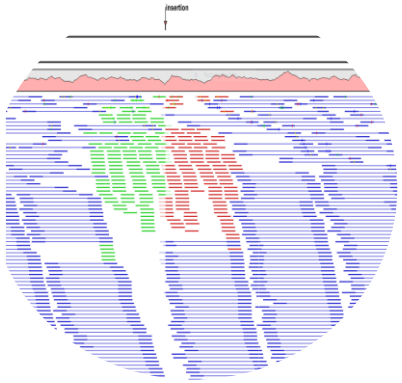
PMB2023

PATHOGEN MULTIOMICS AND BIOINFORMATICS

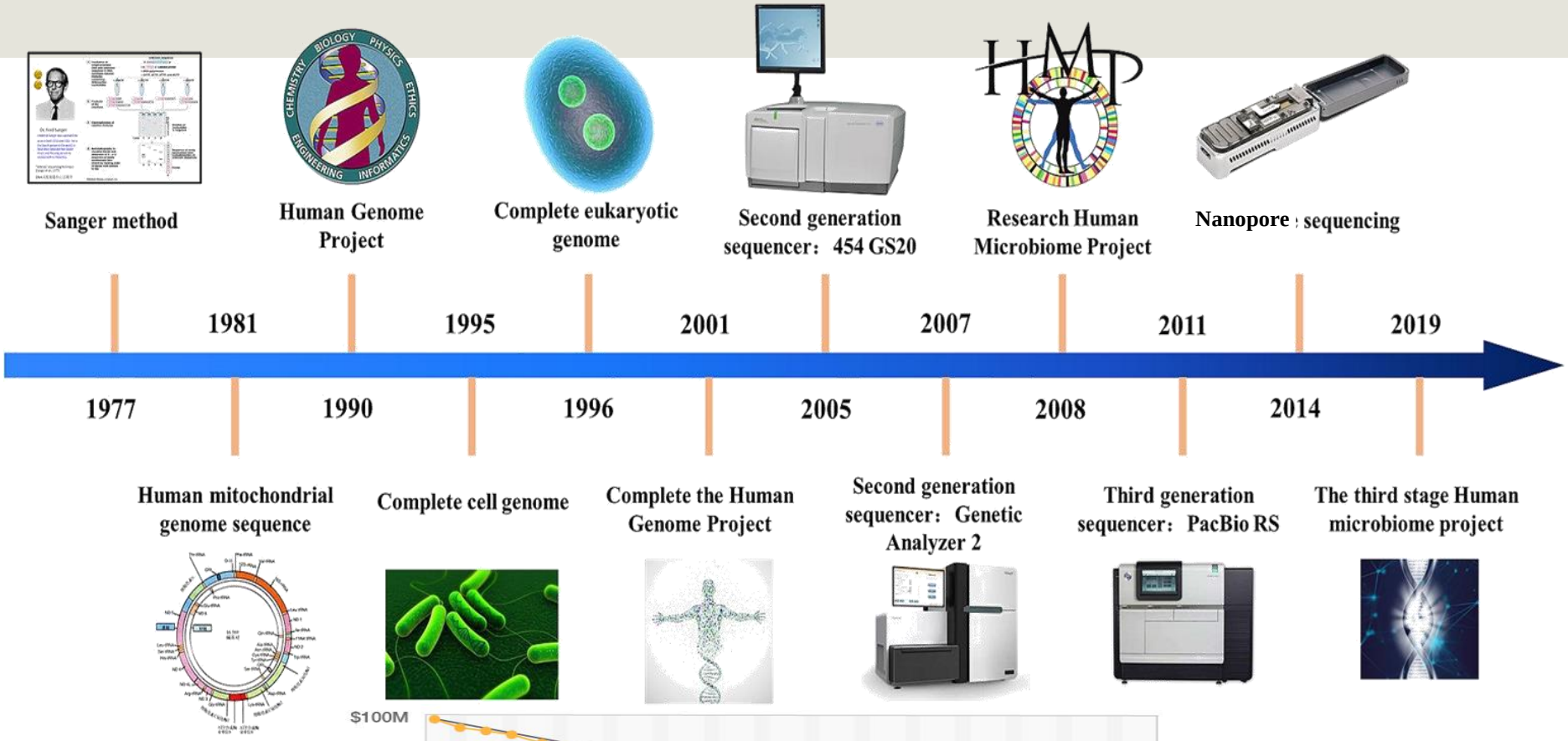
Lisbon 2023

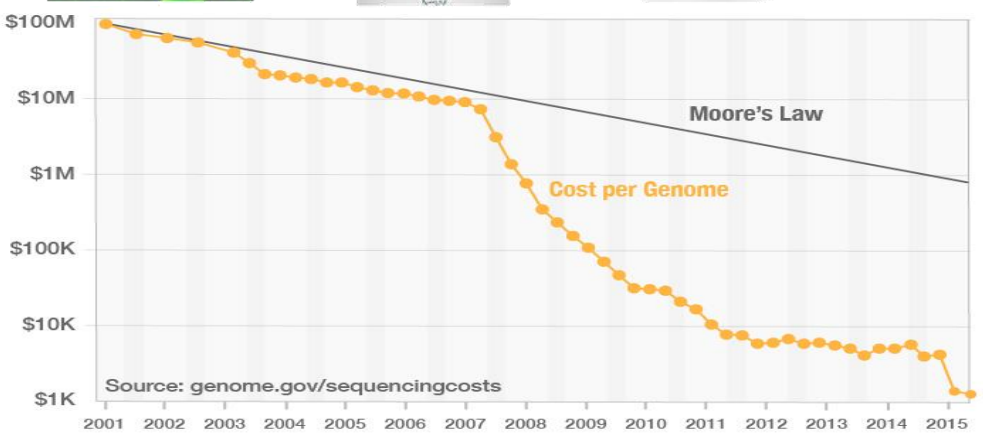
Module 1: Mapping Sequence Data



João Perdigão

Sequencing through time…





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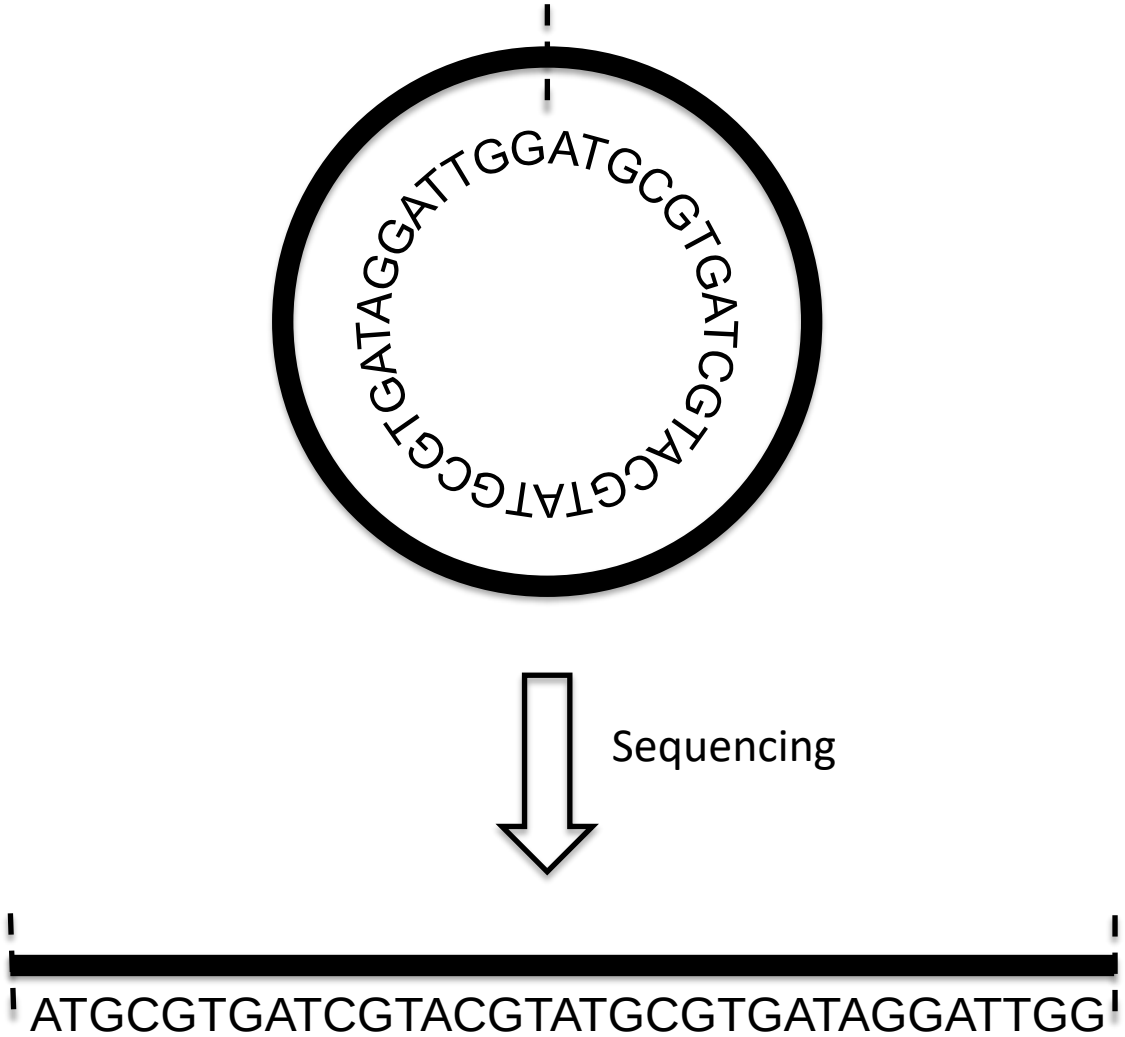
Genome Sequencing: Ideal situation…



