Session- Variant Calling and Consensus Generation

BU-ISCIII

Unidades Comunes Científico Técnicas – SGSAFI-ISCIII

28-02 Junio 2021, 3ª Edición
Programa Formación Continua, ISCIII

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Mapping against reference genome and Variant Calling :

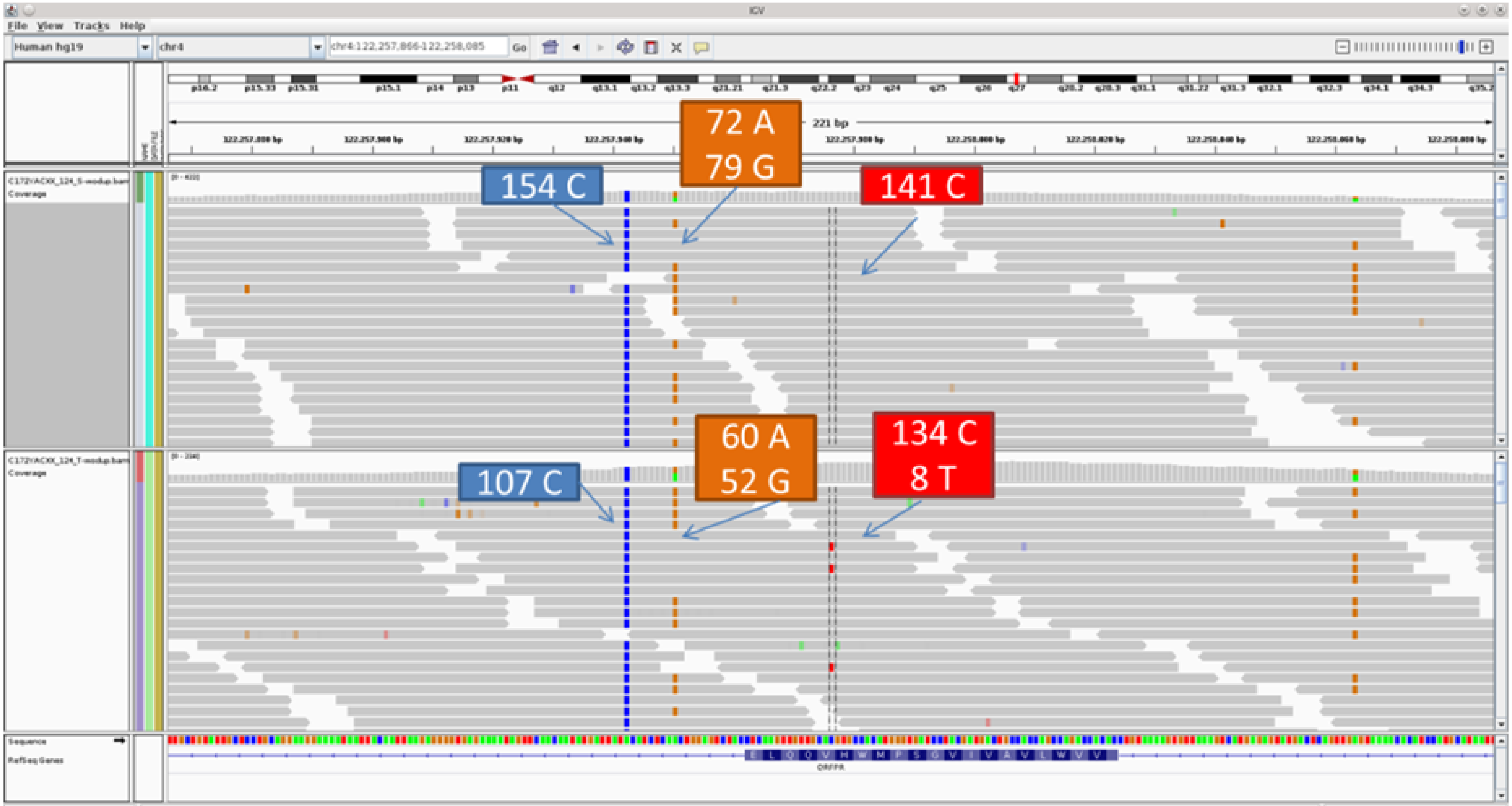
- Variant Calling
- Source of error and mitigation strategies
- VCF and bed format
- IVAR, LOFREQ, VARSCAN2
- Consensus generation: aproximations

Variant Calling

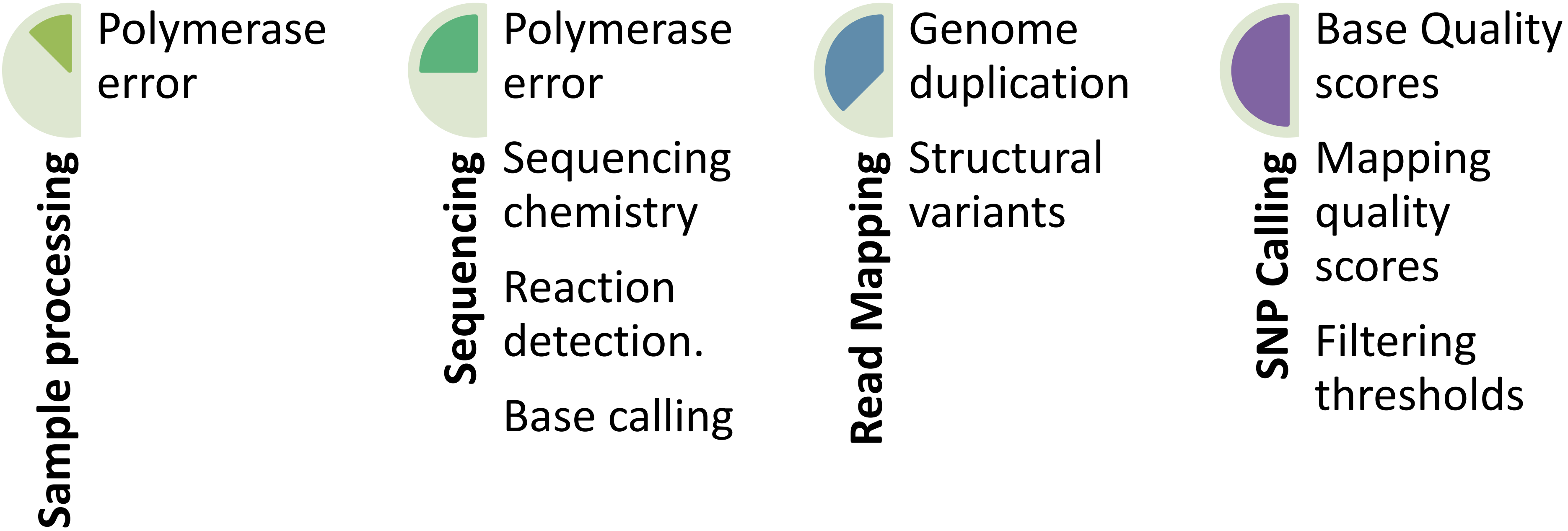
- **Variant calling concept is simple:**

Find positions in our reads different from the reference.

- We start with our secuences mapped against our reference genome, and we walk trough every column of the alignment counting the number of alleles found and comparing them against the reference.


IGV
File View Tracks Help
Human hg19
chr4
chr4:122,257,866-122,258,085
Go
221 bp
72 A
79 G
154 C
141 C
60 A
52 G
107 C
134 C
8 T
Coverage
Coverage
Sequence
RefSeq Genes

Sources of error and mitigation strategies



Sample processing

- Polymerase error

Sequencing

- Polymerase error
- Sequencing chemistry
- Reaction detection.
- Base calling

Read Mapping

- Genome duplication
- Structural variants

SNP Calling

- Base Quality scores
- Mapping quality scores
- Filtering thresholds

Adapted from Olson et al. Frontiers in Genetics. 2015

Sources of error and mitigation strategies

- **Sample processing errors.**
  - Random errors.
  - Associated with polymerase errors . (1 in 10^{2-3} bases)
  - Homopolymers and tandem repeats experience higher indel error rates.
- **Solutions:**
  - Paired-end libraries.
  - Minimization of PCR cycles.

Adapted from Olson et al. Frontiers in Genetics. 2015

Sources of error and mitigation strategies

- **Sequencing:**
  - Dependent on the platform.
  - Can be random and systematic.
  - 6% Illumina, 50% Roche (Ross et al.2013)
  - P.e Illumina commits error in the G/T channels.

- **Solutions:**
  - Strand bias.

Adapted from Olson et al. Frontiers in Genetics. 2015

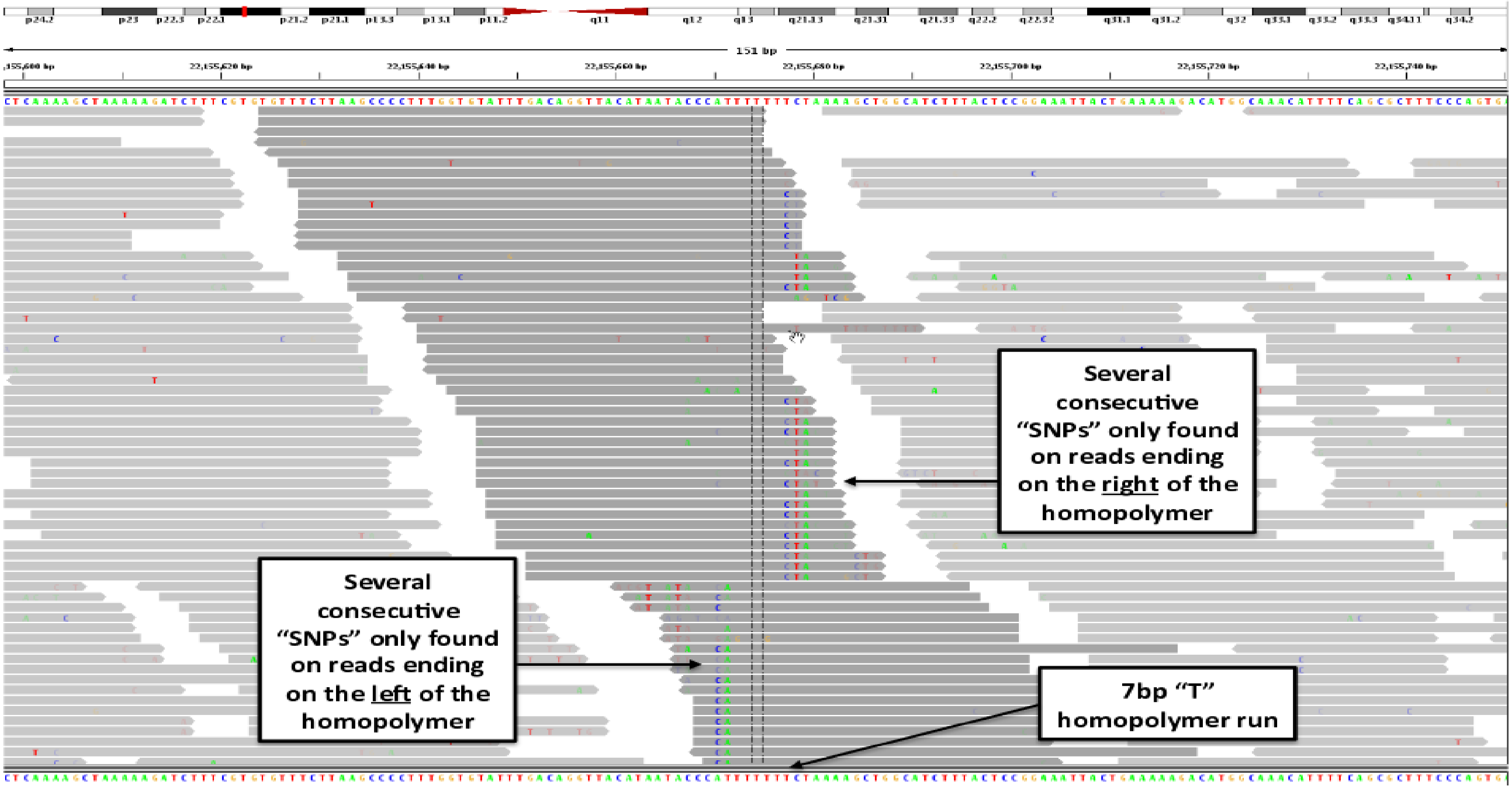
Sources of error and mitigation strategies

- **Mapping errors:**
  - Genomic duplication and structural variation.
  - High diverse areas.

- **Solutions**
  - Paired-end libraries.
  - Long reads / fragments.
  - MAPQ
  - Realignment around indels.

