Pre-analytical aspects and Quality Control in liquid biopsy applications: How important is it?

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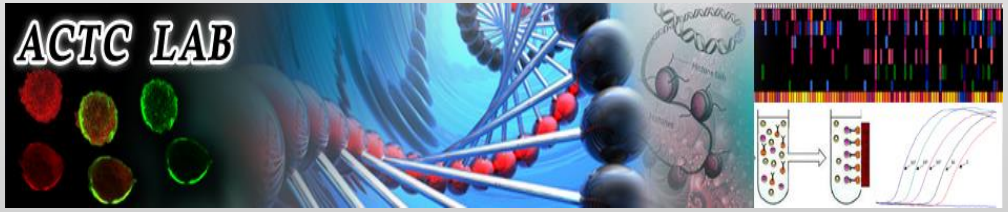
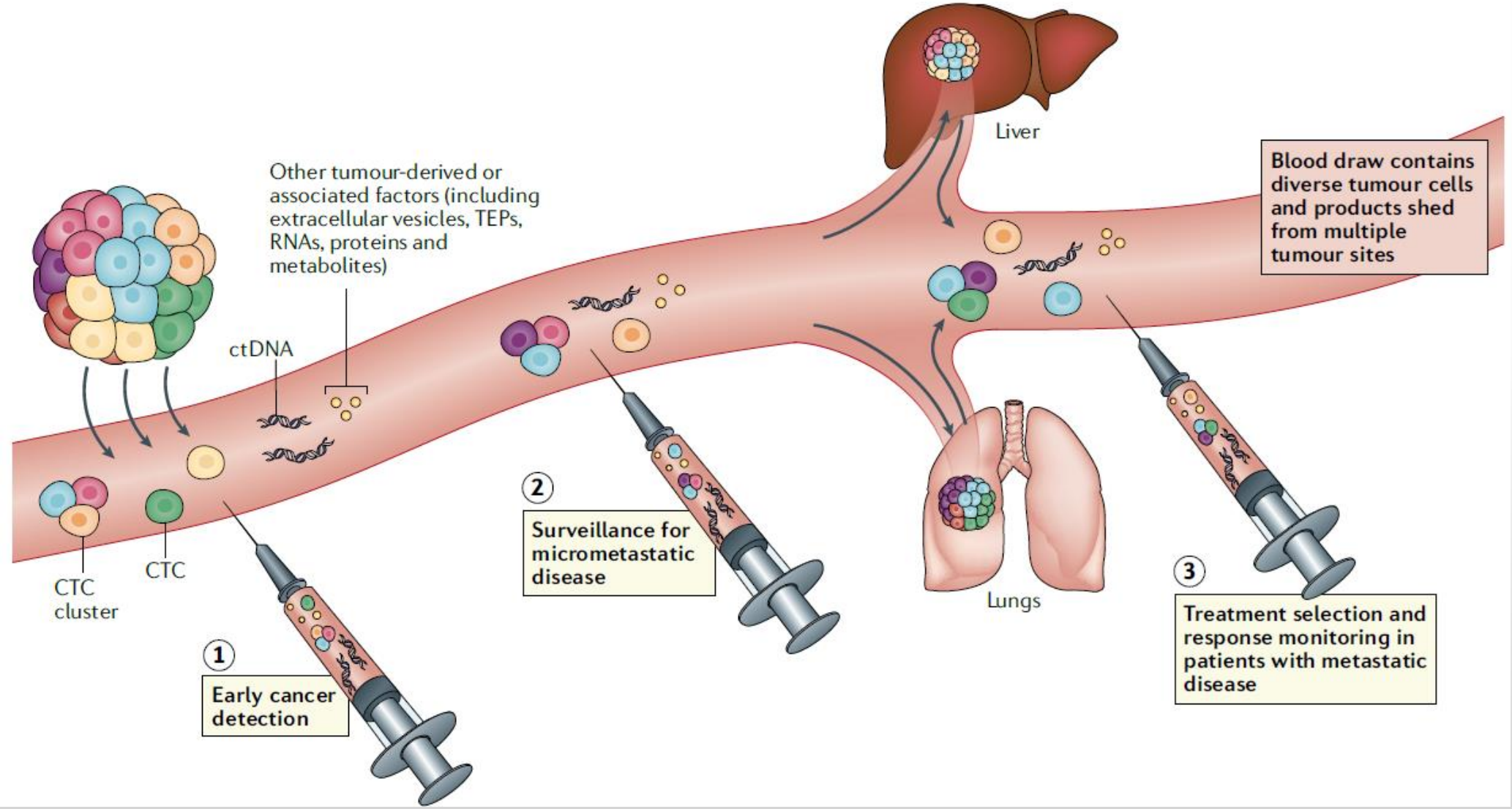
EST. 1837



LIQUID BIOPSY APPLICATIONS IN THE CLINIC



Ignatiadis et al, Nat Rev Clin Oncol, 2021

FDA APPROVED LIQUID BIOPSY TESTS

Table 1. Liquid biopsy tests cleared by the FDA (up to March 2023).

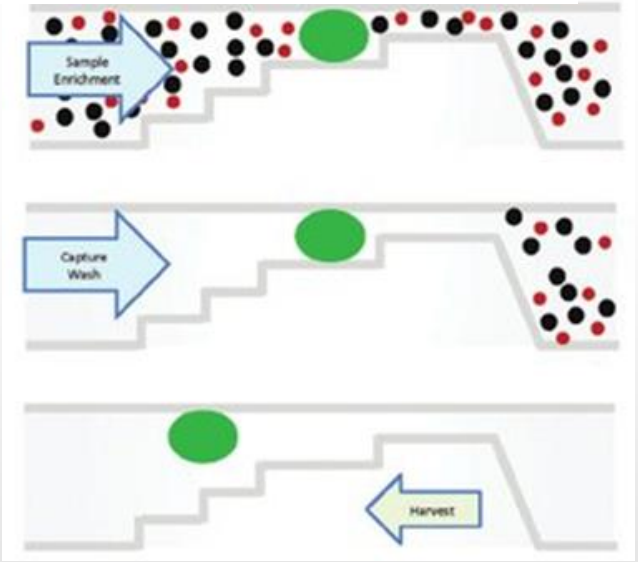
Test	Company	Cancer type	Biomarkers	Intended use	Technology	Year	Matrix
CTC enumeration CellSearch®	Menarini Silicon Biosystems	Metastatic Breast Metastatic Colorectal Metastatic Prostate	CTC detection ($CK^+/DAPI^+/CD45^-$)	Prognostic significance	Ep-CAM based CTC enrichment & CTC detection with IF	2004 2007 2008	Whole blood
cobas® EGFR Mutation Test v2	Roche	Metastatic NSCLC	*EGFR* exon 19 deletions or exon 21 L858R	CDx for erlotinib (TARCEVA, Genentech) and osimertinib (TAGRISSO, AstraZeneca)	Real-time PCR	2016	Plasma
Epi proColon®	Epigenomics AG	Colorectal cancer	*Septin9* gene DNA methylation	Screening test	Bisulfite conversion & Real-time PCR	2016	Plasma
cobas® EGFR Mutation Test v2	Roche	Metastatic NSCLC	*EGFR* exon 19 deletions or exon 21 L858R	CDx for gefitinib (IRESSA, AstraZeneca)	Real-time PCR	2018	Plasma
therascreen® *PIK3CA* RGQ PCR Kit	Qiagen	Advanced or Metastatic Breast Cancer	*PIK3CA* mutations	CDx for alpelisib (PIQRAY, Novartis)	Real-time PCR	2019	Plasma
Guardant360® CDx assay	Guardant Health	Metastatic NSCLC	*EGFR* exon 19 deletions, L858R and T790M	CDx for osimertinib (TAGRISSO, AstraZeneca Pharmaceuticals LP)	NGS	2020	Plasma
Guardant360® CDx assay	Guardant Health	All Solid Cancers	SNVs. Indels, amplifications and fusions in 55 genes	Comprehensive genomic profiling (CGP)	NGS	2020	Plasma
FoundationOne® Liquid CDx	FoundationOne	Metastatic Castration Resistance Prostate Cancer (mCRPC)	*BRCA1* and/ or *BRCA2* mutations	CDx for rucaparib (RUBRACA, Clovis Oncology, Inc.)	NGS	2020	Plasma
FoundationOne® Liquid CDx	FoundationOne	Advanced or Metastatic Breast Cancer	*PIK3CA* mutations	CDx for alpelisib (PIQRAY, Novartis)	NGS	2020	Plasma
FoundationOne® Liquid CDx	FoundationOne	Metastatic NSCLC	*ALK* rearrangements	CDx for alectinib (ALECENSA, Genetech USA, Inc.)	NGS	2020	Plasma
FoundationOne® Liquid CDx	FoundationOne	Advanced Ovarian cancer	*BRCA1* and/ or *BRCA2* mutations	CDx for rucaparib (RUBRACA, Clovis Oncology, Inc.)	NGS	2020	Plasma
cobas® EGFR Mutation Test v2	Roche	Metastatic NSCLC	*EGFR* exon 19 deletions or exon 21 L858R	CDx for expanded EGFR TKIs: osimertinib (TAGRISSO, AstraZeneca), erlotinib (TARCEVA, Genentech), gefitinib (IRESSA, AstraZeneca), afatinib (GILOTRIF, Boehringer Ingelheim), and dacomitinib (VIZIMPRO, Pfizer)	Real-time PCR	2020	Plasma
FoundationOne® Liquid CDx	FoundationOne	Metastatic Castration Resistance Prostate Cancer (mCRPC)	*BRCA1*, *BRCA2* and *ATM* mutations	CDx for olaparib (LYNPARZA, AstraZeneca Pharmaceuticals LP)	NGS	2020	Plasma
Guardant360® CDx assay	Guardant Health	Locally Advanced or Metastatic NSCLC	*EGFR* Exon 20 Insertion Mutations	CDx for amivantamab-vmjw (RYBREVANT, Janssen)	NGS	2021	Plasma
Guardant360® CDx assay	Guardant Health	Locally Advanced or Metastatic NSCLC	*KRAS* G12C mutation	CDx for sotorasib (LUMAKRAS, Amgen Inc.)	NGS	2021	Plasma
FoundationOne® Liquid CDx	FoundationOne	Metastatic NSCLC	*MET* exon 14 skipping	CDx for capmatinib (TABRECTA, Novartis)	NGS	2021	Plasma
FoundationOne® Liquid CDx	FoundationOne	Metastatic NSCLC	*EGFR* exon 19 deletions or exon 21 L858R substitutions	CDx for erlotinib (TARCEVA, Genentech), osimertinib (TAGRISSO), and gefitinib (IRESSA)	NGS	2022	Plasma
therascreen®KRAS RGQ PCR kit	Qiagen	NSCLC	*KRAS* G12C mutation	CDx for adagrasib (KRAZATI, Mirati Therapeutics)	Real-time PCR	2022	Plasma
CTC isolation / enrichment	ANGLE	Metastatic Breast Cancer	*Different biomarkers*	CTC isolation	Size-based enrichment microfluidics	2022	Whole blood
FoundationOne® Liquid CDx	FoundationOne	Metastatic NSCLC	*ROS1* mutations or *NTRK* fusions	CDx for entrectinib (ROZLYTREK, Roche)	NGS	2023	Plasma
Guardant360® CDx assay	Guardant Health	Advanced or Metastatic Breast Cancer	*ESR1* mutations	CDx for elacestrant (ORSERDU, Menarini)	NGS	2023	Plasma

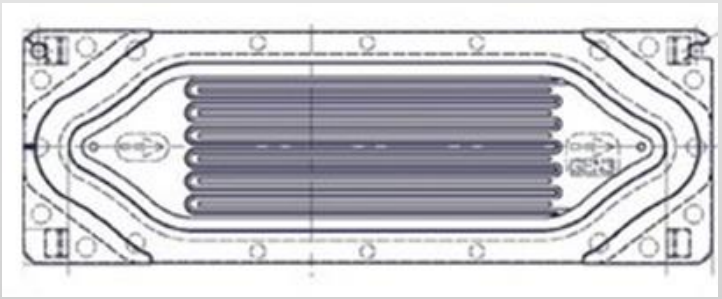
Ntzifa A. & Lianidou E, Crit Rev Clin Lab Sci, 2023

Angle wins first FDA clearance for Parsortix liquid biopsy system

By Catherine Longworth May 26, 2022

U.K.'s Angle plc has become the first company to receive a U.S. FDA product clearance for harvesting intact cancer cells for analysis. Angle reported it scored FDA clearance for its Parsortix system for the capture and harvest of circulating tumor cells (CTCs) from metastatic breast cancer patient blood. Shares in the AIM-listed company soared by more than 50% following the news.



