Characterization of small non-coding RNAs in plasma of breast carcinoma patients by NGS

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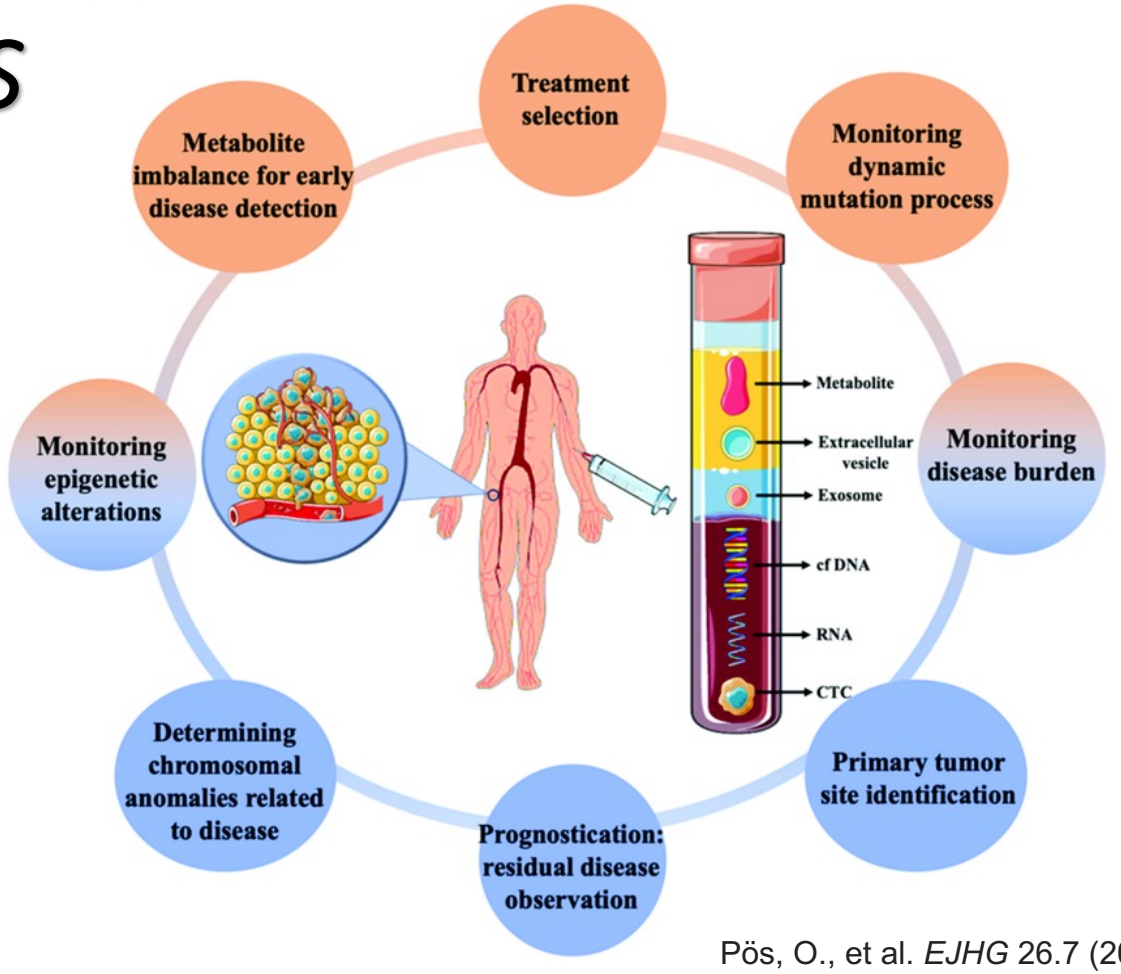
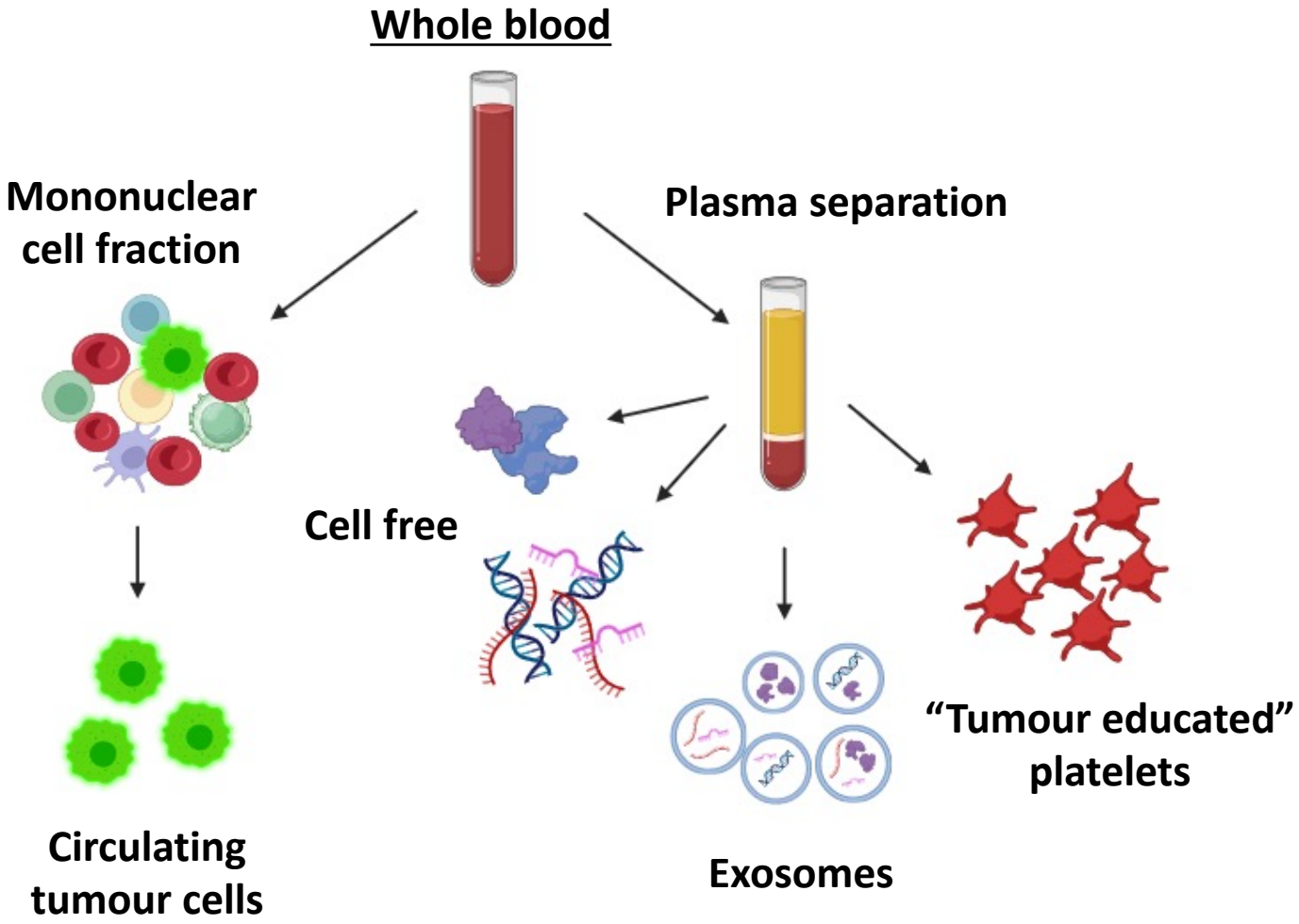


