Preanalytical Considerations about Circulating Tumor DNA

Specimen Improvements, Standards & Technologies

LIQUID BIOPSY SUMMIT

20 – 21 February 2020 Lisbon, Portugal

Dr. Daniel Grölz

Agenda

- Need for pre-analytical workflow standardization to reduce diagnostic errors
- Pre-analytical factors that influence the outcome of ccfDNA analysis
- International initiatives and requirements to standardize pre-analytical workflows
- Liquid biopsy preservation and workflow solutions

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Demand for Improvements and Workflow Standardization

- 150,000 papers documenting thousands of claimed biomarkers, but fewer than 100 have been validated for routine clinical practice

  Bring on the biomarkers, George Poste, Nature 2011

- Diagnostic errors cause about 10% of all patient deaths and about 17% of adverse events

  Institute of Medicine (IOM) Report Sept. 2015

- The pre-analytical phase accounts for 46% to 68% of errors observed during the total testing process

  Medical Laboratory Observer, May 2014

- Unnecessary expenditure caused by pre-analytical errors in a typical U.S. hospital (~ 650 beds) of ~ $1.2 million per year

  Green SF. Clin Biochem. 2013

- Irreproducible preclinical research exceeds 50%, US $28B / year spent on preclinical research that is not reproducible - in the US

  Freedman LP, Cockburn IM, Simcoe TS (2015) PLoS Biol 13(6): e1002165.doi:10.1371/journal.pbio.1002165

An Analytical Test Result is the Result of an Entire Workflow

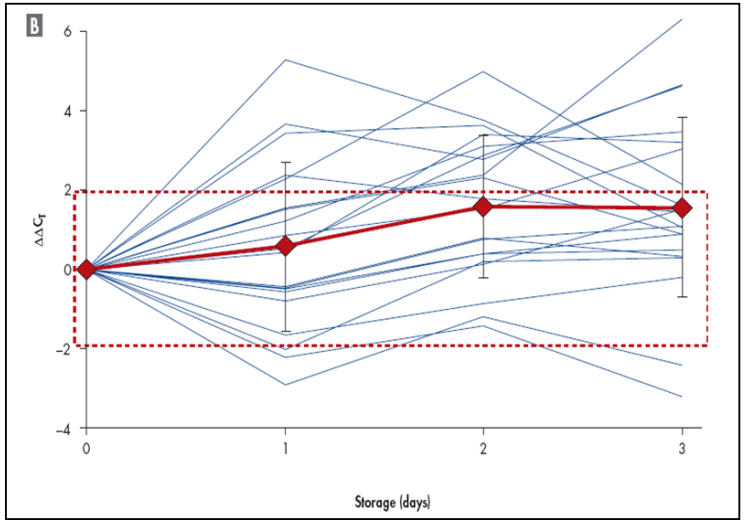
Specifying, developing and verifying preanalytical workflows has to be part of the analytical test development

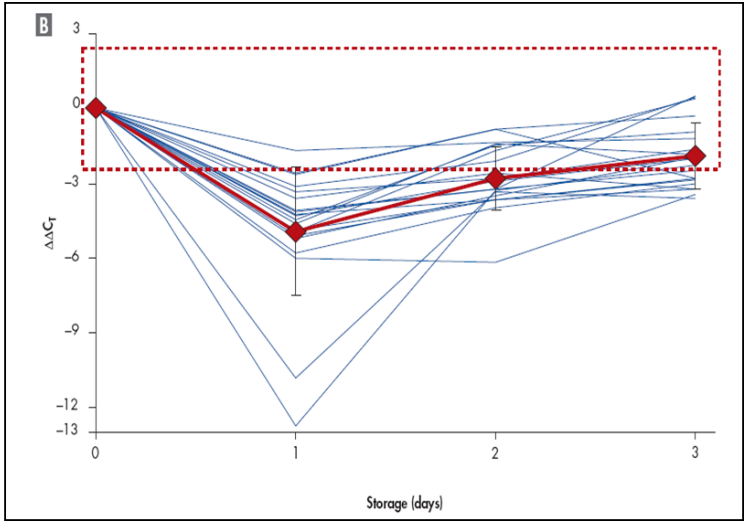
European Conference. Standards: Your Innovation Bridge. Brussels (2014). SPIDIA Booth.

