RE-ALIGNMENT

Alignment errors during mapping require fix

			coor	12345678901234	567890123456789012345 6
9	t	ttt	ref	aggttttataaaac----	aattaagtctacagagcaacta
10	a	aaaC	sample	aggttttataaaacAAAT	aattaagtctacagagcaacta
11	a	aaaaa	read1	aggttttataaaac	aaAtaa
12	a	aaaaaa	read2	ggttttataaaac	aaAtaaTt
13	a	aaaaaa	read3	ttataaaacAAAT	aattaagtctaca
14	c	cccTTT	read4	CaaaT	aattaagtctacagagcaac
15	a	aaaaaa	read5	aaT	aattaagtctacagagcaact
16	a	aaaaaa	read6	T	aattaagtctacagagcaacta
17	t	AAtttt	read1	aggttttataaaacaaataa	
18	t	tttttt	read2	ggttttataaaacaaataatt	
19	a	aaaaaa	read3	ttataaaacaaataattaagtctaca	
20	a	aaaaaa	read4	caaataattaagtctacagagcaac	
21	g	Tgggg	read5	aataattaagtctacagagcaact	
			read6	taattaagtctacagagcaacta	

Source: Heng Li, presentation at GSA workshop 2011

Alignment of an insertion

```
Ref    A A A C A A T T A A G T
                 |
Sample         AAAT

Sample A A A C A A A T A A T T A A G T
```

```
Ref    A A A C - - - - A A T T A A G T

Sample A A A C A A A T A A T T A A G T
       ------------------------------>
```

Correct alignment

```
Sample read A A A C A A A T A A T T
```

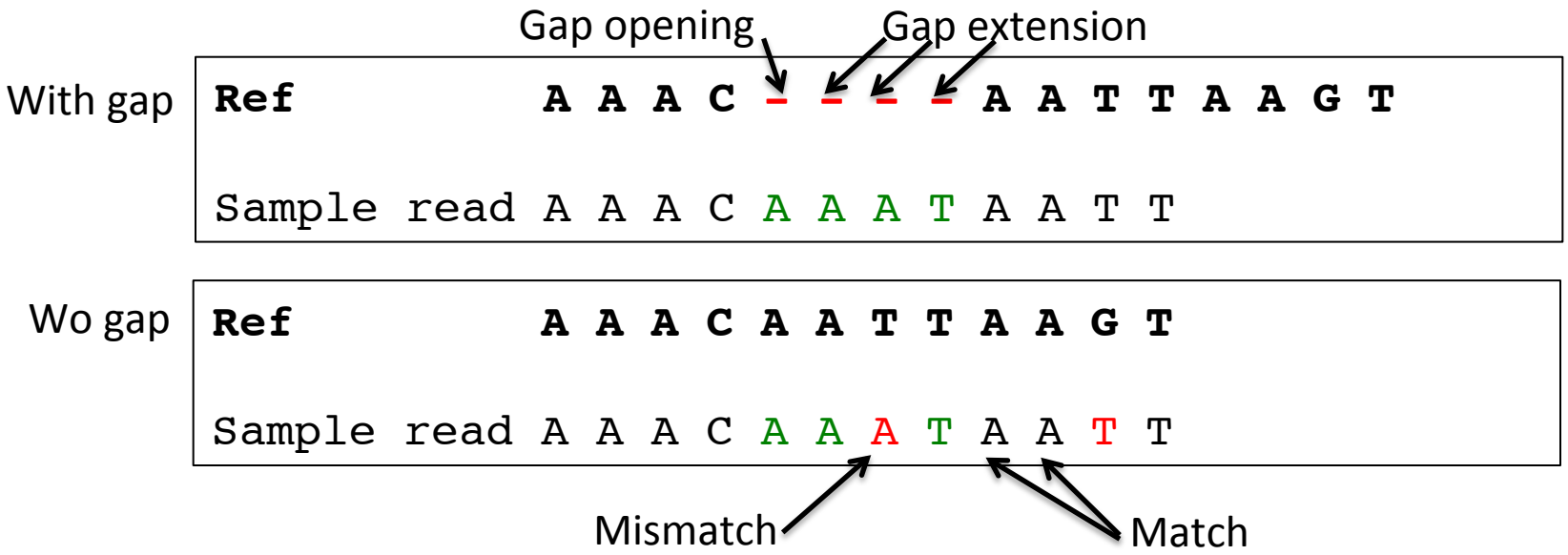
Correct alignment

```
Ref          A A A C - - - - A A T T A A G T

Sample read  A A A C A A A T A A T T
```

Possible alignment

```
Ref          A A A C A A T T A A G T

Sample read  A A A C A A A T A A T T
```

Alignment



- Key component of alignment algorithm is the scoring
  - negative contribution to score
    - opening a gap
    - extending a gap
    - mismatches
  - positive contribution to score
    - matches
- The exact score contributions determine which alignment is chosen
- **Smith-Waterman** is an algorithm for finding optimal alignment given a scoring scheme without exhaustively enumerating and scoring all possible alignments

Longer reads or multiple sequences

Longer reads

With gap

```
Ref           A A A C - - - - A A T T | A A G T

Sample read   A A A C A A A T A A T T | A A G T
```

Wo gap

```
Ref           A A A C A A T T A A G T | C T A C

Sample read   A A A C A A A T A A T T | A A G T
```

Multiple reads

With gap

```
Ref           A A A C - - - - A A T T A A G T C T

Sample read   A A A C A A A T A A T T
                    A C A A A T A A T T A A
                                A A T T A A G T C T
```

Wo gap

```
Ref           A A A C A A T T A A G T C T A C

Sample read   A A A C A A A T A A T T
                    A C A A A T A A T T A A
                          A A T T A A G T C T
```

Match
Mismatch to ref
Mismatch to read

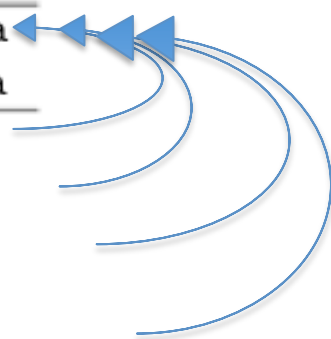
Few mismatches when considering one-to-one

Example: sample has insertion of AAAT relative to reference

Base stacks

9	t	ttt
10	a	aaaC
11	a	aaaaa
12	a	aaaaaa
13	a	aaaaaa
14	c	cccTTT
15	a	aaaaaa
16	a	aaaaaa
17	t	AAtttt
18	t	tttttt
19	a	aaaaaa
20	a	aaaaaa
21	g	Tgggg

```
coor      12345678901234     5678901234567890123456
ref       aggttttataaaac----aattaagtctacagagcaacta
sample    aggttttataaaacAAATaattaagtctacagagcaacta
read1     aggttttataaaac     aaAtaa
read2      ggttttataaaac     aaAtaaTt
read3          ttataaaacAAATaattaagtctaca
read4              CaaaT     aattaagtctacagagcaac
read5                aaT     aattaagtctacagagcaact
read6                 T      aattaagtctacagagcaacta
read1     aggttttataaaacaaataa
read2      ggttttataaaacaaataatt
read3          ttataaaacaaataattaagtctaca
read4                  caaataattaagtctacagagcaac
read5                    aataattaagtctacagagcaact
read6                     taattaagtctacagagcaacta
```

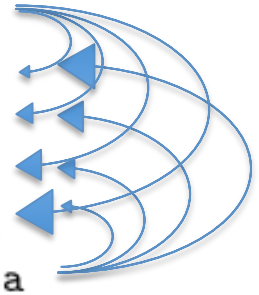


Lots of mismatch in all-to-all if reads mismapped

Base stacks

9	t	ttt
10	a	aaaC
11	a	aaaaa
12	a	aaaaaa
13	a	aaaaaa
14	c	cccTTT
15	a	aaaaaa
16	a	aaaaaa
17	t	AAtttt
18	t	tttttt
19	a	aaaaaa
20	a	aaaaaa
21	g	Tgggg

```
coor      12345678901234     5678901234567890123456
ref       aggttttataaaac----aattaagtctacagagcaacta
sample    aggttttataaaacAAATaattaagtctacagagcaacta
read1     aggttttataaaac    aaAtaa
read2      ggttttataaaac    aaAtaaTt
read3          ttataaaacAAATaattaagtctaca
read4              CaaaT    aattaagtctacagagcaac
read5                aaT    aattaagtctacagagcaact
read6                 T     aattaagtctacagagcaacta
read1     aggttttataaaacaaataa
read2      ggttttataaaacaaataatt
read3          ttataaaacaaataattaagtctaca
read4                caaataattaagtctacagagcaac
read5                  aataattaagtctacagagcaact
read6                   taattaagtctacagagcaacta
```



No mismatches between reads

Mapping vs. alignment

Mapping vs. alignment

Mapping

- A mapping is the region where a read sequence is placed.
- A mapping is regarded to be correct if it overlaps the true region.

Alignment

- An alignment is the detailed placement of each base in a read.
- An alignment is regarded to be correct only if each base is placed correctly.

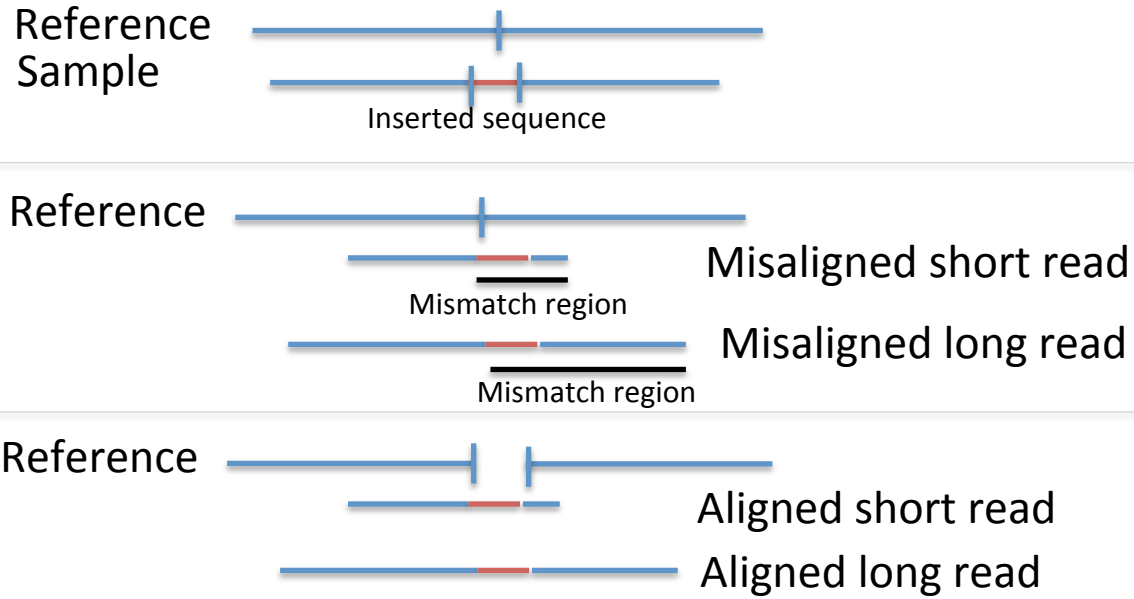
The problem

- A read mapper is fairly good at mapping, may not be good at alignment.
- This is because the true alignment minimizes differences between reads, but the read mapper only sees the reference.

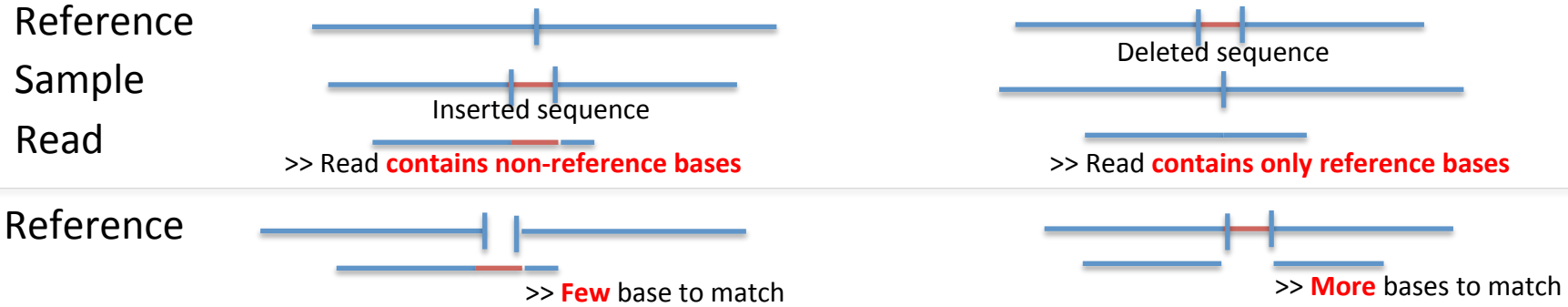
Source: Heng Li, presentation at GSA workshop 2011

Detection of indels

Longer read length facilitates correct alignment



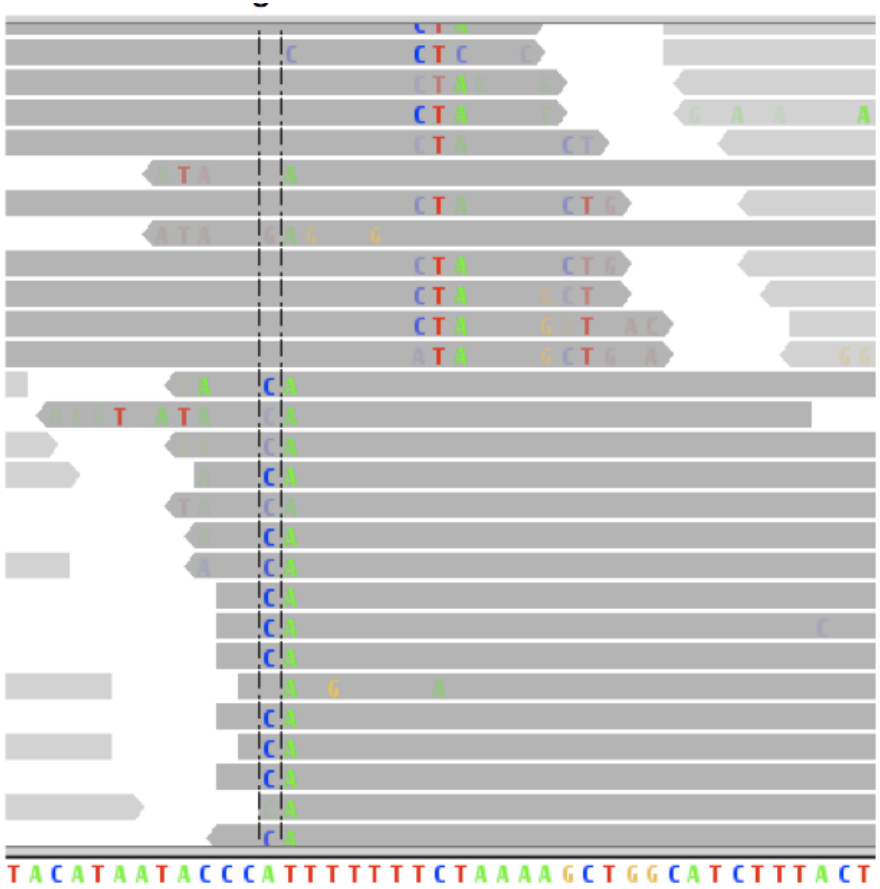
Asymetry between insertions and deletions



