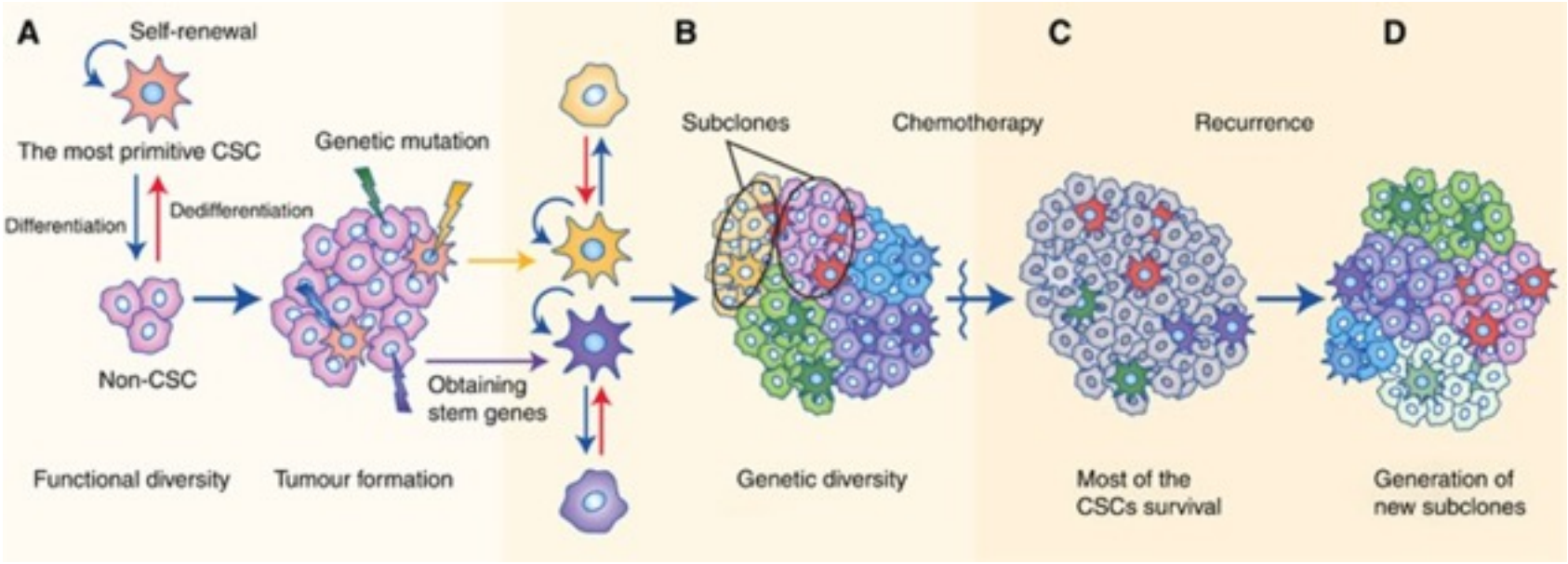
DNA sequencing

FROM SANGER TO NGS: our experience in a molecular pathology lab

Tumour heterogeneity

Stochastic and hierarchical models are reasonable systems that have been hypothesised to describe tumour heterogeneity.

Each model alone inadequately explains tumour diversity.
The two models can be integrated to provide a more comprehensive explanation.



Tumoral mutational burden

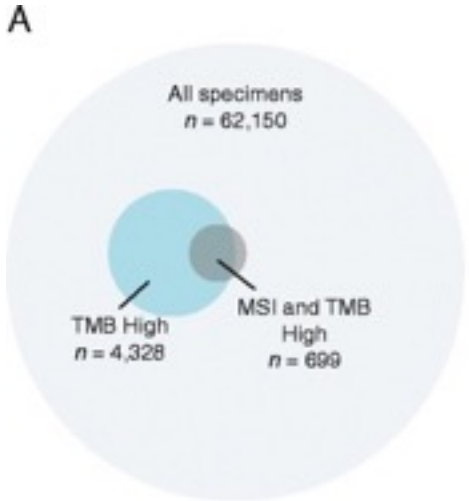
Chalmers et al. Genome Medicine (2017) 9:34
DOI 10.1186/s13073-017-0424-2

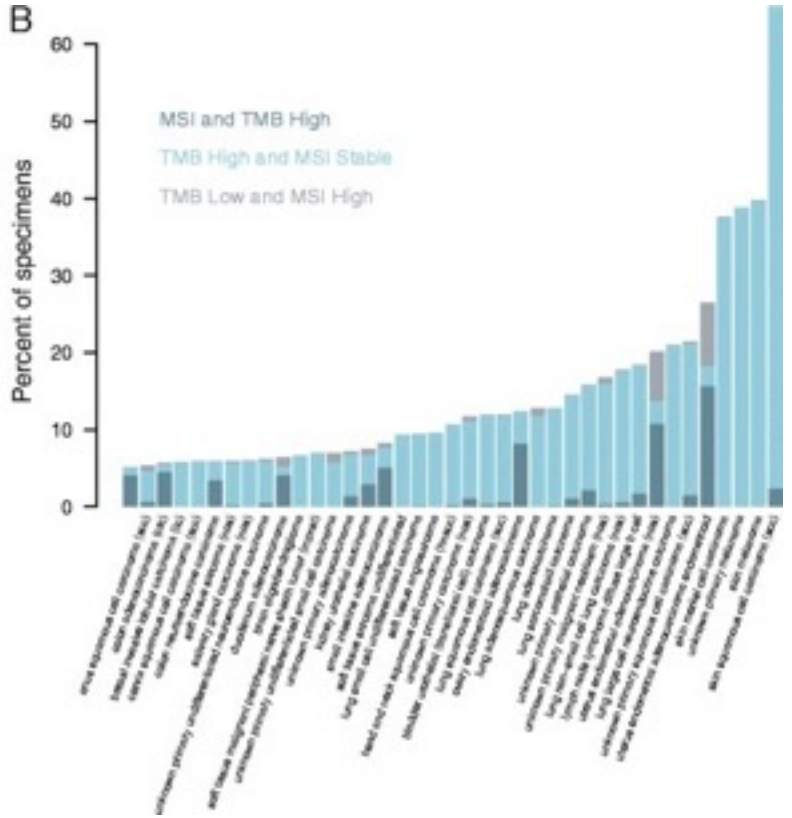
Genome Medicine

RESEARCH Open Access

Analysis of 100,000 human cancer genomes reveals the landscape of tumor mutational burden

Zachary R. Chalmers[1†], Caitlin F. Connelly[1†], David Fabrizio[1], Laurie Gay[1], Siraj M. Ali[1], Riley Ennis[1], Alexa Schrock[1], Brittany Campbell[1], Adam Shlien[1], Juliann Chmielecki[1], Franklin Huang[2], Yuting He[1], James Sun[1], Uri Tabori[1], Mark Kennedy[1], Daniel S. Lieber[1], Steven Roels[1], Jared White[1], Geoffrey A. Otto[1], Jeffrey S. Ross[1], Levi Garraway[2,3], Vincent A. Miller[1], Phillip J. Stephens[1] and Garrett M. Frampton[1*]



