Development of Homogeneous Split-Luciferase Assays for Relative Quantitation of Extracellular Vesicles Through Detection of CD9, CD63, and CD81 in Intact Membranes

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INTRODUCTION

Extracellular vesicles (EVs) are increasingly evaluated as biomarkers, therapeutic agents, and delivery vehicles. Quantitative comparison of EV preparations remains technically challenging because commonly used methods may only measure total particles, and results can be influenced by non-vesicular particles, sample handling, operator-dependent procedures, and instrument-specific analysis.

Methods such as nanoparticle tracking analysis and flow-based particle analysis can provide particle concentration, size distributions, or marker-associated measurements, but they may require specialized instrumentation, extensive sample preparation, wash or separation steps, and complex data analysis. **A complementary approach is therefore needed for rapid, reproducible measurement of EV-associated proteins using standard laboratory instrumentation.**

The assay described here uses proximity-dependent complementation of two luciferase fragments. Antibodies recognizing non-overlapping epitopes on the same tetraspanin are expressed as translational fusions to complementary luciferase fragments. Binding of both antibody fusions within approximately 20 nm reconstitutes enzyme activity, and addition of substrate produces a luminescent signal that can be measured with a plate luminometer. Because signal generation occurs in solution, the assay is homogeneous and does not require immobilization, washing, or physical separation of bound and unbound reagents (Figure 1).

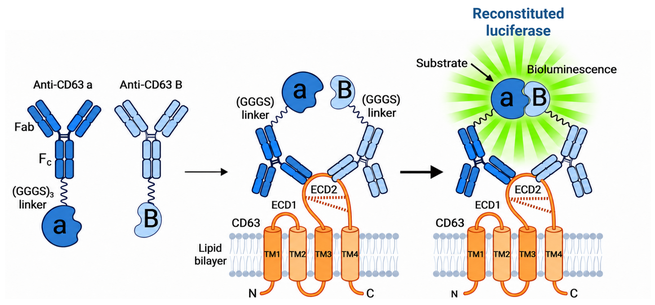


Figure 1. Proximity based complementation for detection of tetraspanins in the EV membrane. Monoclonal antibodies to non-overlapping epitopes of CD9, CD63, and CD81 were developed and expressed as translational fusions to complementary luciferase fragments. When bound to their target, their close proximity (<20 nm) allows complementation of enzyme activity which can process substrate to release light measured by a standard luminometer.

The assay uses recombinant rabbit monoclonal antibodies generated against full-length native CD9, CD63, and CD81 to recognize epitopes, including conformational epitopes, presented by intact tetraspanins. Screening identified compatible antibody pairs that bind non-overlapping epitopes and support luciferase complementation on membrane-associated targets (Figure 3).

We investigated whether antibody pairs in the split-luciferase format could provide **target-specific, reproducible relative quantitation of EVs t**hrough the canonical tetraspanins CD9, CD63, and CD81. The technical features evaluated included homogeneous **no-wash detection, short assay time**, compatibility with standard plate luminometers, **single-target and combined multi-target measurements,** and calibration using particle-based reference materials.

METHODS

Antibodies. Rabbits were immunized against human CD9 (UniProt P21926), CD63 (UniProt P08962), or CD81 (UniProt P60033), and reactive B cell clones were selected and sequenced. Recombinant heavy and light chains were expressed in mammalian cells and purified using Protein A chromatography. Each recombinant antibody was screened against recombinant extracellular domain (rECD) by ELISA to demonstrate specificity towards the intended target.

Selected clones were expressed as translational fusions to complementary portions of luciferase, screened by checkerboard experiment to identify pairs with highest signal to noise ratios, then titrated to identify working concentrations that had low background. The standard EVsure workflow was utilized to screen the antibodies.

