Practical Bioinformatics

Variant Calling 2

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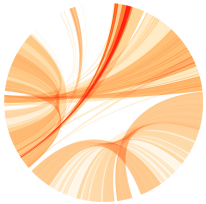
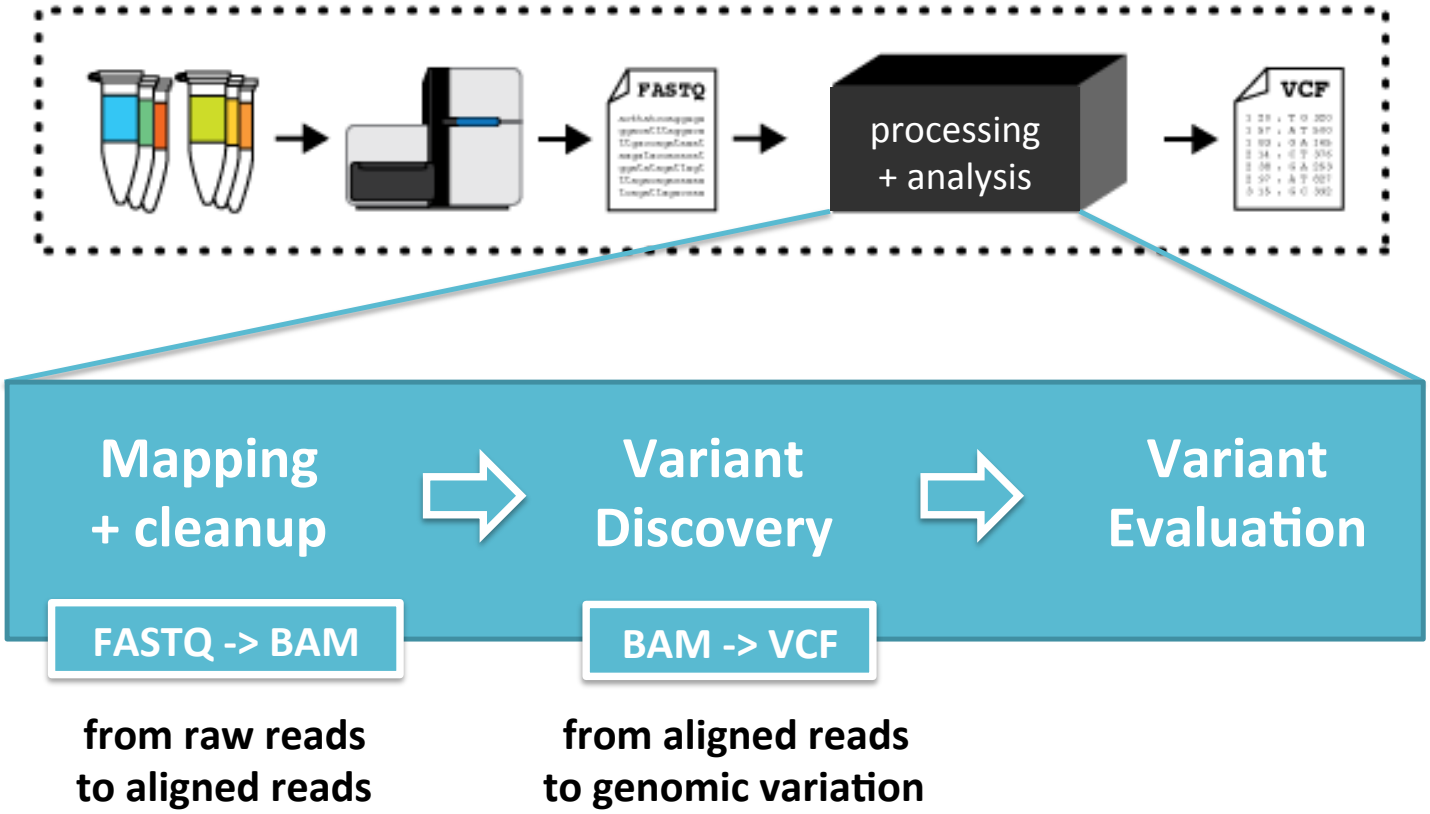


