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Diagnostica Molecolare

Aldo Scarpa
Unità Diagnostica Molecolare
Azienda Ospedaliera Universitaria Integrata di Verona
e
ARC-NET Centro di Ricerca Applicata sul Cancro

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PDTA CARCINOMA POLMONARE

- IL PAZIENTE DI OGGI
- IL PAZIENTE DI DOMANI
- BENCHMARKING REGIONE VENETO

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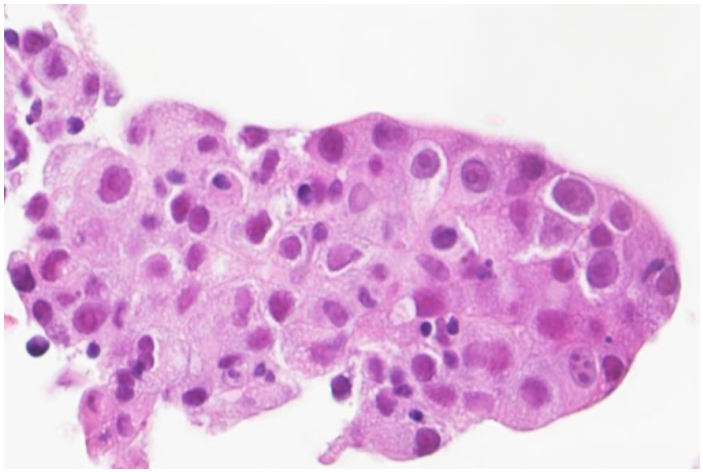
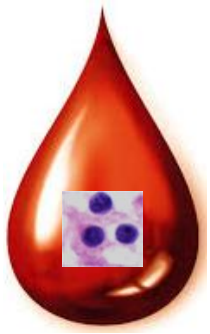
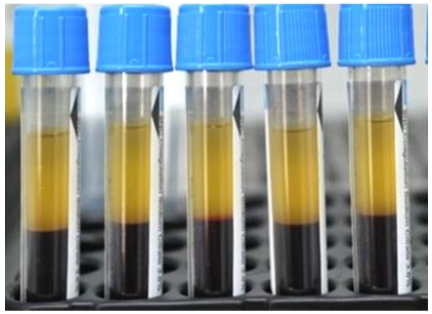
Cancer diagnostics tasks

1. **Diagnosis**
2. **Prognosis**
3. **Predict drug efficacy**
4. **Follow up**







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SIAPEC - IAP

Raccomandazioni AIOM e SIAPEC-IAP per l'analisi mutazionale del gene EGFR nel carcinoma polmonare

Aggiornamento Marzo 2014

A cura del Gruppo di Lavoro di AIOM e SIAPEC-IAP

Raccomandazioni per l'analisi dei riarrangiamenti del gene ALK nel carcinoma polmonare non a piccole cellule

A cura del Gruppo di Lavoro di AIOM e SIAPEC-IAP

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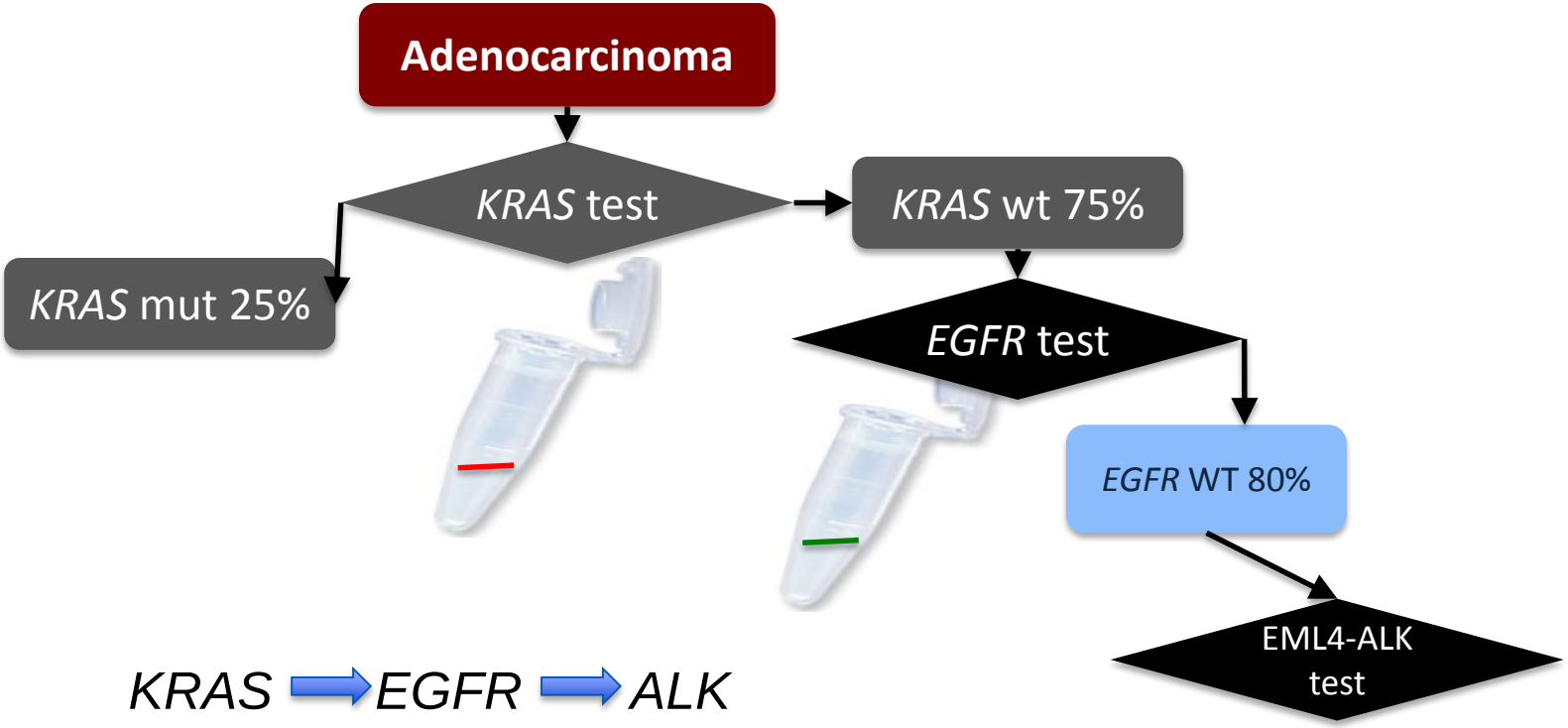
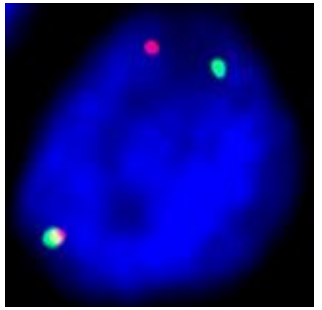
Diagnostic algorithm



KRAS → *EGFR* → *ALK*

One at the time

Time: ~ 15 days



Cheng L et al – Mod Pathol 2012

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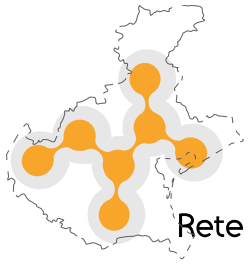
Which method?

