Challenges and Opportunities for Digital PCR in the Cancer CLIA Laboratory
The Moffitt Cancer Center Experience

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Moffitt Cancer Center
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5th qPCR & Digital PCR Congress

DEVELOPING AND APPLYING QPCR & DPCR TECHNOLOGIES AND METHODS FOR APPLICATIONS IN HEALTHCARE

LONDON UNITED KINGDOM DEC 4-5 2017

Opportunities for Digital PCR

- Advantages
  - Superb Sensitivity and Precsion
- Disadvantage
  - Limited mutation panel
  - Special instumentation
  - No FDA applications for Oncology currently

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Current Challenges

- Defining specific clinical applications for use
- “killer applications”
- Regulatory – FDA / CLIA challenges in USA
- Value Proposition – payers

- Moffitt Cancer Center experience

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TAMPA FLORIDA UNITED STATES

Moffitt Cancer Center Overview

- Free standing cancer center
- Private not for profit
- 30 yr history
- Founded by the state of Florida
- Sovereign Immunity
- Annual budget support from state /Tobacco Tax
- Academic Mission: Research, Teaching and Service
- Contributes over $1 billion /yr to local economy

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Moffitt's Singular Mission

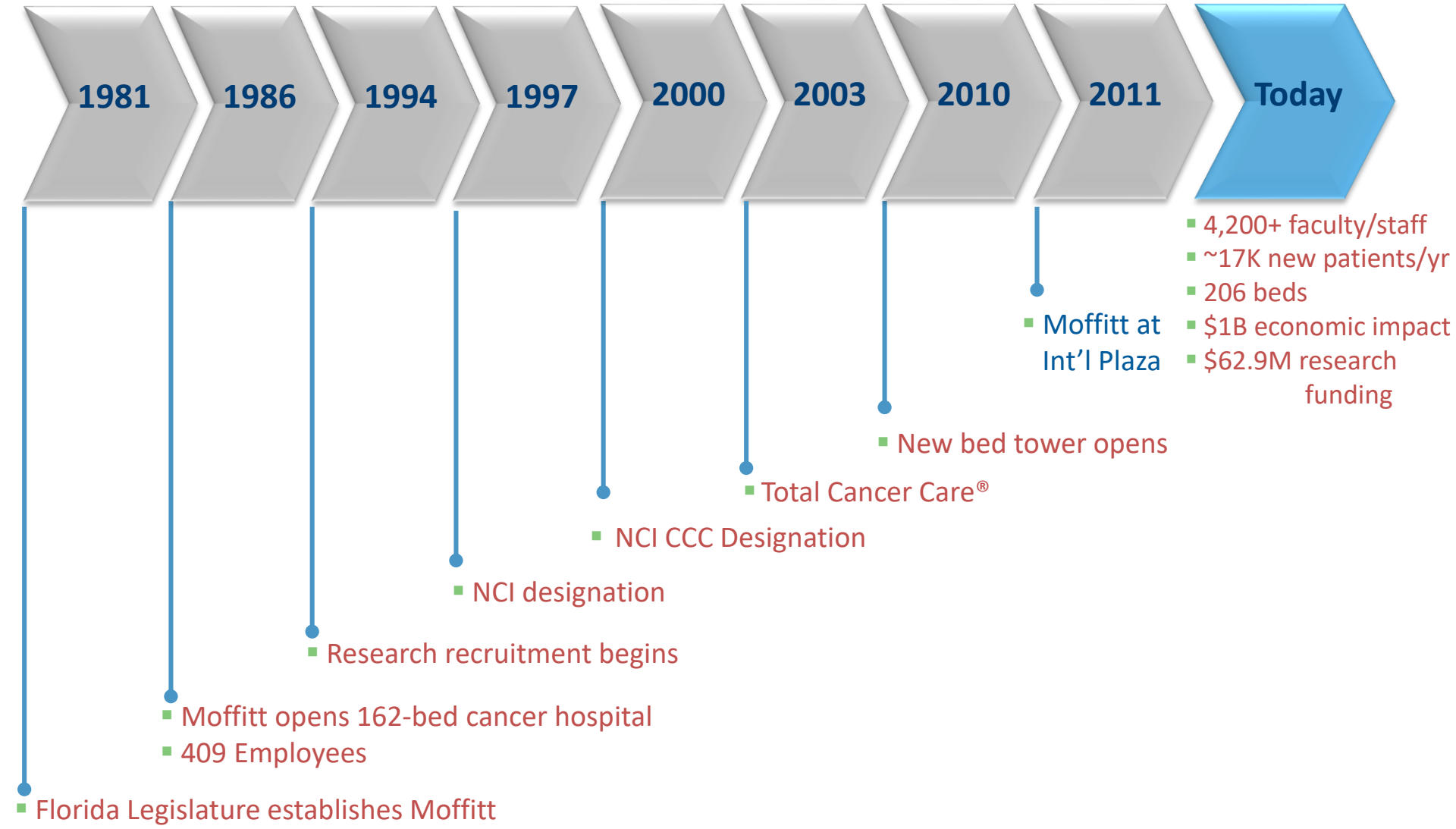
"…To contribute to the prevention & cure of cancer."

- **Statutorily created (1004.43, F.S.) as free-standing instrumentality of state**

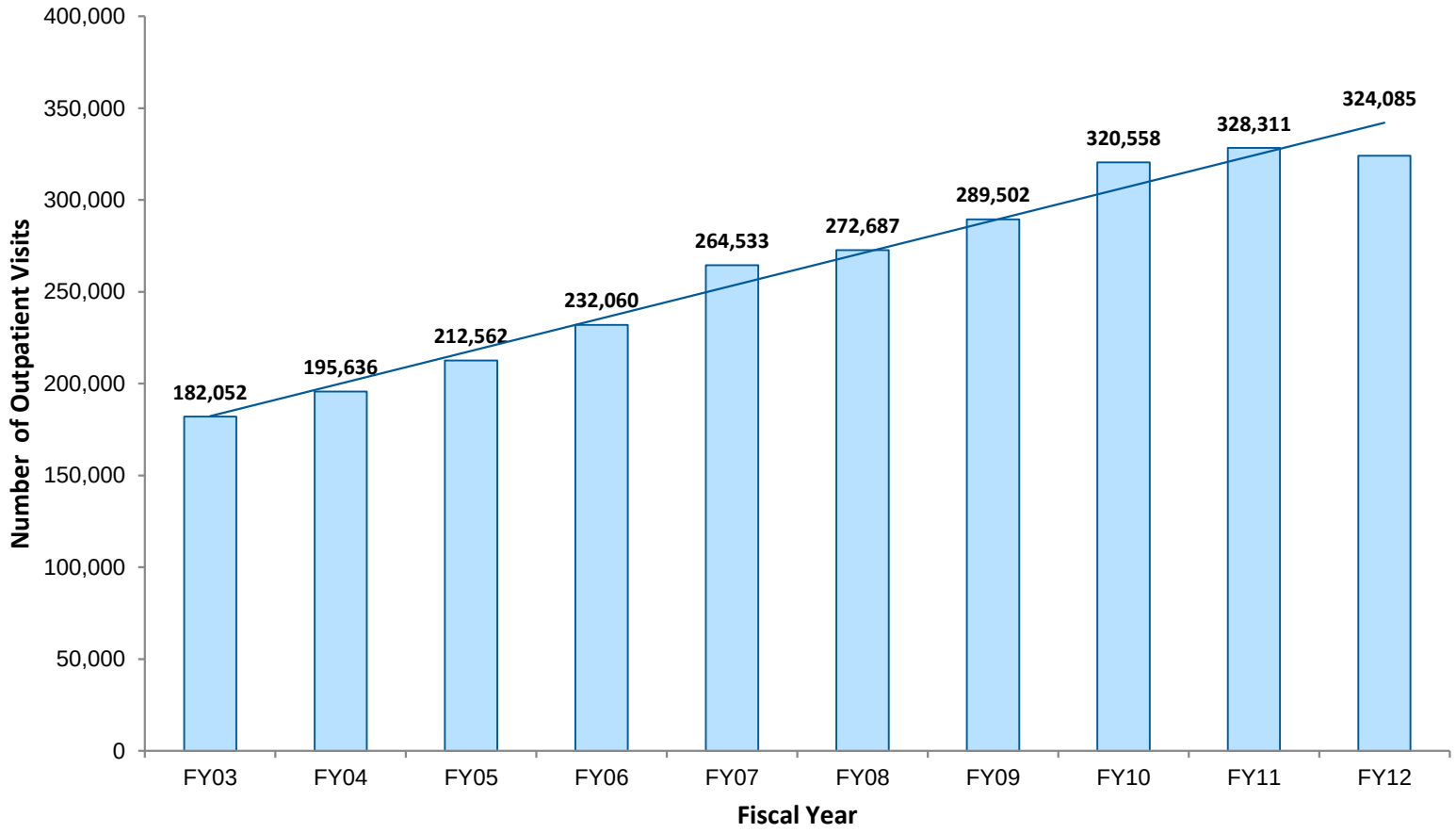
  <u>Legislative Intent</u>: to serve as "statewide research institute" and "perform a statewide function" (Chapter 90-56; 93-167 - Laws of Florida*)*

- **Cigarette tax revenue used for original design, construction and equipment ($50M). Annual-line item appropriation continues.**

Moffitt's Timeline



Total Outpatient Visits



Source: Institutional Stats FY03-12, Decision Support.
Outpatient visits prior to FY08 did not include LCS.
Total Outpatient Visits = Account numbers generated for One-time, Same Day Surgery and Recurring outpatients adjusted for multiple dates of service related to , Chemotherapy and Radiation Therapy.

Multidisciplinary from Inception

Clinical Departments

- Blood & Marrow Transplantation
- Comprehensive Breast Cancer
- Cutaneous Oncology
- Endocrine Tumors
- Gastrointestinal Oncology
- Genitourinary Oncology
- Gynecologic Oncology
- Head & Neck Oncology
- Malignant Hematology
- Neuro-Oncology
- Pain & Palliative Care
- Sarcoma
- Senior Adult Oncology
- Thoracic Oncology

Scientific Programs

- Cancer Biology & Evolution
- Immunology
- Chemical Biology and Molecular Medicine
- Cancer Epidemiology
- Health Outcomes & Behavior

Clinical & Research Expansion

Main Campus

Hospital

- 206 Licensed Beds
  - 32-Bed BMT Unit
  - CRU

Research Space

- Wet Lab: 187,472 sf
- Mouse Barrier Facility: 28,000 sf
- Dry Lab: 36,205 sf
- Moffitt Cancer Screening : 29,846 sf
- Clinical Research Space: 13,416 sf
- Research Admin: 37,096 sf

MOFFITT CANCER CENTER AT INTERNATIONAL PLAZA

Opened July 1, 2011

- Located Near Tampa International Airport
- 2 Floors / 50,630 sf
- Infusion (24 Chairs)
- Radiation Therapy
- Diagnostic Imaging
- Clinical Trials

McKinley Campus

- 30 Acres
- Biorepository Opened 2010
- New outpatient facility under construction for Spring 2015

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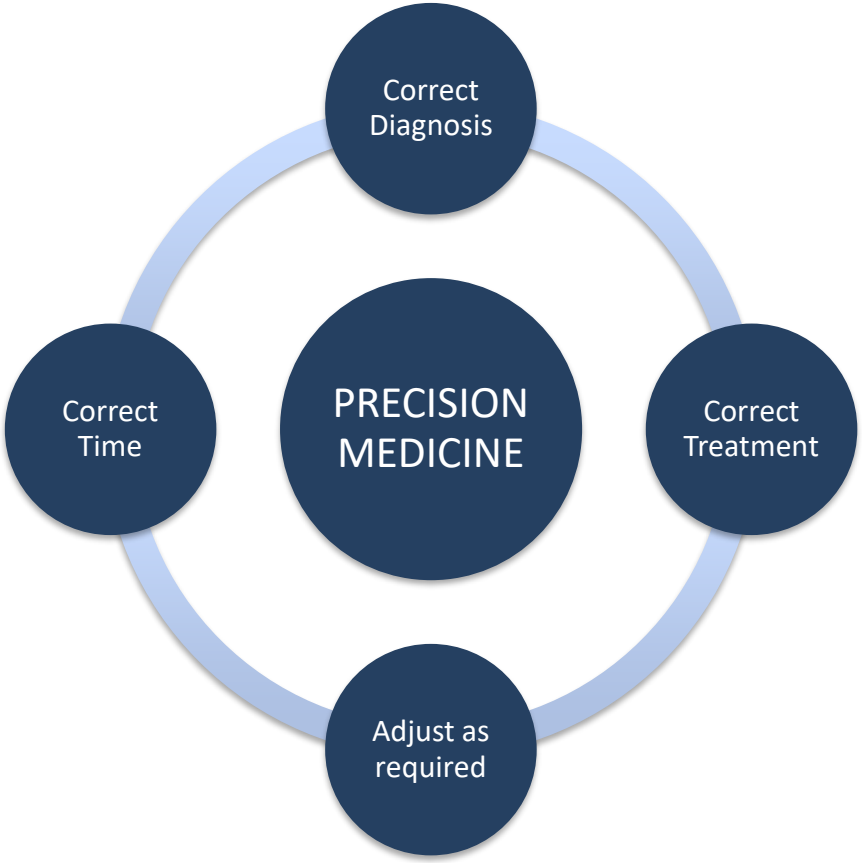
Defining Precision Medicine & Personalized Oncology

Setting the foundations with a common definition



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Personalized oncology *includes the concept that each individual solid tumor and hematologic malignancy in each person is unique in cause, rate of progression and responsiveness to surgery, chemotherapy and radiation therapy.*

PierianDx

Moffitt Molecular Diagnostics

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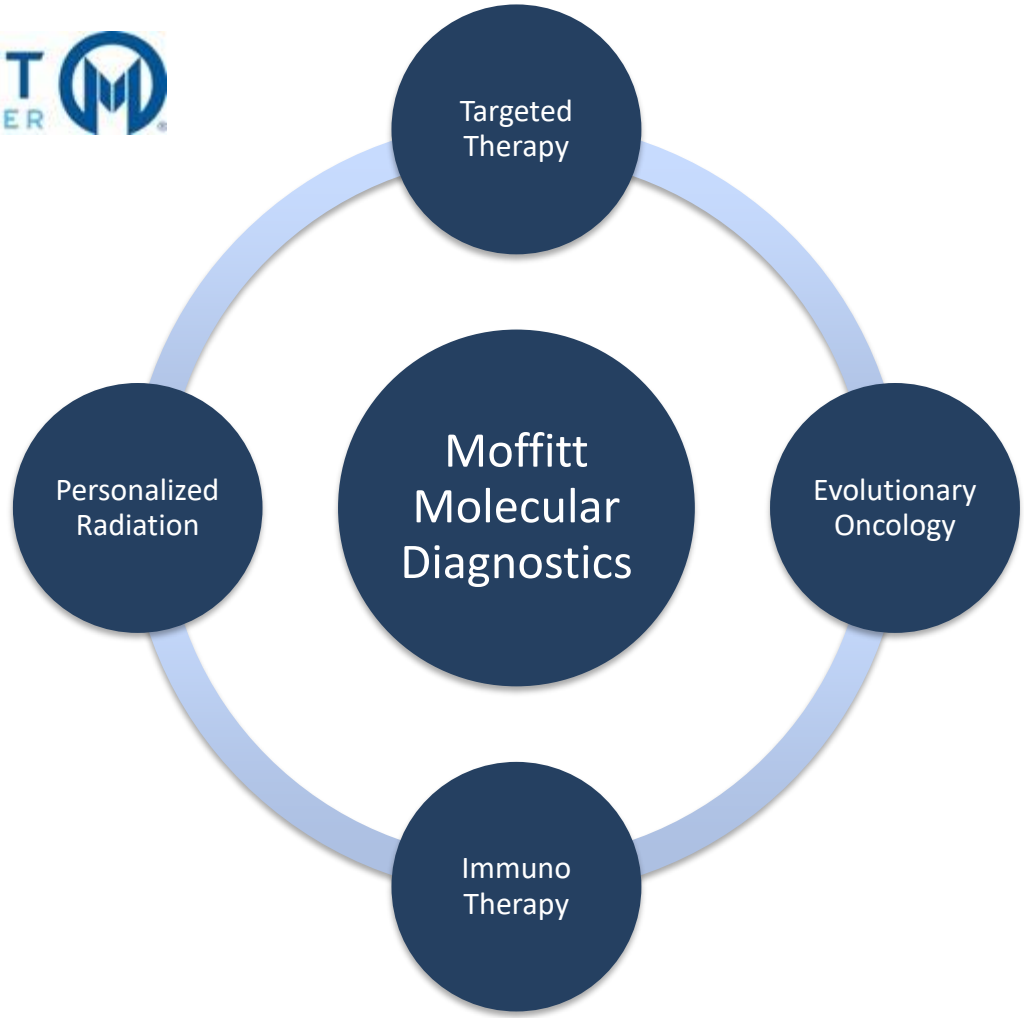

PierianDx

The Moffitt Story

Partnering together to accelerate precision medicine

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Moffitt has done 8,556 cases to date through PierianDx's CGW

May 2014
Moffitt partners with PDx to address key needs:
- Enhanced lab workflow
- Secondary analysis
- EMR integration
- Access to knowledgebase

June 2014
Moffitt adds PDx validation services to launch two assays:
- TruSight Tumor
- TruSeq Myeloid

Oct 2014
Moffitt runs first patient cases in CGW

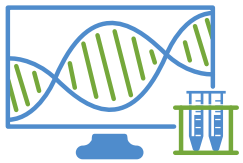
Nov 2015
Moffitt adds PDx Gateway services to handle expanded volume for heme assay

Jan 2016
Moffitt adds TruSight myeloid assay, expands capacity

Aug 2016
Moffitt upgrades secondary analysis for TruSight myeloid and TruSight Tumor assays

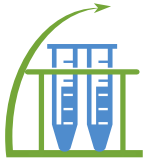
Jan 2017
Moffitt selects PDx for Interpretation Services and adds Fusion assay

Sept 2017
Moffitt works with PDx to launch TruSight Tumor 170 with TMB and MSI



Clinical Genomicist Workspace (CGW)

Professional Services



Gateway Lab Services

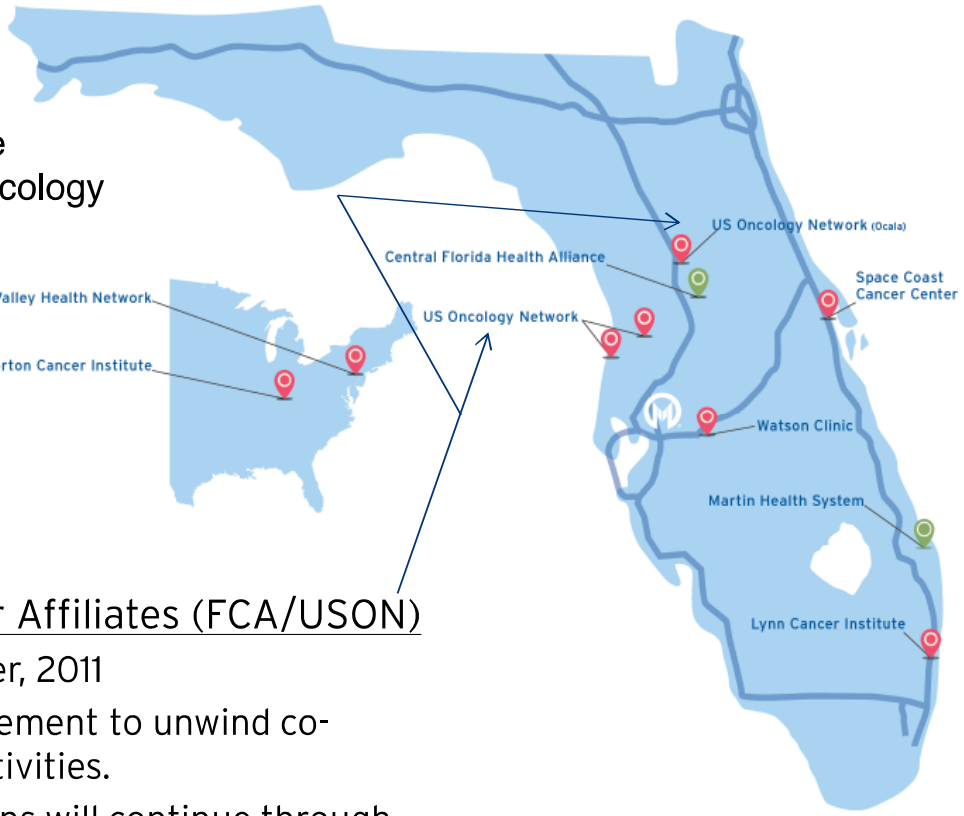
Interpretation Services

PierianDx

PARTNERSHIP CHANGES

Lehigh Valley Health Network

- Affiliate 2011-2013
- MON Member, 2013
- Market and Payer pressures are forcing them to find a tertiary oncology partner closer to their market



Florida Cancer Affiliates (FCA/USON)

- MON Member, 2011
- Mutual agreement to unwind co-branding activities.
- Collaborations will continue through nurse navigator, physician relations, radiology and laboratory services

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Tumor Boards

- All patients are presented at tumor boards
- Comprehensive pathology review
- Discussion of case
- Organized by tumor group
- 13 tumor board rounds per week
- Up to 60 cases presented per rounds

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MOFFITTS TOTAL CANCER CARE IS A MASSIVE BIOBANKING EFFORT TO UNDERSTAND THE MOLECULAR BASIS OF CANCER AT ALL STEPS OF THE PATIENT JOURNEY WITH THE DISEASE

Survivorship

- Behavioral Research
- Psychosocial & Palliative Care
- Family Needs
- Health Outcomes

Populations at Risk

- Risk Factors
- Genetics
- Early Detection
- Health Disparities

Relapsed Disease

- Recurrence Therapy
- Drug Discovery
- Adaptive Trial Design

Intervention

- Prevention
- Lifestyle/Nutrition
- Education

Treatment

- Primary Therapy
  - Multimodality
  - Target Based
- Post Therapy
  - Surveillance
- Clinical Trials Matching