Adapted from Olson et al. Frontiers in Genetics. 2015

Sources of error and mitigation strategies

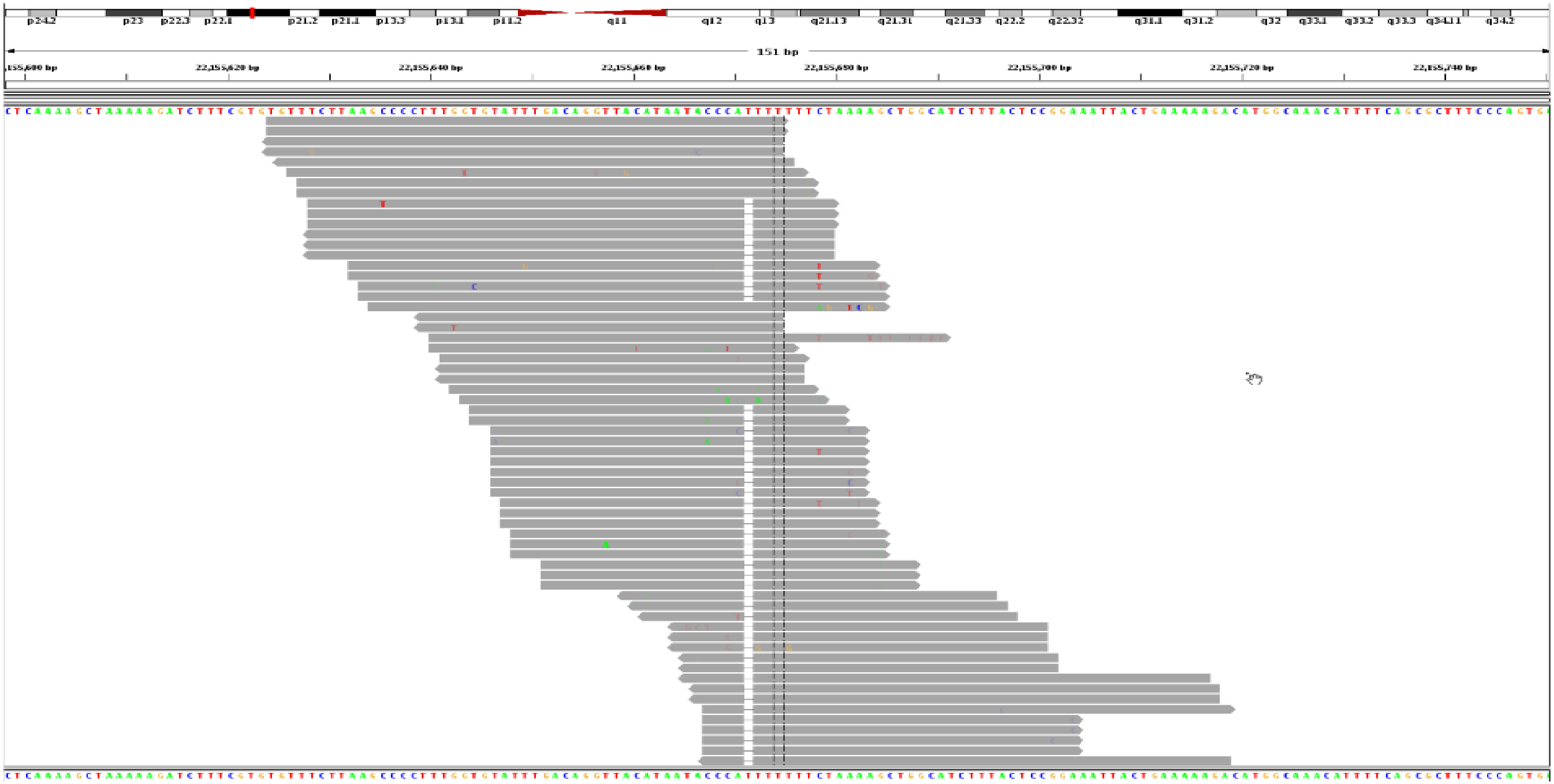


Several consecutive "SNPs" only found on reads ending on the right of the homopolymer

Several consecutive "SNPs" only found on reads ending on the left of the homopolymer

7bp "T" homopolymer run

Sources of error and mitigation strategies



Sources of error and mitigation strategies

- **SNP calling step**
  - Errors may result in base calling errors.
  - FP and FN calls.
- **Solutions**
  - Strand bias
  - Base quality rank sum
  - MAPQ
  - Hard filters:
    - Depth of coverage
    - Minimun base call frequency.

Adapted from Olson et al. Frontiers in Genetics. 2015

Reference selection

- Critial step <- Bias which SNPs are called.
- SNPs in genes not present in the reference **WON'T** be called.
- Less effect in clonal bacteria.
- Number of SNPs called vary **A LOT!**

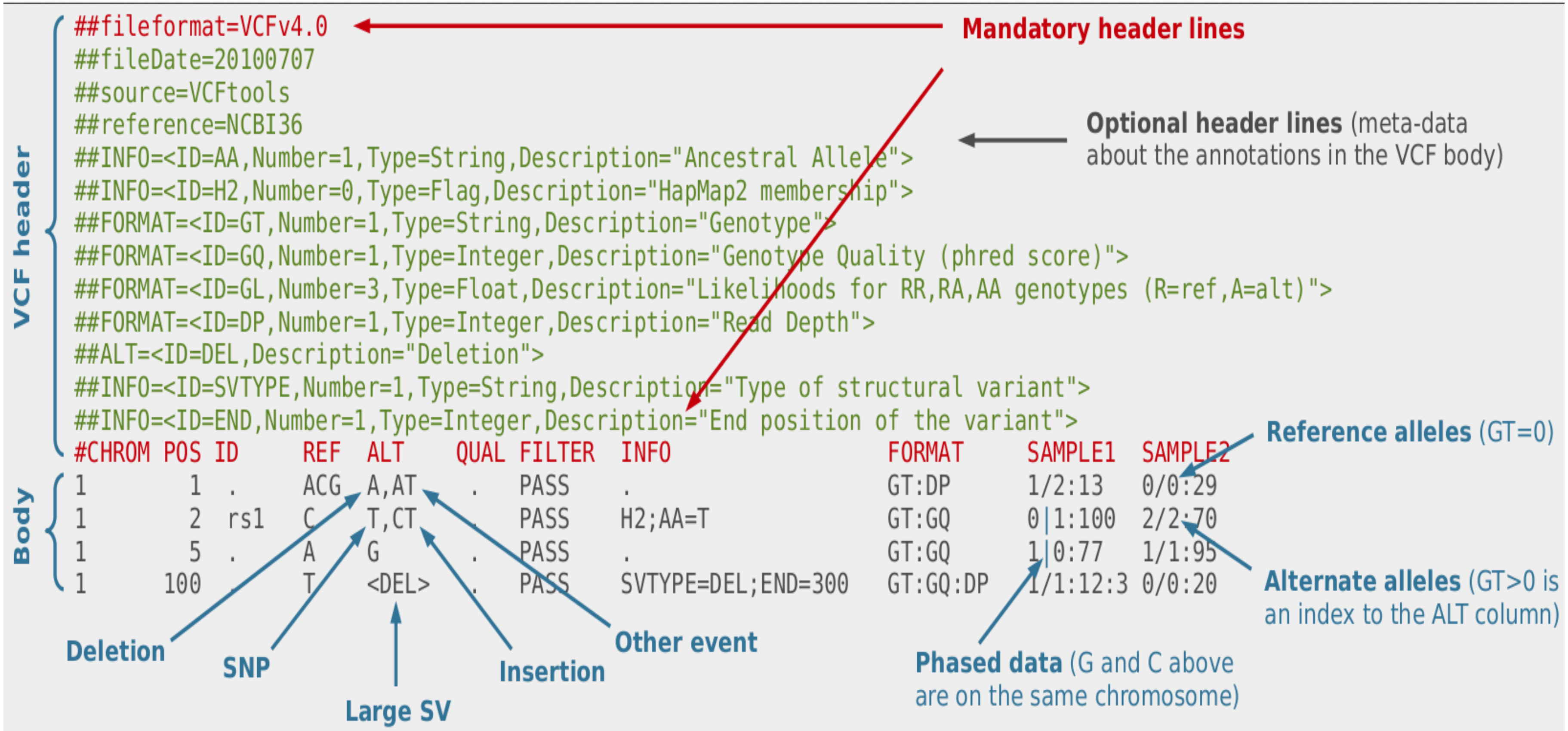
- **Solutions:**
  - Kmerfinder

Repetitive/Phage regions filtering

- **PHASTER**

- We can remove/mask phague/repetitive regions where reads won’t map.
- This way those areas will be out of analysis.
- Problem: those areas could be important!

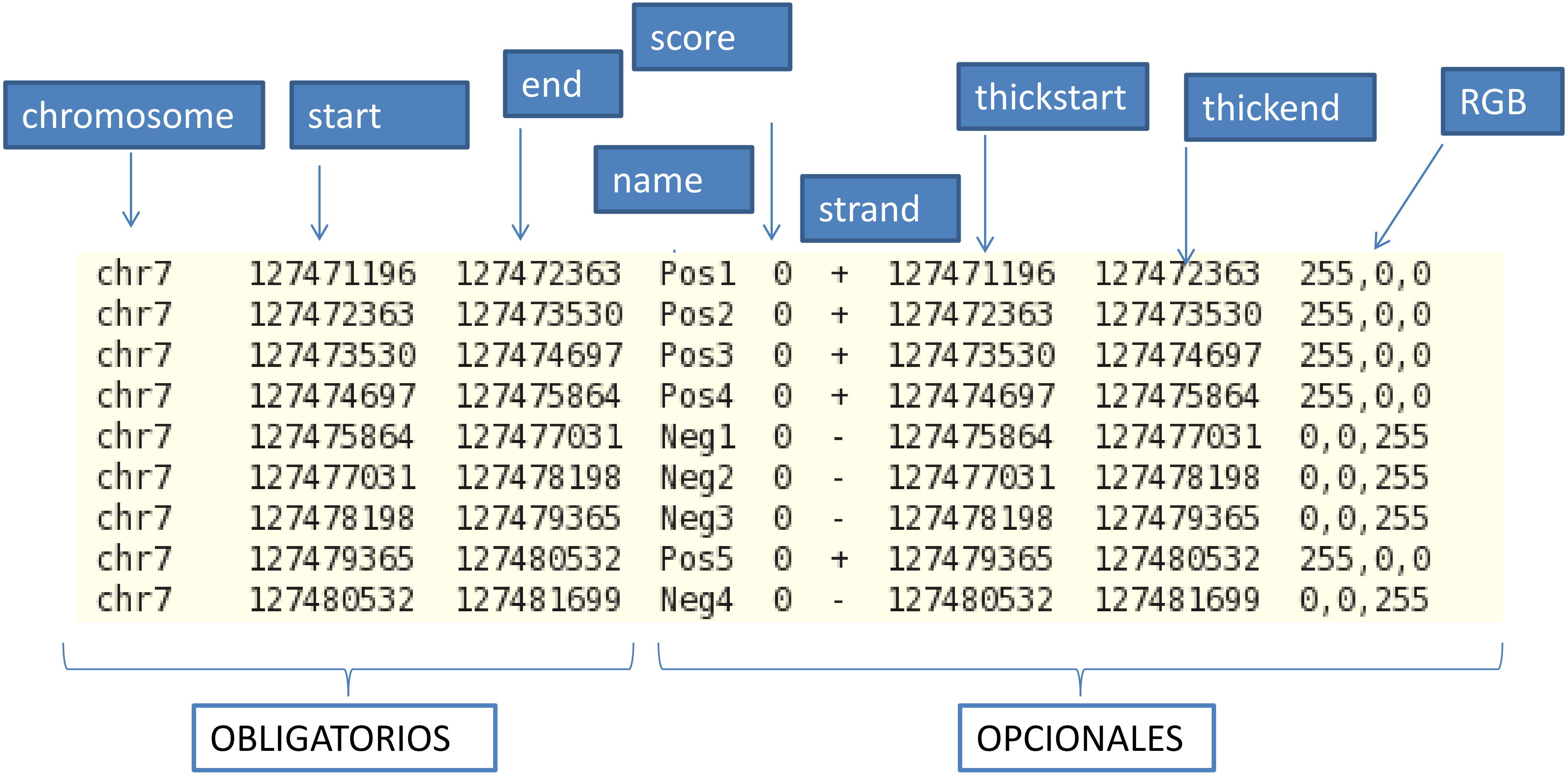
VCF format



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

VCF header
Body
Mandatory header lines
Optional header lines (meta-data about the annotations in the VCF body)
Reference alleles (GT=0)
Alternate alleles (GT>0 is an index to the ALT column)
Deletion
SNP
Large SV
Insertion
Other event
Phased data (G and C above are on the same chromosome)

Bed format



chromosome

start

end

name

score

strand

thickstart

thickend

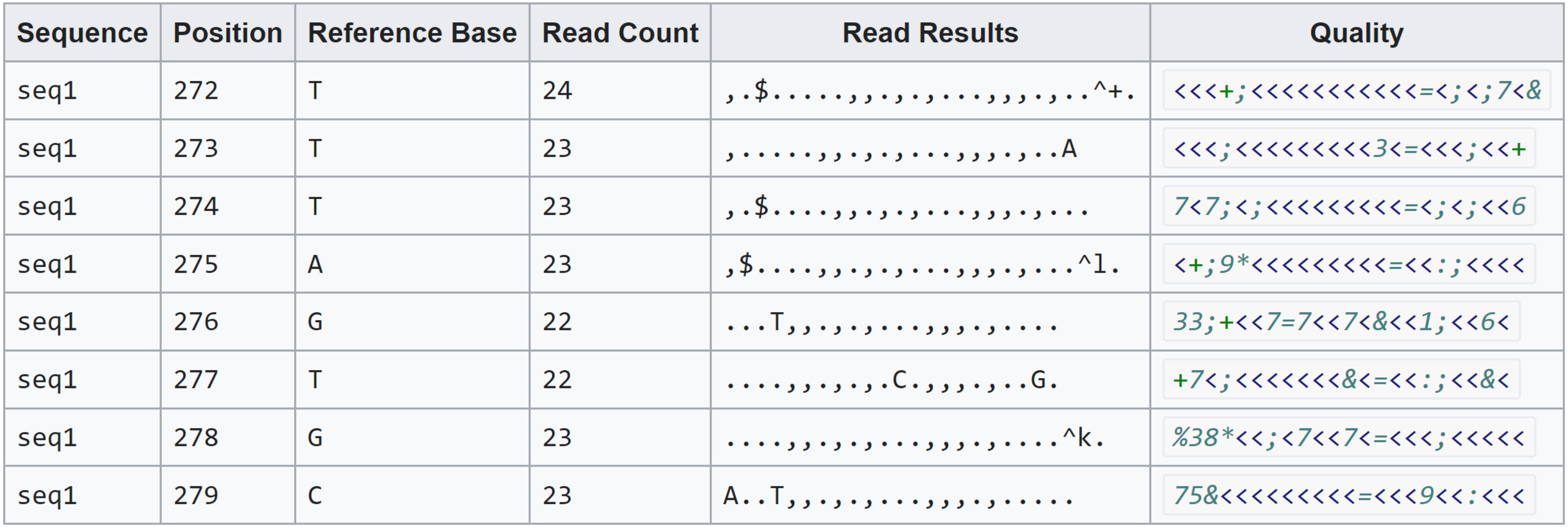
RGB

```
chr7    127471196  127472363  Pos1  0  +  127471196  127472363  255,0,0
chr7    127472363  127473530  Pos2  0  +  127472363  127473530  255,0,0
chr7    127473530  127474697  Pos3  0  +  127473530  127474697  255,0,0
chr7    127474697  127475864  Pos4  0  +  127474697  127475864  255,0,0
chr7    127475864  127477031  Neg1  0  -  127475864  127477031  0,0,255
chr7    127477031  127478198  Neg2  0  -  127477031  127478198  0,0,255
chr7    127478198  127479365  Neg3  0  -  127478198  127479365  0,0,255
chr7    127479365  127480532  Pos5  0  +  127479365  127480532  255,0,0
chr7    127480532  127481699  Neg4  0  -  127480532  127481699  0,0,255
```