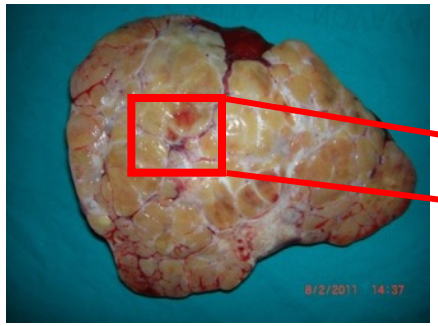


High TMB means high number of neoantigens

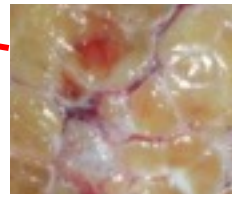
Induction of immunoresponse by the host

INTRO- THE SPECIMENS: FFPE

FORMALIN FIXED TISSUES



TUMOUR DESCRIPTION AND MACRO-SELECTION

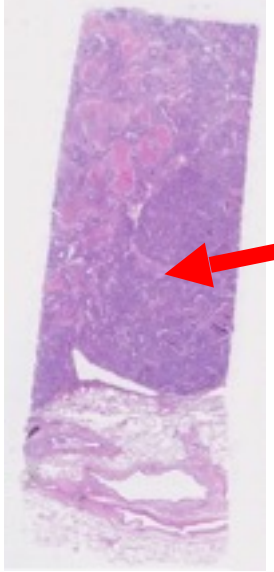


HYTOLOGICAL BLOCK PREPARATION

PARAFFIN INCLUSION

SLICE PREPARATION

Hematoxylin-eosine: MICRO-SELECTION



TUMOUR

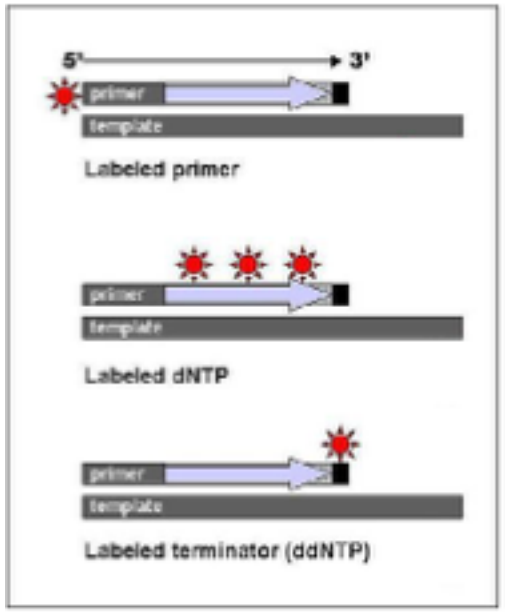
DNA EXTRACTION

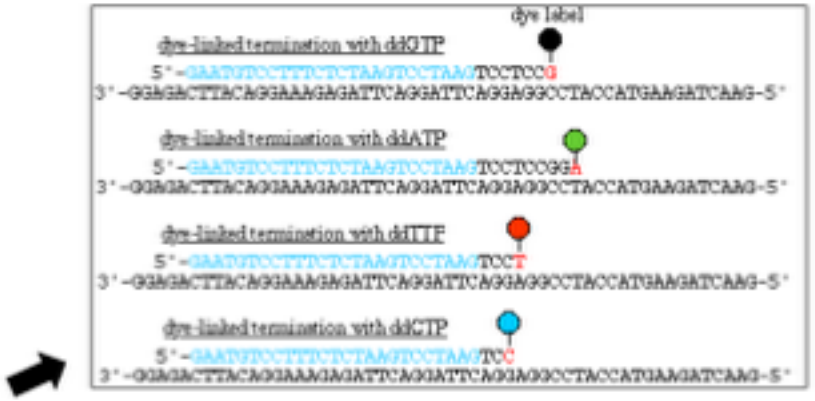
DNA QUANTIFICATION

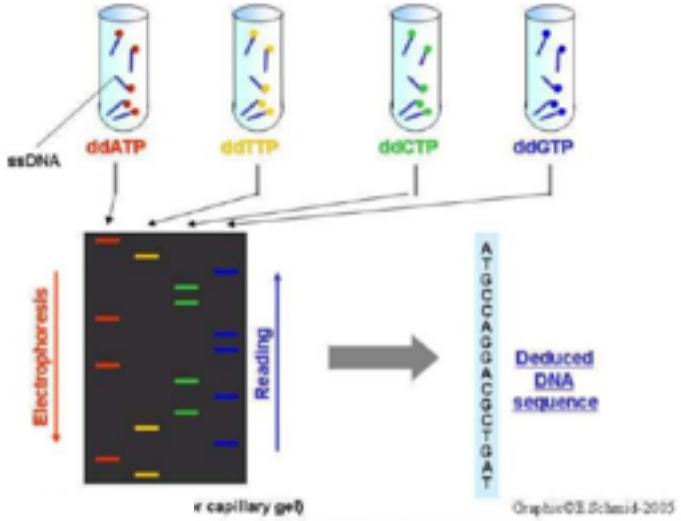
DNA Analysis: SENSITIVITY AND THROUGHPUT

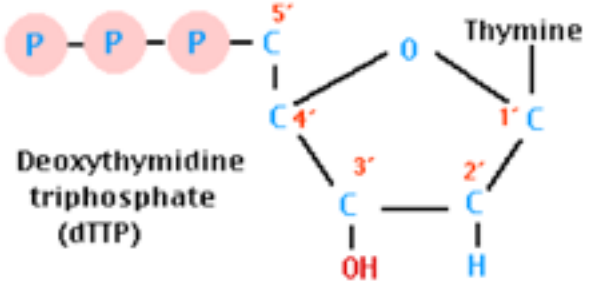
Method	gene	Sensitivity
Sanger Sequencing	Single gene/complete sequence of the exon	20-30%
Pyrosequencing	Single gene/complete sequence of the fragment	10%
Sequenom	Multigene/mutation specific	5-10%
NGS	Multigene/complete sequence	1%-0,1%

Sanger Sequencing

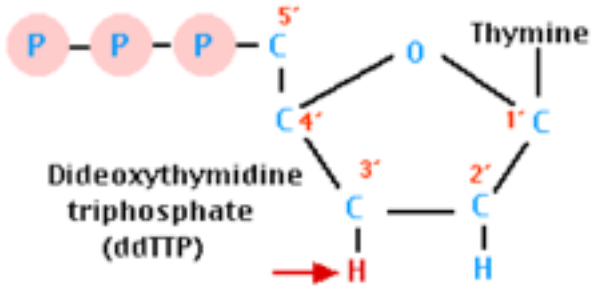






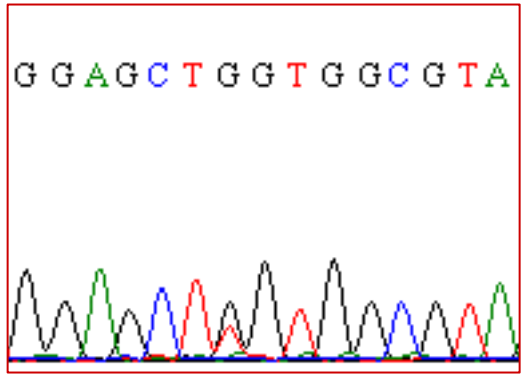


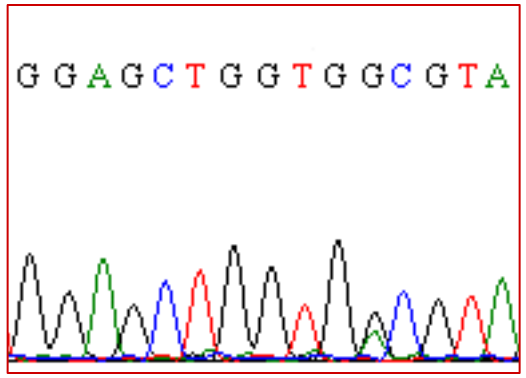
deoxy NTPs have a 3' hydroxyl group
…so chain elongation can occur



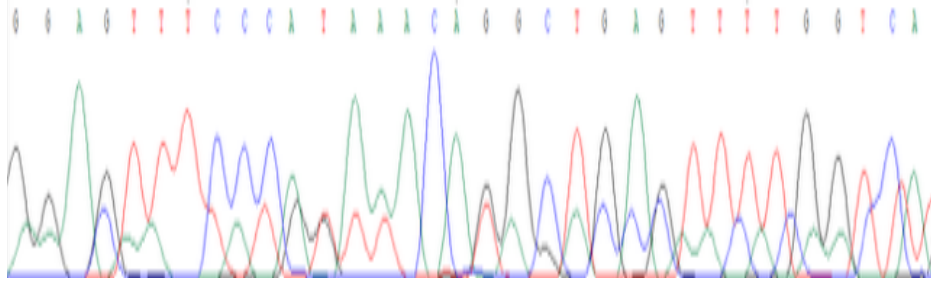
dideoxy NTPs don't have a 3' hydroxyl group
…so when this NTPs is added, chain elongation terminates

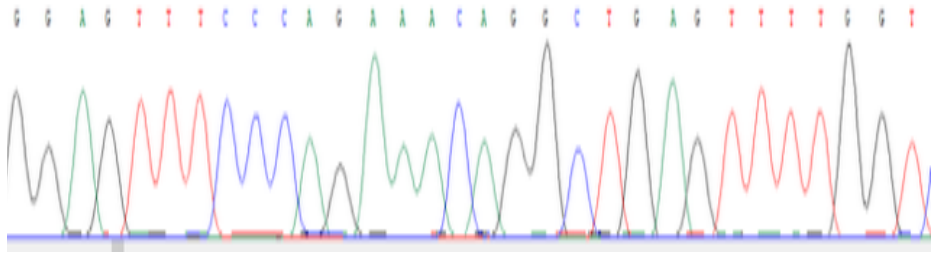
Sanger Sequencing





Missense mutation





Frameshift mutation

PYROSEQUENCING:

THE ORIGINS

Analytical Biochemistry

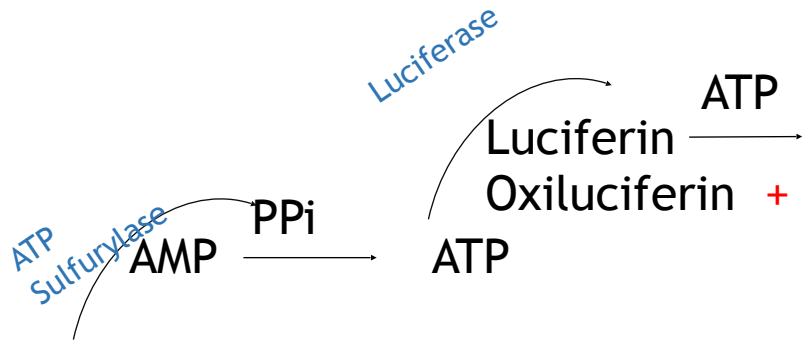
Volume 167, Issue 2, December 1987, Pages 235-238

