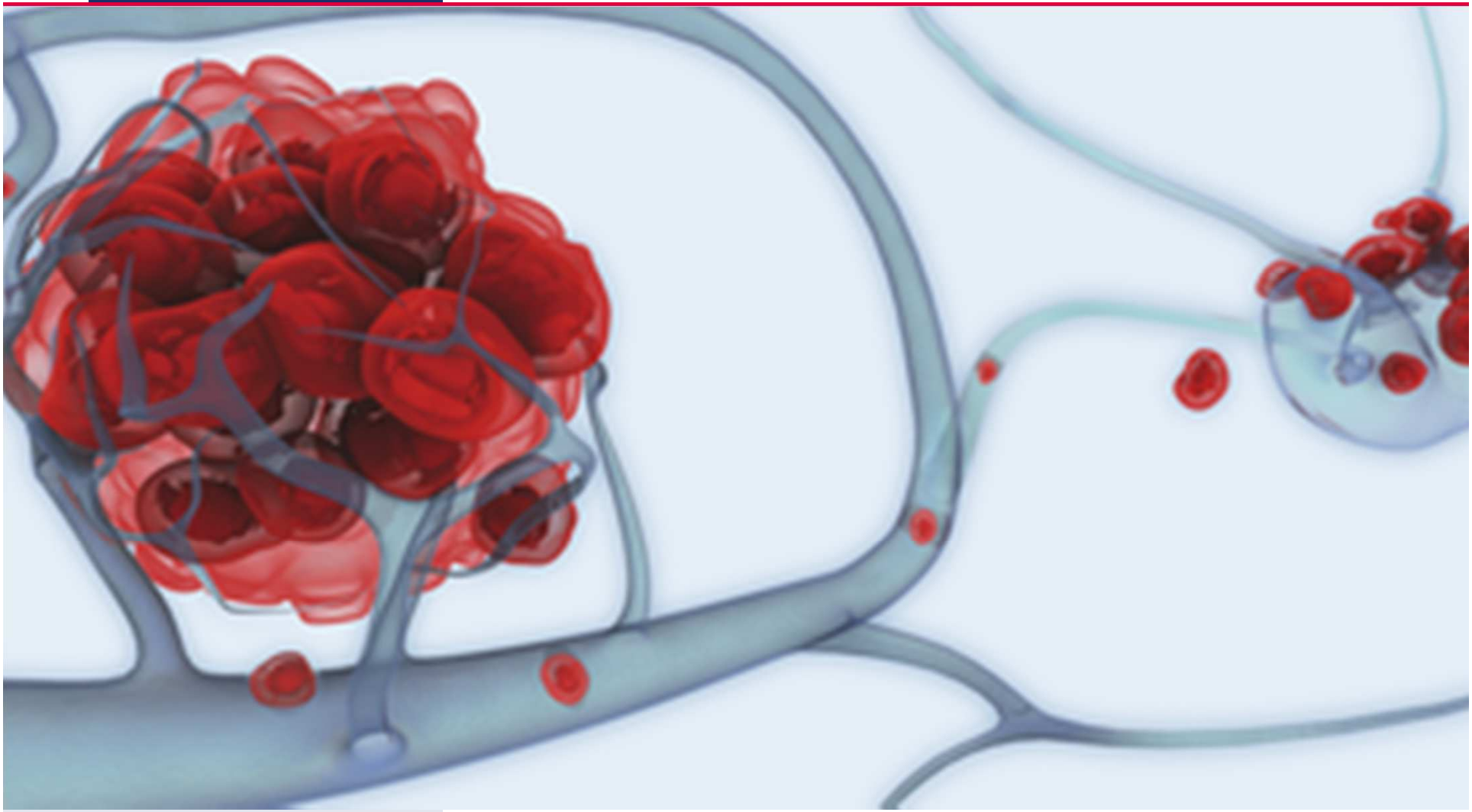
Sample & Assay Technologies



Circulating Nucleic Acids in Cancer

26.10.2012

Marco Polidori

Agenda

- Background on fcNA
  - What is fcNA
- Principle
  - Where does fcNA come from in cancer
- Known Alterations
  - That may be associated with cancer
- Advantages of analyzing fcNA
  - What makes fcNA so interesting as diagnostic marker
- fcNA associated biomarkers
  - What examples exist already
  - What may be preferred detection methods
- Challenges of finding Biomarkers
  - What should be considered when looking for new biomarkers
  - Questions about the baseline
- Extraction methods
  - What we can learn from pre-natal diagnostics
  - Our experience on extraction methods