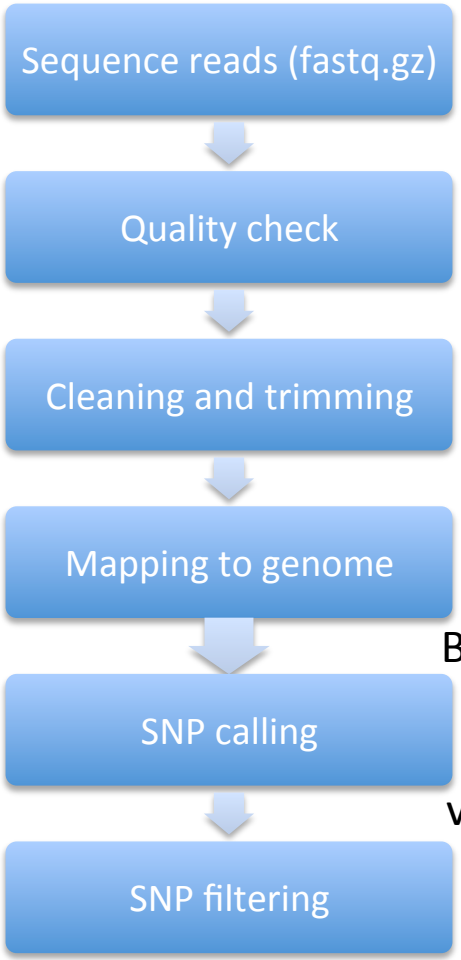
URPP Evolution in Action

From reads to variants



https://www.broadinstitute.org/gatk/guide/best-practices

Simple workflow



fastQC

trimmomatic/
cutadapt

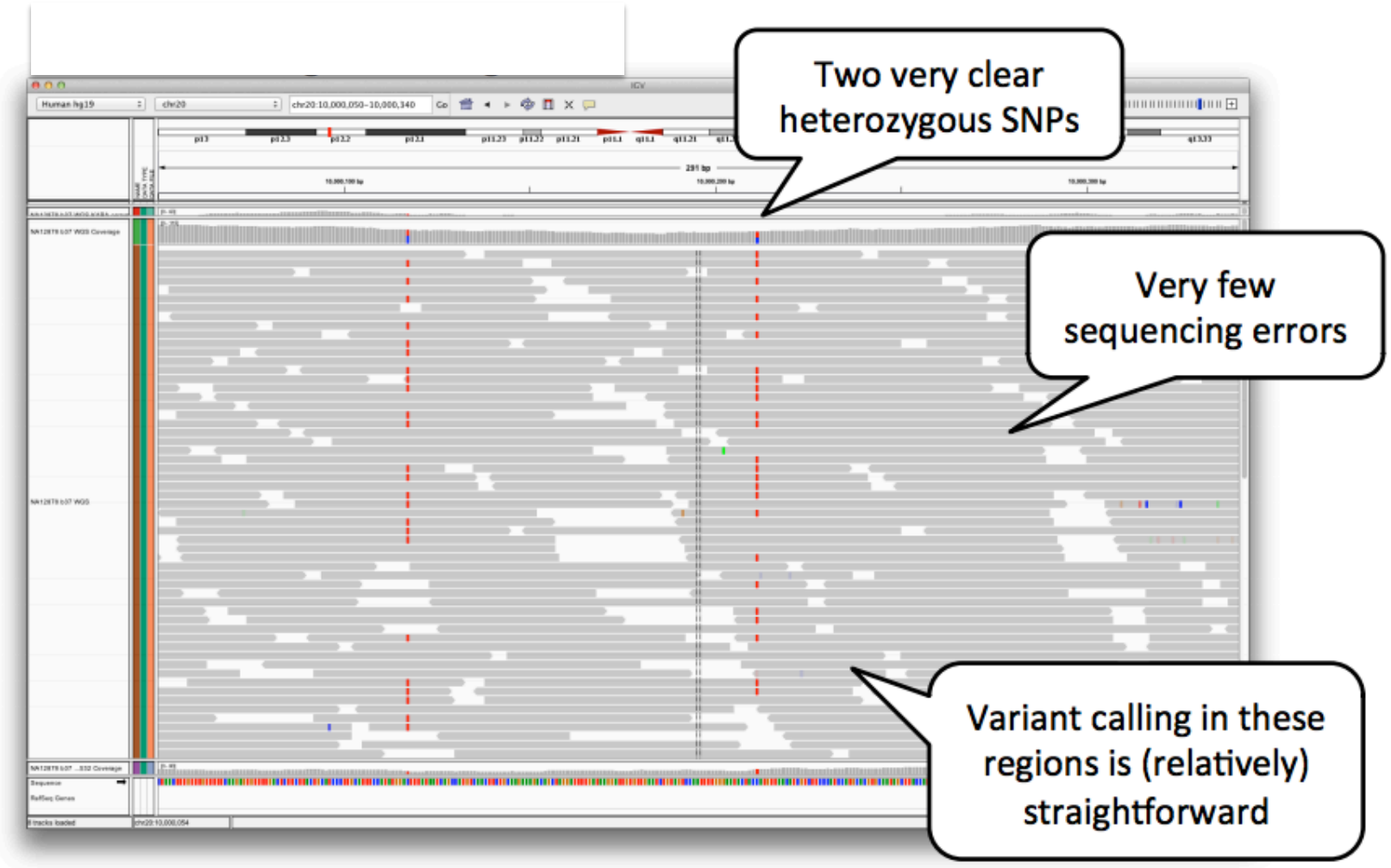
bwa mem

Freebayes /
samtools

bcftools

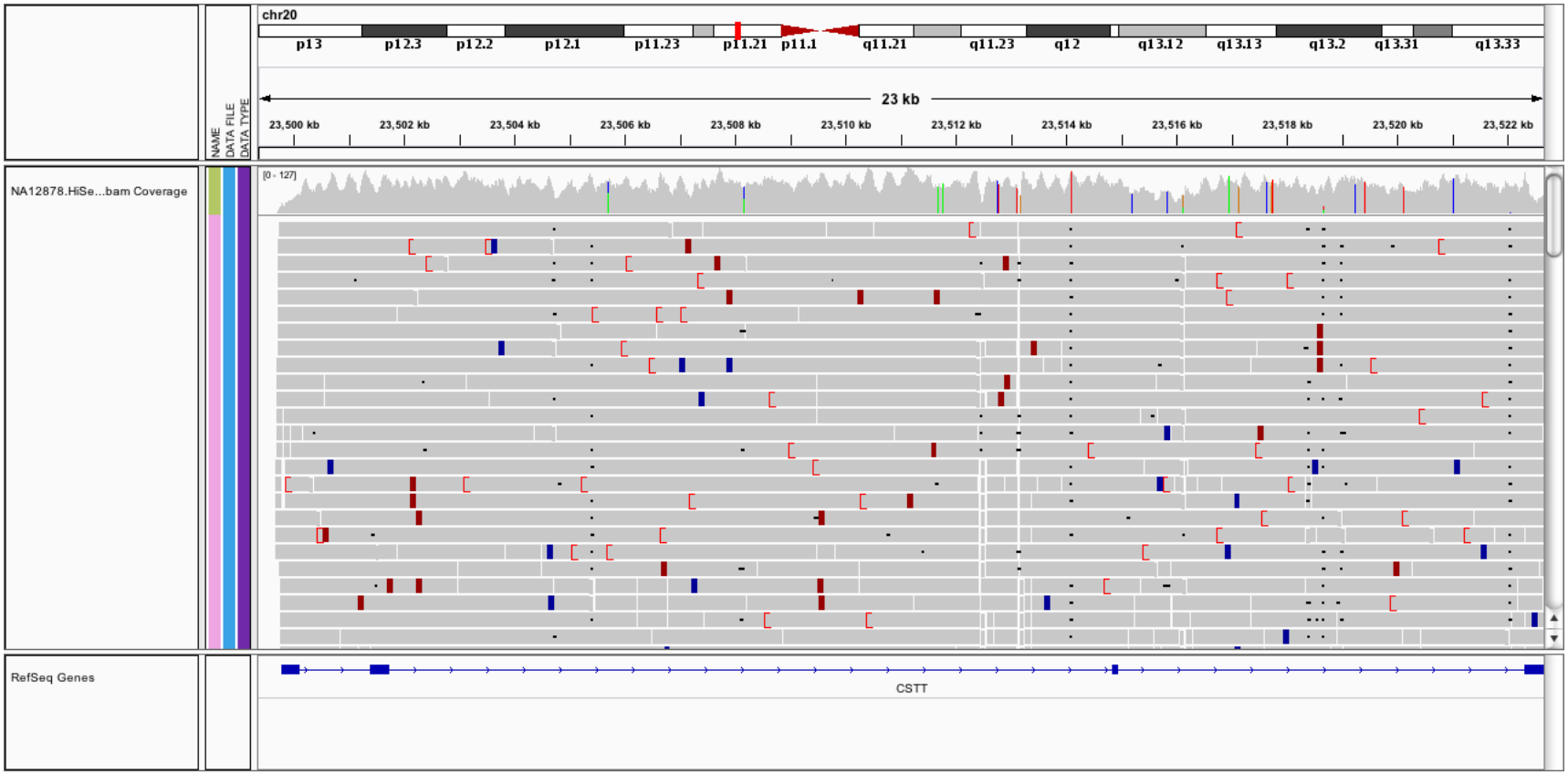
Simply counting?

Analysis of SNPs in well-behaved regions of the genome is pretty simple



http://www.broadinstitute.org/gatk//events/2247/agbt_2013_depristo.pdf

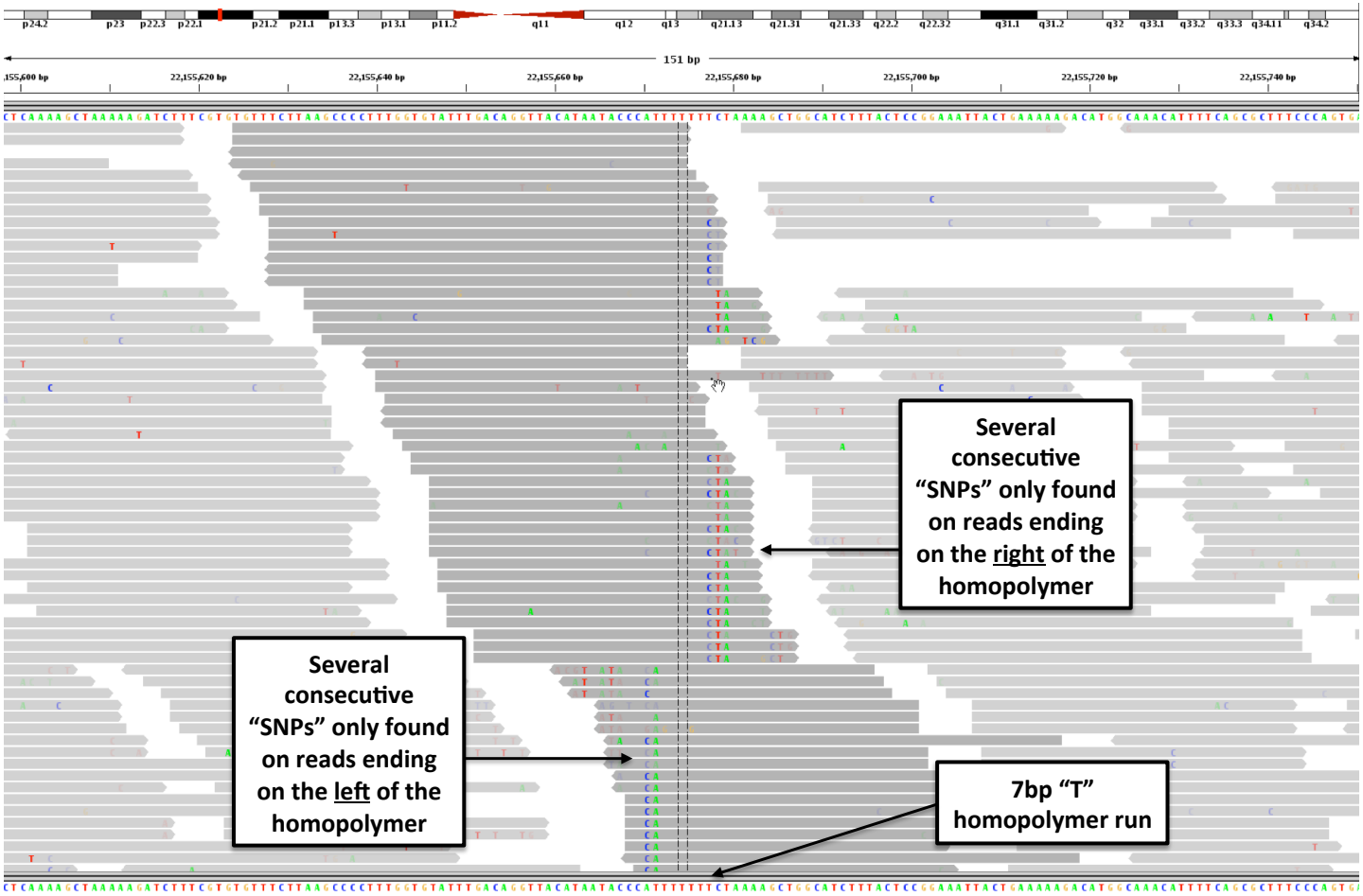
...Messy situations...



