NGS: Read Mapping to Reference Genome

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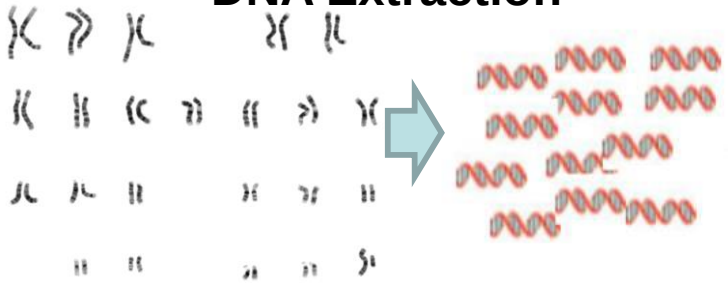
Learning Objectives

- Overal understanding of the data preprocessing steps
  - Alignment of NGS reads (fastq) to a reference sequence
    - The SAM / BAM format
    - Secondary and supplementary alignments
  - Marking Duplicates
  - Base Quality Recalibration
- Visualizing results and assessing quality

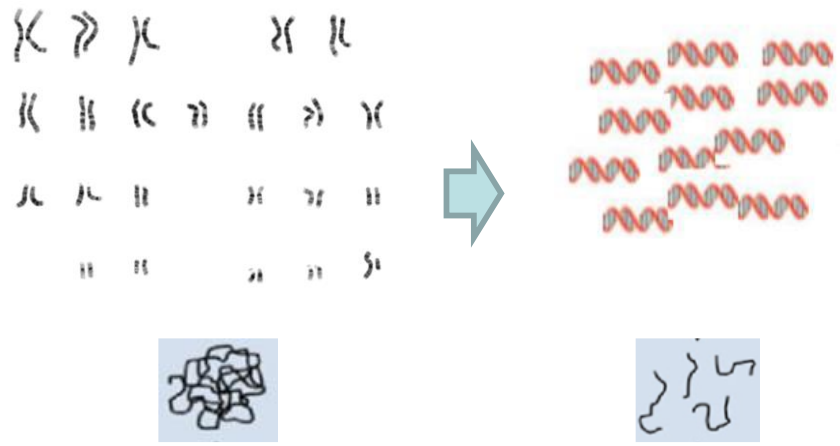
Data pre-processing for variant discovery

DNA Extraction

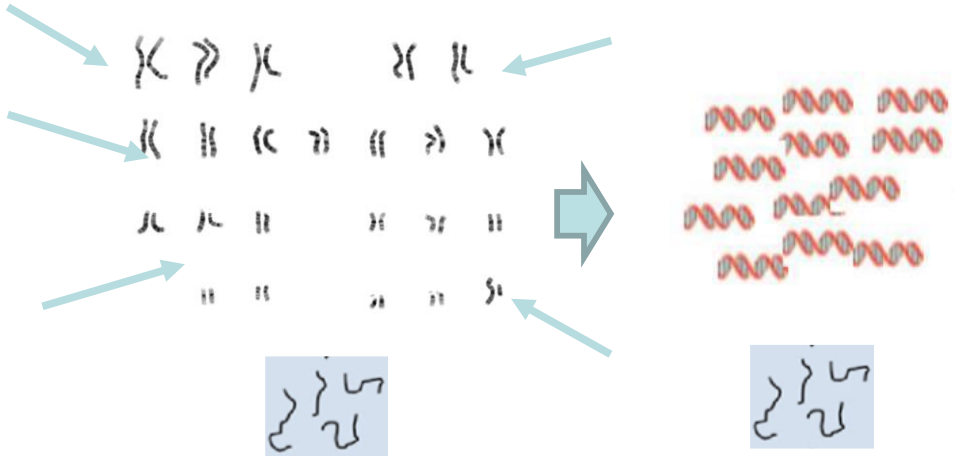


Data pre-processing for variant discovery

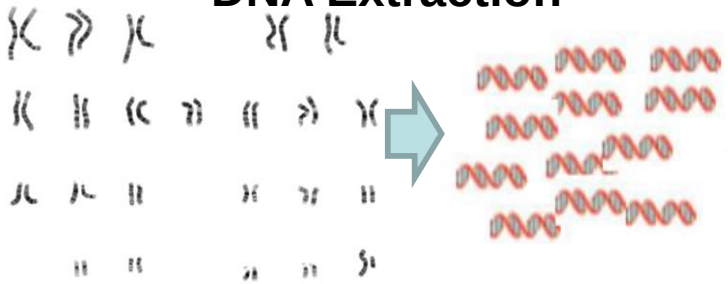
Whole Genome



Targeted (eg. WES)



DNA Extraction



Eg. TruSight One Enrichment Panel

How to calculate coverage

- WGS 30x coverage, 150bp read pairs
  - 30 x $3x10^9$ / (150x2) = $3x10^8$ = 300 million read pairs
- WES (~1-2% genome) 30x coverage, 150bp read pairs
  - 6 million read pairs (theoretical minimum, but usually more)

Genome (WGS) vs Exome (WES)

Advantages of WES over WGS

- WES only focus on potentially actionable regions
  - WES is more economic and less compute intensive

Disadvantages of WES

- WES can have primer-derived artifacts
  - eg. amplification bias, primer dimers, kit-dependent
- WES is very limited for structural variants

Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

DNA Extraction **Sequencing**

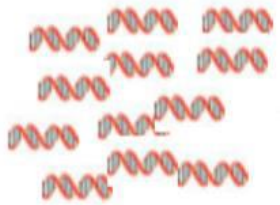
TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

DNA Extraction



Sequencing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

QC and pre-processing

TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

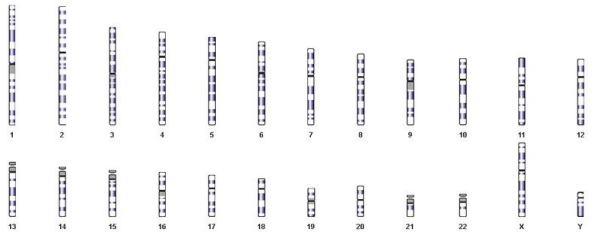
Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

(fasta file)

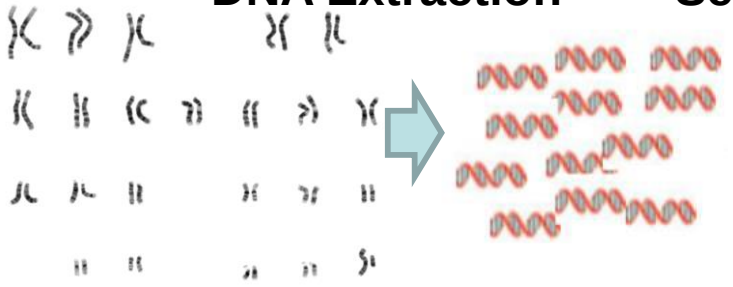
Reference Genome



geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

DNA Extraction **Sequencing**



TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT **(“raw” fastQ)**

What genome reference to use?

- GRCh37 (hg19)
  - The “classical”, for which most resources were built (1000 genomes, etc…)
- **GRCh38 (hg38)**
  - **The recommended one. Most resources have been converted to this now**
- T2T-CHM13
  - Most complete genome, but will take time for resources to be available

Future: pangenome graphs

A lot of investment is going here, but several challenges remain

Mostly relevant for Structural Variants

What genome reference to use?

