

NGS I : VARIANT DETECTION

Javier Perales-Patón
jperales@cnio.es



Translational Bioinformatics Unit
CNIO. Madrid, Spain.

Fátima Al-Shahrour
falshahrour@cnio.es

Elena Piñeiro-Yáñez
epineiro@cnio.es

Pedro Fernandes
pfern@igc.gulbenkian.pt

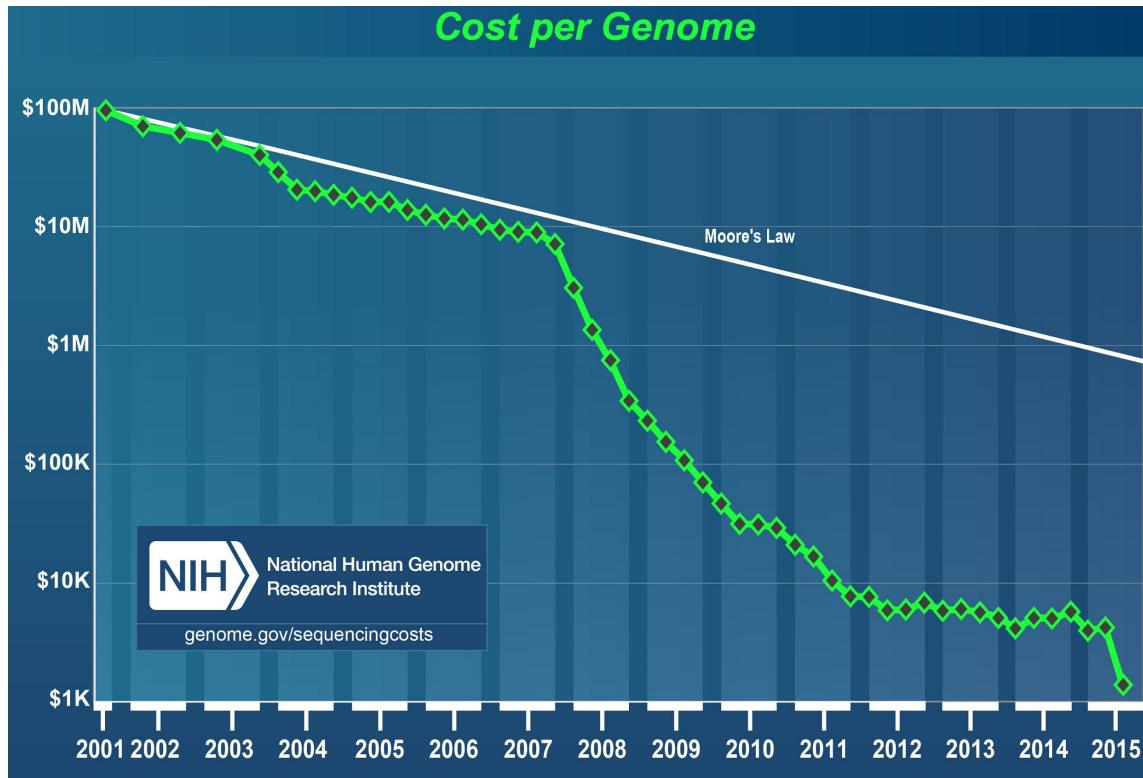


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Today

09:30 - 10:00	Introduction to the course and self presentation of the participants. Personalized medicine.
11:30 - 12:30	NGS I : Variant detection.
14:00 - 16:00	Playing with the data and the methods.
16:30 - 18:00	Practical : Running the pipeline.

Sequencing cost has been coming down



Sequencing cost has been coming down

Cost per Genome

\$10 Mardis *Genome Medicine* 2010, 2:84
<http://genomemedicine.com/content/2/11/84>

 **Genome Medicine**

MUSINGS

The \$1,000 genome, the \$100,000 analysis?

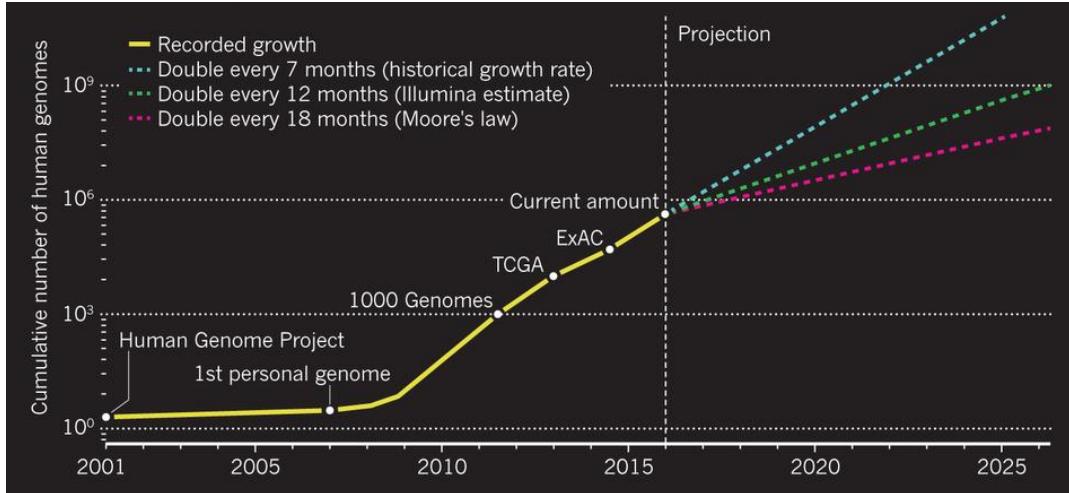
Elaine R Mardis*

Although each presenter emphasized the rapidity with which these data can now be generated using next-generation sequencing instruments, they also listed the large number of people involved in the analysis of these datasets.

[...]

The large number of specialists was critical for the completion of the data analysis, the annotation of variants, the interpretive ‘filtering’ necessary to deduce the causative or ‘actionable’ variants, the clinical verification of these variants, and the communication of results and their ramifications to the treating physician, and ultimately to the patient. At the end of the day, although the idea of clinical whole-genome sequencing for diagnosis is exciting and potentially life-changing for these patients, one does wonder how, in the clinical translation required for this practice to become common-place, such a ‘dream team’ of specialists would be assembled for each case.

DNA sequencing soars



- + 1000 Genomes Project : hundreds of genomes.
- + TCGA : several thousand (genome & exomes).
- + ExAC : > 60,000 exomes.

Data Phase	Astronomy	Twitter	YouTube	Genomics
Acquisition	25 zetta-bytes/year	0.5–15 billion tweets/year	500–900 million hours/year	1 zetta-bases/year
Storage	1 EB/year	1–17 PB/year	1–2 EB/year	2–40 EB/year
Analysis	In situ data reduction	Topic and sentiment mining	Limited requirements	Heterogeneous data and analysis
	Real-time processing	Metadata analysis		Variant calling, ~2 trillion central processing unit (CPU) hours
	Massive volumes			All-pairs genome alignments, ~10,000 trillion CPU hours
Distribution	Dedicated lines from antennae to server (600 TB/s)	Small units of distribution	Major component of modern user's bandwidth (10 MB/s)	Many small (10 MB/s) and fewer massive (10 TB/s) data movement

1EB=1 billion GB

Stephens ZD et al. **Big Data: Astronomical or Genomical?**. PLoS Biol. 2015 Jul 7;13(7)

Eisenstein M. **Big data: The power of petabytes**. Nature. 2015 Nov 5;527(7576):S2-4.

1000 Genomes Project

ARTICLE

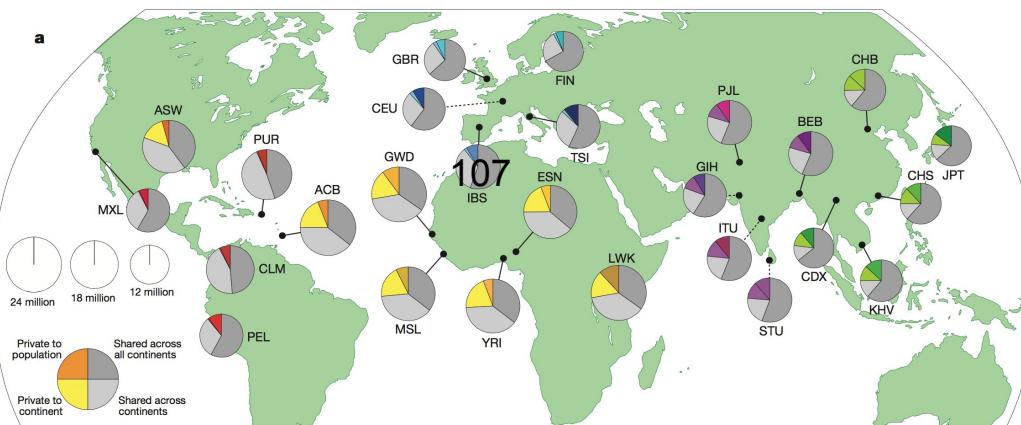
OPEN

doi:10.1038/nature15393

A global reference for human genetic variation

The 1000 Genomes Project Consortium*

The 1000 Genomes Project set out to provide a comprehensive description of common human genetic variation by applying whole-genome sequencing to a diverse set of individuals from multiple populations. Here we report completion of the project, having reconstructed the genomes of 2,504 individuals from 26 populations using a combination of low-coverage whole-genome sequencing, deep exome sequencing, and dense microarray genotyping. We characterized a broad spectrum of genetic variation, in total over 88 million variants (84.7 million single nucleotide polymorphisms (SNPs), 3.6 million short insertions/deletions (indels), and 60,000 structural variants), all phased onto high-quality haplotypes. This resource includes >99% of SNP variants with a frequency of >1% for a variety of ancestries. We describe the distribution of genetic variation across the global sample, and discuss the implications for common disease studies.



Phase 3	WGS	WExS
Raw bases	89 Tb	18 Tb
Samples	2,504	2,504
Region	Genome	Exome
Mean Depth	8.45x	75x
SNPs	85M	1.5M
Indels	3.6M	22K
Structural Variants	60K	6.5K
Het. Concordance (SNPs)	99.4%	99.8%

<http://www.1000genomes.org/about#ProjectSamples> ; Phase 1 n=1092 → Phase 3 n=2504

ARTICLE

OPEN
doi:10.1038/nature19057

Analysis of protein-coding genetic variation in 60,706 humans

Large-scale reference data sets of human genetic variation are critical for the medical and functional interpretation of DNA sequence changes. Here we describe the aggregation and analysis of high-quality exome (protein-coding region) DNA sequence data for 60,706 individuals of diverse ancestries generated as part of the Exome Aggregation Consortium (ExAC). This catalogue of human genetic diversity contains an average of one variant every eight bases of the exome, and provides direct evidence for the presence of widespread mutational recurrence. We have used this catalogue to calculate objective metrics of pathogenicity for sequence variants, and to identify genes subject to strong selection against various classes of mutation; identifying 3,230 genes with near-complete depletion of predicted protein-truncating variants, with 72% of these genes having no currently established human disease phenotype. Finally, we demonstrate that these data can be used for the efficient filtering of candidate disease-causing variants, and for the discovery of human 'knockout' variants in protein-coding genes.



