FACULTAD DE MEDICINA
UNIVERSIDAD DE CHILE

GENOMED-Lab
http://genomed.med.uchile.cl

ICBM
INSTITUTO DE CIENCIAS BIOMÉDICAS

Análisis de datos NGS:
Llamado de variantes

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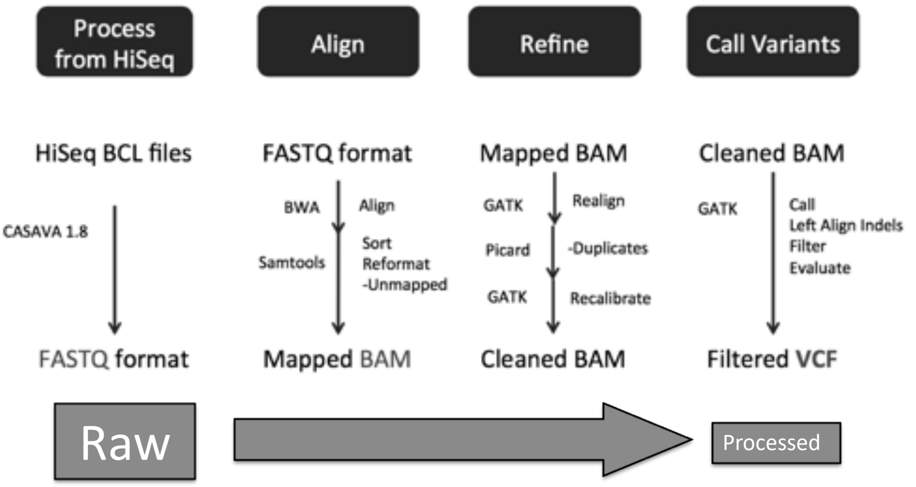
NGS data files



Process from HiSeq

Align

Refine

Call Variants

HiSeq BCL files

CASAVA 1.8

FASTQ format

FASTQ format

BWA Align

Samtools Sort Reformat -Unmapped

Mapped BAM

Mapped BAM

GATK Realign

Picard -Duplicates

GATK Recalibrate

Cleaned BAM

Cleaned BAM

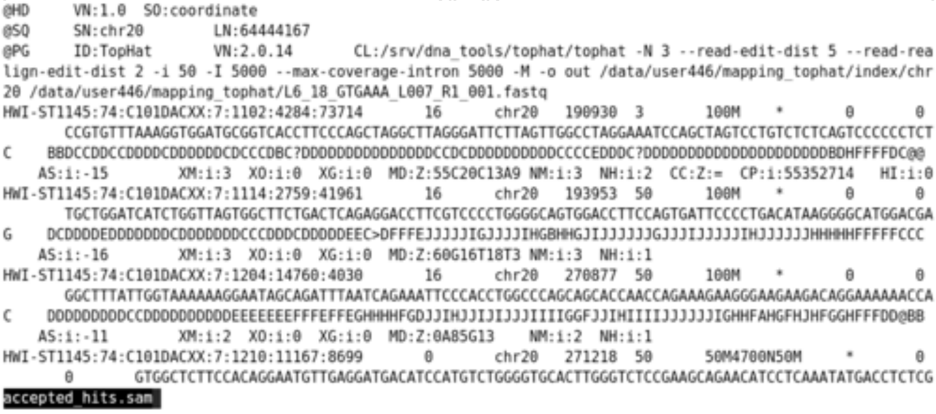
GATK Call Left Align Indels Filter Evaluate

Filtered VCF

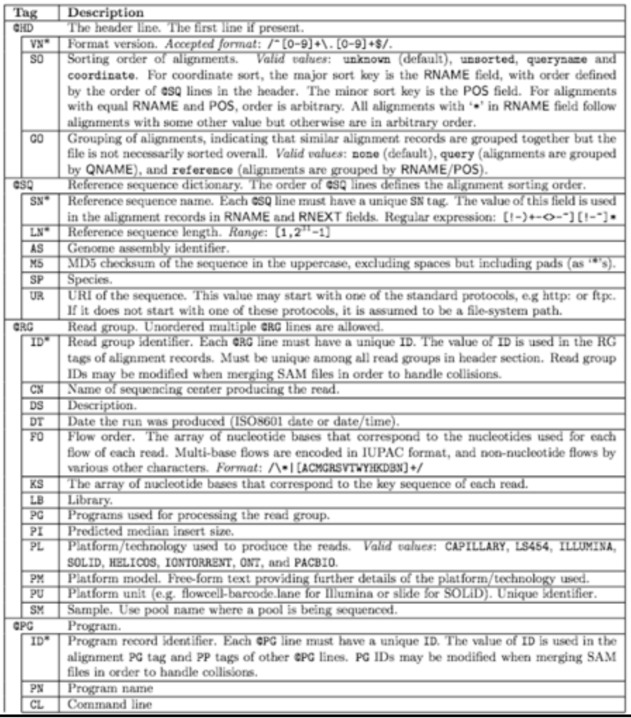
Raw

Processed

SAM file



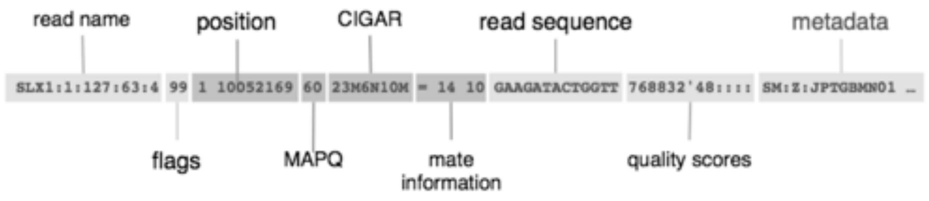
```
@HD     VN:1.0  SO:coordinate
@SQ     SN:chr20        LN:64444167
@PG     ID:TopHat       VN:2.0.14       CL:/srv/dna_tools/tophat/tophat -N 3 --read-edit-dist 5 --read-rea
lign-edit-dist 2 -i 50 -I 5000 --max-coverage-intron 5000 -M -o out /data/user446/mapping_tophat/index/chr
20 /data/user446/mapping_tophat/L6_18_GTGAAA_L007_R1_001.fastq
HWI-ST1145:74:C101DACXX:7:1102:4284:73714       16      chr20   190930  3       100M    *       0       0
        CCGTGTTTAAAGGTGGATGCGGTCACCTTCCCAGCTAGGCTTAGGGATTCTTAGTTGGCCTAGGAAATCCAGCTAGTCCTGTCTCTCAGTCCCCCCTCT
C       BBDCCDDCCDDDDCDDDDDDCDCCCDBC?DDDDDDDDDDDDDDDCCDCDDDDDDDDDDCCCCEDDDC?DDDDDDDDDDDDDDDDDDDDDDBDHFFFFDC@@
    AS:i:-15        XM:i:3  XO:i:0  XG:i:0  MD:Z:55C20C13A9 NM:i:3  NH:i:2  CC:Z:=  CP:i:55352714   HI:i:0
HWI-ST1145:74:C101DACXX:7:1114:2759:41961       16      chr20   193953  50      100M    *       0       0
        TGCTGGATCATCTGGTTAGTGGCTTCTGACTCAGAGGACCTTCGTCCCCTGGGGCAGTGGACCTTCCAGTGATTCCCCTGACATAAGGGGCATGGACGA
G       DCDDDDEDDDDDDDCDDDDDDDCCCDDDCDDDDDEEC>DFFFEJJJJJIGJJJJIHGBHHGJIJJJJJJGJJJIJJJJJIHJJJJJJHHHHHFFFFFCCC
    AS:i:-16        XM:i:3  XO:i:0  XG:i:0  MD:Z:60G16T18T3 NM:i:3  NH:i:1
HWI-ST1145:74:C101DACXX:7:1204:14760:4030       16      chr20   270877  50      100M    *       0       0
        GGCTTTATTGGTAAAAAAGGAATAGCAGATTTAATCAGAAATTCCCACCTGGCCCAGCAGCACCAACCAGAAAGAAGGGAAGAAGACAGGAAAAAACCA
C       DDDDDDDDDCCDDDDDDDDDDEEEEEEEEFFFEFFEGHHHHFGDJJIHJJIJIJJJIIIIGGFJJIHIIIIJJJJJJIGHHFAHGFHJHFGGHFFFDD@BB
    AS:i:-11        XM:i:2  XO:i:0  XG:i:0  MD:Z:0A85G13    NM:i:2  NH:i:1
HWI-ST1145:74:C101DACXX:7:1210:11167:8699       0       chr20   271218  50      50M4700N50M     *       0
        0       GTGGCTCTTCCACAGGAATGTTGAGGATGACATCCATGTCTGGGGTGCACTTGGGTCTCCGAAGCAGAACATCCTCAAATATGACCTCTCG
accepted_hits.sam
```



