ctDNA: mutation detection

Athina Markou
Associate Professor of Analytical Chemistry
National and Kapodistrian University of Athens

Mutations in ctDNA from liquid biopsy samples can be detected via two different approaches.

1. Single, or low numbers of, mutations can be detected using highly sensitive techniques with high specificity and at a rather fast and cost-effective rate

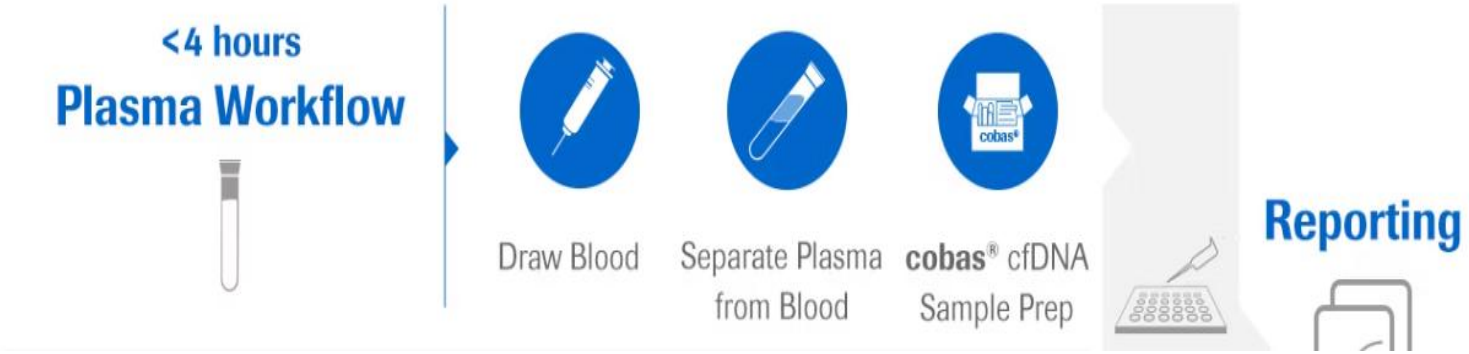
Cobas EGFR mutation Test v2

❑First liquid biopsy-based companion diagnostic to be approved by US Food and Drug Administration (FDA) and the European Medicines Agency for the prescription of EGFR inhibitors in patients with non-small-cell lung cancer (NSCLC) in cases when tumour biopsy tissue is not available

❑ Real time PCR assay

❑ Detects 42 mutations in exons 18,19,20 and 21 of the EGFR gene including the T790M resistance mutation

❑ Delivers results in less than 4 hours with plasma



Regulatory approved EGFR ctDNA testing

Therascreen EGFR Rotor-Gene Q Plasma polymerase chain reaction (PCR) kit (Qiagen)

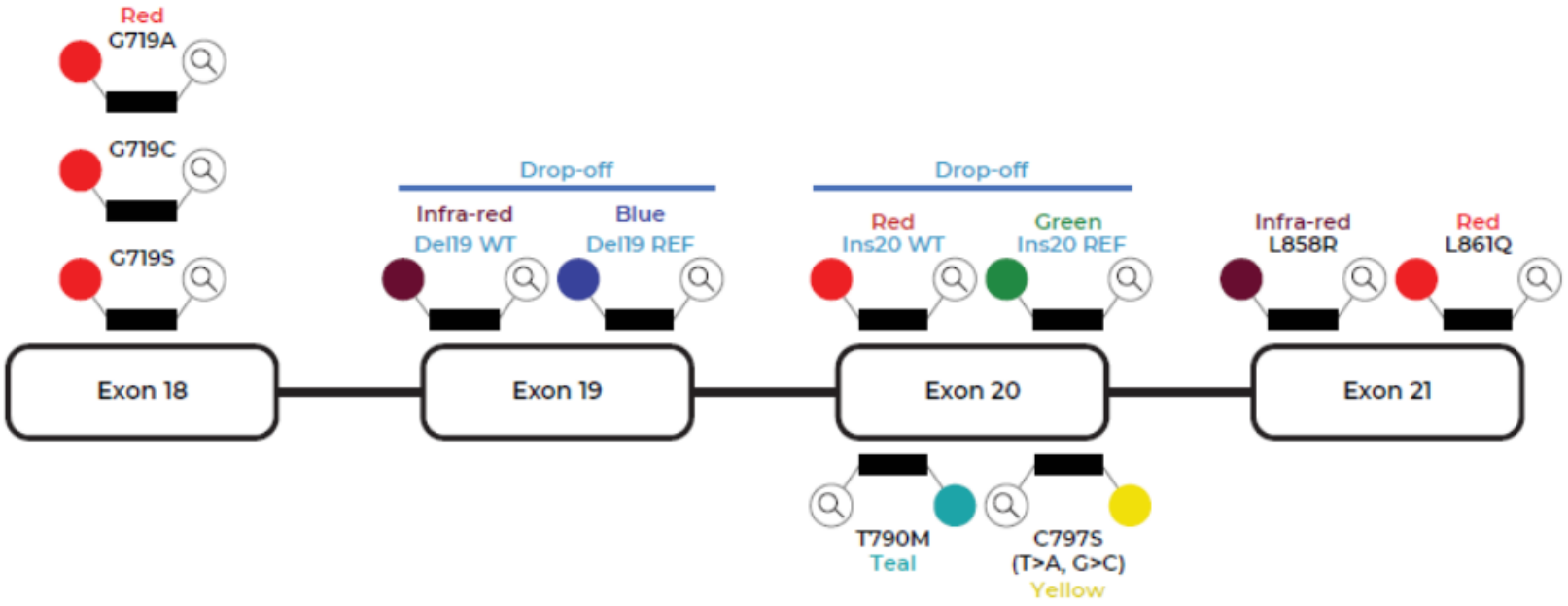
- in vitro diagnostic test for the detection of 21 somatic mutations in the EGFR cancer-related gene
- Using Scorpions® and ARMS® technologies (real time PCR)

