Introduction to Bioinformatics

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Variant Calling I

- Variants: differences between two genomes
- It is now feasible (technical and financial wise) to sequence human samples at large scale for medical and genetic studies
- Major projects, e.g.:
  - 1000 Genomes project (http://www.internationalgenome.org/)
  - The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/)

Variant Calling II

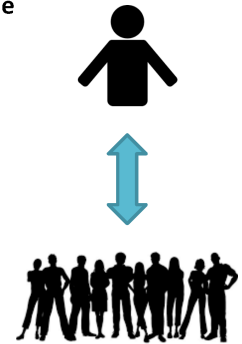
- Clarify the full spectrum of human genetic diversity
- Study the complete genetic architecture of human diseases
- Find mutations that hide links to Mendelian diseases
- Find mutations for which no mapping data is available, e.g.
  - somatic mutations in cancer
  - de novo mutations in autism and schizophrenia

Variant Calling III

- Mapping a fastq file (raw reads) into a genome (fasta)
  - creation of a bam file
- Search (per base) for differences between the bam file and the genome and create a vcf (variant call format) file
  - misaligned reads e.g. because of a low quality read
  - SNP (Single Nucleotide Polymorphism): different nucleotide in just one position
  - INDEL (INsertion/DELetion): a small number of nucleotides has been inserted or deleted
  - CNV (Copy Number Variation): repetition or deletion of larger blocks of nucleotides
- It is hard to distinguish a real polymorphism from artifacts

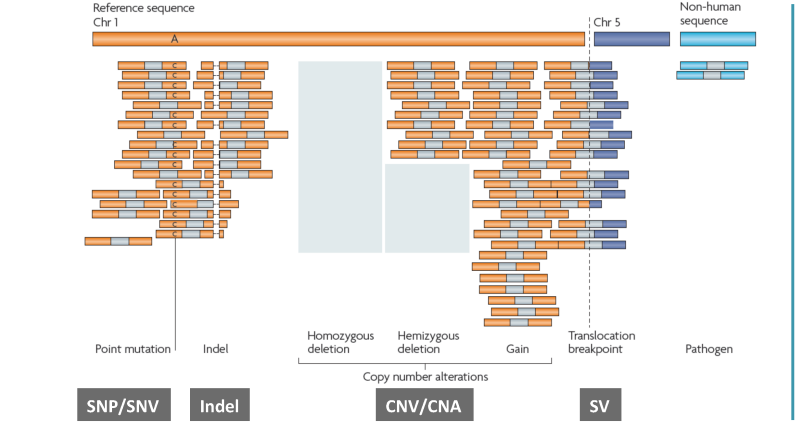
Variant discovery I

- Genetic changes in individuals **relative to a reference genome**
  - Germline (inherited)
  - Somatic (cancer)
- **Reference genome** = a standardized genomic sequence
- Human genome reference sequence
  - Current standard: hg19 / b37
  - New standard (on the rise): hg38
- Other organisms
  - Many have a fully assembled reference available
  - Many still do not -> SOL



https://software.broadinstitute.org/gatk/documentation/presentations

Variant discovery II



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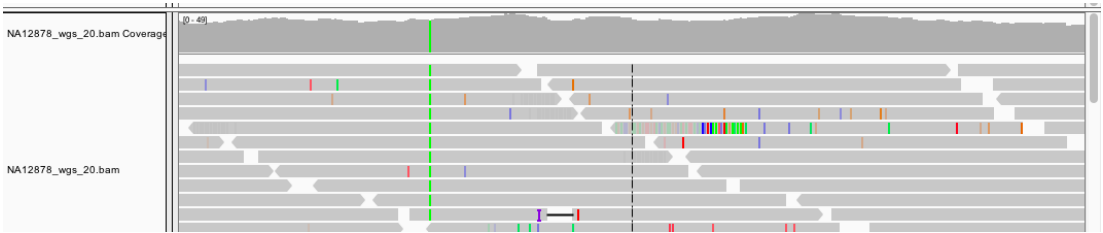
IGV I

Integrative Genomics Viewer - Variant Calling

The Integrative Genomics Viewer (IGV) is a high-performance visualization tool for interactive exploration of large, integrated genomic datasets. It supports a wide variety of data types, including array-based and next-generation sequence data, and genomic annotations.

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

IGV II



Various options for Variant Calling

- Samtools mpileup
- Freebayes
- VarScan
- Atlas2
- **GATK**
- …

GATK

Genome Analysis Toolkit - GATK

A collection of command-line tools for analyzing high-throughput sequencing (HTS) data in formats such as SAM/BAM/CRAM and VCF, with a focus on variant discovery.

