OMICS MASTER

VARIANT CALLING
Detecting variants in NGS data

PRINCIPE FELIPE
CENTRO DE INVESTIGACION
Computational · Genomics

IT4Innovations

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Genomics Data Analysis CIBERER

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Detecting variants

Valencia, 28-30 Sep 2015

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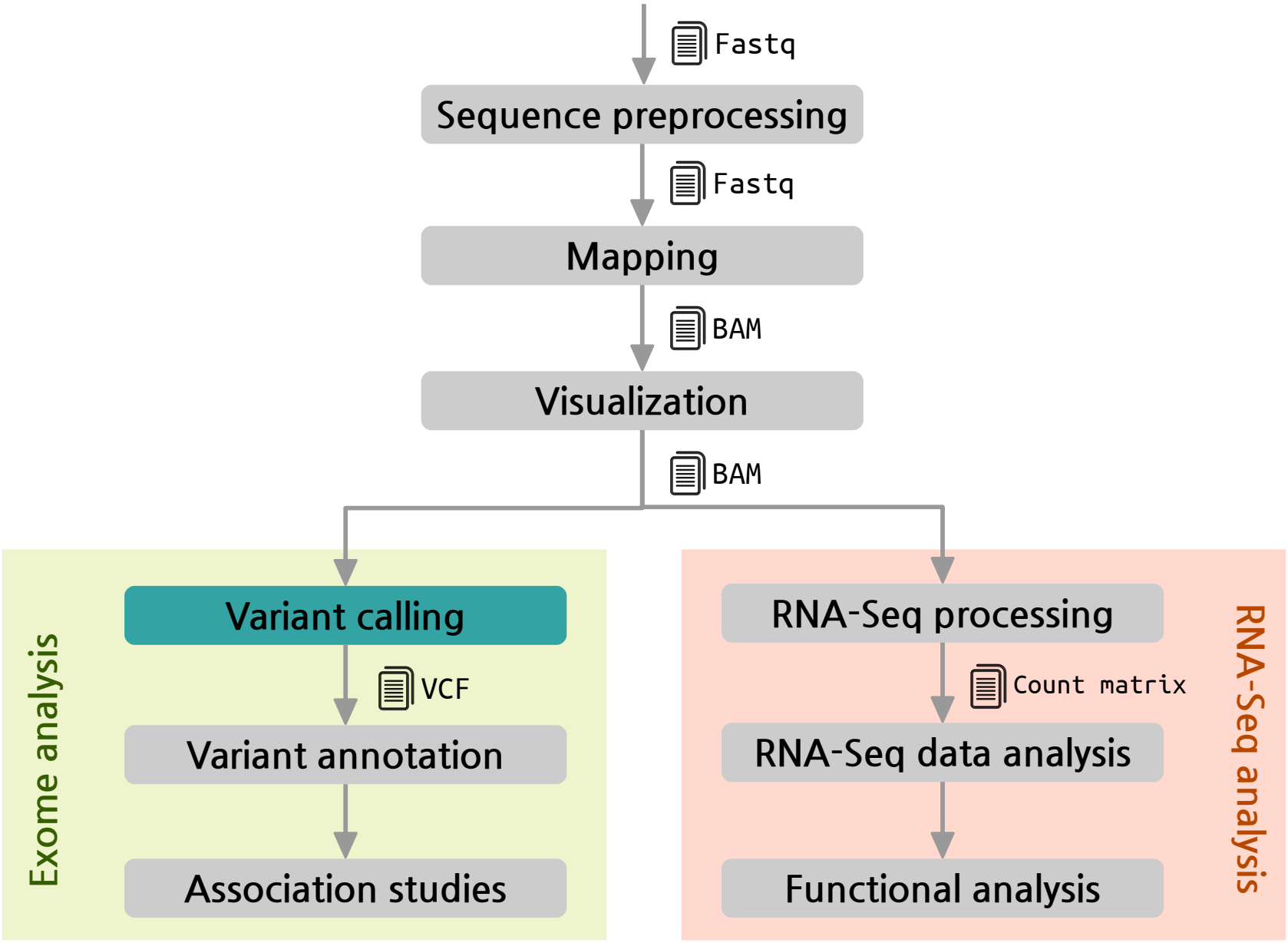
The pipeline



Genomic variation
Terminology

- **Variant**: sequence data difference that exists between individuals in a population
- **Mutation**: molecular event that created a variant
- **Allele**: forms of the bases occupying the same position on matching chromosomes
- **Genotype**: allelic state in a specific individual
  - AA homozygous or AT heterozygous at specific base
- **Polymorphism**: sequence variation that is common within a population
  - "SNP on chromosome 16 associated with obesity"

Types of Genome Sequence Variants

1. **Single Nucleotide Variants (SNVs)**

   Single base changes, e.g., A→T.

2. **Insertions-Deletions (Indels)**

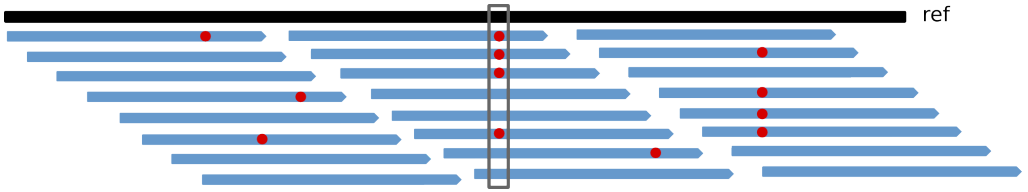
   Consisting of one or a few bases, e.g., +ATGA, ΔT.