AmoyDx Super Amplification Refractory Mutation System (ARMS) EGFR mutation test

- is a real-time PCR assay
- 31 somatic mutations in exons 18-21 of the EGFR gene
- high sensitivity of up to 0.2%

EGFR detection using the Crystal Digital PCR

- molecular analysis based on PCR methods has improved through recent years with the emergence of cutting-edge technologies such as digital PCR (dPCR) and BEAMing .
- EGFR 6-color Crystal Digital PCR™ kit allows the detection from cfDNA of more than 90% of EGFR mutations described in NSCLC
- TaqMan™ probe technology to detect eight, point mutations, as well as twenty-four Exon 19 deletion and Exon 20 insertion alterations using drop-off detection

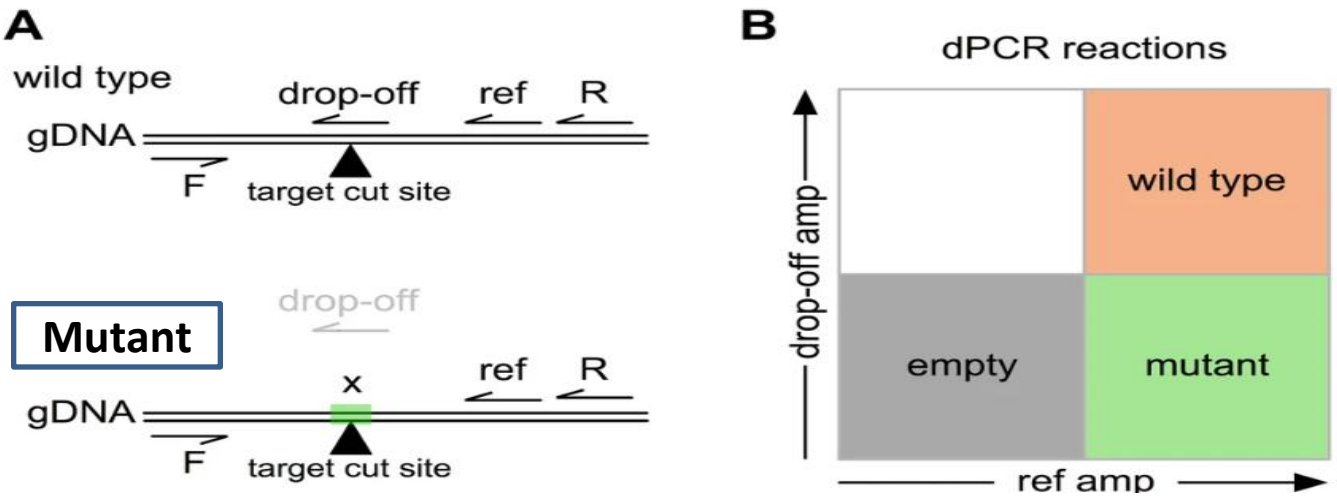


Principle of drop-off digital PCR assay

❑A major advantage of a drop-off digital PCR assay is the simplified detection of numerous proximal genetic lesions within a short genomic interval using minimal reagents.

❑a drop-off assay requires only two TaqMan™ probes targeting the same amplicon: a wild-type probe that spans the genetic lesion site but is uniquely complementary to the wildtype sequence, and a reference probe that hybridizes adjacent to the mutation site and is thus complementary to both the mutant and the wild-type alleles.

❑In the presence of a wild-type allele, both the wild-type and reference probes will hybridize with their targets, leading to a double positive population