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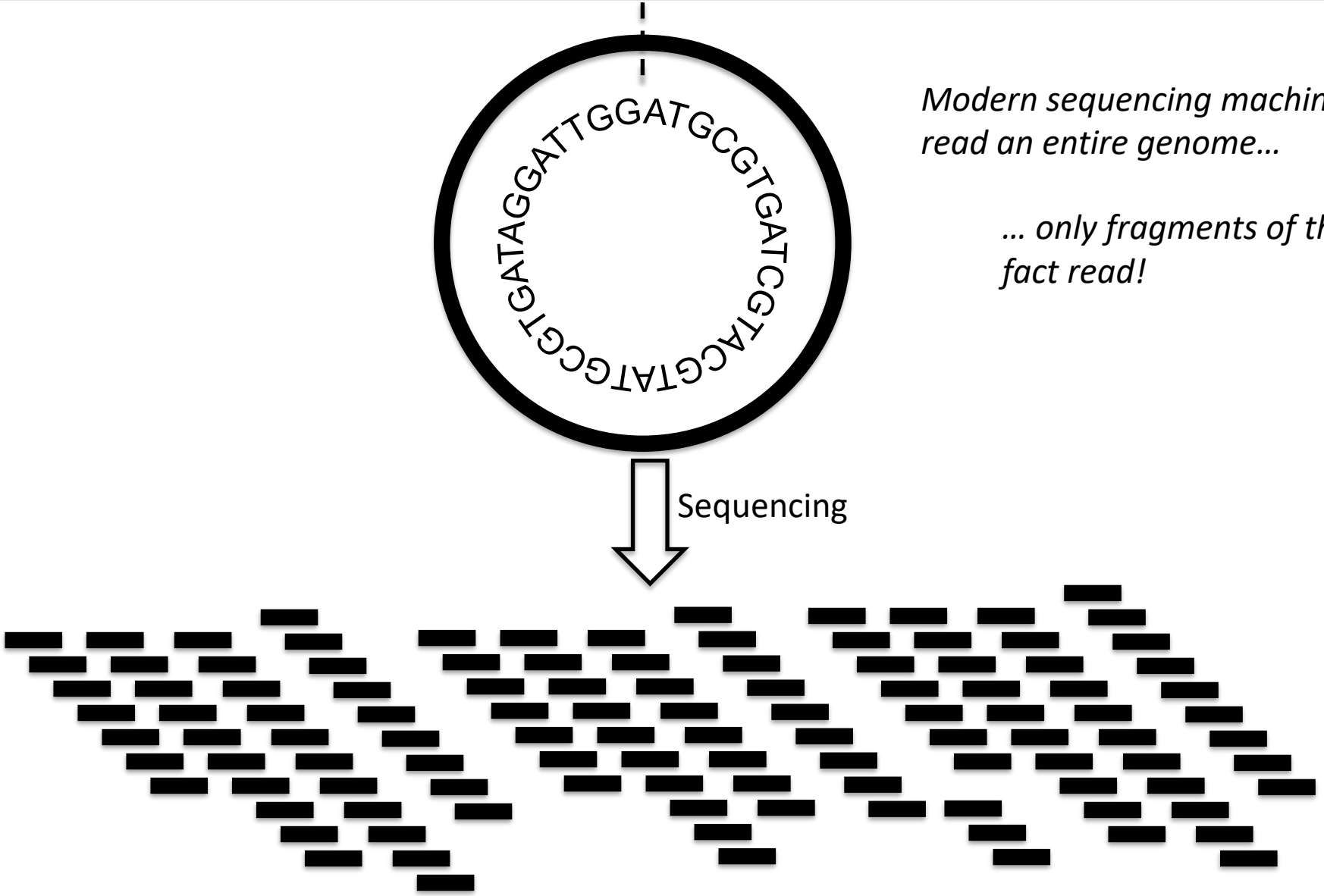
Genome Sequencing: the hard reality…



Modern sequencing machines cannot read an entire genome…

… only fragments of the genome are in fact read!

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NGS Platforms: An overview …

	illumina®	ion torrent	PACIFIC BIOSCIENCES®	Oxford NANOPORE Technologies
Read Length (bp)	50-300	200-400	10000-40000	1Mbp
Output (Gb)	6000	0,05-1	0,5-1	5-40
Cost / Million bp (USD)	0,05-0,15	1	0,13-0,60	variable
Accuracy	99.9%	99.6%	87%	92-97%
Time per run	1-11d	2h	30min-20h	1min-48h

Sanger Cost per MB: 2400USD

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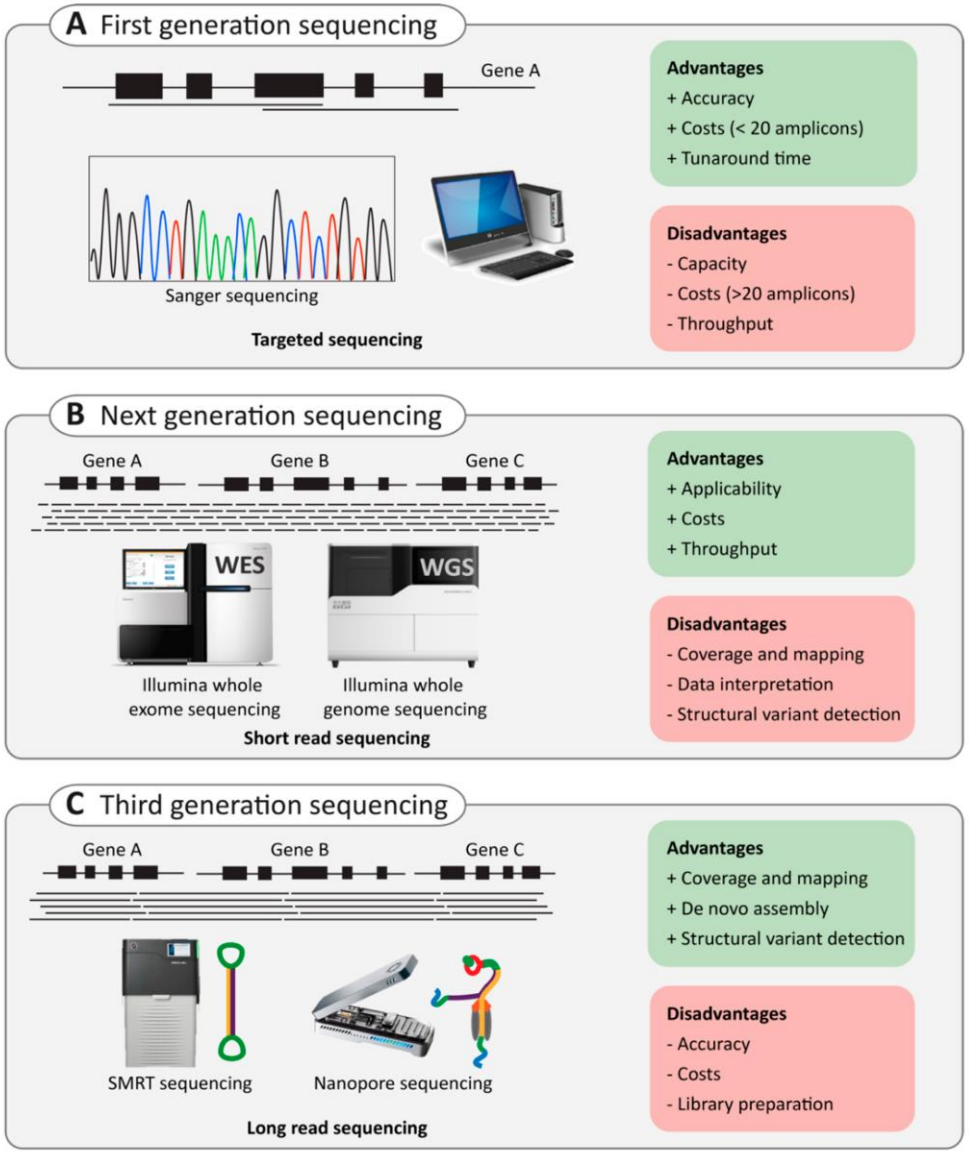
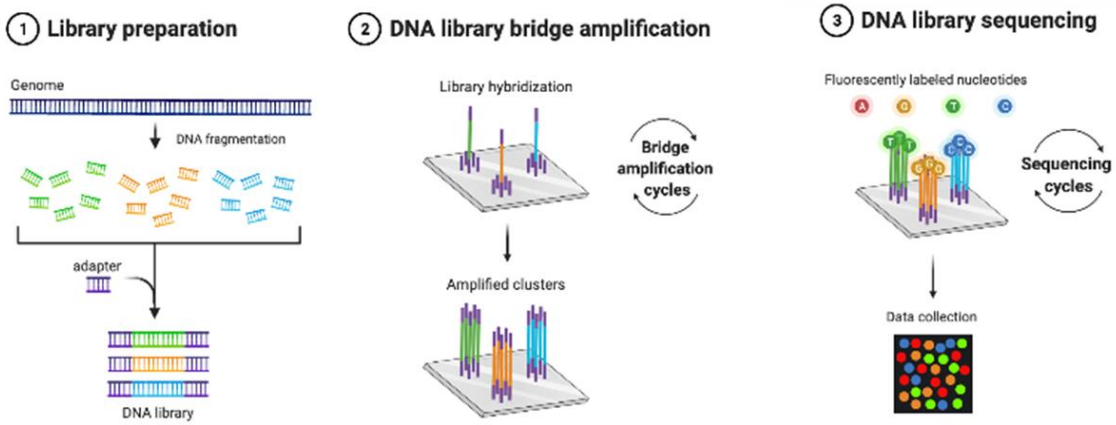
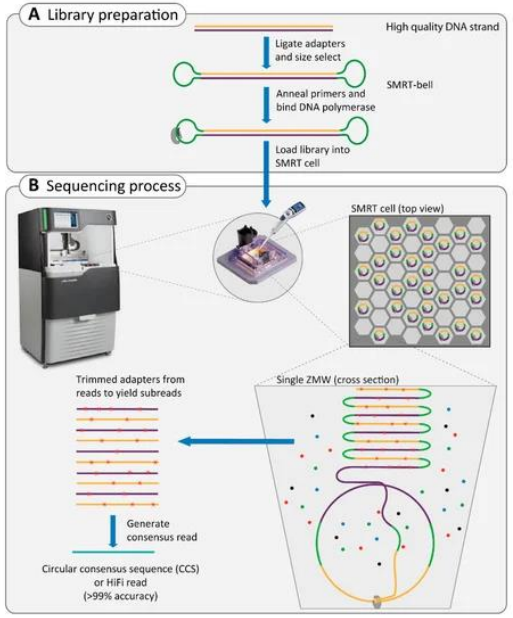
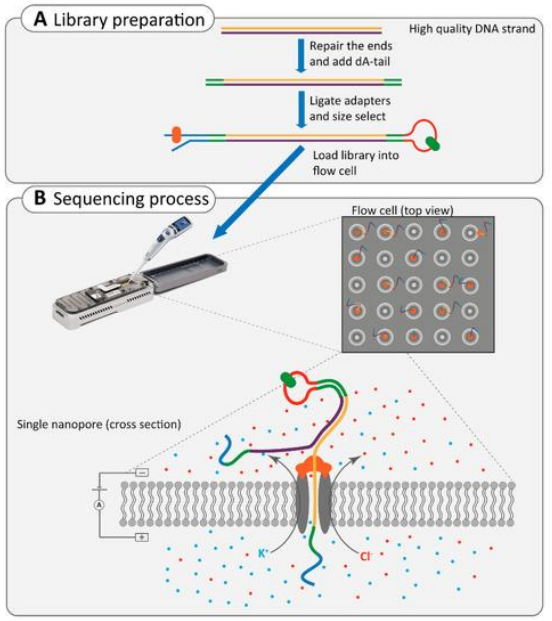
Next Generation Sequencing









