Next Generation Sequencing (NGS): Introduction, Data Formats, Processing & Variant Detection: **Part I**

Dr Stephen J Newhouse
Lead Data Scientist & Senior Bioinformatician @ NHIR BRC-MH SlaM NHS & IoPPN KCL & UCL Farr Institute
stephen.j.newhouse@kcl.ac.uk, @s_j_newhouse

Genomic Medicine MSc
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Overview

1. DNA Sequencing the very basics
2. NGS technologies and terminology
3. Experimental designs
4. Data formats
5. Data processing overview

Special Acknowledgements:

many slides taken from :-

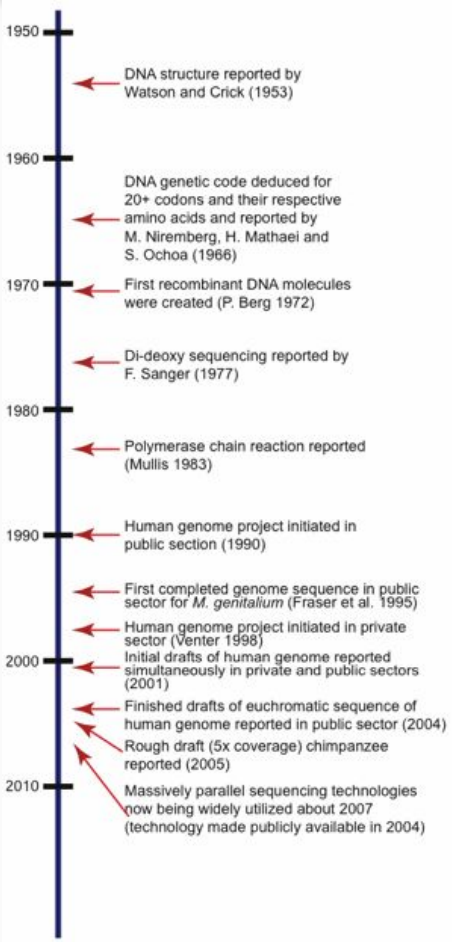
- https://www.broadinstitute.org/gatk/events/slides/1506
- https://www.broadinstitute.org/gatk/events/slides/1506/GATKwr8-A-2-Intro_to_HTS.pdf

(these are the experts & all credit goes to them!)

1. DNA Sequencing

Some Milestones in Molecular Biology

- First Isolation of DNA : 1867 (Freidrich Meisher)
- Composition of nucleic acids; tetranucleotide theory : 1909 - 1940 (Phoebus Levine)
- Base Pair rules: G=C and A=T : 1950 (Edwin Chargaff)
- DNA Structure: 1953 (Watson & Crick)
- Genetic Code deduced, AA>Condons>Proteins: 1966 (Niremberg,Mathael, Ochoa)
- **Sanger Sequencing : 1977 (Frederick Sanger)**
- **Polymerase Chain Reaction: 1983 (Kary Mullis)**
- **Human Genome Project Started: 1990**
- **Next-Generation Sequencing : 2005**

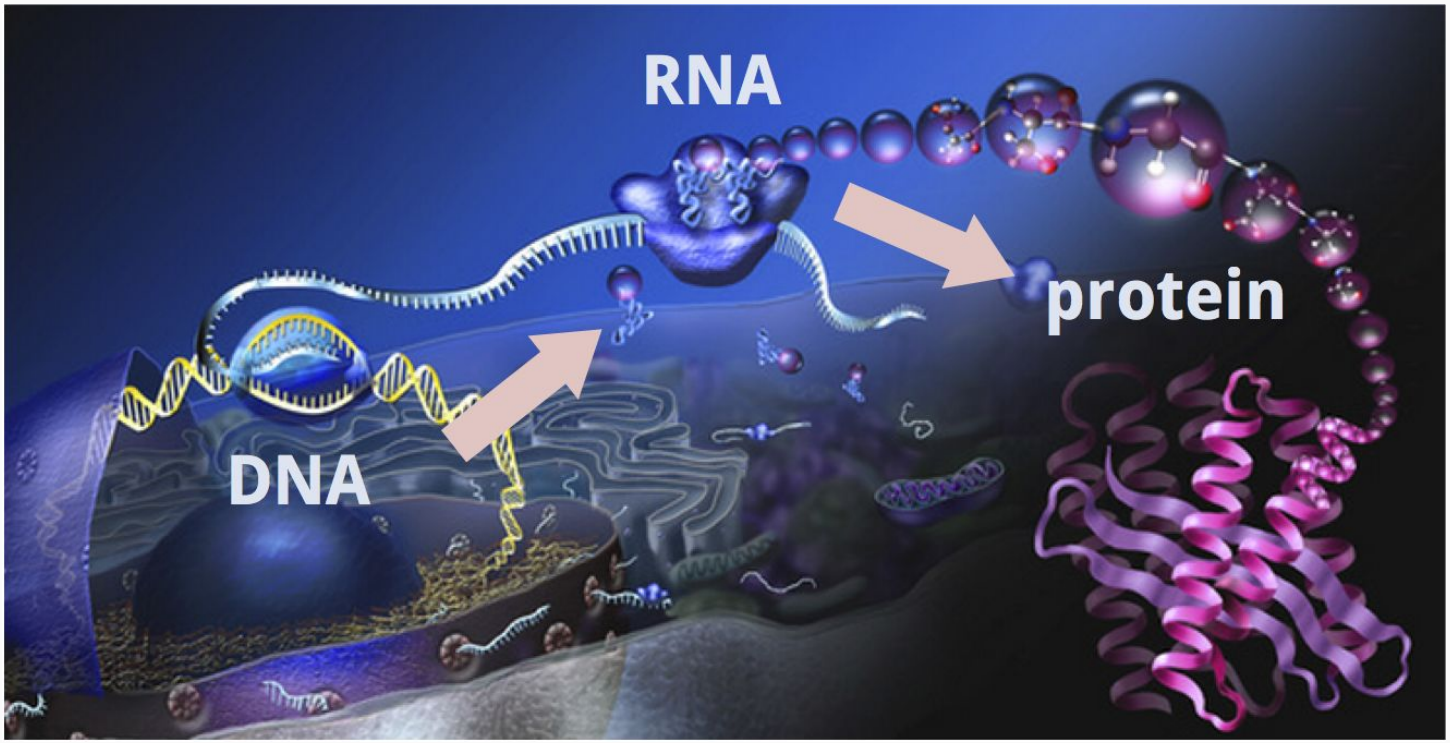


What is DNA Sequencing?

- DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule
- Any method or technology that is used to determine the order of the four (5) bases—adenine, guanine, cytosine, and thymine (A,G,C,T) in a strand of DNA
- DNA can be isolated from cells of animals, plants, bacteria, archaea, or virtually any other source of genetic information.
- The DNA sequencing revolution has impacted nearly every field of biological research!

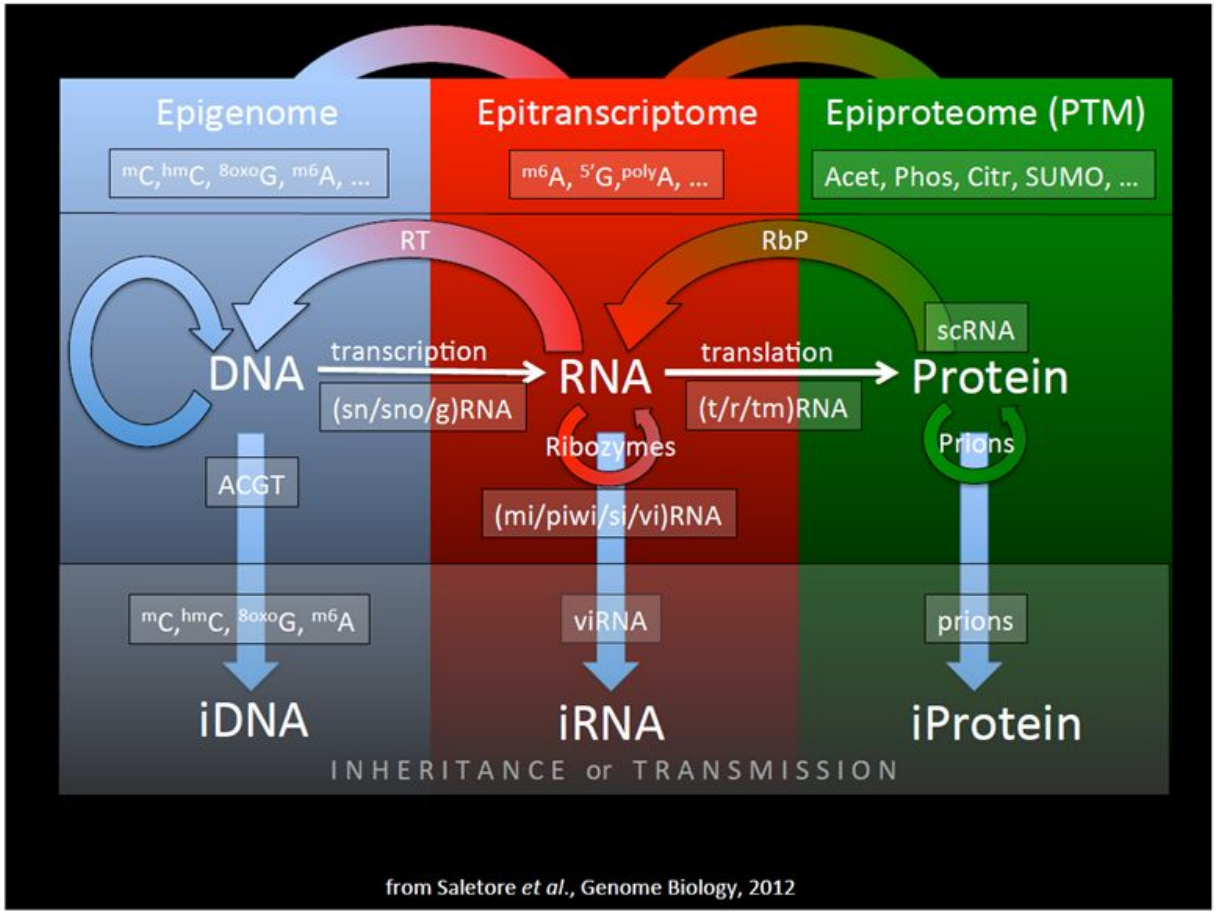
The Central Dogma of Molecular Biology

James Watson version - 1965



So once we have the genomic DNA sequence of a species we have all of the information there is?.....Not Really….

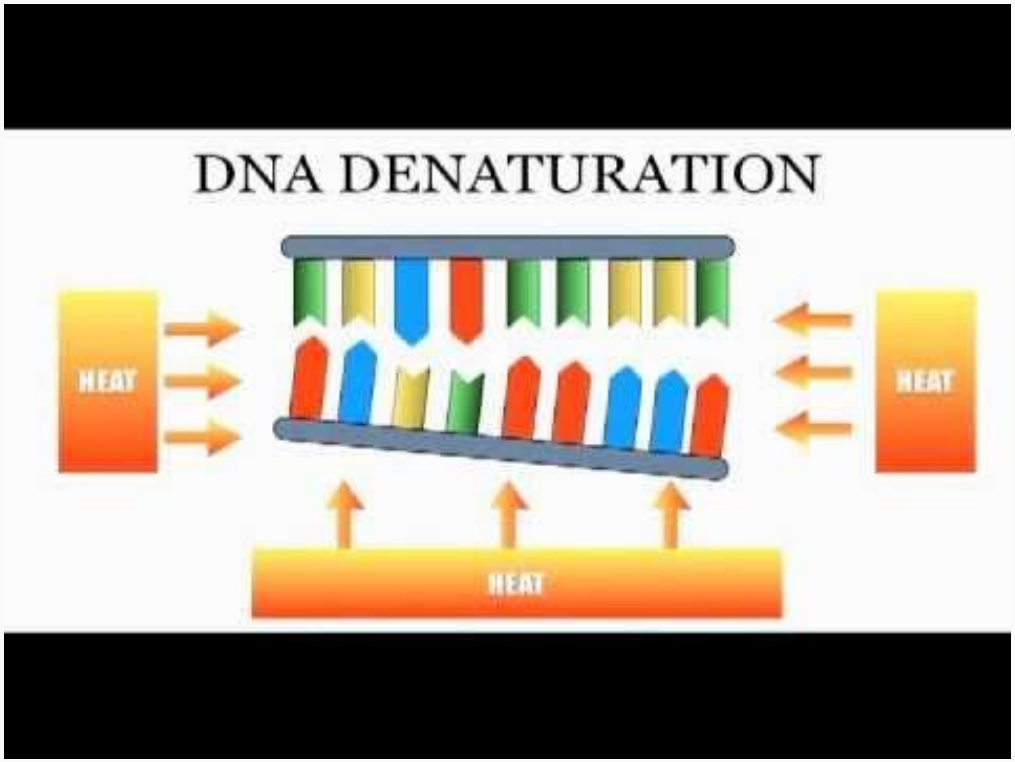
The Central Dogma : 2016



Why Sequence DNA?

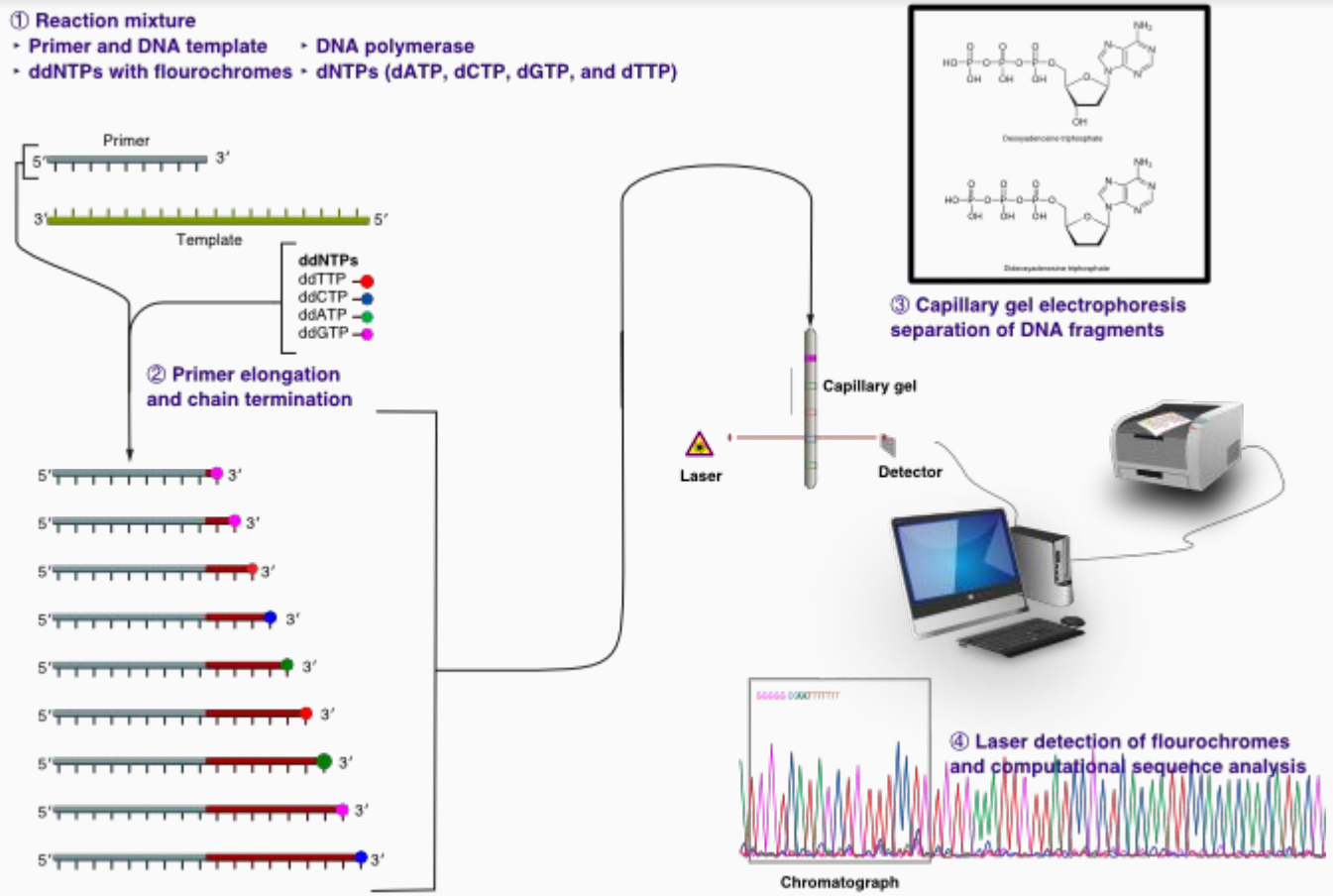
- Determine sequence of:
  - Genes, Gene clusters or pathways
  - Whole Chromosomes
  - Whole Exomes
  - Entire Genomes
- Studying the Genome enables:
  - Identify new genes & new drug targets
  - Look for association of genes/mutations with diseases and phenotypes
  - Personalised/Stratified medicine & Clinical Diagnostics
  - Study Evolutionary biology - relationship between species
  - Plants & Agriculture: Breeding and Improved strains
  - Microbes, Viruses and Infectious diseases
  - Environmental Genomics & Metagenomics - id species present in water, dirt, debris filtered from the air, or swab samples of organisms
  - Forensics, Paternity testing...

Sanger Sequencing: Chain Termination Method (the ‘90s)..