Background

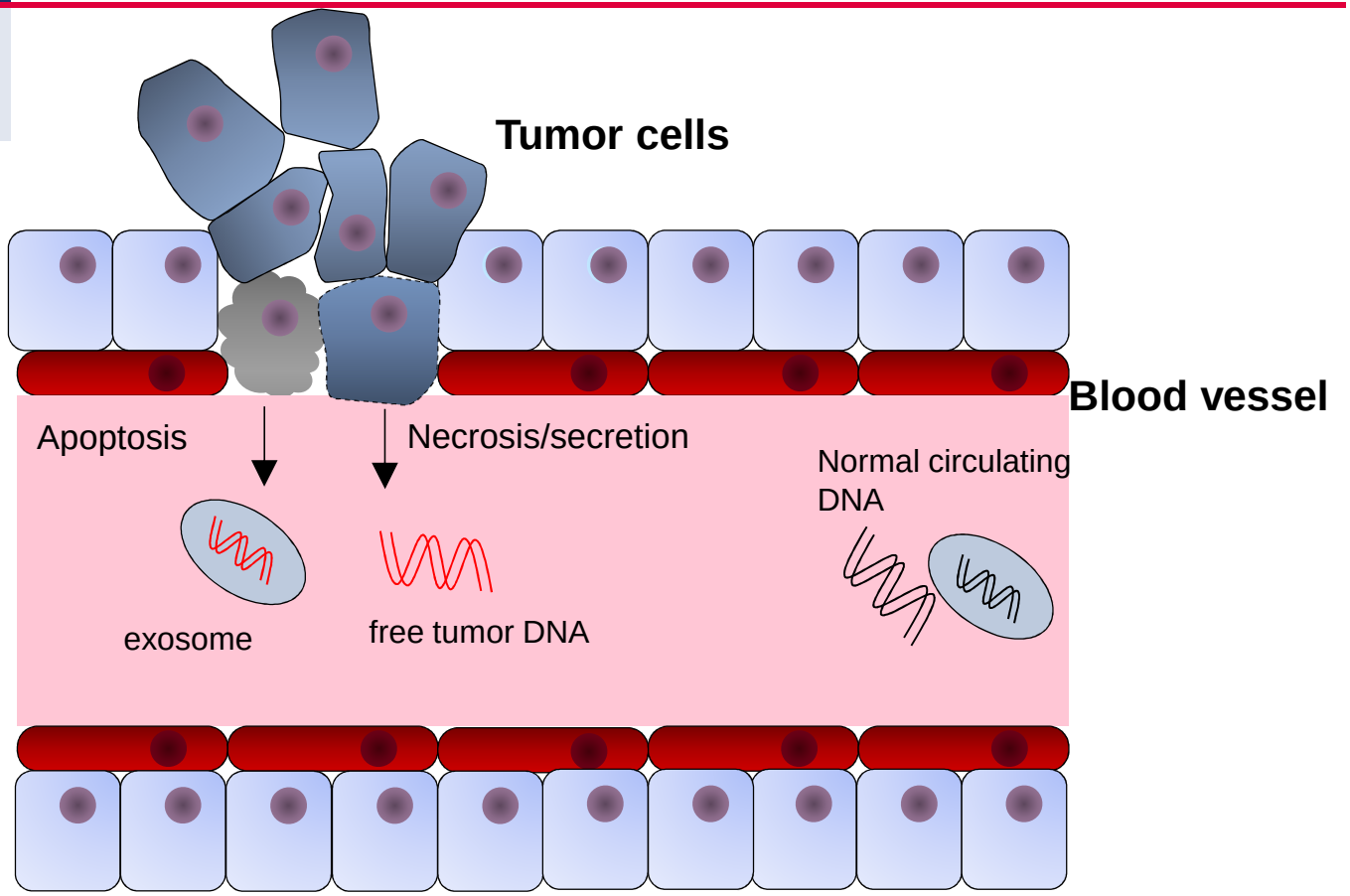
What are free circulating nucleic acids (fcNA) ?

Cell-free nucleic acids present in body fluids like serum/plasma, urine, CSF etc.

Properties

- Cell free DNA, mRNA and miRNA
- Mostly present as shorter fragments (less than 1000 bp or nt)
  - Circulating DNA: predominantly in the range of 140-100 bp
- Can also be several kb long
- Low concentration in plasma and serum (1-100 ng/mL)
- Can be protein-bound (nucleosomes)
- Can be “vesicular” (exosomes)
- Half life of minutes to several days (EDTA plasma)