Provided by courtesy of SPIDIA

Blood RNA Profiling - Challenges with Individual Sample Kinetics

Transcription induction and degradation in human EDTA Blood stored at Room Temperature





IL-1b mRNA

Guenther K. et al. AMP Poster (2005)

c-fos mRNA

Guenther K. et al. CLI 5, 26-28 (2008)

Provided by courtesy of D. Groelz, PreAnalytiX

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Pre-analytical Factors that Influence the Outcome of ccfDNA Analysis

Preanalytical step affecting ccfDNA	Challenge	Recommendation
Blood collection tube	Release of genomic DNA from leukocytes; PCR inhibition	Use of a dedicated ccfDNA stabilization tube Use of EDTA tube, in case no dedicated ccfDNA stabilization tube is available
Time between blood collection and plasma processing	Release of genomic DNA from leukocytes	Must be validated in combination with downstream application In case of unstabilized EDTA blood within 2 to 6 hours
Plasma or serum	Release of genomic DNA from leukocytes	Use of plasma
Plasma processing protocol	Incomplete separation of cellular fraction Mechanical lysis of blood cells	For EDTA blood use double centrifugation protocol with low and high speed centrifugation Follow recommendations of ccfDNA tube manufacturer
Plasma storage	Reduction of yield Increased fragmentation	Do not store plasma at 4°C for longer than 24 hours Freeze at -20°C or -80°C Avoid repeated freezing/ thawing cycles
DNA purification method	Suboptimal compatibility with blood collection tube Low yield	Validation of DNA purification method Follow recommendations of ccfDNA tube manufacturer
DNA quantification	Over-quantification due to absorption of impurities in spectrophotometry	Validation of QC-methods; use of qPCR based methods
DNA storage	Reduction of yield Increased fragmentation	Store ccfDNA at -20°C or below Avoid repeated freezing and thawing

Grölz et al. Curr Pathobiol Rep. 2018;6(4):275-286

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DNA	Low yield	Follow recommendations of ccfDNA tube manufacturer
DNA quantification	Over-quantification due to absorption of impurities in spectrophotometry	Validation of QC-methods; use of qPCR based methods
DNA storage	Reduction of yield Increased fragmentation	Store ccfDNA at -20°C or below Avoid repeated freezing and thawing

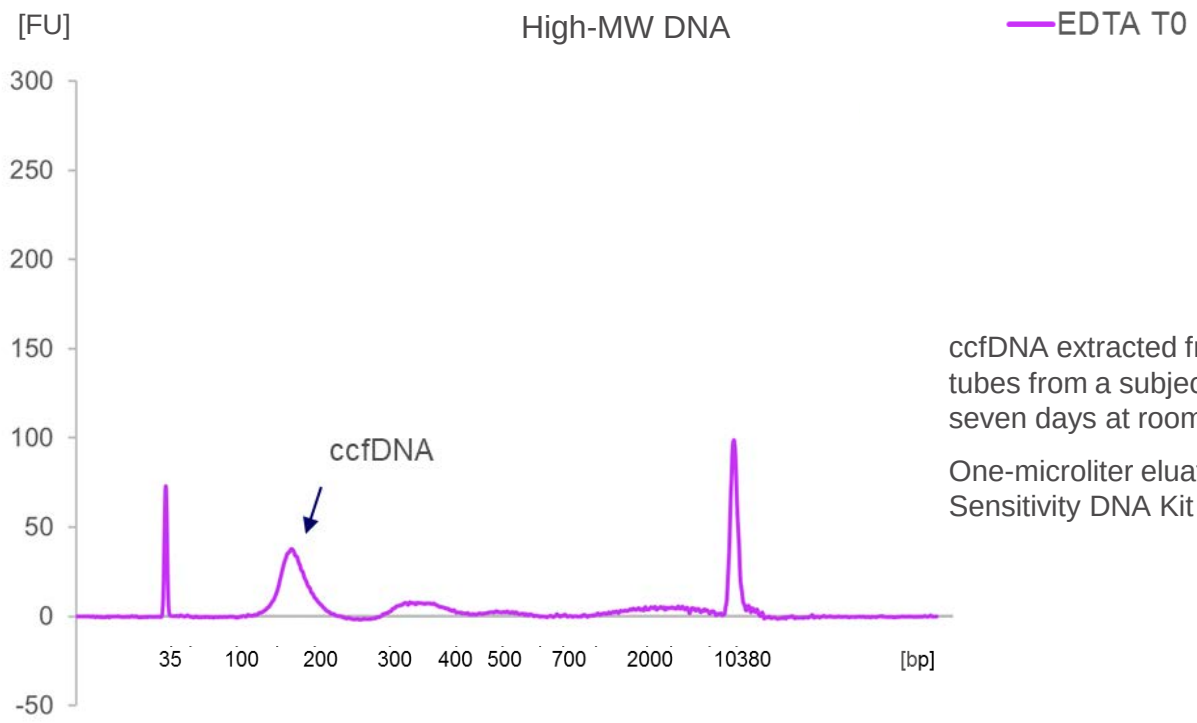
„the entire workflow, including specimen/sample storage and transport conditions, and its impact on the stability of biomolecules intended to be examined shall be verified and validated“

ISO 20186-3:2019
Molecular in vitro diagnostic examinations — Specifications for pre- examination processes for venous whole blood — Part 3: Isolated circulating cell free DNA from plasma.

