Cell-free circulating RNA extraction: Technical hurdles and practical solutions

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INTRODUCTION

Actively released cell-free circulating RNA (cfRNA) within exosomes holds great promise as a source of minimally invasive cancer biomarkers. These extracellular vesicles (30-150 nm) encapsulate diverse RNA species, reflecting cellular states and making them highly relevant for cancer diagnostics and prognostics. However, the clinical utility of exosome-derived biomarkers depends on the standardization and reproducibility of exosome isolation and RNA characterization methods.

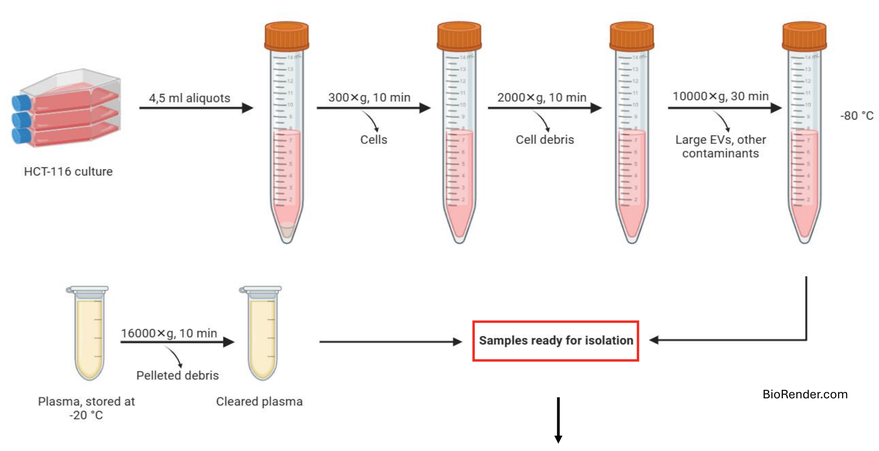
Aim

small EVs (preferentially exosomes) extraction and non-coding RNA extraction from within them

METHODS

Materials

cell culture-conditioned medium (CCM) from HCT-116 cells, 2 – 4 ml of **plasma** from healthy donors



Ultracentrifugation (UC) **100.000 x *g*, 90 min, 4 °C**

- 38,5 ml open-top tubes — 1 x 90 min* / 2 x 90 min* — SW 32 Ti rotor
- 1,5 ml UC tubes with adaptors* — 50.2 Ti Fixed-Angle rotor

Commercial kits

- Norgen ExtraClean Plasma/Serum Exosome Purification (part one) and RNA Isolation Kit
  - Part one (exosome purification)*
  - Complete protocol with RNA extraction
  - Complete protocol + DNase treatment
- Qiagen exoRNeasy
- Qiagen exoEasy*

***RNA isolation**
Qiagen miRNeasy

RNA quantification
Biorad Agilent Bioanalyzer RNA 6000 Pico Kit

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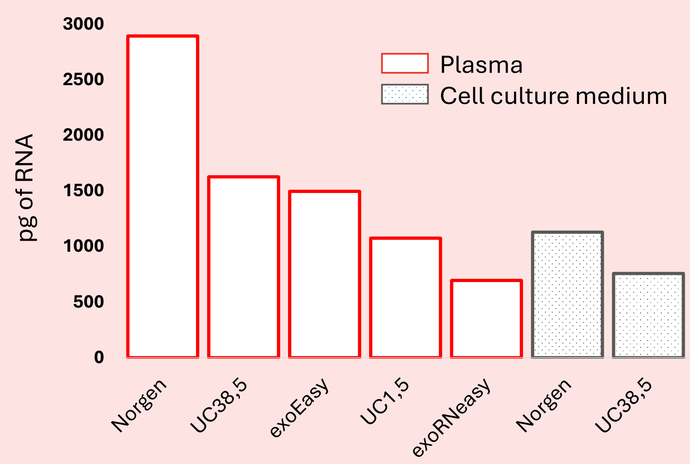
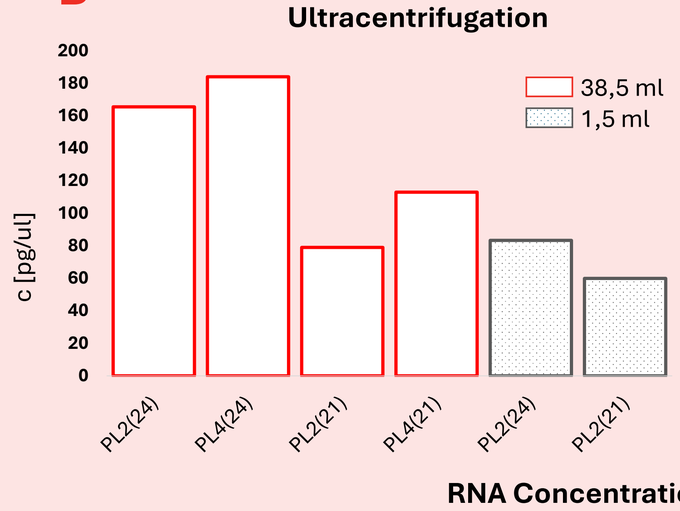
RESULTS