- sensitivity

- ability to quantify



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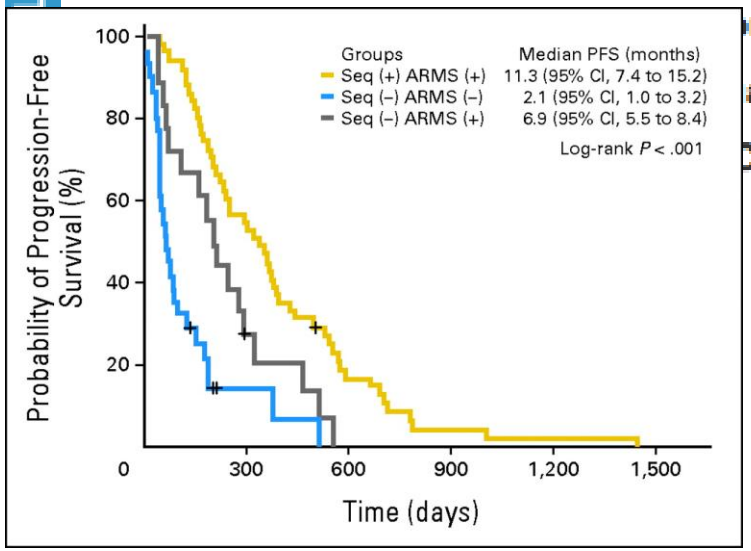
Sensitivity

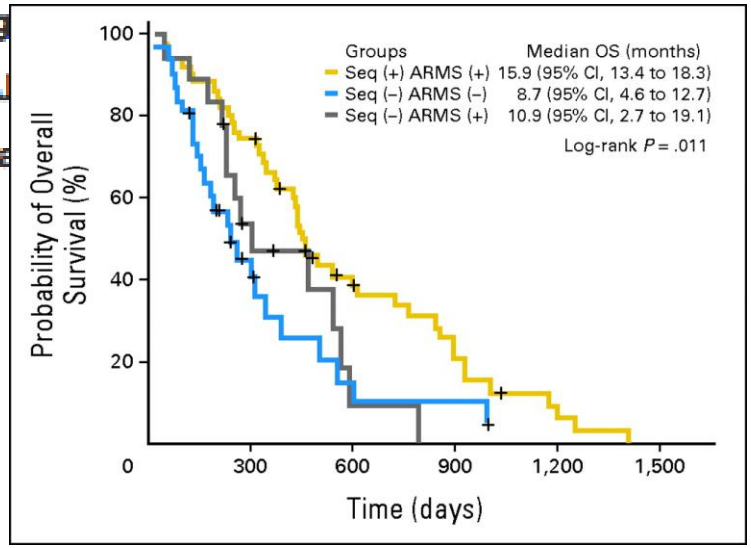
Method	Sensitivity (MT/WT)	Quantification
Sanger sequencing	20%	No
Allele specific probes	10%	No
High-resolution melting	5%	No
Pyrosequencing	5%	No
ARMS/scorpion probes	1%	No
Next Generation Sequencing	1%	Yes

L ONCOLOGY

Relative Abundance of *EGFR* Mutations Predicts Benefit From Gefitinib Treatment for Advanced Non–Small-Cell Lung Cancer



Progression-free survival (PFS)



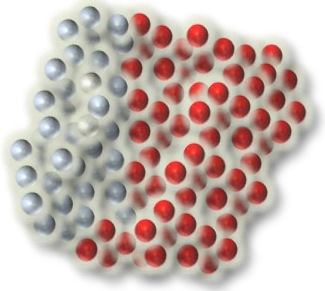
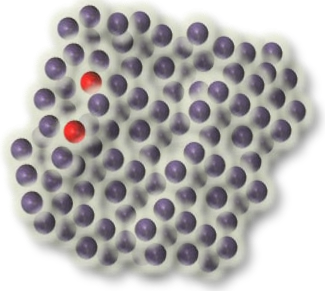
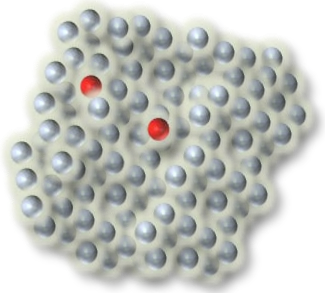
Overall survival (OS)

Quantification of EGFR mutation abundance promotes a better selection of patients for EGFR-TKI treatment but also help develop better treatment strategies for patients with a low abundance of EGFR mutations

Zhou Q et al – JCO 2011

Clonal heterogeneity or sensitivity?

- Normal
- EGFR positive
- EGFR negative

	Sequencing	qRT-PCR	Therapy
 60%?	+	+	YES
 2%?	-	+	NO
 2%?	-	+	YES

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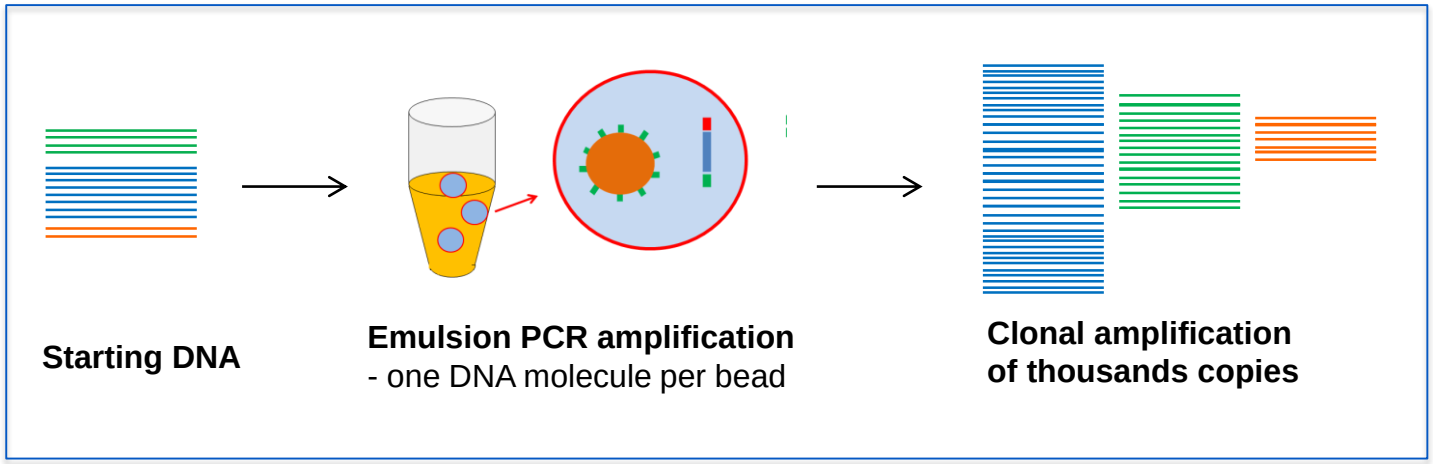
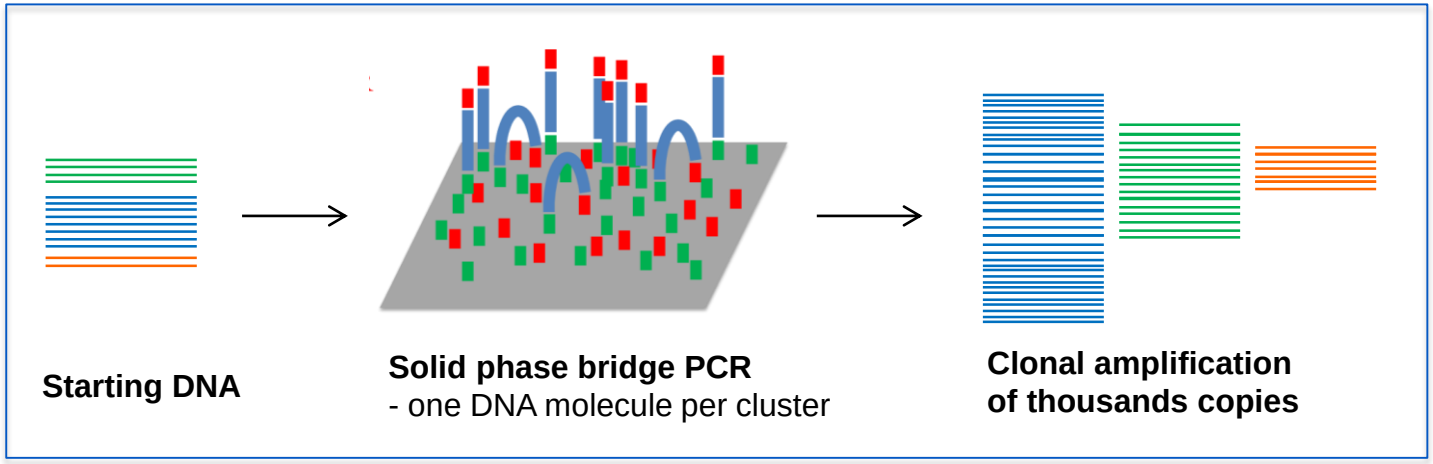
Next generation sequencing





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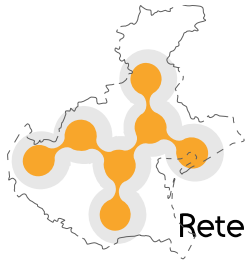
Risparmiare

Metodica capace di analizzare campioni multipli e diversificati



Ion AmpliSeq™ Colon and Lung Cancer Panel

life technologies™

May 2013

Designed with the OncoNetwork consortium

Designed for high utility with a consortium of leading researchers and verified using 155 unique FFPE samples