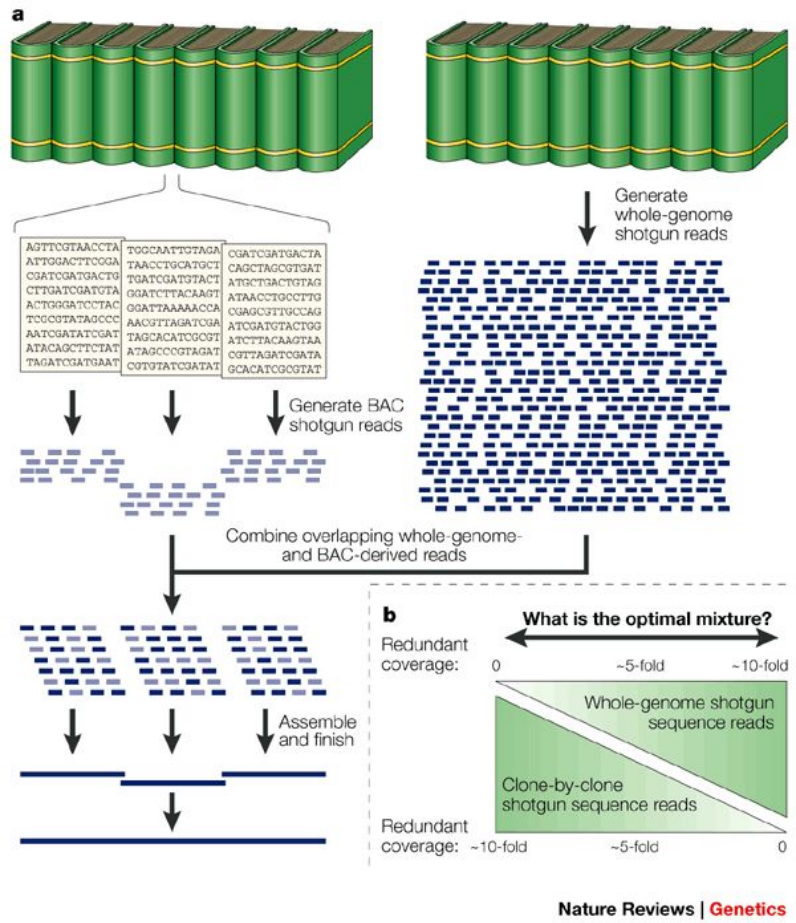


http://youtu.be/vK-HIMaitnE

Sanger Sequencing: Chain Termination Method



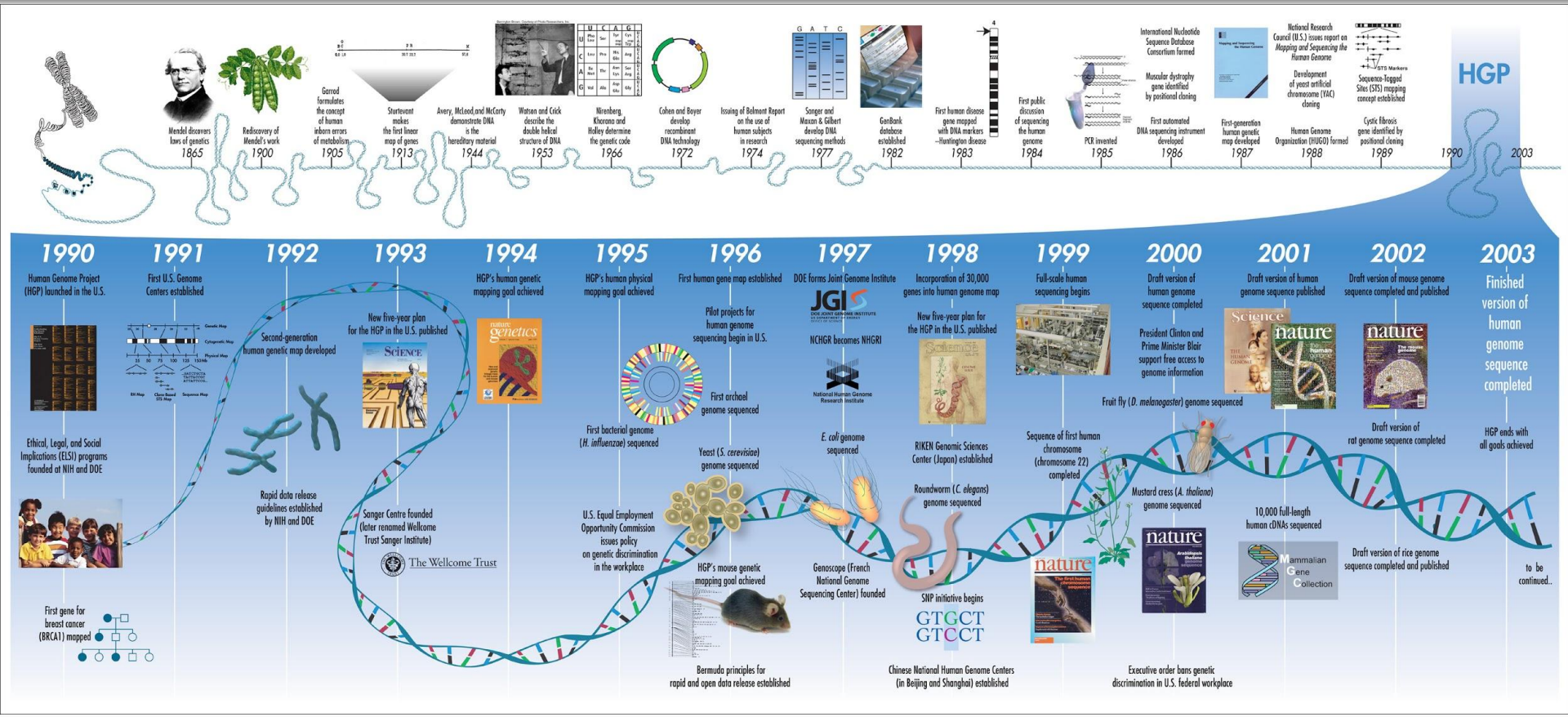
Shotgun-sequencing the human Genome



a | In a hybrid approach, elements of both clone-by-clone and whole-genome shotgun sequencing are amalgamated. A subclone library from a whole genome (represented by an encyclopaedia set) is prepared, and numerous sequence reads (depicted in dark blue) are generated in a genome-wide fashion. Meanwhile, individual mapped BACs are also subjected to shotgun sequencing. The BAC-derived sequence reads (depicted in light blue) can then be used to identify overlapping sequences in the larger collection of whole-genome-derived sequence reads, in essence reducing the complexity of the whole-genome shotgun data set to a series of individual BAC-sized bins. The combined set of sequence reads for each BAC can then be individually assembled and subjected to sequence finishing. ***b*** | Optimal balance of generating clone-by-clone versus whole-genome shotgun sequence reads in a hybrid sequencing strategy. In the hybrid shotgun-sequencing approach illustrated in ***a***, sequence reads derived from individual clones and those generated in a genome-wide fashion are assembled together. Although an overall sequence redundancy of 8–10-fold is typically desired, the optimal mixture of clone-by-clone and whole-genome shotgun sequence reads is at present not well established. Current projects that are using a hybrid approach for sequencing the mouse, rat and zebrafish genomes (seeTable 1) should provide valuable insight into this issue.

http://www.nature.com/nrg/journal/v2/n8/full/nrg0801_573a.html

The Human Genome Project: 13 years in the making!



http://exploreable.wordpress.com/2011/05/03/the-story-of-the-human-genome-project-a-short-narration/

2. NGS Technology & Terminology

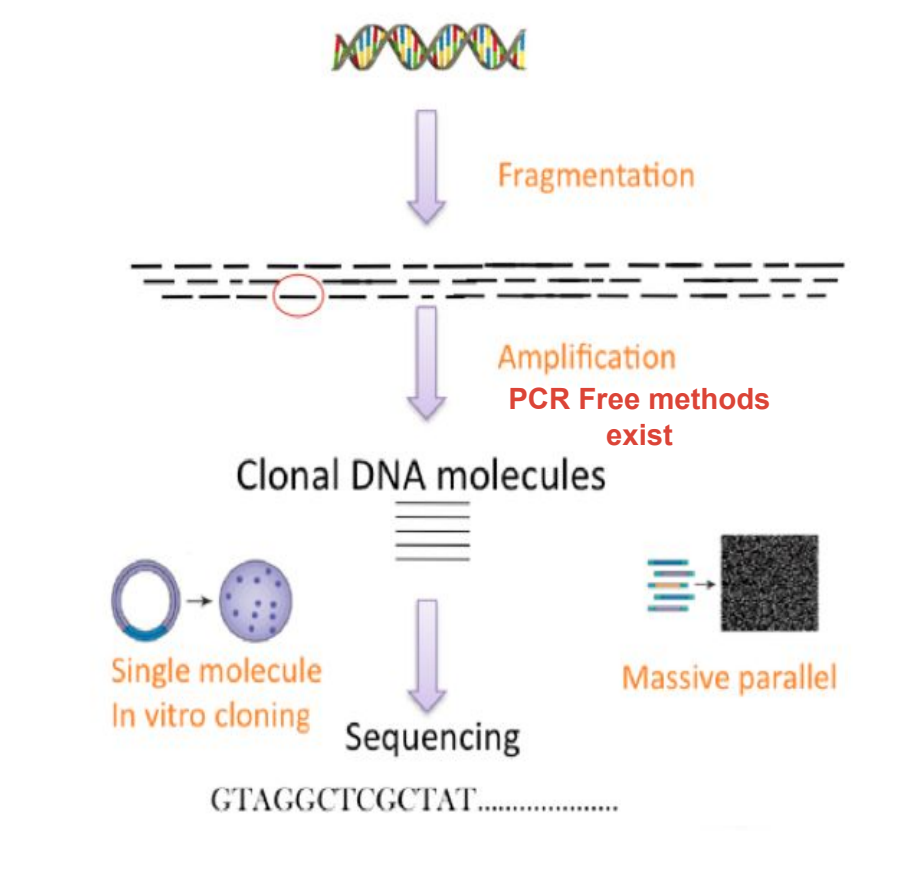
Next Generation Sequencing

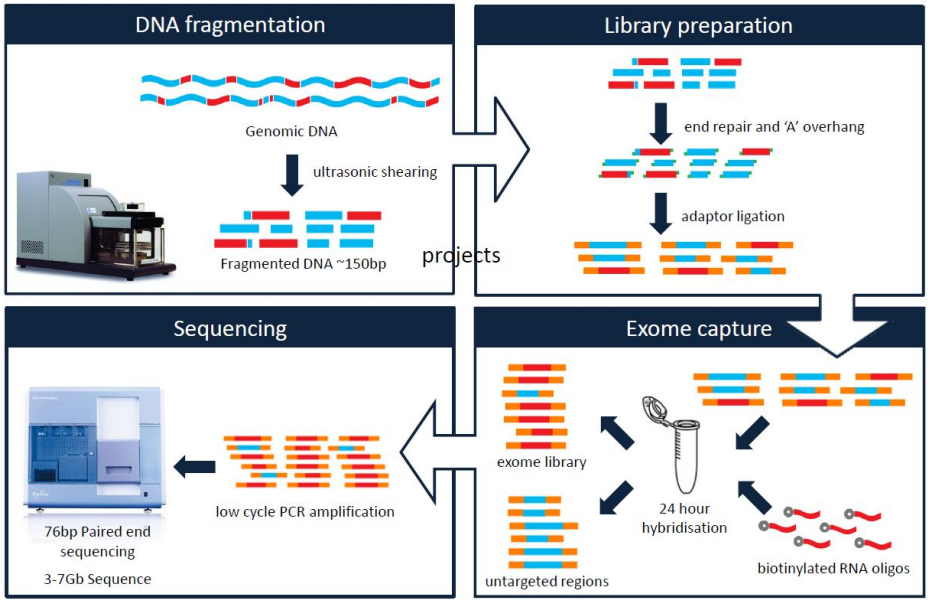
- Shotgun sequencing on steroids!
- Cost effective, fast in comparison, Clone-free, generates millions of short reads that can be assembled and mapped to a reference genome (Human Genome Project)
- A Human Genome Project now takes ~ 1 month vs 13 years!
- NGS has brought high speed, high resolution molecular genetics to science and medicine and the benchtop!
- Will revolutionise medical diagnostics through rapid identification of mutant alleles that cause disease - High hopes for Personalised Medicine!

Terminology: Next Generation Sequencing

- **Second/Third Generation Sequencing**
  - Next Generation Sequencing (NGS)
  - High-Throughput DNA Sequencing (HTS)
  - Massively Parallel Sequencing (MPS)
  - ***Illumina: sequencing by synthesis (SBS):***
    - *http://www.illumina.com/technology/next-generation-sequencing/sequencing-technology.html*
- ***First Generation Sequencing...big in the 90's***
  - *Sanger Sequencing*

How does Next Generation Sequencing work?





Thanks to Dr Mike Simpson (michael.simpson@kcl.ac.uk)

How does Next Generation Sequencing work?

- **generates millions of short nucleotide sequences (reads)**
- **Massive amount of data!**
- Raw files ~2-30GB x 2 per sample (Illumina)

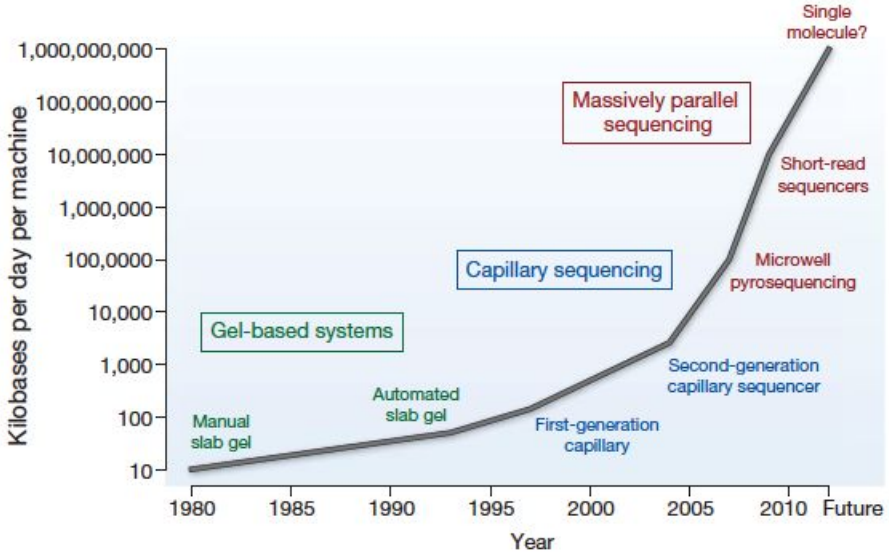
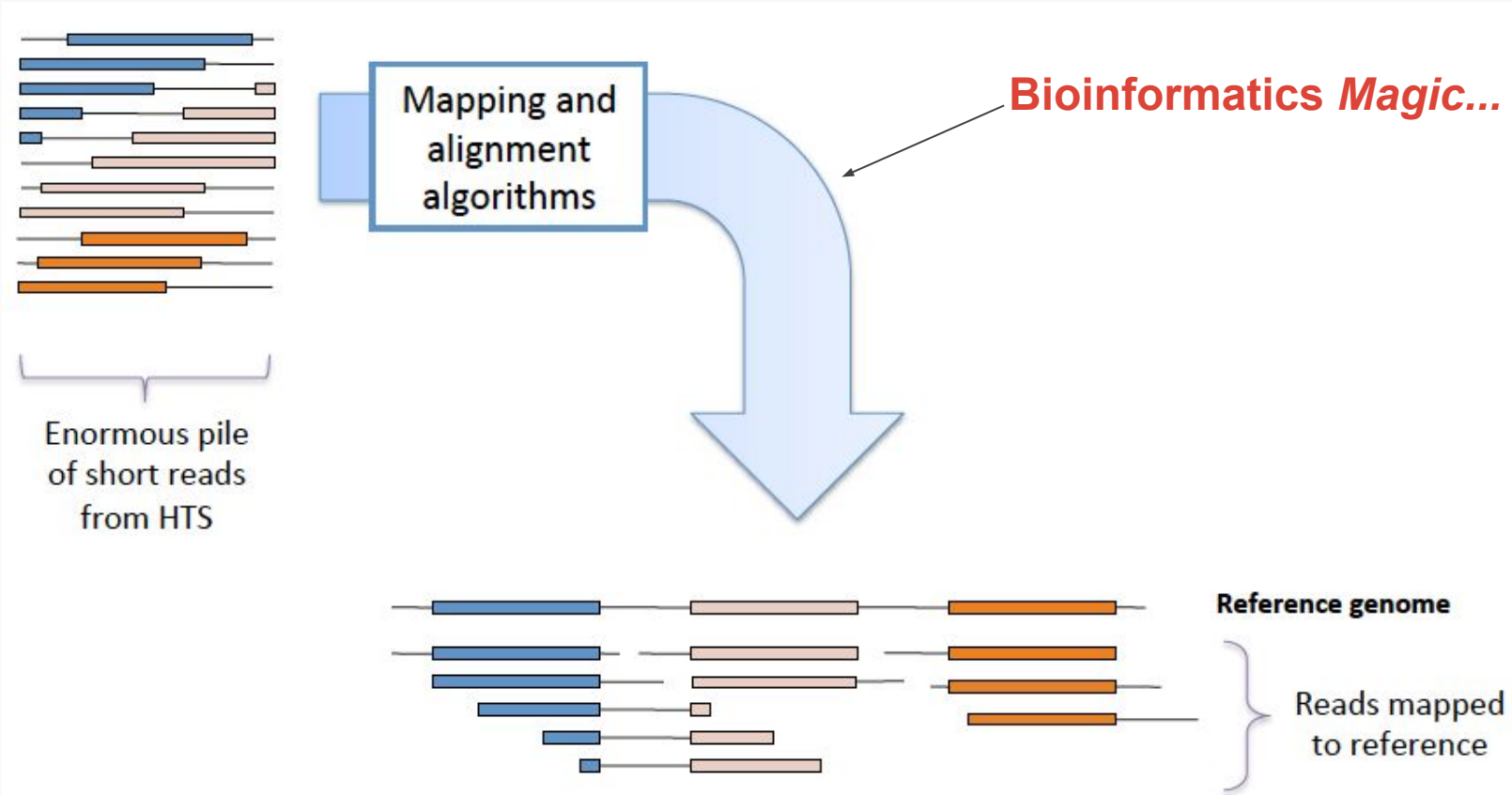


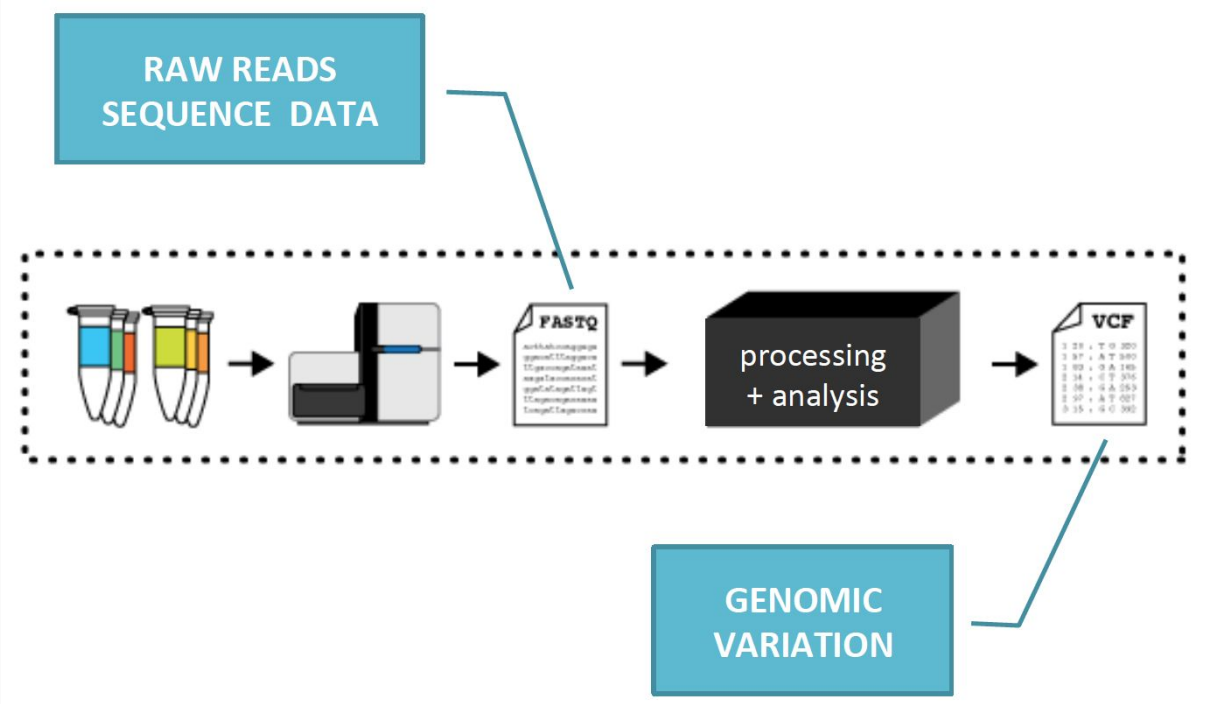
Figure 3 | Improvements in the rate of DNA sequencing over the past 30 years and into the future. From slab gels to capillary sequencing and second-generation sequencing technologies, there has been a more than a million-fold improvement in the rate of sequence generation over this time scale.

How does Next Generation Sequencing work?



