

# IQNPath ctDNA Workshop

From Sample collection to clinical integration of the results - ensuring quality end to end

Florence, 23rd June 2017

## ctDNA extraction methods

Ed Schuuring

Head Laboratory Molecular Pathology, UMCG Groningen  
Professor in Molecular Oncological Pathology  
KMBP (Clinical Scientist in Molecular Pathology)  
Chairman section KMEP in the Dutch Society of Pathology

e.schuuring@umcg.nl



# Disclosures

## Consultant/Advisory Board:

AstraZeneca, Roche, Pfizer, Novartis, Amgen, BioCartis, QCMD, ESP, IQNPATH (WG-ctDNA), Cancer-ID (WG-ctDNA)

## Speaker's fee:

Abbott, Novartis, Roche, Biocartis, Illumina

## Grants/Sponsoring:

Pfizer, Biocartis/Idylla, BMS, Roche/cobas, Boehringer Ingelheim

## Stock/Royalties:

None



All grants/honoraria transferred to UMCG-account

## Tumor-specific mutation testing using DNA from cell-free plasma critical factors/challenges

- Blood collection
- Plasma processing
- Cell free plasma storage
- DNA extraction from cell free plasma
- cfDNA input
- Mutation detection methods
- Interpretation



IQNPath-ctDNA-workshop: From Sample collection to clinical integration of the results - ensuring quality end to end

## DNA extraction assays from cell free plasma

QIAamp® circulating Nucleic Acid Kit (Qiagen)  
QIAamp® DNA Blood Mini Kit (Qiagen)  
EZ1 ccfDNA Kit (Qiagen)  
QIASymphony PAXgene Blood ccfDNA Kit (Qiagen)  
QIASymphony DSP Circulating DNA Kit (Qiagen)  
QIAamp® MinElute® ccfDNA (Qiagen)  
cfDNA isolatie van Quick-cfDNA™ serum & plasma DNA Miniprep kit (Zymo Research)  
Maxwell® RSC circulating cell-free (ccfDNA) Plasma Kit (Promega)  
Cobas cfDNA sample preparation Kit (Roche)  
NucleoSpin Kit (Macherey-Nagel)  
MagNA Pure isolation system (Roche Diagnostics)  
Plasma/serum cf circulating DNA purification kit (Norgen BioTek)  
Chemagic cfNA isolation kit (Perkin-Elmer)  
FitAmp Plasma/Serum DNA isolation kit (Epigentek)  
PureLink Virus KIT (Life technologies)  
PME free circulating DNA extraction KIT (Analytik Jena)  
EpiQuick Circulating Cell-Free DNA (cfDNA) isolation easy kit (Epigentek)  
NEXTprep-Mag cfDNA kit (Bio Scientific/PerkinElmer)  
Biocartis/Idylla (DNA extraction is part of detection-test)  
.....

IQNPath-ctDNA-workshop: From Sample collection to clinical integration of the results - ensuring quality end to end

## Tumor-specific mutation testing using DNA from cell-free plasma critical factors

- Blood

**DNA extraction methods:  
Need for standardisation and  
harmonisation !!!**

extraction methods

interpretation



IQNPath-ctDNA-workshop: From Sample collection to clinical integration of the results - ensuring quality end to end

## cfDNA extraction methods

*what method to use ?*

Choice is depending on:

- Clinical questions/application ?
- Amount of cfDNA needed for ctDNA testing ?
- Detection method used ?

