Recherche de variants génomiques en oncologie clinique

Avec des diapos, données & scripts R de:
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Génétique constitutionnelle

At hospital

Blood sample → Sequence gene panel → Look for specific alteration (BRCA)

Research

Genotype or Sequence → Compare disease and healthy cohorts

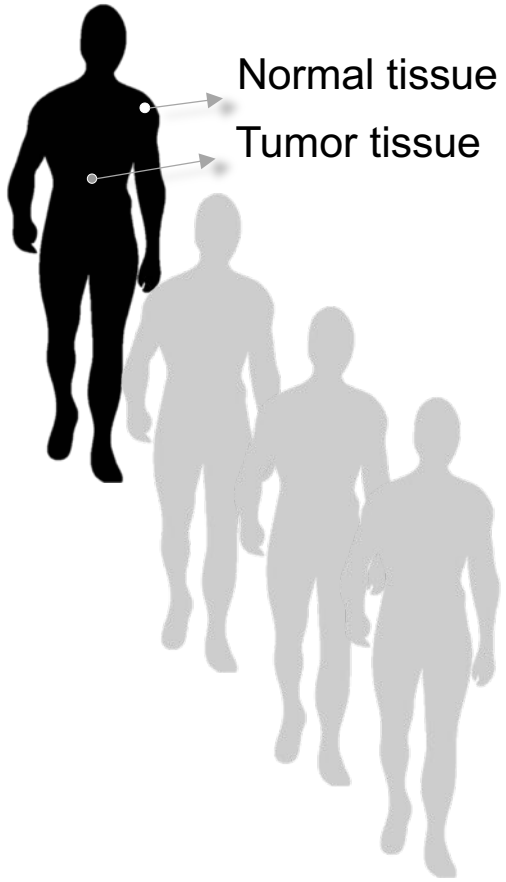
GWAS studies, 1000 Genome Project…

NGS dans le diagnostic de génétique familiale

- BRCA1/2 (breast/ovary cancer)
- XPC, XPV.. (melanoma)
- ERCC1 (colorectal cancer)

Génétique somatique

Finding somatic mutations in the tumor genome



Normal tissue (blood) → Extract and sequence DNA

Tumor tissue → Look for mutations, structural variations

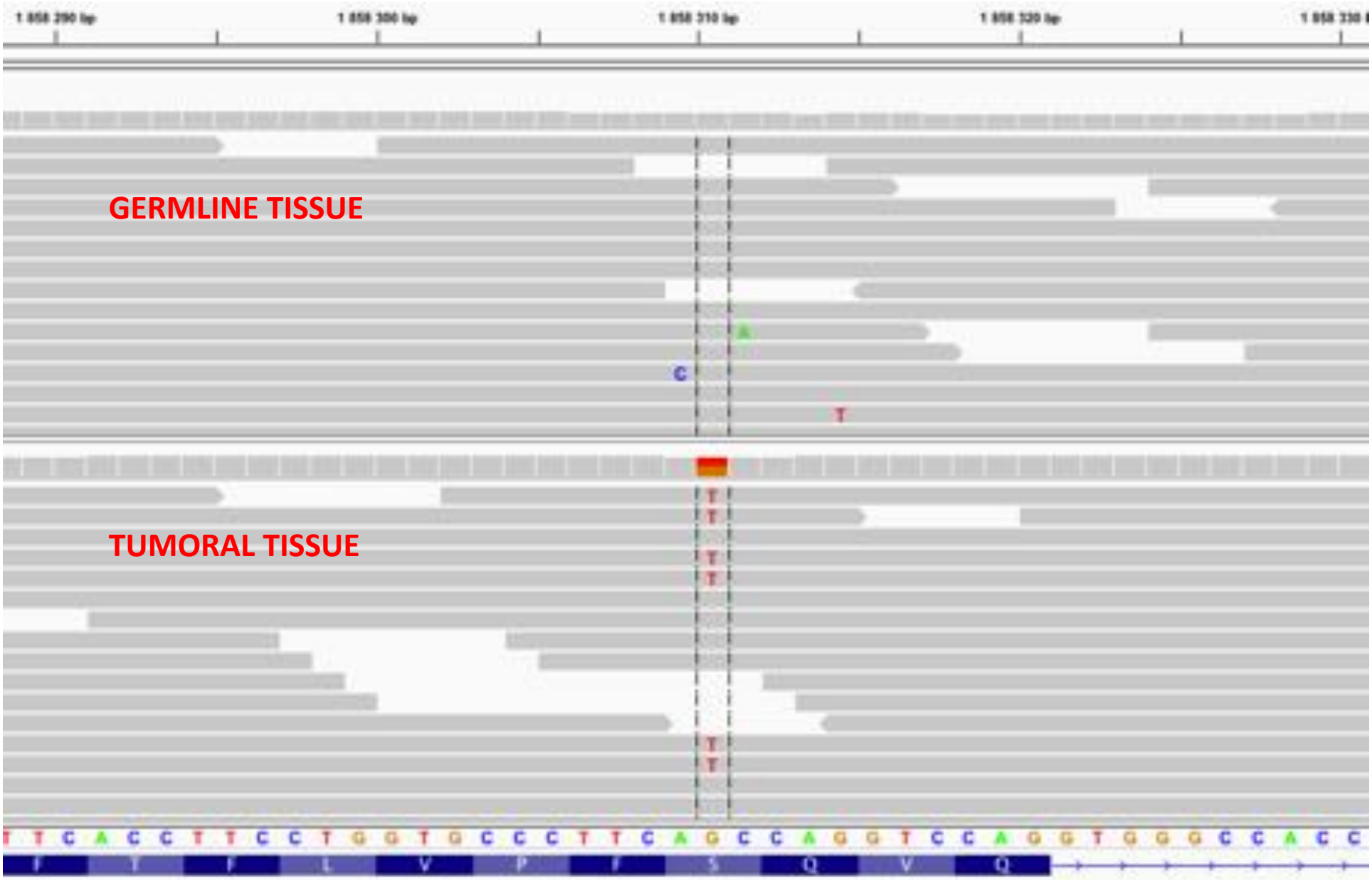
Repeat for several patients → Look for recurrent events

- 100-10,000 differences between a tumor and normal tissue from the same person

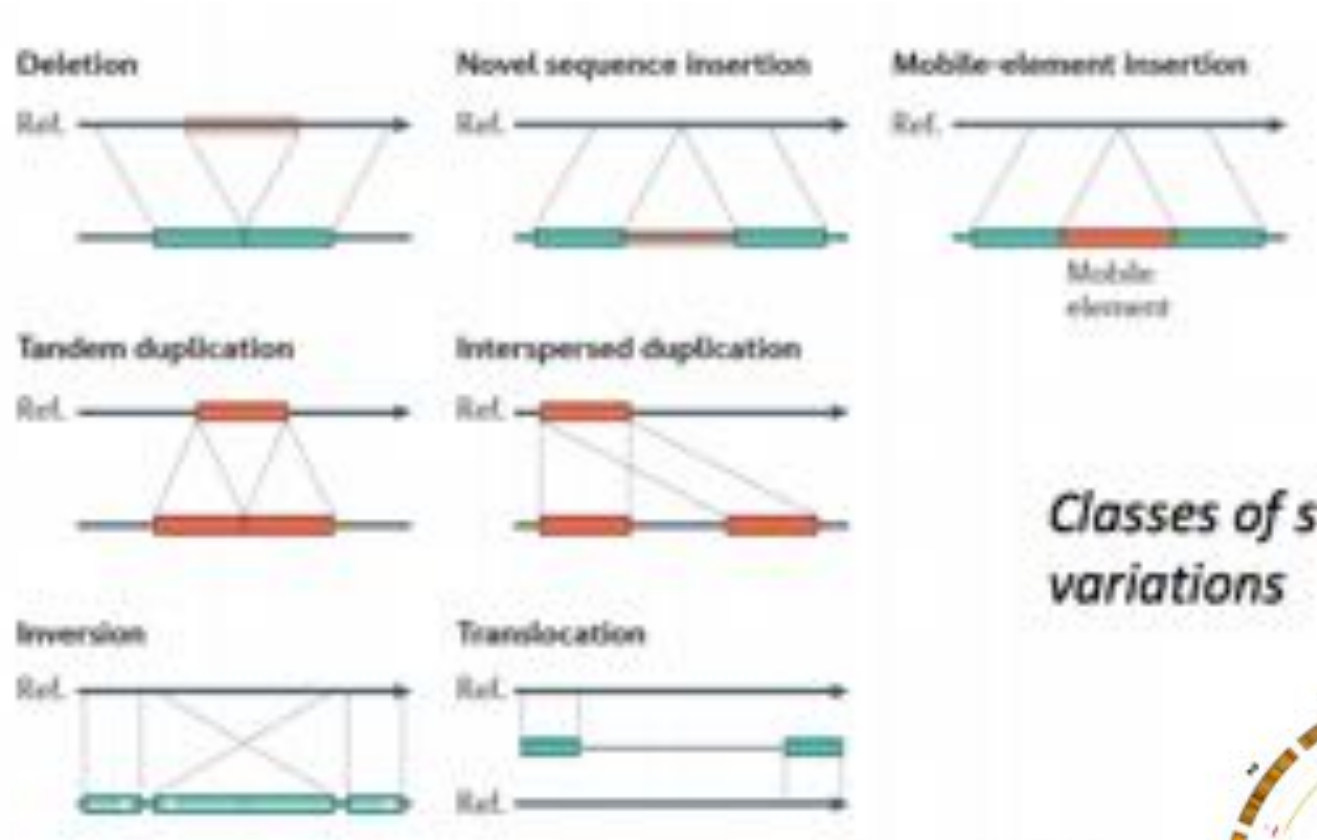
Sequencer quoi?

- Panel de gènes
  - Une série d'exons d'intérêt (gènes de cancer= 100kb)
- Exome
  - Tous les exons du génome (30 Mb)
- Whole genome
  - Le génome complet (3 Gb)

Les mutations somatiques

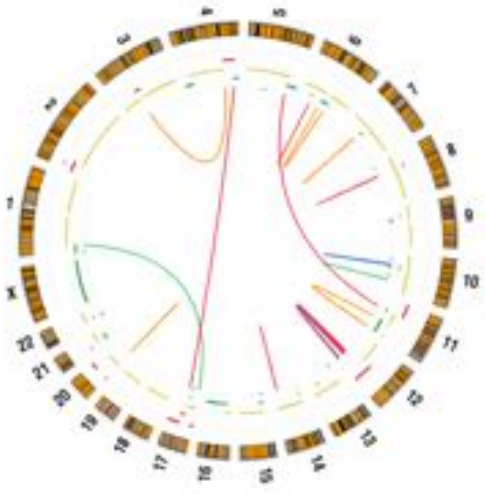


Variants structuraux



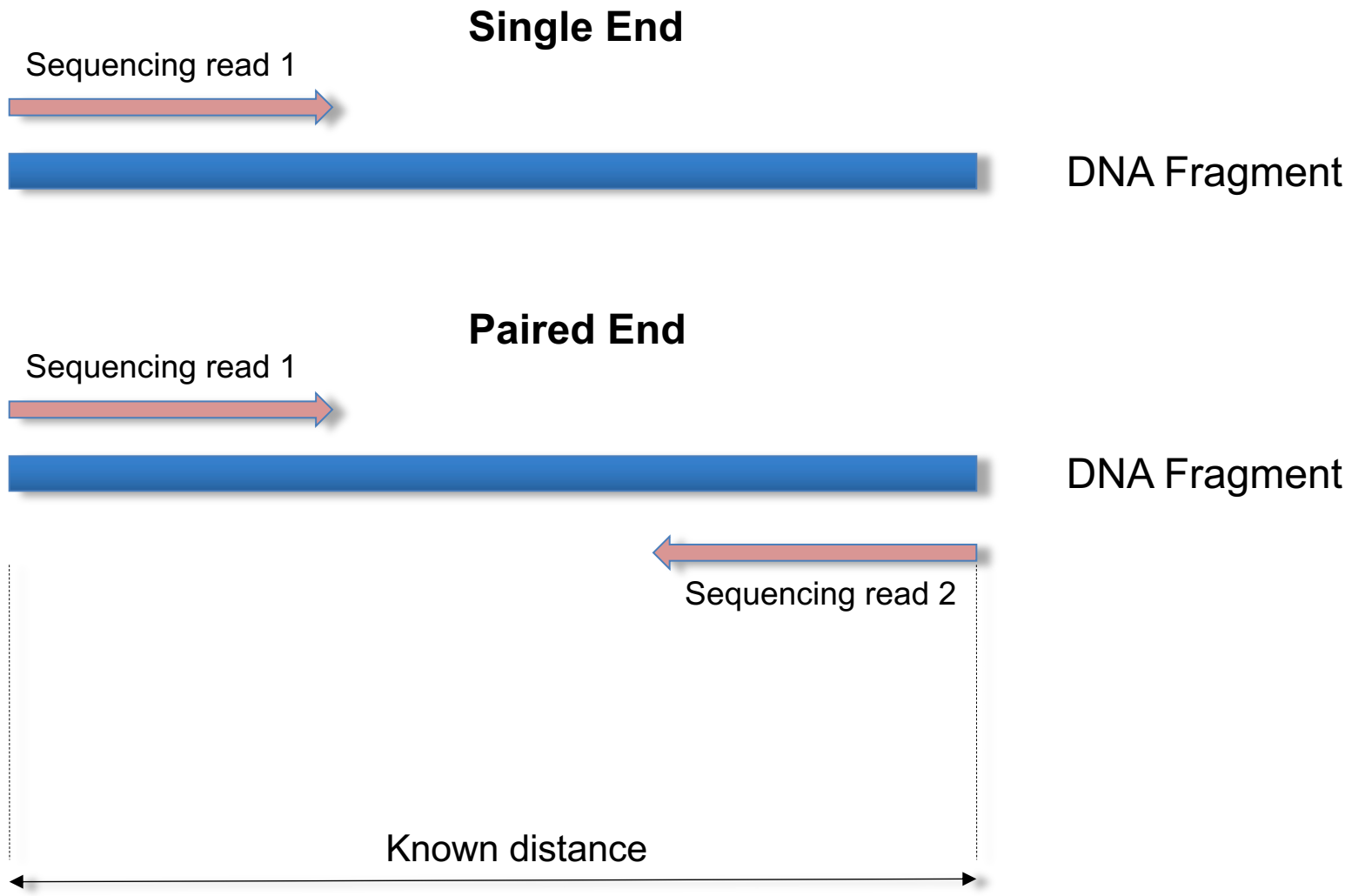
Classes of structural variations

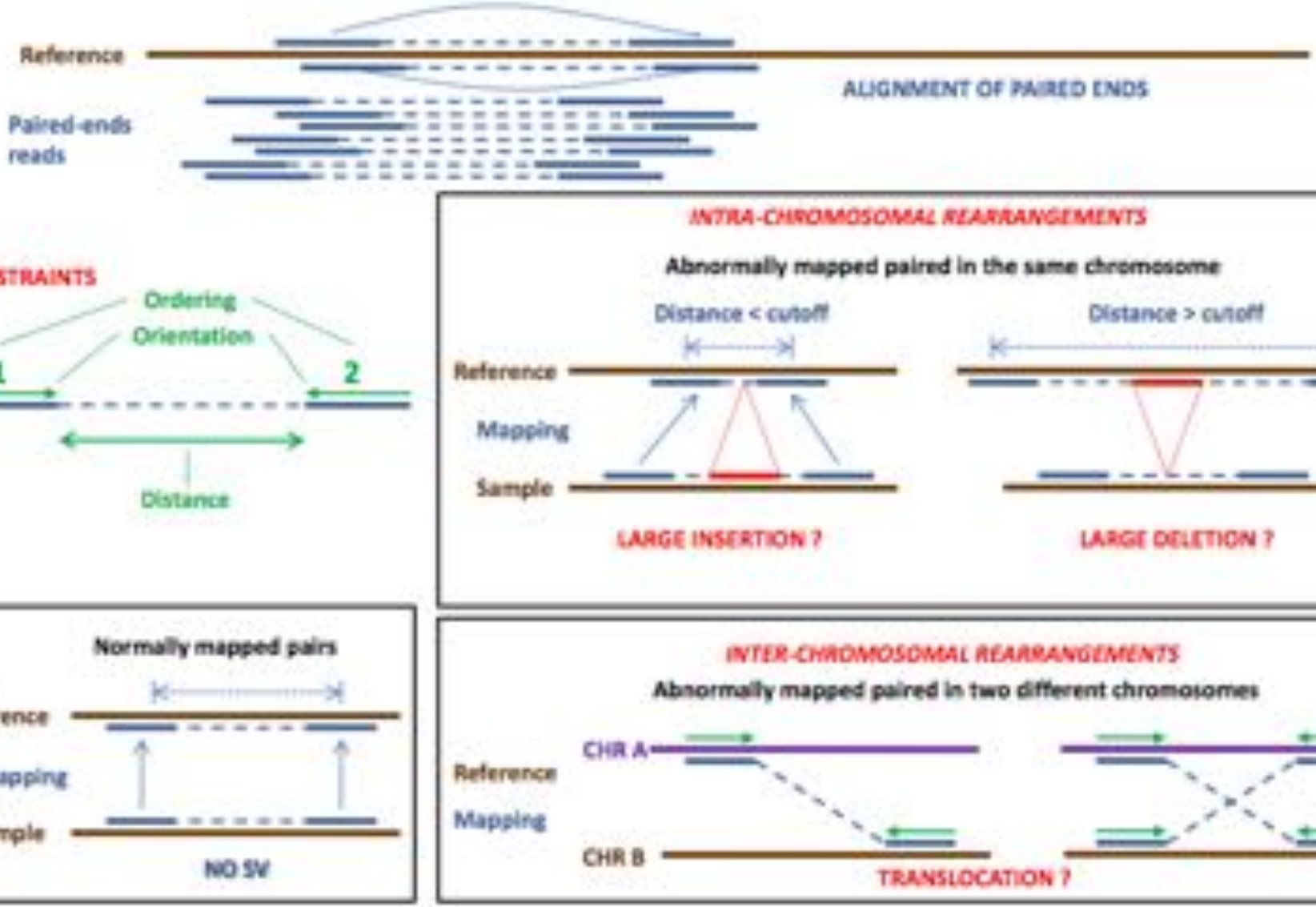
Alkan et al 2012



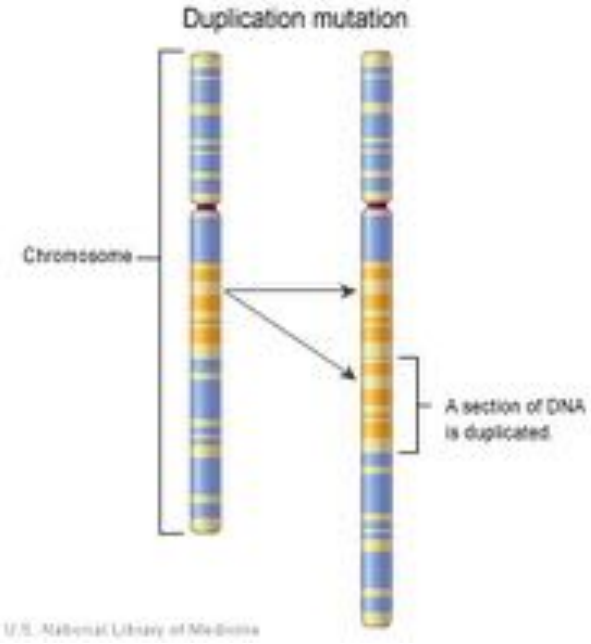
Intérêt du séquençage paired-end:

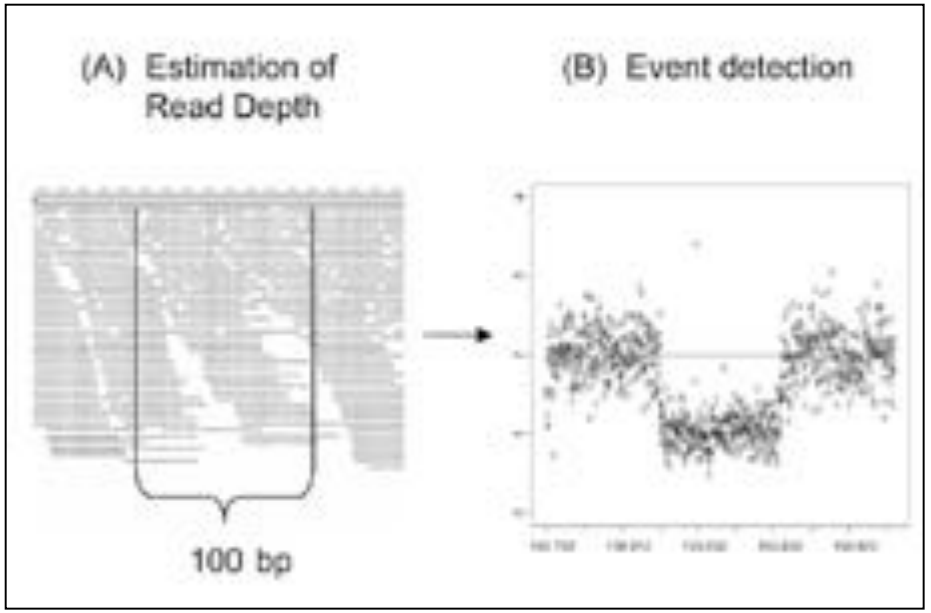
Résolution des repeats et des variants structuraux




Reference
Paired-ends reads
ALIGNMENT OF PAIRED ENDS
CONSTRAINTS
Ordering
Orientation
1
2
Distance
INTRA-CHROMOSOMAL REARRANGEMENTS
Abnormally mapped paired in the same chromosome
Distance < cutoff
Distance > cutoff
Reference
Mapping
Sample
LARGE INSERTION ?
LARGE DELETION ?
Normally mapped pairs
Reference
Mapping
Sample
NO SV
INTER-CHROMOSOMAL REARRANGEMENTS
Abnormally mapped paired in two different chromosomes
CHR A
Reference
Mapping
CHR B
TRANSLOCATION ?

