Total RNA sequencing of liquid biopsies

Jo Vandesompele, Biogazelle CSO, UGent professor

Webinar hosted by Takara Bio

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Acknowledgements

Scientific Reports, 2019

Everaert C, Helsmoortel H, Decock A, Hulstaert E, Van Paemel R, Verniers K, Nuytens J, Anckaert J, Nijs N, Tulkens J, Dhondt B, Hendrix A, Mestdagh P

Extracellular RNA Quality Control study

Avila-Cobos F, Decock A, Deleu J, De Wever O, Dhondt B, Everaert C, Fierro C, Helsmoortel H, Hendrix A, Hulstaert E, Kuersten S, Mestdagh P, Morlion A, Nijs N, Nuytens J, Philippron A, Schoofs K, Schroth G, Vanden Eynde E, Van Paemel R, Verniers K, Yigit N

Human Biofluid RNA Atlas

Hulstaert E, Morlion A, Avila-Cobos F, Verniers K, Nuytens J, Vanden Eynde E, Yigit N, Anckaert J, Mestdagh P

murine PDX experiment

Vermeirssen V, Deleu J, Verniers K, Van Maerken T, De Wilde B, Decock A

Liquid biopsy scientific literature

EV
15%

CTC
27%

microbiome

platelet

metabolite

2579 articles

methylation
5%

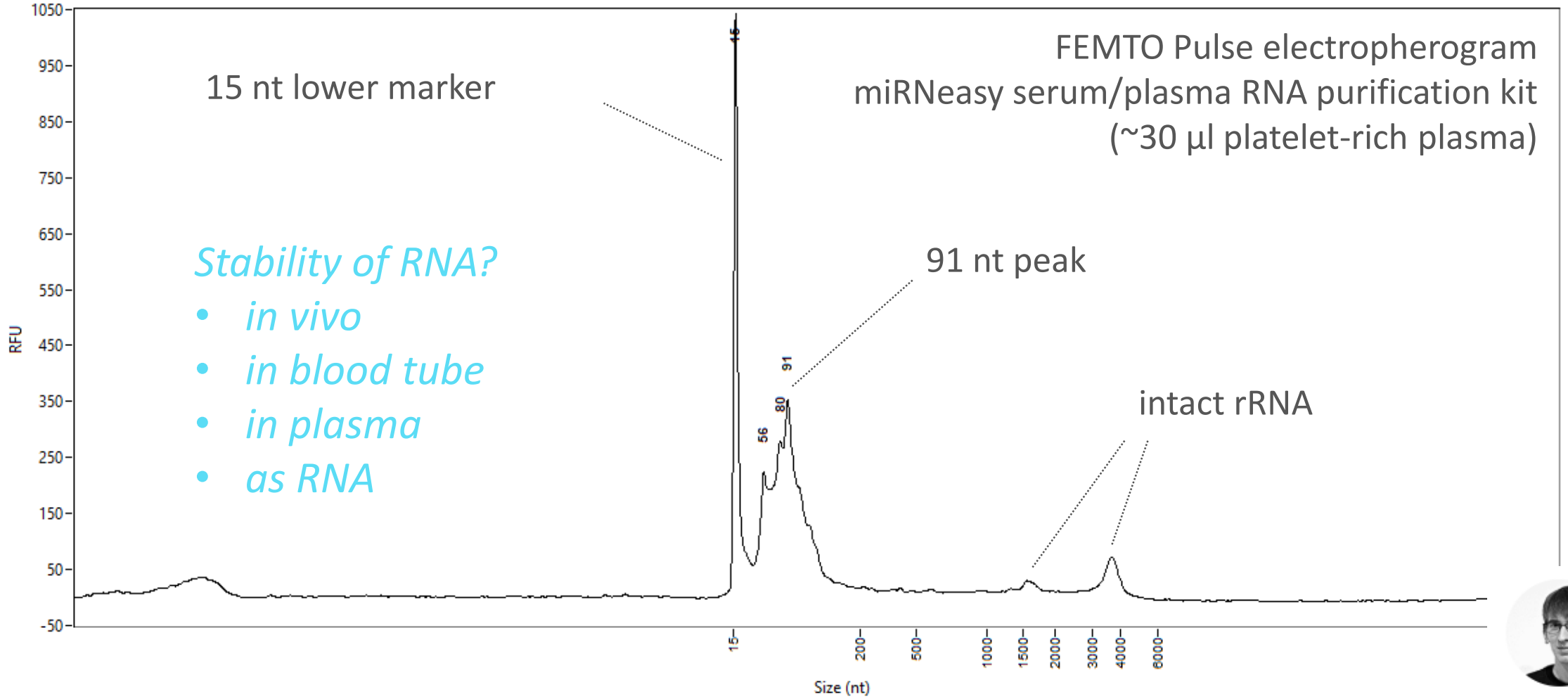
DNA
52%

protein
30%

RNA
16%

degradation
lack of methods
dynamic range

Majority of extracellular RNA is fragmented



Stability of RNA?

- *in vivo*
- *in blood tube*
- *in plasma*
- *as RNA*

platelets, vesicles, (lipo)protein complexes, naked (circular)

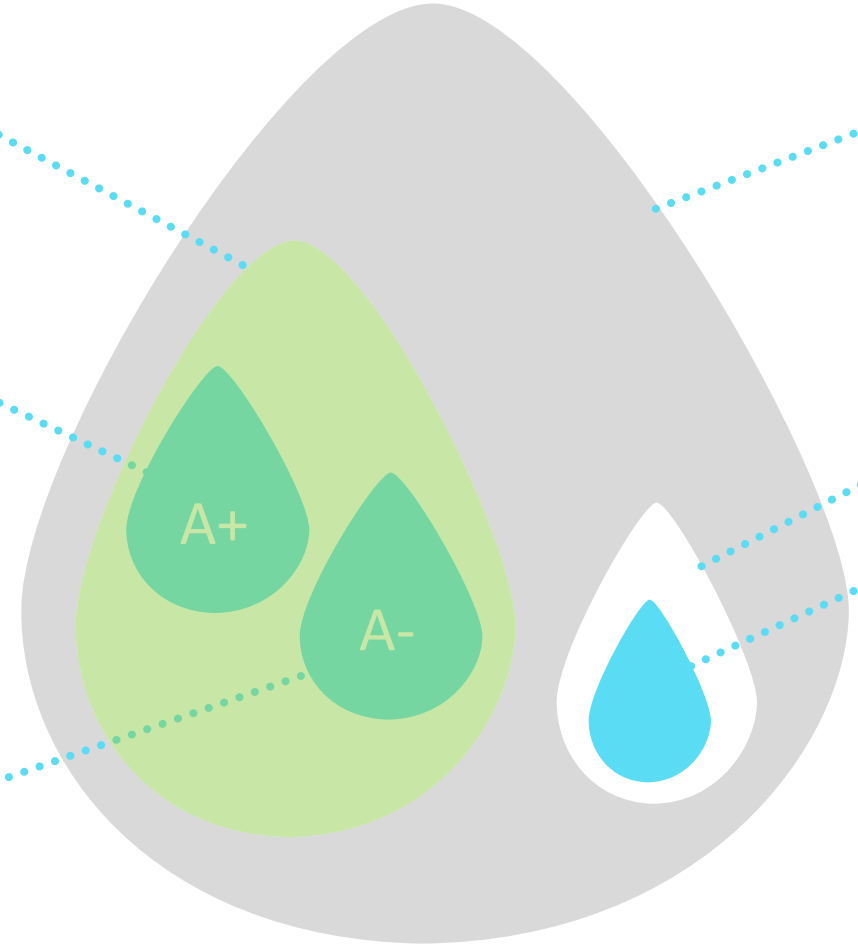
Ruben Van Paemel

Validated RNA seq workflows for any extracellular RNA type

long RNAs[1]
(total RNA)

polyadenylated RNA[2]
- mRNA[1,3]
- lncRNA[1,4]

non-polyadenylated RNA
- rRNA
- lncRNA[4]
- histone mRNAs[1,3]
- circRNAs[1,3,4]



cell-free RNA
extracellular RNA

200 µl
600 microRNAs – 6000 mRNAs

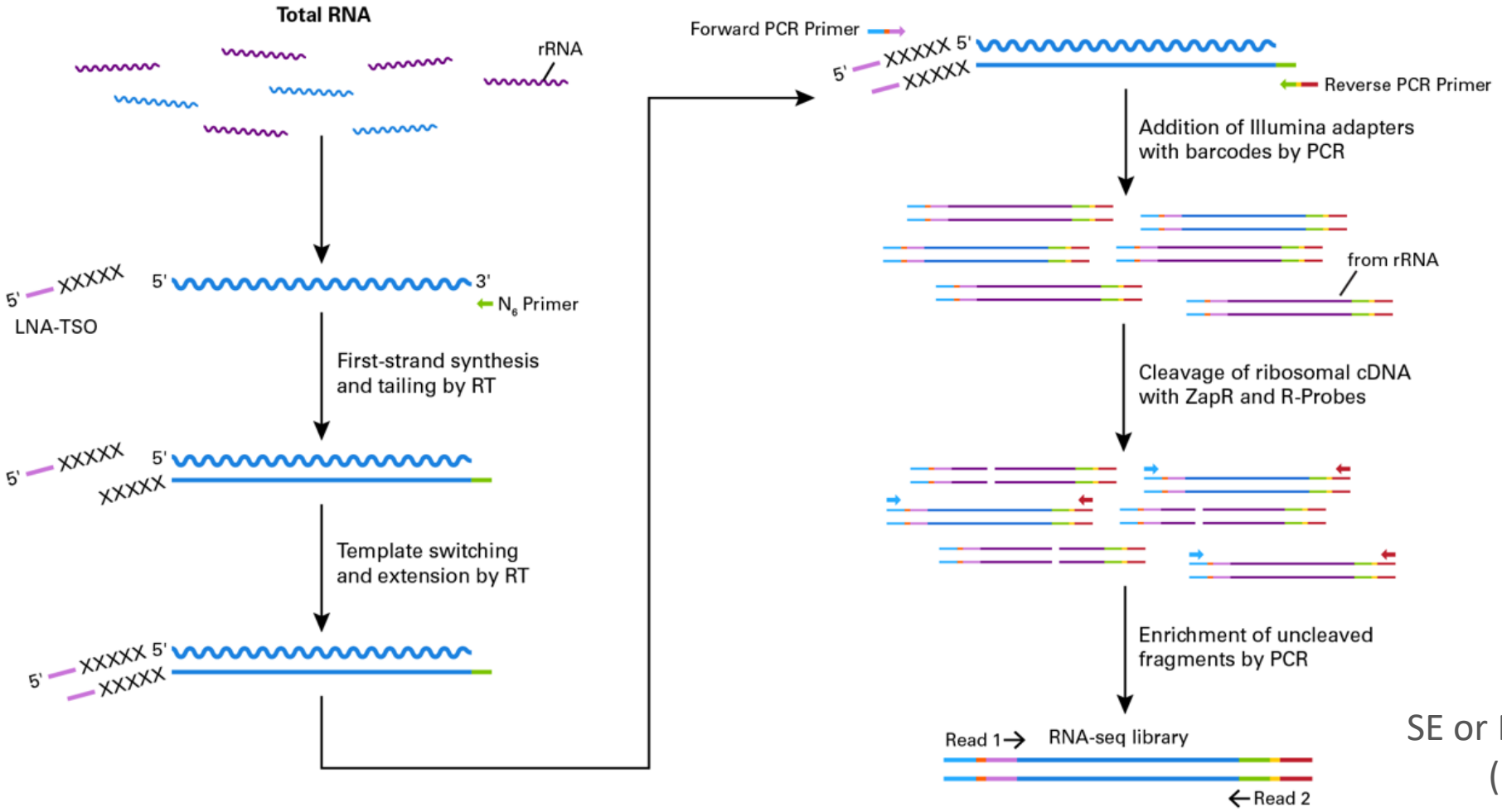
small RNA[5]

microRNA[5]

- total RNA sequencing[1]
- 3’ end sequencing[2]
- mRNA capture sequencing[3]
- lncRNA capture sequencing[4]
- small RNA sequencing[5]

SMARTer stranded total RNA seq kit v2 – pico input mamalian

rRNA
mRNA/lncRNA
circRNA
(microRNA)



SE or PE sequencing
(10-20 M)

How to assess the performance of a method?

