3000788 Intro to Comp Molec Biol

Week 2: DNA sequencing and data analysis

Fall 2024

Sira Sriswasdi, PhD

- Research Affairs
- Center of Excellence in Computational Molecular Biology (CMB)
- Center for Artificial Intelligence in Medicine (CU-AIM)

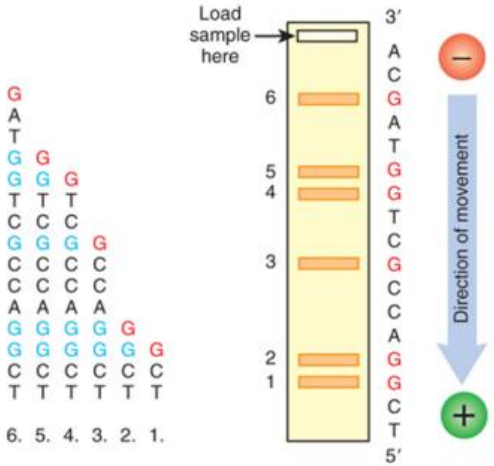
Part I: DNA sequencing platform & applications

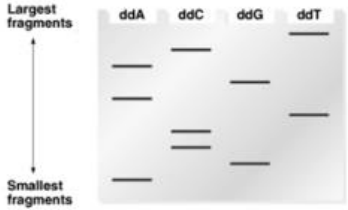
- Unique capability of long-read techniques
- Knowing pros and cons → Pick the best platform for your research
- Integration of experimental design with sequencing

Sanger and NGS

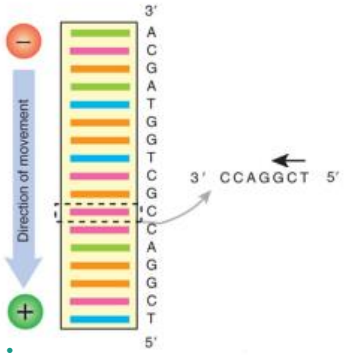
Sanger sequencing

Know only the last nucleotide





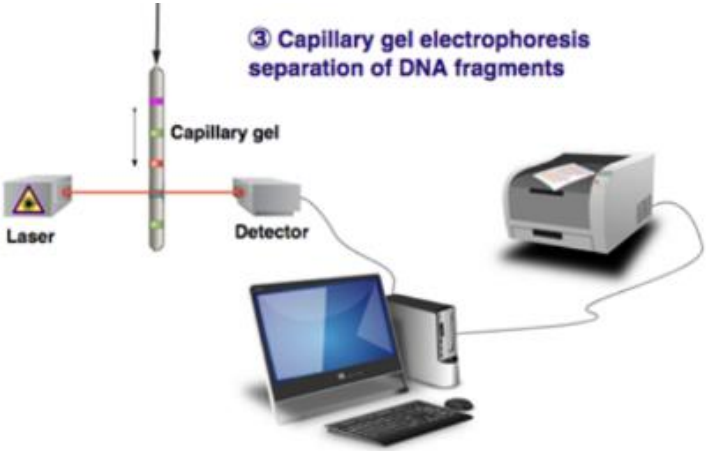
What is the sequence?

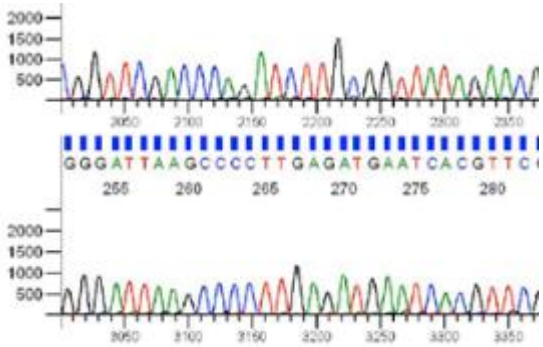


Generate all possible products, each with different length

Fluorescence-labeled ddNTP

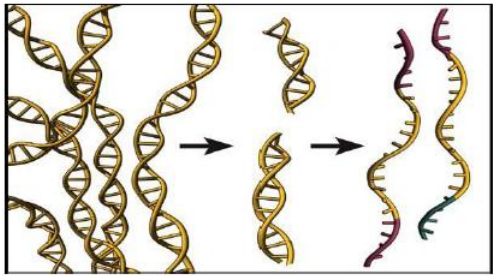
Product length = bp position





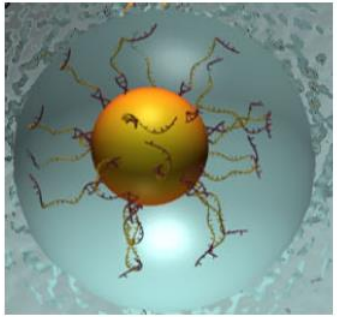
Images from https://en.wikipedia.org/wiki/Sanger_sequencing

High throughput from parallel reactions

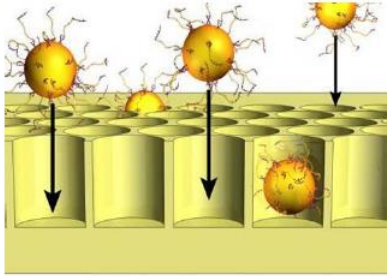


1) Adapter-ligated ssDNA library

Roche & Ion Torrent wells

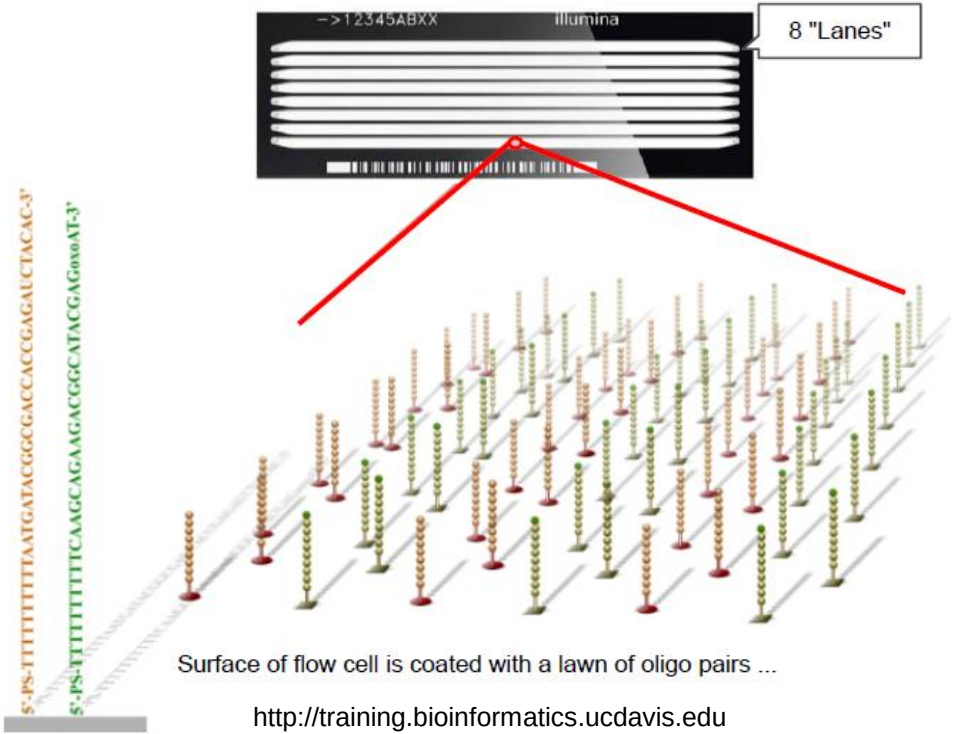


2) Clonal amplification on 28 micron beads ... emulsion PCR



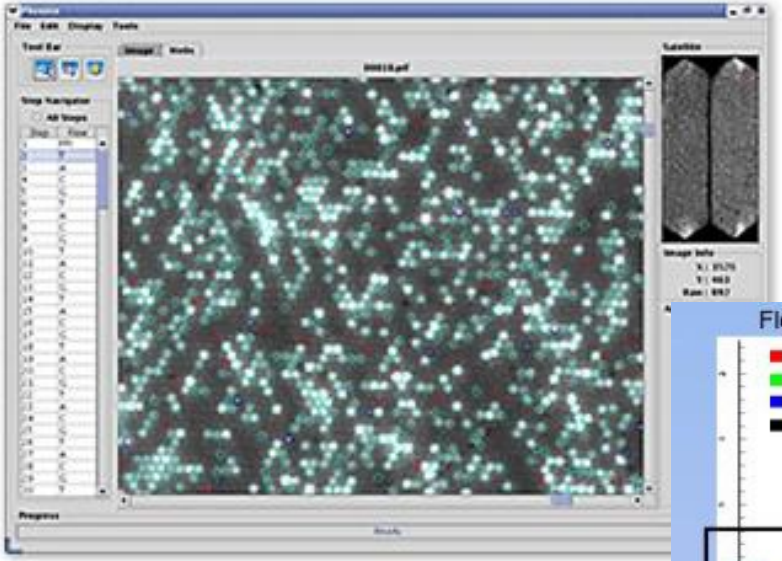
3) Beads deposited on PicoTiterPlate wells

Illumina's flow cell



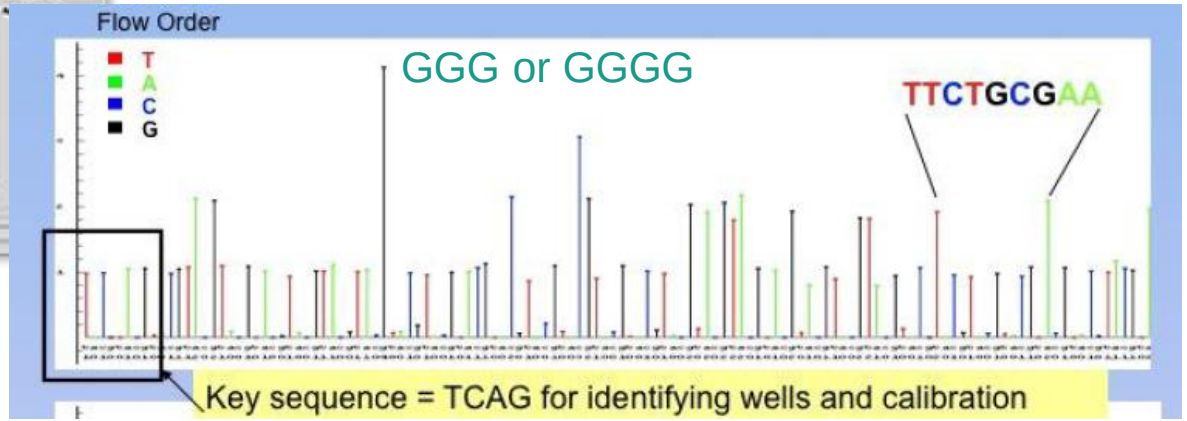
http://training.bioinformatics.ucdavis.edu

Limitation of pyrosequencing

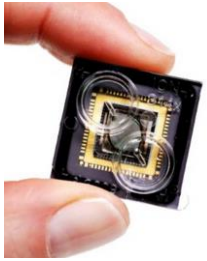


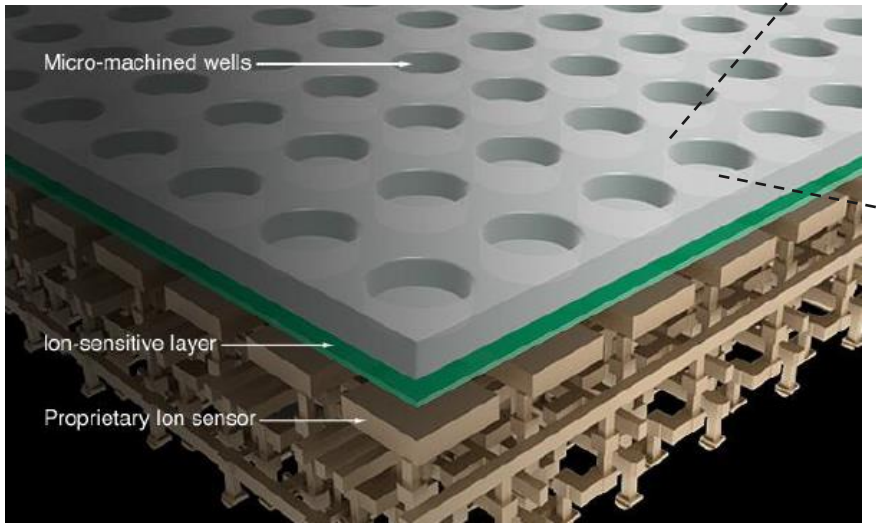
DATA ANALYSIS: OUTPUT PACKAGE

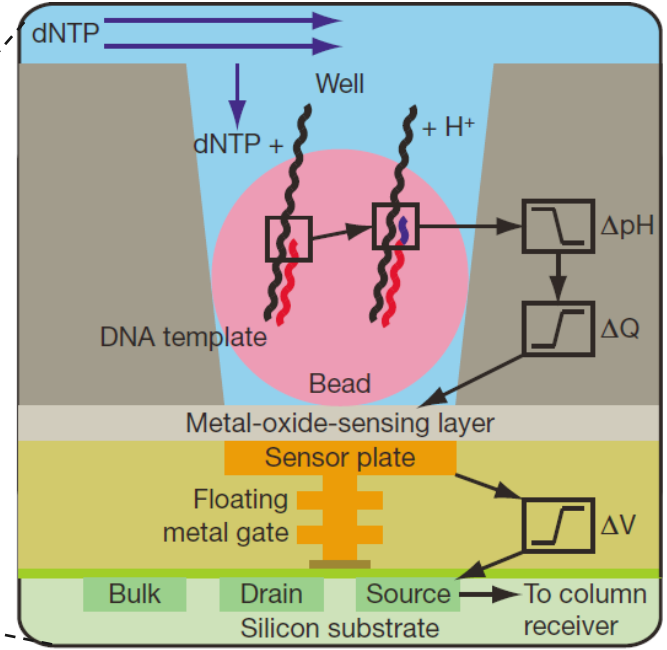
- Difficult to distinguish **homopolymer** of various lengths



