Variant Calling

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MAYO-ILLINOIS COMPUTATIONAL GENOMICS WORKSHOP, JUNE 8, 2023

Overview

Variant calling

Use cases

Variants vs. errors

Experimental design (GATK focus)

Small variant (SNV/Small Indel) analysis

- GATK Pipeline
- Formats encountered within

Variant Calling

As the name implies, we're looking for differences (variations) between:

- **Common reference** – reference genome (for human: hg38, GRCh38)
- **Sample(s)** – one or more comparative samples, each sample from one individual

Start with raw sequence data (FASTQ format)

End with a file listing off differences, recording the variants

Additional information added downstream:

- Filters (quality of the calls)
- Functional annotation

Variations

Difference between 2 individuals : about 1 every 1000 bp

- ~ 2.7 million differences for the human genome

Small (<50 bp)

- SNV – single nucleotide (`**SNPs**`)
- Small insertions or deletions (‘**Indels**’)

Large (structural variations)

- Indels > 50 bp
- Copy Number Variations
- Inversions
- Translocations
- Chromosomal fusions

Variations

Mainly focus on diploid organisms, but this can be polyploid

- Human:
  - 22 pairs of autosomal chromosomes
    - One from mother, one from father
  - 2 sex chromosomes (female XX, male XY)
    - One from mother, one from father (where Y comes from for male offspring)
  - Mitochondrial genome (generally maternally inherited)
    - 100-10,000 copies per cell

Variation can be in

- One chromosome (heterozygous, or ‘het’)
- All chromosomes (homozygous, or ‘hom’)

Use cases

Use cases

Medicine

- Hereditary or genetic diseases, genetic predisposition to disease
- Cancer biology - Normal (germline) vs. tumor (somatic) analyses, driver mutations
- Heteroplasmy

Biotechnology

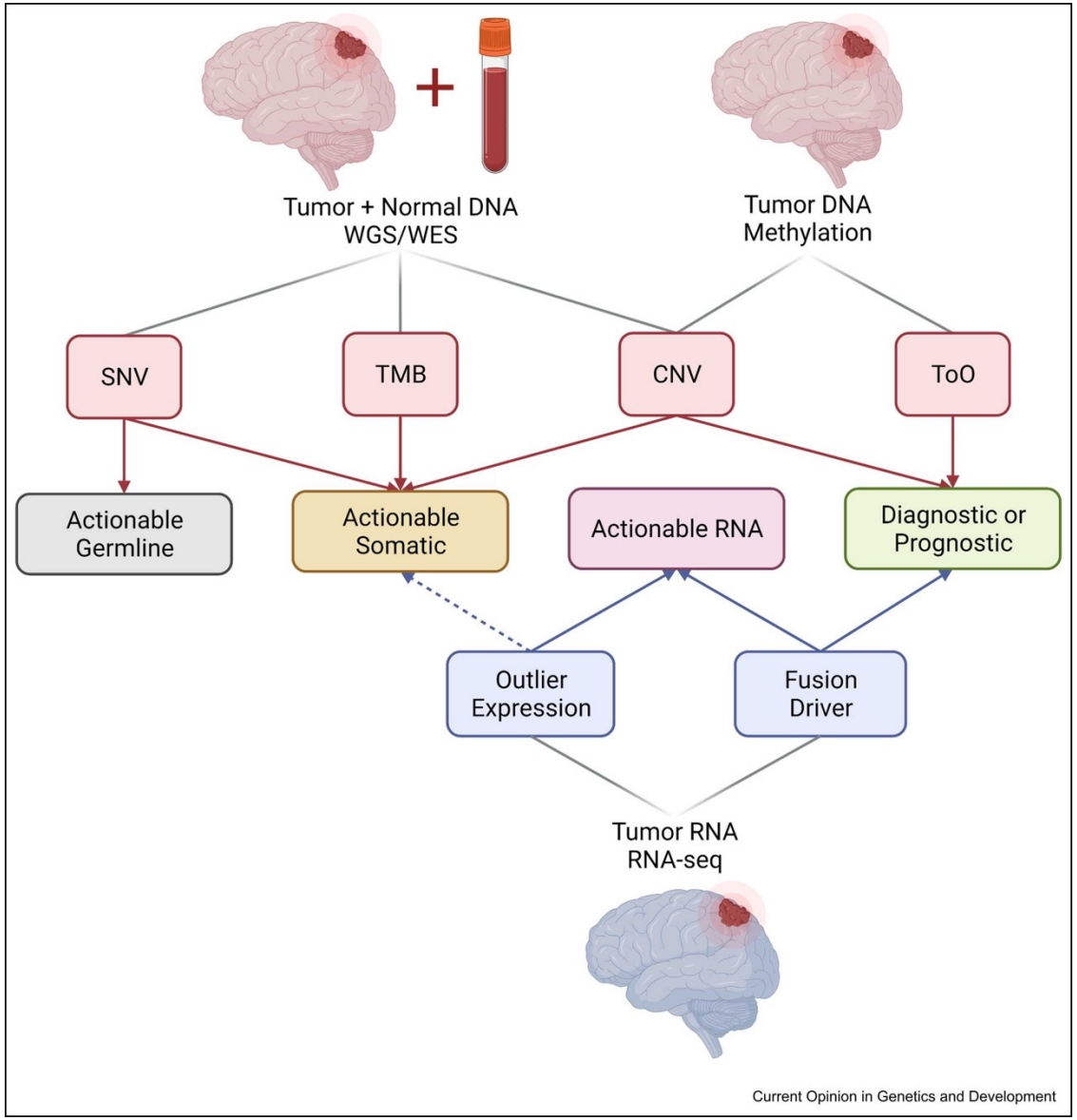
- Detection of genomic modifications (CRISPR/Cas9)

Population genetics

- GWAS

Cancer Biology

- **Germline vs somatic variants**
- **Structural variants**
- **May combine with other types of genomic data (RNA, methylation, etc)**

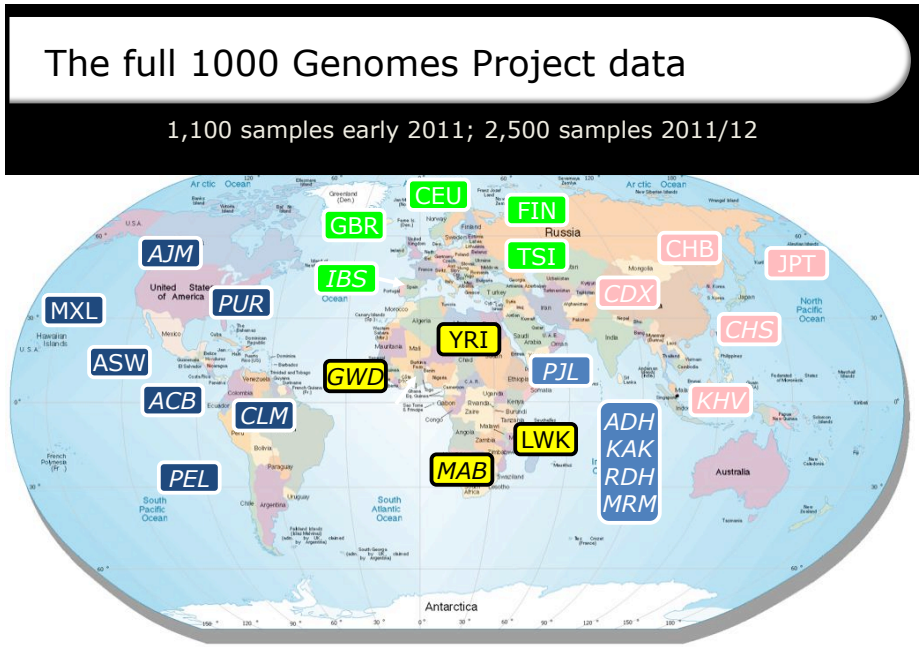


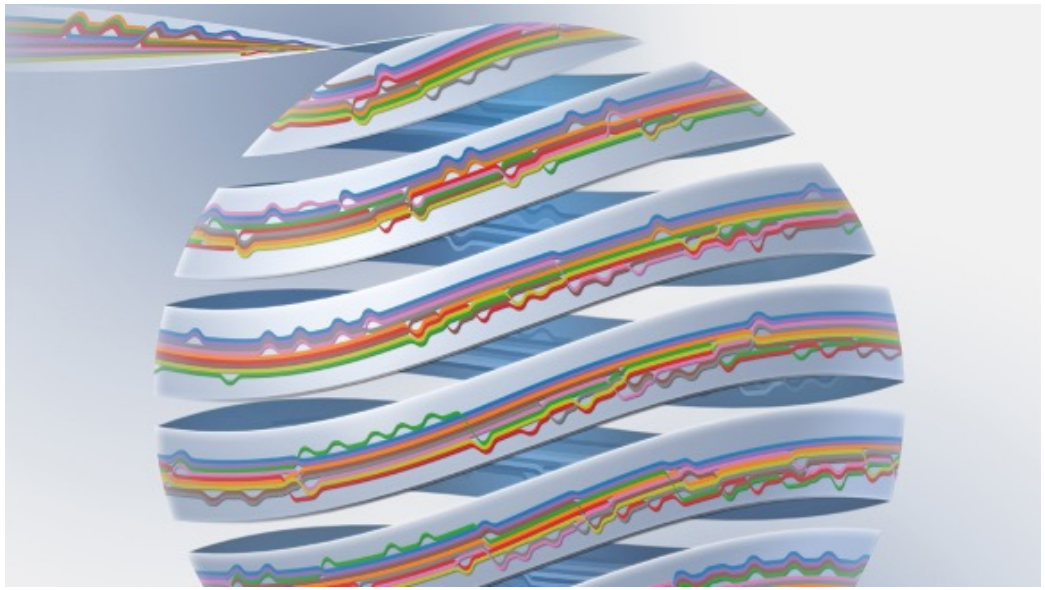
Miller, Mardis, Curr. Op. in Gen. & Dev, v75, Aug. 2022

Population genetics

1000 Genomes Project

Human Pangenome Project





2011

2023

IGSR: The International Genome Sample Resource

HPRC

Nature Special Issue

Variants vs. Errors

Must distinguish between actual **variation** (real change) and **errors** (artifacts) introduced into the analysis

Errors can creep in on various levels:

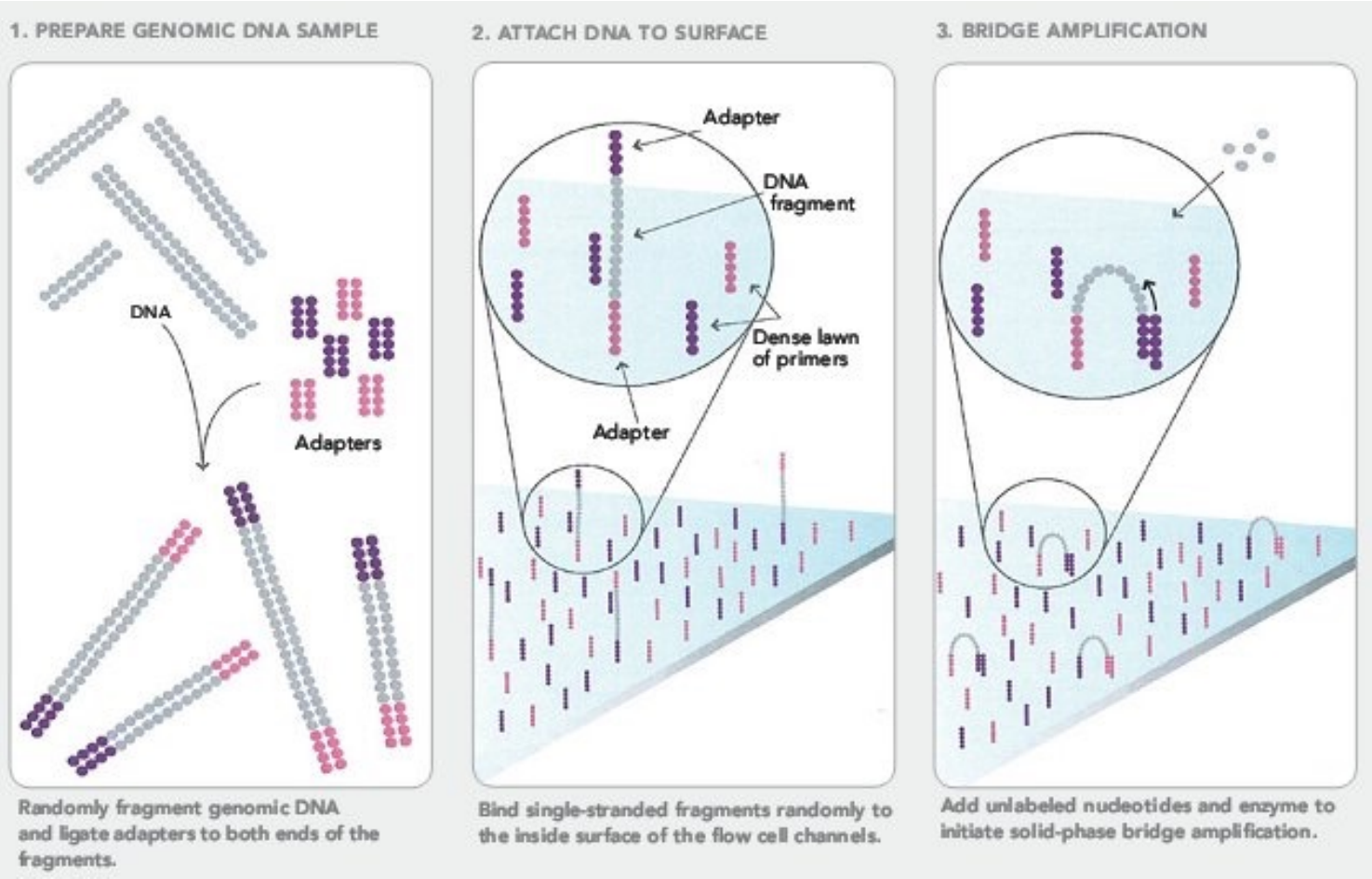
- **PCR artifacts** (amplification of errors)
- **Sequencing** (errors in base calling)
- **Alignment** (misalignment, mis-gapped alignments)
- **Variant calling** (low depth of coverage, few samples)
- **Genotyping** (poor annotation)

Try to control for these when possible to **reduce false positives** w/o incurring (worse) false negatives

How do sequencing errors occur?

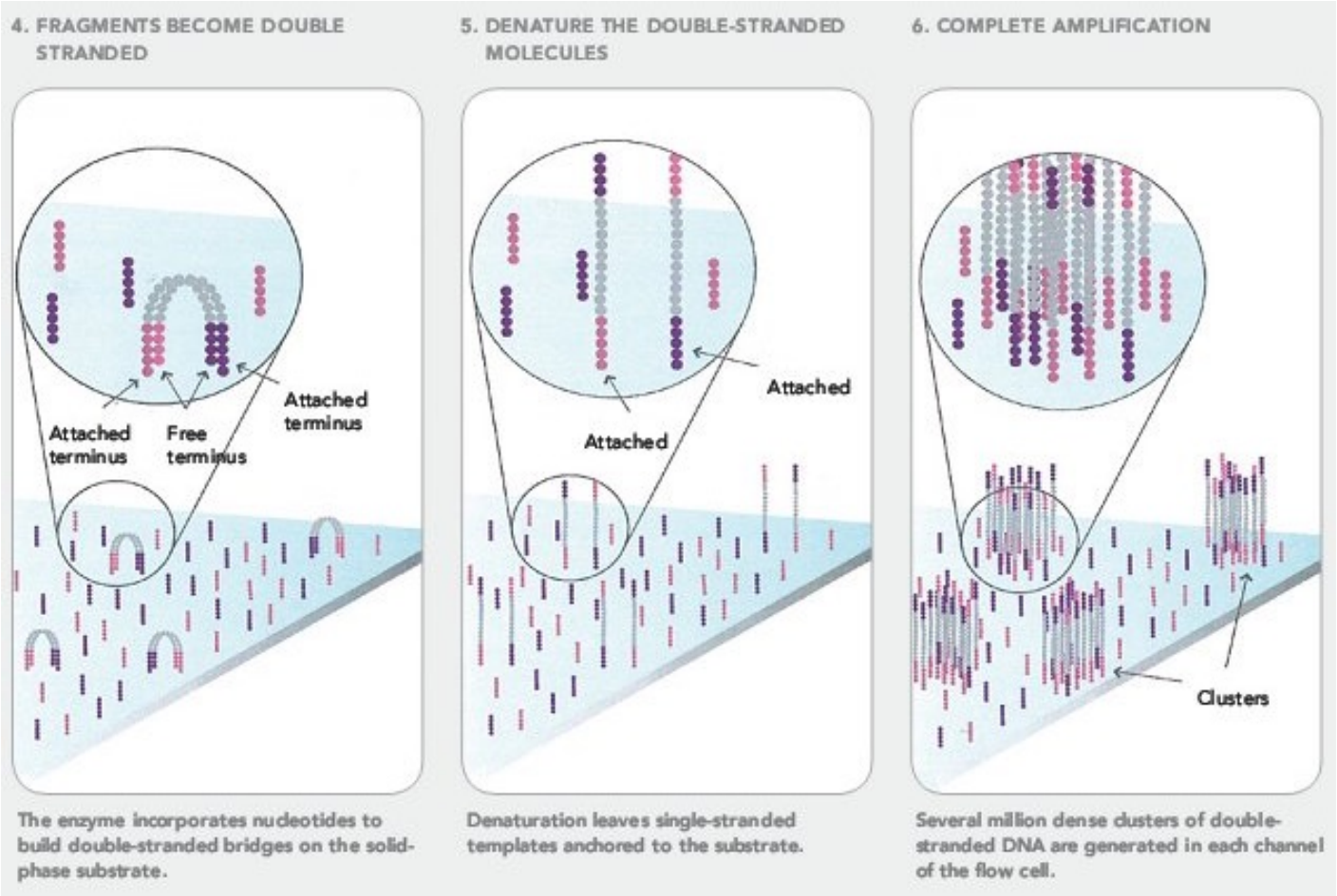
Illumina Sequencing

Video!



