Hôpital Erasme

ULB

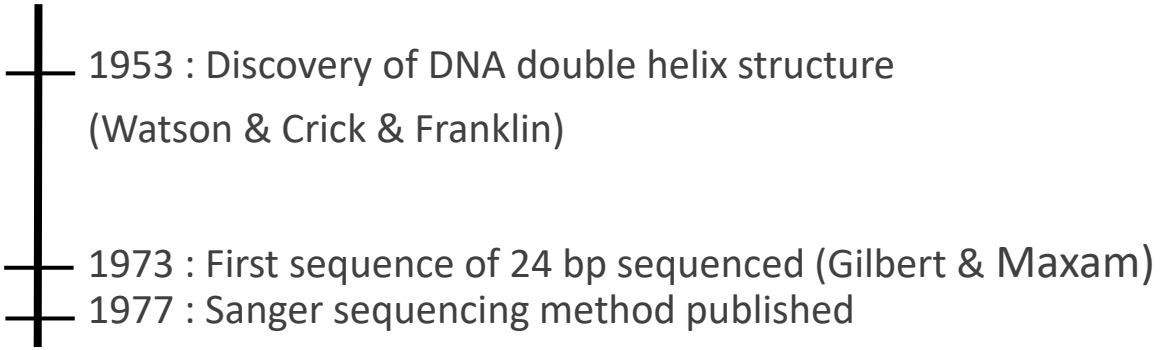
Next generation sequencing: basic principles

Claude Van Campenhout

MOLECULAR PATHOLOGY COURSE

FOR RESIDENTS IN PATHOLOGY

Introduction



Frederick Sanger (13 August 1918 – 19 November 2013) was an English biochemist University of Cambridge, UK

- In 1958, he was awarded a Nobel Prize in Chemistry "for his work on the structure of proteins, especially that of insulin". He identified how the amino acid chains are linked together.
- In 1980, Walter Gilbert and Sanger shared half of the chemistry prize "for their contributions concerning the determination of base sequences in nucleic acids".

Sanger Sequencing (1977)

Proc. Natl. Acad. Sci. USA
Vol. 74, No. 12, pp. 5463–5467, December 1977
Biochemistry

DNA sequencing with chain-terminating inhibitors

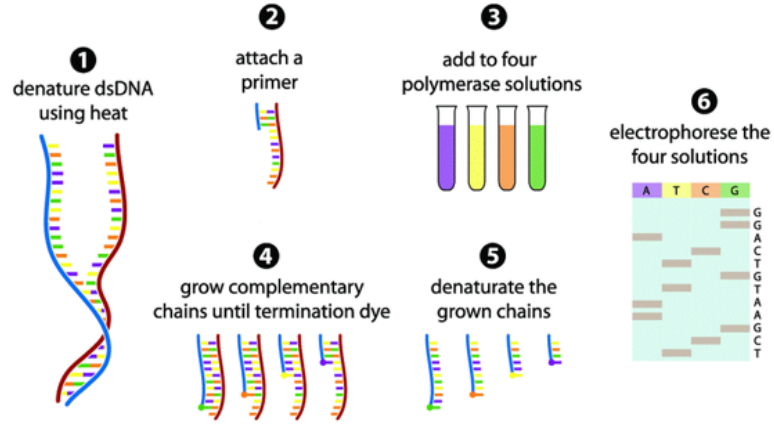
(DNA polymerase/nucleotide sequences/bacteriophage ϕX174)

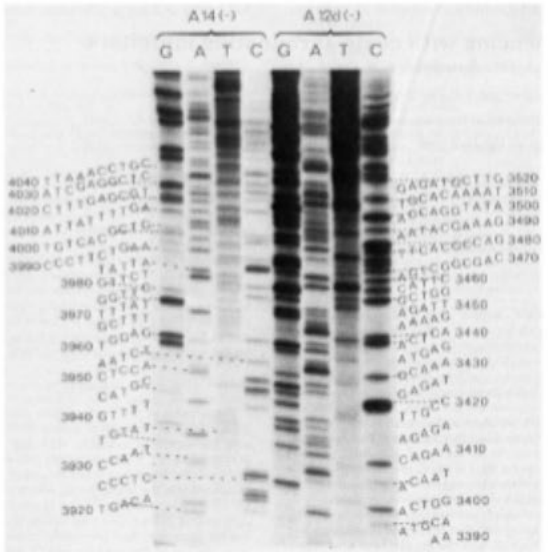
F. SANGER, S. NICKLEN, AND A. R. COULSON

Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England

Contributed by F. Sanger, October 3, 1977

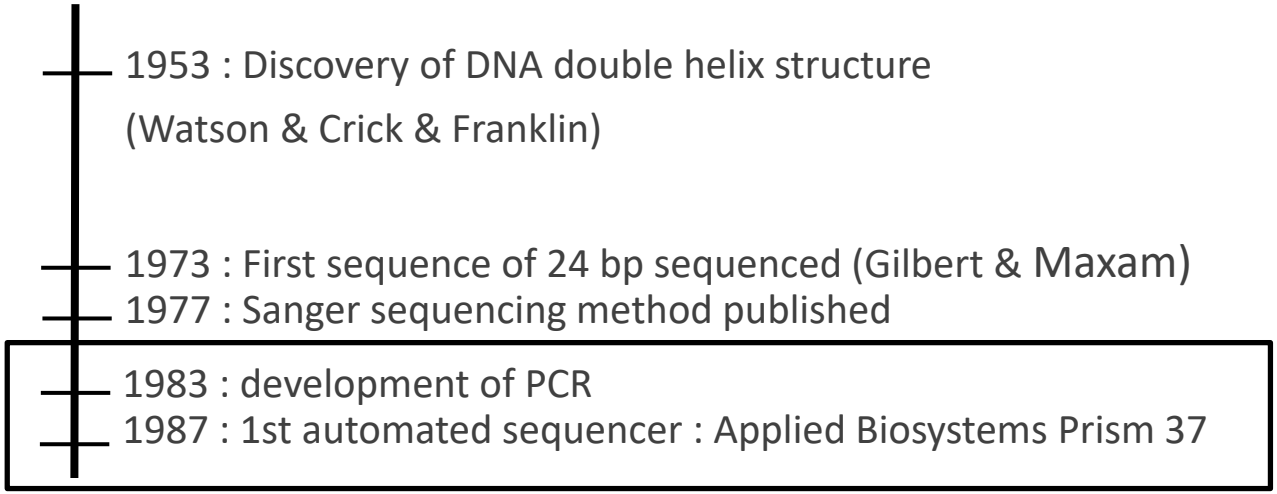
ABSTRACT A new method for determining nucleotide sequences in DNA is described. It is similar to the "plus and minus" method [Sanger, F. & Coulson, A. R. (1975) *J. Mol. Biol.* 94, 441–448] but makes use of the 2′,3′-dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphates, which act as specific chain-terminating inhibitors of DNA polymerase. The technique has been applied to the DNA of bacteriophage ϕX174 and is more rapid and more accurate than either the plus or the minus method.





New method for determining nucleotide sequences in DNA

Introduction



Sanger Sequencing

First generation sequencing (semi-automated)

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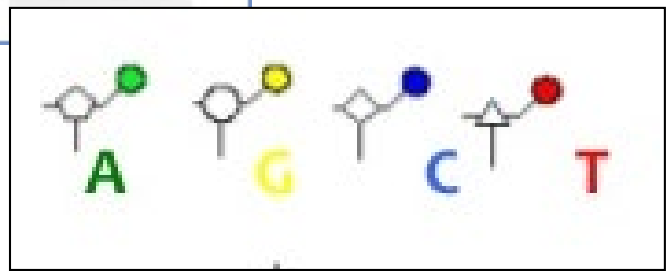
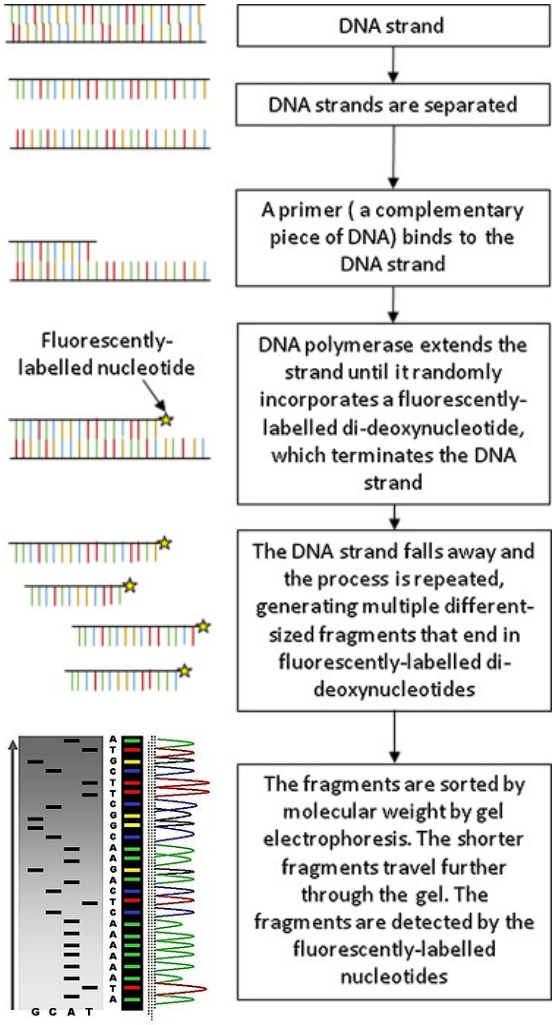


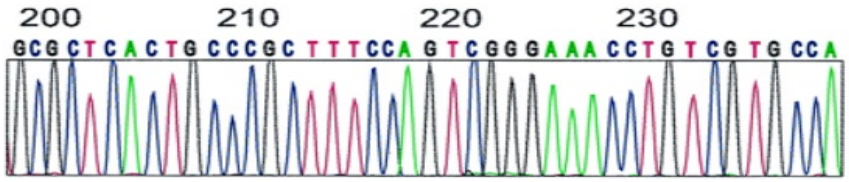


Moorcraft et al. Crit rev oncol 2015

Sanger Sequencing

Advantages and limitations:

- ✓ Standard and most widely used method
- ✓ Long Read length (up to 800 bp)



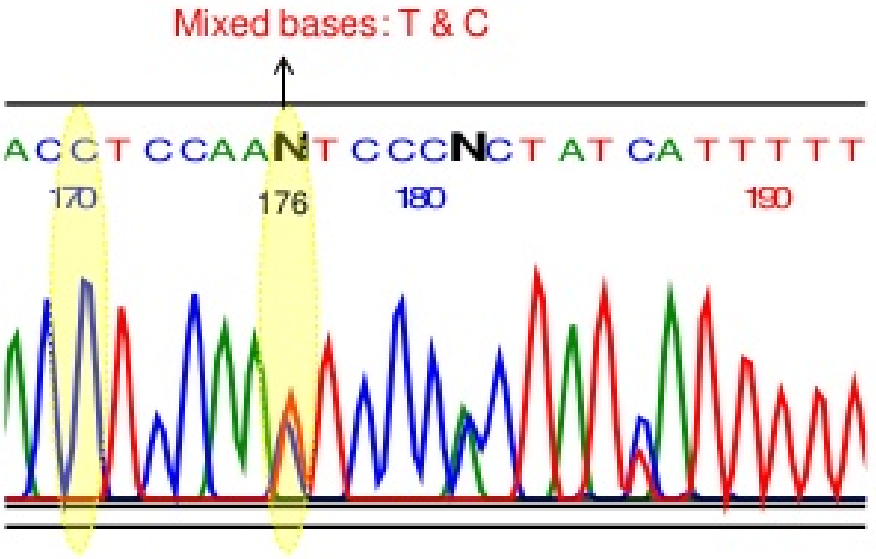
- Low throughput (1-96 reads/run)
- Limited output: +/- 80 000 bases per run

  E.g. 1 single gene for 100 patients or 100 genes for 1 single patient
- Sequence mixture

Sanger Sequencing: limitations

- Sanger sequencing

Position	Genotype
170	C
176	T/C



Up to 1000 bases
1 region at a time
Sensitivity around 20%

Introduction

1953 : Discovery of DNA double helix structure
(Watson & Crick & Franklin)

1973 : First sequence of 24 bp sequenced (Gilbert & Maxam)
1977 : Sanger sequencing method published

1983 : development of PCR
1987 : 1st automated sequencer : Applied Biosystems Prism 37

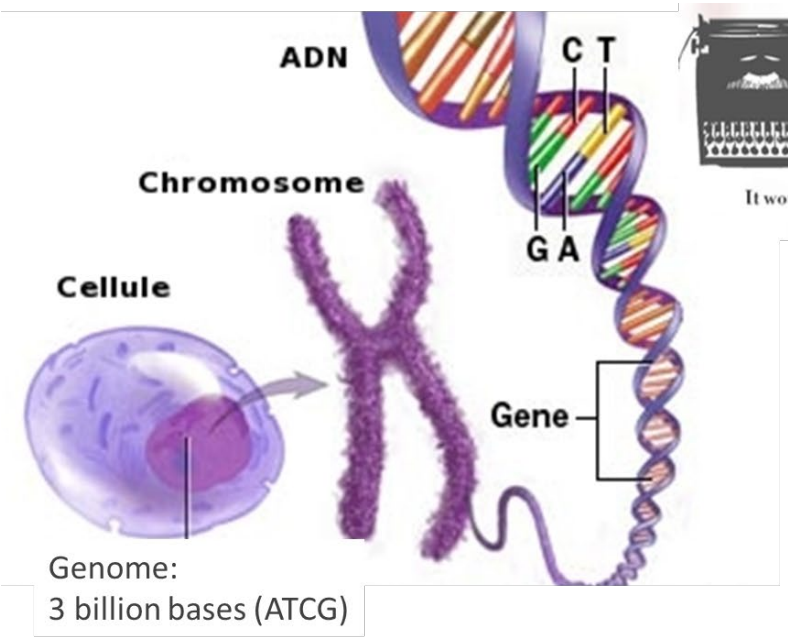
1996 : Capillary sequencer : ABI 310

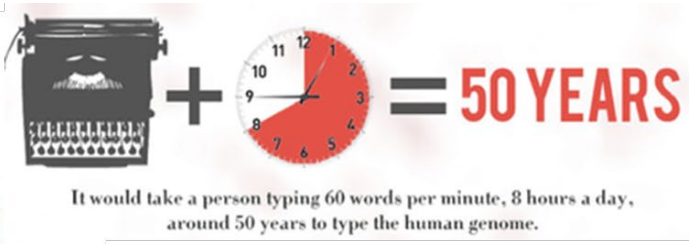
2003 : Human genome sequenced

February 2001

The human genome project

- The human genome project : Started in 1990 by the NIH & the U.S. Department of Energy:
  - Sequence the 3 billion base of the human genome
  - Discover the 20.000 human genes





- Lasted 13 years →Cost 3 billion $ (1$/base)

The human genome project

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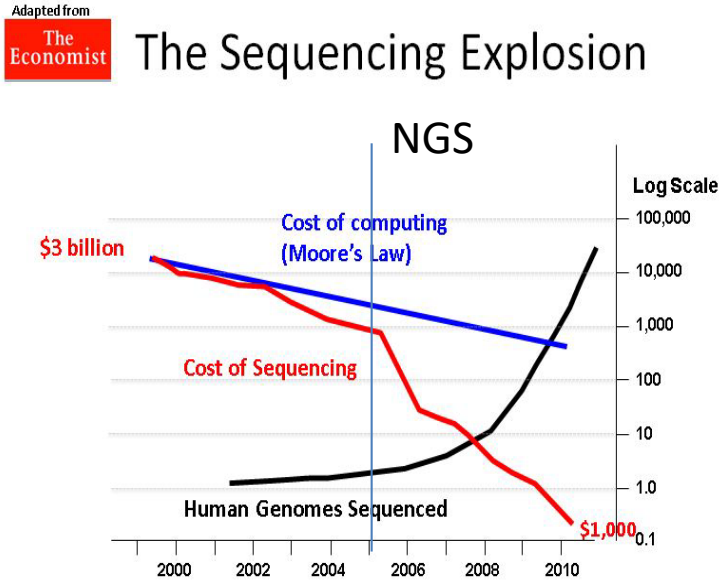
  - Sequence the 3 billion base of the human genome
  - Discover the 20.000 human genes
- Lasted 13 years →Cost 3 billion $ (1$/base)

- 2007: Craig Venter: 4 years, $100 million
- 2008: James Watson: 2 years, $2 million (NGS)
- 2009: 6 months, $200,000
- 2010: 1 month, $20,000
- 2011: 2 weeks, $5,000
- 2012 : 2 weeks, $3,000
- 2015(?): <2 days, <$1,000



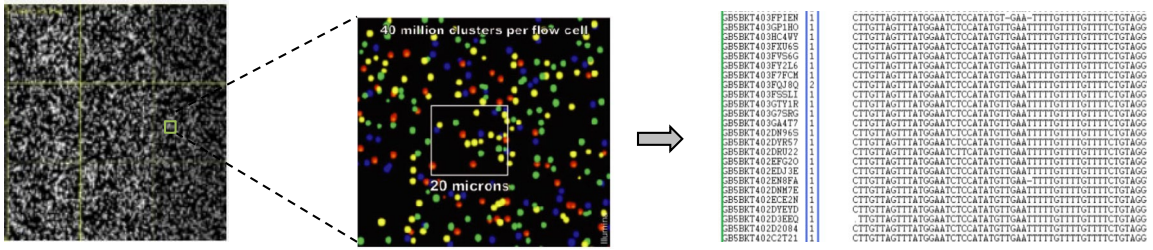
Next Generation Sequencing

“Next-Generation” Sequencing technologies enable:

- ✓ Rapid generation of data
- ✓ By sequencing massive amounts of DNA (shorter read lengths)
- ✓ In parallel on a microchip (in a single reaction)

“Massive Parallel Sequencing“

“High Throughput Sequencing” : +/- 800 000 000 000 bp per run



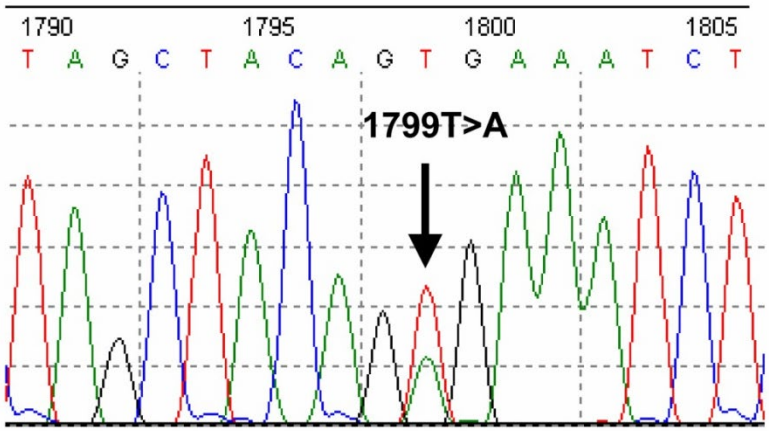
E.g. many gene fragments for many patients or whole exomes/genomes …

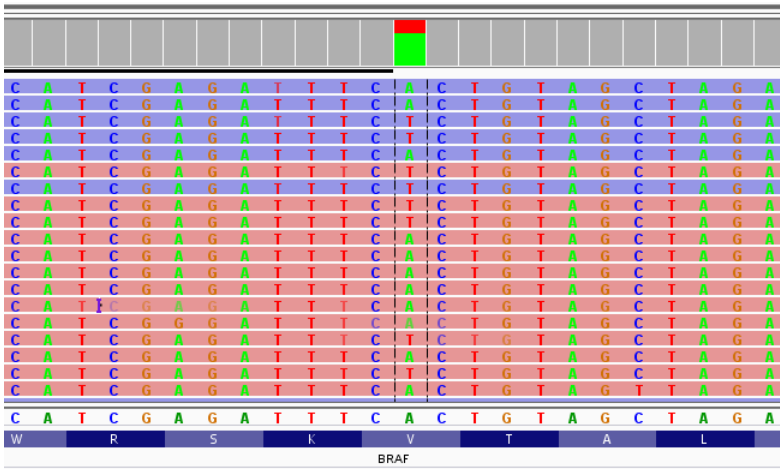
- ✓ Has enabled revolution in cancer research

Source: Jason M. Rizzo and Michael J. Buck. Key Principles and Clinical Applications of "Next-Generation“ DNA Sequencing. Cancer Prev Res; 5(7) 2012.

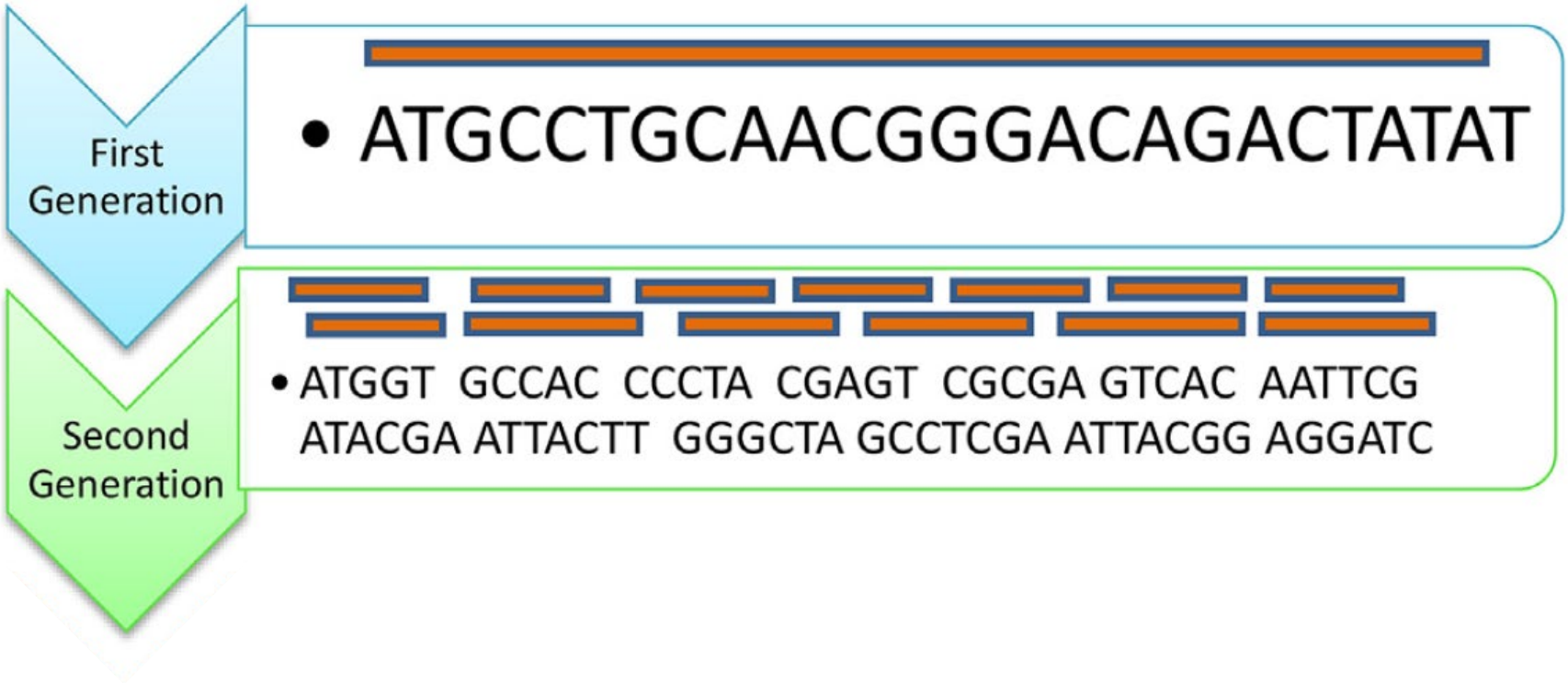
Sanger Sequencing vs NGS

- Sanger Sequencing
  - Low throughput (100kb)
  - High cost
  - Slow
  - Low sensitivity (20-30% of mutant DNA)
    - -> low coverage depth
- Next generation sequencing
  - High throughput (1-100 Gb)
  - Low cost
  - Fast
  - High sensitivity
    - -> high coverage depth





Sanger Sequencing vs NGS



Begin at the beginning and go on till you come to the end: then stop.
Lewis Carroll, *Alice's Adventures in Wonderland.*

Sanger Sequencing vs NGS

Eva détestait cette expression, « garçon manqué ». Elle s'était toujours indignée que les filles, pratiquant des exercices assez physiques, soient apparentées à des garçons. Les êtres humains n'étant pas monolithiques, grimper aux arbres, par exemple, ne représentait pas une contradiction à l'exercice de la féminité. Eva partageait l'opinion qu'hommes et femmes devaient développer une multitude d'activités identiques dans différents registres. En ce domaine, le cinéma du XXIe siècle avait parfois suivi une évolution progressiste. La femme pouvait être forte physiquement, tout en gardant ses attraits ; même Blanche Neige en armure, interprétée par l'adorable Kristen Stewart, arc-en-cielée comme un cœur pur. Gustave se pencha vers Eva, susurrant sur le ton du conspirateur moyen.

— J'ai bien envie de leur balancer une mornifle dans les canines ou de leur claquer le beignet aux pommes.

— Attention de ne pas céder à des désirs incontrôlables. Ne te laisse pas envahir par la haine qui rend aveugle.

— Entre toi et les curés, je finirai donc aveugle et sourd. Pas la haine qui m'a envahi, mais les Pruscos. Je vais narrer à ces lambeaux humains que je leur griffe le nez. Le projet devrait occuper leur attention.

— M'étonnerait bien que cette perspective tigresque suffise, comme dirait l'autre.

— Sait-on jamais, si ces cuistres ont décidé de faire une priorité de leur esthétique.

— Je n'ai pas l'impression que ces gangreneux donnent beaucoup dans le séduisant, rétorqua Eva. Ou alors, taxidermisés éventuellement.

— En fond de couloir de préférence.

— Et puis, à distance, en hauteur, question effluves, a priori, pas triste non plus, une haleine de chacal se roulant dans la charogne faisandée, précisa Eva. On dirait un tas d'immondices vivant. Un relooking radical au programme.

— Tu m'expliqueras plus tard ton affaire avec les radicaux.

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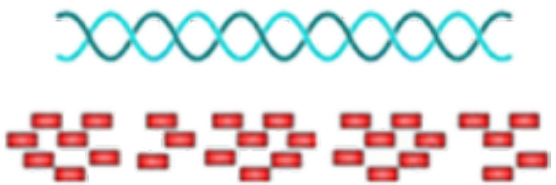
: de p Γu

NGS: from WGS to targeted sequencing

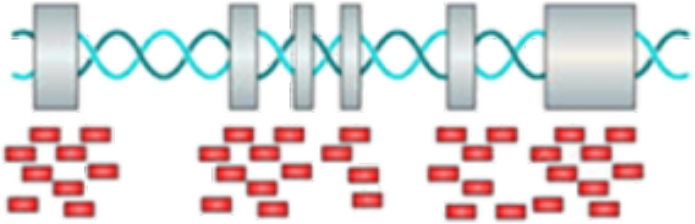
=> whole exomes/genomes or many gene fragments for many patients

Whole genome sequencing



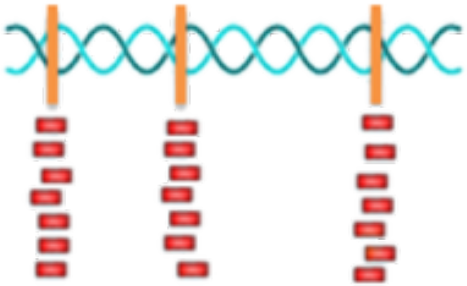
- Sequencing region : whole genome
- Sequencing Depth: >30X
- Covers everything – can identify all kinds of variants including SNPs, INDELs and SV.

Whole exome sequencing



- Sequencing region: whole exome
- Sequencing Depth : >50X ~ 100X
- Identify all kinds of variants including SNPs, INDELs and SV in coding region.
- Cost effective

Targeted sequencing



- Sequencing region: specific regions (could be customized)
- Sequencing Depth : >500X
- Identify all kinds of variants including SNPs, INDELs in specific regions
- Most Cost effective

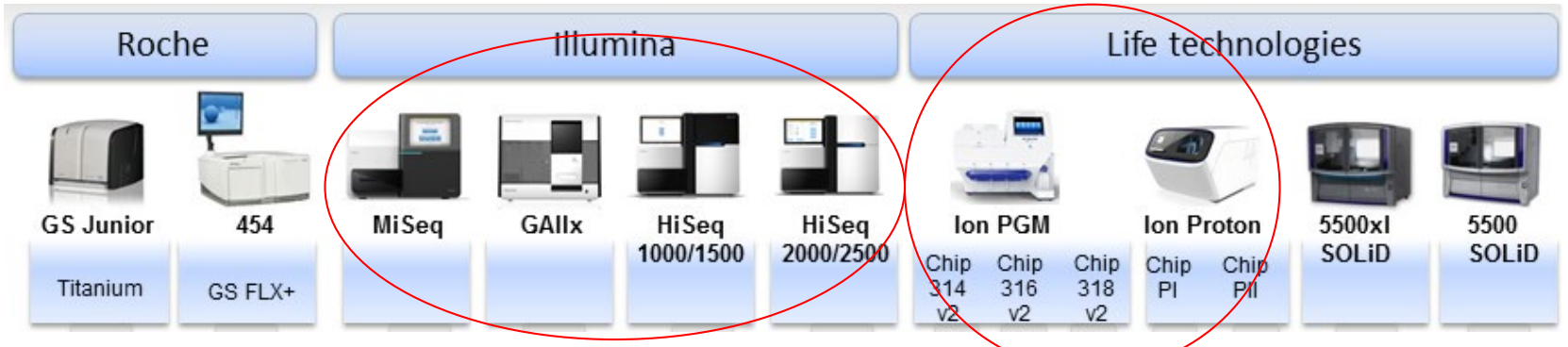
NGS: different technologies

Table 1
NGS platforms currently employed.