An average of 85 heterozygous and 35 homozygous Protein-truncating variants per individual. The majority found in any one person are common, and only 2 are singleton.

Blog Post: <http://blogs.nature.com/freeassociation/2016/08/joint-calling-of-the-exac-publications.html>

Paper : [Lek M et al. Analysis of protein-coding genetic variation in 60,706 humans. \(2016\) Nature.](#)

The objective of the Variant Detection:

Identify the most likely **genotype for each genomic position from the individual**. The genomic regions of interest could be either the whole-genome, the exome or a panel of genes.

- - -

In Cancer genomics, if there is a **matched-normal sample** to be compared against the tumour sample:

- + Identify **somatic variants** (i.e. only in tumour sample).
- + Identify **copy-number alterations** (large genomic aberrations).

Some definitions

- What is a genetic **variant** ?

Genetic differences in individuals as compared to a reference genome (built from a population).

Nomenclature:

- **First level:** Genomic position and nucleotide change.

Chromosome Name:Genomic position (coordinates):**Reference allele**>**Alternative allele**
Chr12:25398284-25398284:**G>A**

- Classes of variants :

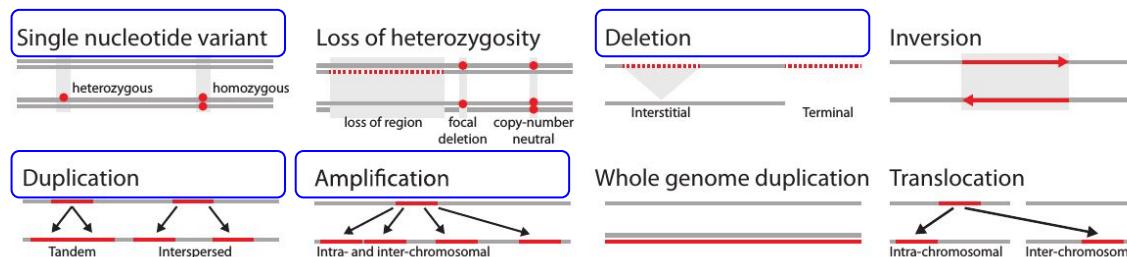
- **Germline** : inherited. E.g. a SNP, or a SNV related to a rare disease.
- **Somatic** : acquired within a cell lineage. E.g. Cancer mutations.

- Polymorphism

common variant in a given population (SNP, Single Nucleotide Polymorphism).

Present in at least 1% in a population.

- Types of genomic variants:



Variants need a context

Example:

chr12:25398284-25398284:G>A → KRAS Gly12Val

White list



DETECTED VARIANTS

Sample

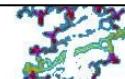
Day 1: Variant Discovery

Prior-knowledge for modeling

1000 Genomes

A Deep Catalog of Human Genetic Variation

dbSNP
Short Genetic Variations



Black list

1000 Genomes

A Deep Catalog of Human Genetic Variation

dbSNP
Short Genetic Variations



ExAC
Exome Aggregation Consortium

DISEASE-RELATED VARIANTS

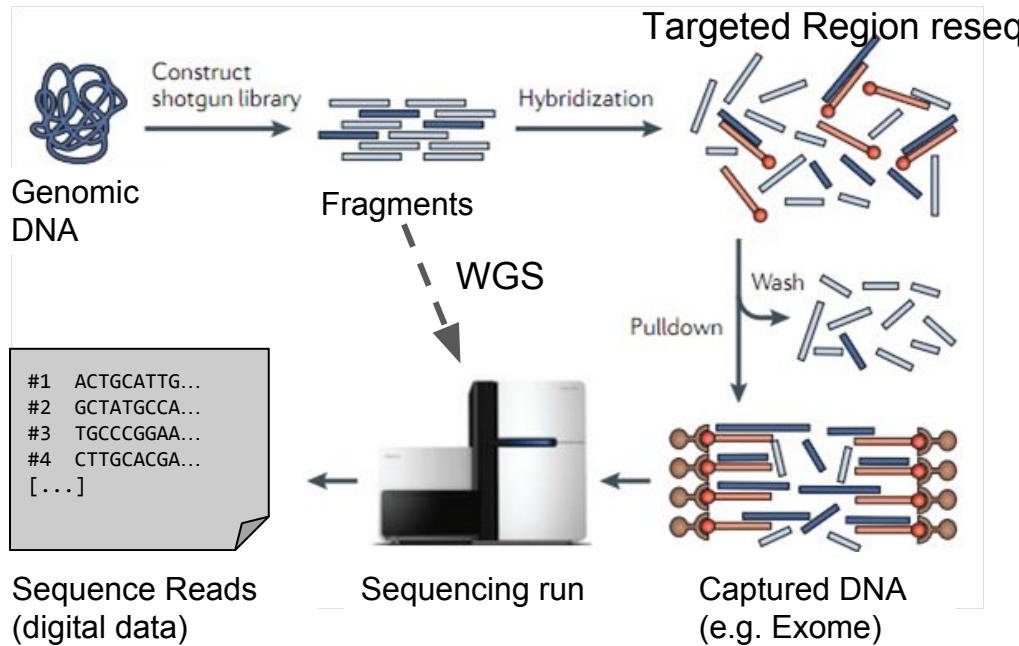
Day 2: Variant Annotation & Filtering

TARGETED THERAPIES

Day 3: in-silico prescription

Day 4: real cases studies!

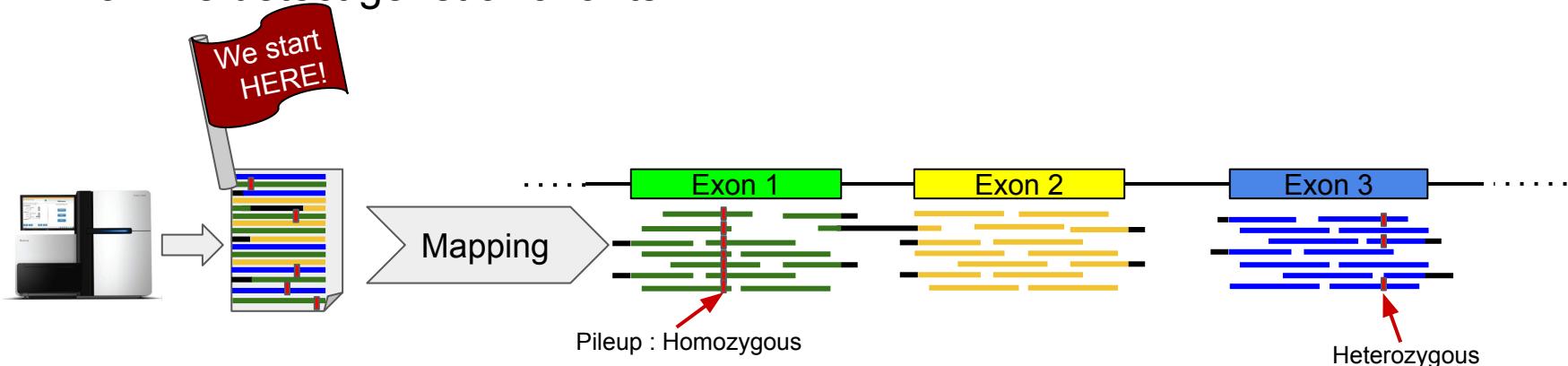
DNA Sequencing data generation



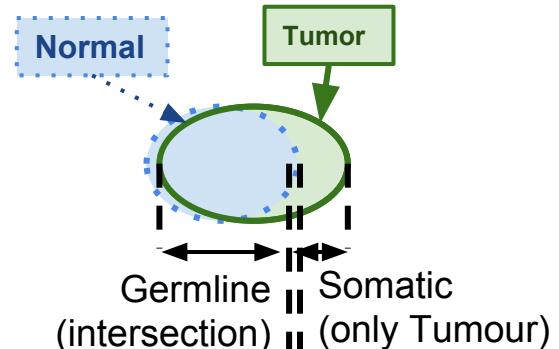
The sequence reads belong from the ends of the fragment.

Fundamentals of variant detection

- How we detect genetic variants ?



Discern somatic mutations by comparison:



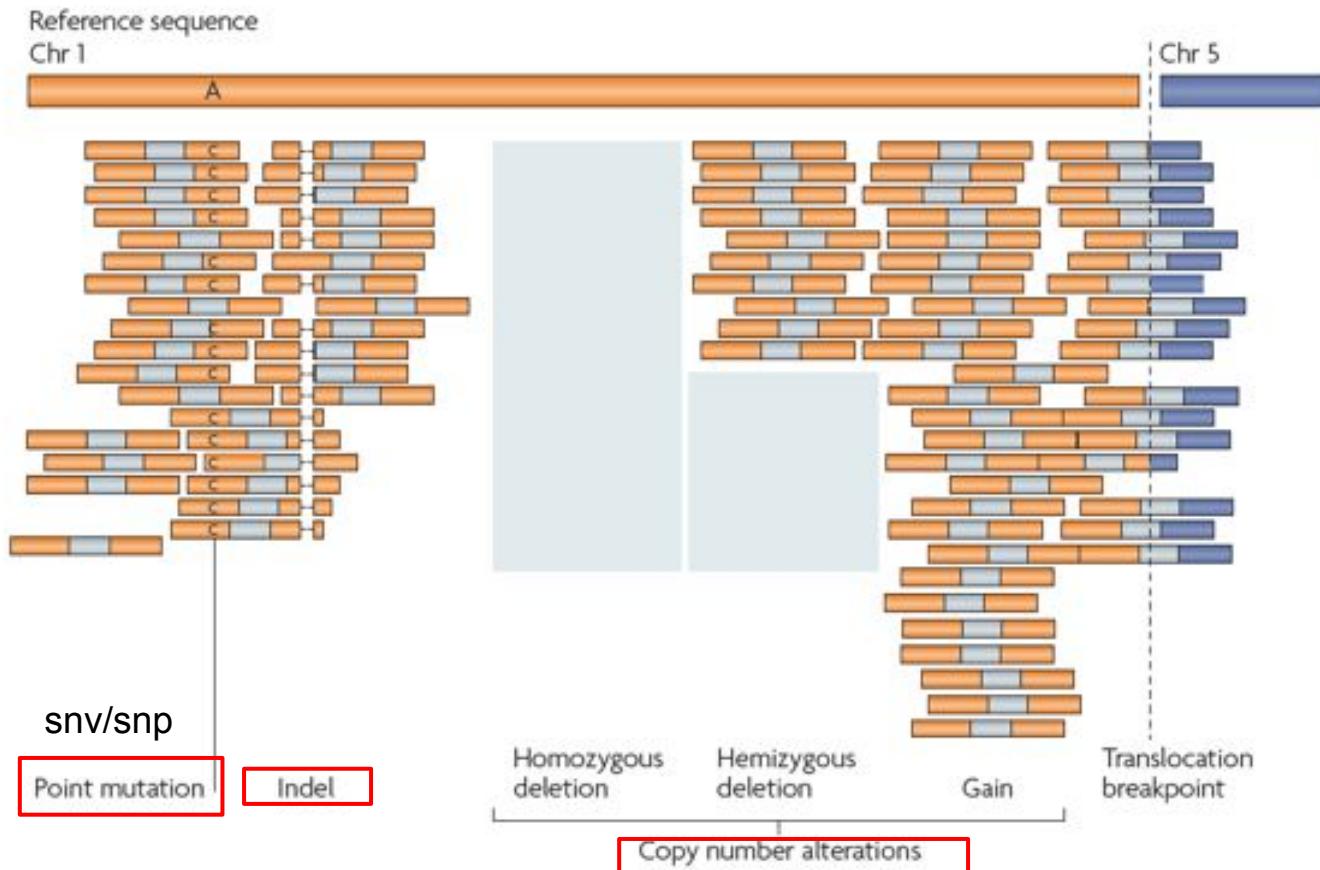
Are these differences true calls?
Statistical method to estimate the most likely genotype.