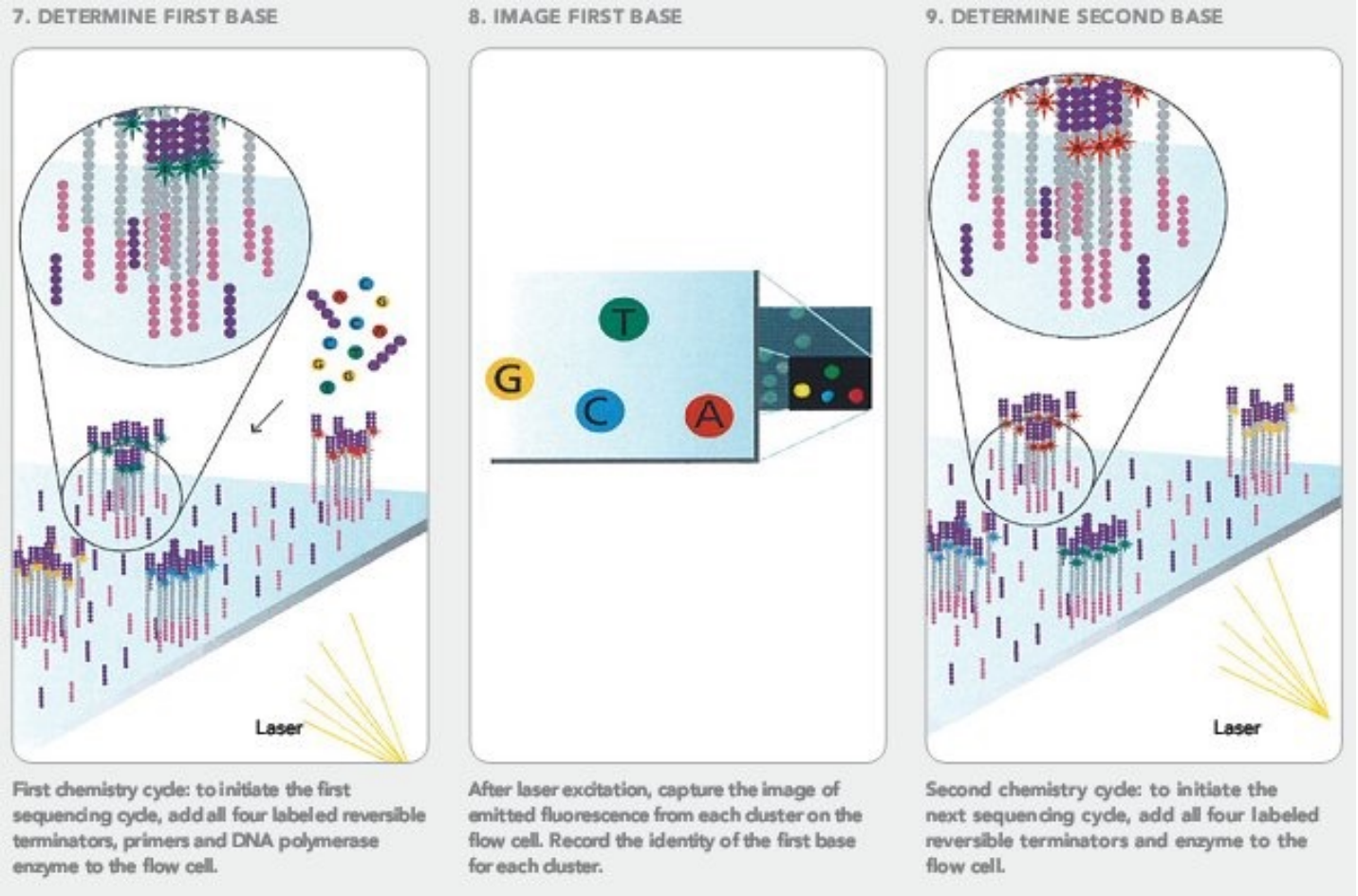
Illumina

Illumina Sequencing



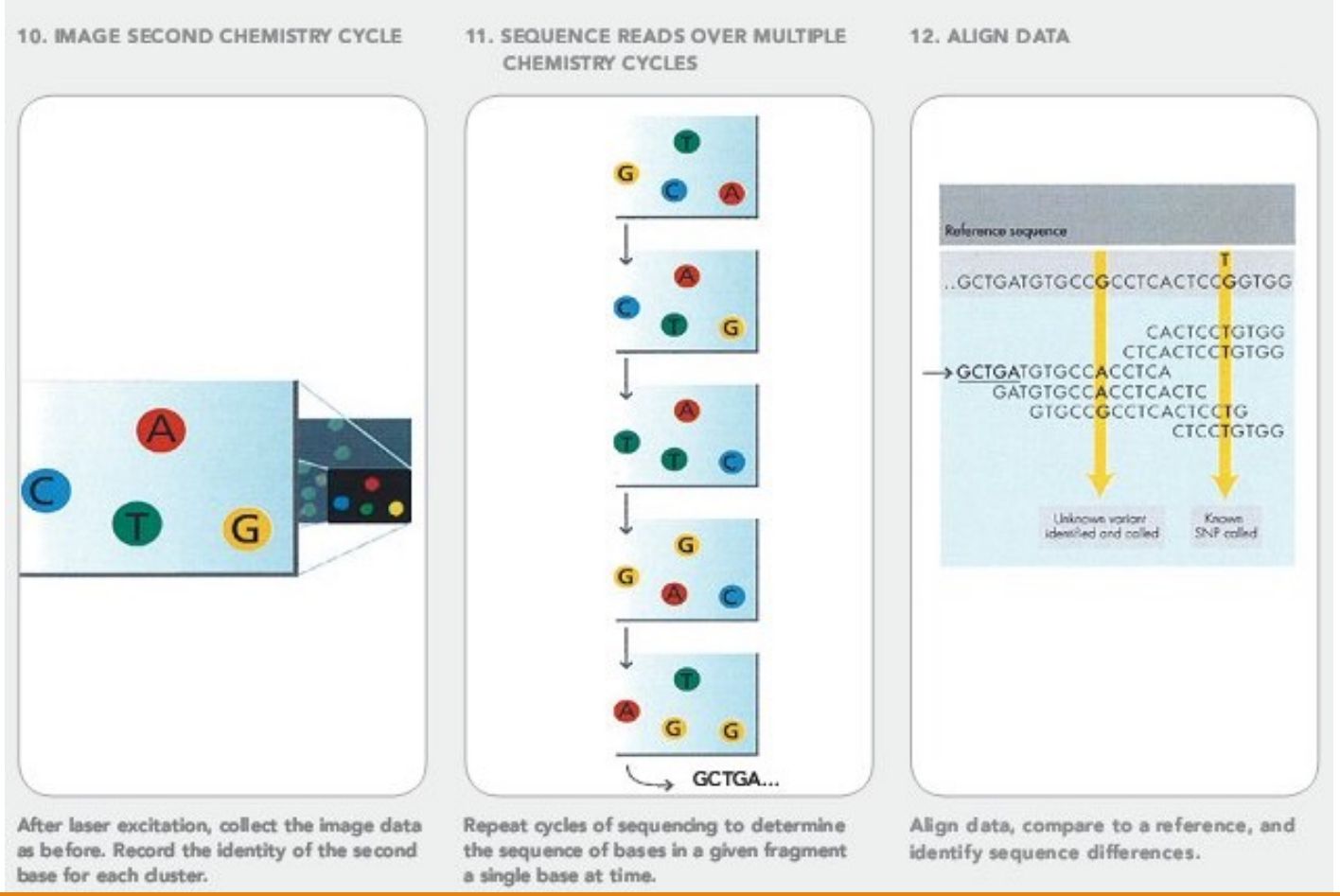
Illumina

Illumina Sequencing



Illumina

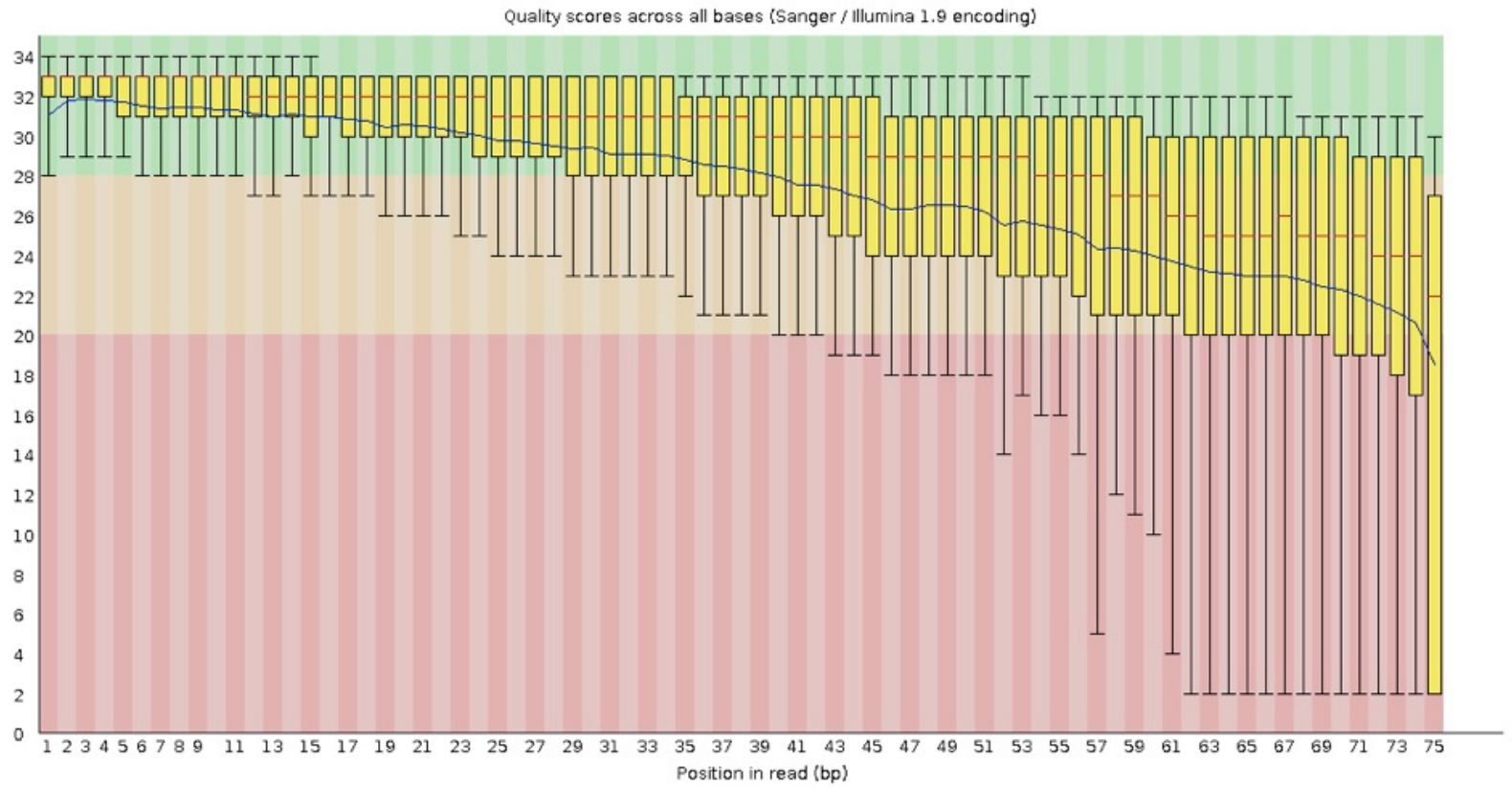
Illumina Sequencing



Illumina

Check sequence data!

Per base sequence quality



FASTQC

Sequence quality

Different technologies have different errors, error rates

- **Illumina** – substitution errors (0.1%)
- **PacBio and Oxford Nanopore** – (10-15%) Indels, primarily around homopolymer track errors

Represented as a quality score (Phred scale)

Probability of incorrect base call (e)	Base call accuracy	log10(e)	Phred Quality Score -10log10(e)
1 in 10 (0.1)	90%	-1	**10**
1 in 100 (0.01)	99%	-2	**20**
1 in 1000 (0.001)	99.90%	-3	**30**
1 in 10000 (0.0001)	99.99%	-4	**40**
1 in 100000 (0.00001)	~100.00%	-5	**50**

Formats: FASTQ – ‘sequence with quality’

```
@HWI-ST1155:109:D0L23ACXX:5:1101:2247:1985 1:N:0:GCCAAT
NTTCCTTTGACAAATATTAAAATTAAGAATCAAATATGGTAGTGTATGCCAAGACCTAGTCTGAGTCAGTAGGAT
+
#1=DDFFFHHHHHJJJJJJIJJJIJJIJIJJJJJJJIJI?FHFHEIJEIIIEGFFHHGIGHIJEIFGIJHGDIII
```

Three general types (Sanger, Illumina, Solexa) Sanger by far most common

May be raw data (straight from sequencer) or processed (trimmed for various reasons)

Can hold 100’s of millions of records **per sample**

Files can be very large (10’s to 100’s of GB) apiece

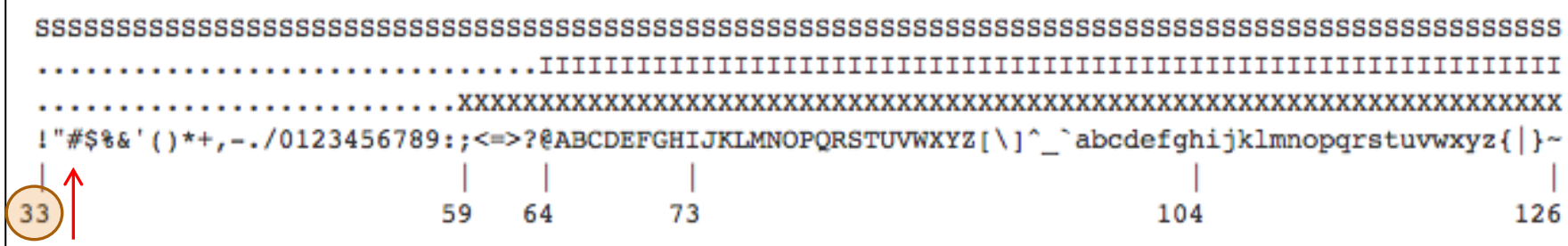
Wikipedia

Formats: FASTQ – ‘sequence with quality’

```
@HWI-ST1155:109:D0L23ACXX:5:1101:2247:1985 1:N:0:GCCAAT
NTTCCTTTGACAAATATTAAAATTAAGAATCAAATATGGTAGTGTATGCCAAGACCTAGTCTGAGTCAGTAGGAT
+
#1=DDFFFHHHHHJJJJJJIJJJIJJIJIJJJJJJJIJI?FHFHEIJEIIIEGFFHHGIGHIJEIFGIJHGDIII
```

Very low Phred score, less than 10 (35 - 33 = 2)

Quality Score Comparison



S - Sanger Phred+33, 93 values (0, 93) (0 to 60 expected in raw reads)
I - Illumina 1.3 Phred+64, 62 values (0, 62) (0 to 40 expected in raw reads)
X - Solexa Solexa+64, 67 values (-5, 62) (-5 to 40 expected in raw reads)

Diagram adapted from http://en.wikipedia.org/wiki/FASTQ_format

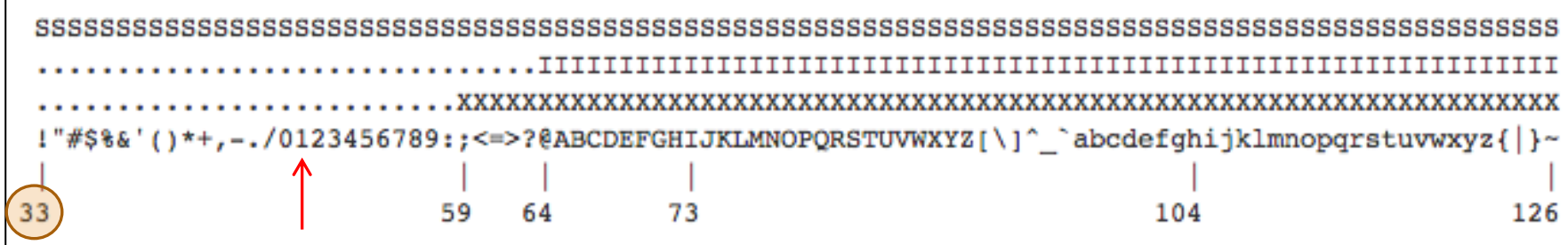
Wikipedia

Formats: FASTQ – ‘sequence with quality’

```
@HWI-ST1155:109:D0L23ACXX:5:1101:2247:1985 1:N:0:GCCAAT
NTTCCTTTGACAAATATTAAAATTAAGAATCAAATATGGTAGTGTATGCCAAGACCTAGTCTGAGTCAGTAGGAT
+
#1=DDFFFHHHHHJJJJJJIJJJIJJIJIJJJJJJJIJI?FHFHEIJEIIIEGFFHHGIGHIJEIFGIJHGDIII
```

Low Phred score, < 20 (49– 33 = 16)

Quality Score Comparison



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Formats: FASTQ – ‘sequence with quality’

```
@HWI-ST1155:109:D0L23ACXX:5:1101:2247:1985 1:N:0:GCCAAT
NTTCCTTTGACAAATATTAAAATTAAGAATCAAATATGGTAGTGTATGCCAAGACCTAGTCTGAGTCAGTAGGAT
+
#1=DDFFFHHHHHJJJJJJIJJJIJJIJIJJJJJJJIJI?FHFHEIJEIIIEGFFHHGIGHIJEIFGIJHGDIII
```

High quality reads, Phred score >= 30 (63 – 33 = 30)

Quality Score Comparison

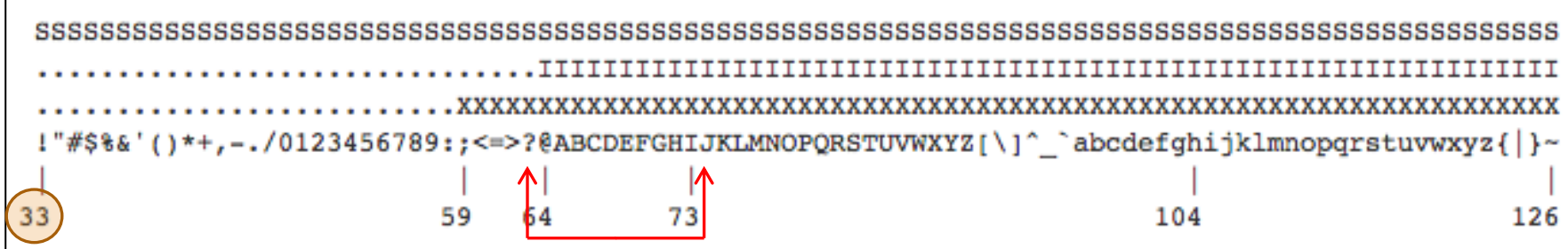

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Diagram adapted from http://en.wikipedia.org/wiki/FASTQ_format

