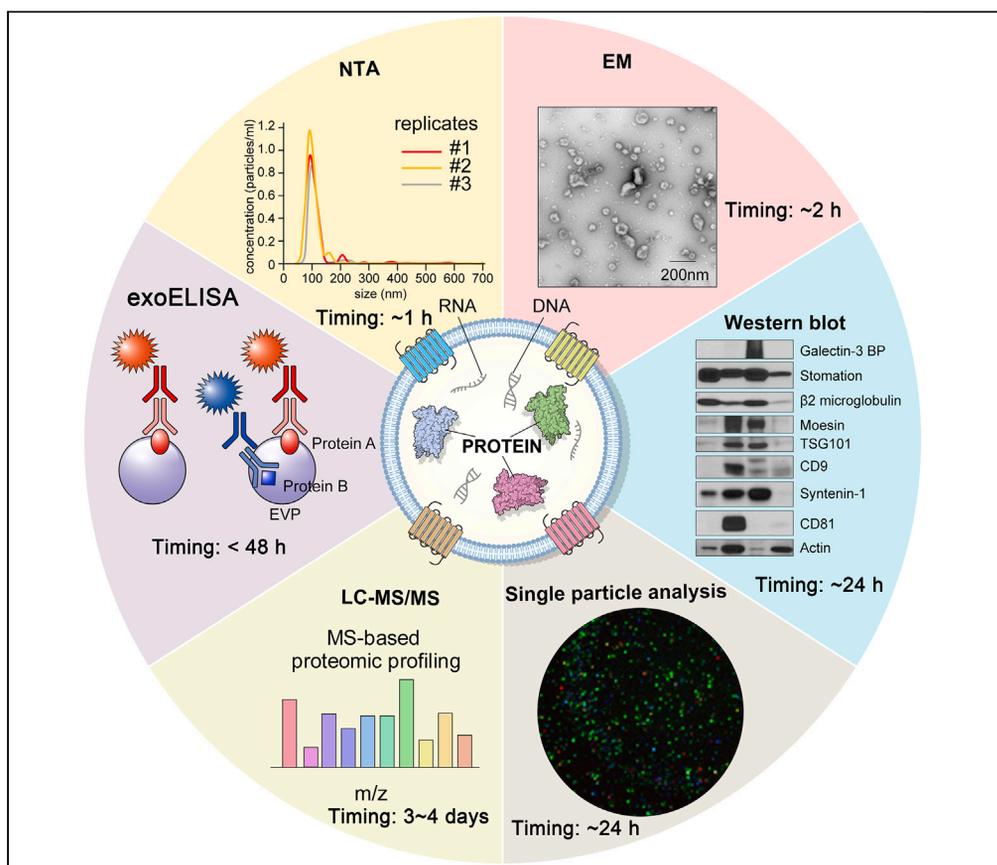


## Protocol

# Protocol for cross-platform characterization of human and murine extracellular vesicles and particles



Characterization of isolated extracellular vesicles and particles (EVPs) is crucial for determining functions and biomarker potential. Here, we present a protocol to analyze size, number, morphology, and EVP protein cargo and to validate EVP proteins in both humans and mice. We describe steps for nanoparticle tracking analysis, transmission electron microscopy, single-EVP immunodetection, EVP proteomic mass spectrometry and bioinformatic analysis, and EVP protein validation by exoELISA and western blot analysis. This allows for EVP cross-validation across different platforms.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Linda Bojmar, Han Sang Kim, Kei Sugiura, ..., Irina R. Matei, David Lyden, Ayuko Hoshino

irm2224@med.cornell.edu (I.R.M.)  
dcl2001@med.cornell.edu (D.L.)  
aynhoshino@g.ecc.u-tokyo.ac.jp (A.H.)