Diagnosis

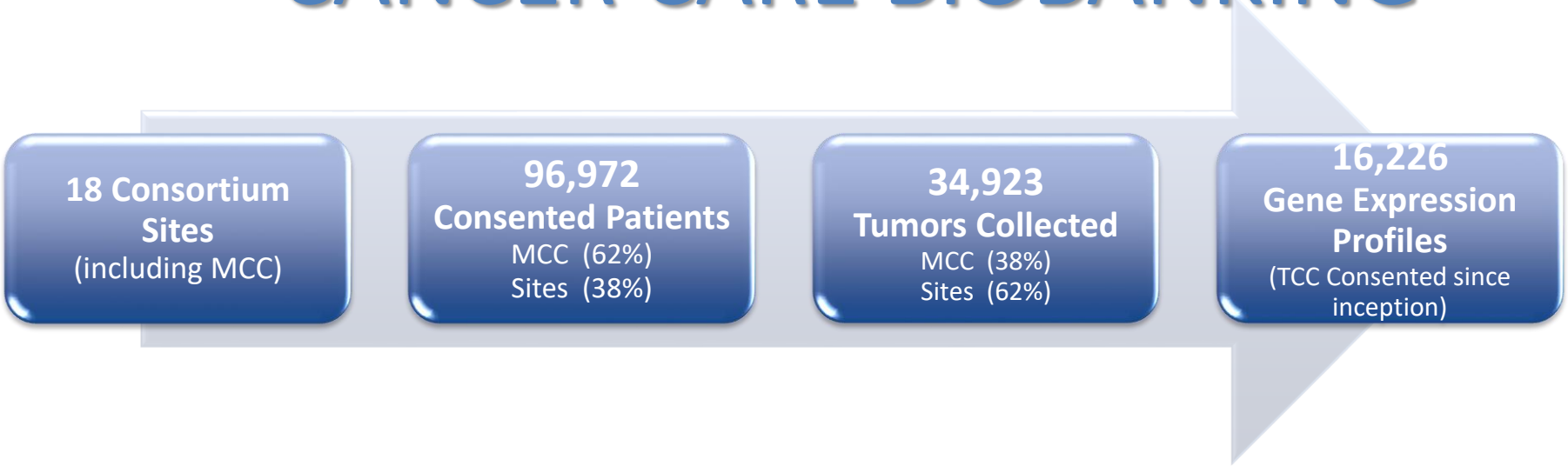
- Genomics/Proteomics
- Imaging Modalities
- Nanotechnology

Prognosis

- Molecular Oncology
- Biomarker Analysis

(http://www.hhs.gov/myhealthcare/news/phc_2008_report.pdf; pg 243)

ENROLLMENT IN MOFFITT TOTAL CANCER CARE BIOBANKING

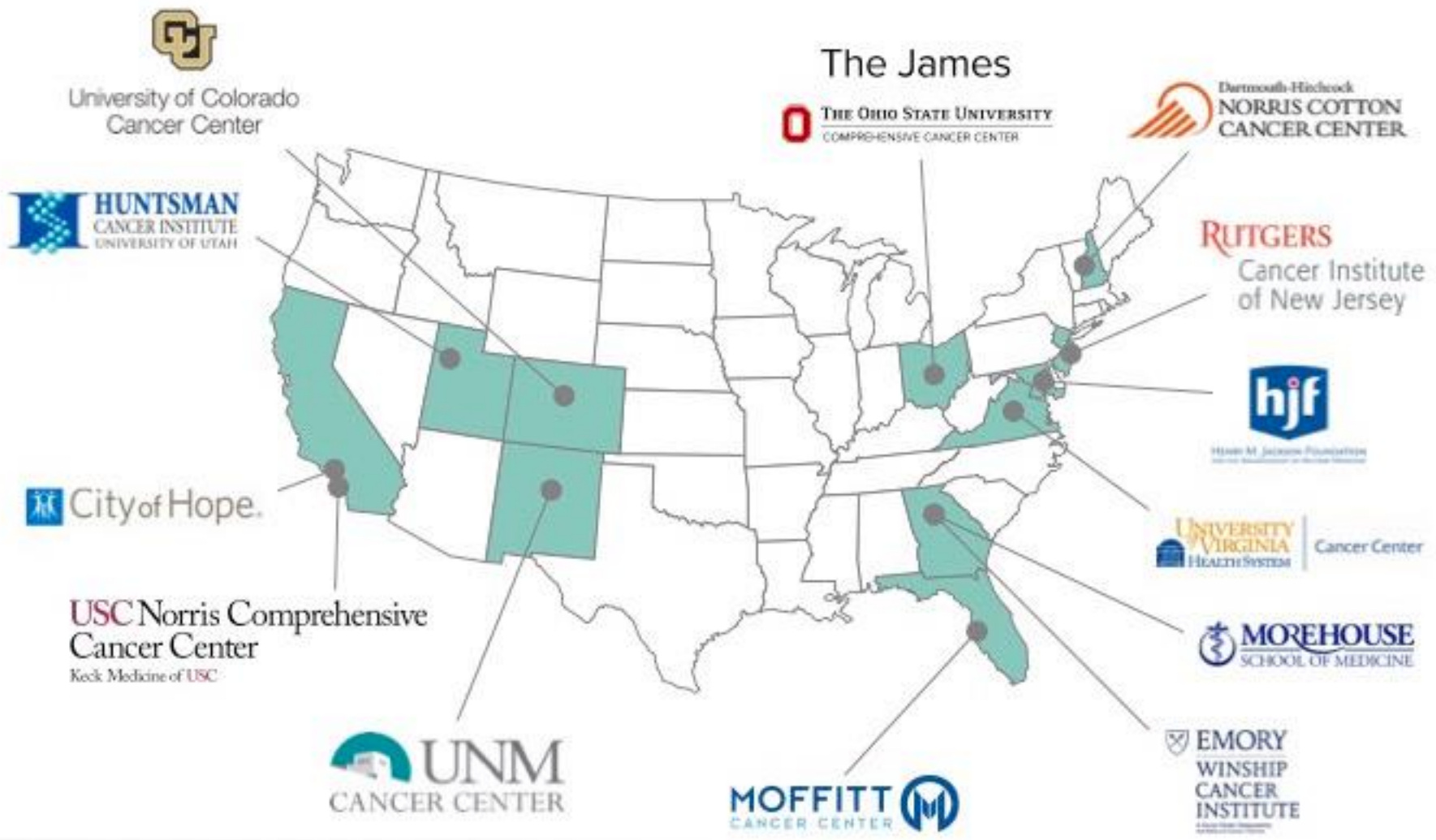


As of May 29, 2013

Data Generated from Specimens	
CEL Files (Gene Expression Data)	16,226 files
Targeted Exome Sequencing	4,016 samples
Whole Exome Sequencing (Ovary, Lung, Colon)	535 samples
Whole Genome Sequencing (Melanoma)	13 samples with normal pairs
SNP/CNV (Lung, Breast, Colon)	559 samples

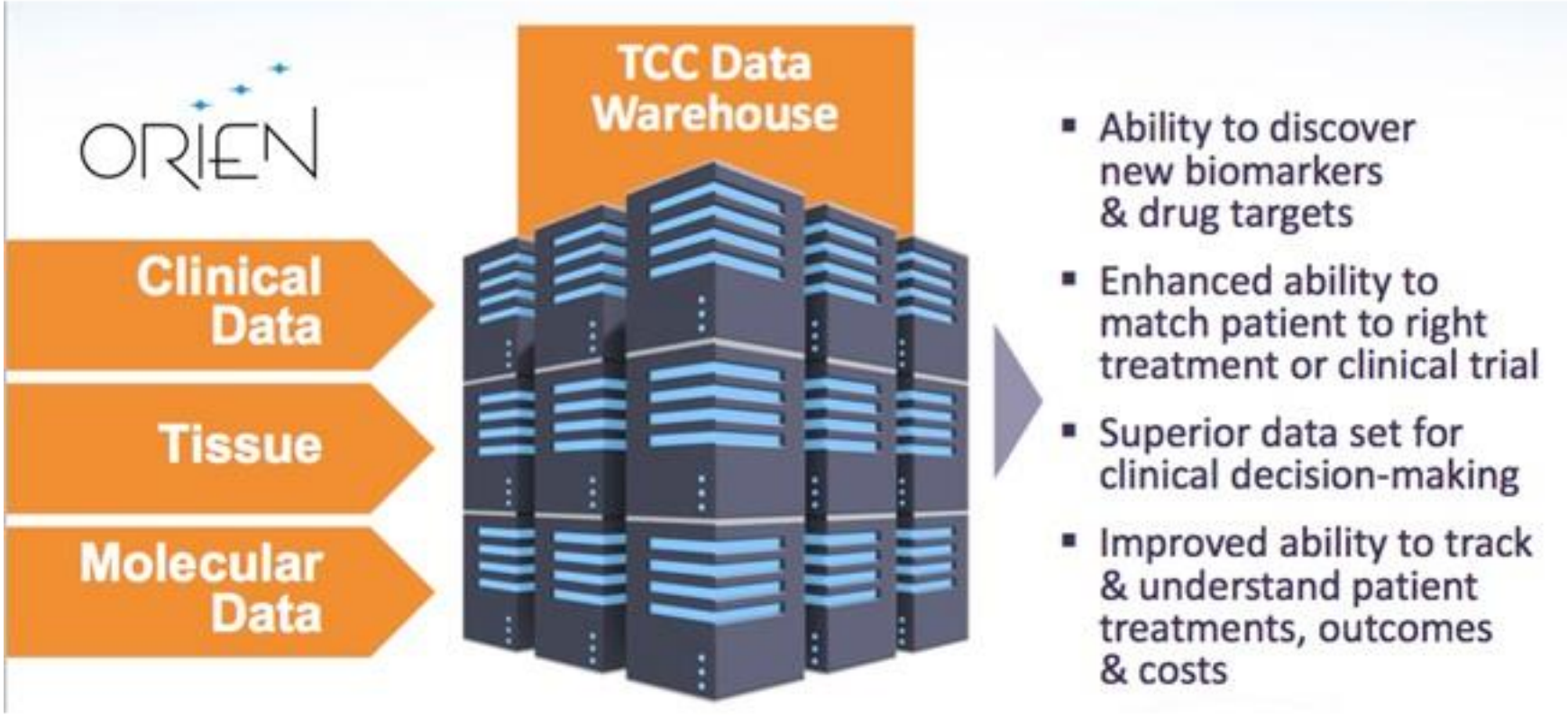
ORIEN
ONCOLOGY RESEARCH
INFORMATION
EXCHANGE NETWORK

ORIEN NETWORK



Continuous improvement based on evidence and value

BIG DATA COLLECTIONS OVER 500,000 patients



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DIAGNOSTIC SERVICES AT MOFFIT
The special "Esoteric Laboratories"

- Flow Cytometry
- HLA – Tissue Typing for Grafts
- FISH – Genetic Analysis
- Routine Molecular
- New Assay Development

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Current Molecular Platforms At Moffitt

- Sanger Sequencing
- Pyrosequencing
- RT-PCR
- MassArray
- MiSeq Next Gen Sequencing
- NexSeq 500
- Luminex
- Nanostring n Counter
- Multiplex Analytical Microscopy / AQUA

Current Assays in MOFFITT CLIA

- MassArray
  - LungCarta
  - Moffitt Neural Tumor Panel (custom)
- NGS
  - Solid Tumor TST26
  - Heme 54 gene
- NanoString nCounter
  - Prosigna (FDA)- Breast Cancer
- CellSearch
  - Breast cancer / Prostate / Colon (FDA)
  - CSF analysis- rare cell isolation
  - Clinical Trials Androgen in Bladder, HER2 Breast, PDL1
- MGMT Promoter Methylation Analysis
- PharmacoGenomics – Luminex Cyp2D6 (FDA)
- CLIA Analytical Microscopy
  - AQUA / Definiens

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2012 MASSARRAY LAUNCH

LungCarta™

26 Oncogenes
214 Selected Mutations

Genes Included in the LungCarta Panel:

AKT1	*JAK2*	*NTRK3*
ALK	*KRAS*	*PIK3CA*
BRAF	*MAP2K1*	*PTCH1*
DDR2	*MET*	*PTEN*
EGFR	*NOTCH1*	*PTPN11*
EPHA3	*NRAS*	*PTPRD*
EPHA5	*NRF2*	*STK11*
ERBB2	*NTRK1*	*TP53*
FGFR4	*NTRK2*	

SEQUENOM®

Gene	Mutations Detected with the LungCarta Panel
AKT1	E17K
ALK	C1156Y, L1196M
BRAF	D594G/V, G469S/E/A/V, L597Q/V, V600E/K/M
DDR2	C580Y, D125Y, G253C, G505S, G774E/V, I120M, I638F, L239R, L63V, T765P
EGFR	R108K, T263P, A289V, G598V, E709K/H, E709A/G/V, G719S/C/A/D, G719S/C/A/D, M766_A767insAI, D761Y/N, S768I, R776C/H, V769_D770insASV, V769_D770insCV, D770_N771>AGG/V769_D770insASV/V769_D770insASV, D770_N771insG, N771_P772>SVDNR, P772_H773insV, H773>NPY, H773_V774insNPH/PH/H, V774L, V774_C775insHV, T790M, L858R/M, L861Q, E746_T751del, E746_A750del, E746_T751del, E746_T751del, S752D, L747_E749del, L747_T750del, L747_S752del, L747_T751del, L747_S752del, P753S, A750P, T751A, T751P, T751I, S752I/F, S752_I759del, L747_Q ins, E746_T751del, I ins (combined), E746_A750del, T751A (combined), L747_E749del, A750P (combined), L747_T750del, P ins (combined), L747_S752del, Q ins (combined), T854A
EPHA3	A435S, D446Y, S449F, D806N, G187R, G518L, K761N, G766E, M269I, N379K, N85S, S229Y, T166N, T37K, T393K, W250R
EPHA5	D493Y, G582E, M1034I, N1032S, R1007Q, S566Y, S810I, T856I
ERBB2	M774_A775insAYVM, A775_G776insAYVM
FGFR4	P672T, H192fs*19
JAK2	L609S, P503L, R1122P, Y931C
KRAS	G12S/V/F/R/A/C/D, G13C/S/A/V/D, Q61L/R/P/H/E/K
MAP2K1	D67N, K57N, Q56P
STK11	A347fs*13, A43_L50del6, D327fs*10, E120*, E165*, E223*, E70*, E70fs*26, F354L, G163C, G188fs*99, G196V, G56fs*4, G56W, G91L, H174R, I26fs*25, K191*, K78E, L285Q, L50_D53del4, M51fs*14, P179L, Q123R, Q137*, Q159*, Q170*,Q220*, Q37L, R426W, R86G, V197fs*69, V236fs*30, Y272Y
MET	N375S, 982_1028del47
NOTCH1	H2276fs*79, D1643H, R2328W, T1997M, V1672I, V2444fs*35
NRAS	Q61E/K/H/L/R/P
NRF2	D29H, D77N/A, E79Q/K/G, G31A, G81D, R34Q
NTRK1	Q80*, R119H, S326R
NTRK2	Q666R, C45F, G261R, L138F, L670M, L755L
NTRK3	I769N, L152I, L248M, L270M, L336Q, S184C, T283K, V307L, R271F
PIK3CA	E542Q/K, E545Q/K, H1047Y/R/L
PTCH1	R1308G, R682L, S1326fs*46
PTEN	R233*
PTPN11	E76V
PTPRD	S1703R, T337A, V483E
TP53	G245C/S, G245D/V, R158C/G/L/P, R175L/H, R248G/L/Q/W, R249S/W/M, R273C/H/L/P, R282G/W, V157F, Y163C, R175L/H, Y220C

Moffitt CUSTOM MassArray Glioma Panel for GLIOMA Over 1000 cases anayzed - 2014

Gene	Mutations
CIC	R201W, R213W, R215Q, V1474F
IDH1	R132H, R132C,R132S, R132L, R132G
TP53	S215I, R273C, H179R, A161T, I573T
CDKN2A	H83Y, P114L, A76V, H98Y, A73T, V106M, A85T, R128W,
PTEN	G129R, D107Y, L345Q, L345Q, A121P,
FUBP1	P281L, A596T,E445* , E411*, H463fs*>182, D570fs*>75
ATRX	S1394fs*95, I737fs*3,H166fs*4, R1302fs*7, R2153C, R1803H , W263*
NF1	R1391S, Q1426K, D1828N, A2315T
EGFR	T263P
PIK3CA	R88Q, H1047R
RB1	N133H, K412N, F650S,
	K130Il, L135P, D421N, T510I, D566N
PIK3R1	L570P, K459E, D464H, G376R, N564D, N564K, D560Y
MSH6	G971E, E201Q, E487D, G932D, K247I, L109V, A1151V, S532A,
	R58S, A1204E, S360N, A1303T, R1024N, T1219I, V1056M, E484K, K187T, G39E
IDH2	R172K, R172G, R172M
CHEK2	P536L, K373E
NOTCH1	V1672I, A2280V, G2153R, S1709S, Q1050L
ERBB2	E914K, T216S, V777A, L49H, C311R, E321G, V750E, N319D
CDKN2C	A2fs*17, R15fs*4
PDGFRA	D842V

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Next Generation Sequencing Panels

NGS Solid Tumor Targeted Mutation Panel
(26 Genes)

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

NGS Myeloid Targeted Mutation Pan
(32Genes)

ABL1	MLL
ASXL1	MPL
CBL	MYD88
CEBPA	NPM1
CSF3R	NRAS
CUX1	PHF6
DNMT3A	RUNX1
ETV6	SETBP1
EZH2	SF3B1
FLT3	SH2B3
IDH1	SRSF2
IDH2	TET2
IKZF1	TP53
JAK2	U2AF1
KIT	WT1
KRAS	ZRSR2

DEMANDS FROM HEME SERVICES– ACUTE LEUKEMIA, AML, MDS

MOFFITT TruSight Myeloid Sequencing Panel Gene List launch 2016

ABL1	CEBPA	HRAS	MYD88	SF3B1
ASXL1	CSF3R	IDH1	NOTCH1	SMC1A
ATRX	CUX1	IDH2	NPM1	SMC3
BCOR	DNMT3A	IKZF1	NRAS	SRSF2
BCORL	ETV6/TEL	JAK2	PDGFRA	STAG2
BRAF	EZH2	JAK3	PHF6	TET2
CALR	FBXW7	KDM6A	PTEN	TP53
CBLFLT3	KIT	PTPN11	U2AF1	
CBLB	GATA1	KRAS	RAD21	WT1
CBLC	GATA2	MLL	RUNX1	ZRSR2
CDKN2A	GNAS	MPL	SETBP1	

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MOFFITT INCREASED DEMANDS FOR LARGER PANELS

- RNA FUSIONS- NTRK
- UNUSUAL MUTATIONS- ie MET 14 skipping
- DEMAND for MSI and Mutational Loads emerging

TruSight® Tumor 170

A comprehensive next-generation sequencing assay that targets DNA and RNA variants from the same FFPE tumor sample.

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TruSight® Tumor 170

A comprehensive next-generation sequencing assay that targets DNA and RNA variants from the same FFPE tumor sample.

Table 1: Gene Content in the TruSight Tumor 170 Assay

SNVs and Indels (from DNA)									
AKT1	*BRCA1*	*FANCI*	*FGF23*	*IDH1*	*MET*	*NBN*	*PIK3CB*	*RB1*	*TP53*
AKT2	*BRCA2*	*FANCL*	*FGFR1*	*IDH2*	*MLH1*	*NF1*	*PIK3CD*	*RET*	*TSC1*
AKT3	*BRIP1*	*FBXW7*	*FGFR2*	*INPP4B*	*MLLT3*	*NOTCH1*	*PIK3CG*	*RICTOR*	*TSC2*
ALK	*BTK*	*FGF1*	*FGFR3*	*JAK2*	*MPL*	*NOTCH2*	*PIK3R1*	*ROS1*	*VHL*
APC	*CARD11*	*FGF2*	*FGFR4*	*JAK3*	*MRE11A*	*NOTCH3*	*PMS2*	*RPS6KB1*	*XRCC2*
AR	*CCND1*	*FGF3*	*FLT1*	*KDR*	*MSH2*	*NPM1*	*PPP2R2A*	*SLX4*	
ARID1A	*ERBB3*	*FGF4*	*FLT3*	*KIT*	*MSH3*	*NRAS*	*PTCH1*	*SMAD4*	
ATM	*ERBB4*	*FGF5*	*FOXL2*	*KMT2A (MLL)*	*MSH6*	*NRG1*	*PTEN*	*SMARCB1*	
ATR	*ERCC1*	*FGF6*	*GEN1*	*KRAS*	*MTOR*	*PALB2*	*PTPN11*	*SMO*	
BAP1	*ERCC2*	*FGF7*	*GNA11*	*MAP2K1*	*MUTYH*	*PAX3*	*RAD51*	*SRC*	
BARD1	*ERG*	*FGF8*	*GNAQ*	*MAP2K2*	*MYC*	*PAX7*	*RAD51B*	*STK11*	
BCL2	*ESR1*	*FGF9*	*GNAS*	*MCL1*	*MYCL1*	*PDGFRA*	*RAD51C*	*TERT*	
BCL6	*EZH2*	*FGF10*	*HNF1A*	*MDM2*	*MYCN*	*PDGFRB*	*RAD51D*	*TET2*	
BRAF	*FAM175A*	*FGF14*	*HRAS*	*MDM4*	*MYD88*	*PIK3CA*	*RAD54L*	*TMPRSS2*	
Amplifications (from DNA)									
AKT2	*BRCA2*	*CHEK1*	*ERCC2*	*FGF5*	*FGF14*	*FGFR4*	*MDM4*	*NRG1*	*RAF1*
ALK	*CCND1*	*CHEK2*	*ESR1*	*FGF6*	*FGF19*	*JAK2*	*MET*	*PDGFRA*	*RET*
AR	*CCND3*	*EGFR*	*FGF1*	*FGF7*	*FGF23*	*KIT*	*MYC*	*PDGFRB*	*RICTOR*
ATM	*CCNE1*	*ERBB2*	*FGF2*	*FGF8*	*FGFR1*	*KRAS*	*MYCL1*	*PIK3CA*	*RPS6KB1*
BRAF	*CDK4*	*ERBB3*	*FGF3*	*FGF9*	*FGFR2*	*LAMP1*	*MYCN*	*PIK3CB*	*TFRC*
BRCA1	*CDK6*	*ERCC1*	*FGF4*	*FGF10*	*FGFR3*	*MDM2*	*NRAS*	*PTEN*	
Fusions and Splice Variants (from RNA)									
ABL1	*BRAF*	*EML4*	*ETV4*	*FGFR4*	*KIF5B*	*MYC*	*NTRK2*	*PIK3CA*	*TMPRSS2*
AKT3	*BRCA1*	*ERBB2*	*ETV5*	*FLI1*	*KIT*	*NOTCH1*	*NTRK3*	*PPARG*	*TP53*
ALK	*BRCA2*	*ERG*	*EWSR1*	*FLT1*	*KMT2A (MLL)*	*NOTCH2*	*PAX3*	*RAF1*	
AR	*CDK4*	*ESR1*	*FGFR1*	*FLT3*	*MET*	*NOTCH3*	*PAX7*	*RET*	
AXL	*CSF1R*	*ETS1*	*FGFR2*	*JAK2*	*MLLT3*	*NRG1*	*PDGFRA*	*ROS1*	
BCL2	*EGFR*	*ETV1*	*FGFR3*	*KDR*	*MSH2*	*NTRK1*	*PDGFRB*	*RPS6KB1*	

