

# Practical Bioinformatics

## Variant Calling 2

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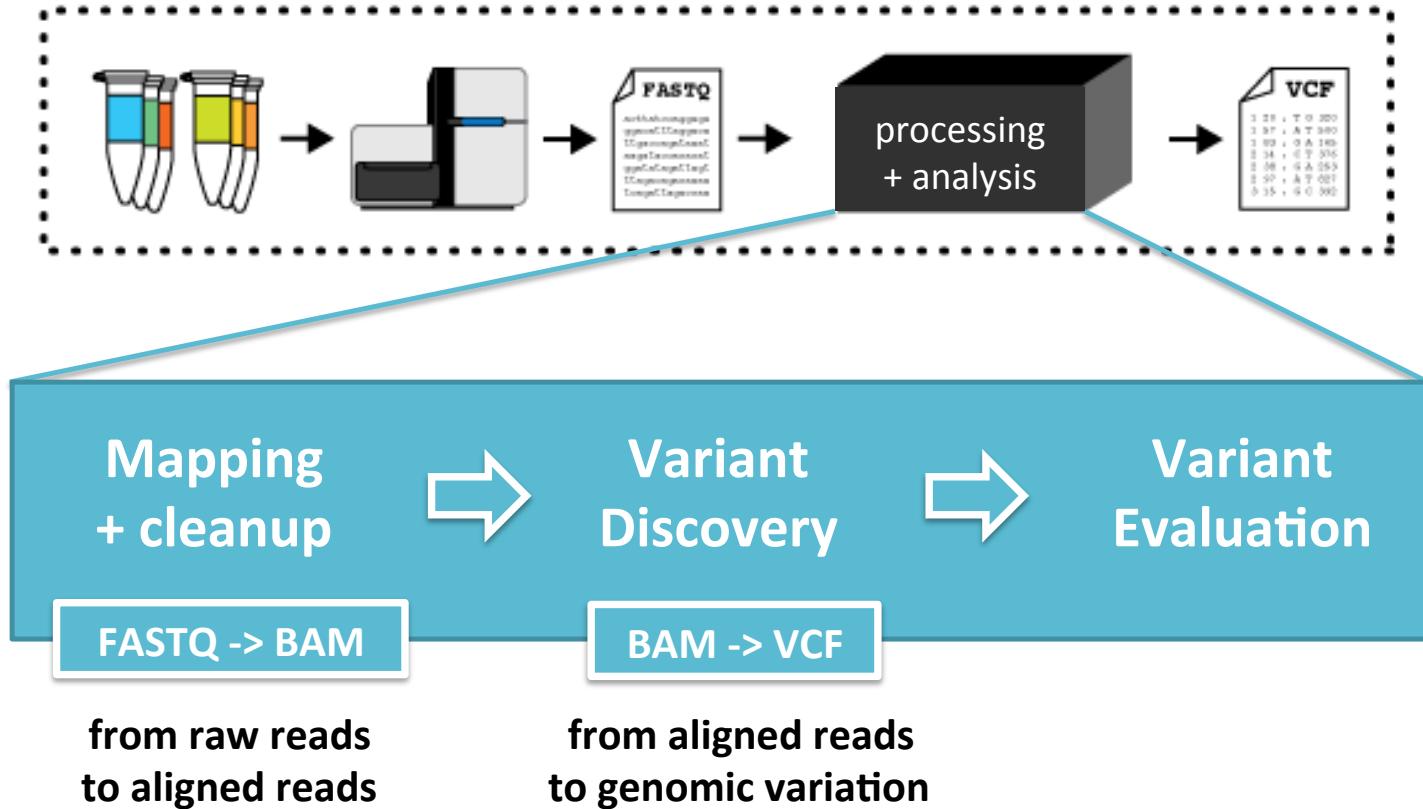