Pös, O., et al. *EJHG* 26.7 (2018)

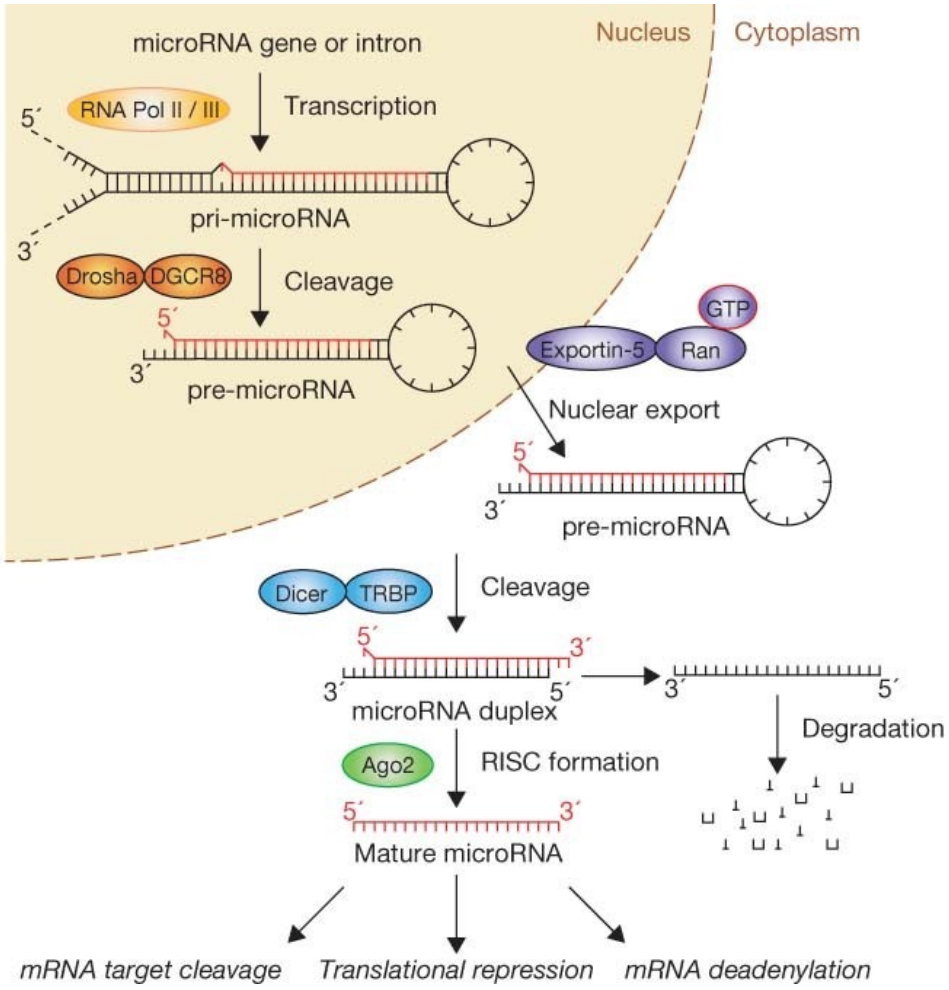
Circulating tumour nucleic acids

- **cfDNA and RNA**
- Plasma + serum
  - **Breast**
  - **Ovarian**
  - **Colorectal**
  - **Pancreatic carcinoma**
- At the time of diagnosis, during treatment and follow-up



Non-coding RNA in the cell-free component of the blood

- small ncRNAs (<200 nucleotides)
  - **microRNAs (miRNAs)**
  - transfer RNAs (tRNAs)
  - piwi-interacting RNAs (piRNAs)
  - transcription initiating RNAs (tiRNAs)
  - endogenous small interfering RNAs (endo-siRNAs)



Winter, J., et al. *Nat. Cell Biol* 11.3 (2009)