Things to consider:

- Mask PAR regions in Y chromosome
- Include decoys (eg. EBV sequences)
- Include alternative sequences such as HLA loci
    - Though these may need to be analized specifically

https://gatk.broadinstitute.org/hc/en-us/articles/360035890951-Human-genome-reference-builds-GRCh38-or-hg38-b37-hg19

https://gatk.broadinstitute.org/hc/en-us/articles/360035890951

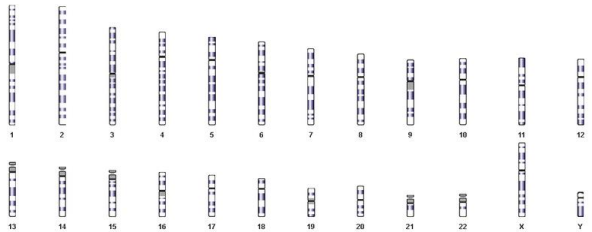
Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

(fasta file)

Reference Genome



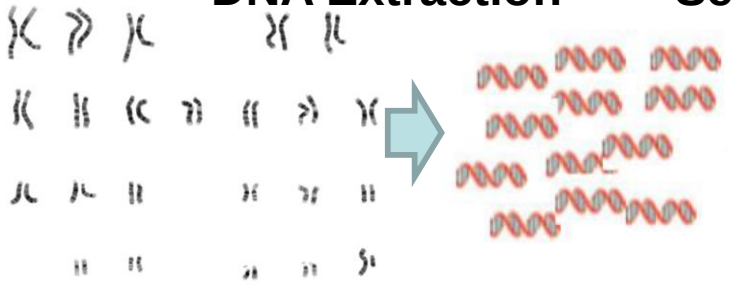
TGCTCAGTT

geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

Alignment

DNA Extraction **Sequencing**



TGCTCAGTT TGAC ATGGAGT GTTGT **(“clean” fastQ)**

QC and pre-processing

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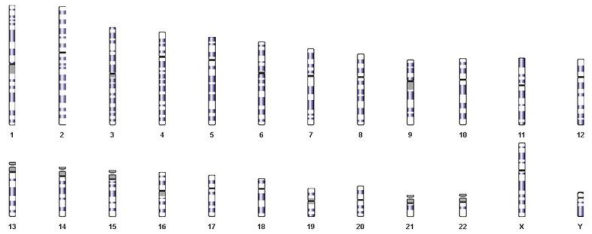
Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

(fasta file)

Reference Genome



TGCTCAGTT

geneA geneB geneC

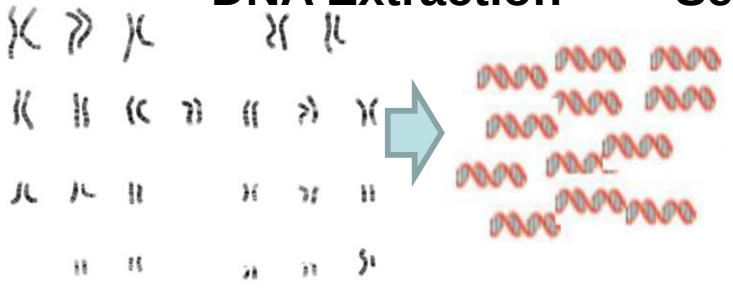
AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

Primary Alignment

Secondary Alignment

DNA Extraction

Sequencing



TGCTCAGTT TGAC ATGGAGT GTTGT

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT

(“clean” fastQ)

(“raw” fastQ)

Data pre-processing for variant discovery

Raw Unmapped Reads

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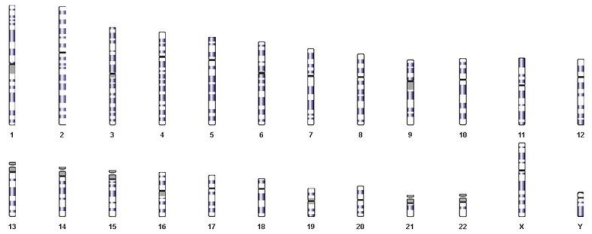
(fasta file)

Reference Genome



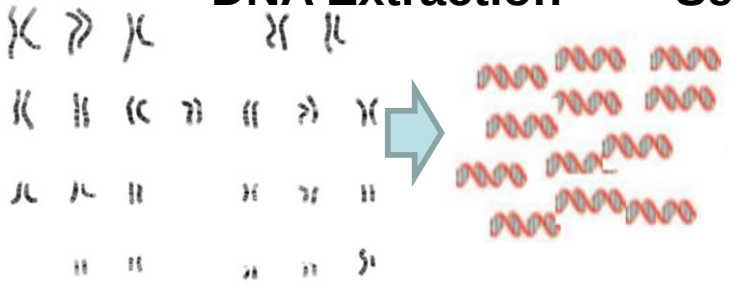
ACTCCAT GTTGT

TGCTCAGTT

geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

DNA Extraction **Sequencing**



TGCTCAGTT TGAC ATGGAGT GTTGT

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QC and pre-processing

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(“clean” fastQ)

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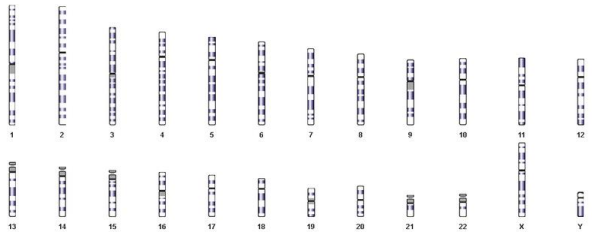
Reference Genome



TCCATGC AGTTGTGT

ACTCCAT GTTGT

AAGCGATG TGCTCAGTT

GTGTGTTT-CA

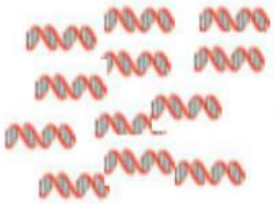
geneA geneB geneC

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

(SAM/BAM)

DNA Extraction

Sequencing



TGCTCAGTT TGAC ATGGAGT GTTGT

(ACTCCAT)

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT

(“clean” fastQ)

(“raw” fastQ)

SAM/BAM format

A file format to represent alignments

BAM -> binary form of SAM

```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

+r001/1        TTAGATAAAGGATA*CTG
+r002         aaaAGATAA*GGATA
+r003       gcctaAGCTAA
+r004                     ATAGCT..............TCAGC
-r003                           ttagctTAGGC
-r001/2                                      CAGCGGCAT
```

```
@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
```

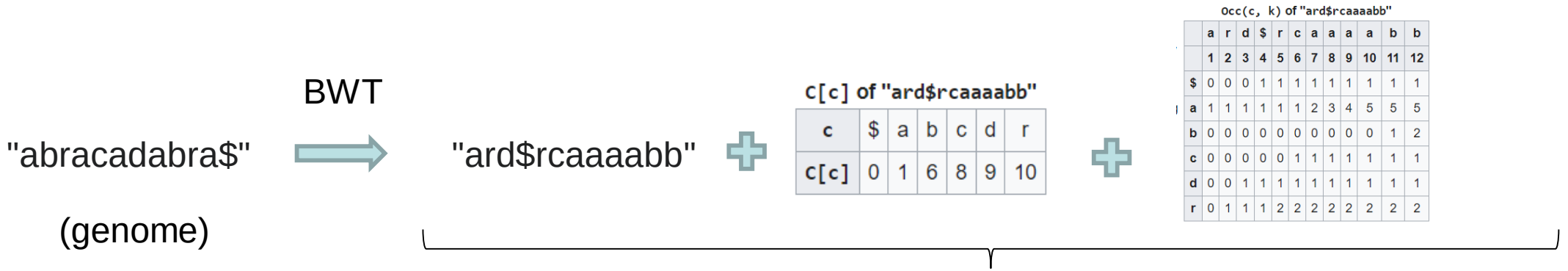
https://samtools.github.io/hts-specs/SAMv1.pdf