Recherche de CNV (copy number variations)





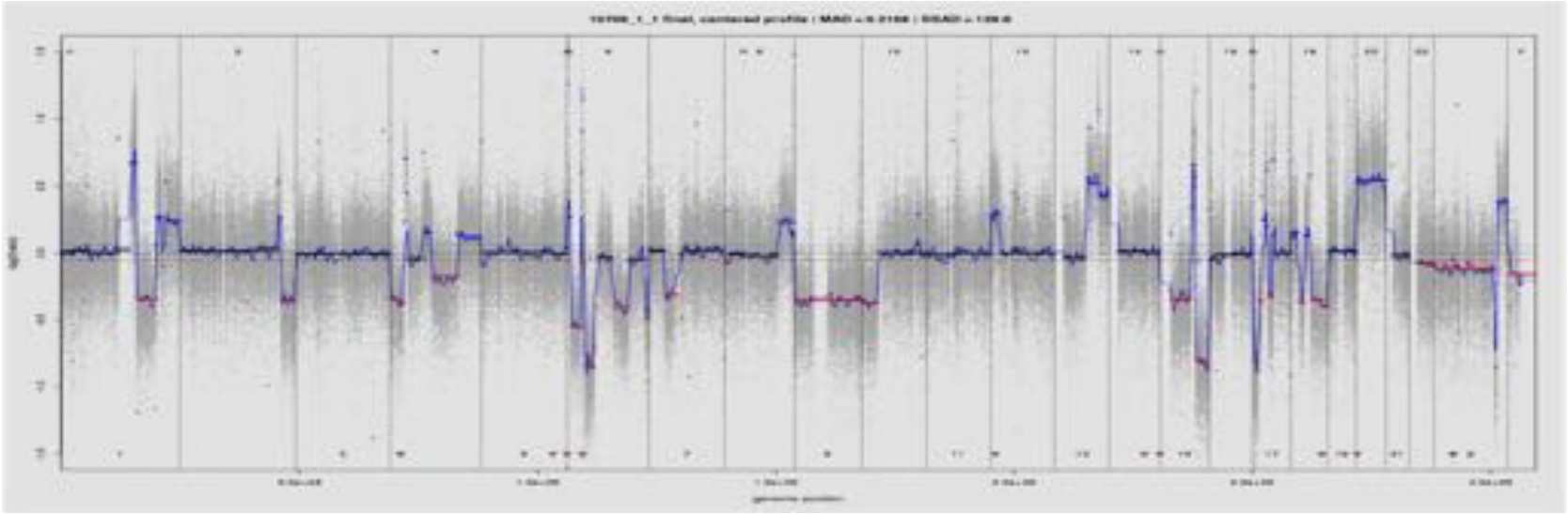
- Attempts to infer variations in copy number from the **local read depth**.
- A strong GC% debiasing is required

Yoon, 2009

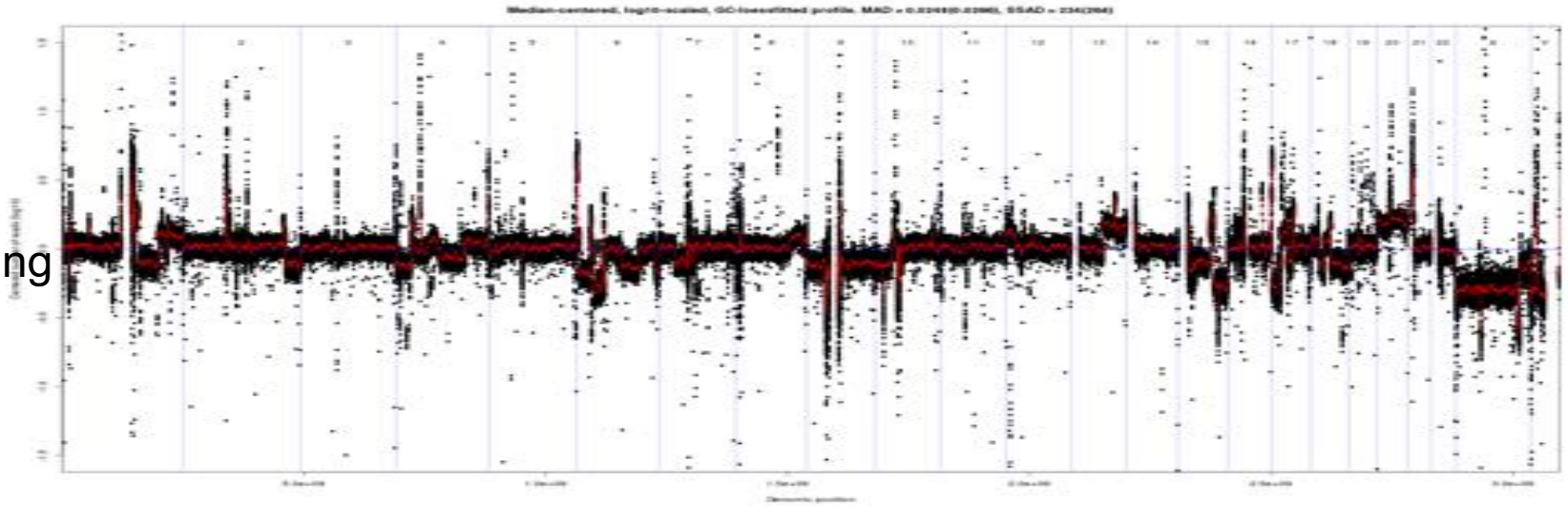
NGS vs CGH

CGH

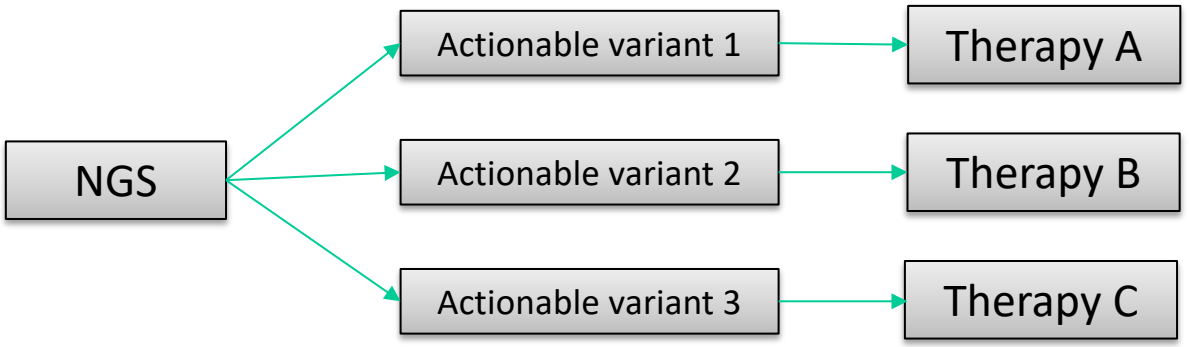
comparative genome hybridization



Sequencing

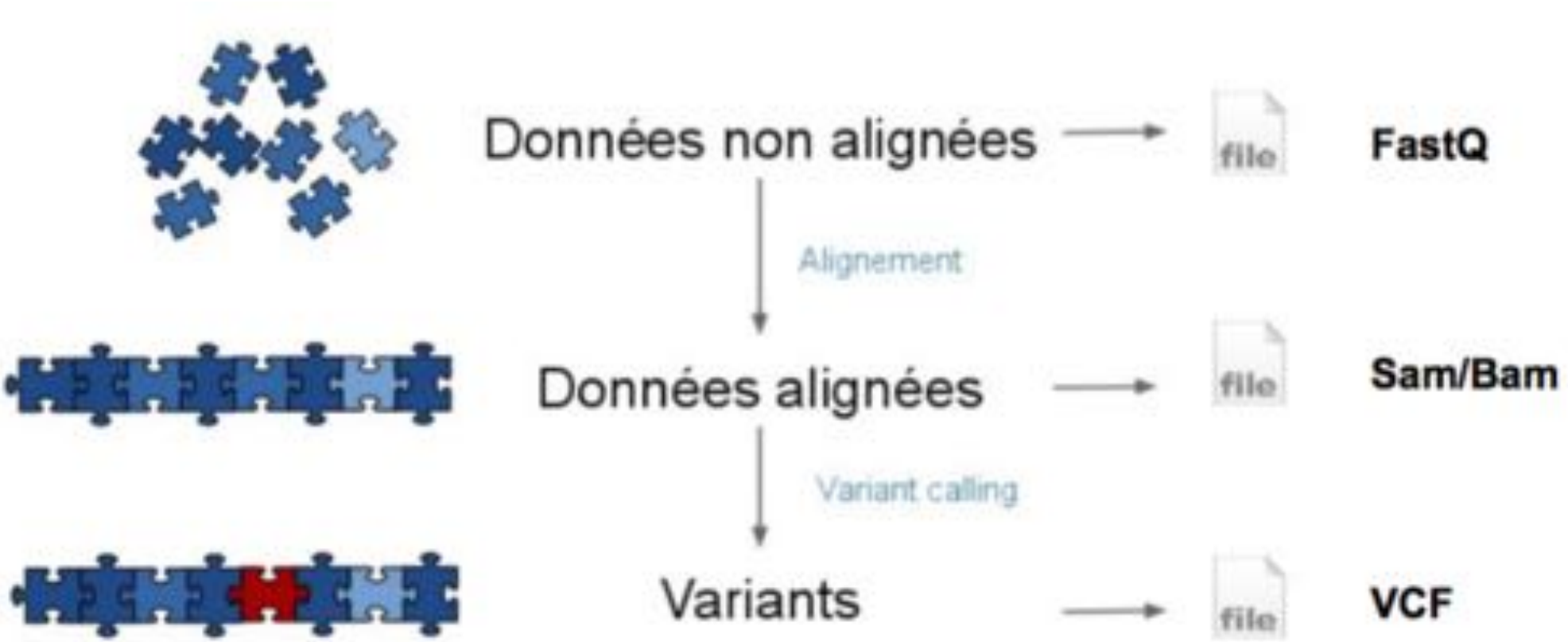


NGS for precision medicine

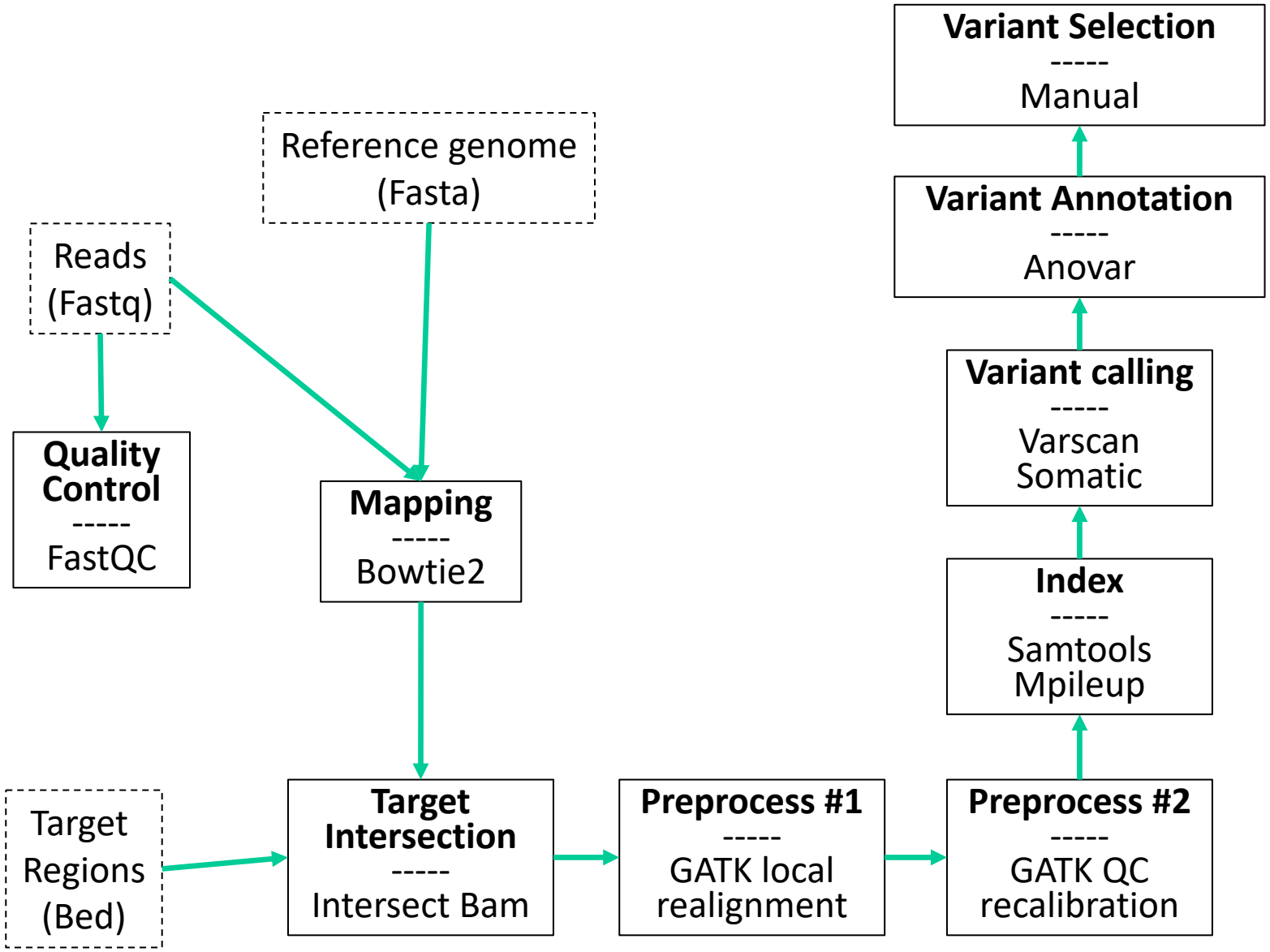


- Clinical trials: MOSCATO (GR), SAFIR (GR), SHIVA (Curie), …
- Ipilimumab (anti-CTLA4), Nivolumab (anti-PD1), Trastuzumab (anti-HER2), Cetuximab (anti-EGFR)

Un pipeline « Variants »

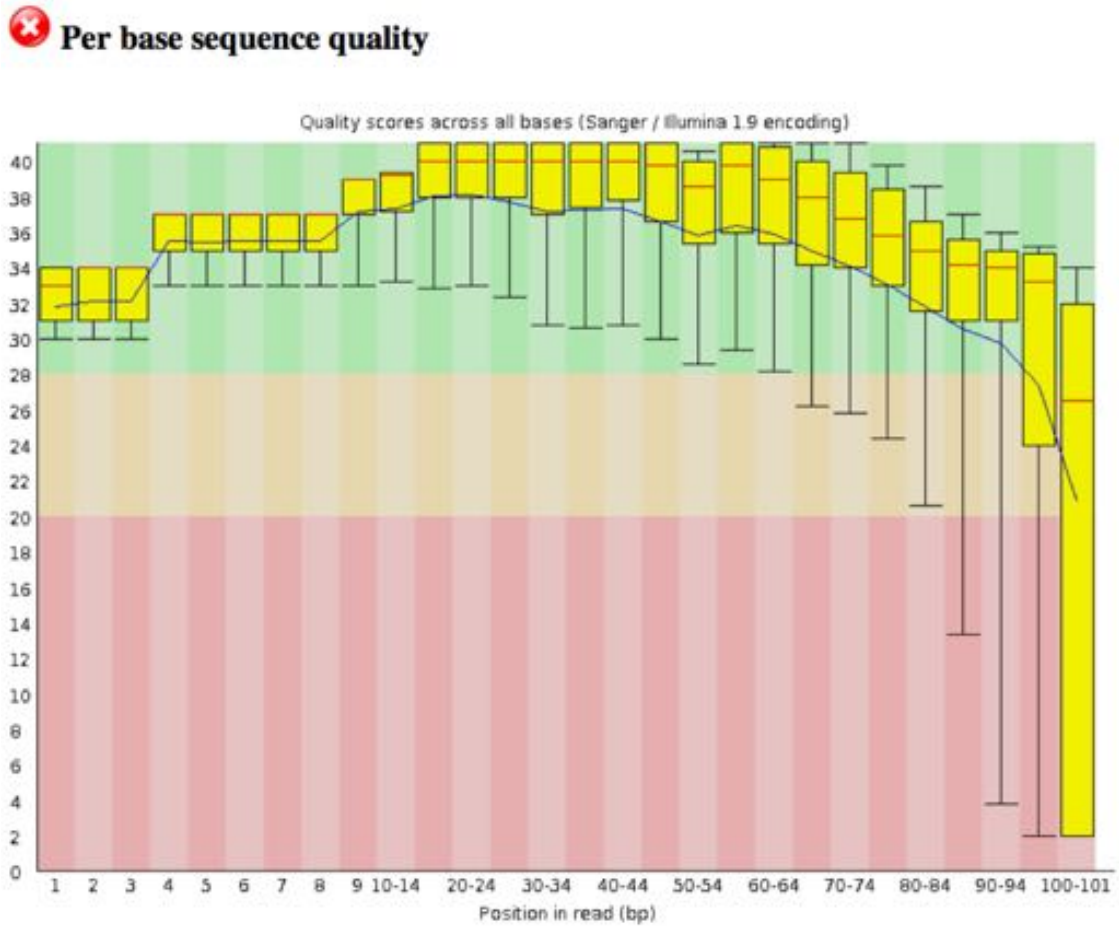


Un vrai pipeline « variants »



FastQC Metrics

- Look at the different metrics for both reads
- **Problem**: the per base sequence quality of the Read2 are quite low towards the end

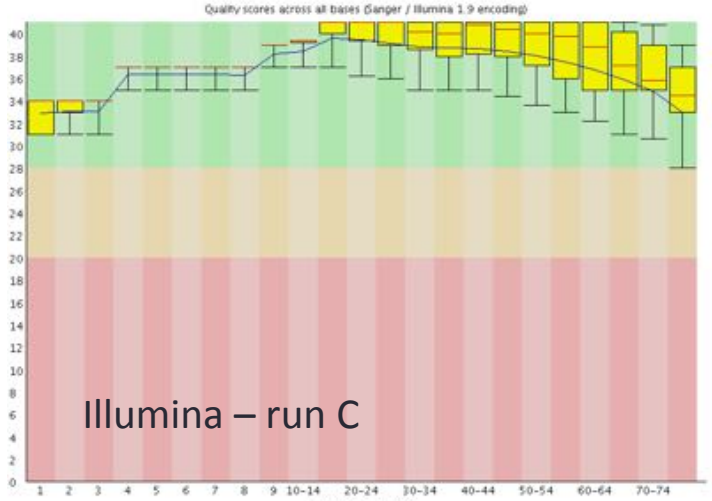


Solution:
Trim the 25bp from
the 3' end
of the reads

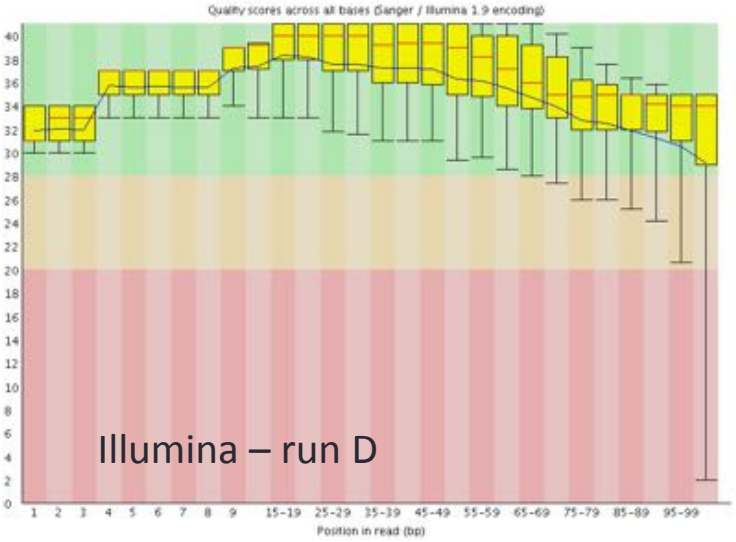
- Higher confidence in the sequenced information

