HTS data formats and Quality Control

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Data Formats

FASTQ

- Unaligned read sequences with base qualities

SAM/BAM

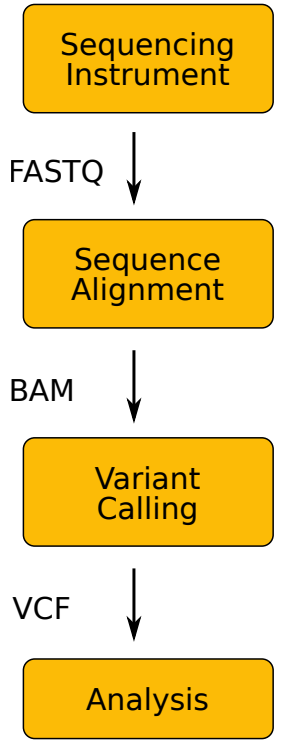
- Unaligned or aligned reads
- Text and binary formats

CRAM

- Better compression than BAM

VCF/BCF

- Flexible variant call format
- Arbitrary types of sequence variation
- SNPs, indels, structural variations



Specifications maintained by the Global Alliance for Genomics and Health

FASTQ

- Simple format for raw unaligned sequencing reads
- Extension to the FASTA file format
- Sequence and an associated per base quality score

```
@ERR007731.739 IL16_2979:6:1:9:1684/1
CTTGACGACTTGAAAAATGACGAAATCACTAAAAAACGTGAAAAATGAGAAATG
+
BBCBCBBBBBBBABBABBBBBBBABBBBBBBBBBBBBBABAAAABBBBB=@>B
@ERR007731.740 IL16_2979:6:1:9:1419/1
AAAAAAAAAGATGTCATCAGCACATCAGAAAAGAAGGCAACTTTAAAACTTTTC
+
BBABBBABABAABABABBABBBAAA>@B@BBAA@4AAA>.>BAA@779:AAA@A
```

- Quality encoded in ASCII characters with decimal codes 33-126
  - ASCII code of "A" is 65, the corresponding quality is Q$= 65 - 33 = 32$
  - Phred quality score: $P = 10^{-Q/10}$

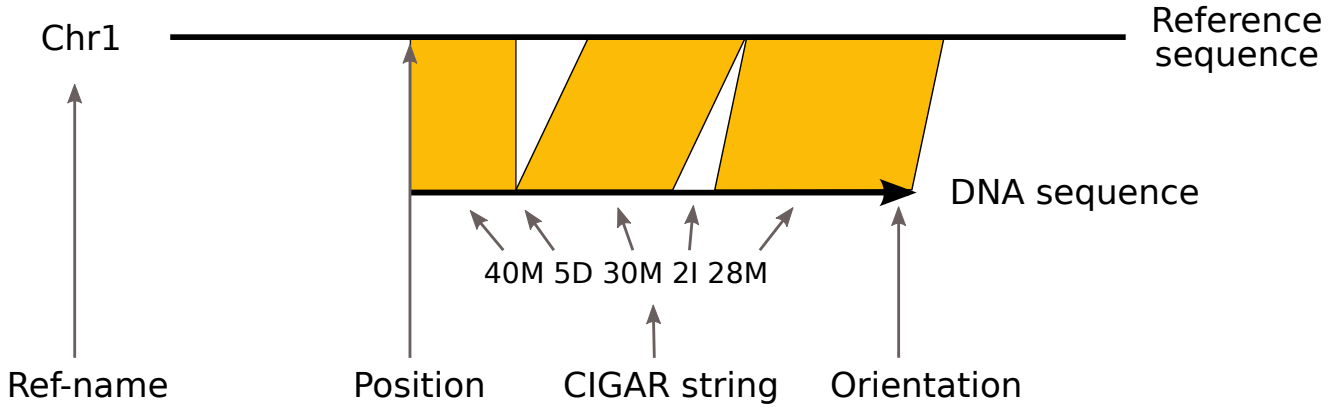
```
perl -e 'printf "%d\n",ord("A")-33;'
```

- Beware: multiple quality scores were in use!
  - Sanger, Solexa, Illumina 1.3+
- Paired-end sequencing produces two FASTQ files

SAM / BAM

SAM (Sequence Alignment/Map) format

- Unified format for storing read alignments to a reference genome
- Developed by the 1000 Genomes Project group (2009)
- One record (a single DNA fragment alignment) per line describing alignment between fragment and reference
- 11 fixed columns + optional key:type:value tuples



Note that BAM can contain

- unmapped reads
- multiple alignments of the same read
- supplementary (chimeric) reads

SAM

SAM fields

1	QNAME	Query NAME of the read or the read pair
2	FLAG	Bitwise FLAG (pairing, strand, mate strand, etc.)
3	RNAME	Reference sequence NAME
4	POS	1-Based leftmost POSition of clipped alignment
5	MAPQ	MAPping Quality (Phred-scaled)
6	CIGAR	Extended CIGAR string (operations: MIDNSHPX=)
7	MRNM	Mate Reference NaMe ('=' if same as RNAME)
8	MPOS	1-Based leftmost Mate POSition
9	ISIZE	Inferred Insert SIZE
10	SEQ	Query SEQuence on the same strand as the reference
11	QUAL	Query QUALity (ASCII-33=Phred base quality)
12-	OTHER	Optional fields

```
$ samtools view -h file.bam | less

@HD VN:1.0  GO:none SO:coordinate
@SQ SN:1    LN:249250621    UR:hs37d5.fa.gz AS:NCBI37   M5:1b22b98cdeb4a9304cb5d48026a85128 SP:Human
@SQ SN:2    LN:243199373    UR:hs37d5.fa.gz AS:NCBI37   M5:a0d9851da00400dec1098a9255ac712e SP:Human
@RG ID:1    PL:ILLUMINA PU:13350_1  LB:13350_1  SM:13350_1  CN:SC
@PG ID:bwa  PN:bwa  VN:0.7.10-r806    CL:bwa mem hs37d5.fa.gz 13350_1_1.fq 13350_1_1.fq
1:2203:10256:56986  97  1   9998    0   106M45S =  10335    0 \
    CCATAACCCTAACCCTAACCCTAACCATAGCCCTAACCCTAACCCTAACCCTAACCCT[...]CAAACCCACCCCCAAACCCAAAACCTCACCAC \
    FFFFFJJJJJJJJFJJJJFJAJJJJJ-JJAAAJFJJFFJJF<FJJFFJJJJFJJJJFF[...]<---F-----A7-J-<J-A--77AF---J7-- \
    MD:Z:1G24C2A76  PG:Z:MarkDuplicates RG:Z:1  NM:i:3  MQ:i:0  AS:i:94 XS:i:94
```

CIGAR string

Compact representation of sequence alignment

M	alignment match or mismatch
=	sequence match
X	sequence mismatch
I	insertion to the reference
D	deletion from the reference
S	soft clipping (clipped sequences present in SEQ)
H	hard clipping (clipped sequences NOT present in SEQ)
N	skipped region from the reference
P	padding (silent deletion from padded reference)

Examples:

```
Ref:   ACGTACGTACGTACGT
Read:  ACGT----ACGTACGA
Cigar: 4M 4D 8M

Ref:   ACGT----ACGTACGT
Read:  ACGTACGTACGTACGT
Cigar: 4M 4I 8M

Ref:   ACTCAGTG--GT
Read:  ACGCA-TGCAGTtagacgt
Cigar: 5M 1D 2M 2I 2M 7S
```

Flags

Hex	Dec	Flag	Description
0x1	1	PAIRED	paired-end (or multiple-segment) sequencing technology
0x2	2	PROPER_PAIR	each segment properly aligned according to the aligner
0x4	4	UNMAP	segment unmapped
0x8	8	MUNMAP	next segment in the template unmapped
0x10	16	REVERSE	SEQ is reverse complemented
0x20	32	MREVERSE	SEQ of the next segment in the template is reversed
0x40	64	READ1	the first segment in the template
0x80	128	READ2	the last segment in the template
0x100	256	SECONDARY	secondary alignment
0x200	512	QCFAIL	not passing quality controls
0x400	1024	DUP	PCR or optical duplicate
0x800	2048	SUPPLEMENTARY	supplementary alignment

