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Basic bioinformatics - from fastq to variants

2nd ERIC workshop on TP53 analysis in Chronic Lymphocytic Leukemia

7/11 - 2017

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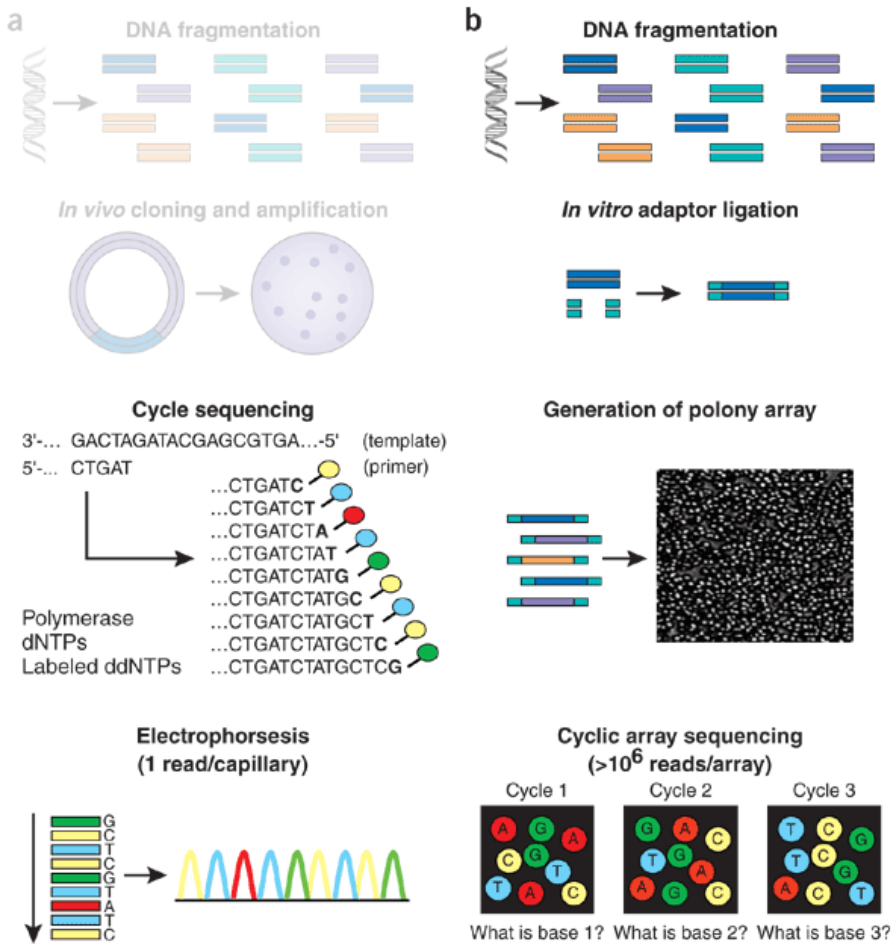
Sanger vs Next-generation sequencing

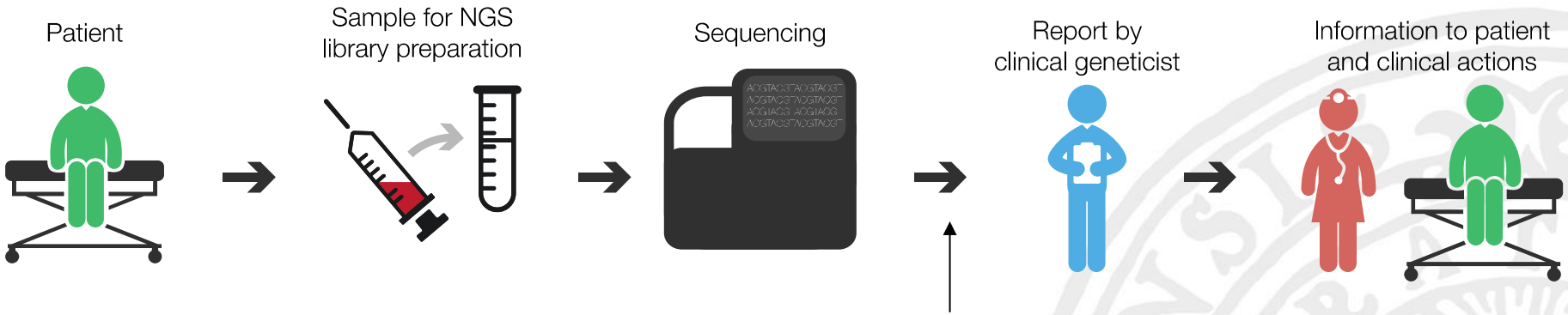


Shender et al., Nature Biotech 2008

Sanger sequencing
- One region in one patient
- Robust
- *Manual* analysis possible