Tag	Description
@HD	The header line. The first line if present.
VN*	Format version. *Accepted format*: /^[0-9]+\.[0-9]+$/.
SO	Sorting order of alignments. *Valid values*: **unknown** (default), **unsorted**, **queryname** and **coordinate**. For coordinate sort, the major sort key is the RNAME field, with order defined by the order of @SQ lines in the header. The minor sort key is the POS field. For alignments with equal RNAME and POS, order is arbitrary. All alignments with '*' in RNAME field follow alignments with some other value but otherwise are in arbitrary order.
GO	Grouping of alignments, indicating that similar alignment records are grouped together but the file is not necessarily sorted overall. *Valid values*: **none** (default), **query** (alignments are grouped by QNAME), and **reference** (alignments are grouped by RNAME/POS).
@SQ	Reference sequence dictionary. The order of @SQ lines defines the alignment sorting order.
SN*	Reference sequence name. Each @SQ line must have a unique SN tag. The value of this field is used in the alignment records in RNAME and RNEXT fields. Regular expression: [!-)+-<>-~][!-~]*
LN*	Reference sequence length. *Range*: $[1,2^{31}-1]$
AS	Genome assembly identifier.
M5	MD5 checksum of the sequence in the uppercase, excluding spaces but including pads (as '*'s).
SP	Species.
UR	URI of the sequence. This value may start with one of the standard protocols, e.g http: or ftp:. If it does not start with one of these protocols, it is assumed to be a file-system path.
@RG	Read group. Unordered multiple @RG lines are allowed.
ID*	Read group identifier. Each @RG line must have a unique ID. The value of ID is used in the RG tags of alignment records. Must be unique among all read groups in header section. Read group IDs may be modified when merging SAM files in order to handle collisions.
CN	Name of sequencing center producing the read.
DS	Description.
DT	Date the run was produced (ISO8601 date or date/time).
FO	Flow order. The array of nucleotide bases that correspond to the nucleotides used for each flow of each read. Multi-base flows are encoded in IUPAC format, and non-nucleotide flows by various other characters. *Format*: /*|[ACMGRSVTWYHKDBN]+/
KS	The array of nucleotide bases that correspond to the key sequence of each read.
LB	Library.
PG	Programs used for processing the read group.
PI	Predicted median insert size.
PL	Platform/technology used to produce the reads. *Valid values*: **CAPILLARY**, **LS454**, **ILLUMINA**, **SOLID**, **HELICOS**, **IONTORRENT**, **ONT**, and **PACBIO**.
PM	Platform model. Free-form text providing further details of the platform/technology used.
PU	Platform unit (e.g. flowcell-barcode.lane for Illumina or slide for SOLiD). Unique identifier.
SM	Sample. Use pool name where a pool is being sequenced.
@PG	Program.
ID*	Program record identifier. Each @PG line must have a unique ID. The value of ID is used in the alignment PG tag and PP tags of other @PG lines. PG IDs may be modified when merging SAM files in order to handle collisions.
PN	Program name
CL	Command line

Archivo SAM: registros

HEADER containing metadata (sequence dictionary, read group definitions etc)
RECORDS containing structured read information (1 line per read record)

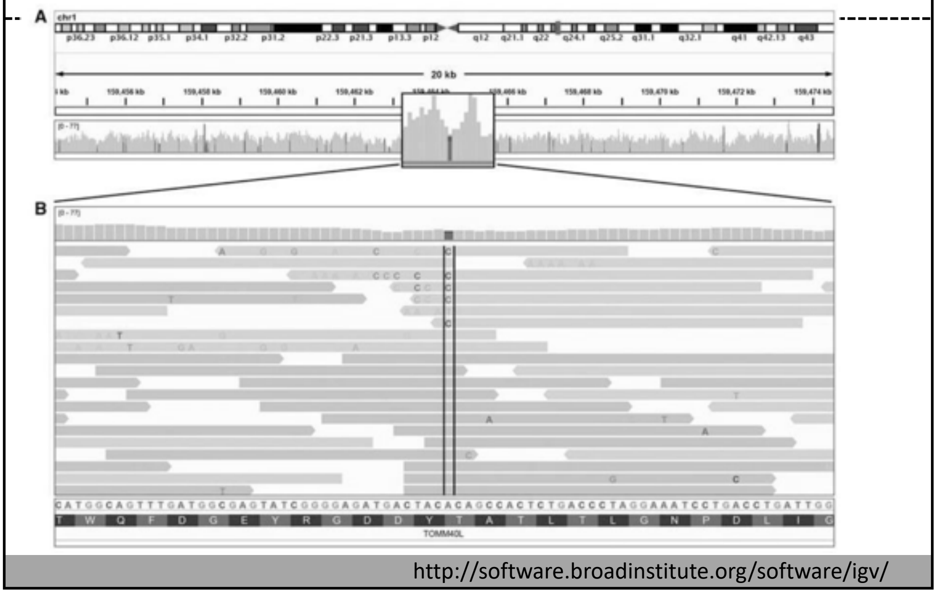


```
SLX1:1:127:63:4 99 1 10052169 60 23M6N10M = 14 10 GAAGATACTGGTT 768832'48:::: SM:Z:JPTGBMN01 …
```

CIGAR

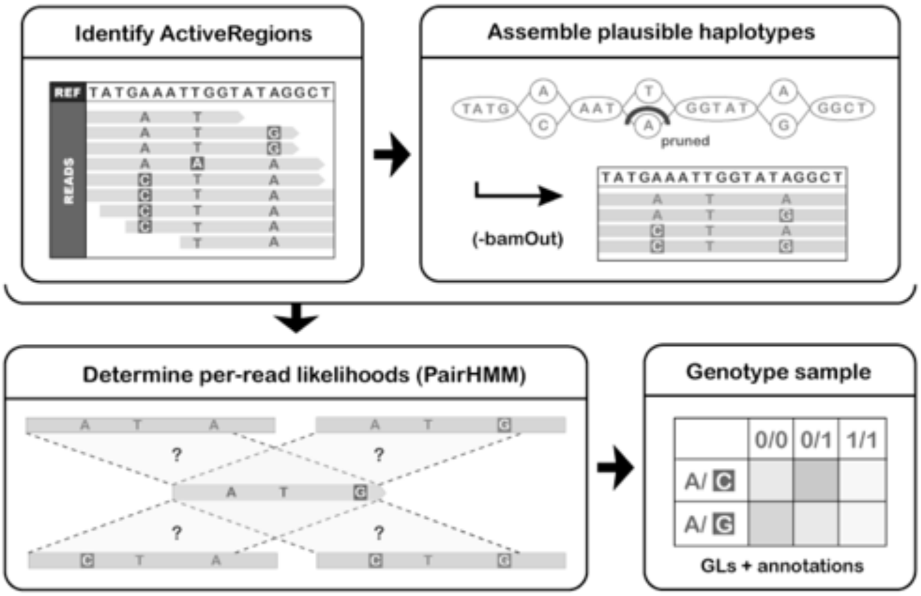
```
RefPos:      1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T  G  A  A  C  T  G  A  C  T  A  A  C
Read:        ACTAGAATGGCT

Aligning these two:
RefPos:      1  2  3  4  5  6  7     8  9 10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T     G  A  A  C  T  G  A  C  T  A  A  C
Read:                    A  C  T  A  G  A  A     T  G  G  C  T

With the alignment above, you get:
POS: 5 CIGAR: 3M1I3M1D5M
```

Integrative Genomic Viewer

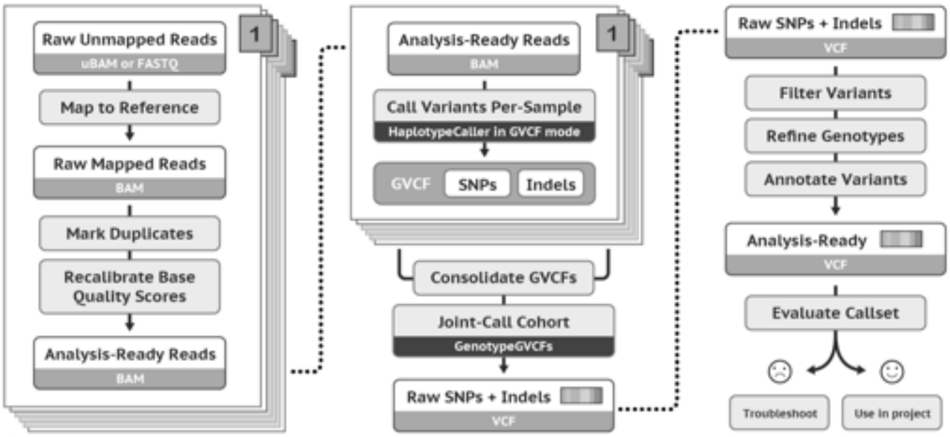


http://software.broadinstitute.org/software/igv/

Llamado de Variantes: GATK HaplotypeCaller



GATK Best Practices workflow for SNP and Indel discovery in germline DNA



1

Raw Unmapped Reads

uBAM or FASTQ

Map to Reference

Raw Mapped Reads

BAM

Mark Duplicates

Recalibrate Base Quality Scores

Analysis-Ready Reads

BAM

1

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

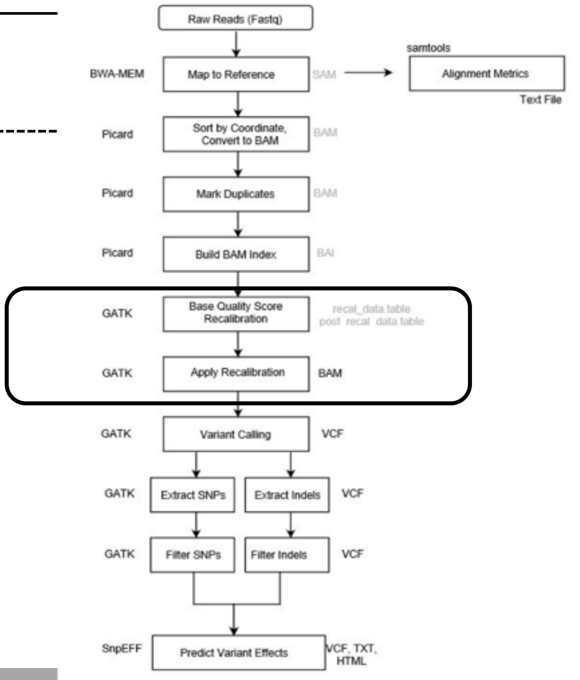
VCF

Evaluate Callset

☹ ☺

Troubleshoot

Use in project



Raw Reads (Fastq)

BWA-MEM Map to Reference SAM

samtools

Alignment Metrics

Text File

Picard Sort by Coordinate, Convert to BAM BAM

Picard Mark Duplicates BAM

Picard Build BAM Index BAI

GATK Base Quality Score Recalibration recal_data.table post_recal_data.table

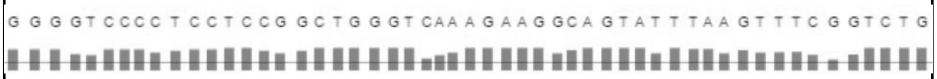
GATK Apply Recalibration BAM

GATK Variant Calling VCF

GATK Extract SNPs Extract Indels VCF

GATK Filter SNPs Filter Indels VCF

SnpEFF Predict Variant Effects VCF, TXT, HTML



Individual quality scores (blue bars) for each position in a single read. The horizontal blue line represents the Q20 phred score value

Why do we care about quality scores so much?