## cfDNA extraction methods

*some facts*

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 methods

## cfDNA extraction methods

*some facts*

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 methods

Detection of mutations in circulating tumor DNA in cell free DNA in plasma  
finding the "abnormal" hay in the haystack

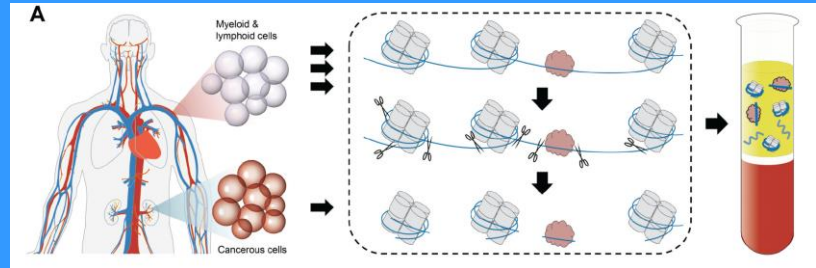


Plasma DNA extraction methods do not discriminate between cfDNA and ctDNA



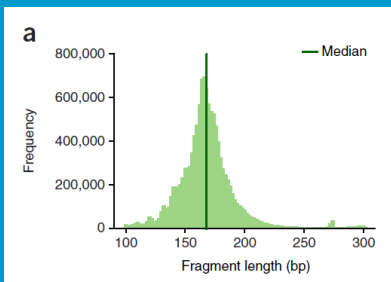
Image from www.palaisdetokyo.com

Nucleosome protected DNA degradation in plasma



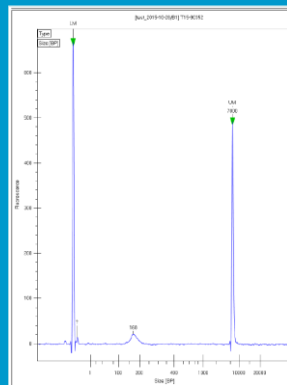
Snyder, Cell 2017

Size of cell free, circulating DNA in plasma



Newman, Nat Med 2014

Plasma DNA extraction methods do not discriminate between cfDNA and ctDNA



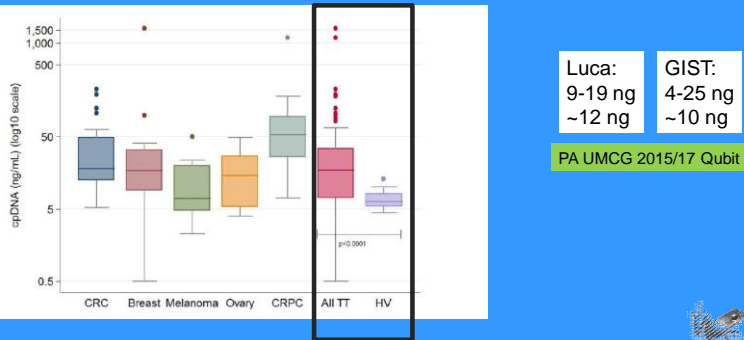
Bioanalyzer Labchip GX: umcg ctDNA sample

cfDNA extraction methods  
*some facts*

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 methods

## Levels of total DNA (cfDNA) in 1ml of plasma

Total DNA concentrations by picogreen (ng/ml plasma) classified per advanced tumor type at baseline



Perkins Plos One 2012



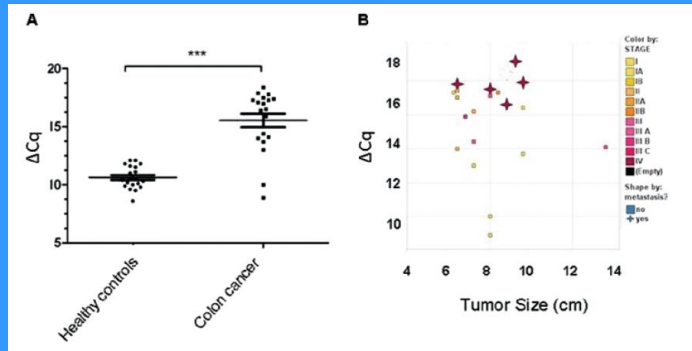
The amount of cell-free DNA in plasma for ctDNA testing

Different assays to determine DNA concentration:

- qPCR
- Picogreen
- Nanodrop
- Qubit (approx 10x lower concentration compared to nanodrop)

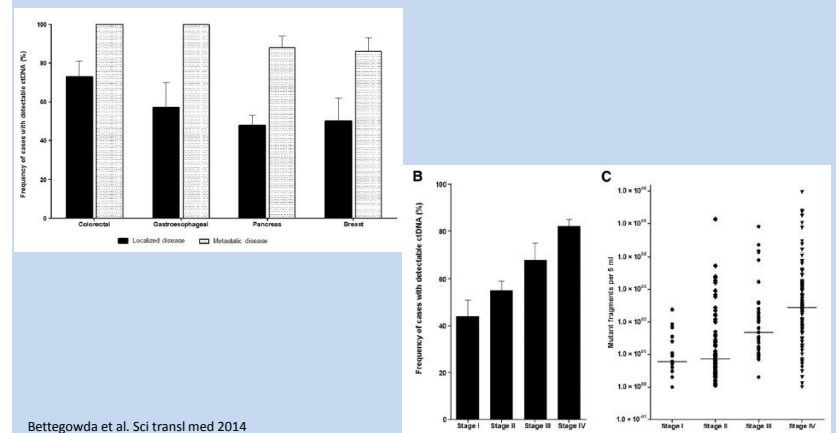


## Total DNA in cell-free plasma associated with tumor load e.g. in colorectal carcinoma



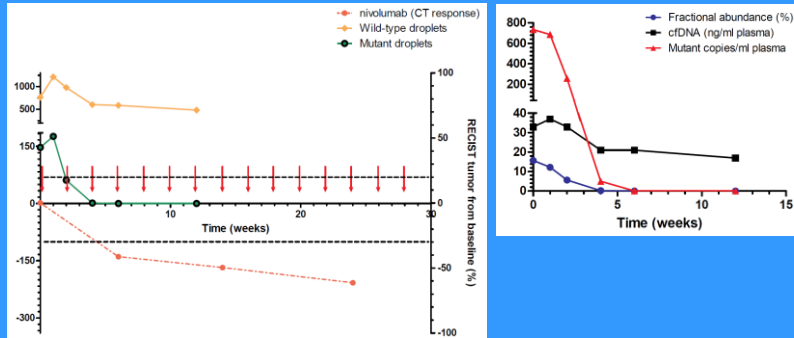
Gorges Biomarkers 2012

## Levels of detectable ctDNA in localized versus metastasized disease



Bettgowda et al. Sci transl med 2014

## NSCLC patient treated with immunotherapy and responds monitored by CT-scanning and tumor-specific mutation



- Mutant drops to non-detectable (following ct-scanning)
- Wt-ddPCR product approx constant during respons
- Qubit-DNA content very similar

Unpublished Hiltnermann, Miedema, Groen, Elst, Schuurin

## Amount of cell-free DNA in plasma for ctDNA testing

Different assays to determine DNA concentration:

- qPCR, Picogreen, Nanodrop, Qubit

Total cfDNA content is related to:

- Treated vs untreated patients (<2 ng/ml plasma (untreated pts !!!))
- Localized disease (no ctDNA detectable)

For optimal/reliable result regarding ctDNA level:

- High levels DNA: ddPCR - 10ng; NGS – 50-200ng (~10ml plasma !!!)
- High concentrations: maximal volume/test (e.g. 8.8 ul/ddPCR)

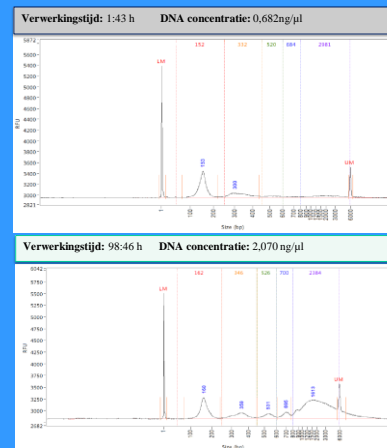
Need for standardisation/harmonisation

## cfDNA extraction methods

*some facts*

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 methods

Bioanalyzer: EDTA tubes stored at 20 °C for <4 hrs and >4 days  
assessment of quality and calculation of DNA yield of cfDNA

