GULBENKIAN SCIENCE

PO: Precision Oncology Course

Variant Detection

BU CNIO BIOINFORMATICS UNIT

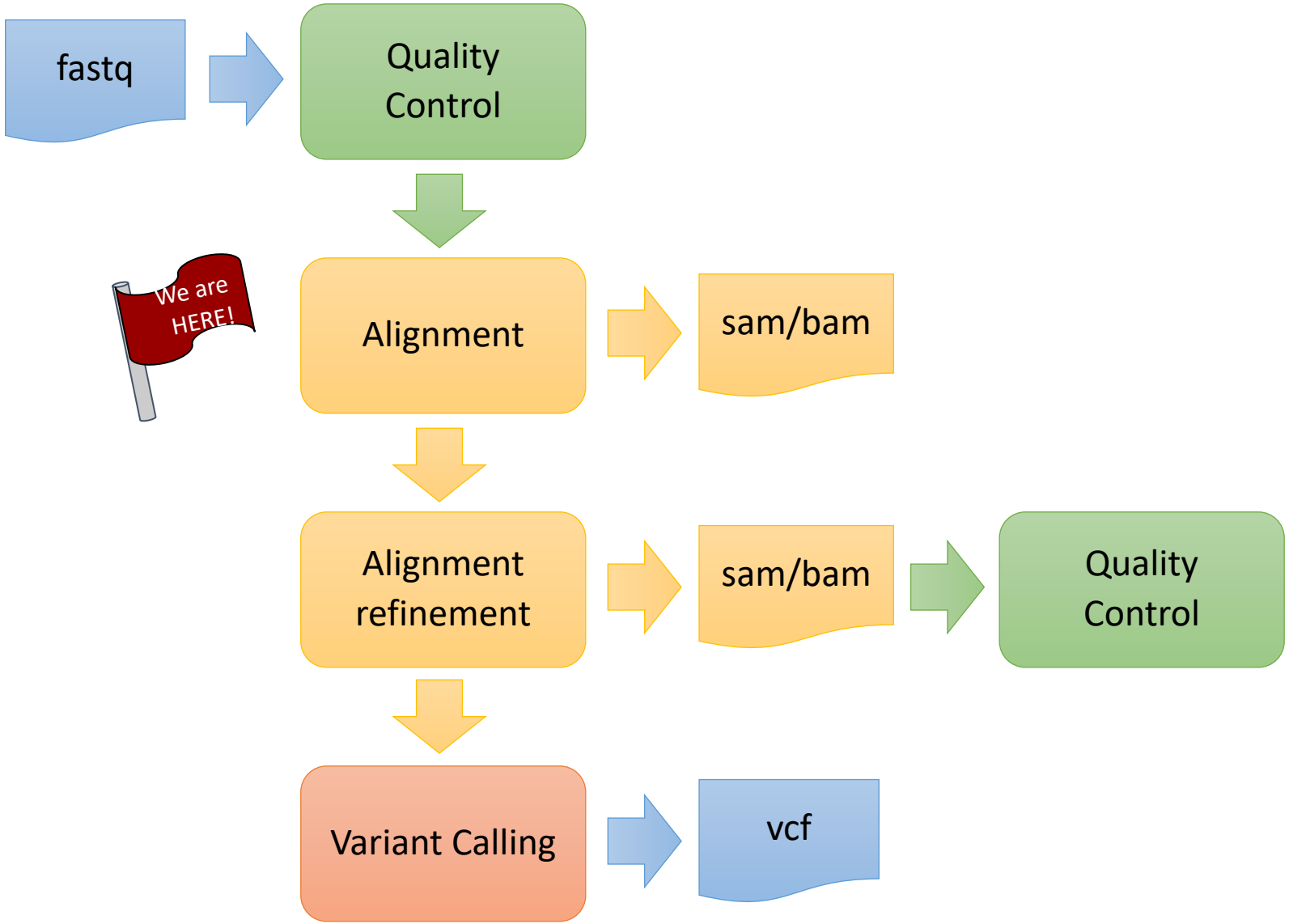
cnio stop cancer

Outline

- Steps for variant detection after alignment
- Algorithms for variant calling
- Pipeline for targeted DNA sequencing: varca