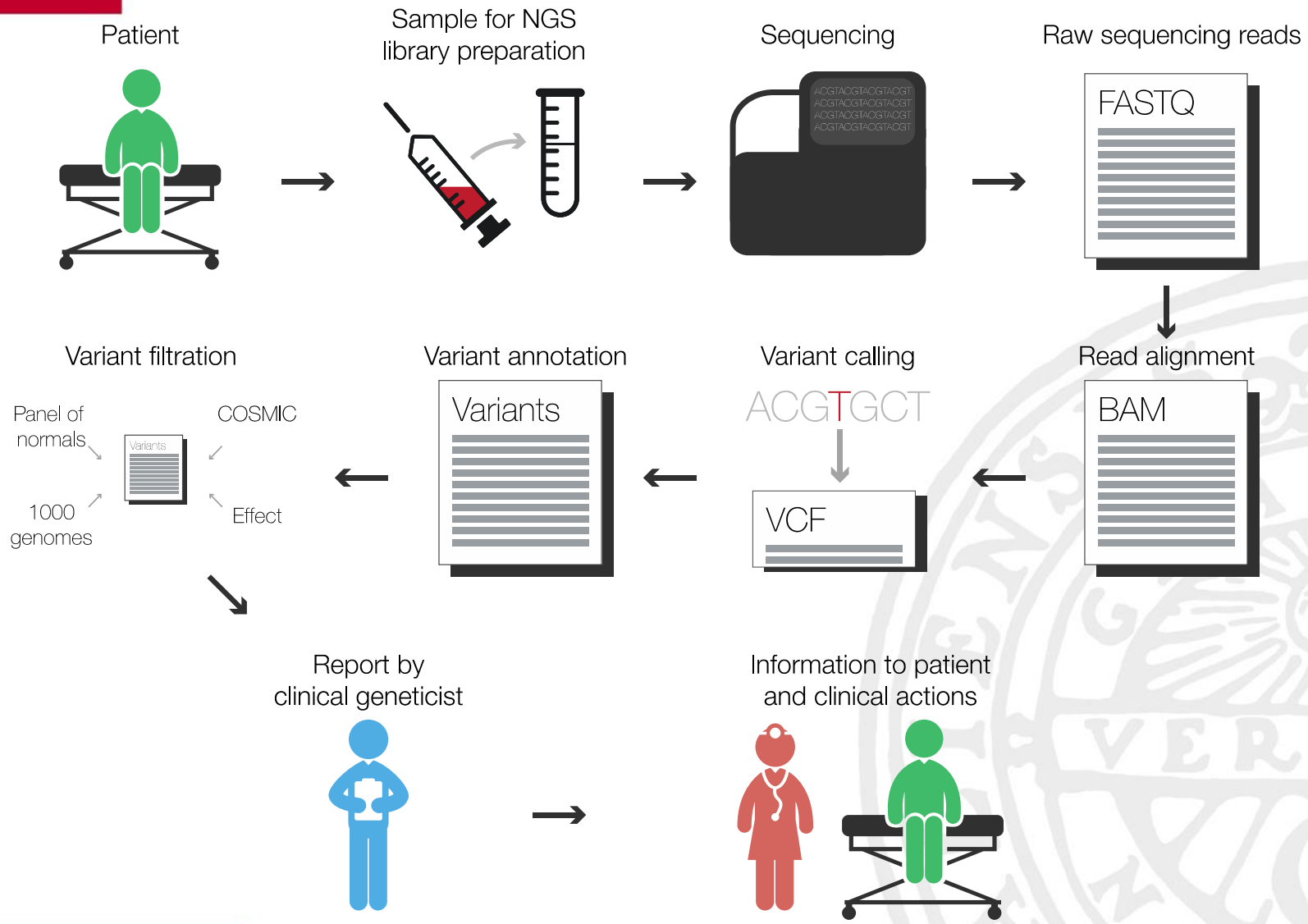
NGS
- Multiplexing regions and patients
- Sensitive
- Need of computational analysis

NGS in the precision medicine workflow



Computational analysis!

NGS in the precision medicine workflow



What is bioinformatics?

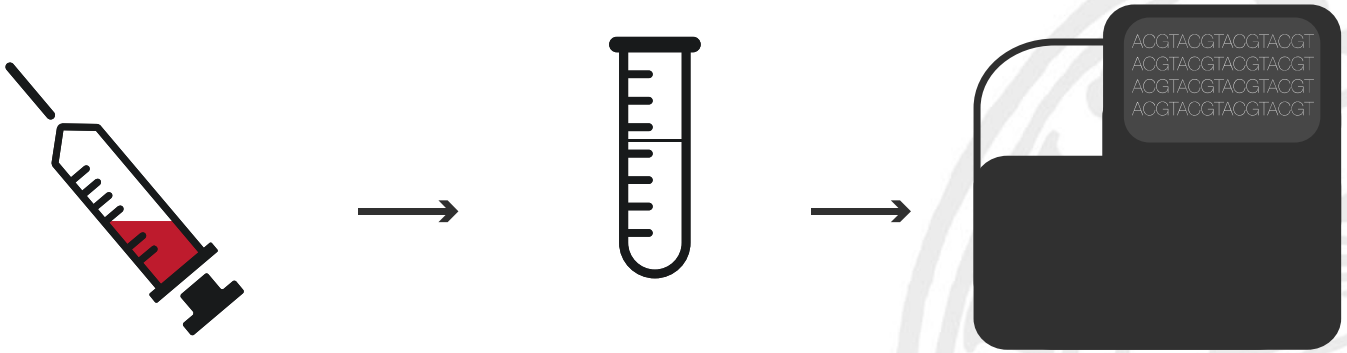
- Broad term
  - From AI to biostatistics
- Here: Computational analysis of NGS data
- From the sequencing machine output to a list of variants that makes sense to the geneticist