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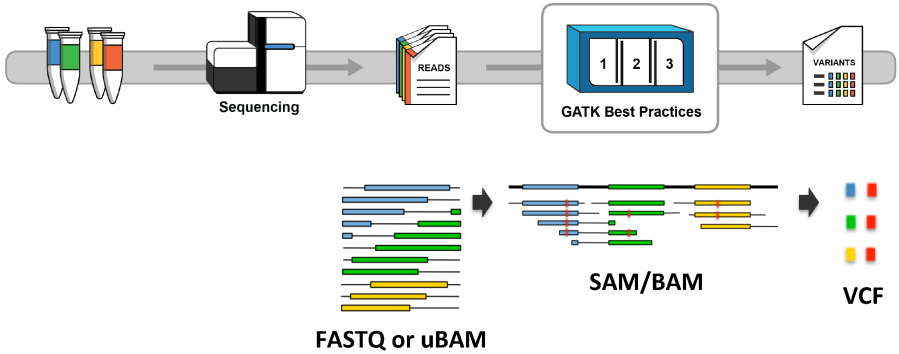
A multi-step procedure divided into 3 parts:

- Pre-processing
- Variant discovery
- Callset refinement

GATK Overview I



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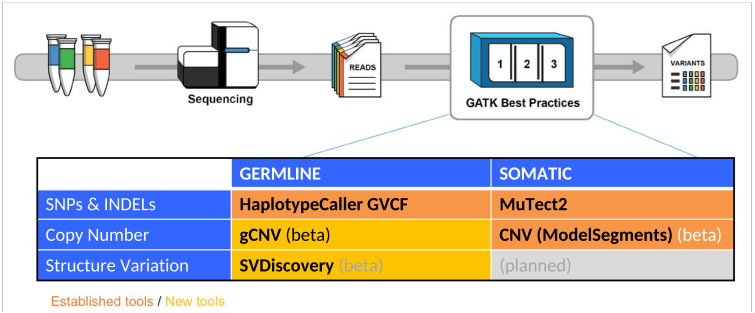
GATK Overview II



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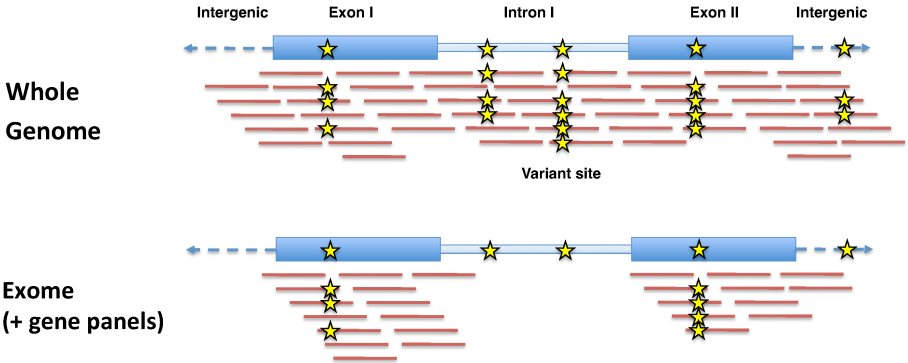
GATK Overview III



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GATK - Technical details

- Java

```
java -jar /path/to/GenomeAnalysisTK.jar
```

- Collection of various tools

```
java jvm-args -jar GenomeAnalysisTK.jar -R reference.fasta -T
GATKToolName -OPTION1 value1 -OPTION2 value2 ...
```

- The jar file is compiled for POSIX systems (i.e. non-Windows)

Pre-processing I

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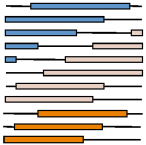
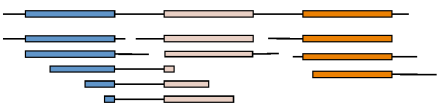
Pre-processing II



Enormous pile of short reads from HTS

Mapping and alignment algorithms

- **BWA for DNA**
- **STAR for RNAseq**



Reference genome

Reads mapped to reference

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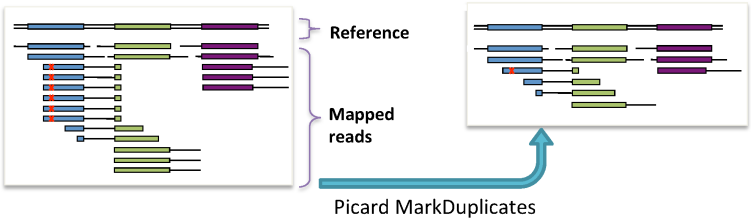
Mark-Duplicates I

Duplicates = **non-independent measurements** of a sequence fragment

-> Must be removed to assess support for alleles correctly



✖ = sequencing error propagated in duplicates

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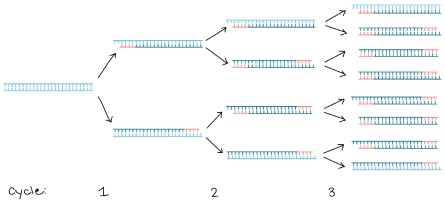
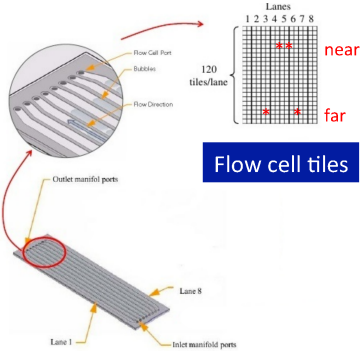
Mark-Duplicates II

- **LIBRARY DUPLICATES**
  - **Increases with PCR cycles**
- **OPTICAL DUPLICATES**
  - **Are nearby clusters on a flow cell lane**