Enzymatic method for continuous monitoring of DNA polymerase activity ☆

Pål Nyrén

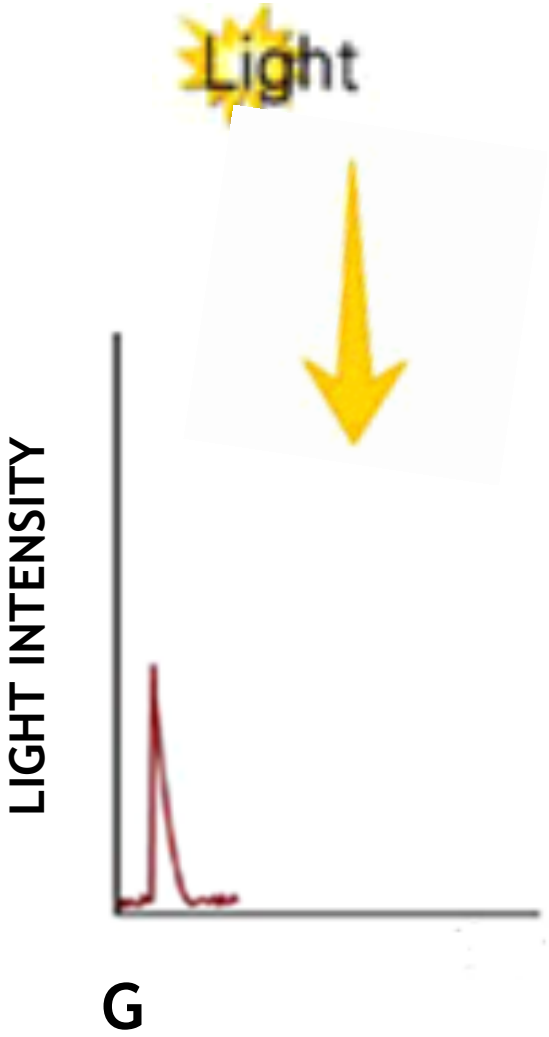
"… One late afternoon in the beginning of January 1986, …… the idea for an alternative DNA sequencing technique came to my mind. The basic concept was to **follow the activity of DNA polymerase during nucleotide incorporation into a DNA strand by analyzing the pyrophosphate released during the process.**

PYROSEQUENCING: THE CHEMISTRY

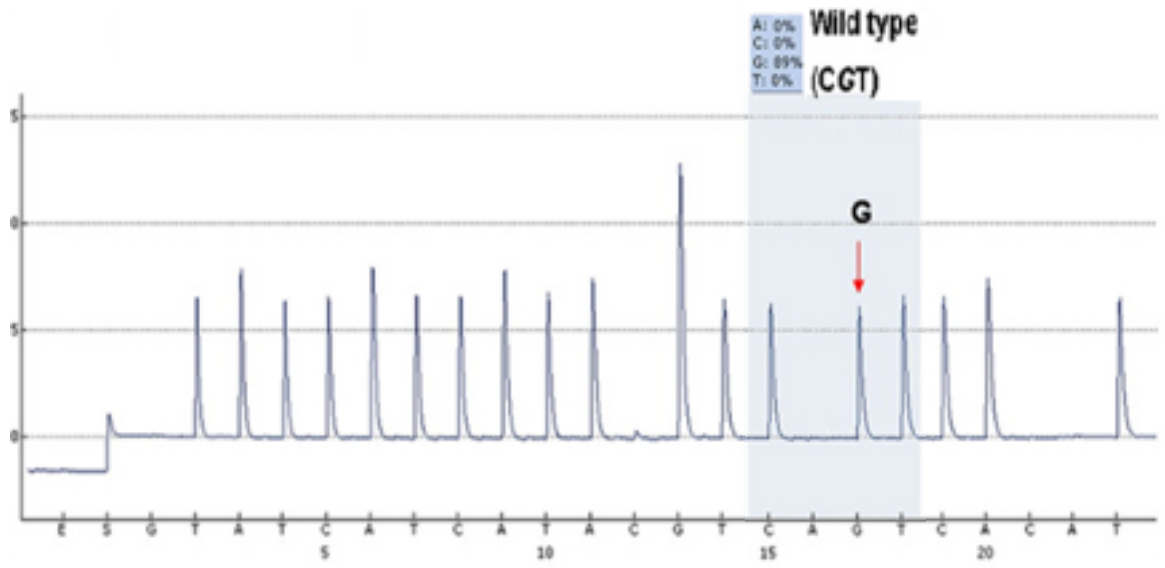


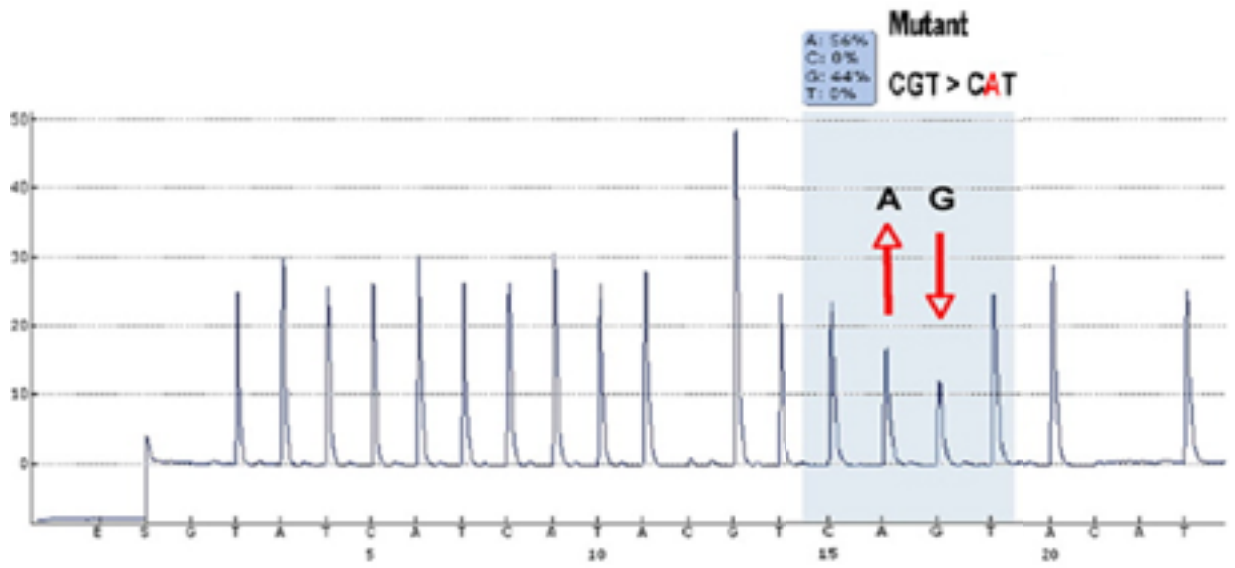
5’-TGGACCTGAGC**G**
3’-
ACCTGGACTCGA**C**GGAGGTGGCTAGATG-5’

DNA polymerase



THE PYROSEQUENCING OUTPUT: THE PYROGRAM





MASS SPECTROMETRY

Detection Method: MASS SPECROMETRY MALDI-TOF

Mass spectrometry (MS) is an analytical technique that ionizes chemical species and sorts the ions based on their mass-to-charge ratio.

- **M**atrix
- **A**ssisted
- **L**aser
- **D**esorption
- **I**onization
- **T**ime **O**f **F**light

Mass Spectrometry: The WorkFlow

5 µl — **PCR**

Amplification of target LOCI by PCR

Thermalcycler

+ 2 µl — **Dephosphorylation SAP**

POST-PCR SAP Reaction CleanUp

+ 2 µl — **Cycled MassEXTEND™ Reaction**

extended Primer —— (N)ddX

Nanodispenser

+ 41 µl — **Sample Conditioning, Nanodispensing**

15 nl — **MS Analysis and Automatic Calling**

MALDI-TOF

Mass Spectrometry: the chemistry

Detection

TimeOfFlight Separation

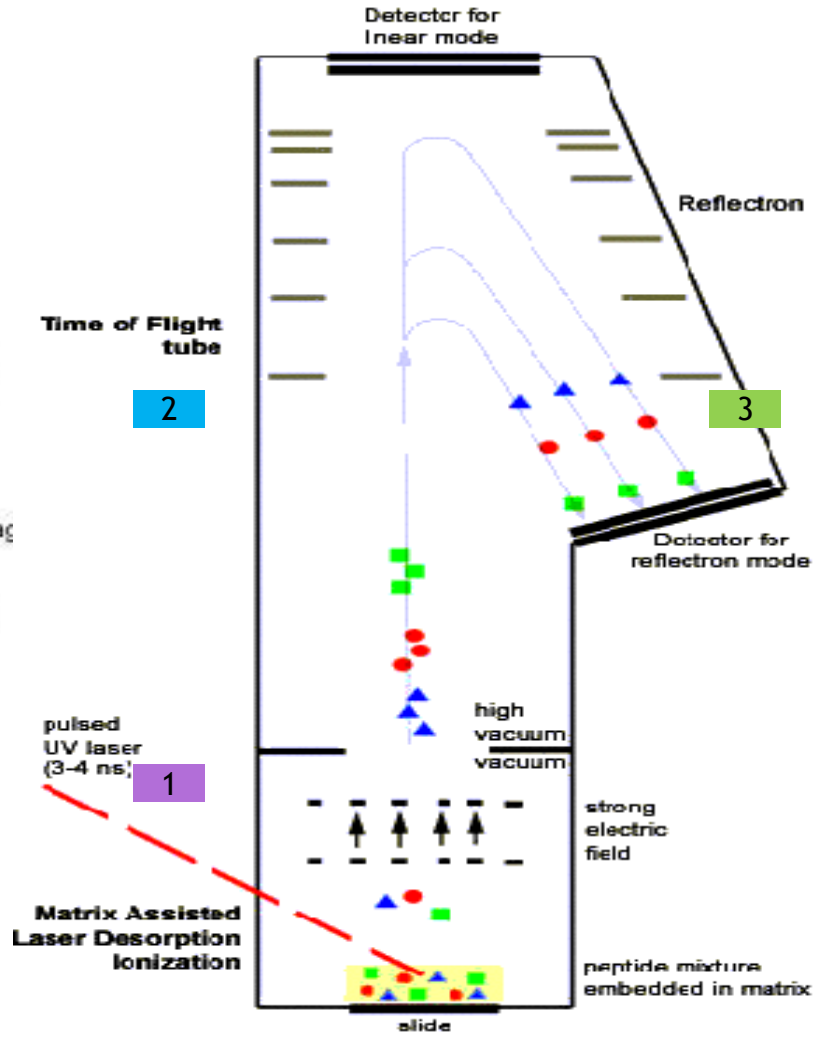
acceleration

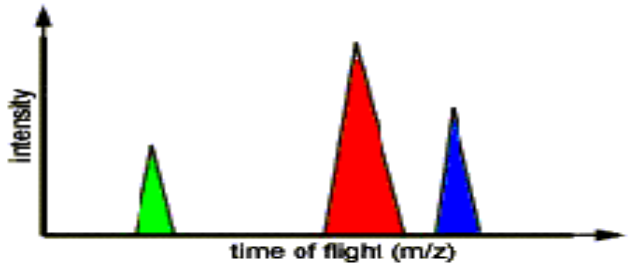
desorption

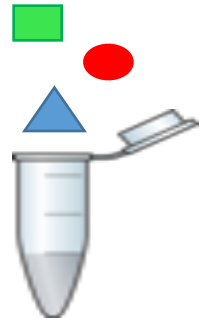
Solid Phase Molecules pass into a Gaseous State

