

Evaluation of plasmatic extracellular vesicles by size

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Method Article

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Abstract

Extracellular vesicles (EVs) play a key role in many physiological and pathological processes [1]. EVs are a heterogeneous group of membrane-confined particles including endosome-derived exosomes and plasma membrane-originated microvesicles. The expanding field of extracellular vesicle research needs reproducible and accurate methods to characterize EVs [2].

EV profiling can be challenging due to the small size and heterogeneity. This protocol aims to provide a method to isolate EVs and facilitate high-precision particle quantitation by Nanoparticle Tracking Analysis (NTA)[3, 4]. NTA is commonly used to determine EV concentration and diameter [5, 6].

The protocol here described refers to the isolation of EVs from blood-plasma samples by using ultracentrifugation and then quantification and sizing of EVs with NTA by NanoSight NS300 system (Malvern Panalytical Ltd., Malvern, UK) provided with a syringe pump module enabling analysis in constant flow for improved sample statistics.

Introduction

In the last decade, the regulatory role of extracellular vesicles (EVs) in pathological and physiological processes has gained increasing interest [1]. Although several methods have been developed for the isolation and purification of EVs, ultracentrifugation (UC) is still considered the gold standard to isolate EVs. UC is widely used to isolate EVs, from both biological fluids (e.g. plasma) and cellular conditioned media [7].

Nanoparticle Tracking Analysis (NTA) is a biophysical method based on optical density tracking of particles. NTA can simultaneously measure the size distribution and concentration of particles, such as EVs, suspended in a liquid. NTA uses laser light scattering microscopy with a Charge-Coupled Device (CCD) or complementary metal-oxide-semiconductor (CMOS) camera. Briefly, based on the Brownian motion of the particles, which are considered as having a spherical shape, the diffusion coefficient of each tracked particle is calculated using the Stokes-Einstein equation; the movement of every single particle is tracked and then used to estimate particle size [8].

This protocol aims to provide a method to isolate EVs from plasma samples and facilitate high-precision particle quantitation by Nanoparticle Tracking Analysis (NTA). Moreover, as the large majority of the papers describing NTA data focus on the mean size or the mean concentration of EVs, we propose a new statistical approach to consider all the sample sizes and a graph designed to report these results.

Reagents

Phosphate buffer saline (PBS)

Equipment

EDTA tubes

15.0ml centrifuge tubes

Refrigerate centrifuge (4°C)

Vacuum pump

Ultracentrifugation tubes (13.5ml Quick-Seal® Round-Top Ultra-Clear Tube-Beckman Coulter)

Red Aluminium Quick-Seal Spacer (Beckman Coulter)

Quick-Seal Cordless Tube Topper (Beckman Coulter)

1.0ml Insulin syringe

2.5ml Syringe (needle dimension 0.9 mm x 25 mm)

10.0ml Syringe (needle dimension 0.9 mm x 25 mm)

0.10-µm pore-size polyether sulfone filter (StericupRVP, Merck Millipore; Burlington, MA, USA).

Ultracentrifuge (Optima™ MAX-XP Beckman Coulter, equipped with ML-55 rotor)

Cutter

Sterile cotton swab

1000µl tips

NS300 NanoSight System (Malvern Instruments, Malvern, United Kingdom)

Procedure

Isolation of extracellular vesicles from blood plasma by ultracentrifugation.

1. Draw blood into 7.5 mL EDTA tubes.
2. Separate plasma by centrifugation at 1300×g for 15 minutes at room temperature.
3. Remove cell debris from plasma by serial centrifugation at 1000, 2000, and 3000×g for 15 minutes at 4 °C.
4. Use a 2.5 ml syringe (needle dimension 0,9 mm x 25 mm) to transfer 1.5 mL of fresh plasma into a Quick-Seal ultra-clear centrifuge tube and fill up with PBS filtered with a 0.10 µm pore size polyether sulfone filter (StericupRVP, Merck Millipore; Burlington, MA, USA). (Figure 1)

5. Seal tube using the cordless tube topper (Beckman Coulter).
6. Ultracentrifuge sample at 110,000×g for 75 minutes at 4 °C on Optima Max-XP Ultracentrifuge, equipped with a MLA-55 rotor (Beckman Coulter).
7. Discard the supernatant using a 10 ml syringe (needle dimension 0.9 mm x 25 mm).
8. Dry the wall of the tube using a sterile cotton swab.
9. Resuspend EVs-rich pellet in 500 µL of 0.10 µm triple-filtered PBS with 0.10-µm pore-size filter.

Nanosight NS300 method

1. Use 100µL of PBS-resuspended EVs for NTA analysis.
2. Bring to a 400µL volume using 0.10 µm triple-filtered PBS.
3. Use 1mL insulin syringe to carry the sample on the syringe pump.
4. Set syringe pump at infusion rate 30 to have the same constant flow for all samples.
5. The acquisition for each sample is performed at camera level 13. Five 30-s recordings for each sample (Supplementary File).
6. Check the analysis setting before going on with the batch process. The number of particles per frame must be 20–120. Set detection threshold to have < 5 not valid track per frame. The ratio between total particles track and valid track must be < 5.
7. Collected data are analyzed with NTA software (Malvern Panalytical Ltd.), which provided high-resolution particle-size distribution profiles as well as measurements of the EVs concentration.