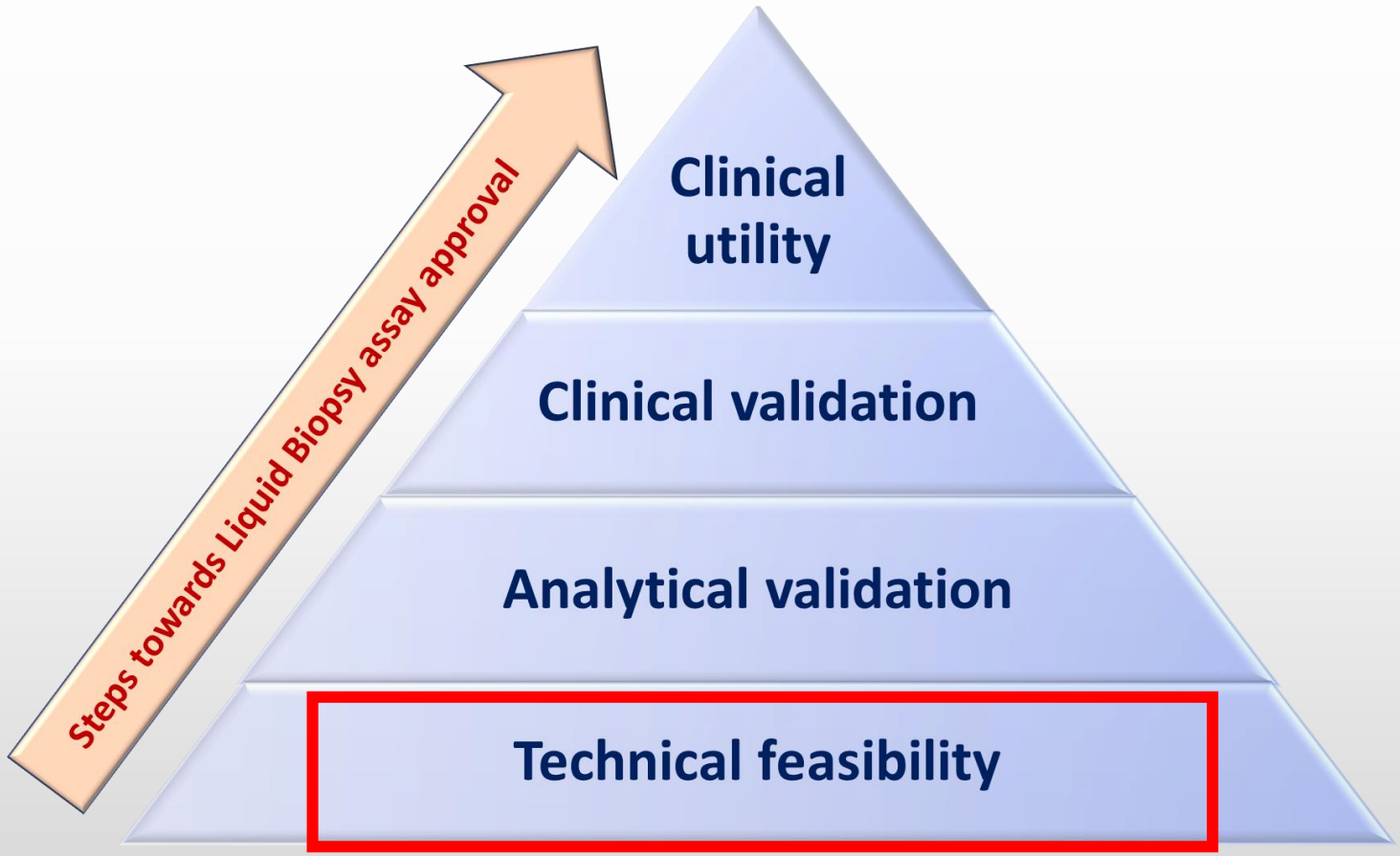


MAIN REQUIREMENTS FOR APPLICATION IN CLINICAL PRACTICE

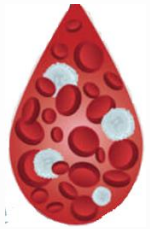
- **Analytical validity** refers to the ability of a test to accurately and reliably detect the variant(s) of interest and includes measures of accuracy, sensitivity, specificity, and robustness.

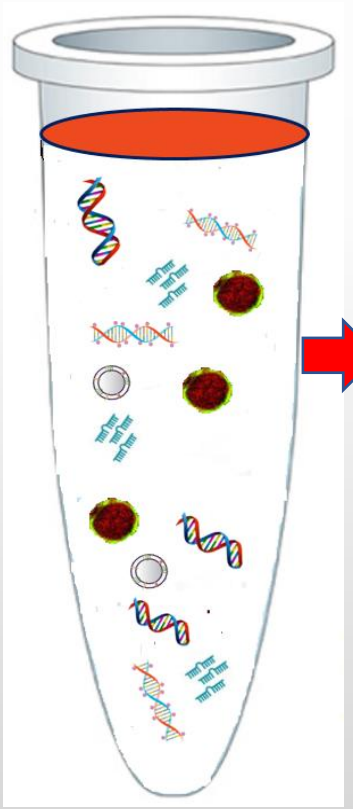
- **Clinical validity** implies that the test may accurately detect the presence or absence of a pathologic state or predict outcomes for groups of patients whose test results differ.

- **Clinical utility** is documented when high levels of evidence exist to demonstrate that the use of the test improves patient outcomes compared with not using it.



Ntzifa A. & Lianidou E, Crit Rev Clin Lab Sci, 2023





CTCs

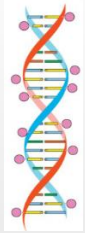
Protein expression
Gene expression
DNA abnormalities
miRNAs
Epigenetic alterations
Functional studies
Single cell analysis
Tumor heterogeneity

ctDNA

Tumor mutational burden
Amplifications/deletions
Translocations
Point mutations
Chromosomal abnormalities
Tumor heterogeneity

ctDNA/methylation

Epigenetic alterations
DNA methylation
Tumor heterogeneity



Circulating miRNAs

Extracellular vesicles

Liquid biopsy Main technologies

CTC

- CTC enumeration
- CTC isolation
- CTC imaging
- Single cell analysis
- Tumor heterogeneity
- RT-qPCR
- ddPCR
- NGS
- FISH

ctDNA

- ARMS-PCR
- Methylation specific PCR
- ddPCR
- NGS

miRNAs

- RT-qPCR
- ddPCR
- NGS

Lianidou and Pantel, Genes Chromosomes and Cancer, 2018

PRE-ANALYTICAL VARIABLES FOR ctDNA ANALYSIS

- **Plasma cfDNA** is released in blood circulation because of **cell death, apoptosis, or necrosis**, but can also arise from fetuses in pregnant women.
- **ctDNA** comprises a **small fraction of cfDNA** and originates **from active tumors** that constantly release their biological information into the bloodstream.
- Different tumor subclones may concurrently constitute the **heterogeneous tumor background of plasma**.
- The size of ctDNA significantly differs from that of cfDNA, **ranging between 160 and 180 bp**, and is packaged around nucleosomes in a specific manner that makes it distinguishable during fragmentation analysis.
- Its **short half-life, usually less than 2 h**, requires careful handling of samples and prompt transit to the laboratory.
- The **levels of ctDNA** in body fluids are affected by various factors such as **tumor stage** or **assigned treatments** that need to be considered during its analysis

PRE-ANALYTICAL VARIABLES FOR ctDNA ANALYSIS

OUTSIDE THE LAB

Blood sampling

avoid e.g. hemolyzed samples

~~Serum~~ or plasma?

Plasma is preferred to serum because clotting in serum increases gDNA

Blood collection tubes

EDTA tubes for immediate sample processing (<2h) or
Tubes with preservatives: anti-coagulant molecules and specific cell stabilizers to prevent cell lysis



or

Transportation

time

-Minimize time intervals between blood collection and sample processing.
-Sample transportation at RT

AT THE LAB

Centrifugation

Double centrifugation protocols: low to avoid cell lysis & higher for max purification

Plasma storage

At −80 °C for long term storage
aliquots to avoid freeze-thaw cycles

cfDNA extraction

commercially available kits or automated systems for high-throughput analysis and better repeatability (e.g. TETHIS)

cfDNA storage

At −20 °C for short term storage
At −80 °C for long term storage
Minimize freeze-thaw cycles

cfDNA quantity

PCR-based techniques are preferred to fluorometric methods

CURRENT GUIDELINES & STANDARDS FOR cfDNA ANALYSIS

SPIDIA4P

CANCER ID

- **developing CEN (European Committee for Standardization) technical preanalytical standards, including those for ccfDNA (CEN/TC 16835-3, https://standards.cen.eu/).**

BloodPAC

BLOOD PROFILING ATLAS IN CANCER

- **11 pre-analytical Minimal Technical Data Elements (MTDE) concerning factors most commonly associated with cell-free DNA (cfDNA) test design and development**
- **generic analytical validation protocols for NGS ctDNA analysis in collaboration with FDA**
- **the future steps - development of standards for analytical validation in multimarker testing, in MRD tests, and in blood tumor mutational burden (bTMB) tests**

CURRENT GUIDELINES & STANDARDS FOR cfDNA ANALYSIS

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

What do we need to obtain high quality circulating tumor DNA (ctDNA) for routine diagnostic test in oncology? – Considerations on pre-analytical aspects by the IFCC workgroup cfDNA

R. Danesi[a], Y.M.D. Lo[b], M. Oellerich[c], J. Beck[d], S. Galbiati[e], M. Del Re[a], E. Lianidou[f], M. Neumaier[g], R.H.N. van Schaik[h,*]

Clin Cancer Res. 2020 July 01; 26(13): 3104–3109. doi:10.1158/1078-0432.CCR-19-3015.

Harmonizing cell-free DNA Collection and Processing Practices through Evidence-based Guidance

Sarah R. Greytak[1], Kelly B. Engel[2], Sonya Parpart-Li[3], Muhammed Murtaza[4], Abel J. Bronkhorst[5], Mark D. Pertile[6], Helen M. Moore[7,*]

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

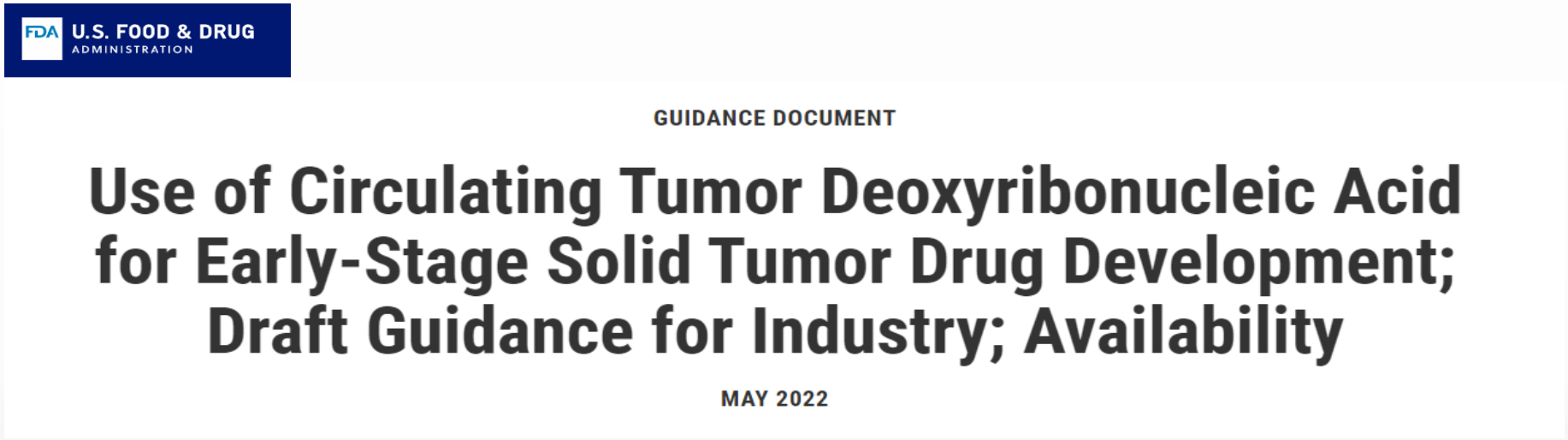
ANNALS OF ONCOLOGY driving innovation in oncology

