Module 6: Variant calling, SNPs and short indels

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Based on slides by:
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HTS workflow

Library preparation

- DNA extraction
- fragmentation
- adapter ligation
- amplification

Sequencing

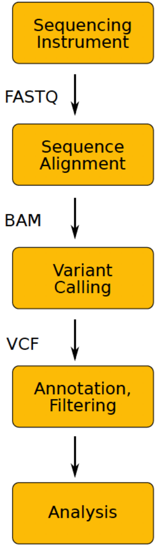
- base calling
- de-multiplexing

Data processing

- read mapping
- variant calling
- variant filtering

Analysis

- Variant annotation
- ...



Variant types

SNPs/SNVs . . . Single Nucleotide Polymorphism/Variation

ACGTTTAGCAT
ACGTTCAGCAT

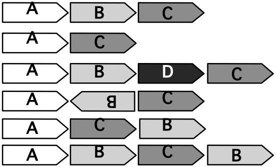
MNPs . . . Multi-Nucleotide Polymorphism

ACGTCCAGCAT
ACGTTTAGCAT

Indels . . . short insertions and deletions

ACGTTTAGCA- TT
ACGTT- AGCAGTT

SVs . . . Structural Variation



Some terminology

The goal is to determine the genotype at each position in the genome

Genotype

- in the broad sense . . . genetic makeup of an organism
- in the narrow sense . . . the combination of alleles at a position

Reference and alternate alleles - R and A

Diploid organism

- two chromosomal copies, three possible genotypes
  - RR .. homozygous reference genotype
  - RA .. heterozygous
  - AA .. homozygous alternate

Germline vs somatic mutation

Germline mutation

- heritable variation in the germ cells

Somatic mutation

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

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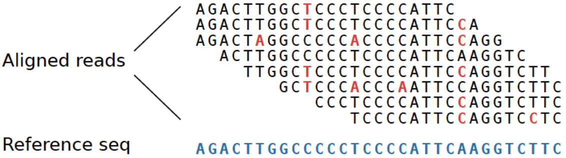
Germline variant calling

- 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

```
Aligned reads          AGACTTGGCTCCCTCCCCATTC
                       AGACTTGGCTCCCTCCCCATTCCA
                       AGACTAGGCCCCCACCCCATTCAAGG
                         ACTTGGCTCCCTCCCCATTCCAGGTC
                           TTGGCTCCCTCCCCATTCCAGGTCTT
                              GCCCCCACCCAATTCAAGGTCTTC
                                 CCCACCCCATTCCAGGTCTTC
                                    TCCCCATTCCAGGTCCTC

Reference seq          AGACTTGGCCCCCTCCCCATTCAAGGTCTTC

Allelic counts       R:3344545562777588878888276655343
                     A:0000010004000300010000500000100
Predicted dosage       0000000001000100000000100000100
```

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

Naive variant calling

Use fixed allele frequency threshold to determine the genotype

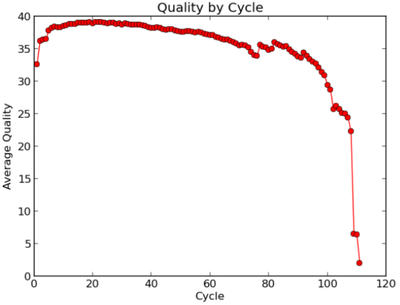
Low base quality →

```
                   AGACTTGGCTCCCTCCCCATTC
                   AGACTTGGCTCCCTCCCCATTCCA
                   AGACTAGGCCCCCACCCCATTCAAGG
                     ACTTGGCTCCCTCCCCATTCCAGGTC
                       TTGGCTCCCTCCCCATTCCAGGTCTT
                          GCCCCCACCCAATTCAAGGTCTTC
                             CCCACCCCATTCCAGGTCTTC
                                TCCCCATTCCAGGTCCTC

Reference seq      AGACTTGGCCCCTCCCCATTCAAGGTCTTC

Allelic counts   R:234452566276758787888276655333
                 A:000001000400030001000050000000
Predicted dosage   000001000100010000000001000000
```

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

Phred quality score
$Q = -10 \log_{10} P\mathrm{err}$

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%



Naive variant calling

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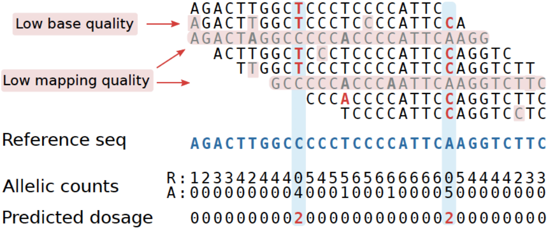

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2) Filter reads with low mapping quality