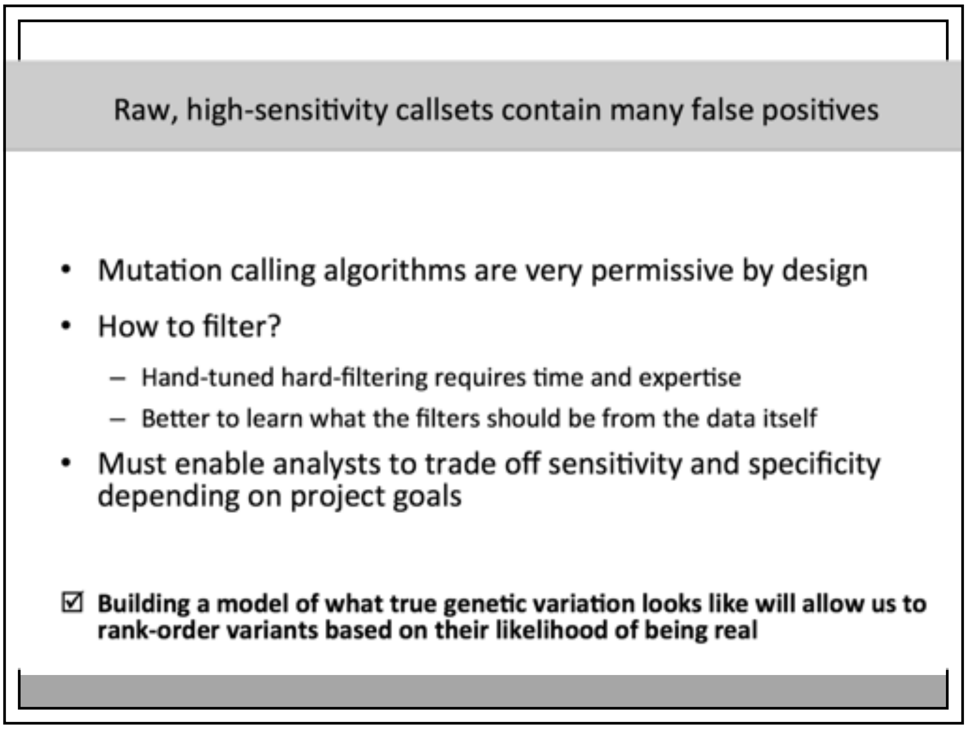
- Variant calling algorithms rely on the quality score assigned to the individual base calls
- Tells us how much we can trust that particular observation to inform us about the biological truth of the site
- If we have a basecall that has a low quality score, that means we're not sure we actually read that A correctly, and it could actually be something else
- So we won't trust it as much as other base calls that have higher qualities
- We use that score to weigh the evidence that we have for or against a variant existing at a particular site

Why Recalibrate?

- Scores produced by the machines are subject to various sources of systematic technical error
- Leads to over- or under-estimated base quality scores in the data.
- Errors can arise due to the physics or the chemistry of how the sequencing reaction works, possibly manufacturing flaws in the equipment.

Why Recalibrate?

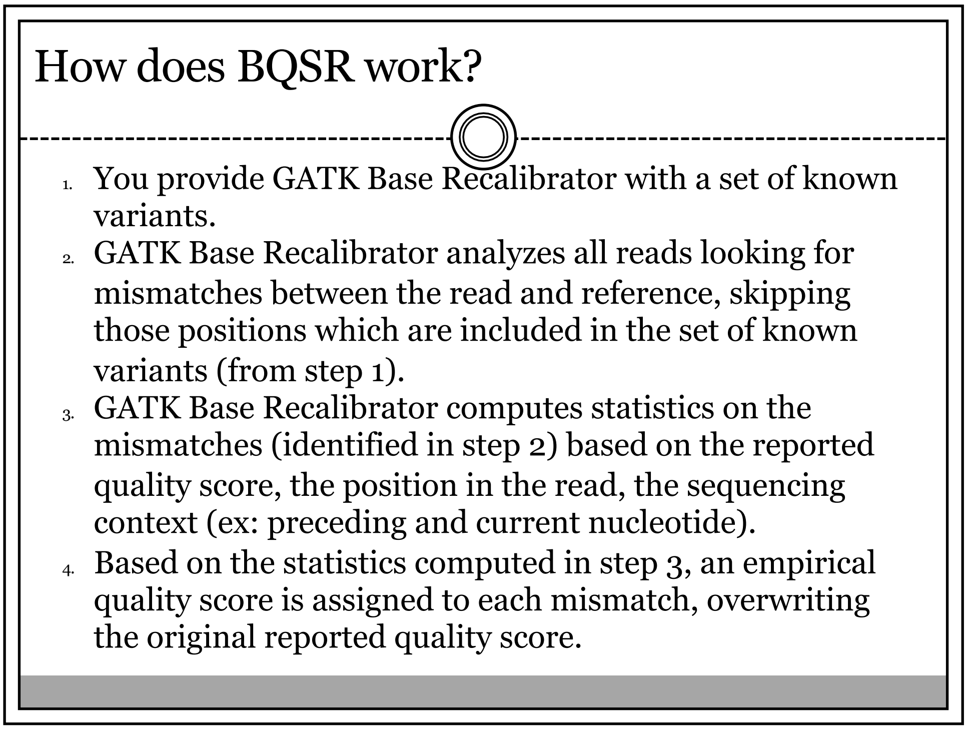
Base quality score recalibration (BQSR) is a process in which we apply machine learning to model these errors empirically and adjust the quality scores accordingly.



Raw, high-sensitivity callsets contain many false positives

- Mutation calling algorithms are very permissive by design
- How to filter?
  - Hand-tuned hard-filtering requires time and expertise
  - Better to learn what the filters should be from the data itself
- Must enable analysts to trade off sensitivity and specificity depending on project goals

☑ **Building a model of what true genetic variation looks like will allow us to rank-order variants based on their likelihood of being real**



How does BQSR work?

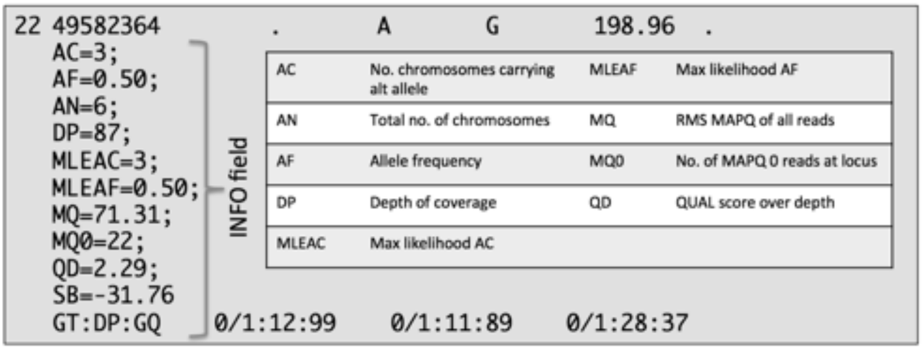
1. You provide GATK Base Recalibrator with a set of known variants.
2. GATK Base Recalibrator analyzes all reads looking for mismatches between the read and reference, skipping those positions which are included in the set of known variants (from step 1).
3. GATK Base Recalibrator computes statistics on the mismatches (identified in step 2) based on the reported quality score, the position in the read, the sequencing context (ex: preceding and current nucleotide).
4. Based on the statistics computed in step 3, an empirical quality score is assigned to each mismatch, overwriting the original reported quality score.

From annotations to mixture models

- Each variant has a diverse set of statistics associated with it.
- These annotations tend to form Gaussian clusters
- We can fit a "Gaussian mixture model" to the annotations known variants in our dataset.
- Any new variant can be scored by evaluating the associated annotations in this model.