Ion Torrent

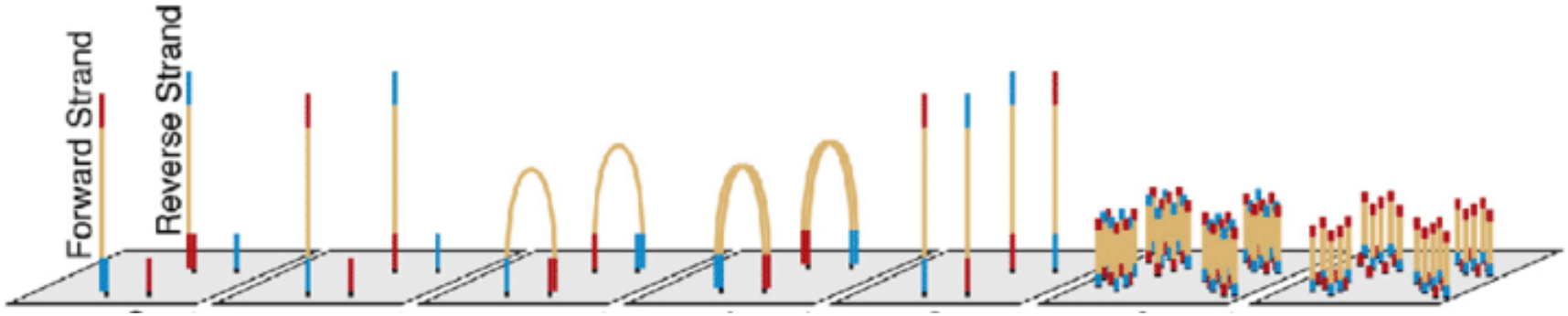






- Measure changes in pH
- Also has **homopolymer** limitation

Illumina / Solexa DNA amplification

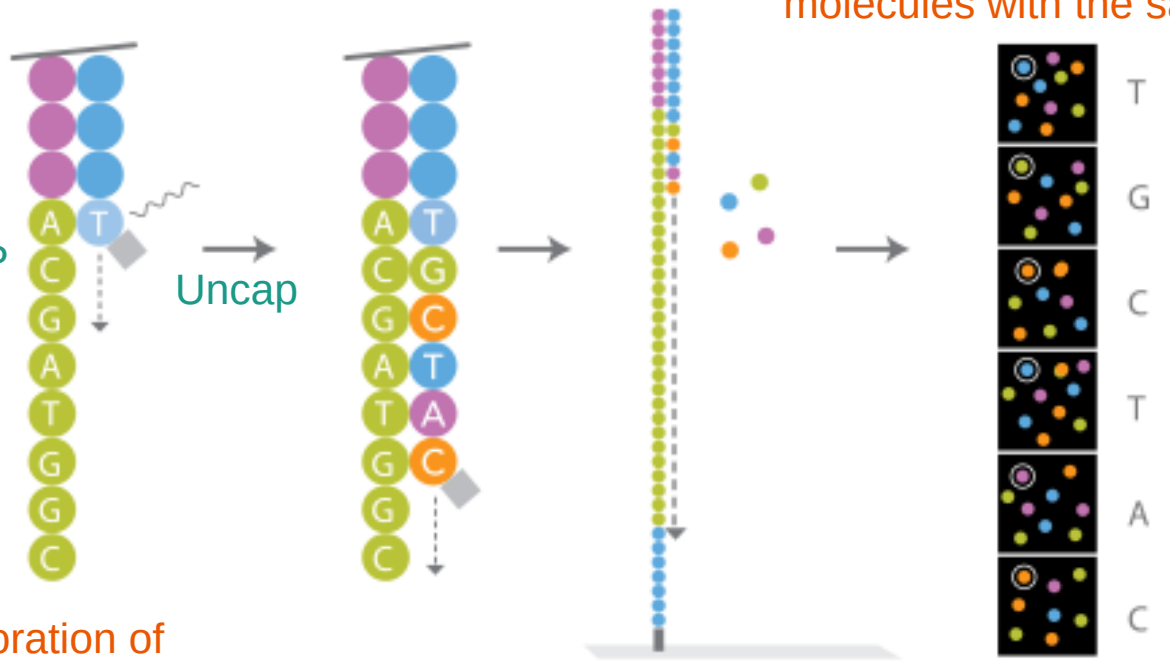


- Improve sensitivity by sequencing clusters of the amplified DNA molecules deriving from the same original DNA

Multi-step DNA polymerization

Each spot = cluster of multiple DNA molecules with the same sequence

Capped dNTP



Prevent incorporation of multiple dNTP

http://www.bioinformatics.nl/

Tradeoffs

Sanger

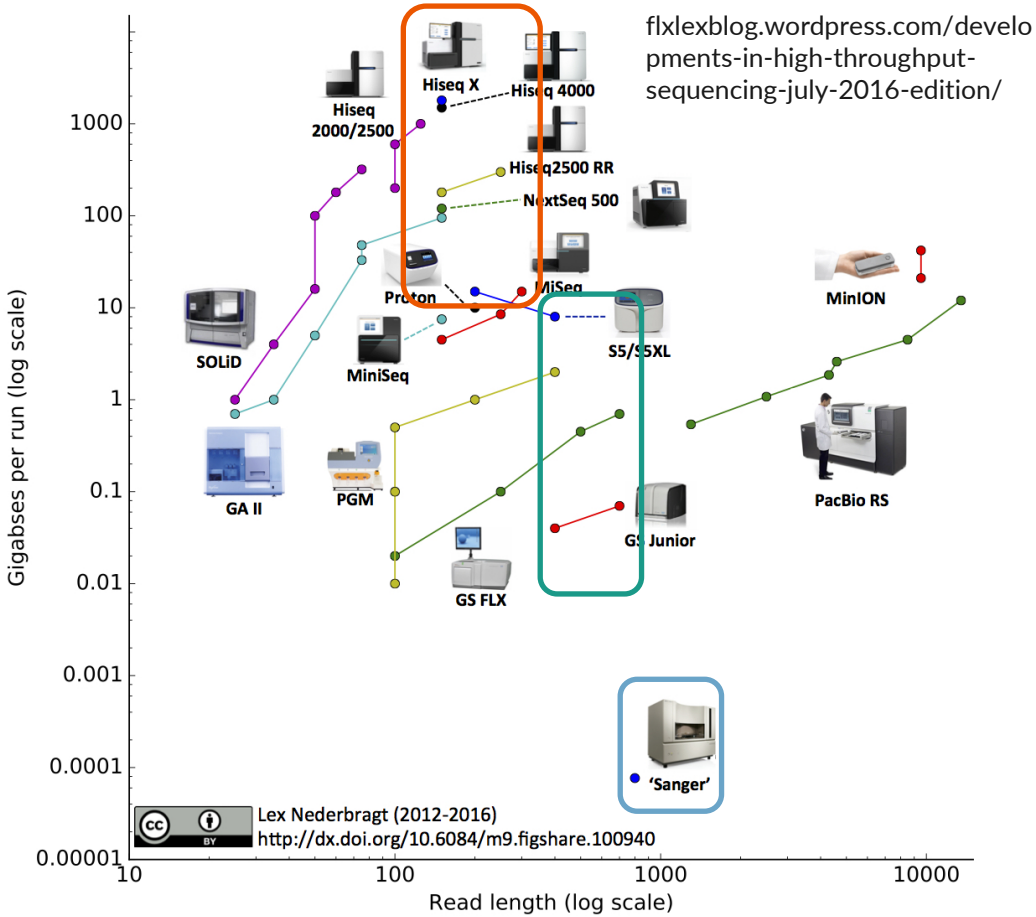
- 1000 bp, low throughput

454 and Ion Torrent

- 400+ bp, medium throughput

Illumina

- <300 bp, high throughput



3rd Generation Sequencing (Long-Read)

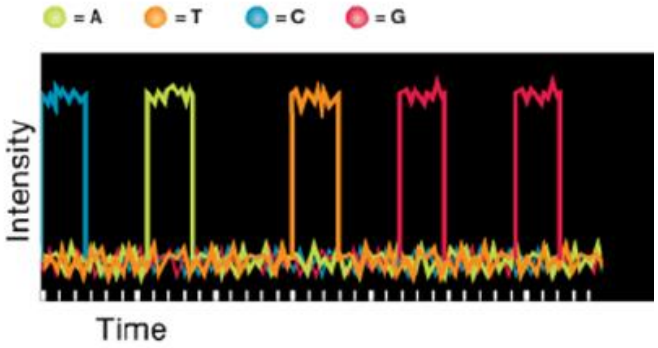
Single-Molecule Real-Time (SMRT) sequencing

Zero-mode waveguide (ZMW) Phospholinked nucleotide

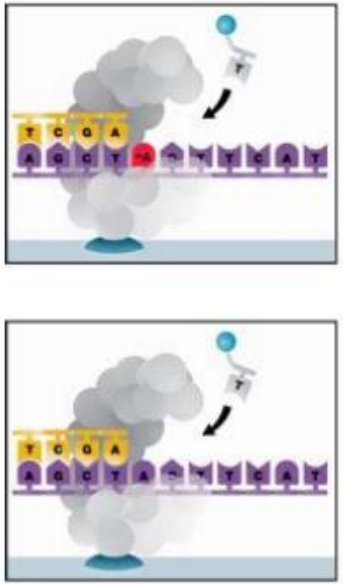
Images from Pacific Biosciences

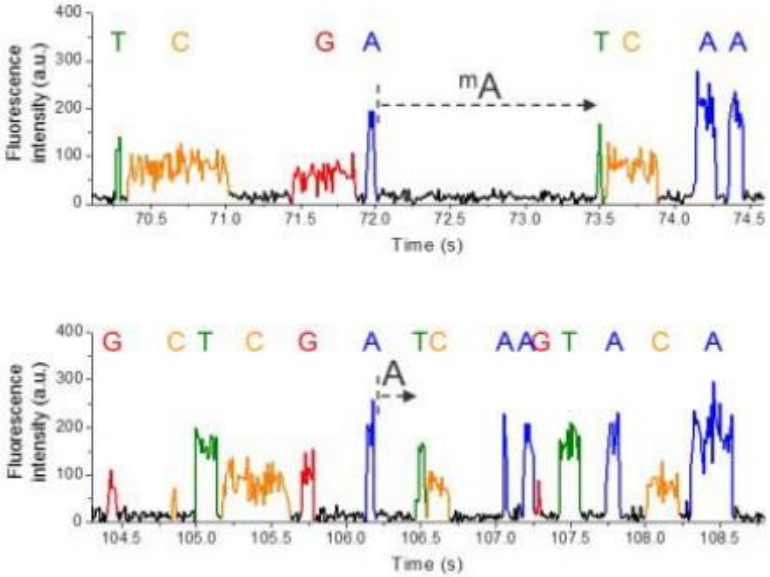
- Faster, more durable DNA polymerase
- Small wells with single DNA molecule
  - Zero-mode waveguide = nanophotonic confinement structure
  - Allow monitoring of fluorescence signal from individual reaction
- No amplification = direct quantification of DNA/RNA abundance