Annotation Day 2

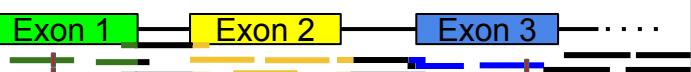
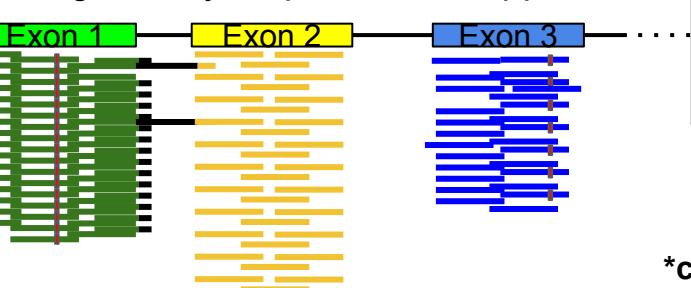
Are these variants polymorphic?
Allele Frequency > 1%



Different types of variants detected by mapping reads

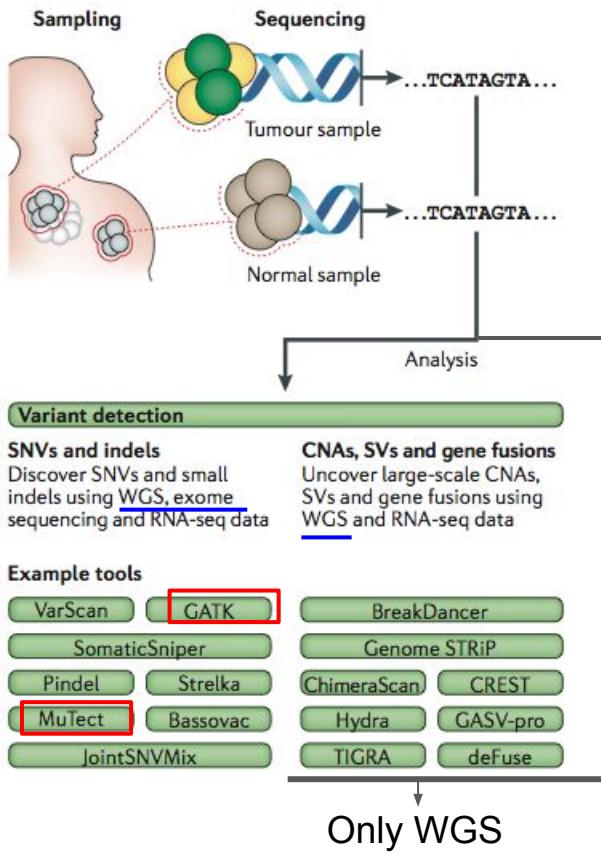


Whole-Genome, and targeted resequencing

	# bp pos seq	Type of variants discovered	Avg Coverage per pos	Cost
<u>Whole-Genome Sequencing</u> 	~100 Gb	- coding variants* , intronic and regulatory sites. - Structural variants - CNA #Variants= 3M - 5M.	30x	High
<u>Whole-Exome Sequencing</u> 	~32Mb 50Mb	- coding variants* . - Some intronic and regulatory sites. - CNA (challenging). #Variants= 20k - 60k.	20x - 80x	Low
<u>Panel of genes by amplicon/PCR approach</u> 	ND	Depends on the design - Particular coding variants* - CNA (challenging) # variants = ND	1000x - 5000x	Low

***coding variants**: missense, stop gained, stop lost, frameshift, splice region...

Methods for Variant Detection



Several Methods have been published.

Tool	Year	Language	Paired or pooled data	Segmentation	Feature
ADTEX	2014	Python, R	Both	HMM	Noise reduction Ploidy estimation
CONTRA	2012	Python, R	Both	CBS	GC correction
Control-FREEC	2011	C++, R	Paired	LASSO	GC correction, mappability
EXCAVATOR	2013	Perl, R	Both	HSLM	GC correction, mappability, exon-size correction
ExomeCNV	2011	R	Paired	CBS	GC correction, mappability
VarScan2	2012	Java, Perl, R	Paired	CBS	GC correction

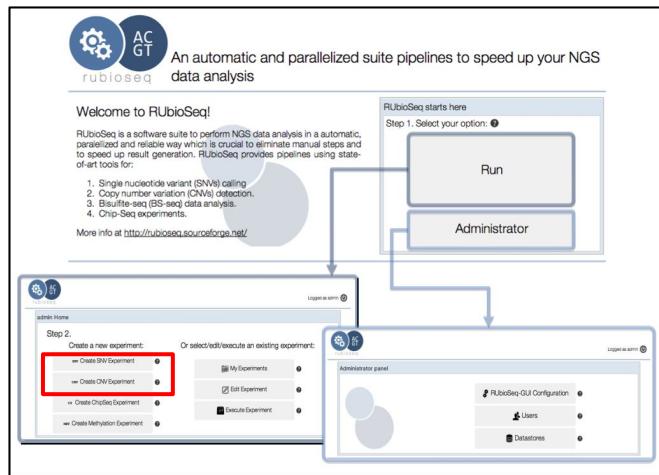
Only WGS
Appropriate methods for Whole-Exome seq

Further reading:

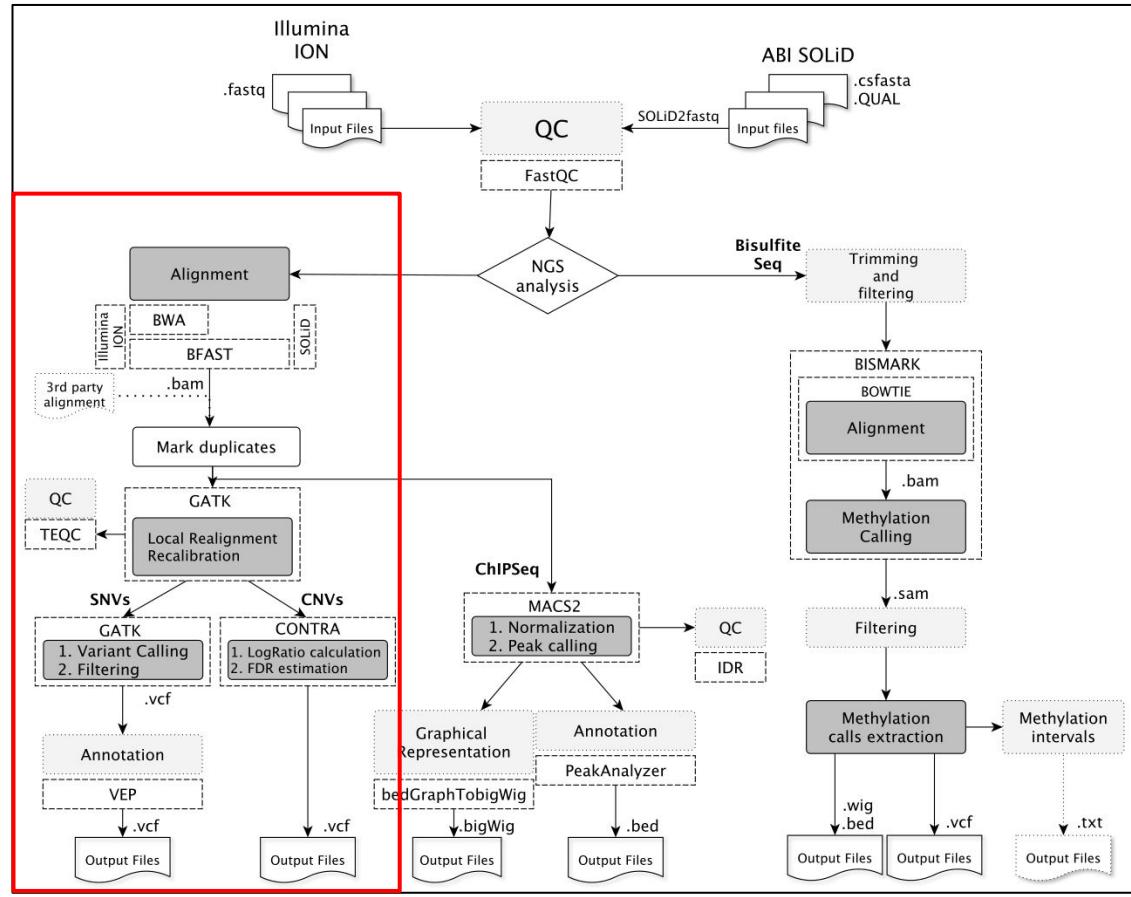
Ding, L., Wendl, M. C., McMichael, J. F. & Raphael, B. J. Nat Rev Genet – (2014). doi:10.1038/nrg3767

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

Our proposed Variant Calling Pipeline



Graphic User Interface



workflow schema

Developed by the **Bioinformatics Unit** at the **Spanish National Cancer Research Centre** (Madrid, Spain).
Paper: Rubio-Camarillo et al. Bioinformatics (2013) 29 (13), 1687-1689

What is Crucial in Variant calling

- For clinical practices, the use of **gold standard methods** and **reproducible analysis** are mandatory.
- The analysis is based on the comparison against the **reference genome** :
A single consensus sequence for the whole genome. It was built up from a high quality set of representative samples of the specie (from different populations). It is the first-line comparison during analysis.

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

- **Human assemblies (Versions):**
 - + **GRCh37/hg19** : former version. Released in 2012. It is still the preference for analysis. Last 1000 Genome Project's paper was published based on GRCh37/hg19.
 - + **CRCh38/hg38** : current version (Sep. 2016). Released in 2014. More accurate, comprehensive (includes Haplotypes) and sophisticated.

“CRCh38 is here now, but still waiting.”