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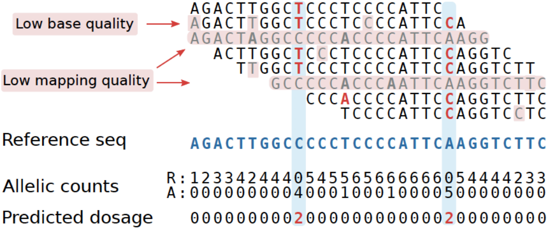

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Problems:

- 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

$$\underbrace{P(G|D)}_{\text{Posterior}} = \frac{\overbrace{P(D|G)}^{\text{Likelihood}}\,\overbrace{P(G)}^{\text{Prior}}}{\underbrace{P(D)}_{\text{Normalization}}}$$

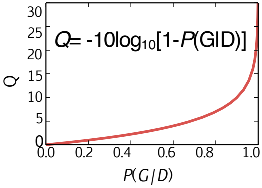
to determine:

1. the most likely genotype $g \in \{RR, RA, AA\}$ 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

    $P(\mathrm{RA}) = 2f(1-f),\ P(\mathrm{RR}) = (1-f)^2,\ P(\mathrm{AA}) = f^2$
- 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**

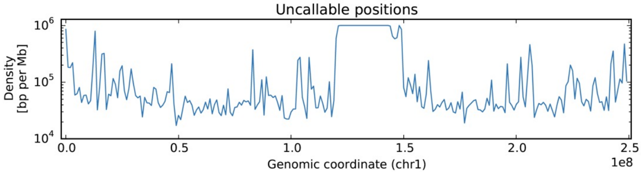
False calls can have many causes

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

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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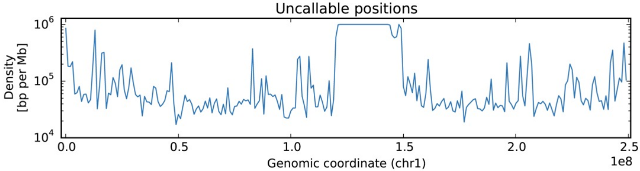
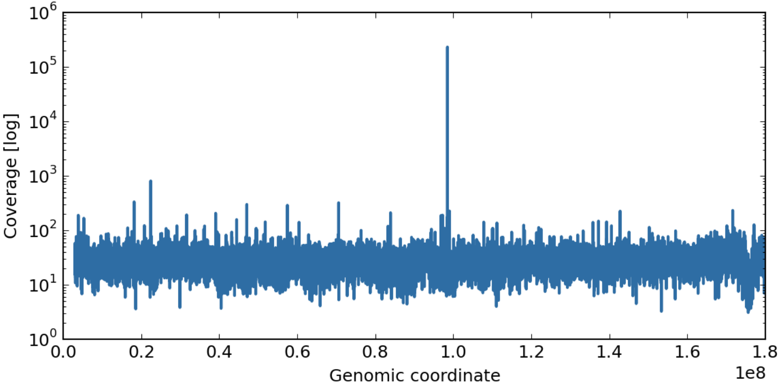


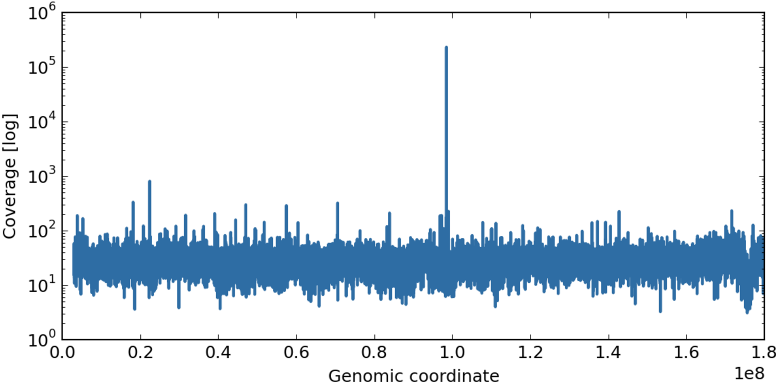
If possible, include only "nice" regions: for many analyses (e.g. population genetic studies) difficult regions can be ignored