Video data



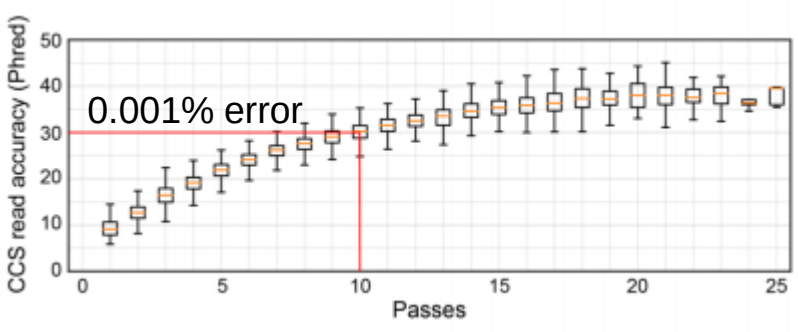
Images from Pacific Biosciences

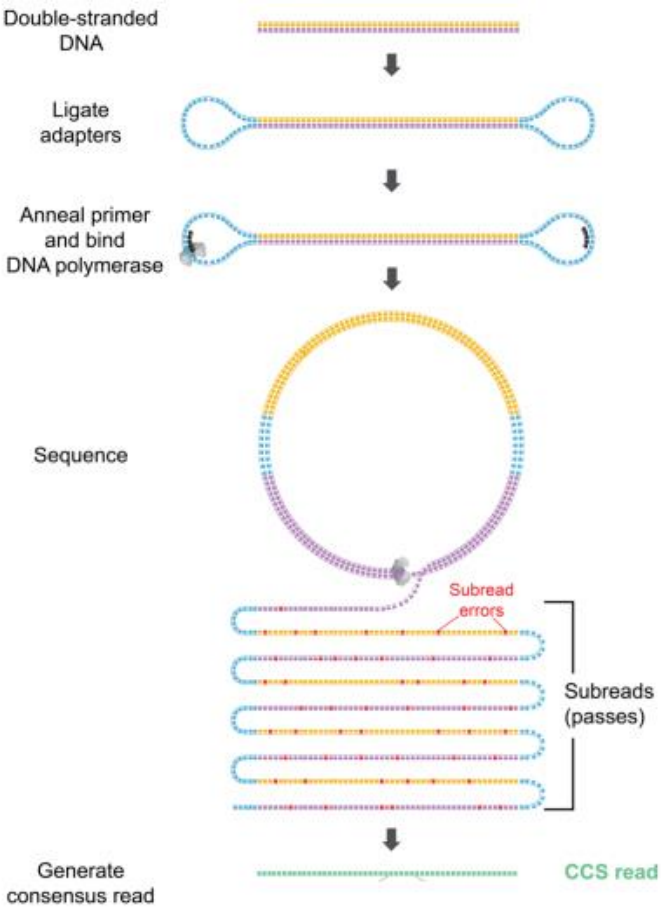




- Compared to image data from Illumina platform
- Video gives time information → identification of modified DNA/RNA

Circular consensus sequencing

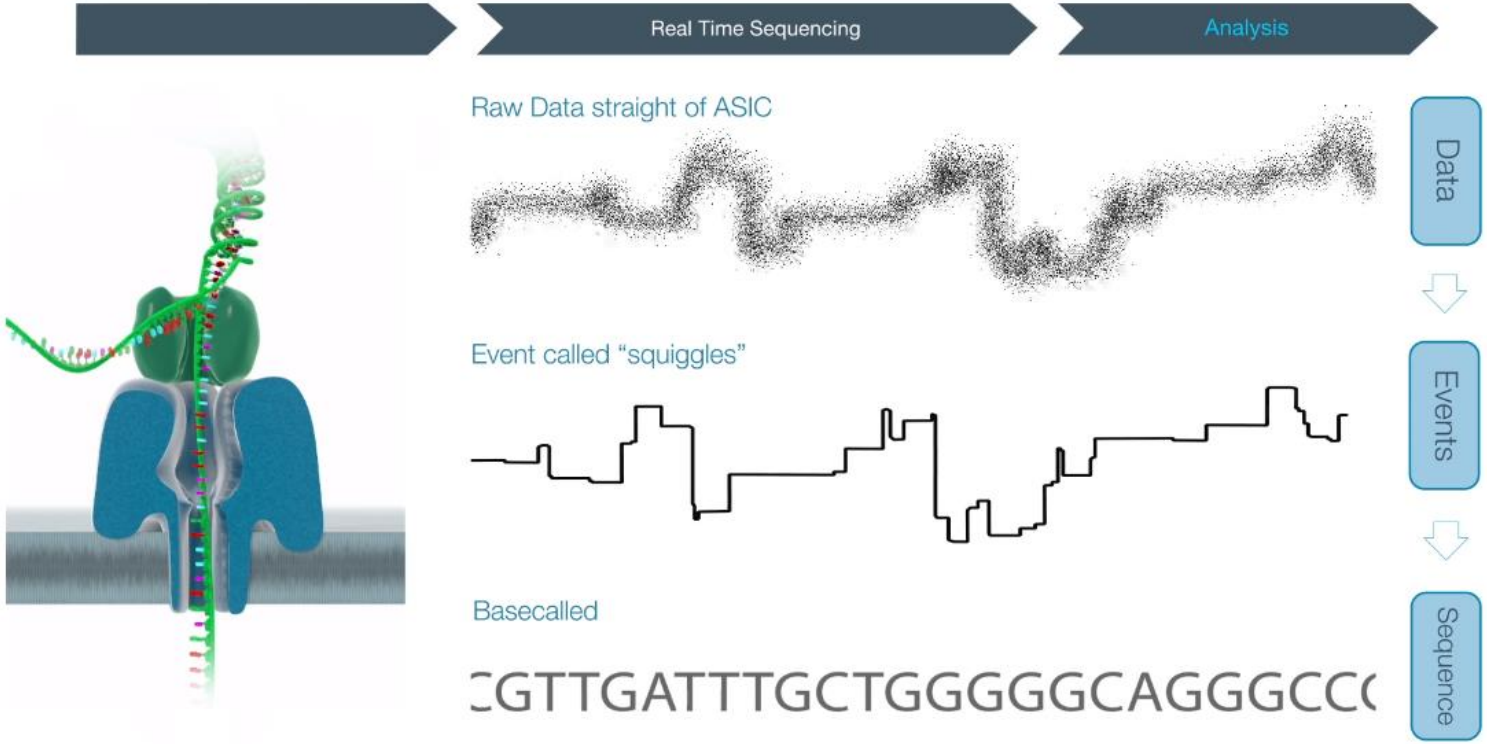




- Circular extension of each DNA molecule
- Read the extended molecules = multiple re-sequencing of the original sequence
- Take the consensus (majority vote)
- P(correct base in >*k* of *N* passes) ~ Binomial

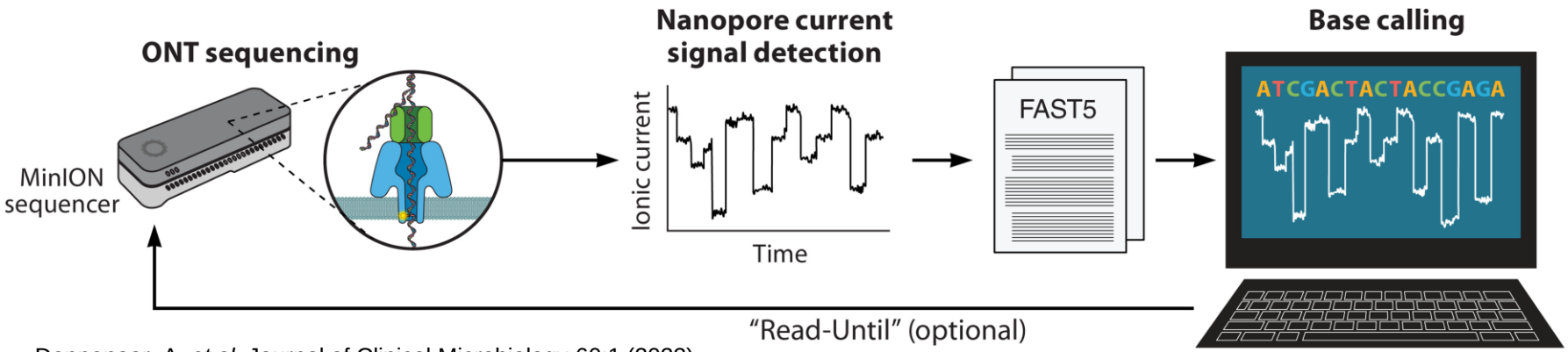
Images from Pacific Biosciences

Nanopore



https://nanoporetech.com/applications/dna-nanopore-sequencing

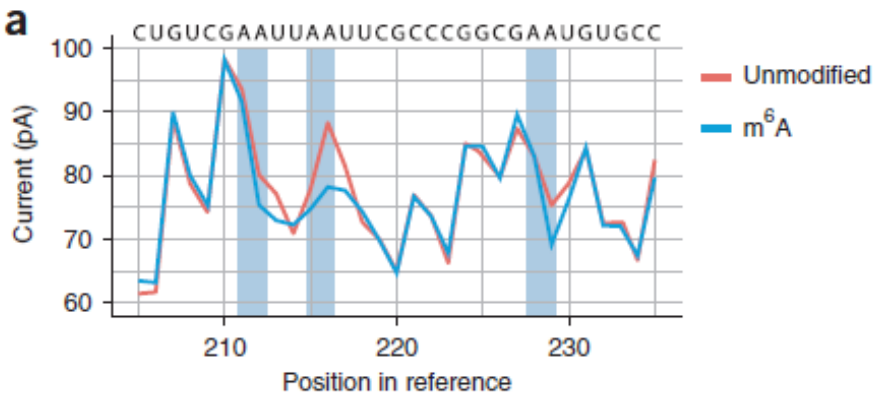
Real-time data

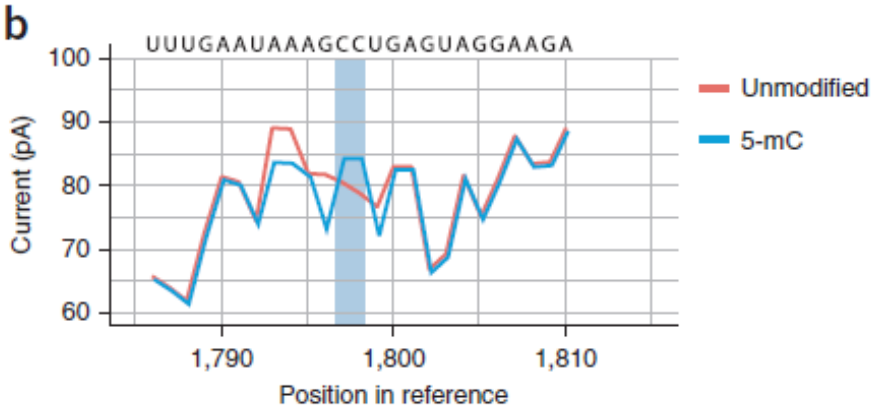


Deppenaar, A. *et al*. Journal of Clinical Microbiology 60:1 (2022)

- Real time ionic flow signals
- Ability to manipulate individual pore and terminate unwanted reads
- Rapid decision making (no need to wait for the full 16-72hr run)