de Bruijn et al 2021

Illumina: Sequencing-by-synthesis

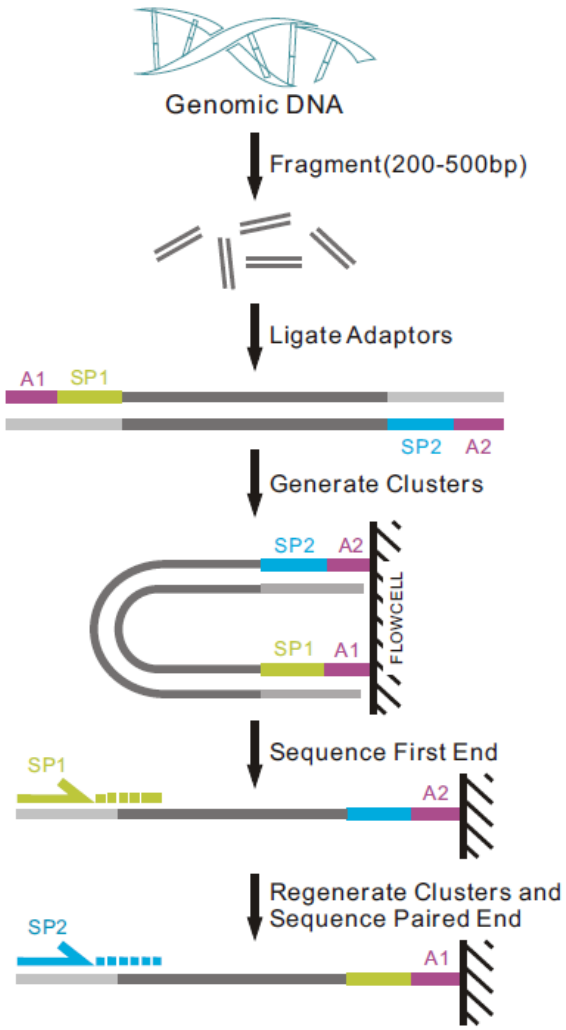
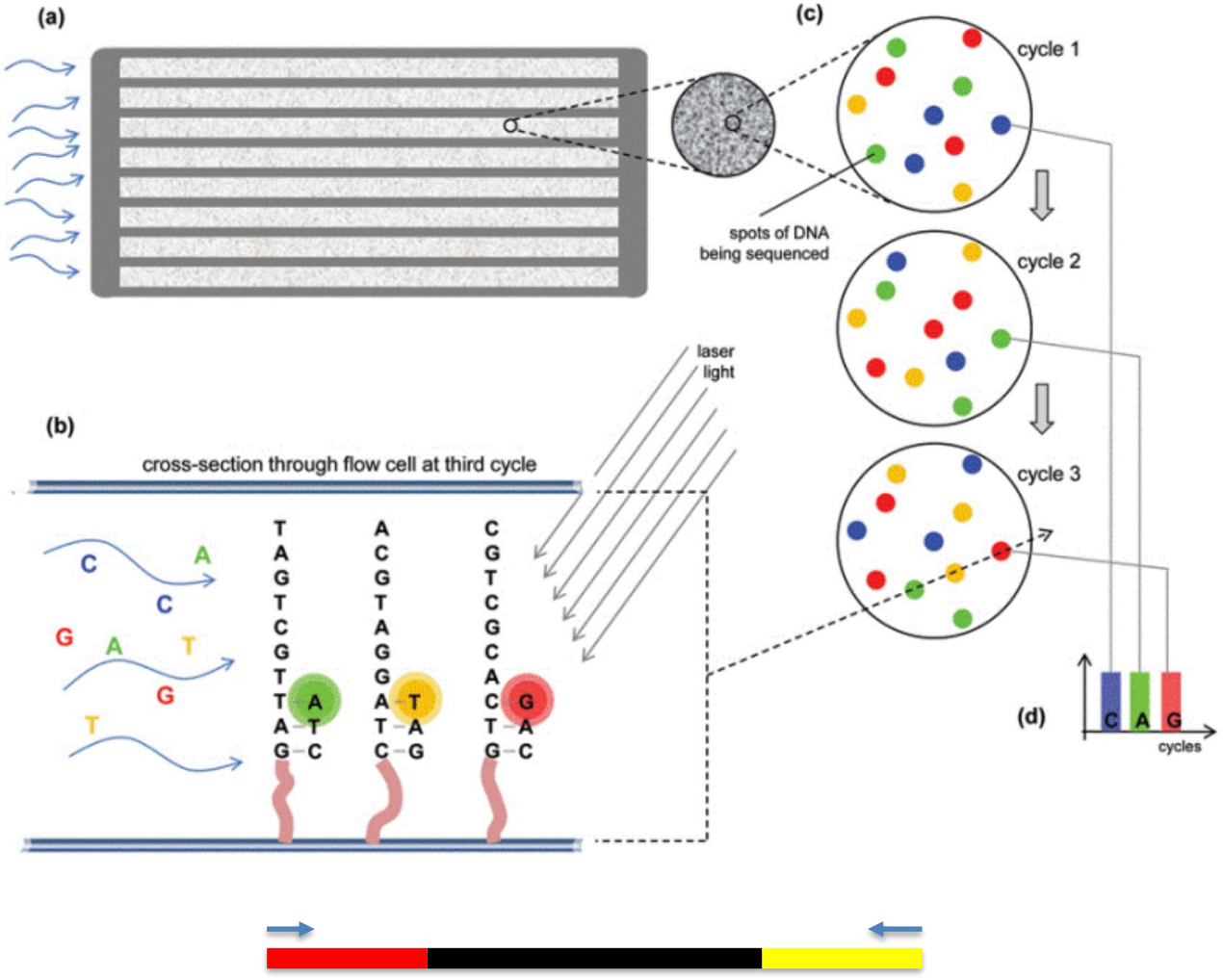


Figure 1-2-1 Pipeline of paired-end sequencing (www.illumina.com)



Read 1 Read 2

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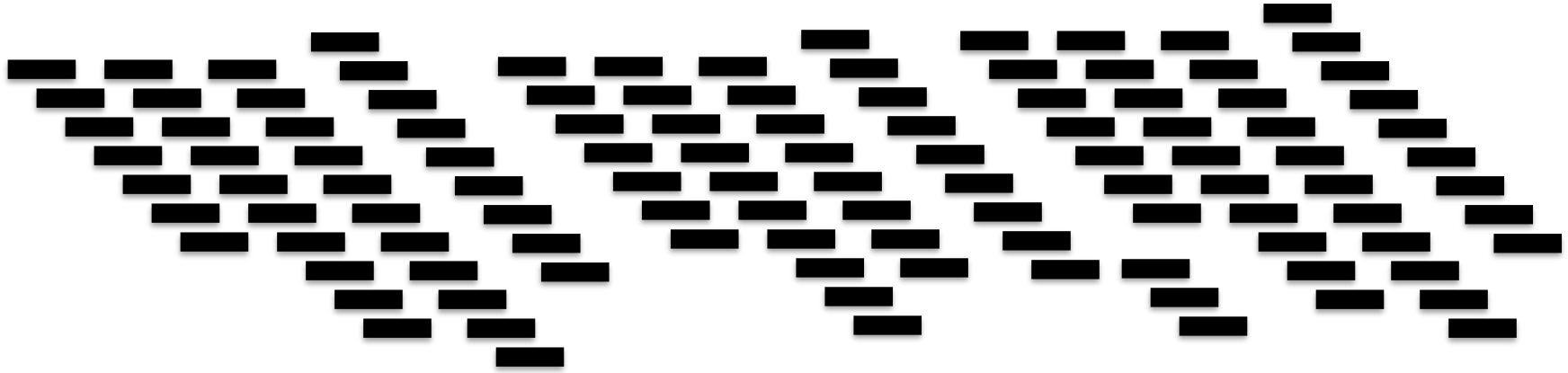
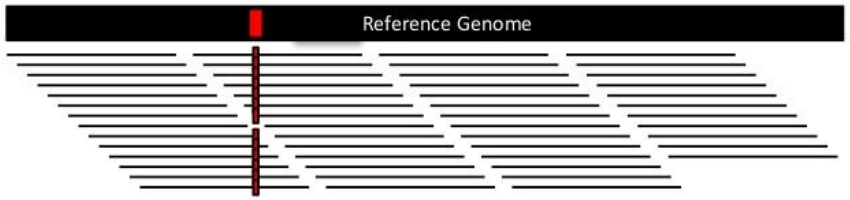
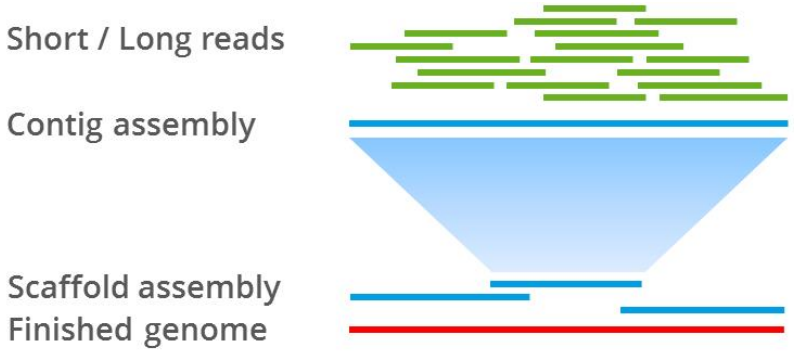
Two main approaches for handling reads…



