hekat fluidics

APPLICATION NOTE

Rapid Evaluation of Vesiculemia from Healthy Volunteers Sera

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ABSTRACT

Extracellular vesicles are rapidly emerging objects of research considered as potential biomarkers in liquid biopsy, yet their rapid quantification and subpopulation characterization in complex biological fluids remain challenging due to their nanoscale size. This study evaluates the performance of HEKAT NanoSorter, a novel optofluidic nanoflow cytometry device, tailored for the direct detection, counting, and sorting of EVs subpopulations from human bodily fluids. Serum EVs from three healthy volunteers were isolated and stained against tetraspanins CD9 and CD63 for dual-color analysis. The system demonstrated high specificity with low noise/signal ratio. HEKAT NanoSorter provided comprehensive quantitative data within 1.5 hours. Results showed high inter-individual variability in total vesiculemia. EVs subpopulation analysis identified distinct profiles of CD9+, CD63+, and double-positive EVs across donors. These results establish that HEKAT technology as a robust, rapid, and accessible tool for EV phenotyping, offering significant advantages for EVs daily research and perspectives for clinical applications.

Extracellular vesicles (EVs), including exosomes and microvesicles, play pivotal roles in intercellular communication and are increasingly recognized as valuable biomarkers for various pathological conditions. Accurate quantification and phenotypic characterization of EVs in cell culture media or biofluids are essential for both basic research and clinical applications. However, the nanoscale size of EVs (30–150 nm) poses significant technical challenges for conventional flow cytometry, which often lacks the sensitivity to resolve these nanoobjects from background noise. Current gold-standard methods for subpopulation analysis, such as co-immunoprecipitation or Western blotting, are labor-intensive, time-consuming, and provide limited quantitative data regarding particle concentration.

The HEKAT NanoSorter represents a new generation of fluorescence-activated flow cytometry designed specifically for the nanoscale (nanoflow cytometry). By leveraging advanced optofluidics technologies, this system enables the detection and sorting of biological nanoobjects, including EVs, viruses, or lipid nanoparticles, with unprecedented sensitivity. Results highlight the system's linearity, specificity, and superior time-to-result compared to classical methodologies. This application note demonstrates the utility of the HEKAT NanoSorter in rapidly measuring vesiculemia and characterizing EV subpopulations based on surface tetraspanin expression (CD9 and CD63) directly from healthy volunteer serum.

Materials and Methods

Sample Collection and Preparation

Blood samples were collected from three volunteers by the Etablissement Français du Sang (EFS, Bordeaux, France) and transferred to HEKAT facilities within 3 hours of sampling. Sera were immediately isolated and prepared following ISEV guidelines (Nieuwland and Siljander, 2023), then aliquoted and stored at -80°C until analysis. On the day of the experiment, 100 µL of serum was thawed at room temperature for 5 minutes. To evaluate technology linearity, serial dilutions (1:10, 1:100, and 1:1000) were performed using PBS (1X, VWR).

Immunostaining

EVs were stained with monoclonal antibodies targeting surface tetraspanins: anti-CD9 conjugated to FITC (BioLegend, dilution 1:50) and anti-CD63 conjugated to AlexaFluor-647 (BioLegend, dilution 1:100). Corresponding isotype controls (BioLegend) were used at similar concentrations to assess non-specific binding. Samples were incubated for 30 minutes at 4°C in the dark.

Isolation and Analysis

Following staining, the EV fraction was isolated using size-exclusion chromatography (qEV single 35nm, Izon Science) and pooling fractions F1 and F2. For quantification, 50 µL of the isolated, immunostained EVs were loaded into a HEKAT counting microfluidic chip. Analysis was performed on the HEKAT NanoSorter equipped with 488 nm and 633 nm lasers in dual-color mode. Each run was fixed at 120 seconds, and measurements were conducted in triplicate.

