

# Variant-calling Workflow

Malin Larsson

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

In separate talk Thursday at 9:

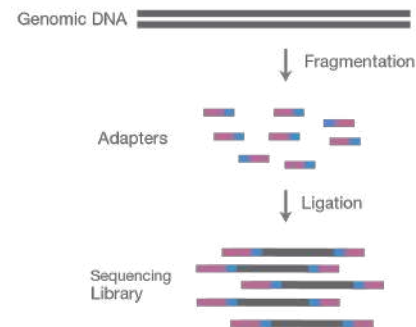
- GATK's Best practices

# Illumina Sequencing



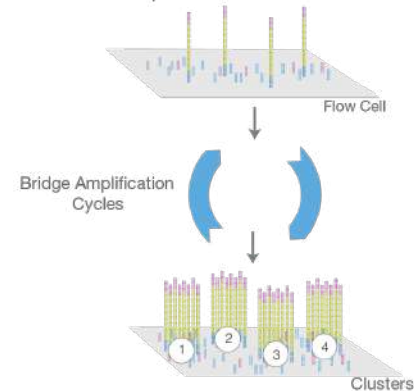
<https://www.youtube.com/watch?v=fCd6B5HRaZ8>

## A. Library Preparation



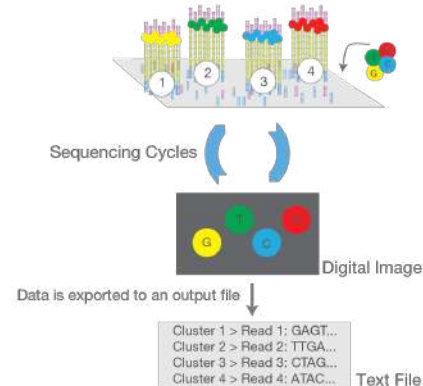
NGS library is prepared by fragmenting a gDNA sample and ligating specialized adapters to both fragment ends.

## 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 Analysis

Reads

```

ATGGCATTGCAATTTGACAT
TGGCATTGCAATTTG
AGATGGTATTG
GATGGCATTGCAA
GCATTGCAATTTGAC
ATGGCATTGCAATT
AGATGGCATTGCAATTTG
    
```

Reference Genome

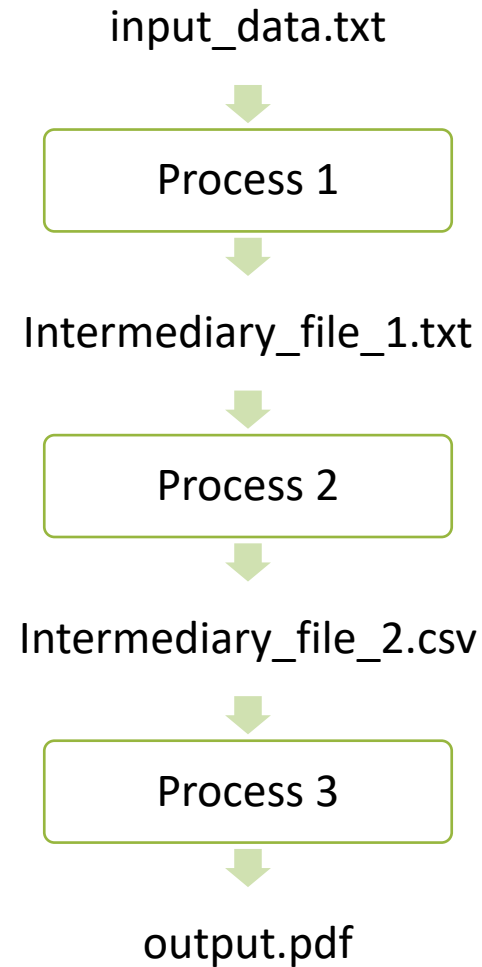
```

AGATGGTATTGCAATTTGACAT
    
```

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.

# Workflows

# What is a workflow



# Workflow conventions

---



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

# Example: Basic variant calling in one sample



HG00097\_1.fastq

HG00097\_2.fastq

FASTQ files

Alignment

HG00097.bam

BAM files

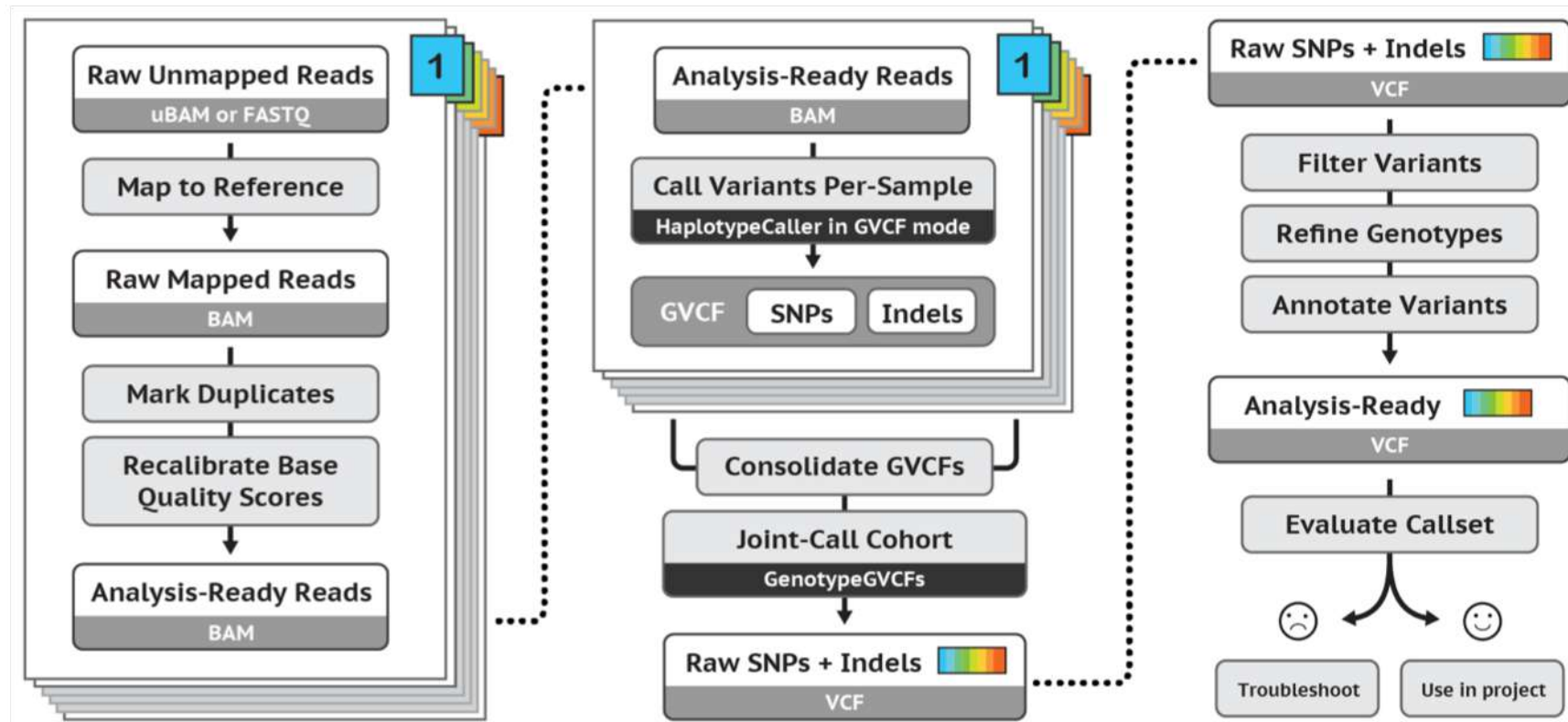
VariantCalling

HG00097.vcf

VCF files



# GATK's best practices workflow for germline short variant discovery





# Basic Variant Calling in one sample

# Alignment



HG00097\_1.fastq

HG00097\_2.fastq

FASTQ files

BWA mem

HG00097.bam

BAM files

HaplotypeCaller

HG00097.vcf

VCF files



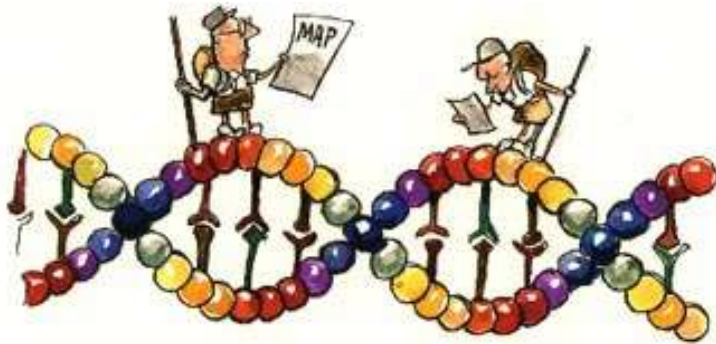
# The reference genome

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

GRCh38 is the latest version of the human reference genome, but 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.