Split luciferase assay. At least one pair of complementary antibodies (A +B) were diluted in Assay Buffer to a volume of 50 µL and added to each well of the plate. Sample (rec. ECD, VLP, EVs, specimen) were diluted with Assay Buffer to a volume of 50 µL and gently mixed in the well for a total volume of 100 µL and incubated with shaking for 60 min. Then 10 µL of Substrate was added to each well, followed by shaking for 1 minute. Reaction was allowed to proceed for 5 minutes before reading on a standard plate luminometer at 490 nm (Figure 2).

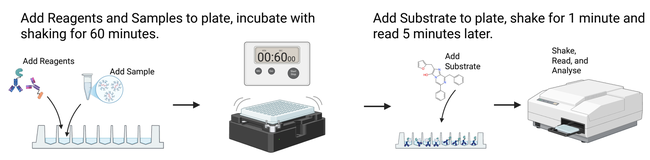


Figure 2. EVsure workflow. Samples are diluted in Assay Buffer and mixed with 1X Antibody Mix in the reaction plate. After incubation with shaking, Substrate is added and mixed briefly before reading on a plate luminometer.

Extracellular Vesicles. EVs were enriched by size exclusion chromatography, tangential flow filtration, or ultracentrifugation as indicated. If from commercial sources, vesicle concentrations were approximated using nanoparticle tracking analysis (NTA) and characterized by the vendor (TEM, fNTA, western, flow cytometry, or ELISA for tetraspanin content. For SEC fractions, 293 Freestyle cells were grown in serum and EV free media for 72 hours before harvest. Recombinant EVs (rEVs) used in the standard curve were enriched by ultracentrifugation and characterized by fluorescent NTA for CD9, CD63, and CD81 content.

RESULTS

B(125 ng/mL)

CD9 A (125 ng/mL)	S/N	CD9-03	CD9-08	CD9-12	CD9-14	CD9-21	CD9-22	CD9-25	CD9-27	CD9-28	CD9-35
	CD9-03	2.8	1.1	1.6	2.4	5.4	2.7	3.9	3.0	6.2	4.1
	CD9-08	1.7	1.4	1.0	1.6	1.5	3.6	3.7	1.8	3.4	3.9
	CD9-12	1.8	1.2	1.0	1.3	1.3	1.9	1.9	1.9	2.8	1.7
	CD9-14	2.6	1.3	1.0	2.8	9.7	10.0	6.2	2.7	4.5	4.8
	CD9-21	5.5	1.3	1.0	15.0	2.0	3.9	4.7	17.1	**97.7**	24.1
	CD9-22	1.5	1.5	0.9	5.4	2.4	4.7	3.7	7.6	**106.5**	16.0
	CD9-25	1.4	1.7	0.9	2.8	1.5	3.1	4.7	3.0	**141.8**	5.2
	CD9-27	2.3	1.5	1.1	2.3	10.7	10.5	5.3	2.9	4.5	3.9
	CD9-28	1.6	1.5	0.8	1.8	**45.9**	**37.7**	**64.9**	1.7	2.9	2.4
	CD9-35	1.9	1.6	0.8	2.1	8.2	9.9	4.9	1.7	3.5	4.1

B (31.3 ng/mL)

CD63 A (62.5 ng/mL)	CD63-02	CD63-03	CD63-05	CD63-06	CD63-09	CD63-10	CD63-12	CD63-13	CD63-14
CD63-02	2.3	2.2	5.3	0.7	4.1	1.7	1.0	5.0	1.6
CD63-03	1.6	1.9	5.1	0.7	3.9	1.6	1.1	3.4	1.2
CD63-05	1.2	1.2	3.3	0.9	3.2	1.2	324.0	3.5	286.3
CD63-06	4.5	2.9	12.4	1.1	12.7	3.6	1.4	12.3	2.5
CD63-09	1.7	1.3	5.2	0.9	3.6	1.4	1.5	3.7	**1.5**
CD63-10	1.9	2.3	7.5	1.1	5.4	1.7	1.4	4.3	**1.4**
CD63-12	0.9	3.3	213.9	1.4	4.2	1.3	1.3	2.2	**1.2**
CD63-13	1.4	1.7	3.8	0.9	3.3	1.5	1.3	2.7	1.2
CD63-14	1.9	1.7	257.1	1.0	**3.7**	**1.7**	**1.1**	3.5	nd

