NATIONAL BIOINFORMATICS INFRASTRUCTURE SWEDEN

SciLifeLab

Variant-calling Workflow

Markus Mayrhofer

Overview

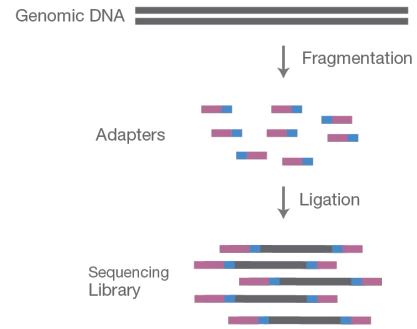
- Workflows
- Basic variant calling in one sample
- Basic variant calling in cohort
- Introduction to exercise

Tomorrow:

- GATK's Best practices

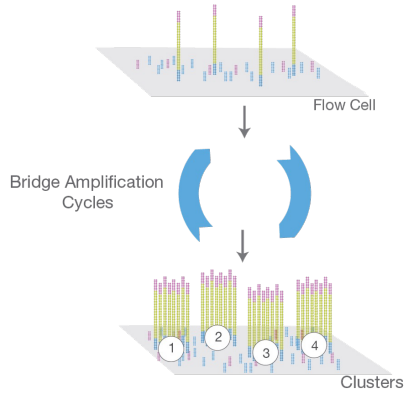
Illumina Sequencing

A. Library Preparation



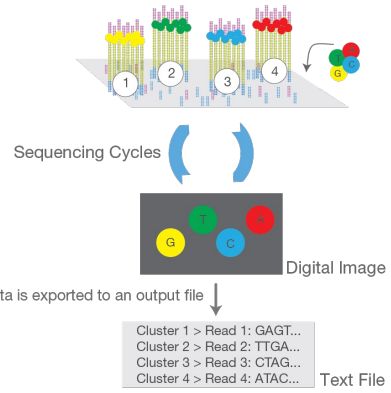
NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.

B. Cluster Amplification



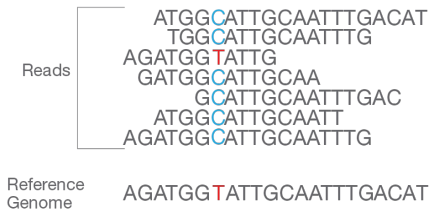
Library is loaded into a flow cell and the fragments are hybridized to the flow cell surface. Each bound fragment is amplified into a clonal cluster through bridge amplification.

C. Sequencing



Sequencing reagents, including fluorescently labeled nucleotides, are added and the first base is incorporated. The flow cell is imaged and the emission from each cluster is recorded. The emission wavelength and intensity are used to identify the base. This cycle is repeated “n” times to create a read length of “n” bases.

D. Alignment and Data Anaylsis



Reads are aligned to a reference sequence with bioinformatics software. After alignment, differences between the reference genome and the newly sequenced reads can be identified.

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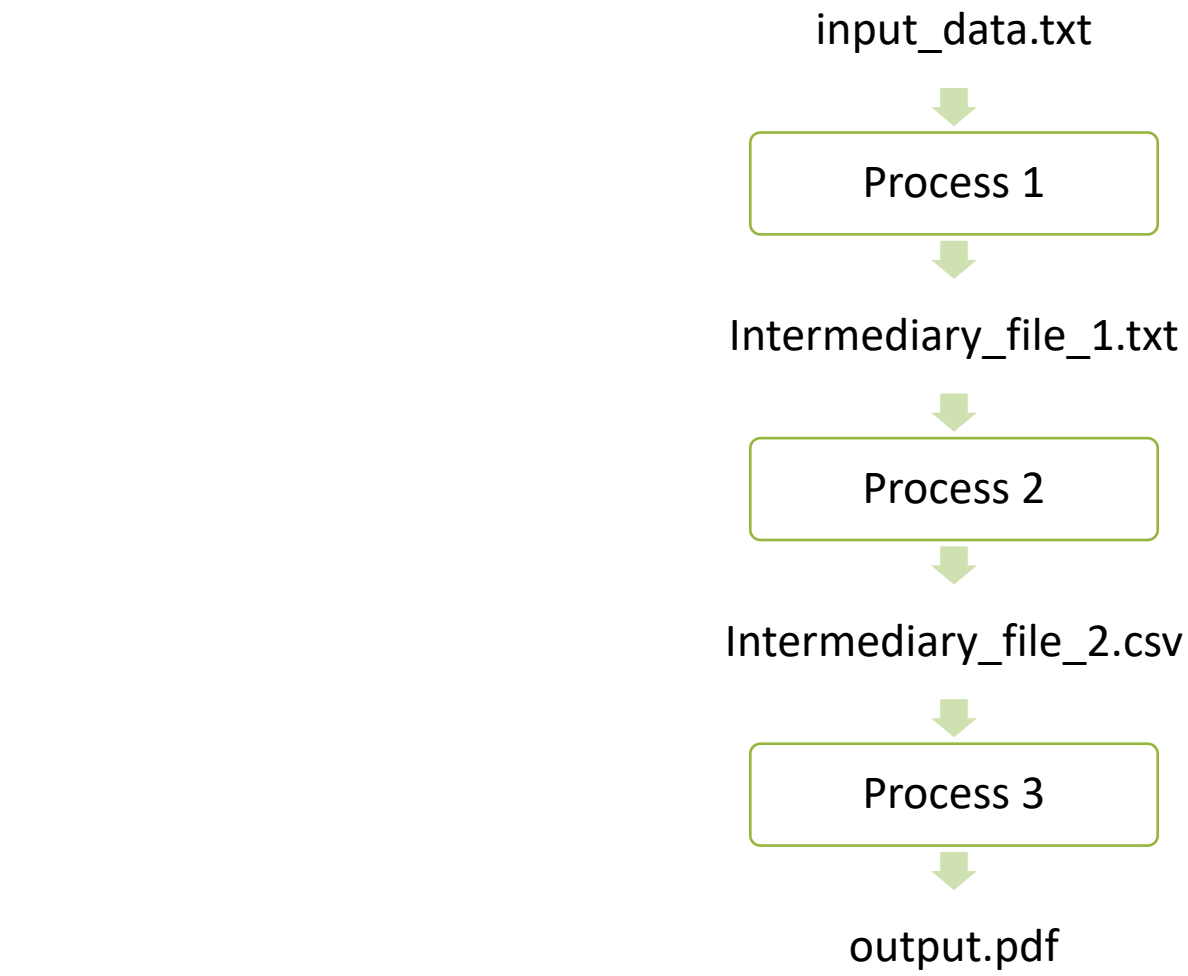
Workflows