Sr. No.	Platform	Read length	Sequencing approach
1	Illumina/ Solexa, Illumina, Inc.	30–40 bp	Sequencing by synthesis with reversible terminators
2	ABI/SOLiD, Applied Biosystems	35 bp	Massively parallel sequencing by Ligation
3	454/Roche FLX system, Roche Applied Science	200–300 bp	Pyrosequencing on solid support
4	Ion Torrent	200 bp	Proton detection

Next Generation Sequencing

- Definition : Technologies that share the ability to massively parallel sequence millions of DNA templates



DNA Library

Clonal amplification → sequencing

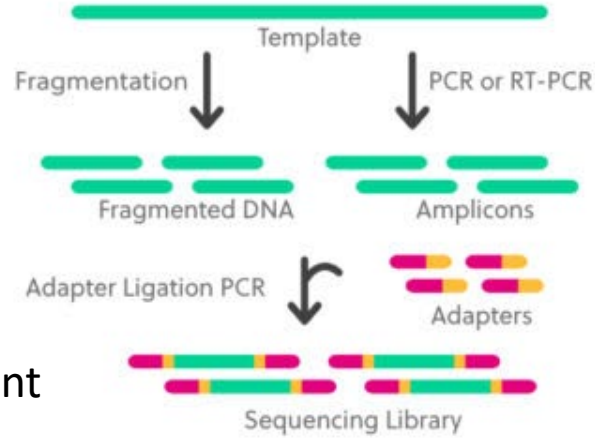
« Polony » PCR → Reversible terminator sequencing