OBLIGATORIOS

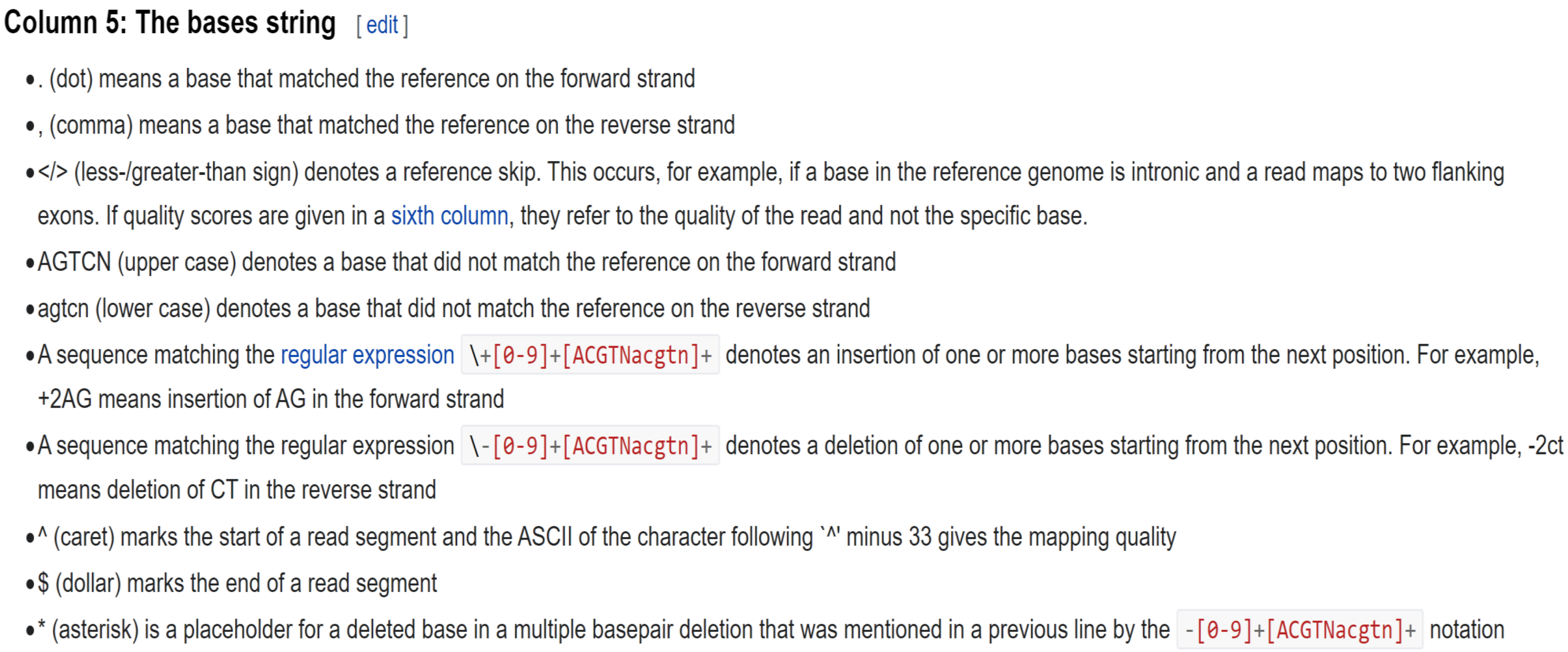
OPCIONALES

Mpileup format



Sequence	Position	Reference Base	Read Count	Read Results	Quality
seq1	272	T	24	,.$.....,,.,.,...,,,.,..^+.	<<<+;<<<<<<<<<<<=<;<;7<&
seq1	273	T	23	,.....,,.,.,...,,,.,..A	<<<;<<<<<<<<<3<=<<<;<<+
seq1	274	T	23	,.$....,,.,.,...,,,.,...	7<7;<;<<<<<<<<<=<;<;<<6
seq1	275	A	23	,$....,,.,.,...,,,.,...^l.	<+;9*<<<<<<<<<=<<:;<<<<
seq1	276	G	22	...T,,.,.,...,,,.,....	33;+<<7=7<<7<&<<1;<<6<
seq1	277	T	22	....,,.,.,.C.,,,.,..G.	+7<;<<<<<<<&<=<<:;<<&<
seq1	278	G	23	....,,.,.,...,,,.,....^k.	%38*<<;<7<<7<=<<<;<<<<<
seq1	279	C	23	A..T,,.,.,...,,,.,.....	75&<<<<<<<<<=<<<9<<:<<<

Mpileup format

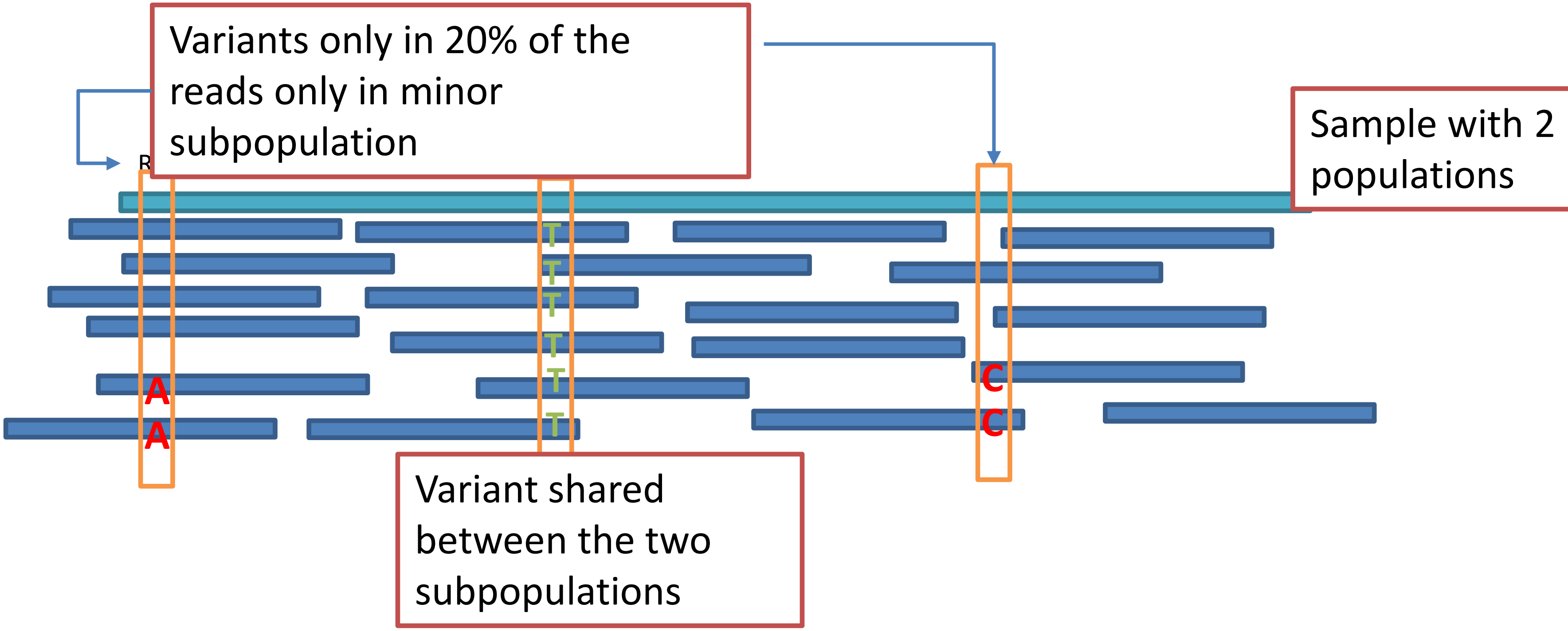


Column 5: The bases string [edit]

- . (dot) means a base that matched the reference on the forward strand
- , (comma) means a base that matched the reference on the reverse strand
- </> (less-/greater-than sign) denotes a reference skip. This occurs, for example, if a base in the reference genome is intronic and a read maps to two flanking exons. If quality scores are given in a sixth column, they refer to the quality of the read and not the specific base.
- AGTCN (upper case) denotes a base that did not match the reference on the forward strand
- agtcn (lower case) denotes a base that did not match the reference on the reverse strand
- A sequence matching the regular expression `\+[0-9]+[ACGTNacgtn]+` denotes an insertion of one or more bases starting from the next position. For example, +2AG means insertion of AG in the forward strand
- A sequence matching the regular expression `\-[0-9]+[ACGTNacgtn]+` denotes a deletion of one or more bases starting from the next position. For example, -2ct means deletion of CT in the reverse strand
- ^ (caret) marks the start of a read segment and the ASCII of the character following `^' minus 33 gives the mapping quality
- $ (dollar) marks the end of a read segment
- * (asterisk) is a placeholder for a deleted base in a multiple basepair deletion that was mentioned in a previous line by the `-[0-9]+[ACGTNacgtn]+` notation

Viral subpopulation - Quasispecies

- Just as in clonal subpopulations in tumor samples, we can have viral subpopulations called quasispecies in viral samples.
- We detect them using the alternative allele frequency.


Variants only in 20% of the reads only in minor subpopulation

Sample with 2 populations

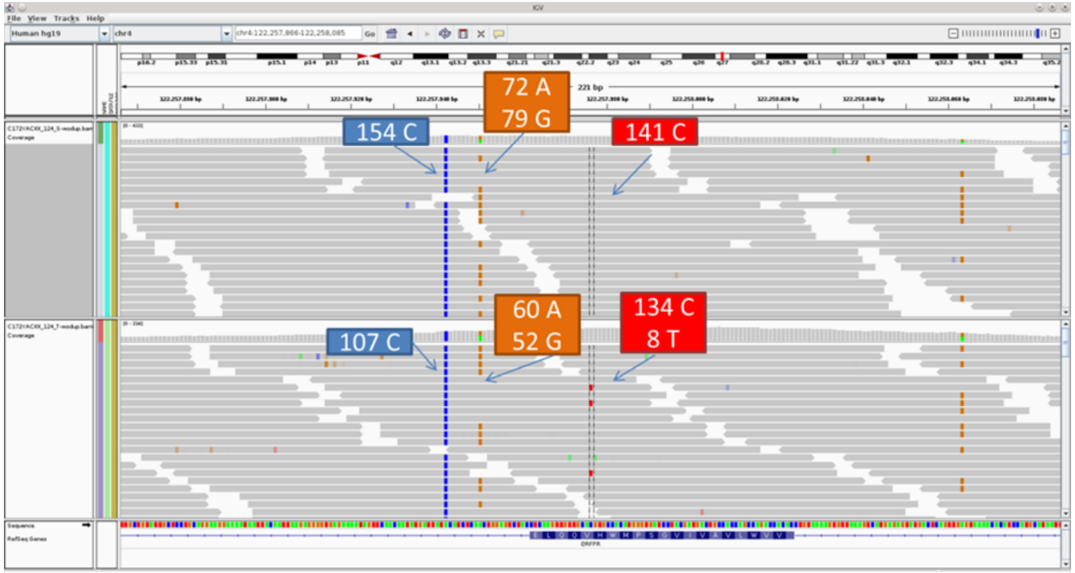
Variant shared between the two subpopulations

Population Allele frequency vs Sample Allele frequency

- **Population allele frequency:** probability of finding an allele in the population. Number of individuals carrying an allele vs total of individuals in the population.

Population Allele frequency vs Sample Allele frequency

- **<u>Alternate/Base allele frequency</u>**: number of reads supporting the alternate allele vs total of reads.