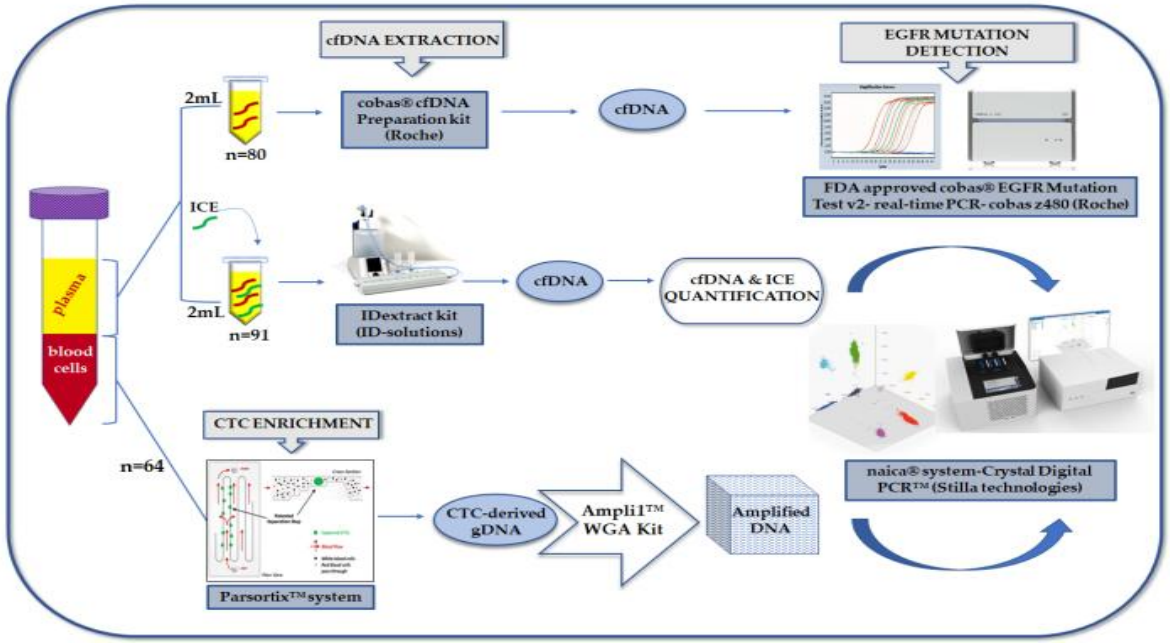
cancers

MDPI

Article

Detection of EGFR Mutations in Plasma cfDNA and Paired CTCs of NSCLC Patients before and after Osimertinib Therapy Using Crystal Digital PCR

Aliki Ntzifa [1], Athanasios Kotsakis [2], Vassilis Georgoulias [3] and Evi Lianidou [1,*]



Direct Comparison of Crystal Digital PCR and the FDA-Approved Cobas® EGFR Mutation Test V2 was performed in 80 identical matched plasma samples. The concordance rates between the two EGFR mutation detection methods were high for all types of mutations

BRAF/NRAS Mutation Test

- Testing for the presence of somatic *BRAF* mutations in melanoma is a crucial step in diagnosis and the qualification for treatment since the presence of activating *BRAF* mutations in melanoma cells is a cornerstone of the antitumor activity of BRAF/MEK inhibitors that was approved from FDA

- allele-specific, real-time PCR test for the qualitative detection and identification of exon 11 and 15 mutations in the proto-oncogene B-Raf (BRAF) gene and exon 2, 3, and 4 mutations in the neuroblastoma RAS viral oncogene homolog (NRAS) gene

KRAS Mutation assays

- KRAS mutations are present in approximately 25% of tumors, making them one of the most common gene mutations linked to cancer.
- KRAS drives 32% of lung cancers, 40% of colorectal cancers, and 85% to 90% of pancreatic cancer cases.
- G12C, G12D and G12R are some of the most common KRAS mutations, based on the specific mutations that are present.
- There are allele-specific, real-time PCR assays for the qualitative detection and identification of exon 2, 3, and 4 mutations in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene
- Mutation of KRAS gene is associated with resistance to anti-EGFR therapies. In addition, although KRAS was thought to be an “undruggable” target, it has become “druggable” after the successful approval of KRAS (G12C) inhibitor (Sotorasib) for the treatment of KRAS G12C-mutated metastatic NSCLC

PIK3CA Mutation assays

- In advanced hormone receptor-positive (HR+) breast cancer, activation of the PI3K downstream pathway is a critical feature of the mechanism of endocrine resistance.
- HR+ advanced breast cancer has been the recent introduction of PI3K inhibitor (PI3Ki) for the treatment of patients with HR+, HER2-negative (HER2-) advanced or metastatic breast cancer that harbors PIK3CA mutations.
- When a patient has PIK3CA mutation, they are likely to be resistant to the chemotherapy.
- There are several real-time PCR available assays such as therascreen®PIK3CA RGQ mutation detection (Qiagen) (detects 11 mutations) and cobas®PIK3CA (Roche) (detects 17 mutations) (recalled the Therascreen PIK3CA RGQ PCR Kit as this product might generate false Q546R mutation positive results caused by nonspecific interactions within Q546R)
- One CE-IVD certified Droplex PIK3CA Mutation Test detects 11 defined mutations within the catalytic a-subunit of the PI3K gene using Digital Droplet TM Technology as a companion diagnostic

PIK3CA Mutation assays

Biology of Human Tumors

Clinical Cancer Research

PIK3CA Mutational Status in Circulating Tumor Cells Can Change During Disease Recurrence or Progression in Patients with Breast Cancer

Athina Markou[1], Sofia Farkona[1], Christina Schiza[1], Tonia Efstathiou[1], Sophia Kounelis[2], Nikos Malamos[2], Vassilis Georgoulias[3], and Evi Lianidou[1]