SPECIAL ARTICLE

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

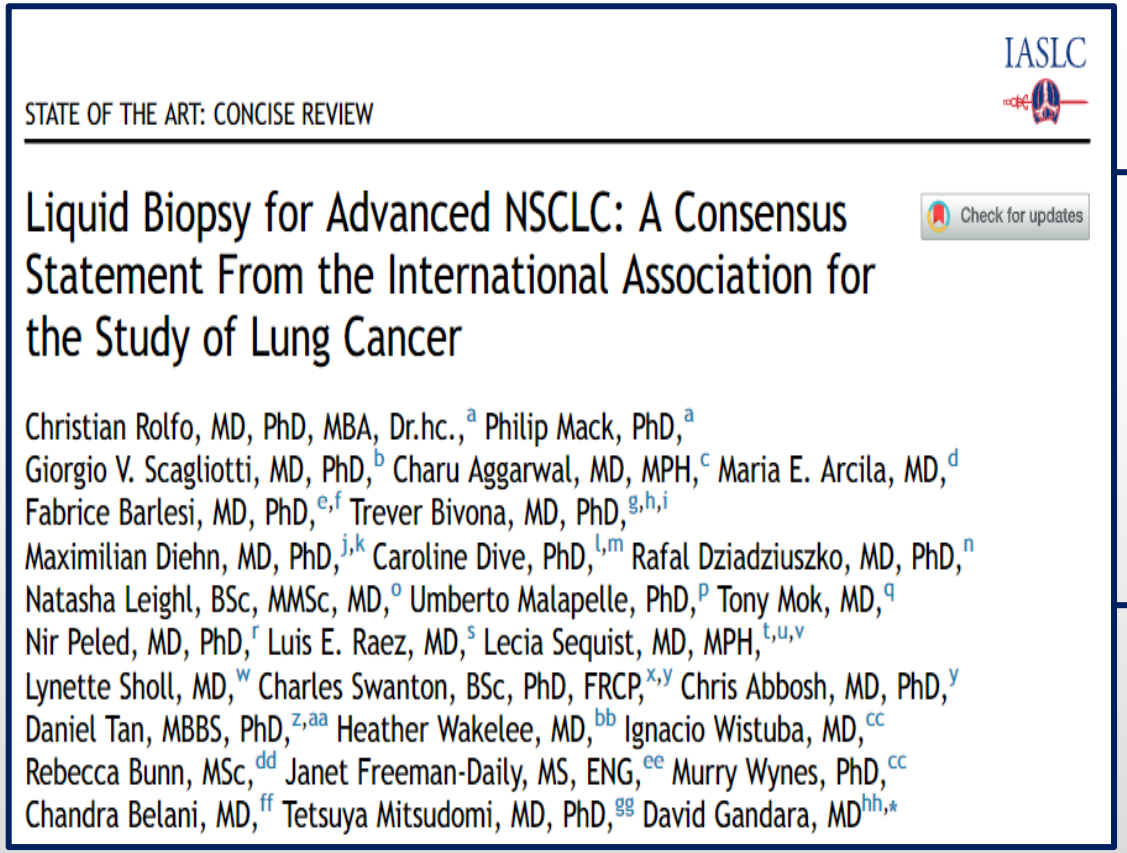
J. Pascual[1], G. Attard[2], F.-C. Bidard[3,4], G. Curigliano[5,6], L. De Mattos-Arruda[7,8], M. Diehn[9], A. Italiano[10,11,12], J. Lindberg[13], J. D. Merker[14], C. Montagut[15], N. Normanno[16], K. Pantel[17], G. Pentheroudakis[18], S. Popat[19,20], J. S. Reis-Filho[21], J. Tie[22,23], J. Seoane[24,25], N. Tarazona[26,27], T. Yoshino[28] & N. C. Turner[19,20*]

CURRENT RECOMMENDATIONS FOR ctDNA ANALYSIS IN SOLID TUMOURS



The Food and Drug Administration (FDA or Agency) is announcing the availability of a **draft guidance for industry** entitled "Use of Circulating Tumor DNA for Early-Stage Solid Tumor Drug Development." This draft guidance is intended to help sponsors planning to use circulating cell-free plasma derived tumor deoxyribonucleic acid (**ctDNA) as a biomarker in cancer clinical trials conducted under an investigational new drug application (IND) and/or to support marketing approval of drugs and biological products for treating solid tumor malignancies in the early-stage setting.**

RECOMMENDATIONS FOR ctDNA TESTING



IASLC

STATE OF THE ART: CONCISE REVIEW

Liquid Biopsy for Advanced NSCLC: A Consensus Statement From the International Association for the Study of Lung Cancer

Check for updates

Christian Rolfo, MD, PhD, MBA, Dr.hc.,[a] Philip Mack, PhD,[a]
Giorgio V. Scagliotti, MD, PhD,[b] Charu Aggarwal, MD, MPH,[c] Maria E. Arcila, MD,[d]
Fabrice Barlesi, MD, PhD,[e,f] Trever Bivona, MD, PhD,[g,h,i]
Maximilian Diehn, MD, PhD,[j,k] Caroline Dive, PhD,[l,m] Rafal Dziadziuszko, MD, PhD,[n]
Natasha Leighl, BSc, MMSc, MD,[o] Umberto Malapelle, PhD,[p] Tony Mok, MD,[q]
Nir Peled, MD, PhD,[r] Luis E. Raez, MD,[s] Lecia Sequist, MD, MPH,[t,u,v]
Lynette Sholl, MD,[w] Charles Swanton, BSc, PhD, FRCP,[x,y] Chris Abbosh, MD, PhD,[y]
Daniel Tan, MBBS, PhD,[z,aa] Heather Wakelee, MD,[bb] Ignacio Wistuba, MD,[cc]
Rebecca Bunn, MSc,[dd] Janet Freeman-Daily, MS, ENG,[ee] Murry Wynes, PhD,[cc]
Chandra Belani, MD,[ff] Tetsuya Mitsudomi, MD, PhD,[gg] David Gandara, MD[hh,*]

IASLC

Recommendations: "In patients with oncogene-addicted NSCLC, liquid biopsy is emerging as not only complementary to tissue-based analysis but also acceptable as the initial "plasma first" approach for biomarker evaluation at the time of diagnosis and for monitoring the efficacy of targeted therapies".

Switch to fulvestrant and palbociclib versus no switch in advanced breast cancer with rising *ESR1* mutation during aromatase inhibitor and palbociclib therapy (PADA-1): a randomised, open-label, multicentre, phase 3 trial

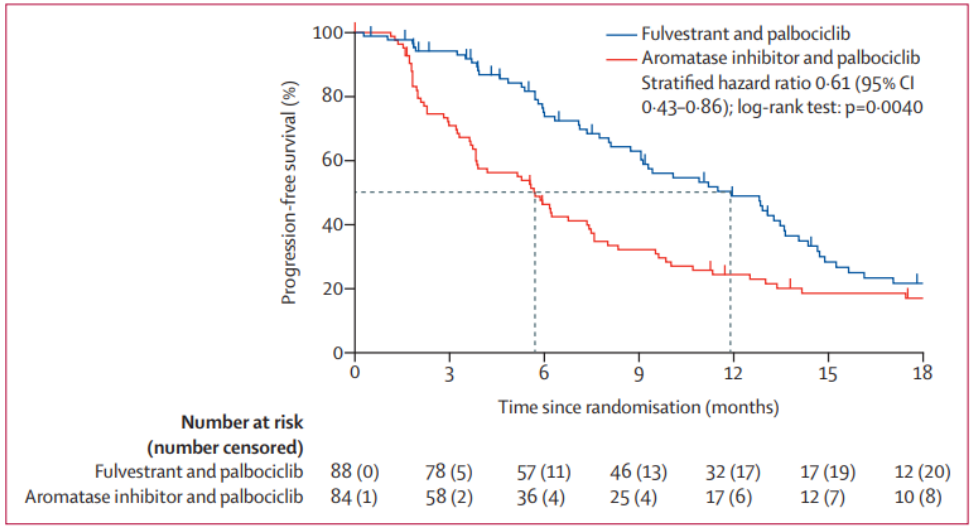
THE LANCET Oncology

François-Clément Bidard, Anne-Claire Hardy-Bessard, Florence Dalenc, Thomas Bachelot, Jean-Yves Pierga, Thibault de la Motte Rouge, Renaud Sabatier, Coraline Dubot, Jean-Sébastien Frenel, Jean Marc Ferrero, Sylvain Ladoire, Christelle Levy, Marie-Ange Mouret-Reynier, Alain Lortholary, Julien Grenier, Camille Chakiba, Laetitia Stefani, Jérôme Edouard Plaza, Florian Clatot, Luis Teixeira, Véronique D'Hondt, Hélène Vegas, Olfa Derbel, Claire Garnier-Tixidre, Jean-Luc Canon, Barbara Pistilli, Fabrice André, Laurent Arnould, Anne Pradines, Ivan Bièche, Céline Callens, Jérôme Lemonnier, Frédérique Berger, Suzette Delaloge, on behalf of the PADA-1 investigators

Summary

Background In advanced oestrogen receptor-positive, HER2-negative breast cancer, acquired resistance to aromatase inhibitors frequently stems from *ESR1*-mutated subclones, which might be sensitive to fulvestrant. The PADA-1 trial aimed to show the efficacy of an early change in therapy on the basis of a rising *ESR1* mutation in blood (b*ESR1*^{mut}), while assessing the global safety of combination fulvestrant and palbociclib.

Lancet Oncol 2022; 23: 1367–77
Published Online September 29, 2022
https://doi.org/10.1016/S1470-2045(22)00555-1



"PADA-1 is the first trial to demonstrate that, in most patients, resistance-associated mutations in the estrogen receptor gene can be detected and targeted before tumor progression through *ESR1* mutation monitoring in blood "

ESMO GOOD SCIENCE BETTER MEDICINE BEST PRACTICE

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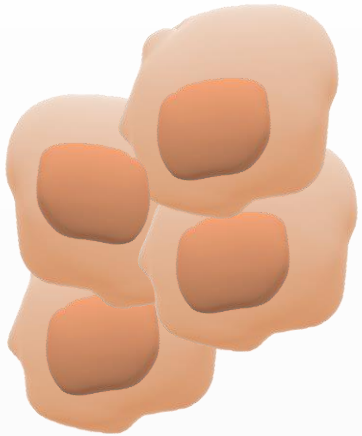
SPECIAL ARTICLE

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

J. Pascual[1], G. Attard[2], F.-C. Bidard[3,4], G. Curigliano[5,6], L. De Mattos-Arruda[7,8], M. Diehn[9], A. Italiano[10,11,12], J. Lindberg[13], J. D. Merker[14], C. Montagut[15], N. Normanno[16], K. Pantel[17], G. Pentheroudakis[18], S. Popat[19,20], J. S. Reis-Filho[21], J. Tie[22,23], J. Seoane[24,25], N. Tarazona[26,27], T. Yoshino[28] & N. C. Turner[19,20*]

Recommendation: ESR1 mutations should preferentially be tested in ctDNA

PRE-ANALYTICAL VARIABLES FOR CTC ANALYSIS



- CTCs constitute a more difficult blood component in respect to ctDNA to handle pre-analytically
- CTCs possess unique characteristics that **reflect the origin of tumors** and make them distinguishable from normal blood cells
- CTCs can artfully be converted from one phenotypic state to another during **epithelial-to-mesenchymal transition (EMT)**
- Moreover, their scarcity in blood, with approximately **1 CTC at 10^7 peripheral blood mononuclear cells (PBMCs)**, often renders them hard to track down as LB components.
- CTCs are, in most types of cancer, **bigger in size** compared to the other blood cells and present **different deformability and electric properties**
- **Standardization** of pre-analytical variables that affect CTC analysis is at its infancy, and guidelines that regulate their processing are **still under investigation**.
- Nevertheless, some of the pre-analytical steps that precede sample processing are also essential for ensuring further CTC analysis. Different factors should be taken into account concerning the type of downstream CTC analysis: CTC counting or CTC molecular characterization on the transcriptomic, genomic, or proteomic level

PRE-ANALYTICAL VARIABLES FOR CTC ANALYSIS

Blood sampling

Discard the first 5 ml of blood draw to avoid contamination of skin epithelial cells, since cytokeratins are used for CTC detection and enumeration

Transportation

time

Transportation and time intervals are also important for the integrity of CTCs

Blood collection tubes

use of specific tubes for CTC staining and counting that contain preservatives, is required because intracellular proteins and cell surface antigens must be preserved for efficient antibody-based or label-independent enrichment and detection by immunofluorescence. However, these are inappropriate for gene expression analysis

or

CTC enrichment

the co-presence of peripheral blood cells significantly affects the sensitivity and specificity of results. The selection of the appropriate CTC enrichment technology is also a crucial step prior to CTC analysis

RECOMMENDATIONS FOR CTC ANALYSIS??