Customizable to meet your research needs

Available for any lab to order on ampliseq.com

OncoNetwork consortium members include Drs:
Aldo Scarpa[1]
Ludovic Lacroix [2]
Marjolijn Ligtenberg, Bastiaan Tops [3]
Christoph Noppen, Henriette Kurth, [4]
Nicola Normanno, [5]
Pierre Laurent Puig [6]
Ian Cree[7]
Orla Sheils[8]

[1] ARC-NET University of Verona, Italy,
[2] Institut Gustave Roussy, Paris, France
[3] Radboud University Nijmegen Medical Centre, The Netherlands
[4] VIOLLIER AG, Basel, Switzerland,
[5] Centro Ricerche Oncologiche

Designed with the leading researchers from the OncoNetwork consortium, the **Ion AmpliSeq™ Colon and Lung Cancer Panel** contains primer pairs that target "hot spot" regions of genes implicated in colon and lung cancers. The OncoNetwork consortium comprises eight cancer research groups from different translational research institutions with many years of experience in adopting the latest DNA sequencing and genotyping technologies to pioneer colon and lung therapy research. The consortium combined the potential of Ion AmpliSeq™ technology and the affordability of Ion semiconductor sequencing to develop a screening solution that meets the following goals:

- **Selective gene content**—panel targets regions of a comprehensive set of genes implicated in colon and lung cancer, including known hot spot regions
- **Low input DNA requirement** — —provides FFPE sample compatibility, requiring as little as 10 ng of DNA, which is critical for colon and lung cancer research
- **Adoptable by any research lab**— accurate, economical, and easy to implement end-to-end solution that can be widely adopted

Design and verification of Ion AmpliSeq™ Colon and Lung Cancer Panel was done with the OncoNetwork consortium on 155 FFPE samples –including control samples and FFPE samples that were previously screened using orthogonal technologies.

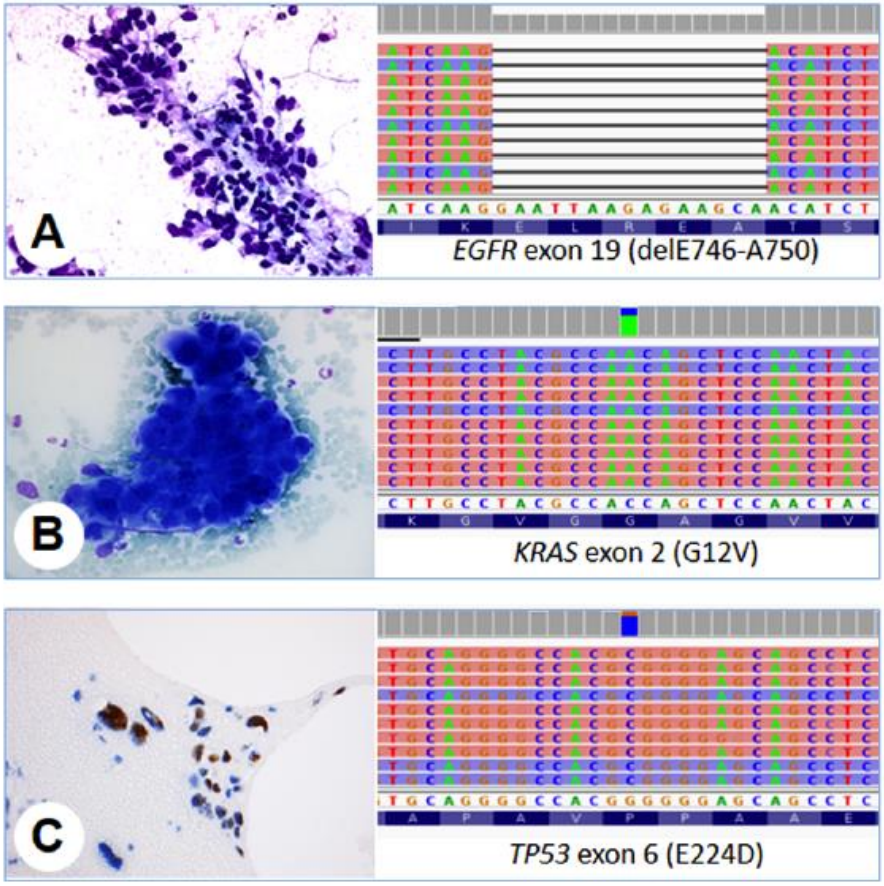
All Ion AmpliSeq™ panels are based on Ion AmpliSeq™ technology that delivers simple and fast library construction for affordable targeted sequencing of specific human genes or genomic regions. Based on ultrahigh-multiplex PCR, Ion AmpliSeq™ panels require as little as 10 ng of input DNA to target regions of interest, making sequencing of FFPE samples routine on Ion PGM™ Systems.

OPEN ACCESS Freely available online

PLOS ONE

Molecular Typing of Lung Adenocarcinoma on Cytological Samples Using a Multigene Next Generation Sequencing Panel

Aldo Scarpa[1,2☯], Katarzyna Sikora[1☯], Matteo Fassan[1,2*], Anna Maria Rachiglio[3], Rocco Cappellesso[4], Davide Antonello[2], Eliana Amato[1], Andrea Mafficini[1], Matilde Lambiase[3], Claudia Esposito[3], Emilio Bria[5], Francesca Simonato[4], Maria Scardoni[2], Giona Turri[2], Marco Chilosi[2], Giampaolo Tortora[5], Ambrogio Fassina[4], Nicola Normanno[6]



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Colon-Lung-Melanoma
CE-IVD NGS Kits

Thermofisher

Oncomine Solid Tumour kit

22 genes

EGFR-KRAS-NRAS-BRAF ……..

4 Bases

BEN-Kit

4 genes

EGFR-KRAS-NRAS-BRAF

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NGS *vs.* Pyrosequencing

the example of the *KRAS, NRAS, BRAF*

NGS

Multiple genes in multiple samples, simultaneously

Time: ~ 5 days

PYRO

KRAS → *NRAS* → *BRAF*

One at the time

Time: ~ 15 days

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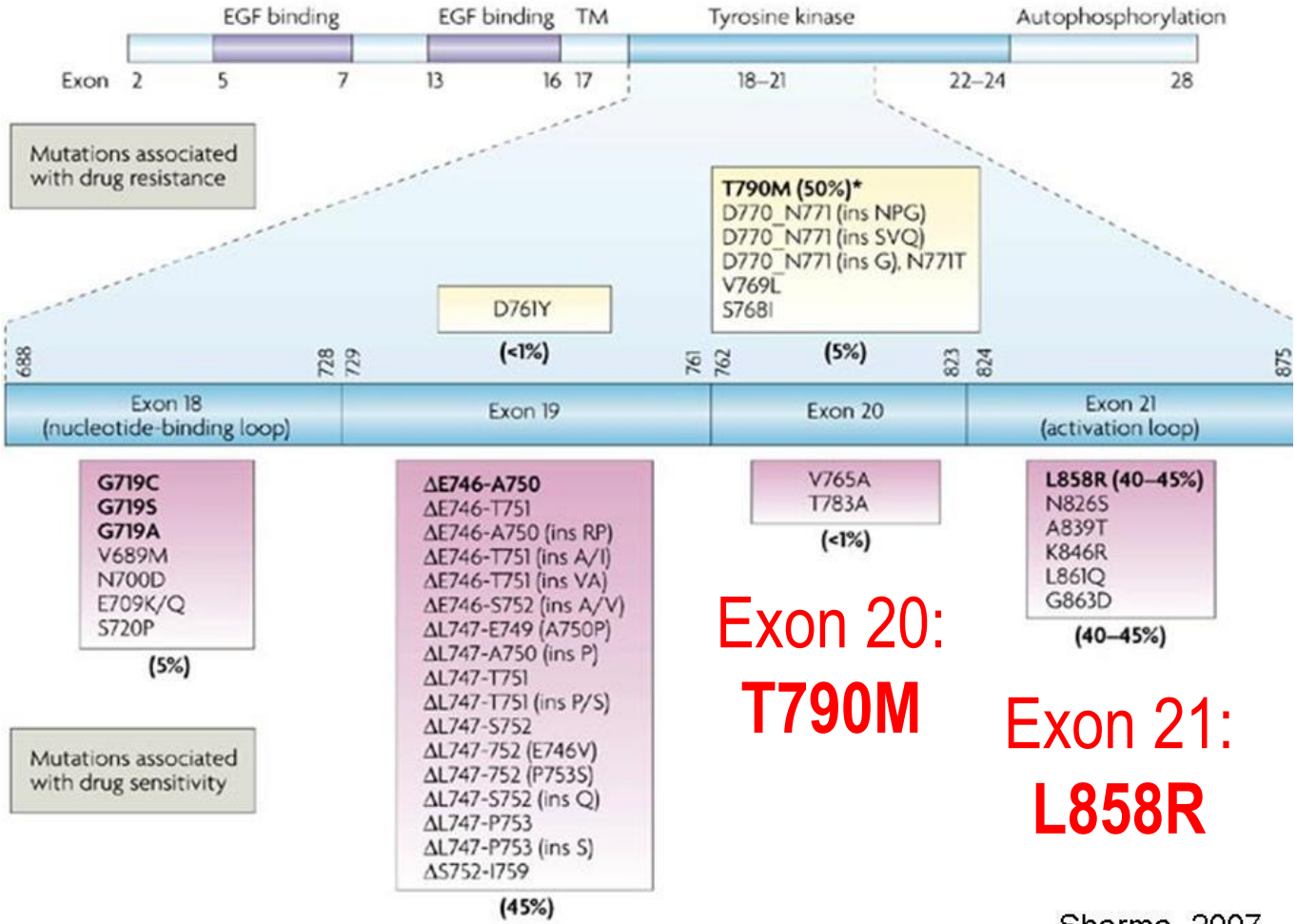
EGFR mutations in lung cancer