PHARMASSIST
CONTRACT RESEARCH ORGANISATION

- A real-time qualitative PCR test that is based on the combination of allele-specific priming, asymmetric PCR, and melting curve analysis for the detection of PIK3CA mutations (E542K, E545K, E545Q, H1047R).
- This is a CE-IVD kit that can be used on DNA samples extracted from FFPE tumor tissues, circulating tumor cells (CTCs) and plasma of breast cancer patients.
- High sensitive: up to 0.05%

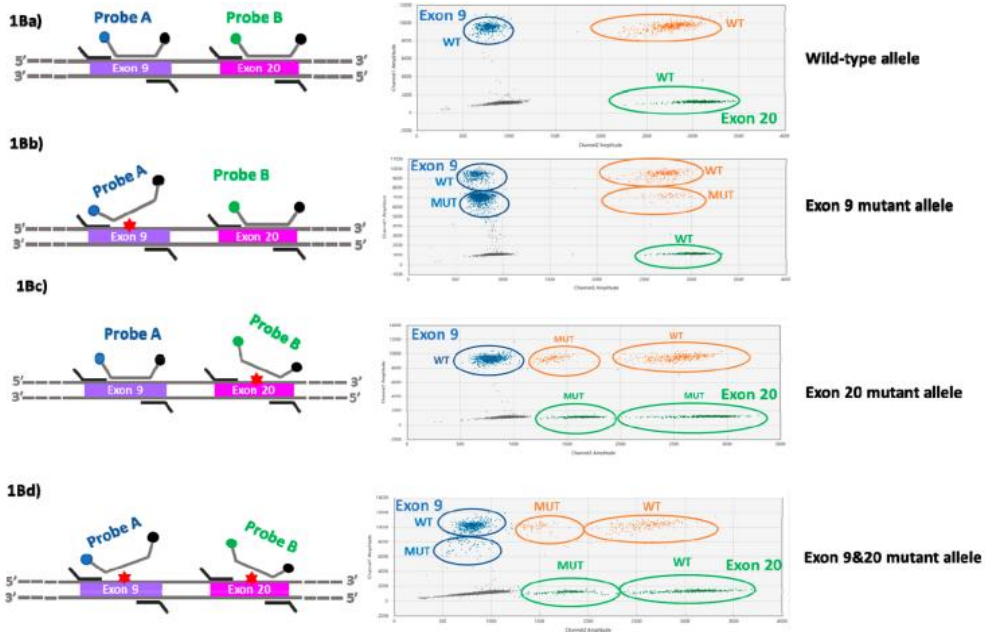
analytical chemistry

pubs.acs.org/ac Article

Development and Validation of a Novel Dual-Drop-off ddPCR Assay for the Simultaneous Detection of Ten Hotspots *PIK3CA* Mutations

Dimitra Stergiopoulou, Stavroula Smilkou, Vasilis Georgoulias, Loukas Kaklamanis, Evi Lianidou, and Athina Markou*

We developed and analytically validated a highly sensitive, specific, cost-effective, and reproducible dual-drop-off ddPCR assay for the simultaneous detection of ten PIK3CA mutations located in exons 9 and 20 in a single reaction



Developed dual-drop-off ddPCR assay applied to 35 plasma-cfDNA samples from patients with ER+ metastatic breast cancer and detected PIK3CA mutations in exon 9 in 9/35(26%) samples, while 9/35 (26%) samples were positive for PIK3CA mutations in exon 20.

Published in final edited form as:
Anal Chem. 2019 October 15; 91(20): 13105–13111. doi:10.1021/acs.analchem.9b03325.

Nuclease-Assisted Minor Allele Enrichment Using Overlapping Probes-Assisted Amplification-Refractory Mutation System: An Approach for the Improvement of Amplification-Refractory Mutation System-Polymerase Chain Reaction Specificity in Liquid Biopsies

Athina Markou[*,†], **Elena Tzanikou**[†], **Ioannis Ladas**[‡], **G. Mike Makrigiorgos**[‡], **Evi Lianidou**[†]
[†]Analysis of Circulating Tumor Cells, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens 15771, Greece
[‡]Department of Radiation Oncology, Dana-Farber Cancer Institute and Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, United States

HARVARD UNIVERSITY

Using ARMS primers in most cases, inefficient priming still occurs on the WT sample, yielding a late "background Cq", originating from WT DNA, i.e., false positive results.

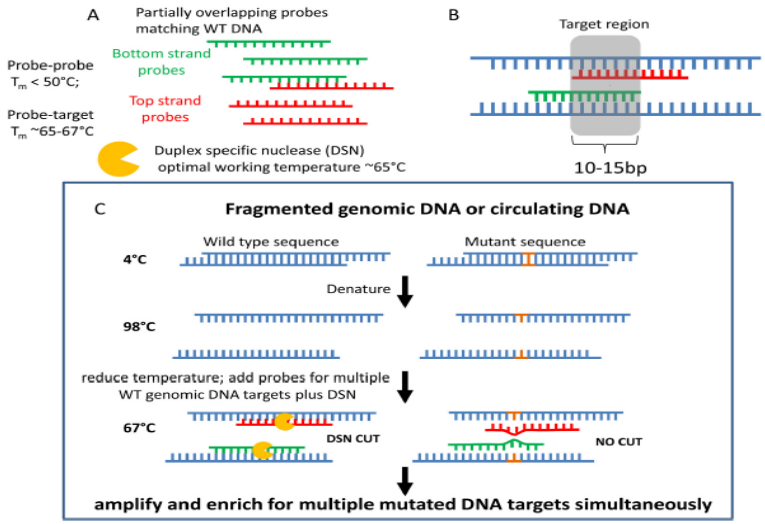
❑NAPA: NaME-PrO-assisted ARMS is based on a combination of **NAME-PrO**, **multiplex ARMS-PCR**, and **melting analysis**.