Detection of modified nucleotides

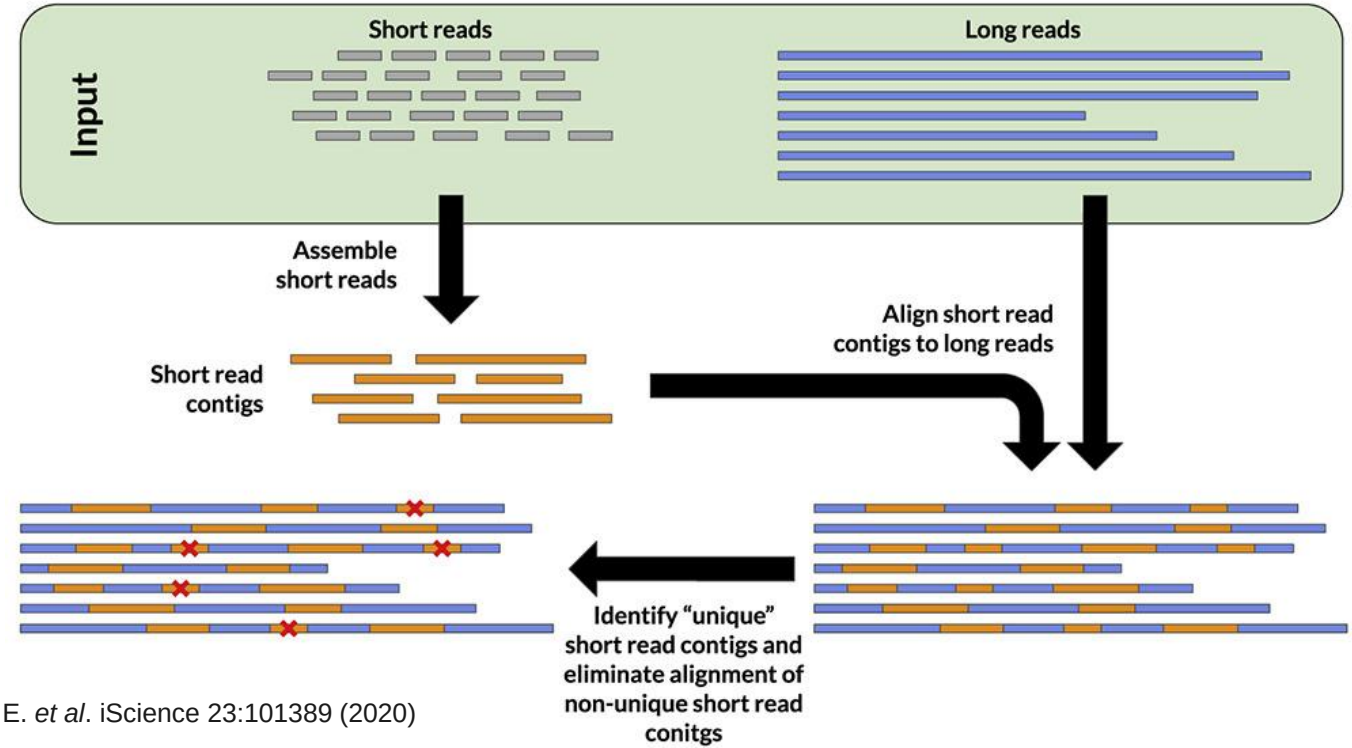




Geralde *et al*. Nature Methods 15, 201-206 (2017)

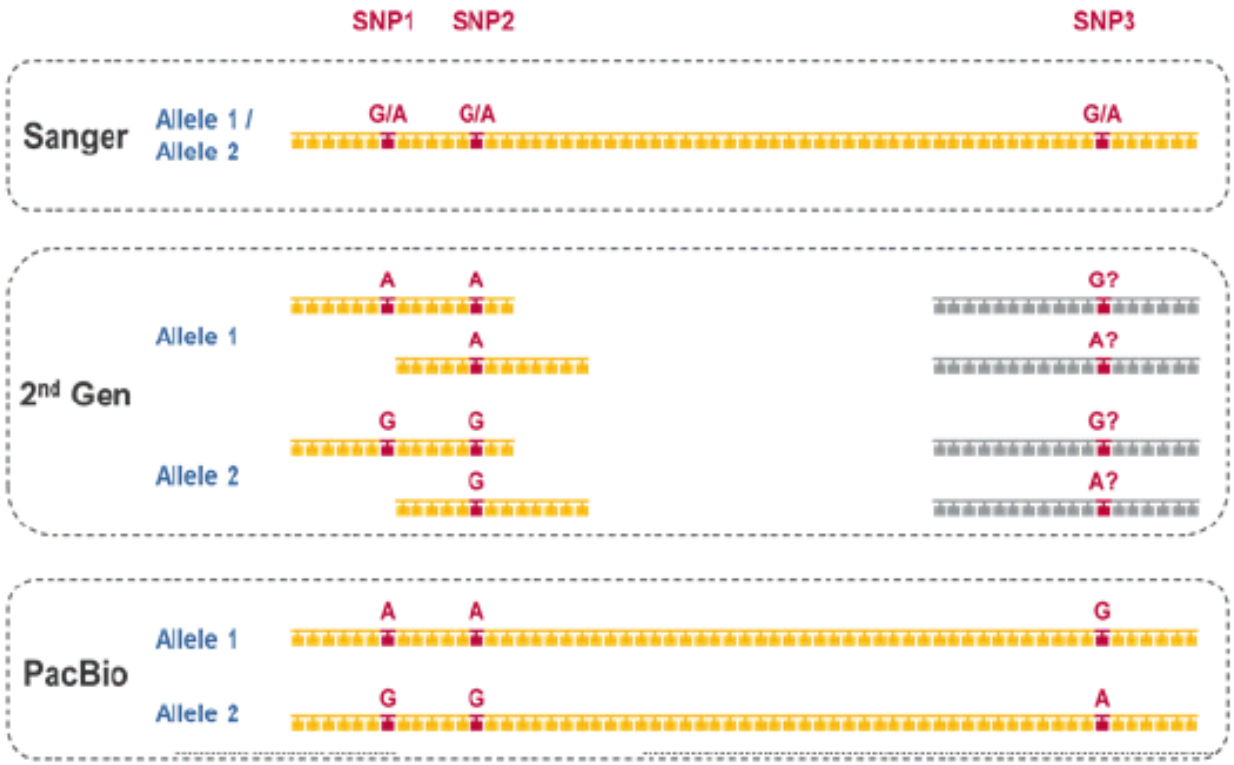
- Modified nucleotides = different 3D structure = different change in ionic flow
- Trained using synthetic nucleotides

Combining short and long read data



Haghshenas, E. *et al*. iScience 23:101389 (2020)

Resolve haplotype



Sanger reads come from mixture of many molecules

2nd generation reads are too short to span the whole haplotype block

3rd generation reads are both single-molecule and long

Pros and cons

Platform	Read Length	Advantage	Disadvantage
Illumina	50-300	High throughput Accurate	Short length
PacBio	10k-25k	Intermediate length Accurate (with more sequencing) Can detect modification	Expensive
Nanopore	10k-1M	Longest length Can detect modification Real-time Portable	High error rate

Applications of DNA/RNA sequencing

Sequencing scope

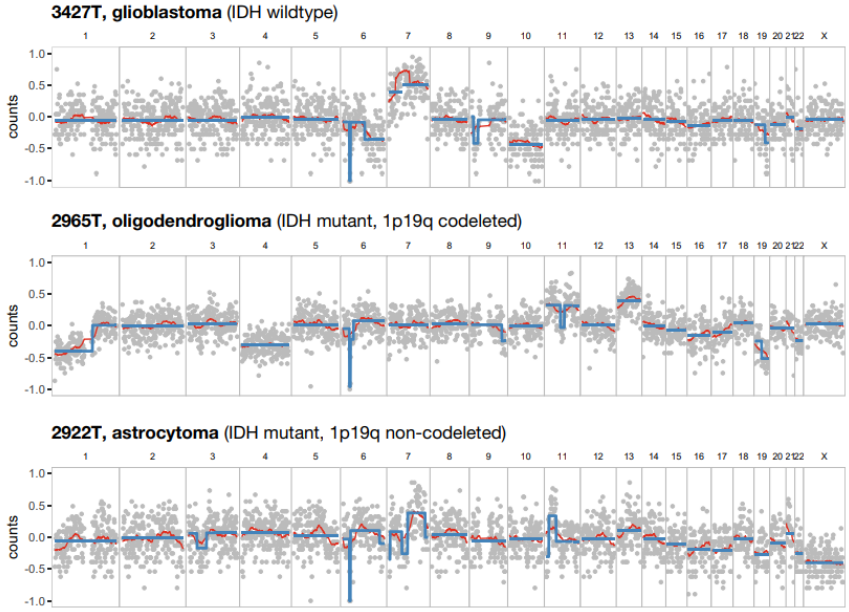
- Cost = Base Pair = Scope x Depth

Reduced scope

- Exome sequencing = exons only
- Amplicon sequencing = selected loci
  - 16S rRNA, RDRP gene
  - (Cancer) gene panels

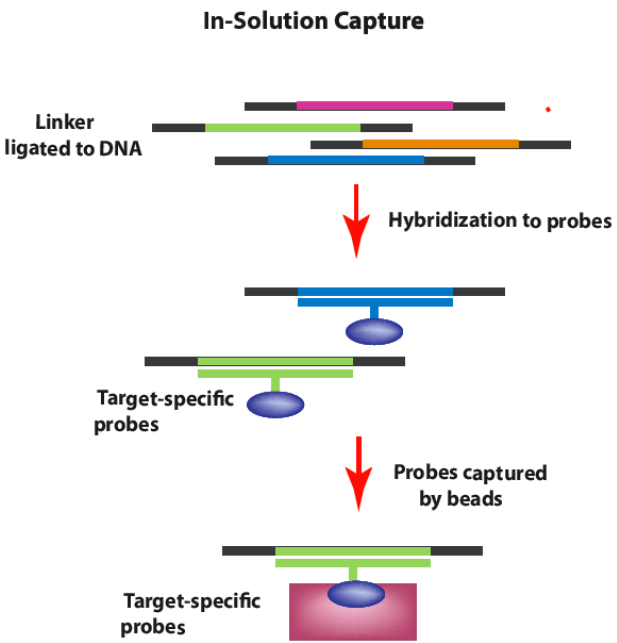
Reduced depth

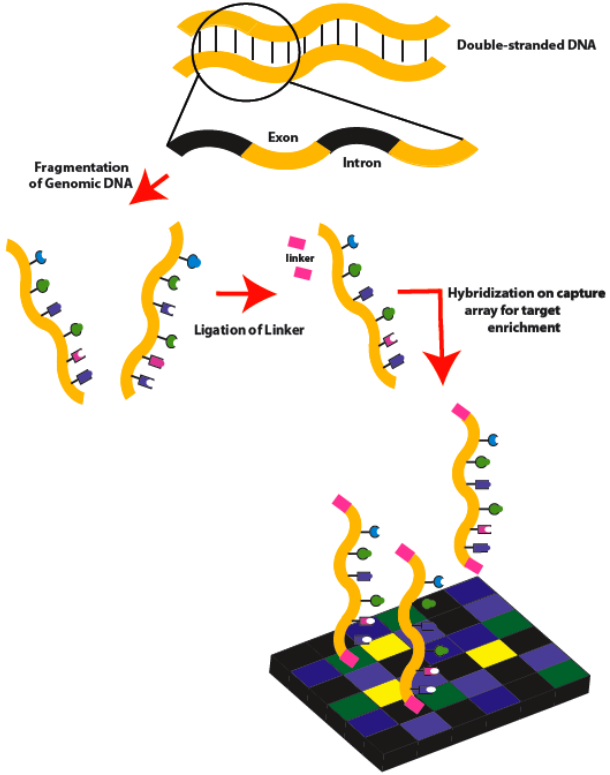
- Ultra-low pass
  - Detect chromosomal copy alternation
  - Estimate tumor fraction



Euskirchen, P. *et al*. Acta Neuropathol 134:691-703 (2017)

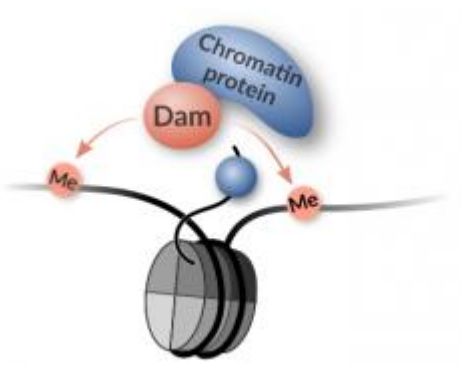
Enrichment for targeted sequencing





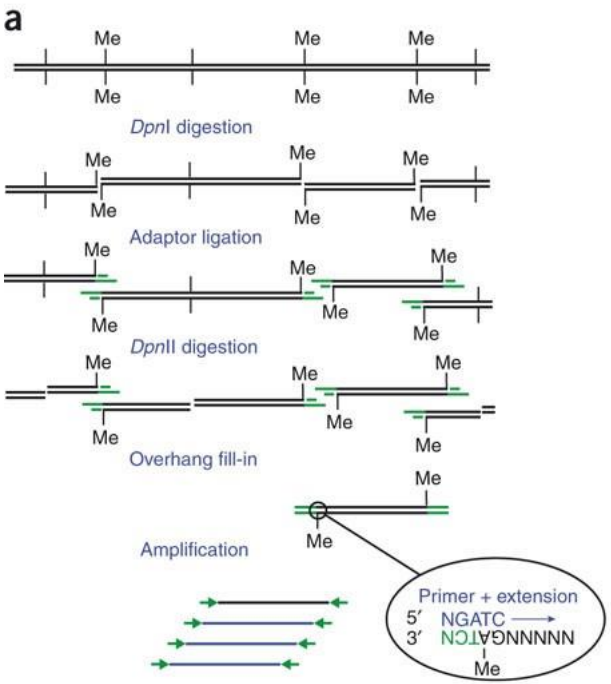
https://en.wikipedia.org/wiki/Exome_sequencing