Wikipedia

Basic Experimental Design

Terminology

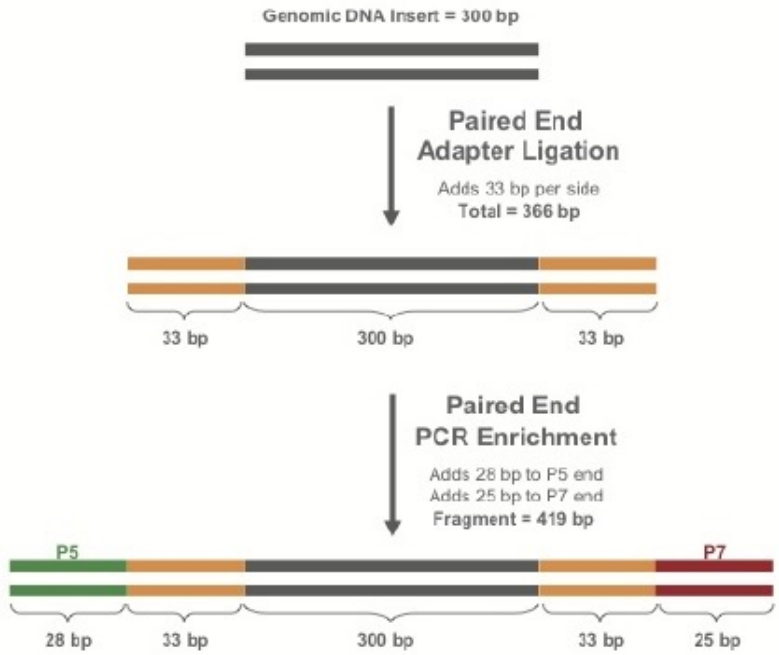
Lane – Physical sequencing lane

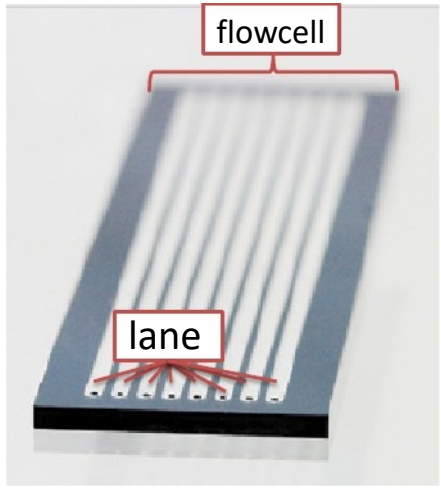
Library – Unit of DNA prep pooled together

Sample – Single individual

Cohort – Collection of samples analyzed together

This information is useful for *read groups*



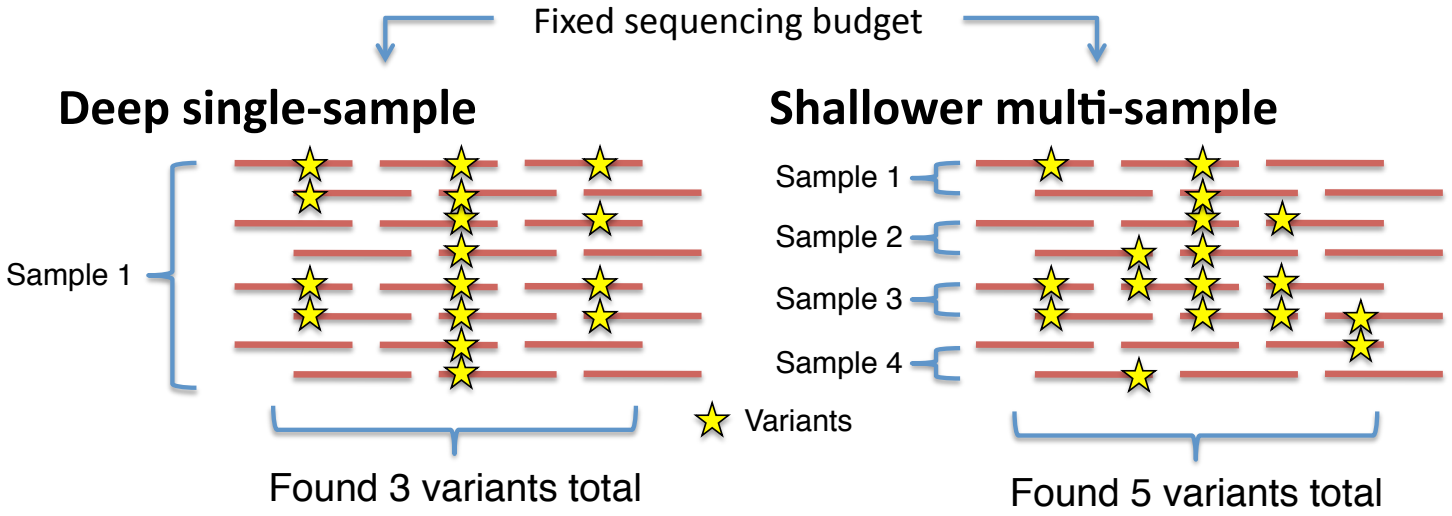


Terminology

WGS vs Exome Capture

- **Whole genome sequencing** – everything
  - High cost if per sample is deep sequence (>25-30x)
  - Can run multisample low coverage samples
- **Exome capture** – targeted sequencing (1-5% of genome)
  - Deeper coverage of transcribed regions
  - Miss other important non-coding regions (promoters, introns, enhancers, small RNA, etc)

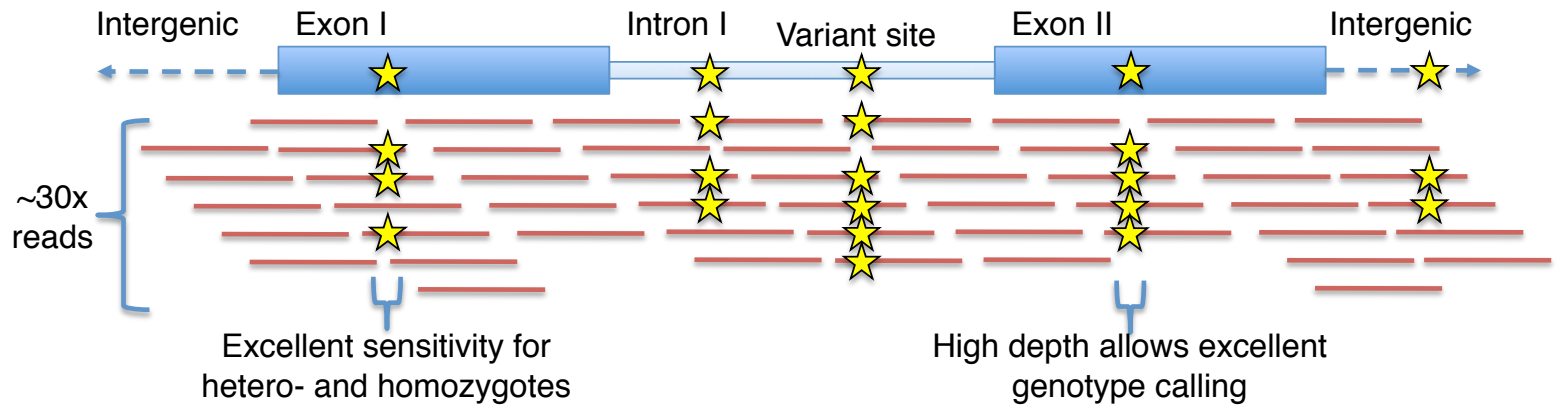
Single vs. multi-sample analysis



- Higher sensitivity for variants in the sample
- More accurate genotyping per sample
- Cost: no information about other samples

- Sensitivity dependent on frequency of variation
- Worse genotyping
- More total variants discovered

High-pass sequencing design



Data requirements per sample

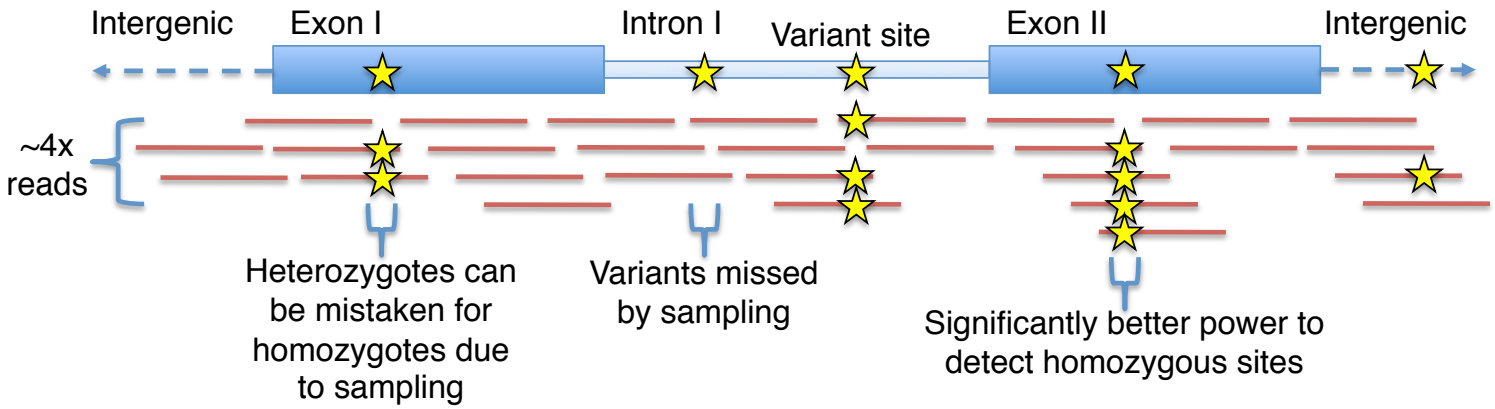
Target bases	3 Gb
Coverage	Avg. 30x
# sequenced bases	100 Gb
# per lane (HiSeq 4000)	~1
# per lane (NovaSeq, S4)	~8-9

Variant detection among multiple samples

Variants found per sample	~4-5M
Percent of variation in genome	>99%
Pr{singleton discovery}	>99%
Pr{common allele discovery}	>99%

NovaSeq 6000 = 750-850 Gb/lane (2x150nt, S4 lane)

Low-pass sequencing design



Data requirements per sample

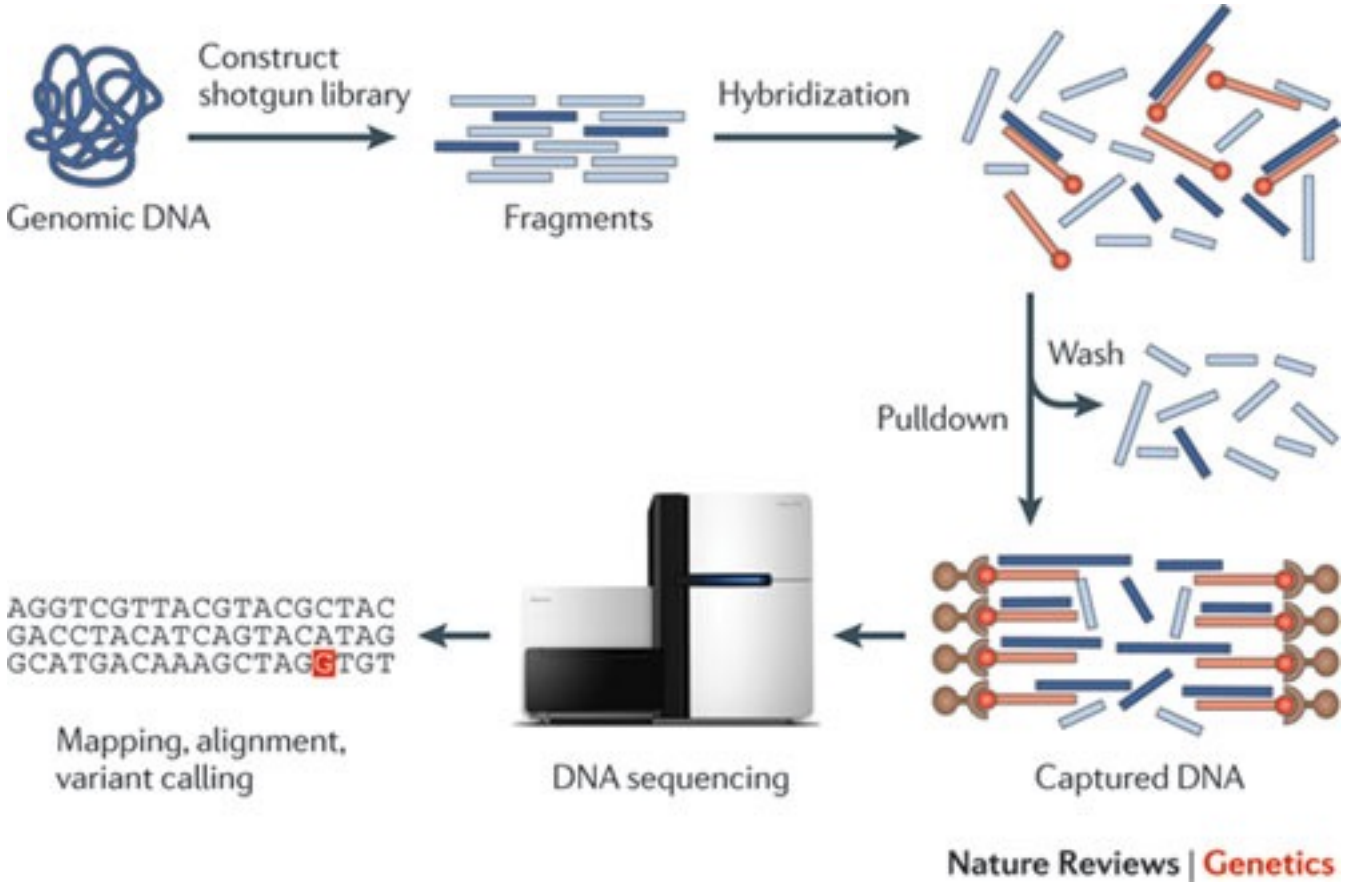
Target bases	3 Gb
Coverage	Avg. 5x
# sequenced bases	15 Gb
# per lane (HiSeq 4000)	~6
# per lane (NovaSeq, S4)	~50

Variant detection among multiple samples

Variants found per sample	~3M
Percent of variation in genome	~90%
Pr{singleton discovery}	< 50%
Pr{common allele discovery}	~99%

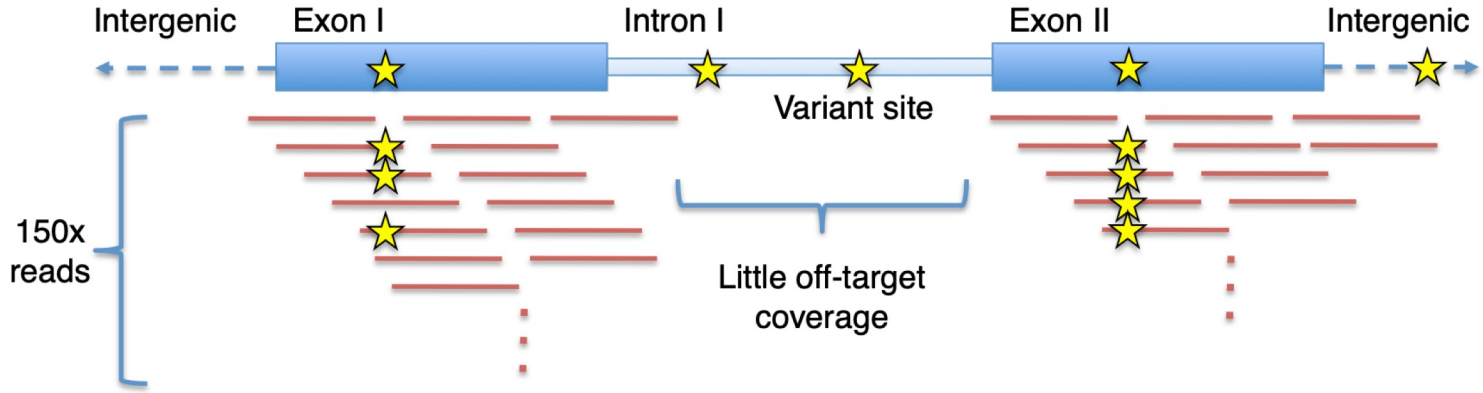
NovaSeq 6000 = 750-850 Gb/lane (2x150nt, S4 lane)

Targeted approach - **Exome Capture**



Exome capture sequencing design

Targeted!