Real mutations or noise?

https://www.broadinstitute.org/gatk

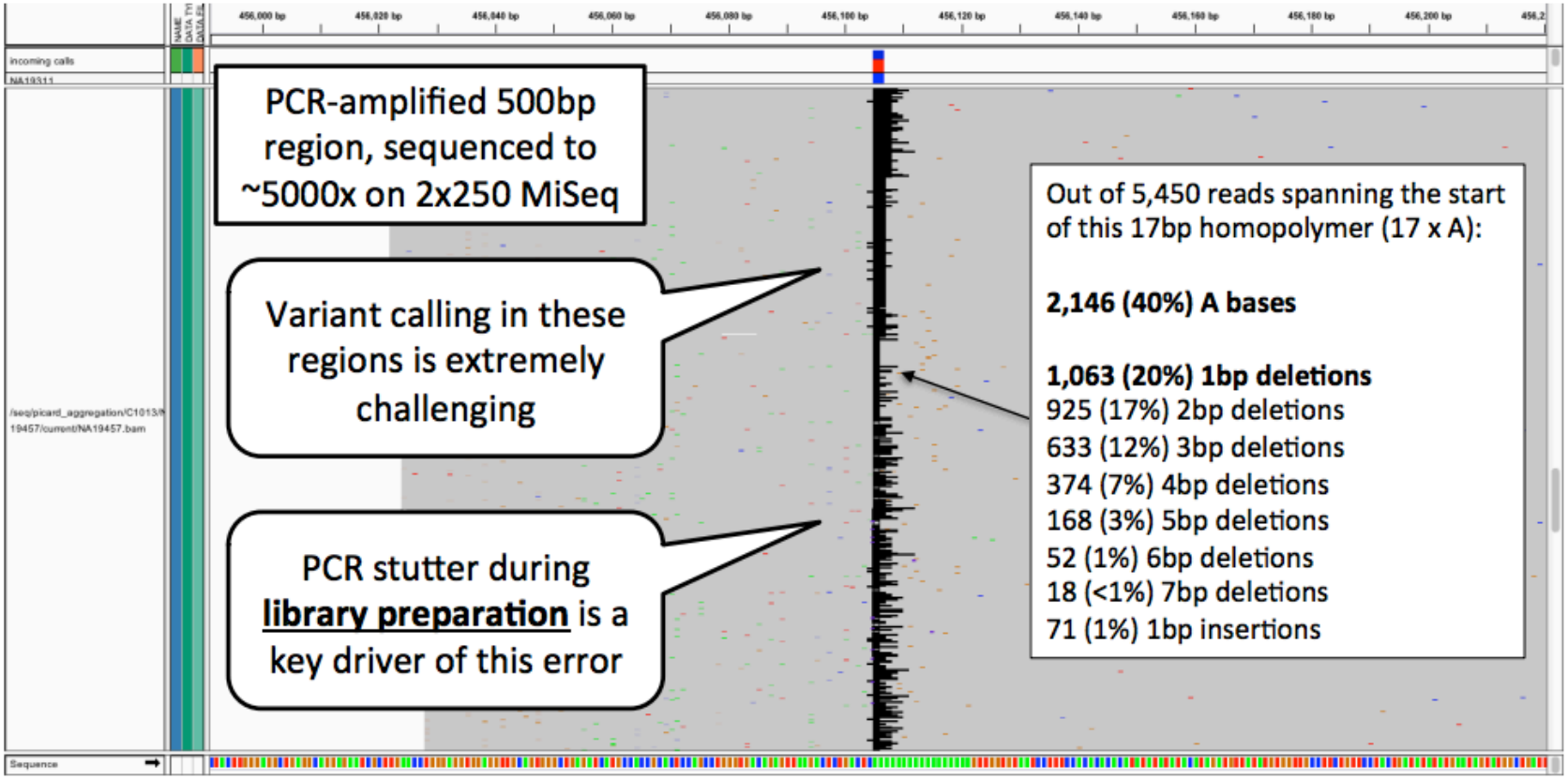
An example of a strand-discordant locus



https://www.broadinstitute.org/gatk

…it can get even worse

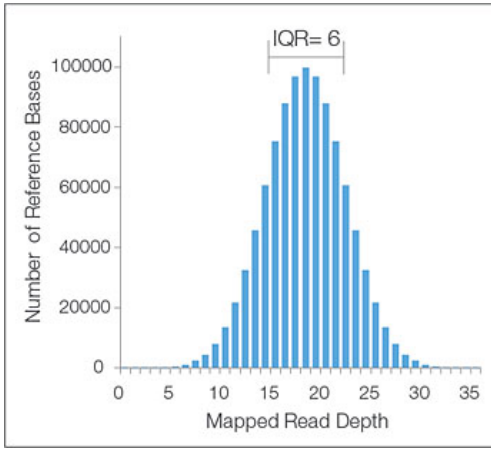
Poorly-behaved region of the genome



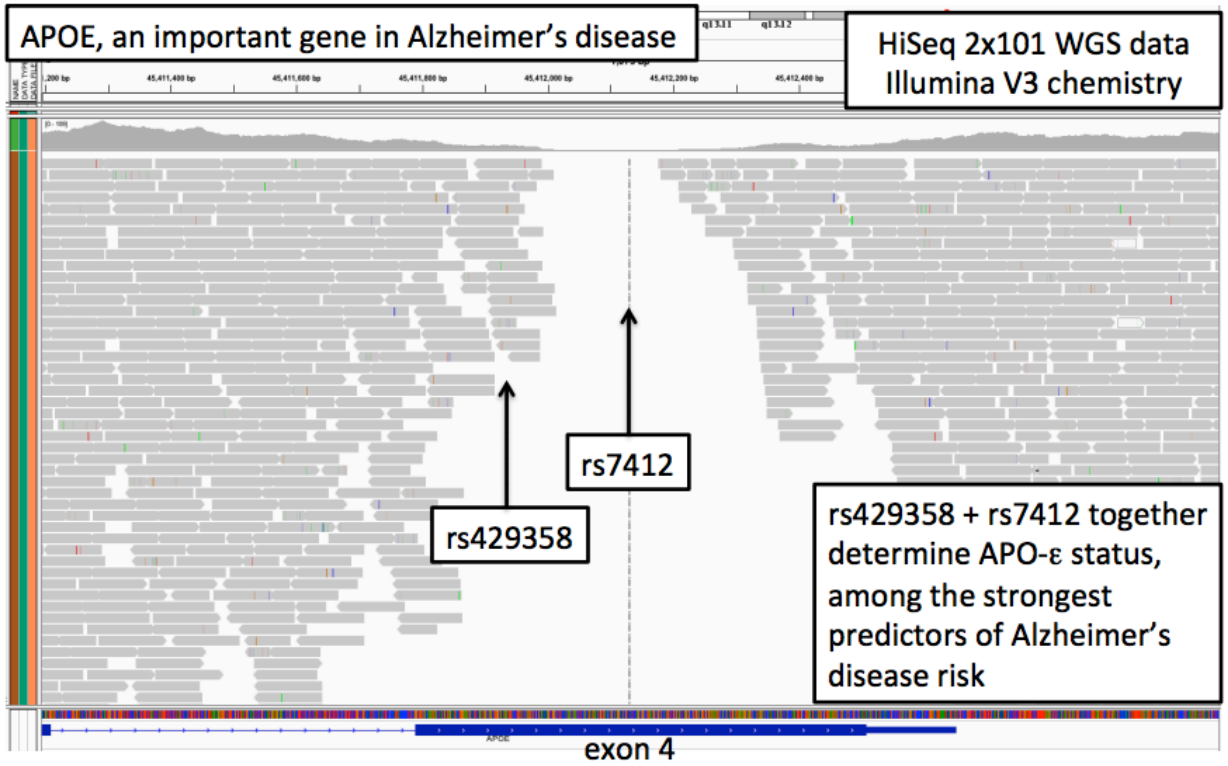
http://www.broadinstitute.org/gatk//events/2247/agbt_2013_depristo.pdf

Problem 1: Lack of coverage

Read depth histogram



Illumina

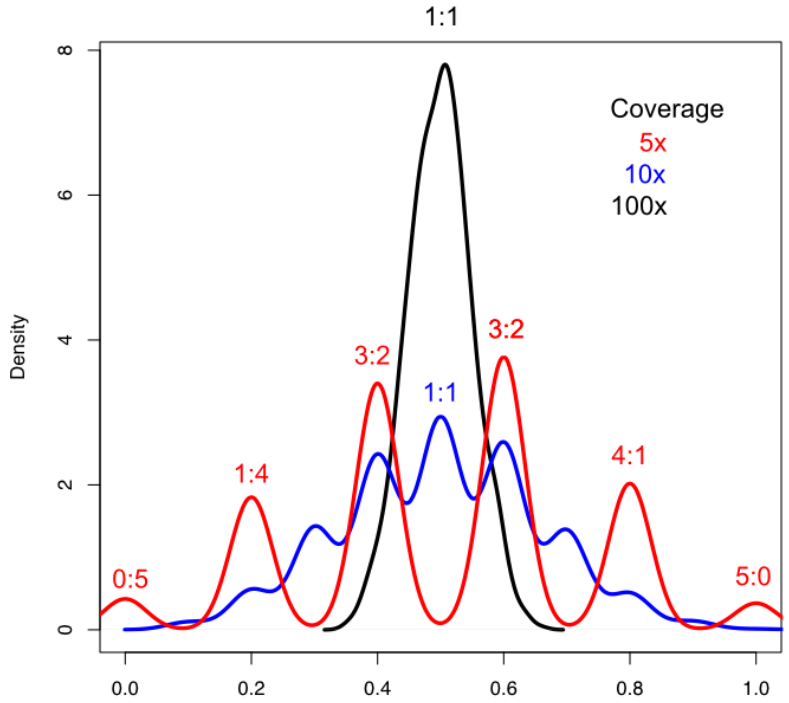


http://www.broadinstitute.org/gatk//events/2247/agbt_2013_depristo.pdf

depends on GC-content, library protocol, sampling effects, mapping problems, ...

