Aligning SGS reads
Calling SNP

Philippe Bardou
Cédric Cabau

Organisation

First day :

Morning (9h00 - 12h00)

- Sequence quality
- Read mapping

Afternoon (13h30 - 17h)

- SAM format
- Visualisation

Second day :

Morning (9h00 - 12h00)

- Variant calling (VCF)
- Variant filtering/annotation

Afternoon (13h30 - 17h)

- Nextflow / Sarek

Organisation

All the course material (slides, input and output data, practical exercises, correction) is available online:

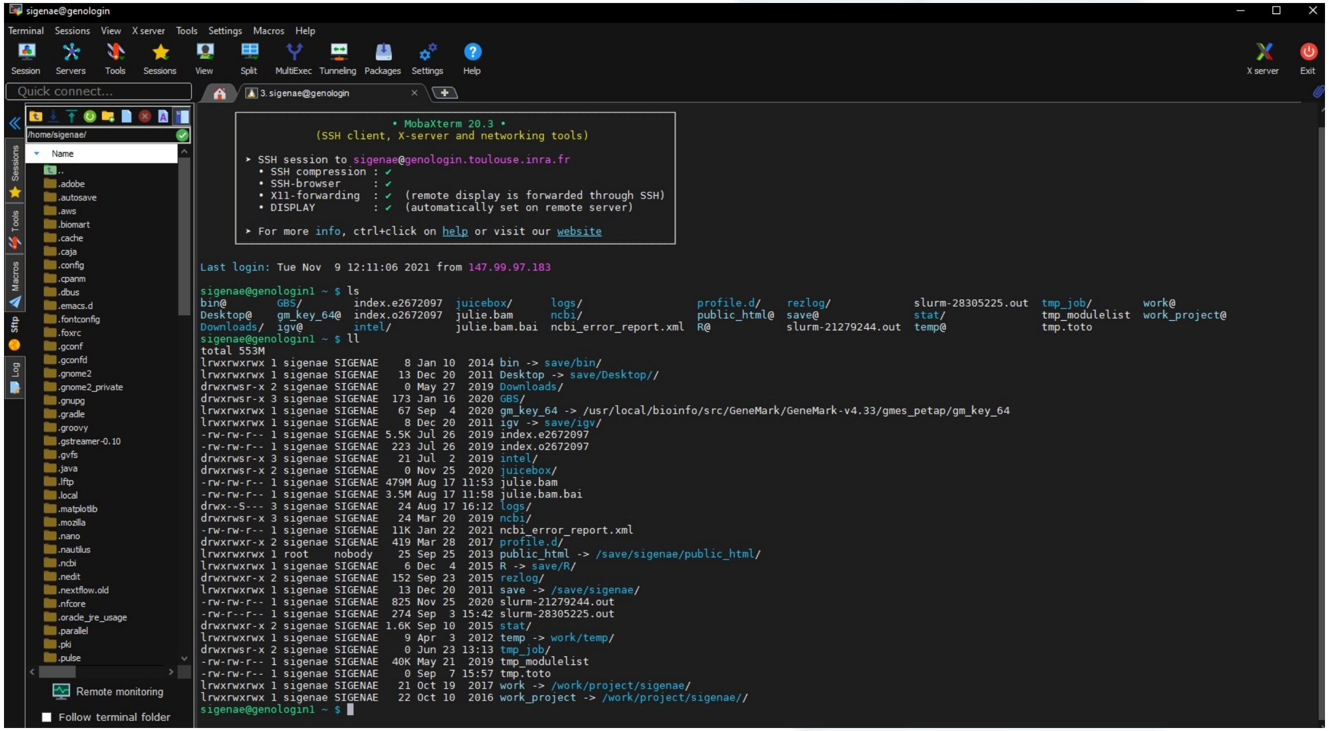
- https://web-genobioinfo.toulouse.inrae.fr/~formation/16_SGS-SNP/
- https://bios4biol.pages.mia.inra.fr/training-aln-variant-calling/

What are you going to learn?

- To extract reads and reference genome from the NCBI
- To verify the read quality
- To align the reads on the reference genome
- To improve alignment and to recalibrate SGS data
- To call variants (SNP and INDEL)
- To visualise the alignments and variations
- To annotate variants
- To filter variants

What you should already know?

- How to connect to a remote unix server (mobaXterm)?
- What a unix command looks like?
- How to move around the unix environment?
- How to submit jobs?
- How to edit a file?

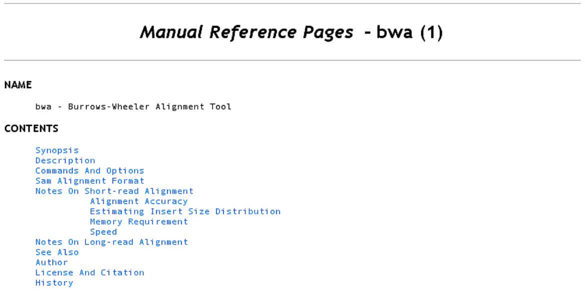


```
                    • MobaXterm 20.3 •
         (SSH client, X-server and networking tools)

 ➤ SSH session to sigenae@genologin.toulouse.inra.fr
   • SSH compression : ✓
   • SSH-browser     : ✓
   • X11-forwarding  : ✓  (remote display is forwarded through SSH)
   • DISPLAY         : ✓  (automatically set on remote server)

 ➤ For more info, ctrl+click on help or visit our website

Last login: Tue Nov  9 12:11:06 2021 from 147.99.97.183

sigenae@genologin1 ~ $ ls
bin@        GBS/        index.e2672097  juicebox/      logs/                  profile.d/     rezlog/              slurm-28305225.out  tmp_job/         work@
Desktop@    gm_key_64@  index.o2672097  julie.bam      ncbi/                  public_html@   save@                stat/               tmp_modulelist   work_project@
Downloads/  igv@        intel/          julie.bam.bai  ncbi_error_report.xml  R@             slurm-21279244.out   temp@               tmp.toto
sigenae@genologin1 ~ $ ll
total 553M
lrwxrwxrwx 1 sigenae SIGENAE    8 Jan 10  2014 bin -> save/bin/
lrwxrwxrwx 1 sigenae SIGENAE   13 Dec 20  2011 Desktop -> save/Desktop//
drwxrwsr-x 2 sigenae SIGENAE    0 May 27  2019 Downloads/
drwxrwsr-x 3 sigenae SIGENAE  173 Jan 16  2020 GBS/
lrwxrwxrwx 1 sigenae SIGENAE   67 Sep  4  2020 gm_key_64 -> /usr/local/bioinfo/src/GeneMark/GeneMark-v4.33/gmes_petap/gm_key_64
lrwxrwxrwx 1 sigenae SIGENAE    8 Dec 20  2011 igv -> save/igv/
-rw-rw-r-- 1 sigenae SIGENAE 5.5K Jul 26  2019 index.e2672097
-rw-rw-r-- 1 sigenae SIGENAE  223 Jul 26  2019 index.o2672097
drwxrwsr-x 3 sigenae SIGENAE   21 Jul  2  2019 intel/
drwxrwsr-x 2 sigenae SIGENAE    0 Nov 25  2020 juicebox/
-rw-rw-r-- 1 sigenae SIGENAE 479M Aug 17 11:53 julie.bam
-rw-rw-r-- 1 sigenae SIGENAE 3.5M Aug 17 11:58 julie.bam.bai
drwx--S--- 3 sigenae SIGENAE   24 Aug 17 16:12 logs/
drwxrwsr-x 3 sigenae SIGENAE   24 Mar 20  2019 ncbi/
-rw-rw-r-- 1 sigenae SIGENAE  11K Jan 22  2021 ncbi_error_report.xml
drwxrwxr-x 2 sigenae SIGENAE  419 Mar 28  2017 profile.d/
lrwxrwxrwx 1 root    nobody    25 Sep 25  2013 public_html -> /save/sigenae/public_html/
lrwxrwxrwx 1 sigenae SIGENAE    6 Dec  4  2015 R -> save/R/
drwxrwxr-x 2 sigenae SIGENAE  152 Sep 23  2015 rezlog/
lrwxrwxrwx 1 sigenae SIGENAE   13 Dec 20  2011 save -> /save/sigenae/
-rw-rw-r-- 1 sigenae SIGENAE  825 Nov 25  2020 slurm-21279244.out
-rw-r--r-- 1 sigenae SIGENAE  274 Sep  3 15:42 slurm-28305225.out
drwxrwxr-x 2 sigenae SIGENAE 1.6K Sep 10  2015 stat/
lrwxrwxrwx 1 sigenae SIGENAE    9 Apr  3  2012 temp -> work/temp/
drwxrwsr-x 2 sigenae SIGENAE    0 Jun 23 13:13 tmp_job/
-rw-rw-r-- 1 sigenae SIGENAE  40K May 21  2019 tmp_modulelist
-rw-rw-r-- 1 sigenae SIGENAE    0 Sep  7 15:57 tmp.toto
lrwxrwxrwx 1 sigenae SIGENAE   21 Oct 19  2017 work -> /work/project/sigenae/
lrwxrwxrwx 1 sigenae SIGENAE   22 Oct 10  2016 work_project -> /work/project/sigenae//
sigenae@genologin1 ~ $
```

The pieces of software

- Fastqc : quality control
- BWA : alignment
- Samtools & Picard-tools : manipulation of SAM/BAM files
- IGV : visualisation
- GATK : preprocess and variant calling
- SNPeff / SNPsift : annotate/filter variants

gatk



Manual Reference Pages - bwa (1)

NAME

bwa - Burrows-Wheeler Alignment Tool

CONTENTS

```
Synopsis
Description
Commands And Options
Sam Alignment Format
Notes On Short-read Alignment
          Alignment Accuracy
          Estimating Insert Size Distribution
          Memory Requirement
          Speed
Notes On Long-read Alignment
See Also
Author
License And Citation
History
```

igv

Babraham Institute Babraham Bioinformatics FastQC

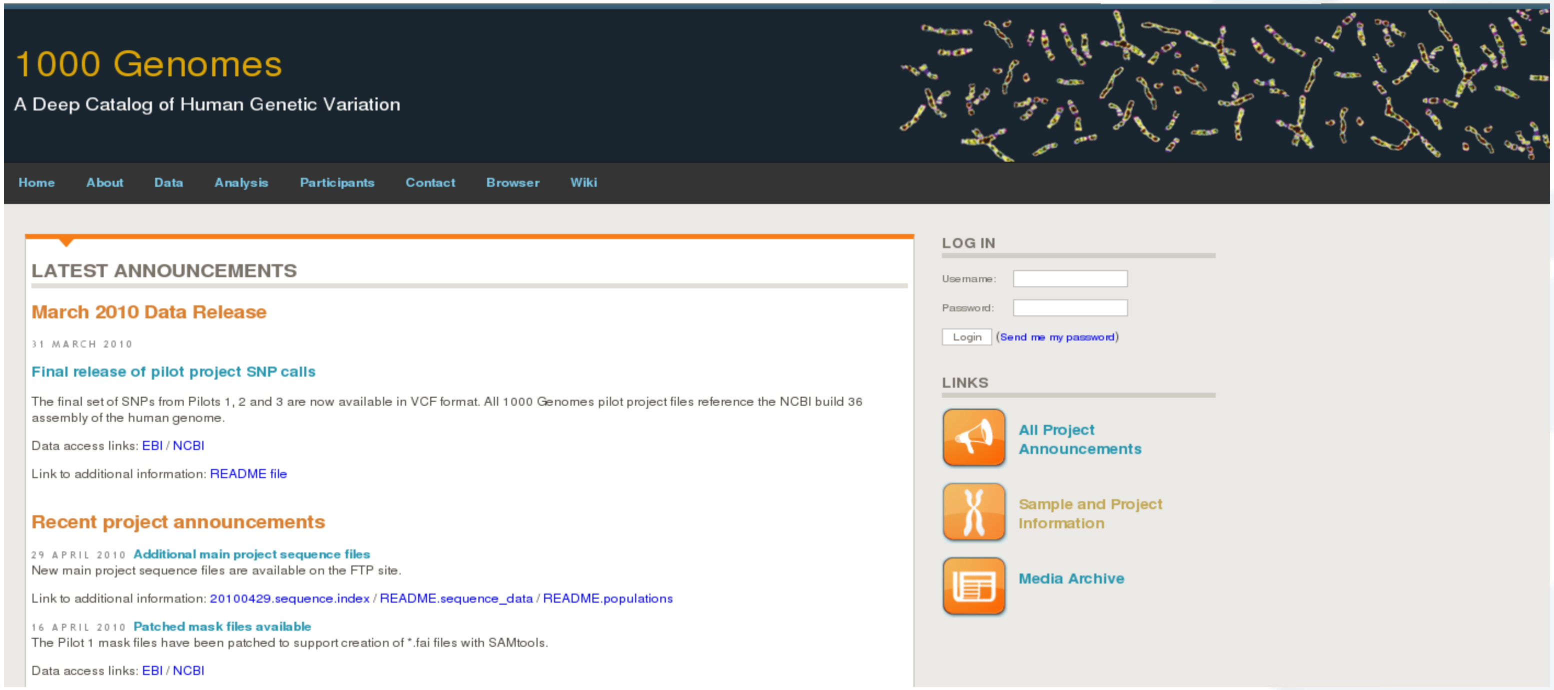
SnpEff & SnpSift

SAMtools

Picard

The 1000 genomes project

- Joint project NCBI / EBI
- Common data formats :
  - FASTQ (Raw data)
  - SAM (Sequence Alignment/Map)
  - VCF (Variant Call Format)



1000 Genomes

A Deep Catalog of Human Genetic Variation

Home About Data Analysis Participants Contact Browser Wiki

LATEST ANNOUNCEMENTS

March 2010 Data Release

31 MARCH 2010

Final release of pilot project SNP calls

The final set of SNPs from Pilots 1, 2 and 3 are now available in VCF format. All 1000 Genomes pilot project files reference the NCBI build 36 assembly of the human genome.

Data access links: EBI / NCBI

Link to additional information: README file

Recent project announcements

29 APRIL 2010 Additional main project sequence files
New main project sequence files are available on the FTP site.

Link to additional information: 20100429.sequence.index / README.sequence_data / README.populations

16 APRIL 2010 Patched mask files available
The Pilot 1 mask files have been patched to support creation of *.fai files with SAMtools.

Data access links: EBI / NCBI

LOG IN

Username:

Password:

Login (Send me my password)

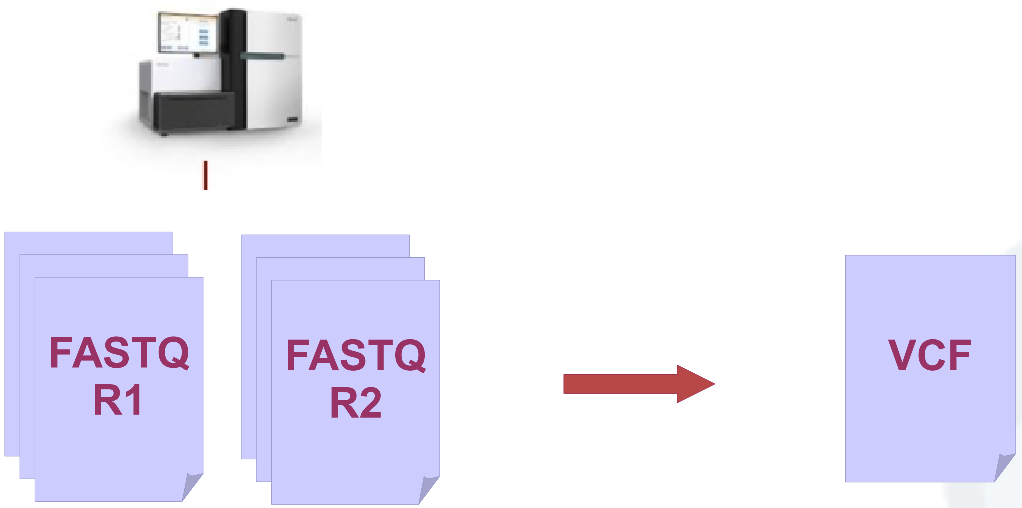
LINKS

All Project Announcements

Sample and Project Information

Media Archive

Overview



FASTQ R1

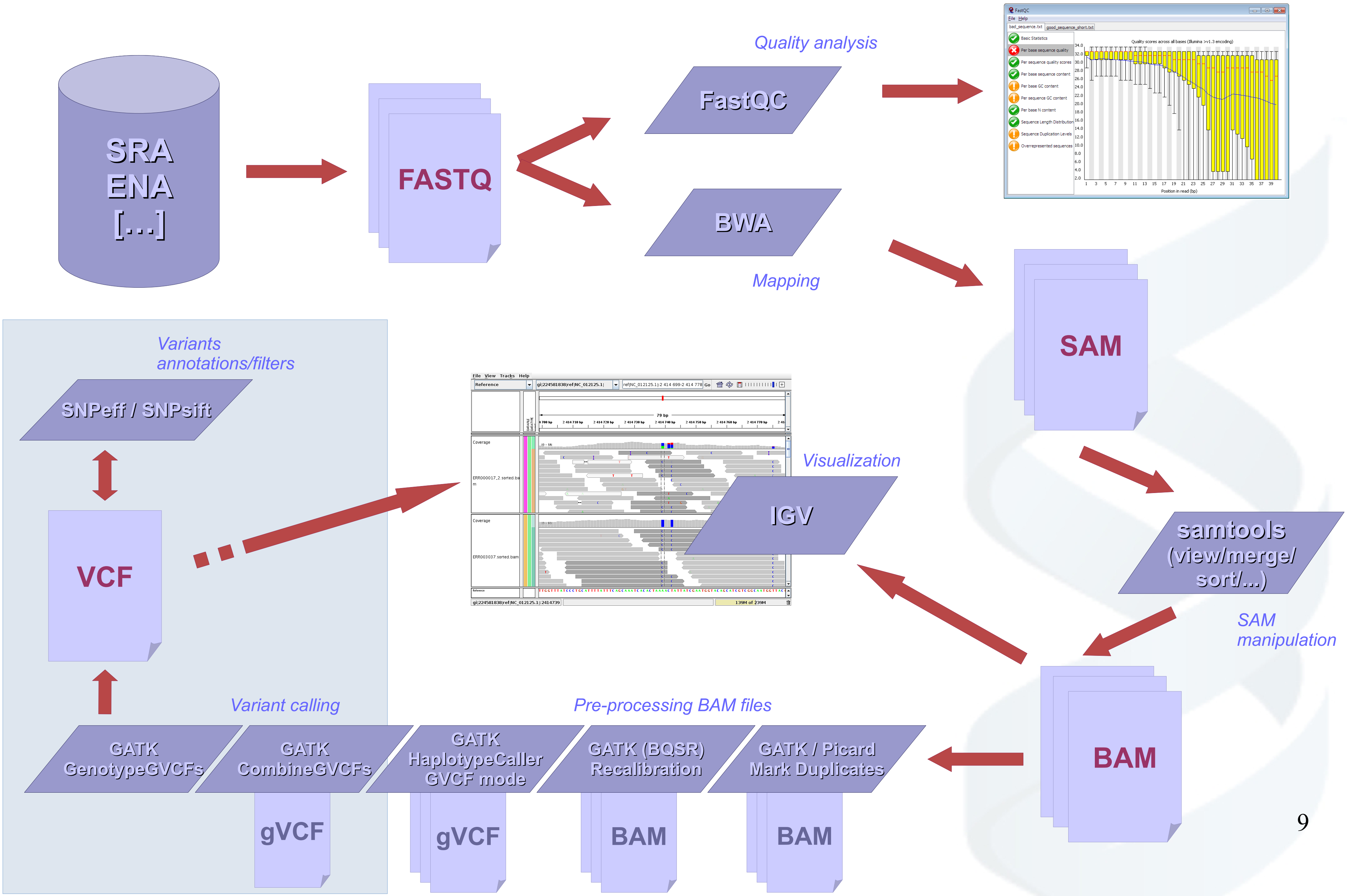
FASTQ R2

VCF

Raw data - Sequences

Variants

Overview



SRA
ENA
[...]

FASTQ

Quality analysis

FastQC

FastQC
File Help
bad_sequence.txt | good_sequence_short.txt

- ✓ Basic Statistics
- ✗ Per base sequence quality
- ✓ Per sequence quality scores
- ✓ Per base sequence content
- ! Per base GC content
- ! Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ! Sequence Duplication Levels
- ! Overrepresented sequences

BWA

Mapping

SAM

samtools
(view/merge/
sort/...)

SAM manipulation

BAM

Visualization

IGV

Pre-processing BAM files

GATK / Picard
Mark Duplicates

BAM

GATK (BQSR)
Recalibration

BAM

GATK
HaplotypeCaller
GVCF mode

gVCF

Variant calling

GATK
CombineGVCFs

gVCF

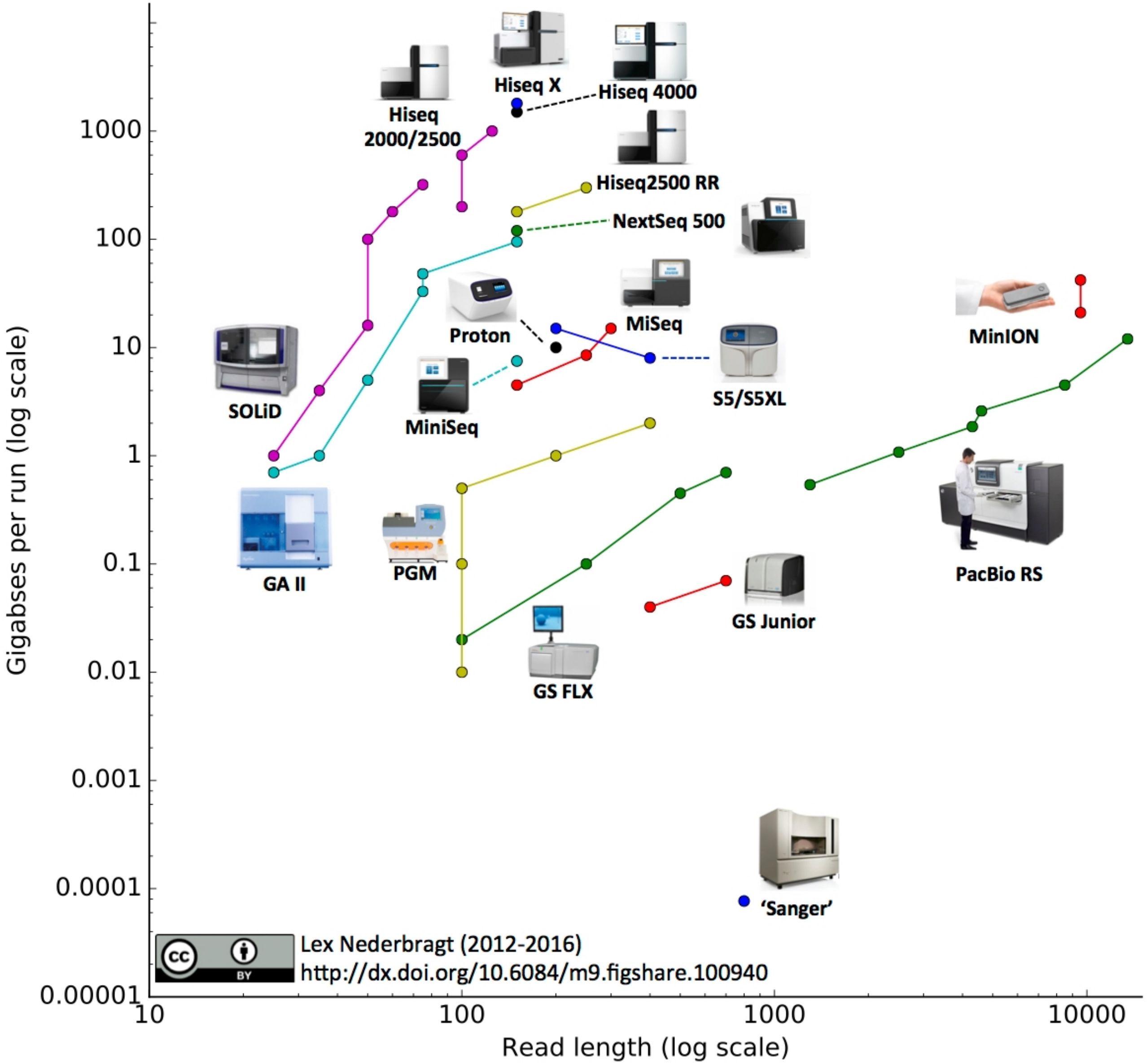
GATK
GenotypeGVCFs

VCF

Variants annotations/filters

SNPeff / SNPsift

SGS platforms

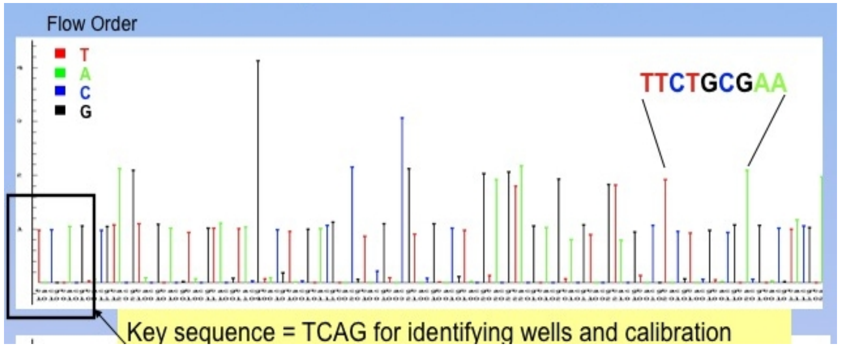


Lex Nederbragt (2012-2016)
http://dx.doi.org/10.6084/m9.figshare.100940

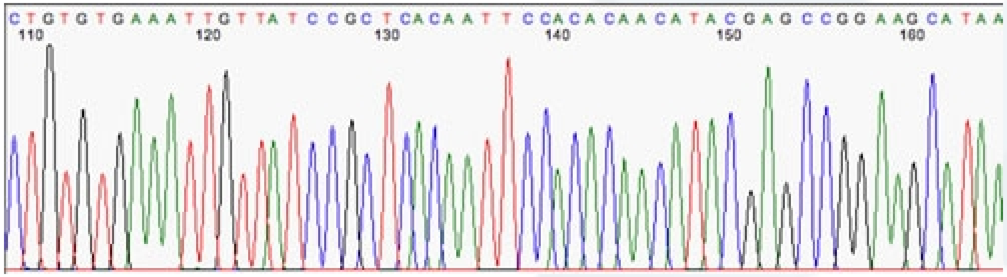
https://flxlexblog.wordpress.com/2016/07/08/developments-in-high-throughput-sequencing-july-2016-edition/#more-790

Sequencing bias

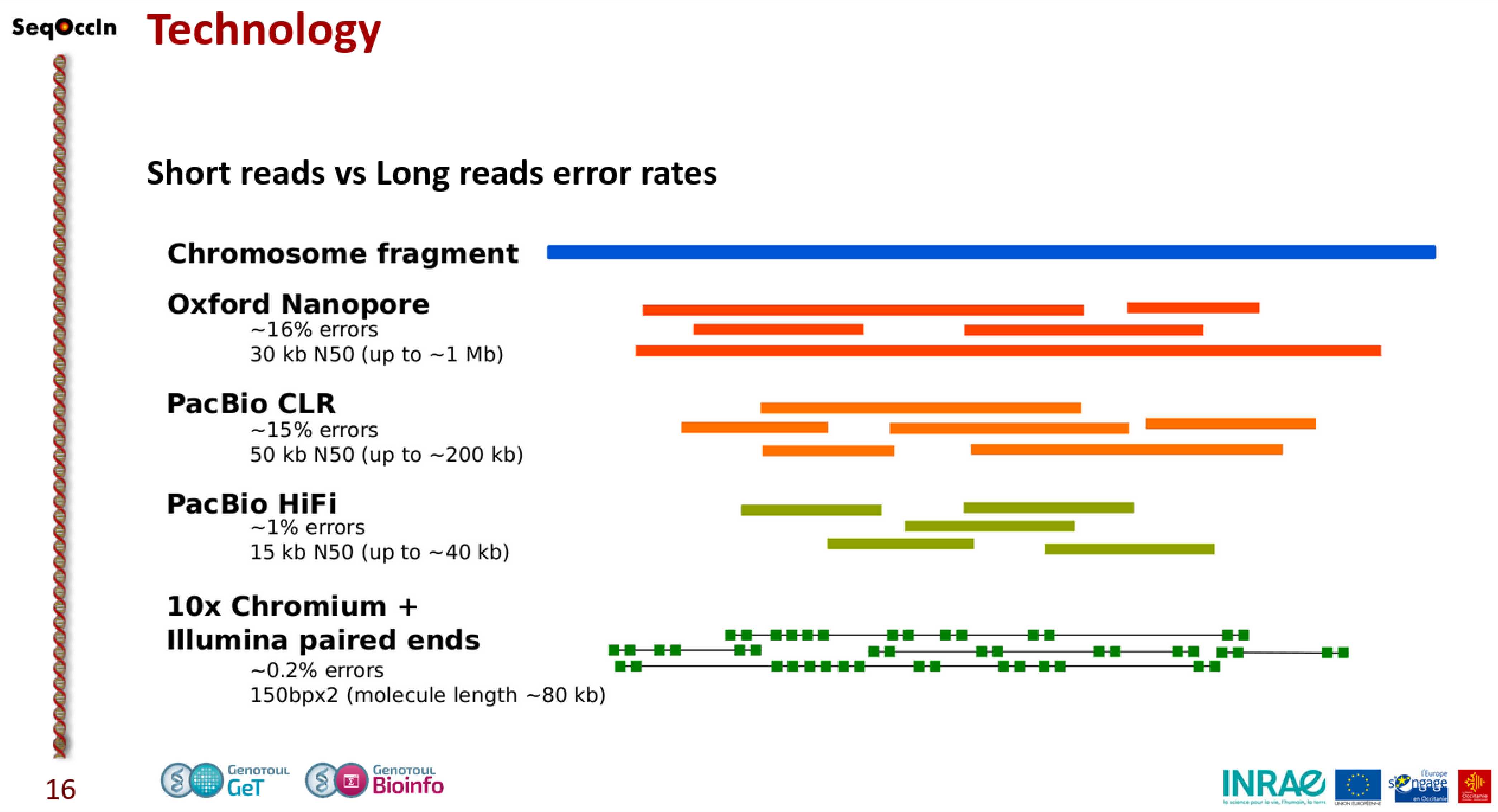
- Platform related
- Illumina (data from Jean-Marc Aury CNS)
  - 98,5% mapped reads
  - Mean error rate : 0,38%
  - 3% deletions, 2% insertions, 95% substitutions
  - GC-rich and AT-rich fragments underrepresented



Key sequence = TCAG for identifying wells and calibration



Sequencing platforms



SeqOccIn

Technology

Short reads vs Long reads error rates

Chromosome fragment

Oxford Nanopore
~16% errors
30 kb N50 (up to ~1 Mb)

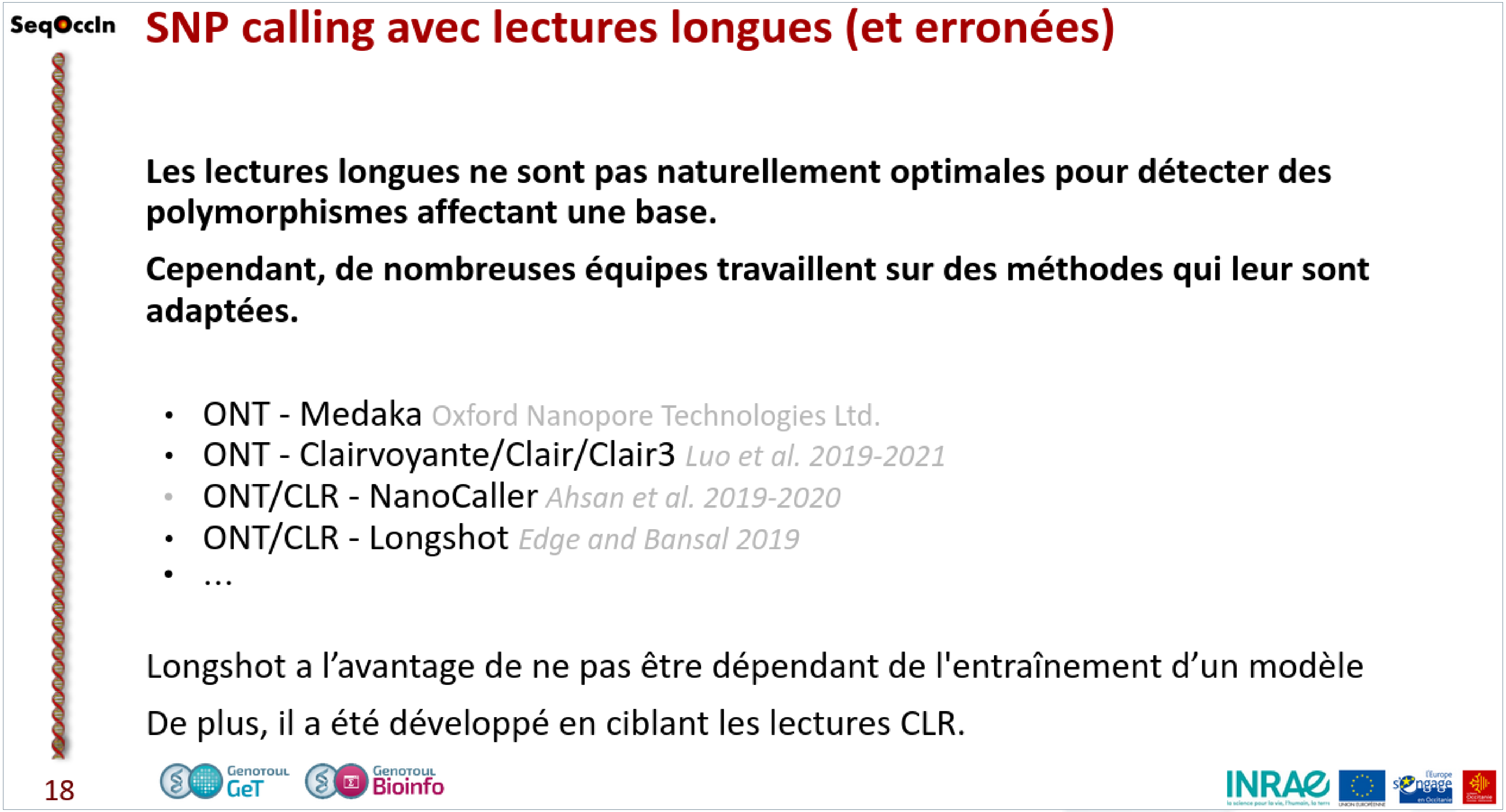
PacBio CLR
~15% errors
50 kb N50 (up to ~200 kb)

PacBio HiFi
~1% errors
15 kb N50 (up to ~40 kb)

10x Chromium +
Illumina paired ends
~0.2% errors
150bpx2 (molecule length ~80 kb)

Extract from Arnaud Di-Franco - SeqOccin - 2020/12
Détection de variants Efficacité de la détection à basse couverture en longue lecture

SGS platforms



SNP calling avec lectures longues (et erronées)

Les lectures longues ne sont pas naturellement optimales pour détecter des polymorphismes affectant une base.

Cependant, de nombreuses équipes travaillent sur des méthodes qui leur sont adaptées.

- ONT - Medaka Oxford Nanopore Technologies Ltd.
- ONT - Clairvoyante/Clair/Clair3 *Luo et al. 2019-2021*
- ONT/CLR - NanoCaller *Ahsan et al. 2019-2020*
- ONT/CLR - Longshot *Edge and Bansal 2019*
- …

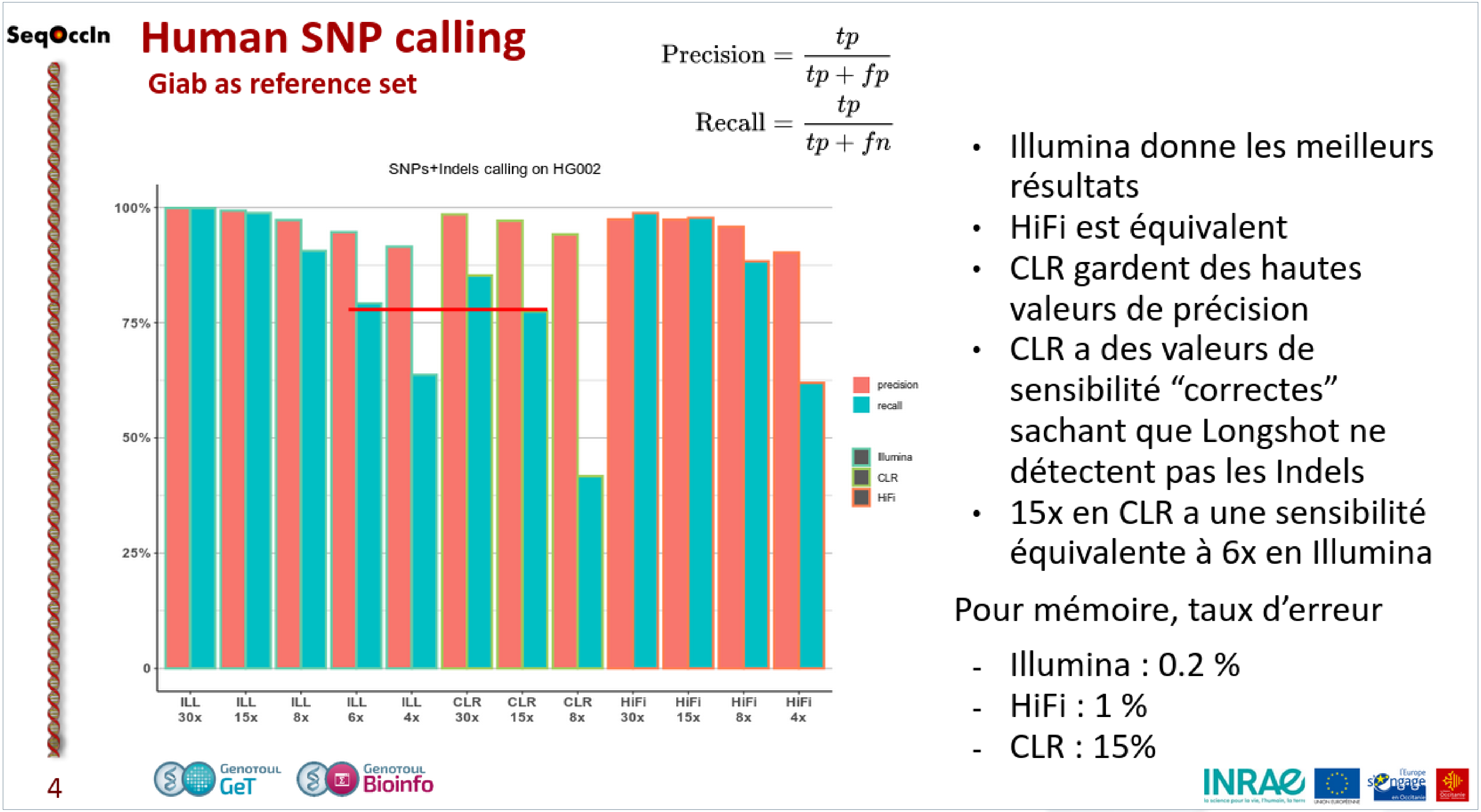
Longshot a l'avantage de ne pas être dépendant de l'entraînement d'un modèle

De plus, il a été développé en ciblant les lectures CLR.

18

Extract from Arnaud Di-Franco - SeqOccin - 2020/12
Détection de variants Efficacité de la détection à basse couverture en longue lecture

SGS platforms



SeqOccIn

Human SNP calling

Giab as reference set

$$\text{Precision} = \frac{tp}{tp + fp}$$

$$\text{Recall} = \frac{tp}{tp + fn}$$

- Illumina donne les meilleurs résultats
- HiFi est équivalent
- CLR gardent des hautes valeurs de précision
- CLR a des valeurs de sensibilité “correctes” sachant que Longshot ne détectent pas les Indels
- 15x en CLR a une sensibilité équivalente à 6x en Illumina

Pour mémoire, taux d’erreur

- Illumina : 0.2 %
- HiFi : 1 %
- CLR : 15%

4

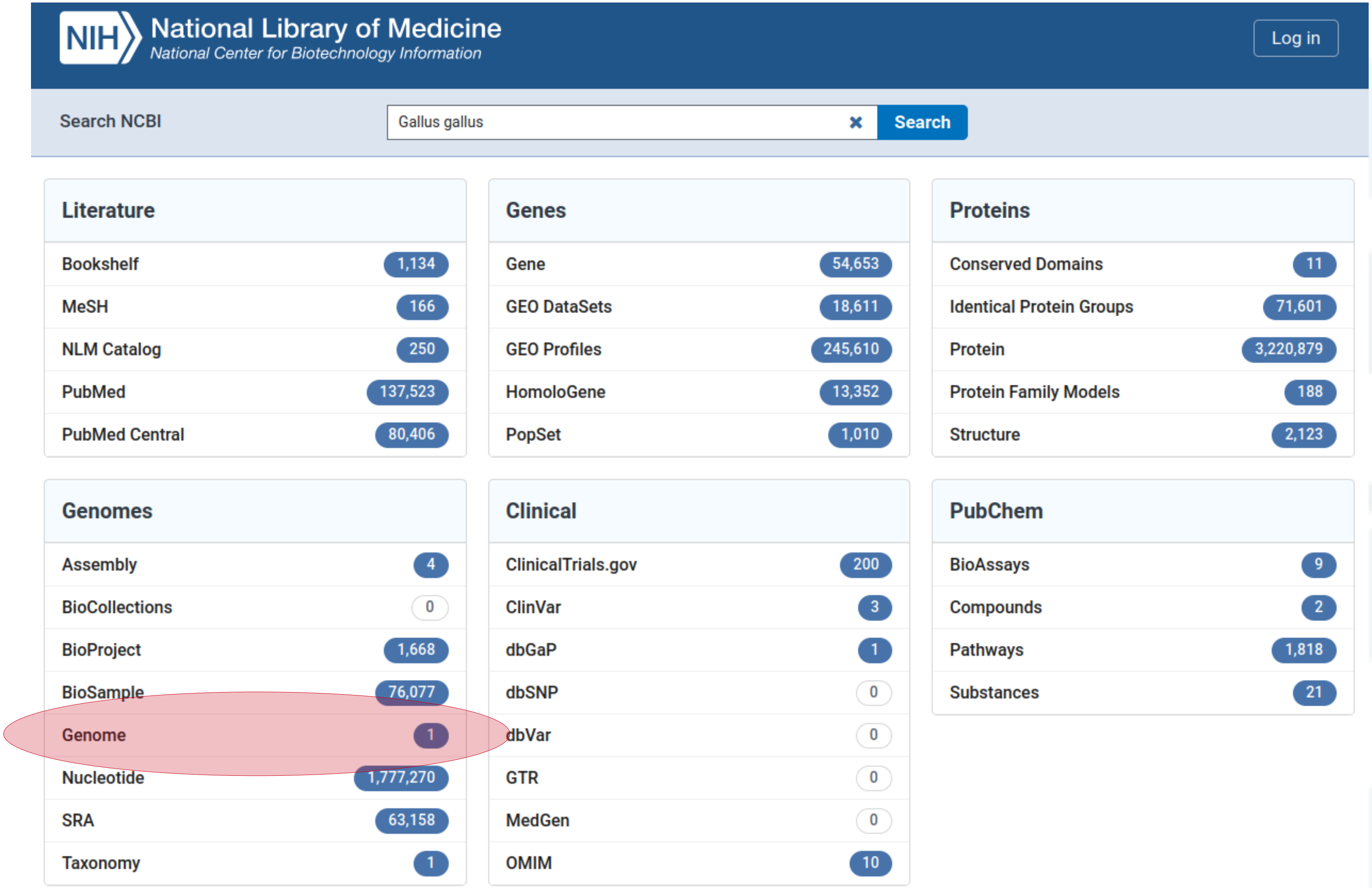
Extract from Arnaud Di-Franco - SeqOccin - 2020/12
Détection de variants Efficacité de la détection à basse couverture en longue lecture

What data will we use?

