LABORATORY ANALYSIS REPORT · FLOW CYTOMETRY

Extracellular Vesicle & Exosome Analysis

Immunophenotyping by Multi-Parameter Flow Cytometry

Report ID	HYB-EV-2026-001
Analysis Date	18 February 2026
Report Date	06 March 2026
Laboratory	Hybrinomics Life Science & Diagnostics LLP, Bengaluru
Platform	Flow Cytometer — FSC / SSC / PE / FITC channels
Assay	EV Surface Marker Immunophenotyping — CD9-PE, CD63-PE, CD45-FITC
Samples	Sample A, Sample B & Sample C — purified through Biotex Autologous Exosome Purification Kit — 18 Feb 2026
Prepared by	Flow Cytometry & EV Analysis Unit, Hybrinomics

01 SCIENTIFIC BACKGROUND

Extracellular vesicles (EVs) — including exosomes (30–150 nm) and microvesicles (100–1000 nm) — are membrane-bound particles secreted by all cell types. They carry surface proteins, nucleic acids and lipids mirroring their cell of origin, making them powerful liquid biopsy biomarkers in oncology, immunology, and infectious disease. Tetraspanin markers CD9 and CD63 are MISEV2018-recommended positive identifiers for EVs; CD45-FITC serves as a leukocyte exclusion marker.

This report presents flow cytometry data from three exosome samples (Sample A, Sample B, and Sample C) purified through the Biotex Autologous Exosome Purification Kit. Each sample comprises an unstained filtered control, a crude 10,000 rpm pellet, and a purified EV fraction. 25,000 events were acquired per tube with FSC-H threshold gating.

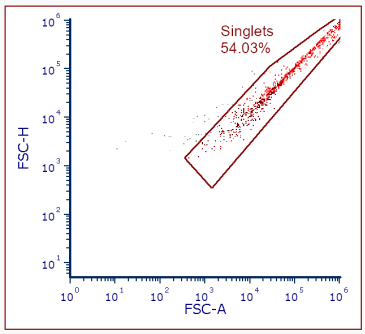
02 PROTOCOL SUMMARY

Step	Condition	Notes
Centrifugation	10,000 rpm × 5 min, 4°C	Low-speed; captures large EVs/microvesicles
Resuspension	500 µL DNS (0.1 µm filtered)	Dextrose Normal Saline — particle-free diluent
Staining	CD9-PE + CD45-FITC or CD63-PE + CD45-FITC	100 µL, 30 min at room temperature
Acquisition	25,000 events, FSC-H threshold	Singlet gate on FSC-H vs SSC-H
Controls	Unstained filtered + Buffer-only	Per ISEV / MISEV2018 guidelines

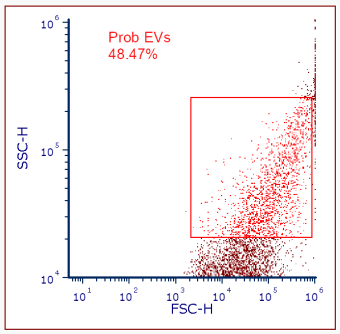
03 SAMPLE A (CD9 PANEL)

3.1 Unstained Filtered Control

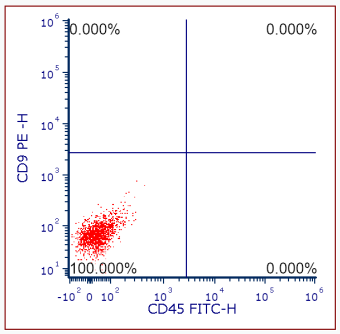
DNS buffer (0.1 µm filtered) acquired without antibody staining. Establishes instrument scatter baseline; confirms zero fluorescent background.