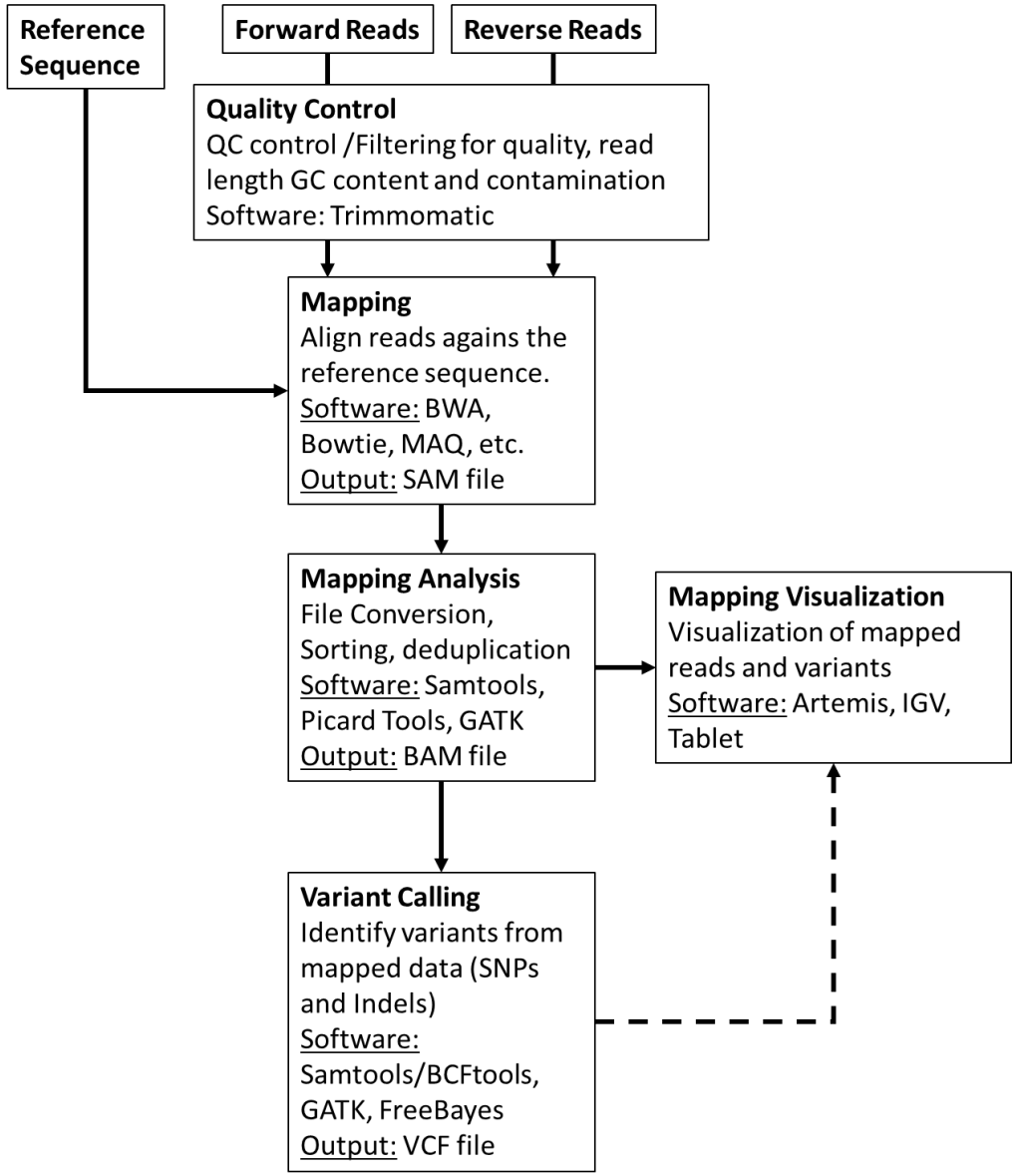
Mapping or Reference Assembly



De novo Assembly



Workflow



Four main analytical stages:

- *Quality-control* – filter out reads/bases associated with poor basecall quality;
- *Mapping* – map reads to a reference genome, obtain sample coverage at each position and read coordinates;
- *Variant Calling* – identify variants existing between sequenced and reference genome, either SNPs or INDELs;
- *Functional Annotation – determine the functional impact of each variant, e.g., which gene is affected? Is the mutation synonymous or non-synonymous? impact at the peptide primary structure?*

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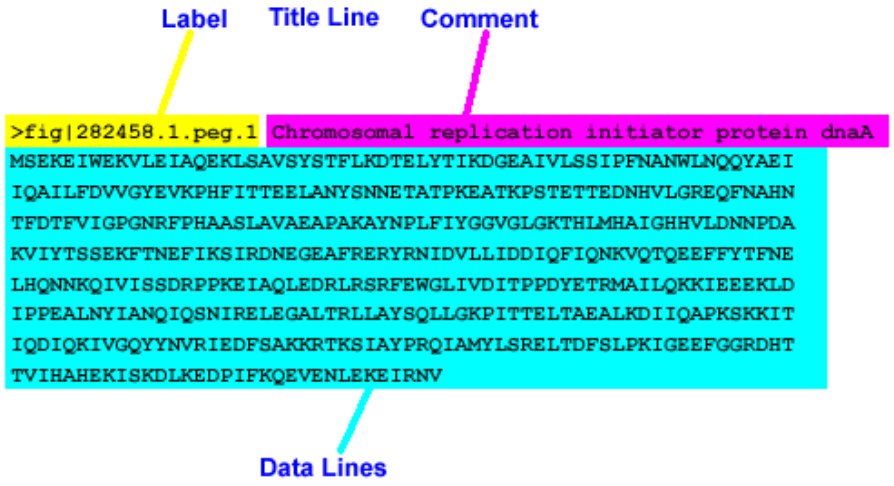
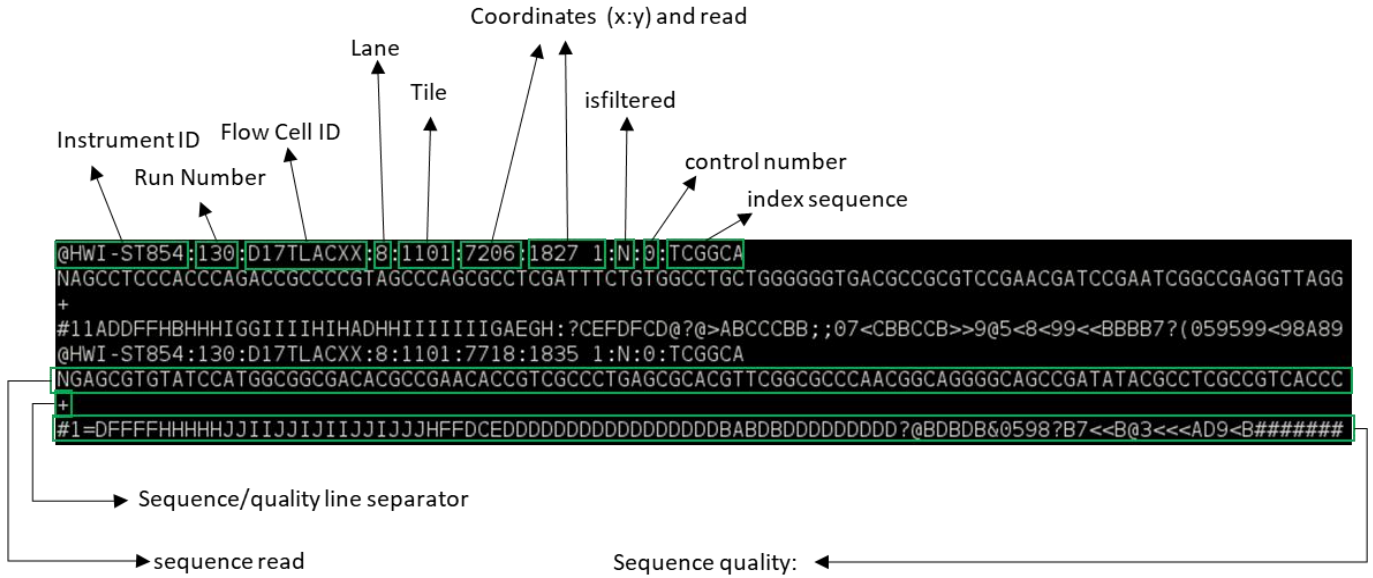
What storage format for Sequencing Reads: FASTA vs FASTQ

FASTA



FASTQ



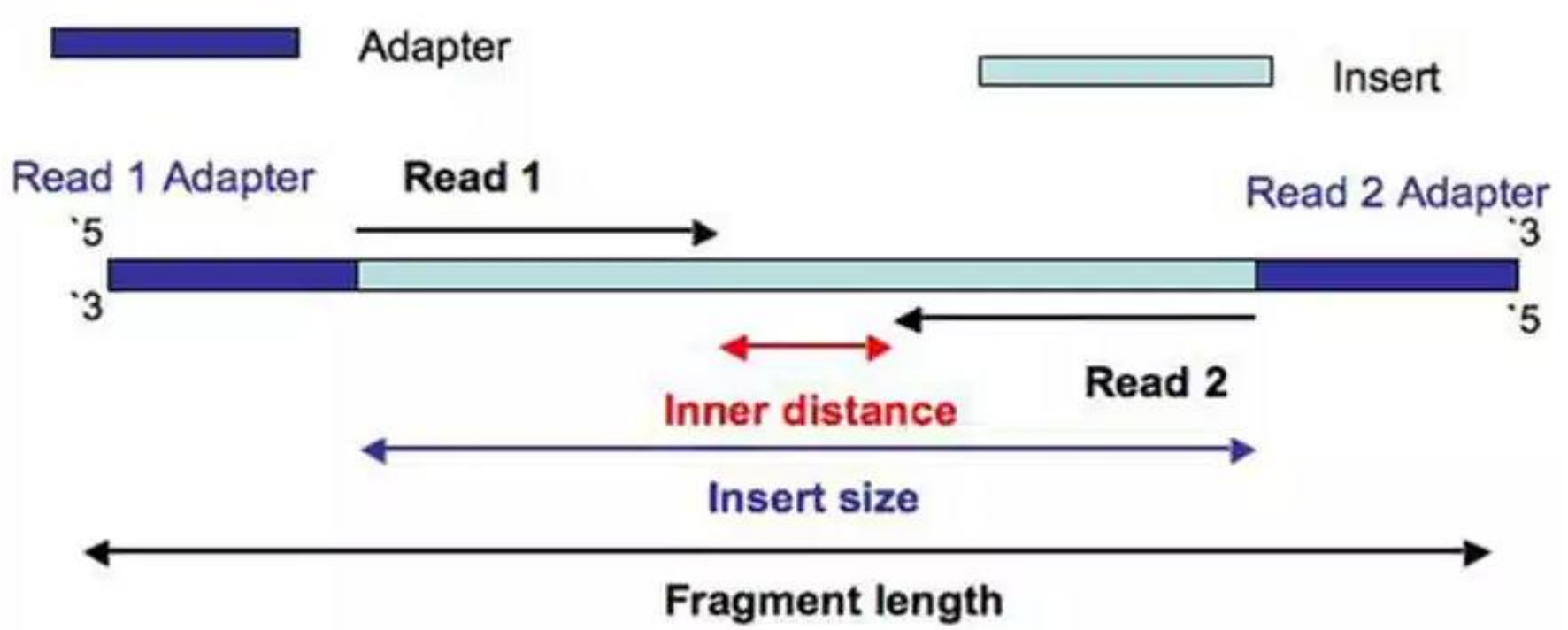
Sequence quality:
ASCII encoded Phred-33 quality score for each nucleotide of the sequence read above. Example: the 3rd nucleotide in the bottom read has a quality (Phred33 Q) of 28. Check the table below.
What about the 5th nucleotide of the same read? _____

ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 $	14	0.03981	47 /	25	0.00316	58 :	36	0.00025	69 E
4	0.39811	37 %	15	0.03162	48 0	26	0.00251	59 ;	37	0.00020	70 F
5	0.31623	38 &	16	0.02512	49 1	27	0.00200	60 <	38	0.00016	71 G
6	0.25119	39 '	17	0.01995	50 2	28	0.00158	61 =	39	0.00013	72 H
7	0.19953	40 (	18	0.01585	51 3	29	0.00126	62 >	40	0.00010	73 I
8	0.15849	41)	19	0.01259	52 4	30	0.00100	63 ?	41	0.00008	74 J
9	0.12589	42 *	20	0.01000	53 5	31	0.00079	64 @	42	0.00006	75 K
10	0.10000	43 +	21	0.00794	54 6	32	0.00063	65 A			

What storage format for Sequencing Reads: FASTA vs FASTQ

Why do I get two FastQ files?