Steps for variant detection after alignment

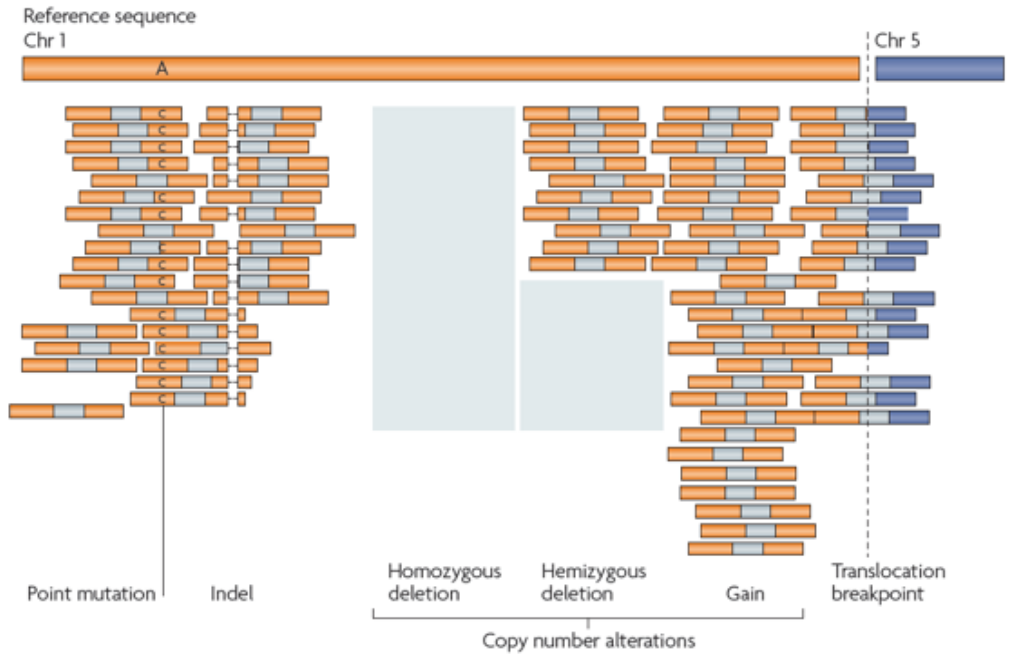
General steps

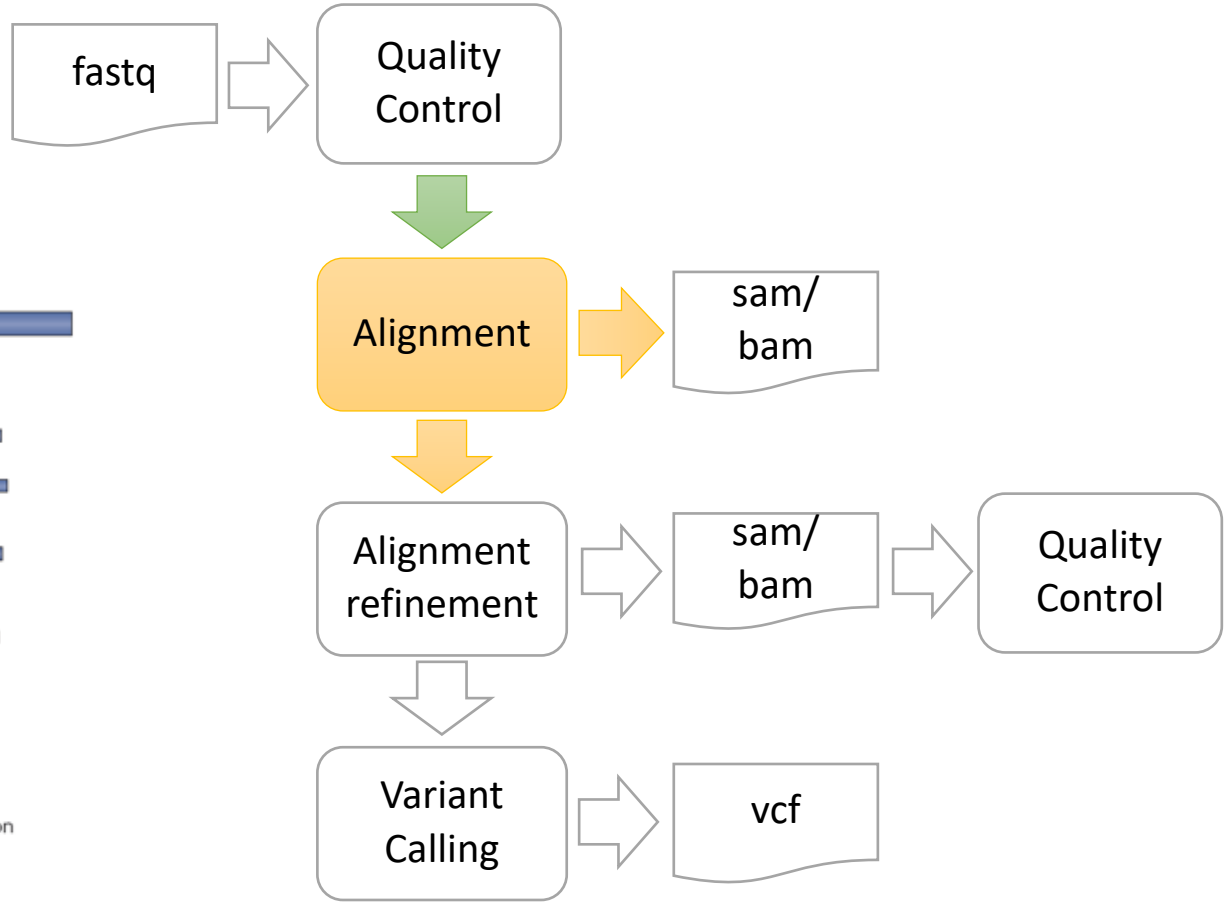


Alignment

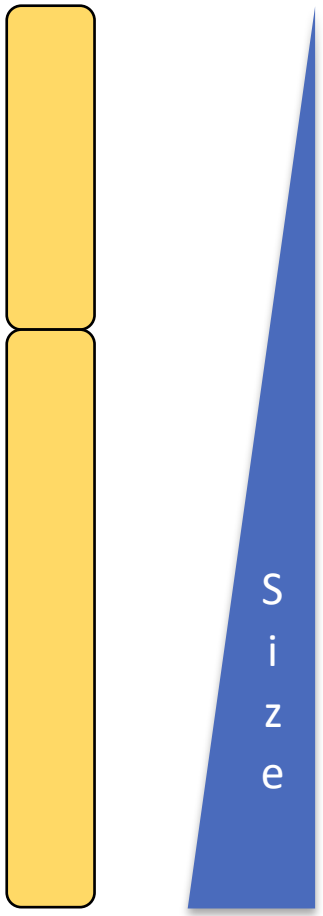
Reference

Sample





Classification of variants according to size



SNVs Single Nucleotide Variants (SNP) - MNV
Indels Small Insertion and Deletions

Small scale variants

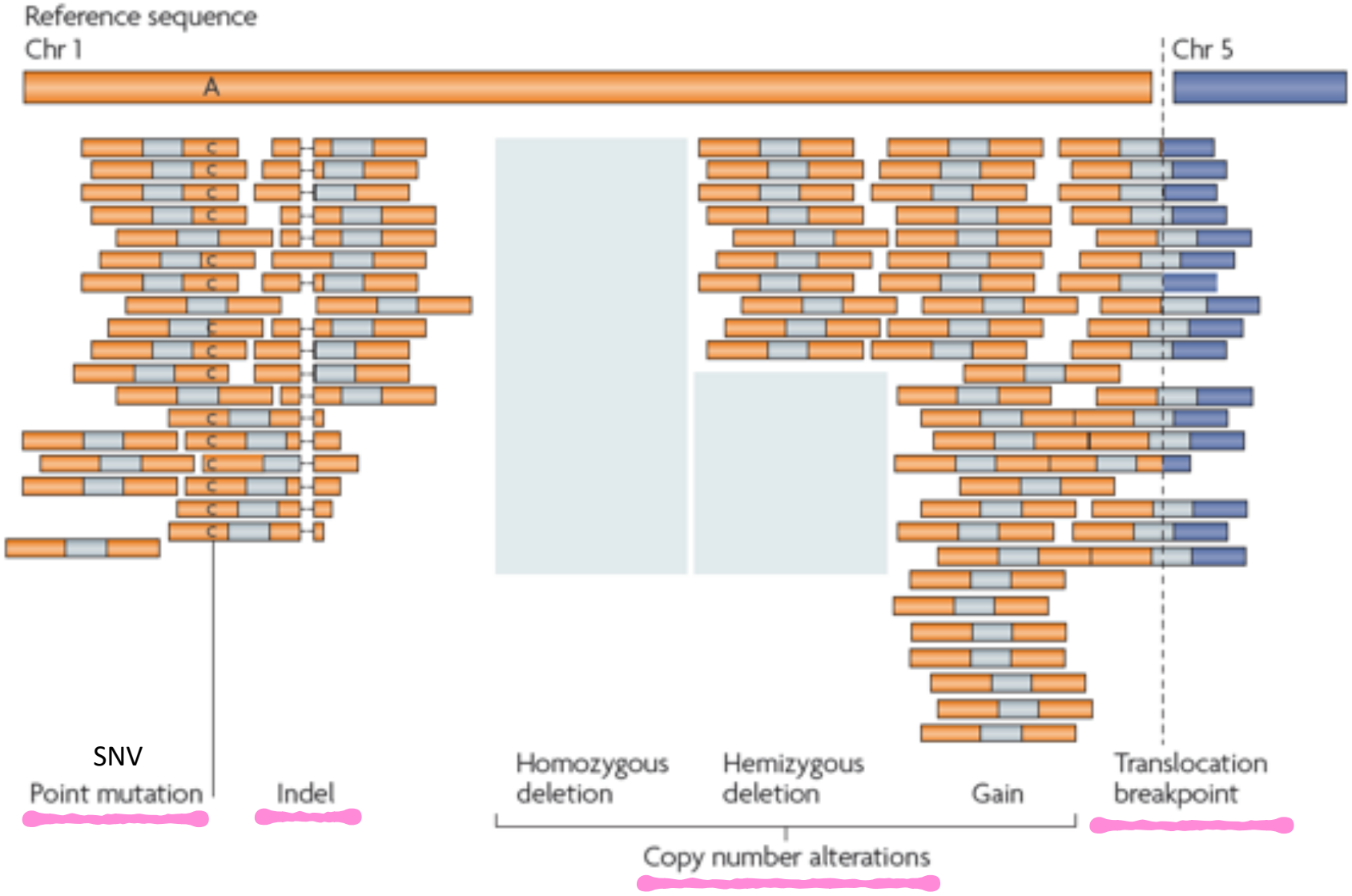
VNTRs (Micro, Minisatellites)
Variable Number of Tandem Repetitions

CNVs Copy Number Variations
Translocations, Inversions

Structural variants

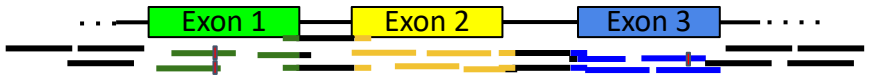
Aneuploidies

Alignment of reads uncovers potential variant sites

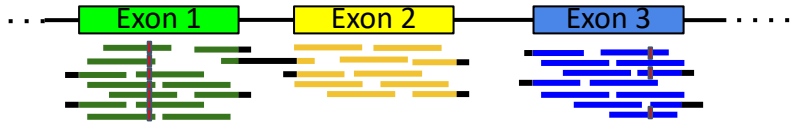


DNA-seq strategies – Sequencing coverage

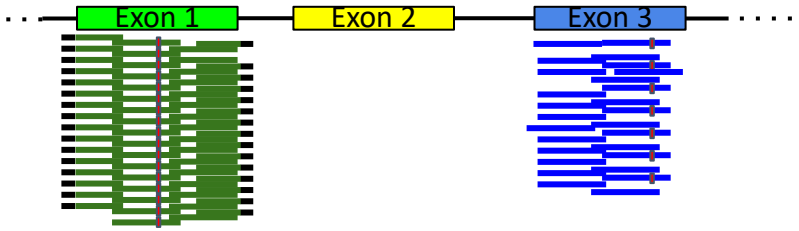
Whole-Genome Sequencing

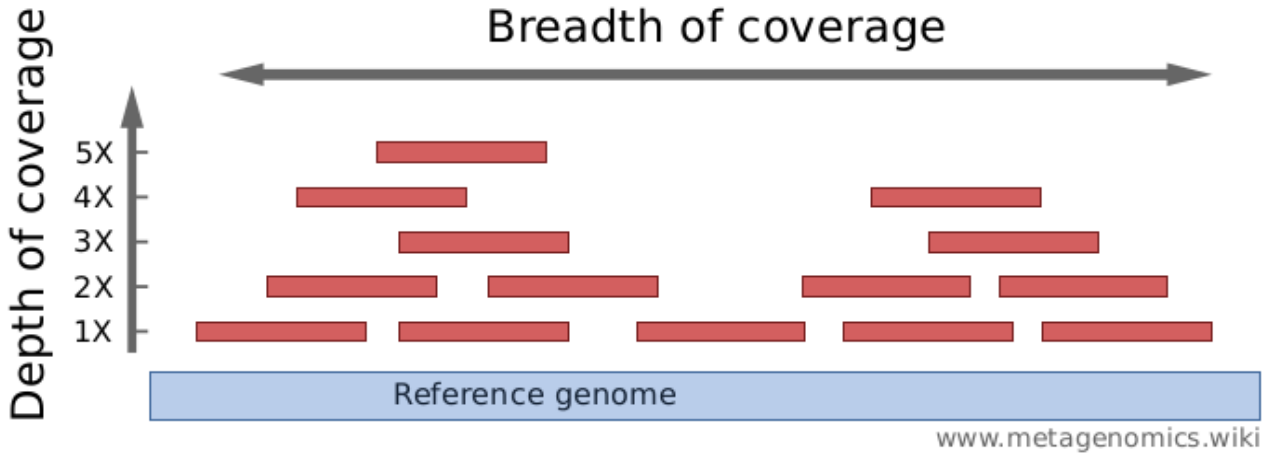


Whole-Exome Sequencing



Panel of genes by amplicon/PCR approach





Depth: times that a base is sequenced

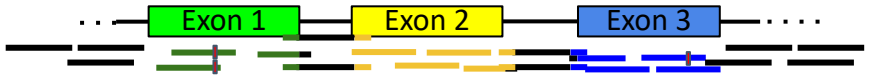
On average:

$$\frac{\sum \textit{Number of times a sequenced base is covered by reads}}{\textit{Length of the sequenced genome}}$$

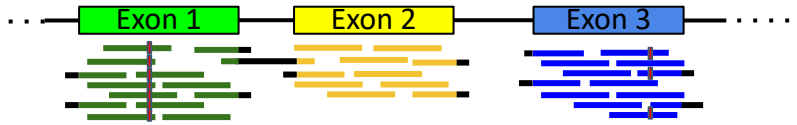
Breadth: percentage of the sequenced genome covered by the reads (at a certain depth)

DNA-seq strategies – Type of variants

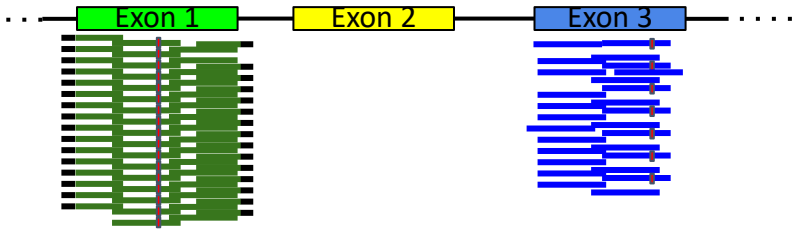
Whole-Genome Sequencing



Whole-Exome Sequencing



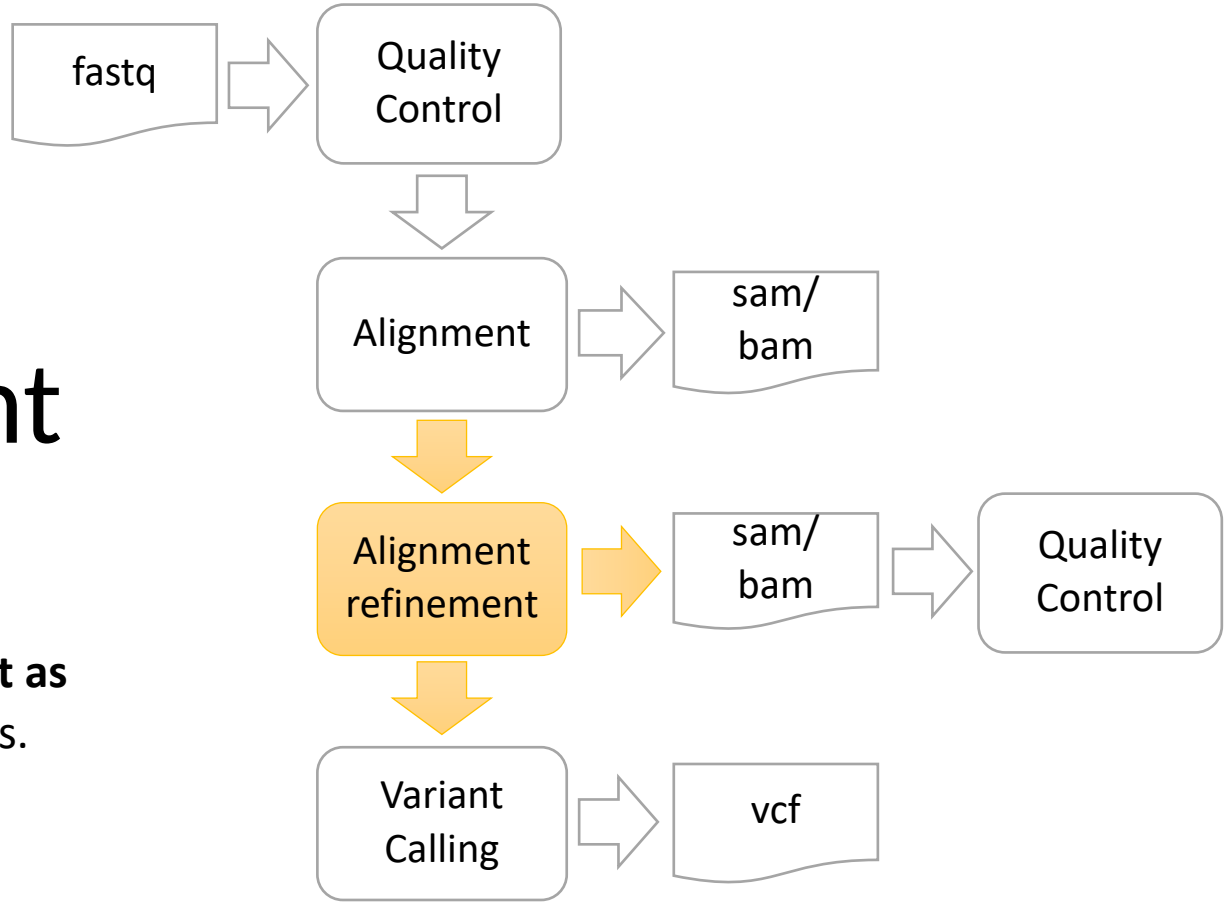
Panel of genes by amplicon/PCR approach



Target	Type of variants discovered	Avg depth per pos	Cost
Entire genome	Coding variants, intronic and regulatory sites. Structural variants #Variants= 3M - 4M.	> 30x Most uniform	High
2% of the genome	Coding variants Some intronic and regulatory sites. Issues in the detection of structural variants **#**Variants= 20k - 60k.	> 50x - 100x	Low
Variable	Depends on the design (customizable) Challenging detection of structural variants # variants = *ND*	> 500x	Lower

Alignment refinement

Variant calling requires the most perfect alignment as possible to avoid False Positives and False Negatives.



Mark/remove duplicates

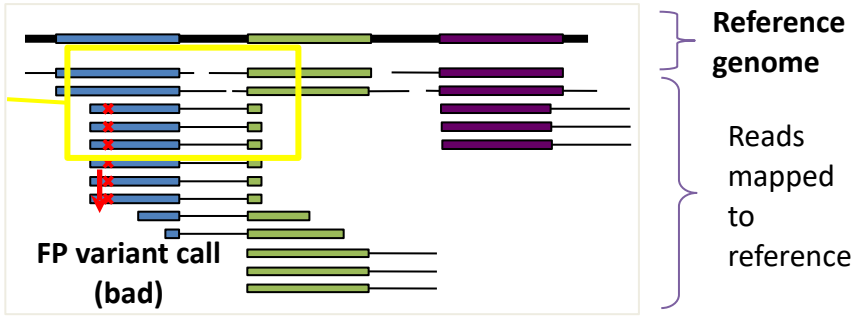
- Duplicates derive from **PCR amplification** (library preparation): one fragment is sequenced multiple times.
- An error at the beginning of the PCR (first steps) is propagated.

- Duplicates in hybrid-seq are **worthless** for the subsequent analysis:
  Duplicates are source of False Positives calls while only provide redundancy.

