Methodology challenges in isolation of circulating cell-free DNA from liquid biopsy samples in patients with colorectal adenoma

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Colorectal cancer (CRC) and liquid biopsy

- *"The blood becomes a window through which we observe what is happening in the body to help diagnose disease, assess efficacy and toxicity of drugs, and assess wellness."* **(Leroy Hood, 2013)**

- **The most common cancer in Croatia**
- **Molecular profiling for personalised approach:**

"Genetic, protein and RNA profiling of colorectal cancer using liquid biopsy" (HRZZ-IP-2019-04-4624)

circulating tumour cells | **ctDNA** | exosomes (proteins, RNAs) | proteins

- **Higher concentrations**
  - 5 – 1500 ng/ml in patients with CRC vs. 1 – 5 ng/ml in healthy individuals (Mouliere et al 2013)
- **Role in metastasis**
  - Transformation of normal cells to tumour cells (Cheng et al, 2016)
- **Reflect changes in tumour**
  - SNPs and mutations, copy number variations, DNA metilation, chromosomal changes, effect on transcription and protein synthesis

Aim

- **Selection of the most suitable method** for the isolation of circulating cell-free DNA (ccfDNA) from liquid biopsy samples of patients with colorectal adenoma (preCRC), which will be used in further assessment of CRC patient samples

Sufficient quality and quantity for NGS!

Liquid biopsy sample collection and plasma preparation

- Peripheral venous blood of 11 preCRC patients
- **CellSave tubes** (Menarini Silicon Biosystems) – stored at +4°C, transported to lab
- Plasma separation by differential centrifugation – aliquots stored at -20°C until ccfDNA isolation

1) 1900 g/10 min/ + 4°C

2) 16000 g/10 min/ + 4°C

11 x → →  **+4°C**

CellSave tubes
(Menarini Silicon Biosystems)

→ 

Lisa refrigerated centrifuge
(AFI)

→  **-20°C**

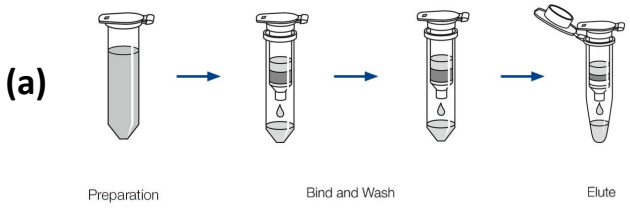
Methods for isolation of ccfDNA

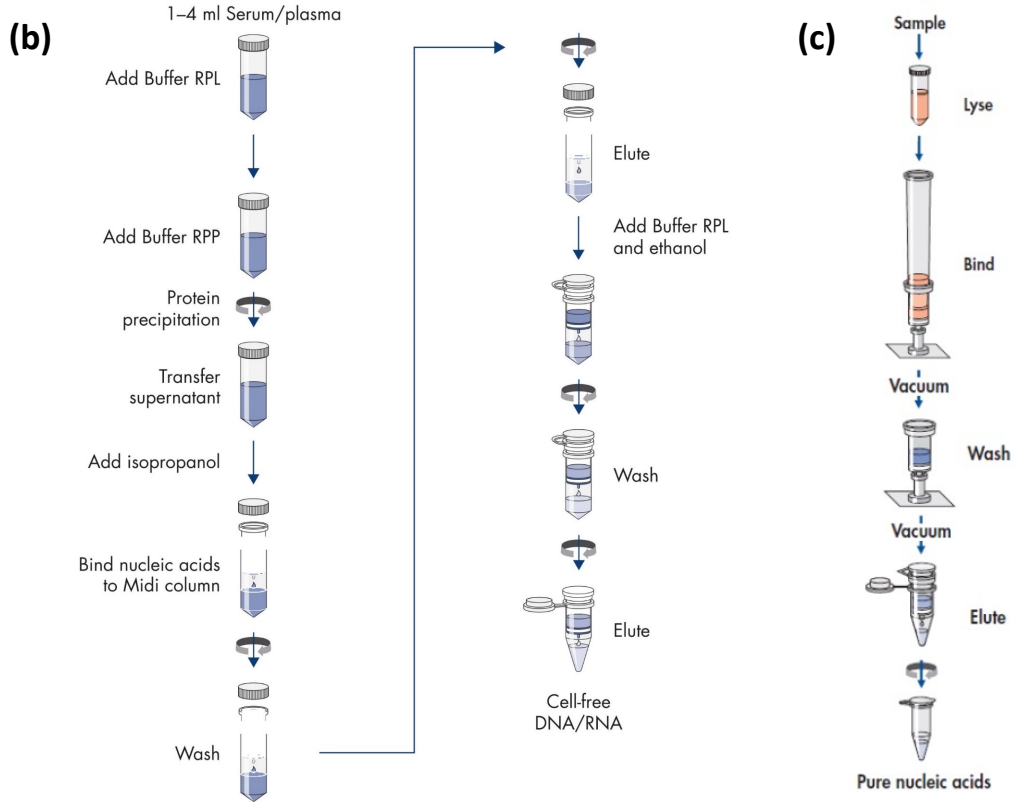
- Cost, duration, availability of equipment!
- Two spin-column-based methods **(a and b*),** one vacuum-based method (**c**) according to manufacturer's instructions
- *additional centrifugation step needed to separate proteins

