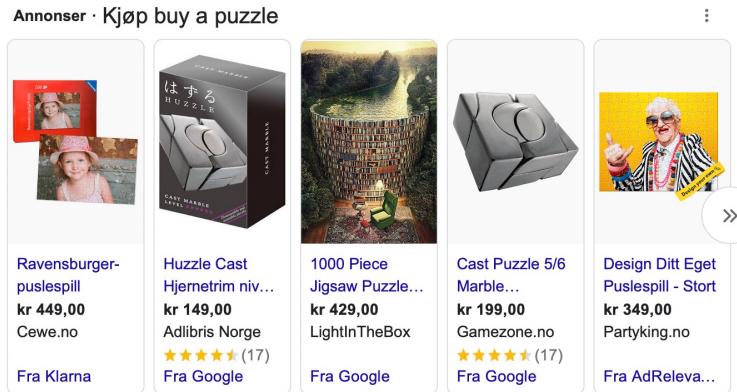


Variant Calling Applications in Avdeling for medisinsk genetikk, OUS

- Inherited disease diagnostic: nuclear DNA, mitochondrial DNA
- Inherited cancer diagnostic
- Noninvasive Prenatal Testing (NIPT)
- Preimplantacional Genetic Diagnosis (PGT)

Variant Calling - steps

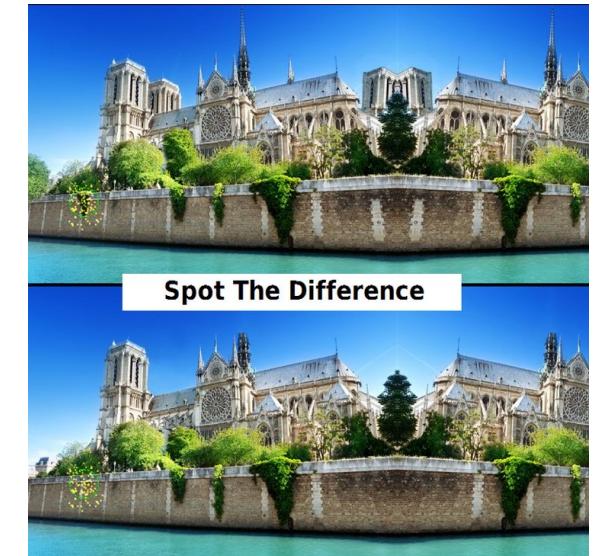
Carry out sequencing
to create FASTQ files



Align the sequences to a
reference genome creating
BAM or CRAM files

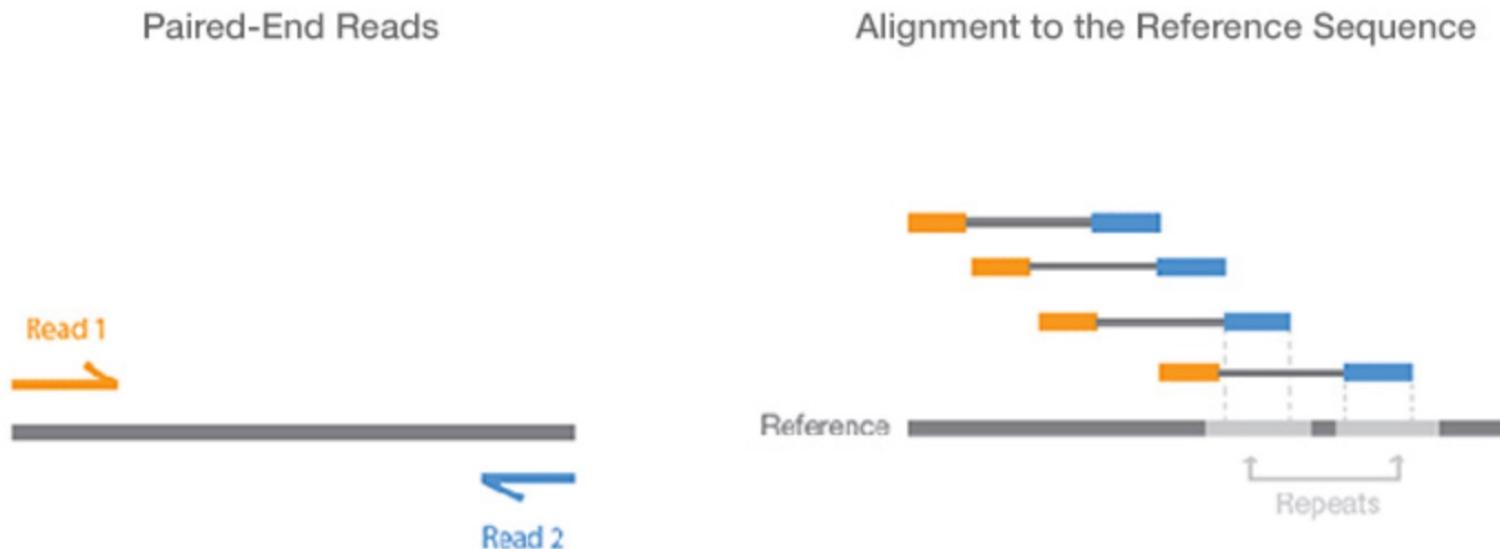


Identify where the
aligned reads differ from
the reference genome
and write to a VCF file



Concepts

- **Short** or long read sequencing (lower yield, higher error rate, higher costs)
- Single end or **paired-end** sequencing
- Target (some regions), exome (all coding regions) or genome sequencing



Genome Analysis Toolkit

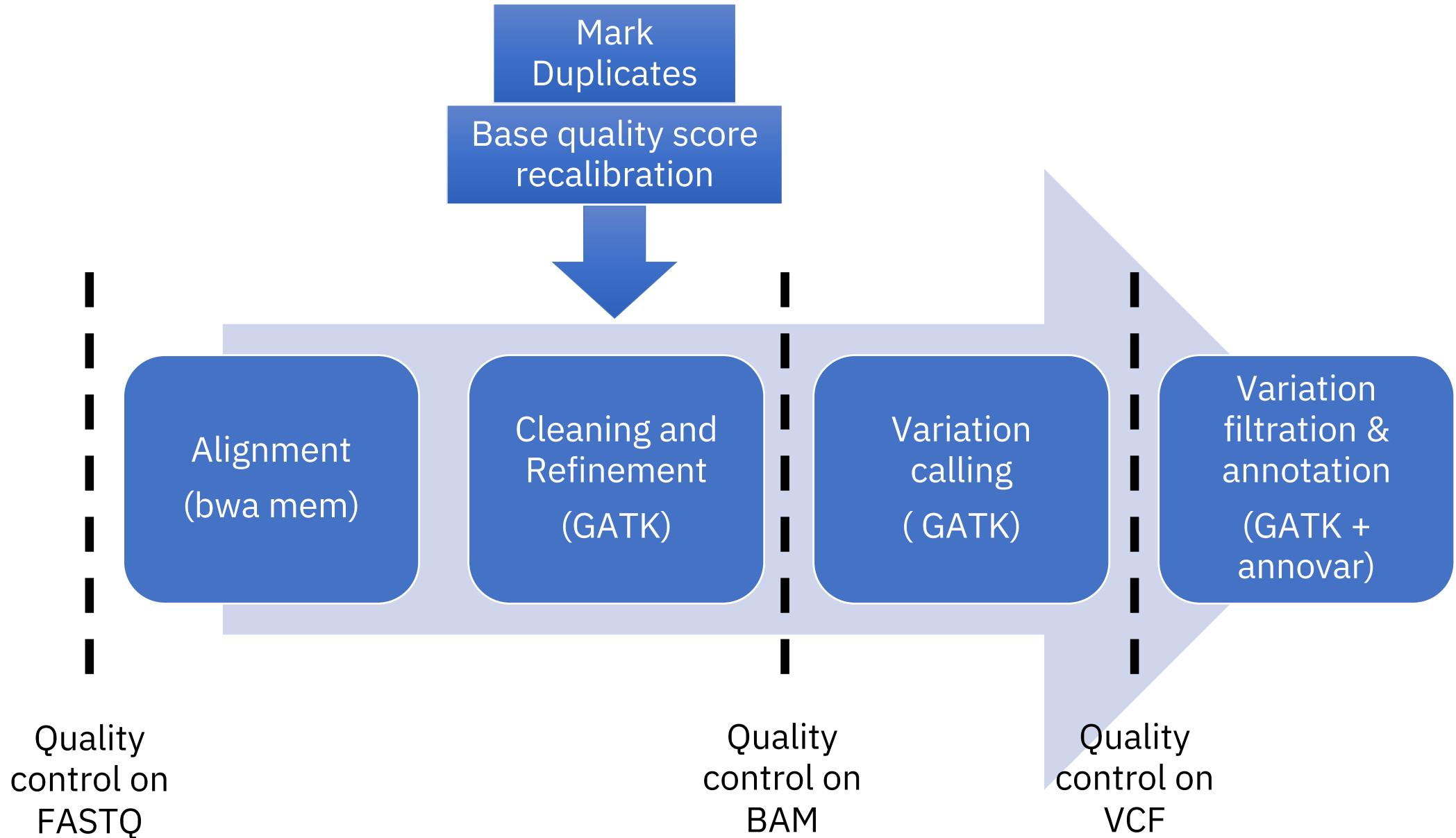
Variant Discovery in High-Throughput Sequencing Data



Developed in the Data Sciences Platform at the [Broad Institute](#), the toolkit offers a wide variety of tools with a primary focus on variant discovery and genotyping. Its powerful processing engine and high-performance computing features make it capable of taking on projects of any size. [Learn more](#)

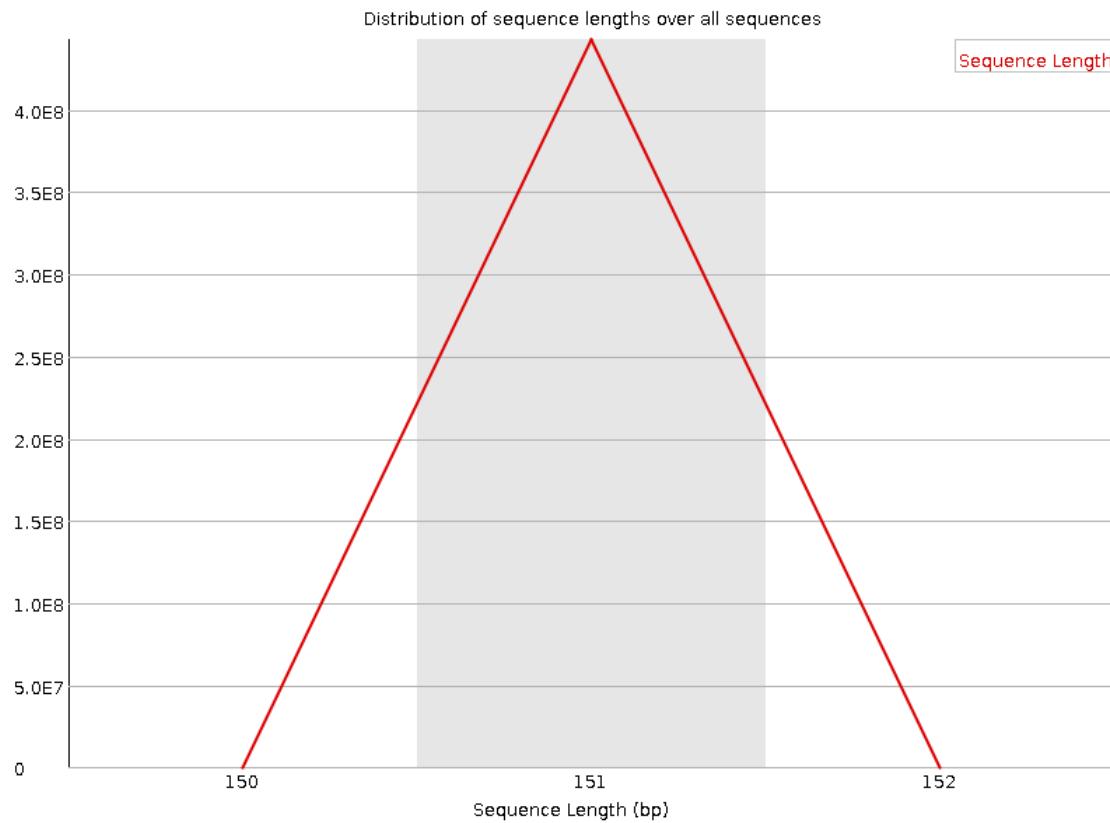
<https://gatk.broadinstitute.org/hc/en-us>