- 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

AACAGGTATATCTTCCCCGCTAGCTAGCTAGCTA**GCTAGCTAGCTAGCTACCCT**C TTCCTTAGGGACTGTAC  
GCTAGCTAGCTAGCTACCCT

---

Figure 6-8 The DNA sequence of the lac operon.

# Burrows-Wheeler Aligner



<http://bio-bwa.sourceforge.net>

## Burrows-Wheeler Aligner

[Home](#)

### Introduction

BWA is a software package for mapping low-divergent sequences against a large reference genome, such as the human genome. It consists of three algorithms: BWA-backtrack, BWA-SW and BWA-MEM. The first algorithm is designed for Illumina sequence reads up to 100bp, while the rest two for longer sequences ranging from 70bp to 1Mbp. BWA-MEM and BWA-SW share similar features for long-read support and split alignment, but BWA-MEM, which is the generally recommended for high-quality queries as it is faster and more accurate. BWA-MEM also has better performance than BWA-backtrack for 70-100bp reads.

### FAQ

#### How can I cite BWA?

The short read alignment component (bwa-short) has been published in:  
Li H. and Durbin R. (2009) Fast and accurate short read alignment with Burrows-Wheeler Transform. *Bioinformatics*, 25:1754-60. [PMID: 19451168]

If you use BWA-SW, please cite:

Li H. and Durbin R. (2010) Fast and accurate long-read alignment with Burrows-Wheeler Transform. *Bioinformatics*, Epub. [PMID: 20111683]

(See also Errata below for a minor correction to the formulae in the above papers.)

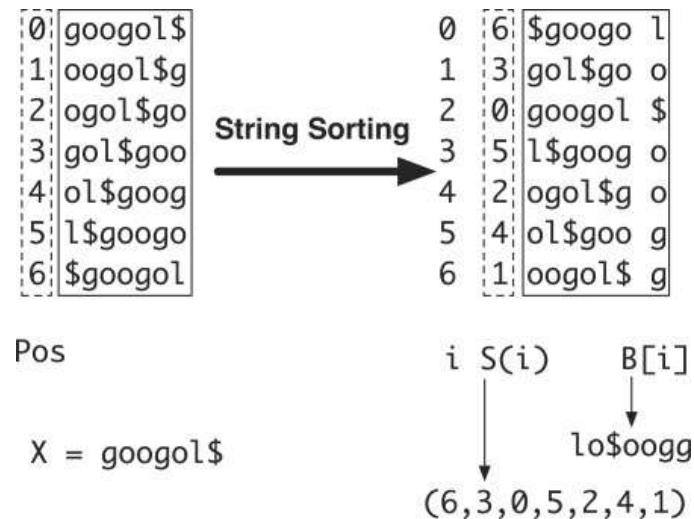
#### There are three algorithms, which one should I choose?

For 70bp or longer Illumina, 454, Ion Torrent and Sanger reads, contigs and BAC sequences, BWA-MEM is usually the preferred algorithm. For short sequences, BWA-backtrack may be better. BWA-SW may be better for longer sequences.

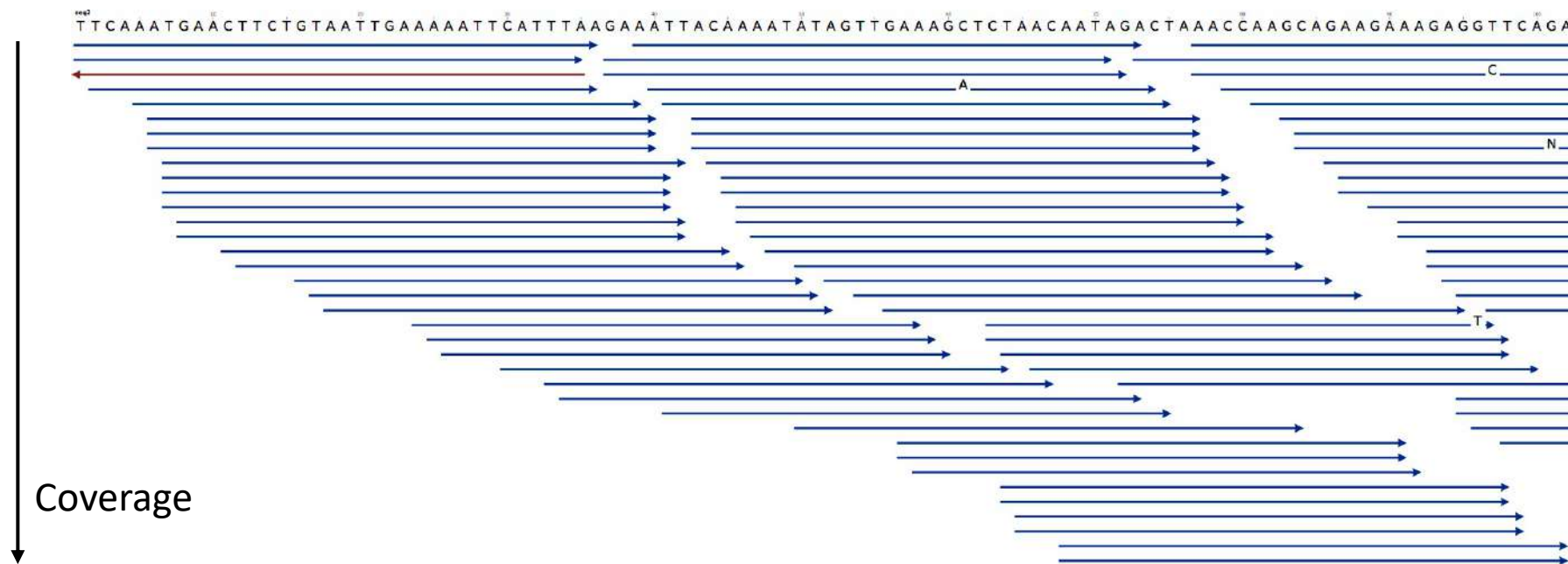
### BWA:

[SF project page](#)

## Burrows-Wheeler transform of reference genome



# 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<FFFBFBFFFBFB
Read_001	147	2	3843625	0	101M	=	3843448	-278	TTATTTTCATTGAGCAGTGGT	FBBI7IIFIB<BBBB<BBFF
Read_002	163	2	4210055	0	101M	=	4210377	423	TGGTACCAAAACAGAGATAT	0IIFBFFFIIIFIFIFFBFB
Read_003	99	2	4210066	0	101M	=	4210317	352	CAGAGATATAGATCAATGGA	0IIFFFIFFFIFIFIIIIIF

Read name  
(usually more  
complicated)

Reference sequence name

Start position

Sequence

Quality

# Convert to Bam



Bam file is a binary representation of the Sam file

# 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

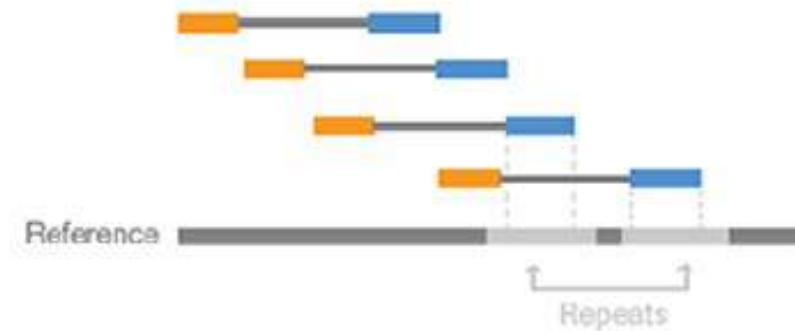
# Paired-End data



Paired-End Reads



Alignment to the Reference Sequence



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@CFFFFFFHHHHHGJJJJJJJJJJJFHHIIIIJJ
JIHGIIJJJJIIJIIJJJJIIJJJJJIIIEIHHIJ
HGHHHHHDFFFEDDDDDCDDDCDDDDDDDCDC
```

ID\_ **R2** \_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:
2197 2:N:0:ATCACG
CTTCGTCCACTTTCATTATTCCTTTCATACATG
CTCTCCGGTTTAGGGTACTCTTGACCTGGCCTT
TTTTCAAGACGTCCTGACTTGATCTTGAAACG
+
CCFFFFFFHHHHHJJJJIIJJJJJJJJJJJJJJ
JJJJJJJJIIJIIJGIJHBGHHIIIIJJJJJJJJ
JJJHFFFFFFDDDDDDDDDDDDDDDEDCDDDD
```