Maximum depth



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

Maximum depth



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

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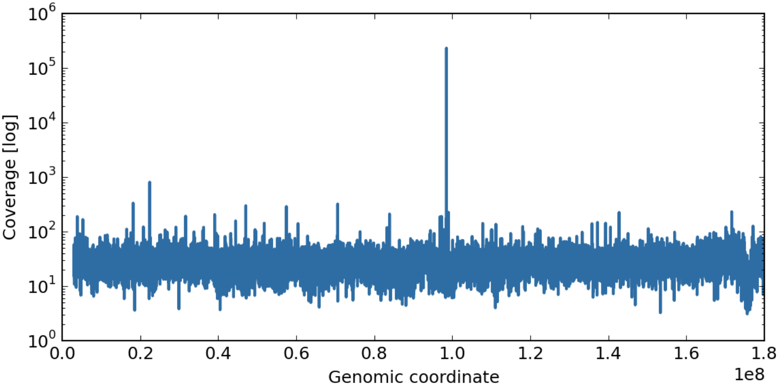
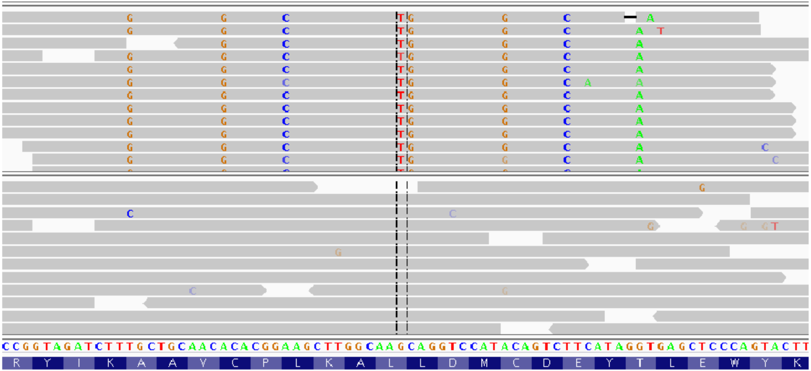


Q: Why is the sequencing depth thousandfold the average in some regions?
A. The reference genome is not complete. This sample was sequenced to 30x coverage, we can infer it has ~30 copies of this region.

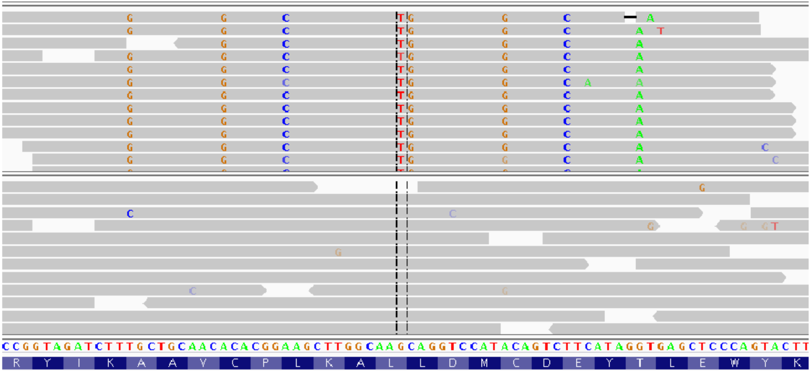
 Filter calls with a too high (for example, 2x the average in WGS)

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?

Mapping errors



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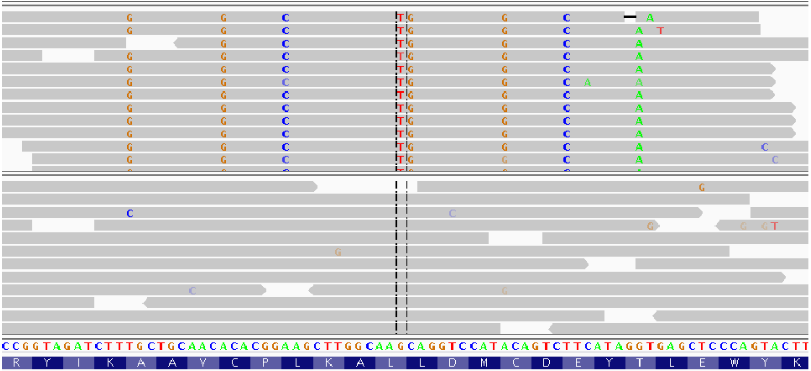
Mapping errors

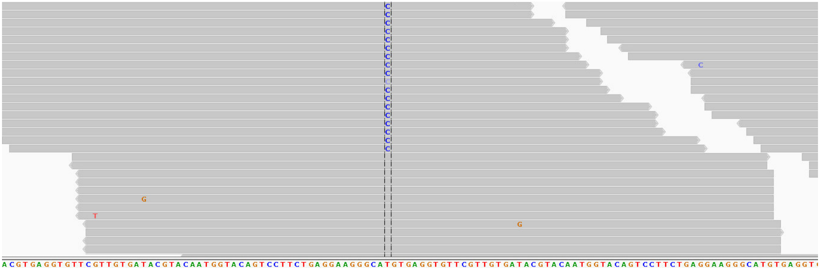


Q: RNA-seq (top) and DNA data (bottom) from the same sample has been mapped onto the reference genome. Can you explain the novel SNVs?