- The needed data :
  - A reference sequence :
    - Genome
    - Parts of the genome
    - Transcriptome
  - Short reads

Where to get reference genome?

- Assemble your own
- Use a public assembly (NCBI / EBI)



NIH National Library of Medicine
National Center for Biotechnology Information

Search NCBI: Gallus gallus ✖ Search

Literature	
Bookshelf	1,134
MeSH	166
NLM Catalog	250
PubMed	137,523
PubMed Central	80,406

Genes	
Gene	54,653
GEO DataSets	18,611
GEO Profiles	245,610
HomoloGene	13,352
PopSet	1,010

Proteins	
Conserved Domains	11
Identical Protein Groups	71,601
Protein	3,220,879
Protein Family Models	188
Structure	2,123

Genomes	
Assembly	4
BioCollections	0
BioProject	1,668
BioSample	76,077
Genome	1
Nucleotide	1,777,270
SRA	63,158
Taxonomy	1

Clinical	
ClinicalTrials.gov	200
ClinVar	3
dbGaP	1
dbSNP	0
dbVar	0
GTR	0
MedGen	0
OMIM	10

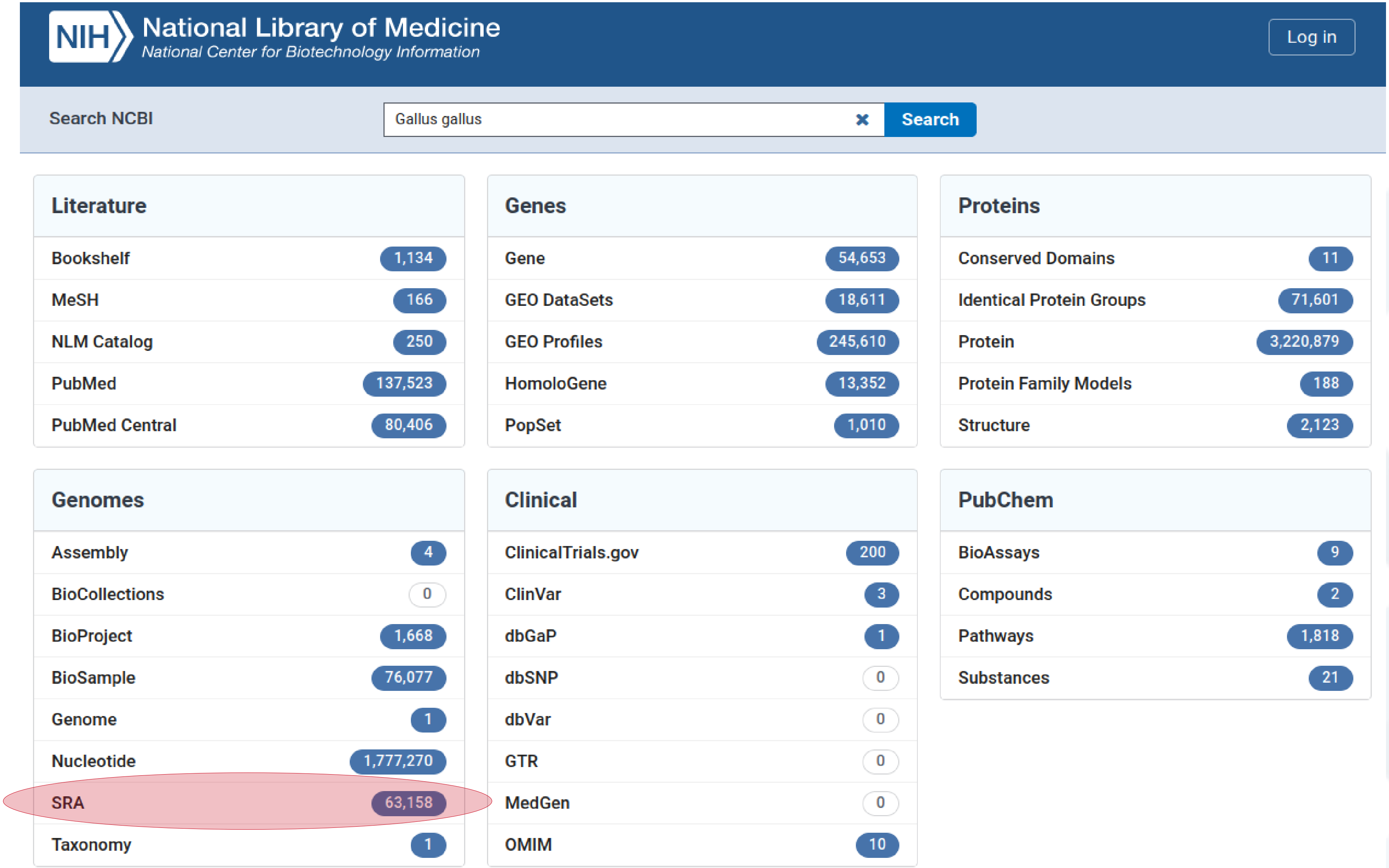
PubChem	
BioAssays	9
Compounds	2
Pathways	1,818
Substances	21

Where to get short reads?

- Produce your own sequences :
  - CNS
  - Local platform
  - Private company

- Use public data :
  - SRA : NCBI Sequence Read Archive
  - ENA : EMBL/EBI European Nucleotide Archive

NCBI SRA?



National Library of Medicine
National Center for Biotechnology Information

Search NCBI: Gallus gallus

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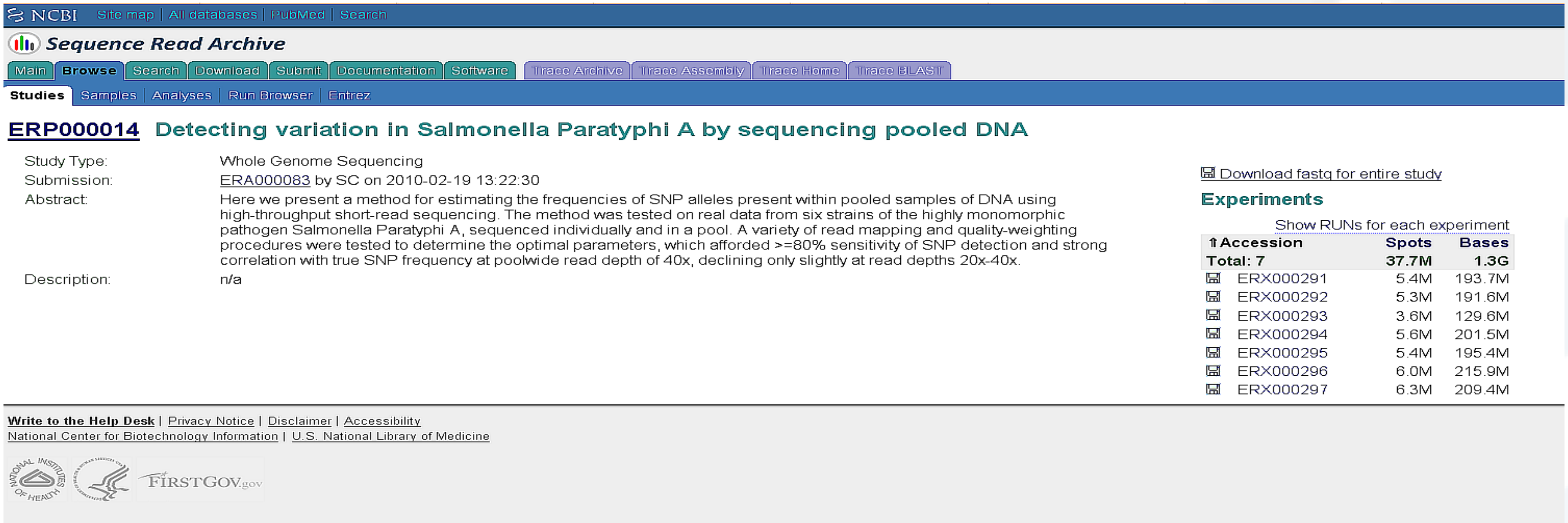
PubChem	
BioAssays	9
Compounds	2
Pathways	1,818
Substances	21

Meta data

- Meta data structure :
  - Experiment
  - Sample
  - Study
  - Run
  - Data file



ERP000014 Detecting variation in Salmonella Paratyphi A by sequencing pooled DNA

Study Type:	Whole Genome Sequencing
Submission:	ERA000083 by SC on 2010-02-19 13:22:30
Abstract:	Here we present a method for estimating the frequencies of SNP alleles present within pooled samples of DNA using high-throughput short-read sequencing. The method was tested on real data from six strains of the highly monomorphic pathogen Salmonella Paratyphi A, sequenced individually and in a pool. A variety of read mapping and quality-weighting procedures were tested to determine the optimal parameters, which afforded >=80% sensitivity of SNP detection and strong correlation with true SNP frequency at poolwide read depth of 40x, declining only slightly at read depths 20x-40x.
Description:	n/a

Download fastq for entire study

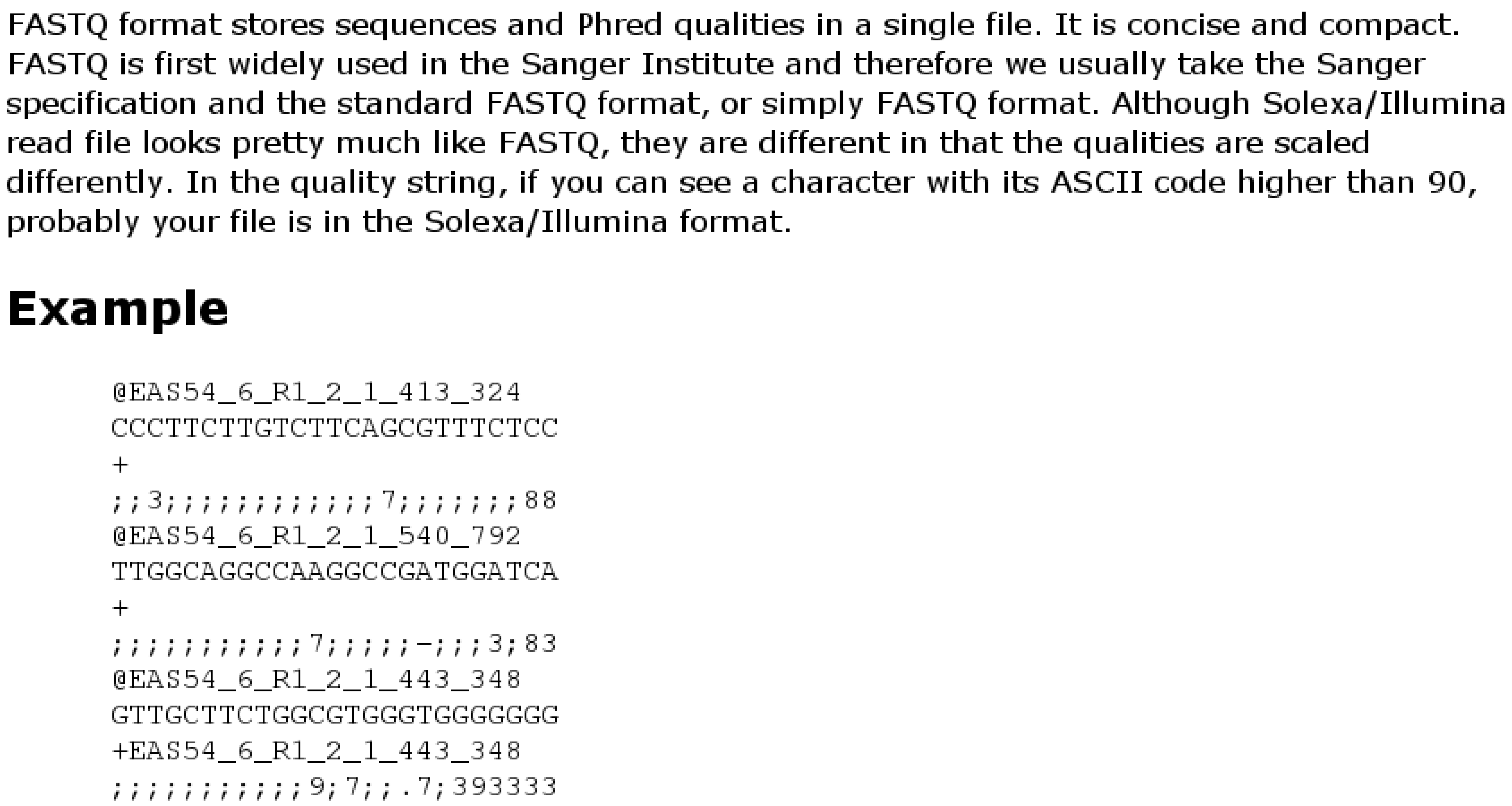
Experiments

Show RUNs for each experiment

⇑Accession	Spots	Bases
Total: 7	37.7M	1.3G
ERX000291	5.4M	193.7M
ERX000292	5.3M	191.6M
ERX000293	3.6M	129.6M
ERX000294	5.6M	201.5M
ERX000295	5.4M	195.4M
ERX000296	6.0M	215.9M
ERX000297	6.3M	209.4M

Write to the Help Desk | Privacy Notice | Disclaimer | Accessibility
National Center for Biotechnology Information | U.S. National Library of Medicine
NATIONAL INSTITUTES OF HEALTH
FIRSTGOV.gov

What is a fastq file?



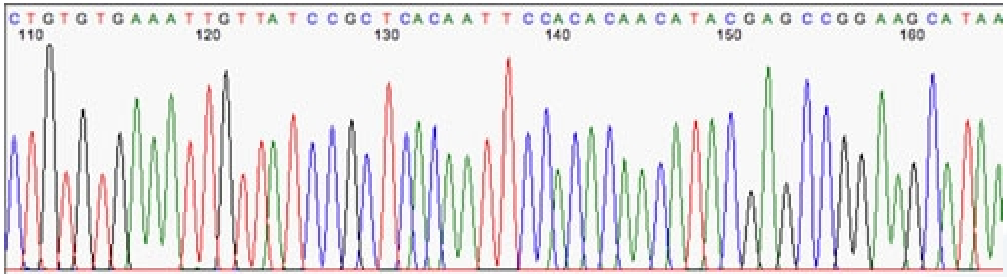
FASTQ format stores sequences and Phred qualities in a single file. It is concise and compact. FASTQ is first widely used in the Sanger Institute and therefore we usually take the Sanger specification and the standard FASTQ format, or simply FASTQ format. Although Solexa/Illumina read file looks pretty much like FASTQ, they are different in that the qualities are scaled differently. In the quality string, if you can see a character with its ASCII code higher than 90, probably your file is in the Solexa/Illumina format.

Example

```
@EAS54_6_R1_2_1_413_324
CCCTTCTTGTCTTCAGCGTTTCTCC
+
;;3;;;;;;;;;;;;7;;;;;;;88
@EAS54_6_R1_2_1_540_792
TTGGCAGGCCAAGGCCGATGGATCA
+
;;;;;;;;;;;7;;;;;-;;;3;83
@EAS54_6_R1_2_1_443_348
GTTGCTTCTGGCGTGGGTGGGGGGG
+EAS54_6_R1_2_1_443_348
;;;;;;;;;;;9;7;;.7;393333
```

Sequence quality

- Phred : base calling



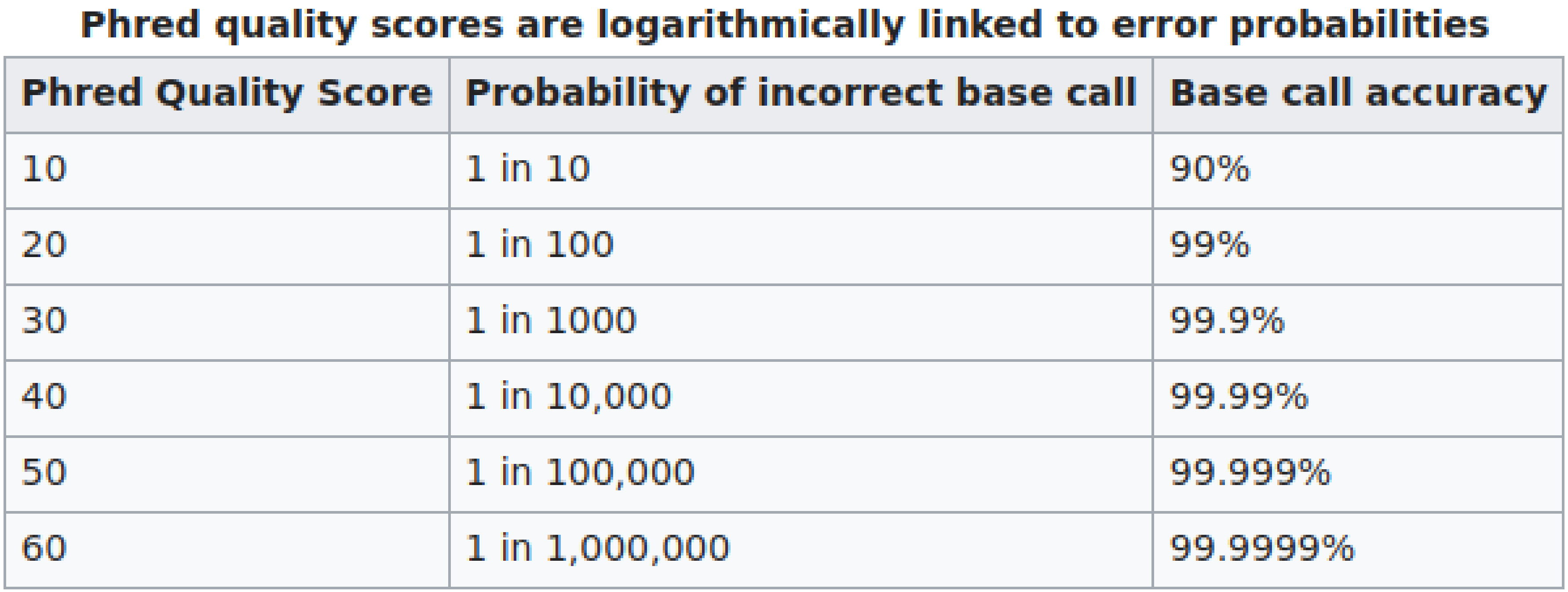
What is Phred Quality?

Traditionally, Phred quality is defined on base calls. Each base call is an estimate of the true nucleotide. It is a random variable and can be wrong. The probability that a base call is wrong is called error probability.

Explanation about the quality values :

source http://maq.sourceforge.net/qual.shtml

Sequence quality



Phred quality scores are logarithmically linked to error probabilities

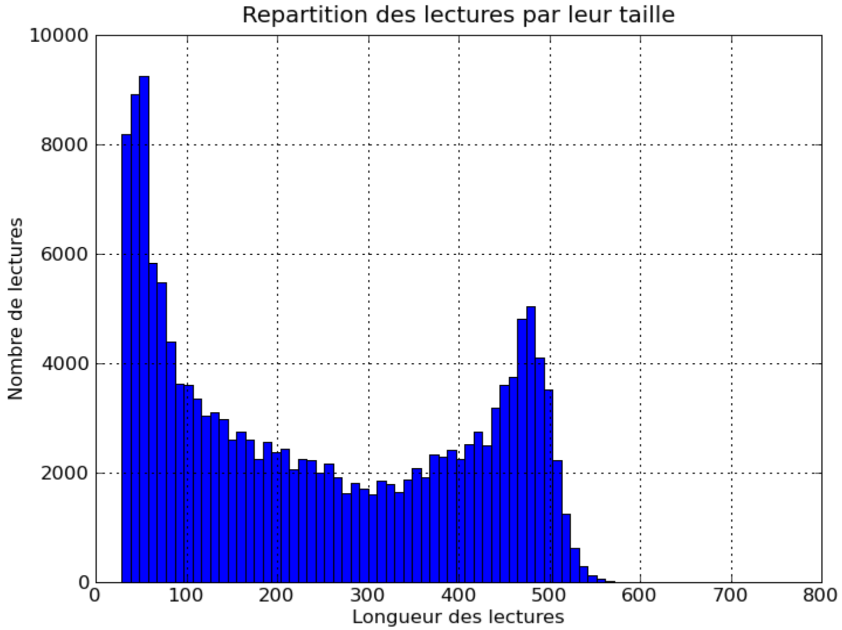
Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

Which reads should I keep?

- All
- Some : what criteria and threshold should I use
  - Composition (number of Ns, complexity, ...)
  - Quality
  - Alignment based criteria
- Should I trim the reads using :
  - Composition
  - Quality

Basic reads statistics

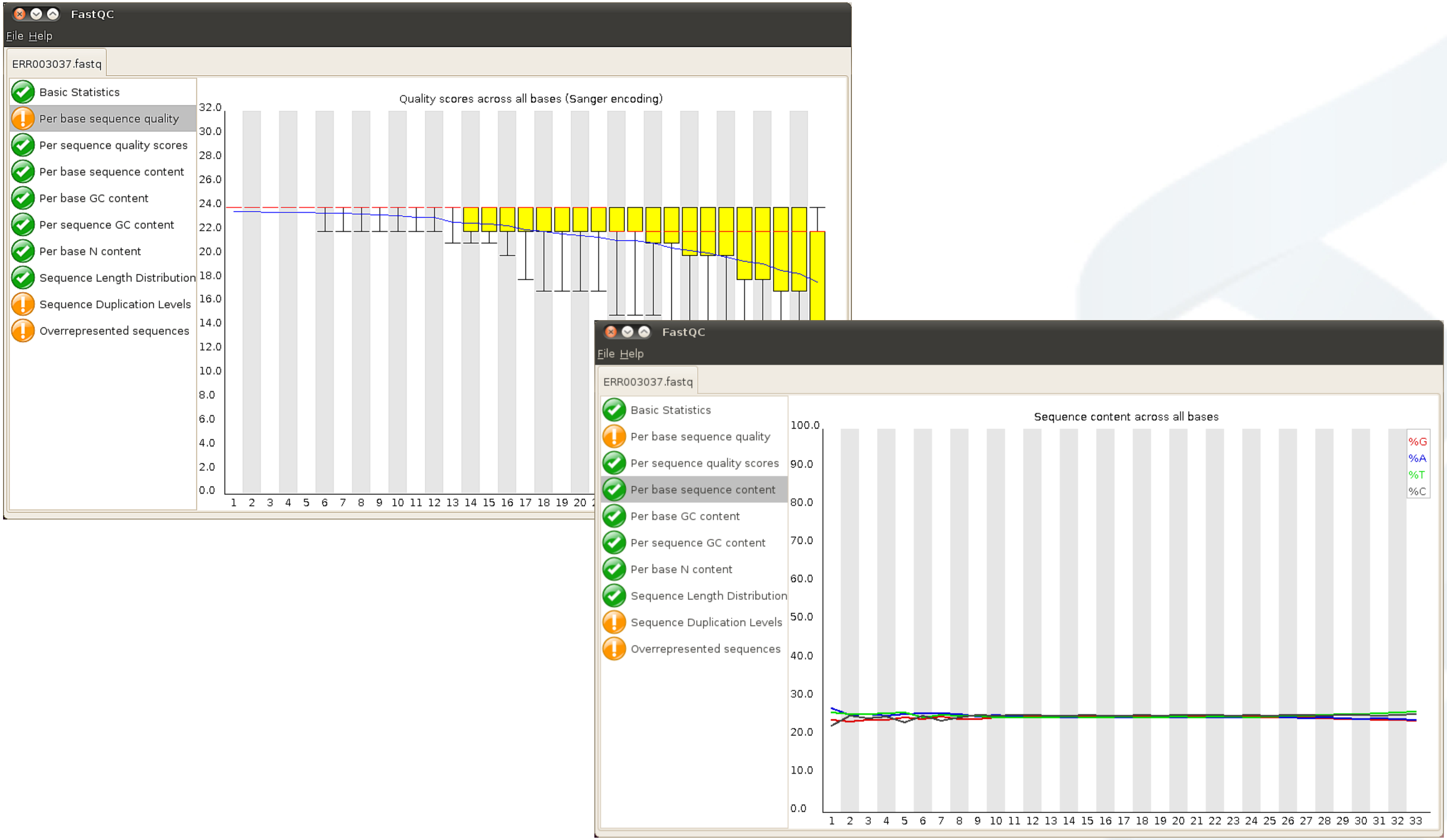
- Number of reads
- Length histogram
- Number of Ns in the reads
- Reads quality
- Reads redundancy
- Reads complexity



Sequence quality analysis

- ➔ FastQC :

Babraham Institute Babraham Bioinformatics FastQC



FastQC

File Help

ERR003037.fastq

- ✓ Basic Statistics
- ! Per base sequence quality
- ✓ Per sequence quality scores
- ✓ Per base sequence content
- ✓ Per base GC content
- ✓ Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ! Sequence Duplication Levels
- ! Overrepresented sequences

Quality scores across all bases (Sanger encoding)

FastQC

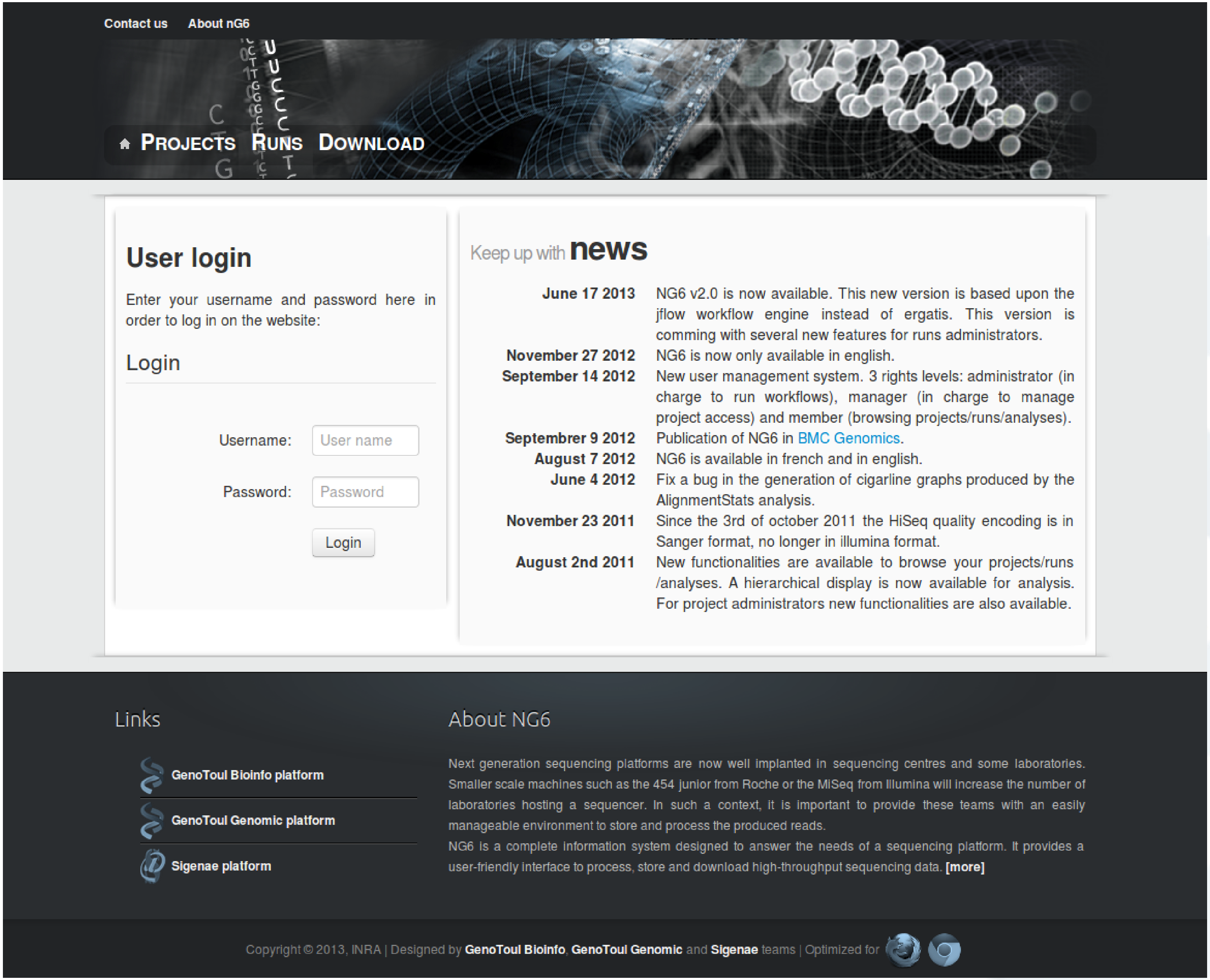
File Help

ERR003037.fastq

- ✓ Basic Statistics
- ! Per base sequence quality
- ✓ Per sequence quality scores
- ✓ Per base sequence content
- ✓ Per base GC content
- ✓ Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ! Sequence Duplication Levels
- ! Overrepresented sequences

Sequence content across all bases

NG6 – Home



Contact us About nG6

User login

Enter your username and password here in order to log in on the website:

Login

Username: User name

Password: Password

Login

Keep up with news

June 17 2013	NG6 v2.0 is now available. This new version is based upon the jflow workflow engine instead of ergatis. This version is comming with several new features for runs administrators.
November 27 2012	NG6 is now only available in english.
September 14 2012	New user management system. 3 rights levels: administrator (in charge to run workflows), manager (in charge to manage project access) and member (browsing projects/runs/analyses).
Septembrer 9 2012	Publication of NG6 in BMC Genomics.
August 7 2012	NG6 is available in french and in english.
June 4 2012	Fix a bug in the generation of cigarline graphs produced by the AlignmentStats analysis.
November 23 2011	Since the 3rd of october 2011 the HiSeq quality encoding is in Sanger format, no longer in illumina format.
August 2nd 2011	New functionalities are available to browse your projects/runs /analyses. A hierarchical display is now available for analysis. For project administrators new functionalities are also available.

Links

- GenoToul Bioinfo platform
- GenoToul Genomic platform
- Sigenae platform

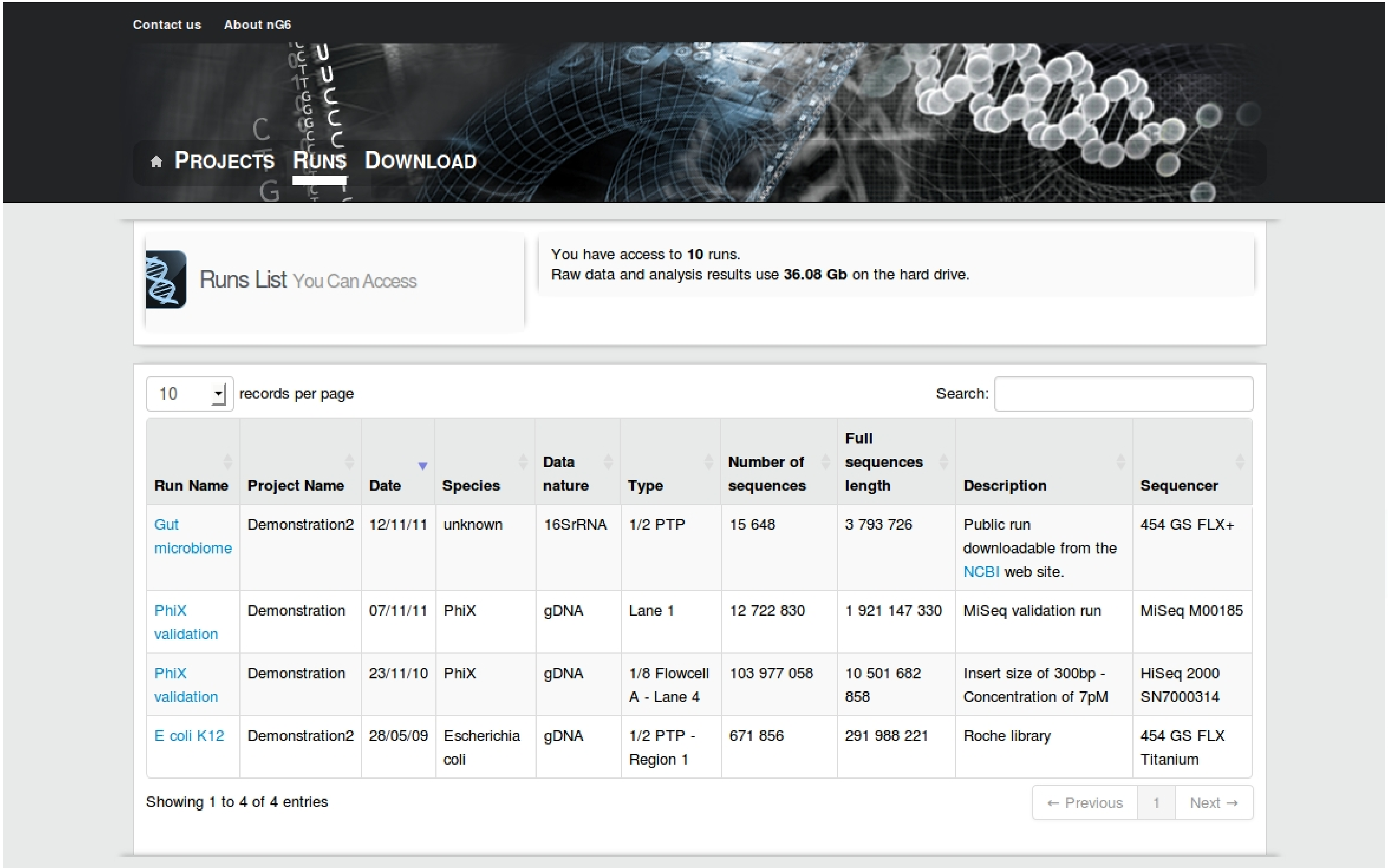
About NG6

Next generation sequencing platforms are now well implanted in sequencing centres and some laboratories. Smaller scale machines such as the 454 junior from Roche or the MiSeq from Illumina will increase the number of laboratories hosting a sequencer. In such a context, it is important to provide these teams with an easily manageable environment to store and process the produced reads.

NG6 is a complete information system designed to answer the needs of a sequencing platform. It provides a user-friendly interface to process, store and download high-throughput sequencing data. **[more]**

Copyright © 2013, INRA | Designed by **GenoToul Bioinfo**, **GenoToul Genomic** and **Sigenae** teams | Optimized for

NG6 - Runs



Runs List You Can Access

You have access to **10** runs.
Raw data and analysis results use **36.08 Gb** on the hard drive.

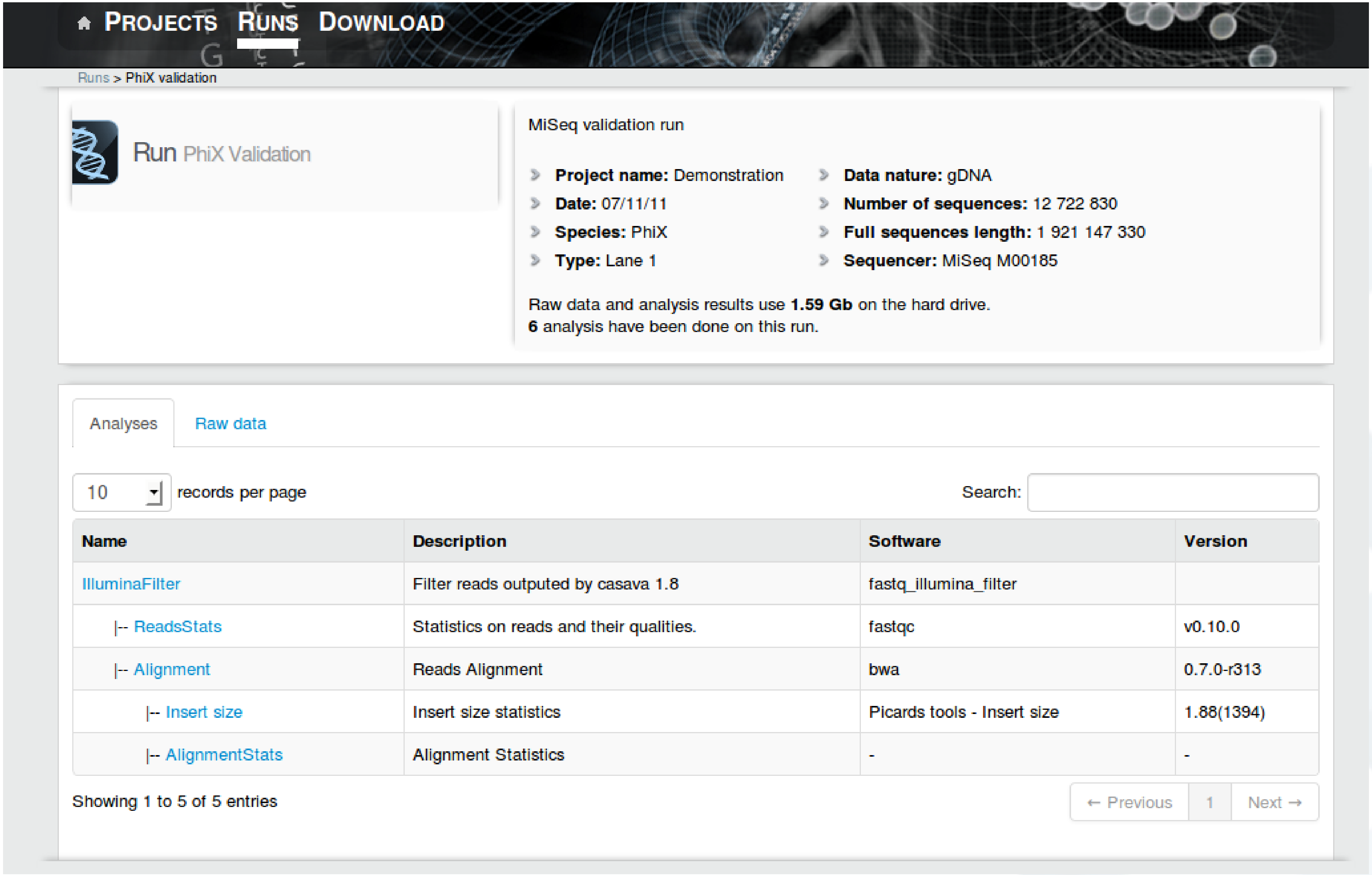
10 records per page

Search:

Run Name	Project Name	Date	Species	Data nature	Type	Number of sequences	Full sequences length	Description	Sequencer
Gut microbiome	Demonstration2	12/11/11	unknown	16SrRNA	1/2 PTP	15 648	3 793 726	Public run downloadable from the NCBI web site.	454 GS FLX+
PhiX validation	Demonstration	07/11/11	PhiX	gDNA	Lane 1	12 722 830	1 921 147 330	MiSeq validation run	MiSeq M00185
PhiX validation	Demonstration	23/11/10	PhiX	gDNA	1/8 Flowcell A - Lane 4	103 977 058	10 501 682 858	Insert size of 300bp - Concentration of 7pM	HiSeq 2000 SN7000314
E coli K12	Demonstration2	28/05/09	Escherichia coli	gDNA	1/2 PTP - Region 1	671 856	291 988 221	Roche library	454 GS FLX Titanium

Showing 1 to 4 of 4 entries

NG6 - Run



MiSeq validation run

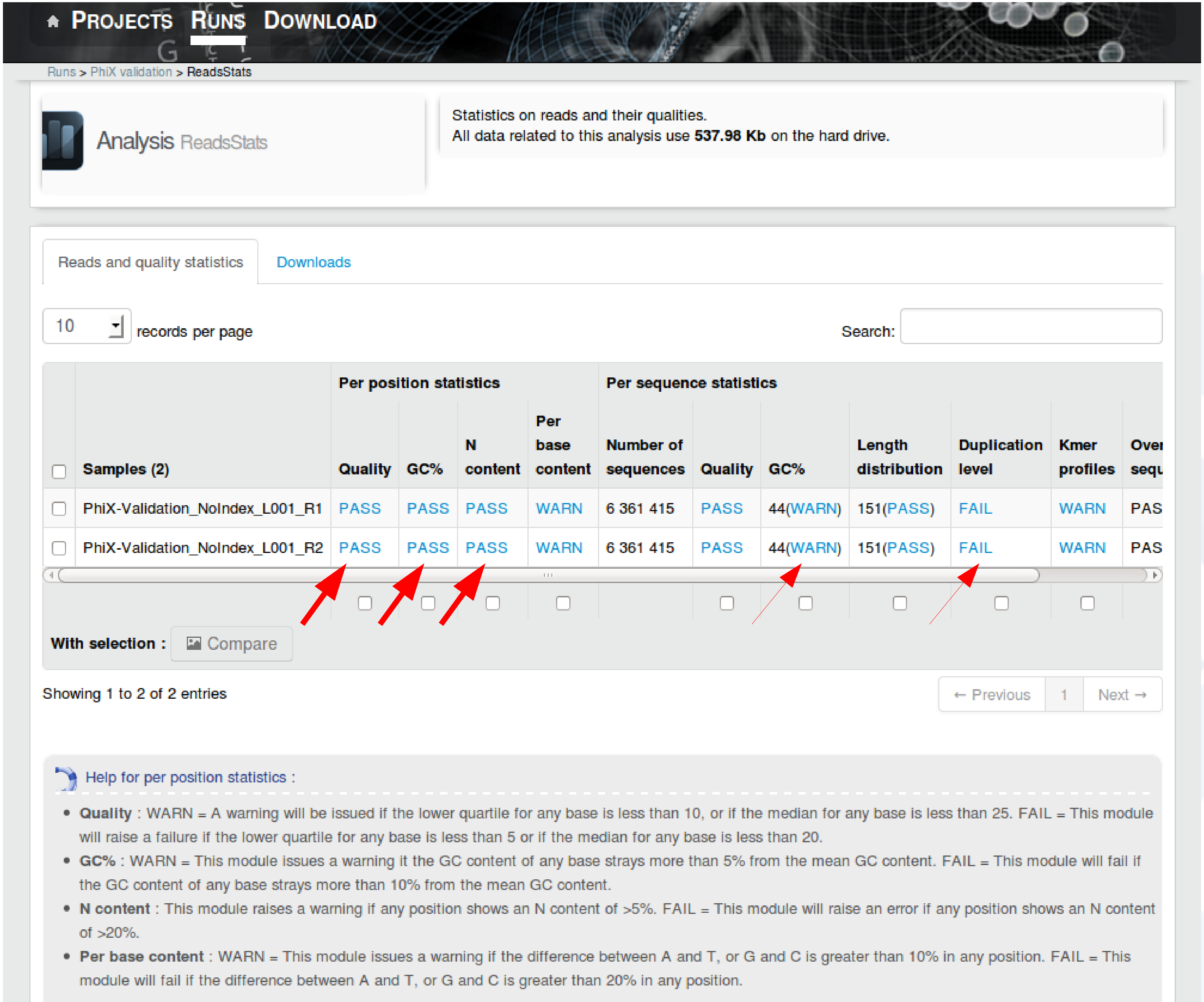
- **Project name:** Demonstration
- **Date:** 07/11/11
- **Species:** PhiX
- **Type:** Lane 1
- **Data nature:** gDNA
- **Number of sequences:** 12 722 830
- **Full sequences length:** 1 921 147 330
- **Sequencer:** MiSeq M00185

Raw data and analysis results use **1.59 Gb** on the hard drive.
6 analysis have been done on this run.

