

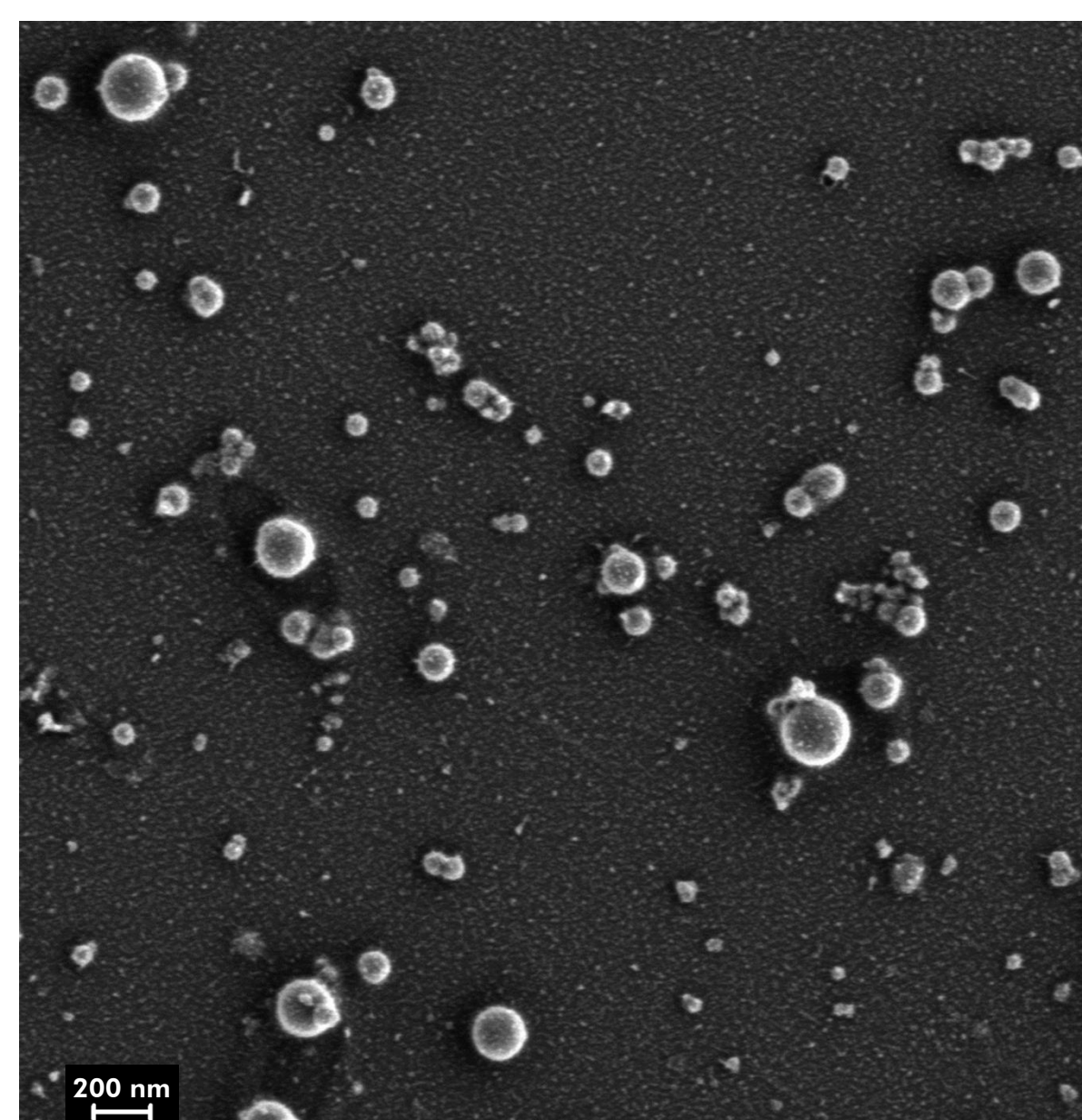
# Comparison of Different Preanalytical Workflows for Isolation of Intact Exosomes and Extracellular Vesicles