Statistical method to analyze data from Nanosight

To compare EVs concentration for each size in different groups (i.e. cases vs controls) we apply generalized multivariable linear models, using the appropriate logit link function. We estimate adjusted EV mean concentration or EV geometric mean concentration as appropriate. For each size, from 30nm to 700nm, we compare the EV mean differences between groups. Due to the high number of comparisons, we apply a multiple comparison method based on Benjamini-Hochberg False Discovery Rate (FDR) to calculate the FDR P-value. Results are reported as a series graph for EV mean concentrations of each size

by group and vertical bar charts to represent the size-specific p-values obtained comparing groups. For all the graphs, X-axis is the size of EVs. In Figure 2 we report an example of this analysis.

Troubleshooting

1. We resuspend the sample with PBS three-time filtered through a 0.10- μ m pore-size polyether sulfone filter (Millipore membrane filters) to avoid interference in NTA analysis as background noise.
2. With NTA-acquisition settings constant between analyses, our approach will enable the mean, mode, and median particle size together with EV concentration to be more precisely compared between different samples.
3. Remove the command “Advanced sample prompt” on NTA software, to allow the syringe pump flow to be continuous among the five recordings.
4. Between samples, push 2 ml of water through the sample chamber, to remove any particles present. After use, the chamber can be disassembled to be cleaned and then reassembled. Rinse the seal and the glass on the underside of the gasket component with a low-pressure source of water and then an up to 10% ethanol-water solution; finally dry with tissue papers without rubbing but gently tapping.

Time Taken

Anticipated Results

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Figures

Beckman Coulter equipment:



Figure 1

Beckman Coulter equipment for EVs isolation.

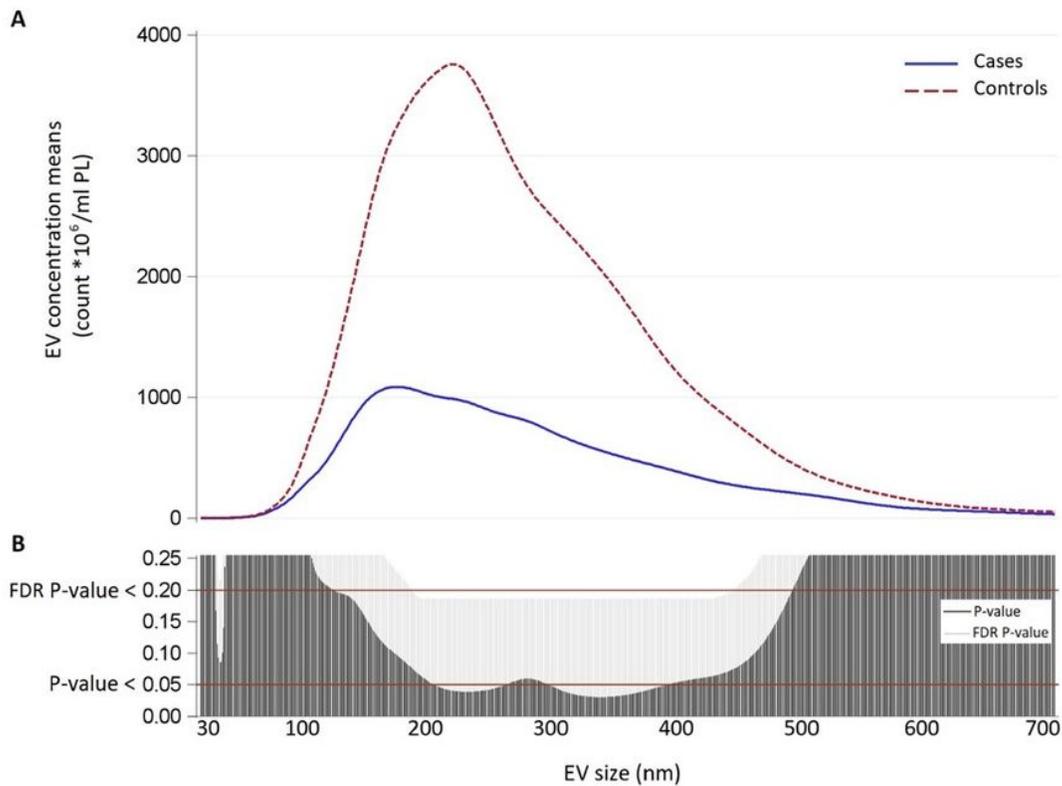


Figure 2

Panel A: Mean concentrations of EVs (*10⁶) for each size (nm) by case or control subjects. Panel B: P-value and False Discovery Rate P-value obtained from the appropriate statistical model (in this example, a Poisson regression models allowing for over dispersion), for each EV size.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [PlasmaEVsNanosight.avi](#)