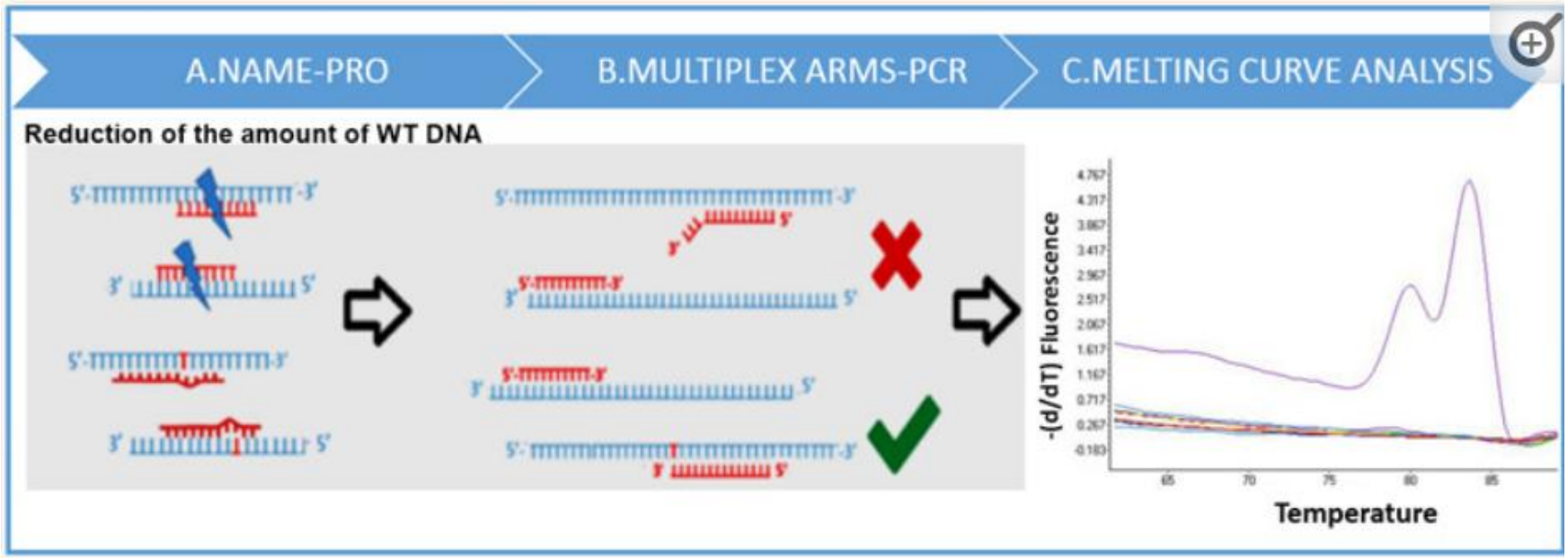
❑By using NAME-PrO, WT DNA removed from DNA targets and the inefficient priming of ARMS primers avoided and the false positive signal that results from this false priming.

❑ Due to the different size of the designed PCR products, discrimination and identification each of two specific *PIK3CA* hotspot mutations simultaneously managed by melting analysis.

1)A thermostable duplex-specific nuclease **(DSN)** that digests dsDNA with high preference over single stranded DNA and requires fully matched template i.e. mismatches near its binding site inhibit enzymatic action. **DSN has no observed sequence specificity**, thereby double stranded regions are digested irrespective of sequence (NaME-PrO harnesses these DSN properties to enable preferential digestion of WT DNA at any desired target.

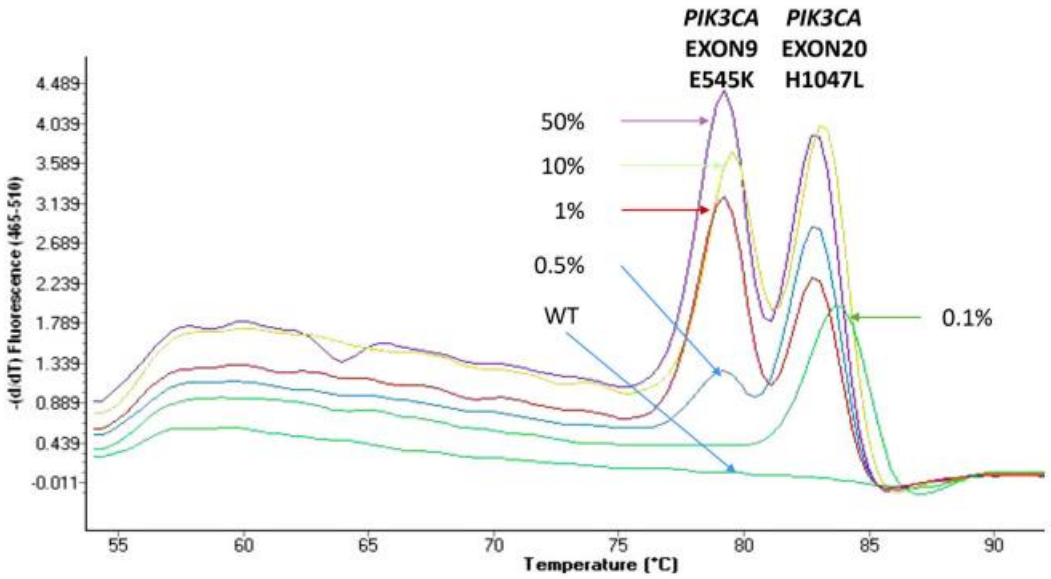
2) For each DNA target interrogated for mutations, **a distinct pair of oligonucleotide probes** overlapping the target region is designed. The probes are complementary to the WT DNA and bind respectively the top and bottom DNA template strands with an overlap 'target' region of about 10–15 bp