Based on Illumina 'DNA Prep with Enrichment' panel

Data requirements per sample

Target bases	45-60 Mb
Coverage	>90% 20x*
# sequenced bases	4 Gb
# per lane (HiSeq 4000)	20
# per lane (NovaSeq, S4)	~384*

Variant detection among multiple samples

Variants found per sample	~25-45k
Percent of variation in genome	0.005
Pr{singleton discovery}	~95%
Pr{common allele discovery}	~95%

NovaSeq 6000 = 750-850 Gb/lane (2x150nt, S4 lane)

General variant calling pipelines

Common pattern:

- Align reads
- Optimize alignment
- Call variants
- Filter called variants
- Annotate

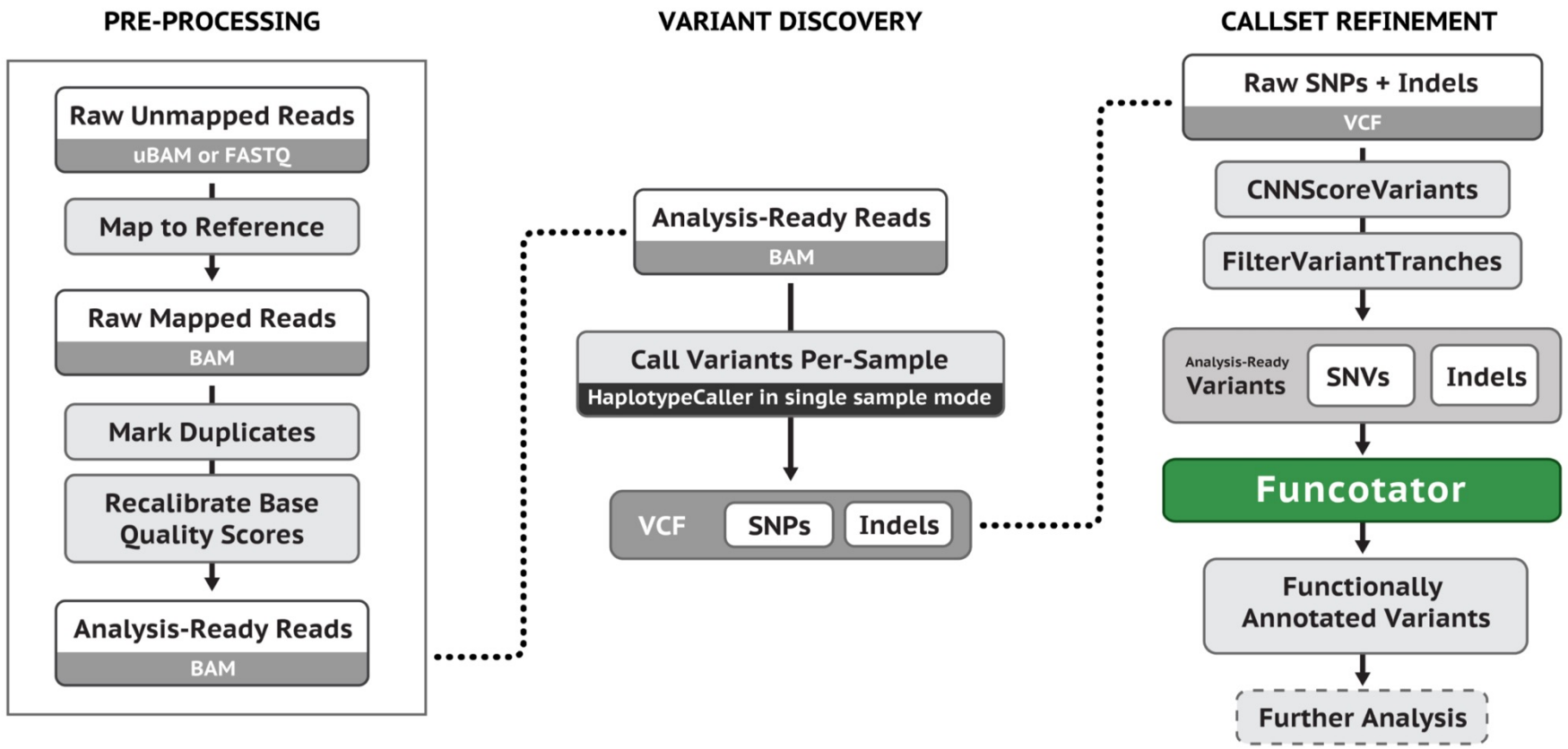
Tool/Workflow examples

Examples (standard variant calling)

- ***Genome Analysis Toolkit (GATK)***
- samtools mpileup
- VarScan2
- freeBayes
- Commercial
  - Illumina DRAGEN – GATK using FPGA (see the NovaSeq X slide!!!)
  - Sentieon – accelerated CPU
  - Parabricks – GPU-based

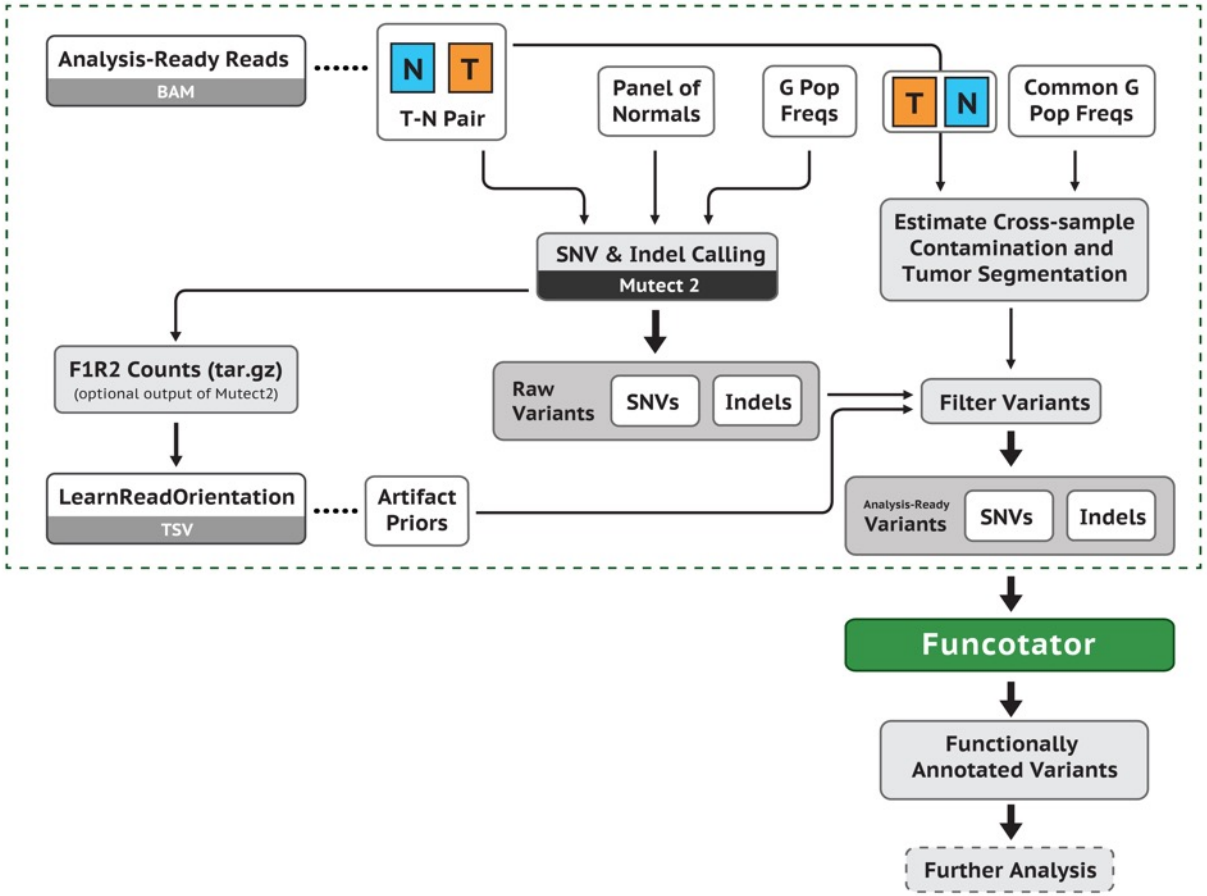
Tool/Workflow examples

GATK – Single Sample Germline Calls



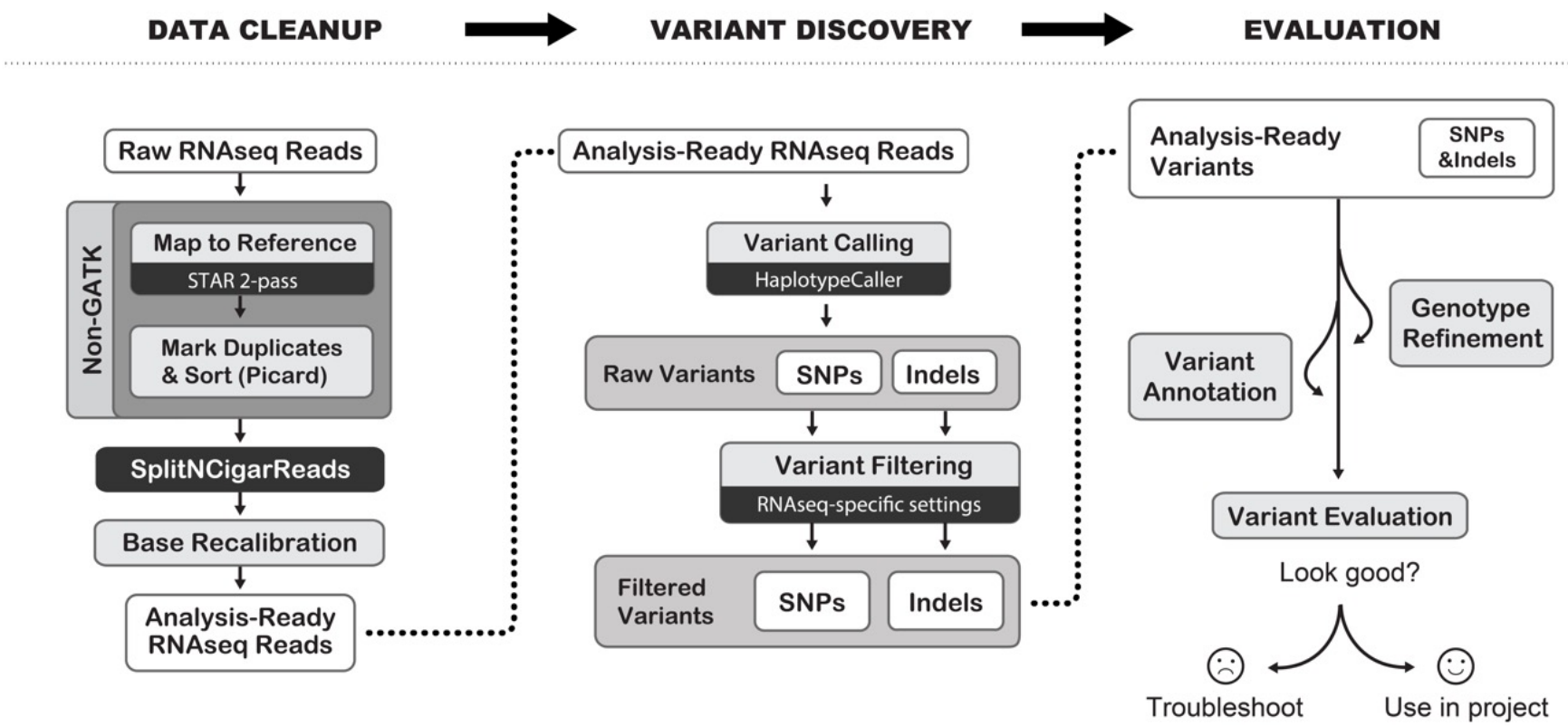
[GATK Germline Calling]

GATK – Somatic Calls (Tumor)



[GATK Somatic Callling]

GATK – RNA-Seq

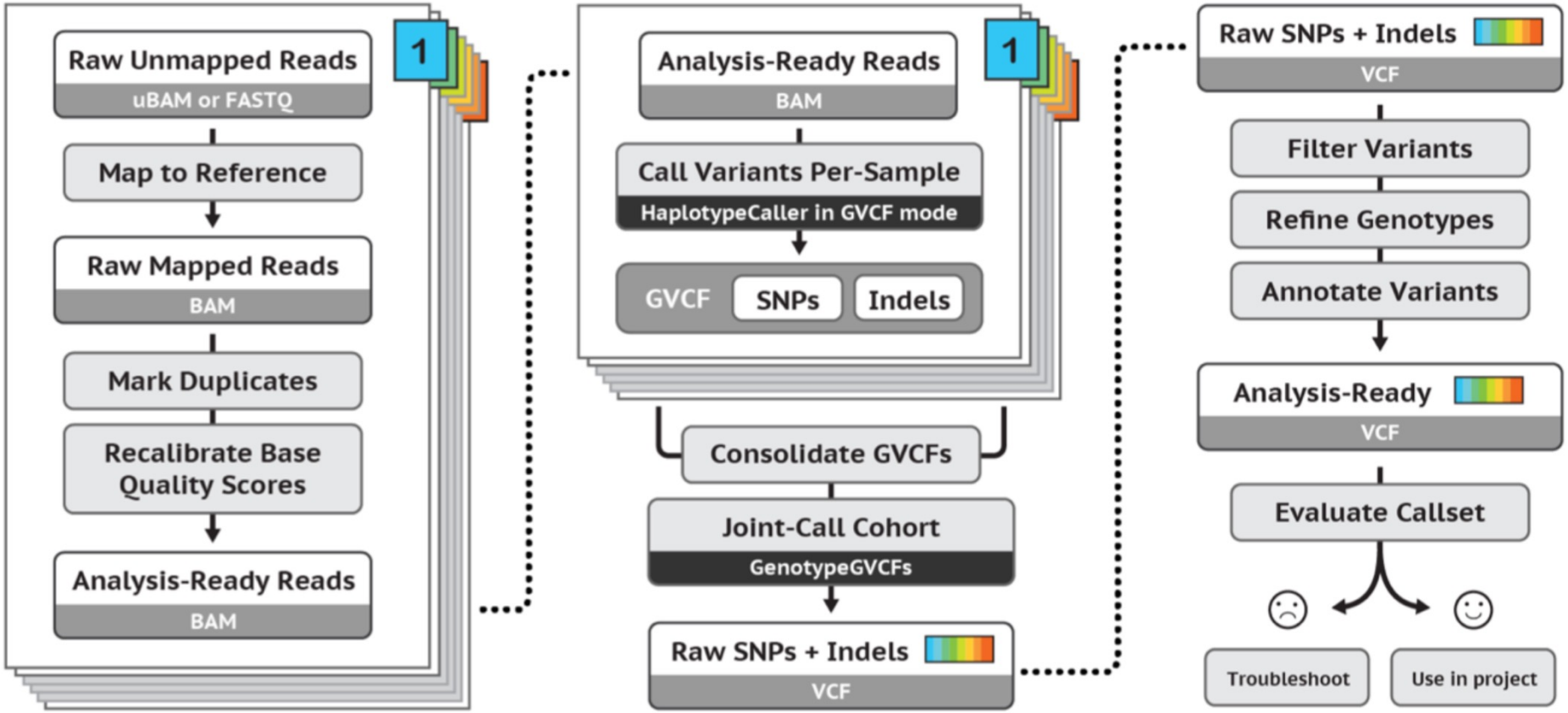


GATK RNA-Seq

Standard GATK Workflow

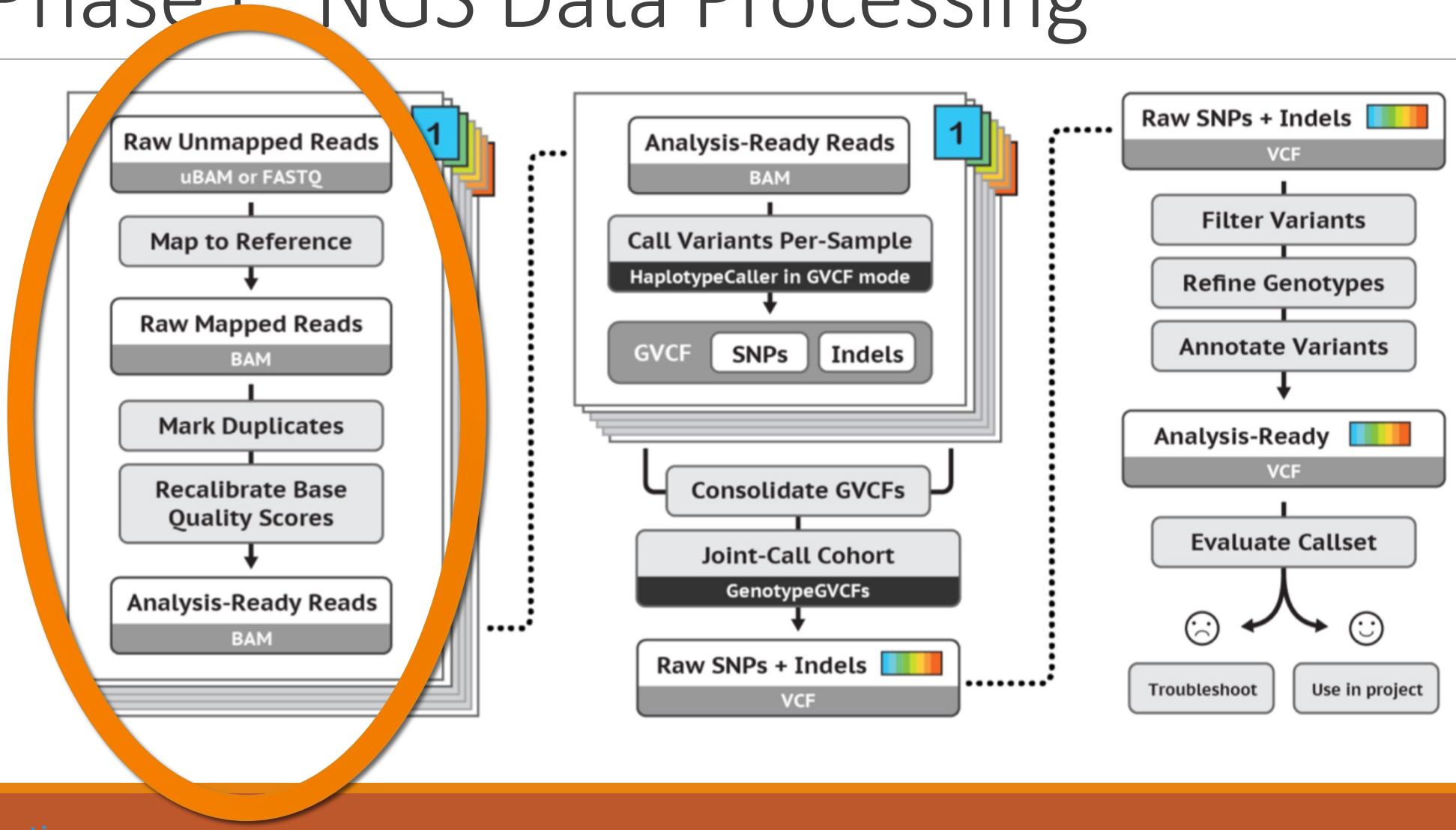
aka 'GATK Best Practices'

GATK Pipeline – Germline Calls



GATK Best Practices

Phase I : NGS Data Processing



GATK Best Practices

Read groups

Information about the samples and how they were run

- **ID** – Simple unique identifier each read belongs to
- **LB** – Library
- **SM** – Sample name
- **PL** – Sequencing platform (Illumina, PacBio, etc)
- **PU** – Platform unit barcode or identifier (flow cell, lane, or similar unit information)
- **PI** – Insert size (fragment size) for library ***(optional)***
- **CN** – Sequencing center name ***(optional)***
- **DS** – Description ***(optional)***
- **DT –** Run date ***(optional)***
- **PM** – Platform model ***(optional)***
- **PG** – Program group ***(optional)***

