From raw reads to variants

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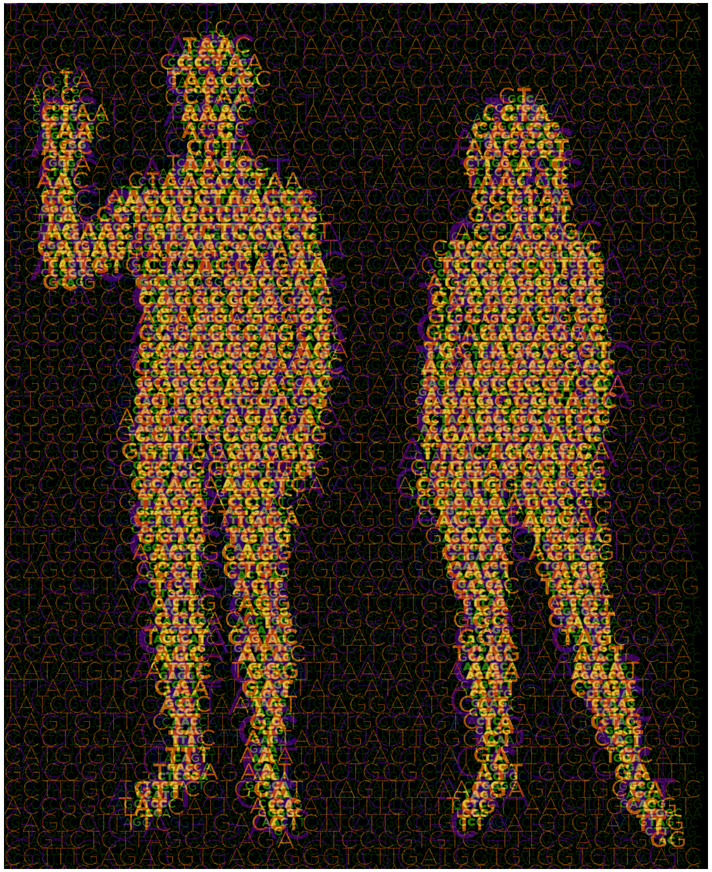
Talk Overview

- Concepts
  - Reference genome
  - Variants
  - Paired-end data
- NGS Workflow
  - Quality control & Trimming
  - Alignment
  - Local realignment
  - PCR duplicates & removal
  - Base Quality Score Recalibration
  - Variant calling
- VCF files
- Joint genotyping & gVCF files
- Annotation & Filtering

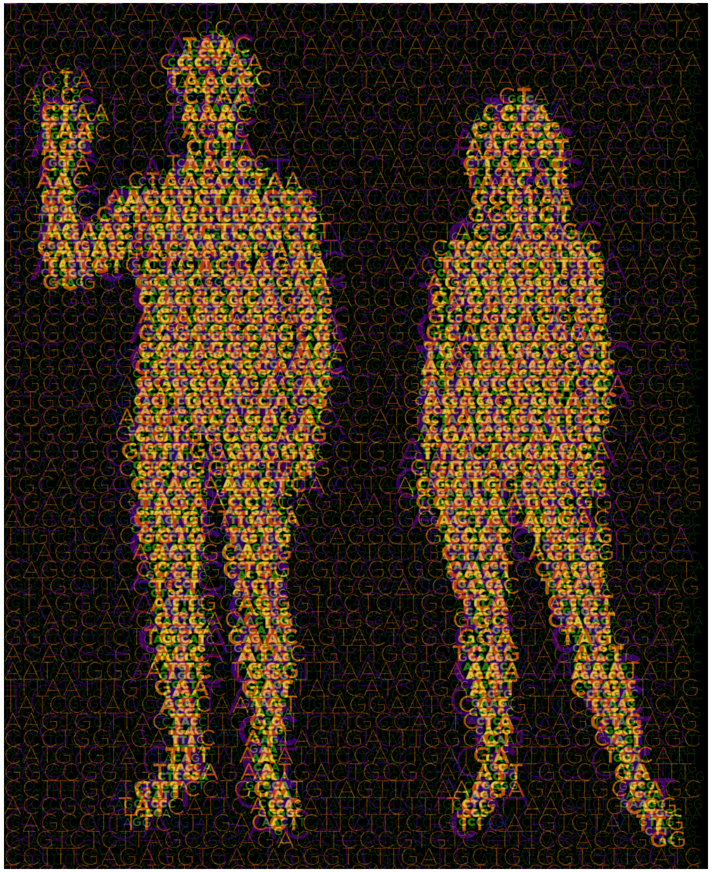
Reference genome

- Genome Reference Consortium
- A mosaic nucleic acid sequence
  - ...GTGCGTAGACTGCTAGATCGAAGA...



Reference genome

- Genome Reference Consortium
- A mosaic nucleid acid sequence
  - ...GTGCGTAGACTGCTAGATCGAAGA...
- What changes between versions?
  - First version: 150,000 gaps
  - HG19: 250 gaps



Variants

A position where sample sequence does not agree with reference genome sequence

Reference: ...GTGCGTAGACTGCTAGATCGAAGA...

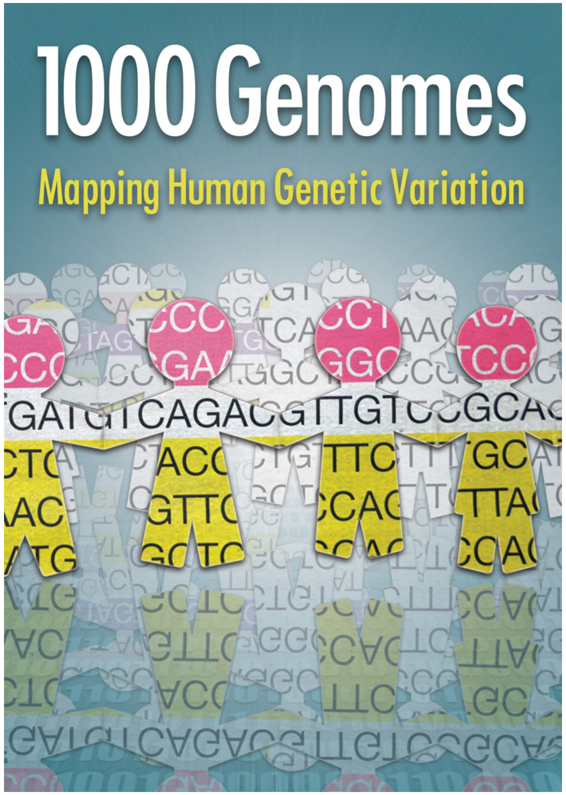
Variants

A position where sample sequence does not agree with reference genome sequence

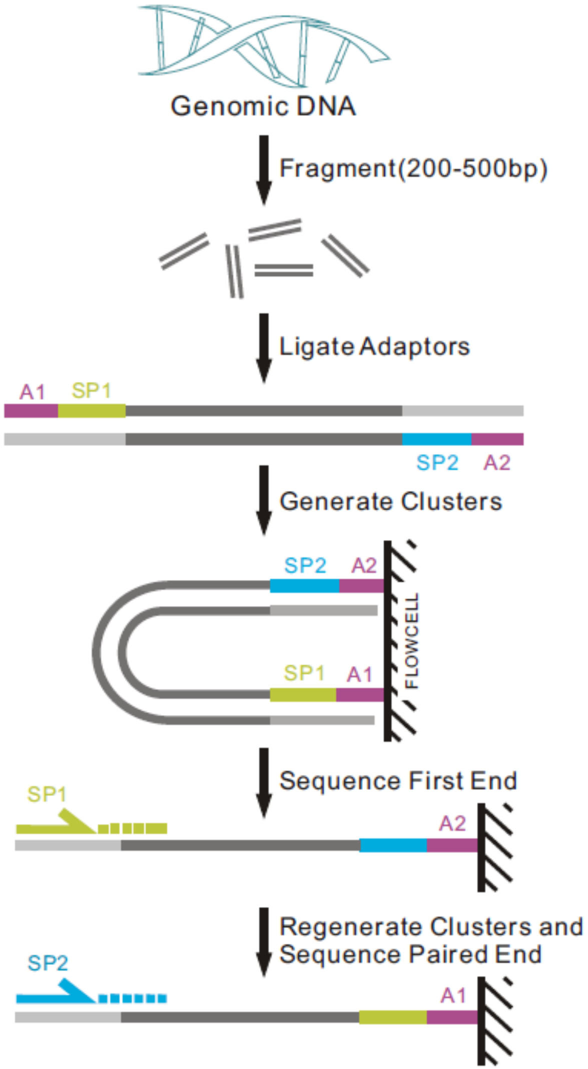
Reference: ...GTGCGTAGACTGCTAGATCGAAGA...
Sample: ...GTGCGTAGACTGATAGATCGAAGA...

Variants

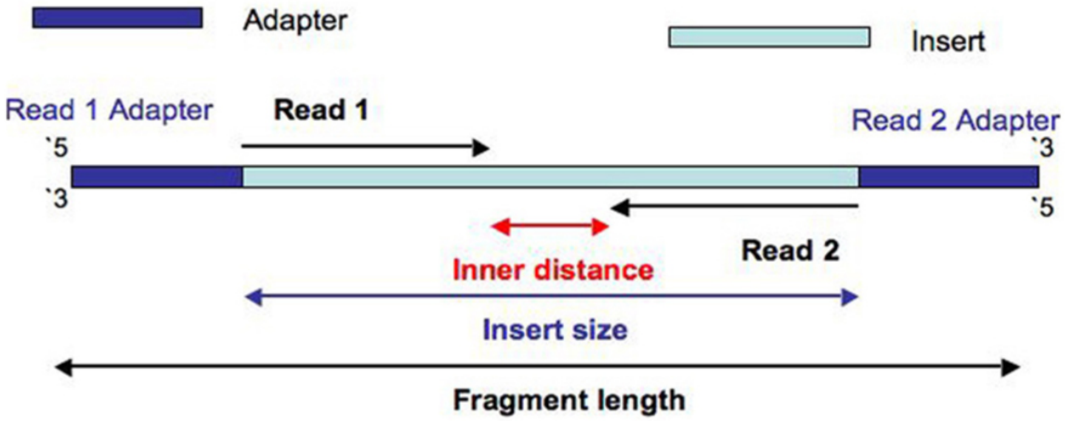
Population based variant projects



Paired-end sequencing



Paired-end data



Paired-end data

The forward and reverse reads are stored in two fastq files.