Several NGS applications today

Different applications and different platforms

Today: Focus on targeted deep sequencing with Illumina technique

Sample | Library preparation | Sequencing



DNA
RNA

TruSight
HaloPlex
Multiplicom

Illumina
Ion torrent
PacBio
Nanopore

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The analysis workflow

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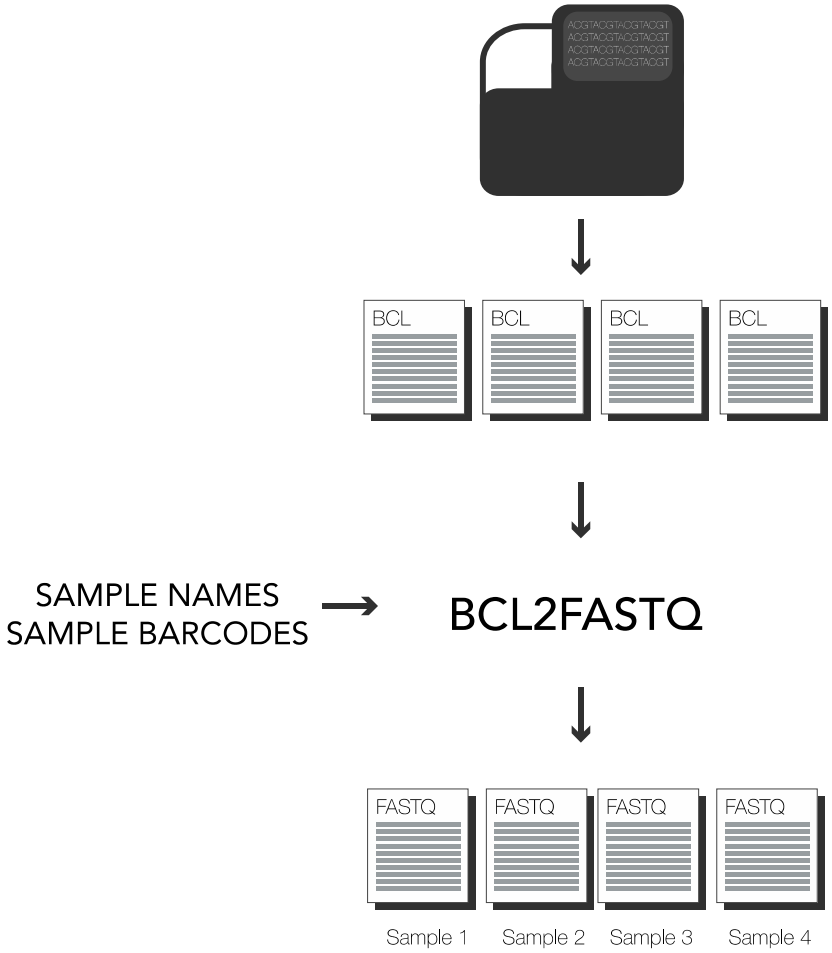
The analysis workflow

1. BCL to FASTQ conversion and demultiplexing



- BCL – raw sequencing data
- Convert to FASTQ and split into sample files
- Sample sheet information, DNA barcodes
- Usually automated on the sequencer

The FASTQ format

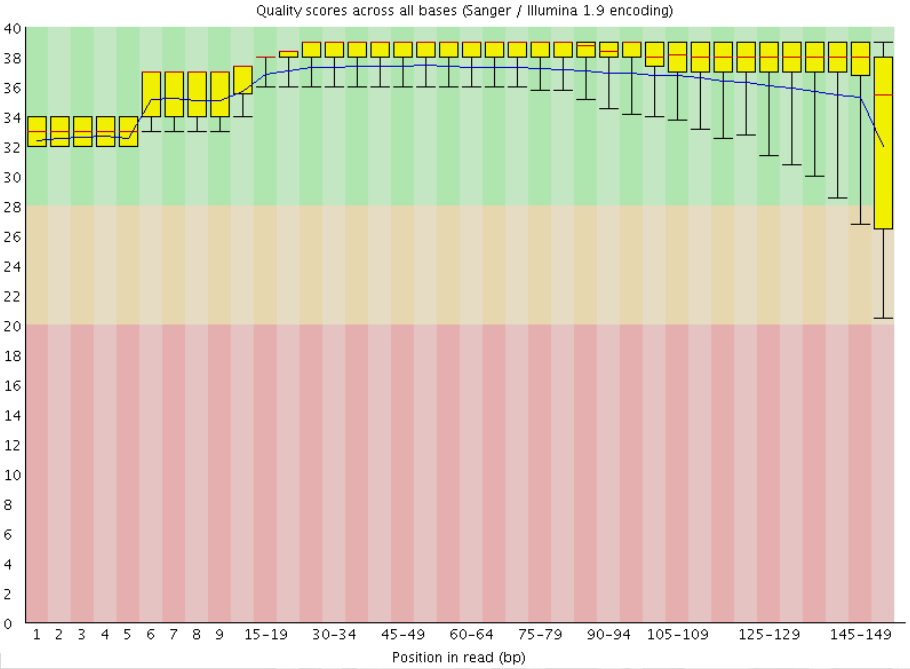
- FASTQ = FASTA + Quality

1. Sequence identifier
2. Nucleotide sequence (the *read*)
3. Phred quality information per base (ASCI encoded)

```
@HISEQ2000-02:420:C2E47ACXX:7:2214:18015:39495/1
CACTCCAGCCTGGGTGACAGAGCGAGATTCCGTCTCAAAAAGTAAAATAAAATAAA
+
EAD@@@?@A@?>>??@@?A?@??>@>ACCAA@A@@@AABAAA?AAAAAAAAAA
```

1. BCL to FASTQ conversion and demultiplexing

- First quality control by eye
  - Are all files present?
  - Are the files of expected size?
- Other quality controls
  - Qscore distribution, GC content, sequence enrichment
- FASTQC



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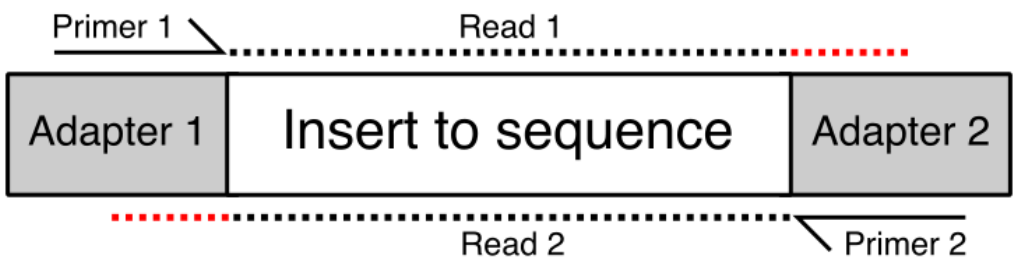
The analysis workflow

2. Read trimming

- Adapter read through
  - Insert shorter than read length
- Low quality bases
- Enzyme footprints (Agilent Haloplex)
- Necessary?



https://sequencing.qcfail.com/articles/read-through-adapters-can-appear-at-the-ends-of-sequencing-reads/