3. **Structural Variants (SVs)**

   Everything else: large deletions, insertions, duplications, inversions, translocations, mobile element insertions, horizontal gene transfer

Objective

Assign a genotype to each position



Problems

Some variation observed in BAM files is caused by mapping and sequencing artifacts:

- **PCR artifacts**:
  - Mismatches due to errors in early PCR rounds
  - PCR duplicates
- **Sequencing errors**: erroneous call, either for physical reasons or to properties of the sequenced DNA
- **Mapping errors**: often happens around repeats or other low-complexity regions

Separate **true variation** from machine artifacts

Variant calling process pipeline

1. **Mark duplicates**

   Duplicates should not be counted as additional evidence

2. **Local realignment around INDELS**

   Reads mapping on the edges of INDELS often get mapped with mismatching bases introducing false positives

3. **Base quality score recalibration (BQSR)**

   Quality scores provided by sequencing machines are generally inaccurate and biased

4. **Variant calling**

   Discover variants and their genotypes

1. Mark duplicates

- All NGS **sequencing platforms are NOT single molecule sequencing** → the same DNA molecule can be sequenced several times
- **PCR** → duplicate DNA fragments in the final library
- If there is a base variation it will have **high depth support**
- Can result in **false variant calls**

Tools

- **Samtools**: samtools rmdup or samtools rmdupse
- **Picard**: MarkDuplicates

1. Mark duplicates

- All NGS **sequencing platforms are NOT single molecule sequencing** → the same DNA molecule can be sequenced several times
- **PCR** → duplicate DNA fragments in the final library
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Tools

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- **Picard**: MarkDuplicates