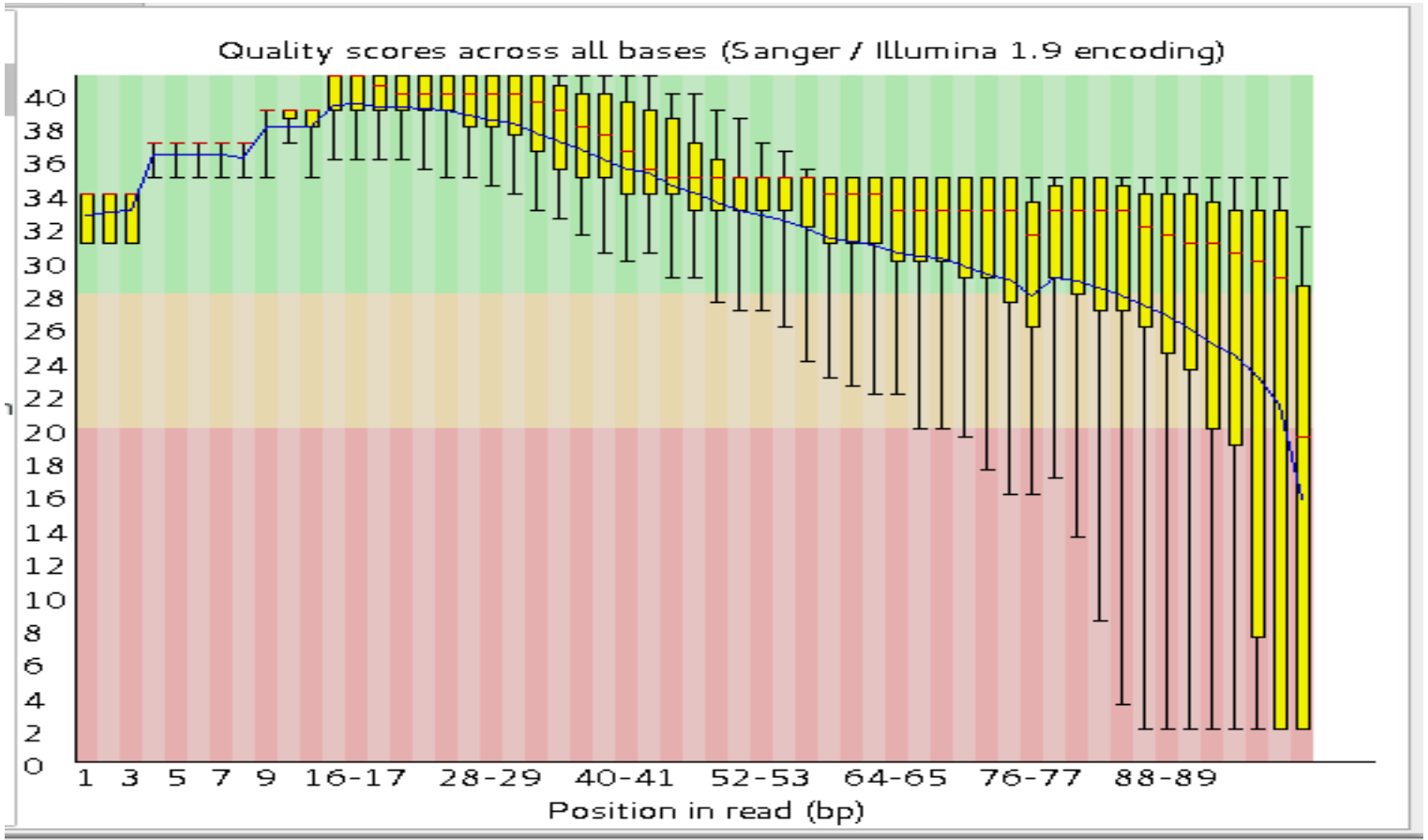
https://thesequencingcenter.com/knowledge-base/what-are-paired-end-reads/

Quality Control: Assessment

Objective:

- Assess overall sequencing quality and assess if sequencing metrics are within expected ranges;
- Remove base calls associated with low quality by removing or trimming sequencing reads;
- Taxonomical read QC – *did you sequence what you though you did*?

Main/Most frequent problem: base quality deterioration along the read length



Quality Control: Assessment

How to assess sequencing metrics?

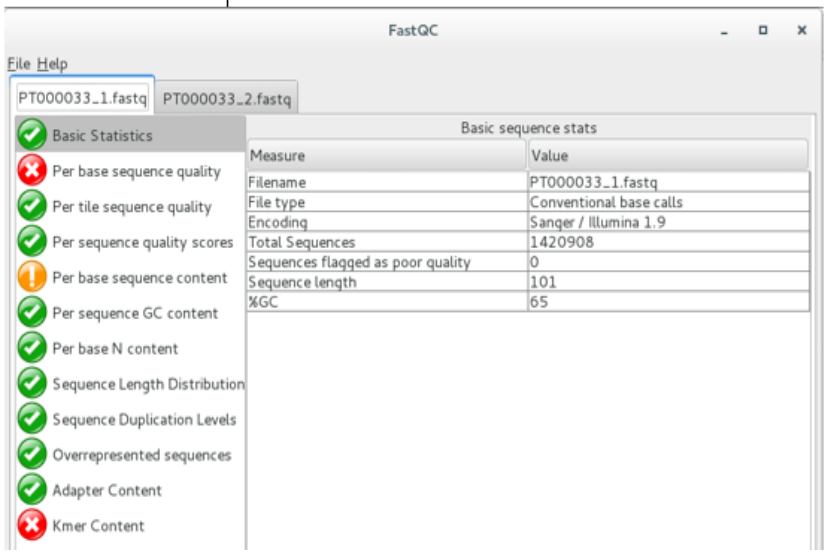
Software: FastQC, AfterQC, fastqp, HTSeq, etc.

FastQC – Java tool with both GUI and command-line as options.

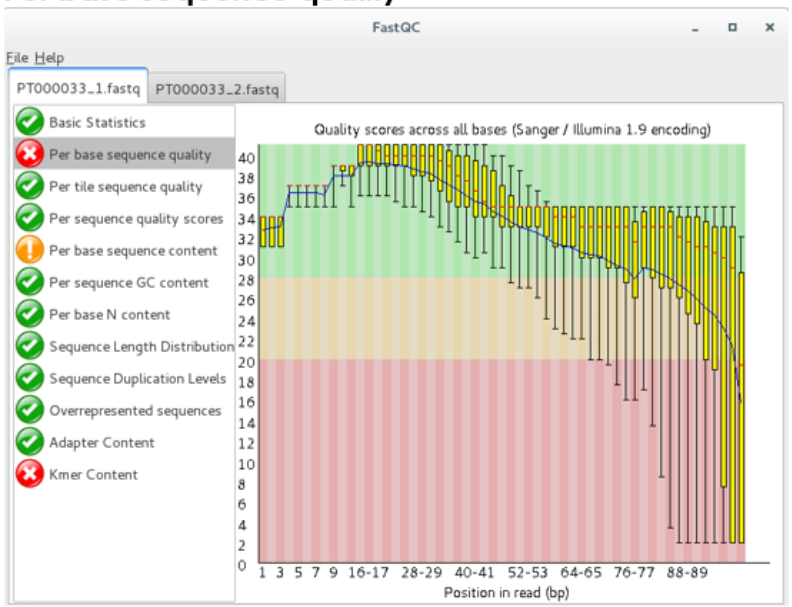
Input: FastQ files (or SAM/BAM files)

Output: sequencing metrics and plots

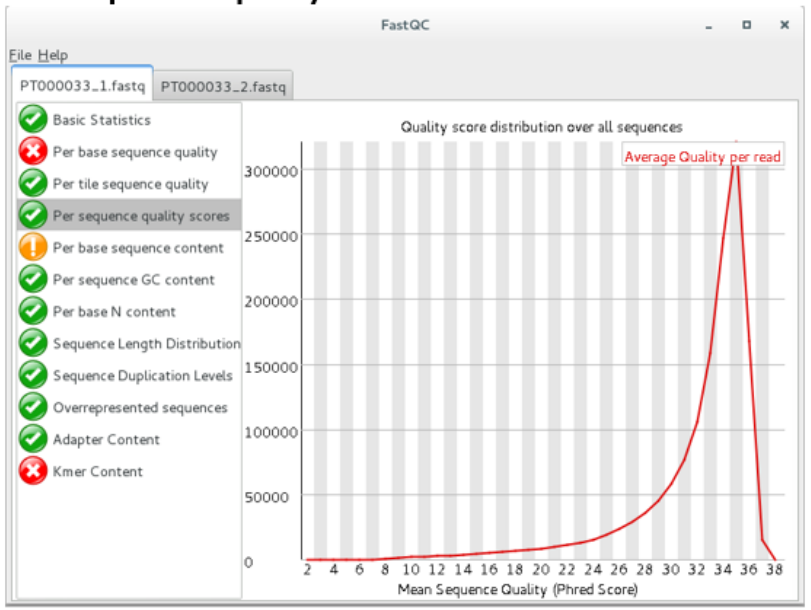
Basic Statistics



Per base sequence quality



Per sequence quality score



Quality Control: Assessment

How to assess sequencing metrics?