B(125 ng/mL)

CD81 A (125 ng/mL)	S/N	CD81-06	CD81-08	CD81-12	CD81-14	CD81-18	CD81-19	CD81-24	CD81-26	CD81-27	CD81-28
	CD81-06	1.1	1.5	1.1	1.1	1.3	1.1	1.1	1.2	1.1	1.6
	CD81-08	2.1	1.1	2.8	1.6	11.4	1.4	1.2	11.2	7.6	1.2
	CD81-12	1.2	5.7	1.2	1.2	20.9	1.1	1.2	10.5	9.7	1.1
	CD81-14	1.2	1.4	1.0	1.2	1.3	1.1	1.1	1.6	1.3	1.1
	CD81-18	1.2	12.0	13.9	1.6	1.7	1.3	1.2	1.6	**1.4**	1.1
	CD81-19	1.1	1.8	1.2	1.1	1.5	1.1	1.1	2.2	**2.0**	1.2
	CD81-24	1.2	1.3	1.1	1.1	1.1	1.2	1.2	1.4	**1.3**	1.2
	CD81-26	1.3	13.4	7.8	1.9	1.6	1.7	1.2	1.5	1.4	1.2
	CD81-27	1.8	10.3	7.8	1.8	**1.5**	**1.9**	**1.3**	1.4	1.7	2.0
	CD81-28	1.2	1.4	1.3	1.3	1.2	1.2	1.2	1.2	2.2	0.6

Table 1. Checkerboard screening for compatible antibody pairs to detect CD9, CD63, or CD81. Individual recombinant antibody clones were expressed with either the A or B portion of luciferase as a translational fusion to the heavy chain. Pairs of A and B were tested in a checkerboard experiment to identify complementing pairs with the addition of the recombinant extracellular domain (ECD) of the corresponding tetraspanin. Fold signal over blank was determined and shown as S/N ratio with higher ratios colored red on a red-grey gradient scale. Pairs were also screened for specificity to off-target tetraspanins; all signals were around background levels (data not shown).

Selected pairs with highest S/N:

- anti-CD9-25A with anti-CD9-28B
- anti-CD63-05A with anti-CD63-12B
- anti-CD81-18A with anti-CD81-12B

These pairs were tested for sensitivity alone and together.