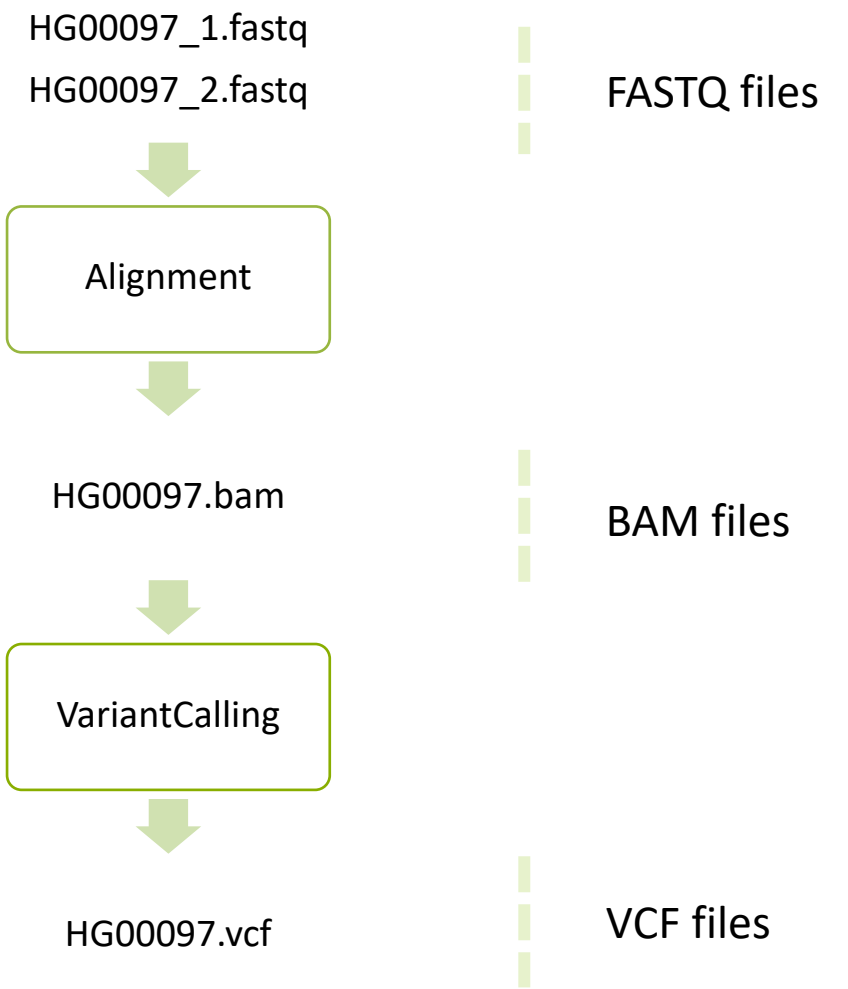
What is a workflow



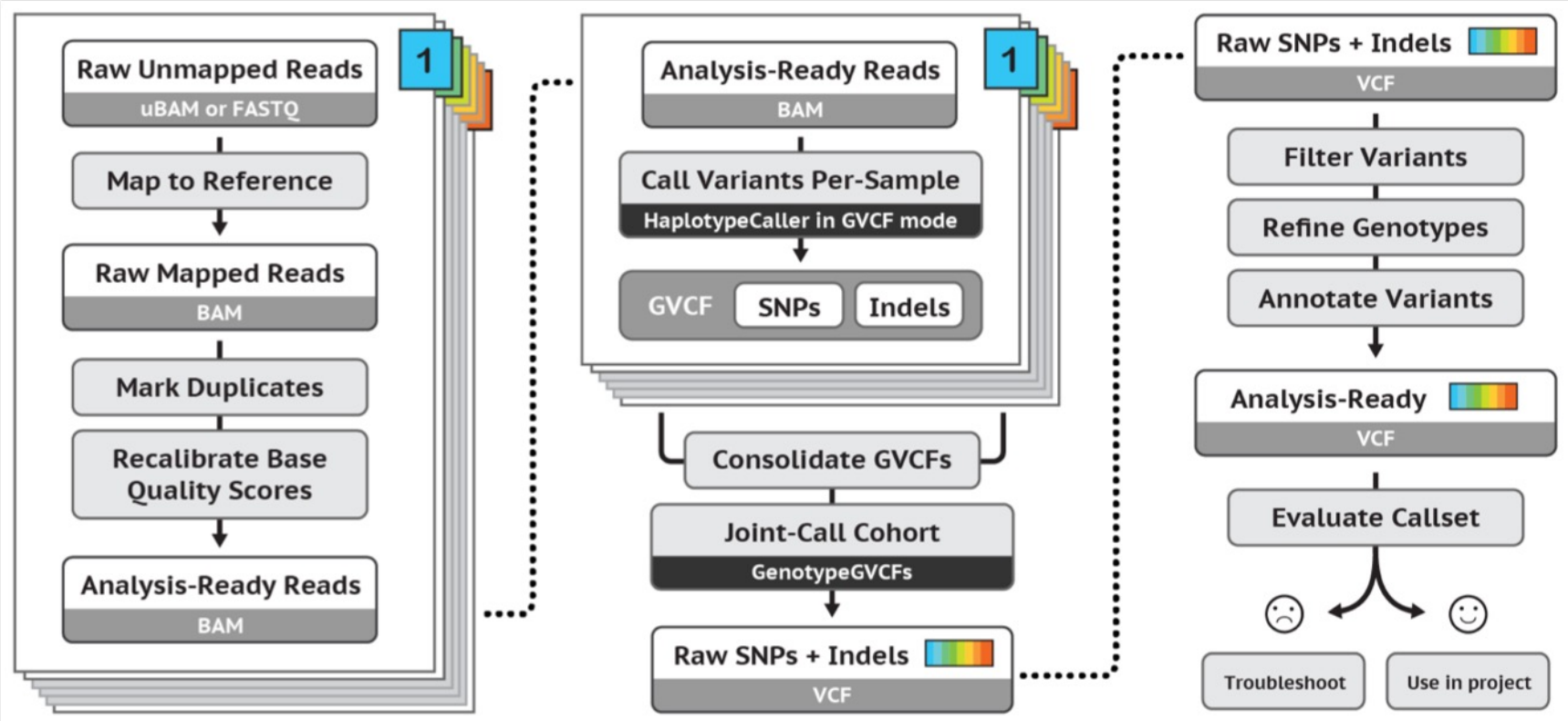
Workflow conventions

- Create a new output file in each process – don't ower write the input file
- Use informative file names
- Include information of the process in output file name

Example: Basic variant calling in one sample



GATK's best practices workflow for germline short variant discovery



https://software.broadinstitute.org/gatk/best-practices/

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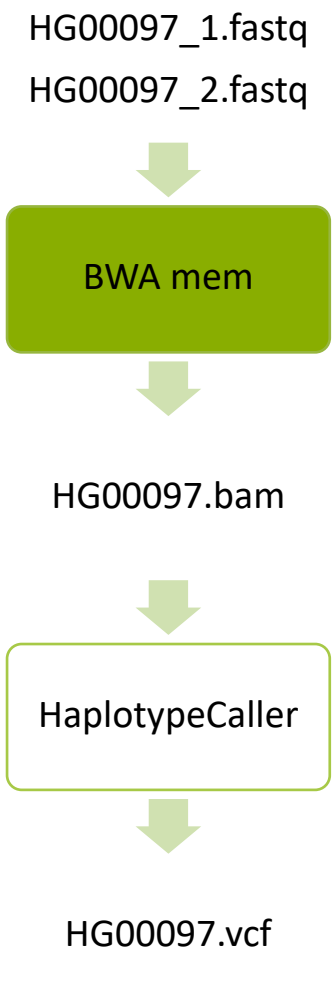
Basic Variant Calling in one sample

Alignment

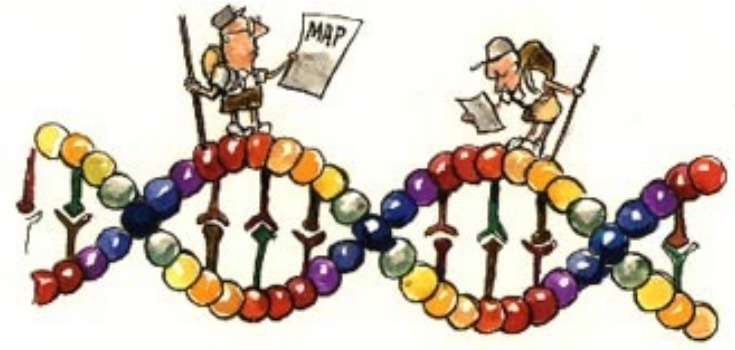


FASTQ files

BAM files

VCF files

The reference genome



A reference genome is a haploid nucleic acid sequence which represents a species genome.

The first draft of the human genome contained 150,000 gaps.

GRCh37: 250 gaps

We will work with GRCh37 in the lab.

Keep track of the reference version!

The reference genome sequence is used as input in many bioinformatics applications for NGS data:

- mapping
- variant calling
- annotation

You must keep track of which version of the reference genome your data was mapped to.

The same version must be used in all downstream analyses.

File indices

- Most large files we work with, such as the reference genome, need an index
- Allows efficient access to the file
- Different indices for different file-types
- Bwa index = Burrows-Wheeler transform of reference genome (several files)
- Needs index: fasta, bam vcf files

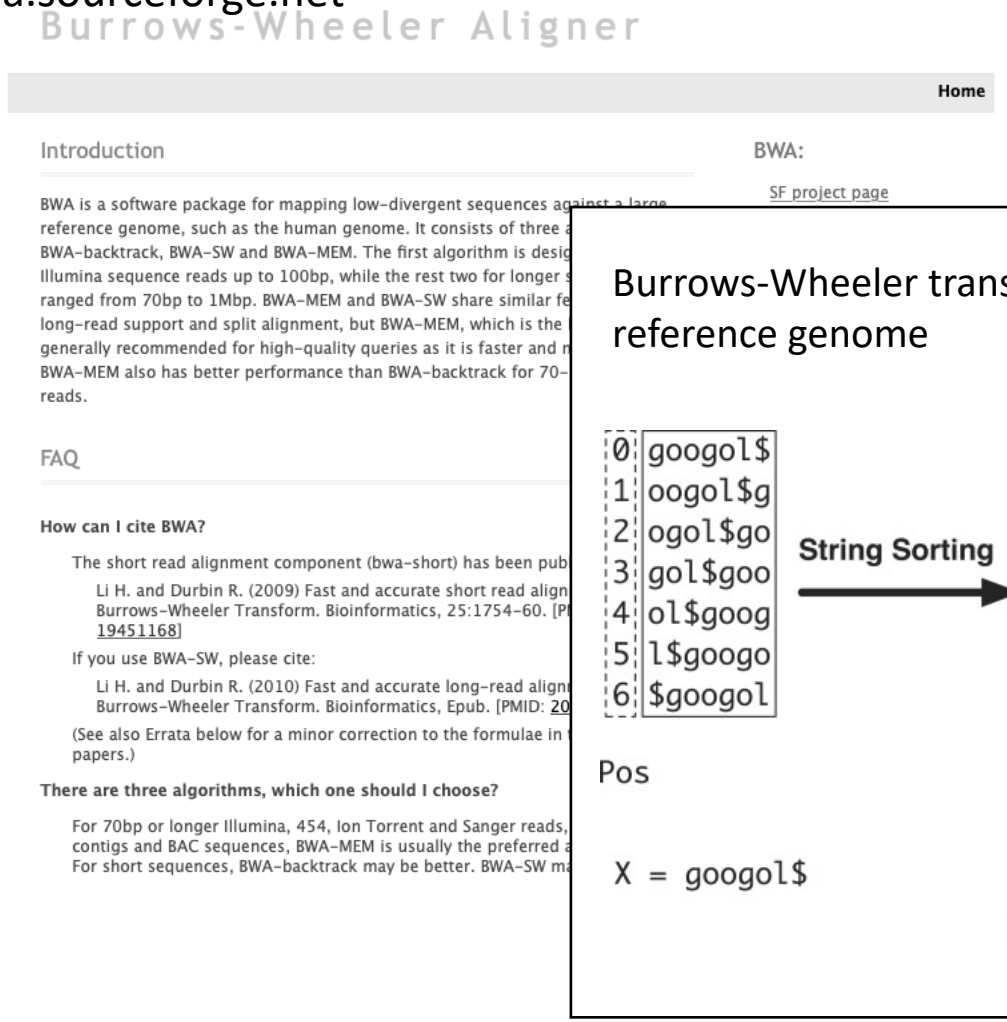
Alignment

AACAGGTATATCTTCCCCGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTAGCTACCCTCTTCCTTAGGGACTGTAC

GCTAGCTAGCTAGCTACCCT

Burrows-Wheeler Aligner

http://bio-bwa.sourceforge.net



Alignment

```
module load bwa
```

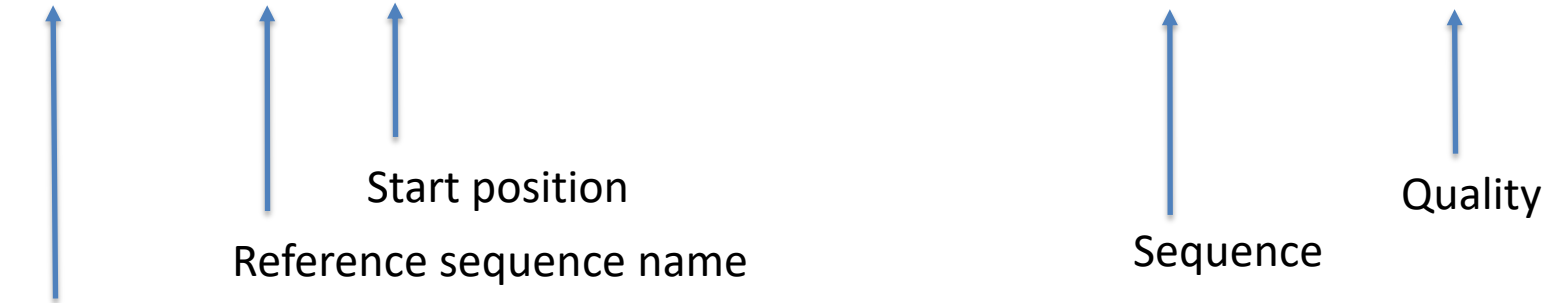
Output from mapping - Sam format

HEADER SECTION

```
@HD    VN:1.6SO:coordinate
@SQ    SN:2  LN:243199373
@PG    ID:bwaPN:bwaVN:0.7.17-r1188    CL:bwa mem -t 1 human_g1k_v37_chr2.fasta HG00097_1.fq HG00097_2.fq
@PG    ID:samtools PN:samtools PP:bwaVN:1.10      CL:samtools sort
@PG    ID:samtools.1      PN:samtools PP:samtools VN:1.10      CL:samtools view -H HG00097.bam
```