(a) NucleoSpin cfDNA XS Kit (Macherey Nagel)
- from **0.7 mL** of plasma

(b) QIAamp ccfDNA/RNA Kit (Qiagen)
- from **2.0 – 3.5 mL** of plasma

(c) QIAamp Circulating Nucleic Acid Kit (Qiagen)
- from **2.0 – 3.5** mL of plasma





Quality of isolated ccfDNA

- **Microvolume automated gel electrophoresis**
  - The presence of fragment corresponding to ccfDNA (160-170 bp)
  - The absence of genomic DNA (gDNA) contamination

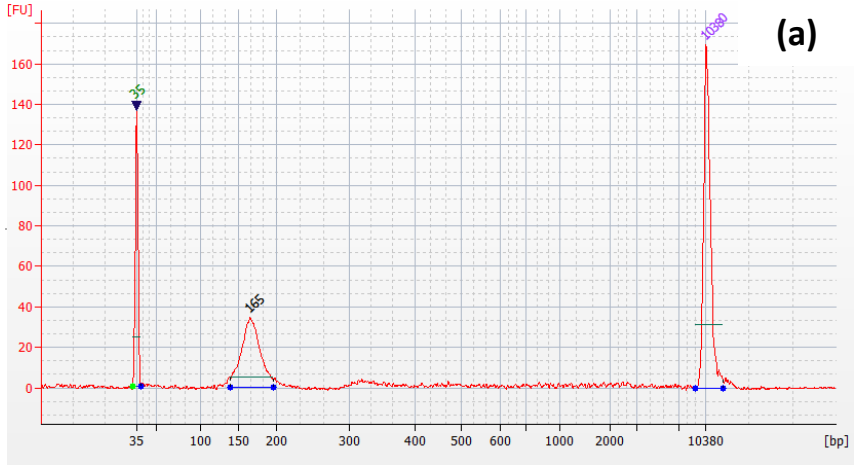
High Sensitivity DNA Kit
(Agilent Technologies)

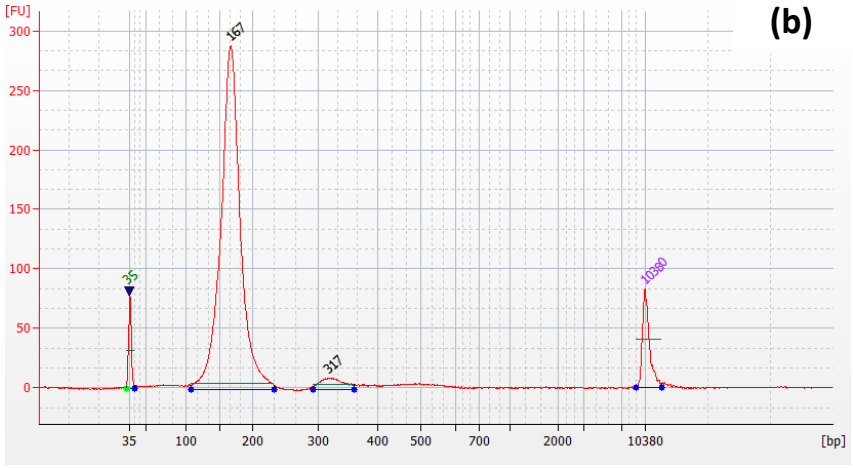
Bioanalyzer 2100
(Agilent Technologies)