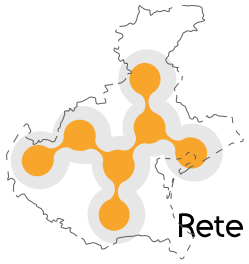


Exon 20:
T790M

Exon 21:
L858R

Exon 19:
Deletions

Sharma, 2007
Nature Reviews | Cancer



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Modularità

Metodica flessibile che permetta di modulare le analisi da eseguire

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Better selection of patients

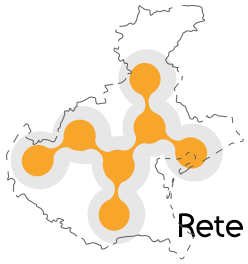
Molecular heterogeneity assessment by next-generation sequencing and response to gefitinib of *EGFR* mutant advanced lung adenocarcinoma

Table 2: Patients' groups according to resistance to Gefitinib and Progression-Free-Survival; 17 evaluable patients (Log-Rank $p<0.0001$).

Group	Definition	Pts (%)	Median PFS (months, 95%CI)
Poor	Progression at 1st assessment	6 (35.2)	1.7 (0.1-3.2)
Intermediate	Progression within 12 months	3 (17.7)	6.1 (3.0-9.2)
Good	Progression ≥ 12 months or treatment ongoing	8 (47.1)	17.3 (9.0-25.5)

Pts: patients; PFS: progression-free-survival; CI: confidence intervals.

Bria et al Oncotarget 2015



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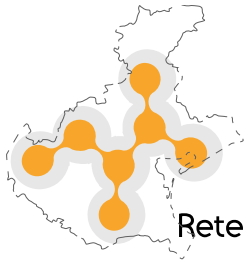
Better selection of patients

Molecular heterogeneity assessment by next-generation sequencing and response to gefitinib of *EGFR* mutant advanced lung adenocarcinoma

Patient	Group	Type of Mutation (PMA)						
		EGFR	***TP53***	***KRAS***	***CTNNB1***	***PIK3CA***	***MET***	***SMAD4***
1	Good	L858R (32%)						
2	Good	p.E746_A750del (31%)						
3	Good	L858R (56%)						
4	Good	L858R (86%)						
5	Good	p.E746_A750del (71%)						
6	Good	p.E746_A750del (88%)						
7	Good	p.L747_T751del (64%)			S33C (20%)			G358E (44%)
8	Good	p.E746_S752delinsA (28%)			S45P (5%)			
9	Intermediate	p.L747QfsTer16 (23%)	R273G (45%)					
10	Intermediate	p.E746_A750del (94%)	R248W (48%)					
11	Intermediate	p.E745_A750del (30%)	R175H (27%)					
12	Poor	L858R (36%)	R273L (47%)					
13	Poor	p.E746_T751delinsIA (11%)		G12C (5%)				
14	Poor	p.E746_A750del (20%)						
15	Poor	L858R (43%)		G12C (22%)		E542K (31%)		
16	Poor	p.E746_A750del (31%)	R248L (40%)					
17	Poor	E746_A750del (94%)	R175L (37%)				N375S (34%)	

Legend – Supplementary Table 1. NGS: next generation sequencing; PMA: Proportion of Mutated Alleles,



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Fusion genes as drivers …

ALK is not alone

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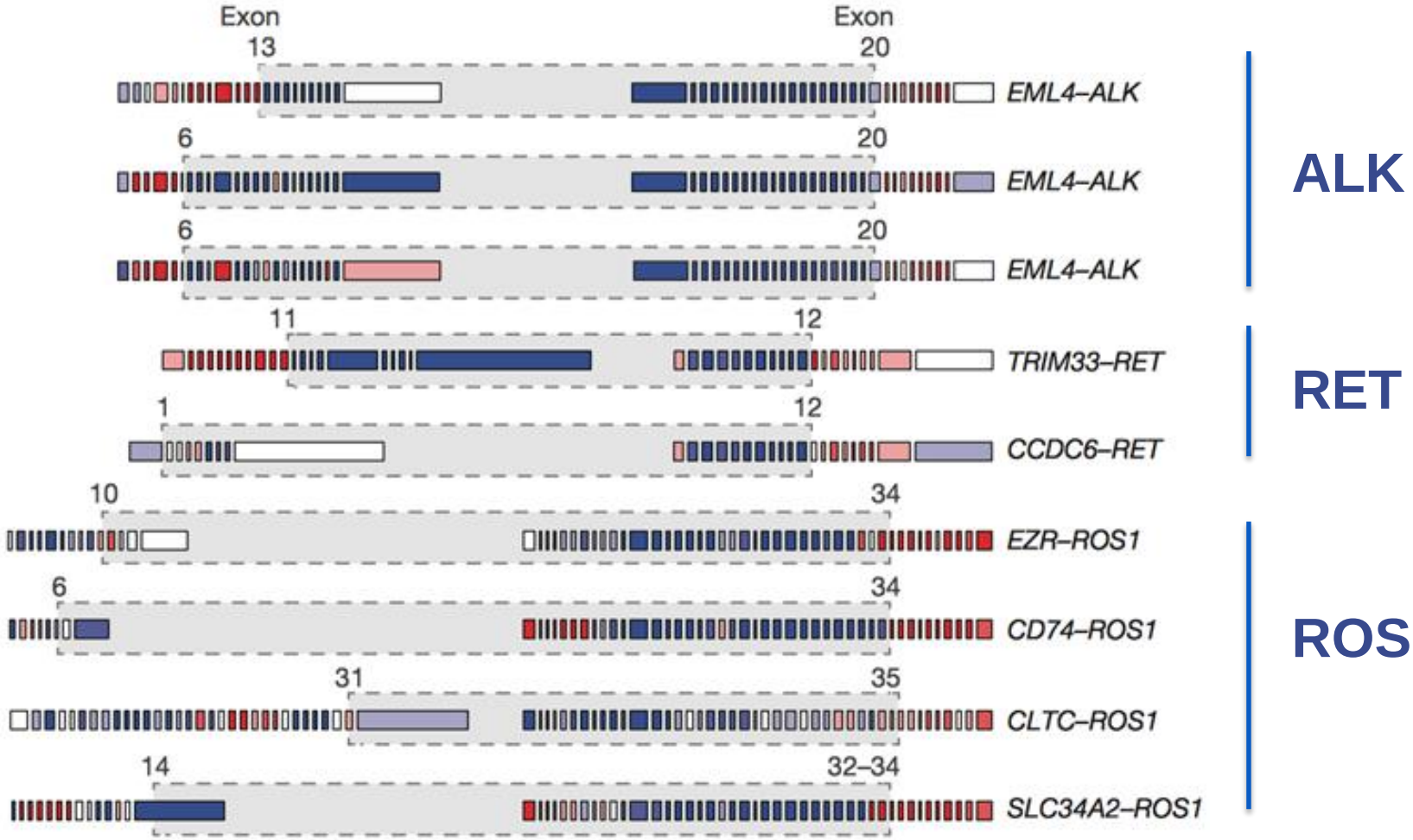
The Cancer Genome Atlas Research Network*



TCGA Network, Nature 2014

Colon – Lung - Melanoma

NGS Kits

DNA Mutations

RNA Translocations

Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2

The Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 (Table 1, Figure 2) has primer pairs in a single pool for hotspots and targeted regions for 22 known genes associated with colon and lung tumor tissue. In addition, the panel now also includes three additional amplicons covering known target regions for *NRAS* and *ALK* genes:

- *NRAS* exon 4 variants (p.117, p.146)
- *ALK* variants (G1269A, p.S1206Y)

These known rare variants are important in colon and lung cancer research. The first version of the panel, the Ion AmpliSeq™ Colon and Lung Cancer Panel, was tested and verified with 155 unique FFPE samples by the OncoNetwork Consortium. The Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 is ready-to-use and optimized for data analysis with Torrent Suite™ and Ion Reporter™ Software.