$$\frac{m}{z} = \frac{2eU}{L^2} t^2$$

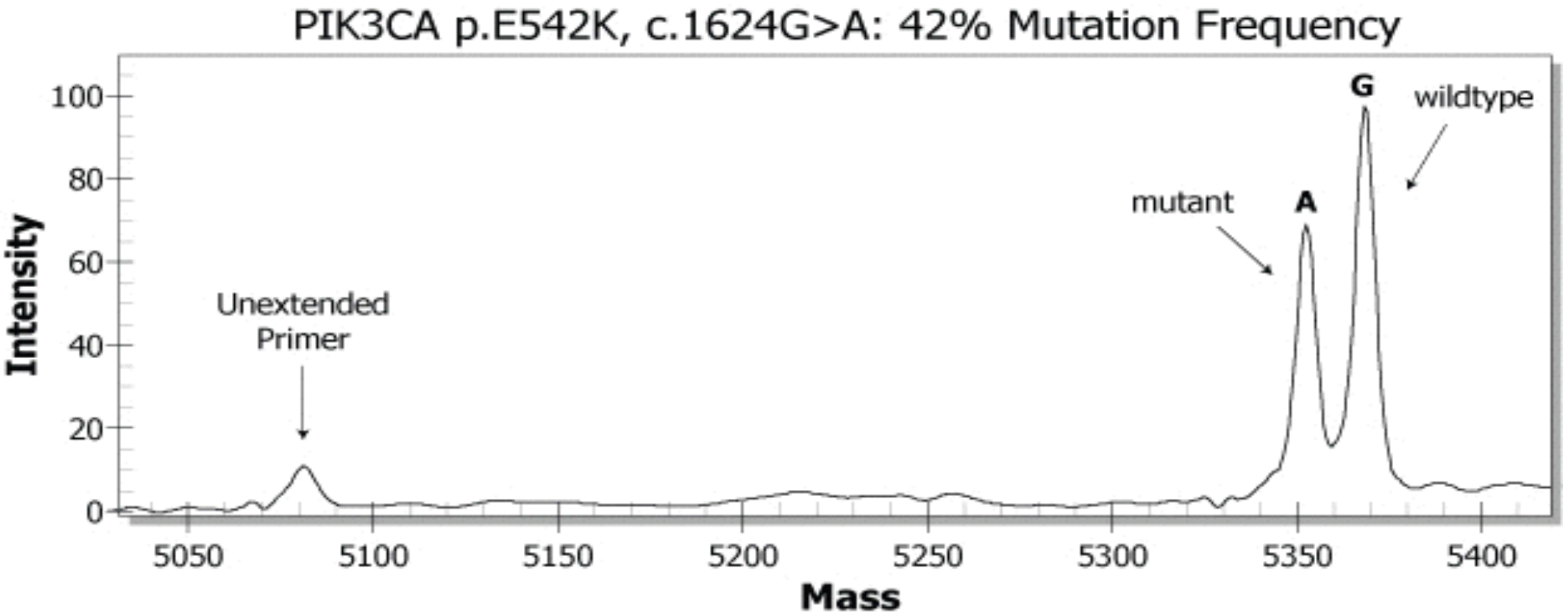
m: mass
z: charge
U: accelaration voltag
L: path length
t: time
e: elemetary charge







Mass Spectrometry: The OutPut



NGS: The Most Diffuse NGS Platforms

Illumina

Output	15 Gb	120 GB	1000 GB	1800 GB
Number of Reads	25 Million	400 Million	4 Billion	6 Billion
Read Length	2x300 bp	2x150 bp	2x125 bp (2x250 update mid-2014)	2x150 bp
Cost	$99K	$250K	$740K	$10M

ion torrent

by life technologies™

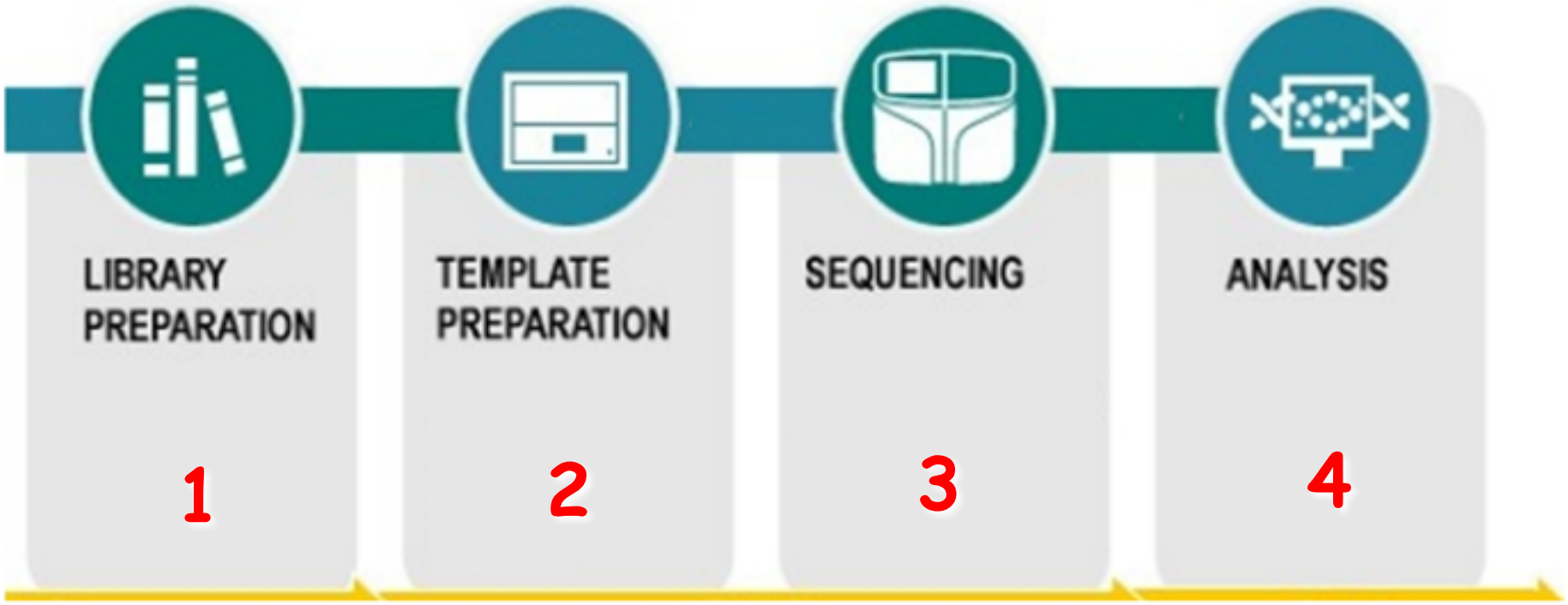
Ion PGM

- 3 types of chips
- 200 or 400 bp reads
- Up to 5.5 million reads / Ion 318 chip
- 4 – 7 h run time

Ion Proton

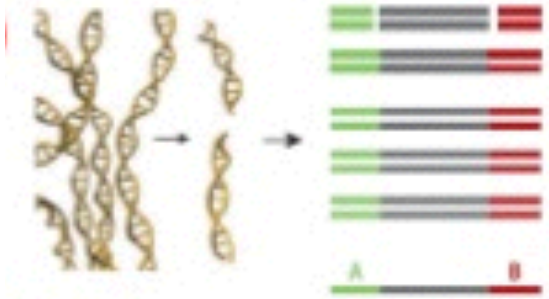
- Up to 200 bp reads
- Up to 60-80 million reads
- 2 – 4 h run time