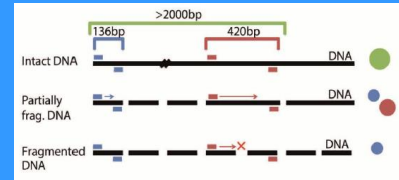


Plasma processed from EDTA-blood tube within 2 hrs after blood drawn (optimal procedure)

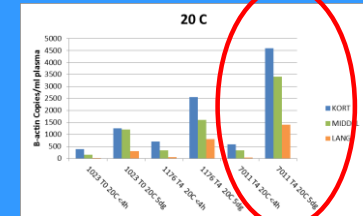
Same EDTA-blood tube stored for several days known resulting in hemolysis

Not yield of DNA but the efficiency of DNA amplification is essential

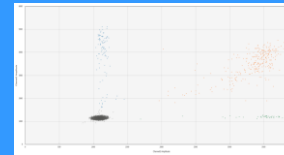
## Quality control plasma processing ddPCR assay to assess quality and quantity of cfDNA



Primers based on Norton Clin Biom 2013; Dessel Mol Oncol 2017



3 plasmas tested after <4hrs and >4days storage



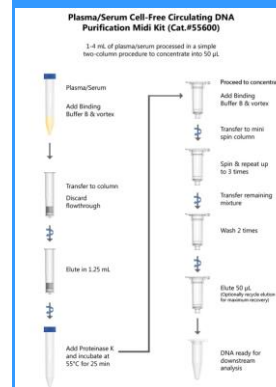
Single ddPCR assay (bACT)  
Boonstra, Miedema, Tibbesma, Elst, Schuurig



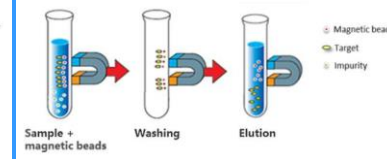
## cfDNA extraction methods some facts

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 methods

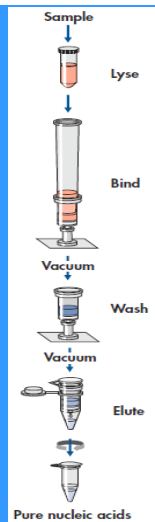
## cfDNA extracted by centrifugation, vacuum or magnetic beads



Norgen-spinning



Promega-magnetic-beads



Qiagen-vacuum

## cfDNA extraction methods

*differences between methods*

- manual vs automatisatie > depending on TAT/costs
- elution (high DNA concentration) > newer kits enable lower elution volume without losing cfDNA on beads (QIAamp MinElute)
- most assays 1-2 ml: newer assays 1-10ml plasma (Zymo cfDNA kit)
- TAT and hands-on-time
- companion diagnostics/FDA (cobas plasma extraction/mutation detection kit)
- DNA extraction is included in assay (Idylla/Biocartis)
- costs

Many/most assays entered the market very recently:  
lack of independent confirmation of proposed efficiencies

## Costs of DNA extractions (examples)

	QIAamp® Circulating Nucleic Acid Kit (Qiagen)	Quick-cfDNA™ serum & plasma DNA Miniprep kit (Zymo)	Maxwell® RSC cfDNA Plasma Kit
Catalogue price per kit	938,00	1160,35	624,00
Costs per extraction	18,76	23,21	13,00
Equipment price	1101,00	1101,00	22000,00
Costs equipment per year	367,00	367,00	7333,00
costs/sample 100 samples	22,53	26,88	86,33
costs/sample 1000 samples	19,12	23,58	20,33