Overview of variant calling

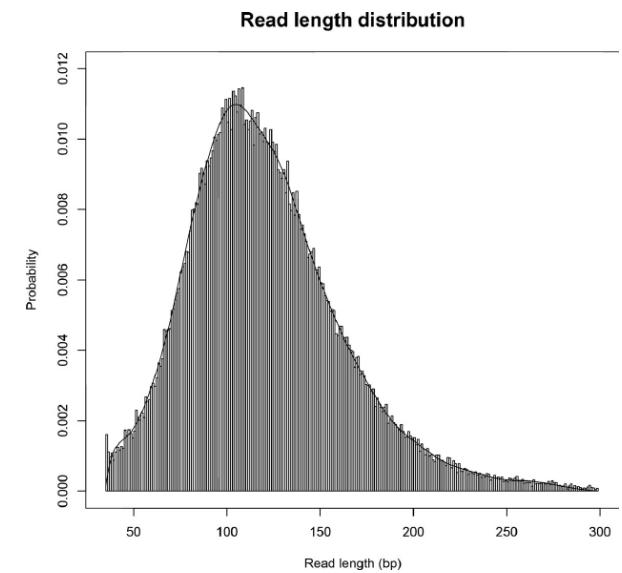


Quality control on fastq files (fastqc)

Sequence length distribution



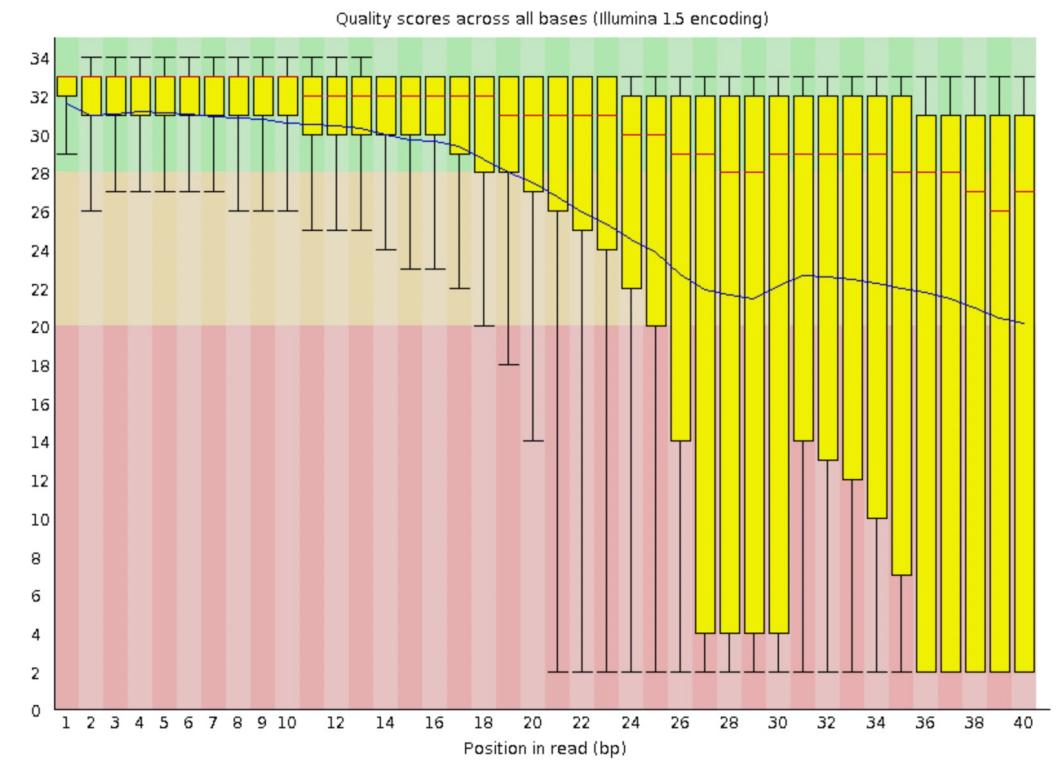
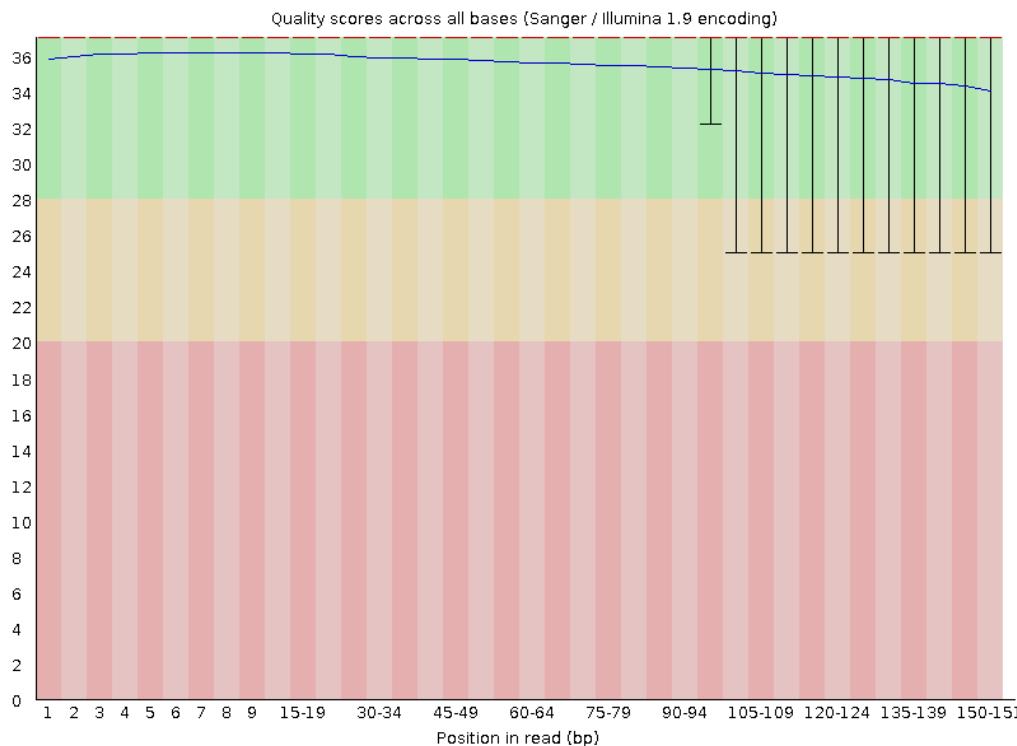
Illumina



Ion Torrent

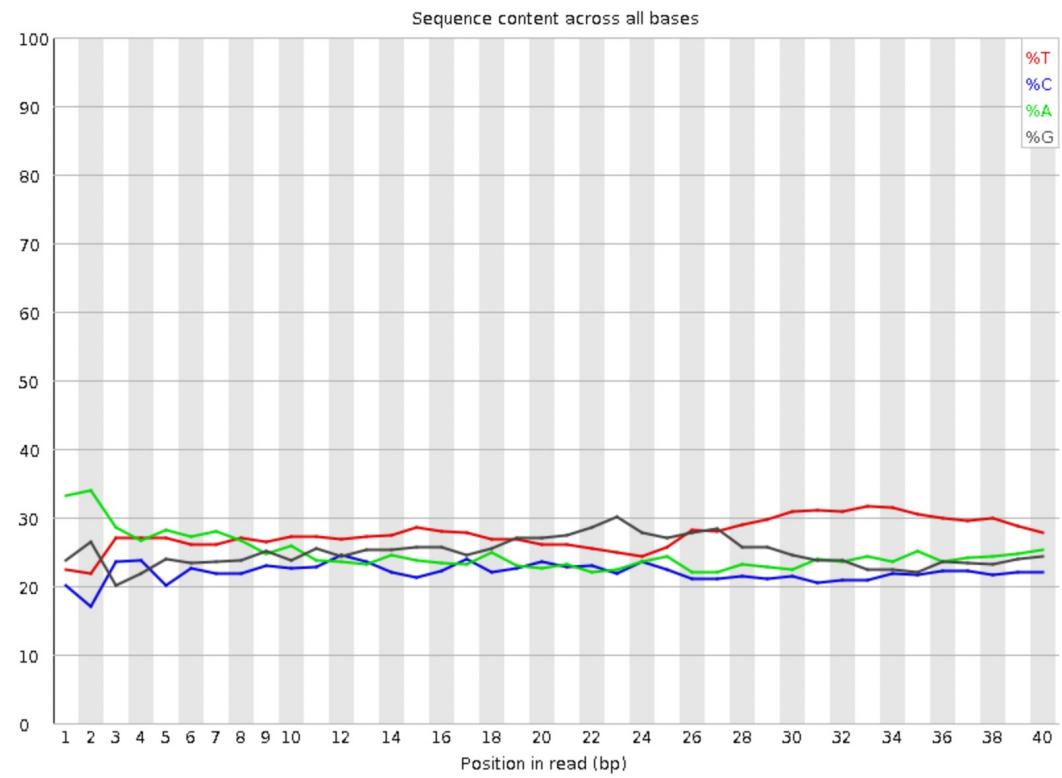
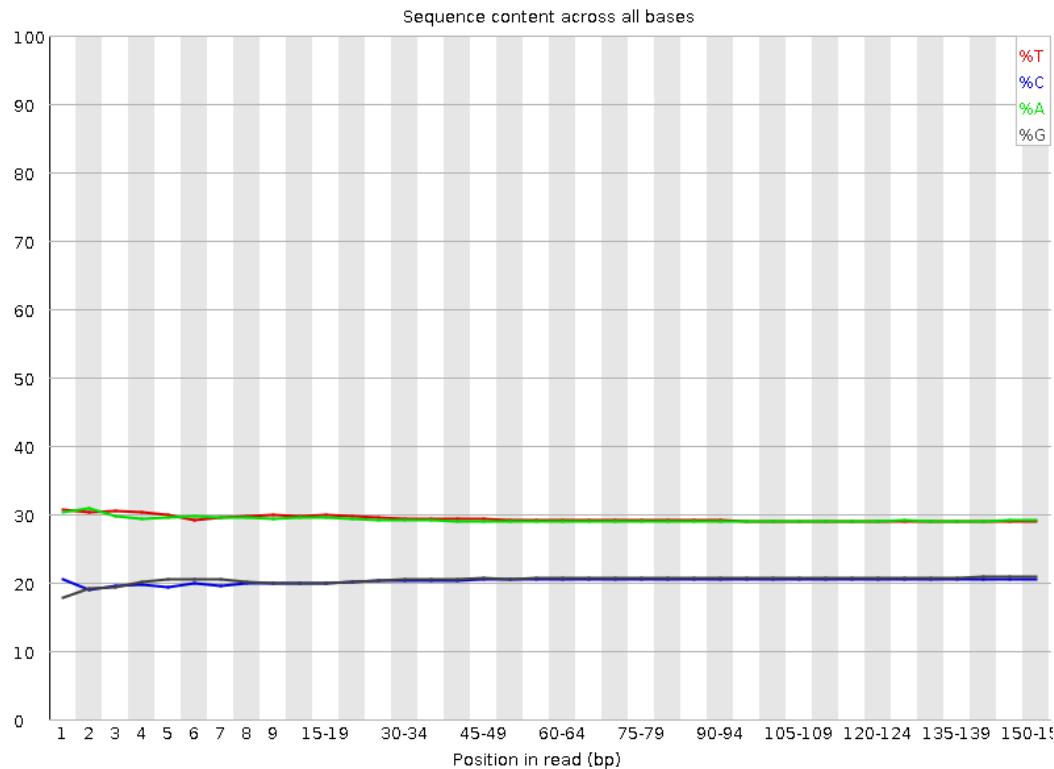
Quality control on fastq files (fastqc)