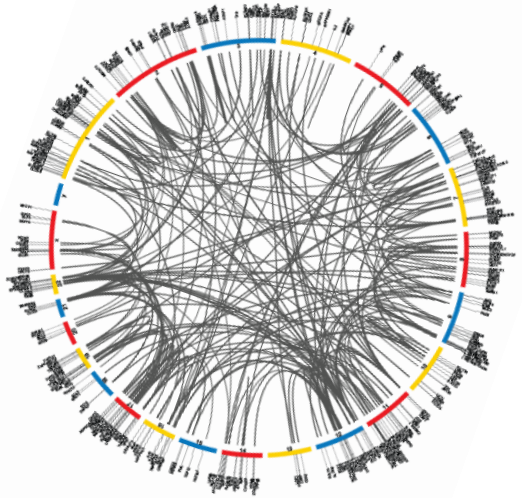
Highlights of TST 170 Gene Panel

- Comprehensive Coverage of Cancer-Related Variants in Single-assay efficiency using **DNA and RNA** for assessment of small variants, amplifications, splice variants, and fusions

- Integrated, Streamlined Workflow DNA and RNA libraries are prepared in parallel with an integrated workflow following **DNA shearing/cDNA synthesis**

- Accurate Results from Low-Quality Samples Variant detection with 40 ng DNA/RNA input, and as low as 5% mutant allele frequency from FFPE samples

RNA FUSION PANEL



ILLLUMINA

507 FUSION ASSOCIATED GENES
RNA WORKFLOW

USES
- Detection of known important variants and also unknown in rare tumors

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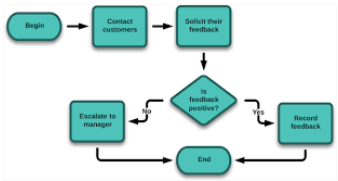
Personalized Medicine Service



Personalized Medicine Consultation

Pathway Development

Molecular Tumor Board

Clinical Trial Matching and off label

Training program

Reimbursiment and payers

LIQUID BIOPSY IN ONCOLOGY

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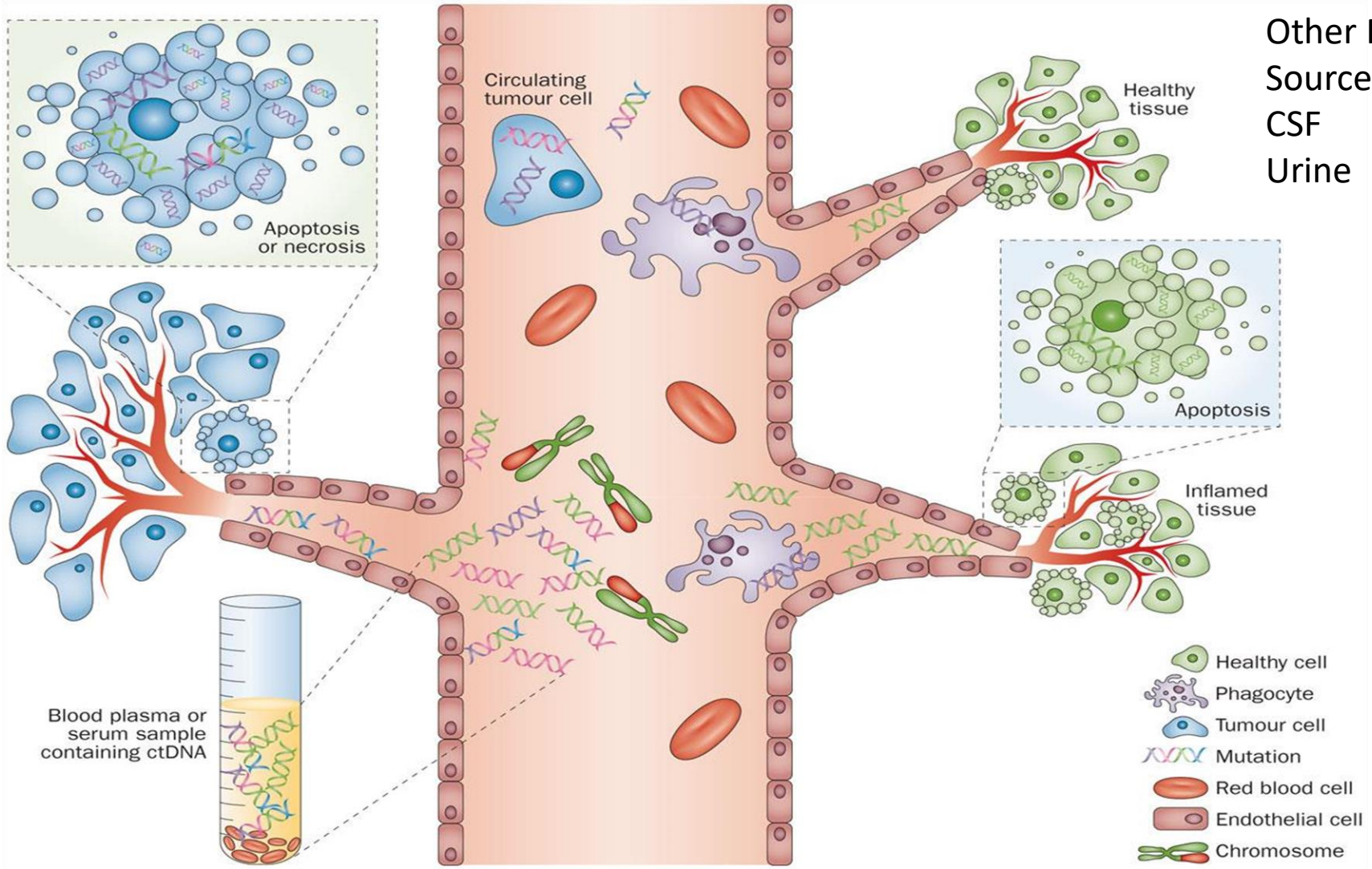
Liquid Biopsy Origin

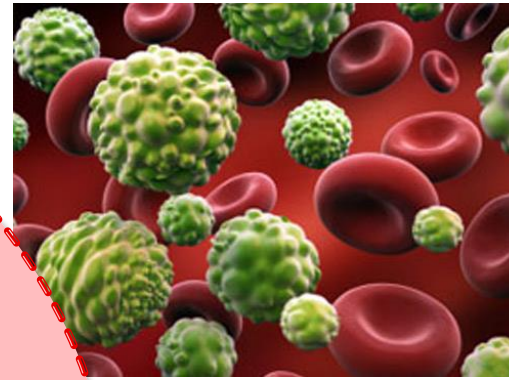


Other Fluid Sources:
CSF
Urine

NATURE REVIEWS | CLINICAL ONCOLOGY VOLUME 10 AUGUST 2013

Liquid Biopsy Clinical Applications

Non-invasive blood sampling



Real time liquid biopsy

Screening and early detection of cancer
EGFR mt ctDNA and CTC counts in (NSCLC)

Real-time monitoring of therapy
CTC counts (BC)
KRAS mt on ctDNA (CRC)
AR mt on ctDNA (PC)

Clinical Applications

Stratification and therapeutic intervention
HER2 or ER expression on CTCs (BC)
CTC counts (BC)-Metabreast trial

Risk for metastatic relapse (prognosis)
CTC counts in solid tumors (e.g. breast, prostate, colorectal, lung and bladder cancers
KRAS mt in ctDNA (CRC)

Therapeutic targets and resistance mechanisms
KRAS mt (CRC)
EGFR my (NSCLC)
Lack of ER expression (BC)
AR mt or ARv7 expression (PC)

CTCs and cfDNA

Personalized Treatment

Adapted from Alix-Panabieres et. al. (2016) Clinical Appilications of Circulating Tumor Cells and Circulating Tumor DNA as Liquid Biopsy . Cancer discovery

CTCs and Cell-Free DNA Pros and Cons

Subject	Advantages	Limitations
CTCs	• Visualization of intact cells for morphological identification of a malignant phenotype • Relevance for the metastatic process and disease progression; • Allow functional *in vitro/in vivo* assays (e.g., xenotransplantation of CTCs into immunodeficient mice) • Opportunity for molecular characterization at both cellular and sub-cellular level (e.g., genomic analysis of single CTCs) • Allows immuno-labeling based approaches • Complementary with ctDNA: CTCs can survive current chemotherapy and might indicate failure of therapeutic interventions • Potentially influence changes in treatment modalities	• Low abundance and fragility • Require extremely sensitive and specific analytic methods • False-negative (due to epithelial-to-mesenchymal transition) and false-positive results • Heterogeneity of the CTC populations (e.g., detection of CTCs with tumor-initiating capacity) • Multiplicity of technologies used for CTC isolation
Cell-free ctDNA	• More sensitive for detection of disease burden • Complementary with CTCs for detection of minimal residual disease after surgery or therapy with curative intent • Might predict acquired drug resistance • Potentially influence changes in treatment modalities	• False-negative and false-positive results (e.g., no specific isolation of tumor DNA unless the detection of tumor-specific mutations; or mutations of tumor-associated genes in normal tissue of aging patients and in frequent benign diseases) • No functional assays • Lack of standardization of preanalytical conditions (e.g., dilution and contamination of ctDNA with normal DNA from dying blood cells after blood collection)

Ann Transl Med. 2014 Nov;2(11):107. doi: 10.3978/j.issn.2305-5839.2014.08.11.

Liquid Biopsy Platforms Available at/or Coming to Moffitt Cancer Center

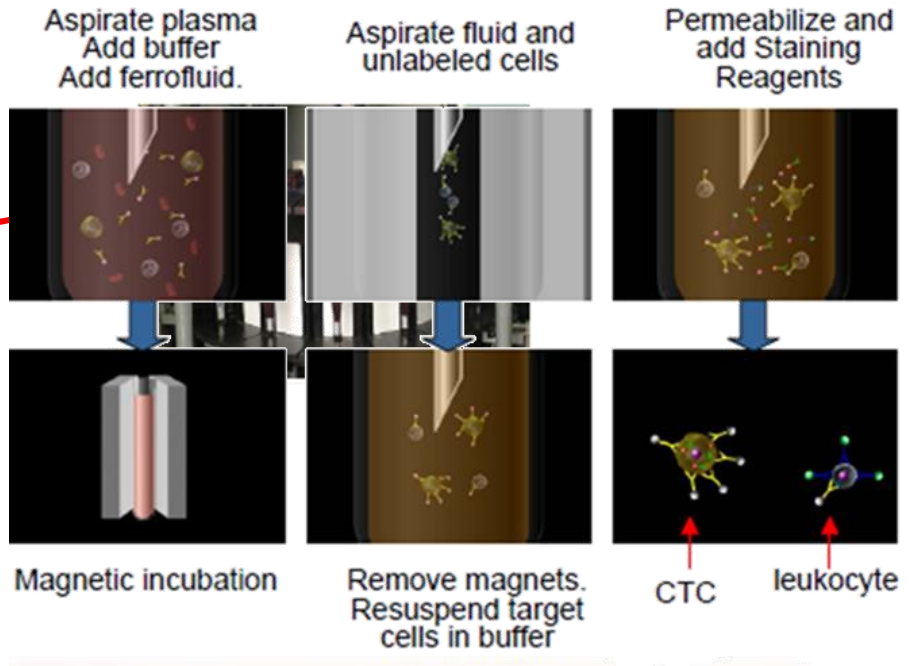
ddPCR

Janssen Diagnostics CellSearch®

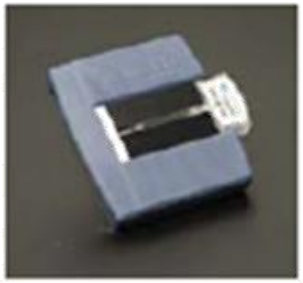
ANGLE Parsortix

Liquid Biopsy CTCs: The CellSearch® System

CellTracks AutoPrep



MagNest®

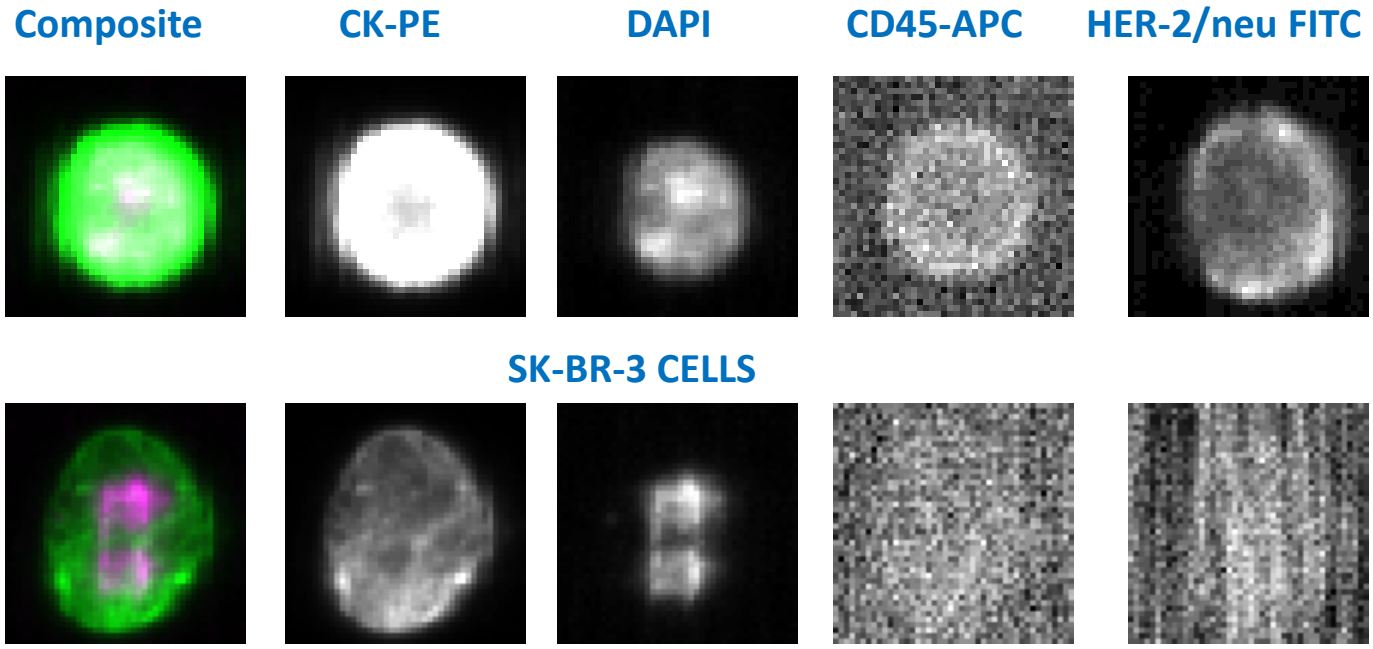


CellTracks Analyzer II

FDA approved for Metastatic Breast, Colorectal and Prostate Cancers

The CellSearch® Antibody Characterization

Her2/neu



Other Fully Characterized Antibodies: ER, AR, Ki67, PD-L1

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The CellSearch® System: Pros and Cons

Pros	Cons
FDA approved	Strict CTC definition: Epcam^{+}, DAPI^{+} CK8, 18, 19^{+} and CD45^{-}
Automated	Limited Available Channels (4)
RUO Applications	Relatively Expensive

Liquid Biopsy CTCs: ANGLE Parsortix PR1

Separates rare circulating cell populations based on size and deformability and is an epitope-independent enrichment method.

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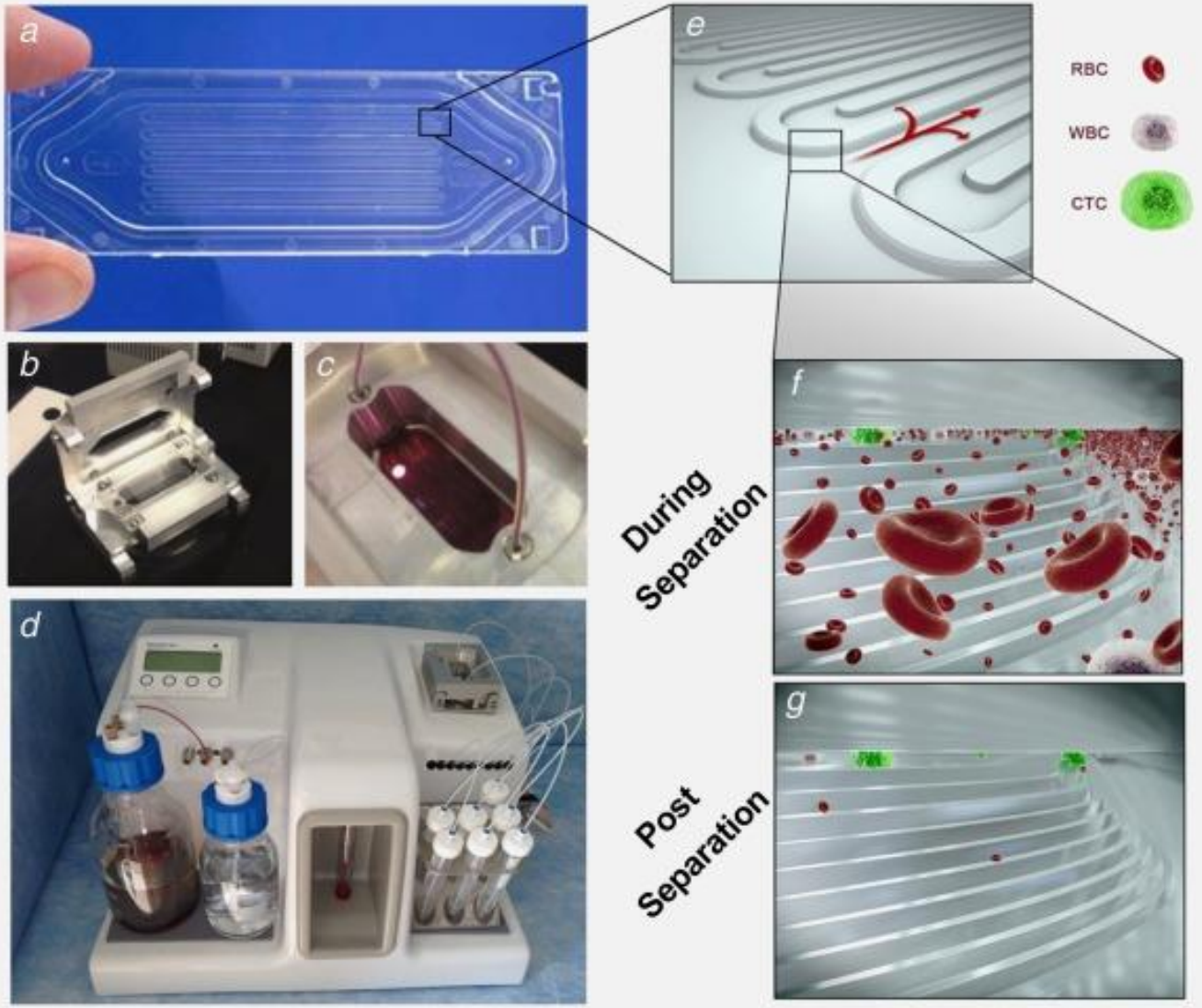
ANGLE Parsortix PR1: How it Works.

6.5, 8 and 10 microns sizes available



Harvest cells by reversing flow

Int J Cancer. 2016 Jun 15; 138(12): 2894–2904.
Published online 2016 Feb 26. doi: 10.1002/ijc.30007

ANGLE Parsortix PR1: Pros and Cons

Pros	Cons
No strict phenotypic definition	Cell enrichment only
Can detect EMT CTCs	Not FDA approved, RUO
Cells are viable	Cell recovery rates ~75%
Color channels defined by user	Visualization not included
Can isolate CTCs and ctDNA from the same sample	Leukocyte contamination still present

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Applications of Digital PCR

- **Circulating Mutations**
  - **BRAF V600E**
  - **EGFR T790M**
- **Sub clones in tissue**
- **Mutational screening, minimal residual disease, tumor evolution**
- **Other fluids-**
  - **Urine, CSF, Pleural**

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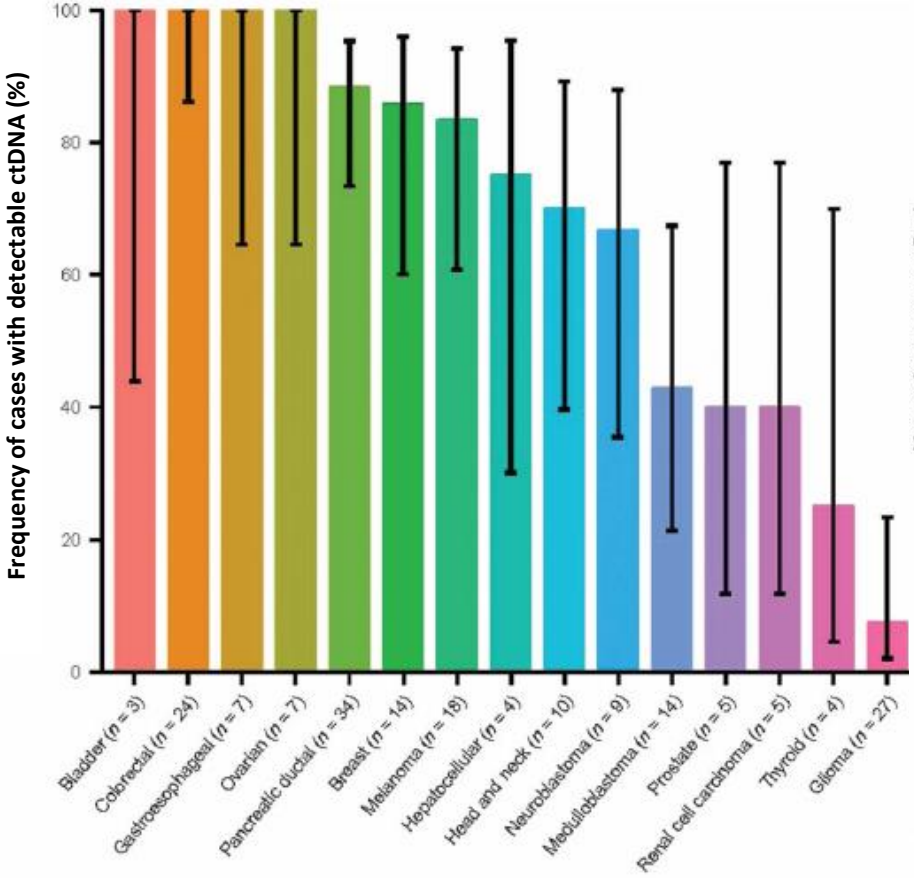
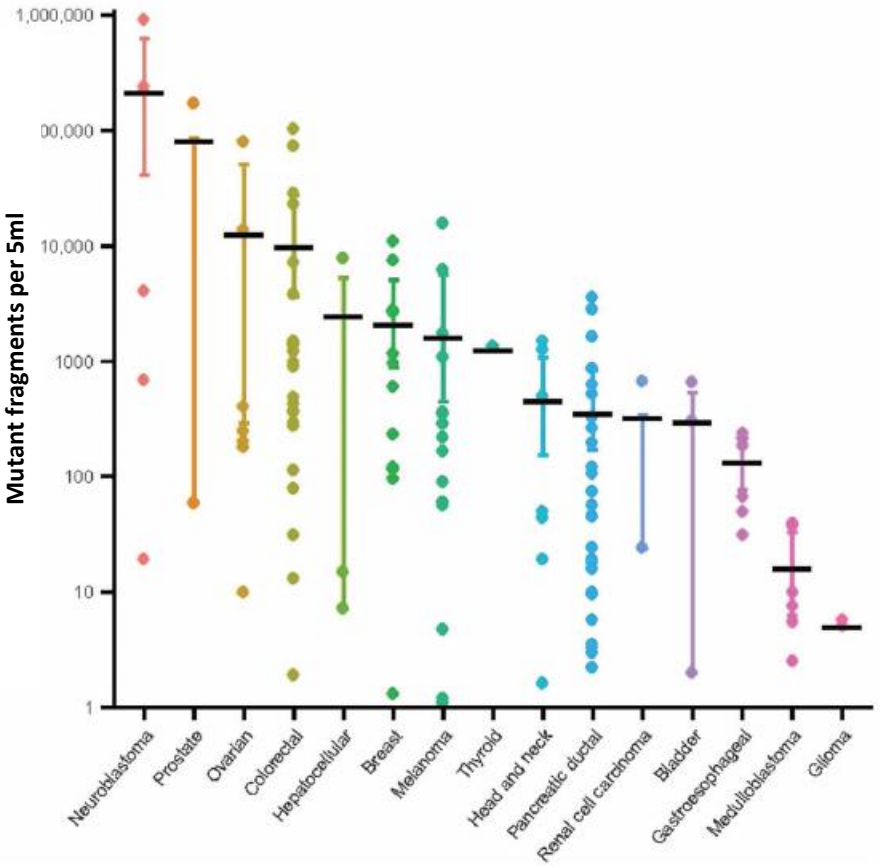
Liquid Biopsy: ctDNA, Why is it Important?