Bit operations made easy

- python

  0x1 | 0x2 | 0x20 | 0x80 .. 163

  bin(163) .. 10100011

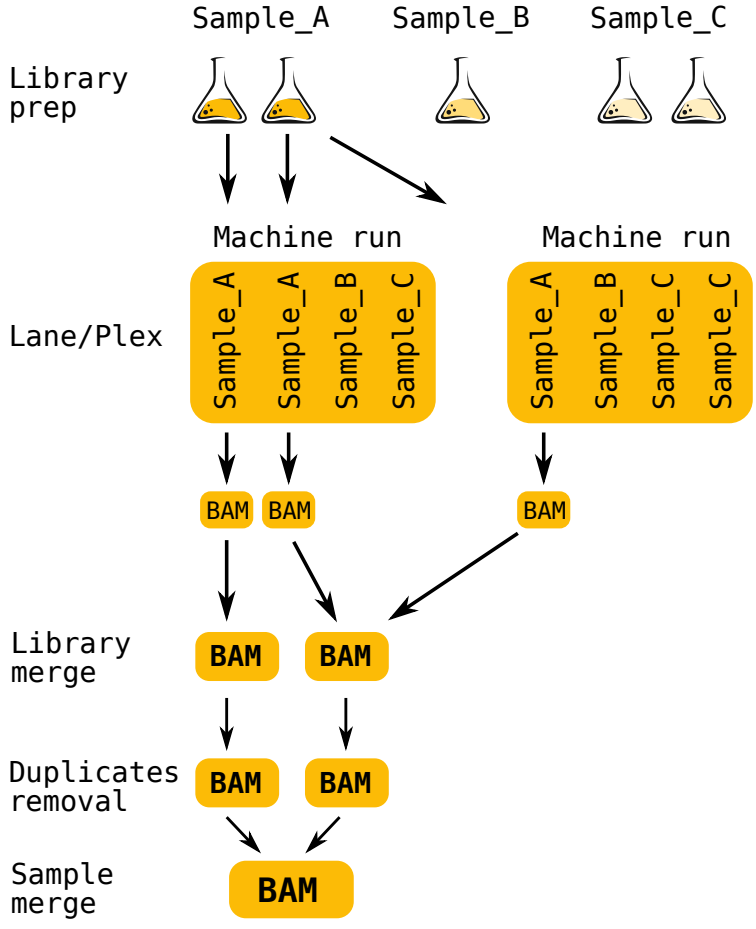
- samtools flags

  0xa3 163 PAIRED,PROPER_PAIR,MREVERSE,READ2

Optional tags

Each lane has a unique RG tag that contains meta-data for the lane
RG tags

- ID: SRR/ERR number
- PL: Sequencing platform
- PU: Run name
- LB: Library name
- PI: Insert fragment size
- SM: Individual
- CN: Sequencing center



BAM

BAM (Binary Alignment/Map) format

- Binary version of SAM
- Developed for fast processing and random access
    - BGZF (Block GZIP) compression for indexing

Key features

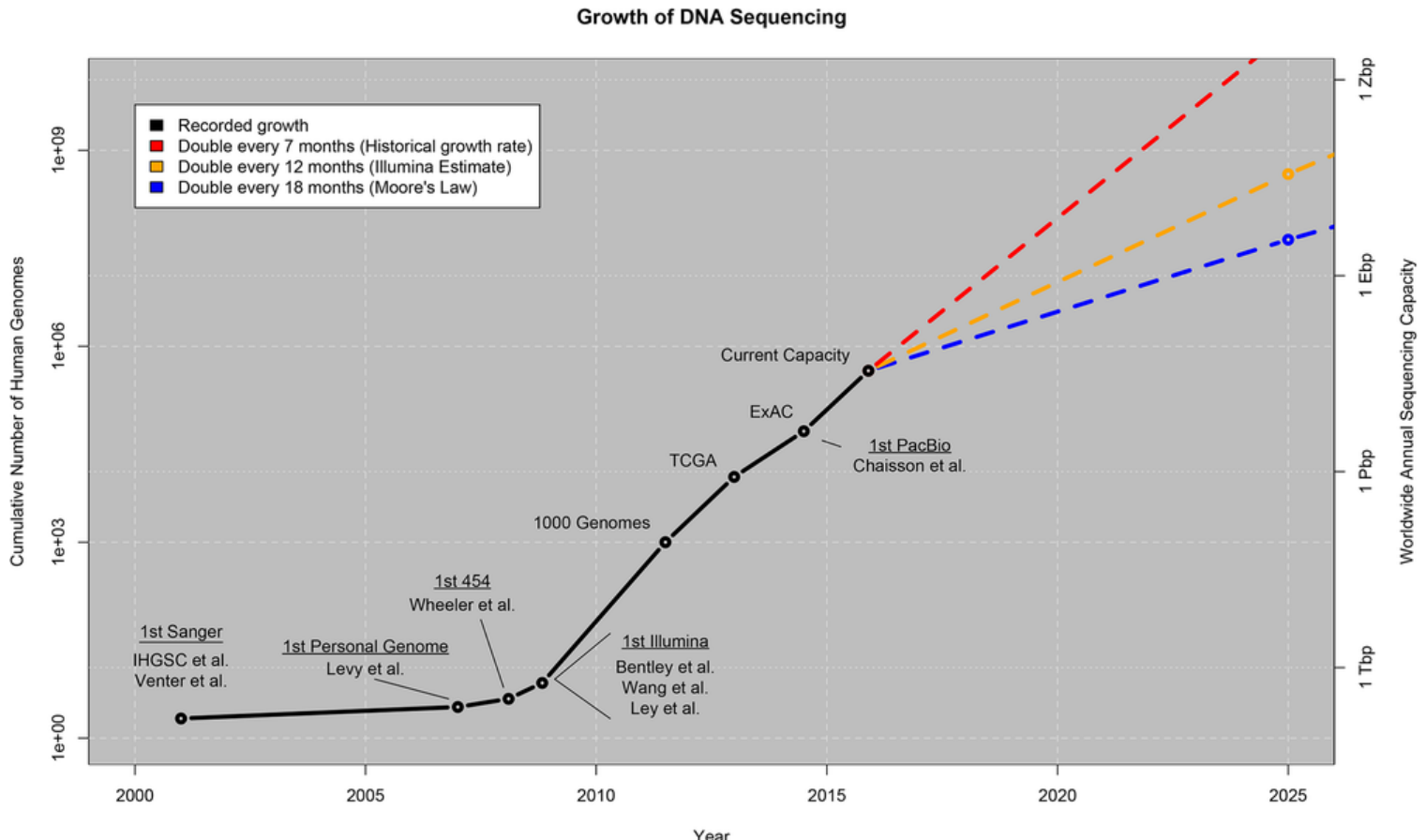
- Can store alignments from most mappers
- Supports multiple sequencing technologies
- Supports indexing for quick retrieval/viewing
- Compact size (e.g. 112Gbp Illumina = 116GB disk space)
- Reads can be grouped into logical groups e.g. lanes, libraries, samples
- Widely supported by variant calling packages and viewers

Reference based Compression

BAM files are too large

- ~1.5-2 bytes per base pair

Increases in disk capacity are being far outstripped by sequencing technologies



Zachary D. Stephens, *et al*, Big Data: Astronomical or Genomical? DOI: 10.1371/journal.pbio.1002195

Reference based Compression

BAM files are too large

- ~1.5-2 bytes per base pair

Increases in disk capacity are being far outstripped by sequencing technologies
BAM stores all of the data

- Every read base
- Every base quality
- Using a single conventional compression technique for all types of data

```
Reference sequence: ACGTACGTACGTACGTACGTACGTACGTACGTAC
read 1:             ACGTACGTACGTACGTACGTGC
read 2:                TACGTACGCACGTACGTGCGTA
read 3:                  CGTACGCACGTACGTACGTACG
read 4:                    TACGTACGTACGTGCGTACGTA
read 5:                      CGCACGTACGTACGTACGTACG
read 6:                            TACGTGCGTACGTACGTAC
```

Reference based Compression

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BAM stores all of the data

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- Every base quality
- Using a single conventional compression technique for all types of data

```
Reference sequence: ACGTACGTACGTACGTACGTACGTACGTAC
read 1:                    ....................G.
read 2:                       ........C............
read 3:                         ......C..............
read 4:                           ............G........
read 5:                             ..C..................
read 6:                                   .....G............
```

