High-throughput, scalable, mixed mode chromatography isolation of extracellular vesicles (EVs) from biofluids and cell cultures

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1 Abstract

Extracellular vesicles (EVs) are secreted by most cells into the extracellular space, eventually passing into circulation. This makes plasma an ideal biofluid for obtaining EVs, though this matrix presents challenges, including efficient isolation of high-purity EVs. While various EV isolation and purification methods have been described, few techniques allow high-throughput removal of soluble molecules that can interfere with accurate EV detection and quantification in low-volume biofluid samples. We developed a high-throughput and scalable method to isolate EVs from plasma samples as small as 20µL in 96-well plate format, using a mixed-mode chromatography (MMC) resin with nominal molecular weight cutoff (MWCO) of 400kDa (Captocore 400). The method, expected to remove soluble proteins below the MWCO, was performed in triplicate on 32 double-spun K2EDTA plasma samples. Soluble protein removal was evaluated concomitantly with EV recovery. Total protein removal averaged 98.5% while albumin removal averaged 99.9%. Similarly, over 99.9% of IgG was depleted. IgM, which has a molecular weight greater than 400kDa was also evaluated, with removal averaging 56.8%. Intact EVs were measured before and after plasma purification with electrochemiluminescence (ECL) immunoassays, and the recovery of six endogenous plasma EV populations averaged 60%. EV recovery was confirmed by measuring fluorescent recombinant EVs spiked into plasma before purification using both super-resolution microscopy and nanoparticle tracking analysis. Comparison of EV quantification by sandwich immunoassays before and after purification indicated a substantial reduction of matrix interference. Variability of EV recovery between three purification replicates averaged 6% CV across all samples, with the highest source of variability attributed to the heterogeneity of the plasma samples, including initial LDL cholesterol concentration. The high-throughput nature of the method was demonstrated by processing 500 plasma samples in one day. We also adapted it to purify EVs from cell culture media and cerebrospinal fluid with similar performance.
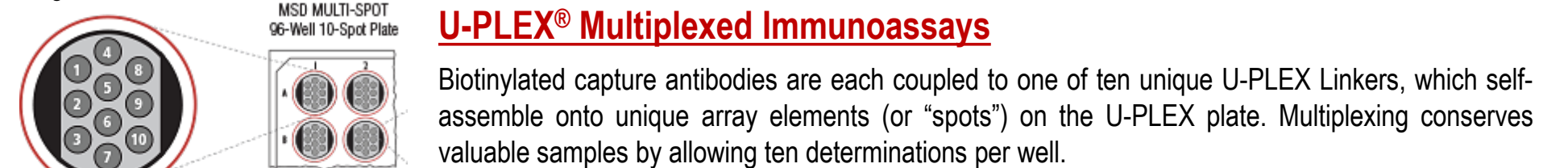
2 Methods

A. High-throughput plasma EV purification using Captocore 400 core-shell resin (Cytiva)

Principle

- **Porous shell** excludes extracellular vesicles and other macromolecular structures with molecular weight (MW) > 400kDa.
- **Ligand-activated core** (octylamine) retains molecules below the MW cutoff.
- **High throughput:** Operator can process 8 plates per day (768 samples). This would take several weeks using gravity SEC columns.

Procedure at a glance

- 20-100 µL plasma per sample diluted and added to washed resin in deep well plates.
- Resin / Samples incubated with mixing for 1 hour.
- Centrifugal processing in 96 well fritted microplates to retain resin with bound protein and elute purified EVs.