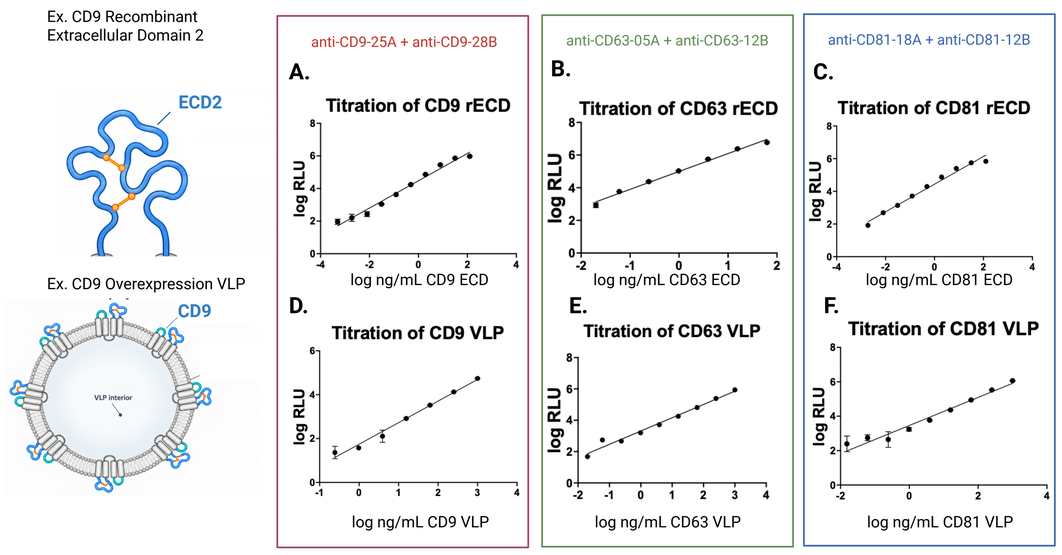


Figure 3. Dose–response curves using single tetraspanin target split luciferase assay for the optimized antibody pairs using their corresponding recombinant extracellular domain (ECD) proteins **(A)** CD9, **(B)** CD63, and **(C)** CD81 or virus like particle (VLP) overexpressing **(D)** CD9, **(E)** CD63, and **(F)** CD81. Data points represent the mean of the triplicate measurements of Relative Light Units (RLU).

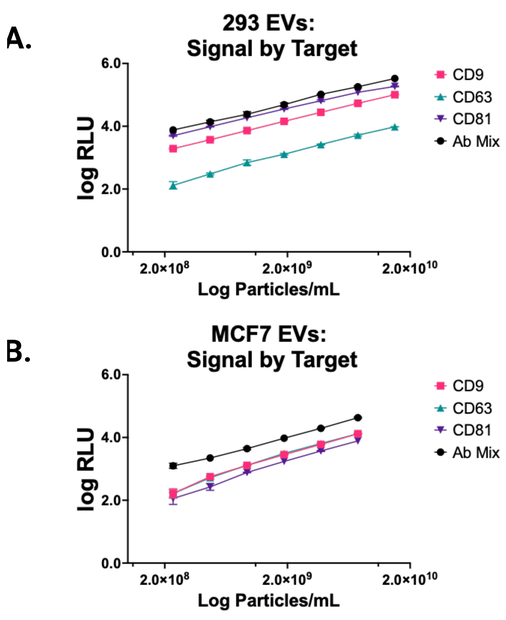


Figure 4. Single tetraspanin antibody and mixed antibody responses to EV concentration. (A) Mean RLU values for UC enriched 293 EVs. Expected particles/mL on the x-axis were determined by NTA. **(B)** Measured RLU values for UC enriched MCF7 EVs. Expected particles/mL on the x-axis were determined by NTA. NTA was performed using scatter mode on ZetaView. In both examples, the Ab mix for the standard EVsure assay detected the fewest particles with highest signal.

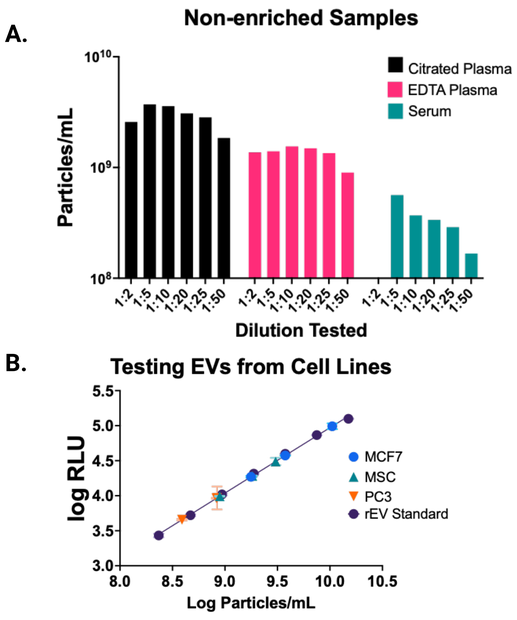


Figure 5. Testing non-enriched biological samples and EVs enriched from different cell lines. (A) Citrated plasma (black), EDTA plasma (pink), or serum (teal) from different donors was diluted and tested using the EVsure standard assay without further enrichment. **(B)** EVs from MCF7 (breast cancer, epithelial, AlphaNanoTech), MSC (human Adipose Mesenchymal Stem Cells, Galen Molecular), PC3 (prostate epithelial cancer, Novus Biologicals) acquired from commercial sources were tested at various concentrations using the standard EVsure assay.