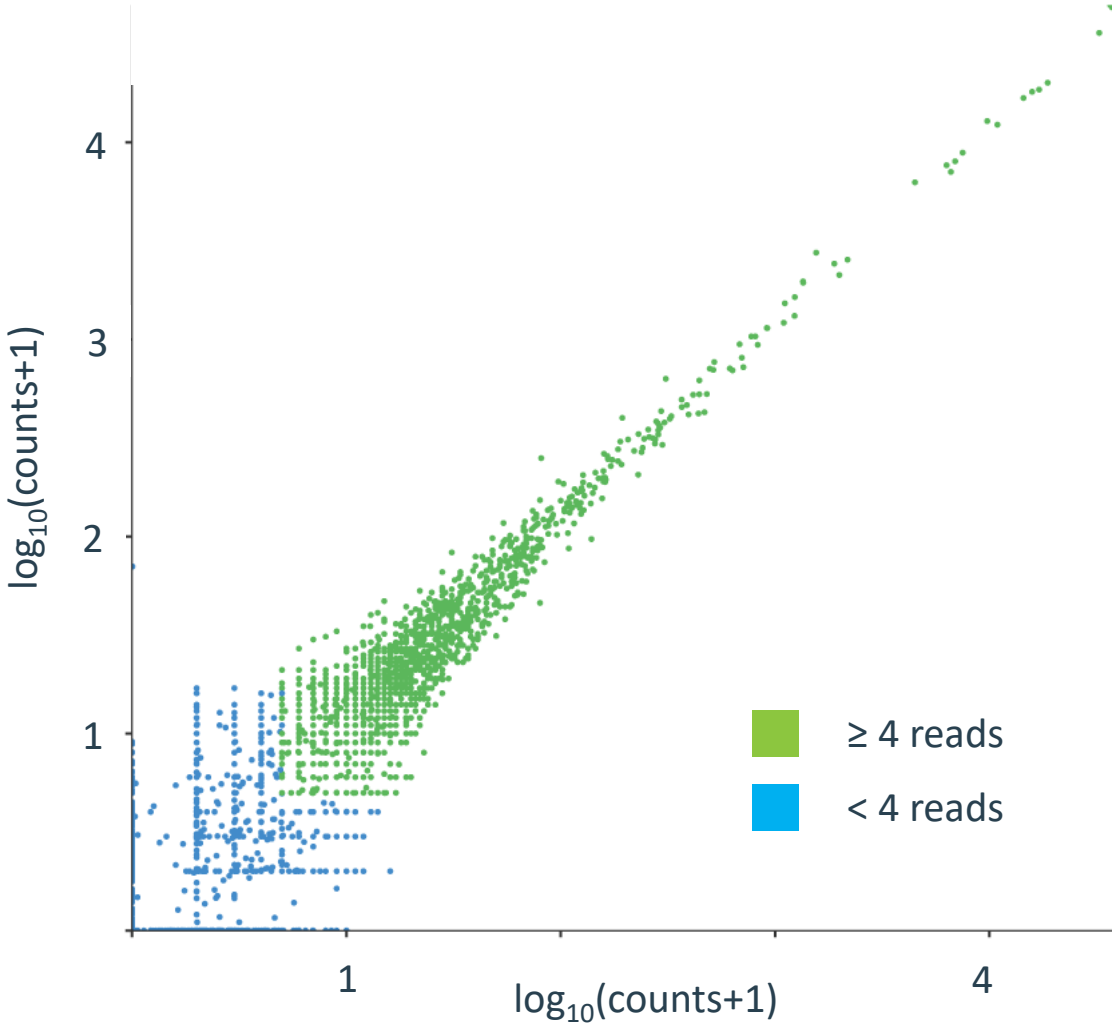
repeatability

analytical sensitivity

quantitative accuracy

fit for purpose

Excellent repeatability

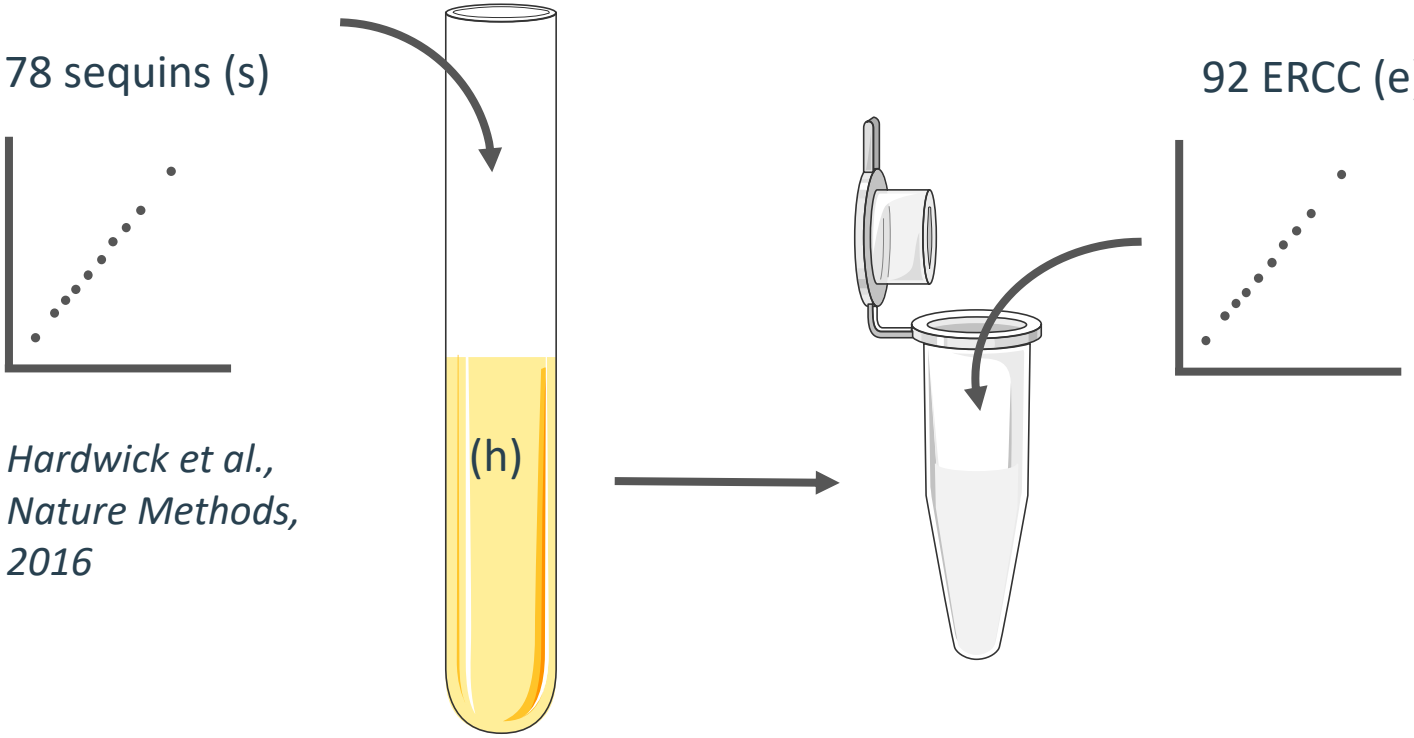


RNA purification replicates
200 µl platelet-free plasma EDTA blood tube
50% of 14 µl RNA eluate used in library prep
Pearson r = 0.948

5000-8000 genes

detection cut-off removes 95% single positive data points, per the miRNAQC study
(Mestdagh et al., Nature Methods, 2014)

170 spike-in RNAs as processing controls and normalization tool



Hardwick et al., Nature Methods, 2016

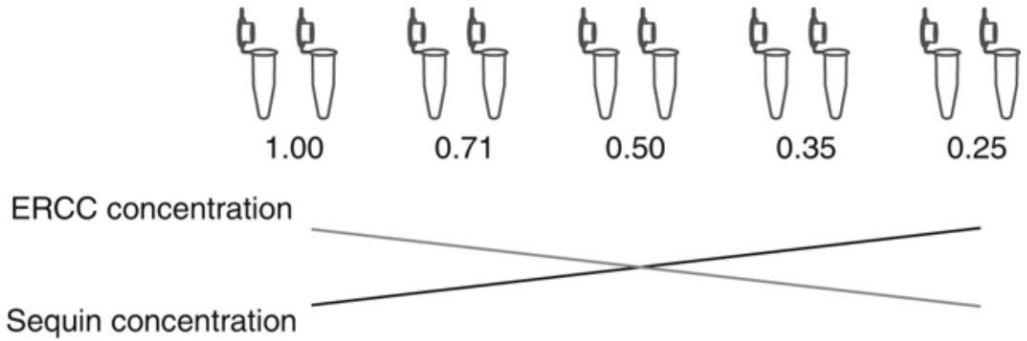
fluid input volume (f) for RNA purification

RNA eluate volume (r) in library prep

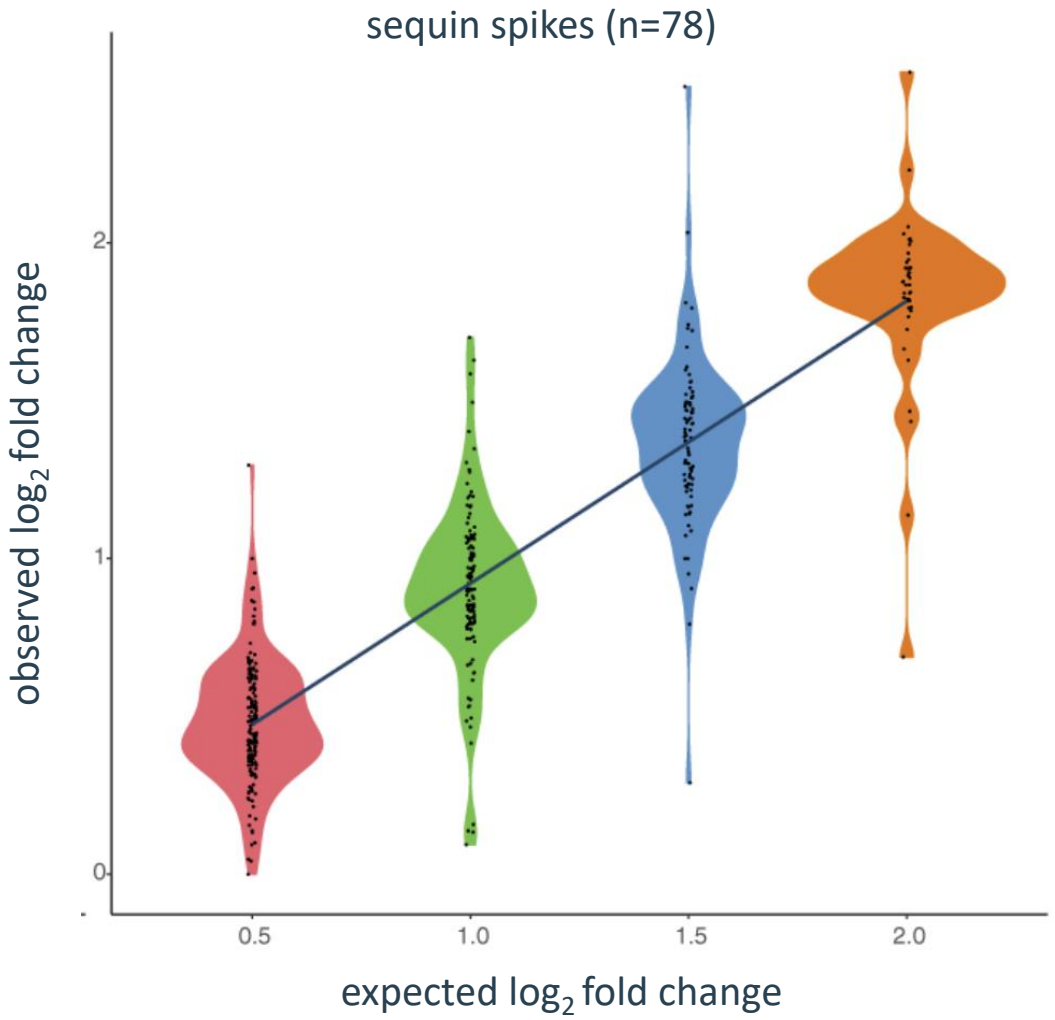
$$\frac{human}{sequin} = relative\ RNA\ conc.$$