Name	Description	Software	Version
IlluminaFilter	Filter reads outputed by casava 1.8	fastq_illumina_filter	
|-- ReadsStats	Statistics on reads and their qualities.	fastqc	v0.10.0
|-- Alignment	Reads Alignment	bwa	0.7.0-r313
|-- Insert size	Insert size statistics	Picards tools - Insert size	1.88(1394)
|-- AlignmentStats	Alignment Statistics	-	-

Showing 1 to 5 of 5 entries

NG6 - Stats



Runs > PhiX validation > ReadsStats

Analysis ReadsStats

Statistics on reads and their qualities.
All data related to this analysis use **537.98 Kb** on the hard drive.

10 records per page

Search:

		Per position statistics				Per sequence statistics						
□	**Samples (2)**	**Quality**	**GC%**	**N content**	**Per base content**	**Number of sequences**	**Quality**	**GC%**	**Length distribution**	**Duplication level**	**Kmer profiles**	**Over sequ**
□	PhiX-Validation_NoIndex_L001_R1	PASS	PASS	PASS	WARN	6 361 415	PASS	44(WARN)	151(PASS)	FAIL	WARN	PAS
□	PhiX-Validation_NoIndex_L001_R2	PASS	PASS	PASS	WARN	6 361 415	PASS	44(WARN)	151(PASS)	FAIL	WARN	PAS
		□	□	□	□		□	□	□	□	□	

With selection : Compare

Showing 1 to 2 of 2 entries

Help for per position statistics :

- **Quality** : WARN = A warning will be issued if the lower quartile for any base is less than 10, or if the median for any base is less than 25. FAIL = This module will raise a failure if the lower quartile for any base is less than 5 or if the median for any base is less than 20.
- **GC%** : WARN = This module issues a warning it the GC content of any base strays more than 5% from the mean GC content. FAIL = This module will fail if the GC content of any base strays more than 10% from the mean GC content.
- **N content** : This module raises a warning if any position shows an N content of >5%. FAIL = This module will raise an error if any position shows an N content of >20%.
- **Per base content** : WARN = This module issues a warning if the difference between A and T, or G and C is greater than 10% in any position. FAIL = This module will fail if the difference between A and T, or G and C is greater than 20% in any position.

Exercises / set 1

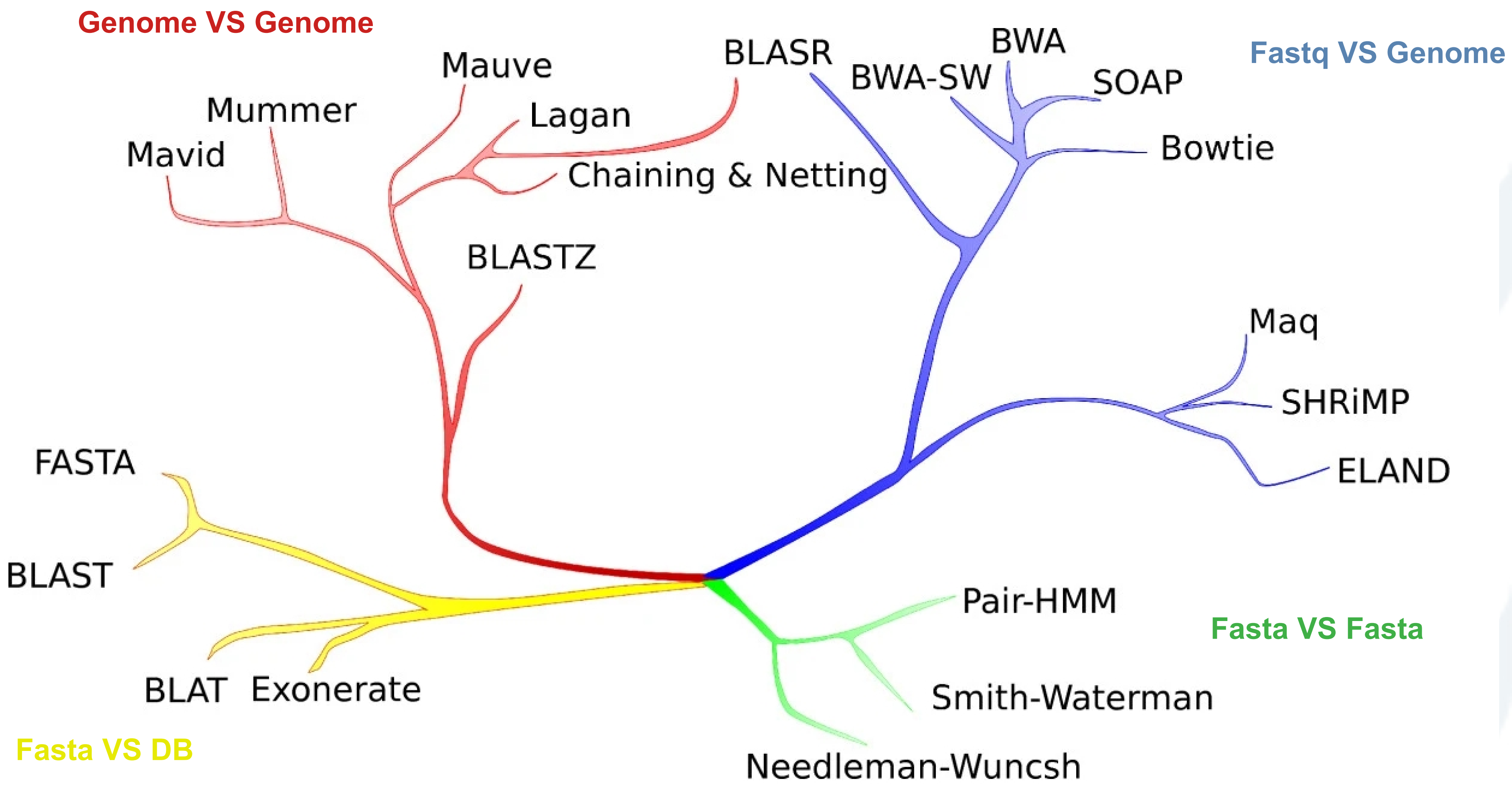
https://bios4biol.pages.mia.inra.fr/training-aln-variant-calling/

Reads alignment

The different software generations :

- Smith-Waterman / Needleman-Wunch (1970)
- BLAST (1990)
- MAQ (2008)
- BWA (2009)
- Minimap2 (2018)

Reads alignment



An illustration of relationships between alignment methods. The applications / corresponding computational restrictions shown are (green) short pairwise alignment / detailed edit model; (yellow) database search / divergent homology detection; (red) whole genome alignment / alignment of long sequences with structural rearrangements; and (blue) short read mapping / rapid alignment of massive numbers of short sequences. Although solely illustrative, methods with more similar data structures or algorithmic approaches are on closer branches. The BLASR method combines data structures from short read alignment with optimization methods from whole genome alignment.

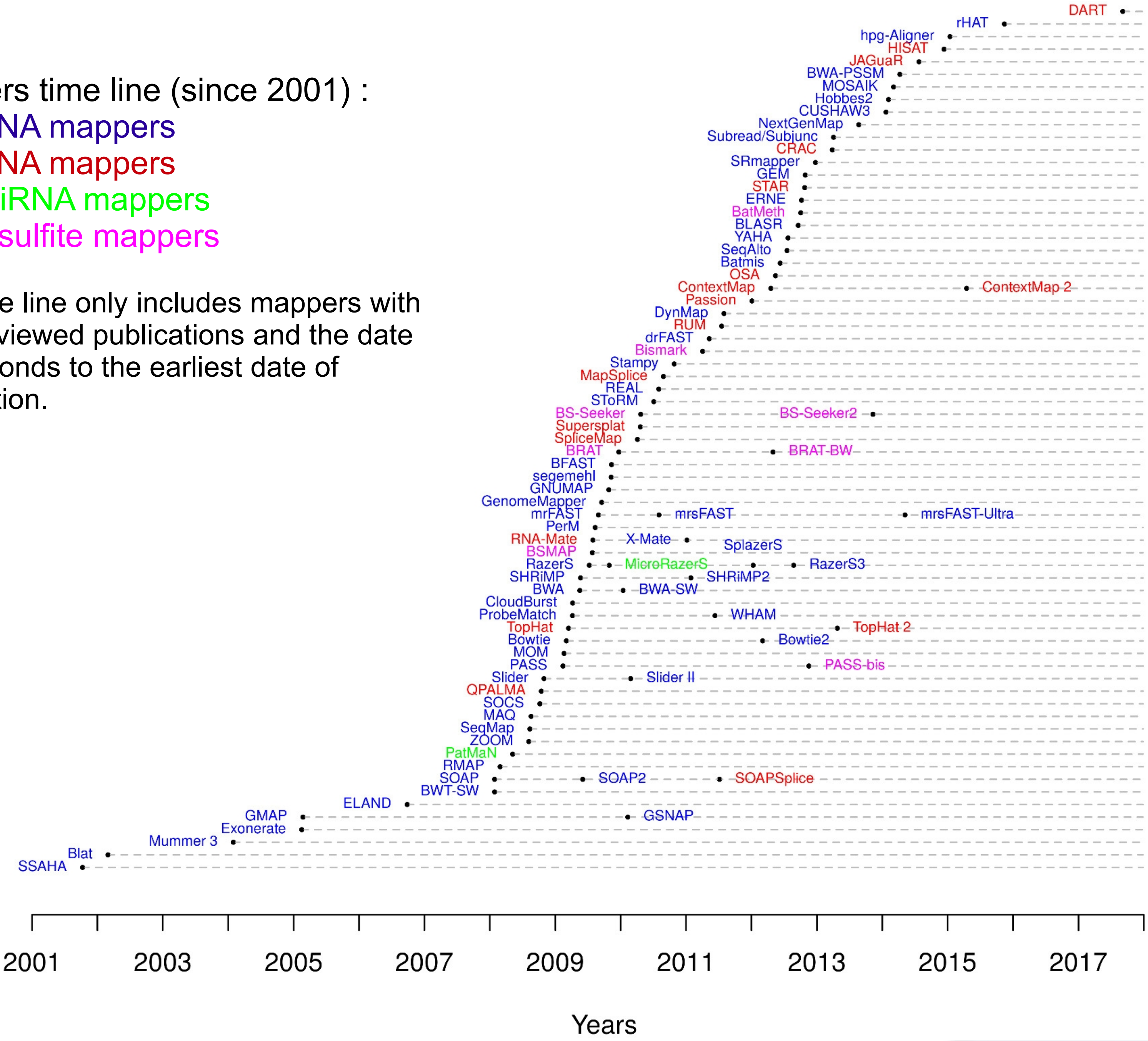
Chaisson, M.J., Tesler, G. BMC Bioinformatics 13, 238 (2012). https://doi.org/10.1186/1471-2105-13-238

Reads alignment

Mappers time line (since 2001) :

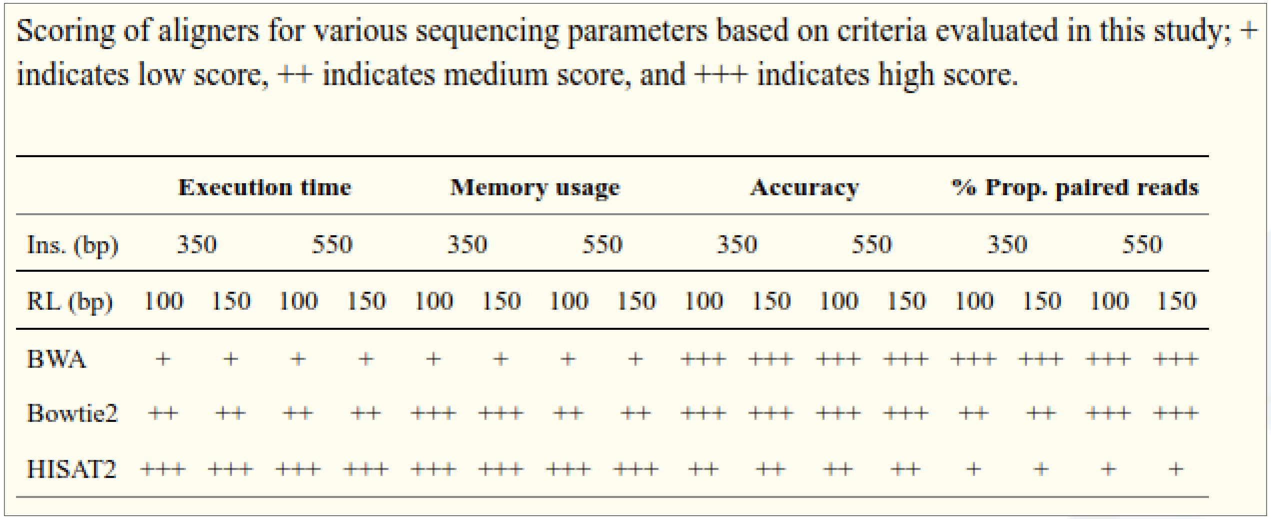
- DNA mappers
- RNA mappers
- miRNA mappers
- bisulfite mappers

The time line only includes mappers with peer-reviewed publications and the date corresponds to the earliest date of publication.



Reads alignment

Most popular tools for mapping to a normal genomic reference (DNAseq, ChIP-Seq, sRNAseq, …) :



Scoring of aligners for various sequencing parameters based on criteria evaluated in this study; + indicates low score, ++ indicates medium score, and +++ indicates high score.

	Execution time				Memory usage				Accuracy				% Prop. paired reads			
Ins. (bp)	350		550		350		550		350		550		350		550	
RL (bp)	100	150	100	150	100	150	100	150	100	150	100	150	100	150	100	150
BWA	+	+	+	+	+	+	+	+	+++	+++	+++	+++	+++	+++	+++	+++
Bowtie2	++	++	++	++	+++	+++	++	++	+++	+++	+++	+++	++	++	+++	+++
HISAT2	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+	+	+	+

https://dx.doi.org/10.3389%2Ffgene.2018.00035

Popular splice read aligners (RNAseq polyA+/total) :

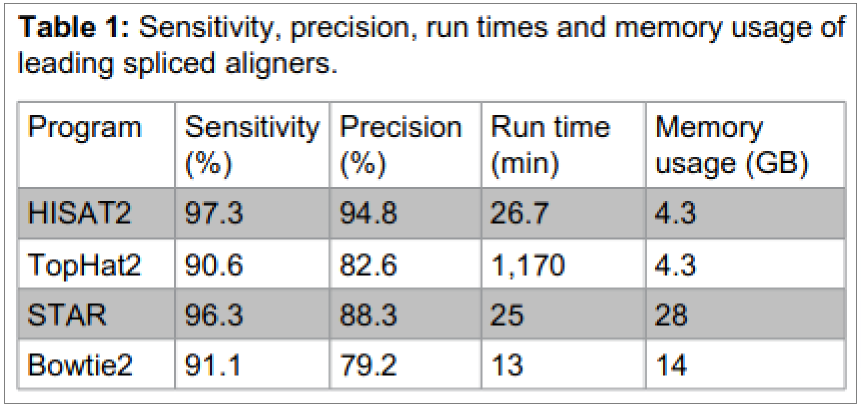


Table 1: Sensitivity, precision, run times and memory usage of leading spliced aligners.

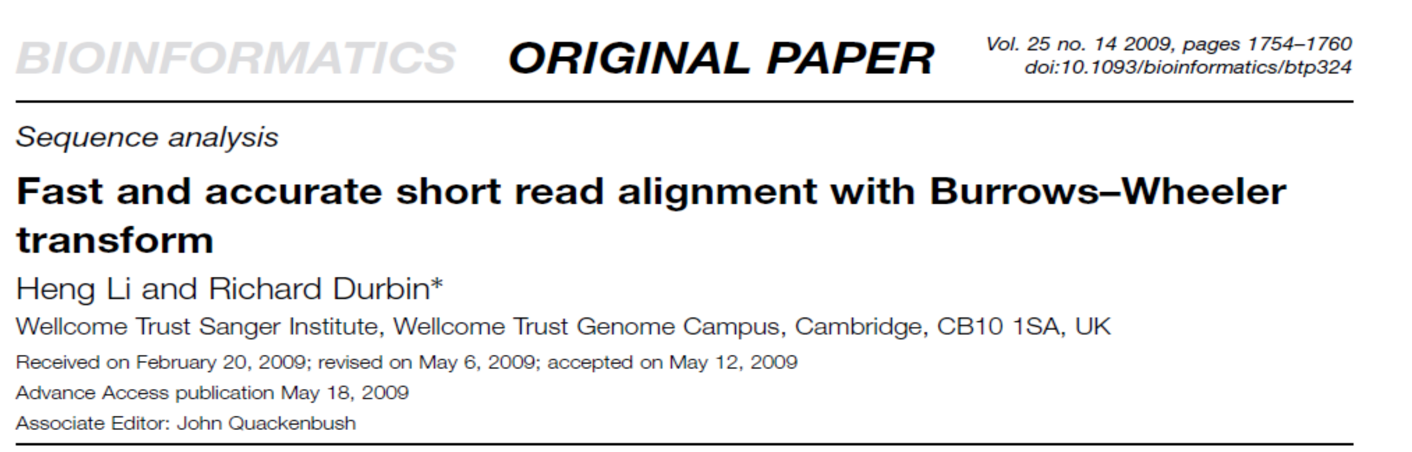
Program	Sensitivity (%)	Precision (%)	Run time (min)	Memory usage (GB)
HISAT2	97.3	94.8	26.7	4.3
TopHat2	90.6	82.6	1,170	4.3
STAR	96.3	88.3	25	28
Bowtie2	91.1	79.2	13	14

https://doi.org/10.23937/2378-3648/1410048

BWA

- Fast and moderate memory footprint (<4GB)
- SAM output by default
- **Gapped** alignment for both SE and PE reads
- Effective pairing to achieve high alignment accuracy; suboptimal hits considered in pairing.
- Non-unique read is placed randomly with a mapping quality 0
- Limited number of errors (2 for 32bp, 4 for 100 bp, ...)
- The default conguration works for most typical input.
  - Automatically adjust parameters based on read lengths and error rates.
  - Estimate the insert size distribution on the fly

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



BIOINFORMATICS **ORIGINAL PAPER**

Vol. 25 no. 14 2009, pages 1754–1760
doi:10.1093/bioinformatics/btp324

Sequence analysis

Fast and accurate short read alignment with Burrows–Wheeler transform

Heng Li and Richard Durbin*

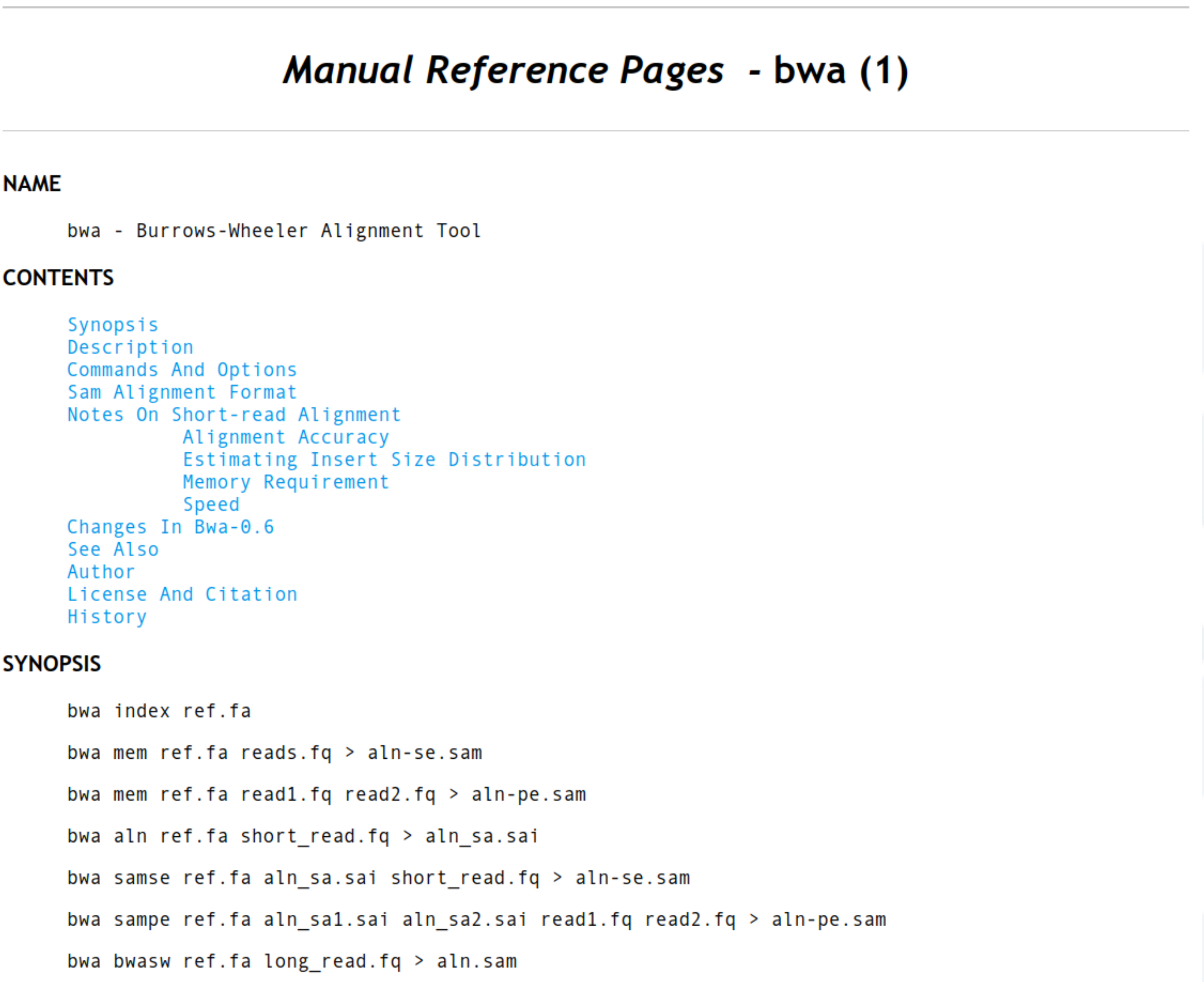
Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK

Received on February 20, 2009; revised on May 6, 2009; accepted on May 12, 2009

Advance Access publication May 18, 2009

Associate Editor: John Quackenbush

http://bio-bwa.sourceforge.net/bwa.shtml



Manual Reference Pages - bwa (1)

NAME

bwa - Burrows-Wheeler Alignment Tool

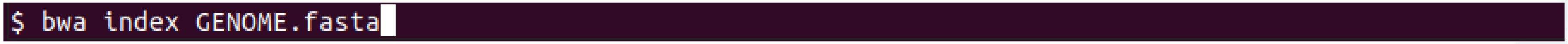
CONTENTS

SYNOPSIS

```
bwa index ref.fa

bwa mem ref.fa reads.fq > aln-se.sam

bwa mem ref.fa read1.fq read2.fq > aln-pe.sam

bwa aln ref.fa short_read.fq > aln_sa.sai

bwa samse ref.fa aln_sa.sai short_read.fq > aln-se.sam

bwa sampe ref.fa aln_sa1.sai aln_sa2.sai read1.fq read2.fq > aln-pe.sam

bwa bwasw ref.fa long_read.fq > aln.sam
```

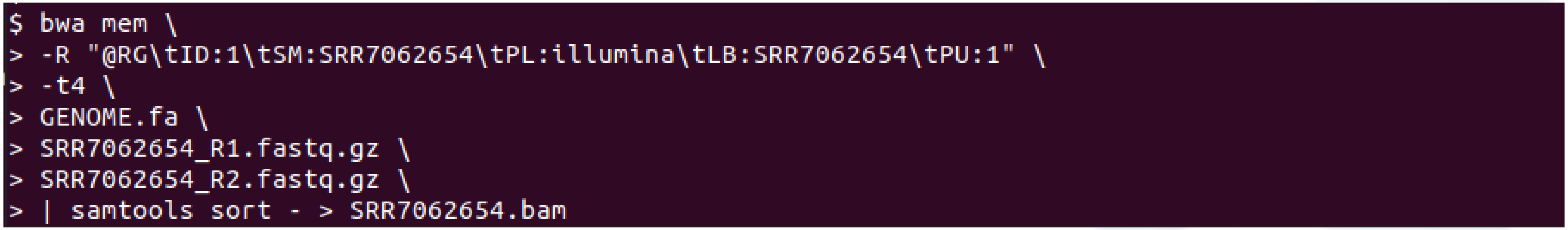
BWA – cmd line

- Two steps :
  - Indexation :



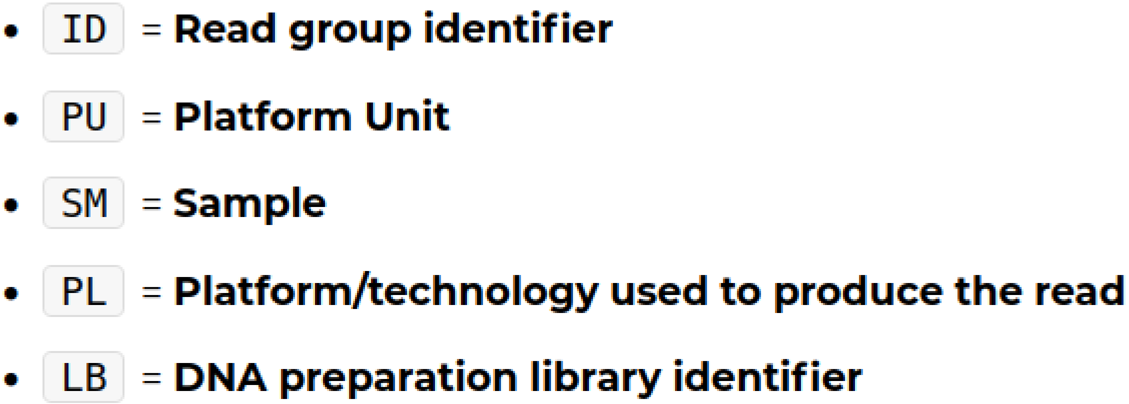
```
$ bwa index GENOME.fasta
```

  - Alignment :



```
$ bwa mem \
> -R "@RG\tID:1\tSM:SRR7062654\tPL:illumina\tLB:SRR7062654\tPU:1" \
> -t4 \
> GENOME.fa \
> SRR7062654_R1.fastq.gz \
> SRR7062654_R2.fastq.gz \
> | samtools sort - > SRR7062654.bam
```

- Meaning of the read group fields required (@RG) :



- `ID` = **Read group identifier**
- `PU` = **Platform Unit**
- `SM` = **Sample**
- `PL` = **Platform/technology used to produce the read**
- `LB` = **DNA preparation library identifier**

https://gatk.broadinstitute.org/hc/en-us/articles/360035890671-Read-groups

Exercises / set 2

Sequence Alignment/Map (SAM) format

- Data sharing was a major issue with the 1000 genomes
- Capture all of the critical information about NGS data in a single indexed and compressed file
- Generic alignment format
- Supports short and long reads (Illumina - Pacbio - ONT)
- Flexible in style, compact in size, efficient in random access

Website :

https://www.htslib.org/

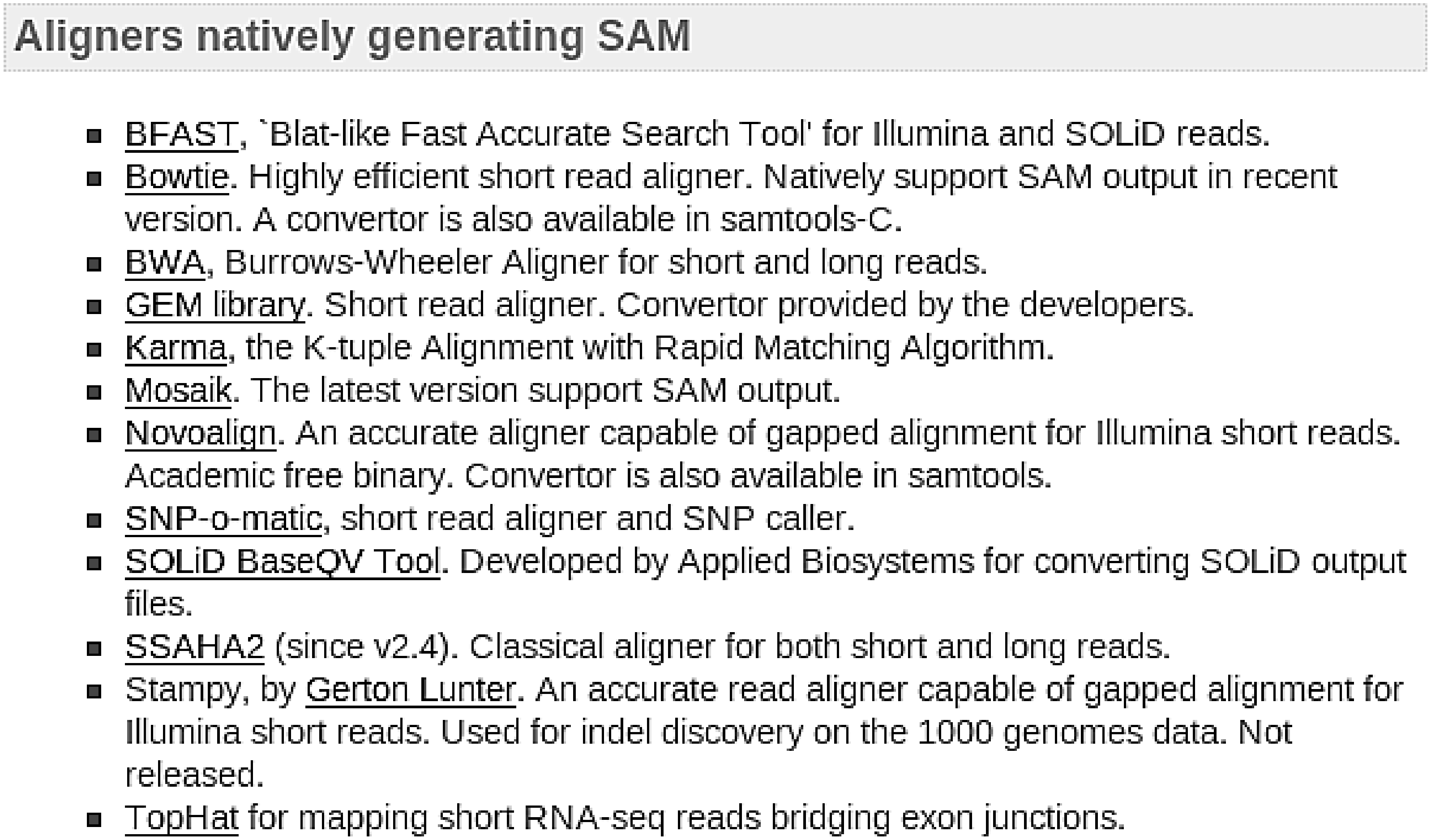
Paper :

Twelve years of SAMtools and BCFtools

Petr Danecek, James K Bonfield, Jennifer Liddle, John Marshall, Valeriu Ohan, Martin O Pollard, Andrew Whitwham, Thomas Keane, Shane A McCarthy, Robert M Davies, Heng Li

GigaScience, Volume 10, Issue 2, February 2021, giab008, https://doi.org/10.1093/gigascience/giab008

Sequence Alignment/Map (SAM) format

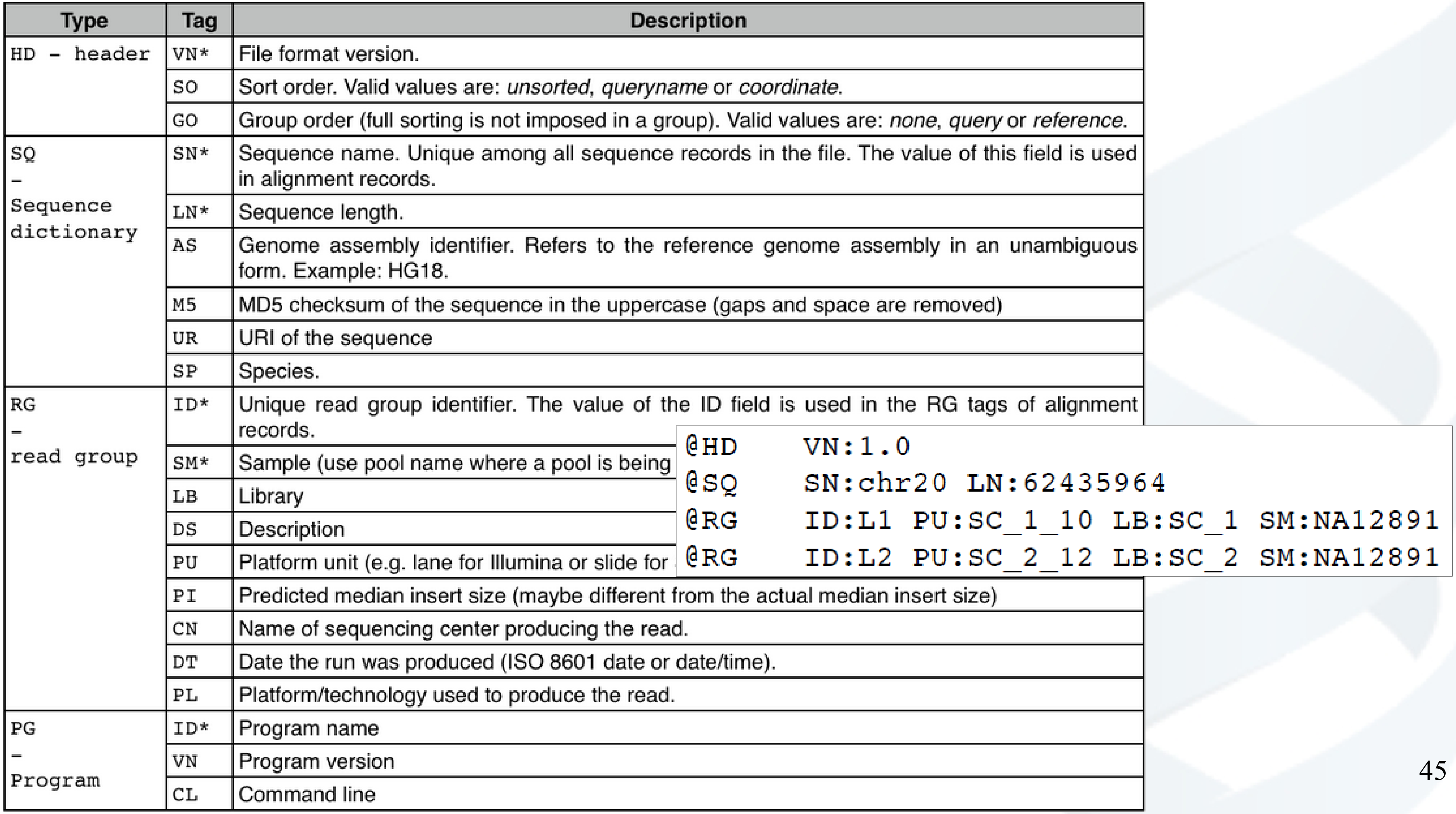


Aligners natively generating SAM

- BFAST, `Blat-like Fast Accurate Search Tool' for Illumina and SOLiD reads.
- Bowtie. Highly efficient short read aligner. Natively support SAM output in recent version. A convertor is also available in samtools-C.
- BWA, Burrows-Wheeler Aligner for short and long reads.
- GEM library. Short read aligner. Convertor provided by the developers.
- Karma, the K-tuple Alignment with Rapid Matching Algorithm.
- Mosaik. The latest version support SAM output.
- Novoalign. An accurate aligner capable of gapped alignment for Illumina short reads. Academic free binary. Convertor is also available in samtools.
- SNP-o-matic, short read aligner and SNP caller.
- SOLiD BaseQV Tool. Developed by Applied Biosystems for converting SOLiD output files.
- SSAHA2 (since v2.4). Classical aligner for both short and long reads.
- Stampy, by Gerton Lunter. An accurate read aligner capable of gapped alignment for Illumina short reads. Used for indel discovery on the 1000 genomes data. Not released.
- TopHat for mapping short RNA-seq reads bridging exon junctions.

SAM format - Header section

- Header lines start with @ followed by a two-letter TYPE
- Header fields are TAG:VALUE pairs



Type	Tag	Description
HD – header	VN*	File format version.
	SO	Sort order. Valid values are: *unsorted*, *queryname* or *coordinate*.
	GO	Group order (full sorting is not imposed in a group). Valid values are: *none*, *query* or *reference*.
SQ – Sequence dictionary	SN*	Sequence name. Unique among all sequence records in the file. The value of this field is used in alignment records.
	LN*	Sequence length.
	AS	Genome assembly identifier. Refers to the reference genome assembly in an unambiguous form. Example: HG18.
	M5	MD5 checksum of the sequence in the uppercase (gaps and space are removed)
	UR	URI of the sequence
	SP	Species.
RG – read group	ID*	Unique read group identifier. The value of the ID field is used in the RG tags of alignment records.
	SM*	Sample (use pool name where a pool is being
	LB	Library
	DS	Description
	PU	Platform unit (e.g. lane for Illumina or slide for
	PI	Predicted median insert size (maybe different from the actual median insert size)
	CN	Name of sequencing center producing the read.
	DT	Date the run was produced (ISO 8601 date or date/time).
	PL	Platform/technology used to produce the read.
PG – Program	ID*	Program name
	VN	Program version
	CL	Command line

```
@HD     VN:1.0
@SQ     SN:chr20 LN:62435964
@RG     ID:L1 PU:SC_1_10 LB:SC_1 SM:NA12891
@RG     ID:L2 PU:SC_2_12 LB:SC_2 SM:NA12891
```

SAM format

Which informations are stored in a SAM file?

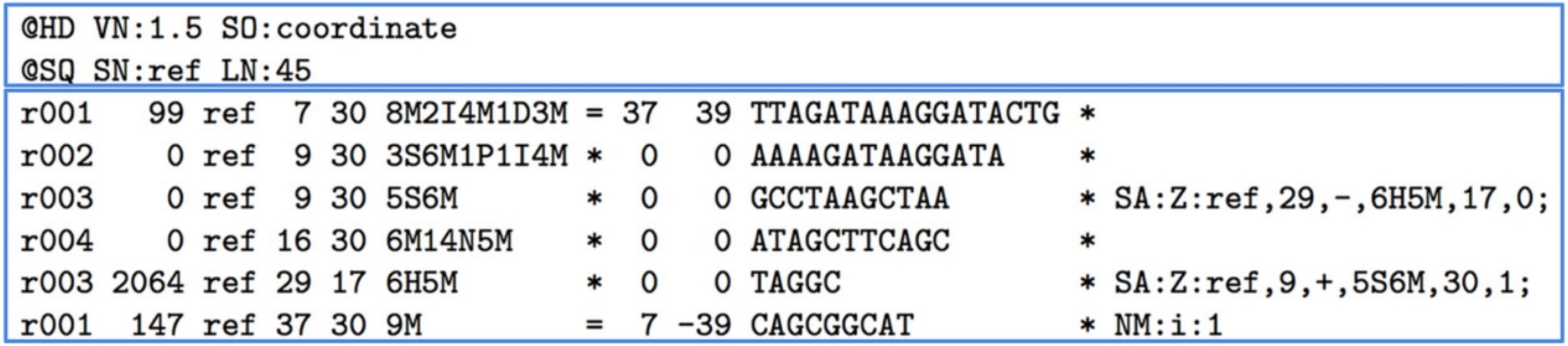
https://web-genobioinfo.toulouse.inrae.fr/~formation/16_SGS-SNP/.formats/sam.html

SAM format - Alignment section

- 11 mandatory fields
- Variable number of optional fields
- Fields are tab delimited

1. **QNAME:** Query name of the read or the read pair
2. **FLAG:** Bitwise flag (pairing, strand, mate strand, etc.)
3. **RNAME:** Reference sequence name
4. **POS:** 1-Based leftmost position of clipped alignment
5. **MAPQ:** Mapping quality (Phred-scaled)
6. **CIGAR:** Extended CIGAR string (operations: MIDNSHP)
7. **MRNM:** Mate reference name ('=' if same as RNAME)
8. **MPOS:** 1-based leftmost mate position
9. **ISIZE:** Inferred insert size
10. **SEQQuery:** Sequence on the same strand as the reference
11. **QUAL:** Query quality (ASCII-33=Phred base quality)

SAM format - Full example



```
@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
```

Header section

Alignment section

Optional fields in the format of TAG:TYPE:VALUE

QUAL: read quality; * meaning such information is not available

SEQ: read sequence

TLEN: the number of bases covered by the reads from the same fragment. Plus/minus means the current read is the leftmost/rightmost read. E.g. compare first and last lines.

PNEXT: Position of the primary alignment of the NEXT read in the template. Set as 0 when the information is unavailable. It corresponds to POS column.

RNEXT: reference sequence name of the primary alignment of the NEXT read. For paired-end sequencing, NEXT read is the paired read, corresponding to the RNAME column.

CIGAR: summary of alignment, e.g. insertion, deletion

MAPQ: mapping quality

POS: 1-based position

RNAME: reference sequence name, e.g. chromosome/transcript id

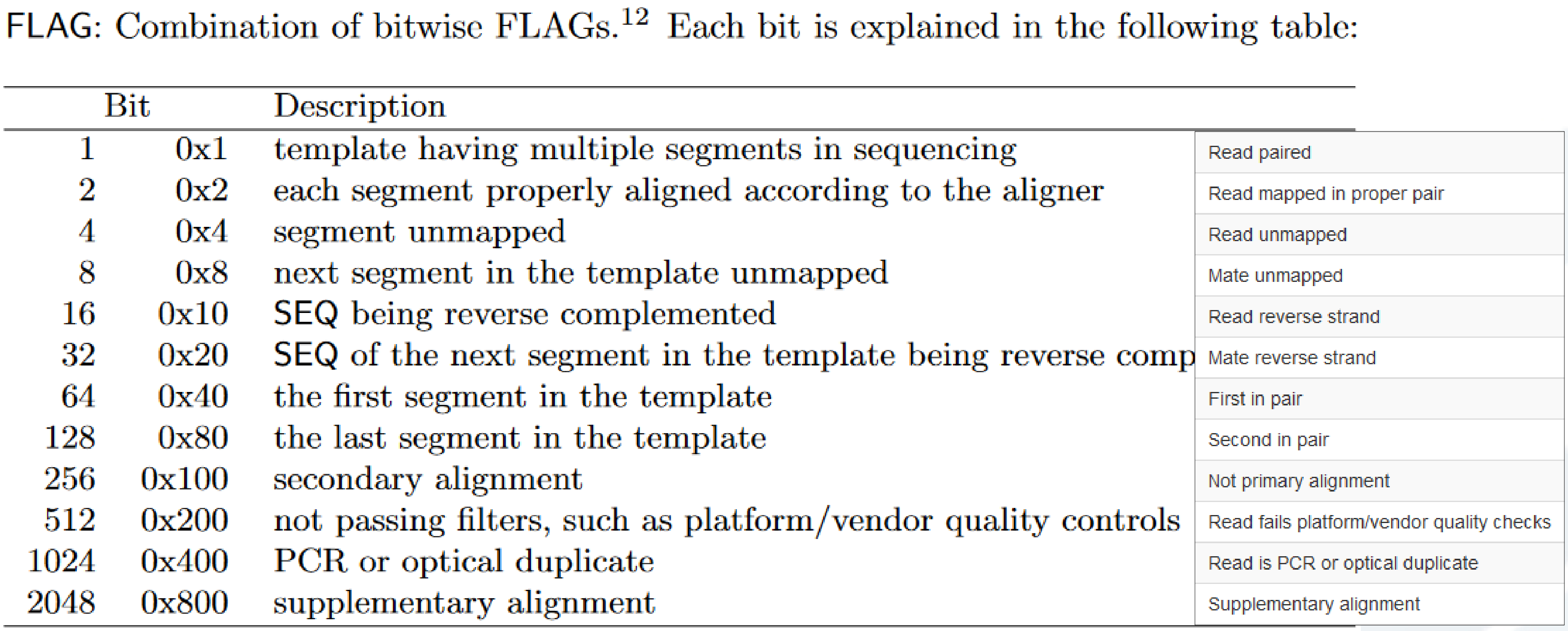
FLAG: indicates alignment information about the read, e.g. paired, aligned, etc.