How alignment is made in practice

BWA (Burrows-Wheeler Aligner) is the most popular tool for WGS/WES

- Align millions of short reads to a human-sized genome in minutes

It is based on the FM-index of the Burrows-Wheeler Transform



c	$	a	b	c	d	r
C[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

https://en.wikipedia.org/wiki/FM-index

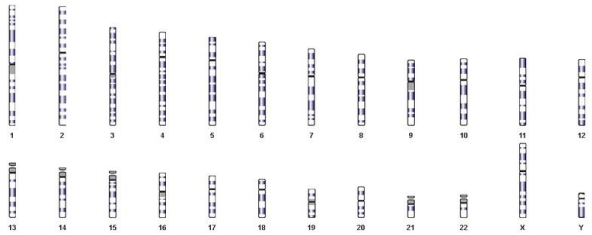
How alignment is made in practice

Genome BWT FM-índex needs to be created (only once)

$ bwa index genome.fasta

(genome.fasta file)

Reference Genome



3x10^9 bases

AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

BWT

c	$	a	b	c	d	r
c[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

FM - index

How alignment is made in practice

Basic command to generate alignments with BWA:

$ bwa mem genome.fasta reads_R1.fastq(.gz) reads_R2.fastq(.gz)>output.sam

reads_R1.fastq
TGCTCAGTT

reads_R2.fastq
ACGTCCGA

BWT

c	$	a	b	c	d	r
C[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

Output in SAM format

TGCTCAGTT Chr2 position xxx

ACGTCCGA Chr2 position yyy

```
@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
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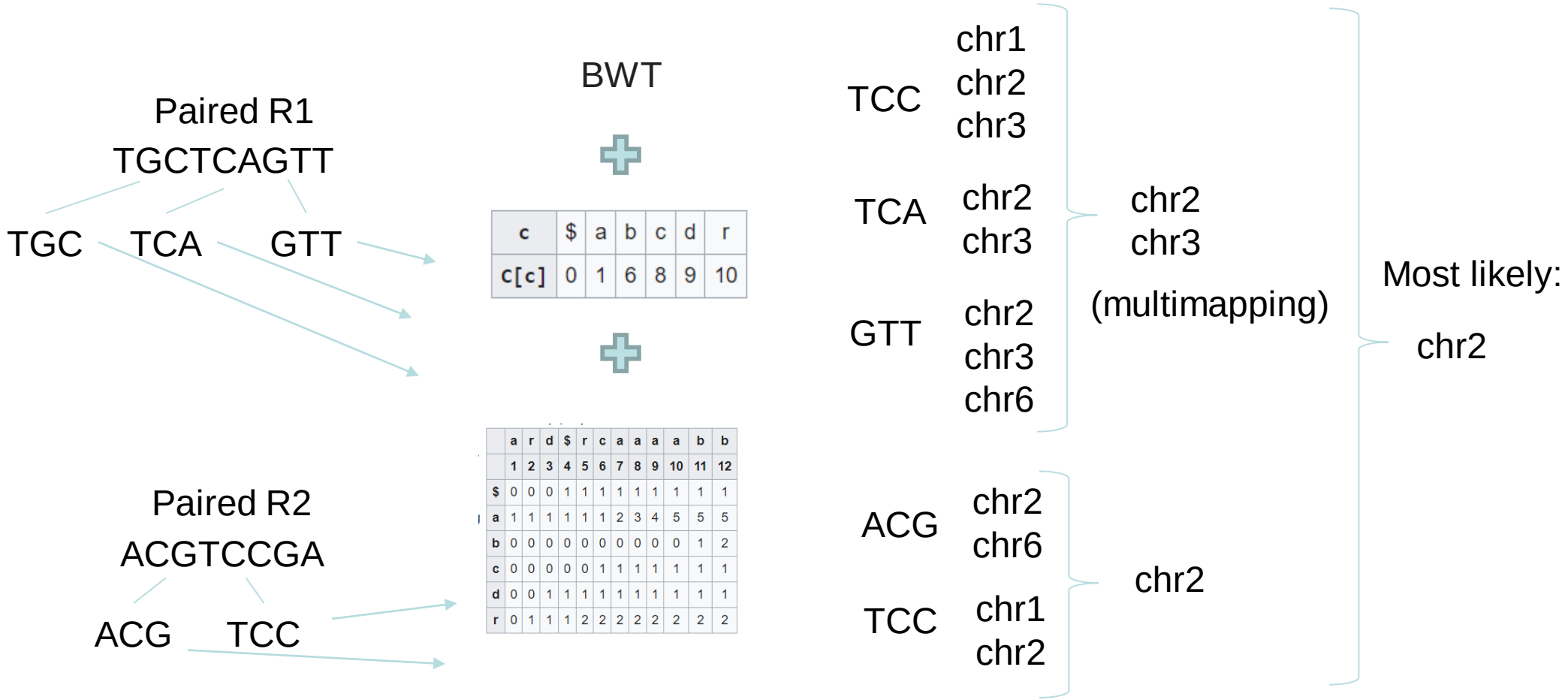
How alignment is made in practice



c	$	a	b	c	d	r
c[c]	0	1	6	8	9	10

	a	r	d	$	r	c	a	a	a	b	b
	1	2	3	4	5	6	7	8	9	10	11	12
$	0	0	0	1	1	1	1	1	1	1	1	1
a	1	1	1	1	1	1	2	3	4	5	5	5
b	0	0	0	0	0	0	0	0	0	0	1	2
c	0	0	0	0	0	1	1	1	1	1	1	1
d	0	0	1	1	1	1	1	1	1	1	1	1
r	0	1	1	1	2	2	2	2	2	2	2	2

How alignment is made in practice: Summary

- Special algorithms are used to have fast alignments
  - They are not guaranteed to be perfect but most of the time they are very good
- A read can map equally well to multiple regions (multimappings)
  - BWA reports **one primary alignment (randomly chosen) with mapping quality of 0**
  - Depending on the software, it can generate **secondary alignments**
  - Information of paired reads are used to disambiguate multimappings if possible
- Alignments are made piece-wise (a read is split in segments)
  - A read alignment can be split in a primary and **supplementary alignment(s)**
    - Eg. splicing in RNA-Seq; large deletions
  - Sometimes, only a part of the read is aligned (the rest is “masked”/hidden)
    - Particularly in repetitive areas this can lead to false alignments

Practical Exercise

You will only need to run alignment using ‘bwa mem’, like this:

$ bwa mem –t 1 –M -R ‘@RG\tID:sampleid\tPU:FlowCell.Lane.Sample\tSM:samplename\tPL:ILLUMINA\tLB:samplename\tCN:UTI’ genoma.fasta reads_R1.fastq.gz reads_R2.fastq.gz > output.sam

-M: Mark split reads as secondary (relevant for detecting duplicates)

-R: Read Group (to associated specific metadata to each read)

ID: Identifier; PU: Platform Code (FlowCell.Lane.Sample); SM: Sample; PN: LB: Library; CN: Center Name (Sequencing Team) ; other fields possible (see SAM specification)

-t: number of “threads” (CPU processors) to use

Practical Exercise