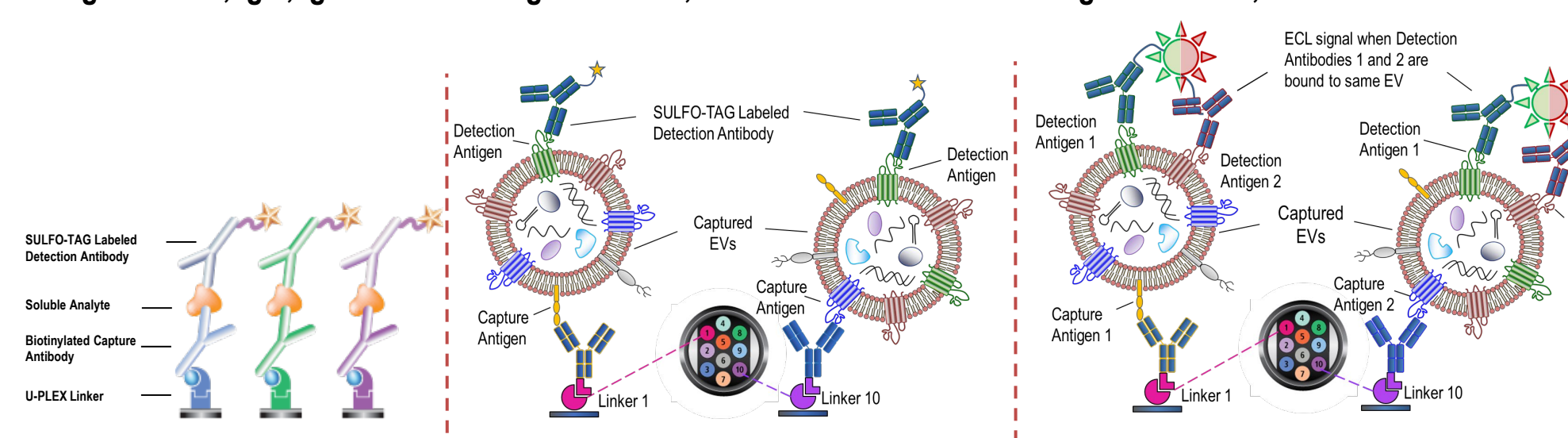### B. MSD® ECL-based assays measure the concentration of soluble proteins and intact EVs in complex biological samples

Electrochemiluminescence (ECL) Technology

MSD's ECL detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

- **High sensitivity:** Multiple excitation cycles amplify signals to enhance light levels.
- **Broad dynamic range:** High and low abundance analytes can be measured without multiple sample dilutions.
- **Low background:** Electronic stimulation of detection label is decoupled from the output signal (light) resulting in extremely low background signals.

U-PLEX® Multiplexed Immunoassays

Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. Multiplexing conserves valuable samples by allowing ten determinations per well.

Assaying soluble analytes: e.g. Albumin, IgM, IgG

Soluble analytes are captured and detected using sandwich immunoassay format with SULFO-TAG ECL label.

Assaying high-abundance intact EVs: e.g. CD9+ EVs, CD36+ EVs

Intact EVs are captured by antibodies targeting distinct surface antigens, e.g. CD9 or CD36. Bound EVs are detected using SULFO-TAG labeled detection antibodies that recognize specific detection antigen(s) on the EVs; usually this is a cocktail of common EV proteins: CD81, CD9, and CD63.

Assaying low-abundance intact EVs: e.g. CD44+ EVs, CD38+ EVs

Bound EVs are detected using a pair of detection antibodies or cocktails targeting at least two distinct detection antigens. The ultrasensitive assay format only generates signal when both detection antibodies are present on the same EV. At least one of the two detection antigens is typically a common EV protein: CD81, CD9, or CD63.

C. Super-resolution microscopy evaluates unbiased EV recovery and unchanged EV properties

Principle and protocol at a glance

- **Direct stochastic optical reconstruction microscopy (dSTORM)** relies on the stochastic switching between a fluorescent "on" state and a dark "off" state that allows temporal separation of individual molecules, enabling high precision localization of each molecule and resolution well below the diffraction limit.
- Recombinant EVs expressing CD63-GFP fusion were spiked into biofluids to assess recovery. EVs from spiked samples before and after purification were captured on a glass surface using anti-CD81 antibodies and stained with anti-CD63-AF647. dSTORM imaging was used to localize clusters of individual AF647 molecules and identify CD81+CD63+ EVs. GFP is non-switching so individual molecules cannot be resolved but the centroid of each cluster of CD63-GFP molecules can be localized with sufficient precision to easily distinguish the spiked recombinant EVs (GFP+) from endogenous EVs (GFP-) in the sample.