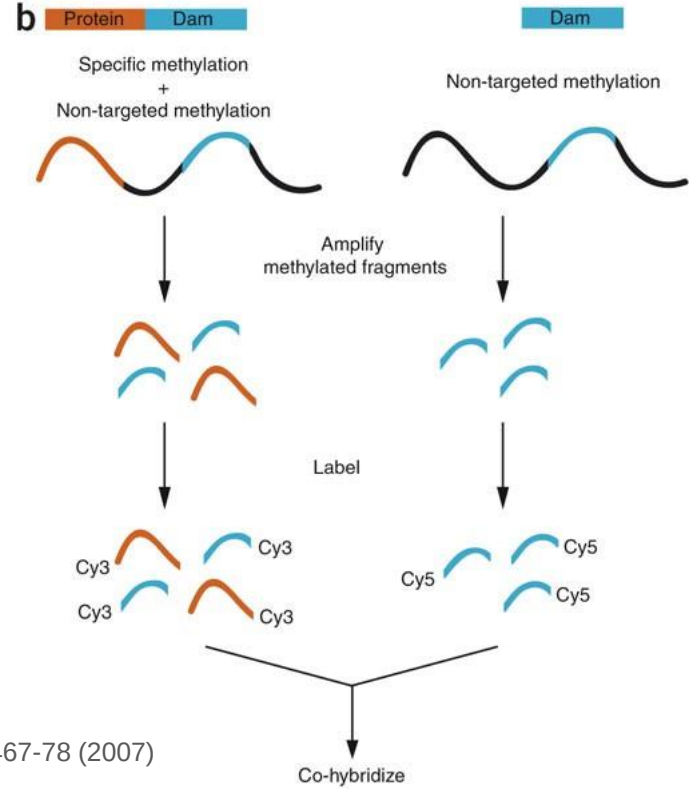
DNA adenine methylatransferase (DamID)



https://marshall-lab.org/damid/

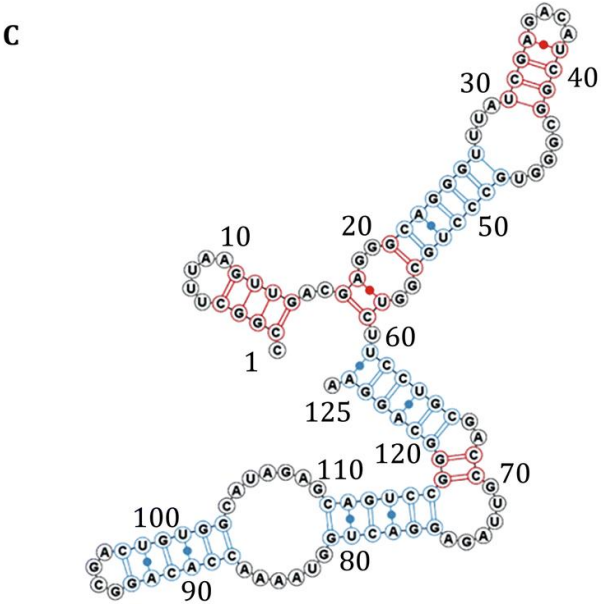
- Dam attached to protein of interest
- Methylation of GATC
- DpnI/DpnII enzymes

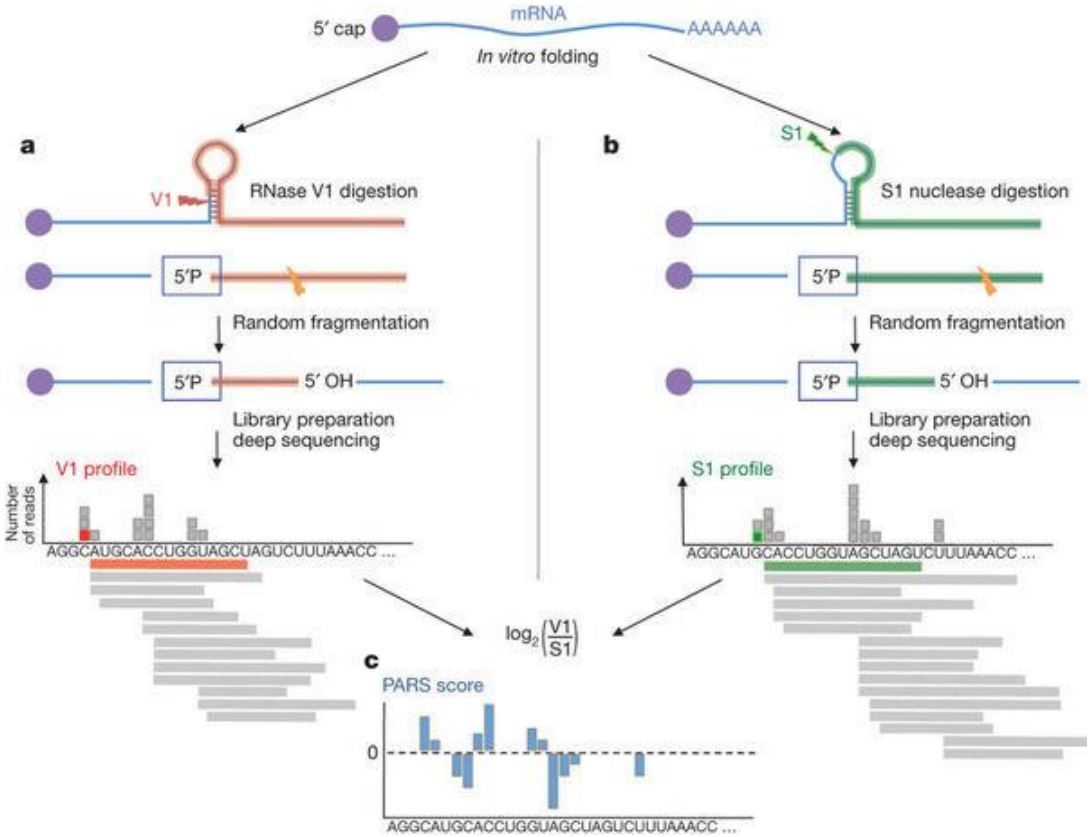




Vogel, M.J. et al. Nat Protocols 2:1467-78 (2007)

RNA secondary structure





Kertesz *et al*. Nature 467: 103-107 (2010)

Any question?

Part II: DNA sequencing data handling

- Key file formats
- QC process
- Basics of sequence alignment and assembly
- Basics of variant calling and annotation

Sequencing data quality control

FastQC tool

Basic Statistics

Measure	Value
Filename	small_rna.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	250000
Sequences flagged as poor quality	0
Sequence length	100
%GC	45

FastQC Report

Summary

- Basic Statistics
- Per base sequence quality
- Per tile sequence quality
- Per sequence quality scores
- Per base sequence content
- Per sequence GC content
- Per base N content
- Sequence Length Distribution
- Sequence Duplication Levels
- Overrepresented sequences
- Adapter Content

FASTQ format

```
@ERR000589.41 EAS139_45:5:1:2:111/1
CTTTCCTCCCTGCTTTCCTGGCCCCACCATTTCCAGGGAACATCTTGTCAT
+
3IIIIIIIIIIIII>1IIIFF9BG08E00I%IG+&?(4)%00646.C1#&(
@ERR000589.42 EAS139_45:5:1:2:1293/1
AGTTGTTAAAATCCAAGCCAATTAAGATAGTCTTATCTTTTTAAAAGAAAT
+
IIIIIGII.AIIII=?I9G-/II=+I=4?761BA2C9I+5A711+&>1$/I
```

- Header: Location of cluster on Illumina's flow cell
- Sequence
- Quality score

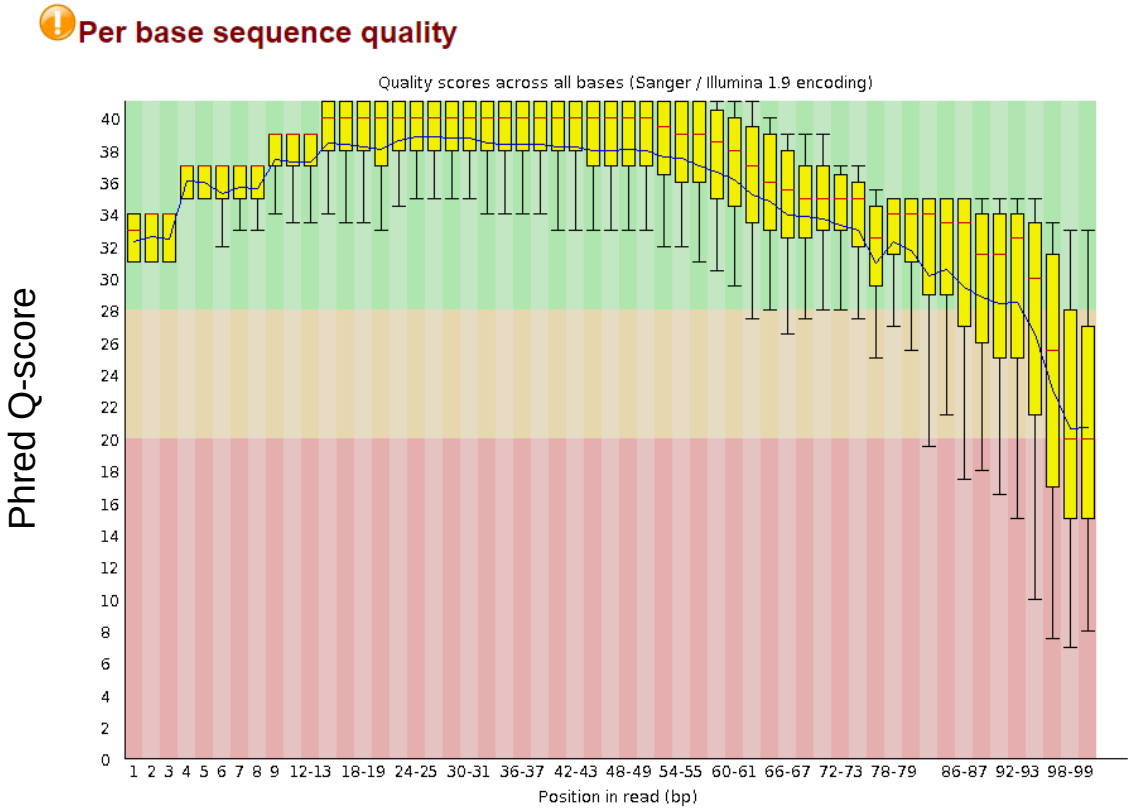
Expected error at the ends of read

```
@ERR000589.41 EAS139_45:5:1:2:111/1
CTTTCCTCCCTGCTTTCCTGGCCCCACCATTTCCAGGGAACATCTTGTCAT
+
3IIIIIIIIIIIII>1IIIFF9BG08E00I%IG+&?(4)%00646.C1#&(
```

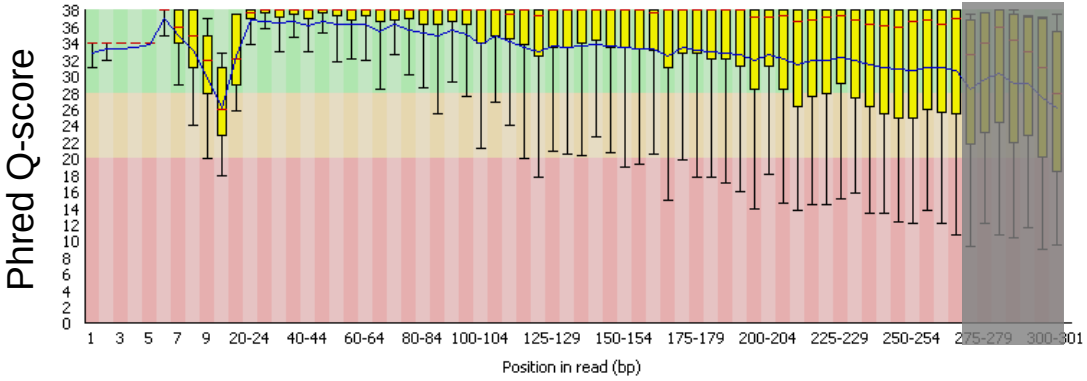
ASCII_BASE=33 Illumina, Ion Torrent, PacBio and Sanger