Emulsion PCR → semiconductor sequencing

NGS workflow

STEP 1:
Extraction

STEP 2:
Library Prep



+enrichment

iRepertoire

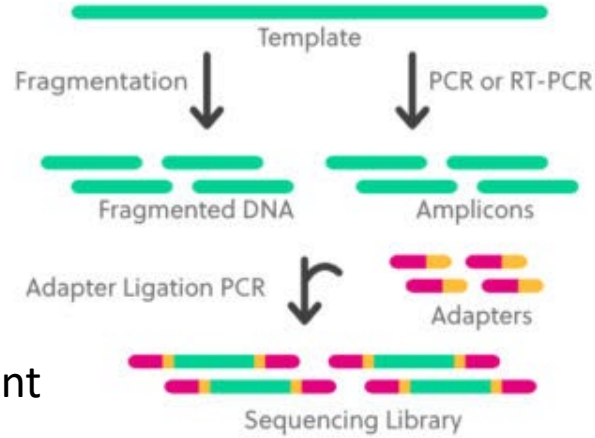
STEP 3:
Sequencing

STEP 4:
Analysis

NGS workflow

STEP 1:
Extraction

STEP 2:
Library Prep



+enrichment

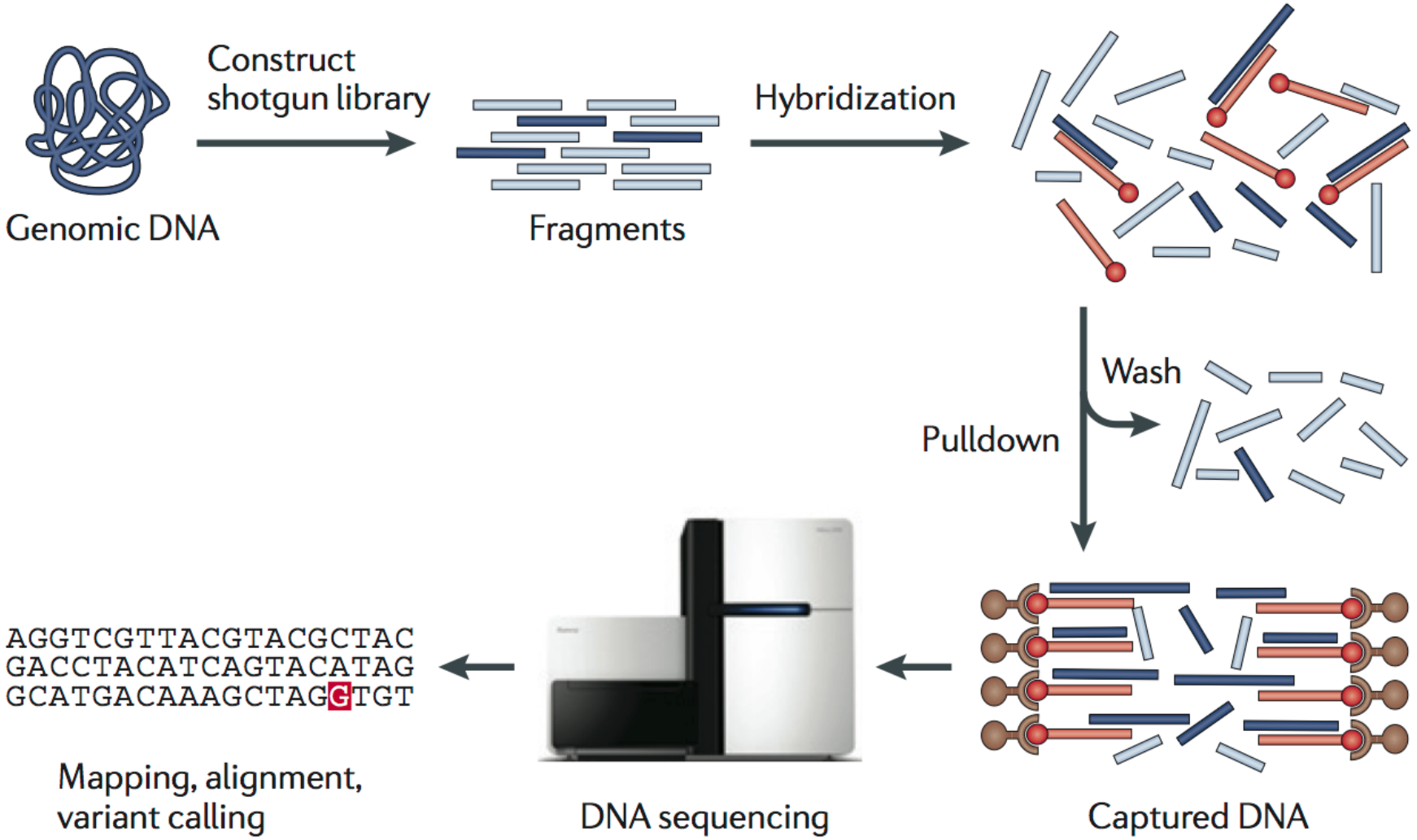
iRepertoire

STEP 3:
Sequencing

STEP 4:
Analysis

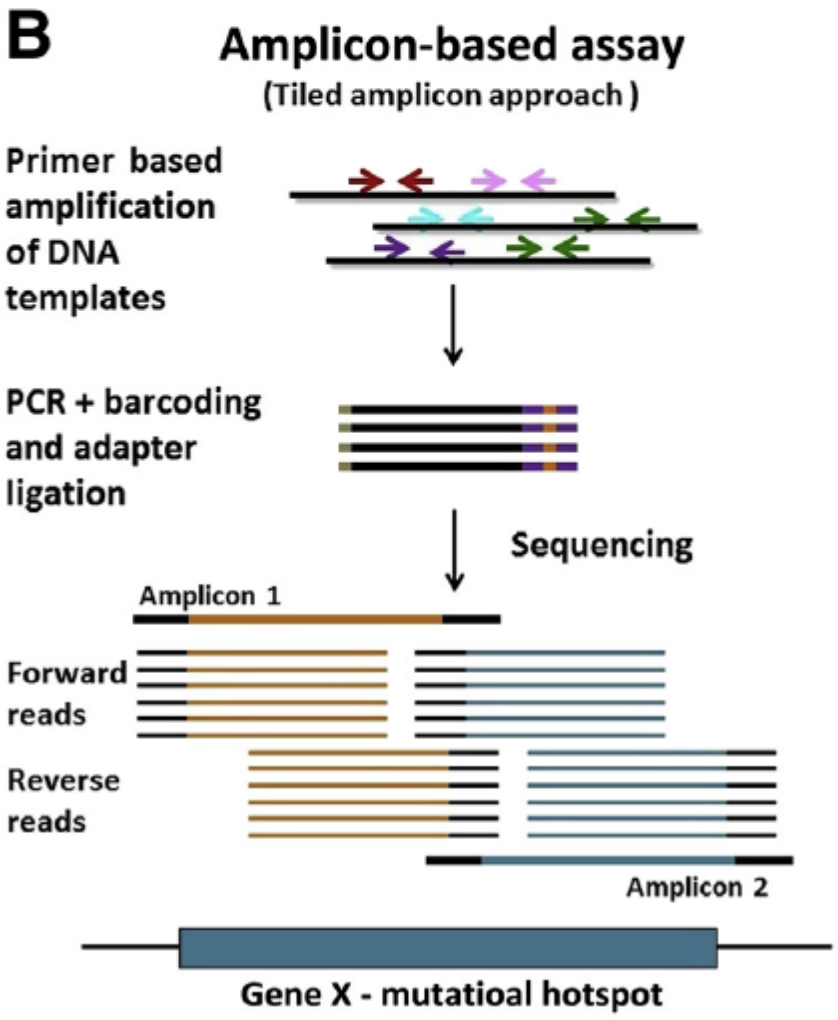
DNA Library: capture

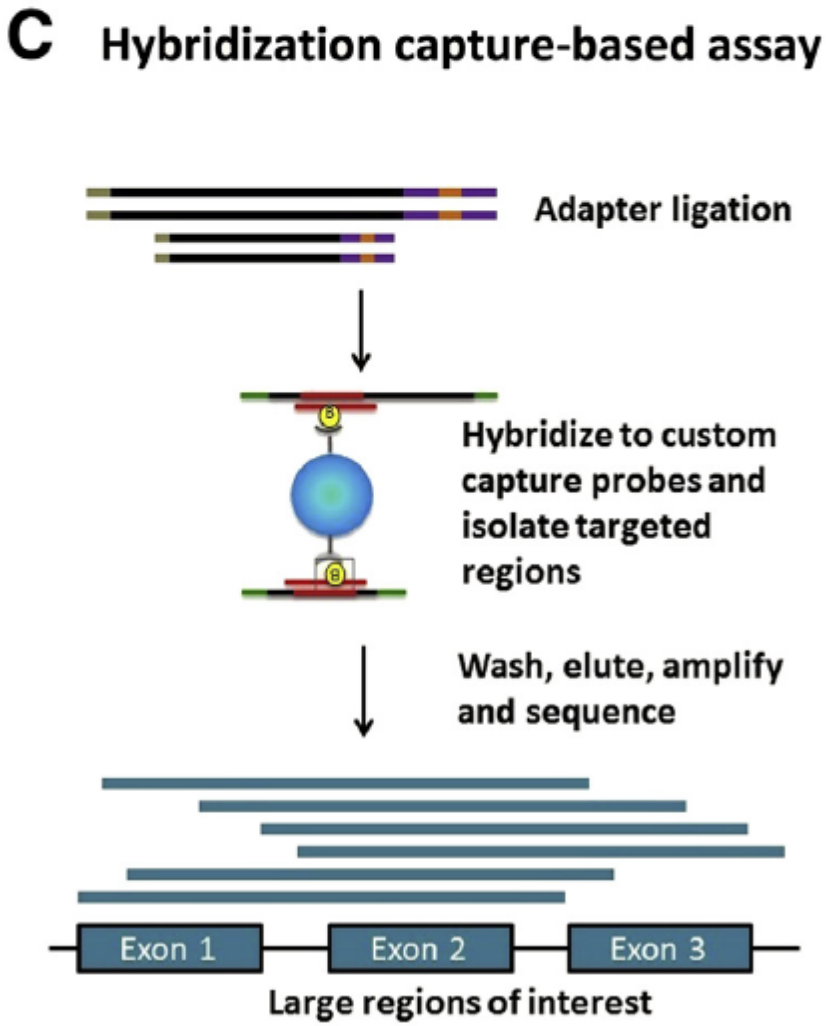
WGS/WES/Targeted sequencing



DNA Library: amplicon or capture

WGS/WES/Targeted sequencing

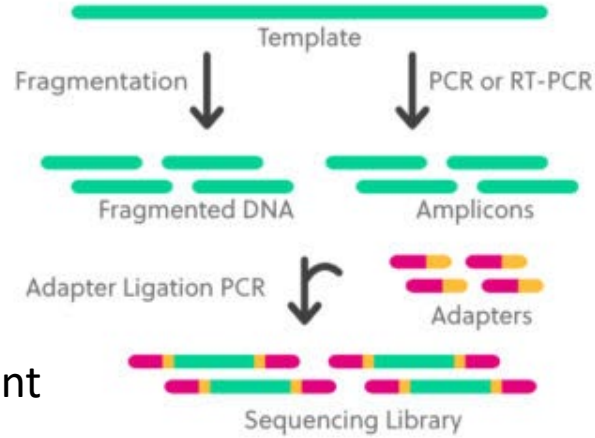




NGS workflow

STEP 1:
Extraction

STEP 2:
Library Prep



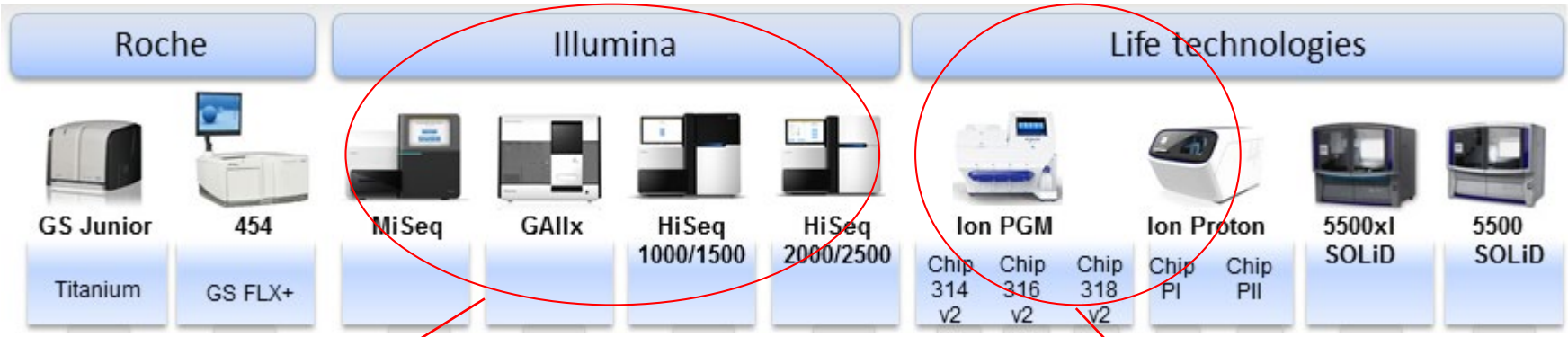
+enrichment

iRepertoire

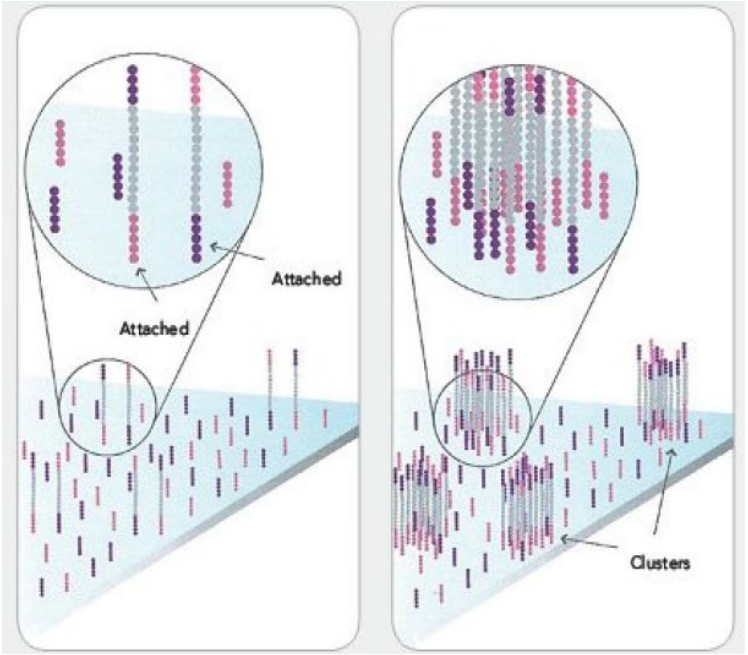
STEP 3:
Sequencing

STEP 4:
Analysis

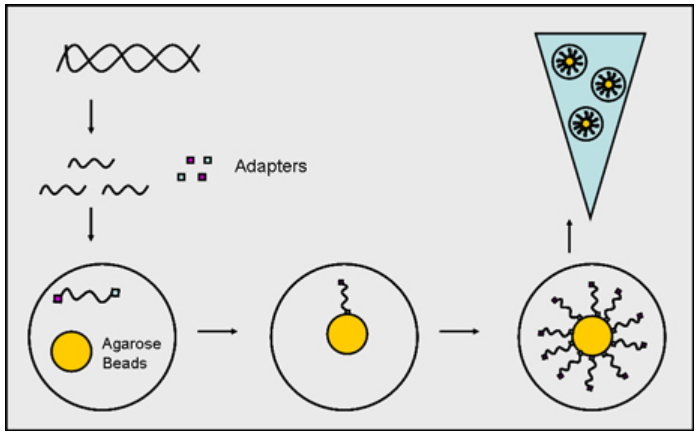
Clonal Amplification



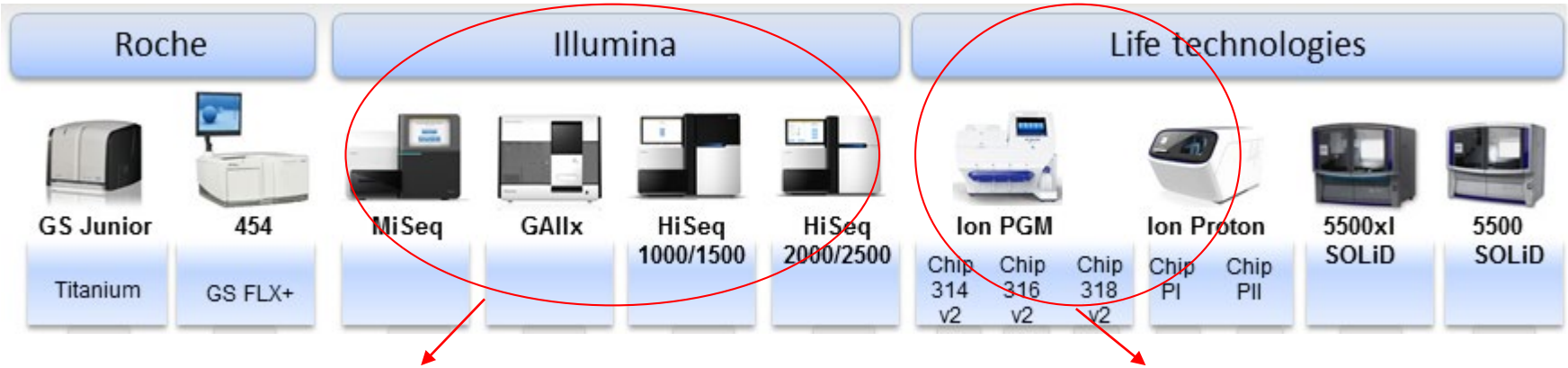
« Polony » PCR

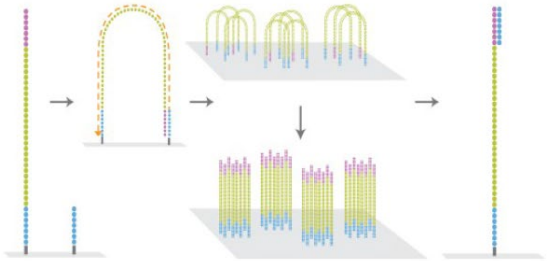


Emulsion PCR

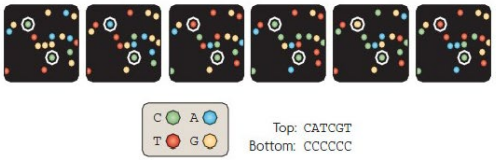


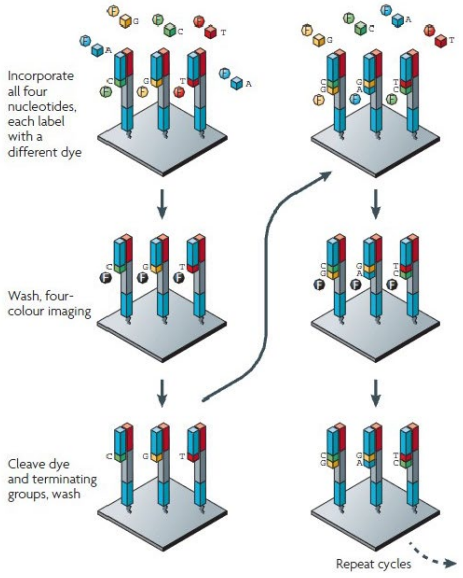
Sequencing



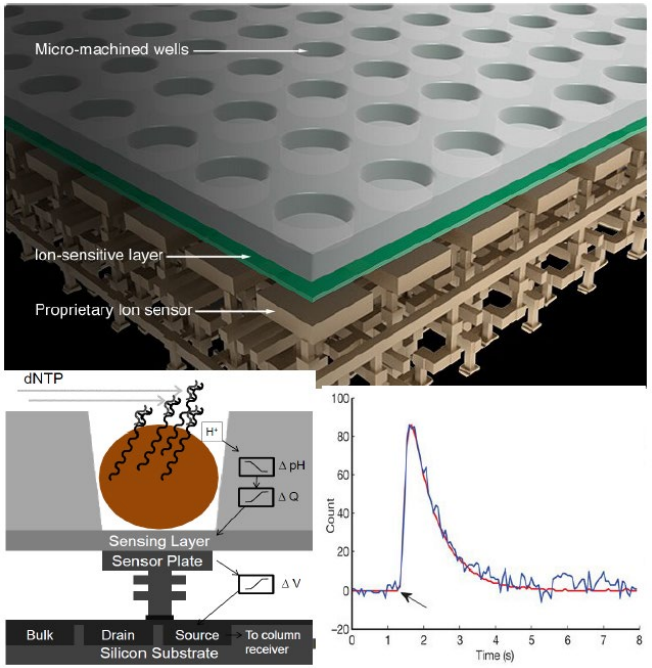


4 nucleotides with different dye flow simultaneous



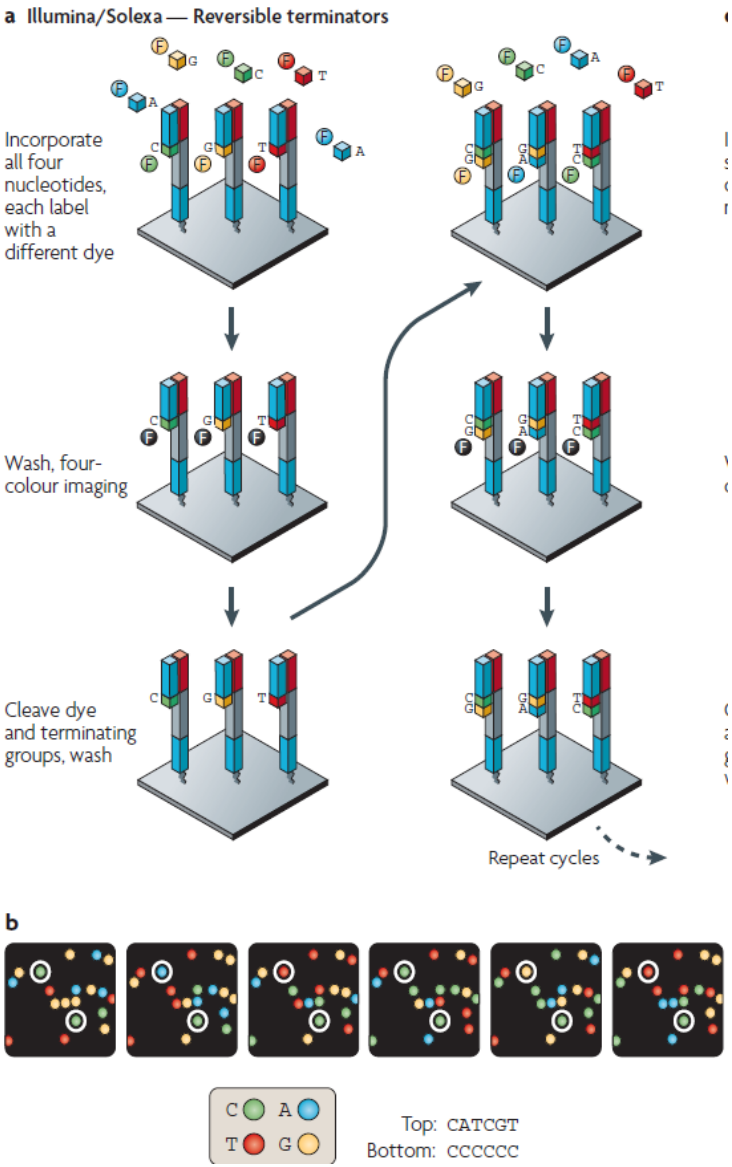


Reversible terminator sequencing

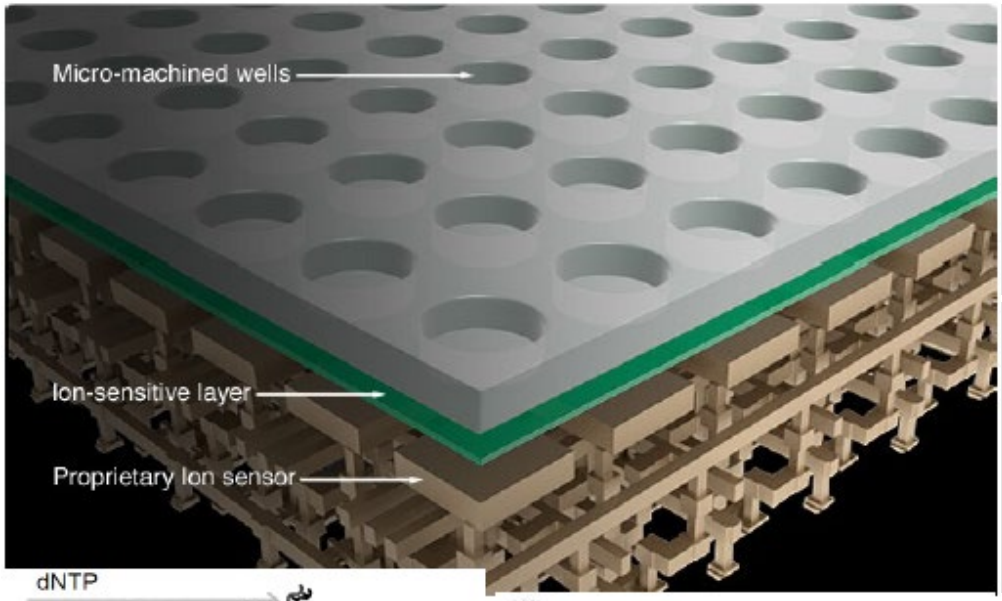


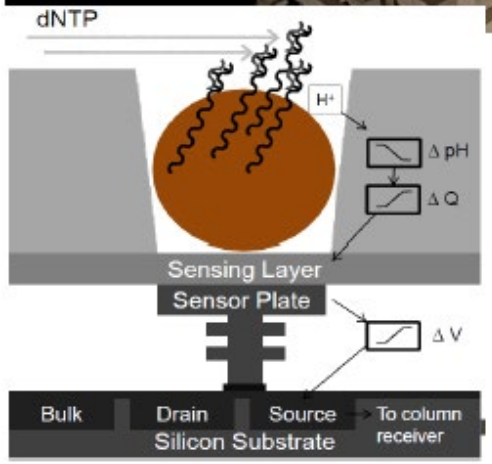
semiconductor sequencing

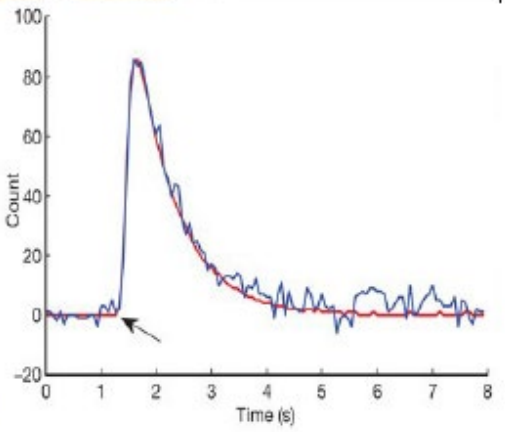
Reversible terminator sequencing



Semiconductor sequencing







Ion 510™ Chip
2–3 M reads

Ion 520™ Chip
3–6 M reads

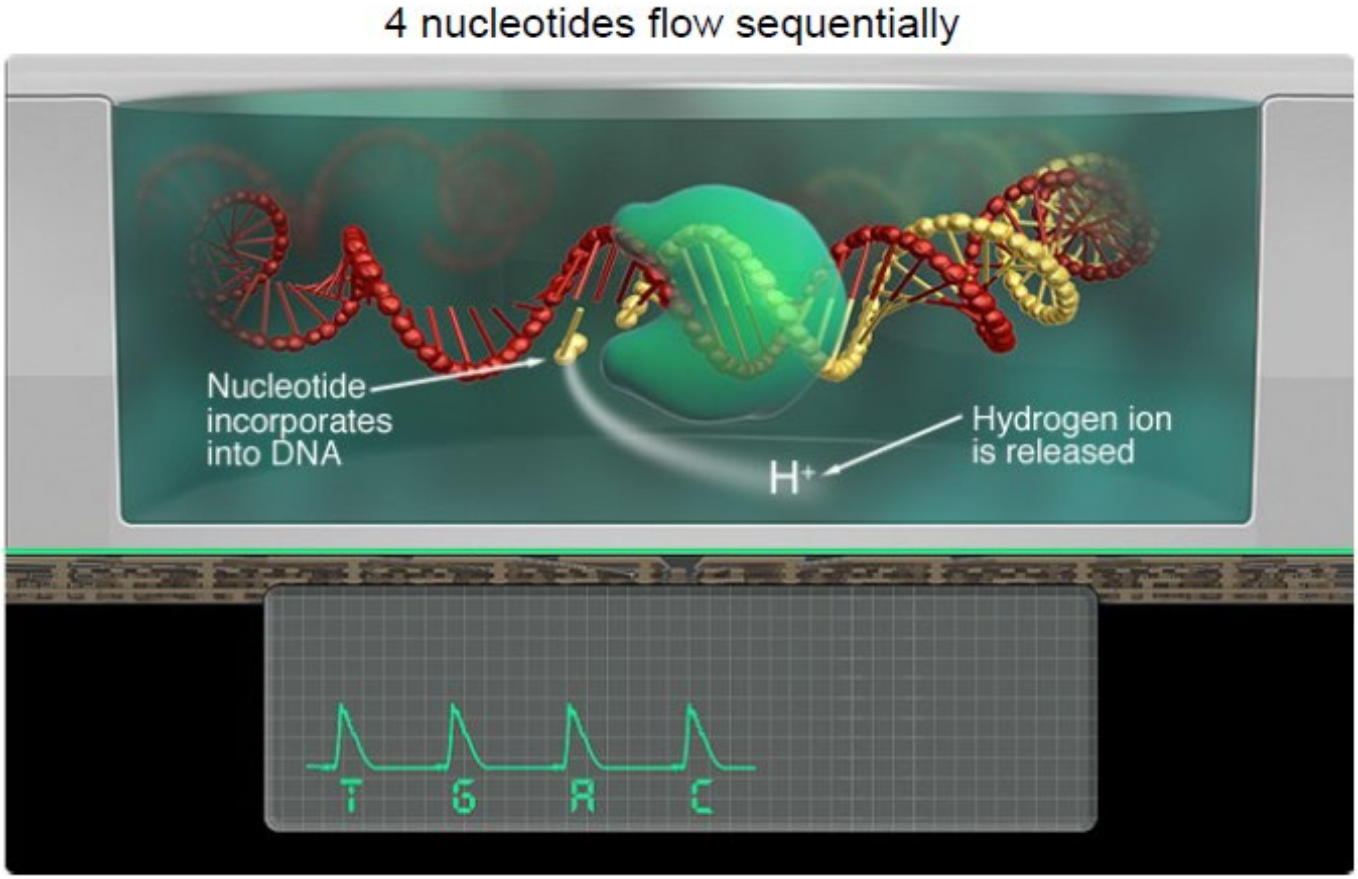
Ion 530™ Chip
15–20 M reads

Ion 540™ Chip
60–80 M reads

New

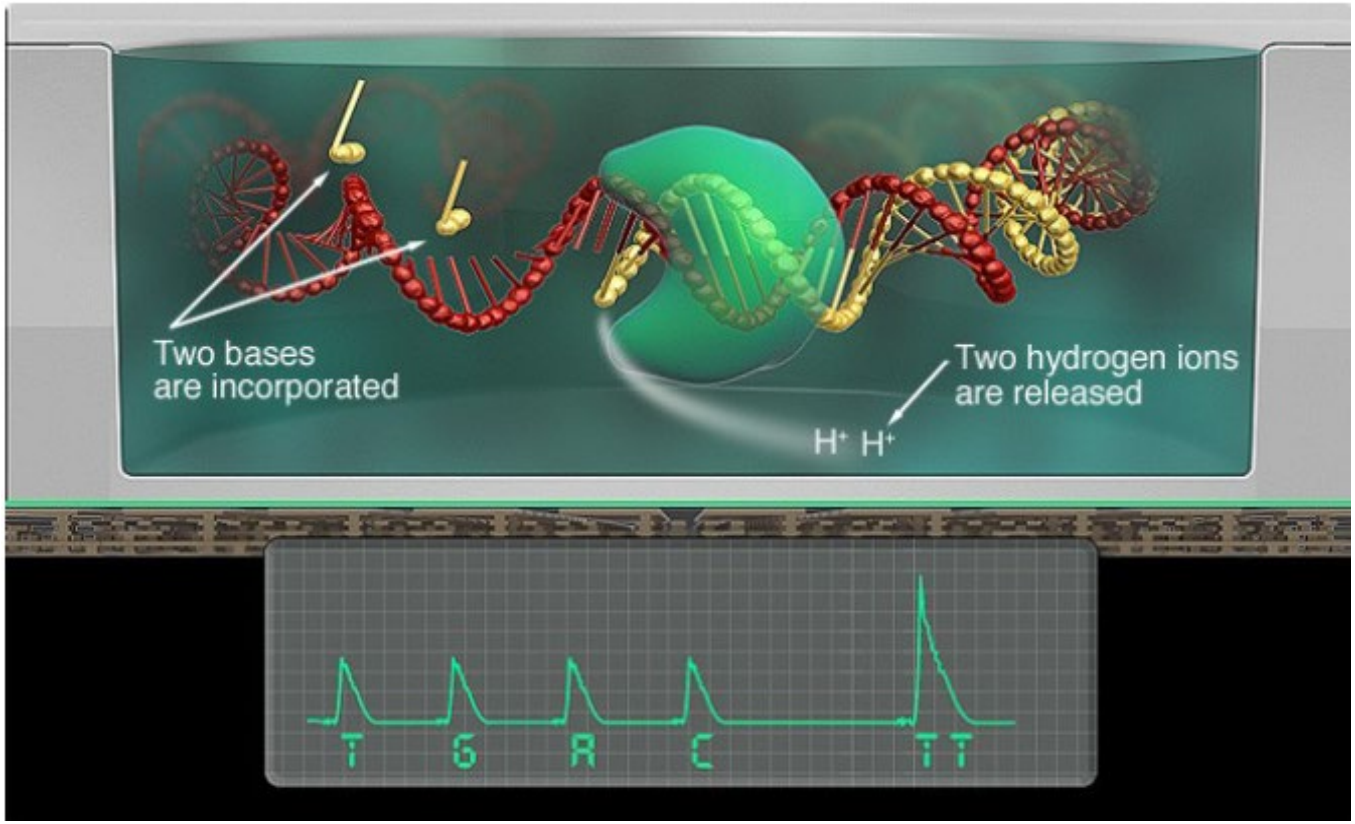
Ion 550™ Chip
100–130 M reads

Semiconductor sequencing

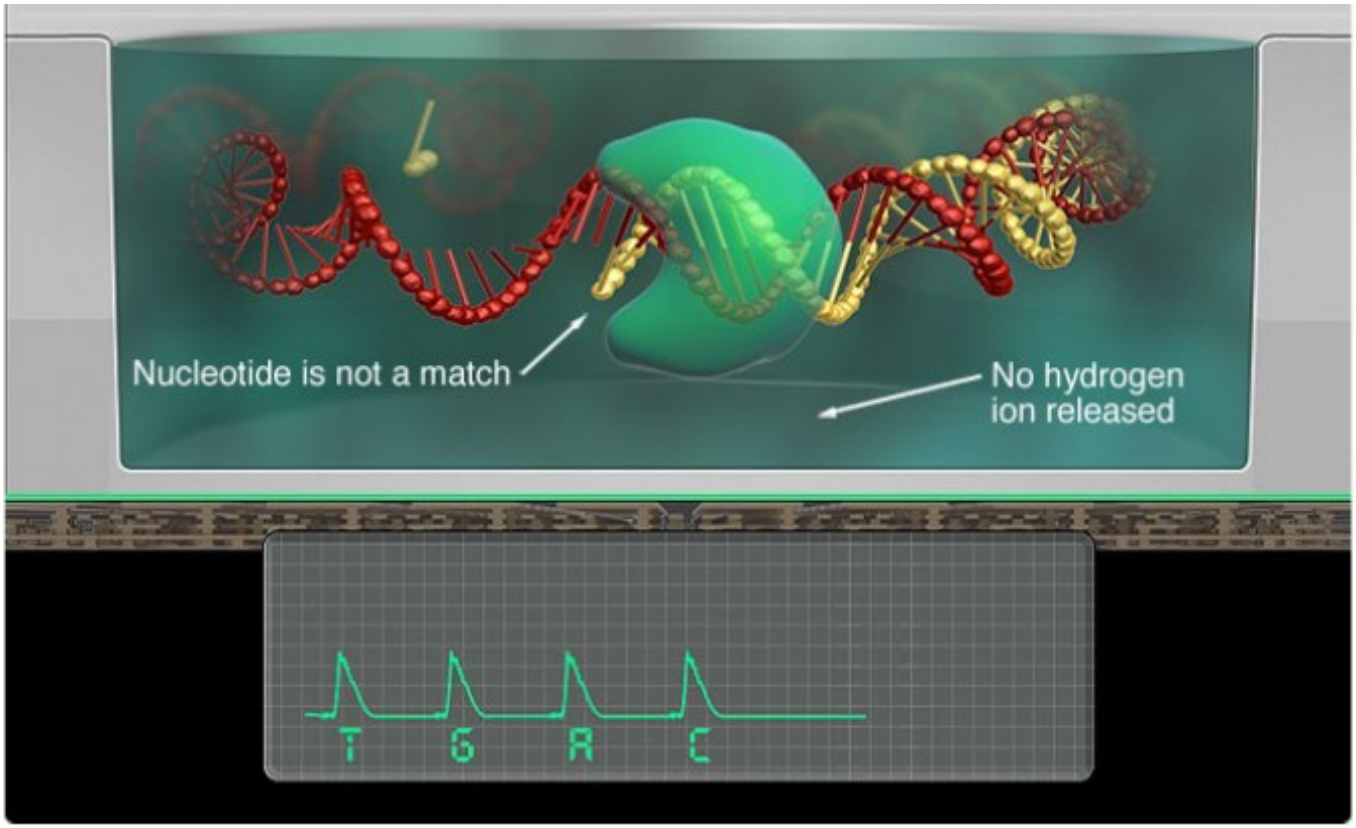


No camera, just a pH sensor

Semiconductor sequencing



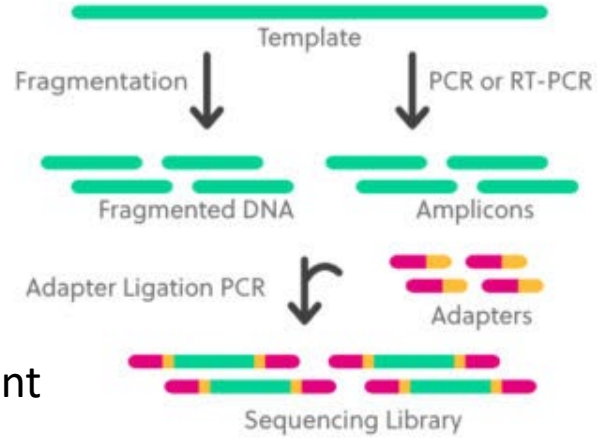
Semiconductor sequencing



NGS workflow

STEP 1:
Extraction

STEP 2:
Library Prep



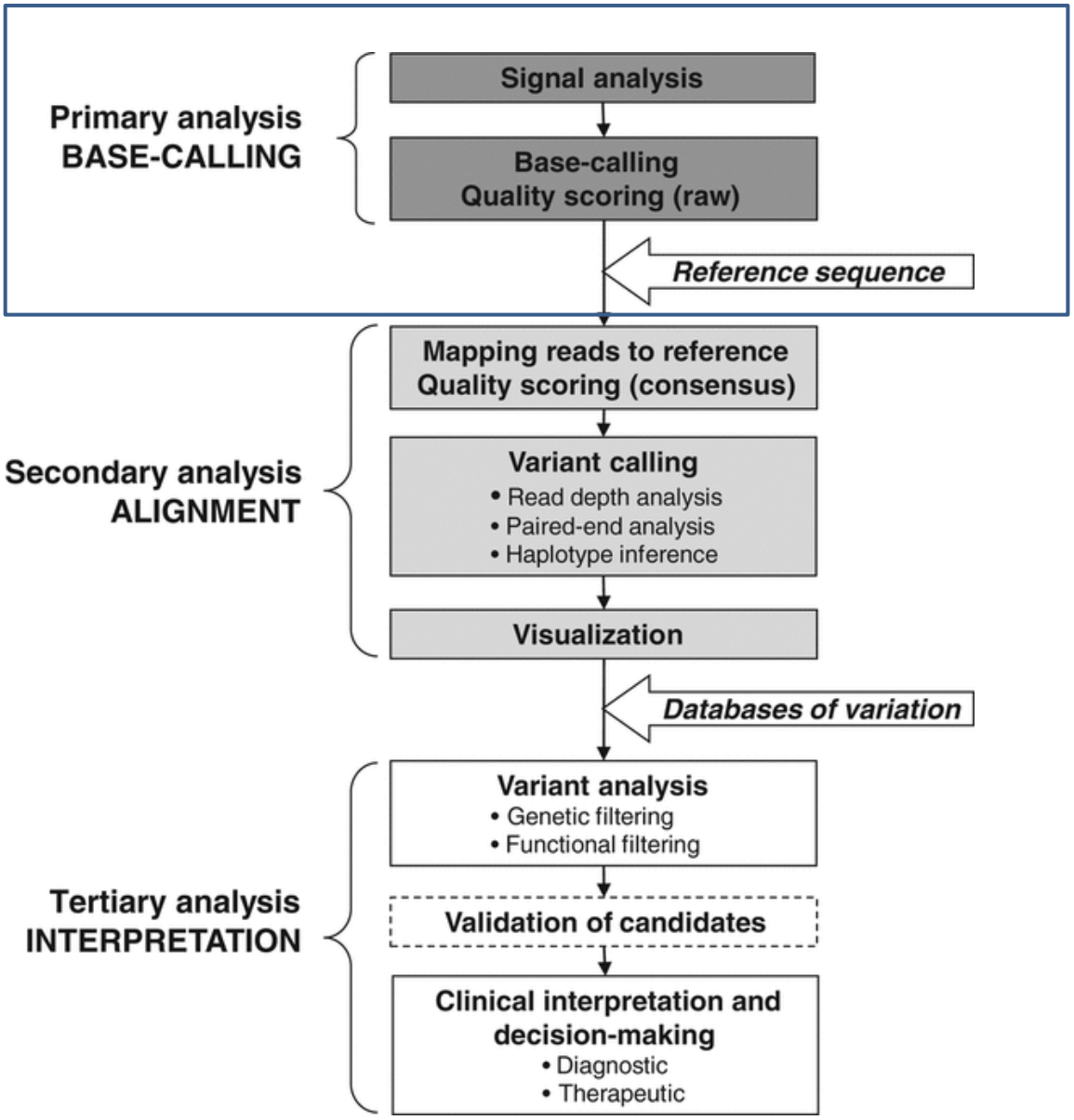
+enrichment

iRepertoire

STEP 3:
Sequencing

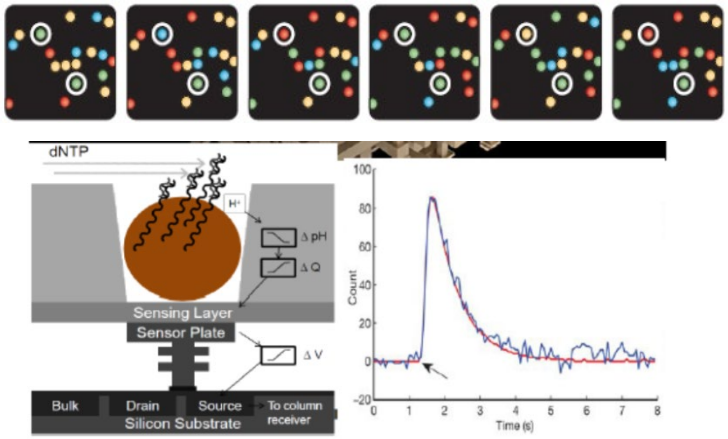
STEP 4:
Analysis

Data analysis (1)



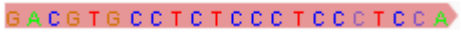
Primary analysis

- Base Calling



Signal

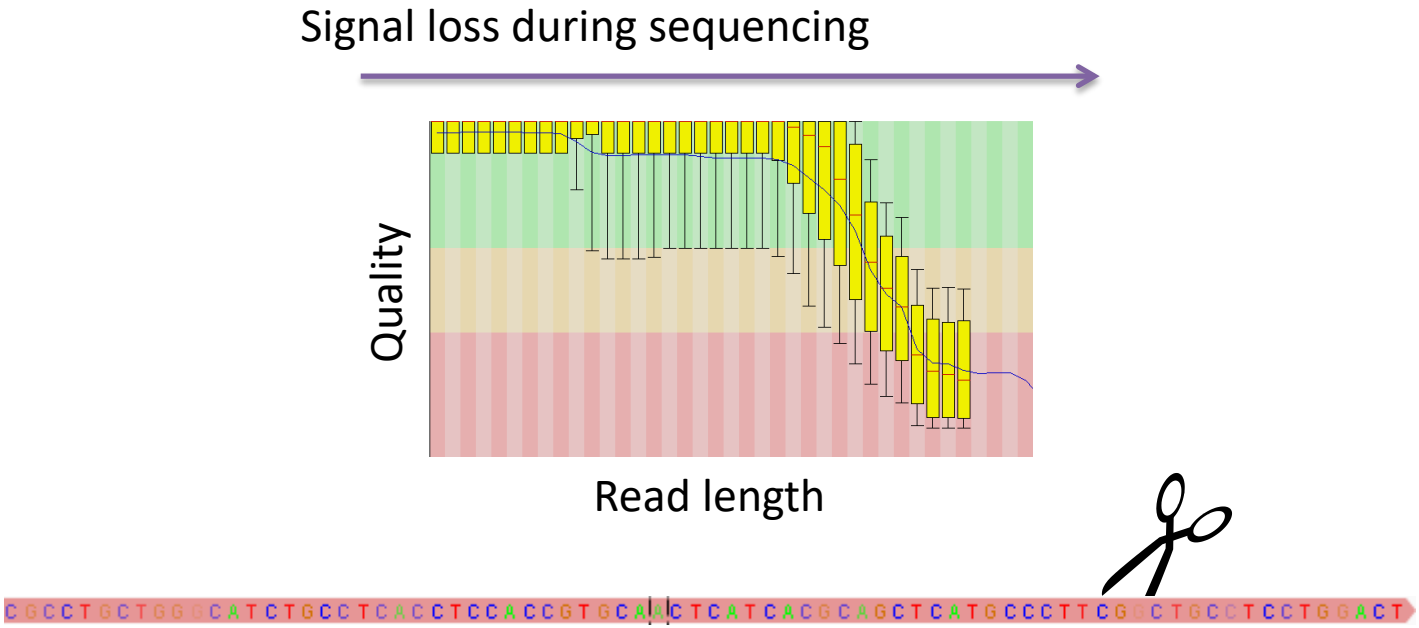
BaseCalling



DNA Read

Primary analysis

- Trimming - removing parts of reads
  - Removing adapters / barcodes
  - Removing parts of reads that have low quality
  - Quality control with FASTQC or Samstats tools



Base Quality Values

- Each base in a read also has assigned base quality value
- Base Quality values are in the Phred scale
  Defined as $-10 \times \log_{10}$ (error probability)
  Predicts the probability of correct base call

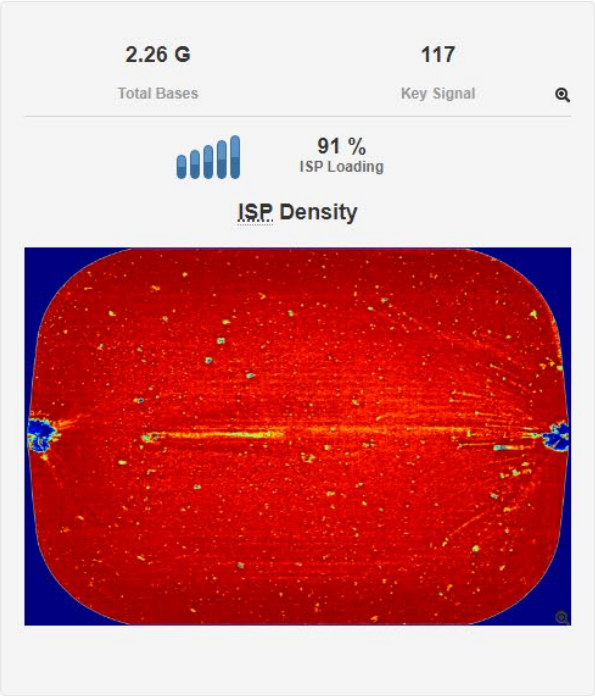
Quality score interpretation

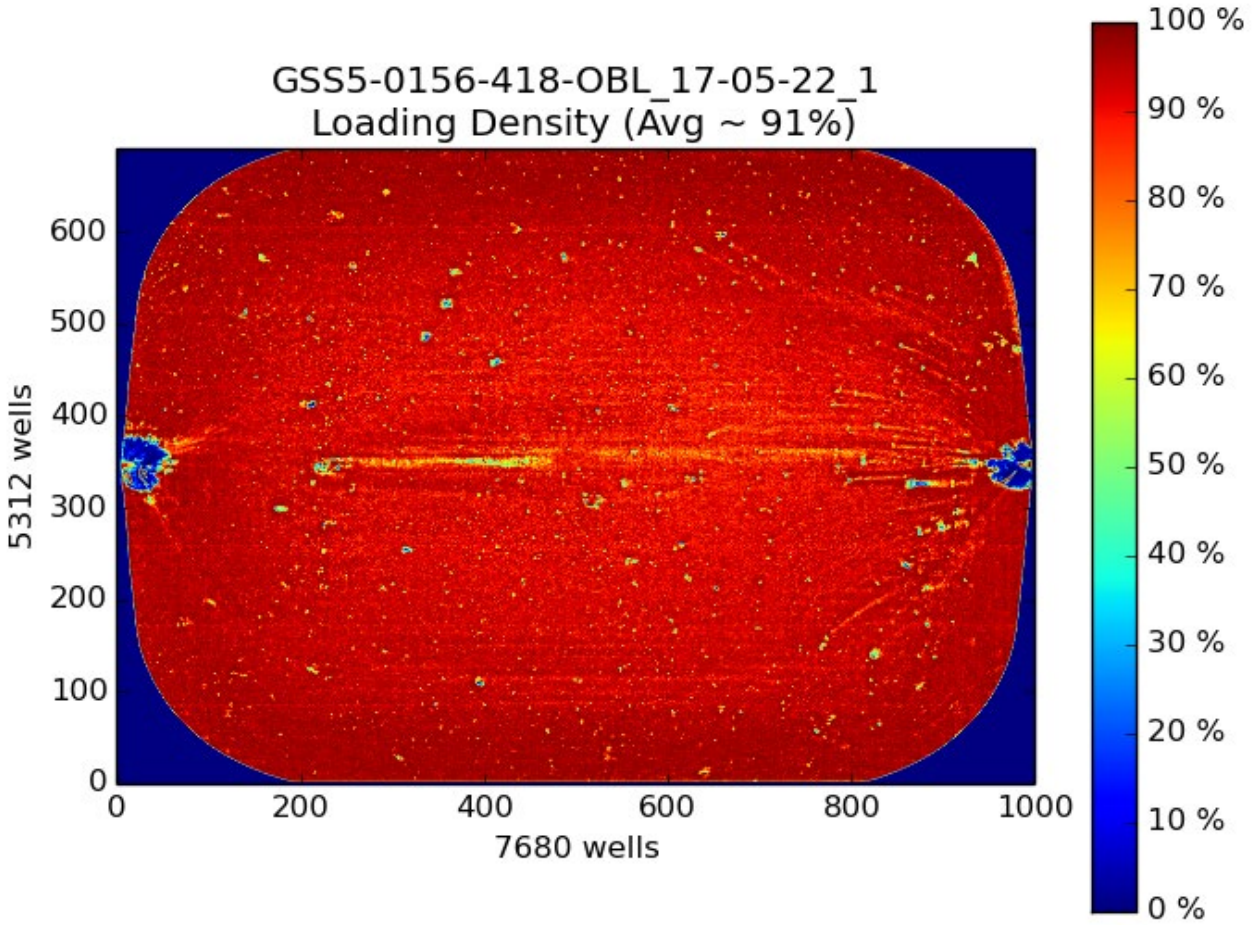
$$Q = -10 \log_{10} P \quad \Longrightarrow \quad P = 10^{\frac{-Q}{10}}$$

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 10000	99.99%
50	1 in 100000	99.999%

Quality Control

Unaligned Reads





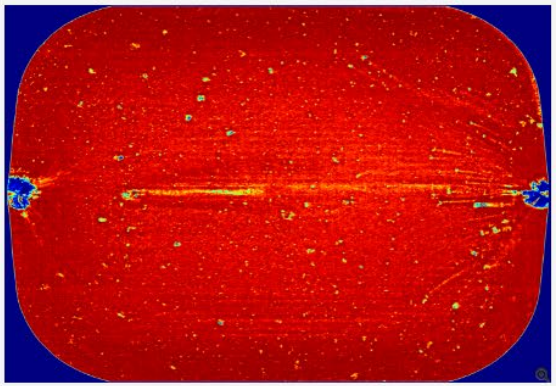
Quality Control

Unaligned Reads

2.26 G Total Bases | **117** Key Signal

91 % ISP Loading

ISP Density



19,391,053 Total Reads

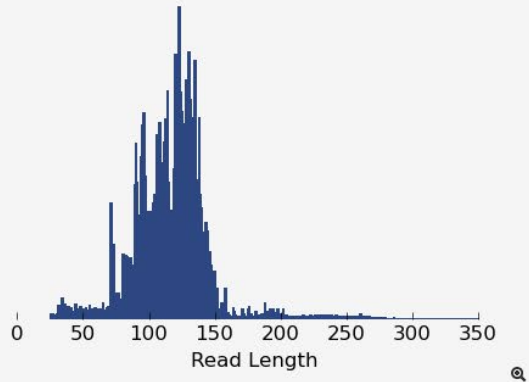
57 % Usable Sequence

Chip well details

91% Loading	34,442,226	9% Empty Wells
100% Enrichment	34,439,207	0% No Template
63% Clonal	21,733,895	37% Polyclonal
89% Final Library	19,391,053	1% Test Fragments, 0% Adapter Dimer, 10% Low Quality

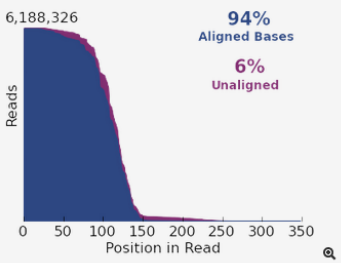
117 bp Mean | **118 bp** Median | **122 bp** Mode

Read Length



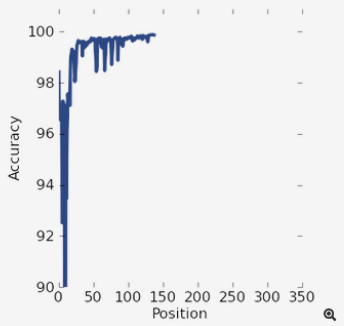
Aligned to Home sapiens hg19

667 M Total Aligned Bases | **0.2X** Reference Coverage



	Count	%
Total Reads	6,188,326	—
Aligned Reads	6,169,266	99.7%
Unaligned Reads	19,060	0.3%

98.8% Mean Raw Accuracy 1x

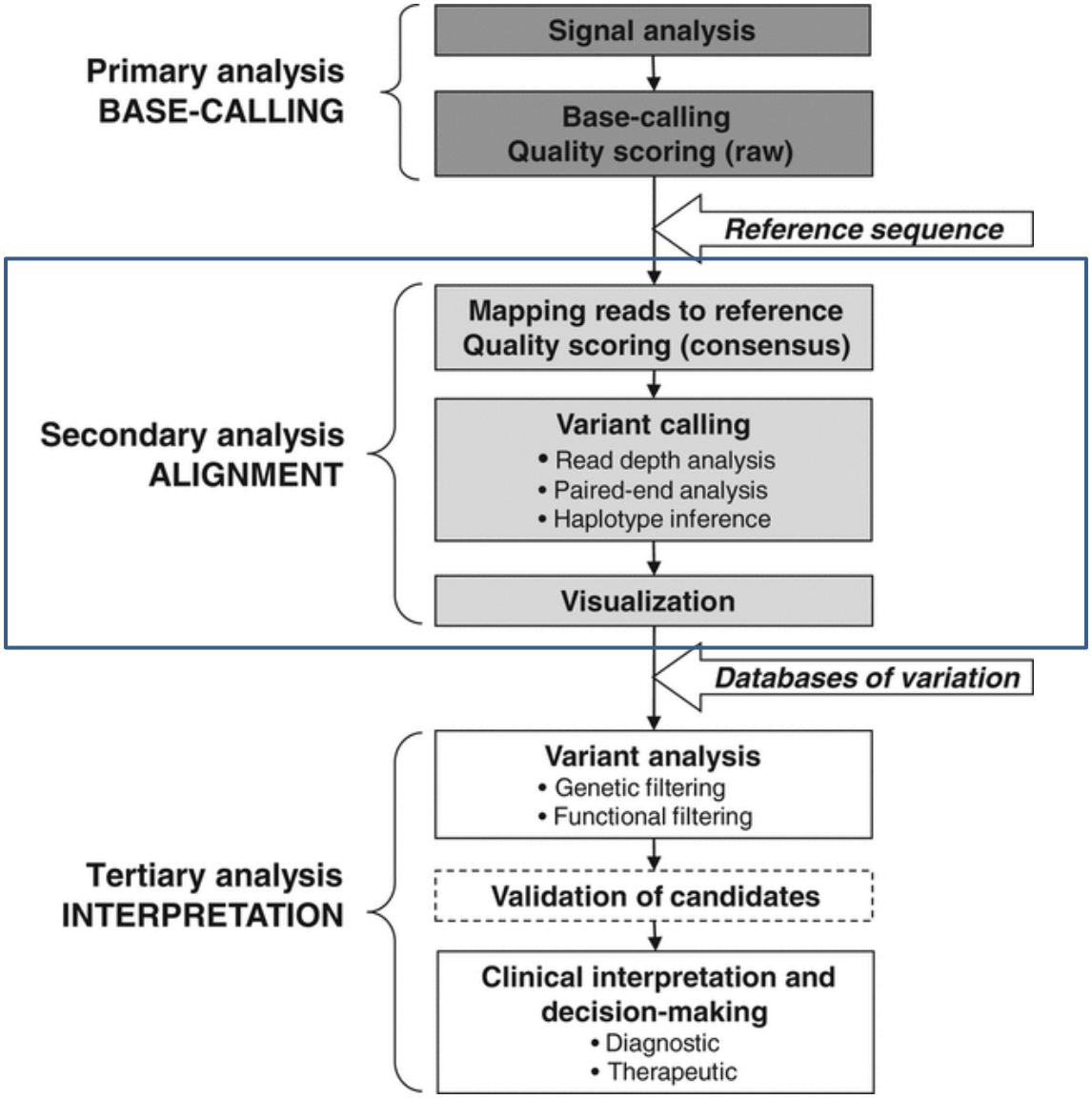


585 M AQ17 Total Bases

Alignment Quality

	AQ17	AQ20	Perfect
Total Number of Bases [bp]	585 M	563 M	522 M
Mean Length [bp]	108	106	100
Longest Alignment [bp]	334	334	334
Mean Coverage Depth [x]	0.2	0.2	0.2

Data analysis (1)



Secondary analysis

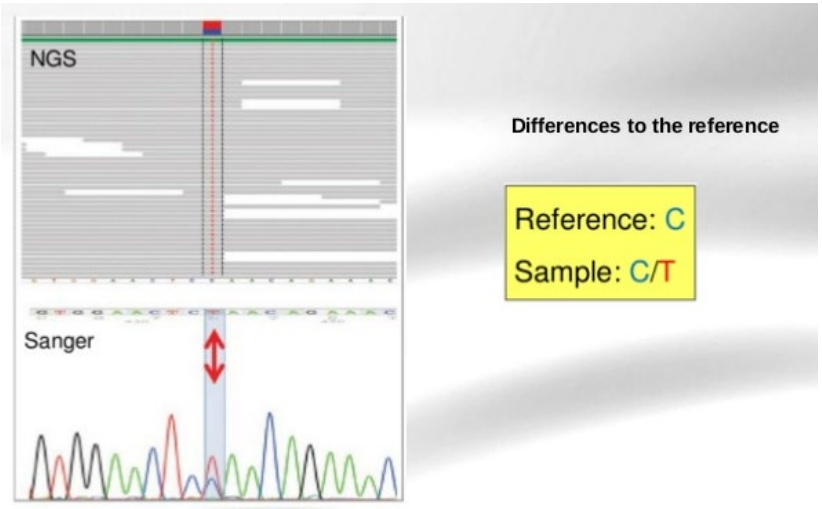
- Alignment

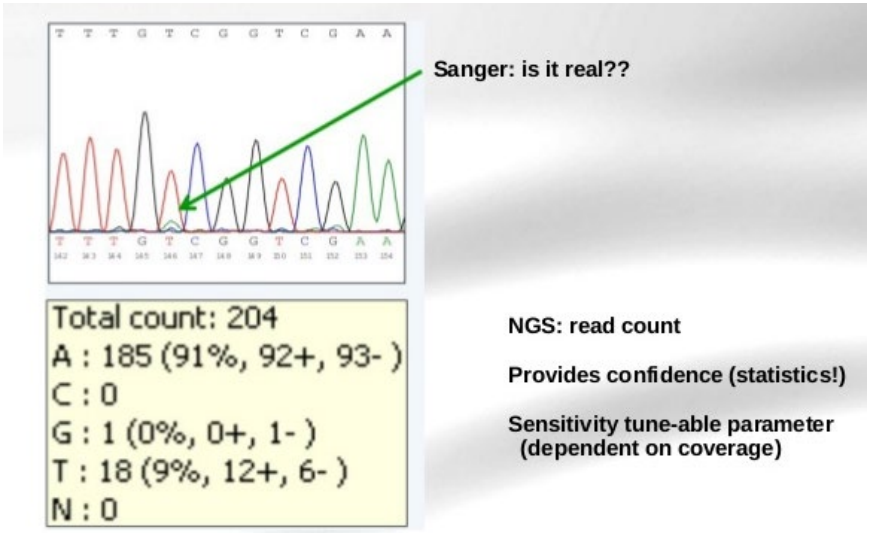
DNA Read

Reference genome (human : hg19)