Phase I

NGS Data Processing

- Alignment of raw reads
- Duplicate marking
- Base quality recalibration
- ***Local realignment no longer required***

Phase I : Alignment of raw reads

Accuracy

- **Sensitivity** – maps reads accurately, allowing for errors or variation
- **Specificity** – maps to the correct region

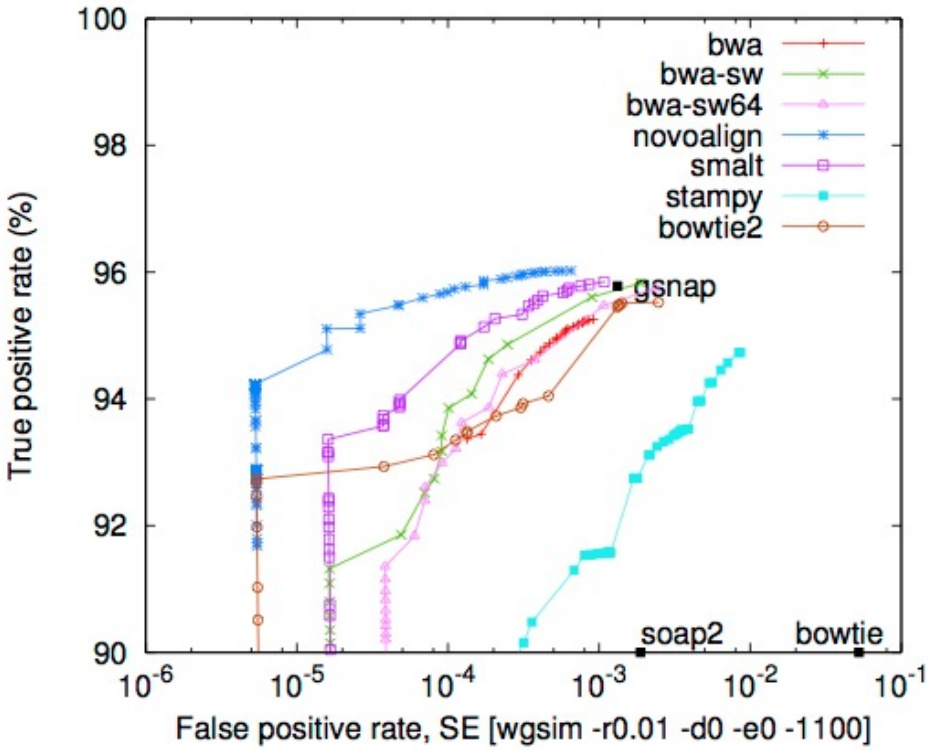
Unique vs. multi-mapped reads

- Should we retain reads mapping to repetitive regions?
- May depend on the application

BWA MEM is currently recommended

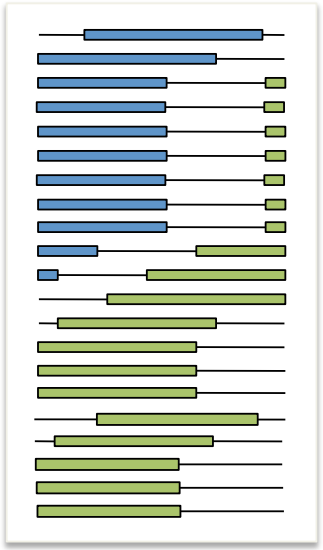
Minimap2 is also good, esp for long reads

You can add read groups at this stage!!!



Heng Li's aligner assessment

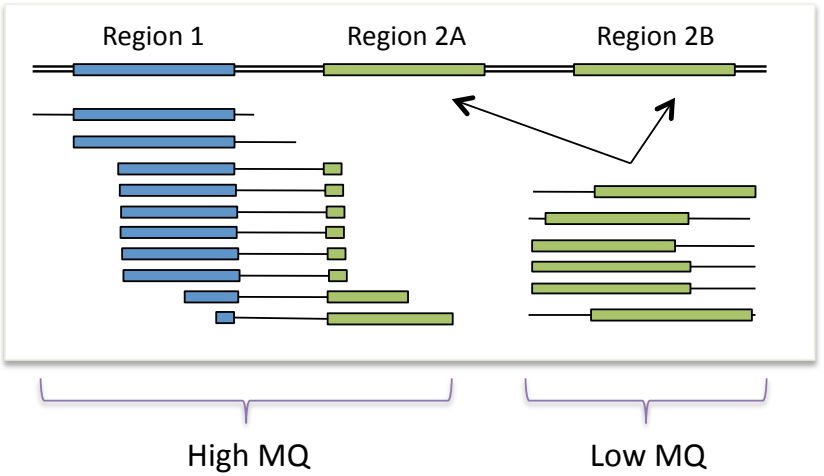
Enormous pile of short reads from NGS



Mapping and alignment algorithms

Mapping algorithms account for this by choosing the most likely placement

→ **mapping quality (MQ)** aka **MAPQ**



For more information see:

Li and Homer (2010). A survey of sequence alignment algorithms for next-generation sequencing. *Briefings in Bioinformatics*.

Alignment output : SAM/BAM

SAM – Sequence Alignment/Map format**; BAM –** BGZF compressed (binary) SAM output

- Stores alignment information
- **Specification**: http://samtools.sourceforge.net/SAM1.pdf
- Contains FASTQ reads, quality information, meta data, alignment information, etc.
- May be unsorted, or sorted by sequence name or genome coordinates
- Sorted BAM may be accompanied by an index file (`.bai`) (only if coord-sorted)
  - Relatively simple format makes it easy to extract specific features, e.g. genomic locations
  - Makes the alignment information easily accessible to downstream applications (large genome file not necessary)

Files are typically very large: 10-100's of GB

Alignment output : SAM/BAM

Alignment

```
Coor     12345678901234  5678901234567890123456789012345
ref      AGCATGTTAGATAA**GATAGCTGTGCTAGTAGGCAGTCAGCGCCAT

+r001/1        TTAGATAAAGGATA*CTG
+r002         aaaAGATAA*GGATA
+r003       gcctaAGCTAA
+r004                       ATAGCT..............TCAGC
-r003                             ttagctTAGGC
-r001/2                                        CAGCGCCAT
```

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

Alignment output : SAM/BAM

1.3 The header section

Each header line begins with character '@' followed by a two-letter record type code. In the header, each line is TAB-delimited and except the @CO lines, each data field follows a format 'TAG:VALUE' where TAG is a two-letter string that defines the content and the format of VALUE. Each header line should match: `/^@[A-Za-z][A-Za-z](\t[A-Za-z][A-Za-z0-9]:[ -~]+)+$/` or `/^@CO\t.*/`. Tags containing lowercase letters are reserved for end users.

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

1.4 The alignment section: mandatory fields

Col	Field	Brief description
1	QNAME	Query template NAME
2	FLAG	bitwise FLAG
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost mapping POSition
5	MAPQ	MAPping Quality
6	CIGAR	CIGAR string
7	RNEXT	Ref. name of the mate/next fragment
8	PNEXT	Position of the mate/next fragment
9	TLEN	observed Template LENgth
10	SEQ	fragment SEQuence
11	QUAL	ASCII of Phred-scaled base QUALity+33

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

1.4 The alignment section: mandatory fields

Col	Field	Brief description
1	QNAME	Query template NAME
2	FLAG	bitwise FLAG
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost mapping POSition
5	MAPQ	MAPping Quality
6	CIGAR	CIGAR string
7	RNEXT	Ref. name of the mate/next fragment
8	PNEXT	Position of the mate/next fragment
9	TLEN	observed Template LENgth
10	SEQ	fragment SEQuence
11	QUAL	ASCII of Phred-scaled base QUALity+33

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

Bit Flags

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001  163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003    0 ref  9 30 5H6M       *  0   0 AGCTAA   *   NM:i:1
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC      *
r003   16 ref 29 30 6H5M       *  0   0 TAGGC    *   NM:i:0
r001   83 ref 37 30 9M         =  7 -39 CAGCGCCAT        *
```

	Hex	0x80	0x40	0x20	0x10	0x8	0x4	0x2	0x1	
	Bit	128	64	32	16	8	4	2	1	
r001		1		1				1	1	= 163

Bit	Description
0x1	template having multiple fragments in sequencing
0x2	each fragment properly aligned according to the aligner
0x4	fragment unmapped
0x8	next fragment in the template unmapped
0x10	SEQ being reverse complemented
0x20	SEQ of the next fragment in the template being reversed
0x40	the first fragment in the template
0x80	the last fragment in the template
0x100	secondary alignment
0x200	not passing quality controls
0x400	PCR or optical duplicate

1.4 The alignment section: mandatory fields

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r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

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8	PNEXT	Position of the mate/next fragment
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10	SEQ	fragment SEQuence
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SAM format

```
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r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
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SAM format

```
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@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

CIGAR

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

Op	BAM	Description
M	0	alignment match (can be a sequence match or mismatch)
I	1	insertion to the reference
D	2	deletion from the reference
N	3	skipped region from the reference
S	4	soft clipping (clipped sequences present in SEQ)
H	5	hard clipping (clipped sequences NOT present in SEQ)
P	6	padding (silent deletion from padded reference)
=	7	sequence match
X	8	sequence mismatch

1.4 The alignment section: mandatory fields

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1	QNAME	Query template NAME
2	FLAG	bitwise FLAG
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost mapping POSition
5	MAPQ	MAPping Quality
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7	RNEXT	Ref. name of the mate/next fragment
8	PNEXT	Position of the mate/next fragment
9	TLEN	observed Template LENgth
10	SEQ	fragment SEQuence
11	QUAL	ASCII of Phred-scaled base QUALity+33

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

1.4 The alignment section: mandatory fields

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5	MAPQ	MAPping Quality
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7	RNEXT	Ref. name of the mate/next fragment
8	PNEXT	Position of the mate/next fragment
9	TLEN	observed Template LENgth
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SAM format

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@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

1.4 The alignment section: mandatory fields

Col	Field	Brief description
1	QNAME	Query template NAME
2	FLAG	bitwise FLAG
3	RNAME	Reference sequence NAME
4	POS	1-based leftmost mapping POSition
5	MAPQ	MAPping Quality
6	CIGAR	CIGAR string
7	RNEXT	Ref. name of the mate/next fragment
8	PNEXT	Position of the mate/next fragment
9	TLEN	observed Template LENgth
10	SEQ	fragment SEQuence
11	QUAL	ASCII of Phred-scaled base QUALity+33

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *  NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *  NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT          *
```

1.5 The alignment section: optional fields

NM	i	Edit distance to the reference, including ambiguous bases but excluding clipping

SAM format

```
@HD VN:1.3 SO:coordinate
@SQ SN:ref LN:45
r001 163 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002   0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA     *
r003   0 ref  9 30 5H6M       *  0   0 AGCTAA    *   NM:i:1
r004   0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003  16 ref 29 30 6H5M       *  0   0 TAGGC     *   NM:i:0
r001  83 ref 37 30 9M         =  7 -39 CAGCGCCAT         *
```

Alignment output : SAM/BAM

1.5 The alignment section: optional fields

Tag[1]	Type	Description
X?	?	Reserved fields for end users (together with Y? and Z?)
AM	i	The smallest template-independent mapping quality of segments in the rest
AS	i	Alignment score generated by aligner
BC	Z	Barcode sequence
BQ	Z	Offset to base alignment quality (BAQ), of the same length as the read sequence. At the i-th read base, $\mathrm{BAQ}_i = Q_i - (\mathrm{BQ}_i - 64)$ where Q_i is the i-th base quality.
CC	Z	Reference name of the next hit; "=" for the same chromosome
CM	i	Edit distance between the color sequence and the color reference (see also NM)
CP	i	Leftmost coordinate of the next hit
CQ	Z	Color read quality on the original strand of the read. Same encoding as QUAL; same length as CS.
CS	Z	Color read sequence on the original strand of the read. The primer base must be included.
E2	Z	The 2nd most likely base calls. Same encoding and same length as QUAL.
FI	i	The index of segment in the template.
FS	Z	Segment suffix.
FZ	B,S	Flow signal intensities on the original strand of the read, stored as (uint16_t) round(value * 100.0).
LB	Z	Library. Value to be consistent with the header RG-LB tag if @RG is present.
H0	i	Number of perfect hits
H1	i	Number of 1-difference hits (see also NM)
H2	i	Number of 2-difference hits
HI	i	Query hit index, indicating the alignment record is the i-th one stored in SAM
IH	i	Number of stored alignments in SAM that contains the query in the current record
MD	Z	String for mismatching positions. *Regex*: [0-9]+(([A-Z]|\^[A-Z]+)[0-9]+)*[2]
MQ	i	Mapping quality of the mate/next segment
NH	i	Number of reported alignments that contains the query in the current record
NM	i	Edit distance to the reference, including ambiguous bases but excluding clipping
OQ	Z	Original base quality (usually before recalibration). Same encoding as QUAL.
OP	i	Original mapping position (usually before realignment)
OC	Z	Original CIGAR (usually before realignment)
PG	Z	Program. Value matches the header PG-ID tag if @PG is present.
PQ	i	Phred likelihood of the template, conditional on both the mapping being correct
PU	Z	Platform unit. Value to be consistent with the header RG-PU tag if @RG is present.
Q2	Z	Phred quality of the mate/next segment. Same encoding as QUAL.
R2	Z	Sequence of the mate/next segment in the template.
RG	Z	Read group. Value matches the header RG-ID tag if @RG is present in the header.
SM	i	Template-independent mapping quality
TC	i	The number of segments in the template.
U2	Z	Phred probility of the 2nd call being wrong conditional on the best being wrong. The same encoding as QUAL.
UQ	i	Phred likelihood of the segment, conditional on the mapping being correct

Too many to go over!!!

Alignment output : SAM/BAM

Tools

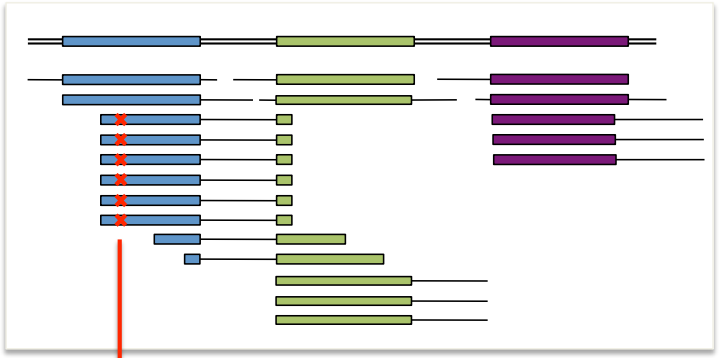
- **samtools**
- **Picard**

Mining information from a properly formatted BAM file:

- Reads in a region (good for RNA-Seq, ChIP-Seq)
- Quality of alignments
- Coverage
- …and of course, differences (variants)

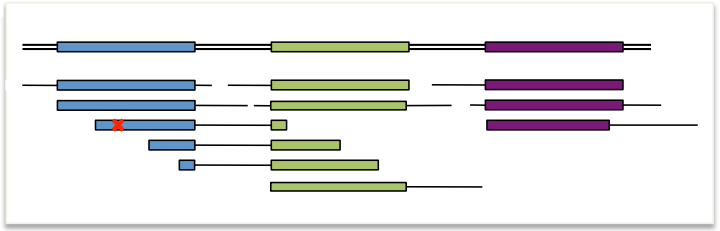
The reason why duplicates are bad

✖ = sequencing error propagated in duplicates



FP variant call (bad)

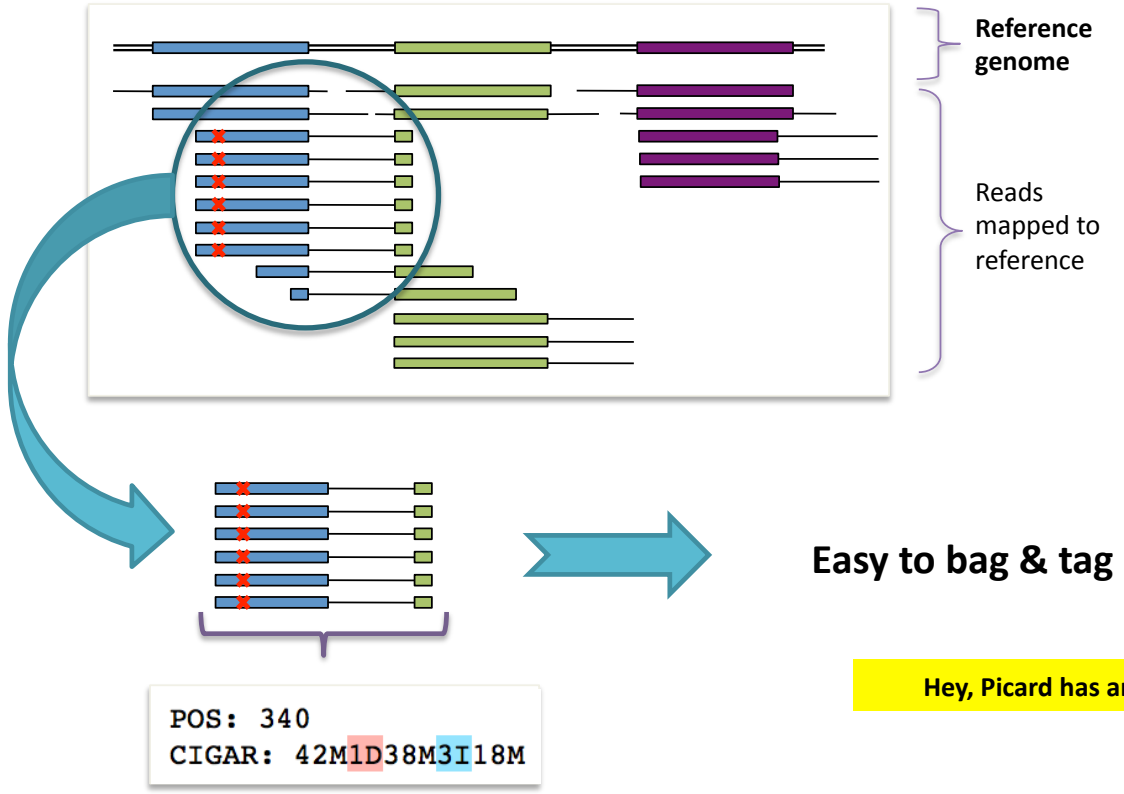
After marking duplicates, the GATK will only see :



