Variant Calling - SNPs and short indels

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Variant types

SNPs/SNVs ... Single Nucleotide Polymorphism/Variation

```
ACGTTTAGCAT
ACGTTCAGCAT
```

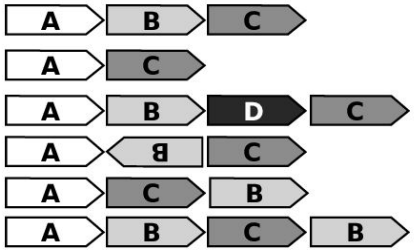
MNPs ... Multi-Nucleotide Polymorphism

```
ACGTCCAGCAT
ACGTTTAGCAT
```

Indels ... short insertions and deletions

```
ACGTTTAGCA-TT
ACGTT-AGCAGTT
```

SVs ... Structural Variation



Germline vs somatic mutation

Germline mutation

- heritable variation in the germ cells

Somatic mutation

- variation in non-germline tissue, tumors...

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Germline variant calling

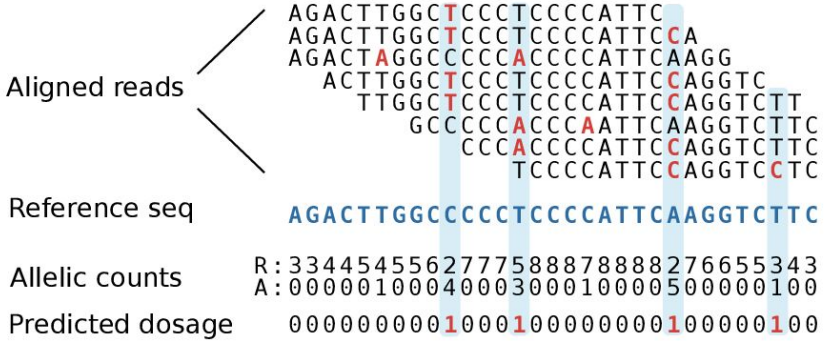
- expect the following fractions of alternate alleles in the pileup:
  - 0.0 for RR genotype (plus sequencing errors)
  - 1.0 for AA (plus sequencing errors)
  - 0.5 for RA (random variation of binomial sampling)

Somatic

- any fraction of alt AF possible - subclonal variation, admixture of normal cells in tumor sample

Naive variant calling

Use fixed allele frequency threshold to determine the genotype



alt AF	genotype
$[0, 0.2)$	RR .. homozygous reference
$[0.2, 0.8]$	RA .. herezogyous
$(0.8, 1]$	AA .. homozygous variant

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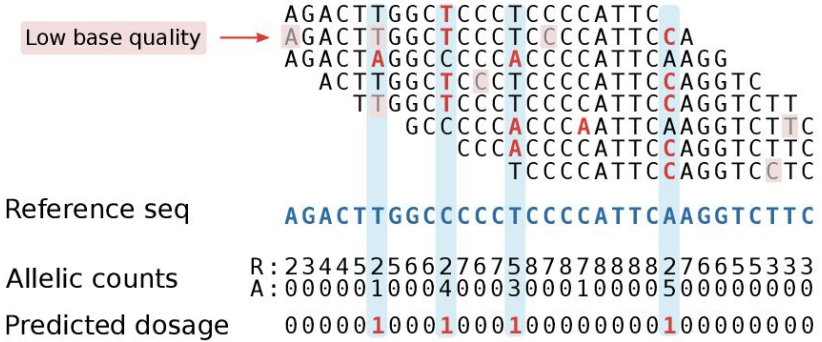
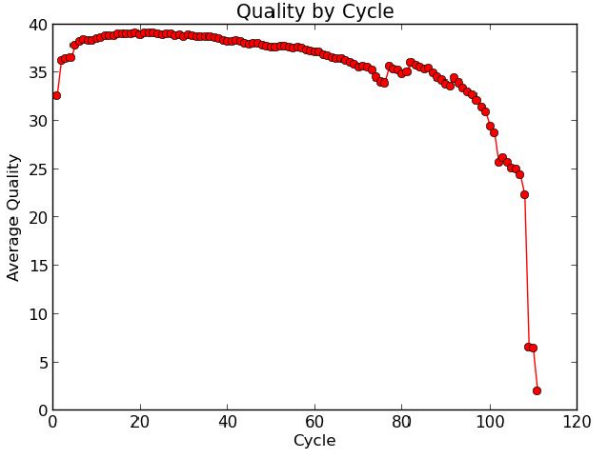


1) Filter base calls by quality
   e.g. ignore bases Q<20

Phred quality score

$$Q = -10 \log_{10} P_{\text{err}}$$

Quality	Error probability	Accuracy
10 (Q10)	1 in 10	90%
20 (Q20)	1 in 100	99%
30 (Q30)	1 in 1000	99.9%
40 (Q40)	1 in 10000	99.99%



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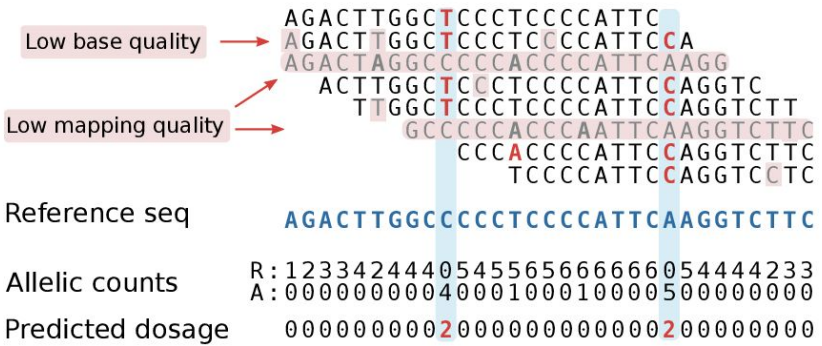

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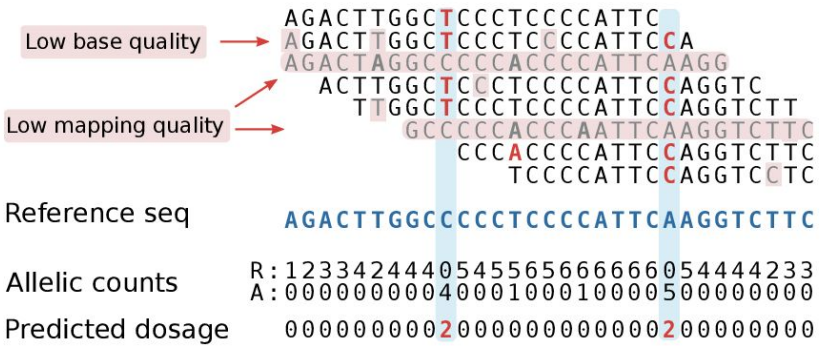

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Problems:

- undercalls hets in low-coverage data
- throws away information due to hard quality thresholds
- gives no measure of confidence

Real life calling models

More sophisticated models apply a statistical framework

Likelihood Prior

$$P(G|D) = \frac{P(D|G)\,P(G)}{P(D)}$$

Posterior Normalization

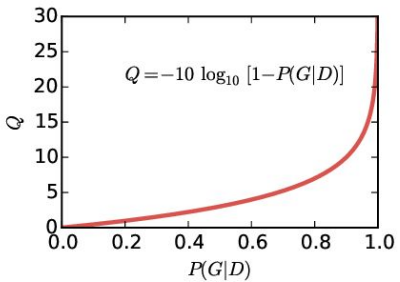
to determine:

1. the most likely genotype $g \in \{\text{RR}, \text{RA}, \text{AA}\}$ given the observed data D

$$g = \underset{G}{\operatorname{argmax}}\, P(G|D)$$

2. and the genotype quality

$$Q = -10 \log_{10}[1 - P(G|D)]$$



Important terms you may encounter

Genotype likelihoods

- which of the three genotypes RR, RA, AA is the data most consistent with?
- calculated from the alignments, the basis for calling
- takes into account:
  - base calling errors
  - mapping errors
  - statistical fluctuations of random sampling
  - local indel realignment (base alignment quality, BAQ)

Prior probability

- how likely it is to encounter a variant base in the genome?
- some assumptions are made
  - allele frequencies are in Hardy-Weinberg equilibrium

    $P(\text{RA}) = 2f(1-f)$, $P(\text{RR}) = (1-f)^2$, $P(\text{AA}) = f^2$
- can take into account genetic diversity in a population

$$P(G|D) = \frac{P(D|G)\,P(G)}{P(D)}$$

Variant calling example

Inputs

- alignment file
- reference sequence

Outputs

- VCF or BCF file

Example

```
bcftools mpileup -f ref.fa aln.bam | bcftools call -mv
```

Tips

`bcftools mpileup`

- increase/decrease the required number (`-m`) and the fraction (`-F`) of supporting reads for indel calling
- the `-Q` option controls the minimum required base quality (30)
- BAQ realignment is applied by default and can be disabled with `-B`
- streaming the uncompressed binary BCF (`-Ou`) is much faster than the default text VCF

`bcftools call`

- decrease/increase the prior probability (`-P`) to decrease/increase sensitivity

General advice

- take time to understand the options
- play with the parameters, see how the calls change

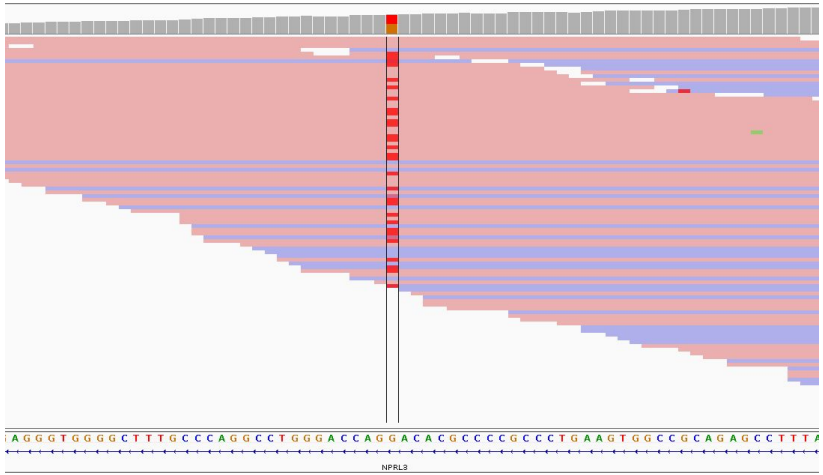
Factors to consider in calling

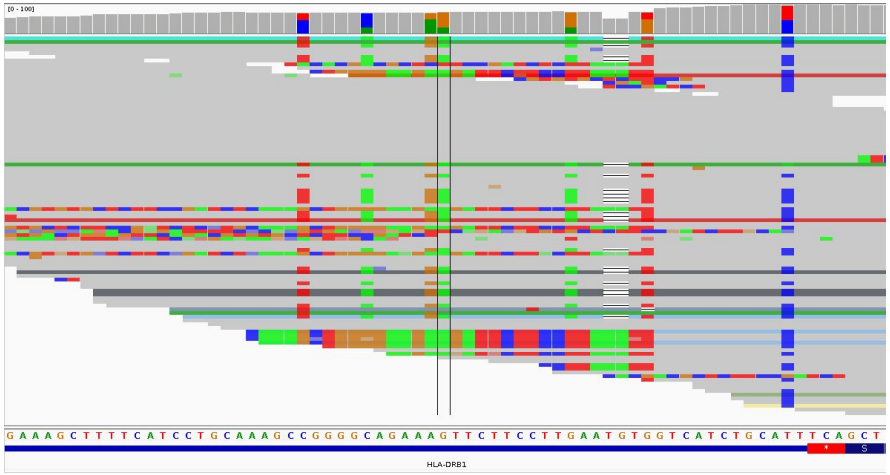
Many calls are not real, a **filtering** step **is necessary**

False calls can have many causes

- contamination
- PCR errors
- sequencing errors
  - homopolymer runs
- mapping errors
  - repetitive sequence
  - structural variation
- alignment errors
  - false SNPs in proximity of indels
  - ambiguous indel alignment

The good, the bad, and the ugly





HLA-DRB1 is a gene with 13 paralogs

Finding a needle in a needle stack

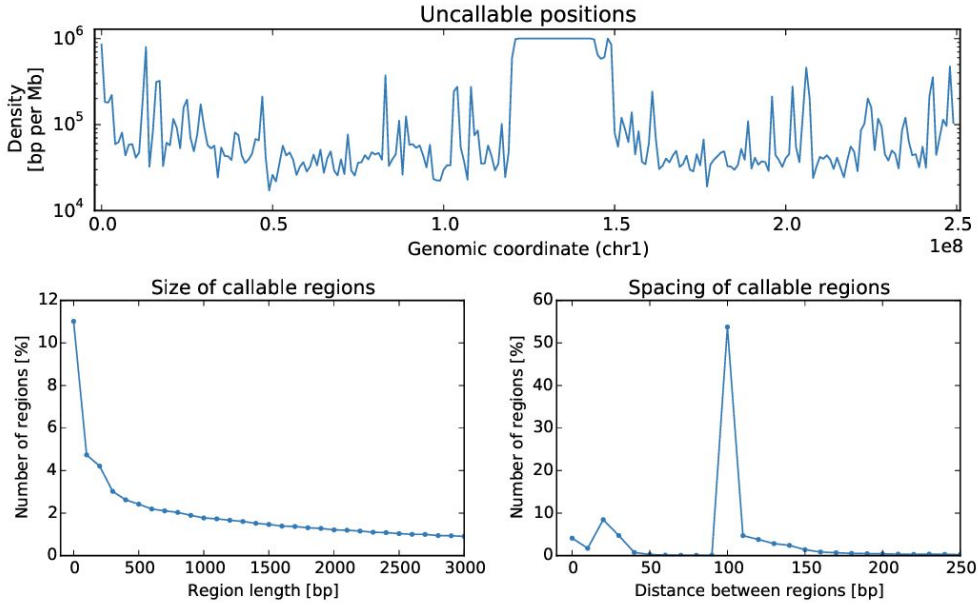
3,494,429 SNVs and short indels

- which ones are real?
- which ones are causal?