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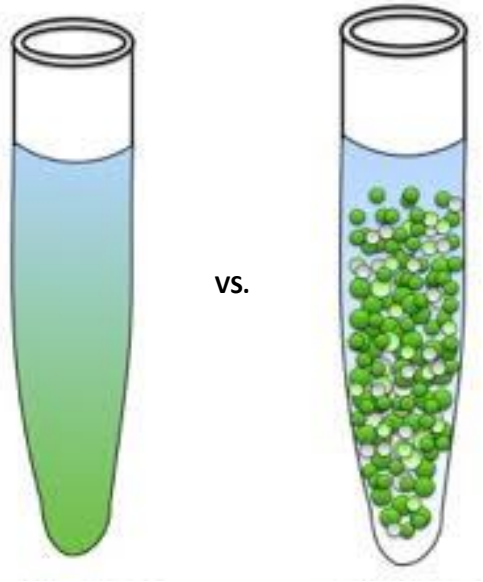
Sci Transl Med. 2014 February 19; 6(224): 224ra24. doi:10.1126/scitranslmed.3007094.

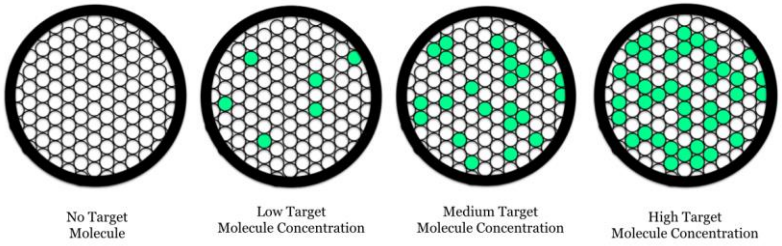
ddPCR Principle: Limiting Dilution PCR

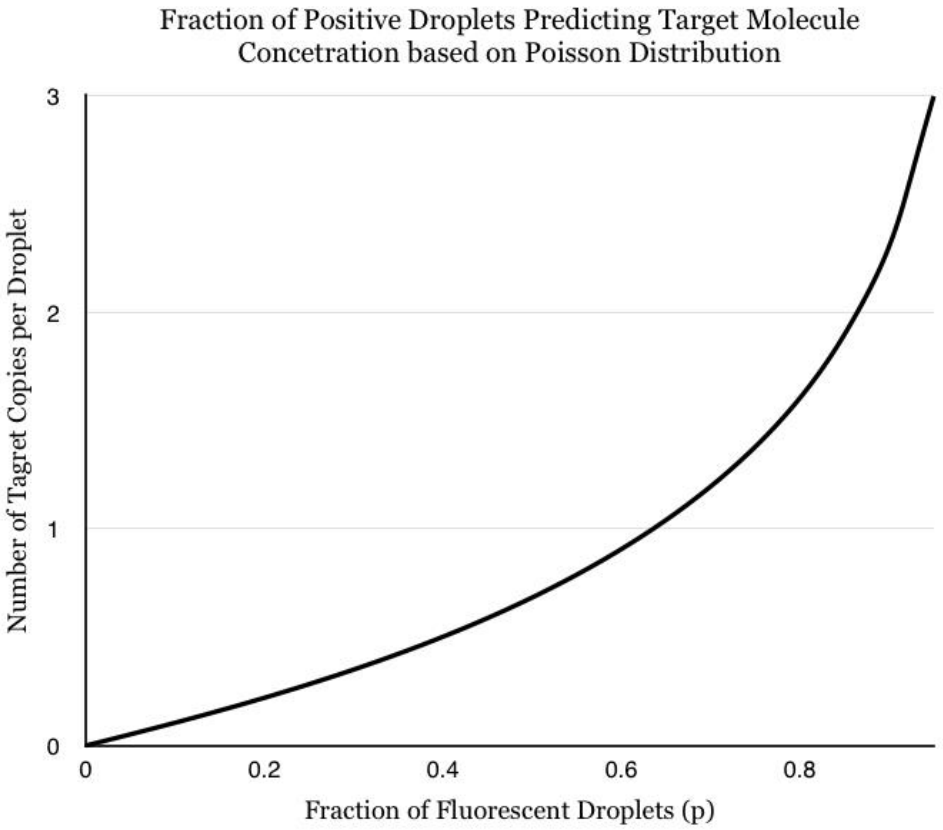


VS.

Traditional PCR:
One fluorescence measurement

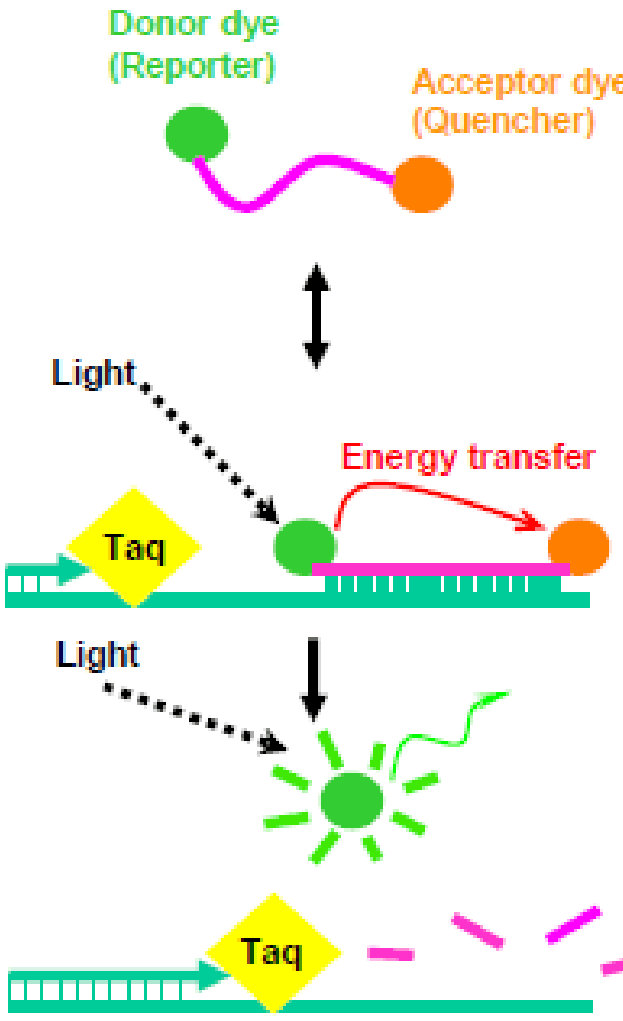
Digital PCR:
Thousands of distinct fluorescence measurements





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

How the assay works



Unbound probe free in solution

Probe and primer bind target, FRET occurs

Taq polymerase extends and hydrolyzes probe, donor dye free to emit fluorescence --> accumulation of signal

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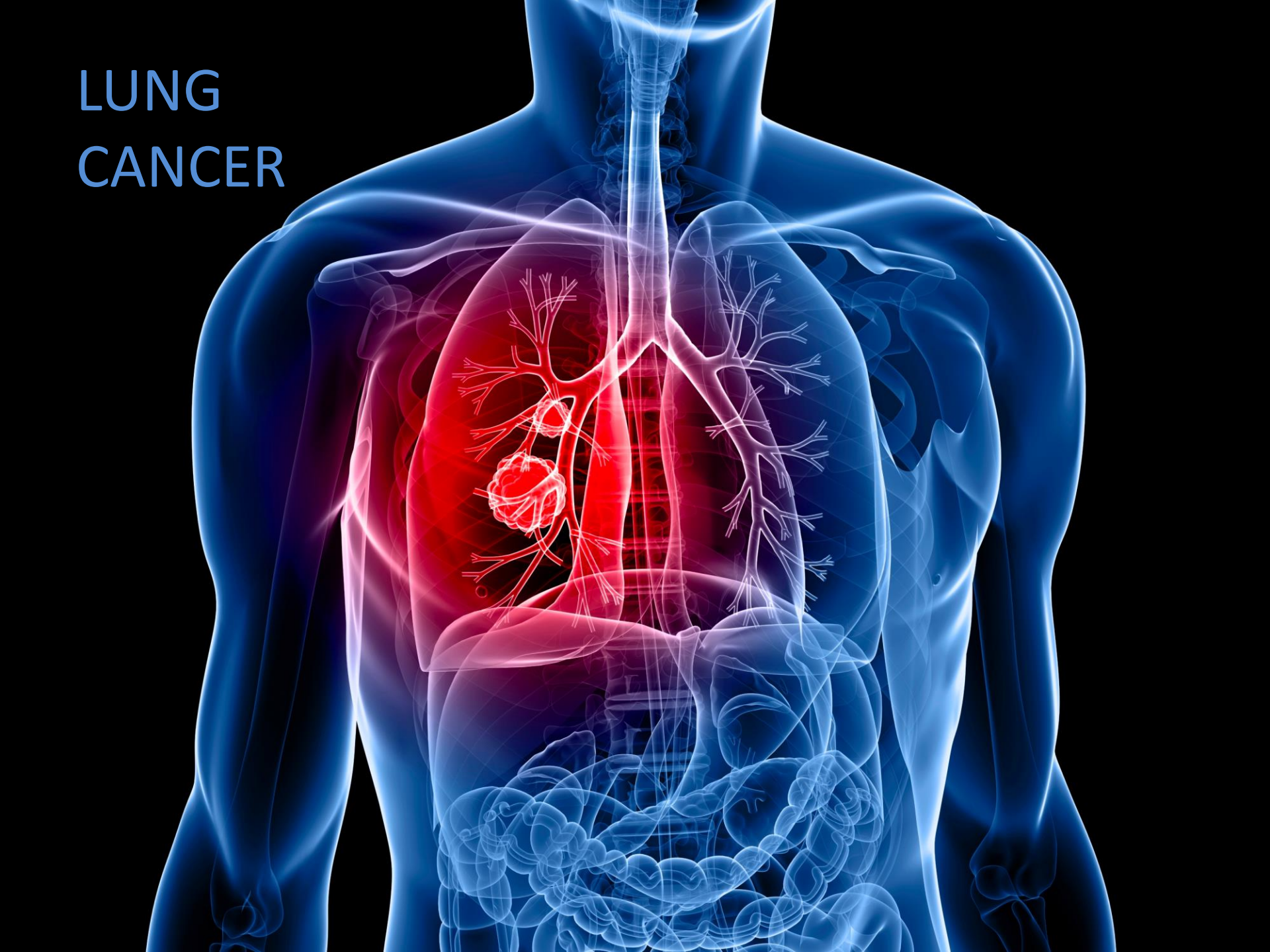
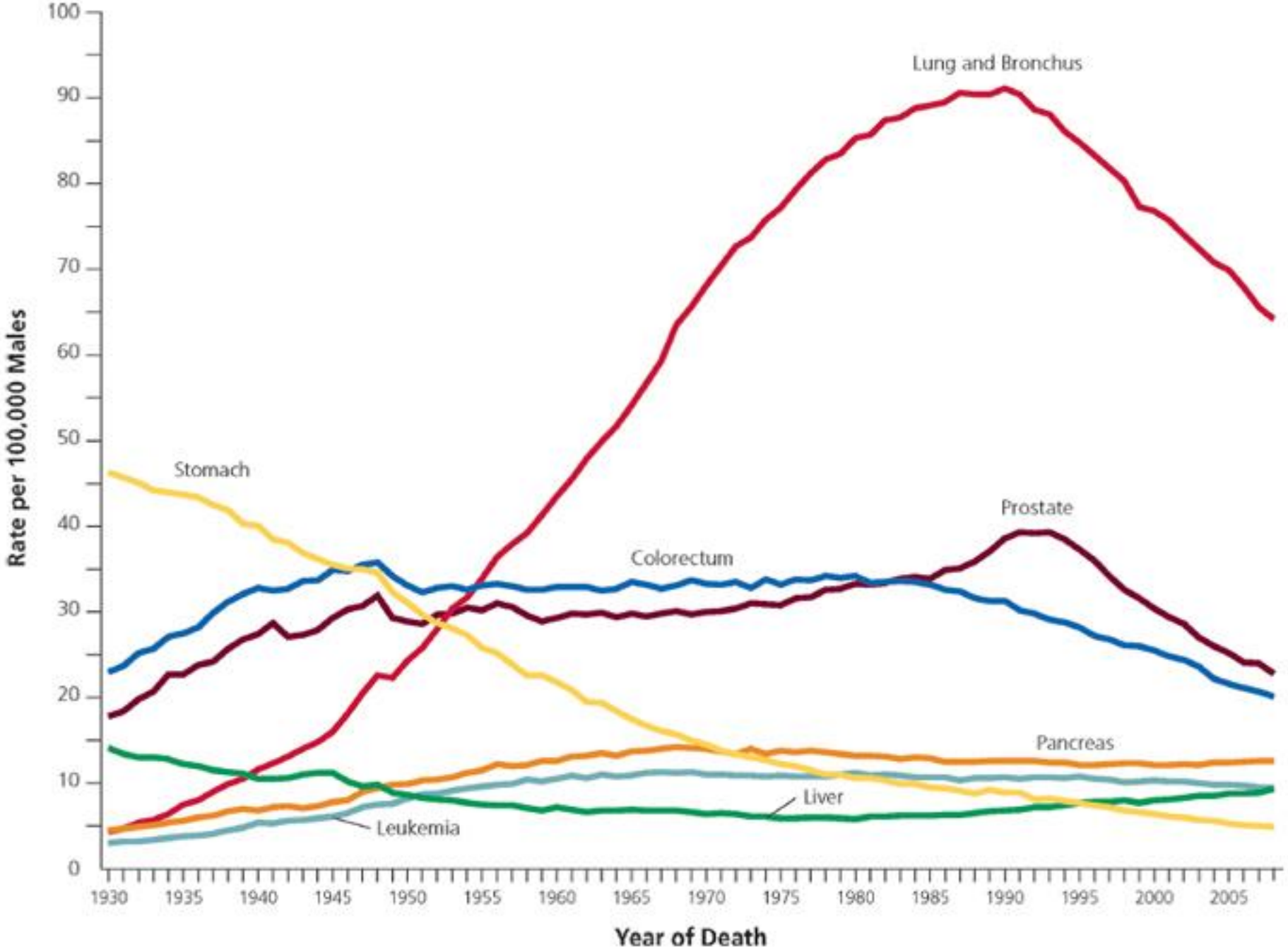
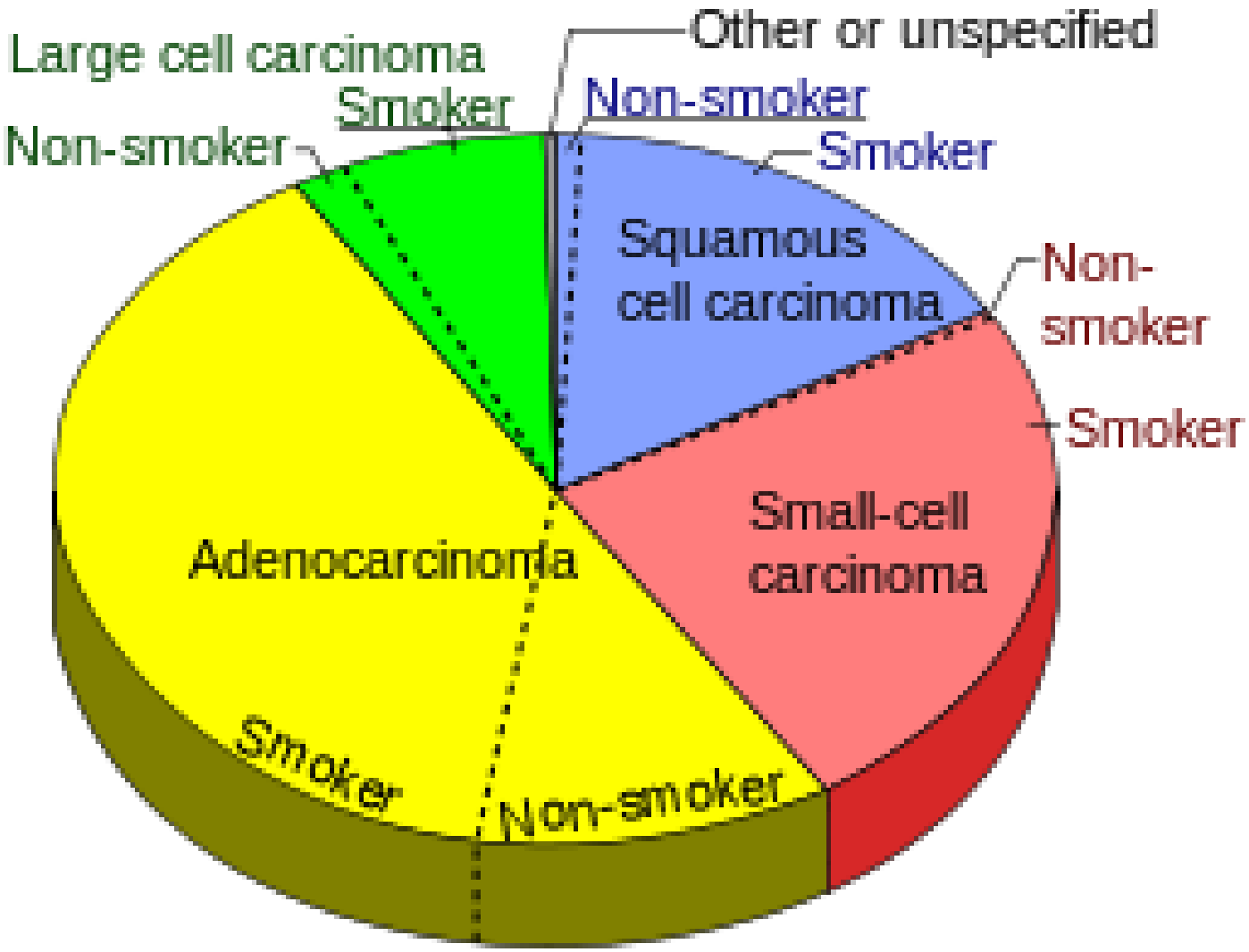
LUNG CANCER

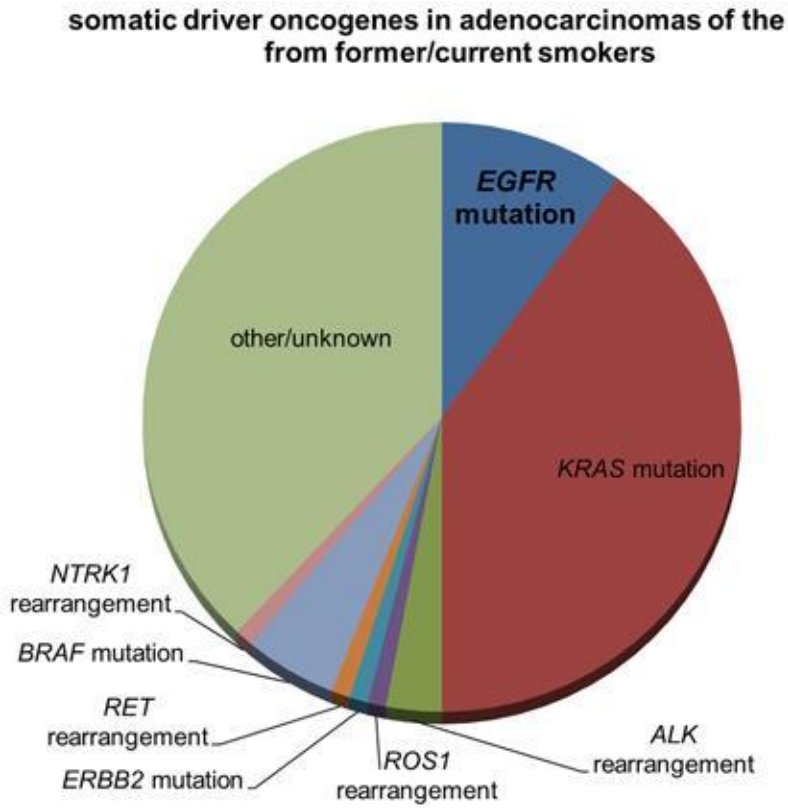


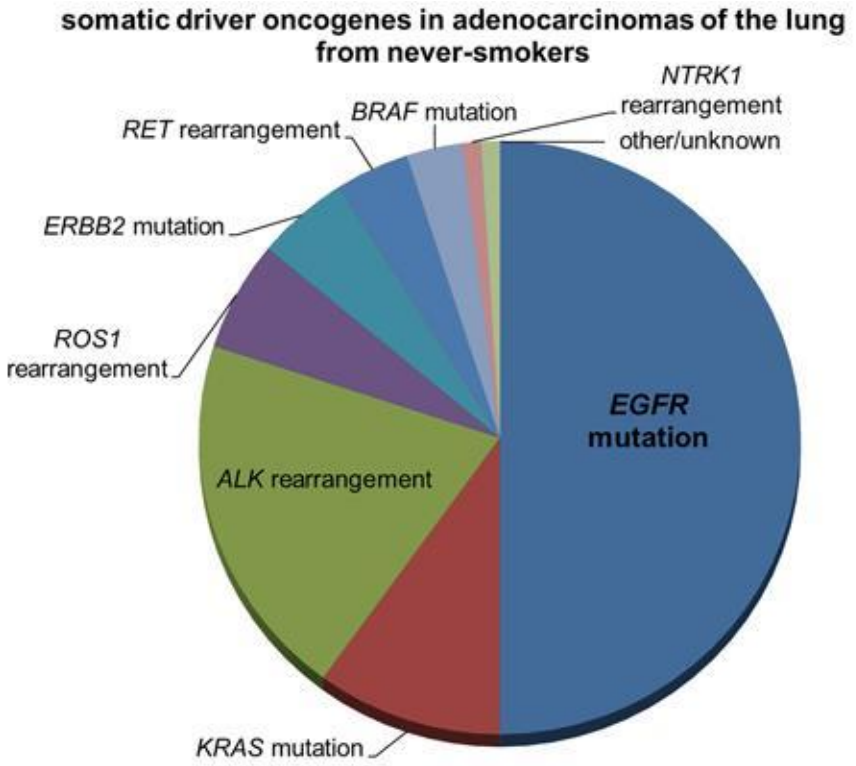

Rate per 100,000 Males
100
90
80
70
60
50
40
30
20
10
0
1930
1935
1940
1945
1950
1955
1960
1965
1970
1975
1980
1985
1990
1995
2000
2005
Year of Death
Lung and Bronchus
Stomach
Prostate
Colorectum
Pancreas
Liver
Leukemia