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## Introduction

The scientific literature on exosomes and other extracellular vesicles (EVs) continues to be characterized by a wide variety of vesicle isolation and characterization procedures, as well as nomenclature, resulting in considerable difficulty comparing results between independent studies. An additional layer of variability is added by sample handling and pretreatment. This may include the type of blood collection tube, time between blood draw and generation of plasma or serum, as well as measures taken to remove residual cells and cell fragments, such as pre-centrifugation or filtration steps. In this study, we compare different handling and pretreatments, and how they affect physical characteristics, as well as RNA content of vesicle preparations resulting from a spin column-based purification approach.

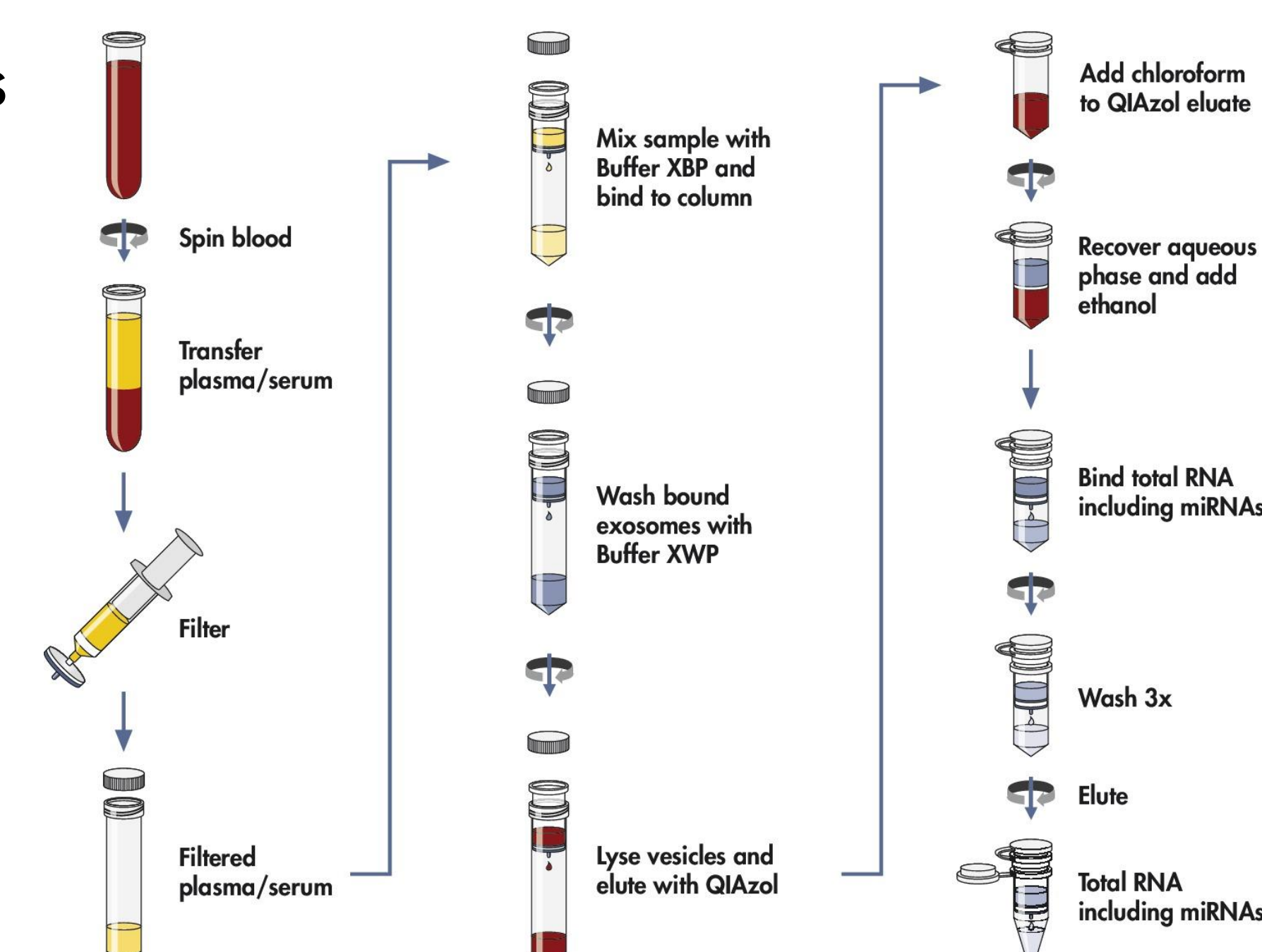


**Figure 1.** Scanning electron micrograph of intact vesicles eluted from the exoEasy spin column (20.000 x magnification).

## Materials and methods

Blood from healthy donors was collected in different collection tubes. After generation of plasma and removal of residual cells and fragments, vesicular RNA was generated using an exoRNeasy kit and relative abundance of selected RNAs compared by qPCR.

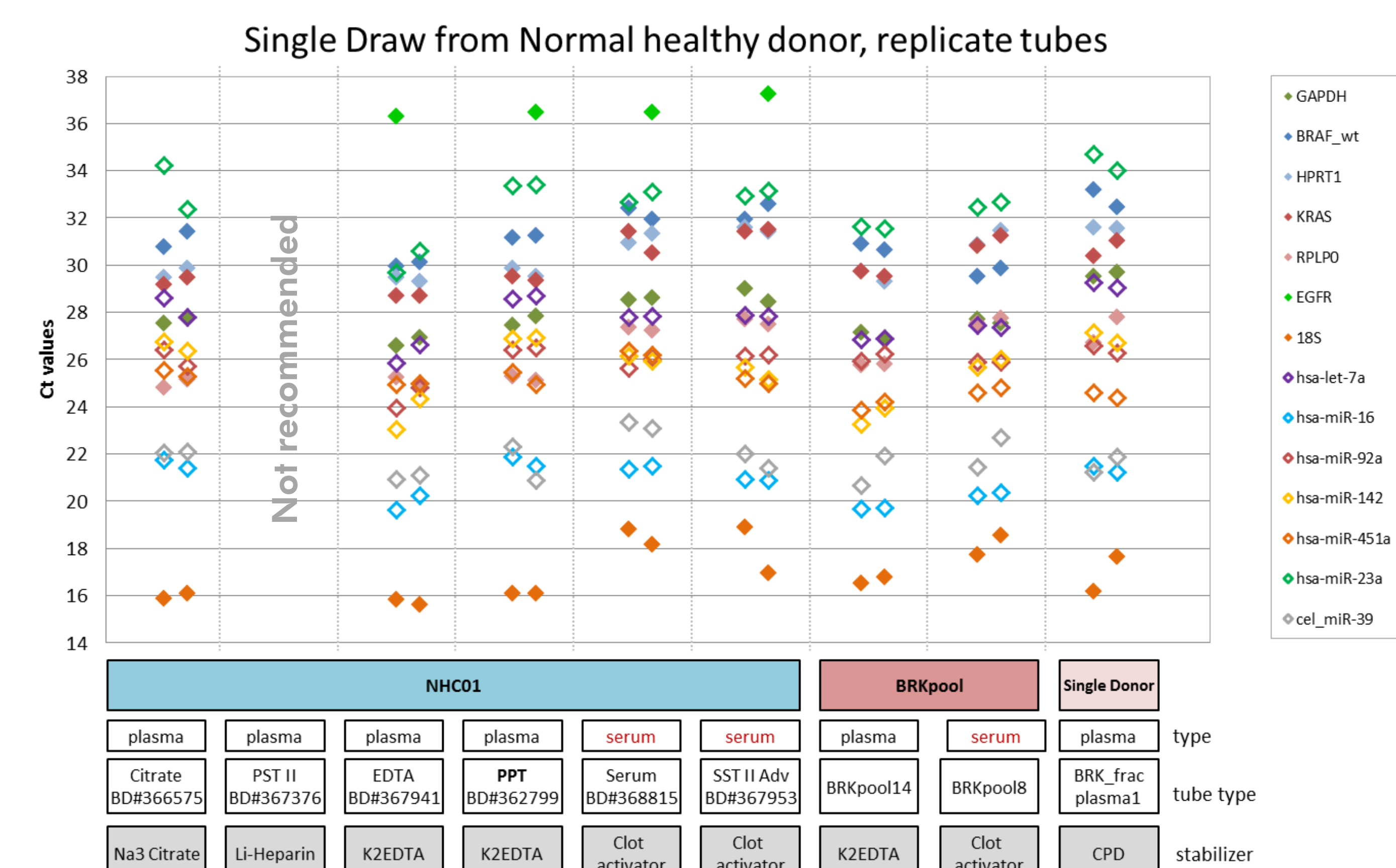
In addition, effects of the time between blood collection and generation of plasma was tested on the RNA level.