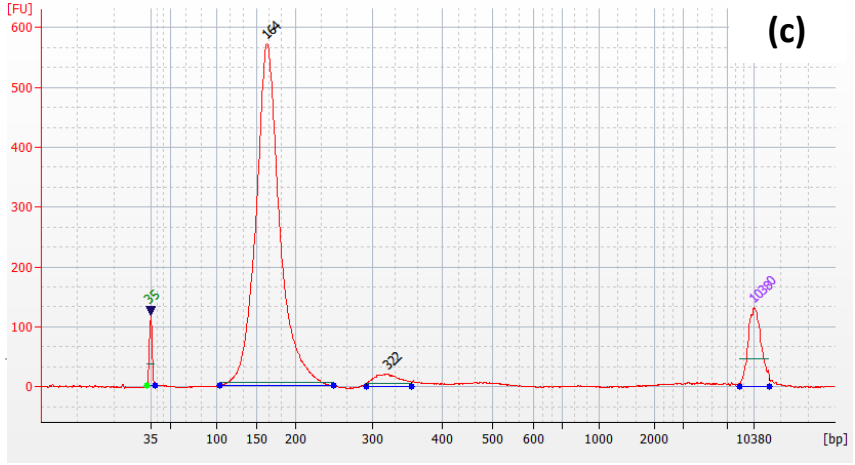
Method	ccfDNA fragment	gDNA fragment
(a)	2	0
(b)	9	2
(c)	11	0

Electropherograms of sample No. 5 isolated using three methods are shown.*

*2100 Expert software, version B.02.11.SI811 (Agilent Technologies)







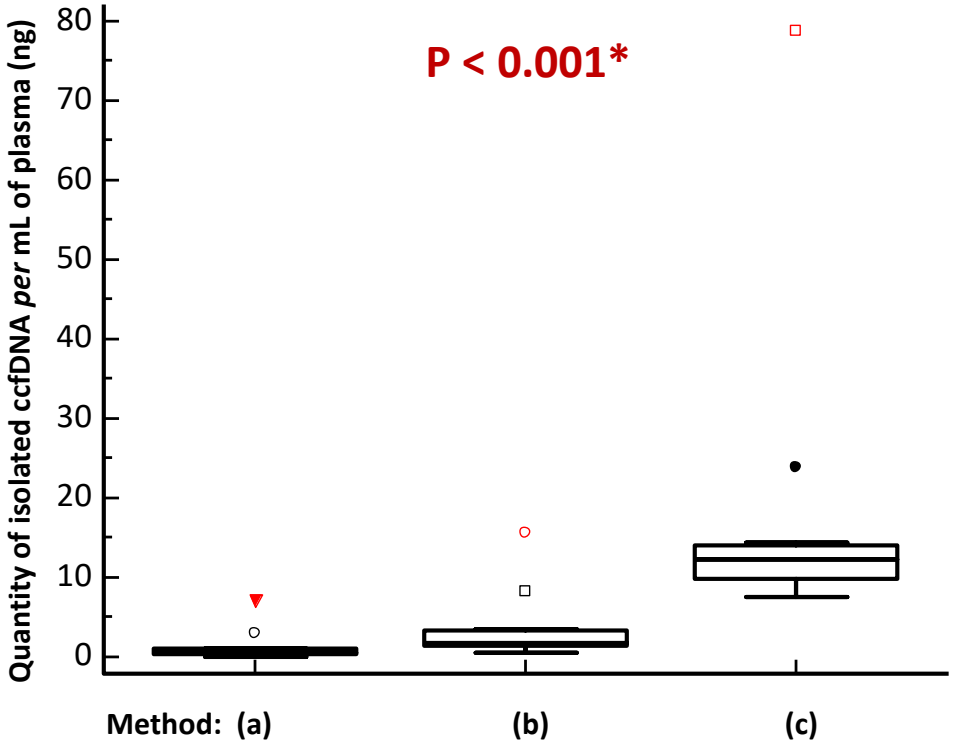
Yeald of ccfDNA *per* mL of plasma using different isolation methods

- **Fluorometric measurement** to determine concentration and calculate quantities of isolated ccfDNA *per* mL of plasma

Qubit dsDNA HS Assay
(Invitrogen)

DS-11 FX
(DeNovix)

Sample number	Quantities of isolated ccfDNA *per* mL of plasma (ng)		
	(a)	(b)	(c)
1	2.914	1.764	12.864
2	0.429	8.200	14.425
3	0.114	1.507	10.442
4	1.000	3.430	9.300
5	7.171	15.556	78.704
6	0.400	2.024	12.235
7	0.229	0.493	7.467
8	0.600	1.209	12.646
9	0.000	1.800	9.729
10	0.886	1.269	10.078
11	0.886	3.152	23.793



*Friedman test (MedCalc software v22.020)

Conclusion

The vacuum-based method (c) proved to be the most suitable for ccfDNA isolation and therefore was selected for further analysis of liquid biopsy samples from CRC patients.

Future

- **NGS panel** → comparison of results with:
  - DNA isolated from tissue samples collected from same patients
  - DNA isolated from tissue samples collected from colorectal adenoma patients

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