3000788 Intro to Comp Molec Biol

Lecture 4: Sequencing data processing

Fall 2025

Sira Sriswasdi, PhD

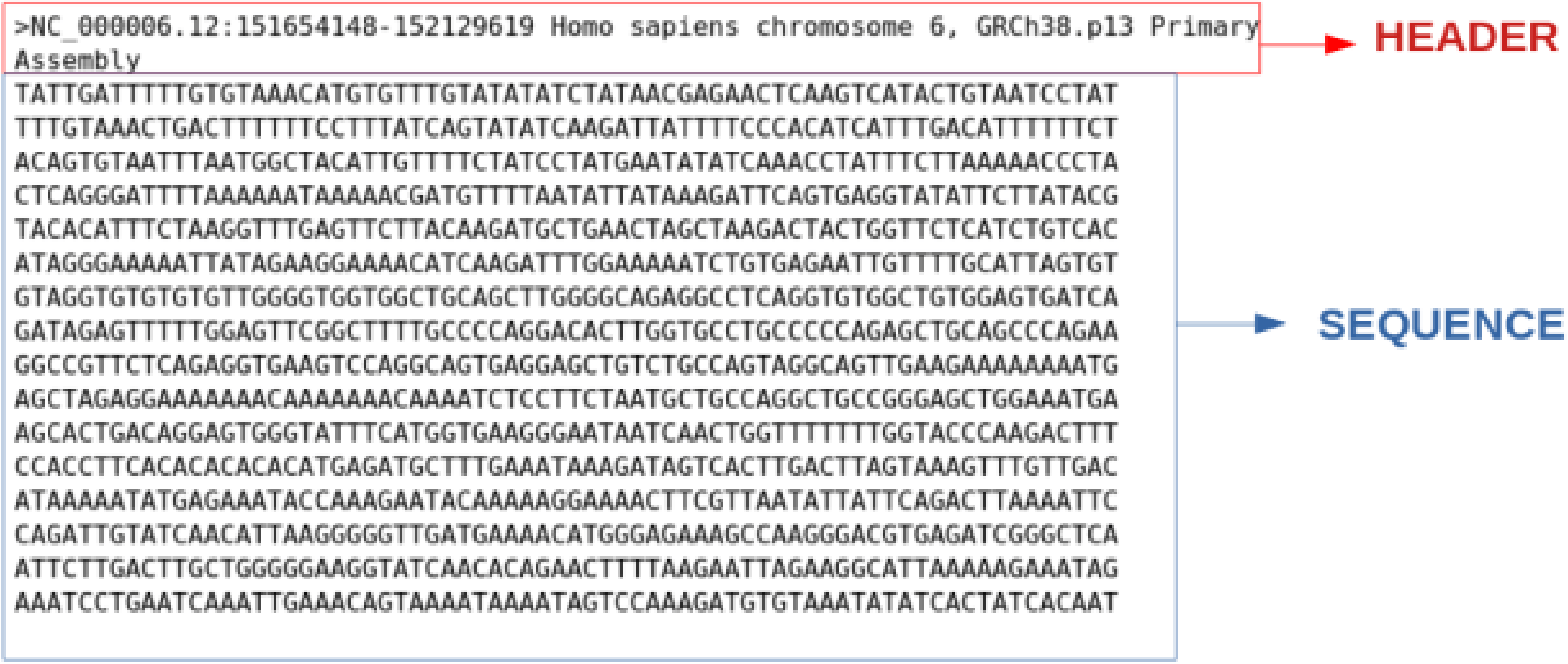
- Research Affairs
- Center of Excellence in Computational Molecular Biology (CMB)
- Center for Artificial Intelligence in Medicine (CU-AIM)

Today’s agenda

- Sequencing data file formats
- Quality check and processing of sequencing reads
- Read-to-genome alignment

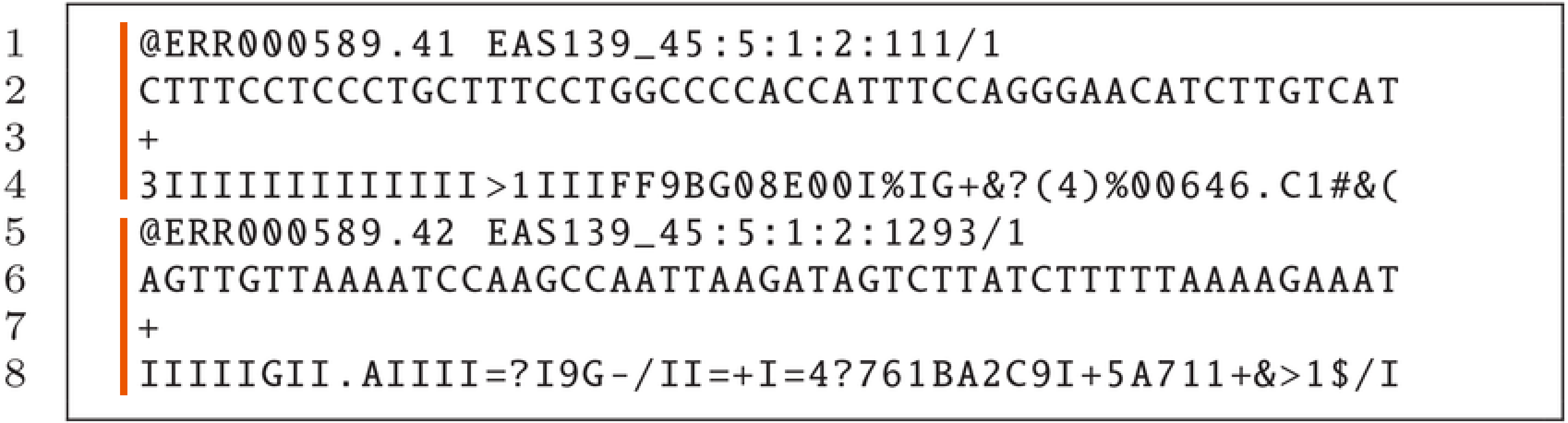
Sequencing data

FASTA format



```
>NC_000006.12:151654148-152129619 Homo sapiens chromosome 6, GRCh38.p13 Primary
Assembly
TATTGATTTTTGTGTAAACATGTGTTTGTATATATCTATAACGAGAACTCAAGTCATACTGTAATCCTAT
TTTGTAAACTGACTTTTTTCCTTTATCAGTATATCAAGATTATTTTCCCACATCATTTGACATTTTTTCT
ACAGTGTAATTTAATGGCTACATTGTTTTCTATCCTATGAATATATCAAACCTATTTCTTAAAAACCCTA
CTCAGGGATTTTAAAAAATAAAAACGATGTTTTAATATTATAAAGATTCAGTGAGGTATATTCTTATACG
TACACATTTCTAAGGTTTGAGTTCTTACAAGATGCTGAACTAGCTAAGACTACTGGTTCTCATCTGTCAC
ATAGGGAAAAATTATAGAAGGAAAACATCAAGATTTGGAAAAATCTGTGAGAATTGTTTTGCATTAGTGT
GTAGGTGTGTGTGTTGGGGTGGTGGCTGCAGCTTGGGGCAGAGGCCTCAGGTGTGGCTGTGGAGTGATCA
GATAGAGTTTTTGGAGTTCGGCTTTTGCCCCAGGACACTTGGTGCCTGCCCCCAGAGCTGCAGCCCAGAA
GGCCGTTCTCAGAGGTGAAGTCCAGGCAGTGAGGAGCTGTCTGCCAGTAGGCAGTTGAAGAAAAAAAATG
AGCTAGAGGAAAAAAACAAAAAAACAAAATCTCCTTCTAATGCTGCCAGGCTGCCGGGAGCTGGAAATGA
AGCACTGACAGGAGTGGGTATTTCATGGTGAAGGGAATAATCAACTGGTTTTTTTGGTACCCAAGACTTT
CCACCTTCACACACACACATGAGATGCTTTGAAATAAAGATAGTCACTTGACTTAGTAAAGTTTGTTGAC
ATAAAAATATGAGAAATACCAAAGAATACAAAAAGGAAAACTTCGTTAATATTATTCAGACTTAAAATTC
CAGATTGTATCAACATTAAGGGGGTTGATGAAAACATGGGAGAAAGCCAAGGGACGTGAGATCGGGCTCA
ATTCTTGACTTGCTGGGGGAAGGTATCAACACAGAACTTTTAAGAATTAGAAGGCATTAAAAAGAAATAG
AAATCCTGAATCAAATTGAAACAGTAAAATAAAATAGTCCAAAGATGTGTAAATATATCACTATCACAAT
```

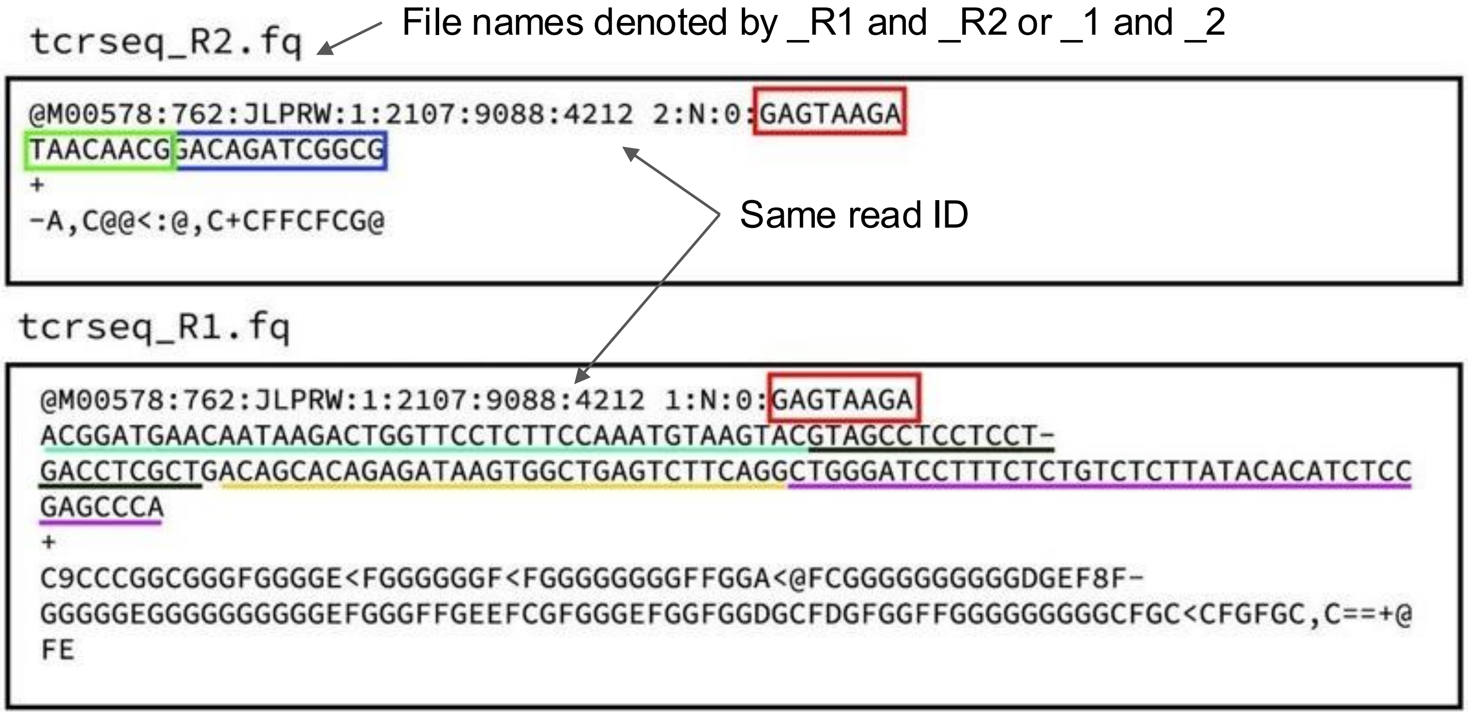
FASTQ format



```
@ERR000589.41 EAS139_45:5:1:2:111/1
CTTTCCTCCCTGCTTTCCTGGCCCCACCATTTCCAGGGAACATCTTGTCAT
+
3IIIIIIIIIIIII>1IIIFF9BG08E00I%IG+&?(4)%00646.C1#&(
@ERR000589.42 EAS139_45:5:1:2:1293/1
AGTTGTTAAAATCCAAGCCAATTAAGATAGTCTTATCTTTTTAAAAGAAAT
+
IIIIIGII.AIIII=?I9G-/II=+I=4?761BA2C9I+5A711+&>1$/I
```

