GULBENKIAN CIÊNCIA

Variant detection: hands on

Precision Oncology Course

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Variant detection case study

1. **Quality control** on sequencing data

2. **Variant calling** of small mutations (SNV and INDELS)
    a. Germline variants
    b. Somatic variants

3. Variant detection **analysis**

Road map

QUALITY CONTROL	Quality Control of raw data		FastQC
TRIMMING	Trimming of fastq reads		Trimmomatic
	Tumor sample	Normal sample	
ALIGNMENT	Indexing of Reference Genome		BWA-index
	Sample alignment	Sample alignment	BWA-MEM
REFINEMENT OF ALIGNMENT	Mark duplicates	Mark duplicates	Picard
	Base quality recalibration	Base quality recalibration	GATK

Road map

	Tumor sample	Normal sample	
VARIANT CALLING	Tumor Genomic positions	Normal Genomic positions	Germinal: Haplotypecaller Somatic: MuTect2
FILTERING	Tumor Calling & Filtering	Normal Calling & Filtering	Germinal: VQRS/Hardfilter Somatic: MuTect2
ANNOTATION	Multisample file annotation		Germinal: SnpEff Both: VEP
VISUALIZATION	Visualization of variants in alignments		IGV
STATISTICS	Quality control and Alignment stats		MultiQC

Data formats cheat sheet

Format	Uses	Example	File type	Software Management	File Extension
Fasta	**Human genome** Define biological sequences (DNA, RNA, cDNA, proteins).	human_genome.fa	Plain text	samtools, picard-tools	.fa; .fasta
FastQ	**Raw sequencing data** Single-end sequencing → 1 file Paired-end sequencing → 2 files (R1 and R2 for each end, respectively)	DNAseq_raw_data.fastq (DNAseq_R1.fastq and DNAseq_R2.fastq)	Plain text	samtools, picard-tools **Aligners**	.fq; .fastq
SAM	Define read alignments. Store alignment meta-info (reference, methods, one- or multi-sample).	mapped_reads.sam	Plain text	samtools, picard-tools	.sam
BAM	**VISUALIZE ALIGNMENTS (IGV)** The same as SAM, but compressed and indexed. Also to store UNMAPPED reads (compressed).	mapped_reads.bam unmapped_reads.bam	Binary	samtools, bcftools, picard-tools, **IGV** (Integrative Genome Viewer)	.bam
VCF	**SNV & Indels calls** Indicates genomic variations. Store Variant calling meta-info (reference, methods,one- or multi-sample).	point_variants.vcf	Plain text	bcftools, Unix	.vcf
BED	**Intervals** Delimit genomic regions (i.e. intervals) w or w/o annotations.	targeted_regions.bed intervals.bed	Plain text	bedtools, Unix **GATK, picard-tools**	.bed
TSV or CSV	Create data matrix (rows X Columns)	annotated_variants.tsv	Plain text	Unix, Microsoft Excel, OpenOffice	.tsv; .csv; .txt

Overview: Exome analysis (OVCA)

Patient suffering ovarian cancer

Whole-exome sequencing data from two samples from the patient:

- Tumour sample.
- Matched normal sample (healthy tissue) from epithelium.