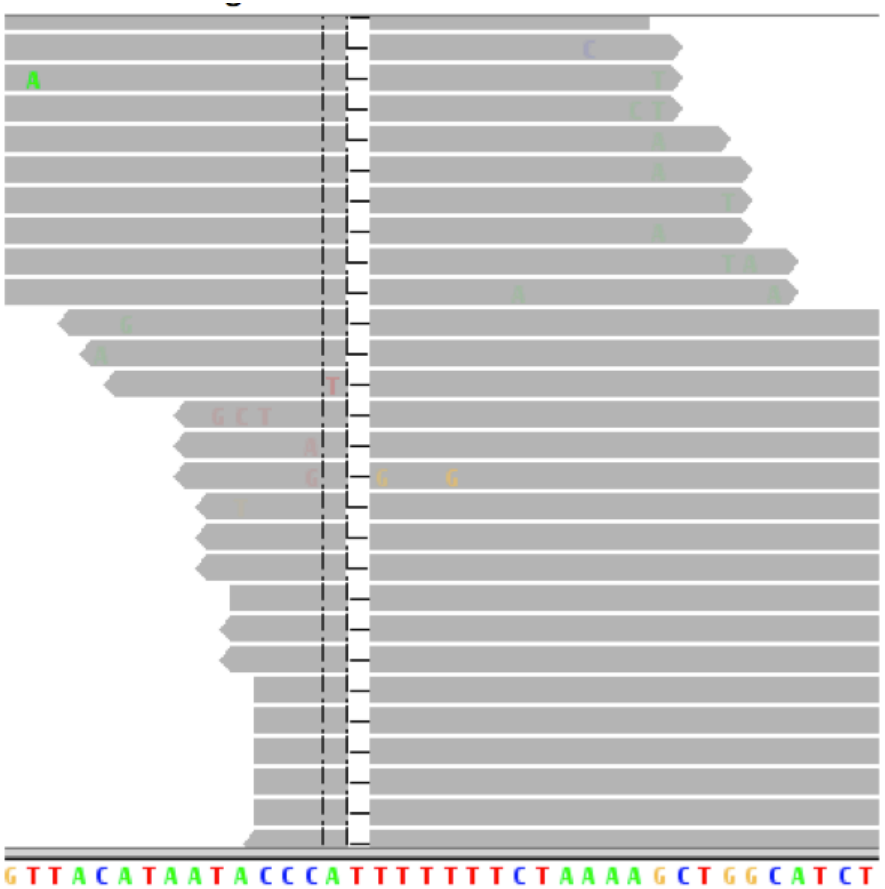
>> insertion and deletion of same size, but more likely to detect the deletion

Local realignment around indels

Before

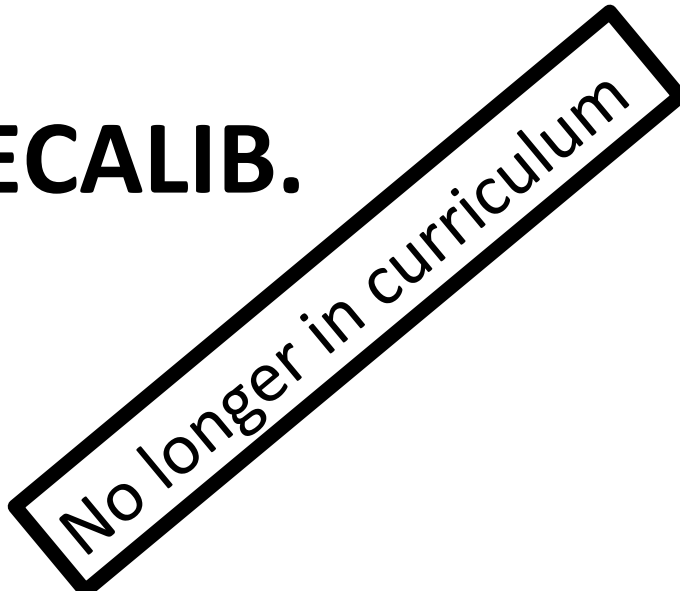


After



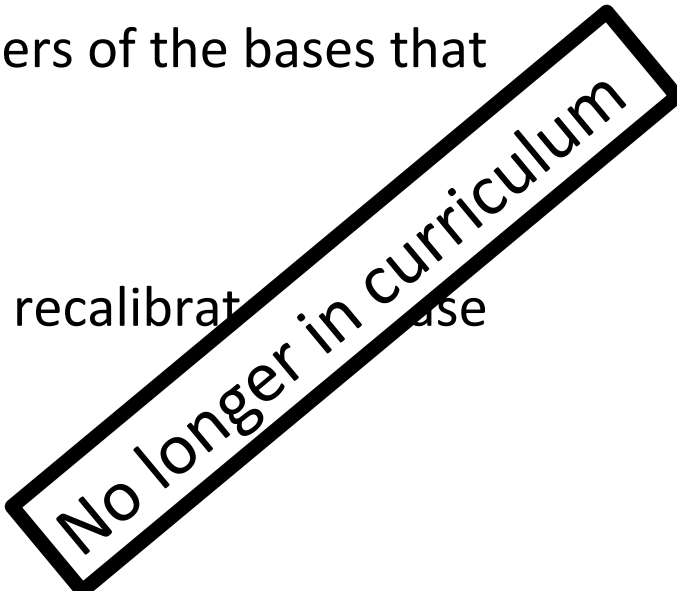
http://www.broadinstitute.org/files/shared/mpg/nextgen2010/nextgen_sivachenko.pdf

BASE QUALITY SCORE RECALIB.

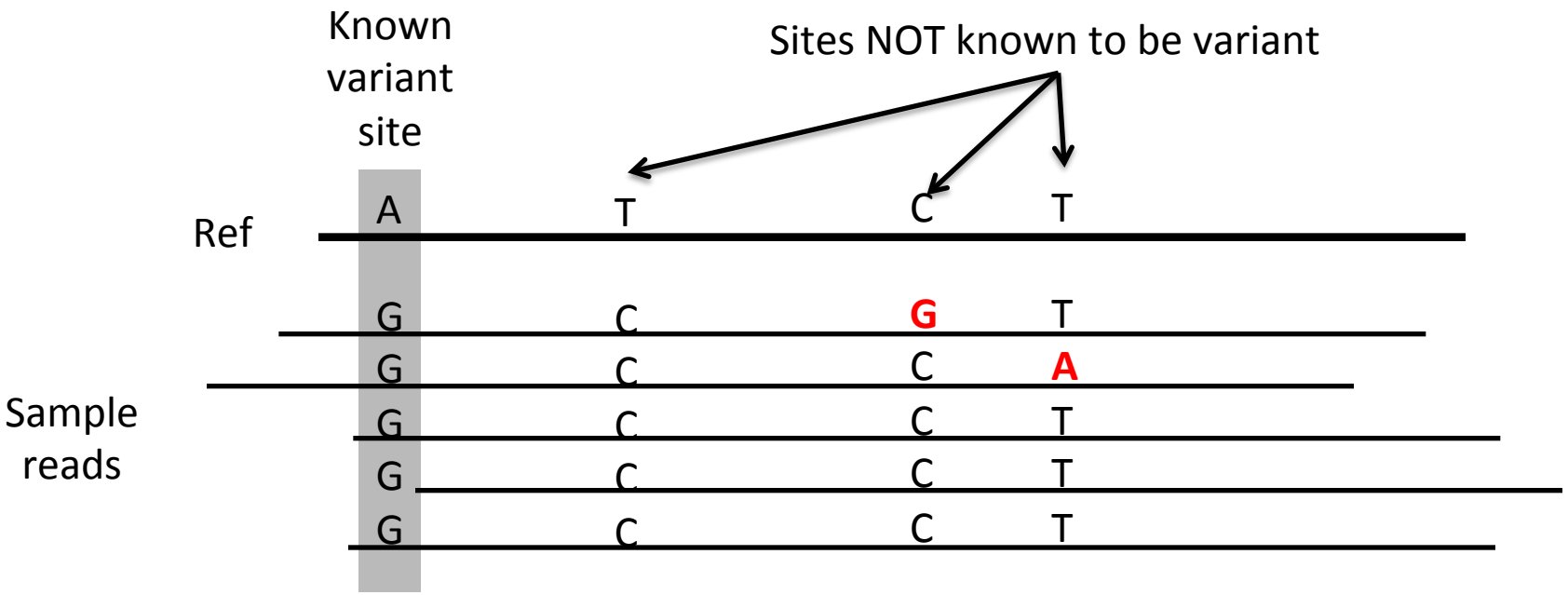


Theoretical vs Empirical error rates / qualities

- The qualities in the fastq file are computed using a model
- This model is not perfect >> there are discrepancies between the model and the empirical error rate
- We can compute a good approximation of the empirical error rate by identifying all sites where there are mismatches between the read and the reference (**being careful to ignore sites with known SNPs**)
- We can analyse whether there are parameters of the bases that covary with the discrepancy
  - e.g. cycle
- We can use these quantified covariances to recalibrate base qualities >> more accurate qualities



Overview of Base Quality recalibration



Sample variant at site **(common)**

Sample variant at site **(rare)**

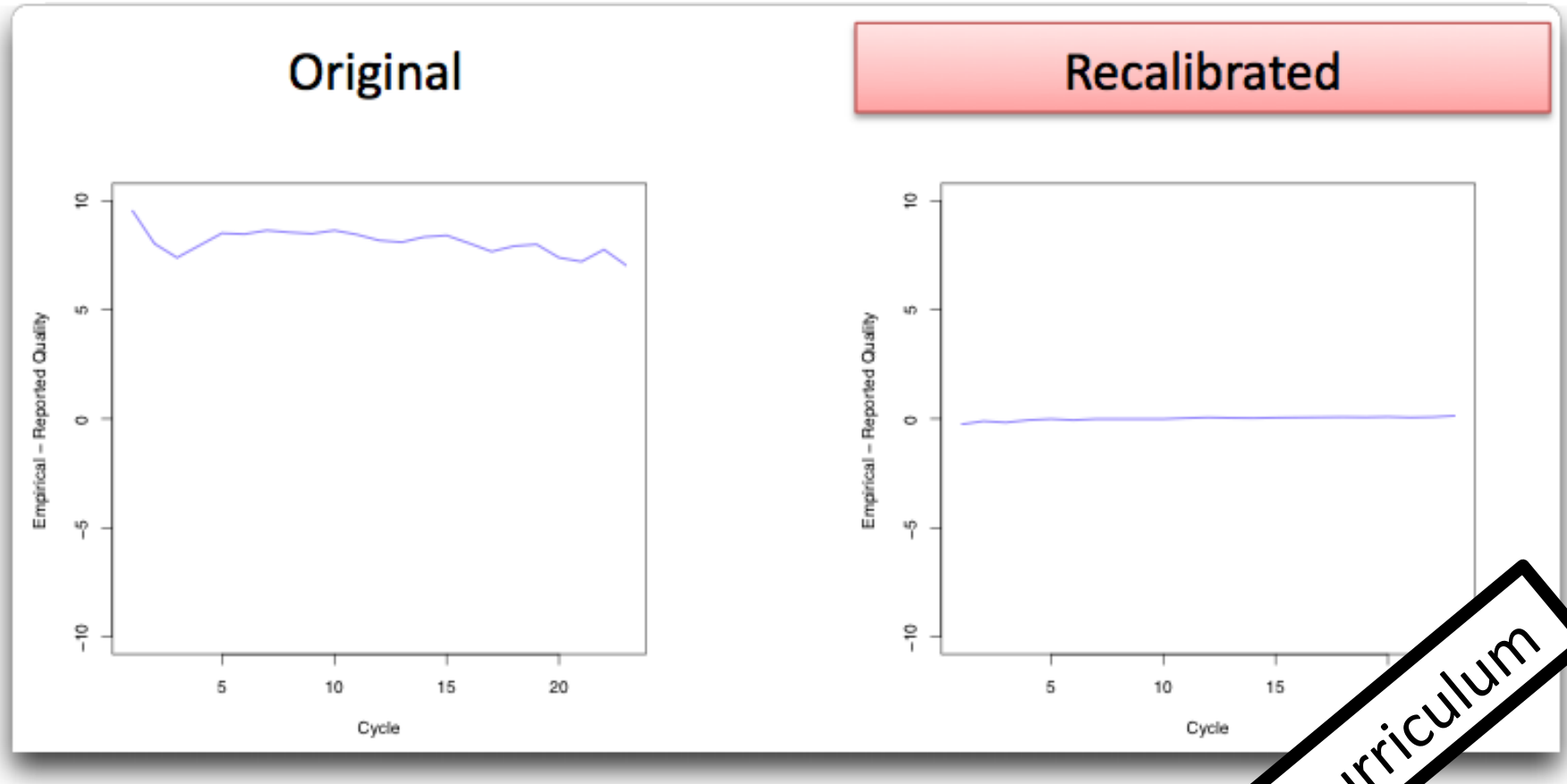
Base call error **(common)**

		Theoretical base Q	Empirical base Q
Cycle	**1**	**28**	**29**
	2	**29**	**30**
	n	**…**	**…**
Dinucleotide	**AG**	**24**	**24**
	AT	**29**	**22**
	AC	**…**	**…**
	…		

Empirical ba… be
comput… e
obse… match rate
b… e read bases
… reference bases
… align **to (at sites that**
are not obviously variant)