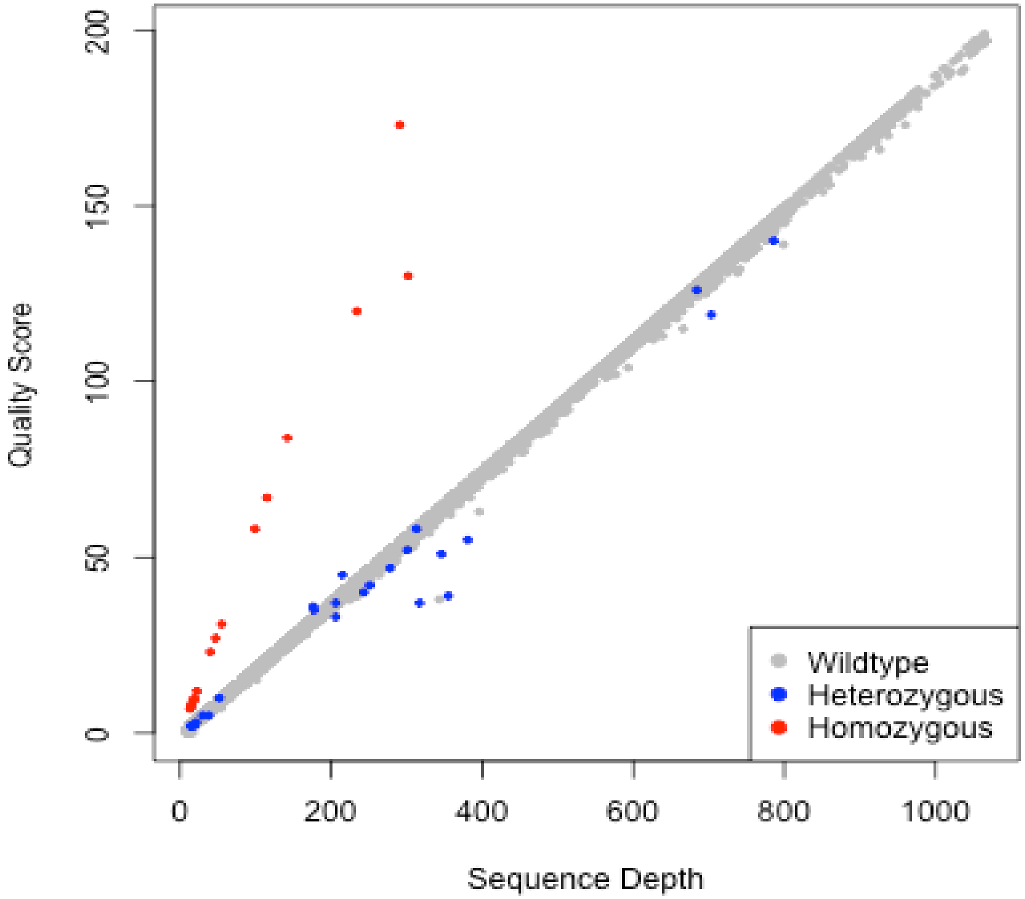
VARSCAN2

- Uses a heuristic/statistic method instead of bayesian.
- Allows more flexibility and hard filters.

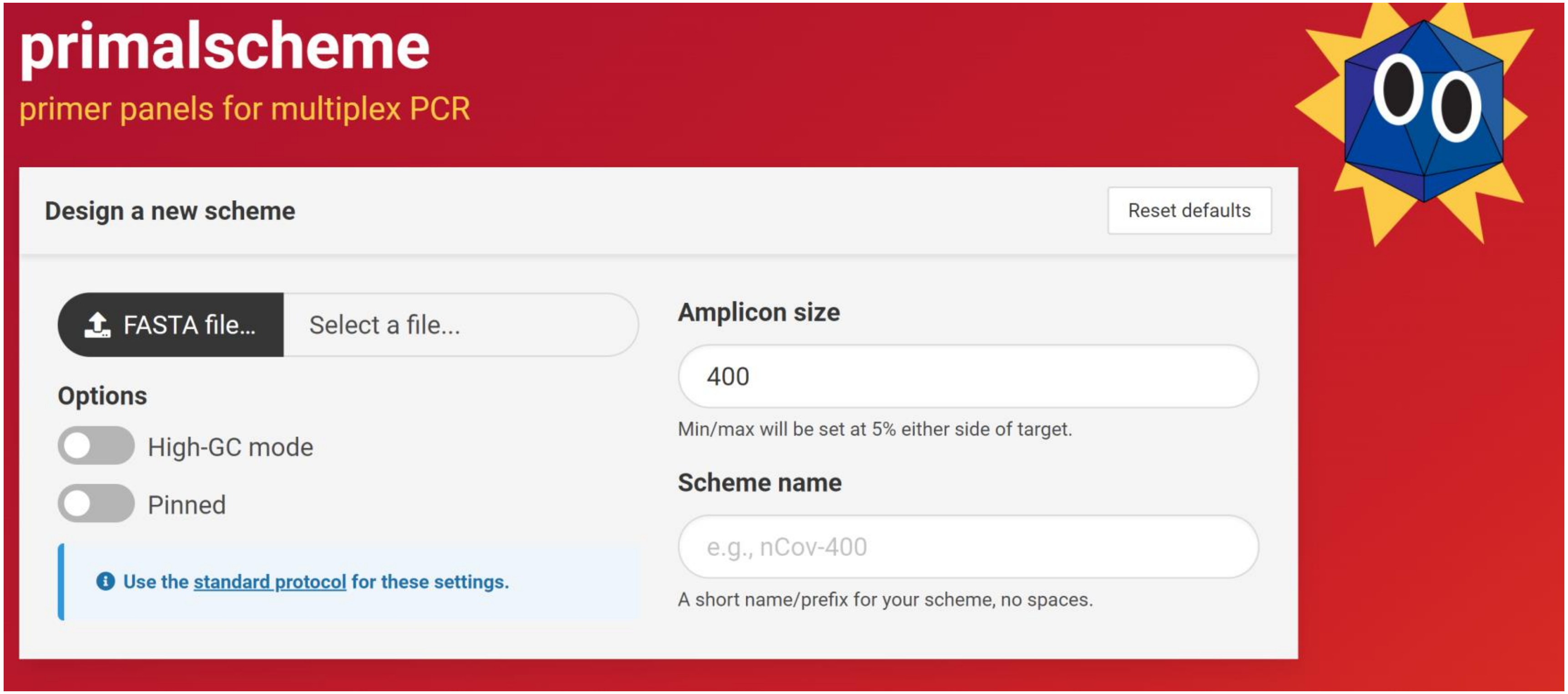
```
samtools pileup -f reference.fasta myData.bam | java -jar VarScan.v2.2.jar pileup2snp
```

http://varscan.sourceforge.net/

VARSCAN2


Quality Score
0
50
100
150
200
0
200
400
600
800
1000
Sequence Depth
● Wildtype
● Heterozygous
● Homozygous

IVAR



primalscheme

primer panels for multiplex PCR

Design a new scheme

Options

○ High-GC mode

○ Pinned

Use the standard protocol for these settings.

Amplicon size: 400

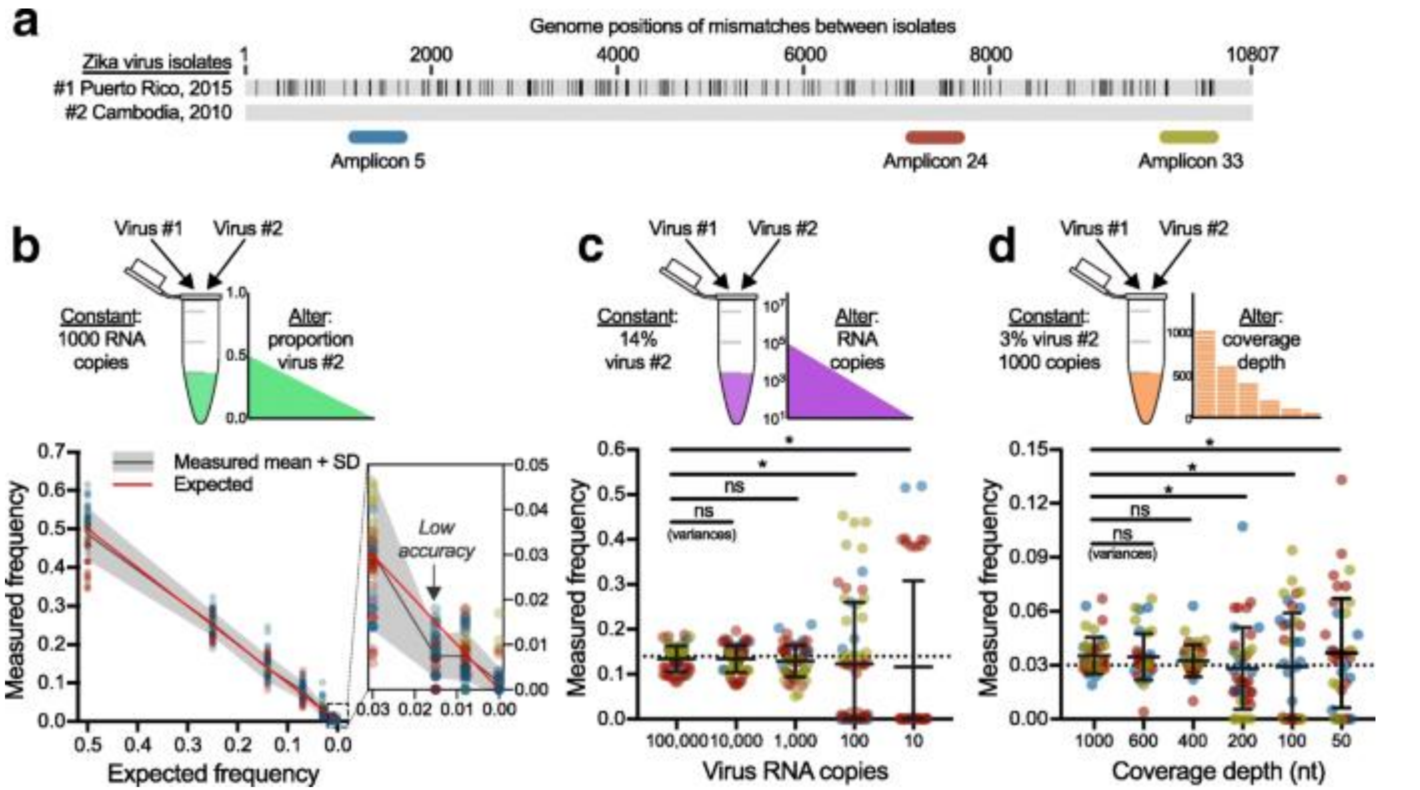
Min/max will be set at 5% either side of target.

Scheme name: e.g., nCov-400

A short name/prefix for your scheme, no spaces.