Genomic Medicine & NGS

- **The Ultimate goal is to detect clinically relevant variants from sequencing data**



Types of Variation Detected by NGS

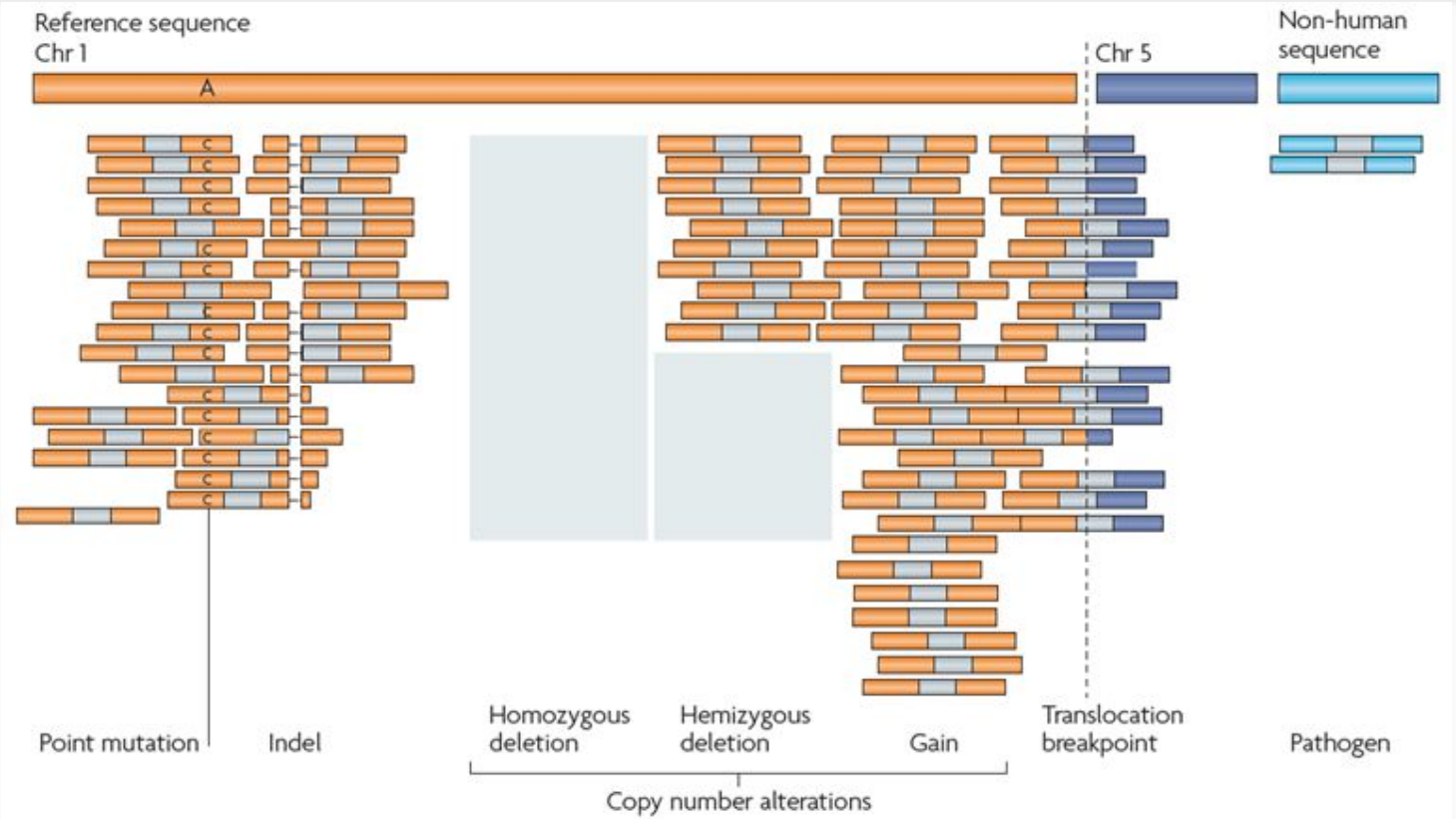
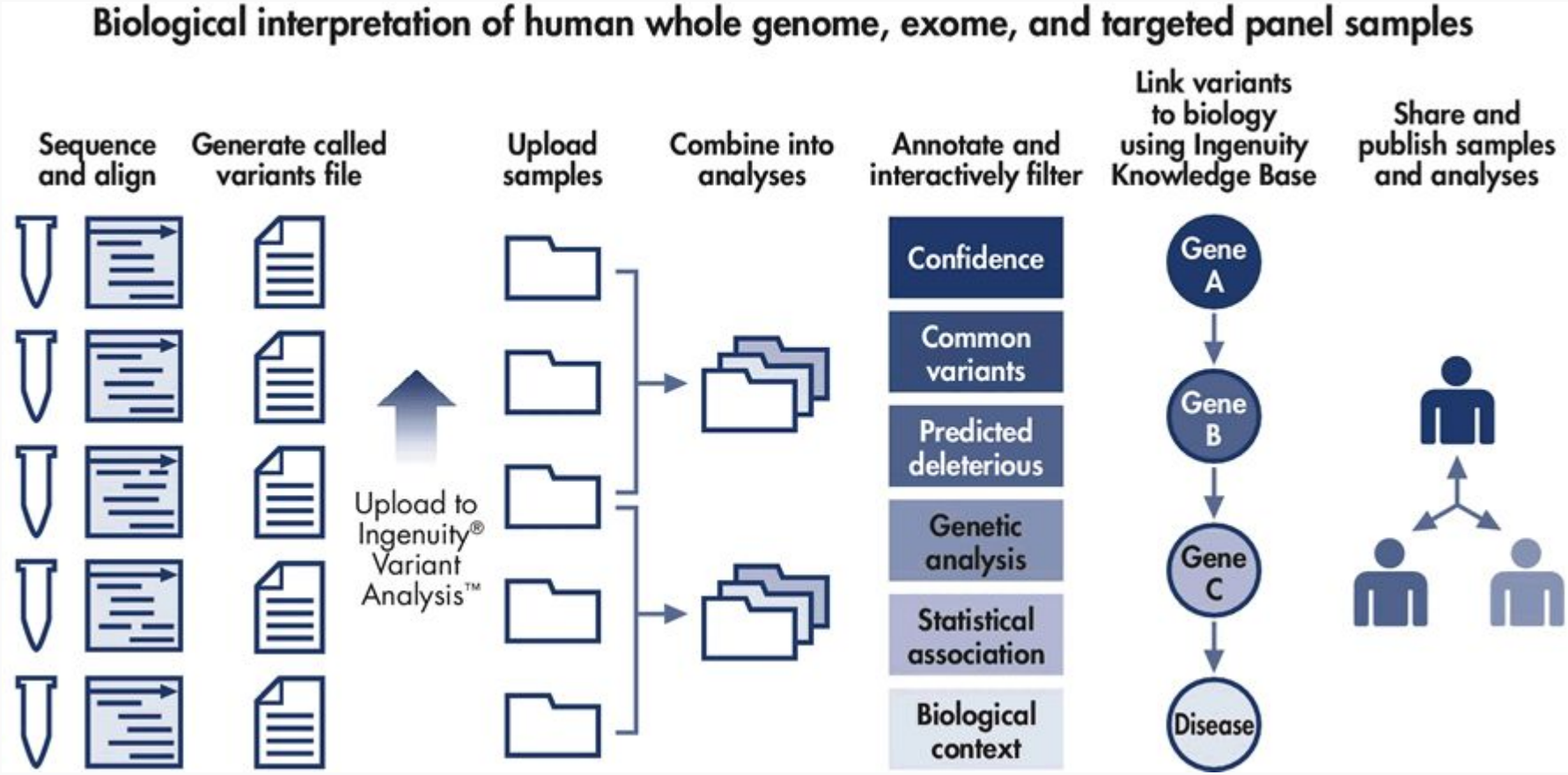


Figure 3 | **Types of genome alterations that can be detected by second-generation sequencing.** Sequenced

Nat Rev Genet. 2010 Oct;11(10):685-96. doi: 10.1038/nrg2841.

Overview: Interpretation of NGS...



http://www.ingenuity.com/products/variant-analysis

NGS Technologies: **Illumina** & Others….

illumina

ion torrent

Roche

illumina · Roche · life technologies · PACIFIC BIOSCIENCES · Helicos BioSciences Corporation · ion torrent · Oxford NANOPORE Technologies

HiSeq2000 / 2500

Ion torrent

Ion Proton

MiSeq

GS-FLX

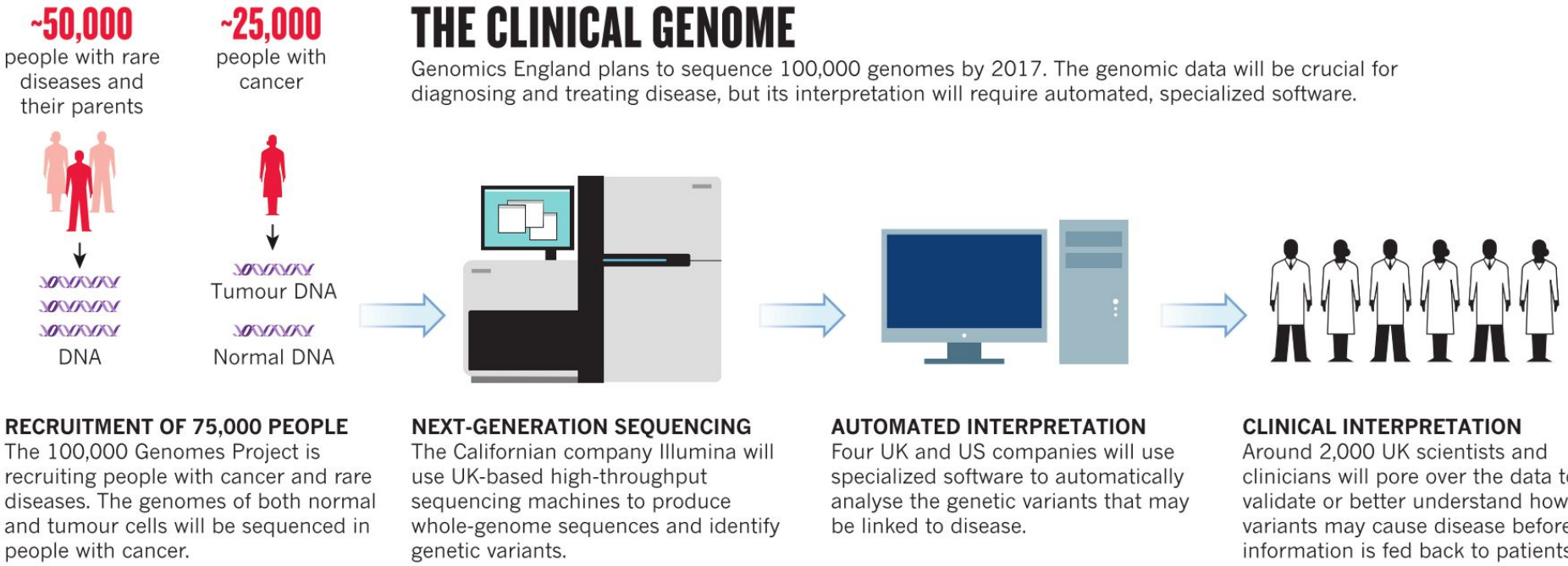
http://flxlexblog.wordpress.com/2012/12/03/developments-in-next-generation-sequencing-a-visualisation/

Genomics England are using illumina

http://www.illumina.com/systems/sequencing-platform-comparison.html

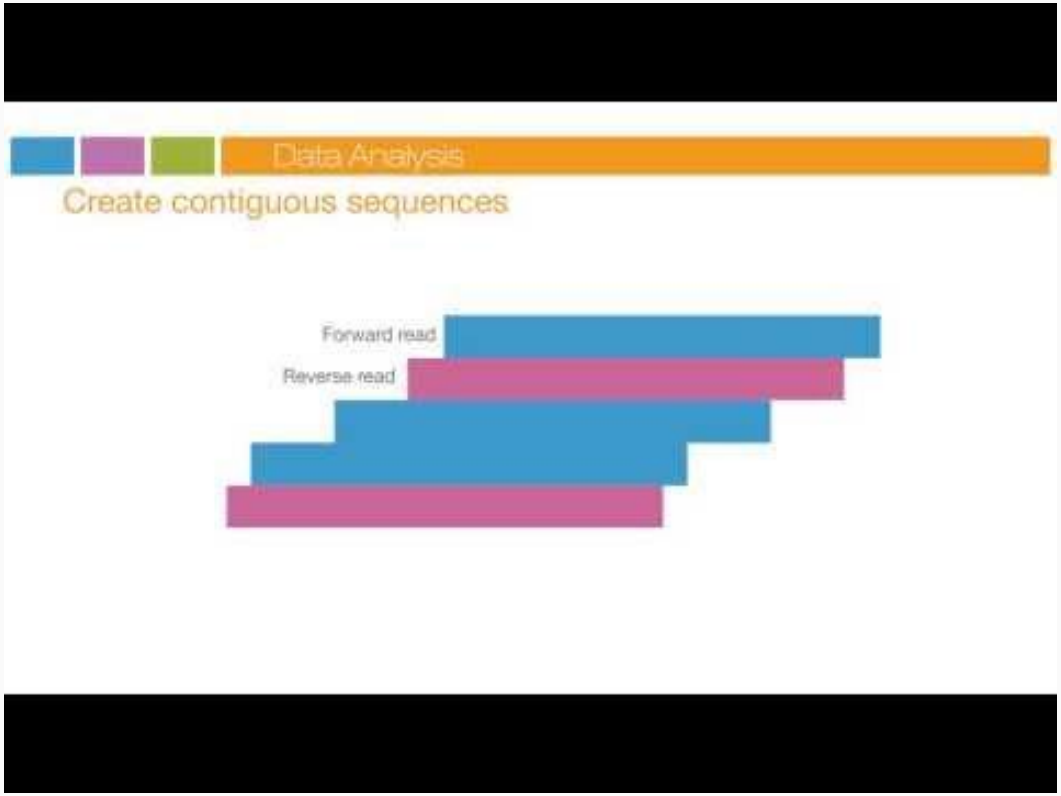
The DNA of A Nation

Vivien Marx
Nature 524, 503–505 (27 August 2015) doi:10.1038/524503a
Published online 26 August 2015



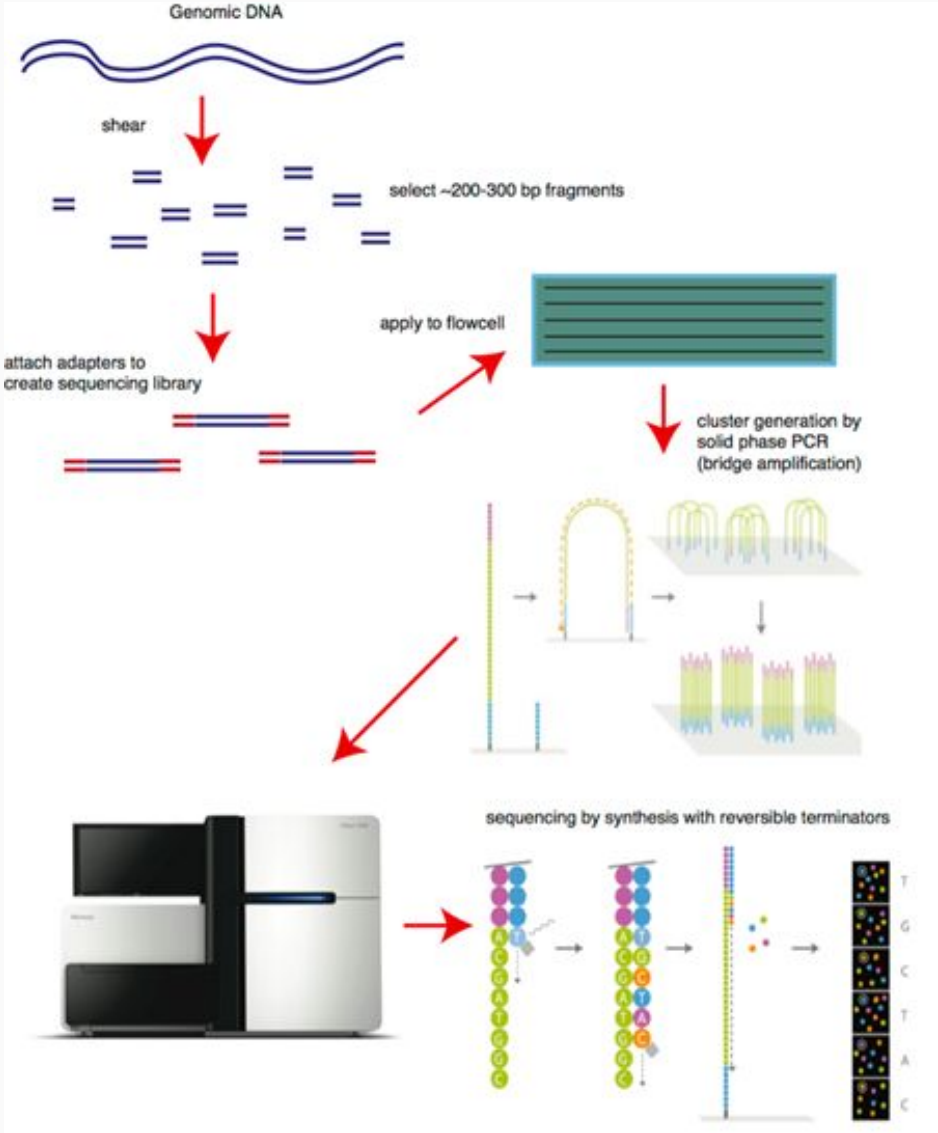
http://www.nature.com/nature/journal/v524/n7566/full/524503a.html

Illumina Sequencing Technology



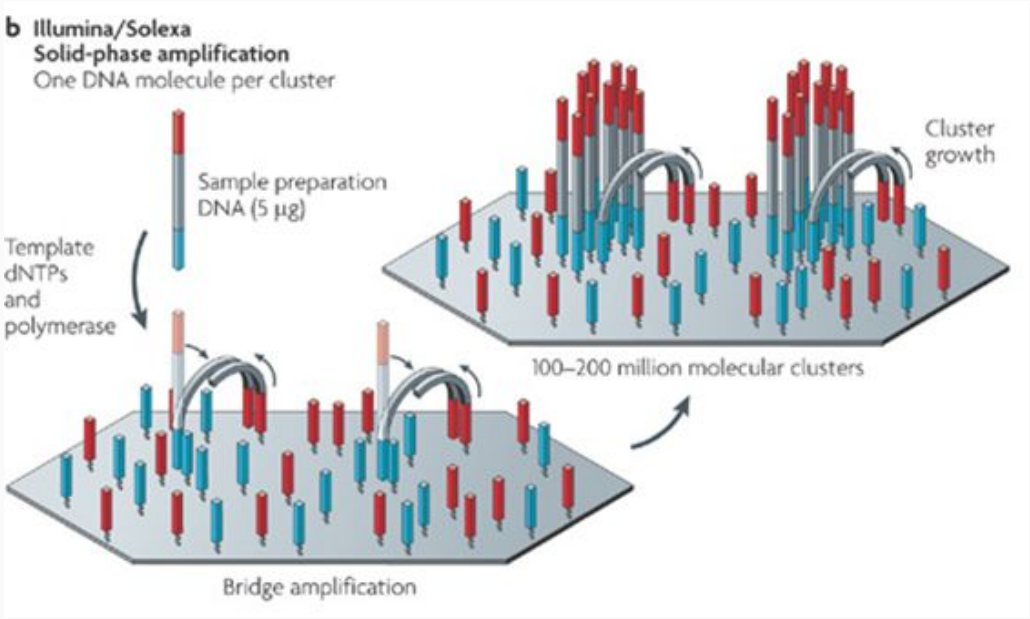
https://youtu.be/womKfikWlxM
5.03mis

Illumina Sequencing Technology: Overview



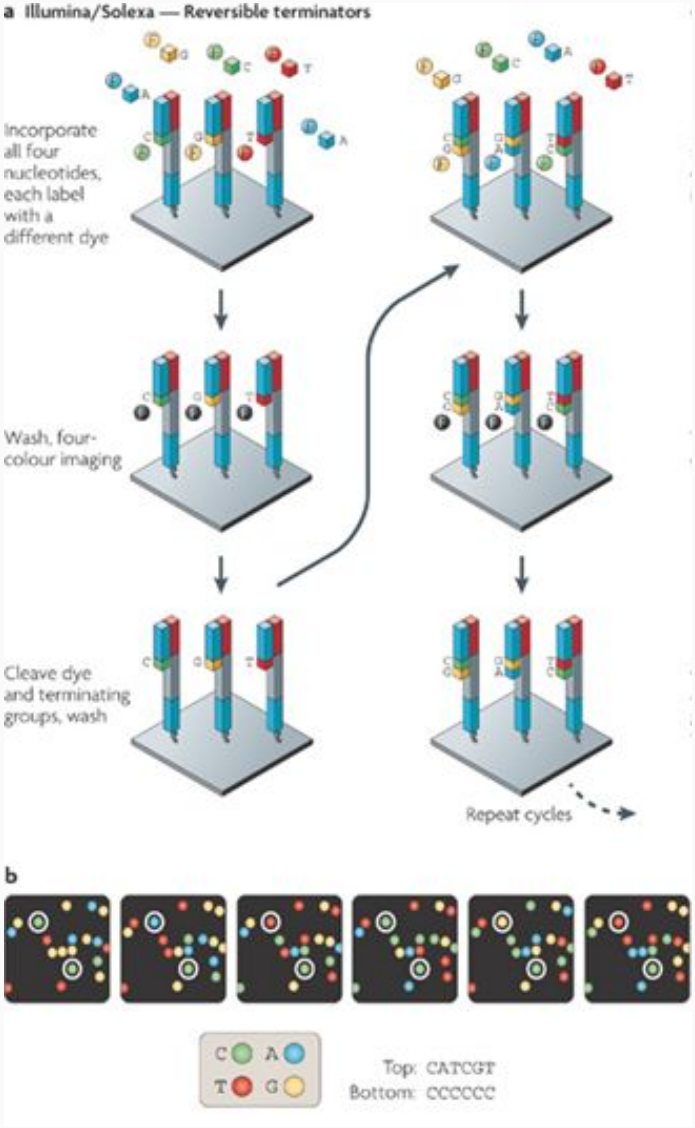
- **Fragmentation** and **tagging** of genomic/cDNA fragments – provides universal primer allowing complex genomes to be amplified with common PCR primers
- Template immobilization – DNA separated into single strands and captured onto beads (1 DNA molecule/bead)
- **Clonal Amplification** – Solid Phase Amplification
- **Sequencing and Imaging – Cyclic reversible termination (CRT) reaction : this is SBS**