Solution: retrieve the best one, discard the duplicates:

METHOD: by **Picard-tools**
http://broadinstitute.github.io/picard/
(alternatives : **samtools**)

Duplicates share the **same alignment properties** : sequence, start and end positions



✖ = sequencing error propagated in duplicates

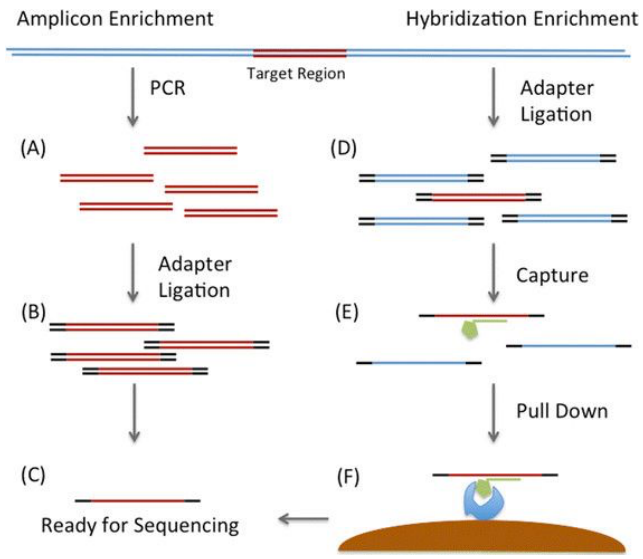
MARK/REMOVE DUPLICATES

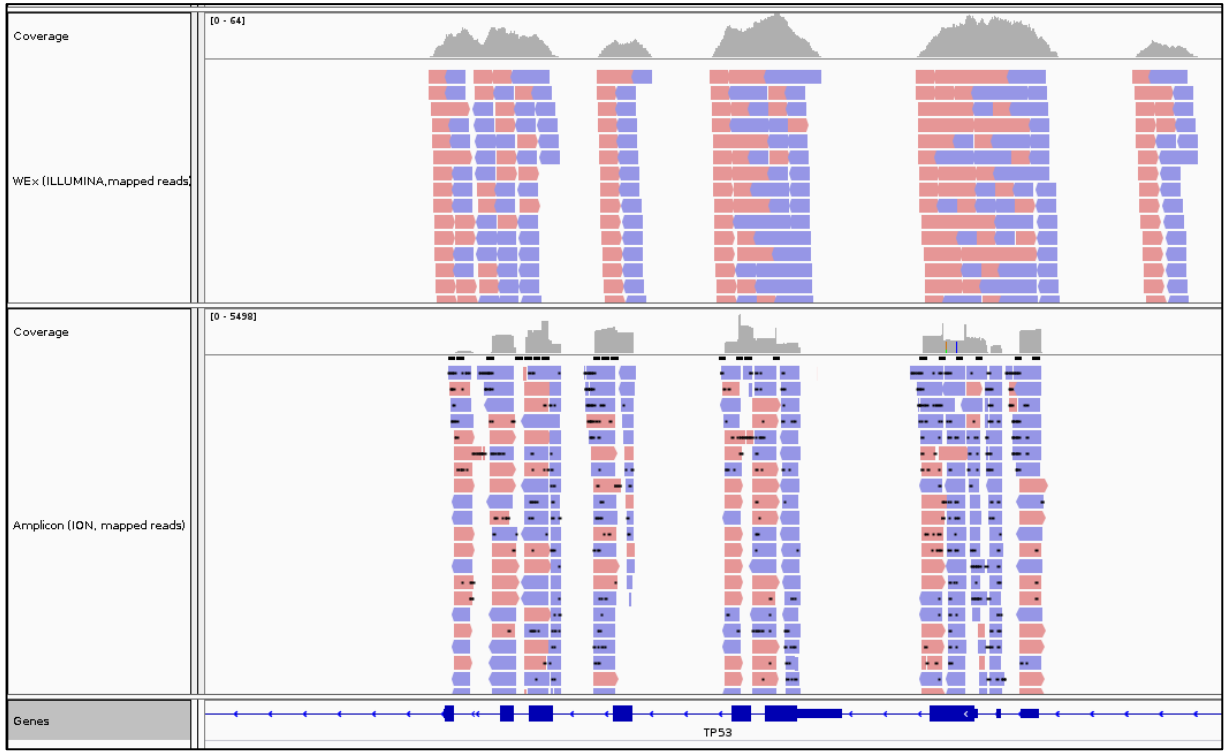
After marking/removing duplicates, the variant caller will only see :

… and thus be more likely to make the right call

Adapted from GATK

Mark/remove duplicates: Amplicon seq





WARNING: **Do NOT remove duplicates in data derived from** amplicon techniques (**Ion Torrent**).
More info.: https://github.com/broadgsa/gatk/blob/master/doc_archive/tutorials/(How_to)_Mark_duplicates_with_MarkDuplicates_or_MarkDuplicatesWithMateCigar.md

Indel realignment

- Algorithms align reads very fast with high accuracy, but not perfectly.

  During alignment, ***penalties on mismatches are much cheaper than gaps*** *(indels).*

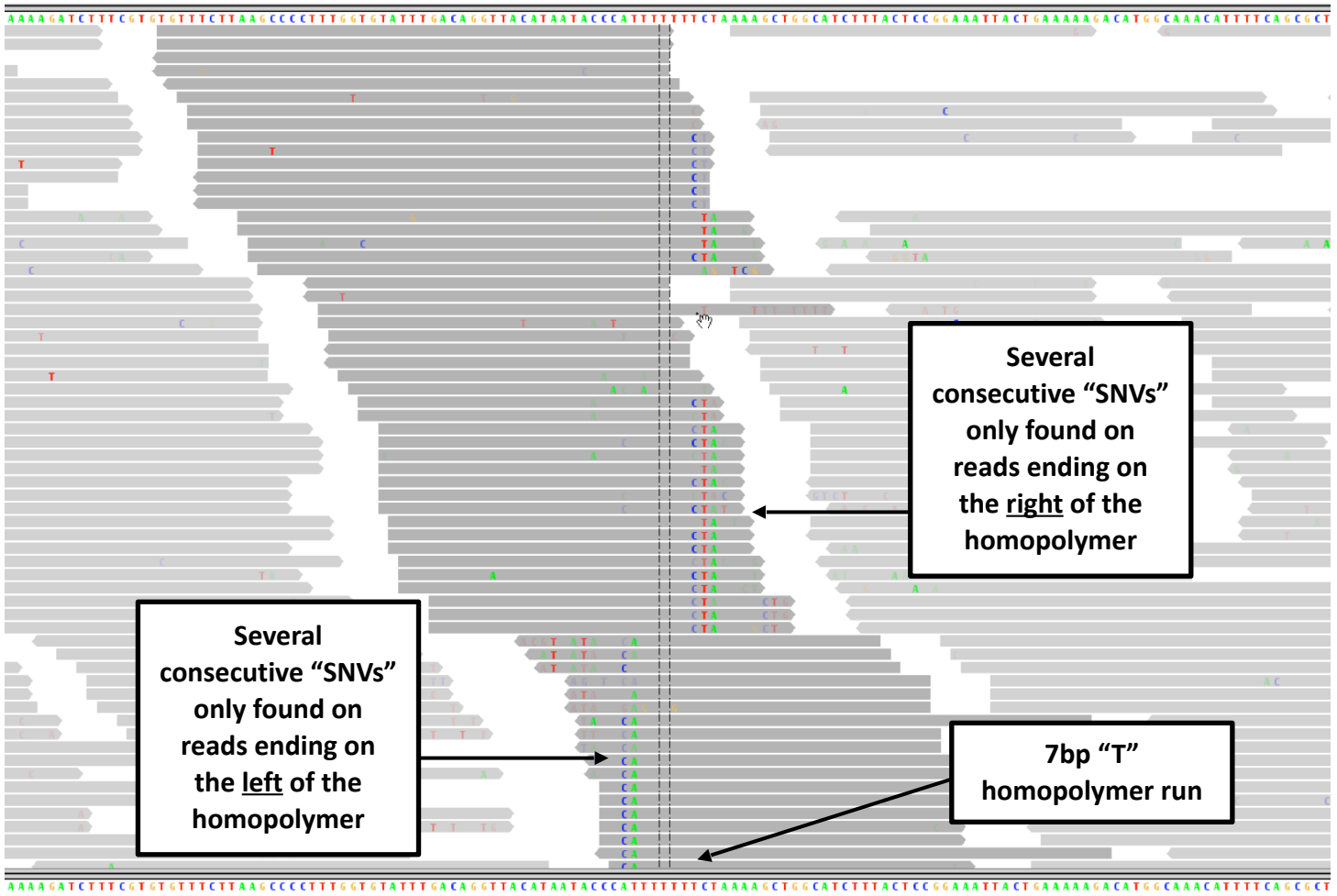
- Also, there are sometimes multiple solutions (alignments) for a given read. **Aligners can choose one randomly**.

- Reads are aligned separately (one by one).

- **Indels can be no properly identified** in the alignment of the read.

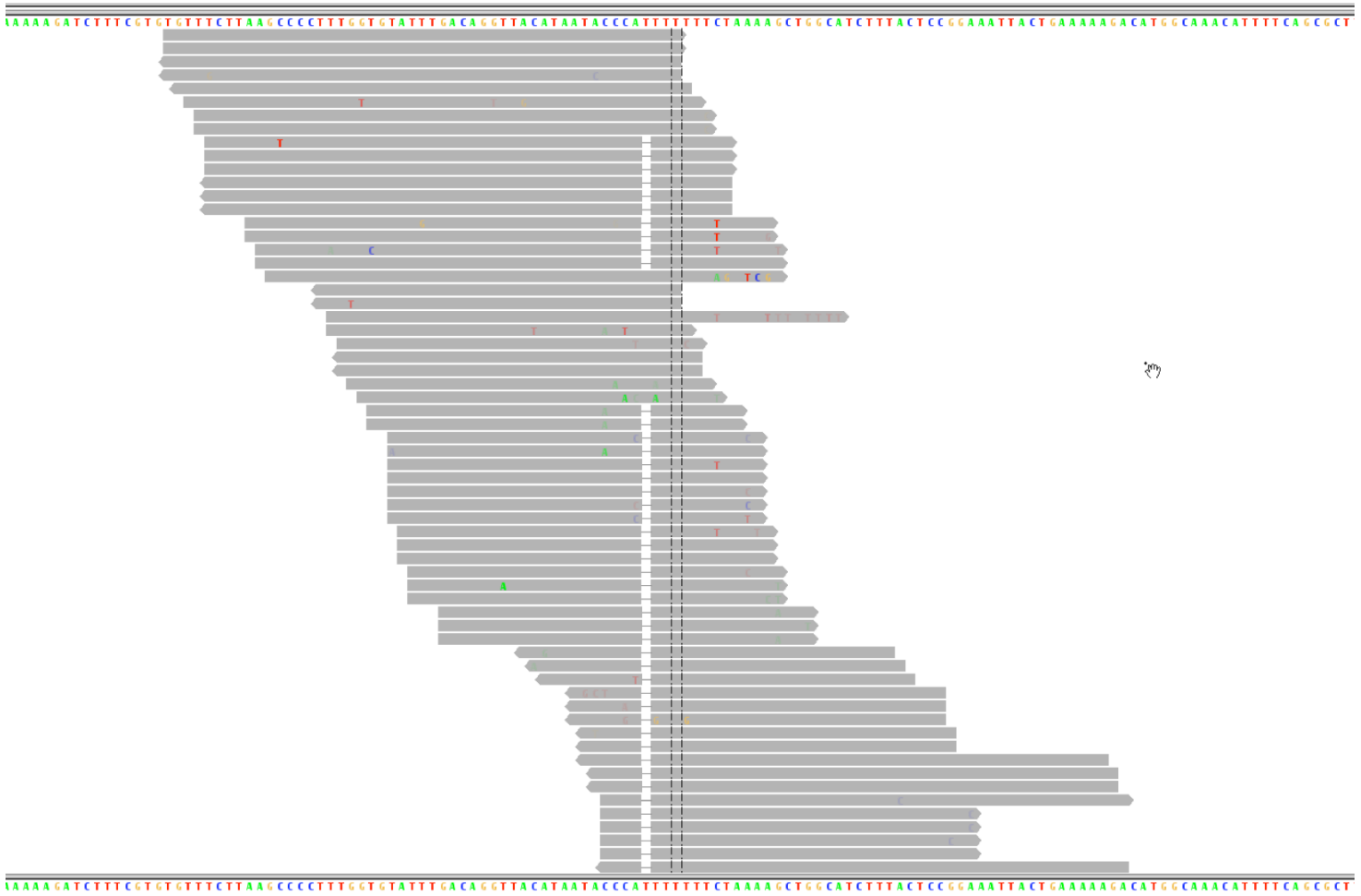
METHOD: by **GATK**
https://github.com/broadinstitute/gatk-docs/blob/master/gatk3-tutorials/(howto)_Perform_local_realignment_around_indels.md

Before indel realignment



Taken from GATK team

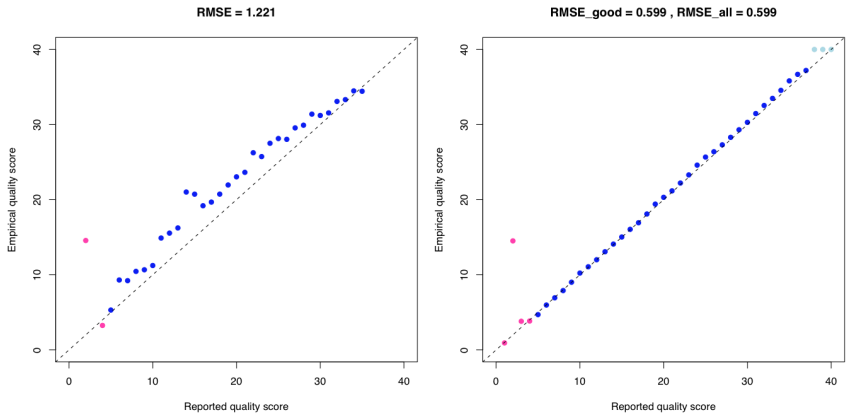
After indel realignment



Taken from GATK team

Base Quality Score Recalibration

- **Phred Quality score**: each position of the sequence has its particular **base quality score**.
- The individual quality measures **are crucial during Variant calling**.
- Different NGS technologies have their particular **bias in Quality Score** depending on the context. Recalibration **correct empirically** these biases.

