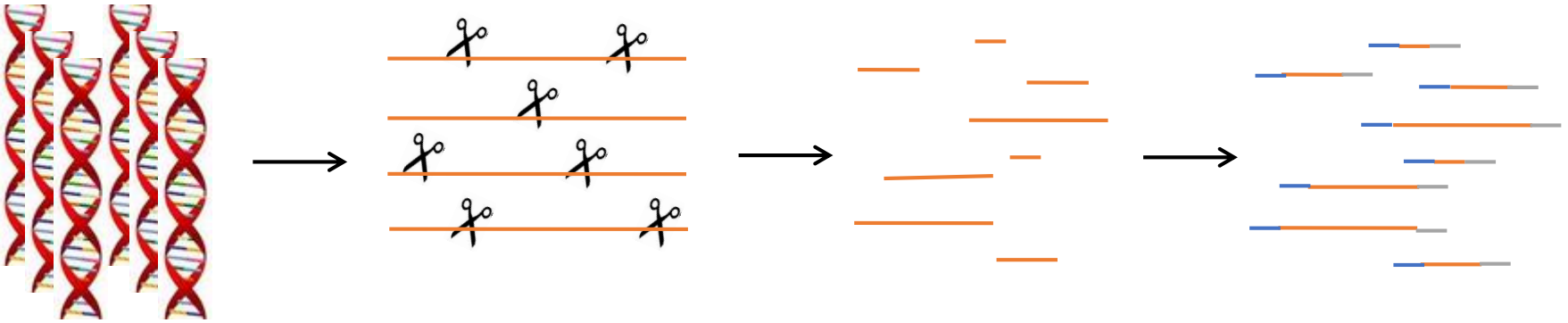
NGS data formats and variant calling

2019 Dragon Star Bioinformatics Course (Day 1)

Sample Preparation



Random shearing of the DNA

Adding adaptors and barcodes

Size selection

Amplification

Sequencing

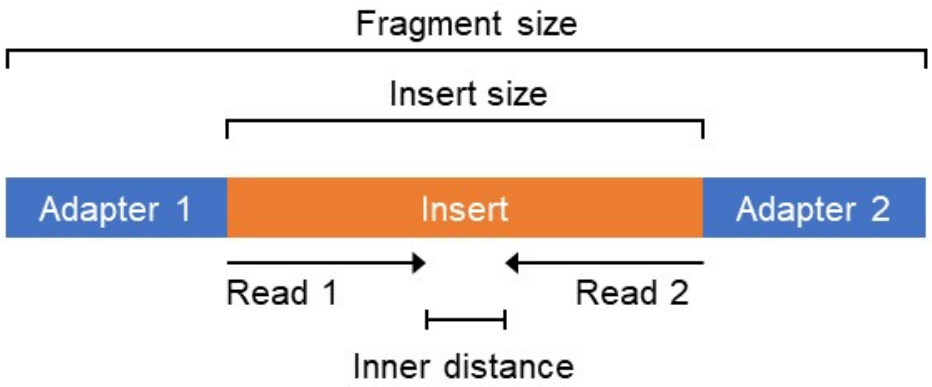
Basic Concepts in NGS

Insert – the DNA portion that is used for sequencing

Read – the part of the insert that is sequenced

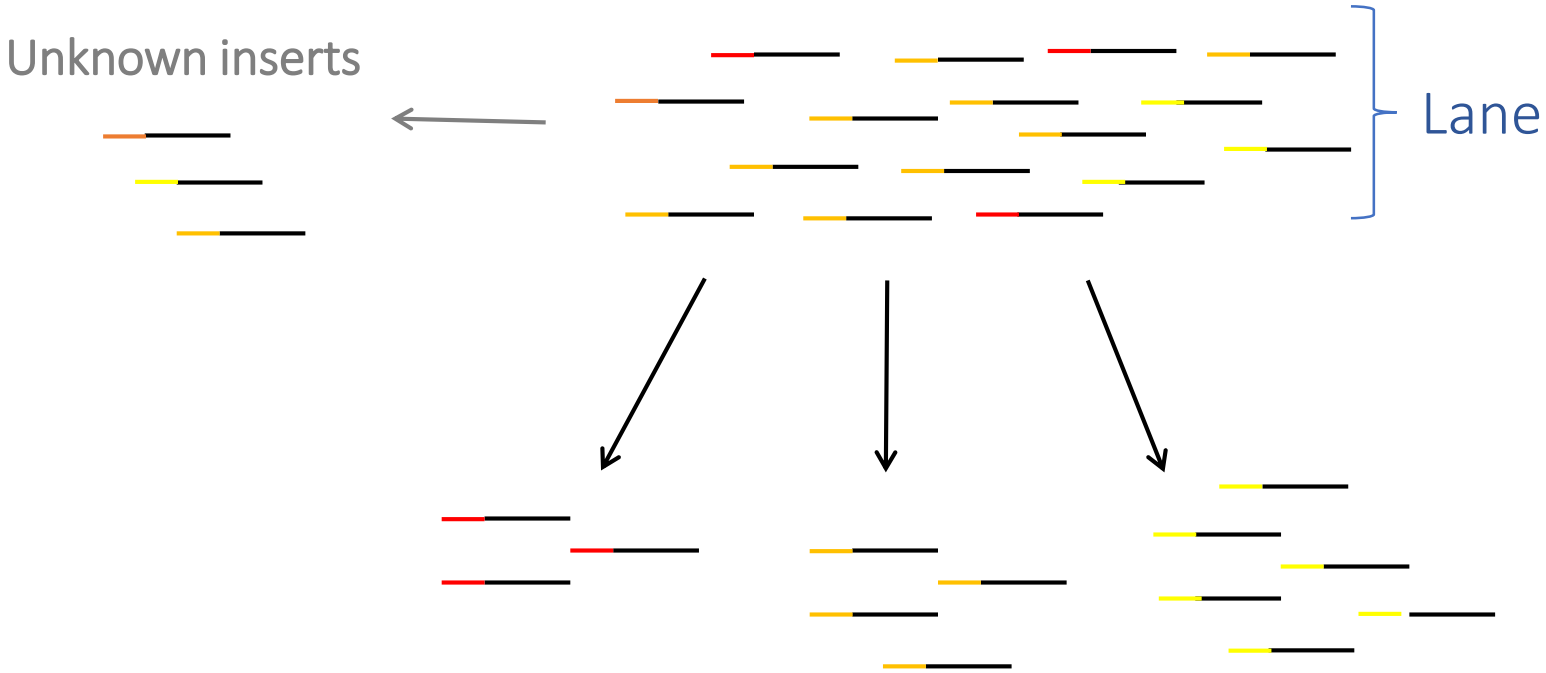
Single End – a sequencing procedure by which the insert is sequenced from one end only

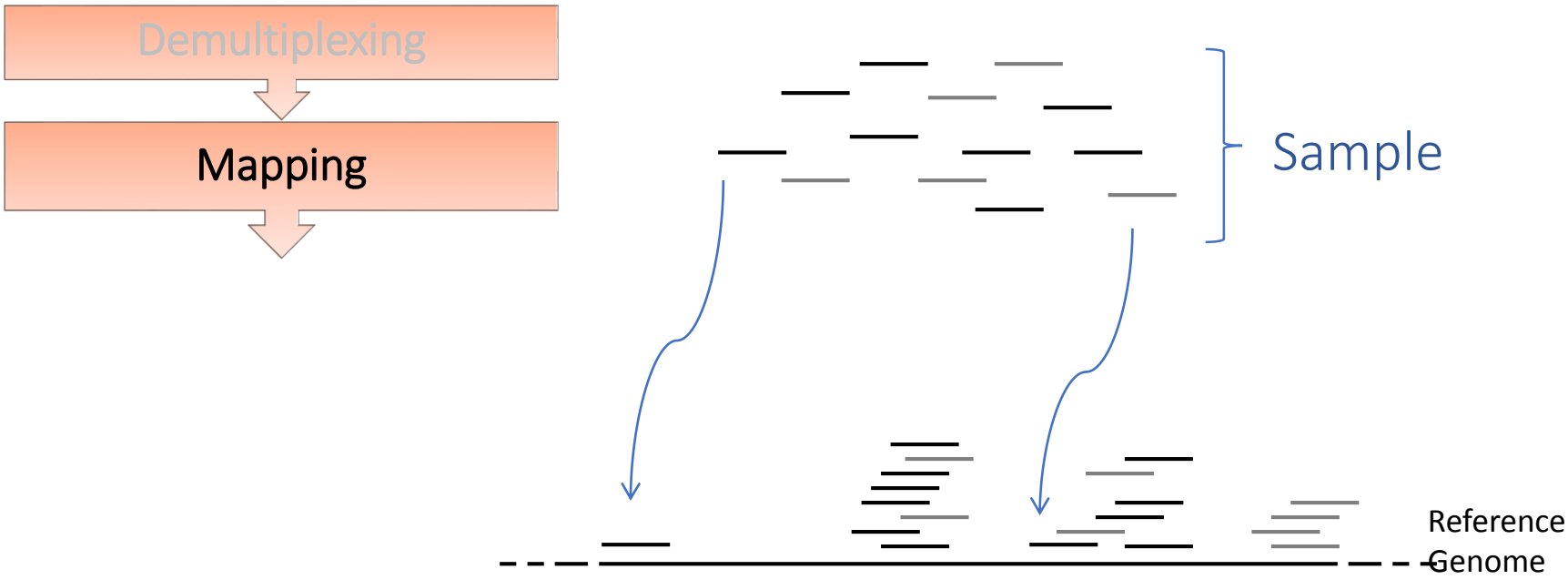
Paired End – a sequencing procedure by which the insert is sequenced from both ends



https://www.badgrammargoodsyntax.com/

Demultiplexing





Example of mapping parameters:

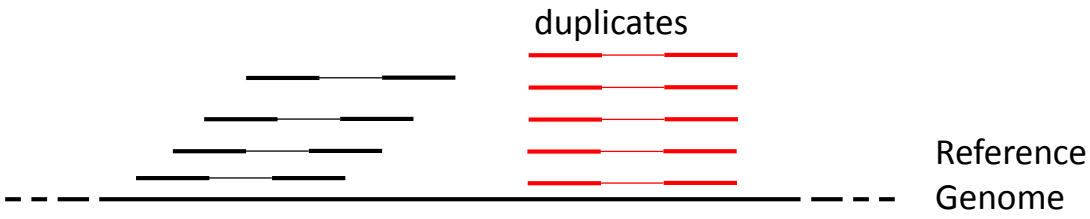
- Number of mismatches per read
- Scores for mismatch or gaps

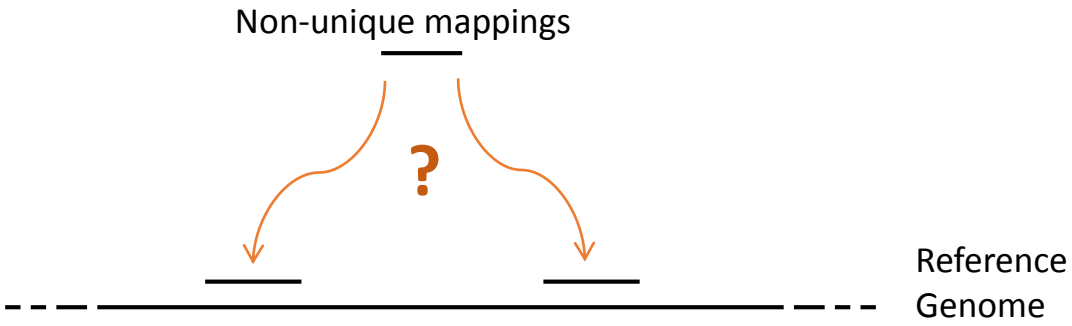
Mapping parameters affect the rest of the analysis

Demultiplexing

Mapping

Removing duplicates and non-unique mappings





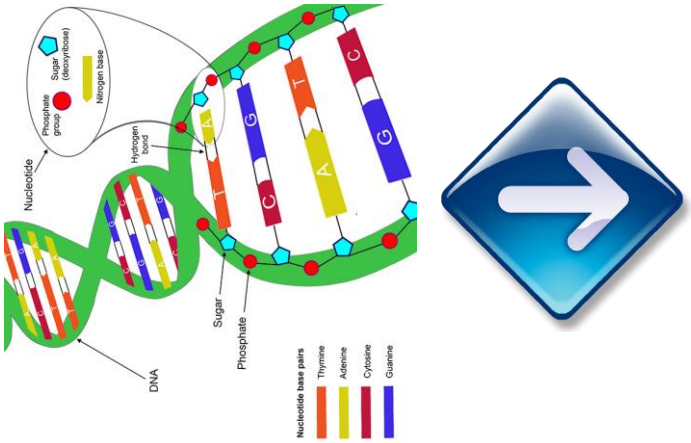
NGS – high-throughput, but

- Shorter reads
  - Sanger sequencing: up to ~1Kb
  - NGS technologies: typically 30-300bp
  - Implication: a lot of computational tasks e.g, assembly, read alignment, haplotyping, detection of SNPs, CNVs, indels etc.
  - Higher per-base sequencing error rate
    - Sanger sequencing: < 0.001%
    - NGS: 0.5-1%
    - Implication: Need redundant sequencing of each base to distinguish sequencing errors from true polymorphisms

Now what does NGS data look like?