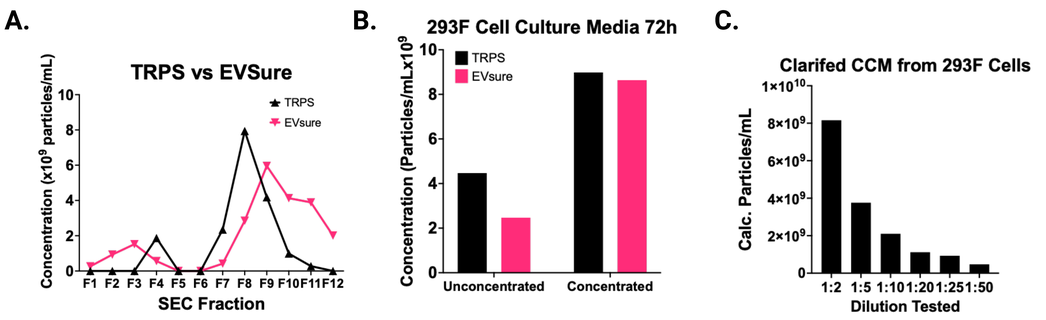


Figure 6. EVsure and TRPS concentration profiles across size exclusion chromatography (SEC) fractions.(A) Dilution-corrected particle concentrations measured by TRPS (Exoid) and independently calibrated EVsure assay was plotted for SEC fractions F1–F12 from 293F cell culture . TRPS and EVsure peak after F7. **(B)** Comparison of unconcentrated and concentrated 293F cell culture media after 72 hours of growth in serum and EV-free media to stimulate EV secretion. **(C)** EVsure measured particles/mL for a dilution series of clarified cell culture media (CCM) after 72 hours of growth under in serum and EV-free media.

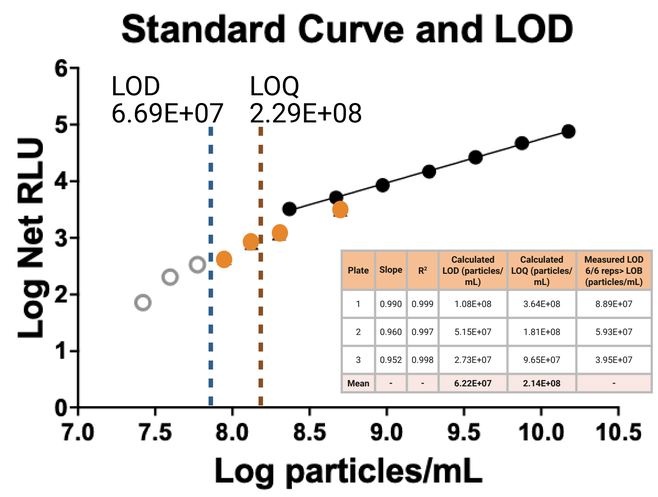


Plate	Slope	R^2	Calculated LOD (particles/mL)	Calculated LOQ (particles/mL)	Measured LOD 6/6 reps> LOB (particles/mL)
1	0.990	0.999	1.08E+08	3.64E+08	8.89E+07
2	0.960	0.997	5.15E+07	1.81E+08	5.93E+07
3	0.952	0.998	2.73E+07	9.65E+07	3.95E+07
Mean	-	-	**6.22E+07**	**2.14E+08**	-

Figure 7. Determination of Limit of Detection (LOD) and Limit of Quantitation (LOQ) using the EVsure assay. Three plates were tested in 3 independent runs. The LOD and LOQ were calculated for each plate. LOD was determined using the equation 3 x (σ/m) and LOQ using 10 x (σ/m) where σ is the standard deviation of the blank and m is the slope of the calibration curve. Calculated LOD (blue) and LOQ (red) are depicted as dotted lines. Low concentration standards with 6 of 6 replicates with values above the LOD are shown in orange to refine the measured LOD. Those below the calculated LOD are shown with grey open circles. The dynamic range spans 4 logs.