Illumina Sequencing Technology: Clonal Amplification



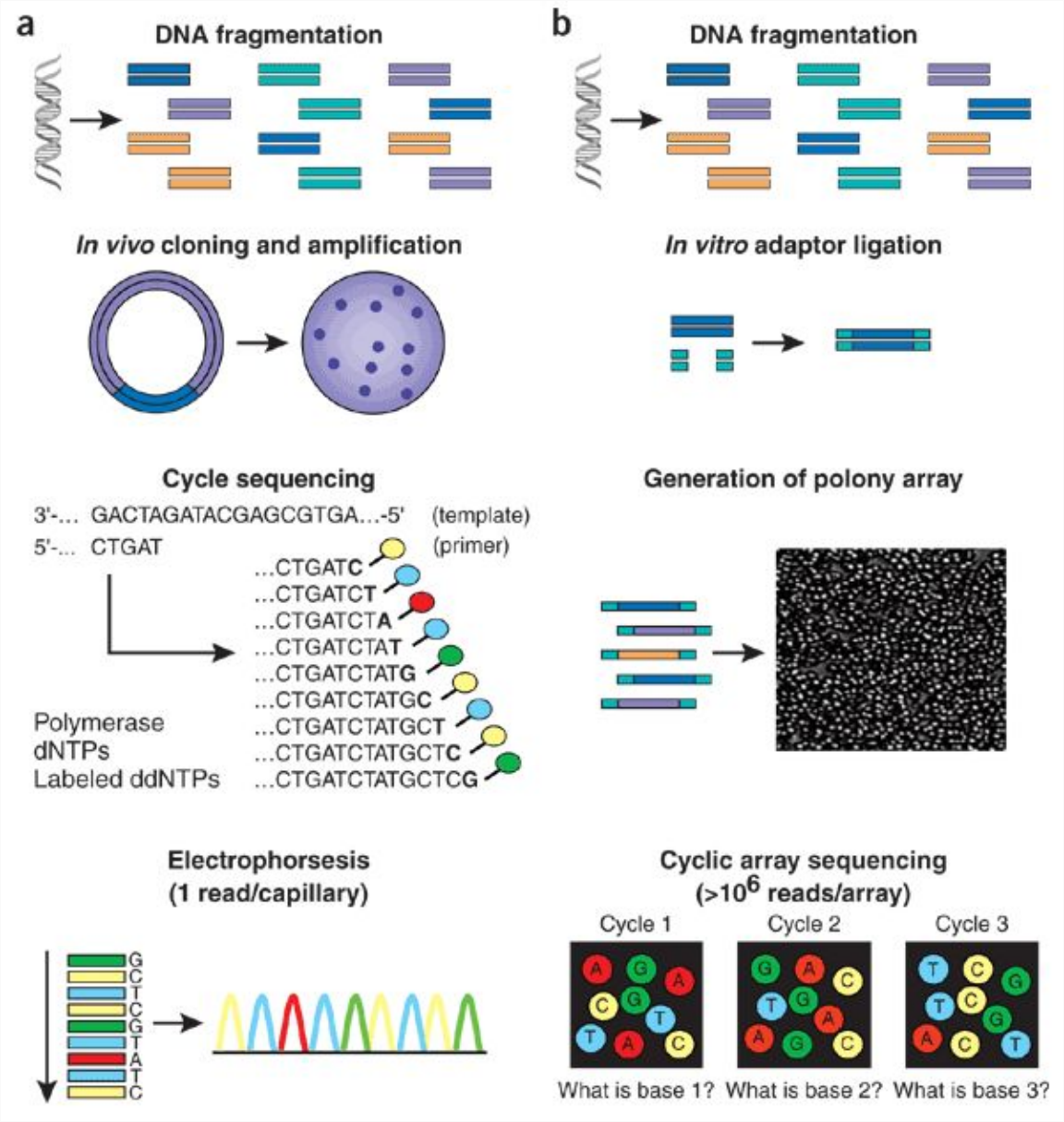
- Clonal Amplification – Solid Phase Amplification
- Priming and extension of single strand, single molecule template
- Bridge amplification of the immobilized template with primers to form clusters (creates 100-200 million spatially separated template clusters)
- Provides free ends to which a universal sequencing primer can be hybridized to initiate NGS reaction – each cluster represents a population of identical templates

Illumina Sequencing Technology : Cyclic Reversible Termination



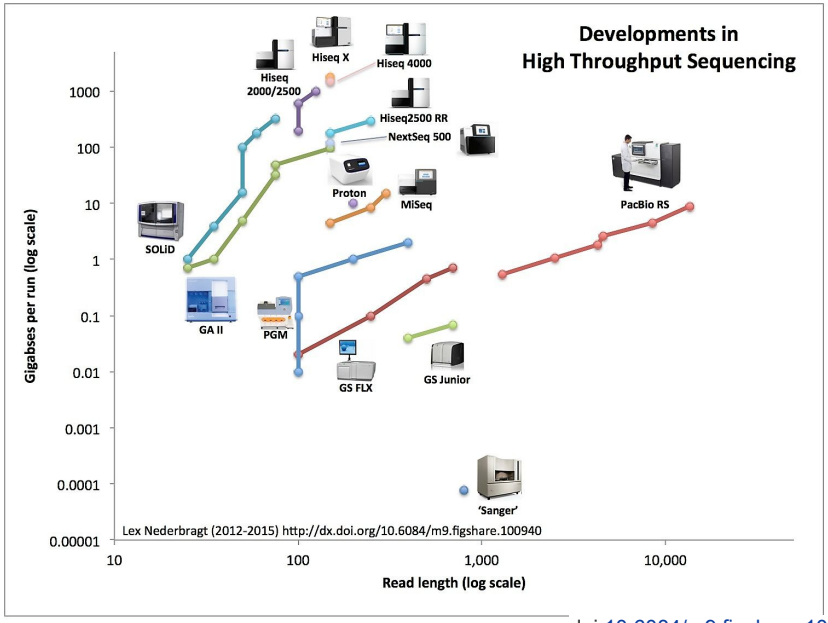
- **Cyclic Reversible Termination** – DNA Polymerase bound to primed template adds 1 (of 4) fluorescently modified nucleotide. 3’ terminator group prevents additional nucleotide incorporation.
- Following incorporation, remaining unincorporated nucleotides are washed away. Imaging is performed to determine the identity of the incorporated nucleotide.
- Cleavage step then removes terminating group and the fluorescent dye. Additional wash is performed before starting next incorporation step
- This is repeated ~250 million times (25Gb) with HiSeq2500 (~4 days)

Sanger *(Shotgun)* vs NGS Sequencing: Overview



Terminology: Short/Long Reads

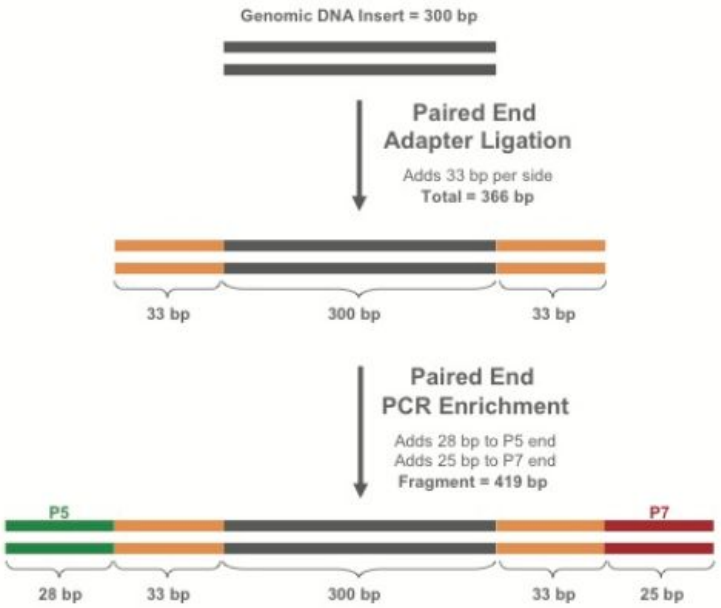
- A **Read**: a sequence of DNA generated (read) by NGS technology
- Short or Long refer to the length of the sequence
- **Short**: 35-150bp (Illumina)
- **Long**: 200bp - 1000's bp (eg Sanger & Pacbio)
- **Read Length:** length of read produced by machine
- *Reads are getting longer as technology improves & with new technologies*



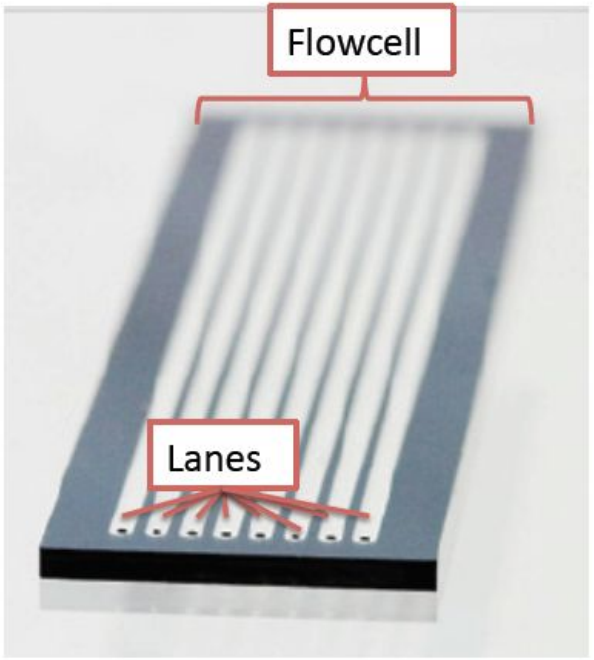
doi 10.6084/m9.figshare.100940

https://github.com/lexnederbragt/developments-in-next-generation-sequencing
https://figshare.com/articles/developments_in_NGS/100940
https://flxlexblog.wordpress.com/2015/06/17/developments-in-high-throughput-sequencing-june-2015-edition/

Terminology: Libraries, Lanes and Flowcells



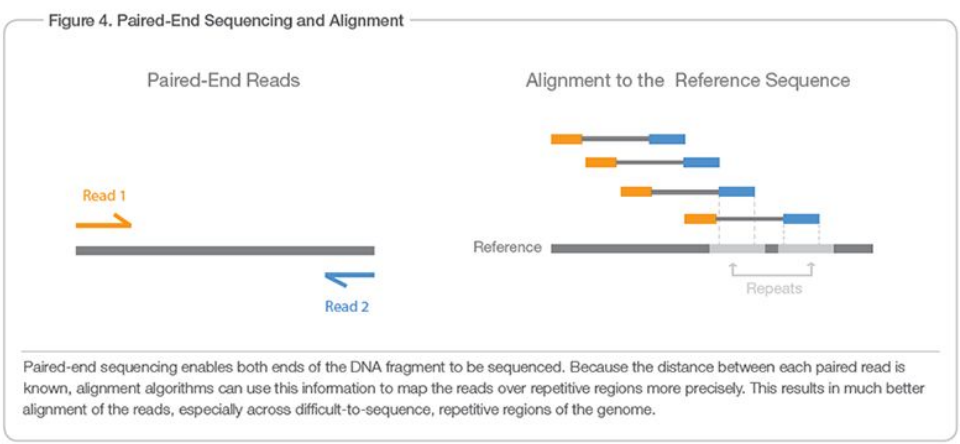
Each reaction produces a unique **library** of DNA fragments for sequencing.



Each NGS machine processes a single **flowcell** containing several independent **lanes** during a single sequencing run

Terminology : Single End (SE) vs Paired End (PE)

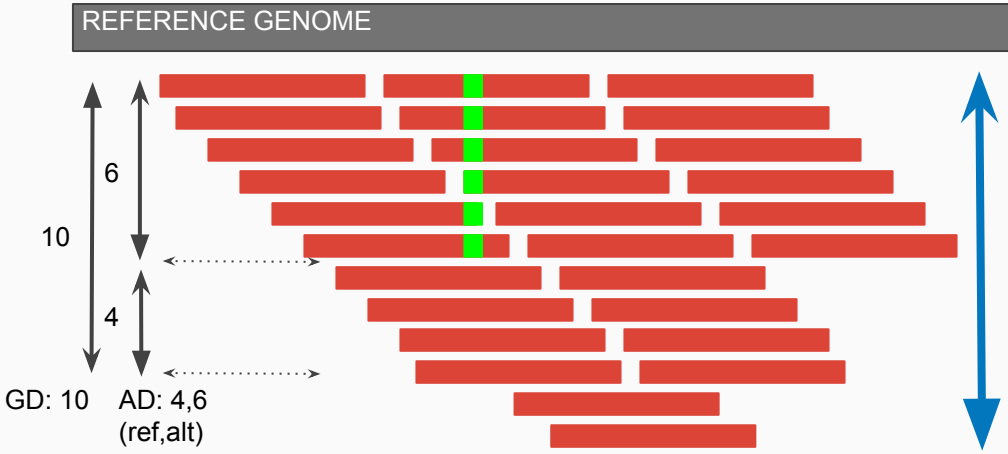
- **Single End:** one read sequenced from one end of each sample DNA insert (Rd1 SP: Read 1 Seqeuncing Primer)
- **Paired End:** two reads (one from each end) sequenced from each sample DNA insert (Rd1 and Rd2 sequencing primer)
  - Akin to F and R reads in Sanger sequencing
  - More robust Variant Calls
  - Provides better resolution of INDELS and Copy-Number Variants



http://www.illumina.com/technology/next-generation-sequencing/paired-end-sequencing_assay.html

Terminology: Depth of Coverage

- **Depth of Coverage:** Number of reads that align to, or "cover," known reference bases.
  - Often reported as an **average** over the whole length of the genome, chromosome, exome...
- **Genotype Depth**: Number of reads at observed **Variant Call** (eg GD:10)
- **Allele Depth:** Number of reads for each observed alleles (eg AD: 4,6)
- Coverage can also refer to the [% region] of genome targeted/sequenced



DEPTH OF COVERAGE

Typically reported as : Nx

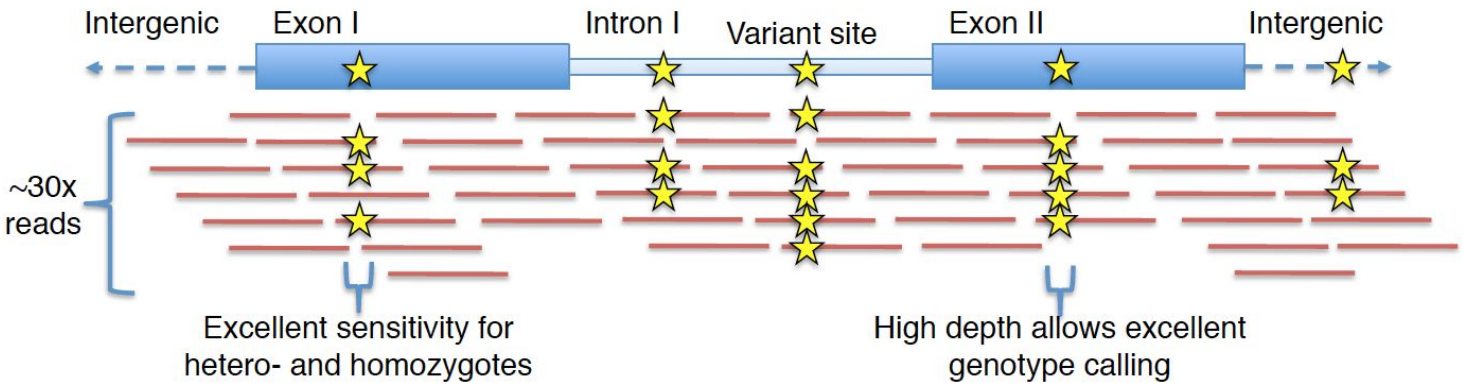
- 4x
- 30x
- 150x

3. Experimental Design

NGS Design Terminology : Some Key Terms

- High depth/coverage (Deep Coverage)
- Low depth/coverage (Shallow Coverage)
- Targeted(Gene Panel) v Exome v Whole Genome
- Single sample v Multiple Sample
- Whole Genome (WGS,WG)
- Whole Exome (WES, WEX, WEx)
- Targeted or Panel (Clinically relevant genes)

High Depth



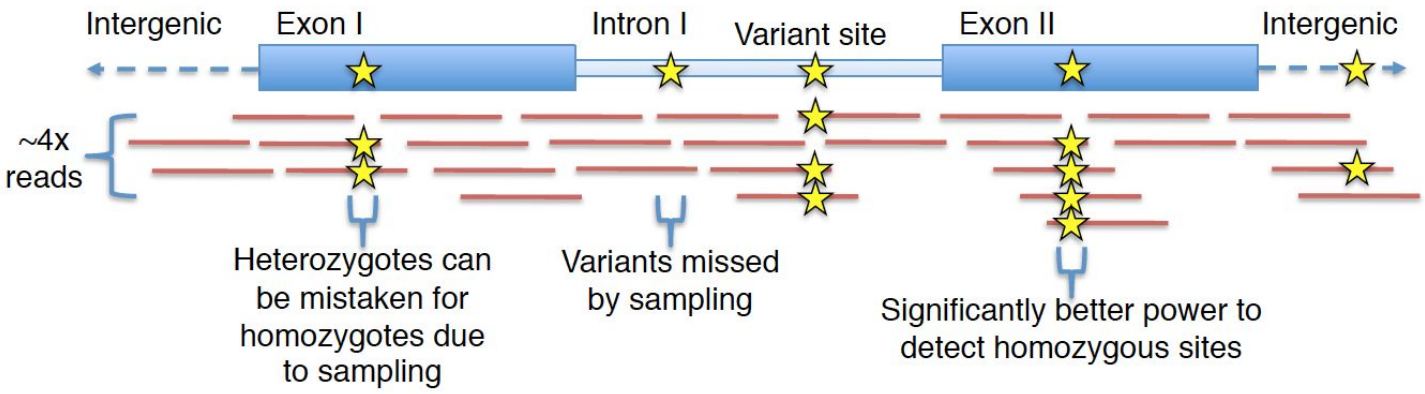
Data requirements per sample

Targeted bases	~3 Gb
Coverage	Avg. 30x
# sequenced bases	100 Gb
# lanes of HiSeq	~8 lanes