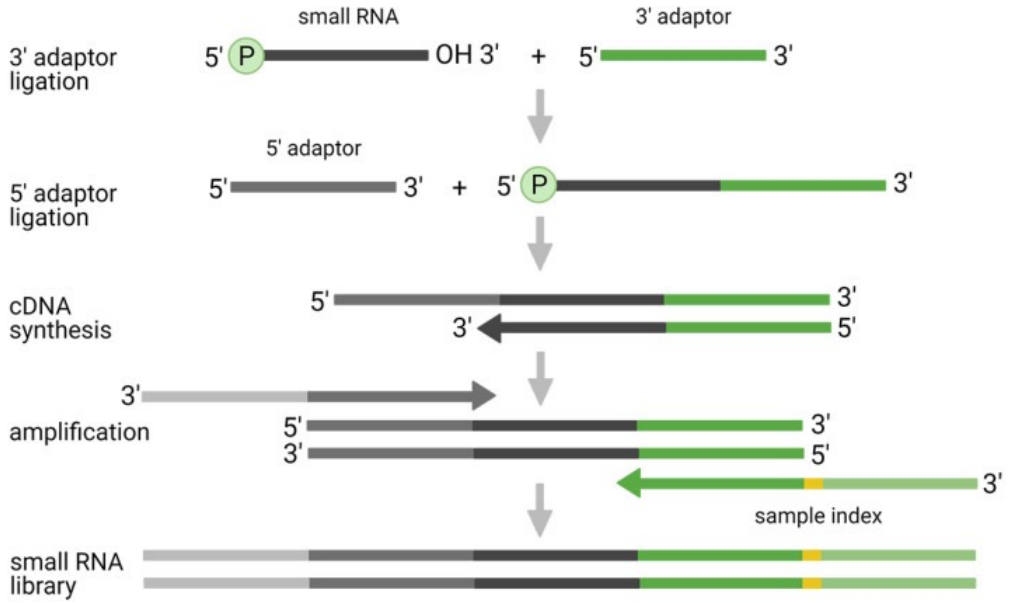
Small RNA sequencing

Advantages

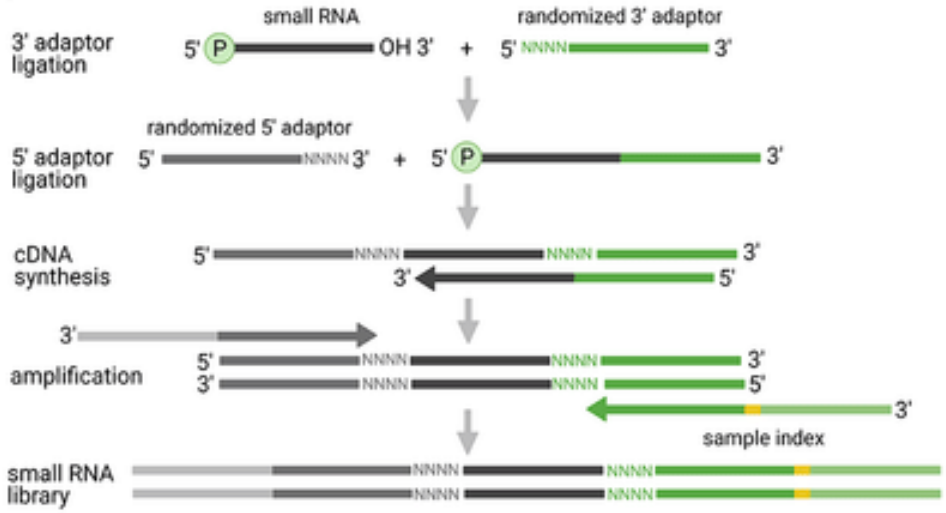
- simultaneous detection of all miRNAs
- including novel and isomiRs

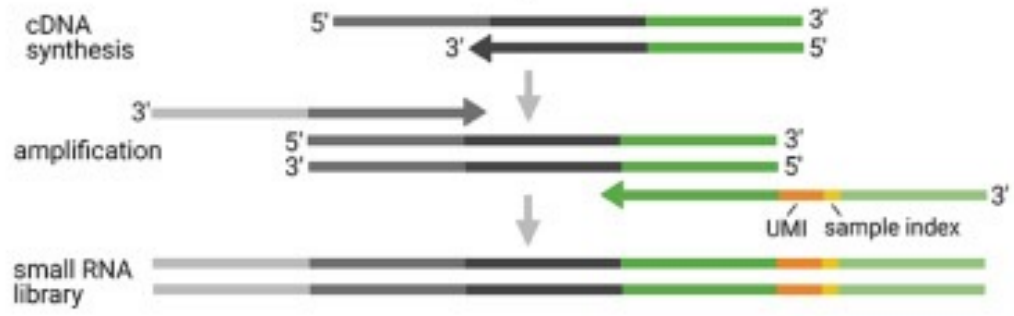
Disadvantages

- various types of biases
- demanding computational analysis



→ *many new small RNA-seq approaches…*





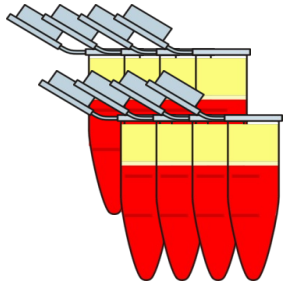
Benesova, S. et al. *Diagnostics* 11.6 (2021)

Small RNA sequencing – sample preparation

1. SAMPLES

8 breast cancer patients

2× 400 µl of plasma



2. ISOLATION

miRNeasy Serum/Plasma Kit

3. QUALITY CONTROL

I. **Isolation homogeneity**
- Cel-miR-39
- Ath-miR-159a

II. **Hemolysis rate**
- A414 < 0.25
- **dCt miR-451a – miR-23a < 10** (*Blondal et al. 2013*)

4. LIBRARY PREPARATION

- **NEXTFLEX Small RNA-Seq Kit (Bioo Scientific)**
- **QIAseq miRNA UDI Library Kit (Qiagen)**

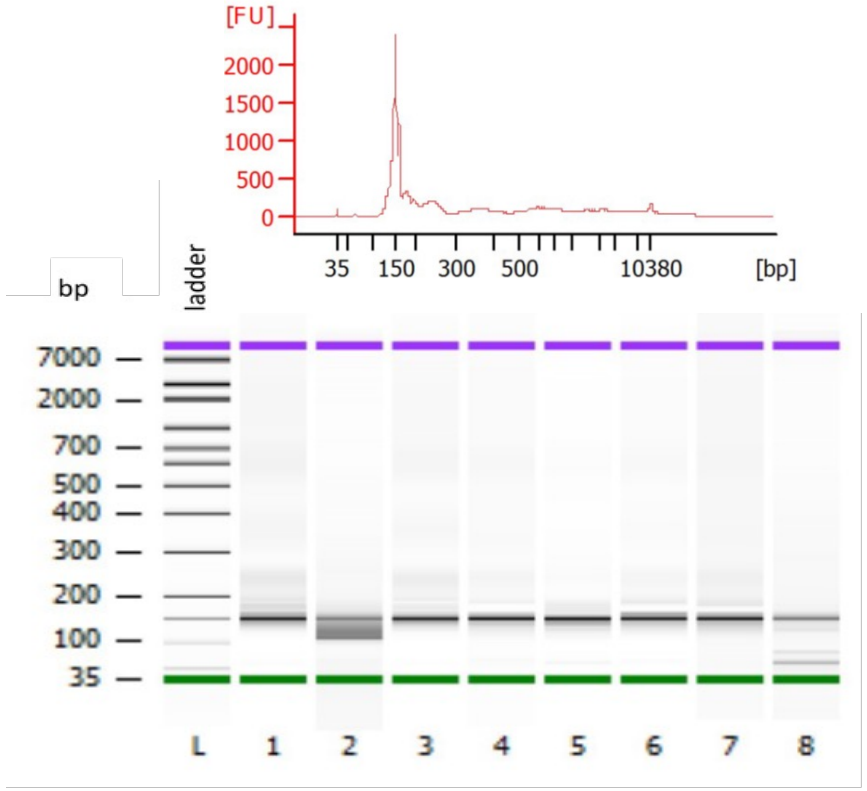
Sample	ng/µl	Ct			Δ Ct miR-451 miR-23a
		cel-39-3p	ath-159a	miR-16-5p	
1	3.0	20.1	27.7	20.5	3.7
2	2.2	20.3	27.9	21.4	7.2
3	2.9	20.2	27.8	20.6	7.4
4	3.0	20.1	27.6	20.9	7.1
5	2.3	19.7	27.3	21.7	6.0
6	2.1	20.5	27.4	21.3	8.2
7	3.0	20.4	27.6	21.5	6.4
8	2.3	20.3	28.3	22.0	8.3