Per Base Sequence Quality



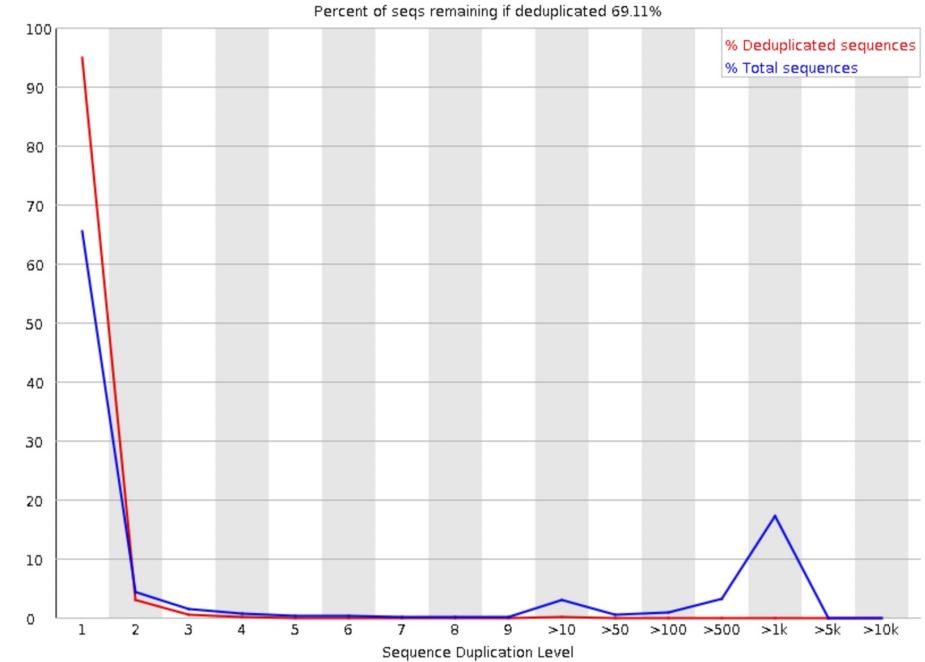
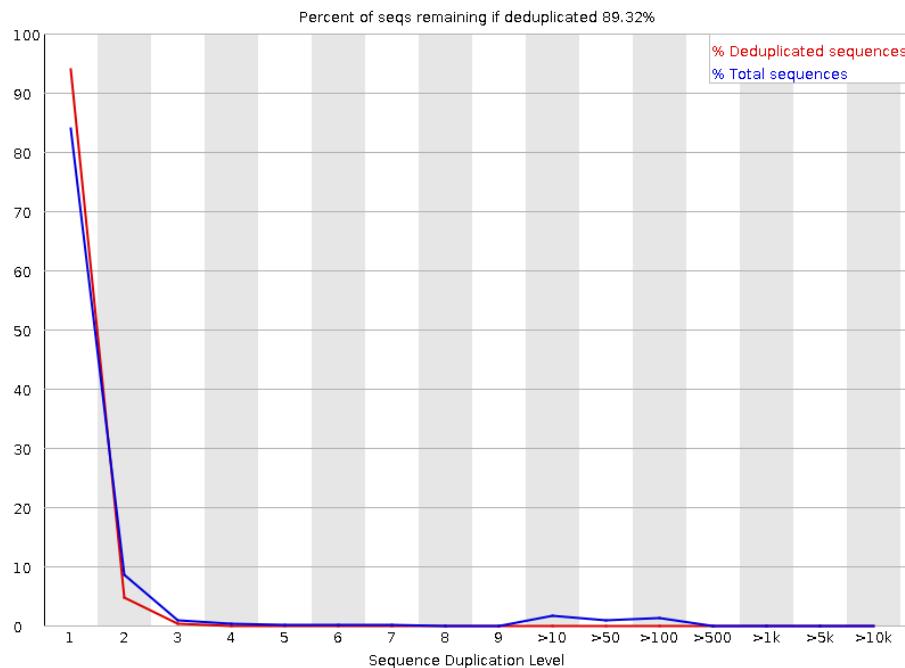
Quality control on fastq files (fastqc) – Cont.

Per Base Sequence Content



Quality control on fastq files (fastqc) – Cont.

Sequence duplication level



Alignment

Find **genomic locations** (genomic coordinates) of the sequences in a **reference genome**, and writing into a **SAM/BAM/CRAM file**.

Requirements:

- Short read aligner: bwa-mem2 (mapping DNA sequences against a large reference genome)
- Reference genome (fasta and its index file)
- Fastq file(s)

Preparation before alignment: index the reference genome by the aligner
bwa-mem2 index human.fasta

Alignment – reference genome

What is a good reference genome:

Because the aligner tries to place the read to a location in the reference genome:

- **It contains all chromosomes:** chromosomes 1–22 (chr1–chr22, **1–22**), X (chrX, **X**), Y (chrY, **Y**) and Mitochondrial (chrM, **MT**).
- **And *unlocalized sequence*:** on a specific chromosome but with unknown order or orientation. Identify by _random suffix.
- **And *unplaced sequence*:** on an unknown chromosome. Identify by chrU_ prefix.

No matter of sizes of the capture, always use the whole genome as the reference genome!

<https://gatk.broadinstitute.org/hc/en-us/articles/360035890711-GRCh37-hg19-b37-humanG1Kv37-Human-Reference-Discrepancies#humanG1Kv37>

Alignment – read group – marker for the reads

A read group: all the reads derived from the same:

library preparation && biological sample && lane && flow cell

Used to not only differentiate samples, but also various technical features associated with artefacts. Can be used to correct errors in downstream analysis, e.g. duplicate marking and base quality score recalibration.

Tags in SAM/BAM/CRAM (connected by tabs, SAM format specification):

@RG

ID : Read group identifier

LB : Library

PL : Platform/technology used to produce the reads (Valid values). E.g. ILLUMINA

CN : Name of sequencing center producing the read

PU : Platform unit (e.g. flowcell-barcode.lane)

SM : Sample

DS : Description

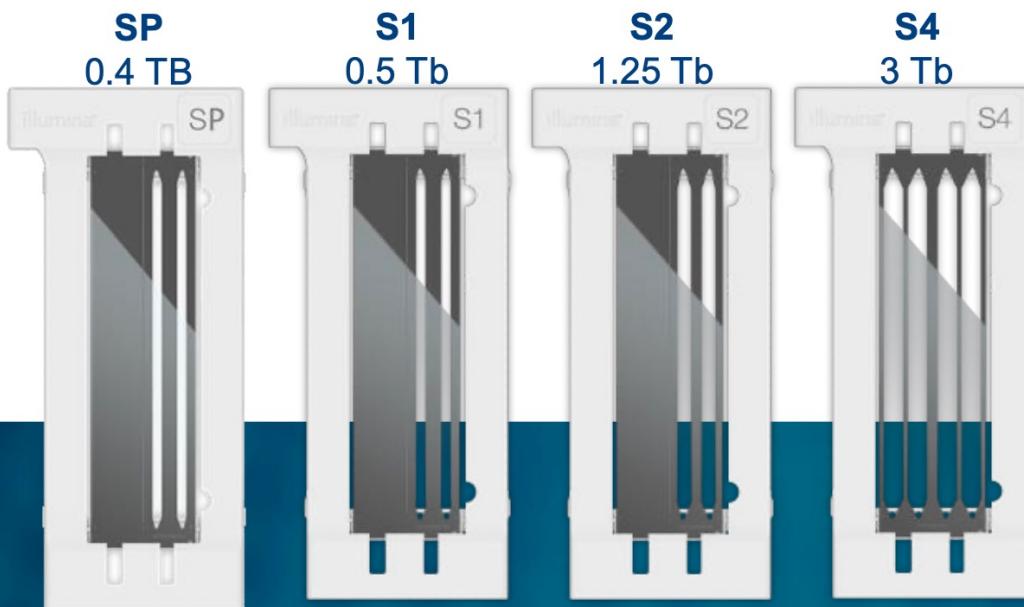
DT : Date the run was produced, ISO8601 date or date/time, e.g. 2022-10-24

@RG\tID:NA12878\tLB:NA12878\tPL:ILLUMINA\tPU:BH35C5DSX5.1\tSM:NA12

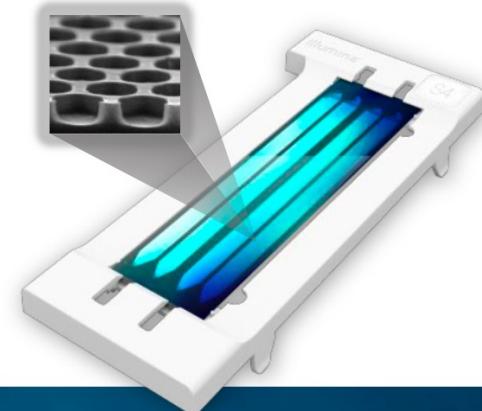
878

NovaSeq 6000 Flow Cell Configurations

Max Output / Flow Cell:



Patterned Flow Cell



Flow Cell Type

	SP	S1	S2	S4
Lane Width	3.8mm	3.8mm	5mm	7mm
Lane Pairs	Single			Dual
Surfaces	Single			Dual

For Research Use Only. Not for use in diagnostic procedures.

Alignment – result

Reads mapped to the unique location: mapping quality > 0

Reads not mapped: unmapped reads, non-human sequences, bad quality bases, big insertion/deletion in the sample

Reads mapped to the multiple locations equally: mapping quality = 0, repeats, pseudogenes

Alignment – commands

```
bwa-mem2 mem \\ # mem is one of the functions in bwa-mem2  
-R 'Read group string' \\ # read group information  
-v 2 \\ # how much log information print out in the screen, 2 means only  
show warnings/error  
-M \\ # Mark shorter split hits as secondary (for Picard compatibility).  
-t CPUs \\ # number of CPUs want to used in the calculation  
-Y indexed reference genome \\ # e.g. human_g1k_v37_decoy.fasta  
R1_FASTQ R2_FASTQ | \\  
samtools view -@ CPUs -Sb - | \\ # convert SAM to BAM file  
samtools sort -@ CPUs -o sorted.bam -T sorted - # sort the BAM file by  
genomic coordinates  
  
samtools index -@ CPUs sorted.bam # index the BAM file
```

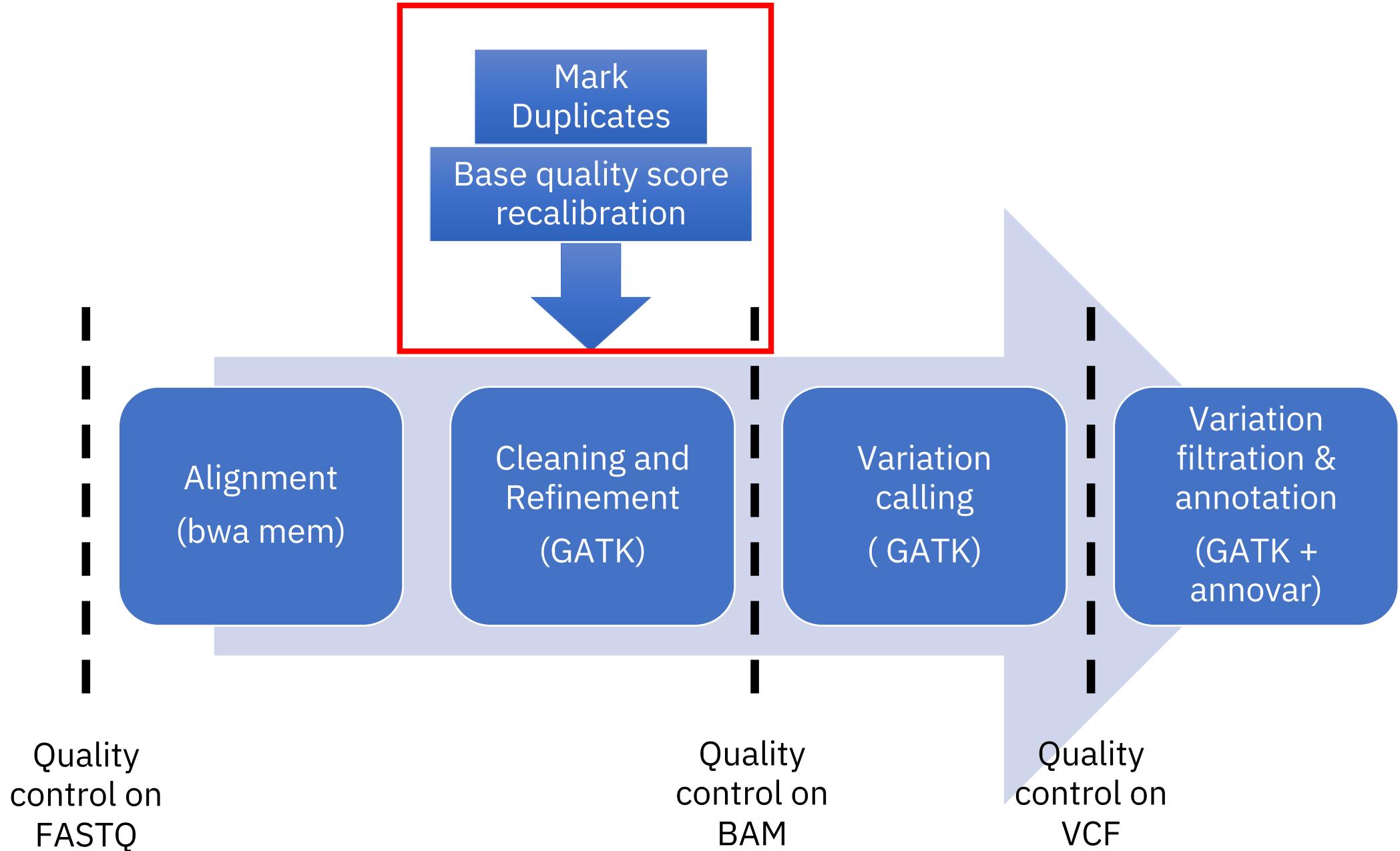
Results will be: sorted.bam and sorted.bam.bai