- ❑ NAME-Probes for two specific areas of PIK3CA genes (exon 9 and 20)
- ❑ ARMS primers for specific mutations (different size of PCR products)

Analytical sensitivity of the NAPA assay



Samples prepared by mixing known concentrations of mutated gDNA (isolated from cell lines), with WT gDNA at ratios of 50%, 10%, 5%, 1%, 0.5%, 0.1%, and 0%.

Direct Comparison between the Developed NAPA Assay and ddPCR for Detecting PIK3CA E545 K Hotspot Mutation

		ddPCR		
		EpCAM-positive CTC fraction		
NAPA PIK3CA assay	+	+	−	total
		2	0	2
	−	2	20	22
	total	4	20	24
	concordance: (22/24) 91.7% ($p = 0.022$ chi-square test)			
		cfDNA		
	+	5	2	7
	−	0	10	10
	total	5	12	17
concordance: (15/17) 88.2% ($p = 0.003$, chi-square test)				

ESR1 Mutation assays

❑ In metastatic estrogen receptor-positive (ER) breast cancer (MBC), ESR1 mutations have been identified as an important mechanism of resistance to estrogen deprivation therapy by aromatase inhibitors (AIs) and can be detected in approximately 30–50% of MBC patients after prior treatment with AIs in the metastatic setting

cancers

MDPI

Article

ESR1 NAPA Assay: Development and Analytical Validation of a Highly Sensitive and Specific Blood-Based Assay for the Detection of *ESR1* Mutations in Liquid Biopsies

Dimitra Stergiopoulou [1], Athina Markou [1,*], Eleni Tzanikou [1], Ioannis Ladas [2], G. Mike Makrigiorgos [2], Vassilis Georgoulias [3,4] and Evi Lianidou [1,*]

We developed and analytically validated a novel, highly sensitive and specific nuclease-assisted minor-allele enrichment with probe-overlap (NaME-PrO)-assisted Amplification refractory mutation system (ARMS) (NAPA) assay for the detection of four ESR1 mutations (Y537S, Y537C, Y537N and D538G)

The Journal of Liquid Biopsy 5 (2024) 100154

Contents lists available at ScienceDirect

The Journal of Liquid Biopsy

journal homepage: www.sciencedirect.com/journal/the-journal-of-liquid-biopsy

Multiplex detection of ten *ESR1* mutations and *AKT1* E17K in breast cancer using digital PCR

Stavroula Smilkou [a], Aliki Ntzifa [a], Dimitra Stergiopoulou [a], Vasilis Georgoulias [b], Evi Lianidou [a,*]

- Evaluation of the performance of a novel multiplex assay (12plex) for the detection of ten ESR1 mutations and AKT1 E17K in plasma-cfDNA based on Crystal Digital PCR® (Stilla Technologies, France
- Comparison to the results with our previously reported ESR1 NAPA assay for D538G, Y537S, Y537C and Y537 N ESR1 mutations

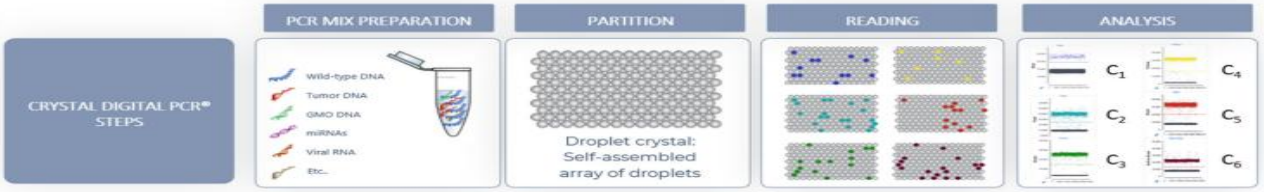
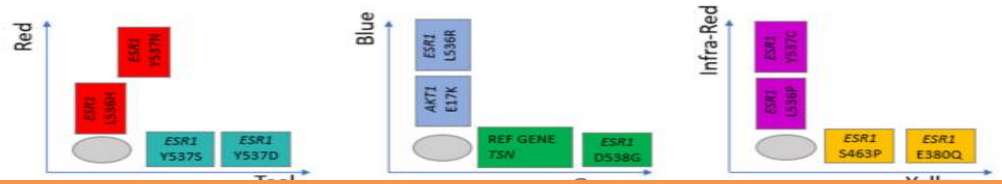


Fig. 1. Workflow for crystal digital PCR®.