Problem 2: Random sampling

Simulation of a heterozygous Site
(Binomial Distribution, 1000 samples each)



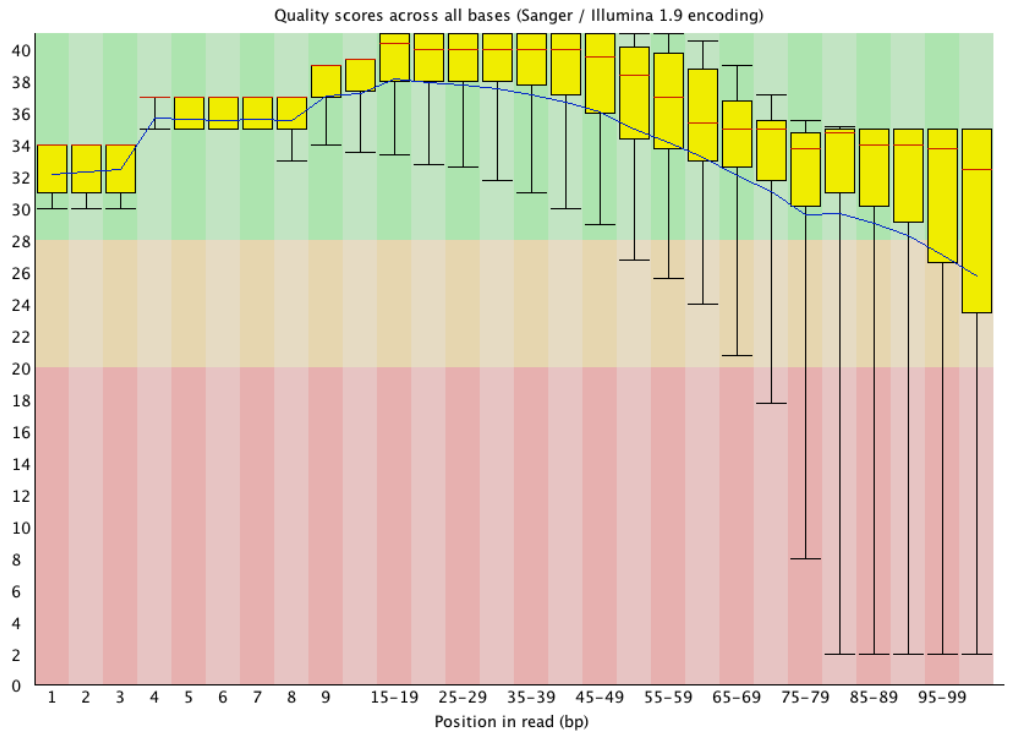
At 5x coverage, ~10% of sites are 0:5 or 5:0 !
At 10x coverage, ~0.4% of sites are 0:10 or 10:0 !

Problem 3: Sequencing errors

Error rate and error profile are technology-specific

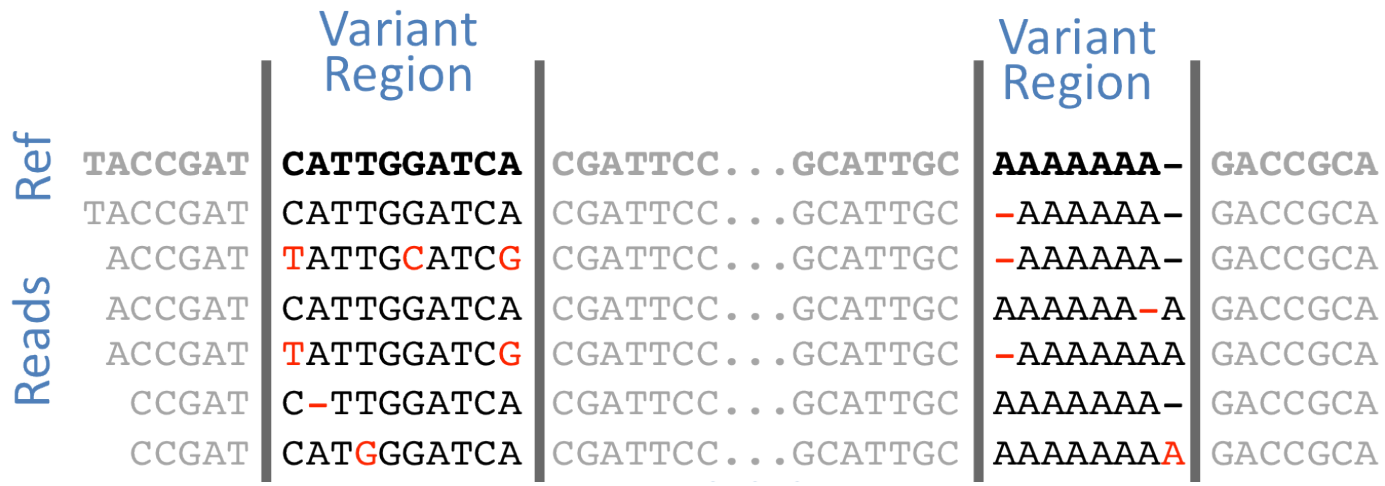
Illumina Sequencing

- Error Rate: > 0.1% (i.e. > 1 in 1000)
- mainly substitutions errors
- errors mostly at read's start and end



Problem 4: Incorrect mapping

With indels multiple alignments are possible



FreeBayes

-> Indel Realignment

Problem 4: Incorrect mapping cont.

- mismapped reads / errors in the alignment
  - segmental duplication
  - processed pseudogenes
  - close paralogs
  - repetitive sequences
  - small but complex indels
  - allelic bias towards reference

- incomplete/missassembled reference genome

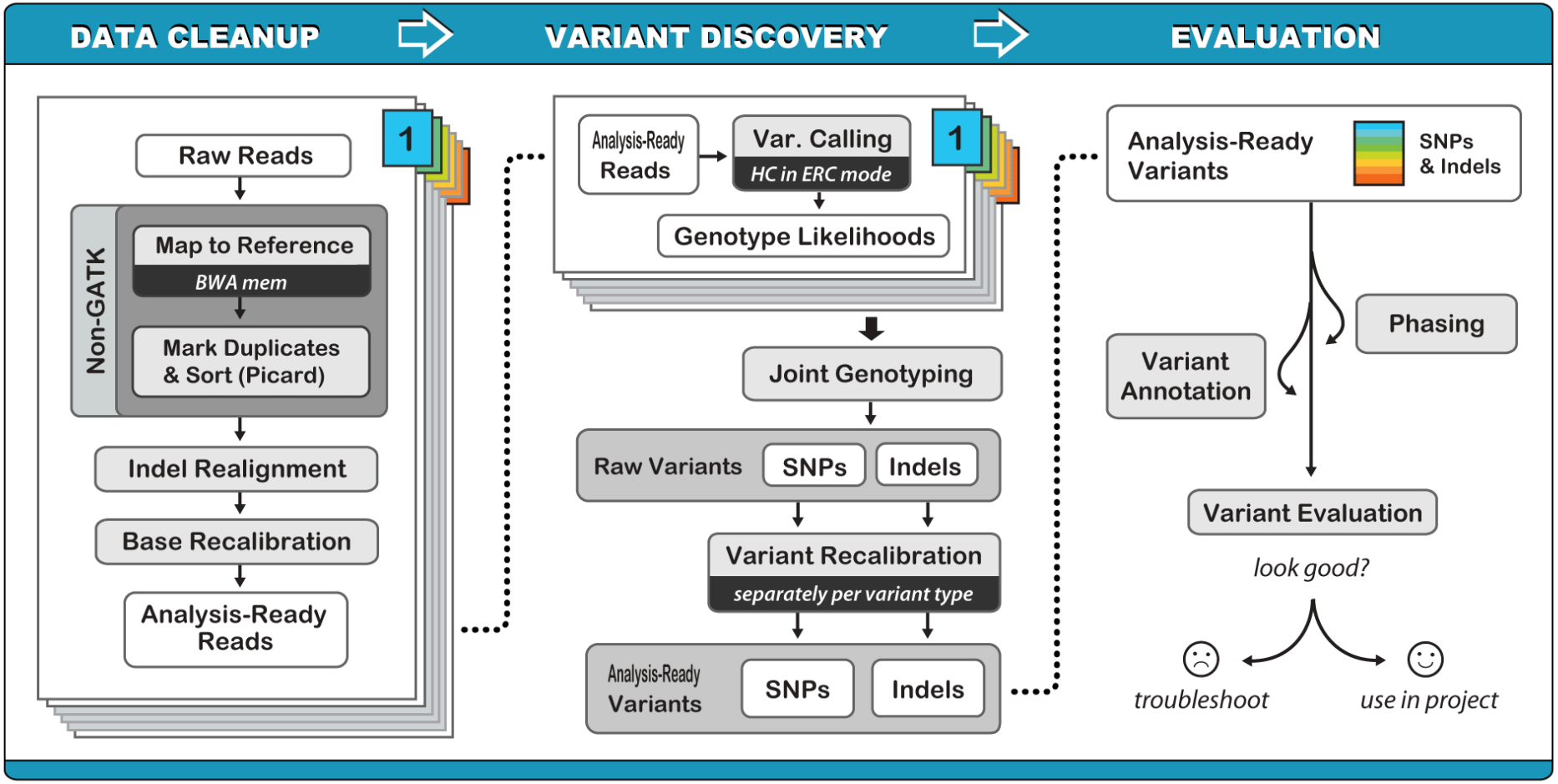
GATK

- Genome Analysis Toolkit (GATK)
- Toolkit focused on variant discovery in DNA and RNA