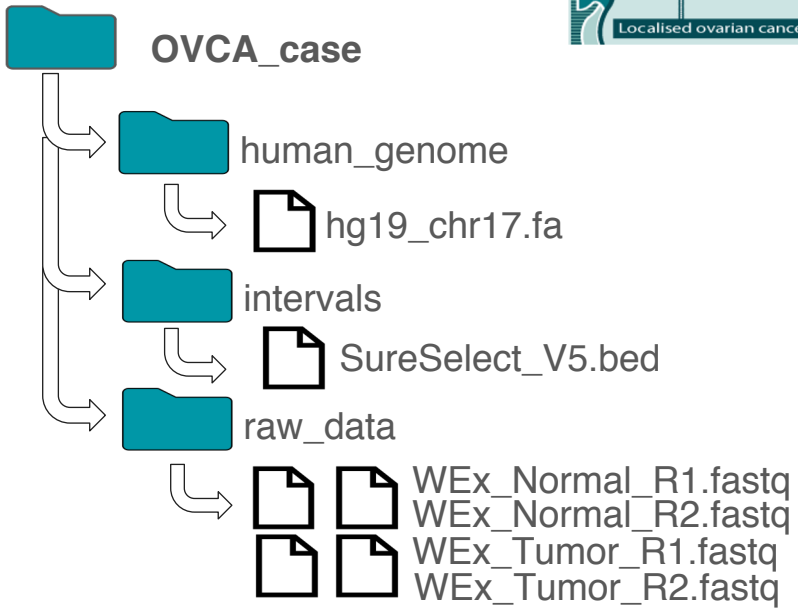
Library protocol: Agilent SureSelect V5 Human All Exons.

Sequencing platform: HiSeq 2000 (Illumina)

Expected outcome:

- ~dozens of germ-line variants.
- A few somatic cancer mutations (SNV, indel).

NOTE: This data was simulated and reduced in order to perform the computational analysis in class time.



Download the data:
https://drive.google.com/drive/u/0/folders/1qolq1kbH8H233vN2Szn0A3NPmPFuPGbH

Getting started: make a local copy of the data

- Get the data into your home
- Un-tar the bundle of files

```
$ cd /home/$USER/
$ tar xvf OVCA_case.tar
```

- Take a look at the data for the exercise

```
$ cd /home/$USER/OVCA_case
$ ls -l *
```

Human_genome:
hg19_chr17.fa

Intervals:
SureSelect_V5_human_all_Exons_chr17.bed

Raw_data:
WEx_Normal_R1.fastq
WEx_Normal_R2.fastq
WEx_Tumour_R1.fastq
WEx_Tumour_R2.fastq

Vep
.vep/homo_sapiens/104_GRCh37/

VEP cache data

Human reference genome.
Alignment on a consensus genome reference.
Source: UCSC / 1000G project

WEx Library design.
Predefined genomic regions of interest.
Source: manufacturer
Useful for : visualize the alignment

Raw exome sequencing data.
Patient's sample data, generated by the sequencing machine.
Source: Collaborator / Consumer provider

Getting started: conda installation

- Go to https://docs.conda.io/en/latest/miniconda.html#linux-installers
- Download the miniconda bundle

```
(optionally)
$ wget https://repo.anaconda.com/miniconda/Miniconda3-py39_4.10.3-Linux-x86_64.sh
$ bash Miniconda-YOUR_VERSION_HERE-Linux-x86_64.sh
```

- Using conda:

```
(optionally)
$ conda create -c CHANNEL_NAME -n ENV_NAME optional_packages
$ conda install -c CHANNEL_NAME PACKAGE_NAME
```

Visit https://anaconda.org/ to find packages of interest

Getting started: snakemake installation

- Install snakemake through mamba:

```
$ conda install -c conda-forge mamba
$ mamba create -c conda-forge -c bioconda -n snakemake snakemake
```

Mamba

https://github.com/mamba-org/mamba

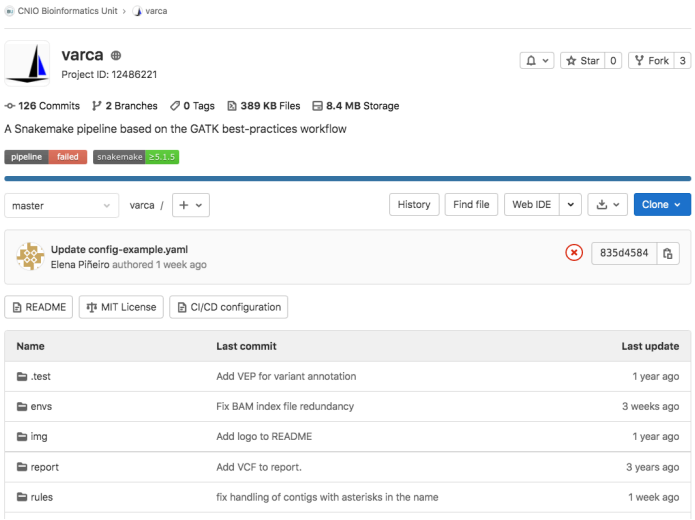
At the same time, mamba utilizes the same command line parser, package installation and deinstallation code and transaction verification routines as conda to stay as compatible as possible.