Small RNA sequencing – NEXTFLEX Small RNA-Seq Kit

- Qubit™ dsDNA HS Assay Kit

Sample	Library conc. (ng/µl)	Fragment lenght (bp)
1	44.2	152
2	1.9	152
3	41.2	150
4	40.8	152
5	22.6	151
6	38.2	152
7	44.0	152
8	1.1	152

- Agilent 2100 Bioanalyzer



- NextSeq 500/550 High Output Kit v2.5, 75 cycles (Illumina)

Small RNA sequences

- **Raw data**

Sample	Number of sequences (mil)	Duplicate (%)	Unique (%)
1	23.9	83.8	16.2
2	4.9	78.3	21.7
3	24.2	84.8	15.2
4	18.2	83.9	16.1
5	17.5	88.9	11.1
6	20.7	83.9	16.1
7	16.8	85.0	15.0
8	9.1	92.4	7.6

Removal and filtering of…

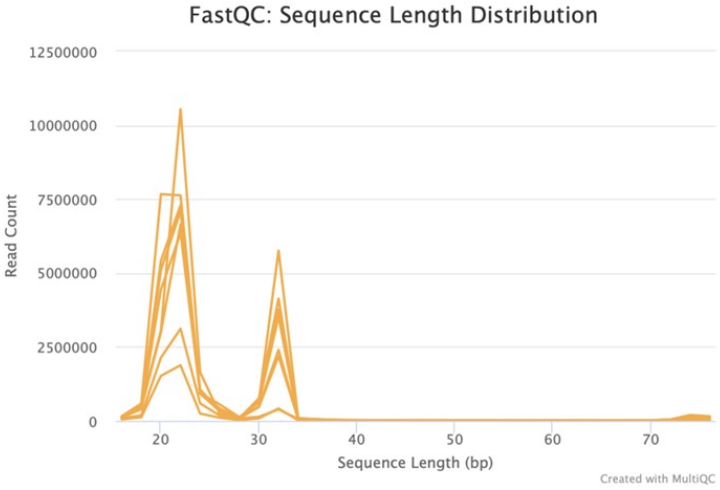
- adapter sequences
- < 16 bp sequences
- 3‘ end with quality < Q20
- mitochondrial rRNA and tRNA

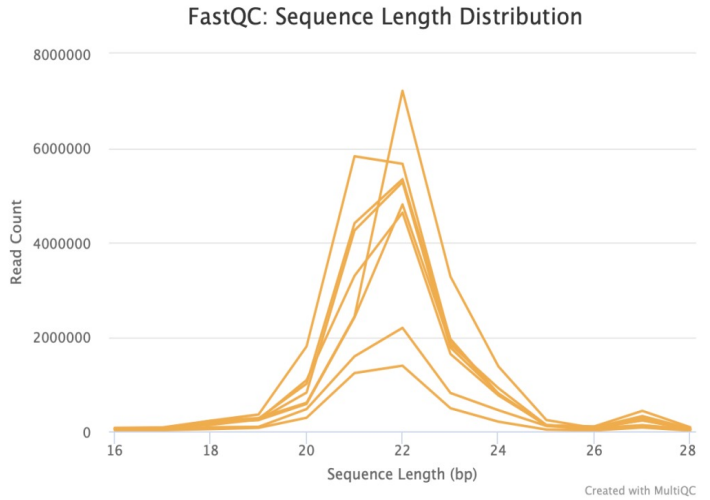
- > 28 bp sequences

… for selection of miRNAs

- **66.3 – 79.6 % of all sequences**

  (71.6 % on avarage)