$$\frac{sequin}{ERCC} = purification\ eff.$$

Good quantitative accuracy



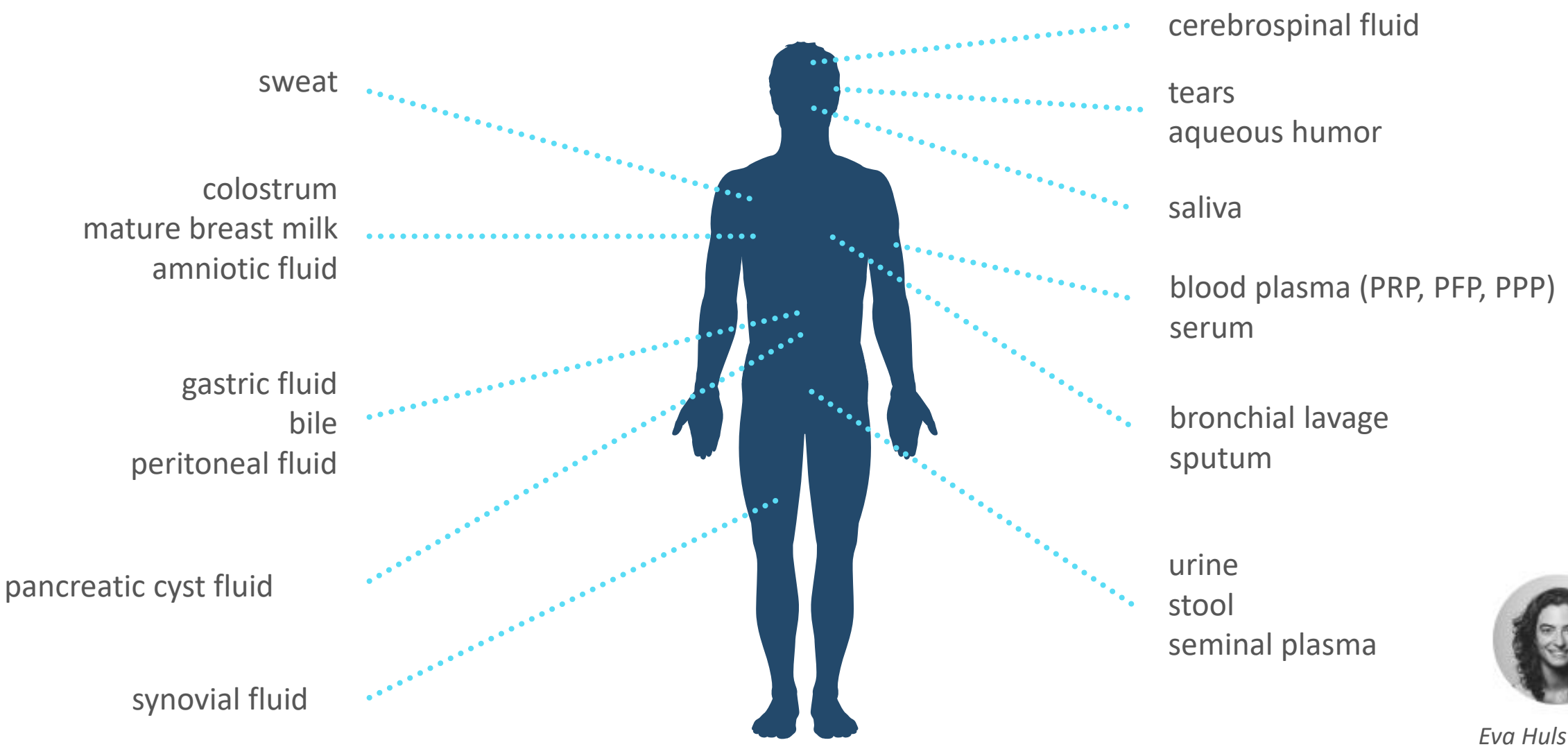
Pearson r = 0.883
slope=0.895



Fit for purpose

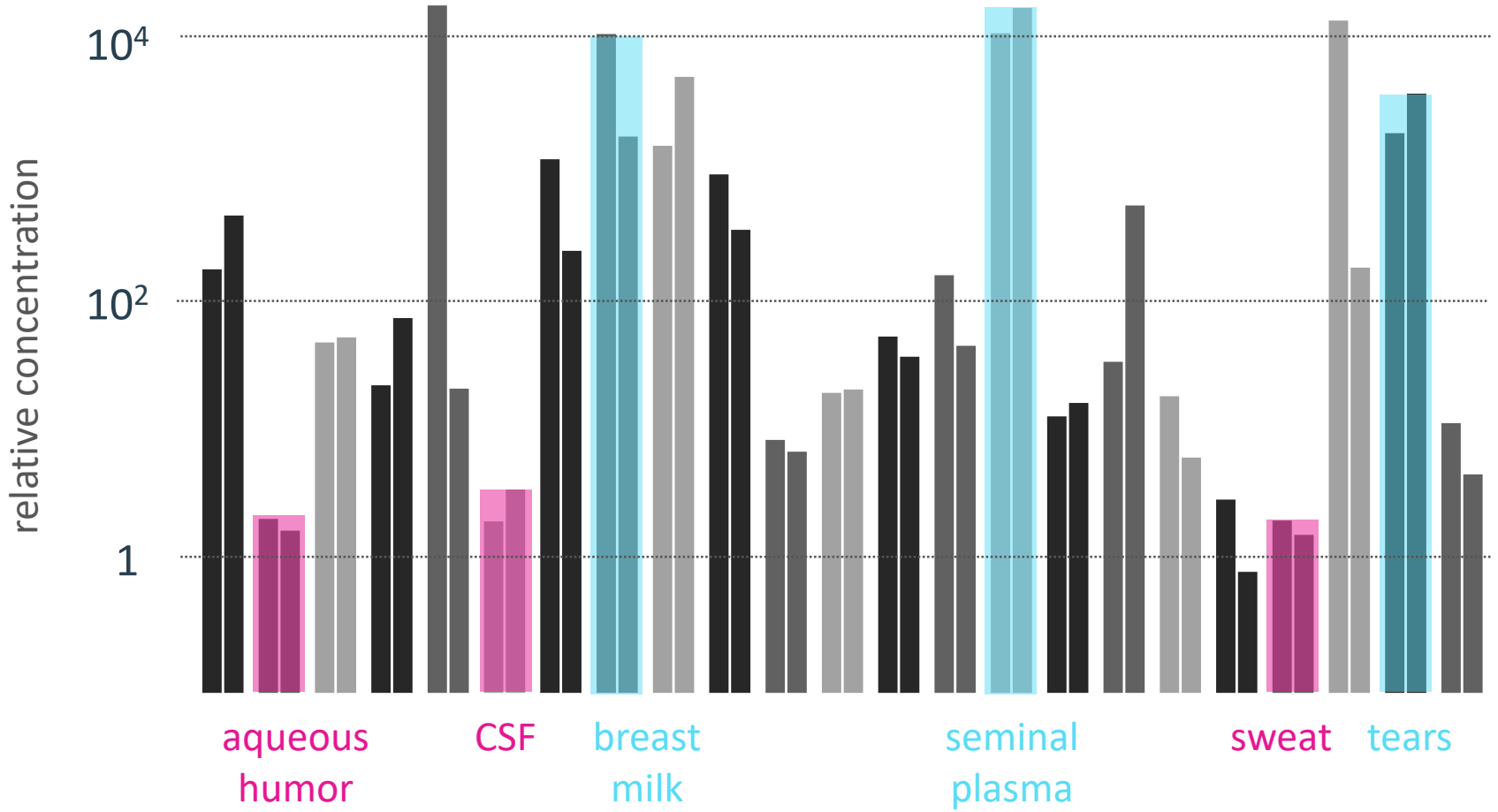
- good (uniquely) mapping rates
  - depending on biofluid type, presence of exogenous RNA
- good strandedness
  - no DNA contamination
  - ability to differentiate sense/antisense overlapping transcripts
- low level of nuclear rRNA reads
  - mitochondrial rRNA may be more problematic, depending on biofluid type
- sizeable intronic read fraction
  - > post-transcriptional regulation
- detection of both polyadenylated and non-polyadenylatd transcripts (e.g. lncRNAs, circRNAs)

Human Biofluid RNA Atlas

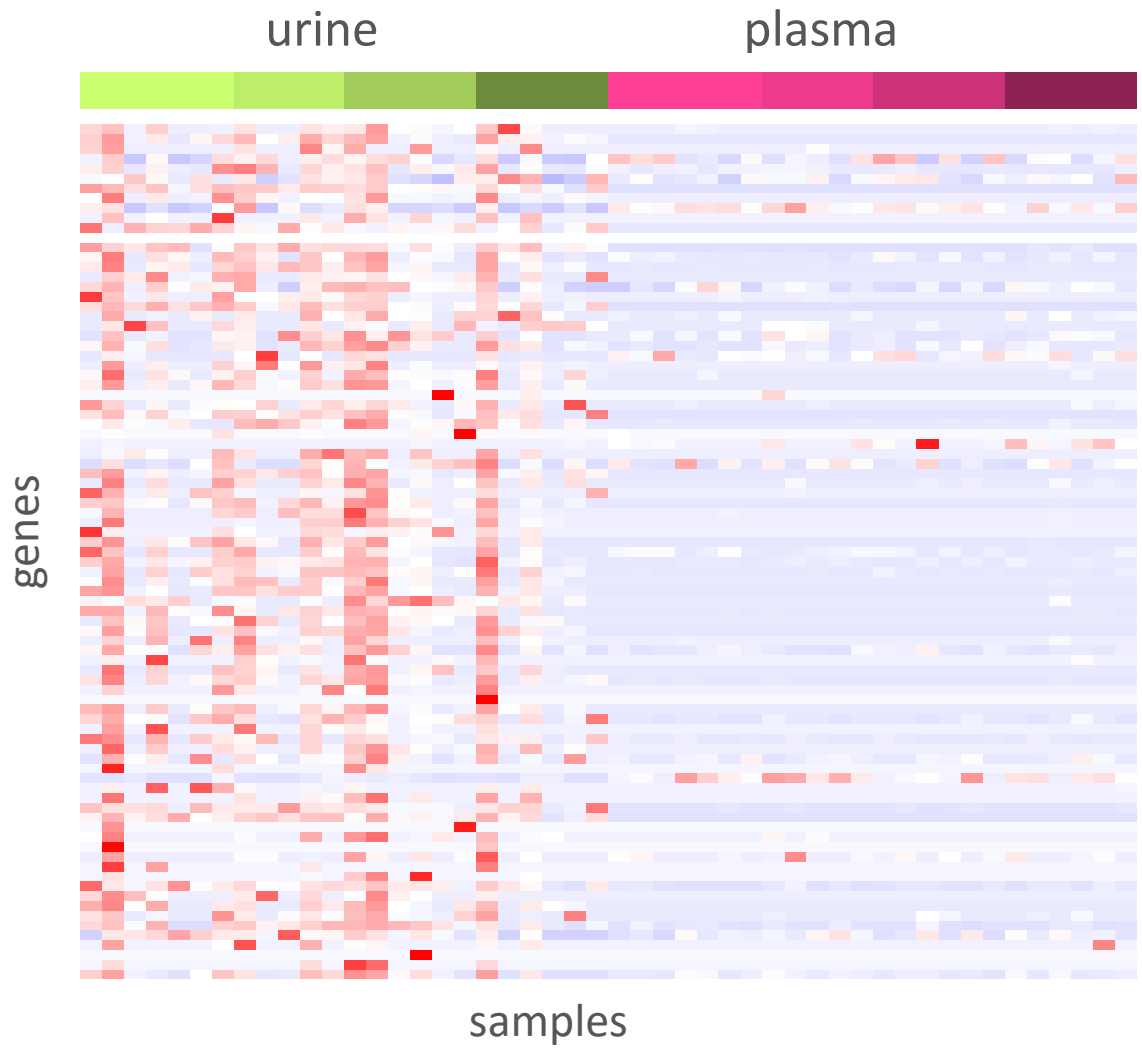


Eva Hulstaert

Thousand-fold difference in mRNA content among biofluids



The choice of biofluid matters



- RNA sequencing of matched urine and plasma from 24 cases and controls
- 88 prostate tissue specific genes are much more abundant in urine compared to blood plasma

high vs. low abundance

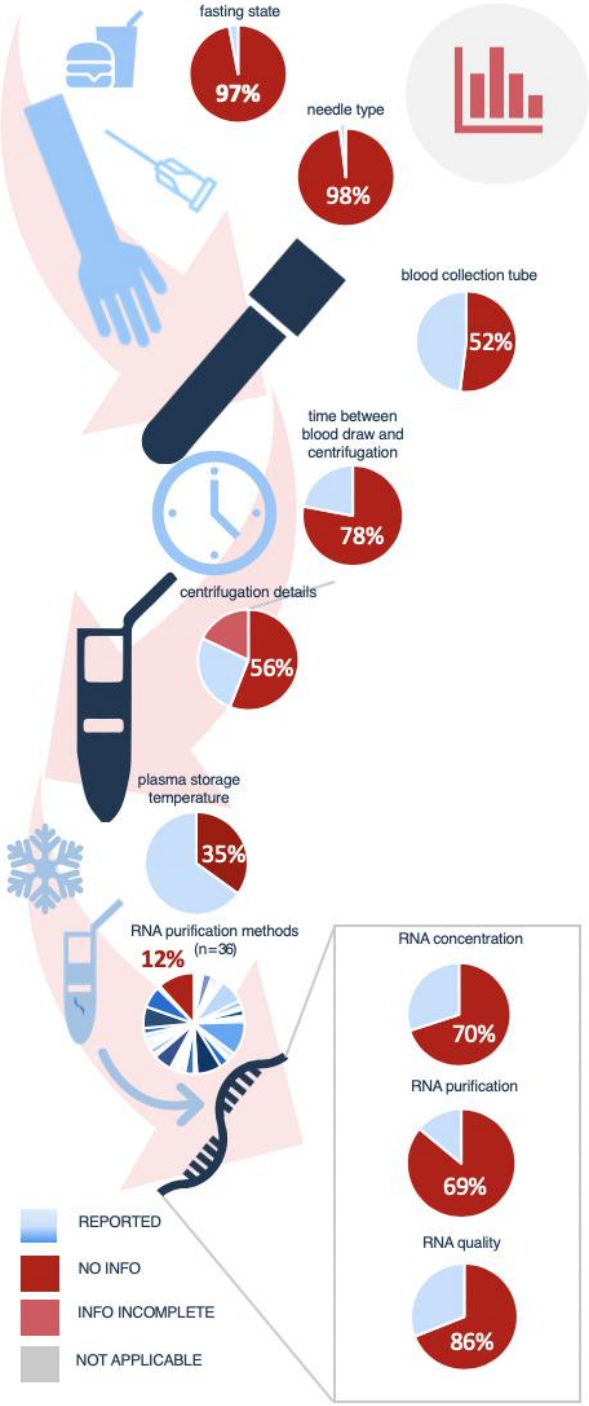
BPH urine
healthy urine
high risk urine
de novo urine
BPH plasma
healthy plasma
high risk plasma
de novo plasma