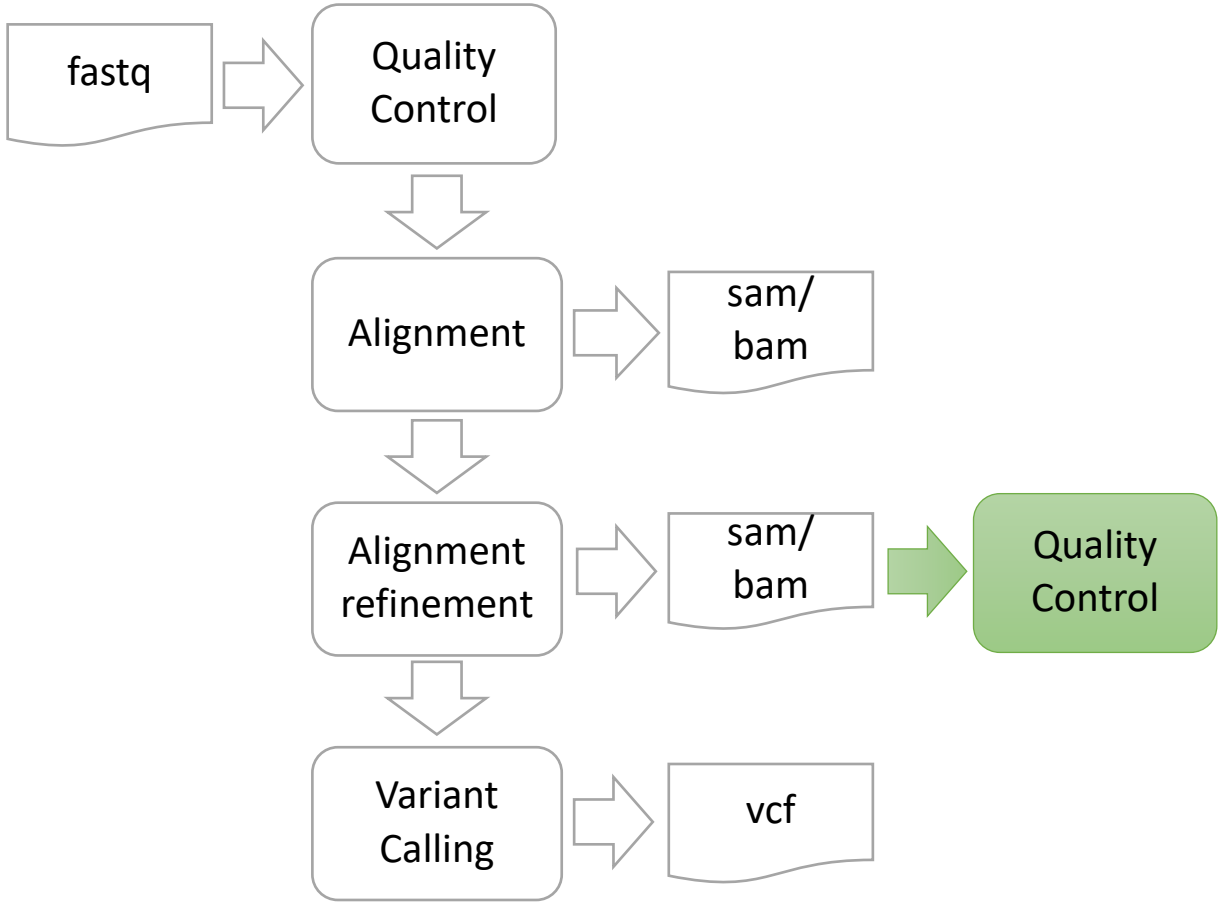


Original

After BQSR recalibration

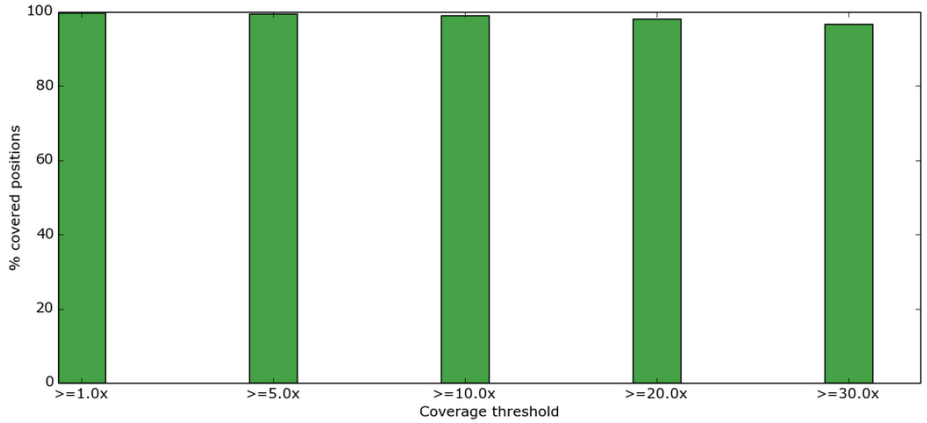
METHOD: by **GATK**
https://gatk.broadinstitute.org/hc/en-us/articles/360035890531-Base-Quality-Score-Recalibration-BQSR-

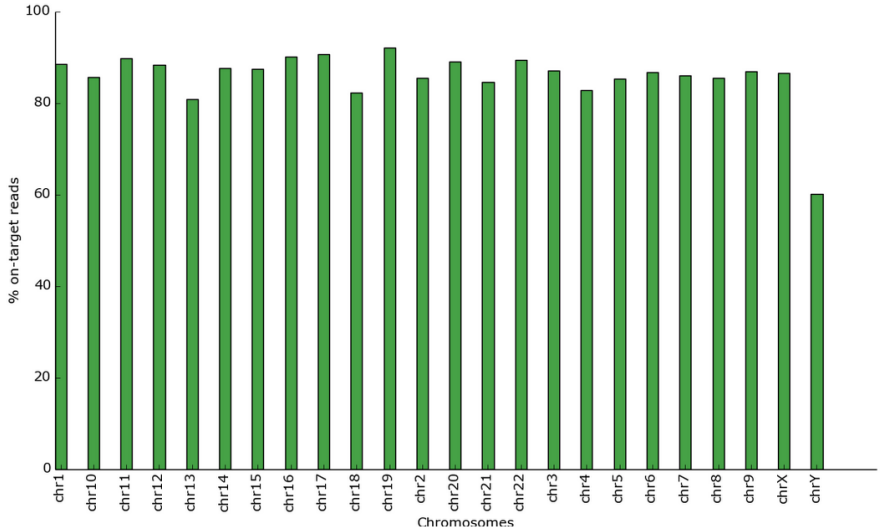
Alignment Quality Control



Alignment Quality Control

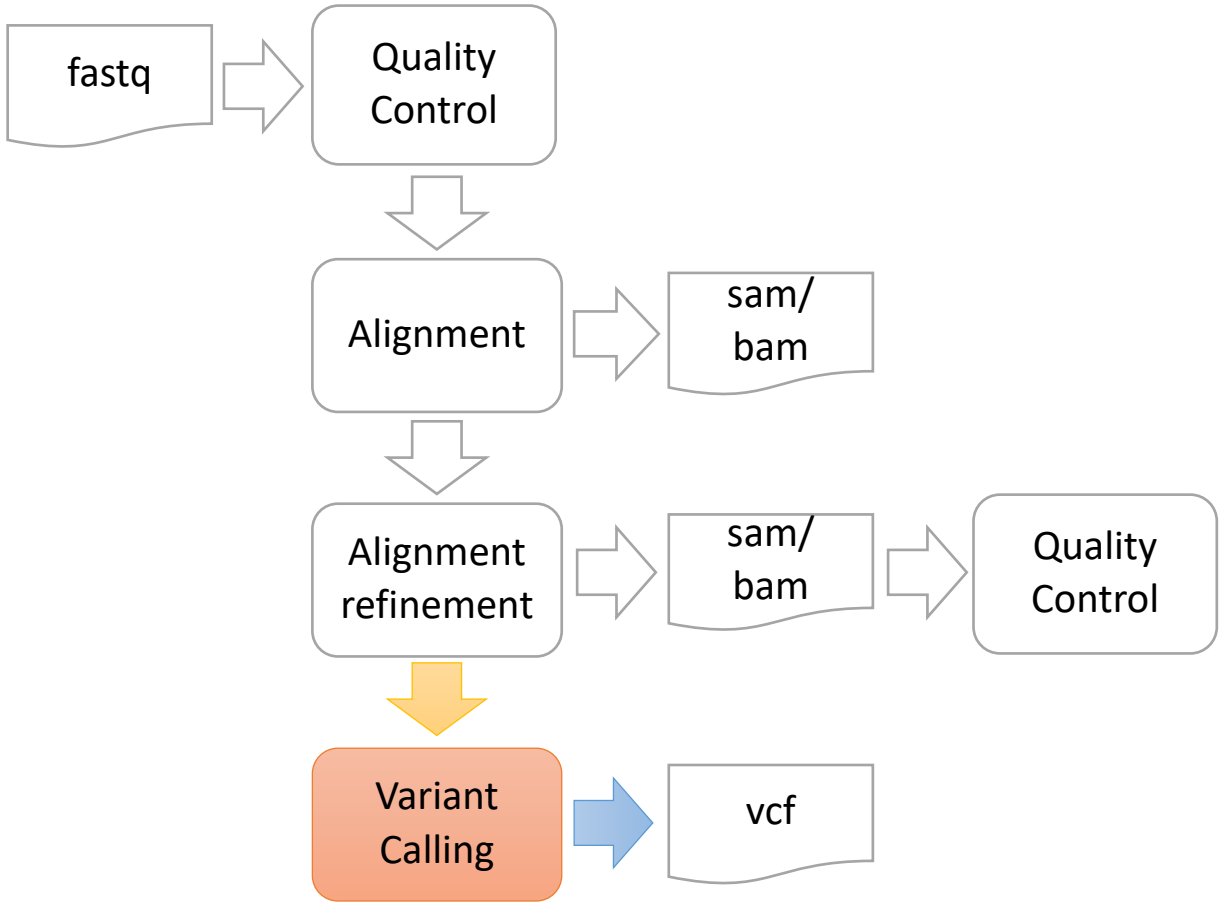
- Mean sequencing depth
- Is there enough coverage in regions of interest?
- Are the reads on-target?



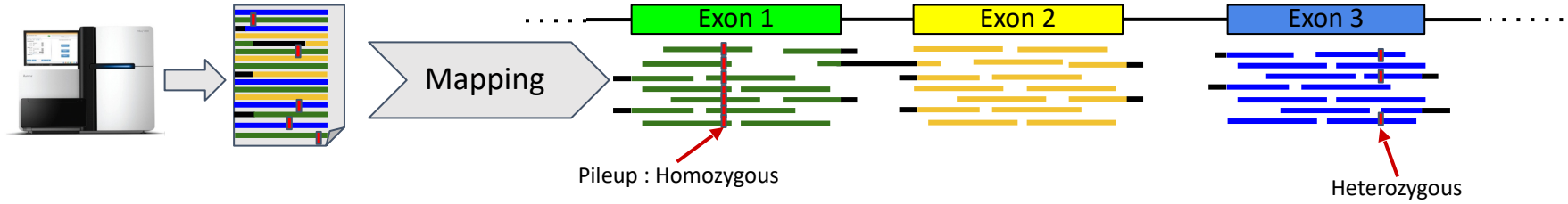


- Examples of **software**: ngsCAT, QualiMap

Variant calling



Fundamentals of Variant Calling



1 Identify the most likely **genotype for each genomic position** using statistical methods.

2 **Identify the differences** by comparing with the reference genome.