ALIGNMENT SECTION

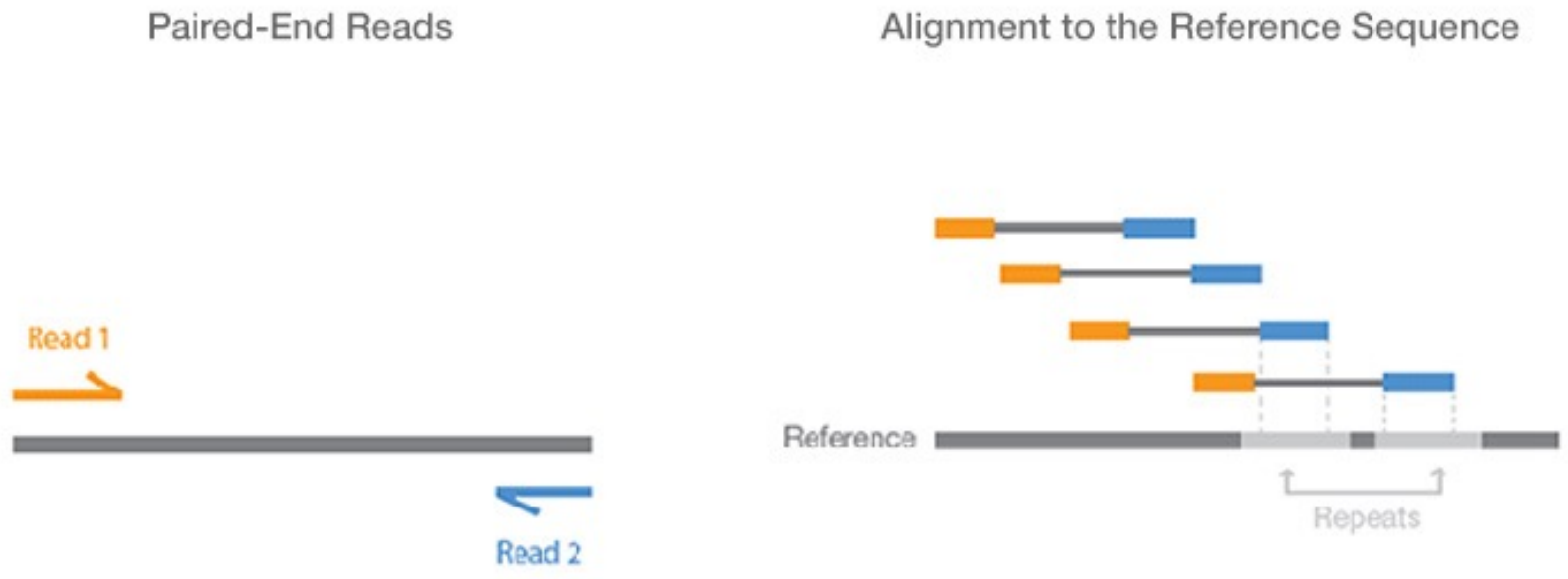
```
Read_001      99      2      3843448      0      101M    =      3843625      278     TTTGGTTCCATATGAACTTT        0F<BFB<FFFBFBFFFBFBB
Read_001      147     2      3843625      0      101M    =      3843448      -278    TTATTTCATTGAGCAGTGGT        FBBI7IIFIB<BBBB<BBFF
Read_002      163     2      4210055      0      101M    =      4210377      423     TGGTACCAAAACAGAGATAT        0IIFBFFFIIIFFIFFFBBF
Read_003      99      2      4210066      0      101M    =      4210317      352     CAGAGATATAGATCAATGGA        0IIFFFIFFFIFIFIIIIIF
```



Convert to Bam

Bam file is a binary representation of the Sam file

Paired-end data



Paired-end sequencing enables both ends of the DNA fragment to be sequenced. Because the distance between each paired read is known, alignment algorithms can use this information to map the reads over repetitive regions more precisely. This results in much better alignment of the reads, especially across difficult-to-sequence, repetitive regions of the genome.

Paired-end data

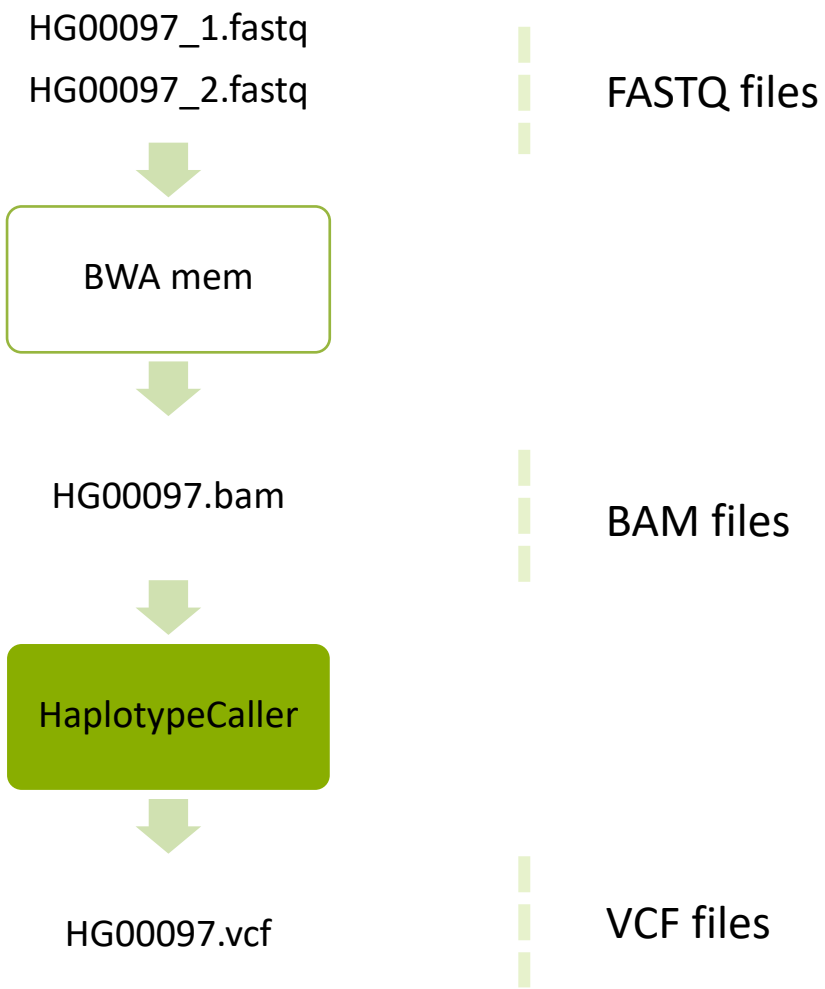
ID_R1_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:2
197 1:N:0:ATCACG
CAGTTGCGATGAGAGCGTTGAGAAGTATAATAGG
AGTTAAACTGAGTAACAGGATAAGAAATAGTGAG
ATATGGAAACGTTGTGGTCTGAAAGAAGATGT
+
B@CFFFFFHHHHHGJJJJJJJJJJFHHIIIIJJ
JIHGIIJJJJIJIJIJJJJIIJJJJJIIEIHHIJ
HGHHHHHDFFFEDDDDDCDDDCDDDDDDDCDC
```

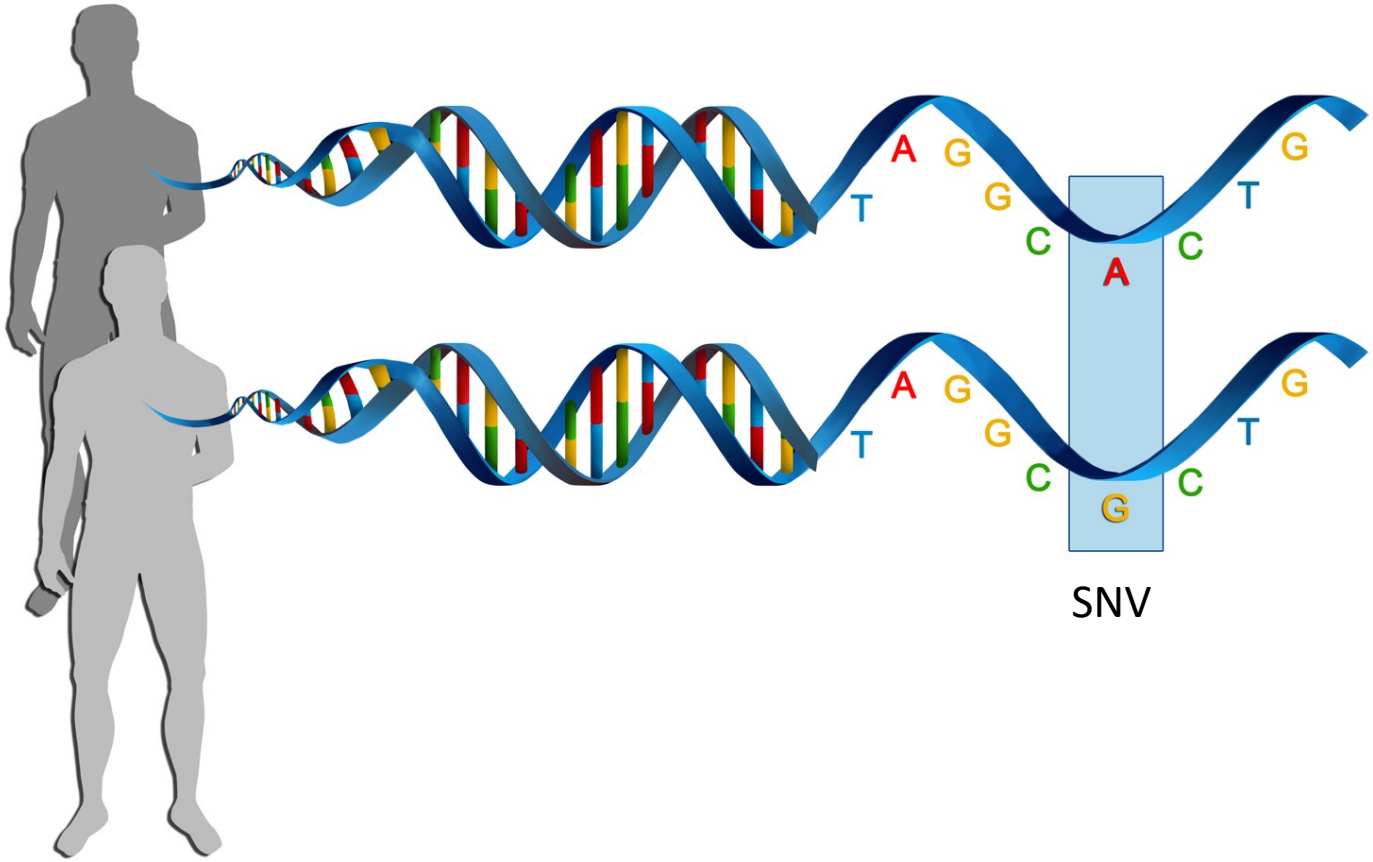
ID_R2_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:
2197 2:N:0:ATCACG
CTTCGTCCACTTTCATTATTCCTTTCATACATG
CTCTCCGGTTTAGGGTACTCTTGACCTGGCCTT
TTTTCAAGACGTCCCTGACTTGATCTTGAAACG
+
CCCFFFFFHHHHHJJJJIJJJJJJJJJJJJJJ
JJJJJJJIJIJGIJHBGHHIIIJIJJJJJJJJI
JJJHFFFFFFDDDDDDDDDDDDDDDDEDCCDDDD
```