Hetty Helsmoortel

Inadequate reporting of pre-analytical variables

100 peer-reviewed articles in 2017-2018 on “plasma” and “RNA”

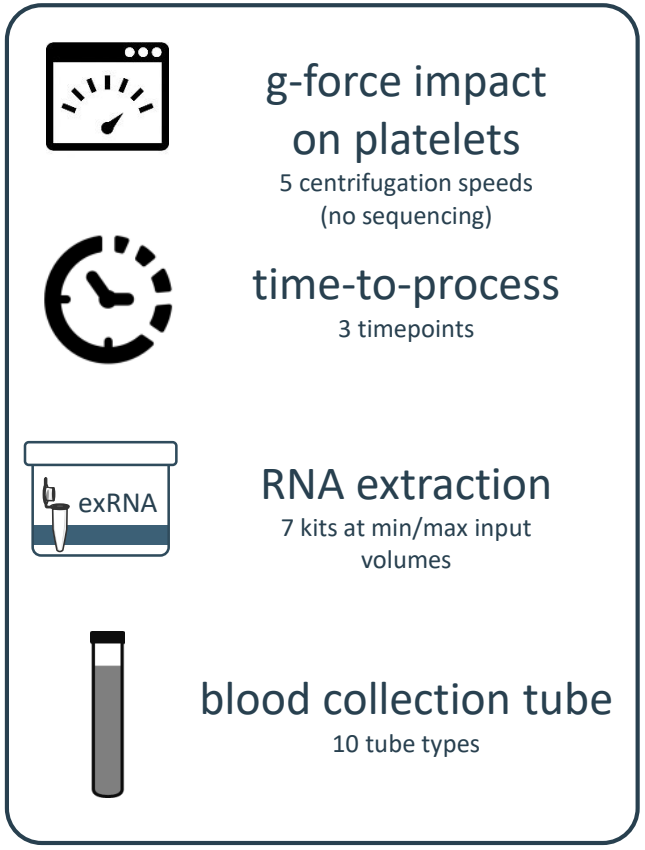
- 3% report on fasting status of donors
- 2% on type of needle
- 48% on type of blood collection tube
- 32% on time between draw and plasma preparation
- 44% on centrifugation details
- 65% on plasma storage
- 88% on RNA purification kit (n=36)
- 30% on RNA concentration, 14% on quality, 31% on purity



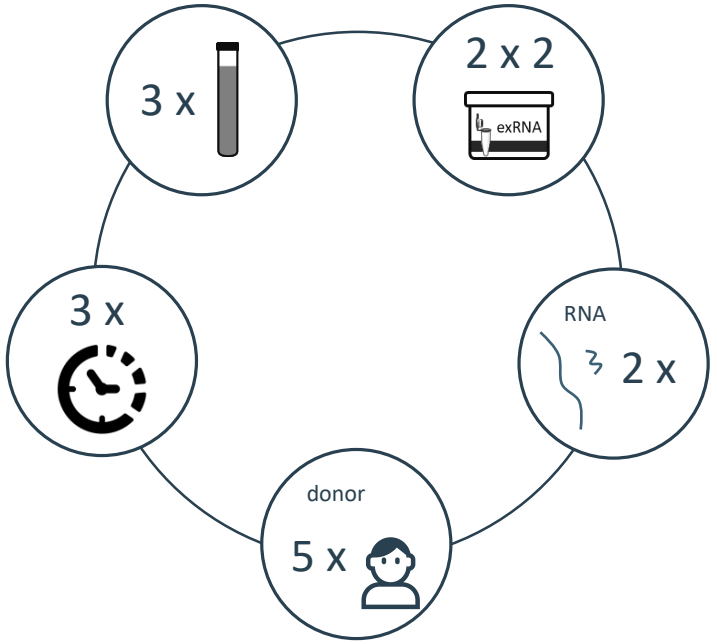
Céleste Van Der Schueren

Extracellular RNA Quality Control study

phase 1
single factor pre-experiments



phase 2
full factorial experiment

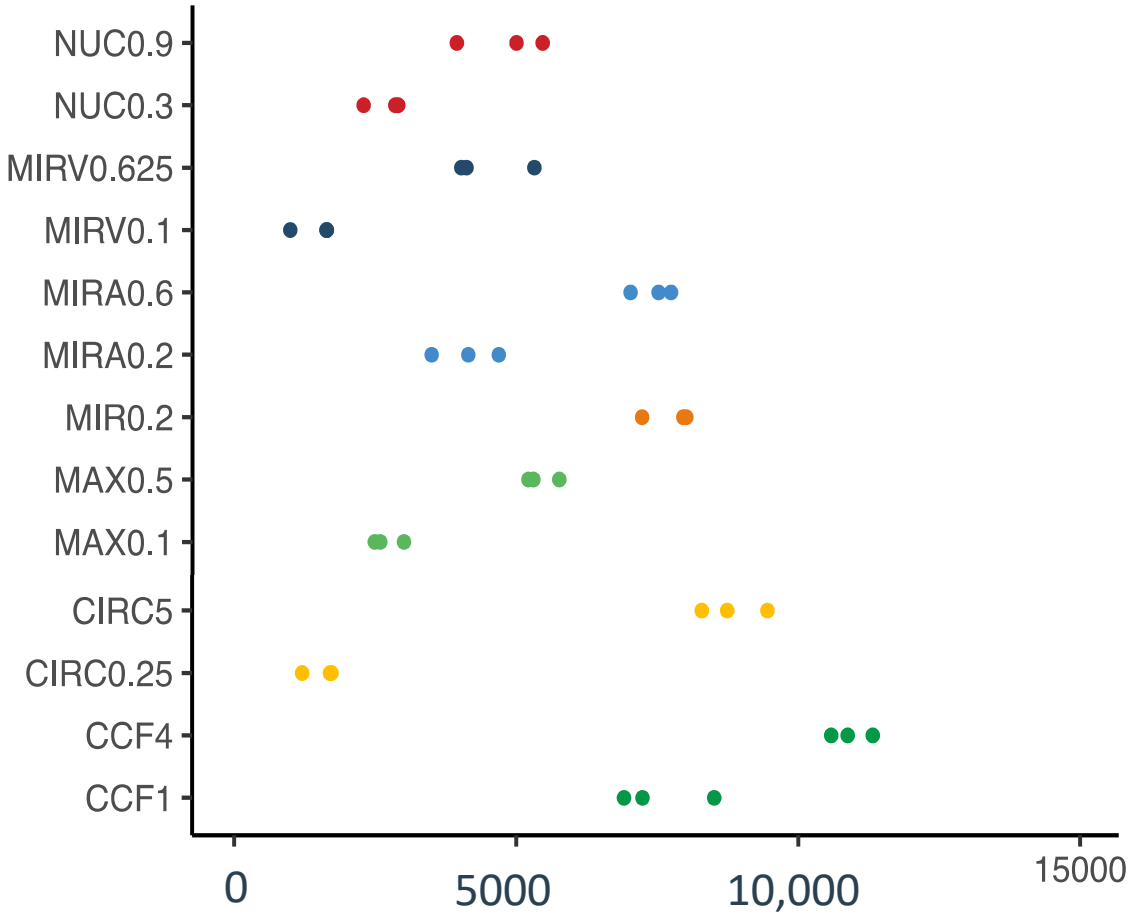


phase 3
post-experiments

- freeze/thaw
- varying plasma and RNA input levels
- variance component analysis
- biomarker study

GHENT UNIVERSITY
biogazelle
illumina

6-fold difference in number of detected genes ~ RNA purification kit



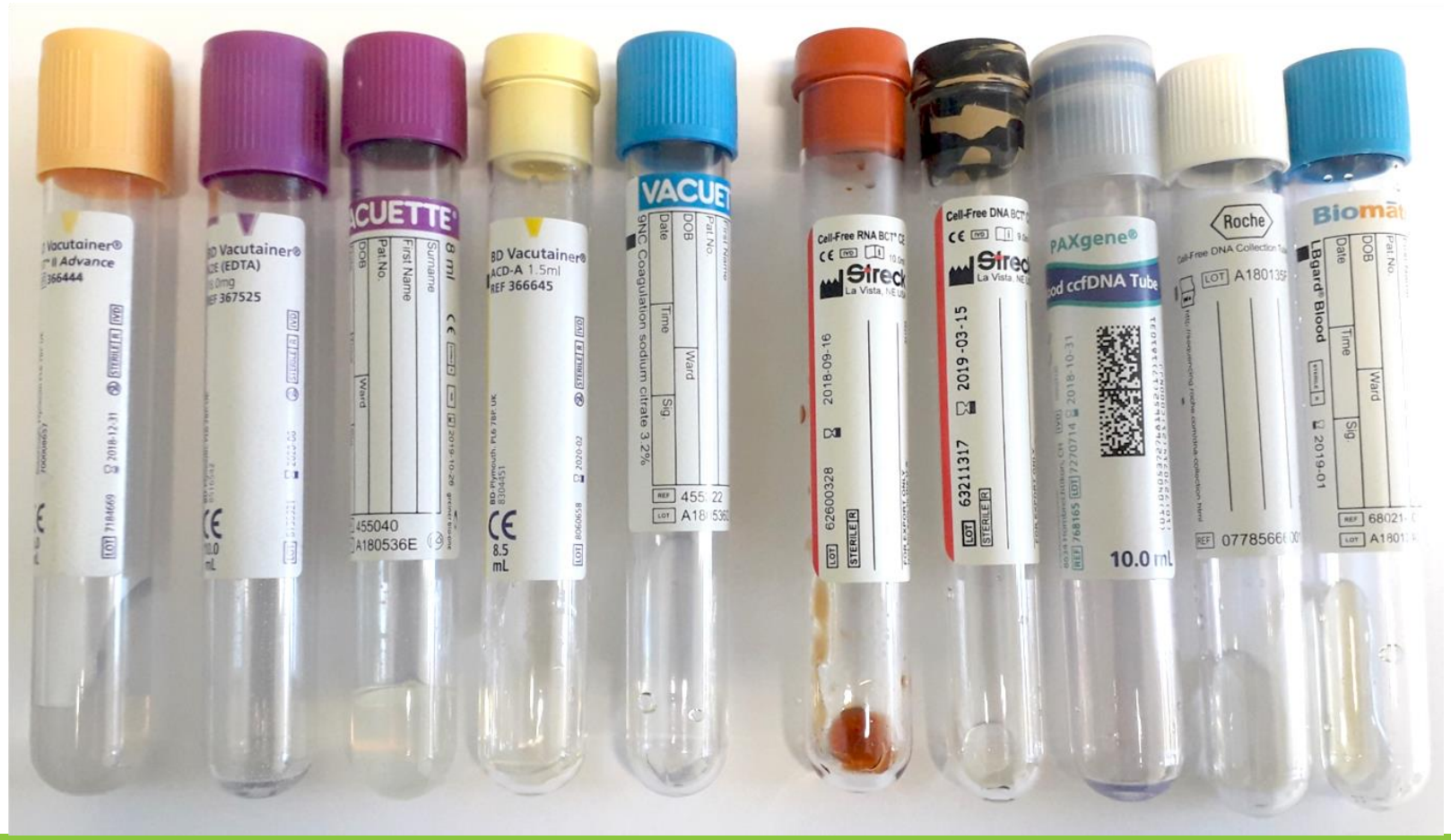
- 0.1 – 5 ml plasma input
- 14 to 100 µl eluate
- mRNA capture sequencing
- 24 M PE reads, 5 read cut-off