ID_R1_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:2
197 1:N:0:ATCACG
CAGTTGCGATGAGAGCGTTGAGAAGTATAATAGG
AGTTAAACTGAGTAACAGGATAAGAAATAGTGAG
ATATGGAAACGTTGTGGTCTGAAAGAAGATGT
+
B@CFFFFFHHHHHGJJJJJJJJJJFHHIIIIJJ
JIHGIIJJJJIJIJIJJJJIIJJJJIIEIHHIJ
HGHHHHDFFFEDDDDDCDDDCDDDDDDDCDC
```

ID_R2_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:
2197 2:N:0:ATCACG
CTTCGTCCACTTTCATTATTCCTTTCATACATG
CTCTCCGGTTTAGGGTACTCTTGACCTGGCCTT
TTTTCAAGACGTCCCTGACTTGATCTTGAAACG
+
CCCFFFFFHHHHHJJJIJJJJJJJJJJJJJJJJ
JJJJJJJIJIJGIJHBGHHIIIJIJJJJJJJJI
JJJHFFFFFFDDDDDDDDDDDDDDDEDCCDDDD
```

Paired-end data

The forward and reverse reads are stored in two fastq files.

The order of pairs and naming is identical, except the designation of forward and reverse.

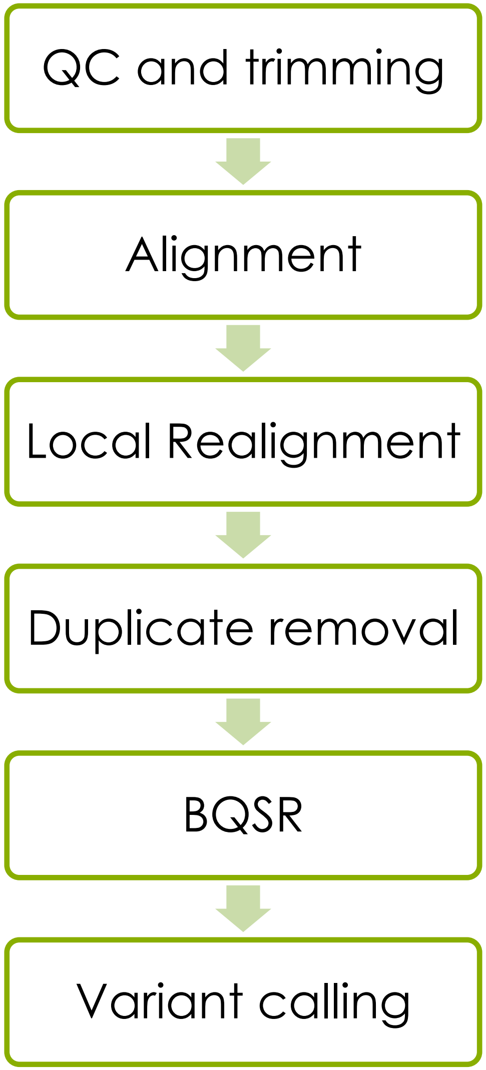
ID_R1_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:2
197 1:N:0:ATCACG
CAGTTGCGATGAGAGCGTTGAGAAGTATAATAGG
AGTTAAACTGAGTAACAGGATAAGAAATAGTGAG
ATATGGAAACGTTGTGGTCTGAAAGAAGATGT
+
B@CFFFFFHHHHHGJJJJJJJJJJFHHIIIIJJ
JIHGIIJJJJIJIJIJJJJIIJJJJJIIEIHHIJ
HGHHHHHDFFFEDDDDDCDDDCDDDDDDDCDC
```

ID_R2_001.fastq

```
@HISEQ:100:C3MG8ACXX:5:1101:1160:
2197 2:N:0:ATCACG
CTTCGTCCACTTTCATTATTCCTTTCATACATG
CTCTCCGGTTTAGGGTACTCTTGACCTGGCCTT
TTTTCAAGACGTCCCTGACTTGATCTTGAAACG
+
CCCFFFFFHHHHHJJJJIJJJJJJJJJJJJJJJ
JJJJJJJIJIJGIJHBGHHIIIJIJJJJJJJJI
JJJHFFFFFFDDDDDDDDDDDDDDDDEDCCDDDD
```

NGS workflow


QC and trimming

Alignment

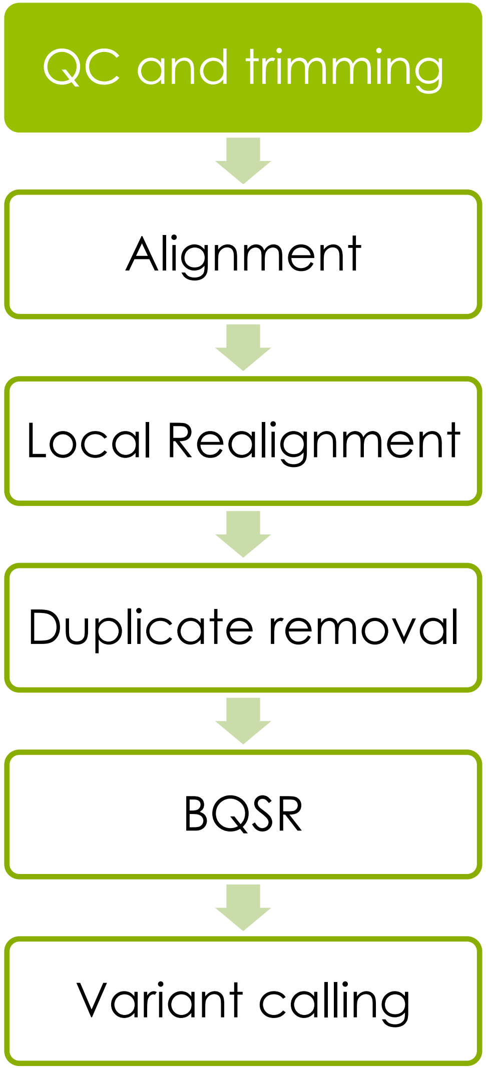
Local Realignment

Duplicate removal

BQSR

Variant calling

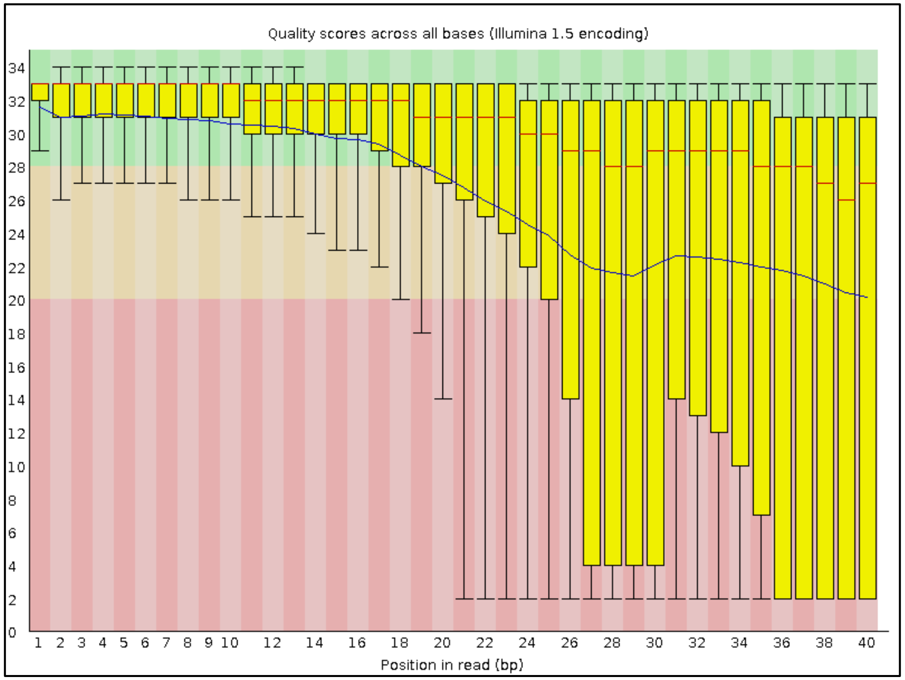
NGS workflow



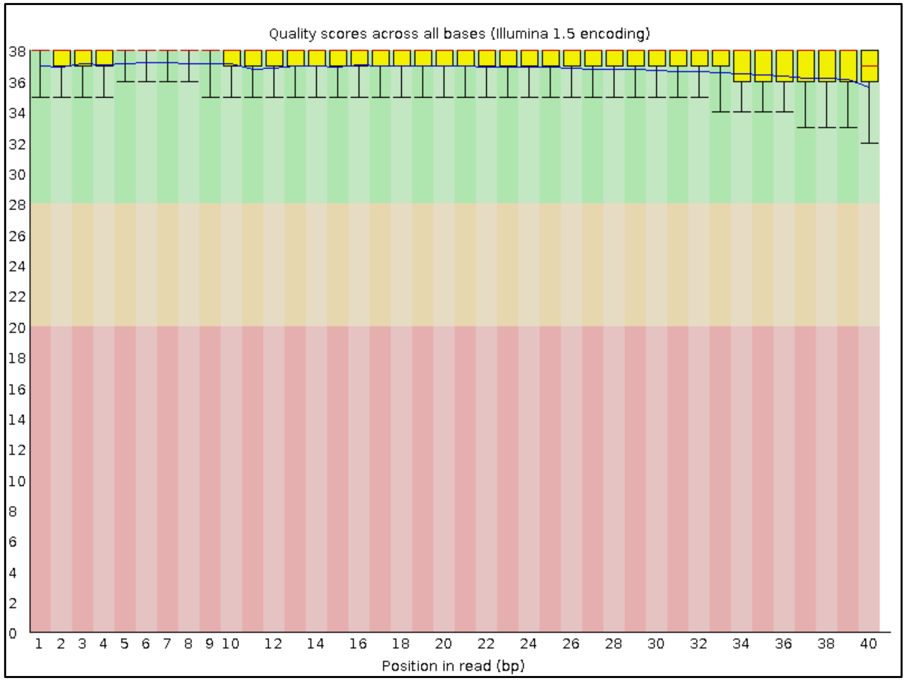
Quality control