# Variant calling

HG00097\_1.fastq

HG00097\_2.fastq

FASTQ files

BWA mem

HG00097.bam

BAM files

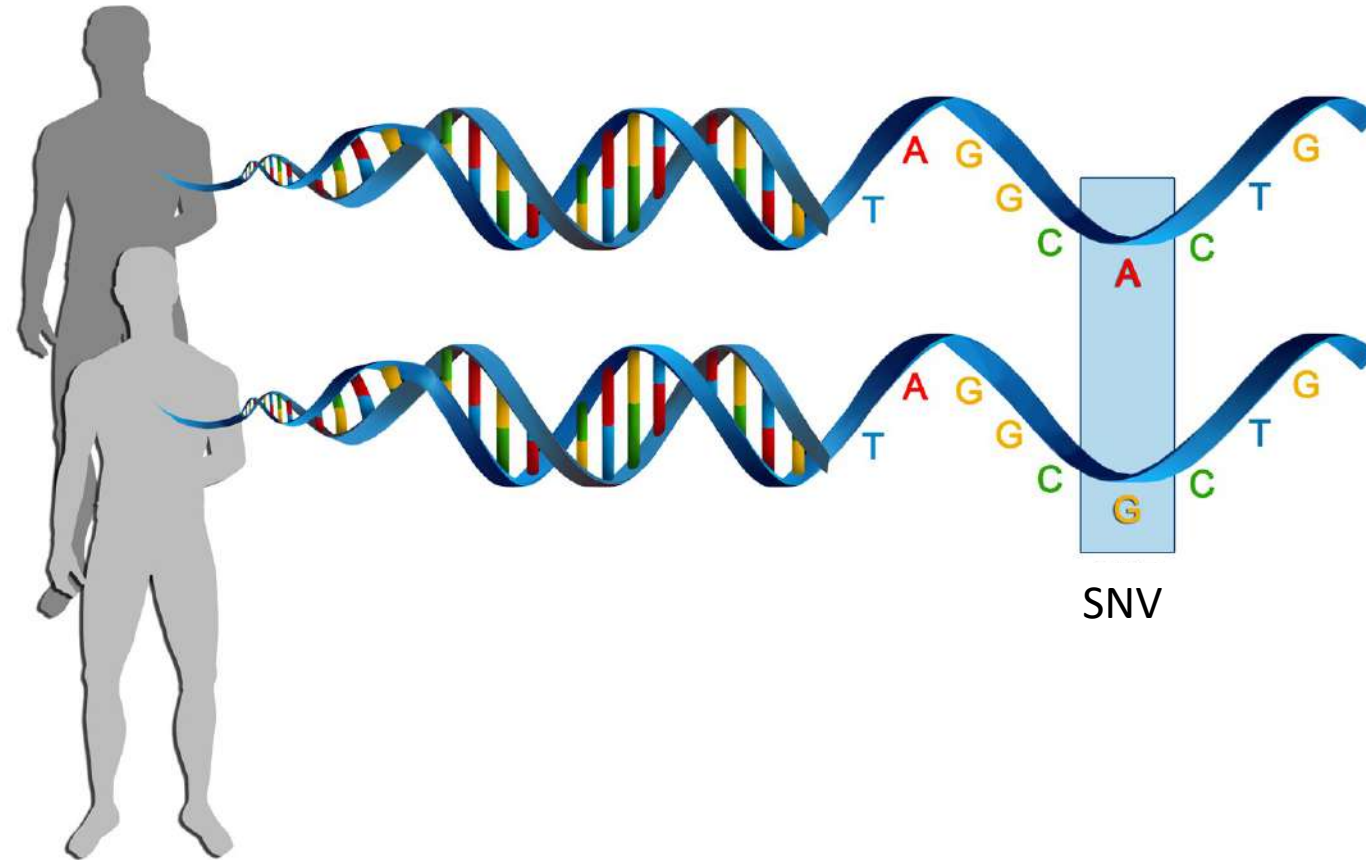
HaplotypeCaller

HG00097.vcf

VCF files



# Genetic variation



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

# Detecting variants in reads



Reference:

...GTGCGTAGACTGCTAGATCGAAGA...

Sample:

...GTGCGTAGACTG**A**TAGATCGAAGA...

...GTGCGTAGACTG**A**TAGATCGAAGA...

...GTGCGTAGACTGCTAGATCGAAGA...

...GTGCGTAGACTGCTAGATCGAAGA...

...GTGCGTAGACTG**A**TAGATCGAAGA...

...GTGCGTAGACTG**A**TAGATCGAAGA...

...GTGCGTAGACTGCTAGATCGAAGA...

...GTGCGTAGACTG**A**TAGATCGAAGA...

...GTGCGTAGACTGCTAGATCGAAGA...

...GTGCGTAGACTG**A**TAGATCGAAGA...