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

Bundle of files for Variant Detection

1. **Genome Reference** (standard 1000 Genomes, fasta).
2. List of **Target beats or intervals** of genomic regions sequenced by the Library protocol.
3. **dbSNP** (VCF file) for a recent dbSNP release (build 138, it includes the 1000 Genomes).
4. HapMap genotypes and sites VCFs
5. **OMNI 2.5 genotypes for 1000 Genomes samples** (VCF).
6. The current best set of **known indels** to be used for local realignment); use both files:
 - 1000G_phase1.indels.b37.vcf (currently from the 1000 Genomes Phase I indel calls)
 - Mills_and_1000G_gold_standard.indels.b37.sites.vcf

Q: How you can get this bundle of files?

A: you could get them from the **Broad Institute's FTP**

<ftp://gsapubftp-anonymous@ftp.broadinstitute.org/bundle/2.8/>

You also need the Intervals file from your NGS provider (Illumina, Ion Torrent,...)

For this workshop, we got these files for you.

More info.:

<http://gatkforums.broadinstitute.org/discussion/1213/whats-in-the-resource-bundle-and-how-can-i-get-it>

Bundle from the GATK's repository



ftp://ftp.broadinstitute.org/bundle/2.8/hg19/

Index of /bundle/2.8/hg19/

Name	Size	Date Modified
[parent directory]		
1000G_omni2.5.hg19.sites.vcf.gz	49.4 MB	12/8/13, 1:00:00 AM
1000G_omni2.5.hg19.sites.vcf.gz.md5	97 B	12/8/13, 1:00:00 AM
1000G_omni2.5.hg19.sites.vcf.idx.gz	464 kB	12/8/13, 1:00:00 AM
1000G_omni2.5.hg19.sites.vcf.idx.gz.md5	101 B	12/8/13, 1:00:00 AM
1000G_phase1.indels.hg19.sites.vcf.gz	42.9 MB	12/8/13, 1:00:00 AM
1000G_phase1.indels.hg19.sites.vcf.gz.md5	103 B	12/8/13, 1:00:00 AM
1000G_phase1.indels.hg19.sites.vcf.idx.gz	326 kB	12/8/13, 1:00:00 AM
1000G_phase1.indels.hg19.sites.vcf.idx.gz.md5	107 B	12/8/13, 1:00:00 AM
1000G_phase1.snp.high_confidence.hg19.sites.vcf.gz	1.7 GB	12/8/13, 1:00:00 AM
1000G_phase1.snp.high_confidence.hg19.sites.vcf.gz.md5	117 B	12/8/13, 1:00:00 AM
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1000G_phase1.snp.high_confidence.hg19.sites.vcf.idx.gz.md5	121 B	12/8/13, 1:00:00 AM
CEUTrio.HiSeq.WGS.b37.bestPractices.hg19.vcf.gz	407 MB	12/8/13, 1:00:00 AM
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CEUTrio.HiSeq.WGS.b37.bestPractices.hg19.vcf.idx.gz	3.2 MB	12/8/13, 1:00:00 AM
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dbSNP_138.hg19_excluding_sites_after_129.vcf.gz	334 MB	12/8/13, 1:00:00 AM
dbSNP_138.hg19_excluding_sites_after_129.vcf.gz.md5	119 B	12/8/13, 1:00:00 AM
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dbSNP_138.hg19.vcf.gz	1.4 GB	12/8/13, 1:00:00 AM
dbSNP_138.hg19.vcf.gz.md5	93 B	12/8/13, 1:00:00 AM
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hapmap_3.3.hg19.sites.vcf.gz.md5	94 B	12/8/13, 1:00:00 AM
hapmap_3.3.hg19.sites.vcf.idx.gz	807 kB	12/8/13, 1:00:00 AM
hapmap_3.3.hg19.sites.vcf.idx.gz.md5	98 B	12/8/13, 1:00:00 AM

Everything you need for the variant calling is stored in the FTP of the GATK team.

It includes the **human genome reference** too!

There are independent bundles for each assembly of the human genome (hg19, hg38,etc). The difference across them is the coordinate system for the annotations, but it is the same data.

You can download the different bundles from GATK's FTP (Broad Institute) visiting this URL with your Internet Browser:

<ftp://gsapubftp-anonymous@ftp.broadinstitute.org/bundle/2.8/>



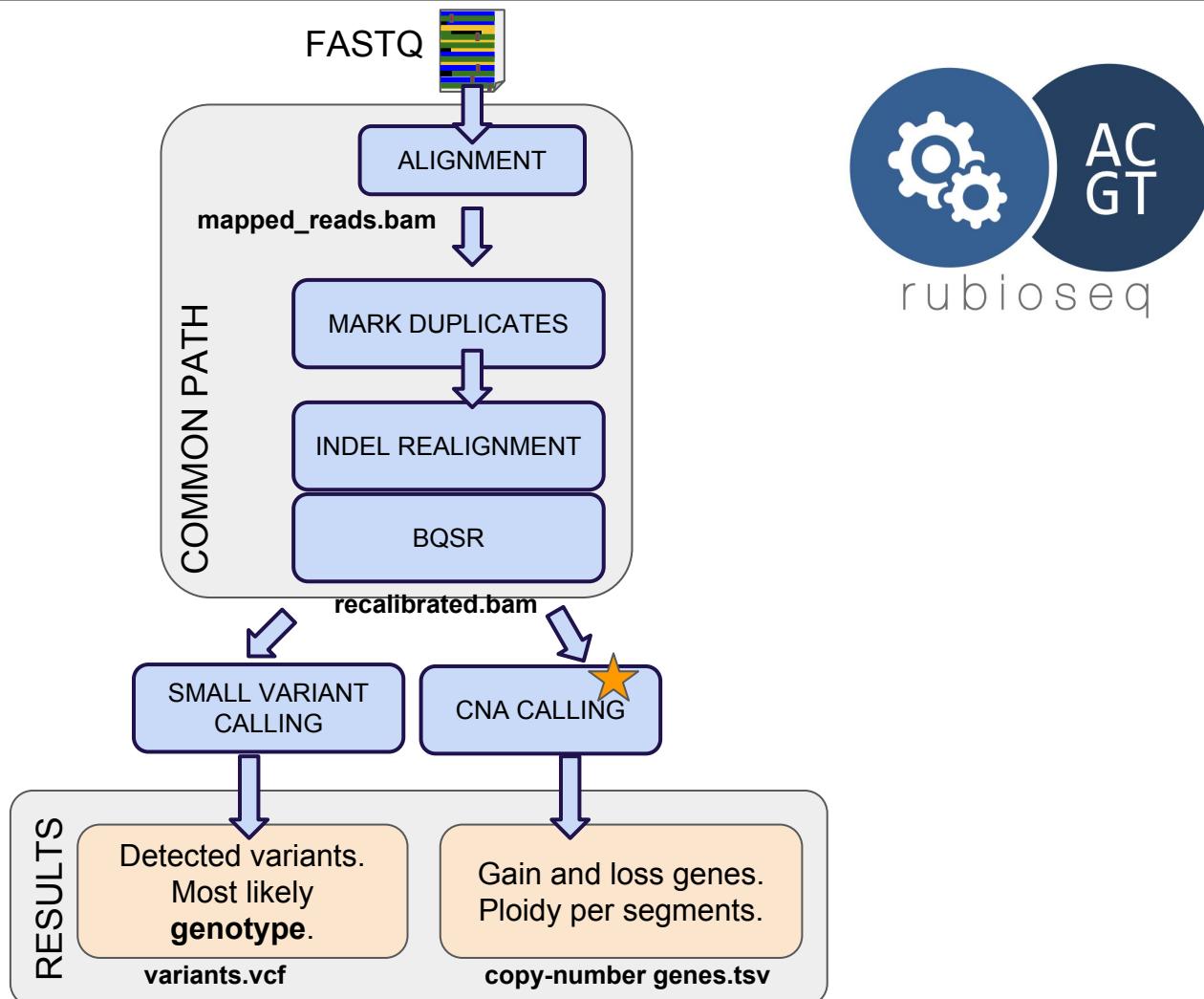
Tip for home: The following UNIX command downloads the whole bundle for **hg19** in one step (~hrs) :

```
$ wget -r -nH --cut-dirs=3 --reject-regex "NA12878|CEUTrio" \
-P /path/to/your_directory/ ftp://gsapubftp-anonymous@ftp.broadinstitute.org/bundle/2.8/hg19/*
```

More info.:

<http://gatkforums.broadinstitute.org/discussion/1213/whats-in-the-resource-bundle-and-how-can-i-get-it>

Point mutations and CNV Calling Process

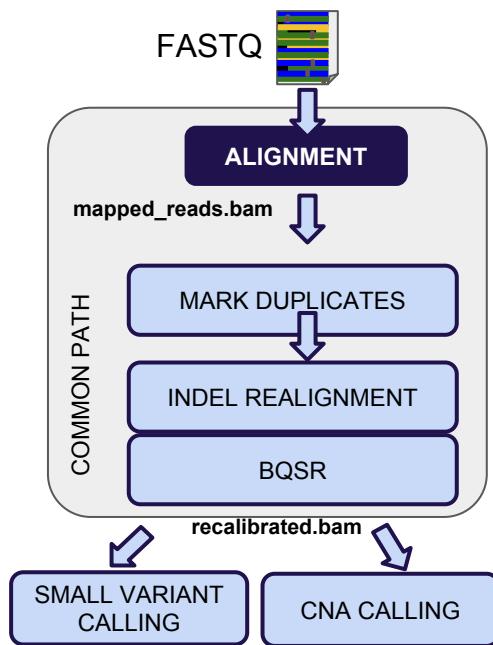


★ It requires **tumour and matched normal sample (or a panel of normals)**

Variant Calling Pipeline

1. Alignment

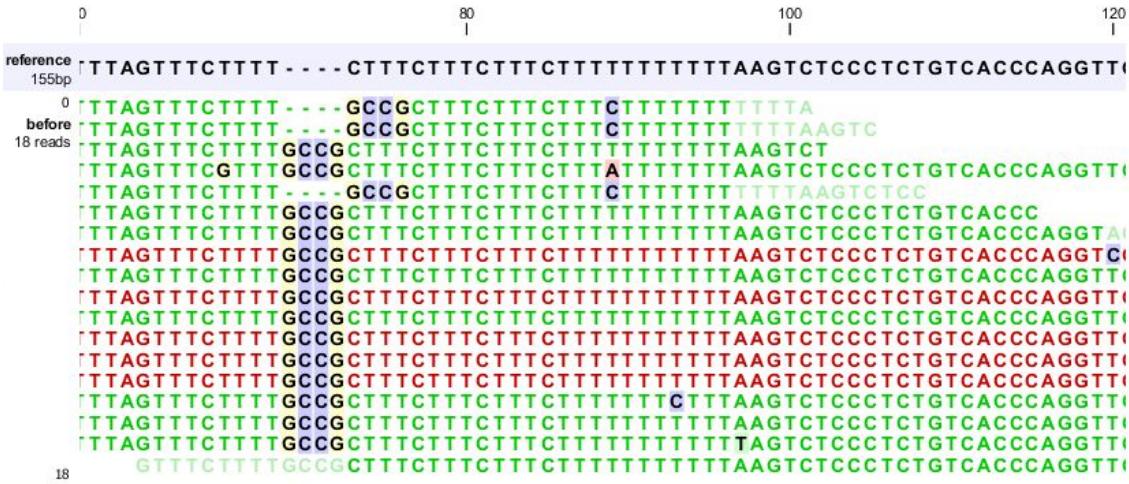
WORKFLOW:



METHOD: by BWA & Bfast+BWA

<https://github.com/lh3/bwa#citing-bwa>

<http://sourceforge.net/projects/bfast/files/bfast%2Bbwa/>

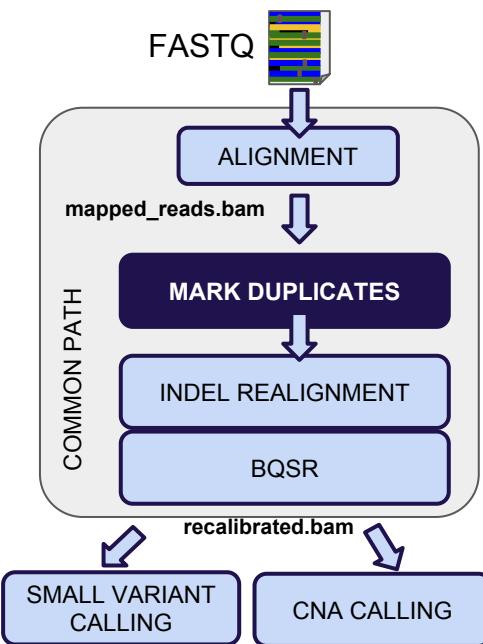


- Fast mapping on the reference genome by creating indexes. It is computationally intensive, but it is done only once.
- Search for candidate sites to align a given read by using seeds (fragments of a read).

Variant Calling Pipeline

2. Mark duplicates

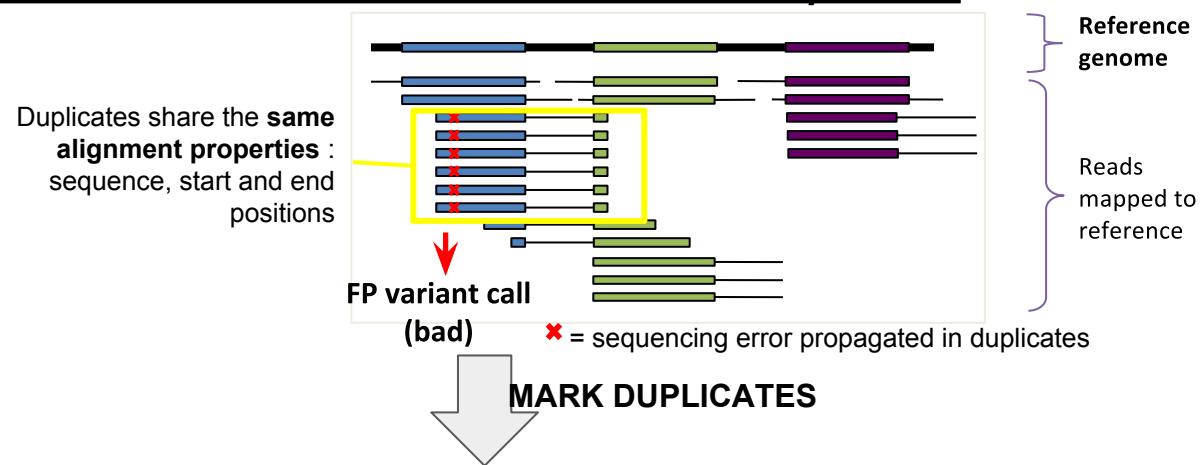
WORKFLOW:



Under the hood:

- Duplicates belong from PCR amplification (library preparation): one fragment is sequenced multiple times.
- An error at the beginning of the PCR (first steps) is propagated.
- Therefore, duplicates are **worthless** for the analysis:
Duplicates are source of False Positives calls while only provide redundancy.

Solution: retrieve the best one, discard the duplicates:



METHOD: by Picard-tools

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

(alternatives : samtools)

Adapted from GATK

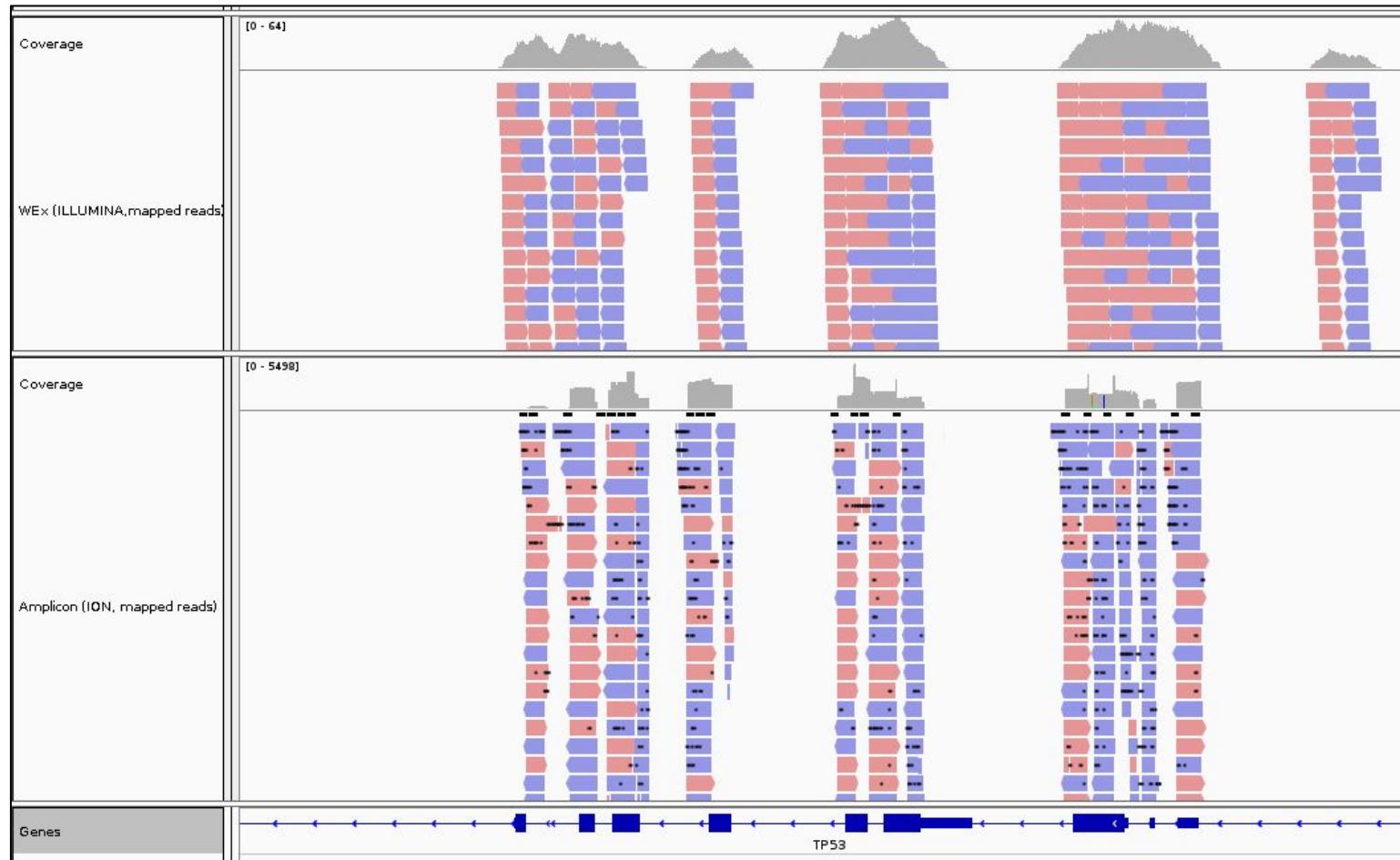
After marking duplicates, the variant caller will only see :



... and thus be more likely to make the right call

Variant Calling Pipeline

2. Mark duplicates: WEx Vs. Amplicon



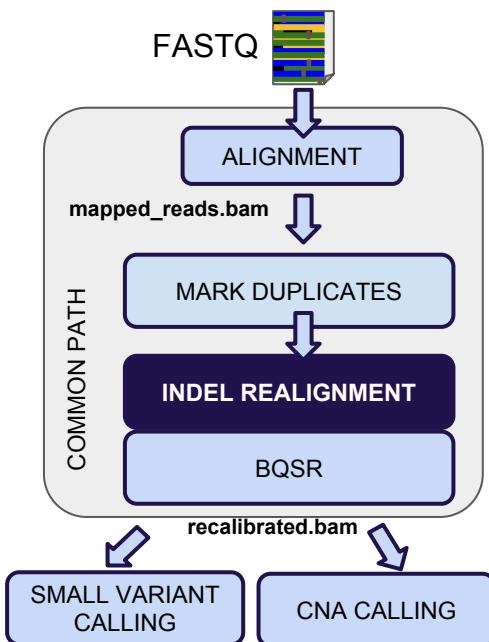
WARNING: Do NOT mark duplicates in data derived from amplicon approach techniques (Ion Torrent**).**

More info.: <http://gatkforums.broadinstitute.org/discussion/5847/remove-duplicates-from-targetted-sequencing-using-amplicon-approach>

Variant Calling Pipeline

3. Indel realignment

WORKFLOW:



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

During alignment, penalties on mismatches are much cheaper than gaps (indels). Aligners will tend to choose Mismatches at the beginning, and locate indels in the rest.

Also, there are sometimes multiple solution (alignments) for a given read. Aligners choose one randomly.