- Header: Location of cluster on Illumina’s flow cell
- Sequence
- Quality score

FASTQ for paired-end sequencing



tcrseq_R2.fq

File names denoted by _R1 and _R2 or _1 and _2

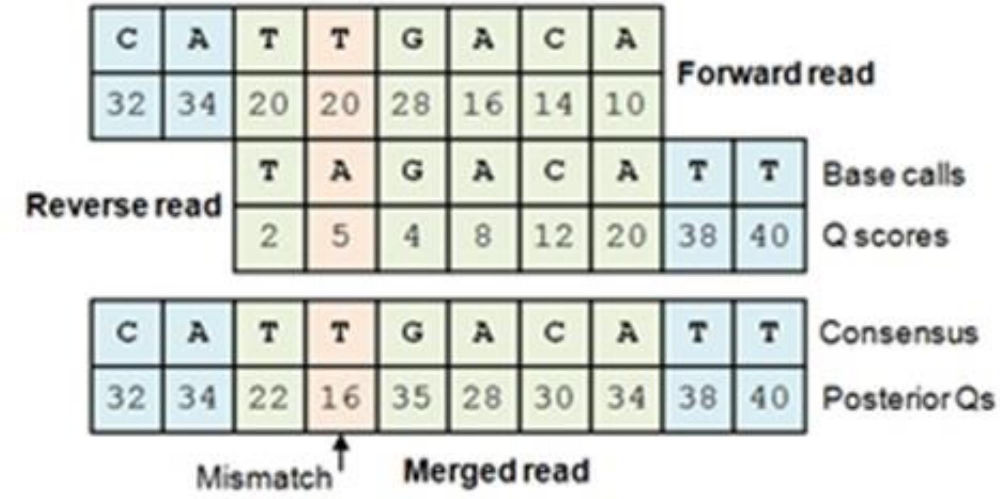
```
@M00578:762:JLPRW:1:2107:9088:4212 2:N:0:GAGTAAGA
TAACAACGGACAGATCGGCG
+
-A,C@@<:@,C+CFFCFCG@
```

Same read ID

tcrseq_R1.fq

```
@M00578:762:JLPRW:1:2107:9088:4212 1:N:0:GAGTAAGA
ACGGATGAACAATAAGACTGGTTCCTCTTCCAAATGTAAGTACGTAGCCTCCTCCT-
GACCTCGCTGACAGCACAGAGATAAGTGGCTGAGTCTTCAGGCTGGGATCCTTTCTCTGTCTCTTATACACATCTCC
GAGCCCA
+
C9CCCGGCGGGFGGGGE<FGGGGGGF<FGGGGGGGGFFGGA<@FCGGGGGGGGGGDGEF8F-
GGGGGEGGGGGGGGGGEFGGGFFGEEFCGFGGGEFGGFGGDGCFDGFGGFFGGGGGGGGGCFGC<CFGFGC,C==+@
FE
```

Merged FASTQ for paired-end sequencing

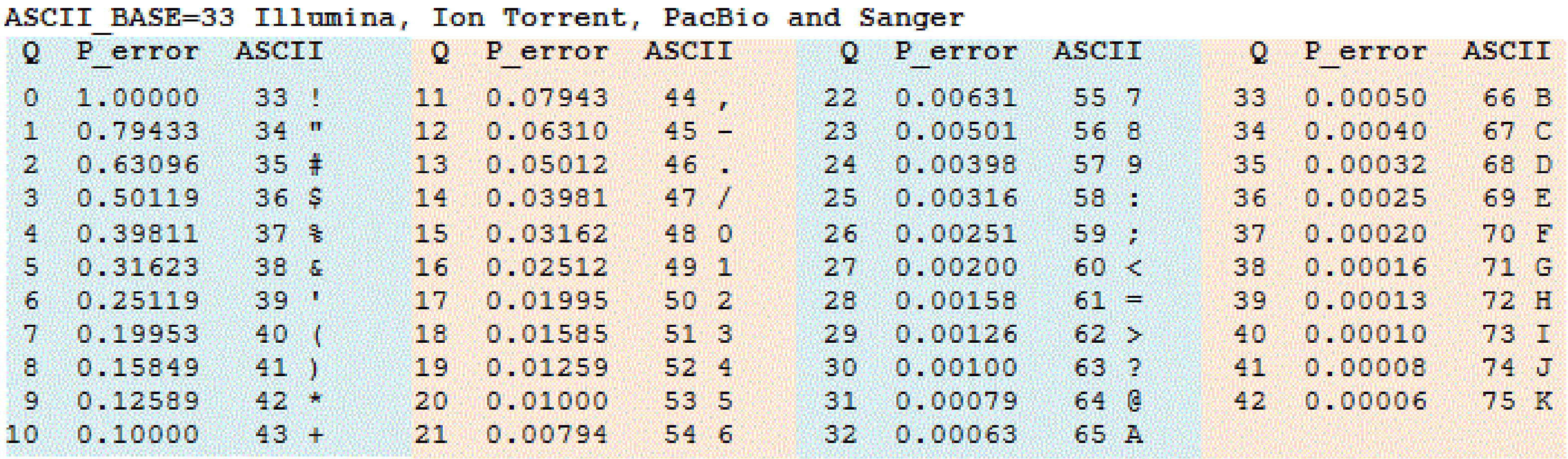


Forward read	C	A	T	T	G	A	C	A		
	32	34	20	20	28	16	14	10		
Reverse read			T	A	G	A	C	A	T	T	Base calls
			2	5	4	8	12	20	38	40	Q scores
	C	A	T	T	G	A	C	A	T	T	Consensus
	32	34	22	16	35	28	30	34	38	40	Posterior Qs

https://drive5.com/usearch/manual8.1/merge_pair.html

- Forward & reverse reads must overlap (read length > fragment length)
- **Example**: 300bp paired-end sequencing of 16S rRNA genes

Sequencing quality score (Phred score)



ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

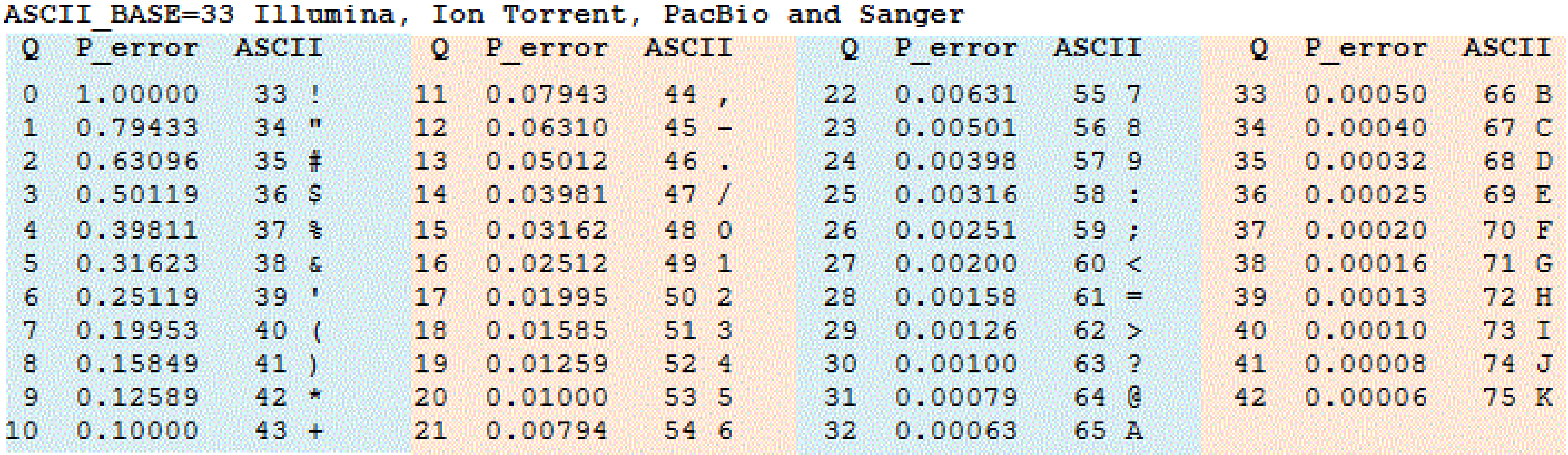
Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 $	14	0.03981	47 /	25	0.00316	58 :	36	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			

https://www.drive5.com/usearch/manual/quality_score.html

- Q score = – 10 x Log$_{10}$ (base call error rate)
- Base call error of 10% → Q score = +
- Base call error of 0.0001 → Q score = I

Increased error toward the ends of read

```
@ERR000589.41 EAS139_45:5:1:2:111/1
CTTTCCTCCCTGCTTTCCTGGCCCCACCATTTCCAGGGAACATCTTGTCAT
+
3IIIIIIIIIIIII>1IIIFF9BG08E00I%IG+&?(4)%00646.C1#&(
```



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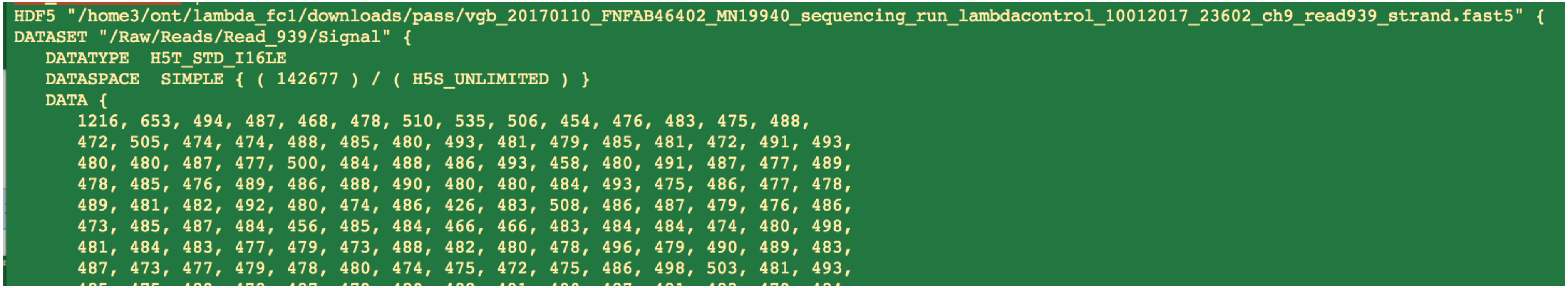
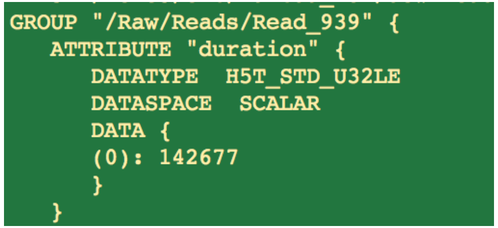
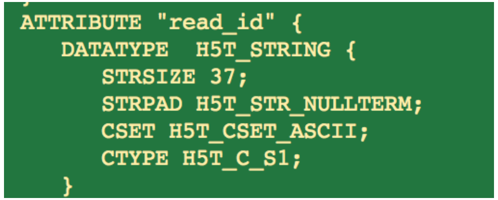
Nanopore FAST5 format



```
HDF5 "/home3/ont/lambda_fc1/downloads/pass/vgb_20170110_FNFAB46402_MN19940_sequencing_run_lambdacontrol_10012017_23602_ch9_read939_strand.fast5" {
DATASET "/Raw/Reads/Read_939/Signal" {
   DATATYPE  H5T_STD_I16LE
   DATASPACE  SIMPLE { ( 142677 ) / ( H5S_UNLIMITED ) }
   DATA {
      1216, 653, 494, 487, 468, 478, 510, 535, 506, 454, 476, 483, 475, 488,
      472, 505, 474, 474, 488, 485, 480, 493, 481, 479, 485, 481, 472, 491, 493,
      480, 480, 487, 477, 500, 484, 488, 486, 493, 458, 480, 491, 487, 477, 489,
      478, 485, 476, 489, 486, 488, 490, 480, 480, 484, 493, 475, 486, 477, 478,
      489, 481, 482, 492, 480, 474, 486, 426, 483, 508, 486, 487, 479, 476, 486,
      473, 485, 487, 484, 456, 485, 484, 466, 466, 483, 484, 484, 474, 480, 498,
      481, 484, 483, 477, 479, 473, 488, 482, 480, 478, 496, 479, 490, 489, 483,
      487, 473, 477, 479, 478, 480, 474, 475, 472, 475, 486, 498, 503, 481, 493,
```



```
GROUP "/Raw/Reads/Read_939" {
   ATTRIBUTE "duration" {
      DATATYPE  H5T_STD_U32LE
      DATASPACE  SCALAR
      DATA {
      (0): 142677
      }
   }
```



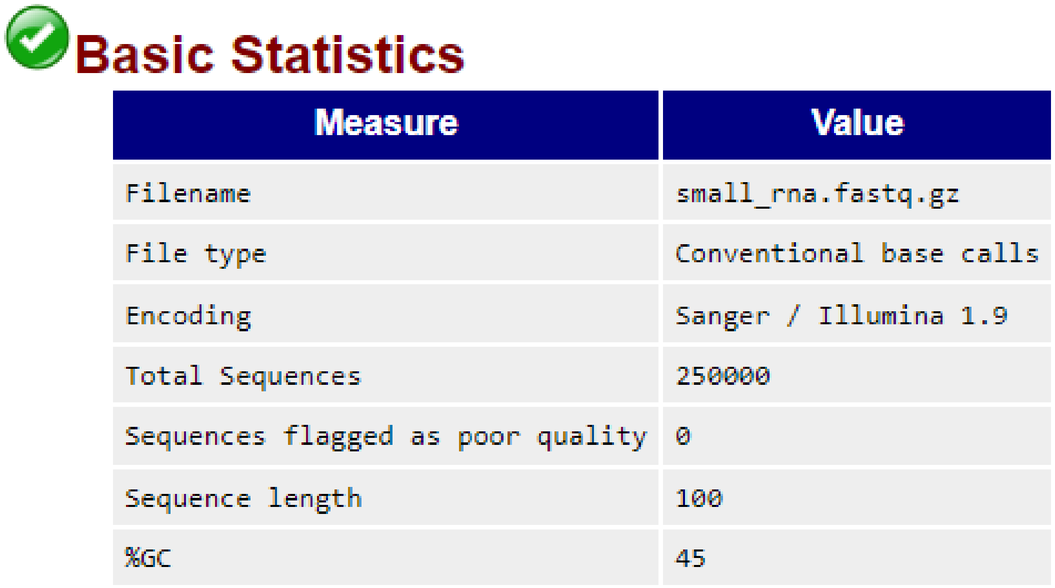
```
ATTRIBUTE "read_id" {
   DATATYPE  H5T_STRING {
      STRSIZE 37;
      STRPAD H5T_STR_NULLTERM;
      CSET H5T_CSET_ASCII;
      CTYPE H5T_C_S1;
   }
```

https://bioinformatics.cvr.ac.uk/exploring-the-fast5-format/

- Ion flow rate data through each nanopore, with time stamps
- **FAST5** is an **HDF5** file (a specialized compression for scientific datasets)