(FastqTrimmer)

Illumina

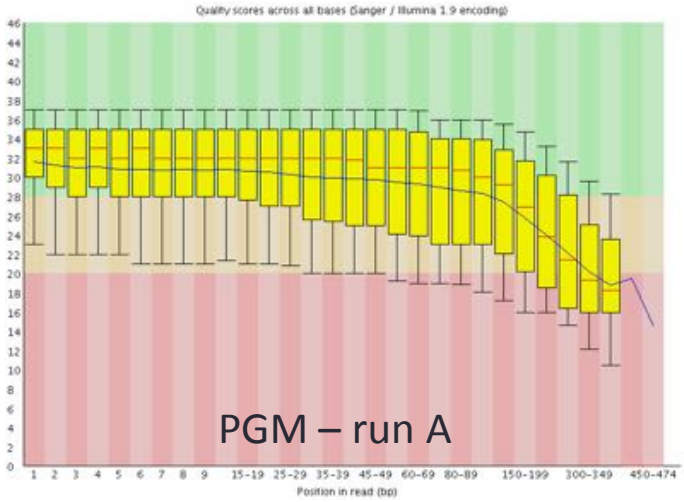

Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Illumina – run C

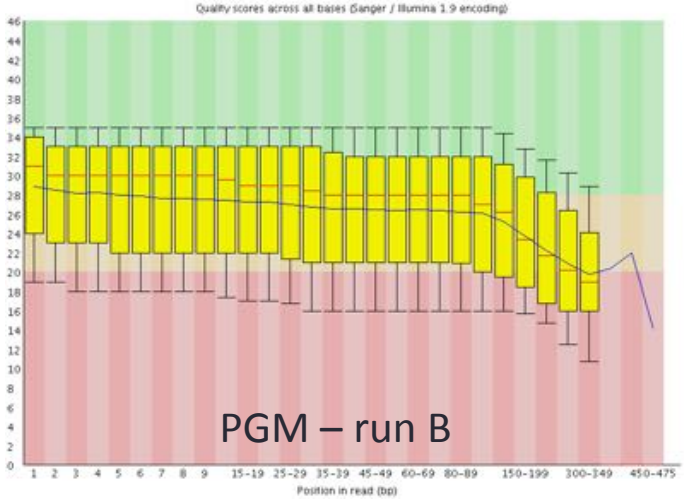

Illumina – run C


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Illumina – run D
Position in read (bp)


Illumina – run D

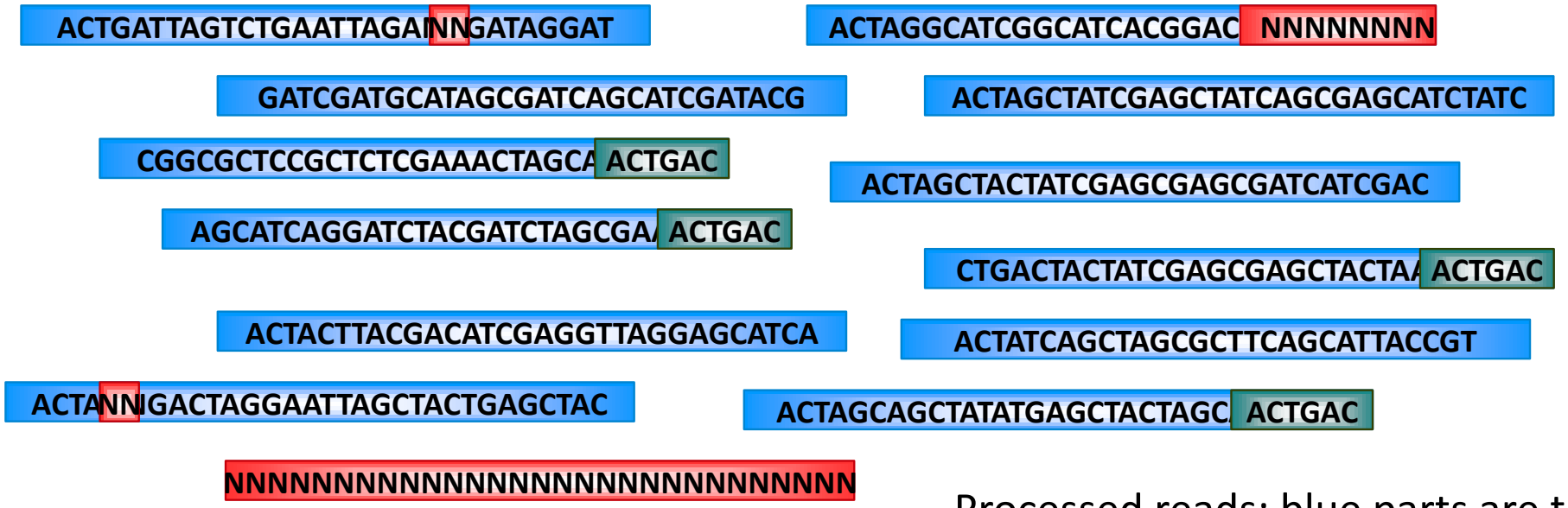
PGM (Ion Torrent)



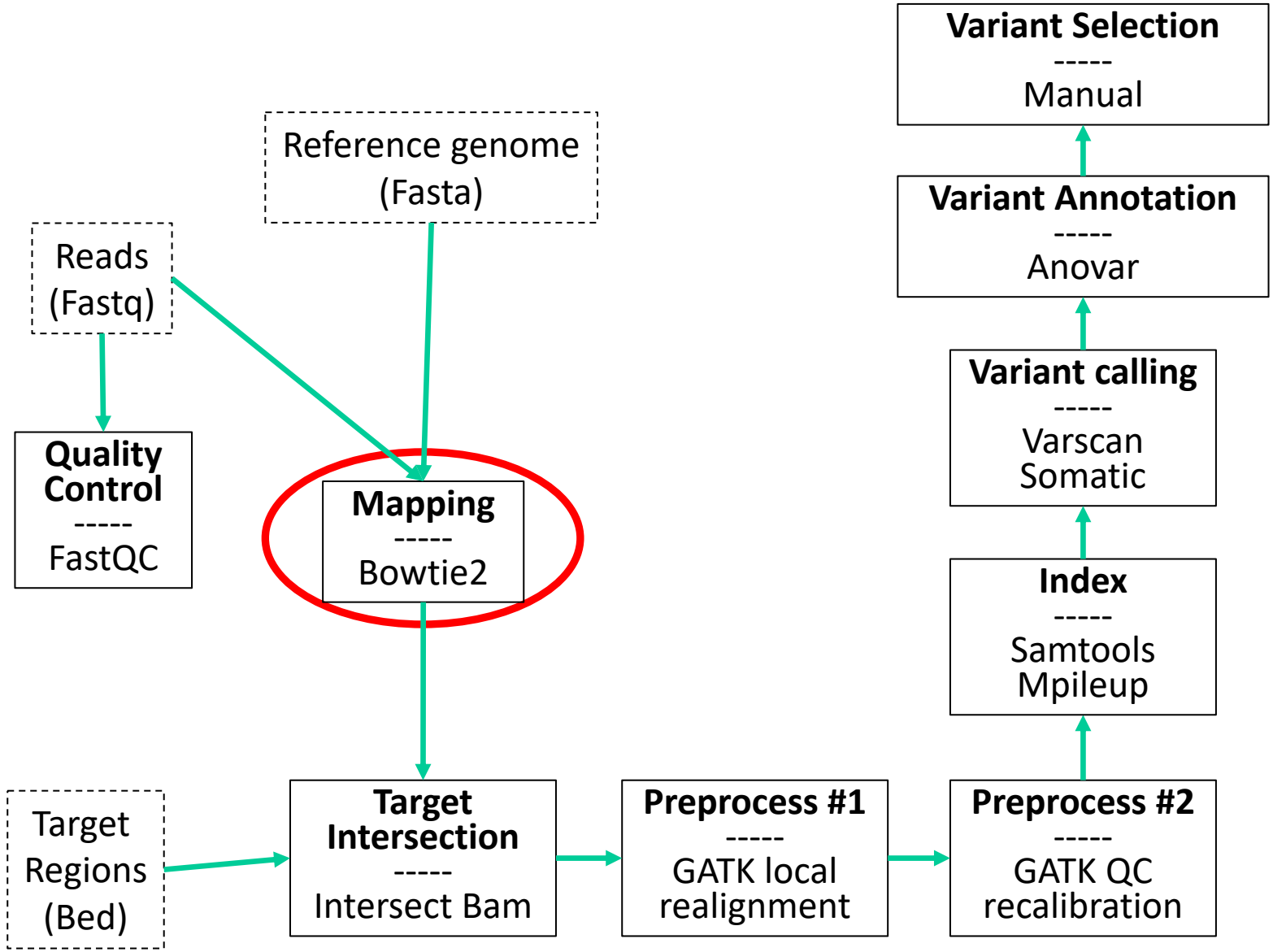


Trimming and discarding low quality reads…

A first Quality Control of raw reads is mandatory and can be established according to the application ('N', adapter sequences, barcode, contamination, etc.)



Processed reads: blue parts are to be kept, green and red parts to be removed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Reference genome
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Alignement des reads sur le génome de référence

Most popular aligners for variant analysis

(support mismatched, gapped, paired-end alignment)

- BWA
  - Li H. and Durbin R. (2009)
- Bowtie2
  - Langmead B, Salzberg S (2012)

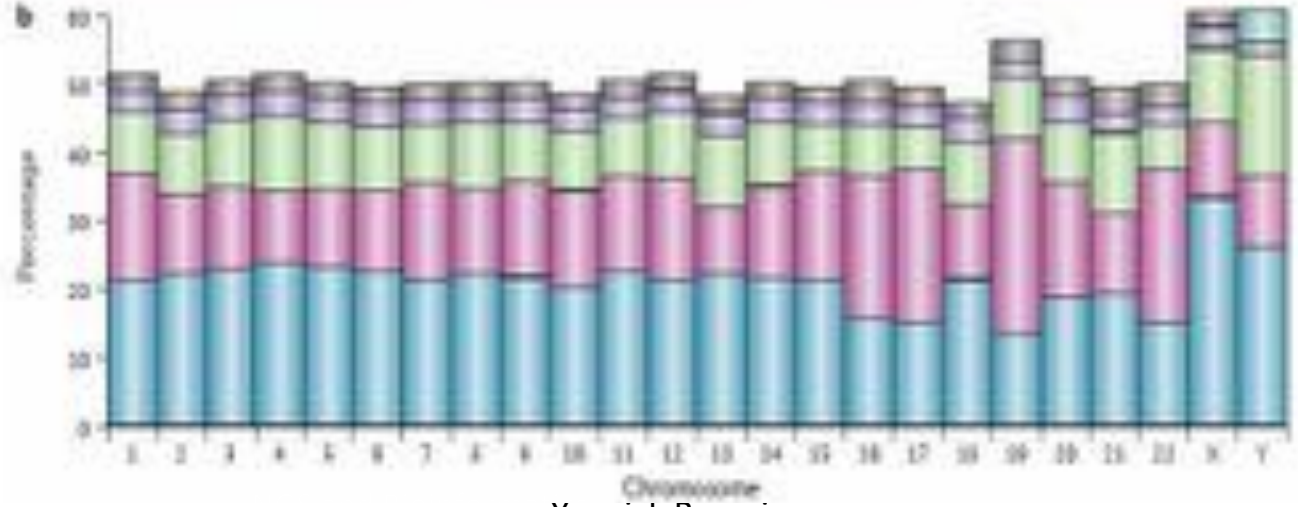
Alignment key parameters - Repeats

Approximately **50%** of the human genome is comprised of repeats

a

Repeat class	Repeat type	Number (bp/%)	Cvg	Length (bp)
Minisatellite, microsatellite or satellite	Tandem	420,918	3%	2–100
LINE	Interspersed	1,797,373	21%	100–300
DNA transposon	Interspersed	453,778	3%	200–2,000
LTR retrotransposon	Interspersed	718,125	8%	200–5,000
LINE	Interspersed	1,508,345	21%	500–8,000
rDNA (18S, 28S, 5.8S and 28S)	Tandem	608	0.01%	1,000–43,000
Segmental duplications and other classes	Tandem or interspersed	2,210	0.23%	1,000–100,000

b



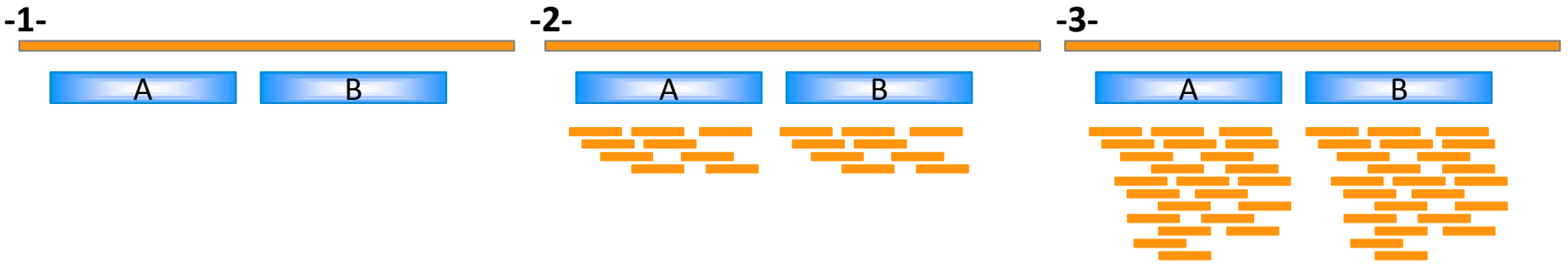
Treangen T.J. and Salzberg S.L. 2012. Nature review Genetics 13, 36-46

Alignment key parameters – Repeats – 3 strategies

-1- Report only unique alignment

-2- Report best alignments and randomly assign reads across equaly good loci

-3- Report all (best) alignments



Treangen T.J. and Salzberg S.L. 2012. Nature review Genetics 13, 36-46

Alignment key parameters – Using single or paired-end reads ?

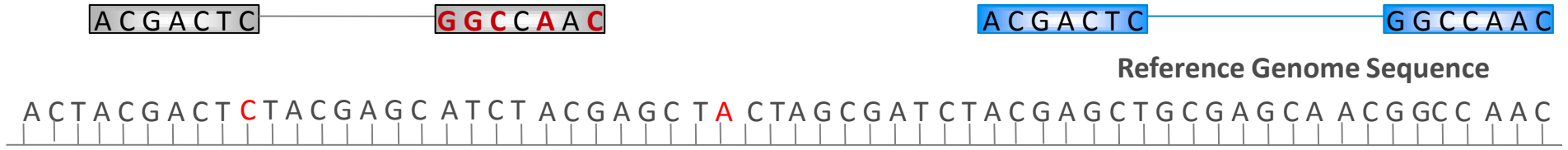
The type of sequencing (i.e. single or paired-end reads) is often driven by the application.

Exemple : Finding large indels, genomic rearrangements, ...

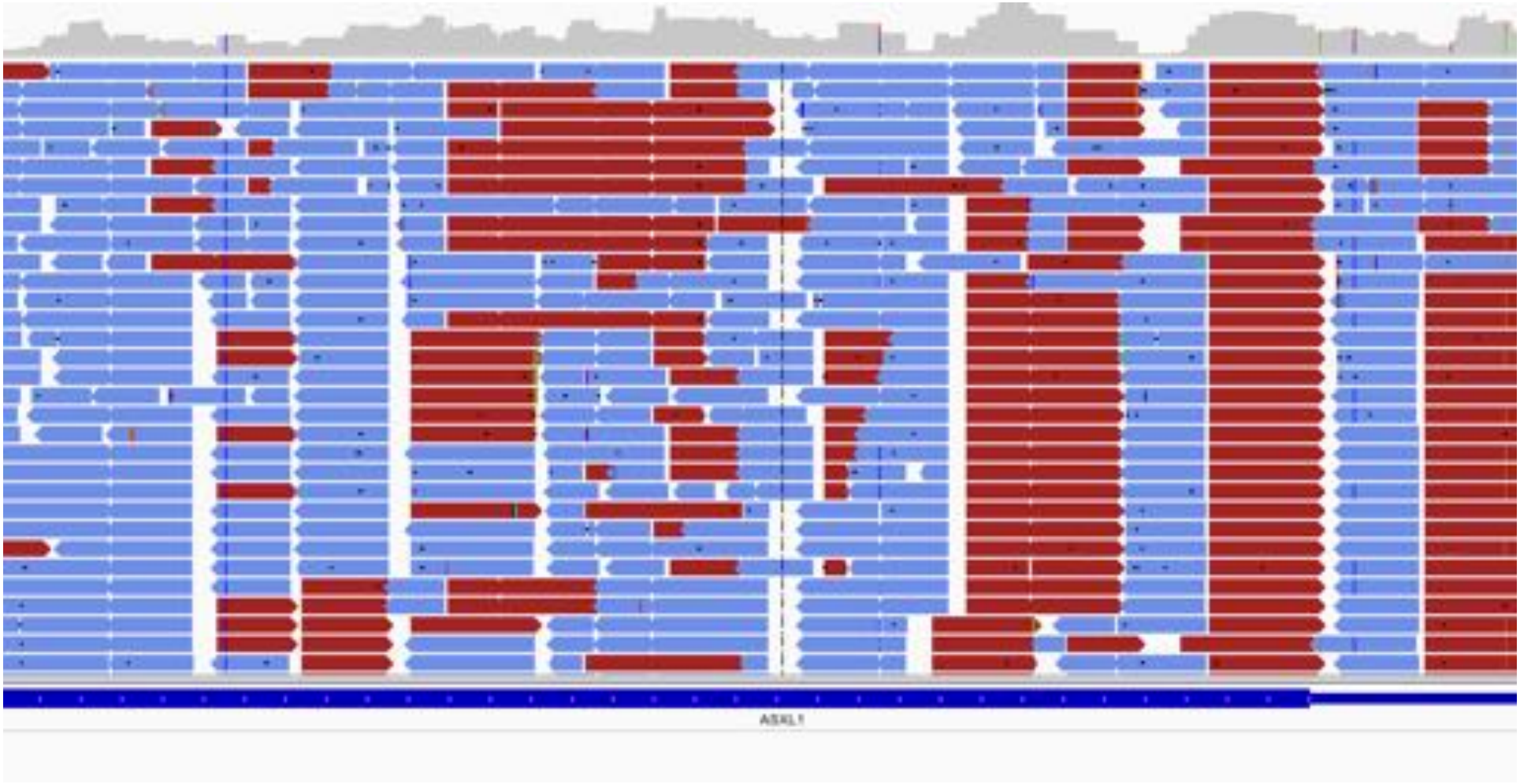
However, in most cases, the pair information can improve mapping specificity