3 Samples tested

- 32 commercially sourced plasma samples from healthy donors were collected in K2EDTA tubes and double spun immediately after collection to remove platelets.
- Expi293 cell line was used to evaluate the purification performance as well as to evaluate EV recovery of fluorescent EVs (transfected with CD63-GFP). In each case, conditioned medium was collected and EVs were purified using 750 kDa tangential flow filtration (TFF).

## 4 Determining EV recovery and purity vs. protein concentration in samples

- Two dilutions of TFF-purified EVs from a cell line were tested for EV purification after spiking a range of 0-160 mg/mL of bovine serum albumin (BSA) to span the range of total protein in relevant sample types. Total protein removal was assessed with BCA assay[1], and EV recovery was assessed using MSD R-PLEX® EV assays assaying CD81+ EVs[2], CD9+ EVs[3], and CD63+ EVs[4].
- EV recovery and protein removal illustrated in the plot show that a minimum of 40 mg/mL of protein is necessary in the sample to allow > 50% of the EVs to be recovered. Above that concentration, EV recovery is improved but the resin reaches saturation, and protein removal performance decreases.
- Plasma samples should be diluted 3-fold to be in the range of resin capacity for protein removal.
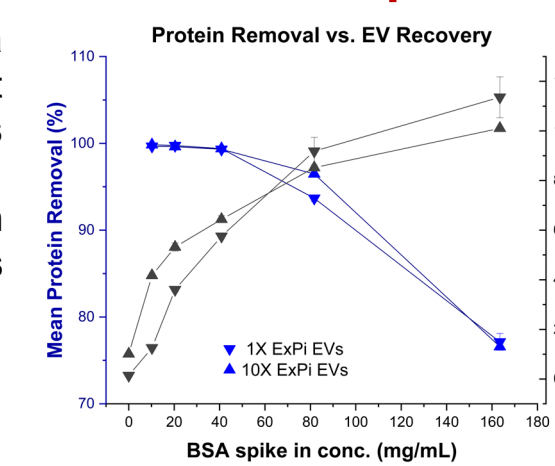
Assay catalog numbers: [1] Pierce A55864, [2] MSD K1515NR, [3] MSD K1515MR, [4] MSD K1515LR

5 Plasma EV purification removes > 99.9% of proteins under the MW cutoff

- Purification efficiency was assessed by measuring protein concentrations in 32 plasma samples before and after purification, each with 3 technical replicates.
- Albumin and IgG removal were typically above 99.9%. Total protein removal was typically greater than 98%. IgM, which is above expected MW cutoff, is not efficiently removed.
- Variability of protein removal between technical replicates of purification on the same sample was much lower than variability of protein removal between different biological samples.
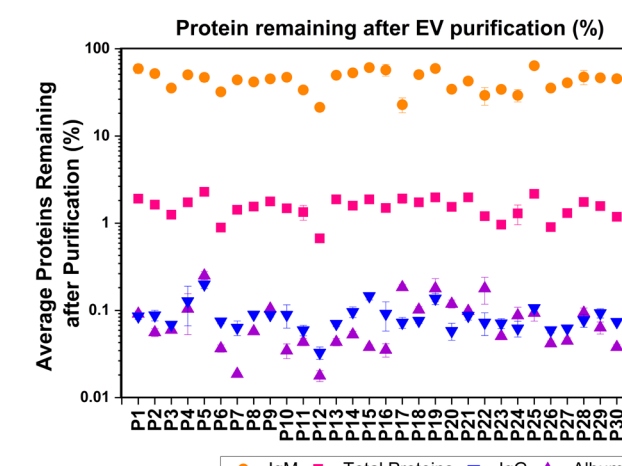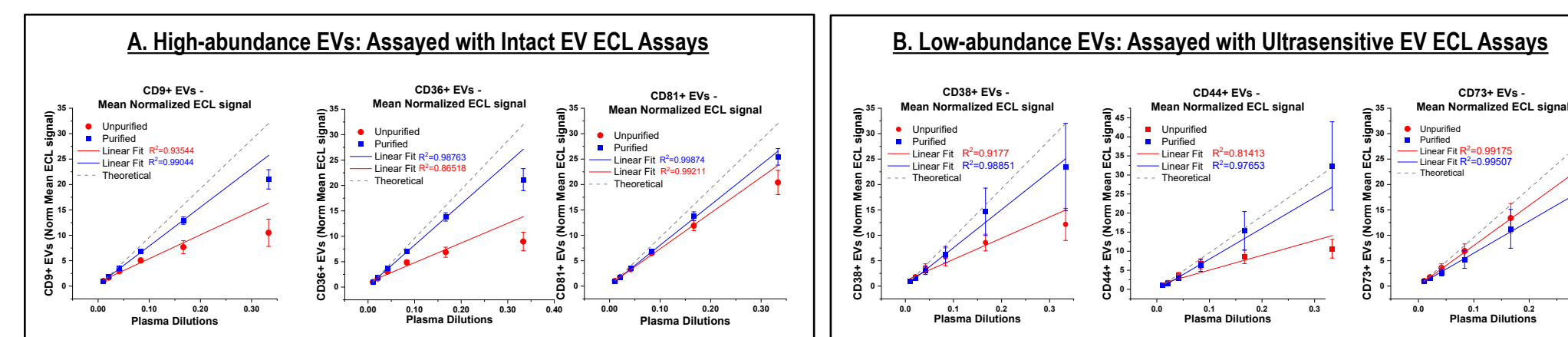