Large cell carcinoma
Smoker
Non-smoker
Other or unspecified
Non-smoker
Smoker
Squamous cell carcinoma
Non-smoker
Smoker
Small-cell carcinoma
Adenocarcinoma
Smoker
Non-smoker

SMOKERS / FORMER SMOKERS

NEVER SMOKERS





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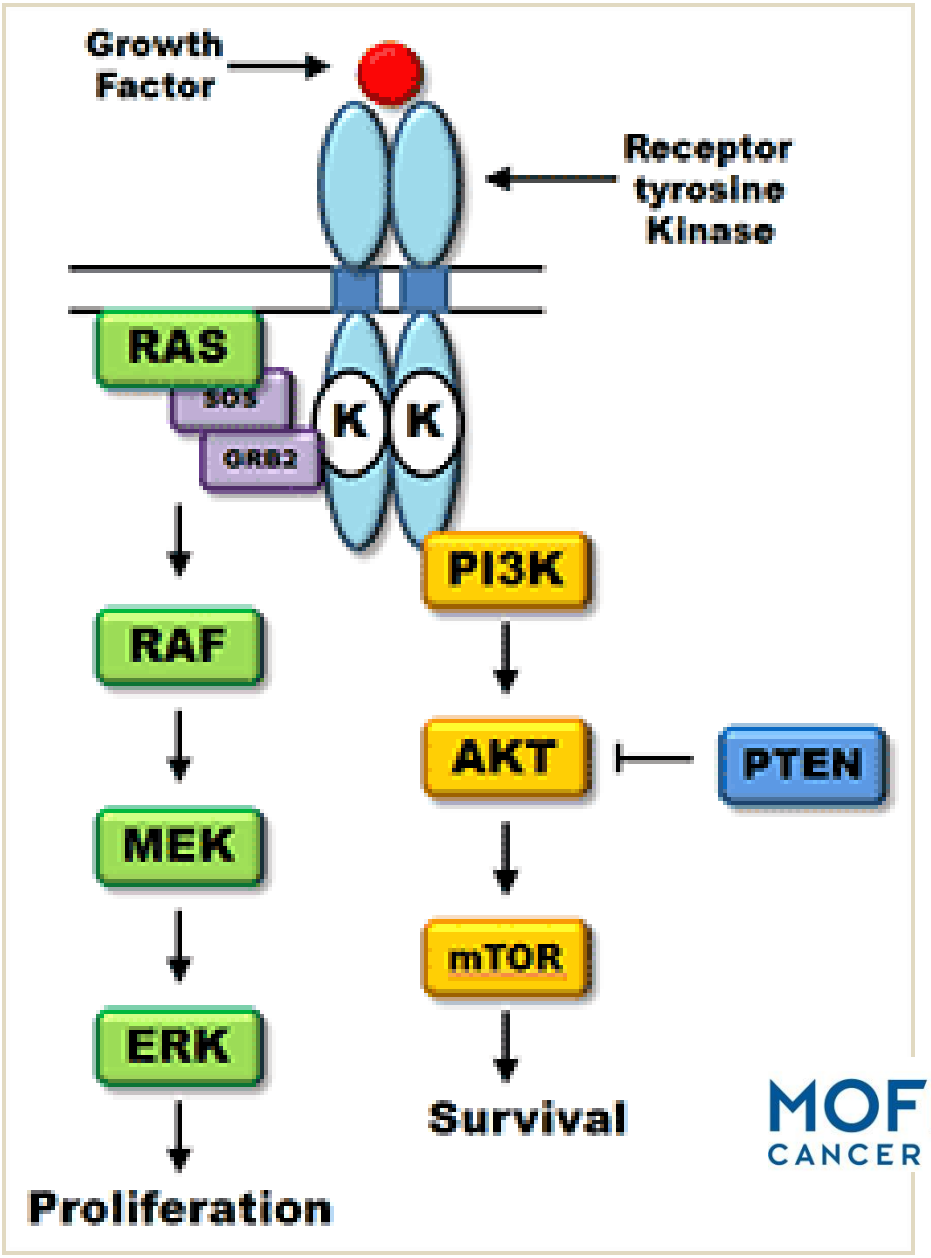
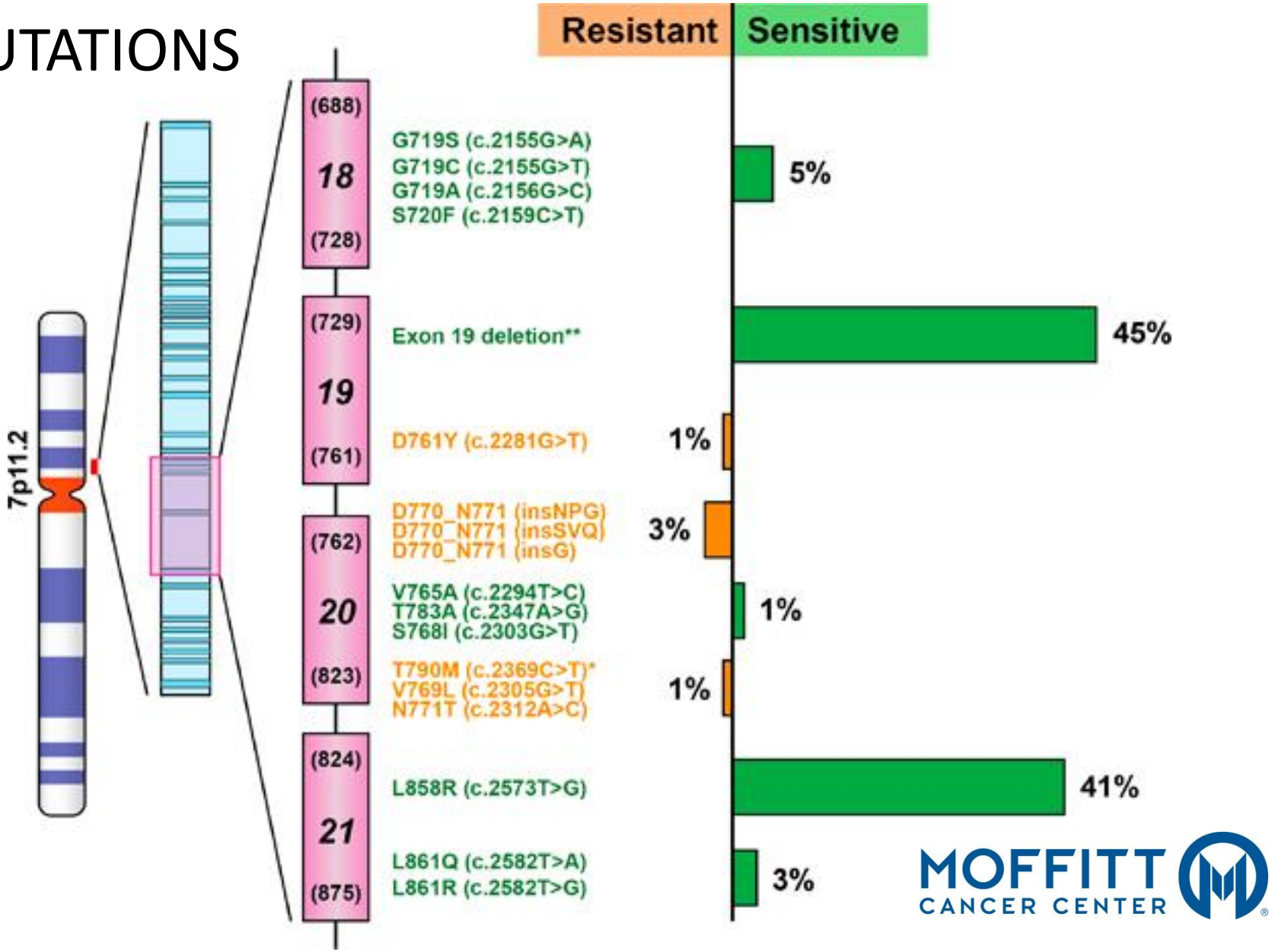
EGFR SIGNALLING

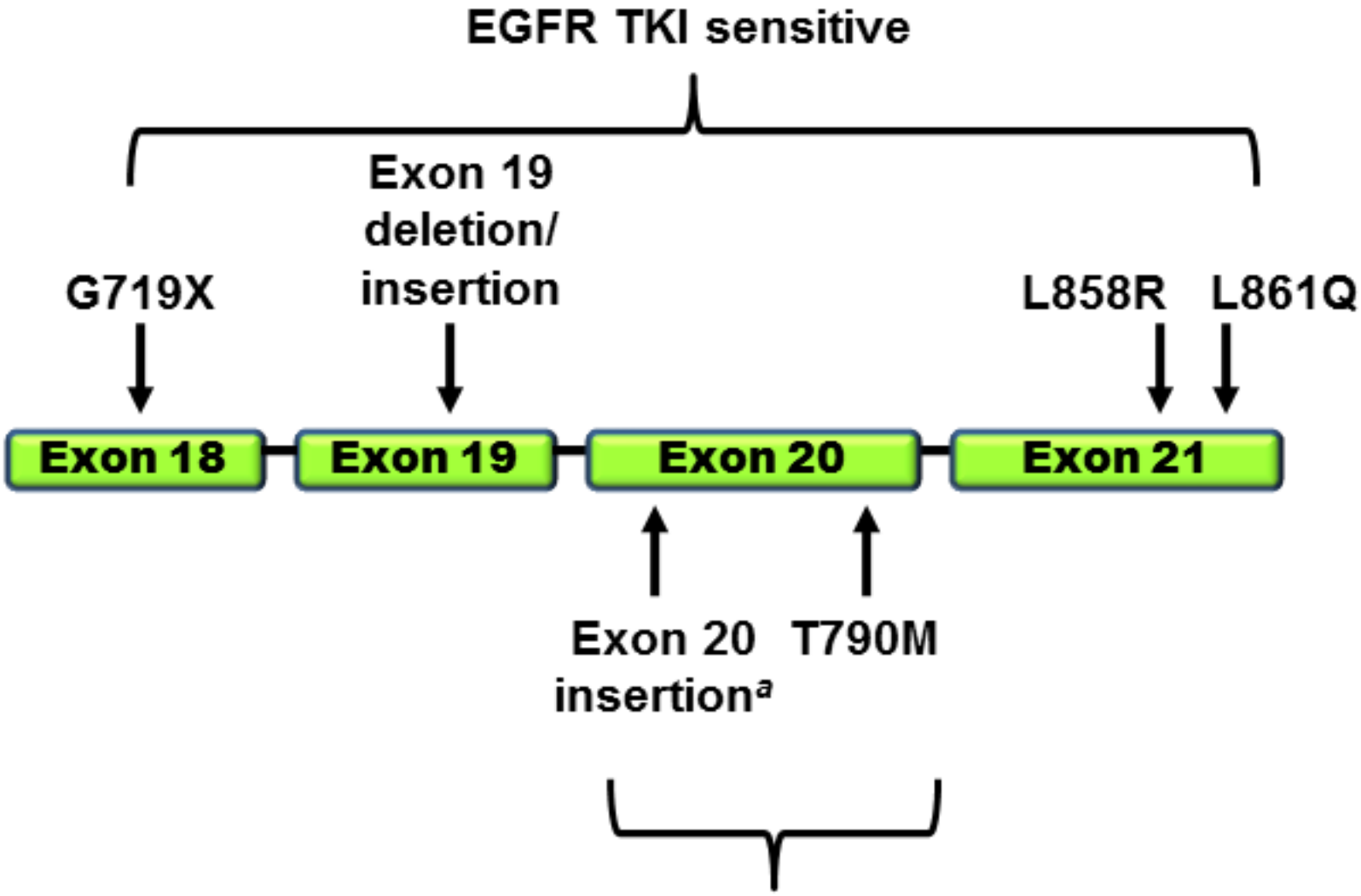


MOFFITT CANCER CENTER

EGFR MUTATIONS



MOFFITT CANCER CENTER



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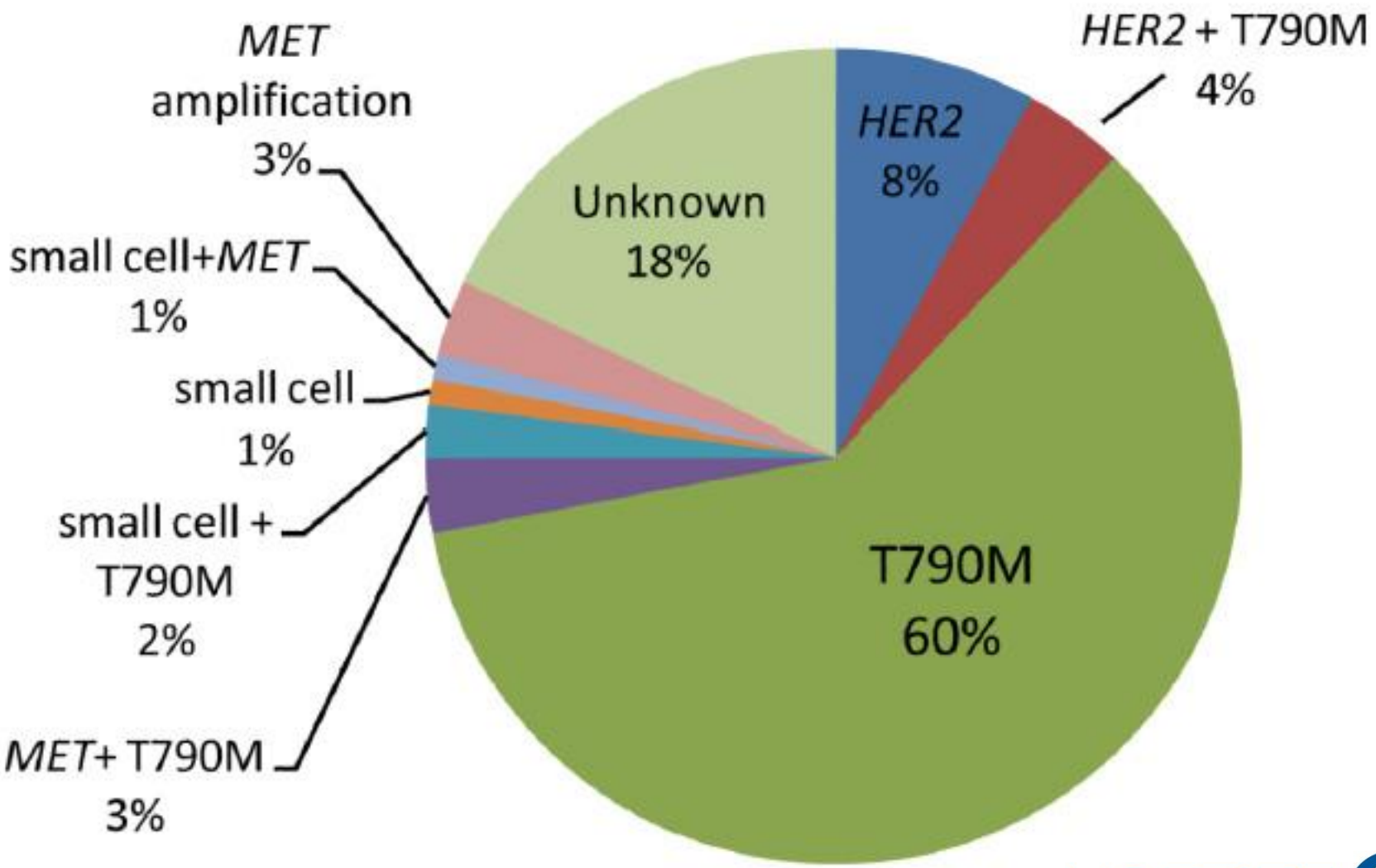

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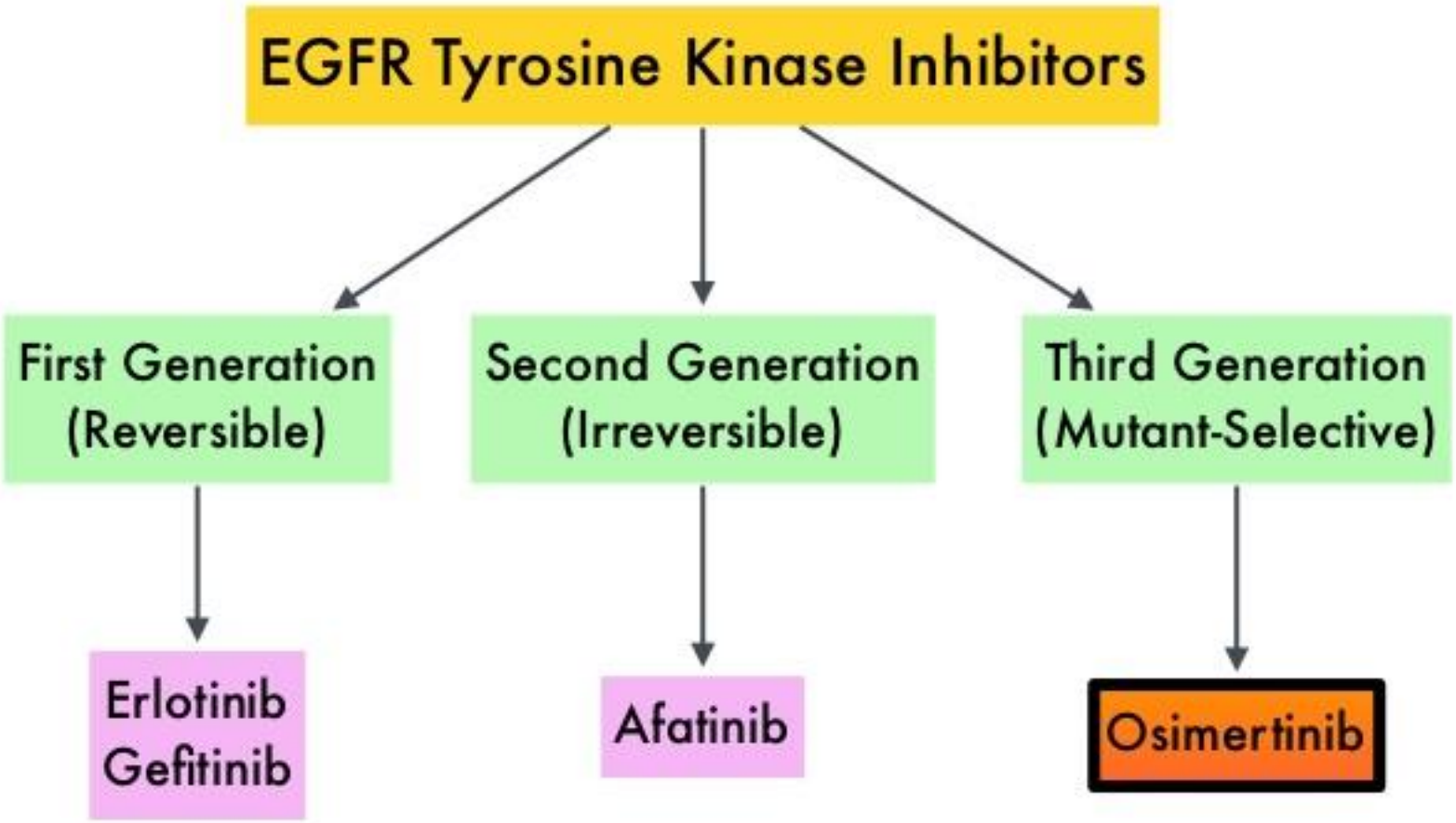
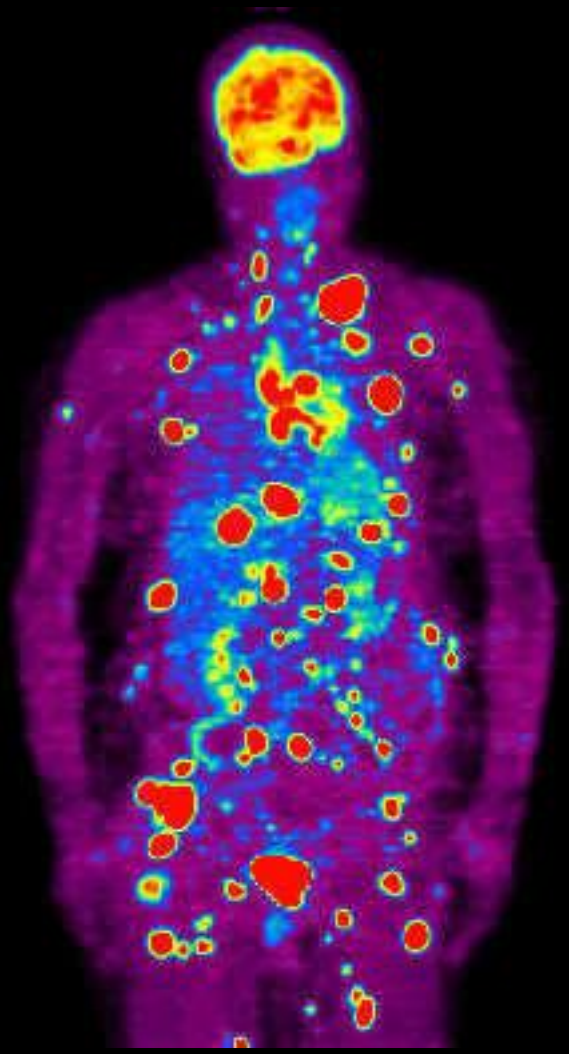