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

Strand bias



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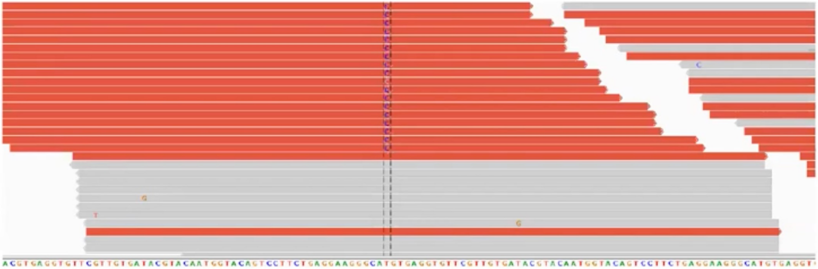
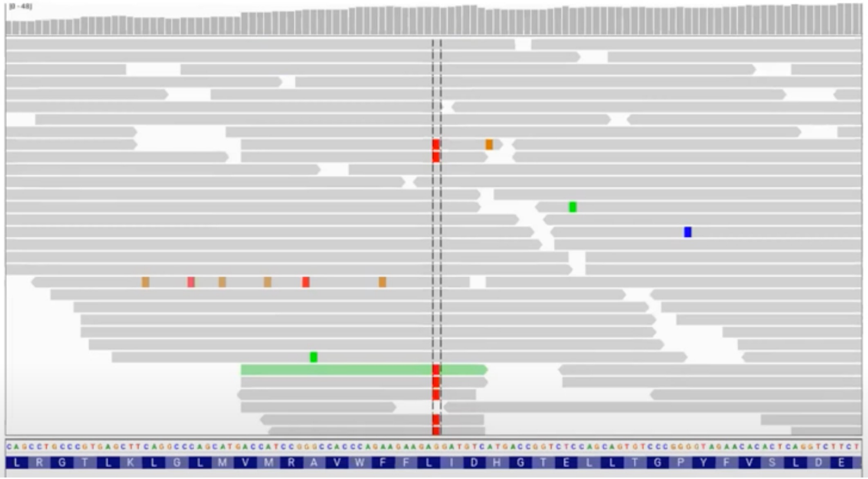


Q: Is this a valid call?

A. No, it is a mapping artifact, 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 artifacts



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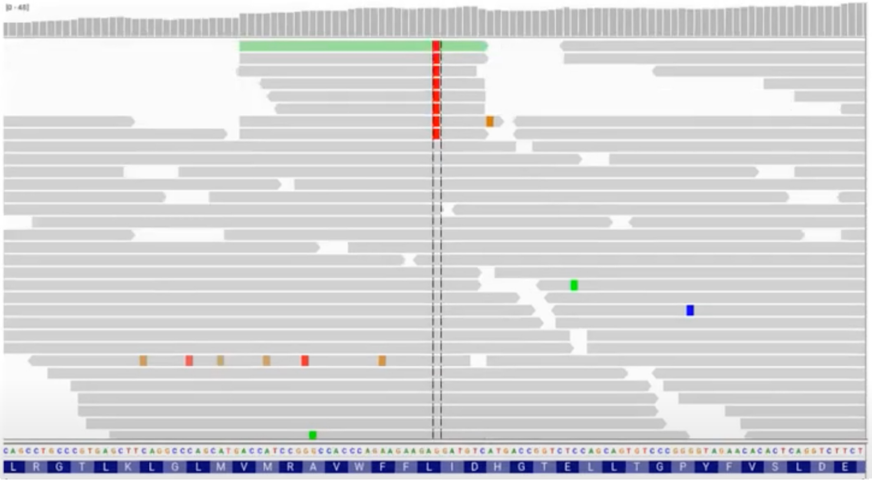
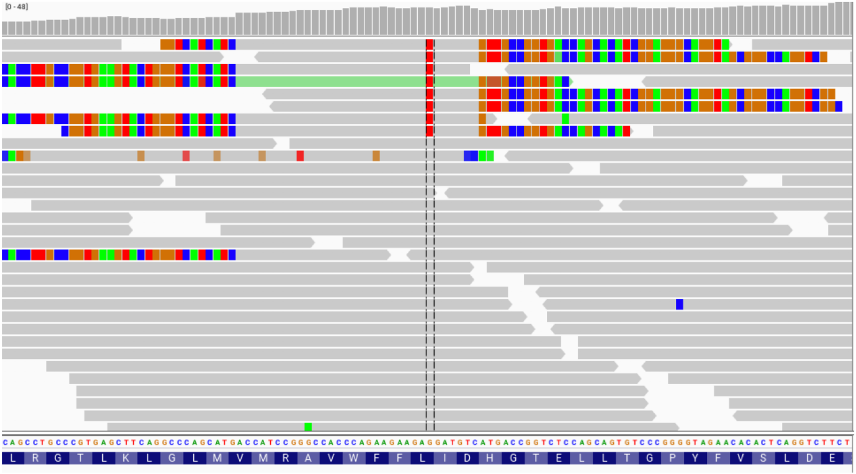


First sort by variant base...