Quality check for sequencing data

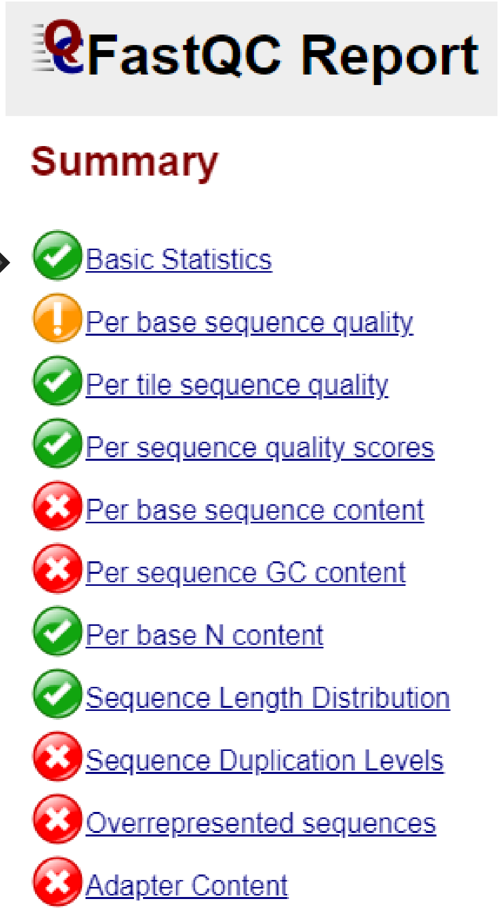
FastQC tool



✓ Basic Statistics

Measure	Value
Filename	small_rna.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	250000
Sequences flagged as poor quality	0
Sequence length	100
%GC	45

Check number of reads and read length

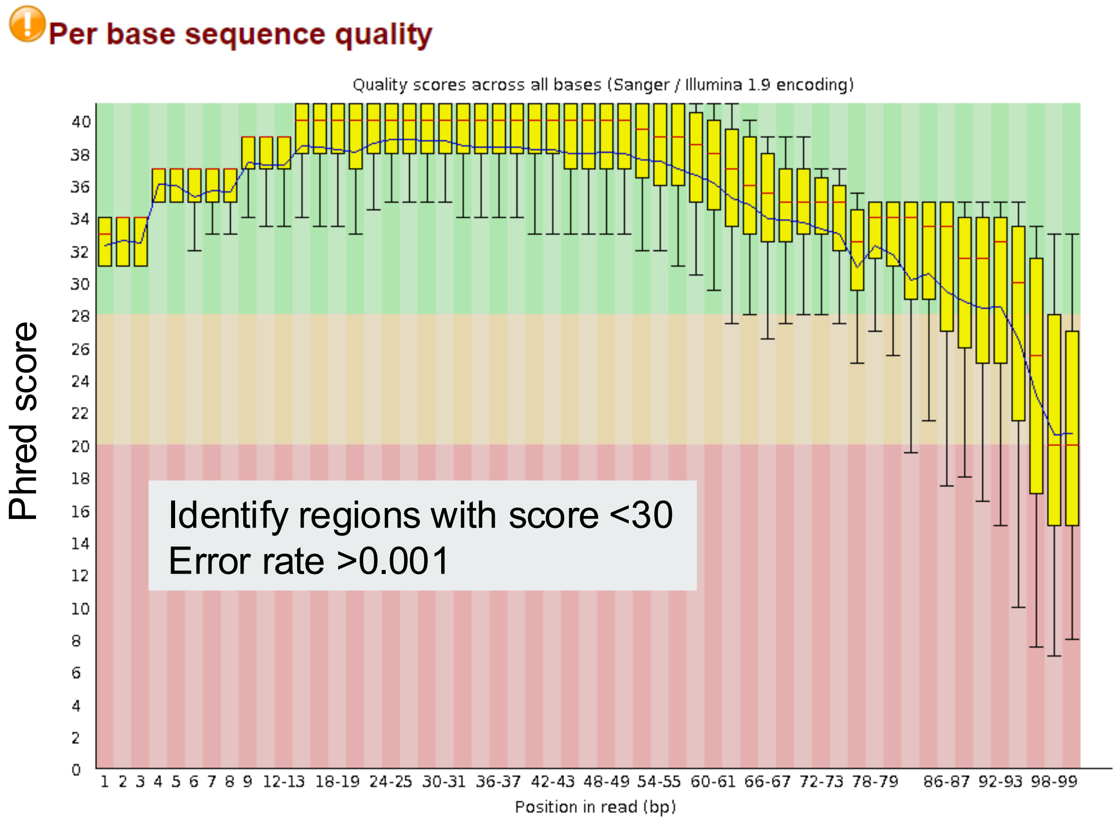


FastQC Report

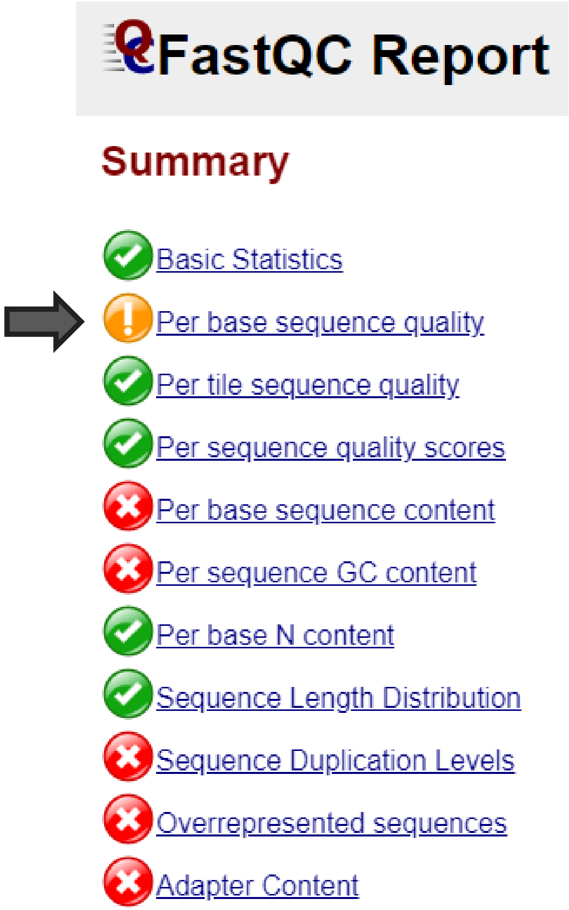
Summary

- ✓ Basic Statistics
- ! Per base sequence quality
- ✓ Per tile sequence quality
- ✓ Per sequence quality scores
- ✗ Per base sequence content
- ✗ Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ✗ Sequence Duplication Levels
- ✗ Overrepresented sequences
- ✗ Adapter Content

Base calling quality



Identify regions with score <30
Error rate >0.001



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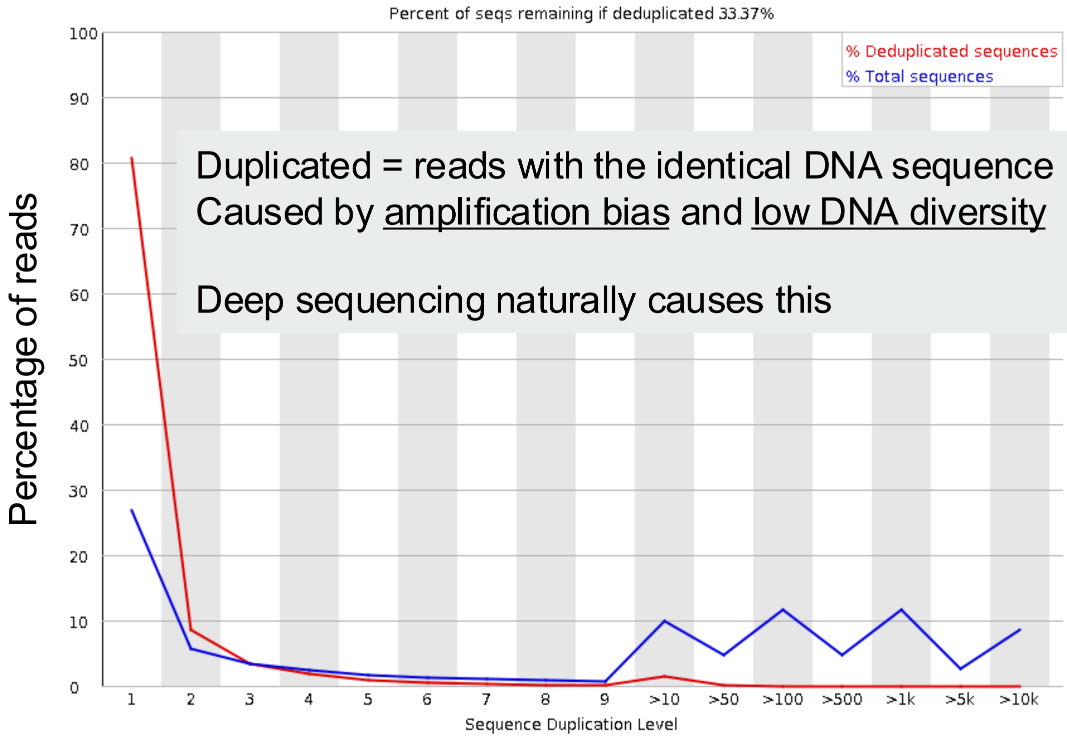
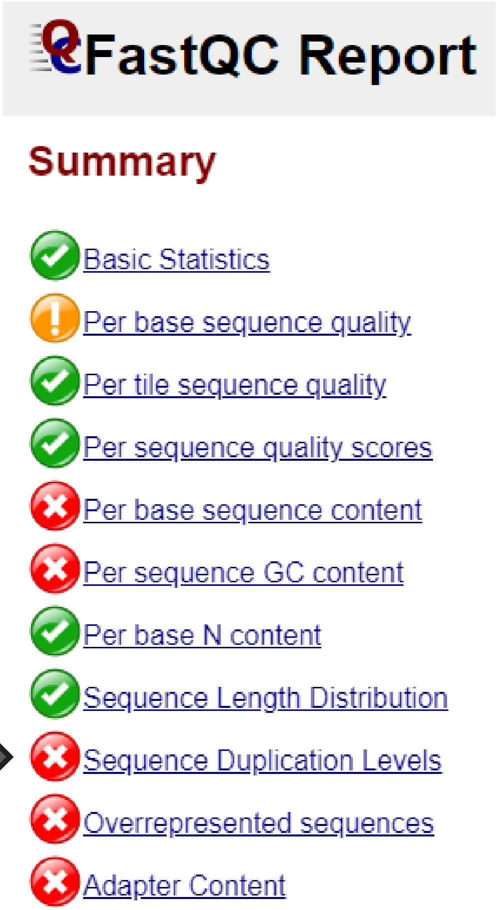
Duplicated reads

☒ Sequence Duplication Levels



Duplicated = reads with the identical DNA sequence
Caused by amplification bias and low DNA diversity