**Figure 2.** exoRNeasy workflow for isolation of vesicular RNA. For isolation of intact vesicles, elution buffer was used instead of QIAzol, and the eluates used for further analysis.

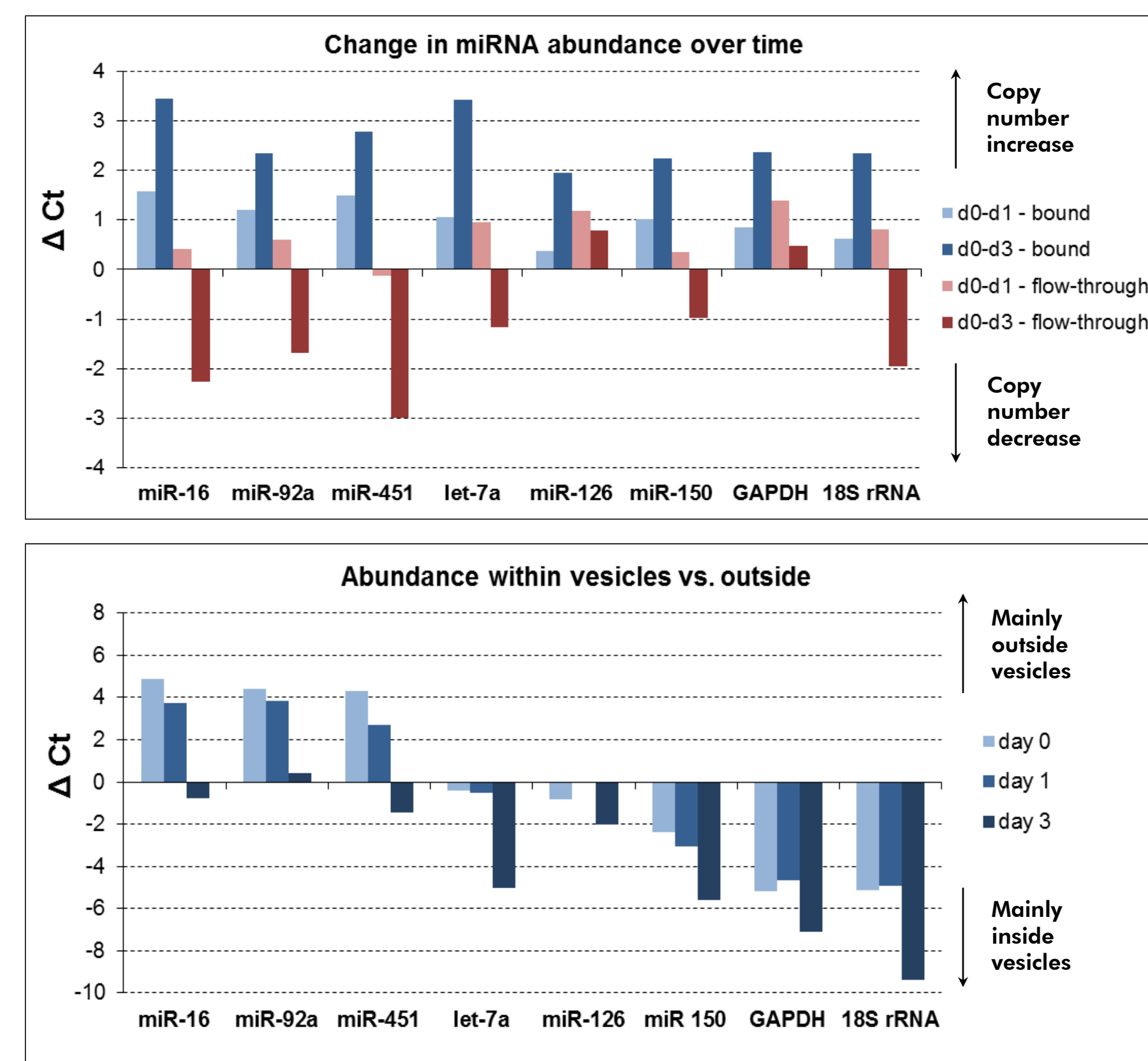
Finally, we compared different approaches to remove residual cells and cell fragments by additional centrifugation and filtration steps prior to vesicle isolation, and analyzed the resulting vesicle size distribution and RNA content. Intact EVs eluted from the exoEasy column were analyzed using the Nanosight NS300.