- What do you want them to look like?

Fantasy Land



DNA

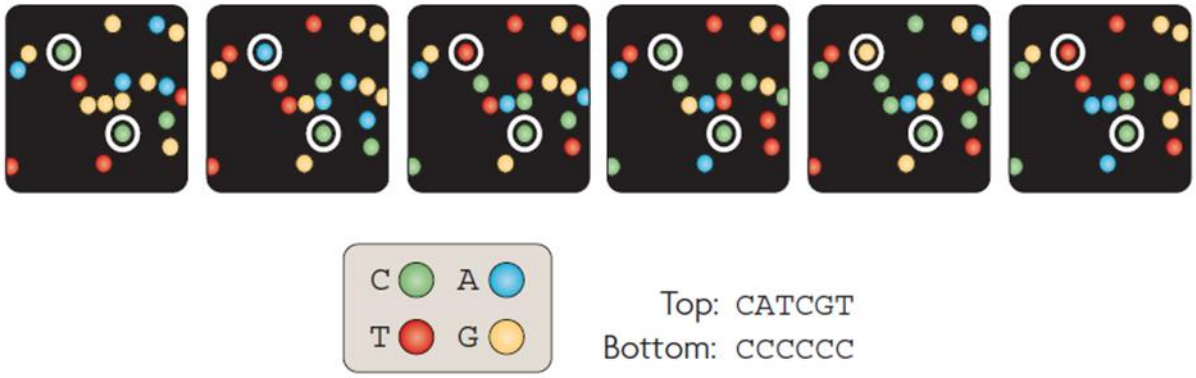
```
Chr1 haplo1: agttataagat...
Chr1 haplo2: agttattagat...
Chr2 haplo1: cctagctggat...
Chr2 haplo2: ccaagctcgat...
Chr3 haplo1: agctctgagcg...
Chr3 haplo2: agctctgagcg...
Chr4 haplo1: atcgttcgatc...
Chr4 haplo2: atcgatcgaac...
          etc...
```

3 billion bases from the beginning of chromosome 1 to the end of the last sex chromosome (2x) in haplotypes

https://mammothmemory.net/biology/dna-genetics-and-inheritance/dna-base-pairing/dna-double-helix.html

The Rawest of Raw Data

- Typically: images



- The first step is to call nucleotides at each base of each read: **base calling**, which is NOT 100% accurate
  - Typically base calling is done by the sequencer itself, and we start analysis after base calling (for example, fastq format file)

FASTQ: The raw sequence data format

What the data *really* look like: FASTQ

```
@IL27_748:3:286:254:231/1
GTGGAATAATGACCATGACGAAGAGGATGACAGTCC
+
BBBDCDED4DEAECEFEF2DC/>>@&*/C6208'<*
@IL27_748:3:285:138:811/1
AAGTGGATTACTACCTACAGAGAGTCAGTAAGAGAG
+
BB3D2D<D>D7DE0+19242?=57?=4%'6%.2.'(
@IL27_748:3:142:204:780/1
AGAAAAAGAAAGAGAGAGACAGACAGACAGAGAAAG
+
26B3C8<DDD>AAA0FCF7DCA012A?(;2?AC(=/
@IL27_748:3:23:252:759/1
TTTTAGATGAAGTTATTTCCTTTACTACCGTAGGCC
+
BB0D;DED>;>CEC:2EFA@69CDC3?@'%=585=‘
…
```

Millions of short reads from unknown genetic locations

What the data *really* look like: FASTQ

```
@IL27_748:3:286:254:231/1
GTGGAATAATGACCATGACGAAGAGGATGACAGTCC
+
BBBDCDED4DEAECEFEF2DC/>>@&*/C6208'<*
```

Read 1

```
@IL27_748:3:285:138:811/1
AAGTGGATTACTACCTACAGAGAGTCAGTAAGAGAG
+
BB3D2D<D>D7DE0+19242?=57?=4%'6%.2.'(
```

Read 2

```
@IL27_748:3:142:204:780/1
AGAAAAAGAAAGAGAGAGACAGACAGACAGAGAAAG
+
26B3C8<DDD>AAA0FCF7DCA012A?(;2?AC(=/
```

Read 3

```
@IL27_748:3:23:252:759/1
TTTTAGATGAAGTTATTTCCTTTACTACCGTAGGCC
+
BB0D;DED>;>CEC:2EFA@69CDC3?@'%=585=‘
```

Read 4

…

Millions of short reads from unknown genetic locations

What the data *really* look like: FASTQ

Millions of short reads from unknown genetic locations

Base Qualities

Short Read Sequence

GCTAGCTGATAGCTAGCTAGCTGATGAGCCCGA

Short Read Base Qualities

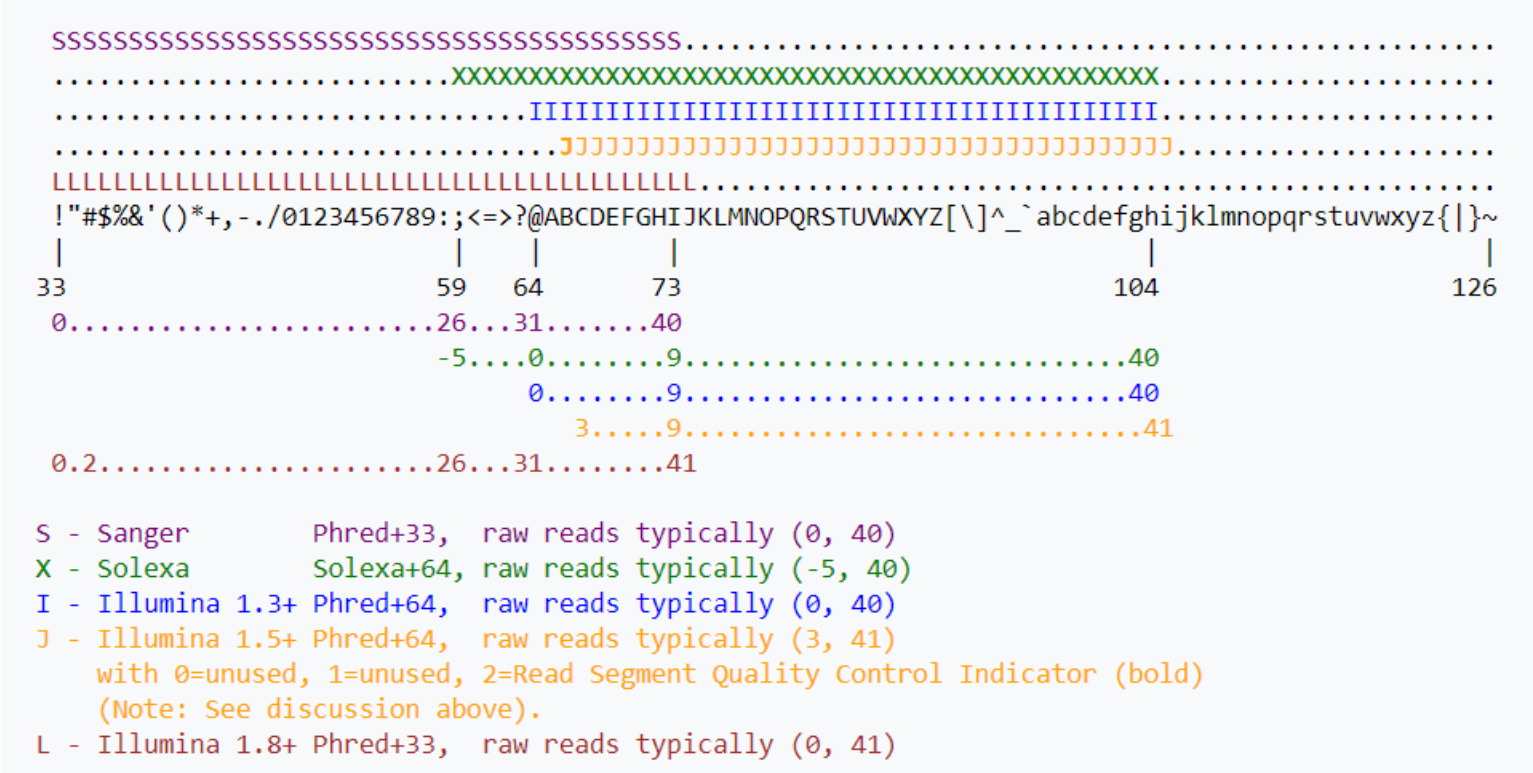
30.30.28.28.29.27.30.29.28.25.24.26.27.24.24.23.20.21.22.10.25.25.20.20.18.17.16.15.14.14.13.12.10

- Each base is typically associated with a quality value
- Measured on a "Phred" scale, which was introduced by Phil Green for his Phred sequence analysis tool

$$BQ = -10log_{10}(\varepsilon) \ where\ \epsilon\ is\ the\ probability\ of\ an\ error$$

Base quality conversion

- Nowadays, we settled on using quality scores on the original Sanger format (Phred+33).



https://en.wikipedia.org/wiki/FASTQ_format

BED: Genomic region format

BED format

BED format Index ▷

BED format provides a flexible way to define the data lines that are displayed in an annotation track. BED lines have three required fields and nine additional optional fields. The number of fields per line must be consistent throughout any single set of data in an annotation track. The order of the optional fields is binding: lower-numbered fields must always be populated if higher-numbered fields are used.

If your data set is BED-like, but it is very large (over 50MB) and you would like to keep it on your own server, you should use the bigBed data format.

The first three required BED fields are:

1. **chrom** - The name of the chromosome (e.g. chr3, chrY, chr2_random) or scaffold (e.g. scaffold10671).
2. **chromStart** - The starting position of the feature in the chromosome or scaffold. The first base in a chromosome is numbered 0.
3. **chromEnd** - The ending position of the feature in the chromosome or scaffold. The *chromEnd* base is not included in the display of the feature. For example, the first 100 bases of a chromosome are defined as *chromStart=0, chromEnd=100*, and span the bases numbered 0-99.

The 9 additional optional BED fields are:

4. **name** - Defines the name of the BED line. This label is displayed to the left of the BED line in the Genome Browser window when the track is open to full display mode or directly to the left of the item in pack mode.
5. **score** - A score between 0 and 1000. If the track line *useScore* attribute is set to 1 for this annotation data set, the *score* value will determine the level of gray in which this feature is displayed (higher numbers = darker gray). This table shows the Genome Browser's translation of BED score values into shades of gray:

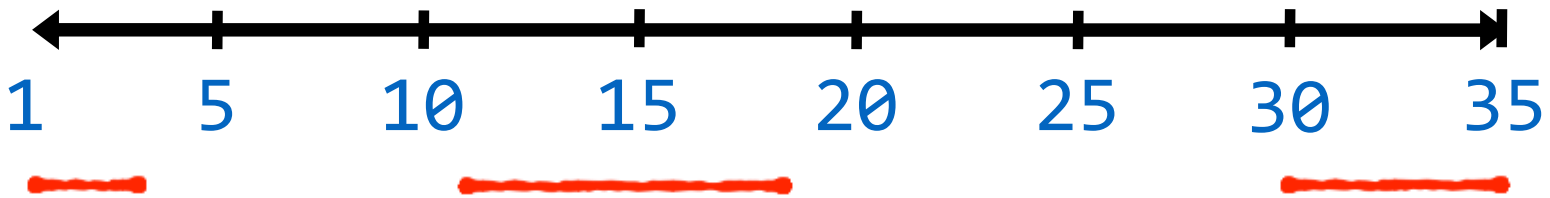
shade								
score in range	≤ 166	167-277	278-388	389-499	500-611	612-722	723-833	834-944	≥ 945

6. **strand** - Defines the strand - either '+' or '-'.
7. **thickStart** - The starting position at which the feature is drawn thickly (for example, the start codon in gene displays). When there is no thick part, thickStart and thickEnd are usually set to the chromStart position.
8. **thickEnd** - The ending position at which the feature is drawn thickly (for example, the stop codon in gene displays).
9. **itemRgb** - An RGB value of the form R,G,B (e.g. 255,0,0). If the track line *itemRgb* attribute is set to "On", this RBG value will determine the display color of the data contained in this BED line. NOTE: It is recommended that a simple color scheme (eight colors or less) be used with this attribute to avoid overwhelming the color resources of the Genome Browser and your Internet browser.
10. **blockCount** - The number of blocks (exons) in the BED line.
11. **blockSizes** - A comma-separated list of the block sizes. The number of items in this list should correspond to *blockCount*.
12. **blockStarts** - A comma-separated list of block starts. All of the *blockStart* positions should be calculated relative to *chromStart*. The number of items in this list should correspond to *blockCount*.

https://genome.ucsc.edu/FAQ/FAQformat.html#format1

Minimal BED format. So-called BED3 format.