Clinical Chemistry 64:10
1522–1533 (2018)

Evaluation of Preanalytical Conditions and Implementation of Quality Control Steps for Reliable Gene Expression and DNA Methylation Analyses in Liquid Biopsies

Martha Zavridou,[1†] Sofia Mastoraki,[1†] Areti Strati,[1] Eleni Tzanikou,[1] Maria Chimonidou,[1] and Evi Lianidou[1*]

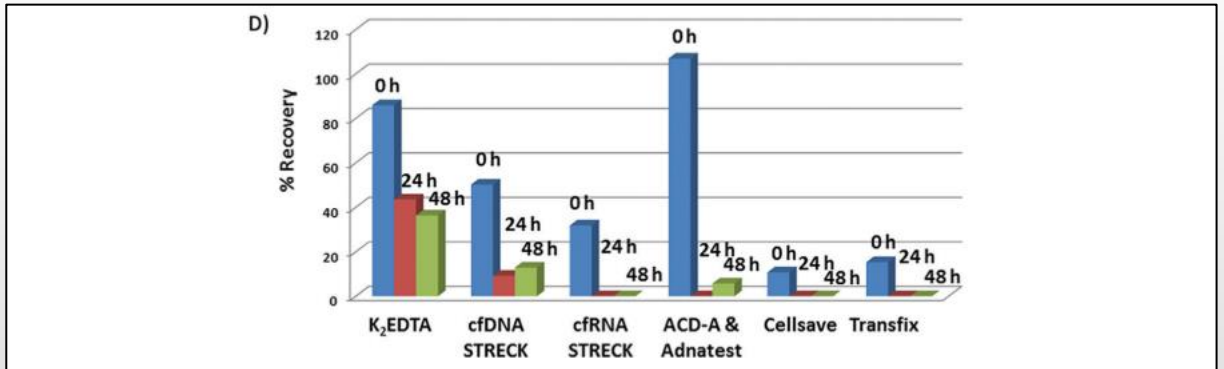


Fig. 4. Evaluation of CTC-RNA stability in 6 different commercially available BCTs at different time points and under different storage conditions.

In all, 100 MCF7 cells were used as recovery control (100%), and results are expressed as Cq values (RT-qPCR). *B2M* (A) and *CK19* (B) RT-qPCR; for *CK19* standard curve (C): Cq plotted vs log (cells/μL), as measured in triplicate, and percentage recovery of *CK19* mRNA transcripts as quantified by RT-qPCR at different time points (D).

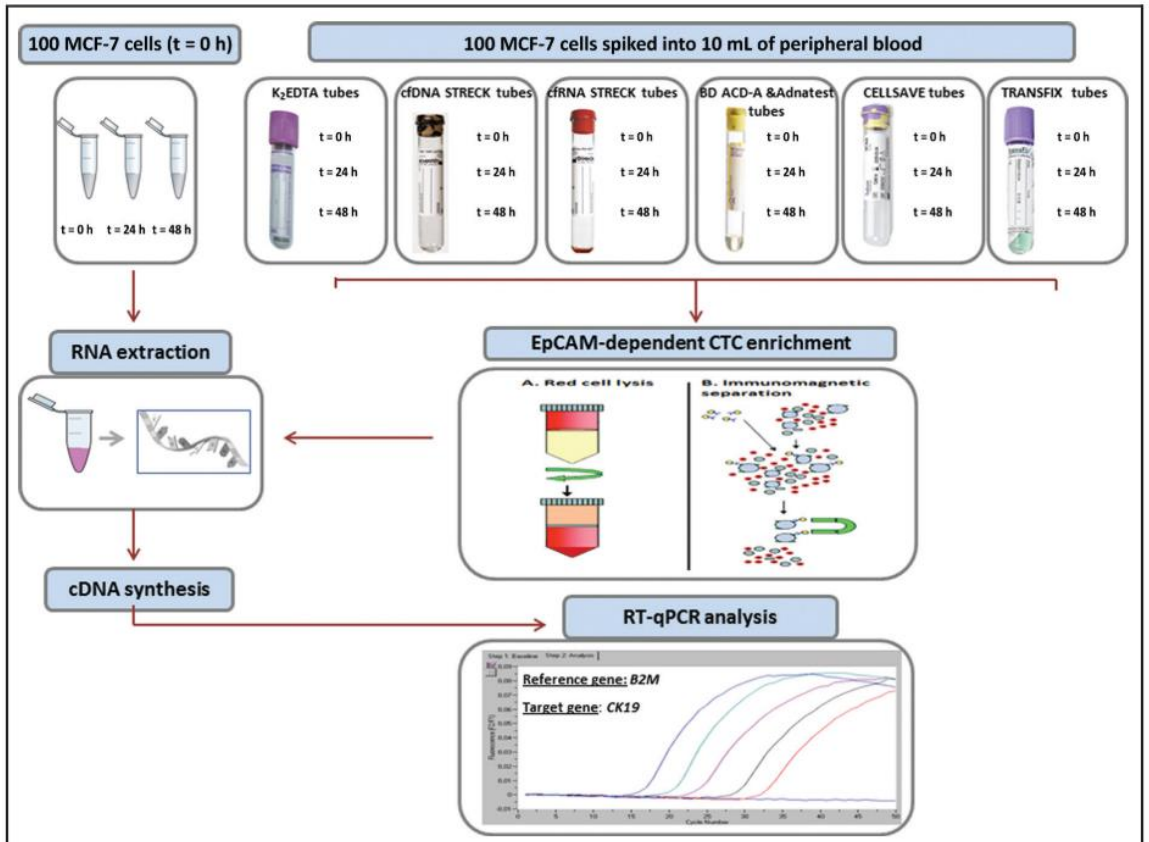


Fig. 3. Evaluation of stability of CTC-mRNA upon collection and storage of PB.

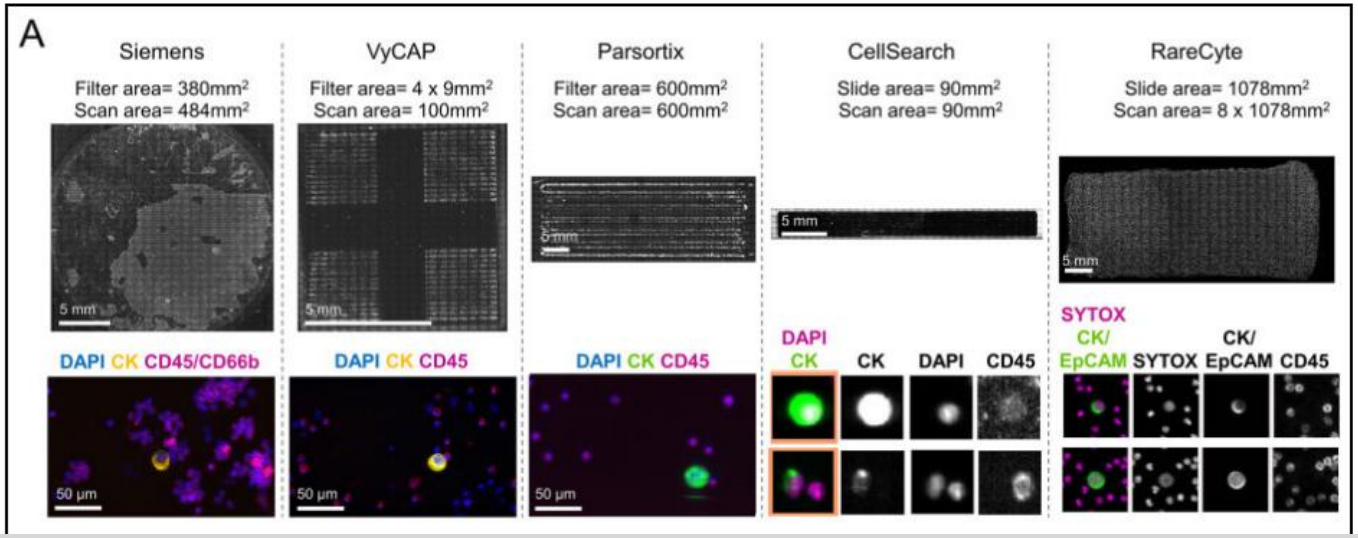
RECOMMENDATIONS FOR CTC ANALYSIS??

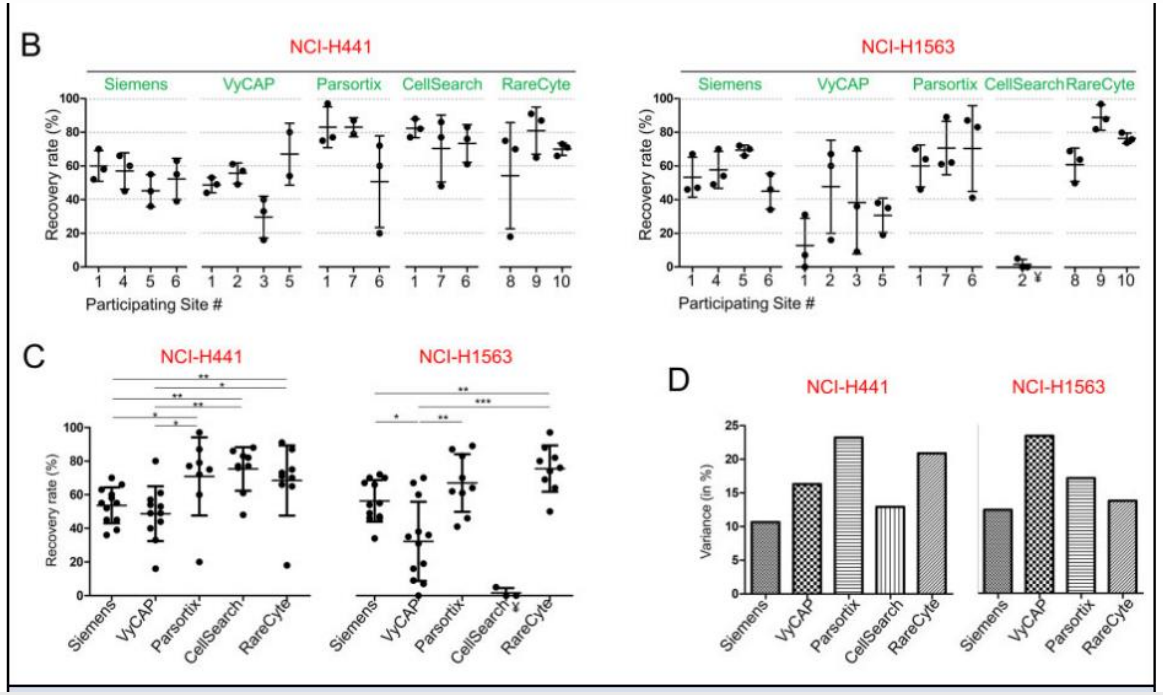
Clinical Chemistry 67:4
631-641 (2021)

Cancer Diagnostics

Proficiency Testing to Assess Technical Performance for CTC-Processing and Detection Methods in CANCER-ID

Rui P.L. Neves,[a,†] Wim Ammerlaan,[b,†] Kiki C. Andree,[c] Sebastian Bender,[d] Laure Cayrefourcq,[e] Christiane Driemel,[a] Claudia Koch,[f] Merlin Verena Luetke-Eversloh,[d] Marianne Oulhen,[g] Elisabetta Rossi,[h,i] Catherine Alix-Panabières,[e] Fay Betsou,[b] Françoise Farace,[g] Sabine Riethdorf,[f] Thomas Schlange,[d] Harriet Wikman,[f] Rita Zamarchi,[i] Klaus Pantel,[f] Leon W.M.M. Terstappen,[c] and Nikolas H. Stoecklein,[a,*] for the CANCER-ID Consortium





RECOMMENDATIONS FOR CTC ANALYSIS??

