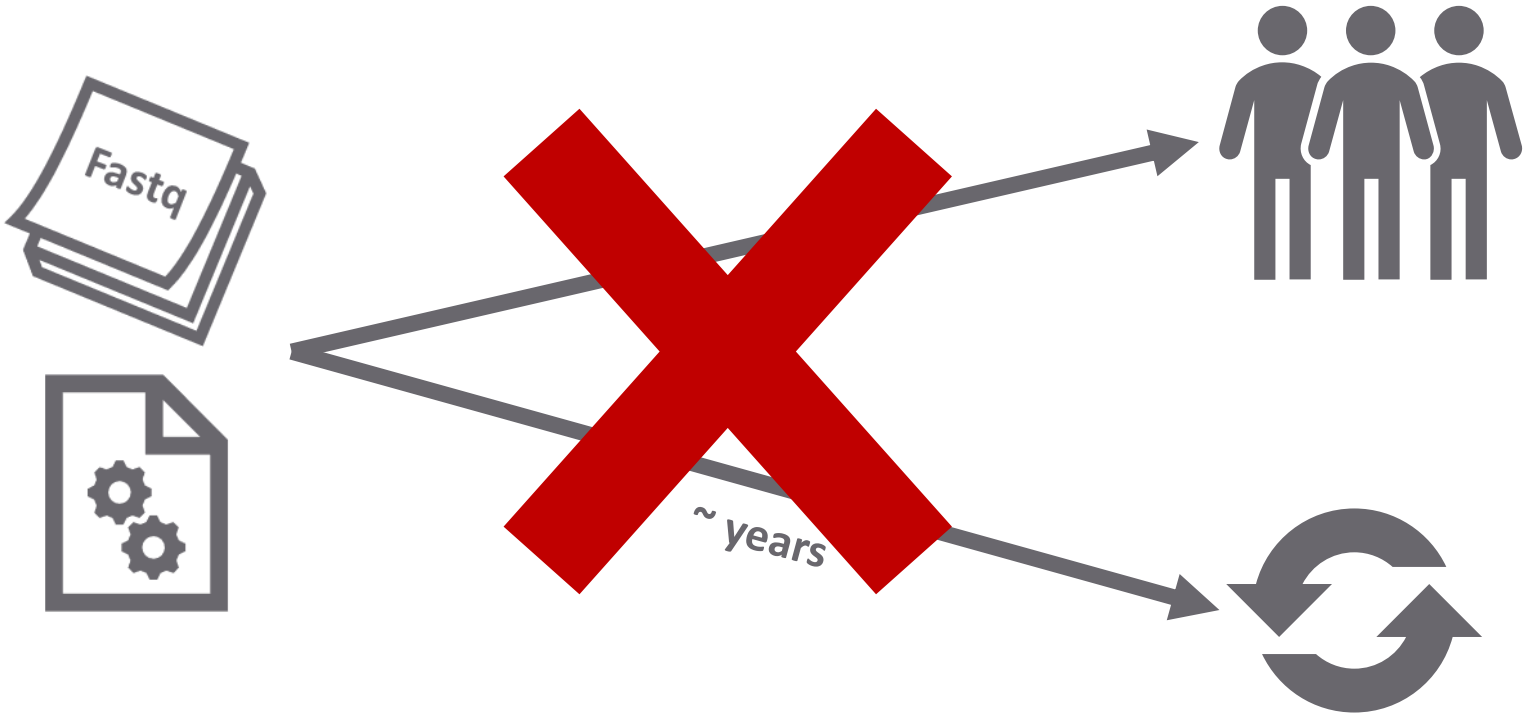


nextflow

Snakemake

nf-core

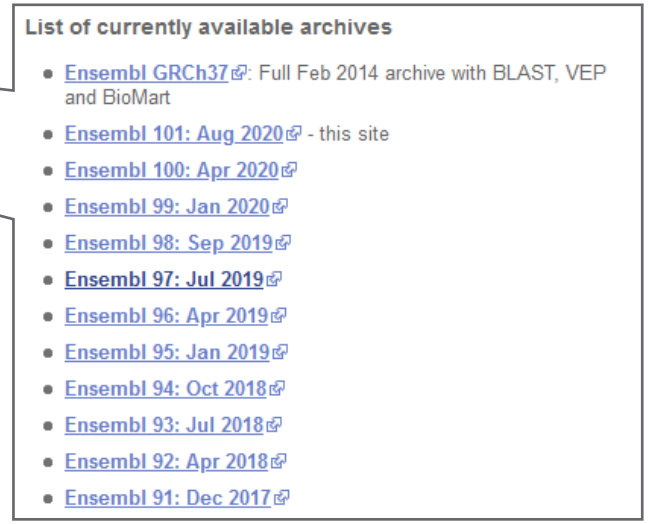
REPRODUCTIBILITY



REPRODUCTIBILITY

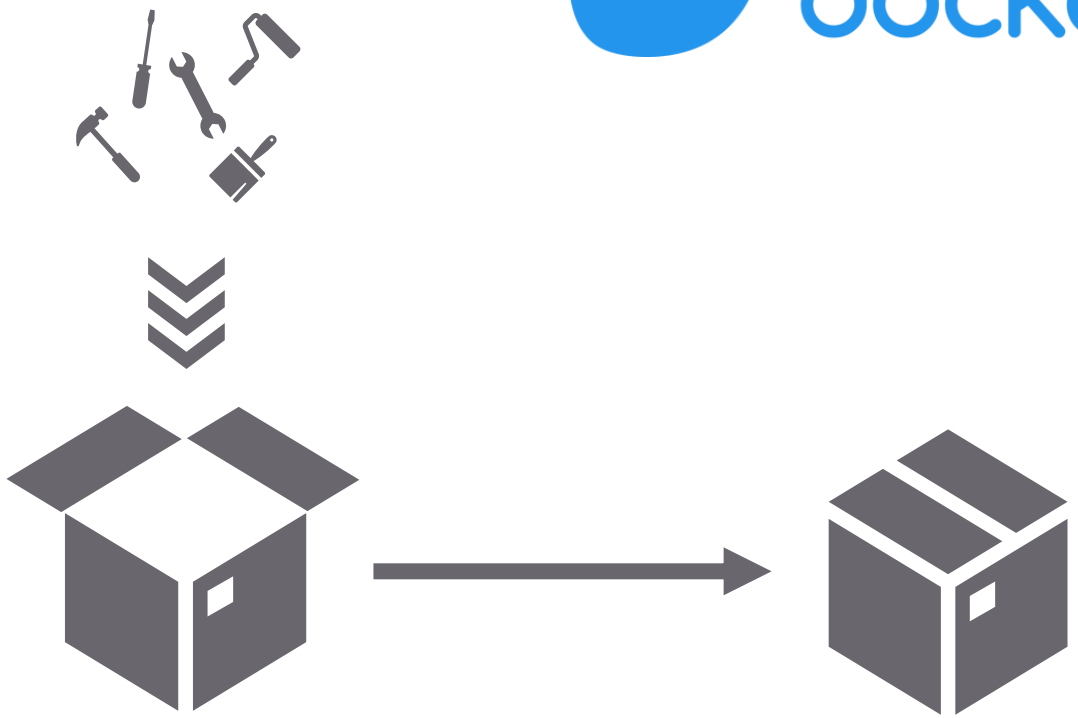
- Variability
  - Updates / Versioning
    - Softwares
    - References
  - Compatibility
  - Format
  - Knowledge