Grölz et al. Curr Pathobiol Rep. 2018;6(4):275-286

Pre-analytical Factor - Blood Collection Type Impact on Size Distribution

Apoptosis of white blood cells leads to release of high molecular weight DNA



ccfDNA extracted from plasma collected using EDTA tubes from a subject directly after blood draw (t0) and after seven days at room temperature (T7d, 25°C)

One-microliter eluates analyzed using Agilent High Sensitivity DNA Kit

Provided by courtesy of D. Groelz, PreAnalytiX

Pre-analytical Factor - Blood Collection Type Impact on Size Distribution

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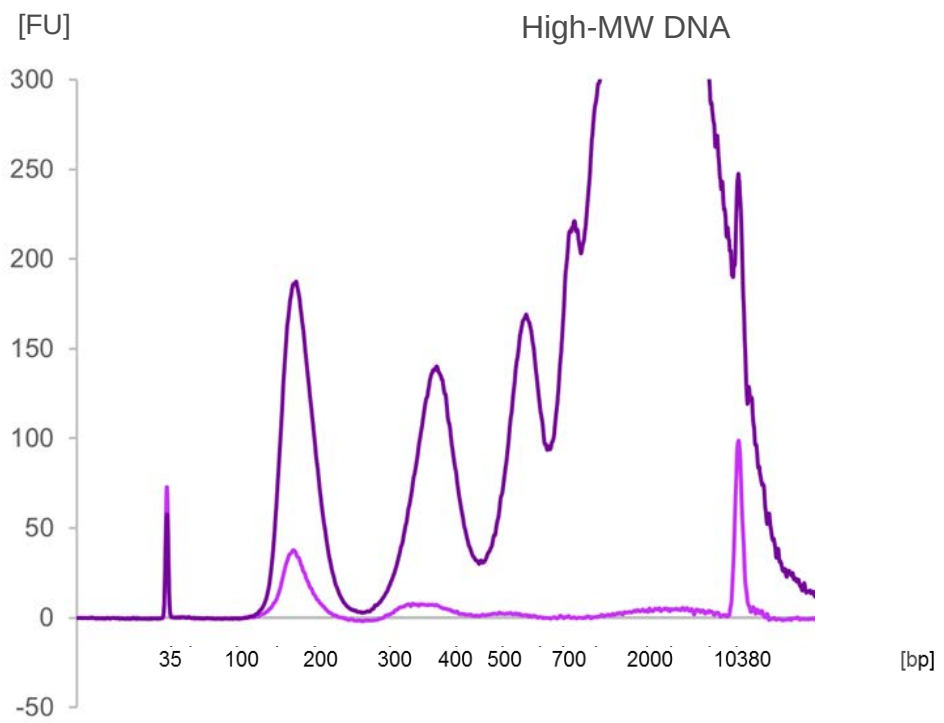

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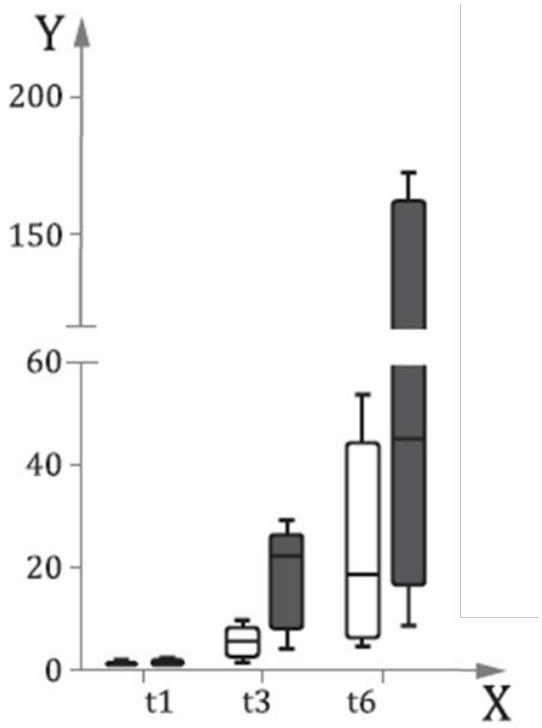
Provided by courtesy of D. Groelz, PreAnalytiX

Pre-analytical Factor – Blood Collection Impact on Yield

Apoptosis of white blood cells leads to increased yield and dilution of the target ccfDNA



18S rDNA gene, 66 bp amplicon

18S rDNA gene, 500 bp amplicon

X whole blood storage duration at room temperature before plasma generation (t1,3,6: 1, 3, and 6 days)

Y ratio of 18S rDNA copy numbers determined in plasma after indicated blood storage durations versus immediately after blood collection (t0)

ISO 20186-3:2019
Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood
— Part 3: Isolated circulating cell free DNA from plasma. Annex A.