Refinement of alignment – data clean up

No perfect world!

- The sample preparation is not perfect – Mark duplicates
- The sequencer is not perfect - Mark duplicates, BQSR
- The aligner is not perfect – re-assembly in variant caller

Overview of variant calling

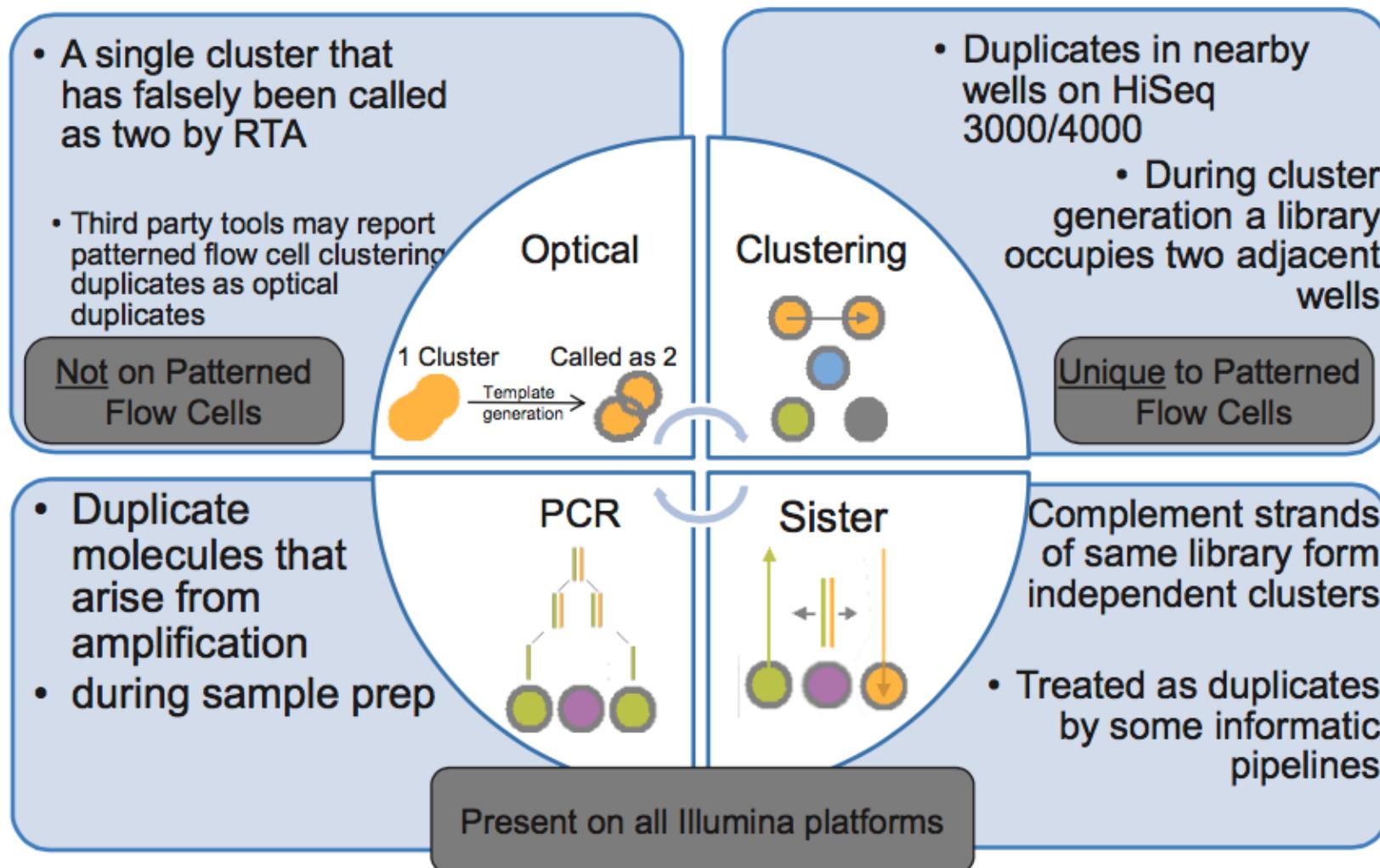


Refinement of alignment – Duplicates

1. Shatter genomic DNA, e.g. with a sonicator.
2. Ligate adapters to both ends of the fragments.
3. PCR amplify the fragments with adapters
4. Create an oil-water emulsion of micrometer beads and DNA molecules (for Roche or Ion Torrent technologies) or spread DNA molecules across flowcells (for Illumina technology). Goal is to get exactly one DNA molecule per bead or per flowcell lawn of primers. This depends purely on probability, based on the concentration of DNA.
5. Use bridge PCR to amplify the single molecule on each bead or each lawn so that you can get a strong enough signal (whether light or pH) to detect. Usually this requires several hundred or low thousands of molecules.
6. Sequence by synthesis of complementary strand: pyrosequencing (Roche), reversible terminator chemistry (Illumina), or ion semiconductor (Ion Torrent).

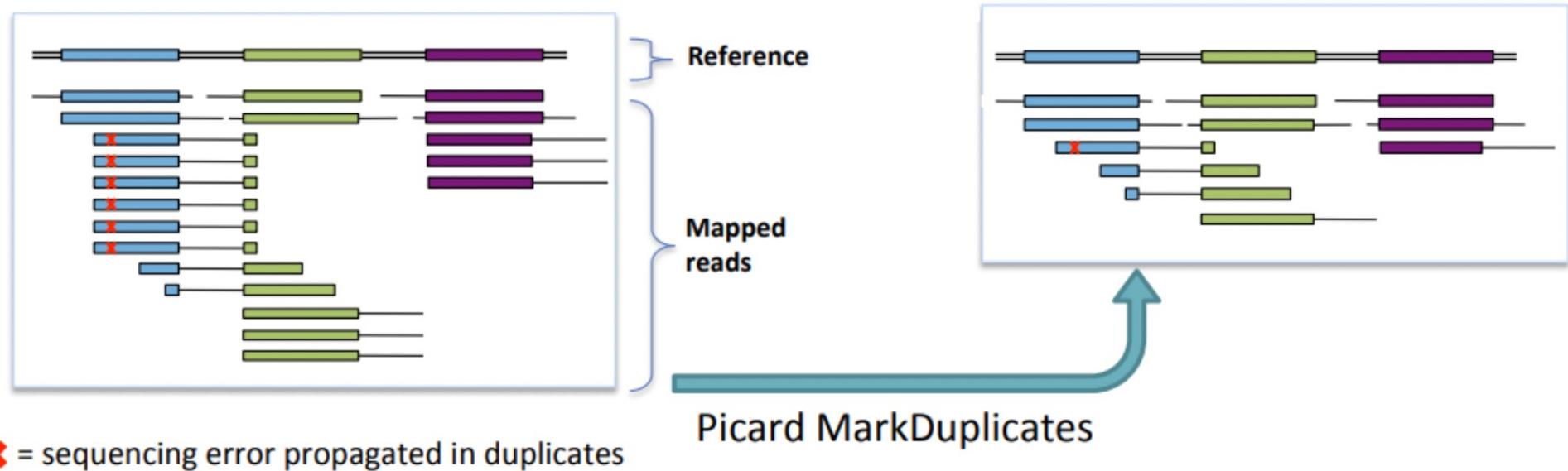
Refinement of alignment – Duplicates

Originating from a single fragment of DNA, but recognized as two reads in the sequencing - PCR duplicates, Optical duplicates



Refinement of alignment – Duplicates - algorithm

Duplicated reads could create fake coverage, which create false variants



By comparing sequences in the 5 prime positions of both reads and read-pairs in a SAM/BAM file.

The tool differentiates the primary and duplicate reads using an algorithm that ranks reads by the sums of their base-quality scores.

main output is a new SAM or BAM file, Duplicates are marked with the hexadecimal value of 0x0400, which corresponds to a decimal value of 1024.

Refinement of alignment – Duplicates - command

```
gatk MarkDuplicates \
-INPUT ${input_file}.bam \
-OUTPUT "${output_file}.bam" \
-METRICS_FILE "${output_file}.markduplicates.metrics" \
-CREATE_INDEX true \
-CREATE_MD5_FILE true \
-VALIDATION_STRINGENCY STRICT
```

Refinement of alignment – Base Quality Score Recalibration

Base quality score (in fastq file): tell how much we can trust the base said by the sequencer. It is important for variant calling.

However, there is systematic (non-random) technical error from sequencer, leading to over- or under-estimated base quality scores in the data.

Steps:

BaseRecalibrator divided bases into bins based on the following features:

- read group
- quality score from the sequencer
- machine cycle
- current base + previous base (dinucleotide)

and calculate error rate by $(\# \text{ mismatches} + 1) / (\# \text{ bases} + 2)$ per bin. The known variants will not be counted as mismatches.

ApplyBQSR adjust each base's score based on which bins it falls in.

Refinement of alignment – BQSR – command 1

```
gatk BaseRecalibrator \
-I ${input_file}.bam \
-R ${reference} \
-L calling_region.interval_list \
--known-sites ${dbsnp_data} \
--known-sites 1000G_phase1.indels.b37.vcf \
--known-sites Mills_and_1000G_gold_standard.indels.b37.vcf \
-O ${output_file}.recal_data.table
```

Refinement of alignment – BQSR – command 2

```
gatk ApplyBQSR \
-I ${input_file}.bam \
-R ${reference} \
--bqsr-recal-file ${output_file}.recal_data.table \
--create-output-bam-md5 true \
-O ${output_file}.bam
```

With `--emit-original-quals`, the original base quality score is stored under `0Q` tag in the `bam` file.