Alignment / mapping

- Variant calling





Secondary analysis

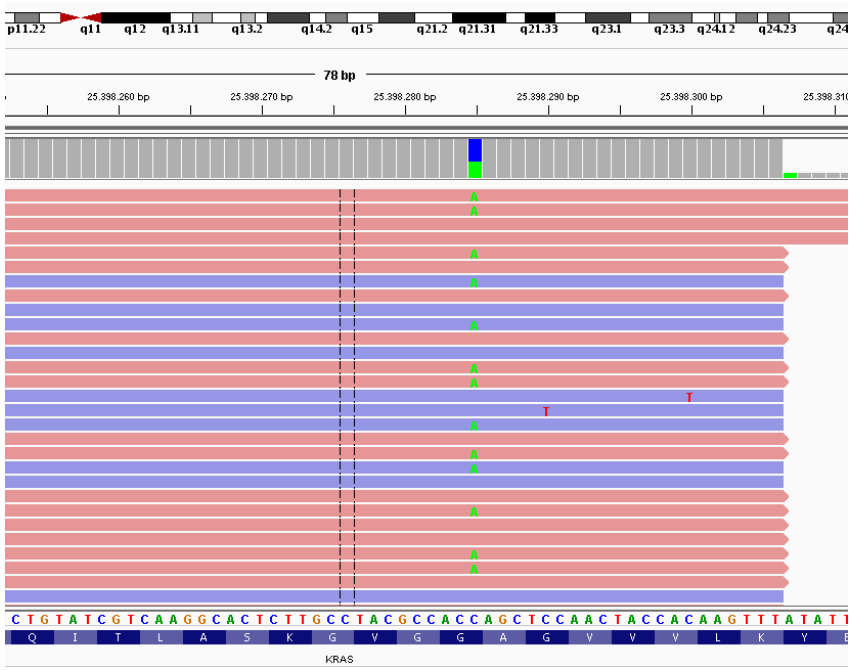
- Annotation

barcode	sample_name	chrom	position	ref	variant	gene_id	type	allele_call	genotype	frequency	quality	coverage	allele_cov	aa_mut_type	cds_mut_syntax
IonXpress_005	22M01490	chr2	212578392	AAG	GAA	ERBB4	MNP	Heterozygous	AAG/GAA	17.1	2772.22	1366	234	none	---
IonXpress_005	22M01490	chr2	212578380	AA	-	ERBB4	DEL	Heterozygous	AA/-	47.8	2772.22	1366	653	Intron	---
IonXpress_005	22M01490	chr4	1807894	G	A	FGFR3	SNP	Homozygous	A/A	99.8	33729.2	2000	1995	Silent	---
IonXpress_005	22M01490	chr4	1807922	G	A	FGFR3	SNP	Heterozygous	G/A	50.1	10742.2	1964	983	Intron.	---
IonXpress_005	22M01490	chr4	55141055	A	G	PDGFRA	SNP	Homozygous	G/G	99.9	30314.8	1796	1795	Silent	---
IonXpress_005	22M01490	chr7	55228053	A	T	EGFR	SNP	Homozygous	T/T	100.0	33779.1	1988	1988	Intron.	---
IonXpress_005	22M01490	chr7	55249063	G	A	EGFR	SNP	Homozygous	A/A	99.8	33792.1	2000	1996	Silent	---
IonXpress_005	22M01490	chr7	116435768	C	T	MET	SNP	Homozygous	T/T	99.6	33621.5	1999	1992	Silent	---
IonXpress_005	22M01490	chr7	140481402	C	G	BRAF	SNP	Heterozygous	C/G	6.8	180.741	1994	135	p.G469A	c.1406G>C
IonXpress_005	22M01490	chr11	534242	A	G	HRAS	SNP	Homozygous	G/G	99.4	7750.83	520	517	Silent	c.81T>C
IonXpress_005	22M01490	chr17	7579472	G	C	TP53	SNP	Heterozygous	G/C	49.8	4768.01	880	438	Polymorphism.	---

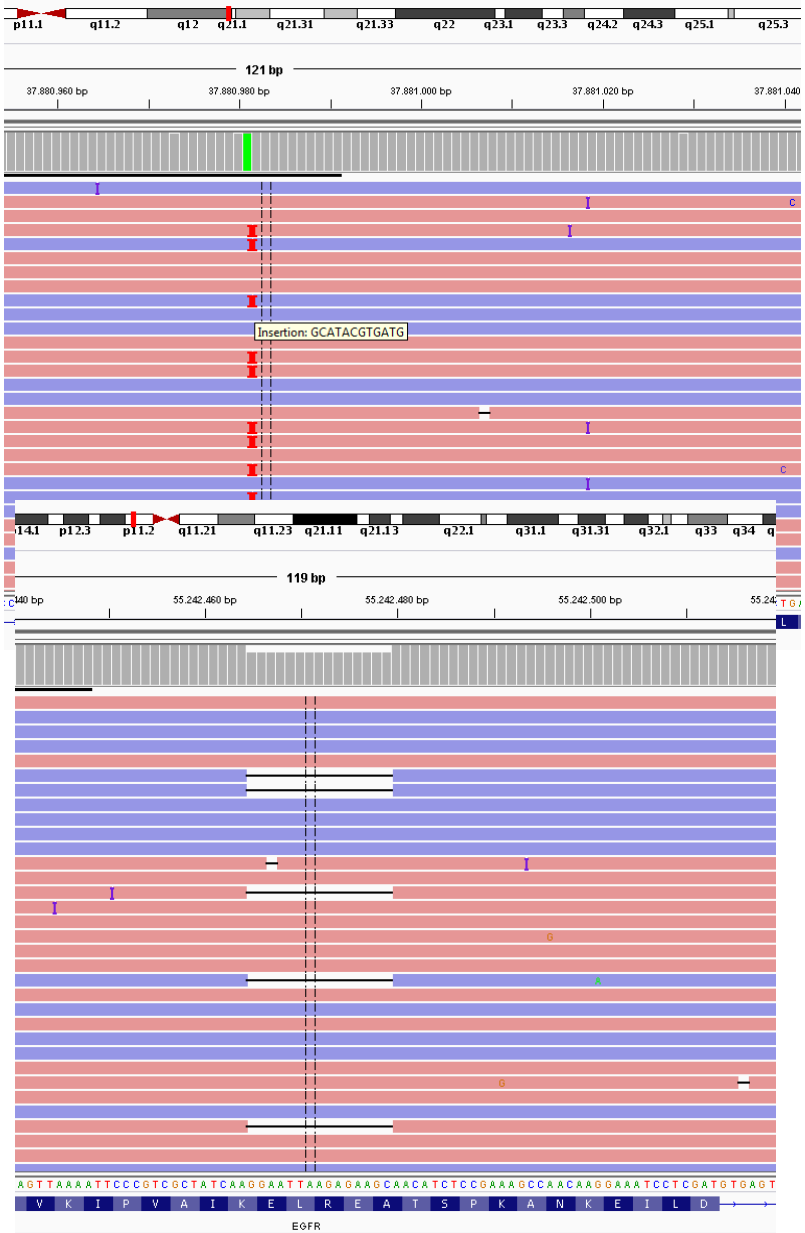
Preferably HGVS nomenclature: http://www.hgvs.org/

Secondary analysis

- Variant calling

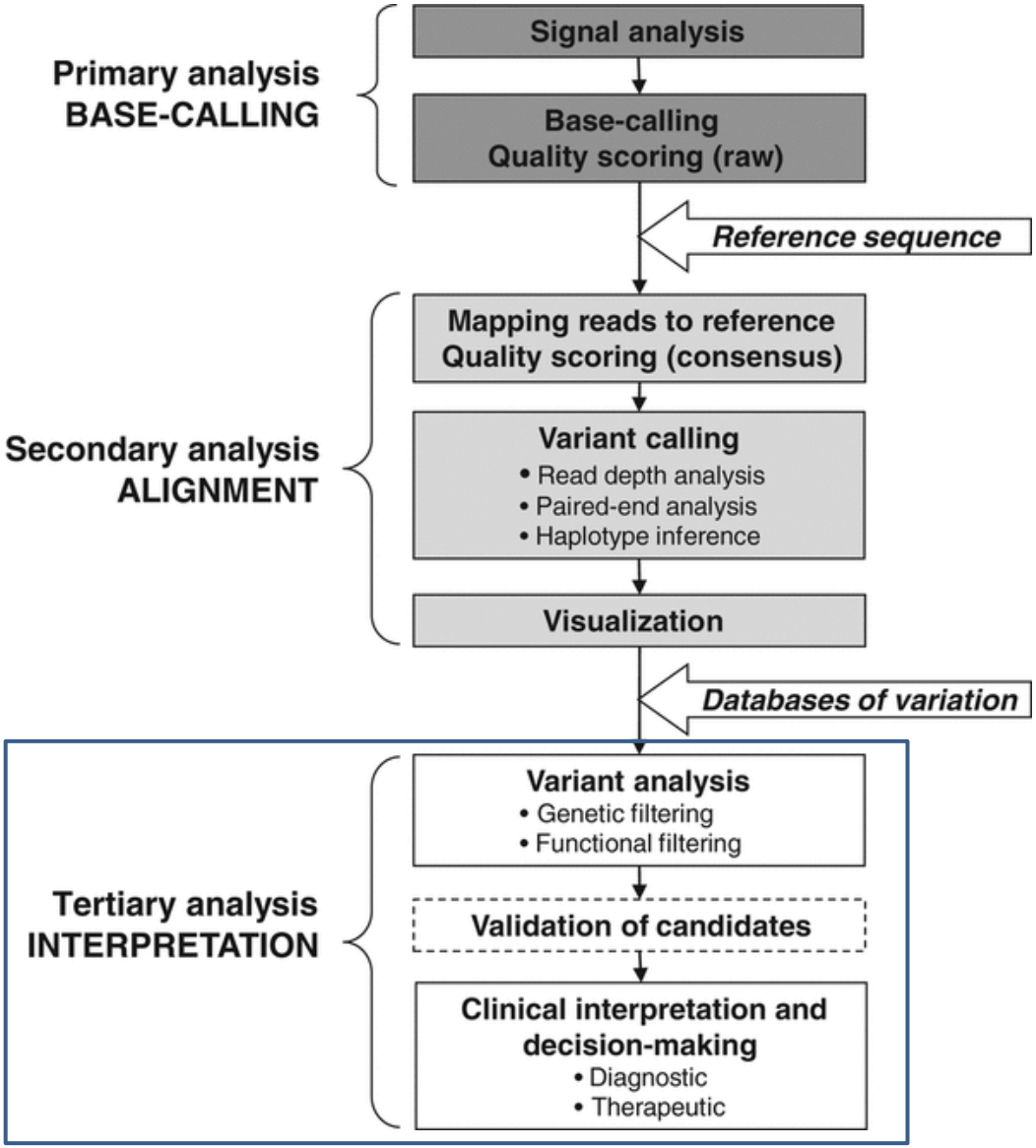


SNP/MNP



Indel

Data analysis



Hôpital Erasme

ULB

Next generation sequencing: data analysis and pratical aspects

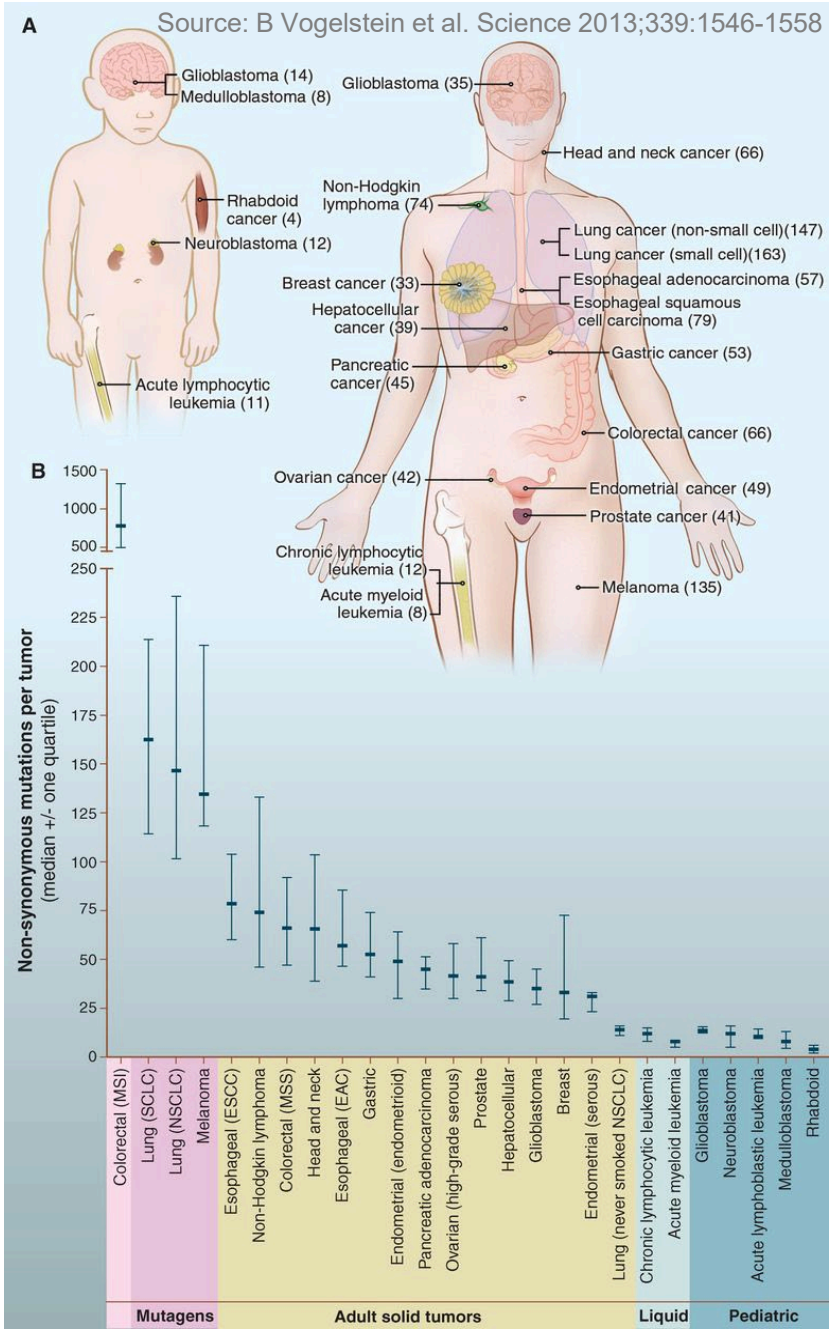
MOLECULAR PATHOLOGY COURSE

FOR RESIDENTS IN PATHOLOGY

Cancer research by NGS

- ✓ Many complete tumor genomes have been sequenced
- ✓ Many mutations in “cancer genes” per tumor
- ✓ **“Cancer is a disease of the genome”**
- ✓ **New insight:** every tumor is different – every patient is different

Number of mutations per tumor demonstrated by NGS



Cancer research by NGS

Translation to the clinic

More refined diagnosis and subtyping of tumors

✓ Clinical findings + medical imaging + histology + immunophenotype

+ genome

Challenge for therapy

✓ New "targets" for therapy – "targeted" therapy

- Revolution in clinical research

✓ Selection of therapy based on the genome of the tumor

➡ **"personalized therapy" of cancer– "precision medicine"**

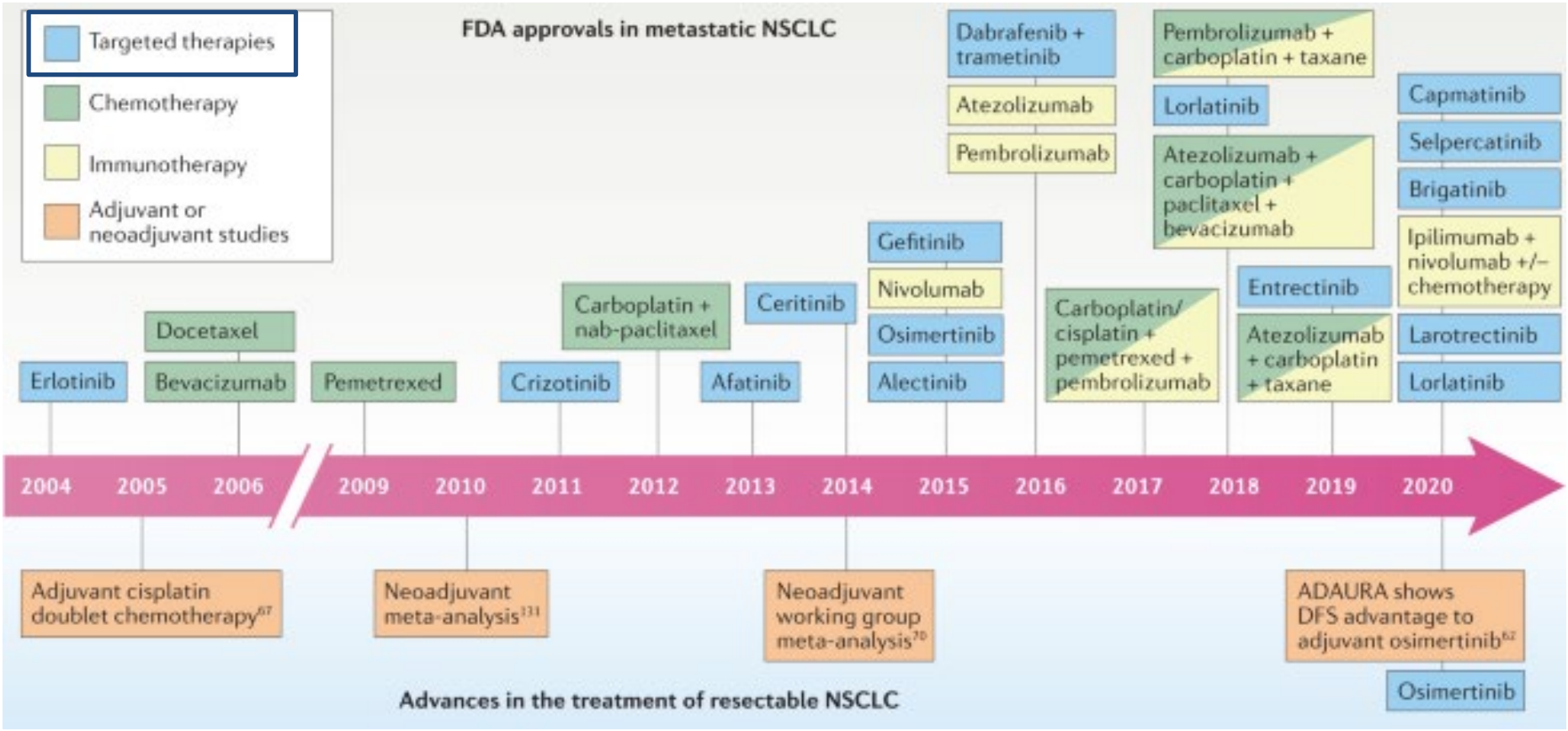
Personalized medicine in oncology

Highly facilitated by NGS detection of DNA variants

The « one biomarker – one drug » scenario → integrated approach with multiple biomarkers and drugs

Lung adenocarcinoma

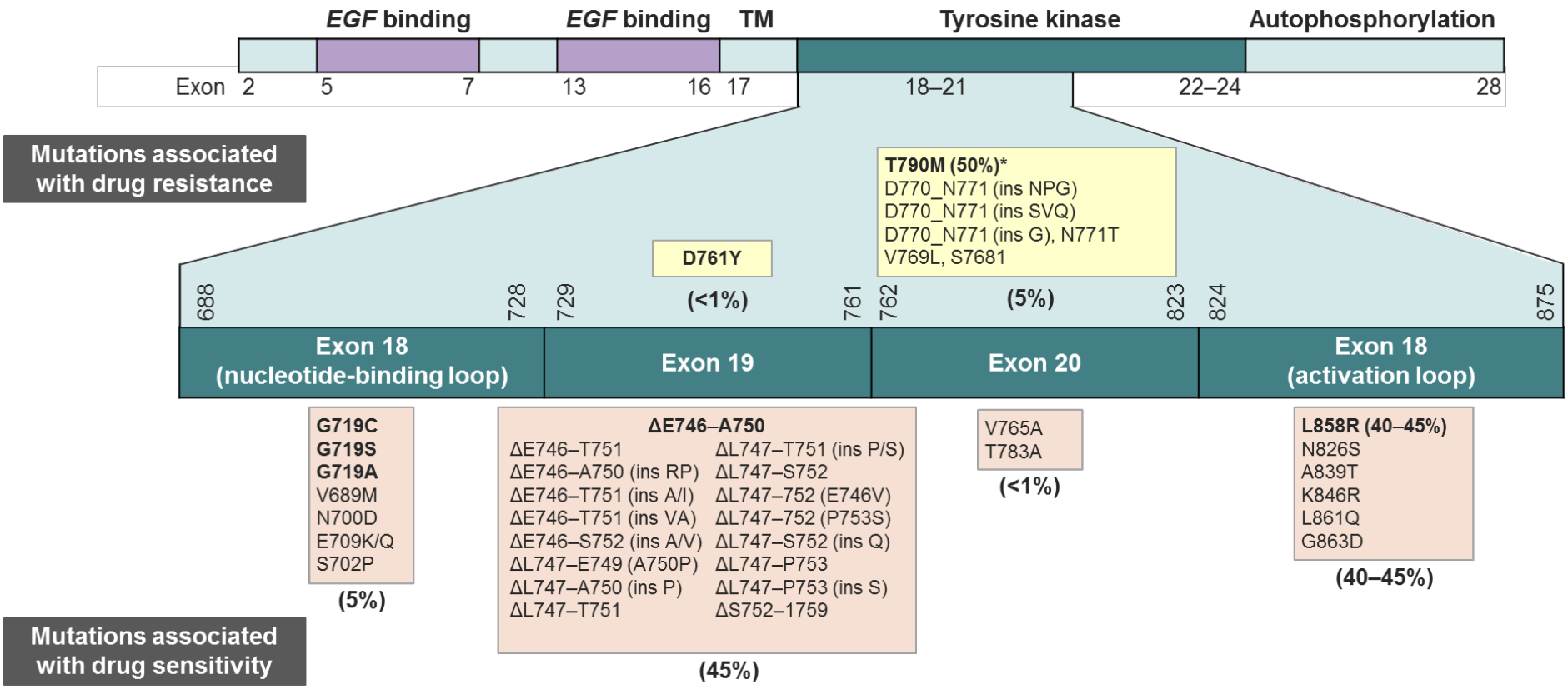
Different genetic subtypes – different cancers



Chaft JE, Rimner A, Weder W, Azzoli CG, Kris MG, Cascone T. Evolution of systemic therapy for stages I-III non-metastatic non-small-cell lung cancer. Nat Rev Clin Oncol. 2021 Sep;18(9):547-557.

Multiple *EGFR* mutations are found in NSCLCs

T790M most associated with drug resistance



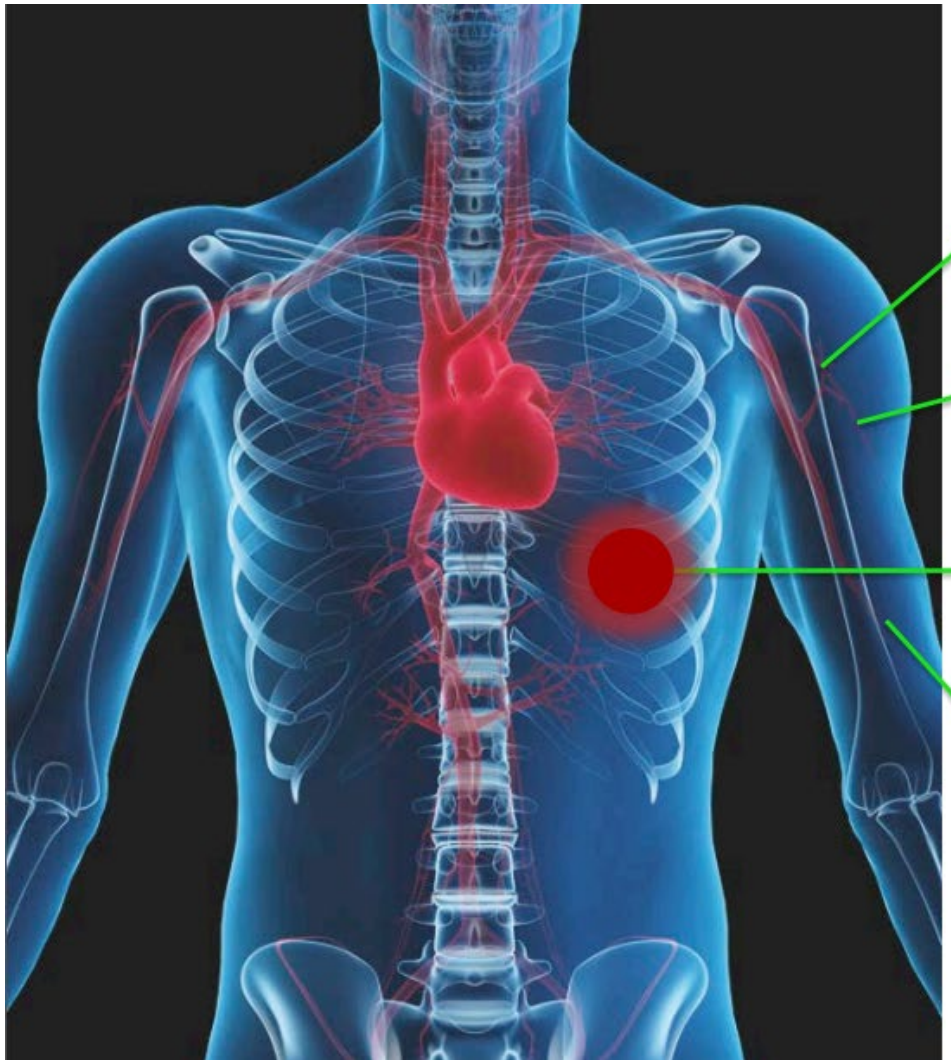
*The most clinically relevant mutation in exon 20.

1. Sharma et al. Nat Rev Cancer 2007;7:169–81.

FDA approved targeted therapies in solid malignancies (2020)

GENE TARGET	NGS	GENOMIC ALTERATION	MALIGNANCY	THERAPEUTIC AGENTS
*EML4-ALK**	+	Rearrangement	Lung Cancer	Crizotinib, Alectinib, Ceritinib, Brigatinib, Lorlatinib
*BRAF**	+	Mutation	Melanoma	Vemurafenib, Dabrafenib, Trametinib, Cobimetinib, Encorafenib, Binimetinib
		Mutation	Anaplastic thyroid cancer, lung cancer	Dabrafenib, trametinib
*BRCA1/2**	+	Mutation	Ovarian Cancer, Prostate Cancer	Olaparib, Niraparib, Talazoparib, Rucaparib
		Mutation	Triple negative breast cancer	Olaparib
*CKIT**	+	Mutation	GIST, Mastocytosis	Imatinib, Sunitinib, Regorafenib
*EGFR**	+	Mutation	Lung Cancer	Erlotinib, Gefitinib, Afatinib, Osimertinib, Dacomitinib
*EGFR**		Expression	Colon	Cetuximab, Panitumumab
			Lung Cancers	Necitumumab
*HER2**		Amplification, overexpression	Breast Cancer	Trastuzumab, Lapatinib, Pertuzumab, Ado-trastuzumab emtansine, Neratinib
		Amplification, overexpression	Gastric Cancer	Trastuzumab
*FGFR3, FGFR2**	+	Mutation	Bladder cancer	Erdafitinib
*Homologous Recombination Deficiency (HRD)**		Composite	Ovarian cancer	Niraparib
*C-KIT**	+	Mutation, expression	GIST	Imatinib, sunitinib, regorafenib
*Mismatch Repair (MMR)**	+	Expression, mutation	Tumor-agnostic, MSI-H Cancers	Pembrolizumab
		Expression, mutation	Colorectal MSI-H Cancers	Nivolumab
*NTRK**	+	Fusion	Tumor-agnostic, NTRK+ cancers	Entrectinib, larotrectinib
*PDGFRA**	+	Mutation	GIST, Sarcoma	Imatinib, Sunitinib, Olaratumab
COL1A1-PDGFB	+	Rearrangement	Dermatofibrosarcoma protuberans	Imatinib
*PDL-1**		Expression	Lung, triple negative breast, urothelial, cervical cancer	Pembrolizumab, atezolizumab
*PI3K**	+	Mutation	Breast Cancer	Alpelisib
SMO and *PTCH1*	+	Mutation	Basal Cell Carcinoma	Vismodegib, Sonidegib
*K-RAS**	+	Mutation	Colon cancer	Cetuximab, panitumumab (in RAS-non mutated)
*RET**	+	Mutation	Thyroid Cancer	Vandetanib, Cabozantinib, Lenvatinib
*ROS-1**	+	Rearrangement	Lung cancer	Crizotinib, Entrectinib
VEGF/VEGFR		Expression	Kidney, Colon, Lung, Gastric, Cervix, Ovarian Cancers	Bevacizumab, Ramucirumab, Regorafenib, Ziv-aflibercept, Axitinib, Pazopanib, Sunitinib, Sorafenib
CDK4/6	+	Amplification	Breast Cancer	Palbociclib, Ribociclib, Abemaciclib
mTOR		Mutation	Breast, Renal, Brain Cancers	Everolimus, Temsirolimus
*Estrogen Receptor**		Expression	Breast Cancer	Tamoxifen, fulvestrant, anastrozole, letrozole, exemestane, everolimus, palbociclib, ribociclib, abemaciclib
Androgen Receptor		Expression	Prostate Cancer	Abiraterone, Enzalutamide, Apalutamide, Darolutamide

Other applications of DNA variant detection



+ microbiology

Inborn genetics:
- Genetic disease
- Risk factors

Disease Genetics:
- Early screening

Disease Genetics:
- Diagnosis
- Prognosis
- Therapy

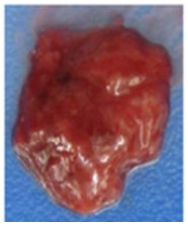
Disease Genetics:
- Residual disease testing
- Resistance mutation surveillance

NGS versus traditional methods

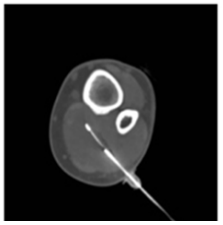
Benefits in oncology

- ✓ Multiple anomalies at different genomic scales can be assayed simultaneously
- ✓ More sensitive than Sanger sequencing
- ✓ Single extraction and single test instead of multiple tests
  - Cost effective
  - Improved turn-around time by avoiding sequential testing
  - Tissue preservation – many genes simultaneously assessed from single extraction
- ✓ Potential for discovery of novel actionable targets
- ✓ Extreme flexibility of analysis types
  - Many different genomic target types can be detected

Pre-analytics



Resection biopsies



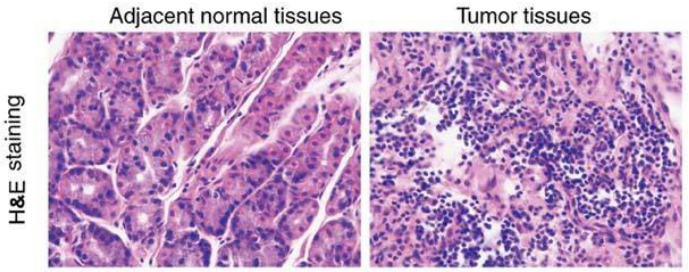
Needle biopsy

FFPE = <u>Formalin Fixed</u> & <u>Paraffin Embedded</u>

preservation tissue structure

storage and handling

Recommendations



- **min. 10% neoplastic cells**
- **tumor enrichment (macrodissection)**
- **no necrotic tissue or normal tissue**
- **fixation: min 6h, max 48h, in 10% neutral buffered formalin solution**
- **FFPE storage: max ± 3years**

INFORMATIONS SUR L'ECHANTILLON

N° identification A-P : …… Diagnostic A-P : ……

…… ……

Date du prélèvement : ……../……../…….. Heure du prélèvement : ……..