Variant detection among multiple samples

Variants found per sample	~3-5M
Percent of variation in genome	>99%
Pr{singleton discovery}	>99%
Pr{common allele discovery}	>99%

Low Depth



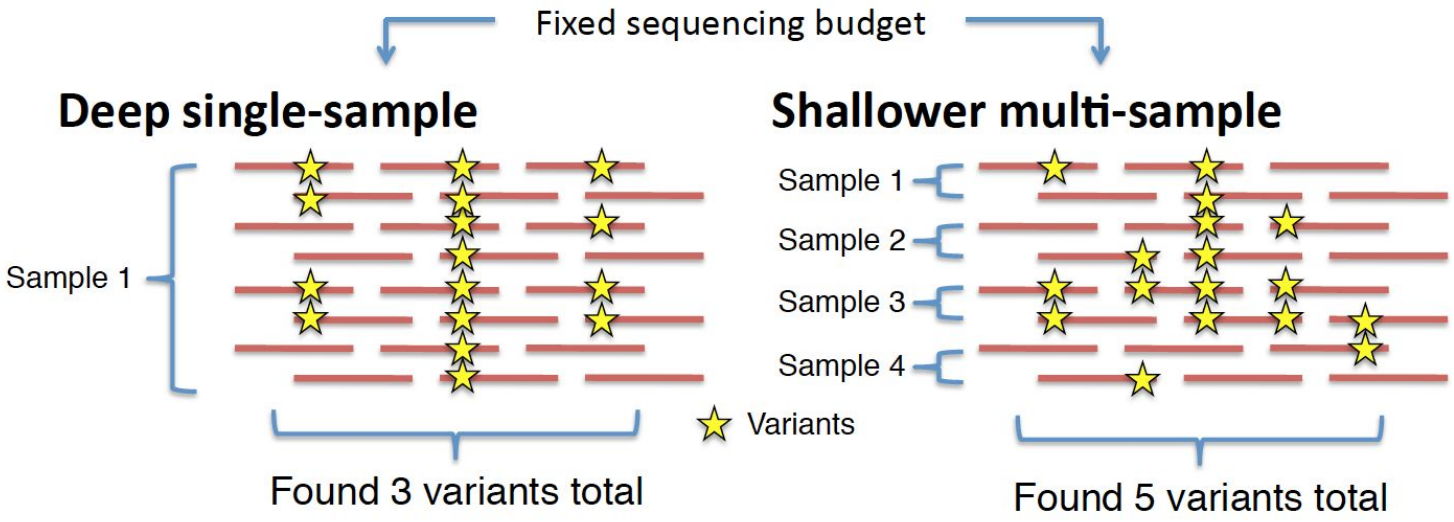
Data requirements per sample

Targeted bases	~3 Gb
Coverage	Avg. 4x
# sequenced bases	20 Gb
# lanes of HiSeq	~1.25

Variant detection among multiple samples

Variants found per sample	~3M
Percent of variation in genome	~90%
Pr{singleton discovery}	<50%
Pr{common allele discovery}	~99%

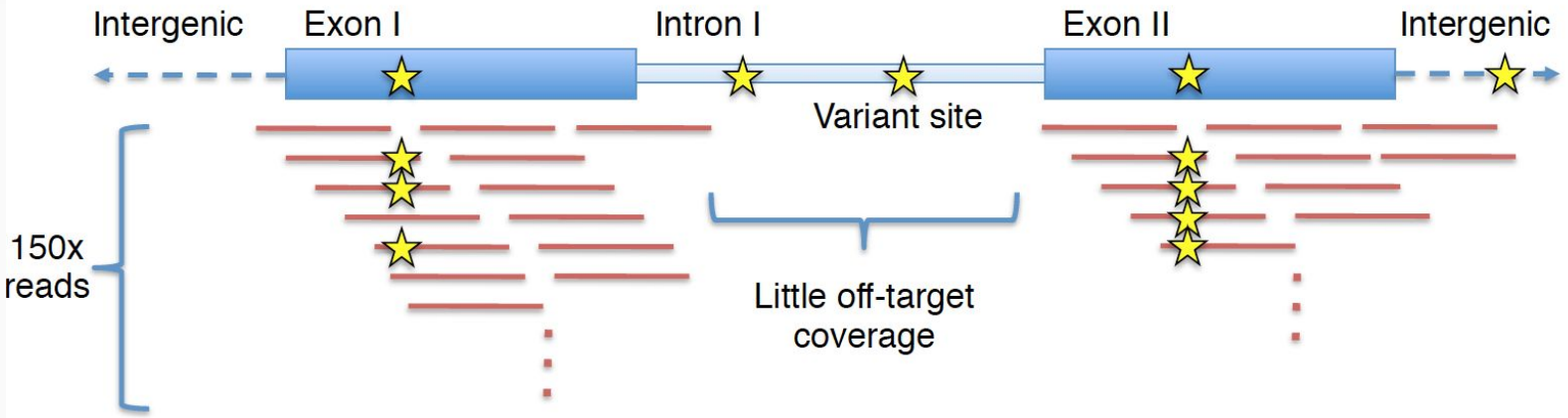
Single Sample v. Multiple Sample



- Higher sensitivity for variants in the sample
- More accurate genotyping per sample
- Cost: no information about other samples

- Sensitivity dependent on frequency of variation
- Worse genotyping
- More total variants discovered

Exome Capture



Data requirements per sample

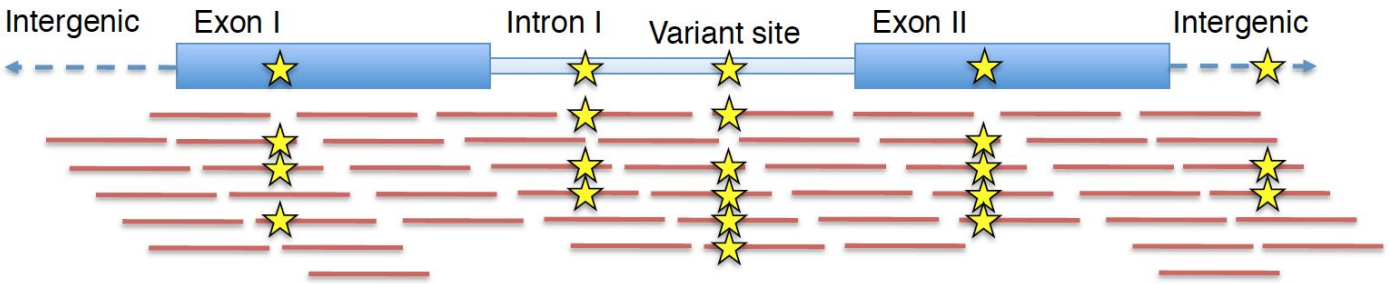
Targeted bases	~32Mb
Coverage	>80% 20x
# sequenced bases	5 Gb
# lanes of HiSeq	~0.33

Variant detection among multiple samples

Variants found per sample	~20K
Percent of variation in genome	0.5%
Pr{singleton discovery}	~95%
Pr{common allele discovery}	~95%

Whole Genome v Exome (Panel/Targeted)
WGS v WEx

Whole genome



Exome

Small targeted experiments, gene panels, RADseq *restriction site-associated DNA sequencing*

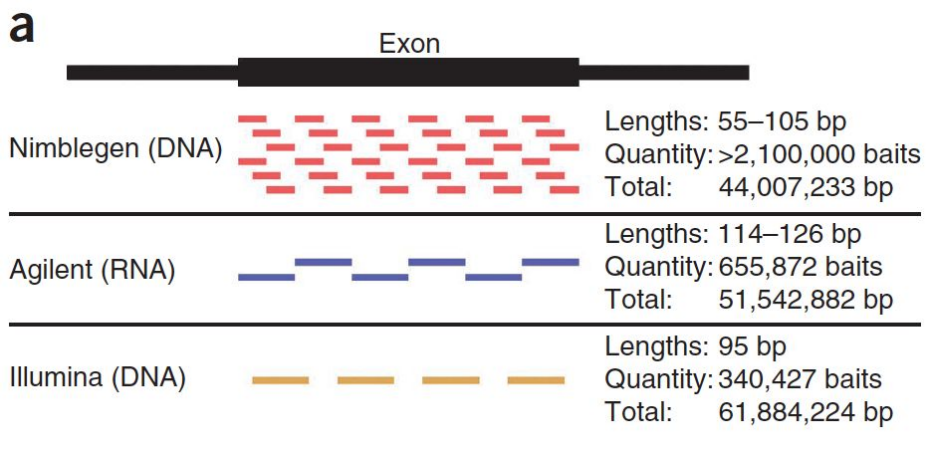
- Similar to exomes for most purposes

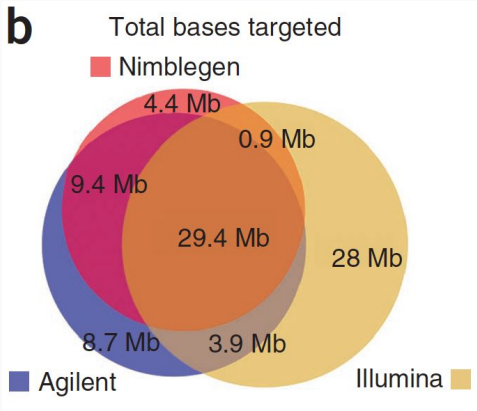
Key Differences between Whole Genome and Exome

- **Whole Genome**
  - Entire genome is prepared
  - PCR free methods possible
  - Higher price
  - Exons + Introns
  - Coverage: Entire Genome
    - Almost: some missing
    - some not “accessible”
- **Exome or Targeted Gene Panel**
  - Capture of target regions
  - PCR Amplification often required
  - Lower price
  - Exons only (+/- adjacent)
  - Coverage: targets only
    - *for regions where we already know the sequence!*
- ***Genome & Targeted...***
  - *We will always miss something*
  - *Never 100% coverage*
  - *based on our current knowledge: the current reference genome is not perfect or complete*

Targeted and Exome Capture Regions

- Involves **BAITS:** complementary sequences designed to “capture” segments of target DNA
- **Resulting covered intervals** are **specific** to capture kit **manufacturer**
- **Capture/Enrichment** designs include **different**
  - biochemical methods
  - bait lengths
  - quality and overlap of baits
  - number of DNA bases targeted



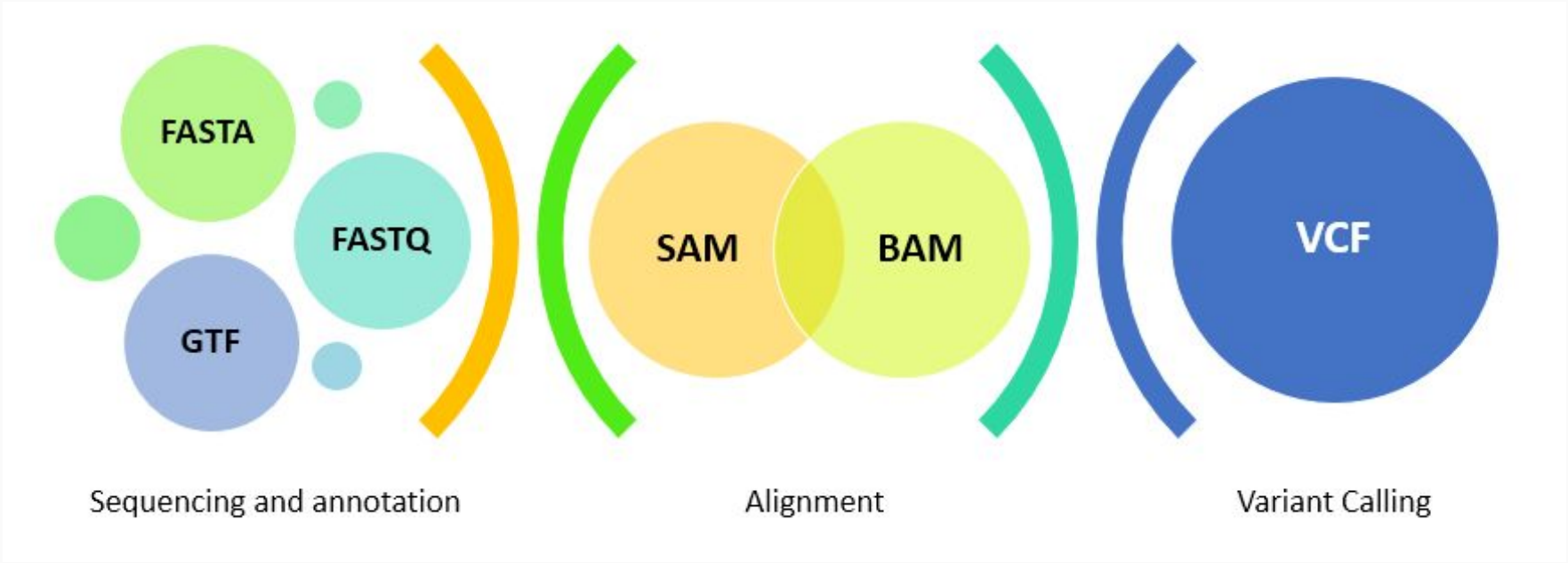


Performance comparison of exome DNA sequencing technologies,Michael J Clark
http://www.nature.com/nbt/journal/v29/n10/abs/nbt.1975.html

4. Data Formats

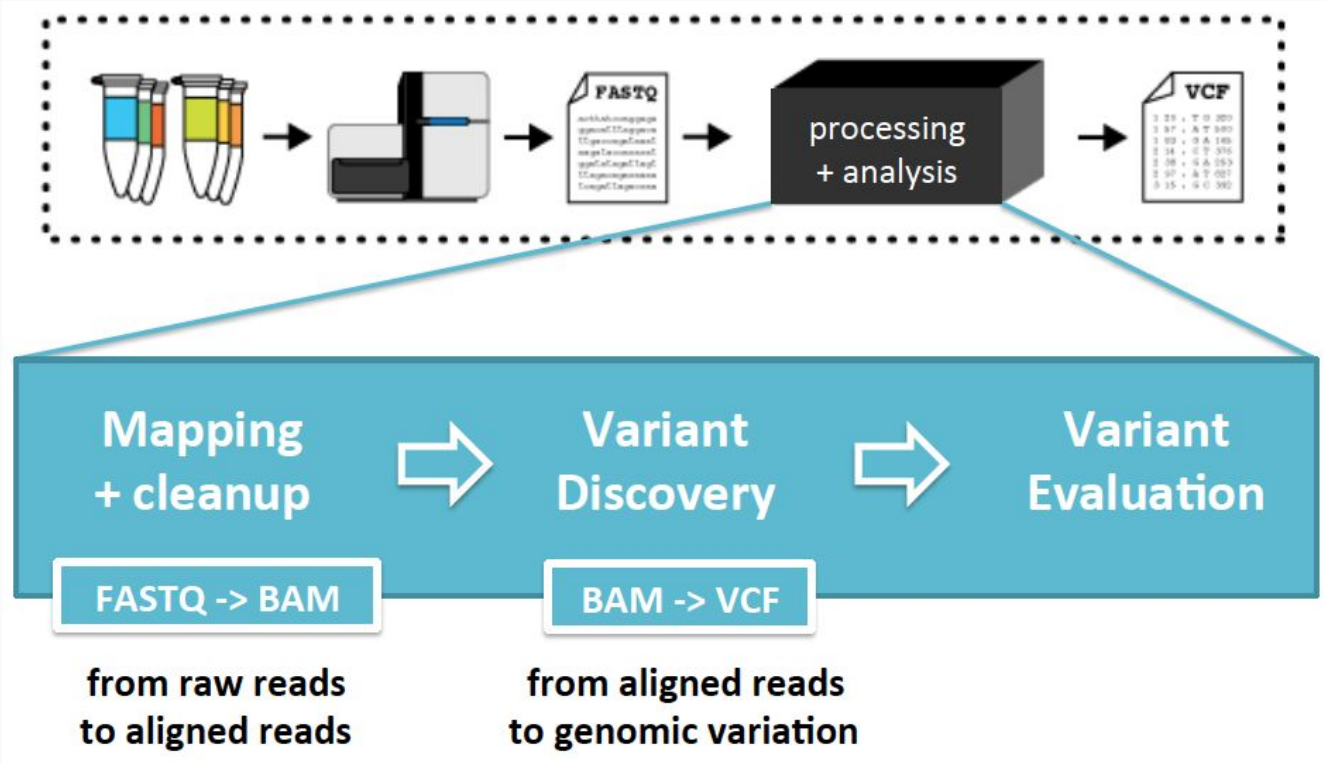
NGS File Formats

- Most common file formats for Next Generation Sequence Analysis
  - https://docs.cyfronet.pl/display/PLGDoc/Most+common+file+formats+for+Next+Generation+Analysis



NGS File Formats: from reads to variants

Major steps involve transforming data and storing results in **SPECIFIC FORMATS**



FASTA Format

- Standard for displaying (nucleotide or protein) sequences in a text file.
- An entry for a sequence takes up two lines in the file:
  - The first line begins with a ">" symbol, followed by the sequence description
  - The second line contains the sequence itself.
  - A sequence file in can contain several FASTA sequences.

```
>1 dna:chromosome chromosome:GRCh37:1:1:249250621:1
GTACGACGGAGTGTTATAAGATGGGAAATCGGATACCAGATGAAATTGTGGATCAGGTGCAAAAGTCGGC
AGATATCGTTGAAGTCATAGGTGATTATGTTCAATTAAAGAAGCAAGGCCGAAACTACTTTGGACTCTGT
CCTTTTCATGGAGAAAGCACACCTTCGTTTTCCGTATCGCCCGACAAACAGATTTTTCATTGCTTTGGCT
GCGGAGCGGGCGGCAATGTTTTCTCTTTTTTAAGGCAGATGGAAGGCTATTCTTTTGCCGAGTCGGTTTC
>2 dna:chromosome chromosome:GRCh37:2:1:243199373:1
TCTTCTGGAGAACAAAAAATGGCTGAGGCACATGAGCTCCTGAAGAAATTTTACCATCATTTGTTAATAA
ATACAAAAGAAGGTCAAGAGGCACTGGATTATCTGCTTTCTAGGGGCTTTACGAAAGAGCTGATTAATGA
ATTTCAGATTGGCTATGCTCTTGATTCTTGGGACTTTATCACGAAATTCCTTGTAAAGAGGGGATTTAGT
GAGGCGCAAATGGAAAAAGCGGGTCTCCTGATCAGACGCGAAGACGGAAGCGGATATTTCGACCGCTTCA
```