Direct comparison between Crystal Digital PCR™ and the ESR1 NAPA assay revealed a high concordance (97.1%, k ¼ 0.871, $p < 0.001$) for the detection of D538G mutation

Mutations in ctDNA from liquid biopsy samples can be detected via two different approaches.

1. Single, or low numbers of, mutations can be detected using highly sensitive techniques with high specificity and at a rather fast and cost-effective rate

2. Multiple mutations in parallel and range from the analysis of several tens of mutations, to a genome-wide analysis of cfDNA by whole-exome sequencing (WES) or whole-genome sequencing (WGS)

Next-generation sequencing (NGS)–based assays such as Illumina® and Oncomine pan-cancer® (ThermoFisher);

Laboratory-developed tests (LDT) such as Foundation Dx® (Foundation Medicine) and Guardant 360® (Guardant Health)

FoundationOne CDx

FDA-approved liquid biopsy comprehensive genomic profiling service

NSCLC

- *EGFR*
- *BRAF*
- *ALK*
- *ROS1*
- *HER2 (ERBB2)*
- *MET*
- *RET*
- *NTRK1/2/3*
- MSI
- TMB

BREAST

- *HER2 (ERBB2)*
- *ESR1*
- *NTRK1/2/3*
- *BRCA1*
- *BRCA2*
- *PIK3CA*
- MSI

PROSTATE

- *BRCA1*
- *BRCA2*
- *ATM*
- *CHEK2*
- *MSH2/6*
- *PALB2*
- *MLH1*
- *PMS2*
- *RAD51D*
- *CDK12*
- *FANCA*
- MSI

OVARIAN

- *BRCA1*
- *BRCA2*
- *RAD51*
- *BRIP1*
- *PALB2*
- *MSH2/6*
- *PMS2*
- MSI

Guardant Health tests

❑First Guardant built out an integrated sequencing platform to measure cfDNA at scale and generate useful data at the low detection rates of cancer cfDNA.

❑ Guardant's first product is called Guardant360- initially focused on patients with advanced-stage solid tumors.

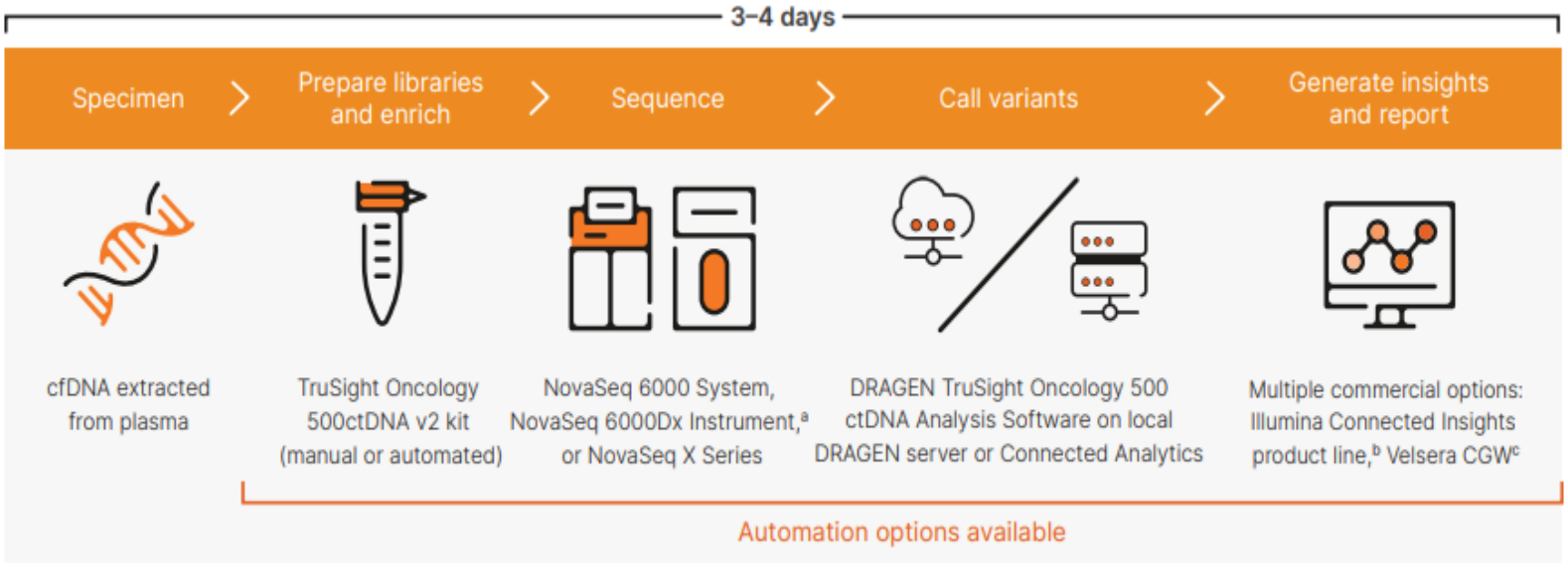
❑The test measures 739 genes with a turnaround time of 7 days where tissue biopsy with around 2 weeks.