Date de fixation : ……../……../…….. Heure de fixation : ……

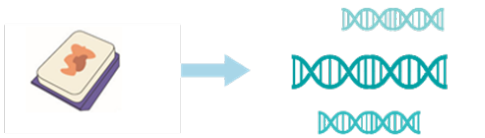
Temps de fixation : ☐ inconnu ☐ < 6 heures ☐ 6 – 48 heures ☐ > 48 heures

Fixateur : ☐ Formol 4% pH neutre ☐ autre fixateur : ……..

Nombre de blocs de paraffine envoyés : …..

Nombre de lames blanches envoyées : …… date de coupe : …./…./…..

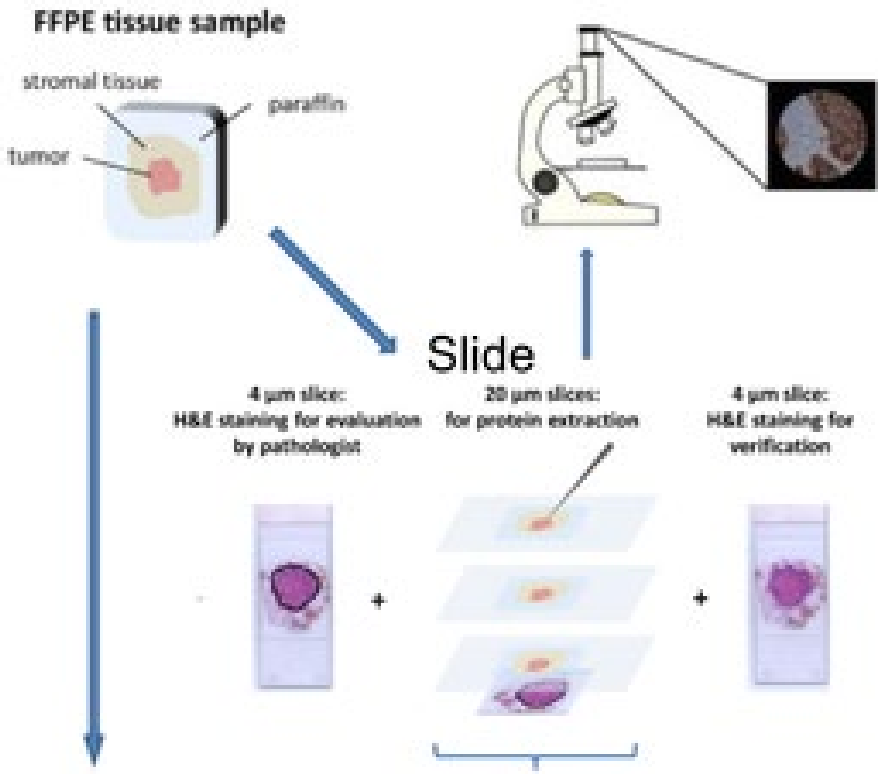
Extraction Workflow

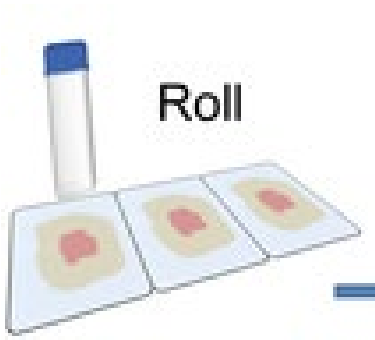


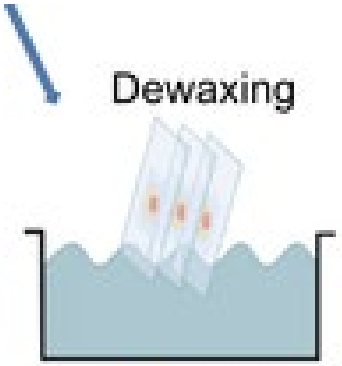
FFPE samples

RNA extraction

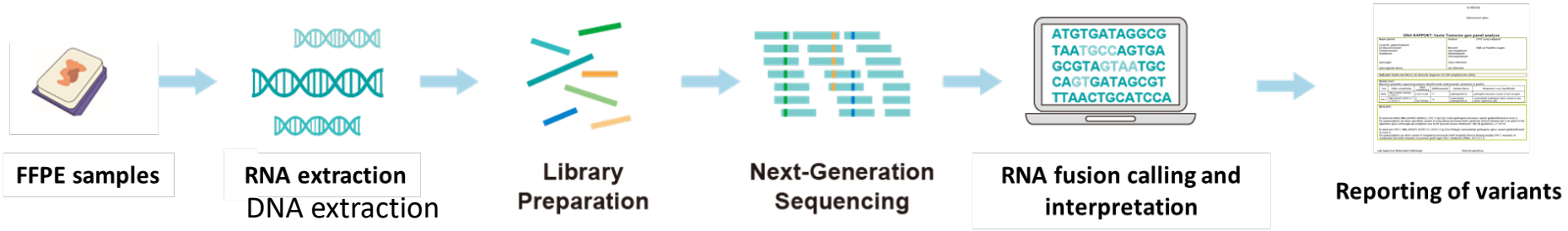
DNA extraction







NGS workflow in Molecular Pathology



FFPE samples

RNA extraction
DNA extraction

Library Preparation

Next-Generation Sequencing

RNA fusion calling and interpretation

Reporting of variants

Nucleic acids extraction + QC 1 day

Library prep 1.5-2 days

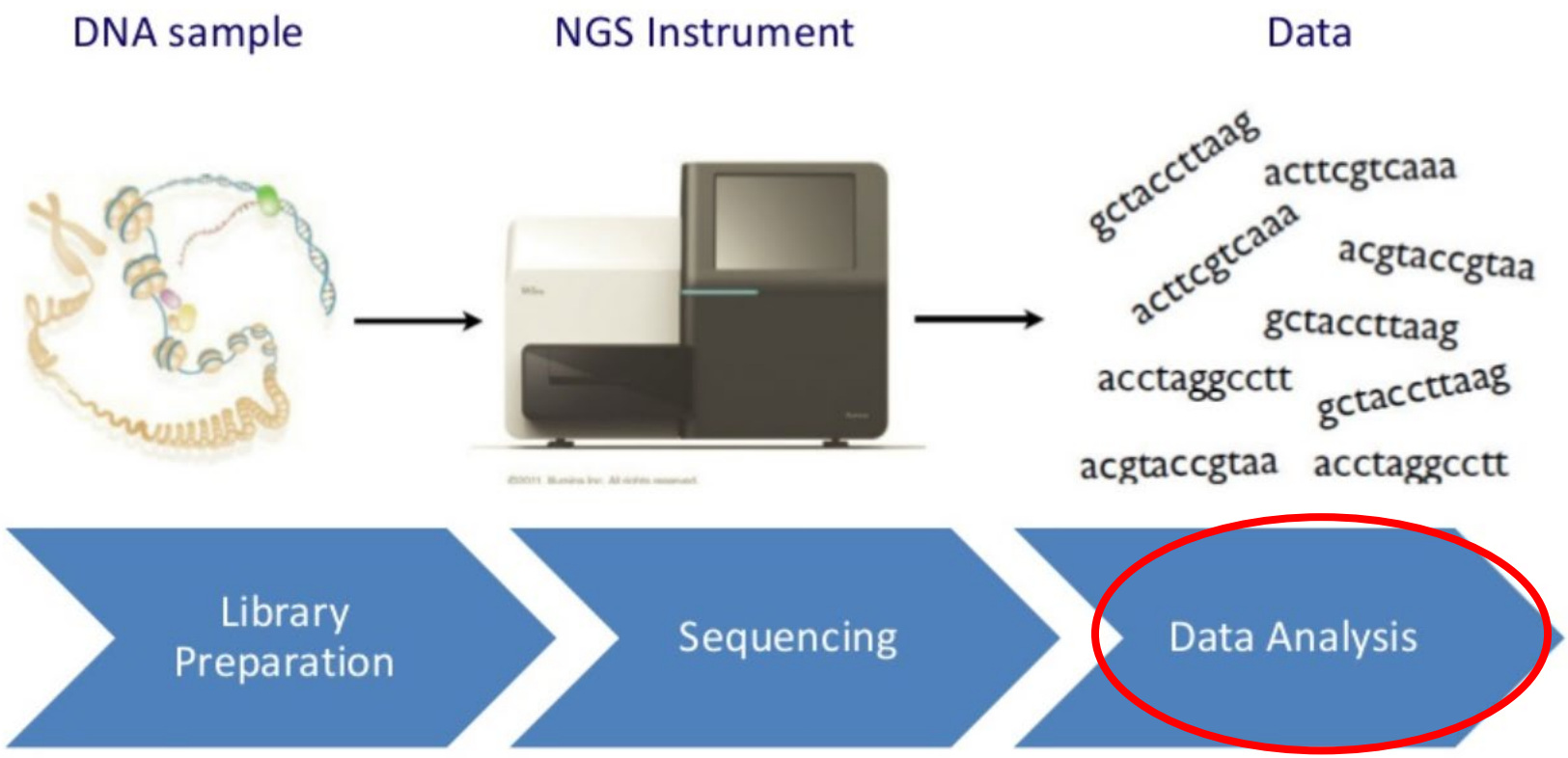
next-generation-sequencing 1-2 days

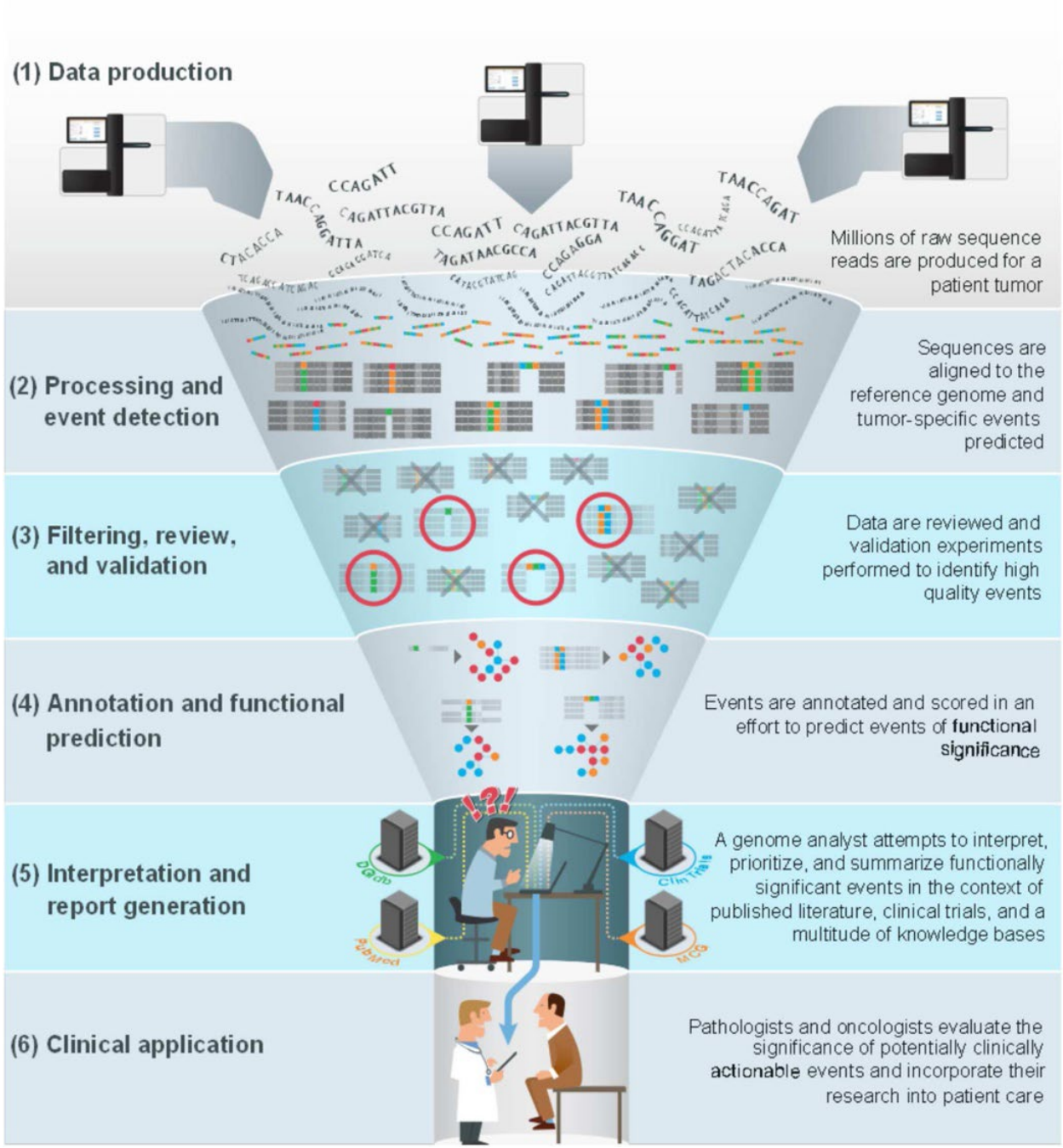
Primaary and secondary analysis 1 day

variant interpretation – reporting 1-2 days/run

15 days TAT recommended

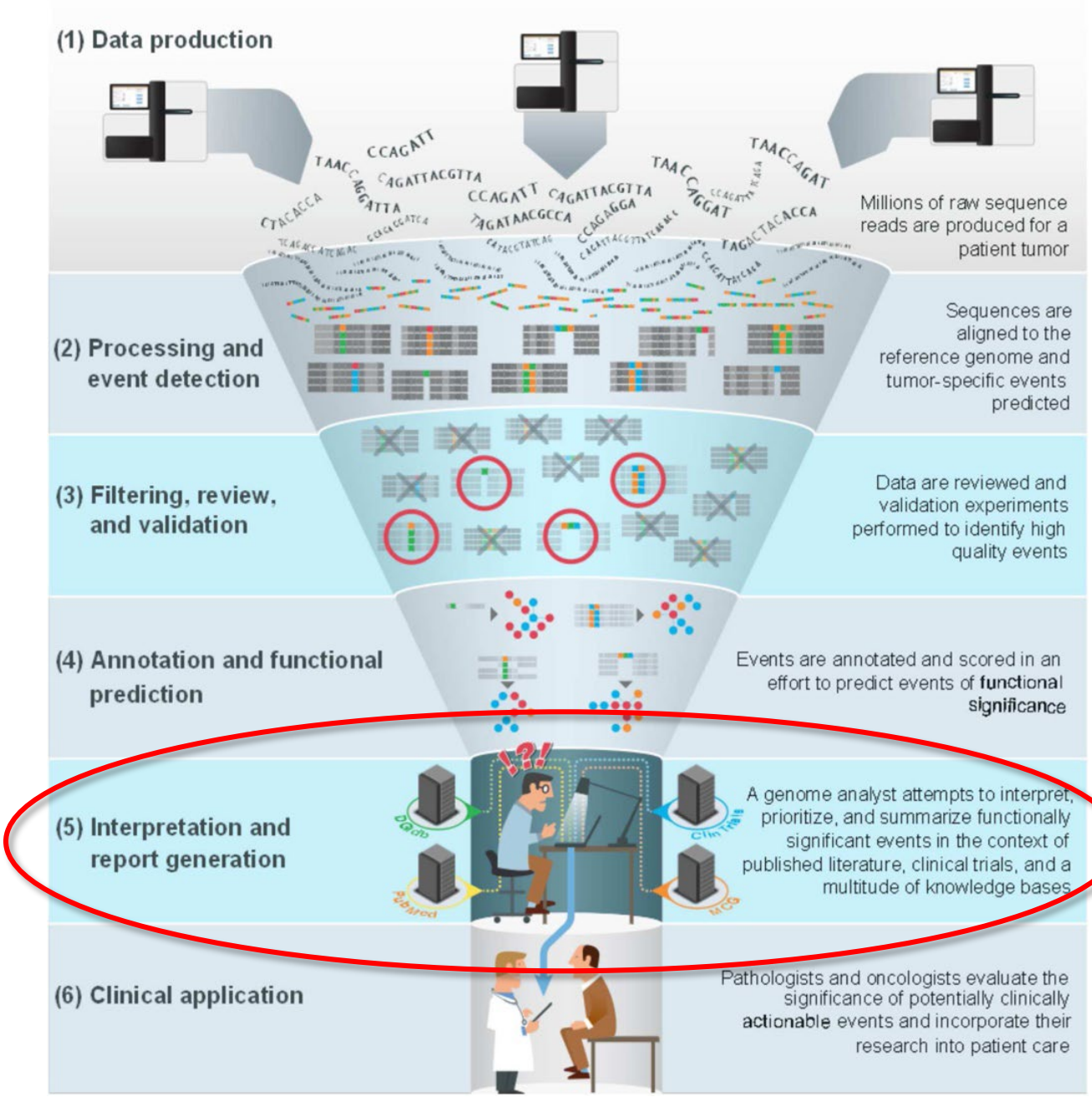
Basic workflow for NGS sequencing technologies





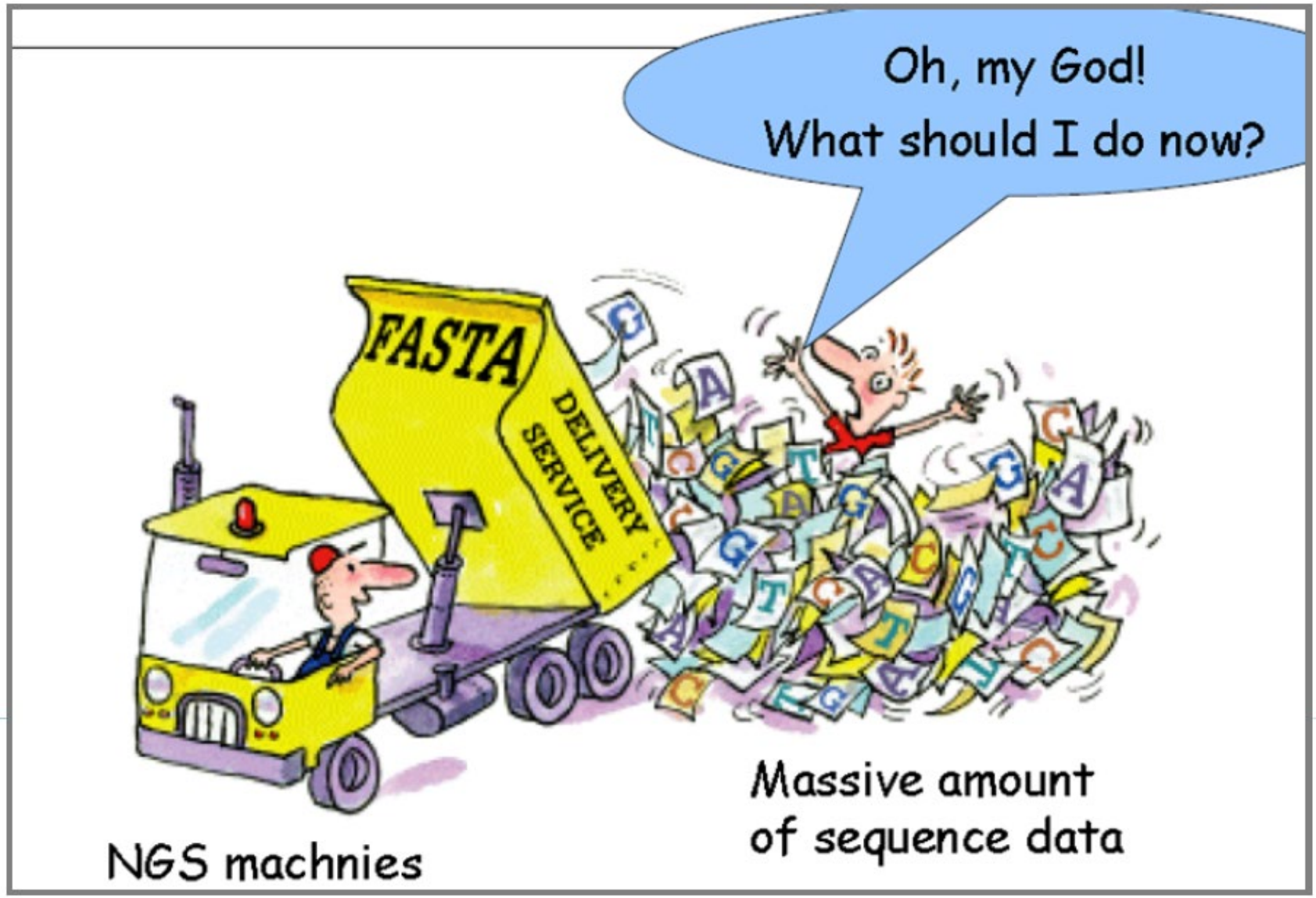
NGS reactions generate huge sequence data sets in the range of megabases (millions) to gigabases (billions)

Source: Good et al. Genome Biology 2014, 15:438



most severe bottleneck of the process

Source: Good et al. Genome Biology 2014, 15:438


Oh, my God!
What should I do now?
FASTA
DELIVERY SERVICE
NGS machnies
Massive amount
of sequence data

Importance of bioinformatics

Need of bioinformatics team / tools for:

- ✓ the sequence alignment
- ✓ variant calling
- ✓ variant filtration

=> to make sense of the huge amount of NGS data.

Bioinformatics…

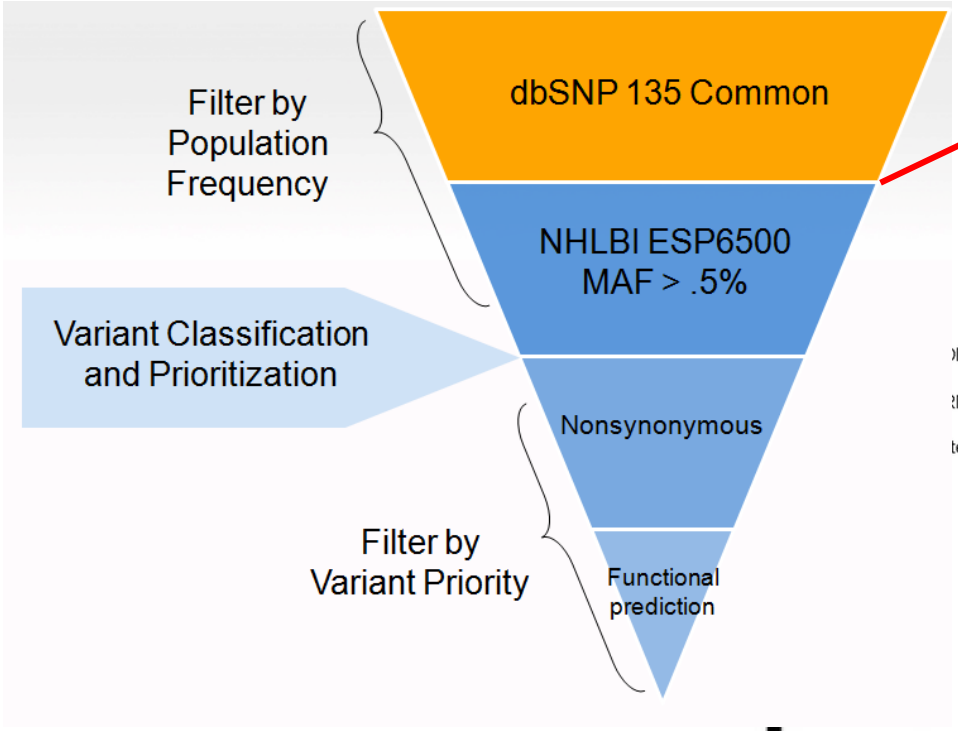
In house OR Commercial softwares available:

- ✓ Highlander
- ✓ SophiaGenetics
- ✓ NetxGNEe (Softgenetics)
- ✓ DataReporter softwares from Illumina, Agilent, QiaGEN,..
- ✓ …

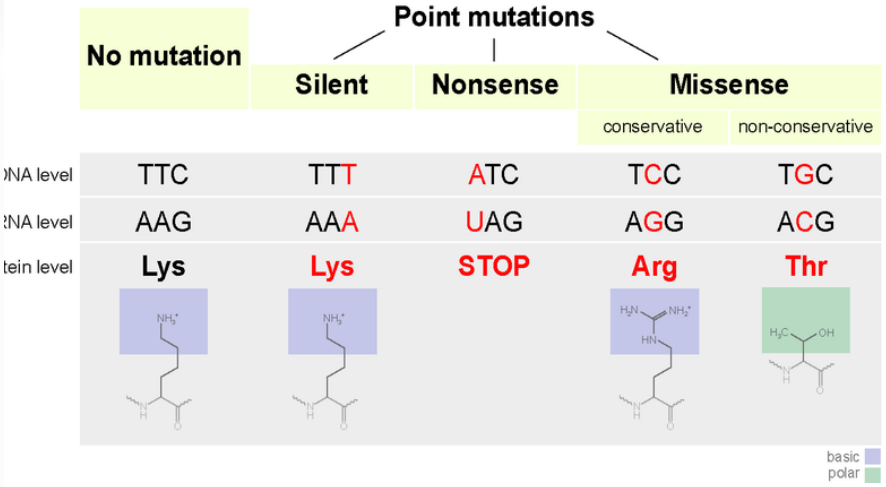
BUT even after bioinformatics data analysis and filtration, NGS data still needs manual interpretation of those identified genes and variants.

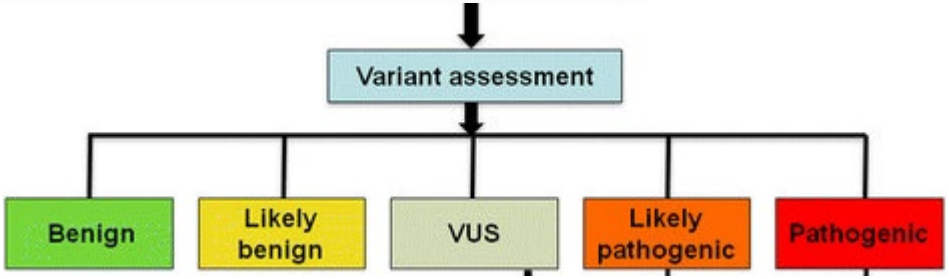
Tertiary analysis

- Variant analysis – Biological Interpretation

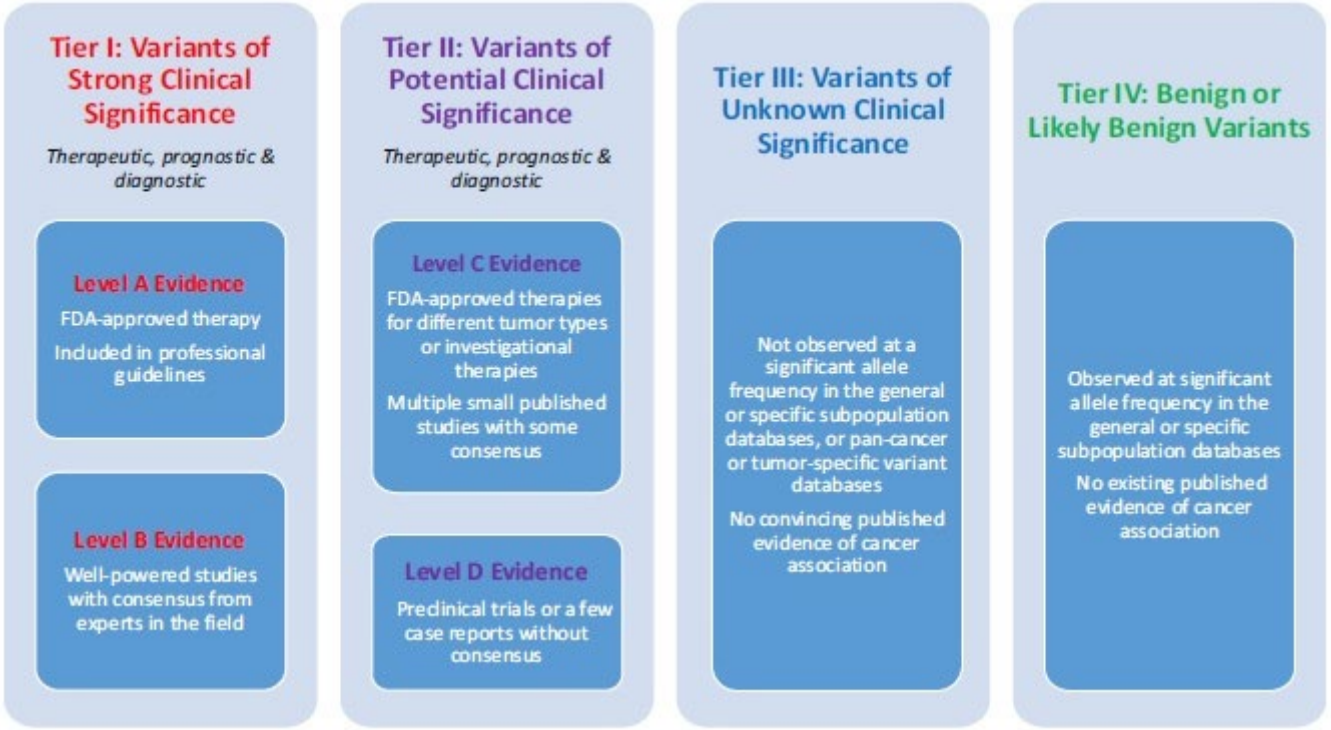


Common polymorphisms





Tertiary analysis



- Li, MM *et al.* Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer. A Joint Consensus Recommendation of the Association for Molecular Pathology, Americal Society of Clinical Oncology, and Collage of American Pathologists. *J Mol Diagn 2017*, 19: 4-23.