Tool examples:
Cutadapt
Trim Galore!
Agilent Agent

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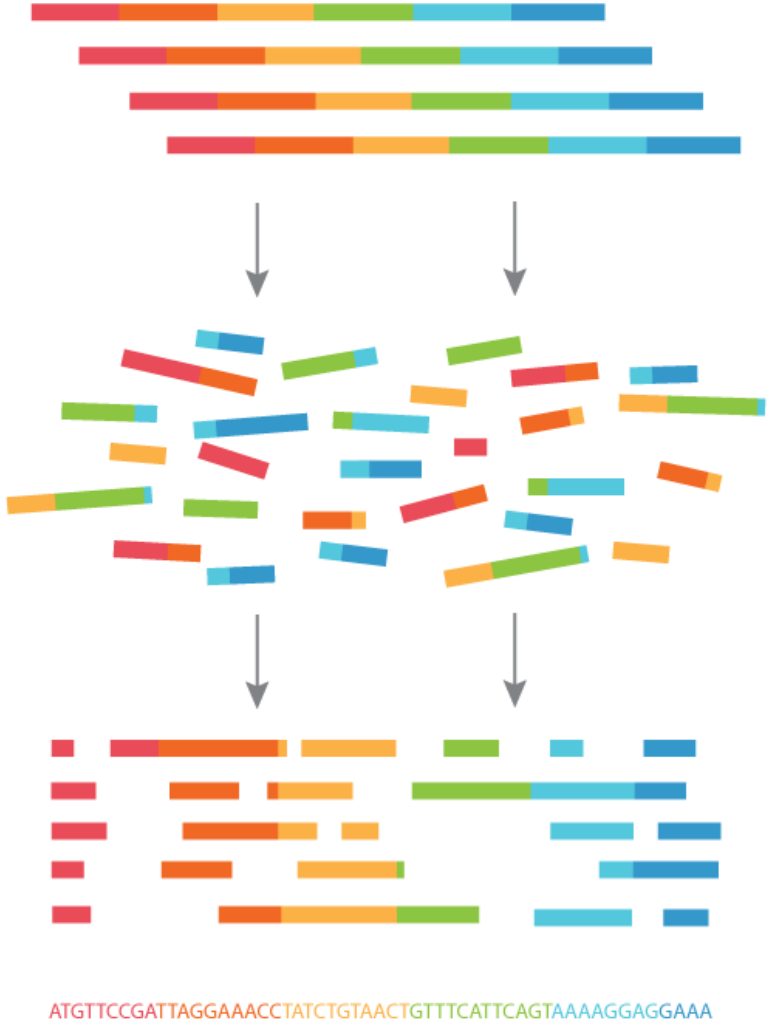
The analysis workflow

3. Read alignment

- Which loci do the read originate from?
- Compare to reference genome
- Technical and biological challenges:
  - The reference is large
  - Somatic and inherited variants?
  Pseudogenes?
- Input: FASTQ files
- Output: SAM/BAM files

Tool examples:
BWA-mem
Novoalign
Bowtie
MOSAIK

The SAM/BAM format



Template DNA

Short reads from Sequencer (FASTQ)

Mapped reads (SAM/BAM-file)

https://www.abmgood.com/marketing/knowledge_base/next_generation_sequencing_data_analysis.php

The SAM/BAM format

- Sequence Alignment/Map format
- Similar to FASTQ but added information

@HISEQ2000-02:420:C2E47ACXX:7:2214:18015:39495. 99. chr1 17644 37 37M = 17919 314
CACTCCAGCCTGGGTGACAGAGCGAGATTCCGTCTCAAAAAGTAAAATAAAATAAAATAAAAAATAAAAGTTTG
EAD@@@?@A@?>>??@@?A?@??>@>ACCAA@A@@@AABAAA?AAAAAAAAAACCCBBBBBAAABA@
RG:Z:UM0098:1. XT:A:R. NM:i:0 SM:i:0. AM:i:0. X0:i:4. X1:i:0. XM:i:0. XO:i:0. XG:i:0. MD:Z:37

Field	
QNAME	@HISEQ2000-02:420:C2E47ACXX:7:2214:18015:39495
FLAG	99
RNAME	chr1
POS	17644
MAPQ	37
CIGAR	37M
MRNM/RNEXT	=
MPOS/PNEXT	17919
ISIZE/TLEN	314
SEQ	CACTCCAGCCTGGGTGACAGAGCG…
QUAL	EAD@@@?@A@?>>??@@?A?@...
TAGs	RG:Z:UM0098:1 XT:A:R NM:i:0 SM:i:0 AM:i:0 X0:i:4 X1:i:0 XM:i:0 XO:i:0 XG:i:0 MD:Z:37

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The analysis workflow

4. Variant calling

- Is there a variation in the tumor sequence compared to the reference?
- Small variants:
  - Single nucleotide variants (SNVs)
  - Insertions and deletions < ~20bp (InDels)
- Input file: BAM-file
- Output file: VCF-file

4. Variant calling

Aligned reads

C>G mutation

```
GTCTGGCCCCTCGTCAGCATCTTATCC
  CTGGCCCCTCCTCAGCATCTTATCC
 TCTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCC
  CTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
    GCCCCTCGCTCAGCATCTTATCCG
  CTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
  CTGGCCCCTCGTCAGCATCTTATCCG
```

GT deletion

```
GAAATTTGCGTGTGGAGTATTTGGATGACAG
GAAATTTGC   GTGGAGTATTTGGATGACAGA
GAAATTTGCGTGTGGAGTATTTGGATGACAGA
GAAATTTGCGTGTGGAGTATTTGGATGACAG
GAAATTTGC   GTGGAGTATTTGGATGACAGA
 AAATTTGC   GTGGAGTATTTGGATGACAGA
 AAATTTGC   GTGGAGTATTTGGATGACAGA
 AAATTTGCGTGTGGAGTATTTGGATGACAG
 AAATTTGC   GTGGAGTATTTGGATGACAG
 AAATTTGC   GTGGAGTATTTGGATGACAG
 AAATTTGCGTGTGGAGTATTTGGATGAC
```

GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGT

Reference sequence

Captured region 1

Captured region 2

4. Variant calling

Tool examples:
VarScan2 (U+P)
Mutect2 (P)
Strelka (U+P)
GATK (U)

- Reports all detectable variation
  - Unaware of effects and gene borders
  - Biological and technical variation
- Paired vs unpaired (somatic / germline)
  - Unpaired: Direct comparison to reference genome
  - Paired: Filter against matched normal sample – germline and noise removal
  - True germline callers may not be best suited for cancer samples

The variant call format - VCF

- Raw output from the variant caller
- Variant and its position + technical data
  - Read depth (11x)
  - VAR (5/11 ≈ 45%)
  - Quality score
- No gene information

```
GTCTGGCCCCTCGTCAGCATCTTATCC
  CTGGCCCCTCCTCAGCATCTTATCC
 TCTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCC
  CTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
    GCCCCTCGCTCAGCATCTTATCCG
  CTGGCCCCTCGTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
  CTGGCCCCTCCTCAGCATCTTATCCG
  CTGGCCCCTCGTCAGCATCTTATCCG

GTCTGGCCCCTCCTCAGCATCTTATCCGAGT
```

#CHROM	POS	ID	REF	ALT	QUAL	FILTER	INFO
chr1	211545955	.	G	A	.	.	AO=34296;DF
chr11	108137985	.	C	T	.	.	';AO=1100;DF
chr17	7578266	.	T	A	.	.	AO=13131;DF

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The analysis workflow

5. Variant annotation

- Information from genomic databases
- Add information to each variant
  - Gene name
  - Transcript
  - Amino acid consequence
  - dbSNP / 1000 genomes
  - COSMIC

Tool examples:
Annovar
Oncotator
Nirvana
SeattleSeq Annotation

5. Variant filtration - biological

- Clinical setting – usually no matched normal
  - Remove *unimportant* variants
- Remove known germline variants in population
  - Improving databases (e.g. dbSNP -> 1000 genomes -> 1000 genomes Europe -> SweGen)
  - Careful with patient samples of other genetic background
- Remove non-coding and synonymous variants
  - UTR3' and 5'?
  - Splice variants?

5. Variant filtration - technical

- Clinical setting – usually no matched normal
  - Remove technical errors/noise
- Technical quality of variants
  - VAR cutoff
  - Read depth cutoff
  - Variant quality score cutoff (?)
- Panel of normals / negative controls?
  - Potentially efficient for recurrent panel errors
  - How many samples?

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The analysis workflow

6. Quality control

- General quality of the sequencing run
  - Base qualities
  - Sequencing yield
  - Over/under clustering
  - Percent on target reads
- Sample specific QC
  - Depth of coverage
  - MAPQ
  - % reads mapped

6. Quality control

- No consensus yet

Quality Measure	ACGS	ACMG	CAP	EuroGentest	RCPA	CDC
Base quality	X		X	X	X	X
GC bias	X			X	X	X
Distinct reads		X		X	X	X
Reads mapped (%)	X			X		
Percentage of reads on target region		X	X	X		
Mapping quality	X		X	X	X	X
Average depth on target region	X			X	X	X
Uniformity		X		X	X	X
Off Target						X
Percent of target captured	X	X	X			

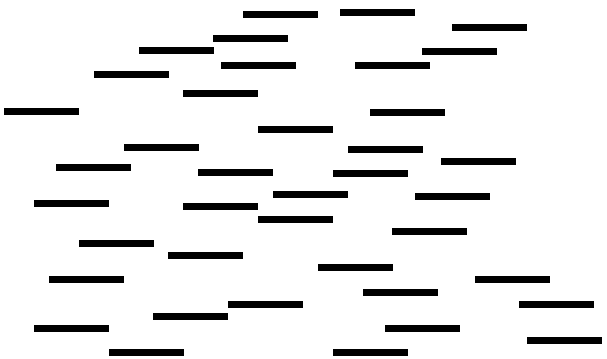
http://euformatics.com/evolving-standards-in-clinical-ngs/

Depth of coverage

- The number of times a base-pair is covered by aligned reads
- Targeted deep sequencing: Mean coverage within the target regions

Depth of coverage

Unaligned reads



X gigabases of sequencing
- Per sample?
- Per region/base?

Depth of coverage

- The number of times a base-pair is covered by aligned reads
- Targeted deep sequencing: Mean coverage within the target regions
- Best cutoff metric
  - Mean coverage?
  - Percent bases covered 100x/1000x?
  - Target specific?