Table 1. Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2.

Sample type	FFPE samples
Application	Somatic mutation detection
Genes	*KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1, FGFR2*
Pairs of primers and amplicon length	92 pairs of primers in a single pool 92 amplicons with an average length of 162 bp
Input DNA required	10 ng
Observed performance	Percent of amplicons with the target base coverage at 500X: >95% Average panel uniformity: 95% Average percent reads on target: 98%
Multiplexing	2 samples per Ion 314™ Chip with at least 500X sequencing coverage 8 samples per Ion 316™ Chip with at least 500X sequencing coverage 16 samples per Ion 318™ Chip with at least 500X sequencing coverage

Ion AmpliSeq™ RNA Fusion Lung Cancer Research Panel

The Ion AmpliSeq™ RNA Fusion Lung Cancer Research Panel targets over 70 fusion transcripts associated with lung cancer research with additional targets for major fusion gene families (Table 2). The panel also includes 5 positive control genes.

With the recent release of Ion Reporter™ Software v4.2, you can quickly and simply detect known and novel gene fusions. With tunable analysis parameters and multisample heat map visualizations, Ion Reporter™ Software makes it easier to customize gene fusion workflows and perform multisample comparisons.

Additional features of the Ion AmpliSeq™ RNA Fusion Lung Cancer Research Panel include:

- RNA quality controls with housekeeping gene expression targets
- 5′ and 3′ *ALK* gene expression assays as indicators of a translocation
- Primer designs that target *ALK*, *ROS1*, *RET*, and *NTRK1* fusions that were previously detected on FFPE archived samples using FISH, immunohistochemistry, or qPCR by the OncoNetwork Consortium

Table 2. Ion AmpliSeq™ RNA Fusion Lung Cancer Research Panel.

Sample type	FFPE samples
Application	Somatic mutation detection
Genes	*ALK, RET, ROS1*, and *NTRK1* fusion transcripts, in addition to targets designed to detect 5′ and 3′ *ALK* gene expression
Pairs of primers and amplicon length	83 pairs of unique primers in a single pool 85 amplicons with an average length of 136 bp
Input RNA required	10 ng of total RNA
Observed performance	Fusion transcript detection down to 1% of the total RNA using cell line dilution
Recommended multiplexing	16 samples per Ion 318™ Chip with at least 20,000 on-target reads per library

May 2013

August 2014

DRIVERS …
EGFR and ALK are not alone …

Table 1 | **Potential important alterations in ADC and SCC**

Gene	Status (M, C or F)*	Frequency (%)		Available GEMMs	Currently available targeted therapies	Selected potential targeted therapies	Refs	
		ADC	SCC				Preclinical evidence	Clinical evidence
Receptor tyrosine kinases								
EGFR	M or C	10 (M)	2–3	L858R, Del19, T790M and Ins20	Erlotinib, gefitinib and afatinib	AZD9291, CO-1686 and HM61713	126	11
FGFR1	C	N/A	20	N/A	N/A	Dovitinib, ponatinib, AZD4547 and BGJ398	150	22
FGFR2	M or C	3 (M)	3	N/A	N/A	Dovitinib, ponatinib, AZD4547 and BGJ398	151	20
ALK	F	3–5	<1	ALK fusion, L1196M and F1174L	Crizotinib and ceritinib	AP26113, alectinib, ganetespib and PF-06463922	125	18
MET	C	2–4	N/A	Overexpression	Crizotinib	Tivantinib, cabozantinib, INC280 and onartuzumab	152	14
ROS1	F	1–2	N/A	N/A	Crizotinib	PF-06463922	153	17
NTRK1	F	1–2	N/A	N/A	N/A	Crizotinib and lestaurtinib	21	21
RET	F	1	N/A	N/A	N/A	Carbozantinib and vandetanib	154	16
HER2	M or C	2–4 (M)	N/A	HER2-YVMA insertion	N/A	Neratinib, afatinib, lapatinib and trastuzumab	155	19
DDR2	M	N/A	2–3	N/A	N/A	Dasatinib	27	27
PDGFRA	M	6–7	4	N/A	N/A	Sunitinib	156	28
Signalling								
KRAS	M	15–25	1–2	G12D, G12C and G12V	N/A	Selumetinib plus docetaxel combination	157	158
NF1	M	12	10	Null	N/A		159	28
BRAF	M	1–6	4–5	V600E	N/A	Vemurafenib, dabrafenib and trametinib	N/A	160
PIK3CA	M	5	15	p110α	N/A	BEZ235, BKM120 and GDC0941	99	161
MEK1	M	1	N/A	N/A	N/A	Selumetinib and trametinib	N/A	162

Chen Z et al. Nature Reviews Cancer 14: 535, 2014

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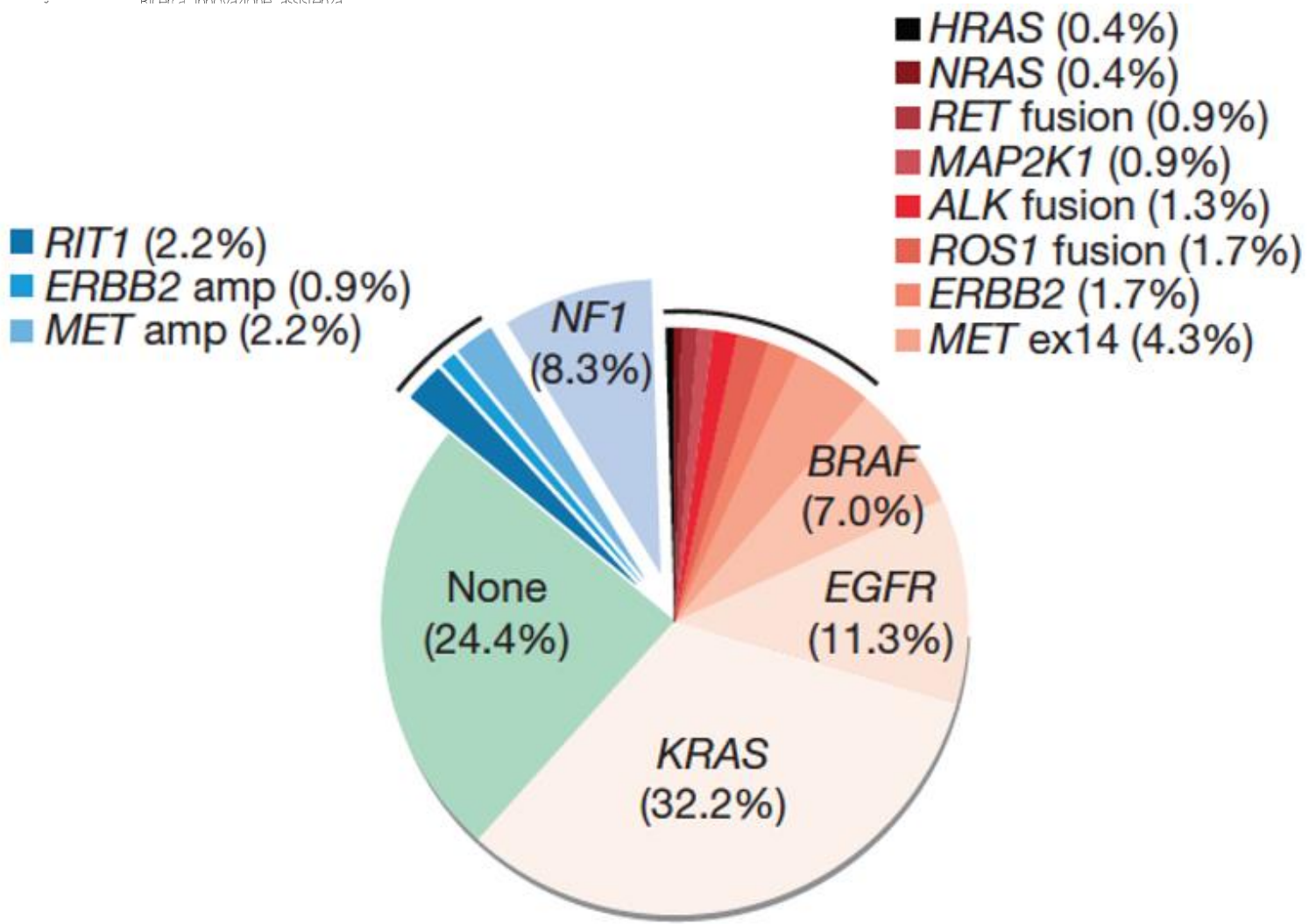
The Cancer Genome Atlas Research Network*