- initially developed for human 1000 genomes project
- handles any organism with any ploidy (<-> samtools/bcftools)
- Java-based command line tool

- Multi-sample SNP calling to increase power
- Automatic filtering ("Variant recalibration") for human data

GATK Workflow for DNA

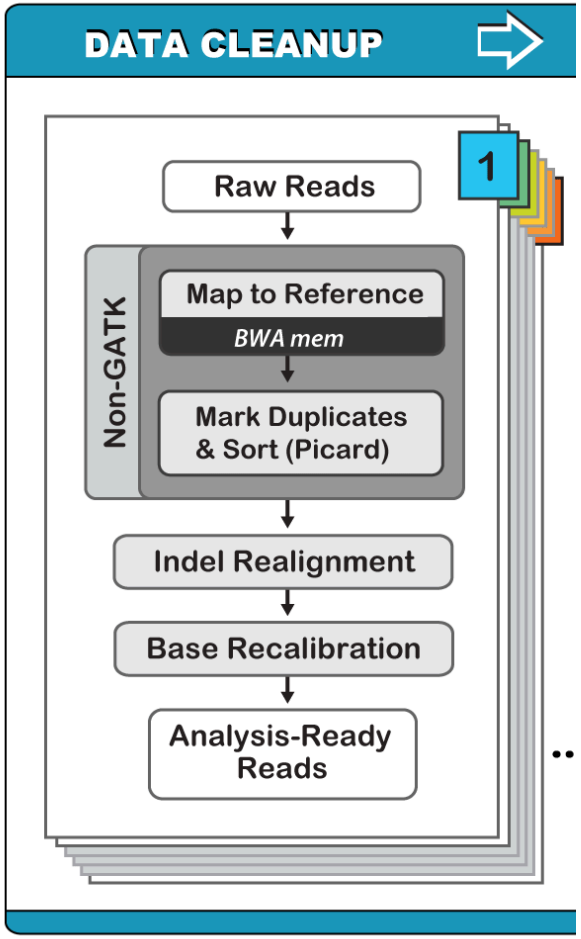


per lane

per sample

https://www.broadinstitute.org/gatk/guide/best-practices

Data Cleanup



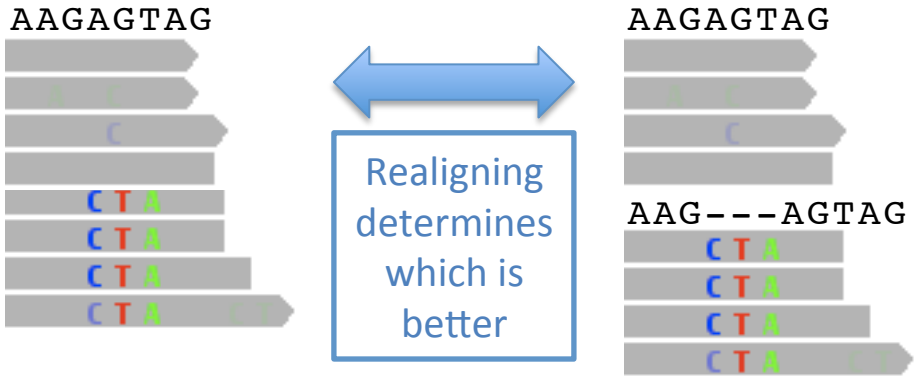
per lane

Mark PCR Duplicates

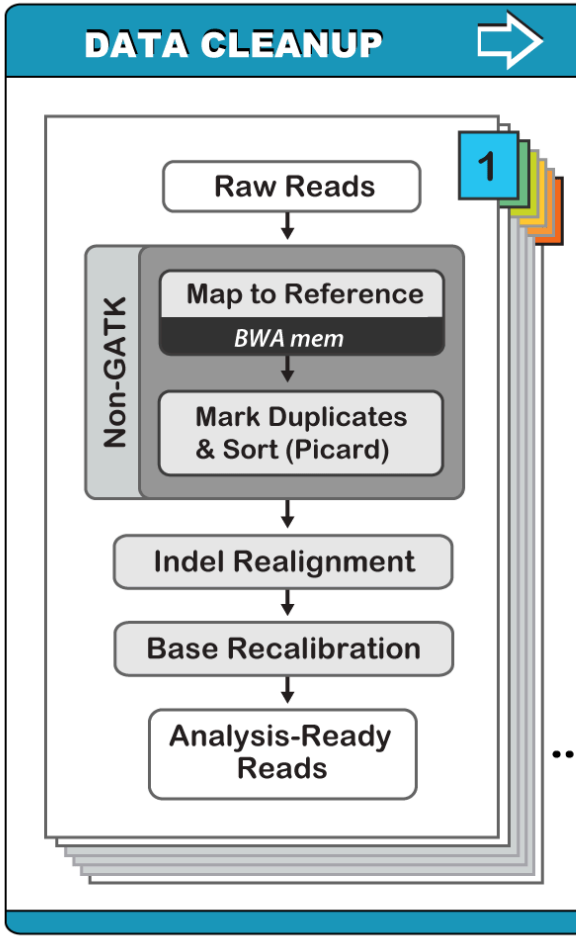
- come from same input DNA template- have same start position on reference
- non-independent measurements violate statistical assumptions
- not applicable in amplicon seq

Indel Realignment (may disappear)

- Indels in reads (especially near the ends) can trick the mappers into mis-aligning with mismatches



Data Cleanup cont.



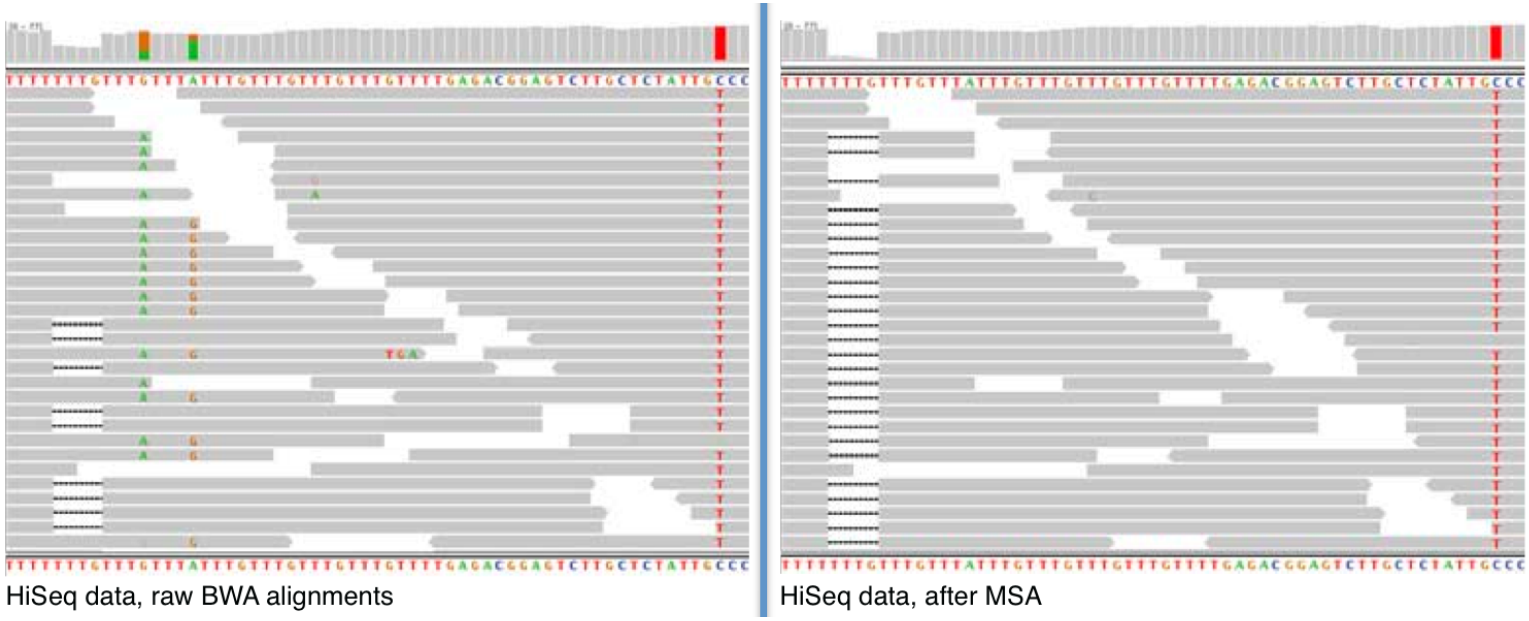
per lane

Base Recalibration

- Base quality scores are per-base estimates of error emitted by the sequencing machines
  -> various sources of systematic error
  -> over- or under-estimated base qualities