Data Analysis

During sample analysis users have live views on signals on NanoSorter oscilloscope tool (Fig. 1A). Output data (.csv file) were processed using Microsoft Excel. Particle concentrations were derived directly from the output files, and initial serum concentrations were estimated based on dilution factors. EV subpopulations were defined based on single-positive (CD9+ or CD63+) and double-positive (CD9+/CD63+) fluorescence signals (Fig. 1B-D). Quantification data represented as mean ± standard deviation. Cytograms were generated using online FCS analysis tool (Floreada.io; https://floreada.io).

Results

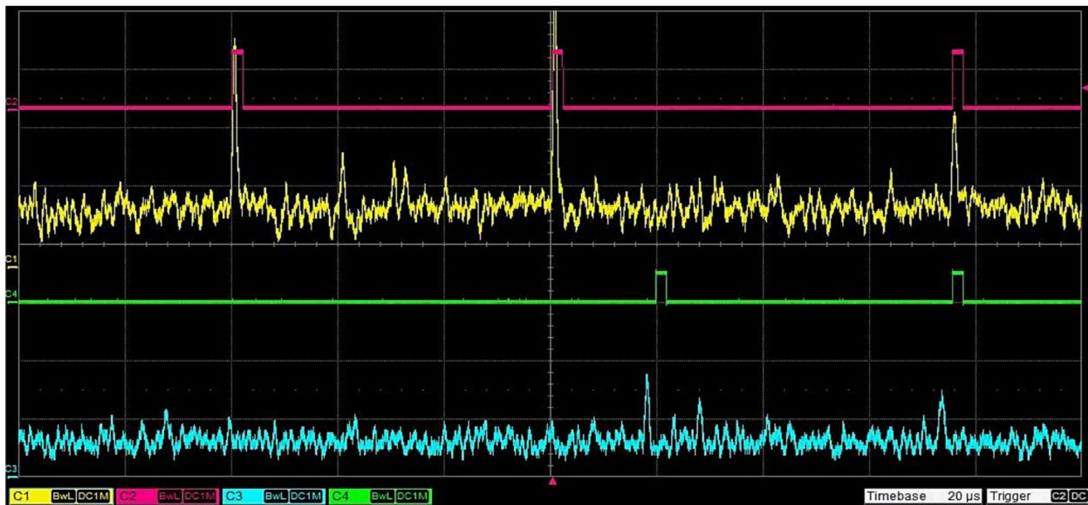
Vesiculemia and Subpopulation Characterization

Analysis of the PBS negative control yielded a low baseline signal (equivalent to a 2.10^5 particles/ml) across both detection channels. Isotypes controls also demonstrated low signal intensity (equivalent to 10^{6-7} particles/ml range, (Fig.2A), indicating appropriate specificity of the anti-CD9 and anti-CD63 antibodies utilized.

For the three volunteers sera tested a relevant signal, corresponding to the range 10^9 particles/ml, was observed for CD9-FITC and CD63-AF647 as a single or dual events indicating the presence of three distinct subpopulations of EVs: $CD9^+$ only, $CD63^+$ only, and $CD9^+/CD63^+$ (Fig.2B).

Adding the concentration of the 3 subpopulations, allowed estimation of serum vesiculemia based on CD9 and CD63

A.



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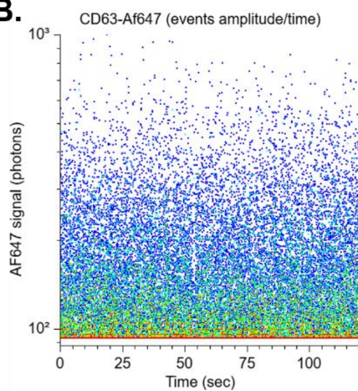

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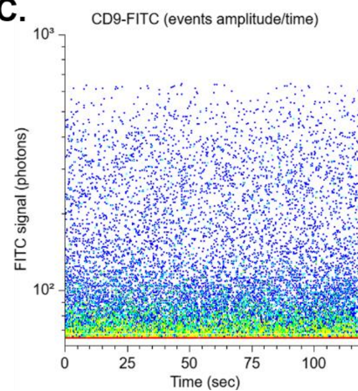