‘bwa mem’ generates a SAM file. To generate a more compact BAM file that is used to visualize and in variant calling, we need to process the SAM:

- Sort SAM by coordinates using ‘samtools sort’

$ samtools sort file.sam –o output_sorted.sam

- Convert SAM to BAM using ‘samtools view’

$ samtools view –Sb file.sam –o output.bam

-S: input file is SAM; -b: output file is bam

- Index the BAM so it can used by other programs using ‘samtools index’

$ samtools sort file.bam –o output_sorted.bam

(note: this has nothing to do with ‘bwa índex’)

Practical Exercise

- There are three samples, use data from any/all:
  - Align selected reads against a specific chromosome
    - What is the identifier of the first aligned read? Where does it align?
  - Sort the aligned reads
    - What is the identifier of the first aligned read? Where does it align?
  - Convert to a bam and index the bam

Commands to run are in the github

Interlude

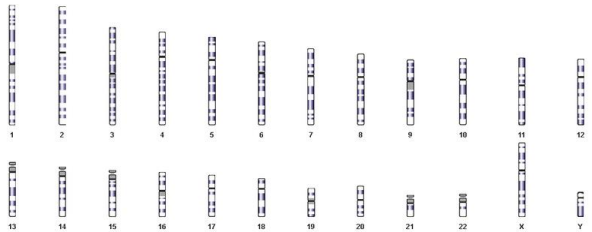
Data pre-processing for variant discovery

Raw Unmapped Reads

uBAM or FASTQ

(fasta file)

Reference Genome



TCCATGC AGTTGTGT

ACTCCAT GTTGT

AAGCGATG TGCTCAGTT

GTGTGTTT-CA

geneA geneB geneC

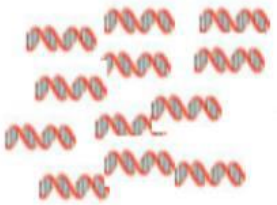
AAGCGATGACTGCATGCACAGTTGTGTGTTTTCACGTGAC

Alignments

(SAM/BAM)

DNA Extraction

Sequencing



TGCTCAGTT TGAC ATGGAGT GTTGT

(ACTCCAT)

QC and pre-processing

TGCTCAGTTA TGAC ATGGAGT TTT CGTTGT

(“clean” fastQ)

(“raw” fastQ)

Data pre-processing for variant discovery
Duplicated Reads

- Duplicate reads (same fragment) can appear
  - In library preparation during amplification (eg. WES)
  - In the amplification process while sequencing (optical duplicates)
  - They can generate “false” mutations, or at a false frequency

Data pre-processing for variant discovery
Duplicated Reads

Read pairs need to be taken in consideration

Data pre-processing for variant discovery
Duplicated Reads

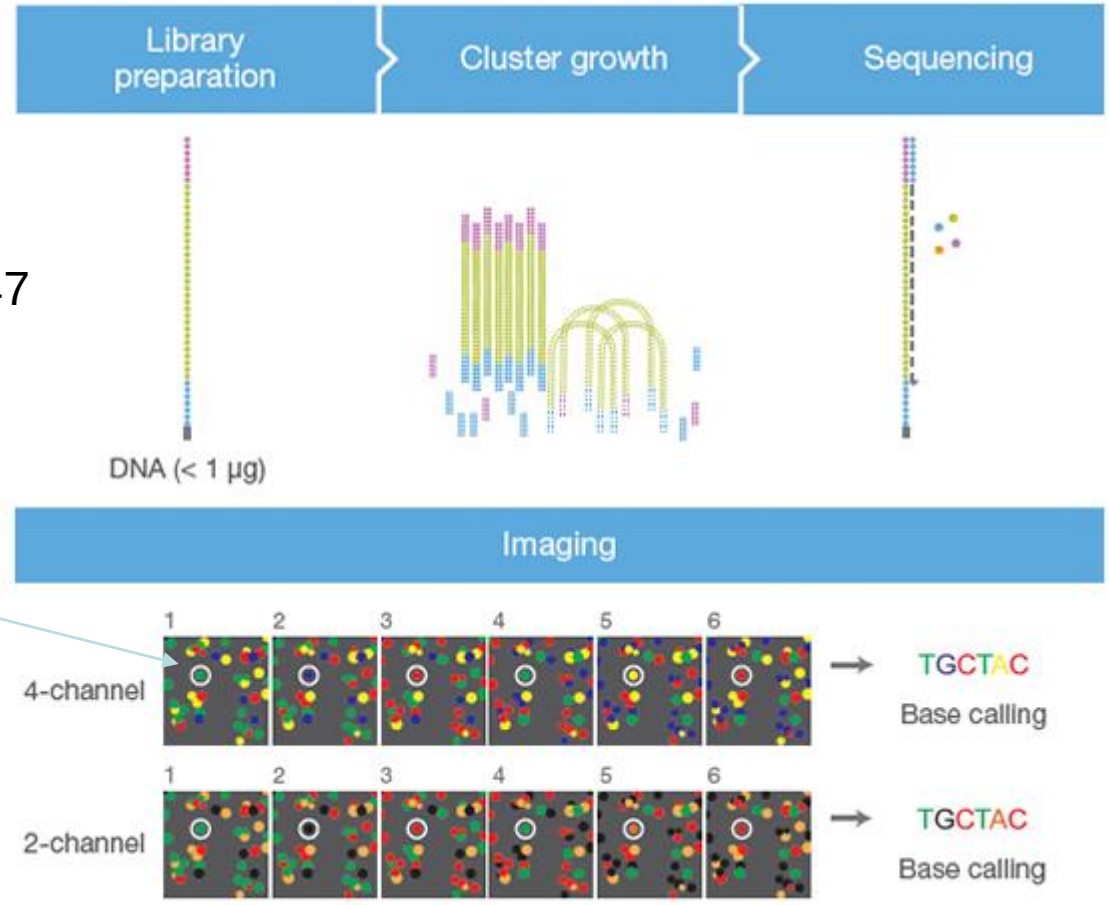
Duplicates are identified by position (alignment and/or flow-cell), not by sequence

Data pre-processing for variant discovery
Duplicated Reads: optical duplicates

@MN00723:33:000H3MCVT:1:11102:7591:1087 1:N:0:47

Machine Flow Cell Lane Position

Optical duplicates are duplicate reads that are very close in the flow cell



Data pre-processing for variant discovery Duplicated Reads

- **The recommended practice is to ignore duplicates**
  - Only consider one of the duplicates for variant calling
    - Usually the one with the best quality
  - This may remove good information (eg. with high coverage, targeted)
  - Duplicates are marked and later ignored (or not)
  - Benefits of marking duplicates not always obvious
    - https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4965708/
    - Eg. one can chose to only ignore reads marker as optical duplicates

Data pre-processing for variant discovery Duplicated Reads

We will run this process using ‘gatk MarkDuplicates’

It takes a BAM file and annotates duplicated reads:

```
> gatk MarkDuplicates -I=sample.bam -O=sample_marked.bam -M=sample_marked_metrics.txt
```

It generates another BAM file with the same number of alignments, but alignments determined as duplicated have an annotation that can be used.

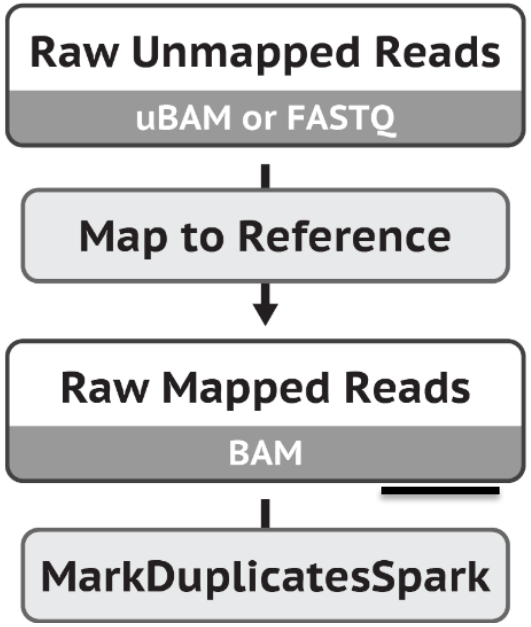
It generates a file with metrics from the analysis (described in next slide)

Data pre-processing for variant discovery Duplicated Reads

Metrics

Field	Description
LIBRARY	The library on which the duplicate marking was performed.
UNPAIRED_READS_EXAMINED	The number of mapped reads examined which did not have a mapped mate pair, either because the read is unpaired, or the read is paired to an unmapped mate.
READ_PAIRS_EXAMINED	The number of mapped read pairs examined. (Primary, non-supplemental)
SECONDARY_OR_SUPPLEMENTARY_RDS	The number of reads that were either secondary or supplementary
UNMAPPED_READS	The total number of unmapped reads examined. (Primary, non-supplemental)
UNPAIRED_READ_DUPLICATES	The number of fragments that were marked as duplicates.
READ_PAIR_DUPLICATES	The number of read pairs that were marked as duplicates.
READ_PAIR_OPTICAL_DUPLICATES	The number of read pairs duplicates that were caused by optical duplication. Value is always < READ_PAIR_DUPLICATES, which counts all duplicates regardless of source.
PERCENT_DUPLICATION	The fraction of mapped sequence that is marked as duplicate.