FSC-A vs FSC-H · Singlets: 54.03%



FSC-H vs SSC-H · Prob EVs: 48.47%

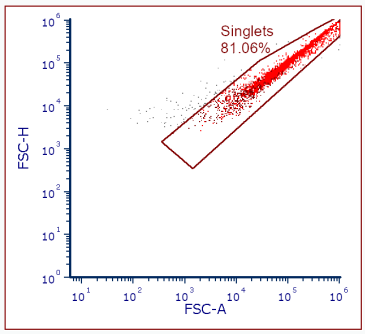


CD9-PE vs CD45-FITC · CD9+CD45-: 0%

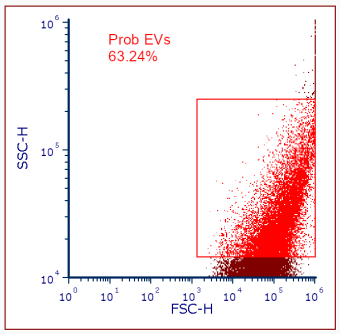
Fig 1 — Unstained filtered control (Sample A). All marker quadrants at 0.000% confirming no non-specific staining.

3.2 Stained Crude Pellet

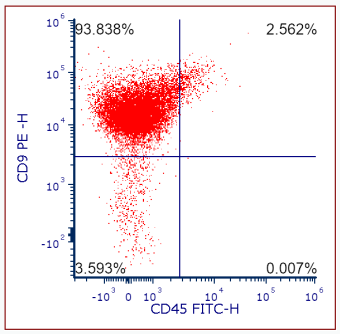
10,000 rpm pellet stained without further purification. Contains EVs, cellular debris, and potentially co-pelleted leukocytes.



FSC-A vs FSC-H · Singlet Gate



FSC-H vs SSC-H · Prob EVs: 63.24%

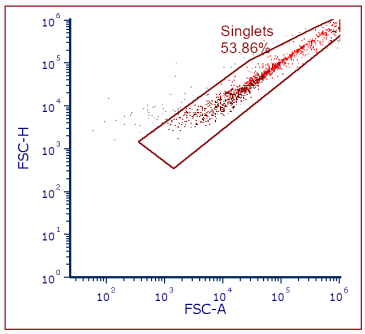


CD9-PE vs CD45-FITC · CD9+CD45-: 93.84%

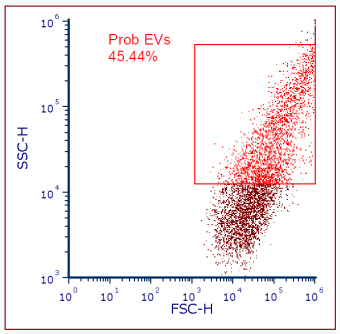
Fig 2 — Stained crude pellet (Sample A). Strong CD9+/CD45- EV population (93.84%) with minor CD9+/CD45+ contamination (2.56%).

3.3 Stained Purified Pellet

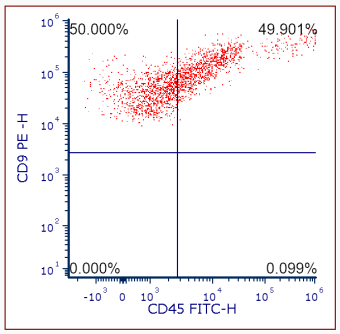
Post-purification EV fraction. CD9/CD45 co-positivity assessed to determine leukocyte-derived EV enrichment vs. true exosome selection.



FSC-A vs FSC-H · Singlet Gate



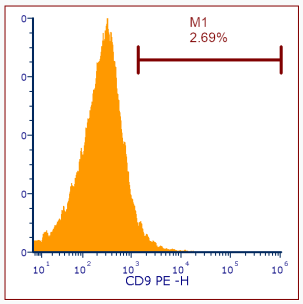
FSC-H vs SSC-H · EV Gate



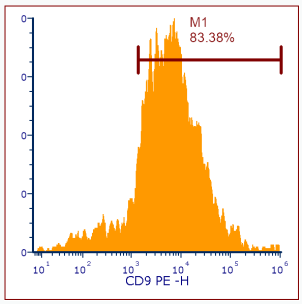
CD9-PE vs CD45-FITC · CD9+CD45-: 50% / CD9+CD45+: 49.9%

Fig 3 — Stained purified pellet (Sample A). CD9+ population splits equally between CD45- and CD45+ fractions after purification.