What steps needed for which sequencing

	Target sequencing	Exome sequencing	Genome sequencing
Mark duplicates	✓	✓	✓
BQSR	✗	✓	(✓)

SAM/BAM file format

Headers

First Record

```
@HD VN:1.3 SO:coordinate
@SQ SN:22 LN:51304566 AS:NCBI37 M5:a718acaa6135fdca8357d5bfe94211dd UR
:file:/home/mktrost/seqshop/example/ref22/human.g1k.v37.chr22.fa
@RG ID:0 SM:HG00551 LB:HG00551 CN:unknown PL:ILLUMINA
@PG ID:bwa PN:bwa VN:0.7.10-r960-dirty CL:/home/mktrost/seqshop/gotcloud/bin/bwa
mem -t 1 -M -R @RG\@ID:0\@SM:HG00551\@LB:HG00551\@CN:unknown\@PL:ILLUMINA /home/mktrost/se
qshop/example/ref22/human.g1k.v37.chr22.fa /home/mktrost/seqshop/example/fastq/HG00551.SRR
190851.fastq
@RG ID:1 SM:HG00551 LB:HG00551 CN:unknown PL:ILLUMINA
SRR190851.48112415 113 22 16918656 3 23M78S = 31650772 1
4732127 TCCTCGACCTCCAAAGTCCTGGTATTAAGCGTTAGAGCCACCGCACCCAGCCAGTTATCTCTTTTTAAAATGTTATT
AATACATTATTTTTATACT ##### Chromosome/position :0A22 NM:i:1 OQ:Z#####
##### G:Z:1 XS:i:21 XA:Z:22,-38586564,7521M735,0;
SRR190851.103013373 121 22 16936847 2 37518M2D46M = 16
936847 0 CACAAGTTCAAAGTCCACAGATCTCAAAGGCAGGTACAAAATCCCACCGACTCTGCTAAAGCATAGCAAGAG
TGACCTTACTCCAGTTCCAACAAAGT ##### 560523/+131335234238-2241+7+-,+/-+89,6 AS:i:46 MD:Z:6G11^TT30T15 NM:i:4 OQ
:Z:##### BC>D?@?>DB?CDEB?CDAE6D@C
C>A8976?=;?A98 RG:Z:1 XS:i:44 XA:Z:22,-36435775,56M1I2M3I39M,12;
```

FLAG (nr. 2): 4 (unmapped reads), 1024 (duplicated reads)

Exercise I

Download IGV (Integrative Genomics Viewer):

<https://software.broadinstitute.org/software/igv/download>

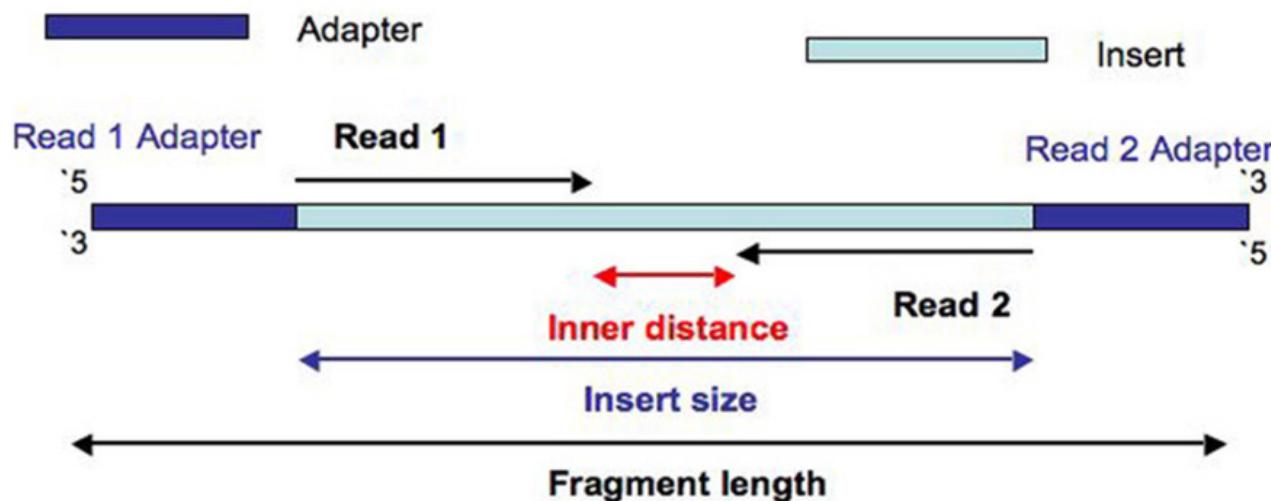
Download the BAM files to view in IGV:

<https://bit.ly/3z0hIuN>

- Folder structures of the exercise
- Running the first two scripts to do alignment, refinement of alignment and quality control on the bam file
- View the bam file in IGV on duplicates, read pairs, reads mapped to multiple locations (click the reads, base, 7:6,021,962-6,023,036, view as pairs, variants 1:17,380,476-17,380,516 (SNP), 2:48,032,854-48,032,893(deletion), 11:108,151,687-108,151,726(insertion))

Quality Control on the BAM file

- **How many reads well mapped to the reference genome**
 - Adaptors
 - Sequences not from the species you sequence
- **The length of the DNA fragment in the sample preparation (insert size)**



- **How many reads mapped on each genomic position (coverage)**
 - Median coverage, percentage of 10X, percentage of 20X, coverage unevenness

Quality Control on the BAM – Supported interval list format

.interval_list, 1-based

```
@HD VN:1.0 S0:coordinate
@SQ SN:1 LN:249250621 AS:GRCh37 UR:http://www.broadinstitute.org/ftp/pub/seq
@SQ SN:2 LN:243199373 AS:GRCh37 UR:http://www.broadinstitute.org/ftp/pub/seq
1 30366 30503 + target_1
1 69089 70010 + target_2
1 367657 368599 + target_3
1 621094 622036 + target_4
1 861320 861395 + target_5
1 865533 865718 + target_6
```

.list or .intervals, 1-based

<chr>:<start>-<end>

.bed, 0-based

<chr>\t<start>\t<end>

.vcf, 1-based

Together with –ip 100, regions with 100 bp on each side of the variant

Quality Control on the BAM – CollectAlignmentSummaryMetrics

```
gatk CollectAlignmentSummaryMetrics \
R=${reference} \
I=${bam_file} \
O=CollectAlignmentSummaryMetrics.txt \
MAX_INSERT_SIZE=600 # used to define chimeras
```

Important results:

- Total number of reads (total, no exclusions)
- High quality aligned PF reads (high quality == mapping quality ≥ 20)
- High quality aligned PF Q20 bases (subset of above where base quality ≥ 20)
- Reads aligned in pairs (vs. reads aligned with mate unaligned/not present)

Metrics are written for the first read of a pair, the second read, and combined for the pair.

Quality Control on the BAM file – CollectInsertSizeMetrics

```
gatk CollectInsertSizeMetrics \
R=${reference} \
I=${input_file}.bam \
O=CollectInsertSizeMetrics.txt \
HISTOGRAM_FILE=CollectInsertSizeMetrics-histogram.pdf
```

Important results:

- MEDIAN_INSERT_SIZE
- MEDIAN_ABSOLUTE_DEVIATION

Quality Control on the BAM file – HsMetrics

For capture sequencing samples (Find out the efficiency of the capture kit):

```
gatk CollectHsMetrics \
R=${reference} \
I=${input_file}.bam \
O=CollectHsMetrics.txt \
BAIT_INTERVALS=${bait_list} \
TARGET_INTERVALS=${target_list} \
PER_TARGET_COVERAGE=CollectHsMetrics-per-target-coverage.txt
```

For whole genome sequencing samples:

```
gatk CollectWgsMetrics \
R=${reference}
I=${input_file}.bam\
O=collect_wgs_metrics.txt
```

Quality Control on the BAM file – HsMetrics

BAIT_DESIGN_EFFICIENCY: TARGET/BAIT, 1 indicates a perfect design

OFF_BAIT_BASES: the number of PF_BASES_ALIGNED that are mapped away from any baited region.

MEDIAN_TARGET_COVERAGE: The median coverage of a target region.

PCT_USABLE_BASES_ON_BAIT/TARGET: The number of aligned, deduped, on-bait/target bases out of the PF bases available.

PCT_EXC_DUPE/MAPQ/BASEQ/OVERLAP/OFF_TARGET: percentage of reads excluded from the coverage calculation because of different reasons

PCT_TARGET_BASES_10X/20X: The fraction of all target bases achieving 10X/20x or greater coverage.

Quality Control on the BAM file – Coverage problems

- Median coverage is low, reason:
The number of reads from sequencing is low,
High duplication rate,
High level of unmapped reads
- The coverage is not even, you will see:
The number of reads from sequencing is probably OK,
the median coverage is also OK,
Percentage of positions covered by more than 10 reads: bad
Percentage of positions covered by more than 20 reads: bad

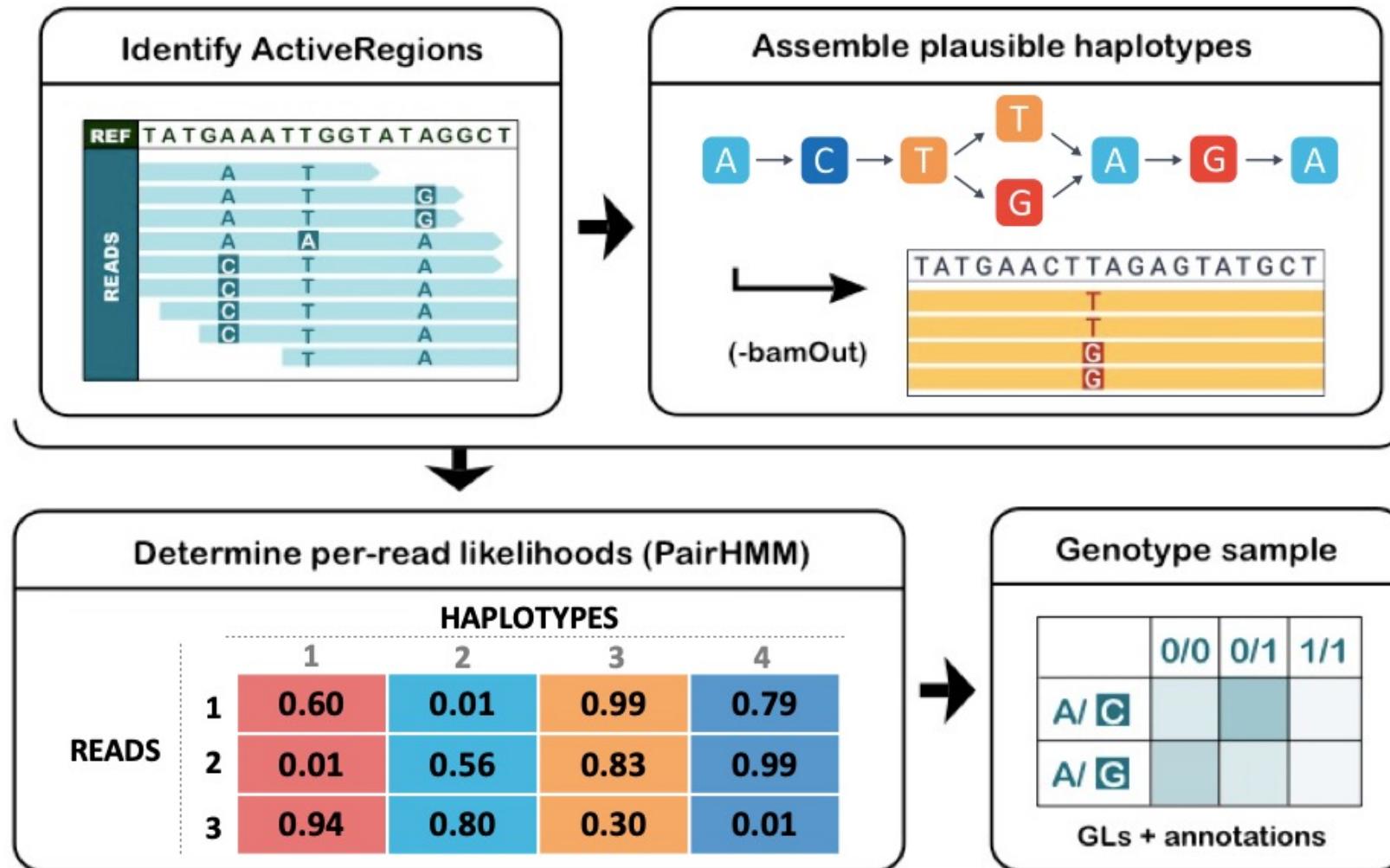
Variant calling – germline variants VS somatic variants

Variant calling is the process by which we identify variants from sequence data.