Costs do not include personal and others costs

Choices are also influenced by your setting  
(screening single sample in diagnostics or analysis of large cohorts)

## cfDNA extraction methods

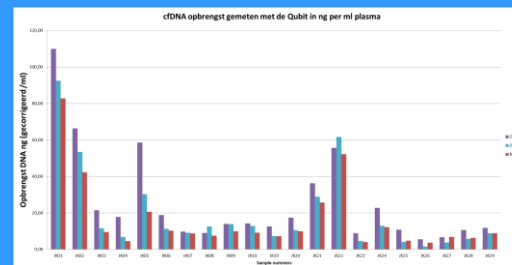
*some facts*

- cfDNA versus ctDNA
- Amount of cfDNA in your plasma
- QC: how to determine quantity (yield) of cfDNA ?
- QC: how to determine quality of cfDNA ?
- Differences between methods ?
- example of comparison of 3 plasma DNA extraction methods

## DNA extraction from cell free plasma comparing manual Qiagen CNA, automatic Maxwell Promega and manual cfDNA Zymo

*DNA yield*

UMCG study:  
same plasma of 21 patients with cancer

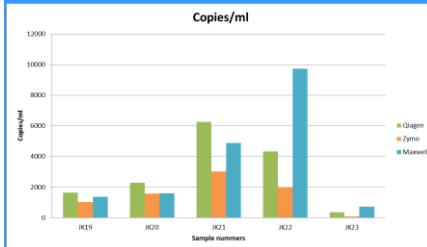


Total DNA concentrations by Qubit (ng/ml plasma)

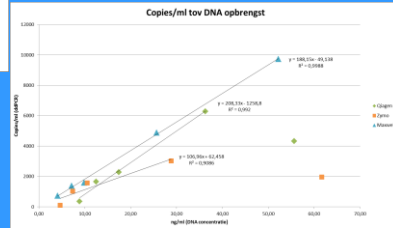
Nr	DNA opbrengst (ng) gecorrigeerd voor 1ml		
	Qiagen	Zymo	Maxwell
JK01	109,92	92,43	82,78
JK02	66,34	26,63	53,27
JK03	21,41	11,49	9,44
JK04	27,76	6,79	4,44
JK05	58,49	30,29	20,56
JK06	18,72	11,28	10,29
JK07	9,71	9,14	8,71
JK08	9,01	12,99	7,42
JK09	13,67	13,79	9,64
JK10	14,20	12,74	9,02
JK19	12,47	7,36	7,13
JK20	17,42	10,50	9,83
JK21	36,27	28,83	25,63
JK22	59,62	61,62	52,17
JK23	8,88	4,65	4,05
JK24	22,69	12,95	12,12
JK25	10,71	4,07	4,71
JK26	5,58	1,53	3,58
JK27	6,61	3,71	6,82
JK28	10,67	5,80	6,24
JK29	11,78	8,77	8,83
Gemiddeld	25,62	17,95	16,99



DNA extraction from cell free plasma comparing manual Qiagen CNA, automatic Maxwell Promega and manual cfDNA Zymo  
*wt-ddPCR assessment*



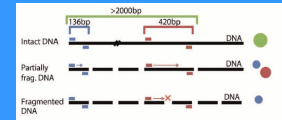
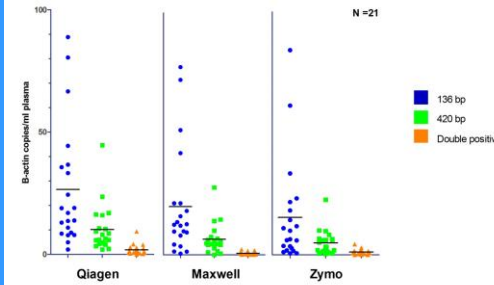
Comparison qubit vs ddPCR-wt