TCGA Network, Nature 2014

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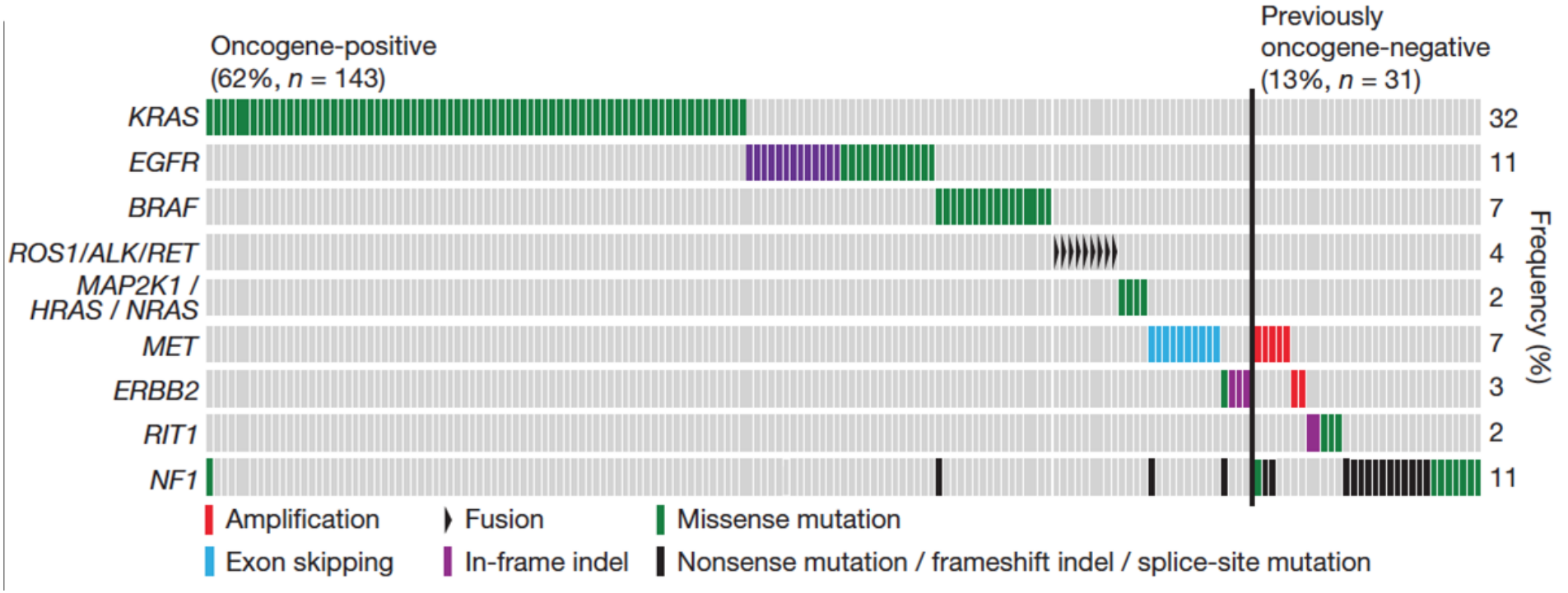
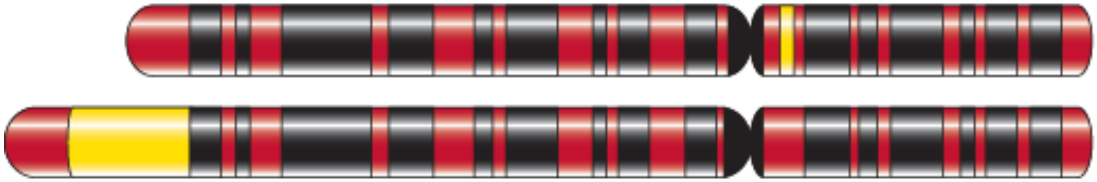
The Cancer Genome Atlas Research Network*