What is Crucial in Variant calling

- For clinical practices, the use of **gold standard methods** and **reproducible analysis** are mandatory.
- The analysis is based on the comparison against the reference genome:

A single consensus sequence for the whole genome. It was built up from a high quality set of representative samples of the species (from different populations).
It is the first-line comparison during analysis.

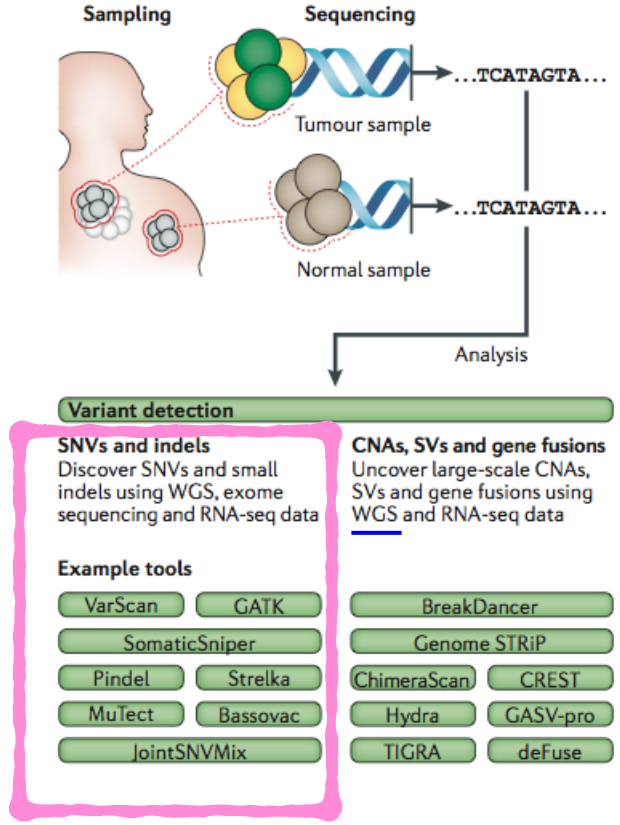
By **Genome Reference Consortium (GRC)** (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/)

- **Human assemblies (Versions):**
  - **GRCh37/hg19** : former version. Released in 2012. It is still used for analysis.
  - **CRCh38/hg38** : current version (Sep. 2017). Released in 2014. More accurate, comprehensive (includes Haplotypes) and sophisticated.

We must **keep consistency in the Genome Reference Version** through the variant analysis.

- We must know what **regions along the genome were sequenced** in the experiment, that is, the sequencing library.

Algorithms for Variant Calling



SNVs and Indels

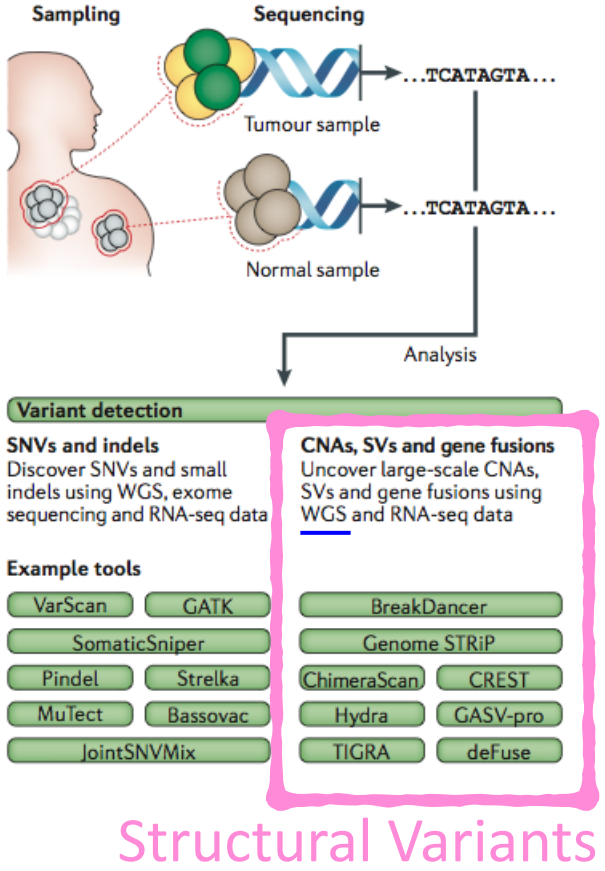
Several Methods have been published.

Further reading:

Ding, L., Wendl, M. C., McMichael, J. F. & Raphael, B. J. Nat Rev Genet – (2014). doi:10.1038/nrg3767

Nam J.N. et al. Evaluation of somatic copy number estimation tools for whole-exome sequencing data. Brief. Bioinformatics (2015)

Algorithms for Variant Calling



Structural Variants

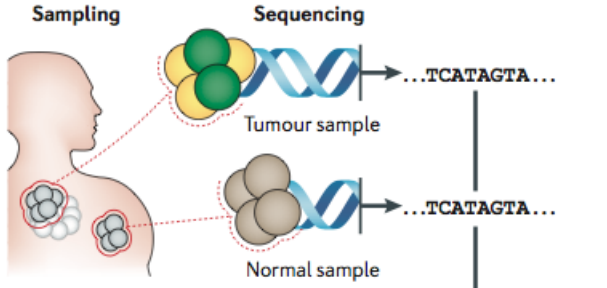
Several Methods have been published.

Further reading:

Ding, L., Wendl, M. C., McMichael, J. F. & Raphael, B. J. Nat Rev Genet – (2014). doi:10.1038/nrg3767

Nam J.N. et al. Evaluation of somatic copy number estimation tools for whole-exome sequencing data. Brief. Bioinformatics (2015)

Algorithms for Variant Calling



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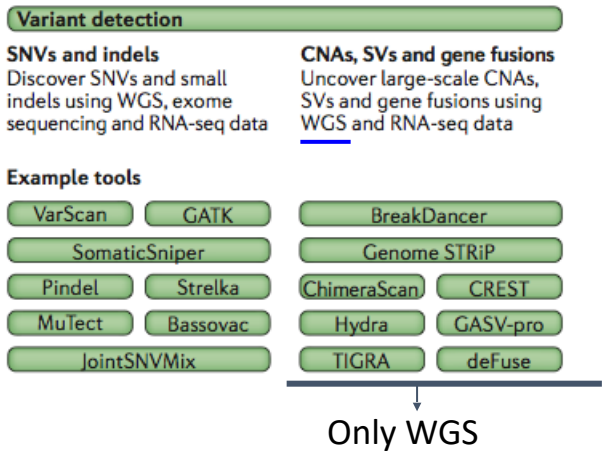


Only WGS

More on CNAs

Tool	Year	Language	Paired or pooled data	Segmentation	Feature
ADTEx	2014	Python, R	Both	HMM	Noise reduction Ploidy estimation
CONTRA	2012	Python, R	Both	CBS	GC correction
Control-FREEC	2011	C++, R	Paired	LASSO	GC correction, mappability
EXCAVATOR	2013	Perl, R	Both	HSLM	GC correction, mappability, exon-size correction
ExomeCNV	2011	R	Paired	CBS	GC correction, mappability
Varscan2	2012	Java, Perl, R	Paired	CBS	GC correction

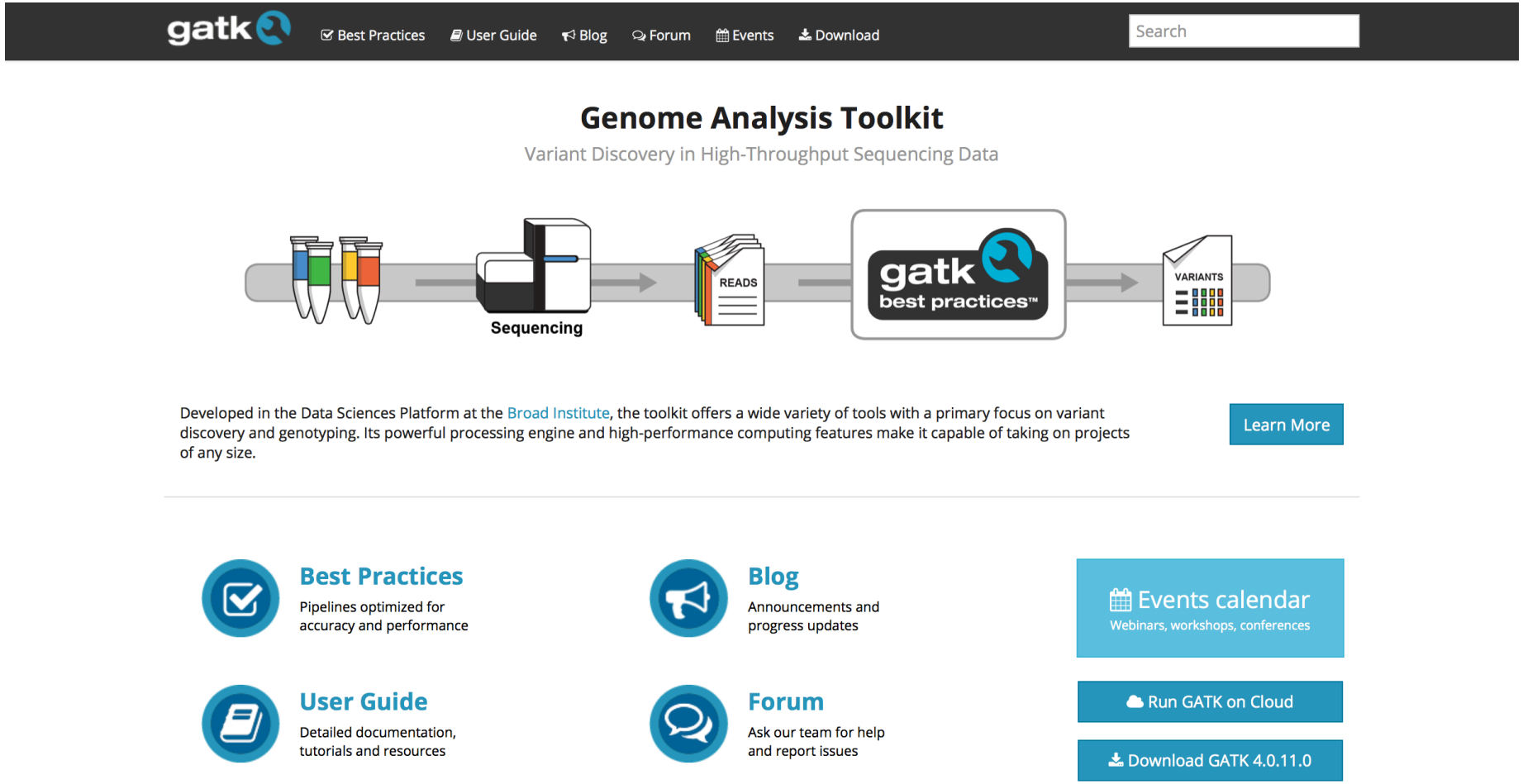
Appropriate methods for Whole-Exome seq

Further reading:

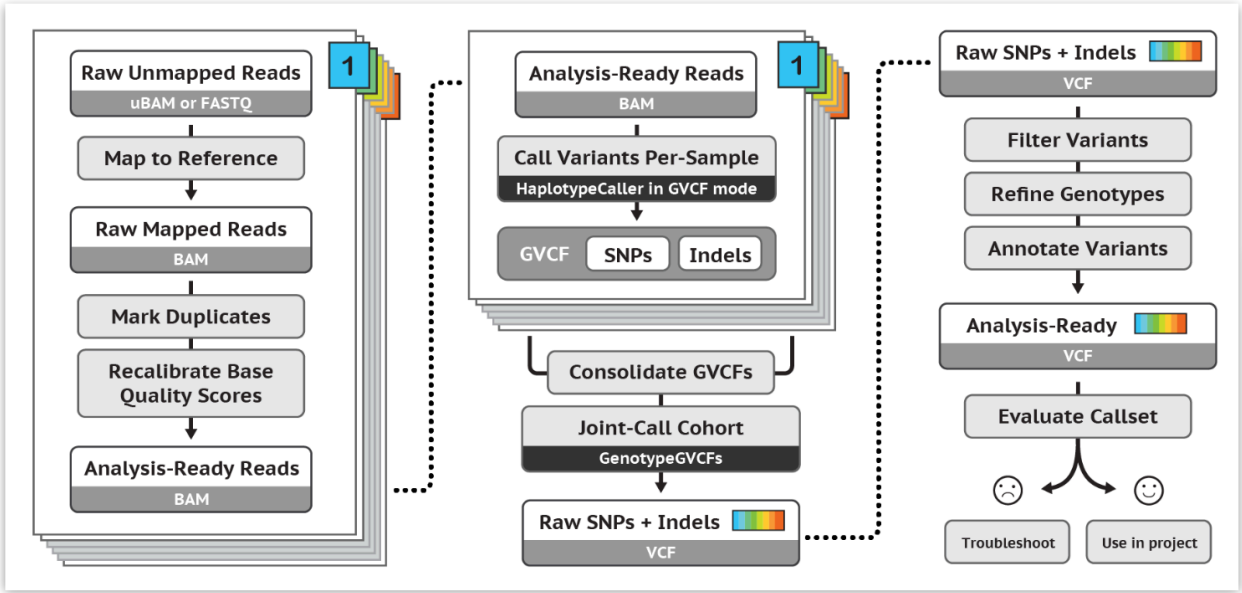
Ding, L., Wendl, M. C., McMichael, J. F. & Raphael, B. J. Nat Rev Genet – (2014). doi:10.1038/nrg3767