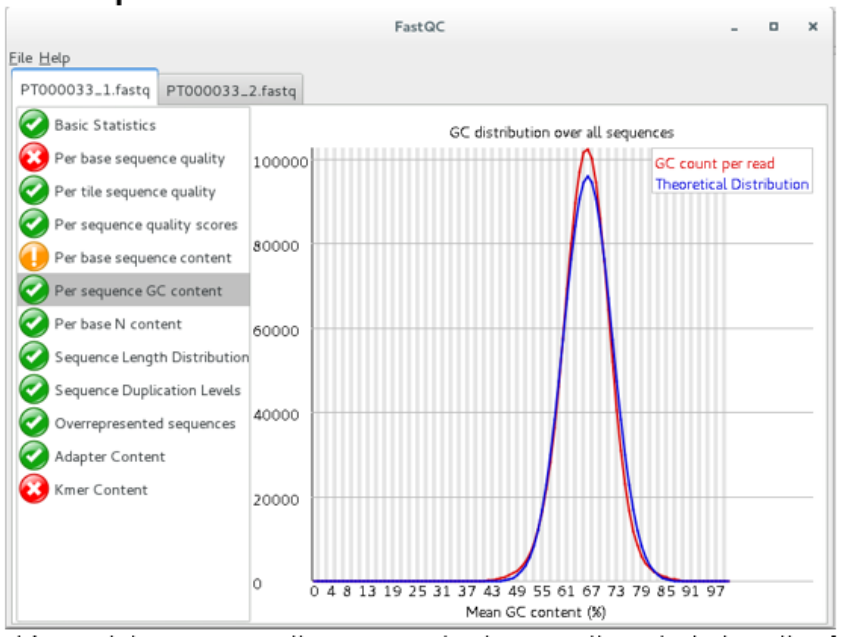
Software: FastQC, AfterQC, fastqp, HTSeq, etc.

FastQC – Java tool with both GUI and command-line as options.

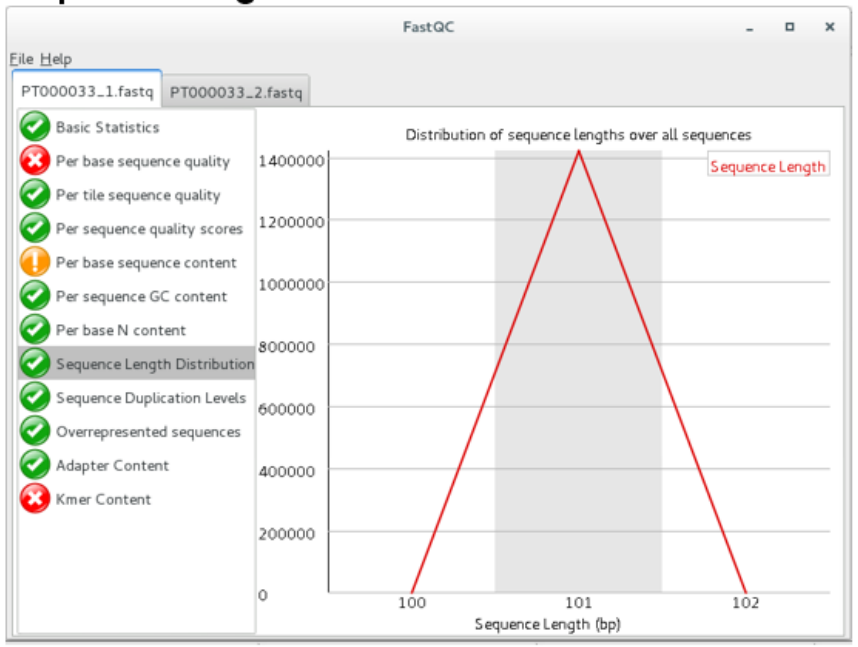
Input: FastQ files (or SAM/BAM files)

Output: sequencing metrics and plots

Per Sequence GC content



Sequence Length Distribution



Quality Control: Correction

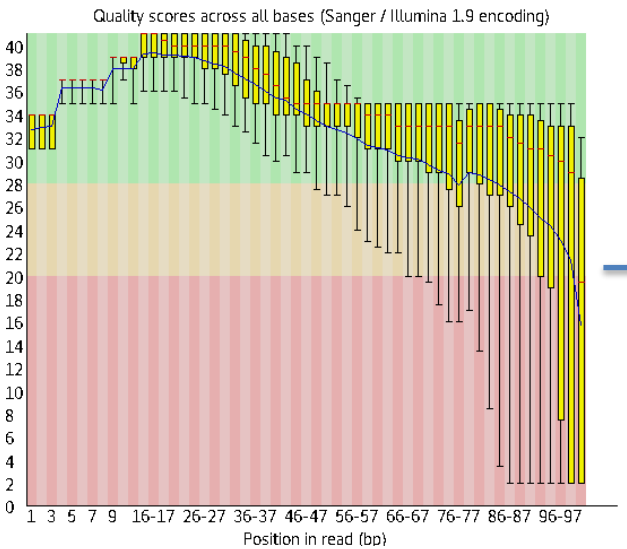
How to correct, cut and filter out sequencing reads?

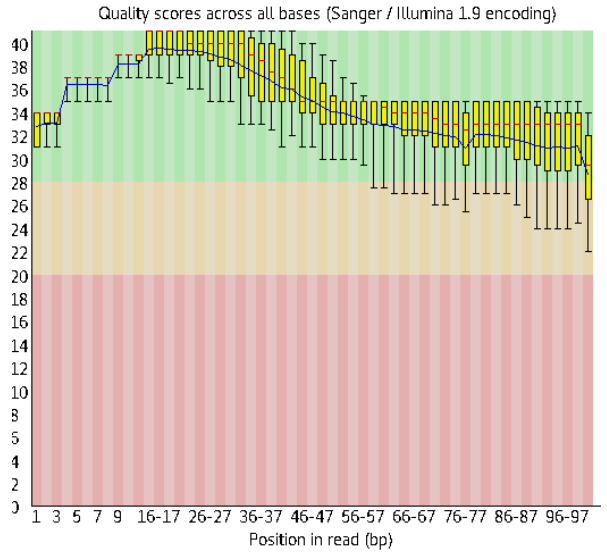
Software: Trimmomatic, FASTX, etc.

Trimmomatic – command-line Java tool capable of handling SE and PE reads.

Input: FastQ files.

Output: FastQ files with trimmed/cut and surviving reads.





Trimmomatic can:

- Remove adapters;
- Remove leading and trailing low quality bases;
- Cut reads upon scanning at user defined sliding Windows when below a specified threshold;
- Remove reads when these don't meet a specified minimum length.

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Quality Control: Taxonomical Read QC

Did I sequence what I thought I did? or ***Why doesn't it map?*** or ***Why does the assembly look strange?***

Software: Kraken

Kraken – command-line tool that assigns reads to different taxonomical clades.

Input: FastQ files.

Output: Text Report.

```
0.89  28702   28702   U      0       unclassified
99.11  3210397 42095   -      1       root
97.81  3168297 1864    -      131567    cellular organisms
97.76  3166433 16142   D      2           Bacteria
97.25  3150142 4158    P      1224          Proteobacteria
97.12  3145930 19183   C      1236            Gammaproteobacteria
94.74  3068819 83719   O      91347             Enterobacterales
92.16  2985015 1245831 F      543                 Enterobacteriaceae
53.46  1731479 124411  G      561                   Escherichia
49.60  1606565 1603919 S      562                     Escherichia coli
```

```
3.65  137472  137472  U      0       unclassified
96.35  3631172 7288    -      1       root
96.16  3623815 2151    -      131567    cellular organisms
96.10  3621664 10917   D      2           Bacteria
95.81  3610594 13835   P      1224          Proteobacteria
95.43  3596429 40211   C      1236            Gammaproteobacteria
92.48  3485213 108814  O      91347             Enterobacterales
89.53  3374249 342184  F      543                 Enterobacteriaceae
79.20  2984764 1375209 G      570                   Klebsiella
38.86  1464425 1415727 S      573                     Klebsiella pneumoniae
```

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Mapping or Reference Assembly

Objective: Find the origin of a sequencing read providing a reference genome is known

Reference genome: Should be a high quality genome, ideally finished, the close as possible to the sequenced genome.

Software: Burrows-Wheeler Aligner (BWA), Bowtie2, HISAT2

Input: FastQ files.

Output: Mapped/Alignment File
SAM/BAM file

Most mapping software implement the Burrows-Wheeler transformation algorithm which enables fast access to sequence data with an acceptable memory footprint.

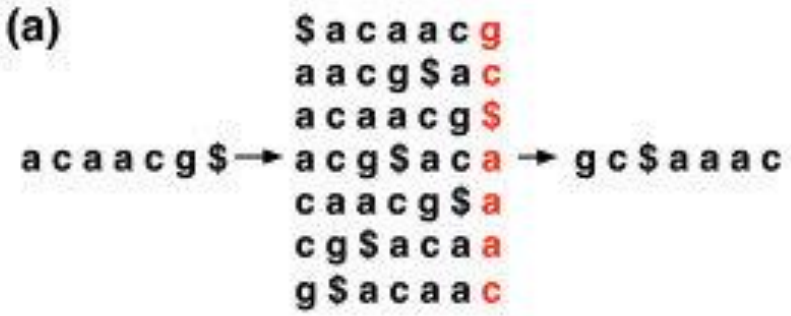
	Execution time				Memory usage				Accuracy				% Prop. paired reads			
Ins. (bp)	350		550		350		550		350		550		350		550	
RL (bp)	100	150	100	150	100	150	100	150	100	150	100	150	100	150	100	150
BWA	+	+	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
Bowtie2	++	++	++	++	+++	+++	++	++	+++	+++	+++	+++	++	++	+++	+++
HISAT2	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	+

"We conclude that there is not a single mapper that is ideal in all scenarios but rather the choice of alignment tool should be driven by the application and sequencing technology."