1. Mark duplicates

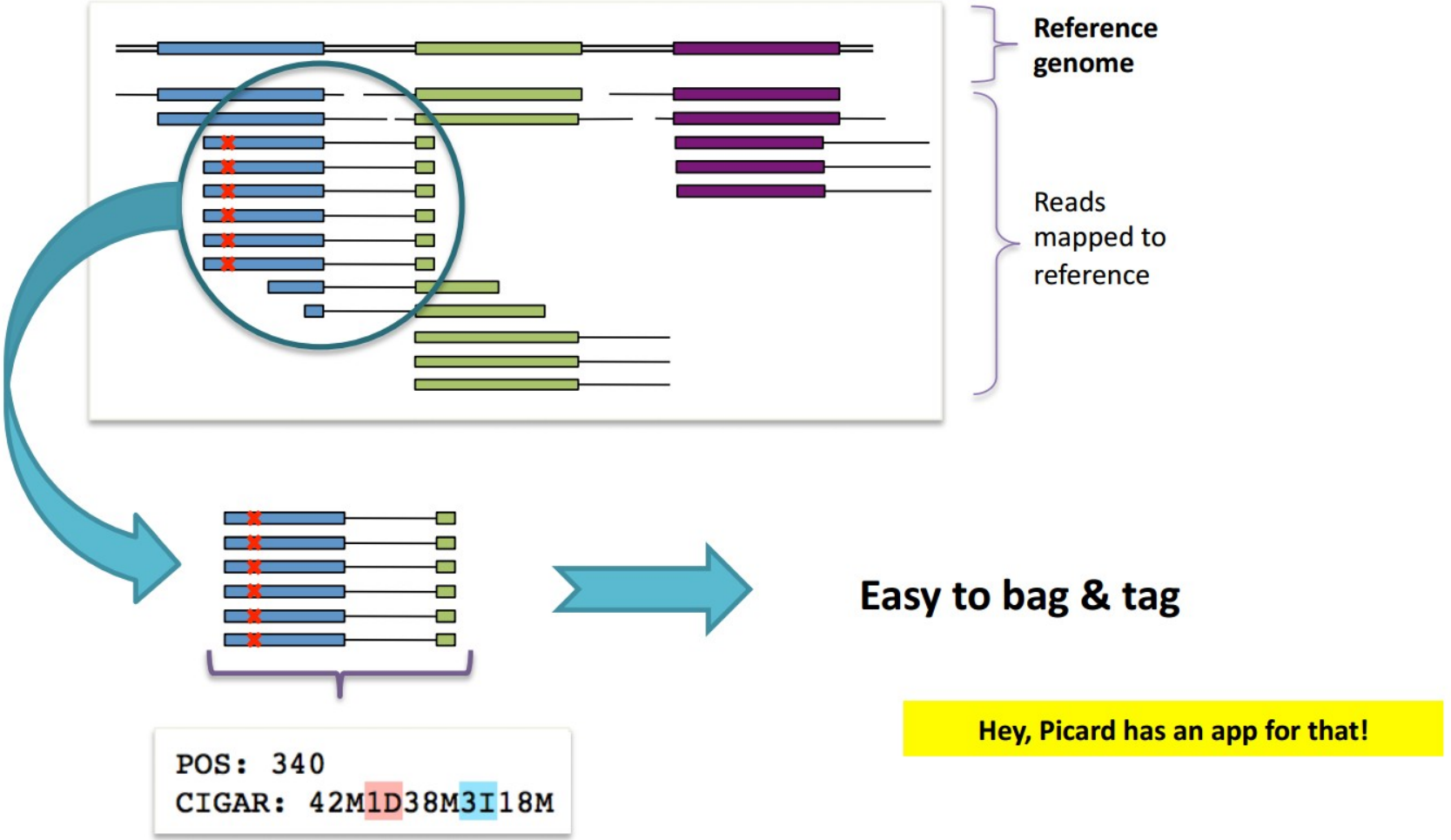
The reason why duplicates are bad

✖ = sequencing error propagated in duplicates

1. Mark duplicates

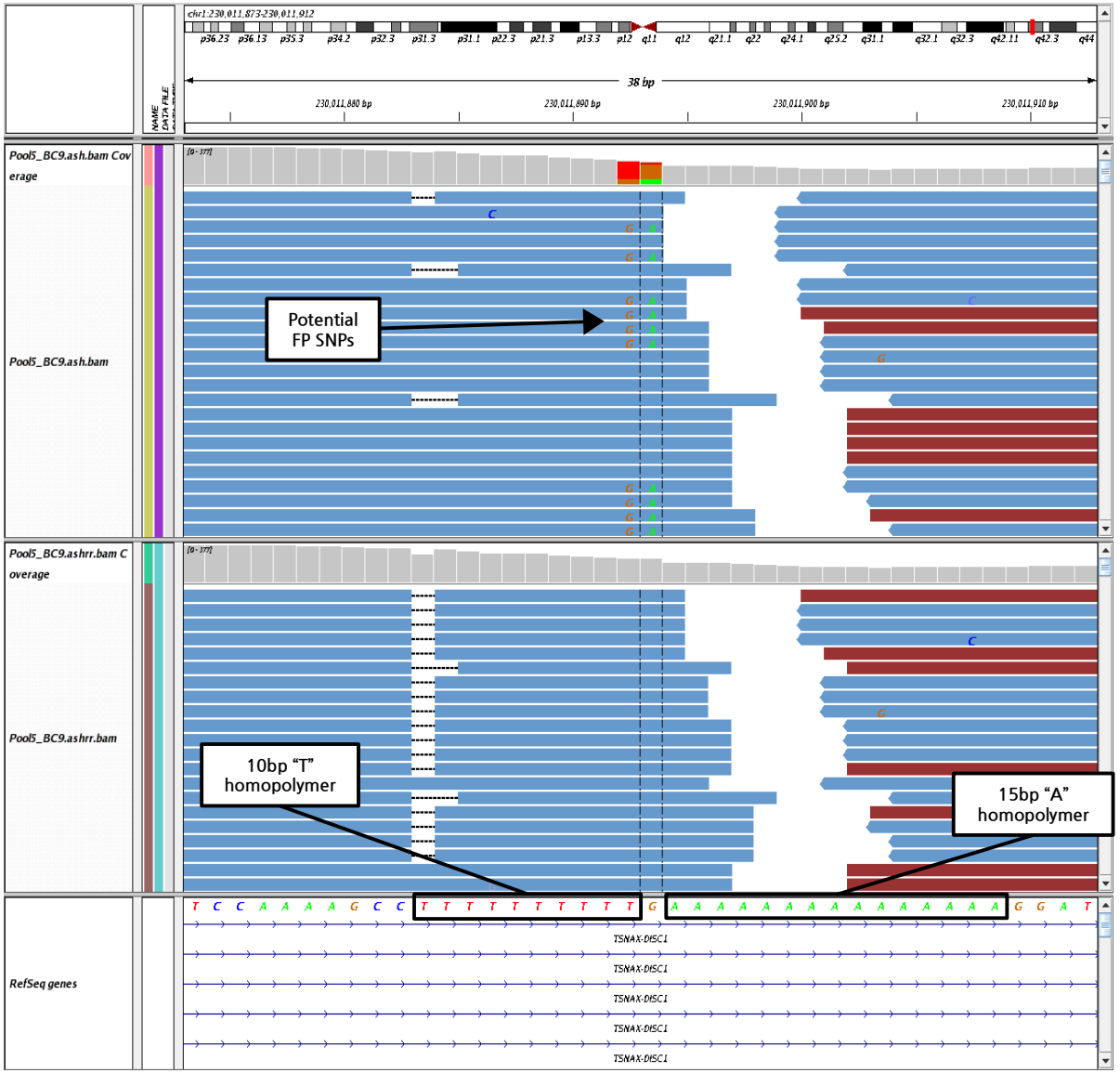
Duplicate identification

Duplicates have the **same starting position** and the **same CIGAR** string



2. Local realignment around INDELS

- Reads **near INDELS** are mapped with mismatches
- **Realignment** can identify the most consistent placement for these reads
  1. **Identify** problematic regions
  2. **Determine the optimal** consensus sequence
- **Minimizes mismatches** with the reference sequence
- **Refines** location of **INDELS**



DePristo MA, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011 May;43(5):491-8. PMID: 21478889

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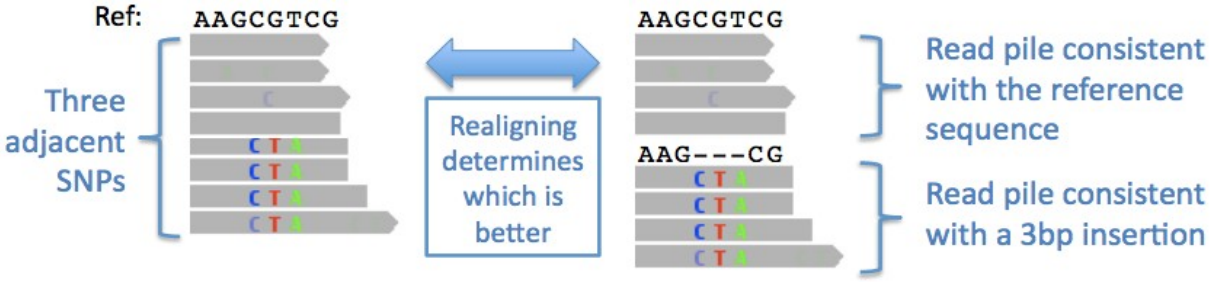