Protein	Size (kD)	Assay	% Remaining in 32 plasma samples Avg	% Remaining in 32 plasma samples Max	% Remaining in 32 plasma samples CV	Purification rep Avg CV
Total Protein	NA	Pierce BCA assay [1]	1.53	2.29	26%	6.2%
Albumin	66	MSD Human Albumin [2]	0.077	0.25	69%	12.3%
IgG	150	MSD Panel 1	0.085	0.20	37%	13.4%
IgM	900 - 1050	Human/NHP [3]	43.3	64.1	28%	7.5%

Assay catalog numbers:
[1]: Pierce A55864; [2]: MSD K1514VR; [3]: MSD K15183B

6 Plasma EV purification eliminates assay interference from soluble proteins

When assaying specific EV populations, the presence of abundant soluble forms of the capture antigen can interfere by binding to the capture antibodies, competing with EVs. In such cases, the EV concentration may be underestimated at low dilutions. High- (A) and low- (B) abundance plasma EV populations were measured with the regular and ultrasensitive assays, respectively. ECL signal is normalized to the signal measured for the most diluted sample (1:96). (A) Average of 32 samples, and (B) average of 16 samples. CD9+ EVs, CD36+ EVs (A), CD38+ EVs and CD44+ EVs (B) show improved dilution linearity after purification, which indicates interference in unpurified plasma. However, CD81+ EVs (A) and CD73+ EVs (B) show comparable dilution linearity before and after purification, suggesting negligible interference for these assays.

A. High-abundance EVs: Assayed with Intact EV ECL Assays

B. Low-abundance EVs: Assayed with Ultrasensitive EV ECL Assays

7 Recovery of high- and low-abundance populations of plasma EVs

A. Variability of endogenous EV recovery is attributed to inherent differences in samples

Efficiency of EV recovery was measured as the ratio of dilution-corrected EV ECL signal in plasma pre-purification measured at 1:24 sample dilution, and post-purification measured at 1:6 sample dilution.