- Single-end alignment – repeated sequence

- Paired-end alignment – unique sequence



Reads alignés: le format BAM/SAM

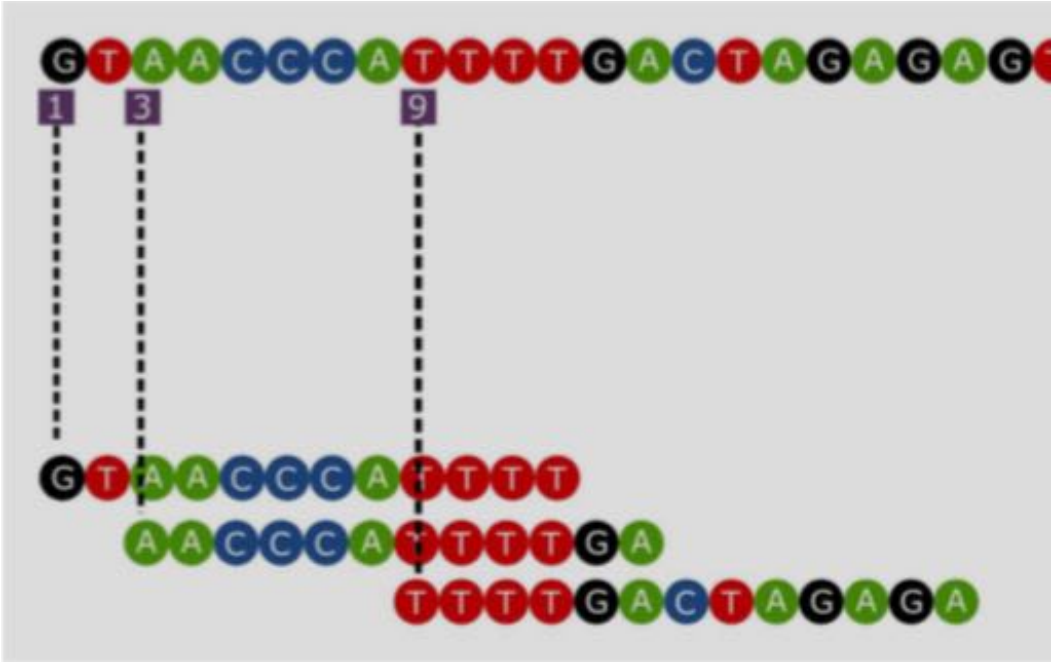


Format SAM

Contient les séquences alignées sur le génome

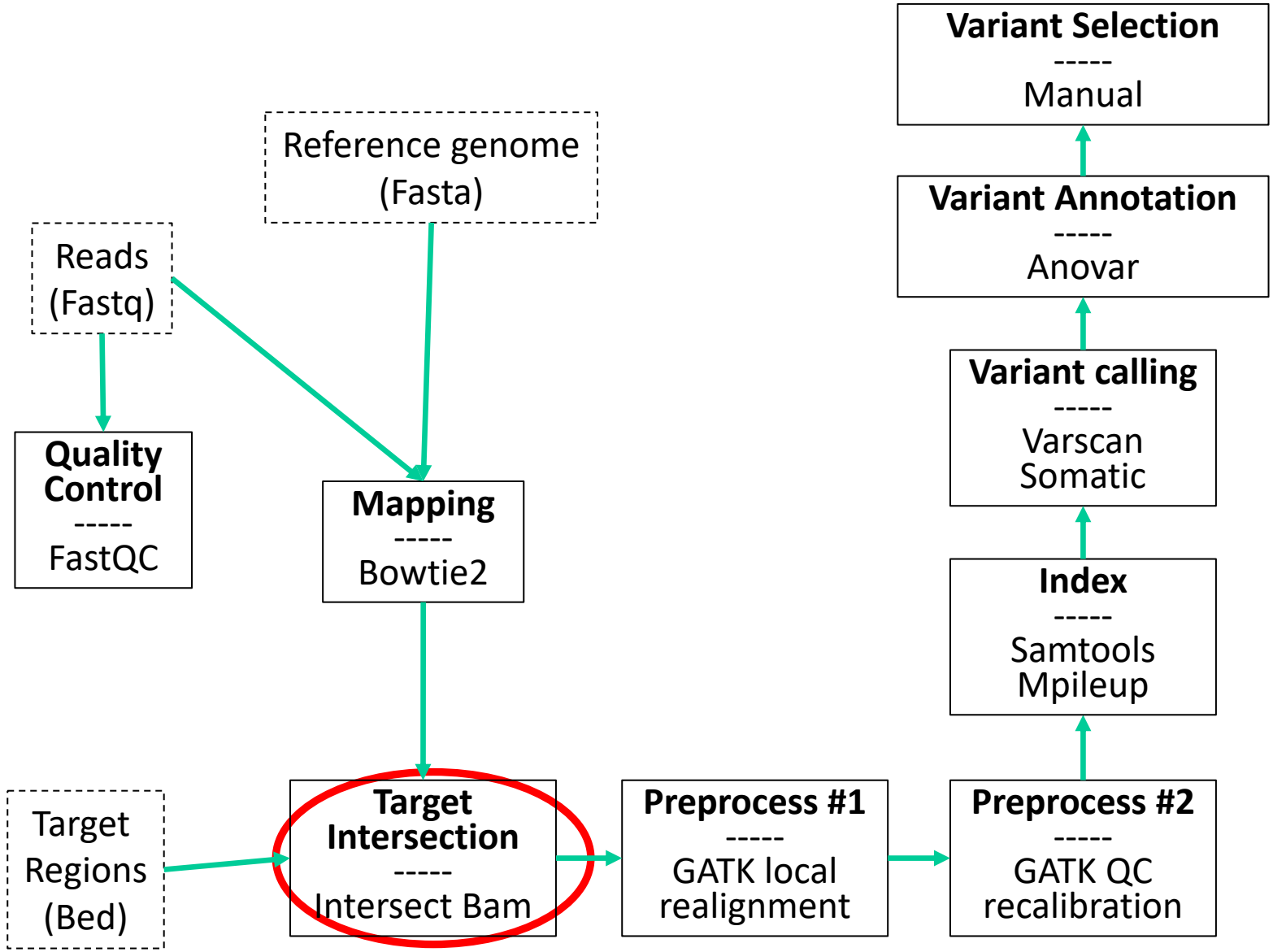
Principe:

```
chr7 1324324 ACGTGCGTTTGCGT
chr8 1424324 GCGTGATGCGTAAG
chr8 1724354 GTATGTTATATGTA
```