Boonstra, Miedema, Tibbesma, Elst, Schuurin

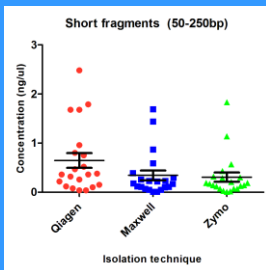
DNA extraction from cell free plasma comparing manual Qiagen CNA, automatic Maxwell Promega and manual cfDNA Zymo  
*single ddPCR assay for QA*

B-actin fragment length in various DNA isolation methods

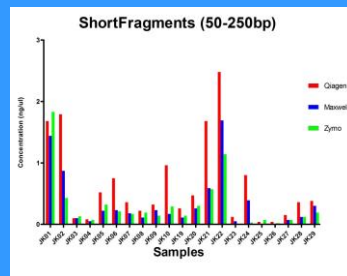


Boonstra, Miedema, Tibbesma, Elst, Schuurin

DNA extraction from cell free plasma comparing manual Qiagen CNA, automatic Maxwell Promega and manual cfDNA Zymo  
*Bioanalyzer for QA*



Fragment-analysis with Bio-analyzer:  
Qiagen has higher yield of 166bp-fractions



DNA extraction from cell free plasma comparing manual Qiagen CNA, automatic Maxwell Promega and manual cfDNA Zymo  
*preliminary conclusions*

- Manual Qiagen CNA test shows best performance
- Automatic Maxwell similar (only cost-effective when using >1000 samples/year)
- The size-length single ddPCR is an optimal assay for assessment of amplificability and quantity of cfDNA extracted from cell free plasma



## DNA extraction assays from cell free plasma

QIAamp® circulating Nucleic Acid Kit (Qiagen)  
QIAamp® DNA Blood Mini Kit (Qiagen)  
EZ1 ccfDNA Kit (Qiagen)  
QIAasymphony PAXgene Blood ccfDNA  
QIAasymphony DSP Circulating  
QIAamp® MinElute®  
cfDNA isolation  
Max

**DNA extraction methods:  
Need for standardisation and  
harmonisation !!!**

CF  
Fi  
Pu  
PML  
EpiC  
NEX  
Bio

IQNPath-ctDNA-workshop: From Sample collection to clinical integration of the results - ensuring quality end to end

## cfDNA extraction methods

*critical factors to come to standardisation ?*

- Need for standardisation of quantity of cfDNA ?
- Need for standardisation of quality assessment of cfDNA ?
- depending on clinical questions/application ?
- depending on amount of cfDNA needed for ctDNA testing ?
- depending on detection method used ?
- harmonisation with other activities (IQNPath, CAP, Cancer-ID, german ?, Italian ?, french?)
- other considerations ?



IQNPath-ctDNA-workshop: From Sample collection to clinical integration of the results - ensuring quality end to end

## Molecular Pathology UMCG-team

### MD-technicians:

- Ingrid de Boer-Huitema
- Annelies ten Caat
- Erik Nijboer
- Paskal van Norel
- Rianne Pelgrim
- Inge Platteel
- Martin Schipper
- Jantine Sietzema
- Klaas Kooistra

### KMBP:

- Elise van der Logt
- Arja ter Elst
- Anke van den Berg
- Ed Schuurung
- Maarten Niemantsverdriet (KMBPio 2016-2020)
- Leon van Kempen (KMBPio 2016-2018)

### Pathologists:

- Wim Timens
- Nils T'Hart
- Arjan Diepstra



### UMCG-Liquid Biopsy team:

GALLOP-study (GIST): Pieter Boonstra, Marco Tibbesma, Arja ter Elst, Ed Schuurung, An Reyners

Lung Cancer studies: Lisette Bosman, Anneke Medema, Arja ter Elst, Jeroen Hiltermann, Harry Groen, Ed Schuurung

International EQA/SOP: IQNPath (ESP, AIOM, EMQN, UK NEQAS), Cancer-ID (Schuurung)