NGS: The WorkFlow

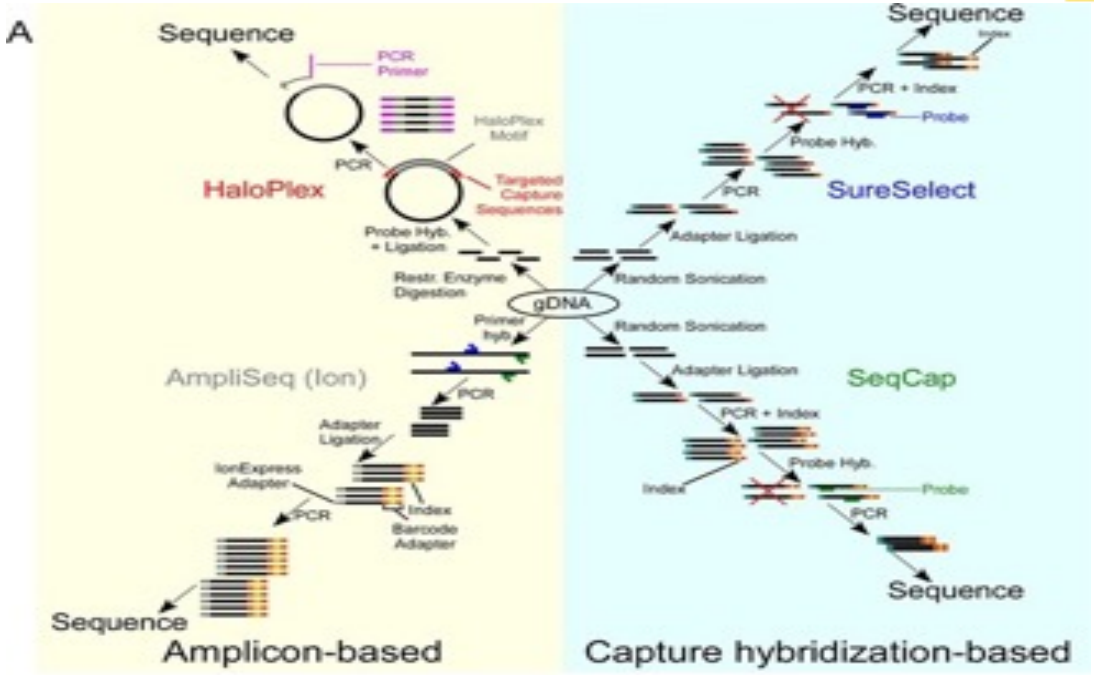


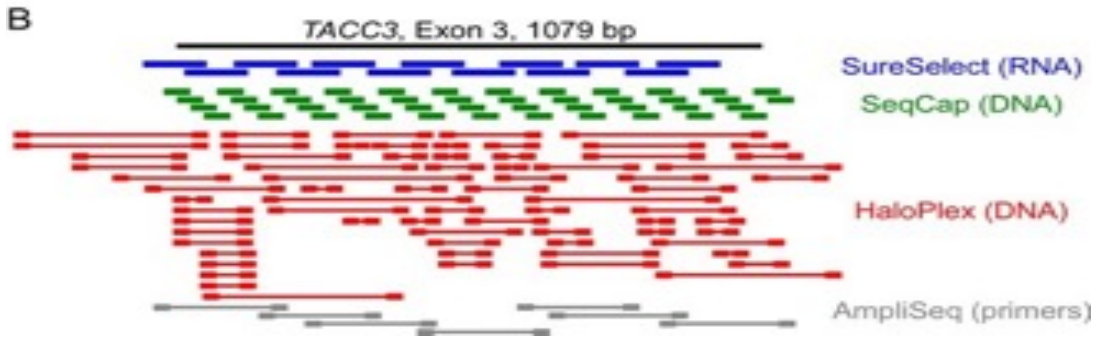
NGS: Library Preparation

A NGS library is a set of nucleic acid fragment with the same termination sequences (ADAPTORs)



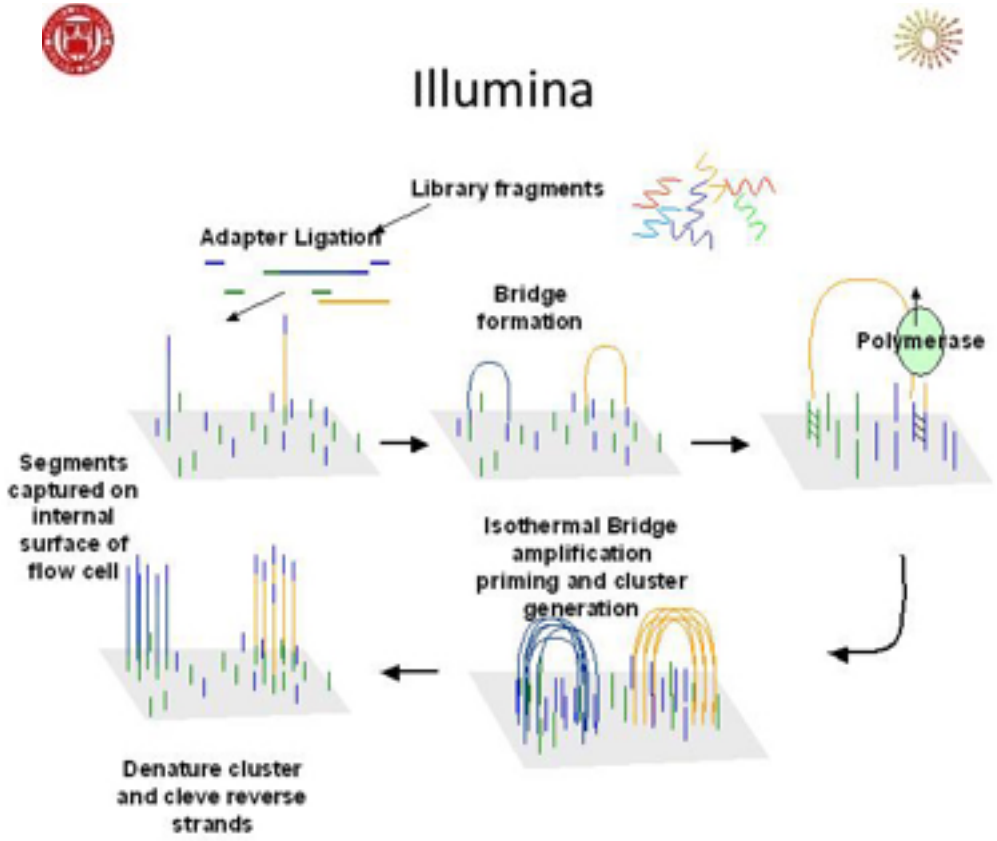
DNA fragmentation and in vitro adaptor ligation





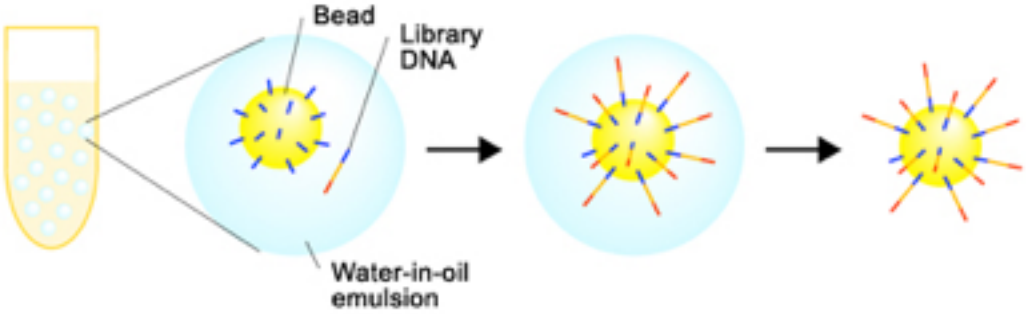
NGS: Template Preparation

The NGS TEMPLATE is a single strand DNA originating by PCR from the molecules of the library, that will be subjected to the sequencing