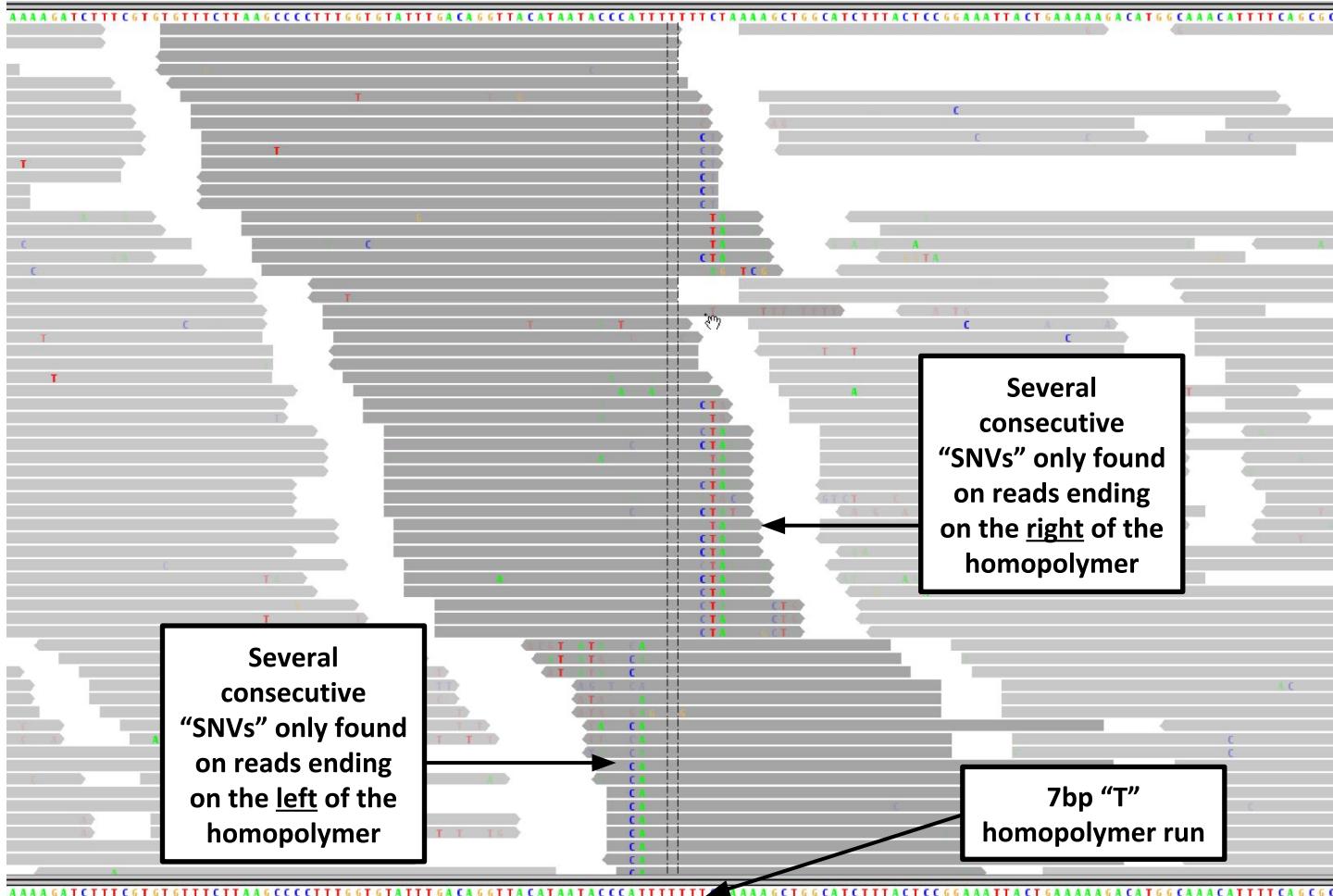
Variant calling requires the most perfect alignment as possible to avoid False Positives.

METHOD: by GATK

https://www.broadinstitute.org/gatk/gatkdocs/org_broadinstitute_gatk_tools_walkers_indels_IndelRealigner.php

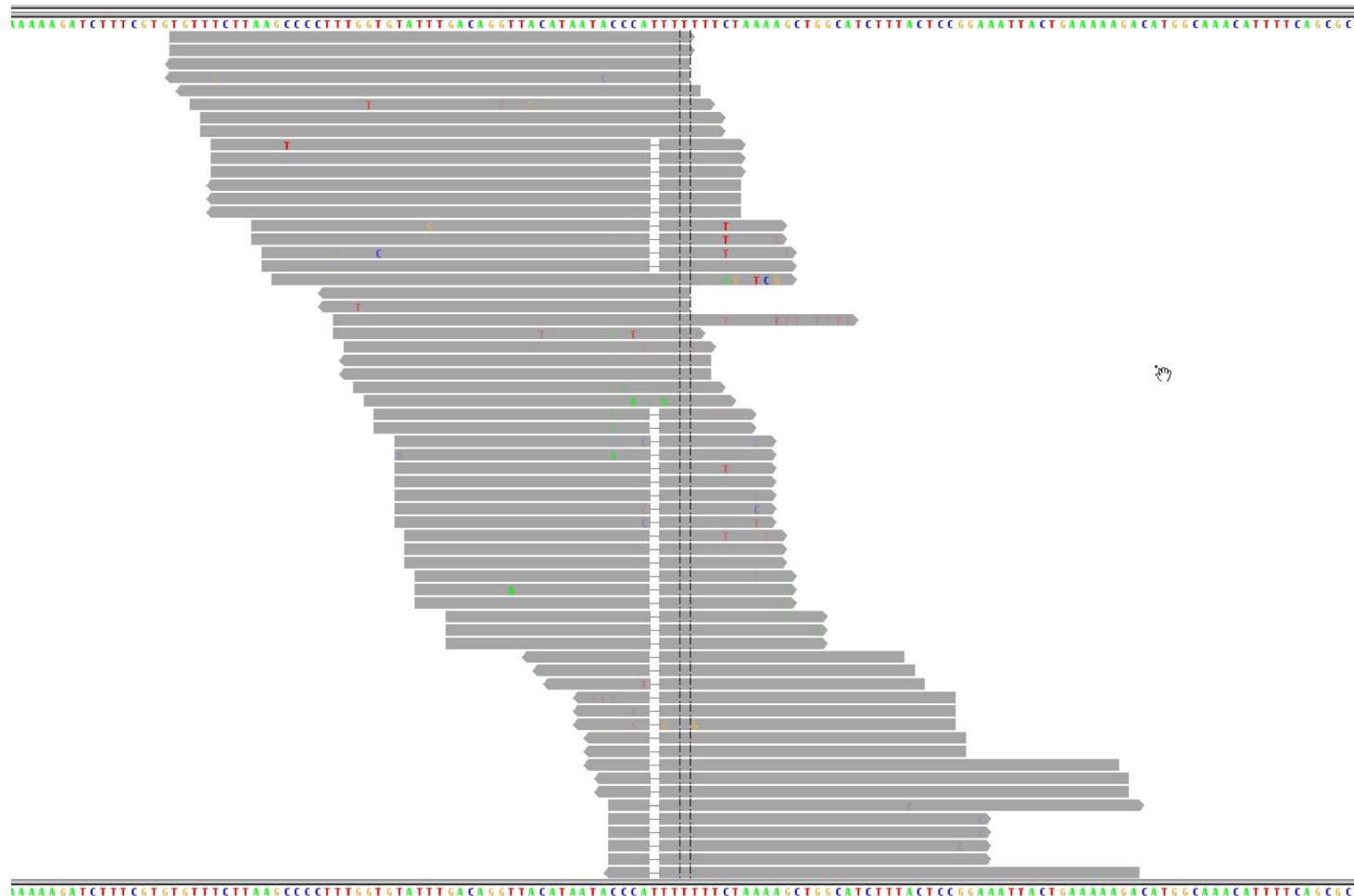
Variant Calling Pipeline

3. Indel realignment



Taken from GATK team

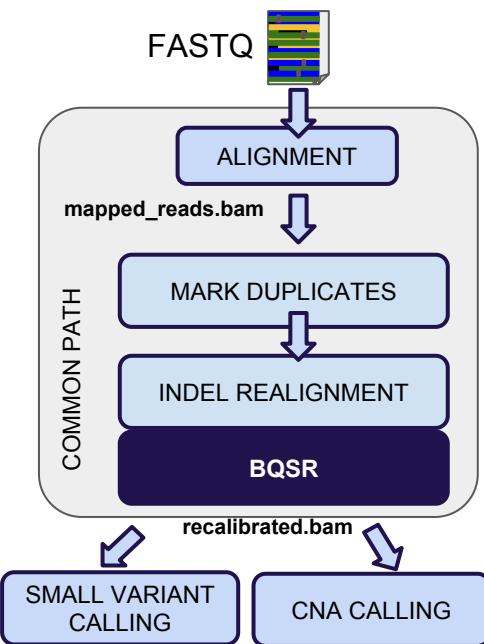
3. Indel realignment



Taken from GATK team

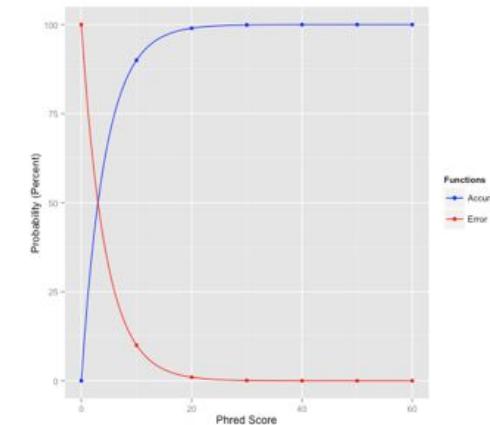
4. Base Quality Score Recalibration

WORKFLOW:

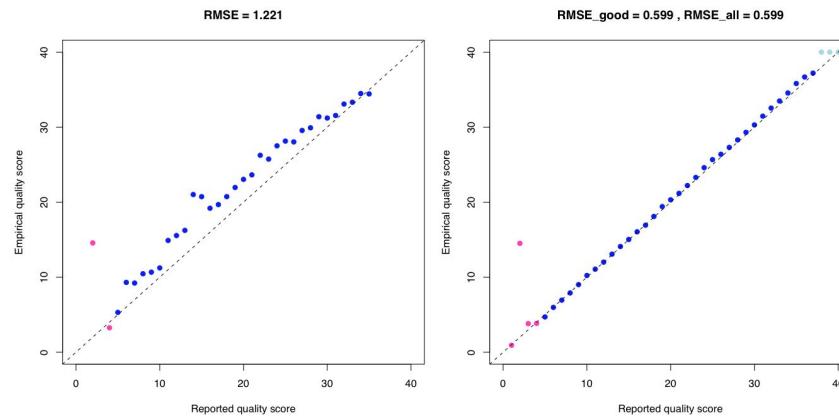


Phred Quality : each position of the sequence has its particular **base Quality score**.

The individual quality measures are NOT very important during the alignment step (mapping), but crucial during Variant calling.



Different NGS technologies have their particular bias in QS depending on the context. **Machine learning techniques** could correct **empirically** these biases.