Universität  
Zürich<sup>UZH</sup>

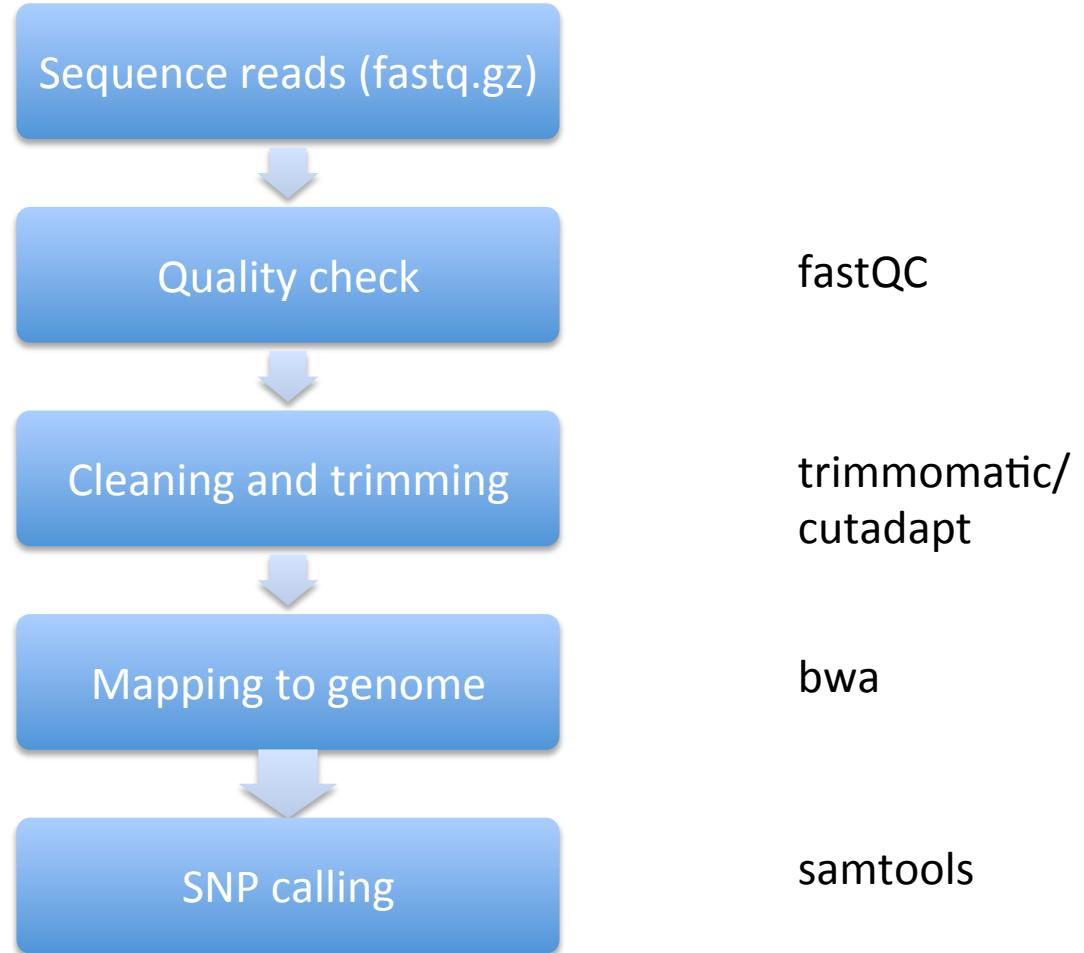


URPP  
Evolution  
in Action

# From reads to variants



# Simple workflow using samtools



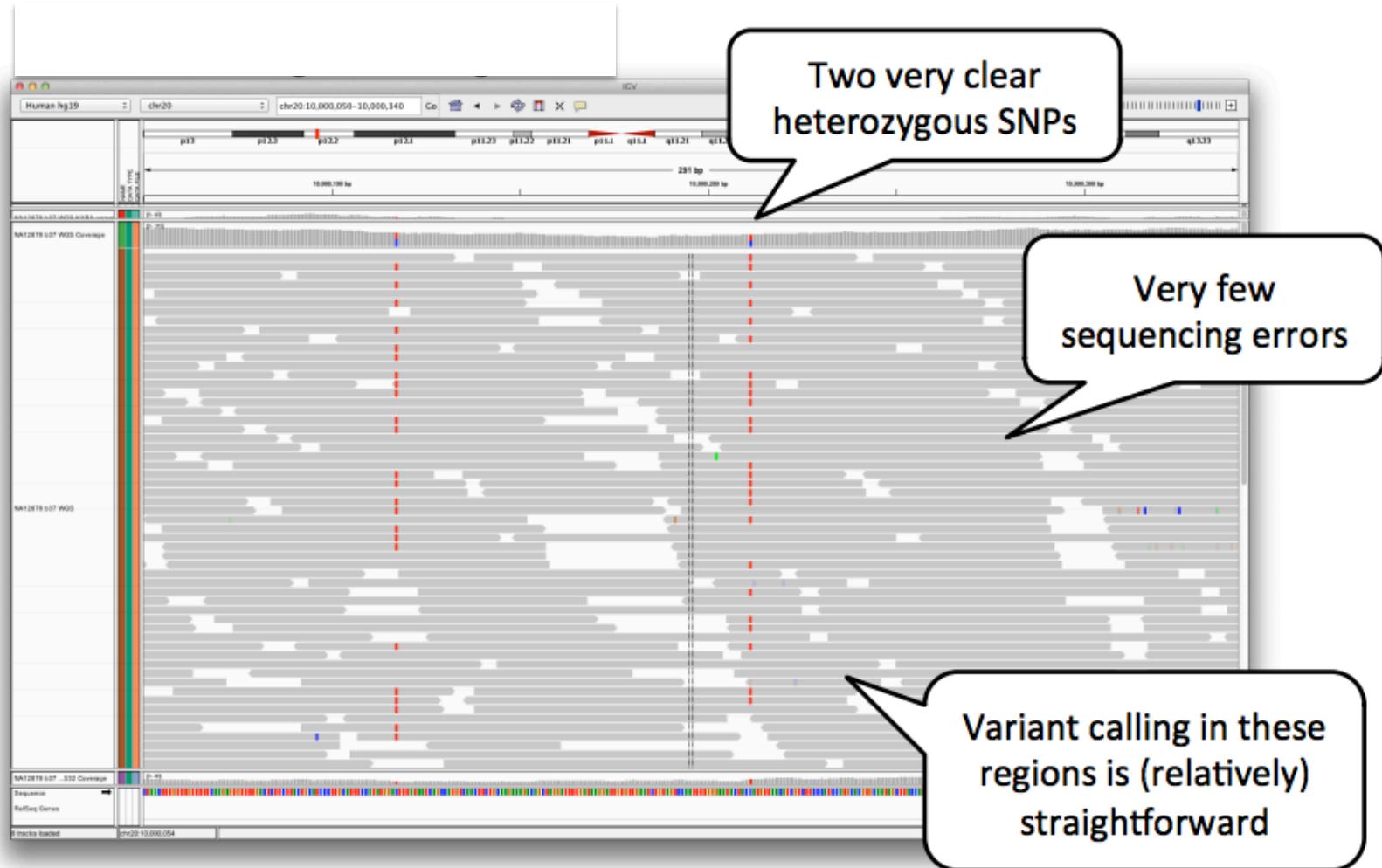
# Simply counting?

GTTACTGTCGTTGTAATACTCCACGATGTC  
GTTACTGTCGTTGTAATATACTCCACGATGTC  
GTTACTGTCGTTGTAATAACTCCACGATGTC  
GTTACTGTCGTTGTAATACCTCCACAATGTC  
GTTACTGTCGTTGTAATgCTCCACGATGTC  
GTTACTGTCGTTGTAATATACTCCACAATGTC  
GTTACTGTCGTTGTAATAACTCCACGATGTC  
GTTACTGTCGTGTAATATACTCCACaATGTC  
GTTACTGTCGTTGTAATATACTCCACaATGTC  
GTTAaTGTCGTTGTAATACTCCACGATGTC  
GTTACTGTCGTTGTAcTACTCCACGATGTC  
GTTACTGTCGTTGTAATACTCCACaATGTC

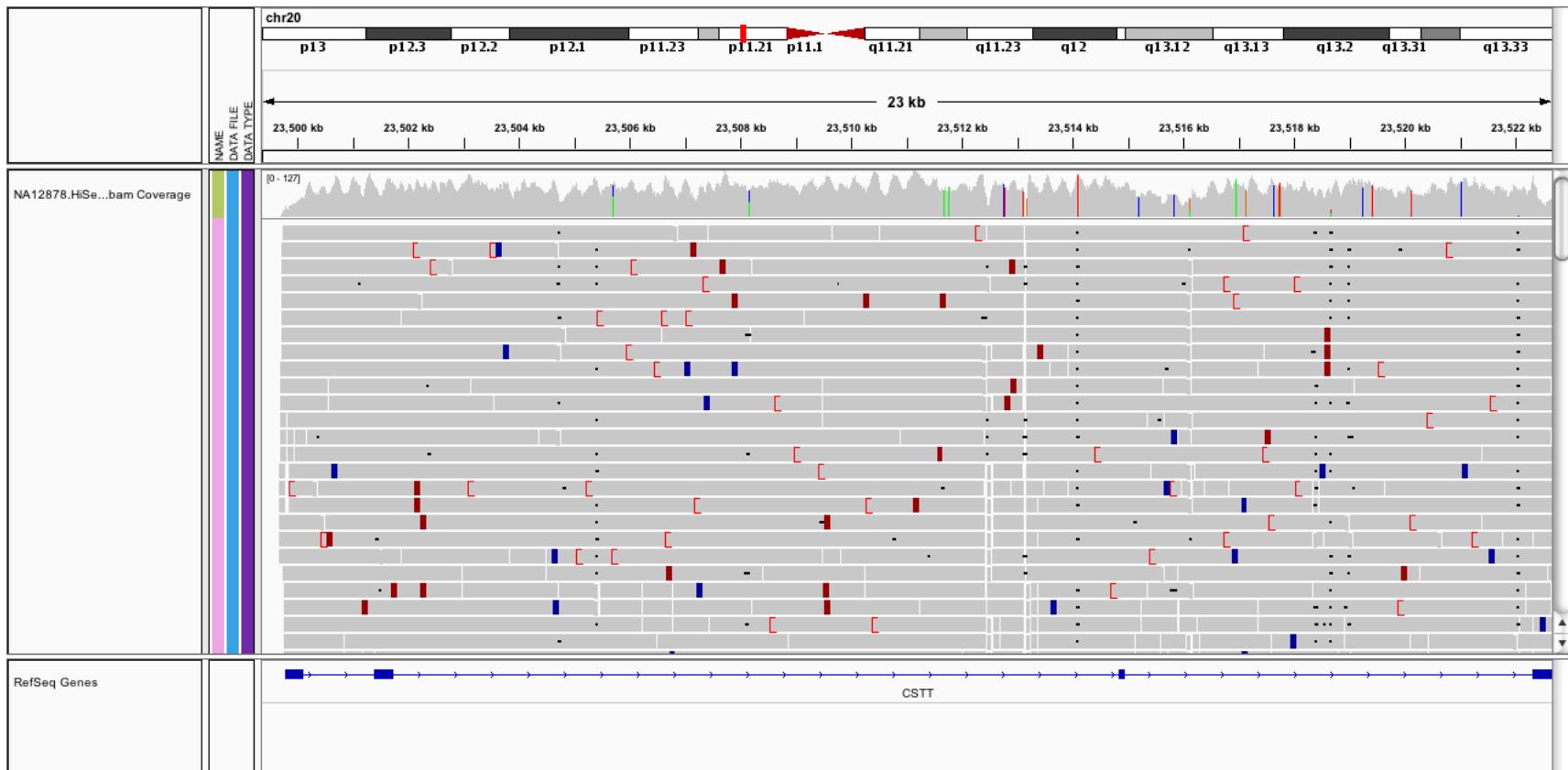
↑      ↑      ↑      ↑      ↑  
sequencing errors