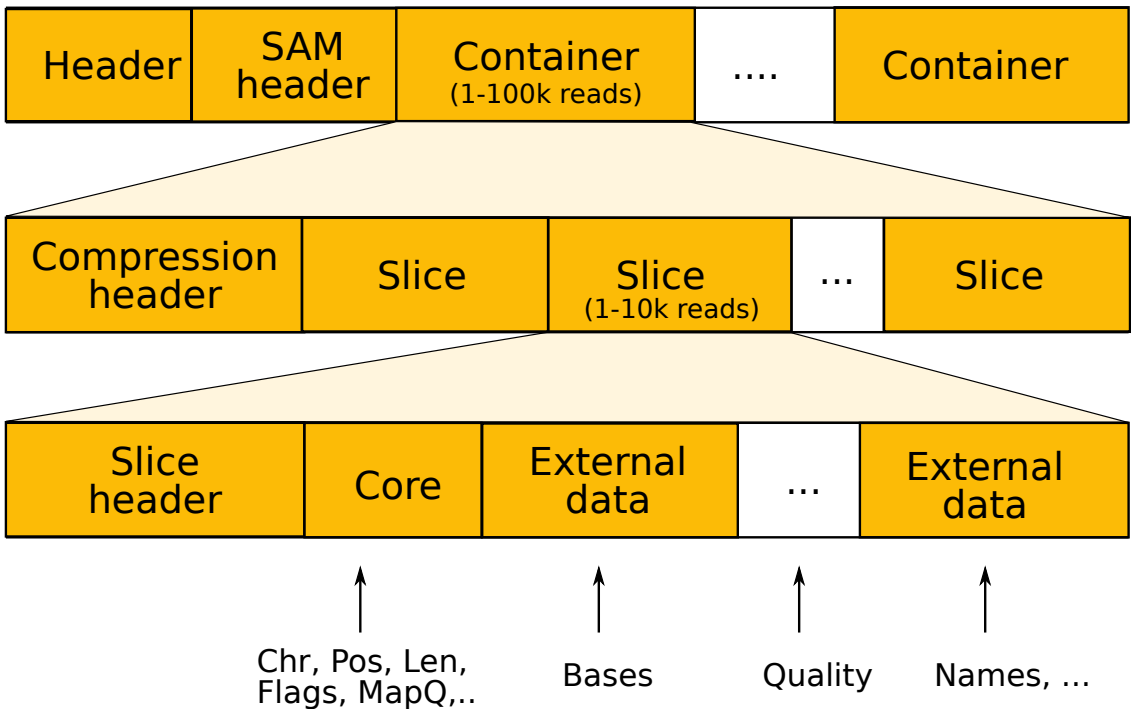
CRAM

Three important concepts

- Reference based compression
- Controlled loss of quality information
- Different compression methods to suit the type of data, e.g. base qualities vs. metadata vs. extra tags

In lossless mode: 60% of BAM size
Archives and sequencing centers moving from BAM to CRAM

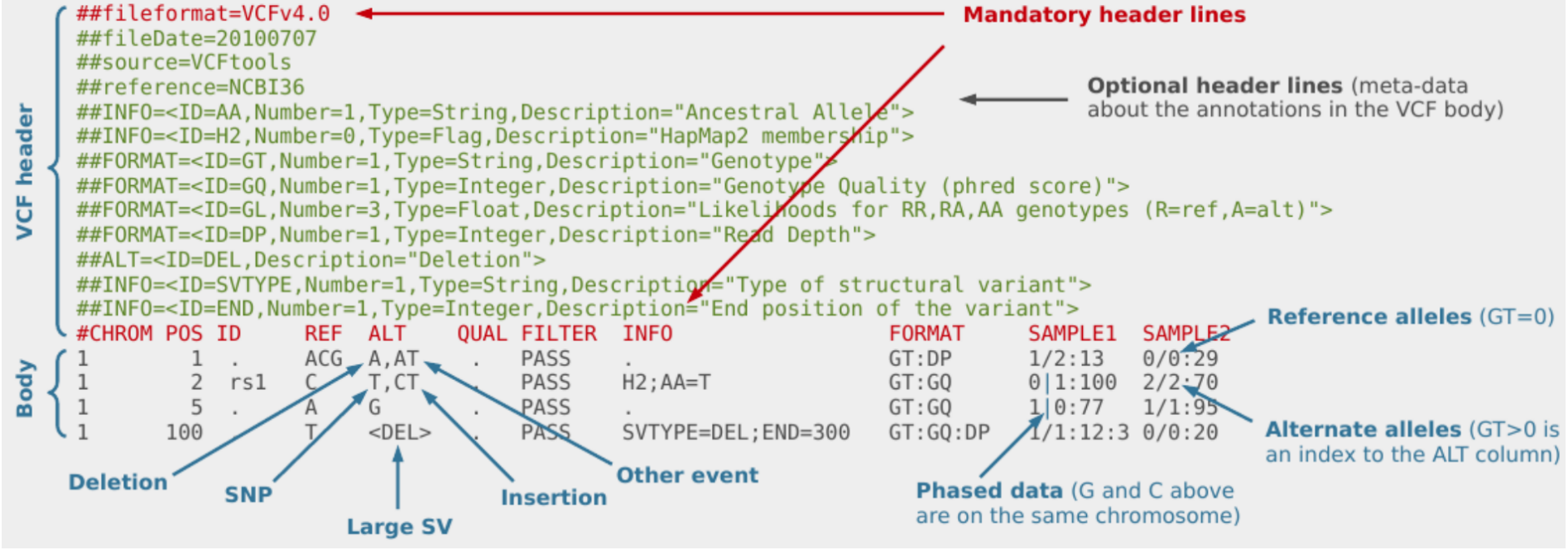
- Support for CRAM added to Samtools/HTSlib in 2014
- Soon to be available in Picard/GATK



VCF: Variant Call Format

File format for storing variation data

- Tab-delimited text, parsable by standard UNIX commands
- Flexible and user-extensible
- Compressed with BGZF (bgzip), indexed with TBI or CSI (tabix)



VCF / BCF

VCFs can be very big

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

VCFs can be slow to parse

- text conversion is slow
- main bottleneck: FORMAT fields

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

BCF

- binary representation of VCF
- fields rearranged for fast access

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

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

gVCF

Often it is not enough not know *variant* sites only

- was a site dropped because of a reference call or because of missing data?
- we need evidence for both variant and non-variant positions in the genome

gVCF

- blocks of reference-only sites can be represented in a single record using the INFO/END tag
- symbolic alleles <*> for incremental calling
  - raw, "callable" gVCF
  - calculate genotype likelihoods only once (an expensive step)
  - then call incrementally as more samples come in