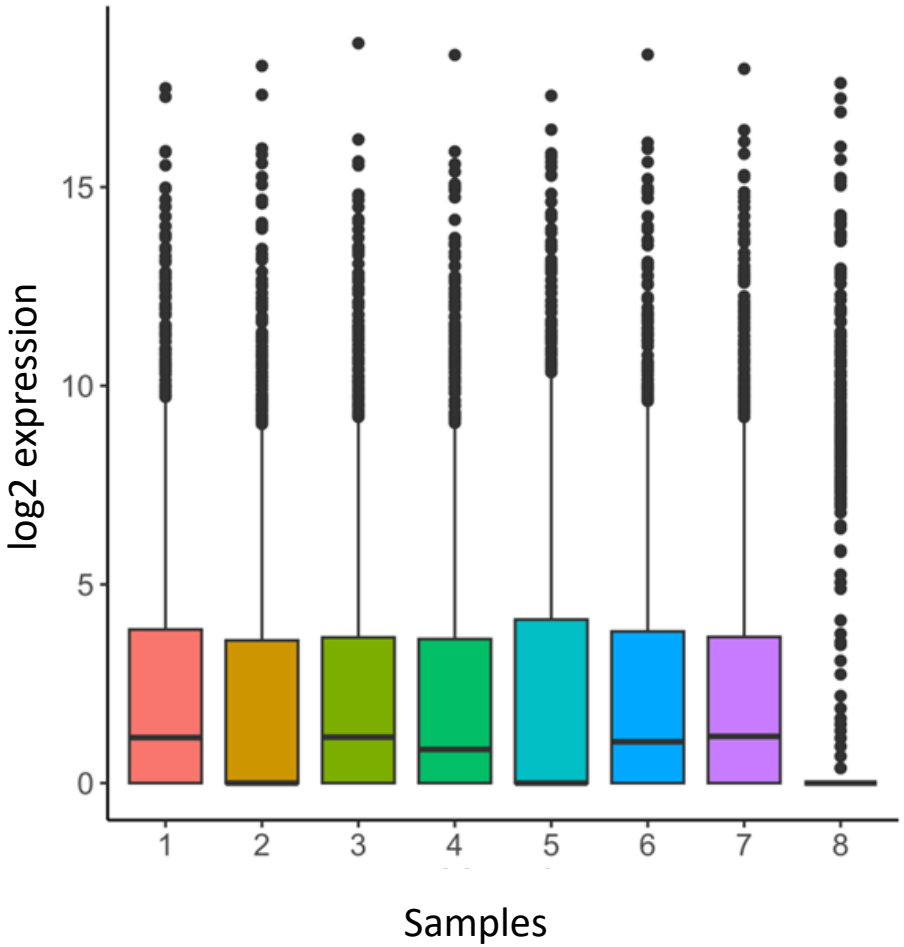


Small RNA sequencing - miRNAs

- **16 – 28 bp in length**
- **Mapping with BWA to miRBase v22.1**

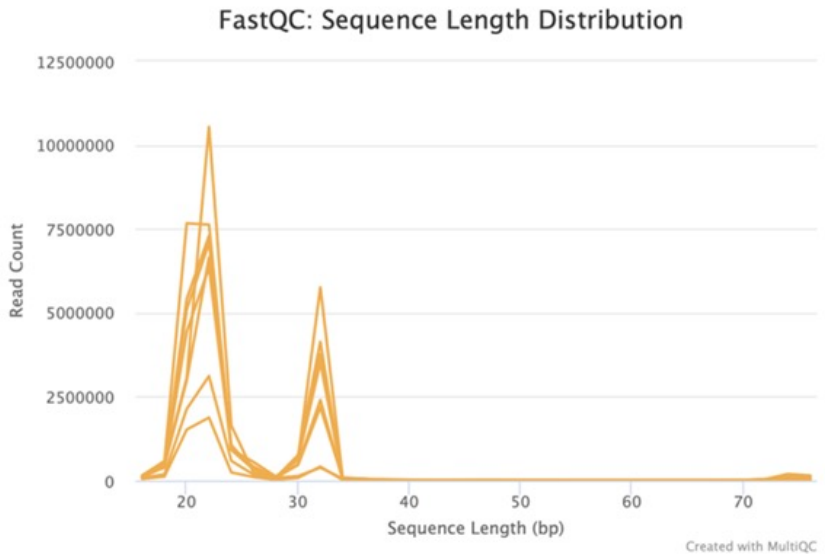
Sample	% of mapped miRNA sequences	% of all sequences	Number of detected unique miRNAs
1	61.9	41.4	651
2	71.5	57.3	530
3	75.5	53.8	640
4	71.4	55.0	598
5	63.6	42.1	543
6	70.2	50.3	617
7	70.6	52.1	648
8	55.0	1.6	161

- **log2 expression levels**
- Normalisation by CPM method



Small RNA sequencing - piRNAs

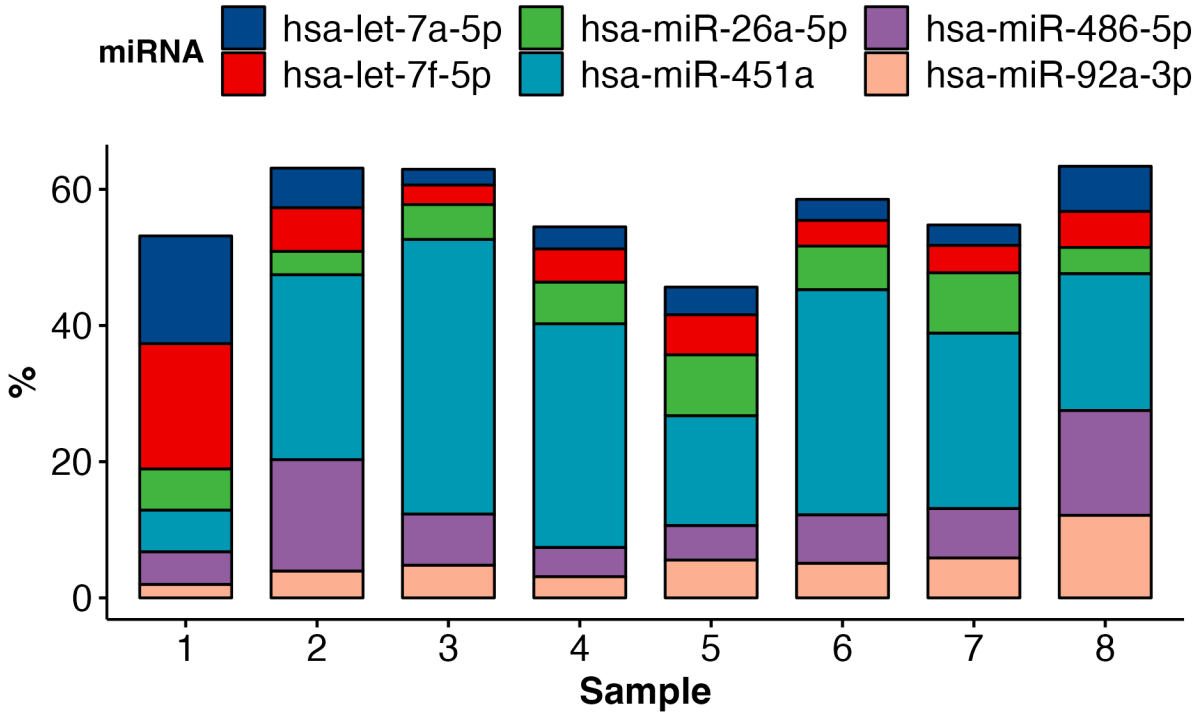
- **10 – 36 bp in length**
- **Mapping with BWA to piRNAdb v1.7.5**



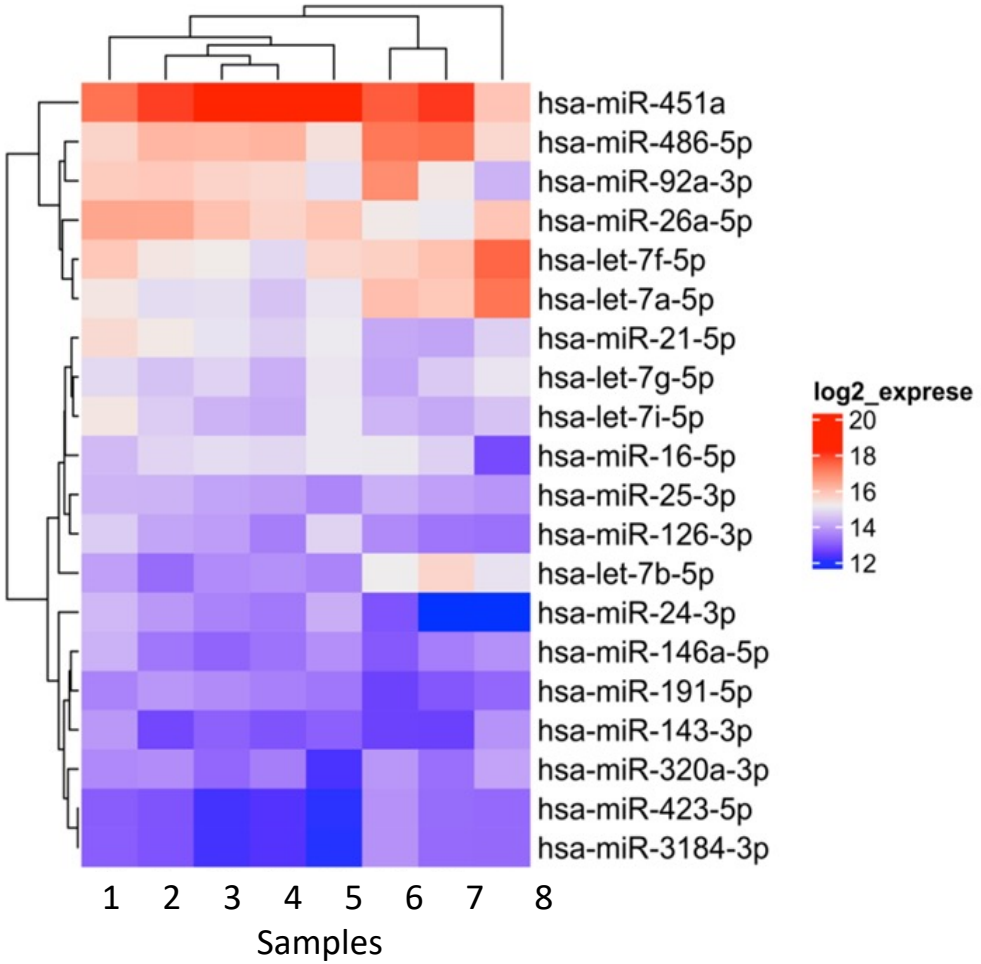
Sample	% of mapped piRNA sequences	% of all sequences	Number of detected unique piRNAs
1	20.5	19.7	459
2	15.1	14.0	304
3	7.0	6.5	493
4	10.4	9.8	397
5	9.2	8.6	288
6	9.1	8.5	470
7	8.8	8.1	380
8	11.3	8.8	79