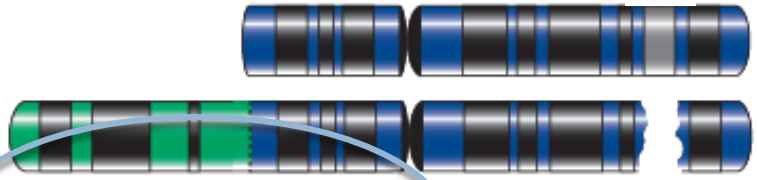
TCGA Network, Nature 2014

Technology advancement

Single nucleotide variations



Amplifications

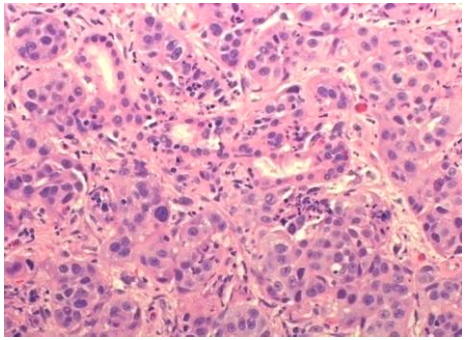


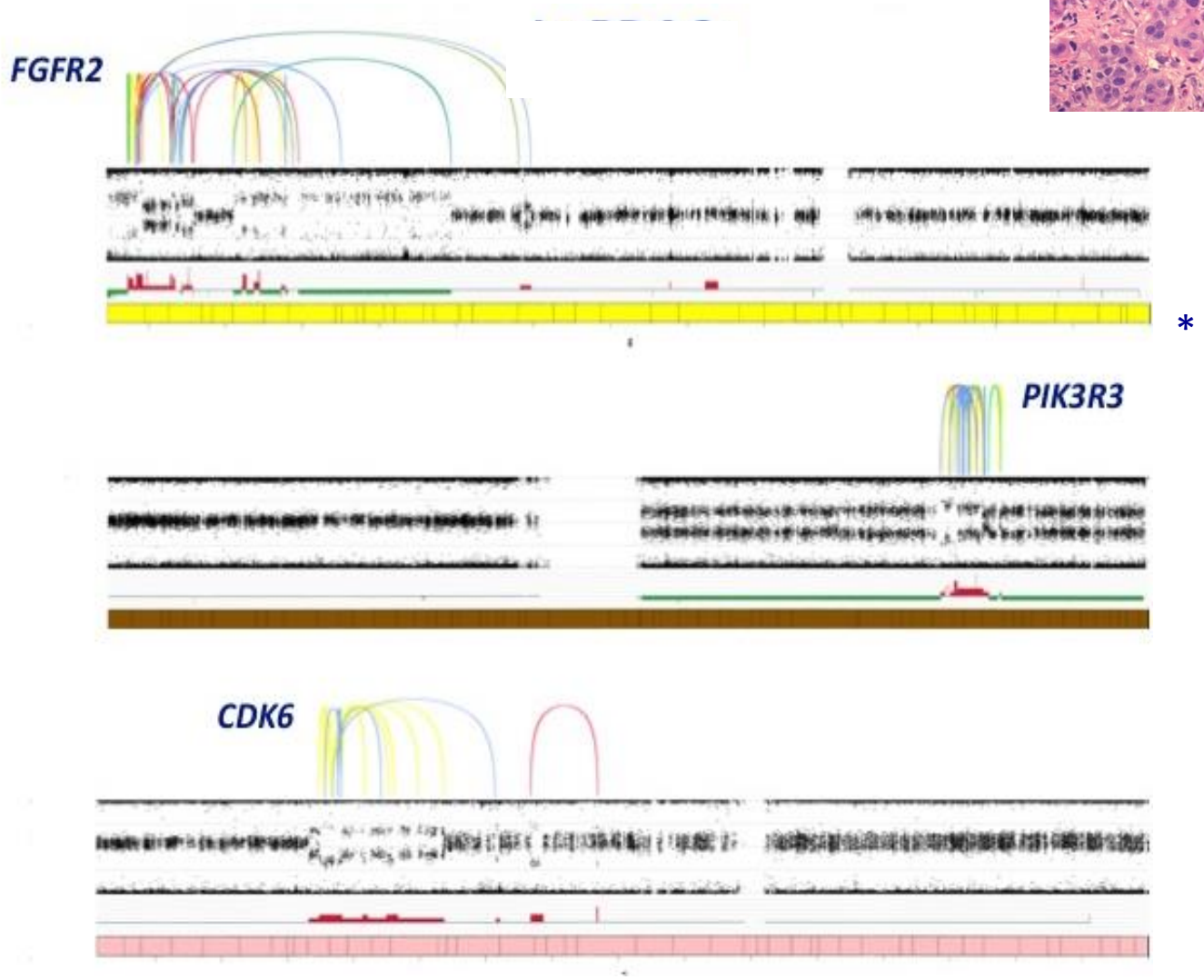
Translocations

Deletions

This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 602783

Focal Amplifications

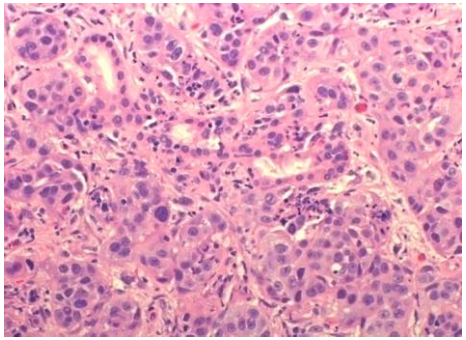




*

Waddell N et al. *Nature* 2015

Oncomine Assay*



Hotspot genes, n=73 (hotspot coverage)

ABL1	GNA11	MYD88
AKT1	GNAQ	NFE2L2
ALK	GNAS	NPM1
AR	HNF1A	NRAS
ARAF	HRAS	PAX5
BRAF	IDH1	PDGFRA
BTK	IDH2	PIK3CA
CBL	IFITM1	PPP2R1A
CDK4	IFITM3	PTPN11
CHEK2	JAK1	RAC1
CSF1R	JAK2	RAF1
CTNNB1	JAK3	RET
DDR2	KDR	RHEB
DNMT3A	KIT	RHOA
EGFR	KNSTRN	SF3B1
ERBB2	KRAS	SMO
ERBB3	MAGOH	SPOP
ERBB4	MAP2K1	SRC
ESR1	MAP2K2	STAT3
EZH2	MAPK1	U2AF1
FGFR1	MAX	XPO1
FGFR2	MED12	
FGFR3	MET	
FLT3	MLH1	
FOXL2	MPL	
GATA2	MTOR	

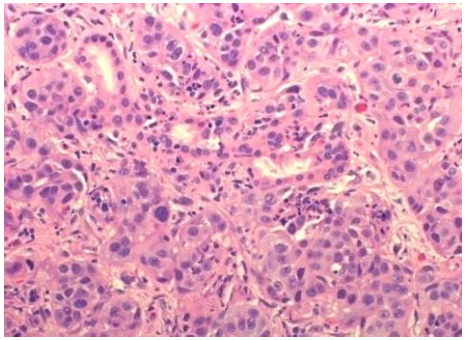
Copy gain, n=49

ACVRL1	IGF1R
AKT1	IL6
APEX1	KIT
AR	KRAS
ATP11B	MCL1
BCL2L1	MDM2
BCL9	MDM4
BIRC2	MET
BIRC3	MYC
CCND1	MYCL
CCNE1	MYCN
CD274	MYO18A
CD44	NKX2-1
CDK4	NKX2-8
CDK6	PDCD1LG2
CSNK2A1	PDGFRA
DCUN1D1	PIK3CA
EGFR	PNP
ERBB2	PPARG
FGFR1	RPS6KB1
FGFR2	SOX2
FGFR3	TERT
FGFR4	TIAF1
FLT3	ZNF217
GAS6	

DNA Panel

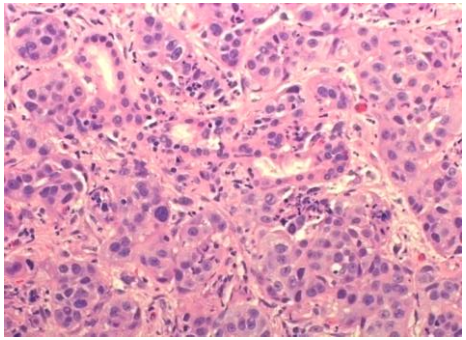
*For Research Use Only. Not for use in diagnostic procedures.

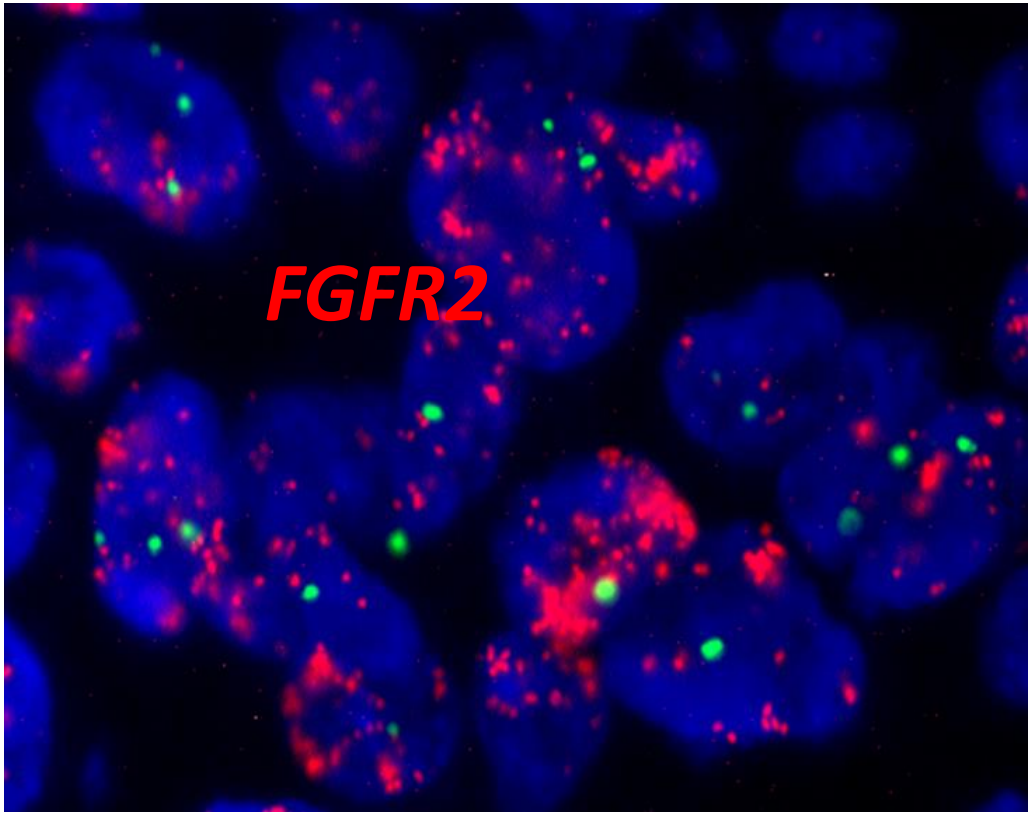
Case 1875 –
ONCOMINE COMPREHENSIVE PGM



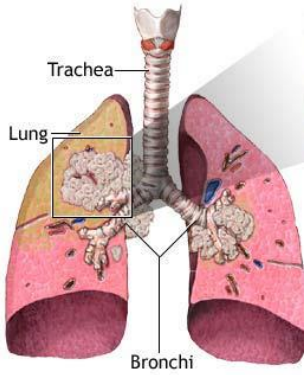
Gene	Class	Functional meaning	Copy Number
FGFR2	CNV_Amplification	Gain-of-function	19.11
ACVRL1	CNV_Amplification	Gain-of-function	9.11
TERT	CNV_Amplification	Gain-of-function	7.44
PTEN	CNV- Deletion	Loss-of-function	0.44