- Base quality score recalibration
  apply machine learning to model errors empirically and adjust the quality scores

Before and after indel realignment



HiSeq data, raw BWA alignments

HiSeq data, after MSA

https://www.broadinstitute.org/gatk

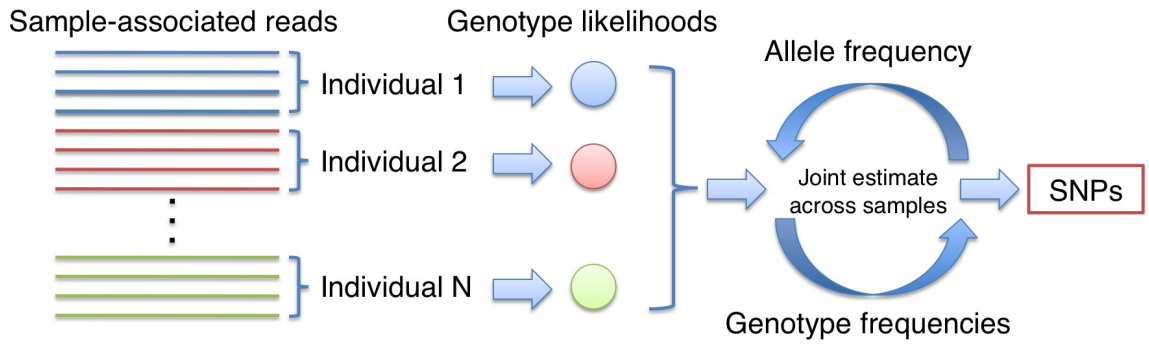
Variant Calling

- modelling various error types
- expected distribution of calls
  (homozygous AA, homozygous variant BB, heterozygous AB)
- from GATK v3.3 HaplotypeCaller is recommended for all cases

HaplotypeCaller
calls SNPs and indels simultaneously
performs a local de-novo assembly
any ploidy
more accurate (especially for indels)
up to 100s samples (-> GVCF mode)

Multi-sample analysis

- To gain sensitivity some SNP callers allow multi-sample variant calling (multiple individuals/samples from the same or closely related species)

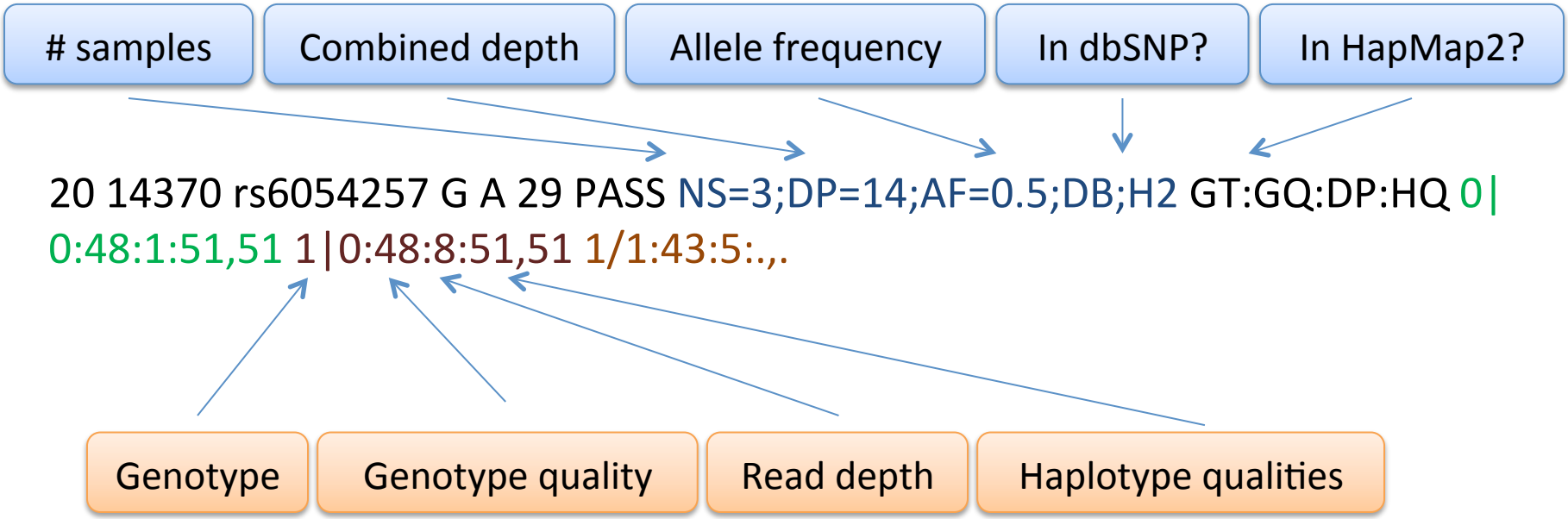


- ~ Hardy-Weinberg Equilibrium
- Genotypings like this: AB, AB, AB, AB, AB, AB have much lower probability than AA, AA, AB, BB, AA, AB, AA
- in reality: multiple alleles,...