Tool examples:
Sambamba
Samtools
Bedtools

6. Quality control and inspection

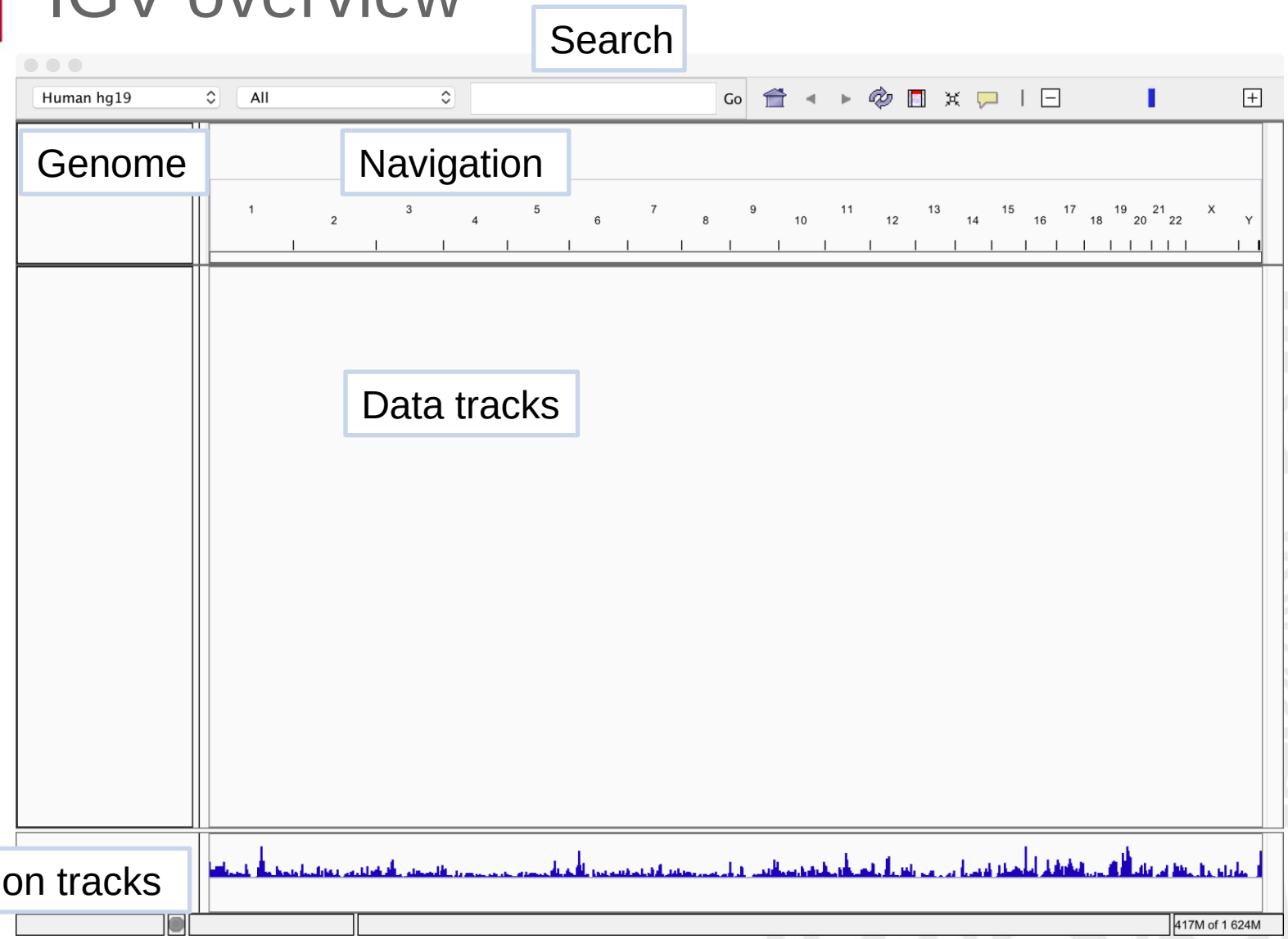
- Variant lists good for big data quantities
- Information about a specific variant?
- Inspect problematic regions and alignment results

- IGV

What is IGV?

- **I**ntegrative **G**enomics **V**iewer
- Desktop genome browser - "visualization tool for interactive exploration of large, integrated genomic datasets”
- Display reads and variants
- Runs locally

IGV overview



IGV input file formats

- **BAM-file**
  - coordinate sorted
  - indexed
- **BED-files**
- **VCF-files**
- **Many others**

What can we do in IGV?

1. **Inspect alignments and coverage**
   - File > Load from file > Select BAM file
   - Reset: File > New session

BAM file overview

Zoom

Coverage track

Alignments

Double click to zoom
Drag to move
Zoom in to show variants
Right click: Collapsed/Expanded

Annotation tracks

What can we do in IGV?

1. **Inspect alignments and coverage**
2. **Inspect SNVs**

Chr	Start	End	Reference_base	Variant_base	Gene	Type	Exonic_type	Variant_allele_ratio%	#reference_alleles	#variant_alleles	Read_depth
chr17	7578466	7578466	G	A	TP53	exonic	nonsynonymous SNV	66,88	52	105	157

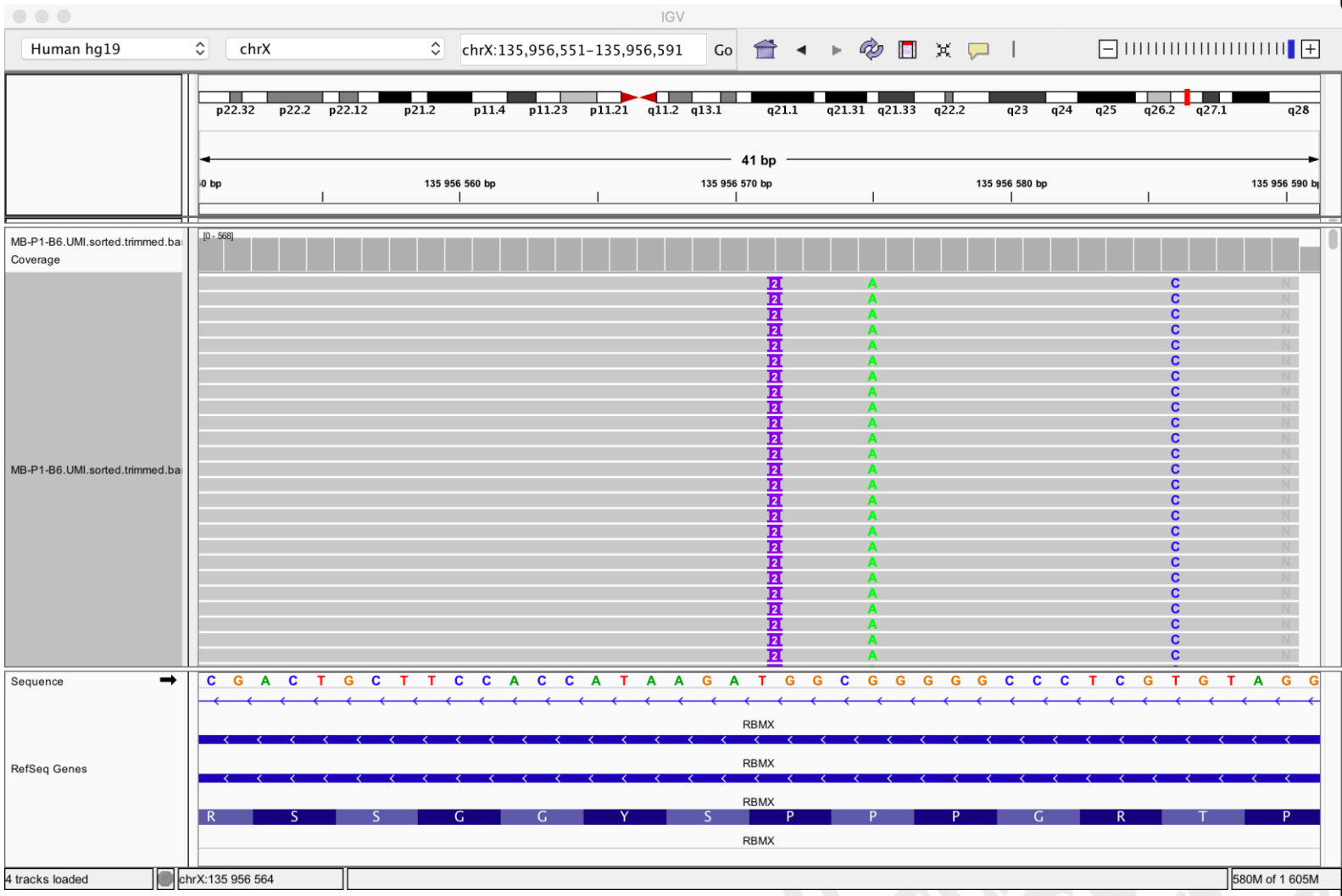
Variant inspection (SNVs)