Variant calling



Genetic variation



Genetic variation = differences in DNA among individuals of the same species

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Detecting variants in reads

```
Reference:      ...GTGCGTAGACTGCTAGATCGAAGA...
Sample:         ...GTGCGTAGACTGATAGATCGAAGA...
                ...GTGCGTAGACTGATAGATCGAAGA...
                ...GTGCGTAGACTGCTAGATCGAAGA...
                ...GTGCGTAGACTGCTAGATCGAAGA...
                ...GTGCGTAGACTGATAGATCGAAGA...
                ...GTGCGTAGACTGATAGATCGAAGA...
                ...GTGCGTAGACTGCTAGATCGAAGA...
                ...GTGCGTAGACTGATAGATCGAAGA...
                ...GTGCGTAGACTGCTAGATCGAAGA...
                ...GTGCGTAGACTGATAGATCGAAGA...
```

Reference- and alternatve alleles

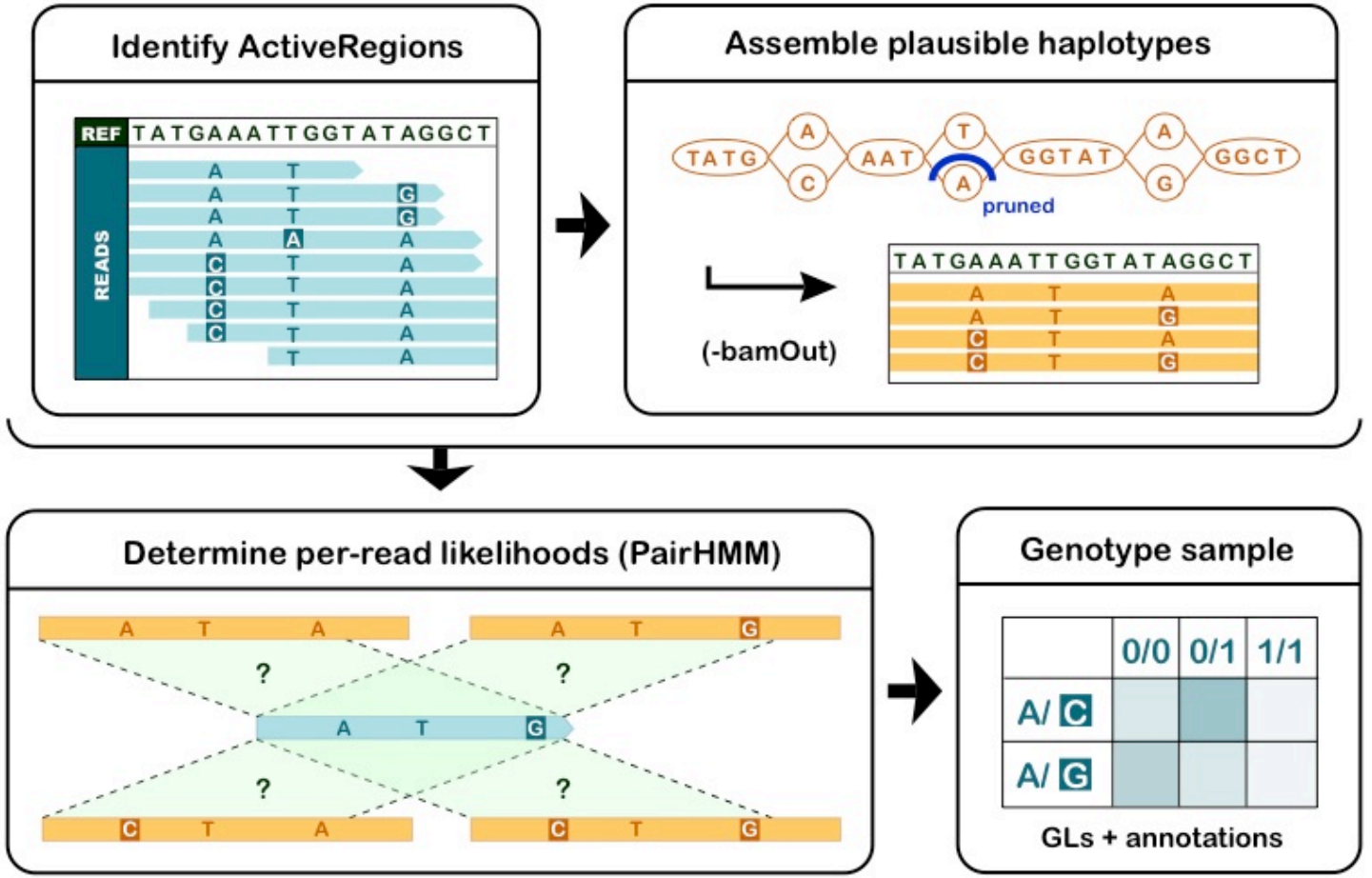
TGGGCTTTTCCAACAGGTATATCTTCCCCGCTAGCTAGCTAGCTACTTCAAATTCCT

Reference allele AGCTAGCTA

Alternative allele AGCTGGCTA

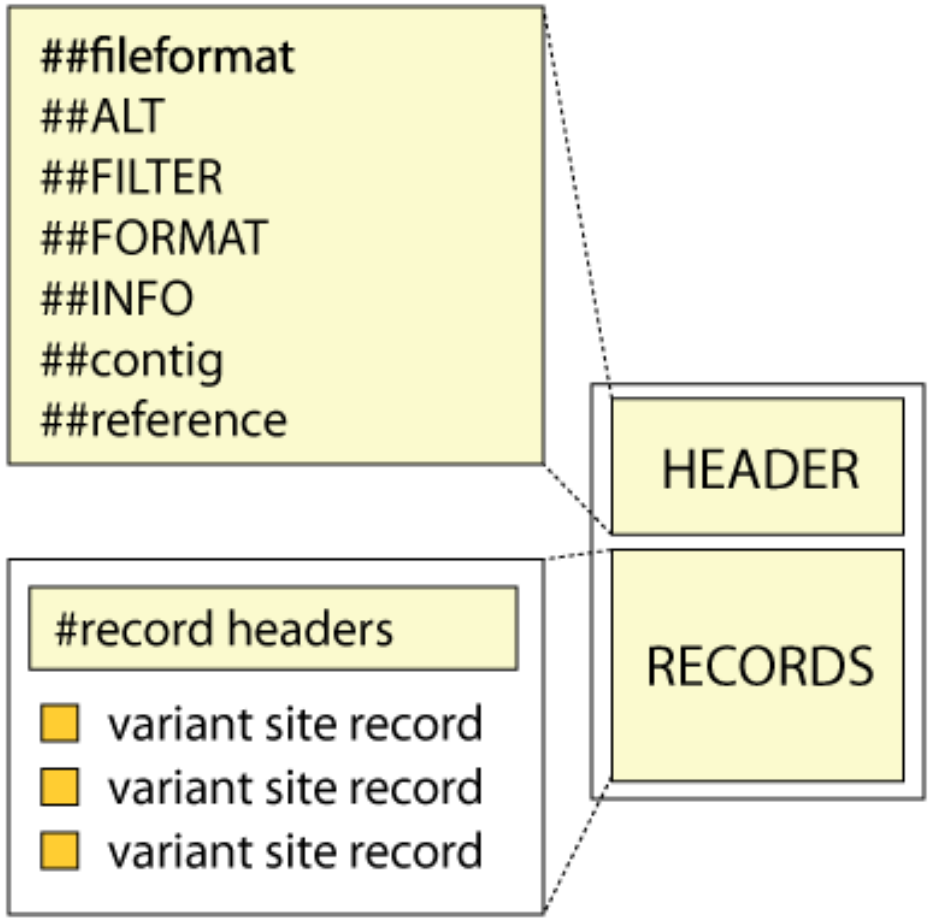
Reference allele = the allele in the refence genome
Alternative allele = the allele NOT in the refence genome