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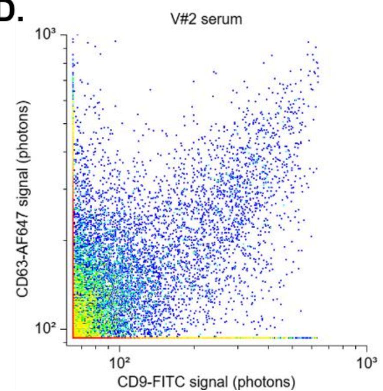


Figure 1. Serum EVs subpopulations detection depending on CD9 and CD63 tetraspanins using NanoSorter. (A) Representative live oscillogram representing analogical CD9-FITC signal (blue) with corresponding fluorescence logical detection signal (green), and CD63-Af647 signal (yellow) with corresponding fluorescence logical detection signal (pink). Density plot of fluorescent signal amplitude (photons) over time for CD63-Af647 **(B)** and CD9-FITC **(C)**. **(D)** CD63 vs. CD9 events fluorescent signals density plot (33299 events). Data obtained from a 120s run.

positive events. Vesiculemia exhibited a notable inter-individual variability, ranging from $5{,}81.10^{9}$ to $1{,}11.10^{10}$ particles/ml (mean = $8{,}60.10^{9} \pm 2{,}64.10^{9}$) corresponding to 30% of variability among the volunteers (Fig.2C).

This phenotypic profiling based on CD9 and CD63 detection revealed distinct EV subpopulation compositions between donors (Fig.2D). For volunteers V#2 and V#3, $CD63^{+}$ EVs represented the main part of the EVs detected (approx. 60% of total), with $CD9^{+}$ and double-positive populations each representing ~20%. In contrast, V#3 displayed a more balanced profile: 47% $CD9^{+}$, 36% $CD63^{+}$, and 17% double positive. These findings confirm the presence of three distinct EV subpopulations in human serum and highlight significant biological variability between individuals.

System linearity

Ten times serial dilutions (1:10, 1:100, 1:1000) of native serum samples resulted in a coherent proportional reduction in the amount of detected $CD9^{+}$, $CD63^{+}$ and