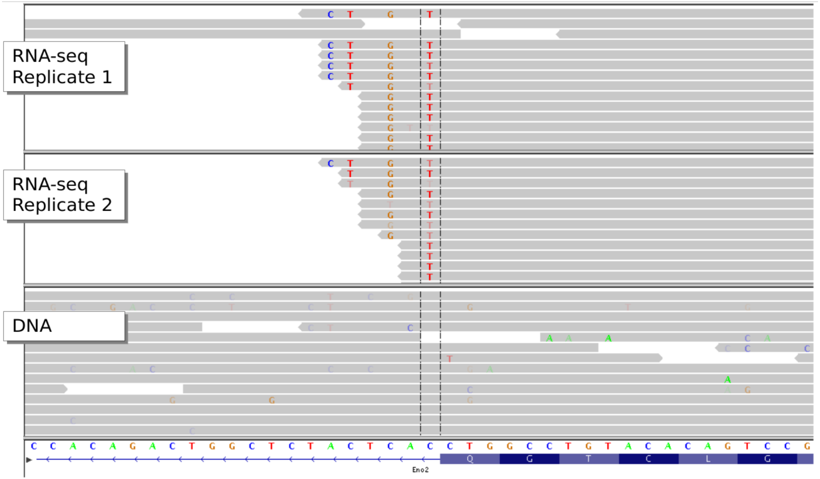
Change the display in IGV to reveal artifacts



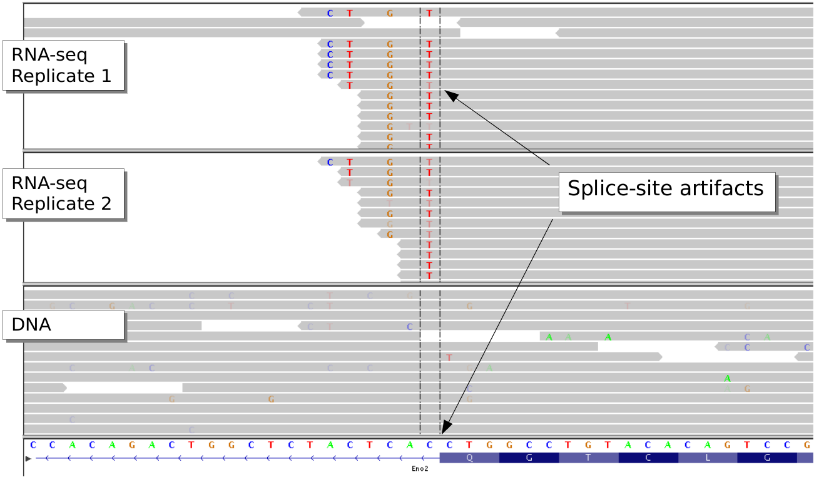
Display soft-clipped bases...

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

Variant distance bias

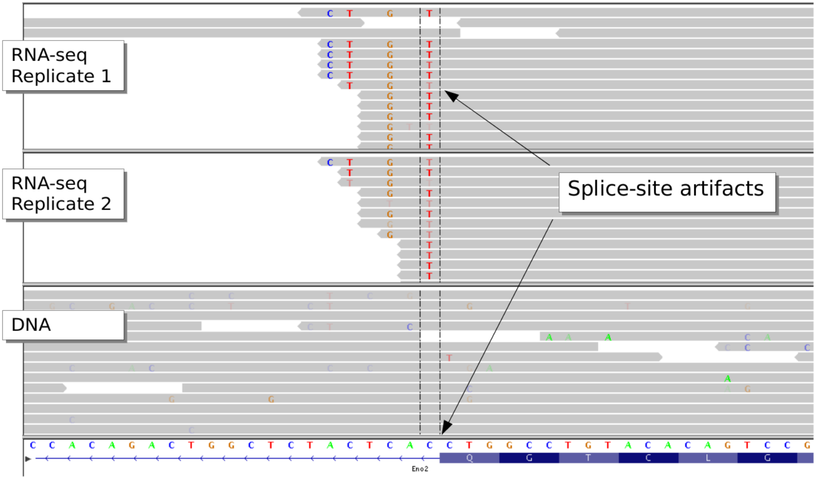


Q: Can you explain what happened here?



Q: Can you explain what happened here?

A: Processed transcript with introns spliced out.