# Reference- and alternative alleles



TGGGCTTTTCCAACAGGTATATCTTCCCCGCTAGCTAGCTAGCTACTTCAAATTCCT

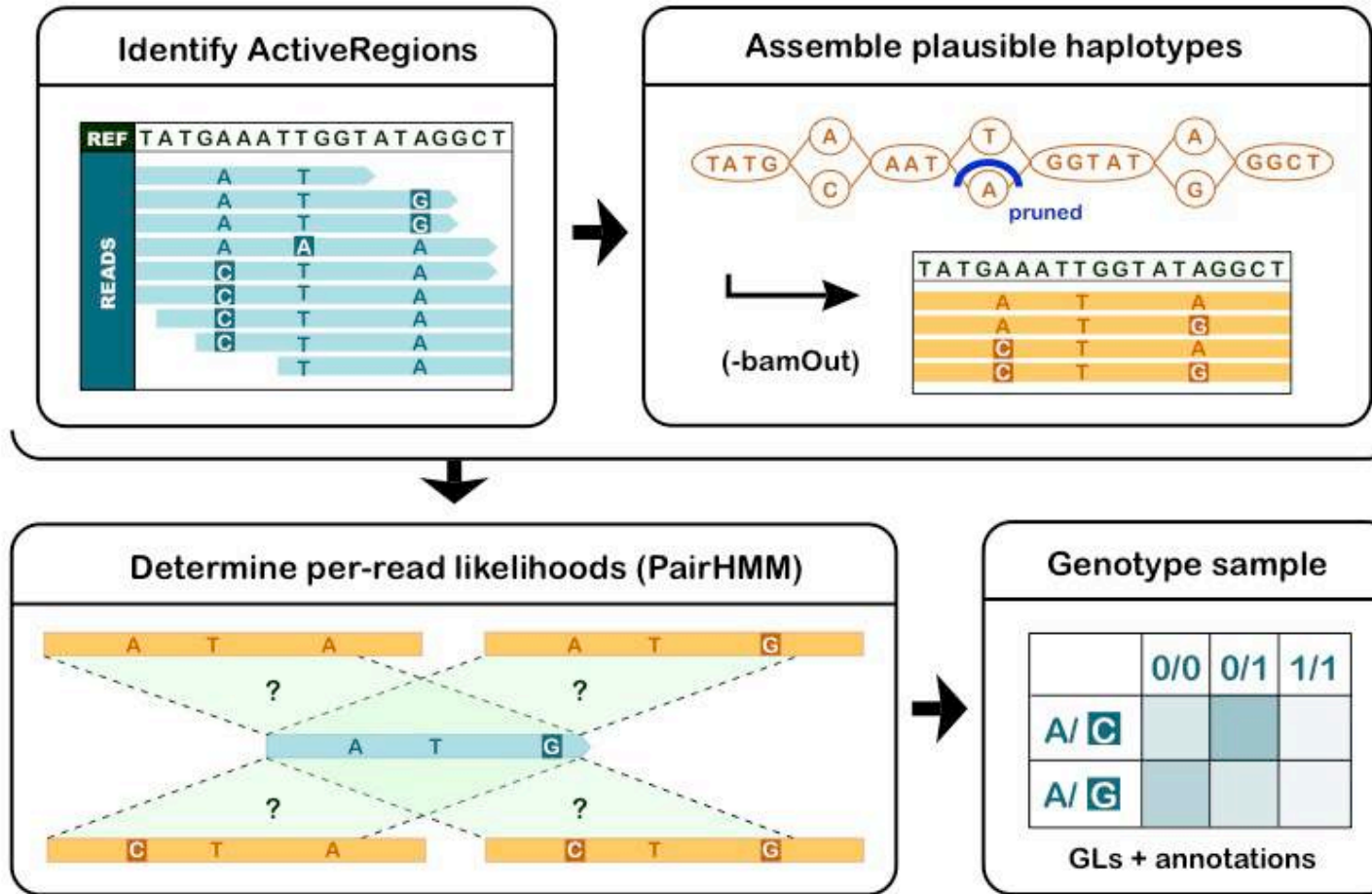
**Reference allele**      AGCTAGCTA

**Alternative allele**      AGCTGGCTA

**Reference allele** = the allele in the reference genome

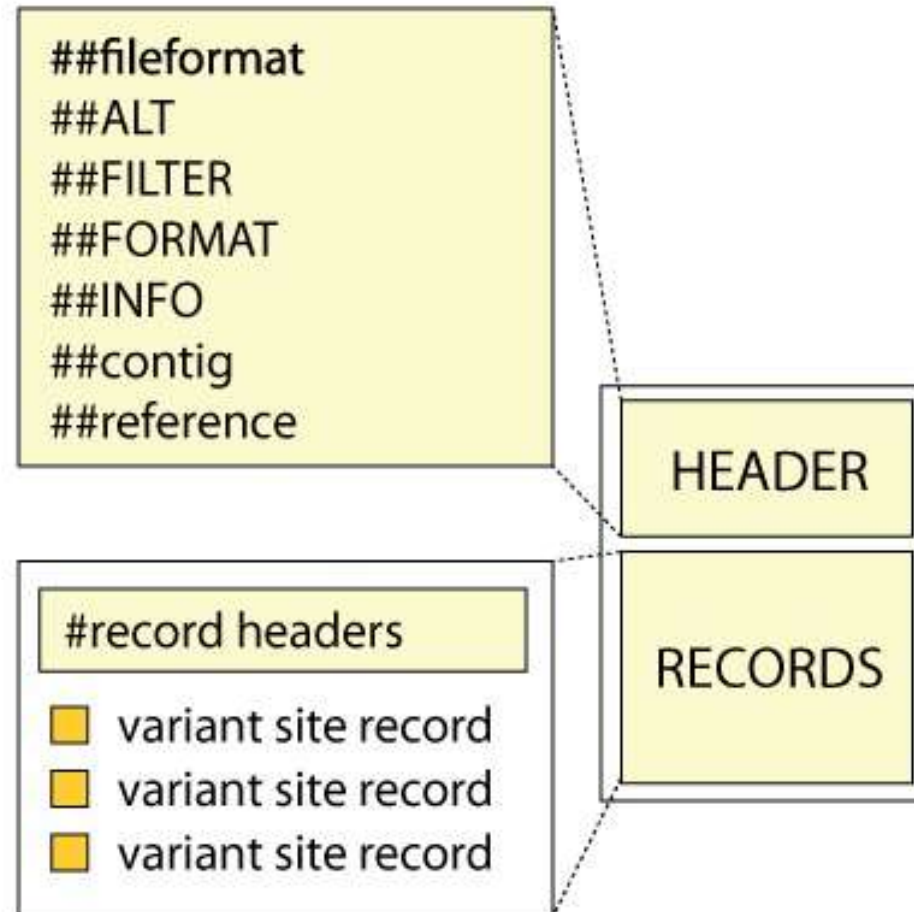
**Alternative allele** = the allele NOT in the reference genome

# Variant Calling HaplotypeCaller





# Variant Call Format (VCF)



# Variant Call Format (VCF)

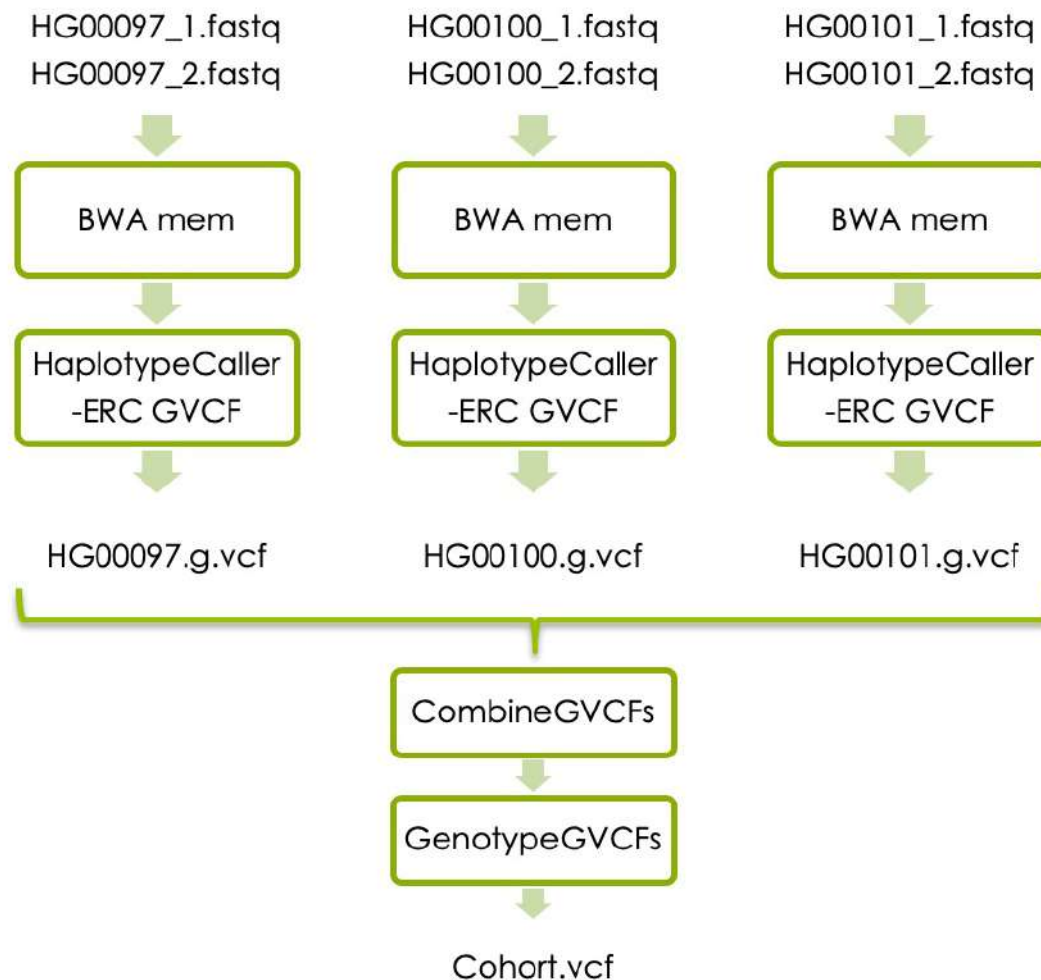


```
##fileformat=VCFv4.2
##FILTER=<ID=LowQual,Description="Low quality">
##FORMAT=<ID=AD,Number=R,Type=Integer,Description="Allelic depths for the ref and alt alleles in the order listed">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth (reads with MQ=255 or with bad mates are 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
...
...
```