Nam J.N. et al. Evaluation of somatic copy number estimation tools for whole-exome sequencing data. Brief. Bioinformatics (2015)

GATK for variant calling analysis

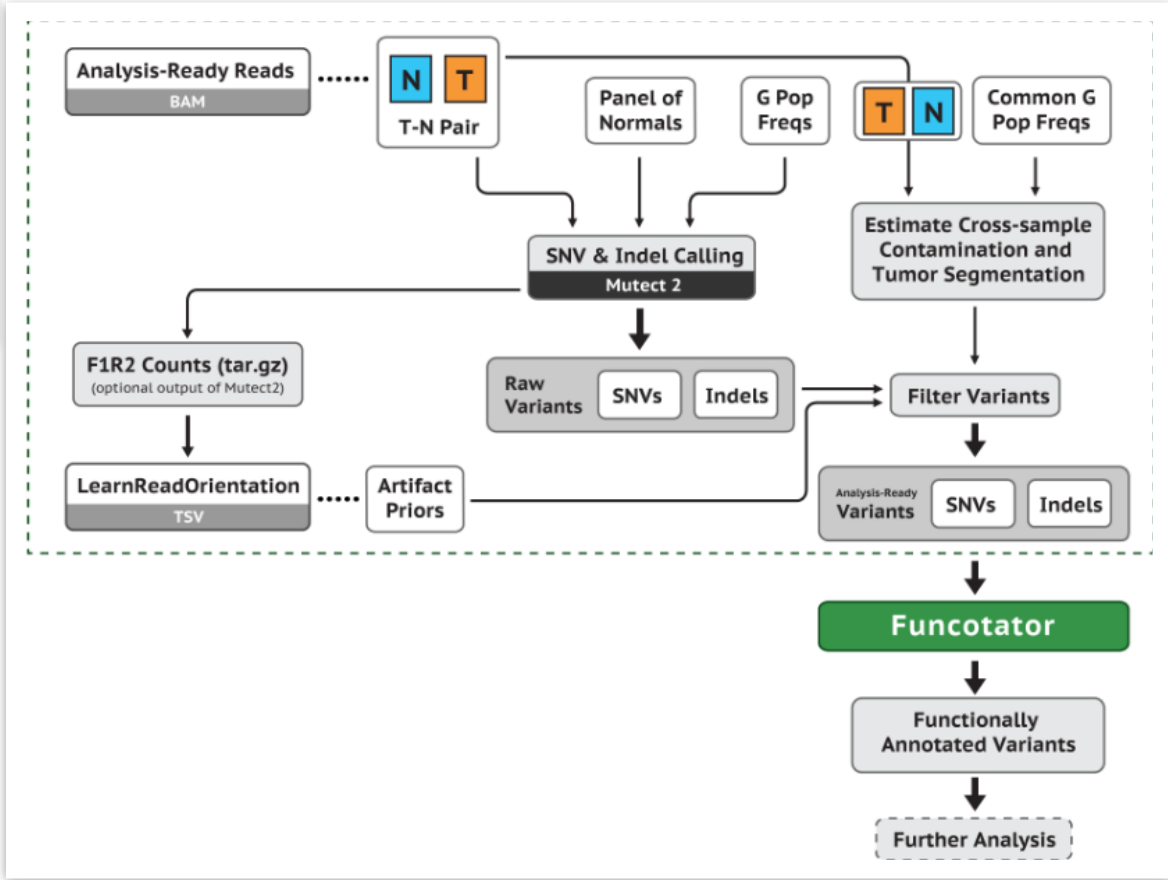


GATK Best Practices



Germline small-scale variants

Somatic small-scale variants

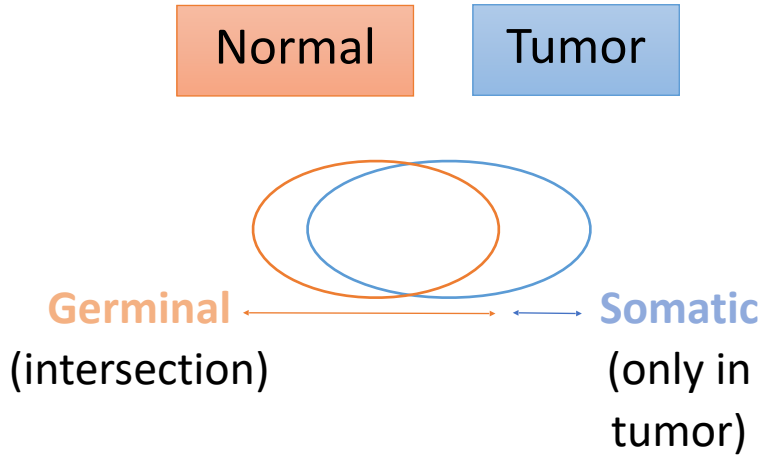


Somatic vs Germline variants

Germline: appear in gametes
Inheritable
Affect to future generations
e.g.: variants involved in rare diseases

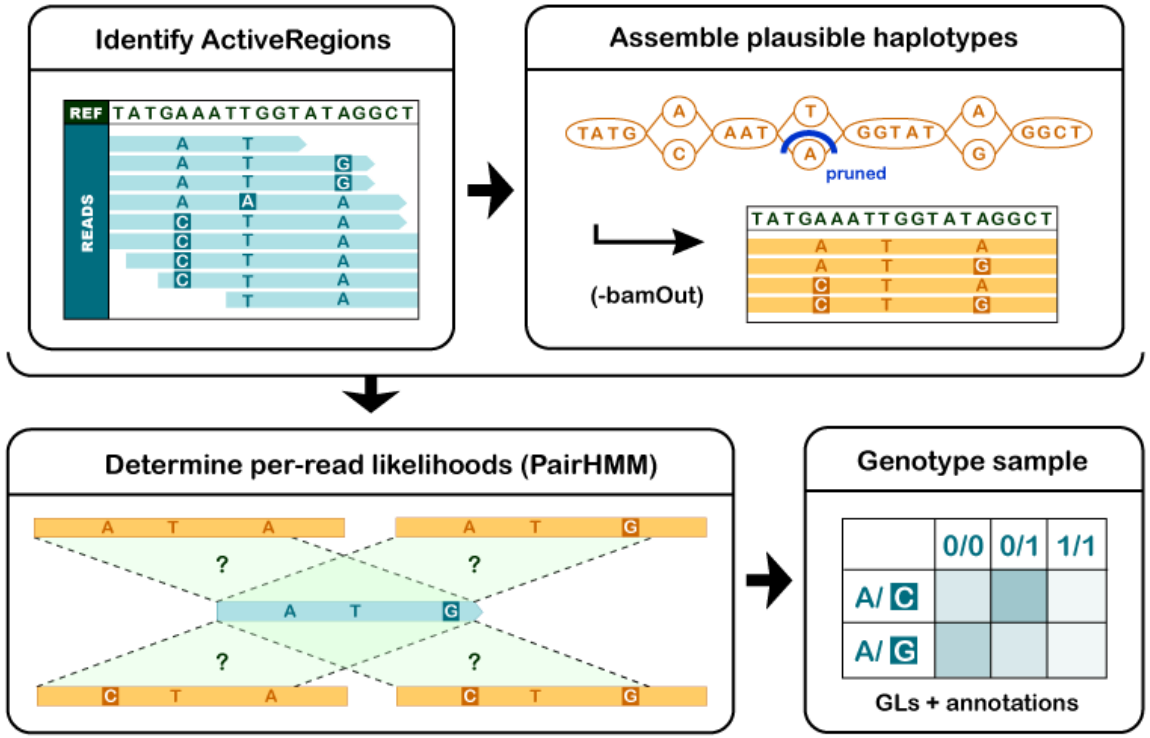
Somatic: appear in different from germline cells
Acquired
Only affect to the linage of the affected cell
e.g.: variants causing cancer

Identified by comparison:



Variant Calling for SNVs and Indels

Haplotype Caller : Variant calling based on the calculation of genotype likelihoods:



Assumptions: It bases the calling in the indicated ploidy (e.g. 2n)

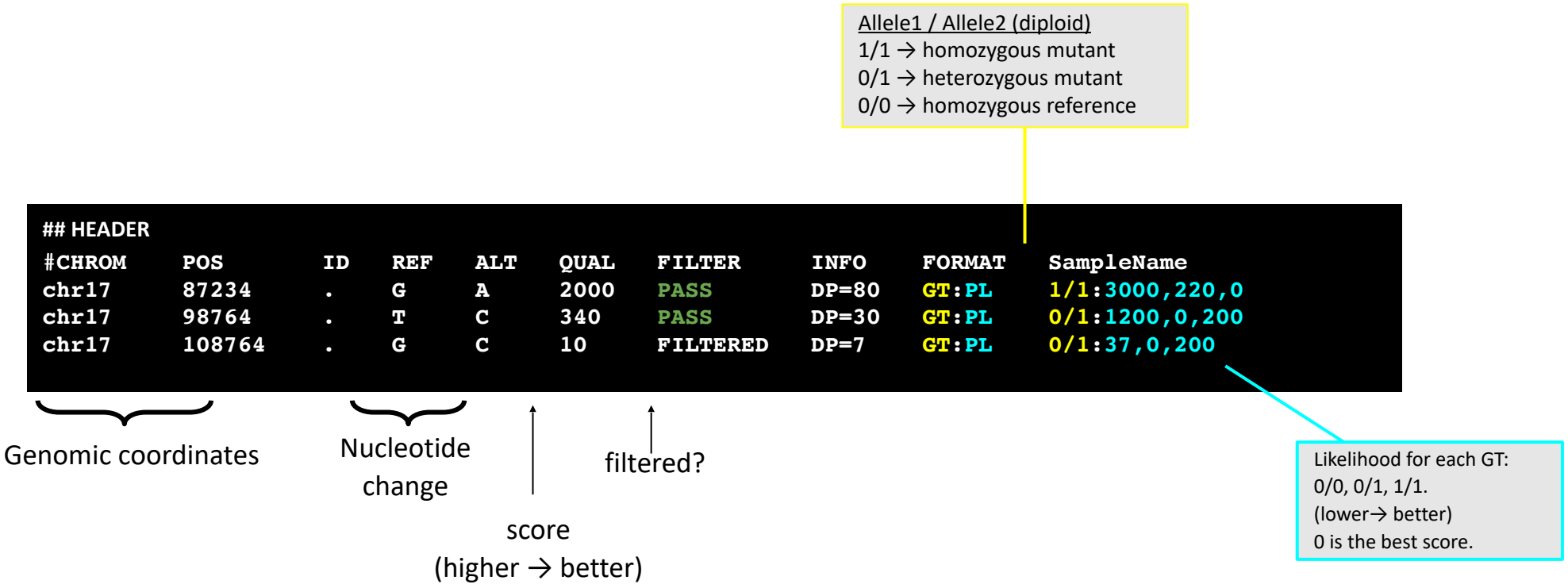
Limited detection of low allele frequencies.

Further reading:

https://github.com/broadgsa/gatk/blob/master/doc_archive/methods/HC_overview:_How_the_HaplotypeCaller_works.md

https://gatk.broadinstitute.org/hc/en-us/sections/360007226771?name=methods

VCF file



More info.:

https://samtools.github.io/hts-specs/VCFv4.2.pdf

https://gatk.broadinstitute.org/hc/en-us/articles/360035531692-VCF-Variant-Call-Format

Intra-tumoral heterogeneity

Variant Allele Frequency

Proportion of DNA molecules in the sample carrying the variant

$$VAF = \frac{sequence\ reads\ with\ a\ DNA\ variant}{overall\ coverage\ at\ that\ locus}$$

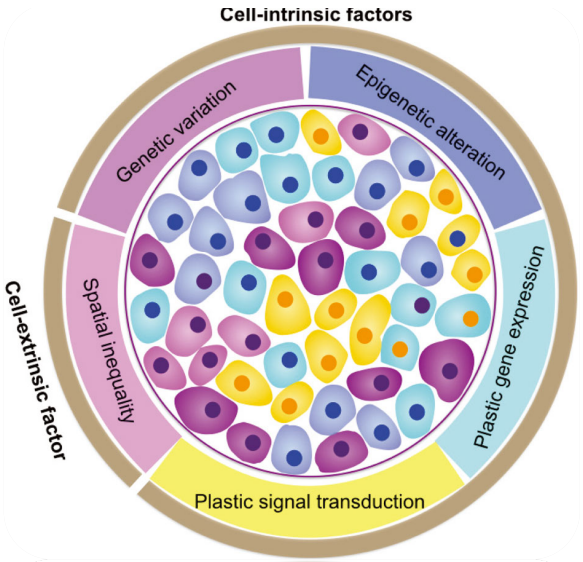
For a diploid organism:

- **heterozygous loci** should be near 0.5 VAF
- **homozygous loci** should be near 1 VAF
- **reference loci** should be near 0 VAF

doi: 10.28092/j.issn.2095-3941.2016.0004

Clonal composition in cancer

changes 0.5/1.0 diploid variant allele frequencies



doi: 10.1038/aps.2015.92

Variant Calling for somatic variants: MuTect2

SNV and Indel caller**.**

Similar logic to Haplotype Caller but:

- It **allows variable allele frequencies**.
- It **includes logic to avoid germline variants**.