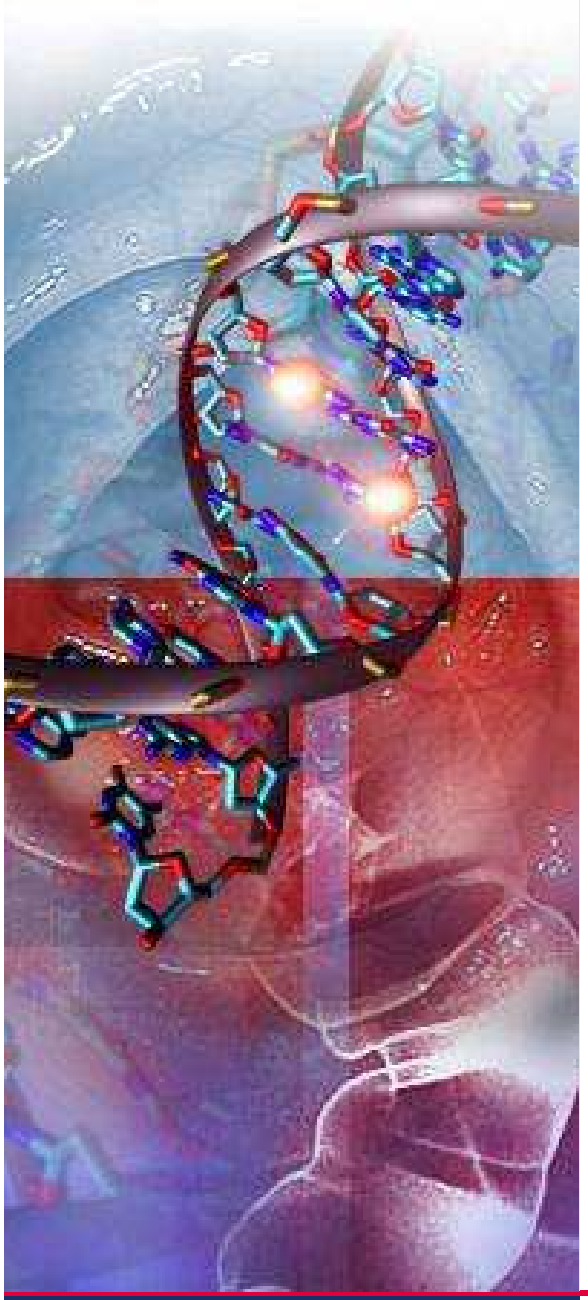
Principle of cancer associated fcDNA



Release of DNA is thought to occur through

- Apoptosis
- Necrosis
- Active secretion

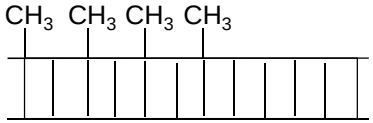
Alterations of fcNA



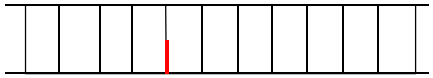
Various alterations of fcNA that may indicate disease MH3

Main alterations known

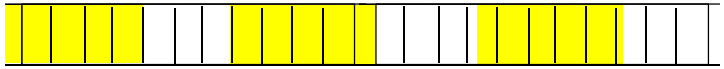
- Methylation (epigenetic alterations)



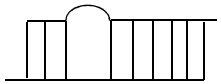
- Mutation (i.e. oncogenes like *KRAS*)



- Microsatellite alterations



- miRNA expression levels

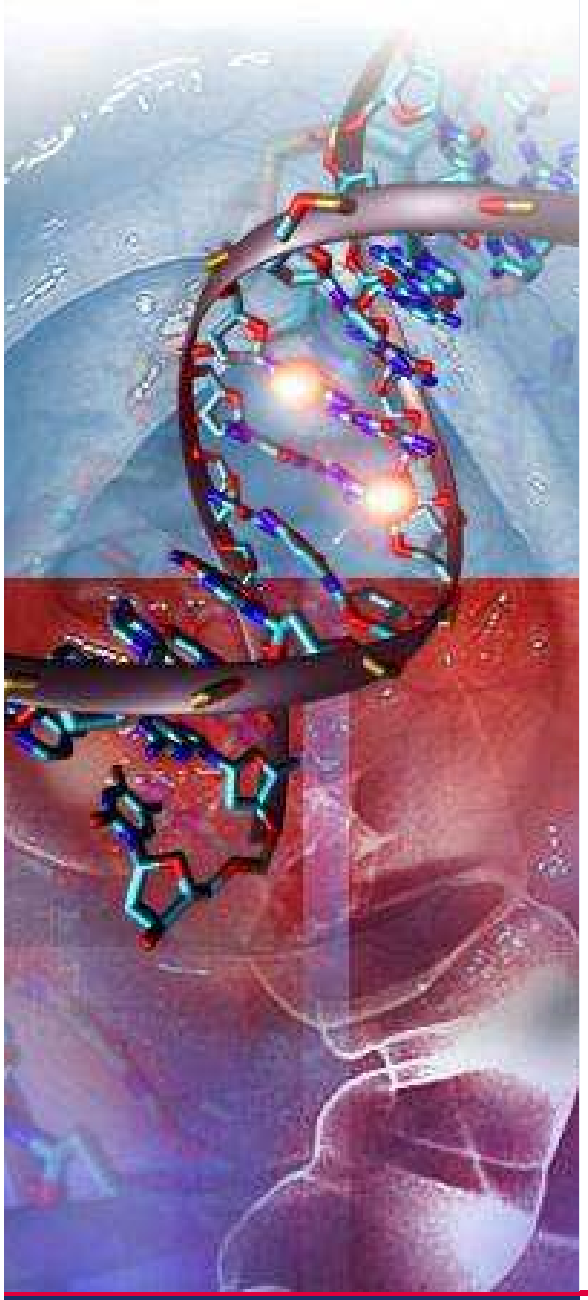


MH3

also:
-- copy number variations
-- chromosomal aneuploidies detectable in fetal ccfDNA in maternal plasma