Variant annotations are the "features" of the model

VCF record for an A/G SNP at 22:49582364

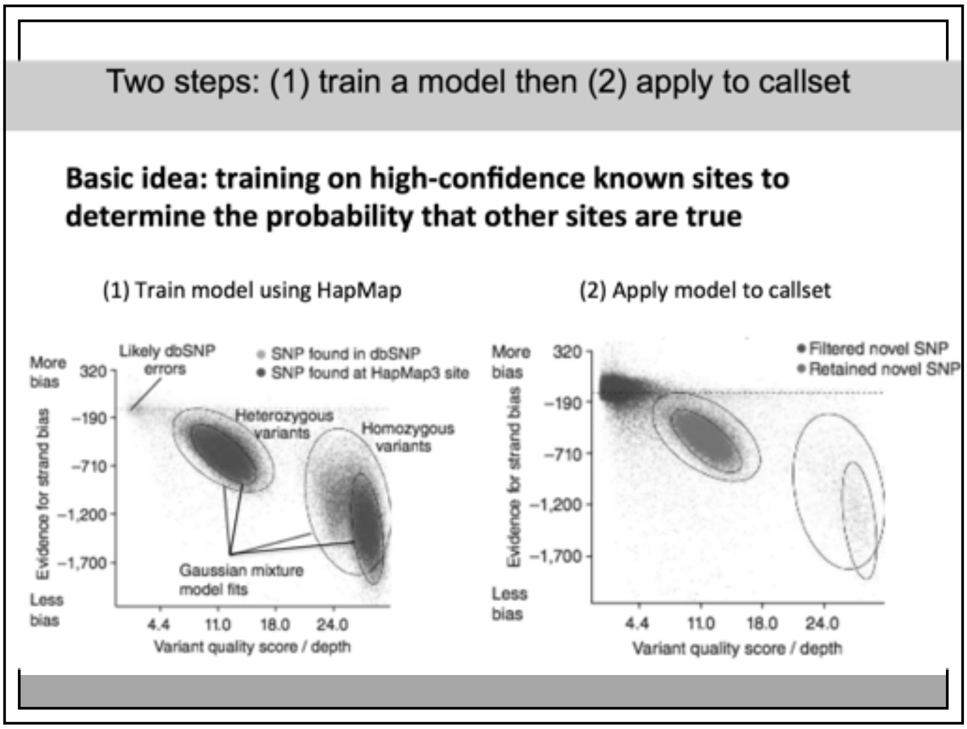


```
22 49582364           .        A        G        198.96  .
   AC=3;
   AF=0.50;
   AN=6;
   DP=87;
   MLEAC=3;
   MLEAF=0.50;
   MQ=71.31;
   MQ0=22;
   QD=2.29;
   SB=-31.76
   GT:DP:GQ     0/1:12:99     0/1:11:89     0/1:28:37
```

INFO field

AC	No. chromosomes carrying alt allele	MLEAF	Max likelihood AF
AN	Total no. of chromosomes	MQ	RMS MAPQ of all reads
AF	Allele frequency	MQ0	No. of MAPQ 0 reads at locus
DP	Depth of coverage	QD	QUAL score over depth
MLEAC	Max likelihood AC		

Note that VQSR will only look at INFO annotations;

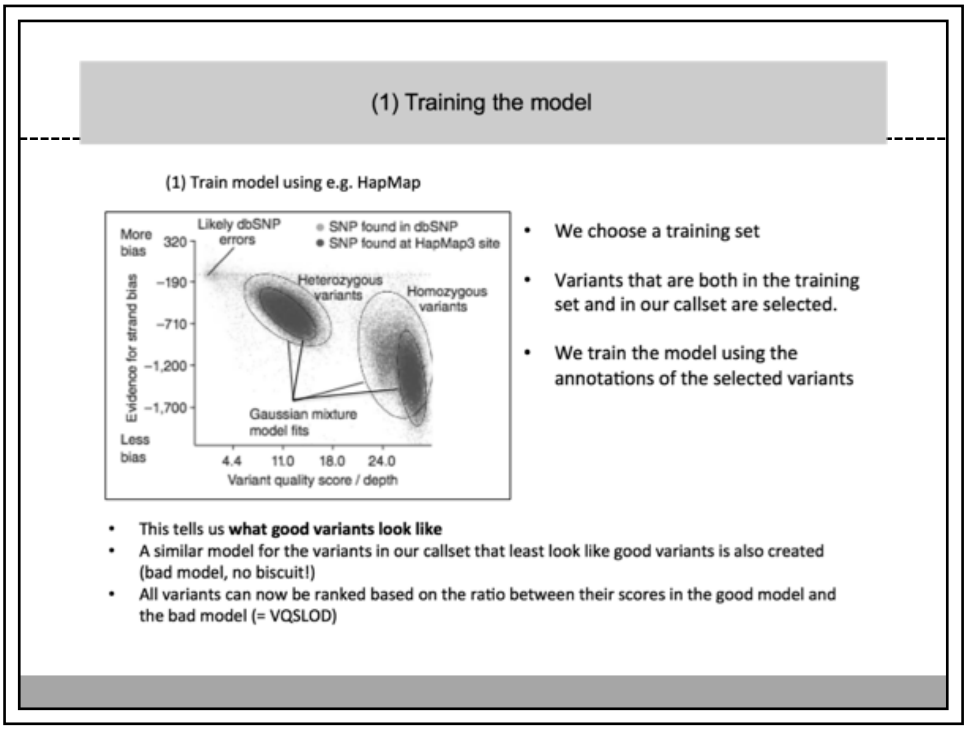


Two steps: (1) train a model then (2) apply to callset

Basic idea: training on high-confidence known sites to determine the probability that other sites are true

(1) Train model using HapMap

(2) Apply model to callset

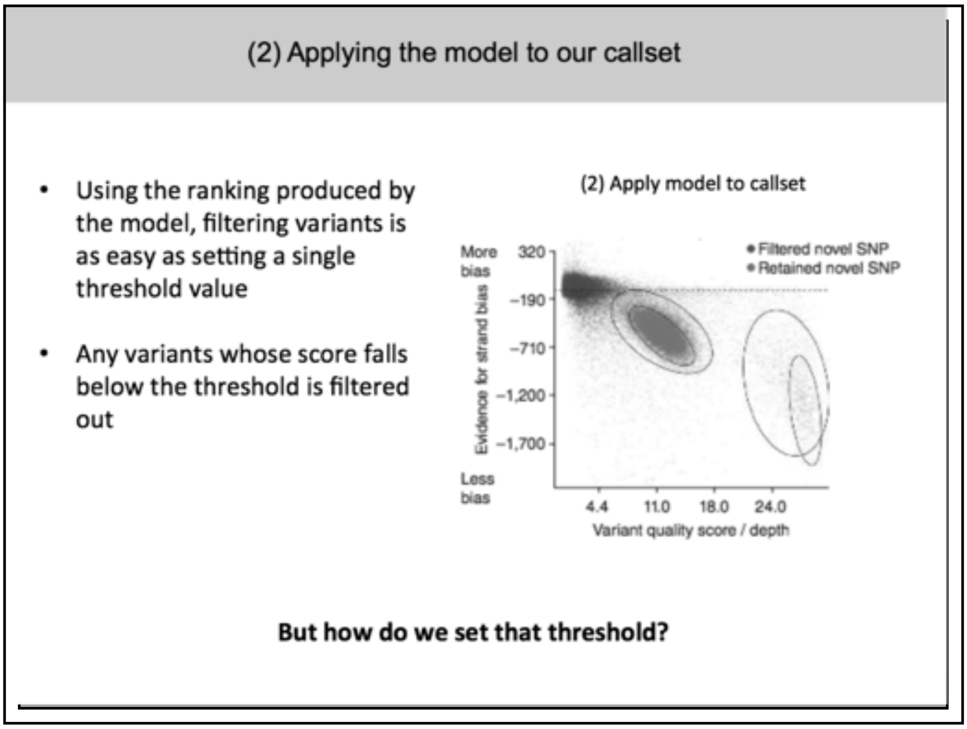


(1) Training the model

(1) Train model using e.g. HapMap

- We choose a training set
- Variants that are both in the training set and in our callset are selected.
- We train the model using the annotations of the selected variants

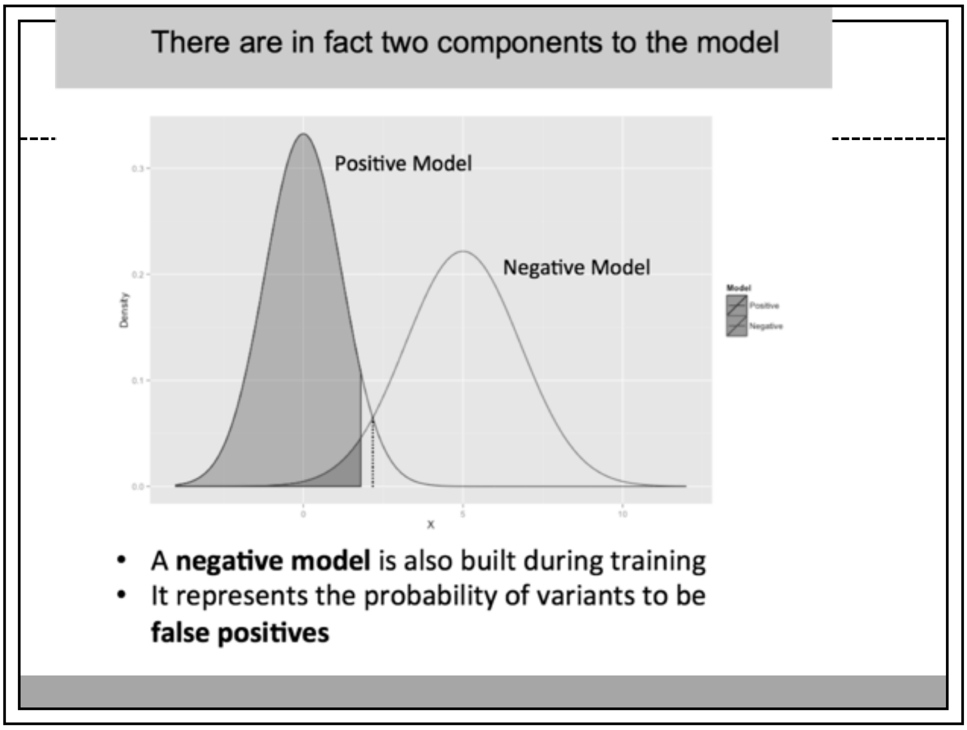
- This tells us **what good variants look like**
- A similar model for the variants in our callset that least look like good variants is also created (bad model, no biscuit!)
- All variants can now be ranked based on the ratio between their scores in the good model and the bad model (= VQSLOD)