Mamba is a reimplementation of the conda package manager in C++. Allows:

- Parallel downloading of repository data and package files using multi-threading
- libsolv for much faster dependency solving, a state of the art library used in the RPM package manager of Red Hat, Fedora and OpenSUSE
- Core parts of mamba are implemented in C++ for maximum efficiency

Getting started: downloading Varca

https://gitlab.com/bu_cnio/varca




```
$ mdkir varca
$ cd varca
$ git clone
https://gitlab.com/bu_cnio/varca.git .
Cloning into '.'...
remote: Enumerating objects: 853, done.
remote: Counting objects: 100% (128/128),
done.
remote: Compressing objects: 100% (74/74),
done.
remote: Total 853 (delta 76), reused 103
(delta 53), pack-reused 725
Receiving objects: 100% (853/853), 1.47
MiB | 0 bytes/s, done.
Resolving deltas: 100% (545/545), done.
Checking connectivity... done.
```

Edit configuration files

Contigs.tsv

It contains the contigs of the reference genome to include in the analysis (one contig per line). If empty, the analysis is performed using all contigs of the fasta index.

In our case, we just need chromosome 17

Edit configuration files

Samples.tsv

Lists all samples to be included in the run, the use of MuTect2 and its execution mode.

HaplotypeCaller will be executed for each sample in the sample column. To activate the execution of MuTect2 and set its execution mode, the control column is used.

If control contains:

- - MuTect2 is not executed for that sample
- The same sample name as in the sample column: MuTect2 is executed for that sample in tumor-only mode.
- A different sample name than in the sample column: MuTect2 is executed in tumor-normal mode; being the tumor sample the one indicated in the sample column, and the normal sample the one indicated in the control column. This way we will detect somatic mutations

sample	control
A	-

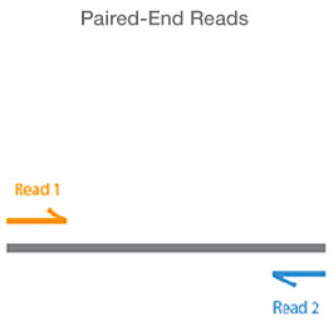
sample	control
A	A

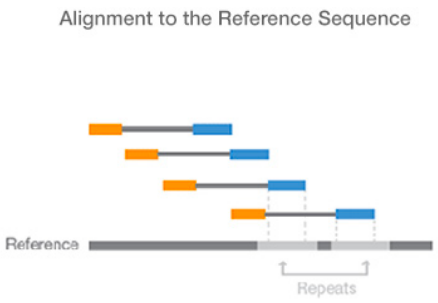
sample	control
A	B
B	-

Edit configuration files

units.tsv
It contains specifications of the samples (sequencing units, sequencing platform and fastq files) listed in samples.tsv.