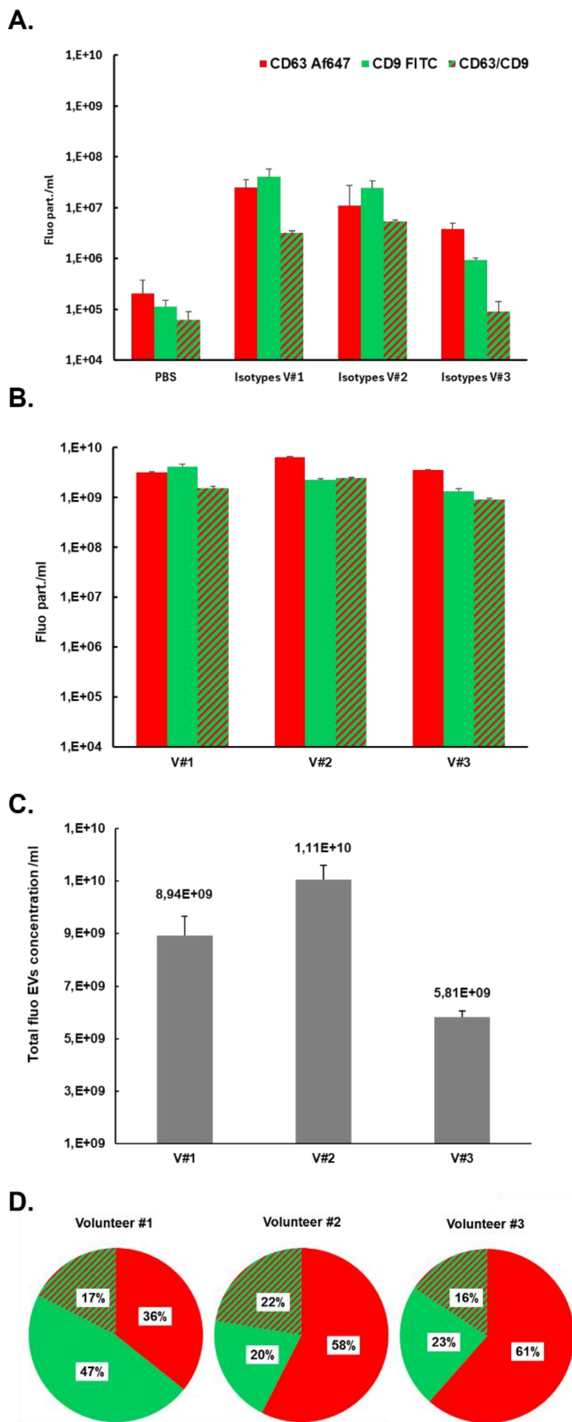


Figure 2. Characterization and quantification of serum EVs subpopulations depending on CD9 and CD63 tetraspanins. **(A)** Quantification of fluorescent particles/ml expressing CD63-AF647, CD9-FITC, or combined CD63/CD9 in PBS controls and isotype controls for three volunteers (V#1–V#3). **(B)** Analysis of CD9 and CD63 marker expression in volunteer serum samples. Bars represent the concentration of fluorescent particles/ml for CD63, CD9, and CD63/CD9 populations. **(C)** Total fluorescent EV concentrations. Data presented as mean ± standard deviation. **(D)** Relative distribution (%) of EVs subpopulations. Percentages are calculated based on

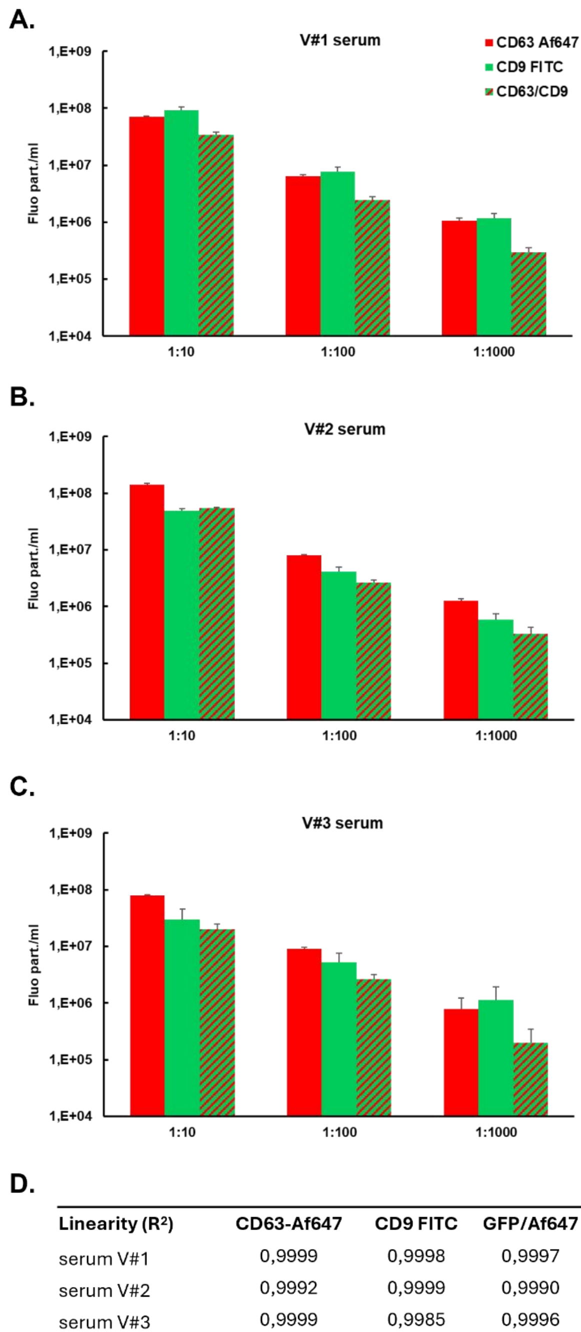
$CD9^+/CD63^+$ particles (Fig.3A-C). The linear regression performed on the dataset exhibited a coefficient of determination (R squared; R^2) approaching 1 (Fig.3D), demonstrating strong linearity across a dynamic range spanning three orders of magnitude.