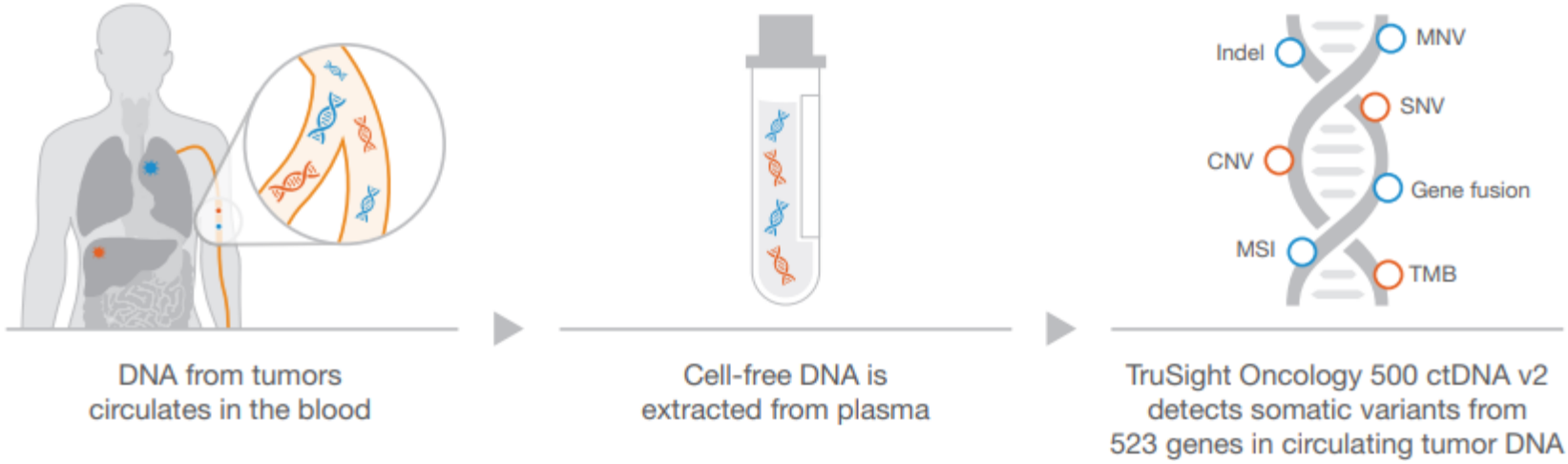
❑ Our Guardant360 CDx test is our FDA-approved liquid biopsy that provides results in less than 7 days to inform treatment decisions.

❑Recently FDA approved Guardant Health's Shield blood test for colorectal cancer screening in adults aged 45 years and older who are at average risk for the disease. It is the first blood test to be approved by the FDA as a primary screening option for colorectal cancer, meaning health-care providers can offer Shield in a manner similar to all other noninvasive methods recommended in screening guidelines. Shield is also the first blood test for colorectal cancer screening that meets the requirements for Medicare coverage.

NGS–based assays such as Illumina

Trusight Oncology





ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

SPECIAL ARTICLE

ANNALS OF ONCOLOGY driving innovation in oncology

ESMO recommendations on the use of circulating tumour DNA assays for patients with cancer: a report from the ESMO Precision Medicine Working Group

Lung Cancer | Breast Cancer | Colorectal Cancer

[illegible] indicated to confirm if the variant is present in germline.

Preanalytical variables

Centralized plasma collection
- Streck (IBBL)
- PAXgene (QIAGEN)
4.25 mL plasma per tube

Centralized mnDNA generation from NCI-H441 and NCI-H1573 (IBBL)

❑Patie
❑ Sam
❑ Colle

Table 1. Important findings and recommendations for ctDNA work flows.

Extraction	Automated approaches should be integrated into the clinical routine to reduce operator-based variability and hands-on time.
	Various extraction kits differ in their efficiency of recovering different fragment lengths.
Quantification	qPCR is more accurate and precise than Qubit.
	qPCR should be used in cases when only a little material is available and accurate quantification is required.
Downstream analysis	Deep sequencing and ddPCR methods can be effectively utilized in a clinical setting in combination with a variety of extraction kits and BCTs.
	The amount of input DNA should be taken into account for assay validation, as it contributes to the variability of the downstream analysis.
Process control	mnDNA closely mirrors the nucleosome-bound nature of ccfDNA and can be used as a process quality control from nucleic acid extraction to mutation detection.
Assay design	Fragment length and amplicon size should also be taken into account for ctDNA assay design.
Validation	A continuous use of the same method based on validated standardization of operating procedures should be pursued.
	Because of unavoidable biases to DNA integrity and mutation detection rate of various work flows, some form of verification is required when changing parts of the work flow to determine whether the new work flow complies with the previous assertions.

Clinic
149–1

Mu

Development o

- ddPCR (2x; Bayer)
- QuantiplexPro (QIAGEN)

- Alu-qPCR (2x; TATAA)

- ddPCR (Bayer)
- NGS (MUG)

Centralized data analysis