### Highlights

Cross-validation of isolated EVPs across different platforms

EVP evaluation using nanoparticle tracking analysis and transmission electron microscopy

LC-MS/MS and subsequent bioinformatic analysis for EVP protein cargo identification

Validation of EVP proteins by single-particle analysis, western blot, and exoELISA

Bojmar et al., STAR Protocols  
5, 102754  
March 15, 2024 © 2023 The Authors.  
<https://doi.org/10.1016/j.xpro.2023.102754>



## Protocol

## Protocol for cross-platform characterization of human and murine extracellular vesicles and particles

Linda Bojmar,<sup>1,2,10</sup> Han Sang Kim,<sup>1,3,10</sup> Kei Sugiura,<sup>4,5,6,10</sup> Søren Heissel,<sup>7,10</sup> Serena Lucotti,<sup>1,10</sup> Michele Cioffi,<sup>1</sup> Kofi Ennu Johnson,<sup>1,8</sup> Leona Cohen-Gould,<sup>9</sup> Haiying Zhang,<sup>1</sup> Henrik Molina,<sup>7</sup> Irina R. Matej,<sup>1,\*</sup> David Lyden,<sup>1,12,\*</sup> and Ayuko Hoshino<sup>1,4,5,11,\*</sup>

<sup>1</sup>Children's Cancer and Blood Foundation Laboratories, Departments of Pediatrics, and Cell and Developmental Biology, Druker Institute for Children's Health, Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA

<sup>2</sup>Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

<sup>3</sup>Yonsei Cancer Center, Division of Medical Oncology, Department of Internal Medicine, Graduate School of Medical Science, Brain Korea 21 FOUR Project, Yonsei University College of Medicine, Seoul, Korea

<sup>4</sup>Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan

<sup>5</sup>School of Life Science and Technology, Tokyo Institute of Technology, Yokohama, Japan

<sup>6</sup>Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

<sup>7</sup>Proteomics Resource Center, The Rockefeller University, New York, NY, USA

<sup>8</sup>Tri-Institutional PhD Program in Computational Biology and Medicine, New York, NY, USA

<sup>9</sup>Microscopy & Image Analysis, Core Facilities, Weill Cornell Medicine, New York, NY, USA

<sup>10</sup>These authors contributed equally

<sup>11</sup>Technical contact: [aynhoshino@g.ecc.u-tokyo.ac.jp](mailto:aynhoshino@g.ecc.u-tokyo.ac.jp)

<sup>12</sup>Lead contact

\*Correspondence: [irm2224@med.cornell.edu](mailto:irm2224@med.cornell.edu) (I.R.M.), [dcl2001@med.cornell.edu](mailto:dcl2001@med.cornell.edu) (D.L.), [aynhoshino@g.ecc.u-tokyo.ac.jp](mailto:aynhoshino@g.ecc.u-tokyo.ac.jp) (A.H.) <https://doi.org/10.1016/j.xpro.2023.102754>

## SUMMARY

Characterization of isolated extracellular vesicles and particles (EVPs) is crucial for determining functions and biomarker potential. Here, we present a protocol to analyze size, number, morphology, and EVP protein cargo and to validate EVP proteins in both humans and mice. We describe steps for nanoparticle tracking analysis, transmission electron microscopy, single-EVP immunodetection, EVP proteomic mass spectrometry and bioinformatic analysis, and EVP protein validation by ExoELISA and western blot analysis. This allows for EVP cross-validation across different platforms.

For complete details on the use and execution of this protocol, please refer to Hoshino et al.<sup>1</sup>

## BEFORE YOU BEGIN

## Institutional permissions

Human specimens and processing: fresh human tumor and adjacent tissues were obtained from patients surgically treated at Memorial Sloan Kettering Cancer Center (MSKCC). All individuals provided informed consent for tissue donation according to protocols approved by the institutional review board of MSKCC (IRB 15–015) and Weill Cornell Medicine (WCM IRB 0604008488). No sex or age selection was performed. The study is compliant with all relevant ethical regulations regarding research involving human participants.

Mouse specimens and processing: all mouse experiments were performed in accordance with Institutional Animal Care and Use Committees (IACUC) and American Association for Laboratory Animal Science (AAALAS) guidelines (Weill Cornell Medicine animal protocol 0709–666A). Female wild-type animals 6–8 weeks old were used. Animals were monitored for stress, illness or abnormal tissue



growth, and euthanized if health deteriorated. Animals were not involved in any previous procedures nor received any drugs. Animals were provided water and chow *ad libitum* and maintained in a pathogen-free facility.

The reader will need to acquire permission from their relevant institution to perform experiments on human and/or animal samples.