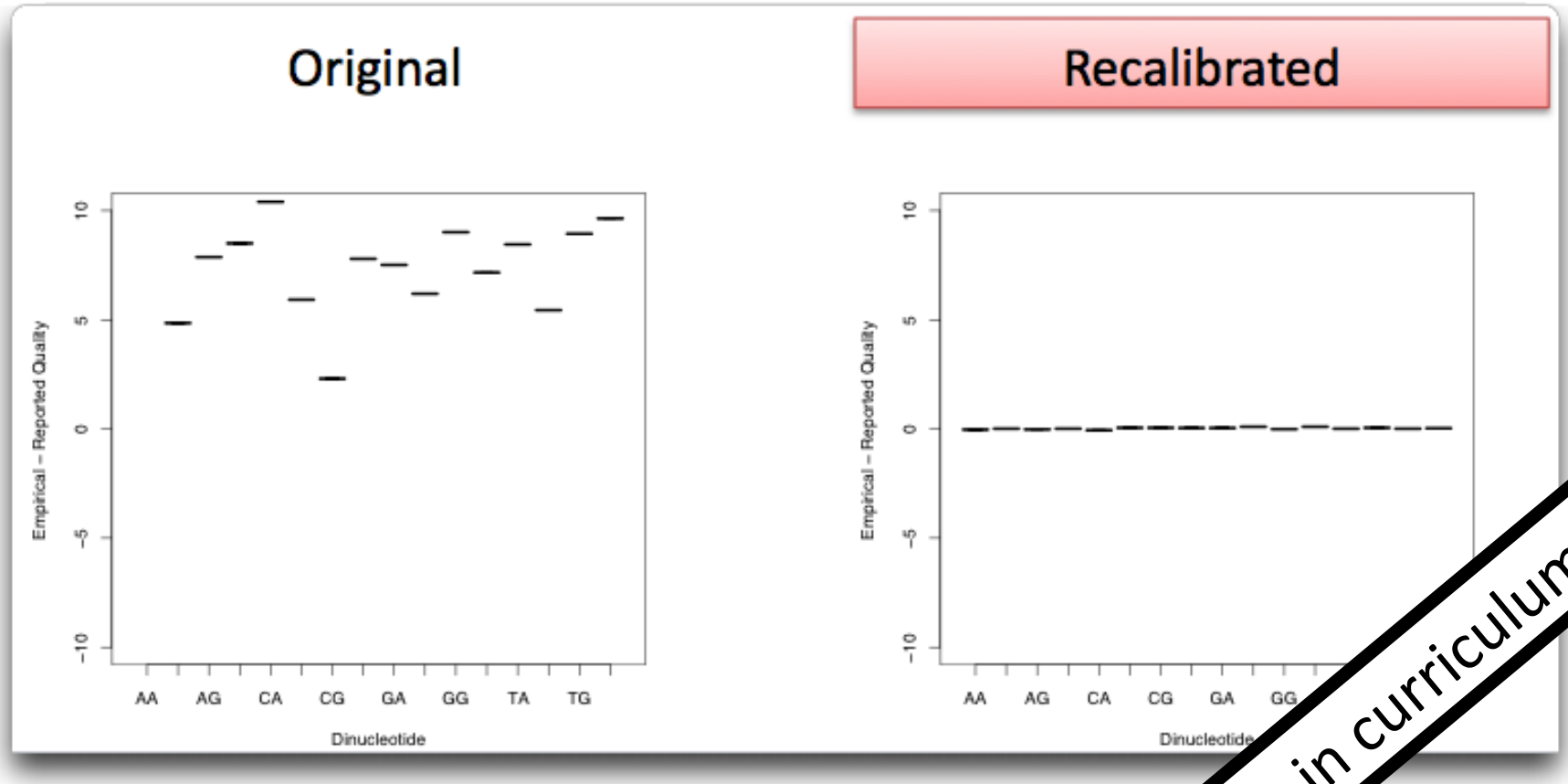
No longer in curriculum

Discrepancy and cycle

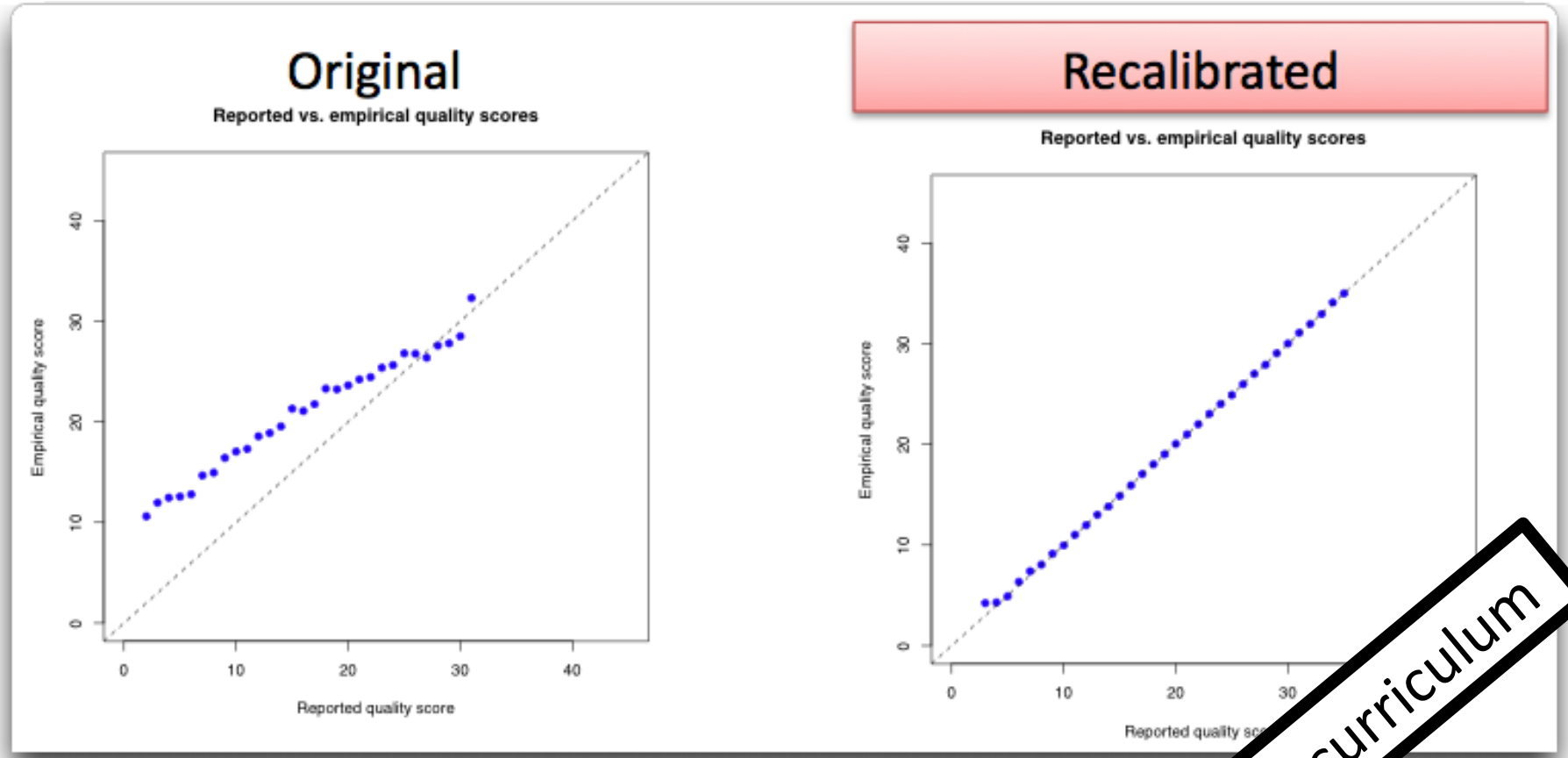


No longer in curriculum

Discrepancy and Dinuc context



Result of recalibration



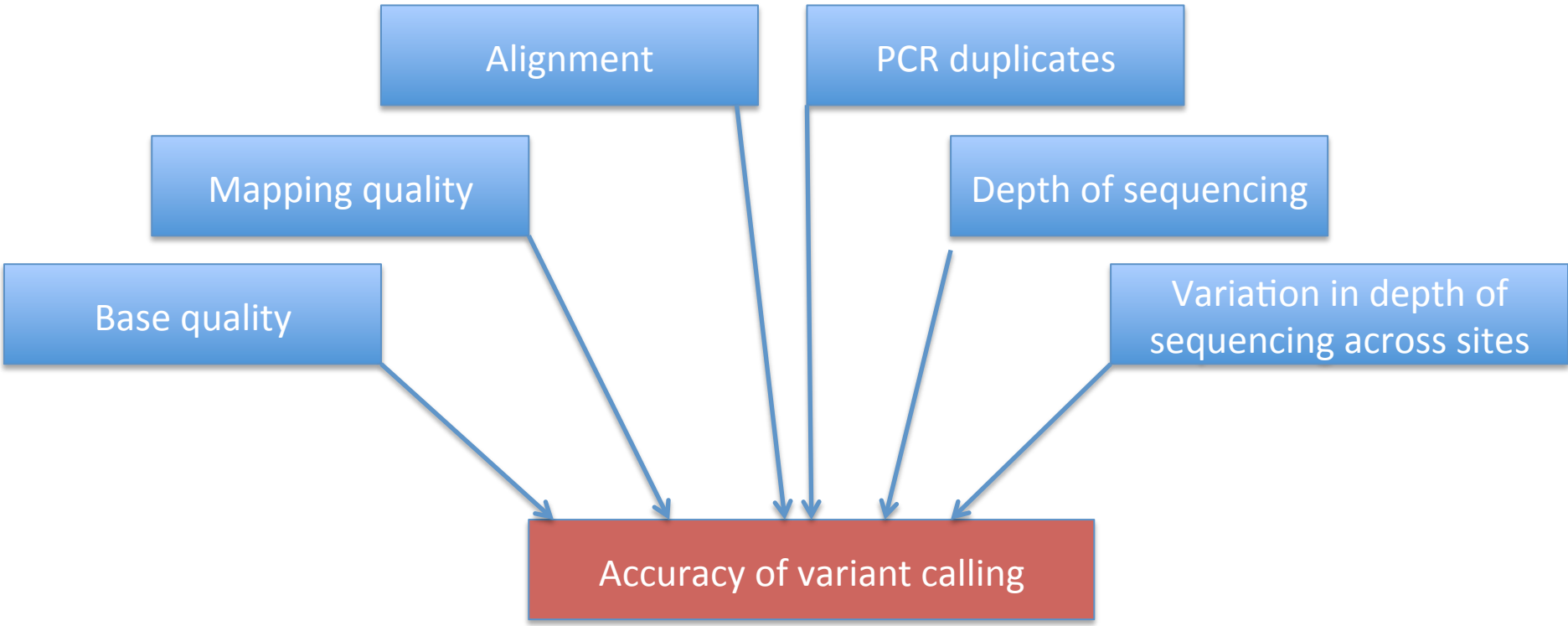
NB: The theoretical base qualities have become very go ere seems to be little to be gained in recalibrating "modern" fa

No longer in curriculum

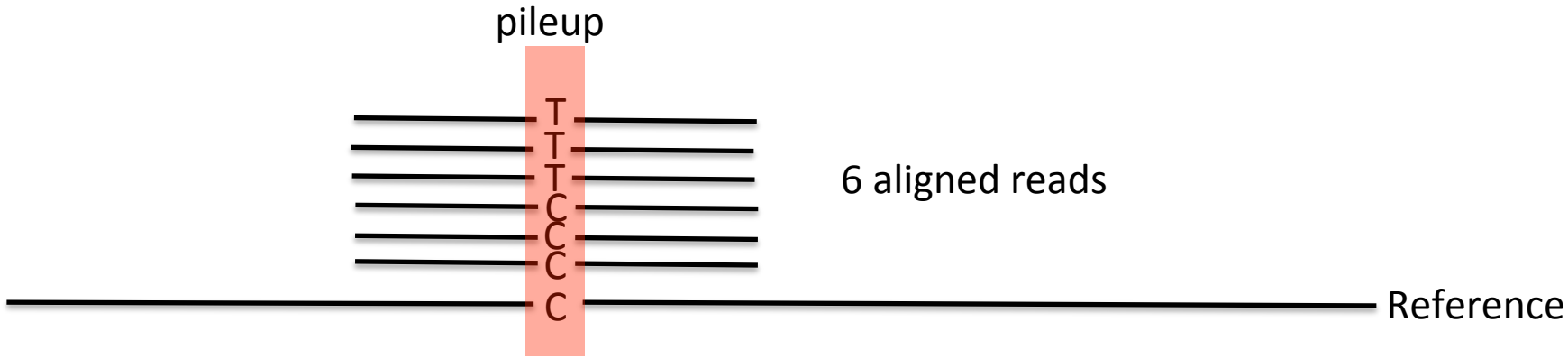
VARIANT CALLING

Recaping source of noise in variant calling

- If we had one long end-to-end read of the chromosome there would be no problem
- BUT we have short reads of imperfect quality



A way of thinking of the variant calling process



- Compute:
  - the probability that the site is **variant**: P(not CC / read data)
  - the probability of the different **genotypes**: P(CC / read data), P(CT / read data), P(TT / read data)

- Intuition of difference between variant site and genotype:
  - **ref is C**, aligned bases are TTTTTTTCC
  - highly likely that the site is variant
  - less clear what the genotype is: T/A or T/T?

- **Only good bases are considered in the pileup**
  - **Minimum base quality**
  - **Read mapping quality**

- The importance of depth of coverage

- Complex mathematical models involved in both allele frequency calculations and genotype likelihoods >> wise to use the recommended option settings in the tool documentation (as we have done in the practicals)

Bayesian variant caller (optional)

Input

Reference is C, observing 4C and 2T, all with base quality 30.

Likelihood of data

- $P(D|CC) = \Pr\{\text{two Q30 errors}\} = 10^{-(30+30)/10} = 10^{-6}$
- $P(D|TT) = \Pr\{\text{four Q30 errors}\} = 10^{-(30*4)/10} = 10^{-12}$
- $P(D|CT) = \Pr\{\text{sample 6 reads from 2 chr}\} = 1/2^6 = 1.56 \times 10^{-2}$

Posterior

- Prior: $P(CC) = 0.9985$, $P(CT) = 0.001$ and $P(TT) = 0.0005$

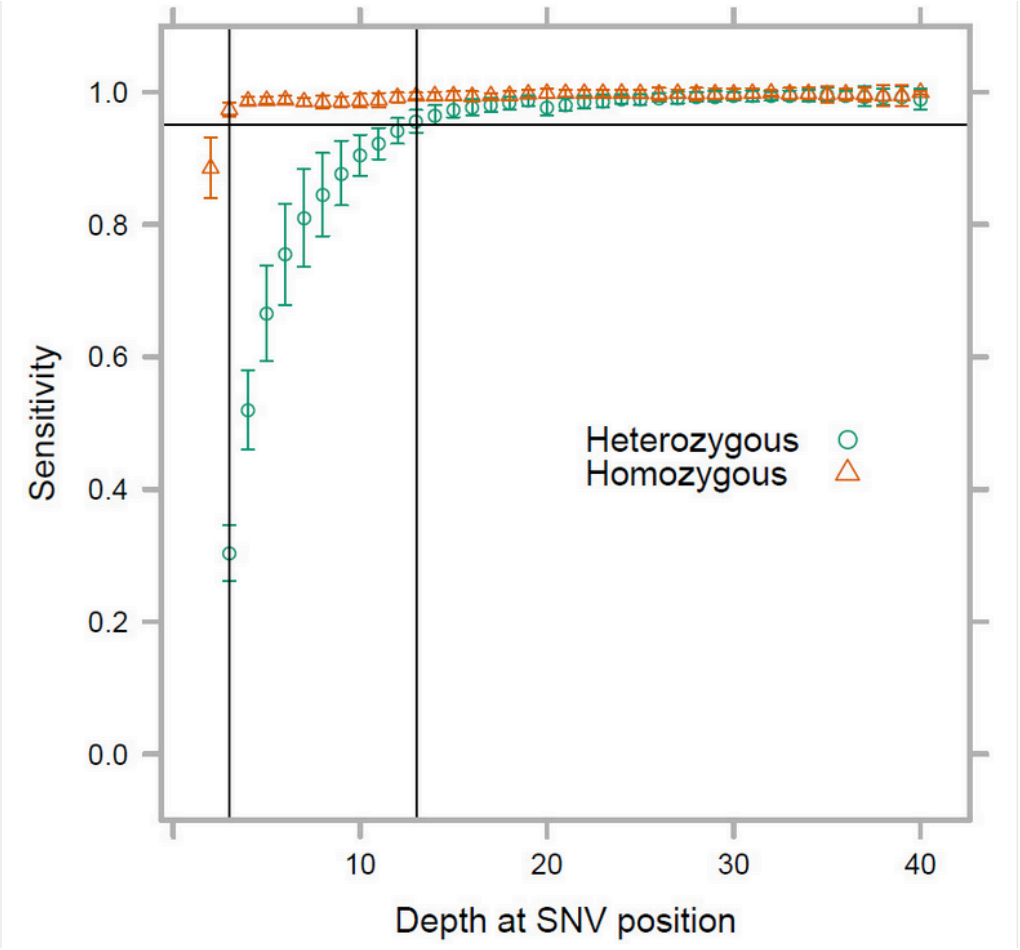
$$P(CC|D) = \frac{P(D|CC)P(CC)}{P(D|CC)P(CC) + P(D|CT)P(CT) + P(D|TT)P(TT)}$$

- Get: $P(CC|D) = 0.06$, $P(CT|D) = 0.94$ and $P(TT|D) = 3 \times 10^{-11}$

Source: Heng Li, presentation at GSA workshop 2011

The effect of depth on errors

- Heterozygotes vs homozygote variant sites
- Equal sampling of alleles



From "Quantifying single nucleotide variant detection sensitivity in exome sequencing"
BMC Bioinformatics 2013

VCF FORMAT – MORE DETAILS

VCF format

```
##fileformat=VCFv4.0
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=1000GenomesPilot-NCBI36
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=.,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF ALT    QUAL FILTER INFO                              FORMAT      NA00001        NA00
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
20     17330   .         T   A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50
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
20     1230237 .         T   .       47   PASS   NS=3;DP=13;AA=T                   GT:GQ:DP:HQ 0|0:54:7:56,60
20     1234567 microsat1 GTCT G,GTACT 50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4
```