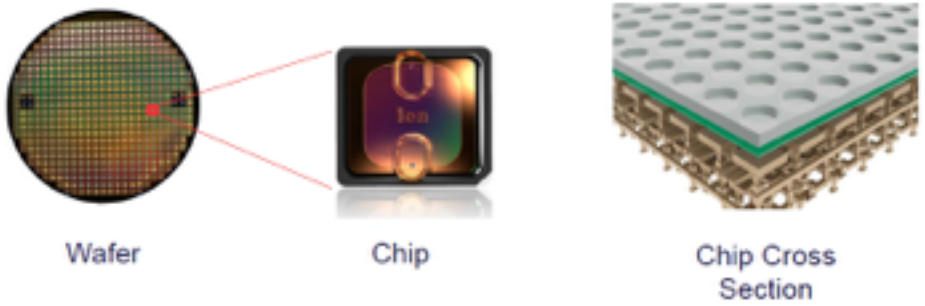


Cluster bridge amplification on flow cells

ion torrent
by life technologies

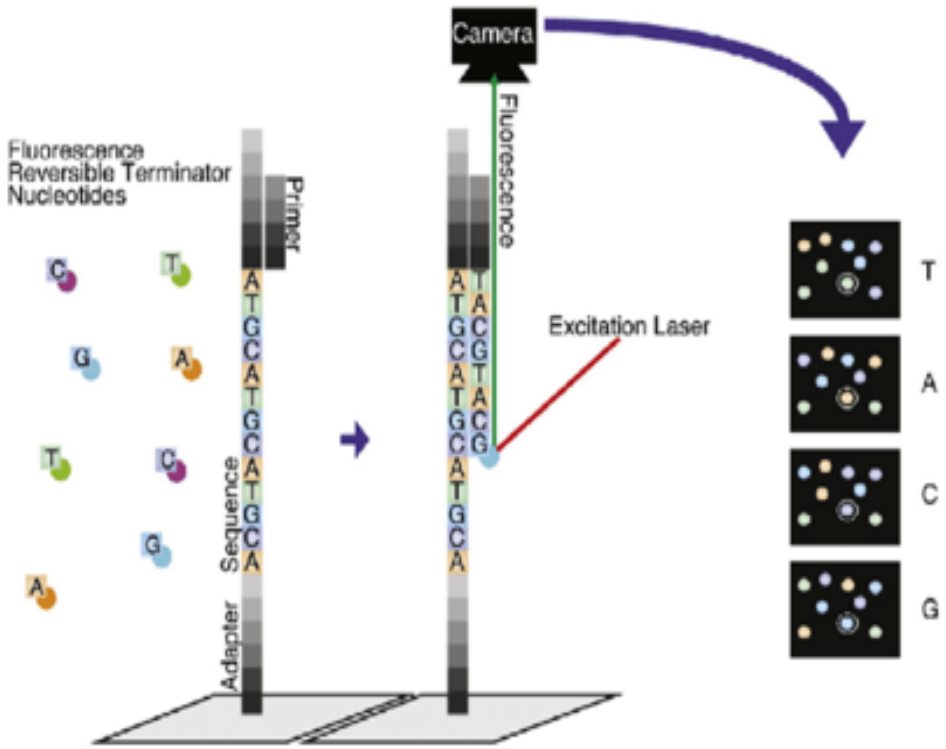


Clonal Emulsion PCR, beads filling and chip preparation



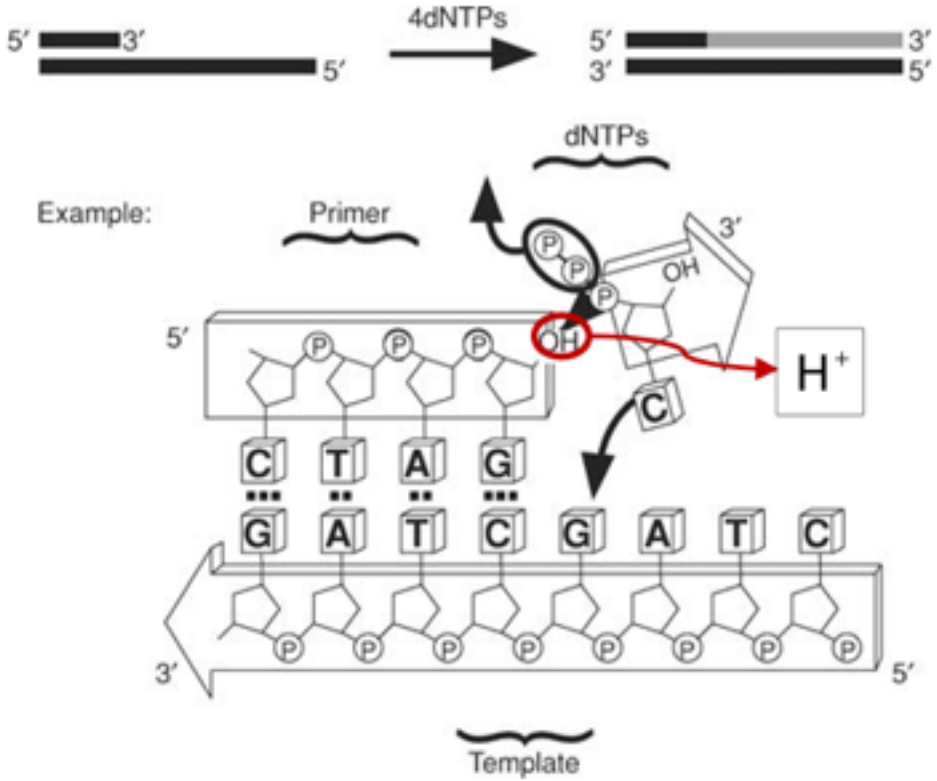
NGS: Sequencing

Illumina



Illumina Dye Sequencing By Synthesis

ion torrent
by life technologies



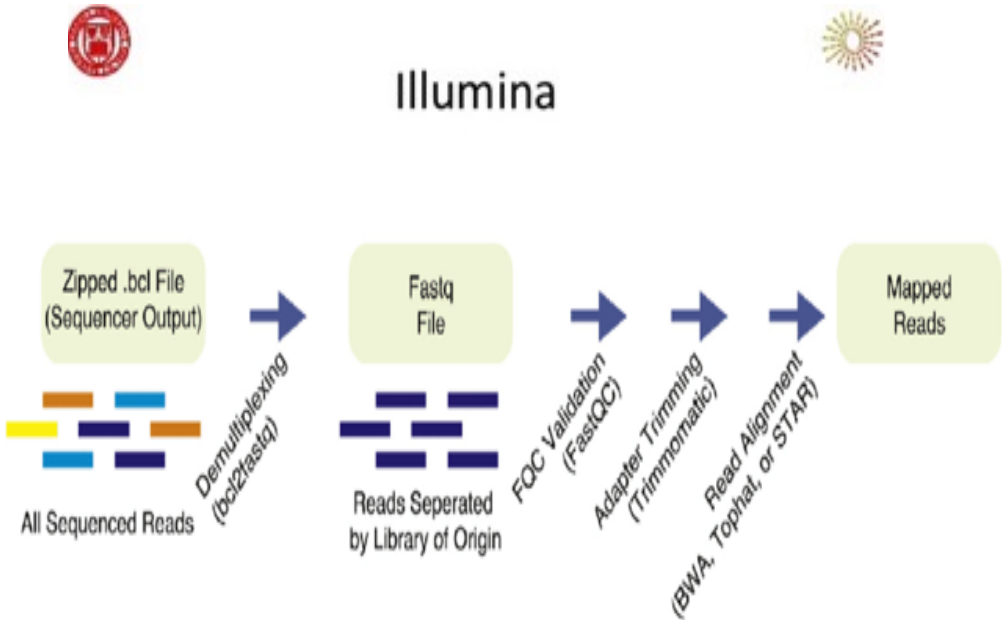
Ion Semiconductor Sequencing

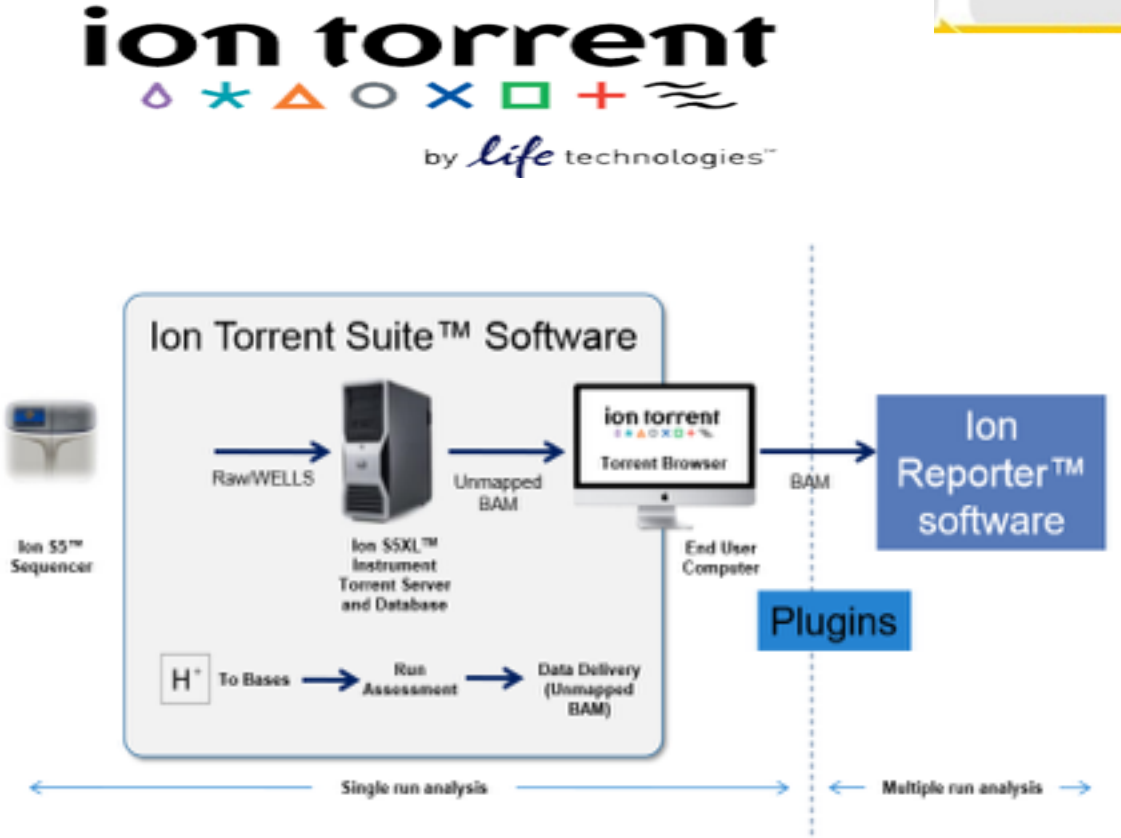
NGS: Analyses

ANALYSIS

4

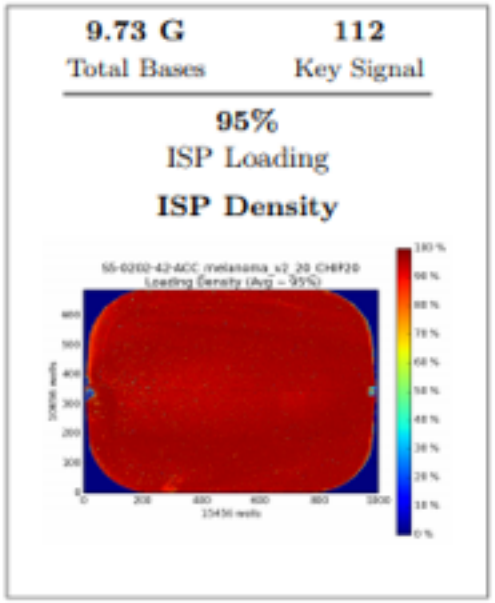
Gb of data were aligned to the hg19 human reference genome. After qualities control, data were analysed by bioinformatics