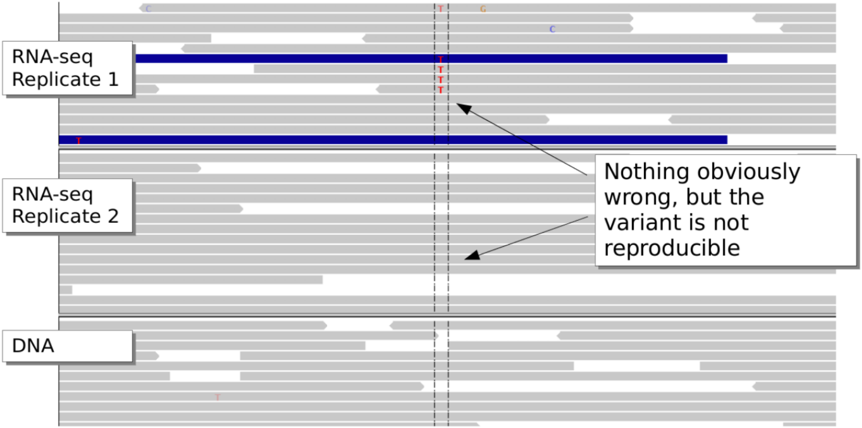


Q: Can you explain what happened here?

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

Reproducibility



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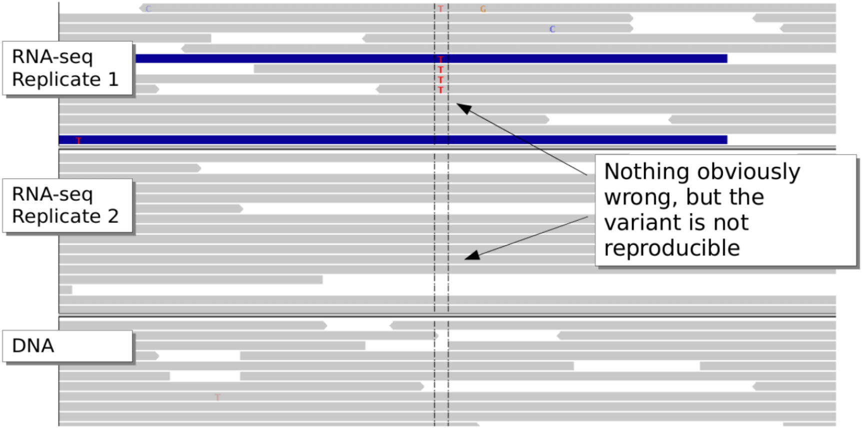

Mind the biological variability. If possible, validate and replicate

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

Q: How many SNPs are real?

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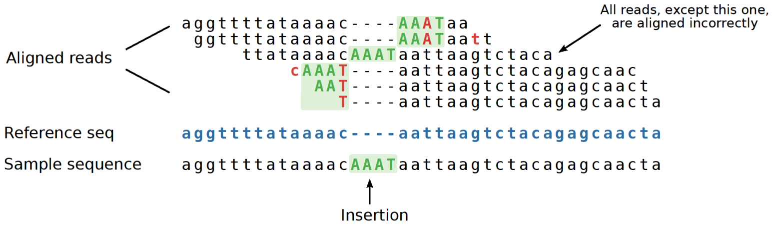
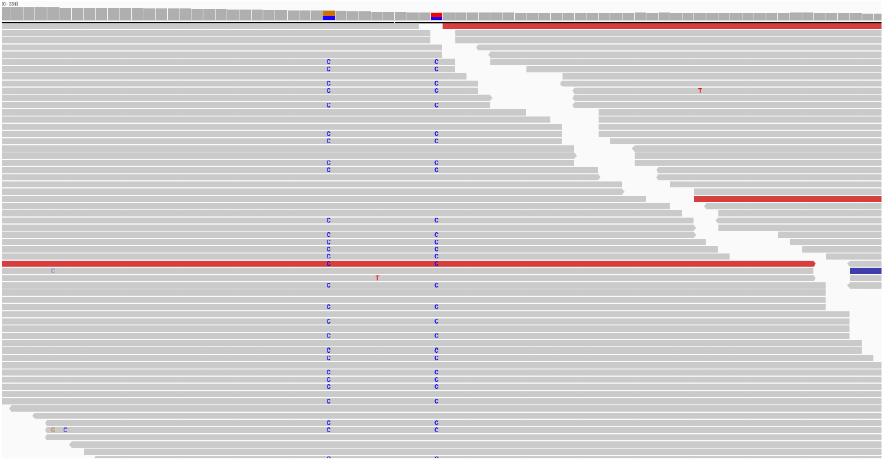


Q: How many SNPs are real?
A: None.

What do good SNPs look like?