heterozygous  
SNP

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

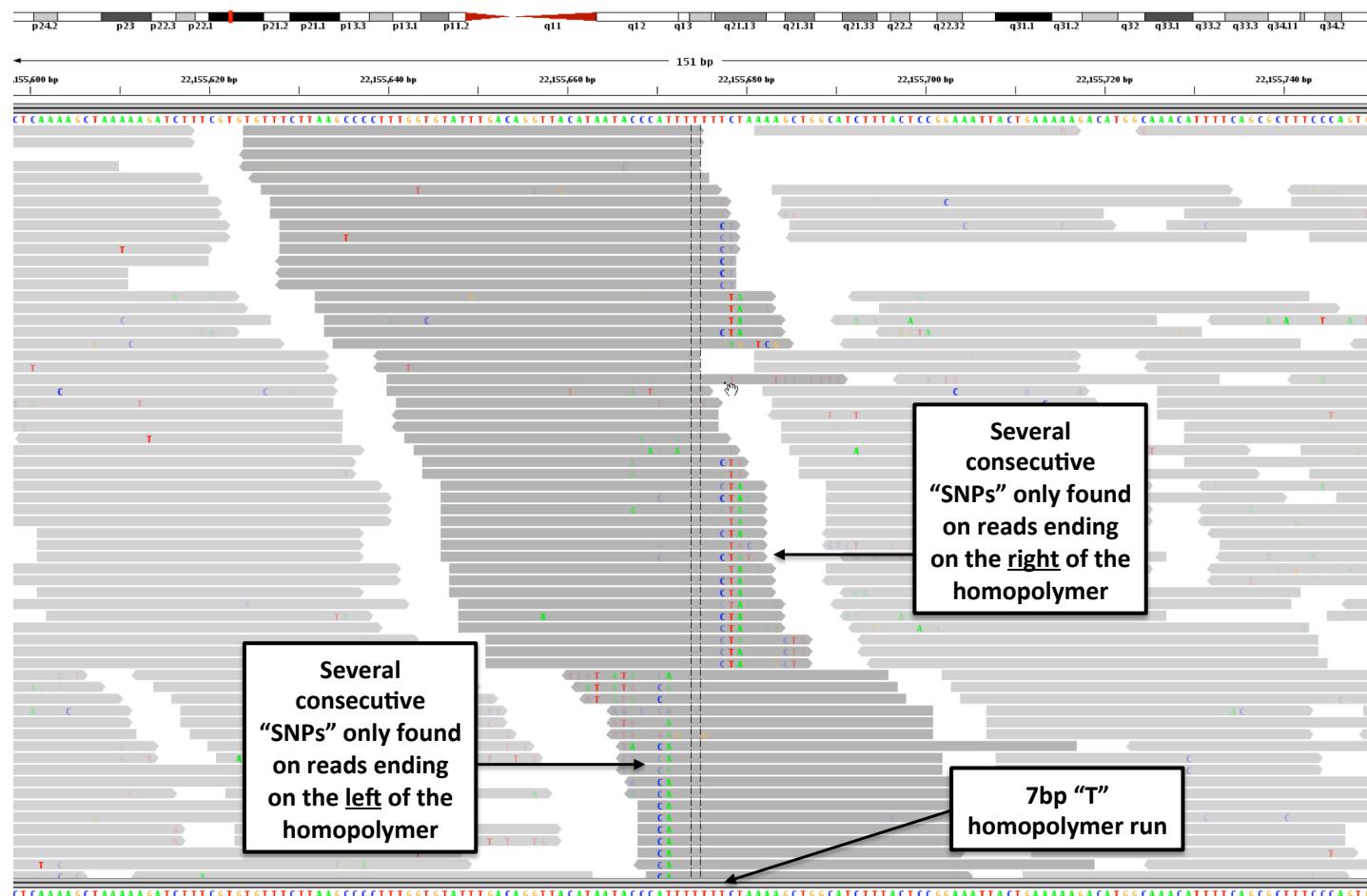


# ...Messy situations...



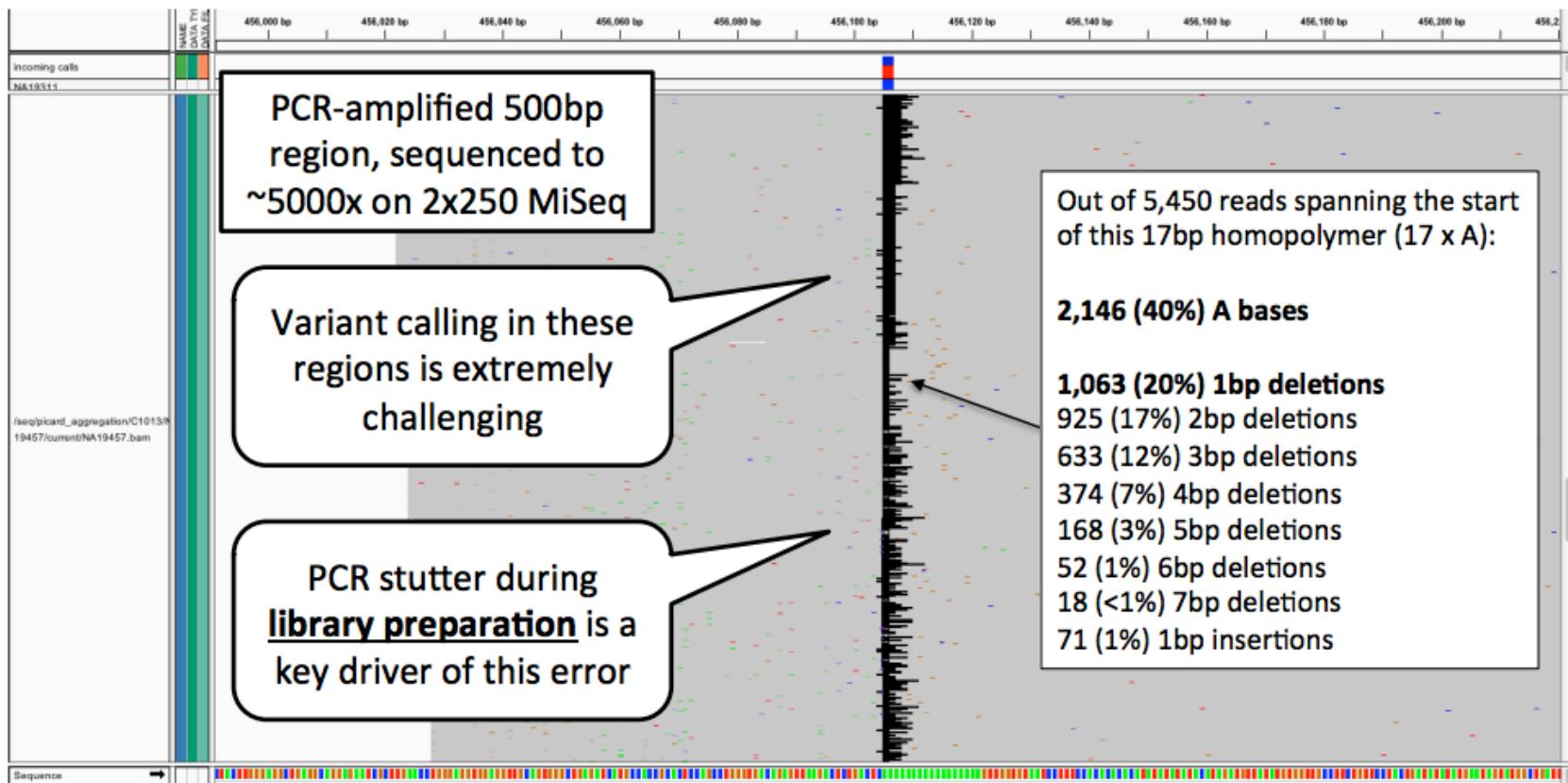
Real mutations or noise?