CGA
Cancer Genome Analysis

Log-in

Search this site:
Search...

CGA
- Home
- Help Forum
- ABSOLUTE
- BreakPointer
- ChainFinder
- ContEst
- Copynumber Pipeline
- D-ToxoG
- dRanger
- Firehose
- GISTIC
- HAPSEG
- Indelocator
- InVEx
- MuTect
  - How do I get MuTect?
  - How do I run MuTect?
  - How do I get help?
- MutSig
- Oncotator
- PathSeq
- RNA-SeQC
- SegSeq
- TranspoSeq

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Home ›

MuTect

What is MuTect?

MuTect is a method developed at the Broad Institute for the reliable and accurate identification of somatic point mutations in next generation sequencing data of cancer genomes.

For complete details, please see our publication in Nature Biotechnology:

Cibulskis, K. et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. Nat Biotechnology (2013).doi:10.1038/nbt.2514

How does it work?

In brief, muTect consists of three steps.

1. Preprocessing the aligned reads in the tumor and normal sequencing data. In this step we ignore reads with too many mismatches or very low quality scores since these represent noisy reads that introduce more noise than signal.
2. A statistical analysis that identifies sites that are likely to carry somatic mutations with high confidence. The statistical analysis predicts a somatic mutation by using two Bayesian classifiers – the first aims to detect whether the tumor is non-reference at a given site and, for those sites that are found as non-reference, the second classifier makes sure the normal does not carry the variant allele. In practice the classification is performed by calculating a LOD score (log odds) and comparing it to a cutoff determined by the log ratio of prior probabilities of the considered events. For the tumors we calculate $LOD_T = \log_{10}\left(\frac{P(\text{observed data in tumor} \mid \text{site is mutated})}{P(\text{observed data in tumor} \mid \text{site is reference})}\right)$, and for the normal $LOD_N = \log_{10}\left(\frac{P(\text{observed data in normal} \mid \text{site is reference})}{P(\text{observed data in normal} \mid \text{site is mutated})}\right)$.

   Since we expect somatic mutations to occur at a rate of ~1 in a Mb, we require $LOD_T > \log_{10}\left(0.5 \times 10^{-6}\right) = 6.3$ which guarantees that our false positive rate, due to noise in the tumor, is less than half of the somatic mutation rate. In the normal, not in dbSNP sites, we require $LOD_N > \log_{10}\left(0.5 \times 10^{-2}\right) = 2.3$ since non-dbSNP germline variants occur roughly at a rate of 100 in a Mb. This cutoff guarantees that the false positive somatic call rate, due to missing the variant in the normal, is also less than half the somatic mutation rate.

3. Post-processing of candidate somatic mutations to eliminate artifacts of next-generation sequencing, short read alignment and hybrid capture. For example, sequence context can cause hallucinated alternate alleles but often only in a single direction. Therefore, we test that the alternate alleles supporting the mutations are observed in both directions.

As muTect attempts to call mutations it also generates a coverage file (in a wiggle file format, which indicates for every base whether it is sufficiently covered in the tumor and normal to be sensitive enough to call mutations). We currently use cutoffs of at least 14 reads in the tumor and at least 8 in the normal (these cutoffs are applied after removing noisy reads in the preprocessing step). In addition, wiggle files can also be generated of the observed depth in the tumor and in the normal.

Most cancer genome studies at the Broad Institute have made use of MuTect and have validated the mutation calls as a part of their cancer biology papers, showing that MuTect has a very low false positive rate. A summary of validation rates from these papers are show below:

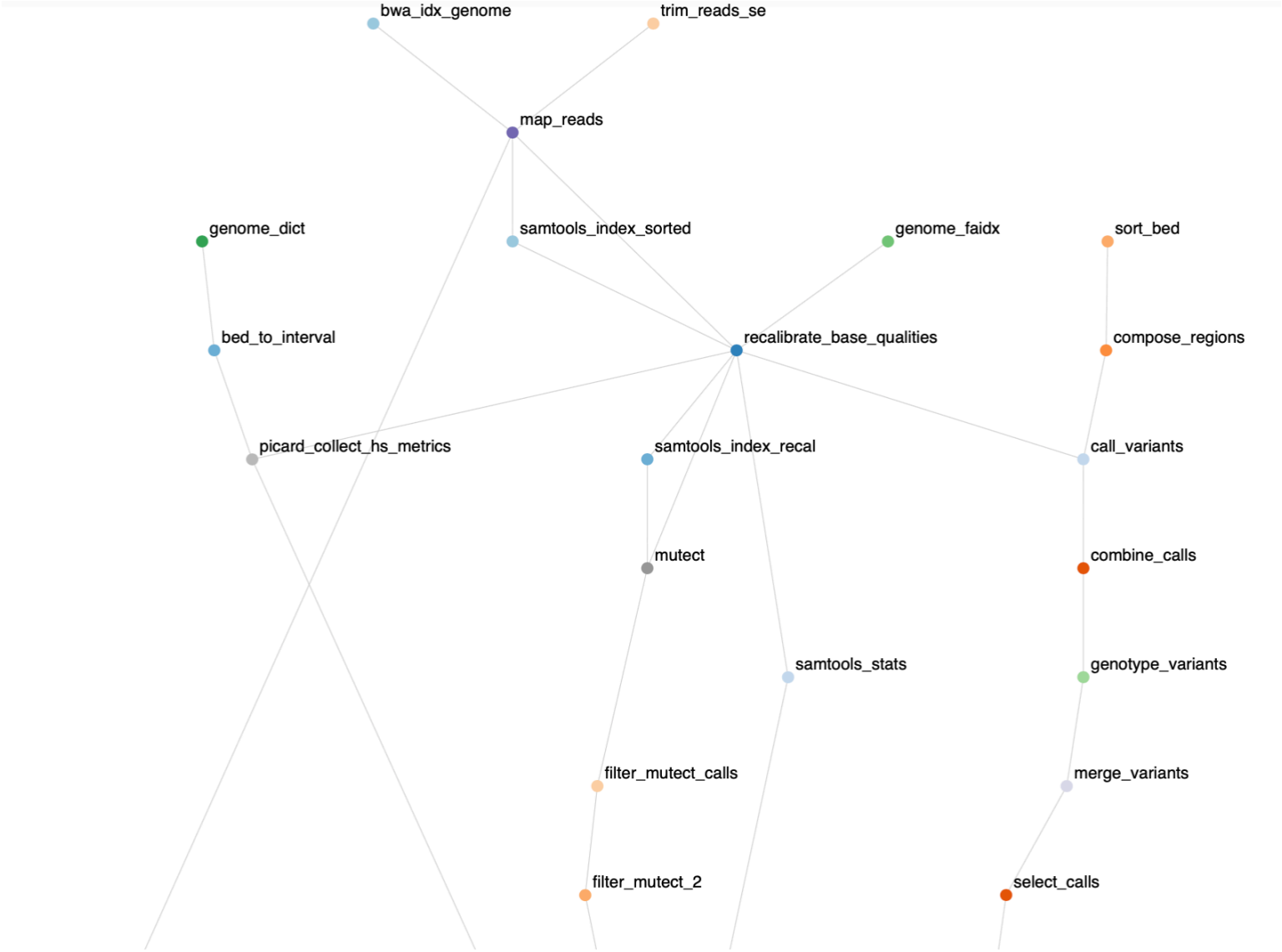
publication	technology	candidates	validated	no result	validation rate
Multiple Myeloma[1]	Sequenom	97	92	5	94.85%
Ovarian[2]	Sequenom/PCR/454	1655	1483	172	89.61%
Ovarian[2]	Capture/Illumina	6497	6232	265	95.92%
Head and Neck[3]	Sequenom	321	288	33	89.72%
Breast[4]	Sequenom/PCR/454	455	428	0	94.07%

https://gatk.broadinstitute.org/hc/en-us/articles/360037593851-Mutect2

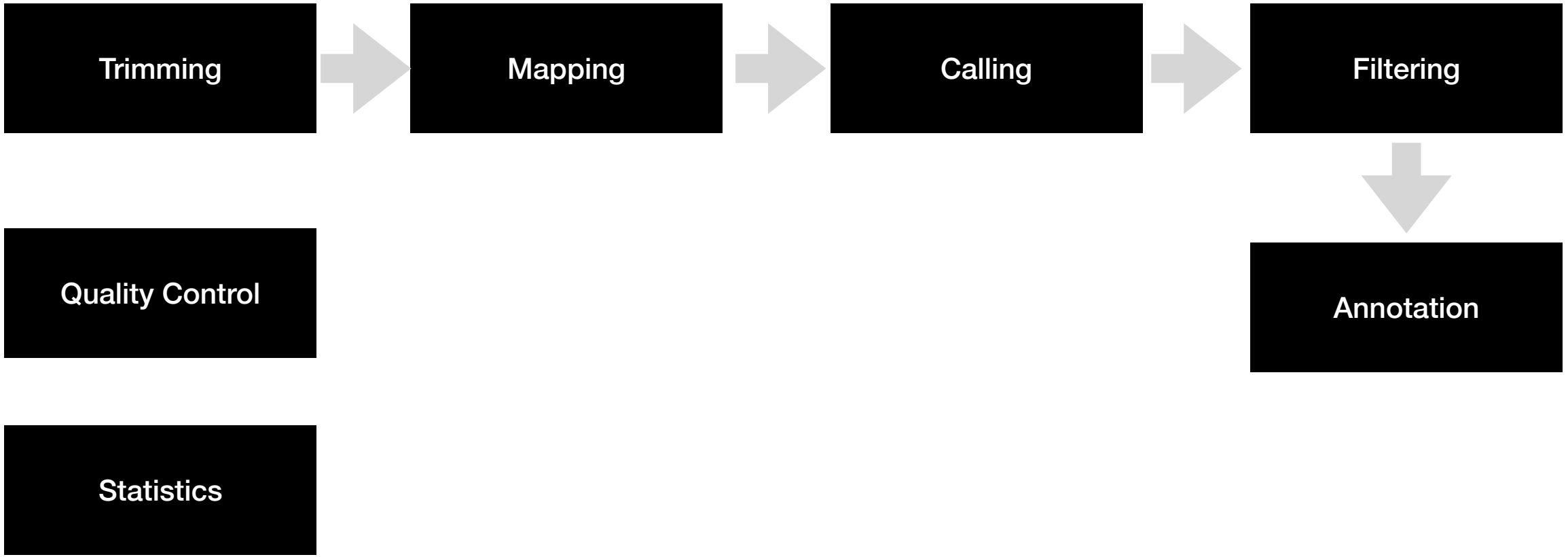
Cibulskis, K. et al.
Nat Biotechnology (2013).doi:10.1038/nbt.2514