Format SAM

```
@HD VN:1.5 SO:coordinate
@SQ SN:ref LN:45
r001    99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002     0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003     0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004     0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001   147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```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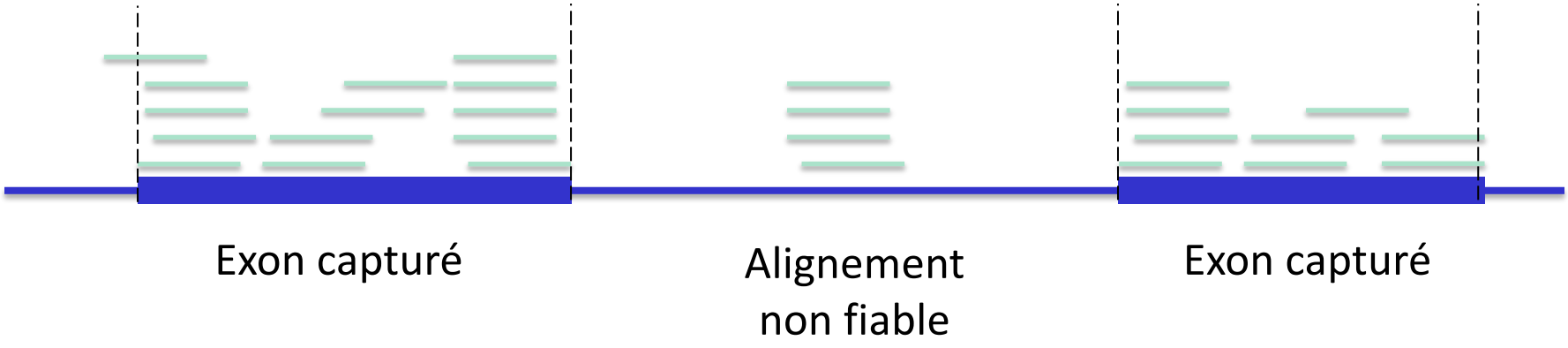
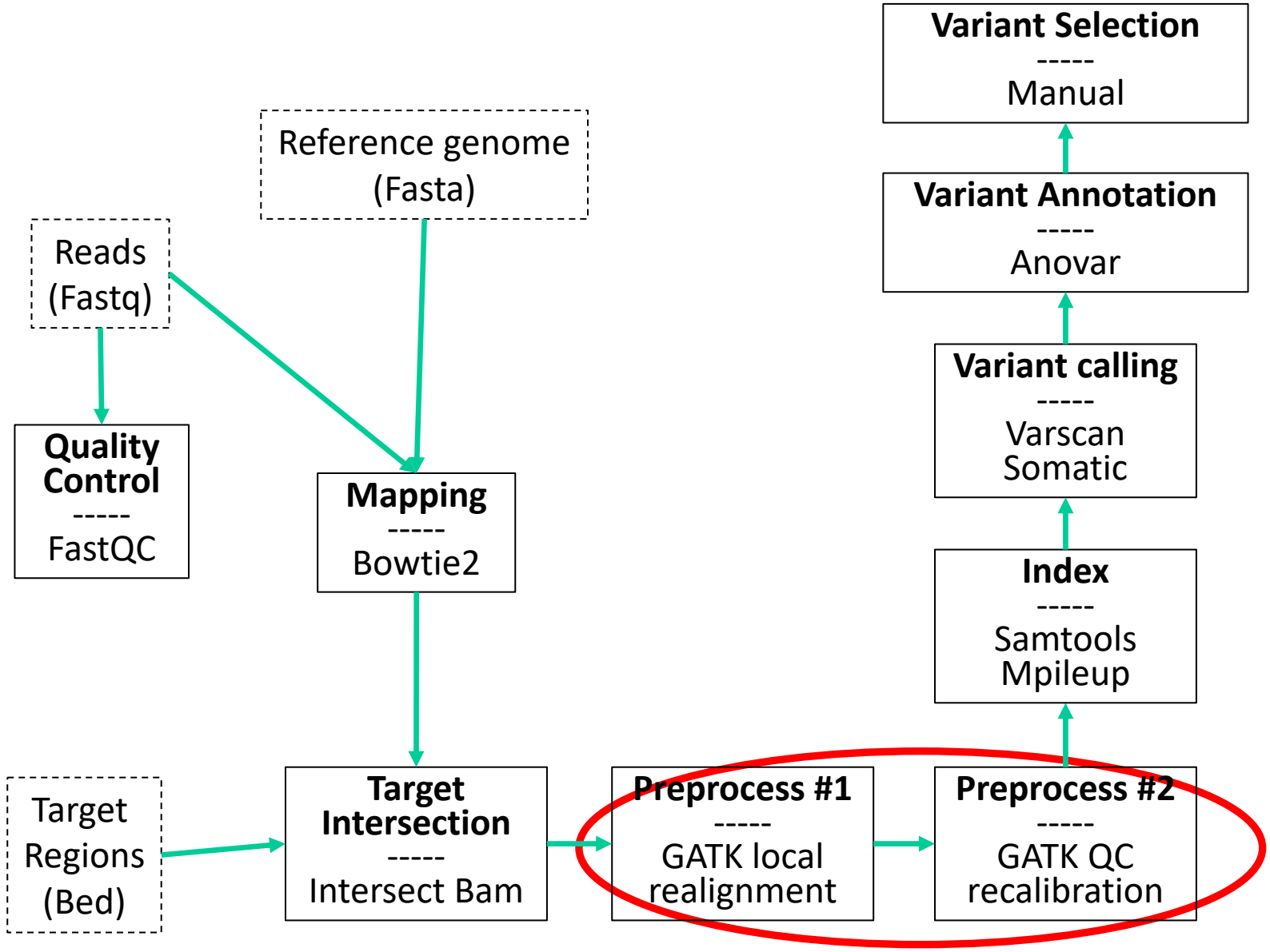
Target intersection

- Comparer l'alignement obtenu à la liste des positions visées par le protocole de capture

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Why realign around indels ?

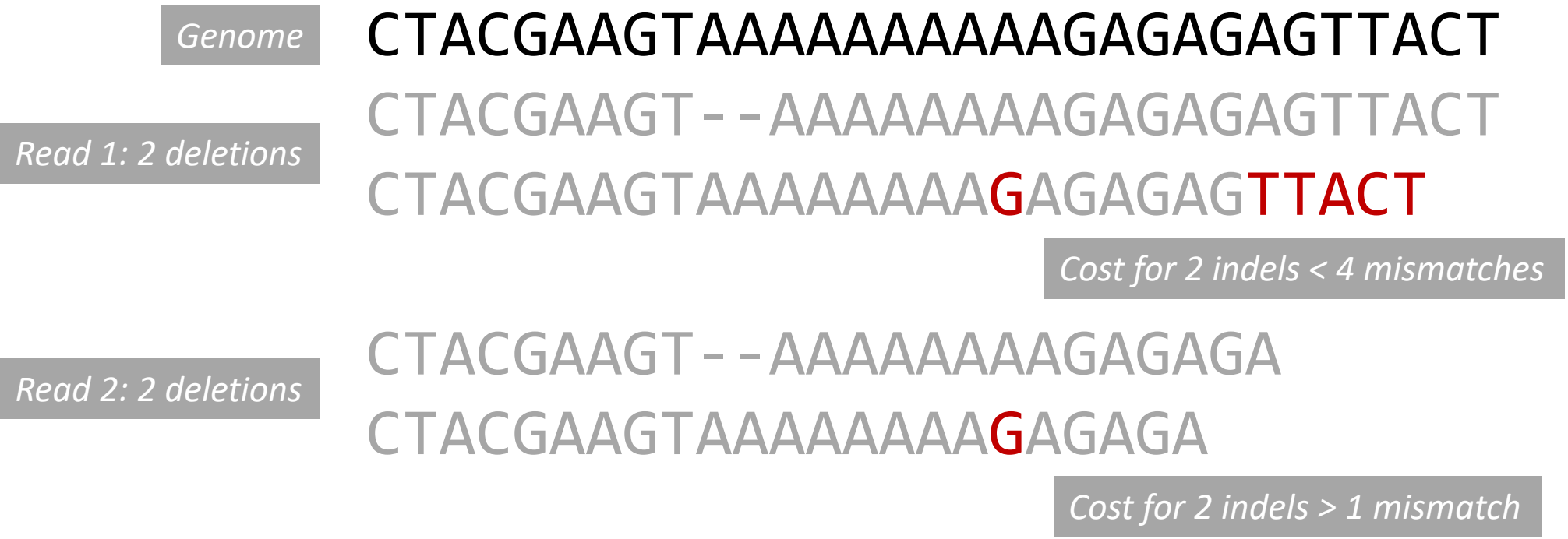
• Small Insertion/deletion (Indels) in reads (especially near the ends) can trick the mappers into wrong alignments

- Alignment scoring – cheaper to introduce multiple Single Nucleotide Variants (SNVs) than an indel: induce a lot of false positive SNVs

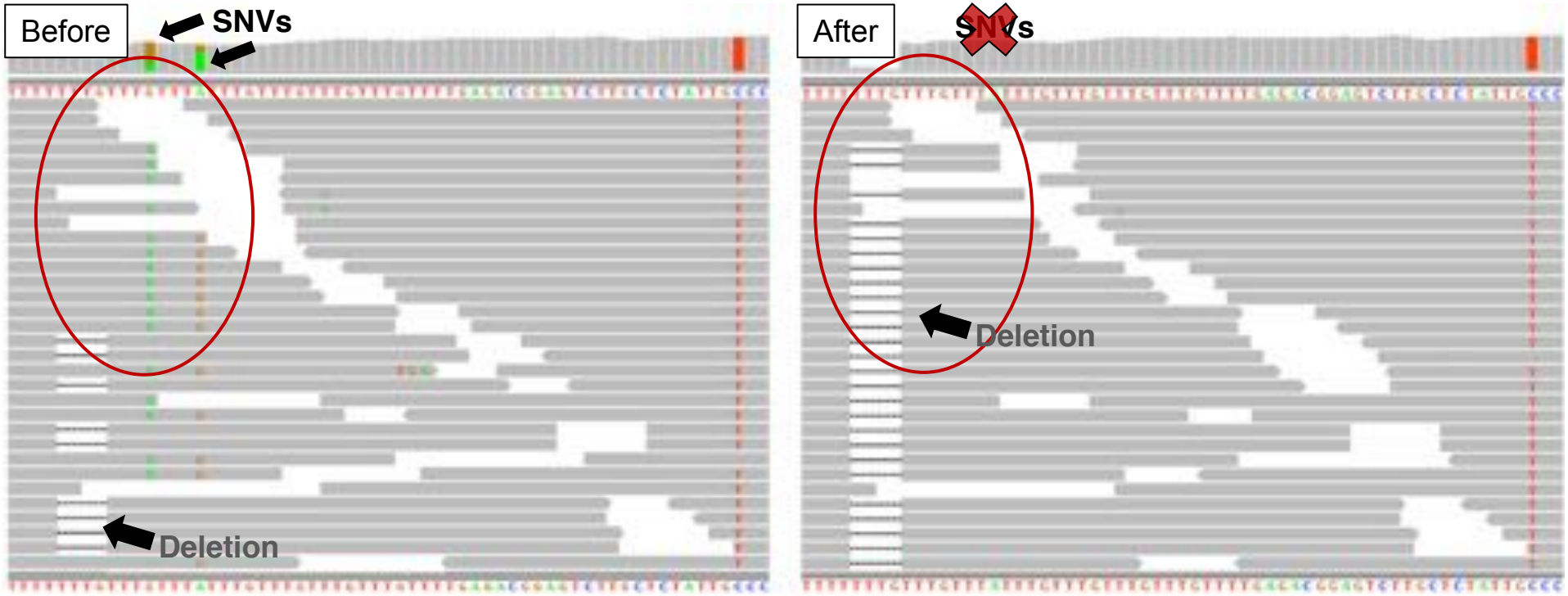
➔ artifactual mismatches

• **Realignment around indels helps improve the downstream processing steps**

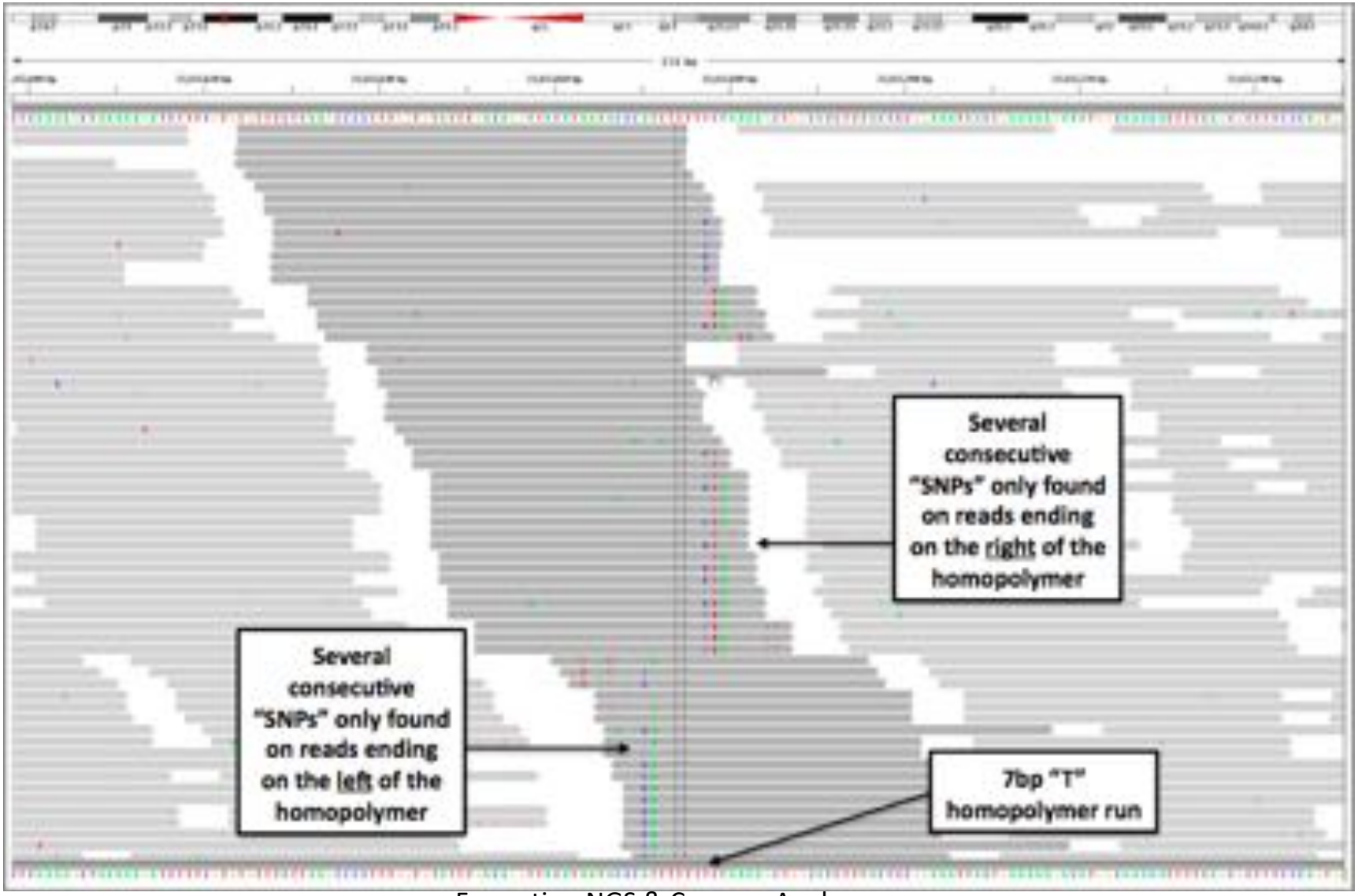
Wrong alignment near indels



Local realignment around indels



Local realignment around indels



Local realignment around indels

Types of realignment targets

1. Indels seen in original alignments (in CIGAR, indicated by I for Insertion or D for Deletion)

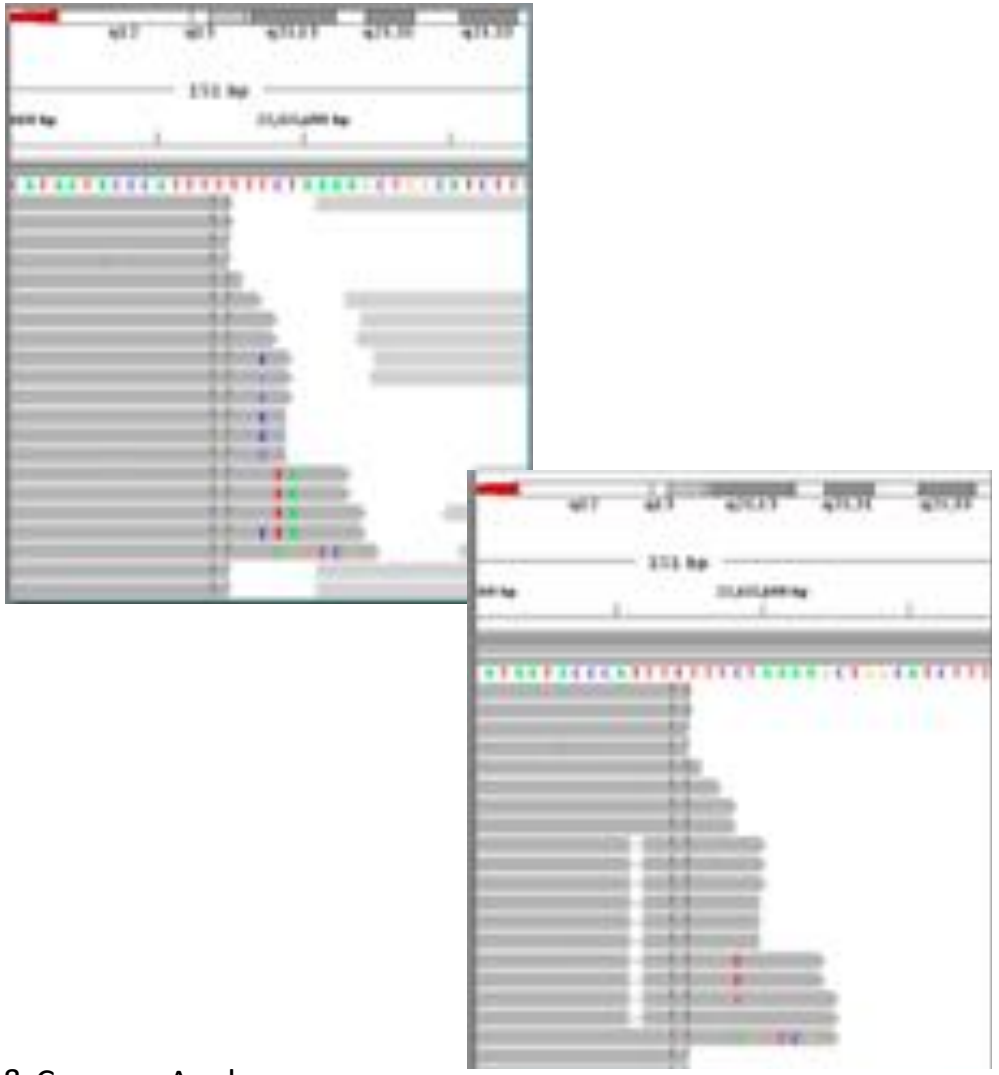
2. Sites where evidences suggest a hidden indel (SNV abundance)

3. Known sites:
   - Common polymorphisms: dbSNP, 1000Genomes

Indel realignment in 2 steps

1. Identify what regions need to be realigned
   - RealignerTargetCreator + known sites

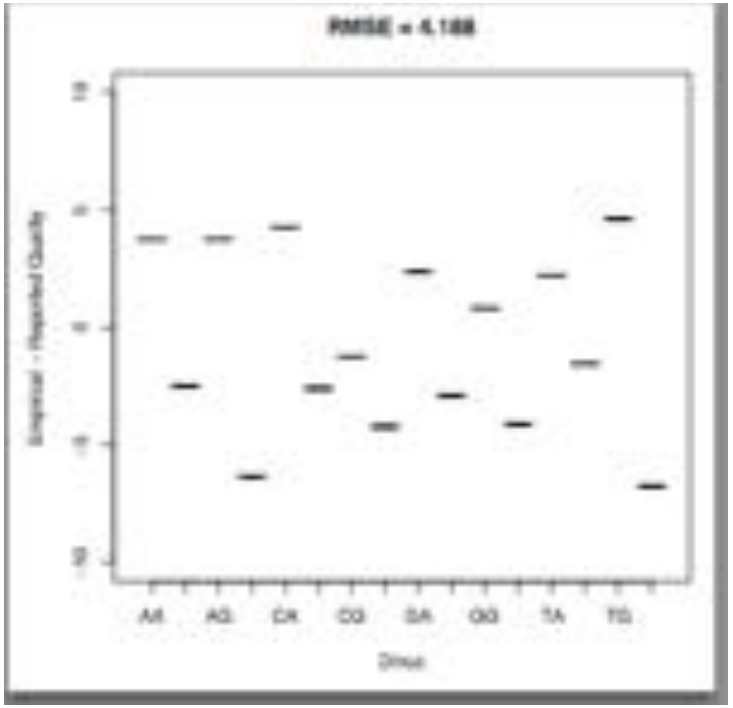
Intervals

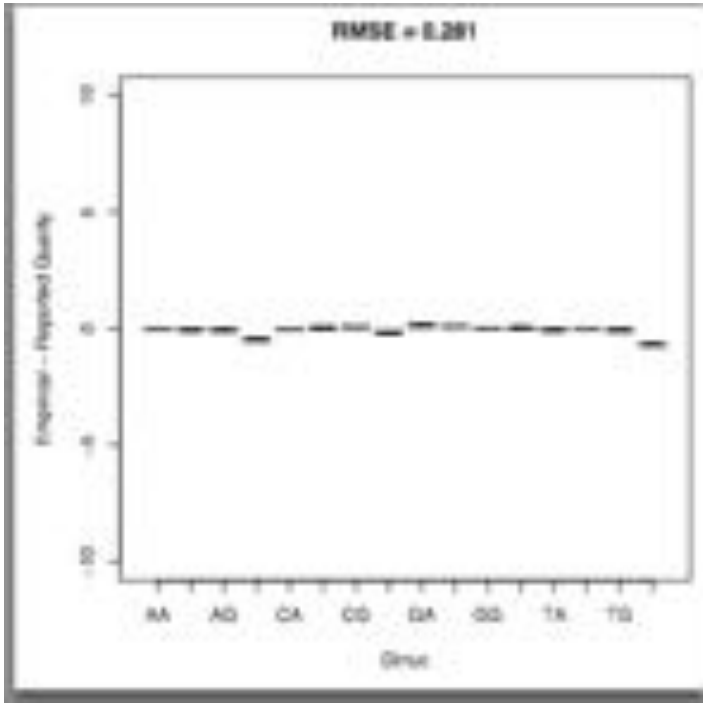


2. Perform the actual realignment (BAM output)
   - IndelRealigner

The quality scores issued by sequencers are biased

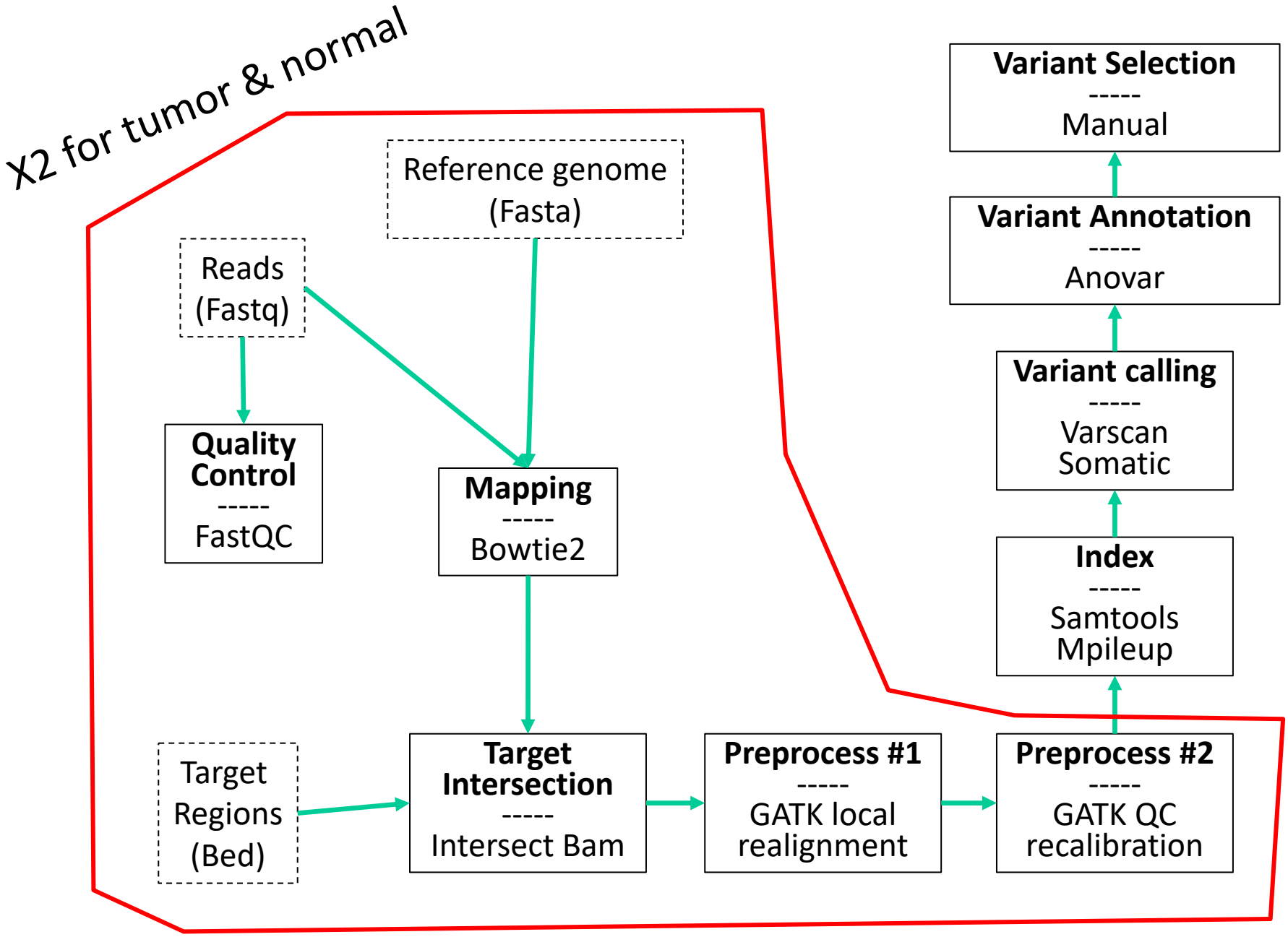
- Quality scores are critical for all downstream analysis
- Systematic biases are a major contributor to bad calls
- Example of sequence context bias in the reported qualities:





before

after


X2 for tumor & normal
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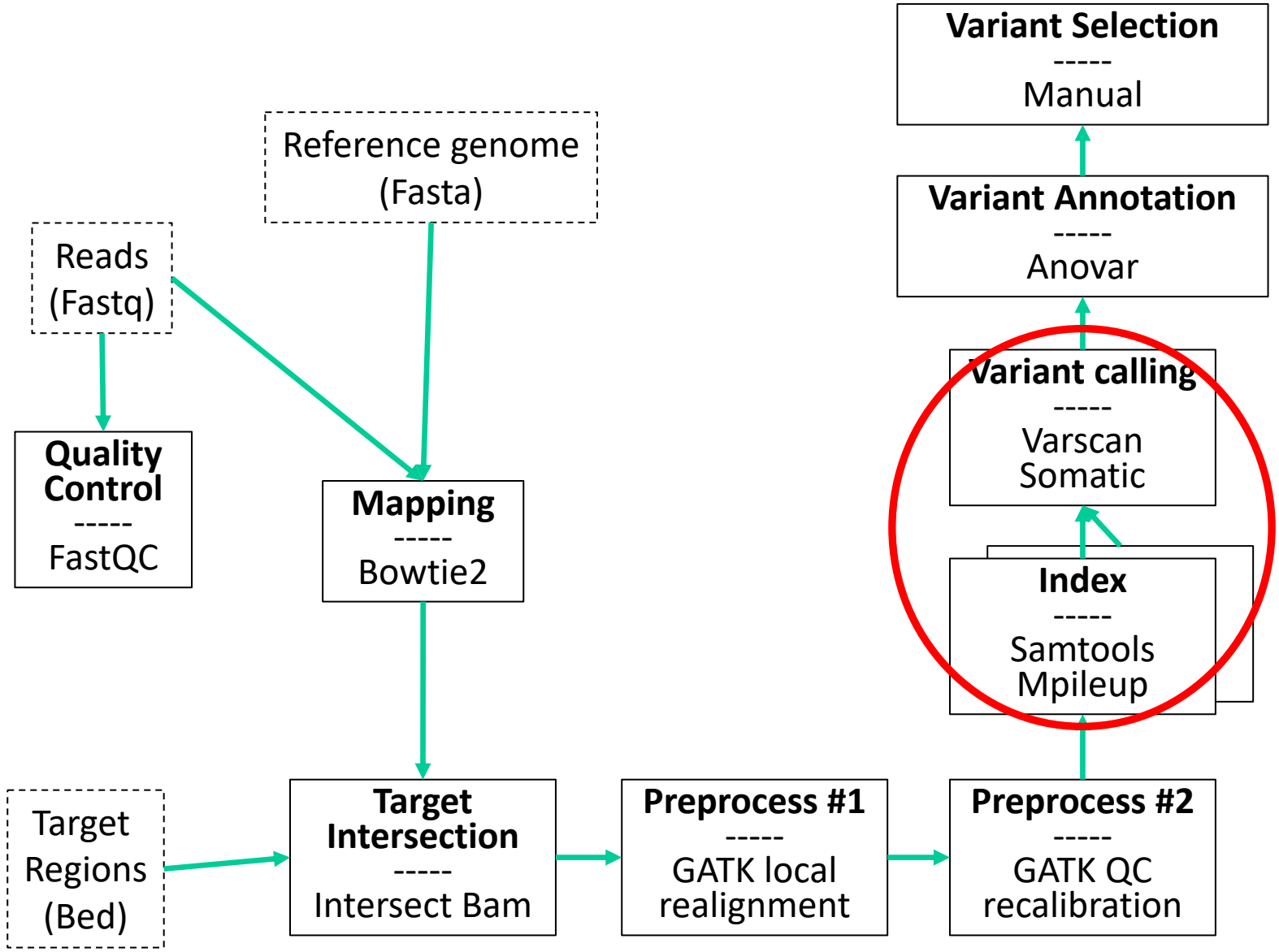
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(Bed)
Target
Intersection

Intersect Bam
Preprocess #1

GATK local
realignment
Preprocess #2

GATK QC
recalibration

Pileup format

Pileup format

Describes base-pair information at each position

Reference base

Base qualities

chr12	112888238	A	108
chr12	112888239	C	108

.$,$.,,.,,..,,.,.,..,.,.,,.,.,,.,..,,.,.,.,.,.,,.,.,,,,.,
,.,.,,,.,.,.,.,.,,.,.,.,.,.,,,,.,.,.,..,,,,,.,.,,,,^F,

=4 =??????@??@?@@ @=@ ??@ ? @? ?
??< ? ??@??????? ? @??? ? ??@??
A???@@ ?@@???AB????= ? @
@@??@@?@ A 00

.$t,.,,.T,tT,.,T.,.,t.tTtt.tTT,t.T,tTt.tT,T,,.t
TtTttt.,.,Tt.ttt.,T,.,.tT,,T,T,.tT,,t,TttTtT,T.
,tt,,T,T,tttt^F.^F,

@6???<?=6 66= ? ???? 6=7??=???<8@7=7=?
936 78??6??6 45<875? ??? ?@6
77??7?8 ??78??????? 8 <8??88 9?8 ?0048

Number of reads covering the site (total depth)

Read bases:

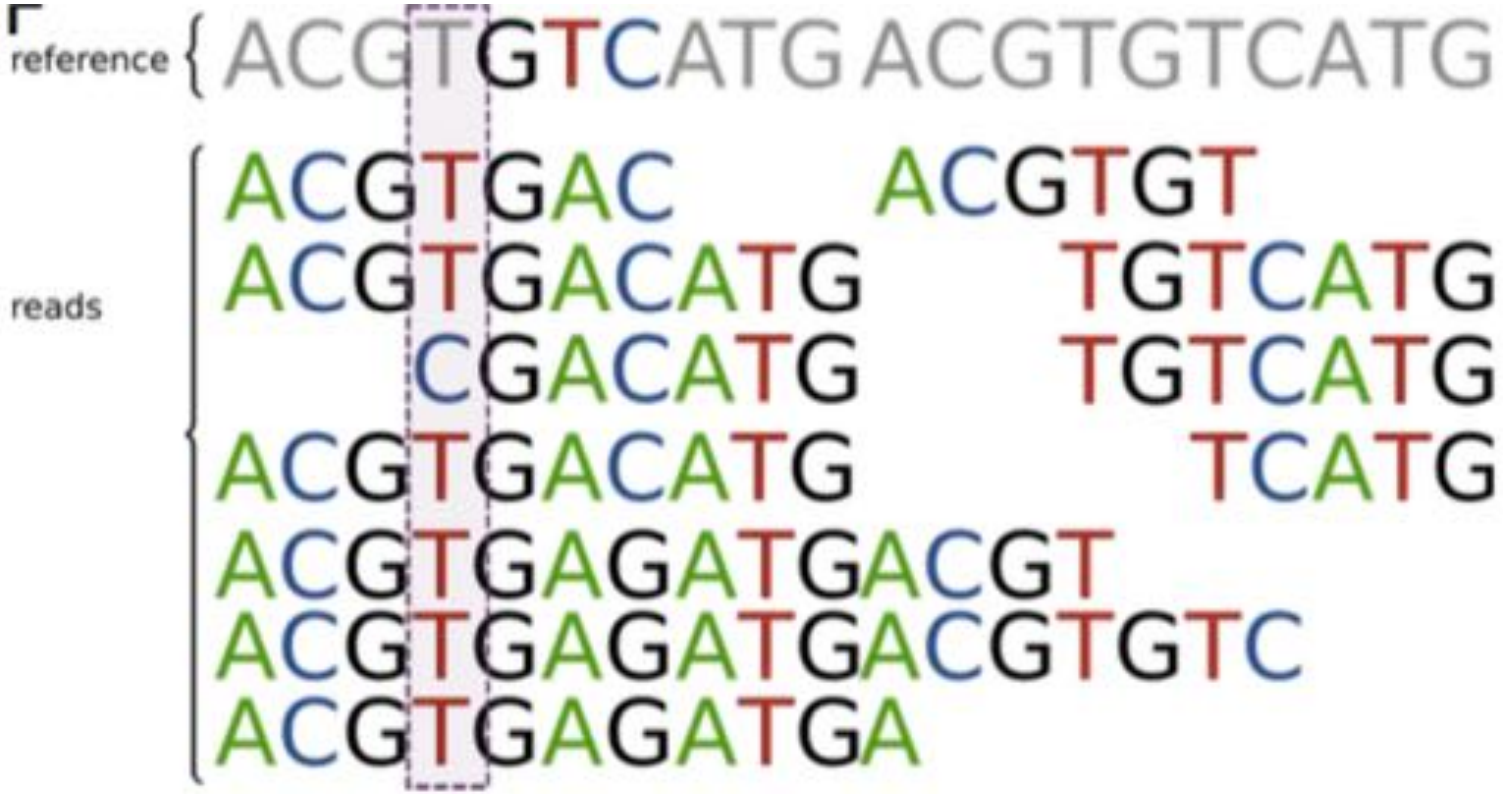
. / , = match on forward/reverse strand

ACGTN / acgtn = mismatch on forward/reverse strand

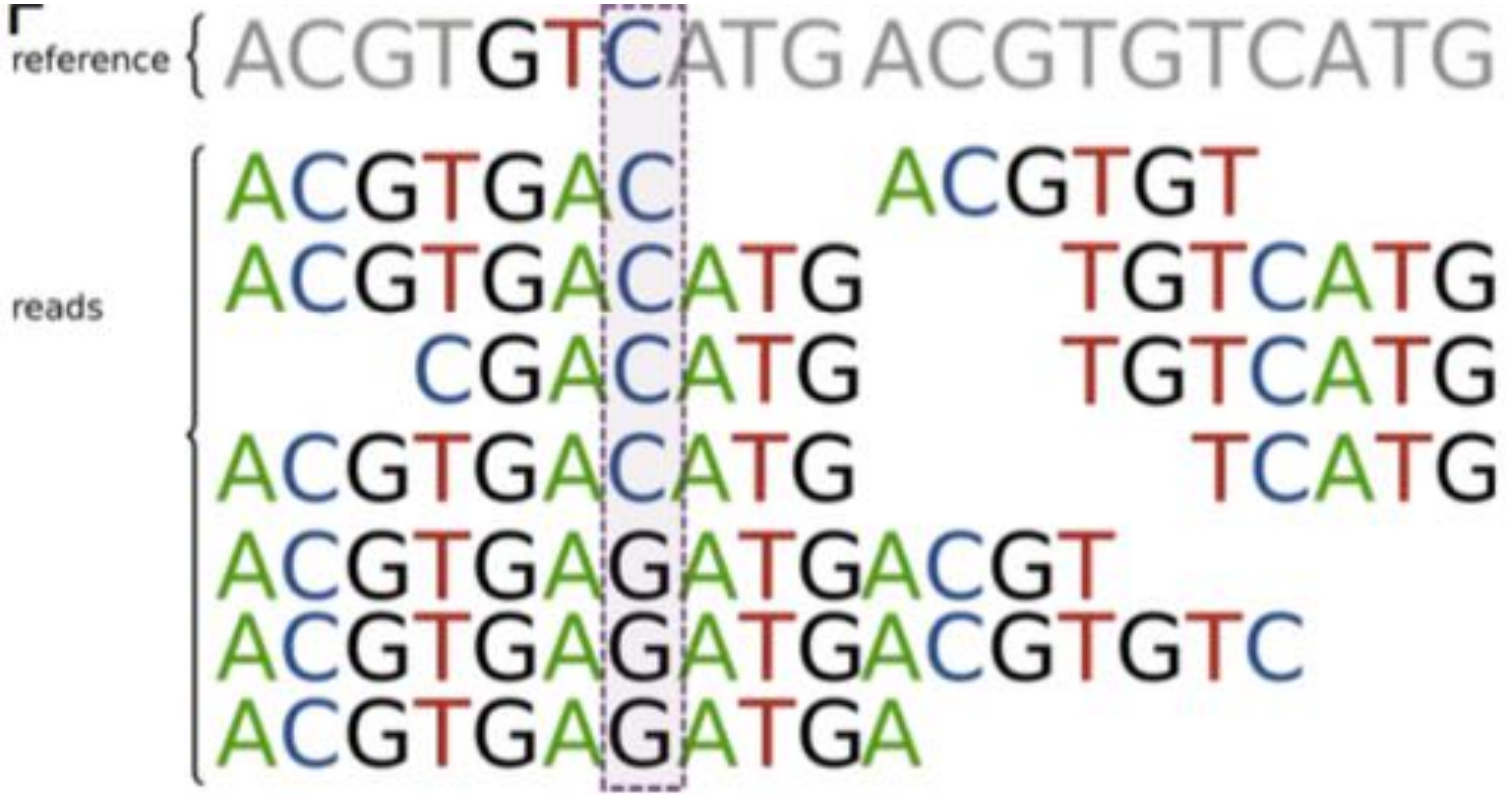
`-\+[0-9]+[ACGTNacgtn]+‘ indicates an indel

Analyse du PileUp

Analyse du PileUp

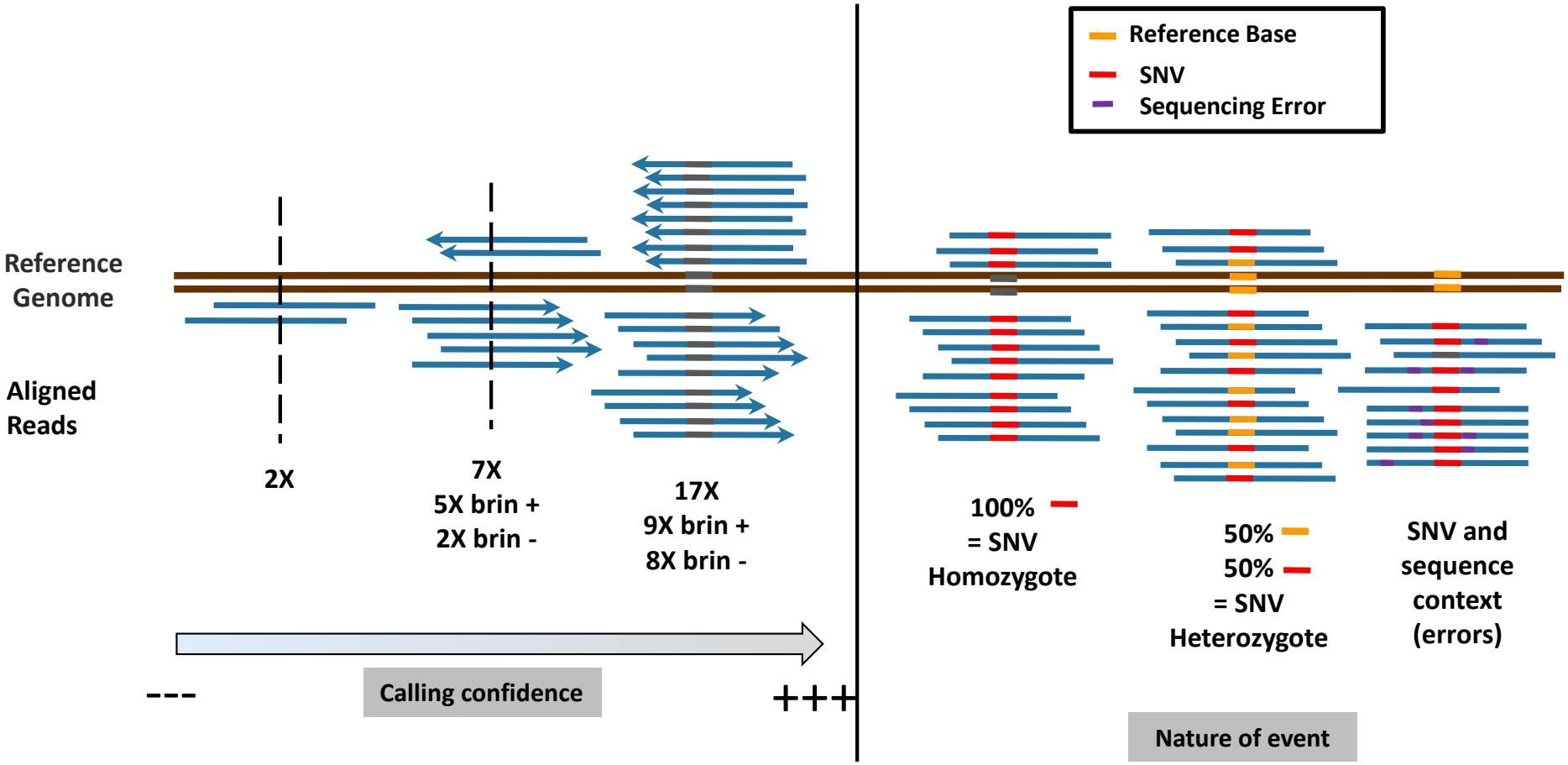


Analyse du PileUp



Depth of Coverage

Depth of Coverage = number of reads supporting one position
ex: 1X, 5X, 100X... >1000X



Variant Calling

- Factors to consider when calling a SNVs:
  - Base call qualities of each supporting base (base quality)
  - Proximity to small indels, or homopolymer run