```
module load FastQC
```

Bad qualities:



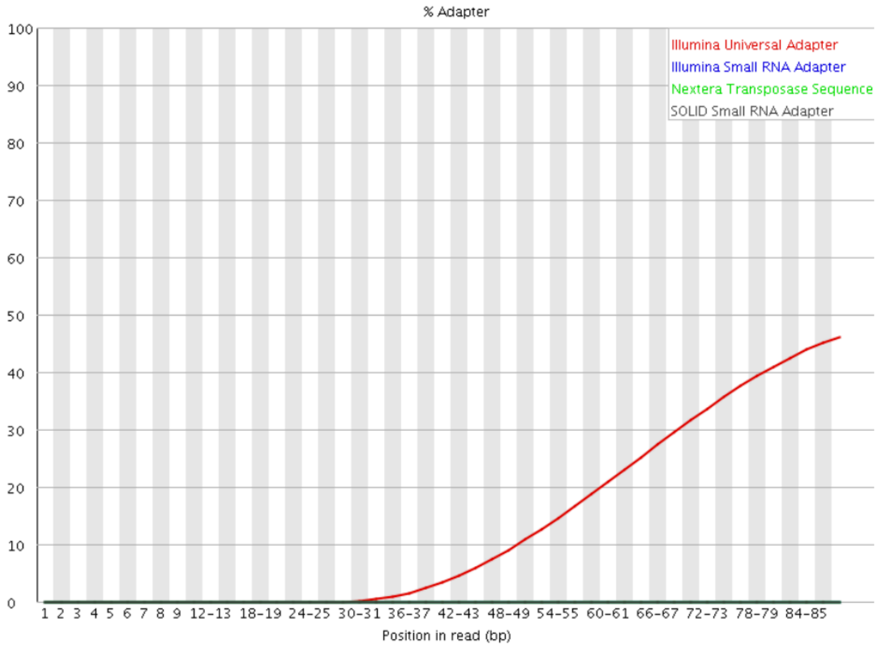
Good qualities:



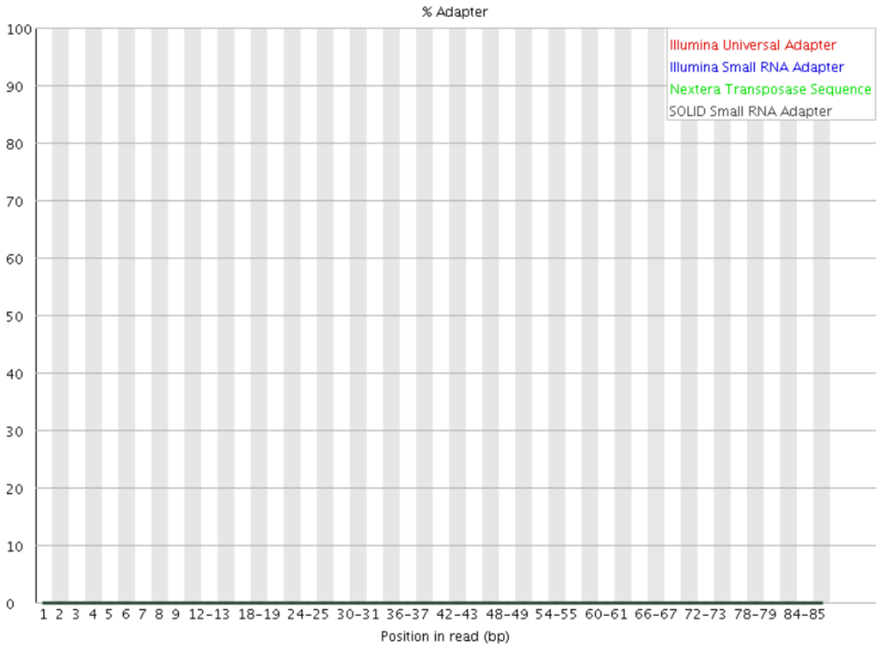
Quality control

```
module load FastQC
```

Adapters present:

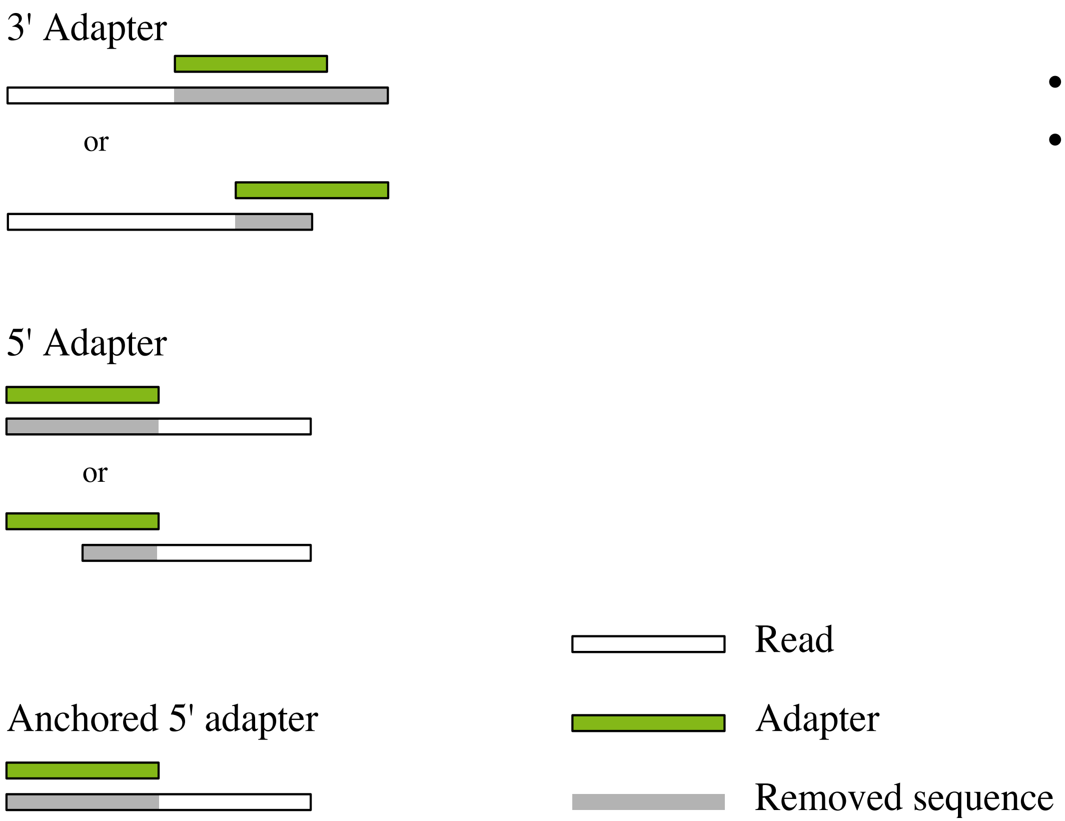


Adapters Absent:



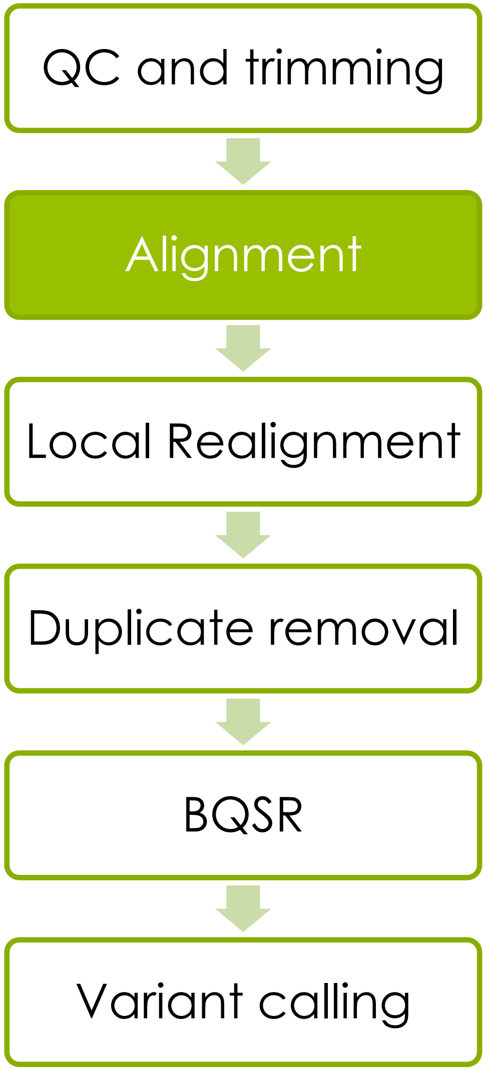
Trimming

```
module load cutadapt / TrimGalore / trimmomatic
```



- Remove bad quality reads
- Remove adapters

NGS workflow



QC and trimming

Alignment

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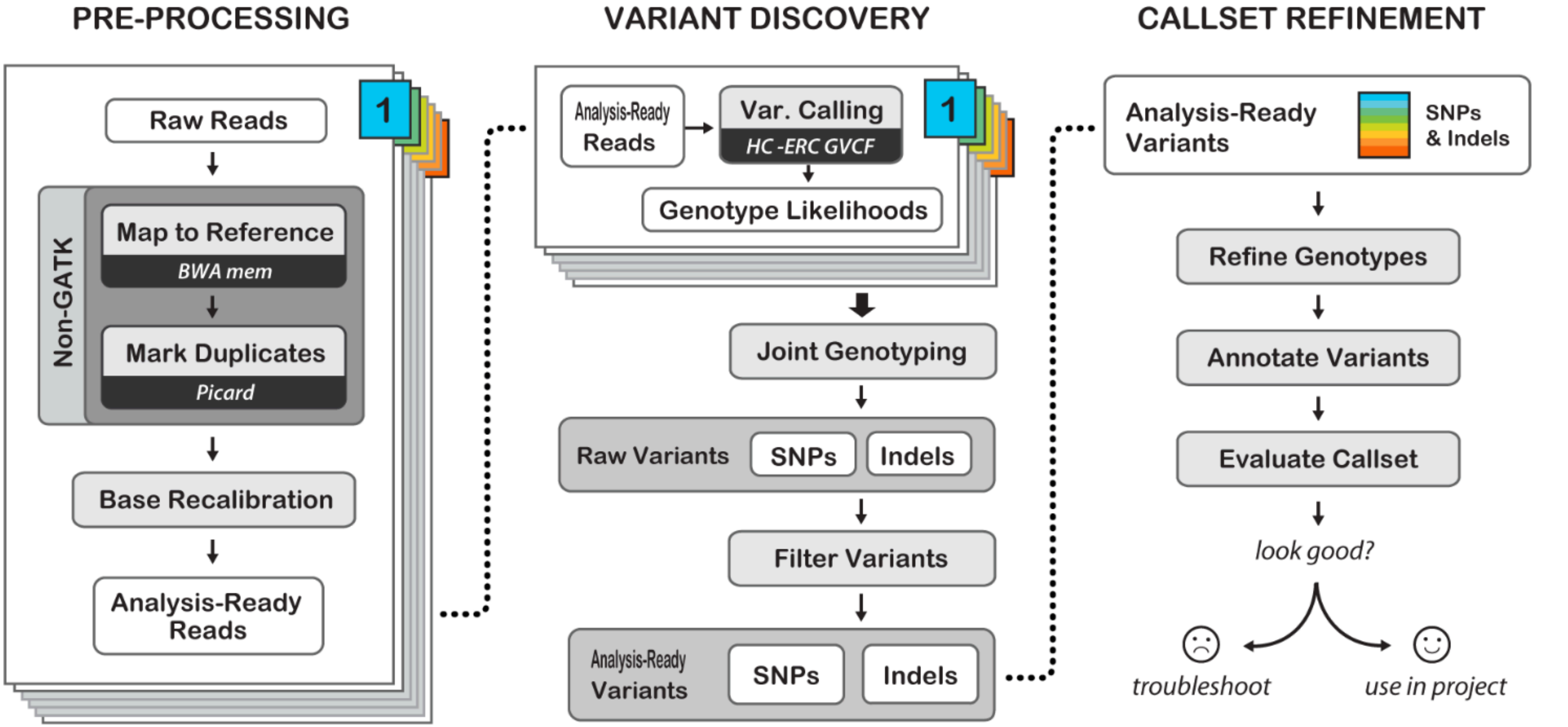
Variant calling

GATK Best Practices



Best Practices for Germline SNPs and Indels in Whole Genomes and Exomes - June 2016

https://software.broadinstitute.org/gatk/best-practices/

Alignment