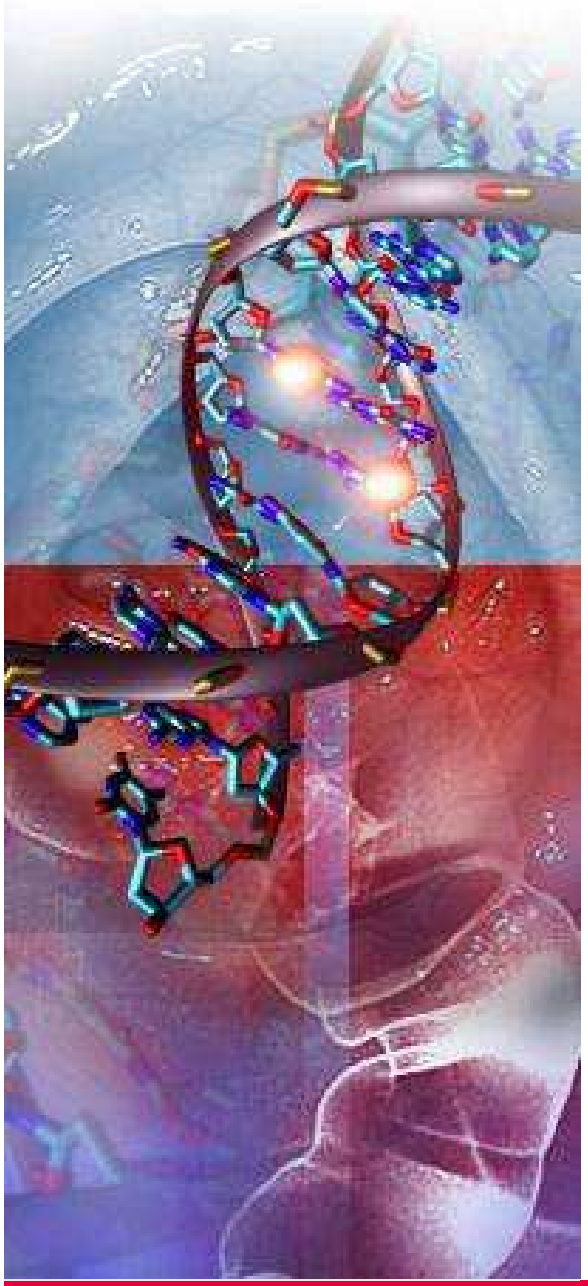
Martin Horlitz; 23.10.2012

Advantages of fcNA as analyte



- Minimally invasive
- Blood is a readily available sample material
- DNA from distant metastatic sites is also being captured
- Can also be used recurrently during treatment monitoring

fcNA associated biomarkers



Many biomarkers have been found to be associated with cancer

Examples include

- Breast (e.g. PIK3CA)
- Prostate (e.g. miR 21/141, GSTP1 methylation)
- Lung (e.g. SHOX2 methylation, EGFR)
- Ovarian (e.g. TP53)
- Colorectal (e.g. SEPT9 methylation, KRAS)
- Pancreatic (methlyation pattern of several markers including SOCS1, CCND2 and others)
- Melanoma (e.g. BRAF)

And several others

Don’t forget about

- CNVs
- Chromosomal rearrangements

Challenges for establishing biomarkers

- "The needle in the haystack"
  - What markers are already known from biopsies for a certain cancer type?
  - Can it also be detected in fcNA
- fcNA concentrations are usually very low
  - assays have to be extremely sensitive
  - Have to be detectable against high background of WT DNA
- fcNA levels increase in advanced stages of cancer
  - Early diagnostics can be challenging using fcNA since concentrations are too low in these stages
- High diversity of cancer genomics may require novel approaches to detect
  - e.g. mutations with low allelic frequency
- Variable sample processing methods are being used
  - Uniform blood collection & extraction method needed

More studies needed with large cohorts

Finding the bigger picture

Do we know enough about the general biology of fcNA?

More studies are required to understand the natural occurrence of fcNA:

- Which factors influence formation of cNA in healthy humans?
- General health, implications of other common diseases (e.g. infections)
- Circadian rhythms
- Age
- Way of life (nutrition, sports, etc.)

Shall help finding better guidelines

- When is it best to draw blood for diagnostic testing?
- Take other implications into account (when to not use fcNA)

Reduce background as much as possible

Extraction methods

Uniform extraction method needed

Blood stabilization agents?

serum or plasma?

How much serum/plasma?

Size cut-offs?

What we can learn from pre-natal DX

Looking beyond the horizon of cancer could be beneficial. Pre-natal Diagnostics is already well established.

K_3EDTA tubes (Barrett et al.)

Use Plasma (if applicable)

As much sample as possible (>1 ml)

- Whole Genome Sequencing shown to work from maternal plasma (Kitzman et al.; Fan et al.)

Also applicable for cancer research!

- Massively parallel sequencing (targeted sequencing using plasma from cancer patients (Forshew et al.; Morgan et al.)

- WGS approach on plasma from cancer patients (Chan et al.)

QIAamp circulating NA Kit

- High input volumes possible (up to 5 ml sample)
- No size bias: small and large fragments are recovered
- Optimized serum/plasma protocol
- Compatible with urine
- miRNA protocols available

QIAamp circulating Nucleic Acid Kit workflow

4 – 5 ml plasma or serum: lysis & protein digestion

add binding buffer to lysate: Bind DNA & RNA to QIAamp Mini column (QIAvac 24 Plus)