VCF

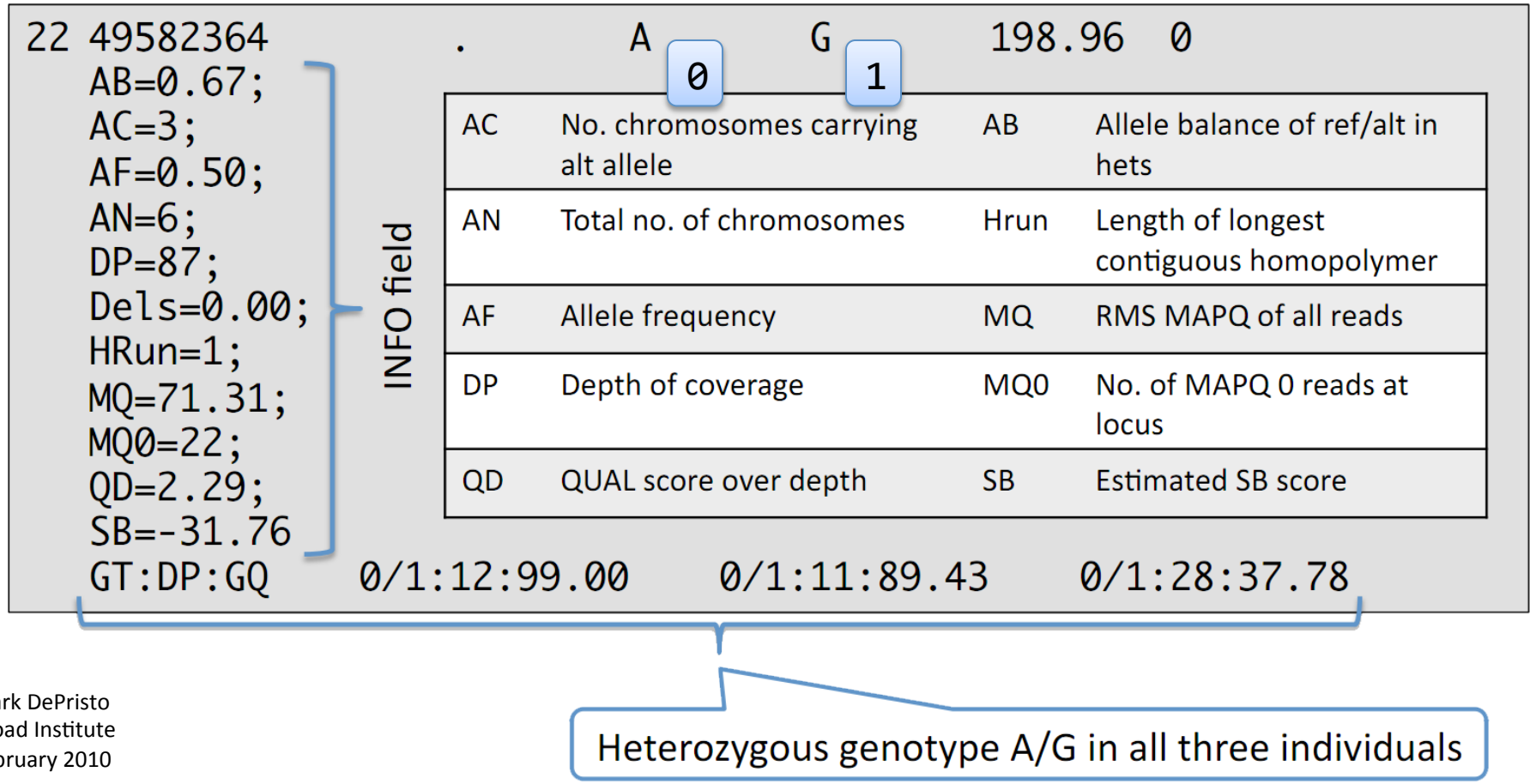
##fileformat=VCFv4.0

#CHROM POS ID REF ALT QUAL FILTER INFO FORMAT NA00001 NA00002 NA00003



VCF info field

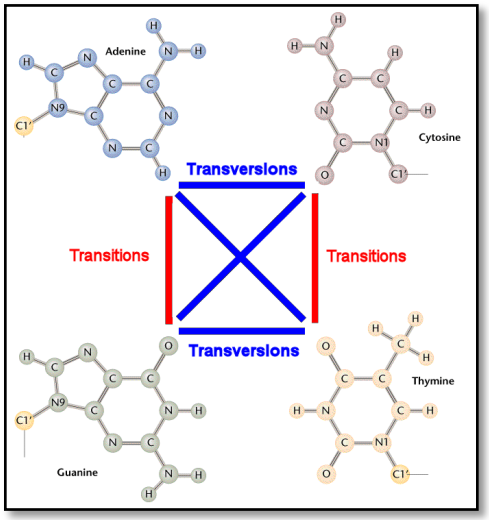
VCF record for an A/G SNP at 22:49582364



Mark DePristo
Broad Institute
February 2010

Variant Filtering

- The optimal threshold for filtering has to be determined empirically
- trade-off sensitivity <-> specificity
- which metric of variant call confidence?



Intrinsic

- Transitions:transversions ratio (Ti/Tv)
  (e.g. nuclear genes in humans close to 2)

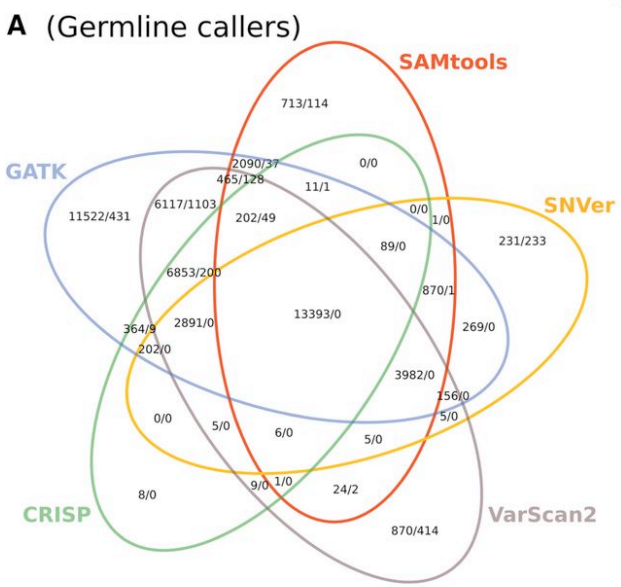
Experimental Validation

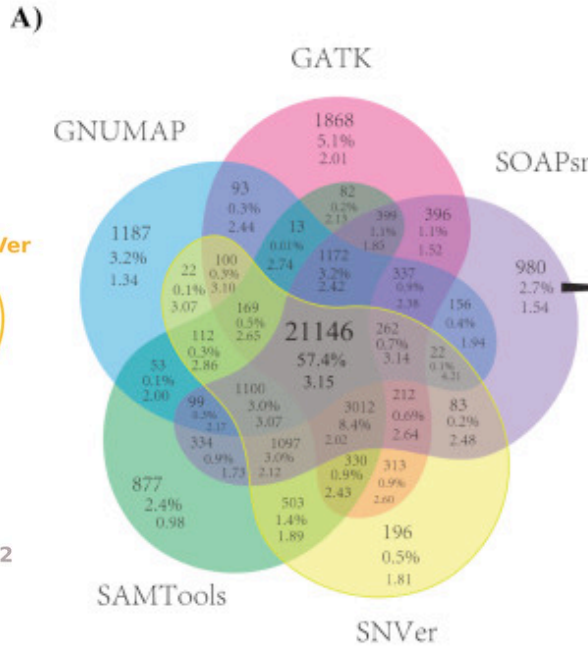
- Small-scale validation (Sanger seq, qPCR, pyrosequencing, ...)
- Orthogonal data (e.g. microarrays, different seq platform)
- Concordance among Trios (2 parents + 1 child)

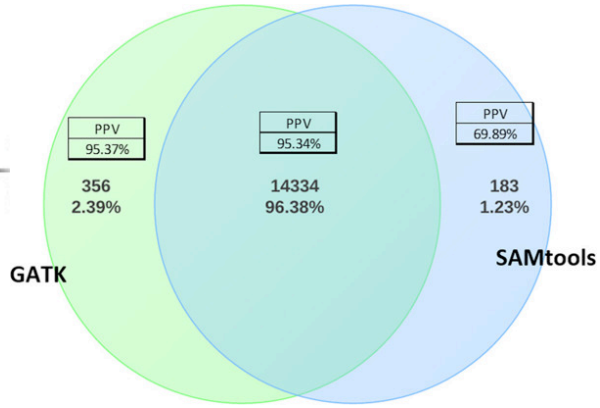
Comparison

- BAM preprocessing steps (e.g., indel realignment and quality score base recalibration using GATK) had only a modest impact on the variant calls (PMID:25289185)
- Realignment of mapped reads and recalibration of base quality scores before SNV calling proved to be crucial to accurate variant calling (PMID: 25078893)

Who performs best?







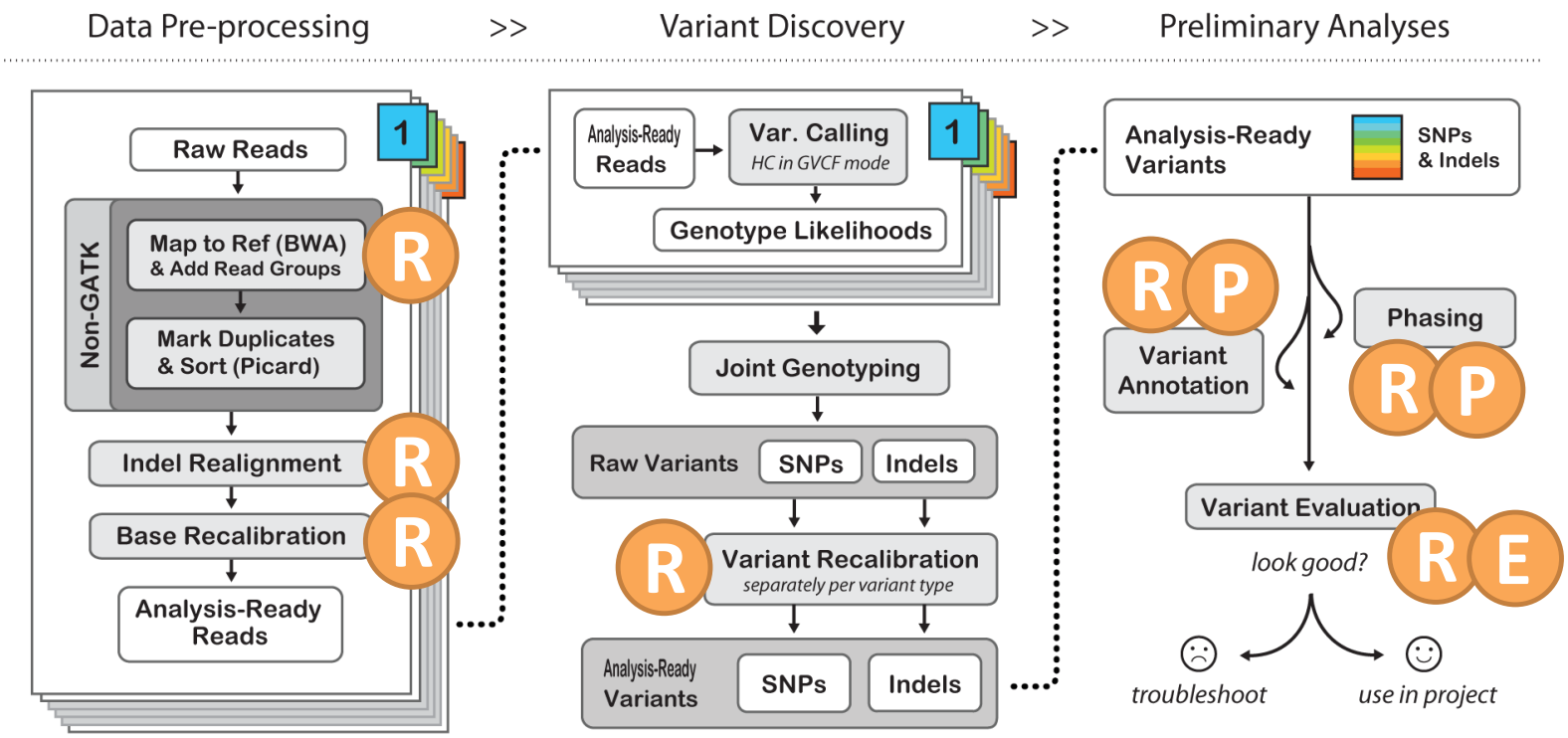
PMID:23341494

PMID:23537139

PMID:25078893

- depends on who you ask
- GATK is 'gold-standard' acc to many but often only sligthly better
- overlap between multiple callers? time-consuming!