ESTIMATED_LIBRARY_SIZE	The estimated number of unique molecules in the library based on PE duplication.

Data pre-processing for variant discovery



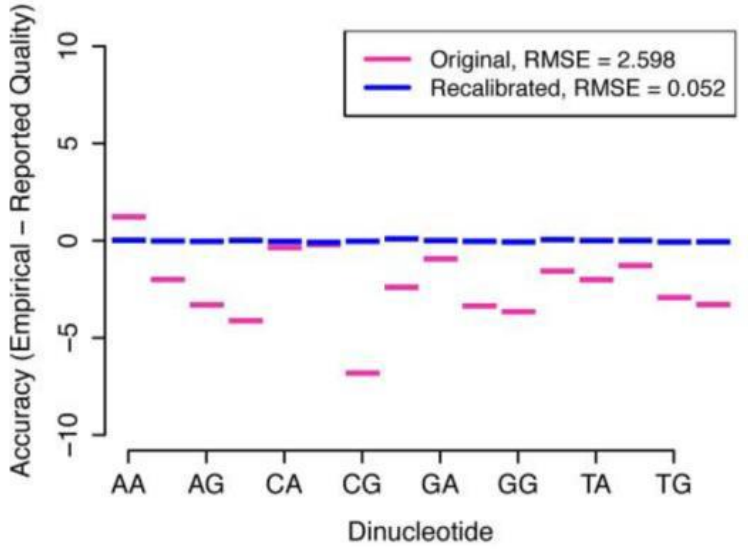
Now we have a BAM with potential duplicated alignments marked

Data pre-processing for variant discovery Base Quality Recalibration

Base Quality not very precise

Depends on several factors:

- Sample Quality (DNA)
- Nucleotide context
- Machine and cycle of sequencing
- Type of variant (SNP or Indel)

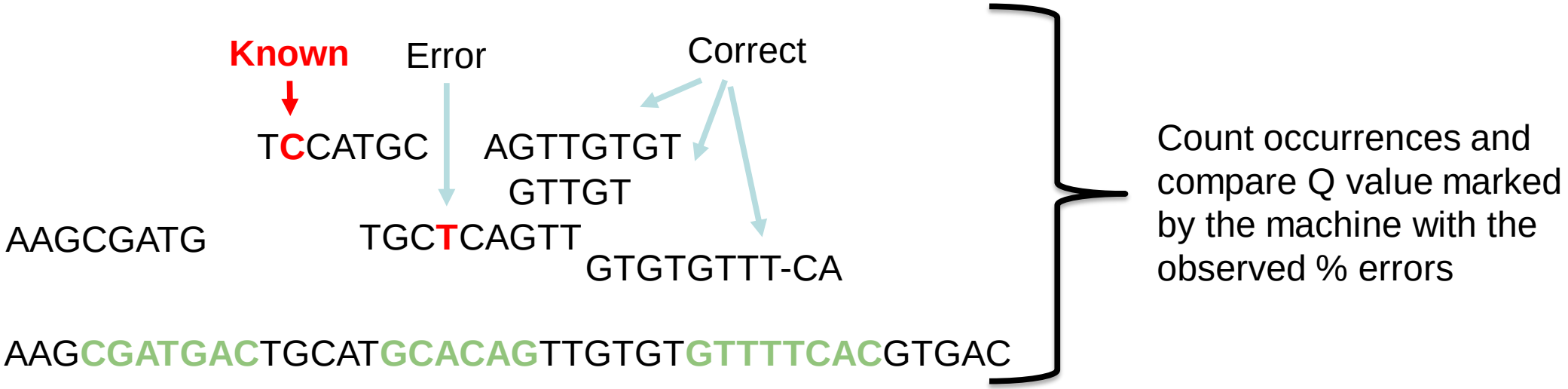


The machine oftens does not estimate well the base quality score

Data pre-processing for variant discovery Base Quality Recalibration

Use list of known variants to estimate correct quality values

- All bases different from reference **not in the provided list** of known variants are considered to be errors

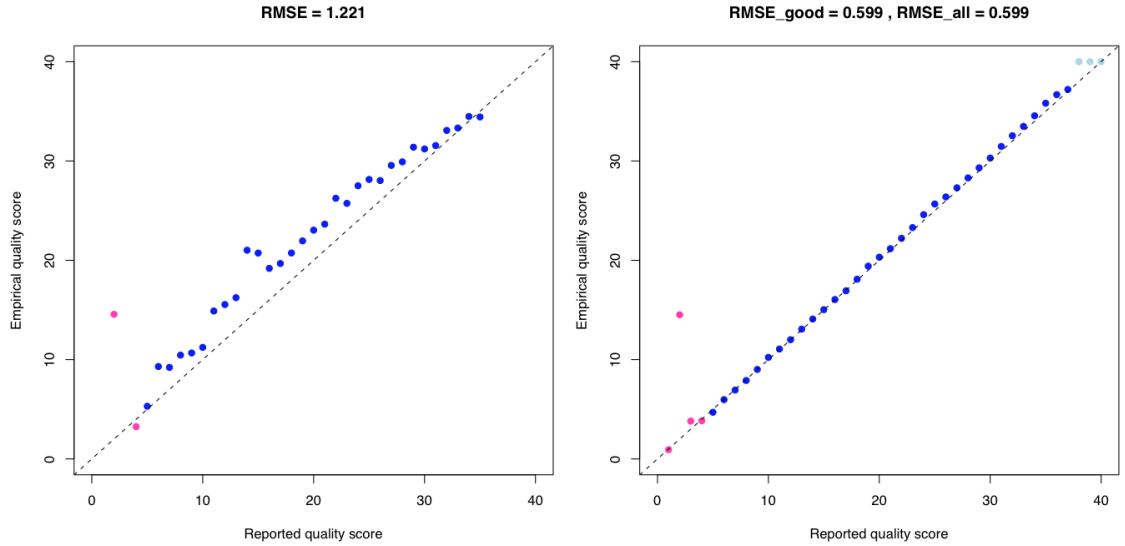


Data pre-processing for variant discovery Base Quality Recalibration

Base Quality Recalibration:

The covariates being used here:

- ReadGroupCovariate
- QualityScoreCovariate
- ContextCovariate
- CycleCovariate



https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3083463/

https://www.youtube.com/watch?v=L4D1dwES9s8

https://gatk.broadinstitute.org/hc/en-us/articles/360035890531-Base-Quality-Score-Recalibration-BQSR-

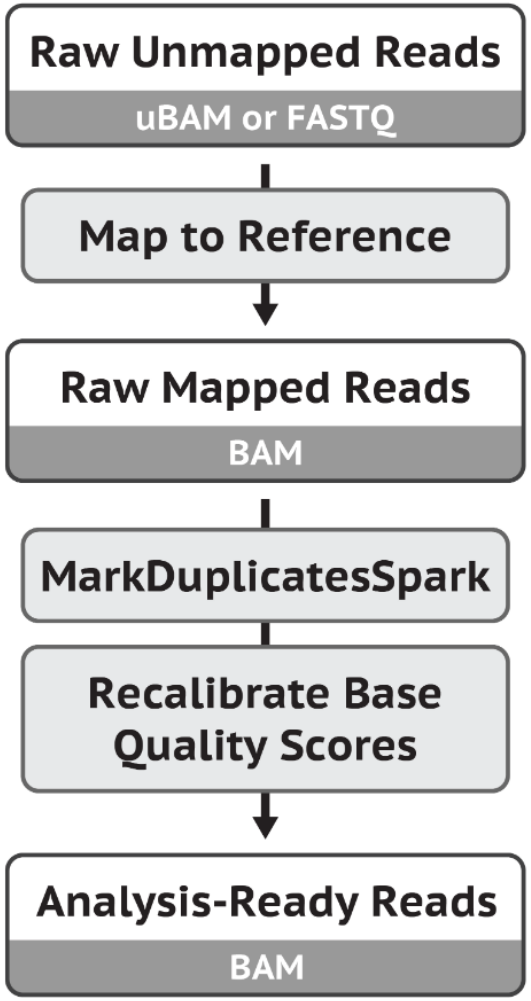
Data pre-processing for variant discovery
Base Quality Recalibration