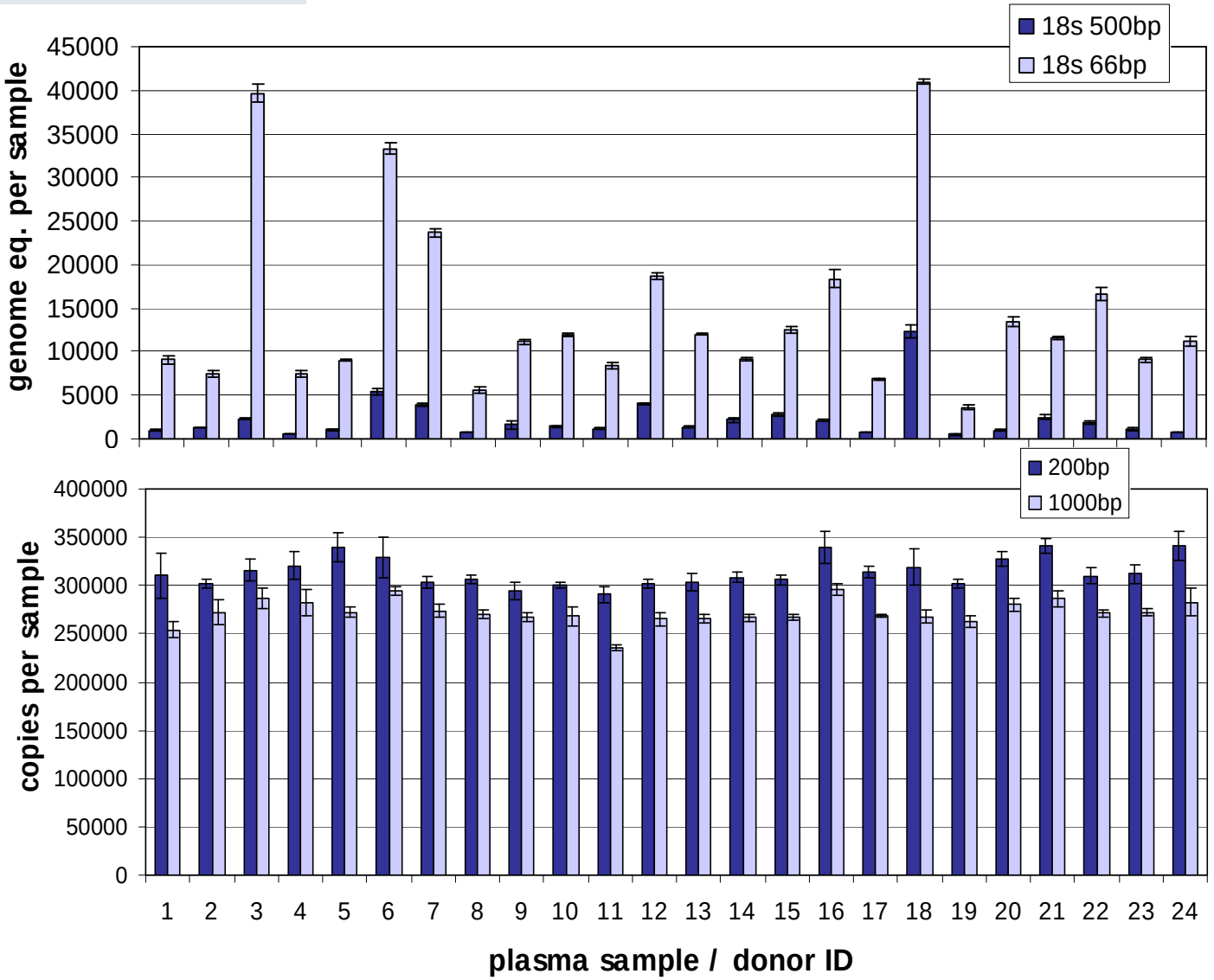
On-column DNase I digestion (optional)