Small RNA sequencing – the most represented miRNAs

- represented more than 5% within the sample

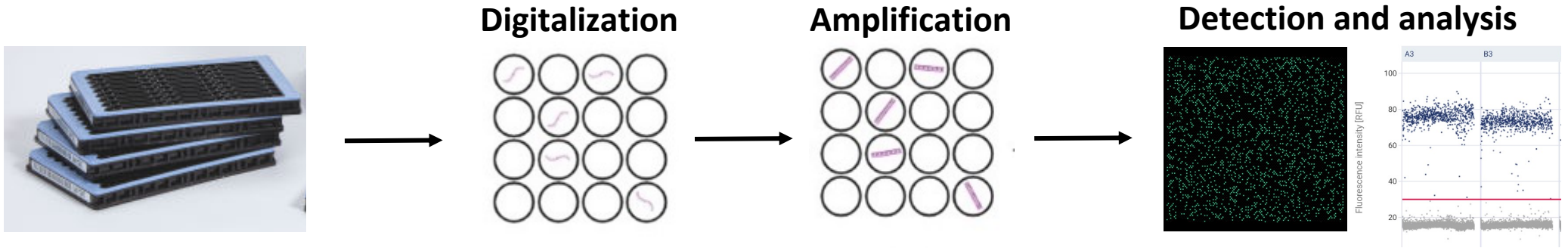


- The 20 most expressed miRNAs



Small RNA sequencing *vs* ddPCR

- QIAcuity (Qiagen), QIAcuity software suite (v2.1.8.23, Qiagen)



ADVANTAGES

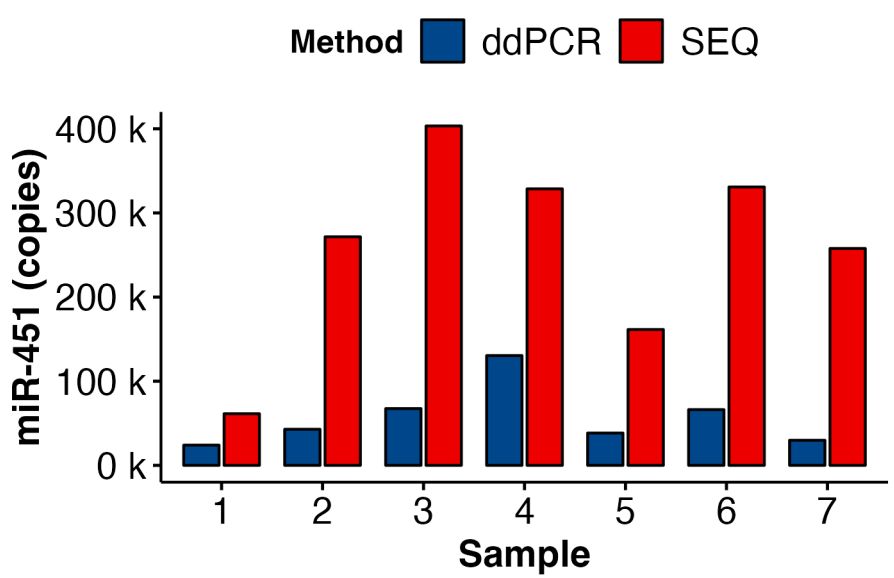
- absolute quantification
- no standard curve required
- more tolerant to some PCR inhibitors
- small fold change differences can be detected

