Circulating miRNAs in human plasma

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Introduction

miRNAs in human plasma are thought to exist either as "free" miRNAs bound to protein complexes (Ago2) or contained within extracellular vesicles (EVs or "vesicles"). These sources of plasma miRNA are generated by different biological processes and the ability to distinguish the two populations may aid in creating meaningful biomarker profiles. Here we dissect the populations of plasma miRNAs using direct lysis, ultracentrifugation, and a new spin-column based method for isolating vesicle RNA (exoRNeasy). Using columns for either depletion or isolation of vesicles, we compare the miRNome of "free" Ago2-bound and vesicle-associated miRNA populations in plasma.

- Bind vesicles to membrane and wash
- QIAzol lysis and release of RNA
- Phenol/chloroform extraction
- Ethanol conditioning
- Bind RNA to RNeasy MinElute® column
- Elute RNA

Figure 1. The new exoRNeasy Serum/Plasma Maxi Kit. Disposable spin column to purify vesicles and workflow of the exoRNeasy Serum/Plasma Maxi Kit.

PCR signal after on-column RNase digestion

% of PCR signal from mock ($\Delta\Delta C_t$)

- GAPDH
- let-7a
- miR-16
- HPRT1

Figure 3. exoRNeasy isolates membrane-protected mRNA and miRNA. Vesicles from 4 ml of pre-filtered plasma were bound to an exoEasy column and washed. The membrane was treated for 30 minutes with either RNase I, the detergent Triton, both, or reaction buffer (mock). The RNA was isolated following exoRNeasy procedure. Only when Triton is used to destabilize the lipid bilayer, is the RNase able to digest the mRNA inside the vesicles (leftmost columns).

miRNA distribution

C_t values, 381 miRNAs

Figure 5. Bulk miRNA signal is found outside extracellular vesicles in plasma. 0.2 ml of pre-filtered plasma was used to isolate vesicle-associated miRNAs using exoEasy columns or ultracentrifugation in triplicate. Flow-through from the exoEasy column and untreated plasma were lysed directly. The RNA from a total of 12 samples was isolated using identical procedures. The samples were reverse transcribed and analyzed using a miScript Serum & Plasma 384HC PCR array. A boxplot of the distribution of all C_t-values reveals the majority of signal to be outside vesicles.

M: $\log_2(C_t^{exoRNeasy}/C_t^{flowthrough})$

Figure 6. Abundance of miRNAs inside and outside extracellular vesicles. 0.2 ml of pre-filtered plasma was used to isolate vesicle-associated miRNAs using exoRNeasy. Remaining miRNAs in the flow-through from the exoEasy columns were isolated with direct lysis. The C_t values from miScript Serum & Plasma 384HC PCR arrays where scaled according to total RNA amount and compared in intensity ratio versus average intensity (MA-Plot). Each circle represents a single miRNA assay as average of three isolation replicates. The broad distribution of data points over 10 C_ts along the M axis shows the strong differential abundance of miRNAs inside versus outside vesicles.