QNAME: query template name, aka. read ID

X?	Reserved for end users
NM	Edit distance to the reference
MD	String for mismatching positions
RG	Read group
SA	Supp. alignment
[...]	

A	Printable character
I	Signed 32-bit integer
F	Single-precision float number
Z	Printable string
H	Hex string (high nybble first)

SAM format - Flag field



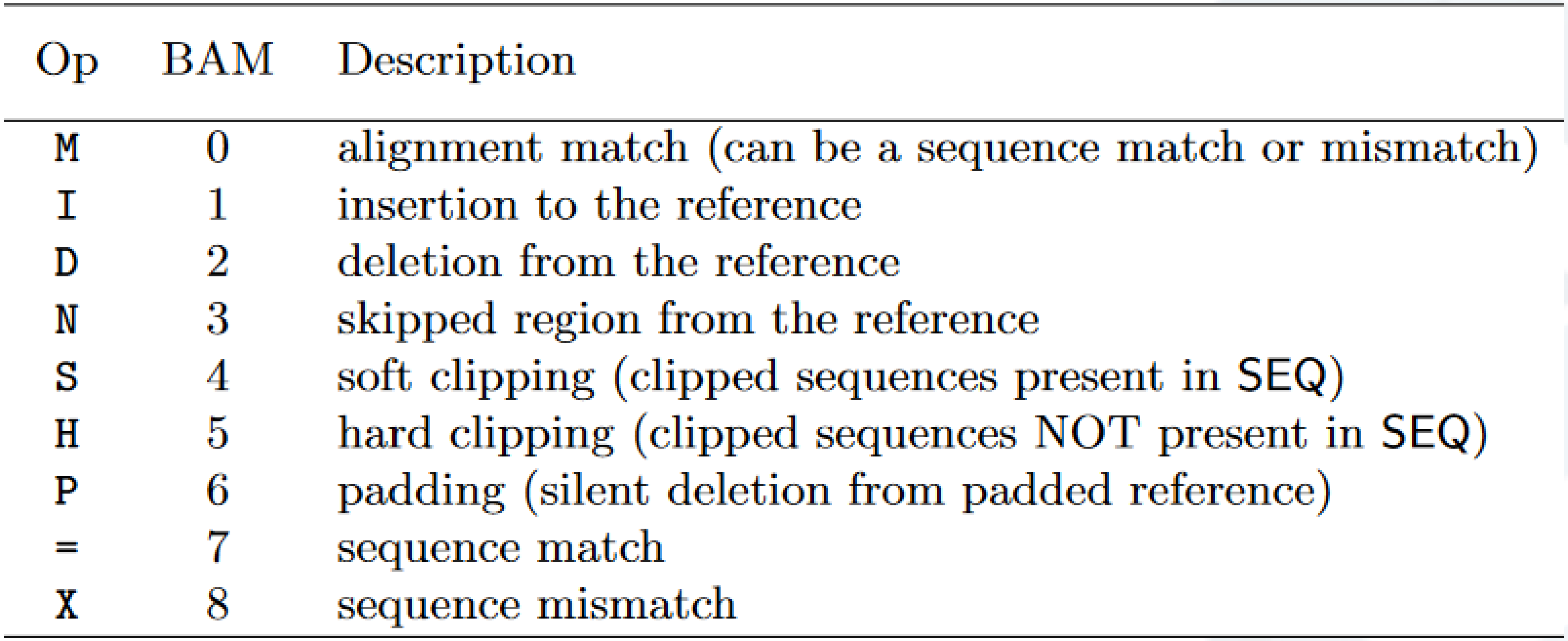
FLAG: Combination of bitwise FLAGs.[12] Each bit is explained in the following table:

Bit		Description	
1	0x1	template having multiple segments in sequencing	Read paired
2	0x2	each segment properly aligned according to the aligner	Read mapped in proper pair
4	0x4	segment unmapped	Read unmapped
8	0x8	next segment in the template unmapped	Mate unmapped
16	0x10	SEQ being reverse complemented	Read reverse strand
32	0x20	SEQ of the next segment in the template being reverse comp	Mate reverse strand
64	0x40	the first segment in the template	First in pair
128	0x80	the last segment in the template	Second in pair
256	0x100	secondary alignment	Not primary alignment
512	0x200	not passing filters, such as platform/vendor quality controls	Read fails platform/vendor quality checks
1024	0x400	PCR or optical duplicate	Read is PCR or optical duplicate
2048	0x800	supplementary alignment	Supplementary alignment

http://broadinstitute.github.io/picard/explain-flags.html

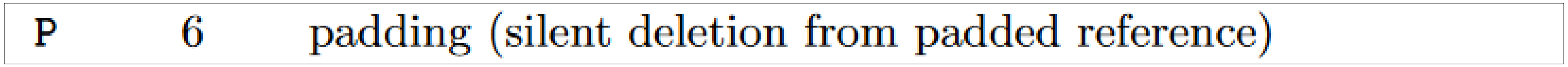
SAM format - Extended CIGAR

```
Ref: GCATTCAGATGCAGTACGC
Read:  ccTCAG--GCATTAgtg
POS  CIGAR
5    2S4M2D6M3S
```

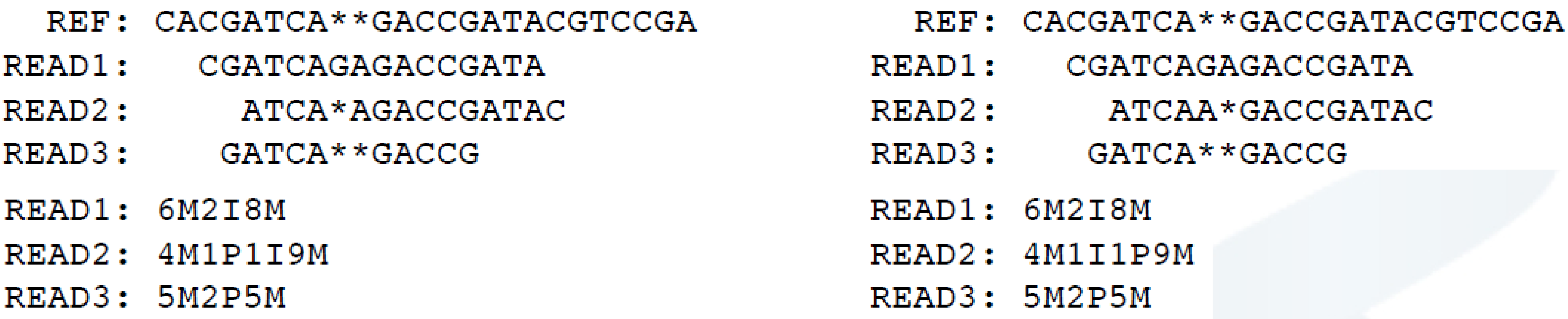


Op	BAM	Description
M	0	alignment match (can be a sequence match or mismatch)
I	1	insertion to the reference
D	2	deletion from the reference
N	3	skipped region from the reference
S	4	soft clipping (clipped sequences present in SEQ)
H	5	hard clipping (clipped sequences NOT present in SEQ)
P	6	padding (silent deletion from padded reference)
=	7	sequence match
X	8	sequence mismatch

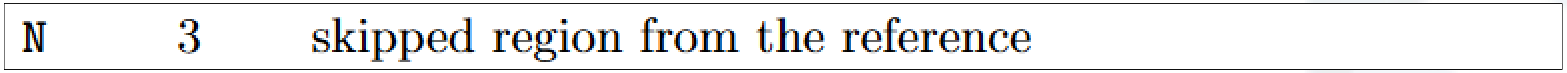
SAM format - Extended CIGAR



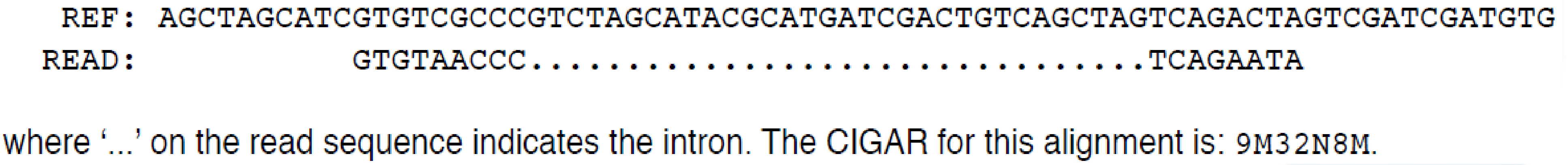
P	6	padding (silent deletion from padded reference)



```
  REF:  CACGATCA**GACCGATACGTCCGA          REF:  CACGATCA**GACCGATACGTCCGA
READ1:    CGATCAGAGACCGATA               READ1:    CGATCAGAGACCGATA
READ2:      ATCA*AGACCGATAC              READ2:      ATCAA*GACCGATAC
READ3:     GATCA**GACCG                  READ3:     GATCA**GACCG

READ1: 6M2I8M                            READ1: 6M2I8M
READ2: 4M1P1I9M                          READ2: 4M1I1P9M
READ3: 5M2P5M                            READ3: 5M2P5M
```



N	3	skipped region from the reference



```
  REF: AGCTAGCATCGTGTCGCCCGTCTAGCATACGCATGATCGACTGTCAGCTAGTCAGACTAGTCGATCGATGTG
READ:           GTGTAACCC................................TCAGAATA
```

where ‘...’ on the read sequence indicates the intron. The CIGAR for this alignment is: `9M32N8M`.

All you need to keep in mind to work with SAM files

https://www.samformat.info/

BAM / CRAM formats

BAM (format spec.)

- Binary representation of SAM
- Compressed by BGZF library
- Greatly reduces size to about 27% of original SAM

CRAM (format spec.)

- More highly compressed alternative to BAM
- Reference based compression
- Only base calls that differ need to be stored

SAMtools

- Library and software package
- Create, sort and index BAM files from SAM files
- Remove PCR duplicates
- Merge alignments
- Visualization of alignments from BAM files
- SNP and short INDELs detection

http://www.htslib.org/doc/samtools.html

Picard

- SAMtools complementary package
- More format conversion than SAMtools
- Numerous tools for manipulating SAM/BAM (n=80)
- Visualization of alignments not available
- SNP calling & short indel detection not available

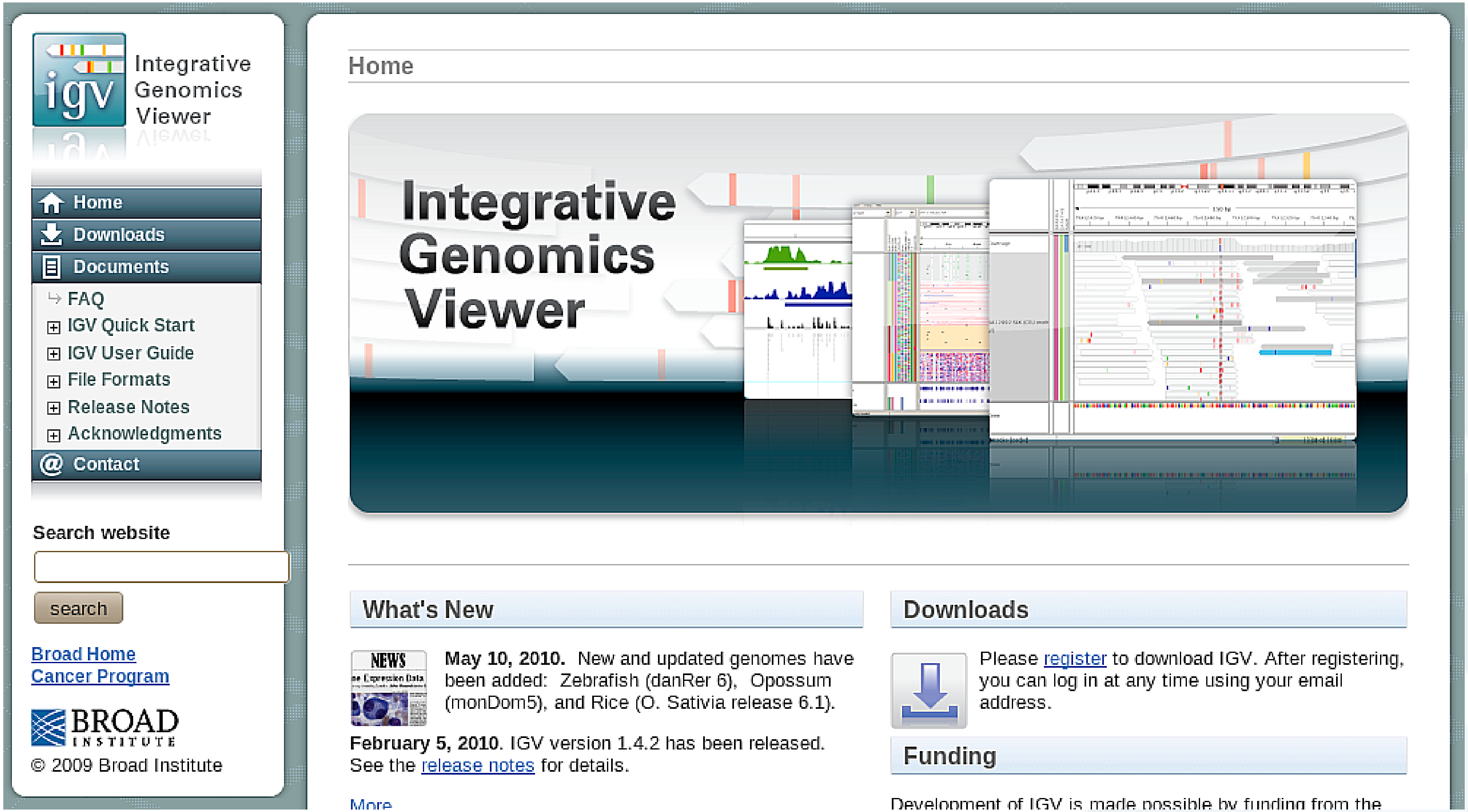
http://broadinstitute.github.io/picard/

Exercise / set 3

Visualizing the alignment - IGV

IGV Integrative Genomics Viewer

https://software.broadinstitute.org/software/igv/



Integrative Genomics Viewer

- Home
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  - FAQ
  - IGV Quick Start
  - IGV User Guide
  - File Formats
  - Release Notes
  - Acknowledgments
- Contact

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Cancer Program

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Home

What's New

May 10, 2010. New and updated genomes have been added: Zebrafish (danRer 6), Opossum (monDom5), and Rice (O. Sativia release 6.1).

February 5, 2010. IGV version 1.4.2 has been released. See the release notes for details.

More

Downloads

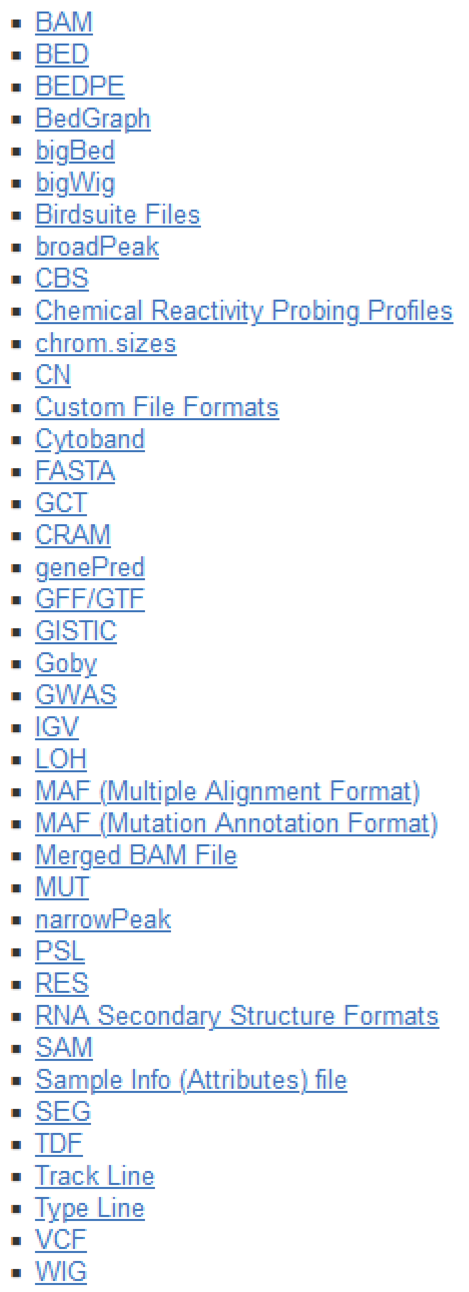
Please register to download IGV. After registering, you can log in at any time using your email address.

Funding

Development of IGV is made possible by funding from the

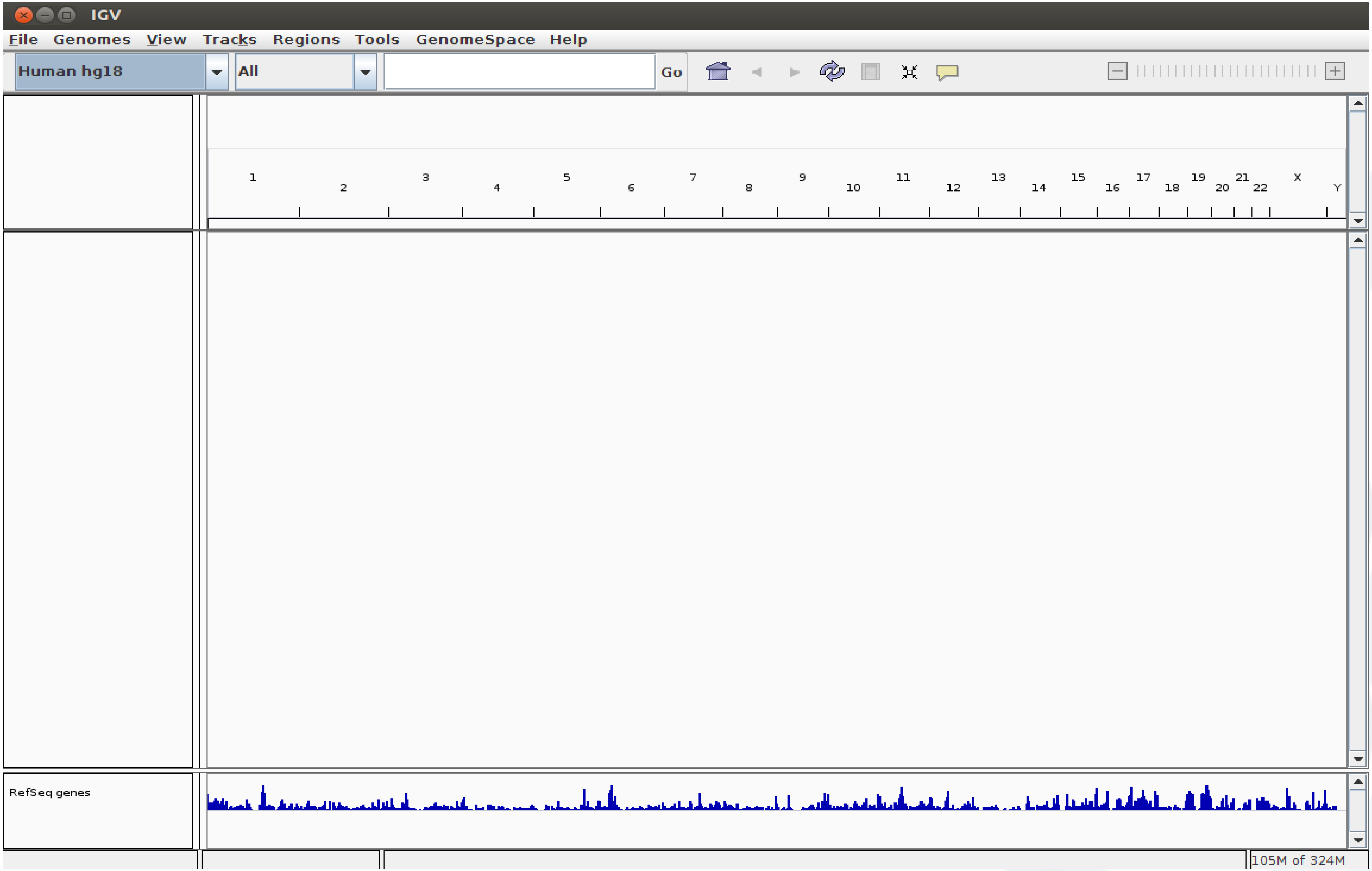
Visualizing the alignment - IGV

- High-performance visualization tool
- Interactive exploration of large, integrated datasets
- Supports a wide variety of data types
- Documentations
- Developed at the Broad Institute of MIT and Harvard

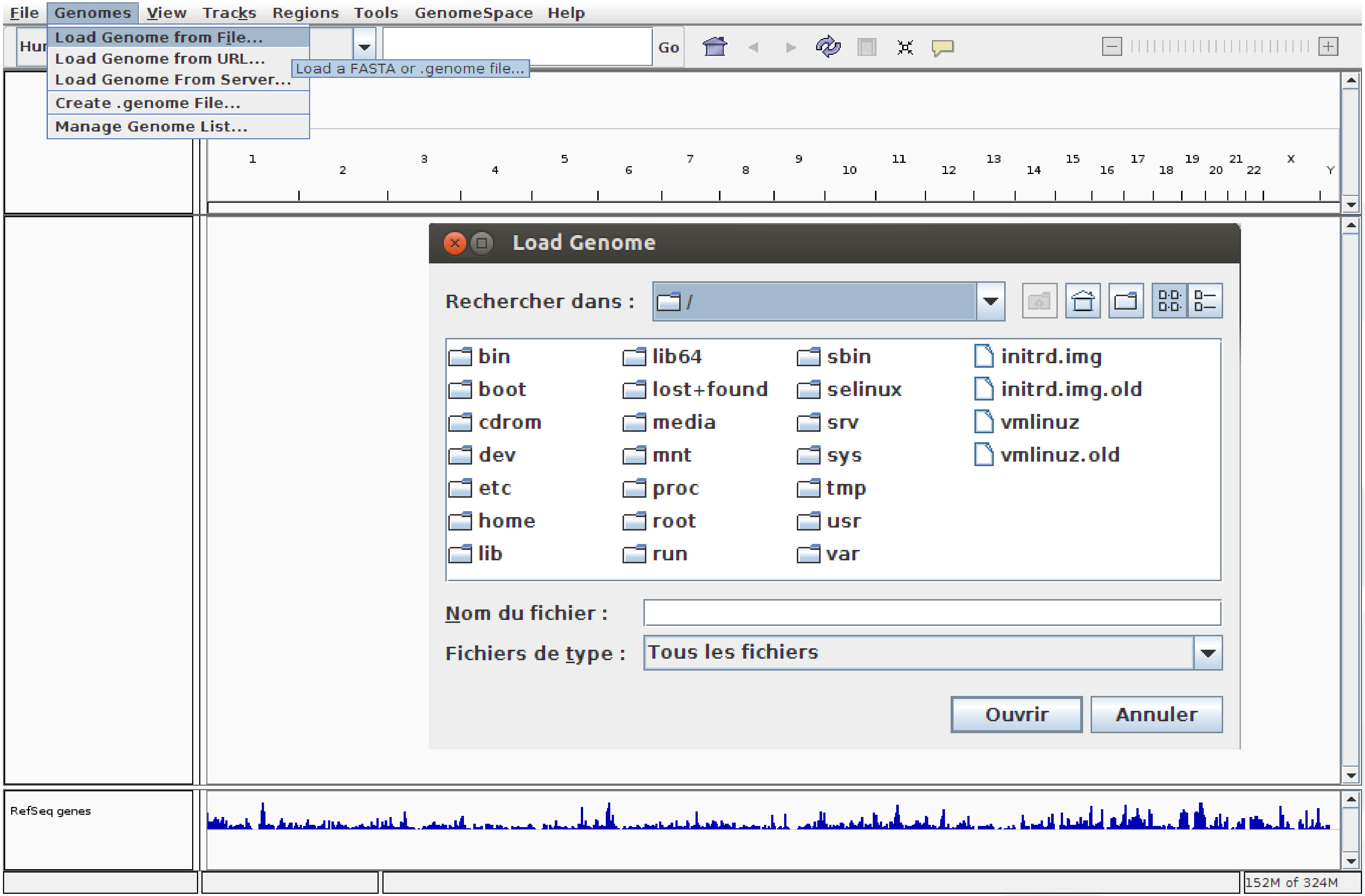


- BAM
- BED
- BEDPE
- BedGraph
- bigBed
- bigWig
- Birdsuite Files
- broadPeak
- CBS
- Chemical Reactivity Probing Profiles
- chrom.sizes
- CN
- Custom File Formats
- Cytoband
- FASTA
- GCT
- CRAM
- genePred
- GFF/GTF
- GISTIC
- Goby
- GWAS
- IGV
- LOH
- MAF (Multiple Alignment Format)
- MAF (Mutation Annotation Format)
- Merged BAM File
- MUT
- narrowPeak
- PSL
- RES
- RNA Secondary Structure Formats
- SAM
- Sample Info (Attributes) file
- SEG
- TDF
- Track Line
- Type Line
- VCF
- WIG

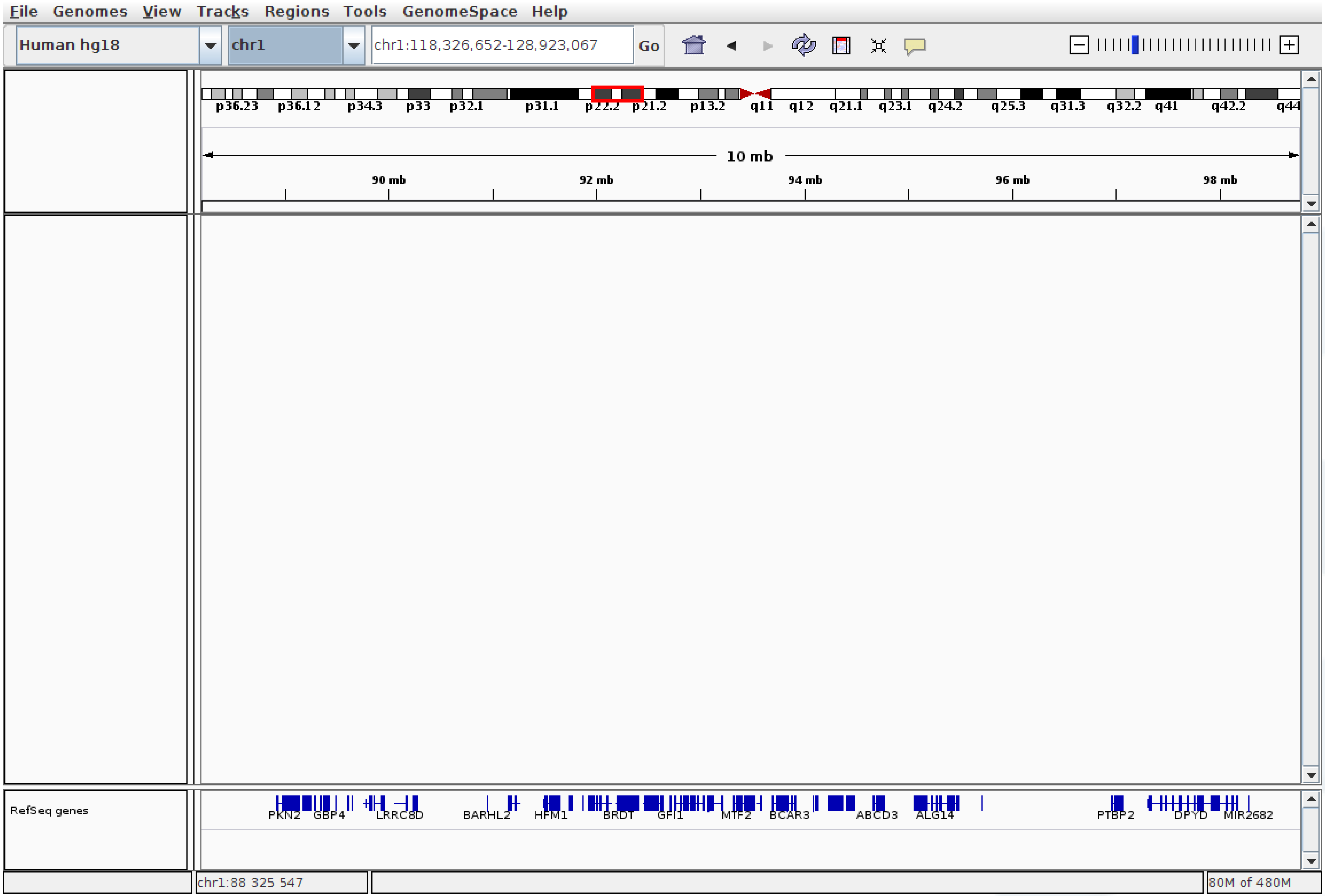
Visualizing the alignment - IGV


IGV
File Genomes View Tracks Regions Tools GenomeSpace Help
Human hg18
All
Go
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 X Y
RefSeq genes
105M of 324M

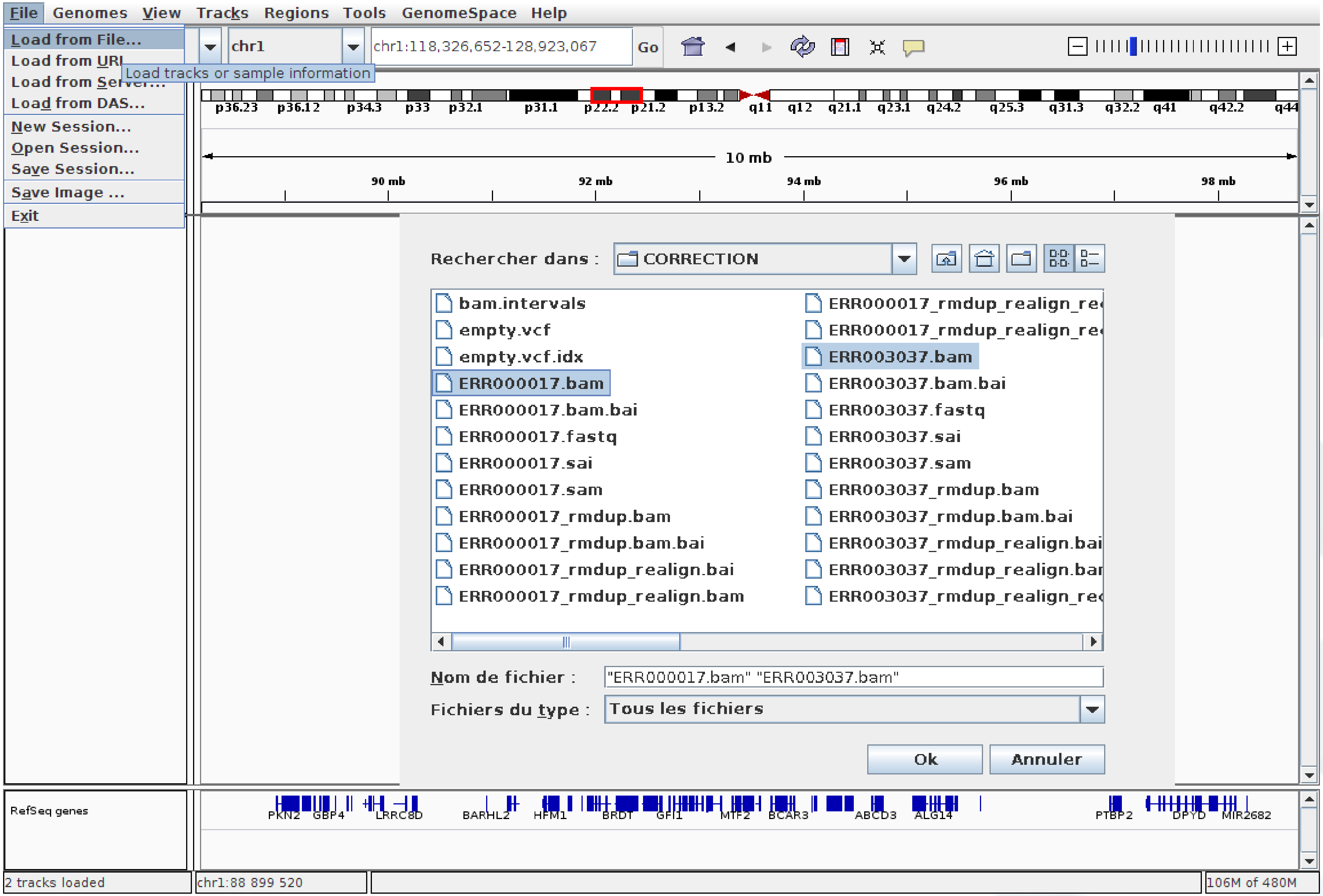
IGV - Loading the reference



IGV - Loading the reference


File Genomes View Tracks Regions Tools GenomeSpace Help
Human hg18
chr1
chr1:118,326,652-128,923,067
Go
p36.23 p36.12 p34.3 p33 p32.1 p31.1 p22.2 p21.2 p13.2 q11 q12 q21.1 q23.1 q24.2 q25.3 q31.3 q32.2 q41 q42.2 q44
10 mb
90 mb
92 mb
94 mb
96 mb
98 mb
RefSeq genes
PKN2 GBP4 LRRC8D BARHL2 HFM1 BRDT GFI1 MTF2 BCAR3 ABCD3 ALG14 PTBP2 DPYD MIR2682
chr1:88 325 547
80M of 480M

IGV - Loading the bam file



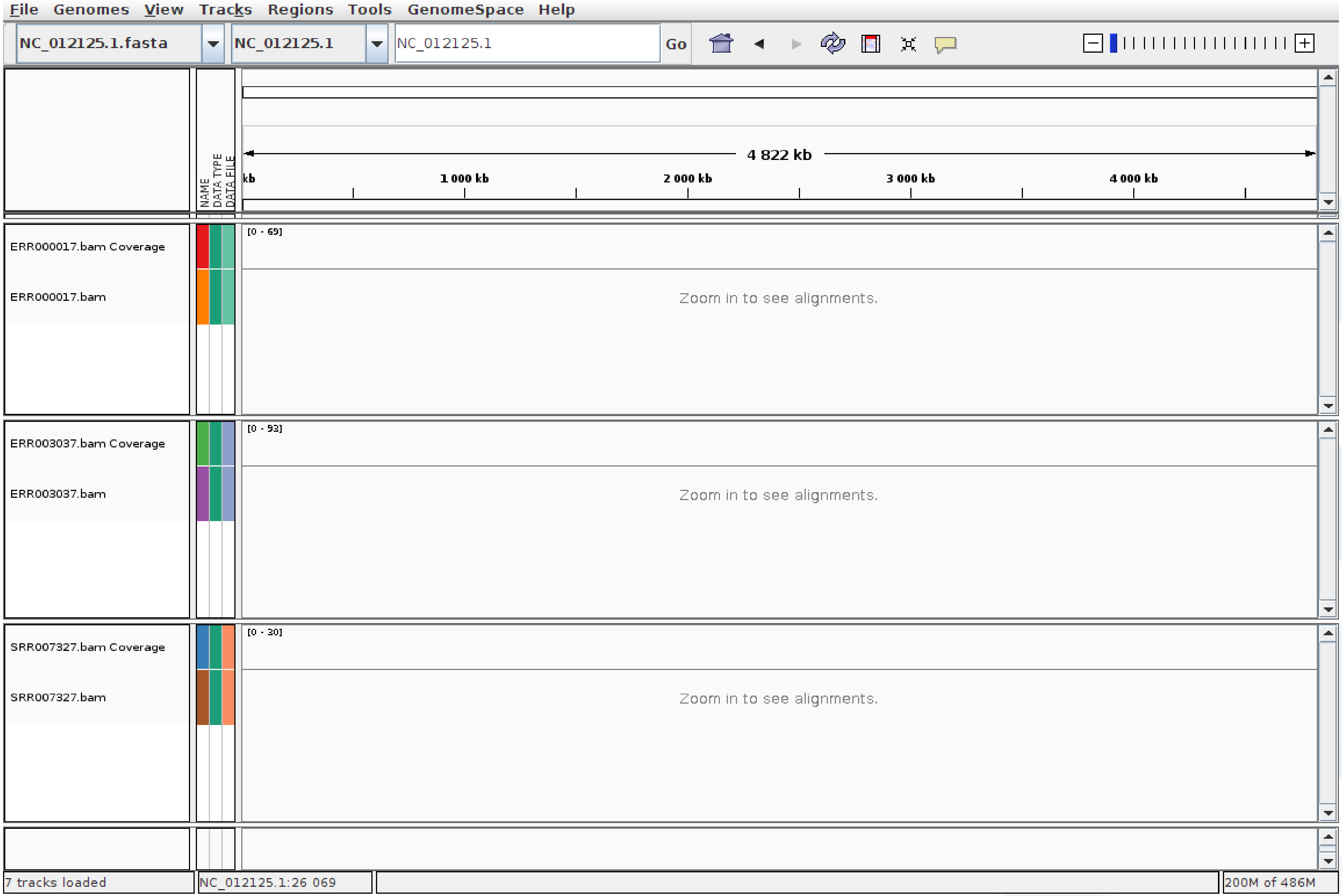
Rechercher dans : CORRECTION

Nom de fichier : "ERR000017.bam" "ERR003037.bam"

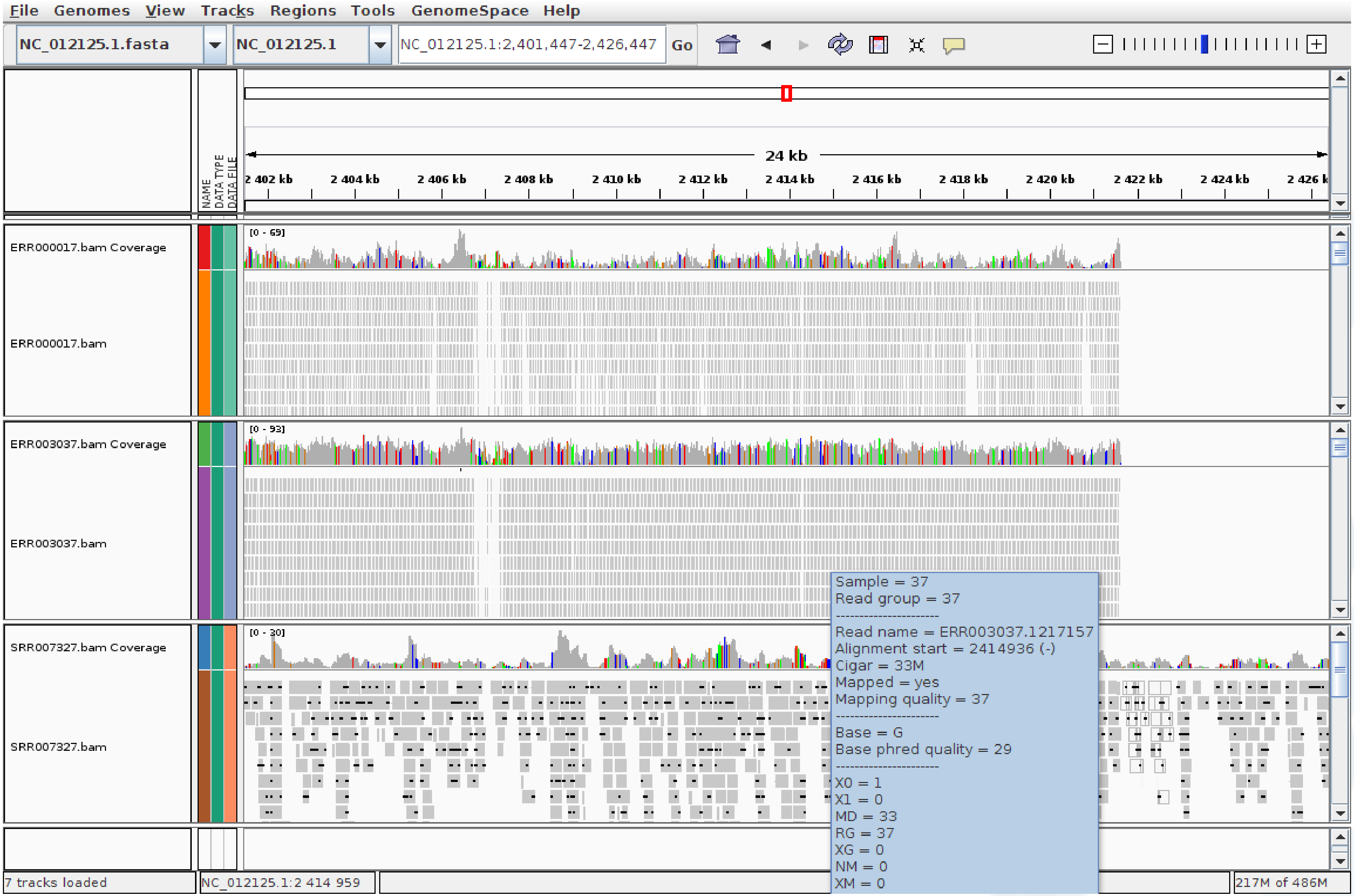
Fichiers du type : Tous les fichiers

2 tracks loaded | chr1:88 899 520 | 106M of 480M

IGV - Loading the bam file



IGV - Zoom



Sample = 37
Read group = 37

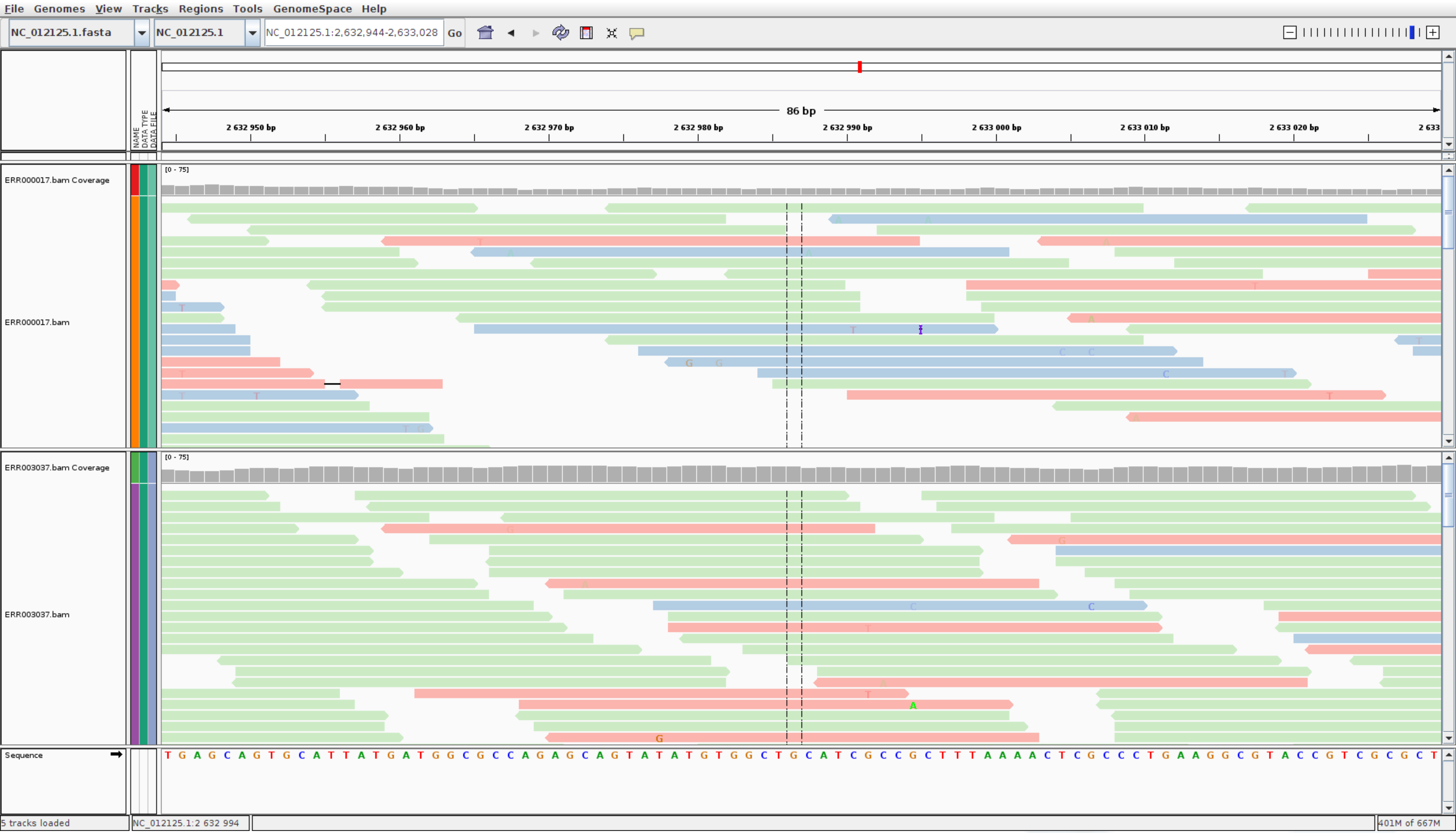
Read name = ERR003037.1217157
Alignment start = 2414936 (-)
Cigar = 33M
Mapped = yes
Mapping quality = 37