- Sukhai, M. A. *et al.* A classification system for clinical relevance of somatic variants identified in molecular profiling of cancer. *Genet. Med.* 1–9 (2015). doi:10.1038/gim.2015.47

Databases for clinical classification

Various ones with various criteria:

- ✓ COSMIC
- ✓ cBioPortal
- ✓ My Cancer Genome
- ✓ ClinVar
- ✓ PubMed
- ✓ …

⇒ Do not limit yourself to one option!

⇒ Look at: date of last update, levels of evidence, variant/tumor type/drug combination, …

cancer.sanger.ac.uk

COSMIC
Catalogue Of Somatic Mutations In Cancer

Projects | Data | Tools | News | Help | About | Search COSMIC... SEARCH | Login

Gene
KIT

GRCh38 · COSMIC v82

- Gene view
- Overview
- External links
- Drug resistance
- Tissue distribution
- Genome browser
- Mutation distribution
- Variants
- References

Reset page

Filters

Show advanced filters

Range Show input fields

1 — 523 — 560 — 977

1 245 489 733 977

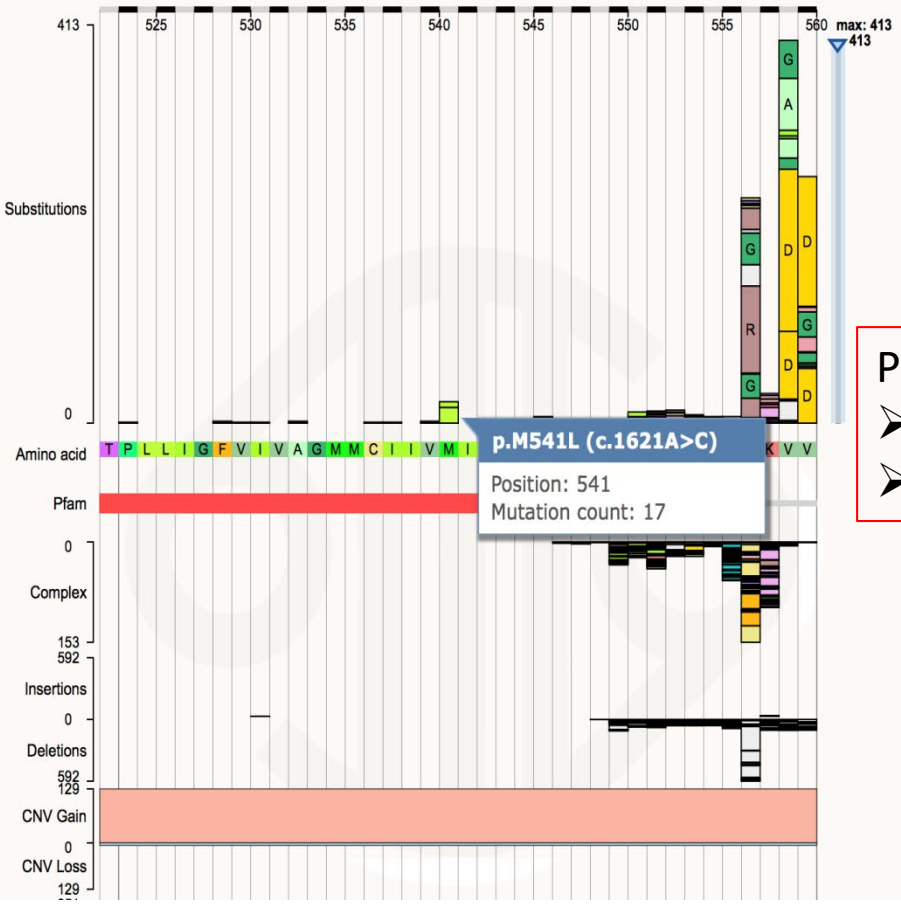
Coordinate system

- Amino-acid
- cDNA

Apply filters Reset filters

Gene view

The gene view histogram is a graphical view of mutations across the gene. These mutations are displayed at the amino acid level across the full length of the gene by default. Restrict the view to a region of the gene by dragging across the histogram to highlight the region of interest, or by using the sliders in the filters panel to the left. Show more

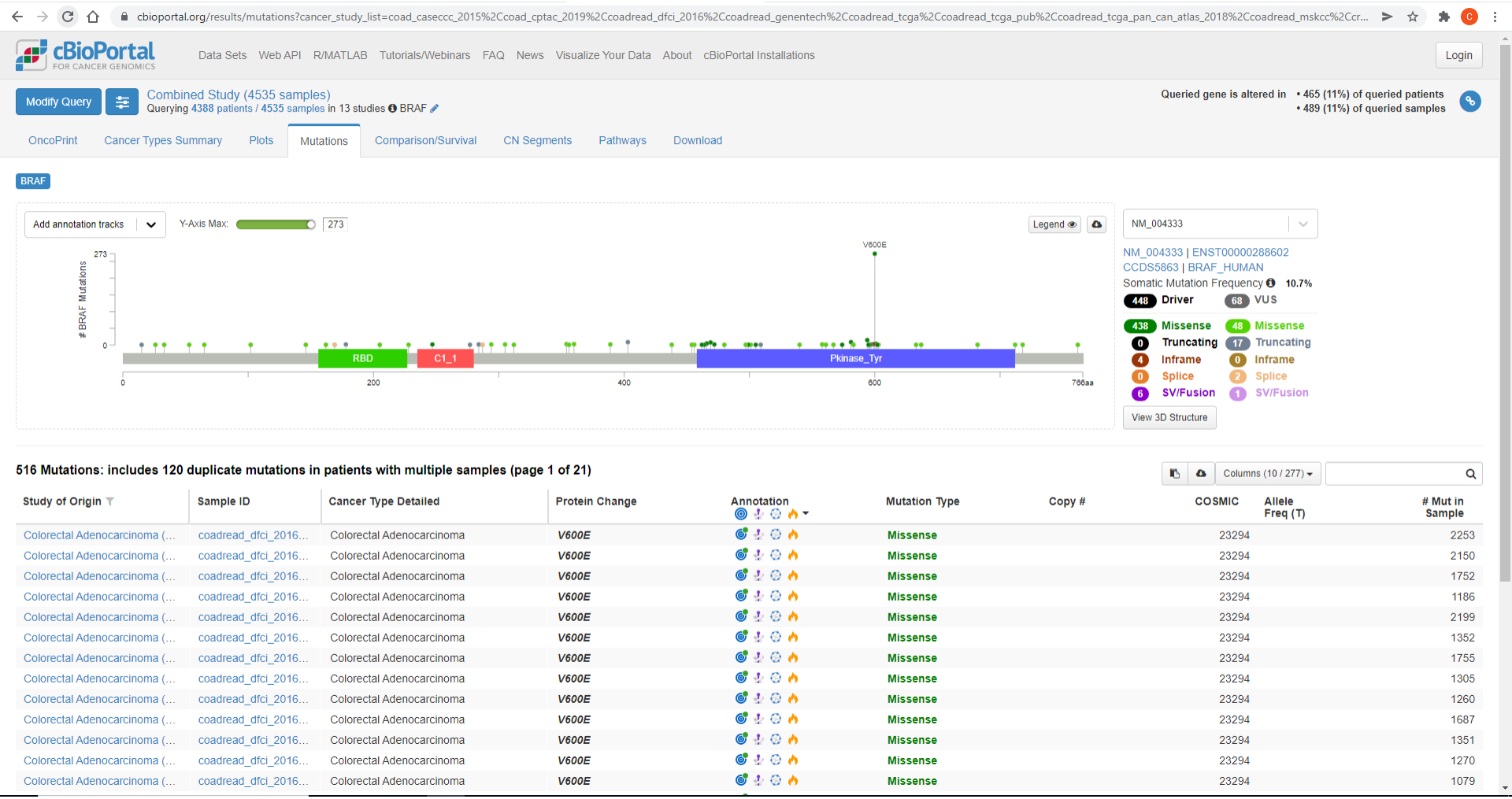


Presumed pathogenic mutation

- Clinical relevance?
- How to report to oncologist?

Databases for clinical classification

https://www.cbioportal.org/



Databases for clinical classification
https://www.mycancergenome.org

MY CANCER GENOME ®
GENETICALLY INFORMED CANCER MEDICINE

Clinical Trials | Diseases | Biomarkers | Drugs | Pathways

Biomarkers / BRAF V600E

Back to Biomarkers List

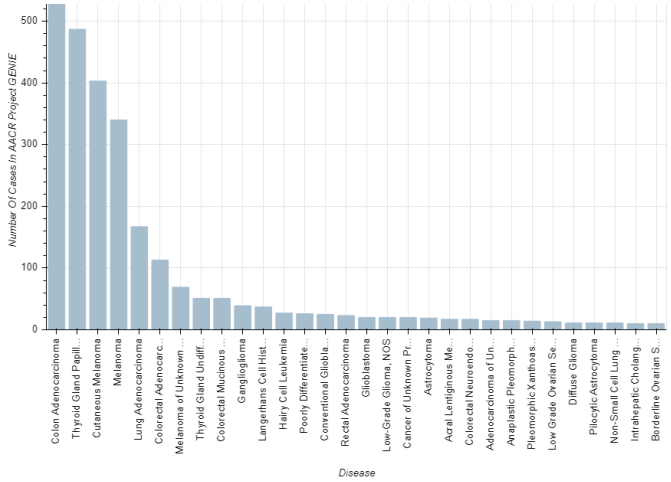
- **Associated Genetic Biomarkers**
- **Associated Diseases**
- **Associated Pathways**

Overview

Gene Location [1]	7q34
Pathways	Kinase fusions, MAP kinase signaling
Variant Type	Substitution - Missense
Affected Exon Number	15
Gene	BRAF
Protein Domain [2]	Protein kinase
SIFT Prediction [3]	Deleterious
ClinVar Prediction [3]	Pathogenic

BRAF V600E is present in 3.05% of AACR GENIE cases, with colon adenocarcinoma, thyroid gland papillary carcinoma, cutaneous melanoma, melanoma, and lung adenocarcinoma having the greatest prevalence [4].

Top Disease Cases with BRAF V600E



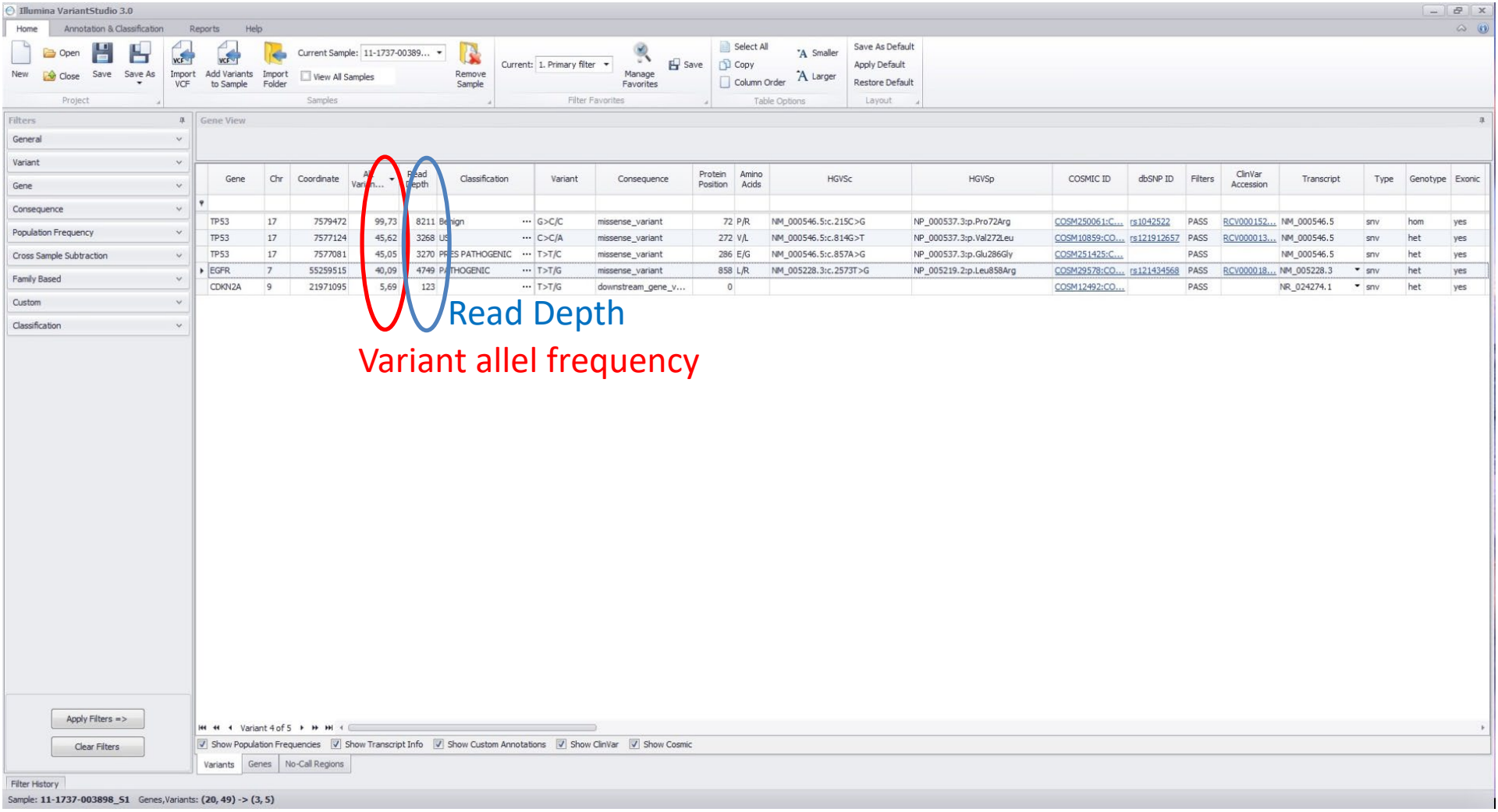
Clinical Trials

View Clinical Trials for BRAF V600E

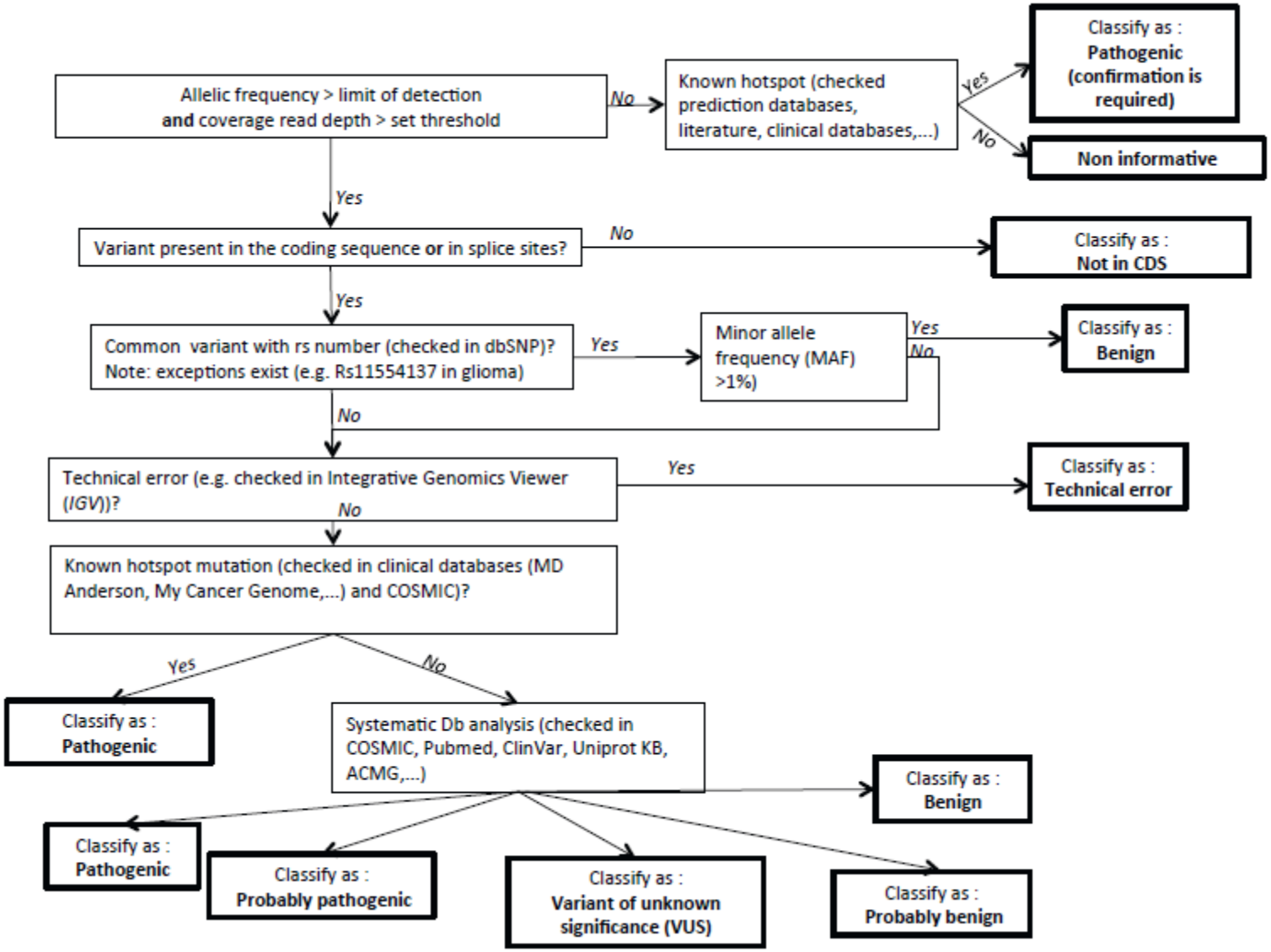
BRAF V600E serves as an inclusion eligibility criterion in 97 clinical trials, of which 71 are open and 26 are closed. Of the trials that contain BRAF V600E as an inclusion criterion, 1 is early phase 1 (1 open), 25 are phase 1 (17 open), 20 are phase 1/phase 2 (17 open), 44 are phase 2 (30 open), and 7 are phase 3 (6 open).

Trials with BRAF V600E in the inclusion eligibility criteria most commonly target melanoma, non-small cell lung carcinoma, colorectal carcinoma, malignant solid tumor, and multiple myeloma [5].

Example of output of commercial bioinformatics tool in a case of lung adenocarcinoma (Variant Studio, Illumina)



Classification of the variants



"Pathogenecity" versus "clinical relevance"

Source: A. Hébrant, G. Froyen, B. Maes, *et al*. Belg J Med Oncol 2017;11(2):56-67 : Modified with permission from Froyen et al., 2016.

Clinically relevant genes in ...

Colorectal cancers:

✓ *KRAS, NRAS, BRAF*

- Actionable mutations
  - Predictive for good response (sensitivity mutation)
  - Predictive for lack of response (resistance mutation)

Melanoma:

✓ *BRAF, NRAS, KIT*

Lung:

✓ *EGFR, KRAS, (ALK, ROS1)*

- Overlap – “solid tumor panel”
- Most gene panels also contain emerging targets or frequently mutated genes without clinical relevance at present
- Typical panel contains 15 to 50 genes

GIST:

✓ *KIT, PDGFRA*

- No world wide consensus on diagnostic gene panel composition
- ComPerMed initiative in Belgium

Importance of multidisciplinary approach

"molecular tumor board" – clinicians, radiologists, pathologists, geneticists, bioinformatician, …

Clinical case information:

- Age, diagnosis, stage, clinical status
- Prior treatments for metastatic cancer
- Measurable disease?
- Sample being tested
- Other clinically relevant information (clinical trial eligibility)
- Specific question

Specimen and/or molecular data:

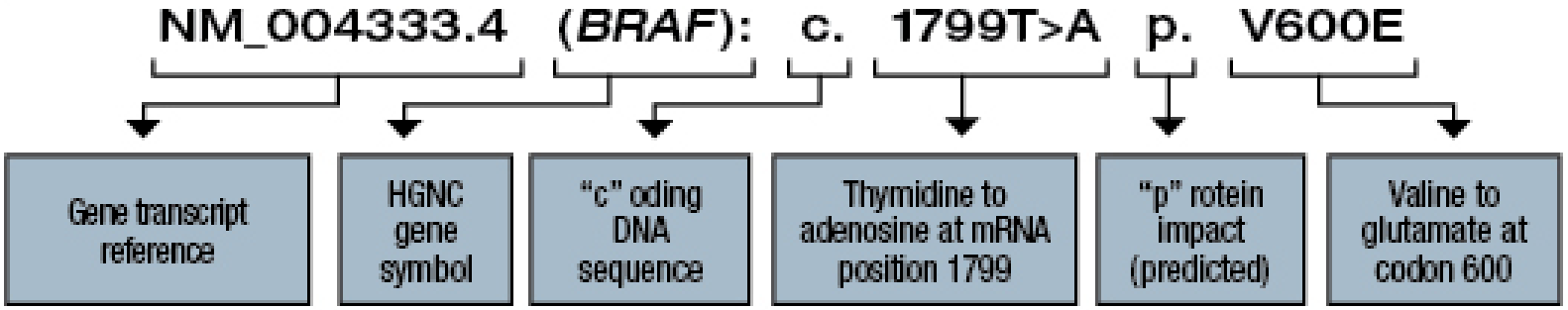
- Specimen for genomic testing
- Genomic test report

Nomenclature of variants

Reporting mutations

- Standardized nomenclature to promote portability, enduring meaning, and accuracy
- Human Genome Variation Society (HGVS): www.hgvs.org/mutnomen/

***BRAF* mutation analysis:**
Mutation detected in codon 600, exon 15 (GTG to GAG) of the *BRAF* gene that would change the encoded amino acid from valine to glutamate (p.Val600Glu)



Guidelines

- ✓ Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer (*American group*). MM. Li, M. Datto, EJ. Duncavage *et al*. The Journal of Molecular Diagnostics 2017;19(1).
- ✓ The **Belgian** next generation sequencing **guidelines** for haematological and solid tumours. A. Hébrant, G. Froyen, B. Maes, *et al*. Belg J Med Oncol 2017;11(2):56-67.
- ✓ Pathological diagnosis and molecular testing in non-small cell lung cancer: **Belgian guidelines**. P. Pauwels, M. Remmelink, D. Hoton, *et al*. Belg J Med Oncol 2016;10(4):123-131.
- ✓ RAS-testing in colorectal cancer: **Belgian guidelines**. A. Jouret-Mourin, C. Cuvelier, P. Demetter, *et al*. Belg J Med Oncol 2015;9(5):183-90.
- ✓ …

⇒ ***Flow charts for variant analysis*** ***(Biological classification of the variants)***

⇒ ***But no world wide standardization – Subjectivity remains***

NGS reporting - Main Challenges

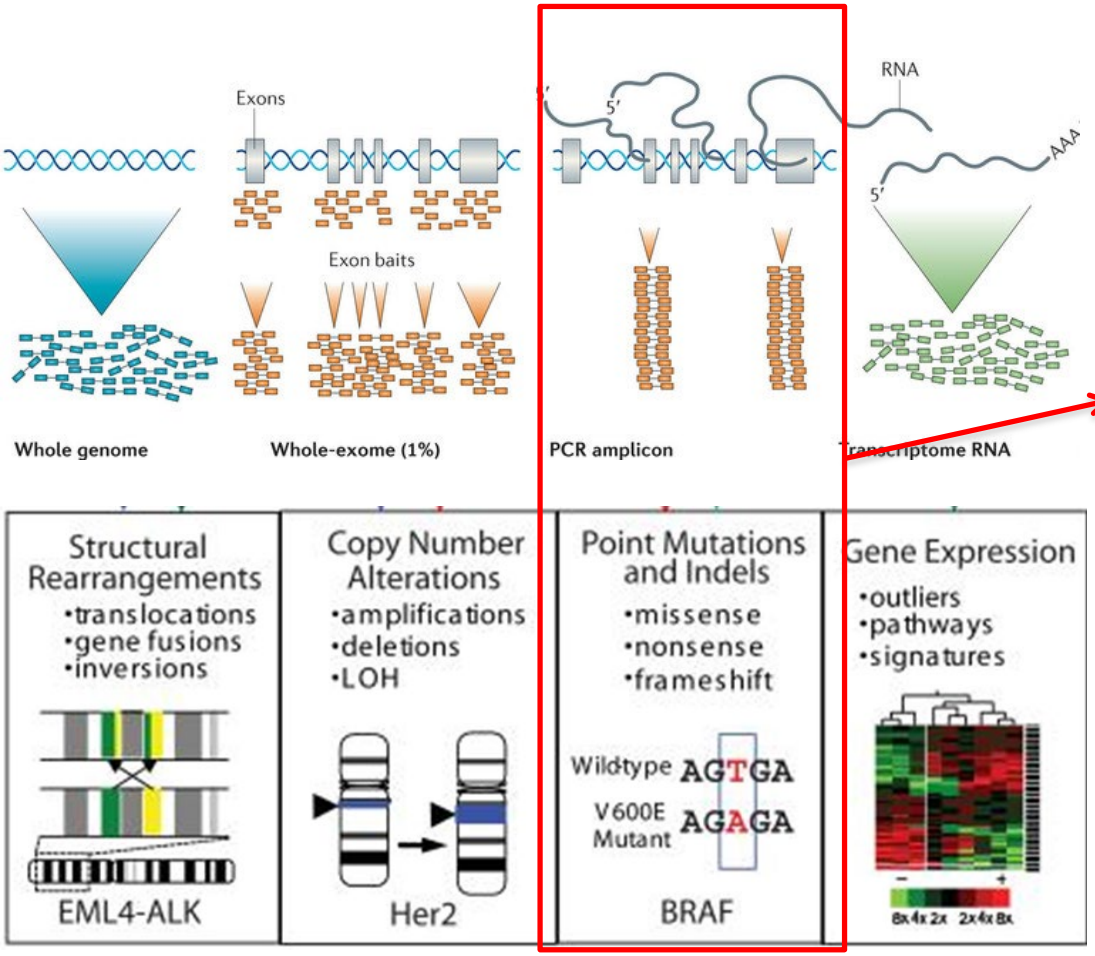
To interpret the clinical genomic data:

- ✓ accurately and unambiguously
- ✓ in a timely manner

- Significant number of mutated genes have been identified in the major tumor types, although only a limited set have been shown to be "driver" mutations
  - Of those, the number of "actionable" mutations remains limited
  - Challenge for report to clinic:
    - Only (future) actionable?
    - All pathogenic?
    - What about 'variant of unknown significance'?
- But knowledge is increasing rapidly – continuous traning of pathologists and molecular biologists required!
- Approved versus non-approved actionable mutation/drug combinations
  - No standardization in reporting, especially not in case of non-approved targets

NGS : application

Applications in Oncology → Clinical practice ???



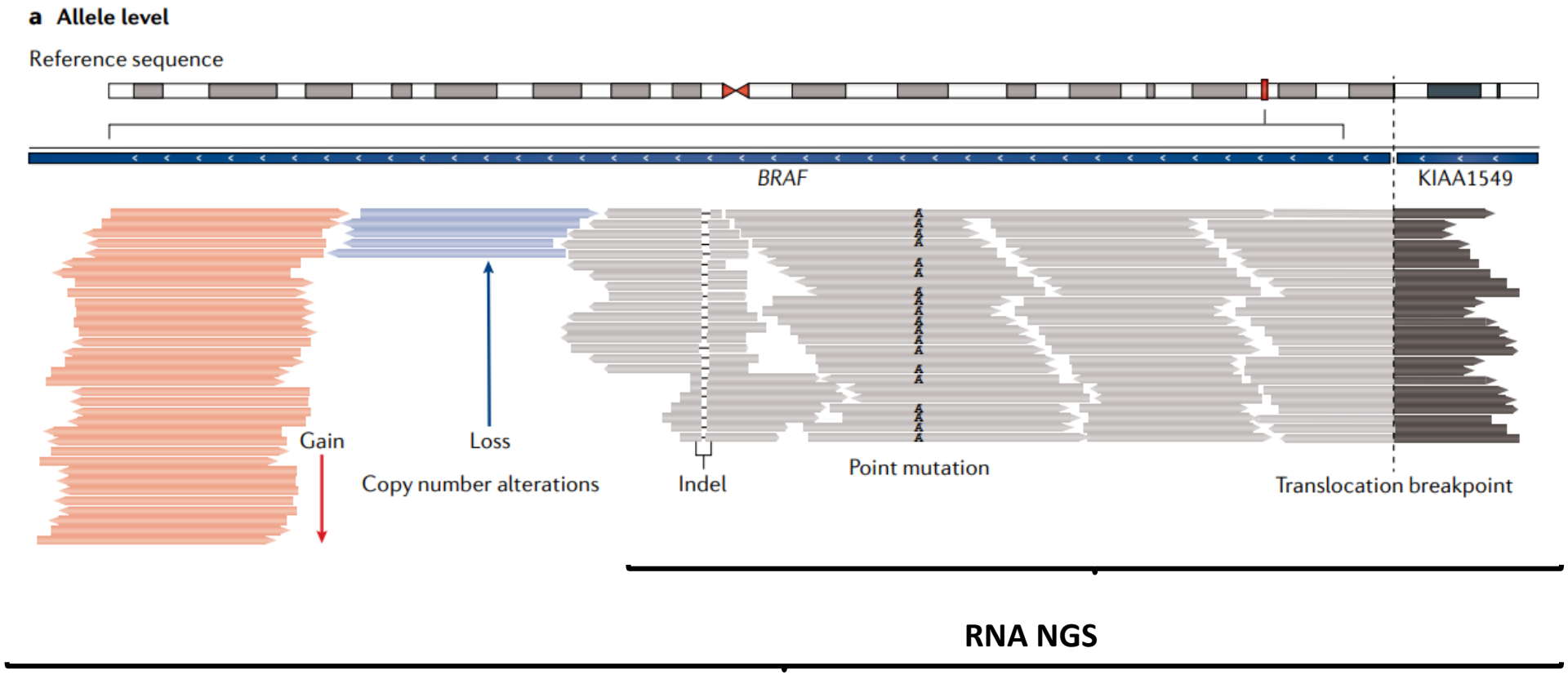
Mutations
EGFR
KRAS
ERBB2 → **Targeted NGS : Limited to a small number of region of interest**
PIK3CA
BRAF
NRAS
MET
.....