DePristo MA, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet. 2011 May;43(5):491-8. PMID: 21478889

3. Base quality score recalibration

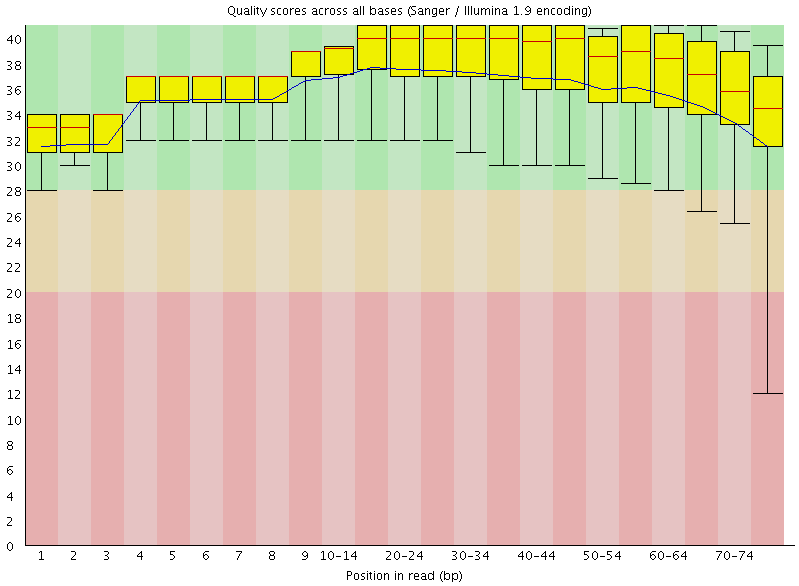
- **Calling algorithms rely** heavily on the **quality scores** assigned to the individual base calls in each sequence read
- Unfortunately, the scores produced by the machines are subject to various sources of **systematic error**, leading to over- or under-estimated base quality scores in the data

How?

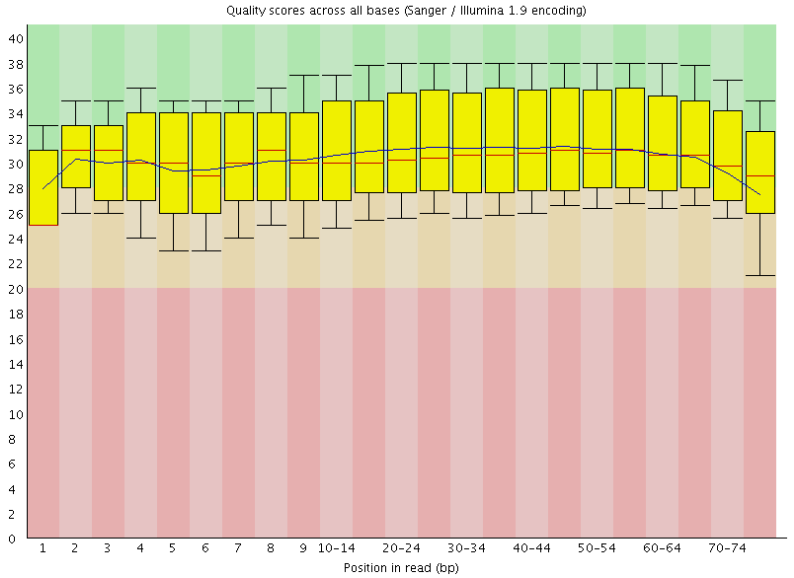
1. **Analyze covariation** among several features of a base:
   - Reported quality score
   - Position within the read
   - Preceding and current nucleotide
2. Use a set of **known variants** (i.e.: dbSNP) to model error properties of real polymorphism and determine the **probability that novel sites are real**
3. **Adjust** the quality scores of all reads in a BAM file

3. Base quality score recalibration

Before



After



Phred Quality score:

$$Q_{\text{Phred}} = -10 \log_{10} P(\text{error}).$$

A score of 20 corresponds to 1% error rate in base calling

4. Variant calling
Variant discovery process

Steps

1. **Variant calling:** Identify the positions that differ from the reference
2. **Genotype calling**: calculate the genotypes for each sample at these sites

Initial approach

Independent base assumption

Counting the number of times each allele is observed

Evolved approach

Bayesian inference → Compute genotype likelihood

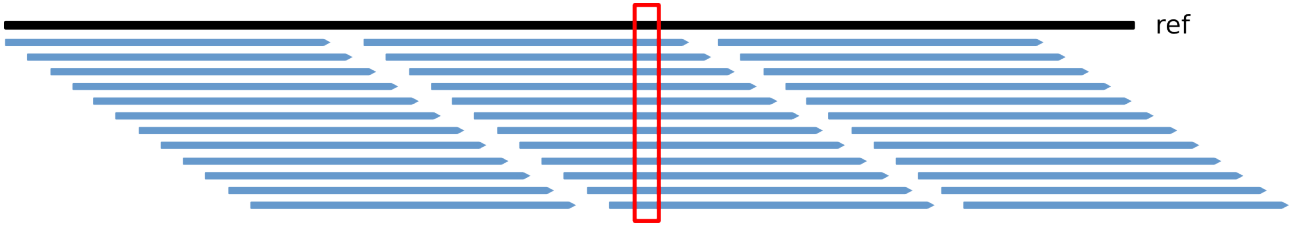
Advantages:

Provide statistical measure of **uncertainty**

Lead to **higher accuracy** of genotype calling

4. Variant calling

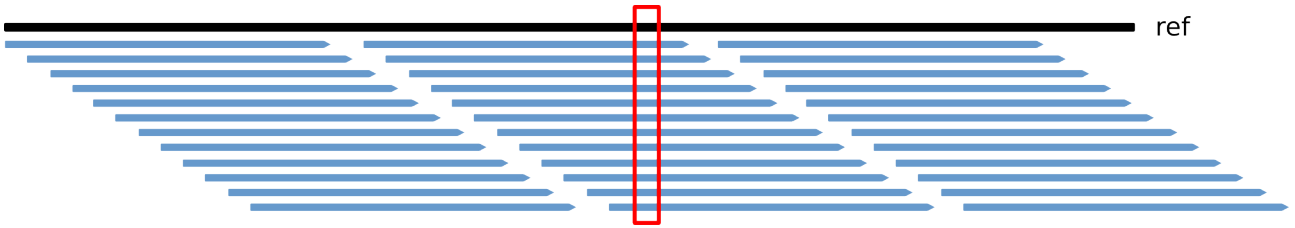
Variant discovery process



Reference = **A**

4. Variant calling

Variant discovery process



Reference = A

AAAAAAAAAAAAAAAAAAAAAAAAAAAAA

GGGGGGGGGGGGGGGGGGGGGGGGGGGGG

AAAAAAAAAAAAAAAGGGGGGGGGGGGGG

AAAAAAAAAAAAAAAGGGGGGGGGGGGCT

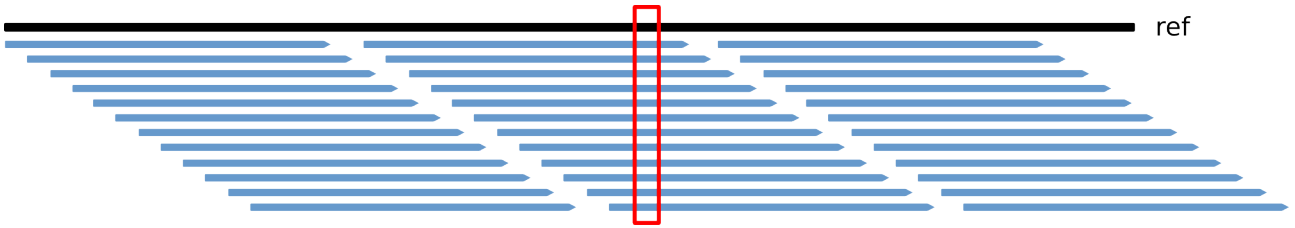
AAAGGGCCTT

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

4. Variant calling

Variant discovery process



Reference = A

AAAAAAAAAAAAAAAAAAAAAAAAAAAA	*N*=30, *X*=0
GGGGGGGGGGGGGGGGGGGGGGGGGGGG	*N*=30, *X*=30
AAAAAAAAAAAAAAGGGGGGGGGGGGGG	*N*=30, *X*=15
AAAAAAAAAAAAAAGGGGGGGGGGGGCT	*N*=30, *X*=12
AAAGGGCCTT	*N*=10, *X*=3

Cutoff for $X \rightarrow$ value or proportion

- $c = 30\%$ $\quad X \leq c \rightarrow$ **RR**, $\quad X > c \rightarrow$ **RV**
- $c_1 = 10\%$, $c_2 = 30\%$

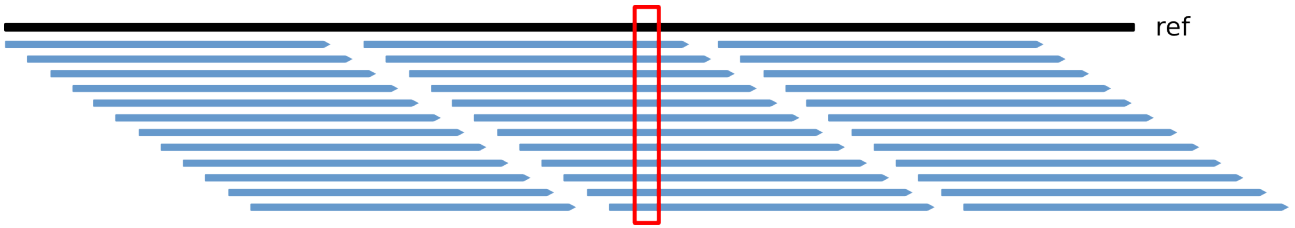
$$X \leq c_1 \rightarrow \textbf{RR}$$
$$c_1 < X < c_2 \rightarrow \textbf{RV}$$
$$X \geq c_2 \rightarrow \textbf{RR}$$

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

4. Variant calling

Variant discovery process



Reference = A

AAAAAAAAAAAAAAAAAAAAAAAAAAAA	N=30, X=0 →	**RR**
GGGGGGGGGGGGGGGGGGGGGGGGGGGG	N=30, X=30 →	**VV**
AAAAAAAAAAAAAAGGGGGGGGGGGGGG	N=30, X=15 →	**RV**
AAAAAAAAAAAAAAGGGGGGGGGGGGCT	N=30, X=12 →	**RV**
AAAGGGCCTT	N=10, X=3 →	**RV?**