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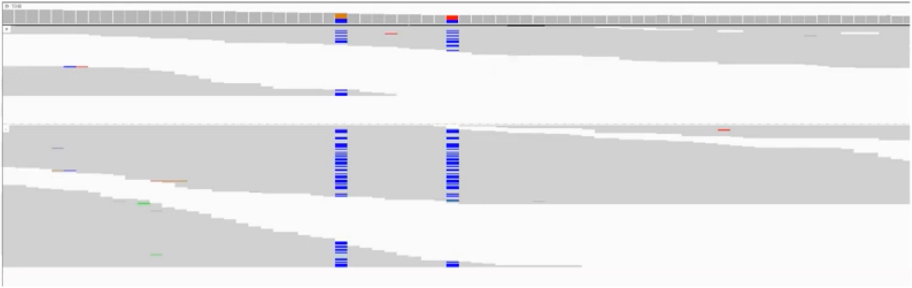

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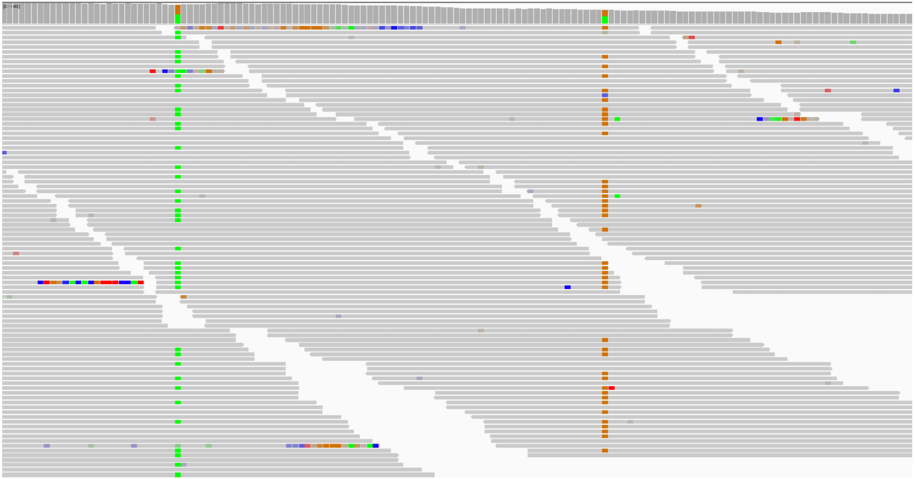

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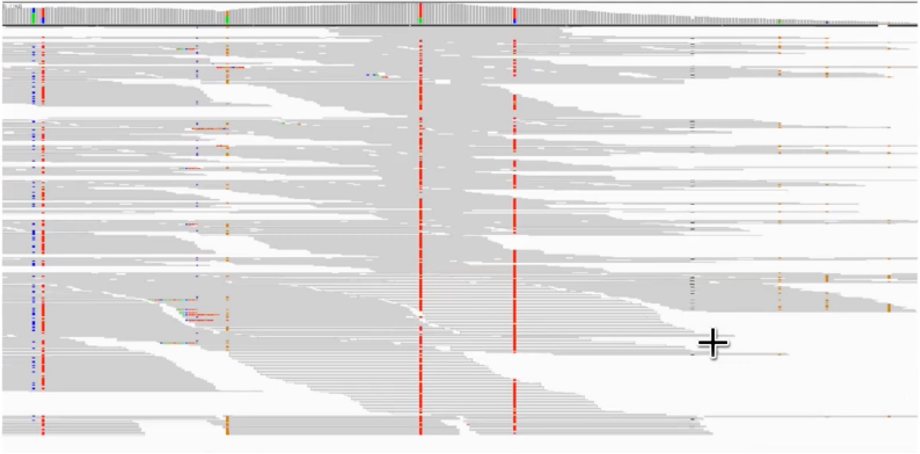


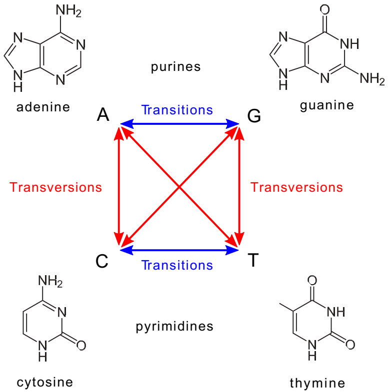
Q: Is this call real? There are many reads with MQ=0.

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

How to estimate the quality of called SNPs?

Transitions vs transversions ratio, known as ts/tv

- transitions are 2-3x more likely than transversions



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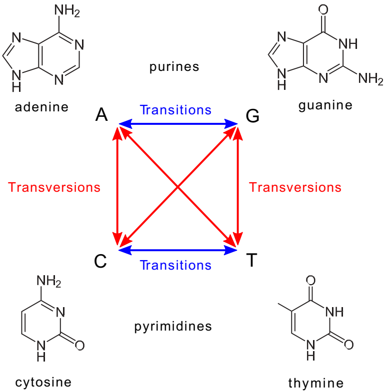