Already ok in clinical practice

Adapted from Roychowdhury et al. Sci Transl Med; 2011

NGS: variants and much more…

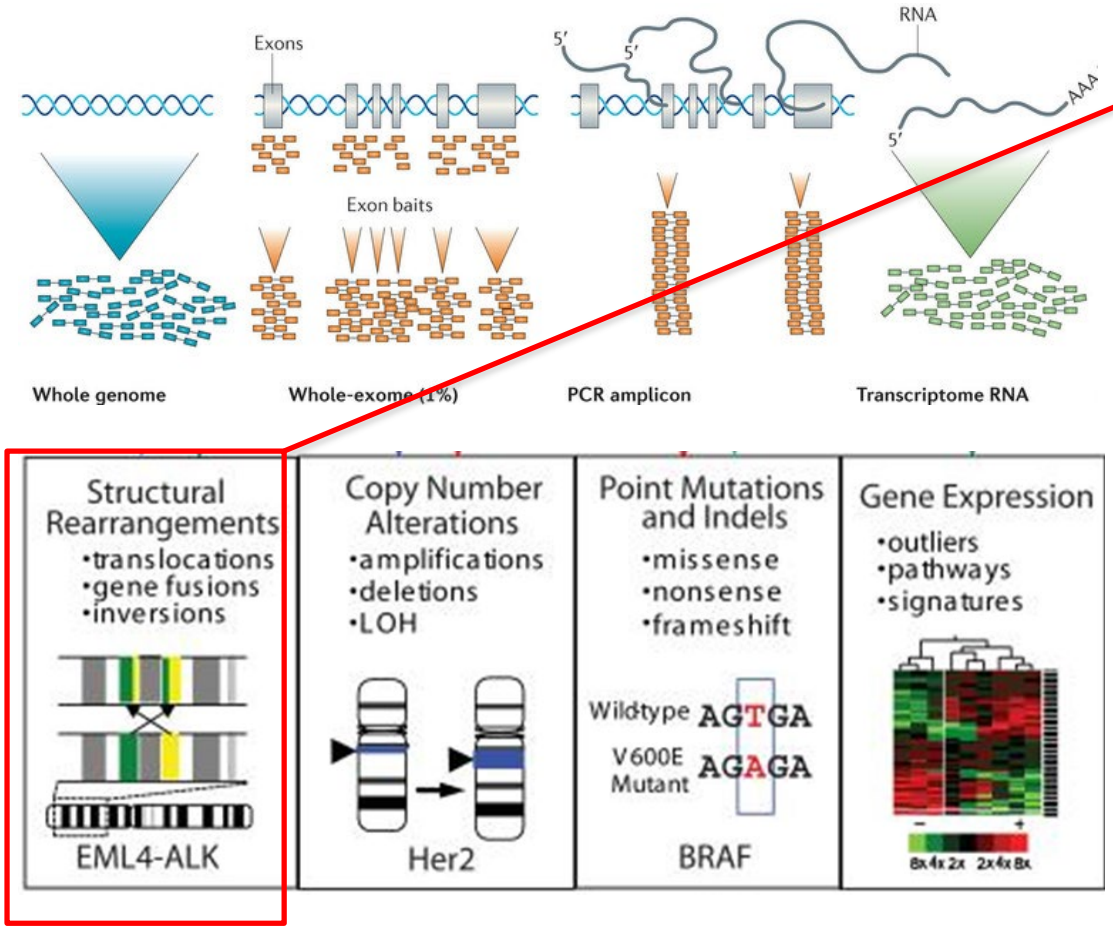
- detection of somatic defects with NGS
- copy number alterations, indels, point mutations, fusions, transcritptomics



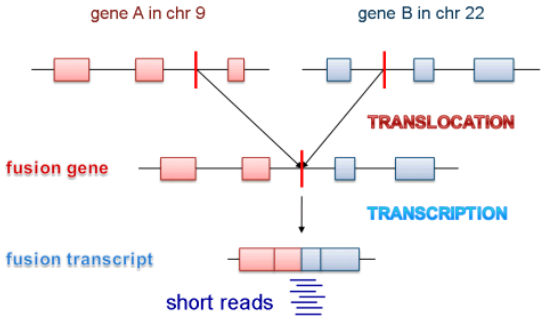
Chakravarty & Solit Nat Rev Genetics 2021

NGS Application in Oncology

Applications in Oncology → Clinical practice ???

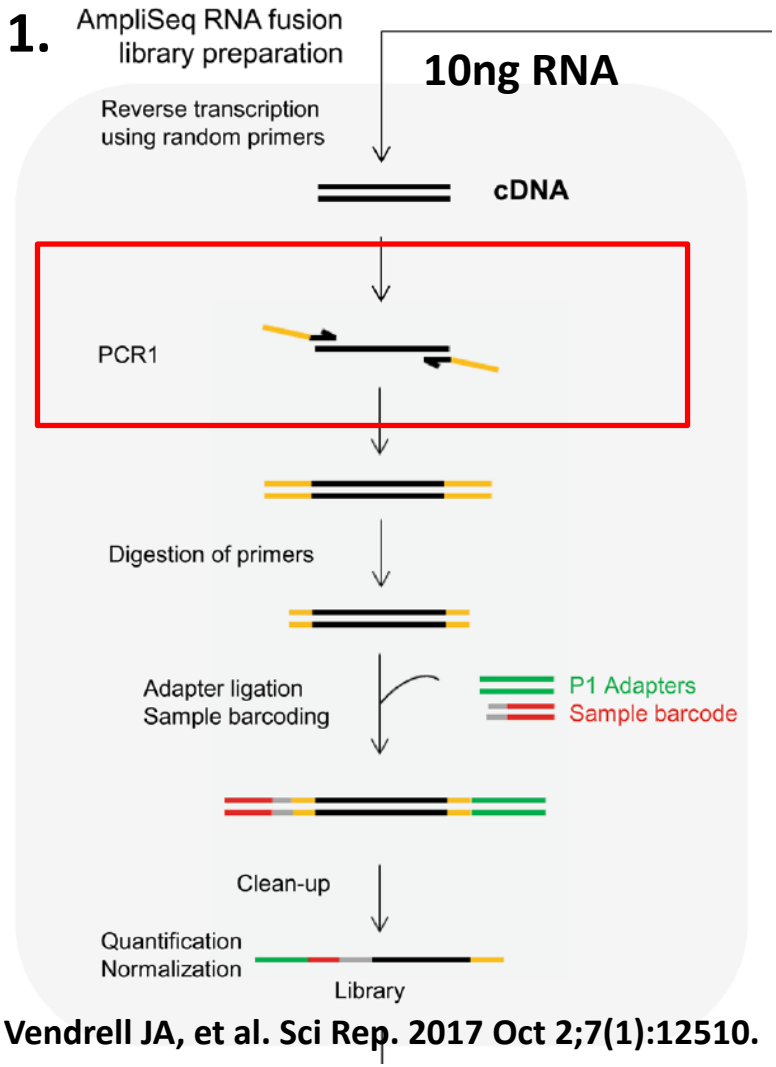


Rearrangements
EML4-ALK
CD74-ROS1 ⟶ **RNA Seq**
KIF5B-RET **Detection of fusion transcript**
……

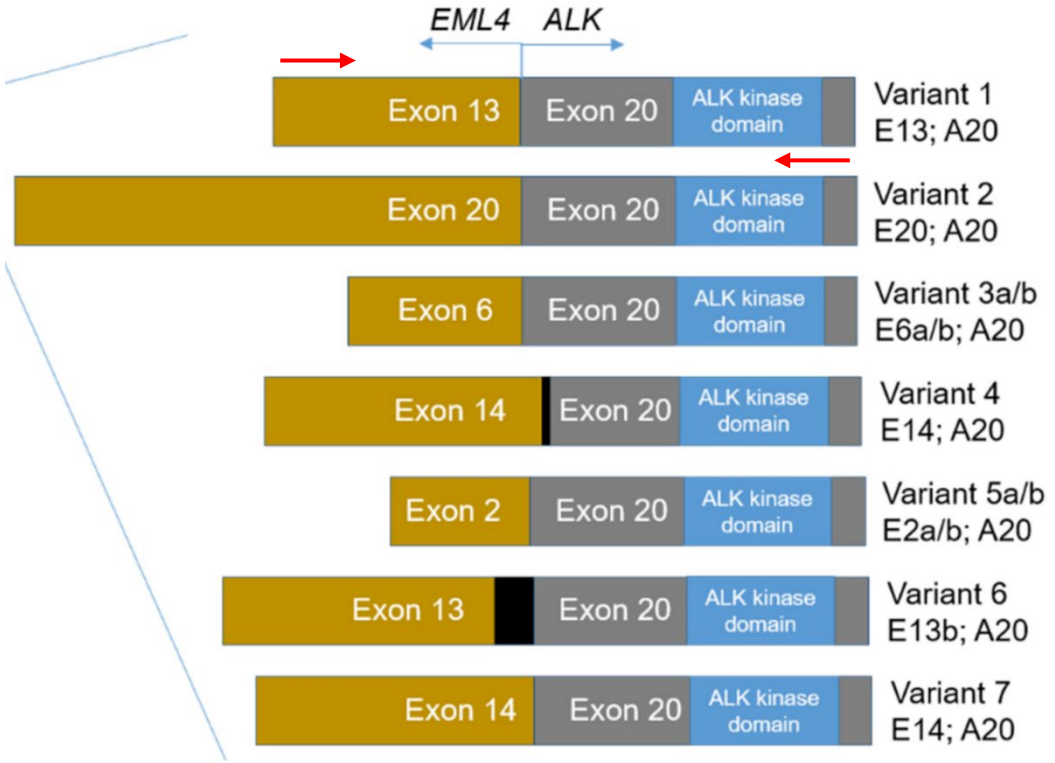


Adapted from Roychowdhury et al. Sci Transl Med; 2011

Fusion dectection using AmpliSeq ThermoFisher Scientific

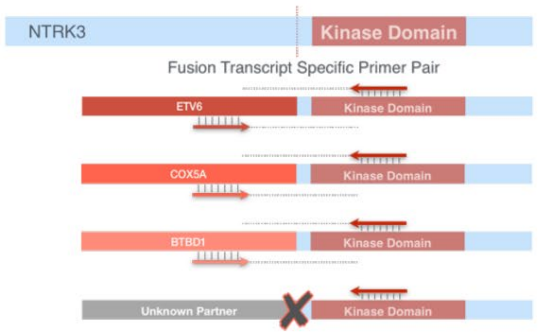


=> Gene specific primers

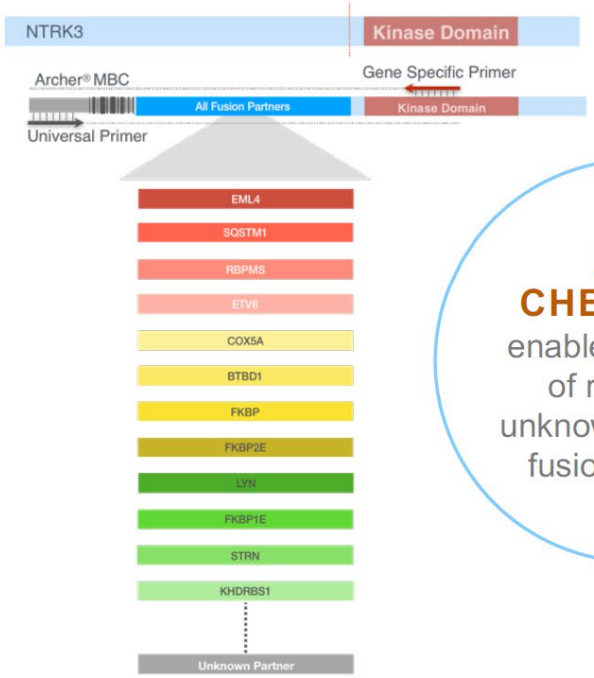


Vendrell JA, et al. Sci Rep. 2017 Oct 2;7(1):12510.

- Archer FusionPlex library prep
- Anchored Multiplex PCR (AMP) technology



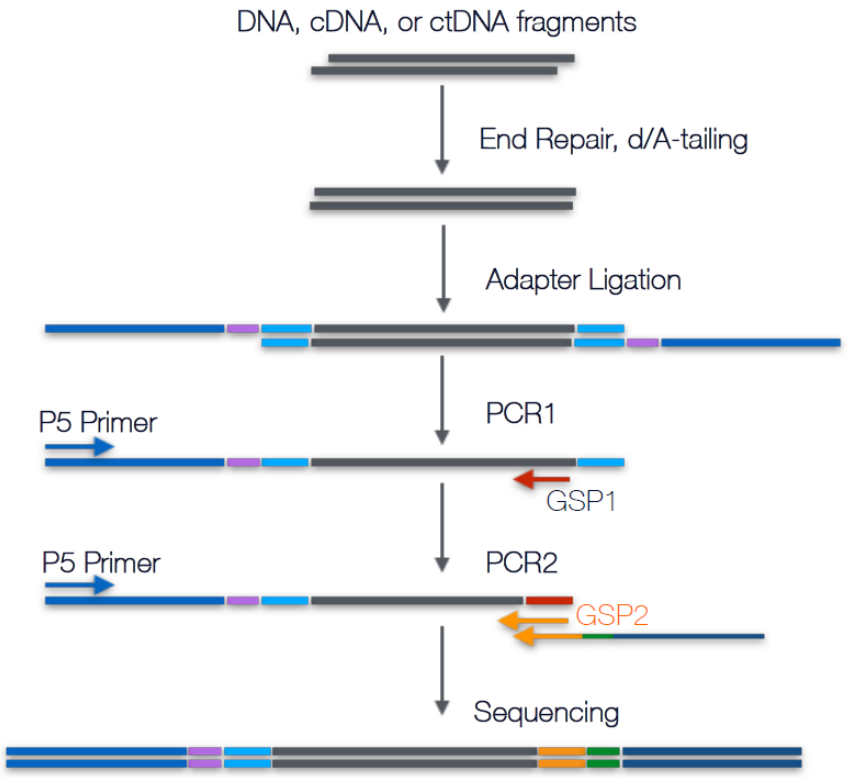
OPPOSING PRIMER TECHNIQUES limit detection to known fusion partners only

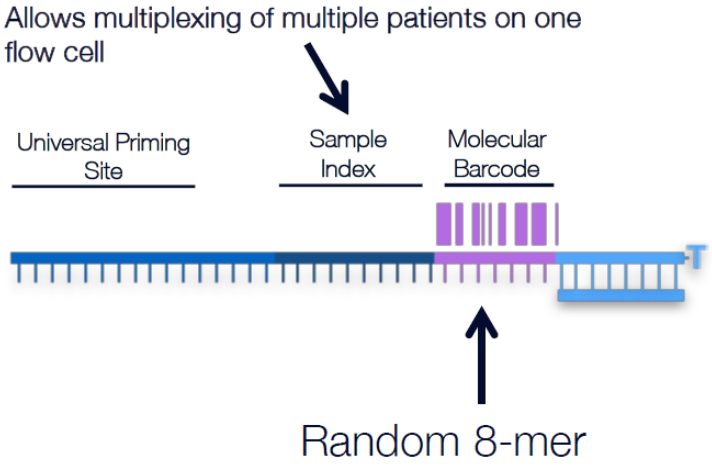


AMP CHEMISTRY enables detection of recurrent, unknown, and rare fusion partners

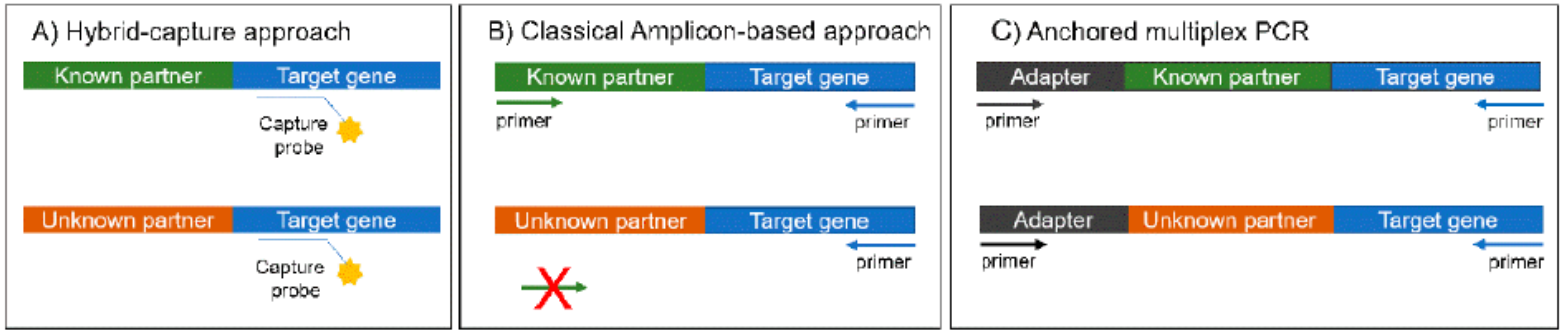
→ only 1 fusion partner needs to be included in the Archer FusionPlex assay to pick up the other fusion partner gene!

- Archer FusionPlex library prep
- Anchored Multiplex PCR (AMP) technology





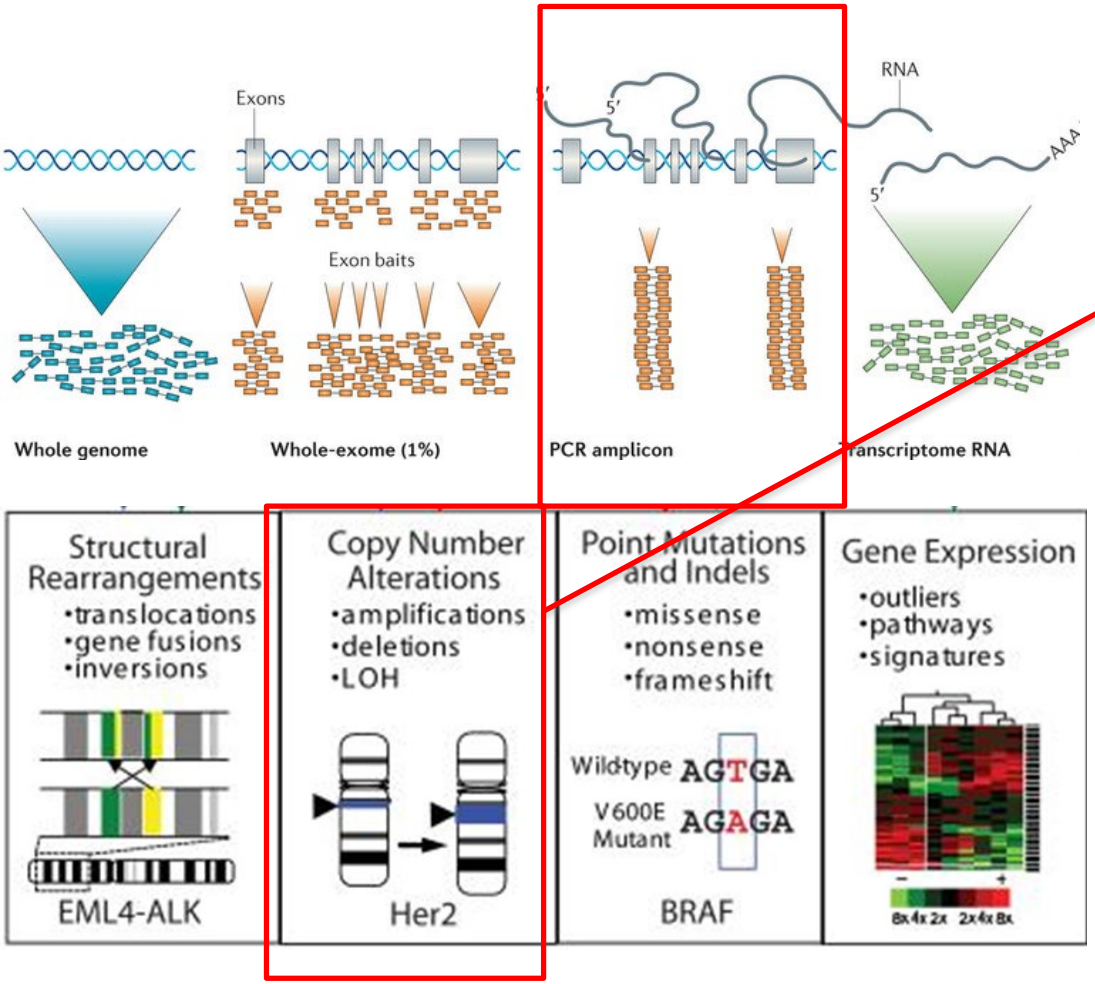
	PROS	CONS
Hybrid-capture	• Characterization of both known and unknown fusion variants of target genes • Easily scalable to large gene panels • Adequate for DNA and RNA gene fusion analysis • At DNA level it does not require RNA purification and allows a simultaneous analysis of different gene variants	• Higher RNA input than amplicon-based methods • Difficulty with fusion variants involving large DNA intronic regions with repetitive sequences
Amplicon-based: ➢ *Classical multiplex PCR (mPCR)* ➢ *Anchored multiplex PCR*	• Low RNA input • Particularly effective with small and mid-size panels • Analysis of both known and unknown fusion variants of target genes (anchored mPCR) • 5′ and 3′ imbalance evaluation can increase test diagnostic accuracy	• Not adequate for gene fusion analysis at DNA level • Primer design can be complex • Characterization of only known fusion variants included in the panel (classical mPCR) • PCR bias like allele dropout can impact on analysis result



Bruno R, et al. Diagnostics (Basel). 2020 Jul

NGS Application in Oncology

Applications in Oncology → Clinical practice ???



Amplification / deletion
ERBB2
EGFR → **Coverage analysis**
MET
....

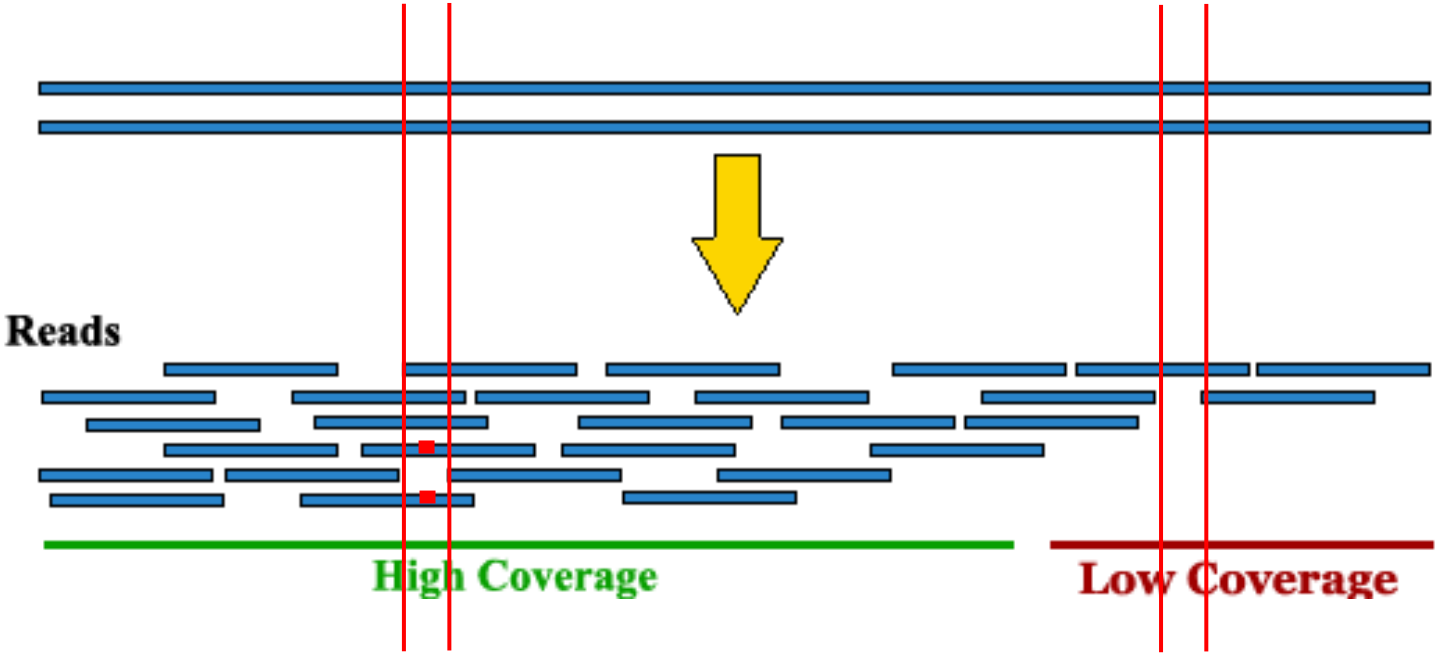
Adapted from Roychowdhury et al. Sci Transl Med; 2011

Coverage

- ✓ Sequence **coverage** (also called "**depth**") refers to the average number of times a base pair is sequenced in a given experiment.
- ✓ Minimum coverage should be determined during the validation to avoid false negative and false positive results

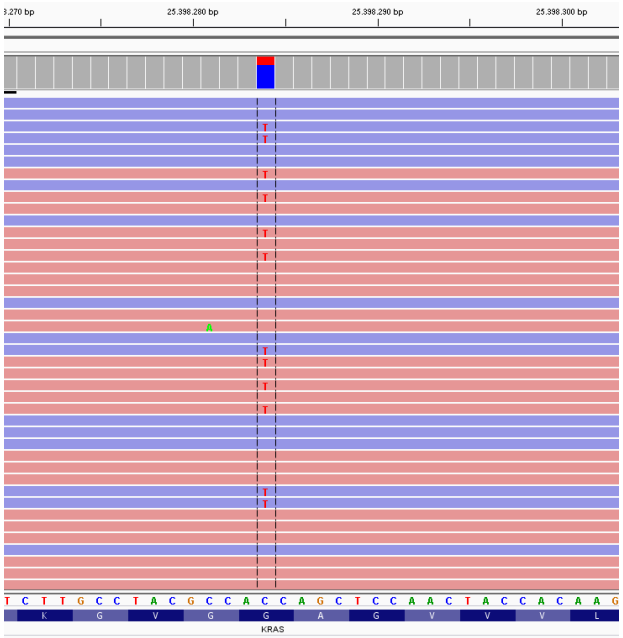
– horizontal coverage

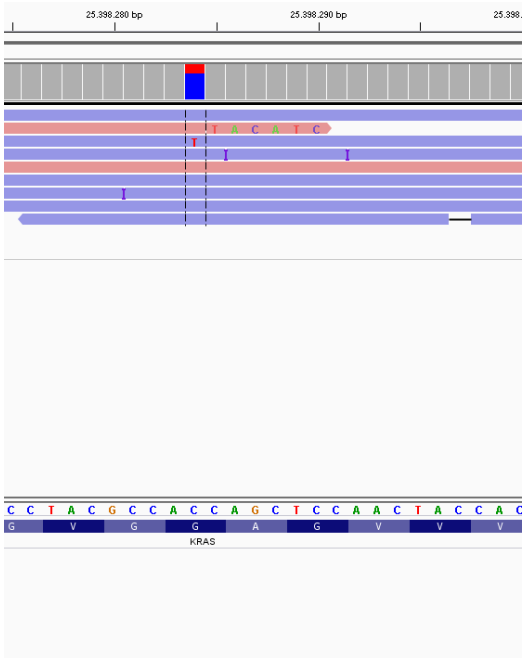
– Vertical coverage : coverage depth (x)



Coverage

Definition : number of times a nucleotide (or a region) is read during the sequencing process.





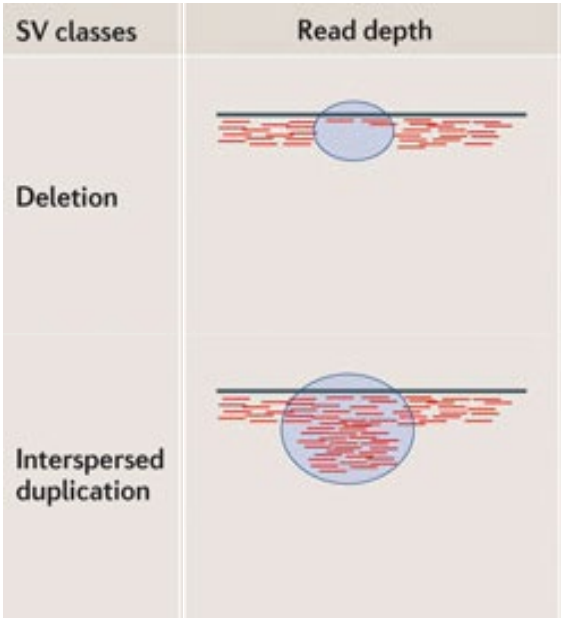
Sensitivity = 5%

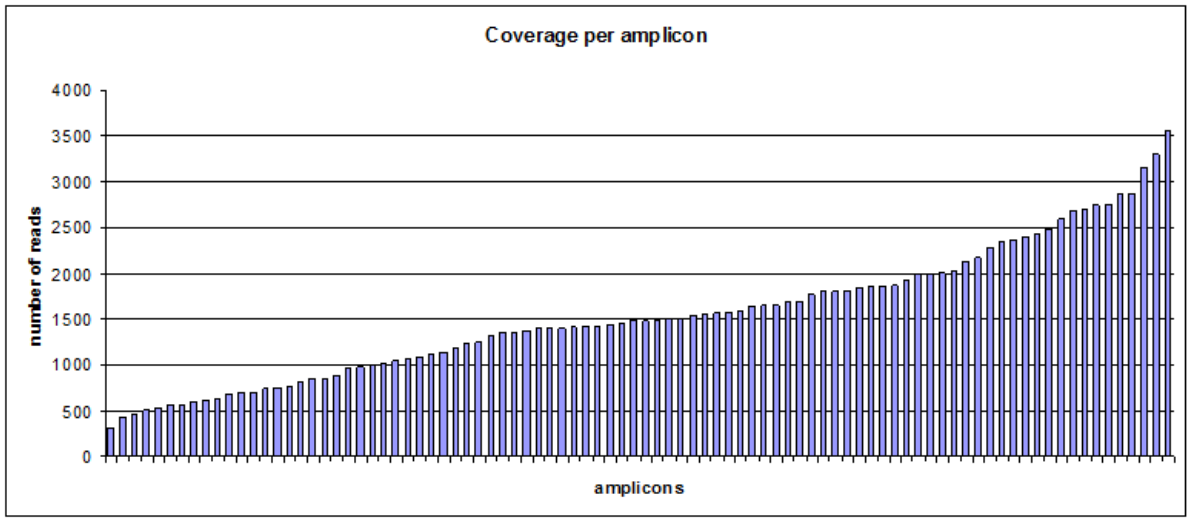
Coverage 20x -> 1 mutated read ???