Callable genome

Large parts of the genome are still inaccessible

- the Genome in a Bottle high-confidence regions:
  - cover 89% of the reference genome
  - are short intervals scattered across the genome



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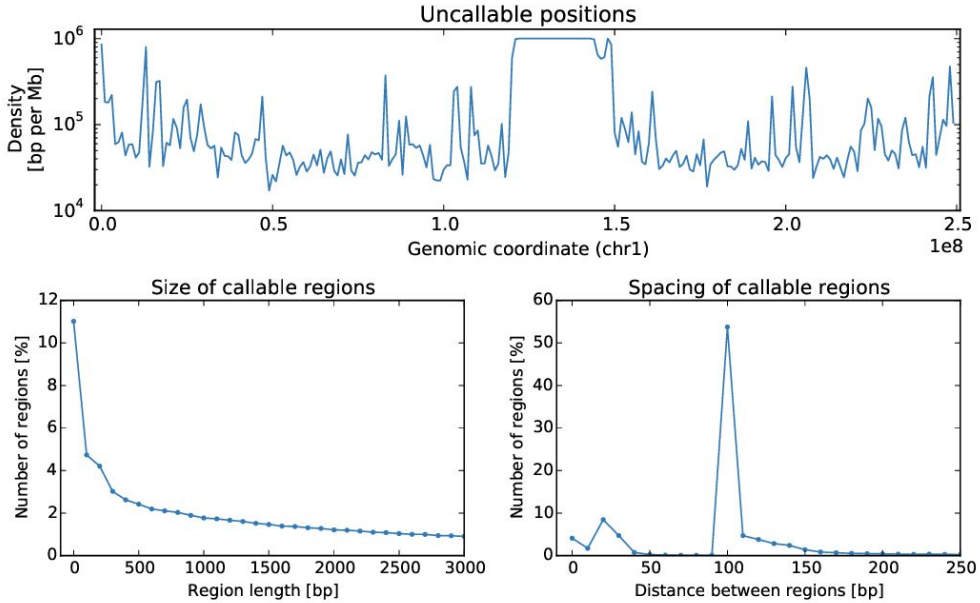
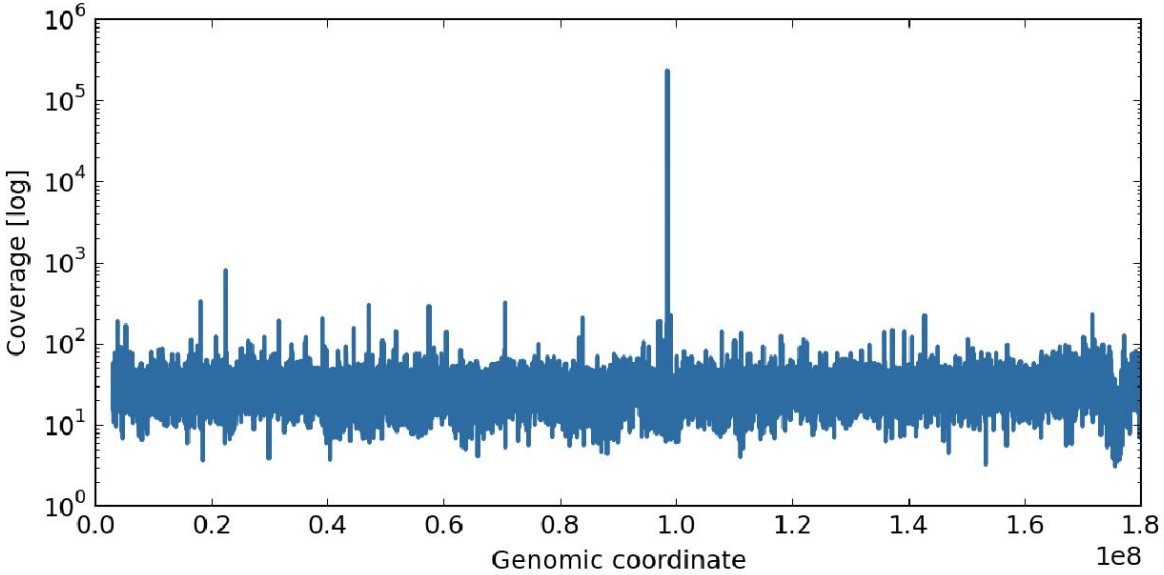


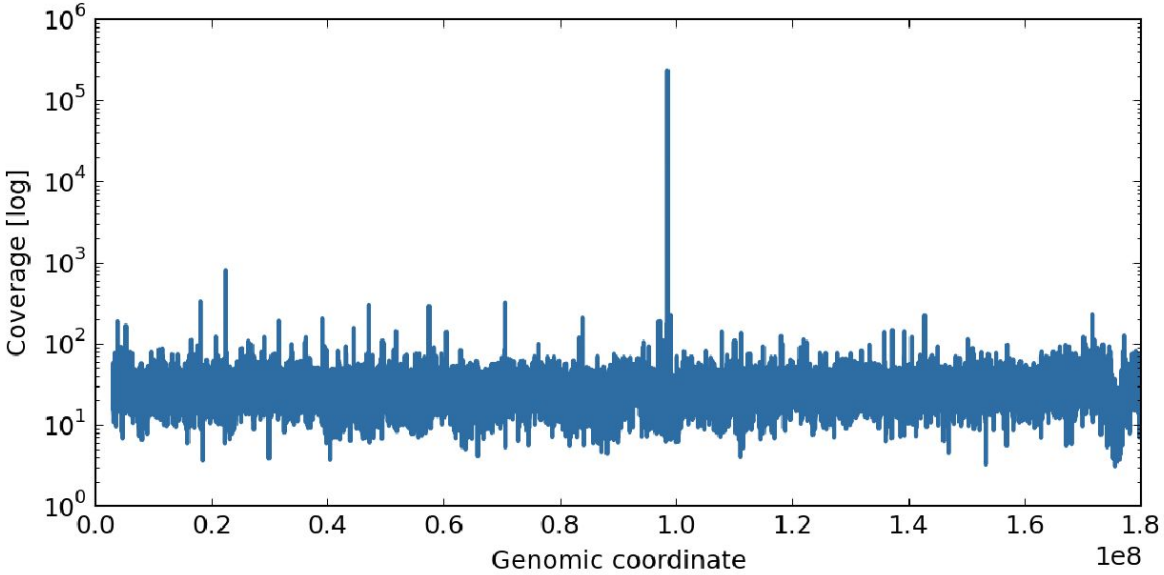
If possible, include only "nice" regions: for many analyses (e.g. population genetics studies) difficult regions can be ignored

Maximum depth



Q: Why is the sequencing depth thousandfold the average in some regions?

Maximum depth



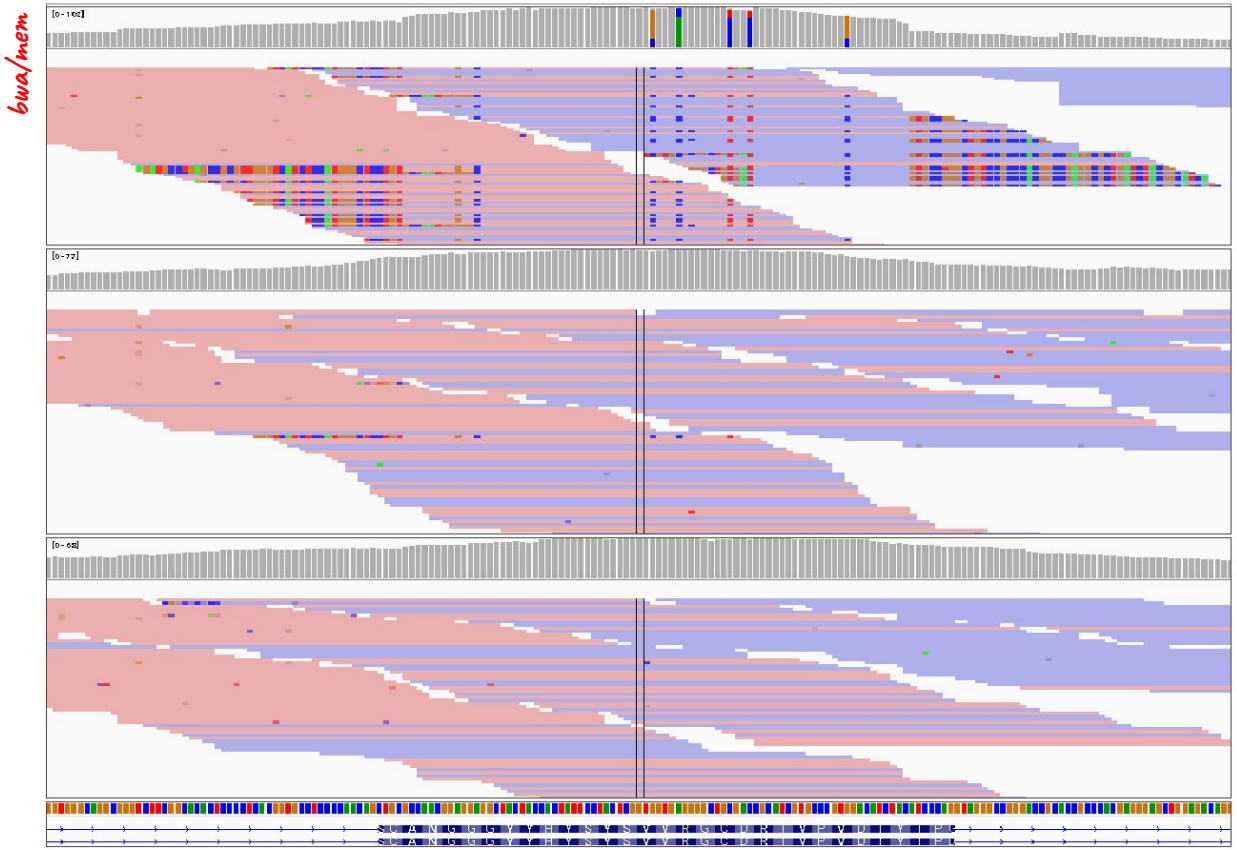
Q: Why is the sequencing depth thousandfold the average in some regions?

A: The reference genome is not complete. This sample was sequenced to 30x coverage, we can infer it has $\sim$ 30 copies of this region.

Filter calls with a too high depth (for example, 2x the average in WGS)

Mapping errors

Different mapping algorithm can lead to different results



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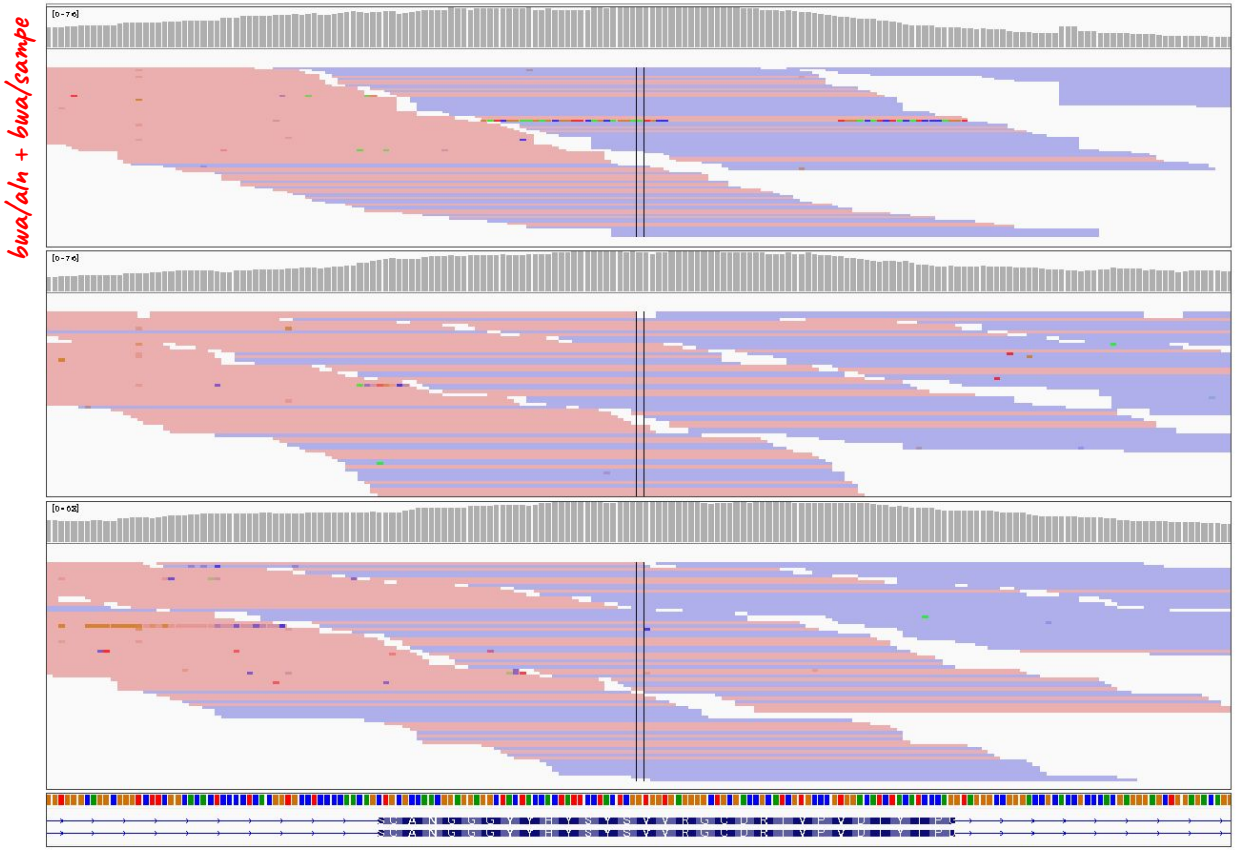