Meta data: definitions of tags used elsewhere in data lines

Header line

Data lines

Variant columns

Genotype columns

Columns of data lines

- **CHROMO**
- **POS**: the reference position with the 1st base having position 1
- **ID**: an id; rs number if dbSNP variant
- **REF**: reference base.
  - The value in POS refers to the position of the first base in the string
  - for indels, the reference string must include the base before the event (and this must be reflected in POS)
- **ALT**: comma sepearated list of alternate non-ref alleles called on at least one of the samples
  - if no alternate alleles then the missing value should be used "."
- **QUAL**: phred-scaled quality score of the assertion made in ALT (whether variant or non-variant)
- **FILTER**: PASS if the position has passed all filters (defined in meta-data).
- **INFO**: additional information

INFO, FORMAT, and genotypes

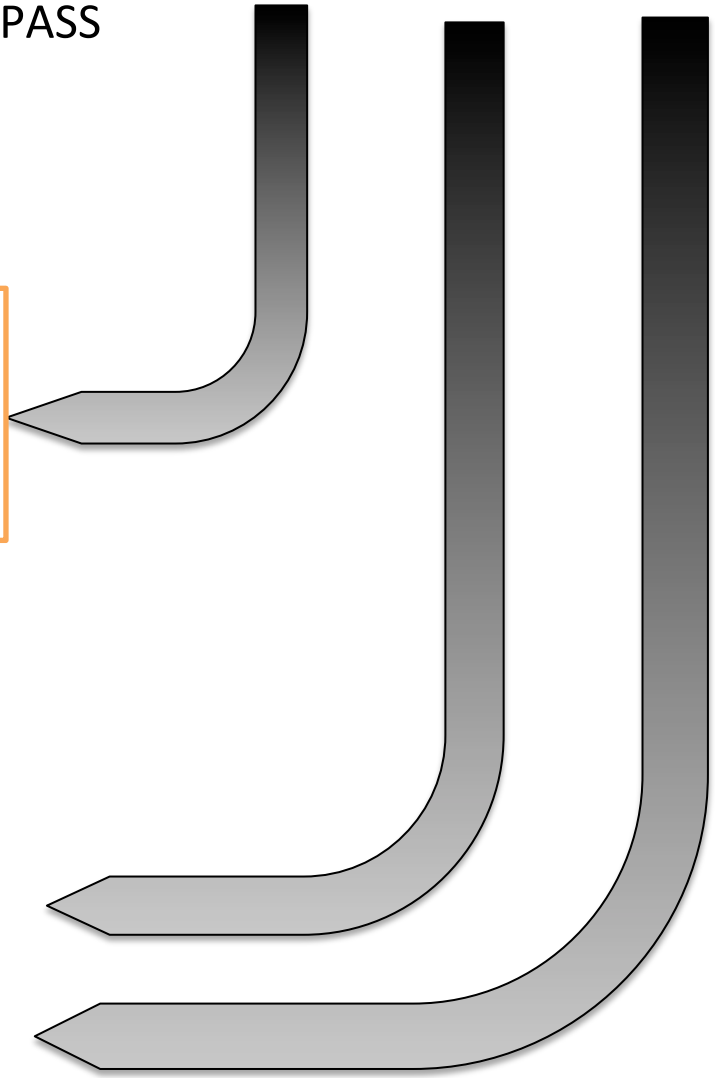
#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT sample1
1 801943 rs7516866 C T 9787.34 PASS

AC=2;
AF=1.00;
AN=2;
BaseQRankSum=1.009;
DB;
DP=556;
FS=18.302;
MQ=44.04;
MQ0=38;
MQRankSum=5.122;
QD=17.60;
ReadPosRankSum=3.375

GT:AD:DP:GQ:PL

1/1:37,518:556:99:9787,685,0

We will explore these fields when we discuss filtering



Genotype fields

- Format field specifies type of data present for each genotype
  - GT:AD:DP:GQ:PL
  - fields defined in metadata header

- GT: genotype, encoded as alleles separated by either | or /
  - 0 for the ref, 1 for the 1st allele listed in ALT, 2 for the second, etc
  - REF=A and ALT=T
    - genotype 0/1 means hetero A/T
    - genotype 1/1 means homo T/T
  - /: genotype unphased and | genotype phased
- DP: read depth at position for sample
- GQ: genotype quality encoded as a phred quality
- etc.....

Homozygous SNP

#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT

1 801943 rs7516866 **C** **T** 9787.34 PASS

AC=2;AF=1.00;AN=2;BaseQRankSum=1.009;DB;DP=556;DS;Dels=0.00;FS=18.302;HRun=1;HaplotypeScore=4.6410;MQ=44.04;MQ0=38;MQRankSum=5.122;QD=17.60;ReadPosRankSum=3.375

GT:AD:DP:GQ:PL **1/1**:37,518:556:99:9787,685,0

Heterozygous SNP

#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT

1 1918488 rs4350140 **A** **G** 233.10 PASS

AC=1;AF=0.50;AN=2;BaseQRankSum=1.349;DB;DP=33;DS;Dels=0.00;FS=0.000;HRun=0;HaplotypeScore=0.0000;MQ=68.18;MQ0=1;MQRankSum=0.436;QD=7.06;ReadPosRankSum=1.547

GT:AD:DP:GQ:PL **0/1**:21,12:33:99:263,0,620

Homozygous deletion

#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT

1 1289367 rs35062587 **CTG** **C** 3139.27 PASS

AC=2;AF=1.00;AN=2;DB;DP=66;DS;FS=0.000;HRun=0;HaplotypeScore=223.1329;MQ=68.34;MQ0=1;QD=47.56

GT:AD:DP:GQ:PL **1/1**:0,66:65:99:3181,196,0

Heterozygous insertion

#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT

1 17948305 . **G** **GGGCCACAGCAG** 3581.32 PASS

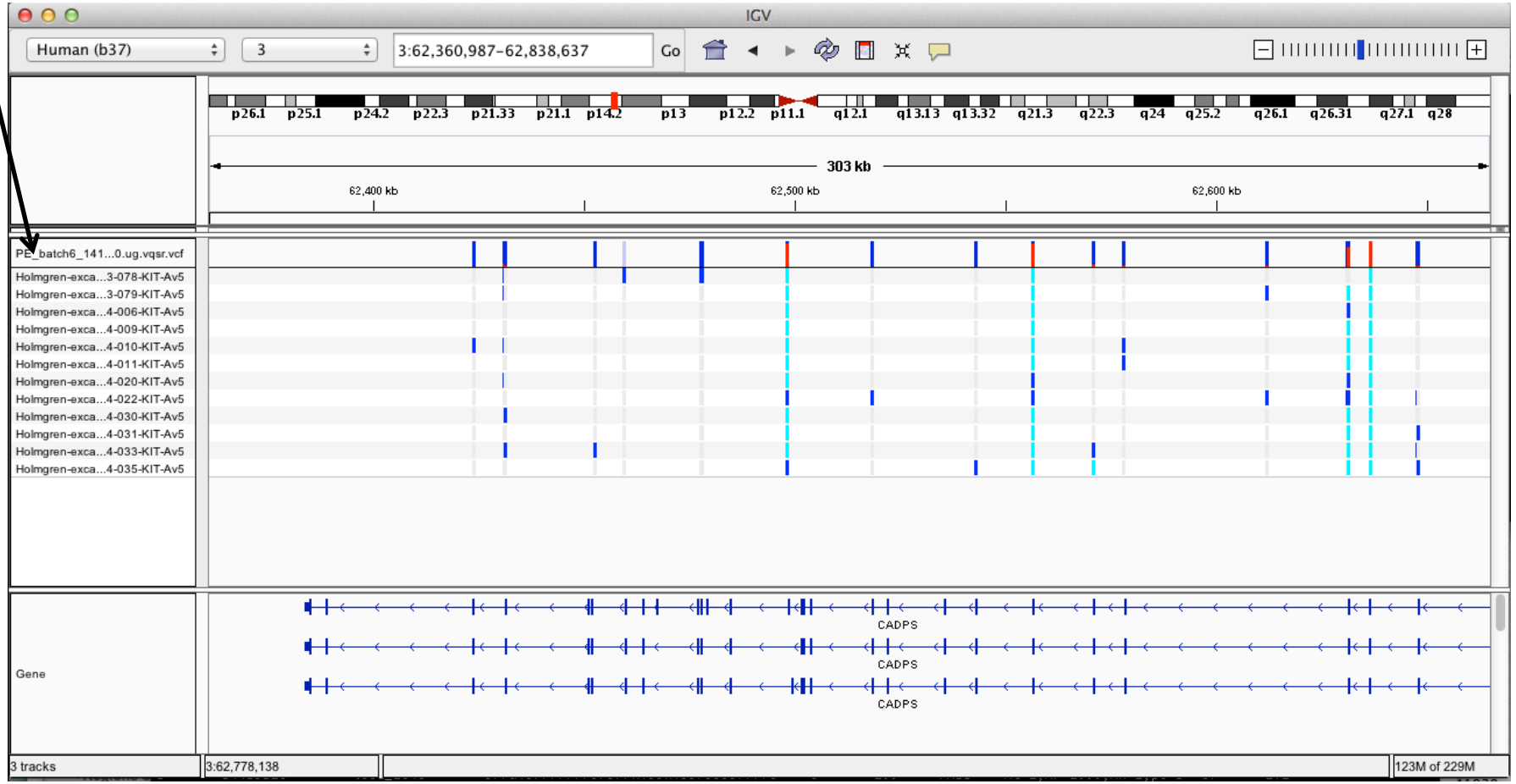
AC=1;AF=0.50;AN=2;BaseQRankSum=-2.638;DP=54;DS;FS=0.000;HRun=0;HaplotypeScore=552.8152;MQ=70.65;MQ0=2;MQRankSum=3.258;QD=66.32;ReadPosRankSum=0.320

GT:AD:DP:GQ:PL **0/1**:44,10:52:99:3581,0,3730

Multi-sample VCF file

Variant sites

Genotypes



Variant sites

Blue: proportion of ref alleles

Red: proportion of variant alleles

Genotypes (**coloured by genotype**)

Grey: reference

Dark blue: heterozygous variant

Cyan: homozygous variant

Multi-sample VCF file - **Close-up**

Variant sites

Blue: proportion of ref alleles

Red: proportion of variant alleles

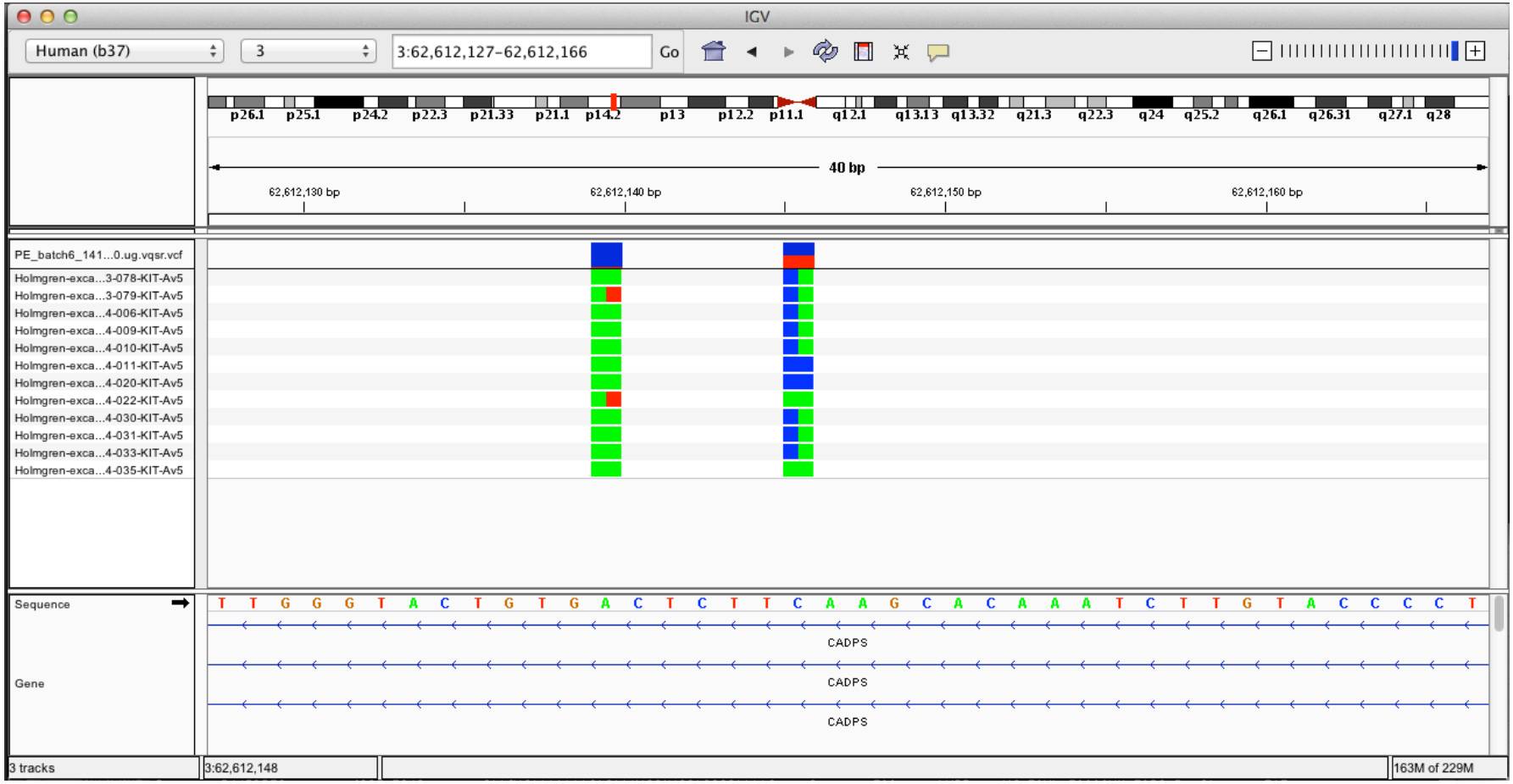
Genotypes (**coloured by genotype**)

Grey: reference

Dark blue: heterozygous variant

Cyan: homozygous variant

Multi-sample VCF file - **Genotypes coloured by allele**



Variant sites

Blue: proportion of ref alleles

Red: proportion of variant alleles

VARIANT FILTERING

The rationale for filtering

- QUAL is a basic measure of variant quality but it assumes that reads are correctly aligned and there are no systematic base call errors

- What causes errors in variant calling?
  - sequencing errors >> should be accounted for by base quality + recalibration + marking of duplicates
  - Incorrect alignment >> **Re-alignment step should have reduced this problem but not eliminated it**

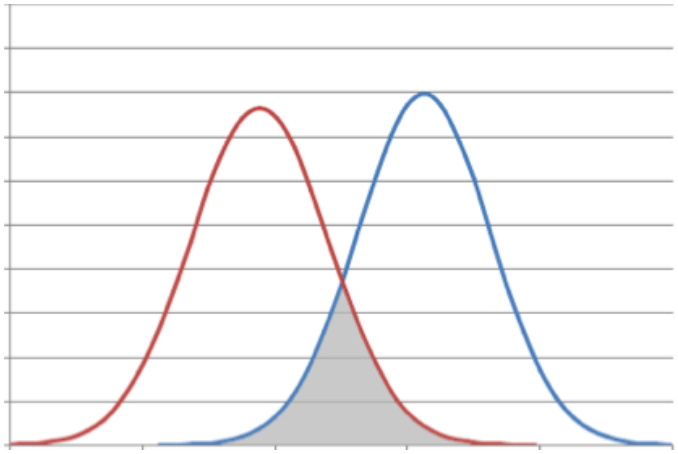
- **Thus although QUAL (which depends on Mapping Quality of reads and Base qualities) is a useful measure, there will still be FP with high QUAL.**
  - **and we wish to reduce the number of FP**