(2) Applying the model to our callset

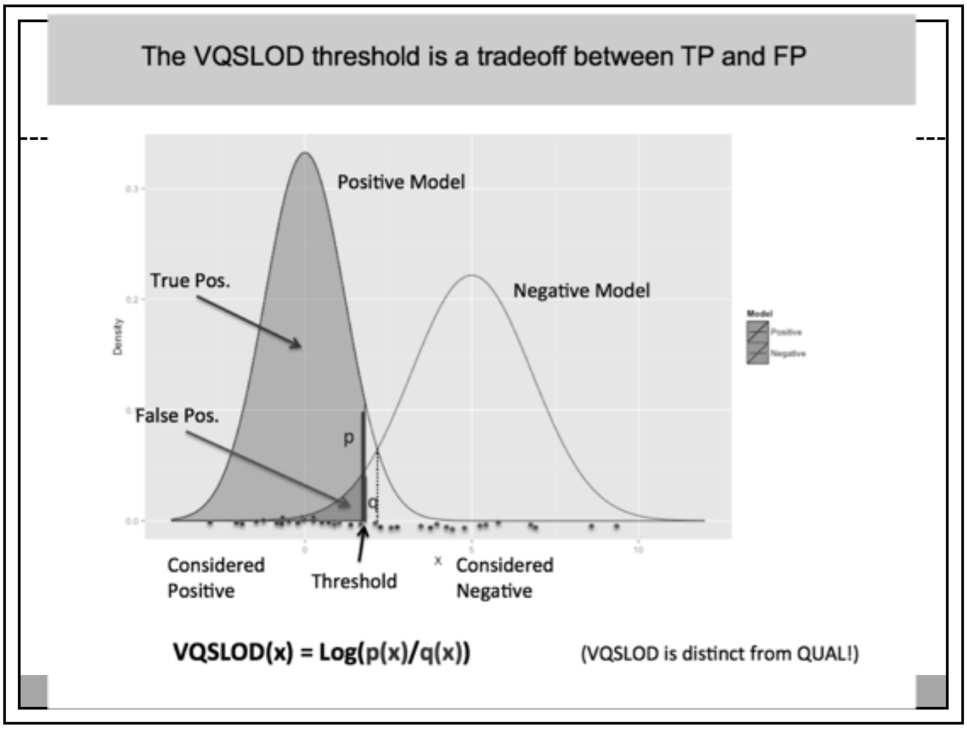
- Using the ranking produced by the model, filtering variants is as easy as setting a single threshold value
- Any variants whose score falls below the threshold is filtered out

But how do we set that threshold?



There are in fact two components to the model

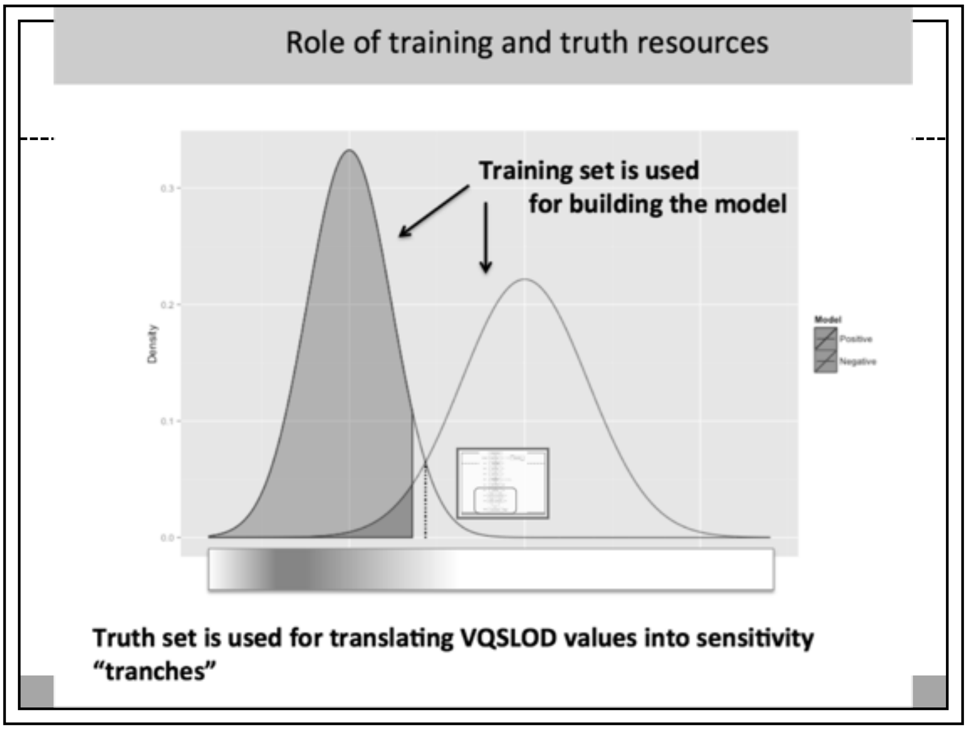
- A **negative model** is also built during training
- It represents the probability of variants to be **false positives**



The VQSLOD threshold is a tradeoff between TP and FP

VQSLOD(x) = Log(p(x)/q(x))

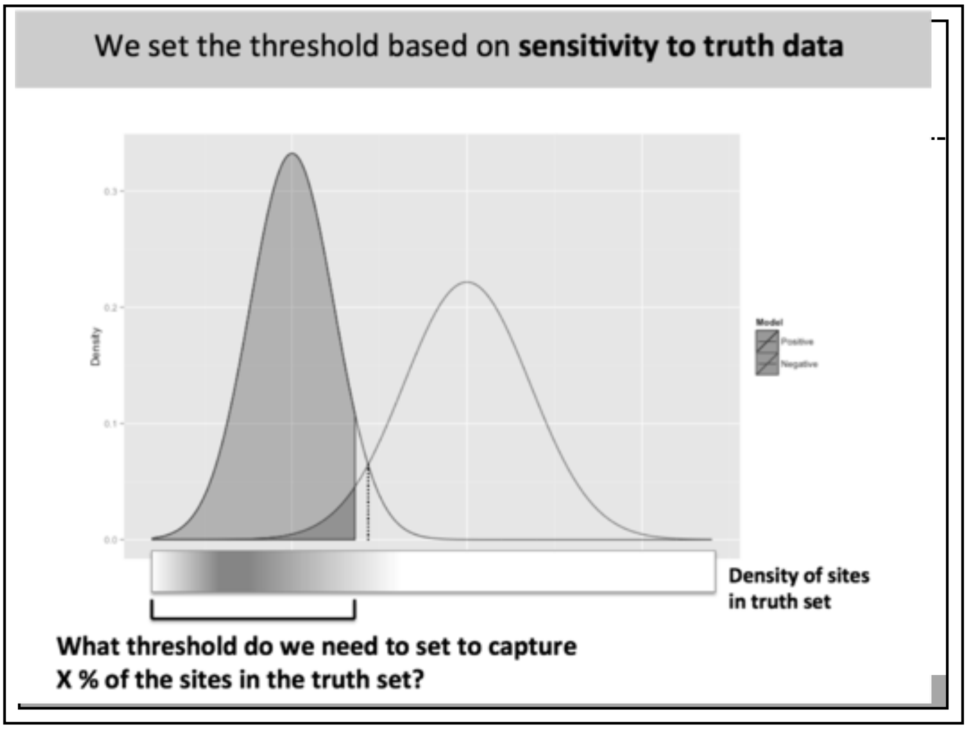
(VQSLOD is distinct from QUAL!)



Role of training and truth resources

Training set is used for building the model

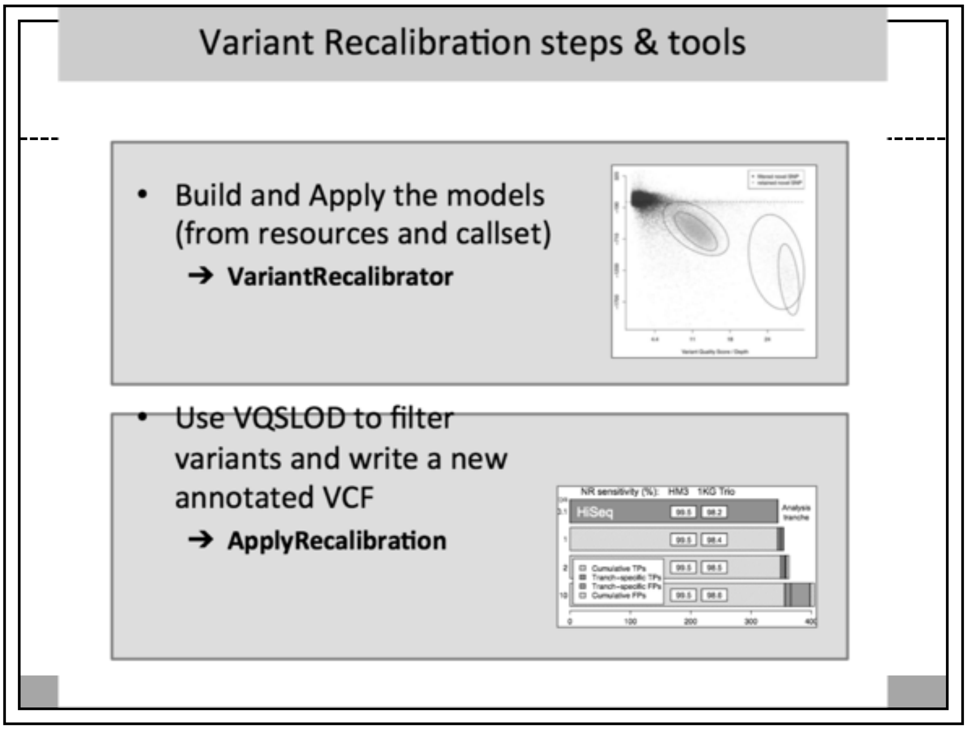
Truth set is used for translating VQSLOD values into sensitivity "tranches"