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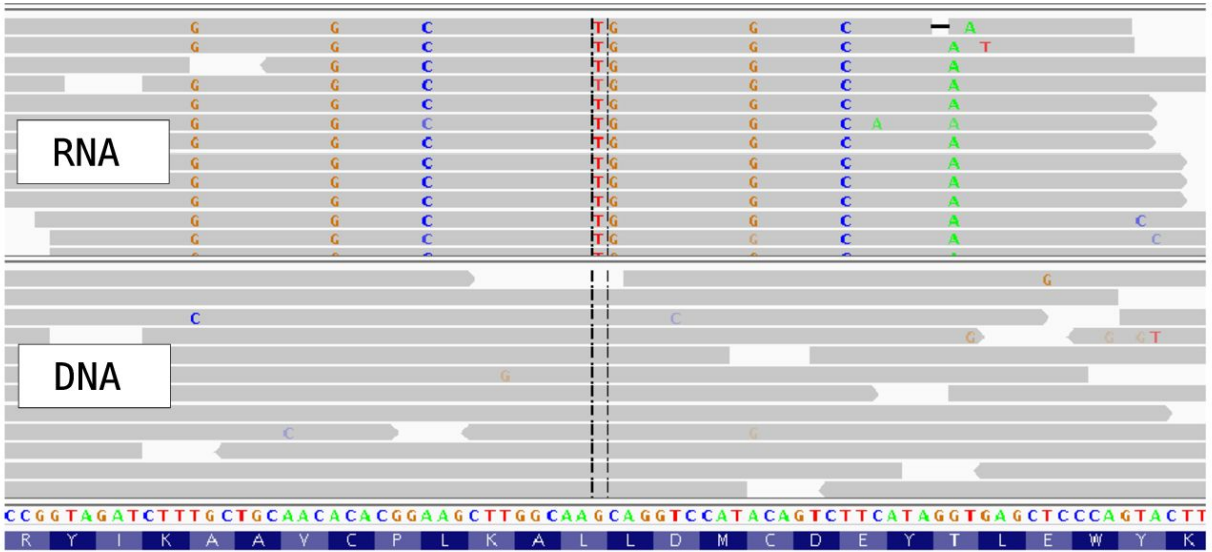
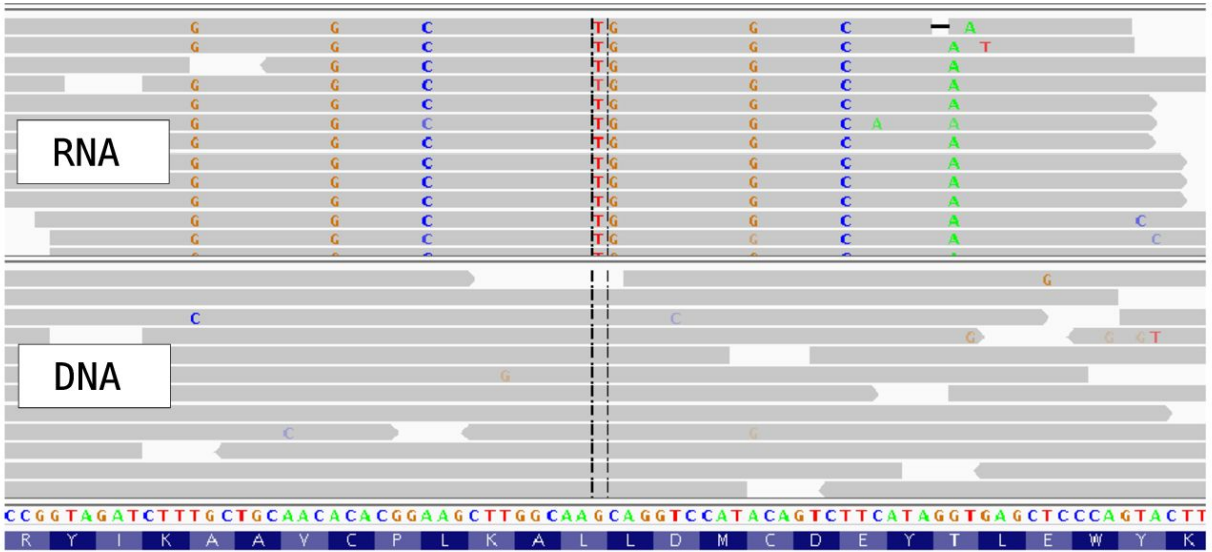


Q: RNA-seq (top) and DNA data (bottom) from the same sample has been mapped onto the reference genome. Can you explain the novel SNVs?

Mapping errors

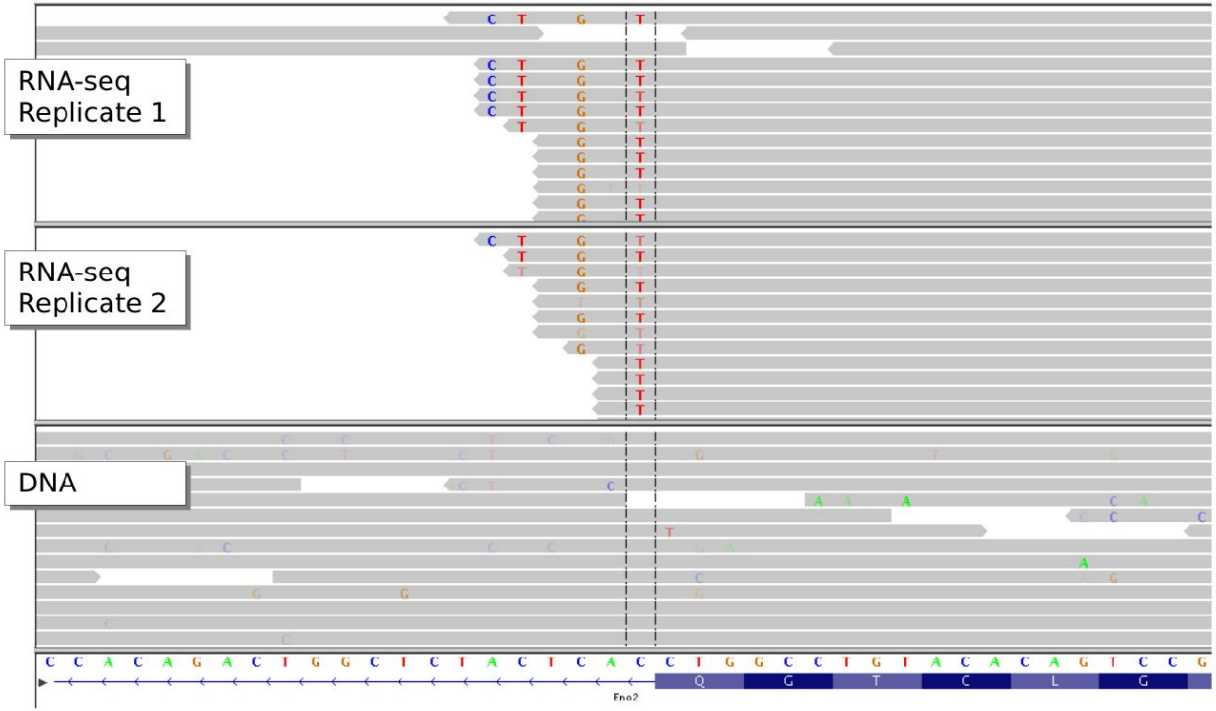


Q: RNA-seq (top) and DNA data (bottom) from the same sample has been mapped onto the reference genome. Can you explain the novel SNVs?

A: The reads were mapped to a pseudogene and originate in a paralog with 92% identity.

Beware of mapping errors, especially when aligning RNA-seq data on the genome.

Variant distance bias



Q: Can you explain what happened here?

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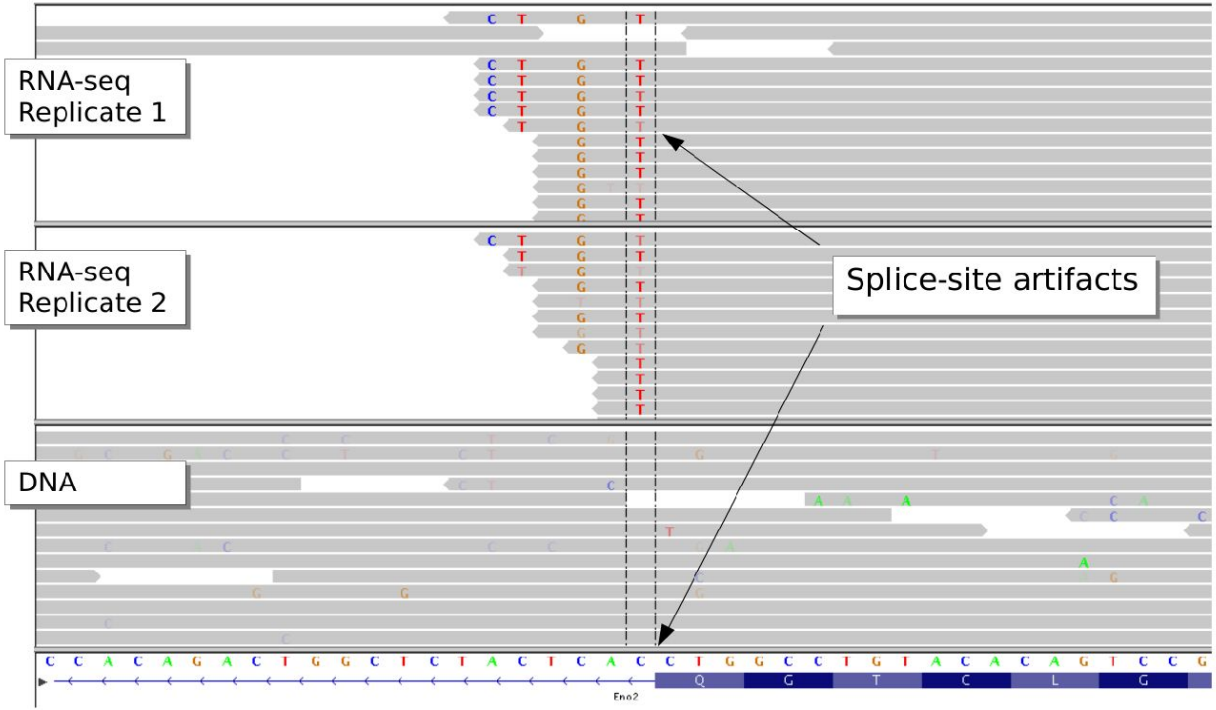
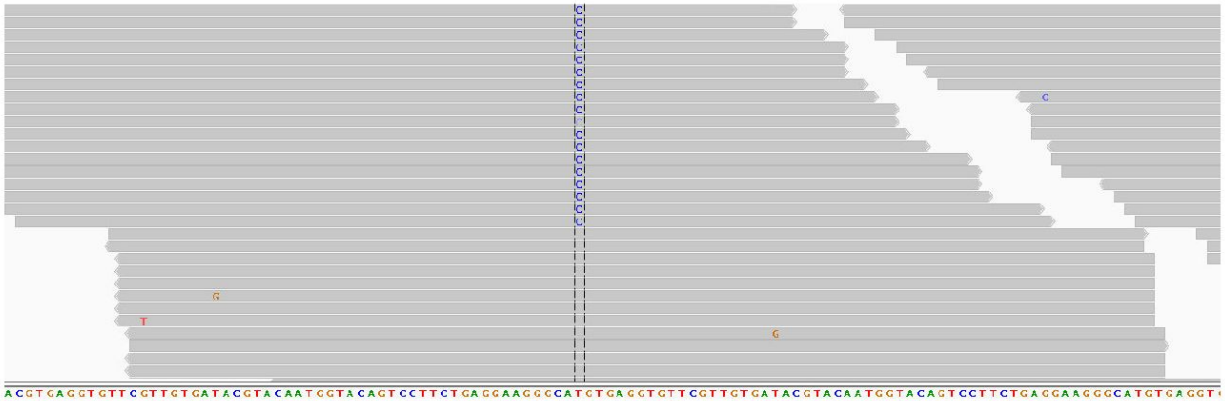


Q: Can you explain what happened here?
A: Processed transcript with introns spliced out.