```
module load bwa
```

Read	TCGATCC
Reference	GACCTCATCGATCCCACTG

Alignment

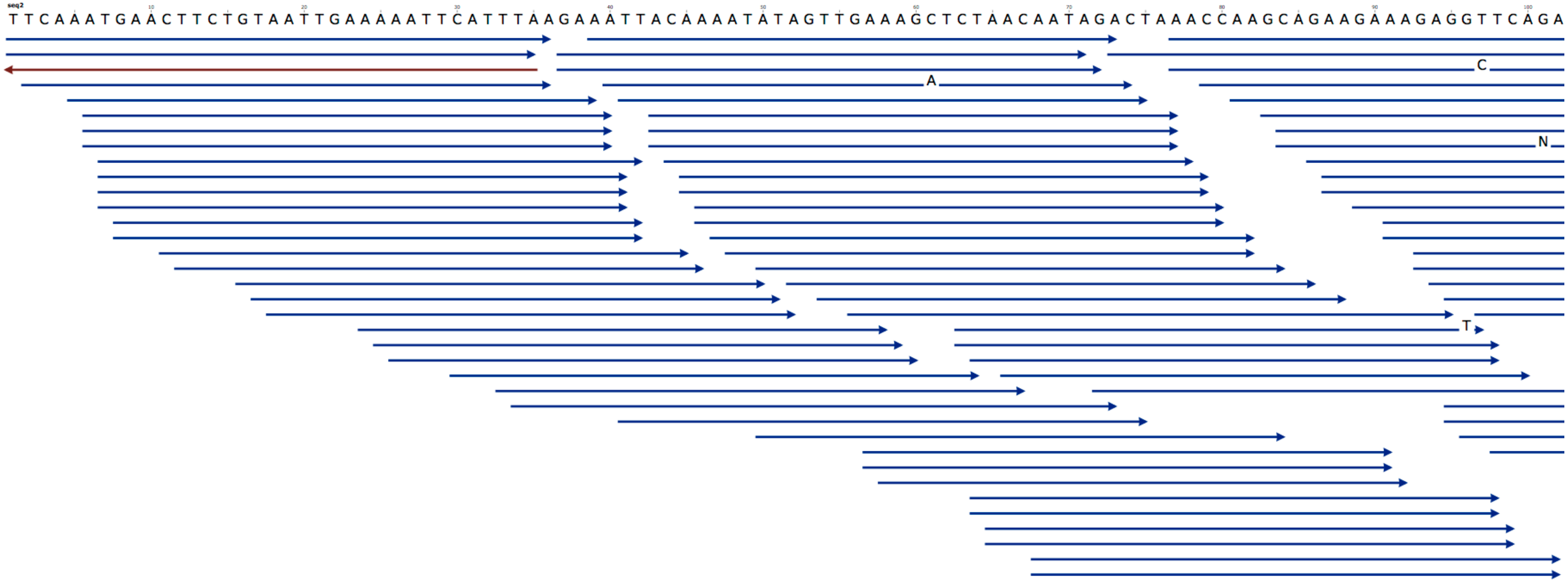
```
module load bwa
```

```
Read       TCGATCC
Reference  GACCTCATCGATCCCACTG
```

```
Read              TCGATCC
Reference  GACCTCATCGATCCCACTG
```

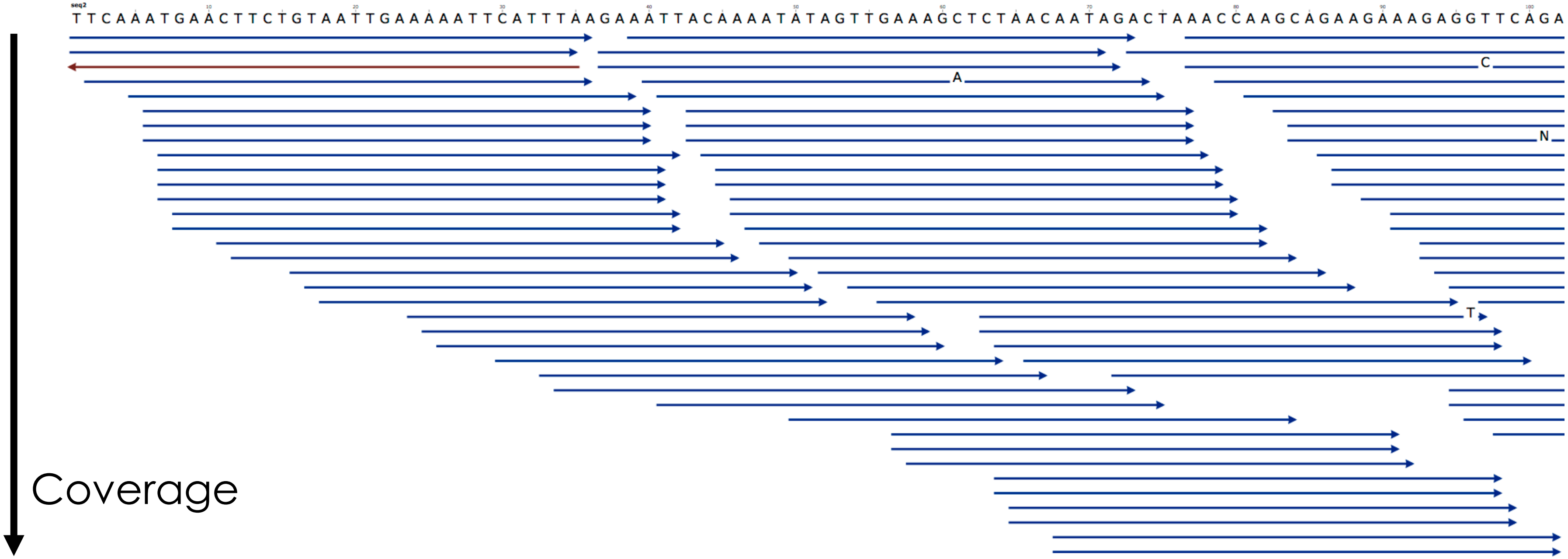
Alignment

```
module load bwa
```


seq2
10
20
30
40
50
60
70
80
90
100
TTCAAATGAACTTCTGTAATTGAAAAATTCATTTAAGAAATTACAAAATATAGTTGAAAGCTCTAACAATAGACTAAACCAAGCAGAAGAAAGAGGTTCAGA
C
A
N
T

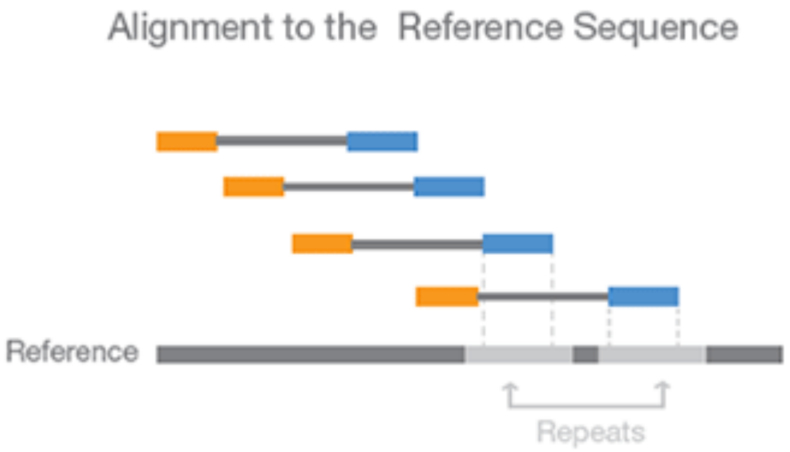
Alignment