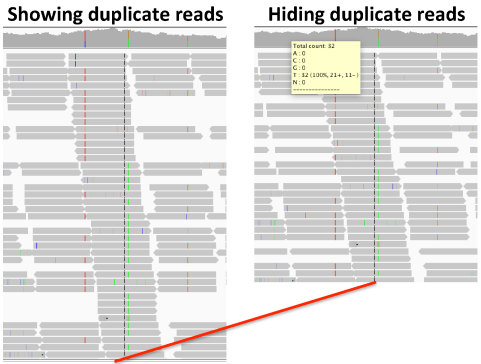


https://www.khanacademy.org/science/biology/biotech-dna-technology/dna-sequencing-pcr-electrophoresis/a/polymerase-chain-reaction-pcr



http://www.slideshare.net/jandot/next-generation-sequencing-course-part-2-sequence-mapping
http://www.slideshare.net/cosentia/illumina-gaiix-for-high-throughput-sequencing

Mark-Duplicates III



- Duplicate status is indicated in SAM flag
- Duplicates are **not removed**, just tagged (unless you request removal)
- Downstream tools can read the tag and choose to ignore those reads
- Most GATK tools ignore duplicates by default

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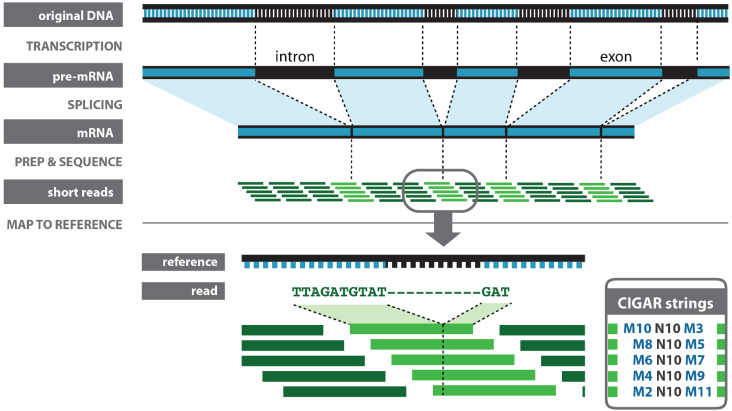
Special handling for RNAseq splice junctions



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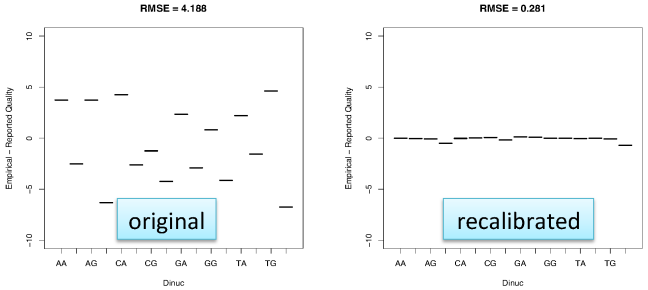
Base Recalibration (BQSR) I

- Sequencers make systematic errors in base quality scores
- BQSR corrects the quality scores (not the bases)

Example of bias: qualities reported depending on nucleotide context



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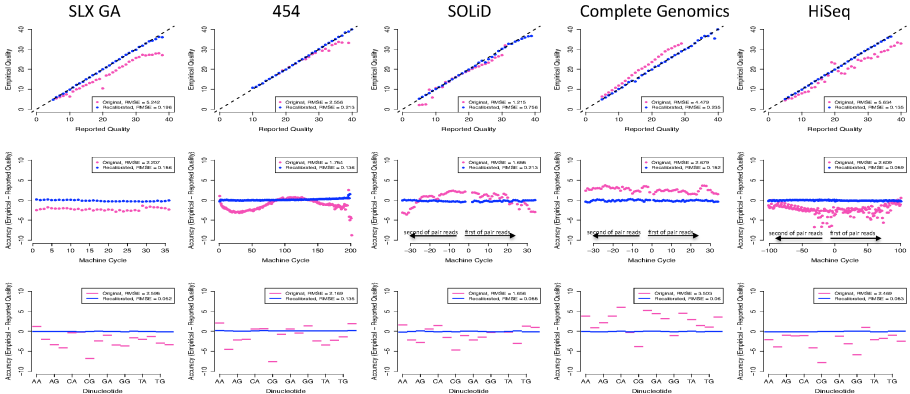
Base Recalibration (BQSR) II

https://software.broadinstitute.org/gatk/documentation/presentations

Base Recalibration (BQSR) III



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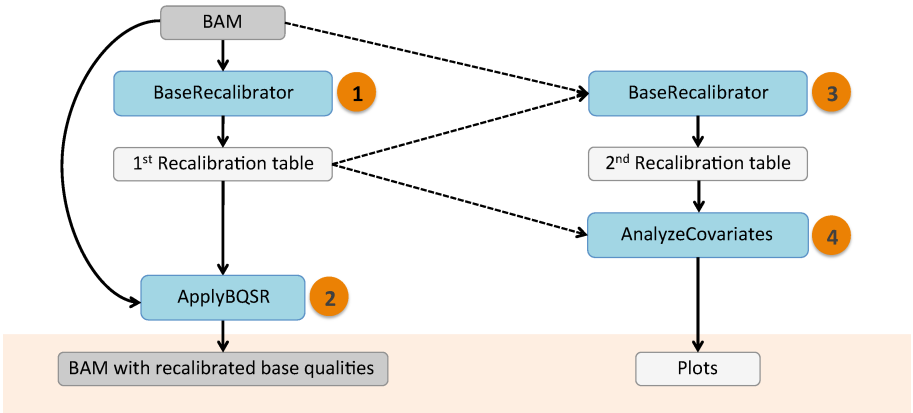
Base Recalibration (BQSR) IV