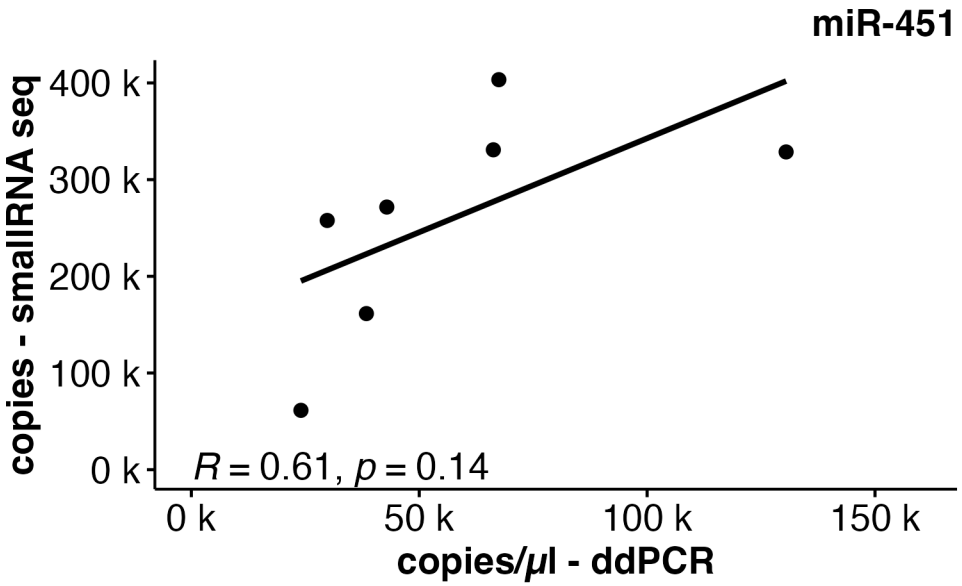
DISADVANTAGES

- targeted analysis
- lower dynamic range
- higher price

Small RNA sequencing *vs* ddPCR

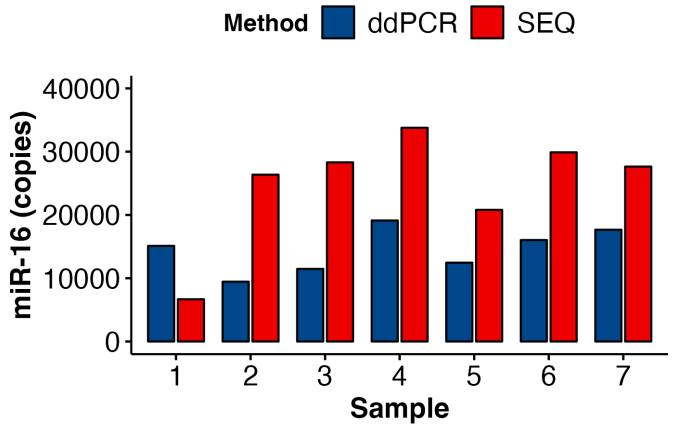
- **The highly expressed miRNAs in plasma**
- hsa-miR-451

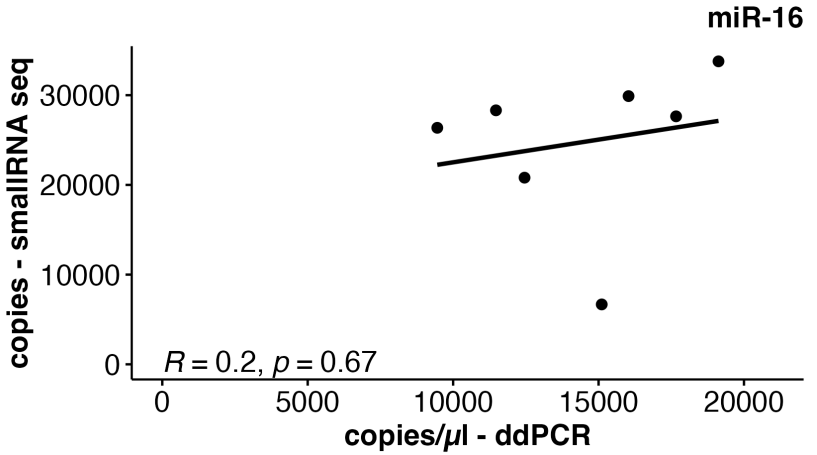




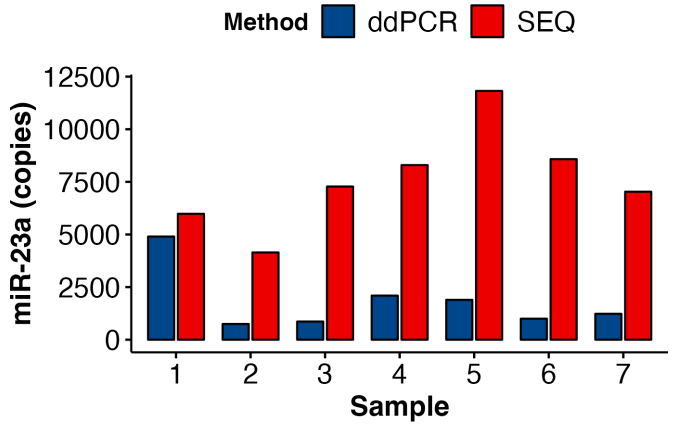
Small RNA sequencing *vs* ddPCR

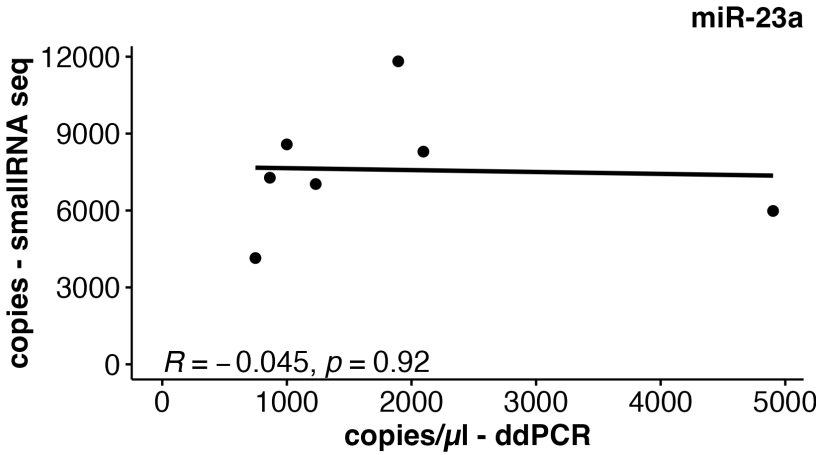
- **miRNAs with medium or low expression in plasma**
- hsa-miR-16