CAGTCGACATAGACTGATATGACACCACACTGAGC...

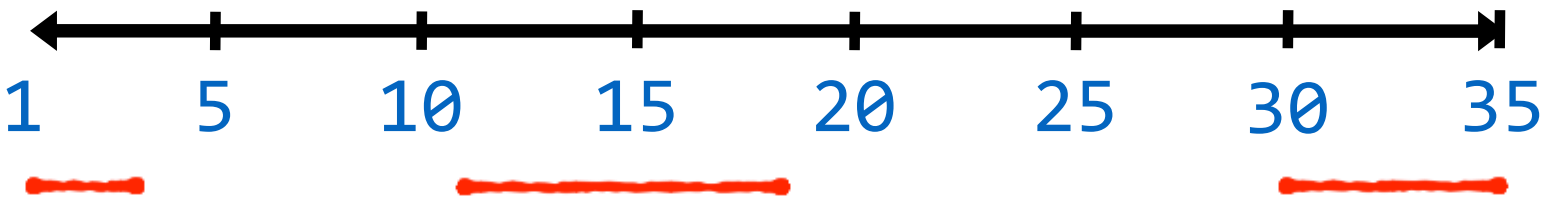



```
chr1    0     3
chr1    11    18
chr1    29    35
```

Slide from Jim Havrilla and Aaron Quinlan

BED format supports “labels”

CAGTCGACATAGACTGATATGACACCACACTGAGC...



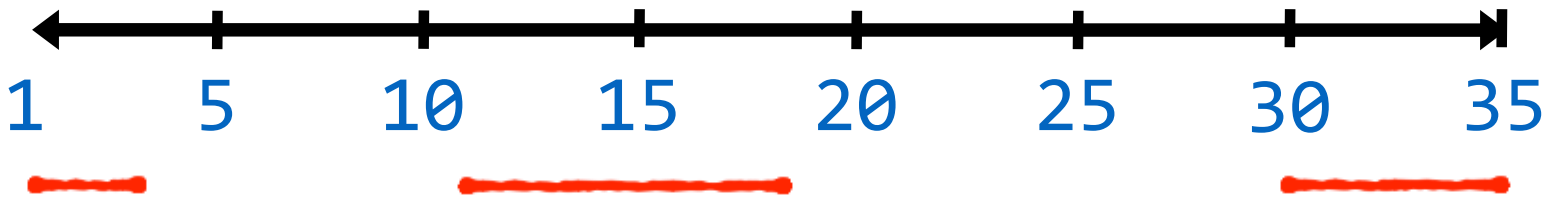
foo bar biz

```
chr1   0    3    foo
chr1   11   18   bar
chr1   29   35   biz
```

Slide from Jim Havrilla and Aaron Quinlan

And scores

CAGTCGACATAGACTGATATGACACCACACTGAGC...



foo 3.1

bar 1.9

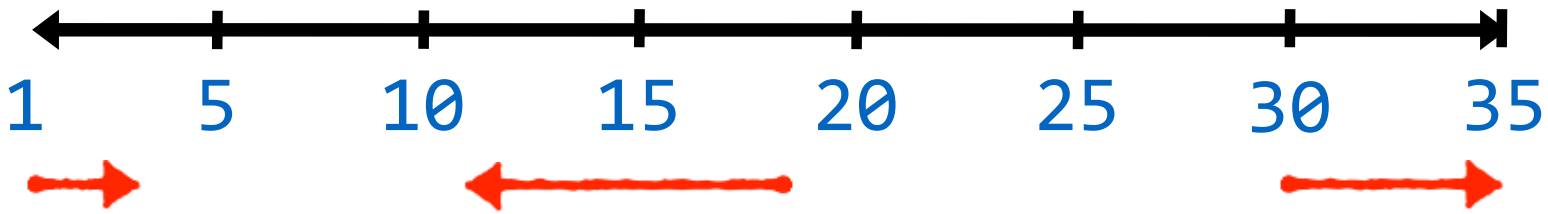
biz 0.1

```
chr1   0    3    foo   3.1
chr1   11   18   bar   1.9
chr1   29   35   biz   0.1
```

Slide from Jim Havrilla and Aaron Quinlan

And strands. This is so-called BED6 format.

CAGTCGACATAGACTGATATGACACCACACTGAGC...



foo 3.1

bar 1.9

biz 0.1

```
chr1    0     3     foo    3.1     +
chr1    11    18    bar    1.9     -
chr1    29    35    biz    0.1     +
```

Slide from Jim Havrilla and Aaron Quinlan

And more! BED12 format

BED format Index ▷

BED format provides a flexible way to define the data lines that are displayed in an annotation track. BED lines have three required fields and nine additional optional fields. The number of fields per line must be consistent throughout any single set of data in an annotation track. The order of the optional fields is binding: lower-numbered fields must always be populated if higher-numbered fields are used.

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3. **chromEnd** - The ending position of the feature in the chromosome or scaffold. The *chromEnd* base is not included in the display of the feature. For example, the first 100 bases of a chromosome are defined as *chromStart=0, chromEnd=100*, and span the bases numbered 0-99.

The 9 additional optional BED fields are:

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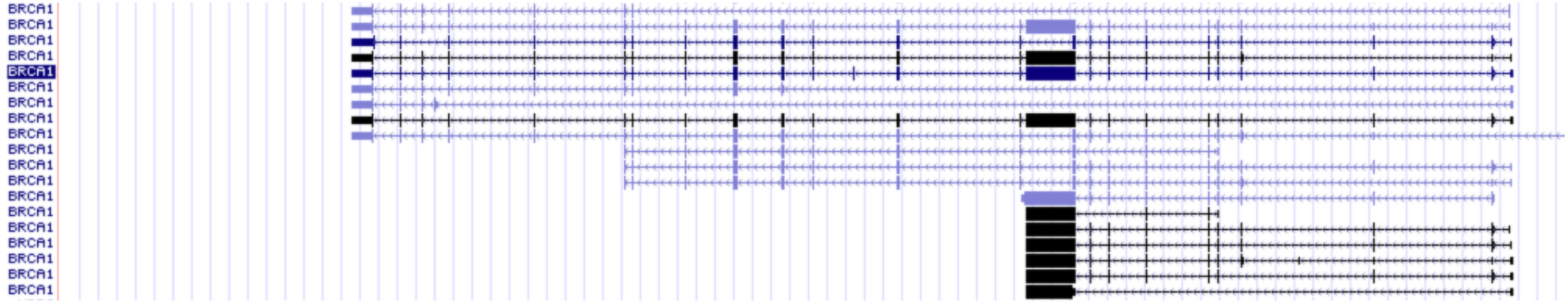
6. **strand** - Defines the strand - either '+' or '-'.
7. **thickStart** - The starting position at which the feature is drawn thickly (for example, the start codon in gene displays). When there is no thick part, thickStart and thickEnd are usually set to the chromStart position.
8. **thickEnd** - The ending position at which the feature is drawn thickly (for example, the stop codon in gene displays).
9. **itemRgb** - An RGB value of the form R,G,B (e.g. 255,0,0). If the track line *itemRgb* attribute is set to "On", this RBG value will determine the display color of the data contained in this BED line. NOTE: It is recommended that a simple color scheme (eight colors or less) be used with this attribute to avoid overwhelming the color resources of the Genome Browser and your Internet browser.
10. **blockCount** - The number of blocks (exons) in the BED line.
11. **blockSizes** - A comma-separated list of the block sizes. The number of items in this list should correspond to *blockCount*.
12. **blockStarts** - A comma-separated list of block starts. All of the *blockStart* positions should be calculated relative to *chromStart*. The number of items in this list should correspond to *blockCount*.

https://genome.ucsc.edu/FAQ/FAQformat.html#format1

BED12 example




```
chr17   41196311        41277340        uc010whm.2      0       -       41197694        41277202        0       8       1508,61,74,55,84,41,78,142,     0,3348,4826,6768,12757,19038,19579,80887,
chr17   41196311        41277340        uc002icp.4      0       -       41197694        41258496        0       23      1508,61,74,55,84,41,78,88,311,191,127,172,89,3426,77,46,103,140,89,56,54,99,142,
0,3348,4826,6768,12757,19038,19579,23313,26633,30036,32193,38109,46649,47140,51551,52949,55480,59827,60573,62183,71431,79722,80887,
chr17   41196311        41277468        uc002icu.3      0       -       41197800        41276113        0       22      1508,61,55,84,41,78,88,311,191,124,172,89,117,77,46,106,140,89,78,54,99,175,
0,3348,6768,12757,19038,19579,23313,26633,30036,32193,38109,46649,50449,51551,52949,55480,59827,60573,62161,71431,79722,80982,
chr17   41196311        41277468        uc010cyx.3      0       -       41197694        41258543        0       22      1508,61,74,55,84,41,78,88,311,191,127,172,89,3426,77,46,106,140,89,78,99,175,
0,3348,4826,6768,12757,19038,19579,23313,26633,30036,32193,38109,46649,47140,51551,52949,55480,59827,60573,62161,79722,80982,
chr17   41196311        41277500        uc002ict.3      0       -       41197694        41276113        0       24      1508,61,74,55,84,41,78,88,311,191,124,66,172,89,3426,77,46,106,140,89,78,54,99,213,
0,3348,4826,6768,12757,19038,19579,23313,26633,30036,32193,35039,38109,46649,47140,51551,52949,55480,59827,60573,62161,71431,79722,80976,
chr17   41196311        41277500        uc010whn.2      0       -       41197694        41226495        0       11      1508,61,74,55,84,41,78,88,311,191,213,  0,3348,4826,6768,12757,19038,19579,23313,26633,30036,80976,
chr17   41196311        41277500        uc010who.3      0       -       41197694        41202109        0       5       1508,61,74,129,213,     0,3348,4826,5767,80976,
chr17   41196311        41277500        uc002icq.3      0       -       41197694        41276113        0       23      1508,61,74,55,84,41,78,88,311,191,127,172,89,3426,77,46,106,140,89,78,54,99,213,
0,3348,4826,6768,12757,19038,19579,23313,26633,30036,32193,38109,46649,47140,51551,52949,55480,59827,60573,62161,71431,79722,80976,
chr17   41196311        41322420        uc010whp.2      0       -       41197694        41258543        0       22      1508,61,74,55,84,41,78,88,311,191,124,172,89,117,77,46,106,140,89,78,54,278,
0,3348,4826,6768,12757,19038,19579,23313,26633,30036,32193,38109,46649,50449,51551,52949,55480,59827,60573,62161,71431,125831,
chr17   41215349        41256973        uc010whq.1      0       -       41215349        41256198        0       12      41,78,88,311,191,127,172,89,117,106,140,89,
0,541,4275,7595,10998,13155,19071,27611,31411,36442,40789,41535,
chr17   41215349        41277468        uc002idc.1      0       -       41215349        41276113        0       18      41,78,88,311,191,127,172,89,117,77,46,103,140,89,78,54,99,175,
0,541,4275,7595,10998,13155,19071,27611,31411,32513,33911,36442,40789,41535,43123,52393,60684,61944,
chr17   41215349        41277468        uc010whr.1      0       -       41215349        41258543        0       17      41,78,88,311,191,127,172,89,117,77,46,106,140,89,78,99,175,
0,541,4275,7595,10998,13155,19071,27611,31411,32513,33911,36442,40789,41535,43123,60684,61944,
chr17   41243116        41276132        uc002idd.3      0       -       41243347        41276113        0       9       3761,77,46,106,140,89,78,54,99, 0,4746,6144,8675,13022,13768,15356,24626,32917,
chr17   41243451        41256973        uc002ide.1      0       -       41243452        41256198        0       4       3426,103,140,89,        0,8340,12687,13433,
chr17   41243451        41277340        uc010cyy.1      0       -       41243452        41276113        0       10      3426,77,46,106,140,89,78,54,99,142,     0,4411,5809,8340,12687,13433,15021,24291,32582,33747,
chr17   41243451        41277468        uc010whs.1      0       -       41243452        41276113        0       10      3426,77,46,106,140,89,78,54,99,175,     0,4411,5809,8340,12687,13433,15021,24291,32582,33842,
chr17   41243451        41277500        uc010cyz.2      0       -       41243452        41258543        0       11      3426,77,46,106,140,89,78,116,54,99,213, 0,4411,5809,8340,12687,13433,15021,19030,24291,32582,33836,
chr17   41243451        41277500        uc010cza.2      0       -       41243452        41276113        0       9       3426,77,46,106,140,89,54,99,213,        0,4411,5809,8340,12687,13433,24291,32582,33836,
chr17   41243451        41277500        uc010wht.1      0       -       41243452        41246659        0       2       3426,213,       0,33836,
chr17   41277599        41292342        uc002idf.3      0       +       41277599        41277599        0       4       188,63,182,1669,        0,5625,7373,13074,
chr17   41277599        41292342        uc010czb.2      0       +       41277599        41277599        0       2       188,1669,       0,13074,
chr17   41277599        41297125        uc002idg.3      0       +       41277599        41277599        0       5       188,63,266,468,381,     0,5625,13074,14233,19145,
chr17   41277599        41305688        uc002idh.3      0       +       41277599        41277599        0       8       188,63,125,182,205,266,120,70,  0,5625,7016,7373,12573,13074,16874,28019,
```