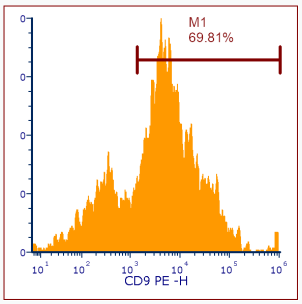
3.4 CD9-PE Histogram Comparison



Unstained · M1: 2.69%



Crude · rightward shift



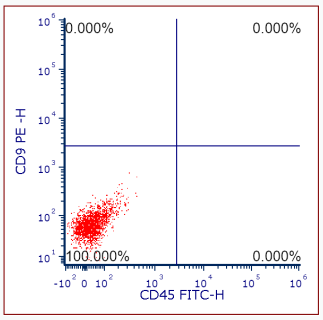
Purified · further shift

Fig 4 — CD9-PE histograms (Sample A). Progressive rightward shift from unstained to crude to purified confirms CD9 surface expression on EVs.

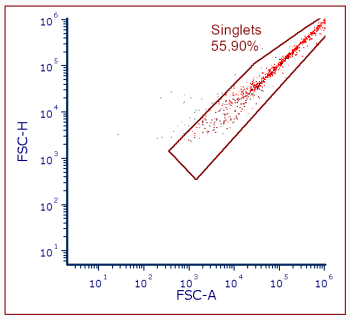
04 SAMPLE B & SAMPLE C FLOW CYTOMETRY DATA

Sample B was analysed using the CD9/CD45 panel and Sample C using the CD63/CD45 panel, both purified through the Biotex Autologous Exosome Purification Kit. A dedicated buffer-only control provides orthogonal EV marker validation.

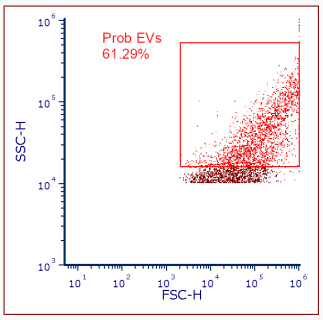
4.1 Unstained Filtered Control



FSC-A vs FSC-H · Singlets



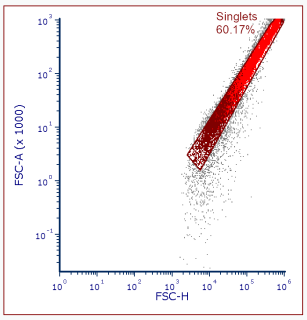
FSC-H vs SSC-H · EV gate



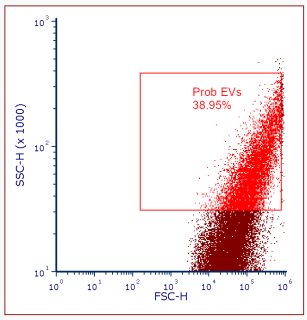
Marker plot · All 0.000%

Fig 5 — Unstained filtered control. Clean baseline; no fluorescent artefacts.

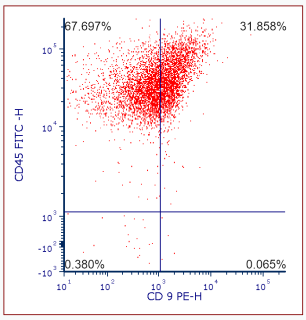
4.2 Sample B — Stained Crude (CD9 Panel)