Base = G
Base phred quality = 29

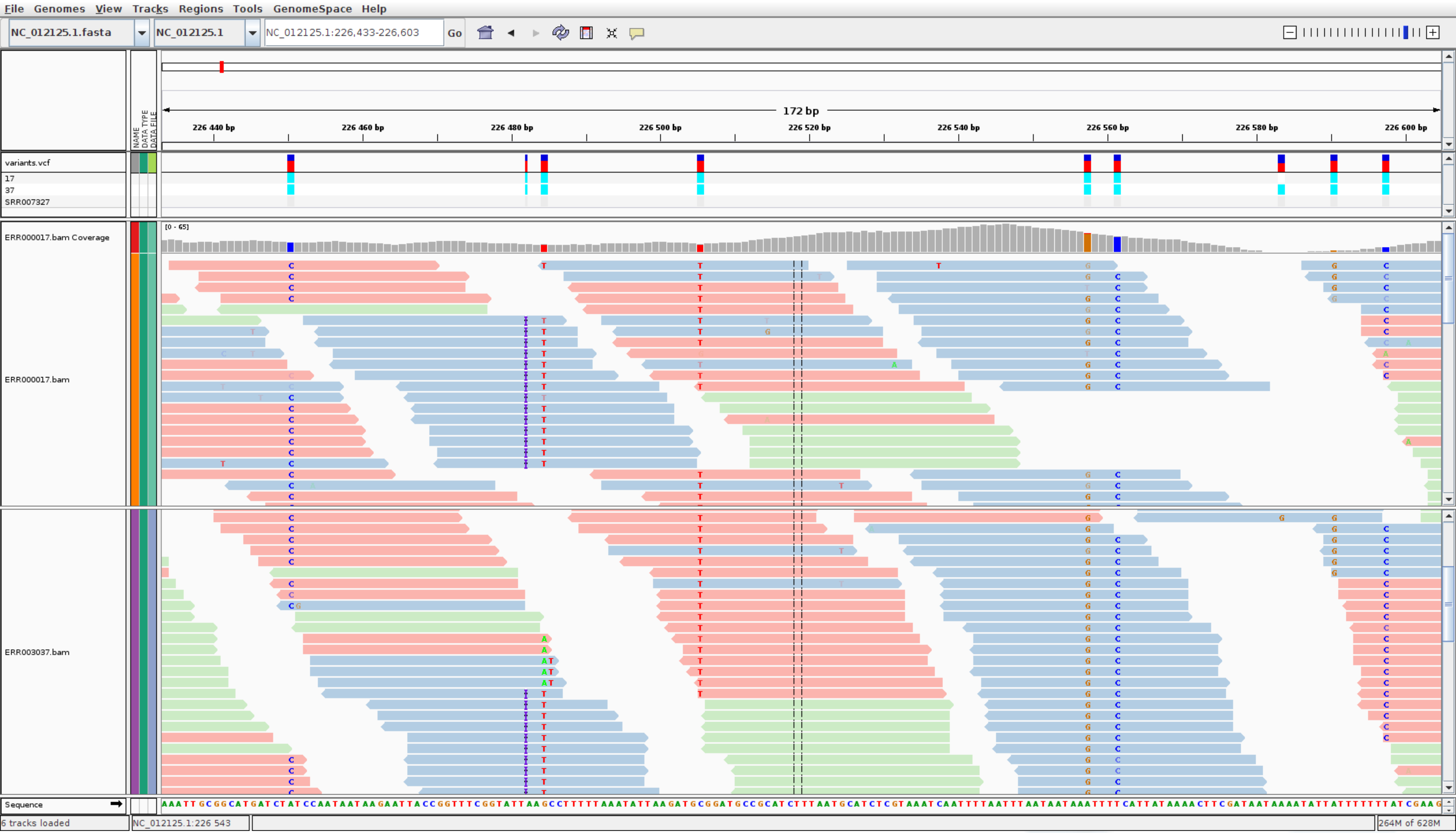
X0 = 1
X1 = 0
MD = 33
RG = 37
XG = 0
NM = 0
XM = 0

7 tracks loaded
NC_012125.1:2 414 959
217M of 486M

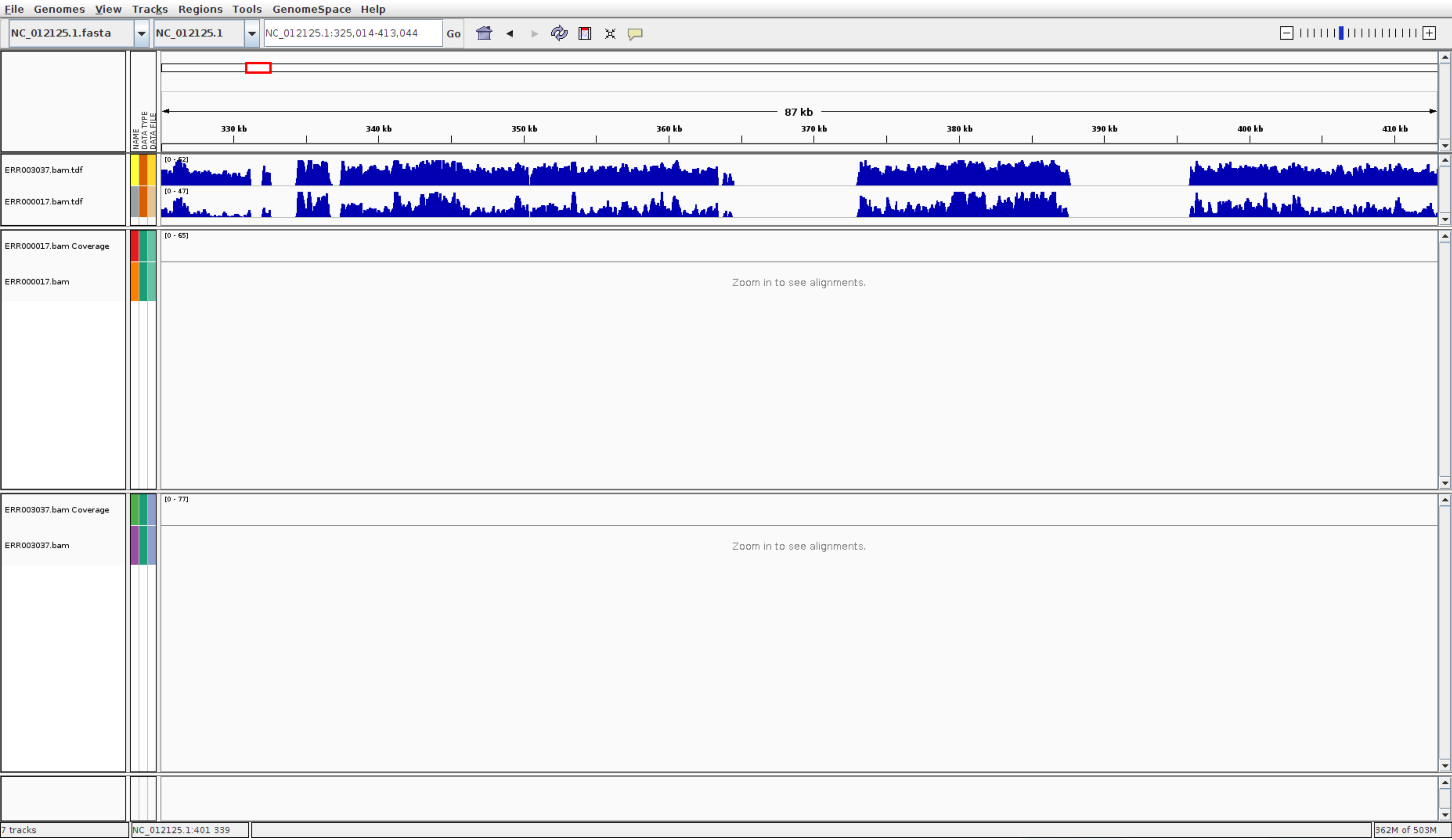
IGV - Zoom


File Genomes View Tracks Regions Tools GenomeSpace Help
NC_012125.1.fasta
NC_012125.1
NC_012125.1:2,632,944-2,633,028
Go
NAME
DATA TYPE
DATA FILE
86 bp
2 632 950 bp
2 632 960 bp
2 632 970 bp
2 632 980 bp
2 632 990 bp
2 633 000 bp
2 633 010 bp
2 633 020 bp
2 633
ERR000017.bam Coverage
[0 - 75]
ERR000017.bam
ERR003037.bam Coverage
[0 - 75]
ERR003037.bam
Sequence
T G A G C A G T G C A T T A T G A T G G C G C C A G A G C A G T A T A T G T G G C T G C A T C G C C G C T T T A A A A C T C G C C C T G A A G G C G T A C C G T C G C G C T
5 tracks loaded
NC_012125.1:2 632 994
401M of 667M

IGV - Loading an annotation file



IGV - Coverage



NC_012125.1.fasta

NC_012125.1

NC_012125.1:325,014-413,044

ERR003037.bam.tdf

ERR000017.bam.tdf

ERR000017.bam Coverage

ERR000017.bam

Zoom in to see alignments.

ERR003037.bam Coverage

ERR003037.bam

Zoom in to see alignments.

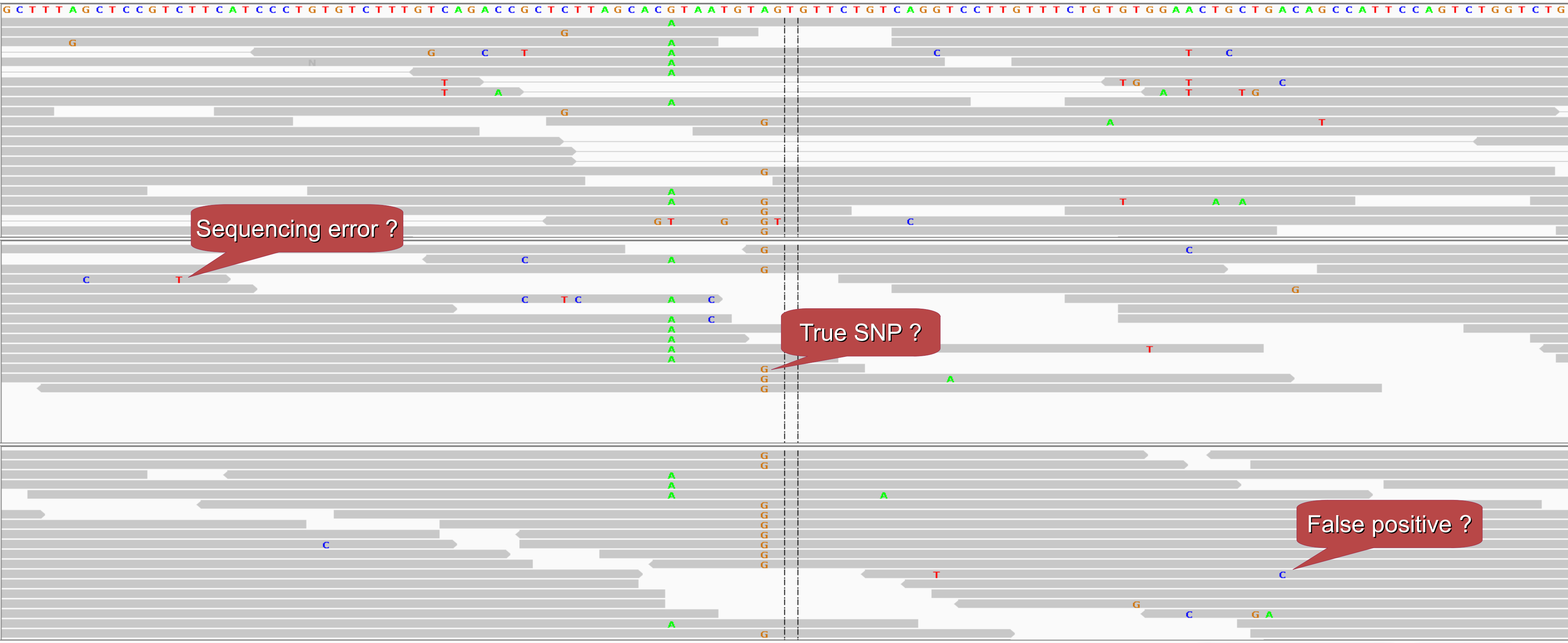
7 tracks

NC_012125.1:401 339

362M of 503M

Exercise / set 4

Variant Calling methodology



Sequencing error ?

True SNP ?

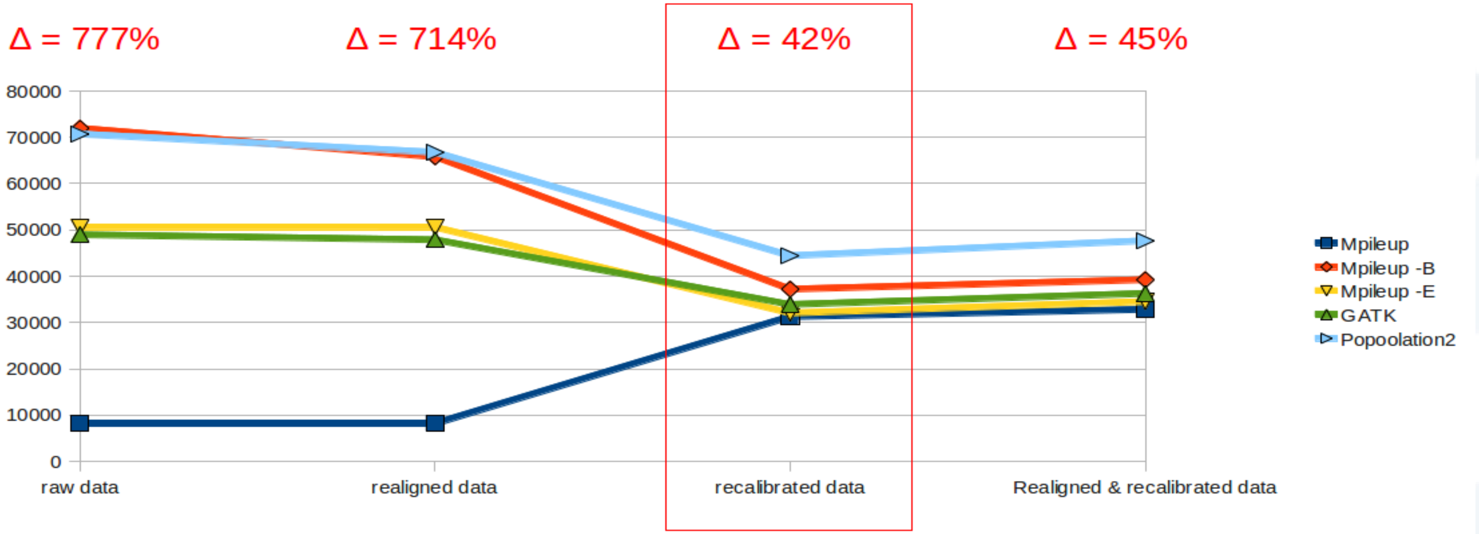
False positive ?

→ Several Variant callers :

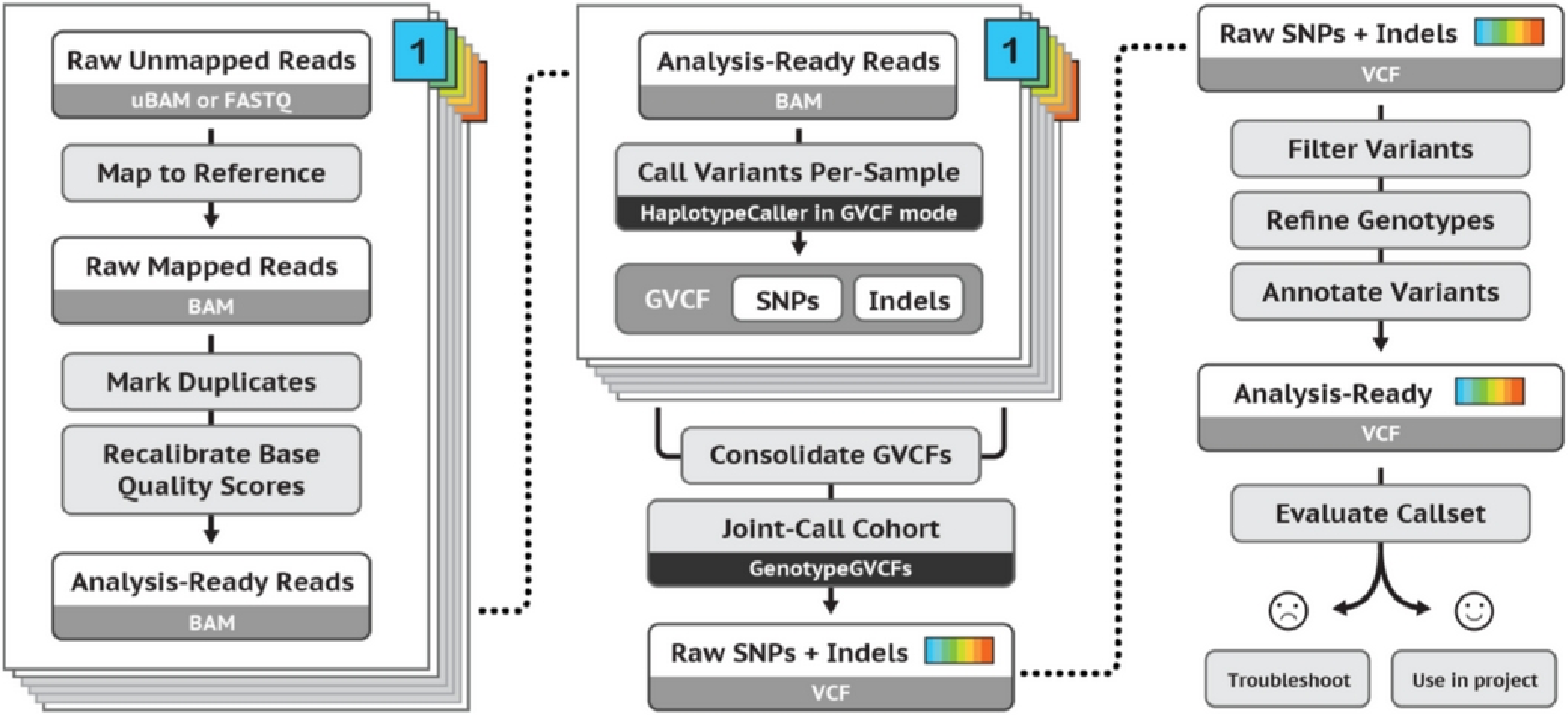
- Samtools mpileup
- GATK
- FreeBayes
- DeepVariant
- ...

Why GATK ?

- 1000 genomes project
- Very used and well documentated
- RNAseq – DNAseq
- Several technologies supported
- Tested on our data :

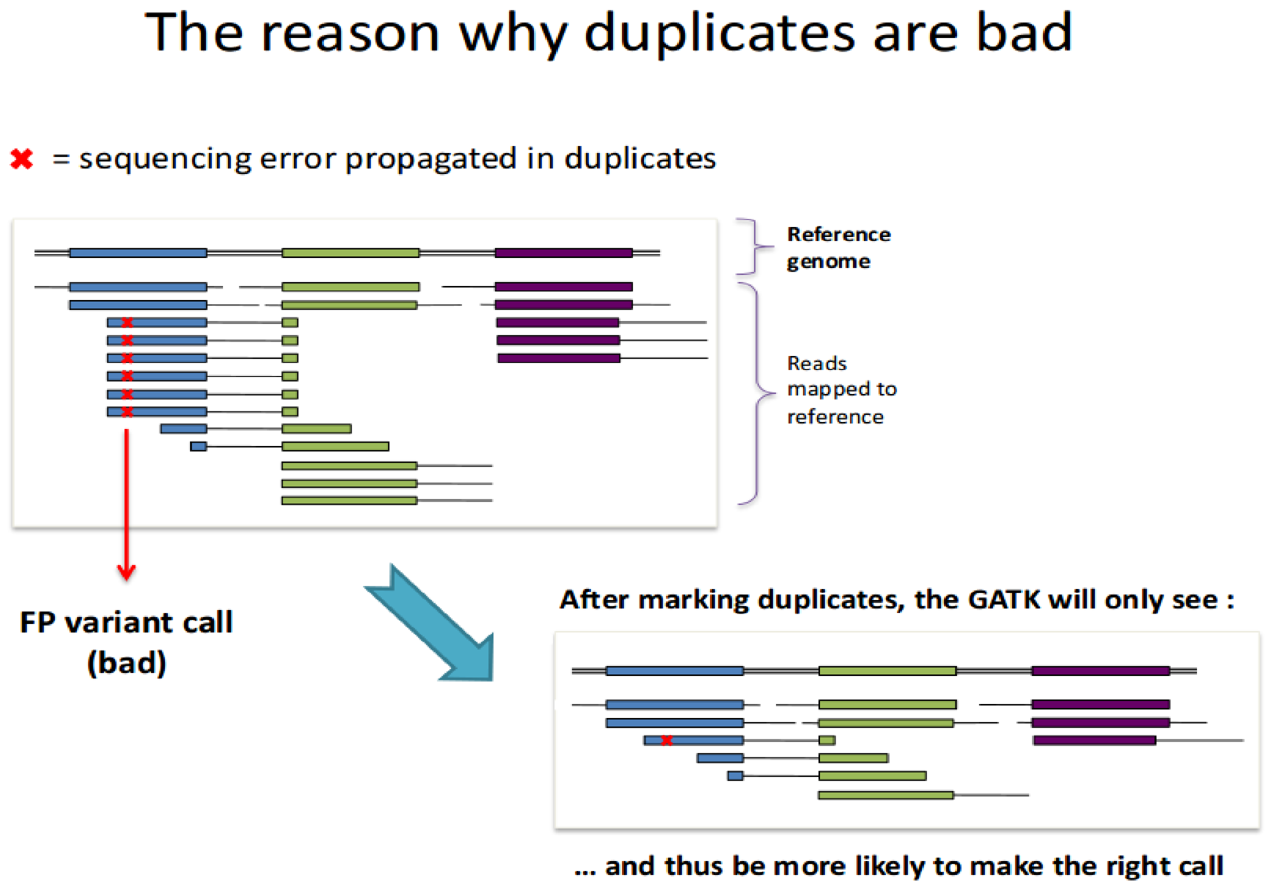


THE GATK best practices



Pre-processing – MarkDuplicates

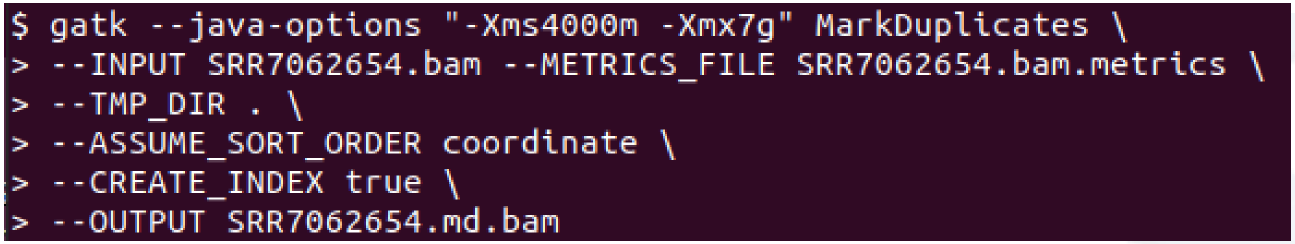
- Remove/mark duplicates (PCR and/or Optical)



The reason why duplicates are bad

✖ = sequencing error propagated in duplicates

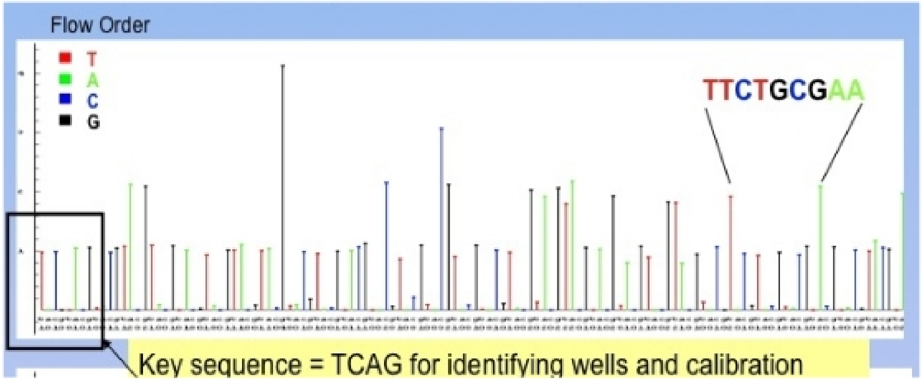
Reference genome
Reads mapped to reference
FP variant call (bad)
After marking duplicates, the GATK will only see :
... and thus be more likely to make the right call

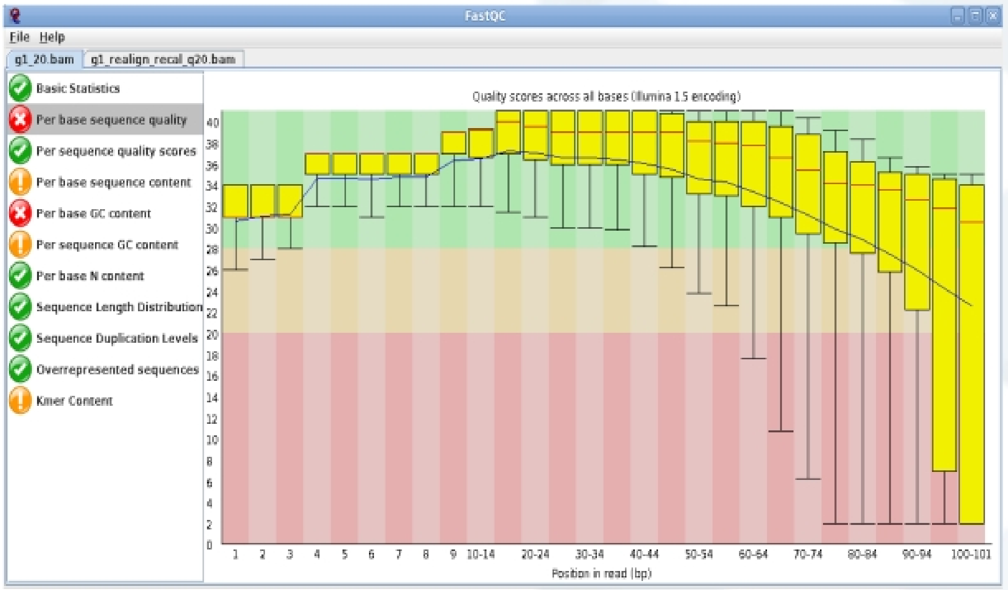


```
$ gatk --java-options "-Xms4000m -Xmx7g" MarkDuplicates \
> --INPUT SRR7062654.bam --METRICS_FILE SRR7062654.bam.metrics \
> --TMP_DIR . \
> --ASSUME_SORT_ORDER coordinate \
> --CREATE_INDEX true \
> --OUTPUT SRR7062654.md.bam
```

Pre-processing – BQSR

- Short variant calling algorithms rely heavily on the quality score
- Scores produced by the machines are subject to various sources of systematic (non-random) technical error
- The base quality provided by the sequencers is too inaccurate to be kept





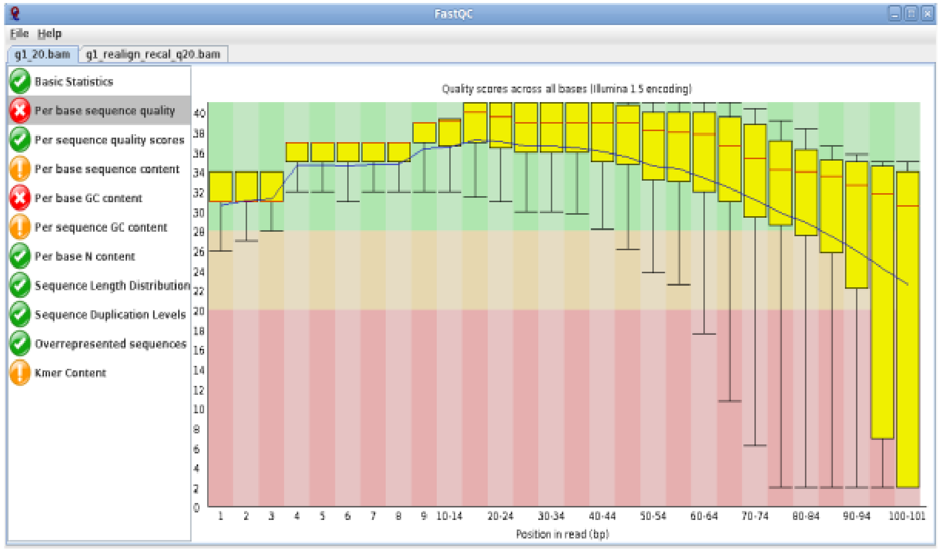
- ✓ Basic Statistics
- ✗ Per base sequence quality
- ✓ Per sequence quality scores
- ! Per base sequence content
- ✗ Per base GC content
- ! Per sequence GC content
- ✓ Per base N content
- ✓ Sequence Length Distribution
- ✓ Sequence Duplication Levels
- ✓ Overrepresented sequences
- ! Kmer Content

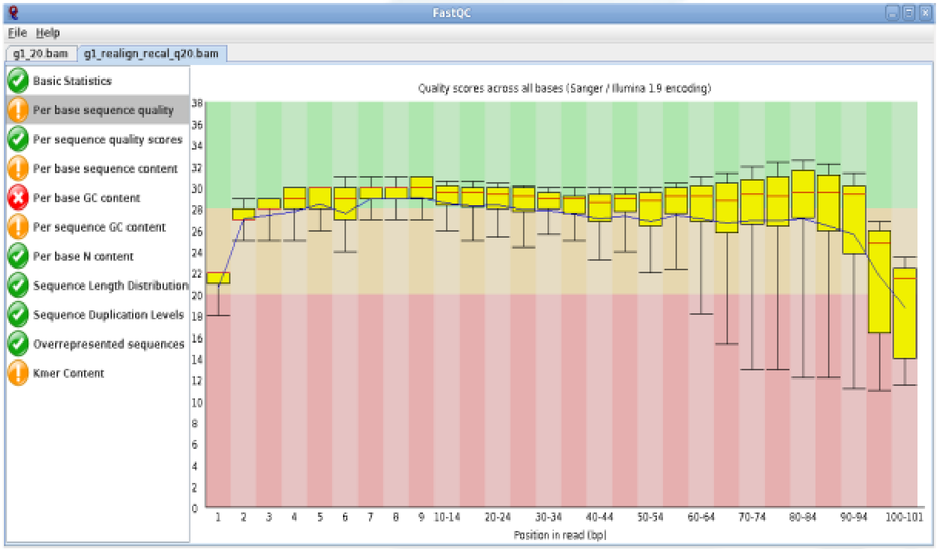
Pre-processing – BQSR step1

- **Needs knowledge of real SNP** => mask out bases at sites of real (expected) variation
- BaseRecalibrator builds the model:
  - Read group the read belongs to
  - Quality score reported by the machine
  - Machine cycle producing this base (Nth cycle = Nth base from the start of the read)
  - Current base + previous base (dinucleotide)

Pre-processing – BQSR step2

- ApplyBQSR adjusts the scores

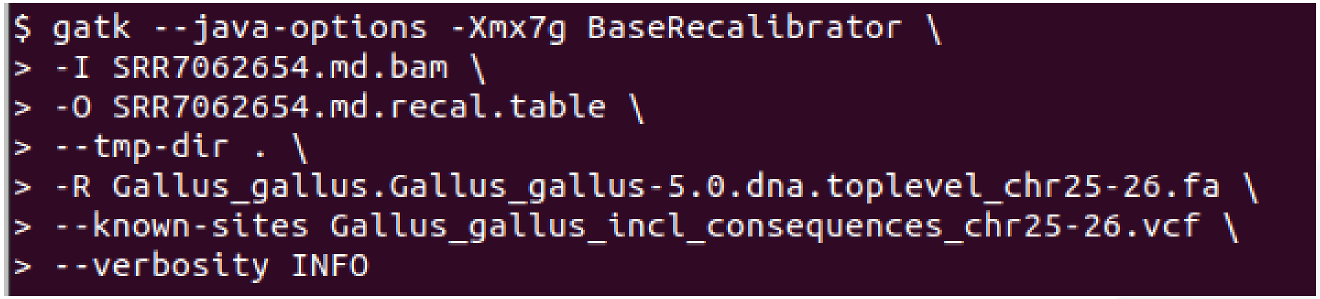

FastQC
File Help
g1_20.bam
g1_realign_recal_q20.bam
Basic Statistics
Per base sequence quality
Per sequence quality scores
Per base sequence content
Per base GC content
Per sequence GC content
Per base N content
Sequence Length Distribution
Sequence Duplication Levels
Overrepresented sequences
Kmer Content
Quality scores across all bases (Illumina 1.5 encoding)
Position in read (bp)


FastQC
File Help
g1_20.bam
g1_realign_recal_q20.bam
Basic Statistics
Per base sequence quality
Per sequence quality scores
Per base sequence content
Per base GC content
Per sequence GC content
Per base N content
Sequence Length Distribution
Sequence Duplication Levels
Overrepresented sequences
Kmer Content
Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)

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

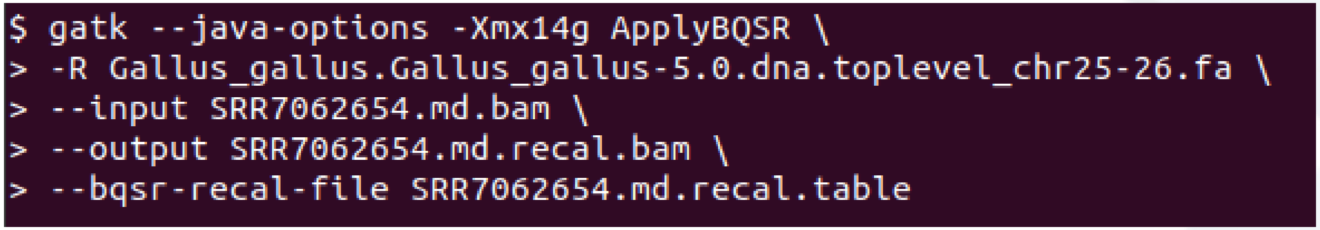
GATK – BQSR

- Two steps :
  - BaseRecalibrator builds the model



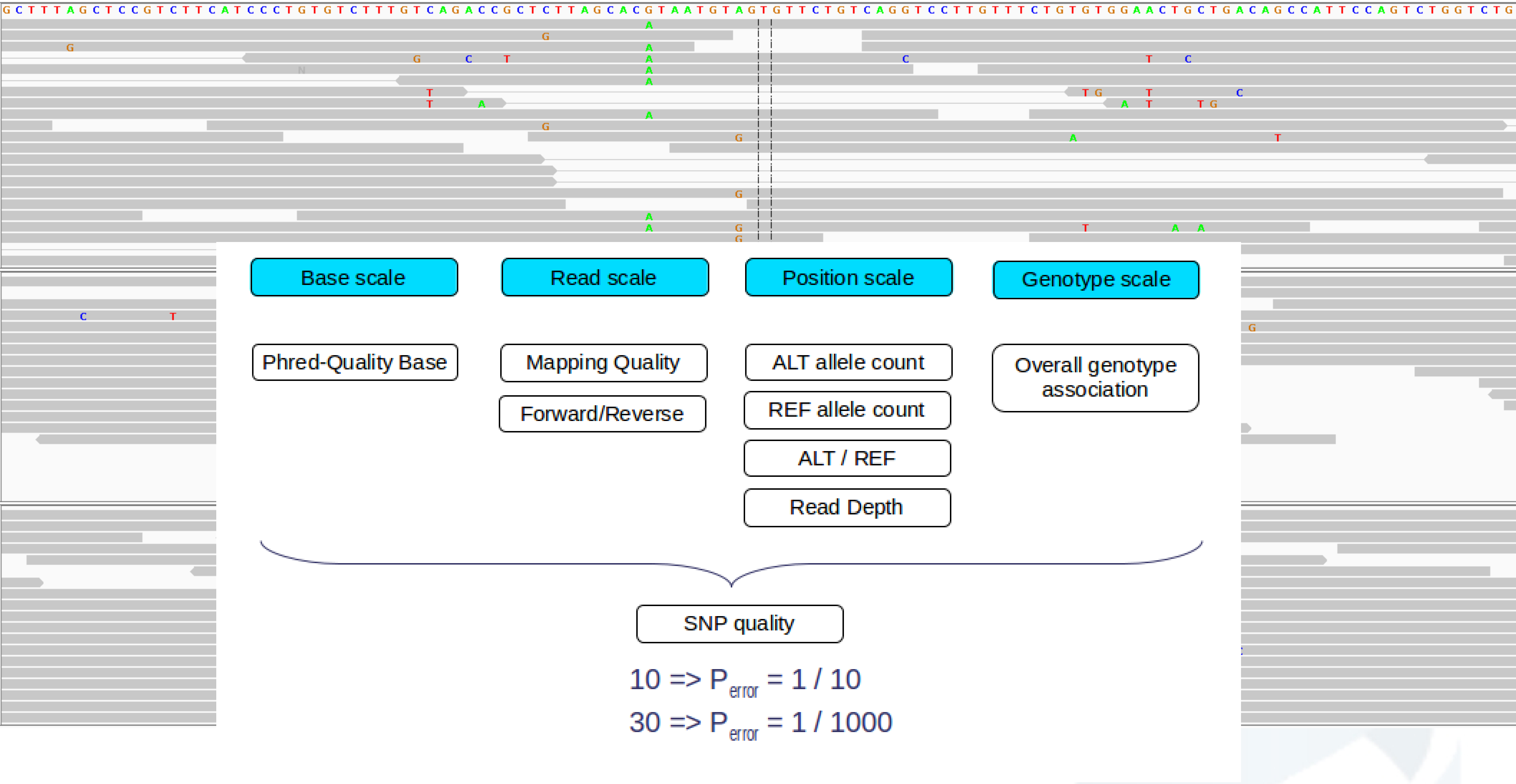
```
$ gatk --java-options -Xmx7g BaseRecalibrator \
> -I SRR7062654.md.bam \
> -O SRR7062654.md.recal.table \
> --tmp-dir . \
> -R Gallus_gallus.Gallus_gallus-5.0.dna.toplevel_chr25-26.fa \
> --known-sites Gallus_gallus_incl_consequences_chr25-26.vcf \
> --verbosity INFO
```

  - ApplyBQSR adjusts the scores :



```
$ gatk --java-options -Xmx14g ApplyBQSR \
> -R Gallus_gallus.Gallus_gallus-5.0.dna.toplevel_chr25-26.fa \
> --input SRR7062654.md.bam \
> --output SRR7062654.md.recal.bam \
> --bqsr-recal-file SRR7062654.md.recal.table
```

Variant discovery



Base scale

Phred-Quality Base

Read scale

Mapping Quality

Forward/Reverse

Position scale

ALT allele count

REF allele count

ALT / REF

Read Depth

Genotype scale

Overall genotype association

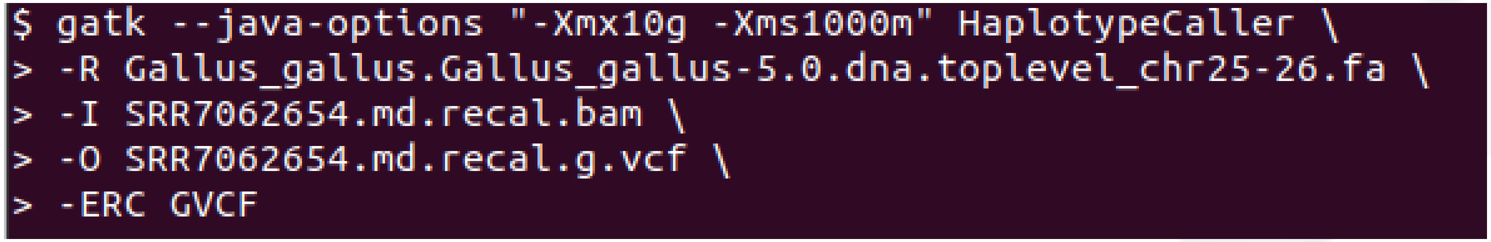
SNP quality

$10 \Rightarrow P_{error} = 1 / 10$

$30 \Rightarrow P_{error} = 1 / 1000$

GATK – HaplotypeCaller

- Call SNPs and indels via local re-assembly of haplotypes
- How HaplotypeCaller work:
    1. Define active regions
    2. Determine haplotypes by assembly of the active regions
        1. Reassemble region and identifies haplotypes (De Bruijn-like graph)
        2. realigns each haplotype against the reference haplotype (Smith-Waterman) in order to identify potentially variant sites
    3. Determine likelihoods of the haplotypes given the read data
    4. Assign sample genotypes