Pre-analytical Factor – Plasma Processing Protocol

Follow manufacturer's centrifugation protocols to avoid gDNA carryover or reduced volume of separated plasma

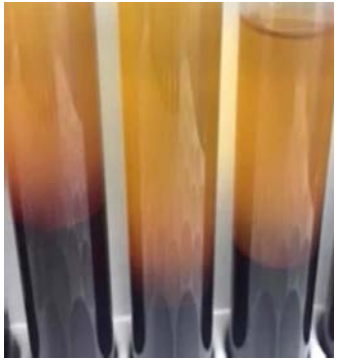
Manufacturer recommendation for PAXgene Blood ccfDNA Tube

- 1st Spin 15 min at 1600-3000 x g
- 2nd Spin 10 min at 1600-3000 x g
- Medium brake

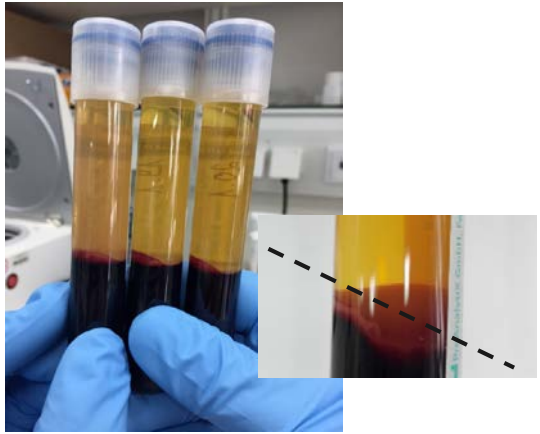
Best outcome: Maximum recovery of clear plasma with compact level interface

Reduced centrifugation time:

5 min 1600 x g

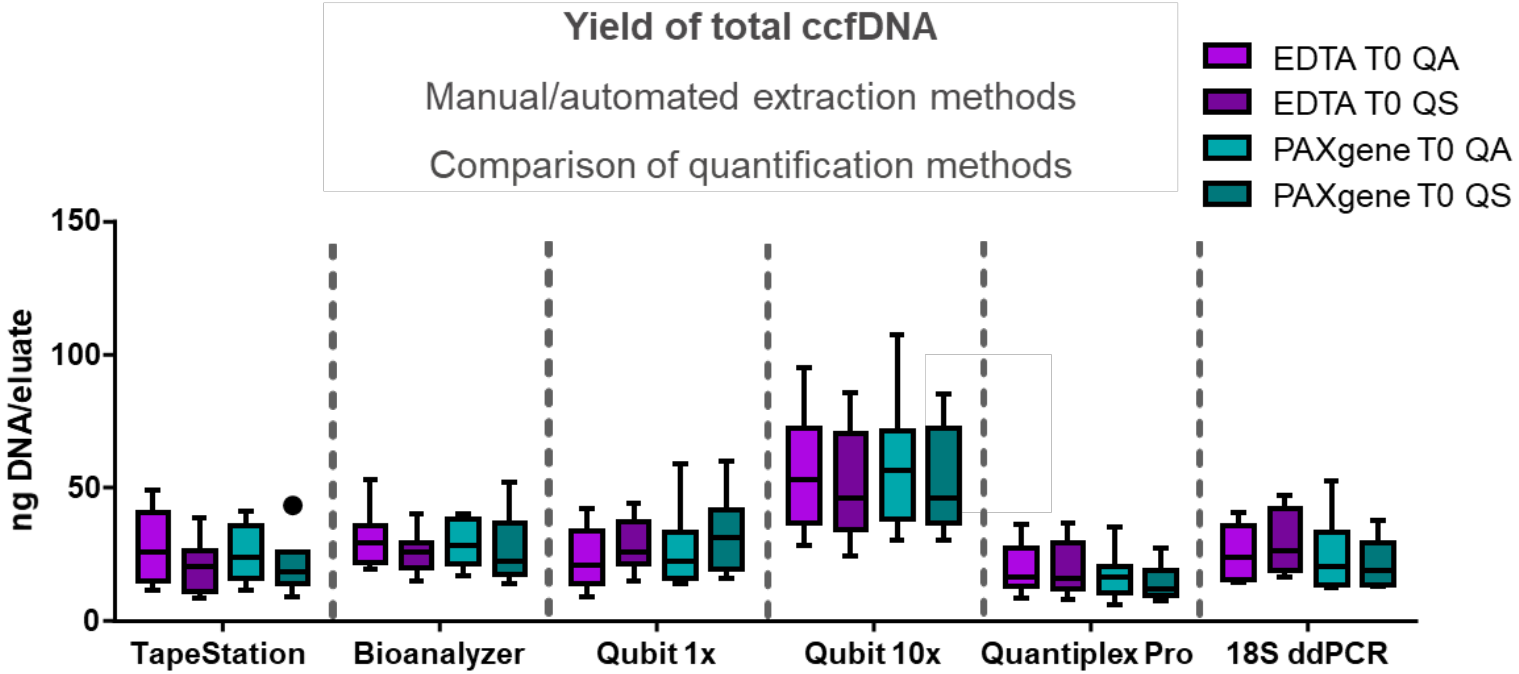


Maximum brake:



Provided by courtesy of D. Groelz, PreAnalytiX

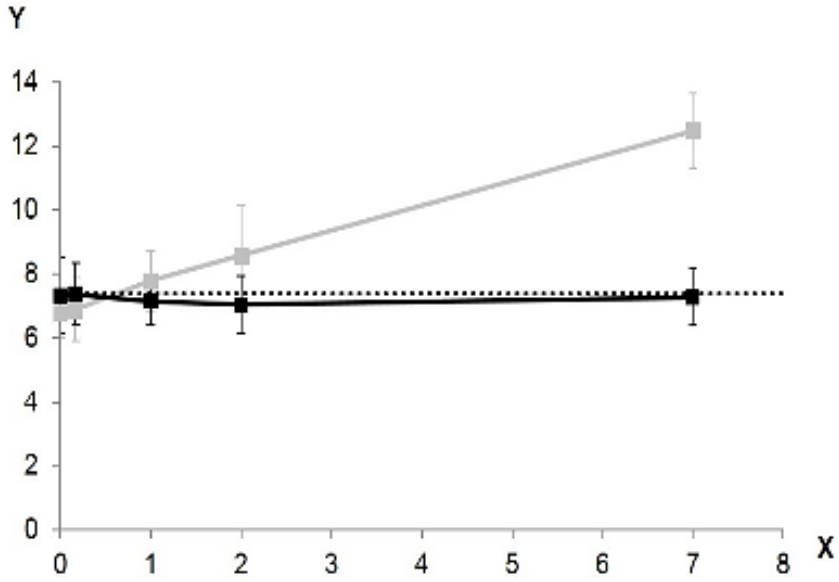
Pre-analytical Factor - DNA Quantification



Blood from 8 donors collected in EDTA and PAXgene Blood ccfDNA Tubes, manual (QIAamp cNA kit) and automated ccfDNA (QIAsymphony) isolation;
DNA quantification with Cell-free DNA ScreenTape Assay (4200 TapeStation System), High Sensitivity DNA Analysis Assay (Bioanalyzer 2100), Qubit® 1x (premixed) and 10x (dilution needed) dsDNA HS Assay (Qubit 2.0), Investigator Quantiplex® Pro Assay (QIAGEN Rotor-Gene® Q), 18S rDNA 66 bp (inhouse assay on QX200 Droplet Digital™ PCR).

Provided by courtesy of D. Groelz, PreAnalytiX

Post Blood Collection ccfDNA Profile Changes - Impact on EGFR Test



X venous whole blood storage duration (in days) before plasma preparation

Y ΔCT = CT (mutant) - CT (wildtype control)

EDTA Blood

Stabilized Blood

···· Threshold (given by the examination provider)

The average of 7 donors is shown

ISO 20186-3:2019
Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood
— Part 3: Isolated circulating cell free DNA from plasma. Annex A.

Blood collected in EDTA and PAXgene Blood ccfDNA tubes
Spiked with restriction enzyme treated EGFR DNA with mutation T790M, equivalent to 200 copies ccfDNA tested with the commercially available EGFR Plasma PCR Kit (RUO)

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SPIDIA: New Technologies and Standards for Pre-analytical Workflows

SPIDIA – FP7 (2008 – 2013)

- ⇨ 16 Partners
- New technologies for sample collection, stabilization, processing, transport, storage (Blood, Tissues)
- 9 EU CEN Standards

SPIDIA4P – H2020 (2017 – 2020)

- ⇨ 19 Partners
- ⇨ 14 associated consortia & stakeholder organizations
- 13 additional new CEN & ISO Standards
- EQAs
- European implementation

www.spidia.eu ⇨ **New Website. Subscribe the Newsletter!**

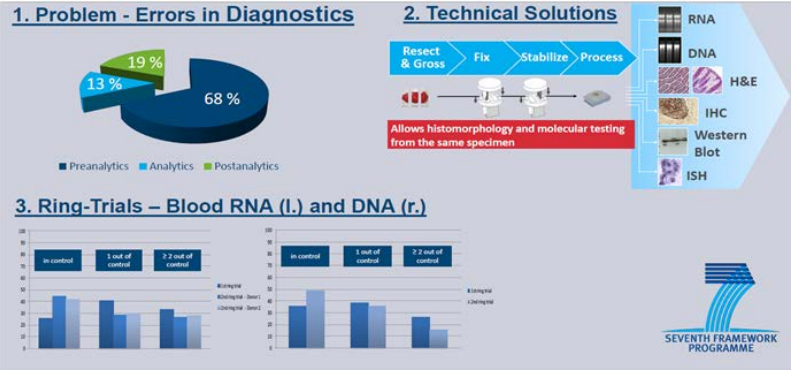
The SPIDIA project has received funding under the Seventh Research Framework Program of the European Union, FP7-HEALTH-2007-1.2.5, under grant agreement no. 222916. The SPIDIA4P project receives funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no. 733112.