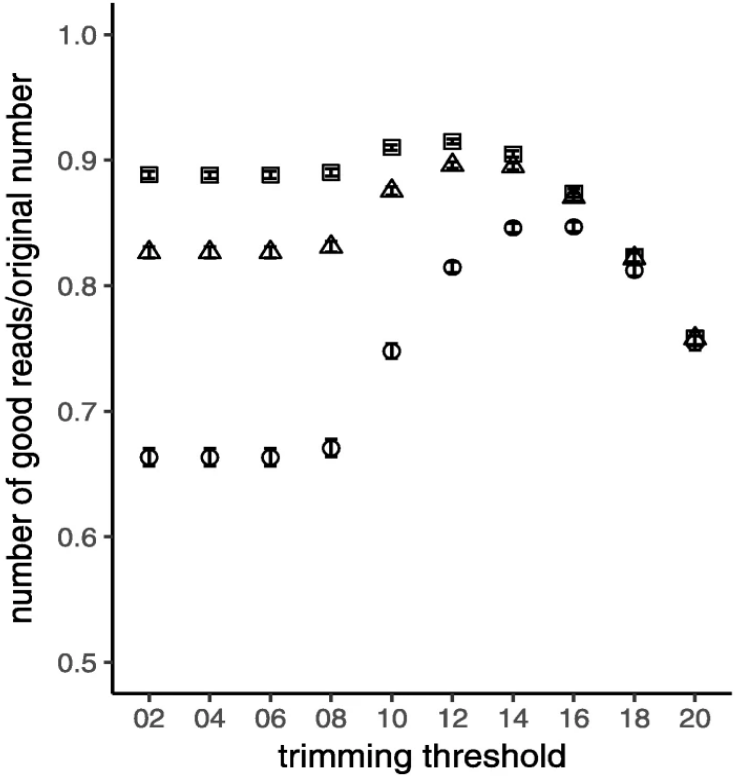
Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII	Q	P_error	ASCII
0	1.00000	33 !	11	0.07943	44 ,	22	0.00631	55 7	33	0.00050	66 B
1	0.79433	34 "	12	0.06310	45 -	23	0.00501	56 8	34	0.00040	67 C
2	0.63096	35 #	13	0.05012	46 .	24	0.00398	57 9	35	0.00032	68 D
3	0.50119	36 $	14	0.03981	47 /	25	0.00316	58 :	36	0.00025	69 E
4	0.39811	37 %	15	0.03162	48 0	26	0.00251	59 ;	37	0.00020	70 F
5	0.31623	38 &	16	0.02512	49 1	27	0.00200	60 <	38	0.00016	71 G
6	0.25119	39 '	17	0.01995	50 2	28	0.00158	61 =	39	0.00013	72 H
7	0.19953	40 (	18	0.01585	51 3	29	0.00126	62 >	40	0.00010	73 I
8	0.15849	41)	19	0.01259	52 4	30	0.00100	63 ?	41	0.00008	74 J
9	0.12589	42 *	20	0.01000	53 5	31	0.00079	64 @	42	0.00006	75 K
10	0.10000	43 +	21	0.00794	54 6	32	0.00063	65 A			

Base calling quality



Quality trimming





- Remove bases from the end until a minimum quality is reached
- May lose reads but lead to better results in downstream analysis

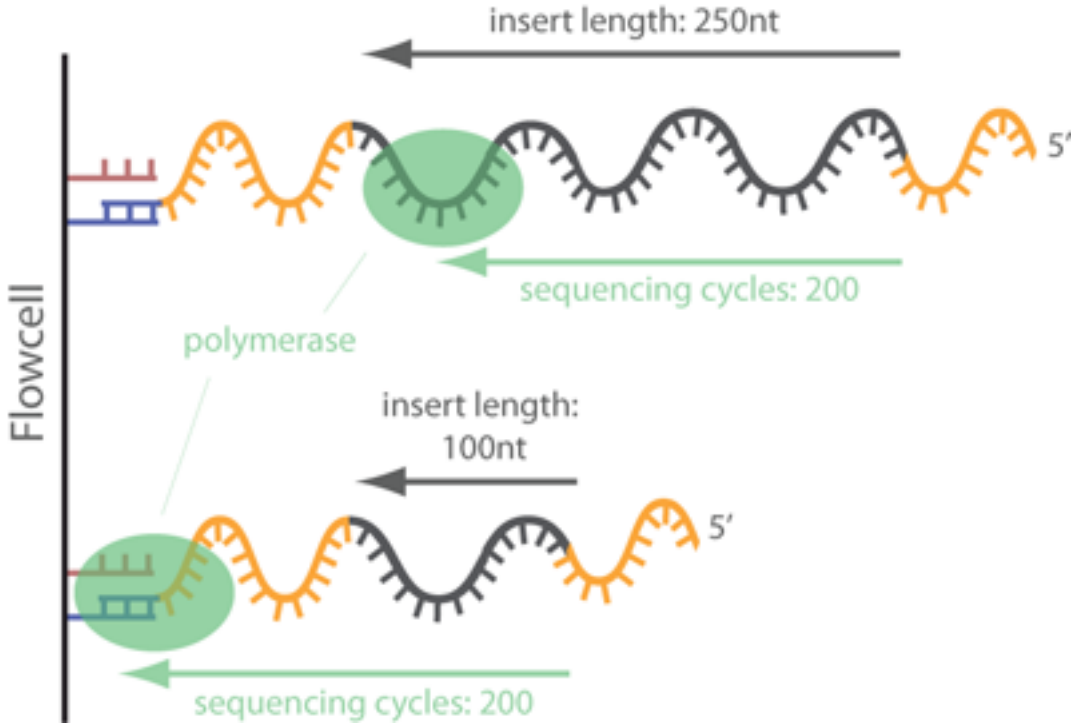
Mohsen, A. et al. BMC Bioinformatics 20:581 (2019)

Possible adapter read-through

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
TGAGGTAGTAGATTGTATAGTTAGATCGGAAGAGCACACGTCTGAACTCC	10865	4.346	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TAGCTTATCAGACTGATGTTGACAGATCGGAAGAGCACACGTCTGAACTC	10845	4.338	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TCTTTGGTTATCTAGCTGTATGAGATCGGAAGAGCACACGTCTGAACTCC	7062	2.8247999999999998	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TCTTTGGTTATCTAGCTGTATGAAGATCGGAAGAGCACACGTCTGAACTC	4056	1.6223999999999998	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TGAGGTAGTAGTTTGTGCTGTTAGATCGGAAGAGCACACGTCTGAACTCC	3737	1.4948	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TGAGGTAGTAGTTTGTACAGTTAGATCGGAAGAGCACACGTCTGAACTCC	3549	1.4196	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TGAGGTAGTAGGTTGTATGGTTAGATCGGAAGAGCACACGTCTGAACTCC	2931	1.1724	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
AACCCGTAGATCCGATCTTGTAGATCGGAAGAGCACACGTCTGAACTCCA	1910	0.764	Illumina Multiplexing PCR Primer 2.01 (100% over 29bp)
CGCGACCTCAGATCAGACGTAGATCGGAAGAGCACACGTCTGAACTCCAG	1749	0.6996	Illumina Multiplexing PCR Primer 2.01 (100% over 30bp)
TGAGGTAGTAGGTTGTATAGTTAGATCGGAAGAGCACACGTCTGAACTCC	1647	0.6588	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
TCTTTGGTTATCTAGCTGTATAGATCGGAAGAGCACACGTCTGAACTCCA	1622	0.6487999999999999	Illumina Multiplexing PCR Primer 2.01 (100% over 29bp)
TAGCTTATCAGACTGATGTTGATAGATCGGAAGAGCACACGTCTGAACTC	1328	0.5312	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)
TTCAAGTAATCCAGGATAGGCTAGATCGGAAGAGCACACGTCTGAACTCC	1248	0.4992	Illumina Multiplexing PCR Primer 2.01 (100% over 28bp)
AGCAGCATTGTACAGGGCTATGAAGATCGGAAGAGCACACGTCTGAACTC	1248	0.4992	Illumina Multiplexing PCR Primer 2.01 (100% over 27bp)

Adapter trimming



main / Trimmomatic / adapters /

TonyBolger Parallel Compression

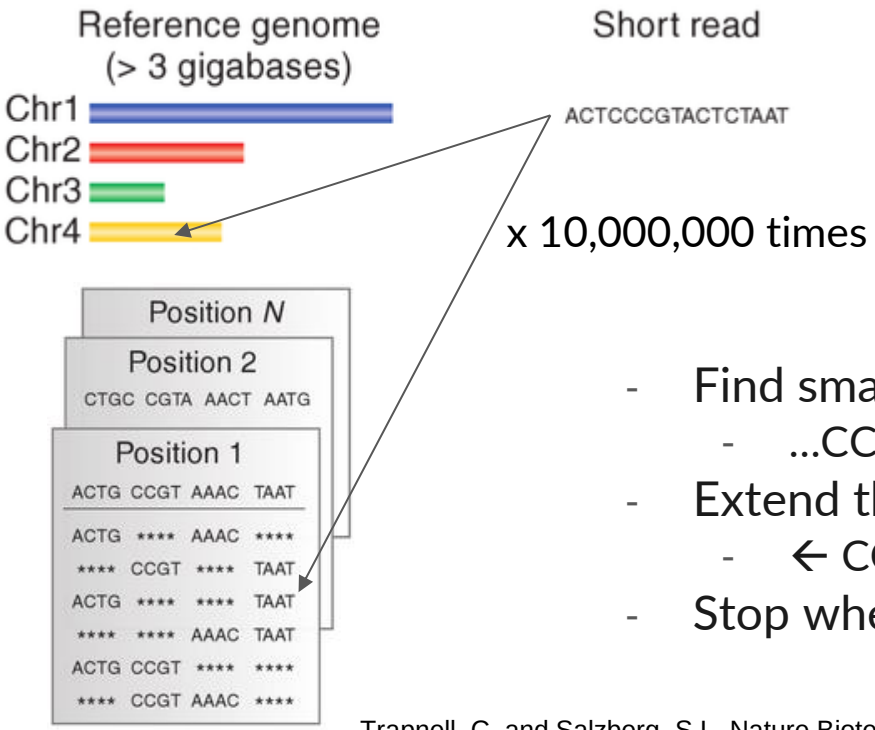
..

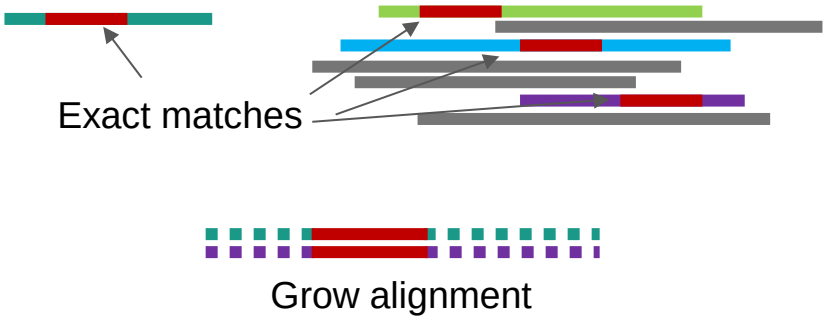
- NexteraPE-PE.fa
- TruSeq2-PE.fa
- TruSeq2-SE.fa
- TruSeq3-PE-2.fa
- TruSeq3-PE.fa
- TruSeq3-SE.fa

https://www.ecseq.com/support/ngs/trimming-adapter-sequences-is-it-necessary

Alignment

Sequence alignment is a form of search





- Find small segments with exact matches
  - ...CCCGTA...
- Extend the alignment to both sides
  - ← CCCGTA →
- Stop when the number of mismatches is too high

Trapnell, C. and Salzberg, S.L. Nature Biotechnology 27:455-7 (2009)