> gatk BaseRecalibrator -I sample_marked.bam -R genome.fasta --known-sites known_snps.vcf --known-sites known_indels.vcf [--intervals target_positions.bed --interval-padding 100] -O sample_marked_baserecalibrator_report.txt

>gatk ApplyBQSR -I sample_marked.bam -R genome.fasta --bqsr-recal-file sample_marked_baserecalibrator_report.txt –O sample_marked_baserecalibrator.bam

Data pre-processing for variant discovery

GATK Best Practices



These steps can be used with other variant callers

https://gatk.broadinstitute.org/hc/en-us/articles/360035535912-Data-pre-processing-for-variant-discovery

Practical Exercise

- There are three samples, use data from any/all:
  - Mark duplicates using “gatk MarkDuplicates”
    - Describe the contents of the Metrics File
  - Apply Base Recalibration: gatk BaseRecalibrator / ApplyBQSR

Commands to run are in the github

Interlude

NGS: Quality Assessment and Visualization of Read Mappings

Ministério da Saúde

Instituto_Nacional de Saúde
Doutor Ricardo Jorge

Quality Assessment of Read Mappings

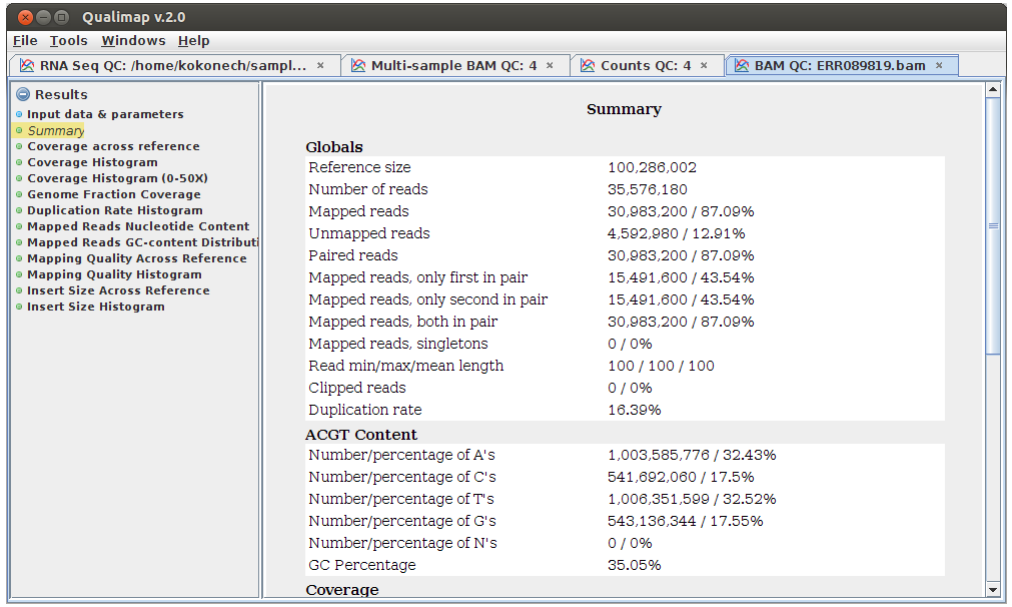
Measures to consider:

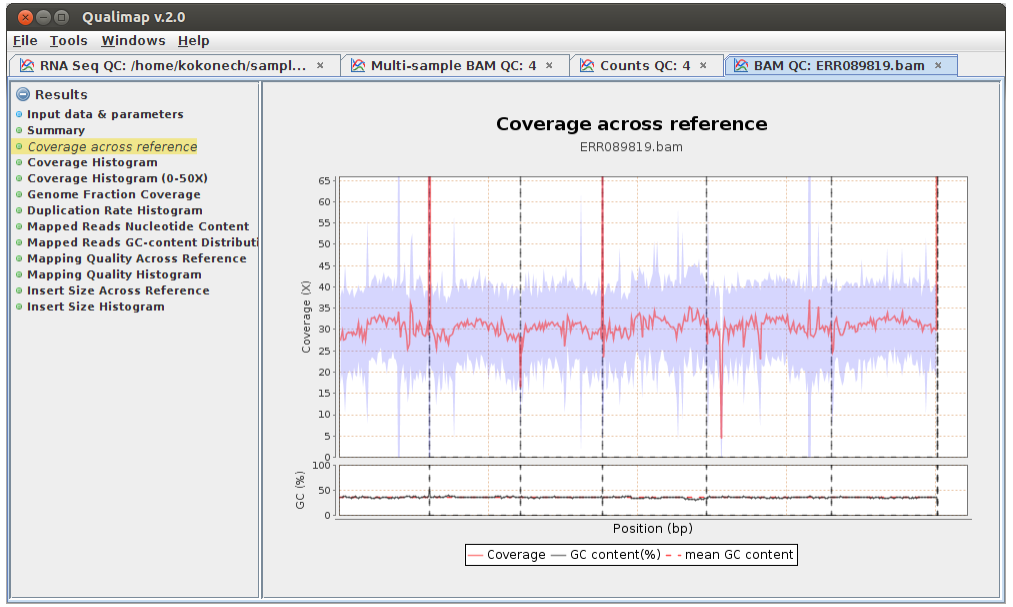
- Mapped Reads / Unmapped Reads
  - Usually >80% for WGS; >90% for WES
- Total Mapped Reads / Coverage
  - Presence of Duplicates (depends on coverage)
- Homogeneity in the coverage
- Distribution of Fragment Size (paired-end)
- Coverage of Targeted Regions (in case of eg. WES)