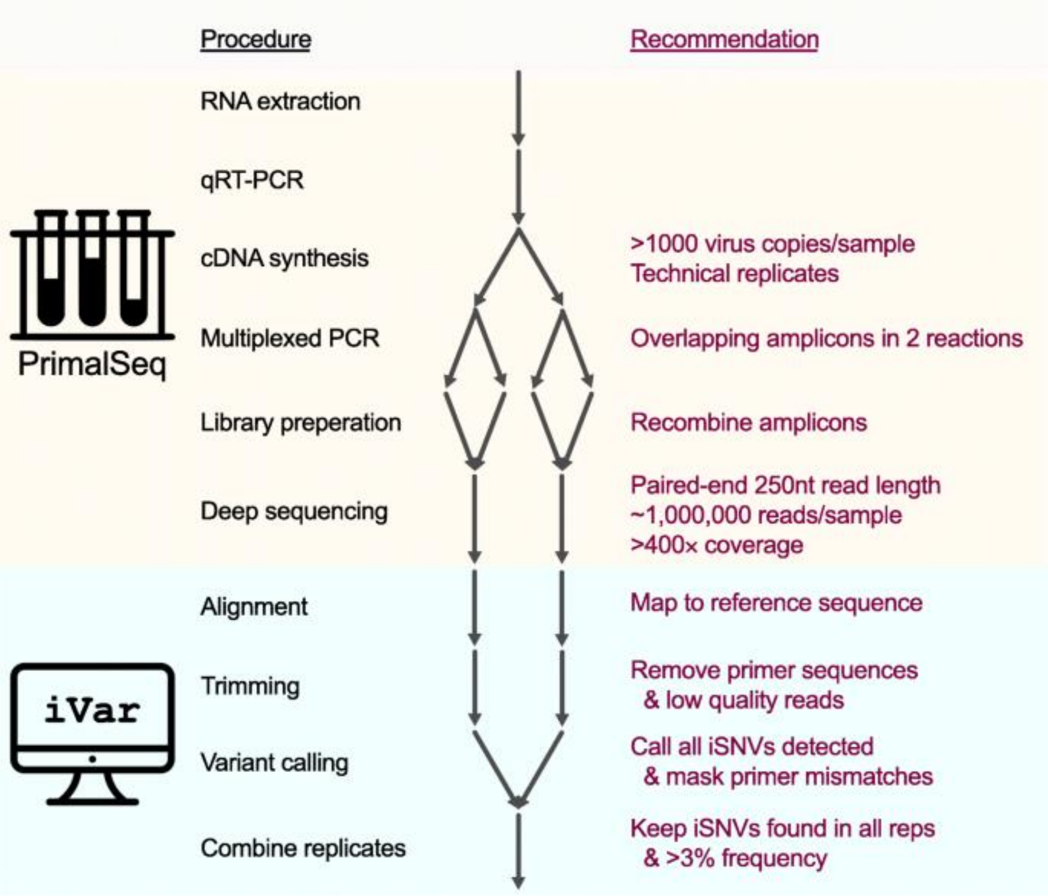
Quick et al. Nature Protoc. 2017

IVAR



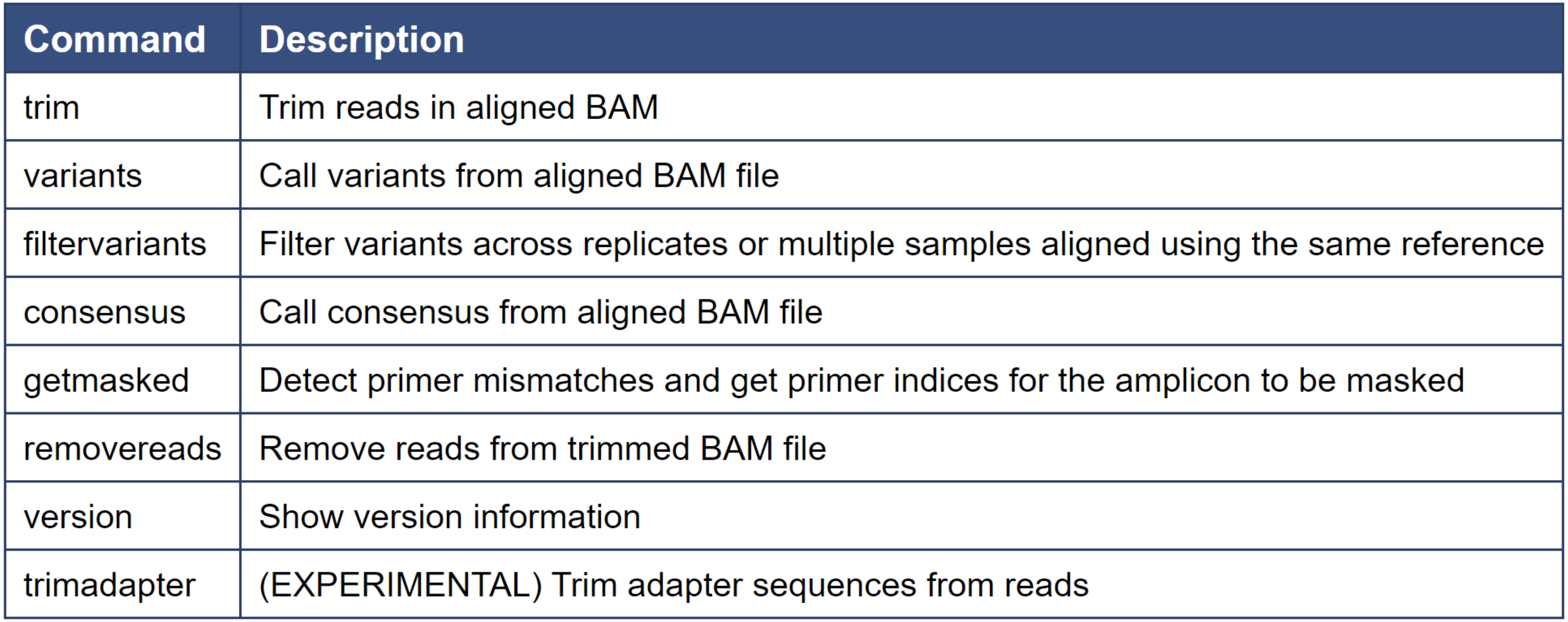
Grubaugh et al. Genome Biology. 2019

IVAR



Grubaugh et al. Genome Biology. 2019

IVAR



Command	Description
trim	Trim reads in aligned BAM
variants	Call variants from aligned BAM file
filtervariants	Filter variants across replicates or multiple samples aligned using the same reference
consensus	Call consensus from aligned BAM file
getmasked	Detect primer mismatches and get primer indices for the amplicon to be masked
removereads	Remove reads from trimmed BAM file
version	Show version information
trimadapter	(EXPERIMENTAL) Trim adapter sequences from reads

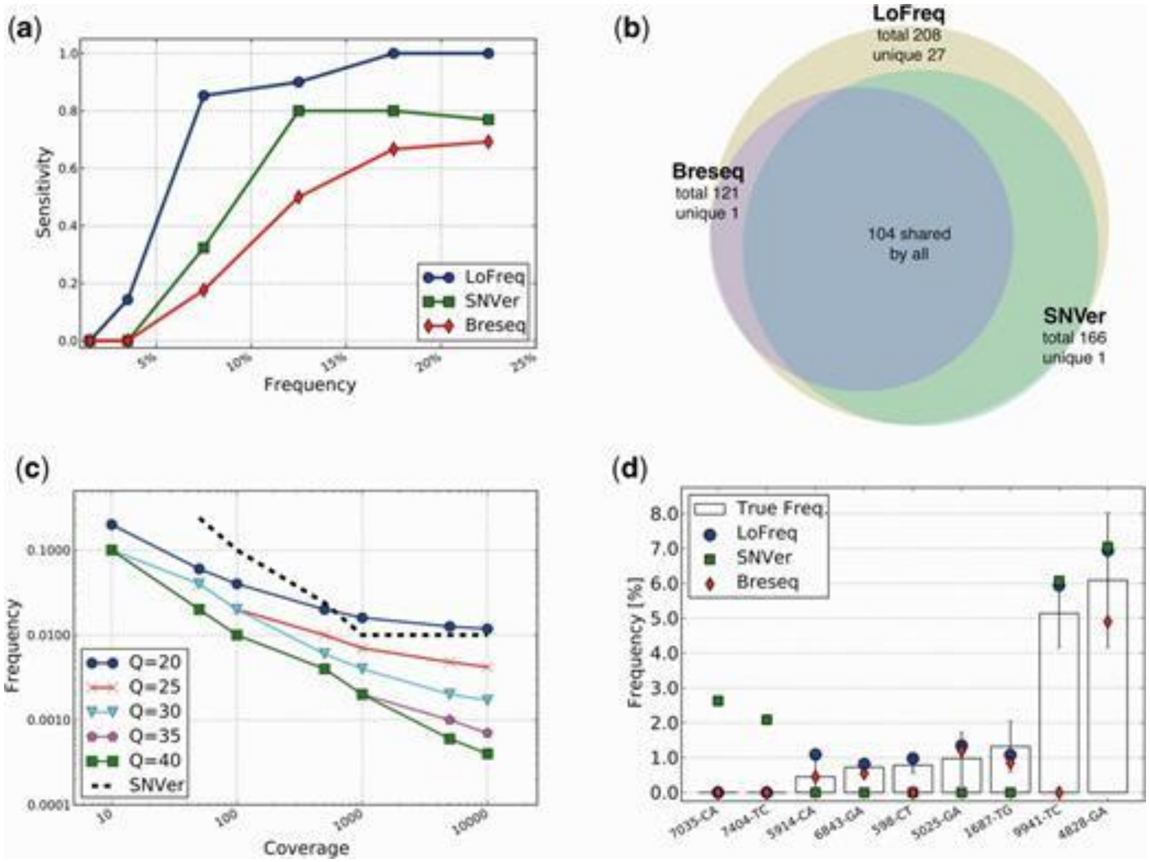
Grubaugh et al. Genome Biology. 2019

IVAR

- Input Options Description
  - -q Minimum quality score threshold to count base (Default: 20)
  - -t Minimum frequency threshold(0 - 1) to call variants (Default: 0.03)
  - -m Minimum read depth to call variants (Default: 0)
  - -r Reference file used for alignment. This is used to translate the nucleotide sequences and identify intra host single nucleotide variants
  - -g A GFF file in the GFF3 format can be supplied to specify coordinates of open reading frames (ORFs). In absence of GFF file, amino acid translation will not be done.
- Output Options Description
  - -p (Required) Prefix for the output tsv variant file

Grubaugh et al. Genome Biology. 2019

LOFREQ



Wilm et al. Nucleic Acids Res. 2012

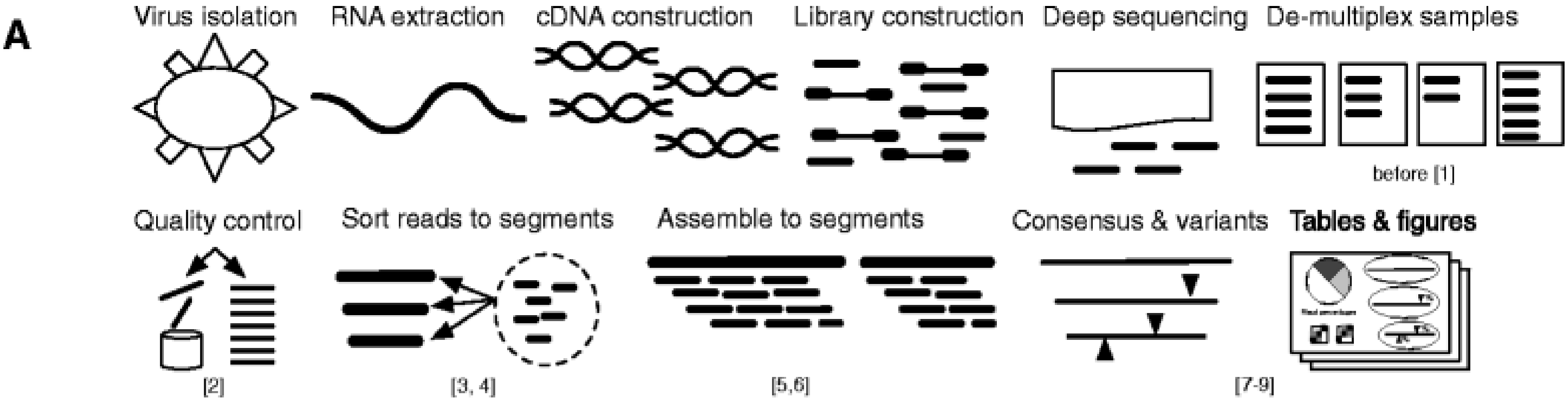
LOFREQ

- **lofreq viterbi:** realignment algorithm
- **lofreq call**: Warning! Only SNPs are called by default.
- **lofreq filter:** vcf filtering.

http://csb5.github.io/lofreq/

IRMA

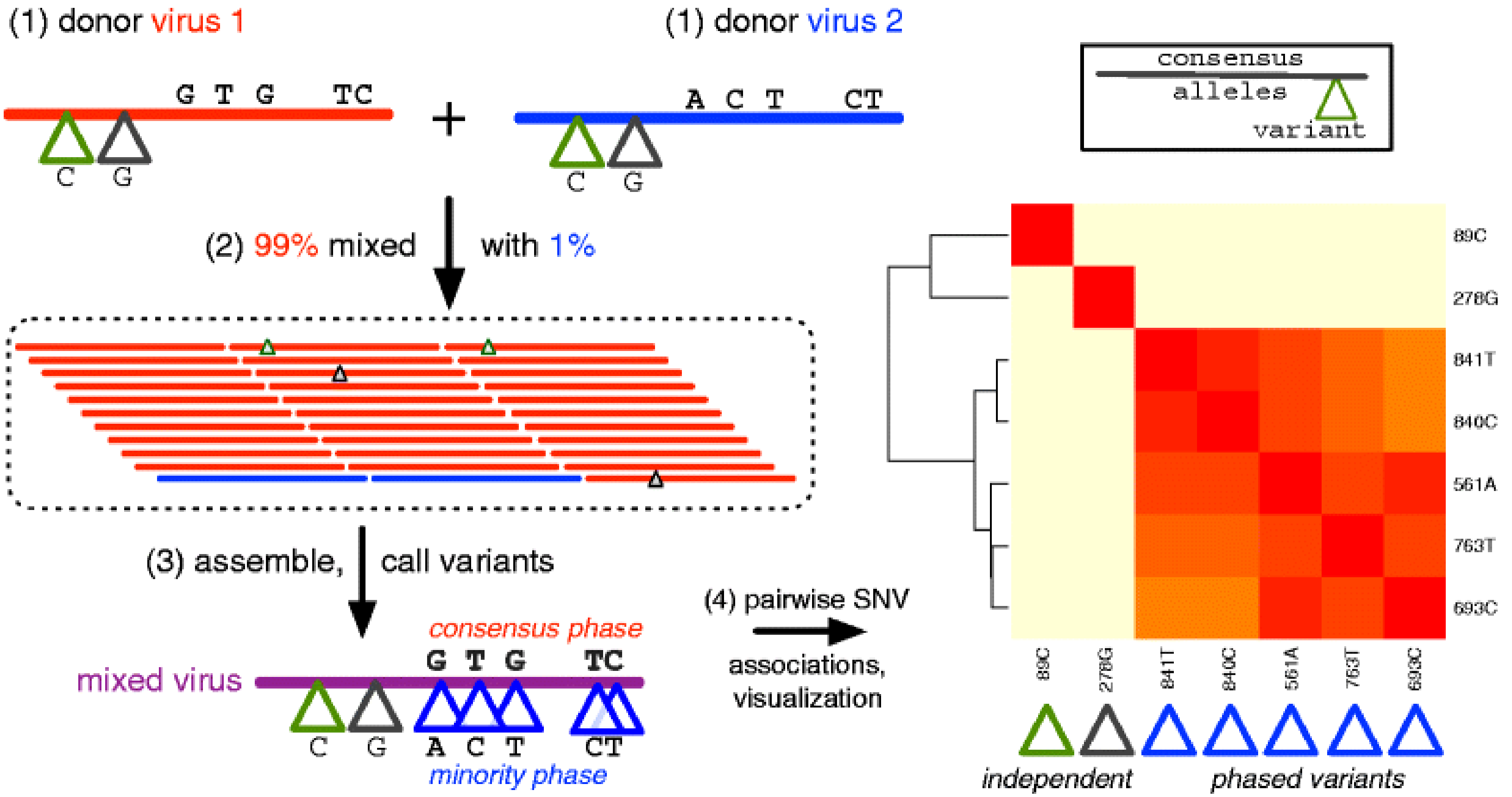
IRMA: Iterative Refinement Meta-Assembler