Cutoff for X → value or proportion

- $c = 30\%$ $\quad X \leq c$ → **RR**, $\quad X > c$ → **RV**
- $c_1 = 10\%$, $c_2 = 30\%$

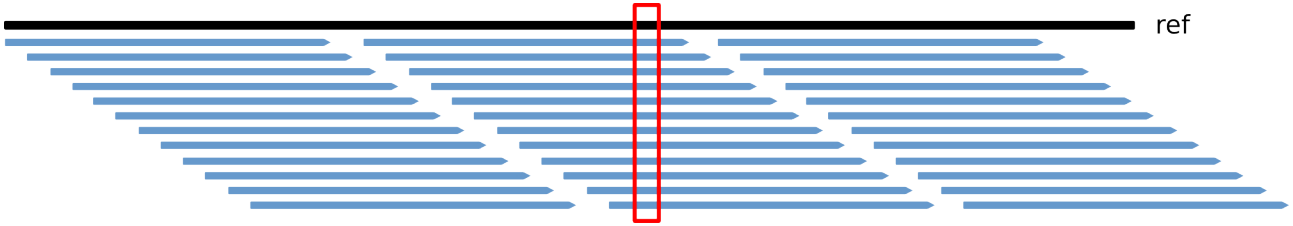
$$X \leq c_1 \rightarrow \textbf{RR}$$
$$c_1 < X < c_2 \rightarrow \textbf{RV}$$
$$X \geq c_2 \rightarrow \textbf{RR}$$

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

4. Variant calling

Variant discovery process



Bayesian approximation

α = nucleotide-base error rate

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

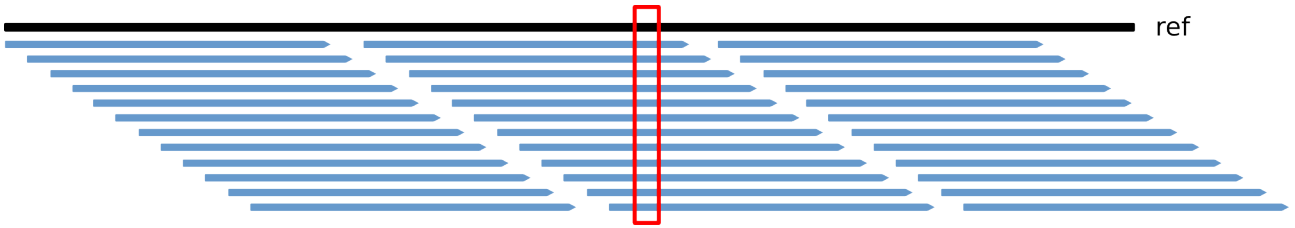
$$P(G=RR, X|N, \alpha) = \text{P of all R calls being correct and all V calls being wrong}$$

$$P(G=VV, X|N, \alpha) = \text{P of all V calls being correct and all R calls being wrong}$$

$$P(G=RV, X|N, \alpha) = \text{P of all R and V calls being correct}$$

4. Variant calling

Variant discovery process



Bayesian approximation

α = nucleotide-base error rate

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

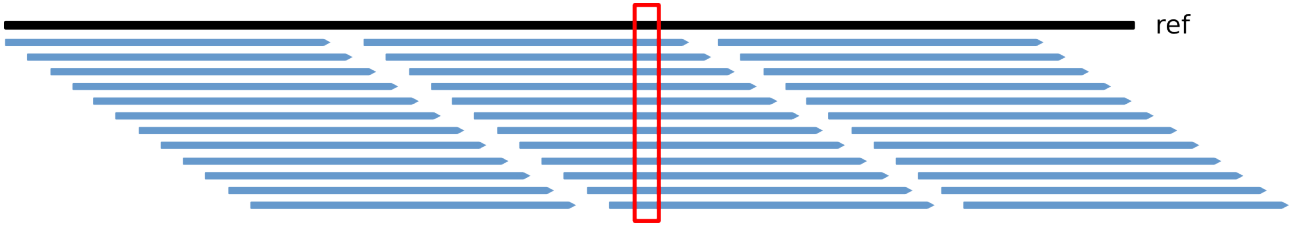
$$P(G=RR, X|N, \alpha) = \binom{N}{X} \alpha^{X} (1-\alpha)^{N-X}$$

$$P(G=VV, X|N, \alpha) = \binom{N}{X} (1-\alpha)^{X} \alpha^{N-X}$$

$$P(G=RV, X|N, \alpha) = \binom{N}{X} \left(\frac{1}{2}\right)^{N}$$

4. Variant calling

Variant discovery process



Bayesian approximation

α = nucleotide-base error rate

p_{VV}

p_{VR}

} Prior probabilities

N = nucleotides
G = true genotype
R = reference base
V = variant base
X = variant nucleotides

