Supporting Information 4: "Calibration of flow cytometers enables reproducible measurements of extracellular vesicle concentrations and reference range establishment"

Protocol for the preparation of the plasma EV test sample (PEVTES) that was used as an EV-containing sample in the interlaboratory comparison study.

1.1 Experimental design

The aim of the experiment was to develop a plasma EV test sample (PEVTES) [1], which was distributed in the interlaboratory comparison study as an EV-containing sample.

1.2 Preparation of the plasma EV test sample (PEVTES)

1.2.1 Preparation of cell-depleted plasma

The collection of blood was performed according to the guidelines of the medical ethical committee of Amsterdam Medical Centre, University of Amsterdam (W18_391#18.450). Blood was collected from 5 healthy and overnight fasting individuals who provided informed consent and denied having a disease and/or using medication. Venous blood was collected using a 21-gauge needle (368607, Becton Dickinson (BD) Biosciences), and the first 3.5 mL of blood was discarded. Three tubes of Ethylenediaminetetraacetic acid (EDTA) blood [2](6 mL, 9203871, BD Biosciences) were collected per donor, mixed gently with the anticoagulant, and processed within 15 minutes. To prepare plasma, whole blood was centrifuged at 2,500× g for 15 minutes at 20 °C, acceleration speed 9, and deceleration speed 1 using a

Rotina 380 R equipped with a swing-out rotor and radius of 155 mm (Hettich Zentrifugen). Plasma was collected 10 mm (determined with a Lego brick) above the buffy coat using a plastic pasteur pipette (86.1171.001, SARSTEDT) and transferred into a new 15-mL polypropylene centrifuge tube (62.9924272, SARSTEDT). Subsequently, the plasma was centrifuged at the same settings used for whole blood. Afterward, plasma was collected to 10 mm above the pellet to reduce platelet contamination and transferred into a new 15-mL polypropylene centrifuge tube (62.9924272, SARSTEDT). Next, plasma was pooled, mixed gently, and transferred to 1.5-mL low protein binding Eppendorf tubes (616201, Greiner Bio-One B.V.).

1.2.2 Staining EVs for flow cytometry

To measure the concentration of platelet-derived (CD61- allophycocyanin (APC)), erythrocyte-derived (CD235a-phycoerythrin (PE)), plasma EVs were immunofluorescently stained. Before staining, aggregates present in the antibodies were removed by centrifugation at 18,890× g for 5 minutes at 20 °C. The supernatant minus 10 µL of the starting volume was collected and used for staining. EVs were stained with anti-human CD61-APC antibody (17-0619-42; VI-PL2; final concentration (f.c.), 8.33 µg/mL; eBioscience), and anti-human CD235a-PE antibody (R7078; JC159; f.c., 25 µg/mL; Dako). Therefore, 10 mL of cell-depleted plasma was incubated with 1375 µL CD61-APC and 1375µL CD235a-PE for 2 hours at room temperature in the dark.

1.2.3 Size-exclusion chromatography

Next, to separate EVs from unbound dye, soluble proteins, and reduce lipoprotein particles, SEC was performed (qEVsingle/70 nm 1004125; Izon Science). Therefore, 1 mL of plasma containing the pre-stained EVs was loaded on each washed SEC column, followed by elution with Dulbecco's phosphate-buffered saline (dPBS; 21-031-CVR, Corning). The first 3.5 mL eluate containing the void volume was discarded, after which the 1-mL fraction containing most EVs was collected and pooled.

1.2.4 Platelet removal with polycarbonate filters

To remove remaining platelets from plasma, plasma was filtered using a 0.8-µm pore-size polycarbonate membrane filter (ATTP02500, Isopore, Merck Millipore) with a diameter of 25 mm[3].

1.2.5 Stabilization

To improve the stability of stained and SEC-isolated EVs, PEVTES was diluted 2x in 1 M D (+)-trehalose dihydrate (T9531; f.c., 0.5 M; Sigma Aldrich) and additionally 10x in 5 % bovine serum albumin ((BSA), f.c. 0.5%; Sigma Aldrich). Next, PEVTES was mixed, aliquoted in 100 µL aliquots, frozen in liquid nitrogen and stored at −80 °C until distribution.

2 References

1. Bettin B, van der Pol E, Nieuwland R: Plasma extracellular vesicle test sample to standardize flow cytometry measurements. Res Pract Thromb Haemost. 2023; 7.
2. Buntsma NC, Gąsecka A, Roos YBWEM, van Leeuwen TG, van der Pol E, Nieuwland R: EDTA stabilizes the concentration of platelet-derived

extracellular vesicles during blood collection and handling. Platelets. 2022; 33:764–771.

3. Bettin B, Gasecka A, Li B, et al.: Removal of platelets from blood plasma to improve the quality of extracellular vesicle research. J Thromb Haemost. 2022; 20:2679–2685.