Shepard et al BMC Genomics 2016, **17**:708

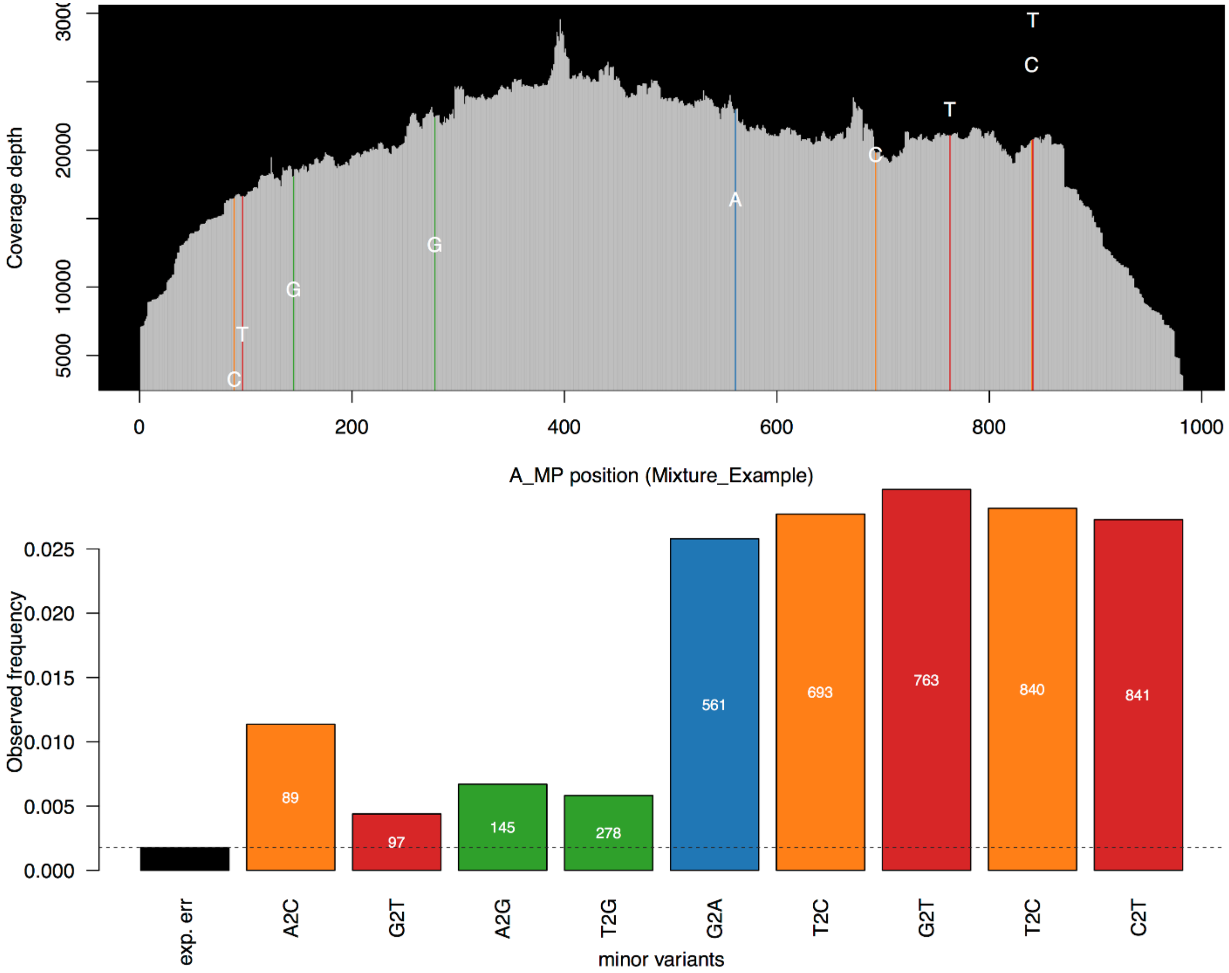
Available for **LU**, ***FLU_AD**, **EBOLA**, & **‡CoV**

IRMA

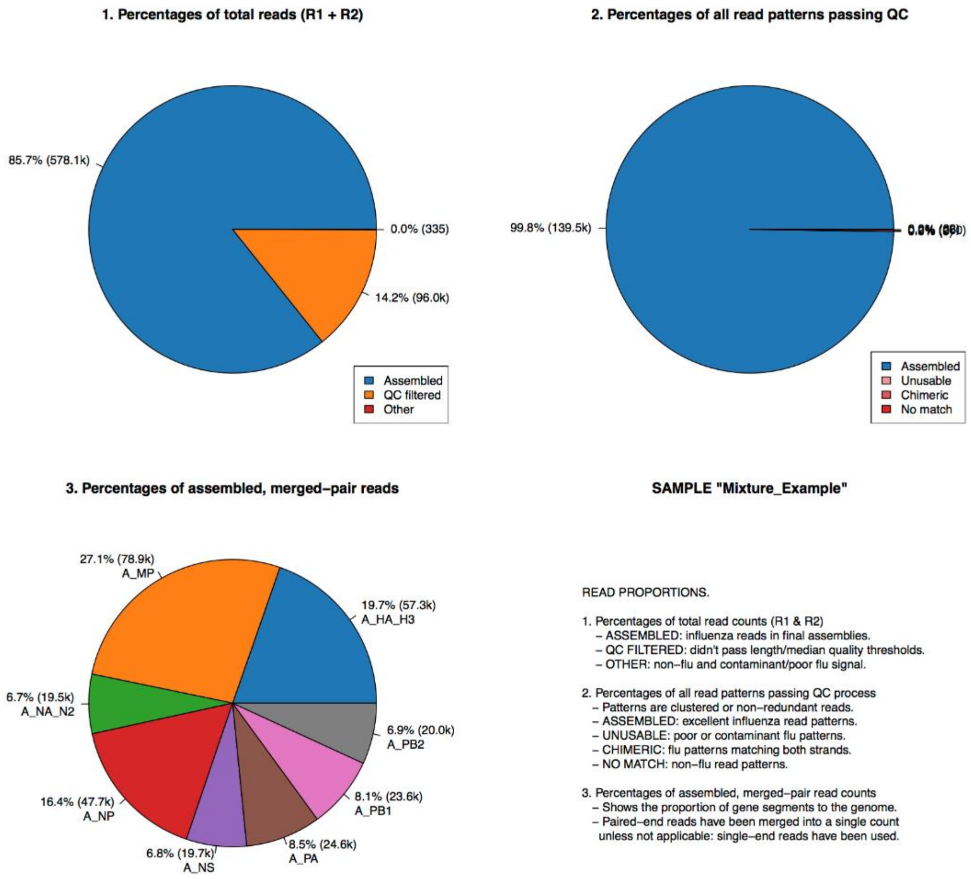


Shepard et al BMC Genomics 2016, **17**:708

IRMA


Coverage depth
300(
20000
10000
5000
T
C
T
C
A
G
G
T
C
0
200
400
600
800
1000
A_MP position (Mixture_Example)
Observed frequency
0.025
0.020
0.015
0.010
0.005
0.000
89
97
145
278
561
693
763
840
841
exp. err
A2C
G2T
A2G
T2G
G2A
T2C
G2T
T2C
C2T
minor variants

IRMA



SAMPLE "Mixture_Example"

READ PROPORTIONS.

1. Percentages of total read counts (R1 & R2)
   - ASSEMBLED: influenza reads in final assemblies.
   - QC FILTERED: didn't pass length/median quality thresholds.
   - OTHER: non–flu and contaminant/poor flu signal.

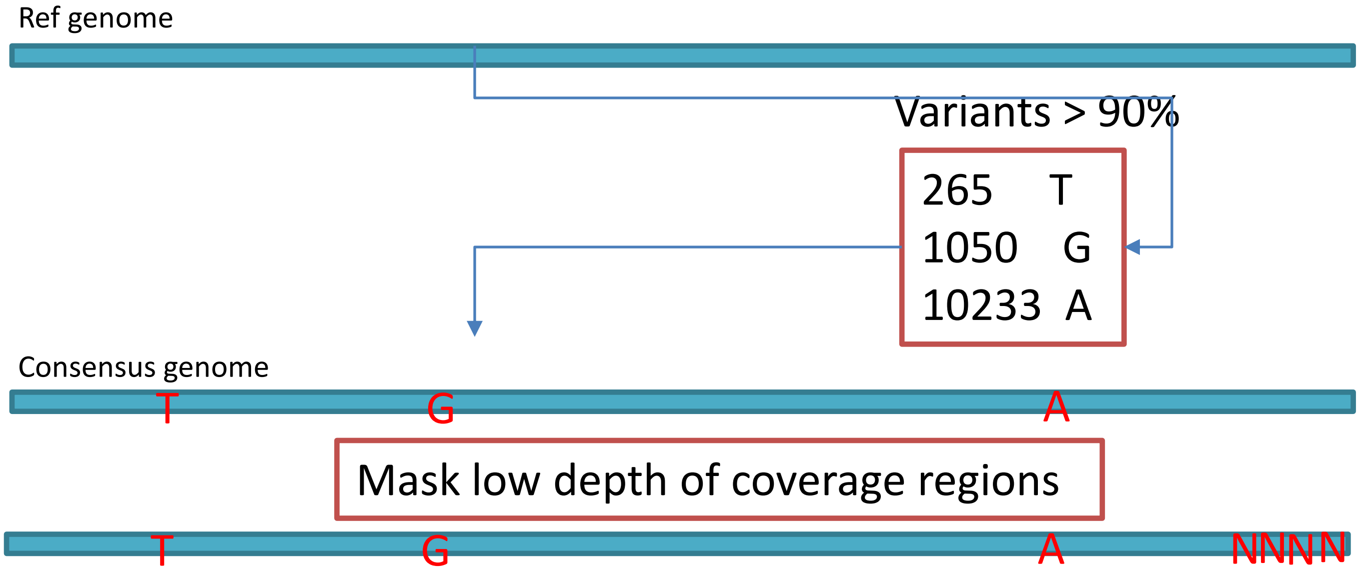
2. Percentages of all read patterns passing QC process
   - Patterns are clustered or non–redundant reads.
   - ASSEMBLED: excellent influenza read patterns.
   - UNUSABLE: poor or contaminant flu patterns.
   - CHIMERIC: flu patterns matching both strands.
   - NO MATCH: non–flu read patterns.

3. Percentages of assembled, merged–pair read counts
   - Shows the proportion of gene segments to the genome.
   - Paired–end reads have been merged into a single count unless not applicable: single–end reads have been used.

Consensus genome

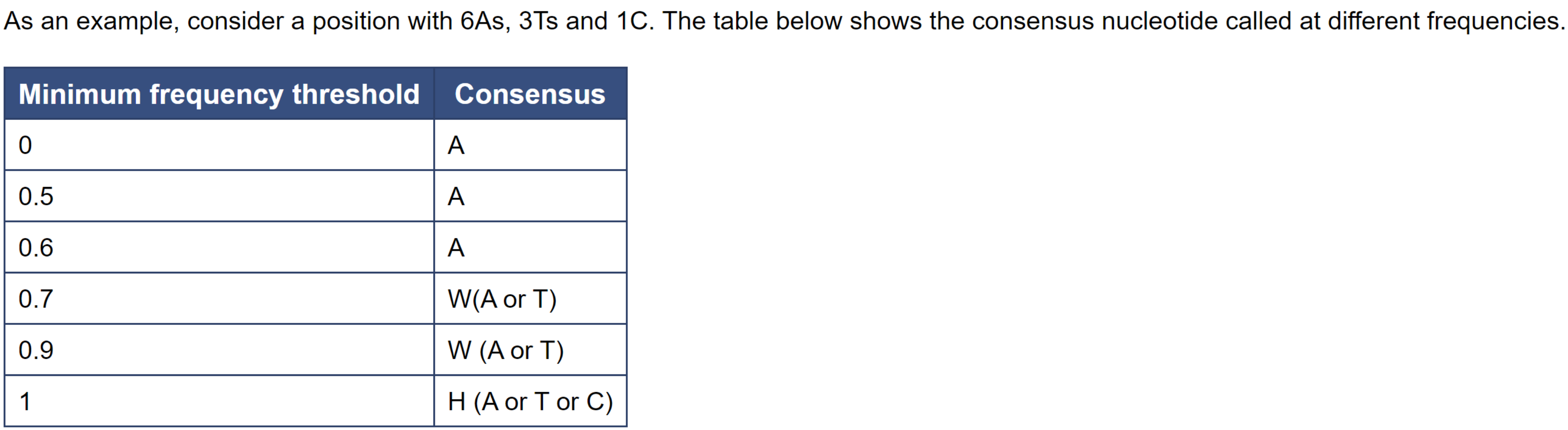
Aproximation 1

- Select variants: > 80% allele frequency
- Include variants in reference genome.
- Mask low frequency positions: <10x.



Consensus genome

- **Approximation 2 (ivar)**
  - Minimum frequency threshold is the minimum frequency that a base must match to be called as the consensus base at a position. If one base is not enough to match a given frequency, then an ambigious nucleotide is called at that position



As an example, consider a position with 6As, 3Ts and 1C. The table below shows the consensus nucleotide called at different frequencies.