Outcomes:
RR RV VV

$$P(G=RR, X|N, \alpha) = \binom{N}{X}\alpha^{X}(1-\alpha)^{N-X}(1-p_{VV}-p_{RV})$$

$$P(G=VV, X|N, \alpha) = \binom{N}{X}(1-\alpha)^{X}\alpha^{N-X}p_{VV}$$

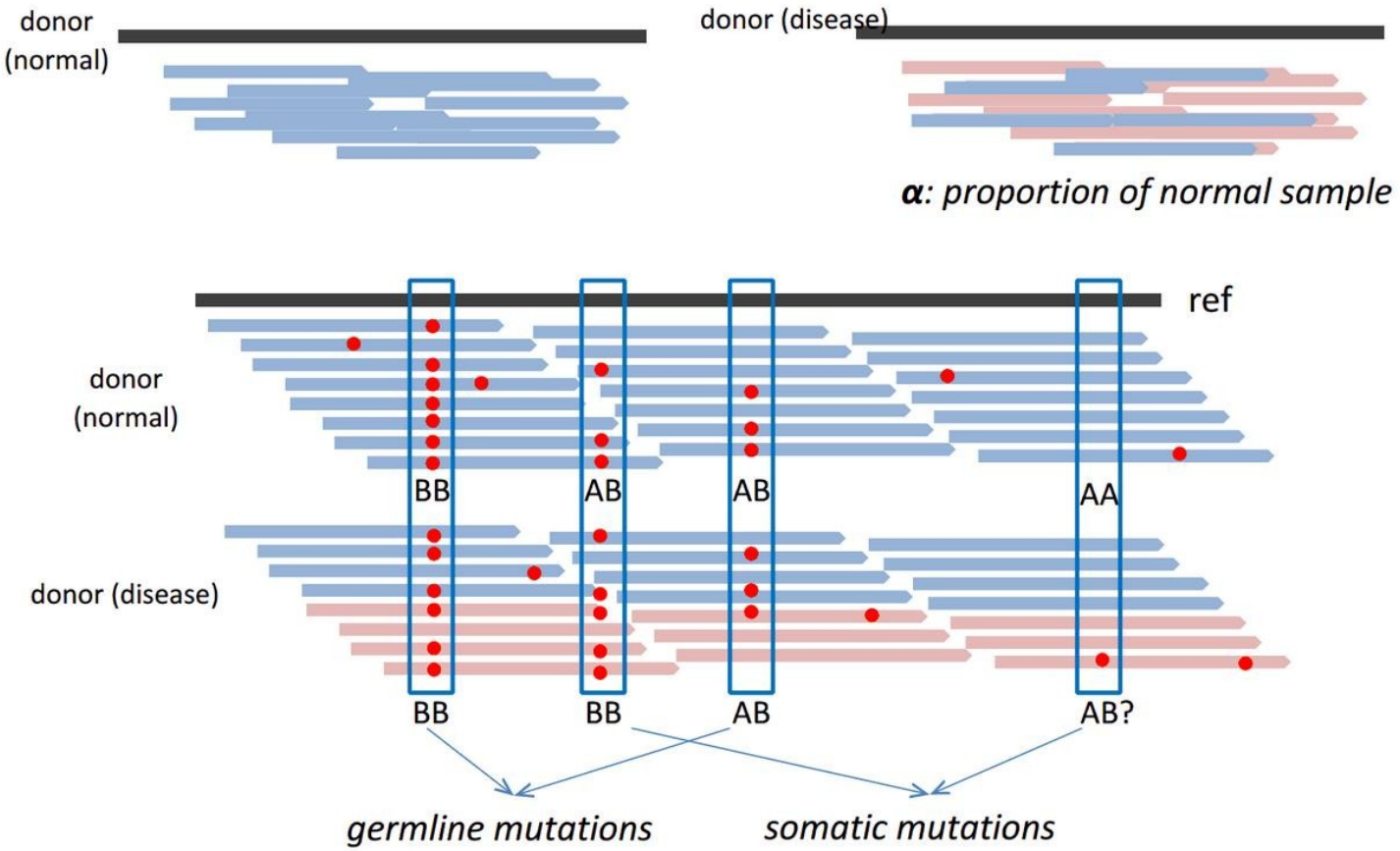
$$P(G=RV, X|N, \alpha) = \binom{N}{X}\left(\frac{1}{2}\right)^{N}p_{RV}$$

Somatic calling

Detecting somatic SNVs in cancer

Challenges:

- Somatic variants occur at low frequency in genome
- Most tumors are impure and heterogeneous



VCF file format

- Specification defined by the 1000 genomes (current version **4.2**):
  http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-41
- Commonly **compressed and indexed** with bgzip/tabix
- Single-sample or multi-sample VCF

```
##fileformat=VCFv4.1
##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">
#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
```

VCF file format

```
#CHROM  POS     ID        REF    ALT     QUAL   FILTER   INFO
20      14370   rs6054257 G      A       29     PASS     NS=3;DP=14;AF=0.5;DB;H2

FORMAT          NA00001           NA00002           NA00003
GT:GQ:DP:HQ     0|0:48:1:51,51    1|0:48:8:51,51    1/1:43:5:.,.
```

genotype genotype quality read depth haplotype qualities

- **CHROM**: chromosome
- **POS**: position
- **ID**: identifier
- **REF**: reference base(s)
- **ALT**: non-reference allele(s)
- **QUAL**: quality score of the calls (phed scale)
- **FILTER**: “PASS” or a filtering tag
- **INFO**: additional information
- **FORMAT**: describes the information given by sample