SPIDIA's Road to Standardization

Vienna Agreement 1991

- 2019: 8 ISO/International Standards
- 2014: 8 new projects for ISO Standards approved in ISO/TC 212 „*Clinical laboratory testing and in vitro diagnostic test systems*"

- 2015: 9 CEN Technical Specifications published
- 2013: 9 new projects approved in CEN/TC 140 „*In vitro diagnostic medical devices*"
- 2010: Start of standardization work



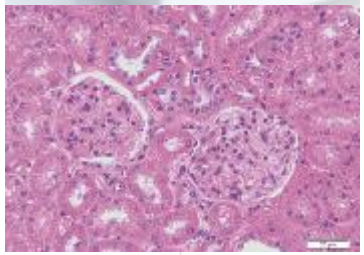
European Conference. Standards: Your Innovation Bridge. Brussels (2014). SPIDIA Booth.

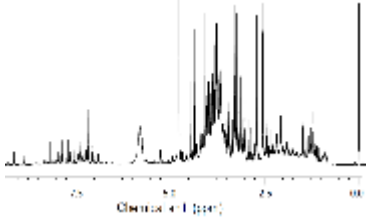
Provided by courtesy of SPIDIA

Highly Consensus Driven Process Needed for Developing Standards

- **CEN**
  - Recognized by the EU and the European Free Trade Association (EFTA) as being responsible for developing standards at European level
  - Development of a European Standard (EN) or International Standard (ISO) is governed by the principles of consensus, openness, transparency, national commitment and technical coherence
  - One European Standard replaces 34 national standards
- **CEN/TC 140** (Committee for in vitro diagnostic medical devices)
  - 34 EU countries National Standards Bodies (NSB)
  - Stakeholders in liaison & cooperations
    - **European Commission** (EC), **ESP** (European Society of Pathology), **EFLM** (European Federation of Laboratory Medicine), **IFCC** (Int. Federation of Clinical Chemistry and Laboratory Medicine), **JISC** (Japanese Industrial Standards Committee), **MedTech** (Alliance of European medical technology industry associations, founded by **EDMA**), **EPBS** (European Association for Professions in Biomedical Science), **BBMRI-ERIC** (Biobanking and BioMolecular resources Research Infrastructure - European Research Infrastructure Consortium), in progress, **ISO/TC 212** (Clinical laboratory testing and in vitro diagnostic test systems), **ISO/TC 276** Biotechnology

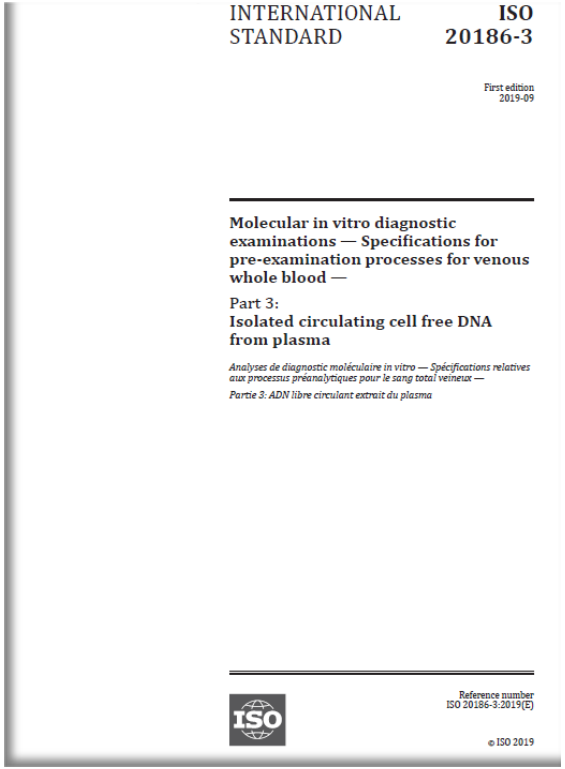
9 CEN Technical Specifications Released in Europe in 2015 / 16





- Molecular in-vitro diagnostic examinations - Specifications for pre-examination processes for
  - Blood — Cellular RNA
  - Blood — Genomic DNA
  - Blood — Circulating cell free DNA
  - FFPE tissue — DNA
  - FFPE tissue — RNA
  - FFPE tissue — Proteins
  - Frozen tissue — RNA
  - Frozen tissue — Proteins
  - Metabolomics in urine, serum and plasma

22 CEN & ISO Standard Documents and EQAs by 2021



INTERNATIONAL STANDARD

ISO 20186-3

First edition
2019-09

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood —

Part 3:
Isolated circulating cell free DNA from plasma

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour le sang total veineux —

Partie 3: ADN libre circulant extrait du plasma

Reference number
ISO 20186-3:2019(E)

© ISO 2019

Provided by courtesy of SPIDIA

Molecular in-vitro diagnostic examinations - Specifications for pre-examination processes for