Variant Calling
HaplotypeCaller



For more info: https://www.youtube.com/watch?v=NQHGkVGICpY

Variant Call Format (VCF)



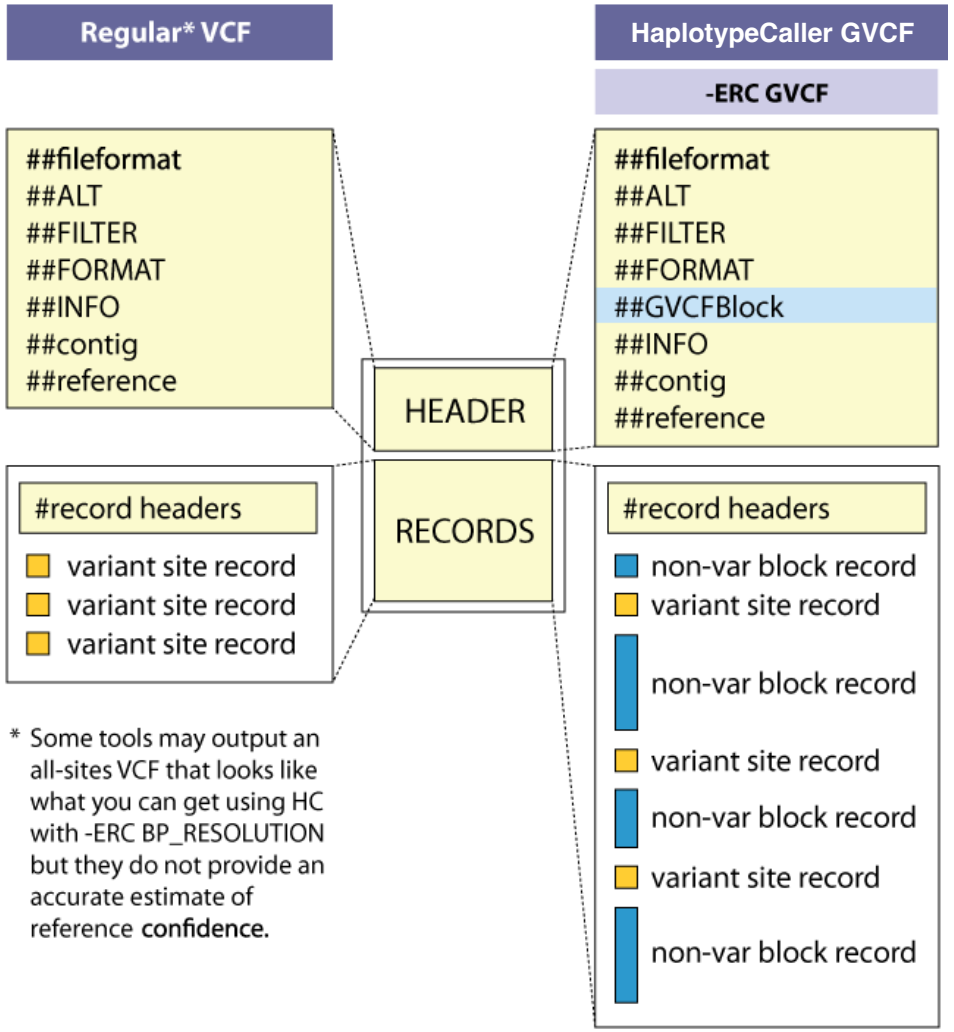
Variant Call Format (VCF)

```
##fileformat=VCFv4.2
##FILTER=<ID=LowQual,Description="Low quality">
##FORMAT=<ID=AD,Number=R,Type=Integer,Description="Allelic depths for the ref and alt alleles in the order listed">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth (reads with MQ=255 or with bad mates are filtered)">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##INFO=<ID=AC,Number=A,Type=Integer,Description="Allele count in genotypes, for each ALT allele, in the same order as listed">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency, for each ALT allele, in the same order as listed">
##INFO=<ID=AN,Number=1,Type=Integer,Description="Total number of alleles in called genotypes">
##contig=<ID=2,length=243199373>
##source=HaplotypeCaller
#CHROM  POS             ID      REF     ALT     QUAL    FILTER          INFO        FORMAT      HG00097
2       136220992       .       G       GT      30.64   .       AC=1;AF=0.500;AN=2  GT:AD:DP    0/1:3,2:5
2       136226814       .       GAC     G       44.60   .       AC=1;AF=0.500;AN=2  GT:AD:DP    0/1:4,2:6
2       136234279       .       C       T       102.60  .       AC=1;AF=0.500;AN=2  GT:AD:DP    0/1:3,4:7
2       136234284       .       C       T       102.60  .       AC=1;AF=0.500;AN=2  GT:AD:DP    0/1:3,4:7
2       136263277       .       T       A       148.60  .       AC=1;AF=0.500;AN=2  GT:AD:DP    0/1:8,5:13
…
…
```

Basic variant calling in cohort

Basic variant calling in cohort

GVCF Files are valid VCFs with extra information



- GVCF has records for all sites, whether there is a variant call there or not.
- The records include an accurate estimation of how confident we are in the determination that the sites are homozygous-reference or not.
- Adjacent non-variant sites merged into blocks

Basic variant calling in cohort

Variant Call Format (VCF)

```
##fileformat=VCFv4.2
##ALT=<ID=NON_REF,Description="Represents any possible alternative allele at this location">
##FILTER=<ID=LowQual,Description="Low quality">
##FORMAT=<ID=AD,Number=R,Type=Integer,Description="Allelic depths for the ref and alt alleles in the order listed">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth (reads with MQ=255 or with bad mates are filtered)">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##INFO=<ID=AC,Number=A,Type=Integer,Description="Allele count in genotypes, for each ALT allele, in the same order as listed">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency, for each ALT allele, in the same order as listed">
##INFO=<ID=AN,Number=1,Type=Integer,Description="Total number of alleles in called genotypes">
##contig=<ID=2,length=243199373>
##source=CombineGVCFs
##source=GenotypeGVCFs
##source=HaplotypeCaller
#CHROM  POS        ID  REF  ALT  QUAL    FILTER  INFO              FORMAT    HG00097     HG00100     HG00101
2       136045826  .   G    A    167.26  .       AC=1;AF=0.167;AN=6  GT:AD:DP  0/0:8,0:8   0/0:13,0:13 0/1:1,5:6
2       136046443  .   CGT  C    129.27  .       AC=3;AF=0.500;AN=6  GT:AD:DP  0/0:8,0:8   0/1:3,1:4   1/1:0,4:4
2       136047387  .   T    C    186.27  .       AC=1;AF=0.167;AN=6  GT:AD:DP  0/0:6,0:6   0/0:16,0:16 0/1:4,6:10
2       136048649  .   C    G    127.26  .       AC=1;AF=0.167;AN=6  GT:AD:DP  0/0:13,0:13 0/0:9,0:9   0/1:1,4:5
2       136052318  .   C    T    107.26  .       AC=1;AF=0.167;AN=6  GT:AD:DP  0/0:7,0:7   0/0:13,0:13 0/1:3,3:6
```

Today's lab

1000 Genomes data

- Low coverage WGS data
- 3 samples
- Small region on chromosome 2

About the samples:
https://www.internationalgenome.org/data-portal/sample

The Lactase enzyme

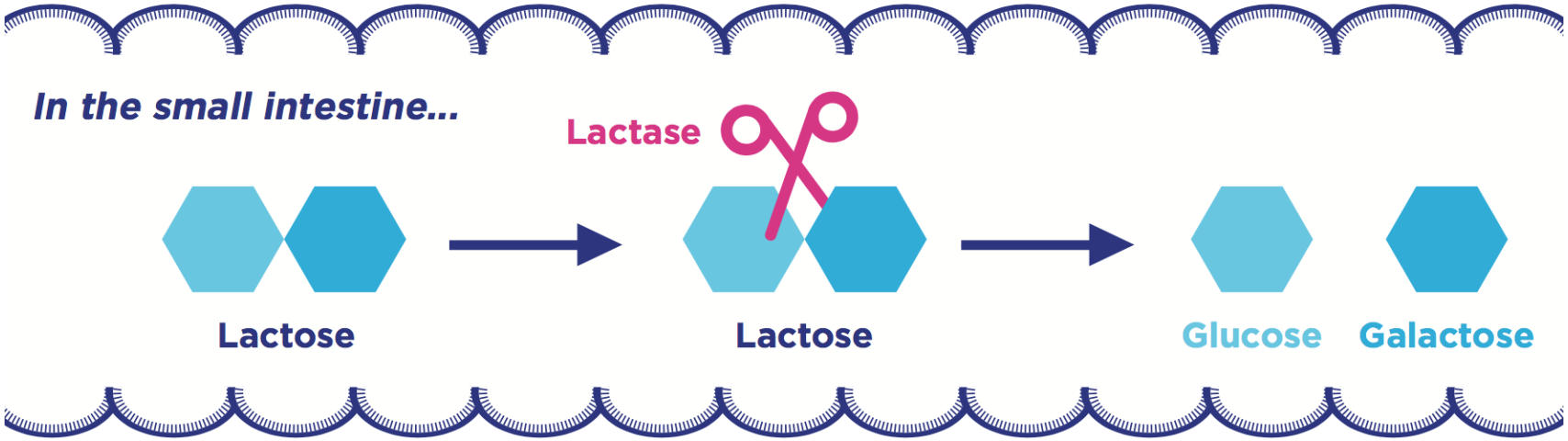


Figure 2. Lactose digestion in the intestine.