In our case, we define A as tumour and B as control samples with two files for each, **read 1** and **read 2**






```
sample   unit     platform fq1      fq2
A        1        ILLUMINA path_to_WEx_Tumour_R1.fastq path_to_Wex_Tumour_R2.fastq
B        1        ILLUMINA path_to_WEx_Normal_R1.fastq path_to_Wex_Normal_R2.fastq
```

Edit configuration files

Config.yaml

This file contains the paths to files required in the analysis, the enabling/disabling of optional steps, as well as additional parameters for some of the programs used in the process. The file is structured in several sections to be customized according to the characteristics of the analysis. The indications for its filling are provided inside the file.

```
$ vi config.yaml
```

Edit configuration files

Config.yaml

```
samples: samples.tsv
units: units.tsv
contigs: contigs.tsv

outdir: "out"
logdir: "log"

ref:
# Genome database of snpeff to be used in the annotation with this resource.
Available databases can be checked with java -jar snpEff.jar databases
name: GRCh37.75
# Path to the reference genome, ideally as it is provided by the GATK bundle.
genome: /path_to_/hg19_chr17.fa
# Path to any database of known variants, ideally as it is provided by the
GATK bundle.
known-variants: /path_to/dbsnp_138.hg19_chr17.vcf.gz
```

Edit configuration files

Config.yaml

```
filtering:
# Set to true in order to apply machine learning based recalibration of
# quality scores instead of hard filtering.
vqsr: false
hard:
# hard filtering as outlined in GATK docs
# (https://gatkforums.broadinstitute.org/gatk/discussion/2806/howto-apply-
hard-filters-to-a-call-set)
snvs:
"QD < 2.0 || QUAL < 100.0 || DP < 50.0 || SOR > 3.0 || FS > 60.0 || MQ < 40.0
|| MQRankSum < -12.5 || ReadPosRankSum < -8.0"
indels:
"QD < 2.0 || QUAL < 100 || DP < 50.0 || FS > 200.0 || ReadPosRankSum < -20.0"
#depth of coverage threshold to apply to variants identified with MuTect2
depth: "DP < 30"
```

Execute the pipeline

Activate snakemake

```
$ conda activate snakemake
```

Test dry run

```
$ snakemake --use-conda -n
```

```
Building DAG of jobs...
Conda environment envs/stats.yaml will be created.
Conda environment https://github.com/snakemake/snakemake-wrappers/raw/0.77.0/bio/samtools/stats/environment.yaml will be created.
Conda environment https://github.com/snakemake/snakemake-wrappers/raw/0.77.0/bio/multiqc/environment.yaml will be created.
Conda environment https://github.com/snakemake/snakemake-wrappers/raw/0.77.0/bio/snpeff/download/environment.yaml will be created.
Conda environment https://github.com/snakemake/snakemake-wrappers/raw/0.77.0/bio/snpeff/annotate/environment.yaml will be created.
Conda environment envs/rbt.yaml will be created.
Job stats:
job                count    min threads    max threads
--------------  -------  -------------  -------------
all                    1              1              1
multiqc                1              1              1
plot_stats             1              1              1
samtools_stats         2              1              1
snpeff                 1              1              1
snpeff_download        1              1              1
vcf_to_tsv             1              1              1
total                  8              1              1


[Thu Oct  7 21:32:06 2021]
rule snpeff_download:
    output: resources/snpeff/GRCh37.75
```