Deep sequencing naturally causes this



FastQC Report

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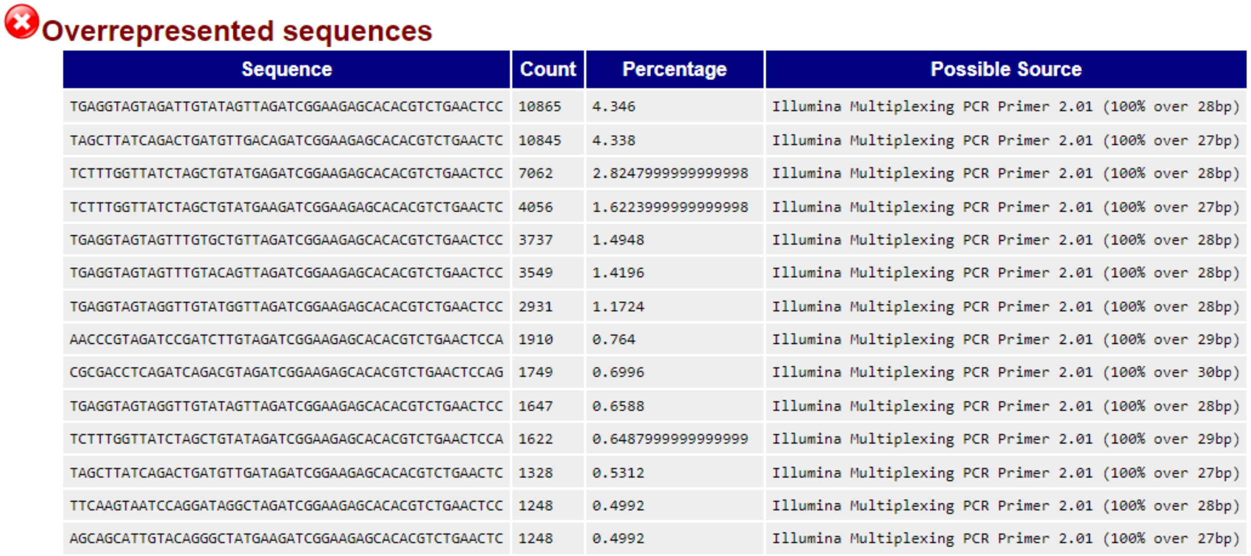
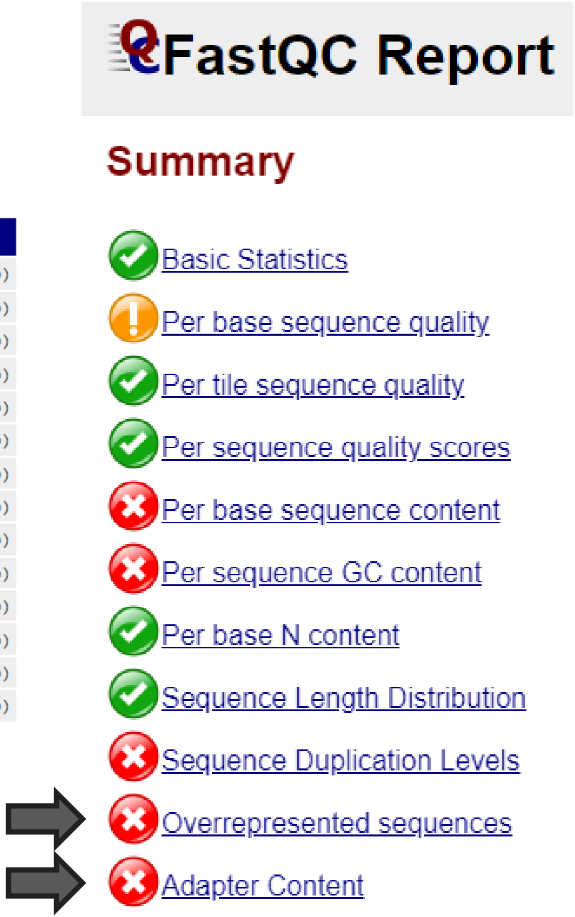
Possible adapter read-through



☒ Overrepresented sequences

Sequence	Count	Percentage	Possible Source
TGAGGTAGTAGATTGTATAGTTAGATCGGAAGAGCACACGTCTGAACTCC	10865	4.346	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TAGCTTATCAGACTGATGTTGACAGATCGGAAGAGCACACGTCTGAACTC	10845	4.338	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TCTTTGGTTATCTAGCTGTATGAGATCGGAAGAGCACACGTCTGAACTCC	7062	2.8247999999999998	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TCTTTGGTTATCTAGCTGTATGAAGATCGGAAGAGCACACGTCTGAACTC	4056	1.6223999999999998	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TGAGGTAGTAGTTTGTGCTGTTAGATCGGAAGAGCACACGTCTGAACTCC	3737	1.4948	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TGAGGTAGTAGTTTGTACAGTTAGATCGGAAGAGCACACGTCTGAACTCC	3549	1.4196	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TGAGGTAGTAGGTTGTATGGTTAGATCGGAAGAGCACACGTCTGAACTCC	2931	1.1724	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
AACCCGTAGATCCGATCTTGTAGATCGGAAGAGCACACGTCTGAACTCCA	1910	0.764	Illumina Multiplexing PCR Primer 2.01 (100% over 29bp)
CGCGACCTCAGATCAGACGTAGATCGGAAGAGCACACGTCTGAACTCCAG	1749	0.6996	Illumina Multiplexing PCR Primer 2.01 (100% over 30bp)
TGAGGTAGTAGGTTGTATAGTTAGATCGGAAGAGCACACGTCTGAACTCC	1647	0.6588	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TCTTTGGTTATCTAGCTGTATAGATCGGAAGAGCACACGTCTGAACTCCA	1622	0.6487999999999999	Illumina Multiplexing PCR Primer 2.01 (100% over 29bp)
TAGCTTATCAGACTGATGTTGATAGATCGGAAGAGCACACGTCTGAACTC	1328	0.5312	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TTCAAGTAATCCAGGATAGGCTAGATCGGAAGAGCACACGTCTGAACTCC	1248	0.4992	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
AGCAGCATTGTACAGGGCTATGAAGATCGGAAGAGCACACGTCTGAACTC	1248	0.4992	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)

Check whether reads contain sequencing adapter
Must be removed!



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- ✓ Per sequence quality scores
- ☒ Per base sequence content
- ☒ Per sequence GC content
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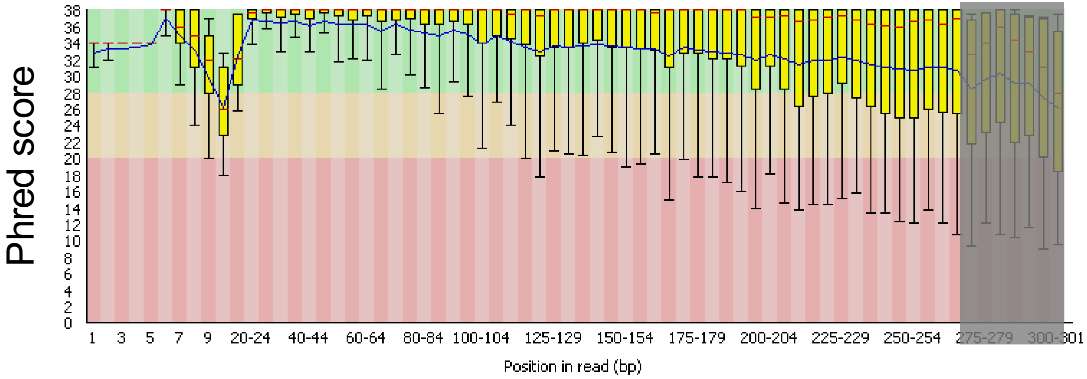
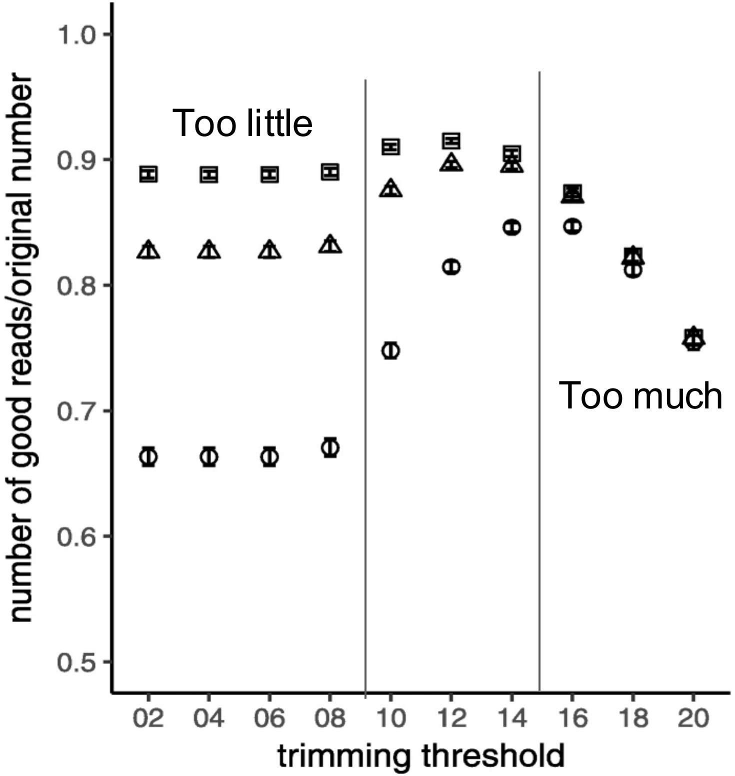
- ✓ Sequence Length Distribution
- ☒ Sequence Duplication Levels
- ☒ Overrepresented sequences
- ☒ Adapter Content

Read trimming

Quality trimming

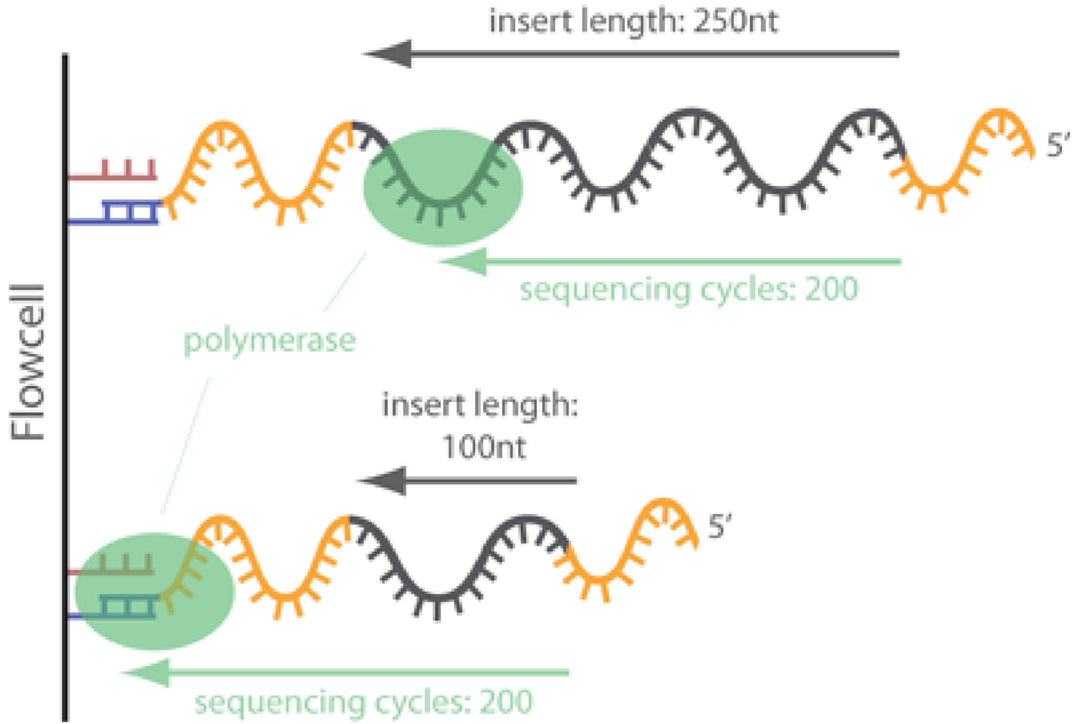


- Remove bases from each end until a minimum quality is reached
    - Or remove a specific number of bases
- Lose some reads but lead to better results

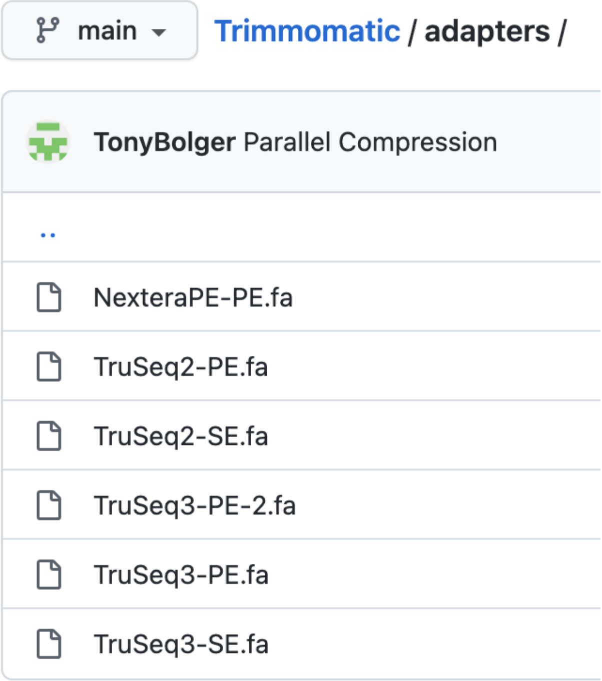


Mohsen, A. et al. BMC Bioinformatics 20:581 (2019)

Adapter trimming



List of known sequencing adapters



Trimmomatic / adapters /

TonyBolger Parallel Compression

..