Scanning electron microscopy of vesicles

SEM of pellet from ultracentrifugation
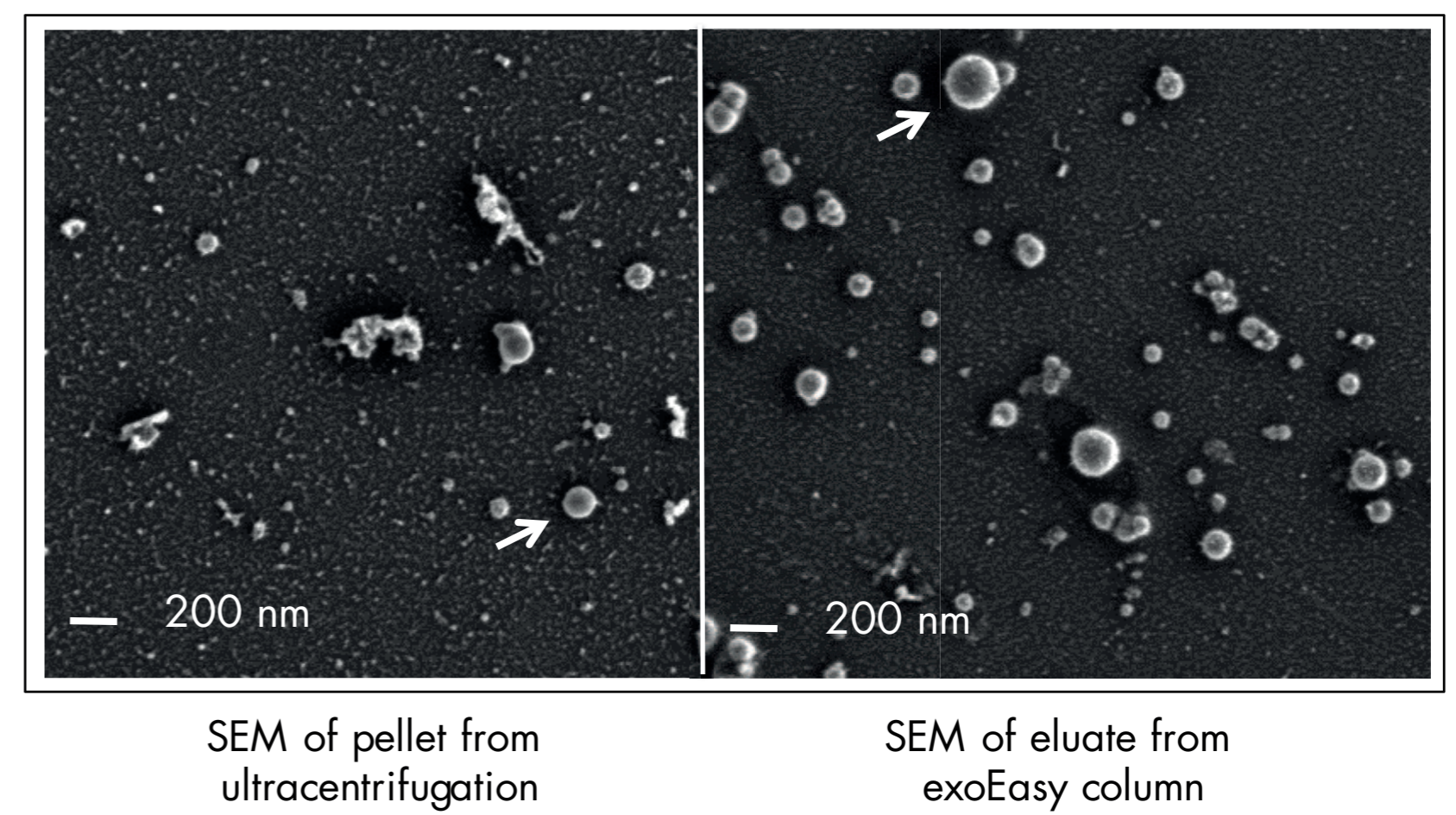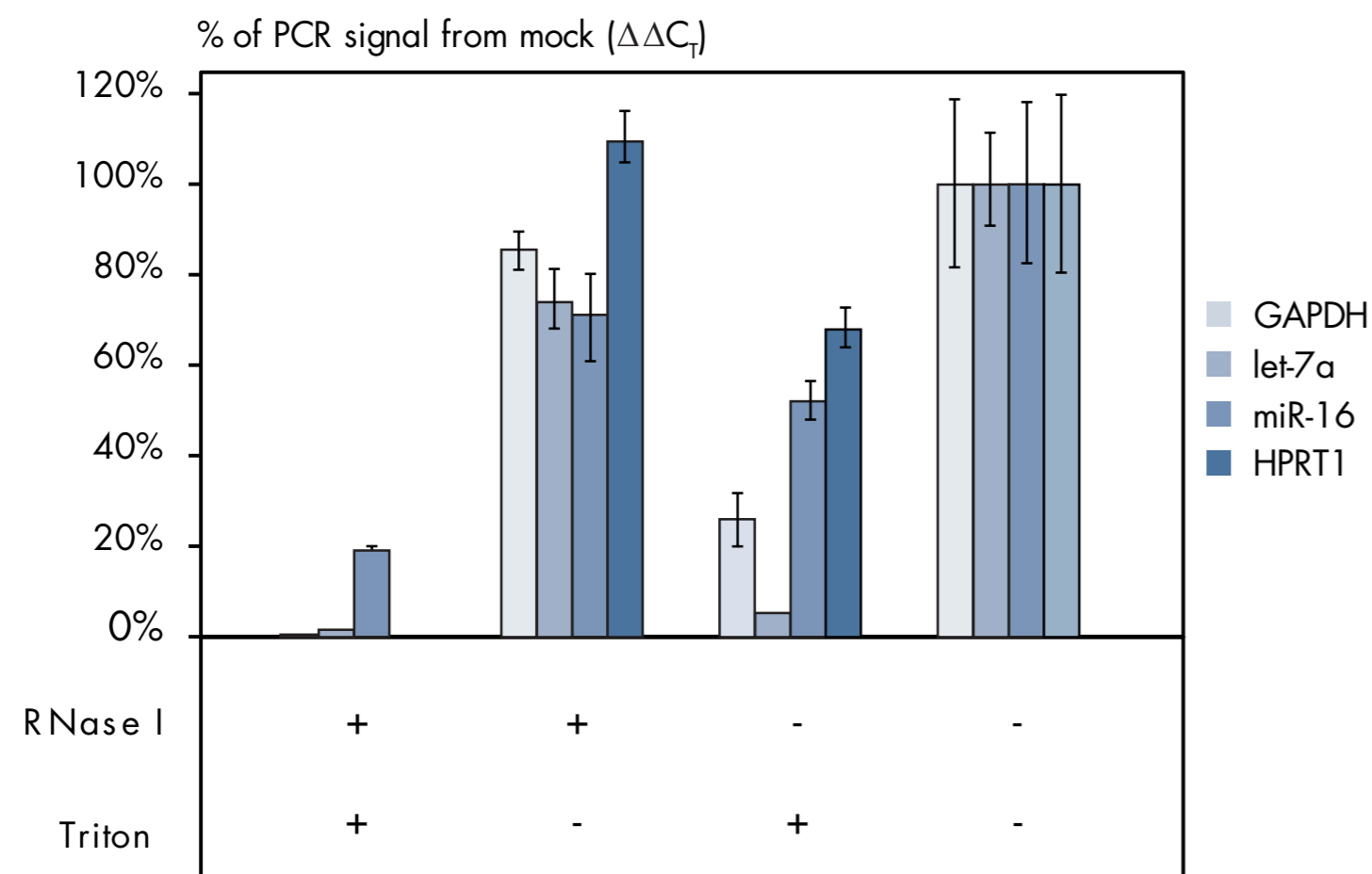
SEM of eluate from exoEasy column