- Recovery of six high-abundance EV populations for 32 plasma samples (each with 3 technical replicates of purification) averaged 57%. (A) EV recovery is consistent across phenotypes but varies between samples (B).
- Recovery of six low-abundance EV populations for 16 plasma samples using a pool of purification triplicates averaged 43% (C), and was more variable between samples than high abundance EVs (D).
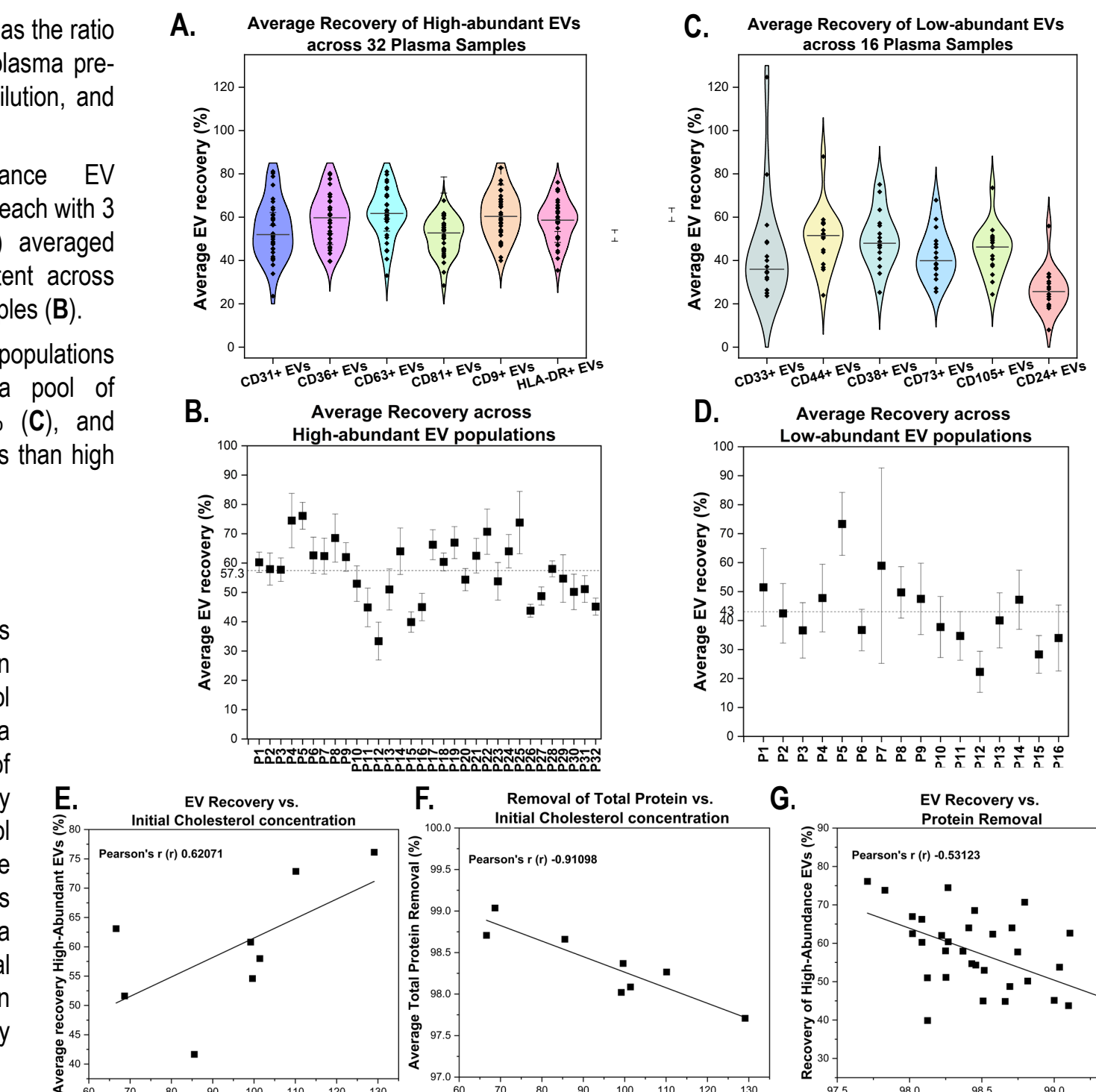
- To investigate differences in samples that might account for variability in recovery, we assessed cholesterol level in the LDL fraction of 8 plasma samples spanning the range of observed recovery. EV recovery positively correlates with initial cholesterol concentration (r=0.62) (E), while protein removal negatively correlates (r=-0.91) (F), suggesting that plasma cholesterol may be a substantial source of variability in purification performance. EV recovery negatively correlates with protein removal (G).