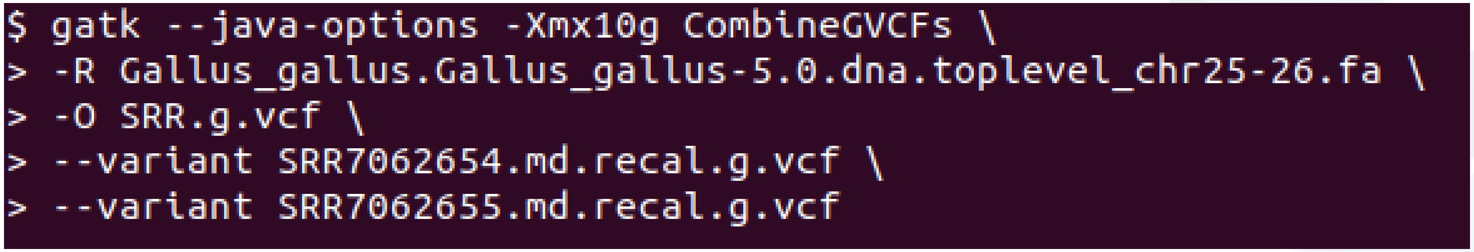


```
$ gatk --java-options "-Xmx10g -Xms1000m" HaplotypeCaller \
> -R Gallus_gallus.Gallus_gallus-5.0.dna.toplevel_chr25-26.fa \
> -I SRR7062654.md.recal.bam \
> -O SRR7062654.md.recal.g.vcf \
> -ERC GVCF
```

https://gatk.broadinstitute.org/hc/en-us/articles/360037225632-HaplotypeCaller

GATK – CombineGVCFs

- Merges one or more HaplotypeCaller GVCF files into a single GVCF
- Combine per-sample gVCF files produced by HaplotypeCaller into a multi-sample gVCF file

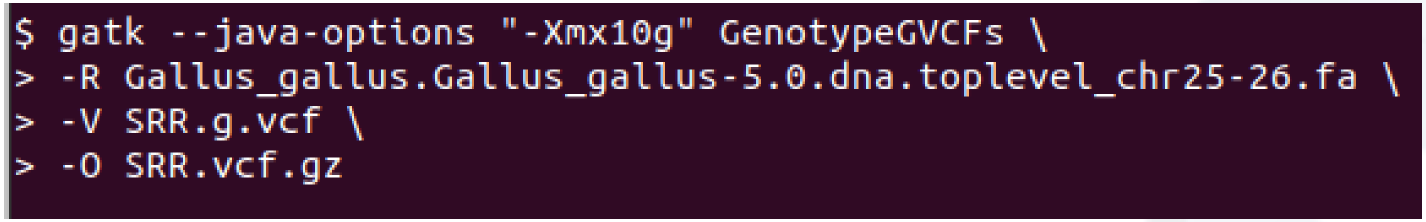


```
$ gatk --java-options -Xmx10g CombineGVCFs \
> -R Gallus_gallus.Gallus_gallus-5.0.dna.toplevel_chr25-26.fa \
> -O SRR.g.vcf \
> --variant SRR7062654.md.recal.g.vcf \
> --variant SRR7062655.md.recal.g.vcf
```

https://gatk.broadinstitute.org/hc/en-us/articles/360037053272-CombineGVCFs

GATK – GenotypeGVCFs

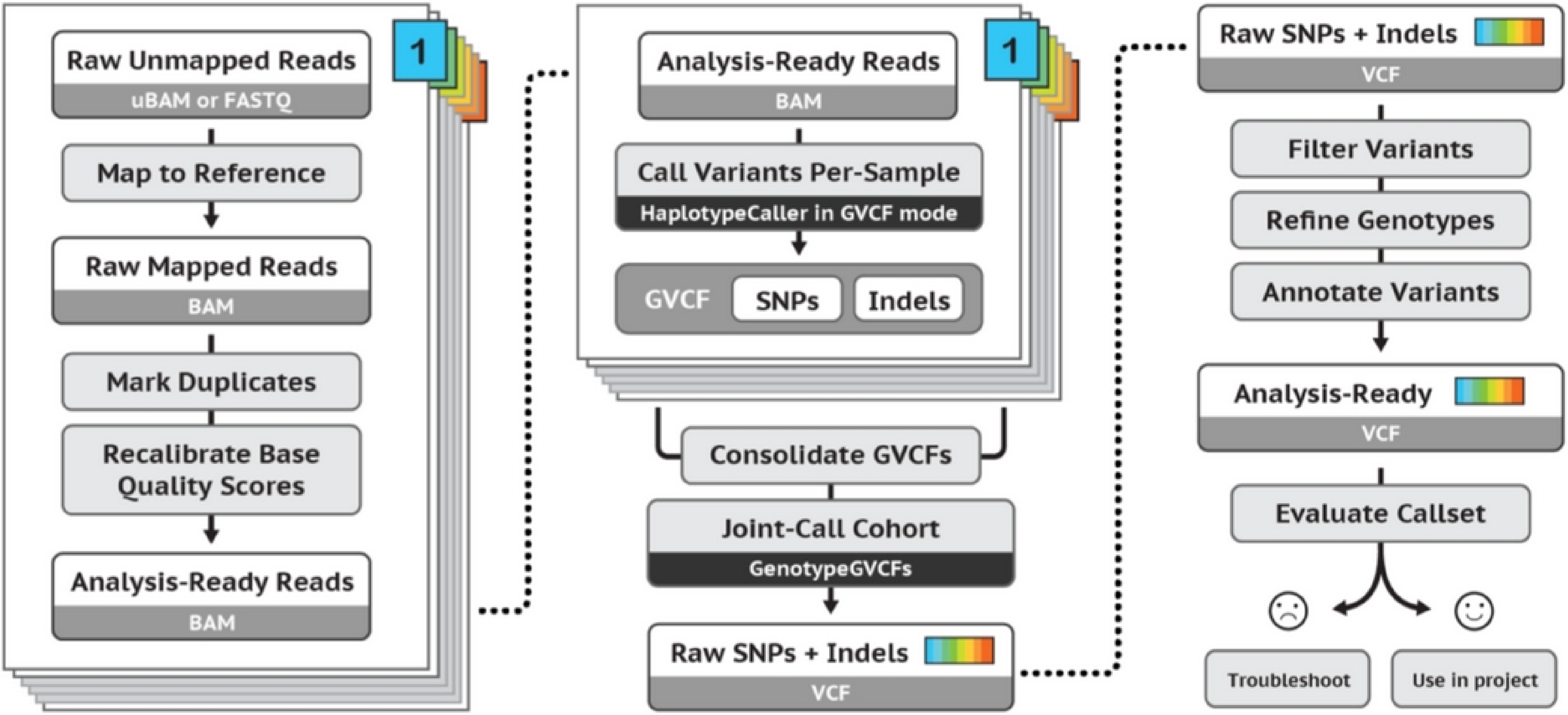
- Perform joint genotyping on one or more samples pre-called with HaplotypeCaller



```
$ gatk --java-options "-Xmx10g" GenotypeGVCFs \
> -R Gallus_gallus.Gallus_gallus-5.0.dna.toplevel_chr25-26.fa \
> -V SRR.g.vcf \
> -O SRR.vcf.gz
```

https://gatk.broadinstitute.org/hc/en-us/articles/360037057852-GenotypeGVCFs

THE GATK best practices



Raw Unmapped Reads

uBAM or FASTQ

Map to Reference

Raw Mapped Reads

BAM

Mark Duplicates

Recalibrate Base Quality Scores

Analysis-Ready Reads

BAM

Analysis-Ready Reads

BAM

Call Variants Per-Sample

HaplotypeCaller in GVCF mode

GVCF SNPs Indels

Consolidate GVCFs

Joint-Call Cohort

GenotypeGVCFs

Raw SNPs + Indels

VCF

Raw SNPs + Indels

VCF

Filter Variants

Refine Genotypes

Annotate Variants

Analysis-Ready

VCF

Evaluate Callset

Troubleshoot

Use in project

Exercise / set 5

VCF format

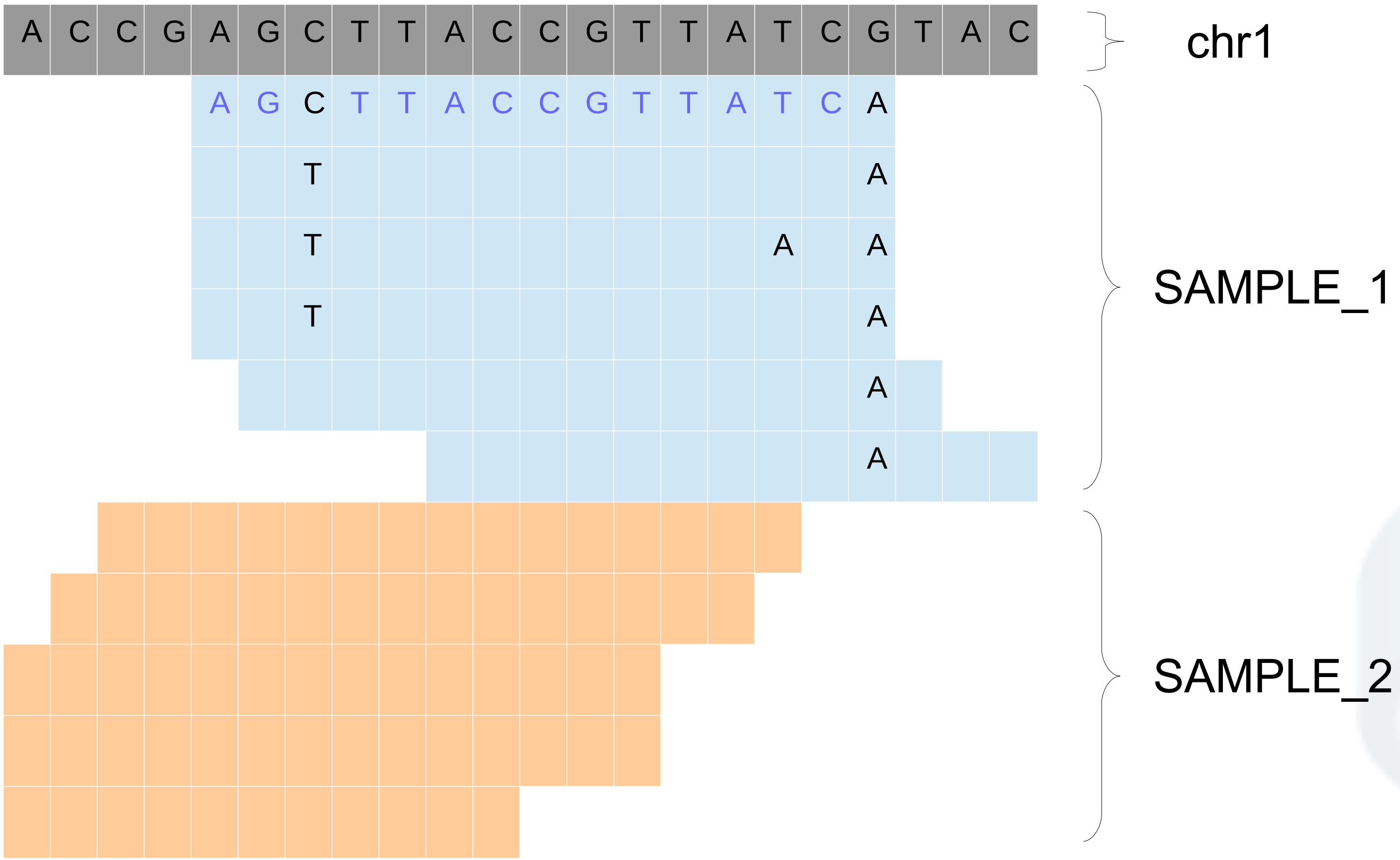
- Which informations have been stored in VCF ?

https://web-genobioinfo.toulouse.inrae.fr/~formation/16_SGS-SNP/.formats/vcf.html

Variant calling Format (VCF)

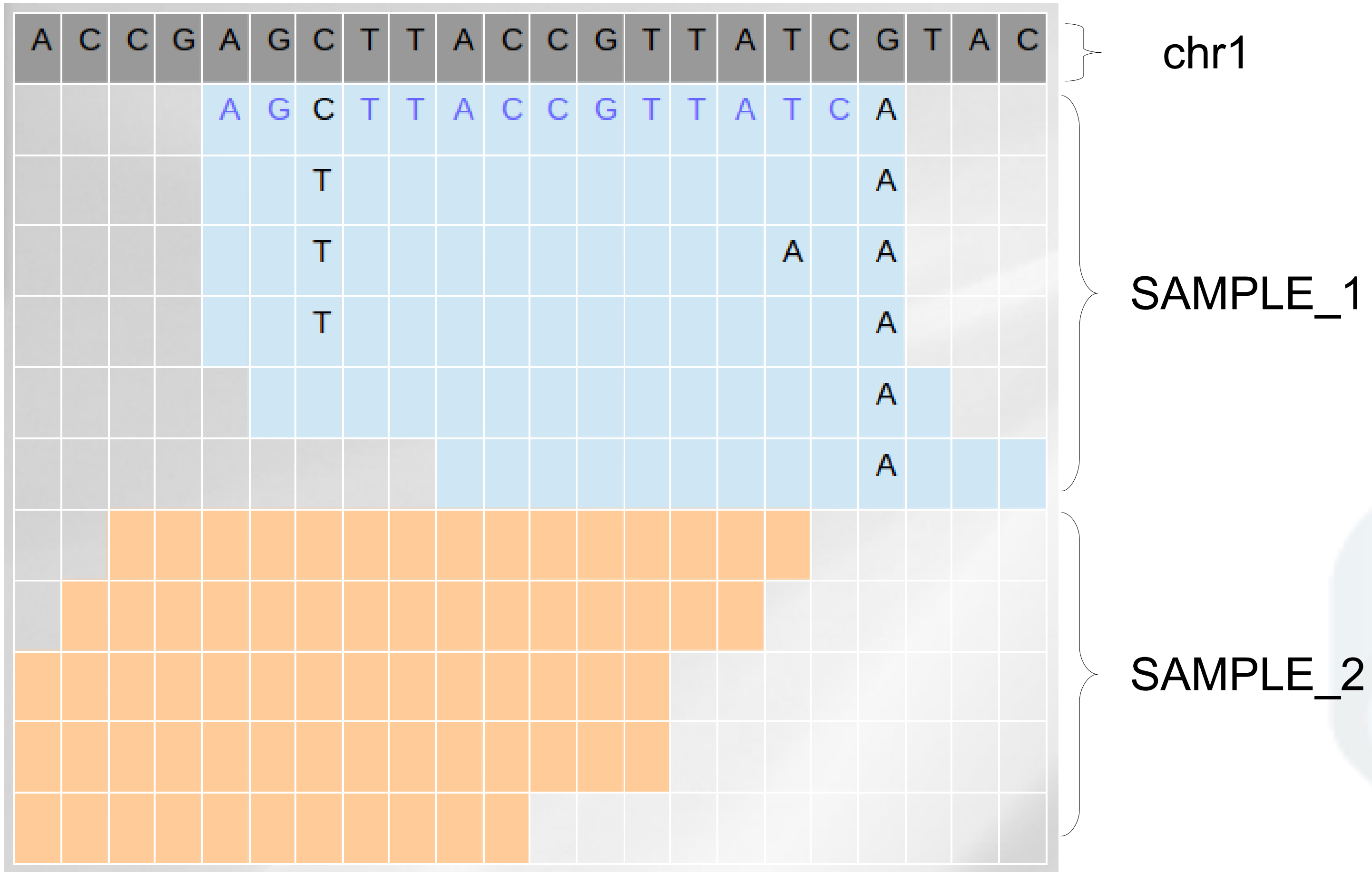
- http://vcftools.sourceforge.net/specs.html
- Tab-delimited file
- Basic documentation inside
- Header lines start with ## or #
- Give informations of data/tools/parameters used
- Variant lines represent a position on the genome

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

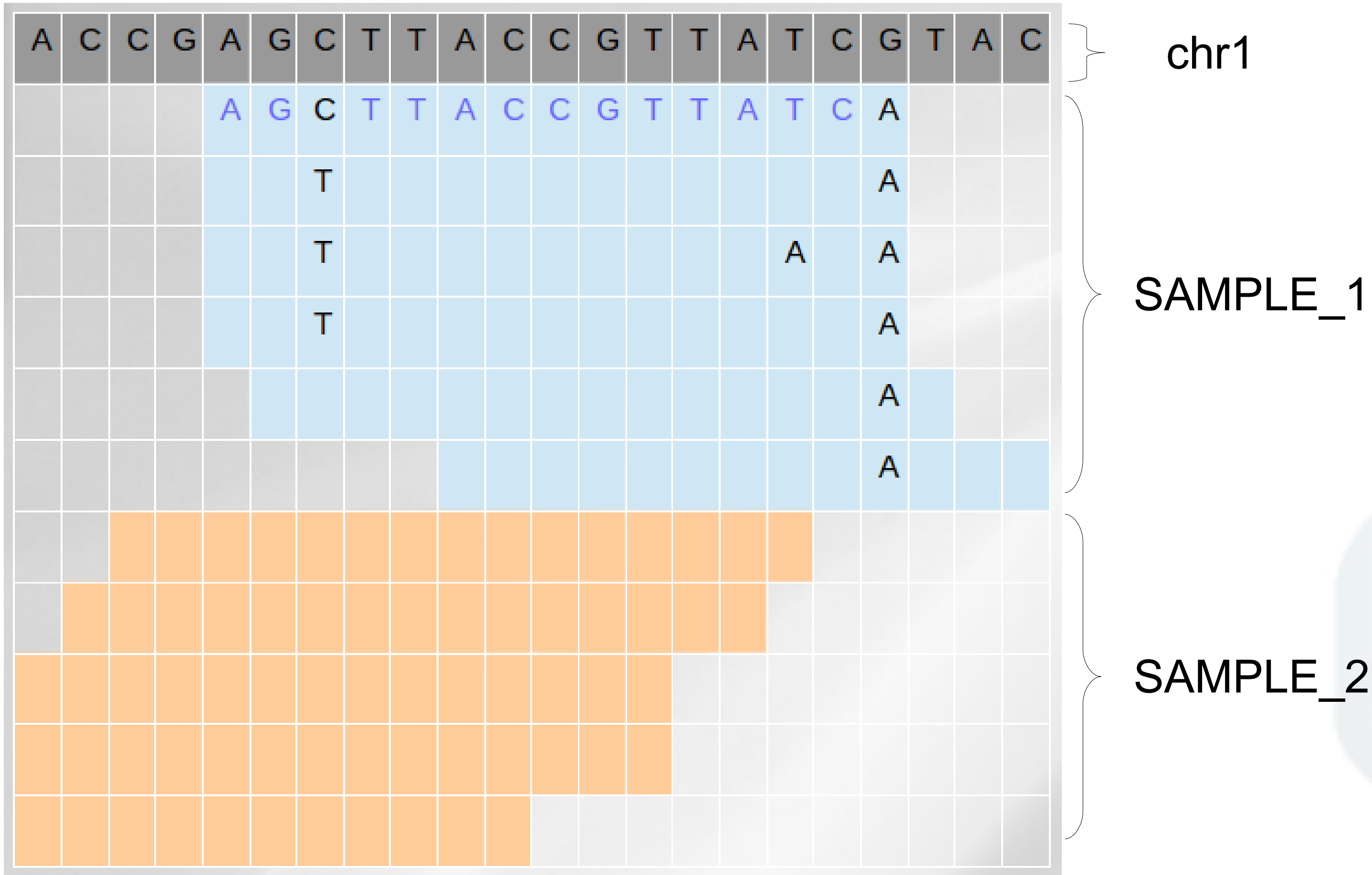
```
#CHR  POS   ID    REF   ALT   QUAL   FILTER    [INFOS]    FORMAT          SAMPLE_1                 SAMPLE_2

chr1  7     .     C     T     247.82      .    [INFOS]    GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15    0/1:2,3:5:9.01:10,1,6

chr1  19    .     G     A     124.34      .    [INFOS]    GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94   ./.
```

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

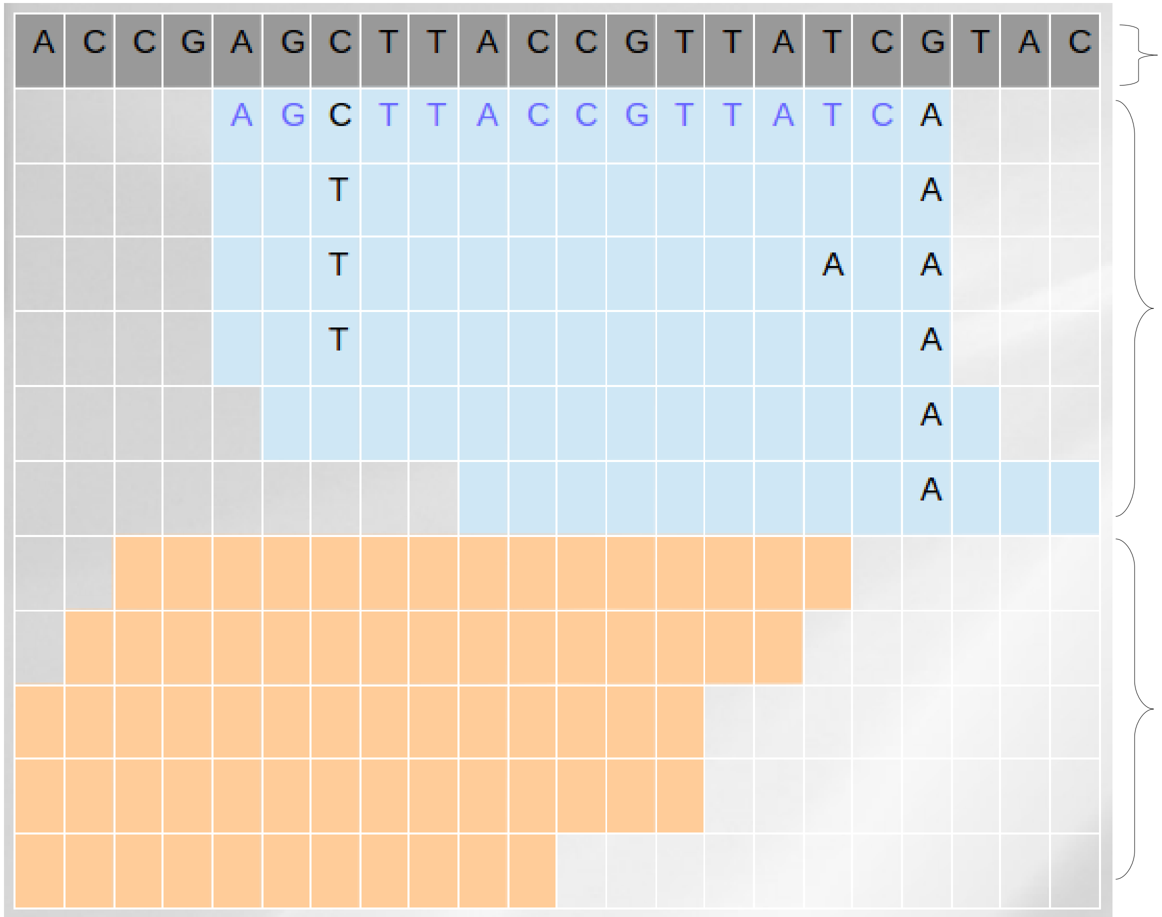
```
#CHR  POS   ID    REF   ALT   QUAL   FILTER     [INFOS]    FORMAT          SAMPLE_1                 SAMPLE_2
chr1  7     .     C     T     247.82      .     [INFOS]    GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15    0/1:2,3:5:9.01:10,1,6

chr1  19    .     G     A     124.34      .     [INFOS]    GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94   ./.
```

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

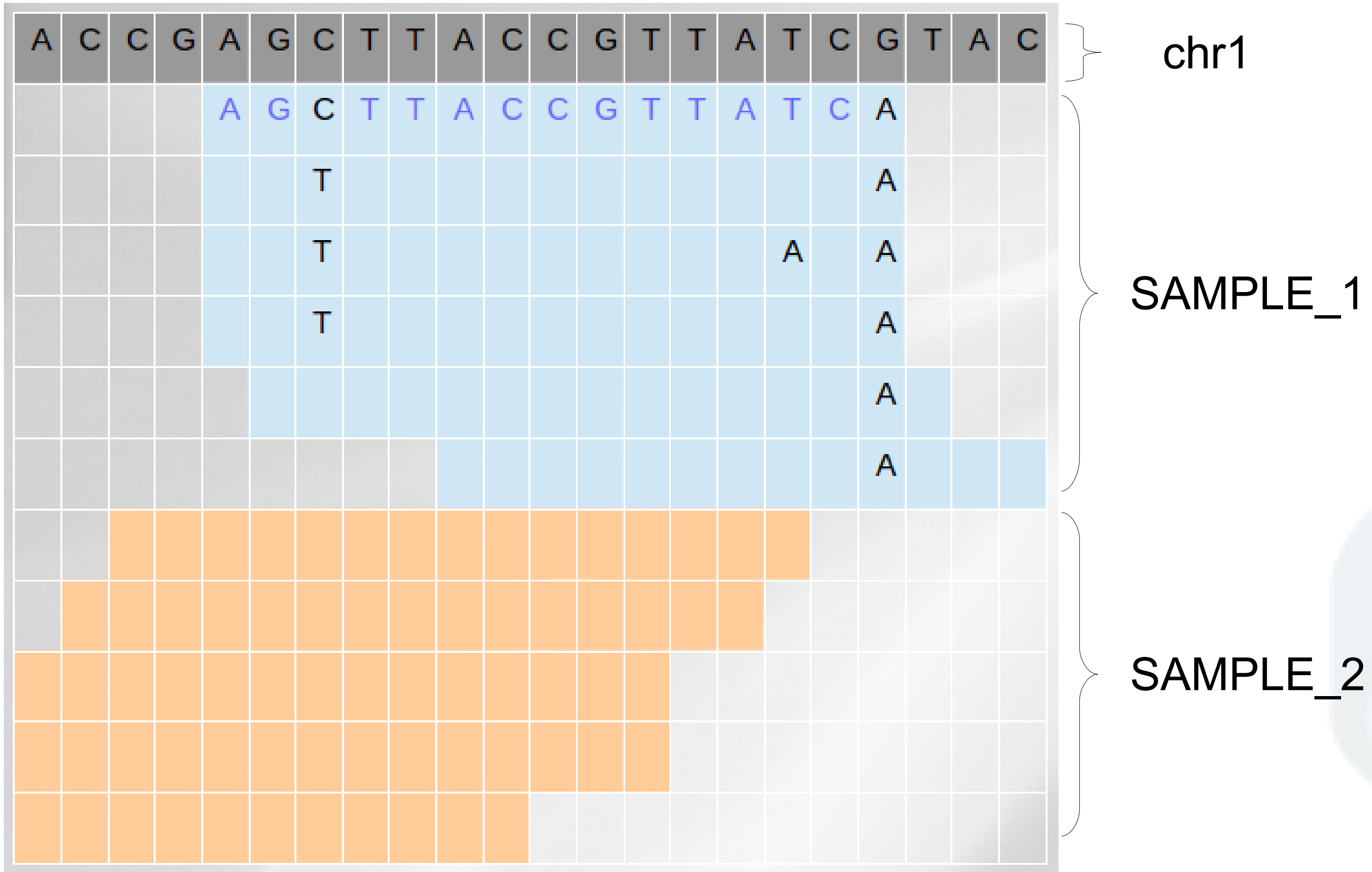
```
#CHR POS   ID    REF   ALT   QUAL  FILTER     [INFOS]   FORMAT          SAMPLE_1                SAMPLE_2
chr1 7     .     C     T     247.82     .     [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15   0/1:2,3:5:9.01:10,1,6

chr1 19    .     G     A     124.34     .     [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94  ./.
```

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

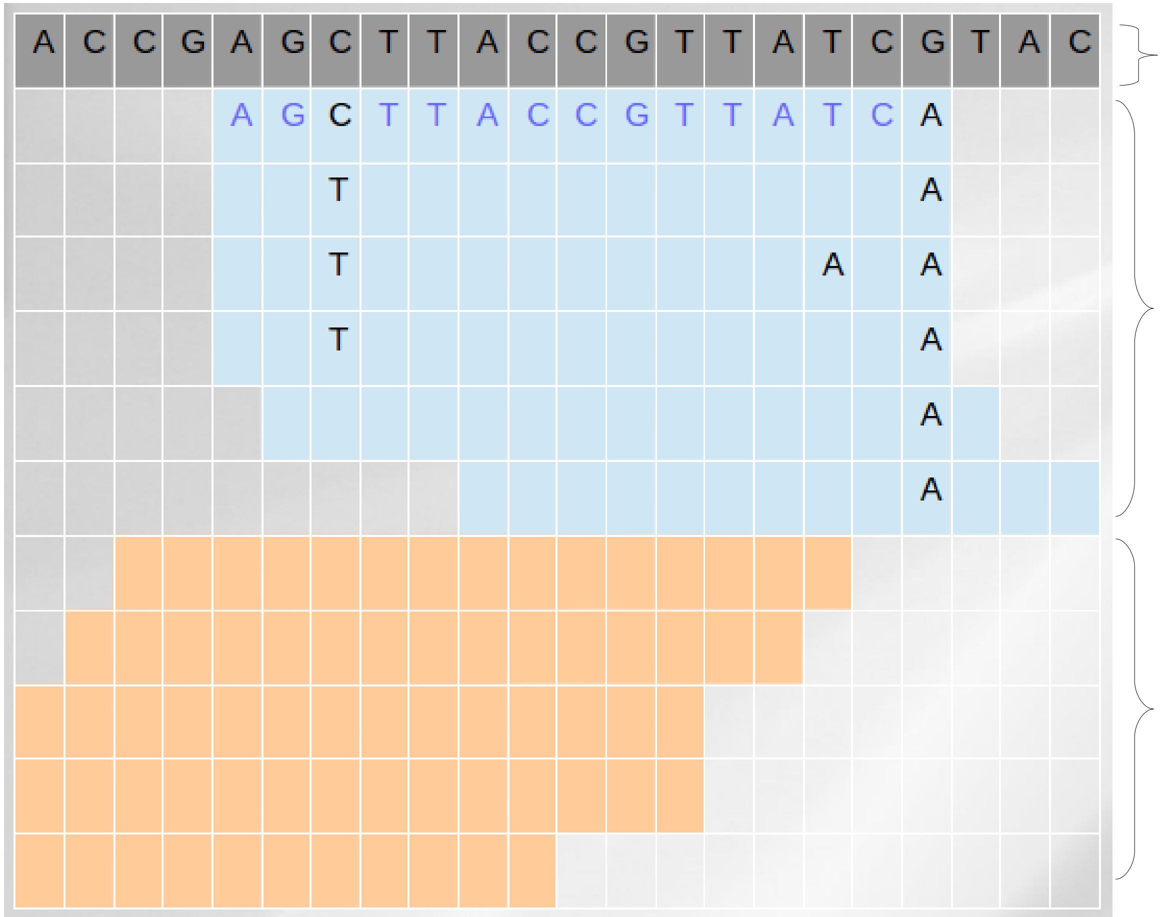
```
#CHR  POS   ID    REF   ALT   QUAL   FILTER     [INFOS]   FORMAT        SAMPLE_1                 SAMPLE_2
chr1  7     .     C     T     247.82      .     [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15    0/1:2,3:5:9.01:10,1,6
chr1  19    .     G     A     124.34      .     [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94   ./.
```

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER     [INFOS]    FORMAT         SAMPLE_1                   SAMPLE_2
chr1  7     .     C     T     247.82     .     [INFOS]    GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15      0/1:2,3:5:9.01:10,1,6
chr1  19    .     G     A     124.34     .     [INFOS]    GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94     ./.
```

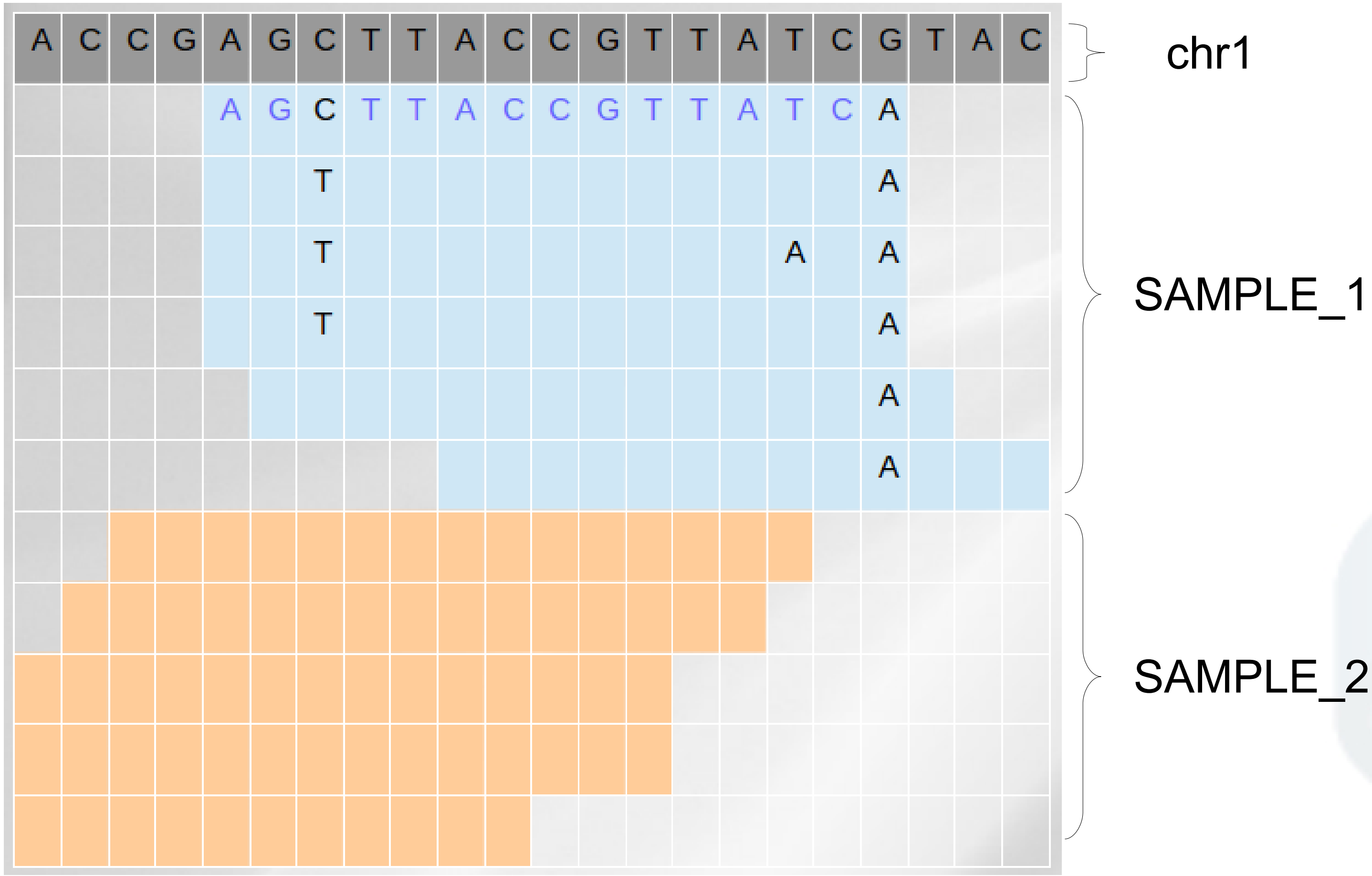
The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL    FILTER    [INFOS]   FORMAT        SAMPLE_1                   SAMPLE_2

chr1  7     .     C     T     247.82    .       [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15     0/1:2,3:5:9.01:10,1,6

chr1  19    .     G     A     124.34    .       [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94    ./.
```

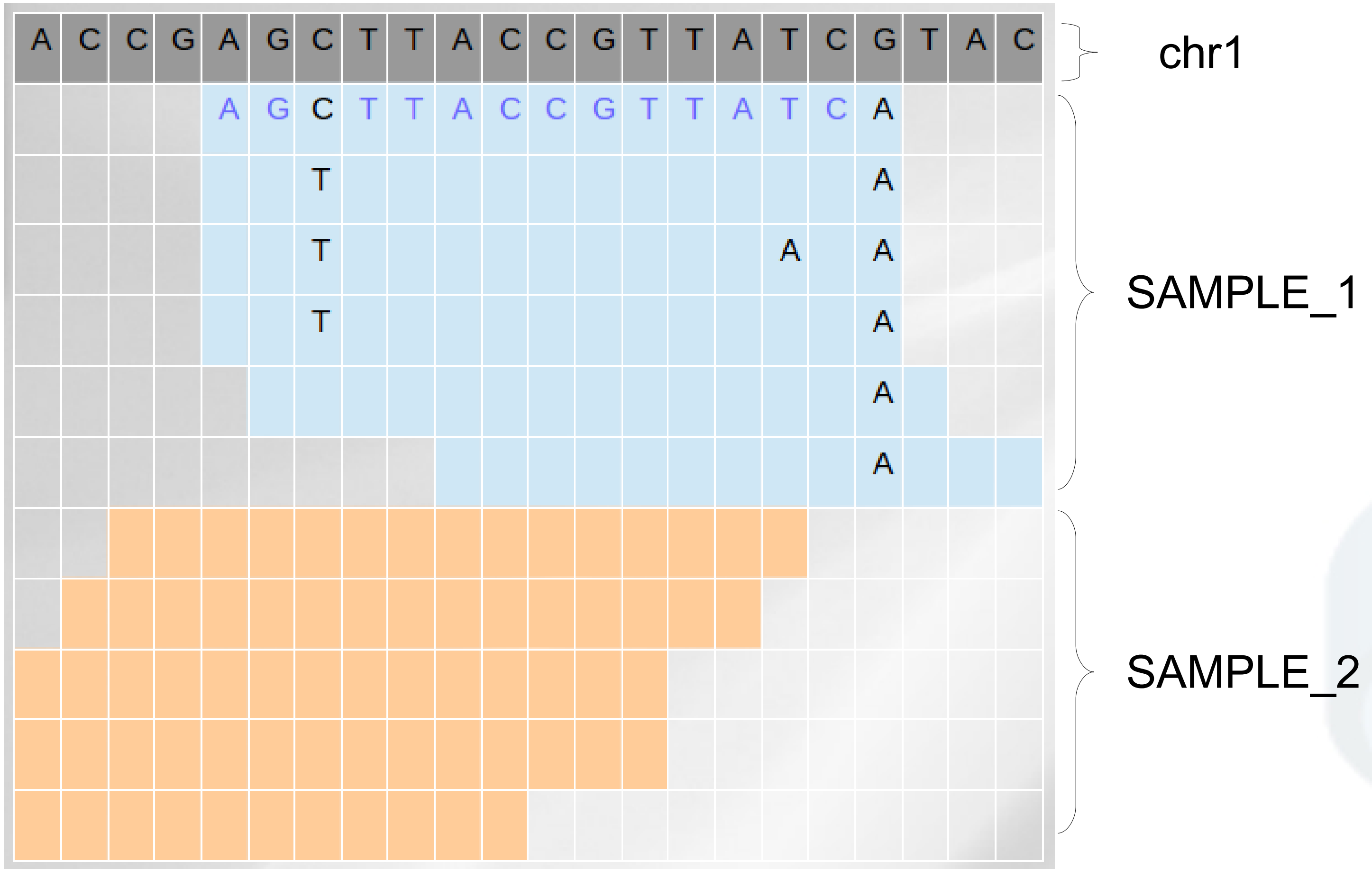
The Phred scaled probability that a
REF/ALT polymorphism exists at this site
given sequencing data

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER     [INFOS]   FORMAT          SAMPLE_1                  SAMPLE_2
chr1  7     .     C     T     247.82      .    [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15     0/1:2,3:5:9.01:10,1,6
chr1  19    .     G     A     124.34      .    [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94    ./.
```

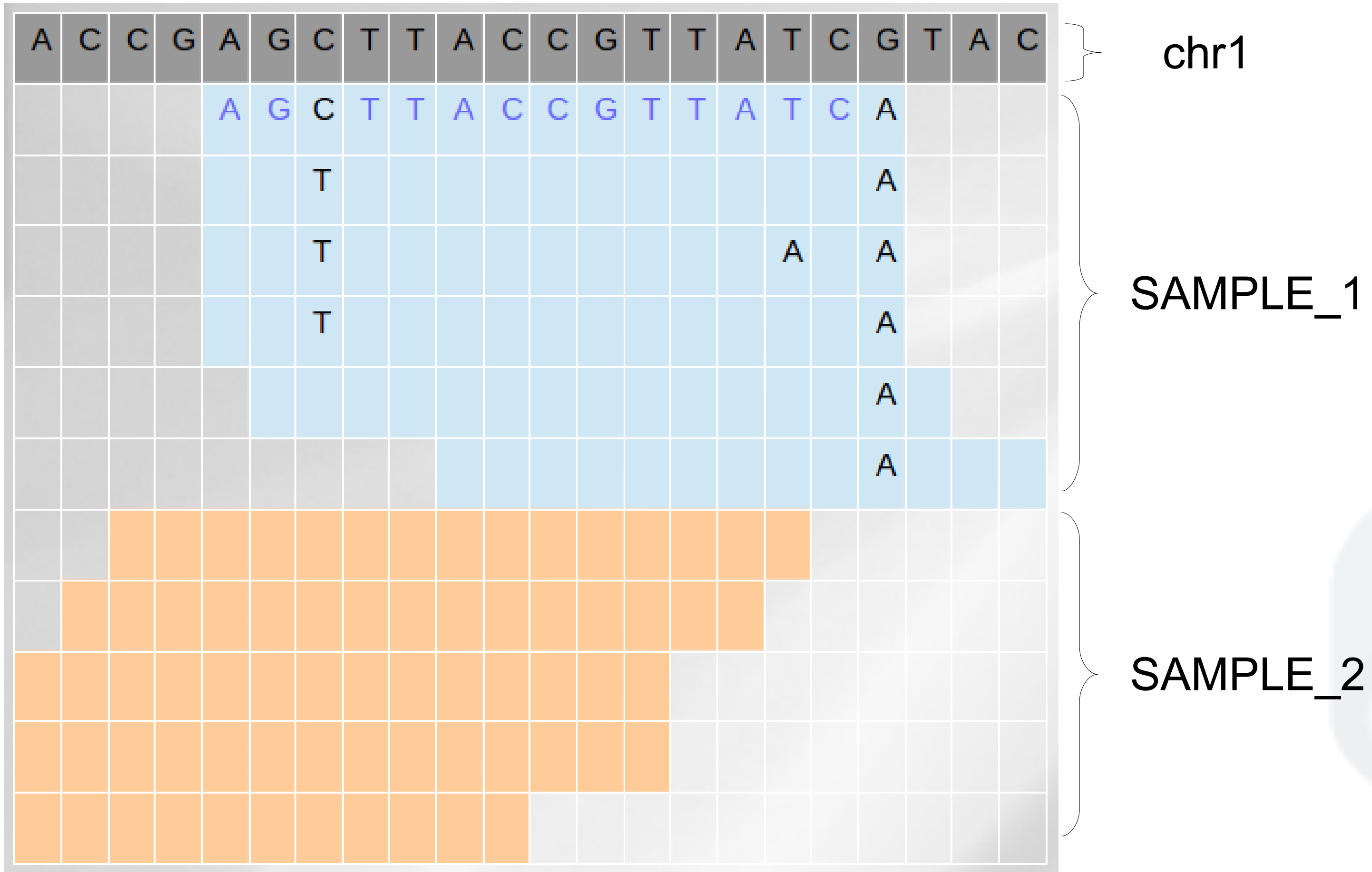
The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL   FILTER     INFOS      FORMAT         SAMPLE_1                   SAMPLE_2
chr1  7     .     C     T     247.82      .     [INFOS]    GT:AD:DP:GQ:PL 0/1:2,3:5:9.2:20,0,15      0/1:2,3:5:9.01:10,1,6
chr1  19    .     G     A     124.34      .     [INFOS]    GT:AD:DP:GQ:PL 0/0:5,0:5:20.2:0,42,94     ./.
```