Execute the pipeline

Launch Varca

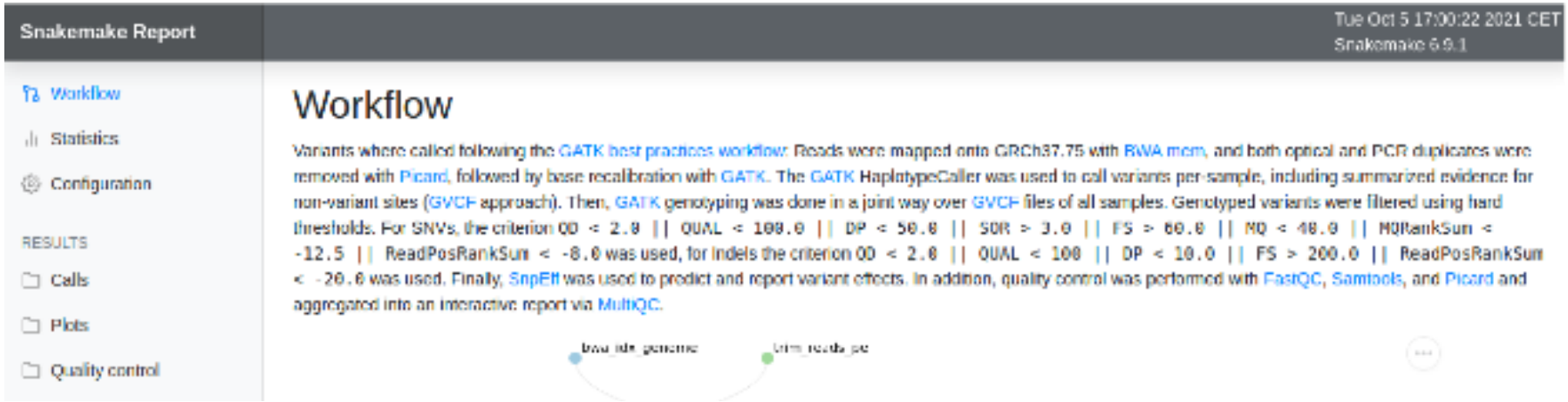
```
$ snakemake --use-conda --cores 2
```

Running just the main steps (no VEP annotation)

```
$ snakemake --use-conda --cores 2 -until merge_calls
$ snakemake --use-conda --cores 2 -until filter_mutect_2
```

Once finished, generate report (this step only works if **all steps** have been previously run)

```
$ snakemake --report report.html
```

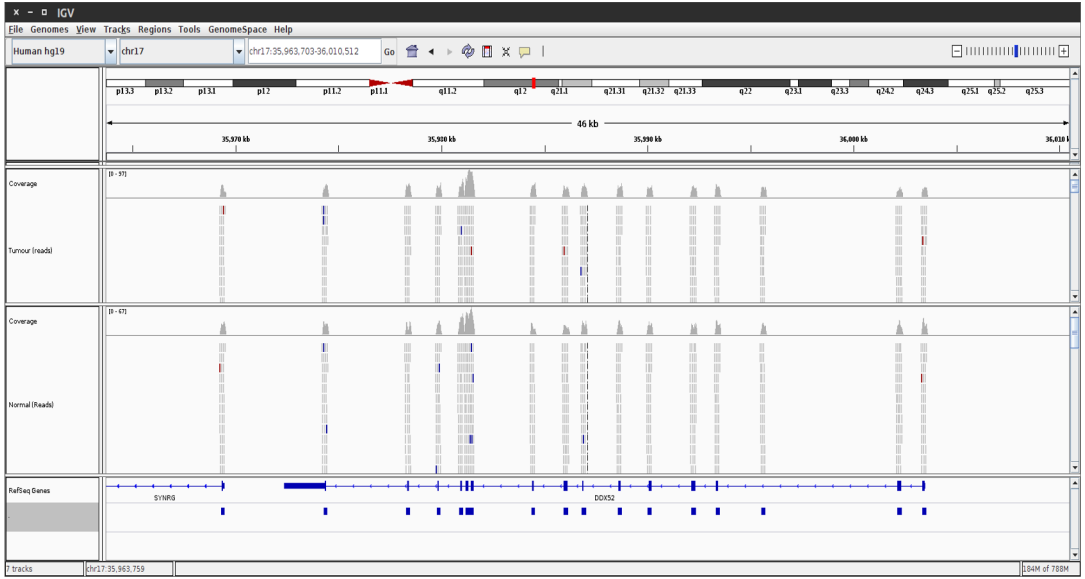


IGV Genome browser

Integrative Genomics Viewer

1. Open a terminal, and execute the command:

```
$ conda install -c bioconda igv
$ igv
```



2. Open the BAM files for each sample

- A-1.bam (tumor sample)
- B-1.bam (normal sample)

3. Open the BED file (intervals file)

SureSelect_V5.bed

Manual: http://software.broadinstitute.org/software/igv/UserGuide

Results

Germline variants

There were detected ___ germline variants in total:

- ___ Single Nucleotide Variants.
- ___ Indels.