Searching with suffix array

Reference Sequence

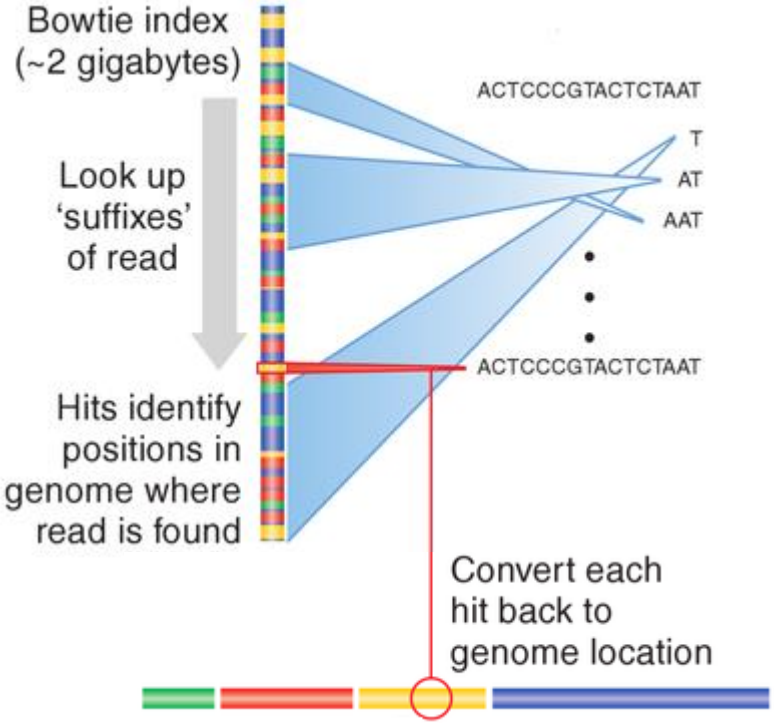
ATTGCAGTCCG

AGTCCG	6
ATTGCAGTCCG	1
CAGTCCG	5
CCG	9
CG	10
G	11
GCAGTCCG	4
GTCCG	7
TCCG	8
TGCAGTCCG	3
TTGCAGTCCG	2

- Suffix = ending part of a string
- Organize suffixes in an easily searchable data structure
- Also record the start positions

Christley, S. et al. BMC Bioinformatics 9:15 (2008)

Genome-scale indexing and searching



- Indexing of all short segments of the genome
- 20x smaller memory than straightforward indexing
- 30x faster search speed

Trapnell, C. and Salzberg, S.L. Nature Biotechnology 27:455-7 (2009)

Dynamic programming

No. of rocks	1	2	3	4	5	6	7	8	9	10
Winner										
No. of rocks	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
Winner										?

- Build the solution of complex problem using on the solutions of simpler ones

- There is a pile of 20 rocks. Two players take turns by removing 1 or 2 rocks from the pile. Whoever removes the last rock(s) win. Who is the winner?

Dynamic programming for sequence alignment

Dynamic programming matrix:

j → (sequence **y**)

i		0	1 T	2 G	3 C	4 T	5 C	6 G	7 T	8 = N A
0		0	−6	−12	−18	−24	−30	−36	−42	−48
1	T	−6	5	−1	−7	−13	−19	−25	−31	−37
2	T	−12	−1	3	−3	−2	−8	−14	−20	−26
3	C	−18	−7	−3	8	2	3	−3	−9	−15
4	A	−24	−13	−9	2	6	0	1	−5	−4
5	T	−30	−19	−15	−4	7	4	−2	6	0
M = 6	A	−36	−25	−21	−10	1	5	2	0	11

(sequence **x**)

Optimum alignment scores 11:

T	**–**	**–**	**T**	**C**	**A**	**T**	**A**
T	**G**	**C**	**T**	**C**	**G**	**T**	**A**
+5	−6	−6	+5	+5	−2	+5	+5

- The best alignments for long sequences depend on the best alignments of shorter sequences
- The best alignment for TTCATA vs TGCTCGTA is either
  - T/T + best alignment for TCATA vs GCTCGTA
  - T/– + best alignment for TCATA vs TGCTCGTA
  - –/T + best alignment for TTCATA vs GCTCGTA

Eddy, S.R. Nature Biotechnology 22:909-10 (2004)

Sequence Alignment Map (SAM)

Sort Order = by genomic coordinate

SN = reference sequence's name (FASTA header)

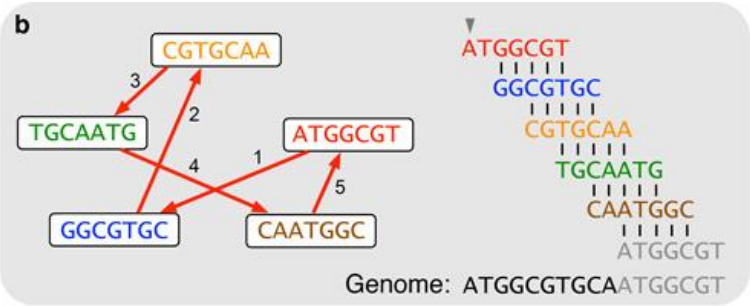
LN = reference sequence's length

```
@HD VN:1.6 SO:coordinate
@SQ SN:ref LN:45
r001   99 ref  7 30 8M2I4M1D3M = 37  39 TTAGATAAAGGATACTG *
r002    0 ref  9 30 3S6M1P1I4M *  0   0 AAAAGATAAGGATA    *
r003    0 ref  9 30 5S6M       *  0   0 GCCTAAGCTAA       * SA:Z:ref,29,-,6H5M,17,0;
r004    0 ref 16 30 6M14N5M    *  0   0 ATAGCTTCAGC       *
r003 2064 ref 29 17 6H5M       *  0   0 TAGGC             * SA:Z:ref,9,+,5S6M,30,1;
r001  147 ref 37 30 9M         =  7 -39 CAGCGGCAT         * NM:i:1
```

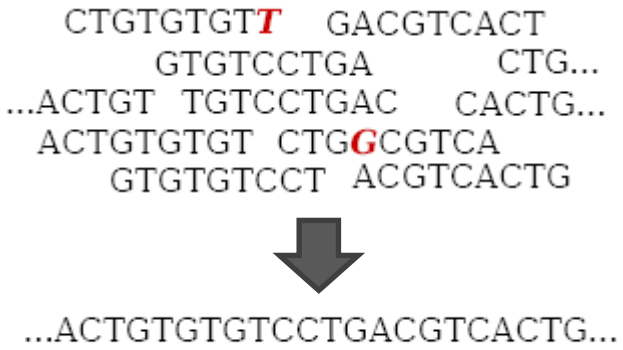
- r001 = read name (from sequencing FASTQ)
- ref = reference sequence name (from genomic FASTA)
- 7 = first position on the reference sequence
- 30 = Mapping quality score = $-10 \times \text{Log}_{10}(\text{error})$
- 8M2I4M1D3M = CIGAR string = matches, insertion, deletion information

Assembly

De novo assembly



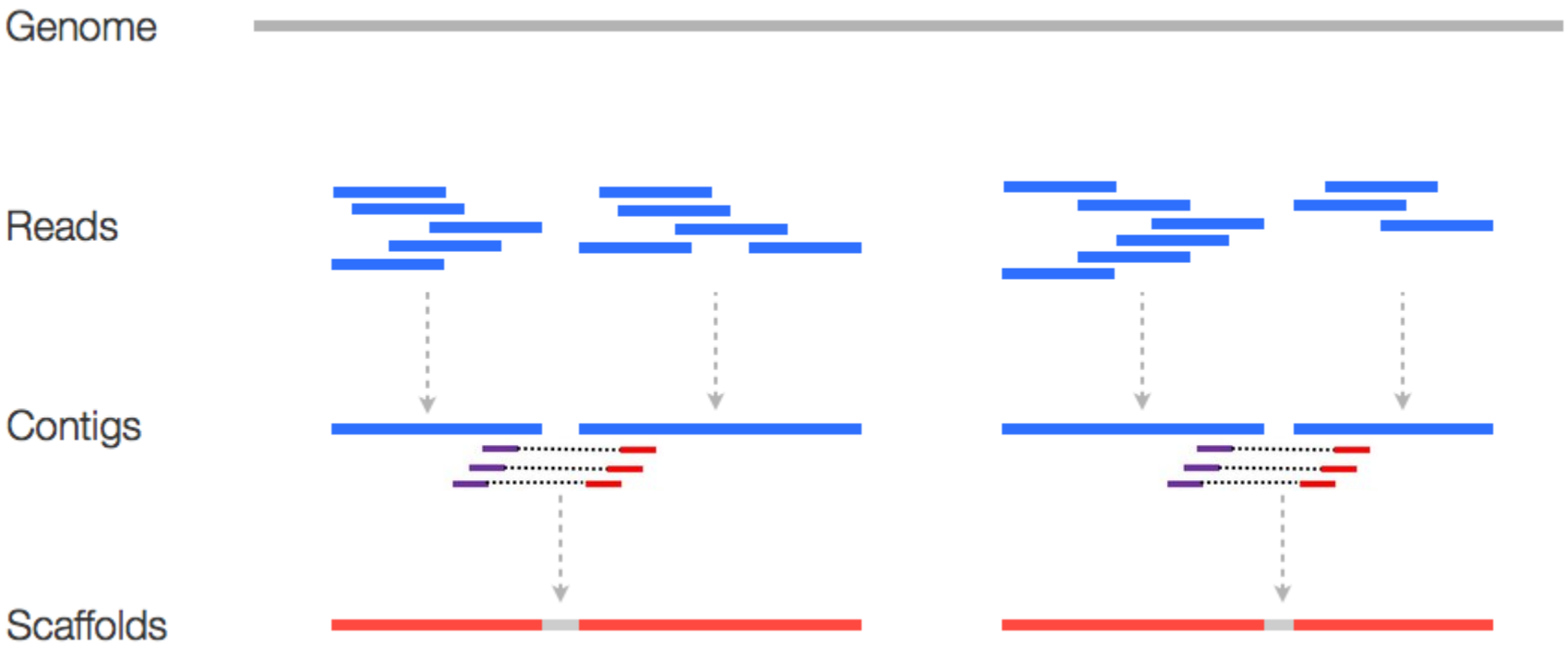
Compeau, P.E. et al. Nature Biotechnology 29:987-991 (2011)



Chandra Varma Bogaraju, S. Int J Embed Syst 9:74 (2017)

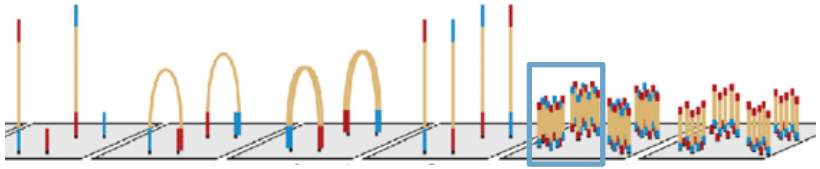
- Each directed path in a de Bruijn graph represents a possible contiguous segment of the genome

Contig and scaffold

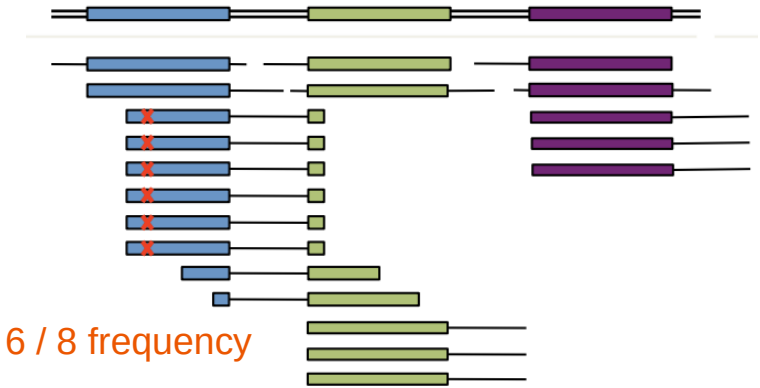


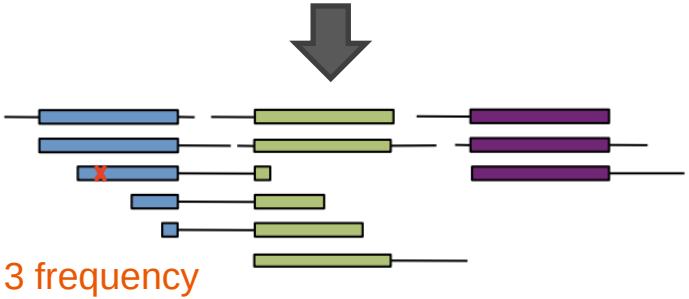
Deduplication

Duplicated = derived from the same molecule



- Similar sequences coming from nearby coordinates in Illumina flow cells
- Reads with the same start and end
  - Highly unlikely to generate the exact same DNA molecules by chance
- Lead to incorrect frequency estimates