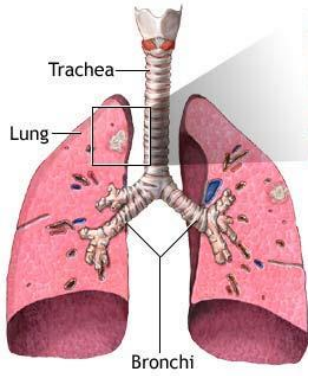
Case 1875 –
ONCOMINE COMPREHENSIVE PGM





CLINICAL PRACTICE

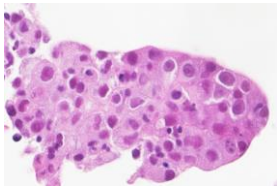




70% **non-resectable** **resectable** 30%

↓ ↓

Cytology or Biopsy **Surgical specimen**



Diagnosis

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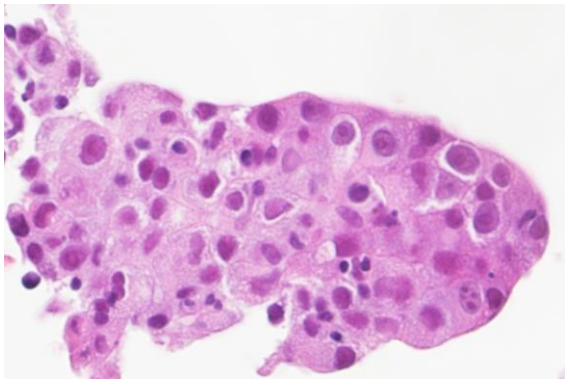
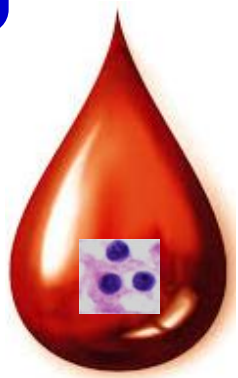
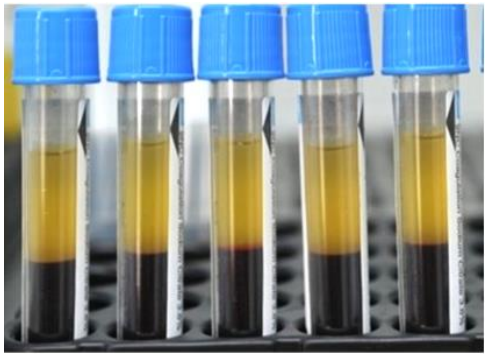
Cancer diagnostics tasks

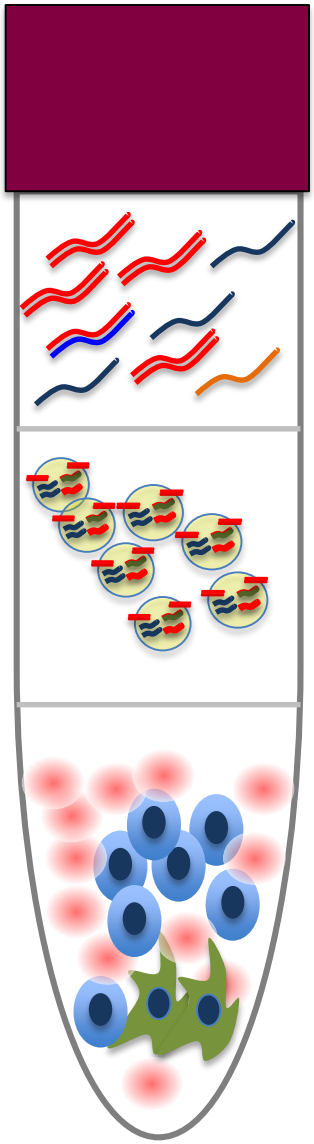
1. **Diagnosis**
2. **Prognosis**
3. **Predict drug efficacy**
4. **Follow up**







Liquid biopsy: what is it?

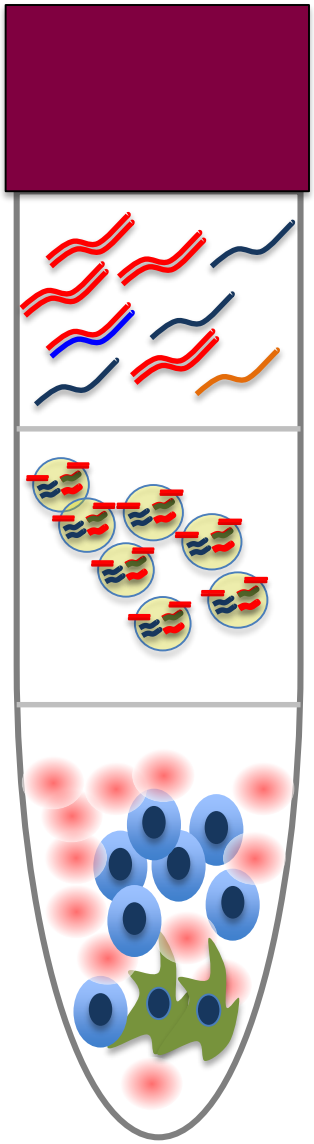


cfNA

Exosomes

CTCs

Liquid biopsy: what is it?



cfNA → • **DNA** • mRNA • miRNA

Exosomes → • DNA • mRNA • miRNA • Protein

CTCs → • DNA • mRNA • miRNA • Protein

Liquid biopsy: what is it for?

Non-invasive access to information through genetics

- early diagnosis
- correlation with the burden load
- minimal residual disease - relapse
- emergence of drug resistance

Liquid biopsy: what is it for?

Non-invasive access to information through genetics

- early diagnosis
- correlation with the burden load
- minimal residual disease - relapse
- emergence of drug resistance

It represents a summary of all the different cancer lesions in a patient

"clonal evolution"

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Liquid biopsy: cfDNA

- small fragments from apoptotic or necrotic cells
- has short half-life
- highly variable level (<0.1% to >50% of total cfDNA)

Technical challenges: **sensitivity** and **dynamic range**

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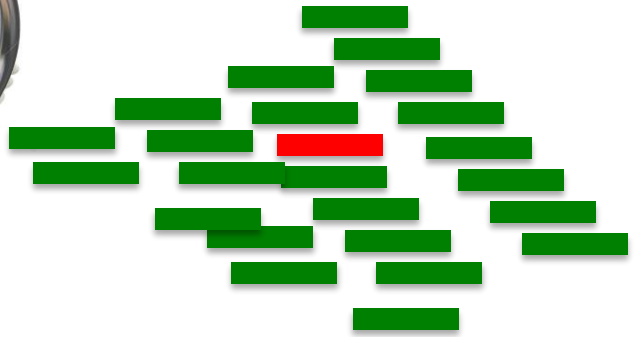
Liquid biopsy: cfDNA

- **Digital PCR**

  BEAMing (**B**eads, **E**mulsions, **A**mplification, and **M**agnetics)
  Droplet digital PCR (ddPCR)

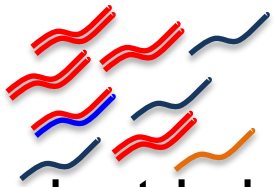
- **Targeted-NGS**

Sensitivity < 0.01%
Dynamic range > 10.000



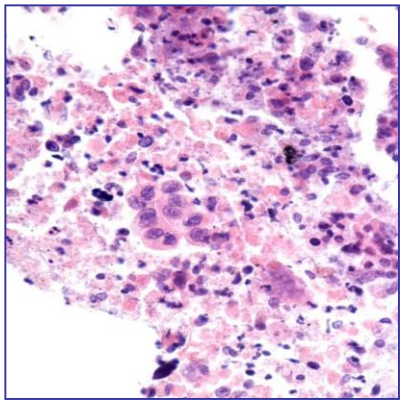
Conventional qPCR Sensitivity: up to 0.1%

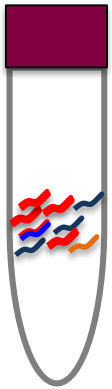
Lung cancer: cfDNA



ddPCR

EGFR status in tissue and matched cfDNA





EGFR status in tissues and matched cfDNA

Case	Stage	ALK	FFPE (Pyroseq)	cfDNA (ddPCR)
1	III	-	wt	wt
2	IV	Rearr	wt	T790M
3	IV	-	wt	T790M
4	III	-	L858R	L858R T790M
5	III	-	wt	wt
6	IV	-	746-750del	746-750del
7	IV	-	746-750del	746-750del
8	IV	-	746-750del	746-750del, T790M
9	IV	-	wt	wt
10	III	-	wt	wt
11	III	-	746-750del	746-750del
12	IV	-	wt	T790M
13	IV	-	wt	wt
14	IV	-	wt	T790M
15	IV	-	746-750del	746-750del

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La diagnostica molecolare

- Standardizzazione
- Scelte strategiche