… and thus be more likely to make the right call

Phase I : Duplicate Marking

Duplicates have the same starting position and the same CIGAR string

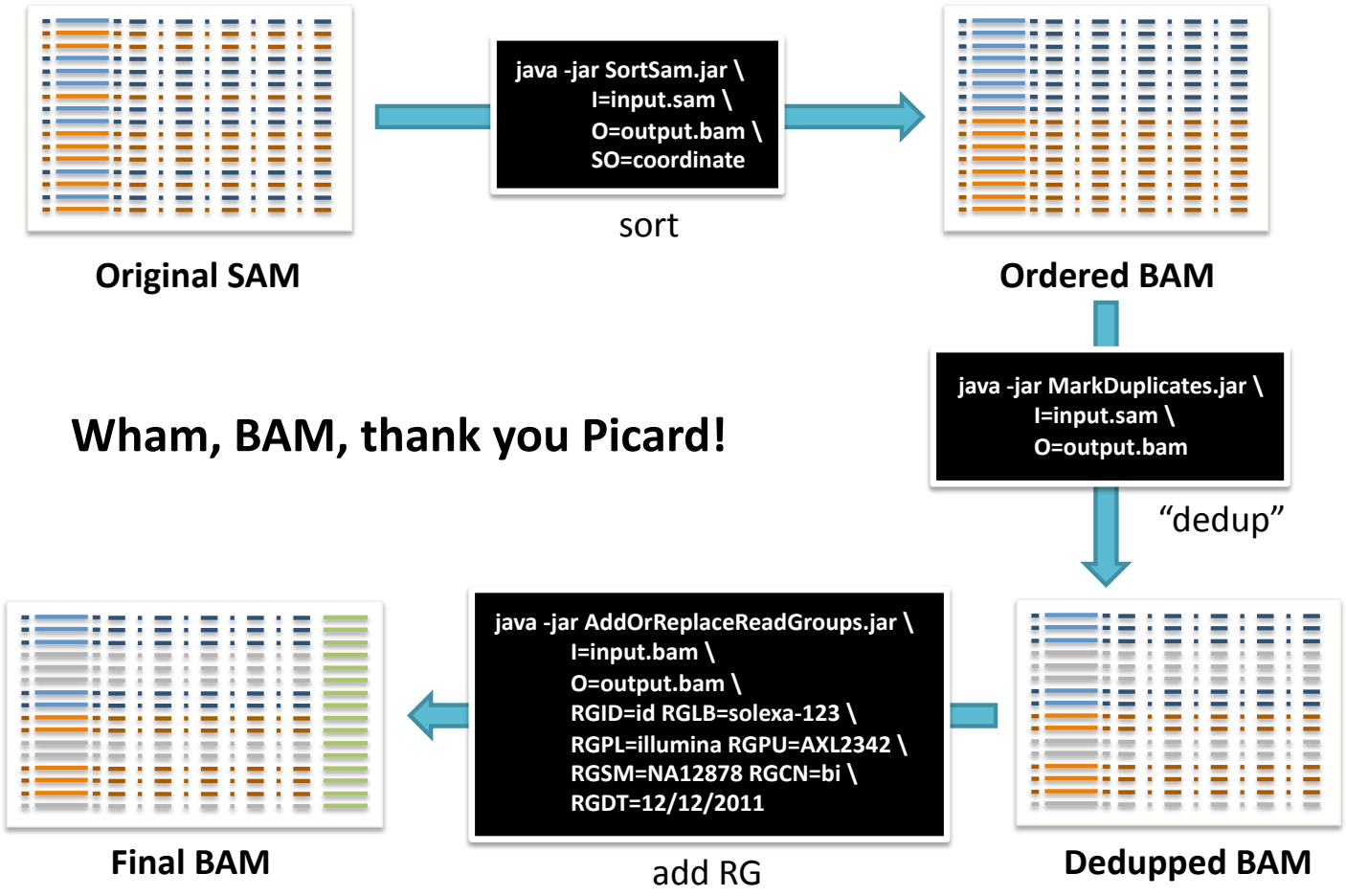


Easy to bag & tag

Hey, Picard has an app for that!

Phase I : Duplicate Marking

Typical workflow using Picard tools to mark duplicates *et al.*



Wham, BAM, thank you Picard!

Phase I : Sorting, Read Groups

Phase I : Base Quality Score Recalibration

Quality scores from sequencers are biased and somewhat inaccurate

Quality scores are critical for all downstream analysis

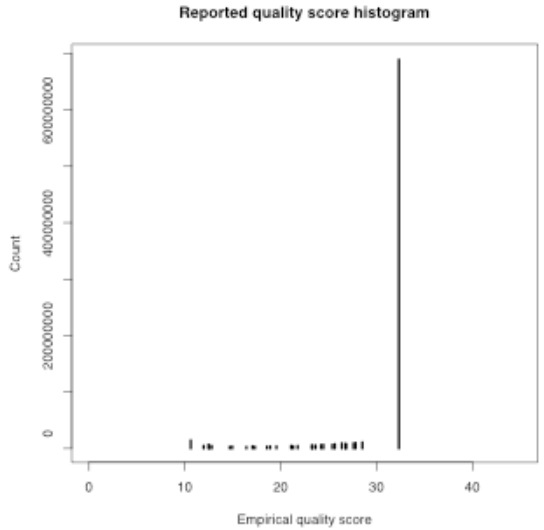
Biases are a major contributor to bad variant calls

Caveat:

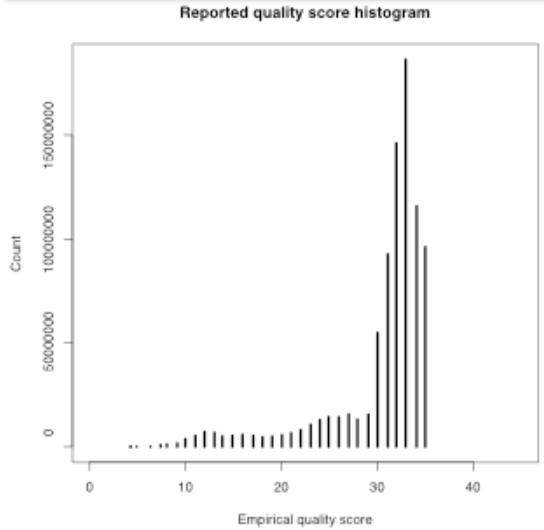
- **In practice, requires having a known set of variants (dbSNP)**

BQSR (GATK Link)

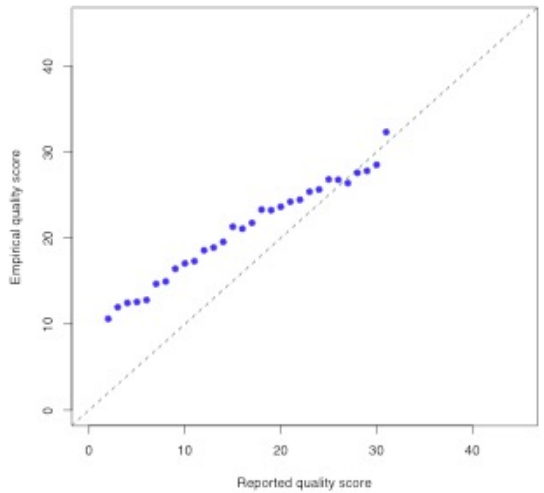
Original



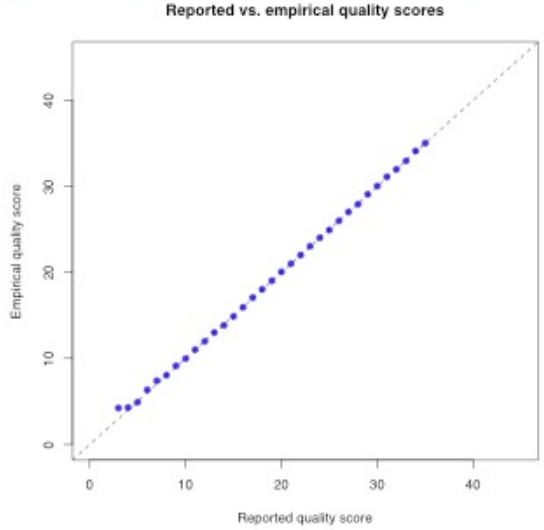
Recalibrated



Original

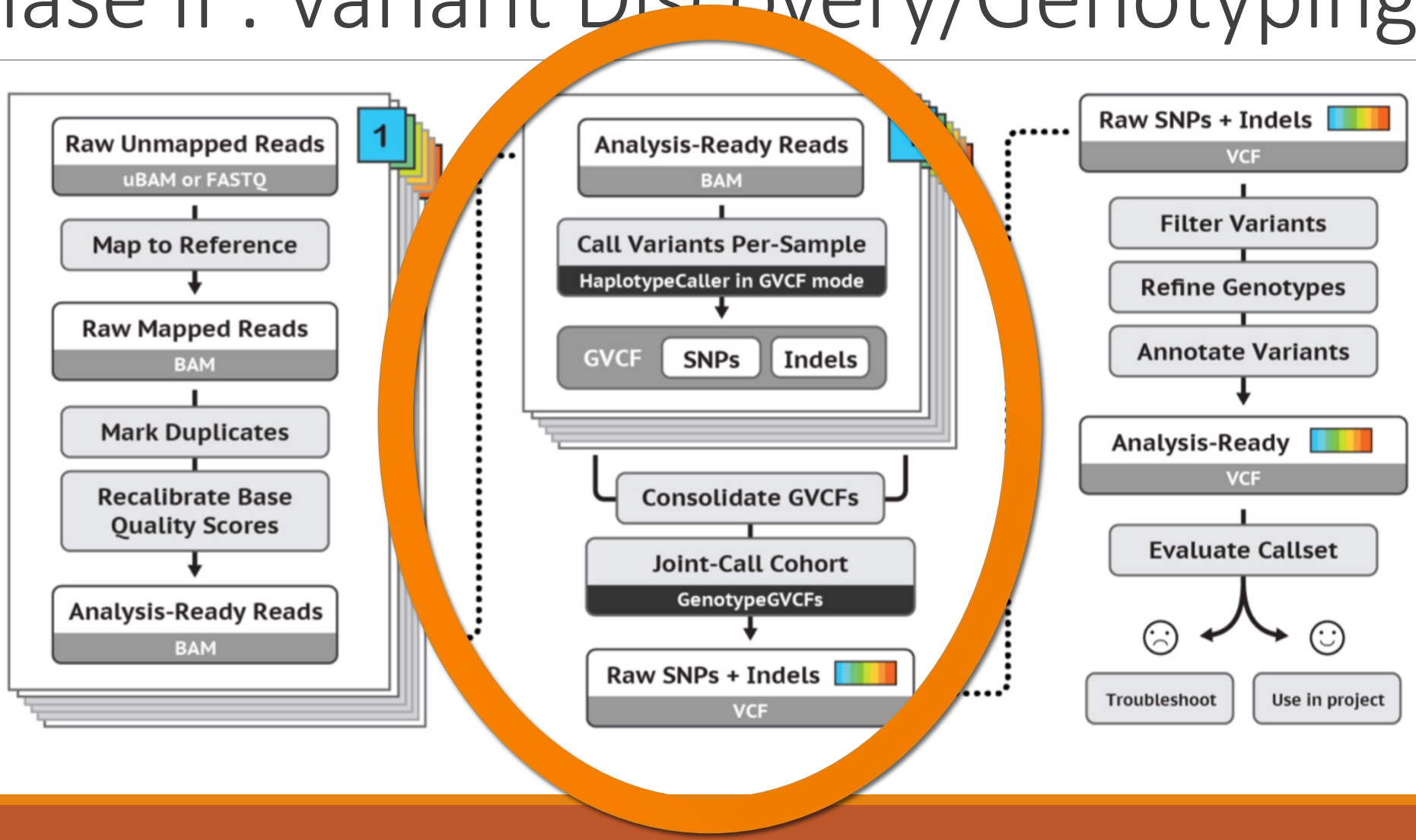


Recalibrated



Also works for binned quality scores (NovaSeq)

Phase II : Variant Discovery/Genotyping



GATK Best Practices

Phase II : Variant Calling

This is where we actually call the variants

Prior steps leading up to this help remove potential causes of variant calling errors

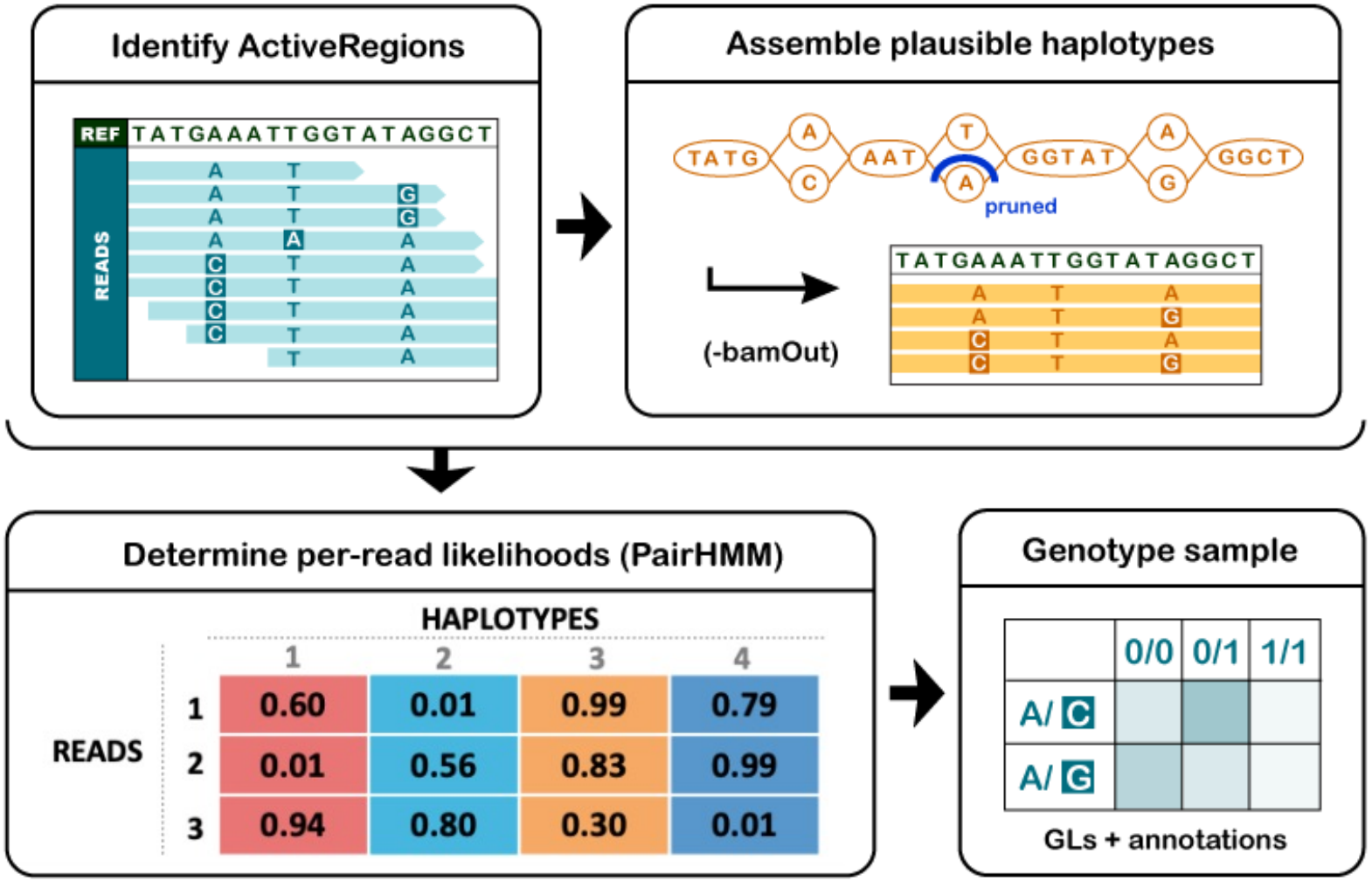
I'll be covering the current recommended caller, the **HaplotypeCaller.** I have a slide on the legacy **UnifiedGenotyper** (not included in GATK v4) included in this talk as well.

Phase II : Variant Calling *HaplotypeCaller*

Assembly-based approach

- Define active regions (evidence for variation)
- Re-assemble active region, align against reference
  - Get a list of *possible* haplotypes
  - No need for local realignment
- Determine likelihoods based on reads compared to haplotypes
- Find most likely genotype at each site, emit as a call

[HaplotypeCaller in a nutshell](#) (Broad Institute)



DeepVariant

'Deep learning' based variant calling tool

NOT PART OF GATK

Considered more accurate than HC

nature biotechnology

Explore content ⌄ Journal information ⌄ Publish with us ⌄

nature › nature biotechnology › letters › article

Published: 24 September 2018

A universal SNP and small-indel variant caller using deep neural networks

Ryan Poplin, Pi-Chuan Chang, David Alexander, Scott Schwartz, Thomas Colthurst, Alexander Ku, Dan Newburger, Jojo Dijamco, Nam Nguyen, Pegah T Afshar, Sam S Gross, Lizzie Dorfman, Cory Y McLean & Mark A DePristo ✉

Nature Biotechnology **36**, 983–987 (2018) | Cite this article

23k Accesses | **144** Citations | **320** Altmetric | Metrics

https://github.com/google/deepvariant

Output?

Variant calling output: VCF

VCF (Variant Call Format)

Like SAM/BAM, also has a versioned specification

- **From the 1000 Genomes Project**
- **http://www.1000genomes.org/wiki/Analysis/Variant%20Call%20Format/vcf-variant-call-format-version-41**

Structure (2 parts):

- **Header (metadata)**
- **Variant calls (one or more samples)**

Variant calls have multiple fields (right)

COL	FIELD	DESCRIPTION
1	CHROM	Chromosome name
2	POS	1-based position. For an indel, this is the position preceding the indel.
3	ID	Variant identifier. Usually the dbSNP rsID.
4	REF	Reference sequence at POS involved in the variant. For a SNP, it is a single base.
5	ALT	Comma delimited list of alternative seuqence(s).
6	QUAL	Phred-scaled probability of all samples being homozygous reference.
7	FILTER	Semicolon delimited list of filters that the variant fails to pass.
8	INFO	Semicolon delimited list of variant information.
9	FORMAT	Colon delimited list of the format of individual genotypes in the following fields.
10+	Sample(s)	Individual genotype information defined by FORMAT.