EGFR Tyrosine Kinase Inhibitors
First Generation (Reversible)
Second Generation (Irreversible)
Third Generation (Mutant-Selective)
Erlotinib
Gefitinib
Afatinib
Osimertinib

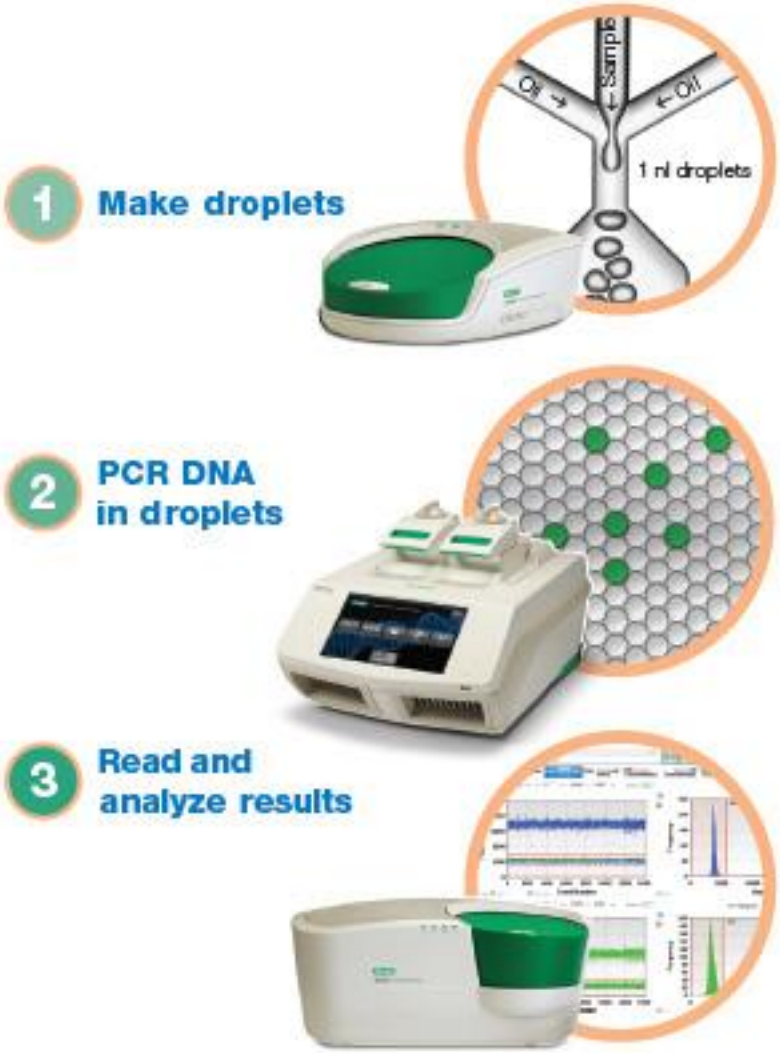
ANTI T790M

METASTATIC CANCER



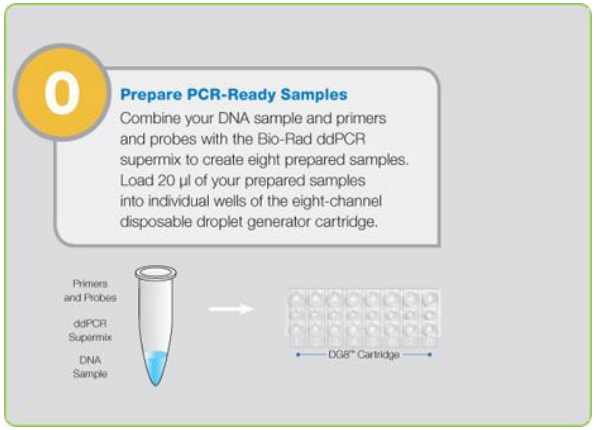
Blood Test

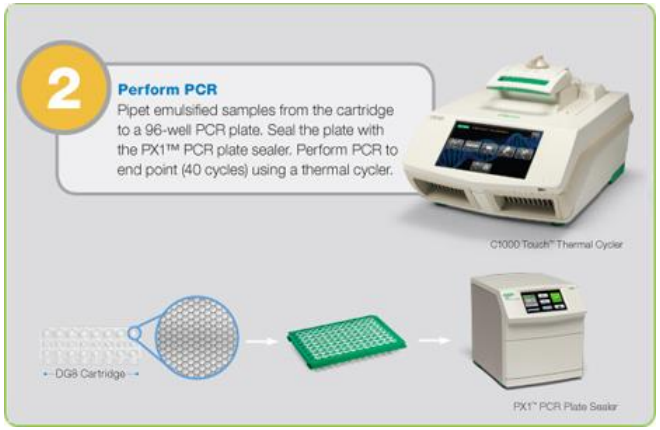
DIGITAL PCR

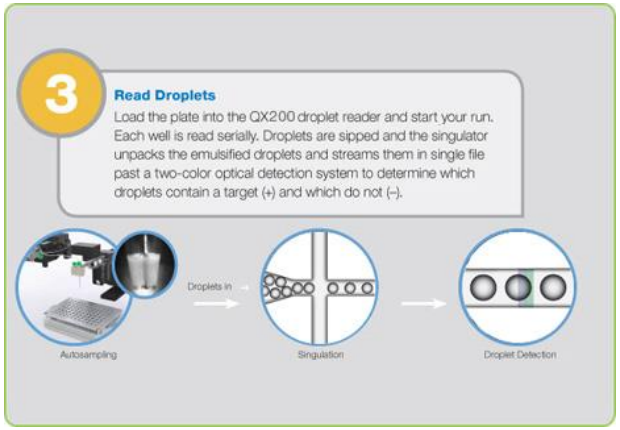

1 Make droplets
Sample
Oil
Oil
1 nl droplets
2 PCR DNA in droplets
3 Read and analyze results

Liquid Biopsy: ddPCR EGFR T790M assay

Process Overview







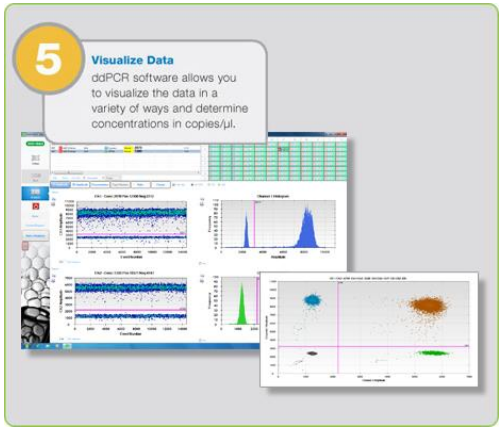
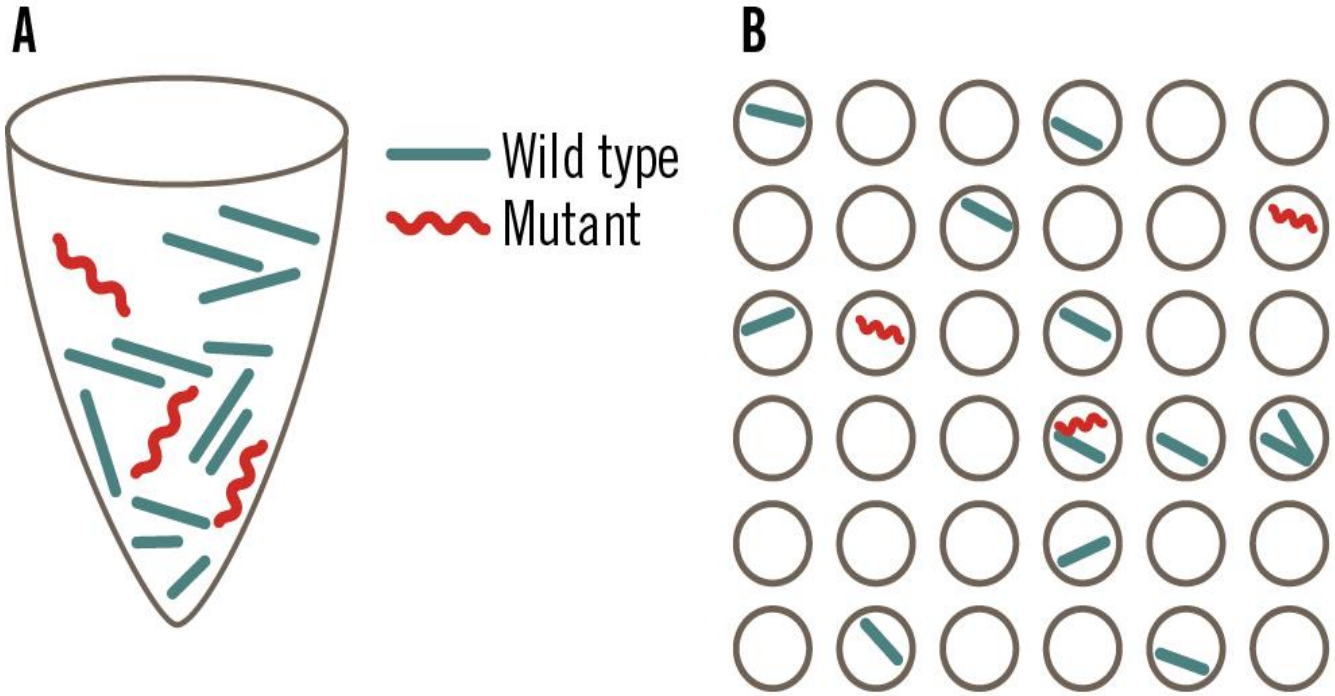


Figure 1.2. Depiction of digital PCR process



Source: (Sedlaka & Keith, 2013).[10]

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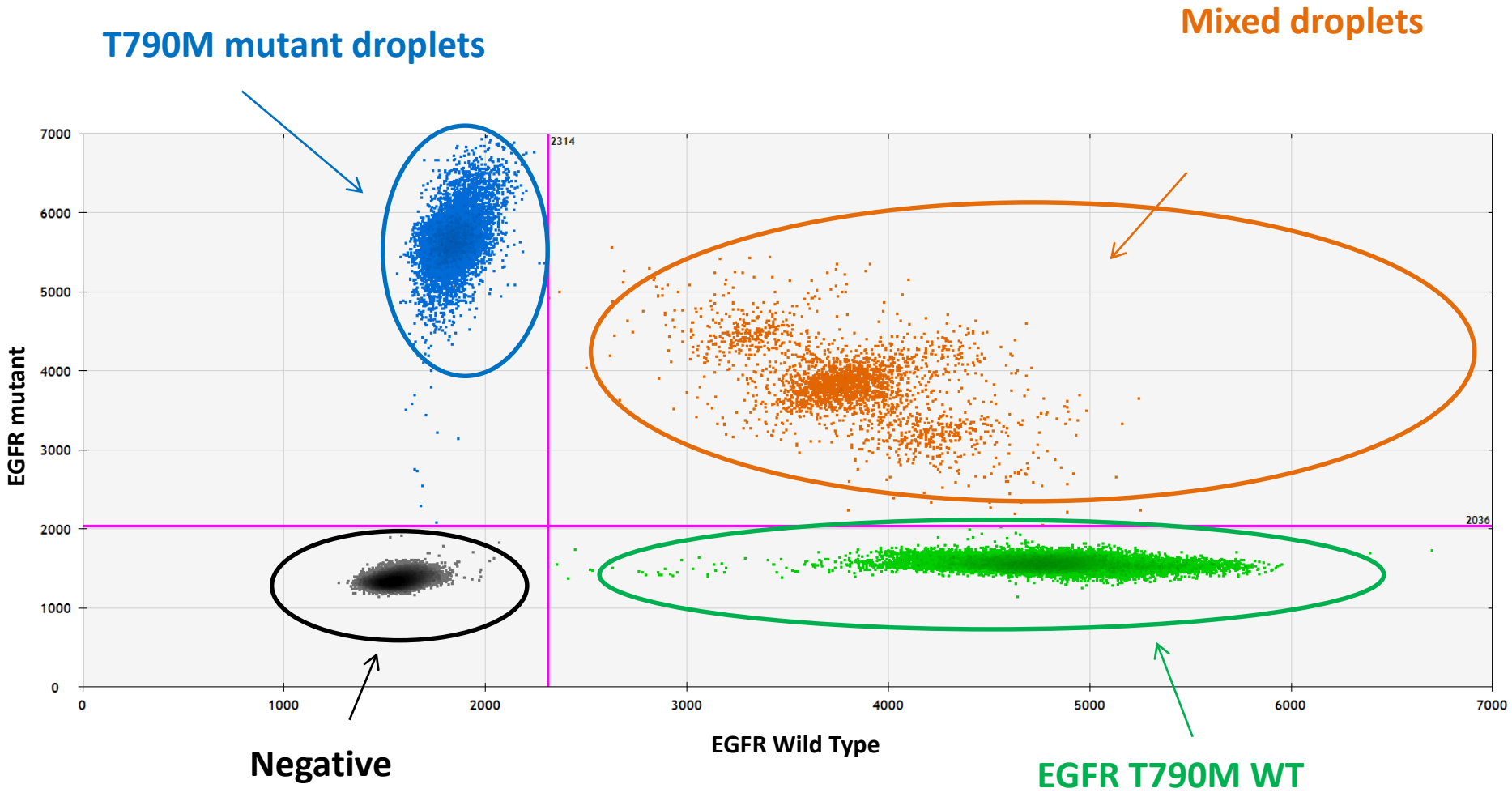
ddPCR EGFR T790M Assay Data Analysis



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CHALLENGES

- CHALLENGES
  - Lack of good wet lab reagents and software
  - Still investigational
  - Challenging to bring to CLIA

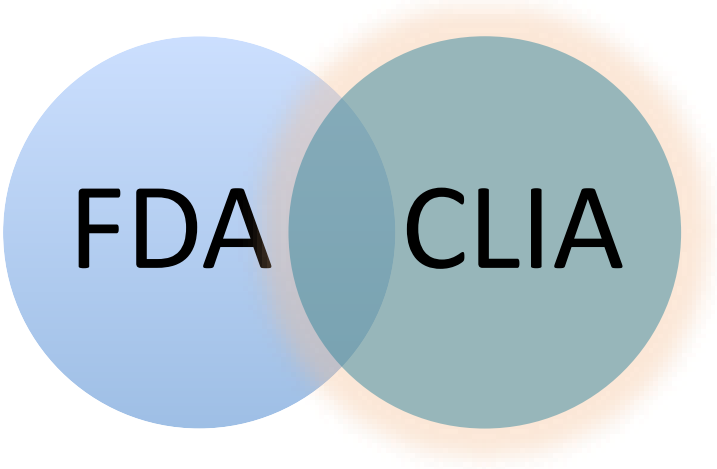
FDA vs CLIA

FDA

- Federal
- Medical Devices
- Kits

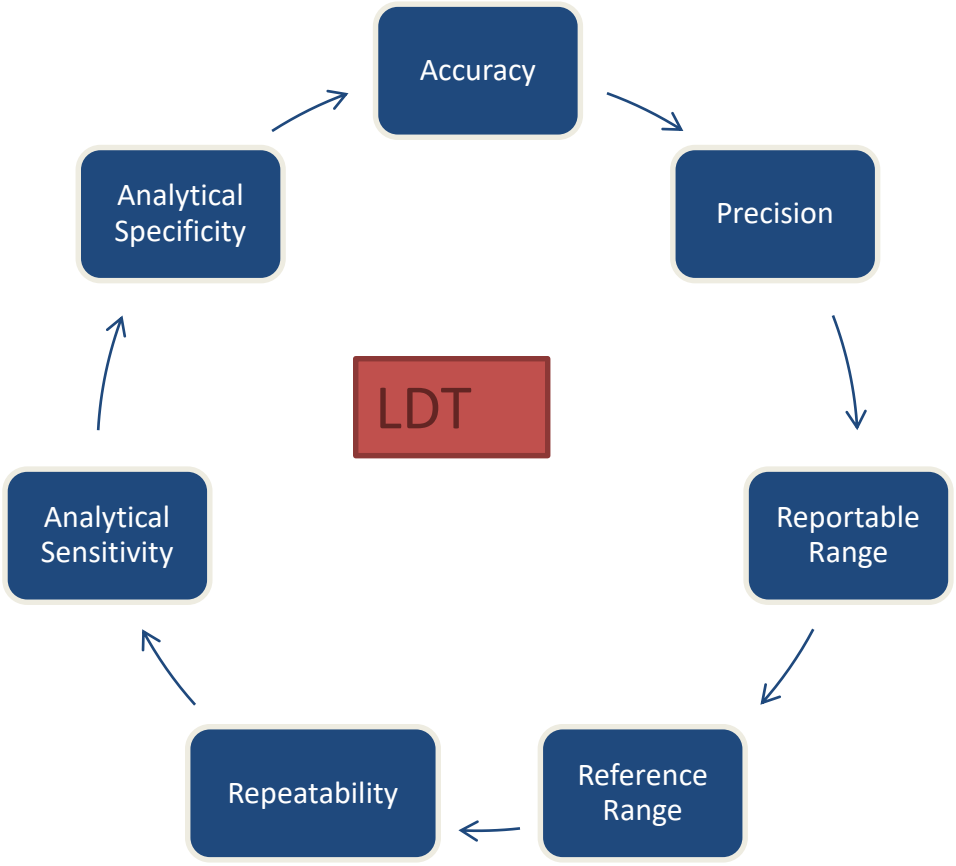
CLIA

- State Authority
- Assays
- Lab operations
- Personnel



CLIA REQUIREMENTS FOR LDT

Laboratory Developed Test



Clinical Laboratory Improvement Amendments

College of American Pathologists

SENSITIVITY LIMIT OF DETECTION DETERMINATION

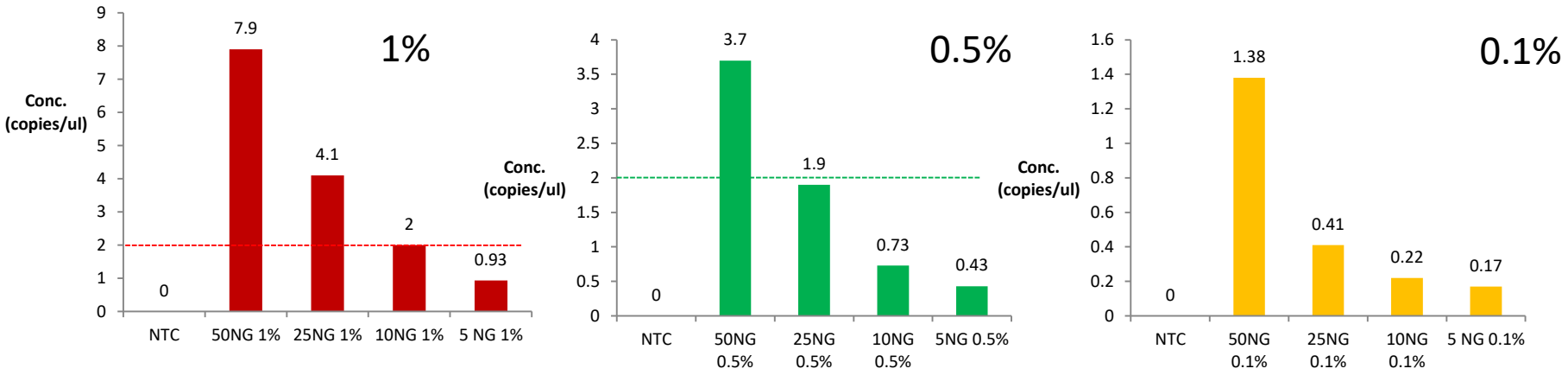


Figure 1 Titration of EGFR T790M standard to determine the Limit of Detection (LOD). 50, 25, 10 and 5ng of each standard (1%, 0.5% and 0.1% T790M MAF) were digested with 20U/100ng HindIII HF enzyme at 37C for 5-15 minutes and then run through the normal ddPCR protocol on the QX200. A water only control was also run (NTC). All samples were run in duplicate and merged during data analysis. The total confidence maximum value for the T790M probe in the NTC well was 0.26 copies/ul. Based on this value, we have sensitivity to 0.5% with 5ng, and 0.1% with 25ng of input ctDNA. Based on this preliminary experiment it was decided to use 50ng of input DNA in the EGFR T790M validation.

MOFFITT CANCER CENTER

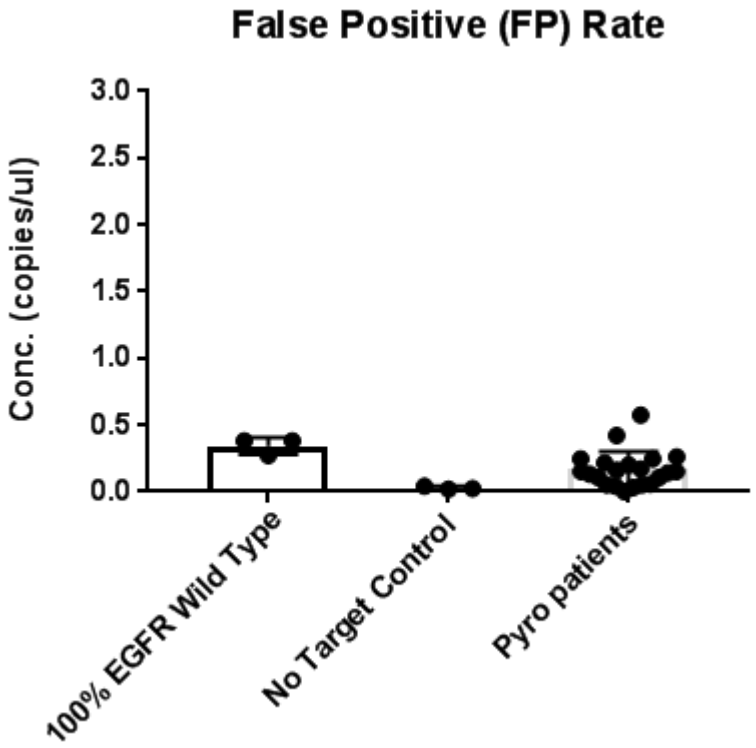


Figure 2. The False Positive Rate (FP) for EGFR T790M WT DNA is low. The FP rate was determined by running 50ng of 100% EGFR T790M Wild Type DNA from Horizon Discovery over a three day period totaling 60 reaction wells and 60 wells of No Target Control (NTC) comprising water only over the same time period. For each day the data for all common conditions were merged, thresholds were set and the concentration of mutant copies were calculated using the QuantaSoft™ software . The overall false positive rate for EGFR T790M WT was 0.3433 copies/µl, 0.028 copies/µl for the NTC, and 0.06 copies/µl for patient samples.