- Tell tale signs of suspicious variants
  - poorly mapped reads (ambiguity)
    - MQ: Root Mean Square of MAPQ of all reads at locus
    - MQ0: Number of MAPQ 0 reads at locus
  - biased support for the **REF** and **ALT** alleles
    - MQRankSum: Mapping quality rank sum test
    - ReadPosRankSum: Read position rank sum test
    - Strand bias and FS

INFO fields – important for filtering

- **QD:** variant quality score over depth of variant allele
  - Confidence in the site being variant should increase with increasing depth
- **MQ:** RMS MAPQ of all reads at locus
  - Regions of excessively low mapping quality are ambiguously mapped and variants called within are suspicious
- **MQ0:** number of MAPQ 0 reads at locus
- **MQRankSum:** Mapping quality rank sum test
  - If the alternate bases are more likely to be found on reads with lower MQ than reference bases then the site is likely mismapped
- **Haplotype score:** Probability that the reads in a window around the variant can be explained by at most two haplotypes
- **FS:** fisher exact test of read strand
  - If the reference-carrying reads are balanced between forward and reverse strands then the alternate-carrying reads should be as well
- **ReadPosRankSum:** Read position rank sum test
  - If the alternate bases are biased towards the beginning or end of the reads then the site is likely a mapping artifact

Details of MQRankSum



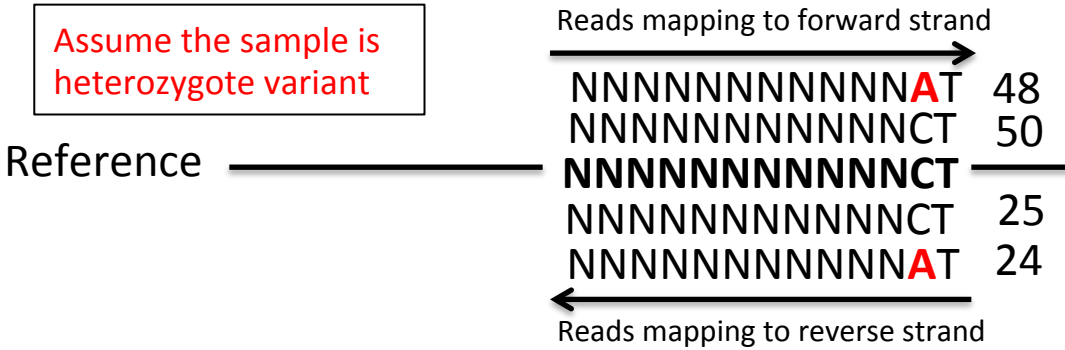
Rank sum test are used to test if two samples come from the same population when the distribution is unknown.

- We test the null hypothesis that the two samples of MQ are the same
- If we reject this null hypothesis, the variant site is likely to be a False Positive

Strand bias - optional

Strand bias **is NOT** about more reads mapping to one of the strands than the other

Assume the sample is heterozygote variant



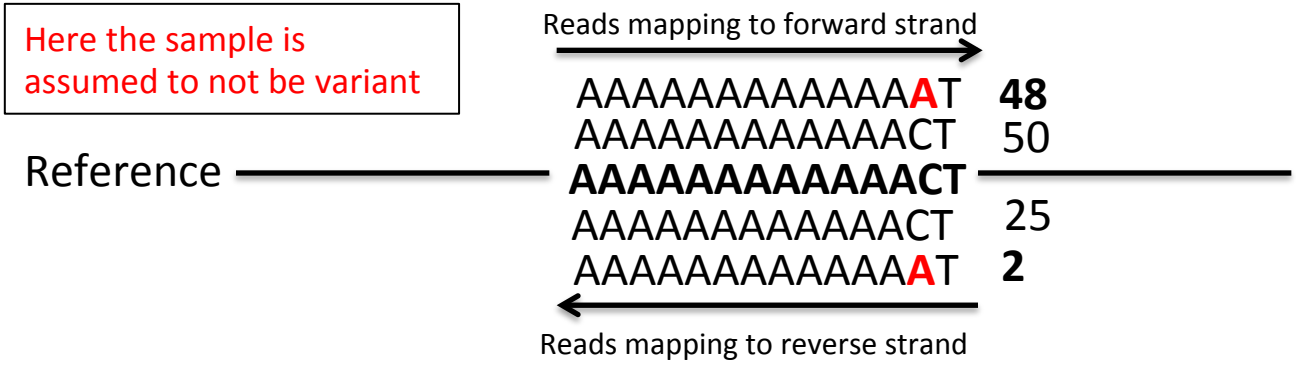
		Fw	Rev
Ref	C	50	25
Alt	A	48	24

Clearly more reads mapping to FW than Rev

But, Fw/Rev ratio is same for Ref allele and Alt: 50/25 = 48/24

This **IS** strand bias

Here the sample is assumed to not be variant



		Fw	Rev
Ref	C	50	25
Alt	A	48	2

50/25 != 48/2

Hard vs. soft filtering

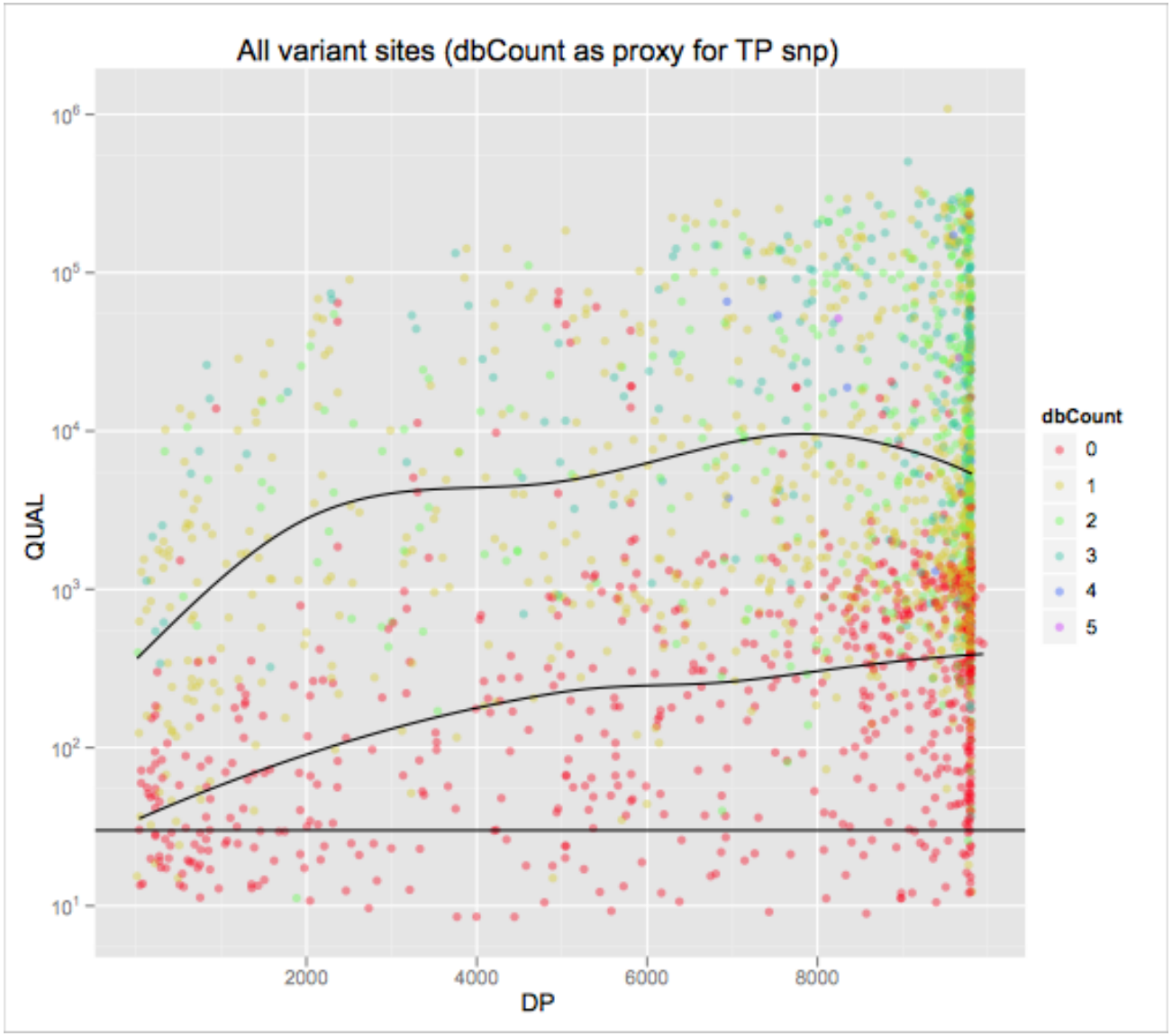
- Can set thresholds for these INFO fields and request that all thresholds are passed for a variant to be considered valid

- Which fields to you use and where do you set the thresholds?
  - use datasets of known SNPs and compare their INFO fields to those likely FP variants
  - fields that provide a good separation can be used as filters

- Disadvantage of **hard filtering**
  - works with hard cut-offs

- Variant Quality Score Recalibration (GATK) or **soft filtering**
  - **use machine learning to learn the features of true variants and distinguish them from false positives**

QUAL provides OK but not great separation



Red: likely false positive SNP

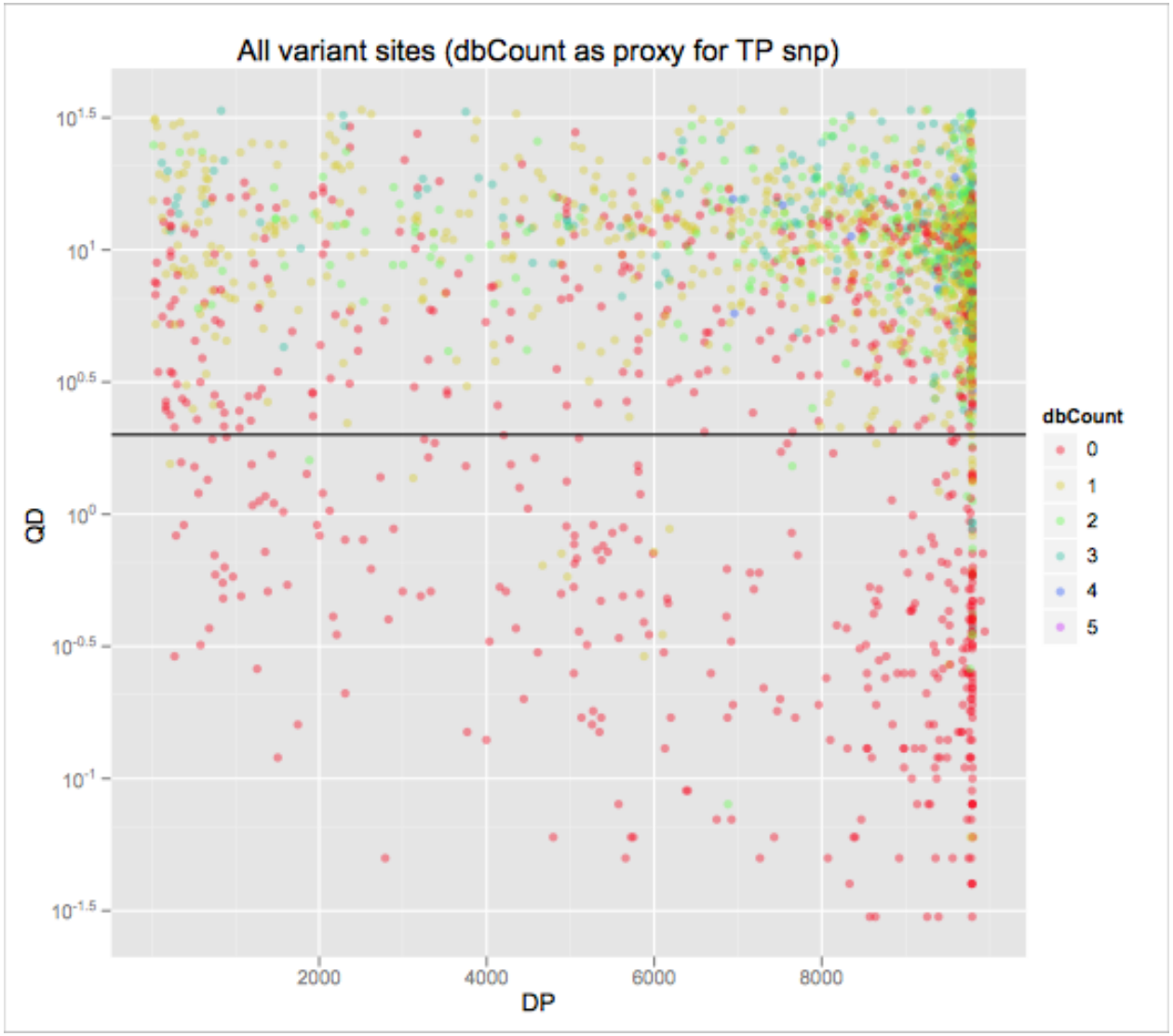
Other colour: likely true positive SNP

Note how:

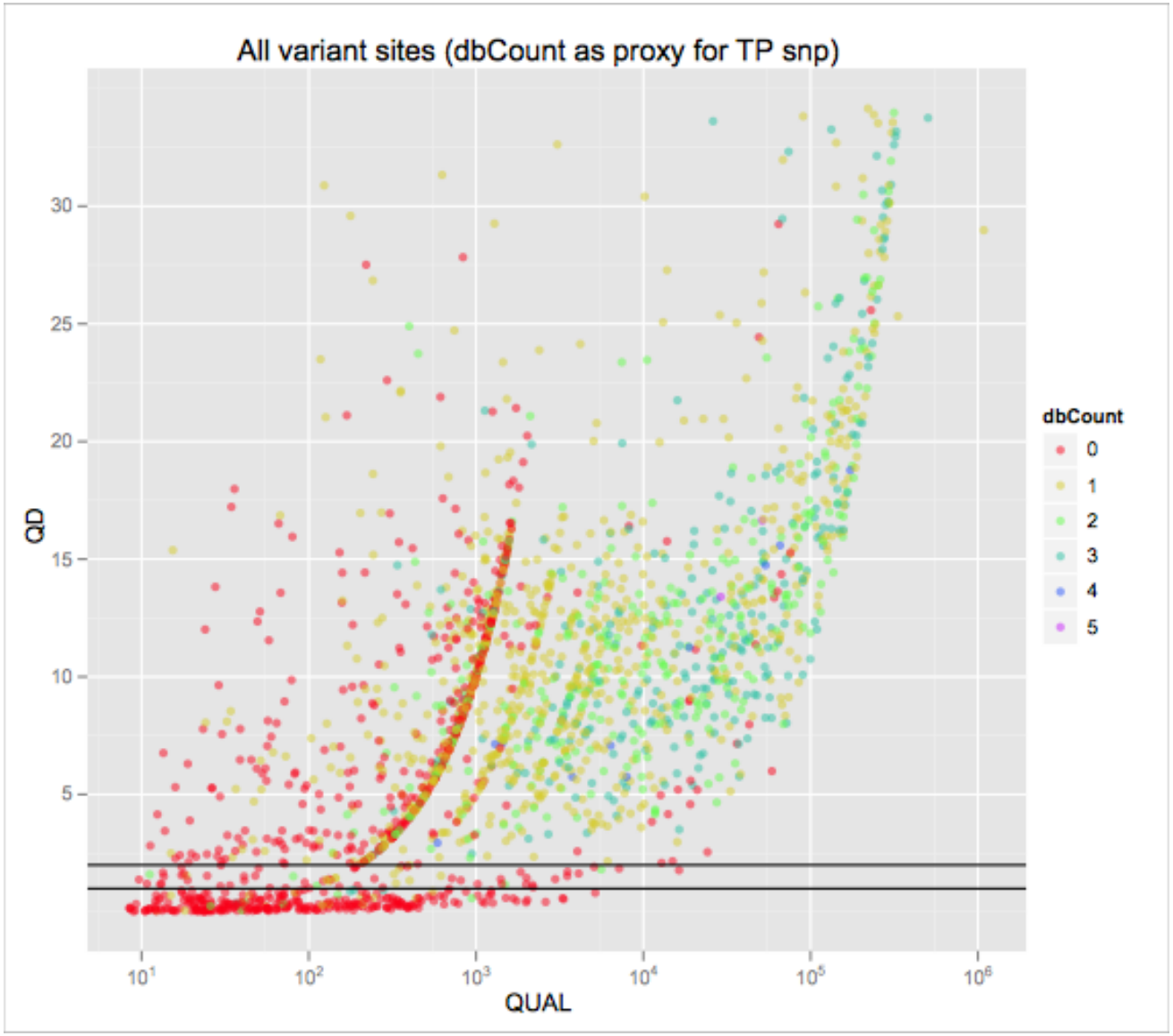
- QUAL increases with depth
- QUAL for known SNPs (green) is higher than for unknown SNPs (red) at any given depth