Time-to-Result Efficiency

The entire workflow, from serum samples thawing to data acquisition, was completed in approximately 1.5 hours. This stands in stark contrast to typical co-immunoprecipitation protocols, which typically require skilled staff and 3 days to yield serum EVs phenotypic data.

Discussion

This study demonstrates the capability of the HEKAT NanoSorter to provide rapid and specific quantitative analysis of EV subpopulations based on canonical tetraspanins in human serum. The system successfully resolved $CD9^+$, $CD63^+$ and $CD9^+/CD63^+$ populations with high sensitivity, overcoming the limitations of conventional flow cytometry regarding nanoparticle detection. The observed total CD9/CD63 based vesiculemia and subpopulation distributions are coherent with data previously published using co-immunoprecipitation methods (Mathieu et al., 2021), validating the accuracy of the HEKAT technology. The inter-individual variability observed in both total vesiculemia and subpopulation ratios underscores the necessity for precise, individualized profiling in future clinical studies.



Linearity (R^2)	CD63-Af647	CD9 FITC	GFP/Af647
serum V#1	0,9999	0,9998	0,9997
serum V#2	0,9992	0,9999	0,9990
serum V#3	0,9999	0,9985	0,9996

Figure 3. Linearity of tetraspanins markers detection in diluted serum samples. (A–C) Concentration of fluorescent particles (fluo part./ml) expressing CD63-AF647, CD9-FITC, or combined CD63/CD9 in diluted serum samples. Data presented as mean ± standard deviation. **(D)** Summary table of determination coefficients (R^2) demonstrating detection linearity for each marker (CD63-Af647, CD9 FITC, and CD63/CD9) across dilutions for each volunteer.

Working with HEKAT NanoSorter offers significant advantages and operational efficiency.

First, sample pre-analytical processing at the bench is limited to one step double immune staining followed by a quick size exclusion chromatography. Allowing rapid access to a marked and cleaned EV-fraction. From that fraction of interest only 50µl to make measures in triplicates (around 15µl/analysis). Once the analytical volume is loaded and the microfluidic chip connected system and place in the chip holder of the NanoSorter, user only must follow a semi-automated procedure to start a measure. At the end of the run (120sec), HEKAT software generates output data (.csv/.fcs file) and a detailed report presenting the most relevant data.

Second, by reducing the time-to-result from days to under two hours, this technology enables high-throughput screening and real-time decision-making, which are critical for nanoscale research and nanoparticles bioproduction processes. Results obtained with NanoSorter can also serve clinical applications such as diagnostic stratification and therapeutic monitoring.

Furthermore, detection and quantification are accessory functions of NanoSorter. This technology has been mostly tailored to sort nanoobjects. After a first quantification step, the rest of the processed sample can be injected into a dedicated microfluidic for a sorting assays based on users choices, here, CD9, CD63 or CD9/CD63 EVs. The duration of the sorting period varies depending and the initial concentration and sorting parameters (droplets frequency, sorting threshold), however after few hours, experimenters will get access to a final volume (between 5 and 40µl depending on users' choice) highly enriched with its particles of interest and ready for selective downstream analysis and functional assays. The HEKAT NanoSorter thus represents a efficient and transformative tool for the field of EVs research and applications,

bridging the gap between complex biological characterization and clinical practicality.

Bibliography

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