NexteraPE-PE.fa

TruSeq2-PE.fa

TruSeq2-SE.fa

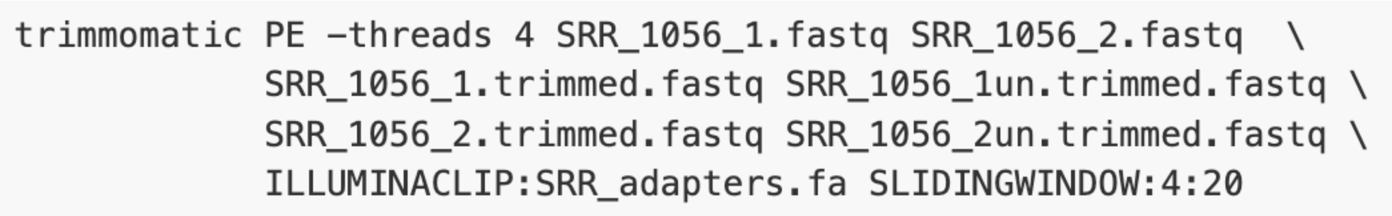
TruSeq3-PE-2.fa

TruSeq3-PE.fa

TruSeq3-SE.fa

https://www.ecseq.com/support/ngs/trimming-adapter-sequences-is-it-necessary

Example of read trimming command



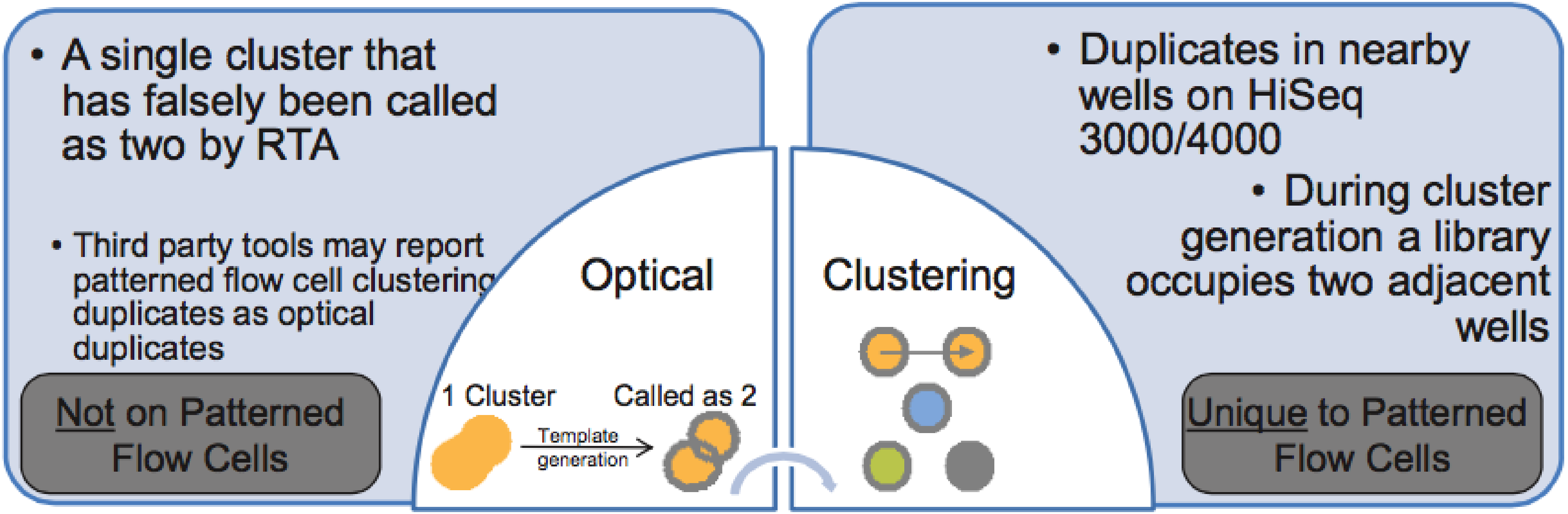
```
trimmomatic PE -threads 4 SRR_1056_1.fastq SRR_1056_2.fastq  \
               SRR_1056_1.trimmed.fastq SRR_1056_1un.trimmed.fastq \
               SRR_1056_2.trimmed.fastq SRR_1056_2un.trimmed.fastq \
               ILLUMINACLIP:SRR_adapters.fa SLIDINGWINDOW:4:20
```

- Use **4 CPU threads**
- Output to trimmed reads to **.trimmed files**
- Output discarded reads to **un.trimmed files**
  - Length became too short after trimming
- Remove adapter sequences listed in **SRR_adapters.fa**
- Check quality score in a sliding window
  - **Average Phred score <= 20** among **4 consecutive nucleotides**

Deduplication

Duplicated reads from technical error

Illumina, 2016



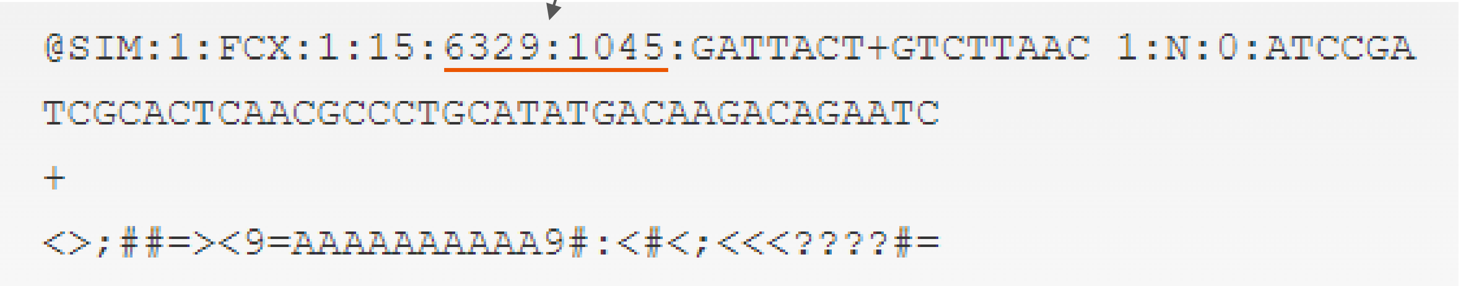
- A single cluster that has falsely been called as two by RTA
  - Third party tools may report patterned flow cell clustering duplicates as optical duplicates

- Duplicates in nearby wells on HiSeq 3000/4000
  - During cluster generation a library occupies two adjacent wells

- Same DNA molecule amplified into adjacent clusters in flow cells
- A large cluster was erroneously read as two clusters

Duplicated reads likely came from the same DNA

(x, y) coordinate on the flow cell

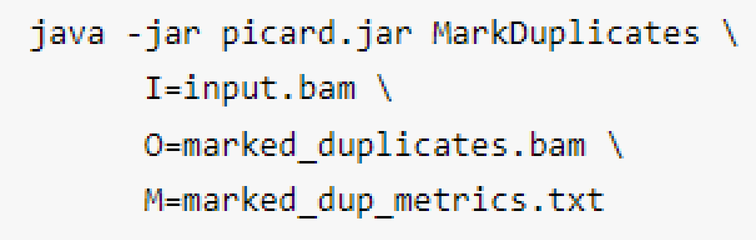


```
@SIM:1:FCX:1:15:6329:1045:GATTACT+GTCTTAAC 1:N:0:ATCCGA
TCGCACTCAACGCCCTGCATATGACAAGACAGAATC
+
<>;##=><9=AAAAAAAAAA9#:<#<;<<<????#=
```

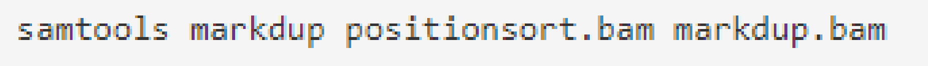
- Reads with identical sequences or mapped positions on the genomes
  - Unlikely to generate multiple identical DNA fragments by chance
  - Retain only one read for each group
- **Illumina**: Nearby cluster coordinates in the header

Problems caused by duplicated reads

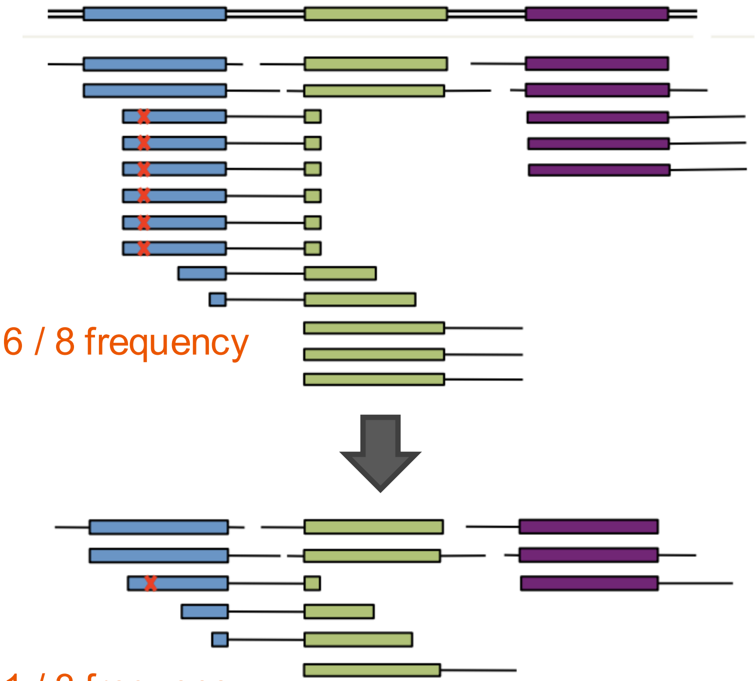
- Lead to incorrect frequency estimates
  - Gene expression level
  - Variant allele frequency
- Many tools can de-duplicate reads
  - Perform after alignment
  - No extra parameters required



```
java -jar picard.jar MarkDuplicates \
      I=input.bam \
      O=marked_duplicates.bam \
      M=marked_dup_metrics.txt
```



```
samtools markdup positionsort.bam markdup.bam
```



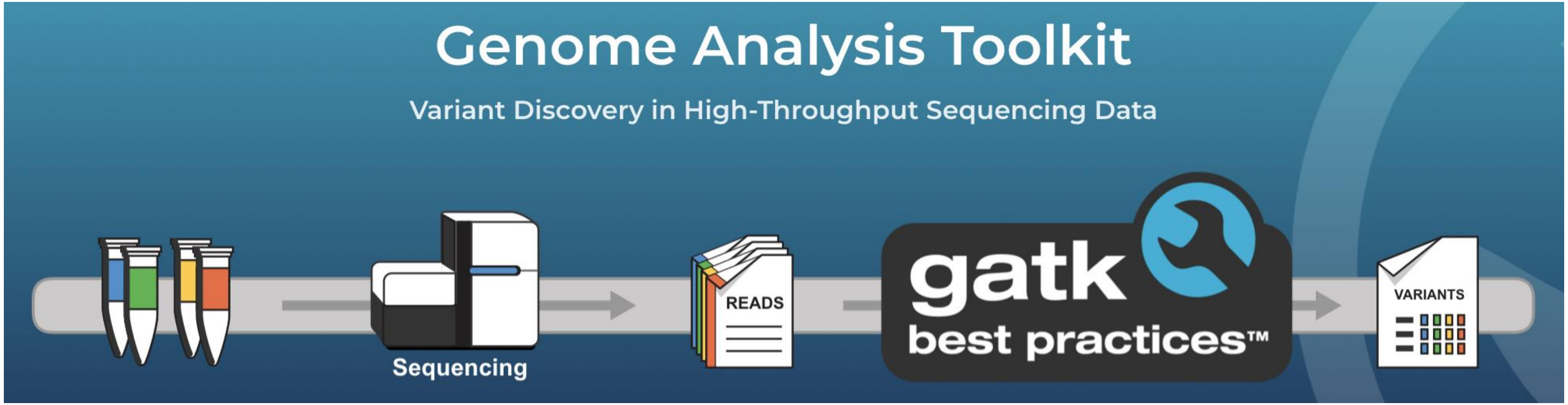
6 / 8 frequency

1 / 3 frequency

Images from qcb.ucla.edu

GATK workflow

The Genome Analysis Toolkit (GATK)

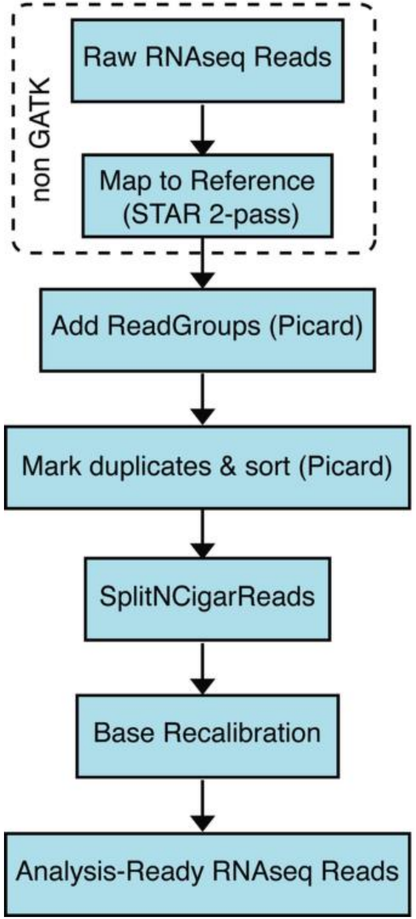


- Developed by Broad Institute since 2010
- Core software uses Java language, with plugins from R and Python
- Industry standard for variant calling workflow

GATK data processing steps

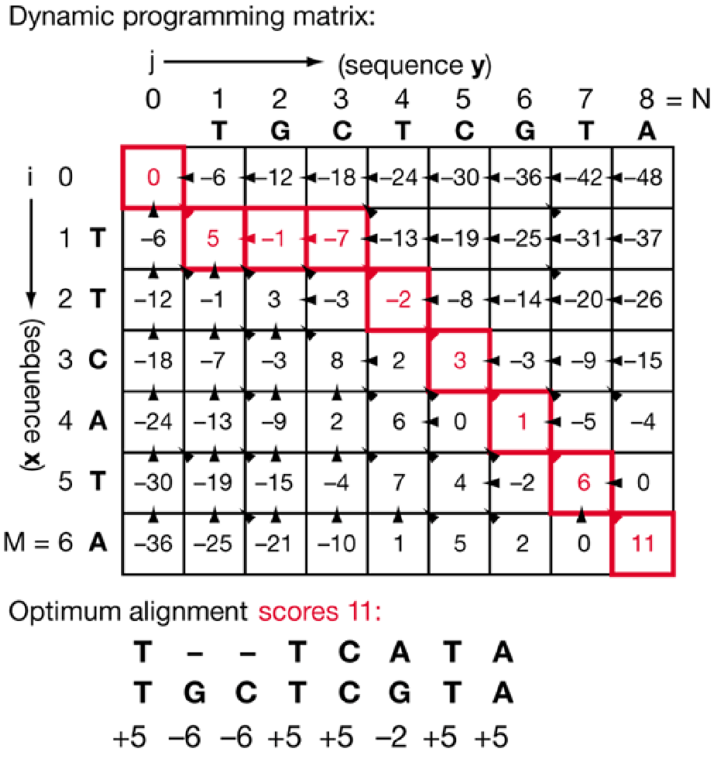
- Performed after sequence alignment

- Key steps:
  - Mark duplicates
  - Split reads based on **N** (unknown base)
    - AACTA**N**CTGAGA → AACTA and CTGAGA
  - Recalibrate quality scores
    - Identify systematic error and apply correction
    - **Example**: 1% more error after reading AAA
    - **Example**: 2% more error after position 120
    - Trained using common variants as truth


non GATK
Raw RNAseq Reads
Map to Reference (STAR 2-pass)
Add ReadGroups (Picard)
Mark duplicates & sort (Picard)
SplitNCigarReads
Base Recalibration
Analysis-Ready RNAseq Reads

