# Agarose Microgel-Based In Situ Cleavable Immuno-Rolling Circle Amplification for Multiplexed Single-Molecule Quantitation on Single Extracellular Vesicles

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**Background:** Extracellular vesicles (EVs) carry clinically informative proteins, but their nanoscale size and heterogeneity make single-vesicle, multiplexed, and high-throughput quantification difficult. Conventional imaging and flow-based assays seldom reach single-molecule sensitivity, and enzymatic amplification performed on EV surfaces is hindered by steric effects and nonspecific binding—especially in complex biofluids—limiting sensitivity and accuracy.

**Objective:** To develop a robust platform—GDEVA (agarose microgel-based, in situ cleavable immuno-rolling circle amplification)—that enables single-molecule, multiplexed protein quantification on individual EVs directly from plasma, with throughput sufficient for detecting rare EV subpopulations.

**Results:** By cleaving RCA templates from EV surfaces inside agarose microgels, GDEVA mitigates steric hindrance and lowers nonspecific binding, achieving single-molecule sensitivity with demonstrated 3-plex multiplexing. The platform detects RCA templates down to ~443 aM and quantifies single EVs at ~5 EV/μL, supporting high-throughput analysis (>60,000 EVs; ~8,800–10,000 EV/min) via flow cytometry or FACS plus imaging for digital counting. Multiplex profiling of tetraspanins (CD9/CD63/CD81) and cancer-immunity markers (PD-L1, CD155, TYRP-1) reveals pronounced single-EV heterogeneity. In melanoma patient plasma, GDEVA outperforms ELISA for dual-marker EV detection, achieving detection across all samples where ELISA identifies only a minority, underscoring superior sensitivity in complex specimens.

**Conclusion:** Integrating in situ cleavable immuno-RCA with agarose microgels and high-throughput readouts, GDEVA enables multiplexed, single-molecule quantification on individual EVs directly from plasma. It overcomes key limitations of surface amplification on EVs, delivers digital single-EV measurements at clinically relevant throughput, and uncovers biologically meaningful heterogeneity in cancer-related markers, positioning GDEVA as a practical tool for rare EV subpopulation detection and translational biomarker studies.