Germline variants: in all cells, for diploid, heterozygosity (Aa) allele ratio should be 50%.

Somatic variants: only in certain cells, heterozygosity (Aa) allele ratio could be very low depends on whether you pick up the right tissue.

Germline Variant Calling



Germline Variant Calling - command

```
gatk HaplotypeCaller \
-R ${reference} \
-I ${input_file}.bam \
--max-alternate-alleles 3 \ # number of alt to the
genotyper
--read-filter OverclippedReadFilter \ # too short after
soft-clipping
--dbsnp ${dbsnp} \
--emit-ref-confidence GVCF \
-L ${intervals} \
-O ${output_file}.g.vcf.gz # Genomic Variant Call Format
```

Germline Variant Calling - command

```
gatk GenotypeGVCFs \
-R ${reference} \
--variant ${output_file}.g.vcf.gz \
--dbsnp ${dbsnp} \
-O ${output_file}.raw.vcf.gz
```

VCF file format

Example

VCF header

```

##fileformat=VCFv4.0
##fileDate=20100707
##source=VCFtools
##reference=NCBI36
##INFO<ID=AA,Number=1>Type=String>Description="Ancestral Allele">
##INFO<ID=H2,Number=0>Type=Flag>Description="HapMap2 membership">
##FORMAT<ID=GT,Number=1>Type=String>Description="Genotype">
##FORMAT<ID=GQ,Number=1>Type=Integer>Description="Genotype Quality (phred score)">
##FORMAT<ID=GL,Number=3>Type=Float>Description="Likelihoods for RR,RA,AA genotypes (R=ref,A=alt)">
##FORMAT<ID=DP,Number=1>Type=Integer>Description="Read Depth">
##ALT<ID=DEL>Description="Deletion">
##INFO<ID=SVTYPE,Number=1>Type=String>Description="Type of structural variant">
##INFO<ID=END,Number=1>Type=Integer>Description="End position of the variant">
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT SAMPLE1 SAMPLE2
1 1 . ACG A,AT PASS .
1 2 rs1 C T,CT PASS H2;AA=T GT:DP 1/2:13 0/0:29
1 5 . A G PASS .
1 100 <DEL> <DEL> PASS SVTYPE=DEL;END=300 GT:GQ 0|1:100 2/2:70
GT:GQ 1|0:77 1/1:95
GT:GQ:DP 1/1:12:3 0/0:20

```

Mandatory header lines

Optional header lines (meta-data about the annotations in the VCF body)

Body

CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	SAMPLE1	SAMPLE2
1	1	.	ACG	A,AT	PASS	.		GT:DP	1/2:13	0/0:29
1	2	rs1	C	T,CT	PASS	H2;AA=T		GT:GQ	0 1:100	2/2:70
1	5	.	A	G	PASS	.		GT:GQ	1 0:77	1/1:95
1	100				PASS	SVTYPE=DEL;END=300		GT:GQ:DP	1/1:12:3	0/0:20

Annotations:

- Deletion:** Variant at position 100 is a deletion ().
- SNP:** Variants at positions 1, 2, and 5 are SNPs.
- Large SV:** Variant at position 100 is a large structural variant ().
- Insertion:** Variant at position 5 is an insertion (G).
- Other event:** Variant at position 1 is an other event (A to AT).
- Reference alleles (GT=0):** The reference alleles for the variants are A, C, G, and T.
- Alternate alleles (GT>0 is an index to the ALT column):** The alternate alleles are A, C, G, and T, corresponding to the values in the ALT column.
- Phased data:** The phased data shows that G and C are on the same chromosome for the variant at position 5.

GVCF - Genomic Variant Call Format

Basically, the same format with the VCF format, but with information on all sites, no matter whether there is a variant. It is used for joint variant calling.

```
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT NA12878
20 10001567 . A <NON_REF> . . END=10001616 GT:DP:GQ:MIN_DP:PL 0/0:38:99:34
20 10001617 . C A,<NON_REF> 493.77 . BaseQRankSum=1.632;ClippingRankSum=0.000;DP=
20 10001618 . T <NON_REF> . . END=10001627 GT:DP:GQ:MIN_DP:PL 0/0:39:99:37
20 10001628 . G A,<NON_REF> 1223.77 . DP=37;ExcessHet=3.0103;MLEAC=2,0;MLEAF=1.00,0
20 10001629 . G <NON_REF> . . END=10001660 GT:DP:GQ:MIN_DP:PL 0/0:43:99:38
```

Quality Control on the VCF file

Whether there are too many false positive variants

- **ti/tv ratio** (transition/tranversion ratio): exome 3.0, genome 2.0
Transition: purine to purine or pyrimidine to pyrimidine, A<->G or C<->T
Tranversion: purine to pyrimidine or vice versa, A<->C or G<->T
- **Number of variants** : exome (35000 - 55000), genome (4.7 M)
- **Contamination**: whether there are other human sequences in the sample (het: allele ratio < 0.25 or >0.75)
- **SNP fingerprinting tests**: check whether there are sample swaps

Variant Quality Filtration – hard filtering and soft filtering

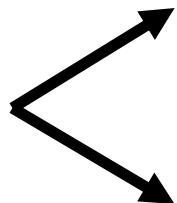
Hard-filtering consists of choosing specific thresholds for one or more annotations and throwing out any variants that have annotation values above or below the set thresholds. Limiting but possible on small set of variants. – used for target or exome sequencing data

Soft-filtering: uses machine-learning algorithms to learn from the data what are the annotation profiles of good variants (true positives) and of bad variants (false positives) in a particular dataset. - Variant quality score recalibration (VQSR) - requires a large number of variants and well-curated known variant resources.

Hard - filtering



$\geq 2.0 \text{ kg}$ PASS



$< 2.0 \text{ kg}$ FAIL

Soft - filtering



Variant Quality Filtration

Little different for the features and thresholds used for SNPs and Indels.



GATK: SelectVariants

GATK: MergeVcfs

Variant Quality Filtration – hard filtering

One of the most helpful ways to approach hard-filtering is to visualize the distribution of annotation values for a truth set called using a particular pipeline.

Quality, strand bias, mapping quality, base position

Several features could be consider:

QualByDepth (QD): QUAL/ the unfiltered depth of non-hom-ref samples. normalized the variant.

FisherStrand (FS): the Phred-scaled probability that there is strand bias at the site.

StrandOddsRatio (SOR): another way to estimate strand bias using a test similar to the symmetric odds ratio test.

RMSMappingQuality (MQ): the root mean square mapping quality over all the reads at the site.

MappingQualityRankSumTest (MQRankSum): It compares the mapping qualities of the reads supporting the reference allele and the alternate allele. A positive value means the mapping qualities of the reads supporting the alternate allele are higher than those supporting the reference allele; a negative value indicates the mapping qualities of the reference allele are higher than those supporting the alternate allele.

ReadPosRankSumTest (ReadPosRankSum): It compares whether the positions of the reference and alternate alleles are different within the reads.

1 17359676 rs1022580 C A 8569.06 . AC=2;AF=1.00;AN=2;DB;DP=303;ExcessHet=0.0000;FS=0.0
 00;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=29.05;SOR=1.358 GT:AD:DP:GQ:PL 1/1:0,295:295:99:8583,881,0
 1 17380497 rs2746462 G T 11539.06 . AC=2;AF=1.00;AN=2;DB;DP=450;ExcessHet=0.000
 0;FS=0.000;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=26.11;SOR=0.711 GT:AD:DP:GQ:PL 1/1:0,442:442:99:11553,1307,0
 1 45796269 rs3219493 G C 11072.06 . AC=2;AF=1.00;AN=2;DB;DP=410;ExcessHet=0.000
 0;FS=0.000;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=27.14;SOR=0.796 GT:AD:DP:GQ:PL 1/1:0,408:408:99:11086,1219,0
 1 45797505 rs3219489 C G 6354.64 . AC=1;AF=0.500;AN=2;BaseQRankSum=0.818;DB;DP=597;Exc
 essHet=0.0000;FS=0.521;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=11.13;ReadPosRankSum=0.303;SOR=0.769 GT:AD:DP:GQ:PL
 0/1:281,290:571:99:6362,0,6018
 1 45798555 rs3219487 T C 18604.06 . AC=2;AF=1.00;AN=2;DB;DP=697;ExcessHet=0.000
 0;FS=0.000;MLEAC=2;MLEAF=1.00;MQ=60.00;QD=27.40;SOR=0.875 GT:AD:DP:GQ:PL 1/1:0,679:679:99:18618,2027,0
 1 183086757 rs2296292 A C 4754.64 . AC=1;AF=0.500;AN=2;BaseQRankSum=-9.380e-01;DB;DP=52
 9;ExcessHet=0.0000;FS=0.000;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=9.16;ReadPosRankSum=1.08;SOR=0.707 GT:AD:
 DP:GQ:PL 0/1:280,239:519:99:4762,0,6002
 2 30381505 rs1137288 T C 3278.64 . AC=1;AF=0.500;AN=2;BaseQRankSum=0.671;DB;DP=362;Exc
 essHet=0.0000;FS=1.251;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=9.08;ReadPosRankSum=1.56;SOR=0.786 GT:AD:DP:GQ:PL
 0/1:206,155:361:99:3286,0,4598
 2 47601106 rs1126497 T C 4930.64 . AC=1;AF=0.500;AN=2;BaseQRankSum=-7.860e-01;DB;DP=54
 8;ExcessHet=0.0000;FS=1.106;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=9.20;ReadPosRankSum=0.268;SOR=0.694 GT:AD:
 DP:GQ:PL 0/1:296,240:536:99:4938,0,6525
 2 47601257 rs4399765 T C 2189.64 . AC=1;AF=0.500;AN=2;BaseQRankSum=0.076;DB;DP=253;Exc
 essHet=0.0000;FS=1.000;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=8.97;ReadPosRankSum=-1.461e+00;SOR=0.764 GT:AD:
 DP:GQ:PL 0/1:139,105:244:99:2197,0,3160
 2 47635523 rs201372136 CT C 519.60 . AC=1;AF=0.500;AN=2;BaseQRankSum=1.26;DB;DP=392;Exc
 essHet=0.0000;FS=0.488;MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=1.57;ReadPosRankSum=1.23;SOR=0.640 GT:AD:DP:GQ:PL
 0/1:281,50:356:99:527,0,5535
 2 47641559 . TAAAAAAA T 68.60 . AC=1;AF=0.500;AN=2;DP=20;ExcessHet=0.0000;FS=0.000;
 MLEAC=1;MLEAF=0.500;MQ=60.00;MQRankSum=0.00;QD=17.15;SOR=0.916 GT:AD:DP:GQ:PL 0/1:2,2:5:76:76,0,100

Variant Quality annotation – hard filtering - command

```
gatk VariantFiltration \  
-R ${reference} \  
--variant ${output_file}.raw.snp/indel.vcf.gz \  
--filter-expression "QD < 2.0" --filter-name "QD_failed" \  
--filter-expression "FS > 60.0" --filter-name "FS_failed" \  
.... \  
-O "variants.${mode}.filtered.vcf"
```

Variant Quality annotation – soft filtering (VQSR)

Steps:

- VariantRecalibrator builds the model(s)**

This model attempts to describe the relationship between variant annotations (e.g. QD, MQ and ReadPosRankSum) and the probability that a variant is a true genetic variant versus a sequencing or data processing artifact.

- ApplyVQSR applies a filtering threshold**

This adaptive error model can then be applied to both known and novel variation discovered in the call set of interest to evaluate the probability that each call is real. The result is a score called the VQSLOD that gets added to the INFO field of each variant. This score is the log odds of being a true variant versus being false under the trained Gaussian mixture model.