A **Average RNA Amount per ml of Sample**

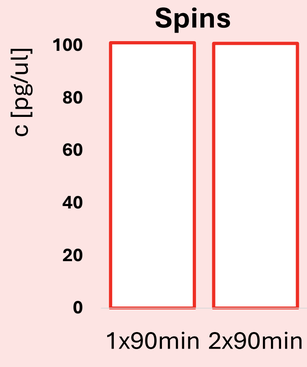


B **RNA Concentration After Ultracentrifugation**



(A) The Norgen kit yielded the highest RNA amount, nearly double that of the second-best method, UC in 38,5 ml open-top tubes, which performed comparably to the exoEasy kit. UC in 1,5 ml tubes ranked fourth, followed by exoRNeasy with the lowest yield, approximately one-third of the RNA amount compared to Norgen. Plasma samples consistently produced higher RNA yields over CCM samples. **(B)** Plasma samples again showed higher RNA yields than CCM samples. Samples derived from 4 ml plasma had higher yields than those from 2 ml plasma, though not proportionally double, with 2 ml still providing relatively good yields. The most significant factor affecting yield was sample age; plasma samples stored for 4 years approximately half the RNA concentration compared to more year old samples. **(C)** Results show no difference between single and double spin conditions, indicating that an additional spin with washing did not increase RNA yield.

C **RNA Concentration After Single vs. Double Ultracentrifugation Spins**



Norgen ExtraClean Plasma/Serum Exosome Purification and RNA Isolation Kit

- Inconsistent results
- Good RNA amounts (larger elution volume), but poor quality
- Great losses with DNase treatment

Qiagen exoEasy

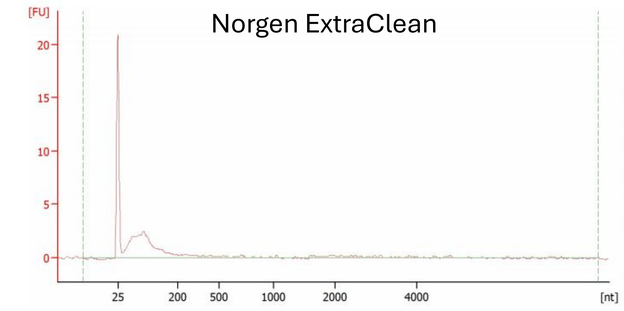
- Mainly short fragments

Qiagen exoRNeasy

- Also yields short fragments

Bioanalyzer as a tool for RNA quality assessment

- Not suitable as RIN (RNA integrity number) algorithm depends on intact 18S and 28S rRNA peaks
- rRNA usually absent or depleted in EV RNA



RNA Electropherogram of Norgen ExtraClean and Qiagen exoRNeasy kits Isolated RNA (Agilent Bioanalyzer)

CONCLUSION & FUTURE PERSPECTIVES

- **Isolation method critical:** Norgen ExtraClean highest yield, but significant losses with DNase treatment
- **Qiagen exoEasy/exoRNeasy:** predominantly enrich short RNA fragments, limiting full-length RNA applications
- **Sample type:** plasma yields superior to CCM; larger plasma volumes improve yield but not proportionally
  Sample age fundamental : older samples show significantly reduced RNA concentrations
- **Ultracentrifugation:** 38,5 ml tubes overall better, extra spin + wash do not improve yield
- **Bioanalyzer:** not the optimal tool for EV RNA quality assessment (no RIN) due to lack of rRNA peaks

Next steps

SEC and **magnetic immunocapture** for higher purity/yield + **PCR**-based quantification to improve sensitivity

References

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