```
module load bwa
```

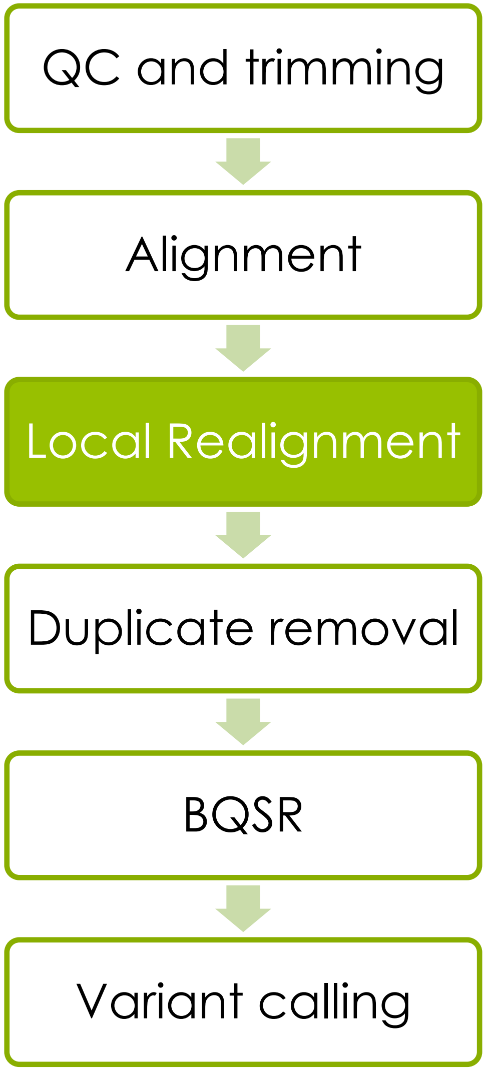

seq2
10
20
30
40
50
60
70
80
90
100
TTCAAATGAACTTCTGTAATTGAAAAATTCATTTAAGAAATTACAAAATATAGTTGAAAGCTCTAACAATAGACTAAACCAAGCAGAAGAAAGAGGTTCAGA
C
A
N
T
Coverage

Paired-end data & Alignment

The known distance between paired reads allows improved mapping over repeat regions



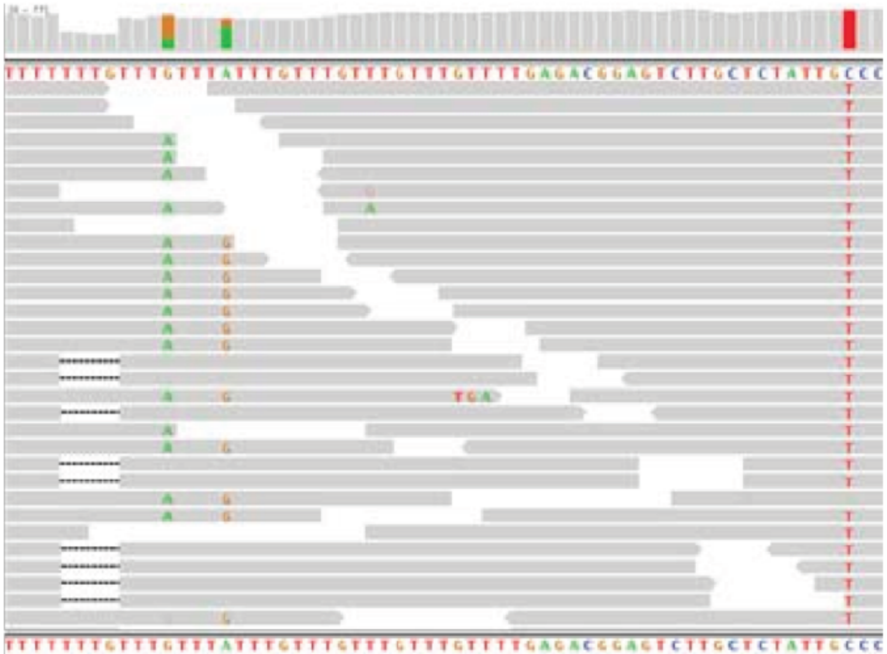
NGS workflow



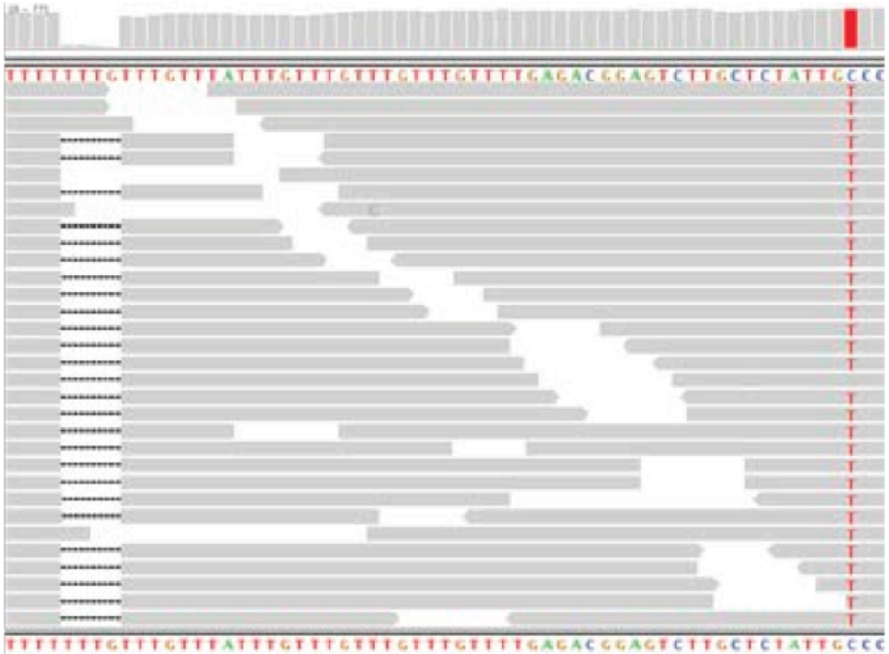
Local realignment

Problem: Reads are mapped **one** read at a time, this sometimes leads to single variants being split into multiple variants

Solution: Realign such a region taking **all** reads into account



HiSeq data, raw BWA alignments



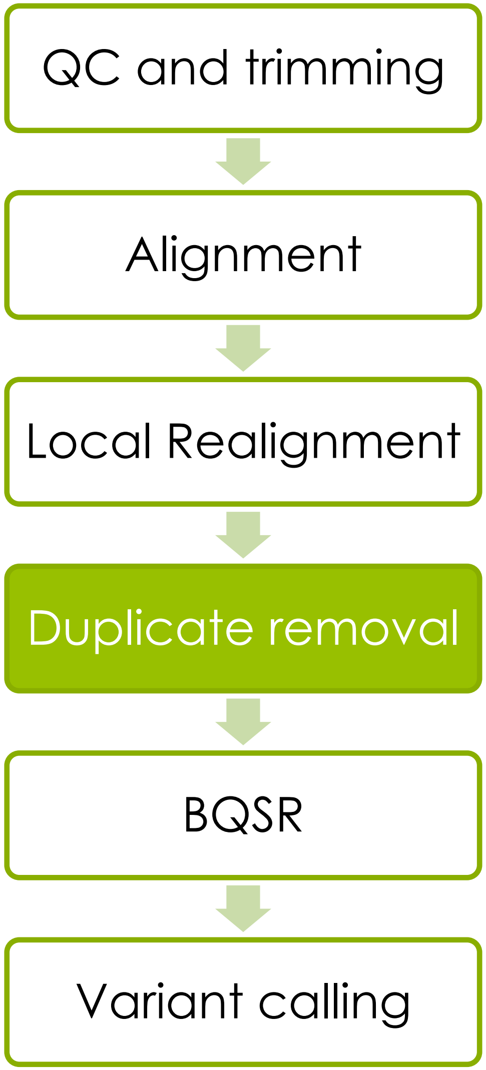
HiSeq data, after MSA

Local realignment

```
module load GATK
```

- `Genome Analysis ToolKit`
  - RealignerTargetCreator
  - IndelRealigner
- Local realignment, still needed?
  - HaplotypeCaller (HC)
  - Mutect2

NGS workflow



QC and trimming

Alignment

Local Realignment

Duplicate removal

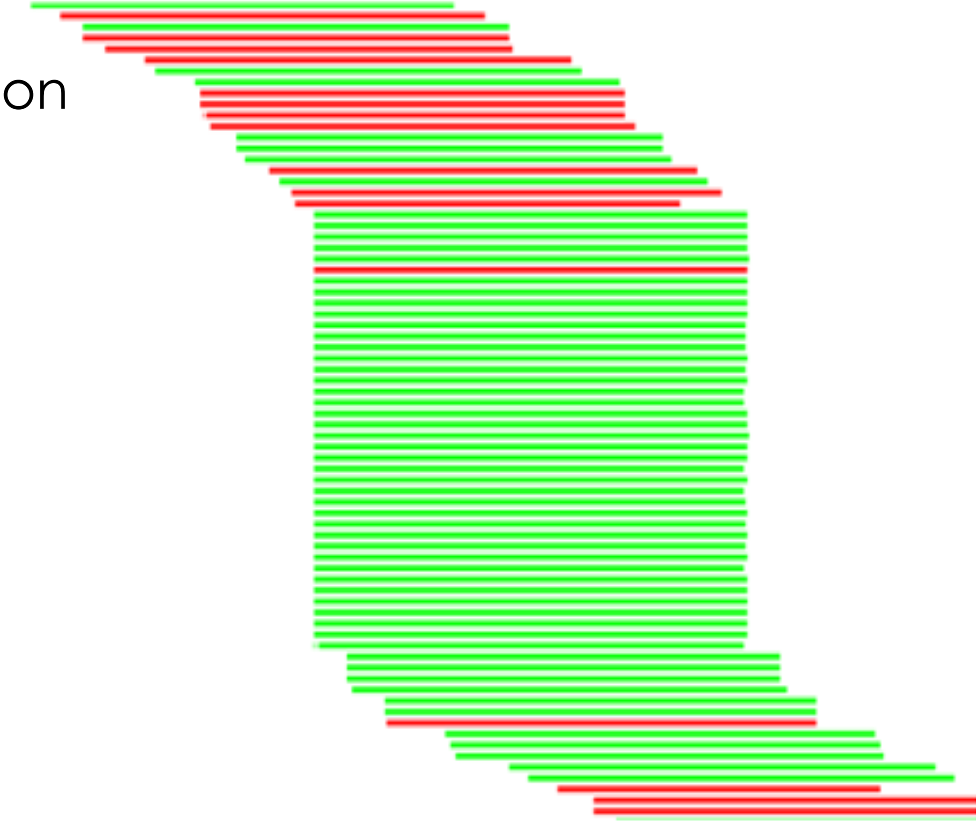
BQSR

Variant calling

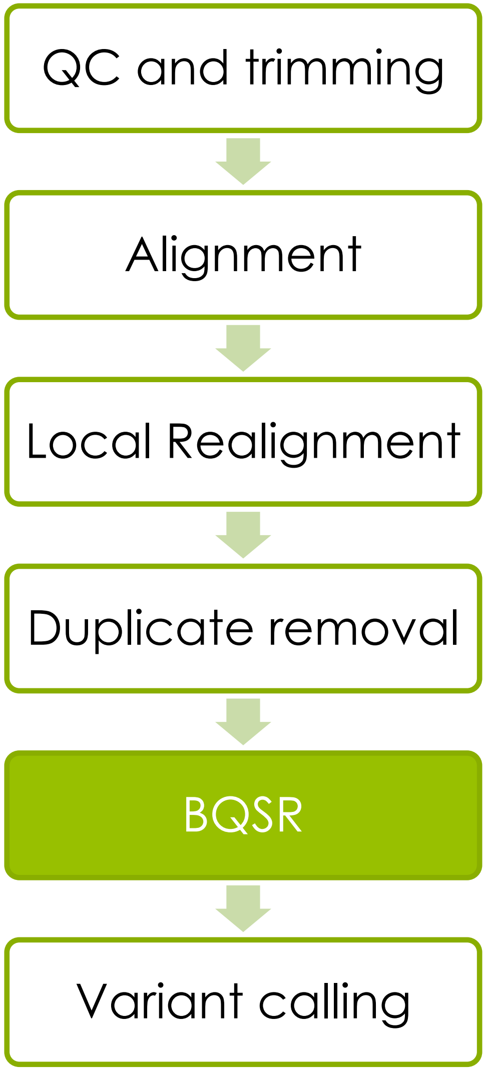
PCR duplicates & removal