Note: in these plots an exremely high depth of sequencing was used

QD vs depth: much better separation



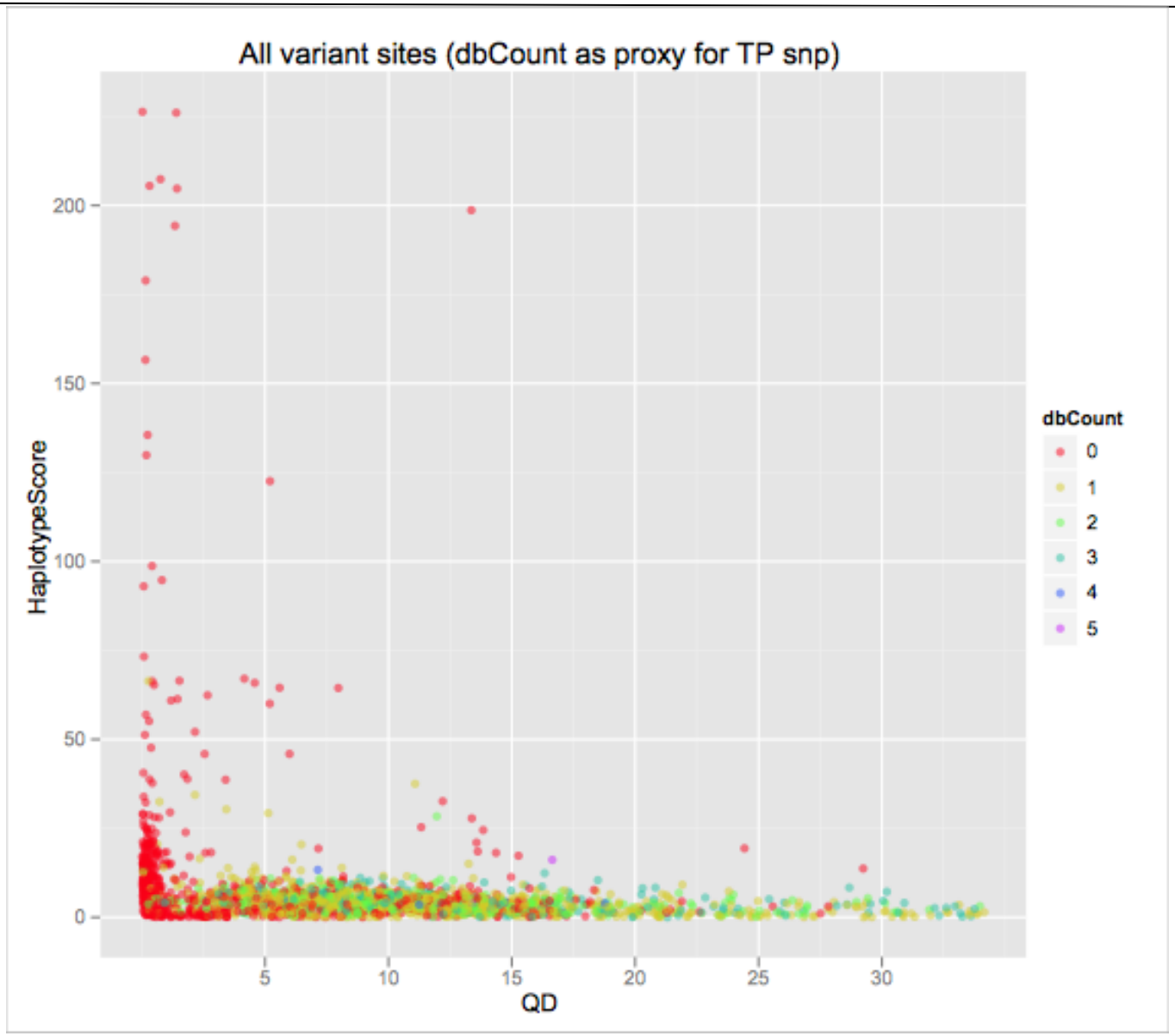
Qual vs QD



Illustrates advantages of soft filtering:

- learning features of true SNPs
- no fixed level cutoffs

Haplotype Score vs QD: advantage of combining



No known SNPs even with QD >3 have high HaplotypeScore, so can remove more likely FP SNPs by filtering on HaplotypeScore

Can require SNPs to have HaplotypeScore less than 20.

practical 03_advancedPipeline.bash

- Now we can try out all the refinements we have discussed

- Please make an effort to progress execute **rapidly** and **without error**
  - **pause to understand new commands (realignment, filtering)**

practical 031_generatingReports.bash

- Let us see if all those refinements made any difference.

- Talk the students through the improvements in IGV

FUNCTIONAL ANNOTATION

Warning

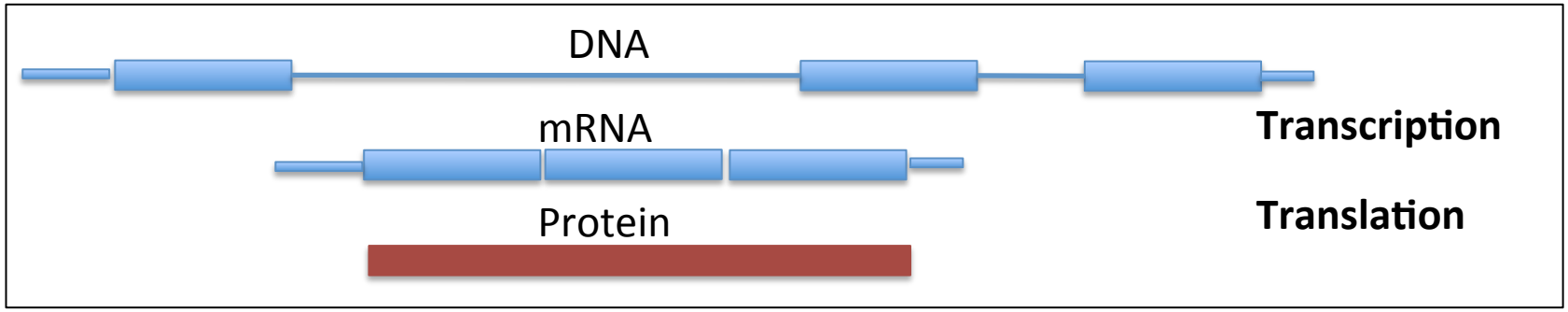
- You will be **rapidly** introduced for many functional annotations in this section

- **Unless you have a good prior background in molecular biology, you will struggle to understand all of these annotations**

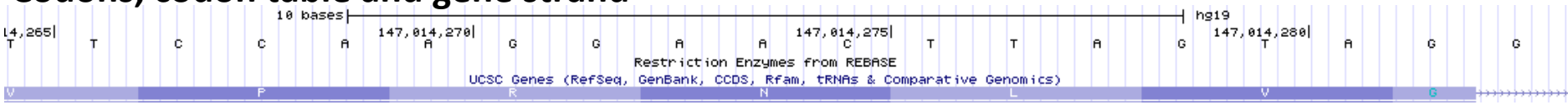
- Keep in mind that the main goal is to use these annotations to distinguish variants with a big phenotypic impact from those with no effect or a very mild effect
  - in the case of monogenic disease, a single variant can be responsible for the entire clinical phenotype.

What is a gene?

Central Dogma

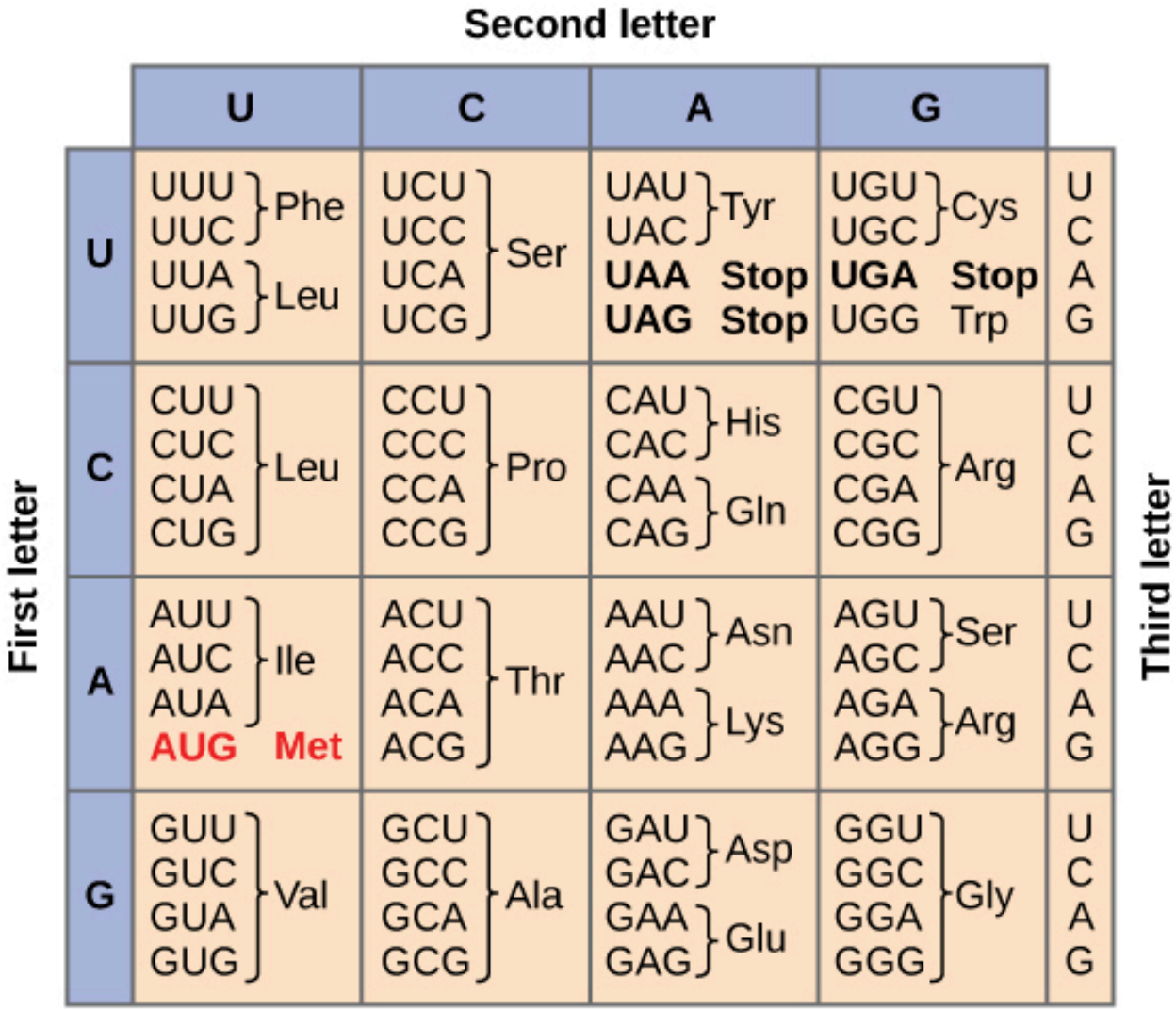


Codons, codon table and gene strand

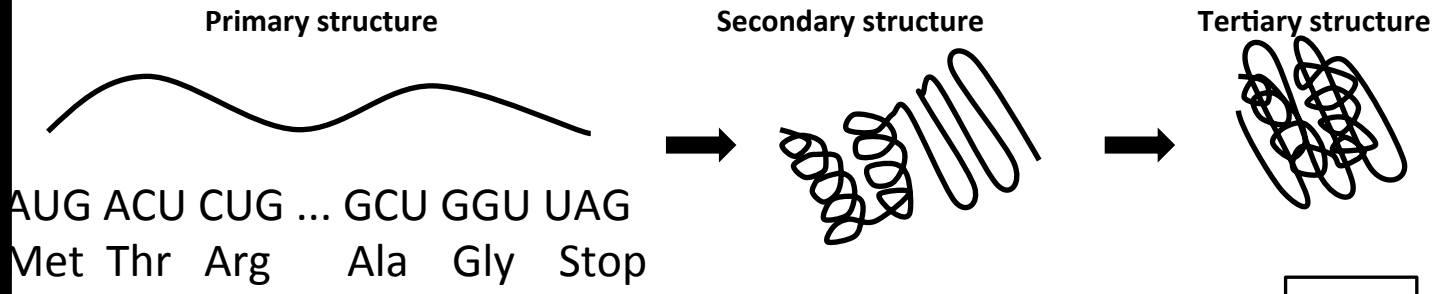


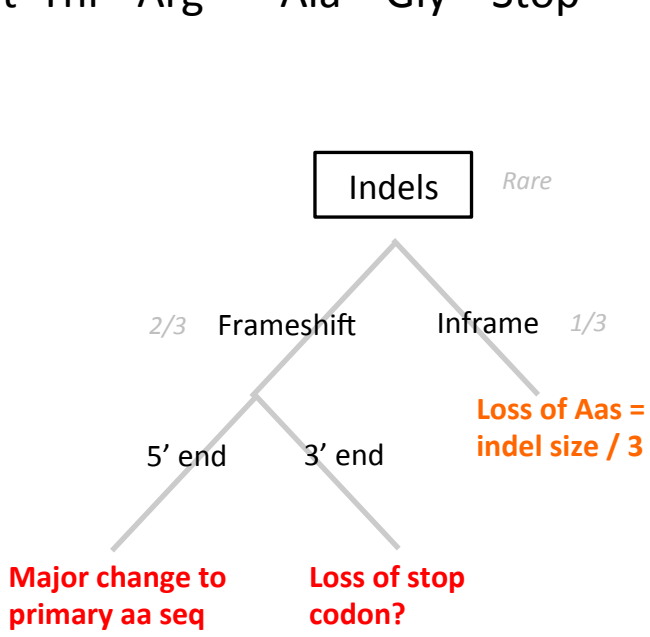
- There are large numbers of genes in multicellular organisms
- Not all organism have multiple exons per gene and thus do not need splicing
- Outside genes are regulatory elements that control when and where genes are expressed