Formats: 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
```

Formats: VCF - Header

```
##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
```

Formats: VCF – Variant calls

```
##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
```

Formats: VCF – Chromosome and position

```
##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
```

Formats: VCF - ID

```
##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
```

Formats: VCF – Reference and alternate alleles

```
##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
```

Formats: VCF – Variant quality

```
##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
```

Formats: VCF – Filter

```
##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
```

Formats: VCF – Variant information (across samples)

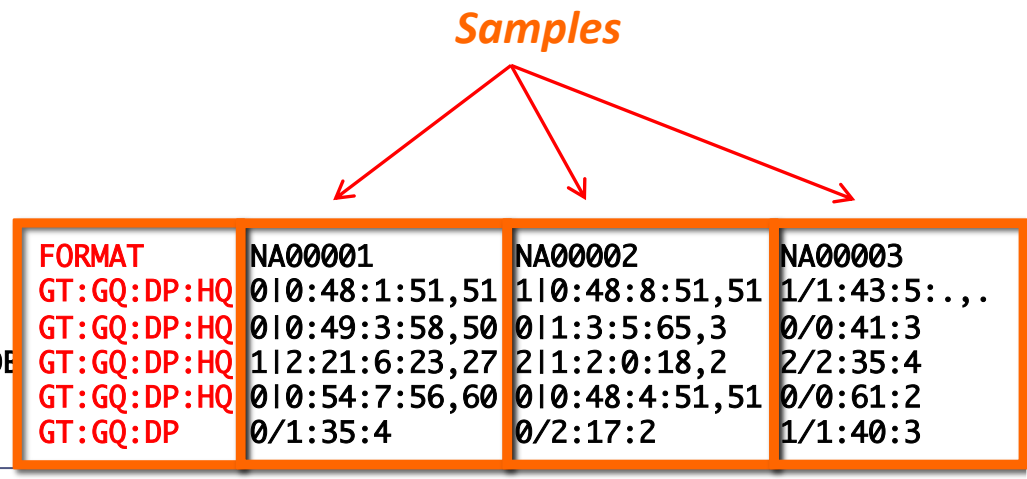
```
##fileformat=VCFv4.1
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number 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
```

Formats: VCF - Per-sample format information

```
##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
```

Formats: VCF – Formats - Variant per-sample information