```
module load picard
```

- Occur during library preparation
- Don’t add unique information
- Optical duplicates



NGS workflow



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Variant calling

Base Quality Score Recalibration

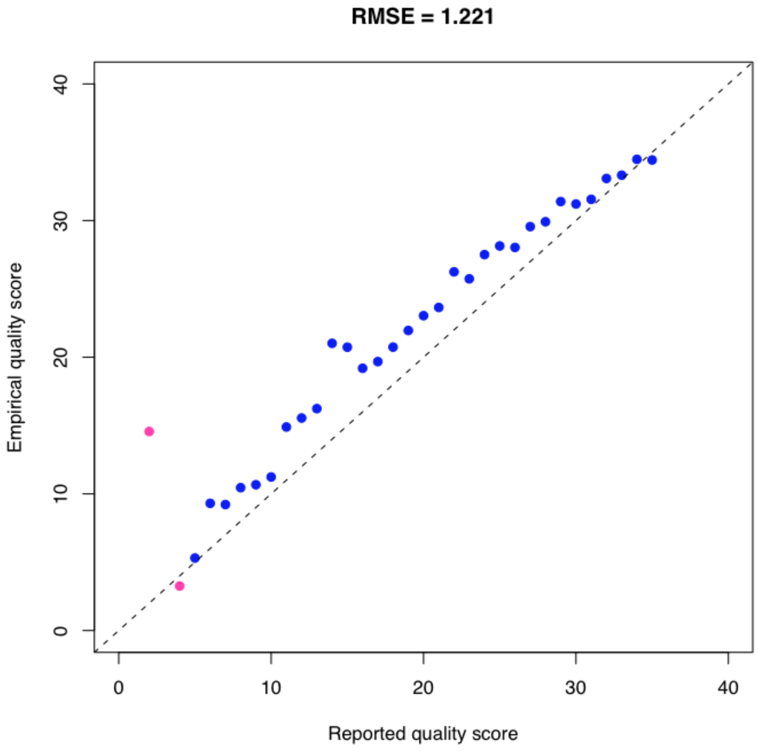
```
module load GATK
```

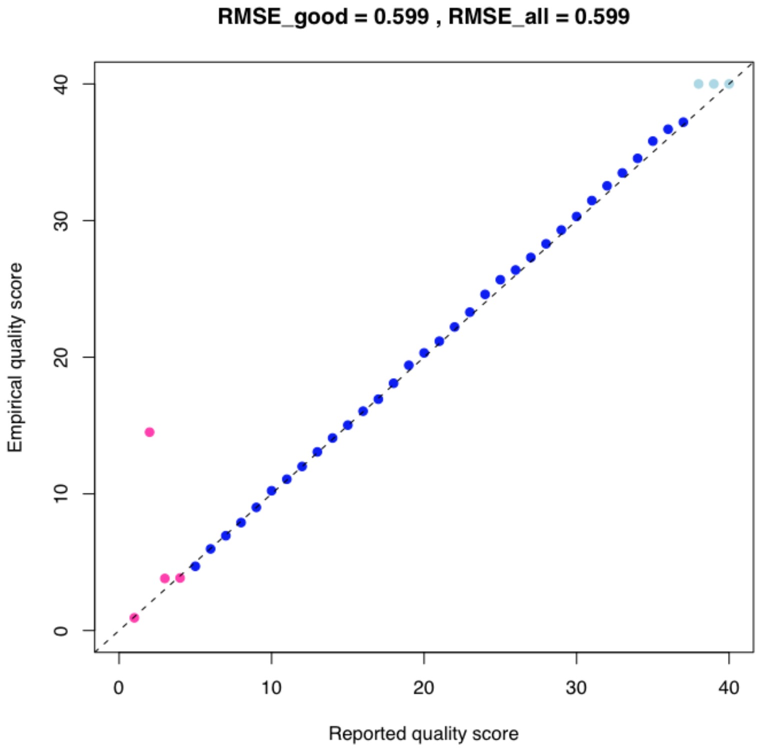
- **Identifies and corrects systematic (non-random) technical errors during sequencing**
- Compares covariation between
  - Reported quality score
  - The position within the read (Machine cycle)
  - The two preceding and current nucleotide (sequencing chemistry effect) observed by the sequencing machine

- Over-/Underestimation of quality scores
  - Helps fight False positives
  - Rescues False negatives

Base Quality Score Recalibration

Reported Quality vs. Empirical Quality



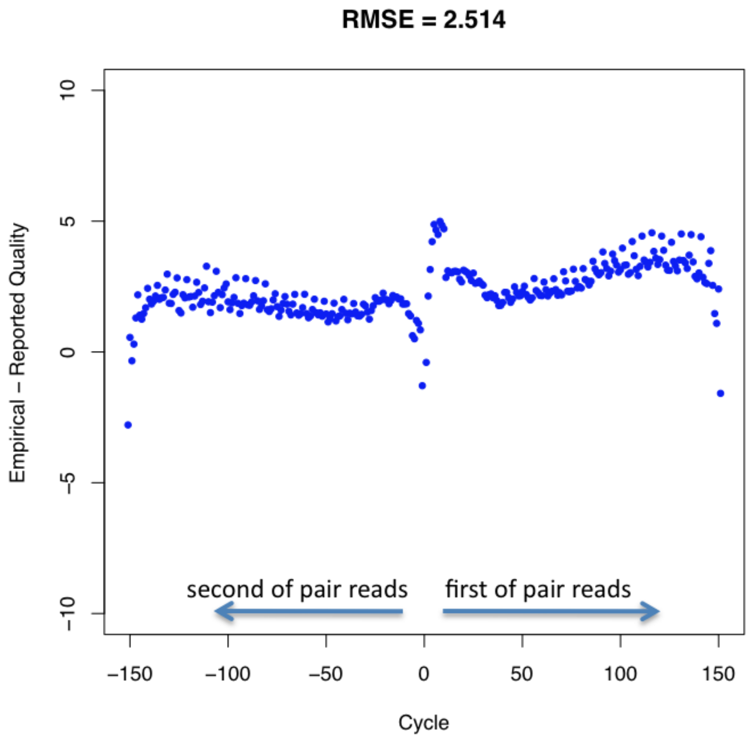


Original Data

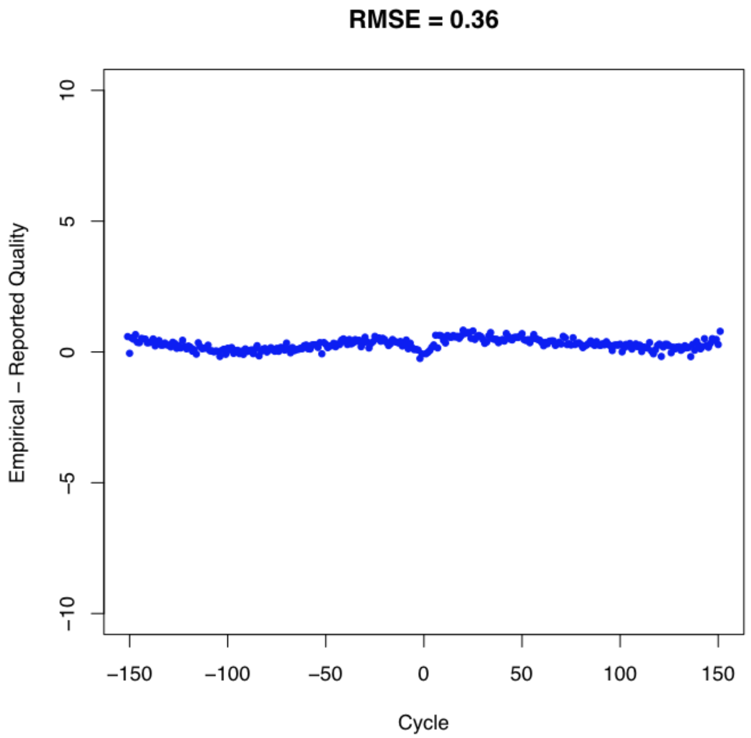
After GATK Recalibration

Base Quality Score Recalibration

Residual Error by Machine Cycle



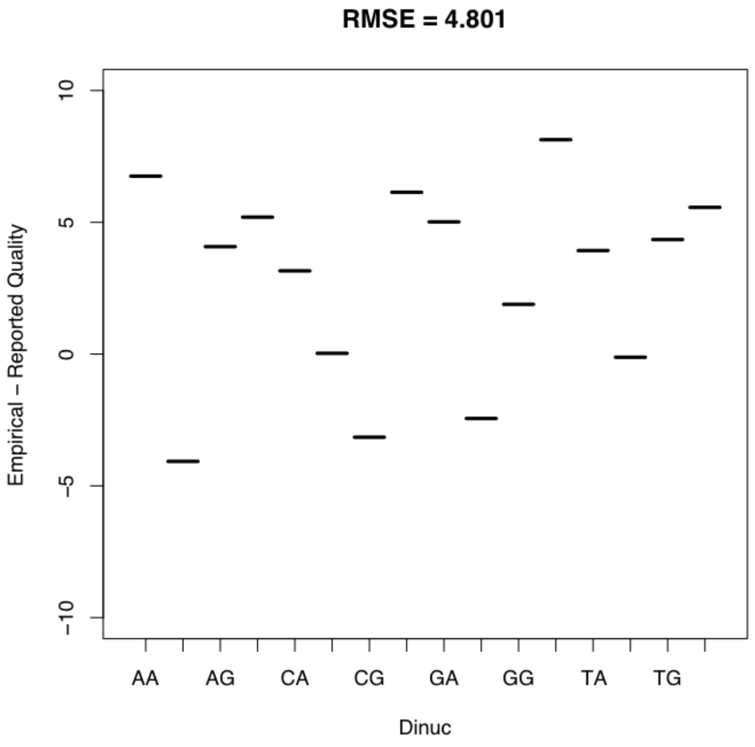
Original Data



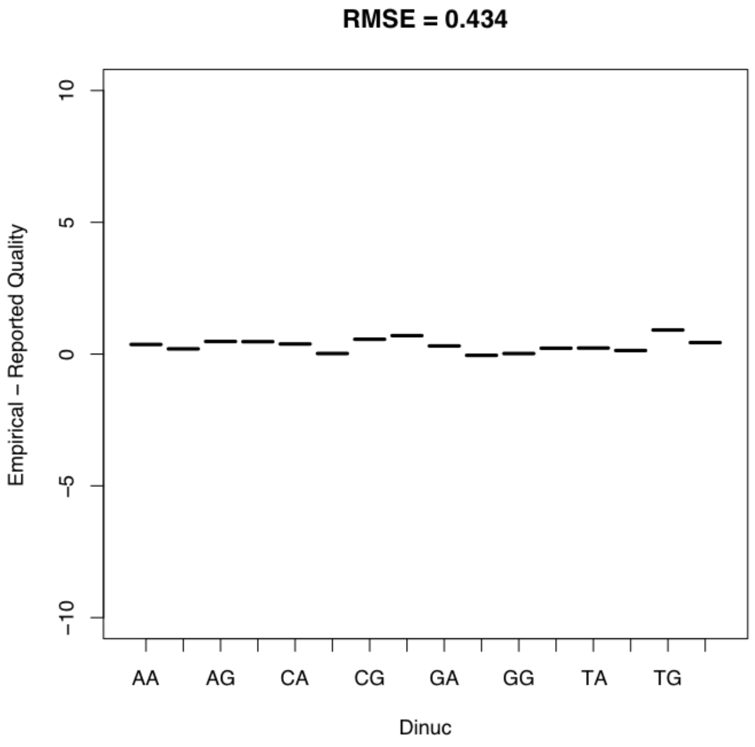
After GATK Recalibration

Base Quality Score Recalibration

Residual Error by Dinucleotide



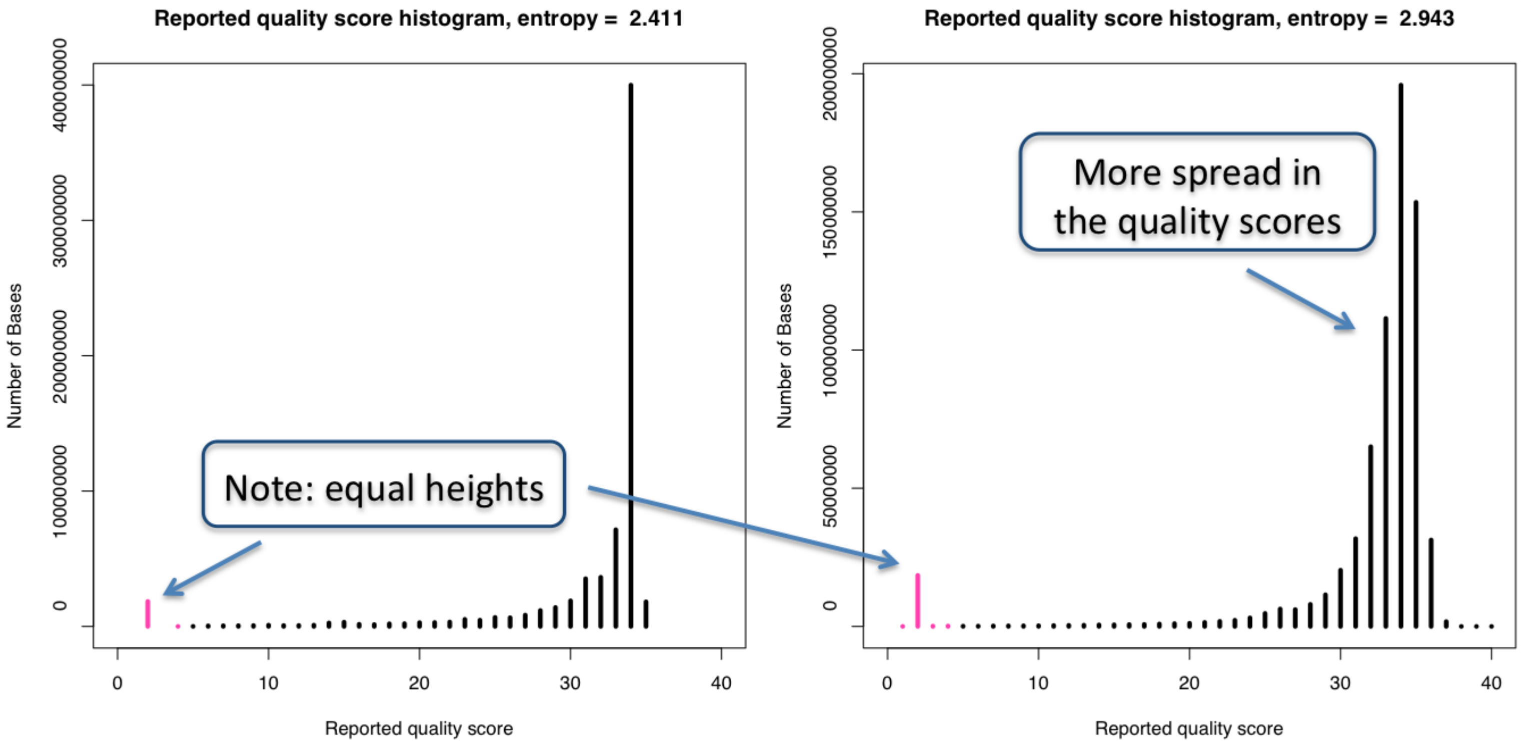
Original Data



After GATK Recalibration

Base Quality Score Recalibration

Distribution of Quality Scores



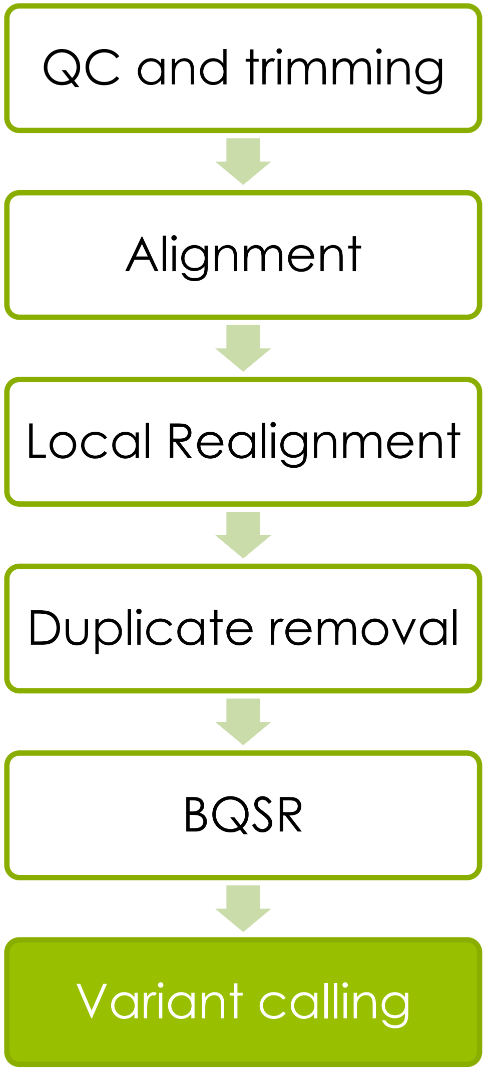