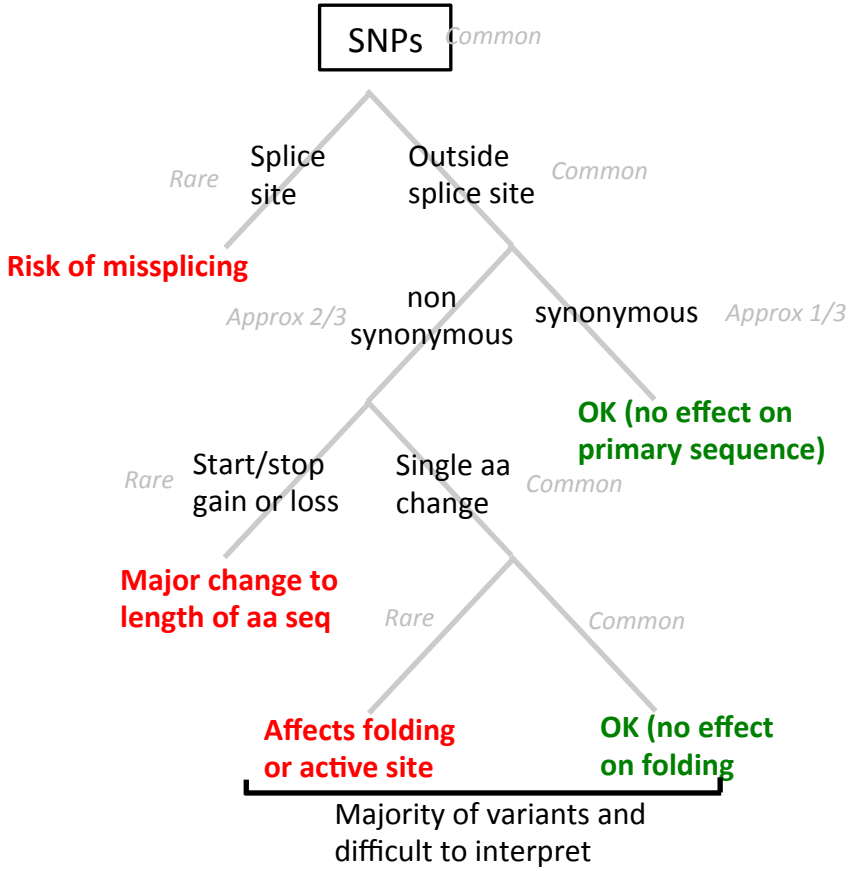
Synonymous and non-synonymous mutations



Effects of variants







http://genetics.bwh.harvard.edu/pph2/dokuwiki/_media/nmeth0410-248.pdf

Polyphen, Sift, etc

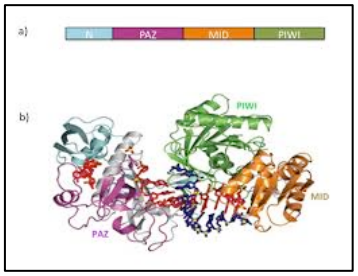
Why are there different isoforms?

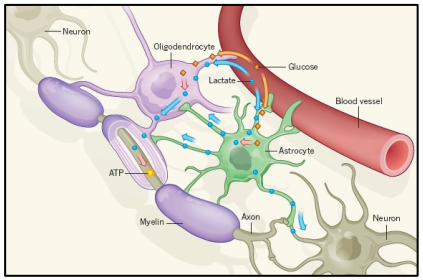
Approx. 25,000 genes in the human genome

DNA >transcription> **RNA** >translation> **PROTEIN**

BUT 1 gene ≠ 1 protein

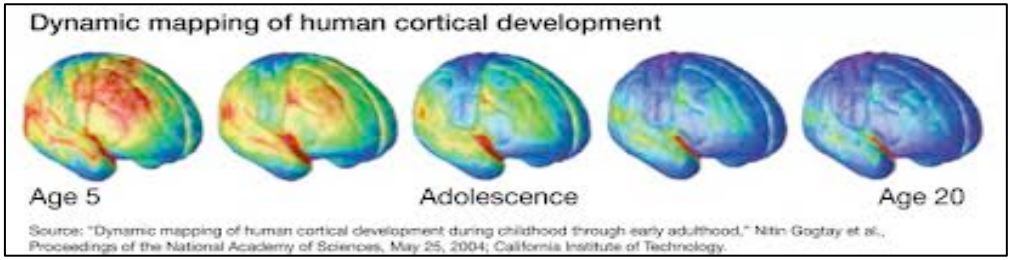
Approx. 25,000 genes in the human genome





Gene A

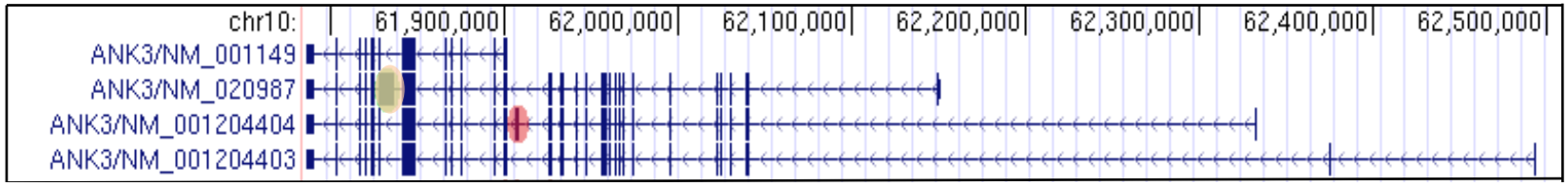
- Transcript A1 → Protein A1
- Transcript A2 → Protein A2
- Transcript A3 → Protein A3
- Transcript A4 → Protein A4



Some transcripts expressed ubiquitously, some highly specific (cell type AND timepoint)

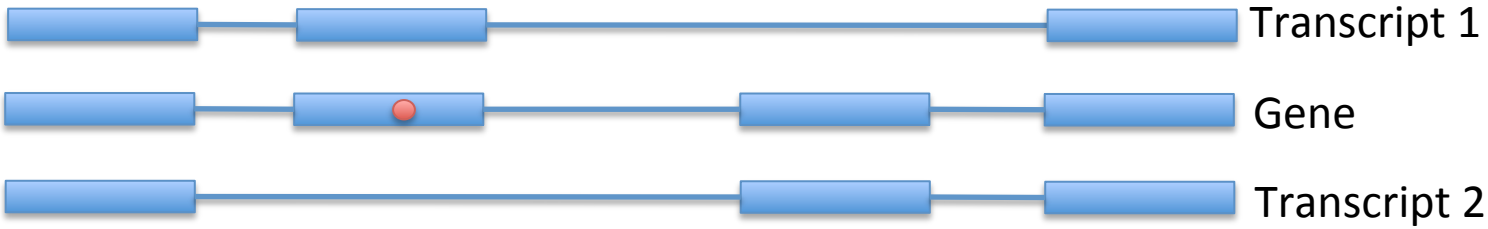
Other domains (spectrin binding)

Ankyrin repeats (membrane binding)



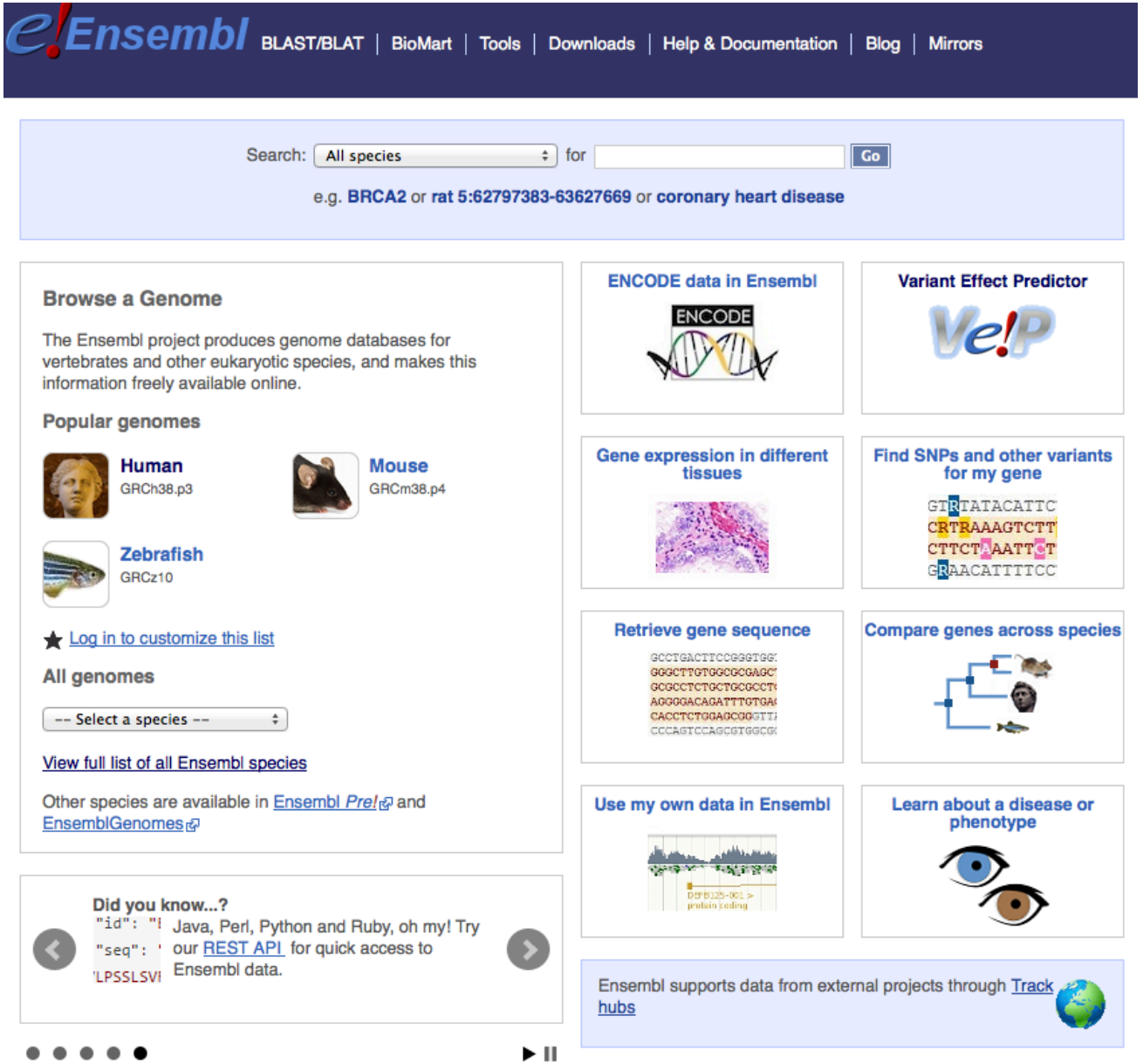
What is annotation?

- Adding information about the variants
- Two broad categories of annotations
  - annotation that **depend on gene models**
    - coding/non-coding
    - if coding: synonymous / non-synonymous
    - if non-synonymous >> what is the impact on protein structure (Polyphen, SIFT, etc)
  - annotations that **do not depend on gene models**
    - variant frequency in different database / different populations
    - degree of conservation across species
- Considerable complications caused by different gene models



- Two approaches to problem
  - decide **ex-ante** what which transcript to use for each gene
  - annotate with all transcript for a given gene and pick the **highest impact effect**

Ensembl – Basis for variant effect prediction



Annotation software

- Two sets of software
  - Annovar
    - provides a wide range of annotations that can be applied with one tool
    - we have experienced some inconsistencies in the results e.g. non-synonymous SNPs without polyphen score
  - SNPEff and dbNSFP (Non-Synoymous Functional Prediction)

- Both tested by GATK team
  - recommended snpEff, but with strict requirements
  - **snpEff version 2.0.5** (not 2.0.5d)
  - db should be **GRCh37.64** (which is the **Ensembl database version 64**)
  - should use the option **-onlyCoding true** (using false can cause erroneous annotation)

- GATKs VariantAnnotator to pick the highest impact.

- Finally, also annotate with **dbNSFP, which contains:**
  - variant frequencies
  - conservation scores
  - protein function effect (Polyphen, SIFT)

snpEff annotation get placed into INFO field

31942920 . G T 683.93 PASS

AC=1;AF=0.50;AN=2;BaseQRankSum=4.358;DP=73;DS;Dels=0.00;FS=0.000;HRun=0;
HaplotypeScore=1.7876;MQ=69.76;MQ0=0;MQRankSum=0.977;QD=9.37;ReadPosR
ankSum=0.508; VQSLOD=1.6292;culprit=QD

SNPEFF_AMINO_ACID_CHANGE=E114*;
SNPEFF_CODON_CHANGE=Gag/Tag;
SNPEFF_EFFECT=STOP_GAINED;
SNPEFF_EXON_ID=exon_22_31942847_31942957;
SNPEFF_FUNCTIONAL_CLASS=NONSENSE;
SNPEFF_GENE_BIOTYPE=processed_transcript;
SNPEFF_GENE_NAME=SFI1;
SNPEFF_IMPACT=HIGH;
SNPEFF_TRANSCRIPT_ID=ENST00000421060;

GT:AD:DP:GQ:PL 0/1:42,31:73:99:714,0,981

Explanation of snpEff fields (bold are important)

Field name	Example	Description
SNPEFF_EFFECT	NON_SYNONYMOUS_CODING	The highest-impact effect resulting from the current variant (or one of the highest-impact effects, if there is a tie)
SNPEFF_IMPACT	MODERATE	Impact of the highest-impact effect resulting from the current variant (**HIGH, MODERATE, LOW, or MODIFIER**)
SNPEFF_FUNCTIONAL_CLASS	MISSENSE	Functional class of the highest-impact effect resulting from the current variant (NONE, SILENT, MISSENSE, or NONSENSE)
SNPEFF_CODON_CHANGE	Tgc/Agc	Old/New codon for the highest-impact effect resulting from the current variant
SNPEFF_AMINO_ACID_CHANGE	C12S	Old/New amino acid for the highest-impact effect resulting from the current variant
SNPEFF_GENE_NAME	SLC6A18	Gene name for the highest-impact effect resulting from the current variant
SNPEFF_GENE_BIOTYPE	protein_coding	Gene biotype for the highest-impact effect resulting from the current variant
SNPEFF_TRANSCRIPT_ID	ENST00000296821	Transcript ID for the highest-impact effect resulting from the current variant
SNPEFF_EXON_ID	exon_5_1225470_1225752	Exon ID for the highest-impact effect resulting from the current variant

Impact classification of all effects

High-Impact effects	Moderate-Impact effe	Low-Impact effects
SPLICE_SITE_ACCEPTOR	NON_SYNONYMOUS_CODING	SYNONYMOUS_START
SPLICE_SITE_DONOR	CODON_CHANGE (note: this ef	NON_SYNONYMOUS_START
START_LOST	CODON_INSERTION	START_GAINED
EXON_DELETED	CODON_CHANGE_PLUS_CODO	SYNONYMOUS_CODING
FRAME_SHIFT	CODON_DELETION	SYNONYMOUS_STOP
STOP_GAINED	CODON_CHANGE_PLUS_CODO	NON_SYNONYMOUS_STOP
STOP_LOST	UTR_5_DELETED	
	UTR_3_DELETED	

All vs. top impact

- SnpEff uses Ensembl gene models and annotates initially with the effect in **all** different transcripts.