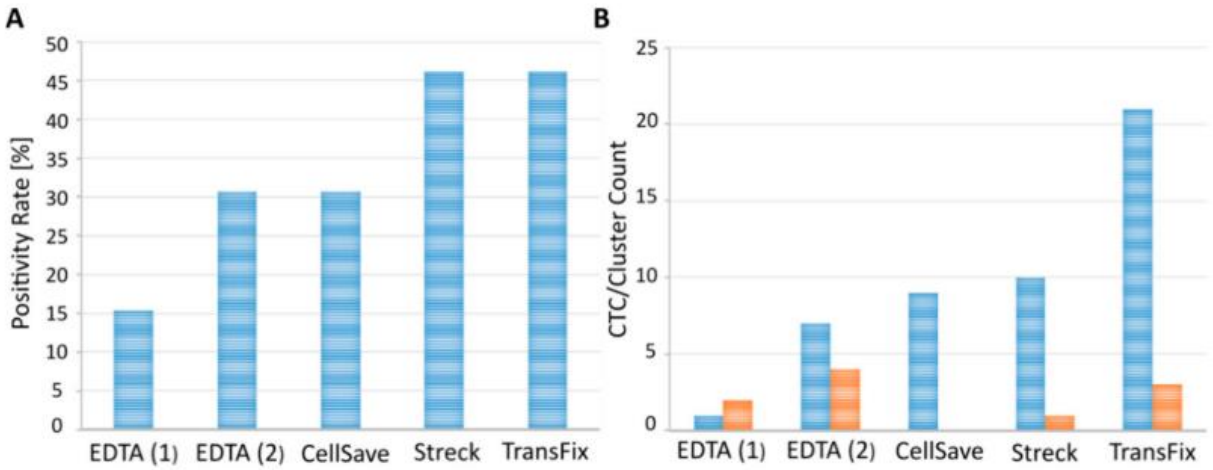
cancers

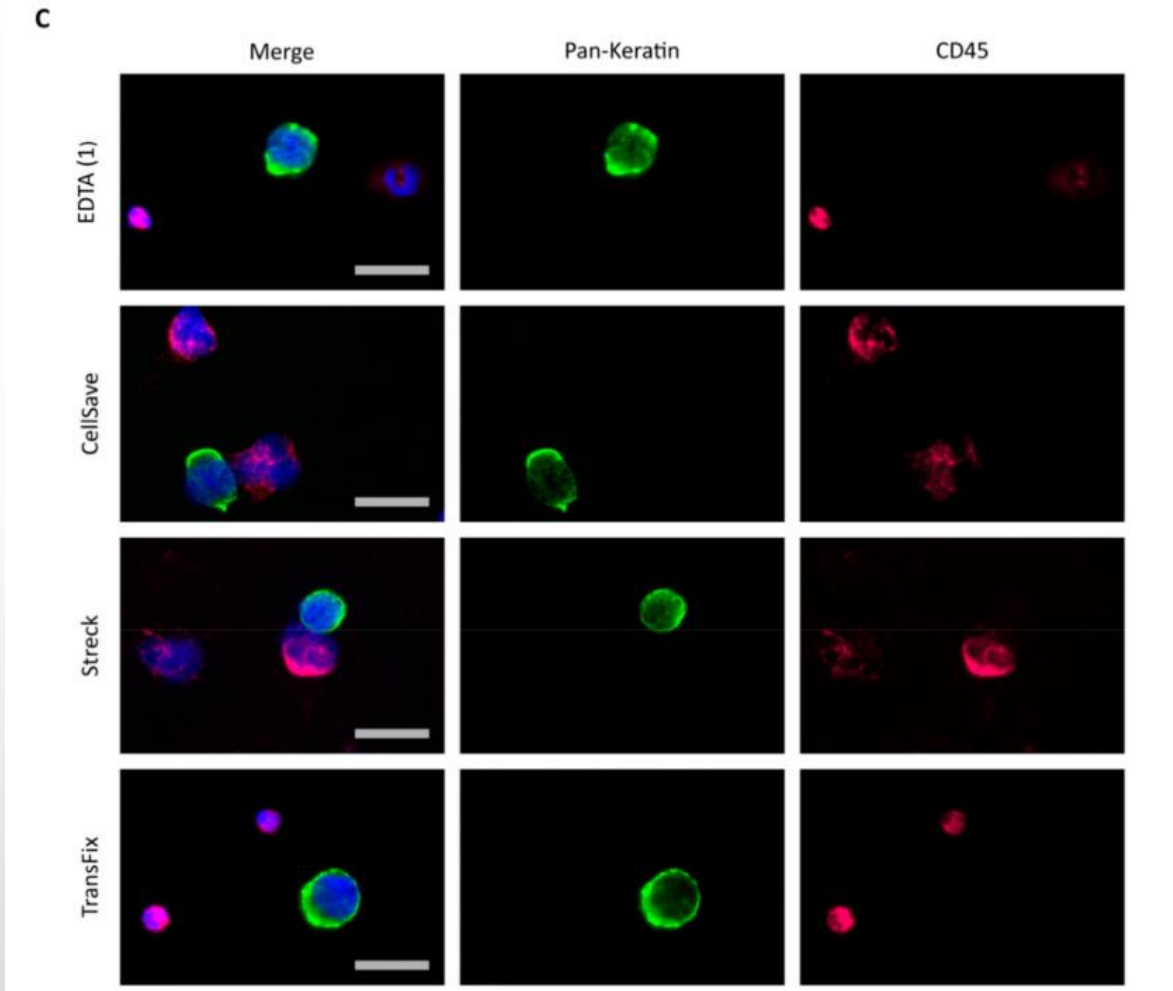
MDPI

Article

Pre-Analytical and Analytical Variables of Label-Independent Enrichment and Automated Detection of Circulating Tumor Cells in Cancer Patients

Claudia Koch [1], Simon A. Joosse [1,†], Svenja Schneegans [1,†], Okka J. W. Wilken [1,†], Melanie Janning [1,2], Desiree Loreth [1], Volkmar Müller [3], Katharina Prieske [3], Malgorzata Banys-Paluchowski [4,5], Ludwig J. Horst [1], Sonja Loges [1,2], Sven Peine [6], Harriet Wikman [1], Tobias M. Gorges [1] and Klaus Pantel [1,*]





EQA SCHEMES

EMQN
The European Molecular Genetics Quality Network

GenQA
GENOMICS QUALITY ASSESSMENT

IQNPath
International Quality Network for Pathology

EQA
European Society of Pathology

DE GRUYTER Clin Chem Lab Med 2017; aop

Verena Haselmann*, Parviz Ahmad-Nejad, Wolf J. Geilenkeuser, Angelika Duda, Merle Gabor, Romy Eichner, Simon Patton and Michael Neumaier*

Results of the first external quality assessment scheme (EQA) for isolation and analysis of circulating tumour DNA (ctDNA)

Keppens *et al. BMC Cancer* (2018) 18:804
https://doi.org/10.1186/s12885-018-4694-x

BMC Cancer

RESEARCH ARTICLE Open Access

International pilot external quality assessment scheme for analysis and reporting of circulating tumour DNA

Cleo Keppens[1,2*], Elisabeth M. C. Dequeker[1,2], Simon J. Patton[3], Nicola Normanno[4], Francesca Fenizia[4], Rachel Butler[5], Melanie Cheetham[3], Jennifer A. Fairley[6], Hannah Williams[6], Jacqueline A. Hall[7,8], Ed Schuuring[2,9], Zandra C. Deans[6] and On behalf of IQN Path ASBL

Virchows Archiv (2019) 474:681–689
https://doi.org/10.1007/s00428-019-02571-3

ORIGINAL ARTICLE

IQN path ASBL report from the first European cfDNA consensus meeting: expert opinion on the minimal requirements for clinical ctDNA testing

Zandra C. Deans[1] · Rachel Butler[2] · Melanie Cheetham[3] · Elisabeth M. C. Dequeker[4,5] · Jennifer A. Fairley[1] · Francesca Fenizia[6] · Jacqueline A. Hall[7] · Cleo Keppens[4] · Nicola Normanno[6] · Ed Schuuring[8] · Simon J. Patton[3]

Fairley *et al. BMC Cancer* (2022) 22:759
https://doi.org/10.1186/s12885-022-09849-x

BMC Cancer

RESEARCH Open Access

Results of a worldwide external quality assessment of cfDNA testing in lung Cancer

Jennifer A. Fairley[1*], Melanie H. Cheetham[2], Simon J. Patton[2], Etienne Rouleau[3], Marc Denis[4], Elisabeth M. C. Dequeker[5], Ed Schuuring[6], Kaat van Casteren[5], Francesca Fenizia[7], Nicola Normanno[8] and Zandra C. Deans[1]

The Journal of Molecular Diagnostics, Vol. 20, No. 4, July 2018

ELSEVIER

the Journal of Molecular Diagnostics
jmd.amjpathol.org

Detection of *EGFR* Variants in Plasma

A Multilaboratory Comparison of a Real-Time PCR EGFR *Mutation Test in Europe*

Cleo Keppens,* John F. Palma,† Partha M. Das,‡ Sidney Scudder,‡ Wei Wen,‡ Nicola Normanno,§ J. Han van Krieken,¶ Alessandra Sacco,§ Francesca Fenizia,§ David Gonzalez de Castro,‖** Selma Hönigschnabl,†† Izidor Kern,‡‡ Fernando Lopez-Rios,§§ Maria D. Lozano,¶¶ Antonio Marchetti,‖‖ Philippe Halfon,*** Ed Schuuring,††† Ulrike Setinek,‡‡‡ Boe Sorensen,§§§ Phillipe Taniere,¶¶¶ Markus Tiemann,‖‖‖ Hana Vosmikova,**** and Elisabeth M.C. Dequeker*

DE GRUYTER Clin Chem Lab M

Letter to the Editor

Aliki Ntzifa, Christos Kroupis[a], Alexander Haliassos[b] and Evi Lianidou[c,*]

A pilot plasma-ctDNA ring trial for the Cobas® EGFR Mutation Test in clinical diagnostic laboratories

Clinical Chemistry 70:5
759–767 (2024)

Cancer Diagnostics

External Quality Assessment on Molecular Tumor Profiling with Circulating Tumor DNA-Based Methodologies Routinely Used in Clinical Pathology within the COIN Consortium

Paul van der Leest,[a,b,†] Pim Rozendal,[a,†] John Hinrichs,[c] Carel J.M. van Noesel,[d] Karen Zwaenepoel,[e] Birgit Deiman,[f,g,h,i] Cornelis J.J. Huijsmans,[j] Ronald van Eijk,[k] Ernst Jan M. Speel,[l] Rick J. van Haastert,[m] Marjolijn J.L. Ligtenberg,[n,o] Ron H.N. van Schaik,[p] Maurice P.H.M. Jansen,[q] Hendrikus J. Dubbink,[r] Wendy W. de Leng,[s] Mathie P.G. Leers,[t] Menno Tamminga,[u] Daan van den Broek,[b] Léon C. van Kempen,[a,e] and Ed Schuuring[a,*]

EQA SCHEMES

- Participation of laboratories in EQA schemes is equally important to the internal control assessment for **testing the good performance of molecular assays** in daily practice or as part of multicentered studies.

- Interlaboratory testing conducted by accredited organizations (ISO17043) compares genotyping results between diverse laboratories that use the same or alternative detection methodologies and aims **to improve quality and strengthen the awareness of any intra-laboratory deficiencies**

- An important aspect that is highlighted through all the above-mentioned inter-laboratory comparisons is the **genotyping inconsistencies observed between laboratories even when they are using exactly the same methodology**.

- **Inter-laboratory differences underline the urgent need for compliance to specific requirements and guidelines regarding the good clinical practice, especially in the field of liquid biopsy where standardization of the pre-analytical variants is significantly important.**

1st EQA SCHEME FOR cfDNA ANALYSIS IN GREECE

DE GRUYTER

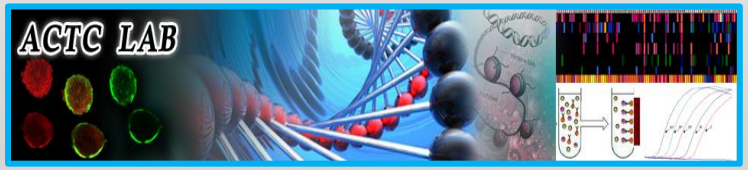
Clin Chem Lab Med 2018; aop

Letter to the Editor

Aliki Ntzifa, Christos Kroupis[a], Alexander Haliassos[b] and Evi Lianidou[c,*]

A pilot plasma-ctDNA ring trial for the Cobas® EGFR Mutation Test in clinical diagnostic laboratories

•**Organized by:**



ΕΣΕΑΠ

❖*ISO17043-accredited External Quality Assessment (EQA) organization*

•**Supported by:**

AstraZeneca

1st EQA SCHEME IN GREECE

➢ **Participation of 8 diagnostic laboratories in Greece**

- 6/8 ISO15189-accredited for EGFR testing in tissues with various techniques
  - 3/6 also in plasma matrix
- Only 1/8 ISO15189-accredited for EGFR testing in plasma ctDNA with Cobas®

➢ **10 different plasma samples:**

- external reference standards
- genetically defined cell-free DNA extracted from human cell lines (400 ng DNA in 2 mL plasma)
- ISO-certified for cfDNA analysis
- purchased from Horizon Discovery, Cambridge, UK

- *EGFR mutations: T790M, E746-A750del,* L858R
- different allelic frequences: 5%, 0.5%, 0.05%, 0% (wild type)
- digital droplet PCR as a reference method

1st EQA SCHEME IN GREECE

➢**Preparation - Distribution**

•Labeling of samples with random numbers (1-100)
•specimen box seal
•shipped on dry ice to each laboratory
•ISO 9001:2015 certified courier for biological samples
•stored at −80 °C until analysis

➢**Within 1 week**

Results: one separate report for each sample, according to the report template they routinely use

➢**Assessment of results**

•Genotyping
•Essential and formal characteristics of result reports (without score marking)

1st EQA SCHEME IN GREECE - RESULTS

Table 1: Plasma *EGFR* mutation results for the eight participating laboratories.[a]

Horizon reference standards	Mutations and percentage in Horizon certified standards	Enrolled laboratories							
		1	2	3	4	5	6	7	8
#1	0.5% T790M	T790M	T790M	T790M	T790M	T790M, **Exon19 Del**	T790M	T790M	T790M
#2	5% T790M	T790M	T790M	**No mutation detected**	T790M	T790M	T790M	T790M	T790M
#3	0.5% L858R	L858R	L858R	L858R	L858R	L858R	**No mutation detected**	L858R	L858R
#4	0.05% E746-A750del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del
#5	0.5% E746-A750del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	**No mutation detected**	Exon19 Del	Exon19 Del
#6	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	**T790M**	Wild type	Wild type
#7	0.5% T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R
#8	5% T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R
#9	0.5% T790M, E746-A750del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del
#10	5% T790M, E746-A750del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del

[a]Cobas® EGFR Mutation Test v2 offers only qualitative genotyping result and also does not refer to the exact nature of exon19 deletion (e.g. e746-750). In bold are the wrong results, that were not expected since these were not the correct answers according to the standards provided.