Note: equal heights

More spread in the quality scores

Original Data

After GATK Recalibration

NGS workflow


QC and trimming

Alignment

Local Realignment

Duplicate removal

BQSR

Variant calling

Variant calling

Reference:	...GTGCGTAGACTGCTAGATCGAAGA...
Sample:	...GTGCGTAGACTGATAGATCGAAGA...

Variant calling

Reference: ...GTGCGTAGACTGCTAGATCGAAGA...

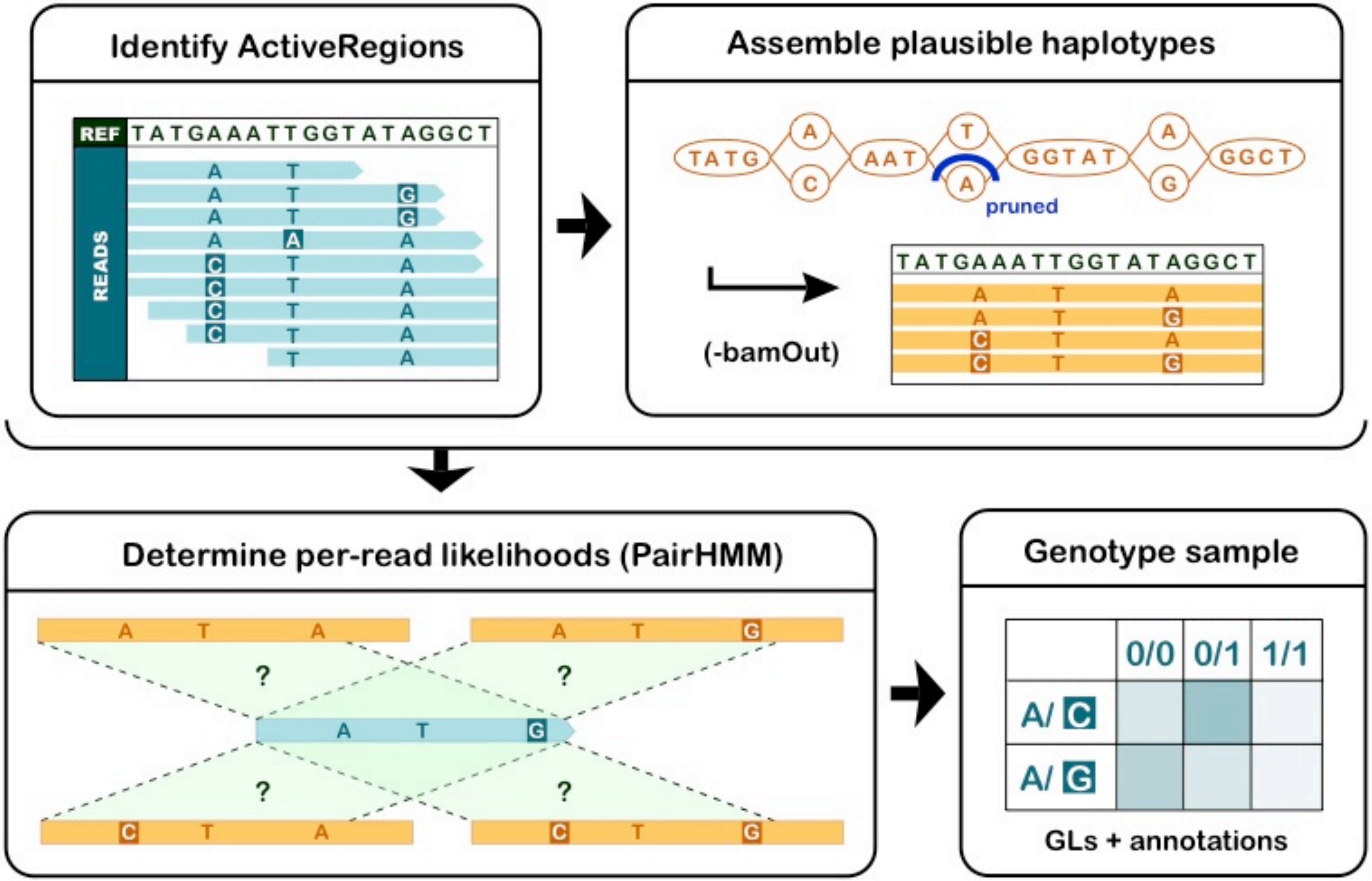
Sample: ...GTGCGTAGACTGATAGATCGAAGA...
...GTGCGTAGACTGATAGATCGAAGA...
...GTGCGTAGACTGCTAGATCGAAGA...
...GTGCGTAGACTGCTAGATCGAAGA...
...GTGCGTAGACTGATAGATCGAAGA...
...GTGCGTAGACTGATAGATCGAAGA...
...GTGCGTAGACTGCTAGATCGAAGA...
...GTGCGTAGACTGATAGATCGAAGA...
...GTGCGTAGACTGCTAGATCGAAGA...
...GTGCGTAGACTGATAGATCGAAGA...

Variant calling

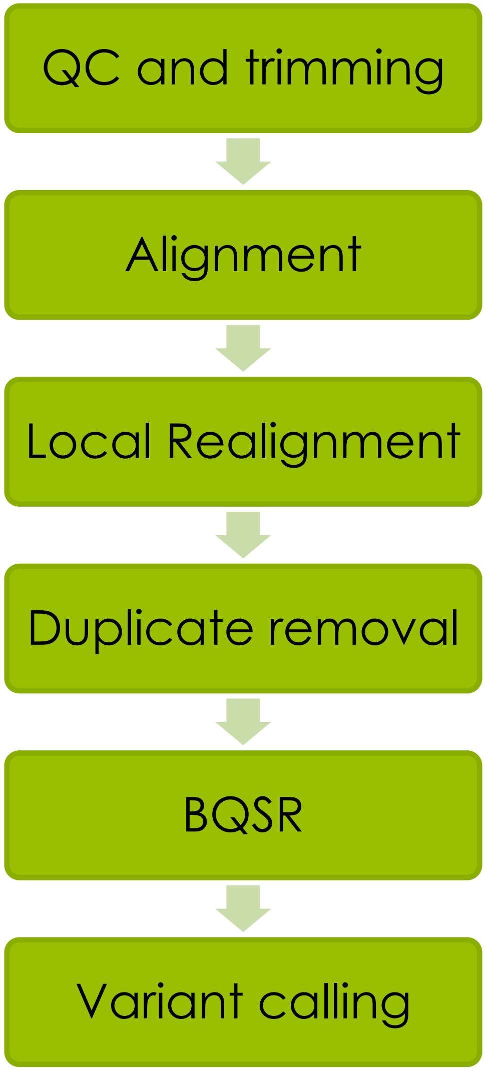
Reference:	...GTGCGTAGACTGCTAGATCGAAGA...
Sample:	...GTGCGTAGACTGATAGATCGAAGA...
	...GTGCGTAGACTGATAGATCGAAGA...
	...GTGCGTAGACTGCTAGATCGAAGA...
	...GTGCGTAGACTGCTAGATCGAAGA...
	...GTGCGTAGACTGATAGATCGAAGA...
	...GTGCGTAGACTGATAGATCGAAGA...
	...GTGCGTAGACTGCTAGATCGAAGA...
	...GTGCGTAGACTGATAGATCGAAGA...
	...GTGCGTAGACTGCTAGATCGAAGA...
	...GTGCGTAGACTGATAGATCGAAGA...

$$\frac{\#Variants\ in\ a\ position}{\#Reads\ in\ a\ position} = A\ variants\ allele\ frequency$$

Variant Calling HaplotypeCaller



NGS workflow



QC and trimming

Alignment

Local Realignment

Duplicate removal

BQSR

Variant calling

VCF Files