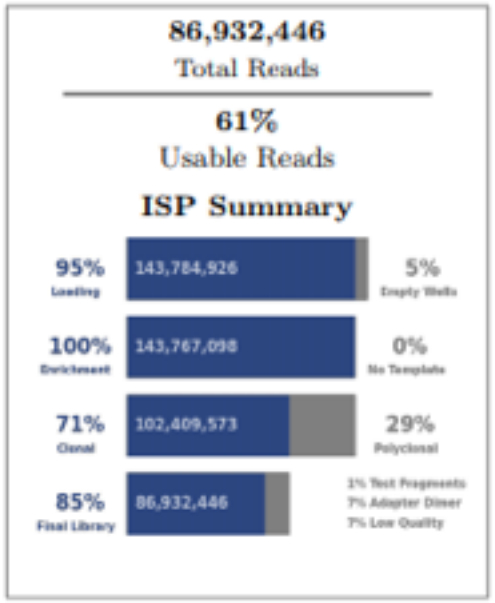


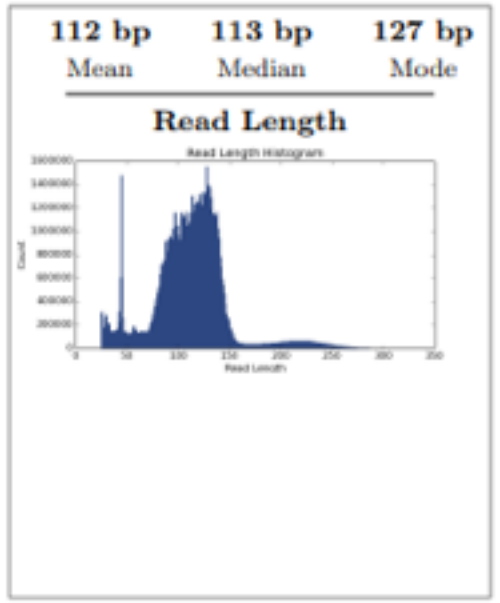


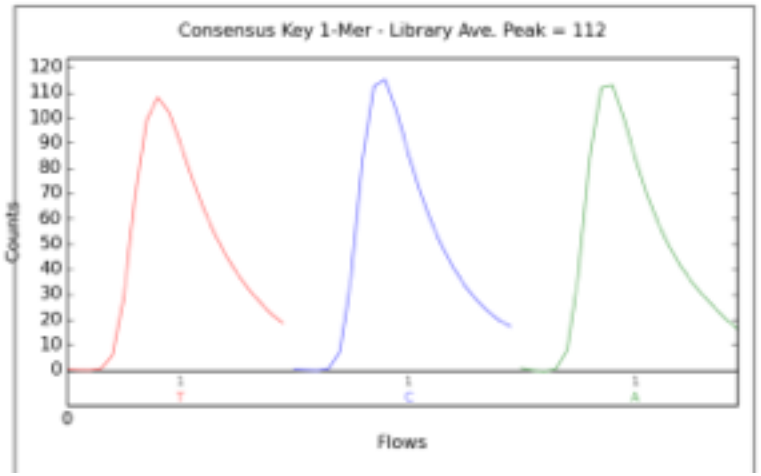
NGS: Example of IonTorrent results

Sequencing Run Summary Report









Addressable Wells	151,539,288	
With ISPs	143,784,926	94.9%
Live	143,767,098	100.0%
Test Fragment	1,194,582	00.8%
Library	142,572,516	99.2%

Library ISPs	142,572,516	
Filtered: Polyclonal	41,357,525	29.0%
Filtered: Low Quality	6,892,194	04.8%
Filtered: Adapter Dimer	7,390,351	05.2%
Final Library ISPs	**86,932,446**	**61.0%**

NGS: Example of IonTorrent results

Sequencing Run Summary Report

Barcode Name	Sample	Bases	≥ Q20	Reads	Mean Read Length	Read Length Histogram
No barcode	none	150,597,632	123,668,029	1,523,181	98 bp	
IonXpress_009	MELA09TBJ7-2NaD105	436,010,239	398,691,755	4,394,094	99 bp	
IonXpress_010	MELA09TBJ7-1TaD105	4,054,167,593	3,612,662,425	33,480,332	121 bp	
IonXpress_011	MELA050H5C-2NaD105	790,614,315	719,638,955	7,876,767	100 bp	
IonXpress_012	MELA050H5C-1TaD105	538,554,727	493,465,612	5,301,414	101 bp	
IonXpress_013	MELA051MYO-2NaD105	579,175,357	529,181,222	5,894,877	98 bp	
IonXpress_014	MELA051MYO-1TaD105	1,439,508,511	1,312,517,841	13,217,274	108 bp	
IonXpress_015	MELA05HOPH-2NaD105	938,456,015	837,347,685	7,887,306	118 bp	
IonXpress_016	MELA05HOPH-1TaD105	802,784,942	727,022,909	7,352,709	109 bp	

NGS: Example of IonTorrent results

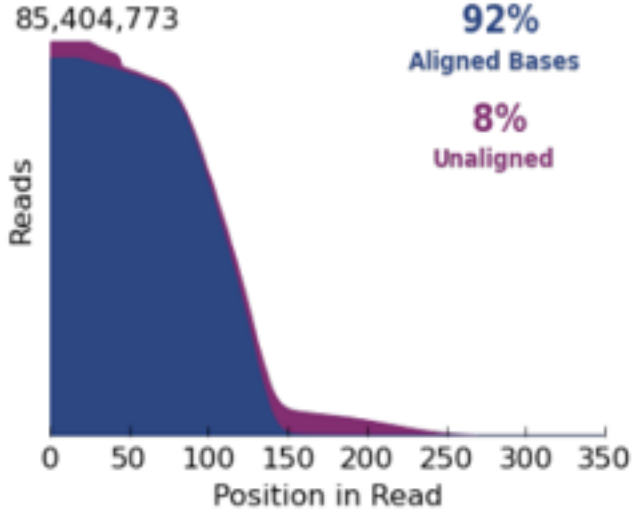
Sequencing Run Summary Report

Hg19 ref genome (Genome Reference Consortium Human Build 37)

Alignment Summary *(aligned to Homo sapiens)*

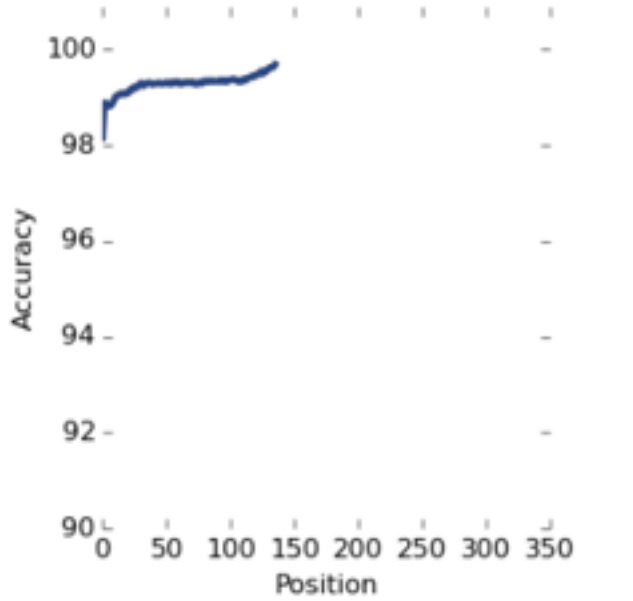
8.83 G
Total Alignment Bases

2.9X
Average Coverage Depth of Reference



	Count	%
Total Reads	85,404,773	–
Aligned Reads	81,819,466	95.8%
Unaligned Reads	3,585,307	4.2%

99.2%
Mean Raw Accuracy 1x



NGS: Example of IonTorrent results

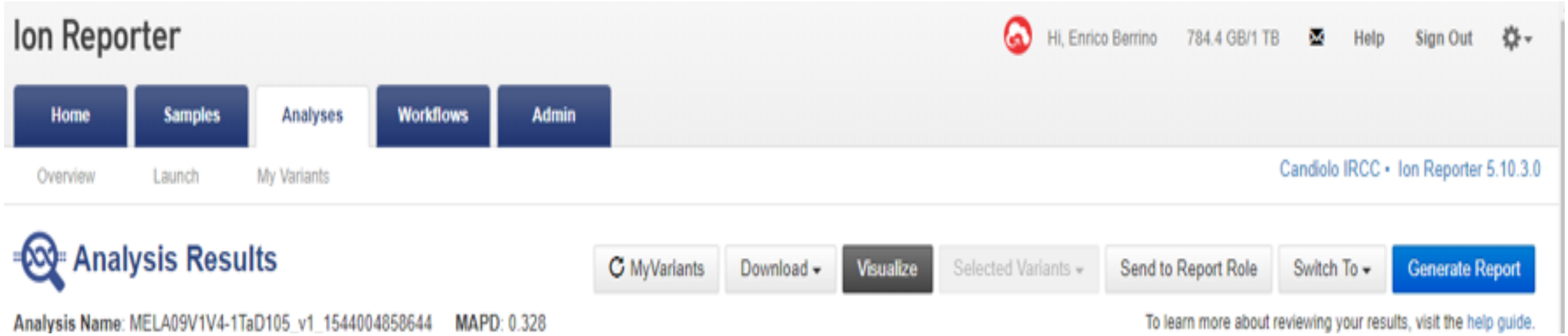
Sequencing Run Summary Report

AmpliSeq CoverageAnalysis

Barcode Name	Sample	Mapped Reads	On Target	Mean Depth	Uniformity
IonXpress_009	MELA09TBJ7-2NaD105	3,888,193	93.11%	443.2	94.33%
IonXpress_010	MELA09TBJ7-1TaD105	32,964,520	97.45%	4,064	95.21%
IonXpress_011	MELA050H5C-2NaD105	7,254,657	94.37%	832.8	76.99%
IonXpress_012	MELA050H5C-1TaD105	4,789,604	94.31%	559	93.46%
IonXpress_013	MELA051MYO-2NaD105	5,261,010	90.19%	573.5	71.03%
IonXpress_014	MELA051MYO-1TaD105	12,599,325	96.16%	1,527	94.00%
IonXpress_015	MELA05HOPH-2NaD105	7,378,571	96.96%	921	57.16%
IonXpress_016	MELA05HOPH-1TaD105	7,016,472	96.79%	836.6	89.17%