```
##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
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
20     1230237 .         T   .       47   PASS   NS=3;DP=13;AA=T
20     1234567 microsat1 GTC G,GTCT  50   PASS   NS=3;DP=9;AA=G
```



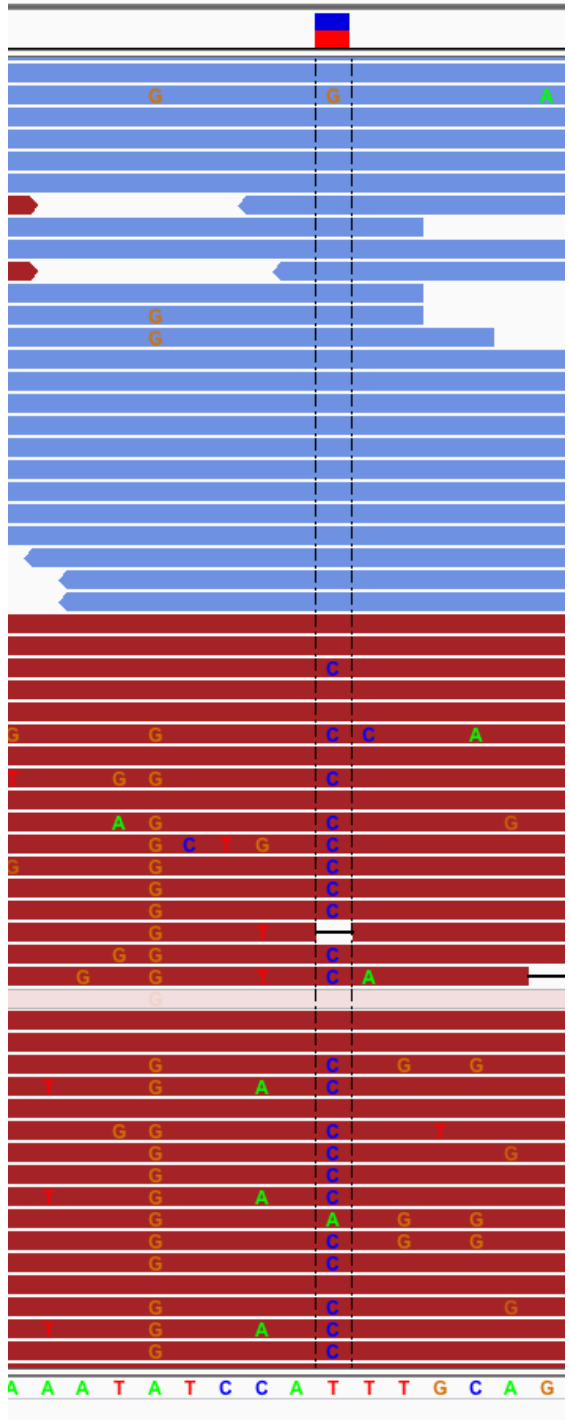
Annotations

Additional data included for variants that help assess quality of the call

Complete list link (GATK)

Can include measures/scores for:

- Quality of variant call (QD)
- Total or allele-specific read depth
- Read base quality metrics
- Strand bias (FS) – at right
- Base quality (QD)
- Consanguinity (InbreedingCoefficient)
- Tumor/normal somatic calling information (TLOD, NLOD)

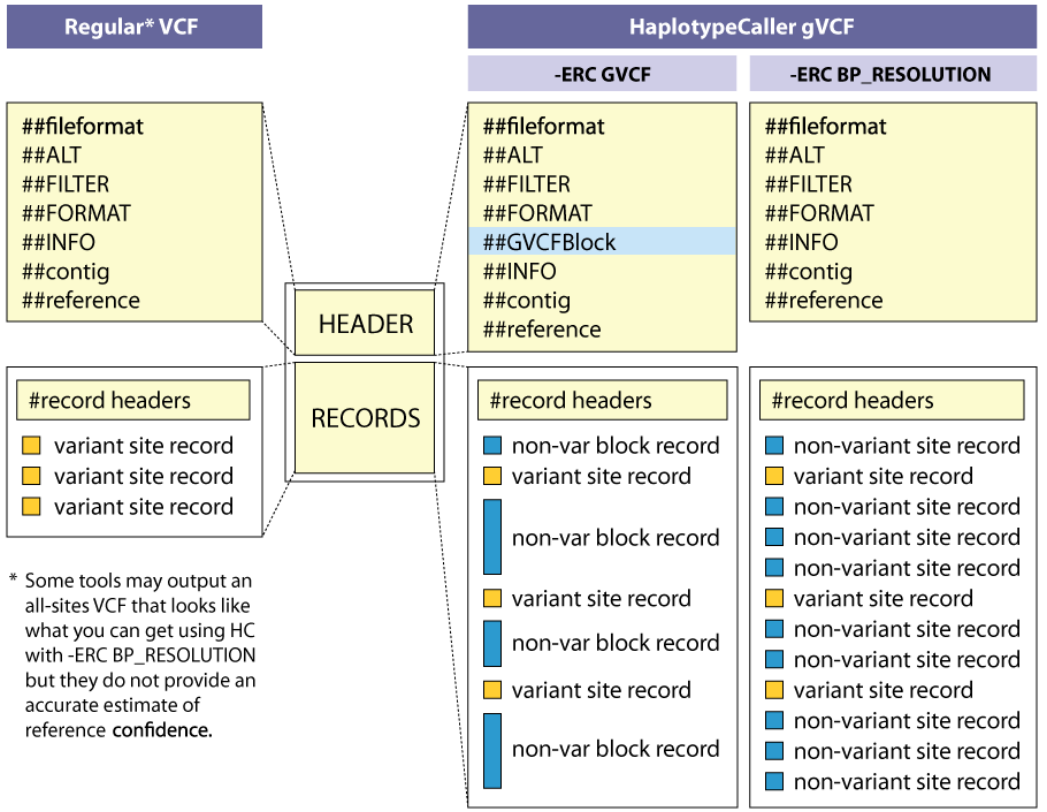


GVCF

Genomic VCF

A VCF file that contains a record for every site (regardless if there is a variant or not)

Highly recommended for multi-sample calling



GATK GVCF

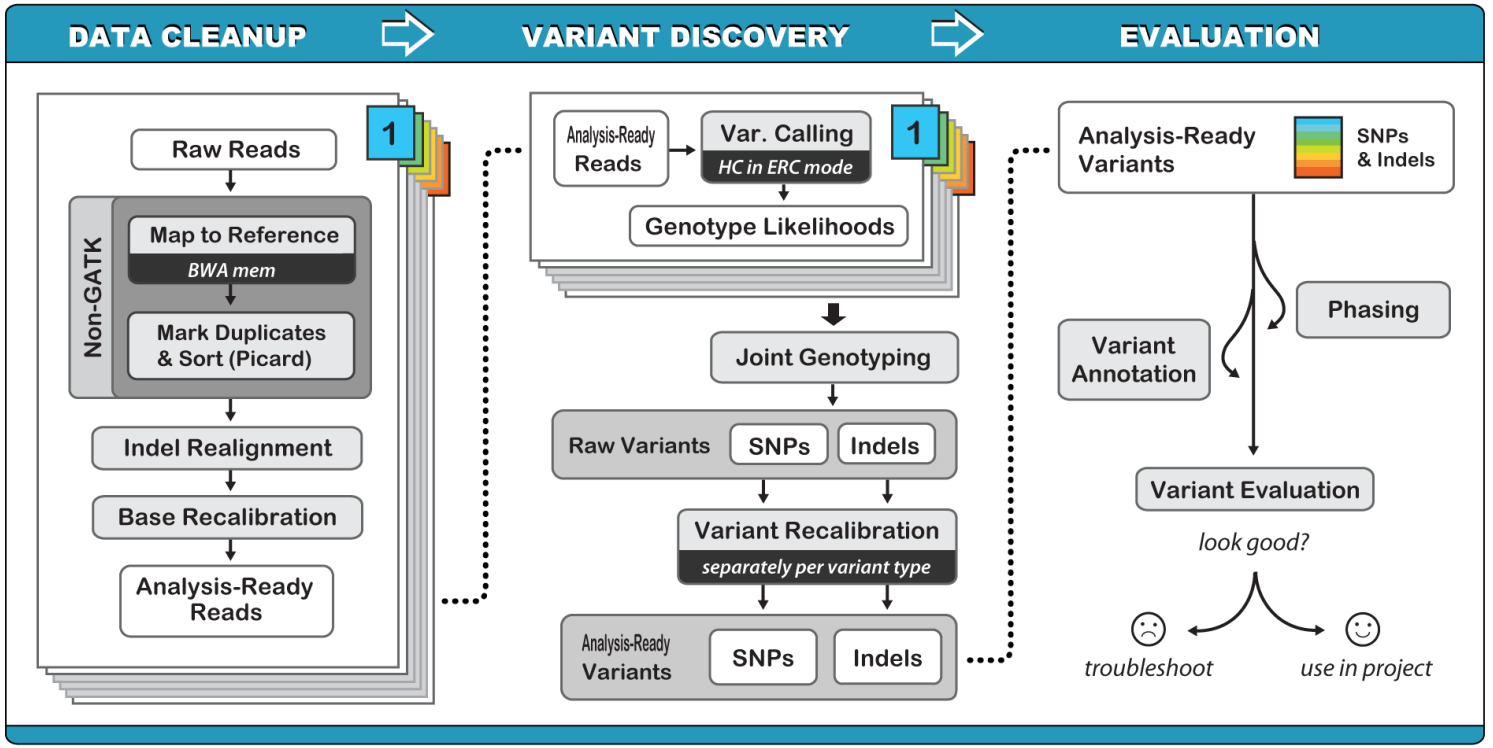
Calling variants on cohorts of samples

Perform joint genotyping calls on cohort

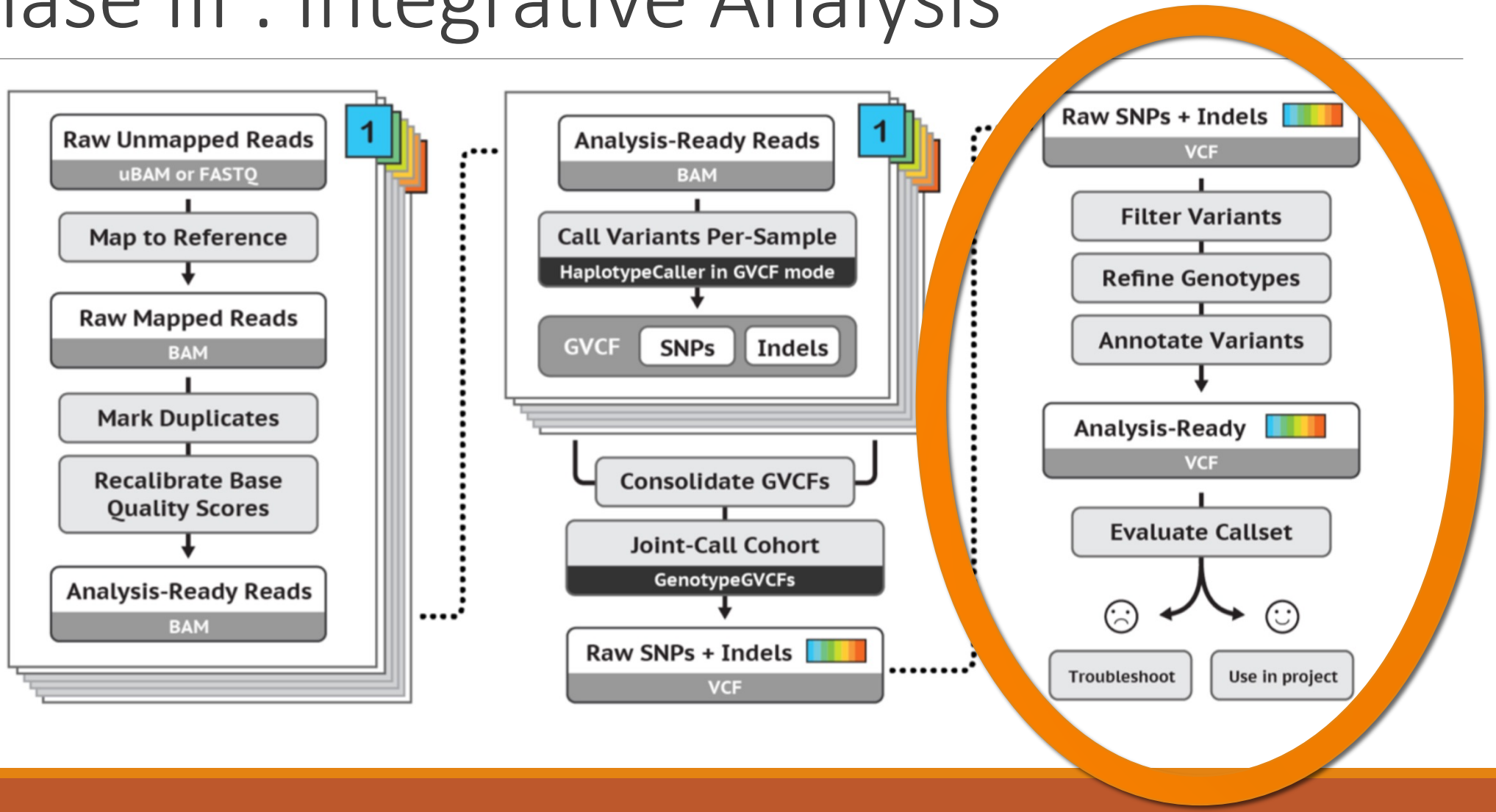
Can rerun as needed if more samples added to cohort

Used for ExAC cohort (92K exomes)

Link!



Phase III : Integrative Analysis



GATK Best Practices

Phase III : Filtering

Two basic methods:

- Hard filtering
- Variant quality score recalibration (VQSR)

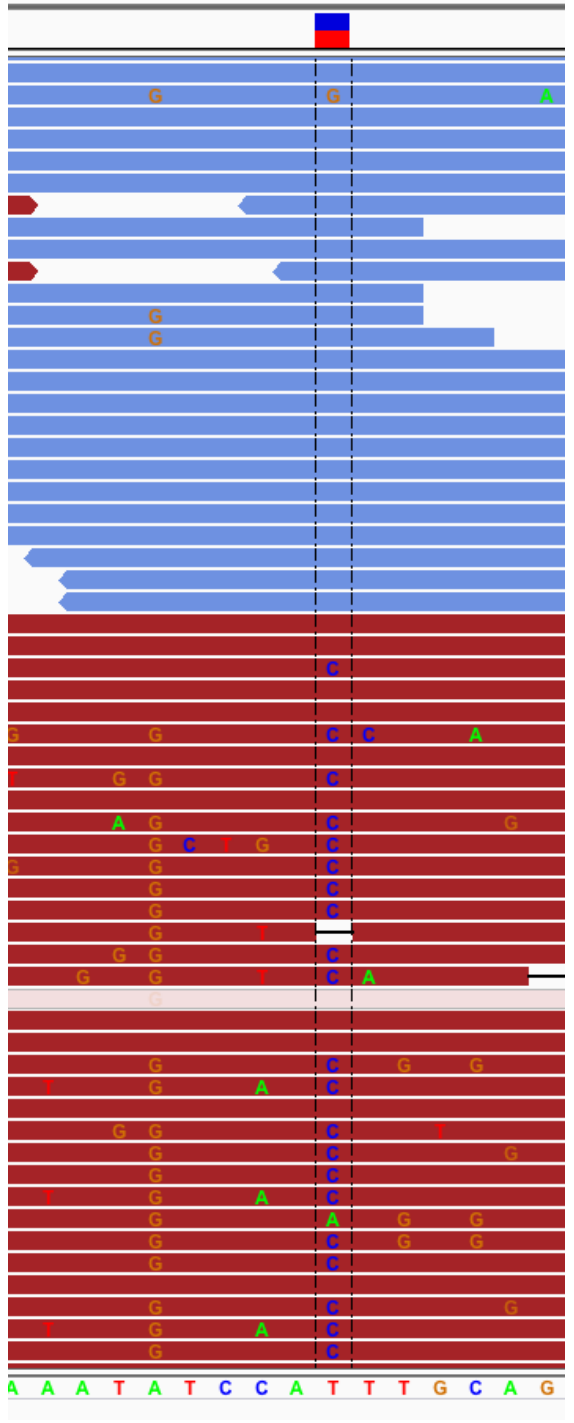
Phase III : Hard Filtering

Reducing false positives by e.g. requiring

- Sufficient Depth
- Variant to be in >30% reads
- High quality
- **Strand balance**
- Etc etc etc

Very high dimensional search space

- … so, very subjective!



Phase III : Hard Filtering

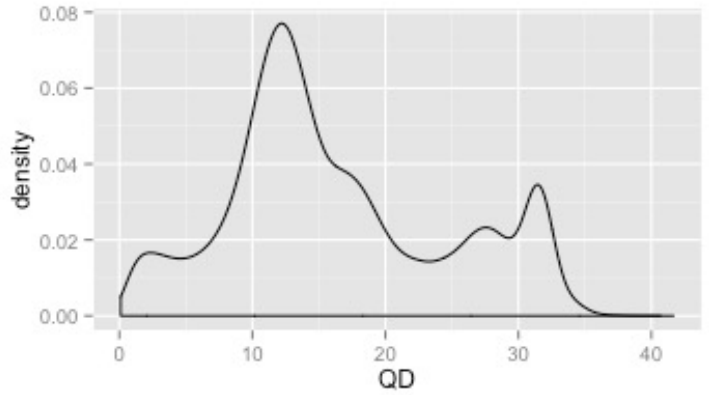
Great overview here

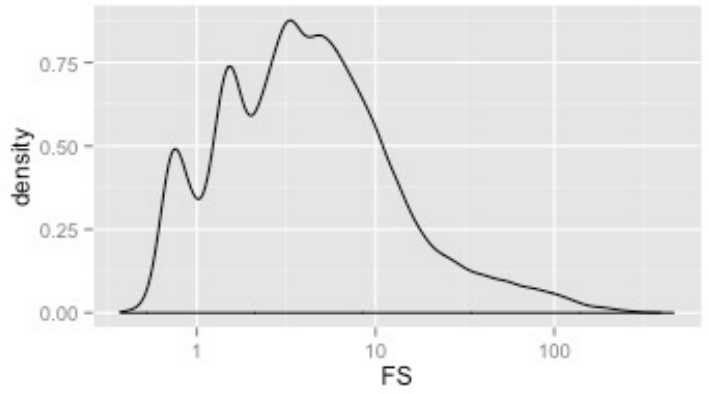
Some starting points here

Visualize distribution of annotation value, pick cutoff

Most informative annotations:

- QD – normalized quality
- FS – strand bias
- SOR - strand bias
- MQ – mapping qual of reads
- MQRankSum - mapping qual of reads
- ReadPosRankSum - position of alleles in read





Phase III : Variant Quality Score Recalibration (VQSR)

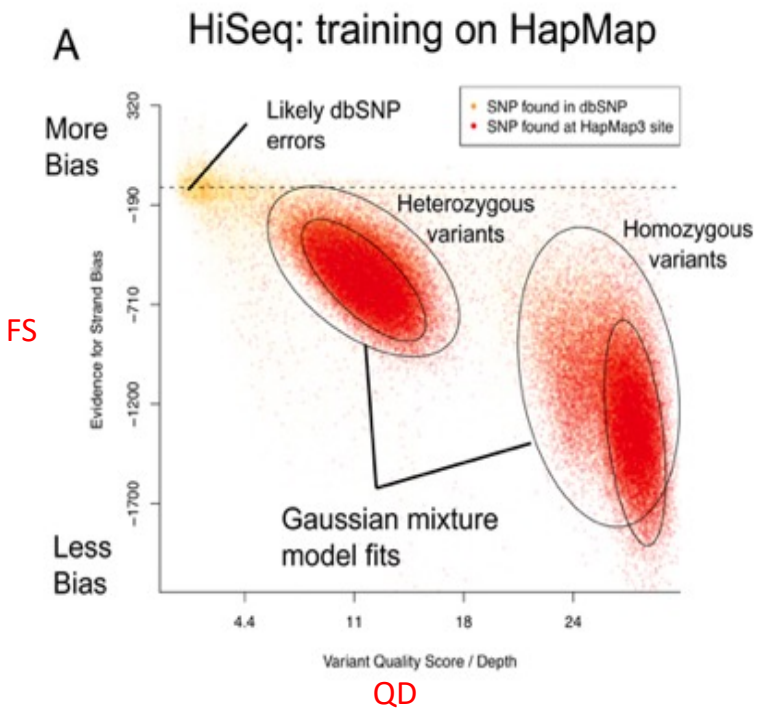
Considered GATK ‘best practice’

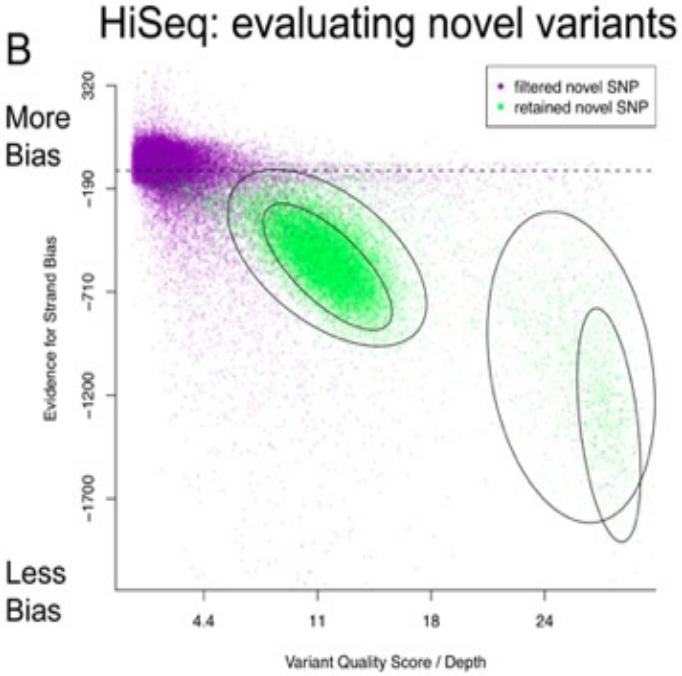
Train on trusted variants (e.g. HapMap)

Require the new variants to live in the same hyperspace

Potential problems:

- Over-fitting
- Biasing to features of known SNPs





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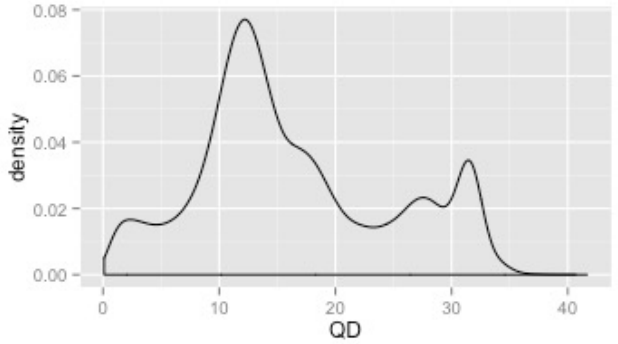
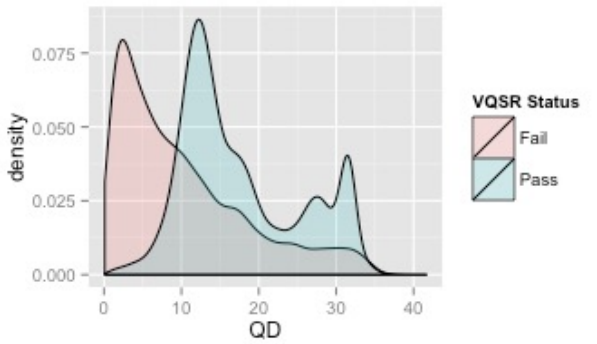
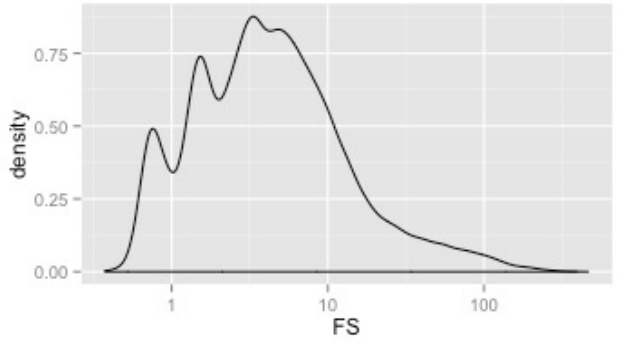
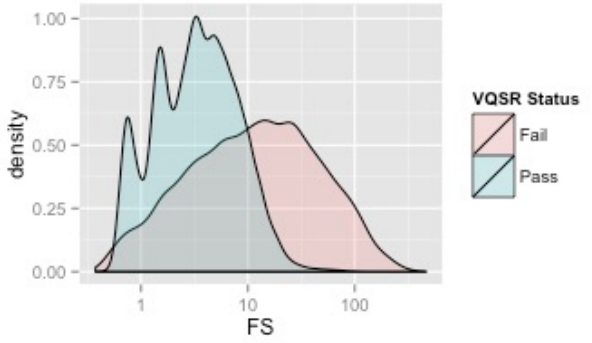
Hard Filtering



VQSR







Phase III : Functional Annotation

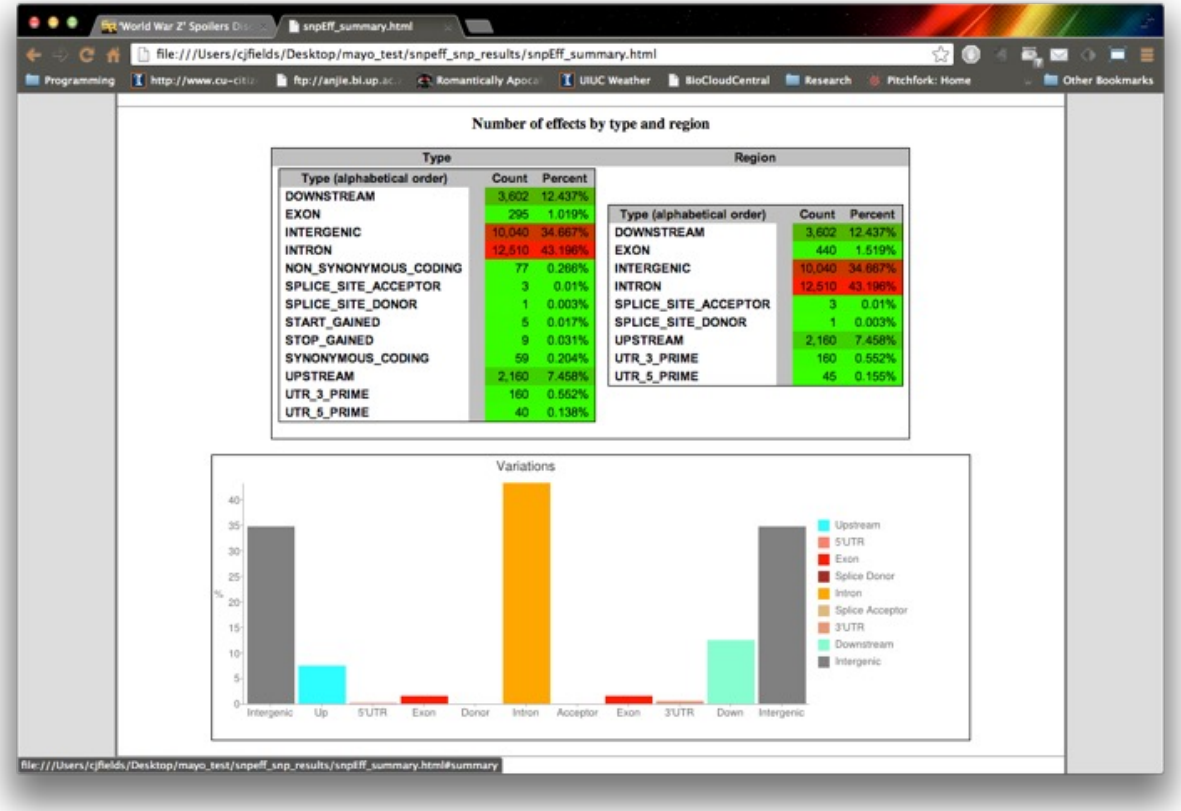
Are these mutations in important regions?

- Genes? UTR?
- Are they changing the coding sequence?

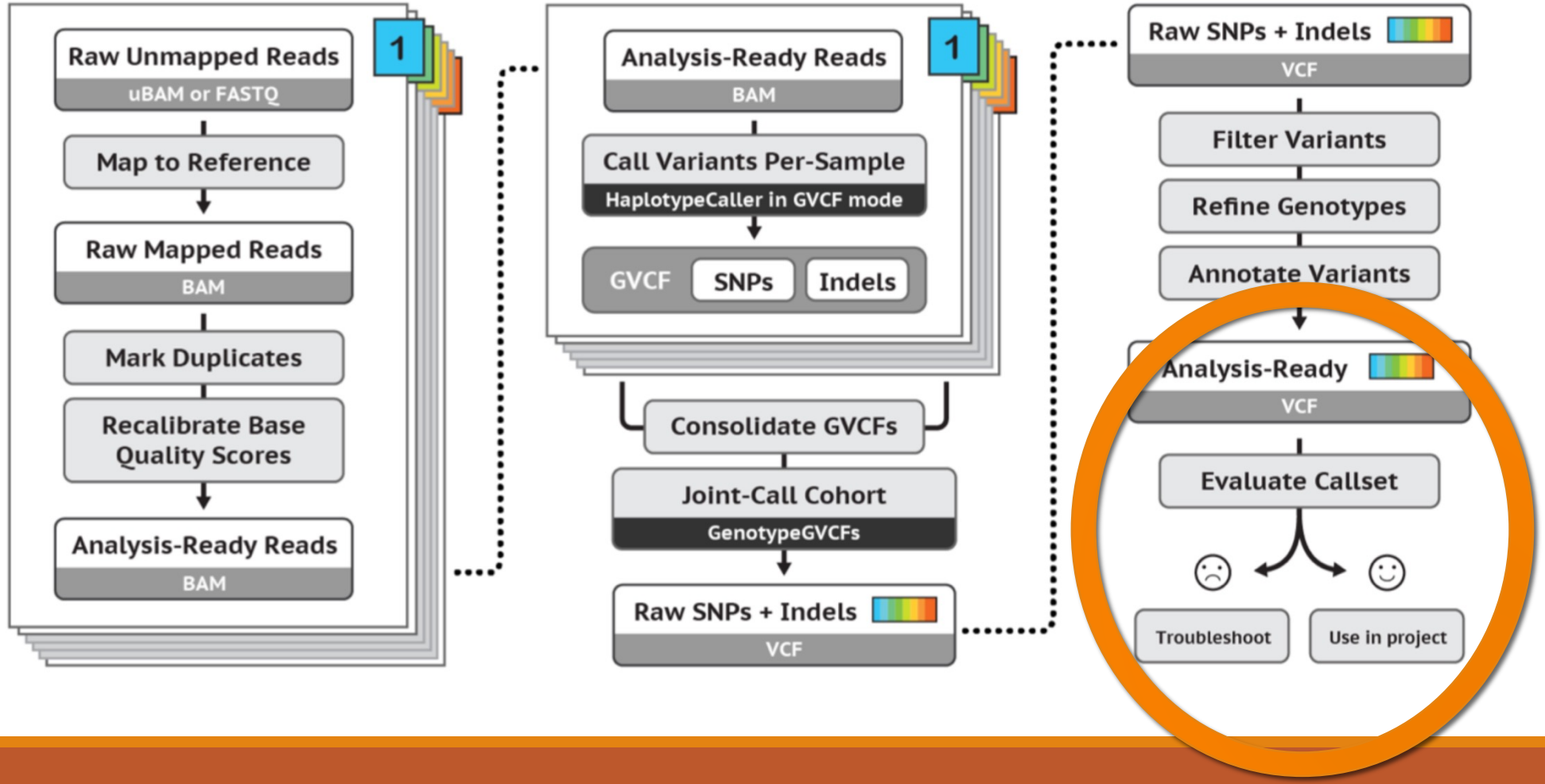
Would these changes have an affect?

Tools:

- SnpEff/SnpSift
- Annovar



The end of the (pipe)line

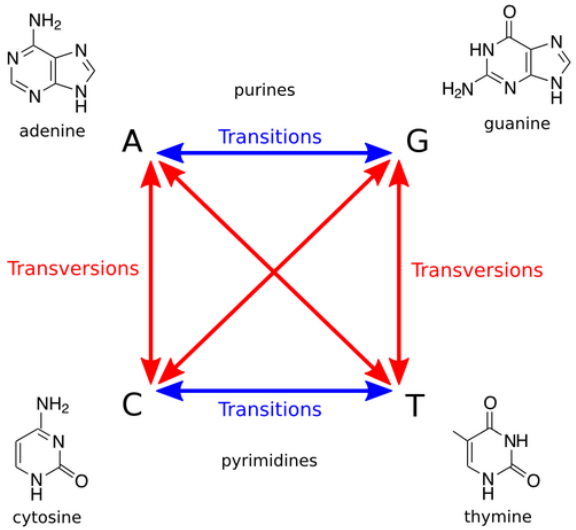


GATK Best Practices

Follow-up Quality Control

Trans**i**tion/**T**rans**v**ersion ratio (T_i/T_v)

Condition	Expected T_i/T_v
random	0.5
whole genome	2.1
exome	3.0-3.3



- ***bcftools*** can help here

Concordance with known variants: dbSNP, HapMap, 1000genomes

Lower than expected – possibly includes more false positives

Higher that expected – indicates potential bias

Acknowledgments

Many figures/slides come from:

- GATK Workshop slides: https://qcb.ucla.edu/collaboratory/resources/gatk-workshop-slide-sets-march-2016/