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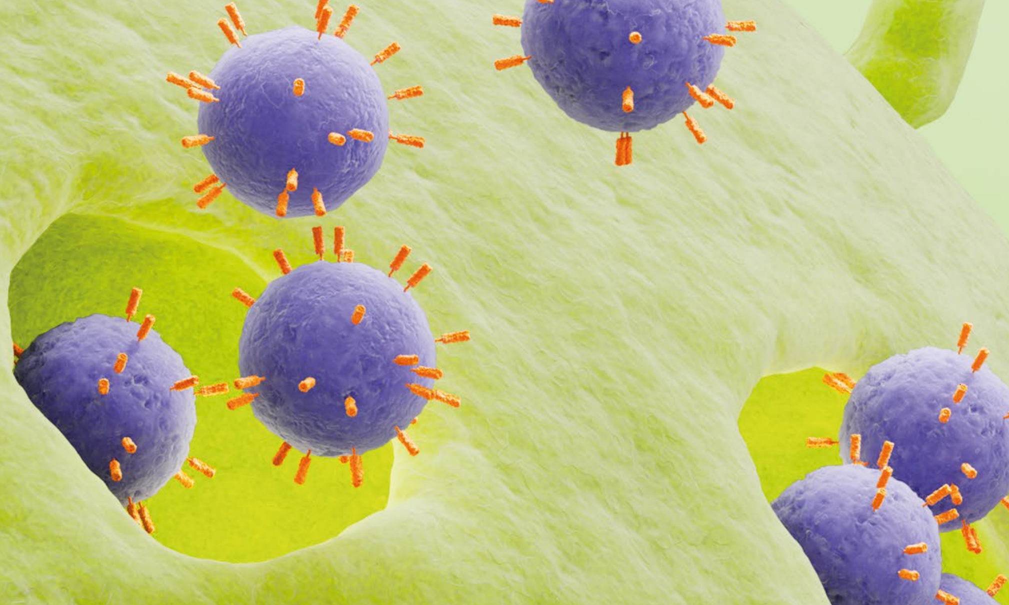

Isolation of highly pure urinary extracellular vesicle fractions suitable for downstream RNA cargo sequencing

Veronika Mussack[a], Georg Wittmann[b], Michael W. Pfaffl[a]
[a]Animal Physiology and Immunology, School of Life Sciences Weihenstephan, Technical University of Munich, Freising, Germany
[b]Department for Transfusion Medicine, Cell therapeutics and Haemostaseology, University Hospital LMU, Munich, Germany

Background

Extracellular vesicles (EVs), which comprise all vesicles that are released by cells, can be detected in various biofluids, including urine. They transmit biological information between close and more distant cells and their molecular cargo (i.e. nucleic acids, proteins, and lipids) is assumed to mirror the conditions of their originating tissues.[1] microRNAs (miRNAs) carried by small urinary EVs (uEVs) have particularly emerged as promising biomarkers to monitor progression of diseases such as bladder cancer.[2] In this study, we investigated the suitability of positive immunomagnetic enrichment for the isolation of small uEVs and compatibility of this method with downstream miRNA screening. To this end, we characterized particle size, concentration, and protein content of purified small uEVs as recommended by MISEV guidelines[3], as well as miRNA cargo of the isolated uEVs. This application note is based on reference 4.

Methods

Urine sampling and processing

Six healthy men (18–36 years) provided urine samples. A volume of 10 mL of each sample was pre-cleared and stored at –80 °C until further processing. Pre-cleared urine samples were then concentrated via ultra-filtration and processed for small uEV isolation with the approaches reported in table 1.

Isolation method	EV isolation based on
A	Spin column chromatography
B	**Immunomagnetic enrichment***
C	Membrane affinity
D	Precipitation
E	Ultracentrifugation with density gradient

Table 1: Isolation strategies applied to extract small uEVs from urine. *Immunomagnetic enrichment with the Exosome Isolation Kit Pan, human.

Isolation of uEVs using the Exosome Isolation Kit Pan, human

The Exosome Isolation Kit Pan, human enables the positive immunomagnetic enrichment of EVs by using MicroBeads recognizing the tetraspanin proteins CD9, CD63, or CD81. EVs are magnetically labeled with Exosome Isolation MicroBeads CD9, CD63, and CD81 and loaded onto a µ Column, which is placed in the magnetic field of a µMACS® Separator. The magnetically labeled EVs are retained within the column, while the unlabeled vesicles and cell components run through. After removing the column from the magnetic field, the intact EVs can be eluted (fig. 1).