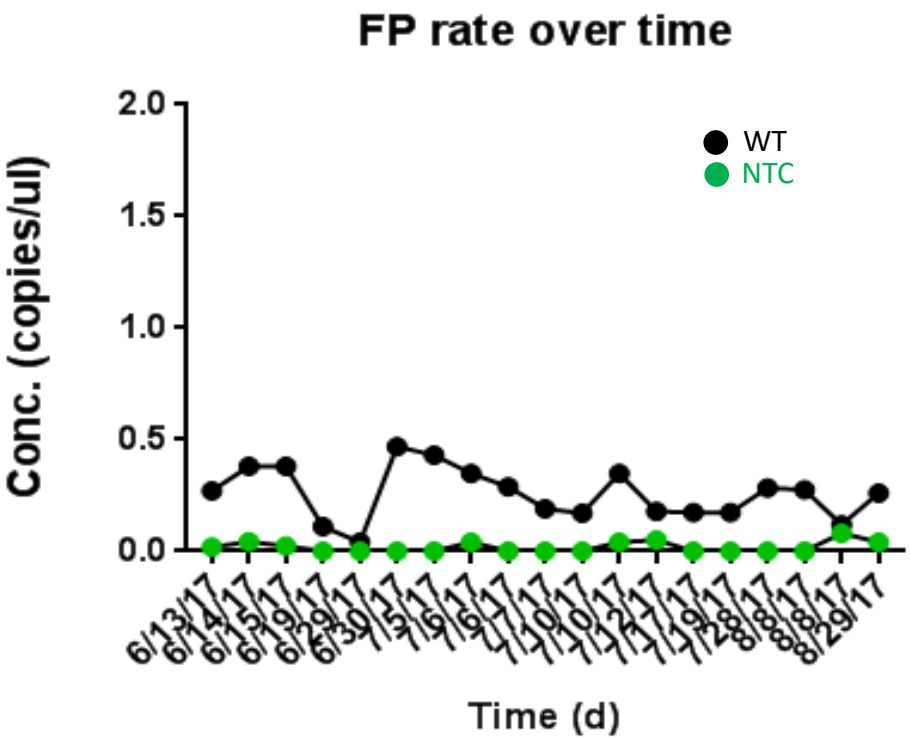


Figure 3. The False Positive Rate (FP) for EGFR T790M WT DNA and NTC is low and stable over time. The FP rate was determined by running 50ng of 100% EGFR T790M WT DNA from Horizon Discovery over time and (NTC) comprising water only over the same time period. For each day the data for all common conditions were merged, thresholds were set and the concentration of mutant copies were calculated using the QuantaSoft™ software .

MOFFITT CANCER CENTER

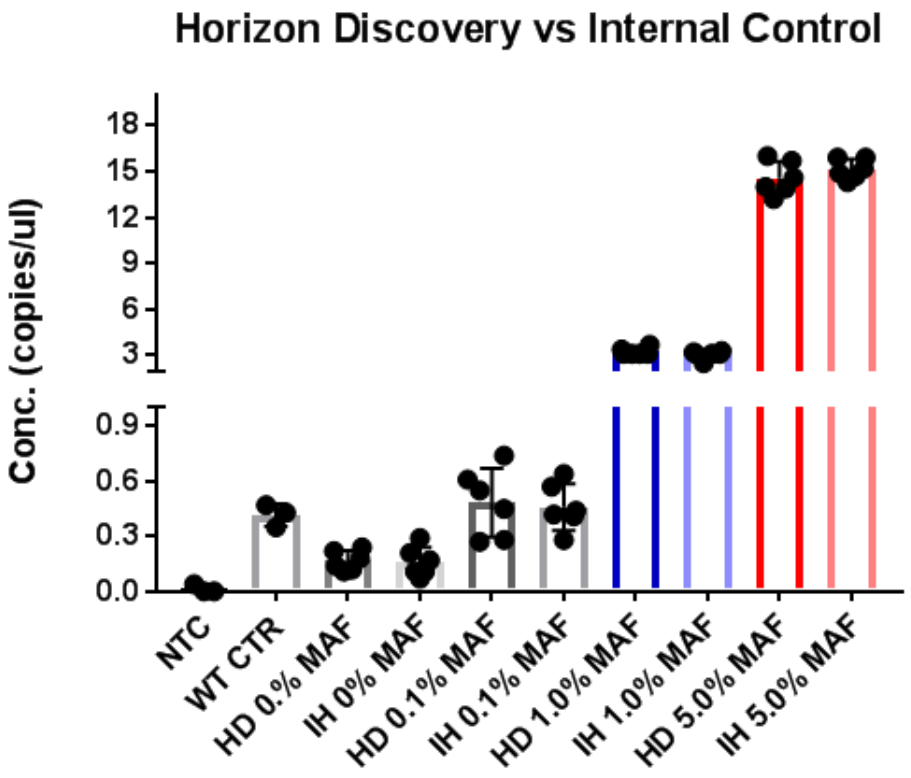


Figure 4. There are no significant differences in Horizon Discovery (HD) synthetic plasma DNA controls versus DNA from cell lines diluted to the same mutant allele frequencies (MAF). DNA isolated from synthetic plasma at 0%, 0.1%, 1.0% and 5.0% EGFR T790M MAF was compared to Horizon Discovery cell line DNA diluted to the same MAF and run on the EGFR T790M ddPCR assay. Each condition was tested over several days with 25ng of DNA per well run in duplicate each day. The WT positive control was 50ng/well 100% EGFR T790M wild type DNA, NTC is water only no DNA control. Statistical values for each condition are shown in table 1

Table 1

Sample	HD 0.% MAF	IH 0% MAF	HD 0.1% MAF	IH 0.1% MAF	HD 1.0% MAF	IH 1.0% MAF	HD 5.0% MAF	IH 5.0% MAF
Mean	0.2072	0.235	0.4939	0.53	**3.45**	**3.183**	15.16	17.72
SD	0.07726	0.1322	0.151	0.1457	**0.3674**	**0.3**	1.248	3.886

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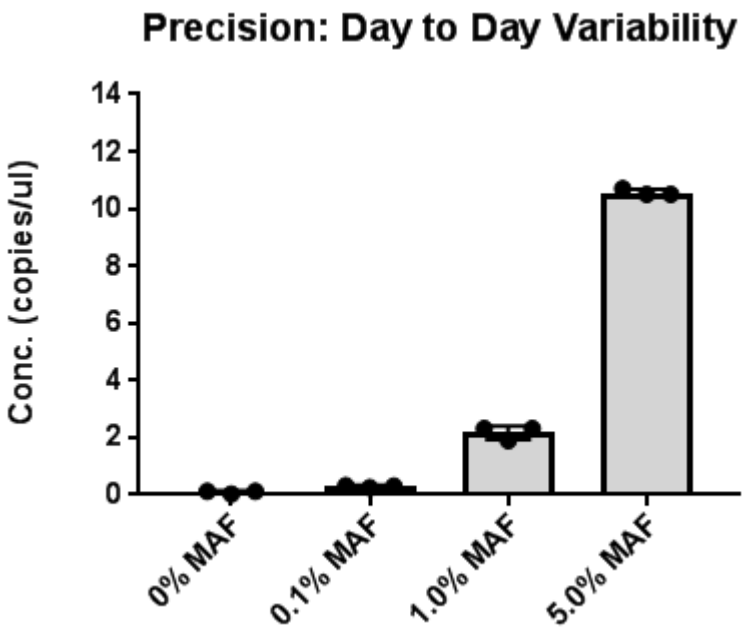

Figure 5. There is no significant variability from day to day with Synthetic Plasma DNA Controls at set Mutant Allele Frequencies. DNA from Horizon Discovery Synthetic Plasma controls containing Mutant Allele Frequencies (MAF) of 0%, 0.1%, 1.0% and 5.0% T790M were extracted using the Qiagen circulating nucleic acid kit, quantitated on Qubit and run in triplicate over a three day period. All common wells were merged during data analysis.

Table 2

Sample	0% MAF	0.1% MAF	1.0% MAF	5.0% MAF
Mean	0.09	0.2933	2.173	10.57
Std. Deviation	0.05196	0.03786	0.2367	0.1155
Coefficient of variation	57.74%	12.91%	10.89%	1.09%

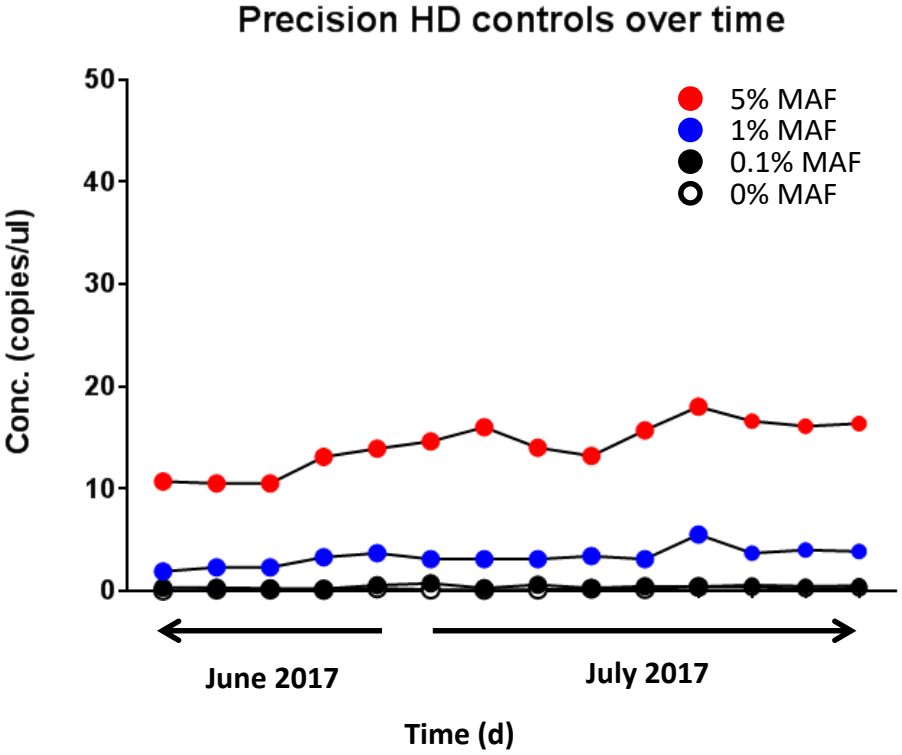

Figure 6. The precision of the Horizon Discovery synthetic plasma DNA was isolated and tracked over time with different operators and different lots. The cfDNA isolated from synthetic plasma was extracted using the Qiagen circulating nucleic acid kit and quantitated on Qubit. Then these control DNA were run with other experiments during the validation of the assay. These data were then plotted versus time.

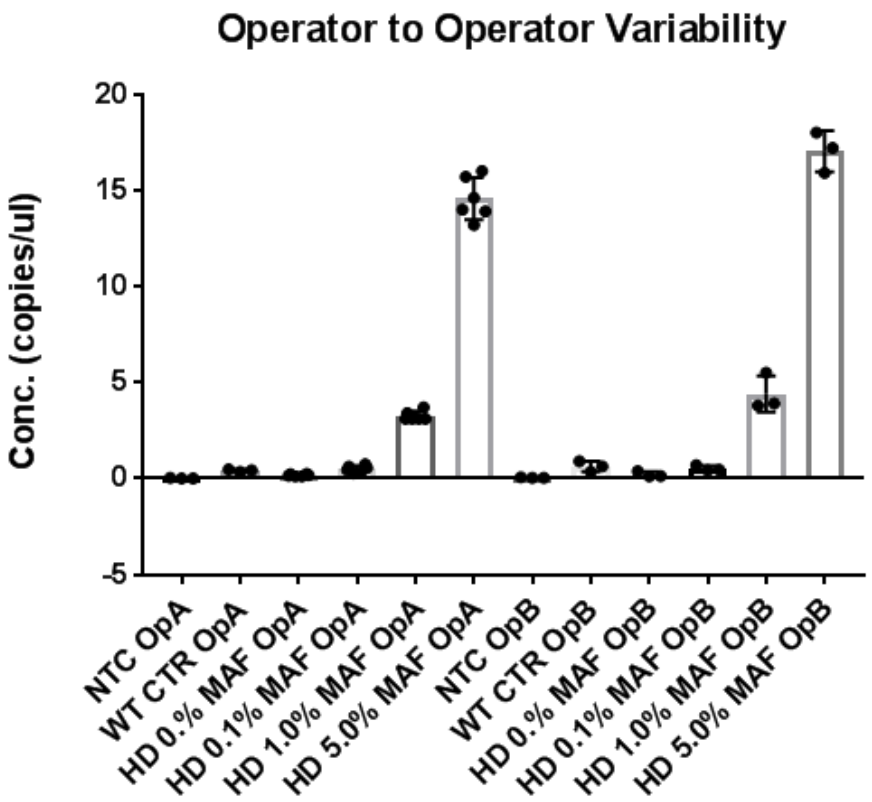

Figure 7. Precision: There is no significant difference from operator to operator. Different operators isolated Horizon Discovery cfDNA from synthetic plasma and tested each of the mutant allele frequencies of 0%, 0.1%, 1.0% and 5.0% using the same reagents as well as negative controls of NTC and 100% WT EGFR cell line DNA.

Table 3

Sample	NTC OpA	WT CTR OpA	HD 0% MAF OpA	HD 0.1% MAF OpA	HD 1.0% MAF OpA	HD 5.0% MAF OpA	NTC OpB	WT CTR OpB	HD 0% MAF OpB	HD 0.1% MAF OpB	HD 1.0% MAF OpB	HD 5.0% MAF OpB
Mean	0.01333	0.4167	0.1683	0.4833	**3.25**	14.57	0.05	0.6233	0.2067	0.5233	**4.4**	17.03
Std. Deviation	0.02309	0.0611	0.05382	0.1867	**0.251**	1.093	0.01732	0.2701	0.1504	0.1457	**0.9539**	1.06
Coefficient of variation	173.21%	14.66%	31.97%	38.63%	**7.72%**	7.50%	34.64%	43.33%	72.80%	27.84%	**21.68%**	6.22%

LOT TO LOT

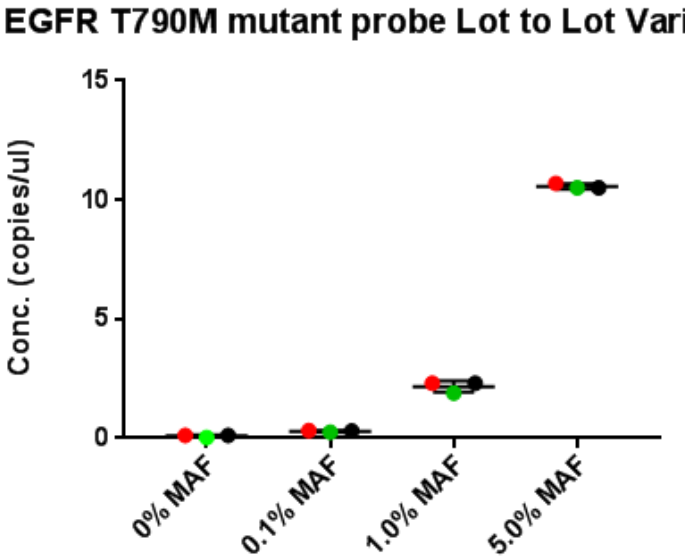

Figure 8. Precision: There is no significant difference in EGFR T790M mutant concentration values different probe lots are used. Three different EGFR T790M mutant probes lots were used over three consecutive days with 50ng of Horizon Discovery synthetic plasma isolated DNA samples containing mutant allele frequencies of 0%, 0.1%, 1.0% and 5.0%. Each lot of probes is designated by the three different colors at each data point.

Table 4. Lot to lot variability statistical data analysis

Sample	0% MAF	0.1% MAF	1.0% MAF	5.0% MAF
Mean	0.09	0.2933	2.173	10.57
Std. Deviation	0.05196	0.03786	0.2367	0.1155
Coefficient of variation	57.74%	12.91%	10.89%	1.09%

INTRADAY VARIABILITY

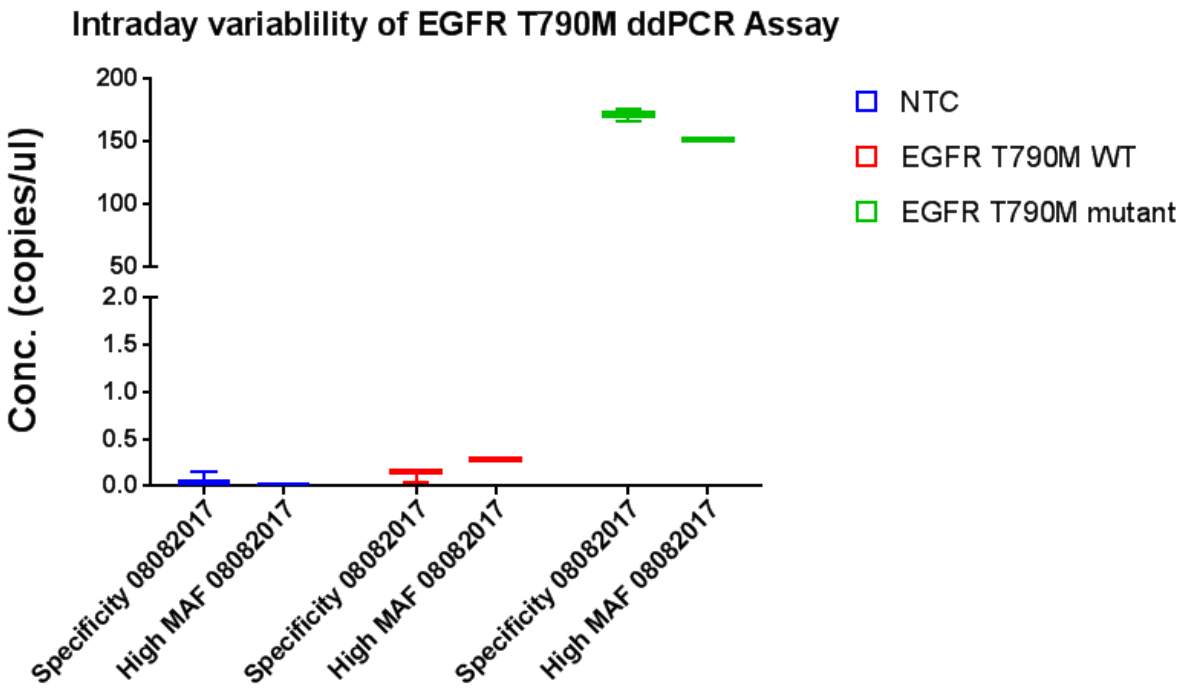

Figure 9. Precision: Intraday variability of the EGFR T790M assay was assessed by running the same cfDNA samples in two subsequent runs on the same day. The samples were a NTC, 50ng of 100% WT EGFR, and 50ng of 50% MAF EGFR T790M mutant in duplicate using the same reagents for both experiments

Table 5. Intraday variability statistical data analysis

	NTC	EGFR T790M WT	EGFR T790M mutant
Mean	0.03833	0.1958	161.9
Std. Deviation	0.05421	0.112	14.27
Coefficient of variation	141.42%	57.17%	8.82%

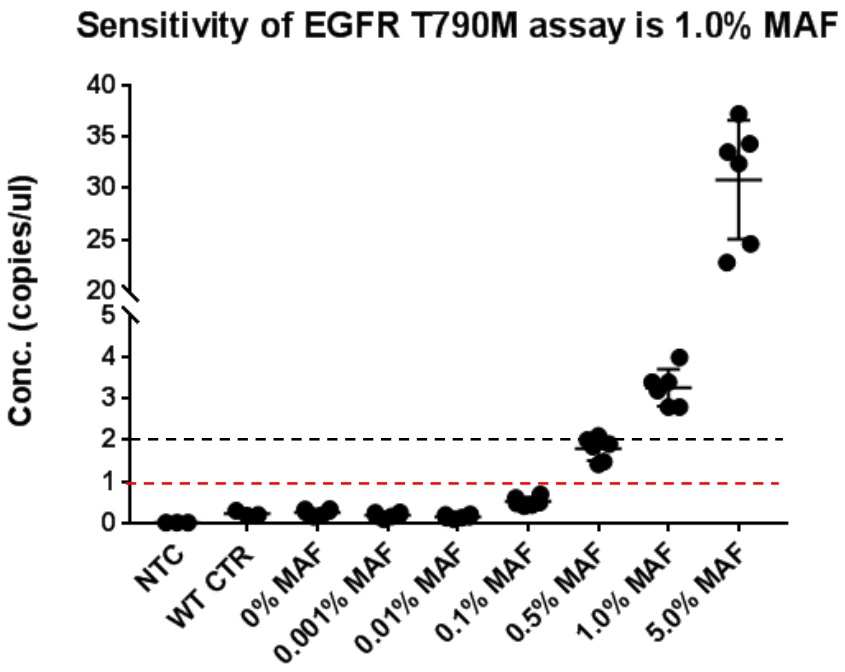

Figure 10. The sensitivity of the EGFR T790M ddPCR assay is 1.0% mutant allele frequency (MAF). EGFR T790M cell line Horizon Discovery DNA with 50% MAF was diluted with 100% EGFR wild type DNA to 0%, 0.001%, 0.01%, 0.1%, 0.5%, 1.0% and 5.0%. Wild type DNA and NTC is water only no DNA control. Here the cutoff of 2 copies/ul is shown by the black dotted line. The red dotted represents 2x the average of the 95% Poisson confidence maximum confidence interval value 0.95 copies/ul. The sensitivity of the assay would then be 0.5% MAF.