```
##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
```

VCF Files

```
##fileformat=VCFv4.0 ##fileDate=20090805
##source=myImputationProgramV3.1
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##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
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##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:.,.
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VCF Files

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##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
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#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:.,.
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##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
```

VCF Files

```
##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
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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
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##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
20      14370    rs6054257  G    A    29   PASS   NS=3;DP=14;AF=0.5;DB;H2
20      17330    .           T   A    3    q10    NS=3;DP=11;AF=0.017
20      1110696  rs6040355  A   G,T   67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB
```

VCF Files

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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
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##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">
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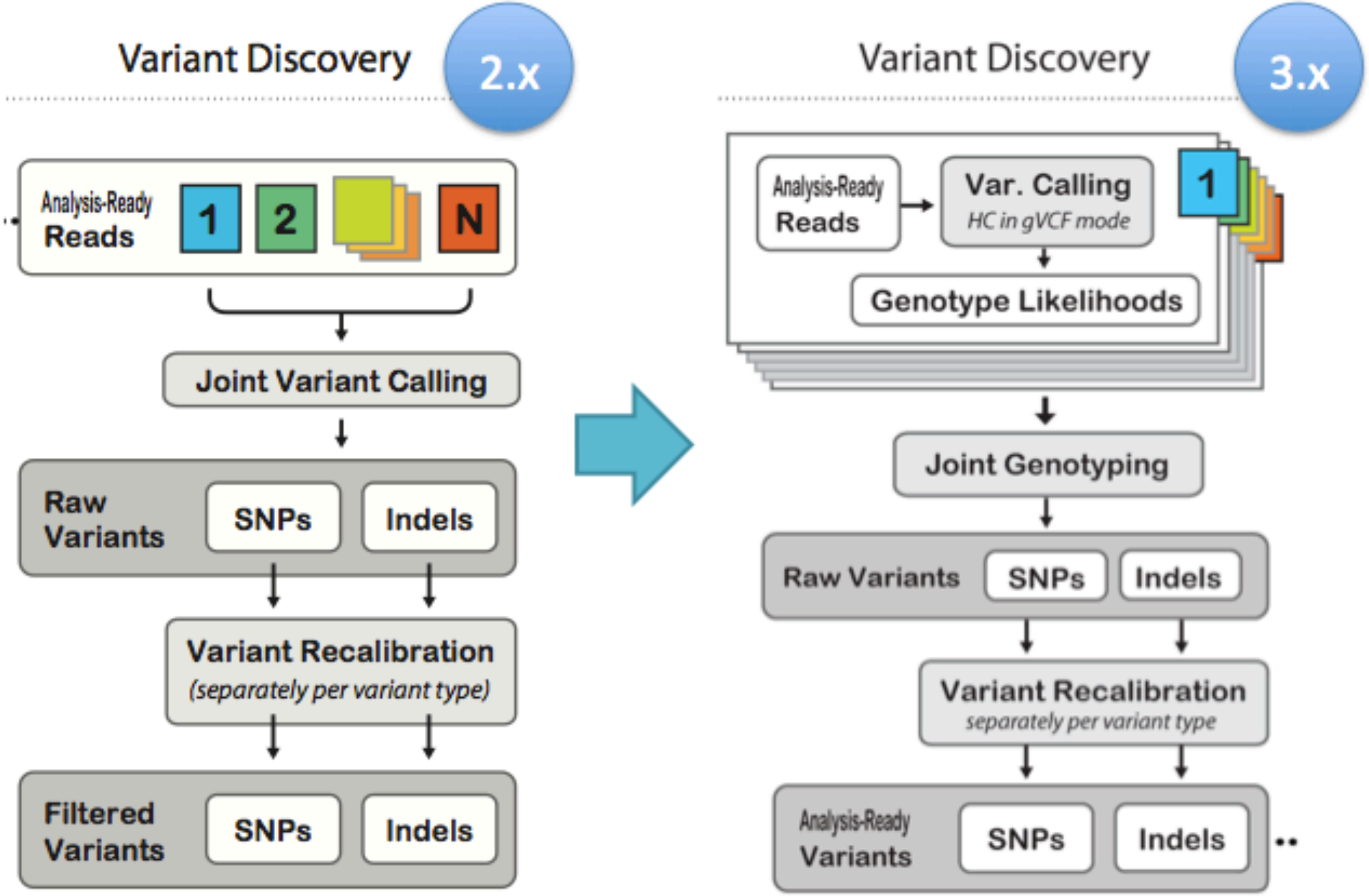
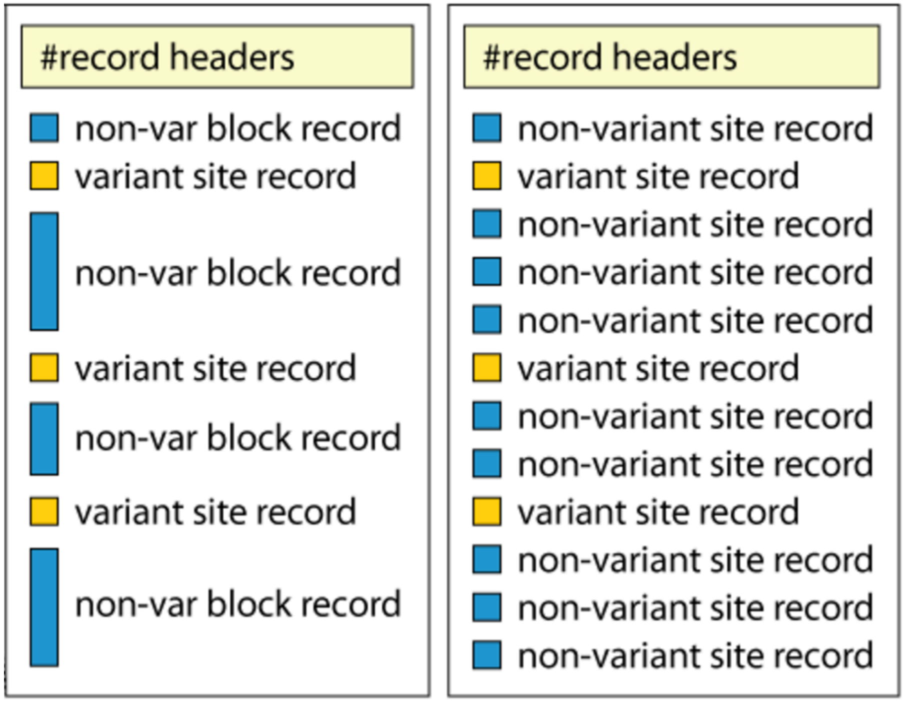
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#FORMAT       NA00001        NA00002        NA00003
GT:GQ:DP:HQ   0|0:48:1:51,51 1|0:48:8:51,51 1/1:43:5:.,.
GT:GQ:DP:HQ   0|0:49:3:58,50 0|1:3:5:65,3   0/0:41:3
GT:GQ:DP:HQ   1|2:21:6:23,27 2|1:2:0:18,2   2/2:35:4
```

Joint genotyping



gVCF Files

New gVCF **Old gVCF**



New gVCF:

- #record headers
- 🟦 non-var block record
- 🟨 variant site record
- 🟦 non-var block record
- 🟨 variant site record
- 🟦 non-var block record
- 🟨 variant site record
- 🟦 non-var block record

Old gVCF:

- #record headers
- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟨 variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record
- 🟦 non-variant site record

```
##GVCFBlock=minGQ=0(inclusive),maxGQ=5(exclusive)
##GVCFBlock=minGQ=20(inclusive),maxGQ=60(exclusive)
##GVCFBlock=minGQ=5(inclusive),maxGQ=20(exclusive)
```

Annotation & Filtering

```
module load annovar /snpEff / vep
```

```
#CHROM POS ID REF ALT QUAL
20 14370 rs6054257 G A 29
```

- Gene-based
  - Non-synonymous/synonymous
- Region-based
  - CpG-islands
  - Conserved regions
  - Predicted transcription factor binding sites
- Filter-based
  - dbSNP
  - 1000G
  - COSMIC

Annotation & Filtering

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module load annovar /snpEff / vep
```

```
#CHROM POS ID REF ALT QUAL
20 14370 rs6054257 G A 29
```

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- Region-based
  - CpG-islands
  - Conserved regions
  - Predicted transcription factor binding sites
- Filter-based
  - dbSNP
  - 1000G
  - COSMIC

USE THE SAME REFERENCE!

Annotation & Filtering

```
module load GATK
```

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

```
VariantFiltration
    --filterExpression ”QUAL > 30”
    --filterName QUAL_filter
    --filterExpression  "QUAL / DP < 10.0"
    --filterName QUALDP_filter
```

Questions?

Questions?

Work like a professional bioinformatician – Google errors!