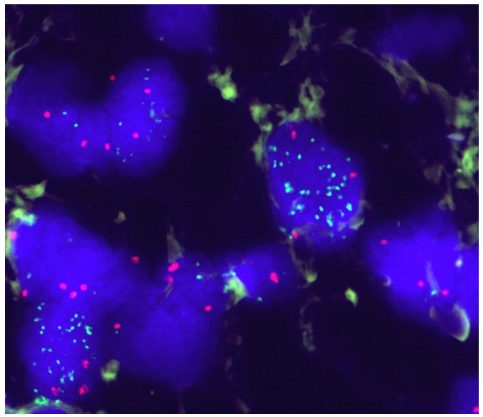
Coverage 1000x -> 50 mutated reads

CNV analysis

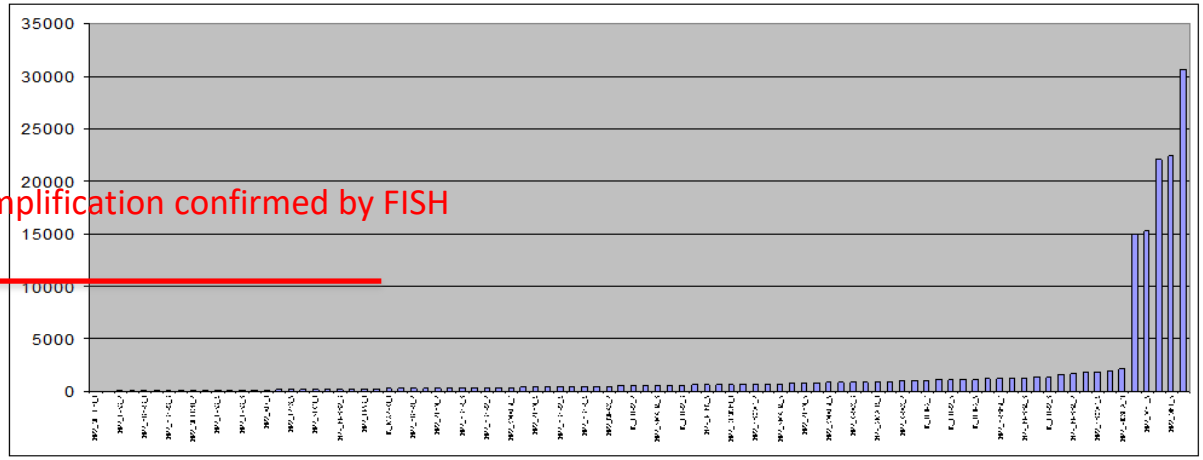
Nb of sequencing reads per region







MET amplification confirmed by FISH

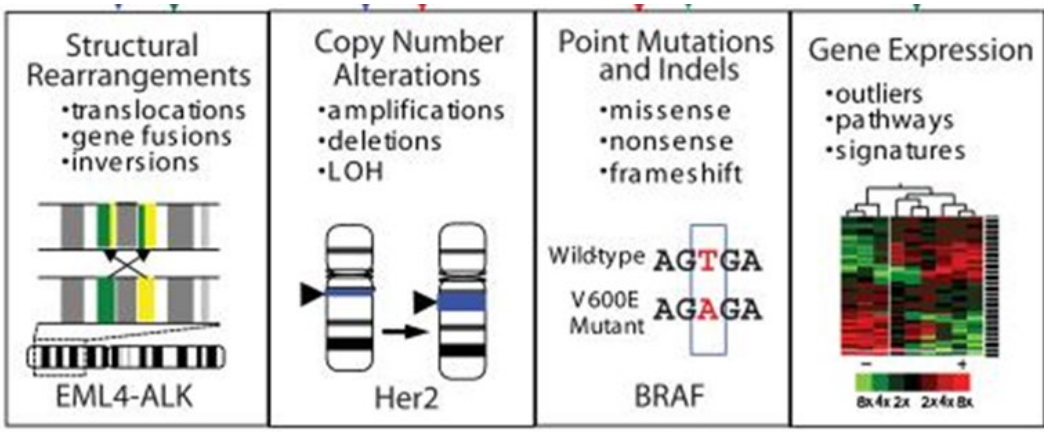


CNV analysis ?

- Need validations
  - Comparison with FISH gold standard
  - Can we detect smaller copy number variation ???
- Size of the panel
  - Number of total regions
  - Number of region per gene

NGS Application in Oncology

- Theoretically:
  - Almost everything is possible
- In Clinical Practice:



OK **+/- OK** **BUT** **Need more validations** **OK**

Roychowdhury et al. Sci Transl Med; 2011

Single test Vs WGS

Single test

BRAF V600E
1h30

Single test Vs WGS

Single test | WES | WGS

BRAF V600E
1h30

Coding sequence 20 000 genes

Coding AND non coding sequence

- Challenging on FFPE
- Works on frozen tissues
- Several 100 ng
- Blood sequencing in parallel
- Huge bioinformatics needs

Costs
TAT
Technical expertise

NGS cost does not equal wet lab cost!

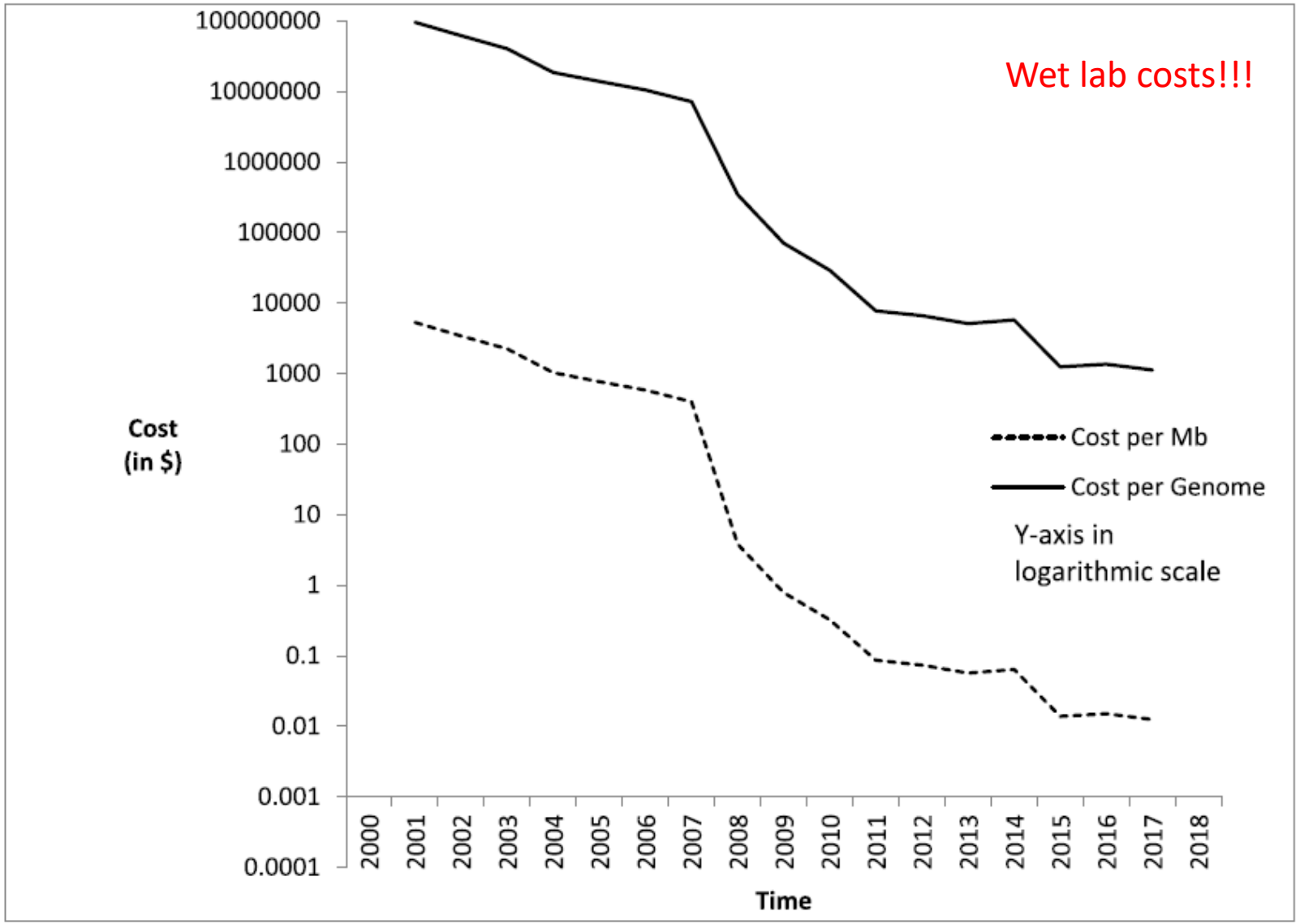


Fig. 2. Graph depicting DNA sequencing cost per Mb and genome for past 20 years.

Single test Vs WGS

Single test | NGS targeted panels | WES | WGS

BRAF V600E
1h30

Which genes?
How many genes?

10?
50?
500?

Coding sequence 20 000 genes

Coding AND non coding sequence

- Challenging on FFPE
- Works on frozen tissues
- Several 100 ng
- Blood sequencing in parallel
- Huge bioinformatics needs

Costs
TAT
Technical expertise

Targeted DNA Sequencing

Table 1: TruSight Tumor Genes

AKT1	*EGFR*	*GNAS*	*NRAS*	*STK11*
ALK	*ERBB2*	*KIT*	*PDGFRA*	*TP53*
APC	*FBXW7*	*KRAS*	*PIK3CA*	
BRAF	*FGFR2*	*MAP2K1*	*PTEN*	
CDH1	*FOXL2*	*MET*	*SMAD4*	
CTNNB1	*GNAQ*	*MSH6*	*SRC*	

Genes selected from NCCN[1] and CAP[2] guidelines, late-stage clinical trials[3], and relevant publications for lung, colon, melanoma, gastric and ovarian[4].

Table 1: TSACP Cancer-Related Genes

ABL1	*EGFR*	*GNAS*	*MLH1*	*RET*
AKT1	*ERBB2*	*HNF1A*	*MPL*	*SMAD4*
ALK	*ERBB4*	*HRAS*	*NOTCH1*	*SMARCB1*
APC	*FBXW7*	*IDH1*	*NPM1*	*SMO*
ATM	*FGFR1*	*JAK2*	*NRAS*	*SRC*
BRAF	*FGFR2*	*JAK3*	*PDGFRA*	*STK11*
CDH1	*FGFR3*	*KDR*	*PIK3CA*	*TP53*
CDKN2A	*FLT3*	*KIT*	*PTEN*	*VHL*
CSF1R	*GNA11*	*KRAS*	*PTPN11*	
CTNNB1	*GNAQ*	*MET*	*RB1*	

Cancer-related genes represented in the TSACP. For a full list of target regions, see the manifest file[1] (MyIllumina login required).

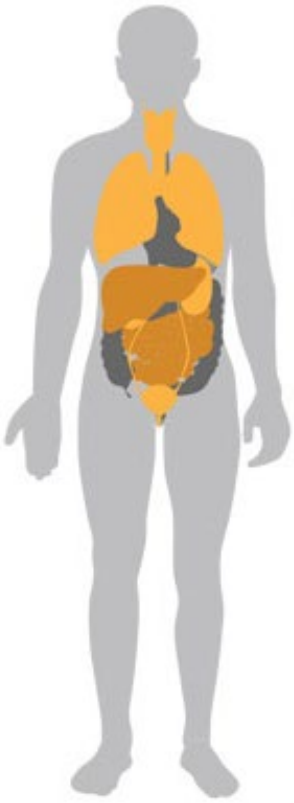
The Ion AmpliSeq™ Cancer Panel targets 50 genes

ABL1	*EZH2*	*JAK3*	*PTEN*
AKT1	*FBXW7*	*IDH2*	*PTPN11*
ALK	*FGFR1*	*KDR*	*RB1*
APC	*FGFR2*	*KIT*	*RET*
ATM	*FGFR3*	*KRAS*	*SMAD4*
BRAF	*FLT3*	*MET*	*SMARCB1*
CDH1	*GNA11*	*MLH1*	*SMO*
CDKN2A	*GNAS*	*MPL*	*SRC*
CSF1R	*GNAQ*	*NOTCH1*	*STK11*
CTNNB1	*HNF1A*	*NPM1*	*TP53*
EGFR	*HRAS*	*NRAS*	*VHL*
ERBB2	*IDH1*	*PDGFRA*	
ERBB4	*JAK2*	*PIK3CA*	

Ion Ampliseq™ Colon & lung Panel = 22 genes

AKT1	ERBB2	KRAS	PTEN
ALK	ERBB4	MAP2K1	SMAD4
BRAF	FBXW7	MET	STK11
CTNNB1	FGFR1	NOTCH1	TP53
DDR2	FGFR2	NRAS	
EGFR	FGFR3	PIK3CA	

TSO 500 panel



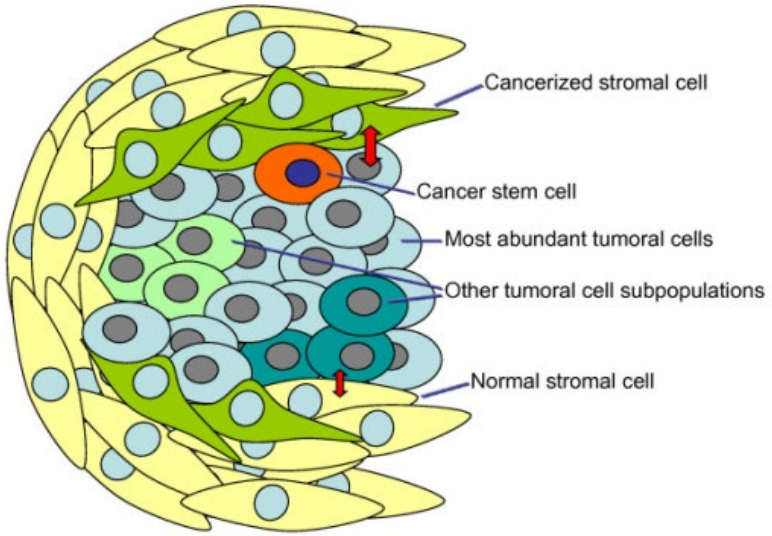
NTRK1, *NTRK2*, *NTRK3* (pan-cancer) | MSI (pan-cancer) | TMB (pan-cancer)								
Lung	Melanoma	Colon	Ovarian	Breast	Gastric	Bladder	Myeloid	Sarcoma
AKT1 *ALK* *BRAF* *DDR2* *EGFR* *ERBB2* *FGFR1* *FGFR3* *KRAS* *MAP2K1* *MET* *NRAS* *PIK3CA* *PTEN* *RET* *TP53*	*BRAF* *CTNNB1* *GNA11* *GNAQ* *KIT* *MAP2K1* *NF1* *NRAS* *PDGFRA* *PIK3CA* *PTEN* *TP53*	*AKT1* *BRAF* *HRAS* *KRAS* *MET* *MLH1* *MSH2* *MSH6* *NRAS* *PIK3CA* *PMS2* *PTEN* *SMAD4* *TP53*	*BRAF* *BRCA1* *BRCA2* *KRAS* *PDGFRA* *FOXL2* *TP53*	*AKT1* *AR* *BRCA1* *BRCA2* *ERBB2* *FGFR1* *FGFR2* *PIK3CA* *PTEN*	*BRAF* *KIT* *KRAS* *MET* *MLH1* *PDGFRA* *TP53*	*MSH6* *PMS2* *TSC1*	*ABL1* *ASXL1* *CALR* *CEBPA* *ETV6* *EZH2* *FLT3* *GATA2* *IDH1* *IDH2* *JAK2* *KIT* *MPL* *NPM1* *RUNX1* *SF3B1* *SRSF2* *TP53*	*ALK* *APC* *BRAF* *CDK4* *CTNNB1* *ETV6* *EWSR1* *FOXO1* *GLI1* *KIT* *MDM2* *MYOD1* *NAB2* *NF1* *PAX3* *PAX7* *PDGFRA* *PDGFRB* *SDHB* *SDHC* *SMARCB1* *TFE3* *WT1*

Limitations

- Samples characteristics
  - Quantity : small biopsy, cytology
  - Quality : FFPE -> DNA/RNA integrity (short DNA/RNA fragments)

- Inherent characteristics of tumor samples

  - Contamination with normal tissue
  - Aneuploidy
  - Tumor heterogeneity
    - Low frequency variant detection
    - High coverage necessity



Limitations

- TAT
  - wet-bench (from sample reception to sequencing : 3-4 days)
  - dry-bench (4 – 24 hours)
  - Analysis
    - Answer within 10 working days
- COST
  - INAMI/RIZIV reimbursement

Technical issues to consider while reporting (1)

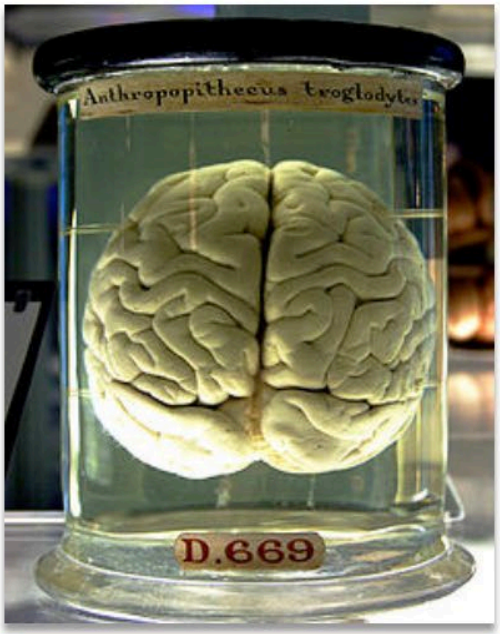
- ✓ Type of starting material – difficult tissues
- ✓ Composition of the starting material – macrodissection may have been necessary to enrich the neoplastic tissue zone
- ✓ Neoplastic cell percentage whithin the neoplastic tissue zone (e.g. background of normal cells)
  - Minimum required (e.g. 10 %, determined by the limit of detection of NGS (often +/- 5 %))
- ✓ Complicating issues:
  - Intratumor heterogeneity
    - ✓ Distinct clones and subclones
    - ✓ Particular mutation may be present in only part of the neoplastic cells
    - ✓ May explain differential response to therapy
  - Neoplastic cells are often hyperdiploid: higher DNA content compared to background normal cells

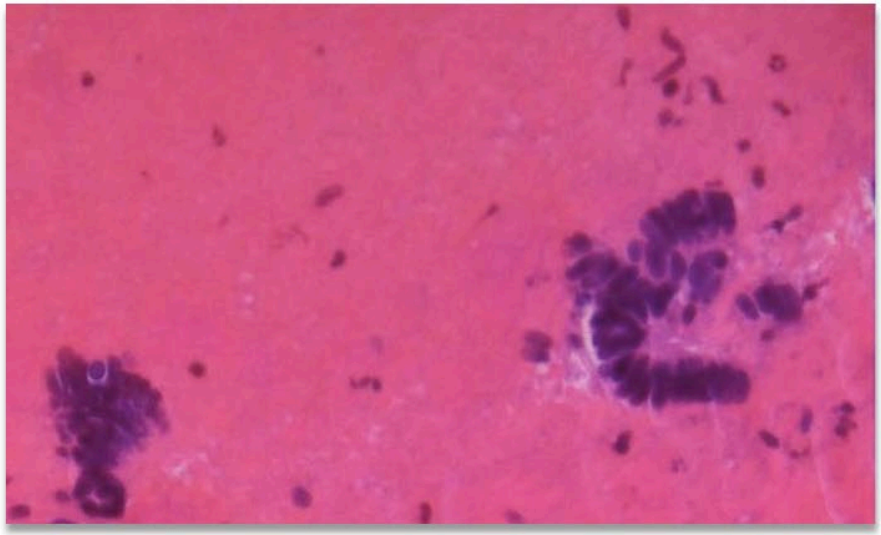
Technical issues to consider while reporting (2)

- ✓ DNA yield and quality
  - Highly variable
  - Look at deamination
- ✓ Coverage – read depth
  - Determines sensitivity of the analysis
  - Minimum required for optimal sensitivity and specificity (e.g. 500 x)
- ✓ Variant allele frequency (VAF)
  - % of DNA sequences with the mutation
  - Consider all the above when interpreting VAF

!!! Take care of … !!!

Difficult samples





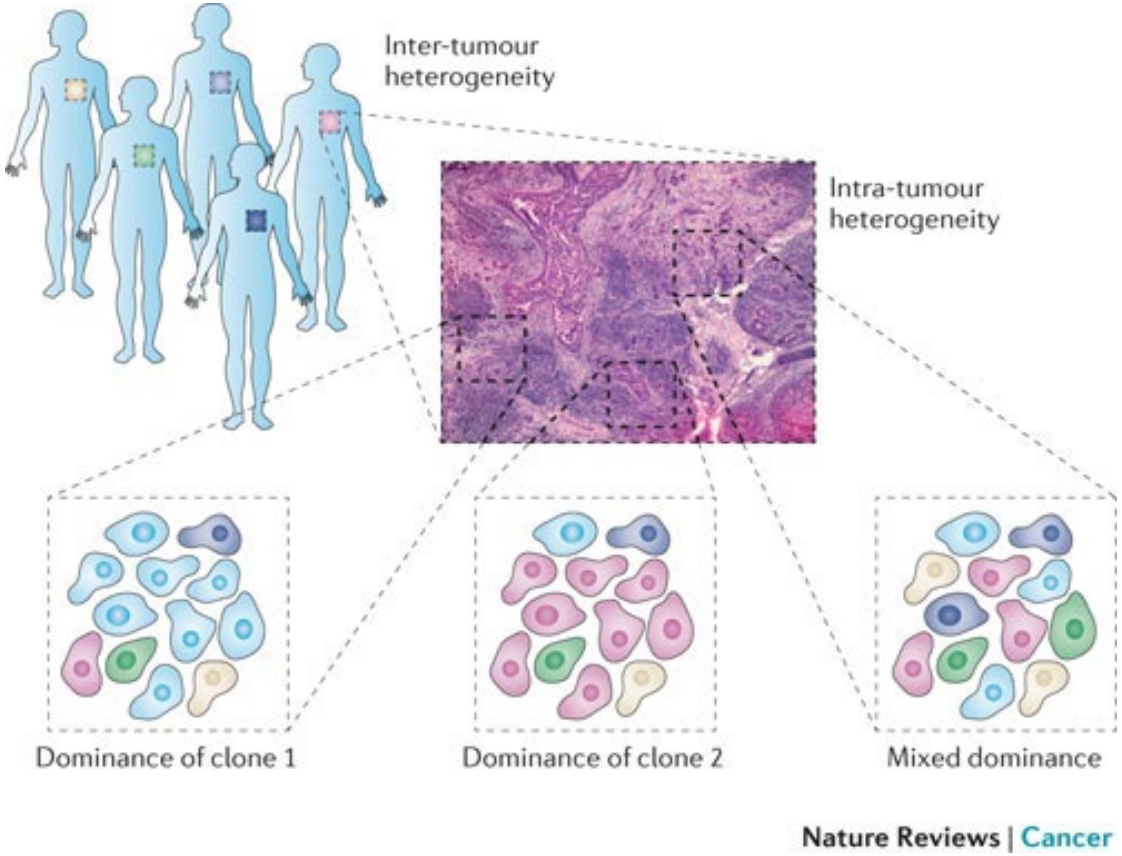
Formalin Fixation

Fragmented, nicked, crosslinked, end-damaged DNA

Scant Tissue

Low yield of DNA

Intra-tumor heterogeneity



- Different (sub)clones in one tumor
- Importance of sampling – impact on mutation profile and VAFs (potential of liquid biopsy!)
- Different mutations in a single sample may have different VAFs

Source: Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer. 2012;12(5):323-34.

Present and Future applications

- ✓ Oncogenic driver identification and resistance detection
- ✓ Mutational burden for sensitivity detection to immunotherapy
- ✓ RNA-seq, Epigenetic changes, ….
- ✓ Early cancer screening (liquid biopsy)
- ✓ …

In Belgium: INAMI / RIZIV

Article 33 bis de la nomenclature

"A.R. 17.5.2019" (en vigueur 1.7.2019)

587915 587926 Dépistage d'une mutation ponctuelle acquise au moyen d'une méthode de biologie moléculaire dans la phase d'investigation diagnostique d'une tumeur solide non lymphoïde et non-myéloïde B 1800
(Règle de cumul 5) (Règle diagnostique 1, 13)"

79 euros

"A.R. 7.6.2007" (en vigueur 1.8.2007) + "A.R. 4.5.2010" (en vigueur 1.8.2010) + "A.R. 17.5.2019" (en vigueur 1.7.2019)

588534 588545 Dépistage d'anomalies chromosomiques ou géniques acquises à l'exception d'une mutation ponctuelle au moyen d'une méthode de biologie moléculaire, dans la phase d'investigation diagnostique d'une tumeur solide non-lymphoïde et non-myéloïde B 3000
"(Règle de cumul 5) (Règle diagnostique 1, 13)"

132 euros

B= 0.044

In Belgium: INAMI / RIZIV

- concept:
  - **NEW article 33ter:**
    - new “generic” nomenclature codes for predictive tests linked to a drug *(diagnostic/prognostic: article 33bis)*
    - defined by TMC
    - published by royal decree
  - **NEW chapter “VIII”:**
    - list of “personalised” drugs
    - + list with “companion” tests

if the Minister decides to reimburse the drug, the marker will be added to the list by the same Ministerial Decree

In Belgium: INAMI / RIZIV

- methodology
  - providers: idem art 33bis
    - Clinical biology
    - Pathology
    - Centre for human genetics
  - content: end-to-end process
  - quality: ISO15189 + control by ISP-WIV + EQA
  - fee: 3 levels of complexity
    - complexity of test
    - complexity of sample
    - prevalence
    - consistent with existing nomenclature 33 and 33bis

Level 1: 88 euros
Level 2: 147 euros
Level 3: 196 euros

In Belgium: INAMI / RIZIV

- methodology
  - follow-up: separate codes
  - diagnostic rules:
    - 1/diagnostic phase
    - follow-up: 1/follow-up period
  - cumulative rules:
    - on the list for art33ter = tarification through 33ter
    - no double tarification (with 33bis)
  - registration mandatory
    - start with “light” registry
    - reports for data providers, health insurance and
    - future: link with MOC/COM and outcome data

In Belgium: INAMI / RIZIV

Annexe 4 : Modalités de financement pour 2022

Les honoraires ont été ajustés dès 1/1/2020 conformément à l'Accord national médico-mutualiste 2020 (1,25%). Cela était de nouveau ajusté à partir du 1/1/2021 conformément à l'Accord national médico-mutualiste 2021 (0,80%) et à partir du 1/1/2022 conformément à l'Accord national médico-mutualiste 2021 (0,73%).

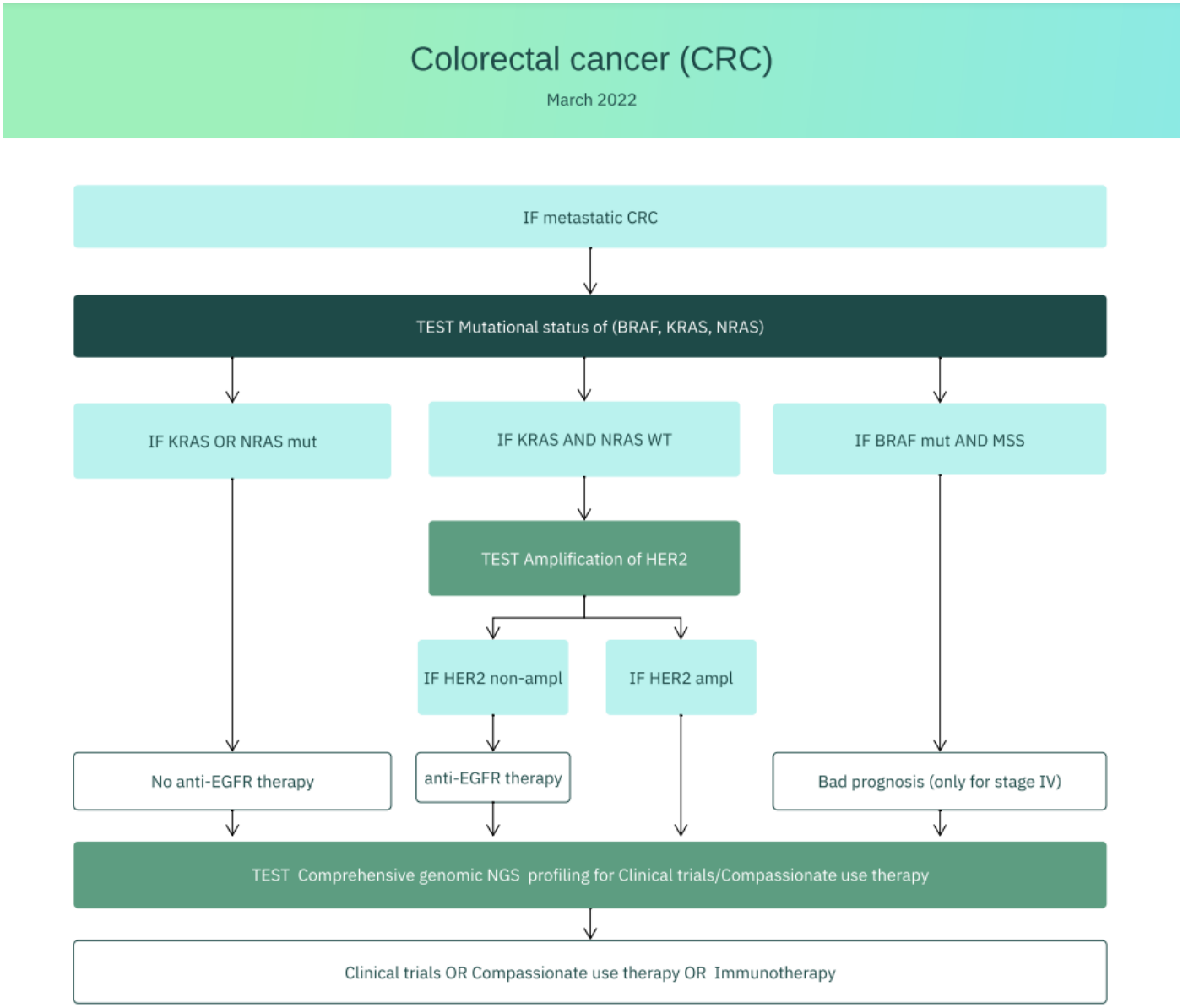
Le remboursement total initiale de 350 € a été ajusté à l'indice santé (1,95%) à 356,83 € dès 1/1/2020, conformément à l'article 3 de la Convention. Cela était de nouveau ajusté à partir du 1/1/2021 avec 1,01% à 360,43 € et à partir du 1/1/2022 avec 0,79% à 363,28 €. Le remboursement total de 550 € pour RNA-seq était ajusté à partir du 1/1/2021 avec 1,01%, à 555.56 € et à partir du 1/1/2022 avec 0,79%, à 559.95 €.

Le montant complémentaire est la différence entre les deux montants.

Indicatie/*Indication*	Art33ter		Art33bis		Honorarium in Euro/ Honoraire en Euro	Toeslag in Euro/Montant Complémentaire en Euro	Populatie aan 100%/ Population à 100%
Gemetastaseerd Colorectaal carcinoma *Carcinome colorectal métastatique*	594053- 594064	B3000	587915-587926	B1800	~~224.42~~ 226,06	~~136.01~~ 137.22	3000
~~Gevorderd~~ Adenocarcinoma Long *Adénocarcinome pulmonaire ~~avancé~~*	594053-594064	B3000	588534-588545 587915-587926	B3000+ B1800	~~356.43~~ 359.04	~~4.00~~ 4.24	5600
Long: progressie binnen 1j na positief advies MOC *Poumon : progression avant 1 an après avis positif d'une COM*					0	~~360.43~~ 363.28	180
Long zonder driver mutatie (RNA-seq) *Poumon sans mutation driver (RNA-seq)*	594090-594101 (ROS, ALK, 2x niveau 3)	B4000			~~387.24~~ 390.06	~~168.32~~ ~~(totaal 555.56€)~~ 169.89 (totaal 559.95€)	1800

In Belgium: ComPerMed

Commission de Médecine Personnalisée



"*Take home*" message

NGS interpretation – NOT so EASY

- ✓ Interpreting NGS data requires a **team approach**
- ✓ Understanding the **clinical context** and how NGS report may impact the management of the patient is critical
- ✓ Each case is **UNIQUE**
- ✓ Each variant must be interpreted in the context of the **tumor type**
- ✓ Lot of **variables** can impact the result (pre-analytic, analytic, post-analytic)
- ✓ Be always **CRITICAL** with the results

=> Advice for **CLINICAL** interpretation : **STAY ON THE GROOMED TRAILS**