Table 6. Analytical Sensitivity statistical data analysis

	NTC	WT CTR	0% MAF	0.001% MAF	0.01% MAF	0.1% MAF	0.5% MAF	**1.0% MAF**	5.0% MAF
Mean	0	0.2167	0.2483	0.1767	0.1417	0.5133	1.788	**3.267**	30.8
Std. Deviation	0	0.06429	0.07834	0.06282	0.04579	0.1104	0.2812	**0.4502**	5.753
Coefficient of variation	+infinity%	29.67%	31.55%	35.56%	32.32%	21.51%	15.72%	**13.78%**	18.68%

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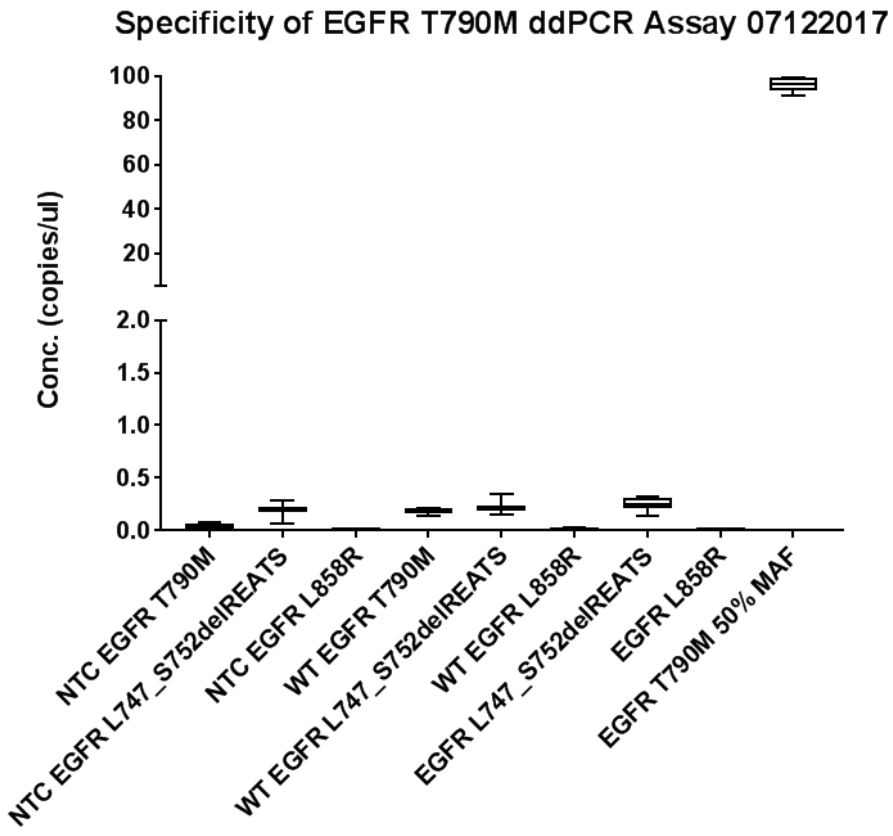

Figure 12. The EGFR T790M ddPCR assay is specific for only the T790M mutation. Twenty five nanograms of EGFR T790M 50% mutant allele frequency DNA or EGFR T790M wild type were added to duplicate wells of a 96 well plate and analyzed with probes specific for EGFR T790M, L858R or L747_S752delREATS. Only the probes specific for EGFR T790M were detected above background levels. The experiment was repeated two times on separate days with similar results.

Table 9. Analytical Specificity statistical data analysis

Sample	NTC EGFR T790M	NTC EGFR L747_S752 delREATS	NTC EGFR L858R	WT EGFR T790M	WT EGFR L747_S752del REATS	WT EGFR L858R	EGFR L747_S752del REATS	EGFR L858R	EGFR T790M 50% MAF
Mean	0.04667	0.18	0	0.18	0.2333	0.01	0.25	0	**96.07**
Std. Deviation	0.02082	0.1114	0	0.03606	0.09713	0.01732	0.06603	0	**3.207**
Coefficient of variation	44.61%	61.86%	+infinity%	20.03%	41.63%	173.21%	26.41%	+infinity%	**3.34%**

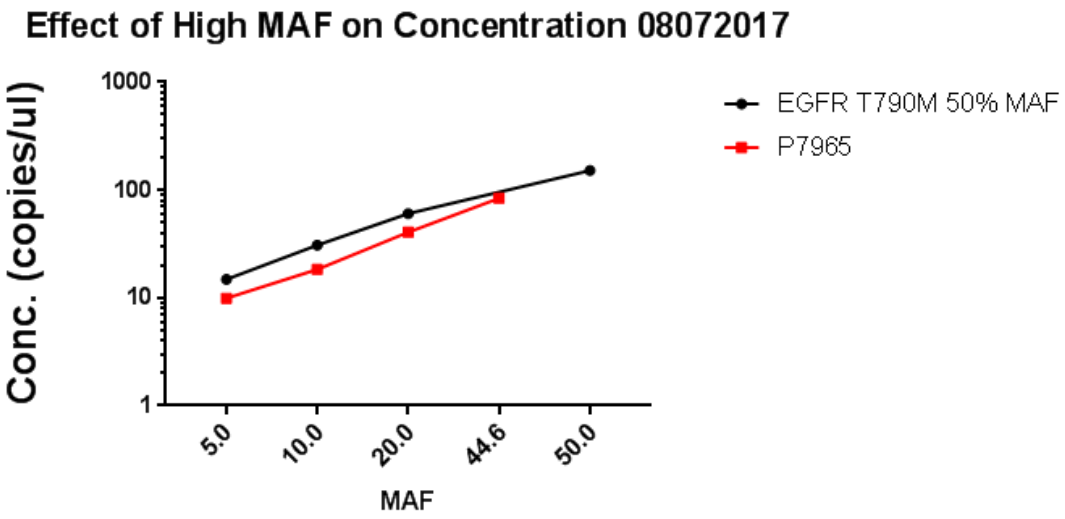

Figure 13. The concentration readout appears linear as the MAF increases. EGFR T790M cell line Horizon Discovery DNA (black) with 50% MAF was tested neat and diluted with 100% EGFR wild type DNA to 20%, 10% and 5%. Also a pyrosequencing patient sample (red) with a MAF of 44.6% was tested neat and diluted to 20%, 10%, 5%, 1%, 0.5% and 0.1% with wild type DNA. This experiment was run twice with similar results.

Table 10. High MAF statistical data analysis

MAF	EGFR T790M 50% MAF		P7965	
	Mean	SD	Mean	SD
5	14.85	0.64	9.9	0.28
10	30.9	0.42	18.35	0.49
20	60.4	0.71	40.55	1.77
44.6			83.8	3.82
50	151.8	0.42		

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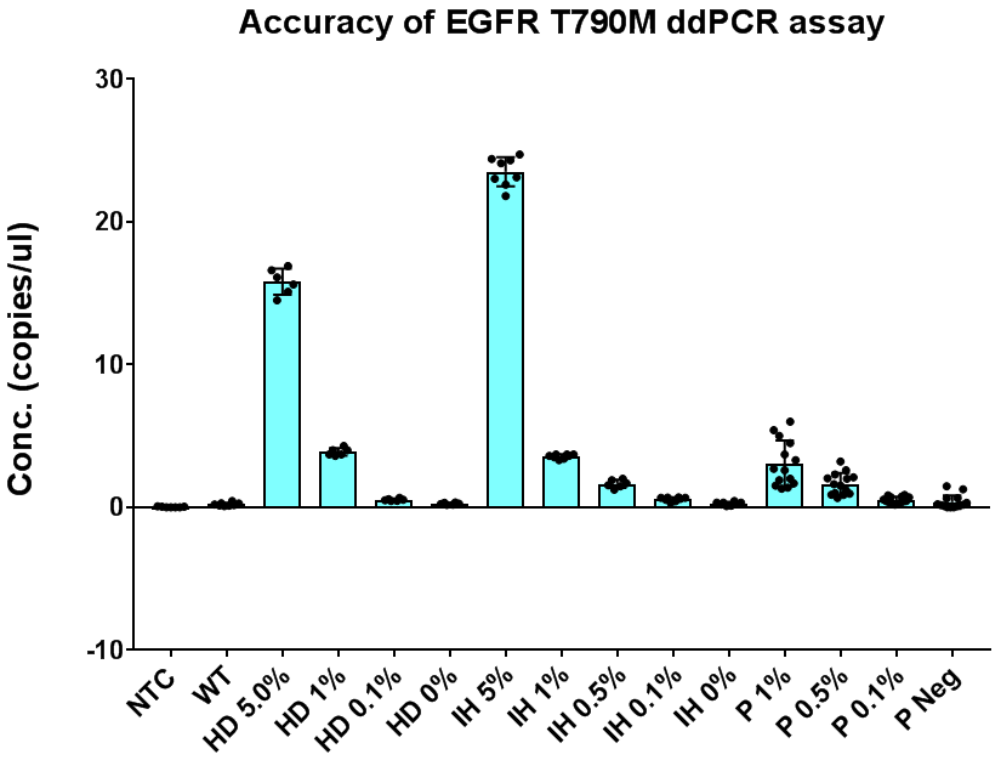

Figure 12. Accuracy of the EGFR T790M assay at different MAFs. EGFR T790M positive patient samples (P) as determined by NGS or pyrosequencing were diluted down with 100% WT EGFR Horizon Discovery DNA to 1%, 0.5%, and 0.1% MAF and run in duplicate with 25ng per well each and merged for analysis. These values were compared to either Horizon Discovery synthetic DNA standards (HD) isolated using the Qiagen nucleic acid isolation kit and In-house (IH) derived standards prepared by diluting Horizon Discovery cell line DNA to the appropriate MAFs.

Table 9. Accuracy statistical data analysis

	NTC	WT	HD 5.0%	HD 1%	HD 0.1%	HD 0%	IH 5%	IH 1%	IH 0.5%	IH 0.1%	IH 0%	P 1%	P 0.5%	P 0.1%	P Neg
n	9	10	6	6	6	6	10	10	8	10	10	18	18	18	14
Mean	0.026	0.218	15.8	**3.883**	0.525	0.25	21.95	**3.55**	1.666	0.605	0.212	**2.726**	1.506	0.4778	0.3721
SD	0.026	0.1085	0.9121	**0.2639**	0.08044	0.06663	3.391	**0.1434**	0.2414	0.1501	0.1385	**1.552**	0.7115	0.2144	0.4711
CV	101.87%	49.77%	5.77%	**6.80%**	15.32%	26.65%	15.45%	**4.04%**	14.49%	24.81%	65.35%	**56.93%**	47.24%	44.88%	126.60%

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Liquid Biopsy: ddPCR Pros and Cons

Pros	Cons
Quantitative	50ng sample/well (150ng)
Fast	Little or No Multiplexing
Inexpensive	Need to know mutation
Sensitivity	

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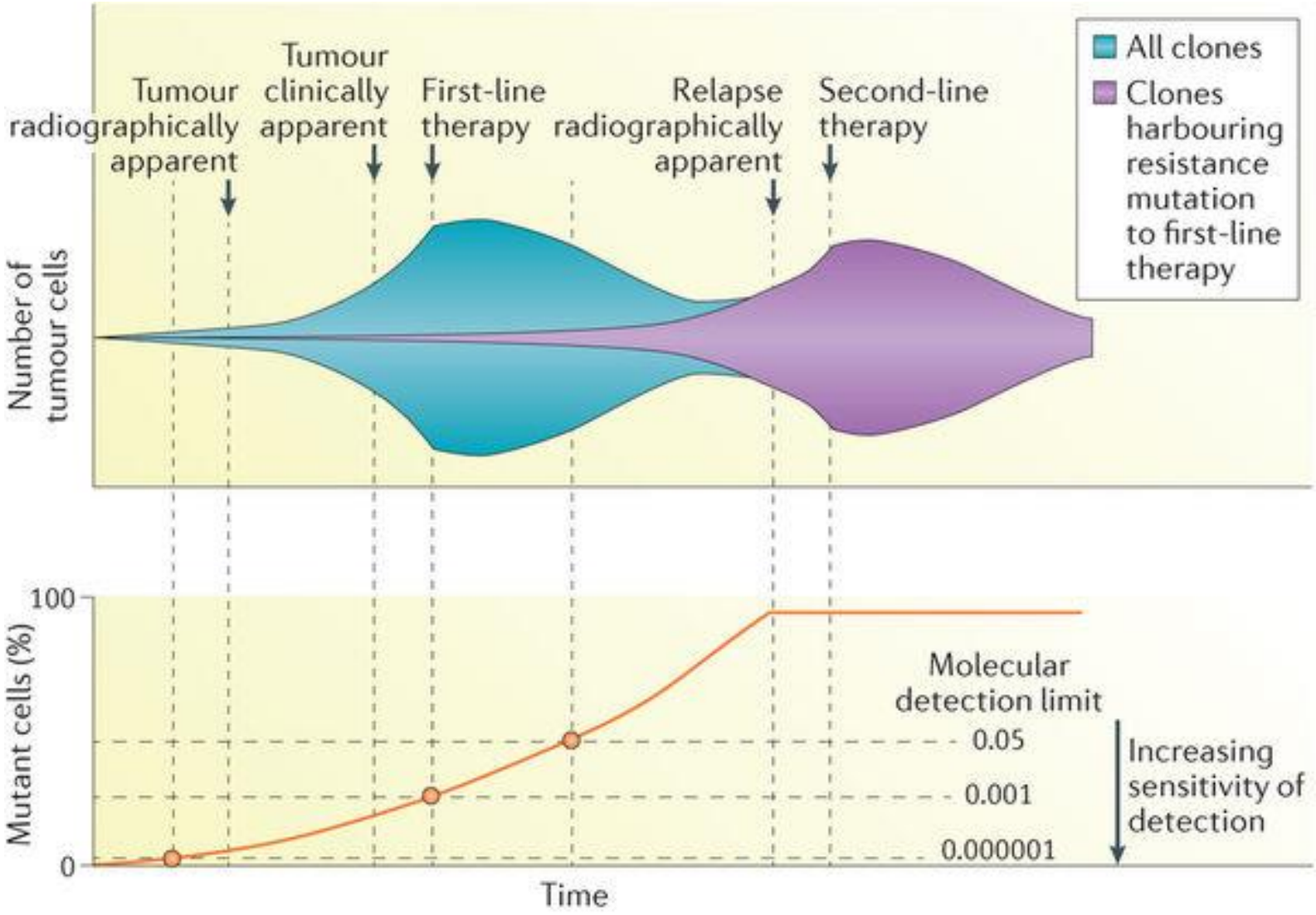
CANCER EVOLUTION BEFORE AND AFTER TREATMENT IN PATIENTS



Nature Reviews | Clinical Oncology

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Osimertinib or Platinum–Pemetrexed in EGFR T790M–Positive Lung Cancer- AURA

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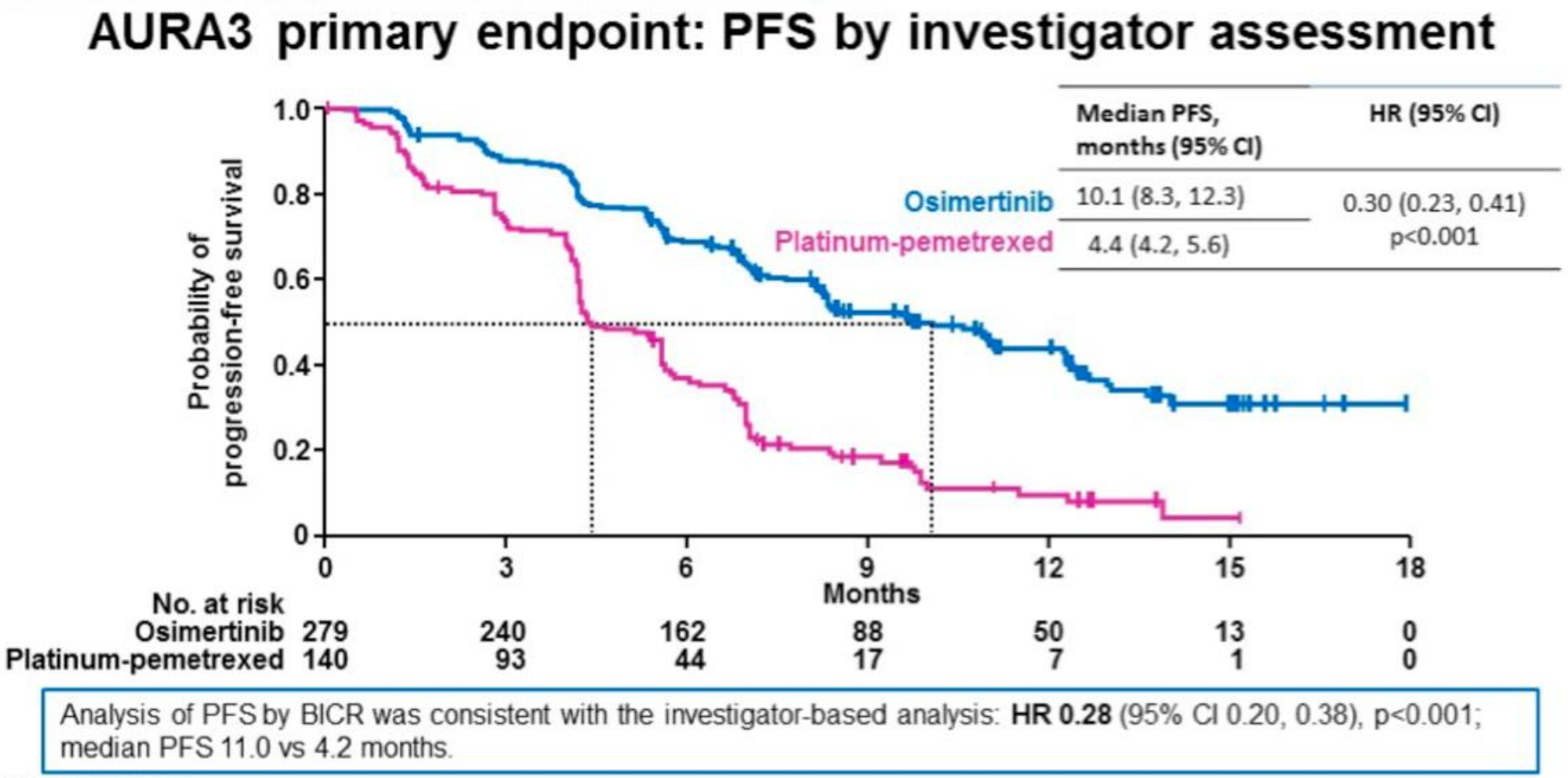

Analysis of PFS by BICR was consistent with the investigator-based analysis: **HR 0.28** (95% CI 0.20, 0.38), p<0.001; median PFS 11.0 vs 4.2 months.

Population: intent-to-treat
Progression-free survival defined as time from randomisation until date of objective disease progression or death; calculated using the Kaplan-Meier approach. Progression included deaths in the absence of RECIST progression.
Tick marks indicate censored data; CI, confidence interval
Papadimitrakopoulou V, et al. AURA3. To be presented at 17th WCLC, hosted by IASLC. December 2016.

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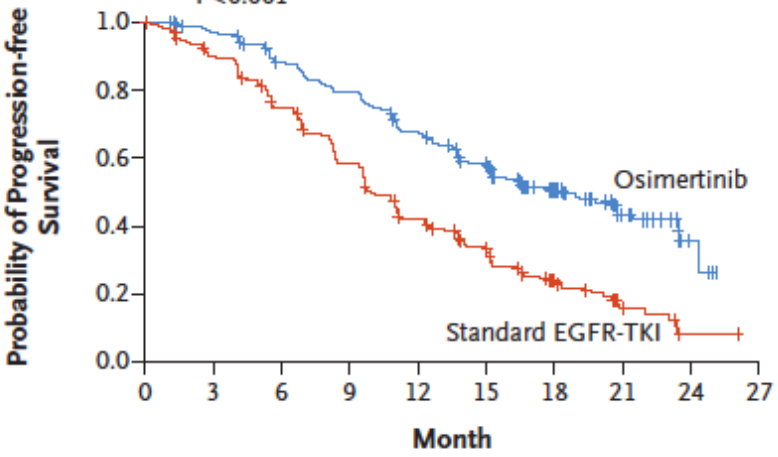
Osimertinib in First Line untreated NSCLC with EGFR MUT

A Progression-free Survival in Full Analysis Set

	No. of Patients	Median Progression-free Survival (95% CI)
		mo
Osimertinib	279	18.9 (15.2–21.4)
Standard EGFR-TKI	277	10.2 (9.6–11.1)

Hazard ratio for disease progression or death, 0.46 (95% CI, 0.37–0.57)
P<0.001



No. at Risk

Osimertinib	279	262	233	210	178	139	71	26	4	0
Standard EGFR-TKI	277	239	197	152	107	78	37	10	2	0

The NEW ENGLAND JOURNAL of MEDICINE

ORIGINAL ARTICLE

Osimertinib in Untreated *EGFR*-Mutated Advanced Non–Small-Cell Lung Cancer

J.-C. Soria, Y. Ohe, J. Vansteenkiste, T. Reungwetwattana, B. Chewaskulyong, K.H. Lee, A. Dechaphunkul, F. Imamura, N. Nogami, T. Kurata, I. Okamoto, C. Zhou, B.C. Cho, Y. Cheng, E.K. Cho, P.J. Voon, D. Planchard, W.-C. Su, J.E. Gray, S.-M. Lee, R. Hodge, M. Marotti, Y. Rukazenkov, and S.S. Ramalingam, for the FLAURA Investigators*

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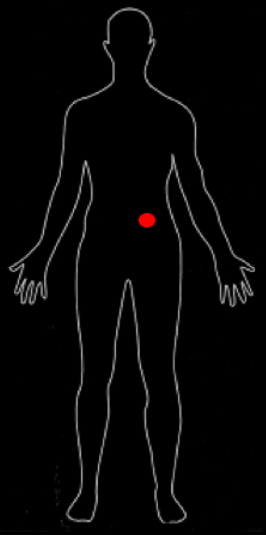
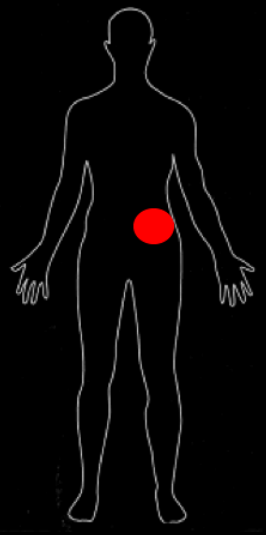
Three Areas of Cancer Diagnostics

Most Suited TO ddPCR applications

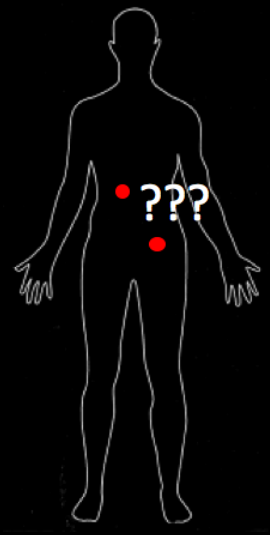
Early Detection:
Individuals without symptoms



Treatment Selection:
What therapy?
(or no therapy?)



Recurrence Monitoring:
Is the therapy working?
Is disease coming back?



Summary

- Digital PCR is a robust method that can be used to creat LDTs in CLIA laboratories

- One example is measuring EGFR T790M mutation in cell free DNA circulating in blood.

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Elena Ryzhova Ph.D.
Ravi Kothapalli Ph.D.
Liang Nong MD

Moffitt Cancer Center

Jhanelle Gray MD

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WATCH FOR ANNOUNCEMENT REGARDING MOFFITT PATHOLOGY SYMPOSIUM FALL 2018 in CLEARWATER BEACH FLORIDA- DATES TO BE DETERMINED

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