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

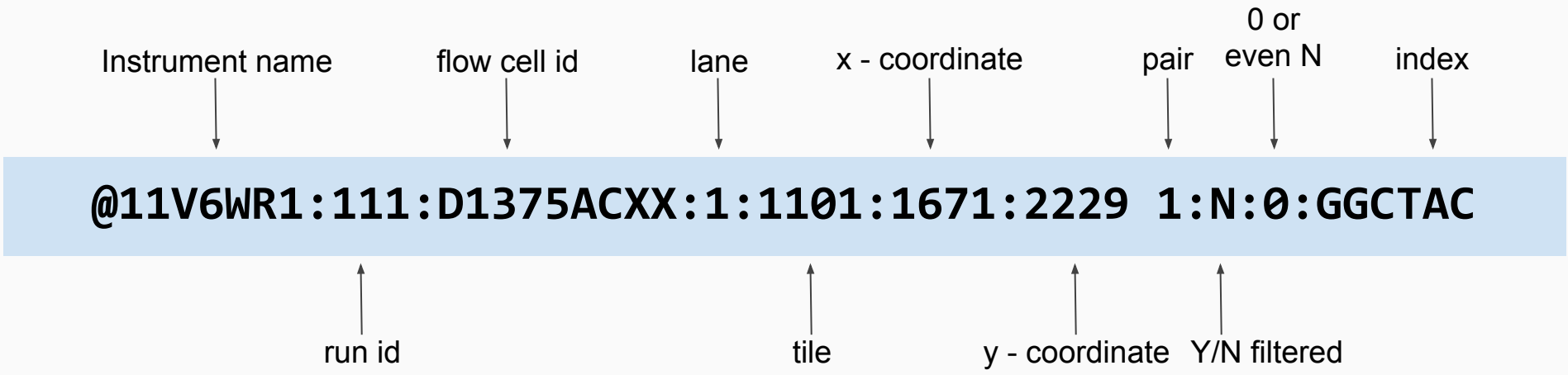
FASTQ Format

- **Raw Reads**
- **DNA sequence** and **PHRED Quality Score** in a single text file
- 4 lines per sequence:
  - **Line 1** - begins with a '@' character and is followed by a sequence identifier and an optional description (like a sequence description),
  - **Line 2** - is the raw sequence letters,
  - **Line 3** - begins with a '+' character,
  - **Line 4** - encodes the quality values for the sequence in Line 2. **Base Qualities (ASCII 33 + Phred scaled Q)**

```
@11V6WR1:111:D1375ACXX:1:1101:1671:2229 1:N:0:GGCTAC ← Identifier
TTTTATAAAAATATTTCACATGTGACTAACATTACTCACATTGTTGAGATGGGGATAAAAACAGAAGGAACATAACCTTT ← sequence
+
=?@DAB22A?FDBBGHF@E@EAAIG9CEFH9CHHIIEFI3CFCHBFDFFHDFCGB6??8*?ABG)8CC(7=2=)7=?>?E ← base qualities
```

original spec: http://maq.sourceforge.net/fastq.shtml
More details at: https://en.wikipedia.org/wiki/FASTQ_format

Illumina Sequence Identifiers



More details at: https://en.wikipedia.org/wiki/FASTQ_format

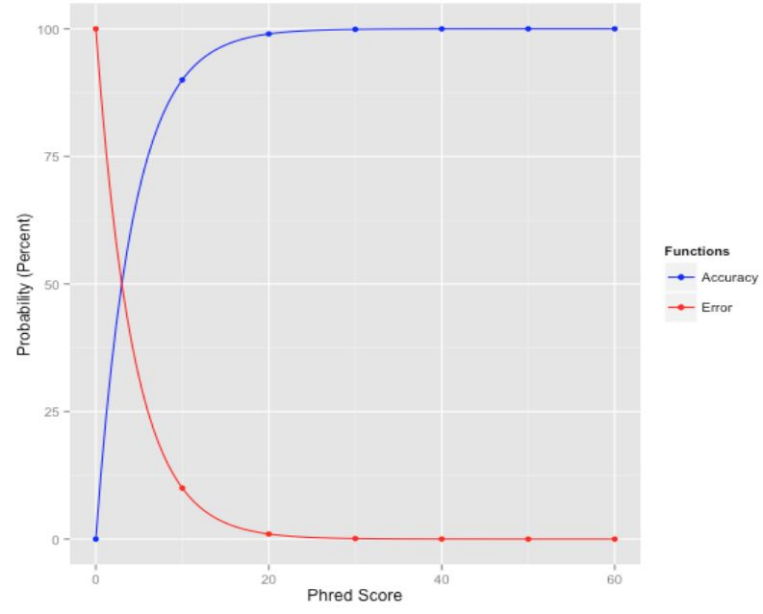
original spec: http://maq.sourceforge.net/fastq.shtml

Phred Scores

- **Phred** value or **Q score** : **Probability that the corresponding base call is incorrect**
- **Q = -10 * *log*10(*e*)**
  - e: probability of a base being wrong
- **Examples**
  - Q10 = 90% Confident = 10% error rate
  - Q30 = 99.9% Confident = 0.1% error rate

Phred quality scores are logarithmically linked to error probabilities

Phred Quality Score	Probability of incorrect base call	Base call accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%
60	1 in 1,000,000	99.9999%

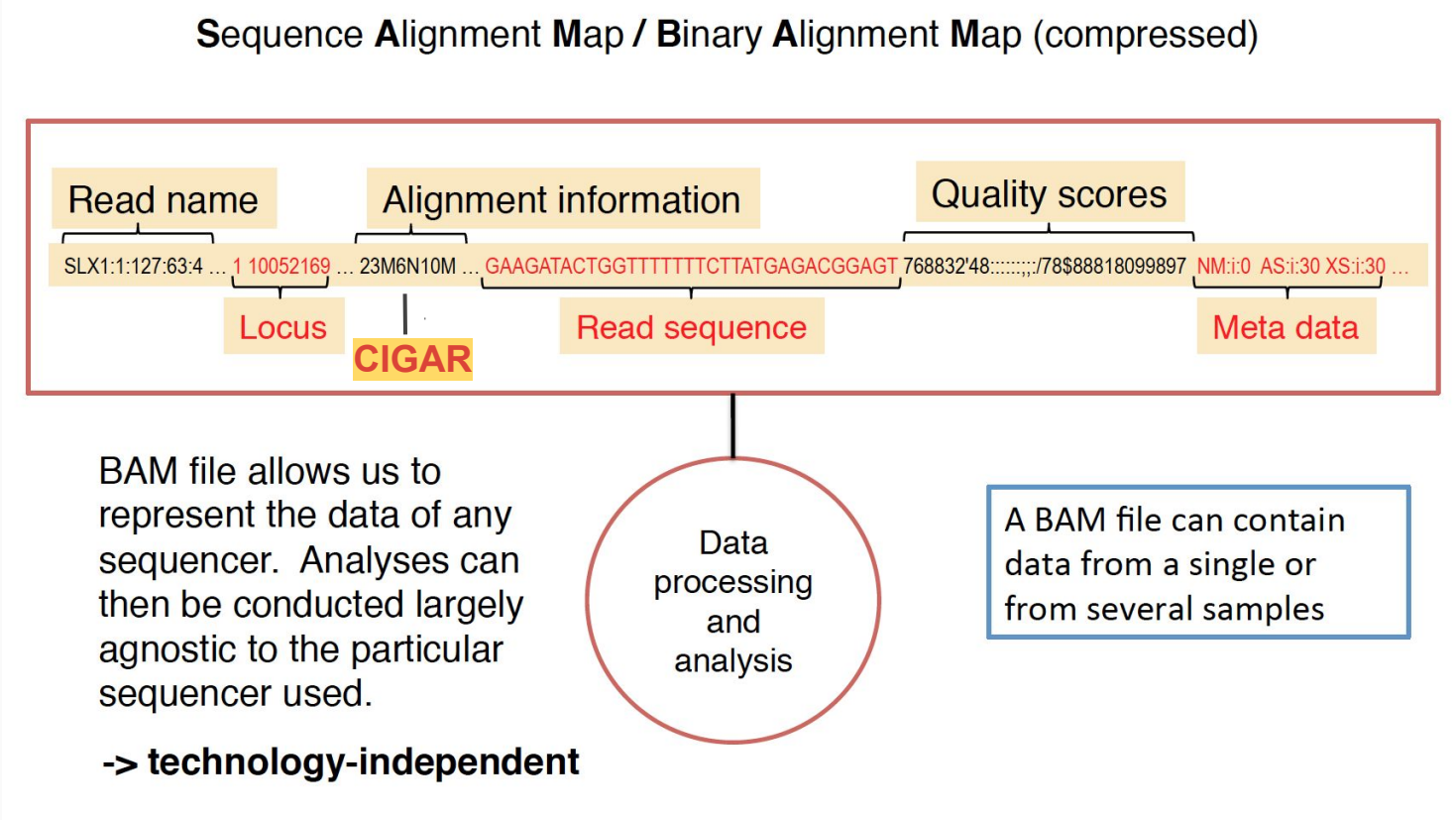


https://en.wikipedia.org/wiki/Phred_quality_score
https://en.wikipedia.org/wiki/FASTQ_format#Quality

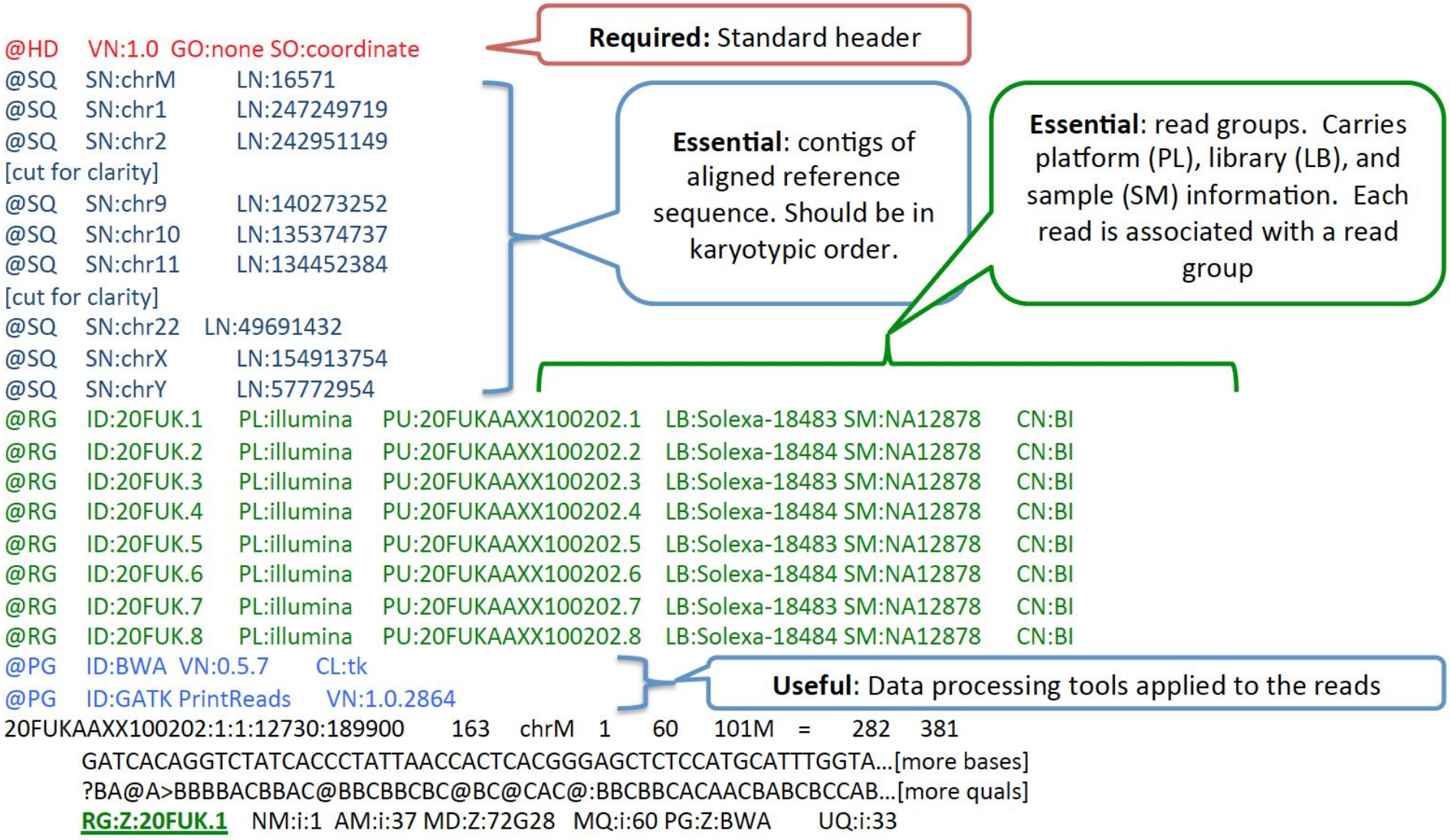
SAM & BAM Format

Offical Specification : https://samtools.github.io/hts-specs/SAMv1.pdf

- Text format for storing information about aligned reads in a series of tab delimited ASCII columns



BAM Headers: An essential part of a BAM file



The CIGAR String: describes how a read aligns to the reference

The sequence being aligned to a reference may have additional bases that are not in the reference or may be missing bases that are in the reference. The CIGAR string is a sequence of of base lengths and the associated operation. They are used to indicate things like which bases align (either a match/mismatch) with the reference, are deleted from the reference, and are insertions that are not in the reference

Operator Description

- **D** Deletion; the nucleotide is present in the read but not in the reference
- **H** Hard Clipping; the clipped nucleotides are not present in the read.
- **I** Insertion; the nucleotide is present in the reference but not in the read
- **M** Match; can be either an alignment match or mismatch. The nucleotide is present in the reference.
- **N** Skipped region; a region of nucleotides is not present in the reference
- **P** Padding; padded area in the read and not in the reference
- **S** Soft Clipping; the clipped nucleotides are present in the read
- **X** Read Mismatch; the nucleotide is present in the reference
- **=** Read Match; the nucleotide is present in the reference

The CIGAR String: An Example

http://genome.sph.umich.edu/wiki/SAM#What_is_a_CIGAR.3F

```
RefPos:     1  2  3  4  5  6  7  8  9 10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T  G  A  A  C  T  G  A  C  T  A  A  C
Read: ACTAGAATGGCT
```

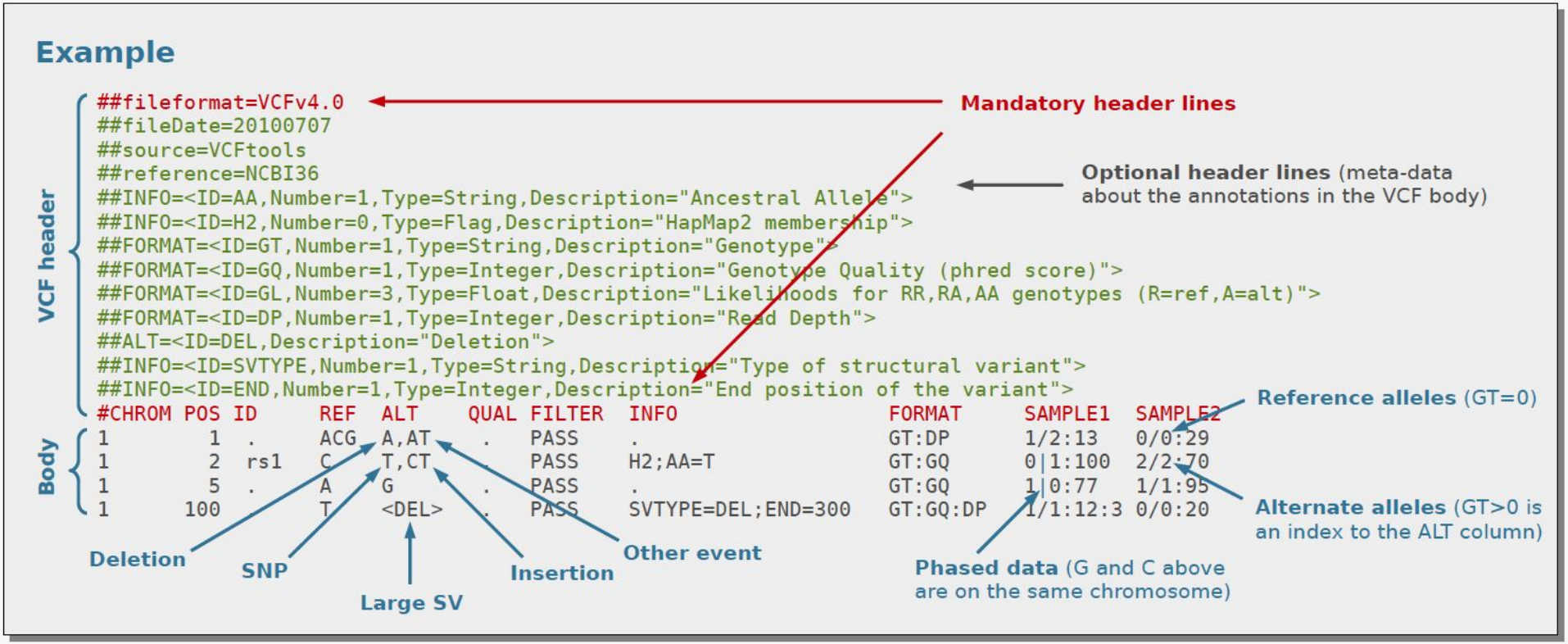
```
RefPos:     1  2  3  4  5  6  7     8  9 10 11 12 13 14 15 16 17 18 19
Reference:  C  C  A  T  A  C  T     G  A  A  C  T  G  A  C  T  A  A  C
Alignment:              A  C  T  A  G  A  A     T  G  G  C  T
CIGAR:        3M       1I     3M     1D        5M
```

```
POS: 5
CIGAR: 3M1I3M1D5M
```

The **POS** indicates that the read **aligns starting at position 5** on the reference. The CIGAR says that the **first 3 bases in the read sequence align with the reference**. The next base in the read does not exist in the reference. Then 3 bases align with the reference. The next reference base does not exist in the read sequence, then 5 more bases align with the reference. **Note that at position 14, the base in the read is different than the reference, but it still counts as an M since it aligns to that position**.