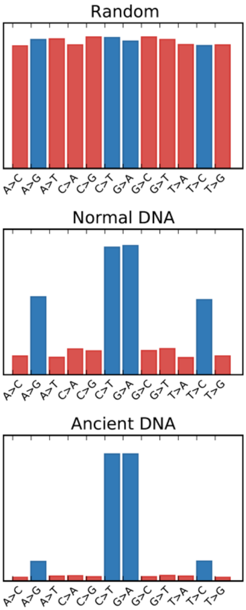

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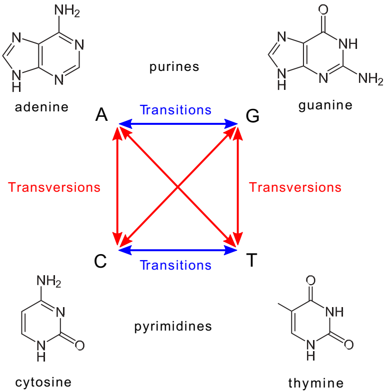
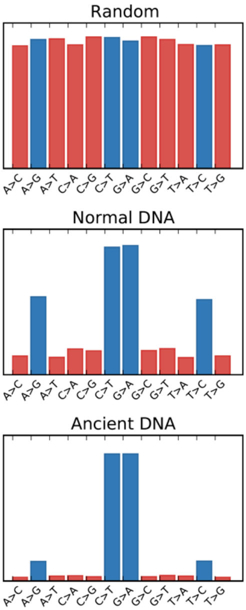




Whole human genome ~ 2.1
Whole human exome ~ 2.8

Indel calling challenges

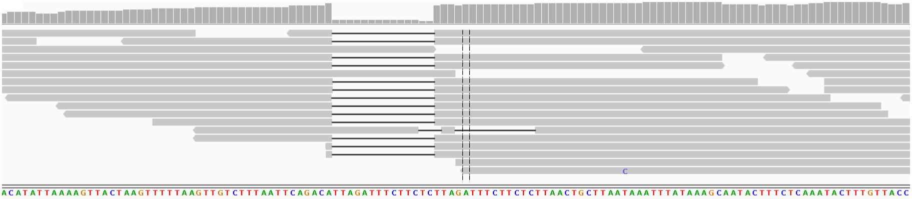
The sequencing error rate is elevated in microsatellites

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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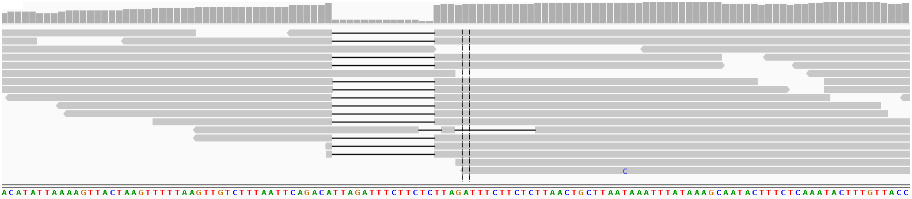


```
CTTTAATTCAGACA~~~~~~~~~~~~~~TTAGATTTCTTCTC
CTTTAATTCAGACA~~~~~~~~~~~~~~TTAGATTTCTTCTCTTA
CTTTAATTCAGACA--------------TTAGATTTCTTCTCTTAACTGCTT
CTTTAATTCAGACA~~~~~~~~~~~~~~TTAGATTTCTTCTATTAACTGCTT

CTTTAATTCAGACATTAGATTTCTTCTCTTAGATTTCTTCTCTTAACTGCTT
```

Future of variant calling

Current approaches

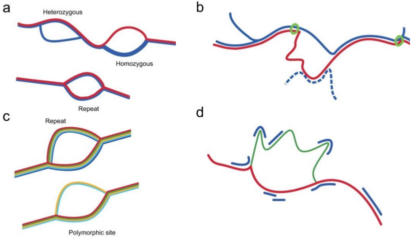
- rely heavily on the supplied alignment, but aligners see one read at a time
- largely site based, do not examine local haplotype and linked sites

Local *de novo* assembly based variant callers

- call SNPs, indels, MNPs and small SV simultaneously
- can remove alignment artefacts
- *eg* GATK haplotype caller, Scalpel, Octopus

Variation graphs

- align to a graph rather than a linear sequence



Iqbal *et al* (2012) *Nat Genet* **44**(2):226

Functional annotation

VCF can store arbitrary INFO tags (per site) and FORMAT tags (per sample)

- Describe genomic context of the variant (e.g. coding, intronic, UTR)
- Predict functional consequence (e.g. synonymous, missense, start lost)

Several tools for annotating a VCF, only few are haplotype-aware

- BCFtools/csq http://github.com/samtools/bcftools
- VEP Haplosaurus http://github.com/willmclaren/ensembl-vep

Excercise time!

- Open your VM
- Open a terminal window.
- Go to course_data/variant_calling

```
cd course_data/variant_calling/
```

- Open the exercises, which are in Github or in:

```
/home/manager/course_data/variant_calling/practical/ \
variant_calling.pdf
```

- Follow the instructions!