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Base Recalibration (BQSR) V

Build base recalibration model in GATK3:

```
java -jar GenomeAnalysisTK.jar \
     -T BaseRecalibrator \
     -R ref.fasta \
     -I sample.bam \
     -knownSites snps.vcf.gz \
     -knownSites indels.vcf.gz \
     -o recal.table
```

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Base Recalibration (BQSR) VI

Recalibrate base qualities in GATK3:

```
java -jar GenomeAnalysisTK.jar \
     -T PrintReads \
     -R ref.fasta \
     -I sample.bam \
     -BQSR recal.table \
     -o sample_bqsr.bam
```

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Base Recalibration (BQSR) VII

Generate before and after plots in GATK3:

```
java -jar GenomeAnalysisTK.jar \
    -T AnalyzeCovariates \
    -R ref.fasta \
    -before 1st_recal.table \
    -after 2nd_recal.table \
    -plots plots.pdf
```

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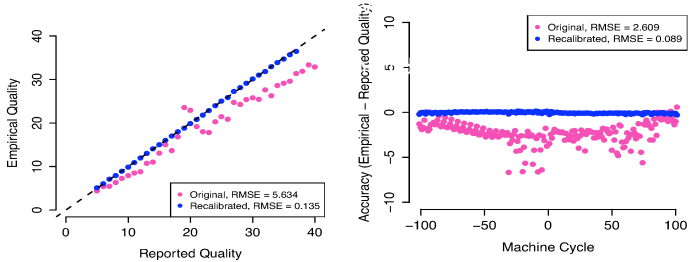
Base Recalibration (BQSR) VIII



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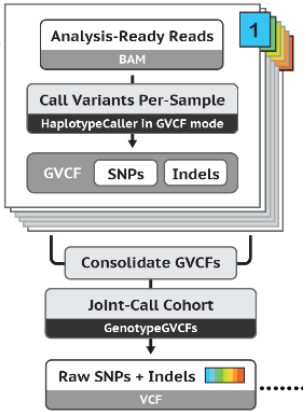
GATK - Variant discovery



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Variant discovery I

- Single genome in isolation: almost never useful
- Family or population data
  add valuable information
  - rarity of variants
  - *de novo* mutations
  - ethnic background

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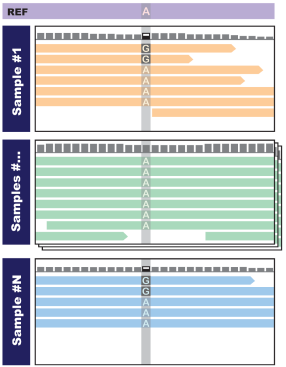
Variant discovery II

https://software.broadinstitute.org/gatk/documentation/presentations

Variant discovery III



- Sample #1 or Sample #N alone:
  - **weak evidence for variant**
  - **may miss calling the variant**
- Both samples seen together:
  - **unlikely to be artifact**
  - **call the variant more confidently**

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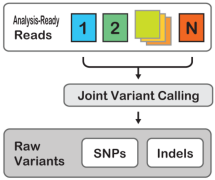
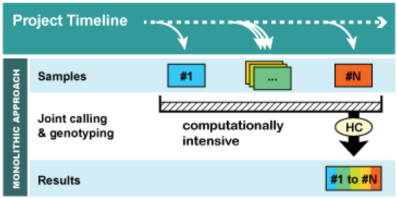
Variant discovery - UnifiedGenotyper



Compute requirements scale very badly with number of samples!!!

It gives us the right answers, but…



Want to add new samples?

Got to re-run pipeline from scratch! The N+1 problem!

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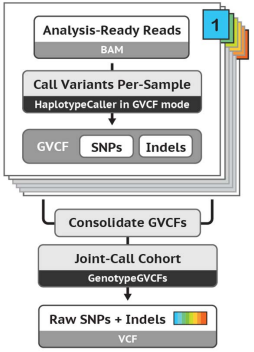
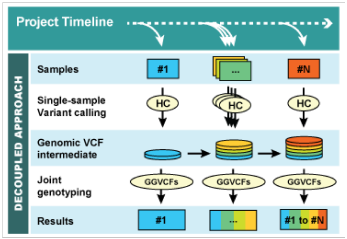
Variant discovery - HaplotypeCaller





Scales linearly with number of samples!

Want to add a new sample? Make a GVCF for that sample then re-call the cohort at will!