# An example of a strand-discordant locus



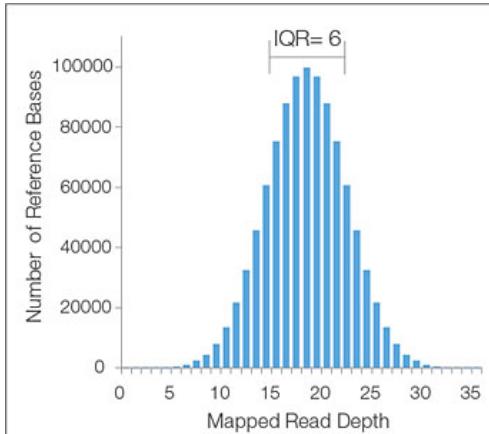
# ...it can get even worse

Poorly-behaved region of the genome

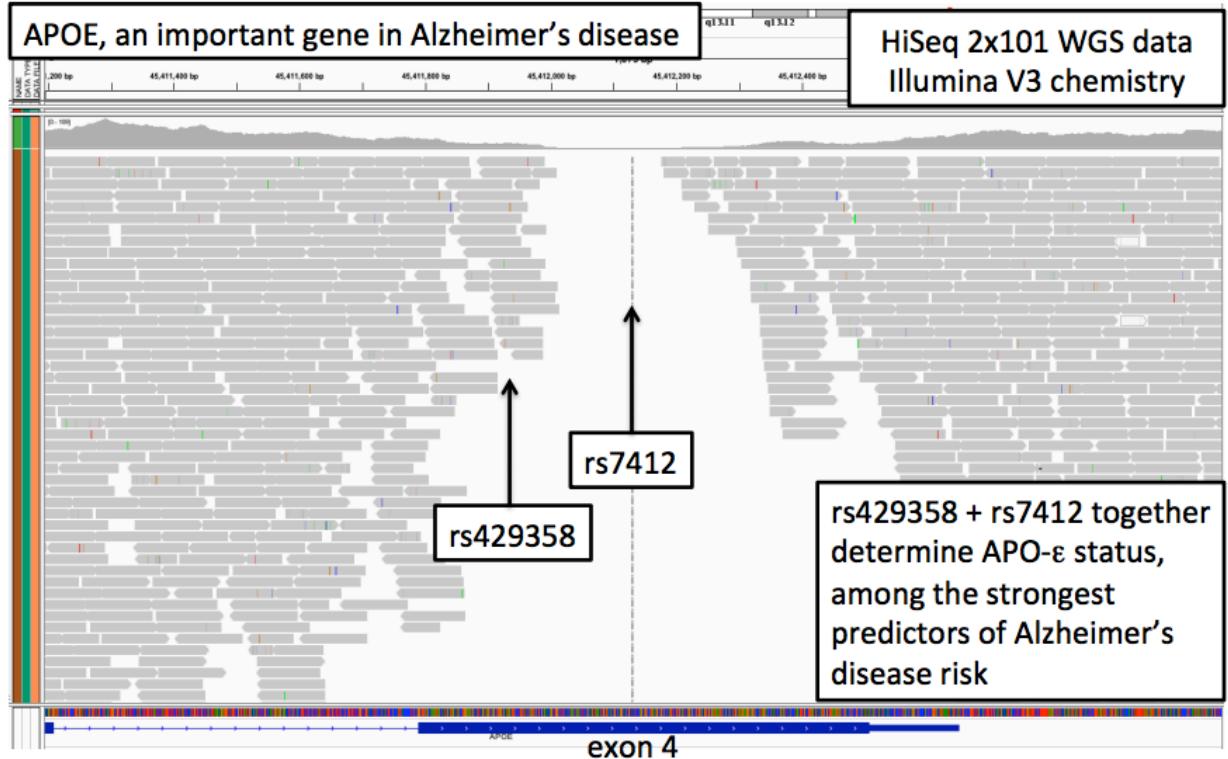


# Problem 1: Lack of coverage

Read depth histogram



Illumina

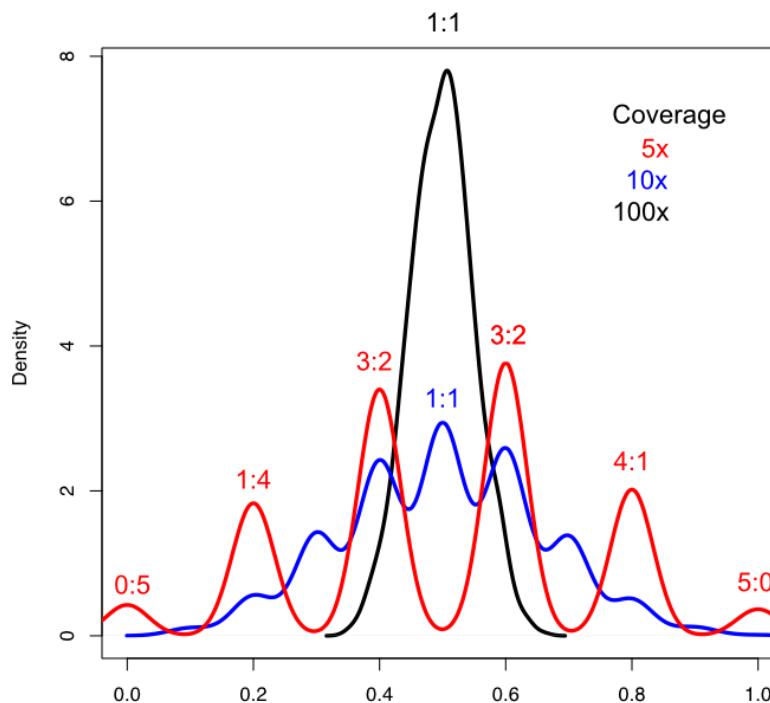


[http://www.broadinstitute.org/gatk//events/2247/agbt\\_2013\\_depristo.pdf](http://www.broadinstitute.org/gatk//events/2247/agbt_2013_depristo.pdf)

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 !

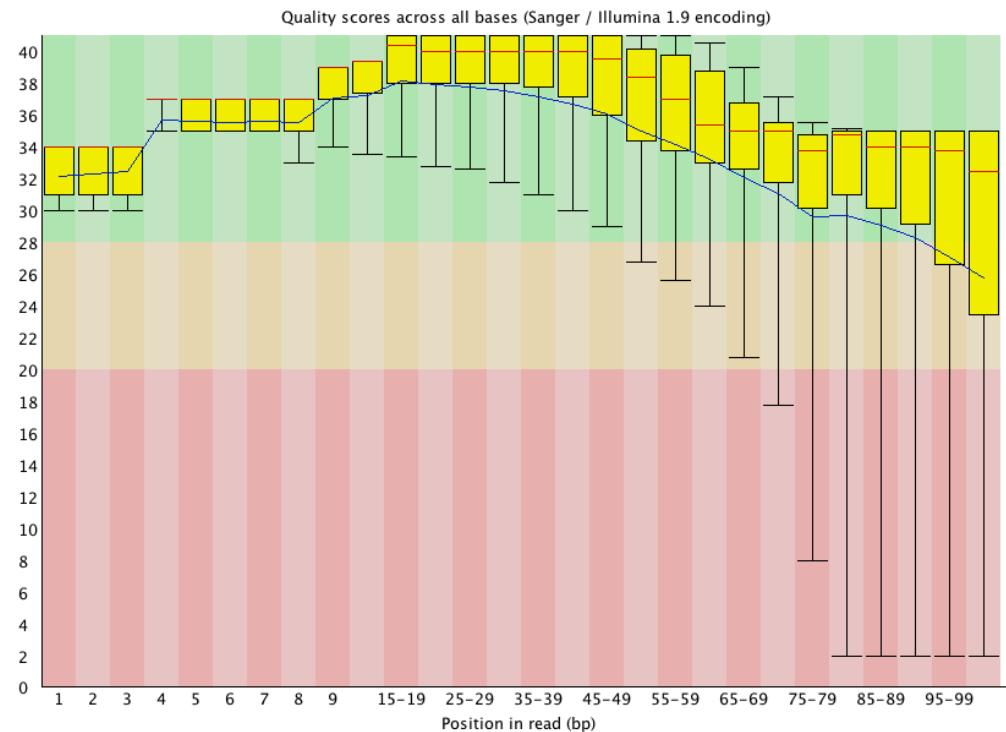
At 10x coverage, ~0.4% of sites are 0:10 or 10:0 !