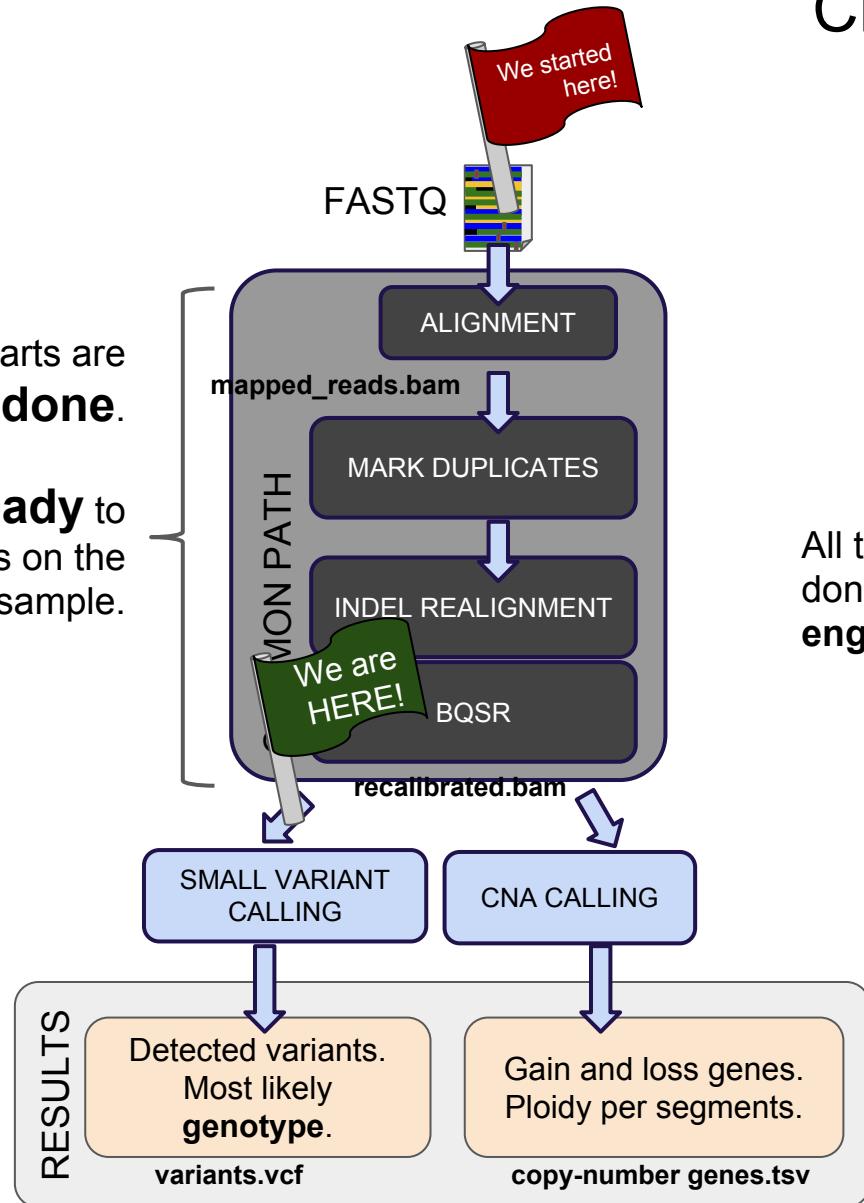
METHOD: by GATK

<http://gatkforums.broadinstitute.org/discussion/44/base-quality-score-recalibration-bqsr>

Point mutations and CNV Calling Process



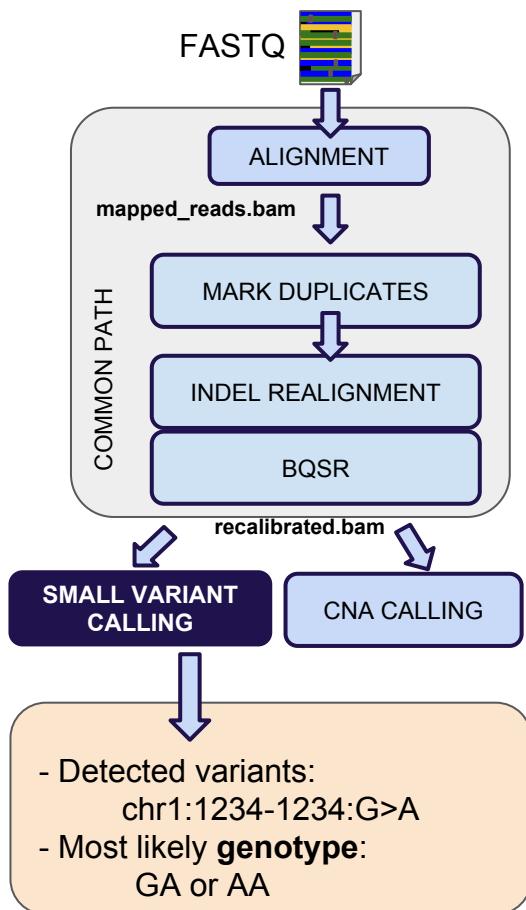
The first two parts are **done**.
We are **ready** to discover variants on the sample.



All these steps are **automatically** done by the pipeline (**R**UbioSeq's **e**ngine).

5. GATK Variant Calling Process : SNV & Indels

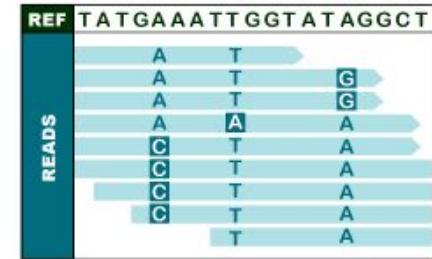
WORKFLOW:



Assumptions:

- Diploid genome (2n).

Limitation: Allele freq > 10%.

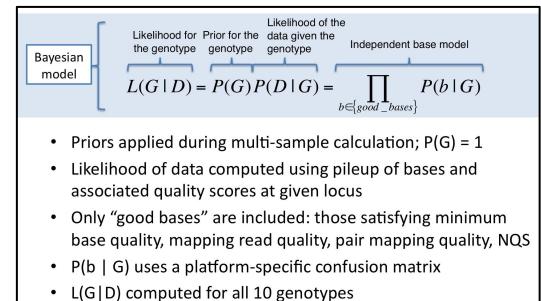


1. Identification of **ActiveRegions**.

2. Variant calling based on the calculation of genotype likelihoods. GATK have had two callers:

- **Unified Genotyper** (old, not longer used): It is included in **RUBioSeq v3.7**. We will work using this caller.
pros: Faster, good with SNV calls | **cons:** retired, many Indel FP

- **Haplotype Caller** (new, improved): It is included in **RUBioSeq v3.8** (beta, coming soon).
pros: Better with indels calls | **cons:** really computationally intensive



Further reading:

<http://gatkforums.broadinstitute.org/discussion/1237/how-unified-genotyper-works-retired>
<http://gatkforums.broadinstitute.org/discussion/4148/hc-overview-how-the-haplotypecaller-works>

GATK is in active development

 <https://www.broadinstitute.org/gatk/>

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The Genome Analysis Toolkit or GATK is a software package for analysis of high-throughput sequencing data, developed by the [Data Science and Data Engineering](#) group at the [Broad Institute](#). The toolkit offers a wide variety of tools, with a primary focus on variant discovery and genotyping as well as strong emphasis on data quality assurance. Its robust architecture, powerful processing engine and high-performance computing features make it capable of taking on projects of any size.

[Learn more »](#)



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GATK is in active development

<https://www.broadinstitute.org/gatk/>

The GATK is a toolkit for high-throughput sequencing analysis, developed at the Broad Institute. The toolkit offers a wide variety of tools for variant discovery and analysis, with a strong emphasis on data quality and performance.

GATK Best Practices

Recommended workflows for variant discovery analysis with GATK

What can you do with this?

The GATK Best Practices workflows provide step-by-step recommendations for performing variant discovery analysis in high-throughput sequencing (HTS) data. They enable discovery of SNPs and small indels (no size limit in theory but adjustments may be required to call indels > 50 bp) in DNA and RNAseq. They do not yet enable discovery of structural variants (SVs) or copy number variants (CNVs). Although originally designed for human genome research, the GATK Best Practices are widely used (with adaptations as described in the documentation) for analysis of non-human organisms of all kinds, including non-diploids.

Break it down

We currently have two separate workflows for Germline DNA and for RNAseq, and we are developing a workflow for Somatic DNA. All of them are divided into three sequential phases:

1. PRE-PROCESSING
Pre-processing starts from raw sequence data, either in FASTQ or uBAM format, and produces analysis-ready BAM files. Processing steps include alignment to a reference genome as well as some data cleanup operations to correct for technical biases and make the data suitable for analysis.

2. VARIANT DISCOVERY
Variant Discovery starts from analysis-ready BAM files and produces a callset in VCF format. Processing involves identifying sites where one or more individuals display possible genomic variation, and applying filtering methods appropriate to the experimental design.

3. CALLSET REFINEMENT
Callset Refinement starts and ends with a VCF callset. Processing involves using meta-data to correct for known sequencing artifacts.

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GATK Support Forum

Announcements
Announcements regarding new tools and features, as well as user support service and forum features

Ask the GATK team
Errors, bugs, problems and usage questions for the developers of the GATK or the community at large

GATK Documentation Guide
This is an exact mirror of the GATK website Guide pages, with the added possibility of commenting on the documentation articles.

Cancer Tools
Tools developed by the Broad Institute for cancer research.

Third-party Tools
Tools built on top of GATK.

Ask the GATK team
Errors, bugs, problems and usage questions for the developers of the GATK or the community at large

Gziped gVCF files
Tools built on top of GATK.

In a VCF file, when DP=0, why it stills give a Genotype?
Answered ✓ 27 views 4 comments Most recent by mike_boursnell 10:31AM

About variant calling of single sample ..
Question 5 views 0 comments Started by namcheolkim 2:41AM

GATK HaplotypeCaller run
Answered 215 views 7 comments Most recent by Geraldine_VdAuwera November 23

How can I use command to genotype refinement of population priors(no family groups)
Answered 22 views 3 comments Most recent by namcheolkim November 23

Variant calling issue
39 views 3 comments Most recent by Geraldine_VdAuwera November 23

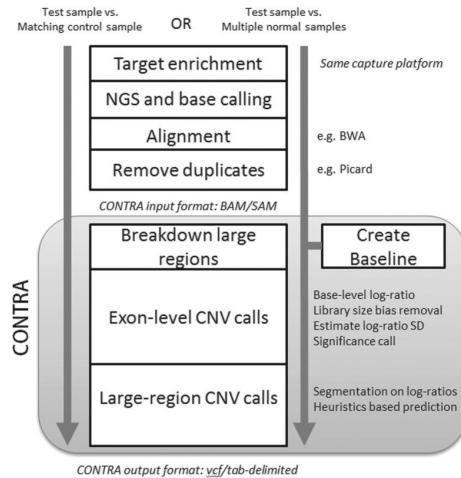
GenotypeGVCF removes PL field from output