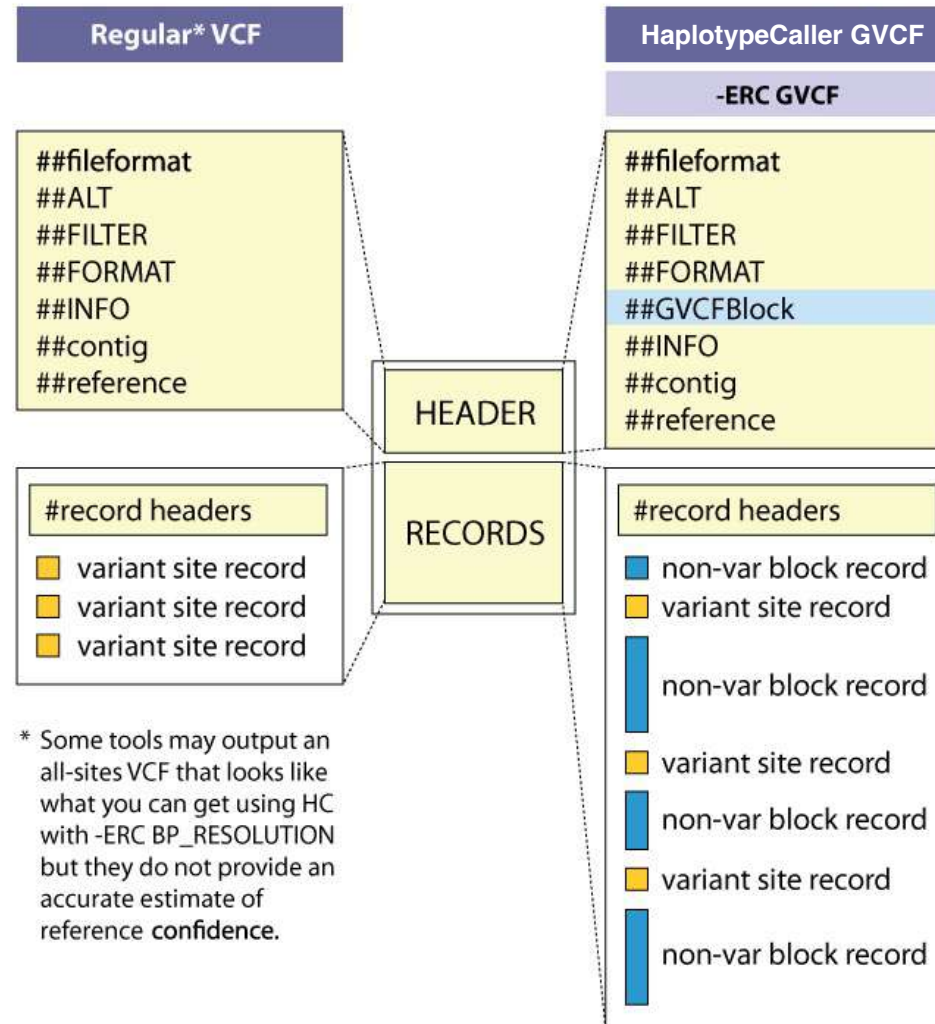
# Variant calling in cohort



# Basic variant calling in cohort



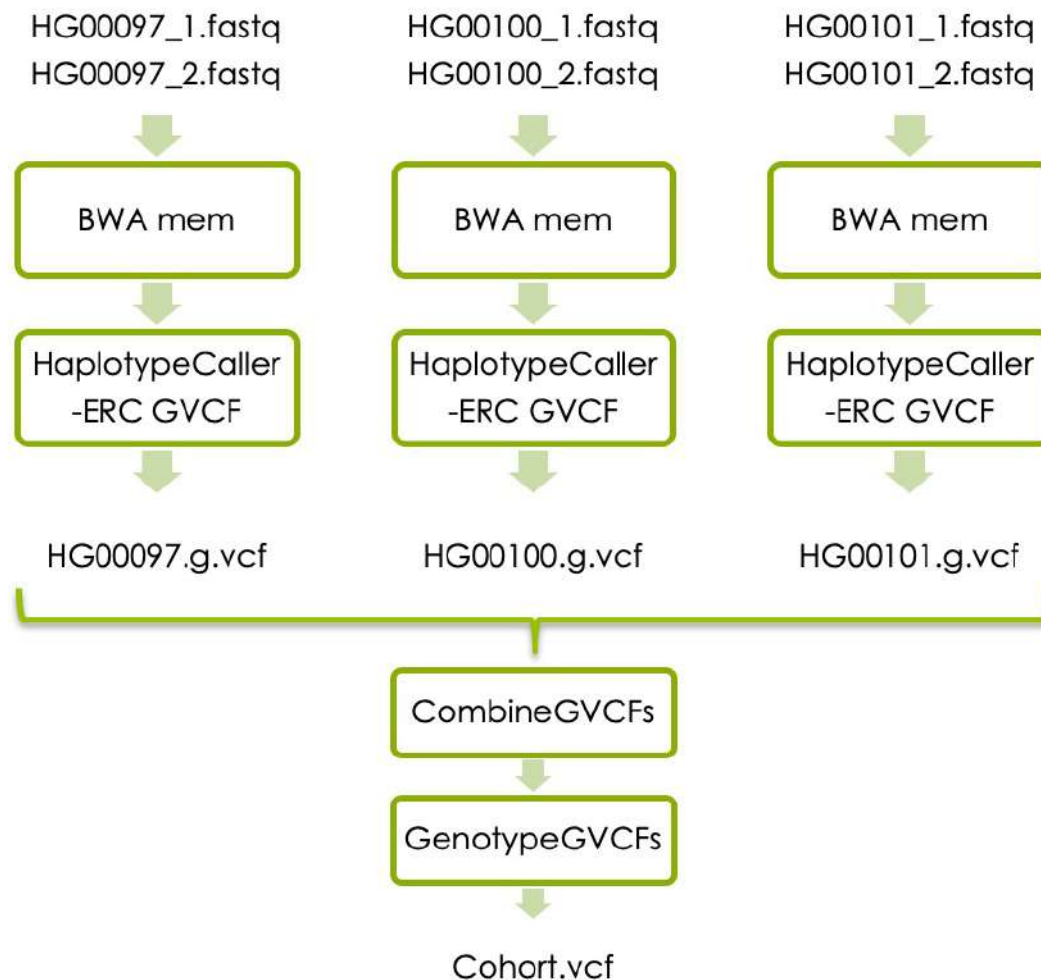
# GVCF Files are valid VCFs with extra information



- 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

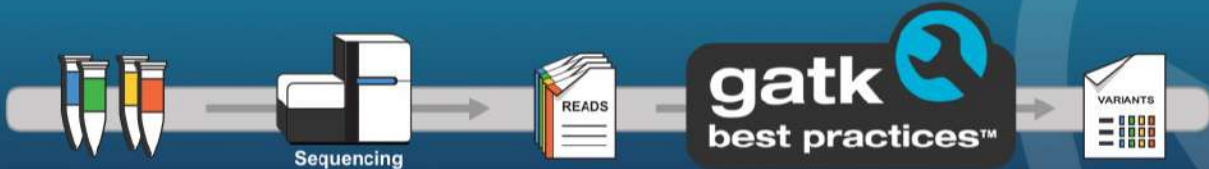
# **GATK's best practices for germline short variant discovery**



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## 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](#)


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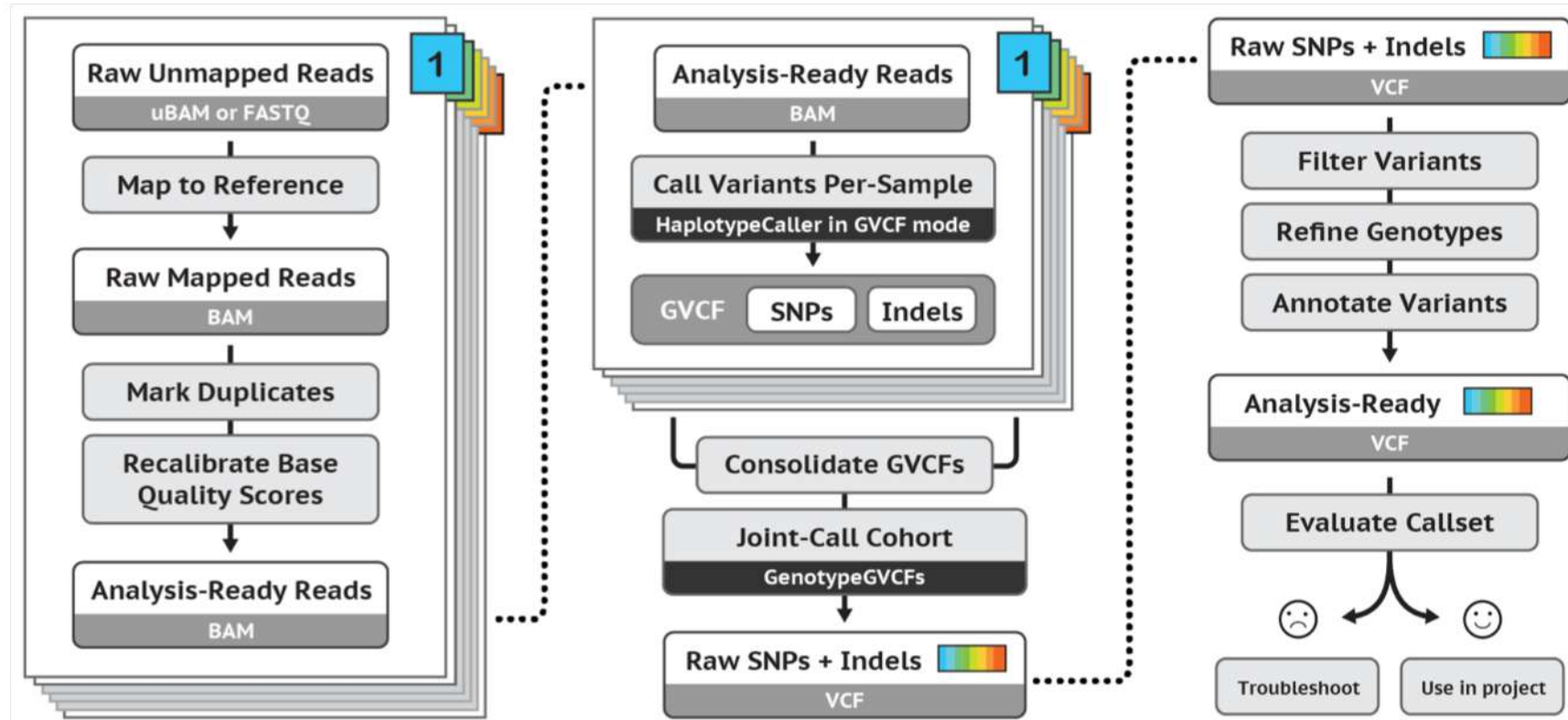
**DRAGEN-GATK**  
Learn more about DRAGEN-GATK