Wash steps on QIAvac 24 Plus

DNA / RNA containing all fragment sizes

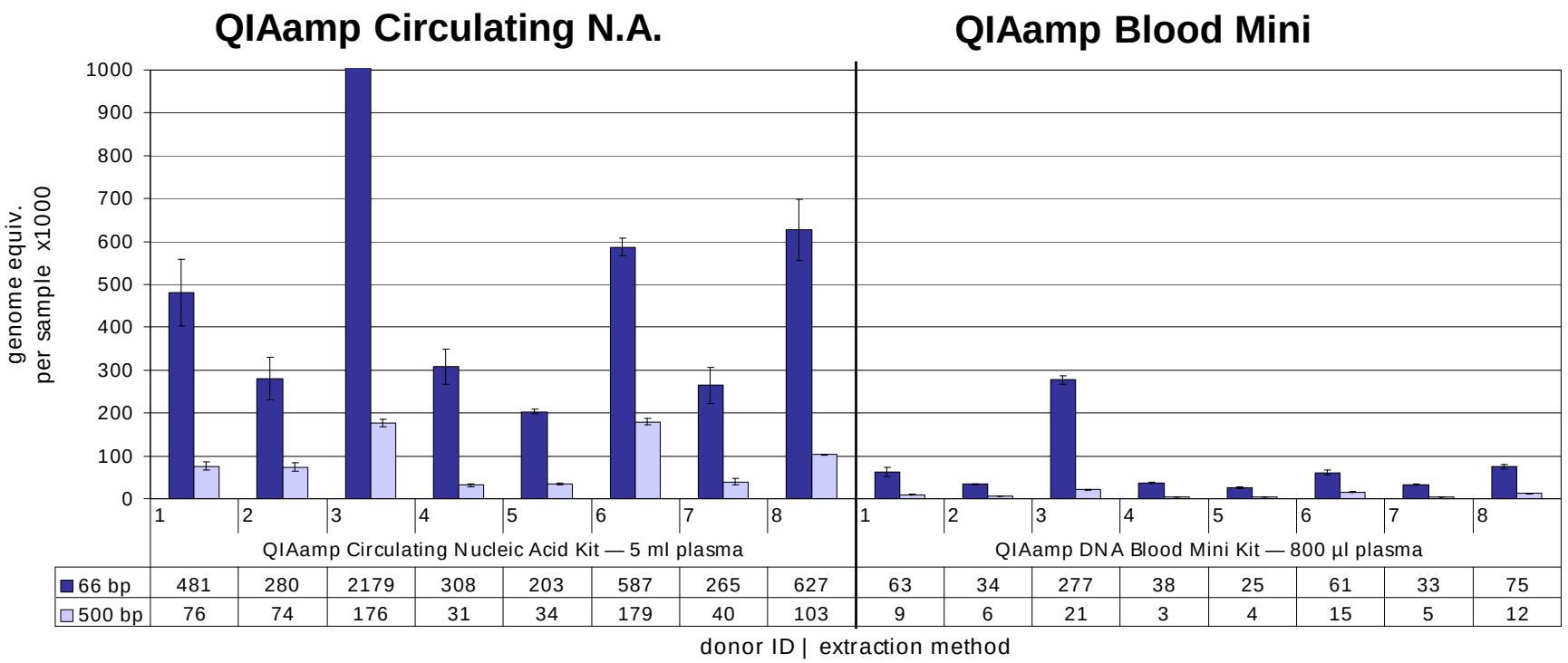
20 – 150 µl

Recovery rates of fcNA



QIAamp cNA vs. QIAamp Blood Mini

Enabling large volume preparations increases performance drastically

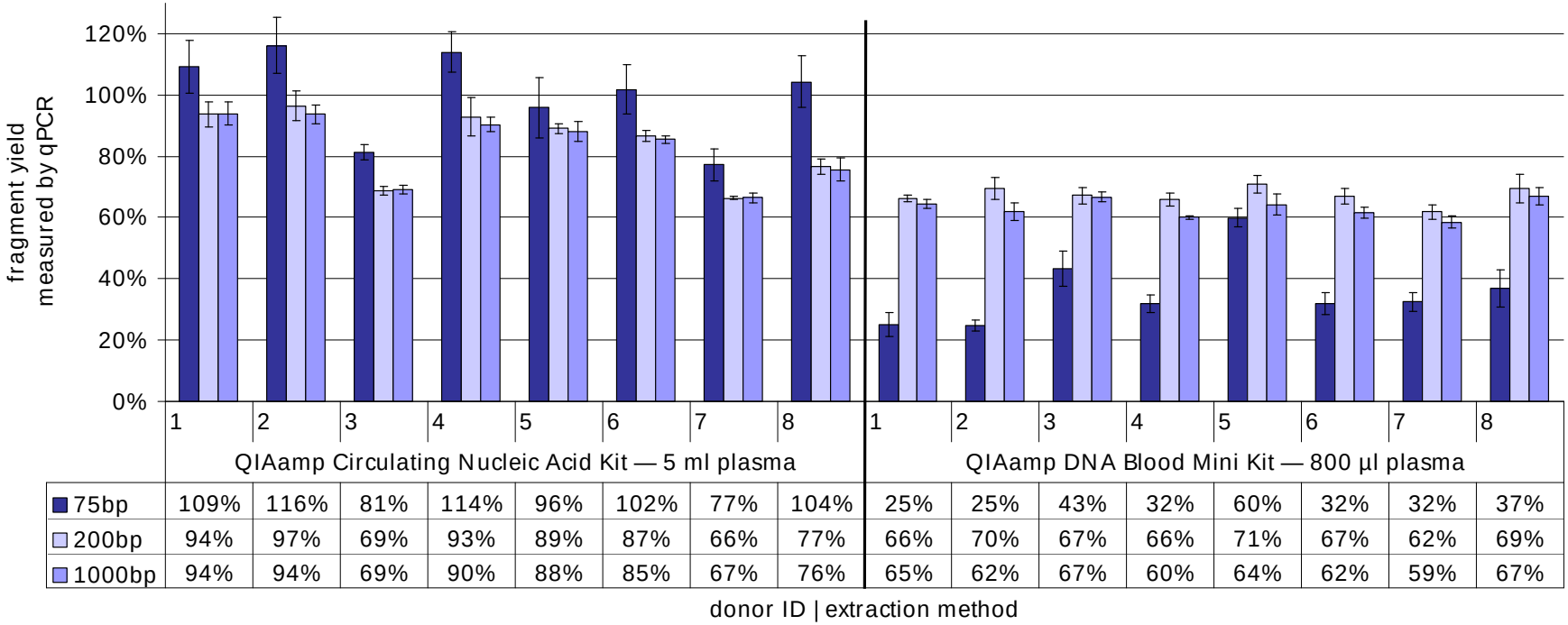


Circulating DNA in EDTA plasma — individual donors

- **Circulating DNA yield: 18S rDNA PCR — 66 bp and 500 bp amplicons**
- **Comparison to QIAamp DNA Blood Mini Kit (800 µl protocol)**
  - **Chemistry of QIAamp C.NA kit improves recovery of circulating DNA beyond the effect of higher sample volume**