VCF Format: Stores variant information

https://vcftools.github.io/specs.html



http://vcftools.sourceforge.net/VCF-poster.pdf
https://samtools.github.io/hts-specs/VCFv4.2.pdf

gVCF Format: Stores Genome variant information

Human clinical applications require sequencing information for both variant and non-variant positions, yet there is currently no common exchange format for such data. Genome VCF (gVCF) was developed to address this issue

The following is a segment of a VCF file following the gVCF conventions for representation of non-variant sites, and more specifically using the gvcftools block compression and filtration levels.

gVCF example segment

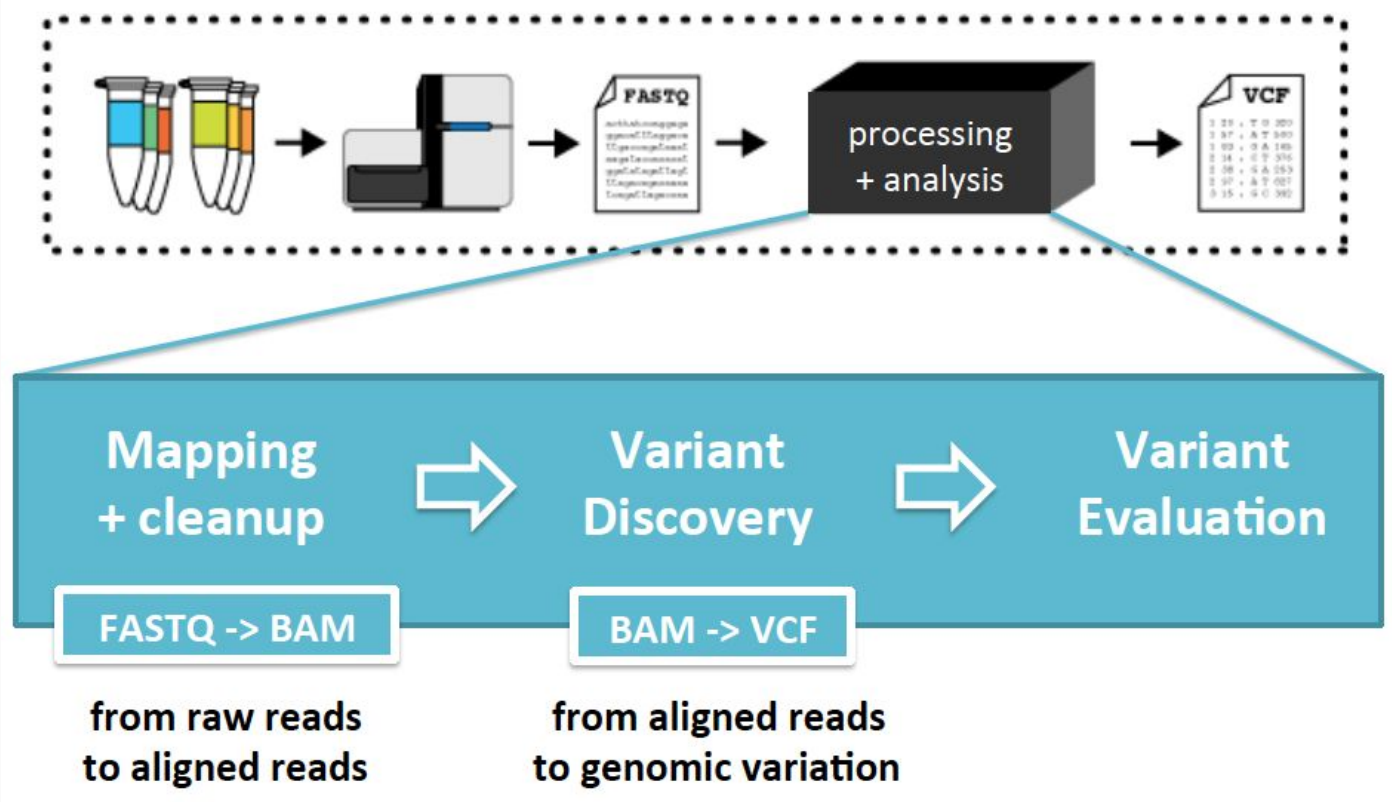
```
chr20  287125  .  T  .  .  PASS  END=287136;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:40:78:40
chr20  287137  .  G  .  .  LowGQX  .  GT:DP:GQX:MQ  0/0:42:11:42
chr20  287138  .  C  .  .  PASS  END=287178;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:36:96:42
chr20  287179  .  C  T  310.01  PASS  BaseQRankSum=-0.721;DP=37;Dels=0.00;FS=14.994;HaplotypeScore=0.0000;MLEAC=1;MLEAF=0.500;MQ=52.29;MQ0=
0;MQRankSum=-1.091;QD=8.38;ReadPosRankSum=-1.963;SB=-1.901e+01  GT:AD:DP:GQ:PL:MQ:GQX  0/1:24,13:37:99:340,0,810:52:99
chr20  287180  .  G  .  .  PASS  END=287245;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:32:78:49
chr20  287246  .  G  A  567.01  PASS  BaseQRankSum=-0.718;DP=33;Dels=0.00;FS=5.093;HaplotypeScore=3.2995;MLEAC=1;MLEAF=0.500;MQ=49.01;MQ0=0
;MQRankSum=1.050;QD=17.18;ReadPosRankSum=0.129;SB=-2.920e+02  GT:AD:DP:GQ:PL:MQ:GQX  0/1:13,20:33:99:597,0,343:49:99
chr20  287247  .  C  .  .  PASS  END=287259;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:27:75:46
chr20  287260  .  C  .  .  PASS  END=287270;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:26:69:38
chr20  287271  .  T  G  778  PASS  DP=26;Dels=0.00;FS=0.000;HaplotypeScore=0.0000;MLEAC=2;MLEAF=1.00;MQ=38.49;MQ0=0;QD=29.92;SB=-2.930e+
02 GT:AD:DP:GQ:PL:MQ:GQX  1/1:0,26:26:72:811,72,0:38:72
chr20  287272  .  A  .  .  PASS  END=287285;BLOCKAVG_min30p3a  GT:DP:GQX:MQ  0/0:26:69:34
```

In the example segment non-variant regions are shown in blue, and variants are shown in red. Note that the variant lines can be extracted from a gVCF file to produce a conventional variant VCF file.

https://sites.google.com/site/gvcftools/home/about-gvcf
https://sites.google.com/site/gvcftools/home/about-gvcf/gvcf-conventions

5. NGS Data Processing

The Basic Pipeline



Bioinformatic Challenges of NGS

- NGS pushes bioinformatics needs way up!
- Large amount of CPU power
- Informatics groups must manage and maintain large compute clusters and storage
- Challenges in parallelizing software/algorithms for NGS
- VERY large text files (> 10 million lines)
- Standard tools face problems with memory usage, execution times and impossible to browse for problems

Bioinformatic Challenges of NGS

- Data management issues
- Raw data is large (5GB - 30GB per patient)
- How long should they be kept?
- Processed data are manageable for most small studies
- More of an issue for NGS facilities and Bioinformatics services running multiple projects
- WGS 900 samples > 200TB processed data!
- 100K Genomes >>> PETABYTES or More!

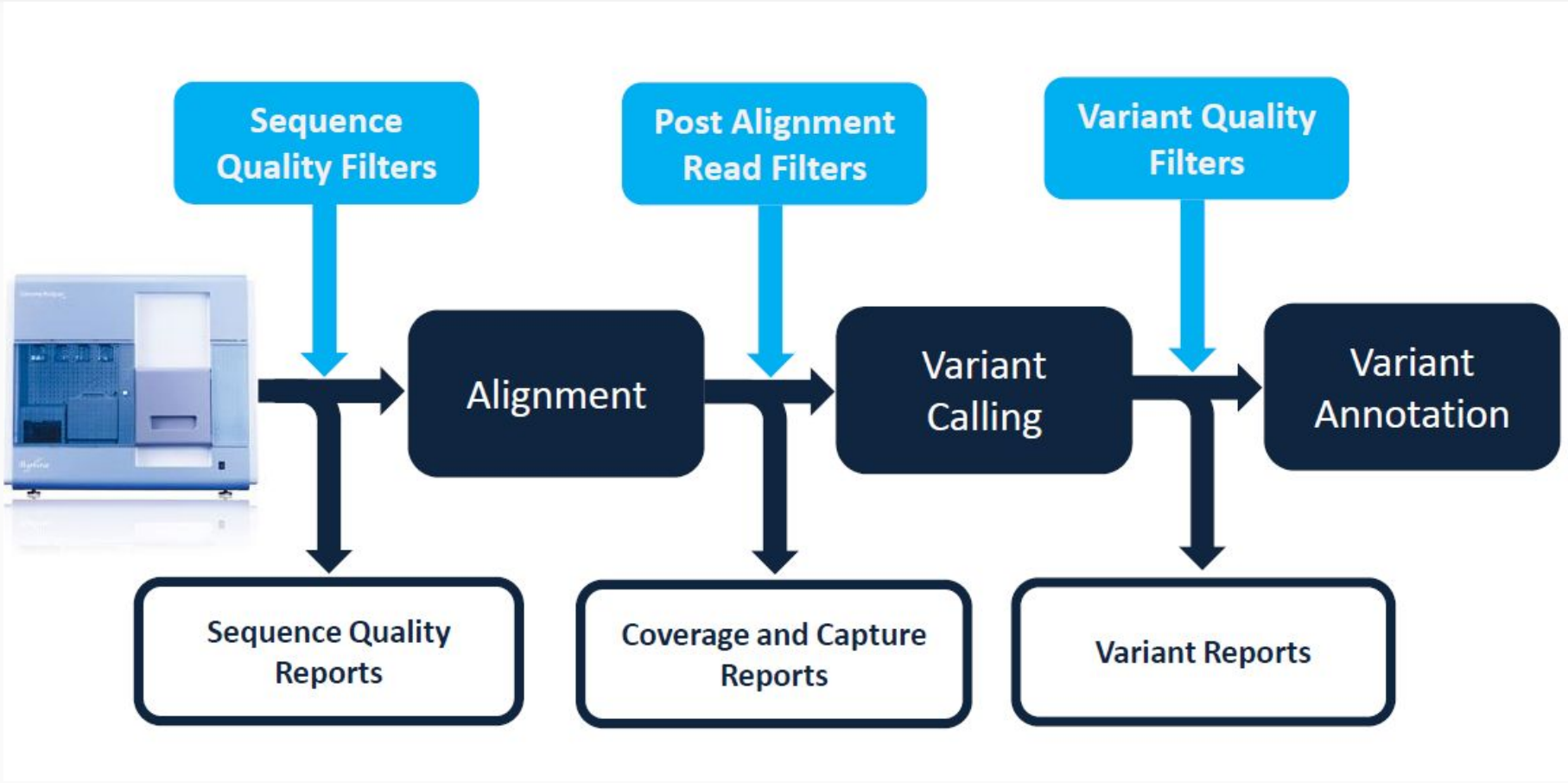
Bioinformatic Challenges of NGS

- In NGS we have to process LARGE amounts of data - non trivial compute time
- Big NGS projects require supercomputing infrastructures and specialist software
- Can't run applications on a standard desktop
- Not everyone can study everything and small facilities,research labs must carefully choose/run projects inline with their compute capabilities & expertise

Computational infrastructure for NGS

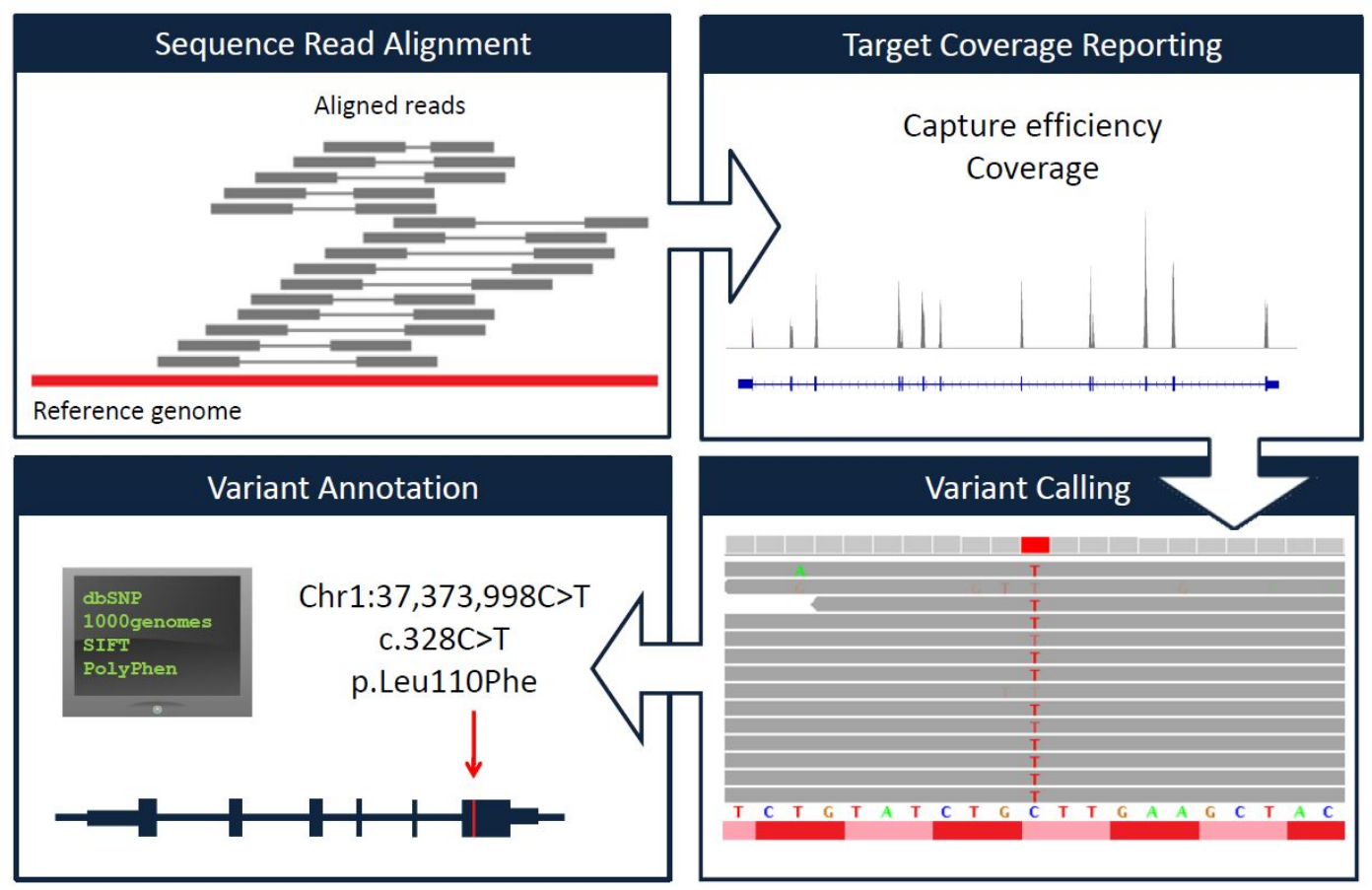
- General Requirements
  - Large Compute cluster
  - Multiple nodes (servers) with multiple cores (500 cores, 50-128 GB RAM)
  - High performance storage (TB,PB levels)
  - Fast network
  - Skilled Bioinformaticians (sysadmin, developers)
- Bare minimum for a small project
  - EG. Families Exome Seq, single sample WGS
  - 1-2 Workstations
  - 8-12 cores, 48-64GB RAM, at least 4TB
  - Skilled Bioinformaticians (sysadmin, developers)

NGS Data Analysis: Basic Bioinformatic Workflow I



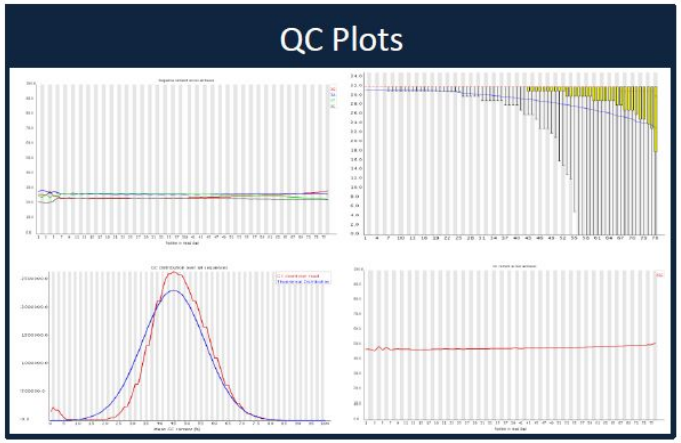
Thanks to Dr Mike Simpson (michael.simpson@kcl.ac.uk)

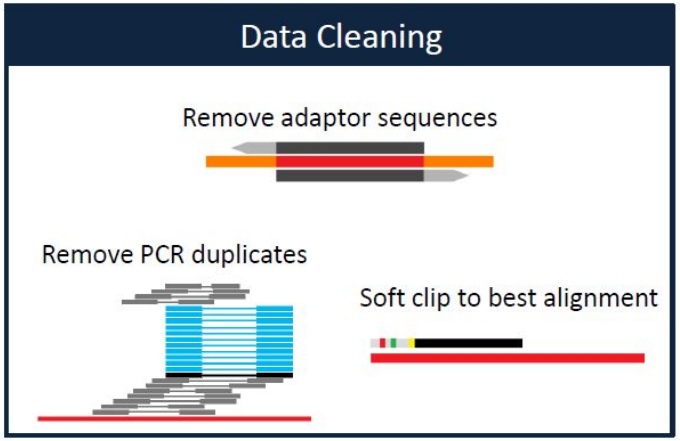
NGS Data Analysis: Basic Bioinformatic Workflow II

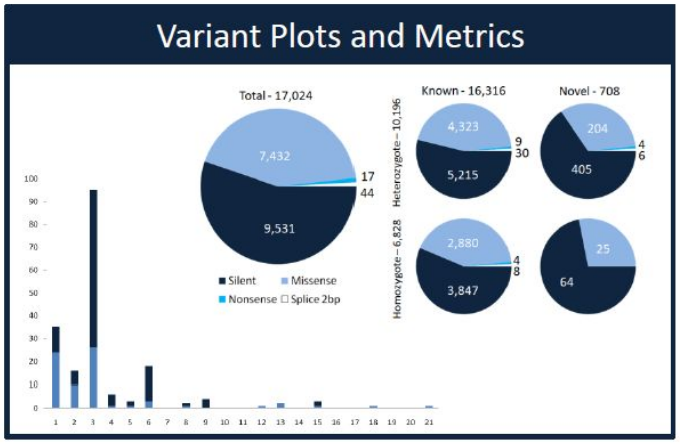


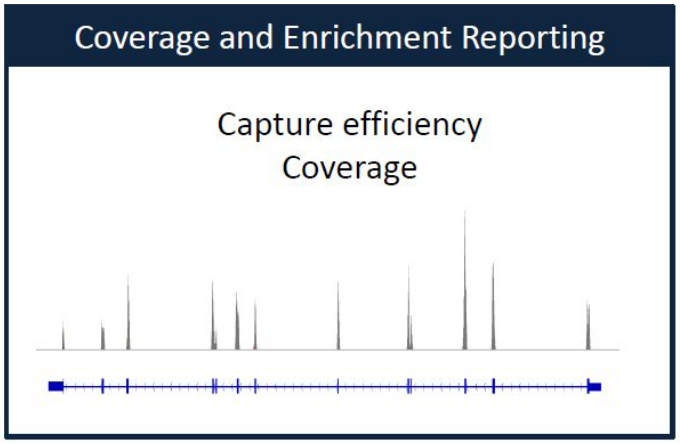
Thanks to Dr Mike Simpson (michael.simpson@kcl.ac.uk)

