

The quick reference card “Storage of urinary EVs” – A practical guideline tool for research and clinical laboratories

Dear Editor,

The high diagnostic potential of urinary extracellular vesicles (uEVs) for urogenital disease has been recognized for more than a decade. This is emphasized by the identification of different molecular biomarkers (i.e. protein, mRNA, miRNA, lipids and metabolites) in uEV preparations that may assist the clinical management of prostate, bladder, and renal cancer (Junker et al., 2016). uEV biomarkers for other pathologies like acute and chronic kidney disease of various etiologies, cystic and tubule-interstitial disease, or for kidney transplantation are also under active investigation (Grange & Bussolati, 2022).

Apart from the growing need for validation studies, the translational potential of uEV biomarkers is hampered by several biological factors. Such factors include the diverse cellular origins of uEVs throughout the renal and urogenital tract, but also the dynamic molecular composition of urine due to hydration status, diet, salt regulation, exercise, and circadian rhythm. In addition to these inherent factors, the reproducibility of uEV analysis is also strongly influenced by logistic variables like the differences in the time of sampling or the preanalytical procedures for handling of urine samples (Erdbrügger et al., 2021).

The general reporting recommendations for EV sample processing and analysis are covered in detail in the Minimal Information for Studies of Extracellular Vesicles (MISEV 2018) position paper (They et al., 2018). However, a community consensus on best methodological practices that is tailored to the biofluid-specific characteristics and requirements is of particular importance for the success of preclinical and clinical studies on biomarker discovery, validation and future use in clinical decision making. To address this need in uEVs research, the Urine Task Force of the Rigor and Standardization Subcommittee of the *International Society for Extracellular Vesicles* (ISEV) published a position paper summarizing the current *state of the art* and listing detailed recommendations for improved rigor, reproducibility and inter-operability in uEV research (Erdbrügger et al., 2021).

To support the implementation of the published recommendations, and enhance their application in daily research practices, here we provide a Quick Reference Card on *STORAGE of urinary EVs* (Figures 1 and 2, Supplementary File 1). The Quick Reference Card does not substitute a uEVs protocol for storage, isolation or processing but it summarizes the expert community consensus recommendations on the most critical factors affecting storage of fresh or biobank urine and uEVs samples as discussed in the uEV position paper (Erdbrügger et al., 2021). The Card is organized according to six critical stages: Biobanking, Storage of urine prior to processing, Preprocessing, Storage of urinary supernatant and uEVs, Defrosting, and Transportation. Evidence level and reporting priority for each stage are color-coded in accordance to the findings as described in the ISEV uEVs position paper (Erdbrügger et al., 2021) and according to the MISEV 2018 guidelines (They et al., 2018). The Card is intended as an easily accessible guideline tool that can be used during study planning and manuscript preparation, but also as a “bench top” reference during everyday laboratory work.

To conclude, we present a novel format of communication for EV study guidelines and recommendations that can also be applied to other topics within, but importantly also outside the field of urinary EVs. Ultimately, by using this format, we endeavor to enhance adherence to pre-analytical best practice guidelines in order to promote reproducibility and, above all, the translational potential of uEV studies.

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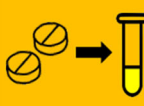




CONFLICTS OF INTEREST

The authors report no conflict of interest.

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Parameter		Recommendation	
Reporting priority		Evidence level	
Existing biobanks	All parameters  Max. 800 x g ...etc.	Report as many parameters as possible.	HIGH: Archive urine samples from existing urine biobanks, are often collected according to protocols that are not optimal for uEV preservation and uEV research . Collect all below-mentioned parameters and determine if sample collection is appropriate for your research purpose. Perform tests to determine urine quality, number and characteristics of EVs.
Storage of urine prior to processing	Time  8 h	Max. 8 h	HIGH: Longer storage time may lead to microbial growth, cell debris, sedimentation, and degradation of more labile biomolecules (e.g. RNA).
	Temperature  +4°C	Max. +4°C, avoid freezing.	HIGH: Freshly collected urine samples should be cooled promptly to avoid microbial growth or biomolecule degradation. Avoid freezing at this step.
	Light 	Protect from light.	LOW: Some urinary analytes may be light sensitive (e.g. bilirubin, porphyrins); impact on uEVs is unknown. Use amber-colored or dark collection tubes.
	Quality control 	Use dipstick. Report brand.	HIGH: The presence of cells, microbes as well as high levels of protein and other factors affect the purity and composition of uEV population. Use dipsticks to examine urine quality and identify sample outliers. Report dipstick brand, tested parameters, and sample inclusion criteria and cutoffs.
Preprocessing	Protease inhibitors 	Use fresh or frozen aliquots of protease inhibitors.	MEDIUM: Preservative might be affected by time and storage in collection container. If protease inhibitors are used at the time of collection, it is recommended that sample containers are prepared by adding protease inhibitor cocktail. Protease inhibitor cocktail aliquots should be kept frozen at -20°C for a maximum of 6 months.
	Time  4-6 h	4-6 h	HIGH: Freshly collected urine samples should be processed promptly to avoid microbial growth or biomolecule degradation. Consider addition of protease inhibitors or preservatives when fast processing (faster than 6 hours) is not possible (see above).
	Urine Centrifugation 	Max. 800 x g Max. +4°C	MEDIUM: Centrifuge at a maximum of 800 x g to sediment cells and debris present in urine without damaging them. Report centrifuge and rotor model, G-force, volume, temperature, and duration.
	Supernatant Recovery 	Report volume (ml) and method.	MEDIUM: Operator-dependent. Report volume. Report method (e.g. pipetting, decanting).
	Other fractions 	Report type and volume (ml).	MEDIUM-HIGH: Collection and storage of pellet and whole urine aliquots is recommended to monitor the uEVs purification process.