Slide in this section are from Jim Havrilla and Aaron Quinlan

GFF: annotates one line per feature

GFF format

GFF format Index ▷

GFF (General Feature Format) lines are based on the Sanger GFF2 specification. GFF lines have nine required fields that *must* be tab-separated. If the fields are separated by spaces instead of tabs, the track will not display correctly. For more information on GFF format, refer to Sanger's GFF page.

Note that there is also a GFF3 specification that is not currently supported by the Browser. All GFF tracks must be formatted according to Sanger's GFF2 specification.

If you would like to obtain browser data in GFF (GTF) format, please refer to Genes in gtf or gff format on the Wiki.

Here is a brief description of the GFF fields:

1. **seqname** - The name of the sequence. Must be a chromosome or scaffold.
2. **source** - The program that generated this feature.
3. **feature** - The name of this type of feature. Some examples of standard feature types are "CDS", "start_codon", "stop_codon", and "exon".
4. **start** - The starting position of the feature in the sequence. The first base is numbered 1.
5. **end** - The ending position of the feature (inclusive).
6. **score** - A score between 0 and 1000. If the track line *useScore* attribute is set to 1 for this annotation data set, the *score* value will determine the level of gray in which this feature is displayed (higher numbers = darker gray). If there is no score value, enter ".".
7. **strand** - Valid entries include '+', '-', or '.' (for don't know/don't care).
8. **frame** - If the feature is a coding exon, *frame* should be a number between 0-2 that represents the reading frame of the first base. If the feature is not a coding exon, the value should be '.'.
9. **group** - All lines with the same group are linked together into a single item.

Example:
Here's an example of a GFF-based track. This example can be pasted into the browser without editing. NOTE: Paste operations on some operating systems will replace tabs with spaces, which will result in an error when the GFF track is uploaded. You can circumvent this problem by pasting the URL of the above example (http://genome.ucsc.edu/goldenPath/help/regulatory.txt) instead of the text itself into the custom annotation track text box. If you encounter an error when loading a GFF track, check that the data lines contain tabs rather than spaces.

```
browser position chr22:10000000-10025000
browser hide all
track name=regulatory description="TeleGene(tm) Regulatory Regions"
visibility=2
chr22  TeleGene enhancer  10000000  10001000  500 +  .  touch1
chr22  TeleGene promoter  10010000  10010100  900 +  .  touch1
chr22  TeleGene promoter  10020000  10025000  800 -  .  touch2
```

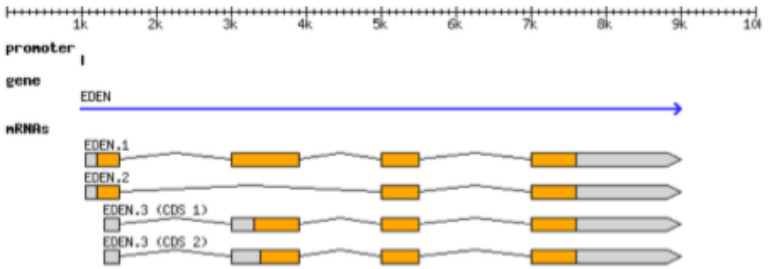
Click here to display this track in the Genome Browser.

```
chr22  TeleGene enhancer  10000000  10001000  500 +  .  touch1
chr22  TeleGene promoter  10010000  10010100  900 +  .  touch1
chr22  TeleGene promoter  10020000  10025000  800 -  .  touch2
```

https://genome.ucsc.edu/FAQ/FAQformat.html#format3

GFF example

spliced transcripts, isoform 3 has two alternative translation start sites




```
##gff-version 3
##sequence-region   ctg123 1 1497228
ctg123 . gene            1000  9000  .  +  .  ID=gene00001;Name=EDEN

ctg123 . TF_binding_site 1000  1012  .  +  .  ID=tfbs00001;Parent=gene00001

ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00001;Parent=gene00001;Name=EDEN.1
ctg123 . mRNA            1050  9000  .  +  .  ID=mRNA00002;Parent=gene00001;Name=EDEN.2
ctg123 . mRNA            1300  9000  .  +  .  ID=mRNA00003;Parent=gene00001;Name=EDEN.3

ctg123 . exon            1300  1500  .  +  .  ID=exon00001;Parent=mRNA00003
ctg123 . exon            1050  1500  .  +  .  ID=exon00002;Parent=mRNA00001,mRNA00002
ctg123 . exon            3000  3902  .  +  .  ID=exon00003;Parent=mRNA00001,mRNA00003
ctg123 . exon            5000  5500  .  +  .  ID=exon00004;Parent=mRNA00001,mRNA00002,mRNA00003
ctg123 . exon            7000  9000  .  +  .  ID=exon00005;Parent=mRNA00001,mRNA00002,mRNA00003

ctg123 . CDS             1201  1500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             3000  3902  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             5000  5500  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1
ctg123 . CDS             7000  7600  .  +  0  ID=cds00001;Parent=mRNA00001;Name=edenprotein.1

ctg123 . CDS             1201  1500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS             5000  5500  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2
ctg123 . CDS             7000  7600  .  +  0  ID=cds00002;Parent=mRNA00002;Name=edenprotein.2

ctg123 . CDS             3301  3902  .  +  0  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS             5000  5500  .  +  1  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3
ctg123 . CDS             7000  7600  .  +  1  ID=cds00003;Parent=mRNA00003;Name=edenprotein.3

ctg123 . CDS             3391  3902  .  +  0  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS             5000  5500  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
ctg123 . CDS             7000  7600  .  +  1  ID=cds00004;Parent=mRNA00003;Name=edenprotein.4
```

Slide in this section are from Jim Havrilla and Aaron Quinlan

BAM/SAM: Genome alignment format

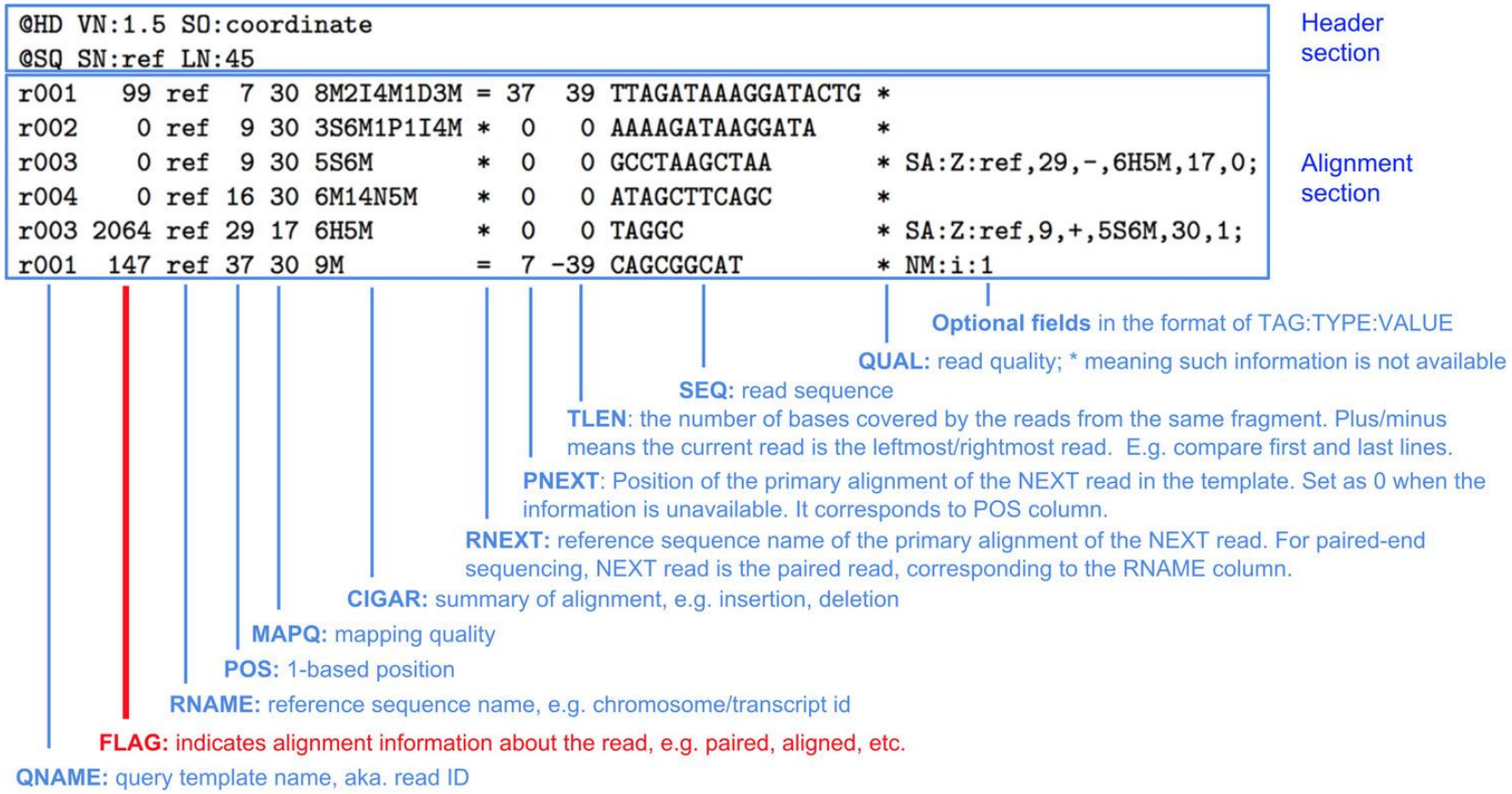
BAM/SAM format

- SAM: Sequence Alignment/Map format (tab-delimited text file).
- BAM: The binary equivalent of a SAM file, which stores the same data in a compressed binary representation

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

Li et al, Bioinformatics, 2009

Example of a SAM file



https://www.samformat.info/sam-format-flag

CRAM

- CRAM was designed to be an efficient reference-based alternative to SAM/BAM file formats
- Better lossless compression than BAM, but also allow for controlled loss of BAM data
- Typically used for large-scale population-based genome/exome sequencing project (for example, CRAMs has ~50TB for 50K exomes in UK Biobank).