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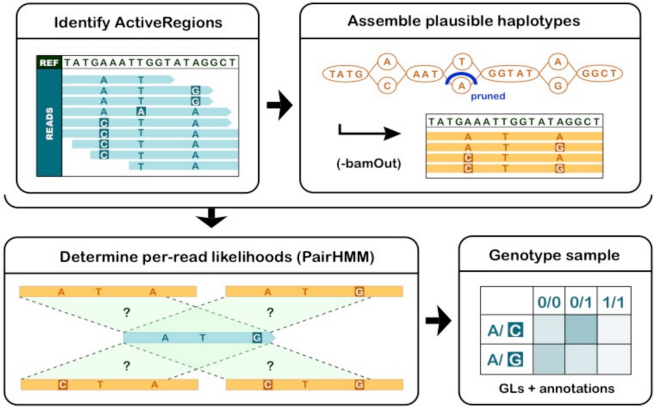
HaplotypeCaller I



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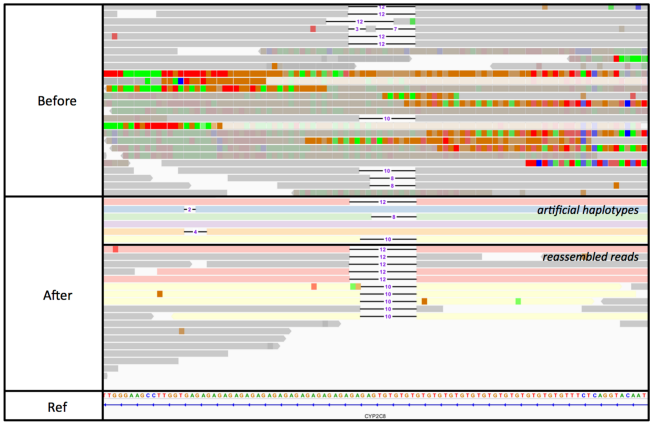
HaplotypeCaller II



Showing 100bp region starting at 10:96,825,862 for NA12878. IGV is a snapshot version from 2017/8/28

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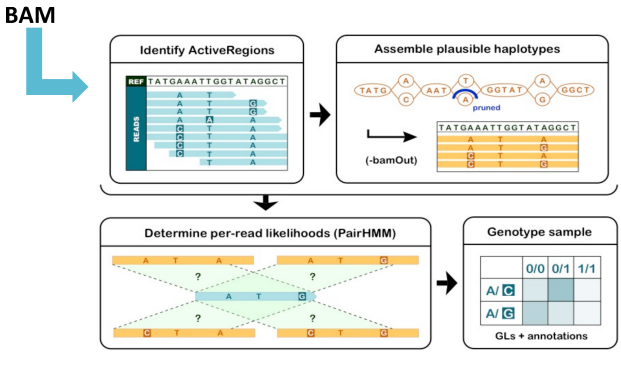
HaplotypeCaller III



This is all you need for a **single sample** or **traditional multi-sample** analysis

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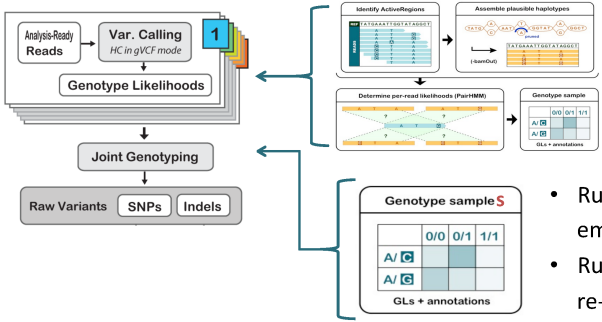
HaplotypeCaller IV



- Run HC in **GVCF mode** to emit GVCF
- Run GenotypeGVCFs to re-genotype samples with **multi-sample model**

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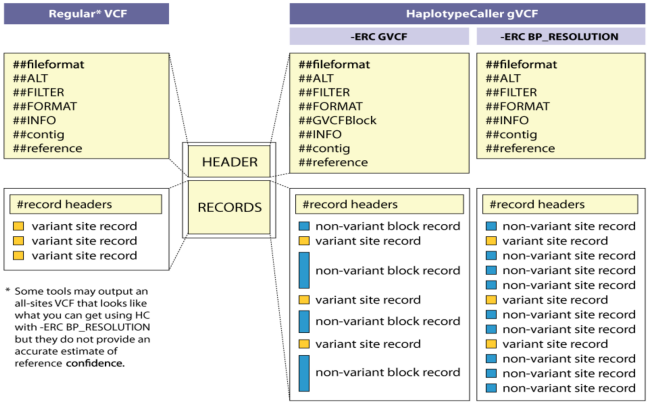
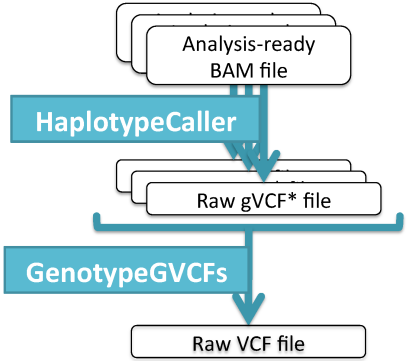
HaplotypeCaller V



https://software.broadinstitute.org/gatk/documentation/presentations

HaplotypeCaller VI




```
java -jar GenomeAnalysisTK.jar \
     -T HaplotypeCaller \
     -R human.fasta \
     -I sample1.bam \
     -o sample1.g.vcf \
       [ -L exome_targets.intervals \ ]
     -ERC GVCF
```

```
java -jar GenomeAnalysisTK.jar \
     -T GenotypeGVCFs \
     -R human.fasta \
     -V sample1.g.vcf \
     -V sample2.g.vcf \
     -V sampleN.g.vcf \
     -o output.vcf
```