- 138-fold difference in RNA concentration
- 37-fold difference in RNA yield

- microRNA differences are less pronounced

Assessment of blood collection tubes and time-to-process

serum non-preservation plasma preservation plasma

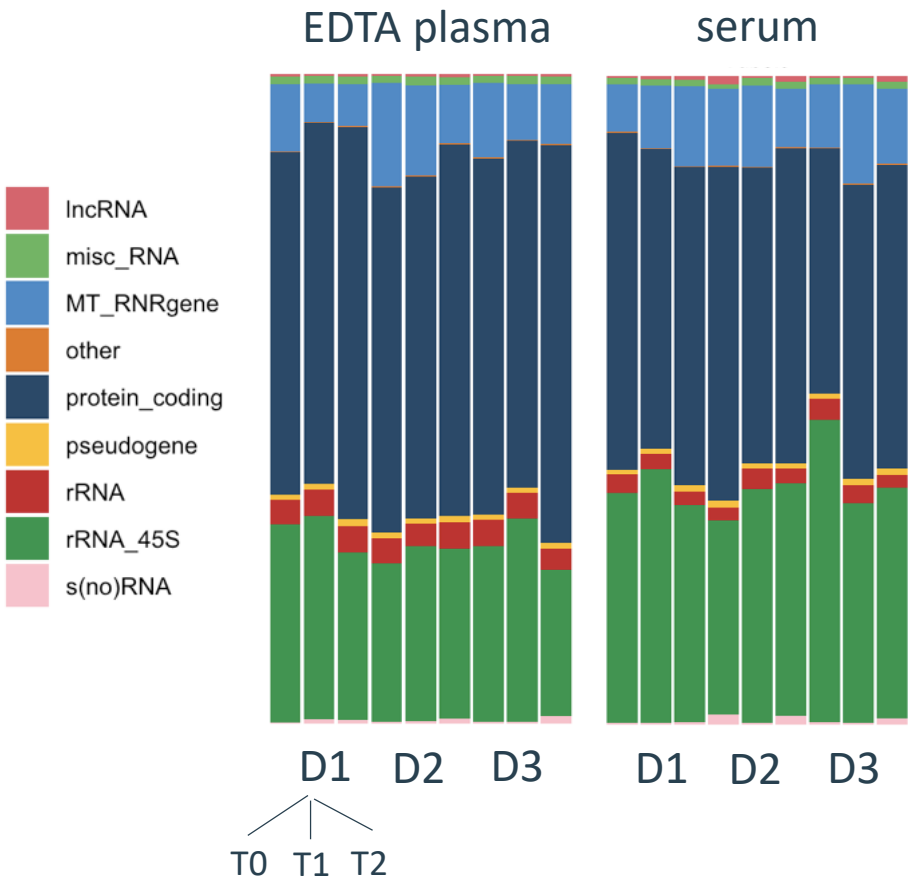


3 donors
x
3 time points
x
10 tubes

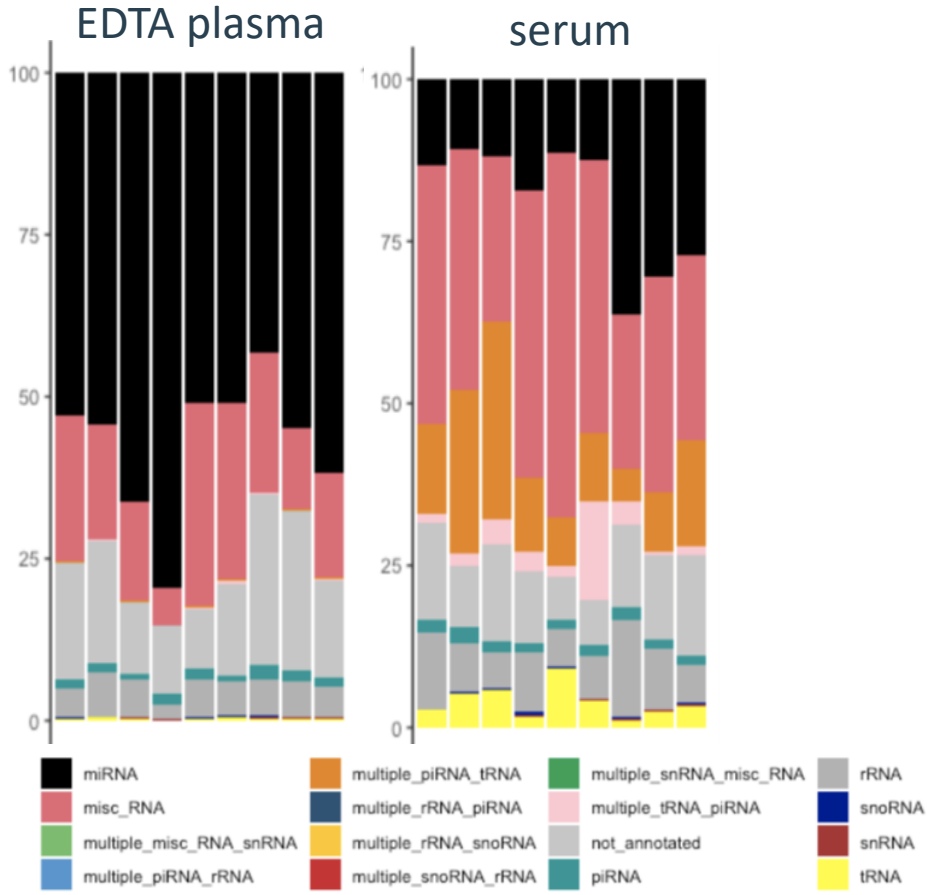
small RNA
+
mRNA

Serum and EDTA plasma are (dis)similar with respect to RNA biotype composition

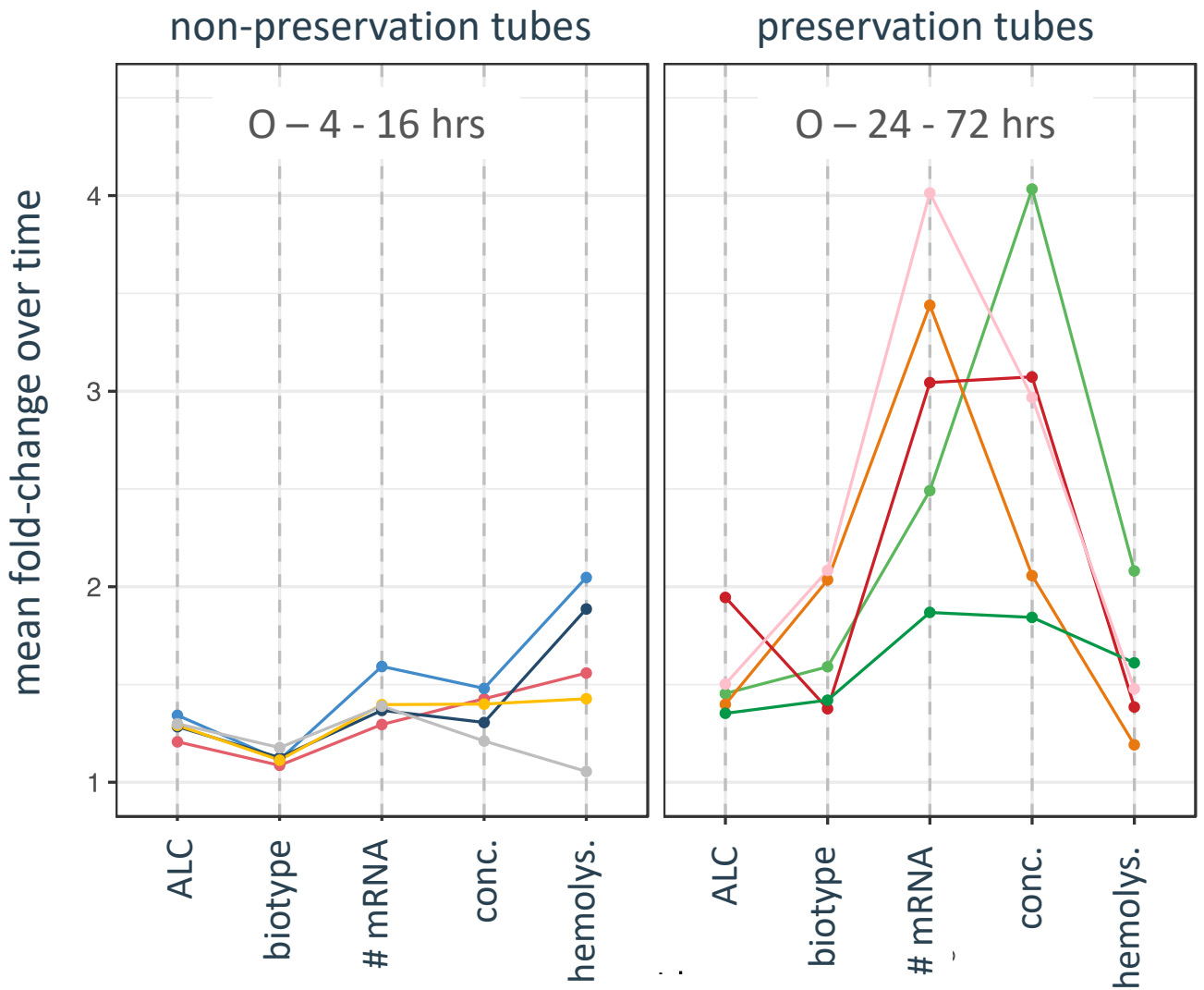
long RNA



small RNA



Preservation of mRNA, say what?



- reproducibility (ALC)
- RNA biotype fractions
- # detected mRNAs
- RNA concentration
- hemolysis

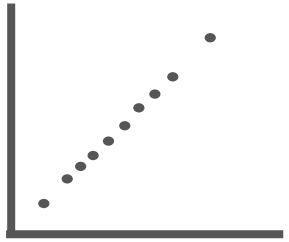
No prime time yet for blood collection preservation tubes

- compromised precision
- low and varying RNA levels over time
- different RNA biotype composition over time
- problems with removal of contaminating DNA
- higher and increasing levels of hemolysis

- quickly prepared serum or EDTA plasma (<4 hrs) are currently recommended for total RNA sequencing studies

Case 1: tumor educated platelets as a novel concept in liquid biopsies

- *Nilsson et al., Blood, 2011*
- *Best et al., Cancer Cell, 2015*
- *Best et al., Cancer Cell, 2017*