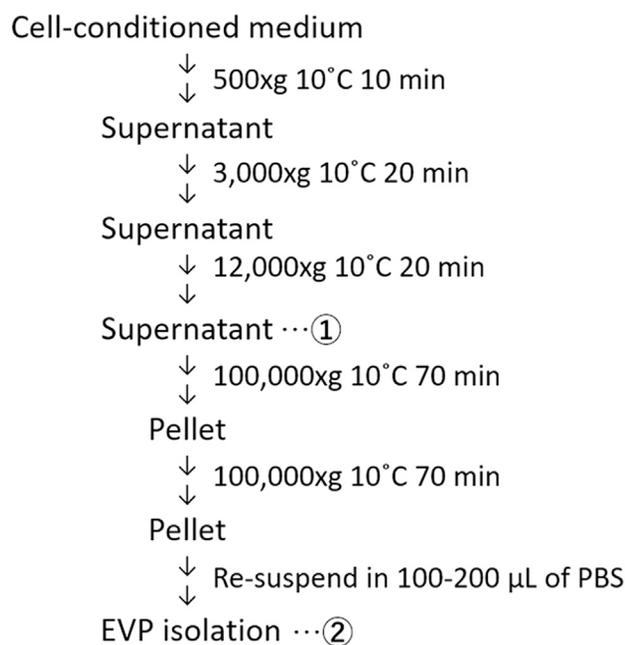
### EVP sample preparation

⌚ Timing: ≤5 h

Please refer to the previous protocol for a step-by-step description of the EVP isolation process from cell lines, tissues or biofluids.<sup>2</sup> A brief description is shown in [Figure 1](#).

Based on [Figure 1](#), aliquots from supernatant ① are used for nanoparticle tracking analysis (NTA), ExoELISA, and single EVP immuno-detection (i.e., ExoView). Samples from step ② are used for TEM, western blot, ExoELISA, NTA, single EVP immuno-detection (i.e., ExoView), and liquid chromatography mass spectrometry (LC-MS/MS). The recommended sample starting amount varies depending on sample type and analytical method. For plasma analyses, 10–15  $\mu\text{L}$  of supernatant ① are required for NTA, 10  $\mu\text{L}$  should be used for ExoView, and 10–50  $\mu\text{L}$  should be used for ExoELISA. A minimum of 50  $\mu\text{L}$  of supernatant ① should be used for NTA when tissue culture medium is used. The amount of tissue culture supernatant ① required for ExoView analysis has not been optimized. For more details regarding recommendations for specific sample amounts, see respective method and [Tables 1](#) and [2](#).

Samples may be handled sterily in a safety cabinet when of human origin or to be used for *in vitro* or *in vivo* downstream applications.



**Figure 1. Workflow for EVP isolation using serial differential ultracentrifugation**

For detailed procedures for various sample types, please refer to the previous STAR Protocol. 2020;2(1):100225<sup>2</sup> and Hoshino et al., 2020.<sup>1</sup>

**Table 1. Recommended dilutions of EVP samples from different sources for NTA, related to Step 1**

EVP source	Supernatant after 12,000 × g (Figure 1 step ①)	EVPs after 100,000 × g (Figure 1, step ②)
Bodily fluid (plasma)	1:500-1:3000	2–5 µg/mL
Cell line conditioned media	1:50–1:200	1 µg/mL
Tissue explant conditioned media	1:500-1:2000 <sup>a</sup>	1 µg/mL

<sup>a</sup>Range depends on tissue type. Explant (<2 mm<sup>3</sup>) tissue samples such as liver, which produce more EVPs, may require further dilutions, beyond 1:2000.

△ **CRITICAL:** Keep all samples on ice during processing. Store at –80°C.

### NTA (NanoSight NS300/NS500) initialization

⌚ **Timing:** 20 min

This section describes how to prepare the instrument before acquiring EVP samples.

- Switch on the NS500 instrument, then switch on the computer. Initiate the NanoSight 3.1 software.
- Wash the instrument fluidics and gasket with deionized (Milli-Q) water before use.
  - Connect the tubing to the Milli-Q water bottle, ensure that the lid is tightly closed and the tube is submerged in the liquid.
  - Click on PRIME FLUIDICS to fill the system with Milli-Q water.
  - Disconnect the tube from the Milli-Q water bottle and click on EMPTY FLUIDICS.
- Fill the tubing with PBS. The solvent used to suspend EVPs should be used here.
  - Connect the PBS bottle, ensure that the lid is tightly closed, and the tube is submerged in the liquid.
  - Click on PRIME FLUIDICS to fill the system with PBS.
- Check that the machine is clean. Ensure that no particles are present in the imaging field. One or two particles are acceptable, but if more particles are detected, repeat steps 2 and 3 until no debris/particles are detected in the PBS.