We set the threshold based on **sensitivity to truth data**

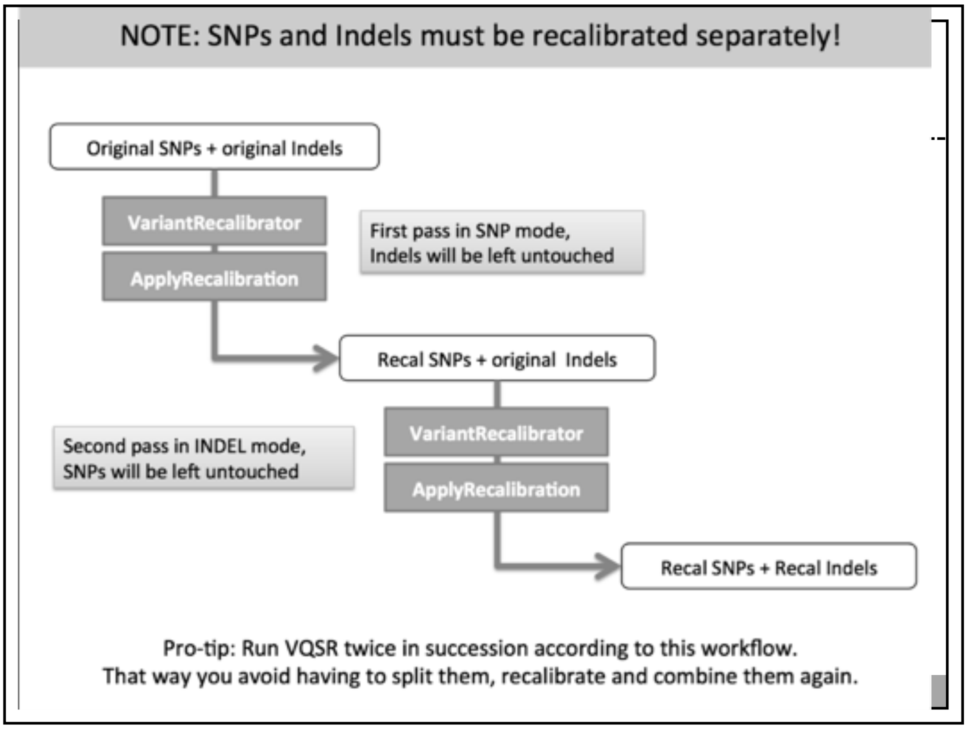
Density of sites in truth set

What threshold do we need to set to capture X % of the sites in the truth set?



Variant Recalibration steps & tools

- Build and Apply the models (from resources and callset)
  ➔ **VariantRecalibrator**
- Use VQSLOD to filter variants and write a new annotated VCF
  ➔ **ApplyRecalibration**



NOTE: SNPs and Indels must be recalibrated separately!

Original SNPs + original Indels

VariantRecalibrator

ApplyRecalibration

First pass in SNP mode,
Indels will be left untouched

Recal SNPs + original Indels

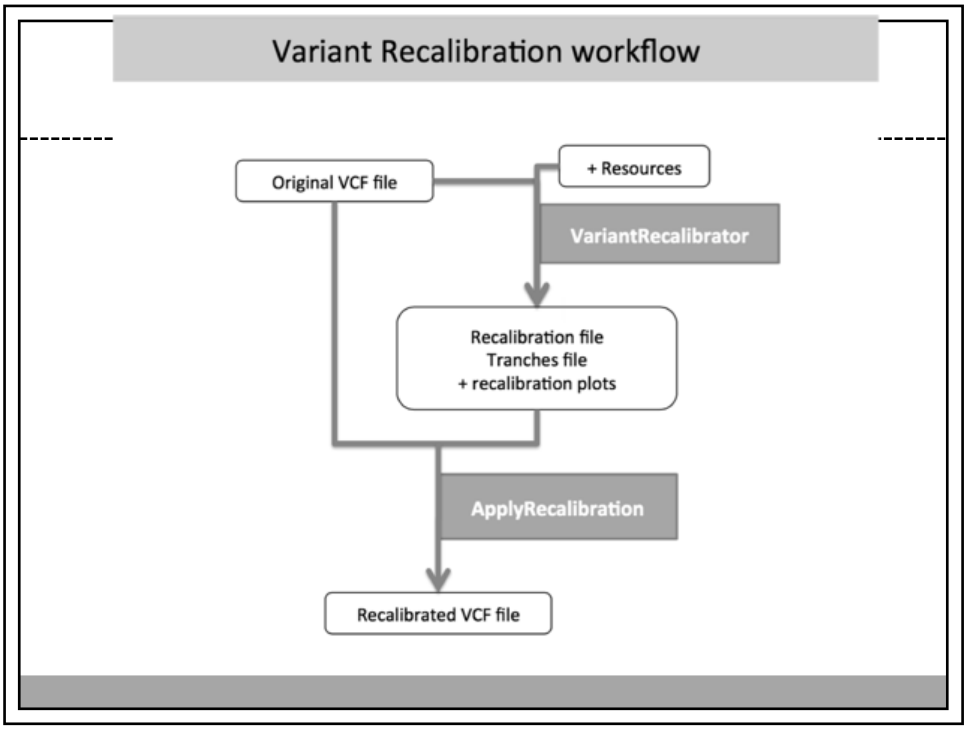
Second pass in INDEL mode,
SNPs will be left untouched

VariantRecalibrator

ApplyRecalibration

Recal SNPs + Recal Indels

Pro-tip: Run VQSR twice in succession according to this workflow.
That way you avoid having to split them, recalibrate and combine them again.