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PM16 @GTPB

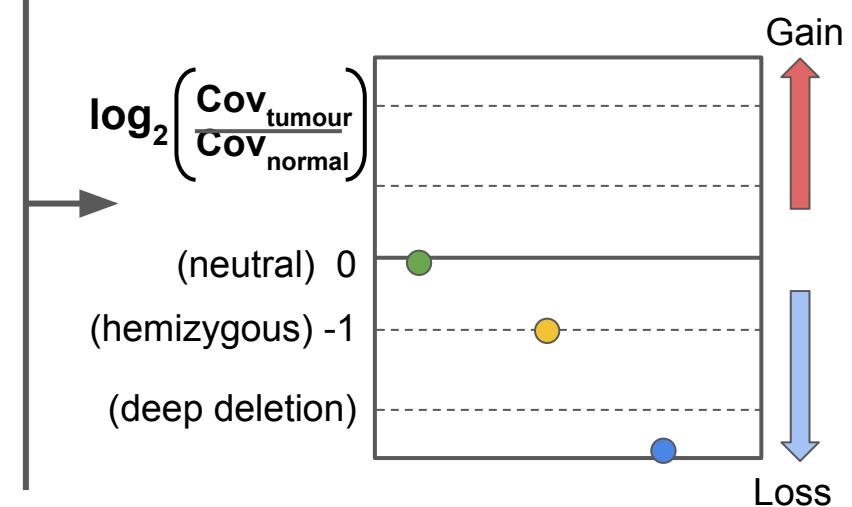
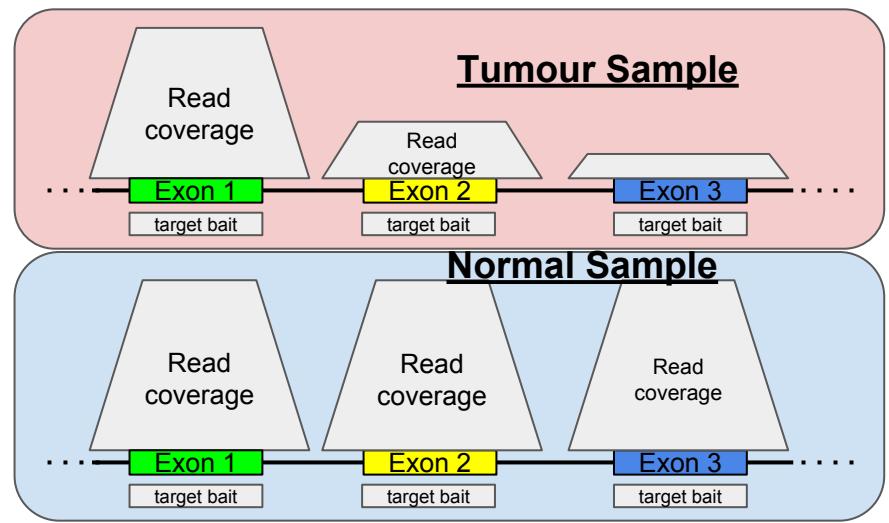
Day #1 - NGS I : Variant Detection

6. CNA Variant Calling



- **Normalization:** Split large regions. GC-content bias, unbalanced library size effect on log-ratios.
- Read-depth coverage & log₂ CN ratio are corrected.
- Significance:
Assumption: log₂-transformed coverage fits a normal distribution:

$RLR \sim N(\mu_d, \sigma_d)$; Two-tailed P-value.
multiple testing correction (FDR).



Li J et al. CONTRA: copy number analysis for targeted resequencing. (2012) Bioinformatics

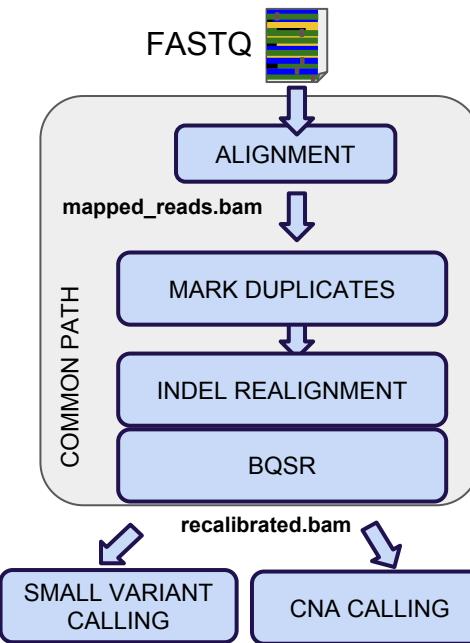
● What have we learnt?

Main concepts:

- Variants.
- How to detect them.
- Differences between platforms.

Requirements:

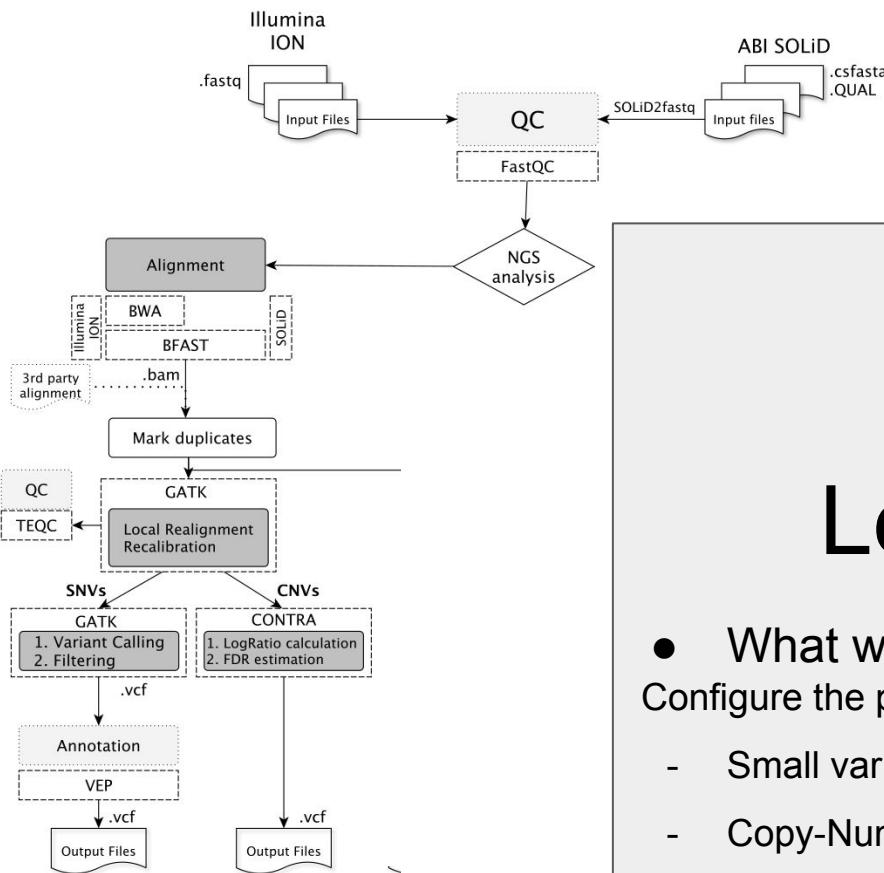
- Files.
- Methods



● Questions?

Thanks to Miriam Rubio-Camarillo & Gonzalo Gómez for the support and development of RUBioSeq.

First hands-on :: Configure RUbioSeq+ Configuration files



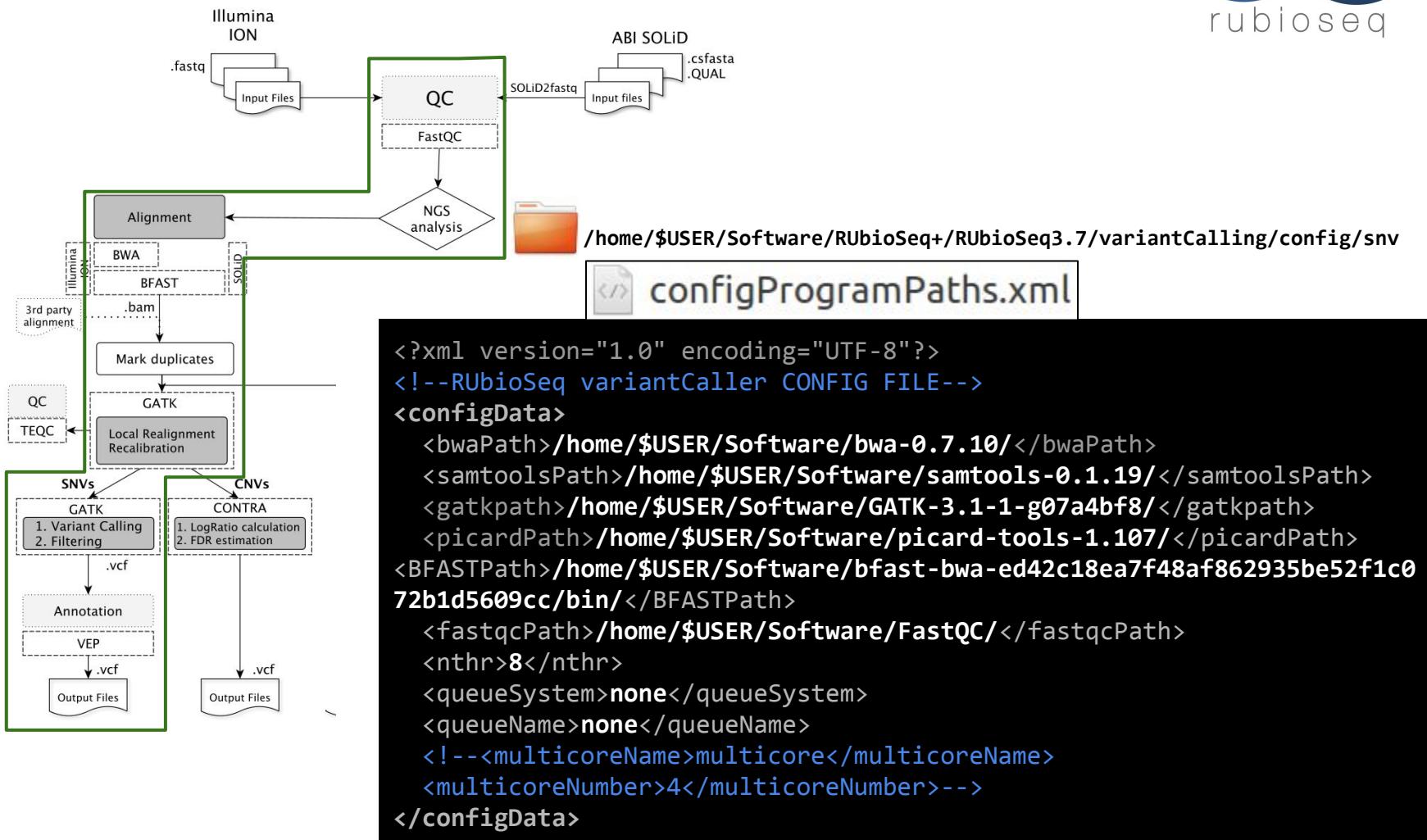
It is already installed.
Let's configure it!

- What will we do?

Configure the pipeline for the detection of:

- Small variants (SNV + indels)
- Copy-Number Variants

First hands-on :: Configure RUBioSeq+ Configuration for Small variant analysis



First hands-on :: Configure RUBioSeq+ Configuration for CNV analysis