Images from qcb.ucla.edu

Variant calling

Type of variants

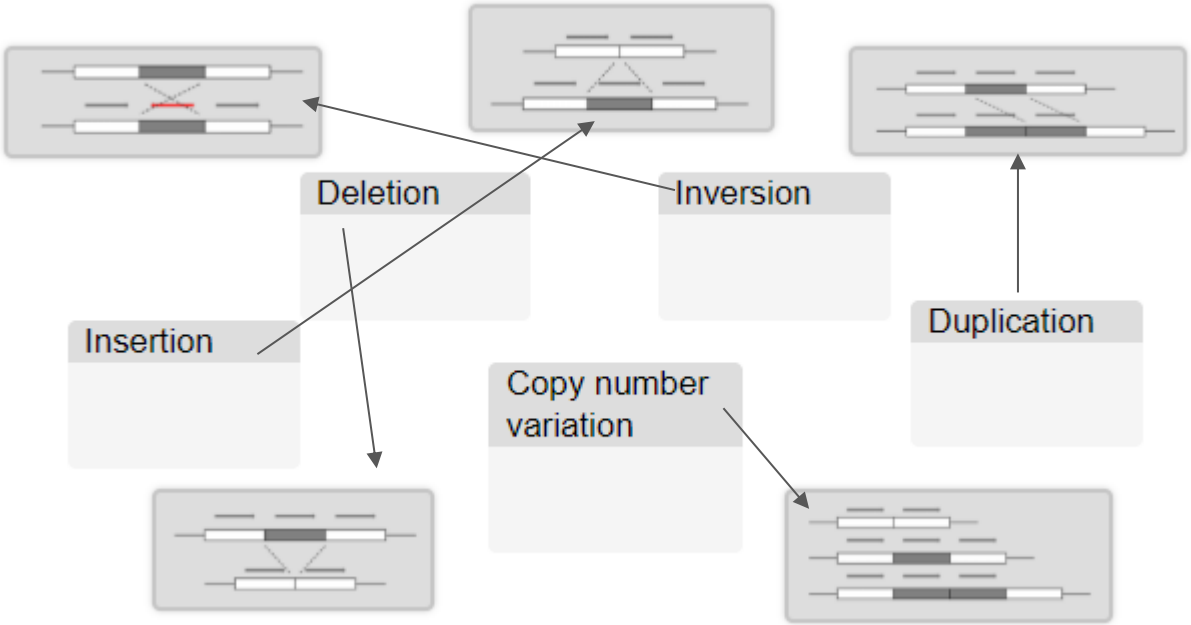


Image from EBI

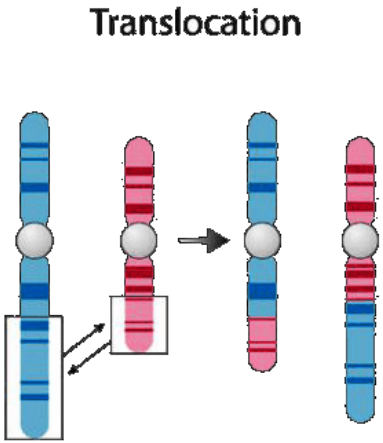


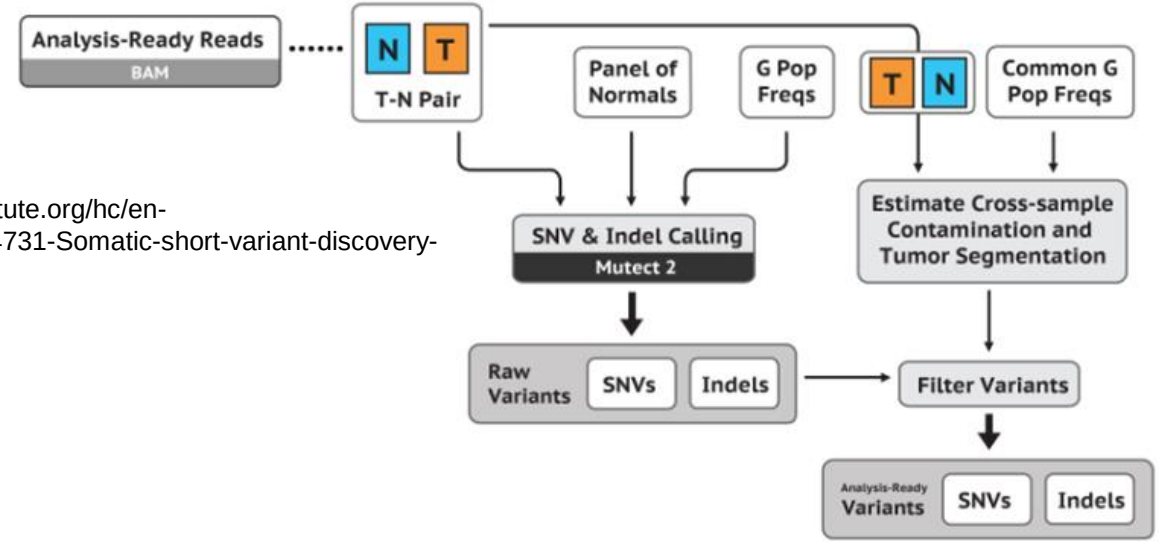
Image from wikipedia

Germline vs somatic variants

- Germline mutations = inherited and appear in every cell of the offspring
  - Compare DNA from normal tissues to reference genomes
  - Fixed ploidy

- Somatic mutations = occur during lifetime
  - Compare DNA from disease tissues to normal tissues
  - Also compare to DNA from other healthy individuals
  - Allow variation in ploidy (different disease cells can have different mutations)

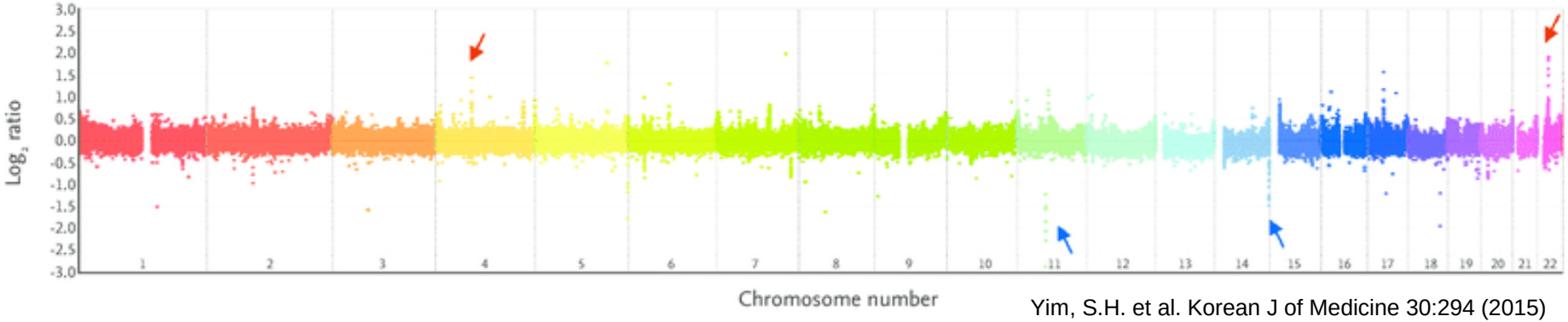
Genome Analysis Toolkit (GATK) somatic workflow



https://gatk.broadinstitute.org/hc/en-us/articles/360035894731-Somatic-short-variant-discovery-SNVs-Indels-

- Inclusion of matched normal (N), panels of healthy individual (Panels of Normals), and allele frequency in the general population (G Pop Freqs)
- Also estimate contamination = normal cells in disease sample

Copy number variations



Yim, S.H. et al. Korean J of Medicine 30:294 (2015)

- Look for loci with high or low read frequencies compared to others

Variant Call Format (VCF)

```
##fileformat=VCFv4.2
##fileDate=20090805
##source=myImputationProgramV3.1
##reference=file:///seq/references/1000GenomesPilot-NCBI36.fasta
##contig=<ID=20,length=62435964,assembly=B36,md5=f126cdf8a6e0c7f379d618ff66beb2da,species="Homo sapiens",taxonomy=x>
##phasing=partial
##INFO=<ID=NS,Number=1,Type=Integer,Description="Number of Samples With Data">
##INFO=<ID=DP,Number=1,Type=Integer,Description="Total Depth">
##INFO=<ID=AF,Number=A,Type=Float,Description="Allele Frequency">
##INFO=<ID=AA,Number=1,Type=String,Description="Ancestral Allele">
##INFO=<ID=DB,Number=0,Type=Flag,Description="dbSNP membership, build 129">
##INFO=<ID=H2,Number=0,Type=Flag,Description="HapMap2 membership">
##FILTER=<ID=q10,Description="Quality below 10">
##FILTER=<ID=s50,Description="Less than 50% of samples have data">
##FORMAT=<ID=GT,Number=1,Type=String,Description="Genotype">
##FORMAT=<ID=GQ,Number=1,Type=Integer,Description="Genotype Quality">
##FORMAT=<ID=DP,Number=1,Type=Integer,Description="Read Depth">
##FORMAT=<ID=HQ,Number=2,Type=Integer,Description="Haplotype Quality">
#CHROM POS     ID        REF    ALT     QUAL FILTER INFO                              FORMAT      NA00001        NA00002
20    14370   rs6054257 G      A       29   PASS   NS=3;DP=14;AF=0.5;DB;H2           GT:GQ:DP:HQ 0|0:48:1:51,51 1|0:48:8:51,51
20    17330   .         T      A       3    q10    NS=3;DP=11;AF=0.017               GT:GQ:DP:HQ 0|0:49:3:58,50 0|1:3:5:65,3
20    1110696 rs6040355 A      G,T     67   PASS   NS=2;DP=10;AF=0.333,0.667;AA=T;DB GT:GQ:DP:HQ 1|2:21:6:23,27 2|1:2:0:18,2
20    1230237 .         T      .       47   PASS   NS=3;DP=13;AA=T                  GT:GQ:DP:HQ 0|0:54:7:56,60 0|0:48:4:51,51
20    1234567 microsat1 GTC    G,GTCT  50   PASS   NS=3;DP=9;AA=G                    GT:GQ:DP    0/1:35:4       0/2:17:2
```

| = phased

/ = unphased

Variant annotation

Understanding the importance of a variant

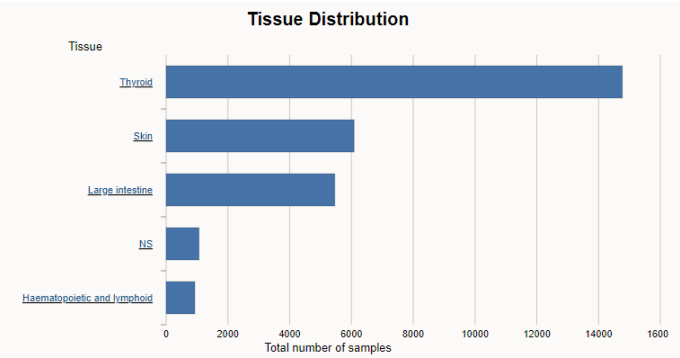
- Impact on sequence
  - Non-synonymous, splice site, frameshift, regulatory element

- Is it known?
  - Genome Aggregation Database: gnomAD
  - dbSNP

- Clinical implication: observed in patients, treatment response, drug target
  - ClinVar, COSMIC, PharmGKB

- Variant effect predictor (VEP)
- Funcotator/Oncotator

Online databases



NM_007294.3(BRCA1):c.*6207C>T

Allele ID: 206177
Variant type: single nucleotide variant
Variant length: 1 bp
Cytogenetic location: 17q21.31
Genomic location: 17: 43039471 (GRCh38) GRCh38 UCSC
17: 41191488 (GRCh37) GRCh37 UCSC

HGVS:

Nucleotide	Protein	Molecular consequence
NC_000017.11:g.43039471G>A		
NC_000017.10:g.41191488G>A		
NG_005905.2:g.178513C>T		

... more HGVS

Protein change: -
Other names: 11918 C>T
Canonical SPDI: NC_000017.11:43039470:G:A
Functional consequence: -
Global minor allele frequency (GMAF): 0.00679 (A)
Allele frequency: Trans-Omics for Precision Medicine (TOPMed) 0.00211

VARIANT	LITERATURE	DRUGS	GENES	ASSOCIATION
rs2069502	PMCID:PMC3959225	somatropin recombinant	CDK4	Genotype CC is associated with decreased respc to somatropin recombinant in children with Tur Syndrome as compared to genotypes CT + TT.

Any question?