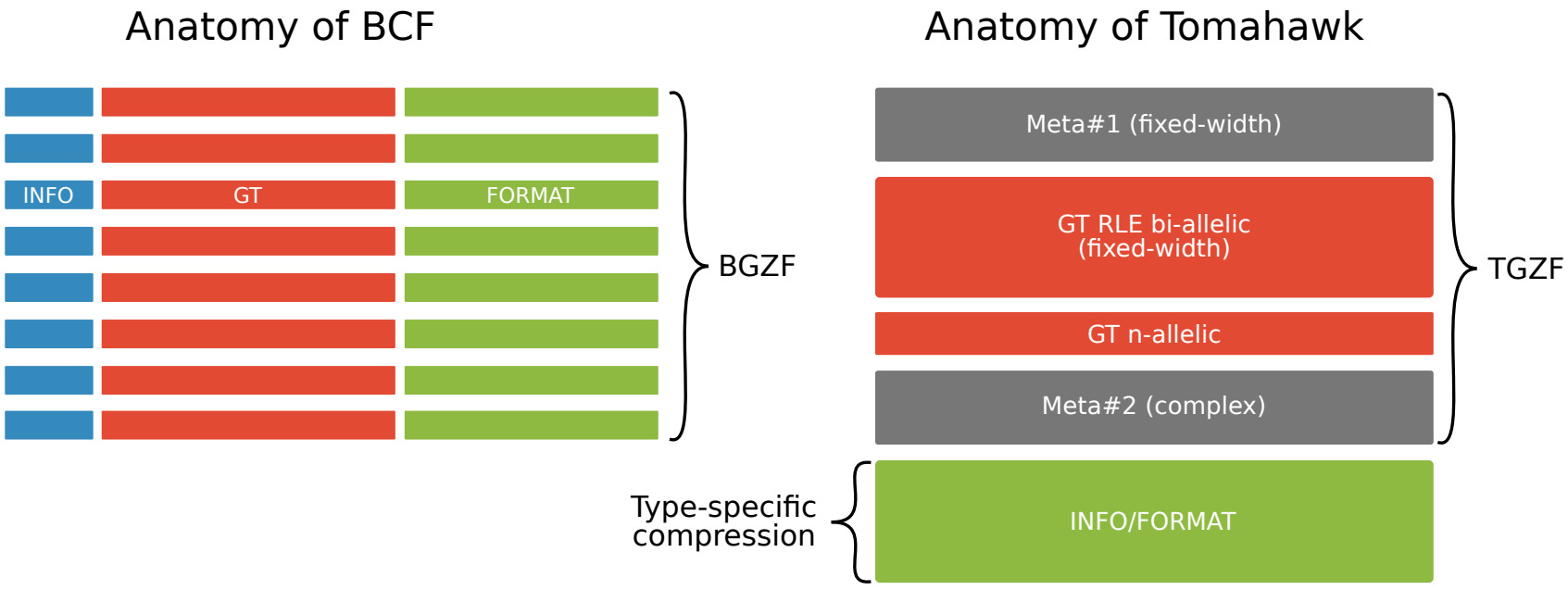
```
#CHROM POS  ID REF ALT  QUAL FILTER    INFO              FORMAT Sample
19    9902 .  G   <*>   .    .      END=9915;MinDP=0   PL:DP  0,0,0:0
19    9916 .  C   <*>   .    .      END=9922;MinDP=5   PL:DP  0,15,137:5
19    9923 .  G   <*>   .    .      END=9948;MinDP=10  PL:DP  0,30,214:10
19    9949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

Symbolic "unobserved" allele
Represents any other possible alternate allele

A block of 10 sites with
at least 34 reference reads

Genotype likelihoods
for CC, C*, **

Optimizing variant calls for speed

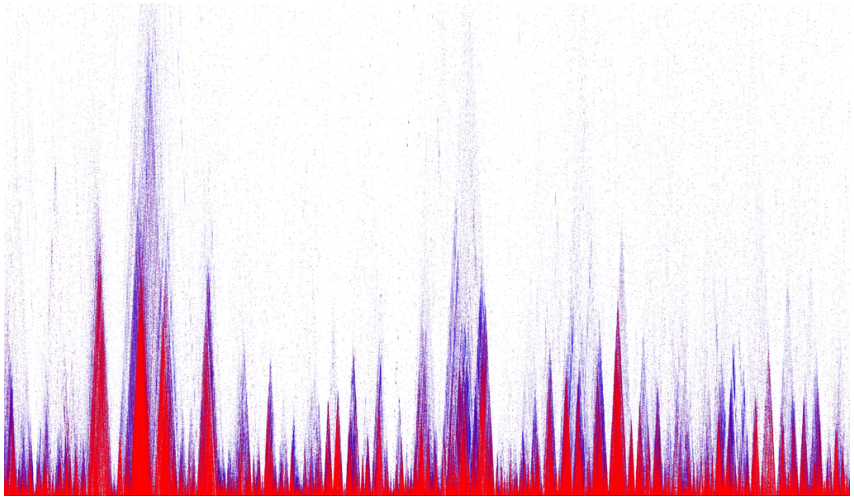


New TWK format by Marcus Klarqvist (under development)

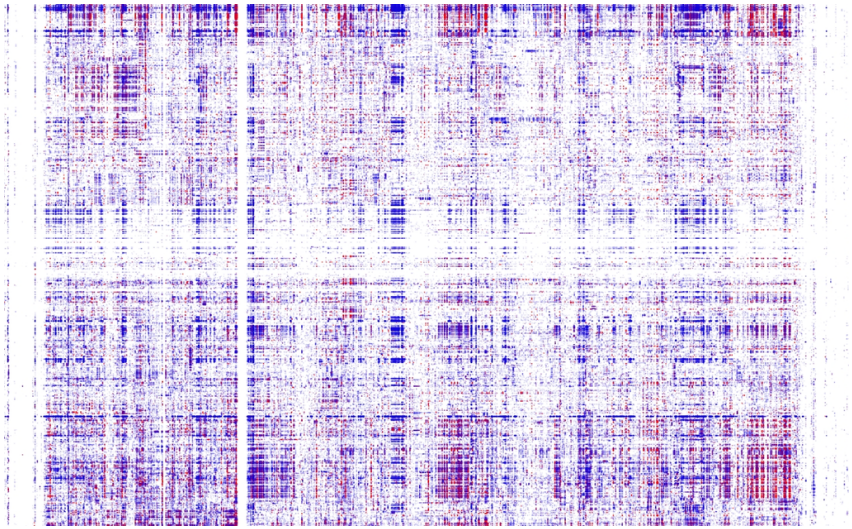
- BCF still too slow for querying hundreds of thousands and millions of samples
- bigger but 100x faster for certain operations on GTs

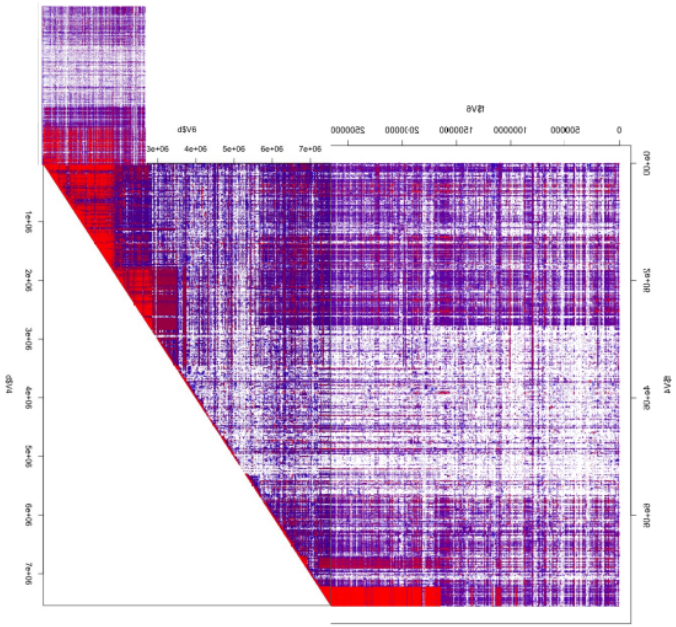
Custom formats for custom tasks

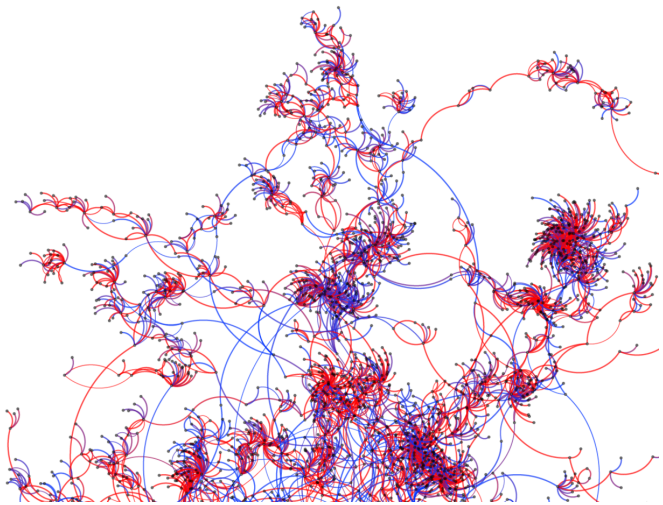