QIAamp cNA vs. QIAamp Blood Mini

No size bias of the QIAamp circulating NA Kit

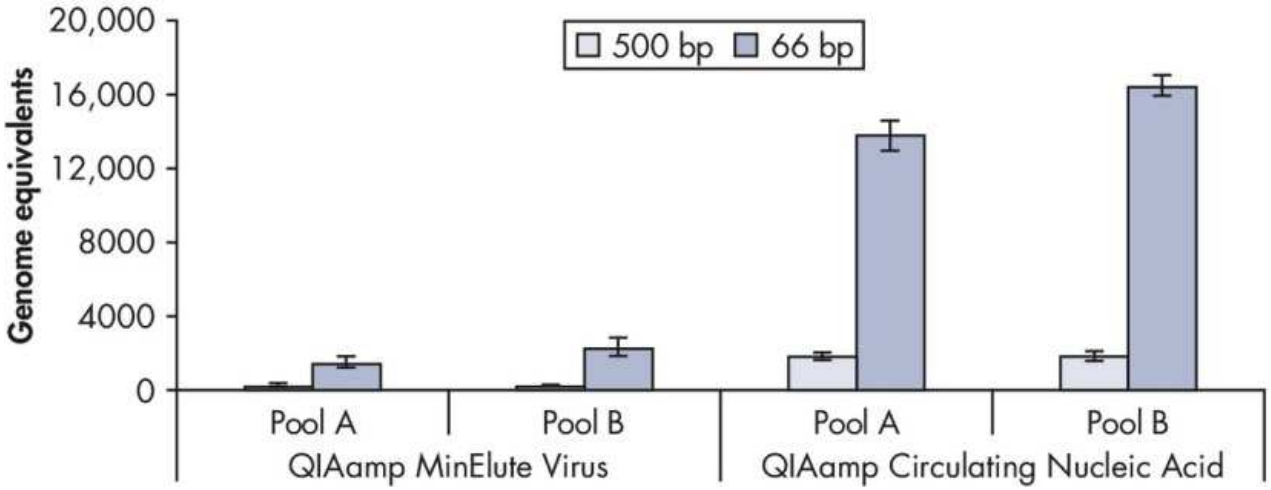


	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
75bp	109%	116%	81%	114%	96%	102%	77%	104%	25%	25%	43%	32%	60%	32%	32%	37%
200bp	94%	97%	69%	93%	89%	87%	66%	77%	66%	70%	67%	66%	71%	67%	62%	69%
1000bp	94%	94%	69%	90%	88%	85%	67%	76%	65%	62%	67%	60%	64%	62%	59%	67%

Recovery of spiked DNA fragments from EDTA plasma — individual donors

- **75 bp, 200 bp 1000 bp DNA added to plasma before extraction (200,000 copies)**
  - **Chemistry of QIAamp C.NA kit improves recovery of DNA fragments shorter than 200 bp**

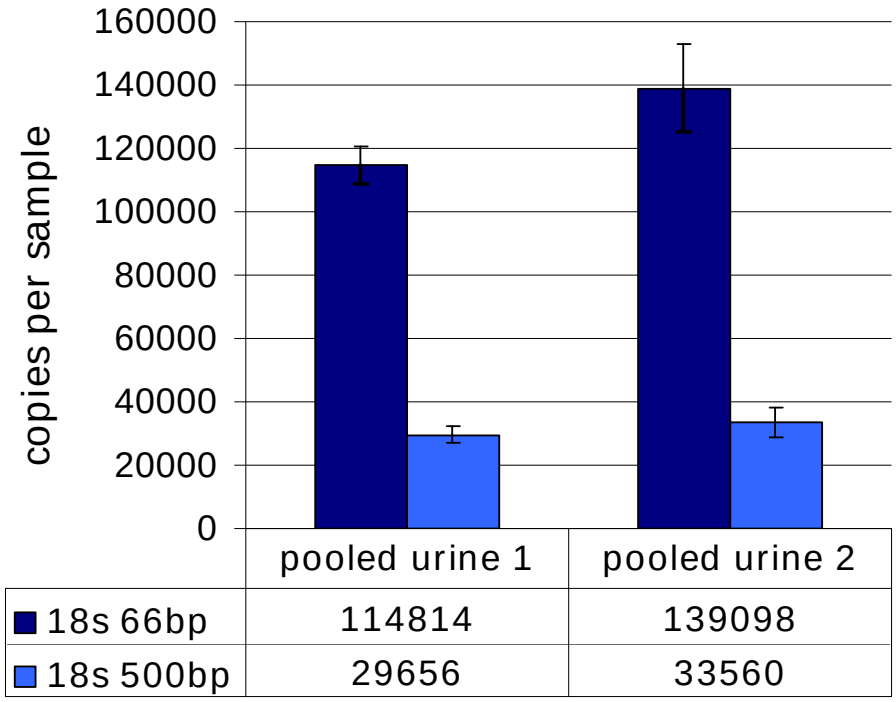
QIAamp cNA vs. QIAamp MinElute Virus



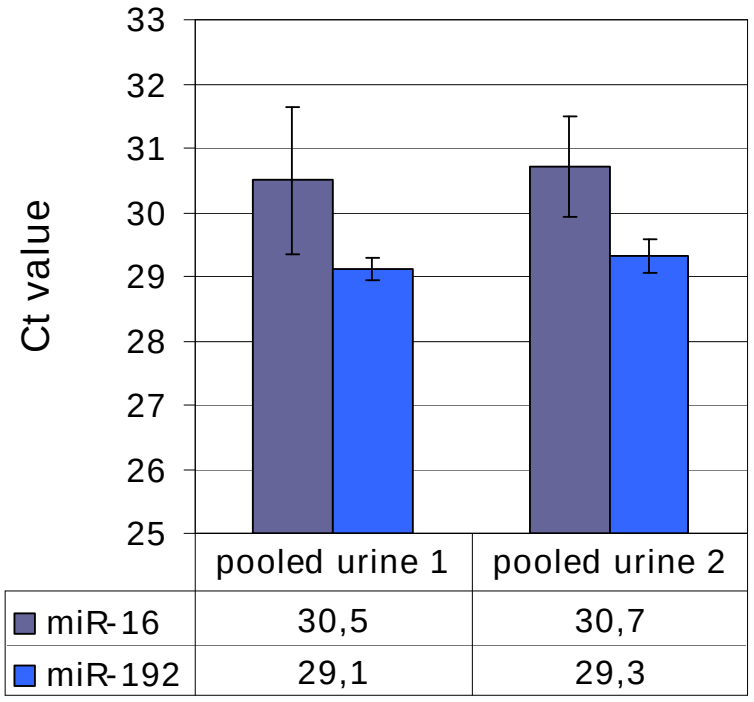
Circulating DNA in EDTA plasma — pooled plasma