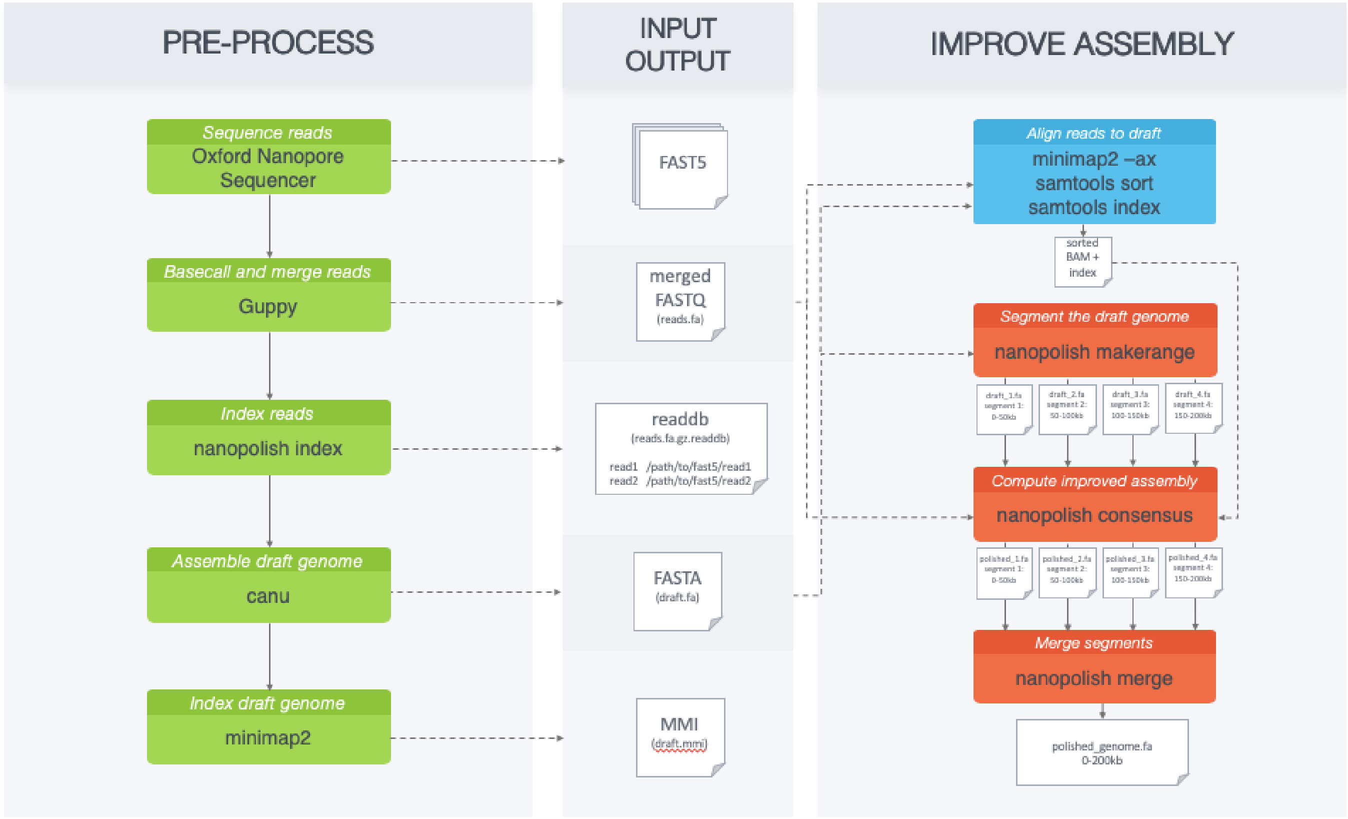
Minimum frequency threshold	Consensus
0	A
0.5	A
0.6	A
0.7	W(A or T)
0.9	W (A or T)
1	H (A or T or C)

Variant Calling in Nanopore

- **Medaka** is a tool to create consensus sequences and variant calls from nanopore sequencing data. This task is performed using neural networks applied a pileup of individual sequencing reads against a draft assembly.
- medaka consensus: Creates a consensus from a draft assembly in fasta format and the nanopore corrected fastq reads.

Variant Calling in Nanopore

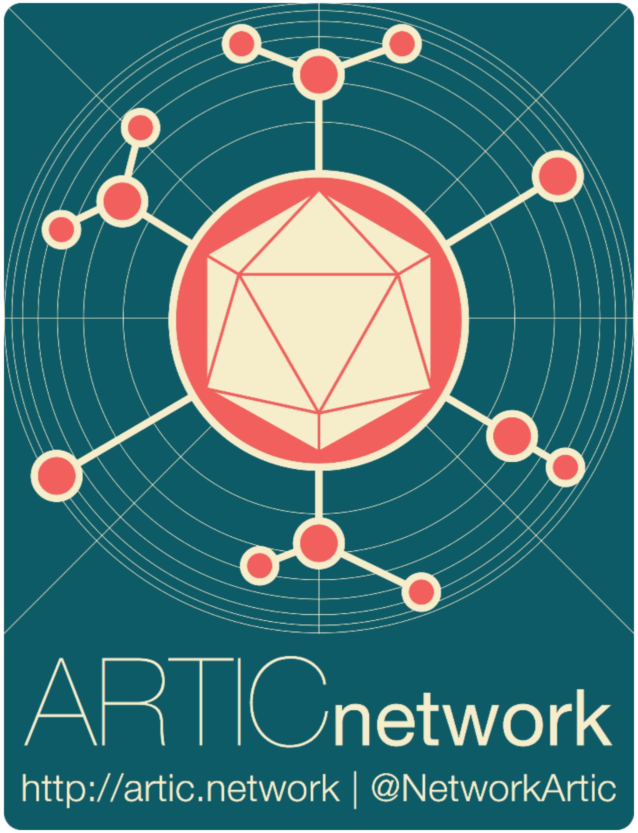
- **Nanopolish** can calculate an improved consensus sequence for a draft genome assembly, detect base modifications, call SNPs and indels with respect to a reference genome and more



Variant Calling in Nanopore

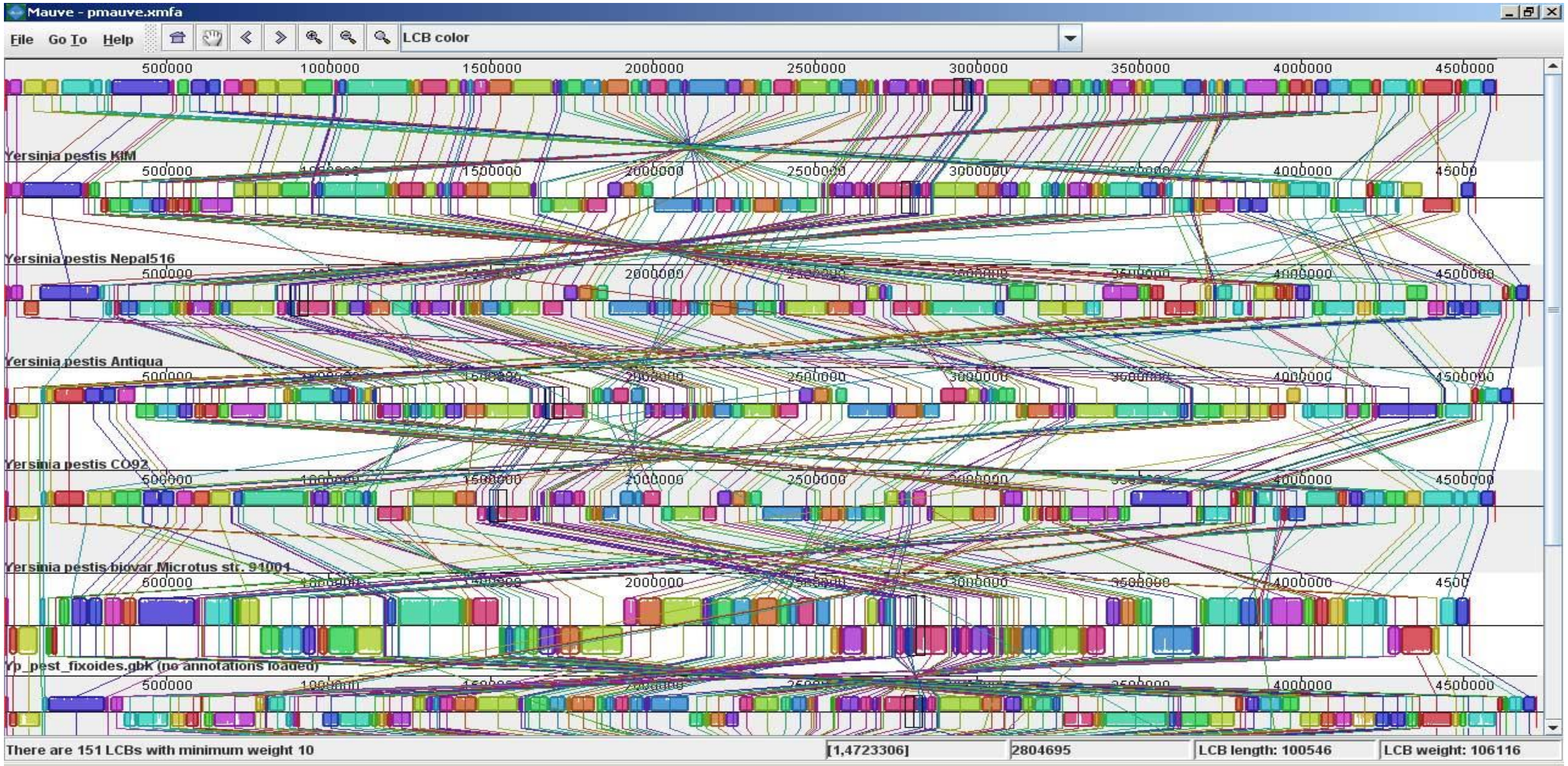
- **Artic protocol**

Full protocol from wet to drylab. Includes basecalling, preprocessing, mapping and consensus generation for Artic primers.



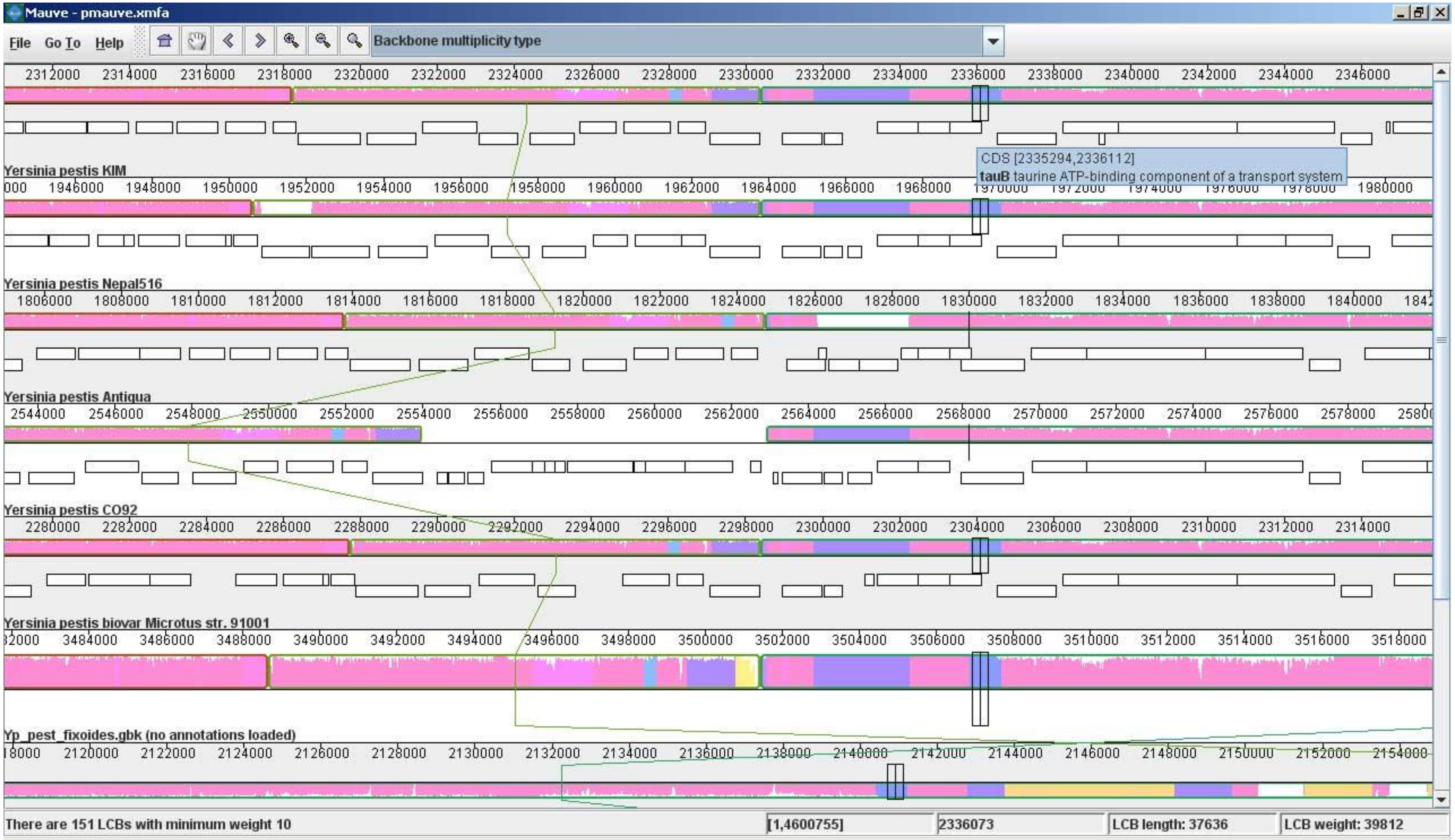
Consensus genome comparison: Mauve

Mauve is a complete genoma aligner



There are 151 LCBs with minimum weight 10 | [1,4723306] | 2804695 | LCB length: 100546 | LCB weight: 106116