# 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

		Variant Region	Variant Region	
Ref	TACCGAT	CATTGGATCA	CGATTCC...GCATTGC	GACCGCA
Reads	TACCGAT	CATTGGATCA	CGATTCC...GCATTGC	GACCGCA
	ACCGAT	TATTGCATCG	CGATTCC...GCATTGC	GACCGCA
	ACCGAT	CATTGGATCA	CGATTCC...GCATTGC	GACCGCA
	ACCGAT	TATTGGATCG	CGATTCC...GCATTGC	GACCGCA
	CCGAT	C-TTGGATCA	CGATTCC...GCATTGC	GACCGCA
	CCGAT	CATGGGATCA	CGATTCC...GCATTGC	GACCGCA

FreeBayes

-> Indel Realignment

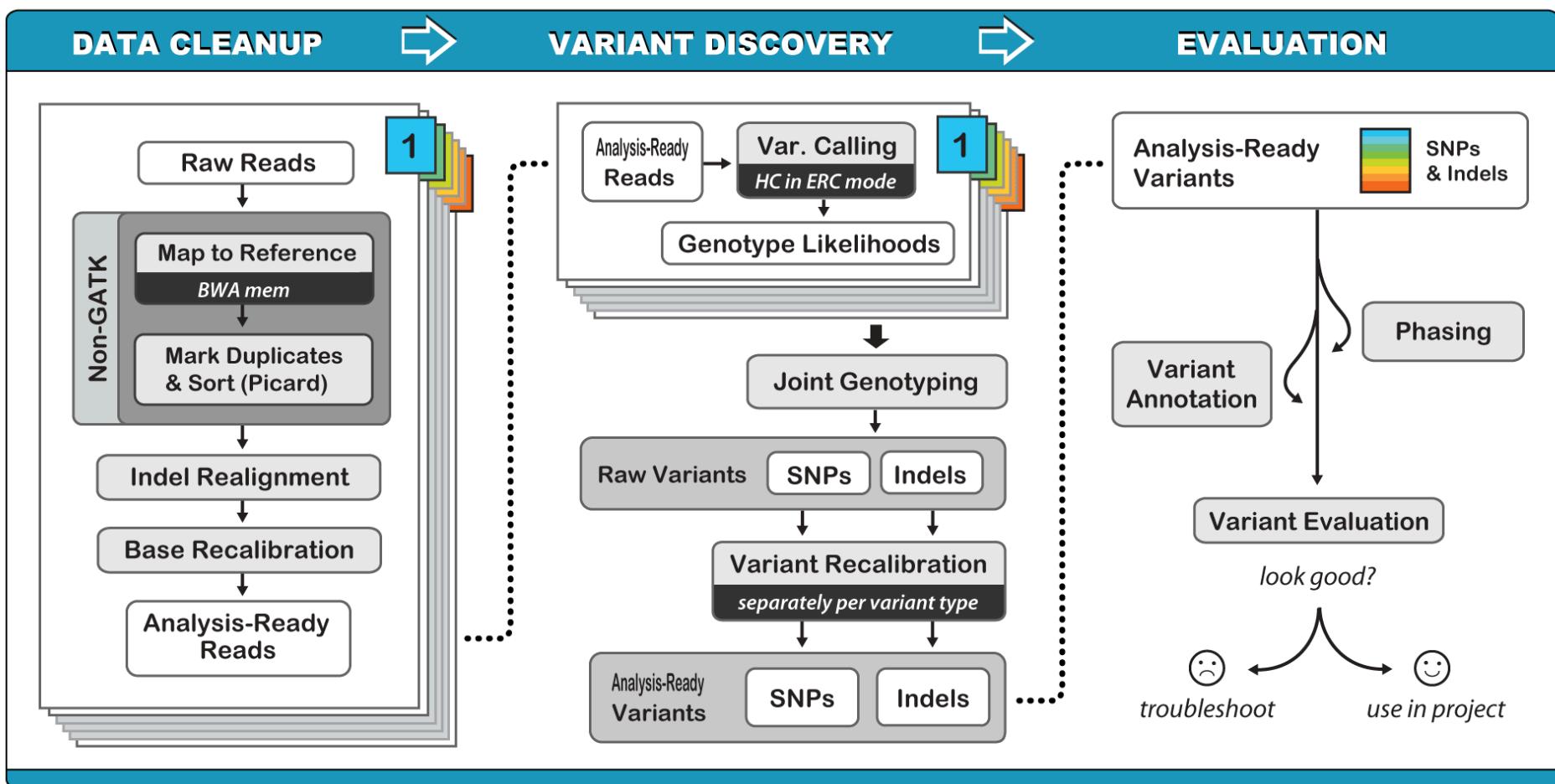
# Problem 4: Incorrect mapping cont.

- mismapped reads / errors in the alignment
  - segmental duplication
  - processed pseudogenes
  - close paralogues
  - 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