If >200 samples, combine in batches first using CombineGVCFs

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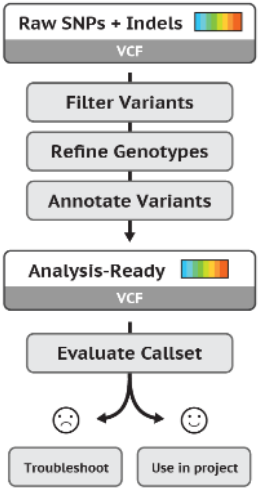
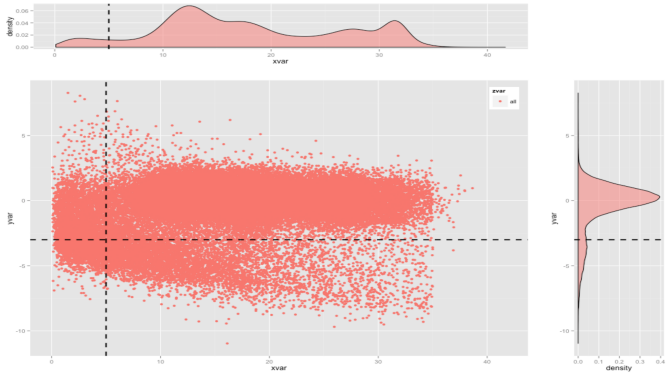
VCF Filtering



VCF Filtering - Hard filter



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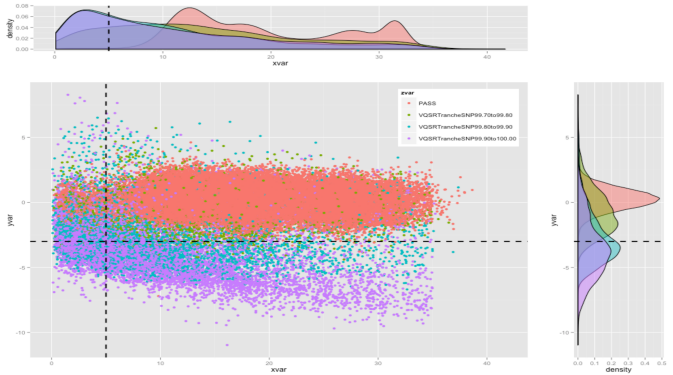
VCF Filtering - Variant recalibration I



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VCF Filtering - Variant recalibration II

Train on high-confidence known sites to determine the probability that other sites are true or false

- Assume annotations tend to form **Gaussian clusters**
- Build a "Gaussian mixture model" from annotations of **known variants** in our dataset
- Score **all variants** by where their annotations lie relative to these clusters
- Filter base on **sensitivity to truth set**

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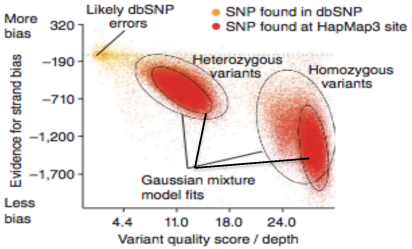
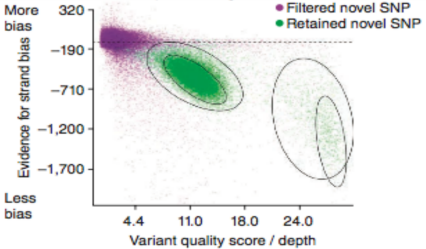
VCF Filtering - Variant recalibration III

Model trained on HapMap



Model applied to new SNPs



Modified from DePristo et al. Nature Genetics. 2011

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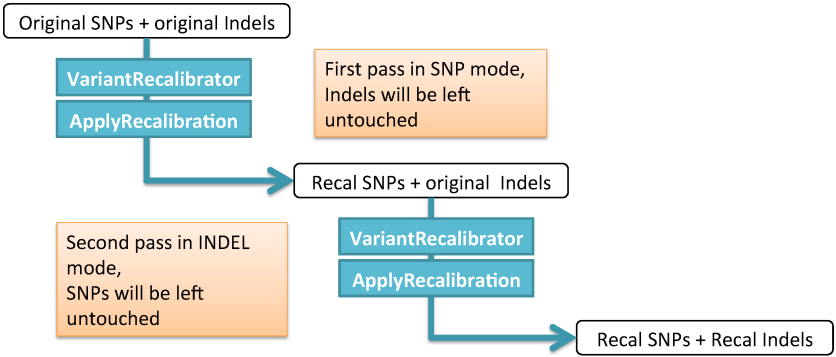
VCF Filtering - Variant recalibration IV



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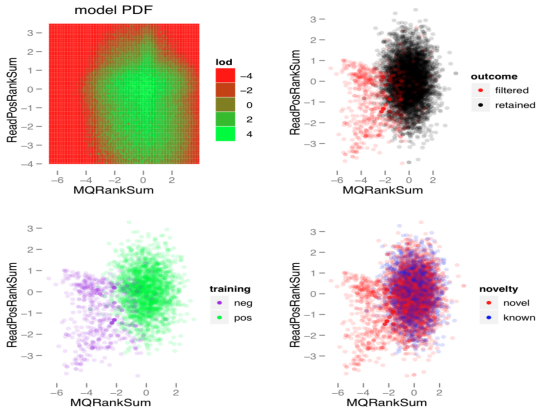
VCF Filtering - Variant recalibration V