- All mammals produce lactase as infants
- Some human produce lactase in adulthood
- Genetic variation upstream of the *LCT* gene cause the lactase persistent phenotype (lactose tolerance)

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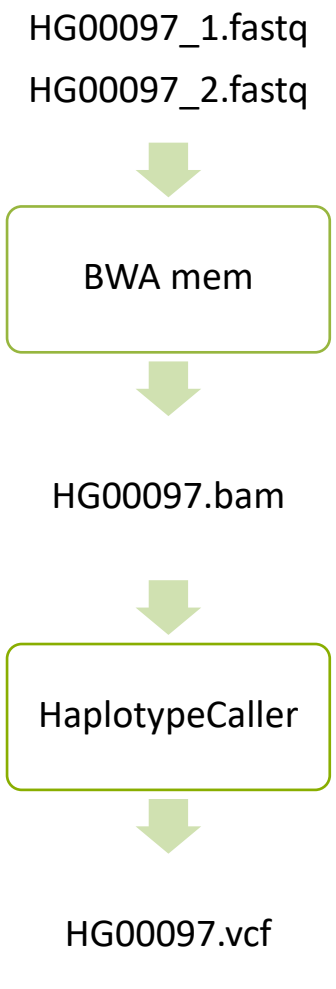
Part 1:

Variant calling in one sample

Basic variant calling in one sample



FASTQ files

BAM files

VCF files

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Part 2:

Variant calling in cohort

Joint variant calling workflow

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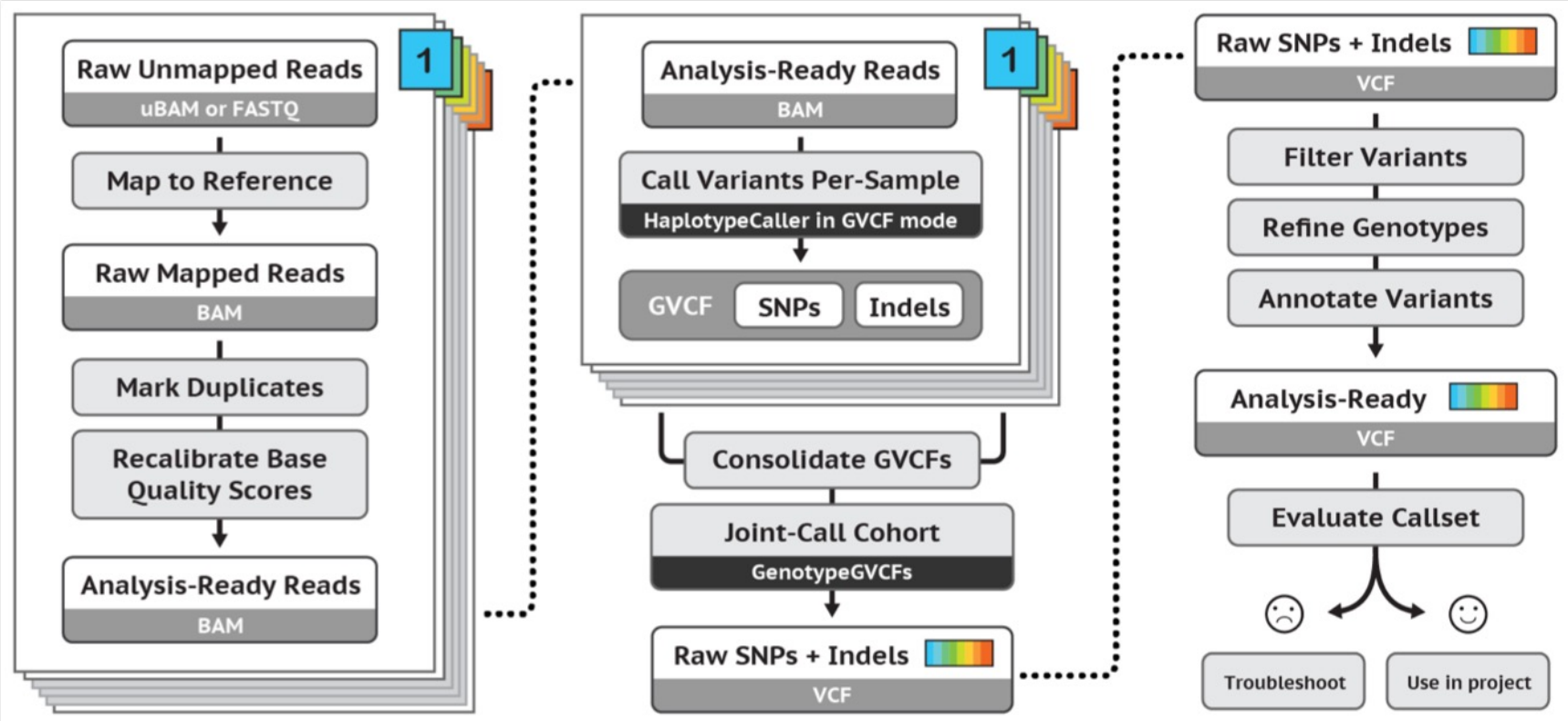
Part 3:

Follow GATK best practices for short variant discovery

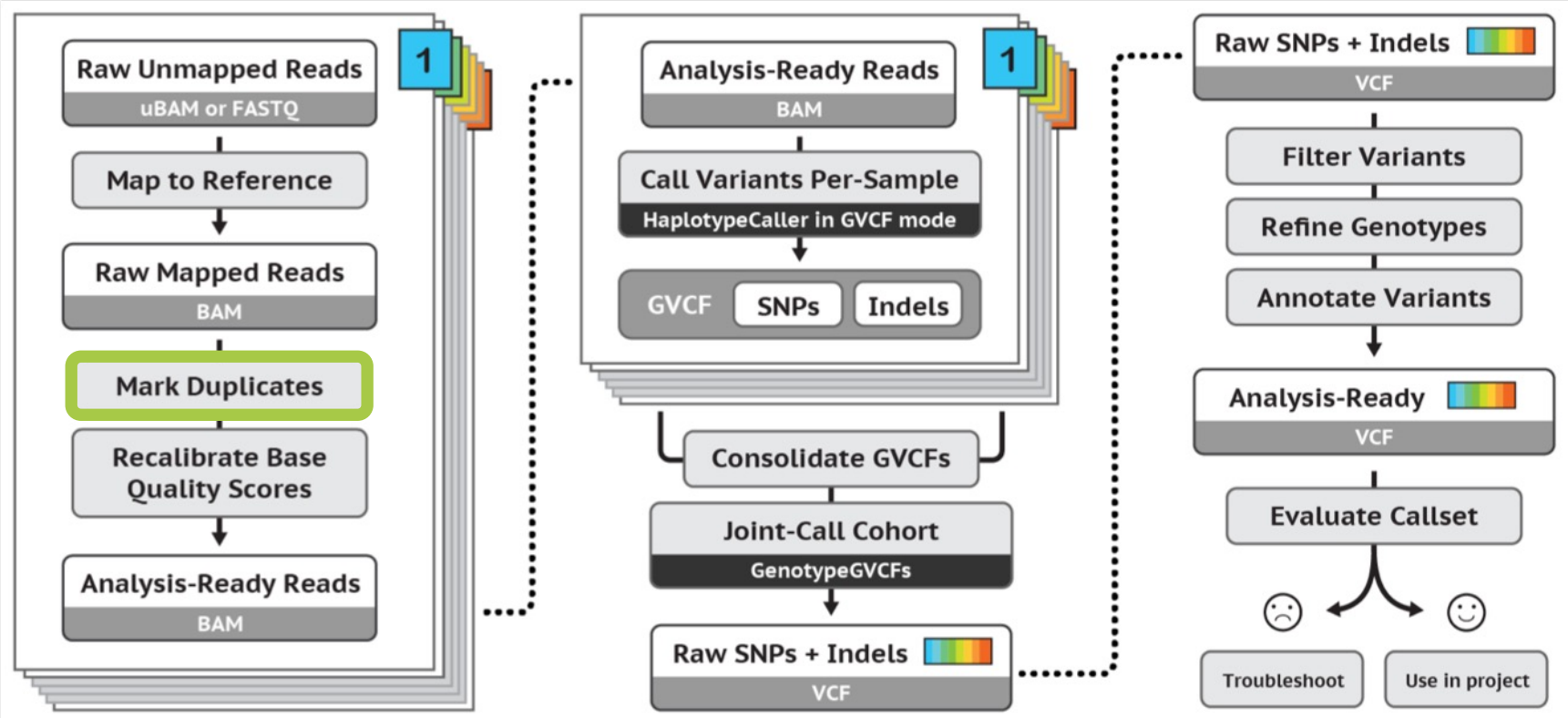
https://gatk.broadinstitute.org

GATK's best practices workflow for germline short variant discovery



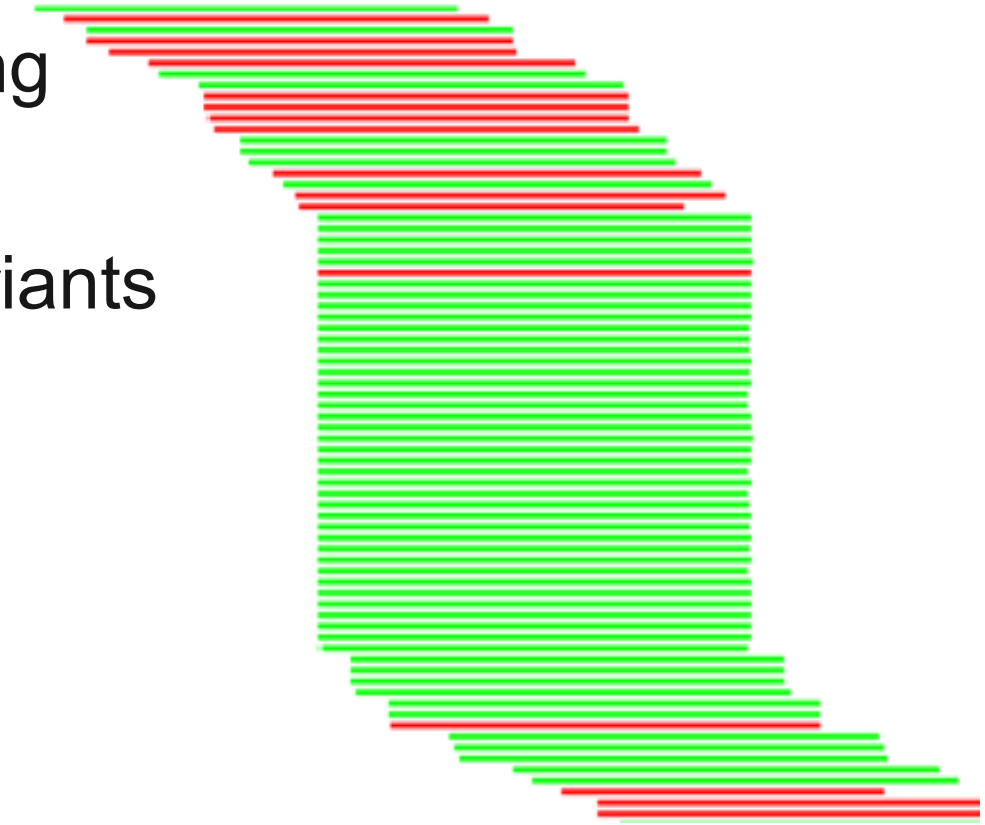
https://software.broadinstitute.org/gatk/best-practices/