Sequence alignment:
Read-to-genome

Dynamic programming alone is not enough



Dynamic programming matrix:

		0	1	2	3	4	5	6	7	8 = N
			T	**G**	**C**	**T**	**C**	**G**	**T**	**A**
i 0		0	–6	–12	–18	–24	–30	–36	–42	–48
1	**T**	–6	5	–1	–7	–13	–19	–25	–31	–37
2	**T**	–12	–1	3	–3	–2	–8	–14	–20	–26
3	**C**	–18	–7	–3	8	2	3	–3	–9	–15
4	**A**	–24	–13	–9	2	6	0	1	–5	–4
5	**T**	–30	–19	–15	–4	7	4	–2	6	0
M = 6	**A**	–36	–25	–21	–10	1	5	2	0	11

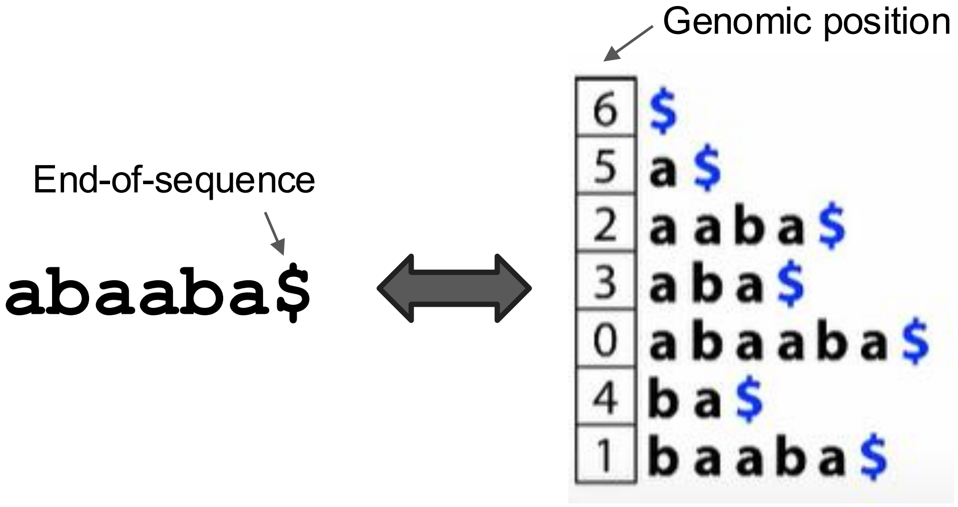
Optimum alignment scores 11:

T	**–**	**–**	**T**	**C**	**A**	**T**	**A**
T	**G**	**C**	**T**	**C**	**G**	**T**	**A**
+5	–6	–6	+5	+5	–2	+5	+5

- Aligning a 150 bp read to a human genome would create a 150 by 3 x10^9 table!
- 10 million reads = 10 million searches
- We need a faster strategy

Eddy, S.R. Nature Biotechnology 22:909-10 (2004)

Indexing



Genomic position

End-of-sequence

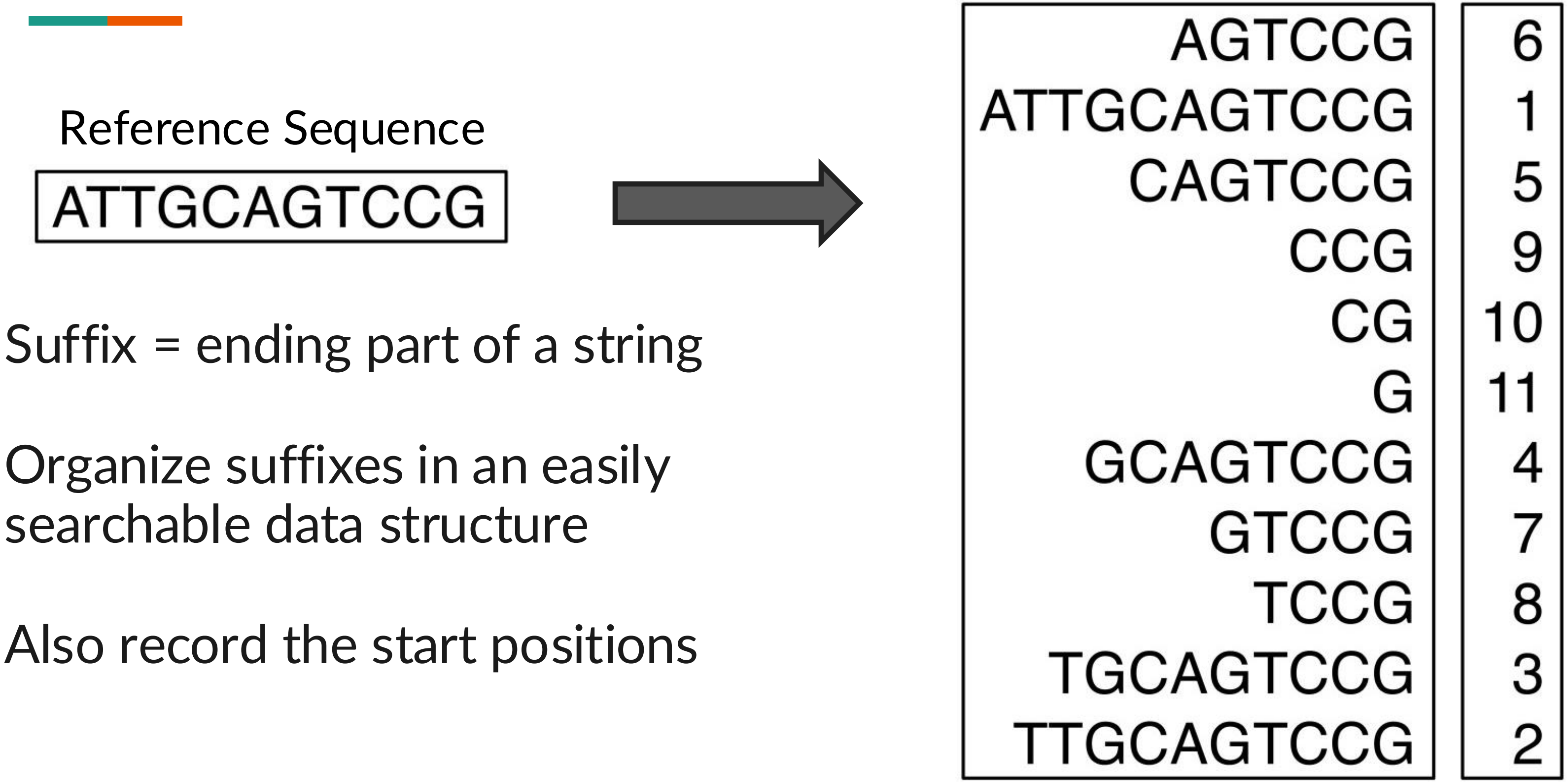
abaaba$

6	$
5	a $
2	a a b a $
3	a b a $
0	a b a a b a $
4	b a $
1	b a a b a $

FM index by Ben Langmead

- The genome is static → We can preprocess beforehand and reuse
- Indexing create a lookup table like alphabetical order in dictionary
    - Generate all possible short DNA fragments and sort

Suffix array



Reference Sequence

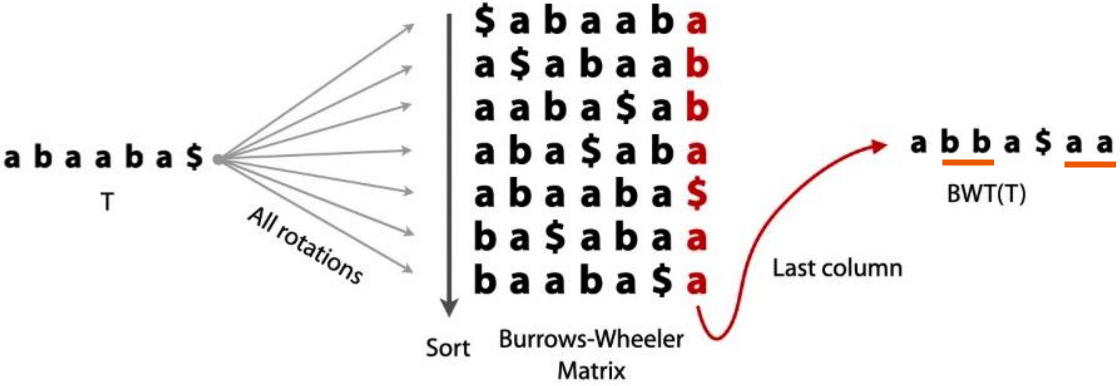
ATTGCAGTCCG

AGTCCG	6
ATTGCAGTCCG	1
CAGTCCG	5
CCG	9
CG	10
G	11
GCAGTCCG	4
GTCCG	7
TCCG	8
TGCAGTCCG	3
TTGCAGTCCG	2

- Suffix = ending part of a string
- Organize suffixes in an easily searchable data structure
- Also record the start positions

Christley, S. et al. BMC Bioinformatics 9:15 (2008)

Burrows-Wheeler transform

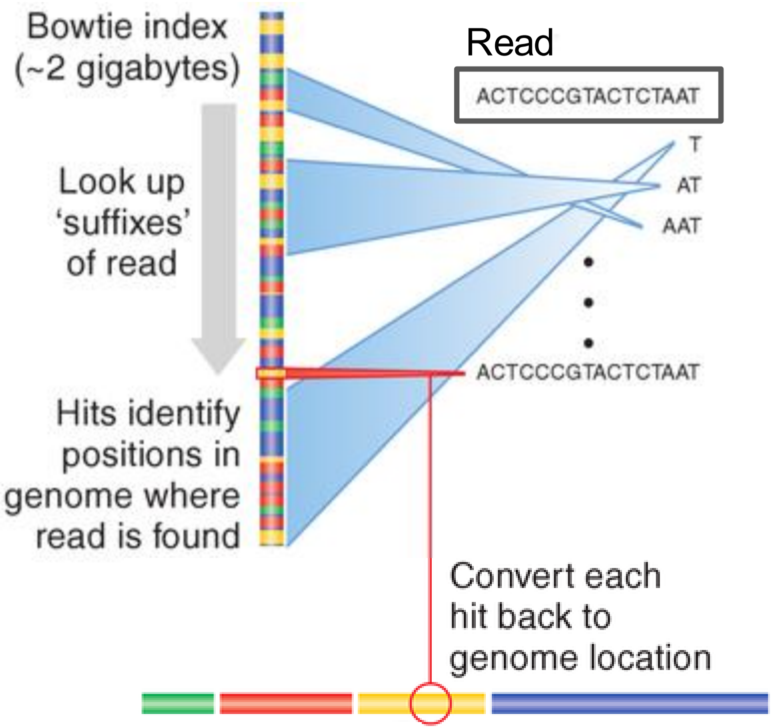


```
$ a b a a b a
a $ a b a a b
a a b a $ a b
a b a $ a b a
a b a a b a $
b a $ a b a a
b a a b a $ a
```

Burrows, M. and Wheeler, D.J. A block sorting lossless data compression algorithm. 1994

- BWT tends to group the same character consecutively
    - Make the data easier to describe/compress: **ab2a$a2**
- BWT is reversible: can recover the original position