https://samtools.github.io/hts-specs/CRAMv3.pdf

VCF: Genetic variant file format

VCF file format

- Variant Call Format, established > 10 years ago and is now a gold standard for describing variants.
- One locus per line, and it may contain more than one mutations, but most lines contain one variant only.
- Additional header lines starts with “#” to explain the meaning of the various tags in the file

Example of a VCF file

```
##fileformat=VCFv4.3
##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
```

As of June 2019, the latest version is 4.3

https://en.wikipedia.org/wiki/Variant_Call_Format

The header line of a VCF file

- The header line names the 8 fixed, mandatory columns. These columns are as follows:
  - #CHROM POS ID REF ALT QUAL FILTER INFO

- If genotype data is present in the file, these are followed by a FORMAT column header, then an arbitrary number of sample IDs

The INFO line of a VCF file

- INFO fields are encoded as a semicolon-separated series of short keys with optional values in the format: key[=data[,data]].
- Some keys are reserved:

Key	Number	Type	Description
AA	1	String	Ancestral allele
AC	A	Integer	Allele count in genotypes, for each ALT allele, in the same order as listed
AD	R	Integer	Total read depth for each allele
ADF	R	Integer	Read depth for each allele on the forward strand
ADR	R	Integer	Read depth for each allele on the reverse strand
AF	A	Float	Allele frequency for each ALT allele in the same order as listed (estimated from primary data, not called genotypes)
AN	1	Integer	Total number of alleles in called genotypes
BQ	1	Float	RMS base quality
CIGAR	A	String	Cigar string describing how to align an alternate allele to the reference allele
DB	0	Flag	dbSNP membership
DP	1	Integer	Combined depth across samples

The genotypes in a VCF file

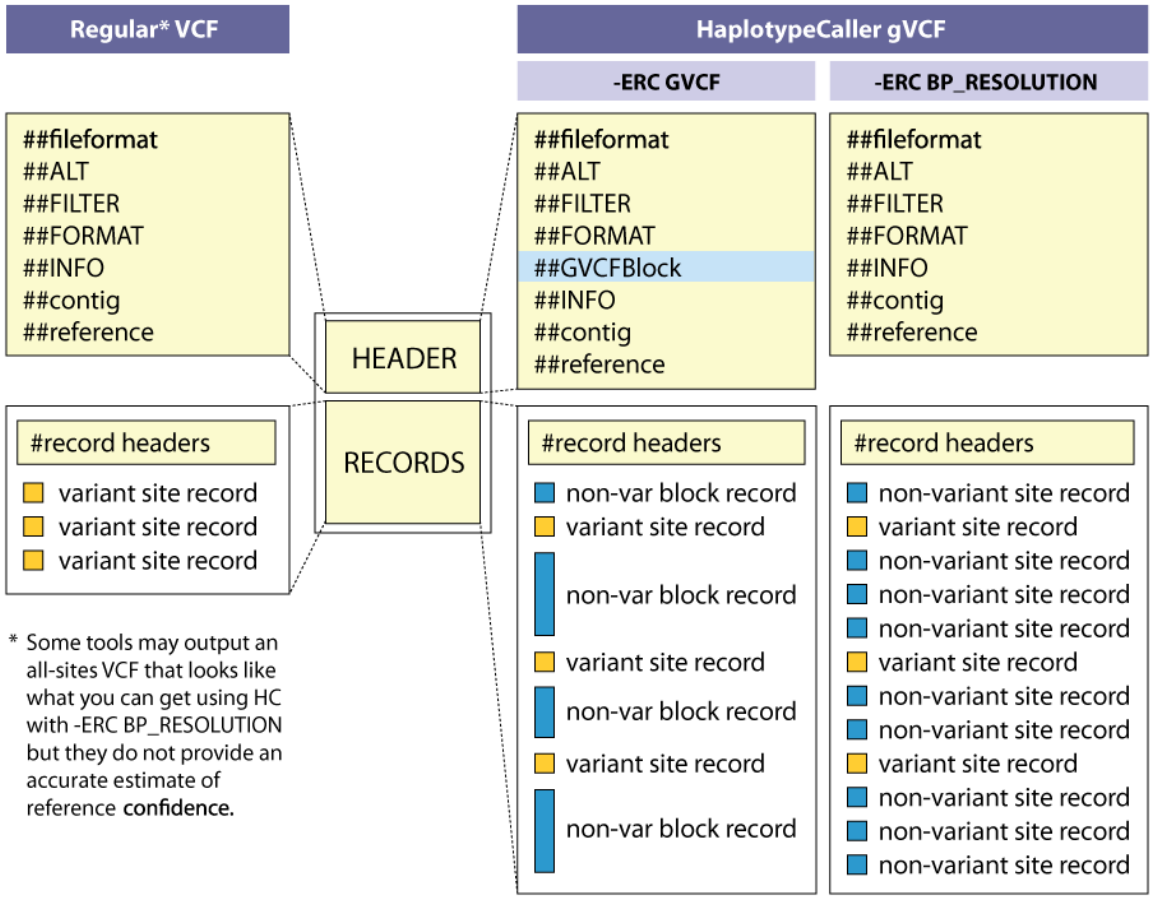
- A FORMAT field is given specifying the data types and order
- This is followed by one data block per sample, with the colon-separated data corresponding to the types specified in the format.

FORMAT	NA00001	NA00002	NA00003
GT:GQ:DP:HQ	0|0:48:1:51,51	1|0:48:8:51,51	1/1:43:5:.,.
GT:GQ:DP:HQ	0|0:49:3:58,50	0|1:3:5:65,3	0/0:41:3
GT:GQ:DP:HQ	1|2:21:6:23,27	2|1:2:0:18,2	2/2:35:4
GT:GQ:DP:HQ	0|0:54:7:56,60	0|0:48:4:51,51	0/0:61:2
GT:GQ:DP	0/1:35:4	0/2:17:2	1/1:40:3

gVCF format

- gVCF (Genomic VCF): the basic format specification is the same as for a regular VCF, but gVCF contains extra information.
- gVCF was developed to store sequencing information for both variant and non-variant positions, which is required for human clinical applications.

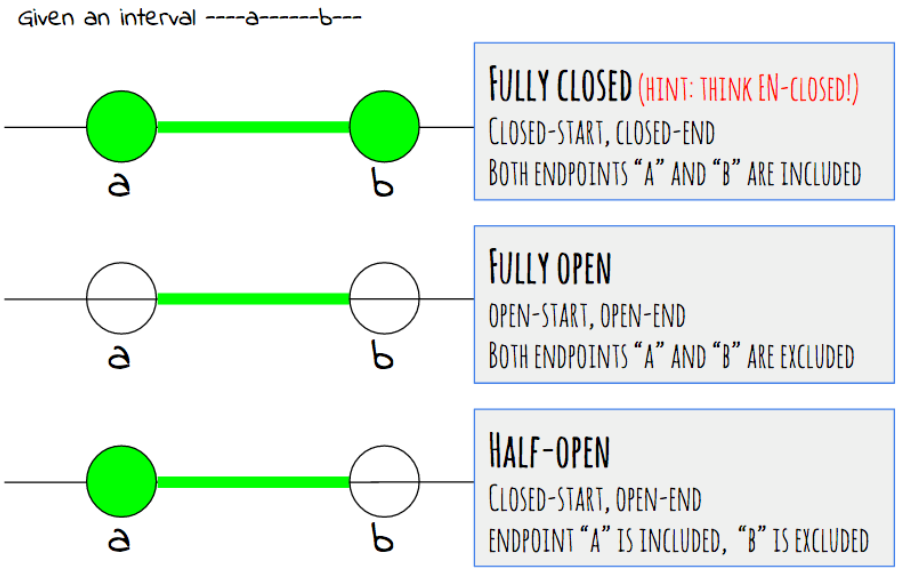
VCF versus gVCF



https://software.broadinstitute.org/gatk/documentation/article.php?id=4017

Formats use different coordinate systems, which adds confusion

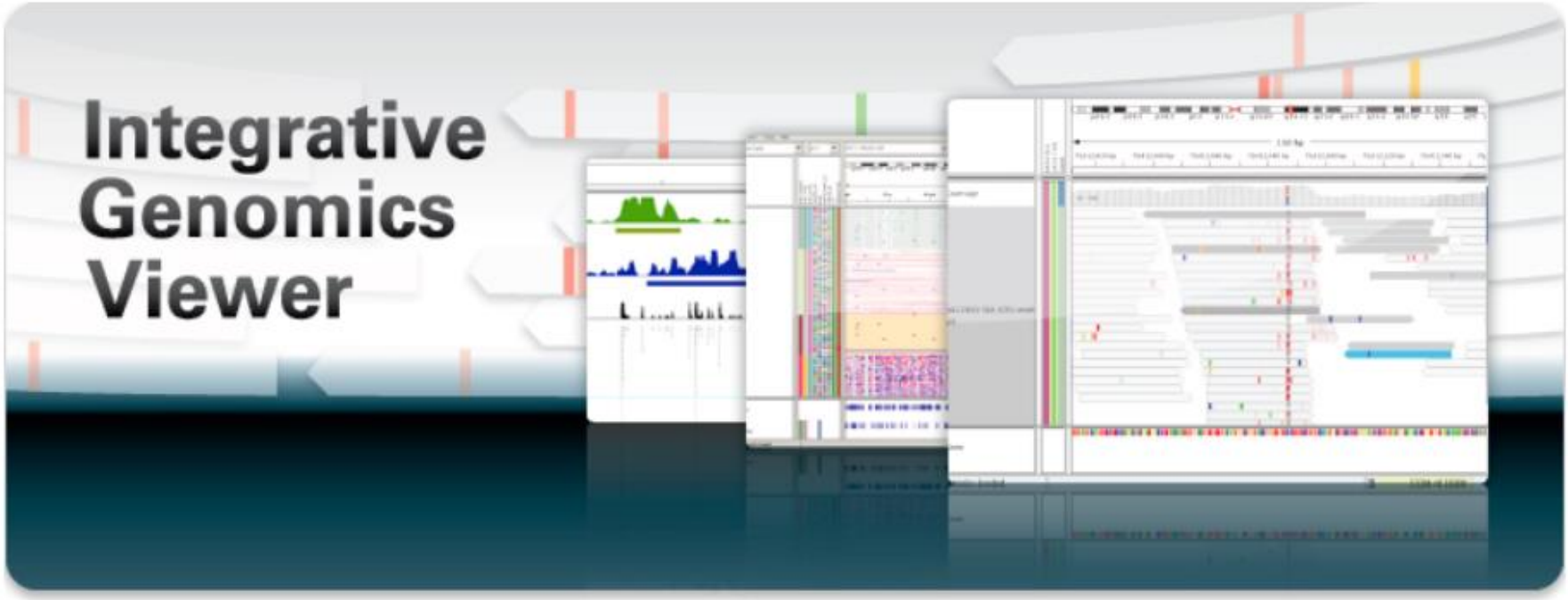
BED: 0-based, half-open
GFF: 1-based, fully closed
SAM: 1-based, fully closed
BAM: 0-based, half-open
VCF: 1-based, fully closed
…