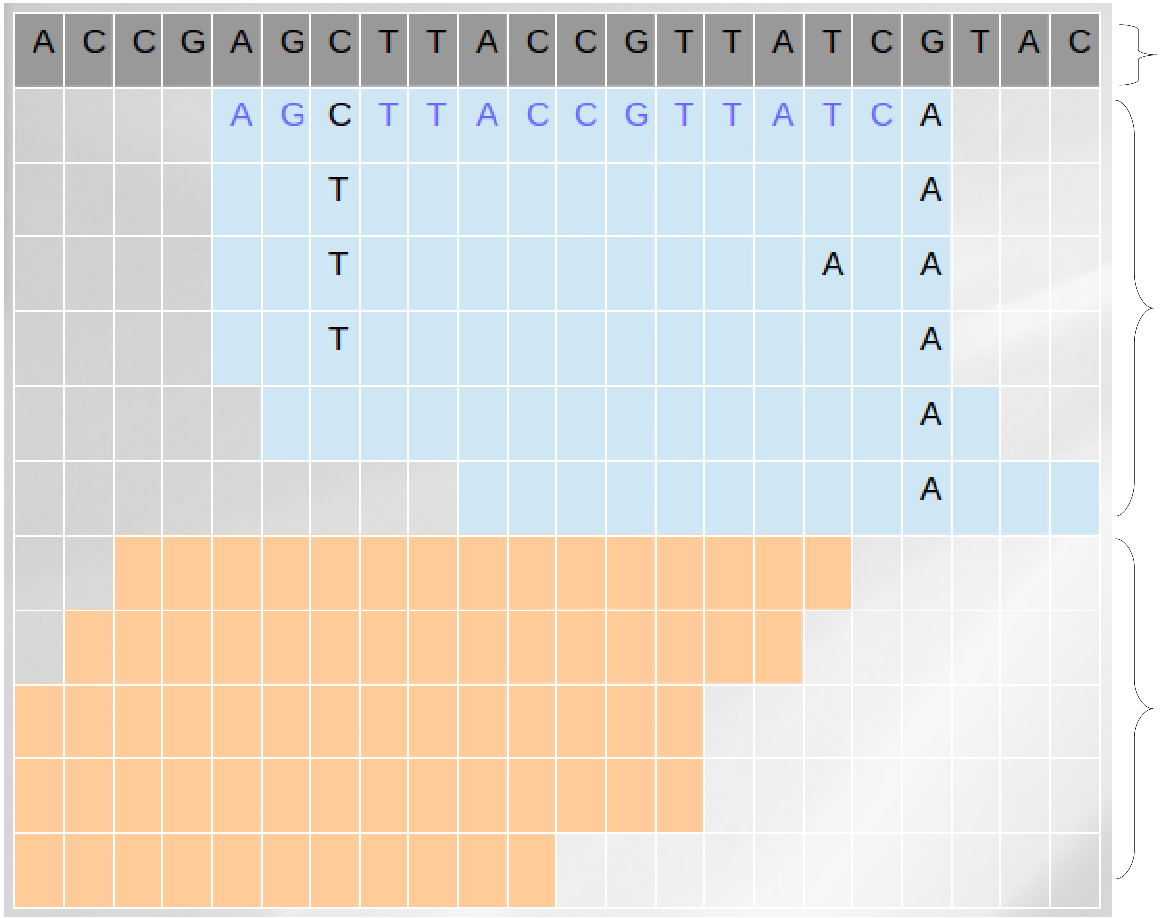
[TAG=VALUE]
DP=45

The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL   FILTER     [INFOS]   FORMAT         SAMPLE_1               SAMPLE_2

chr1  7     .     C     T     247.82      .     [INFOS]   GT:AD:DP:GQ:PL 0/1:2,3:5:9.2:20,0,15  1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34      .     [INFOS]   GT:AD:DP:GQ:PL 0/0:5,0:5:20.2:0,42,94 ./.
```

The VCF format : example

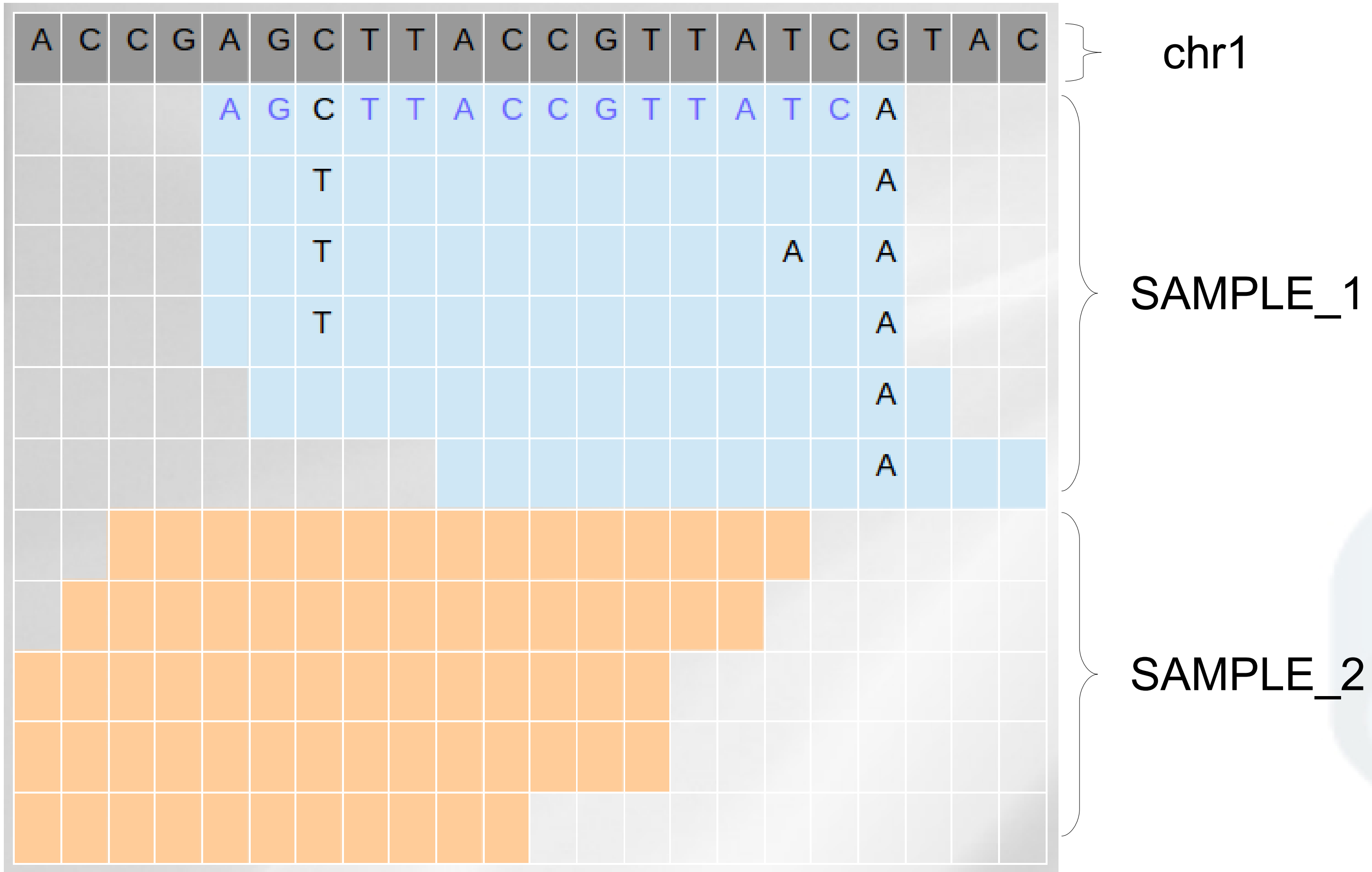


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER     [INFOS]   FORMAT         SAMPLE_1                  SAMPLE_2

chr1  7     .     C     T     247.82     .     [INFOS]   GT:AD:DP:GQ:PL 0/1:2,3:5:9.2:20,0,15     1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34     .     [INFOS]   GT:AD:DP:GQ:PL 0/0:5,0:5:20.2:0,42,94    ./.
```

```
0/1 : homozygous reference
0/1 : heterozygous
1/1 : homozygous alternative
```

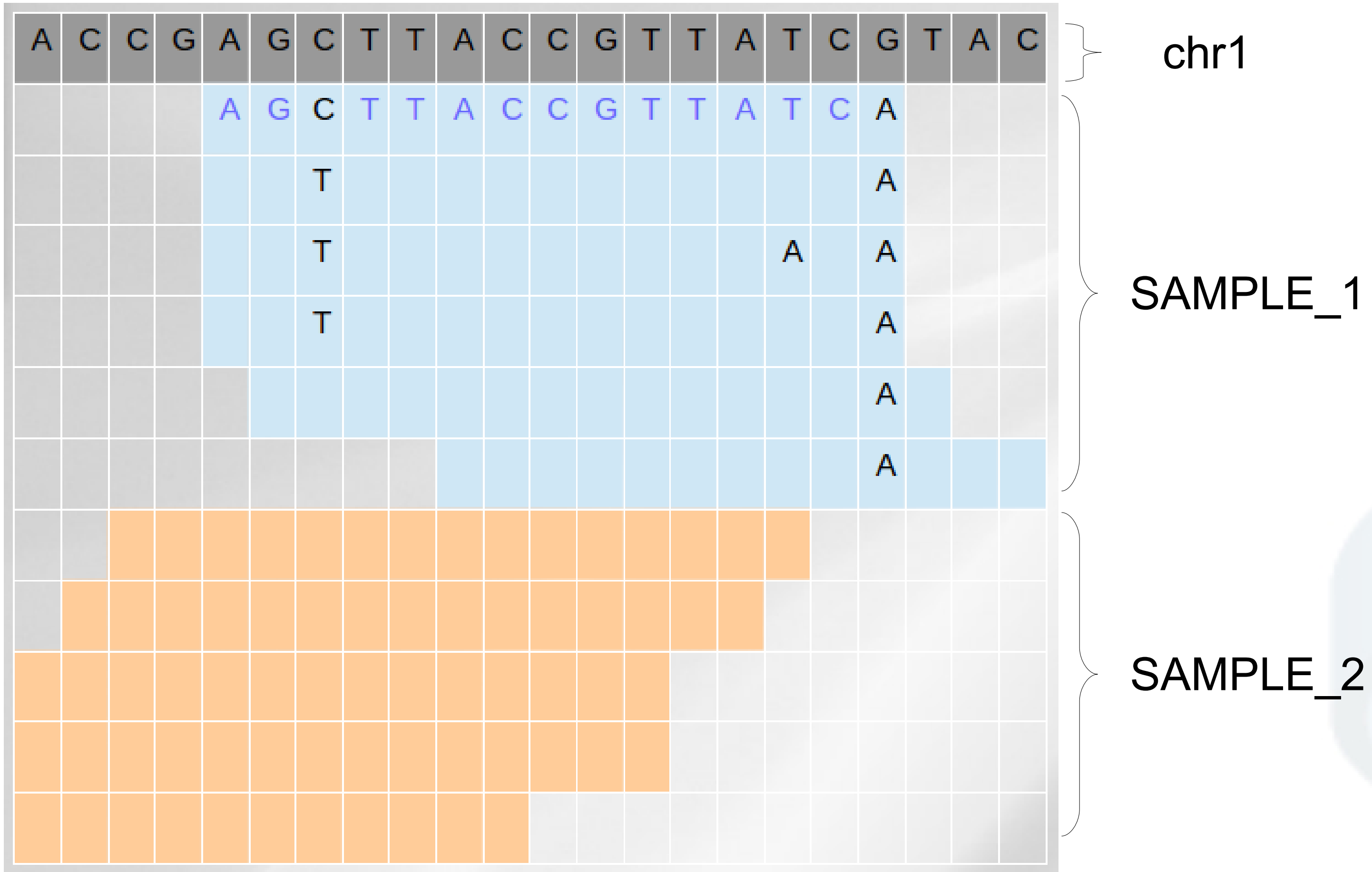
The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER     [INFOS]   FORMAT         SAMPLE_1                  SAMPLE_2
chr1  7     .     C     T     247.82     .     [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15     1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34     .     [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94    ./.
```

count REF,count ALT *[,count ALT2...]*

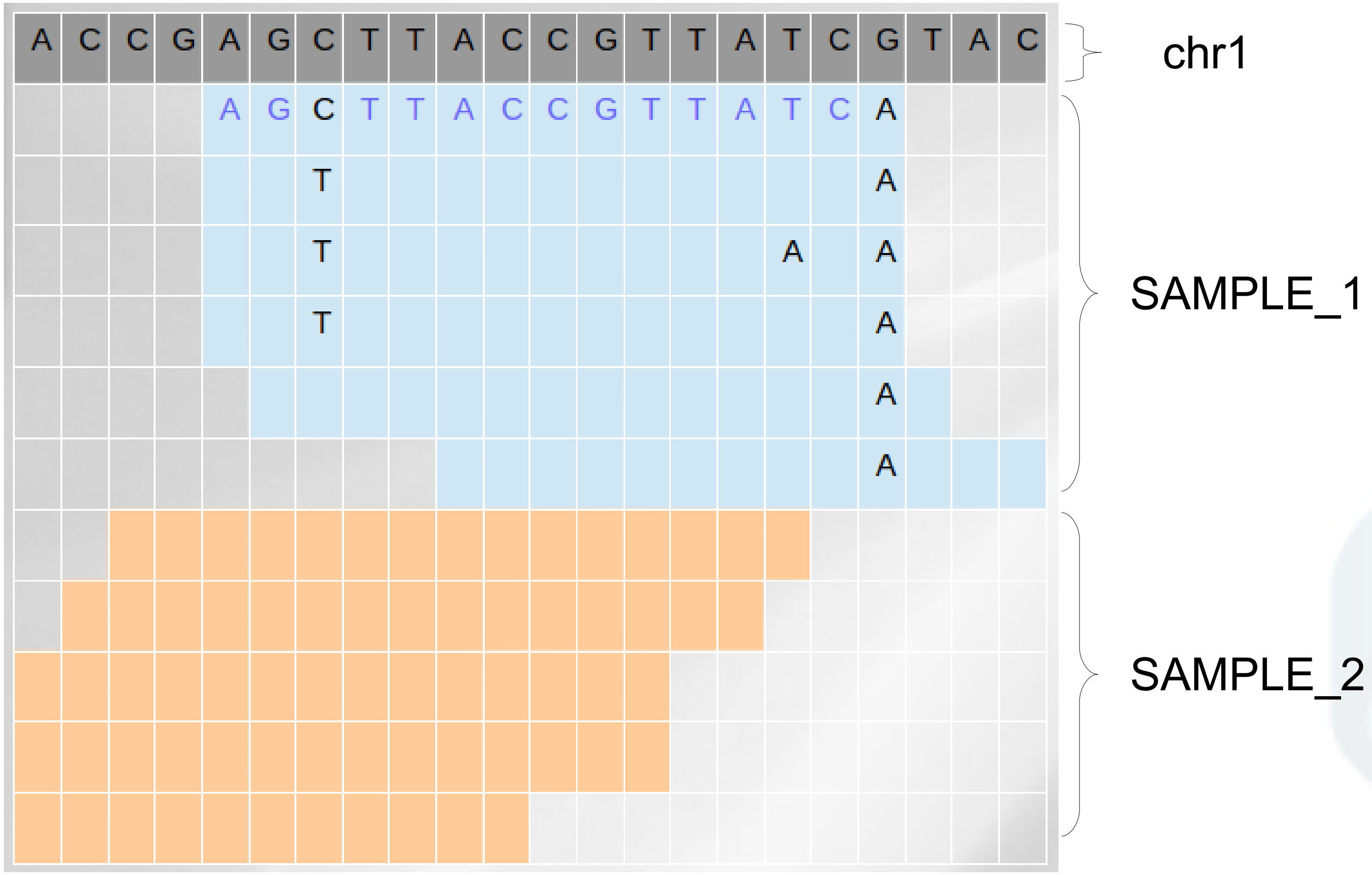
The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER    [INFOS]   FORMAT         SAMPLE_1                 SAMPLE_2

chr1  7     .     C     T     247.82     .    [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15    1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34     .    [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94   ./.
```

Depth position

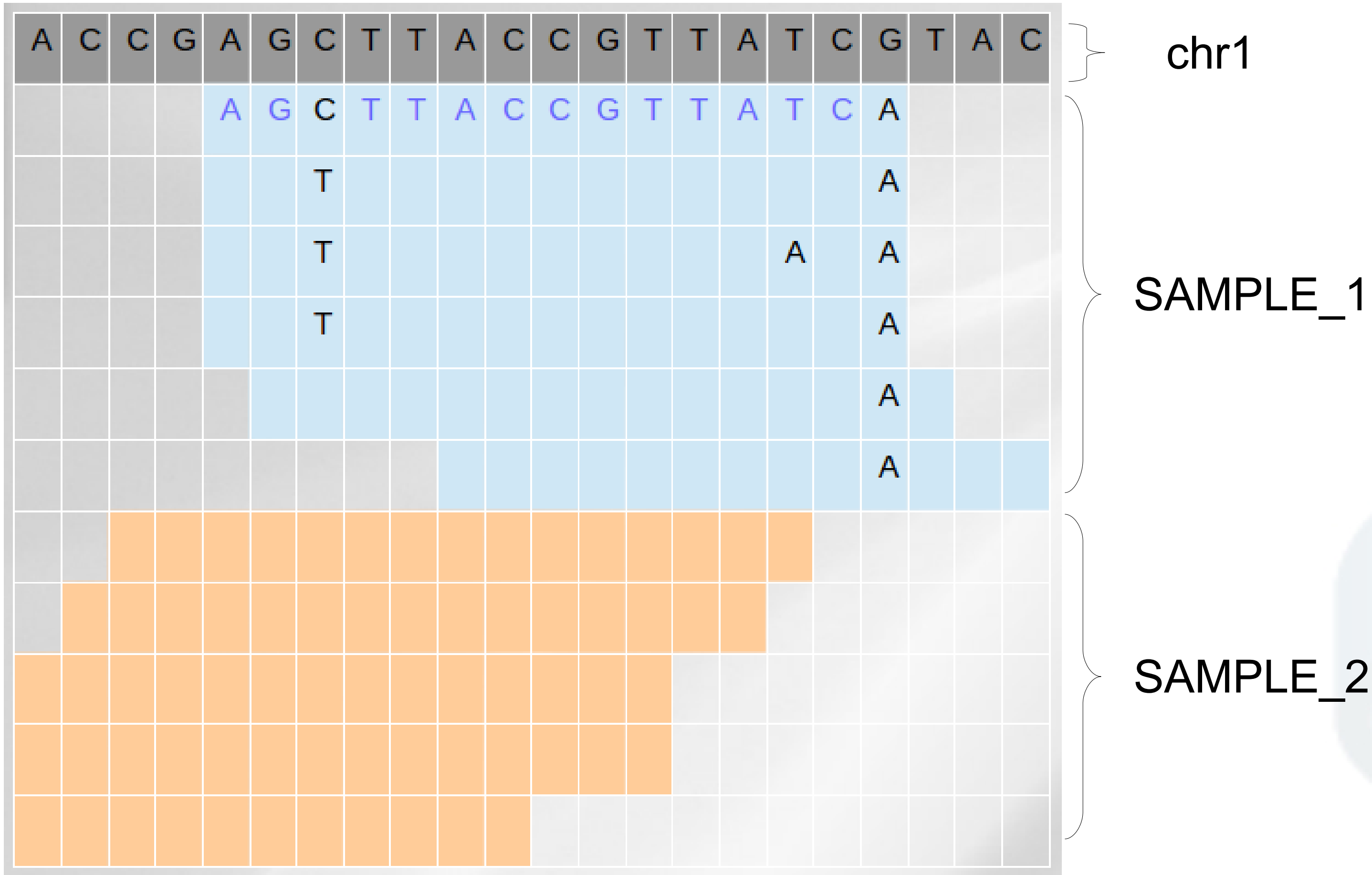
The VCF format : example


A C C G A G C T T A C C G T T A T C G T A C
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
chr1
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER    [INFOS]   FORMAT          SAMPLE_1                SAMPLE_2

chr1  7     .     C     T     247.82    .     [INFOS]   GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15   1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34    .     [INFOS]   GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94  ./.
```

The Genotype Quality, or Phred-scaled confidence that the true genotype is the one provided in GT.

The VCF format : example

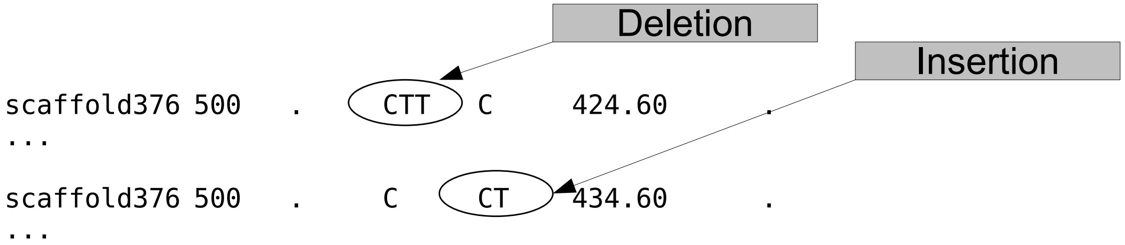

A C C G A G C T T A C C G T T A T C G T A C
chr1
A G C T T A C C G T T A T C A
T A
T A A
T A
A
A
SAMPLE_1
SAMPLE_2

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER     [INFOS]    FORMAT          SAMPLE_1                 SAMPLE_2

chr1  7     .     C     T     247.82     .     [INFOS]    GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:48,0,26    1/1:0,6:6:9.4:75,19,0

chr1  19    .     G     A     124.34     .     [INFOS]    GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94   ./.
```

These are normalized, Phred-scaled likelihoods for each of the 0/0, 0/1, and 1/1, without priors.
Ex : PL(0/0) = 26, which corresponds to 10^(-2.6), or 0.0025

The VCF format

- Small INDELs



```
scaffold376 500   .    CTT  C      424.60      .
...
scaffold376 500   .      C    CT   434.60      .
...
```

- Multi-allelic variants

```
scaffold376 577   .      C    A,T  2303.19     .
...
GT:AD:DP:GQ:PL    1/2:0,10,6:16:99:394,145,118,249,0,234    1/2:0,20,6:26:99:658,160,106,498,0,480
```

The VCF header

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM       POS    ID     REF    ALT    QUAL   FILTER INFO   FORMAT l1     l2
```

The VCF header

VCF version

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM       POS    ID     REF    ALT    QUAL    FILTER INFO    FORMAT l1     l2
```

The VCF header

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM       POS    ID     REF    ALT    QUAL   FILTER INFO   FORMAT l1    l2
```

Fields description

The VCF header

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM       POS    ID     REF    ALT    QUAL   FILTER INFO   FORMAT l1     l2
```

Tools & options used

The VCF header

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM       POS     ID      REF     ALT     QUAL    FILTER INFO    FORMAT l1     l2
```

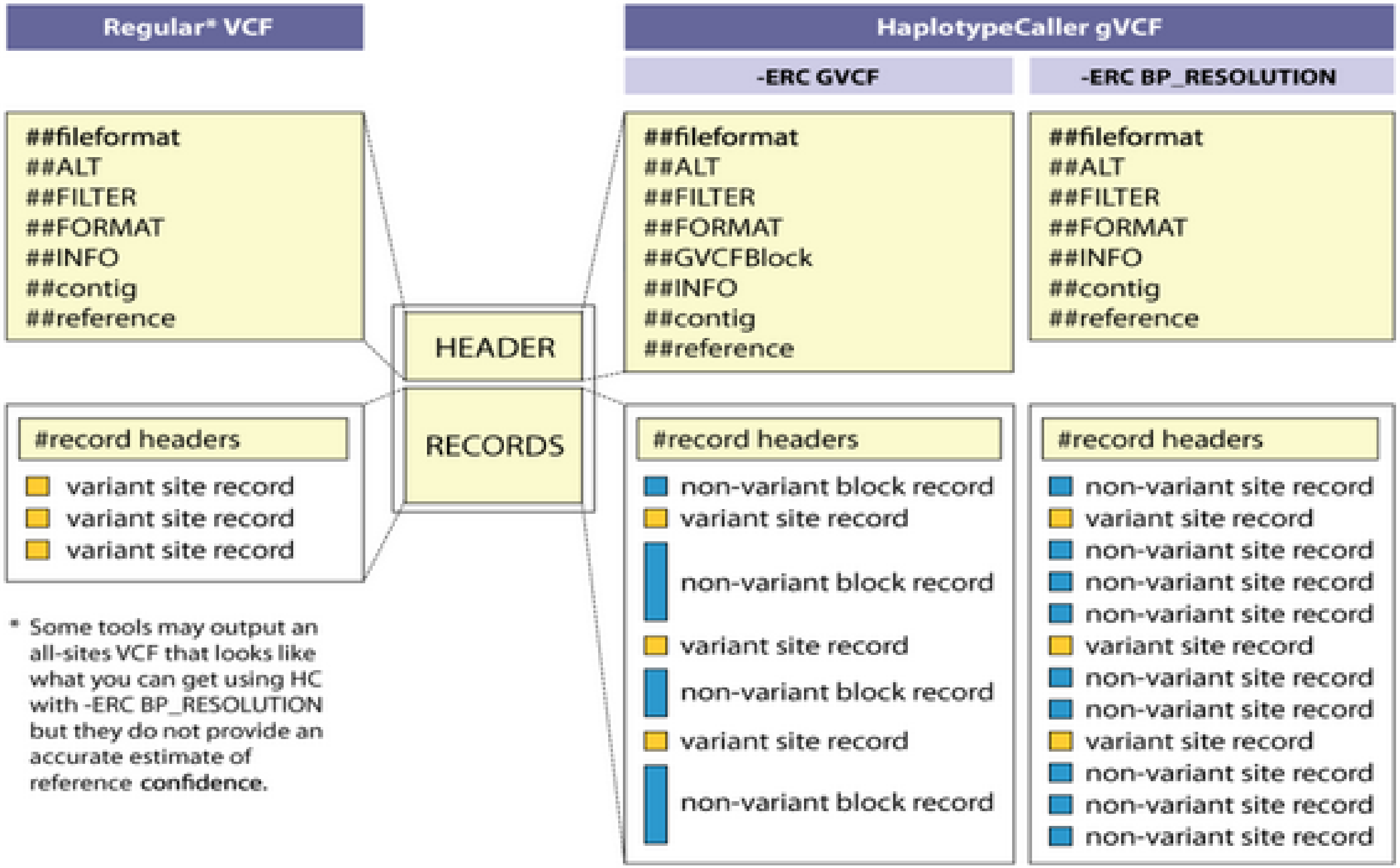
Genome informations

The VCF header

```
##fileformat=VCFv4.1
##FORMAT=<ID=AD,Number=.,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=GQ,Number=1,Type=Float,Description="Genotype Quality">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=PL,Number=G,Type=Integer,Description="Normalized, Phred-scaled likelihoods for genotypes as defined in the VCF specification">
##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">
##INFO=<ID=BaseQRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt Vs. Ref base qualities">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Approximate read depth; some reads may have been filtered">
##INFO=<ID=DS,Number=0,Type=Flag,Description="Were any of the samples downsampled?">
##INFO=<ID=Dels,Number=1,Type=Float,Description="Fraction of Reads Containing Spanning Deletions">
##INFO=<ID=FS,Number=1,Type=Float,Description="Phred-scaled p-value using Fisher's exact test to detect strand bias">
##INFO=<ID=HRun,Number=1,Type=Integer,Description="Largest Contiguous Homopolymer Run of Variant Allele In Either Direction">
##INFO=<ID=HaplotypeScore,Number=1,Type=Float,Description="Consistency of the site with at most two segregating haplotypes">
##INFO=<ID=InbreedingCoeff,Number=1,Type=Float,Description="Inbreeding coefficient as estimated from the genotype likelihoods per-sample when
compared against the Hardy-Weinberg expectation">
##INFO=<ID=MQ,Number=1,Type=Float,Description="RMS Mapping Quality">
##INFO=<ID=MQ0,Number=1,Type=Integer,Description="Total Mapping Quality Zero Reads">
##INFO=<ID=MQRankSum,Number=1,Type=Float,Description="Z-score From Wilcoxon rank sum test of Alt vs. Ref read mapping qualities">
##INFO=<ID=QD,Number=1,Type=Float,Description="Variant Confidence/Quality by Depth">
##INFO=<ID=ReadPosRankSum,Number=1,Type=Float,Description="Z-score from Wilcoxon rank sum test of Alt vs. Ref read position bias">
##INFO=<ID=SB,Number=1,Type=Float,Description="Strand Bias">
##UnifiedGenotyper="analysis_type=UnifiedGenotyper input_file=[L1_RG_s_realign_recal_q30.bam, L2_RG_s_realign_recal_q30.bam]
read_buffer_size=null phone_home=STANDARD gatk_key=null read_filter=[] [...]
##contig=<ID=scaffold376,length=1000>
##reference=file:///work/banks/genome.fasta
#CHROM        POS    ID     REF    ALT    QUAL   FILTER INFO   FORMAT l1    l2
```

Header line

The VCF vs gVCF



Regular* VCF

##fileformat
##ALT
##FILTER
##FORMAT
##INFO
##contig
##reference

#record headers

- 🟨 variant site record
- 🟨 variant site record
- 🟨 variant site record

HEADER

RECORDS

HaplotypeCaller gVCF

-ERC GVCF

##fileformat
##ALT
##FILTER
##FORMAT
##GVCFBlock
##INFO
##contig
##reference

#record headers

- 🟦 non-variant block record
- 🟨 variant site record
- 🟦 non-variant block record
- 🟨 variant site record
- 🟦 non-variant block record
- 🟨 variant site record
- 🟦 non-variant block record

-ERC BP_RESOLUTION

##fileformat
##ALT
##FILTER
##FORMAT
##INFO
##contig
##reference

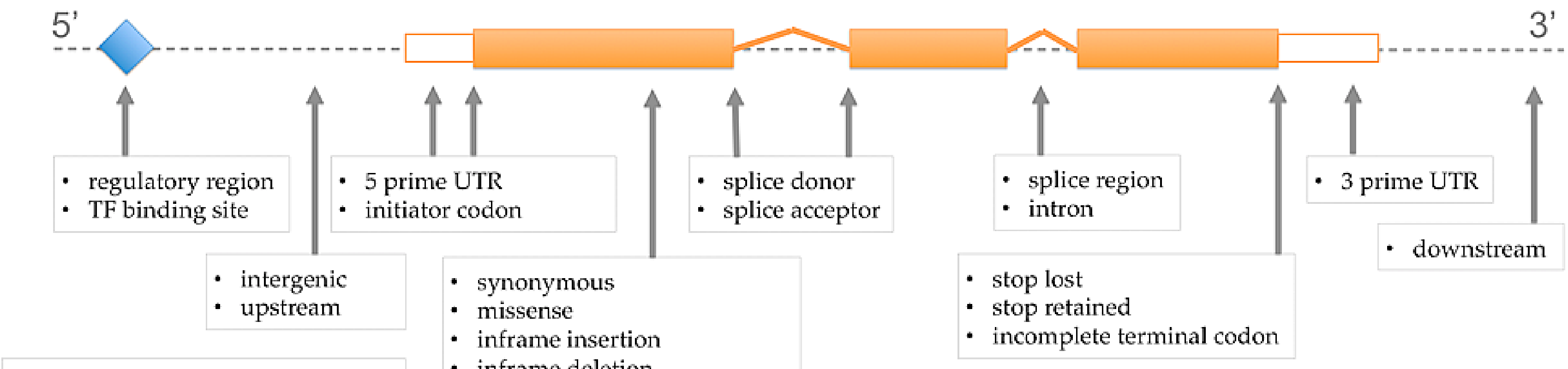
#record headers

- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record

* Some tools may output an all-sites VCF that looks like what you can get using HC with -ERC BP_RESOLUTION but they do not provide an accurate estimate of reference confidence.

Variant annotation

- ➔ Where are my SNPs ?
- ➔ Known or unknown ?
- ➔ Which effects ?



- regulatory region
- TF binding site

- intergenic
- upstream

- 5 prime UTR
- initiator codon

- synonymous
- missense
- inframe insertion
- inframe deletion
- stop gained
- frameshift
- coding sequence variant

- splice donor
- splice acceptor

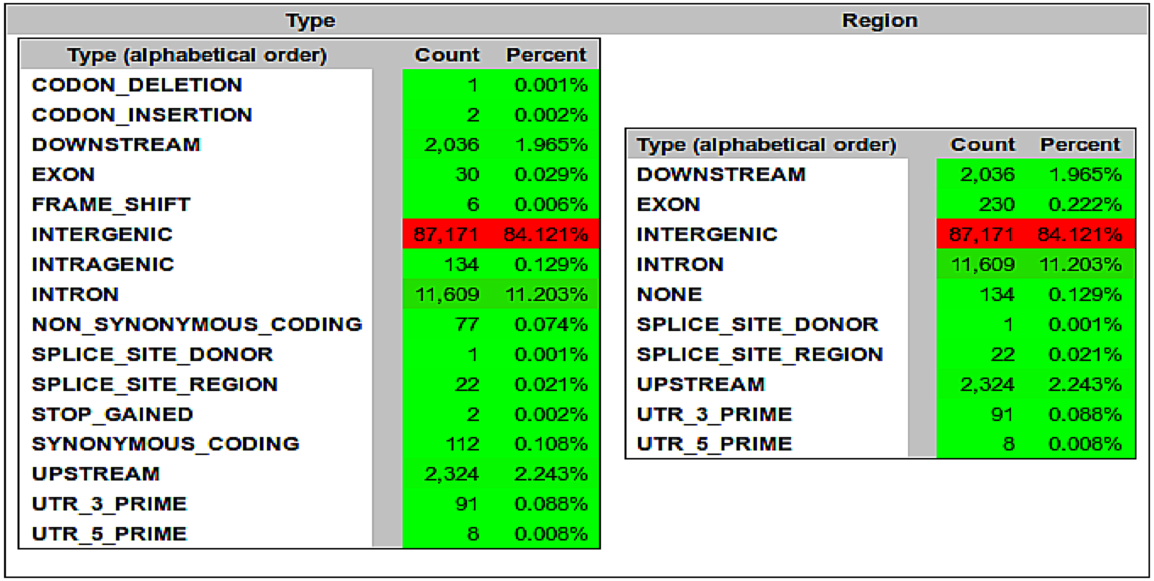
- splice region
- intron

- stop lost
- stop retained
- incomplete terminal codon

- 3 prime UTR

- downstream

- transcript/regulatory region /TF binding site ablation
- mature miRNA
- NMD transcript
- non-coding exon / transcript



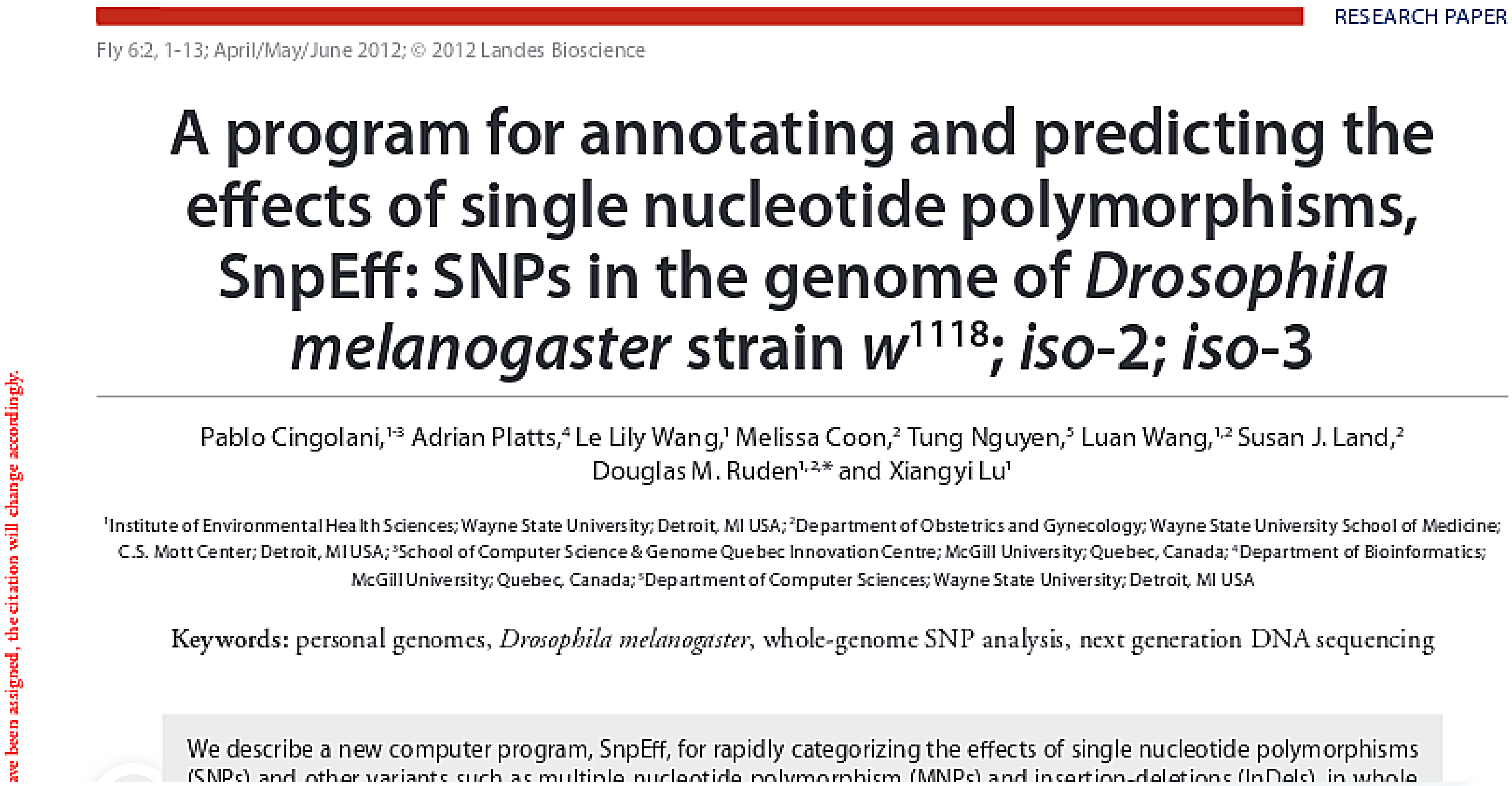
Type

Type (alphabetical order)	Count	Percent
CODON_DELETION	1	0.001%
CODON_INSERTION	2	0.002%
DOWNSTREAM	2,036	1.965%
EXON	30	0.029%
FRAME_SHIFT	6	0.006%
INTERGENIC	87,171	84.121%
INTRAGENIC	134	0.129%
INTRON	11,609	11.203%
NON_SYNONYMOUS_CODING	77	0.074%
SPLICE_SITE_DONOR	1	0.001%
SPLICE_SITE_REGION	22	0.021%
STOP_GAINED	2	0.002%
SYNONYMOUS_CODING	112	0.108%
UPSTREAM	2,324	2.243%
UTR_3_PRIME	91	0.088%
UTR_5_PRIME	8	0.008%

Region

Type (alphabetical order)	Count	Percent
DOWNSTREAM	2,036	1.965%
EXON	230	0.222%
INTERGENIC	87,171	84.121%
INTRON	11,609	11.203%
NONE	134	0.129%
SPLICE_SITE_DONOR	1	0.001%
SPLICE_SITE_REGION	22	0.021%
UPSTREAM	2,324	2.243%
UTR_3_PRIME	91	0.088%
UTR_5_PRIME	8	0.008%

Variant annotation - SnpEff



RESEARCH PAPER

Fly 6:2, 1-13; April/May/June 2012; © 2012 Landes Bioscience

A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w^{1118}; *iso-2*; *iso-3*

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Keywords: personal genomes, *Drosophila melanogaster*, whole-genome SNP analysis, next generation DNA sequencing

We describe a new computer program, SnpEff, for rapidly categorizing the effects of single nucleotide polymorphisms (SNPs) and other variants such as multiple nucleotide polymorphism (MNPs) and insertion-deletions (InDels) in whole

ave been assigned, the citation will change accordingly.

http://snpeff.sourceforge.net/

Variant annotation - SnpEff

SnpEff input(s) :
- vcf file
- (annotation => over 2500 genomes pre-built databases / build one yourself)

SnpEff outputs :
- html report
- vcf file (information added to the INFO fields)

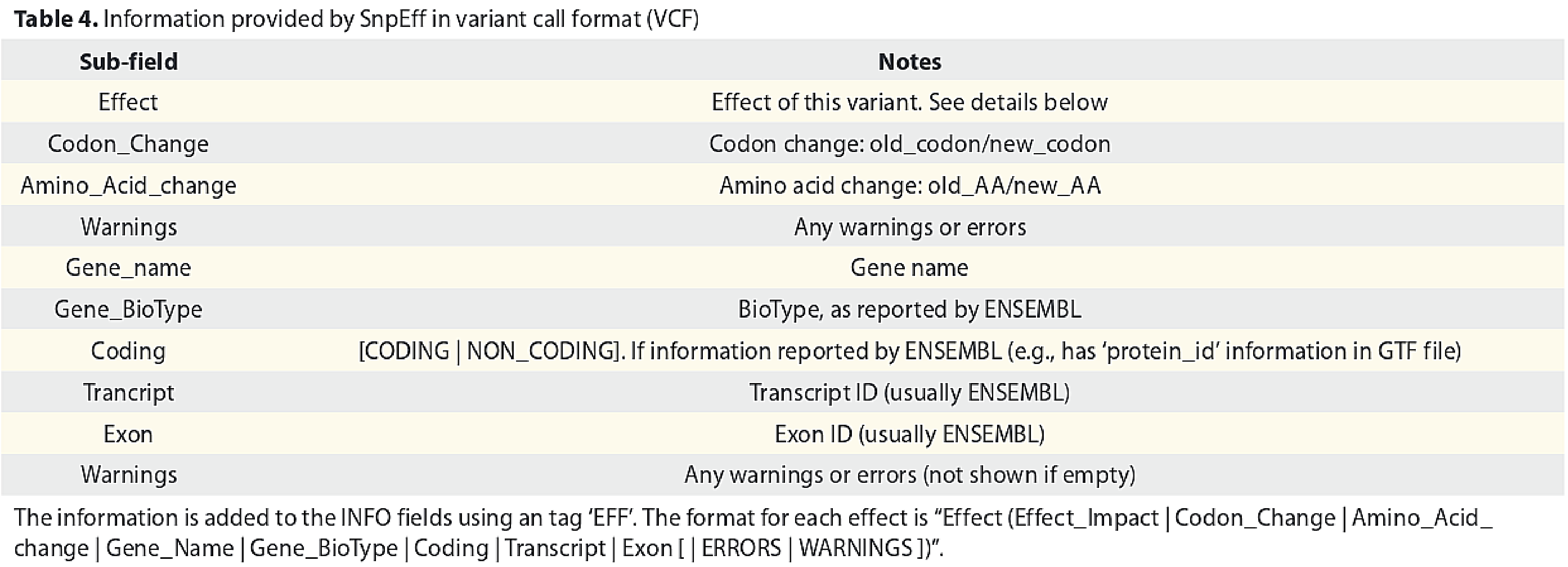


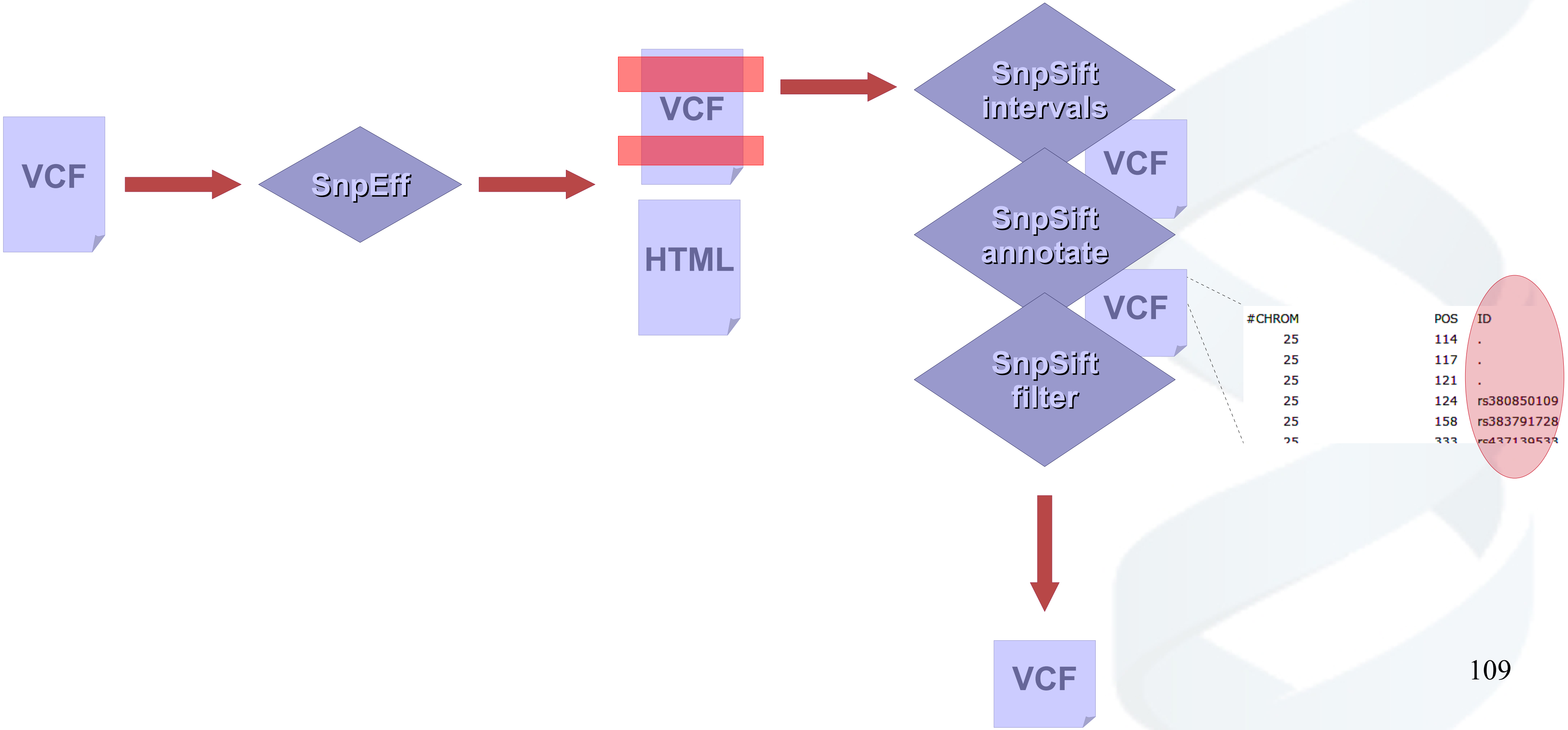
Table 4. Information provided by SnpEff in variant call format (VCF)

Sub-field	Notes
Effect	Effect of this variant. See details below
Codon_Change	Codon change: old_codon/new_codon
Amino_Acid_change	Amino acid change: old_AA/new_AA
Warnings	Any warnings or errors
Gene_name	Gene name
Gene_BioType	BioType, as reported by ENSEMBL
Coding	[CODING | NON_CODING]. If information reported by ENSEMBL (e.g., has 'protein_id' information in GTF file)
Trancript	Transcript ID (usually ENSEMBL)
Exon	Exon ID (usually ENSEMBL)
Warnings	Any warnings or errors (not shown if empty)

The information is added to the INFO fields using an tag 'EFF'. The format for each effect is "Effect (Effect_Impact | Codon_Change | Amino_Acid_change | Gene_Name | Gene_BioType | Coding | Transcript | Exon [| ERRORS | WARNINGS])".

Variant annotation – pipeline

- SnpEff : Variant effect and annotation
- SnpSift Intervals : Filter variants using intervals
- SnpSift Annotate SNPs from dbSnp
- SnpSift Filter : Filter variants using arbitrary expressions

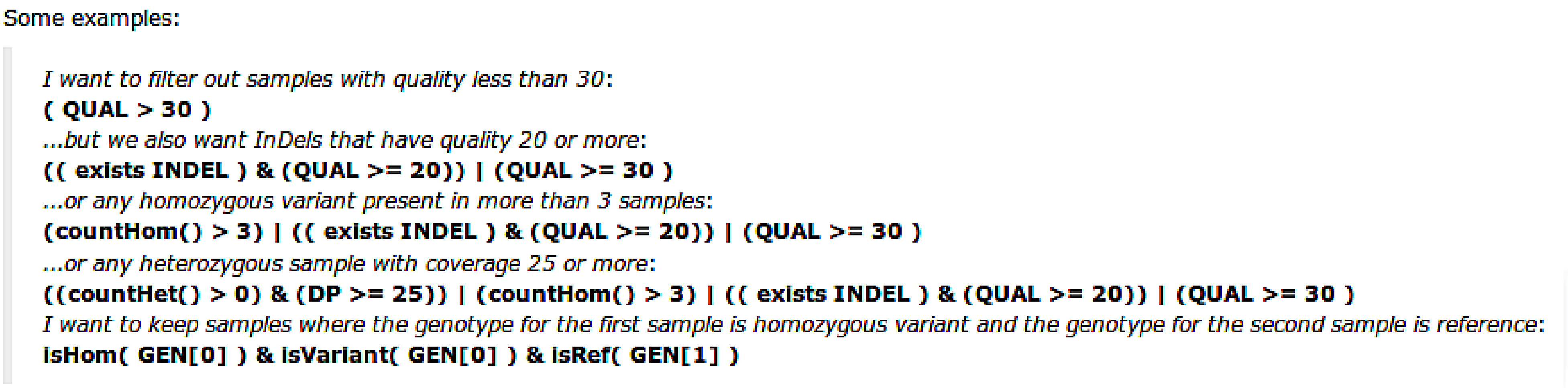