  - Mapping qualities of the reads supporting the SNP
  - Sequencing **depth**: >=30x for constit ; >=100 for tumor
  - SNVs position within the reads: Higher error rate at the reads ends
  - Look at strand bias (SNVs supported by only one strand are more likely to be artifactual)
  - **Allelic frequency**: Tumor cellularity will reduce the % of an heterozygous variant
- Higher stringency when calling indels (and Sanger validation often needed)

La fraction allélique

- Vocabulaire:
  - Germline/population: Allelic **frequency**, MAF (minor allele frequency). Par ex. dans données 1000Genomes.
  - Somatic: Allelic **fraction** (mais souvent on utilise VAF or BAF: variant allele frequency)
- Où trouver l'info?
  - Colonne info#AF dans VCF

VarScan2

- Mutation caller written in **Java** (no installation required) working with **Pileup files** of Targeted, Exome, and Whole-Genome sequencing data (DNAseq or RNAseq)

- **Multi-platforms**: Illumina, SOLiD, Life/PGM, Roche/454

- Detection of different kinds of Germline SNVs/Indels (classical mode):
  - Variants in individual samples
  - Multi-sample variants **shared or private** in multi-sample datasets

- VarScan is able to work with **Tumor/Normal pairs (somatic mode):**
  - Somatic and germline mutation, LOH events in tumor-normal pairs
  - Somatic copy number alterations (CNAs) in tumor-normal exome data

VarScan2 Performance

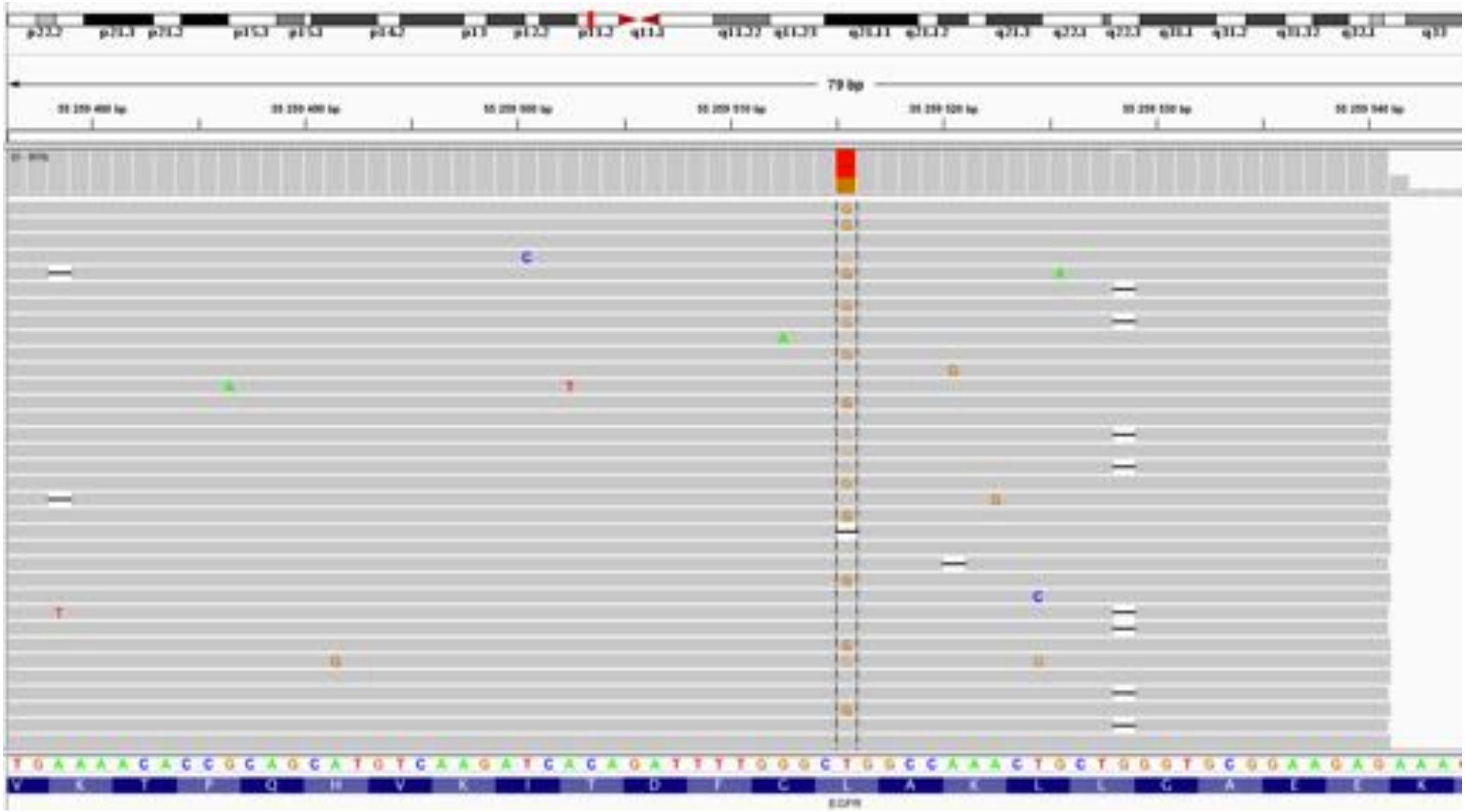
- VarScan uses a robust **heuristic/statistic** approach to call variants that meet desired thresholds for read depth, base quality, variant allele frequency, and statistical significance

- Stead *et* al. (2013) compared 3 different **somatic callers** : MuTect, Strelka, VarScan2
  - **VarScan2 performed best** overall with sequencing depths of 100x, 250x, 500x and 1000x required to accurately identify variants present at 10%, 5%, 2.5% and 1% respectively

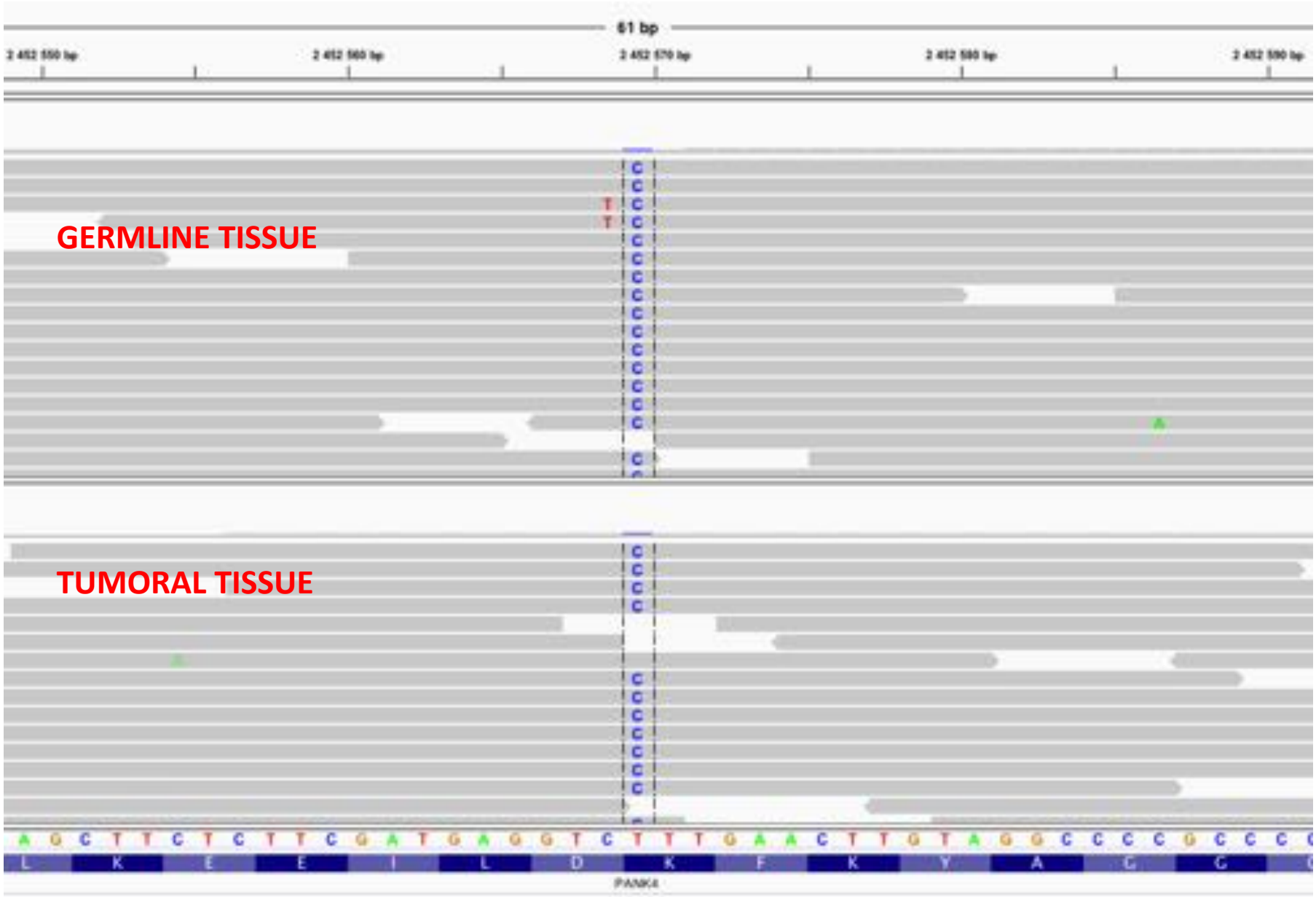
- Other widely used tool: **GATK**

Somatic calling criteria

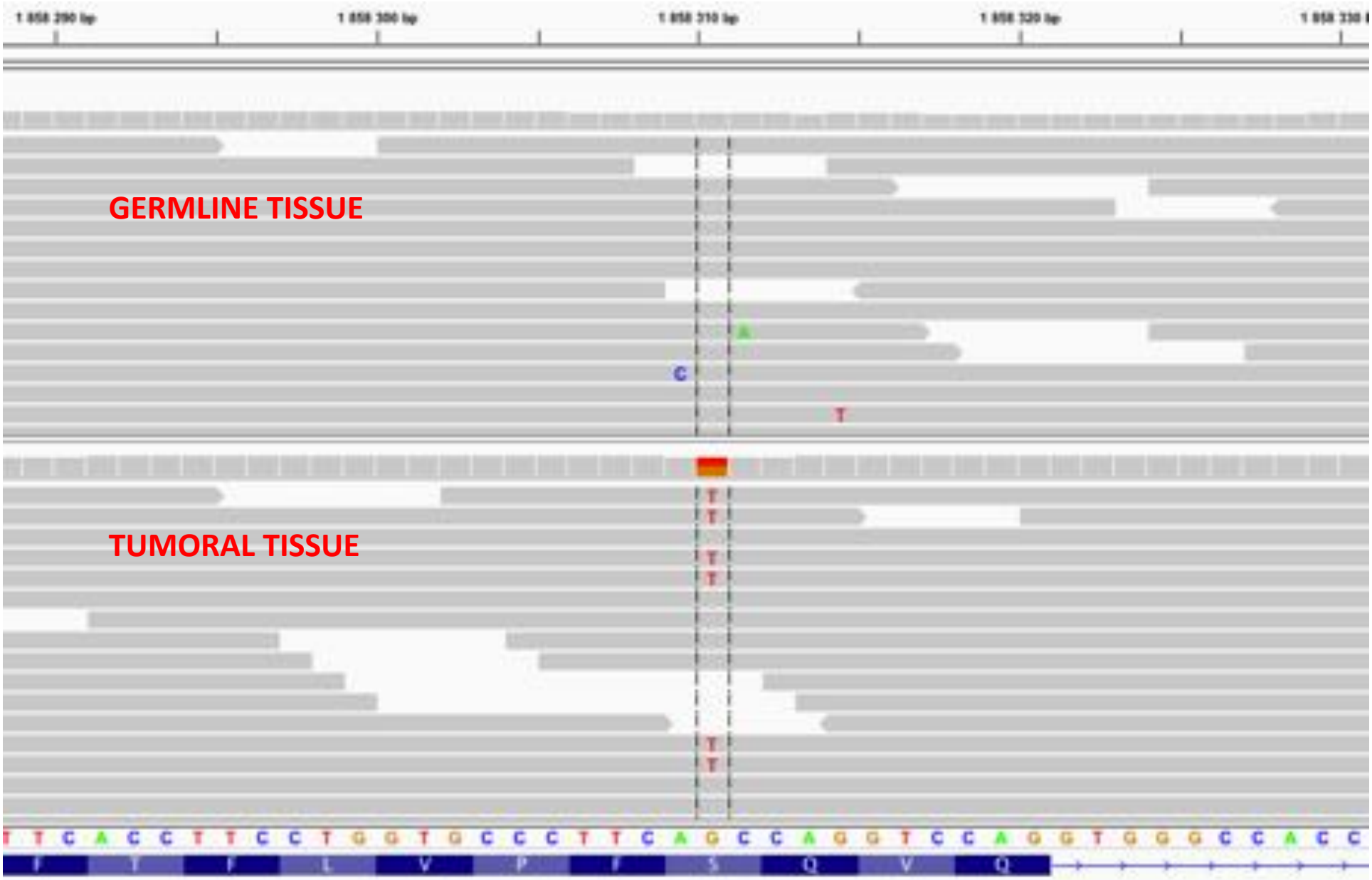
Un variant tumoral: polymorphisme ou mutation?



Un polymorphisme (SNP)



Une mutation somatique



Varscan's Somatic P-value

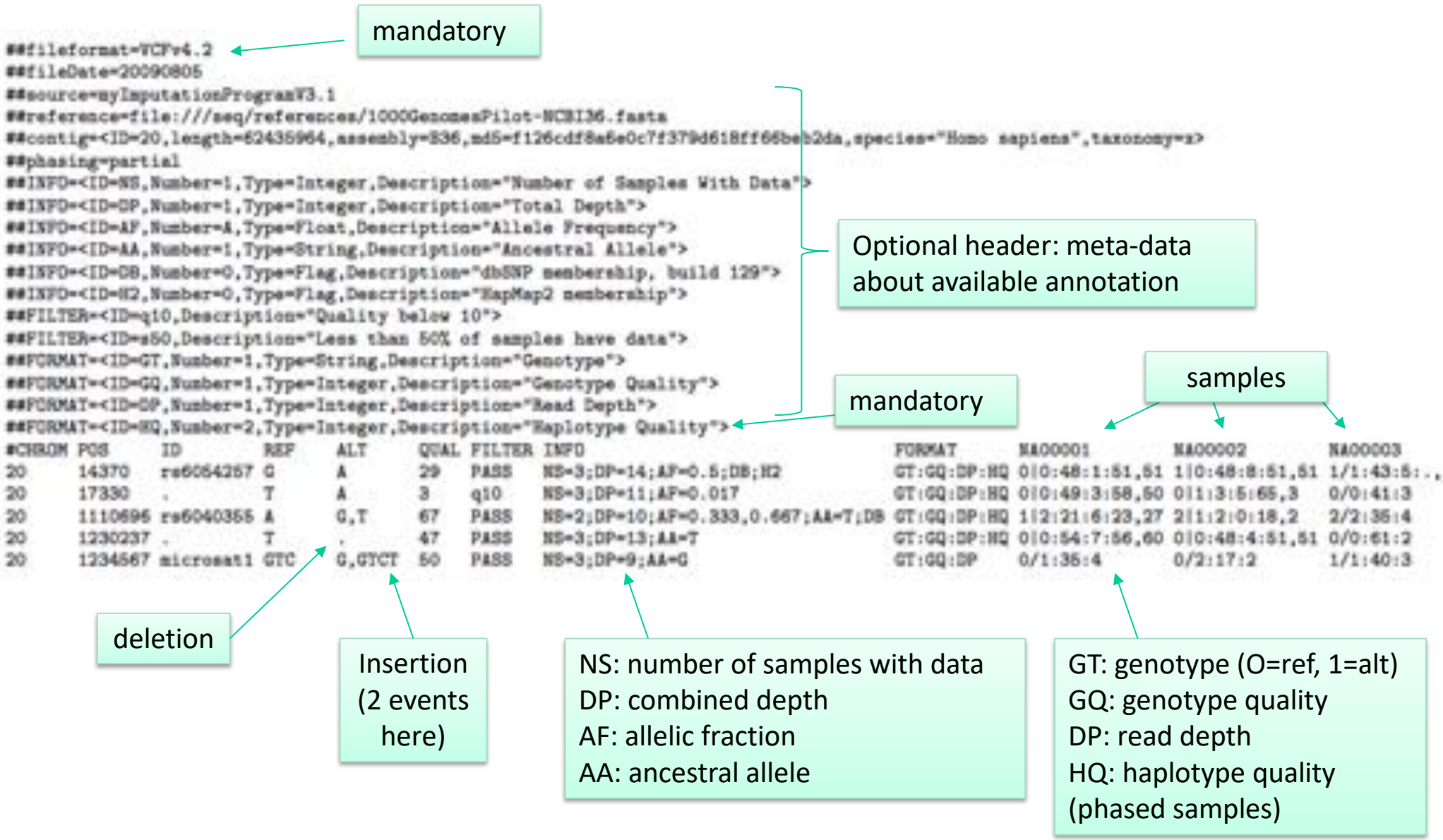
Variant Calling and Comparison
At every position where both normal and tumor have sufficient coverage, a comparison is made. First, normal and tumor are called independently using the germline consensus calling functionality. Then, their genotypes are compared by the following algorithm:

If **tumor does not match normal:**
Calculate significance of allele frequency difference by Fisher's Exact Test
If difference is significant (p-value < threshold):
If normal matches reference
==> Call Somatic
Else If normal is heterozygous
==> Call LOH
Else normal and tumor are variant, but different
==> Call IndelFilter or Unknown
If difference is not significant:
==> Call Germline

	Ref	Var
N	50	0
T	70	30

N	25	25
T	10	90

Format VCF


mandatory
##fileformat=VCFv4.2
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT NA00001 NA00002 NA00003
20 14370 rs6054257 G A 29 PASS NS=3;DP=14;AF=0.5;DB;H2 GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20 17330 . T A 3 q10 NS=3;DP=11;AF=0.017 GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3 0/0:41:3
20 1110696 rs6040355 A G,T 67 PASS NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2 2/2:35:4
20 1230237 . T . 47 PASS NS=3;DP=13;AA=T GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20 1234567 microsat1 GTC G,GTCT 50 PASS NS=3;DP=9;AA=G GT:GQ:DP 0/1:35:4 0/2:17:2 1/1:40:3
Optional header: meta-data about available annotation
mandatory
samples
deletion
Insertion (2 events here)
NS: number of samples with data