Region in LD



Random region







Method and figures by Marcus Klarqvist

Global Alliance for Genomics and Health

International coalition dedicated to improving human health
Mission

- establish a common framework to enable sharing of genomic and clinical data

Working groups

- clinical
- regulatory and ethics
- security
- data

Data working group

- beacon project .. test the willingness of international sites to share genetic data
- BRCA challenge .. advance understanding of the genetic basis of breast and other cancers
- matchmaker exchange .. locate data on rare phenotypes or genotypes
- reference variation .. describe how genomes differ so researchers can assemble and interpret them
- benchmarking .. develop variant calling benchmark toolkits for germline, cancer, and transcripts
- file formats .. CRAM, SAM/BAM, VCF/BCF

File formats

- http://samtools.github.io/hts-specs/

Quality Control

Biases in sequencing

- Base calling accuracy
- Read cycle vs. base content
- GC vs. depth
- Indel ratio

Biases in mapping

Genotype checking

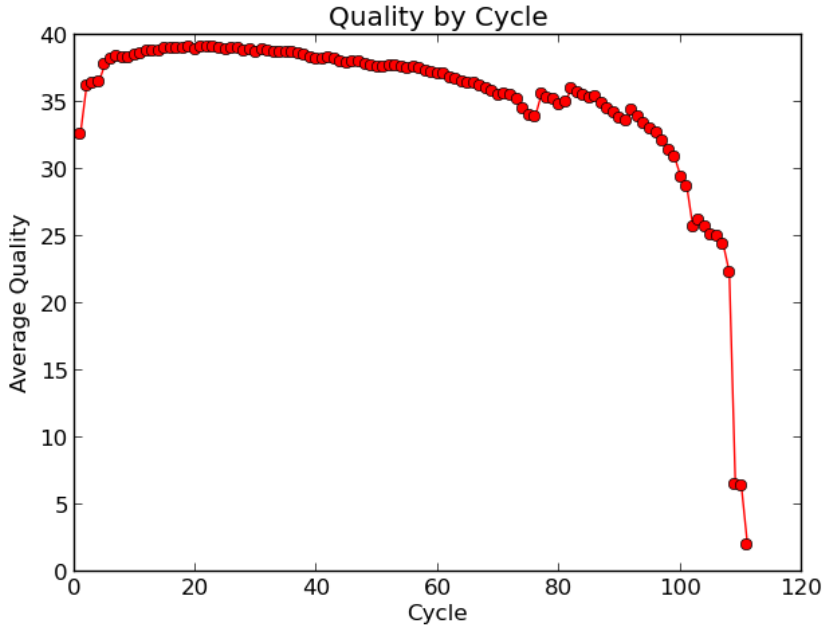
- Sample swaps
- Contaminations

Base quality

Sequencing by synthesis: dephasing

- growing sequences in a cluster gradually desynchronize
- error rate increases with read length

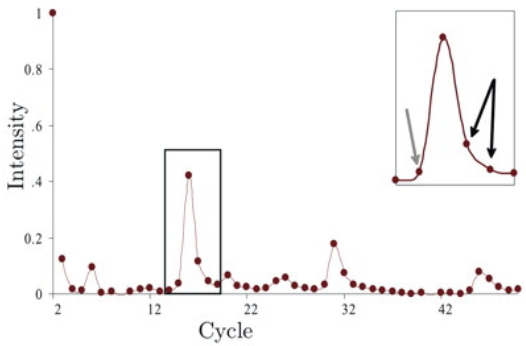
Calculate the average quality at each position across all reads



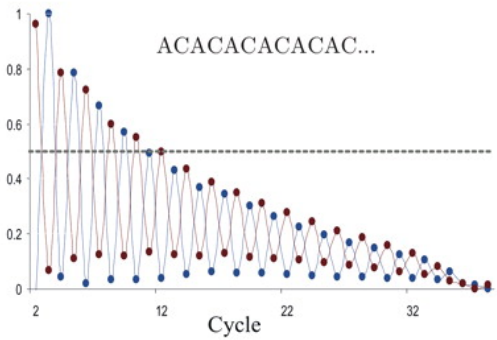
Quality	Probability of error	Call Accuracy
10 (Q10)	1 in 10	90%
20 (Q20)	1 in 100	99%
30 (Q30)	1 in 1000	99.9%
40 (Q40)	1 in 10000	99.99%

Base calling errors

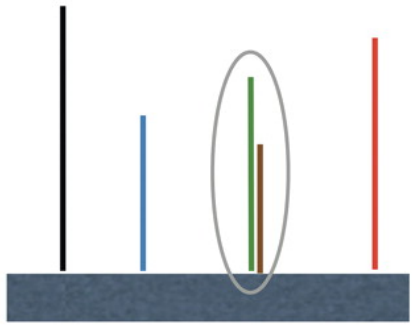
Phasing noise ϕ



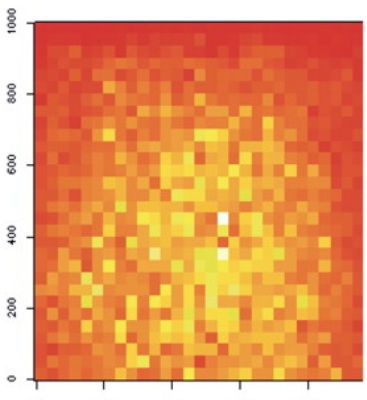
Signal Decay δ



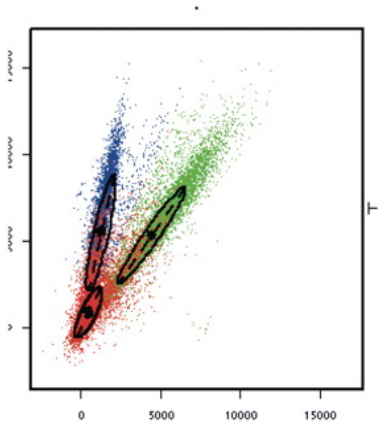
Mixed Cluster μ



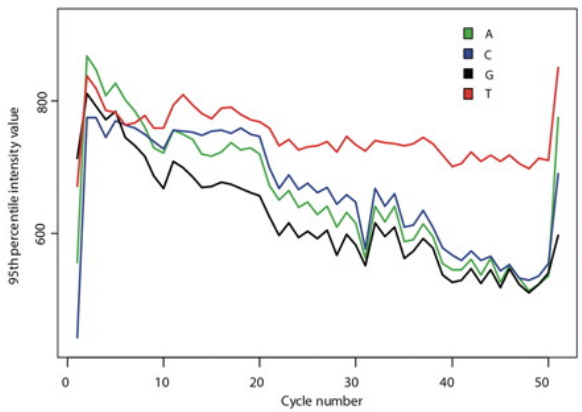
Boundary effects ω



Cross-talk Σ

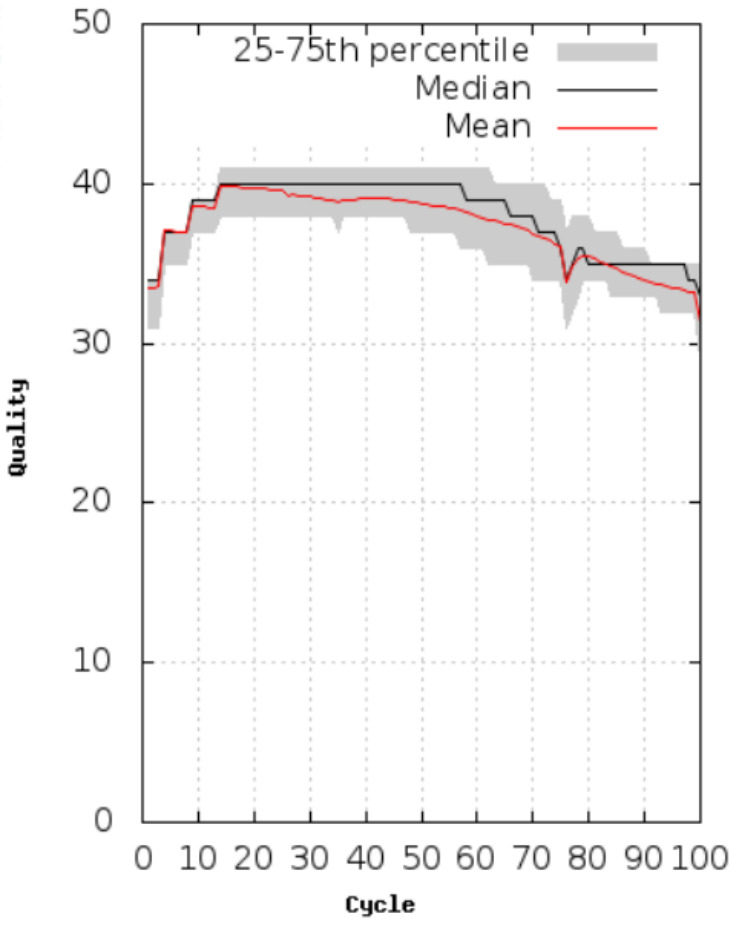


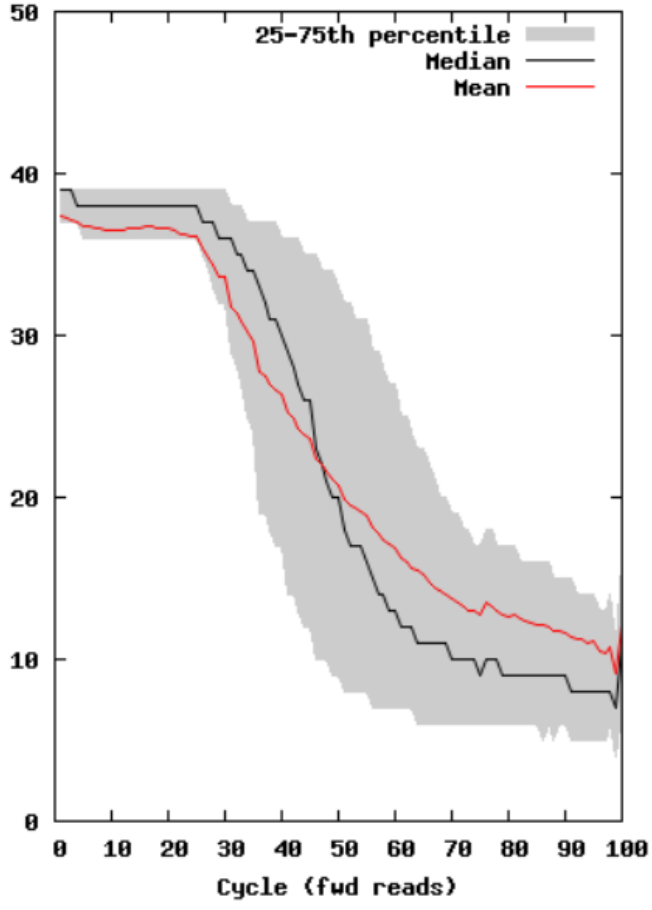