## Results — Influence of Blood Collection Device on RNA



**Figure 3.** A wide variety of blood collection tubes is compatible with EV RNA isolation, but representation of individual transcripts may differ. Use of heparin tubes is not recommended.

## Results — Influence of Blood Storage



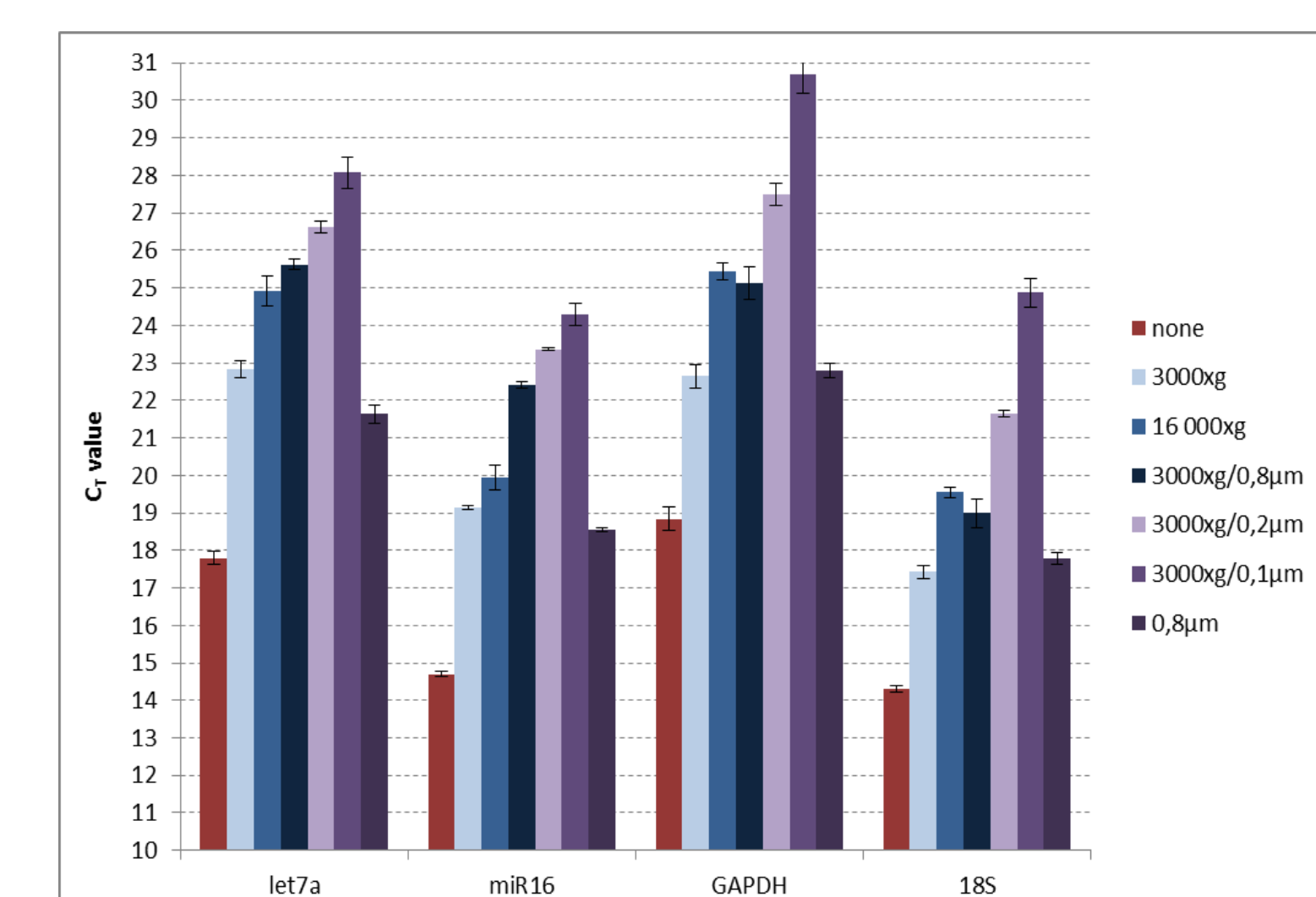
**Figure 4.** Change in RNA abundance during storage of blood samples at room temperature, prior to generation of plasma.