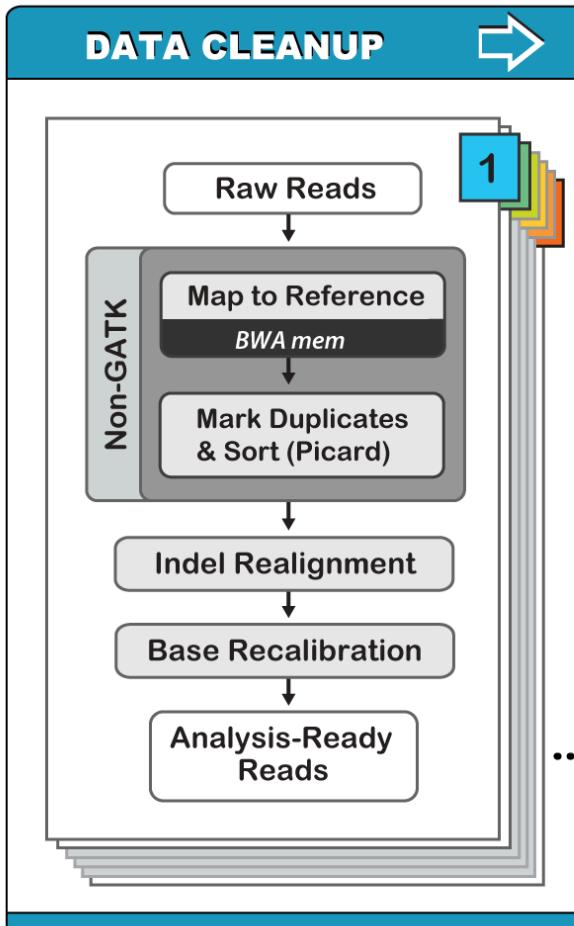
# GATK Workflow for DNA



per lane

per sample

# Data Cleanup



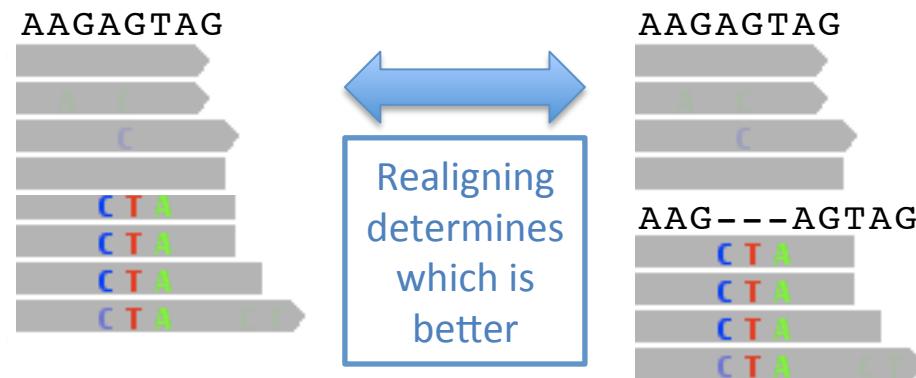
per lane

## 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

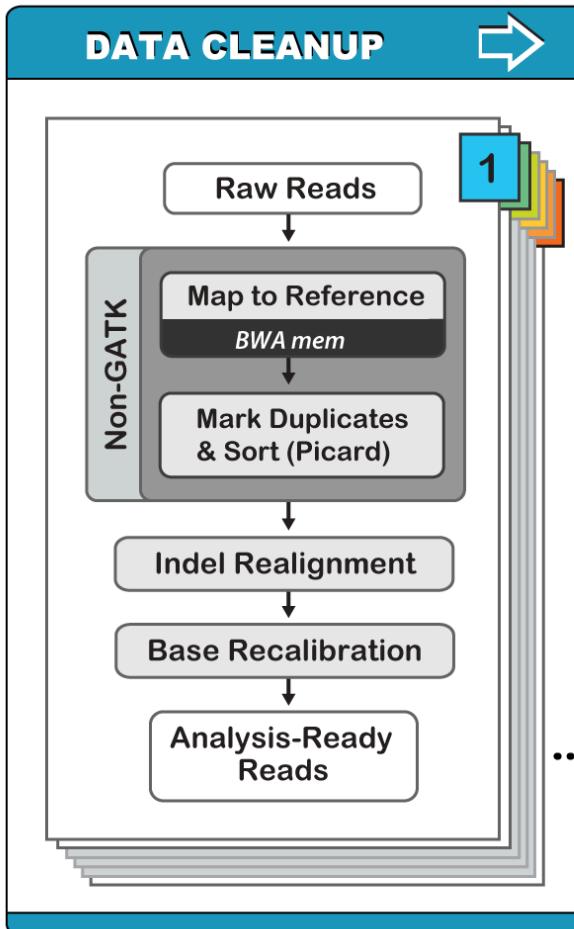
## Indel Realignment (mainly UnifiedGenotyper)

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



Realigning  
determines  
which is  
better

# Data Cleanup cont.

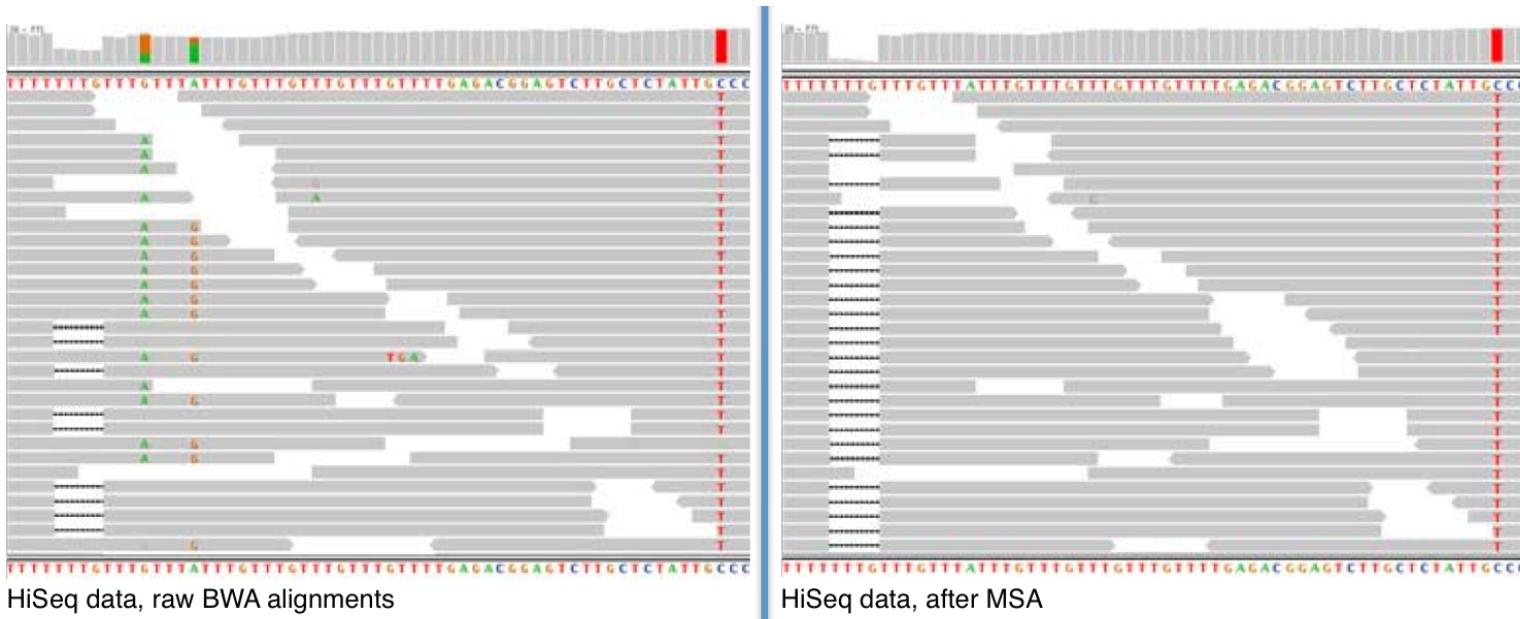


per lane

## 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



# 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

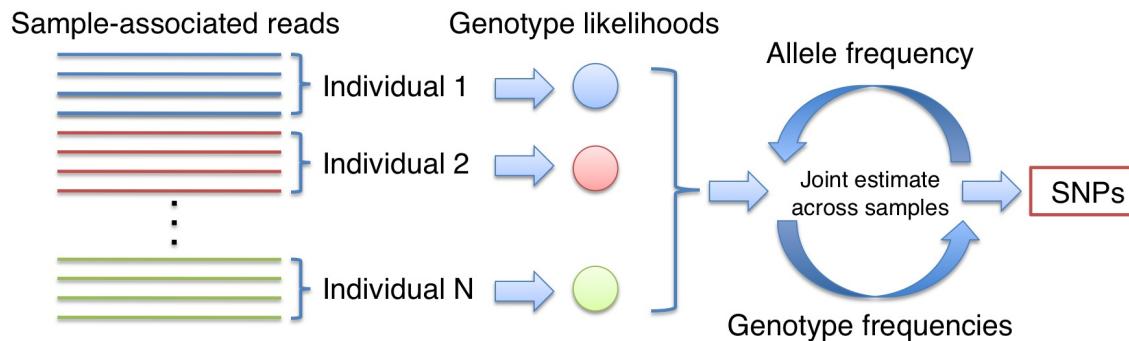
any ploidy

more accurate (especially for  
indels)

up to hundreds (-> GVCF mode)

# Multi-sample analysis

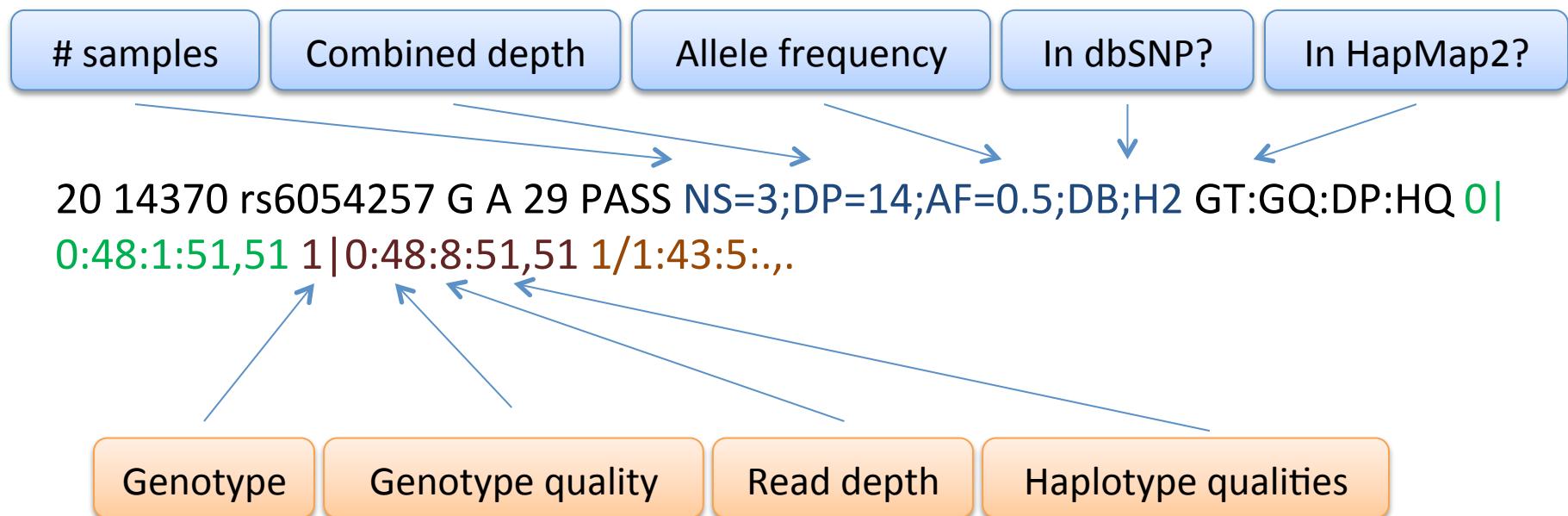
- To gain sensitivity some SNP callers allow **multi-sample** variant calling (multiple individuals/samples from the same or closely related species)