T fluophore accumulation τ



Base-calling for next-generation sequencing platforms, doi: 10.1093/bib/bbq077

Base quality

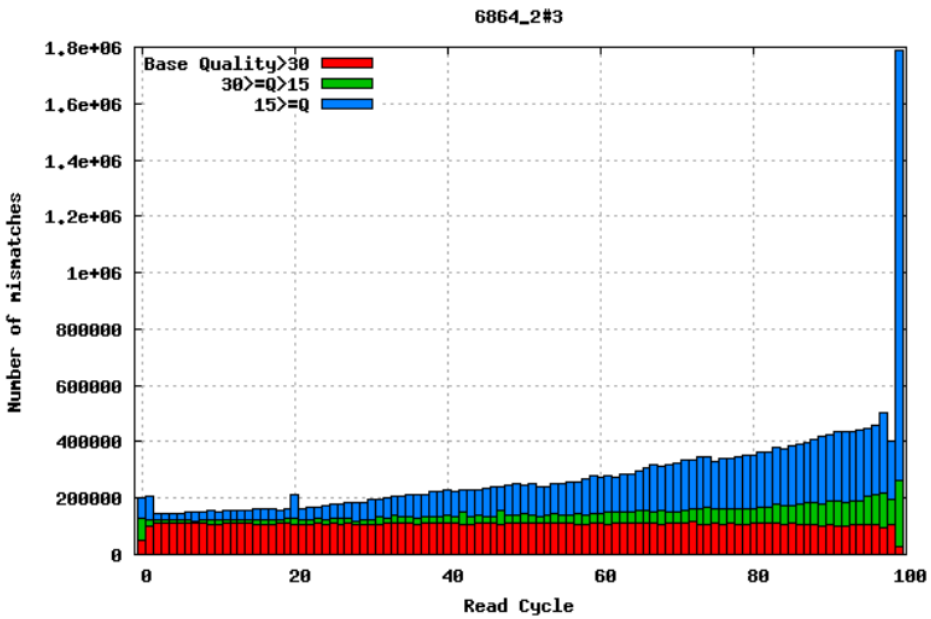


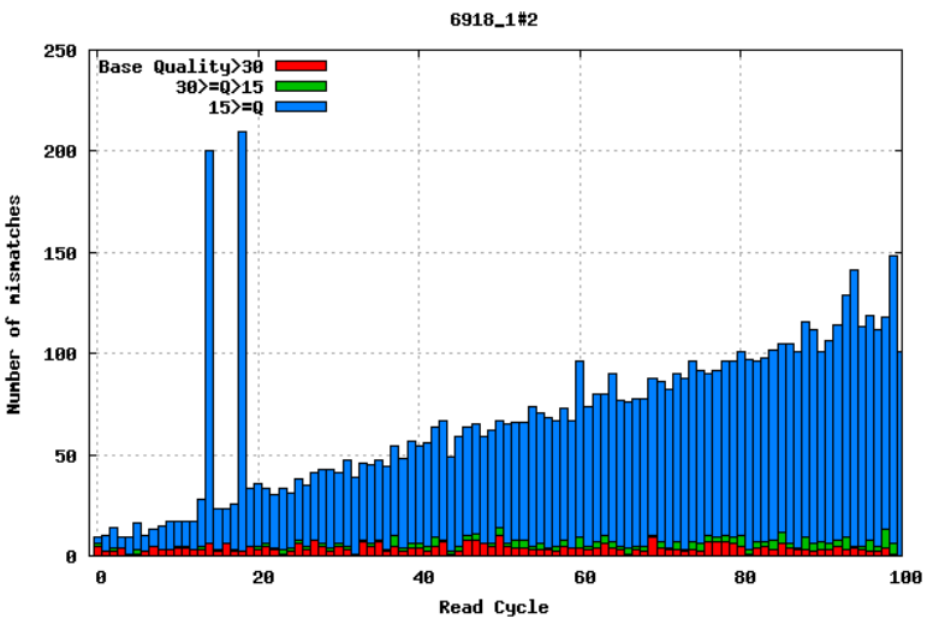


Mismatches per cycle

Mismatches in aligned reads (requires reference sequence)

- detect cycle-specific errors
- base qualities are informative!

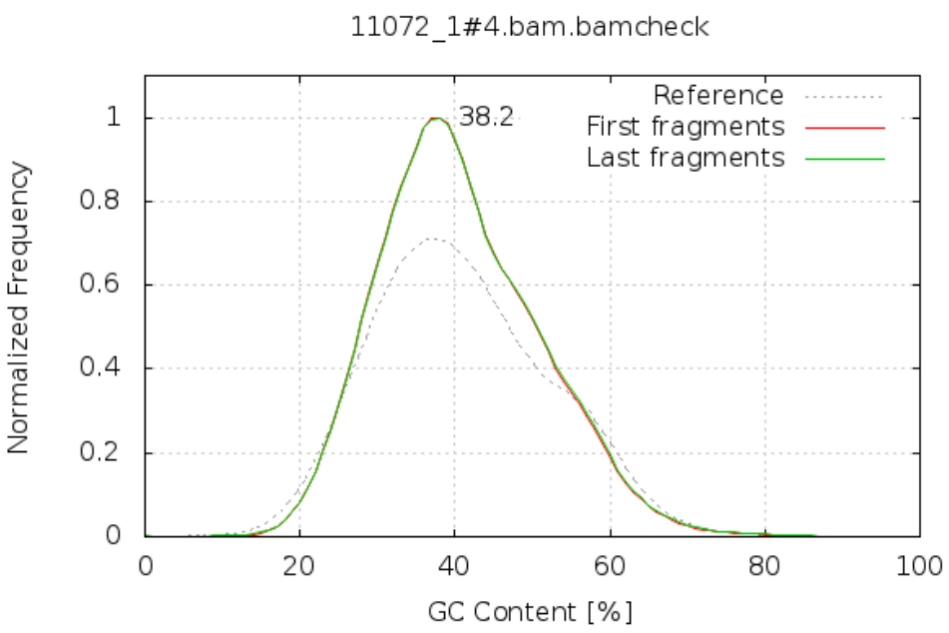


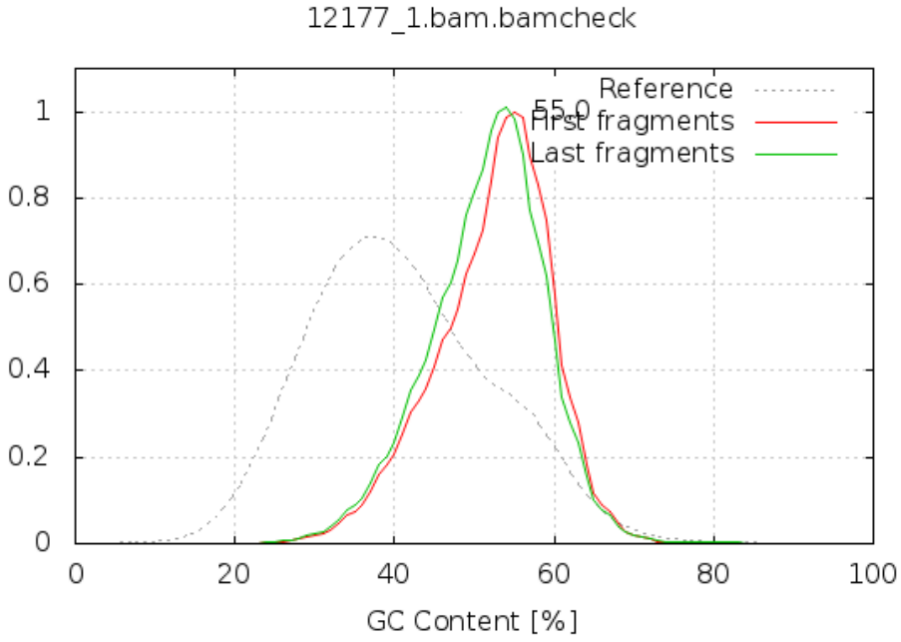


GC bias

GC- and AT-rich regions are more difficult to amplify

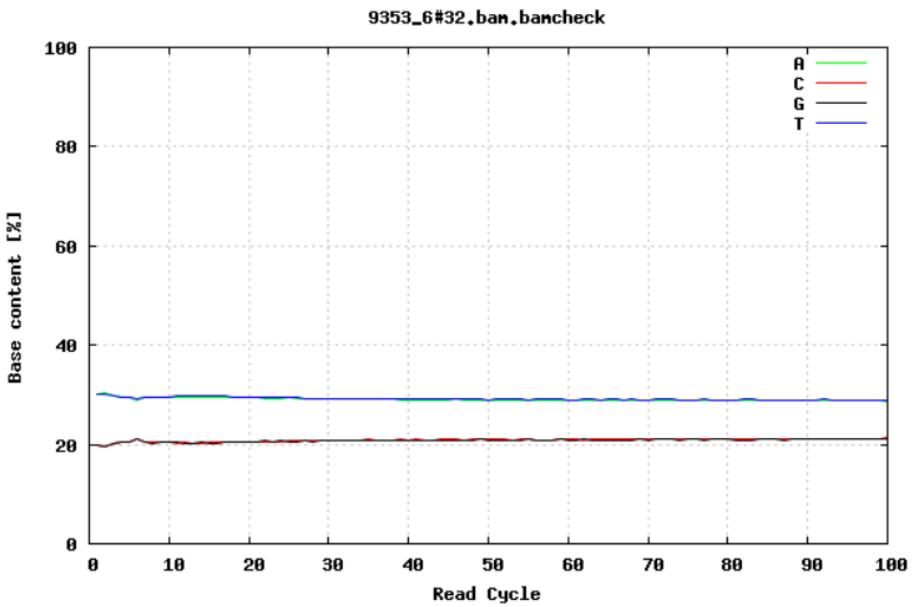
- compare the GC content against the expected distribution (reference sequence)

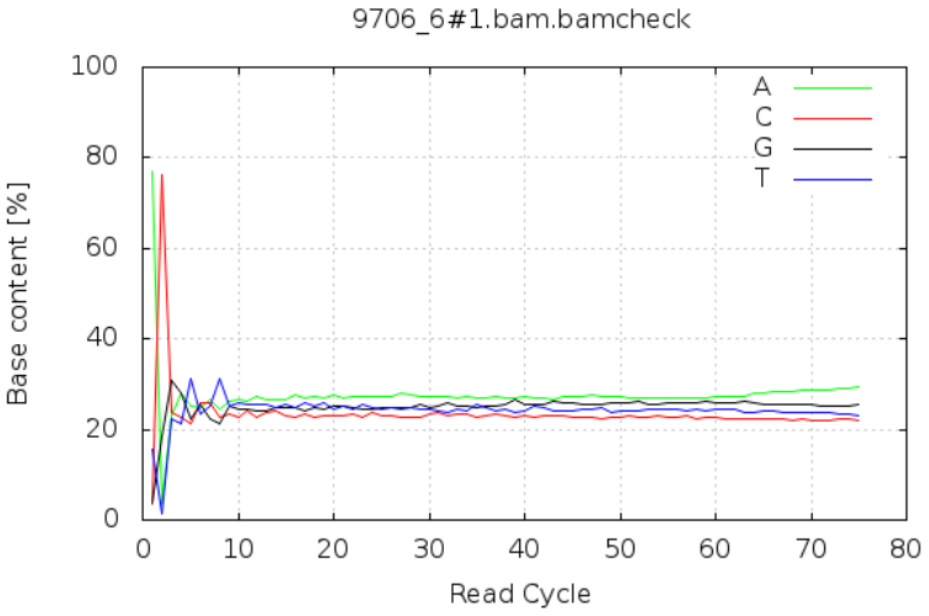




GC content by cycle

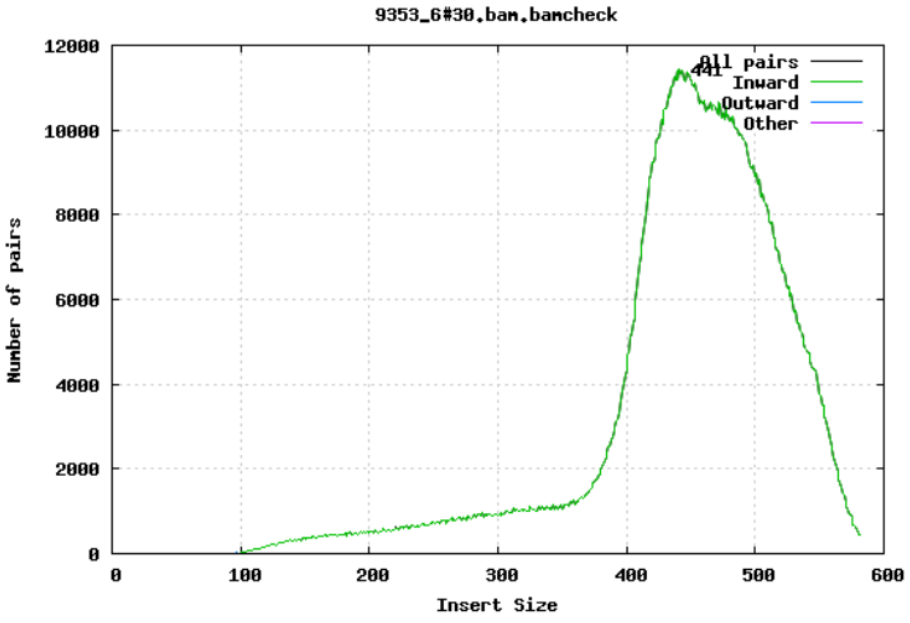
Was the adapter sequence trimmed?

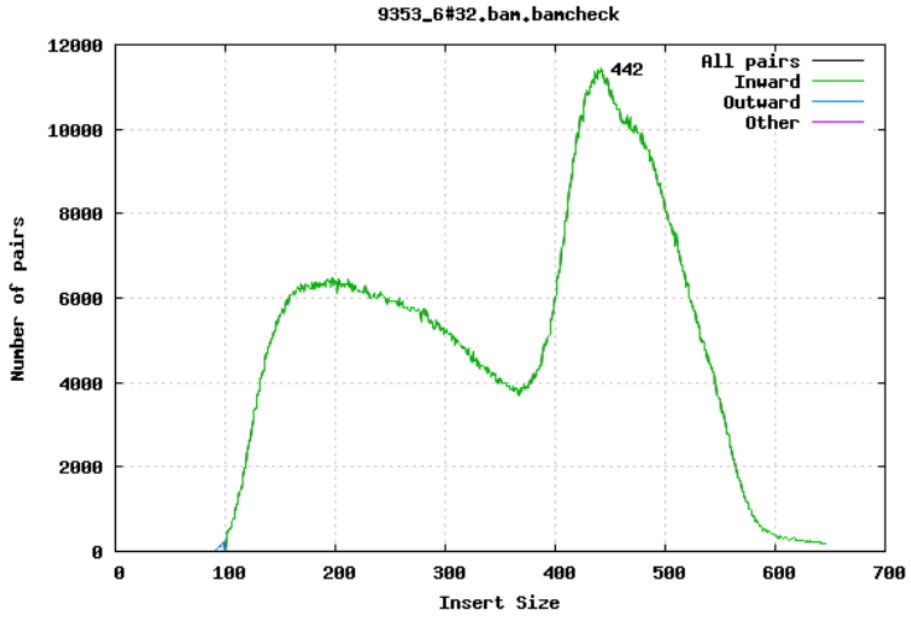




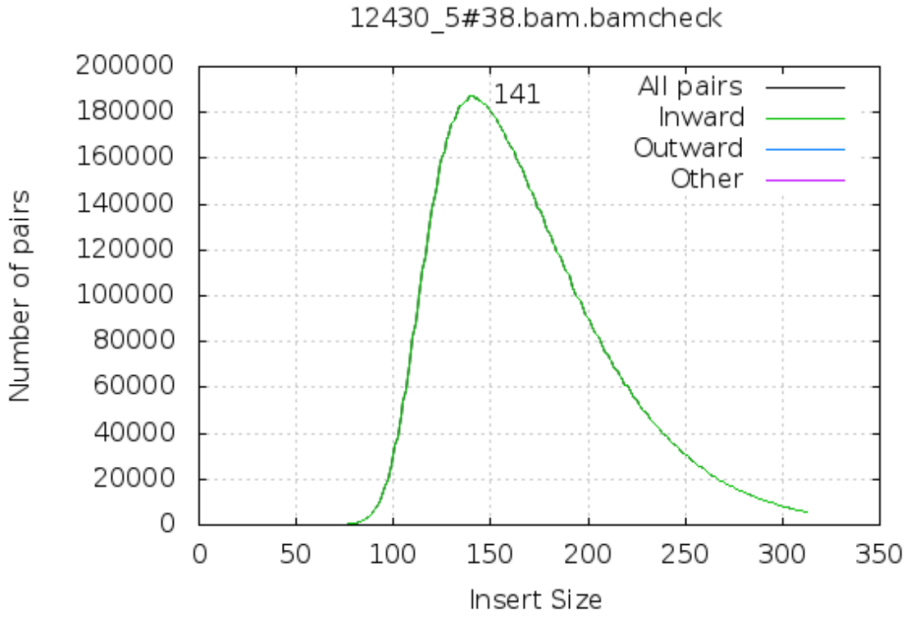
Fragment size

Paired-end sequencing: the size of DNA fragments matters





Quiz

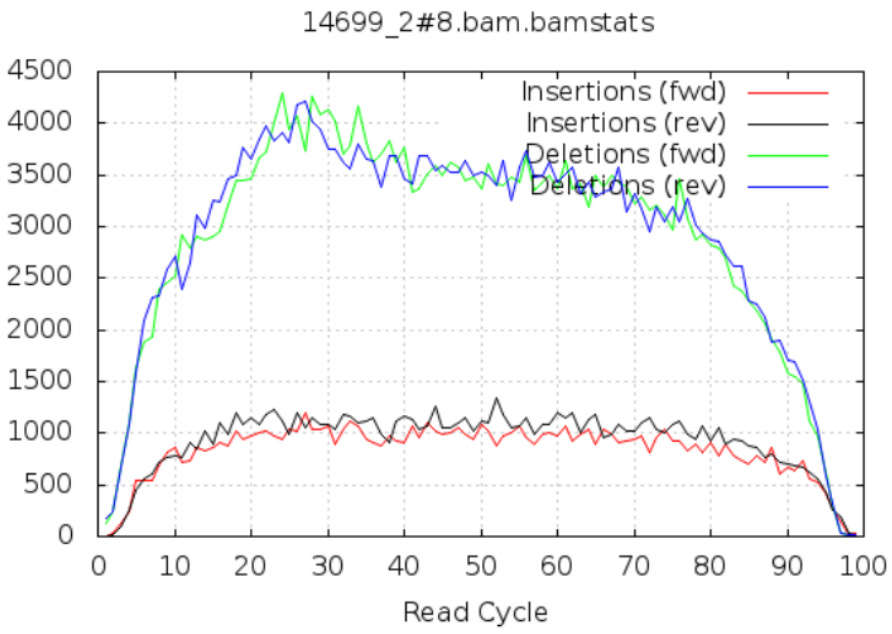