We previously demonstrated that the exoRNeasy protocol selects for vesicular RNA (e.g. let-7a), whereas 'free' Ago2-associated miRNA (e.g. miR-16) remains in the column flowthrough.

Blood samples were stored for 0-3 days prior to generating plasma, followed by EV RNA isolation and analysis.

- Vesicular RNA increases during storage, up to 10-fold; presumably due to continued production of vesicles by blood cells
- Non-vesicular RNA increases after 1 day, but strongly decreases after 3 days; probably due to RNA degradation

## Results — Influence of Pre-Handling



**Figure 5.** Effect of centrifugation and filtration steps for removal of residual cells and cell fragments from plasma on RNA abundance.

Filtration or centrifugation is required to exclude residual cells and cell fragments from plasma prior to isolation of EVs and vesicular RNA. More than 90% of plasma RNA content is removed by a 0.8  $\mu$ m filter. Different centrifugation speeds or filter pore sizes also affect representation of different RNA transcripts, even though particle concentration and size distribution is not significantly affected, except for the smallest filter pore size used.

	Part. Size mean [nm]	Part. Size D50 [nm]	Part. Conc. (per ml plasma)
3000xg	143.3	133.7	5,34E+10
16000xg	162.2	161.3	4,32E+10
3000xg, 0.8 $\mu$ m	159.2	155.6	4,80E+10
3000xg, 0.2 $\mu$ m	165.1	161.9	5,10E+10
3000xg, 0.1 $\mu$ m	123.1	110.9	2,34E+10
0.8 $\mu$ m	153.8	143.2	4,54E+10

**Figure 6.** Effect of centrifugation and filtration steps for removal of residual cells and cell fragments from plasma on particle concentration and size distribution (det. by nanoparticle tracking analysis).

## Conclusions

- In all tested preprocessing workflows, intact vesicles and vesicular RNA could be isolated. However, the choice of collection tube, anticoagulant, etc. does have an influence on RNA representation, so it is strongly recommended to not switch between different collection tubes within the same study. Use of heparin tubes is not recommended for molecular biology applications, because heparin is known to strongly inhibit enzymatic reactions, including PCR.
- Storage of blood prior to plasma generation can result in release of additional vesicular RNA from blood cells, which in most cases represents unwanted background. In contrast, cell-free, non-vesicular RNA decreases after prolonged storage of blood. This results in a major shift in the ratio of vesicular versus non-vesicular RNA over time.
- Centrifugation and filtration steps are essential to remove cell fragments, but also affect representation of different classes of vesicles and RNA. The effect on vesicle concentration and size distribution is not proportional to the effect on RNA representation, indicating that RNA is not evenly distributed in vesicles.