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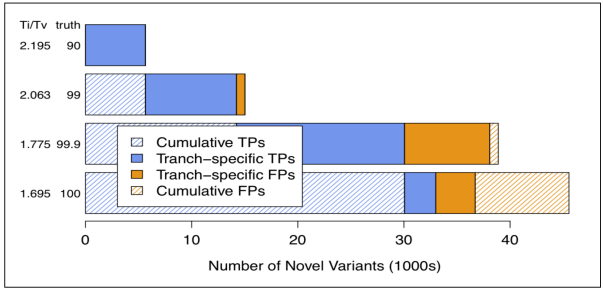
VCF Filtering - Variant recalibration VI



Estimation is based on Ti/Tv ratio of novel variants

Default target Ti/Tv is for WGS and must be adapted for exomes

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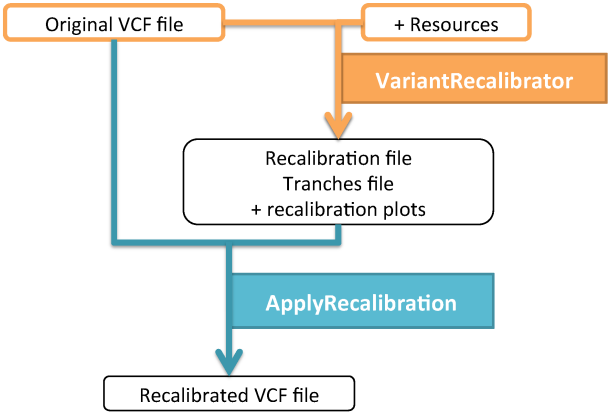
VCF Filtering - Variant recalibration VII



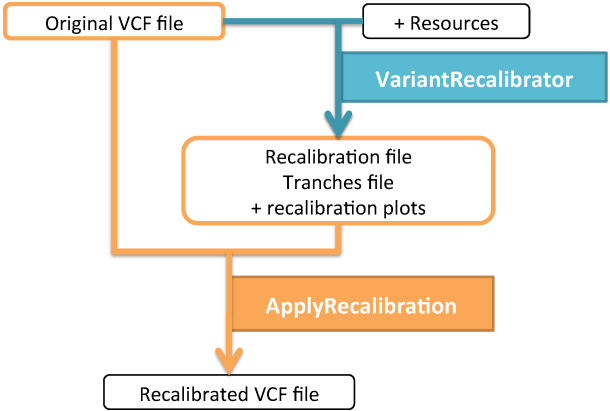
https://software.broadinstitute.org/gatk/documentation/presentations

VCF Filtering - Variant recalibration VIII

```
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
```

https://software.broadinstitute.org/gatk/documentation/presentations

VCF Filtering - Variant recalibration IX



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VCF Filtering - Variant recalibration X

```
java -jar GenomeAnalysisTK.jar -T ApplyRecalibration \
    -R human.fasta \
    -input raw.vcf \
    -mode SNP \
    -recalFile raw.SNPs.recal \
    -tranchesFile raw.SNPs.tranches \
    -o recal.SNPs.vcf \
    -ts_filter_level 99.0
```

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VCF Filtering - Variant recalibration XI

- Before VQSR (input vcf):

#CHROM	POS	FILTER	INFO
1	10146	.	AC=1;DP=32;FS=9.208; MQ=31.96;MQRankSum=0.085;...
1	10403	.	AC=1;DP=64;FS=1.645;MQ=41.86;MQRankSum=1.87;...
1	234313	.	AC=1;DP=239;FS=12.675;MQ=38.19;MQRankSum=-0.122;...

- After VQSR (output vcf):

#CHROM	POS	FILTER	INFO
1	10146	VQSRTrancheINDEL99.30to99.50	AC=1...;NEGATIVE_TRAIN_SITE;VQSLOD=-1.328;culprit=SOR
1	10403	PASS	AC=1;...;QD=0.60; VQSLOD=0.794;culprit=QD
1	234313	VQSRTrancheSNP99.90to100.00	AC=1;...;POSITIVE_TRAIN_SITE;VQSLOD=-5.356;culprit=MQ

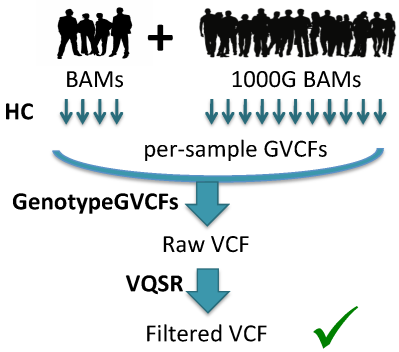
- Hard filtered vcf:

#CHROM	POS	FILTER	INFO
1	10146	PASS	AC=1;DP=32;FS=9.208; MQ=31.96;MQRankSum=0.085;...
1	10403	INDEL_Filter	AC=1;DP=64;FS=1.645;MQ=41.86;MQRankSum=1.87;...
1	234313	SNP_Filter	AC=1;DP=239;FS=12.675;MQ=38.19;MQRankSum=-0.122;...