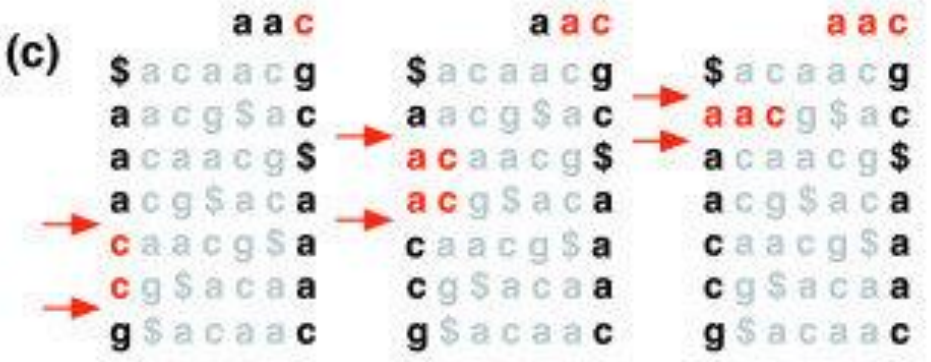
Keel et al 2018

The Burrows-Wheeler Transform

Reference compression:

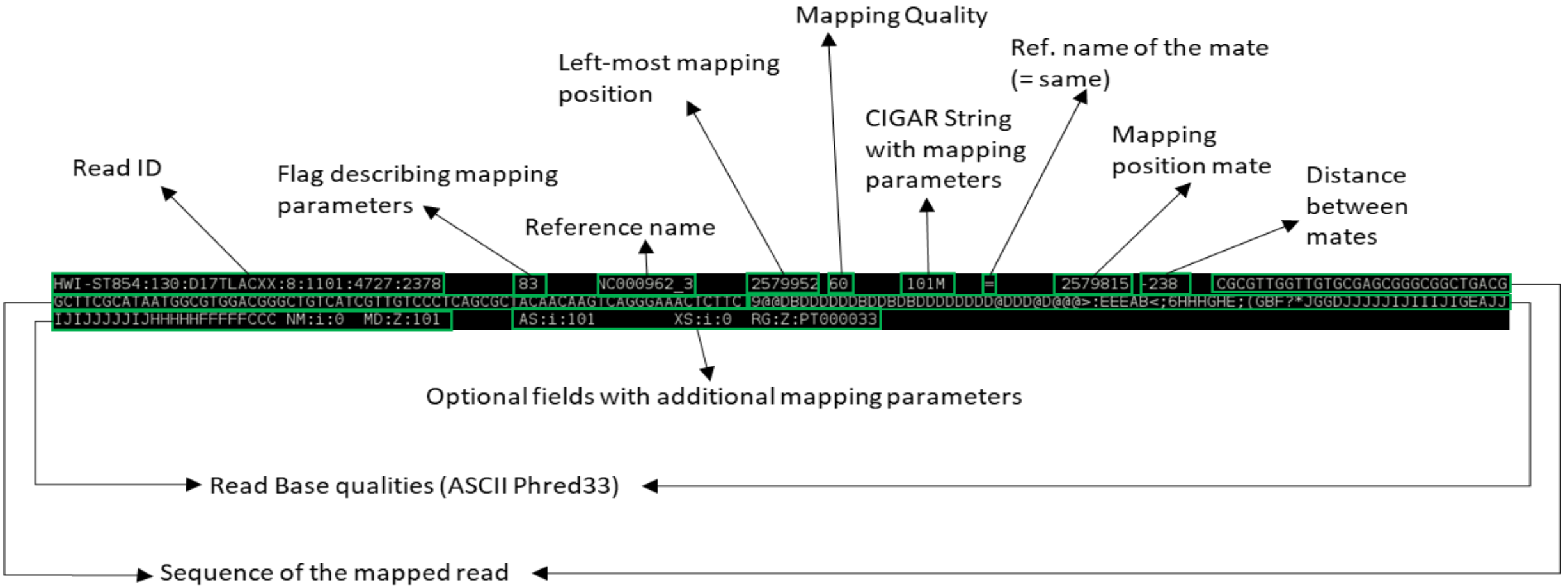


Searching for *aac* string:



Reconstructing original sequence:

The SAM Format…



The BAM files are a binary version of the SAM files (text format)

Both BAM and SAM files can be manipulated and viewed with SAMtools or Picard Tools

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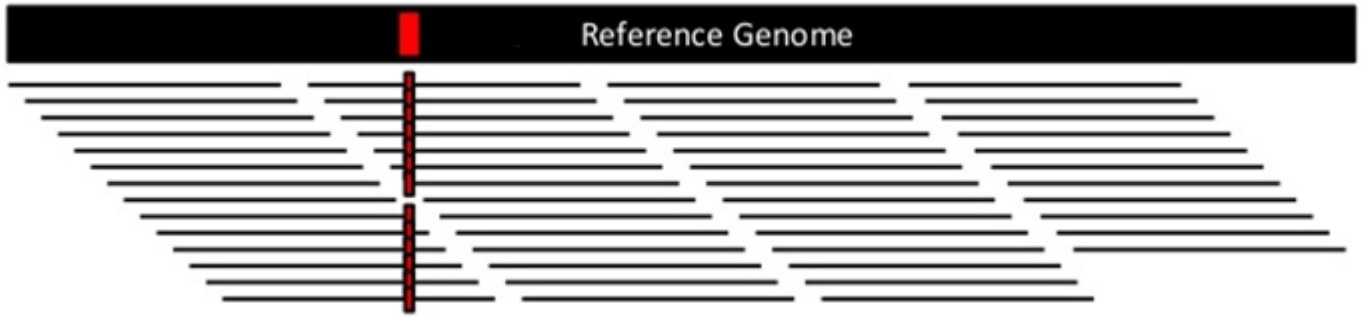
Variant Calling

Objective: Identify, list and store genomic variants, either SNPs or INDELs.

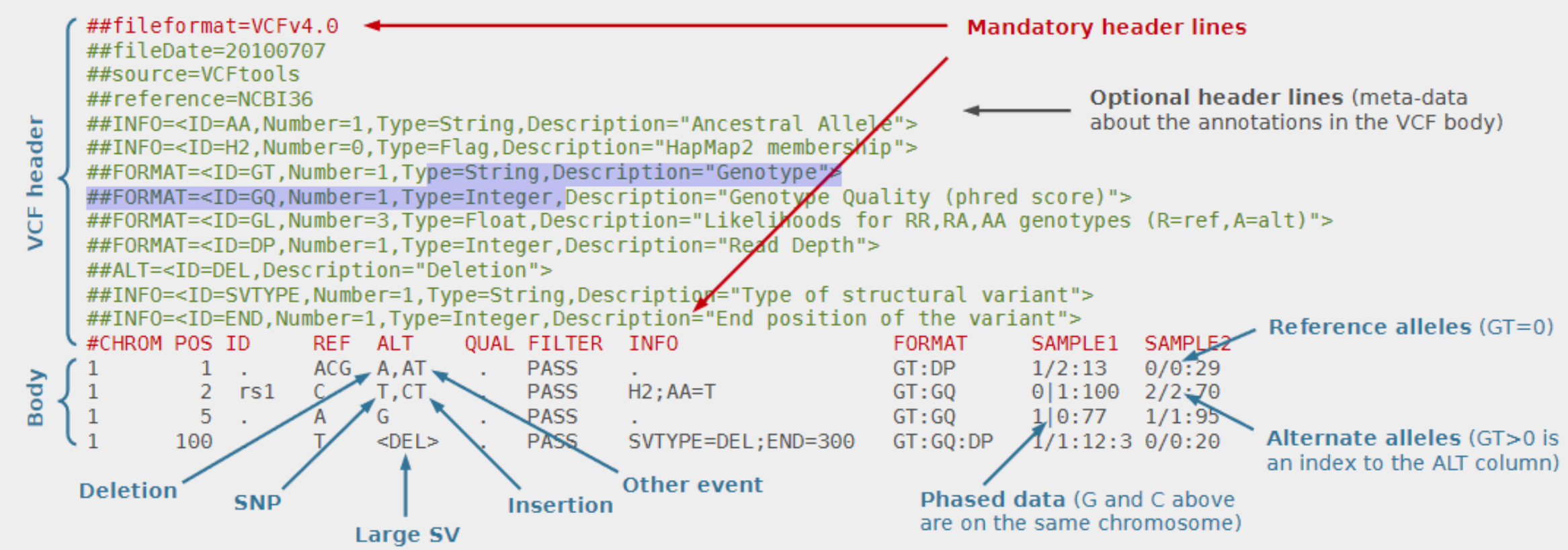
Software: SAMtools/BCFtools; Genome Analysis Toolkit (GATK), FreeBayes, LoFreq

Input: BAM/SAM files.

Output: VCF files



VCF Format



http://vcftools.sourceforge.net/VCF-poster.pdf

See full specs:
https://samtools.github.io/hts-specs/VCFv4.2.pdf

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VCF Format

Format differences between variant callers - Examples

Finding allelic depth and filtering

SAMTools

NC000962_3 69871 . C T 225 . DP=23;VDB=0.641395;SGB=-0.69168;MQSB=0.537242;MQ0F=0;AC=2;AN=2;DP4=0,0,11,8;MQ=43 GT:PL 1/1:255,57,0

bcftools view --include 'QUAL>=20 && INFO/DP>=10 && **(INFO/DP4[2]+INFO/DP4[3])/(sum(INFO/DP4))>=0.9**'c

GATK

NC000962_3 69871 . C T 810 . AC=1;AF=1.00;AN=1;BaseQRankSum=1.988;DP=24;Dels=0.00;FS=0.000;HaplotypeScore=0.9469;MLEAC=1;MLEAF=1.00;MQ=60.00;MQ0=0;MQRankSum=0.000;QD=33.75;ReadPosRankSum=1.592;SOR=0.353 GT:AD:DP:GQ:PL 1:1,23:24:99:840,0

bcftools view --include 'QUAL>=20 && FORMAT/DP>=10 && **(FORMAT/AD[*:1])/(FORMAT/DP)>=0.9**'

Freebayes

NC000962_3 69871 . C T 656.091 . AB=0;ABP=0;AC=1;AF=1;AN=1;AO=23;CIGAR=1X;DP=24;DPB=24;DPRA=0;EPP=3.10471;EPPR=5.18177;GTI=0;LEN=1;MEANALT=1;MQM=60;MQMR=60;NS=1;NUMAL
T=1;ODDS=151.07;PAIRED=0.956522;PAIREDR=0;PAO=0;PQA=0;PQR=0;PRO=0;QA=760;QR=14;RO=1;RPL=14;RPP=5.3706;RPPR=5.18177;RPR=9;RUN=1;SAF=12;SAP=3.10471;SAR=11;SRF=0;SRP=5.18177;SRR=1;TYPE=snp;technology.illumina=1 GT:DP:AD:RO:QR:AO:QA:GL 1:24:1,23:1:14:23:760:-67.3027,0

bcftools view --include 'QUAL>=20 && FORMAT/DP>=10 **&& (FORMAT/AO)/(FORMAT/DP)**>=0.9