STAR 2.7.5b - 2020/08/01
STAR 2.7.5c - 2020/08/16
STAR 2.7.6a - 2020/09/19

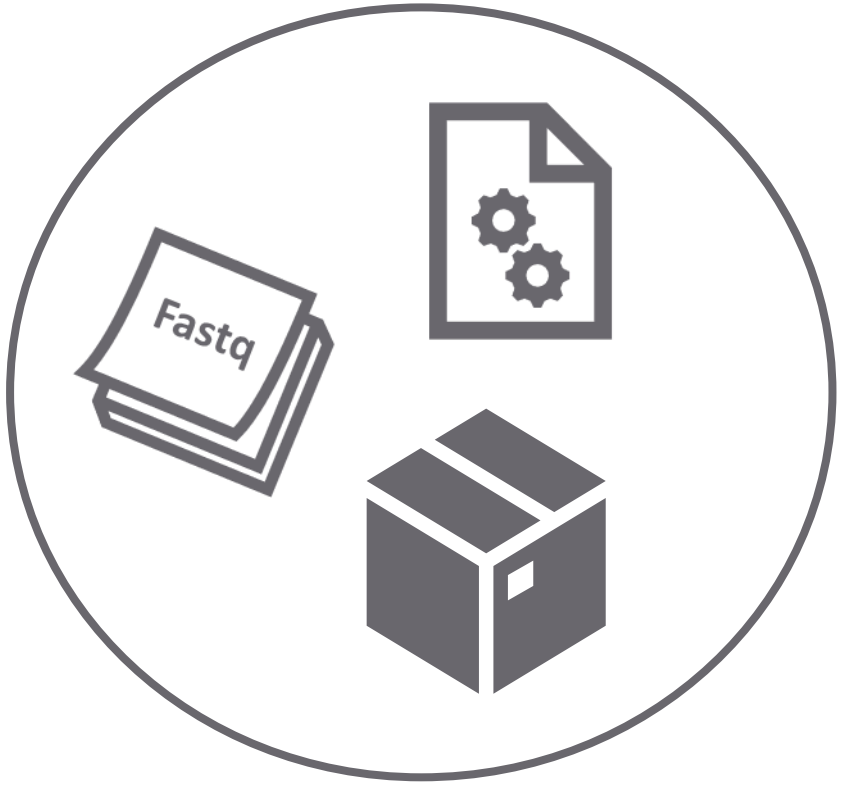


REPRODUCTIBILITY

- CONTAINERS
  - Docker
  - Singularity
- Softwares
  - Versions



REPRODUCTIBILITY



DATA DEPOSITORY

- Gene Expression Omnibus (NCBI)
- ArrayExpress (EMBL-EBI)

THANK YOU FOR YOUR ATTENTION