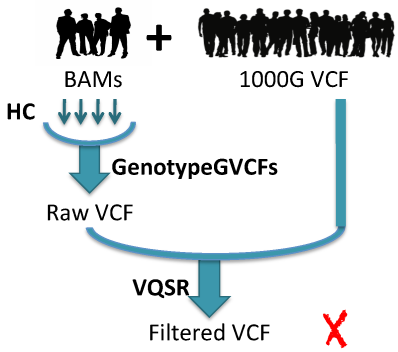
https://software.broadinstitute.org/gatk/documentation/presentations

VCF Filtering - Variant recalibration XII

ALWAYS do this:

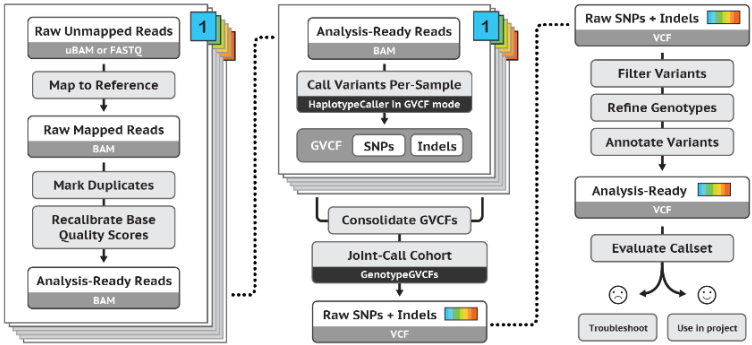


NEVER do this :



https://software.broadinstitute.org/gatk/documentation/presentations

Presented GATK pipeline



https://software.broadinstitute.org/gatk/documentation/presentations

Variant Annotation I

- Variant annotation is a very important step in the analysis
- Functional annotation can have a strong impact on the final conclusions of the studies
- Inaccurate or incorrect annotation can lead to the skipping of polymorphisms potentially responsible for a disease or to conceal interesting variations in a group of false positives

Variant Annotation II

Various tools for annotation:

- Funcotator (GATK)
- SnpEff
- Annovar
- VEP

Funcotator I

Funcotator

Funcotator (FUNCtional annOTATOR) analyzes given variants for their function (as retrieved from a set of data sources) and produces the analysis in a specified output file. This tool is a functional annotation tool that allows a user to add annotations to called variants based on a set of data sources, each with its own matching criteria.

https://gatk.broadinstitute.org/hc/en-us/articles/360042912011-Funcotator

Funcotator II

- For **somatic** data sources:

```
./gatk FuncotatorDataSourceDownloader --somatic --validate-integrity --extract-after-download
```

- For **germline** data sources:

```
./gatk FuncotatorDataSourceDownloader --germline --validate-integrity --extract-after-download
```

SnpEff

SnpEff

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

http://snpeff.sourceforge.net/SnpEff.html

SnpEff:Basic example

```
java -Xmx4g -jar snpEff.jar GRCh37.75 examples/test.chr22.vcf >
test.chr22.ann.vcf
```

SnpEff:Basic example

```
java -Xmx4g -jar snpEff.jar GRCh37.75 examples/test.chr22.vcf >
test.chr22.ann.vcf
```

SnpEff adds functional annotations in the ANN field (8^{th} column in the VCF file test.chr22.ann.vcf)

- Putative_impact: A simple estimation of putative impact / deleteriousness : HIGH, MODERATE, LOW, MODIFIER

  frameshift_variant, stop_gained, stop_lost, start_lost, . . .
- Gene Name: Common gene name (HGNC). Optional: use closest gene when the variant is “intergenic”
- Gene ID: Gene ID
- . . .

Annovar

ANNOVAR

ANNOVAR is an efficient software tool to utilize update-to-date information to functionally annotate genetic variants detected from diverse genomes (including human genome hg18, hg19, hg38, as well as mouse, worm, fly, yeast and many others.

http://annovar.openbioinformatics.org/en/latest/

check also wANNOVAR

Variant Effect Predictor

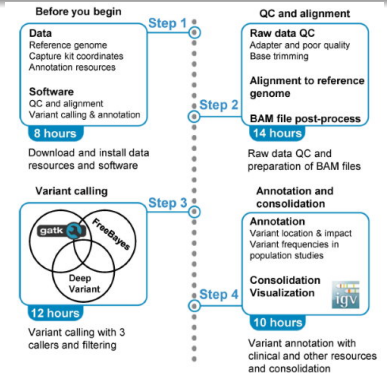
Variant Effect Predictor - VEP

VEP determines the effect of your variants (SNPs, insertions, deletions, CNVs or structural variants) on genes, transcripts, and protein sequence, as well as regulatory regions.

- Standalone perl script
- Web interface

https://www.ensembl.org/info/docs/tools/vep/index.html

Combining three variant callers (HaplotypeCaller, FreeBayes, and DeepVariant)



› STAR Protoc. 2022 May 30;3(2):101418. doi: 10.1016/j.xpro.2022.101418. eCollection 2022 Jun 17.

Protocol for unbiased, consolidated variant calling from whole exome sequencing data

Kleio-Maria Verrou [1], Georgios A Pavlopoulos [1 2], Panagiotis Moulos [1 2]

Affiliations — collapse

Affiliations

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PMID: 35669050 PMCID: PMC9163752 DOI: 10.1016/j.xpro.2022.101418
Free PMC article

https://pubmed.ncbi.nlm.nih.gov/35669050/

Hands on

Lab Exercise 6 - GATK TUTORIAL :: Variant Discovery

Questions ?