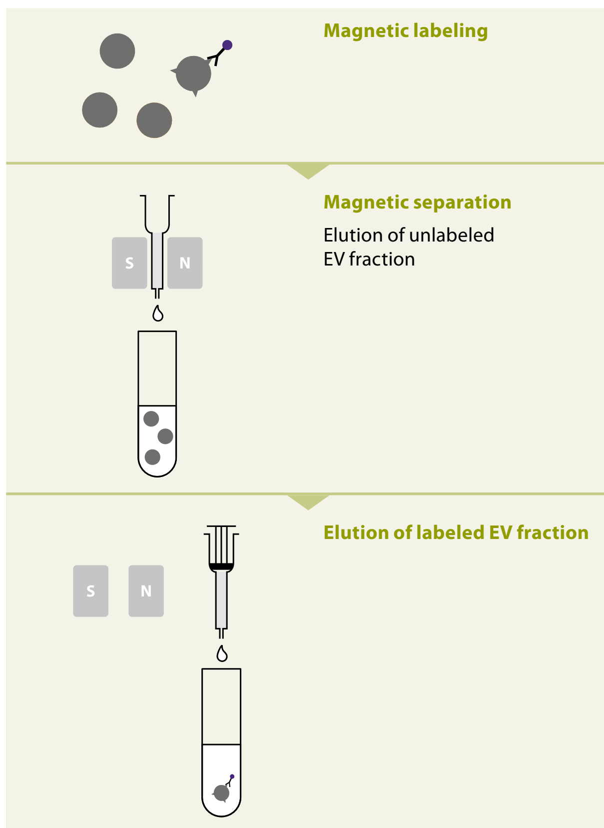

Figure 1: Principle of magnetic isolation of EVs using the Exosome Isolation Kit Pan, human.

Characterization of purified uEVs
The presence of EV-associated proteins as well as uEV quantities were evaluated by Western blotting and nanoparticle tracking analysis as described in reference 4.

RNA extraction and sequencing
Total RNA was extracted from isolated small uEVs as described in reference 4 and stored at –80 °C until further processing. Small RNA libraries were prepared and analyzed by single-end sequencing-by-synthesis reactions as described in reference 4 considering sequencing recommendations for liquid biopsies[5].

Results

The Exosome Isolation Kit Pan, human isolates a highly pure and concentrated fraction of uEVs
Nanoparticle tracking analysis (ZetaView® PMX110, Particle Metrix) revealed a highly concentrated fraction of enriched small uEVs (fig. 2). The discrepancy in particle concentration measured in scattering mode (SM) versus fluorescence mode (FM) after immunomagnetic enrichment can be explained by the presence of kit-derived MicroBeads. MicroBeads are only detected in SM, whereas FM solely detects membrane-enclosed particles like EVs.

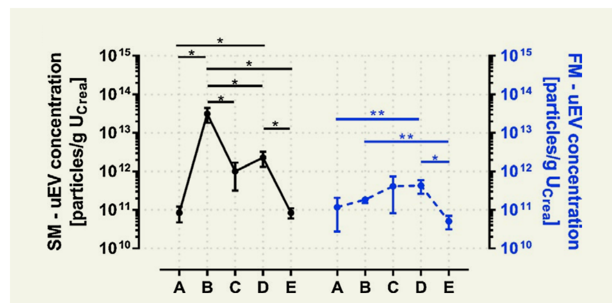

Figure 2: Nanoparticle tracking analysis of small uEVs. Small uEVs were extracted by isolation strategies A–E as indicated in table 1. Particle concentration was determined by nanoparticle tracking analysis in SM (black) and FM (purple). Data and figure adapted from reference 4.

Investigation of EV-associated proteins and possibly contaminating proteins indicated the high purity of small uEVs after enrichment with the Exosome Isolation Kit Pan, human. The latter express high amounts of the three common exosome markers CD9, CD63, and CD81, as well as syntenin, Alix, and TSG101 (fig. 3). Furthermore, the presence of contaminating non-EV structures including uromodulin and calnexin could be excluded, highlighting the specificity of the assay and the high purity of the small uEVs after immunomagnetic enrichment.