Clean reads?
Surrounding reads?
Surrounding indels?

What can we do in IGV?

1. **Inspect alignments and coverage**
2. **Inspect SNVs**
3. **Inspect InDels**

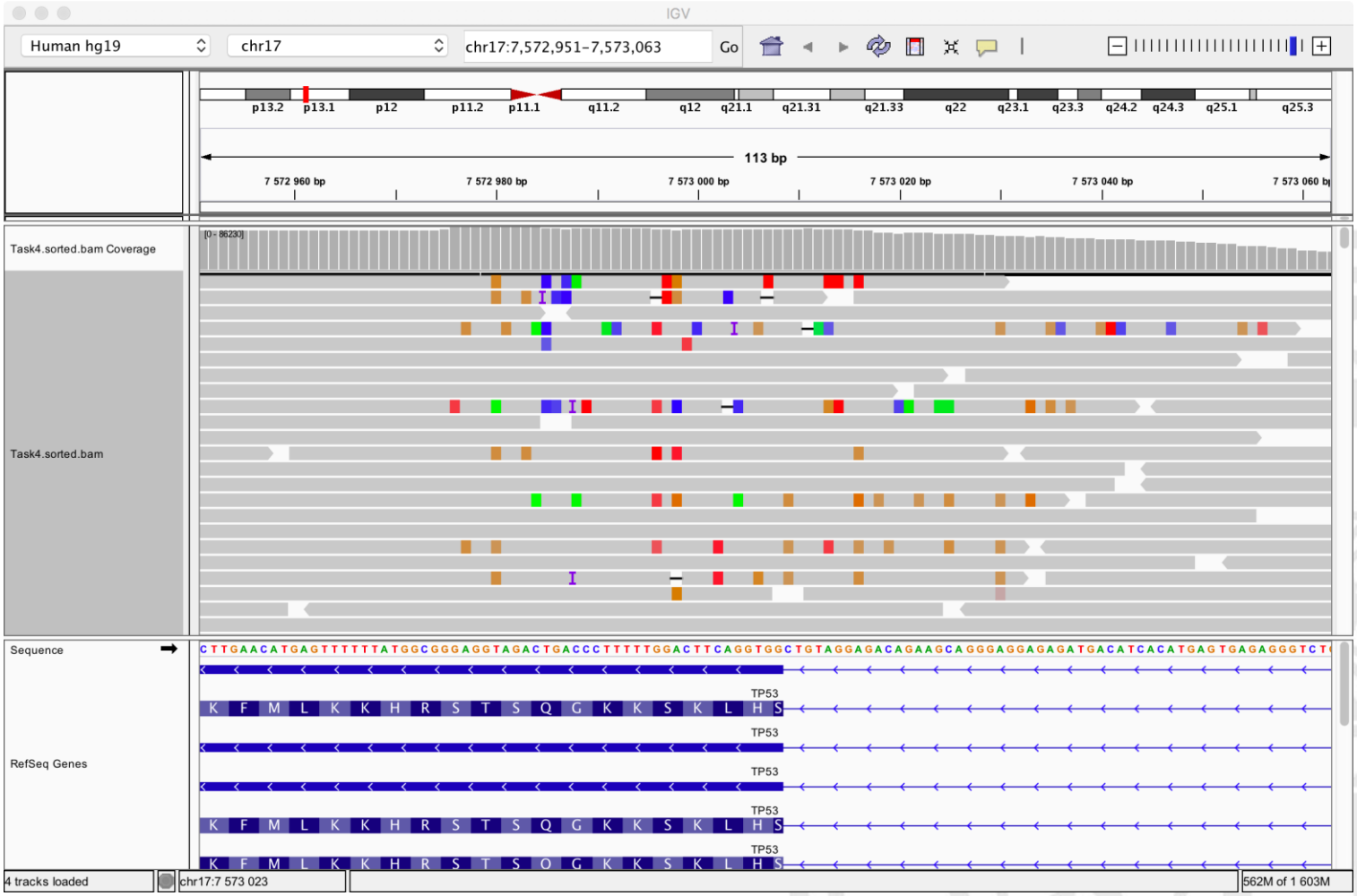
Variant inspection (insertion)



What can we do in IGV?