△ **CRITICAL:** Milli-Q and PBS should be filtered through a 0.2 µm filter unit.

**Alternatives:** Initialization for NS300 is the same, wash two times (1 mL × 2) with Milli-Q water, followed by three PBS washes (1 mL × 3), using a syringe.

### LC-MS/MS reagent preparation

This section describes how to prepare reagents and material for LC-MS/MS, including enzymes for peptide digestion, denaturing urea buffer, and micro-purification tips.

**Table 2. Example of concentration and other information of each type of sample, related to Step 33**

	Recommended starting dilution	Remarks	Corresponding result of BCA assay after isolation
Plasma after 12,000 × g (Figure 1, step ①)	1:50	Maximum dilution should be 1:5	N/A
Plasma after 100,000 × g (Figure 1, step ②)	1:50		1800–2150 µg/mL <sup>a</sup>
Supernatant of cultured cells after 100,000 × g (Figure 1, step ②)	1:7500	Maximum dilution should be 1:2500	2140 µg/mL <sup>b</sup>

<sup>a</sup>EVPs prepared from 2 mL of human plasma from a healthy donor and resuspended in 100 µL of PBS.

<sup>b</sup>EVPs prepared from 100 mL of MDA-MB-231-LM2 conditioned media (see Bojmar et al., Star Protocol, 2020 for recommended culture conditions) and resuspended in 100 µL of PBS.

### Preparation of 1,4-Dithiothreitol (DTT) aliquots

⌚ Timing: 30 min

5. Prepare 1 M 1,4-Dithiothreitol.
  - a. Dissolve 50 mg 1,4-Dithiothreitol (DTT) in 325  $\mu\text{L}$  of LCMS-grade water. Vortex until fully dissolved.
  - b. Create 10  $\mu\text{L}$  aliquots of 1 M DTT in 1.5 mL Eppendorf vials for further experiments. Store 1 M DTT aliquots at  $-20^{\circ}\text{C}$  until ready to use.

**Note:** 1 M DTT is stable for 3 months if stored at  $-20^{\circ}\text{C}$ .

### Preparation of endopeptidase lys C (Lys-C) aliquots

⌚ Timing: 20 min

**Note:** Endopeptidase Lys-C can also be purchased in ready-to-use form.

6. Dissolve 10 AU (amidase units) Endopeptidase Lys-C.
  - a. Keep vial of lyophilized Lys-C on ice.
  - b. Prepare 5 mL of 50 mM triethylammonium bicarbonate (TEAB) in LCMS-grade water and place on ice.
  - c. Pre-chill 130  $\times$  1.5 mL Eppendorf tubes at  $-20^{\circ}\text{C}$  for 5 min.
  - d. Add 3 mL of cold 50 mM TEAB to Lys-C and vortex.
  - e. Use a mechanical pipette with a 500  $\mu\text{L}$  pre-chilled tip to pipet 20  $\mu\text{L}$  of the Lys-C from step d into each of the 130  $\times$  1.5 mL Eppendorf from step c. Keep the aliquots cold.
  - f. Store Lys-C aliquots at  $-20^{\circ}\text{C}$ . Aliquots are stable for 3 months.

### Preparation of denaturing buffer

⌚ Timing: 30 min

7. Prepare 1 M ammonium bicarbonate (ABC) stock:
  - a. Weigh out 791 mg ABC.
  - b. Dissolve, using vortex, in 10 mL LCMS-grade water.
8. Prepare 8 M urea, 50 mM ABC:
  - a. Weigh out 2.4 g urea and place in a 15 mL test tube.
  - b. Add 250  $\mu\text{L}$  ABC stock and vortex.
  - c. Slowly add LCMS-grade water to a final volume of 5 mL and vortex until all urea is dissolved.
9. Add DTT to 8 M urea, 50 mM ABC solution:
  - a. Add 990  $\mu\text{L}$  8 M urea, 50 mM ABC solution to a 10  $\mu\text{L}$  1 M DTT aliquot.

**Note:** The denaturing buffer can be stored at room temperature ( $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ ).

**⚠ CRITICAL:** Urea may take several minutes to dissolve, but DO NOT heat. When vortexing at the highest speed, urea should dissolve within 10 minutes.