Ntzifa et al, Clin Chem Lab Med, 2018

1st EQA SCHEME IN GREECE - RESULTS

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#2	5% T790M	T790M	T790M	**No mutation detected**	T790M	T790M	T790M	T790M	T790M
#3	0.5% L858R	L858R	L858R	L858R	L858R	L858R	**No mutation detected**	L858R	L858R
#4	0.05% E746-A750del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del
#5	0.5% E746-A750del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	Exon19 Del	**No mutation detected**	Exon19 Del	Exon19 Del
#6	Wild type	Wild type	Wild type	Wild type	Wild type	Wild type	**T790M**	Wild type	Wild type
#7	0.5% T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R
#8	5% T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R	T790M, L858R
#9	0.5% T790M, E746-A750del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del
#10	5% T790M, E746-A750del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del	T790M, Exon19 Del

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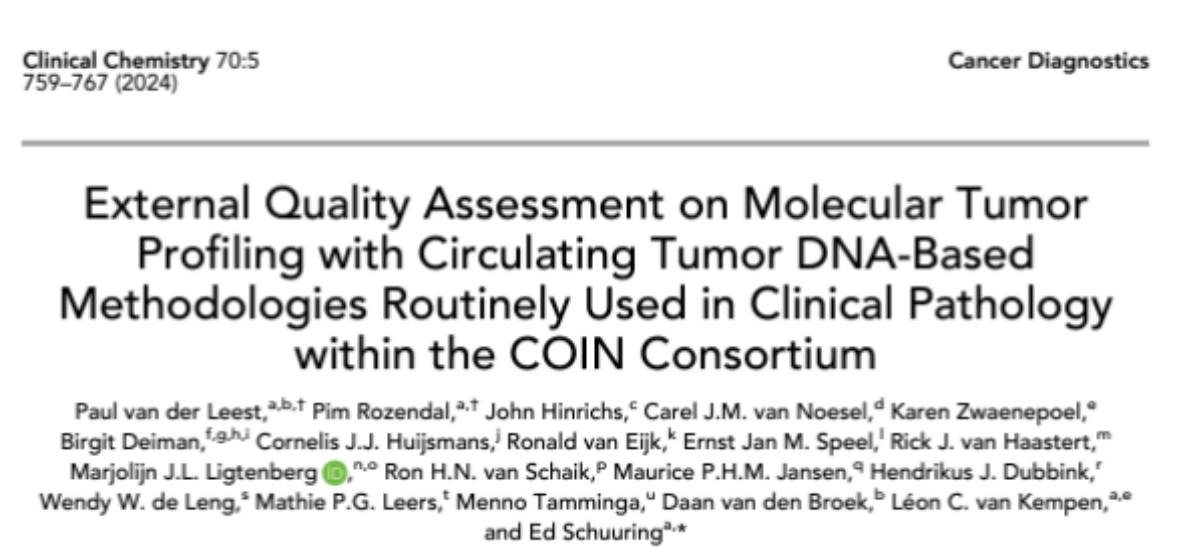
Ntzifa et al, Clin Chem Lab Med, 2018

EQA- Dutch COIN consortium



Clinical Chemistry 70:5
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Cancer Diagnostics

External Quality Assessment on Molecular Tumor Profiling with Circulating Tumor DNA-Based Methodologies Routinely Used in Clinical Pathology within the COIN Consortium

Paul van der Leest,[a,b,†] Pim Rozendal,[a,†] John Hinrichs,[c] Carel J.M. van Noesel,[d] Karen Zwaenepoel,[e] Birgit Deiman,[f,g,h,i] Cornelis J.J. Huijsmans,[j] Ronald van Eijk,[k] Ernst Jan M. Speel,[l] Rick J. van Haastert,[m] Marjolijn J.L. Ligtenberg,[n,o] Ron H.N. van Schaik,[p] Maurice P.H.M. Jansen,[q] Hendrikus J. Dubbink,[r] Wendy W. de Leng,[s] Mathie P.G. Leers,[t] Menno Tamminga,[u] Daan van den Broek,[b] Léon C. van Kempen,[a,e] and Ed Schuuring[a,*]

Dutch COIN consortium (ctDNA on the road to implementation in The Netherlands)

- ✓ Aliquots of 3 high-volume diagnostic leukapheresis (DLA) plasma samples
- ✓ 3 artificial reference plasma samples with predetermined mutations
- ✓ 16 Dutch laboratories.
- ✓ ctDNA analysis for BRAF exon 15, EGFR exon 18–21, and KRAS exon 2–3 using their regular circulating cell-free DNA (ccfDNA) analysis work flow.
- ✓ Laboratories were assessed based on adherence to the study protocol, overall detection rate, and overall genotyping performance.

EQA- Dutch COIN consortium

Table 2. Laboratory preanalytical conditions.

Lab	Plasma volume, mL	Elution volume, µL	Extraction method[a]	ccfDNA quantification method	Remarks
1	4	100	CNA	None	None
2	4	40	CNA	Qubit	None
3	4 (2 × 2)	120 (2 × 60)	DSP	Qubit	None
4	3	80	ME	None	None
5	4 (2 × 2)	200 (2 × 100)	COB	Qubit	None
6	4 (2 × 2)	160 (2 × 80)	COB	None	None
7	3	50	CNA	None	None
8	4	75	RSC	Nanodrop	None
9	4 (2 × 2)	200 (2 × 100)	COB	None	None
10	4	55	Custom[b]	ddPCR	None
11	2	52	CNA	Qubit	None
12	4	60	AVE	TapeStation	Yes[c]
13	4	25	MMA	Qubit	None
14	3	50	CNA	Qubit	None
15	4	50	CNA	Qubit	None
16	4	70	RSC	Qubit	None

[a]Abbreviations: CNA, QIAamp Circulating Nucleic Acid Kit; DSP, QIAsymphony DSP Circulating DNA Kit; ME, QIAamp MinElute ccfDNA Mini Kit; COB, Cobas ccfDNA Sample Preparation Kit; RSC, Maxwell RSC LV ccfDNA Kit; AVE, AVENIO cfDNA Isolation Kit; MMA, MagMAX Cell-Free Total Nucleic Acid Kit.
[b]Applied a modified protocol (see Supplemental Appendix 1).
[c]Plasma Samples 4 to 6 were nearly thawed upon arrival at the site.

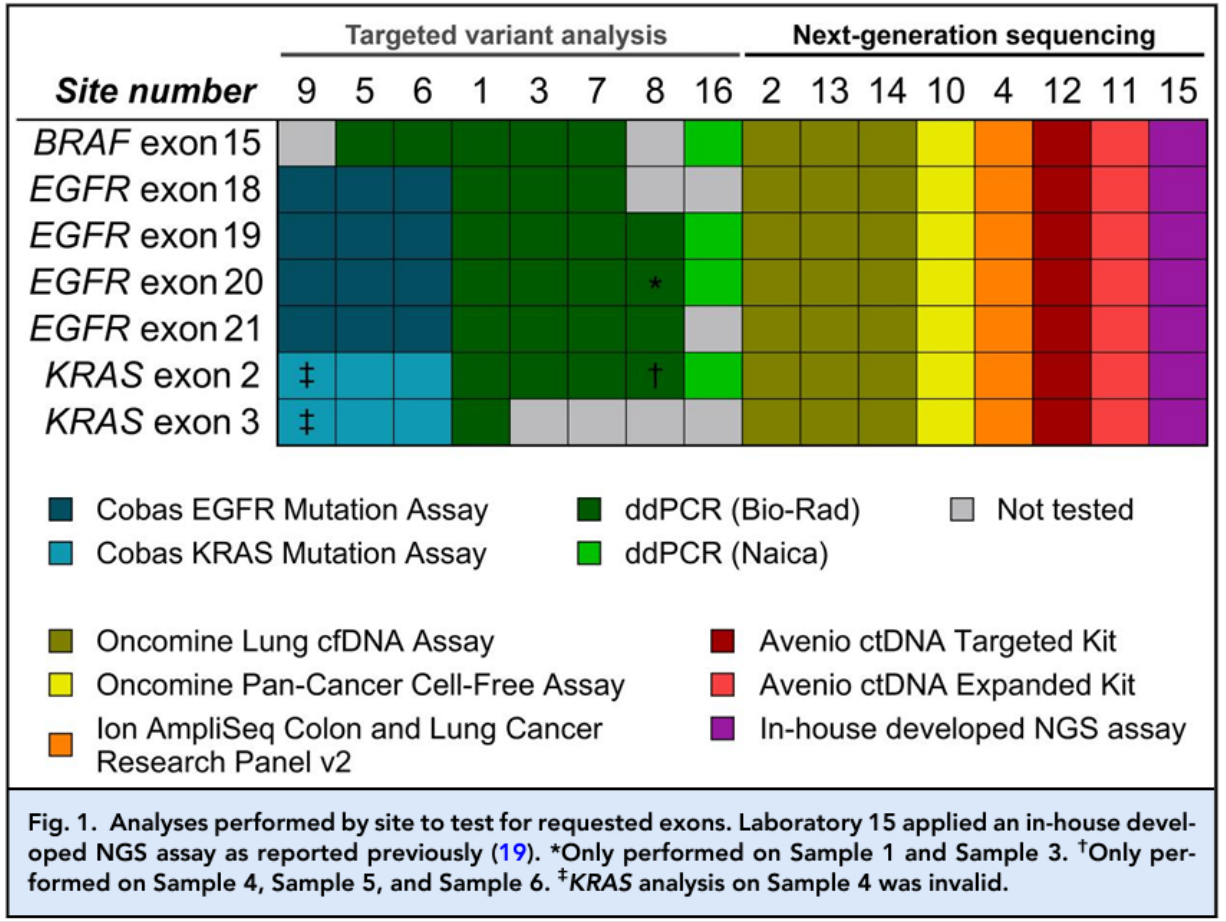
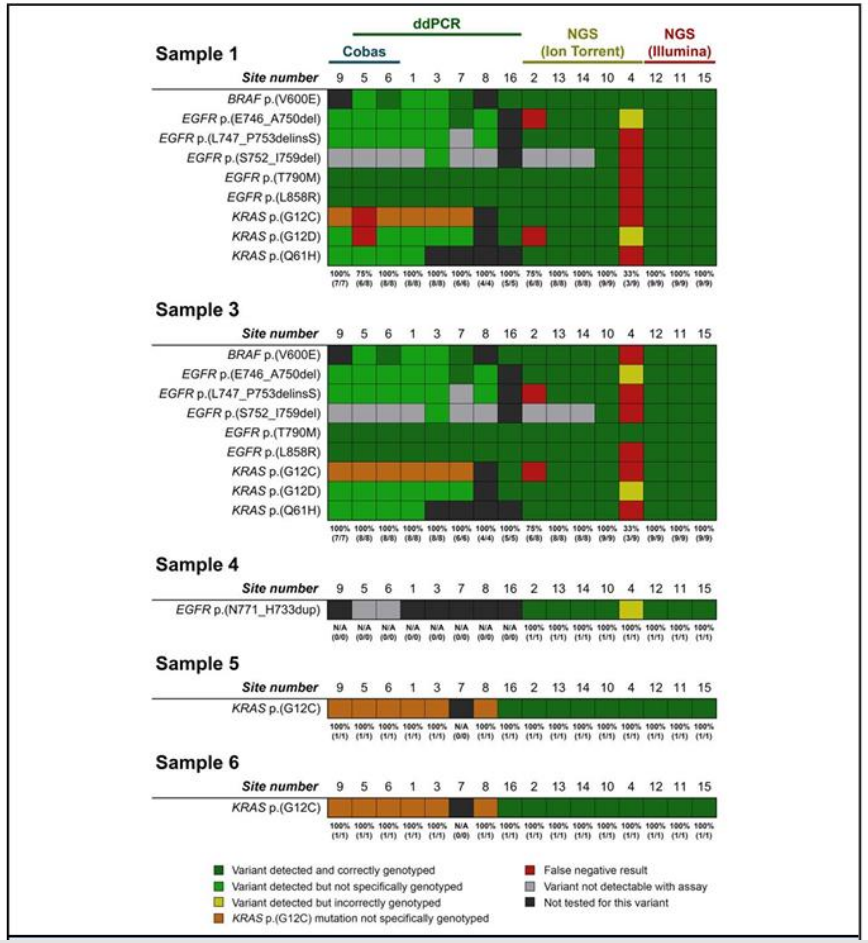
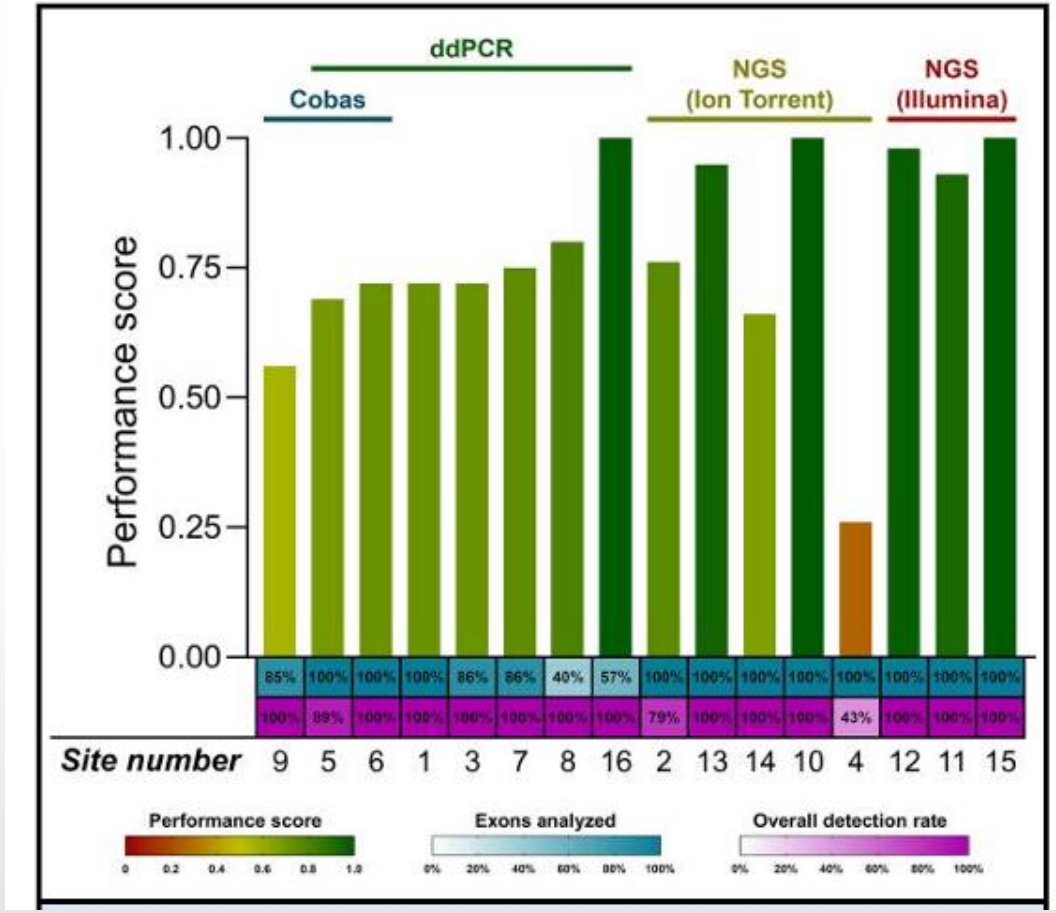