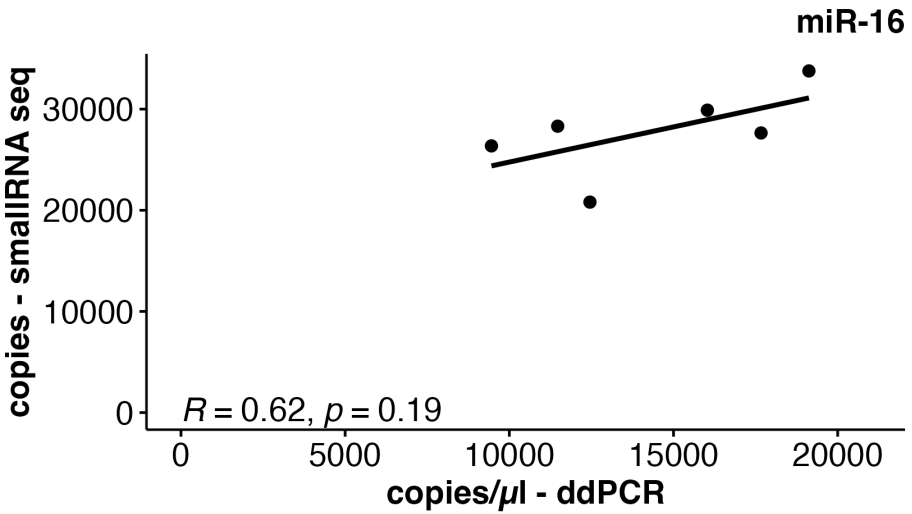
- hsa-miR-23a



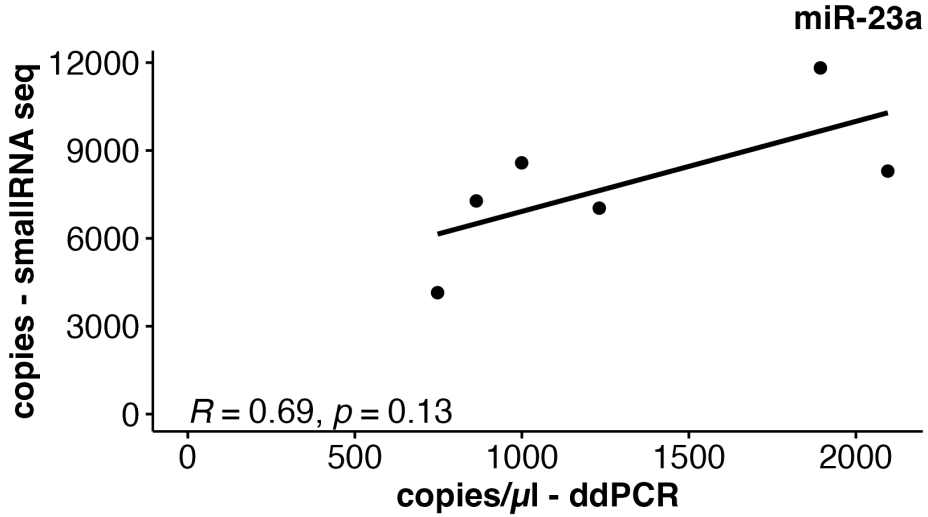


Small RNA sequencing *vs* ddPCR

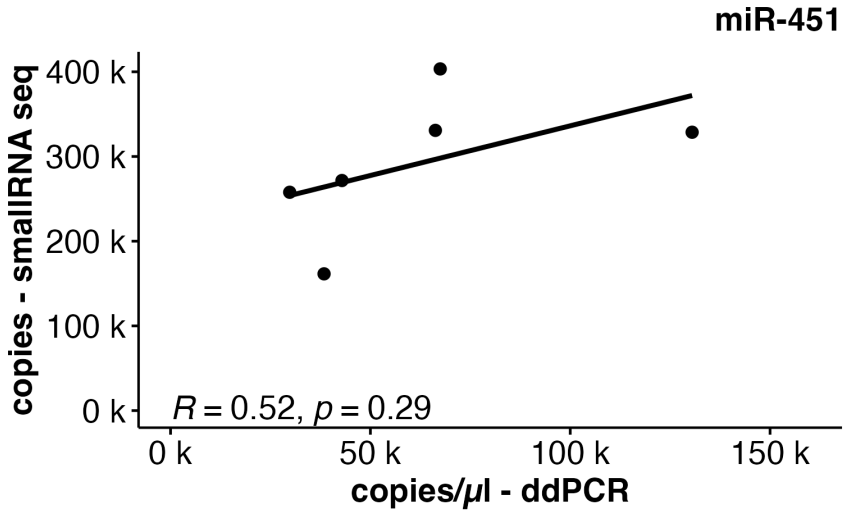
- **hsa-miR-16**



- **hsa-miR-23a**



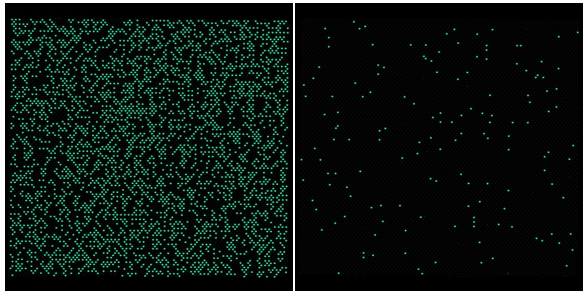
- **hsa-miR-451**



Conclusions

- Approximately **50% of the detectable small RNA sequences** in plasma consist of **miRNA**.
- The average proportion of detectable sequences represented by **piRNA molecules is 10%**.
- **The careful choice of methods** for verification of the quality of samples and for small RNA sequencing results is essential.
- **Accuracy of quantification of miRNAs by NGS differs for high versus medium and low abundant miRNA molecules.**

Mutation detection in cfDNA using ddPCR



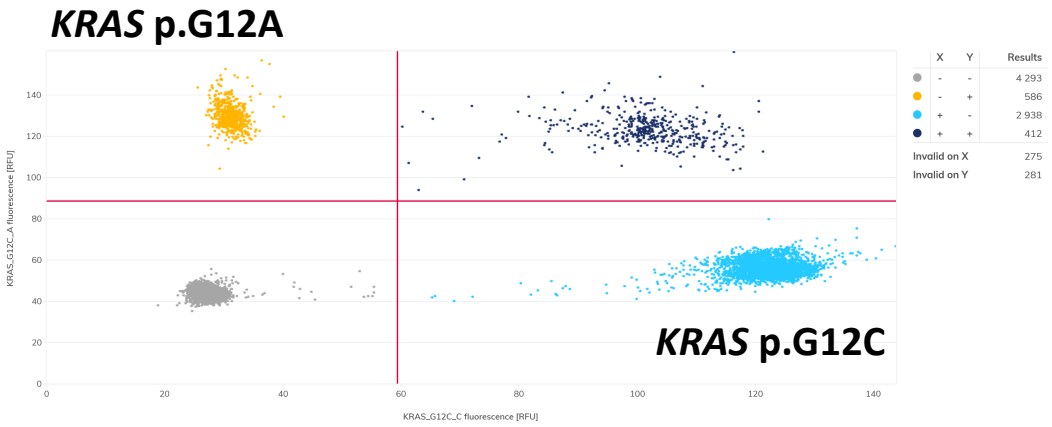
- **Optimization of dPCR for detecting *KRAS* and *TP53* mutants**
- **Colorectal and ovarian cancer patients**

KRAS c.34G>T; p.G12C
KRAS c.35G>A; p.G12D
KRAS c.35G>T; p.G12V
KRAS c.38G>A; p.G13D

TP53 43687; c.641A>G
TP53 10758; c.659A>G
TP53 10660; c.818G>A

METHODOLOGICAL WORKFLOW

- 1 ml of plasma sample
- QIAamp Circulating Nucleic Acid Kit
- Qubit dsDNA HS Assay Kits
- QIAcuity dPCR



Acknowledgements

STÁTNÍ ZDRAVOTNÍ ÚSTAV
SZÚ
BIOMEDICÍNSKÉ CENTRUM

Pavel Souček
Radka Václavíková
Marie Ehrlichová
Viktor Hlaváč
Simona Šůsová
Petr Holý
Alžběta Kloudová
Karolína Šeborová
Tereza Tesařová
Ivona Krus
Tomáš Sychra

PŘÍRODOVĚDECKÁ FAKULTA
Univerzita Karlova

Lukáš Kobrle

ÚPMD
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Thank you!

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