- **Circulating DNA yield: 18S rDNA PCR — 66 bp and 500 bp amplicons**

cfDNA in Urine



	pooled urine 1	pooled urine 2
18s 66bp	114814	139098
18s 500bp	29656	33560



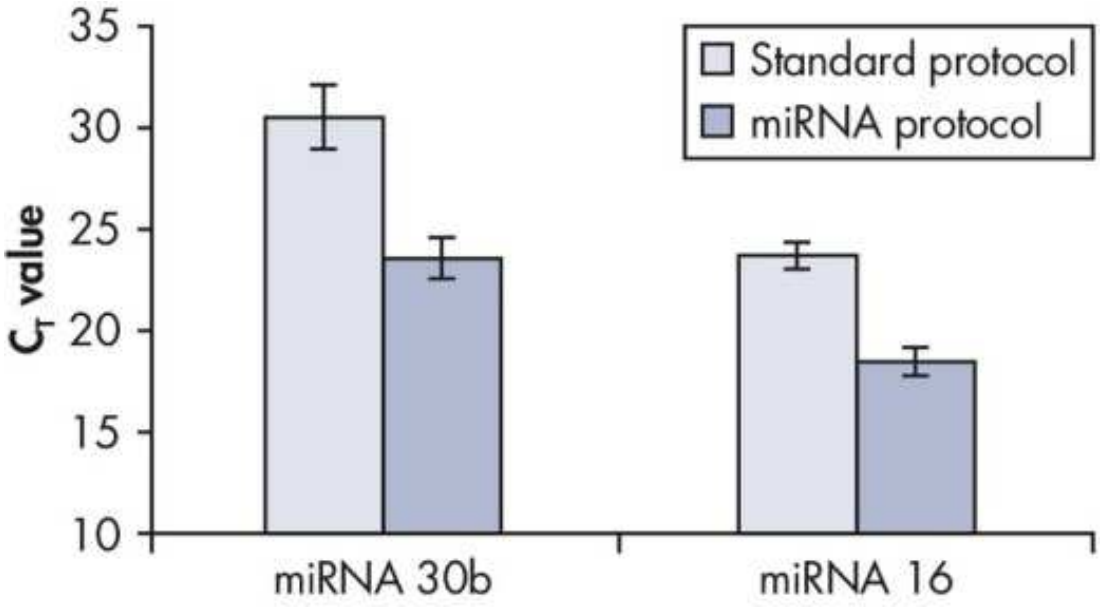
	pooled urine 1	pooled urine 2
miR-16	30,5	30,7
miR-192	29,1	29,3

Circulating DNA and miRNA in pooled urine samples:

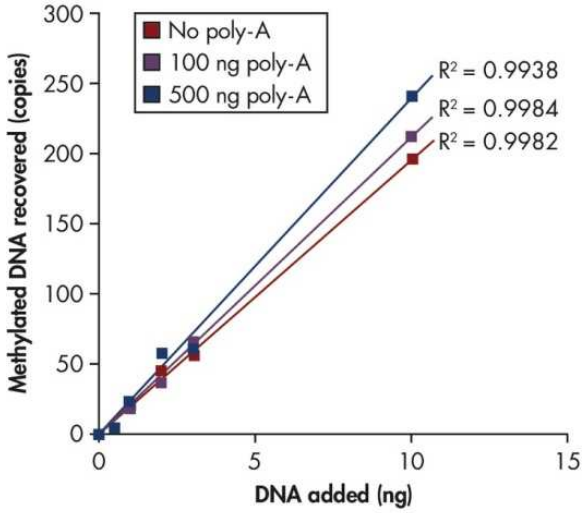
- **ccfDNA is present in urine as fragments mostly <500 bp**
- **miRNAs can be easily detected in samples of 3 ml urine**

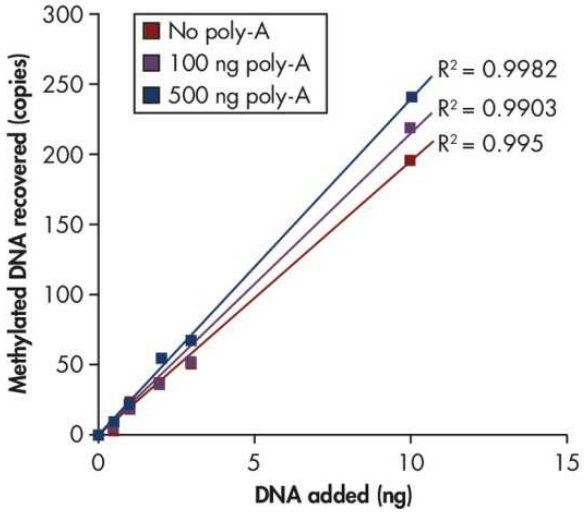
Purification of free circulating miRNA

Purification of miRNA can be enhanced using a specialized protocol



Recovery of methylated DNA





We are looking for you

Please reach out to us if you are interested in collaborations:

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Questions & answers

Thank you for your attention!

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