Quality Assessment of Read Mappings

- Qualimap

http://qualimap.conesalab.org/





Practical example

- Look at Metrics from GATK

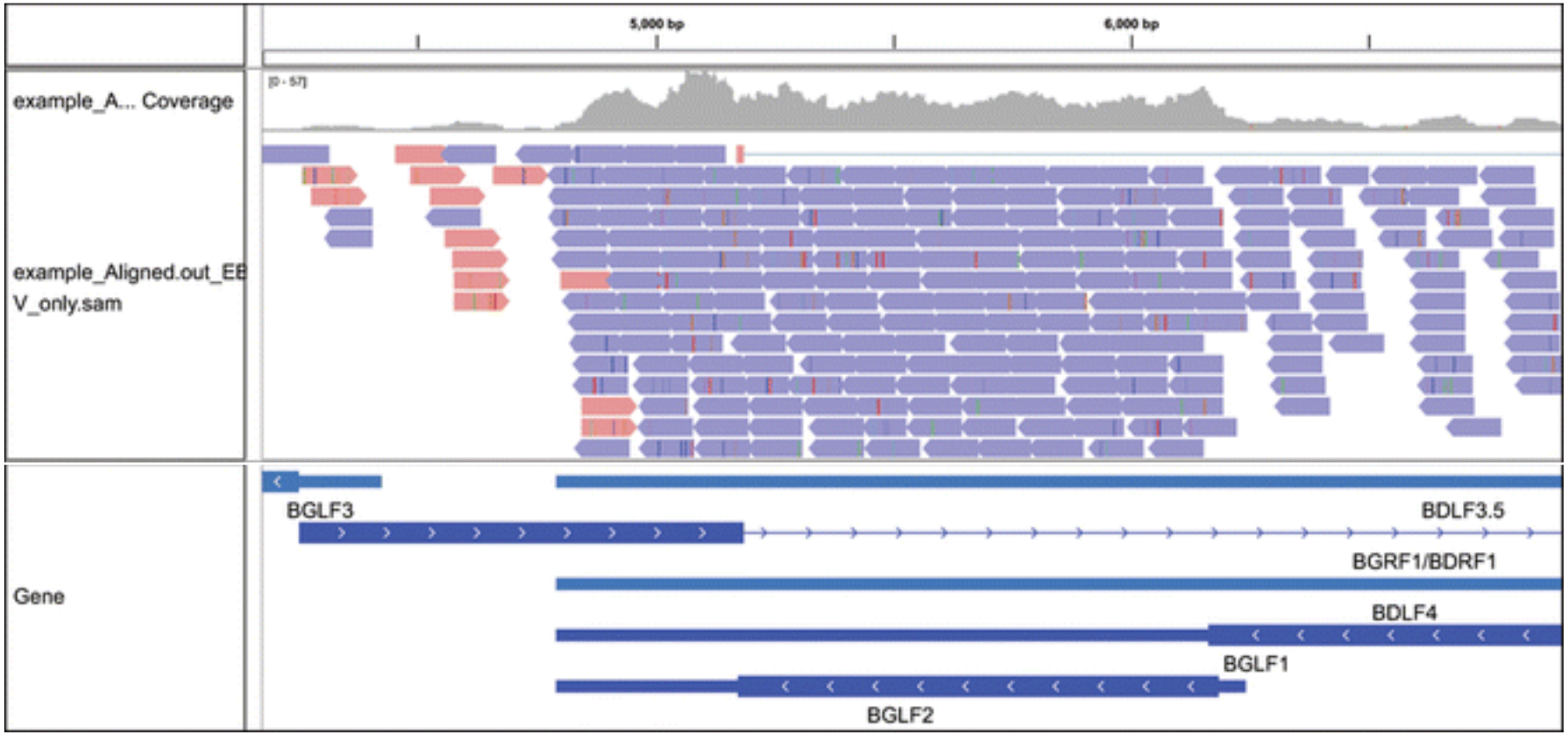
>qualimap bamqc –bam sample_marked_recalibrated.bam –outdir . –outfile sample_marked_recalibrated_qualimap.pdf –outformat PDF [-gff targets_position.bed]

Exercise

- Look at Metrics from GATK for your sample
- Run qualimap bamqc for your sample and see output

Visualization of Read Mappings

Integrative Genomics Viewer



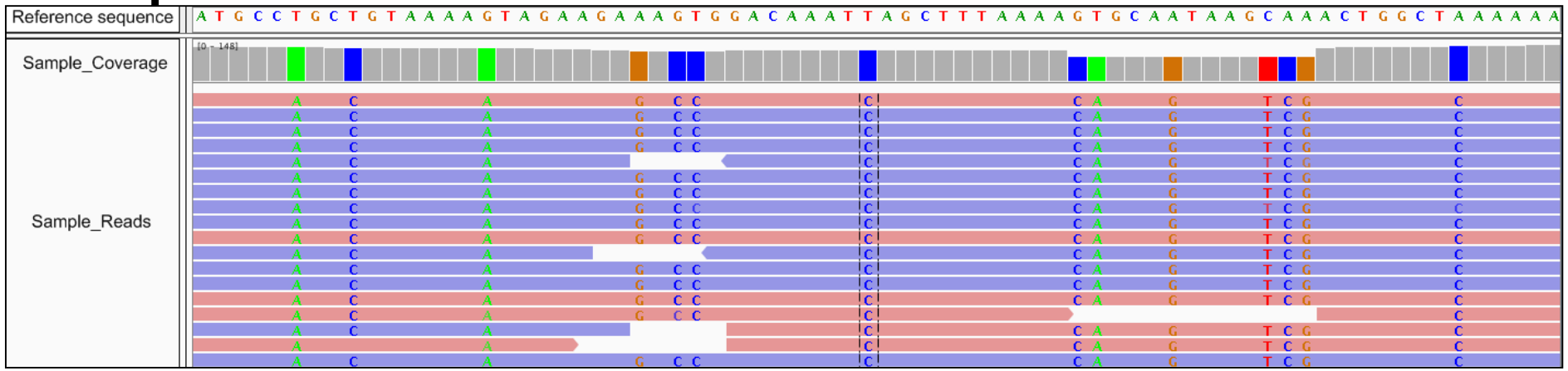
Coverage

Alignments

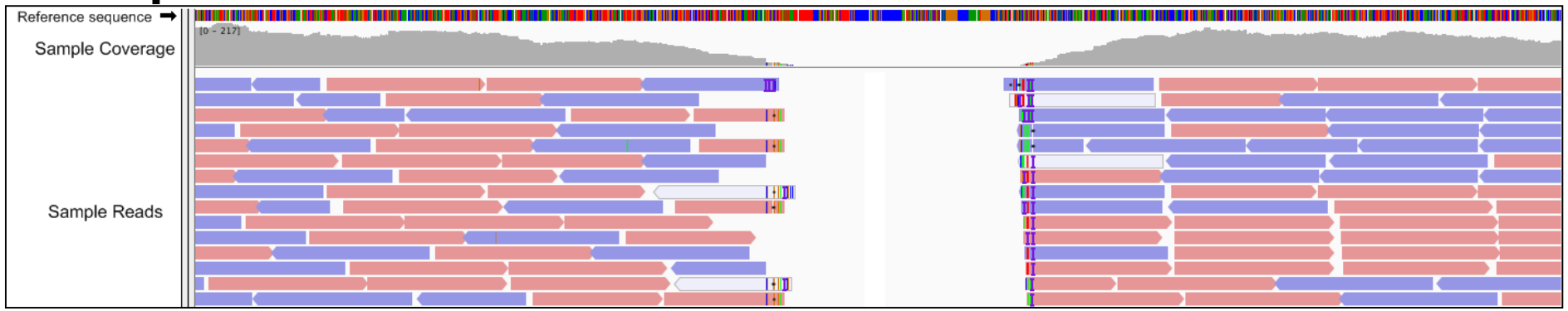
Annotation (genes)

Visualization of Read Mappings

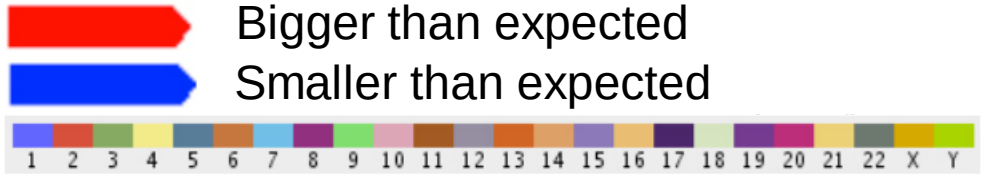
Example mutations



Example deletion

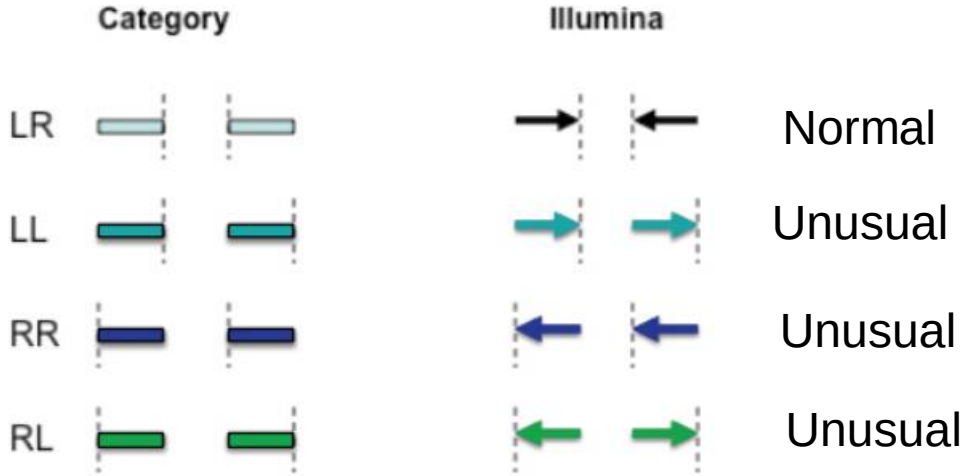


- IGV provides colors to signal unusual situations
  - Besides mutations, information from paired-end is also there



Pairs on different chromosomes

Insert Size Lengths

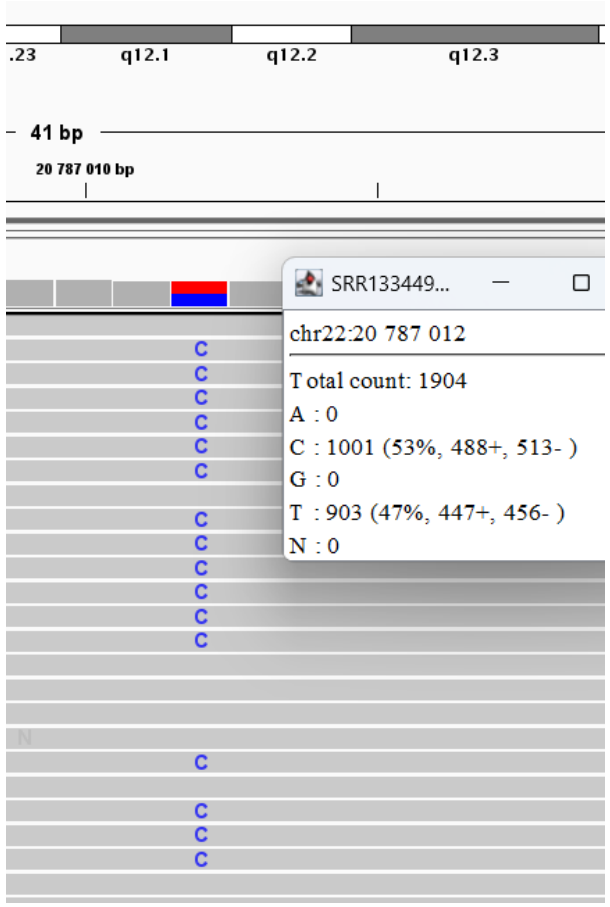


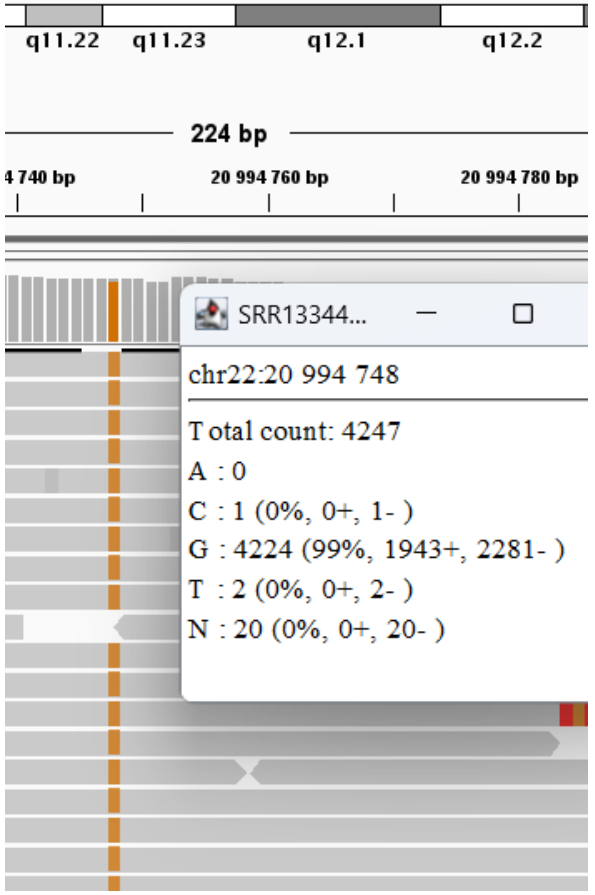
Pair orientation

https://software.broadinstitute.org/software/igv/

Visualization of Read Mappings

- Example of mutations, showing evidence





Practical Exercise

- Look at the alignments in IGV in specific positions:
  - Sample 1: 2:171868791
  - Sample 2: 6:121216049
  - Sample 3: X:129746638

Gather evidence that you think is relevant for the mutation:

- Number of reads, strand of reads, quality etc…