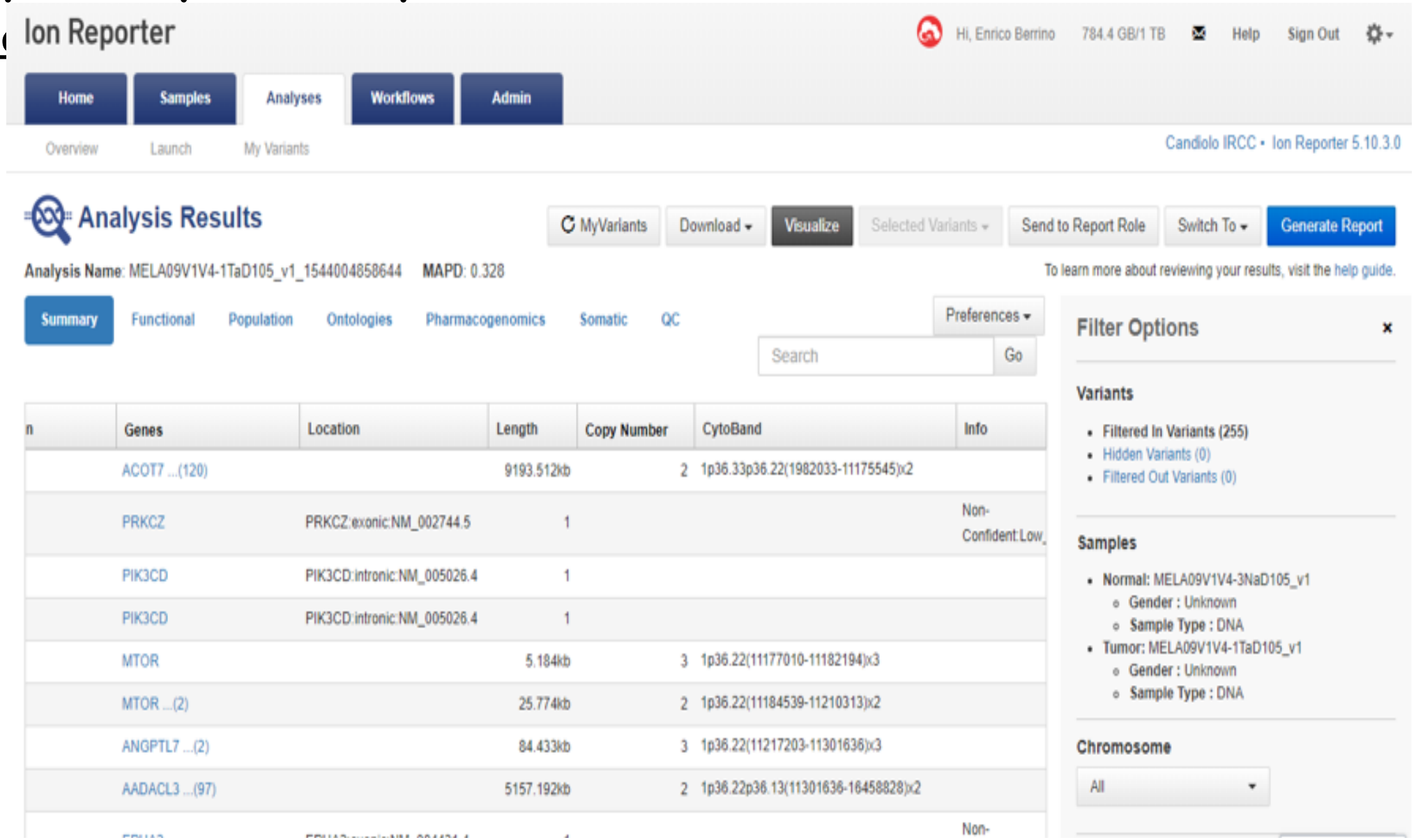
NGS: Example of IonTorrent results

Sample Analyses: Software



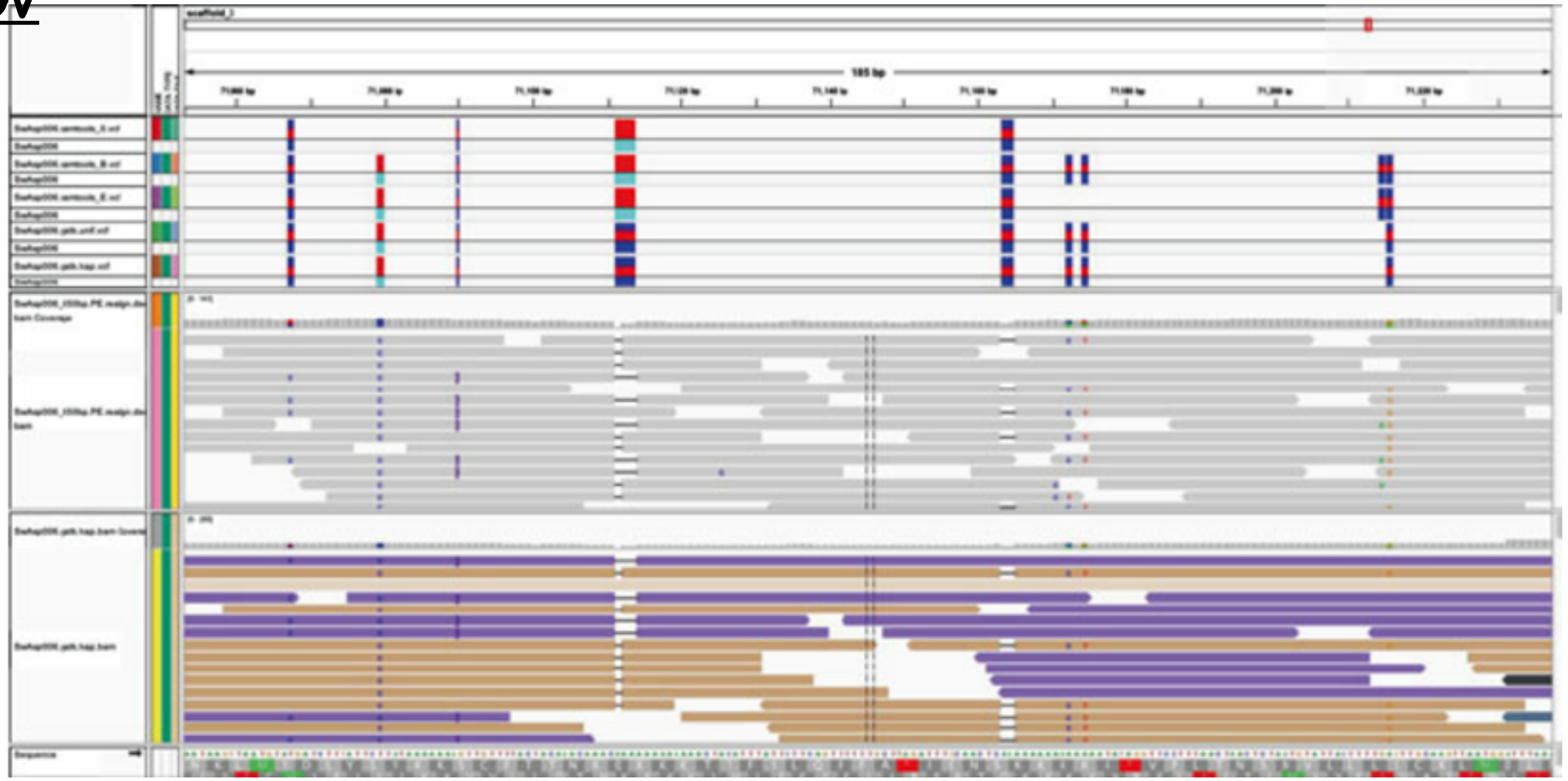
NGS: Example of IonTorrent results

Sample Analyses: Sample Vari



NGS: Example of IonTorrent results

Sample Analyses: Sample Variants on IGV



«Viviamo in una Hiroshima culturale dove la scienza è spesso confusa con la tecnologia. C'è bisogno di creare un legame tra le esigenze delle istituzioni impegnate nel campo tecnologico industriale e le attività per la ricerca scientifica. Oggi questo legame è molto debole. Quasi inesistente».
Prof. Antonino Zichichi