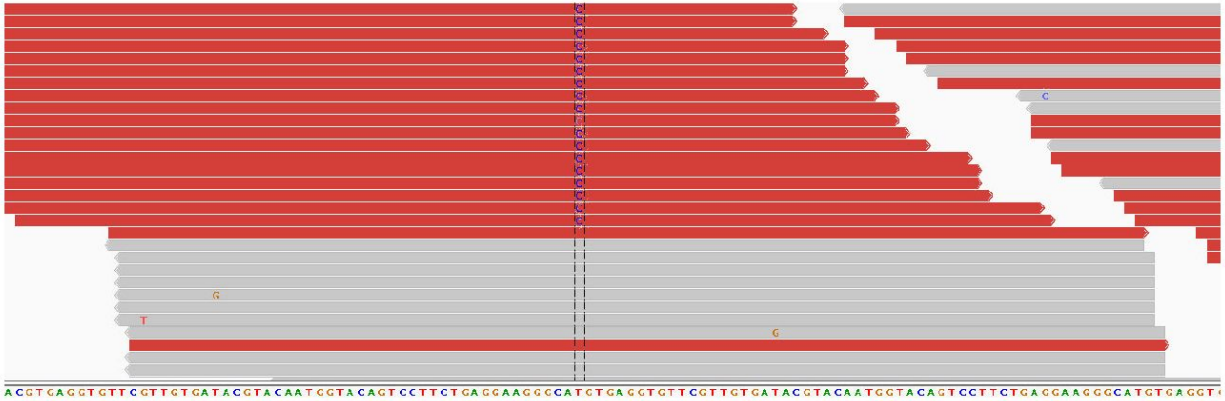
Better to use a splice-aware mapper when working with RNA-seq data, or filter most extreme cases using annotations such as VDB

Strand bias



Q: Is this a valid call?

Strand bias

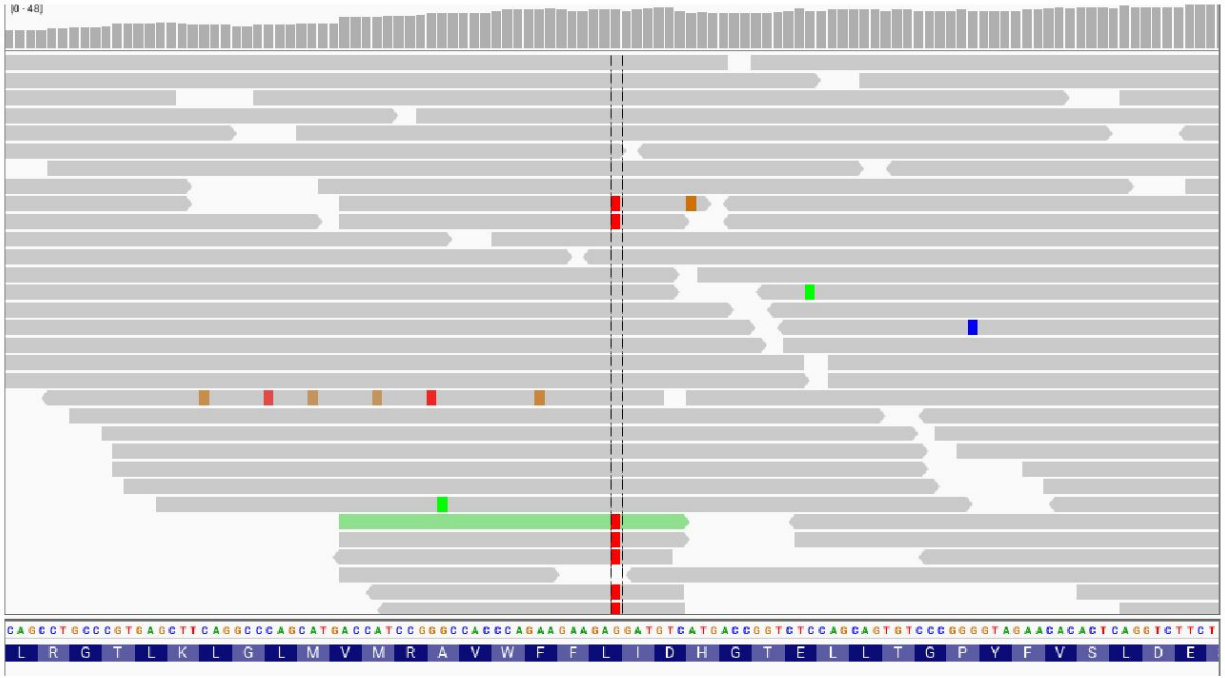


Q: Is this a valid call?

A: No, it is a mapping artefact, the call is supported by forward reads only.

Filter extremely biased calls using annotations generated by your caller (e.g. Fisher or rank-sum test)

Change the display in IGV to reveal artefacts



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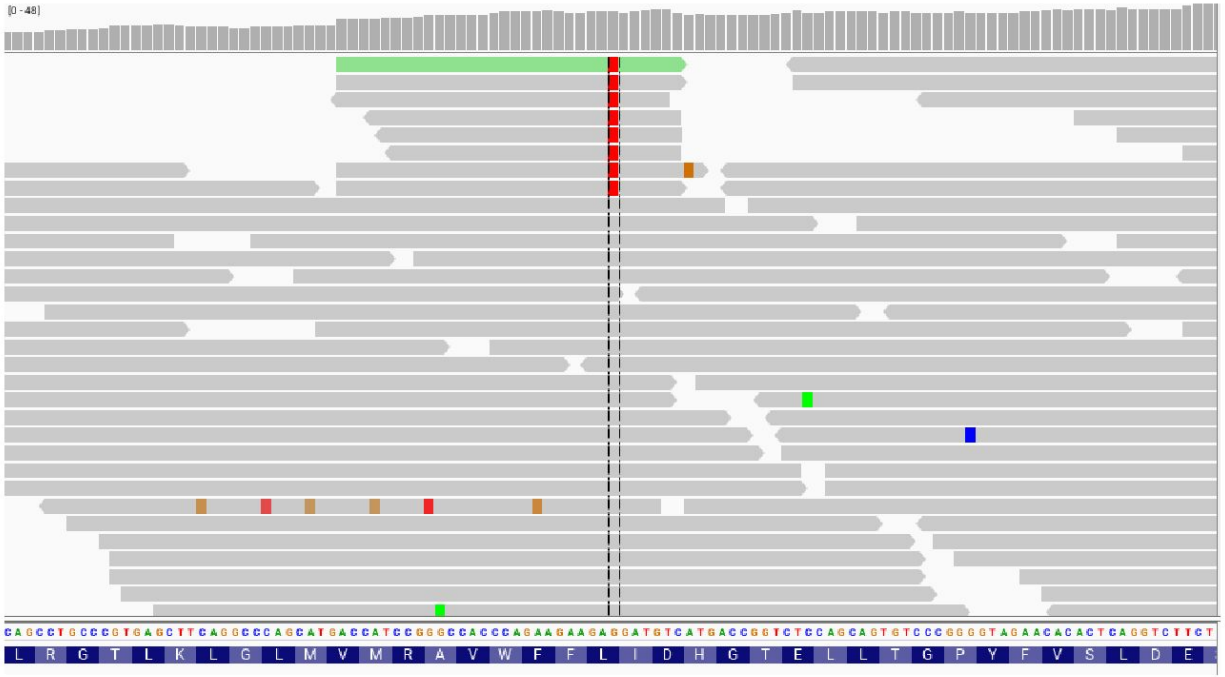
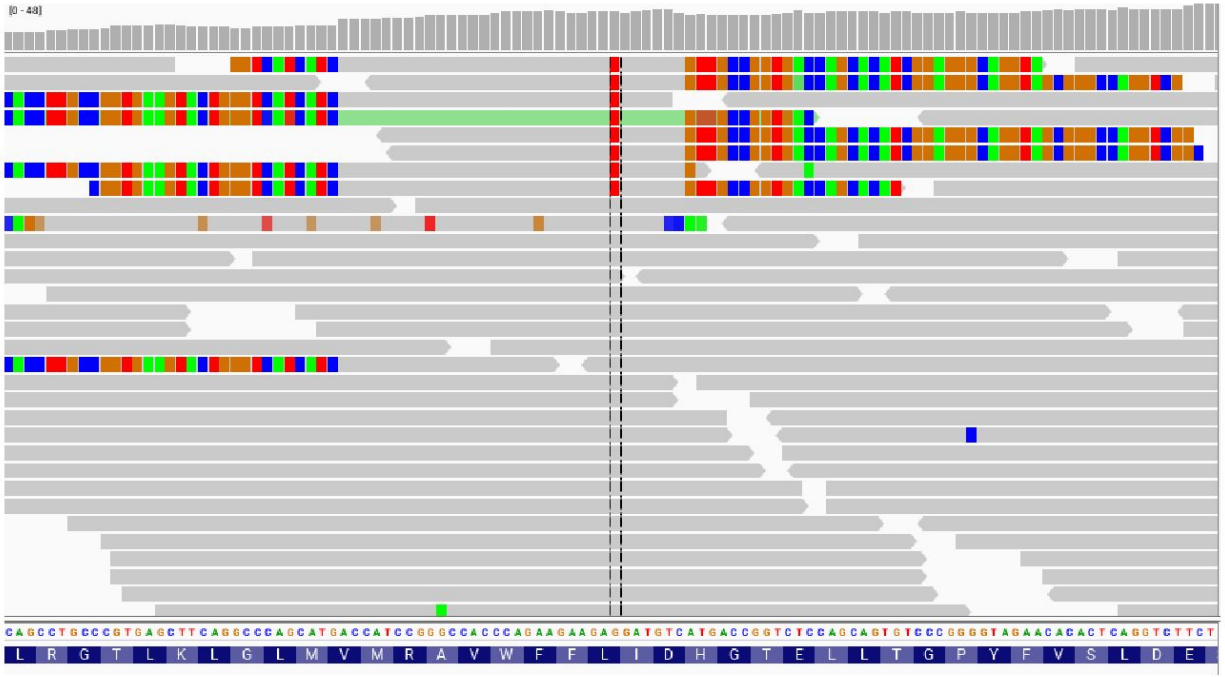


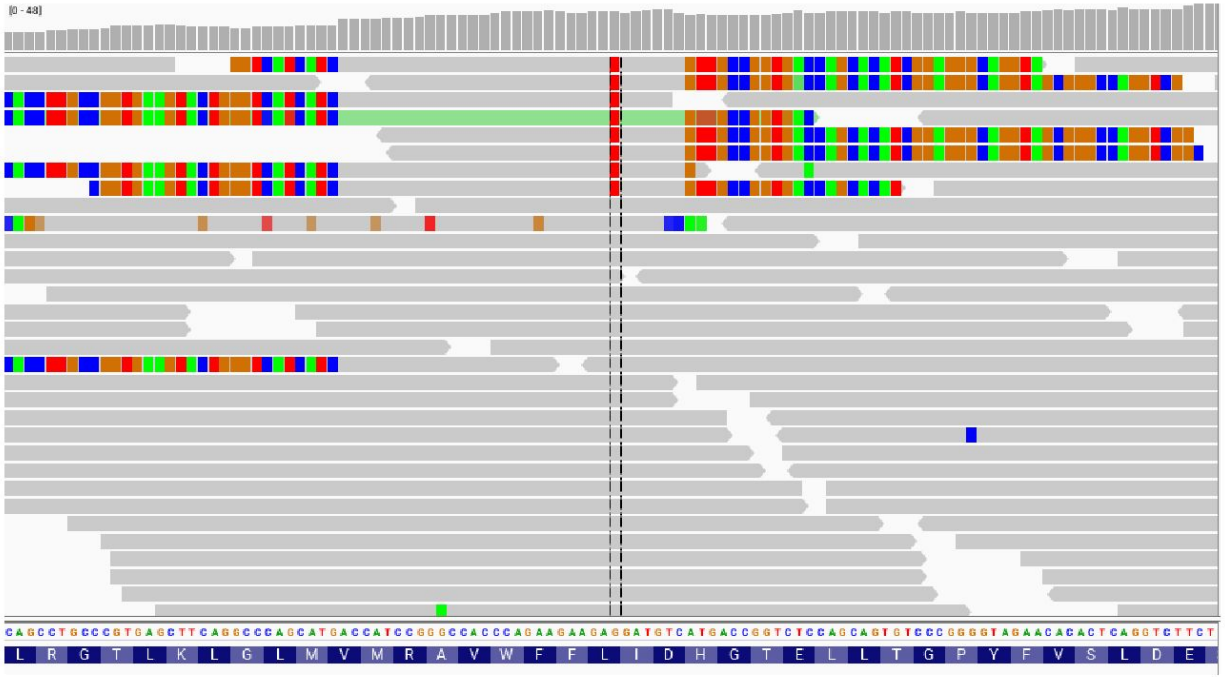
Sort by base...

Change the display in IGV to reveal artefacts



Display soft-clipped bases...