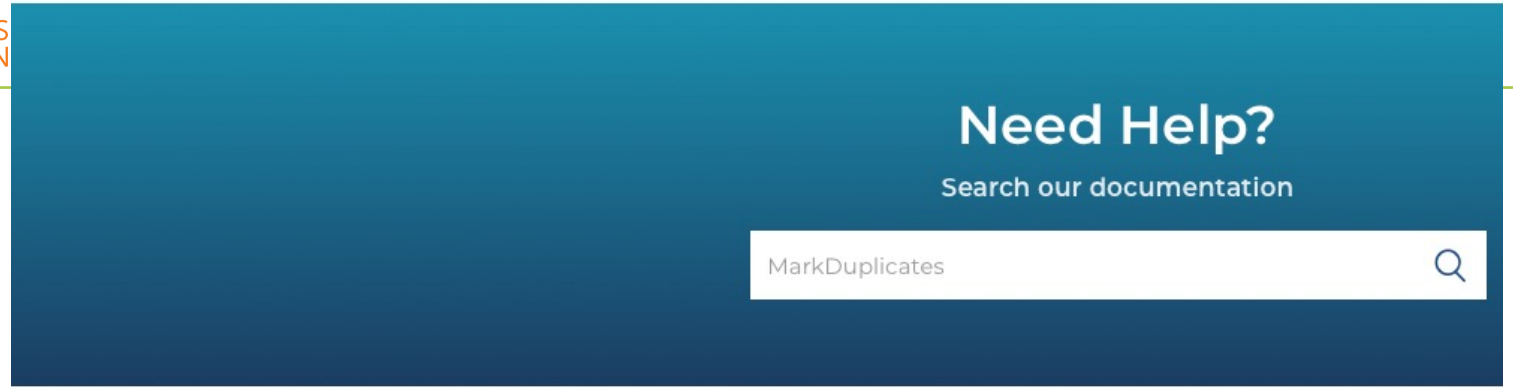
Mark Duplicates



Duplicate reads

- PCR duplicates - library preparation
- Optical duplicates - sequencing
- Don’t add unique information
- Gives false allelic ratios of variants
- Should be removed/marked





MarkDuplicates (Picard) Follow

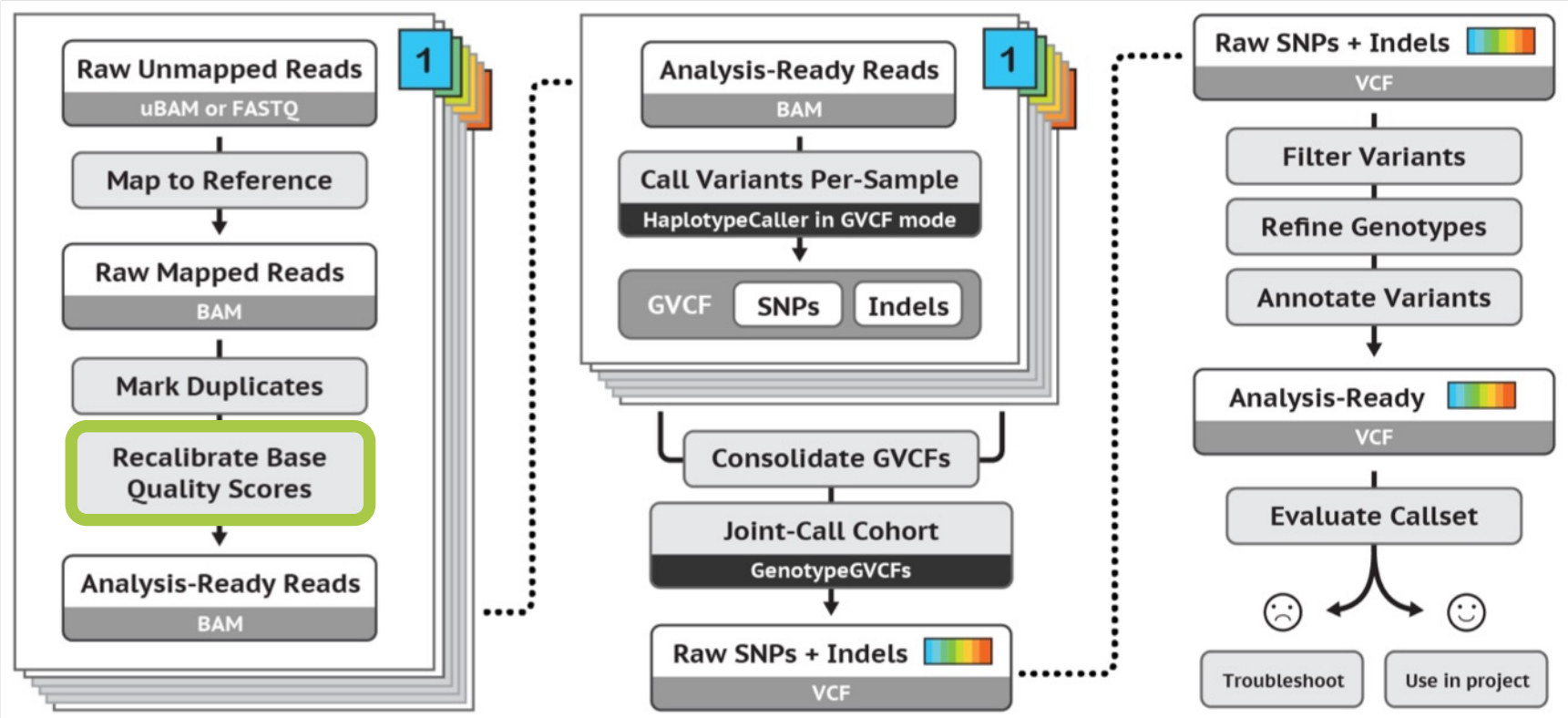
GATK Team
10 months ago · Updated

Identifies duplicate reads.

This tool locates and tags duplicate reads in a BAM or SAM file, where duplicate reads are defined as originating from a single fragment of DNA. Duplicates can arise during sample preparation e.g. library construction using PCR. See also EstimateLibraryComplexity for additional notes on PCR duplication artifacts. Duplicate reads can also result from a single amplification cluster, incorrectly detected as multiple clusters by the optical sensor of the sequencing instrument. These duplication artifacts are referred to as optical duplicates.

```
gatk --java-options -Xmx7g MarkDuplicates \
      -I input.bam \
      -O marked_duplicates.bam \
      -M marked_dup_metrics.txt
```

Base Quality Score Recalibration (BQSR)



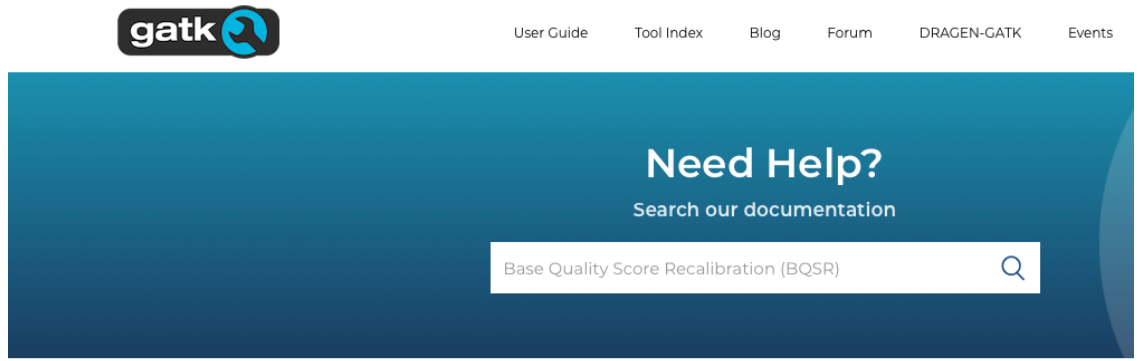
Base Quality Score Recalibration (BQSR)

- During base calling, the sequencer estimates a quality score for each base. This is the quality scores present in the fastq files.

- Systematic (non-random) errors in the base quality score estimation can occur.
  - due to the physics or chemistry of the sequencing reaction
  - manufacturing flaws in the equipment
  - etc

- Can cause bias in variant calling

- **Base Qualtiy Score Recalibration** helps to calibrate the scores so that they correspond to the real per-base sequencing error rate (phred scores)



GATK / Technical Documentation / Algorithms

Base Quality Score Recalibration (BQSR) Follow

GATK Team
5 days ago · Updated

BQSR stands for Base Quality Score Recalibration. In a nutshell, it is a data pre-processing step that detects systematic errors made by the sequencing machine when it estimates the accuracy of each base call.