**Download latest version of GATK**  
The GATK package download includes all released GATK tools

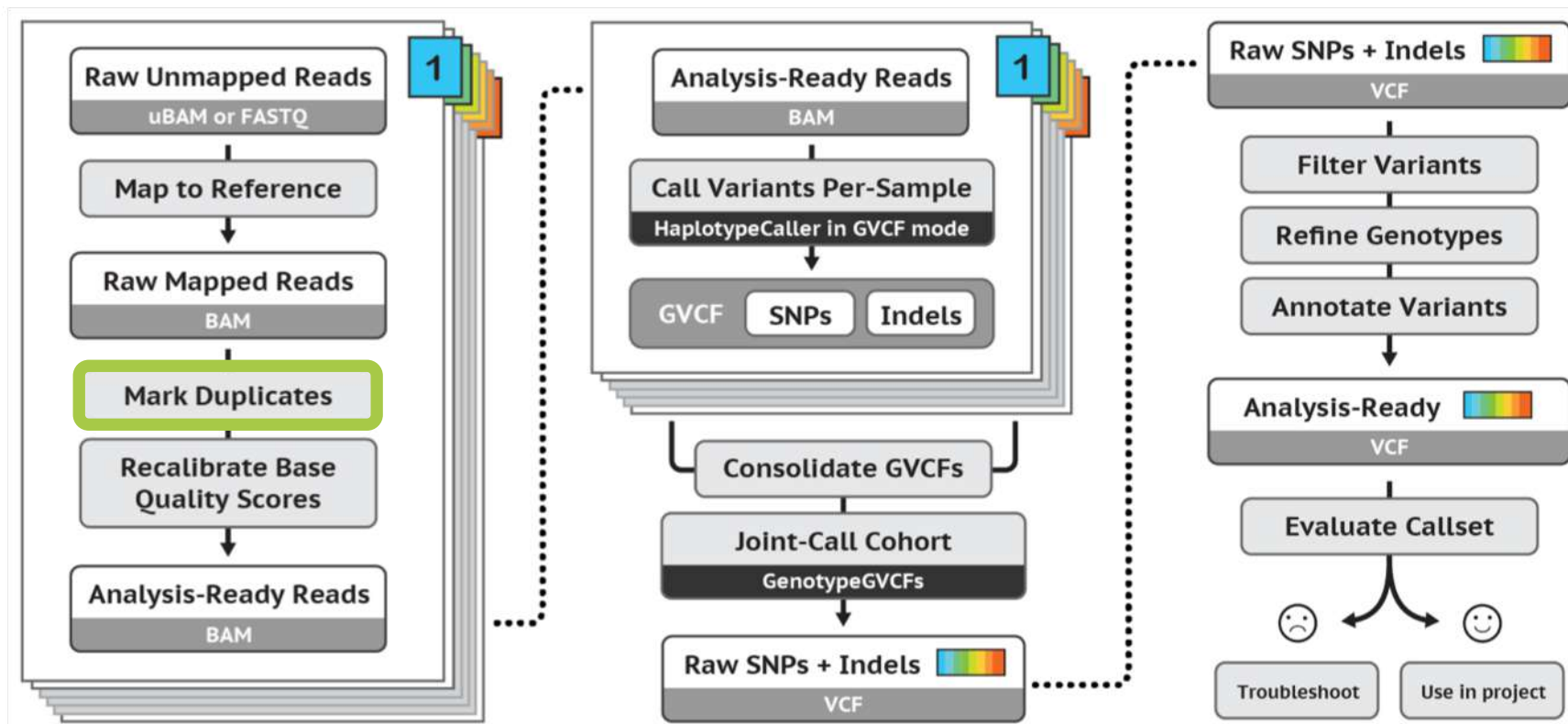
**Run on Cloud**

**Run on HPC**

# GATK's best practices workflow for germline short variant discovery



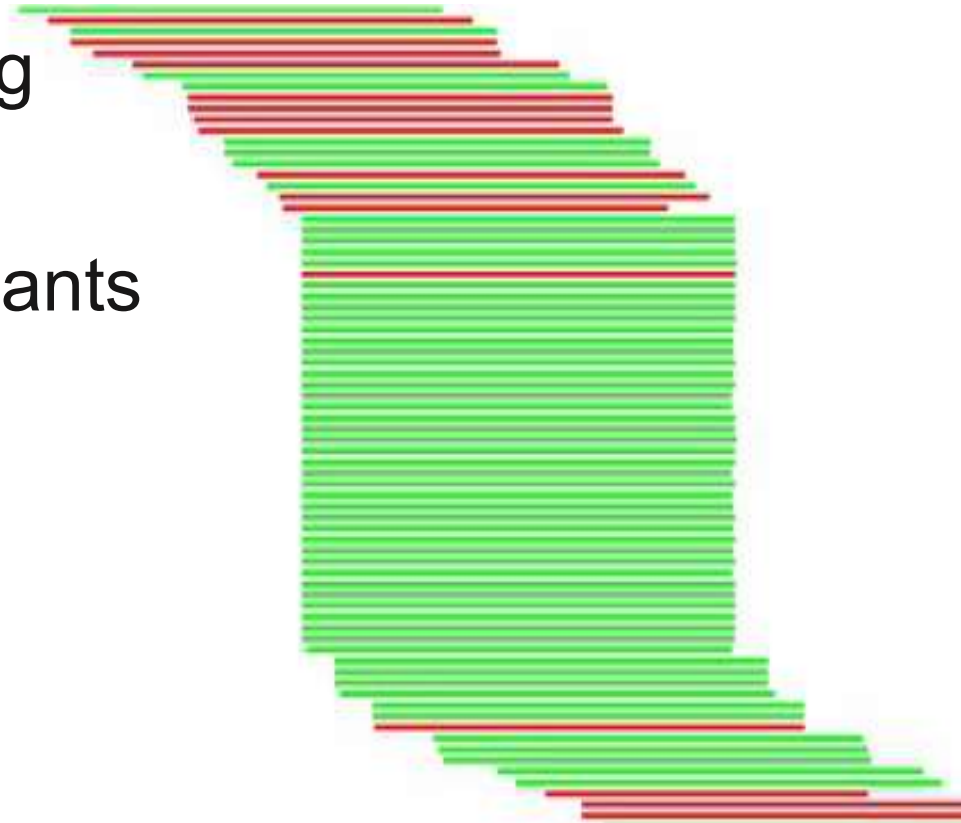
# Mark Duplicates



# Duplicate reads



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





## Need Help?

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MarkDuplicates



[GATK](#) / [Tool Index](#) / 4.0.1.1

### MarkDuplicates (Picard)

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GATK Team

10 months ago · Updated

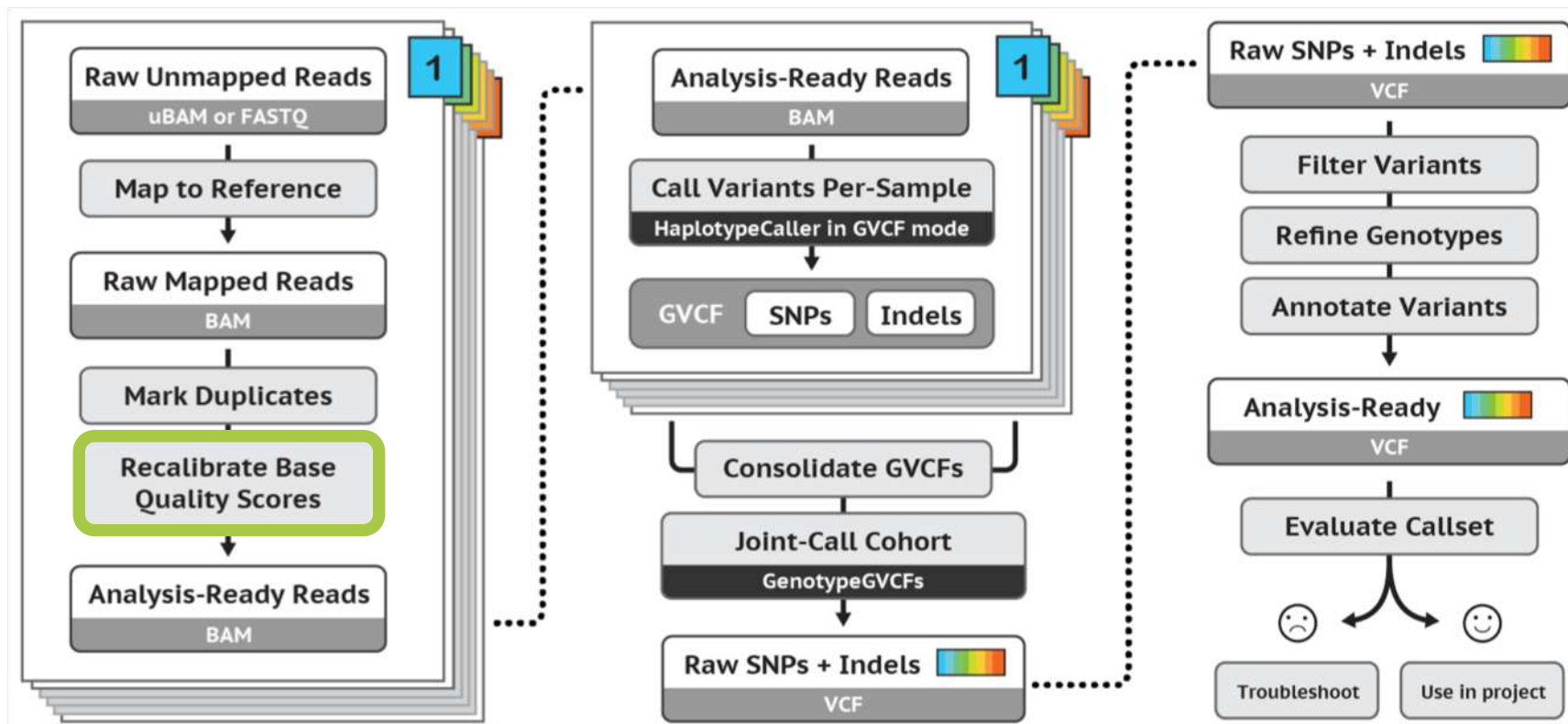
Identifies duplicate reads.