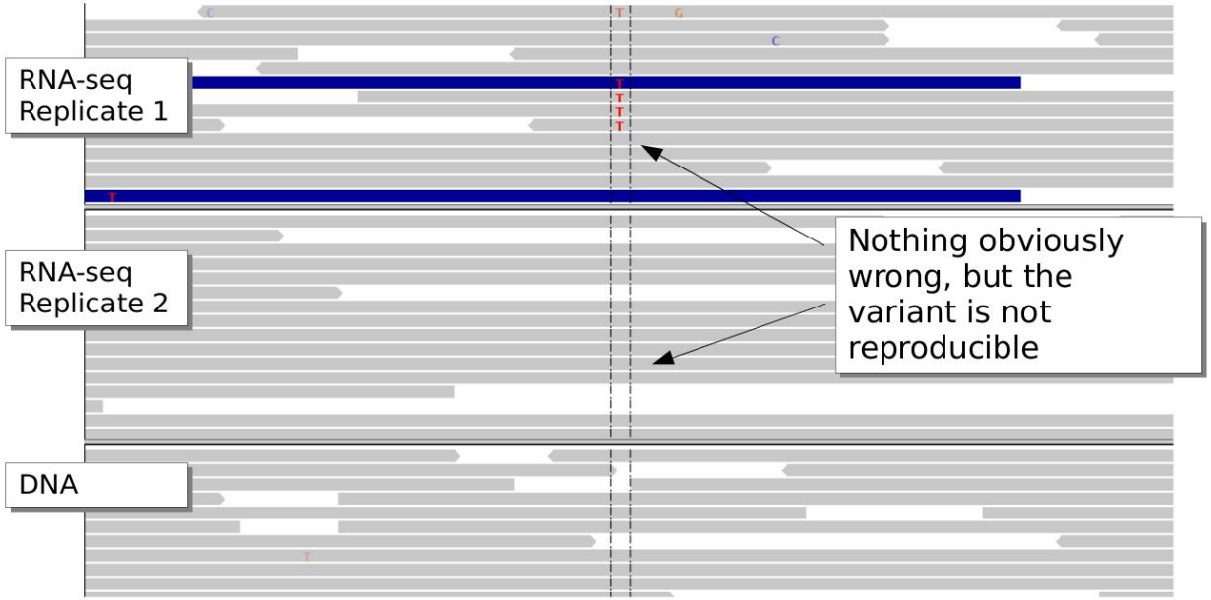
Change the display in IGV to reveal artefacts



Display soft-clipped bases...

Too many soft-clipped reads in a region suggest mapping errors, beware!

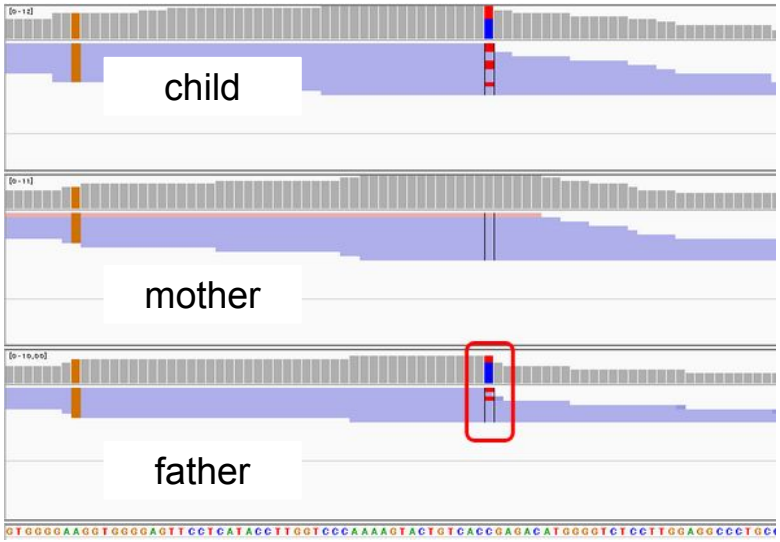
Reproducibility



Mind the biological variability. If possible, validate and replicate.

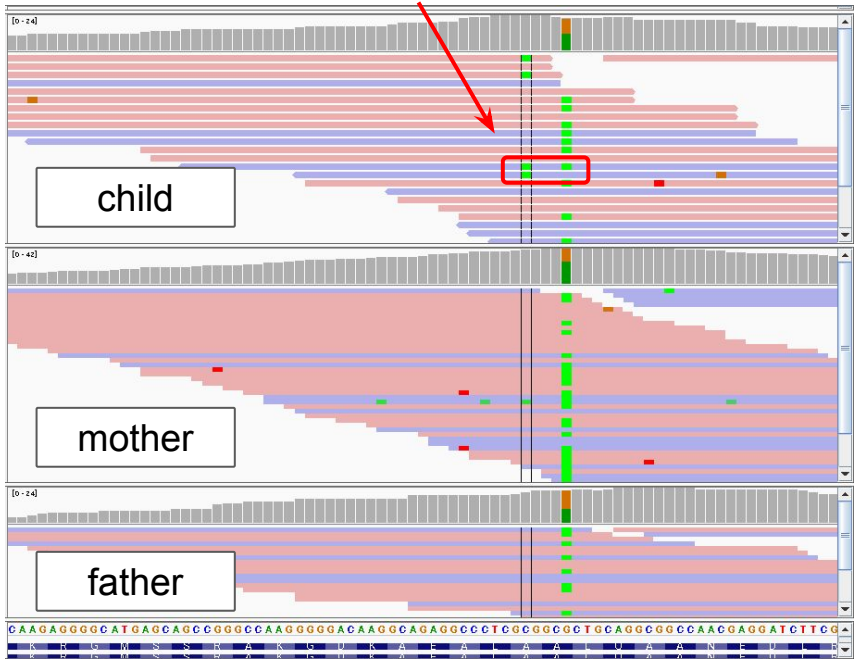
De novo mutations

Not a real DNM



Haplotype consistency

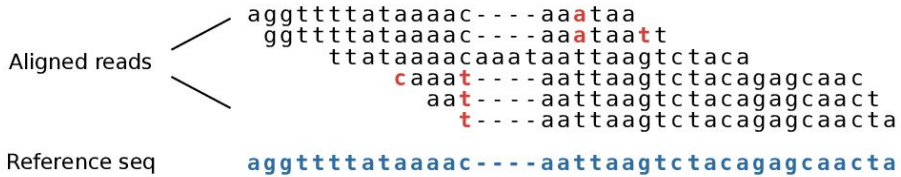
Both chromosomal copies affected, very likely a false positive!



False SNPs caused by incorrect alignment

Pairwise alignemnt artefacts can lead to false SNPs

- multiple sequence alignment is better, but very expensive
- instead: base alignment quality (BAQ) to lower quality of misaligned bases



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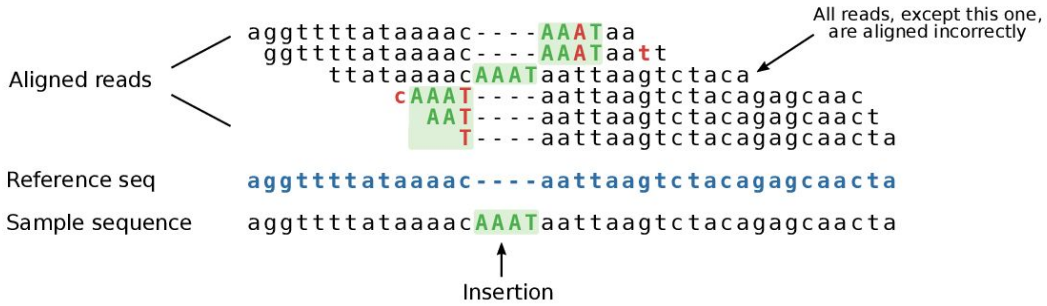


Q: How many SNPs are real?

A: None.

Be careful when looking at SNPs close to indels.

Indel calling challenges

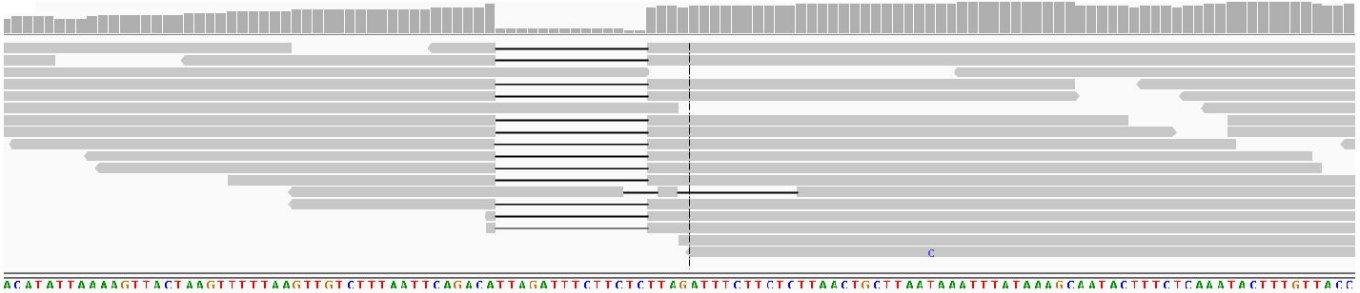
The sequencing error rate is elevated in microsatellites

Low reproducibility across callers

- 37.1% agreement between HapCaller, SOAPindel and Scalpel
  Narzisi et al. (2014) Nat Methods, 11(10):1033

Reads with indels are more difficult to map and align

- the aligner can prefer multiple mismatches rather than a gap
- indel representation can be ambiguous



```
CTTTAATTCAGACATTAGATTTCTTCTC
CTTTAATTCAGACATTAGATTTCTTCTCTTA
CTTTAATTCAGACA--------------TTAGATTTCTTCTCTTAACTGCTT
CTTTAATTCAGACATTAGATTTCTTC---TA----------TTAACTGCTT

CTTTAATTCAGACATTAGATTTCTTCTCTTAGATTTCTTCTCTTAACTGCTT
```

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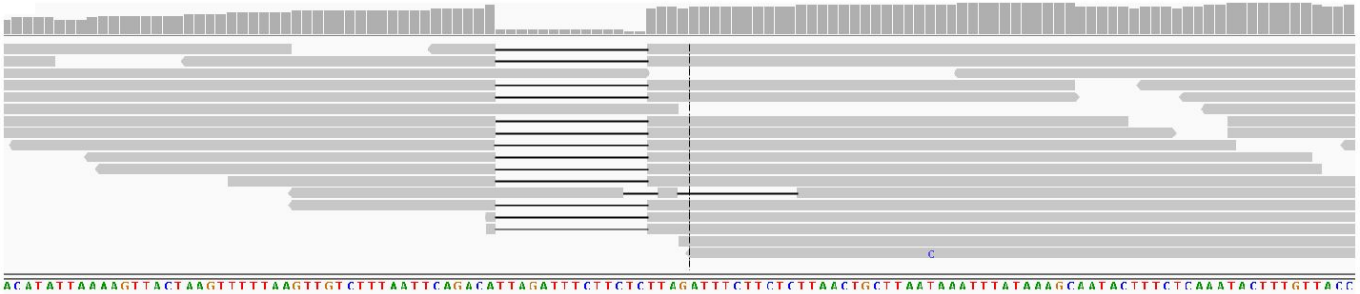
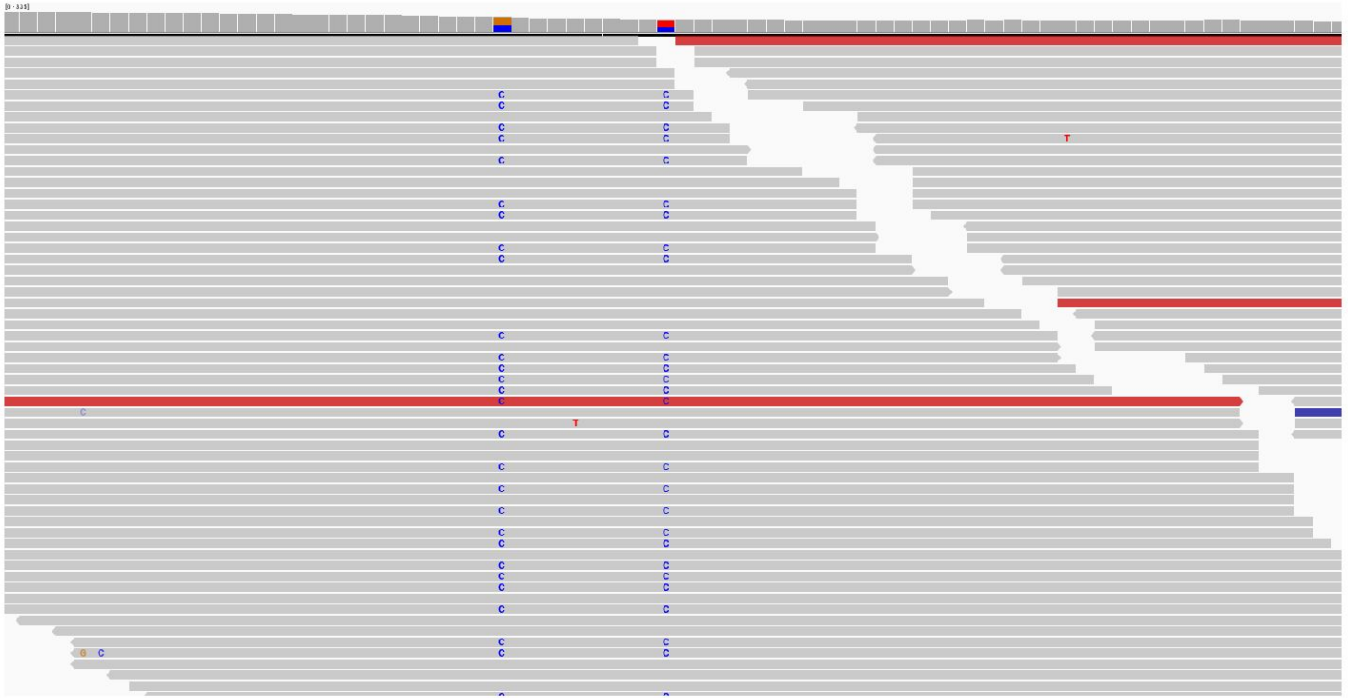


```
CTTTAATTCAGACA~~~~~~~~~~~~~~~TTAGATTTCTTCTC
CTTTAATTCAGACA~~~~~~~~~~~~~~~TTAGATTTCTTCTCTTA
CTTTAATTCAGACA---------------TTAGATTTCTTCTCTTAACTGCTT
CTTTAATTCAGACA~~~~~~~~~~~~~~~TTAGATTTCTTCTATTAACTGCTT

CTTTAATTCAGACATTAGATTTCTTCTCTTAGATTTCTTCTCTTAACTGCTT
```

What good SNPs look like?