FSC-A vs FSC-H · Singlets



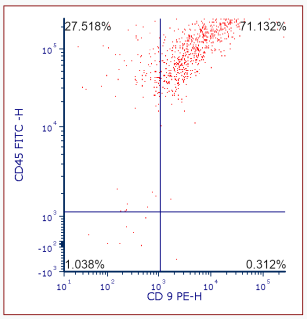
FSC-H vs SSC-H · EV gate



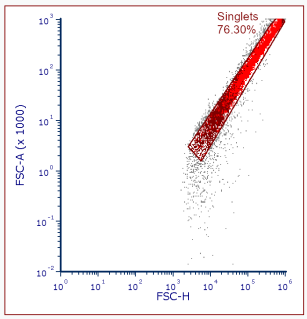
CD9-PE vs CD45-FITC · CD9+CD45+: 31.86%

Fig 6 — Sample B, crude pellet (CD9 panel). CD9+/CD45+ fraction (31.86%) indicates leukocyte-EV co-purification.

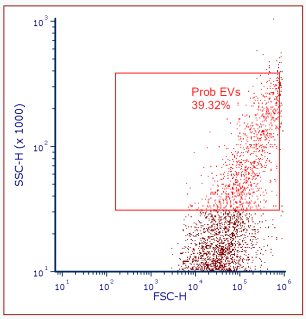
4.3 Sample B — Stained Purified (CD9 Panel)



FSC-A vs FSC-H



FSC-H vs SSC-H · Prob EVs: 39.32%

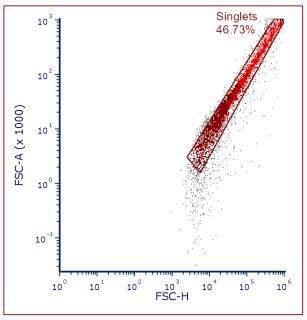


CD9-PE vs CD45-FITC · post-purification

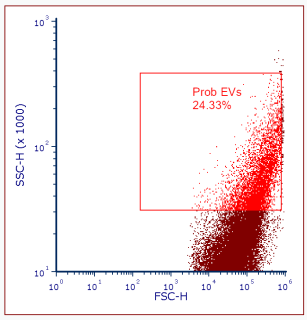
Fig 7 — Sample B, purified pellet (CD9 panel). EV gate 39.32%; selective particle enrichment confirmed.

4.4 Sample C — Stained Crude (CD63 Panel)

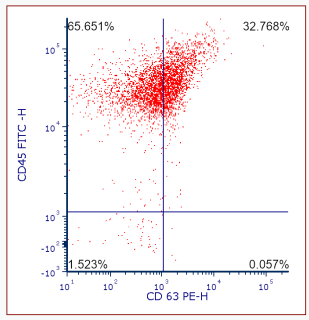
CD63 is an endosomal tetraspanin enriched on classical exosomes; its detection orthogonally validates the CD9 findings.



FSC-A vs FSC-H · Singlets



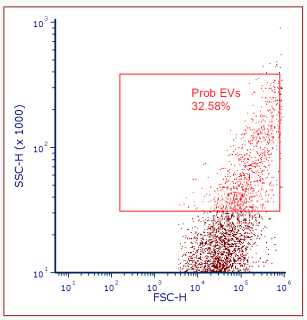
FSC-H vs SSC-H · EV gate



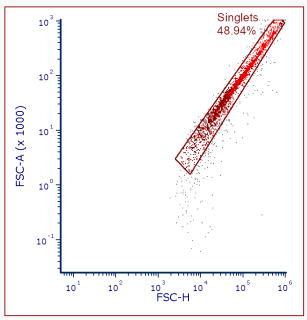
CD63-PE vs CD45-FITC · CD63+CD45+: 32.77%

Fig 8 — Sample C, crude pellet (CD63 panel). CD63+/CD45+: 32.77%; CD63+/CD45-: 65.65%.