Variant Recalibration workflow

Original VCF file

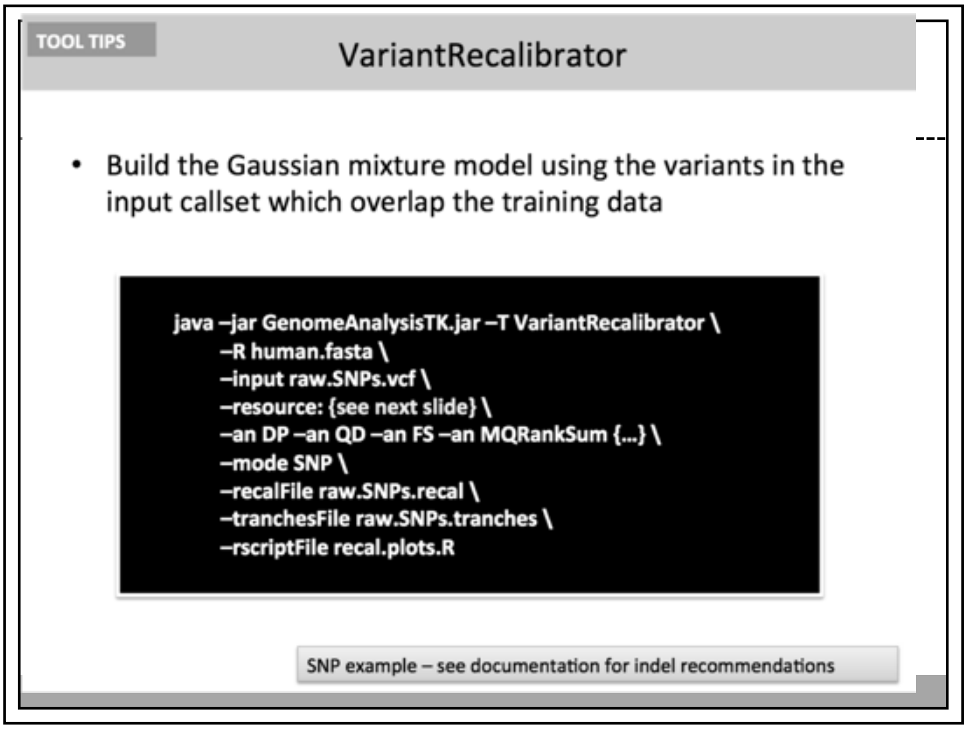
+ Resources

VariantRecalibrator

Recalibration file
Tranches file
+ recalibration plots

ApplyRecalibration

Recalibrated VCF file



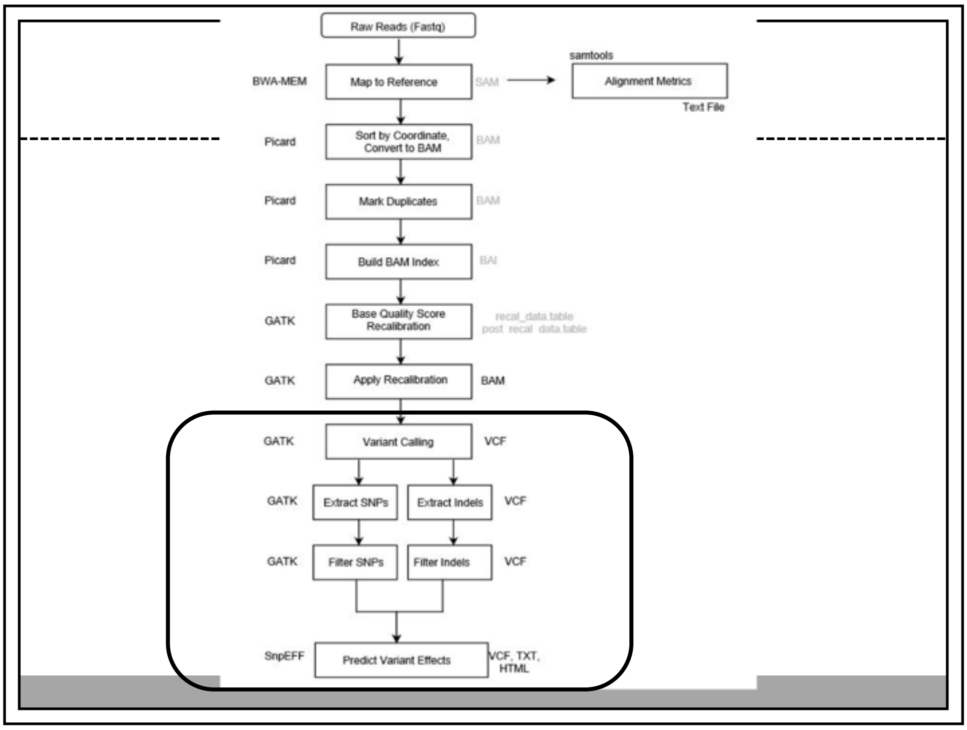
TOOL TIPS

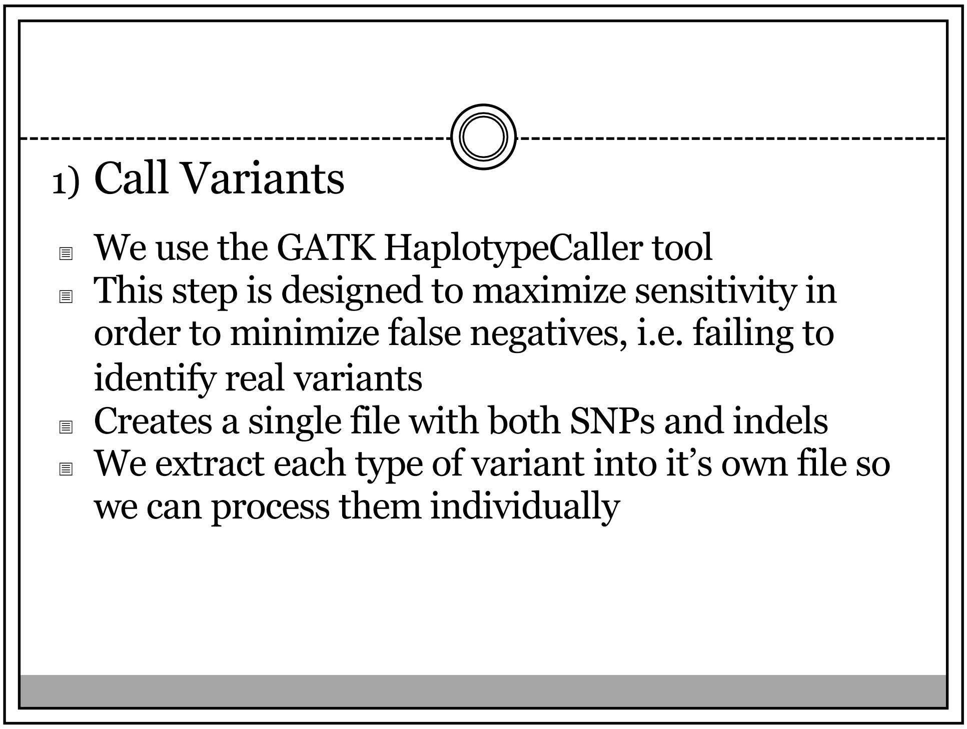
VariantRecalibrator

- Build the Gaussian mixture model using the variants in the input callset which overlap the training data

```
java –jar GenomeAnalysisTK.jar –T VariantRecalibrator \
        –R human.fasta \
        –input raw.SNPs.vcf \
        –resource: {see next slide} \
        –an DP –an QD –an FS –an MQRankSum {…} \
        –mode SNP \
        –recalFile raw.SNPs.recal \
        –tranchesFile raw.SNPs.tranches \
        –rscriptFile recal.plots.R
```

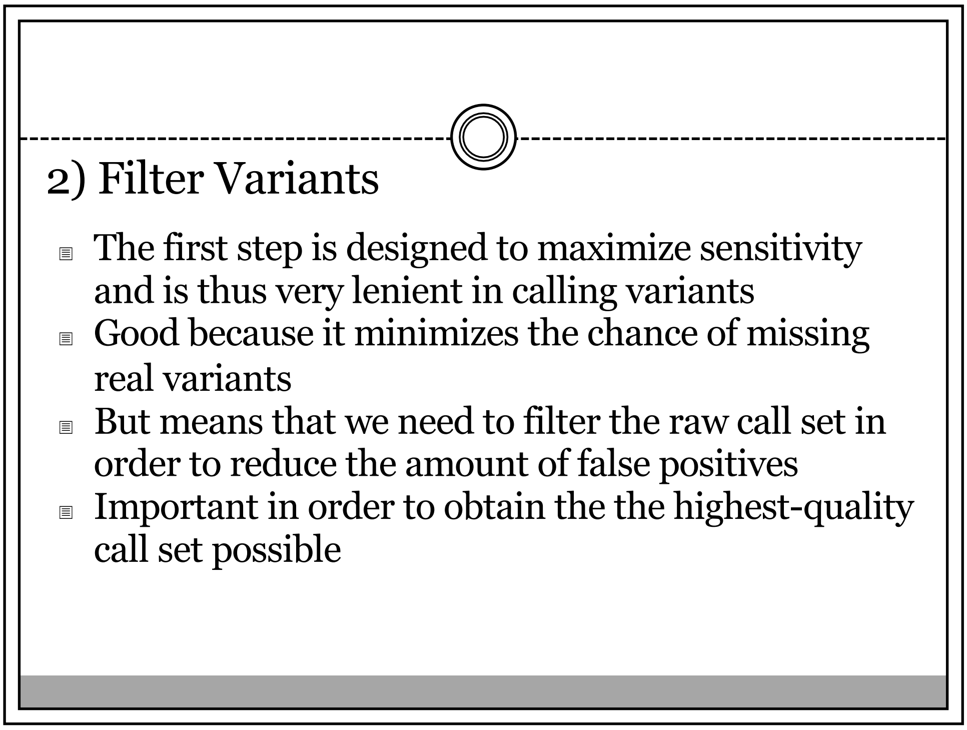
SNP example – see documentation for indel recommendations





1) Call Variants

- We use the GATK HaplotypeCaller tool
- This step is designed to maximize sensitivity in order to minimize false negatives, i.e. failing to identify real variants
- Creates a single file with both SNPs and indels
- We extract each type of variant into it's own file so we can process them individually



2) Filter Variants

- The first step is designed to maximize sensitivity and is thus very lenient in calling variants
- Good because it minimizes the chance of missing real variants
- But means that we need to filter the raw call set in order to reduce the amount of false positives
- Important in order to obtain the the highest-quality call set possible

3) Annotation

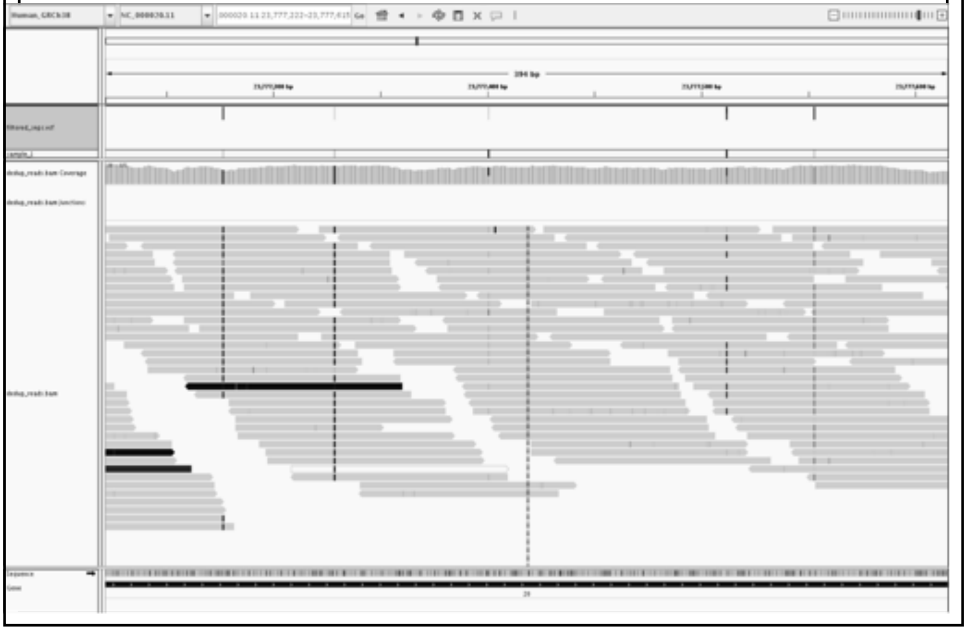
- We use SnpEff
- Annotates and predicts the effects of variants on genes
  - Codon changes
  - Amino acid changes
  - Genomic region
  - Functional effect (silent, missense)
- SnpEff has pre-built databases for thousands of genomes

Archivo VCF

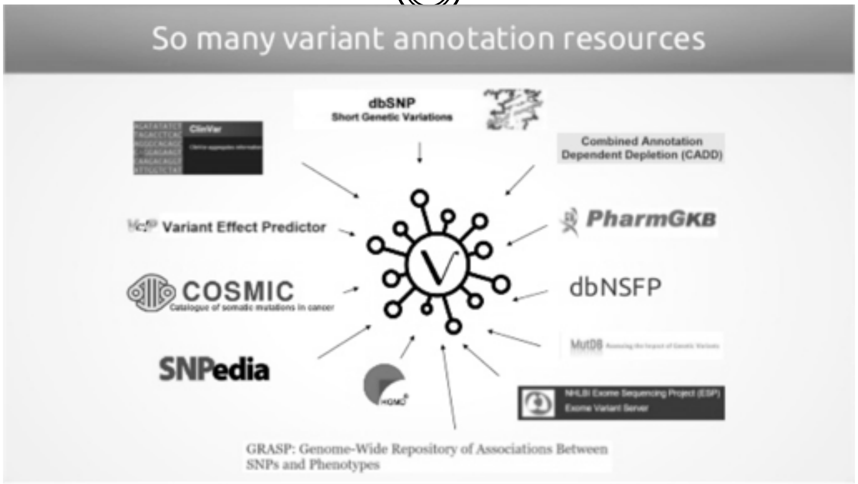
```
##fileformat=VCFv4.0
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=1000GenomesPilot-NCBI36
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=.,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF ALT    QUAL FILTER INFO                              FORMAT      NA00001        NA00002        NA00003
20     14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
20     17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
20     1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
20     1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51 0/0:61:2
20     1234567 microsat1 GTCT   G,GTACT 50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4       0/2:17:2       1/1:40:3
```

http://www.internationalgenome.org/wiki/Analysis/vcf4.0/

4) Visualization - IGV



Variant Annotation



So many variant annotation resources

dbSNP
Short Genetic Variations

ClinVar

Combined Annotation Dependent Depletion (CADD)

Variant Effect Predictor

PharmGKB

COSMIC
Catalogue of somatic mutations in cancer

dbNSFP

SNPedia

MutDB

HGMD

NHLBI Exome Sequencing Project (ESP)
Exome Variant Server

GRASP: Genome-Wide Repository of Associations Between SNPs and Phenotypes

Variant Annotation: SnpEff

- Variant annotation and effect prediction tool. It annotates and predicts the effects of genetic variants (such as amino acid changes).
- Many effects are calculated: such as SYNONYMOUS_CODING, NON_SYNONYMOUS_CODING, FRAME_SHIFT, STOP_GAINED just to name a few.