DP: combined depth
AF: allelic fraction
AA: ancestral allele
GT: genotype (O=ref, 1=alt)
GQ: genotype quality
DP: read depth
HQ: haplotype quality (phased samples)

VarScan Tabulated Format

Chrom	Position	Ref	Cons	Reads1	Reads2	VarFreq	Strands 1	Strands 2	Qual1	Qual2	Pvalue	Map Qual1	Map Qual2	R1 +	R1 -	R2 +	Rs2 -	Alt
chr12	113348849	C	Y	31	30	**49.18%**	2	2	27	27	0.98	1	1	19	12	25	5	**T**
chr12	113354329	G	R	72	2	**2.70%**	2	2	31	26	0.98	1	1	48	24	1	1	**A**
chr12	113357193	G	A	2	72	**97.30%**	1	2	28	24	0.98	1	1	2	0	45	27	**A**
chr12	113357209	G	A	0	77	**100%**	0	2	0	29	0.98	0	1	0	0	51	26	**A**

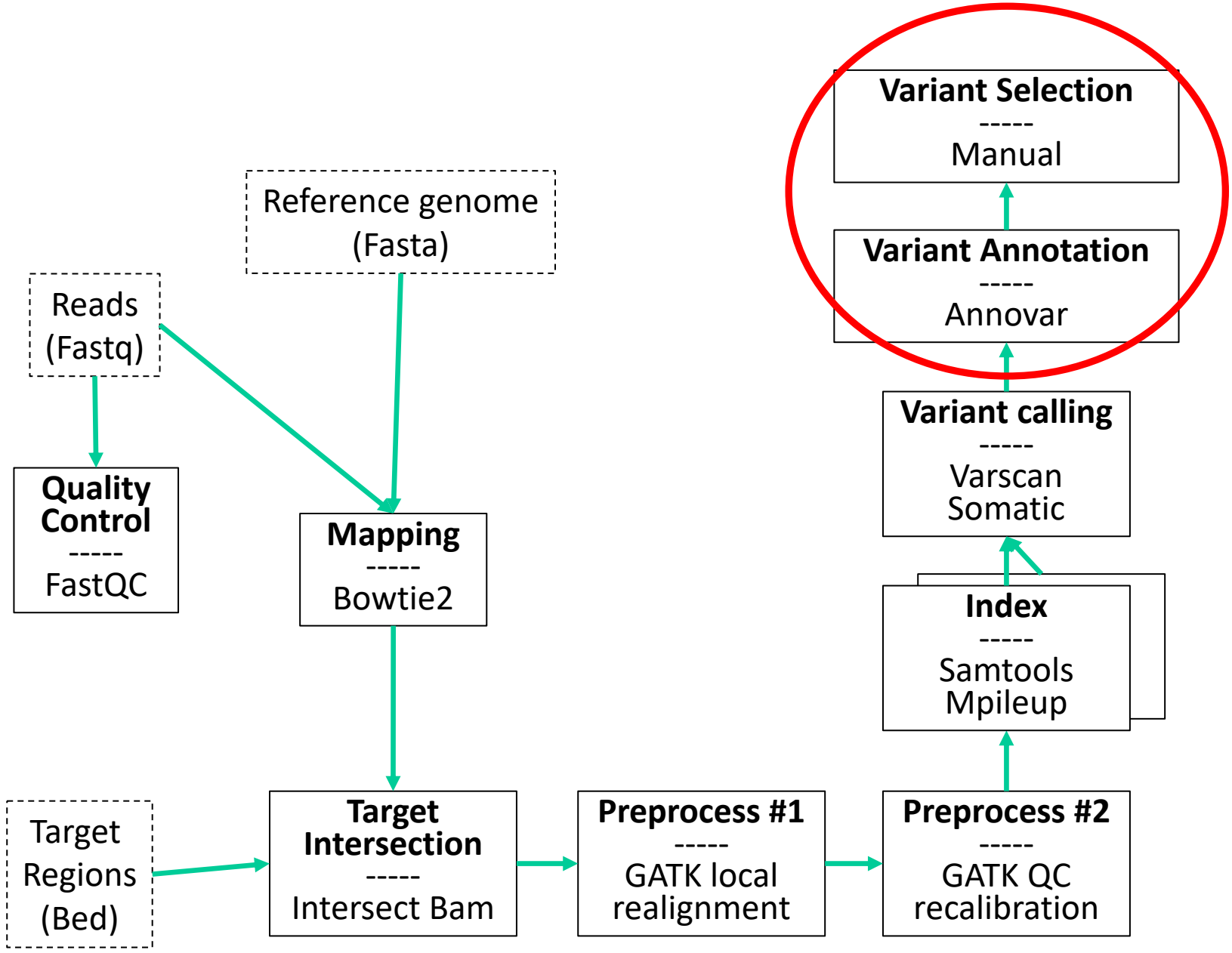
Cons : Consensus Genotype of Variant Called (IUPAC code):

M -> A or C	Y -> C or T	D -> A or G or T	W -> A or T	V -> A or C or G
R -> A or G	K -> G or T	B -> C or G or T	S -> C or G	H -> A or C or T

MAF format

Mutation Annotation Format (MAF) is a tab-delimited text file with aggregated mutation information from VCF Files and are generated on a project-level.

Column	Description
1 - Hugo_Symbol	HUGO symbol for the gene (HUGO symbols are always in all caps). "Unknown" is used for regions that do not correspond to a gene
2 - Entrez_Gene_Id	Entrez gene ID (an integer). "0" is used for regions that do not correspond to a gene region or Ensembl ID
3 - Center	One or more genome sequencing center reporting the variant
4 - NCBI_Build	The reference genome used for the alignment (GRCh38)
5 - Chromosome	The affected chromosome (chr1)
6 - Start_Position	Lowest numeric position of the reported variant on the genomic reference sequence. Mutation start coordinate
7 - End_Position	Highest numeric genomic position of the reported variant on the genomic reference sequence. Mutation end coordinate
8 - Strand	Genomic strand of the reported allele. Currently, all variants will report the positive strand: '+'
9 - Variant_Classification	Translational effect of variant allele
10 - Variant_Type	Type of mutation. TNP (tri-nucleotide polymorphism) is analogous to DNP (di-nucleotide polymorphism) but for three consecutive nucleotides. ONP (oligo-nucleotide polymorphism) is analogous to TNP but for consecutive runs of four or more (SNP, DNP, TNP, ONP, INS, DEL, or Consolidated)
11 - Reference_Allele	The plus strand reference allele at this position. Includes the deleted sequence for a deletion or "-" for an insertion
12 - Tumor_Seq_Allele1	Primary data genotype for tumor sequencing (discovery) allele 1. A "-" symbol for a deletion represents a variant. A "-" symbol for an insertion represents wild-type allele. Novel inserted sequence for insertion does not include flanking reference bases
13 - Tumor_Seq_Allele2	Tumor sequencing (discovery) allele 2
14 - dbSNP_RS	The rs-IDs from the dbSNP database, "novel" if not found in any database used, or null if there is no dbSNP record, but it is found in other databases
15 - dbSNP_Val_Status	The dbSNP validation status is reported as a semicolon-separated list of statuses. The union of all rs-IDs is taken when there are multiple
16 - Tumor_Sample_Barcode	Aliquot barcode for the tumor sample
17 - Matched_Norm_Sample_Barcode	Aliquot barcode for the matched normal sample
18 - Match_Norm_Seq_Allele1	Primary data genotype. Matched normal sequencing allele 1. A "-" symbol for a deletion represents a variant. A "-" symbol for an insertion represents wild-type allele. Novel inserted sequence for insertion does not include flanking reference bases (cleared in somatic MAF)
19 - Match_Norm_Seq_Allele2	Matched normal sequencing allele 2


Variant Selection

Manual
Variant Annotation

Annovar
Reference genome
(Fasta)
Reads
(Fastq)
Variant calling

Varscan
Somatic
Quality
Control

FastQC
Mapping

Bowtie2
Index

Samtools
Mpileup