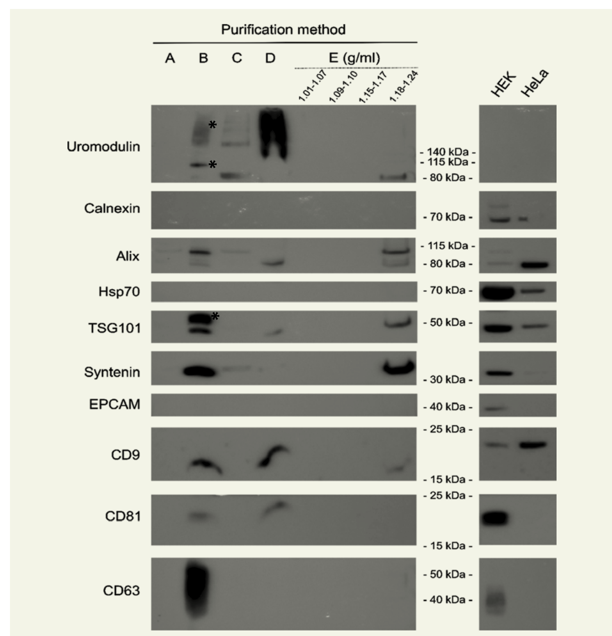

Figure 3: Detection of EV-associated proteins by Western blotting. EV-associated protein expression is shown for small uEVs extracted by isolation strategies A–E as indicated in table 1. Unspecific bands indicated by asterisks could be identified as isolation kit–derived mouse IgG proteins. Data and figure adapted from reference 4.

Small uEVs isolated with the Exosome Isolation Kit Pan, human are suitable for downstream RNA sequencing

In order to analyze RNA cargo of isolated small uEVs, total RNA was extracted and small RNA sequencing (RNA-Seq) was performed. Comparable miRNA amounts could be detected in small uEVs isolated by all isolation strategies (fig. 4). Immunomagnetic isolation with the Exosome Isolation Kit Pan, human therefore proved to be compatible with downstream RNA-Seq, especially for the analysis of miRNA cargo in the pure CD9+/CD63+/CD81+ small uEV population.

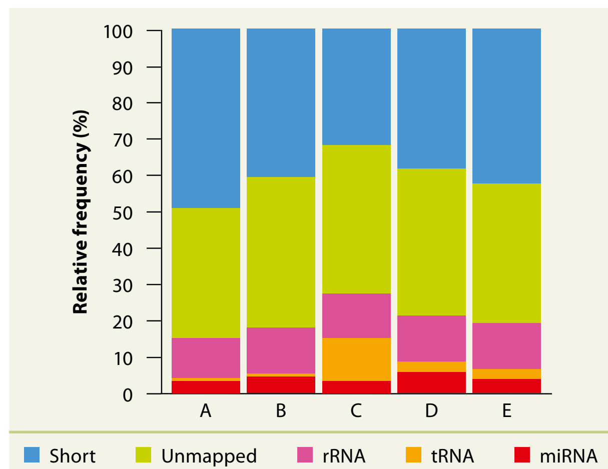

Figure 4: Characterization of RNA cargo in isolated small uEVs. Total RNA was extracted from uEV samples isolated with methods A–E as indicated in table 1 for subsequent small RNA-Seq. Mean mapping percentages for small RNA classes: microRNA (miRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). Short: reads shorter than 16 nucleotides. Data and figure adapted from reference 4.

Conclusions

- Exosome Isolation Kit Pan, human is suitable for the isolation of small EVs from urine samples.
- Exosome Isolation Kit Pan, human results in the isolation of a highly pure small uEV population, expressing the common EV-associated proteins Alix, TSG101, syntenin, CD9, CD63, and CD81.
- The assay is compatible with small RNA sequencing and thus can be used to investigate RNA cargo derived from CD9+/CD63+/CD81+ small uEVs.

References

This application note is based on the paper V. Mussack, G. Wittmann, M.W. Pfaffl, Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research, Biomolecular Detection and Quantification 17 (2019) 100089, Copyright © 2019 The Authors and Elsevier. Reproduced by kind permission of the authors and Elsevier.

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