- ~ 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

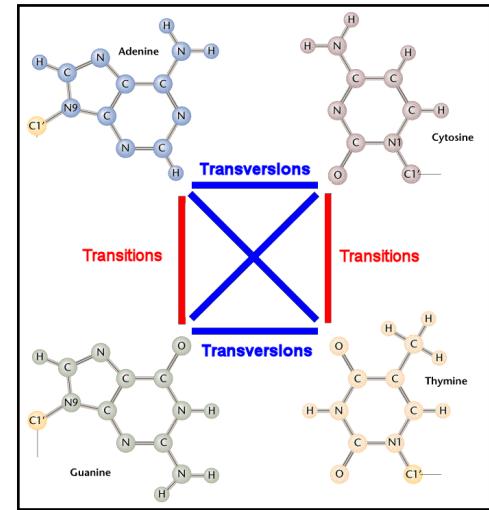
22 49582364	.	A	0	G	1	198.96	0
AB=0.67;	AC=3;	AF=0.50;	AN=6;	DP=87;	Dels=0.00;	HRun=1;	MQ=71.31;
MQ0=22;	QD=2.29;	SB=-31.76	GT:DP:GQ	0/1:12:99.00	0/1:11:89.43	0/1:28:37.78	
INFO field	AC	No. chromosomes carrying alt allele	AB	Allele balance of ref/alt in hets			
	AN	Total no. of chromosomes	Hrun	Length of longest contiguous homopolymer			
	AF	Allele frequency	MQ	RMS MAPQ of all reads			
	DP	Depth of coverage	MQ0	No. of MAPQ 0 reads at locus			
	QD	QUAL score over depth	SB	Estimated SB score			

# 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 ( $T_i/T_v$ )  
(e.g. nuclear genes in humans close to 2)



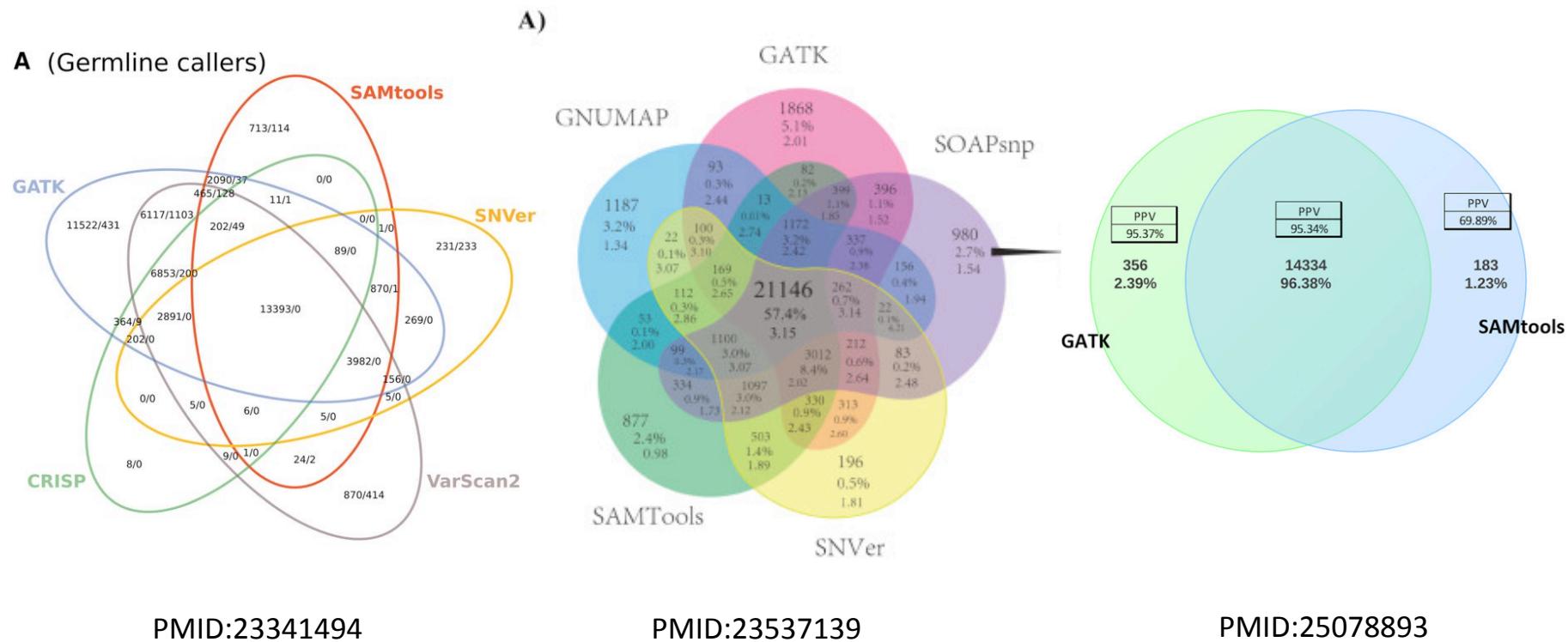
## Experimental Validation

- 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)

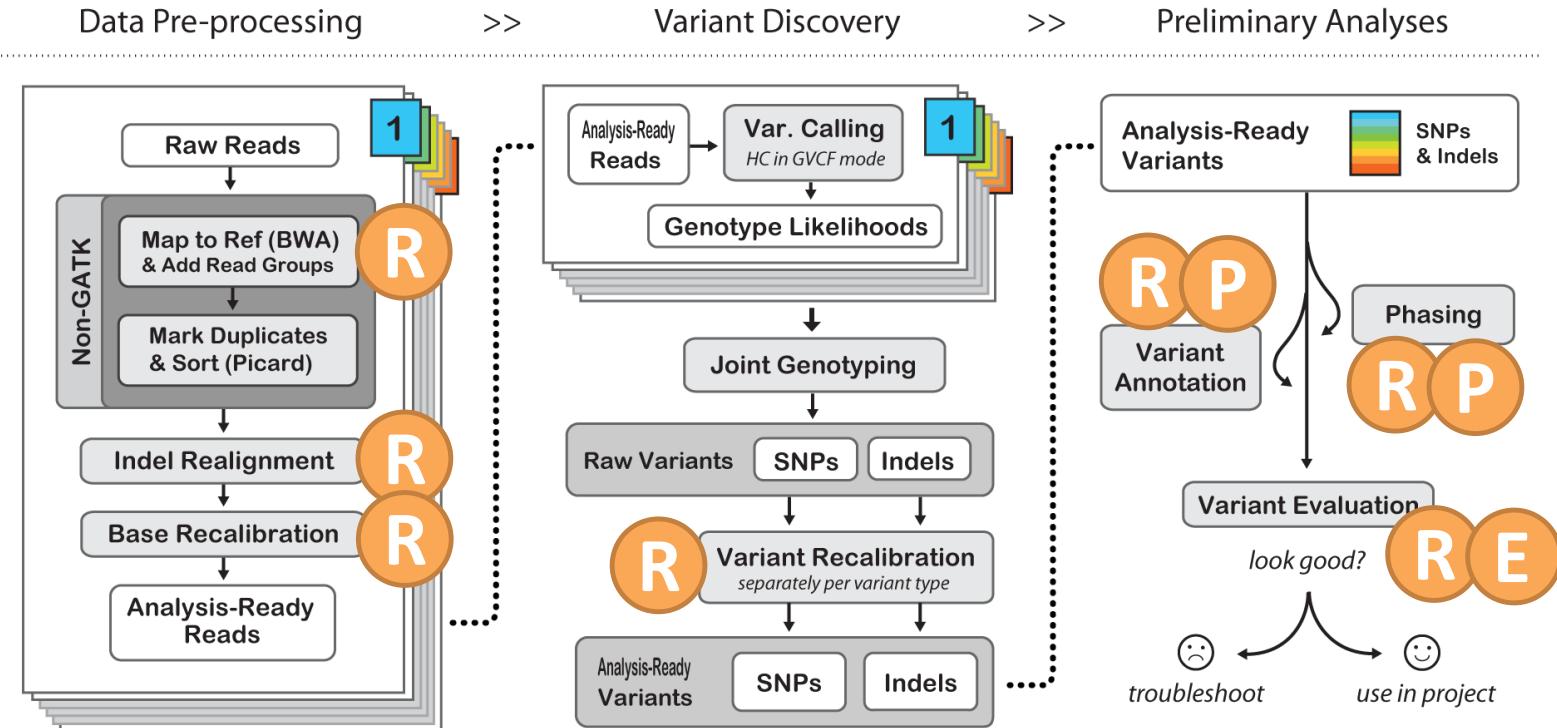
# Who performs best?