Figure 2. Intact vesicles can be eluted from the exoRNeasy membrane. Scanning electron microscopy (SEM; 20,000 × magnification) of a solubilized pellet from ultracentrifugation of pre-filtered (0.8 μm) plasma compared to a non-lysed eluate of an exoEasy column. Both preparations contain vesicle-shaped structures with an expected size range from 50–200 nm (white arrows; scale bar 200 nm), but there is less non-vesicular background in the material eluted from the exoEasy spin column.

miRNAs inside vesicles are protected

miRNAs outside vesicles
PCR signal ($\Delta\Delta C_t$)

- miR-16 mock
- miR-16 ProtK
- Sensitive to Proteinase K
- miR-92a mock
- miR-92a ProtK

miRNAs inside vesicles
PCR signal ($\Delta\Delta C_t$)

- let-7a mock
- let-7a ProtK
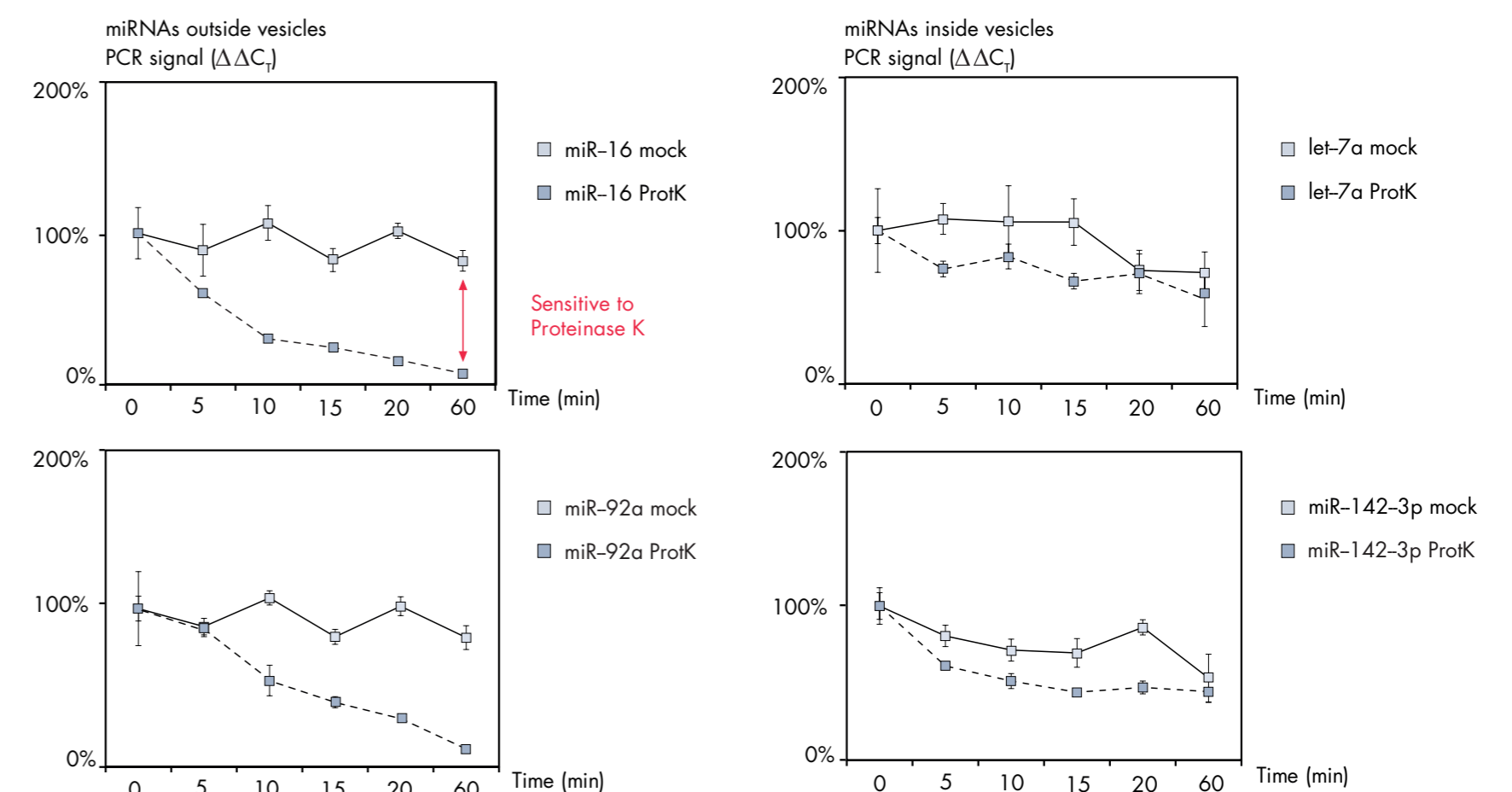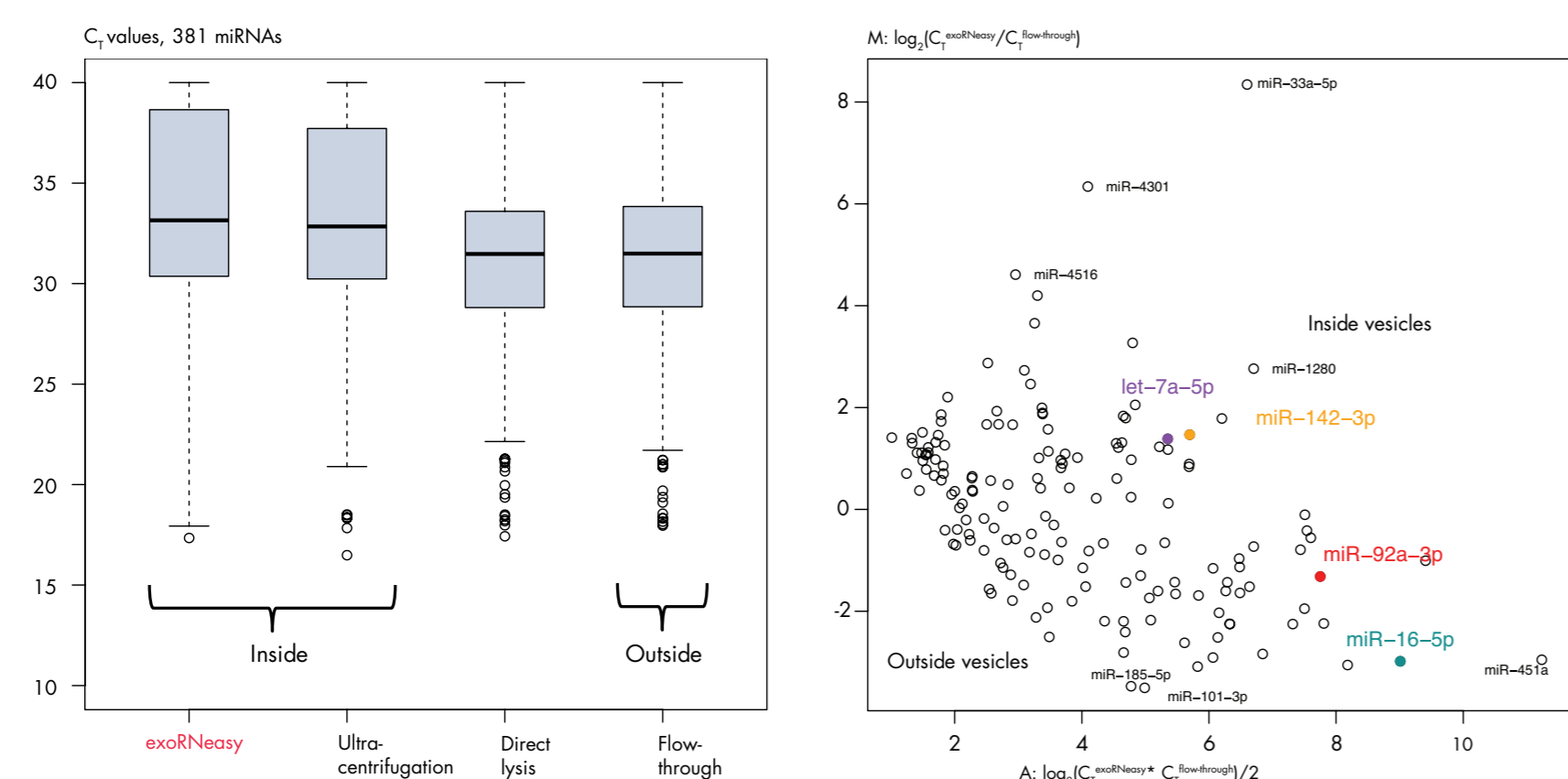- miR-142-3p mock
- miR-142-3p ProtK

Figure 4. miRNAs inside vesicles are protected from proteinase digestion and degradation. Pre-filtered plasma was either incubated at 55°C with proteinase K (ProtK) or with buffer only (mock). Aliquots of 0.2 ml where sampled at various time points and RNA extracted using direct lysis with QIAzol. The miRNAs which are not protected by a vesicle membrane lose their associated protein and subsequently are degraded by plasma RNases.

Conclusions

- The exoRNeasy Serum/Plasma Maxi Kit can be used to extract high-quality RNA from plasma EVs using a fast and convenient spin-column procedure.

- exoEasy columns can be used to separately investigate vesicle-associated miRNAs and "free" circulating Ago2-associated miRNAs.

- EVs carry a small, defined fraction of all plasma miRNAs, which may allow for the detection of specific biological signals.

The applications presented here are for molecular biology applications. They are not intended for the diagnosis, prevention or treatment of a disease.

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