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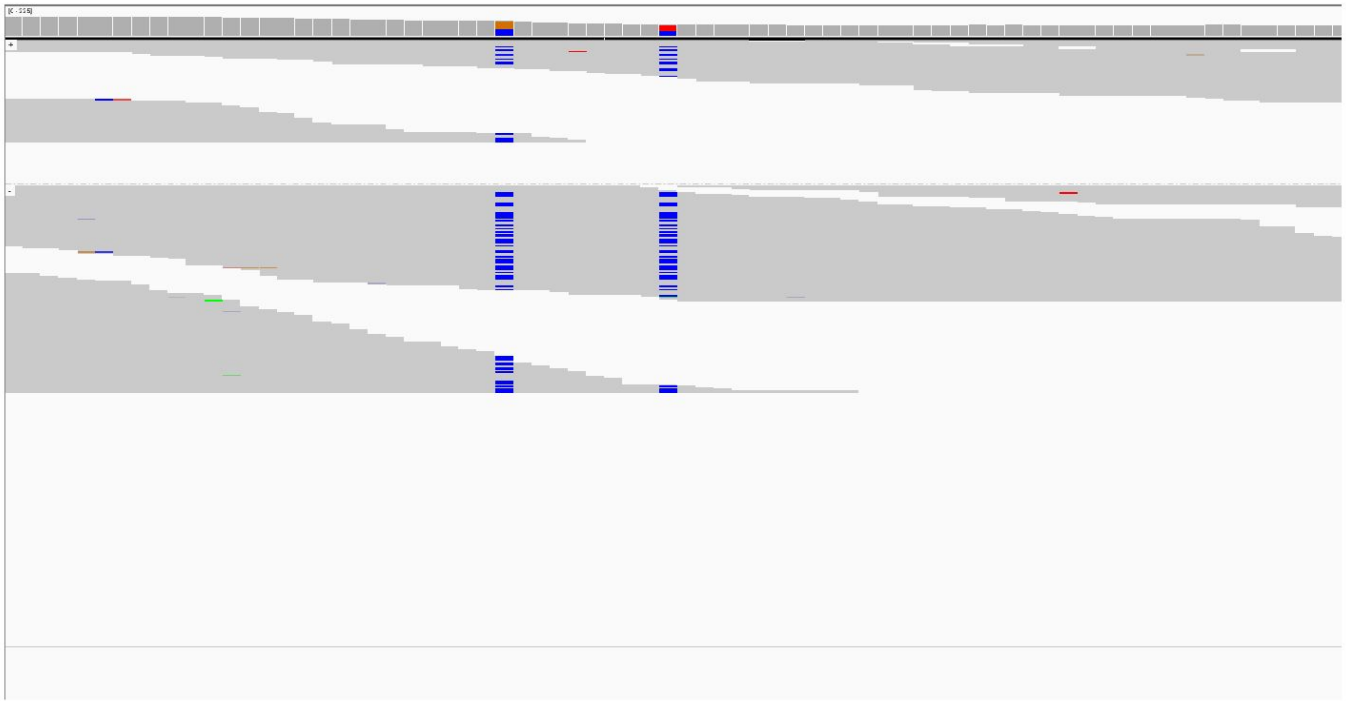

Change the view IGV to inspect possible biases. Here the reads were squished and grouped by read strand to confirm two clean unbiased calls.

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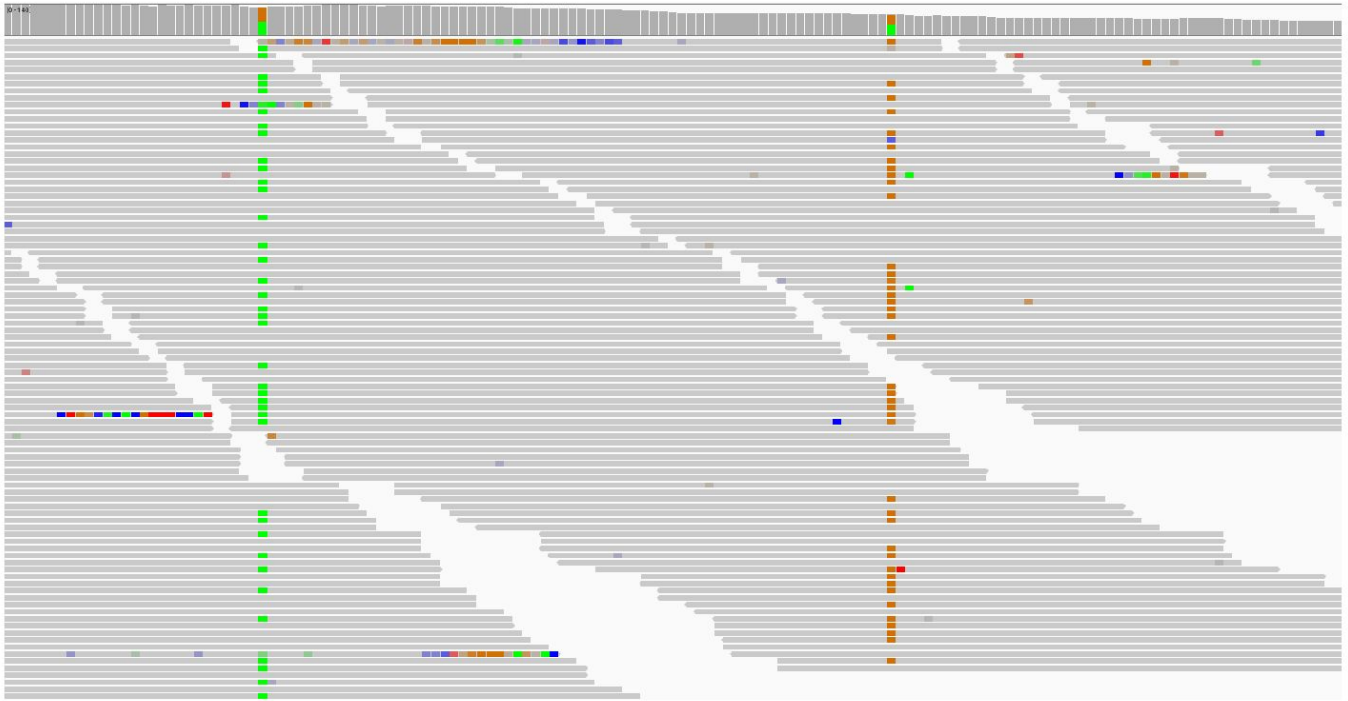

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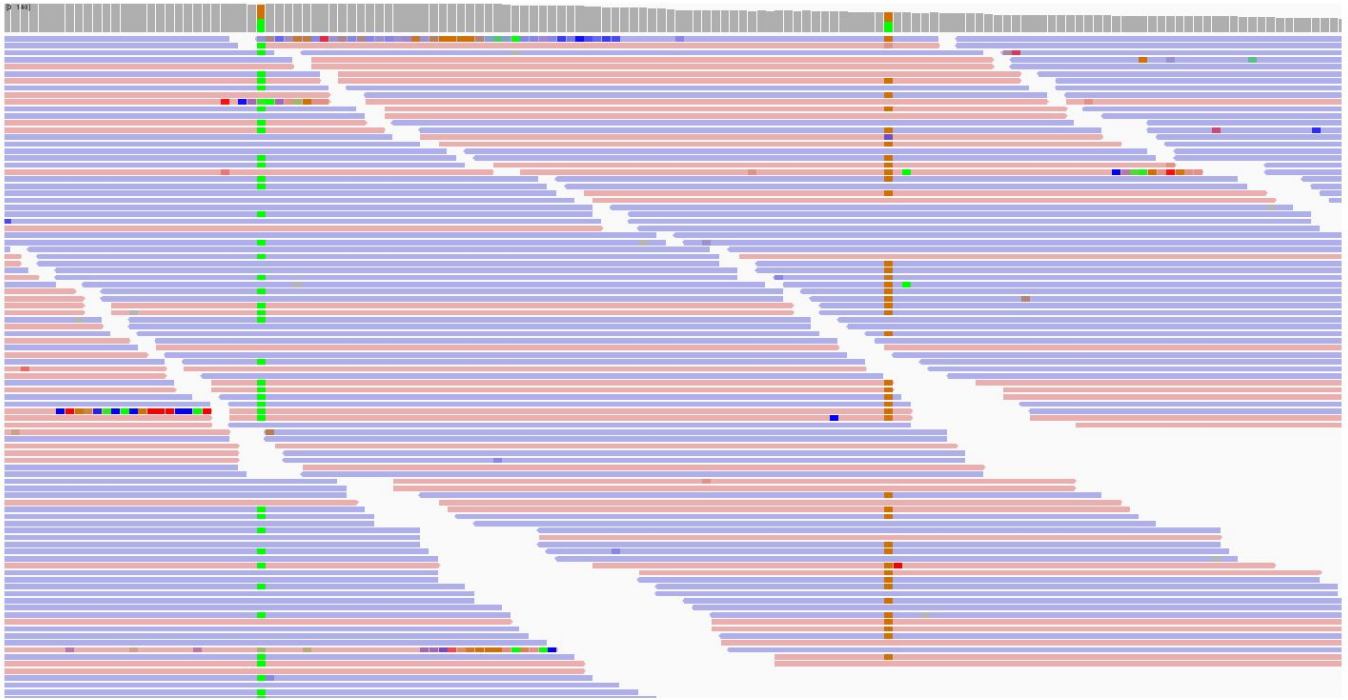

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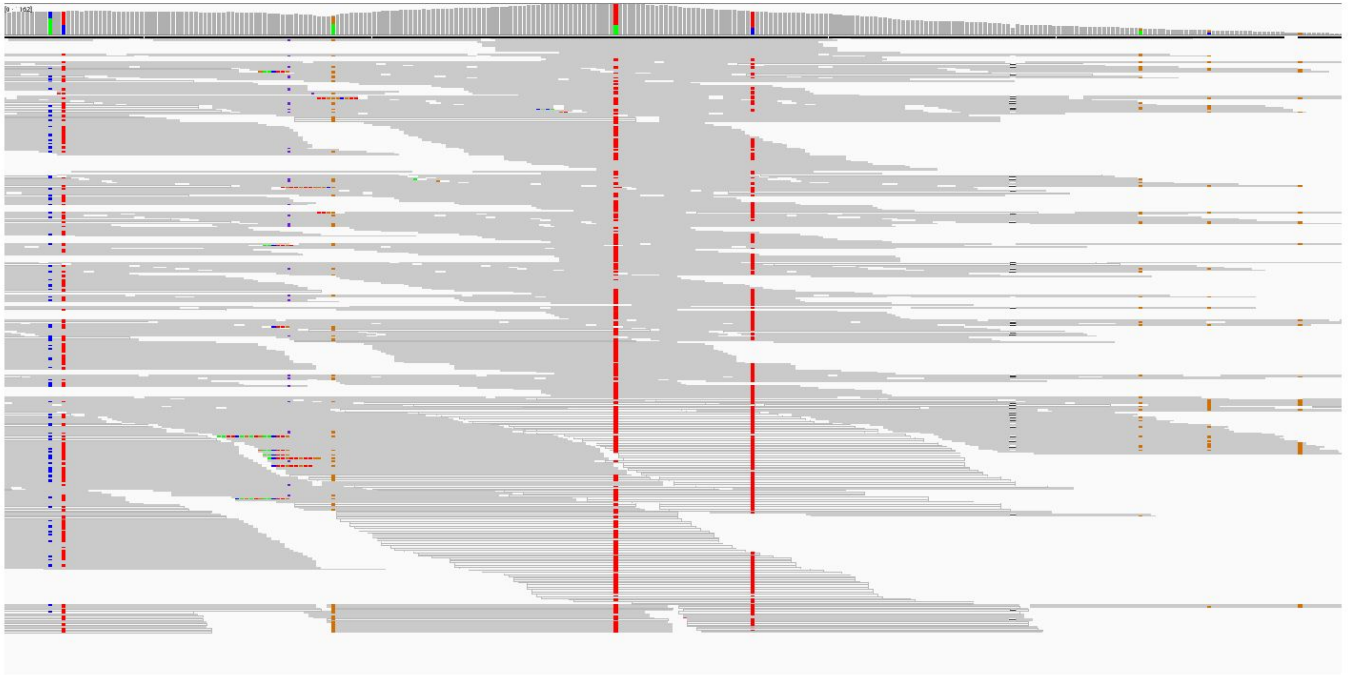


Q: Is this call real? There are many reads with MQ=0.

Sorting the reads by MQ reveals the variant is also supported by many high-quality reads.

How to estimate overall callset quality?