Somatic variants

There were detected ___ somatic SNVs.

Genes affected and type of mutations (see alignment using IGV on chr17):

- _____.
- _____.

Integrative Genomics Viewer

Extra: VCF format

Header

```
##fileformat=VCFv4.2
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
```

single Variant calls

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO	FORMAT	NA00001	NA00002	NA00003
20	14370	rs6054257	G	A	29	PASS	NS=3;DP=14;AF=0.5;DB;H2	GT:GQ:DP:HQ	0|0:48:1:51,51	1|0:48:8:51,51	1/1:43:5:.,.
20	17330	.	T	A	3	q10	NS=3;DP=11;AF=0.017	GT:GQ:DP:HQ	0|0:49:3:58,50	0|1:3:5:65,3	0/0:41:3
20	1110696	rs6040355	A	G,T	67	PASS	NS=2;DP=10;AF=0.333,0.667;AA=T;DB	GT:GQ:DP:HQ	1|2:21:6:23,27	2|1:2:0:18,2	2/2:35:4
20	1230237	.	T	.	47	PASS	NS=3;DP=13;AA=T	GT:GQ:DP:HQ	0|0:54:7:56,60	0|0:48:4:51,51	0/0:61:2
20	1234567	microsat1	GTC	G,GTCT	50	PASS	NS=3;DP=9;AA=G	GT:GQ:DP	0/1:35:4	0/2:17:2	1/1:40:3

Genotype variables ↔ Individual Samples

valid?

Genotype specifications

- VCF records are oriented to provide details of single variant calls.
- Not all records in a VCF are true calls, the **FILTER** column specifies those which passed the calling.
- **QUAL** is the score assigned to a given call. The greater QUAL is, the more reliable is. It is in log-scale.
- **ID** is an identifier. E.g. a dbSNP id.

A PDF with the v4.2 specifications: http://samtools.github.io/hts-specs/VCFv4.2.pdf

What is a VCF and how to interpret it : https://software.broadinstitute.org/gatk/guide/article?id=1268

Extra: VCF from callers

Allele1 / Allele2 (diploid)
1/1 → homozygous mutant
0/1 → heterozygous mutant
0/0 → homozygous reference

```
## HEADER
#CHROM    POS       ID   REF   ALT   QUAL   FILTER     INFO    FORMAT   SampleName
chr17     87234     .    G     A     2000   PASS       DP=80   GT:PL    1/1:3000,220,0
chr17     98764     .    T     C     340    PASS       DP=30   GT:PL    0/1:1200,0,200
chr17     108764    .    G     C     10     FILTERED   DP=7    GT:PL    0/1:37,0,200
```

Genomic coordinates

Nucleotide change

score
(higher → better)

filtered?

Likelihood for each GT:
0/0, 0/1, 1/1.
(lower→ better)
0 is the best score.

More info.:
https://samtools.github.io/hts-specs/VCFv4.2.pdf
https://www.broadinstitute.org/gatk/guide/article?id=1268

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Thanks!

Credits for many class materials to:

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Vep annotation cache

MENU

Variant Effect Predictor Download and install

Download

Download ensembl-vep package (see below the different ways to download it) and then follow the installation instructions.

https://m.ensembl.org/info/docs/tools/vep/script/vep_download.html#installer

```
$ mamba install -c bioconda perl-dbi
```

```
$ git clone https://github.com/Ensembl/ensembl-vep.git
$ cd ensembl-vep
$ git pull
$ git checkout release/104
$ perl INSTALL.pl --AUTO cf --ASSEMBLY GRCh37 --SPECIES "Homo_sapiens"
```