http://genome.ucsc.edu/blog/the-ucsc-genome-browser-coordinate-counting-systems/

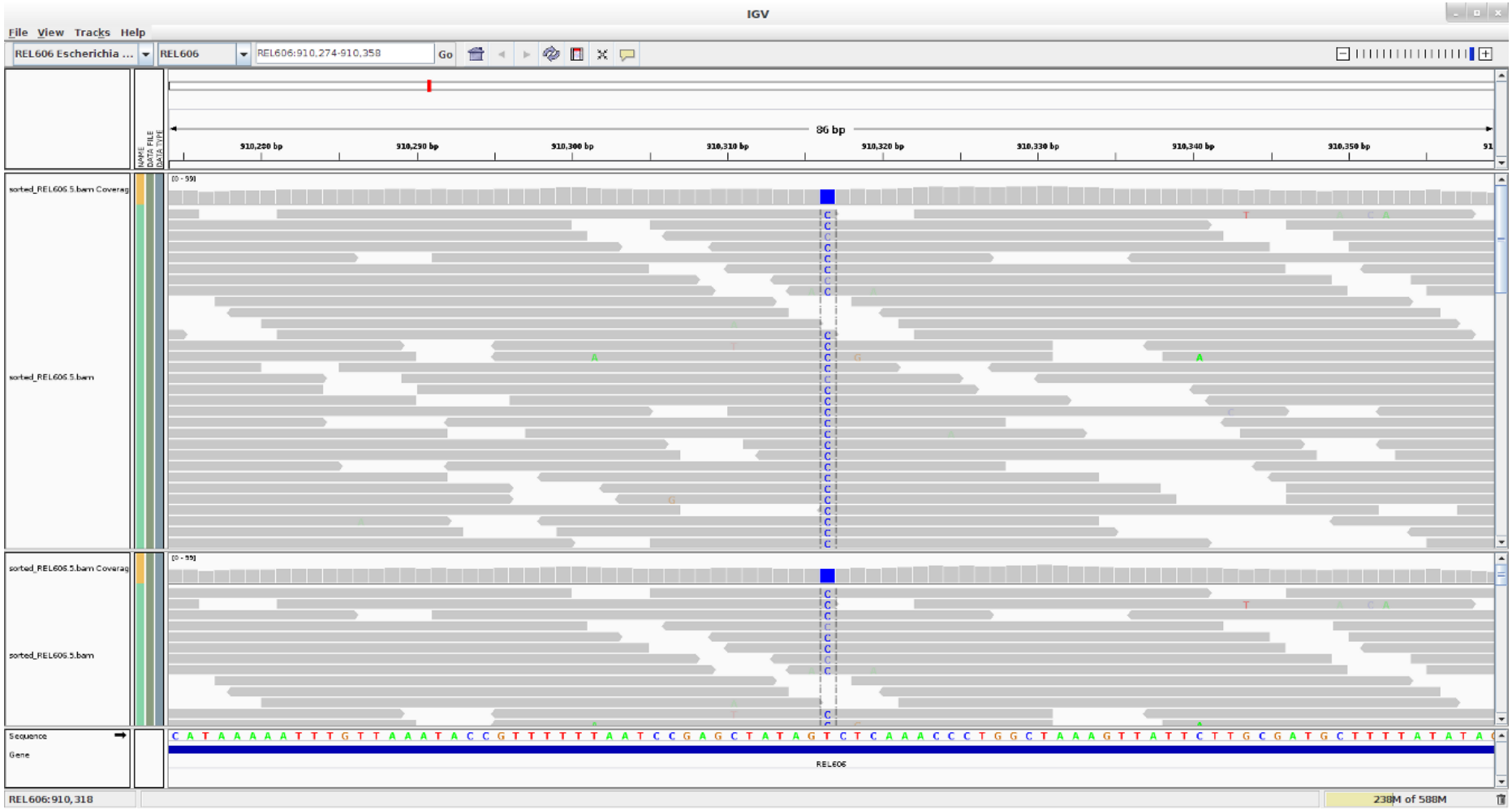
Visualization of genomic data

- Integrative Genomics Viewer (IGV) is a high-performance visualization tool for interactive exploration of large, integrated genomic datasets.

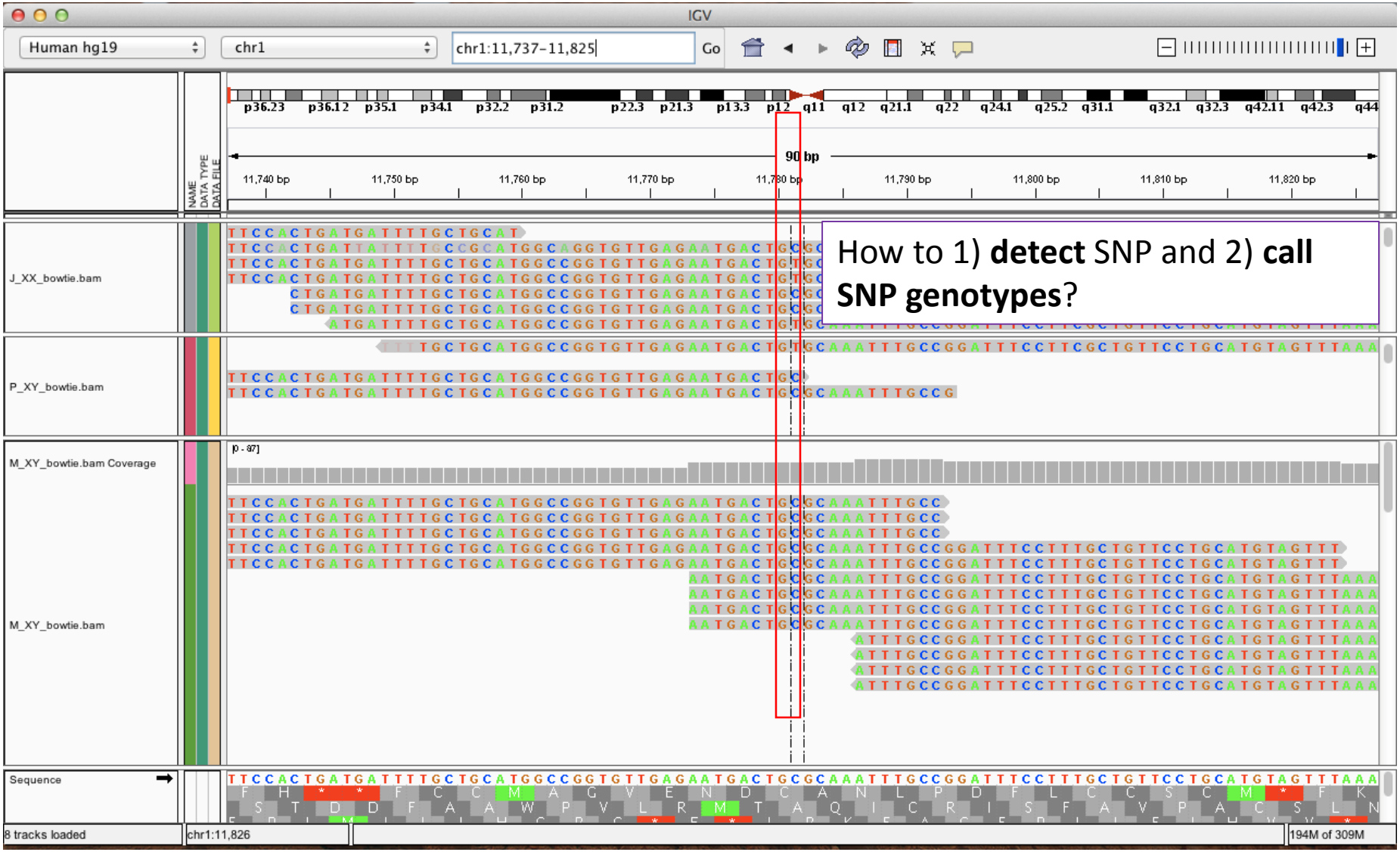


https://software.broadinstitute.org/software/igv/

Visualization: IGV Viewer

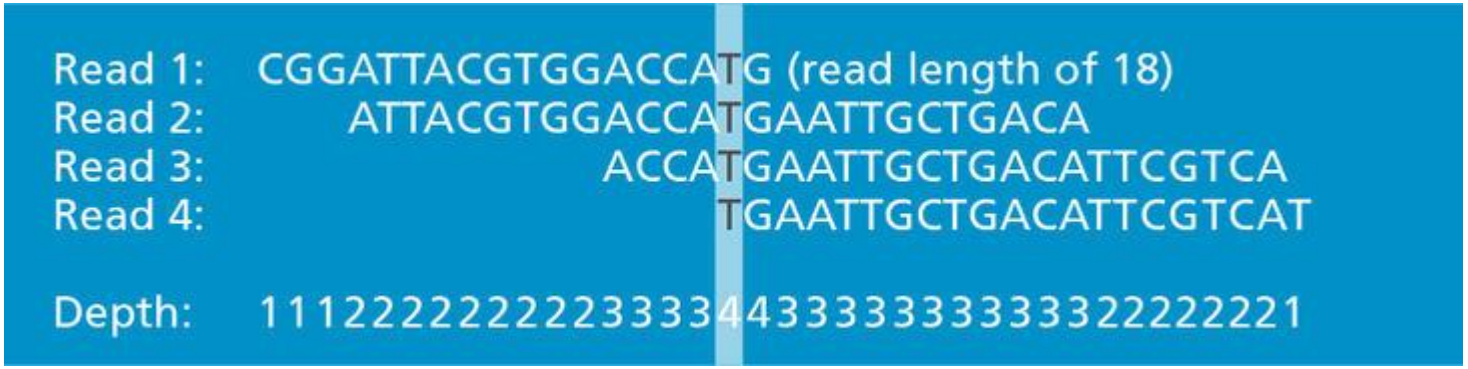


Visualization: IGV Viewer



What is coverage?

- depth of sequencing coverage can be defined theoretically as *LN/G*, where *L* is the read length, *N* is the number of reads and *G* is the haploid genome length.
- The breadth of coverage is the percentage of target bases that have been sequenced for a given number of times.
- The accuracy of variant calling is affected by sequence quality, uniformity of coverage and the threshold of false-discovery rate that is used.



Sims et al, Nature Reviews Genetics, 2014

Coverage: how many reads we need to cover the genome?

Depth of coverage model

In a shotgun sequencing, genome size ***G***, read size ***S***, ***N*** reads

Probability of a read starts at an interval ***L*** is $p=L/G$

D: Number of reads start in an interval of length L:

$D \sim$ **Binomial**$(N, L/G)$

Let $L = S$, then D is also the number of reads that cover the last position of the interval → D is depth of coverage.

$S \ll G$, N very large, therefore depth of coverage is approximated by a Poisson distribution:

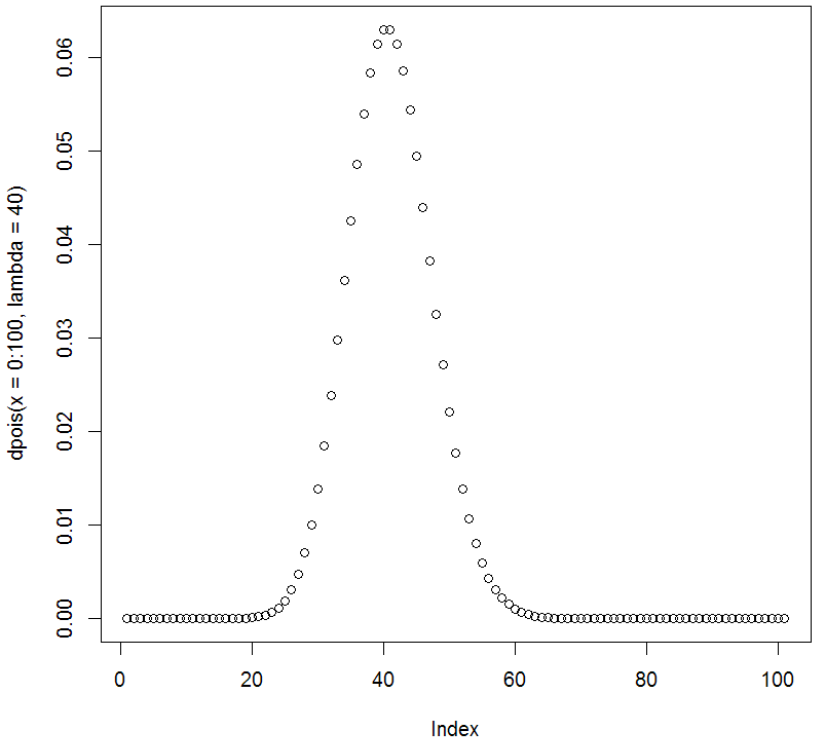
$\boldsymbol{D} \sim$ **Poisson**(λ)

$\lambda = SN/G$ (average depth of coverage)