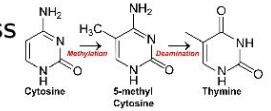
- ti/tv .. proportion of transitions vs transversions
- VAFxx .. proportion of calls with small VAF (variant allele frequency)
- het/hom .. proportion of heterozygous vs homozygous calls

In trios

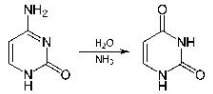
- transmission rate .. ~50% of parental singletons should be transmitted to the child

Detour: Some causes of SNPs

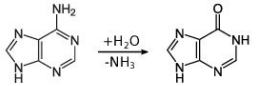
Spontaneous chemical processes which lead to base modification or loss



- Deamination
  - methylated CpG dinucleotides: 5-methylcytosine → T
  - hydrolytic deamination of C → U (400 cytosines daily in each cell)

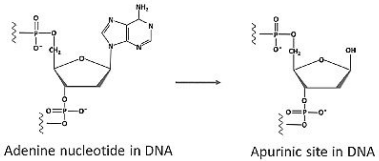


  - A → hypoxanthine (pairs with C, A-to-G mutation)
- Depurination (loss of A or G)
  - purines are cleaved from the backbone (10^2-10^3 daily in each cell)
  - if base excision repair fails, random base is inserted



DNA damage by mutagens

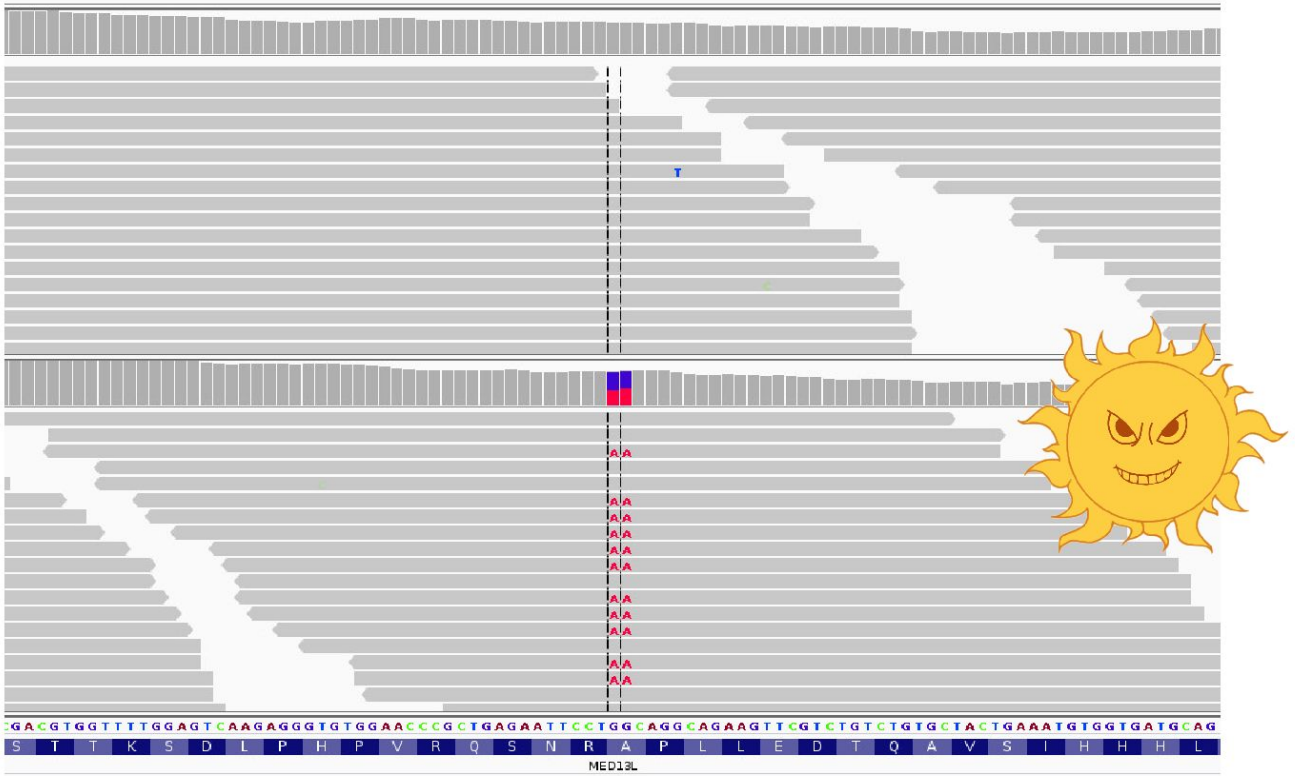
- base analogs
  - incorporation of chemicals with different properties
- base-modifying agents



Radiation

Some causes of MNPs

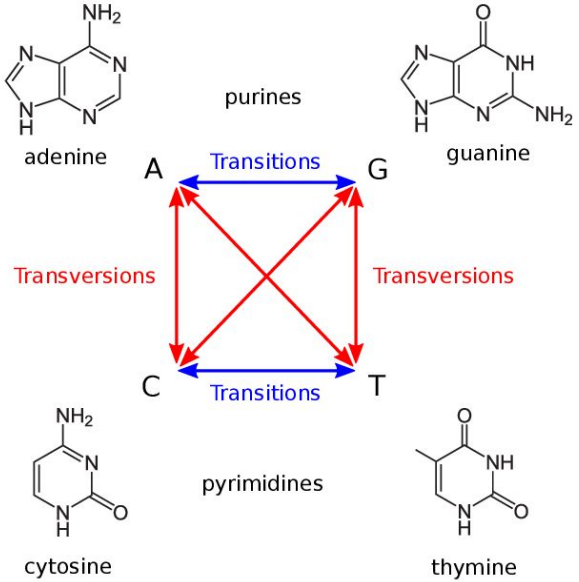
UV-induced mutations (CC → TT in skin cells)

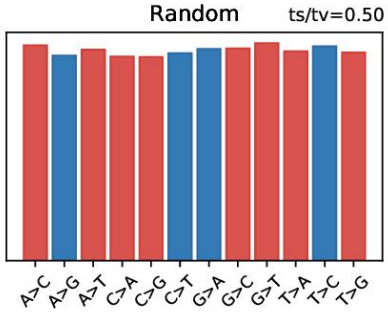


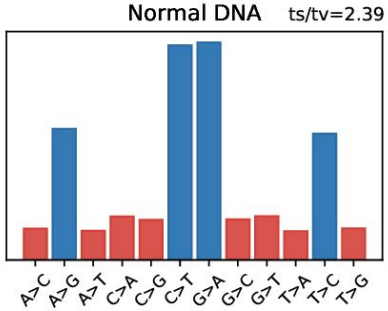
Also known as ti/tv

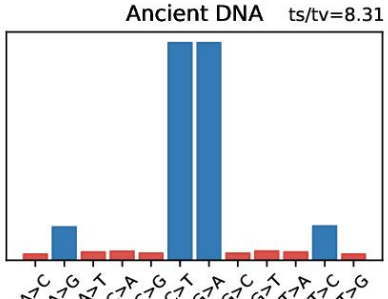
Transitions vs transversions ratio, known as ts/tv

- transitions are 2-3× more likely than transversions



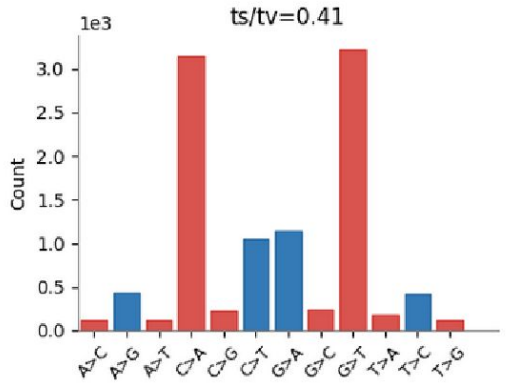


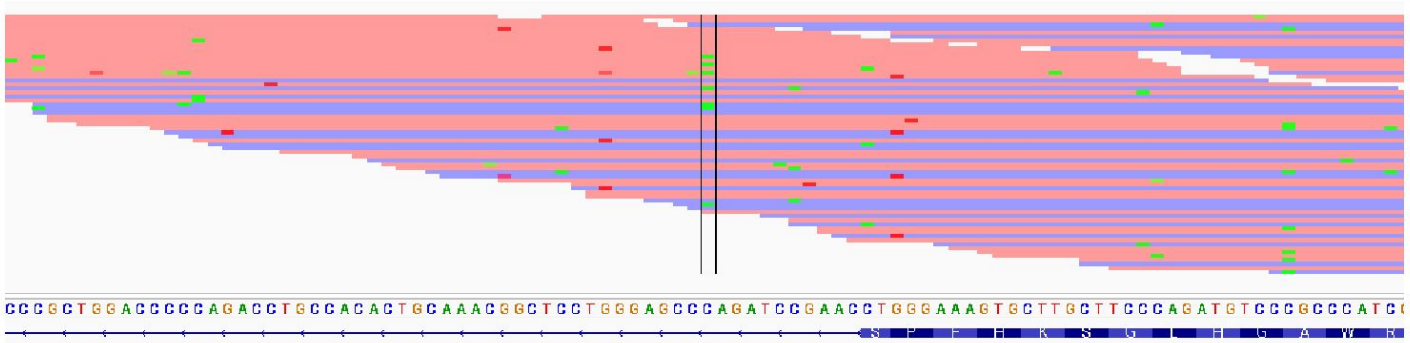




Example: false C>A transversions due to a failed library prep

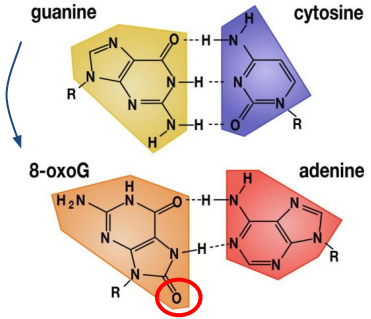
Cause unknown, likely oxidative damage





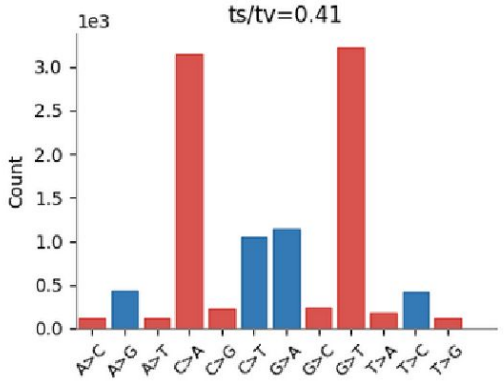
Example: false C>A transversions due to a failed library prep

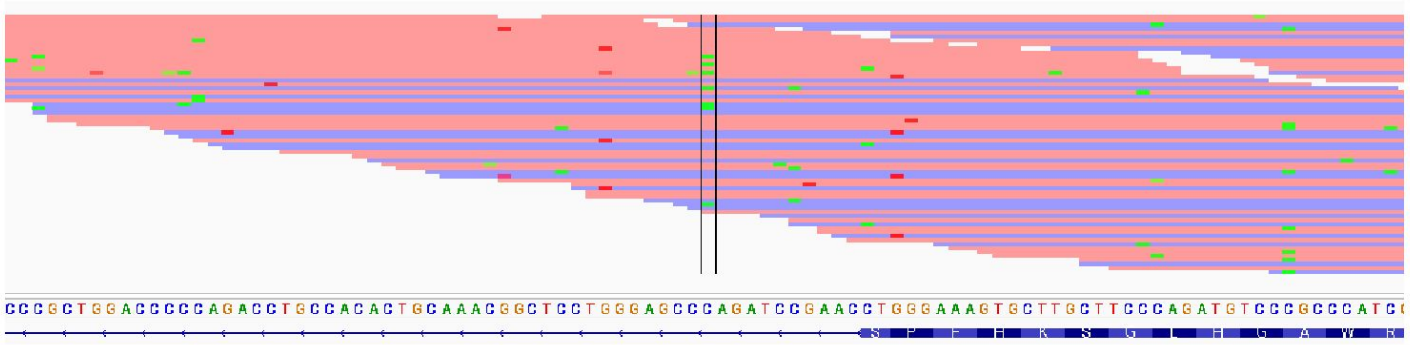
Cause unknown, likely oxidative damage



8-oxo-7,8-dihydroguanine likes to pair with adenine

doi: 10.1016/j.csbj.2019.12.013

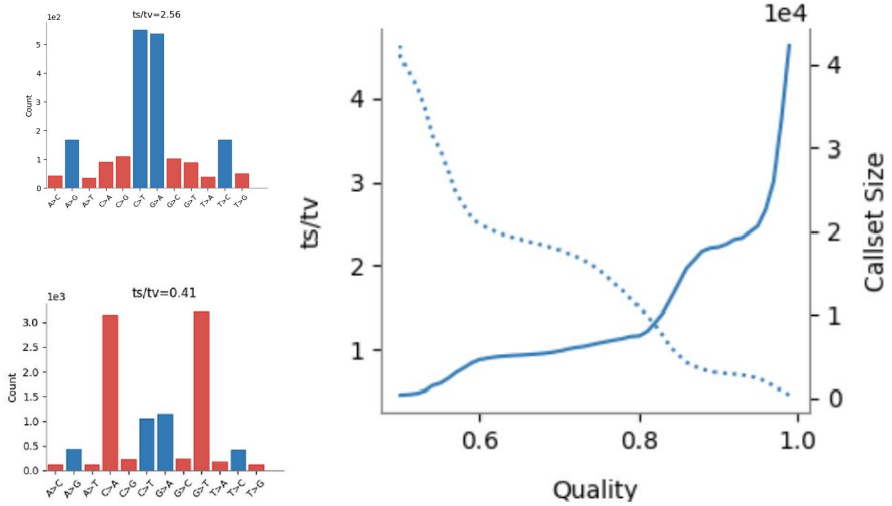




ts/tv by size

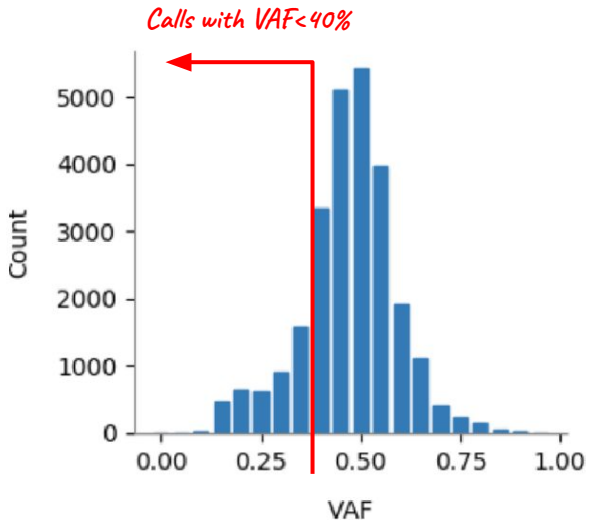
Ts/tv is a convenient metric to compare callsets