LoFreq

NC000962_3 69871 . C T 732 PASS DP=24;AF=0.958333;SB=0;DP4=0,1,12,11

bcftools view --include 'QUAL>=20 && INFO/DP>=10 && **(INFO/DP4[2]+INFO/DP4[3])/(sum(INFO/DP4))>=0.9**'c

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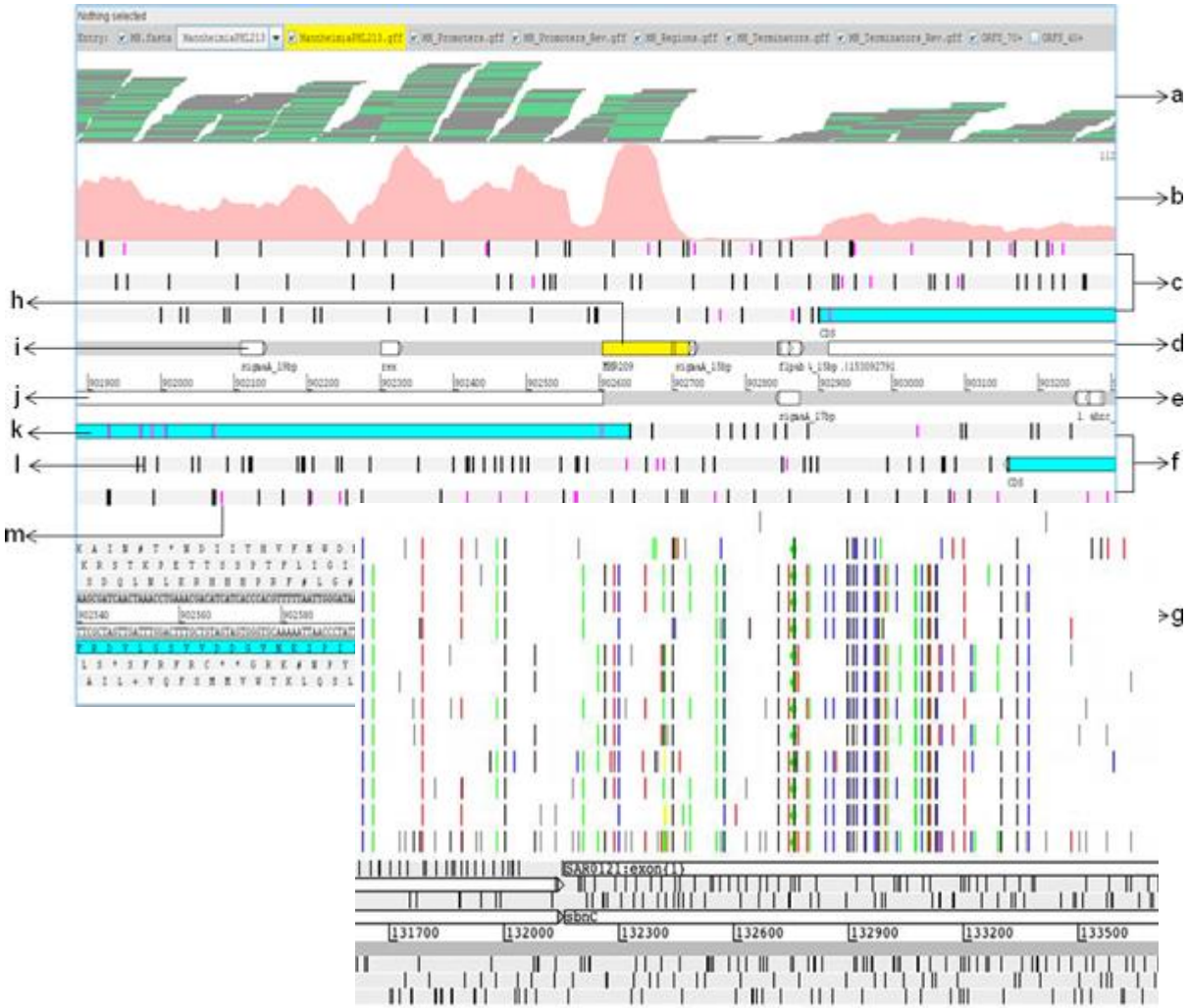
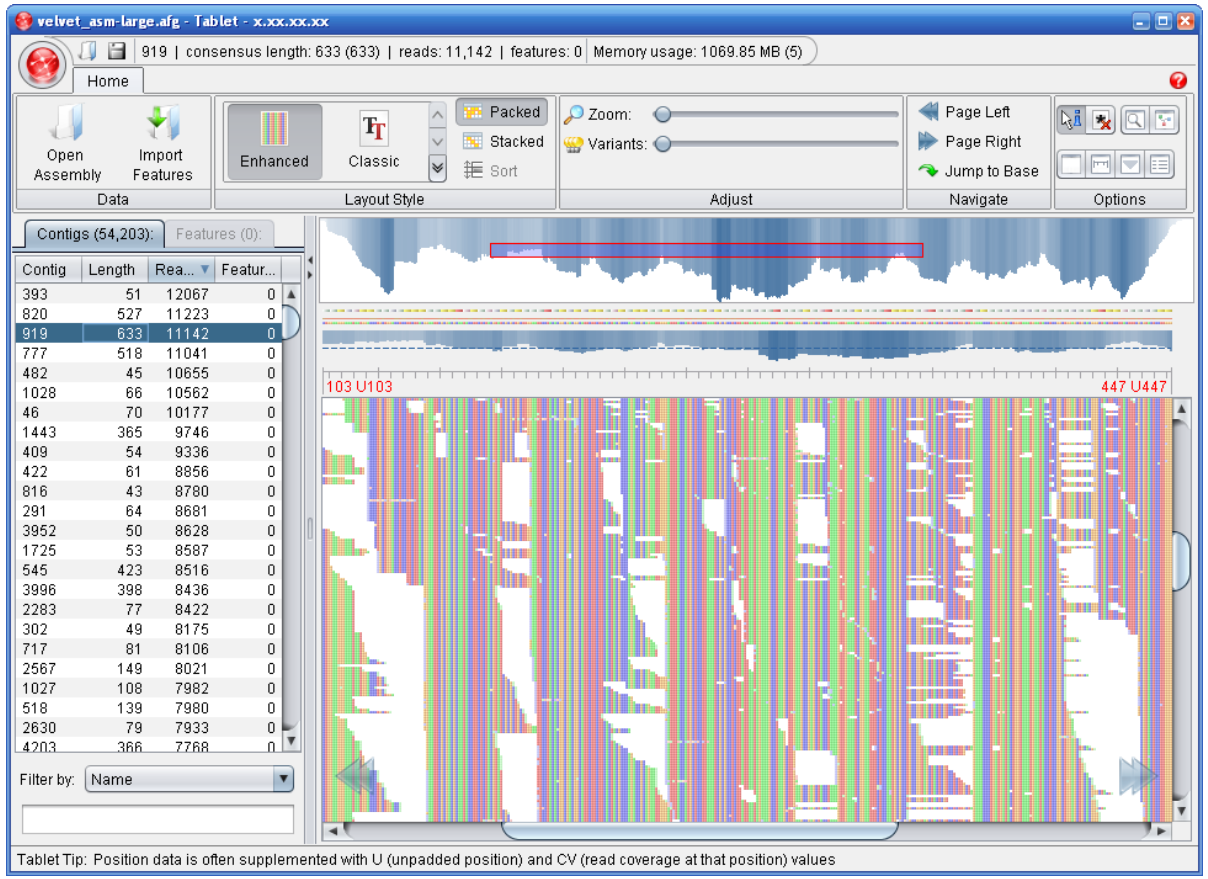
BAM and VCF Visualizatiom

Artemis



Tablet



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Functional Annotation

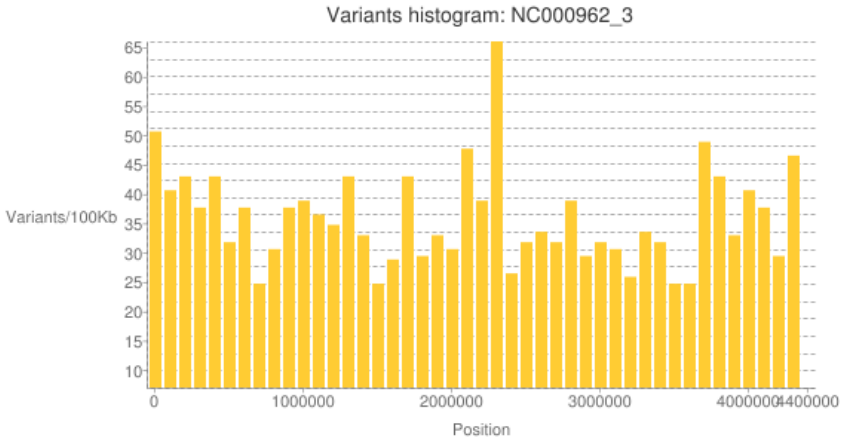
Genome	NC000962_3
Date	2021-06-17 01:39
SnpEff version	SnpEff 5.0e (build 2021-03-09 06:01), by Pablo Cingolani
Command line arguments	SnpEff -no-downstream -no-upstream NC000962_3 PT000033.filt.vcf
Warnings	124
Errors	0
Number of lines (input file)	1,597
Number of variants (before filter)	1,598
Number of not variants (i.e. reference equals alternative)	0
Number of variants processed (i.e. after filter and non-variants)	1,598
Number of known variants (i.e. non-empty ID)	0 (0%)
Number of multi-allelic VCF entries (i.e. more than two alleles)	1
Number of effects	16,074
Genome total length	4,411,532
Genome effective length	4,411,532
Variant rate	1 variant every 2,760 bases

Objective: Annotate each variant with tis functional impact/consequence.

Software: SnpEff, GATK etc

Input: VCF files.

Output: Annotated VCF files



Number of effects by functional class

Type (alphabetical order)	Count	Percent
MISSENSE	786	61.025%
NONSENSE	13	1.009%
SILENT	489	37.966%

Missense / Silent ratio: 1.6074

'Allele | Annotation | Annotation_Impact | Gene_Name | Gene_ID | Feature_Type | Feature_ID | Transcript_BioType | Rank | HGV S.c | HGVS.p | cDNA.pos / cDNA.length | CDS.pos / CDS.length | AA.pos / AA.length | Distance | ERRORS / WARNINGS / INFO' ">

ANN=G|missense_variant|MODERATE|katG|Rv1908c|transcript|Rv1908c|protein_coding|1/1|c.944G>C|p.Ser315Thr|944/2223|944/2223|315/740||