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)








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





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## 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

1. Overview
2. Base recalibration procedure details
3. Important factors for successful recalibration
4. Examples of pre- and post-recalibration metrics
5. Recalibration report

### Articles in

ActiveRe  
(Haplotype)

Evaluatir  
and varia  
Mutect2)

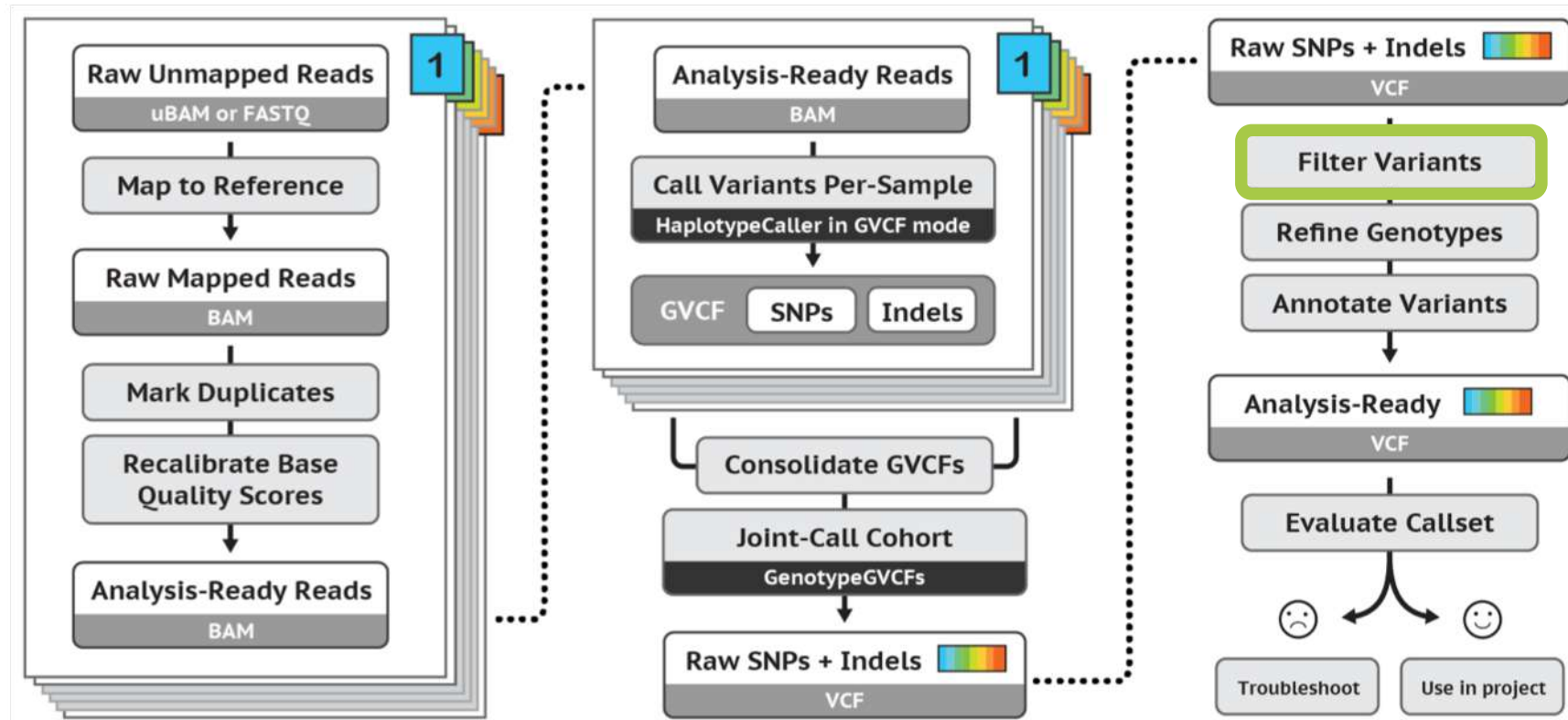
Local re-  
determin  
Mutect2)

Allele-sp  
germline

Variant C

Evaluatir  
variant c

# Filter variants

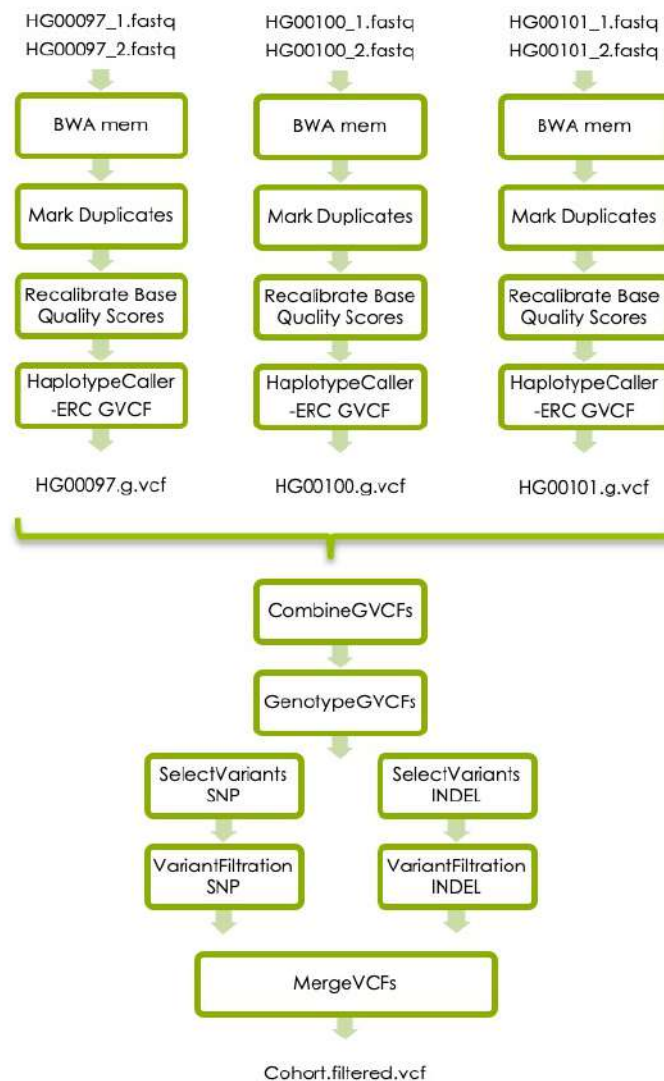


<https://software.broadinstitute.org/gatk/best-practices/>  
Germline short variant discovery (SNPs + Indels)