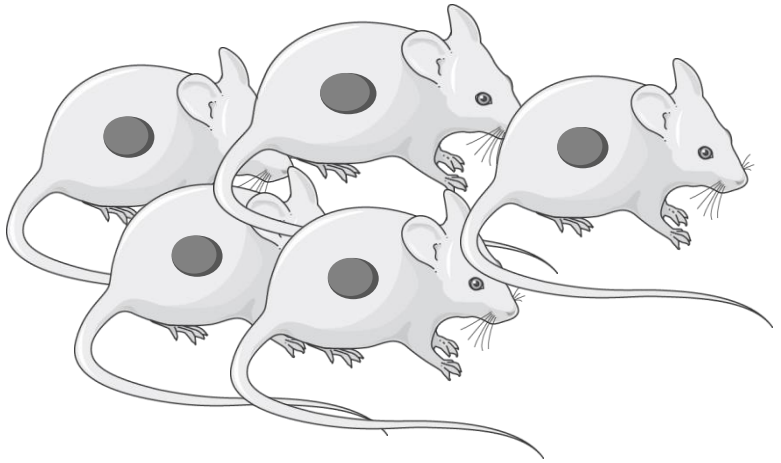


sequin
ERCC

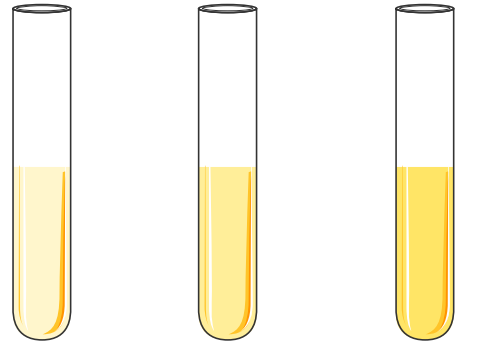
Jill Deleu

Anneleen Decock

Vanessa Vermeirssen



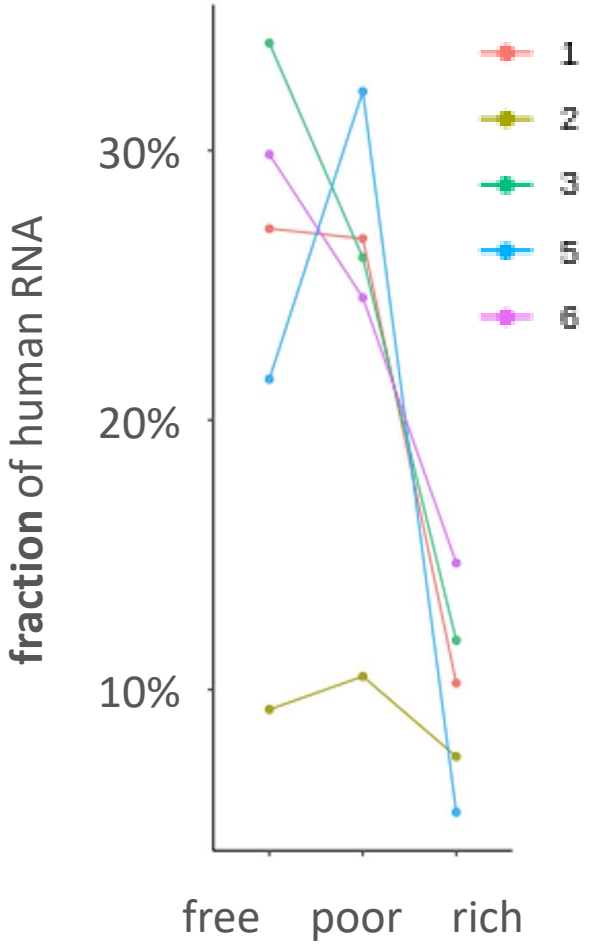
breast cancer PDX

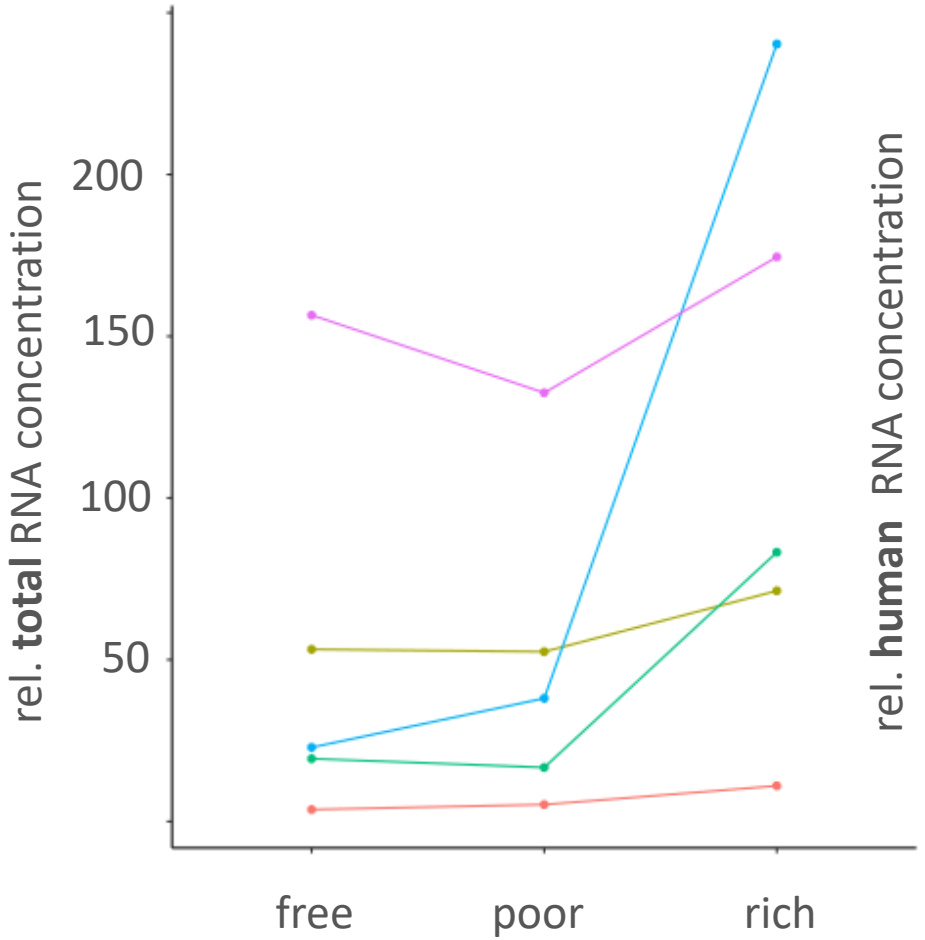


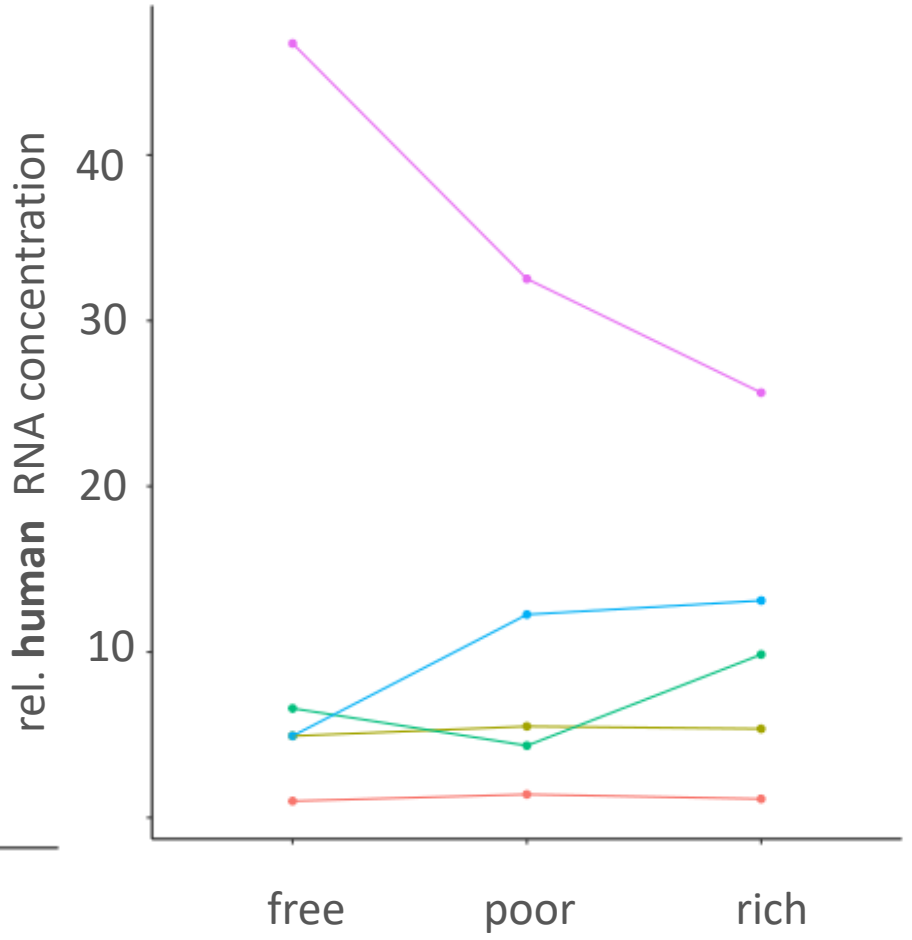
free poor rich

70 µl plasma (5 x 3)

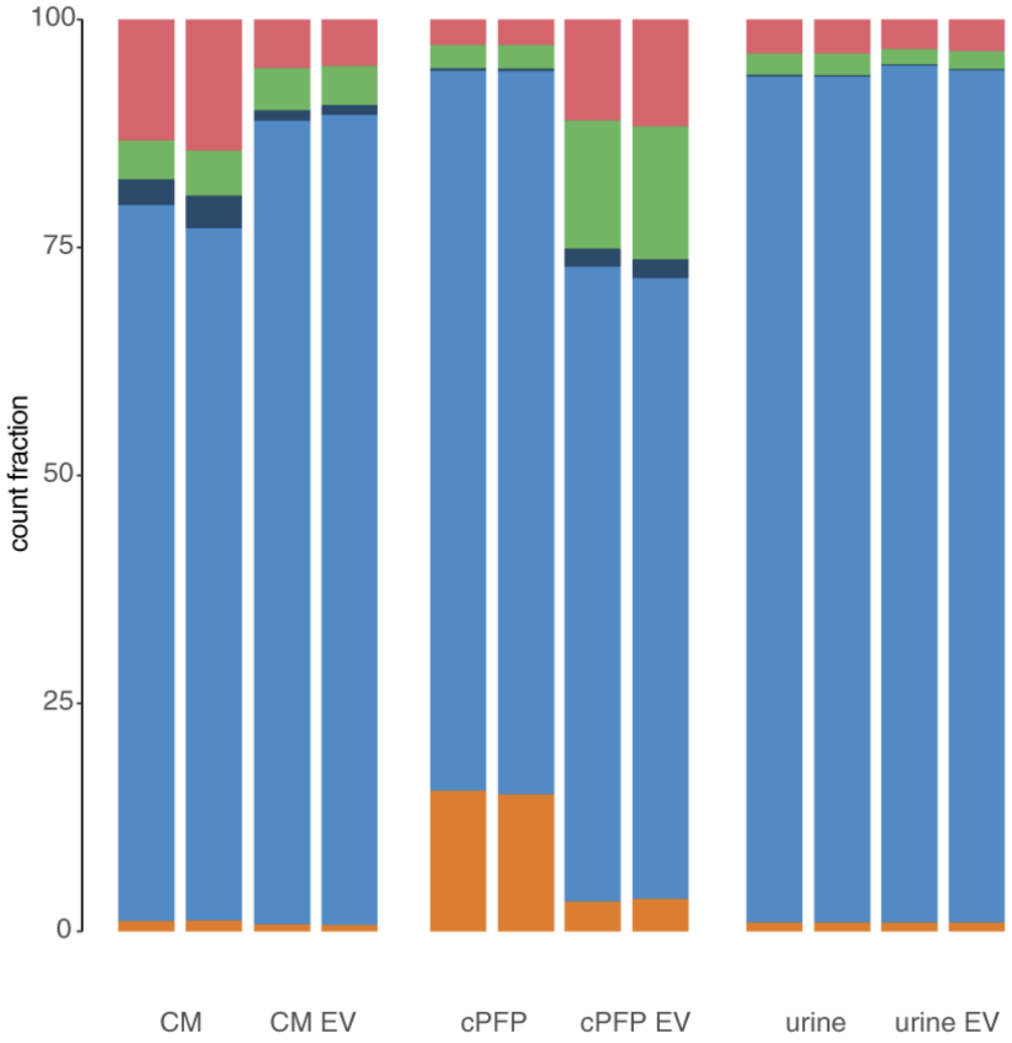
Majority of tumor derived RNA may not end up in platelets







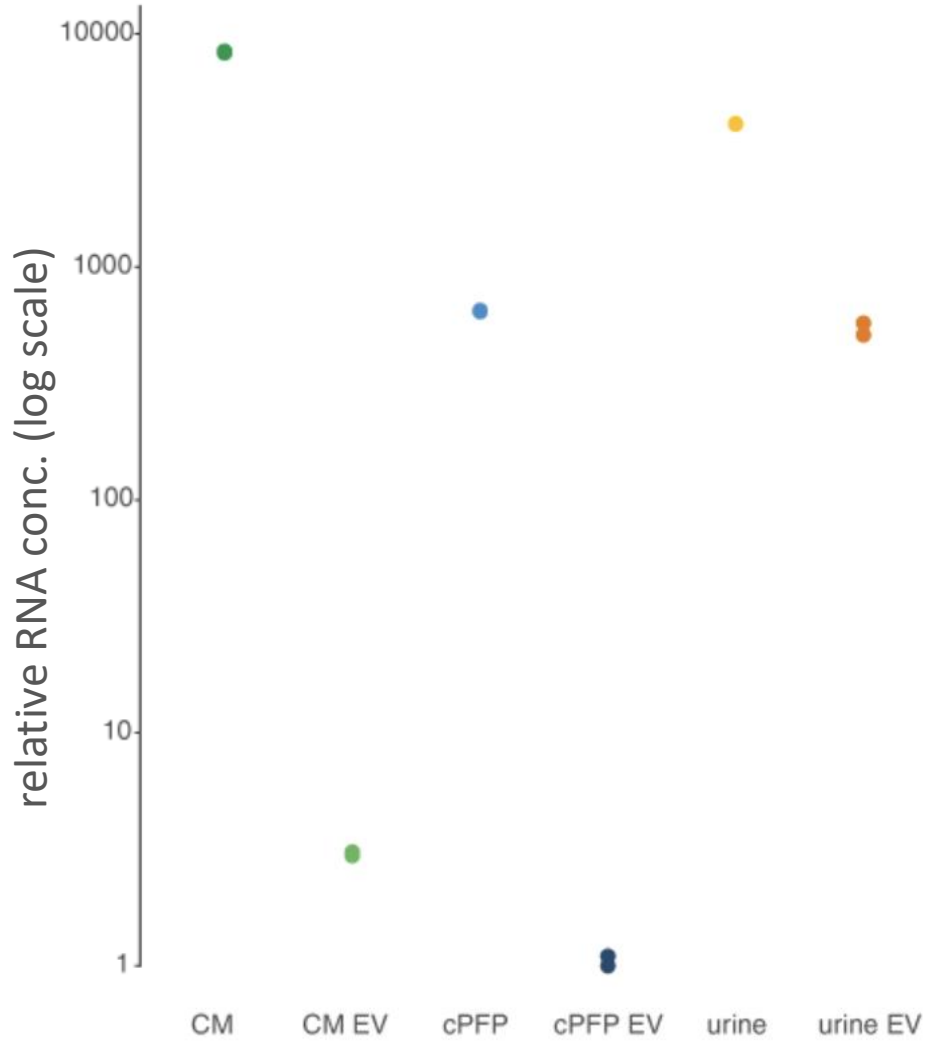
Case 2: extracellular vesicles



- matched fluid and derived Evs
- density and size based purification of EVs (Hendrix lab)
- varying biotype contributions