Variant Quality annotation – soft filtering - command

```
gatk VariantRecalibrator \
-R ${reference} \
--variant ${output_file}.raw.snp/indel.vcf.gz \
--resource:hapmap,known=false,training=true,truth=true,prior=15.0
${hapmap_ressource} \
..... (other resources)
-an QD \
-an MQRankSum \
-an ReadPosRankSum \
..... (other features)
-tranche 100.0 -tranche 99.5 -tranche 99.0 -tranche 90.0 \
-o ${output_file}.vqsr.output.recal \
--tranches-file ${output_file}.vqsr.output.tranches \
--rscript-file ${output_file}.vqsr.output.plots.R \
--mode SNP/INDEL
```

Variant Quality annotation – soft filtering - command

```
gatk ApplyVQSR \
-R ${reference} \
--variant ${output_file}.raw.snp/indel.vcf.gz \
-O variants.snp/indel.filtered.vcf \
--truth-sensitivity-filter-level 99.0 \
--tranches-file ${output_file}.vqsr.output.tranches \
--recal-file ${output_file}.vqsr.output.recal \
--mode SNP/INDEL
```

Exercise II

- Running the 03,04,05 scripts to do bam quality control, variant calling and variant filtration
- Find **PCT_PF_READS_ALIGNED** in
[NA12878F.CollectAlignmentSummaryMetrics.easy.txt](#)
- Find **MEDIAN_INSERT_SIZE**, **MEDIAN_ABSOLUTE_DEVIATION** in
[NA12878F.CollectInsertSizeMetrics.easy.txt](#)
- Find **BAIT DESIGN EFFICIENCY**, **OFF_BAIT_BASES** , **MEDIAN_TARGET_COVERAGE**,
PCT_TARGET_BASES_10X, **PCT_TARGET_BASES_20X** in
[NA12878F.CollectHsMetrics.easy.txt](#)
- Find how many variants in **04_variantfiltration/NA12878F.final.vcf** (`grep -v '^#'`
`NA12878F.final.vcf|wc -l`)
- Find how many PASS variants in **04_variantfiltration/ NA12878F.final.vcf** (`grep 'PASS'`
`NA12878F.final.vcf|wc -l`)

Variant Functional annotation – Annovar - Consequence

A tool (written with perl) with providing different data source.

Gene based annotations:

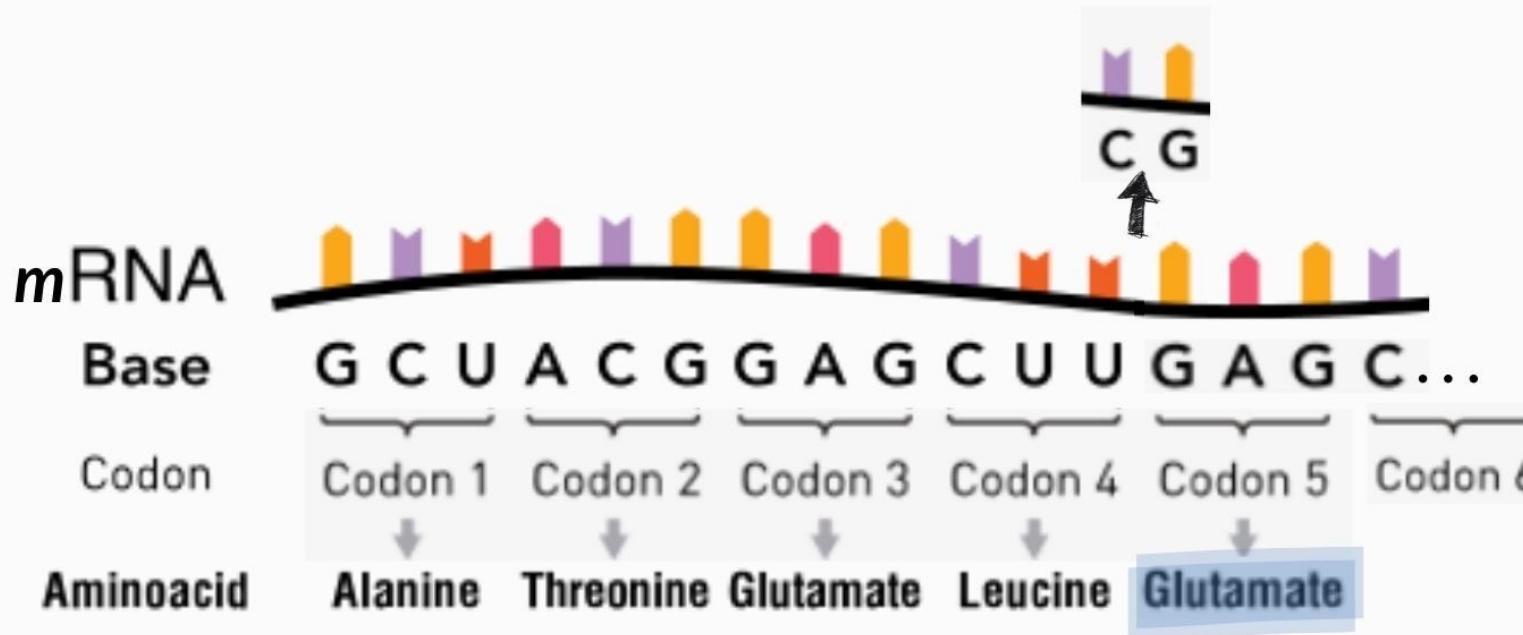
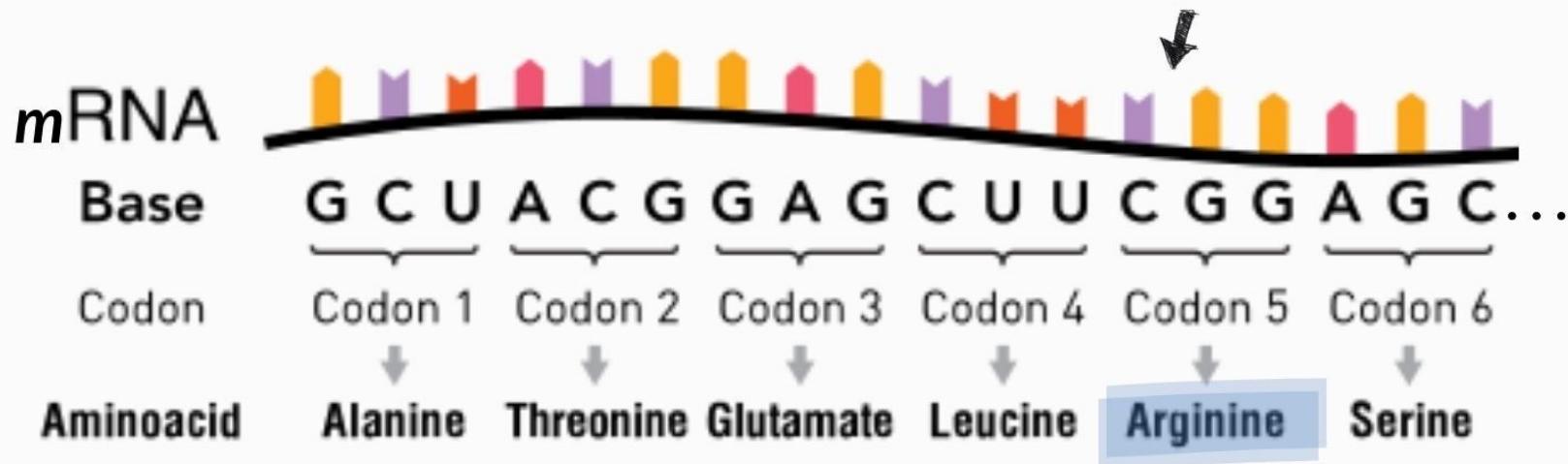
Where are the variants:

exonic = splicing > ncRNA > UTR5/UTR3 > intronic > upstream/downstream (1kb) > intergenic

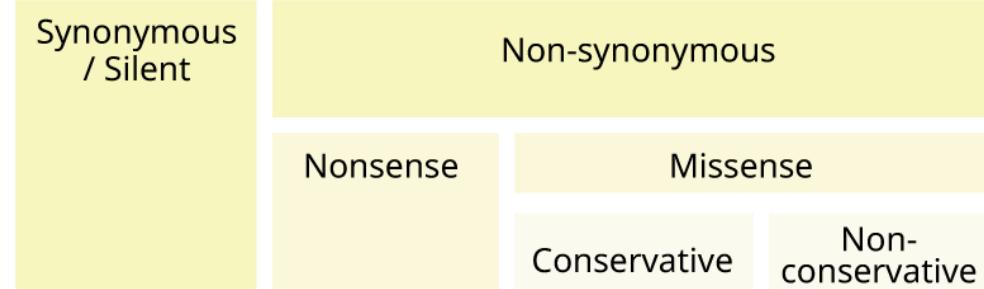
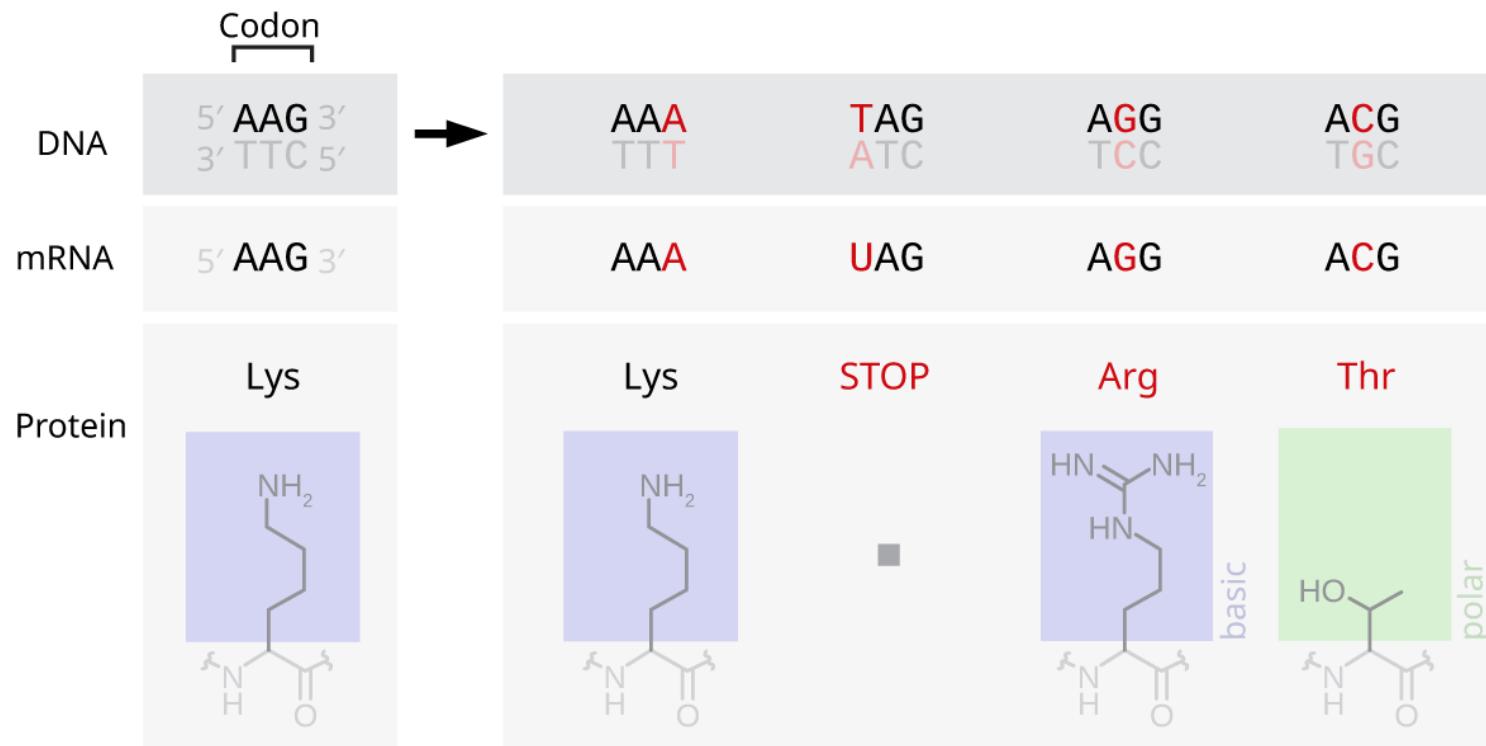
What are the consequence of the variants:

frameshift insertion > frameshift deletion > frameshift block substitution > stopgain > stoploss > nonframeshift insertion > nonframeshift deletion > nonframeshift block substitution > nonsynonymous SNV > synonymous SNV > unknown

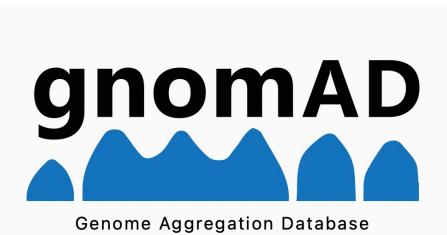
Variant Functional annotation – Frameshift mutations



Variant Functional annotation – nonsynonymous mutations



Variant Functional annotation – frequencies - gnomAD



The **Genome Aggregation Database** (gnomeAD):

- V2.1.1 (GRCh37/hg19) contains 125,748 healthy exome sequences and 157,078 healthy genome sequencing data
- Free to use
- Analyze all samples with the same pipeline and joint variant calling to improve consistency across the projects
- Quality control on each variant
- It also contains structural variants, mitochondrial variants
- It contains population variant frequencies (e.g. European, East Asian etc.)