varca

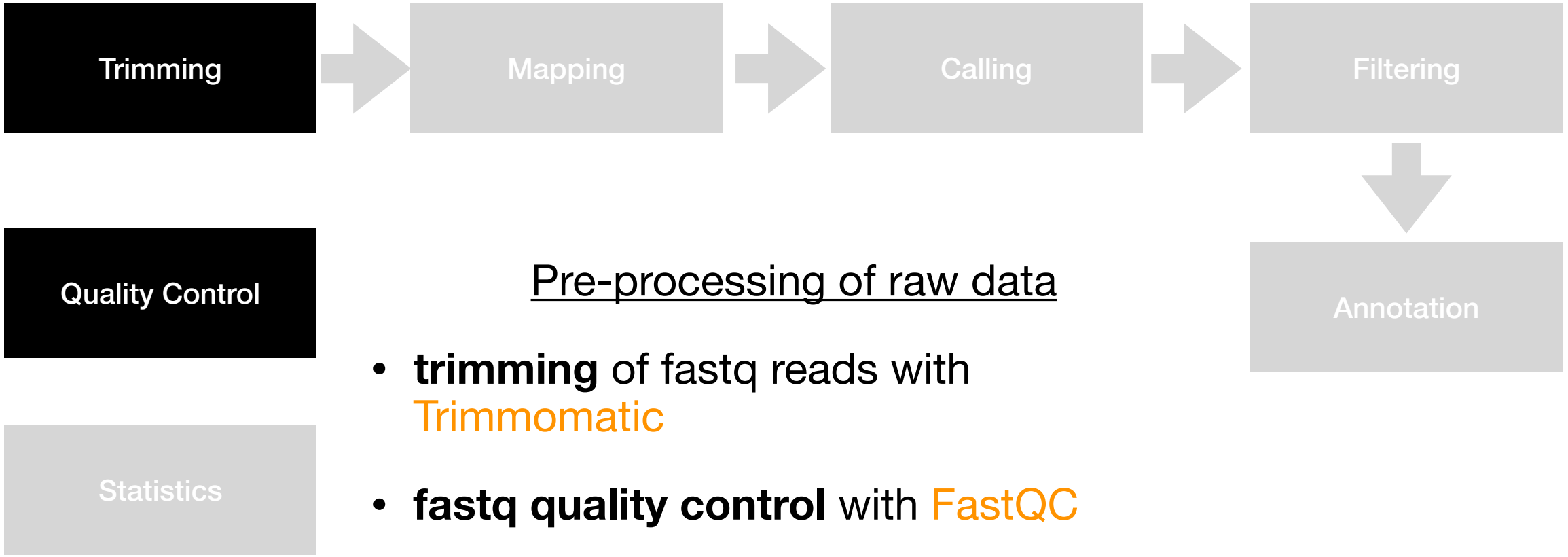
https://gitlab.com/bu_cnio/varca



Steps in the pipeline



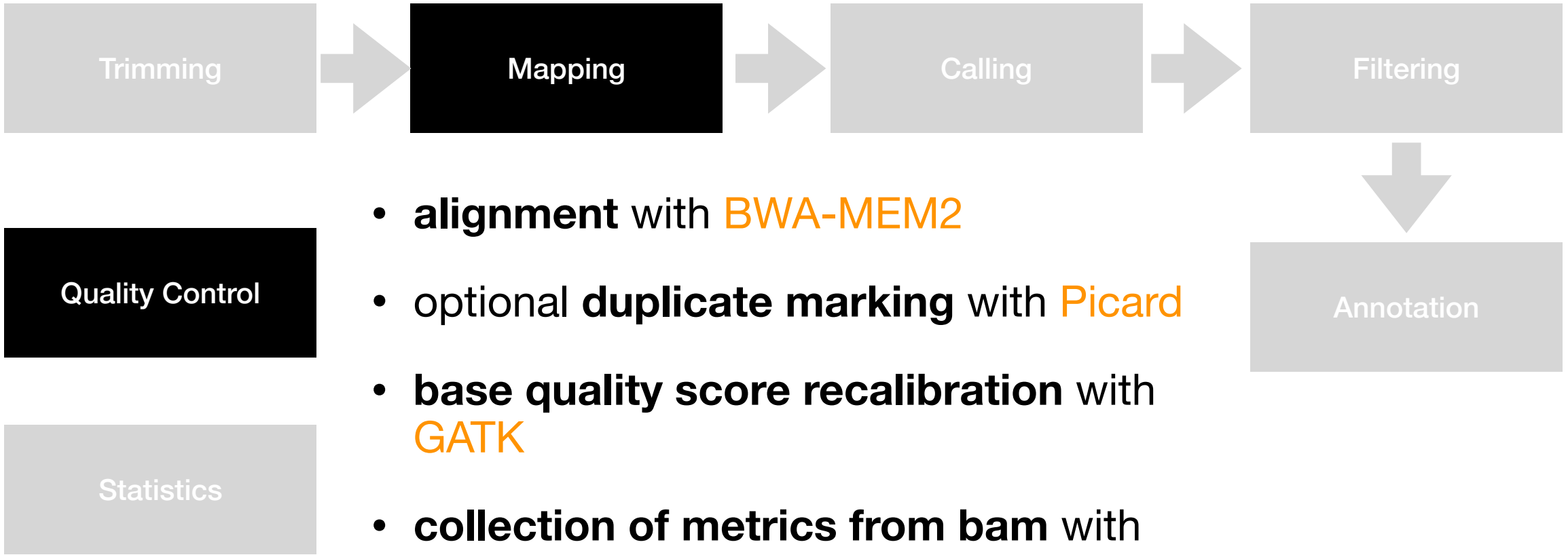
Steps in the pipeline



Pre-processing of raw data

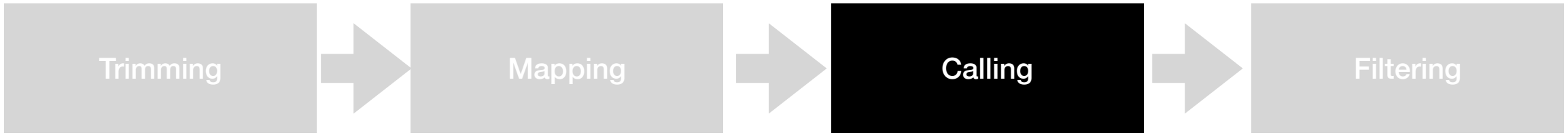
- **trimming** of fastq reads with Trimmomatic
- **fastq quality control** with FastQC

Steps in the pipeline



- **alignment** with BWA-MEM2
- optional **duplicate marking** with Picard
- **base quality score recalibration** with GATK
- **collection of metrics from bam** with samtools and Picard

Steps in the pipeline

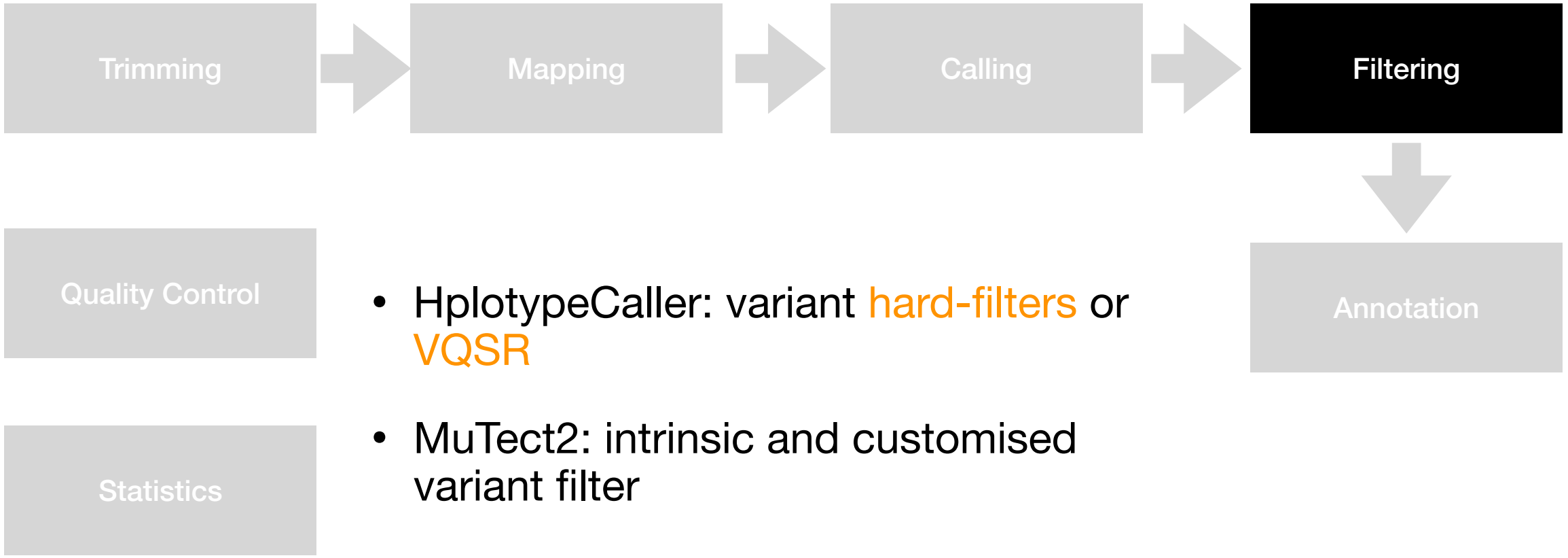


Quality Control

Statistics

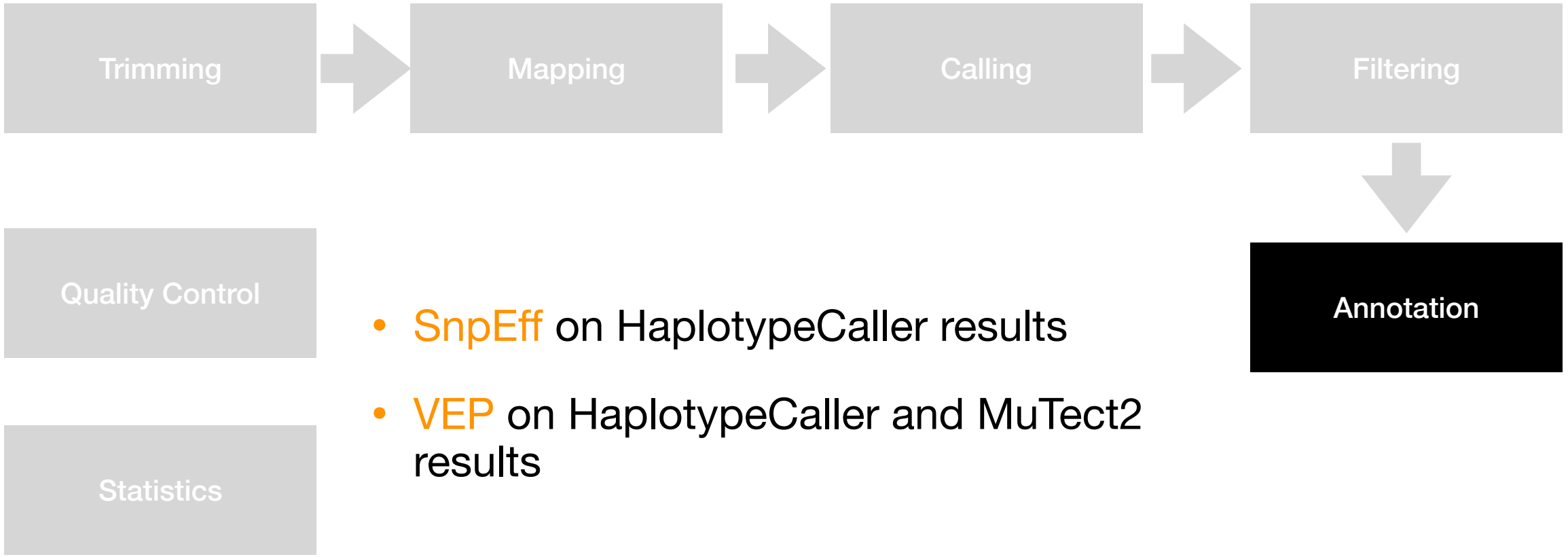
- Germline and medium-to-high allele frequency somatic variants with HaplotypeCaller (GATK)
- Optional extension to somatic low-frequency variants with MuTect2

Steps in the pipeline



- HplotypeCaller: variant hard-filters or VQSR
- MuTect2: intrinsic and customised variant filter

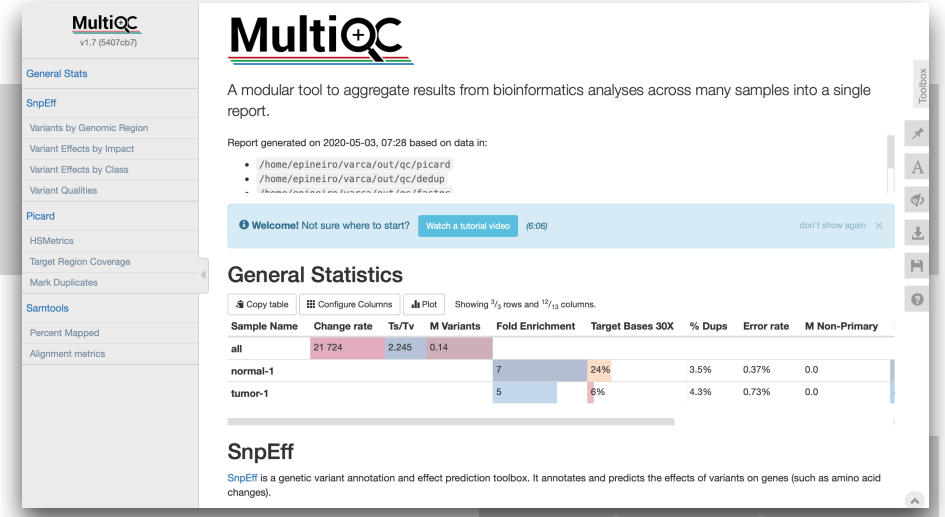
Steps in the pipeline



- SnpEff on HaplotypeCaller results
- VEP on HaplotypeCaller and MuTect2 results

Steps in the pipeline

Trimming → Mapping → Annotation



Quality Control

- Quality report with MultiQC

Statistics

- Statistic Plots: allele frequencies and depth of sequencing

Steps in the pipeline: parallelization