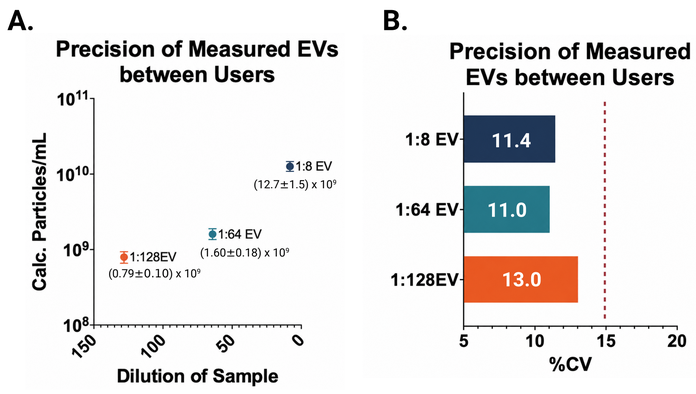


Figure 8. Precision of EVsure measurements across users and plates. EV preparations were tested at 1:8, 1:64, and 1:128 dilutions by three users over 3 days. Particles/mL were interpolated from standard curves run on each plate. **(A)** Mean measured particle concentrations ± Standard Deviation (SD), plotted on a logarithmic scale. **(B)** Pooled coefficients of variation (CV = 100 × SD/mean) were 11.4%, 11.0%, and 13.0%, respectively. Analyses included 27 measurements each for EV 1:8 and EV 1:64 and 24 measurements for EV 1:128. Pooled variability includes user, plate, and replicate effects.



Figure 9 Components of the EVsure kit. Materials included: 500X Antibody Mix, Substrate, Resuspension buffer, Assay Buffer, Assay Plate, and Plate Seals. Enough reagents are provided to use kit twice with the included standards. Samples should be tested with at least 2 dilutions in at least duplicate wells. All you need are samples, a multi-channel pipette, a plate shaker, and a luminometer.

CONCLUSIONS

EVsure enables rapid relative quantitation of extracellular vesicles through a combined CD9, CD63, and CD81 luminescent signal from tetraspanins in membranes.

- **No wash protocol and rapid workflow:** Mix-and-read detection eliminates immobilization, washing, and separation steps and uses a standard plate luminometer for results in 75 minutes or less (Figs 1 and 2).
- **Novel recombinant antibodies** expressed as translational fusions can be combined for sensitive and specific EV quantification or characterization (Table 1 and Fig 3).
- **Broad sample compatibility:** Detects EVs enriched from cell culture or biological samples, or directly from cell culture media or from diluted biological samples (Figs 4 and 5).
- **Cumulative signal for detection:** Relative quantitation using EVsure is possible despite differences in individual tetraspanin distribution in the sample (Figs 4 and 5).
- **Sensitive EV quantification:** The mean calculated limit of detection (LOD) was 6.22E+07particles/mL across three independent runs. An experimentally confirmed threshold of 8.89E+07 particles/mL yielded 6 of 6 replicates above the limit of blank (Fig 7).
- **Repeatability across users and plates:** Pooled CVs were 11.4%, 11.0%, and 13.0% for EVs across 3 users and 3 dilutions demonstrating repeatable measurements. (Fig 8).

EVsure is a practical assay for rapid comparison of EVs either in enriched samples or directly in specimens in an easy and high-throughput format.

More information

www.IntuitiveEV.com US Patent Application No: 64/035358