- Blood — Cellular RNA, gDNA, ccfDNA, *ccfRNA*
- Blood – *Exosomes, ccfRNA*
- Circulating Tumor Cells – **DNA, RNA, staining**
- Tissue (FFPE) — DNA, RNA, Proteins
- Tissue (Frozen) – RNA, Proteins, **DNA**
- Tissue (FFPE) - *staining*
- Fine Needle Aspirates – *DNA, RNA, Proteins*
- Saliva – **DNA**
- Urine & Body Fluids – *cfDNA*
- Microbiome – *Stool, Saliva etc.*

published CEN

published ISO

in development

https://www.spidia.eu/

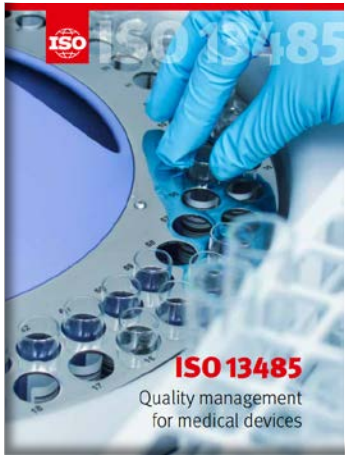
New In Vitro Diagnostic Regulations 2017 (IVDR)

- New European In Vitro Diagnostic Regulation in force since May 2017
- Pre-analytical workflow parameters in several sections
  - 6. PRODUCT VERIFICATION AND VALIDATION (Annex II)
  - 6.1. Information on analytical performance of the device
  - 6.1.1. Specimen type

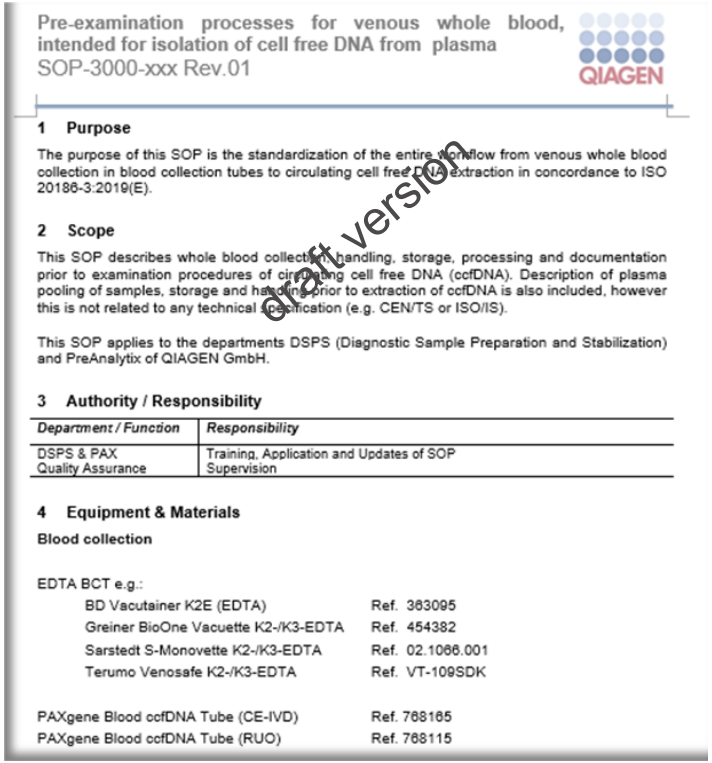
    This Section shall describe the different specimen types that can be analysed, including their stability such as storage, where applicable specimen transport conditions and, with a view to time-critical analysis methods, information on the timeframe between taking the specimen and its analysis and storage conditions such as duration, temperature limits and freeze/thaw cycles

Implementation of Preanalytical Standards

PreAnalytiX a QIAGEN/BD Company and SPIDIA4P partner



Product Development Process



Pre-examination processes for venous whole blood, intended for isolation of cell free DNA from plasma
SOP-3000-xxx Rev.01

QIAGEN

1 Purpose

The purpose of this SOP is the standardization of the entire workflow from venous whole blood collection in blood collection tubes to circulating cell free DNA extraction in concordance to ISO 20186-3:2019(E).

2 Scope

This SOP describes whole blood collection, handling, storage, processing and documentation prior to examination procedures of circulating cell free DNA (ccfDNA). Description of plasma pooling of samples, storage and handling prior to extraction of ccfDNA is also included, however this is not related to any technical specification (e.g. CEN/TS or ISO/IS).

This SOP applies to the departments DSPS (Diagnostic Sample Preparation and Stabilization) and PreAnalytix of QIAGEN GmbH.