- 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>

# GATK's best practices workflow



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

# 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

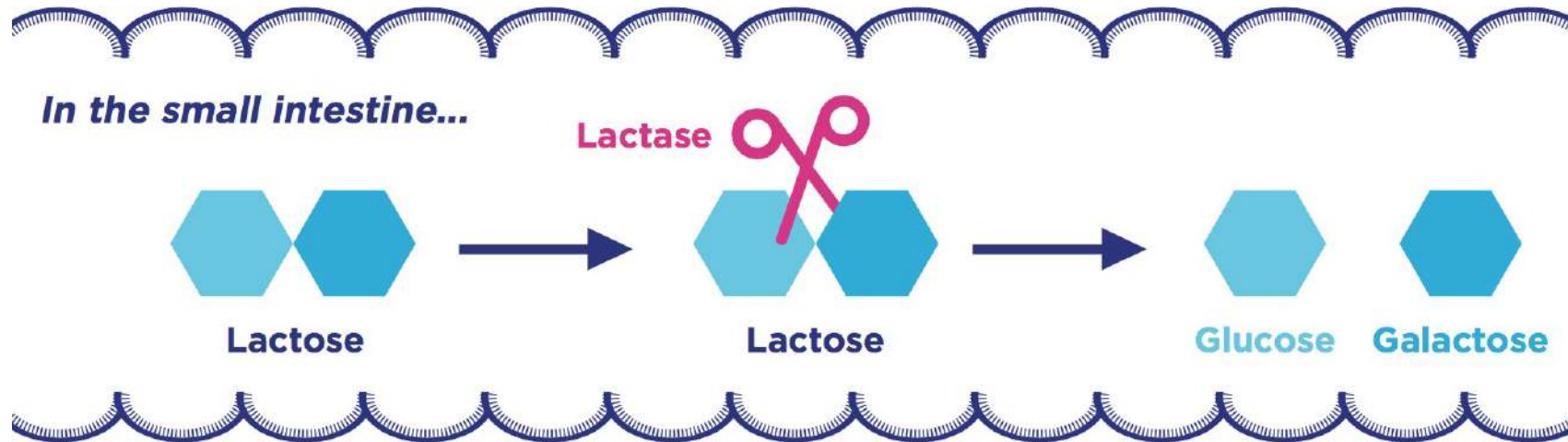


Figure 2. Lactose digestion in the intestine.

- 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)



**part one:**

**variant calling in one sample**



# Basic variant calling in one sample

HG00097\_1.fastq

HG00097\_2.fastq

FASTQ files

BWA mem

HG00097.bam

BAM files

HaplotypeCaller

HG00097.vcf

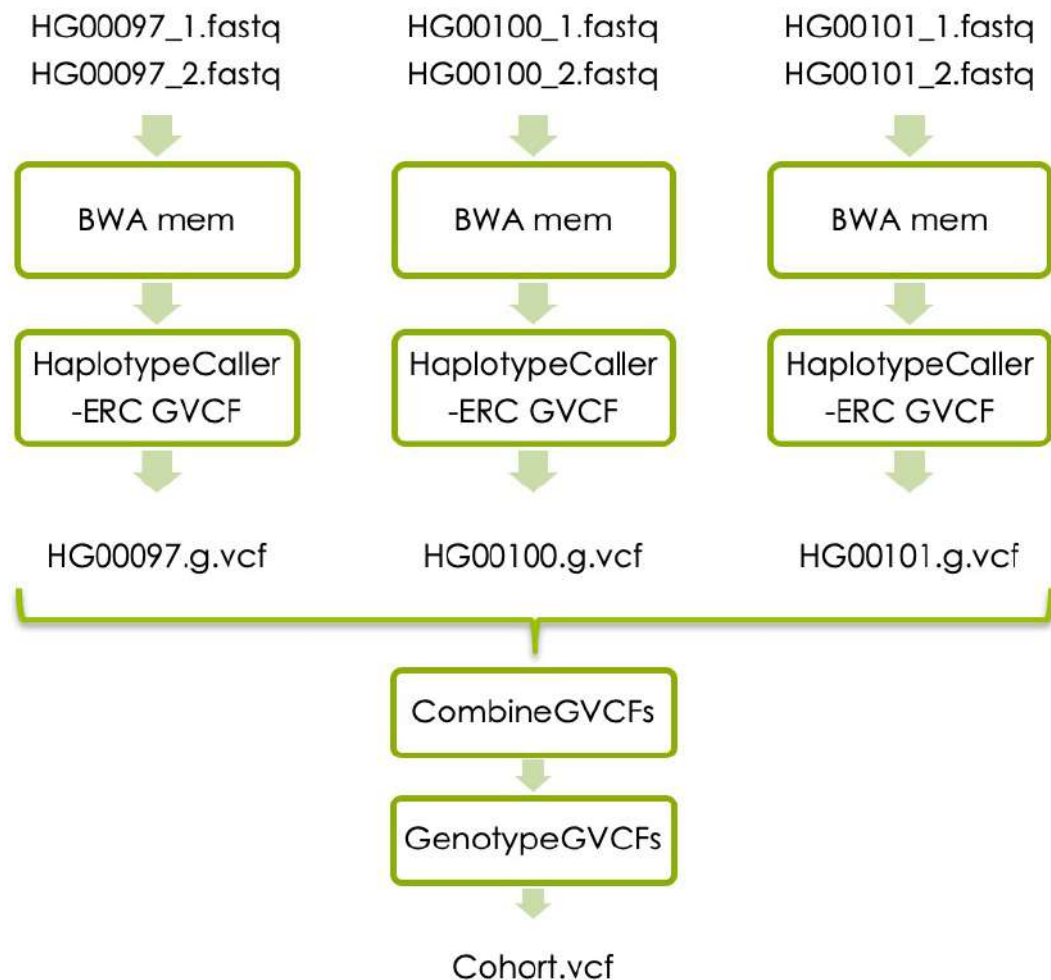
VCF files



# **Part two (if you have time):**

## **variant calling in cohort**

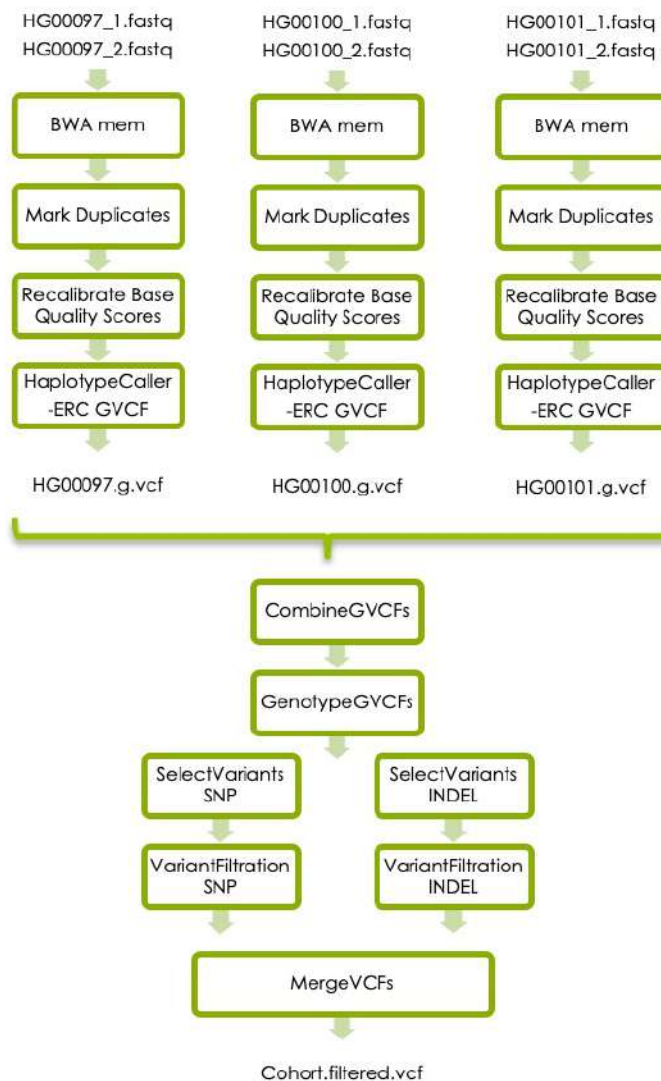
# Joint variant calling workflow



**Part three (if you have time):**


**Follow GATK best practices for short  
variant discovery**

# GATK's best practises



First look at video  
about this linked from  
schedule!

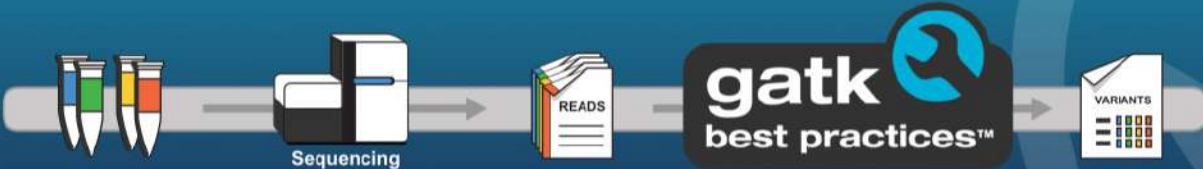




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## Genome Analysis Toolkit


Variant Discovery in High-Throughput Sequencing Data




Sequencing → READS → **gatk best practices™** → VARIANTS

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](#)


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