Software

Software	Available from	Calling method	Prerequisites	Comments	Refs
SOAP2	http://soap.genomics.org.cn/index.html	Single-sample	High-quality variant database (for example, dbSNP)	Package for NGS data analysis, which includes a single individual genotype caller (SOAPsnp)	15
realSFS	http://128.32.118.212/thorfinn/realSFS/	Single-sample	Aligned reads	Software for SNP and genotype calling using single individuals and allele frequencies. Site frequency spectrum (SFS) estimation	-
Samtools	http://samtools.sourceforge.net/	Multi-sample	Aligned reads	Package for manipulation of NGS alignments, which includes a computation of genotype likelihoods (samtools) and SNP and genotype calling (bcftools)	53
GATK	http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit	Multi-sample	Aligned reads	Package for aligned NGS data analysis, which includes a SNP and genotype caller (Unifed Genotyper). SNP filtering (Variant Filtration) and SNP quality recalibration (Variant Recalibrator)	32,33
Beagle	http://faculty.washington.edu/browning/beagle/beagle.html	Multi-sample LD	Candidate SNPs, genotype likelihoods	Software for imputation, phasing and association that includes a mode for genotype calling	42
IMPUTE2	http://mathgen.stats.ox.ac.uk/impute/impute_v2.html	Multi-sample LD	Candidate SNPs, genotype likelihoods	Software for imputation and phasing, including a mode for genotype calling. Requires fine-scale linkage map	44
QCall	ftp://ftp.sanger.ac.uk/pub/rd/QCALL	Multi-sample LD	'Feasible' genealogies at a dense set of loci, genotype likelihoods	Software for SNP and genotype calling, including a method for generating candidate SNPs without LD information (NLDA) and a method for incorporating LD information (LDA). The 'feasible' genealogies can be generated using Margarita (http://www.sanger.ac.uk/resources/software/margarita)	54
MaCH	http://genome.sph.umich.edu/wiki/Thunder	Multi-sample LD	Genotype likelihoods	Software for SNP and genotype calling, including a method (GPT_Freq) for generating candidate SNPs without LD information and a method (thunder_glf_freq) for incorporating LD information	-

A more complete list is available from http://seqanswers.com/wiki/Software/list. LD, linkage disequilibrium; NGS, next-generation sequencing.

GATK (Genome Analysis ToolKit)

http://www.broadinstitute.org/gatk/

- Probabilistic method: **Bayesian estimation** of the most likely genotype
- Calculates many **parameters** for each position of the genome
- INDEL realignment
- Base quality recalibration
- SNP and INDEL calling
- **Multi-sample** calling
- Uses standard input and output files
- Used in **many NGS projects**, including the 1000 Genomes Project, The Cancer Genome Atlas, etc.

GATK prerequisites

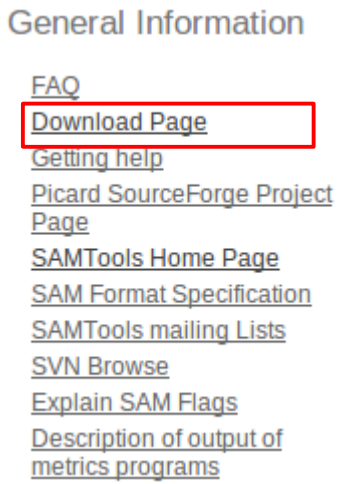
- **Requires Java** (http://www.oracle.com/technetwork/java/javase/downloads/index.html)
  - Check your java version

```
java -version
```

  GATK ≥ 2.6 → Requires Java version 1.7

- **Picard**
  - Website: http://picard.sourceforge.net/
  - Go to Download page and select

    Download picard-tools-1.114.zip (48.0 MB)



  - Testing:

```
java -jar AddOrReplaceReadGroups.jar -h
```

  - Usage

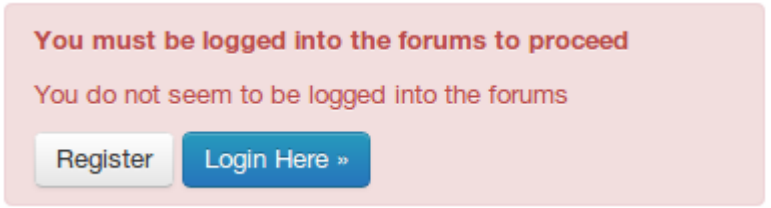
```
java -jar <ToolName> [options]
```

GATK installation

- **GATK 3.1 download**

  http://www.broadinstitute.org/gatk/

  - We need to register before download
  - Go to Downloads and click 
  - Accept the license agreement
  - Extract the file in the applications folder



- **Check if GATK is working**

Show GATK help

```
java -jar GenomeAnalysisTK.jar -h
```

- **Usage**

```
java -jar GenomeAnalysisTK.jar -T <ToolName> [arguments]
```

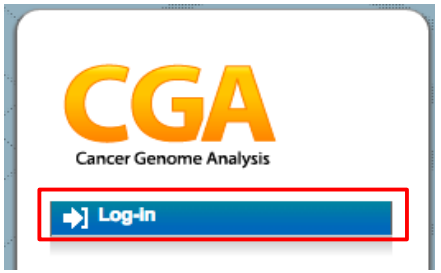
MuTect installation

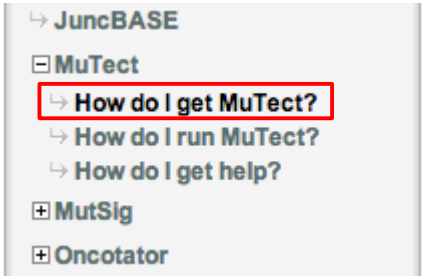
- **MuTect download**

  http://www.broadinstitute.org/cancer/cga/mutect

  - Click ***Log-in*** and go to the ***Create new account*** tab
  - Fill the form
  - Go to ***How do I get mutect*** and accept the license agreement
  - Download the latest version

    muTect-1.1.4-bin.zip
  - Extract the file in the applications folder





- **Check if MuTect is working**

```
java -jar muTect-1.1.4.jar -h
```

- **Usage**

```
java -jar muTect-1.1.4.jar --analysis_type MuTect [arguments]
```

THANK YOU.