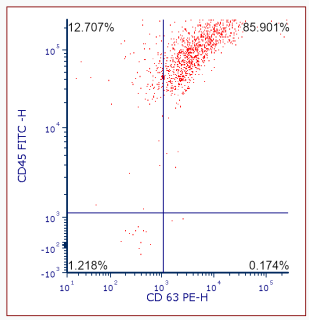
4.5 Sample C — Stained Purified (CD63 Panel)



FSC-A vs FSC-H



FSC-H vs SSC-H · EV gate

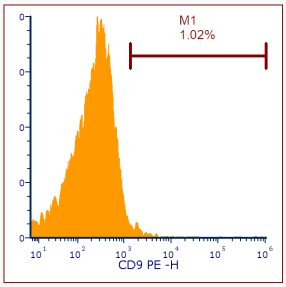


CD63-PE vs CD45-FITC · CD63+CD45+: 85.90%

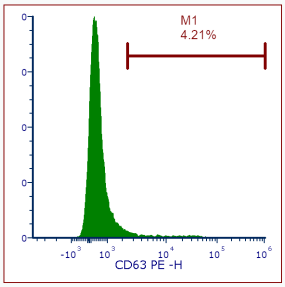
Fig 9 — Sample C, purified pellet (CD63 panel). CD63+/CD45+ rises to 85.90% — suggests lymphocyte-derived EV enrichment.

4.6 Histogram Comparison — Sample B (CD9) & Sample C (CD63)

Unstained baseline:



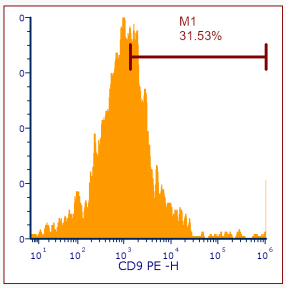
CD9-PE · Unstained



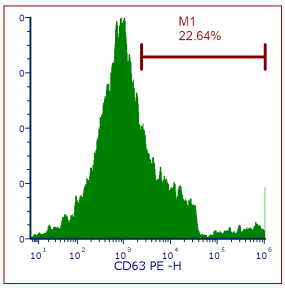
CD63-PE · Unstained

Fig 10 — Unstained histograms. Narrow dim peaks establish M1 gate position.

Crude vs Purified:

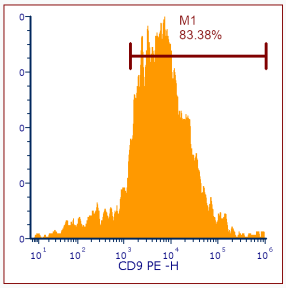


CD9-PE · Crude (M1: 31.53%)

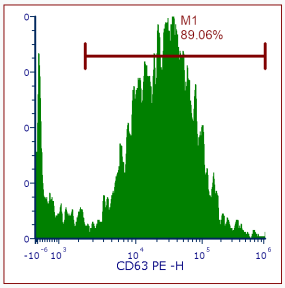


CD63-PE · Crude

Fig 11 — Sample B crude histogram. CD9 M1 gate = 31.53%; clear shift from unstained baseline.



CD9-PE · Purified (M1: 83.38%)

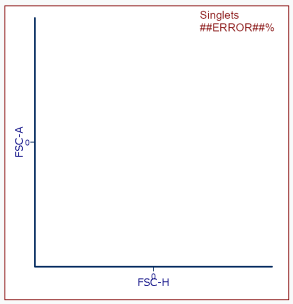


CD63-PE · Purified (M1: 89.06%)

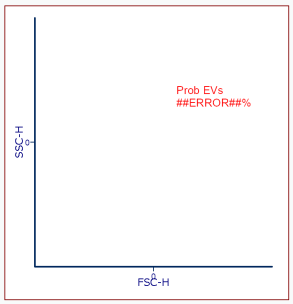
Fig 12 — Purified histograms. Sample B CD9 M1 = 83.38%; Sample C CD63 M1 = 89.06%. Robust EV enrichment confirmed.

4.7 Buffer-Only Control

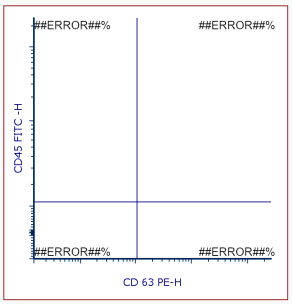
DNS diluent acquired without any sample or antibody staining. Confirms the buffer contributes zero particles and zero fluorescent background to the analysis. All scatter gates and marker quadrants show no detectable events, validating the purity of the DNS diluent used throughout the experiment.