<https://gnomad.broadinstitute.org/news/2018-10-gnomad-v2-1/>

Variant Functional annotation – frequencies – in-house database

If you have large number of samples (> 1000 individuals):

- Prepared with the same sample preparation method
- Sequenced in the same sequencer
- Analyzed with the same pipeline

Build in-house database

Advantage:

Filter out artefacts in your settings.

Variant Functional annotation – classification – Clinvar



- Archive of interpretations of variants relative to conditions
- Variant-level interpretations
 - Assertion/Clinical significance/Interpretation/Classification/
- Fully public and freely available
- Submission-driven database
- Curation support from NCBI staff

CLINSIG: clinical significant

Variant Functional annotation – classification – HGMD



The Human Gene Mutation Database (HGMD) represents an attempt to collate all known (published) gene lesions responsible for human inherited disease.

New release every quarter.

Free (3 year old data, no downloading, limited, accessible from VEP) and licence version.

<https://www.hgmd.cf.ac.uk/ac/all.php>

Variant Functional annotation – variant functional effect predictors

SIFT (Sorting Intolerant from Tolerant) scores: SIFT uses sequence homology to compute the likelihood that an amino acid substitution will have an adverse effect on protein function. The underlying assumption is that evolutionarily conserved regions tend to be less tolerant of mutations, and hence amino acid substitutions or insertions/deletions in these regions are more likely to affect function (**D: Deleterious; T: tolerated**)

PolyPhen-2 (Polymorphism Phenotyping v2) scores: predicts the possible impact of amino acid substitutions on the stability and function of human proteins using structural and comparative evolutionary considerations. (**D: Probably damaging, P: possibly damaging; B: benign**)

.....

CADD (Combined Annotation Dependent Depletion) scores: a method for objectively integrating many diverse annotations into a single measure (C score) for each variant. C scores correlate with allelic diversity, annotations of functionality, pathogenicity, disease severity, experimentally measured regulatory effects and complex trait associations, precompute C scores for all 8.6 billion possible human single-nucleotide variants and enable scoring of short insertions-deletions. (The higher the worst consequence)

Variant Functional annotation – annovar command

```
perl annovar_2020June07/table_annovar.pl \
    ${input_vcf} \
    annovar_2017Jul16/humandb/ \
    --vcfinput \
    --remove \ # remove all temporary files
    --buildver hg19 \
    --outfile ${output_marker} \
    --otherinfo \ # print out columns after QUAL
    --gff3dbfile repeatMasker_hg19_all.gff3 \
    -protocol
"gff3,refGene,avsnp150,dbnsfp33aReduced,clinvar_20210123,c
add13gt10,gnomad_exomePOPMAX,gnomad_genomePOPMAX" \
    --operation 'r,g,f,f,f,f,f' \
```

View the annotated vcf file under 05_variantannotation (41203088):

Joint Variant Calling

Variant Calling on multiple samples – higher sensitivity and better genotype

1. Clearer distinction between homozygous reference sites and sites with missing data

Batch-calling does not output a genotype call at sites where no member in the batch has evidence for a variant; it is thus impossible to distinguish such sites from locations missing data. In contrast, joint calling emits genotype calls at every site where any individual in the call set has evidence for variation.

2. Greater sensitivity for low-frequency variants

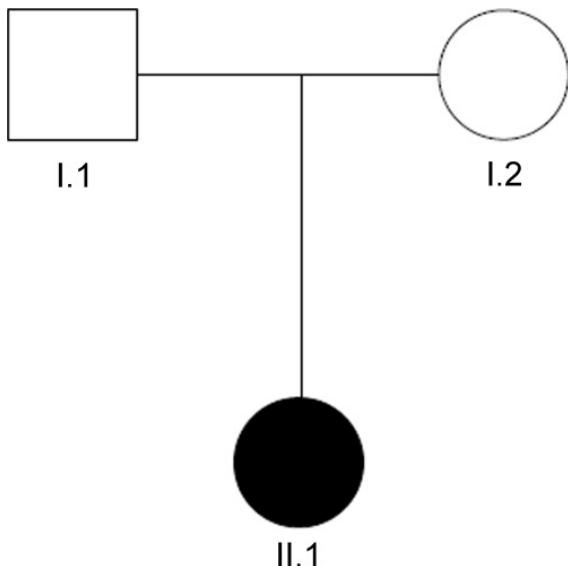
By sharing information across all samples, joint calling makes it possible to “rescue” genotype calls at sites where a carrier has low coverage but other samples within the call set have a confident variant at that location. However this does not apply to singletons, which are unique to a single sample. To minimize the chance of missing singletons, we increase the cohort size -- so that singletons themselves have less chance of happening in the first place.

3. Greater ability to filter out false positives (VQSR works better with bigger data)

Joint Variant Calling – trio pedigree file

A pedigree is a structured description of the familial relationships between samples. One row = one person

Family ID Individual ID Paternal ID Maternal ID Sex Phenotype



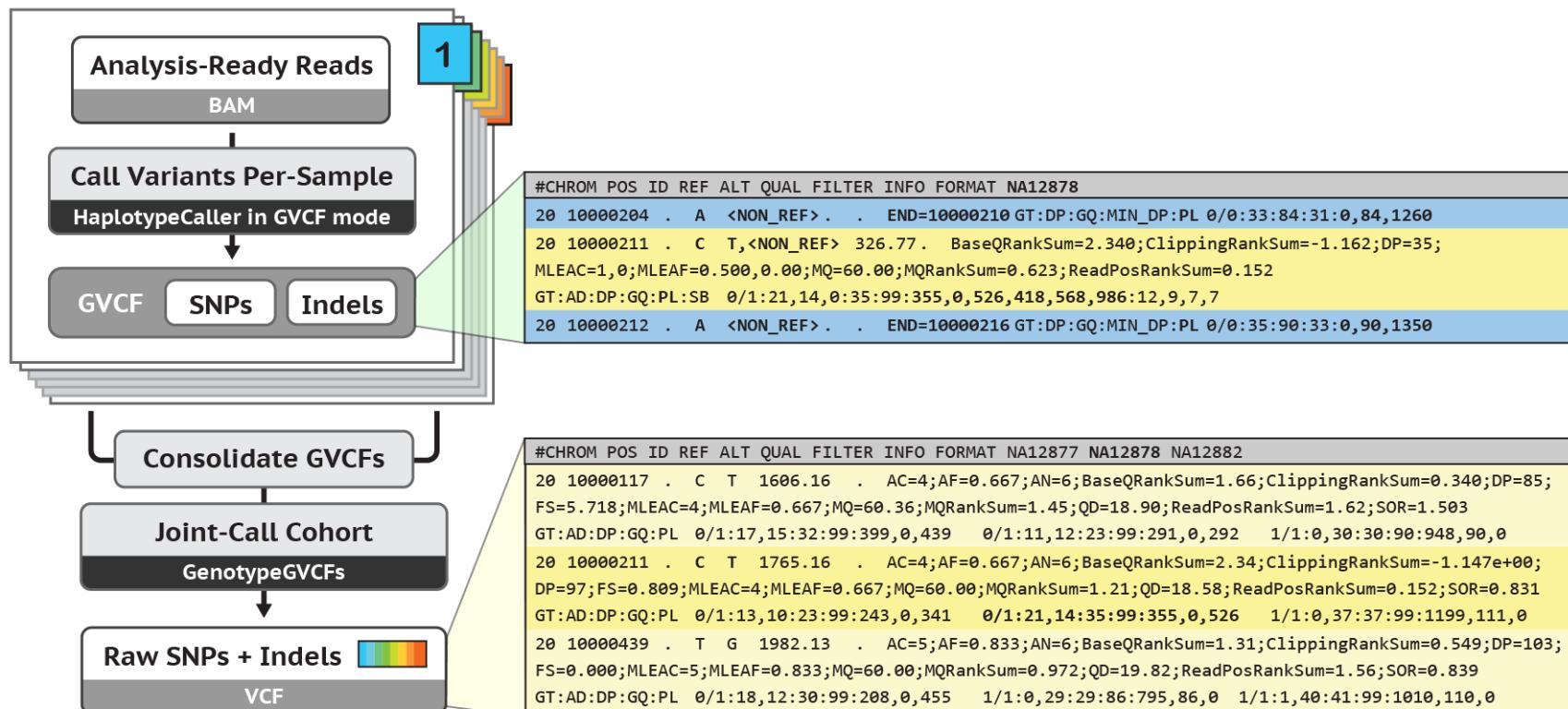
Family ID	Individual ID	Paternal ID	Maternal ID	Sex	Phenotype
Family_1	II.1	I.1	I.2	2	2
Family_1	I.1	0	0	1	1
Family_1	I.2	0	0	2	1

Family_1 II.1 I.1 I.2 2 2
Family_1 I.1 0 0 1 1
Family_1 I.2 0 0 2 1

Sex: 1=male; 2=female; other=unknown

Phenotype: 1=unaffected, 2=affected, 0,-9=missing

Joint Variant Calling - workflow



Joint Variant Calling - command

```
g.vcf.gz per individual
```

```
gatk CombineGVCFs \
-R ${reference} \
--variant proband.g.vcf.gz \
--variant father.g.vcf.gz \
--variant mother.g.vcf.gz \
-ped family_1.ped \
-O family_1.combined.g.vcf.gz
```

```
GenotypeGVCFs on the
combined.g.vcf.gz with
-ped
```

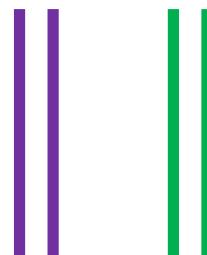
Trio analysis - De novo variants and Recessive variants

Suppose both parents are healthy, the child has the disease. Try to find differences between the child and the parents.

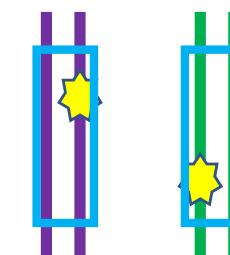
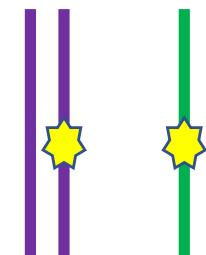
Parents don't have: De novo

Parents just have one while the child have two: recessive variants, compound heterozygosity

De novo
variants



Recessive
variants



Pedigree check and gender detection – Quality Control

Pedigree check: whether the three individuals coming from the same family, by comparing with genotypes, e.g. AA+BB=AB

Gender detection: Coverage on chrX,Y compared those to autosomes

View the joint variant calling vcf file under: 06_jointvc/HG002.filter.vcf

Genome in a bottle (GIAB) – control samples and validation

<https://www.nist.gov/programs-projects/genome-bottle>

Benchmark (or "High-confidence") variant calls and regions.

For the same control samples, collect variant calls from different sequencing platform and different analysis pipelines. Find consensus calls and confident regions to create a 'true' set.

Free for downloading.

Have both single samples and trio samples.

Storage, Pipeline, etc.

Storage

	exome	genome
Fastq files	15 G	80 G
BAM files	10 G	80 G

Pipelines: instead of running commands step by step, it runs the whole workflow without interaction, also consider parallelization and catching errors.

Pipeline language: nextflow (nf-core) and WDL (GATK)