SnpEff: Public databases

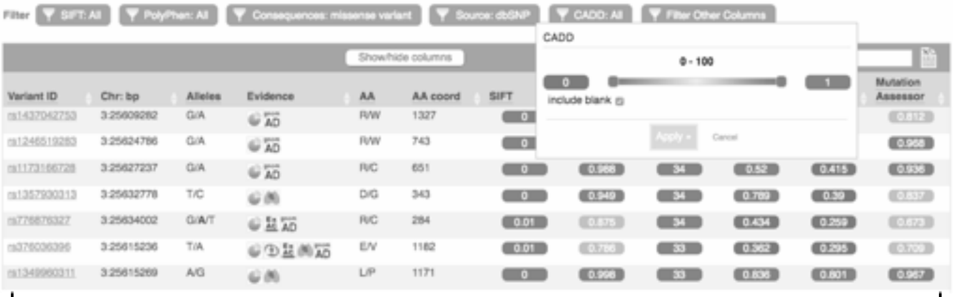
- **ENCODE** datasets are supported by SnpEff (by means of BigWig files provided by ENCODE project).
- **Epigenome Roadmap** provides data-sets that can be used with SnpEff.
- **TFBS** Transcription factor binding site predictions can be annotated. Motif data used in this annotations is generates by Jaspar and ENSEBML projects
- **NextProt** database can be used to annotate protein domains as well as important functional sites in a protein (e.g. phosphorilation site)

CADD - Combined Annotation Dependent Depletion

- Framework that integrates multiple annotations into one metric by contrasting variants that survived natural selection with simulated mutations
- C-scores strongly correlate with allelic diversity, pathogenicity of both coding and non-coding variants, and experimentally measured regulatory effects, and also highly rank causal variants within individual genome sequences.
- C-scores of complex trait-associated variants from genome-wide association studies (GWAS) are significantly higher than matched controls and correlate with study sample size, likely reflecting the increased accuracy of larger GWAS.

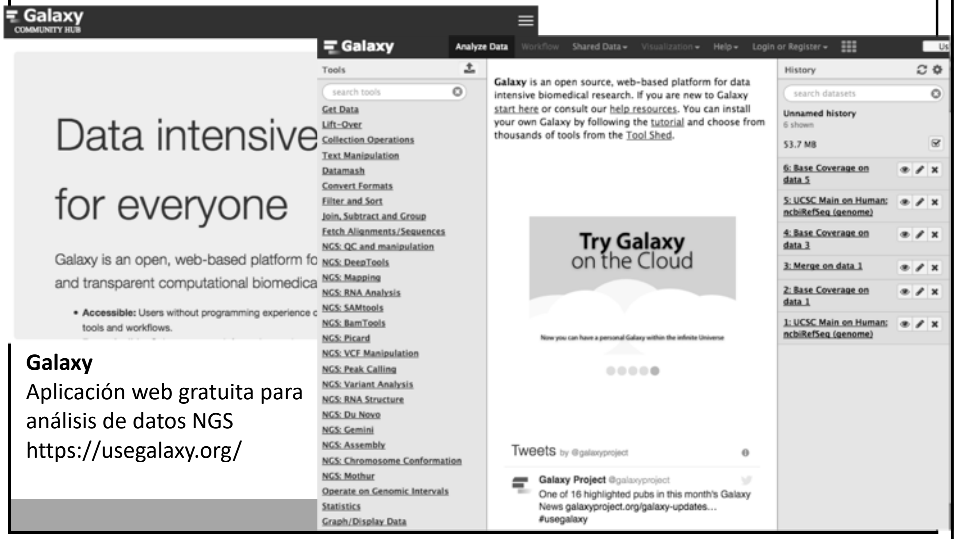
https://cadd.gs.washington.edu/

Puntajes CADD



Variant ID	Chr: bp	Alleles	Evidence	AA	AA coord	SIFT					Mutation Assessor
rs1437042753	3:25609282	G/A	AD	R/W	1327	0					0.812
rs1246519283	3:25624786	G/A	AD	R/W	743	0					0.968
rs1173166728	3:25627237	G/A	AD	R/C	651	0	0.988	34	0.52	0.415	0.936
rs1357930313	3:25632778	T/C		D/G	343	0	0.949	34	0.789	0.39	0.837
rs776876327	3:25634002	G/A/T	AD	R/C	284	0.01	0.875	34	0.434	0.259	0.673
rs376036396	3:25615236	T/A	AD	E/V	1182	0.01	0.786	33	0.362	0.295	0.709
rs1349960311	3:25615269	A/G		L/P	1171	0	0.998	33	0.836	0.801	0.967

Software Libre



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3: Merge on data 1
2: Base Coverage on data 1
1: UCSC Main on Human: ncbiRefSeq (genome)

Galaxy
Aplicación web gratuita para análisis de datos NGS
https://usegalaxy.org/

Resources in NGS data analysis

Public forums:

SEQanswers
the next generation sequencing community

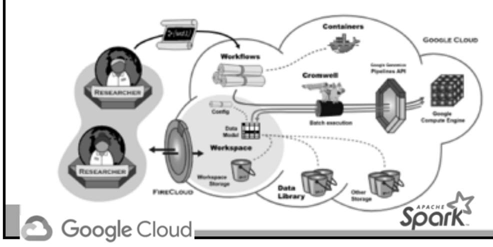
Bioconductor
OPEN SOURCE SOFTWARE FOR BIOINFORMATICS

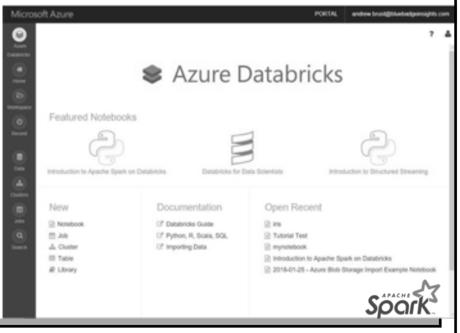
BioBits: A Blog about bioinformatics

Biostars
BIOINFORMATICS EXPLAINED

BROAD INSTITUTE FireCloud

An open platform for secure and scalable analysis on the cloud





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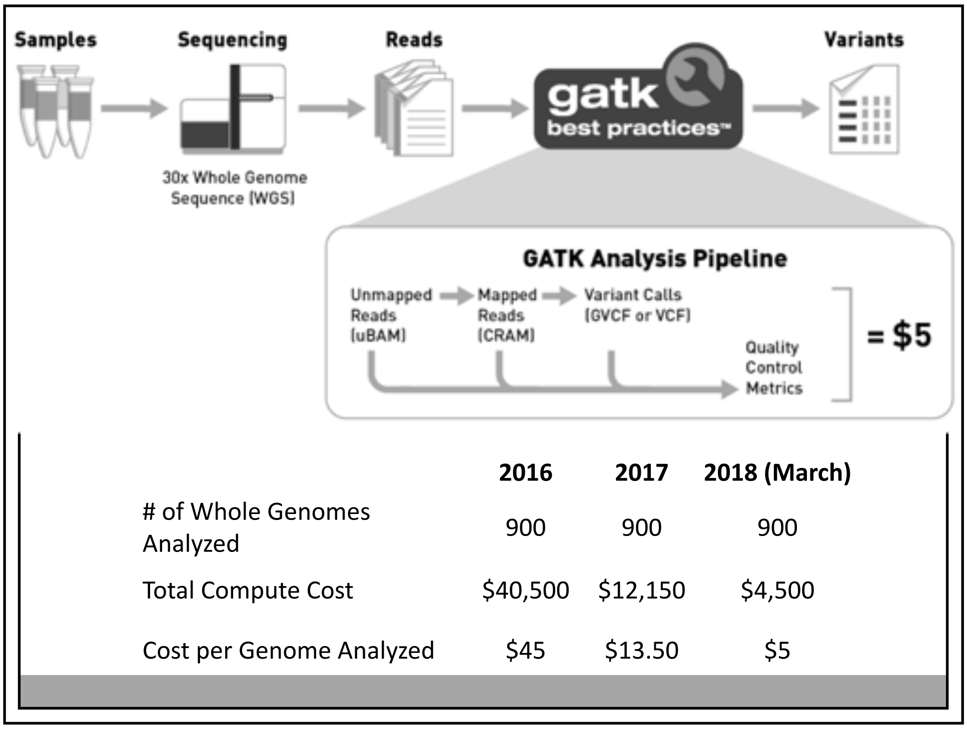

	2016	**2017**	**2018 (March)**
# of Whole Genomes Analyzed	900	900	900
Total Compute Cost	$40,500	$12,150	$4,500
Cost per Genome Analyzed	$45	$13.50	$5