Scatter · No events detected



EV gate · No events detected



Marker plot · No events detected

Fig 13 — Buffer-only control. No particle events across all gates; DNS diluent is particle-free and non-fluorescent.

05 QUANTITATIVE RESULTS SUMMARY

Parameter	Samp A Crude	Samp A Purified	Samp B Crude CD9	Samp B Purif CD9	Samp C Crude CD63	Samp C Purif CD63
Prob EVs gate (%)	—	—	—	39.32	—	—
CD9+/CD45- (%)	93.84	50.00	0.38	—	N/A	N/A
CD9+/CD45+ (%)	2.56	49.90	31.86	—	N/A	N/A
CD63+/CD45- (%)	N/A	N/A	N/A	N/A	65.65	12.71
CD63+/CD45+ (%)	N/A	N/A	N/A	N/A	32.77	85.90
CD9 M1 gate (%)	—	—	31.53	83.38	N/A	N/A
CD63 M1 gate (%)	N/A	N/A	N/A	N/A	—	89.06

Table 1. Gate statistics from flow cytometry analysis. — = not directly readable; N/A = panel not applicable.

06 INTERPRETATION & FINDINGS

DETECTED ✓
CD9+ EVs

DETECTED ✓
CD63+ EVs

VALID ✓
CD45 Exclusion

PASS ✓
Buffer Controls

EV Detection

- **CD9 and CD63 tetraspanin expression confirmed in both samples.**
- Sample A crude: CD9+/CD45- = 93.84% — abundant EV capture at 10,000 rpm.
- Sample B purified: CD9 M1 = 83.38%; Sample C purified: CD63 M1 = 89.06% — strong dual-marker positivity.

Controls

- **All unstained filtered controls show 0.000% marker expression — antibody specificity confirmed.**
- Buffer-only control: no events across all gates — DNS diluent is particle-free.
- Singlet gating on FSC-A/FSC-H effectively excludes doublets and aggregates.

Purification Efficiency

- **Sample A: CD9+/CD45+ rises from 2.56% (crude) to 49.9% (purified) — co-enrichment of leukocyte-derived EVs.**
- Sample C: CD63+/CD45+ increases from 32.77% to 85.90% after purification — preferential enrichment of lymphocyte-derived exosomes (T-cell EVs).
- Recommend review of purification strategy — consider SEC or density-gradient ultracentrifugation.

Technical Notes

- 10,000 rpm captures large EVs (200 nm–1 µm); classical small exosomes require 100,000 × g.
- Prob EVs gate (39–48%) indicates heterogeneous preparation — expected for differential centrifugation.

07 CONCLUSIONS & RECOMMENDATIONS

Conclusions

- EVs expressing CD9 and CD63 confirmed in both samples — EV isolation by differential centrifugation is effective.
- Both crude and purified fractions show strong marker positivity; purification selectively enriches CD9+ and CD63+ populations.
- All negative controls (unstained, buffer-only) perform as expected, confirming assay specificity.

Recommendations

- **Upgrade isolation:** 100,000 × g ultracentrifugation or size exclusion chromatography (SEC) for classical small exosome enrichment.
- **CD45 depletion:** Magnetic bead CD45-depletion prior to EV capture to reduce leukocyte-EV co-purification.
- **NTA/DLS validation:** Complement flow data with Nanoparticle Tracking Analysis for size and concentration verification.
- **Expand panel:** Add CD81-APC for full MISEV2018 three-tetraspanin characterisation.

Disclaimer: This report is for research and investigational purposes only. Results should be interpreted in conjunction with clinical history and additional validation studies. For queries: Flow Cytometry Unit, Hybrinomics, Bengaluru · info@hybrinomics.com