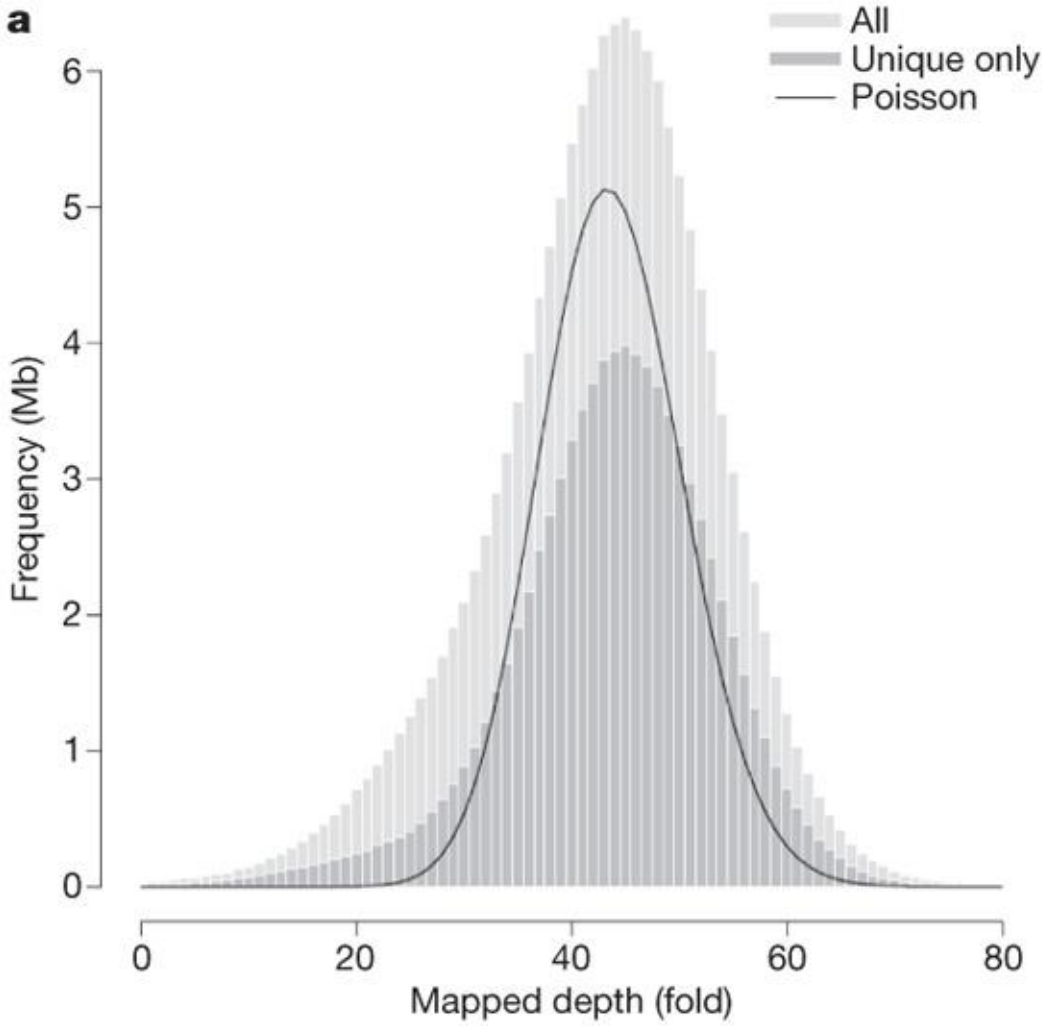
Fraction of genome that are covered

- Given λ=40, the fraction of genome that are covered more than 30x (*D*>30) is: 0.938

- Given λ=40, the fraction of genome that are covered more than 20x (*D*>20) is: 0.9996

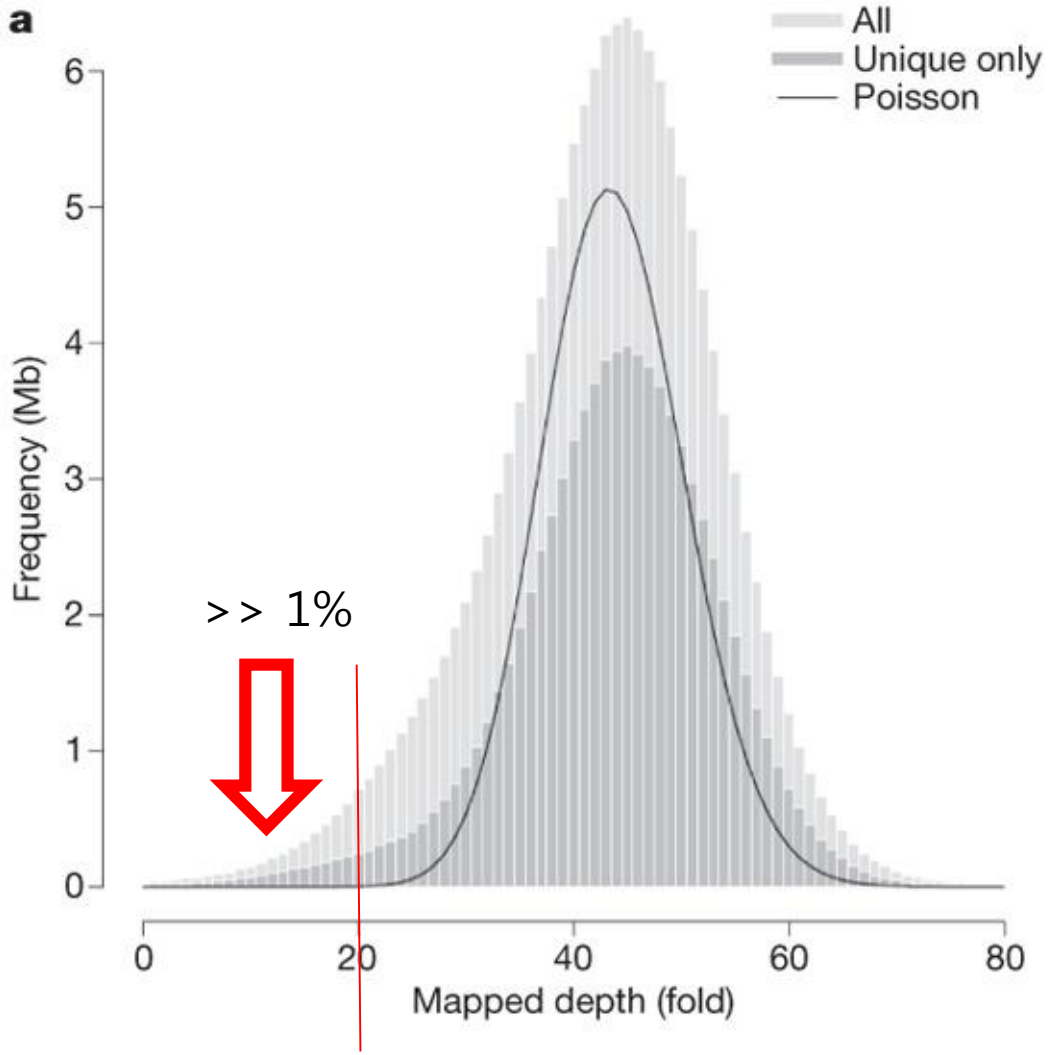


Empirical results



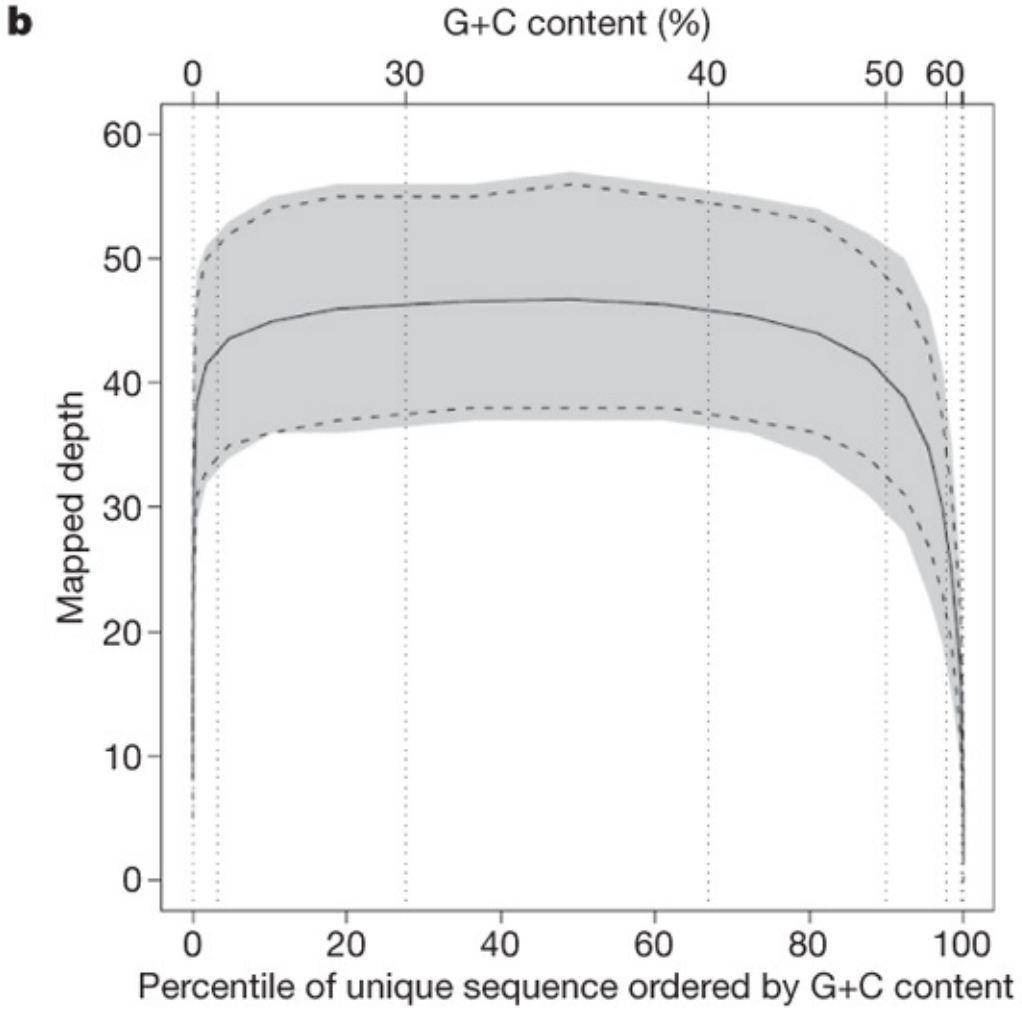
Bentley et al, Nature, 2008

Empirical results



Bentley et al, Nature, 2008

Main cause of overdispersion



Bentley et al, Nature, 2008

How to model overdispersion

GC bias and other technical factors lead to systematic bias in coverage, resulting in overdispersion

Ideal situation (Poisson distrition):

$\mathrm{Var}(D) = \mu$

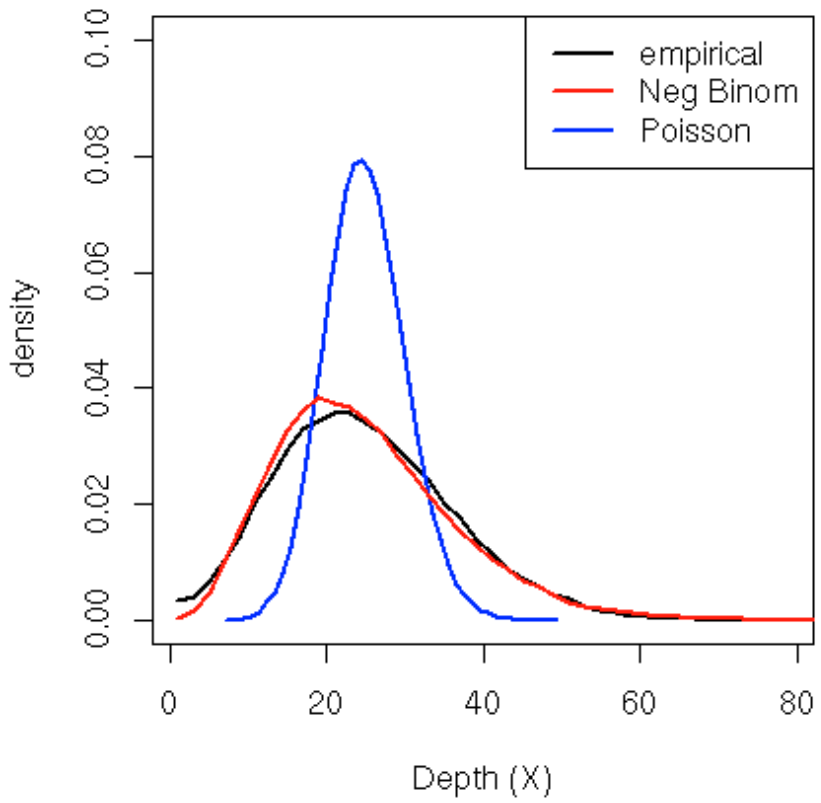
Gamma-Poisson is equivalent to Negative Binomial, which is a commonly used model for dealing with overdispersion in count data:

$\mathrm{Var}(D) = \mu + \mu^2/k$

It is generalizable to model overdispersion caused by other factors, e.g. biological noise.