3 Authority / Responsibility

Department / Function	*Responsibility*
DSPS & PAX Quality Assurance	Training, Application and Updates of SOP Supervision

4 Equipment & Materials

Blood collection

EDTA BCT e.g.:

BD Vacutainer K2E (EDTA)	Ref. 363095
Greiner BioOne Vacuette K2-/K3-EDTA	Ref. 454382
Sarstedt S-Monovette K2-/K3-EDTA	Ref. 02.1066.001
Terumo Venosafe K2-/K3-EDTA	Ref. VT-109SDK

PAXgene Blood ccfDNA Tube (CE-IVD)	Ref. 768165
PAXgene Blood ccfDNA Tube (RUO)	Ref. 768115

Draft version

Certification according to ISO 13485

Company Quality Manual: Process Landscape

Global Process SOPs incl. legal requirements

Technical SOPs for pre-analytical workflows based on ISO & CEN standards

Provided by courtesy of D. Groelz, PreAnalytiX

ISO 20186-3 – Pre-examination Processes for Blood ccfDNA

Contents

ISO 20186-3:2019 - Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood — Part 3: Isolated circulating cell free DNA from plasma

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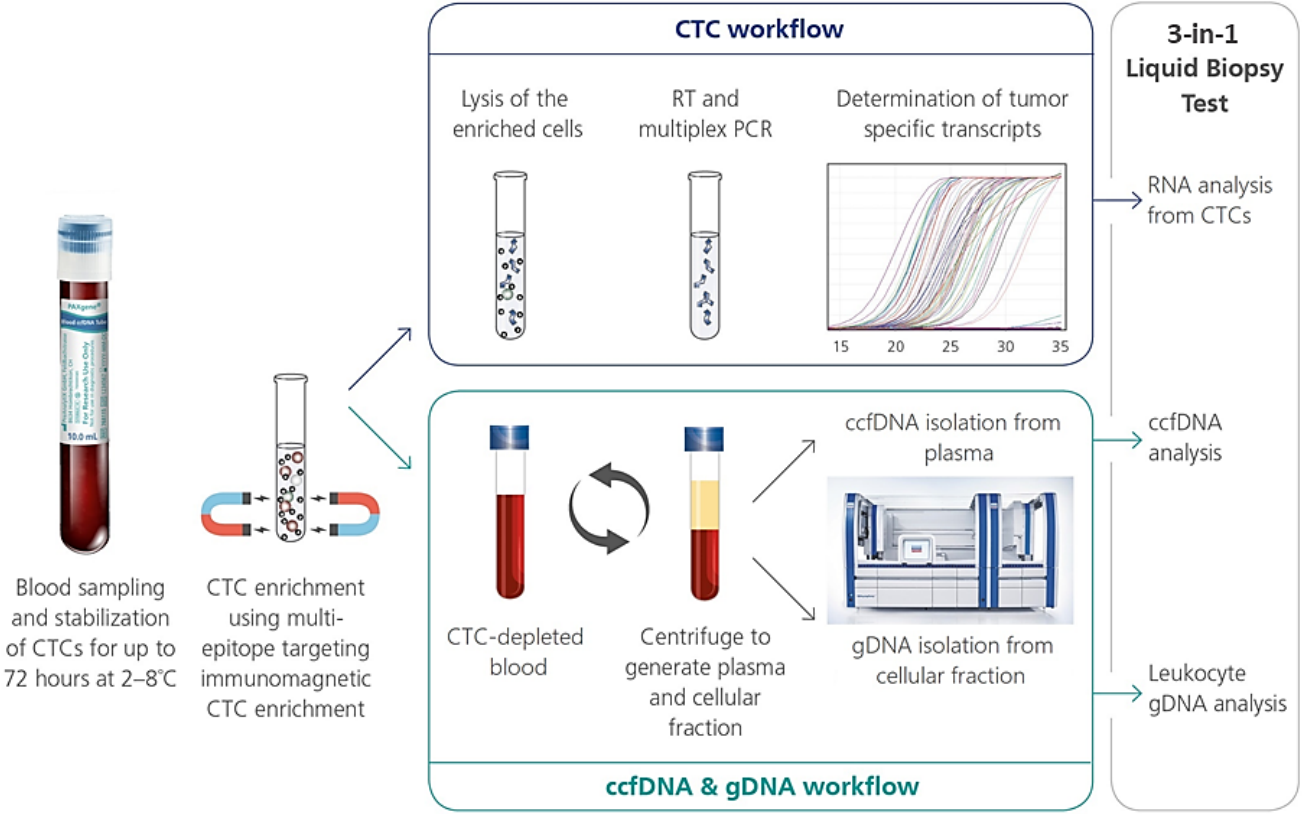
Pre-analytical Steps: Part of a Whole Diagnostic Test Workflow

Provided by courtesy of D. Groelz, PreAnalytiX

Multimodal Analysis: Extraction and Analysis of CTC RNA, ccfDNA and gDNA from a Single Blood Sample



Babayan et al. [abstract]. Proceedings: AACR Advances in Liquid Biopsies 2020; January 13-16, 2020; Miami, FL.

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

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- A CE-marked PAXgene Blood ccfDNA Tube for in vitro diagnostics is available in certain European countries. The performance characteristics of the RUO and the IVD tubes are equivalent.