- sort calls by a quality metric
- calculate ts/tv at various thresholds
- bigger ts/tv indicates fewer false positives in the callset

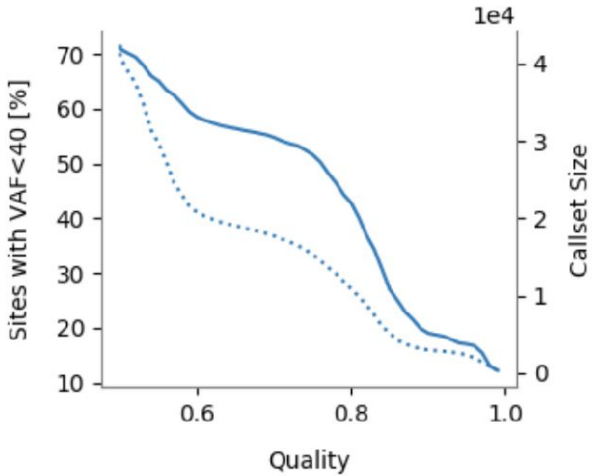


VAF by size

VAF = variant allele frequency (fraction of reads with the alternate allele)



VAF distribution of the final callset



Fraction of sites with VAF<40% at various quality cutoffs

Sensitivity vs Specificity

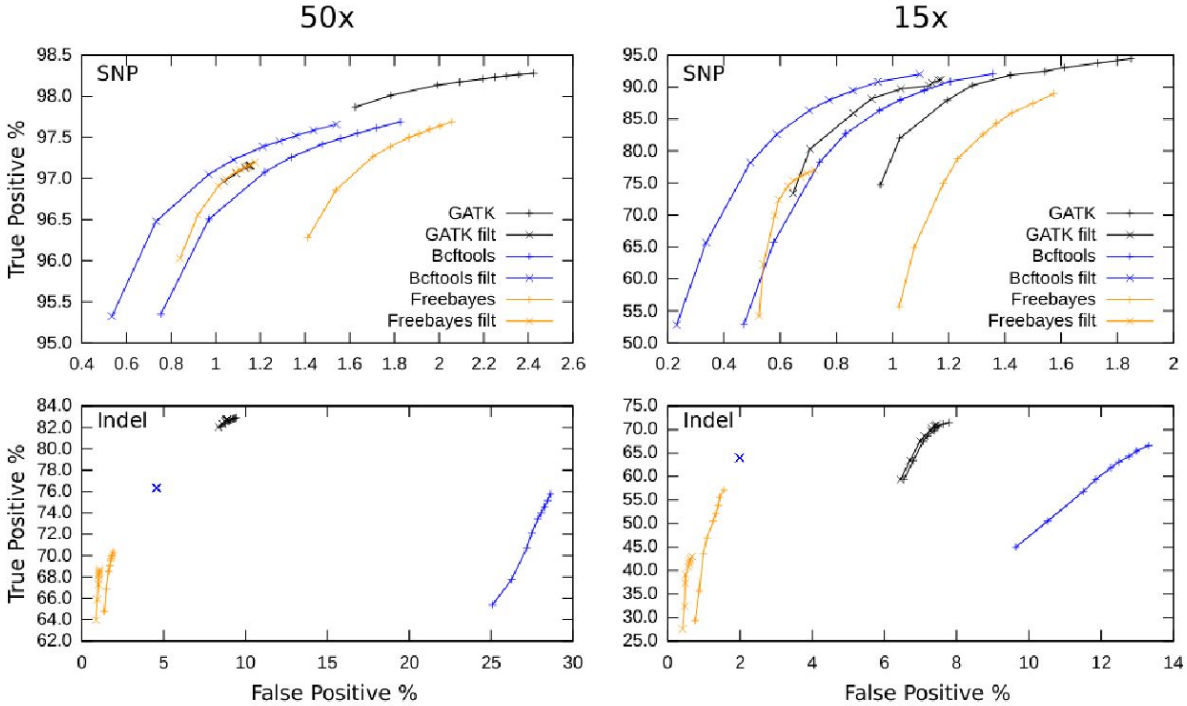


Figure 4: *A summary of True Positive vs False Negative rates of GATK HaplotypeCaller, Bcftools and Freebayes at multiple quality thresholds, with and without filtering.*

Single vs multi-sample and gVCF calling

VCF files can be **very** big, therefore we often store only variant sites[1]

- however, variant-only VCFs are difficult to compare - was a site dropped because of a reference call or because of low coverage?
- we need evidence for both variant and non-variant positions in the genome

gVCF

- represents blocks of reference-only calls in a single record using the END tag
- symbolic allele in raw "callable" gVCFs allows to calculate genotype likelihoods only once (an expensive step), then do calling repeatedly as more samples come in

```
#CHROM POS  ID REF ALT  QUAL FILTER    INFO              FORMAT Sample
19    9902 .  G   <*>   .    .      END=9915;MinDP=0    PL:DP  0,0,0:0
19    9916 .  C   <*>   .    .      END=9922;MinDP=5    PL:DP  0,15,137:5
19    9923 .  G   <*>   .    .      END=9948;MinDP=10   PL:DP  0,30,214:10
19    9949 .  G   A,<*> .    .      DP=28               PL:DP  0,60,255,78,255,255:27
19    9950 .  C   <*>   .    .      END=9958;MinDP=28   PL:DP  0,84,255:28
19    9959 .  G   T,<*> .    .      DP=34               PL:DP  0,82,255,99,255,255:34
19    9960 .  C   <*>   .    .      END=9969;MinDP=34   PL:DP  0,102,255:34
```

Symbolic "unobserved" allele
Represents any other possible alternate allele

A block of 10 sites with at least 34 reference reads

Genotype likelihoods for CC, C*, **

[1] Annotated VCF with 3,781 samples, variant sites only, UK10k project . . . 680GB

Genome VCF (gVCF)

VCF

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO
19	9902	.	G	.	.	.	DP=0
19	9903	.	T	.	.	.	DP=0
19	9904	.	A	.	.	.	DP=0
19	9905	.	C	.	.	.	DP=0
19	9906	.	G	.	.	.	DP=5
19	9907	.	T	.	.	.	DP=7
19	9908	.	A	.	.	.	DP=10
19	9909	.	C	.	.	.	DP=13
19	9910	.	G	A	.	.	DP=15
19	9911	.	T	.	.	.	DP=14
19	9912	.	A	.	.	.	DP=19
19	9913	.	C	.	.	.	DP=23
19	9914	.	G	.	.	.	DP=22
19	9915	.	T	.	.	.	DP=17
19	9916	.	G	T	.	.	DP=18
19	9917	.	A	.	.	.	DP=19
19	9918	.	C	.	.	.	DP=16
19	9919	.	G	.	.	.	DP=25
19	9920	.	T	.	.	.	DP=23

→

gVCF

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO
19	9902	.	G	.	.	.	MinDP=0;END=9905
19	9906	.	G	.	.	.	MinDP=5;END=9909
19	9910	.	G	A	.	.	DP=15
19	9911	.	T	.	.	.	MinDP=14;END=9915
19	9916	.	G	T	.	.	DP=18
19	9917	.	A	.	.	.	MinDP=16;END=9920

Often it is not sufficient to keep only *variant* sites:

- is there **no alternate allele** or is there **no coverage**???
- need evidence for both variant and non-variant positions in the genome

VCF vs BCF

VCFs can be very big

- compressed VCF with 3781 samples, human data:
  - 54 GB for chromosome 1
  - 680 GB whole genome

VCFs can be slow to parse

- text conversion is slow
- main bottleneck: FORMAT fields

```
##fileformat=VCFv4.0
##fileDate=20100707
##source=VCFtools
##ALT=<ID=DEL,Description="Deletion">
##INFO=<ID=END,Number=1,Type=Integer,Description="End position of the variant">
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT SAMPLE1 SAMPLE2
1  3  .  A  G  .  PASS  AC=67;AN=5400;DP=2809  GT:PL:DP:GQ  1/1:0,9,73:26:22    0/0:0,9,73:13:31    0/0:0,9,73:48:99  1/0:255,0,75:32:15  1/0:255,0,75:32:15
1  4  .  A  T  .  PASS  AC=15;AN=6800;DP=6056  GT:PL:DP:GQ  0/0:0,9,73:13:31    1/0:255,0,75:32:15  0/0:0,2,80:14:90  1/1:0,9,73:26:22    0/0:0,9,73:13:31
1  5  .  C  T  .  PASS  AC=20;AN=6701;DP=5234  GT:PL:DP:GQ  1/0:255,0,75:32:15  0/0:0,2,170:14:90   1/1:0,9,73:13:31  0/0:0,6,50:13:80    0/0:0,2,80:14:90
1  6  .  A  G  .  PASS  AC=67;AN=5400;DP=2809  GT:PL:DP:GQ  1/1:0,9,73:26:22    0/0:0,9,73:13:31    0/0:0,9,73:48:99  1/0:255,0,75:32:15  1/0:255,0,75:32:15
1  7  .  A  T  .  PASS  AC=15;AN=6800;DP=6056  GT:PL:DP:GQ  0/0:0,9,73:13:31    1/0:255,0,75:32:15  0/0:0,2,80:14:90  1/1:0,9,73:26:22    0/0:0,9,73:13:31
```

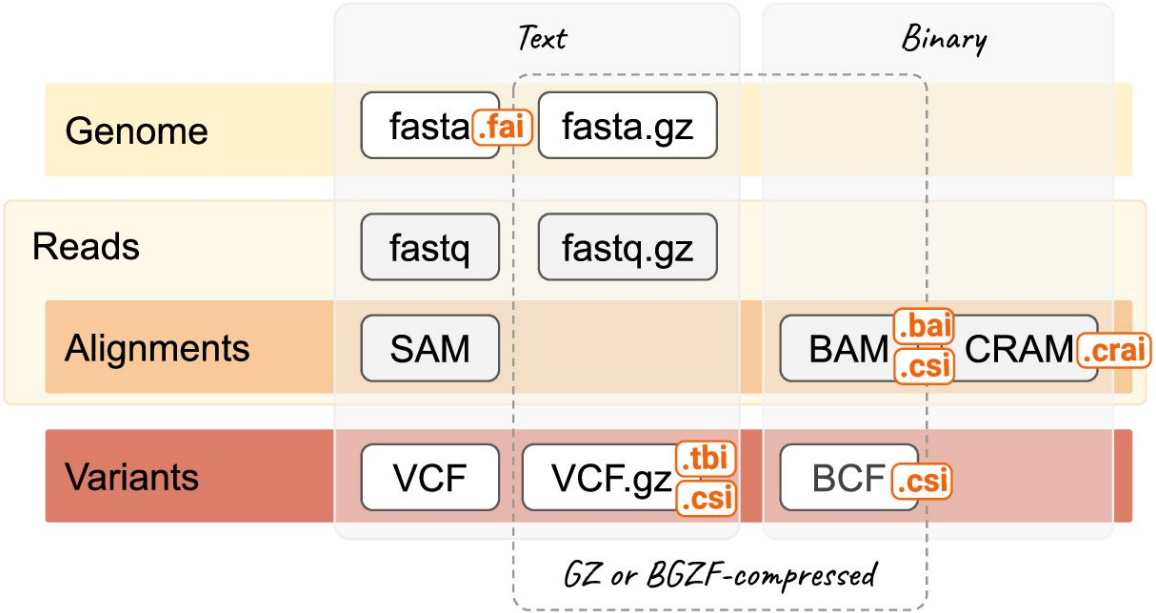
BCF

- binary representation of VCF
- fields rearranged for fast access

```
#CHROM POS ID REF ALT QUAL FILTER INFO          FORMAT      SAMPLE1          SAMPLE2          SAMPLE3          SAMPLE4          SAMPLE5
1      6   .  A   G   .    PASS   AC=67;AN=540  GT:PL:DP:GQ  1/1:0,9,73:26:22  0/0:0,9,73:13:31  0/0:0,9,73:48:99  1/0:255,0,75:32:15  1/0:255,0,75:32:15

1      6   .  A   G   .    PASS   AC=67;AN=540  GT:1/1:0/0:0/0:1/0:1/0  PL:0,9,73:0,9,73:0,9,73:255,0,75:255,0,75  DP:26:13:48:32:32  GQ:22:31:99:15:15
```

File formats summary



Note: BCF can be compressed (`bcftools view -Ob`) or uncompressed (`bcftools view -Ou`). Use the latter for streaming, it is much faster!