Target
Regions
(Bed)
Target
Intersection

Intersect Bam
Preprocess #1

GATK local
realignment
Preprocess #2

GATK QC
recalibration

Different types of SNVs

- SNVs and short indels are the most frequent events:
  - Intergenic
  - Intronic
  - *cis*-regulatory
  - splice sites
  - frameshift or not
  - synonymous or not
  - benign or damaging etc...

- Example of SNV one want to pinpoint:
  - non-synonymous + highly deleterious + somatically acquired

Resources dedicated to human genetic variation

- dbSNP and 1000-genomes
  - Population-scale DNA polymorphisms
- COSMIC
  - Catalogue Of Somatic Mutations In Cancer
- Non synonymous SNVs predictions
  - SIFT, Polyphen2 (damaging impact)... PhyloP, GERP++ (conservation)

Annovar

« Annovar » annotates SNVs and Indels

Takes Multi sample VCF (Tumor &+normal samples)

- RefGene: Gene & Function & AminoAcid Change (HGVS format: c.A155G ; p.Lys45Arg)
- 1000g2012apr_all: Minor Allele Frequency for all ethnies
- ESP6500: Exome Sequencing Project
- Predictions: (**SIFT**, **Polyphen2**, LRT, MutationTaster, PhyloP, GERP++)

✧ Tabulated file

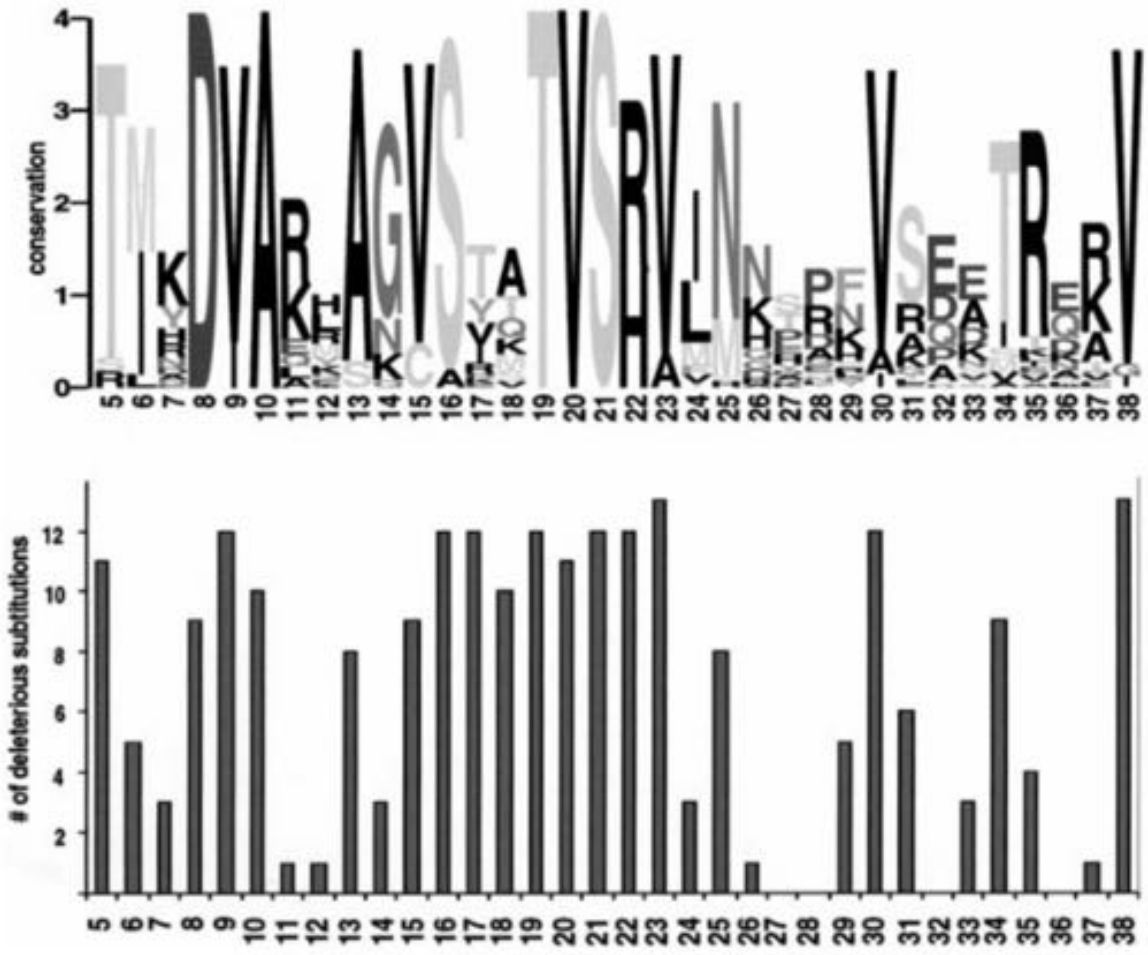
Chr	Start	End	Ref	Alt	Func.refGene	Gene.refGene	ExonicFunc.refGene	AAChange.refGene	1000g2012apr_all	snp137	cosmic68	esp6500_all	LJB_PhyloP	LJB_PhyloP_Pre
chr1	160251792	160251792	A	G	intronic	PEX19	NA	NA						
chr1	167082869	167082869	G	A	intronic	DUSP27	NA	NA						
chr1	167095163	167095163	G	C	exonic	DUSP27	nonsynonymous SNV	DUSP27:NM_001080426:exon5:c.G795C:p.E265D						
chr1	167095881	167095881	G	A	exonic	DUSP27	nonsynonymous SNV	DUSP27:NM_001080426:exon5:c.G1513A:p.A505T						
chr1	167097739	167097739	C	A	exonic	DUSP27	nonsynonymous SNV	DUSP27:NM_001080426:exon5:c.C3371A:p.T1124N						
chr1	214803969	214803969	G	C	exonic	CENPF	nonsynonymous SNV	CENPF:NM_016343:exon9:c.G1287C:p.K429N						

Sorting Intolerant From Tolerant

Ng & Henikoff,
Genome Res. 2001

Utilise la conservation des domaines protéiques comme indication du caractère délétère d'une substitution

Classe en
D(eleterious),
T(olerant),
.(unknown)



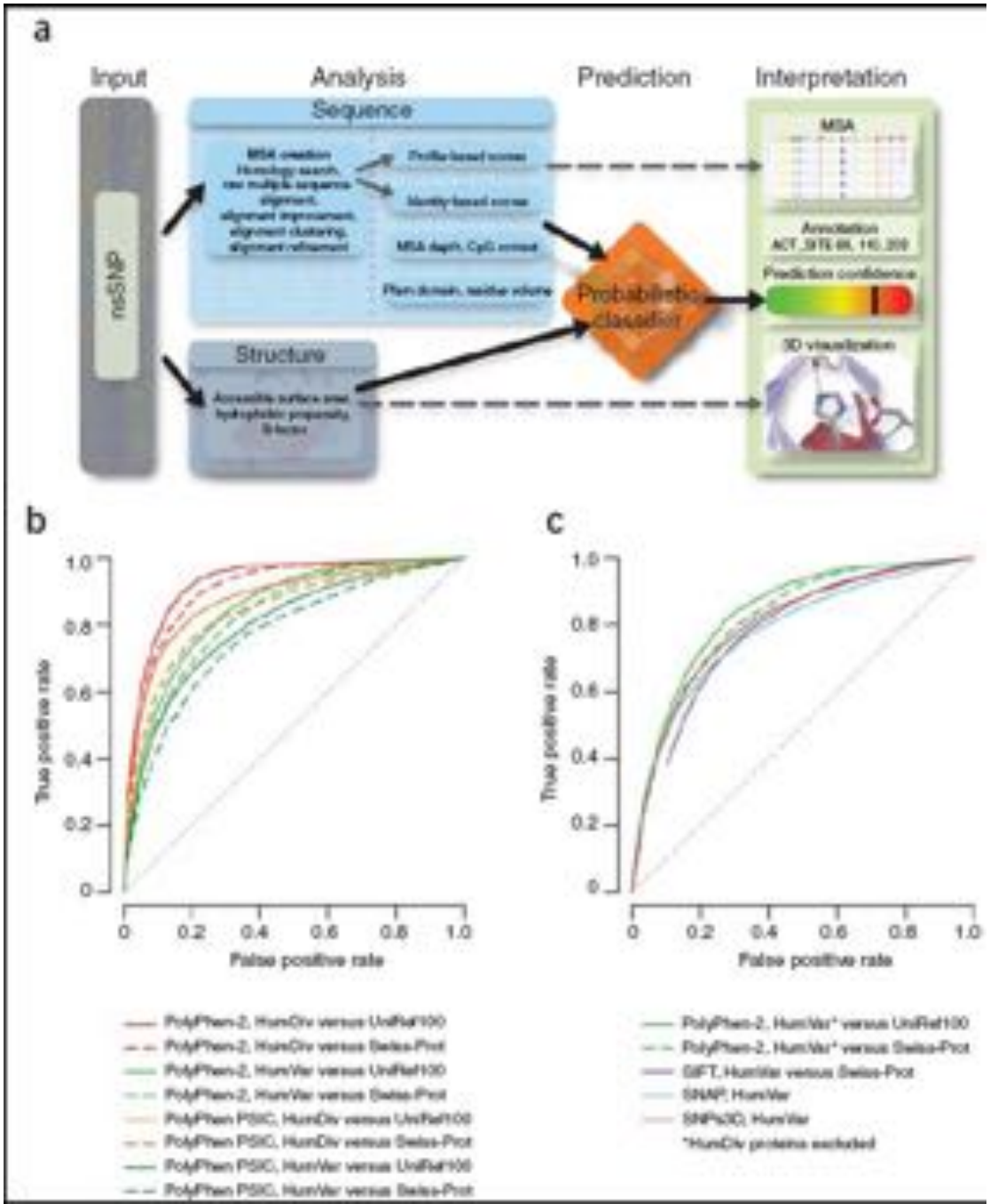
effet réél: LacI

PolyPhen2

Adzhubei et al. *Nature Methods* 2010.

Probabilistic classifier: Estimates the probability of the missense mutation being damaging based on a combination of seq+struct properties.

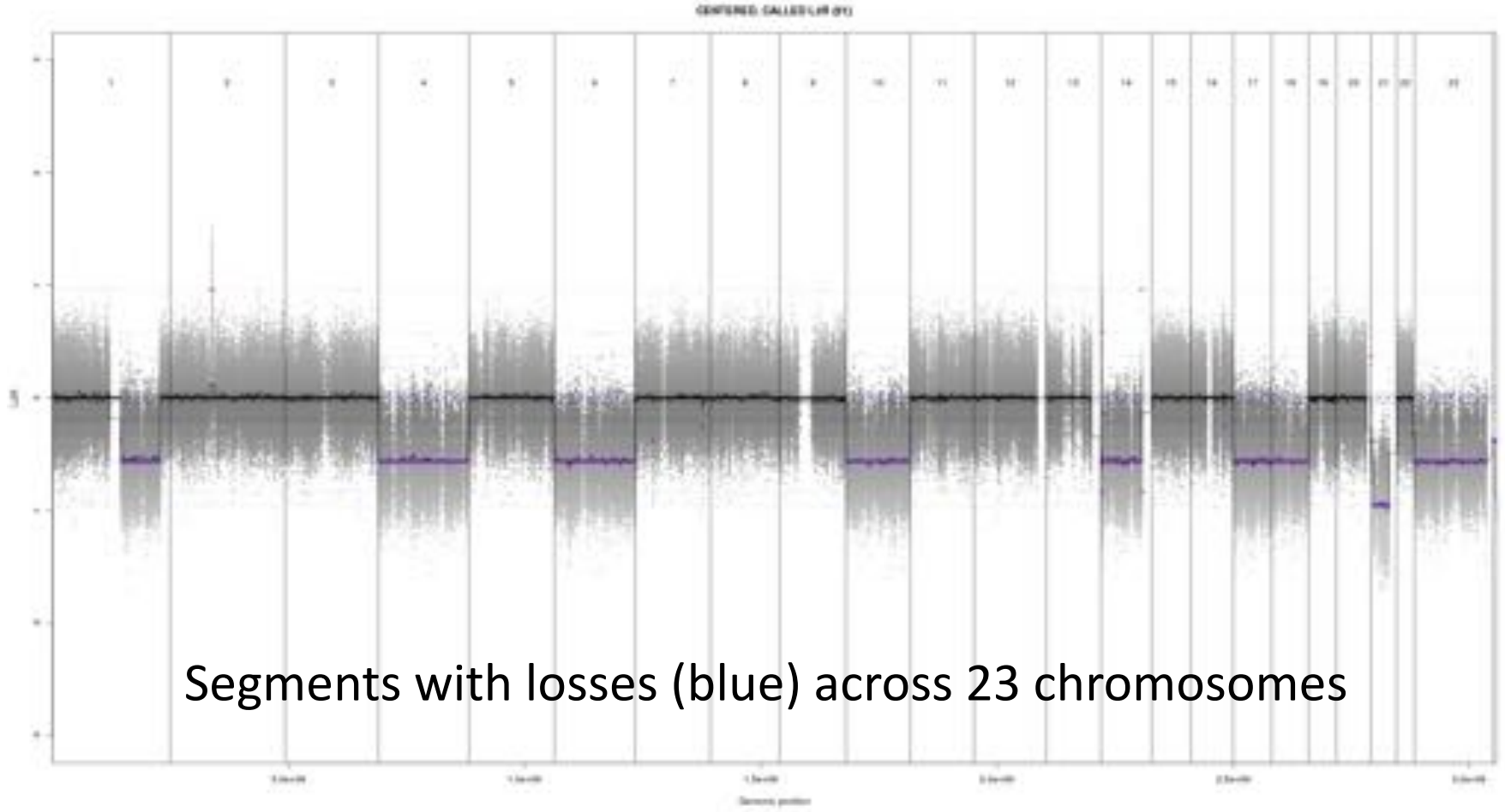
Classe en: **B**enign, **P**ossibly damaging, or probably **D**amaging



Coverage & Allelic Frequencies For CNV detection

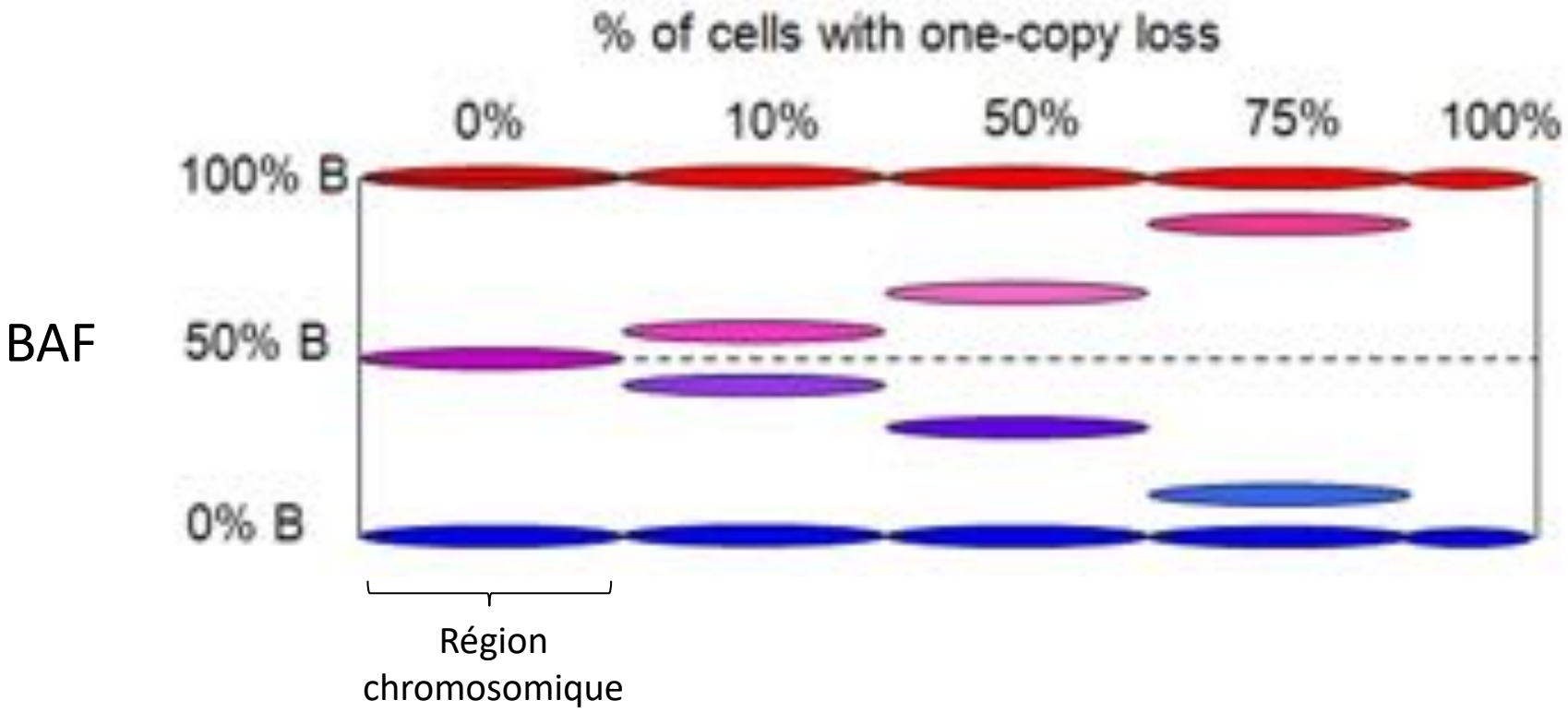
Detection of copy-number variations

Copy-number alterations (gain or loss of chomosomal regions, amplifications …) are key drivers of tumorigenesis.

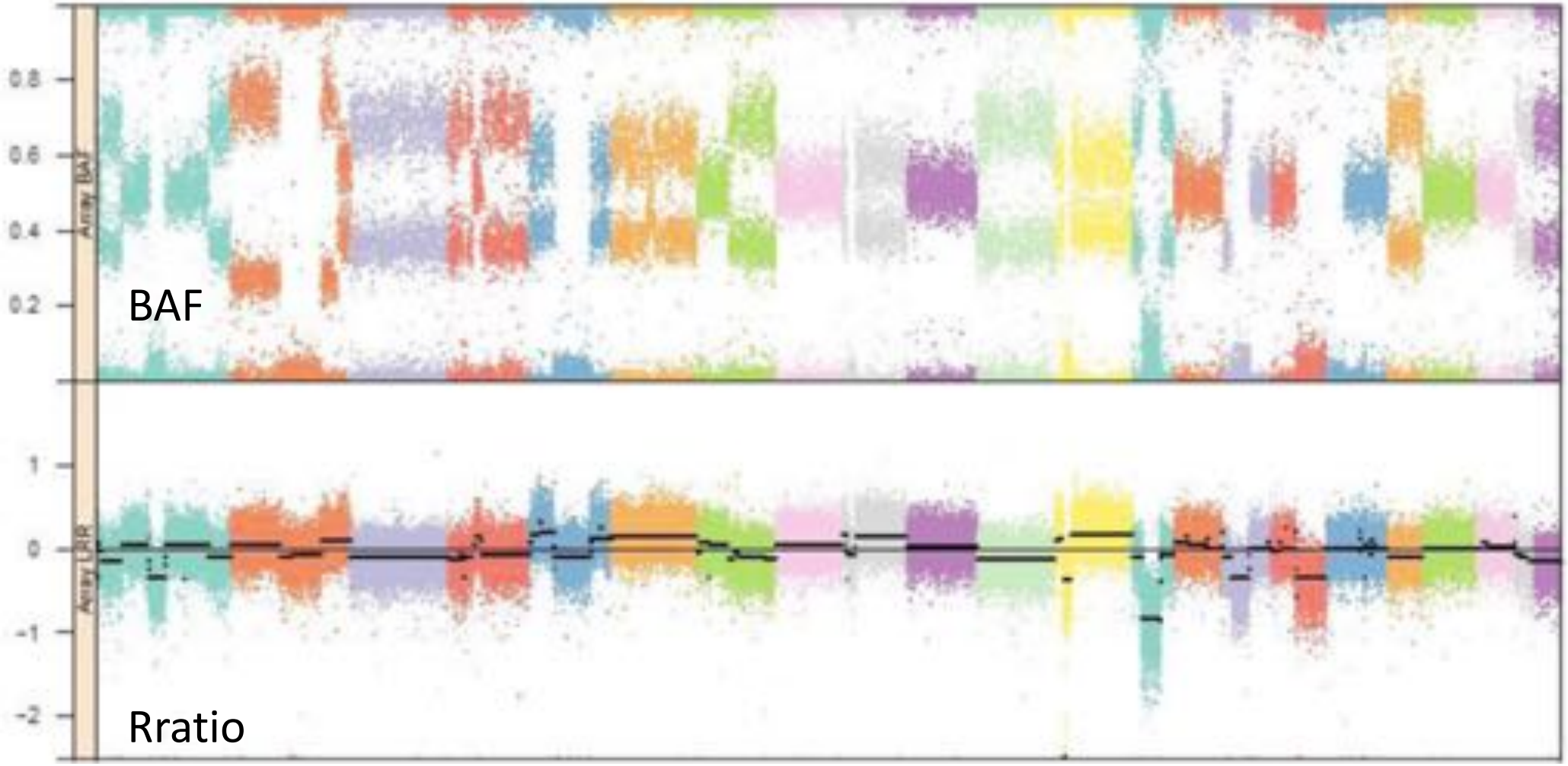


Segments with losses (blue) across 23 chromosomes

Cellularité et Fréquence Allelique



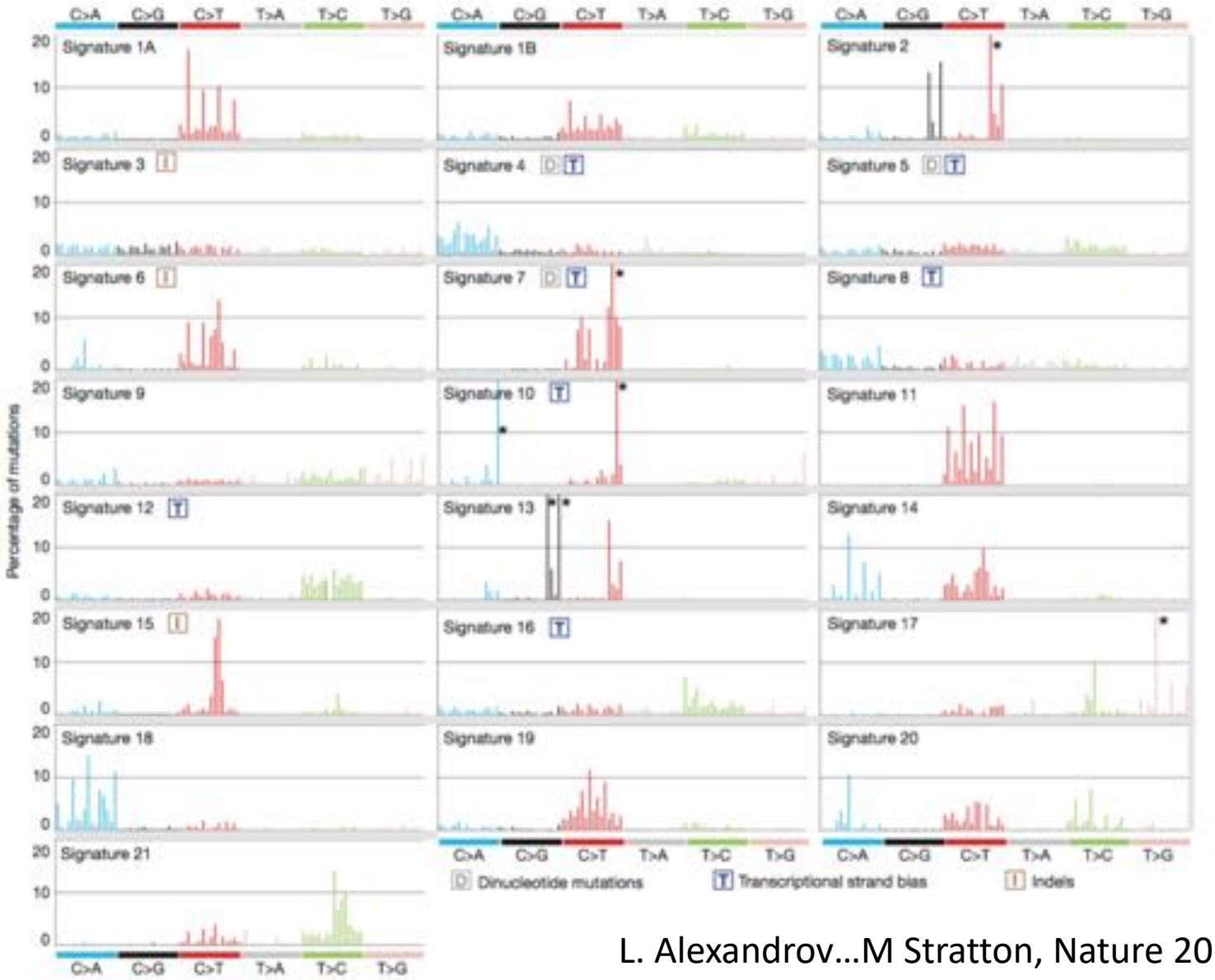
Segmentation et fréquence allélique



R ratio=utilisé en CGH, =couverture en NGS

Scott et al. Gene 2014

Les Signatures Mutationnelles



L. Alexandrov…M Stratton, Nature 2013. [69]

Signatures et origine des tumeurs

L. Alexandrov…M Stratton, Nature 2013.

Données exercice

- Données de cancer ovarien
  - WES Normal + Tumeur
  - Original BAM: 10Go x 2
- 2 fichiers tabulés:
  - ovc-Tpre_varscan2_annot.tab: Tous les variants de la tumeur (somatique ou non)
  - ovc-somatic-variants.tsv : Les variants déclarés somatiques (par comparaison avec normal) et annotés

Pipeline:

Alignement
bwa
Post traitement bam
Picartools Sort Sam
GATK indelRealigner
GATK variantRecalibration
Variant Calling
samtools mpileup
varscan2 somatic
Filtering
variants somatiques ou LOH
& Somatic P-value < 10^-3
Annotation
SNPeff, SNPSIFT
Script VCF>Tabulé

Atelier pratique

- Voir *TP-variant*