Fig. 1. Analyses performed by site to test for requested exons. Laboratory 15 applied an in-house developed NGS assay as reported previously (19). *Only performed on Sample 1 and Sample 3. †Only performed on Sample 4, Sample 5, and Sample 6. ‡*KRAS* analysis on Sample 4 was invalid.

van der Leest P et al. Clin Chem. 2024 May 2;70(5):759-767

EQA- Dutch COIN consortium





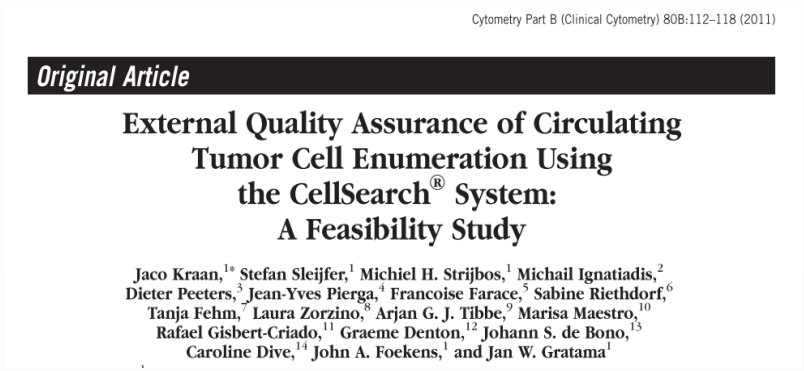
- ✓ **Most laboratories were unable to appropriately characterize mutations of clinical significance (KRAS p.(G12C) and EGFR p.(N771_H773dup)**
- ✓ **Sample 1 contained variants with <0.5% and were detected by all labs except for Lab 4**

❖ **divergent (pre)analytical protocols could lead to discrepant clinical outcomes when using the same plasma samples**

van der Leest P et al. Clin Chem. 2024 May 2;70(5):759-767

EQA SCHEMES

- ***To date, official bodies certified according to ISO17043 standard for organizing EQA schemes for CTC analysis do not exist***

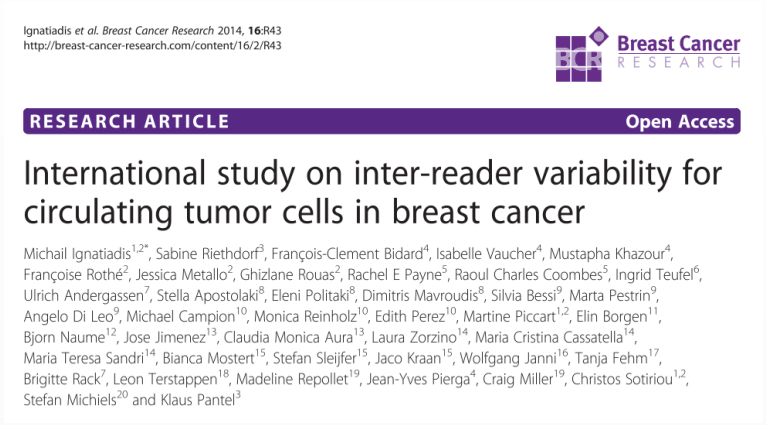


Cytometry Part B (Clinical Cytometry) 80B:112–118 (2011)

Original Article

External Quality Assurance of Circulating Tumor Cell Enumeration Using the CellSearch® System: A Feasibility Study

Jaco Kraan,[1*] Stefan Sleijfer,[1] Michiel H. Strijbos,[1] Michail Ignatiadis,[2] Dieter Peeters,[3] Jean-Yves Pierga,[4] Francoise Farace,[5] Sabine Riethdorf,[6] Tanja Fehm,[7] Laura Zorzino,[8] Arjan G. J. Tibbe,[9] Marisa Maestro,[10] Rafael Gisbert-Criado,[11] Graeme Denton,[12] Johann S. de Bono,[13] Caroline Dive,[14] John A. Foekens,[1] and Jan W. Gratama[1]



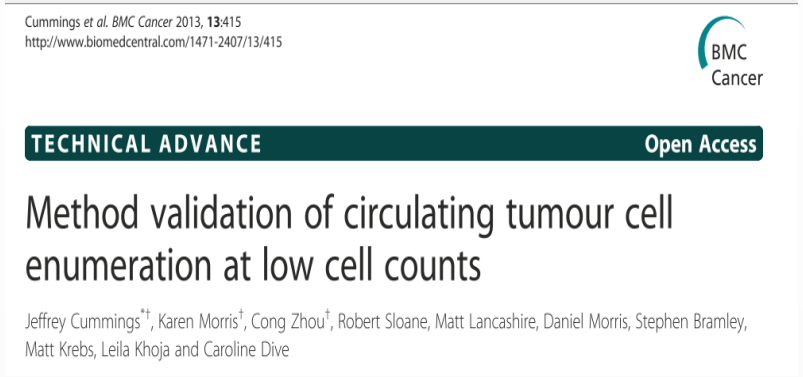
Ignatiadis *et al. Breast Cancer Research* 2014, **16**:R43
http://breast-cancer-research.com/content/16/2/R43

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

International study on inter-reader variability for circulating tumor cells in breast cancer

Michail Ignatiadis[1,2*], Sabine Riethdorf[3], François-Clement Bidard[4], Isabelle Vaucher[4], Mustapha Khazour[4], Françoise Rothé[2], Jessica Metallo[2], Ghizlane Rouas[2], Rachel E Payne[5], Raoul Charles Coombes[5], Ingrid Teufel[6], Ulrich Andergassen[7], Stella Apostolaki[8], Eleni Politaki[8], Dimitris Mavroudis[8], Silvia Bessi[9], Marta Pestrin[9], Angelo Di Leo[9], Michael Campion[10], Monica Reinholz[10], Edith Perez[10], Martine Piccart[1,2], Elin Borgen[11], Bjorn Naume[12], Jose Jimenez[13], Claudia Monica Aura[13], Laura Zorzino[14], Maria Cristina Cassatella[14], Maria Teresa Sandri[14], Bianca Mostert[15], Stefan Sleijfer[15], Jaco Kraan[15], Wolfgang Janni[16], Tanja Fehm[17], Brigitte Rack[7], Leon Terstappen[18], Madeline Repollet[19], Jean-Yves Pierga[4], Craig Miller[19], Christos Sotiriou[1,2], Stefan Michiels[20] and Klaus Pantel[3]



Cummings *et al. BMC Cancer* 2013, **13**:415
http://www.biomedcentral.com/1471-2407/13/415

BMC Cancer

TECHNICAL ADVANCE Open Access

Method validation of circulating tumour cell enumeration at low cell counts

Jeffrey Cummings[*†], Karen Morris[†], Cong Zhou[†], Robert Sloane, Matt Lancashire, Daniel Morris, Stephen Bramley, Matt Krebs, Leila Khoja and Caroline Dive

Variations were observed between laboratories, between instruments and between operators