*Note that this **base** recalibration process (BQSR) should not be confused with **variant** recalibration (VQSR), which is a sophisticated filtering technique applied on the variant callset produced in a later step. The developers who named these methods wish to apologize sincerely to anyone, especially Spanish-speaking users, who get tripped up by the similarity of these names.*

Contents

Articles ir

ActiveRe
(Haploty

Evaluatir
and varia
Mutect2)

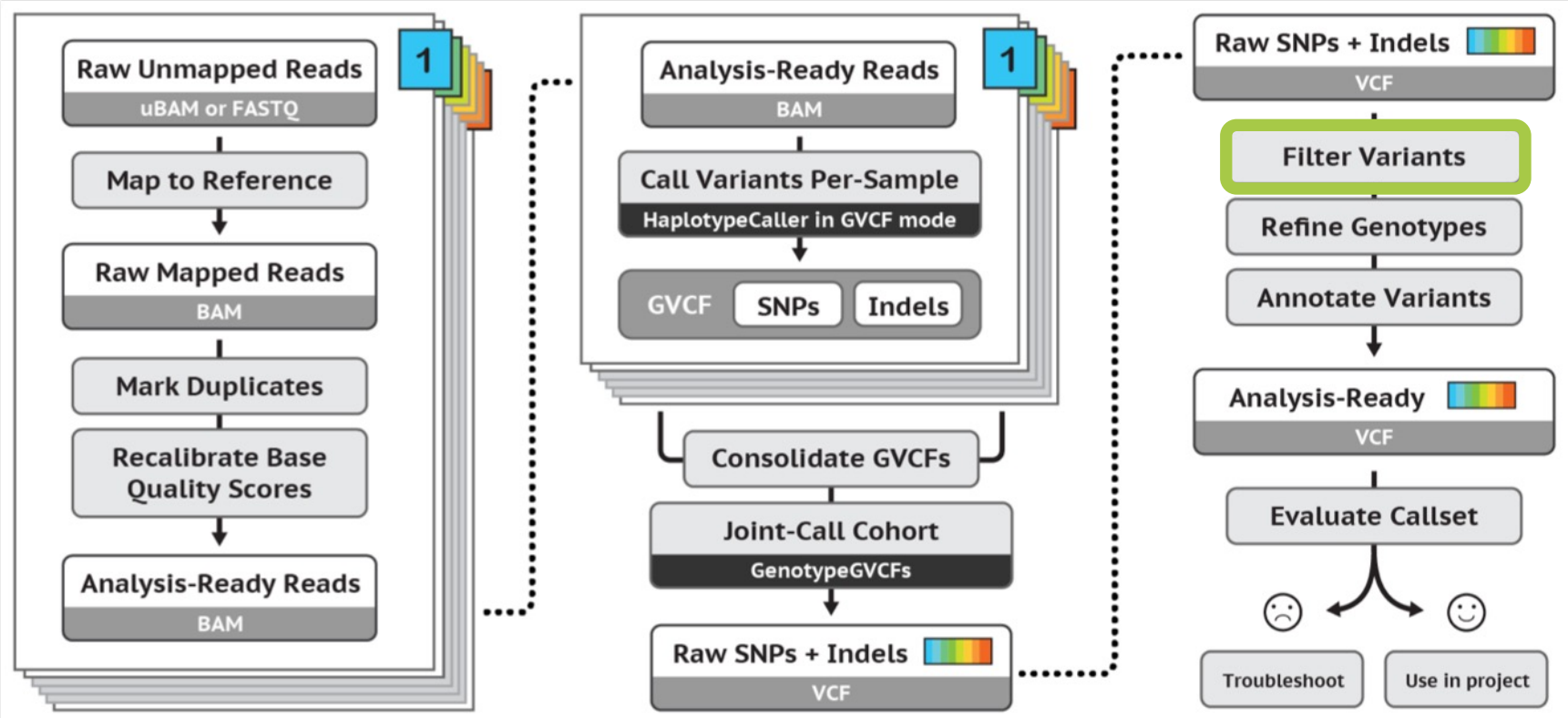
Local re-
determir
Mutect2)

Allele-sp
germline

Variant C

Evaluatir
variant c

Filter variants



https://software.broadinstitute.org/gatk/best-practices/

Germline short variant discovery (SNPs + Indels)

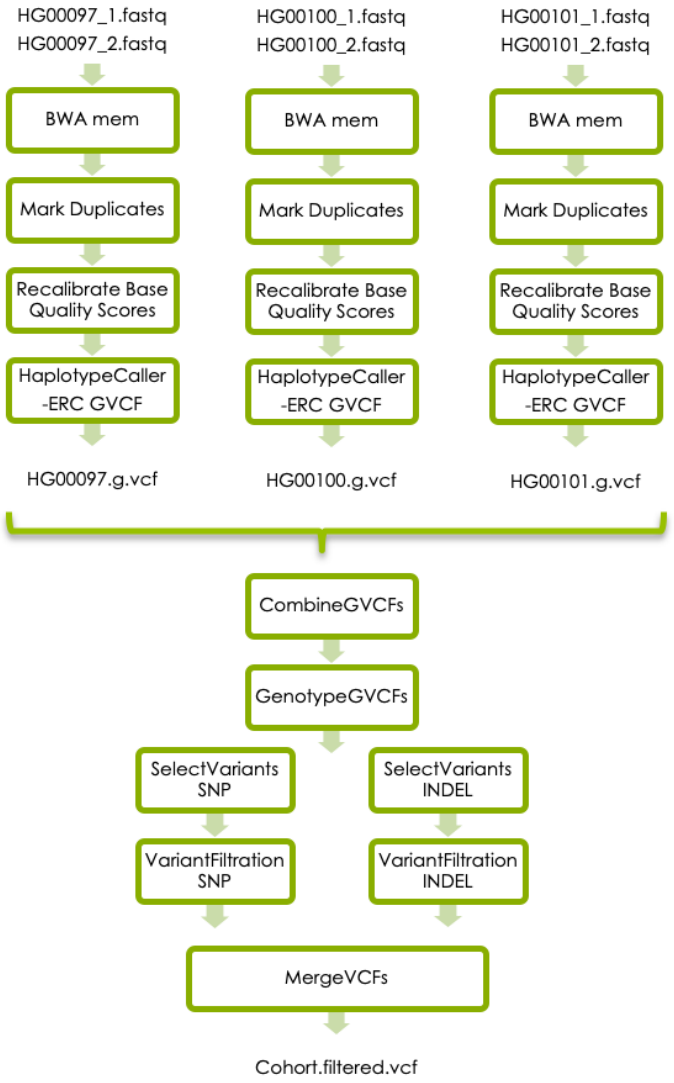
Filtering

- Remove low quality variants

- Variant quality score recalibration (VQSR):
  - For large data sets (>1 WGS or >30WES samples)
  - GATK has a machine learning algorithm that can be trained to recognise ”likely false” variants
  - **We do recommend to use VQSR when possible!**

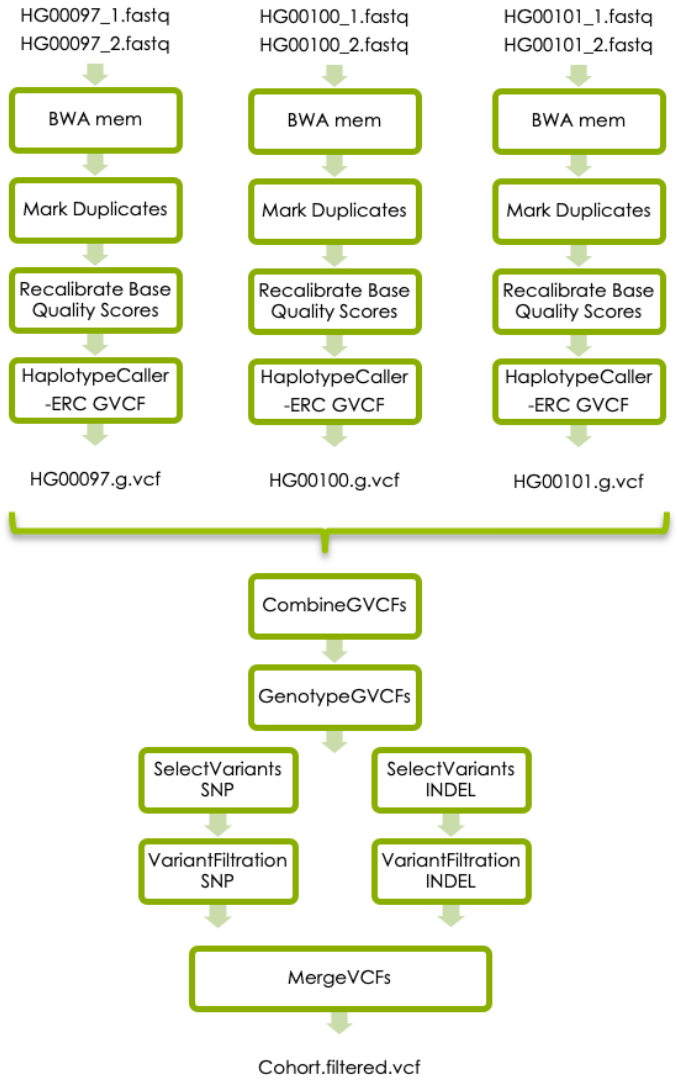
- Hard filters:
  - For smaller data sets
  - Hard filters on information in the VCF file
  - For example: Flag variants with ”QD < 2” and ”MQ< 40.0”
  - GATK discussion on hard filters: https://gatkforums.broadinstitute.org/gatk/discussion/2806/howto-apply-hard-filters-to-a-call-set

Part 3:



More details and links to GATK for each step is found in the lab instructions.

GATK's best practises



First look at video about this linked from schedule!

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Questions?

https://gatk.broadinstitute.org

Read groups

- Link *sample id*, *library prep*, *flowcell* and *sequencing run* to the reads.
- Good for error tracking!
- Often needed for variant calling
- Detailed description at https://gatkforums.broadinstitute.org/gatk/discussion/6472/read-groups

RGID = *combination of the sample id and run id*
RGLB = Library prep
RGPL = Platform (for us ILLUMINA)
RGPU = Run identifier *usually barcode of flowcell*
RGSM = Sample name