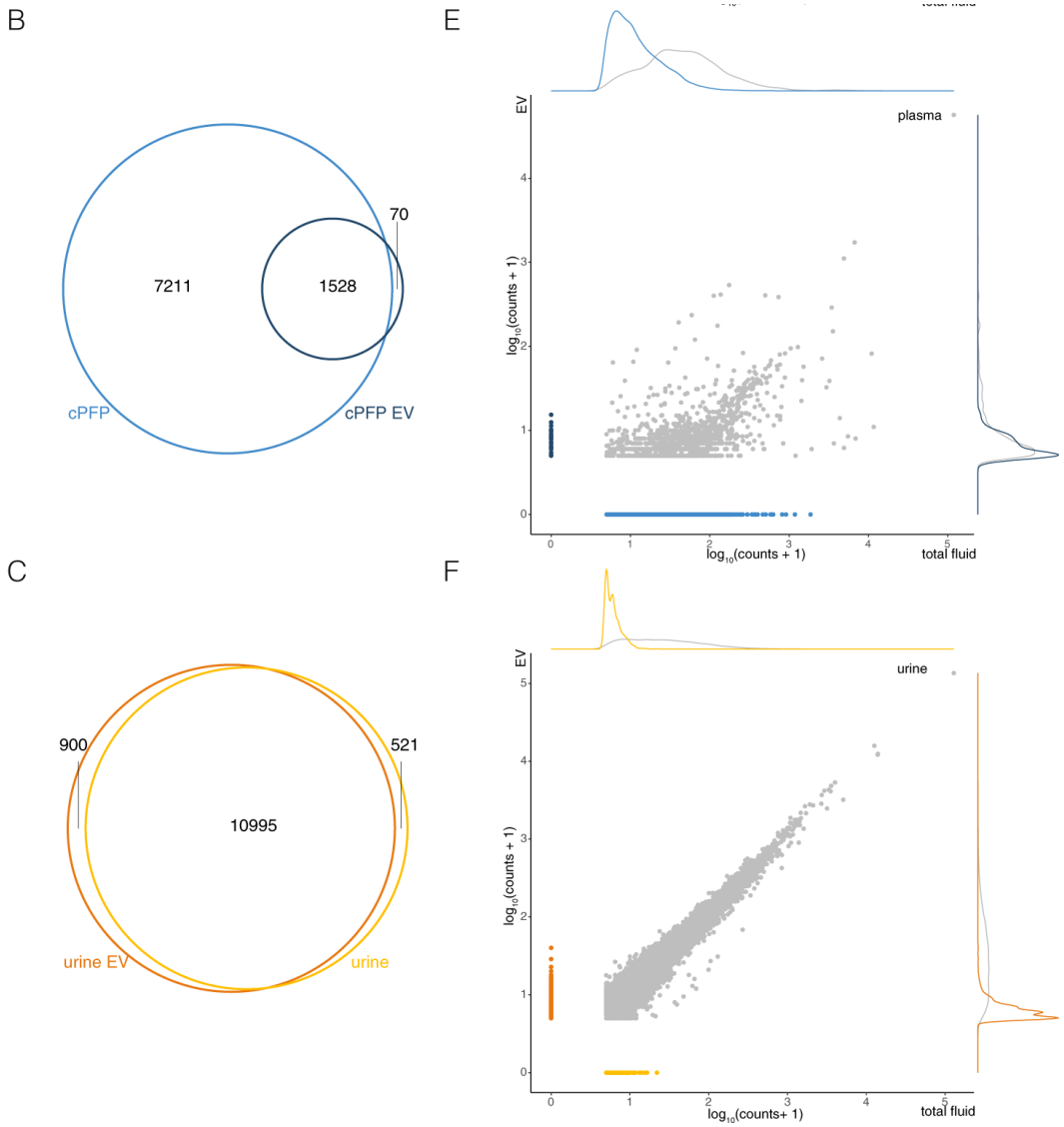
- conditioned medium breast cancer cells (CM)
- platelet-free plasma (citrate tube) (cFPF)
- urine

Variable EV RNA cargo concentrations



- volume standardized spike-based relative RNA concentration
- differences among fluids and between fluids and EVs

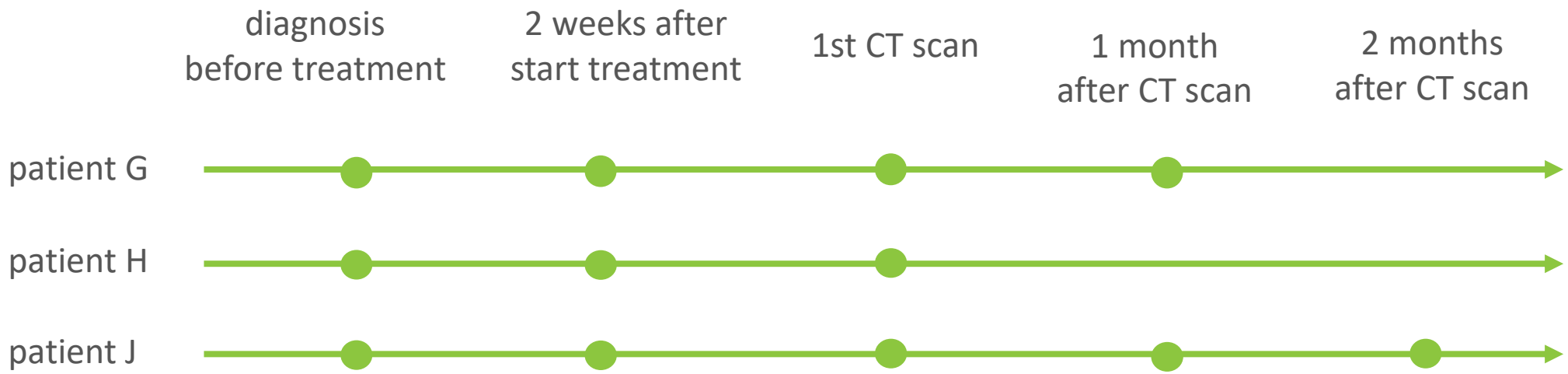
Variable RNA cargo



- urine and urine EVs have very similar RNA cargo
- platelet-free plasma and PFP EVs are very different

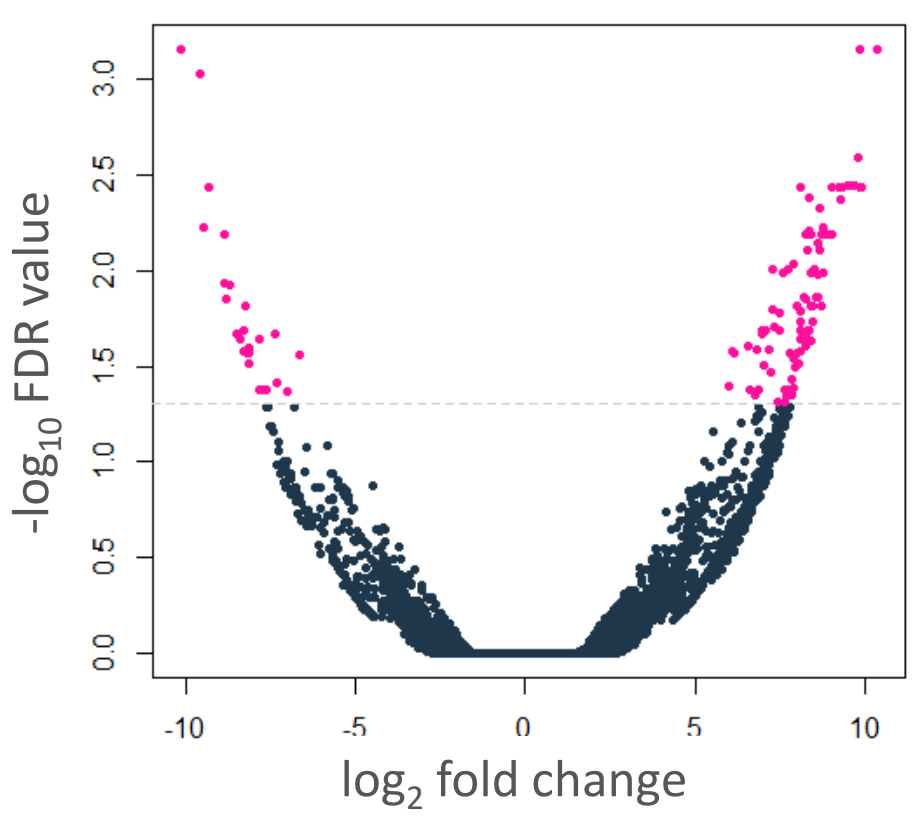
Case 3: colon cancer

- platelet-poor plasma
- metastatic patients (chemo + anti-VEGF or anti-EGFR)

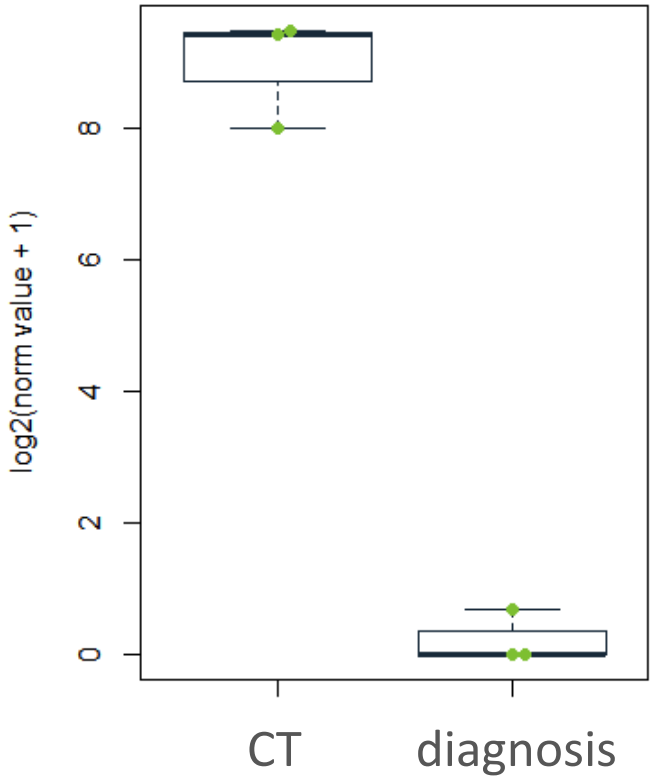


Large RNA abundance differences in plasma over time

large fold changes
(CT scan vs diagnosis)



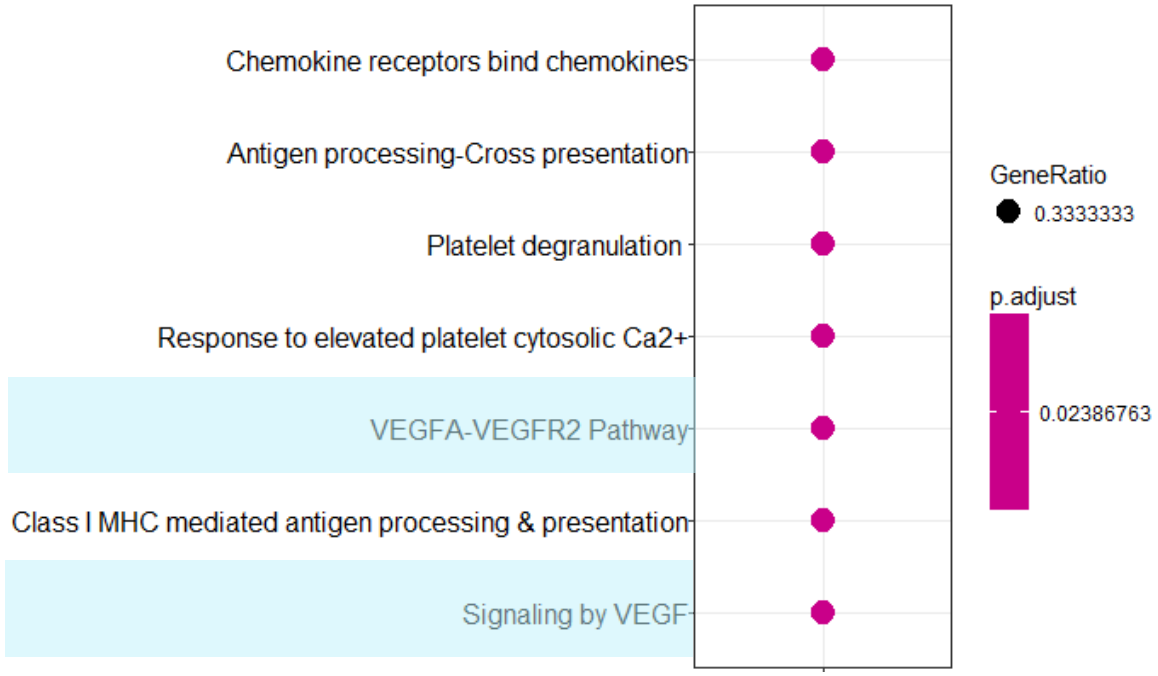
POLB is the most
upregulated gene



- involved in mismatch repair
- upregulated upon chemotherapy
- mutated in colon cancer