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FIGURE 1 Quick Reference Card “Storage of urinary EVs”, page 1 *Storage of urine prior to processing* and *Pre-processing steps*. Priority and Evidence levels are as reported in (Erdbrügger et al., 2021) and represent expert consensus opinion of the current level of confidence that the parameter is a variable to consider during sample biobanking and data analysis and interpretation.

Parameter		Recommendation	
Reporting priority		Evidence level	
Storage of urinary supernatant and uEVs	Supernatant Aliquots 	Report number, Date, and volume (ml).	MEDIUM: As samples may be used for several experiments, when possible, collect aliquots of different volumes to avoid repeated freeze/thawing. <i>Large</i> , up to 30 ml; <i>Medium</i> , 5 - 10 ml; <i>Small</i> , 1 - 2 ml.
	Container 	Use max. ¾ of container volume.	MEDIUM: Use max ¾ of container volume to accommodate sample expansion. Storage container should resist pH range of urine and not shed any particles. Low EV binding properties are generally beneficial.
	Freezing Time 	Sec. Min. Seconds, minutes.	LOW: Quick freezing is generally recommended to preserve biological specimens, but impact of freezing speed or cryoprotective agents on uEVs is unknown. Freeze quickly in -70°C freezer or snap freeze in liquid nitrogen. Report freezing method and sample volume.
	Temperature 	Max. -70°C	MEDIUM: Particle counts may decrease and lead to loss of antigenicity of EV proteins after storage at -20°C. EV yield from samples stored at -20°C may be lower. Freeze immediately and store at -70°C or lower.
Defrosting	Method 	The same for all samples.	LOW: Heating pad, water bath, incubator, room temperature, refrigerator. Standardize defrosting method and use the same technique for all samples.
	Temperature 	~ 37°C Avoid prolonged warming.	LOW: The effect on thawing temperature on uEVs has not been studied extensively. However, high temperatures might affect heat labile biomolecules or lead to sediment formation.
	Time 	Max. 1 h	LOW: Longer thawing times may require addition of preservatives.
Transportation of uEV	Temperature 	Check temperature during transport and at arrival.	MEDIUM-HIGH: EV quality and quantity diminish during long-term exposure at room temperature and during multiple freeze-thaw cycles. Use cooling system whenever possible. Preservatives can prevent protein/RNA breakdown and bacterial outgrowth. Transport uEVs and processed supernatant frozen ($\leq -70^\circ\text{C}$) and whole urine at $+4^\circ\text{C}$.
	Time and Method 	Duration in hours. Check container for integrity & damage.	MEDIUM-HIGH: uEV quality and quantity diminish with long-term storage at room temperature. Container leakage could introduce contamination. Inspect containers for integrity and damage.

Reporting Priority level: ■ Obligatory ■ High ■ Medium ■ Low

Evidence level: ■ High ■ Medium ■ Low

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FIGURE 2 Quick Reference Card “Storage of urinary EVs”, page 2 *Storage of urinary supernatant and uEVs, Defrosting, and Transportation of uEVs*. Priority and Evidence levels are as reported in (Erdbrügger et al., 2021) and represent expert consensus opinion of the current level of confidence that the parameter is a variable to consider during sample biobanking and data analysis and interpretation.

AUTHOR CONTRIBUTIONS

Conceptualization: M.v.R., C.S., C.G., J.W., T.T., M.D., A.B., B.G., A.L., C.B., D.B., U.E., E.M.U. Writing, original draft preparation: M.v.R., E.M.U.; Writing, review and editing: M.v.R., C.S., C.G., J.W., T.T., M.D., A.B., B.G., A.L., C.B., D.B., U.E., E.M.U.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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