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

# GATK for non-human organisms

## Potential problems



R: Lack of known resources

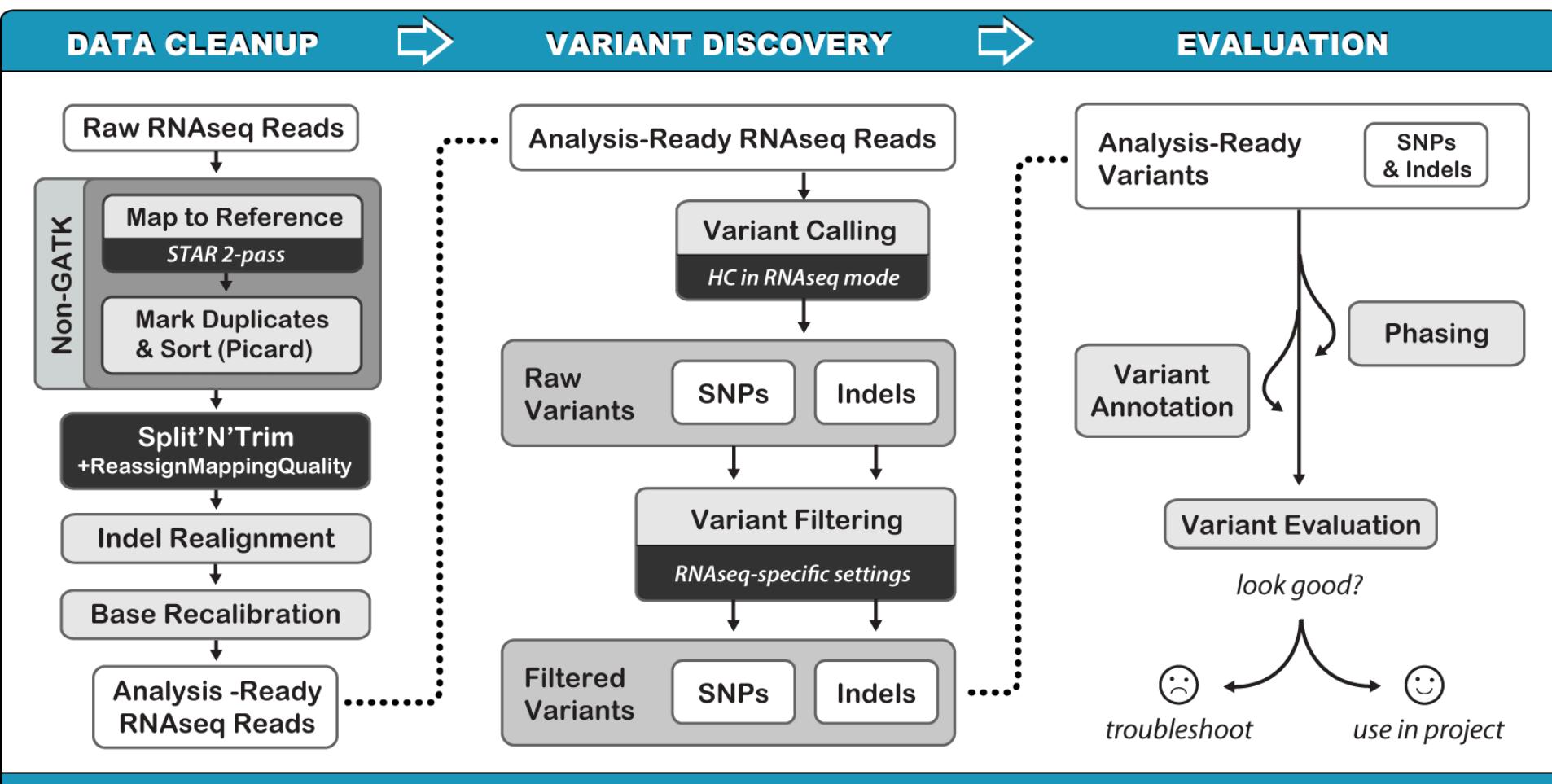
P: Ploidy assumptions in calculations

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



# 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

TTGTGTTCAAAATATATAATTATTTATAAGCTATAATCTTATGNNNNNNNCTCCTTAGCTT

+

@C@DDDDDFHHHHJJJDHIIII@HHGGIDGEBDEIEIIIIJJII#####008BGGGHIIGGH>



@ = ASCII code 64

BQ = ASCII code – 33 = **31**

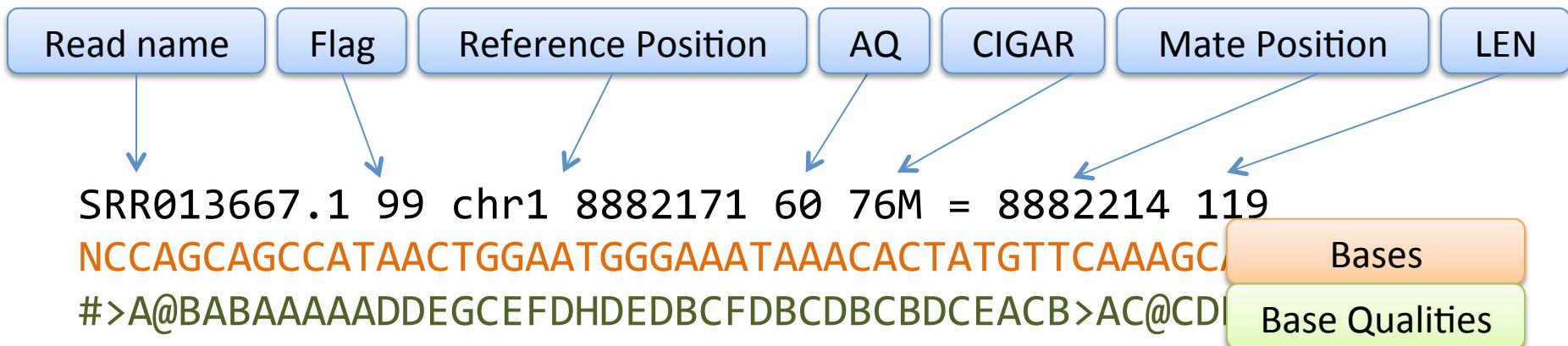
Base Quality: Phred Score  $Q_{\text{phred}}$

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

Base Quality	$P_{\text{error}}$
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