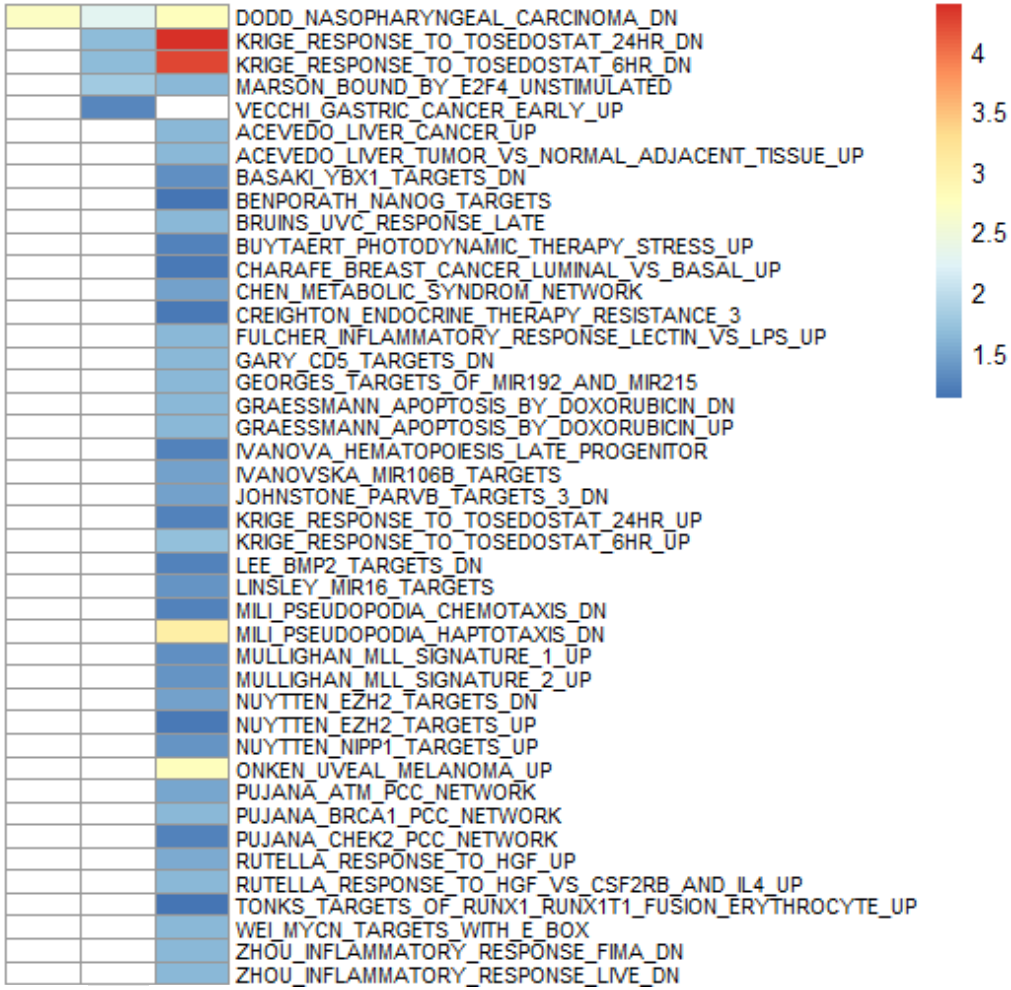
Plasma holds signal of VEGF pathway inhibition

pathway enrichment
(CT scan vs 1st treatment)



- n=1
- power of enrichment analysis
- hints at a putative pharmacodynamic biomarker

Plasma holds signal of stroma and host



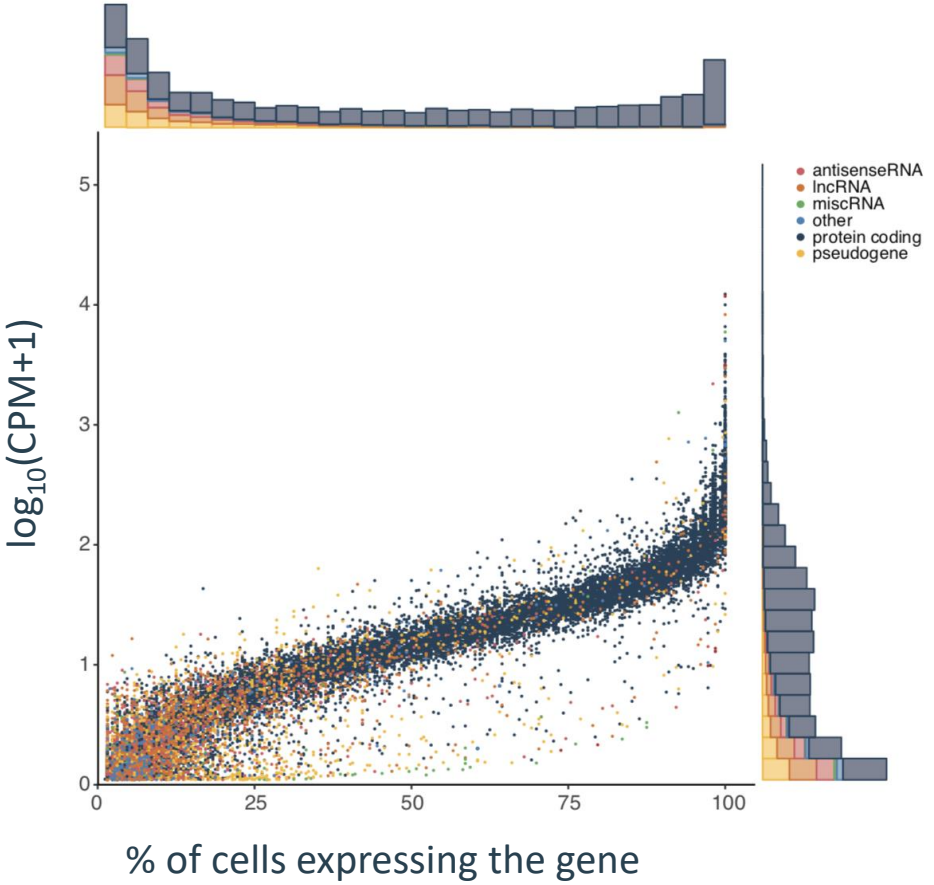
gene set enrichment analysis

- DNA mismatch repair
- (anti-)proliferation
- T-cell stimulation
- inflammatory response

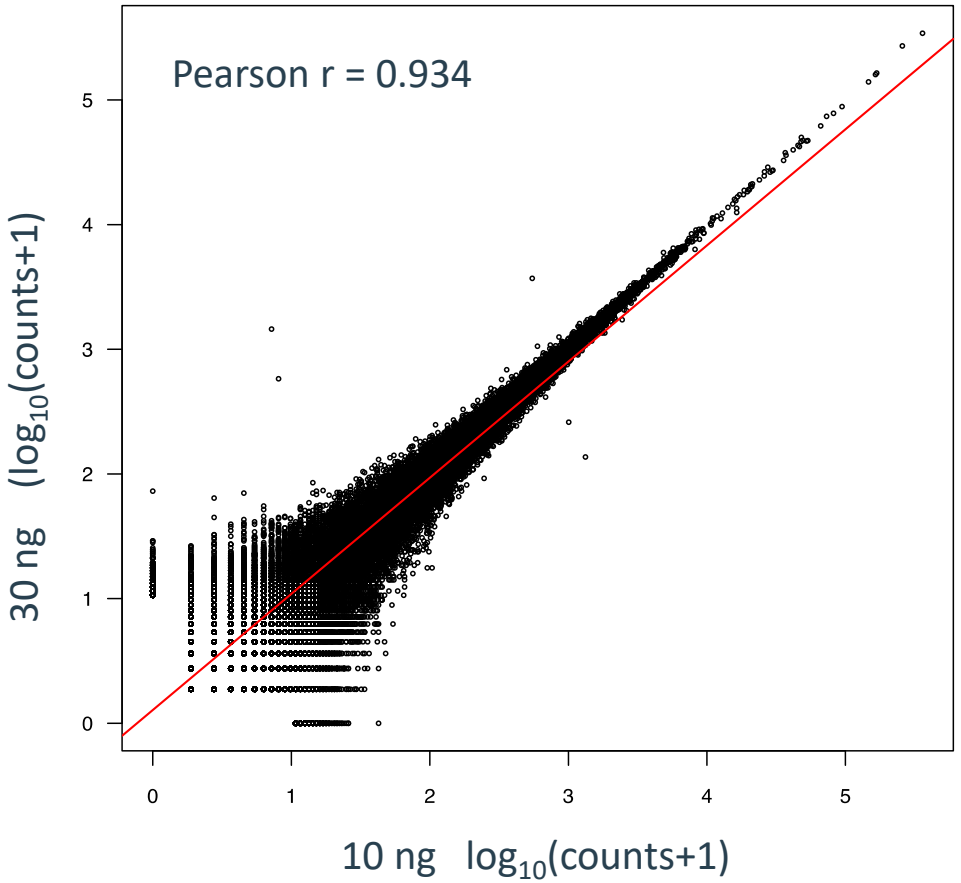
- *tumor stroma*
- *immune system*
- *organ toxicity*

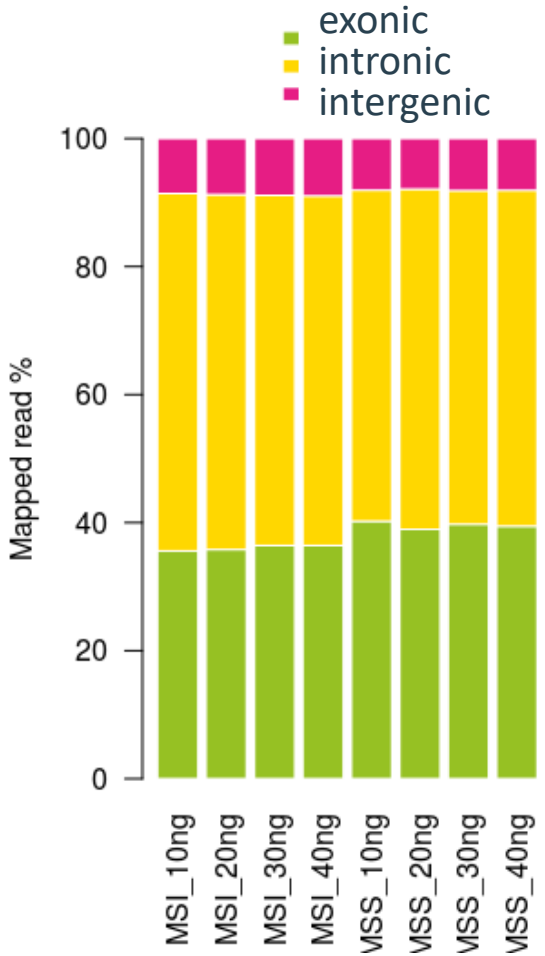
SMARTer single cell total RNA seq

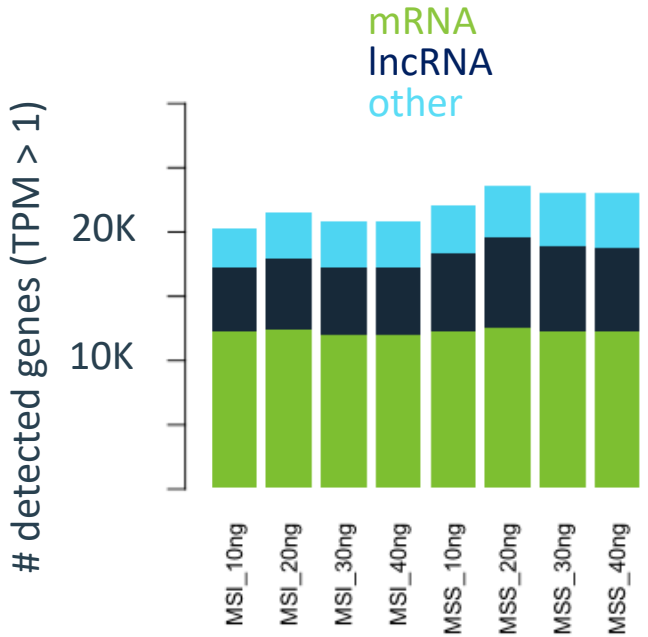
- *Verboom et al., Nucleic Acids Research, 2019*
- 458 cells – 1528 million reads
- <3% reads mapping to rRNA
- 20% intronic reads, enabling ‘velocity’ analysis
- with 1 million reads per cell, >5360 genes detected by at least four reads per cell
  - majority mRNAs, but also novel genes, polyA[–] genes, and circular RNAs



SMARTer FFPE tissue total RNA seq







10-17% reads mapping to rRNA
(on par with other methods)

Conclusions

- All human biofluids contain RNA, likely reflecting health and specific disease states
- We developed and benchmarked SMARTer pico v2 for exRNA profiling
- Optimization and standardization of pre-analytical steps is key to success
- A case study hints at early clinical validity for exRNA as pharmacodynamic biomarker
- SMARTer pico v2 is a Swiss knife (single cells, FFPE tissue, biofluids)

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