GATK for non-human organisms
Potential problems

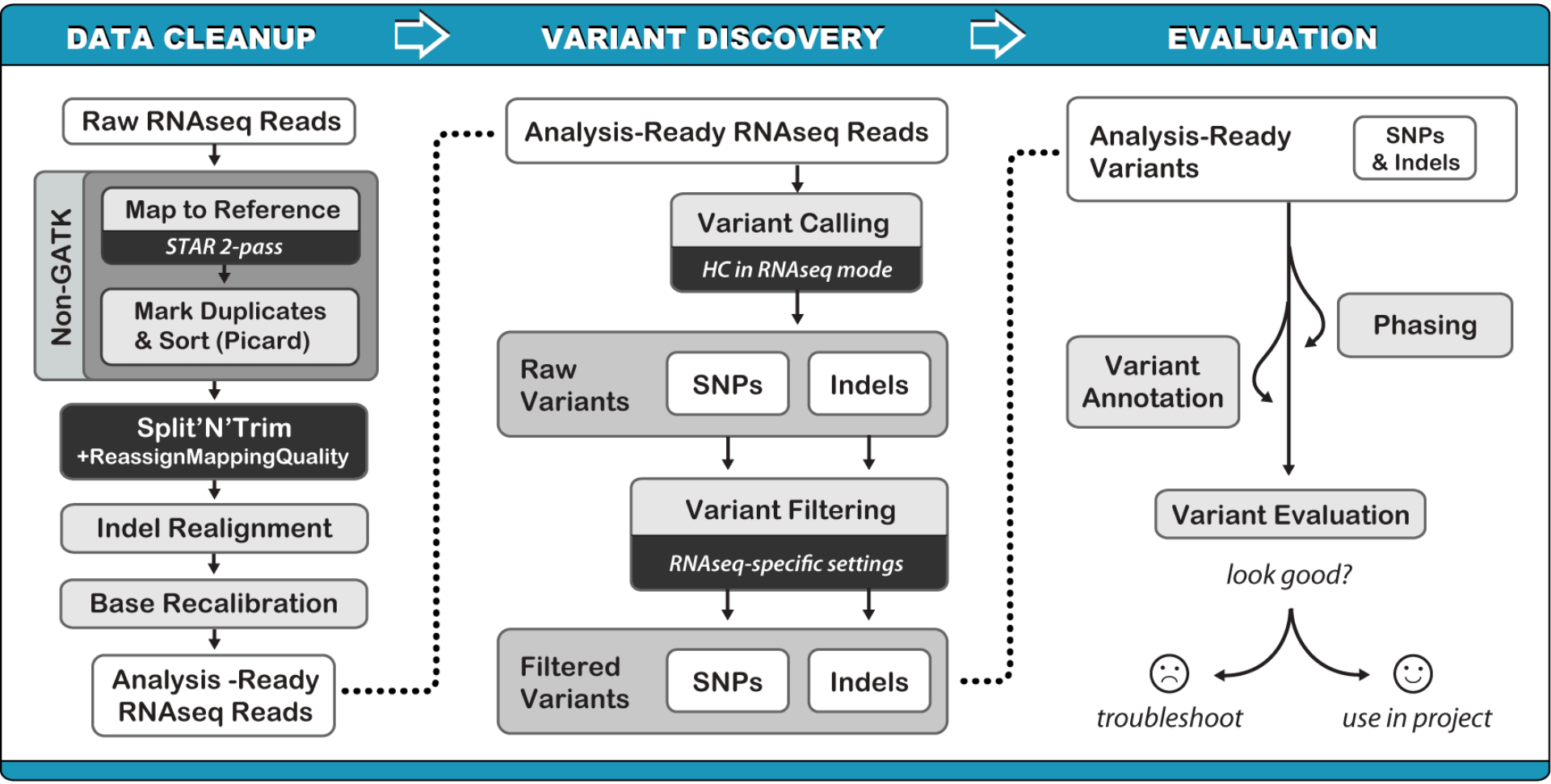


R: Lack of known resources
P: Ploidy assumptions in calculations
E: Lack of clear expectations

Non-human organisms

- GATK needs a reference genome
- very slow with many contigs (make supercontigs, remove/mask transposons/repeats)
- Indel Realignment by default uses indels identified in reads (known inDels not required)
- Base Quality Score Recalibration: Bootstrap until convergence:
  1. Call variants on realigned, unrecalibrated data
  2. Filter resulting variants with stringent filters
  3. Use variants that pass filters as known for BQSR
- Ploidy: HaplotypeCaller has --ploidy argument since v3.2
- Use hard filtering (No Variant quality score recalibration)
- Variant Annotation/Phasing only work for diploid organisms

GATK variant discovery for RNA-seq



https://www.broadinstitute.org/gatk/guide/best-practices

Summary

- Variants tend to be enriched with artifacts because
  - Short reads are noisy
  - Alignments are noisy
  - Sampling effects
- BUT when careful, we still get mostly correct SNP calls
- BAM preprocessing is recommended, but the effect is disputed in some publications
- Calling indels is error-prone, calling structural variants from short-reads even more (we miss many)
- Filtering variants is key (and an art): Hard-filtering for non-human organisms

Sources & Links

GATK

- Presentations https://www.broadinstitute.org/gatk/guide/presentations
- Documentation https://www.broadinstitute.org/gatk/guide/
- Ask the GATK team http://gatkforums.broadinstitute.org/categories/ask-the-team

Article Collections

- Review Articles from Nature Reviews Genetics
- PLoS Computational Biology: Education

Material

- SEQanswers NGS forum http://seqanswers.com/
- Biostar http://biostars.org/
- List of Applications http://seqanswers.com/wiki/Special:BrowseData/

FASTQ format & base qualities

```
@read1
TTGTGTTCAAAATATATAATTTATTTATAAGCTATAATCTTATGNNNNNNNCTCCTTCTTAGCTT
+
@C@DDDDDFHHHHJJDHIIIJI@HHGGIDGEBDEIEIIIIJJII#######008BGGGHIIGGH>
```

@ = ASCII code 64
BQ = ASCII code – 33 = **31**

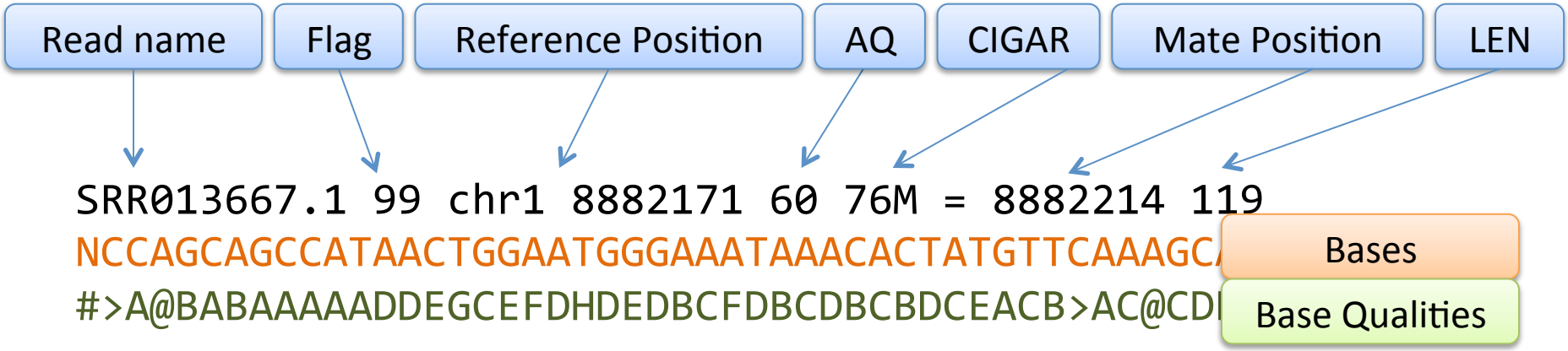
Base Quality: Phred Score Q_{phred}

$Q_{phred} = -10 * \log_{10} (P_{error})$

Base Quality	P_{error}
3	50 %
5	32 %
10	10 %
20	1 %
30	0.1 %
40	0.01 %

Output Formats: SAM & BAM

- SAM http://samtools.sourceforge.net/SAMv1.pdf



- BAM
  - binary version of SAM