Consensus genome comparison: Mauve

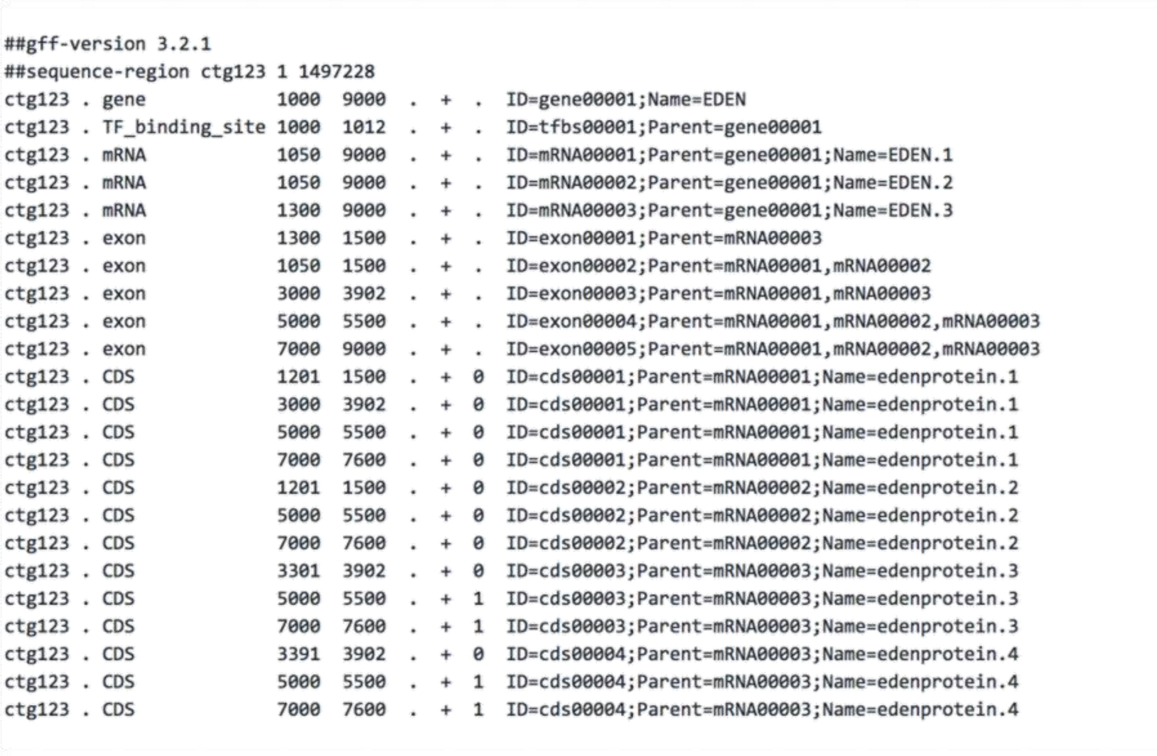


CDS [2335294,2336112]
tauB taurine ATP-binding component of a transport system

There are 151 LCBs with minimum weight 10 | [1,4600755] | 2336073 | LCB length: 37636 | LCB weight: 39812

Annotation format: gff3

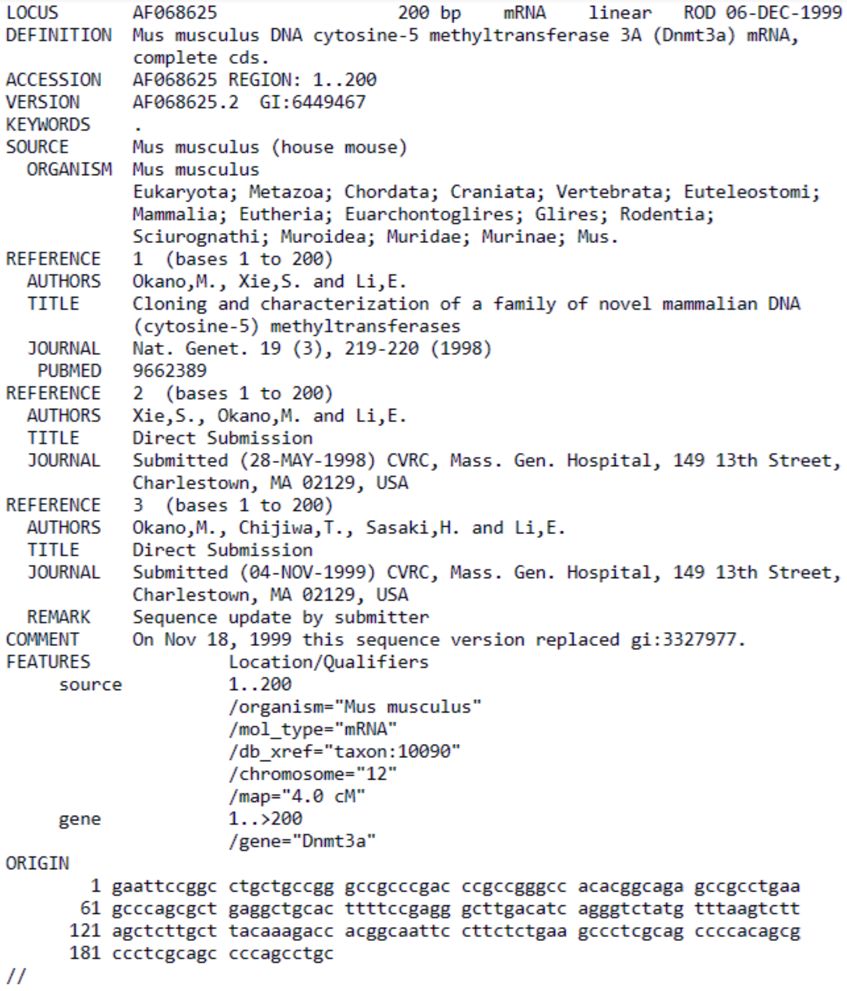
1. Seqid - name
2. Source - program
3. Type – term or SOFA sequence ontology
4. Start
5. End
6. Score
7. Strand – (+/-)
8. Phase – (0/1/2)
9. Attributes
    - Name
    - Alias
    - Parent
    - Target
    - Gap
    - Derives_from
    - Note
    - Dbxref
    - Ontology_term



```
##gff-version 3.2.1
##sequence-region ctg123 1 1497228
ctg123 . gene           1000  9000  .  +  .  ID=gene00001;Name=EDEN
ctg123 . TF_binding_site 1000  1012  .  +  .  ID=tfbs00001;Parent=gene00001
ctg123 . mRNA           1050  9000  .  +  .  ID=mRNA00001;Parent=gene00001;Name=EDEN.1
ctg123 . mRNA           1050  9000  .  +  .  ID=mRNA00002;Parent=gene00001;Name=EDEN.2
ctg123 . mRNA           1300  9000  .  +  .  ID=mRNA00003;Parent=gene00001;Name=EDEN.3
ctg123 . exon           1300  1500  .  +  .  ID=exon00001;Parent=mRNA00003
ctg123 . exon           1050  1500  .  +  .  ID=exon00002;Parent=mRNA00001,mRNA00002
ctg123 . exon           3000  3902  .  +  .  ID=exon00003;Parent=mRNA00001,mRNA00003
ctg123 . exon           5000  5500  .  +  .  ID=exon00004;Parent=mRNA00001,mRNA00002,mRNA00003
ctg123 . exon           7000  9000  .  +  .  ID=exon00005;Parent=mRNA00001,mRNA00002,mRNA00003
ctg123 . CDS            1201  1500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS            3000  3902  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS            5000  5500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS            7000  7600  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS            1201  1500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS            5000  5500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS            7000  7600  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS            3301  3902  .  +  0  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS            5000  5500  .  +  1  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS            7000  7600  .  +  1  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS            3391  3902  .  +  0  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS            5000  5500  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS            7000  7600  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
```

Annotation format: gbk

- LOCUS – Annotated sequence
- DEFINITION
- ACCESION
- FEATURES
  - source
  - gene
  - CDS
    - Locus tag
    - function
    - Product
    - protein_id
    - Translation (sequence)



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LOCUS       AF068625                 200 bp    mRNA    linear   ROD 06-DEC-1999
DEFINITION  Mus musculus DNA cytosine-5 methyltransferase 3A (Dnmt3a) mRNA,
            complete cds.
ACCESSION   AF068625 REGION: 1..200
VERSION     AF068625.2  GI:6449467
KEYWORDS    .
SOURCE      Mus musculus (house mouse)
  ORGANISM  Mus musculus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Glires; Rodentia;
            Sciurognathi; Muroidea; Muridae; Murinae; Mus.
REFERENCE   1  (bases 1 to 200)
  AUTHORS   Okano,M., Xie,S. and Li,E.
  TITLE     Cloning and characterization of a family of novel mammalian DNA
            (cytosine-5) methyltransferases
  JOURNAL   Nat. Genet. 19 (3), 219-220 (1998)
   PUBMED   9662389
REFERENCE   2  (bases 1 to 200)
  AUTHORS   Xie,S., Okano,M. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (28-MAY-1998) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
REFERENCE   3  (bases 1 to 200)
  AUTHORS   Okano,M., Chijiwa,T., Sasaki,H. and Li,E.
  TITLE     Direct Submission
  JOURNAL   Submitted (04-NOV-1999) CVRC, Mass. Gen. Hospital, 149 13th Street,
            Charlestown, MA 02129, USA
  REMARK    Sequence update by submitter
COMMENT     On Nov 18, 1999 this sequence version replaced gi:3327977.
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /db_xref="taxon:10090"
                     /chromosome="12"
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     gene            1..>200
                     /gene="Dnmt3a"
ORIGIN
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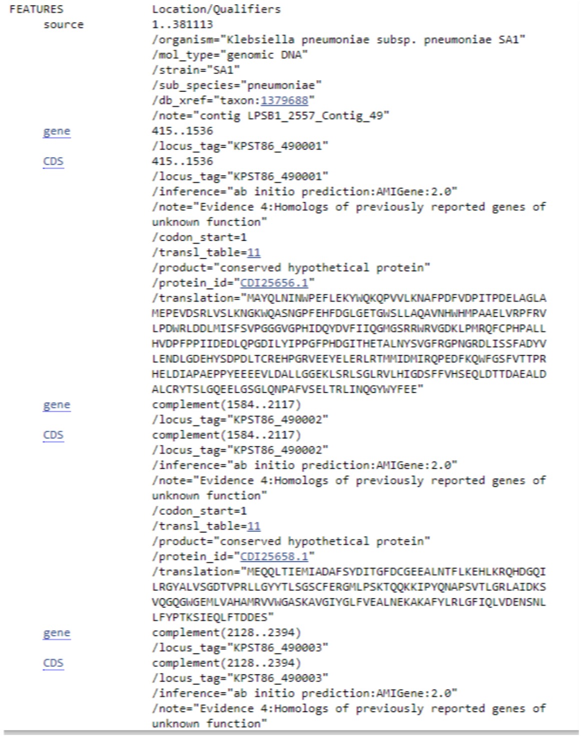
       61 gcccagcgct gaggctgcac ttttccgagg gcttgacatc agggtctatg tttaagtctt
      121 agctcttgct tacaaagacc acggcaattc cttctctgaa gccctcgcag ccccacagcg
      181 ccctcgcagc cccagcctgc
//
```

Annotation format: gbk

- LOCUS – Annotated sequence
- DEFINITION
- ACCESION
- FEATURES
  - source
  - gene
  - CDS
    - Locus tag
    - function
    - Product
    - protein_id
    - Translation (sequence)



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                     /strain="SA1"
                     /sub_species="pneumoniae"
                     /db_xref="taxon:1379688"
                     /note="contig LPSB1_2557_Contig_49"
     gene            415..1536
                     /locus_tag="KPST86_490001"
     CDS             415..1536
                     /locus_tag="KPST86_490001"
                     /inference="ab initio prediction:AMIGene:2.0"
                     /note="Evidence 4:Homologs of previously reported genes of
                     unknown function"
                     /codon_start=1
                     /transl_table=11
                     /product="conserved hypothetical protein"
                     /protein_id="CDI25656.1"
                     /translation="MAYQLNINWPEFLEKYWQKQPVVLKNAFPDFVDPITPDELAGLA
                     MEPEVDSRLVSLKNGKWQASNGPFEHFDGLGETGWSLLAQAVNHWHMPAAELVRPFRV
                     LPDWRLDDLMISFSVPGGGVGPHIDQYDVFIIQGMGSRRWRVGDKLPMRQFCPHPALL
                     HVDPFPPIIDEDLQPGDILYIPPGFPHDGITHETALNYSVGFRGPNGRDLISSFADYV
                     LENDLGDEHYSDPDLTCREHPGRVEEYELERLRTMMIDMIRQPEDFKQWFGSFVTTPR
                     HELDIAPAEPPYEEEEVLDALLGGEKLSRLSGLRVLHIGDSFFVHSEQLDTTDAEALD
                     ALCRYTSLGQEELGSGLQNPAFVSELTRLINQGYWYFEE"
     gene            complement(1584..2117)
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                     /note="Evidence 4:Homologs of previously reported genes of
                     unknown function"
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                     /transl_table=11
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                     LFYPTKSIEQLFTDDES"
     gene            complement(2128..2394)
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     CDS             complement(2128..2394)
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                     /note="Evidence 4:Homologs of previously reported genes of
                     unknown function"
```

Variants annotation

SnpEff is a variant annotation and effect prediction tool. It annotates and predicts the effects of genetic variants (such as amino acid changes).

It needs an annotation database, there are few for virus as default, commonly you need to build it using a gff file if available.

Output: annotated vcf

SnpSift converts snpeff output to a table.

Thanks for your attention!