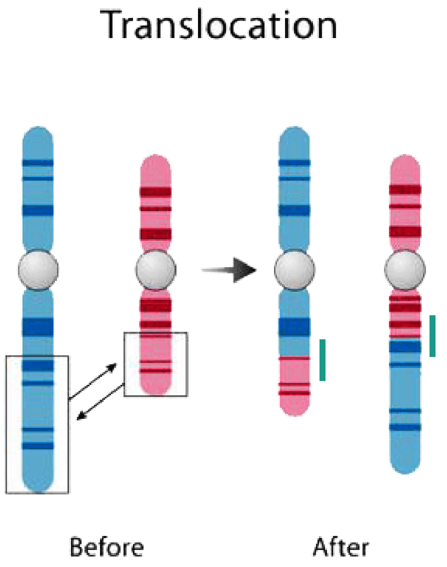
Genome-scale alignment



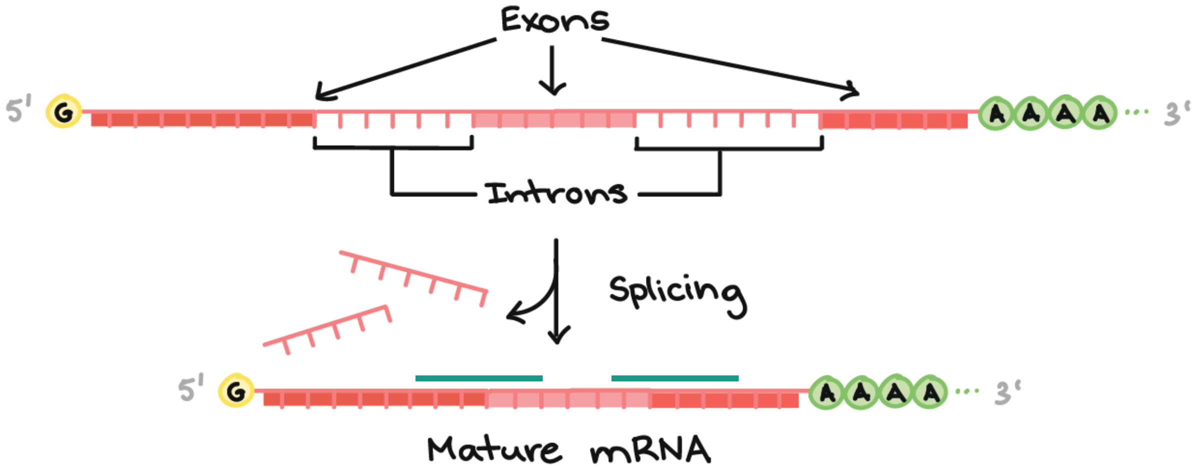
- Use BWT to index the genome
- 20x smaller memory than simple indexing for human genome
- 30x faster search speed

Trapnell, C. and Salzberg, S.L. Nature Biotechnology 27:455-7 (2009)

The need for gapped alignment



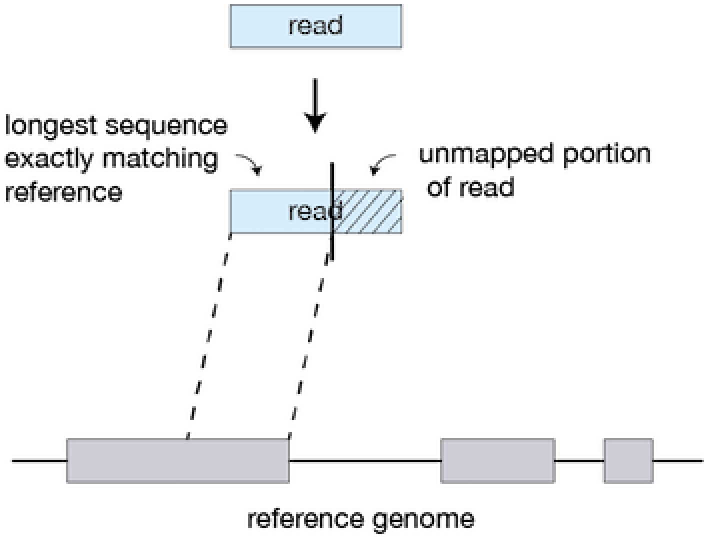
Non-homologous chromosomes exchange segments



- A read can span a long genomic region due to translocation (DNA) or splicing (RNA)

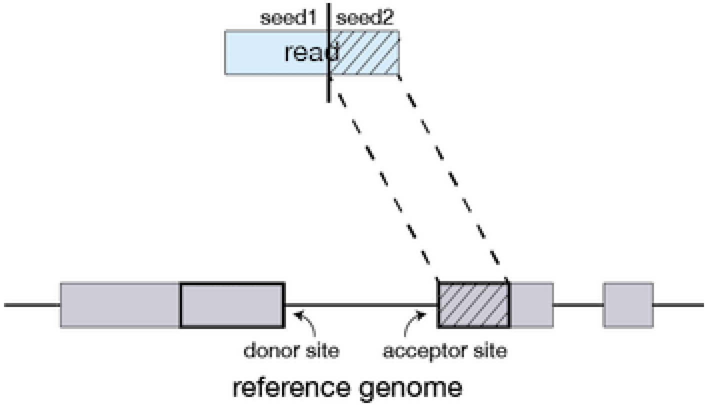
https://www.paragongenomics.com/targeted-sequencing/rna-fusion-detection/

Gapped alignment



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

Essential for aligning RNA-seq data (spliced) and identifying translocation/gene fusion



- Split read into two segments
- Align each segment separately → Look for nearby hits

Typical genome alignment commands

- Bowtie2
  - Index a genome database (FASTA file)

    ```
    bowtie-build GRCh38.fasta GRCh38_db
    ```

  - Perform alignment

    ```
    bowtie -x GRCh38_db -1 sample1_R1.fastq -2 sample1_R2.fastq
            -sam --threads 8 sample1.sam
    ```

- BWA
  - Index a genome

    ```
    bwa index GRCh38.fasta
    ```

  - Perform alignment

    ```
    bwa mem sample1_R1.fastq sample1_R2.fastq > sample1.sam
    ```

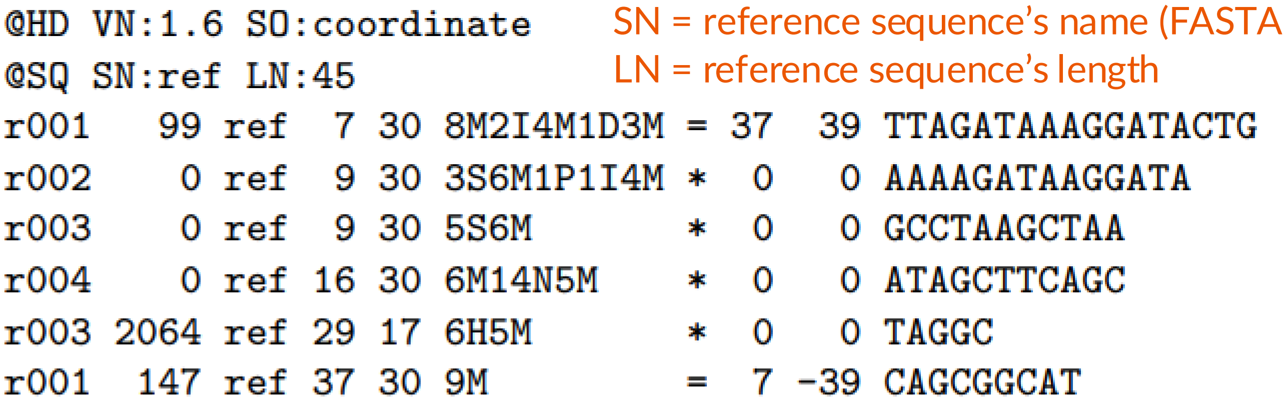
Sequence alignment results

Sequence Alignment Map (SAM)

Sort Order = by genomic coordinate

SN = reference sequence’s name (FASTA header)

LN = reference sequence’s length



```
@HD VN:1.6 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA
r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT
```

- r001 = read name (from sequencing FASTQ)
- ref = reference sequence name (from genomic FASTA)
- 7 = first position on the reference sequence
- 30 = Mapping quality score = $-10 \times \text{Log}_{10}(\text{error})$
- 8M2I4M1D3M = **CIGAR string** = matches, insertion, deletion information

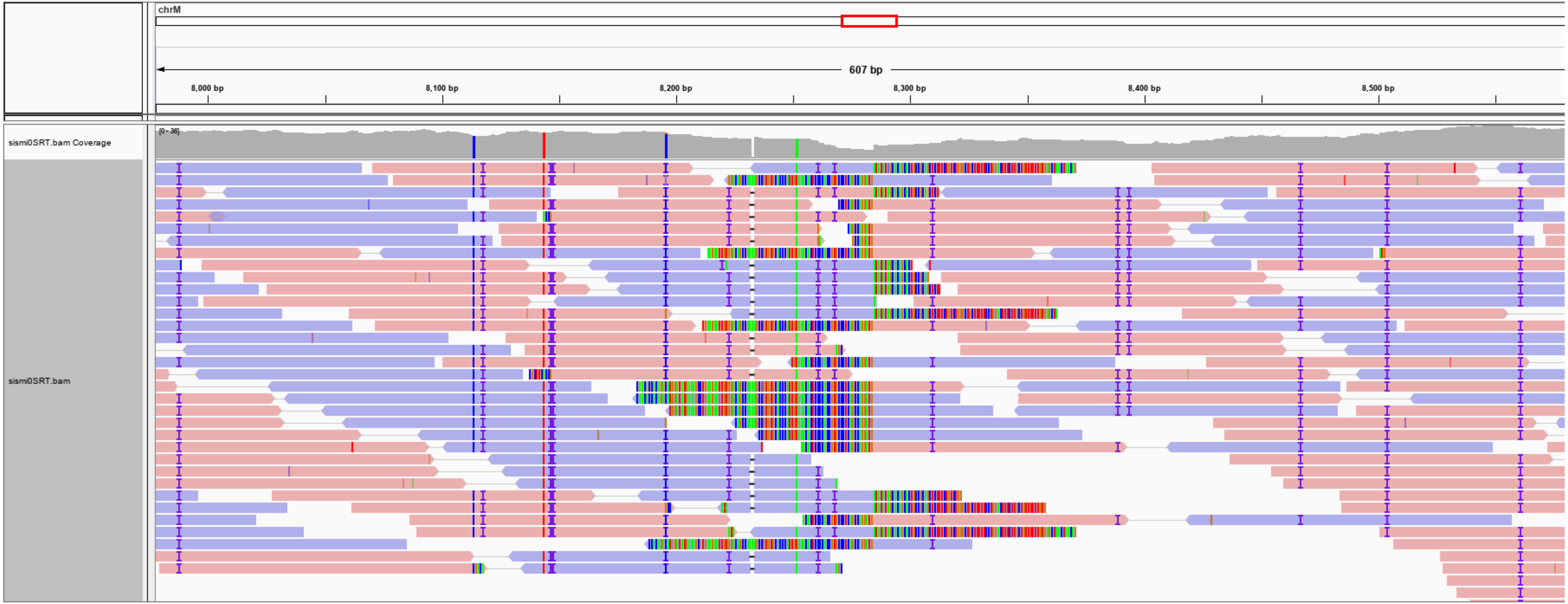
SAM file manipulation

- Sequence alignment results can be sorted, indexed, filtered, and zipped
- BAM is a zipped version of SAM (~40% of the size)

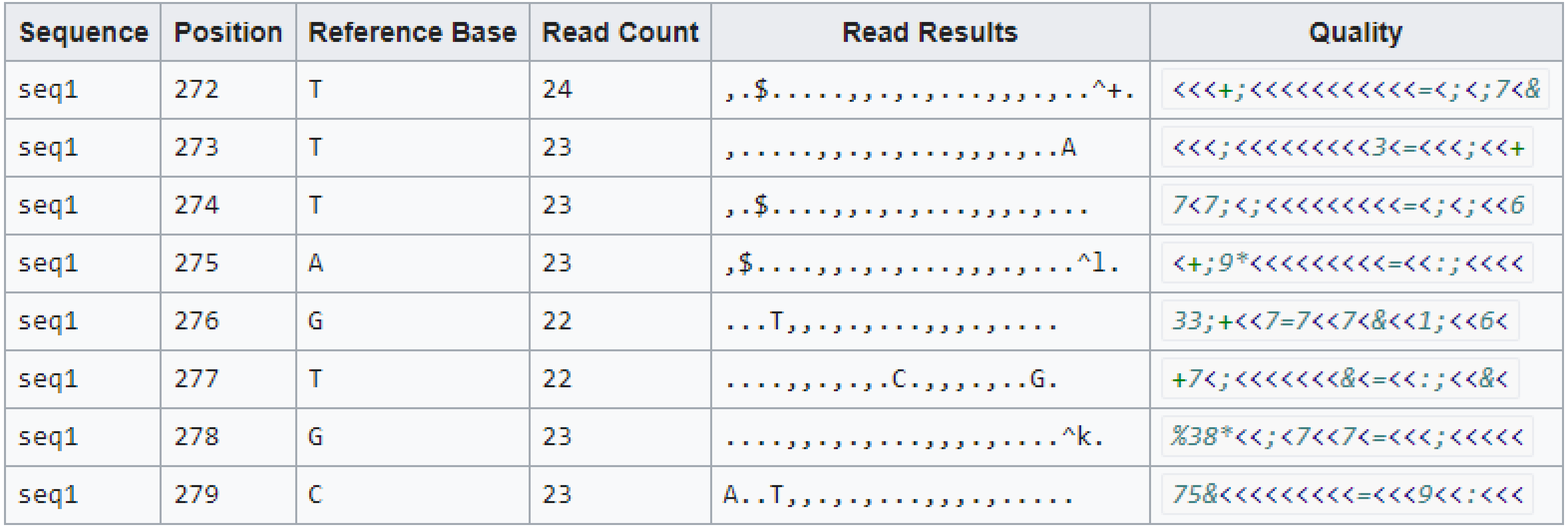
- Sorting and indexing makes alignment results in BAM file easier to be located and analyzed

- Performed with **samtools**

Integrated Genomics Viewer (IGV)