1. **Inspect alignments and coverage**
2. **Inspect SNVs**
3. **Inspect InDels**
4. **Inspect low quality variants**

Variant inspection (low quality SNV)



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More IGV in the hands-on workshop tomorrow

Read the email and download IGV tonight

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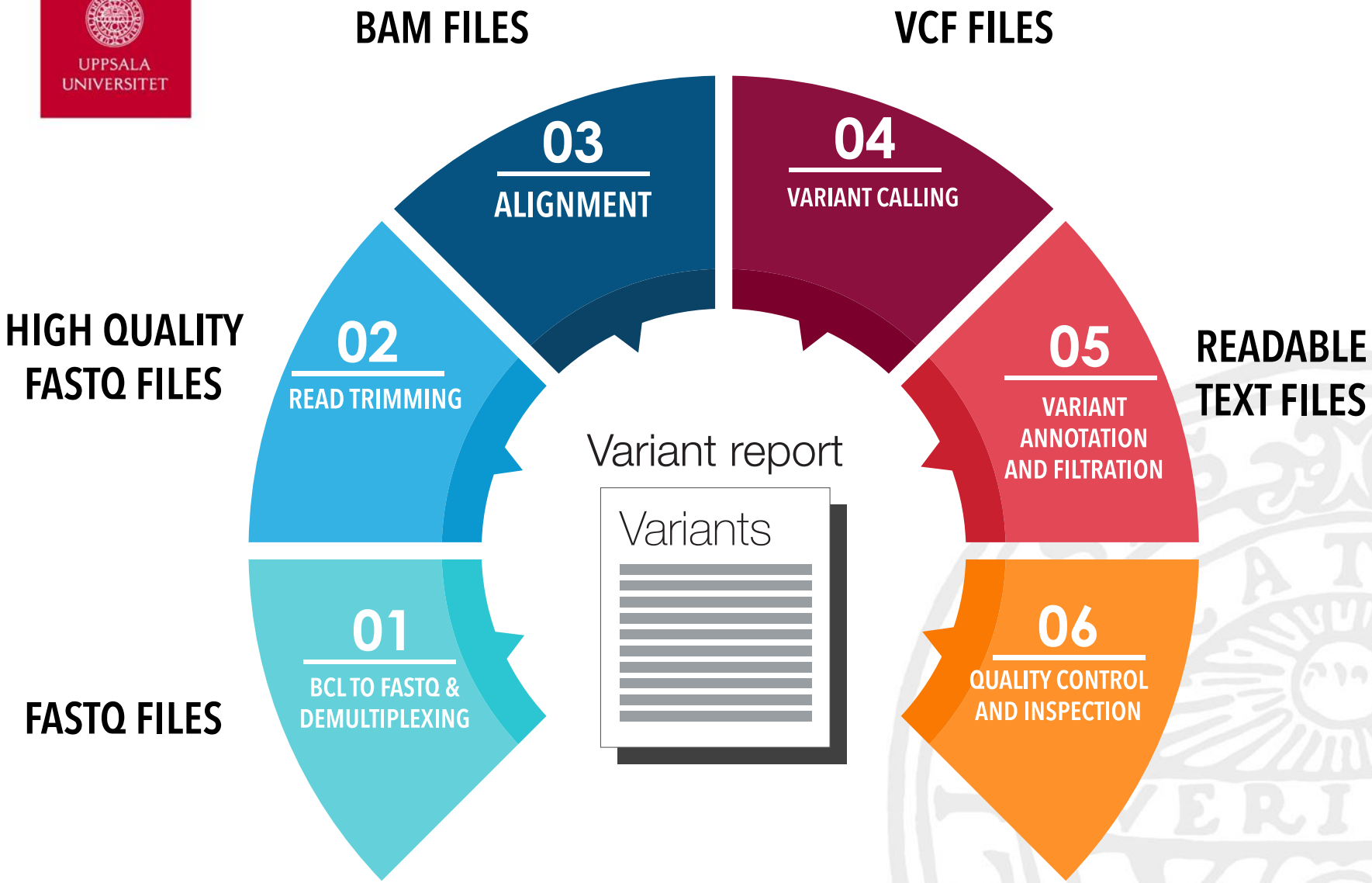
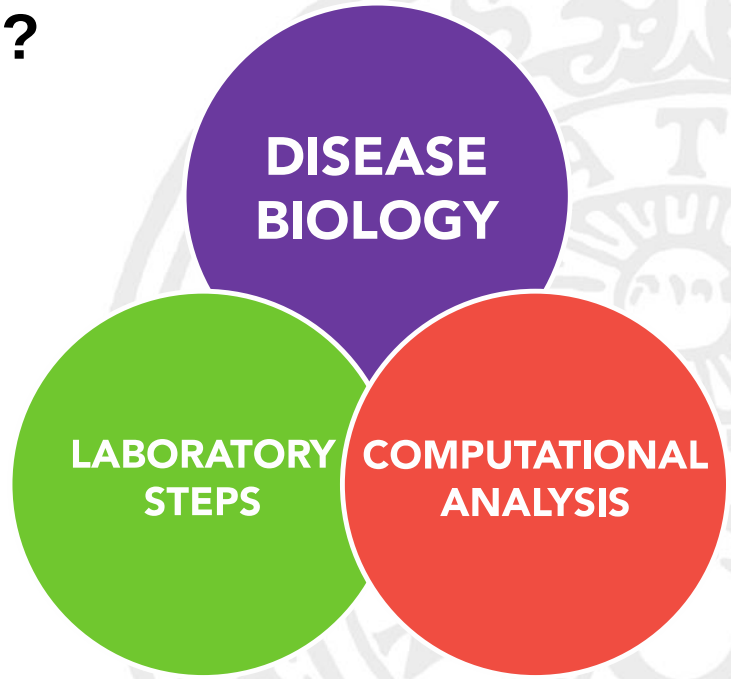


Final remarks

- **Which tools to use**
  - Open source vs proprietary software
  - Still no best practice on the somatic side
- **Bioinformatics pipelines**
  - Feeding from one tool to another
  - Can we agree on one?
- **Cloud solutions**
- **Bioinformatics**
  - Part of the puzzle
- **Future**
  - UMI analysis
  - CNV analysis



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Thank you!