Larger k → smaller overdispersion

How to model overdispersion



- Over-dispersion: **var >** μ
- Due to sequencing bias:
  - **GC content**
  - Other context dependent factors

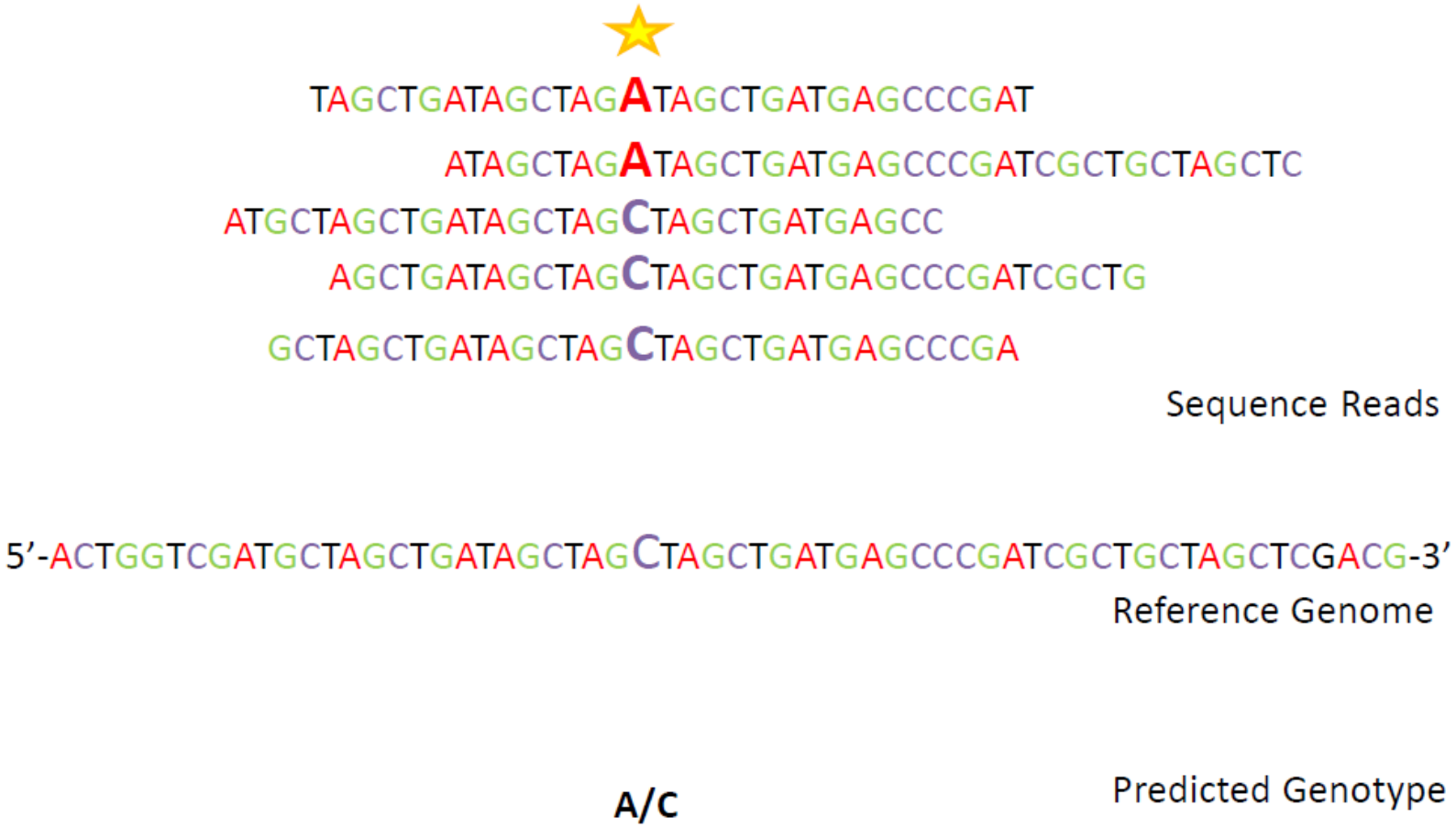
Shen et al, Nature, 2008

Question on coverage

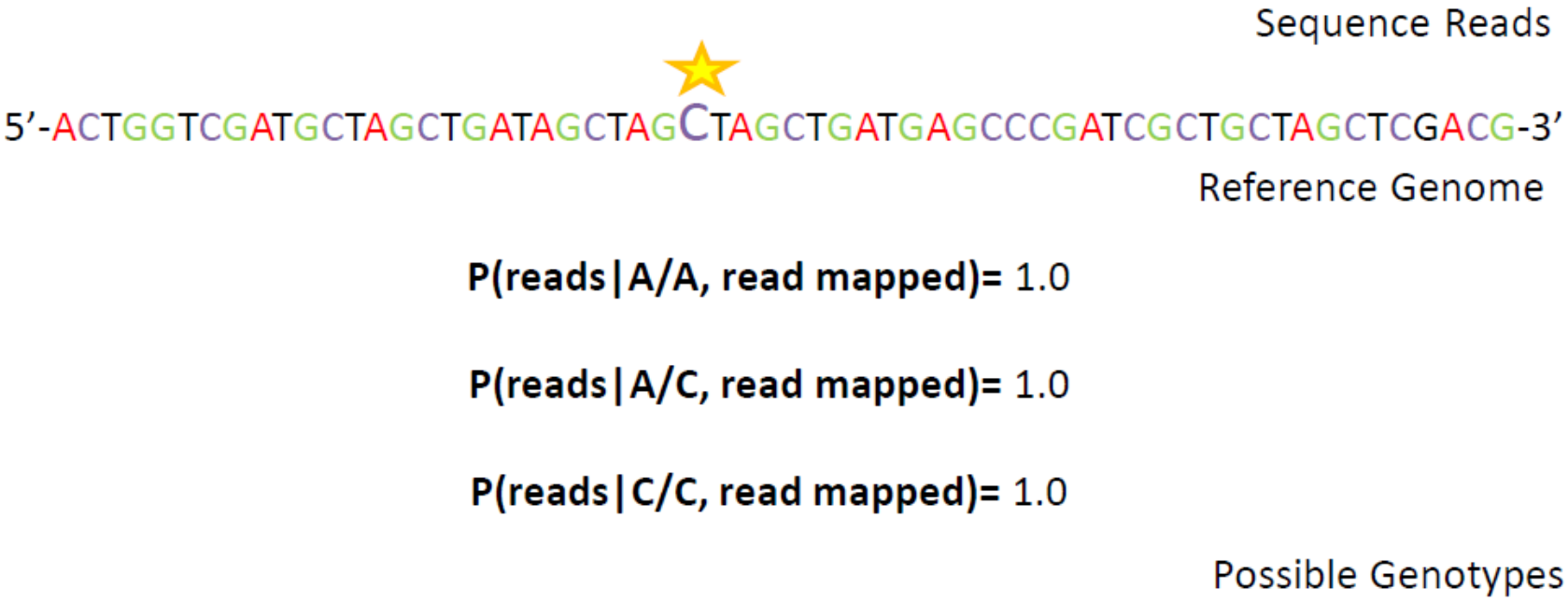
- Why do we need average 30-50x in a typical WGS experiment, and 100x in WES?

General strategy for variant calling

- Reads piled up at each base of interest
- With per-base qualities and mapping quality



NGS Data


Sequence Reads
5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'
Reference Genome
P(reads|A/A, read mapped)= 1.0
P(reads|A/C, read mapped)= 1.0
P(reads|C/C, read mapped)= 1.0
Possible Genotypes

NGS Data

Sequence Reads

5’-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3’

Reference Genome

P(reads|A/A, read mapped)= P(C observed|A/A, read mapped)

P(reads|A/C, read mapped)= P(C observed|A/C, read mapped)

P(reads|C/C, read mapped)= P(C observed|C/C, read mapped)

Possible Genotypes

NGS Data

Assuming that sequencing error rate is 0.01

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A, read mapped)= 0.01

P(reads|A/C, read mapped)= 0.50

P(reads|C/C, read mapped)= 0.99

Possible Genotypes

NGS Data

AGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTG

GCTAGCTGATAGCTAGCTAGCTGATGAGCCCGA

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A, read mapped)= 0.0001=0.01*0.01

P(reads|A/C , read mapped)= 0.25=0.5*0.5

P(reads|C/C , read mapped)= 0.98=0.99*0.99

Possible Genotypes

NGS Data

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A , read mapped)= 0.000001=0.01*0.01*0.01

P(reads|A/C , read mapped)= 0.125=0.5*0.5*0.5

P(reads|C/C , read mapped)= 0.97=0.99*0.99*0.99

Possible Genotypes

NGS Data

ATAGCTAGATAGCTGATGAGCCCGATCGCTGCTAGCTC

ATGCTAGCTGATAGCTAGCTAGCTGATGAGCC

AGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTG

GCTAGCTGATAGCTAGCTAGCTGATGAGCCCGA

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A , read mapped)= 0.00000099=0.01*0.01*0.01*0.99

P(reads|A/C , read mapped)= 0.0625=0.5*0.5*0.5*0.5

P(reads|C/C , read mapped)= 0.0097=0.99*0.99*0.99*0.01

Possible Genotypes

NGS Data

TAGCTGATAGCTAGATAGCTGATGAGCCCGAT

ATAGCTAGATAGCTGATGAGCCCGATCGCTGCTAGCTC

ATGCTAGCTGATAGCTAGCTAGCTGATGAGCC

AGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTG

GCTAGCTGATAGCTAGCTAGCTGATGAGCCCGA

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A , read mapped)= 0.00000098=0.01^3*0.99^2

P(reads|A/C , read mapped)= 0.03125=0.5*0.5*0.5*0.5*0.5

P(reads|C/C , read mapped)= 0.000097=0.99^3*0.01^2

Possible Genotypes

NGS Data

P(reads|A/A, read mapped)= 0.00000098

P(reads|A/C, read mapped)= 0.03125

P(reads|C/C, read mapped)= 0.000097

Combine these likelihoods with a prior incorporating information from other individuals and flanking sites to assign a genotype.

NGS Data

$$P(Genotype|reads) = \frac{P(reads|Genotype)Prior(Genotype)}{\sum_G P(reads|G)Prior(G)}$$

Combine these likelihoods with a prior incorporating information from other individuals and flanking sites to assign a genotype.

From Sequence to Genotype: Individual Based Prior

TAGCTGATAGCTAGATAGCTGATGAGCCCGAT

ATAGCTAGATAGCTGATGAGCCCGATCGCTGCTAGCTC

ATGCTAGCTGATAGCTAGCTAGCTGATGAGCC

AGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTG

GCTAGCTGATAGCTAGCTAGCTGATGAGCCCGA

Sequence Reads

5'-ACTGGTCGATGCTAGCTGATAGCTAGCTAGCTGATGAGCCCGATCGCTGCTAGCTCGACG-3'

Reference Genome

P(reads|A/A)= 0.00000098 **Prior(A/A) =** 0.00034 **Posterior(A/A) =** <.001

P(reads|A/C)= 0.03125 **Prior(A/C) =** 0.00066 **Posterior(A/C) =** 0.175

P(reads|C/C)= 0.000097 **Prior(C/C) =** 0.99900 **Posterior(C/C) =** 0.825

Individual Based Prior: Every site has 1/1000 probability of varying.

From Sequence To Genotype: Population Based Prior

P(reads|A/A)= 0.00000098	**Prior(A/A) =** 0.04	**Posterior(A/A) =** <.001
P(reads|A/C)= 0.03125	**Prior(A/C) =** 0.32	**Posterior(A/C) =** 0.999
P(reads|C/C)= 0.000097	**Prior(C/C) =** 0.64	**Posterior(C/C) =** <.001

Population Based Prior: Use frequency information from examining others at the same site.

In the example above, we estimated P(A) = 0.20

More on Prior

- **Population Based Prior**
  - Uses frequency information obtained from examining other individuals
  - Calling very rare polymorphisms still requires 20-30x coverage of the genome
  - Calling common polymorphisms requires much less data