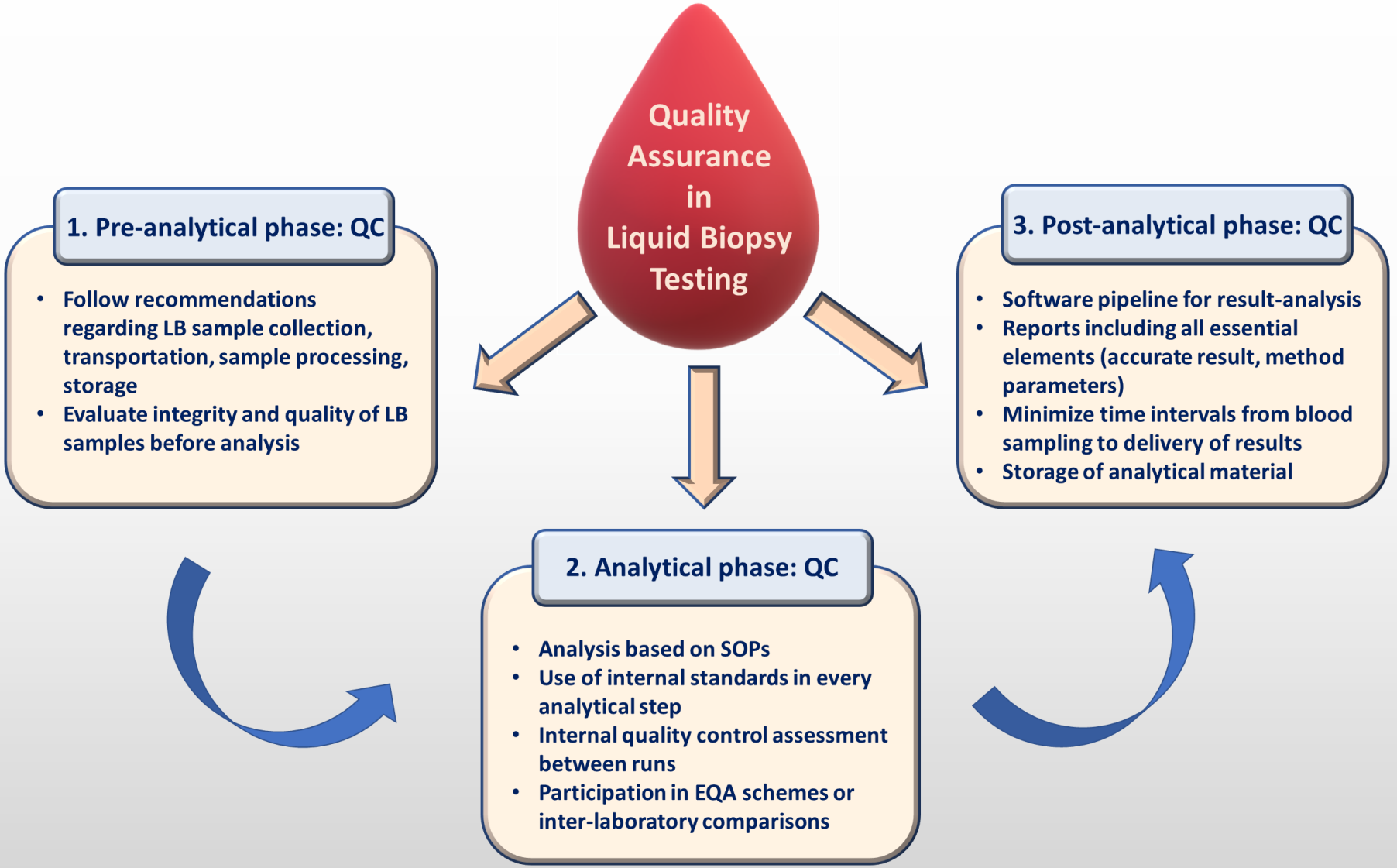
The main contributor to these inter-laboratory variations was the variability among reviewers of CellSearch images

Lower agreement was observed in the non-metastatic setting of M0 breast cancer patients in contrast to the metastatic setting

The lower number of CTCs or the presence of granular CTCs due to administration of therapy were two of the major contributors to inter-reader disagreement

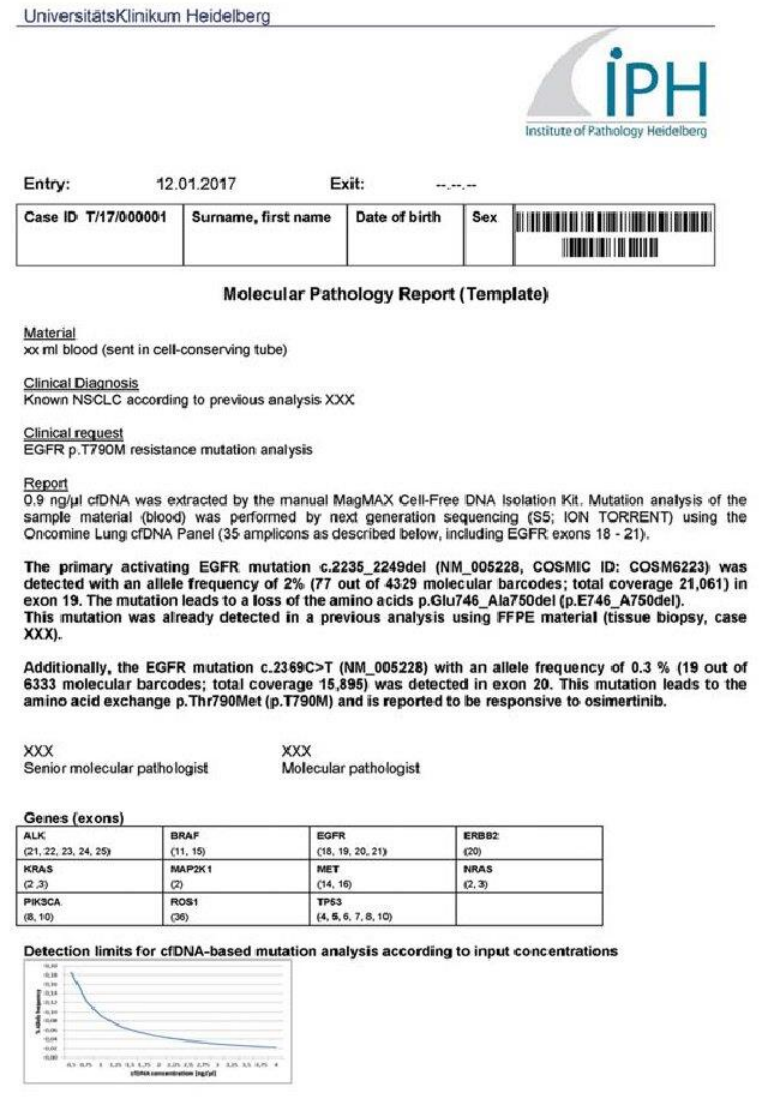
The pivotal role of standardization of image interpretation through regular training of the readers and through well-designed external quality schemes

QUALITY CONTROL ISSUES IN LIQUID BIOPSY TESTING



Ntzifa A. & Lianidou E, Crit Rev Clin Lab Sci, 2023

REPORTING OF RESULTS



UniversitätsKlinikum Heidelberg

IPH
Institute of Pathology Heidelberg

Entry: 12.01.2017 Exit: --.--.--

Case ID T/17/000001	Surname, first name	Date of birth	Sex

Molecular Pathology Report (Template)

Material
xx ml blood (sent in cell-conserving tube)

Clinical Diagnosis
Known NSCLC according to previous analysis XXX

Clinical request
EGFR p.T790M resistance mutation analysis

Report
0.9 ng/µl cfDNA was extracted by the manual MagMAX Cell-Free DNA Isolation Kit. Mutation analysis of the sample material (blood) was performed by next generation sequencing (S5; ION TORRENT) using the Oncomine Lung cfDNA Panel (35 amplicons as described below, including EGFR exons 18 - 21).

The primary activating EGFR mutation c.2235_2249del (NM_005228, COSMIC ID: COSM6223) was detected with an allele frequency of 2% (77 out of 4329 molecular barcodes; total coverage 21,061) in exon 19. The mutation leads to a loss of the amino acids p.Glu746_Ala750del (p.E746_A750del). This mutation was already detected in a previous analysis using FFPE material (tissue biopsy, case XXX).

Additionally, the EGFR mutation c.2369C>T (NM_005228) with an allele frequency of 0.3 % (19 out of 6333 molecular barcodes; total coverage 15,895) was detected in exon 20. This mutation leads to the amino acid exchange p.Thr790Met (p.T790M) and is reported to be responsive to osimertinib.

XXX
Senior molecular pathologist

XXX
Molecular pathologist

Genes (exons)

ALK (21, 22, 23, 24, 25)	BRAF (11, 15)	EGFR (18, 19, 20, 21)	ERBB2 (20)
KRAS (2,3)	MAP2K1 (2)	MET (14, 16)	NRAS (2, 3)
PIK3CA (8, 10)	ROS1 (36)	TP53 (4, 5, 6, 7, 8, 10)	

Detection limits for cfDNA-based mutation analysis according to input concentrations

- According to the ISO15189, the results shall be reported "**accurately, clearly, unambiguously, in accordance with any specific instruction**", sample quality/ suitability/adequacy shall be commented, and interpretive comments shall be included
- All reported recommendations suggest the use of "**mutation detected/not detected**" instead of "positive/ negative" result
- **False-positive results** could be attributed to the presence of clonal hematopoiesis or concurrent germline mutations. Therefore, it is advisable to **report the variants detected by the assay and underline genes that are commonly implicated in CHIP**
- **False-negative results** might be subjected to **pre-analytical variables** that could lead to insufficient ctDNA input or to the lower analytical sensitivity of the methodology used.
- **It is essential to include these quality control metrics regarding DNA yield or DNA quality and LOD of the assay to avoid over-interpretation of results.**

Volckmar AL et alGenes Chromosomes Cancer. 2018 Mar;57(3):123-139.

ISO15189 STANDARD-ACCREDITATION

The **aim of ISO15189 standard** is

- to specify the requirements about the total testing procedure including pre-analytical, analytical and post-analytical phase,
- to give recommendations and
- to offer guidance to laboratories for improving their quality system.

The **two main components** of the ISO15189 standard are
a) the management and
b) the technical requirements,
the fulfilment of which ensures the generation of technically valid results.

- Laboratories are asked to develop and define **their own quality management system that meets ISO15189 requirements** adapted to their total testing process thus aiming to the **quality of services**. Undoubtedly, accredited laboratories often reach more optimal results in contrast to the labs that don't work under standardized procedures

- All the quality metrics mentioned above, including pre-analytical variables, quality control issues, regular participation to EQA schemes or conformity to recommendations about reporting and interpretation of results are well-defined in the requirements of the ISO15189 standard.

ISO15189 STANDARD-ACCREDITATION

- ***Accreditation*** *is a procedure by which an authoritative body gives formal recognition that a diagnostic laboratory is competent to carry out specific analyses and deliver valid and reliable results*

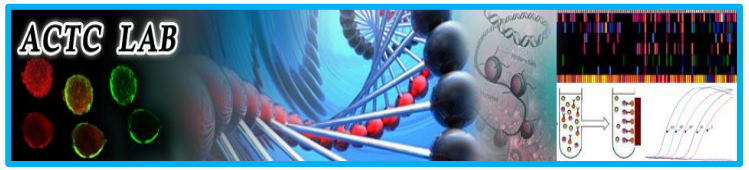
- Accreditation is an effective way to demonstrate competence of the laboratory, a tool to recognize laboratories world-wide, is linked to periodical audits, to stimulate the maintenance and improvement of the quality, which leads to high standard of services for clients (patients, health care providers, etc.)

- Accreditation offers the laboratories **opportunities for continuous improvement**. This constant effort to remain attentive and cautious against the possible errors that may occur renders laboratories to be more preventive and reliable **ensuring quality of patient care**.

ISO15189 STANDARD-ACCREDITATION

- In liquid biopsy, **harmonization to standardized procedures** can be achieved **through compliance to ISO15189 standard and laboratory accreditation.**
- ISO15189 certification to laboratories that offer liquid biopsy testing will **upgrade the quality systems** of the labs by enhancing the competence.
- ISO certification constitutes **a prerequisite for reimbursement** of liquid biopsy tests. One of the main activities of European Liquid Biopsy Society (ELBS) is to encourage and support laboratories to fulfil ISO15189 requirements regarding liquid biopsy testing (https://www.uke.de/english/departments-institutes/institutes/tumor-biology/european-liquid-biopsy-society-elbs/index.html).
- The **recent in vitro diagnostics regulation (IVDR)** included an important requirement regarding the use of laboratory-developed tests (LDT) that are limited **only to the laboratories compliant to the ISO15189 standard** to guarantee a proper validation of such tests

ACCREDITATION IN LIQUID BIOPSY ANALYSIS



Κλινικές Δοκιμές
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ISO15189 certified for:

- ***EGFR*** **mutations in ctDNA in COBAS, ROCHE (FDA cleared assay)**

- **CTCs enumeration using the CellSearch system (FDA cleared) for metastatic:**
  - **Breast cancer**
  - **Colorectal cancer**
  - **Prostate cancer**

- ***PD-L1*** **mRNA expression in CTCs –Oncolipsy kit (Pharmassist)**

INTERNATIONAL LIQUID BIOPSY ASSOCIATION (ILSA)

Several organizations and committees worldwide are working towards the implementation of liquid biopsy in clinical practice covering various aspects of this multifaceted procedure. An important effort is guided by the International Liquid Biopsy Standardization Alliance (ILSA) that gathered organizations and foundations to systematically working towards the global use of liquid biopsy in oncology practice.

CONCLUSIONS

- Liquid biopsy is a valuable tool for real-time monitoring of cancer patients during therapy, for treatment selection, and cancer diagnosis and prognosis
- To date, there are guidelines and recommendations mainly for ctDNA testing in solid cancers
- There are still technical challenges to overcome to achieve standardization of liquid biopsy testing and implementation to clinical practice
- Pre-analytical considerations and quality control issues are important steps to consider for the implementation of liquid biopsy assays
- Accreditation of laboratories according to ISO15189 standard is the best way to reassure valid and reliable results in favor of cancer patients

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Pre-analytical conditions and implementation of quality control steps in liquid biopsy analysis

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ANY
QUESTIONS?