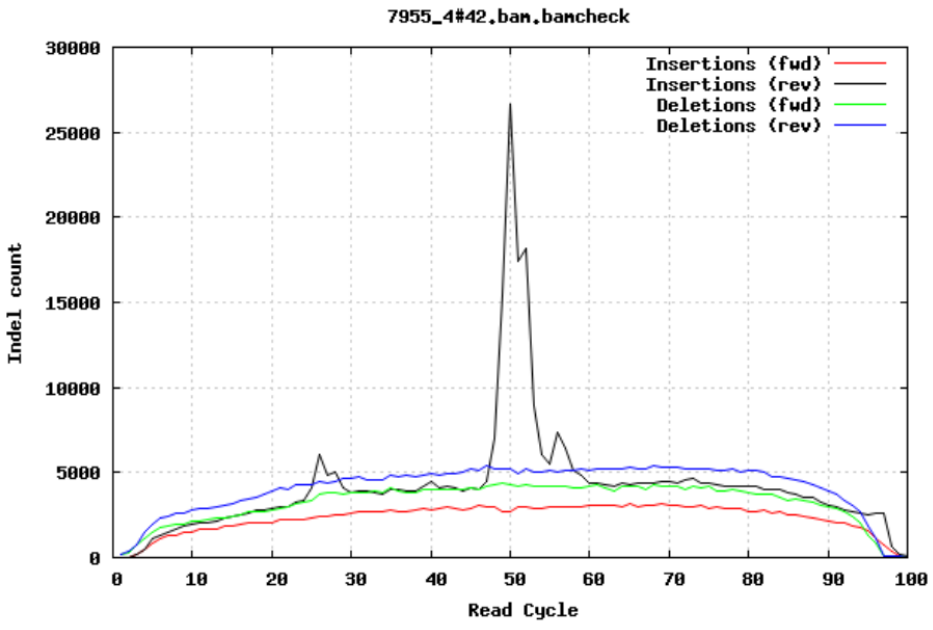
This is 100bp paired-end sequencing. Can you spot any problems??

Insertions / Deletions per cycle

False indels

- air bubbles in the flow cell can manifest as false indels

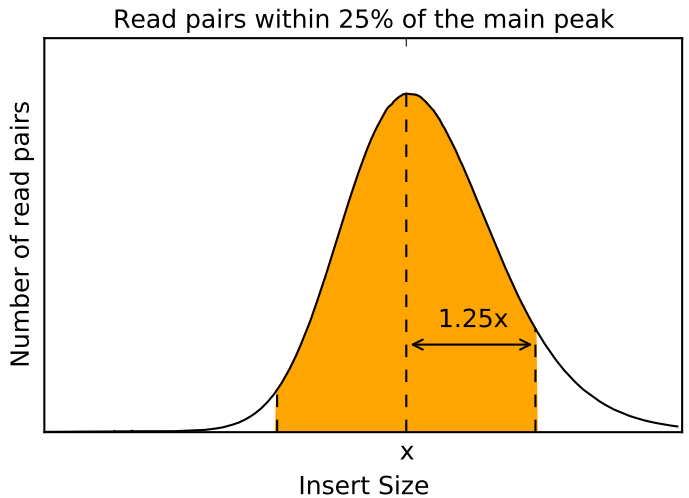




Auto QC tests

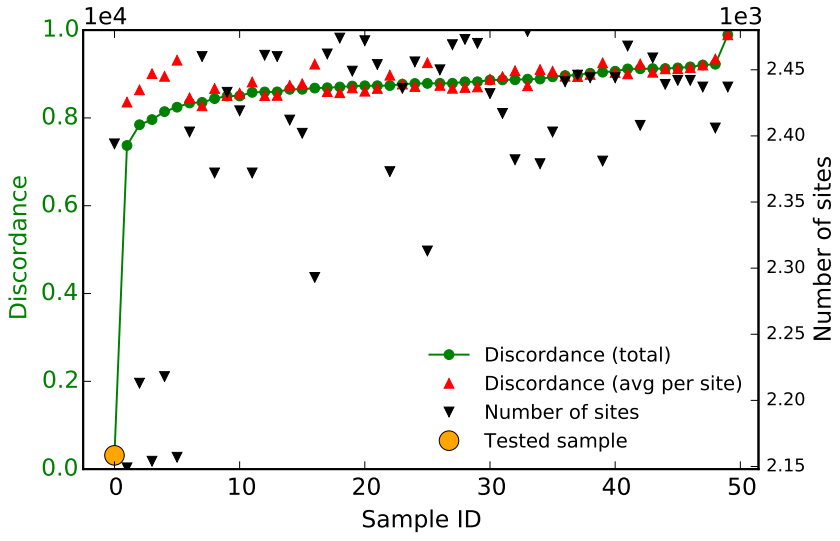
A suggestion for human data:

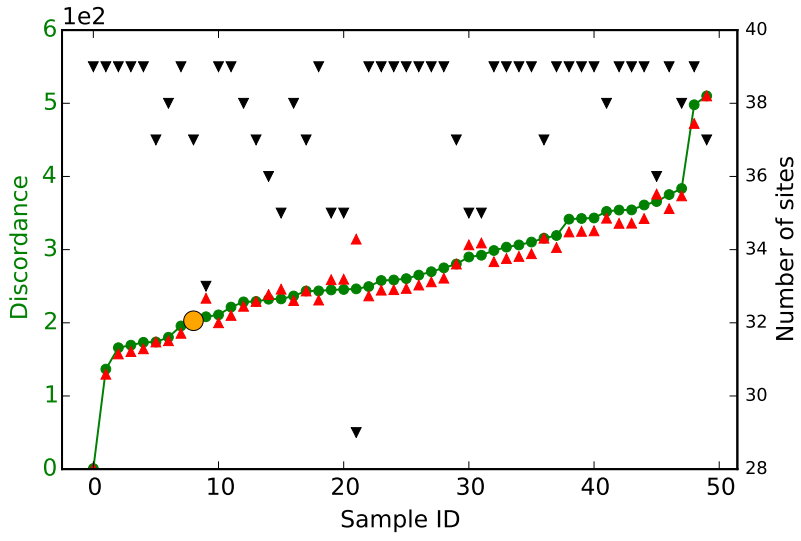
Minimum number of mapped bases	90%
Maximum error rate	0.02%
Maximum number of duplicate reads	5%
Minimum number of mapped reads which are properly paired	80%
Maximum number of duplicated bases due to overlapping read pairs	4%
Maximum in/del ratio	0.82
Minimum in/del ratio	0.68
Maximimum indels per cycle, factor above median	8
Minimum number of reads within 25% of the main peak	80%



Detecting sample swaps

Check the identity against a known set of variants





Software

Software used to produce graphs in these slides

- `samtools stats` and `plot-bamstats`
- `bcftools gtcheck`
- `matplotlib`