NGS Data Analysis: Basic Bioinformatic Workflow III



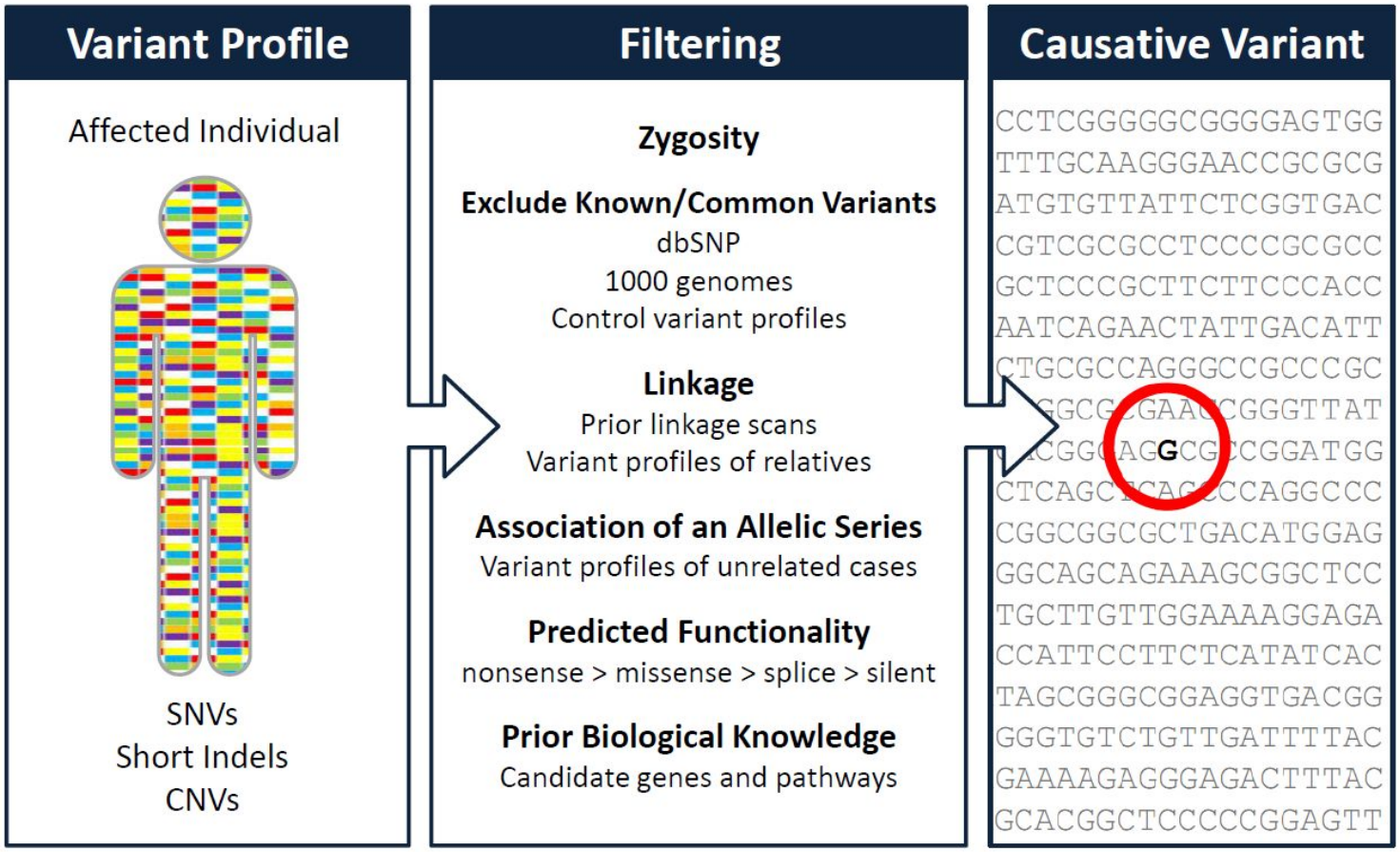






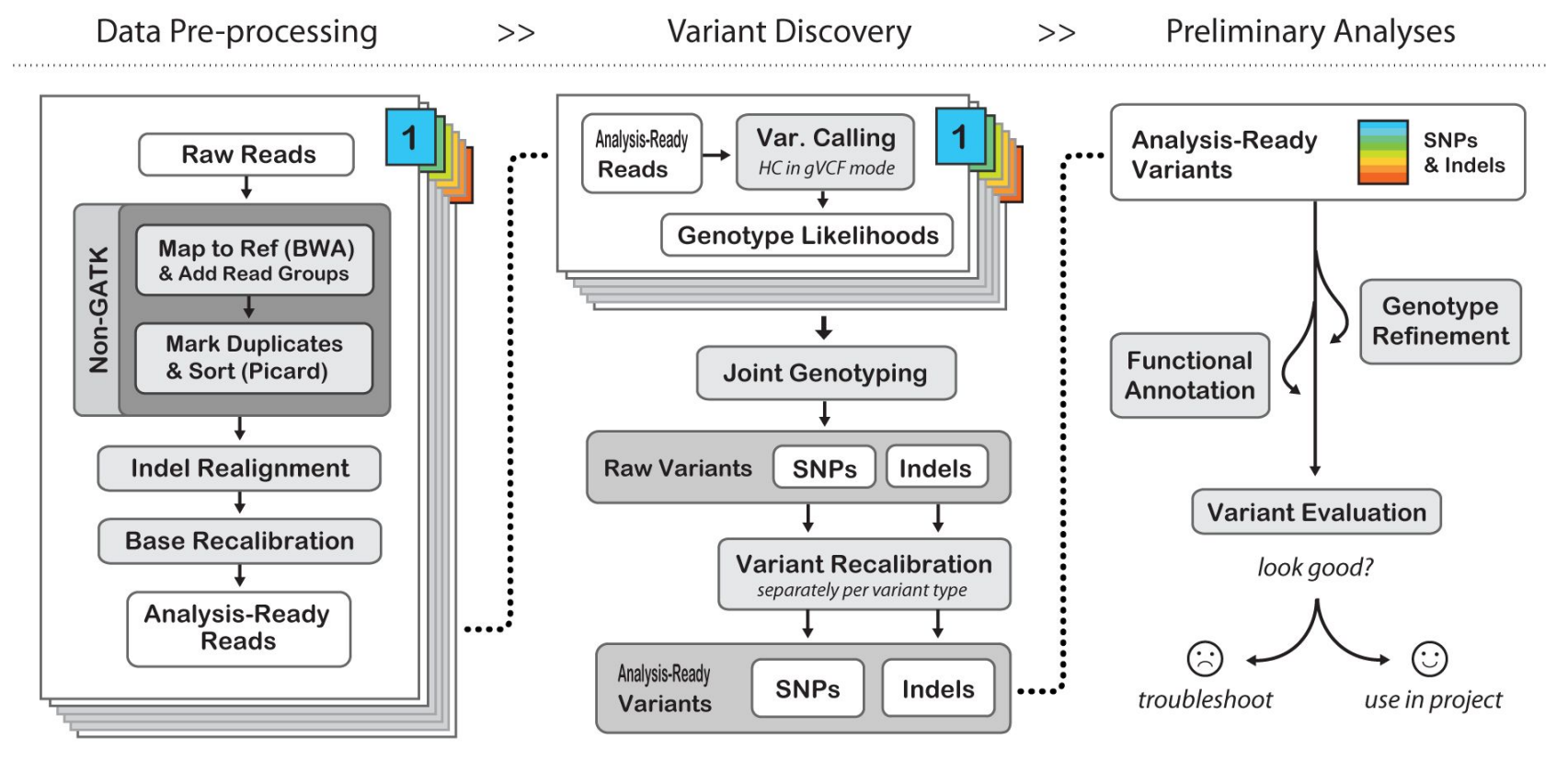
Thanks to Dr Mike Simpson (michael.simpson@kcl.ac.uk)

NGS Data Analysis: Basic Bioinformatic Workflow IV



Thanks to Dr Mike Simpson (michael.simpson@kcl.ac.uk)

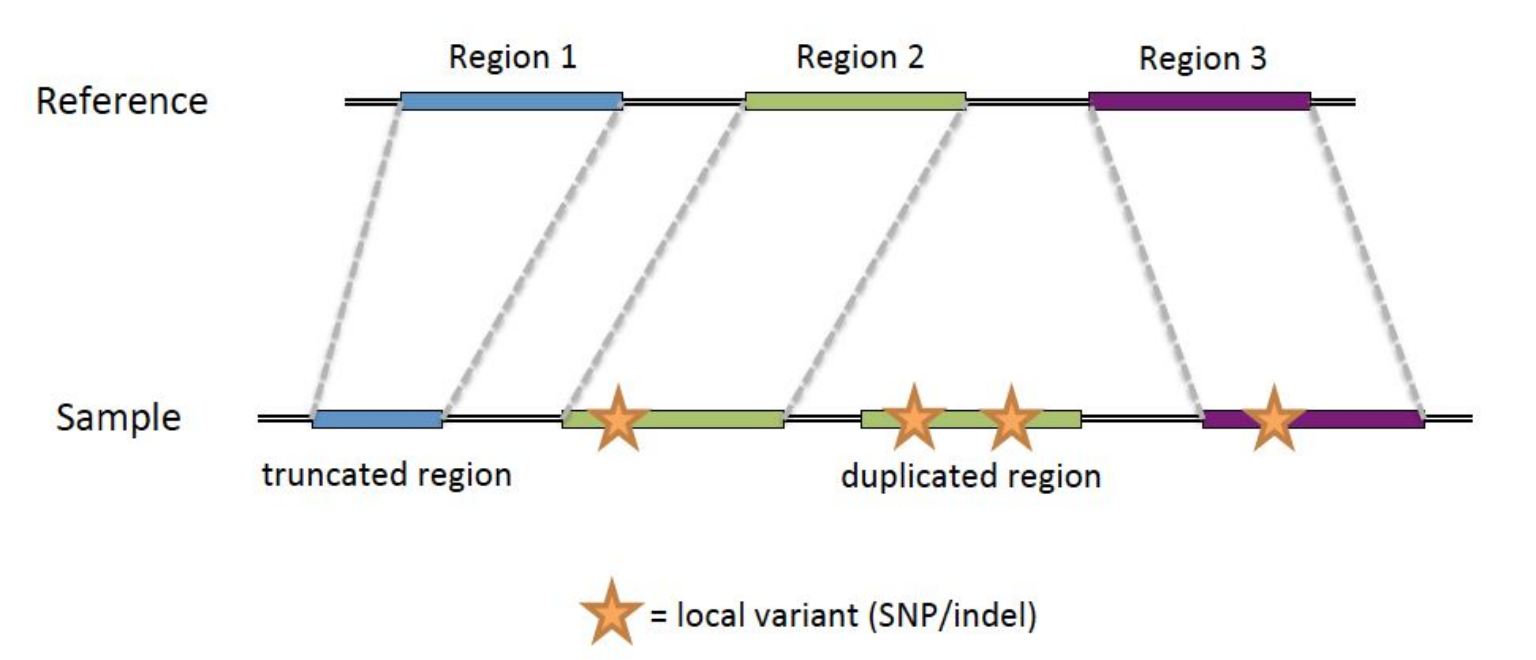
GATK “Genome Analysis Toolkit” : Community Driven Best Practices



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

Alignment: the most important step in the pipeline

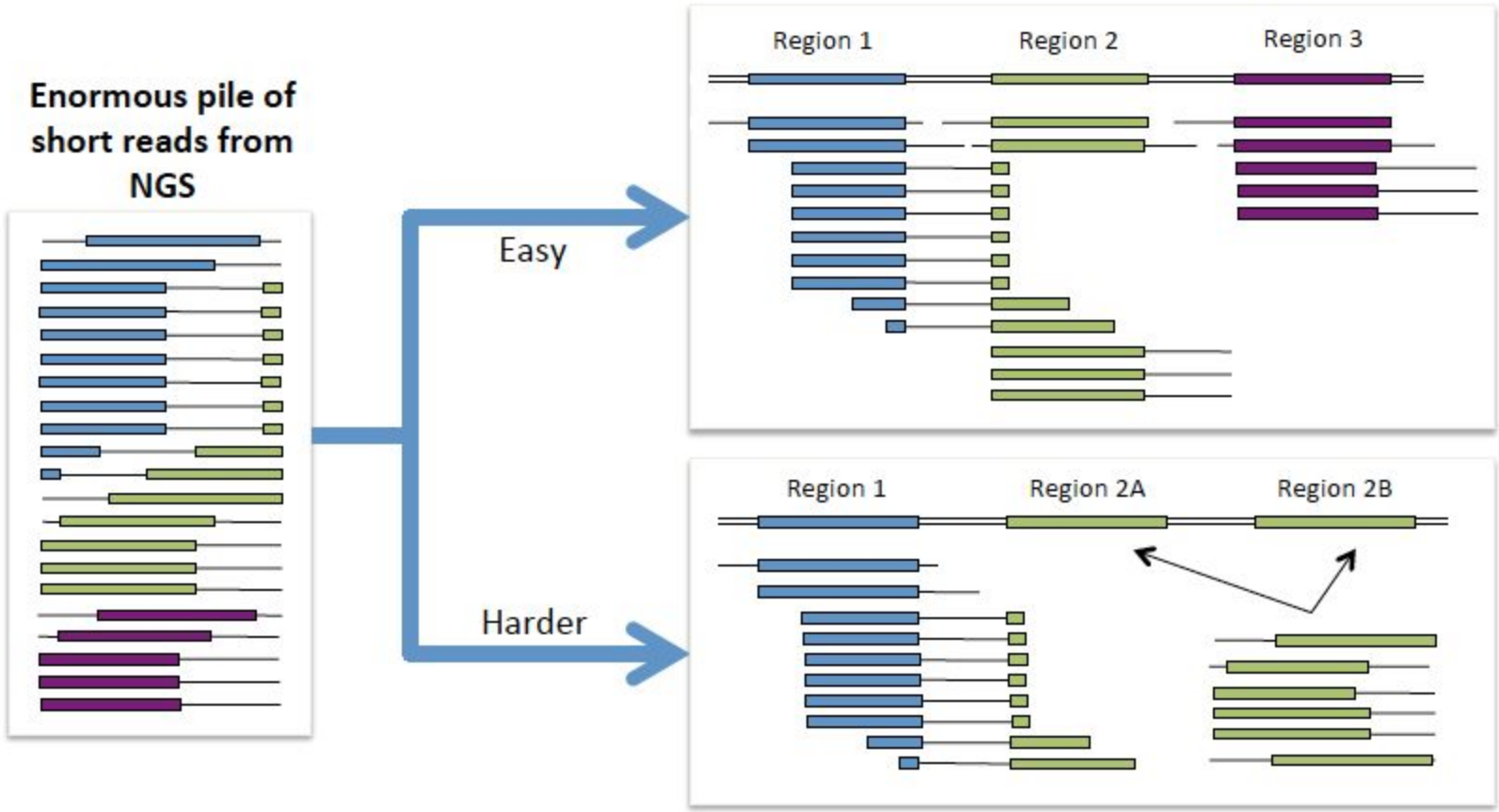
- Ideally we'd align the sample genome to the reference genome….



...But we don't have the whole sample...just a bunch of short reads...

We have a pile of reads that need to be mapped individually

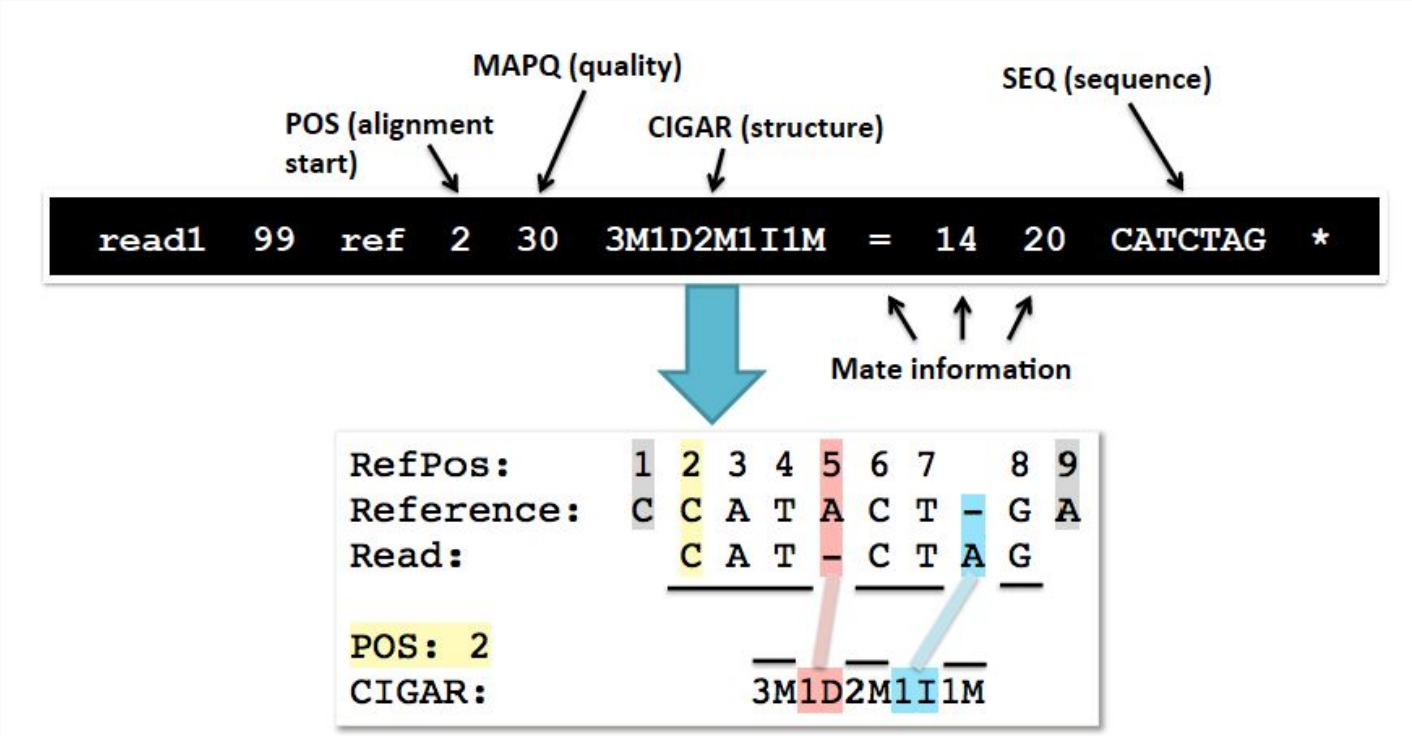
Mapping is complicated by mismatches (true mutations or sequencing errors), indels, duplicated regions etc.



Many alignment tools available...

- All concerned with finding where short reads map to the reference genome
- Details outside the scope of this course...
- **Popular aligners**
  - bwa http://bio-bwa.sourceforge.net/
  - bowtie http://bowtie-bio.sourceforge.net/index.shtml
  - novoalign http://www.novocraft.com/products/novoalign/
  - stampy http://www.well.ox.ac.uk/project-stampy
  - **isaac** (illumina) https://github.com/Illumina/isaac2
  - many, many more.....

SAM revisited: Mapping produces SAM summarizing position, quality and structure for given sequence alignment..

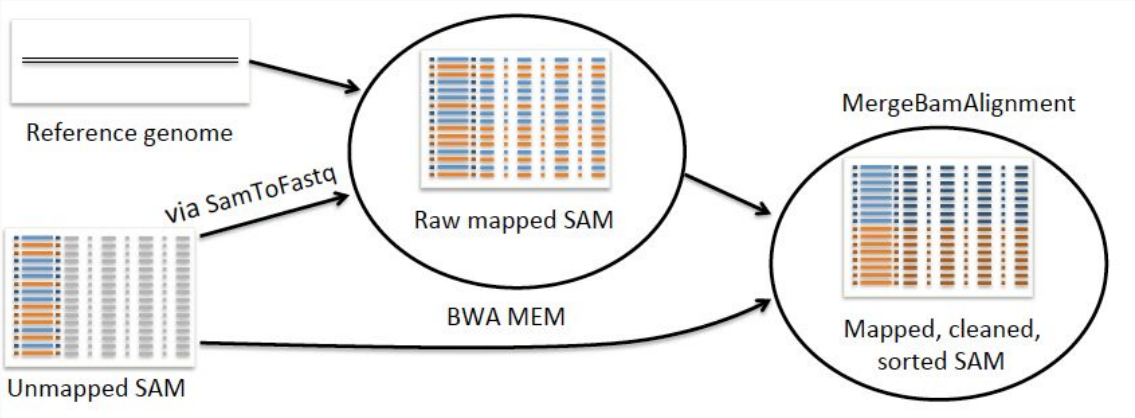


See also:

- SAM format spec: http://samtools.github.io/hts-specs/SAMv1.pdf
- Explain SAM flags: http://broadinstitute.github.io/picard/explain-flags.html

Post-processing of aligned files

- **Marking of PCR duplicates**
  - PCR amplification errors can cause some sequences to be over-represented
  - Chances of any two sequences aligning to the same position are unlikely
  - Caveat: obviously this depends on amount of the genome you are capturing
  - Such reads are marked but not usually removed from the data
  - Most downstream methods will ignore such reads
  - Typically, picard is used
- **Sorting**
  - Reads can be sorted according to genomic position
  - samtools
- **Indexing**
  - Allow efficient access
  - samtools
- **Mapping artefacts**
  - picard : CleanSam
  - picard: FixMateInformation



Genomics England “Pipeline”

as of Feb 2016...

- NGS Provider: Illumina Ltd
- NGS Technology: HiSeq x10
  - Paired End
  - 150bp
- Single Sample Whole Genome
- Depth: min average depth 30x (Tumour 75x)
- Pipeline: **ISSAC2** (Aligner), **Starling** (SNPs), **Canvas** (CNV), **Manta** (SV)
- Alignment: **BAM**
- Variant Calls: **VCF** or gVCF
  - SNP, CNV, Small
- Fast Track Whole Genome Sequencing Services: https://goo.gl/uMgvgR

https://github.com/Illumina/isaac2
https://github.com/Illumina/manta
https://github.com/Illumina/canvas

Further Reading

- See all slide links & Handbook