- Multiple functional annotations for the same variant make interpretation difficult

- One can then use GATK to pull out the TOP impact ie the most damaging effect

A second source of functional annotation: dbNSFP

- NSFP = Non-synonymous functional prediction
- Limited to non-synonymous variants
- Has many data fields. We use only:
  - dbnsfpSIFT_score
  - dbnsfpPolyphen2_HVAR_pred
  - dbnsfp29way_logOdds
  - dbnsfp1000Gp1_AF

Example of annotation with dbNSFP

766910 rs1809933 C T 556.42 PASS

AC=1;AF=0.50;AN=2;BaseQRankSum=1.366;DB;DP=30;Dels=0.00;FS=0.000;HRun=0;HaplotypeScore=1.8675;MQ=47.46;MQ0=0;MQRankSum=-0.651;QD=18.55;ReadPosRankSum=-1.757;SB=-109.24;

SNPEFF_AMINO_ACID_CHANGE=R42Q;SNPEFF_CODON_CHANGE=cGg/cAg;SNPEFF_EFFECT=NON_SYNONYMOUS_CODING;SNPEFF_EXON_ID=exon_5_766813_767034;SNPEFF_FUNCTIONAL_CLASS=MISSENSE;SNPEFF_GENE_BIOTYPE=processed_transcript;SNPEFF_GENE_NAME=ZDHHC11B;SNPEFF_IMPACT=MODERATE;SNPEFF_TRANSCRIPT_ID=ENST00000382776;

dbnsfp29way_logOdds=3.0289; SiPhy score based on 29 mammals genomes. The **larger** the score, the **more** conserved the site.

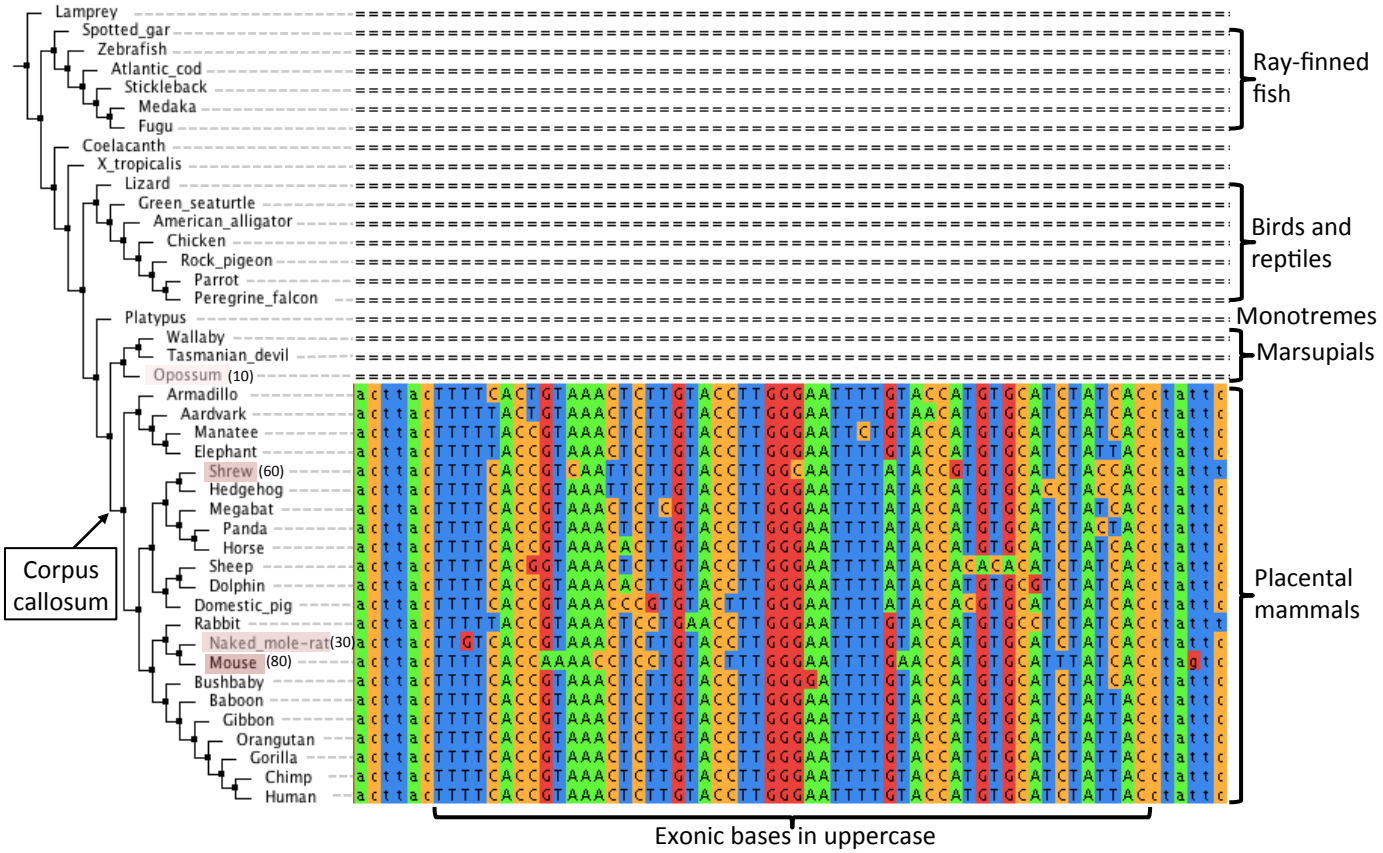
dbnsfp1000Gp1_AF=0.76; Alt. allele frequency in the whole 1000Gp1 data.

dbNSFP_Polyphen2_HVAR_pred=B; Polyphen2 prediction based on HumVar, "D" (”probably damaging"), "P" ("possibly damaging") and "B" ("benign"). Multiple entries separated by ";".

dbNSFP_SIFT_score=0.560000; SIFT score, If a score is smaller than 0.05 the corresponding NS is predicted as "D(amaging)"; otherwise it is predicted as "T(olerated)". SIFT predicts whether an amino acid substitution affects protein function.

GT:AD:DP:GQ:PL 0/1:5,25:30:98:586,0,98

Variation between species



Multiz DNA sequence alignment for the little exon from the UCSC genome browser (note: DNA from the forward strand, but ANK3 is encoded on the reverse strand). Species selected from 100 vertebrate alignments in order to have representative species throughout the phylogeny. Pink shading of four mammalian species with similarly sized cortices approximately proportional to cortical neuron densities (in millions of cells per gram in parenthesis) (Seelke 2014)

Some human variant databases

ExAC Browser (Beta) | Exome Aggregation Consortium

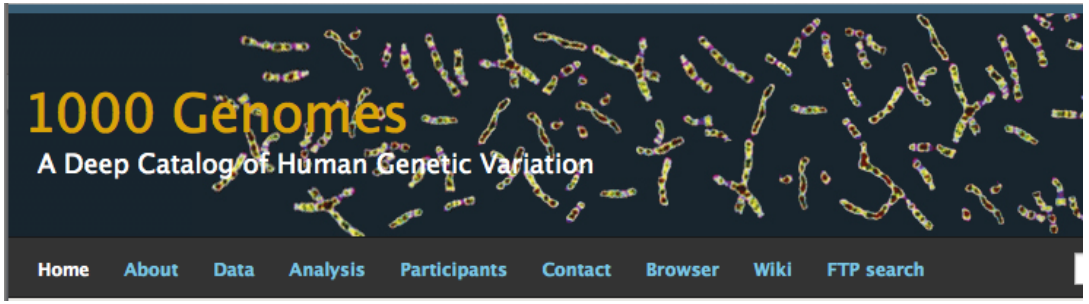
Search for a gene or variant or region

Examples - Gene: PCSK9, Transcript: ENST00000407236, Variant: 22-46615880-T-C, Multi-allelic variant: rs1800234, Region: 22:46615715-46615880

About ExAC

The Exome Aggregation Consortium (ExAC) is a coalition of investigators seeking to aggregate and harmonize exome sequencing data from a wide variety of large-scale sequencing projects, and to make summary data available for the wider scientific community.

The data set provided on this website spans 60,706 unrelated individuals sequenced as part of various disease-specific and population genetic studies. The ExAC Principal Investigators and groups that have contributed data to the current release are listed here.



What are the most useful fields

- The tradeoff
  - Use very strong filters and get very short lists but risk that the causal variant has been excluded
  - Use weaker filters to keep the causal variant “in” but risk getting very long lists

- A typical strategy is to start with very strong filters
  - See whether you can identify a good candidate
  - If not loosen some of the filters

- Strong filters are for example:
  - SNPEFF IMPACT: HIGH or HIGH and MEDIUM
  - Low frequency in 1000G or in Exac
  - Polyphen damaging

HAVING A GO ON YOUR OWN WITH THE REAL DATASET

A full pipeline from fastq to annotated VCF

- 040_functionalAnnotation.bash
- This is a recap of everything we have done in the course
- Again try to be precise.
- **Be quick** on the sections you fully understand
- Ask questions on the sections where you are still unsure.
- Do not copy and paste commands that you do not understand

Hunt for the causal mutant

- Maybe the most fun part of the course

- In **running out of time**: use filtering at the command line in **040_functionalAnnotation.bash**

- **If sufficient time**: work in spreadsheet following the instructions in: **041_findCausalVariantExercise.txt**

MULTIPLE SAMPLES

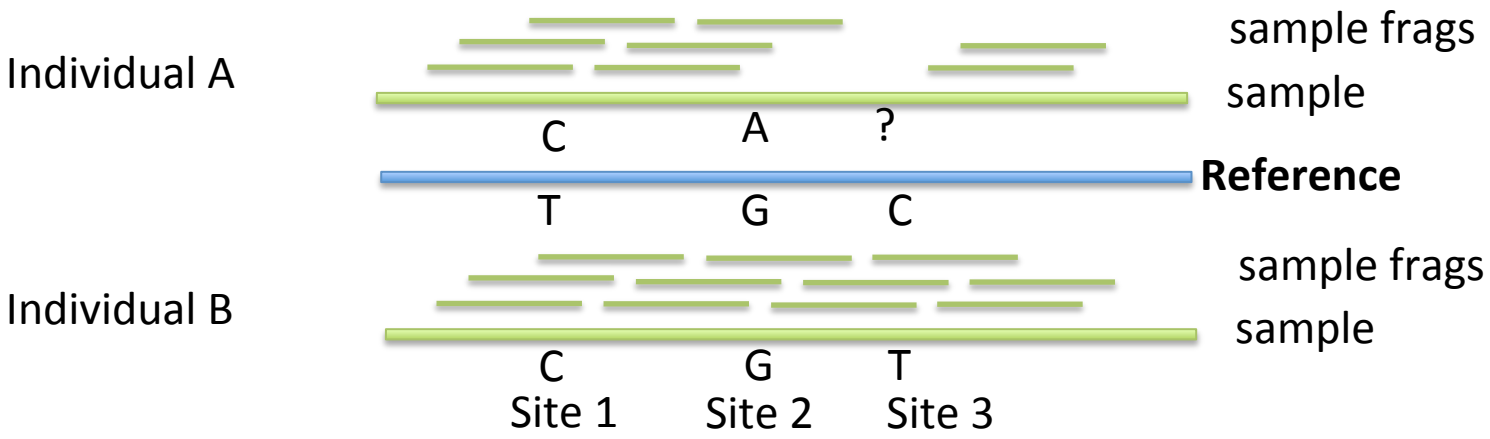
FROM HERE ON IT IS JUST USEFUL INFO

Batch calling / Joint calling

- Better specificity through filtering
  - more variant sites to train on in soft filtering
  - increased power of tests like FS
- Better sensitivity for regions of low coverage (Need to graphically illustrate)
- **Squaring up**
  - **genotyping all samples at all sites where at least one of the samples is variant (see next slide)**

The squaring off problem

Variant files only contain variant sites!



Merging singles

	Indiv. A	Indiv. B
Site 1	Variant	Variant
Site 2	Variant	
Site 3		Variant

Indiv. A	Indiv. B
Variant	Variant
Variant	-
-	Variant

Both sites lack information but we have information on individual B at site 2 (REF G)

Joint calling

Indiv. A	Indiv. B
Variant	Variant
Variant	Ref
No call	Variant

ALL individuals characterised at ALL sites where at least ONE individual is variant

Additional important applications and methods

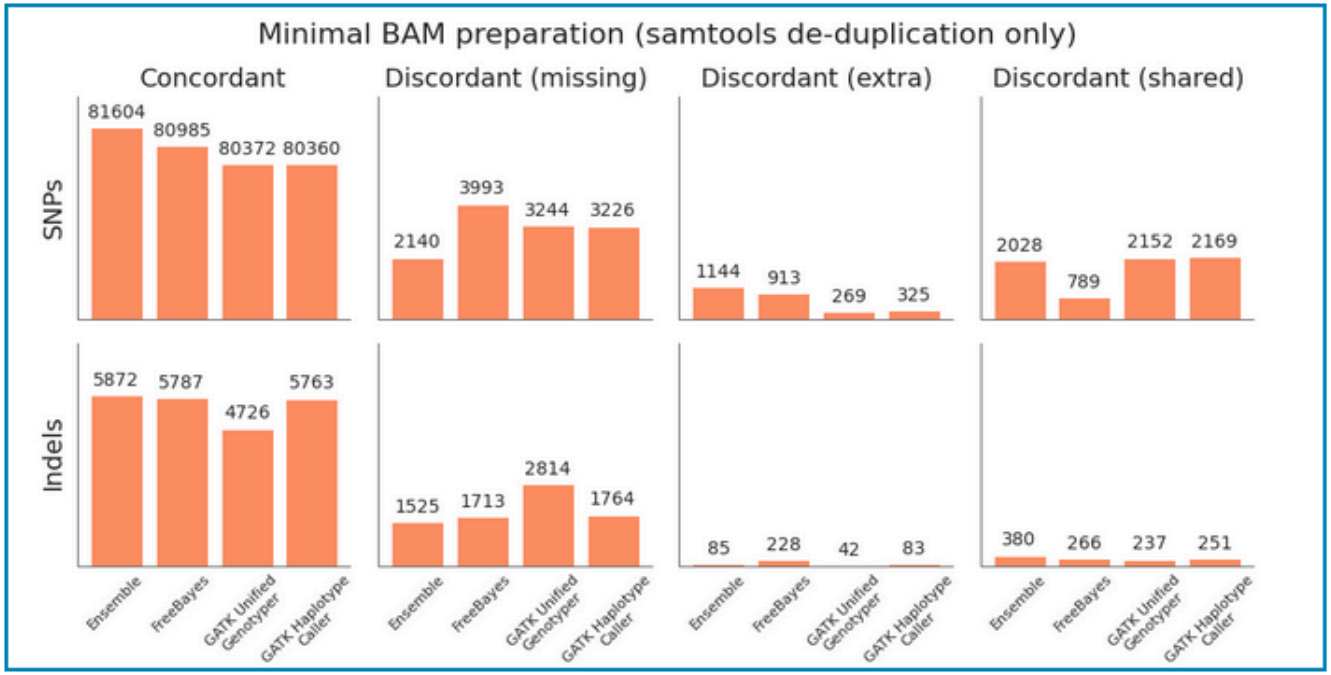
- Somatic variant calling (e.g. cancer cells)
- Pooled sequencing and allele count estimation
- Trios and denovo variants
- Bioplanet website for direct comparison of different methods (different read technologies, different mappers, different SAM processing, different Callers, different variant filtering)
- Pipelines like nextgen bcbio
- Structural variation programs like XHMM and BreakDancer

RECENT ADVANCES

HaplotypeCaller and FreeBayes

- HaplotypeCaller (in GATK) is an alternative to UnifiedGenotyper which is better at detecting insertions and deletions
  - performs local assembly
- Possibility of dropping the realignment and the recalibration
- FreeBayes is another similar variant caller that does well without realignment and base quality score recalibration.

From bcbio.wordpress.com (Brad Chapman)



CONCLUDING REMARKS

Reading list

Nielsen et al., Genotype and SNP calling from next-generation sequencing data. Nature Reviews Genetics 2011

Ng et al., Exome sequencing identifies the cause of a mendelian disorder. Nature Genetics 2010

Summary in plenum of the whole process and all the key concepts

Good luck with the other mountains!!