B. Recovery of exogenous fluorescent EVs shows unchanged EV characteristics

- Fluorescent CD63-GFP EVs spiked into four plasma samples were imaged using localization microscopy (A) to assess recovery after purification. The ratio of spiked EVs to endogenous EVs (B) is relatively unchanged before and after purification, suggesting that these populations have similar recovery. There is no observable change in the size (C) or number of localizations (D) per EV after purification.
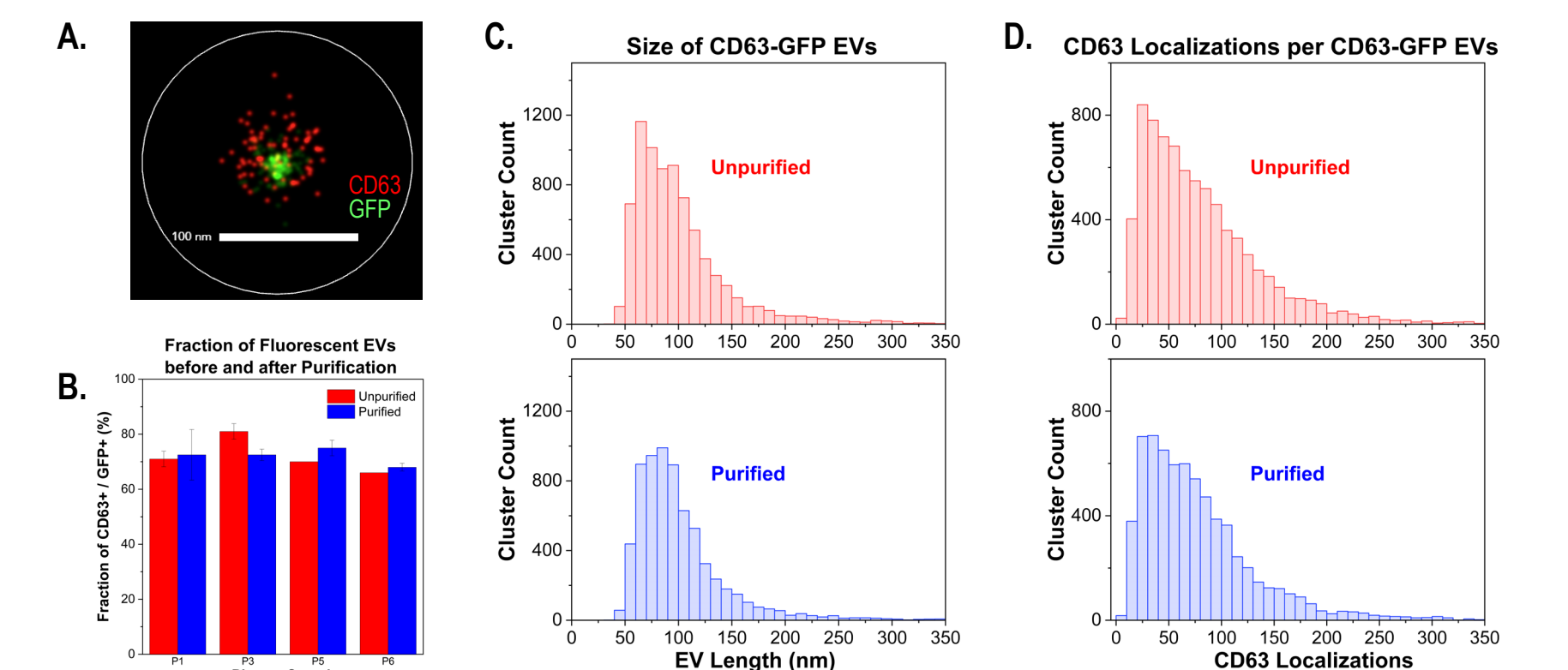
8 Conclusion

We demonstrated a high-throughput isolation of plasma EVs that consistently removes over 99.9% of soluble proteins below 400 kDa in a fraction of the time of conventional EV purification methods including SEC or ultracentrifugation. The removal of proteins, including the soluble forms of capture antigens, allows more accurate quantitation of both high- and low-abundance EV populations across a wider range of sample dilutions than unpurified samples. The EV isolation method described is able to recover an average of 57% and 43% of high- and low-abundance EVs, respectively. We also showed that the level of cholesterol in plasma samples is a significant correlate of EV recovery and protein removal.

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