```
#CHROM            POS  ID
    25            114  .
    25            117  .
    25            121  .
    25            124  rs380850109
    25            158  rs383791728
    25            333  rs437139533
```

SnpSift - Filter

Filter variants using arbitrary expressions

http://snpeff.sourceforge.net/SnpSift.html#filter



Some examples:

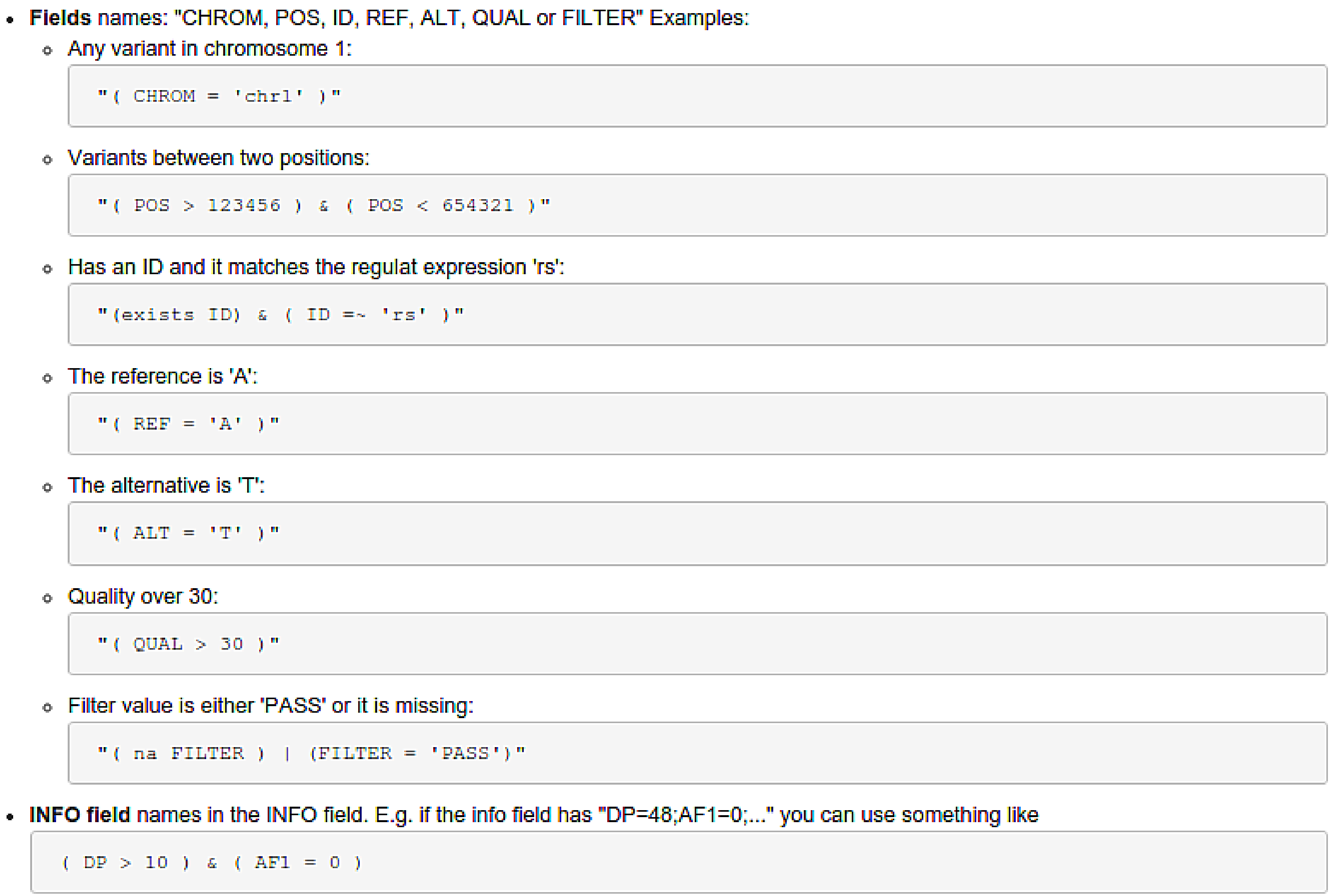
> *I want to filter out samples with quality less than 30:*
> **(QUAL > 30)**
> *...but we also want InDels that have quality 20 or more:*
> **((exists INDEL) & (QUAL >= 20)) | (QUAL >= 30)**
> *...or any homozygous variant present in more than 3 samples:*
> **(countHom() > 3) | ((exists INDEL) & (QUAL >= 20)) | (QUAL >= 30)**
> *...or any heterozygous sample with coverage 25 or more:*
> **((countHet() > 0) & (DP >= 25)) | (countHom() > 3) | ((exists INDEL) & (QUAL >= 20)) | (QUAL >= 30)**
> *I want to keep samples where the genotype for the first sample is homozygous variant and the genotype for the second sample is reference:*
> **isHom(GEN[0]) & isVariant(GEN[0]) & isRef(GEN[1])**

```
#CHR  POS   ID    REF   ALT   QUAL  FILTER    [INFOS]    FORMAT         SAMPLE_1                   SAMPLE_2

chr1  7     .     C     T     247.82    .     [INFOS]    GT:AD:DP:GQ:PL  0/1:2,3:5:9.2:20,0,15      0/1:2,3:5:9.01:10,1,6

chr1  19    .     G     A     124.34    .     [INFOS]    GT:AD:DP:GQ:PL  0/0:5,0:5:20.2:0,42,94     ./.
```

SnpSift Filter – Variables



- **Fields** names: "CHROM, POS, ID, REF, ALT, QUAL or FILTER" Examples:
  - Any variant in chromosome 1:

    ```
    "( CHROM = 'chr1' )"
    ```

  - Variants between two positions:

    ```
    "( POS > 123456 ) & ( POS < 654321 )"
    ```

  - Has an ID and it matches the regulat expression 'rs':

    ```
    "(exists ID) & ( ID =~ 'rs' )"
    ```

  - The reference is 'A':

    ```
    "( REF = 'A' )"
    ```

  - The alternative is 'T':

    ```
    "( ALT = 'T' )"
    ```

  - Quality over 30:

    ```
    "( QUAL > 30 )"
    ```

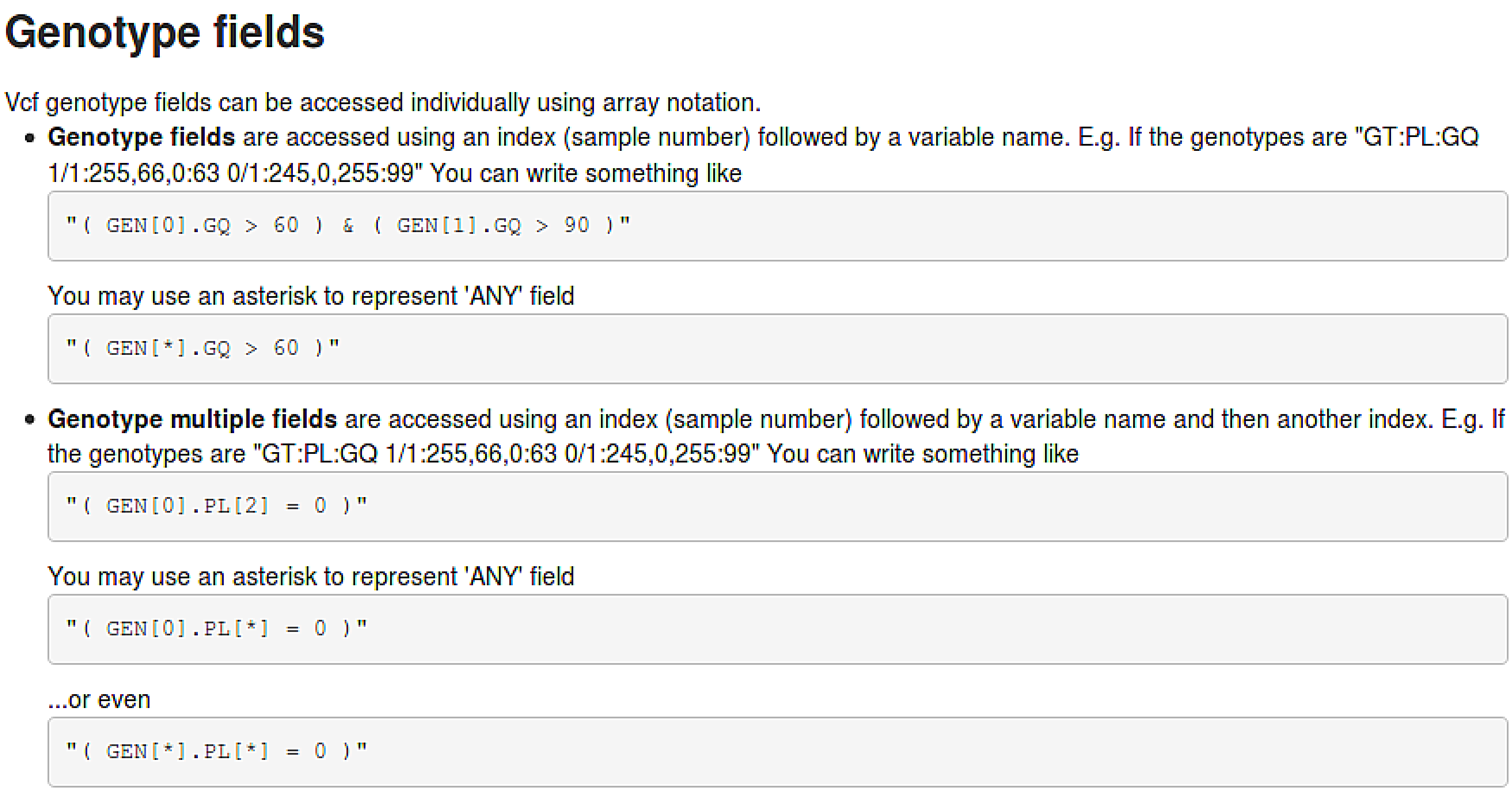
  - Filter value is either 'PASS' or it is missing:

    ```
    "( na FILTER ) | (FILTER = 'PASS')"
    ```

- **INFO field** names in the INFO field. E.g. if the info field has "DP=48;AF1=0;..." you can use something like

  ```
  ( DP > 10 ) & ( AF1 = 0 )
  ```

SnpSift - Filter on genotype fileds



Genotype fields

Vcf genotype fields can be accessed individually using array notation.

- **Genotype fields** are accessed using an index (sample number) followed by a variable name. E.g. If the genotypes are "GT:PL:GQ 1/1:255,66,0:63 0/1:245,0,255:99" You can write something like

  ```
  "( GEN[0].GQ > 60 ) & ( GEN[1].GQ > 90 )"
  ```

  You may use an asterisk to represent 'ANY' field

  ```
  "( GEN[*].GQ > 60 )"
  ```

- **Genotype multiple fields** are accessed using an index (sample number) followed by a variable name and then another index. E.g. If the genotypes are "GT:PL:GQ 1/1:255,66,0:63 0/1:245,0,255:99" You can write something like

  ```
  "( GEN[0].PL[2] = 0 )"
  ```

  You may use an asterisk to represent 'ANY' field

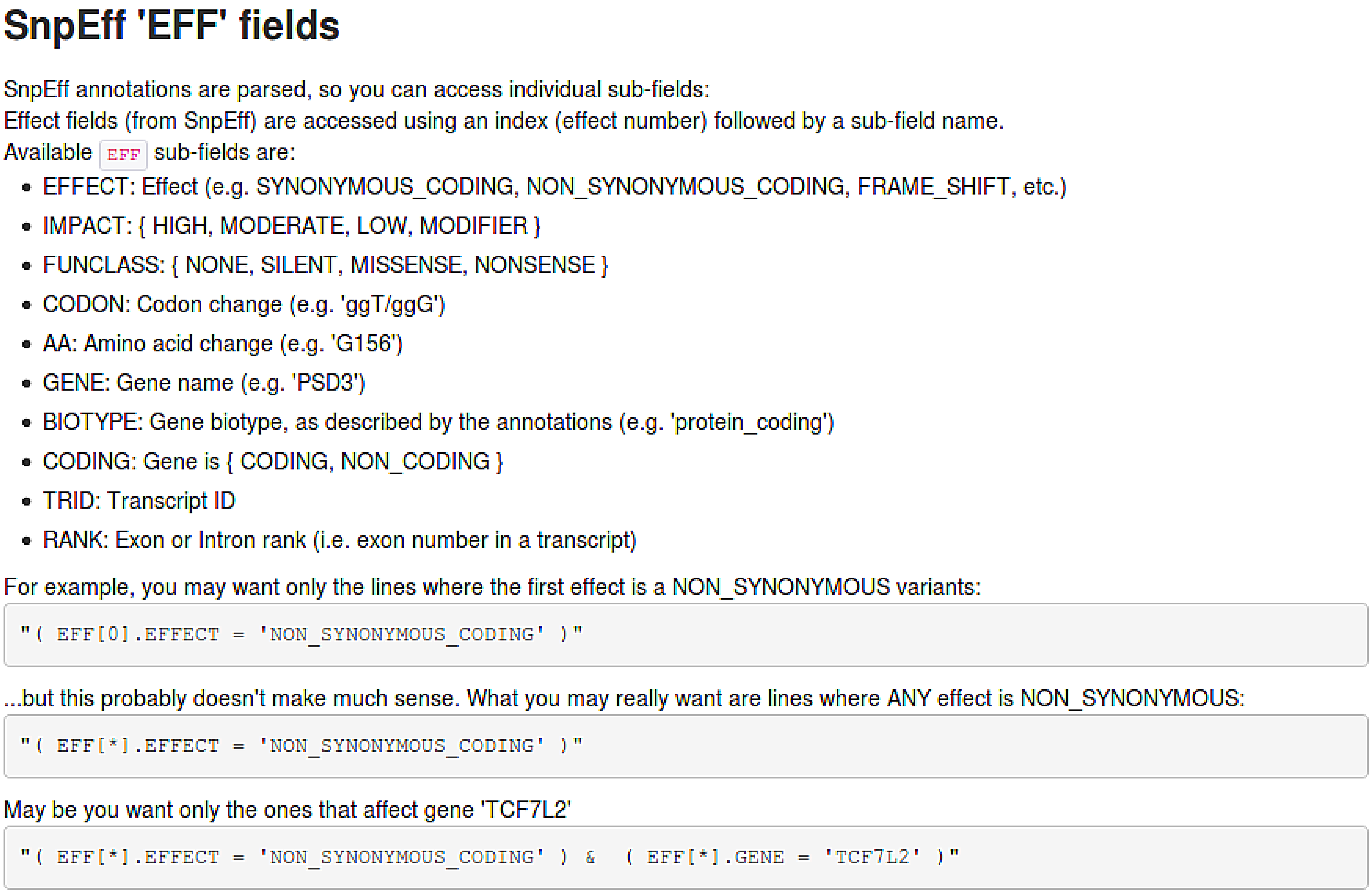
  ```
  "( GEN[0].PL[*] = 0 )"
  ```

  ...or even

  ```
  "( GEN[*].PL[*] = 0 )"
  ```

Rq : You can create an expression using sample names instead of genotype numbers !

SnpSift - Filter on EFF fileds



SnpEff 'EFF' fields

SnpEff annotations are parsed, so you can access individual sub-fields:
Effect fields (from SnpEff) are accessed using an index (effect number) followed by a sub-field name.
Available `EFF` sub-fields are:

- EFFECT: Effect (e.g. SYNONYMOUS_CODING, NON_SYNONYMOUS_CODING, FRAME_SHIFT, etc.)
- IMPACT: { HIGH, MODERATE, LOW, MODIFIER }
- FUNCLASS: { NONE, SILENT, MISSENSE, NONSENSE }
- CODON: Codon change (e.g. 'ggT/ggG')
- AA: Amino acid change (e.g. 'G156')
- GENE: Gene name (e.g. 'PSD3')
- BIOTYPE: Gene biotype, as described by the annotations (e.g. 'protein_coding')
- CODING: Gene is { CODING, NON_CODING }
- TRID: Transcript ID
- RANK: Exon or Intron rank (i.e. exon number in a transcript)

For example, you may want only the lines where the first effect is a NON_SYNONYMOUS variants:

```
"( EFF[0].EFFECT = 'NON_SYNONYMOUS_CODING' )"
```

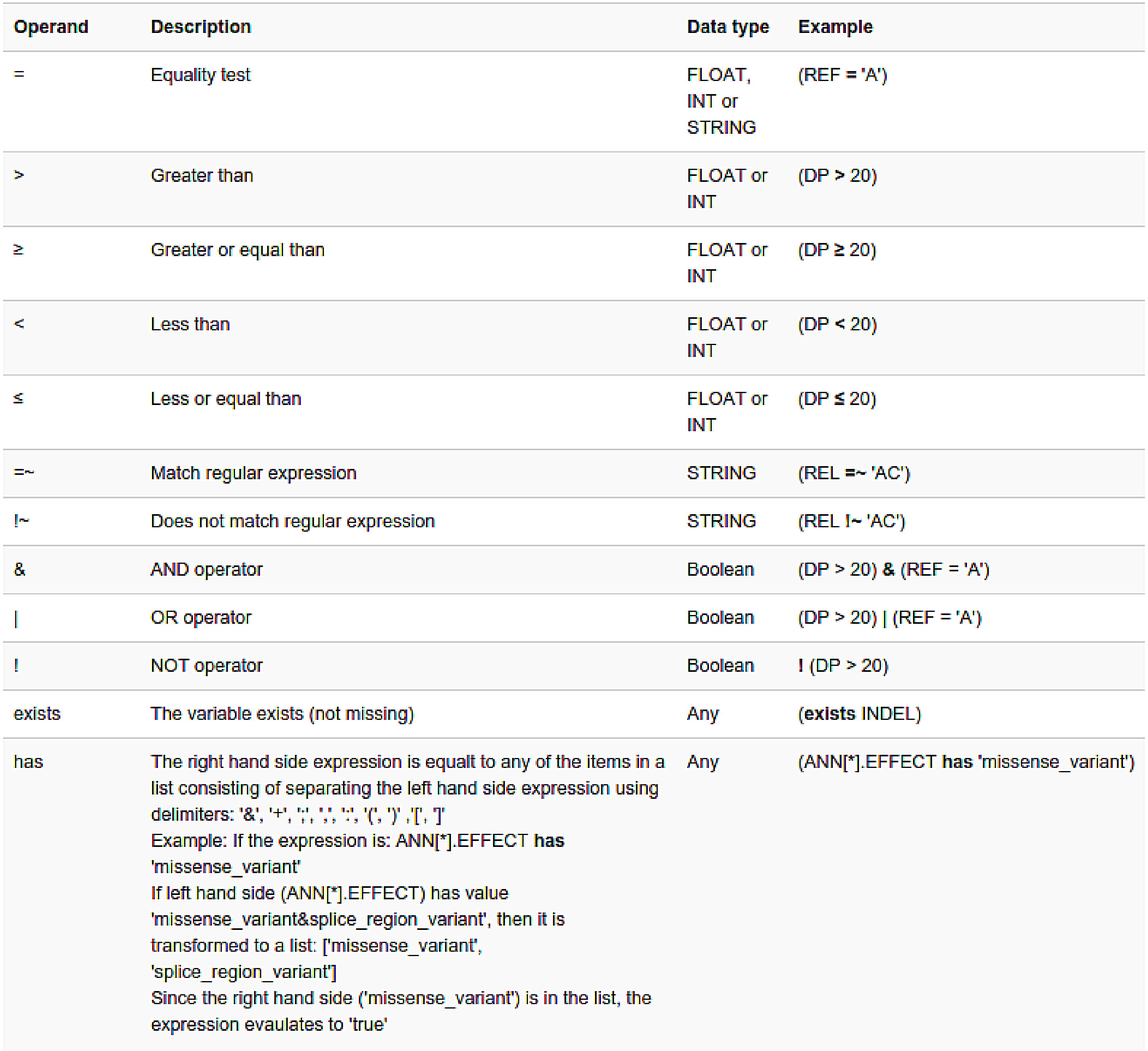
...but this probably doesn't make much sense. What you may really want are lines where ANY effect is NON_SYNONYMOUS:

```
"( EFF[*].EFFECT = 'NON_SYNONYMOUS_CODING' )"
```

May be you want only the ones that affect gene 'TCF7L2'

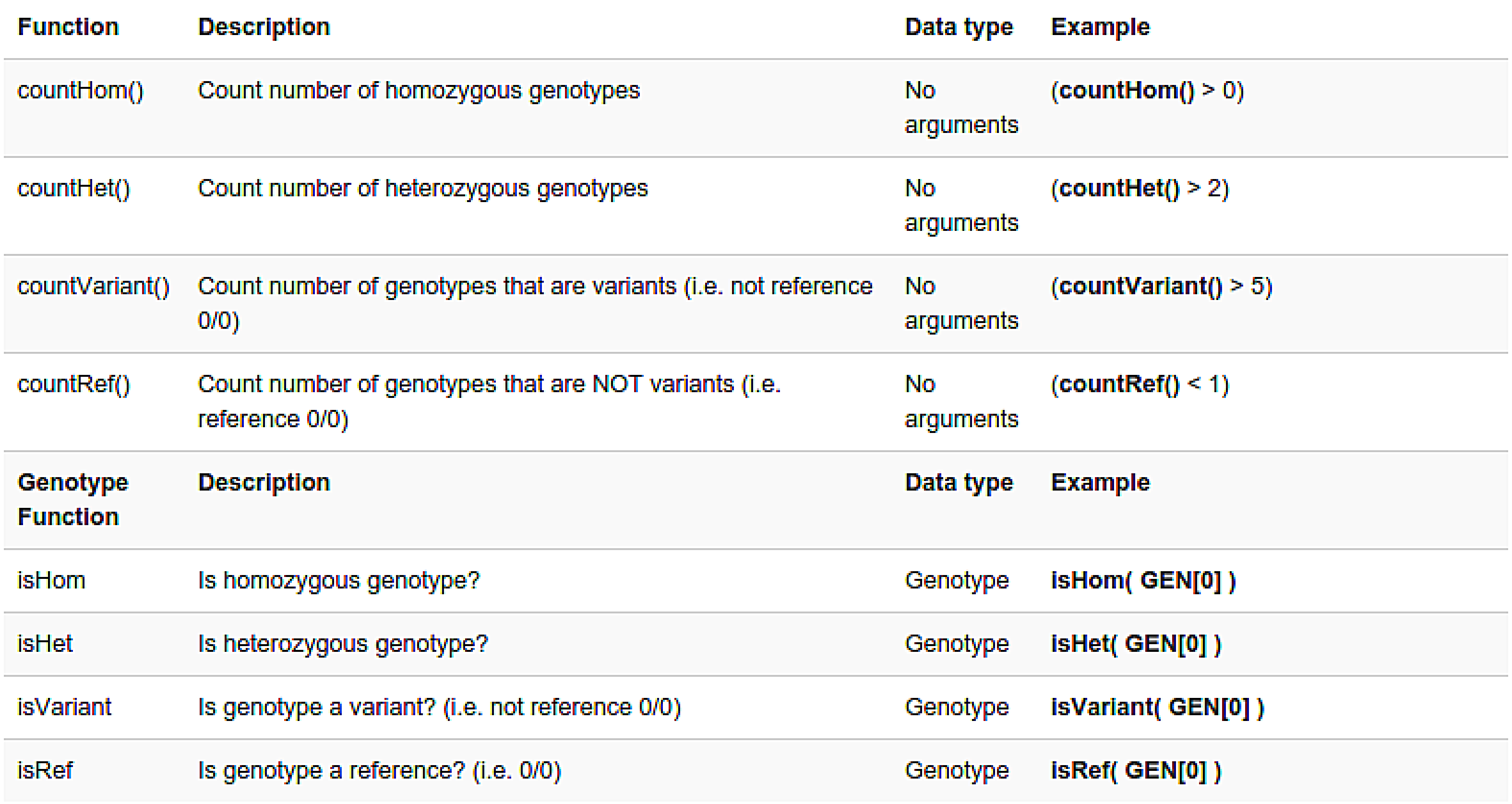
```
"( EFF[*].EFFECT = 'NON_SYNONYMOUS_CODING' ) &  ( EFF[*].GENE = 'TCF7L2' )"
```

SnpSift Filter - Available operands and functions



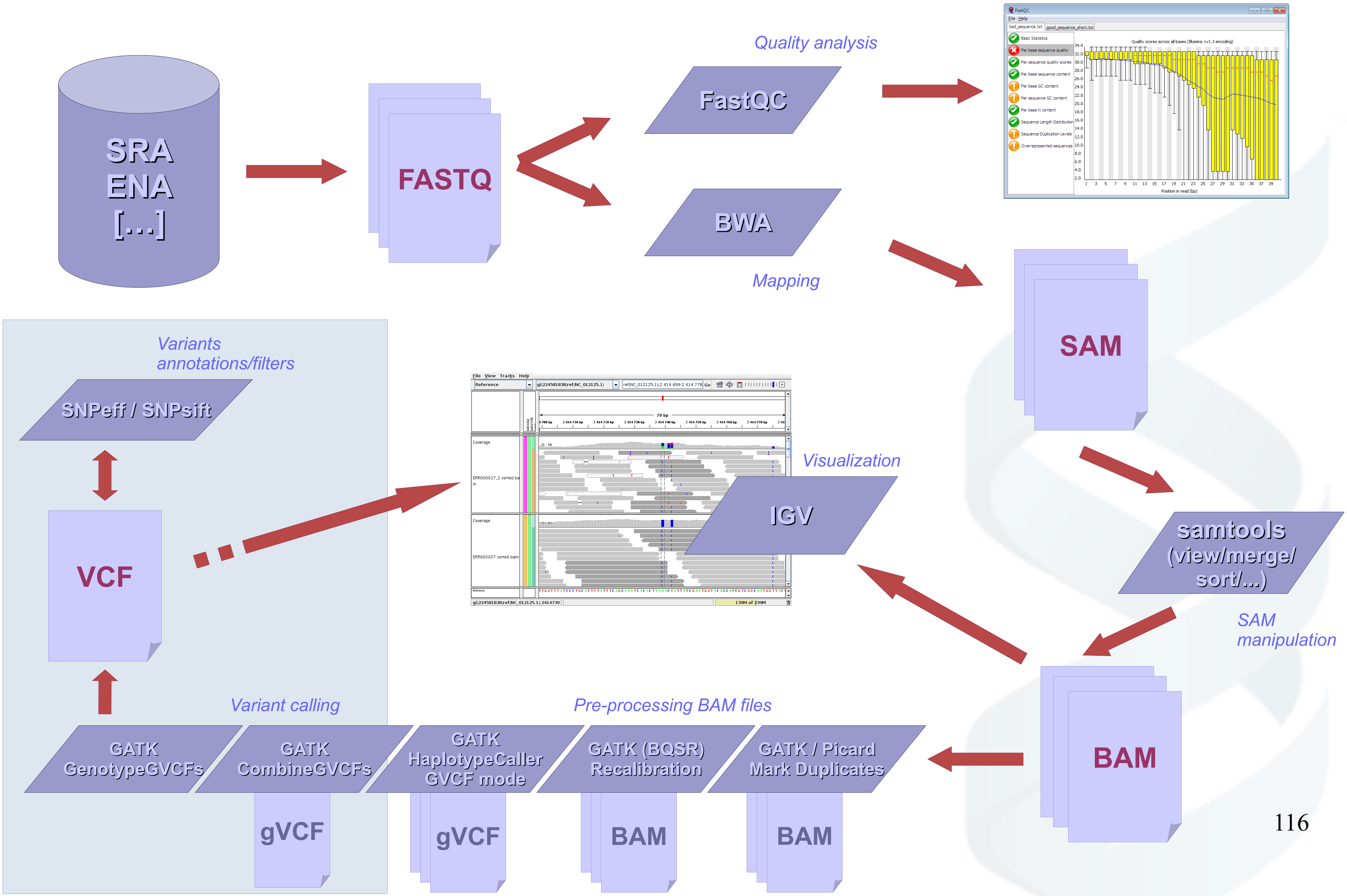
Operand	Description	Data type	Example
=	Equality test	FLOAT, INT or STRING	(REF **=** 'A')
>	Greater than	FLOAT or INT	(DP **>** 20)
≥	Greater or equal than	FLOAT or INT	(DP **≥** 20)
<	Less than	FLOAT or INT	(DP **<** 20)
≤	Less or equal than	FLOAT or INT	(DP **≤** 20)
=~	Match regular expression	STRING	(REL **=~** 'AC')
!~	Does not match regular expression	STRING	(REL **!~** 'AC')
&	AND operator	Boolean	(DP > 20) **&** (REF = 'A')
|	OR operator	Boolean	(DP > 20) **|** (REF = 'A')
!	NOT operator	Boolean	**!** (DP > 20)
exists	The variable exists (not missing)	Any	(**exists** INDEL)
has	The right hand side expression is equalt to any of the items in a list consisting of separating the left hand side expression using delimiters: '&', '+', ';', ',', ':', '(', ')' ,'[', ']' Example: If the expression is: ANN[*].EFFECT **has** 'missense_variant' If left hand side (ANN[*].EFFECT) has value 'missense_variant&splice_region_variant', then it is transformed to a list: ['missense_variant', 'splice_region_variant'] Since the right hand side ('missense_variant') is in the list, the expression evaulates to 'true'	Any	(ANN[*].EFFECT **has** 'missense_variant')

SnpSift Filter - Available operands and functions



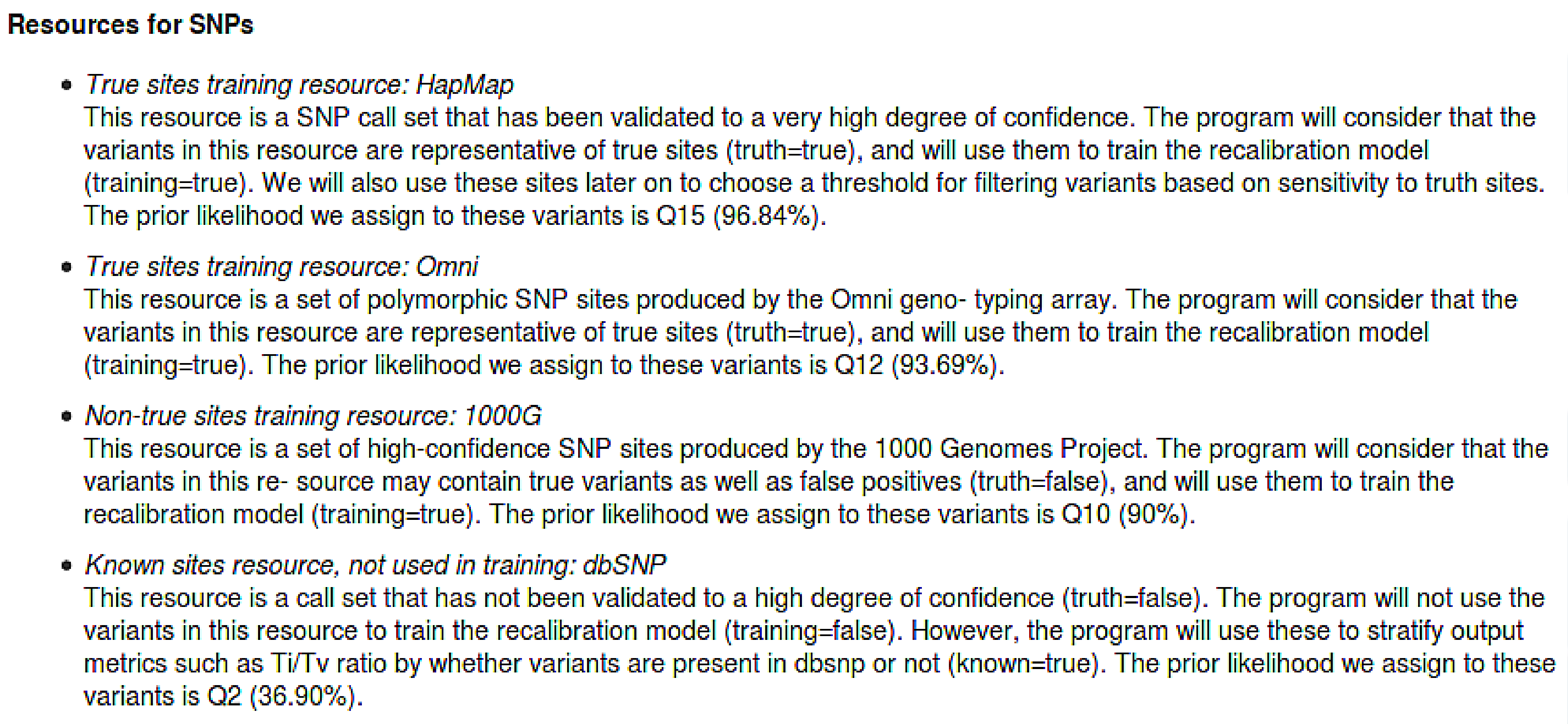
Function	**Description**	**Data type**	**Example**
countHom()	Count number of homozygous genotypes	No arguments	(**countHom()** > 0)
countHet()	Count number of heterozygous genotypes	No arguments	(**countHet()** > 2)
countVariant()	Count number of genotypes that are variants (i.e. not reference 0/0)	No arguments	(**countVariant()** > 5)
countRef()	Count number of genotypes that are NOT variants (i.e. reference 0/0)	No arguments	(**countRef()** < 1)
Genotype Function	**Description**	**Data type**	**Example**
isHom	Is homozygous genotype?	Genotype	**isHom(GEN[0])**
isHet	Is heterozygous genotype?	Genotype	**isHet(GEN[0])**
isVariant	Is genotype a variant? (i.e. not reference 0/0)	Genotype	**isVariant(GEN[0])**
isRef	Is genotype a reference? (i.e. 0/0)	Genotype	**isRef(GEN[0])**

Synthesis


SRA
ENA
[…]
FASTQ
Quality analysis
FastQC
FastQC
File Help
bad_sequence.txt
good_sequence_short.txt
Basic Statistics
Per base sequence quality
Per sequence quality scores
Per base sequence content
Per base GC content
Per sequence GC content
Per base N content
Sequence Length Distribution
Sequence Duplication Levels
Overrepresented sequences
Quality scores across all bases (Illumina >v1.3 encoding)
Position in read (bp)
BWA
Mapping
SAM
samtools
(view/merge/
sort/...)
SAM
manipulation
BAM
Visualization
IGV
File View Tracks Help
Reference
Coverage
ERR000017_2.sorted.bam
ERR003037.sorted.bam
79 bp
Variants
annotations/filters
SNPeff / SNPsift
VCF
Variant calling
Pre-processing BAM files
GATK
GenotypeGVCFs
GATK
CombineGVCFs
GATK
HaplotypeCaller
GVCF mode
GATK (BQSR)
Recalibration
GATK / Picard
Mark Duplicates
gVCF
gVCF
BAM
BAM

Exercise / set 6

Variant recalibration



Resources for SNPs

- *True sites training resource: HapMap*
  This resource is a SNP call set that has been validated to a very high degree of confidence. The program will consider that the variants in this resource are representative of true sites (truth=true), and will use them to train the recalibration model (training=true). We will also use these sites later on to choose a threshold for filtering variants based on sensitivity to truth sites. The prior likelihood we assign to these variants is Q15 (96.84%).
- *True sites training resource: Omni*
  This resource is a set of polymorphic SNP sites produced by the Omni geno- typing array. The program will consider that the variants in this resource are representative of true sites (truth=true), and will use them to train the recalibration model (training=true). The prior likelihood we assign to these variants is Q12 (93.69%).
- *Non-true sites training resource: 1000G*
  This resource is a set of high-confidence SNP sites produced by the 1000 Genomes Project. The program will consider that the variants in this re- source may contain true variants as well as false positives (truth=false), and will use them to train the recalibration model (training=true). The prior likelihood we assign to these variants is Q10 (90%).
- *Known sites resource, not used in training: dbSNP*
  This resource is a call set that has not been validated to a high degree of confidence (truth=false). The program will not use the variants in this resource to train the recalibration model (training=false). However, the program will use these to stratify output metrics such as Ti/Tv ratio by whether variants are present in dbsnp or not (known=true). The prior likelihood we assign to these variants is Q2 (36.90%).

http://gatkforums.broadinstitute.org/gatk/discussion/1259/which-training-sets-arguments-should-i-use-for-running-vqsr