Pileup format



Sequence	Position	Reference Base	Read Count	Read Results	Quality
seq1	272	T	24	,.$.....,,.,.,...,,,.,..^+.	<<<+;<<<<<<<<<<<=<;<;7<&
seq1	273	T	23	,.....,,.,.,...,,,.,..A	<<<;<<<<<<<<<3<=<<<;<<+
seq1	274	T	23	,.$....,,.,.,...,,,.,...	7<7;<;<<<<<<<<<=<;<;<<6
seq1	275	A	23	,$....,,.,.,...,,,.,...^l.	<+;9*<<<<<<<<<=<<:;<<<<
seq1	276	G	22	...T,,.,.,...,,,.,....	33;+<<7=7<<7<&<<1;<<6<
seq1	277	T	22	....,,.,.,.C.,,,.,..G.	+7<;<<<<<<<&<=<<:;<<&<
seq1	278	G	23	....,,.,.,...,,,.,....^k.	%38*<<;<7<<7<=<<<;<<<<<
seq1	279	C	23	A..T,,.,.,...,,,.,.....	75&<<<<<<<<<=<<<9<<:<<<

Image from wikipedia

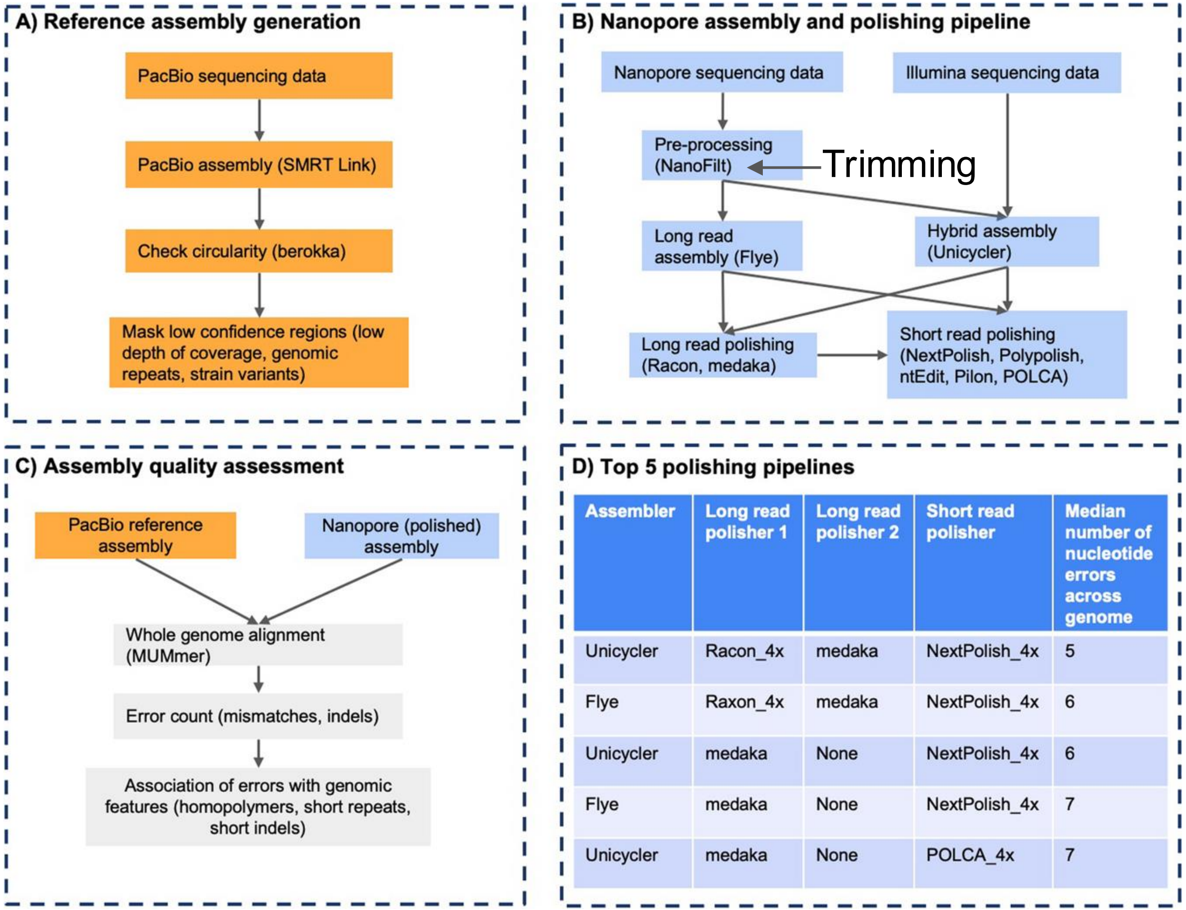
- Focus on each base pair position
  - Summarize match, mismatch, and gap from multiple reads

Long-read data handling

Assembly-polish

- With long-read, **assembly** was performed before **alignment** to genome

- **Polishing** = correct error using raw signals and consensus sequences from multiple reads



A) Reference assembly generation

PacBio sequencing data → PacBio assembly (SMRT Link) → Check circularity (berokka) → Mask low confidence regions (low depth of coverage, genomic repeats, strain variants)

B) Nanopore assembly and polishing pipeline

Nanopore sequencing data

Illumina sequencing data

Pre-processing (NanoFilt)

Trimming

Long read assembly (Flye)

Hybrid assembly (Unicycler)

Long read polishing (Racon, medaka)

Short read polishing (NextPolish, Polypolish, ntEdit, Pilon, POLCA)

C) Assembly quality assessment

PacBio reference assembly

Nanopore (polished) assembly

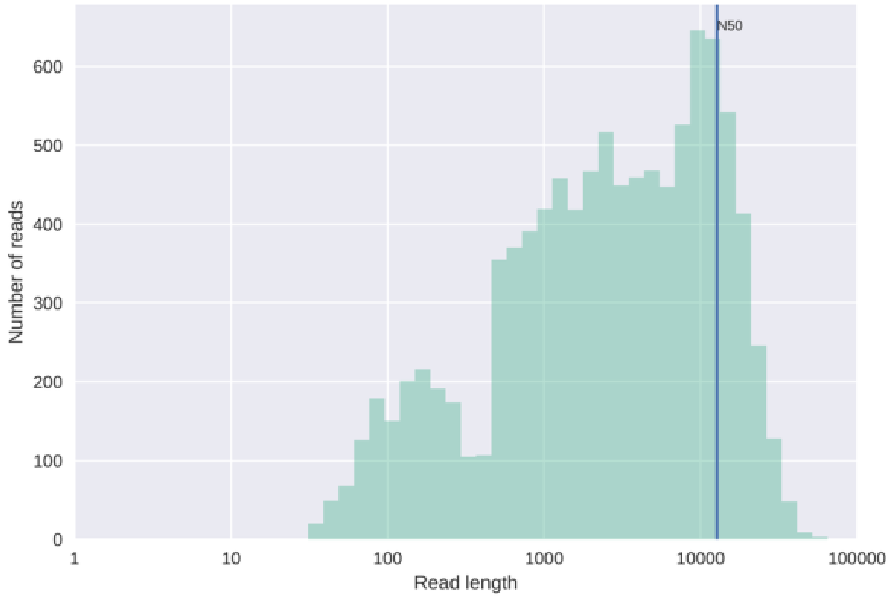
Whole genome alignment (MUMmer) → Error count (mismatches, indels) → Association of errors with genomic features (homopolymers, short repeats, short indels)

D) Top 5 polishing pipelines

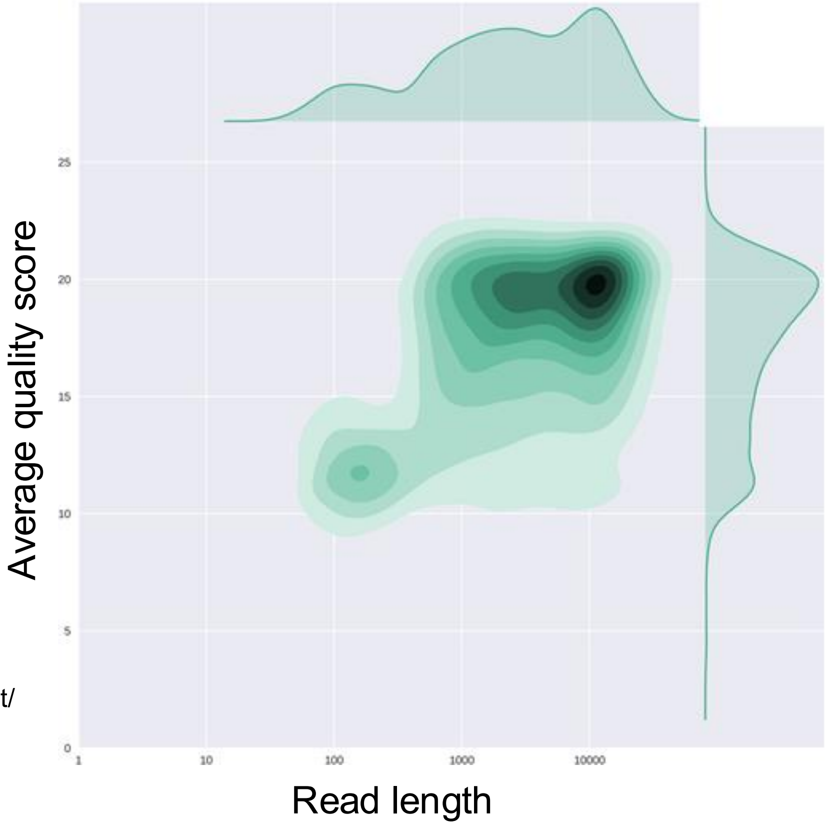
Assembler	Long read polisher 1	Long read polisher 2	Short read polisher	Median number of nucleotide errors across genome
Unicycler	Racon_4x	medaka	NextPolish_4x	5
Flye	Raxon_4x	medaka	NextPolish_4x	6
Unicycler	medaka	None	NextPolish_4x	6
Flye	medaka	None	NextPolish_4x	7
Unicycler	medaka	None	POLCA_4x	7

Luan, T. et al. BMC Genomics 25:679 (2024)

QC with NanoPlot



https://gigabaseorgigabyte.wordpress.com/2017/06/01/example-gallery-of-nanoplot/



- Read length and quality scores

Nanopore polishing algorithms and tools

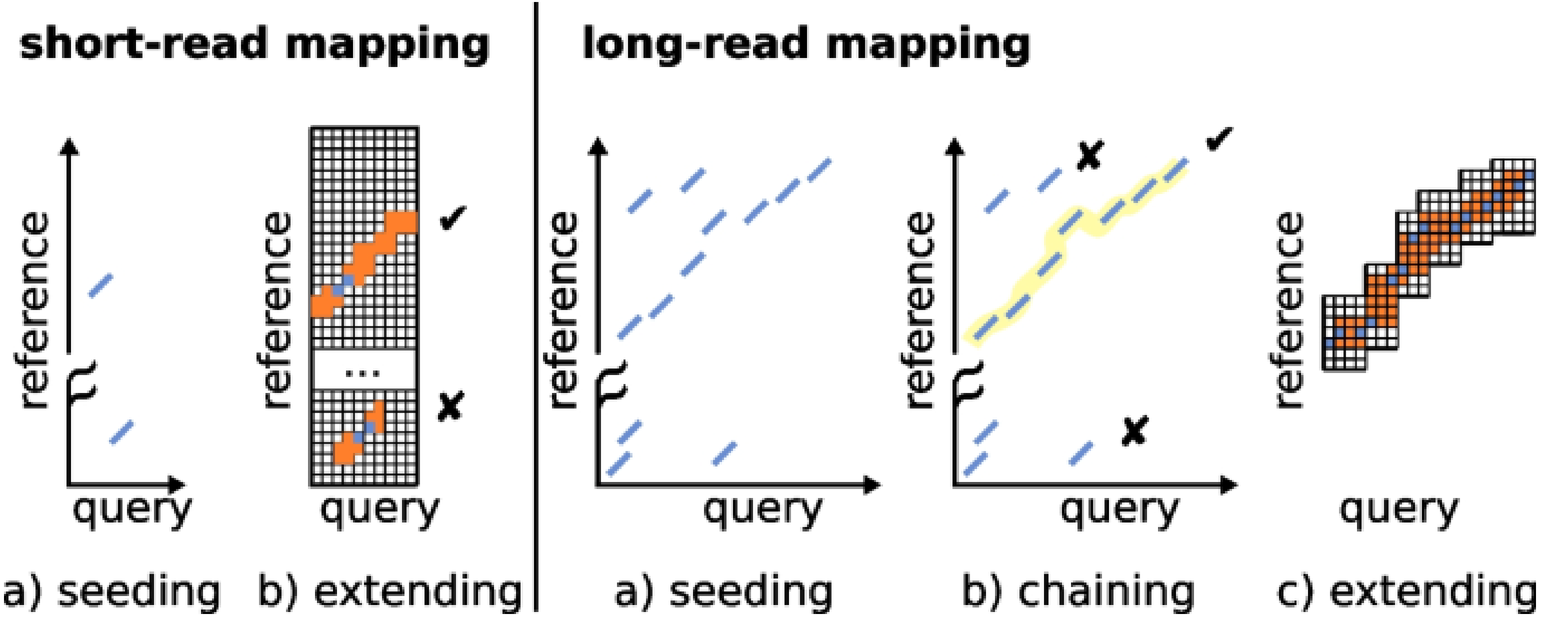
- Many tools: Nanopolish, Medaka, Racon, etc.

- Consensus sequence called by performing multiple sequence alignment among reads

- Repeatedly edit the consensus sequence and compare theoretical signal to the observed signal, maximizing the probability

- Polish using long-read or short-read data (if available)

Seed-and-chain alignment for long-read data



Sahlin, K. et al. Genome Biology 24:133 (2023)

- **Minimap2**: Index unique DNA subsequences (minimizers)

Any question?

- See you next time