### Preparing micro-purification tips

⌚ Timing: 15 min

**Note:** Micro-purification tips can also be purchased (STAGETIP C18 MAT 200UL 96PC Part Number: NC0028383, Thermo Scientific).

10. Preparation of micro-purification tips (Figure 2).

- a. Cut two pieces from a C<sub>18</sub> extraction disk (3M) by placing the disk on a clean (detergent-free) surface and pressing a gauge 16 Kel-F hub needle through the disk against the surface.

**Alternatives:** Fold the disk in half and cut into two pieces.

- b. Stomp the piece(s) into a 200- $\mu$ l pipette tip (Rainin) by placing the needle inside the pipette tip and pushing the C<sub>18</sub> plug using a 1702 plunger assembly.

**Note:** Construct as many purification tips as the number of samples to be analyzed (for additional details on construction of needle-plunger assembly, refer to Rappsilber et al.<sup>3</sup>).

- c. Clearly mark the tips with a marker using either a number or abbreviation of the sample name.
- d. Prepare dispenser for elution.
- e. Using a pair of scissors, cut 1 inch of the tip of an Eppendorf 5 mL combitip.
- f. Prepare 25 mL of each of the following solvents:
  - i. Activation solvent: acetonitrile, 100%.
  - ii. Equilibration/washing solvent: 0.1% trifluoroacetic acid in LCMS-grade water.
  - iii. Elution solvent: 70% acetonitrile, 0.1% trifluoroacetic acid in LCMS-grade water.

### Preparation for single EVP immuno-detection

Single EVP immuno-detection is used for detecting specific protein cargo on the surface or inside of single EVPs. In this section, we are going to describe the ExoView, which is one of the single EVP immuno-detection methods.

Before starting ExoView analysis, prepare the antibody mix and pre-scan chips to record the background, see below for further details.

**Note:** Reagents for ExoView analysis are commercially available (Unchained Labs, <https://www.unchainedlabs.com/leprechaun/>).

### Preparation of antibody mix for ExoView

⌚ Timing: 15 min

11. Prepare antibody mix.

- a. To prepare immunofluorescence (IF) Blocking solution, add 3 mL of the blocking solution to 3 mL of solution A (both supplied by the manufacturer).



**Figure 2.** Picture showing a micro-purification tip

**Note:** IF blocking solution is stable and can be stored at 4°C until the expiration date of the kit.

- b. To prepare the antibody mixture, dilute each antibody in “IF Blocking solution”.
  - i. To use the antibodies provided with the ExoView kit (CD9, CD63, and CD81), dilute antibodies in IF Blocking solution to a ratio of 1:600 as suggested by the company.
  - ii. To use antibodies that are not provided with the kit, start with the recommended concentration (0.2 µg/mL).

△ **CRITICAL:** It is highly recommended that antibodies not provided in the kit be titrated and tested prior to the experiment.

**Note:** A total volume of 250 µL of antibody mixture is required per chip.

△ **CRITICAL:** The antibody mixture should be prepared fresh on the day of the experiment. Store on ice in the dark until use.

**Note:** The use of conjugated antibodies is encouraged.

### Pre-scan of ExoView chips

⌚ **Timing:** 30 min for 4 chips

12. Pre-scan chip.
  - a. Start the ExoScan 2.5.5 acquisition software.
    - i. Plug the USB supplied in the ExoView kit into the computer.
    - ii. Choose the stage and corresponding chip ID number.
  - b. Place chips on the stages. Start scanning.
  - c. Start ExoViewer 2.5.0 software.
    - i. Choose the correct folder and chip ID to display the pictures of pre-scanned chips.

**Note:** Use chips only if there are no obvious streak or background patterns in the scanned picture.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
CD9 Alexa Fluor 488 conjugated, 1:600 dilution	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
CD81 Alexa Fluor 555 conjugated, 1:600 dilution	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
CD63 Alexa Fluor 647 conjugated, 1:600 dilution	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
CETP polyclonal antibody, Alexa Fluor 647, 0.1 µg/mL	Bioss	bs-3694R-A647
S100A9 Alexa Fluor 488, 0.1 µg/mL	Abcam	Ab200972
B2-microglobulin Alexa Fluor 647, 0.1 µg/mL	Santa Cruz Biotechnology	sc-13565
Mouse monoclonal anti-CD9 (clone MM2/57), 0.1 µg/mL	Millipore	CBL162; RRID: AB_2075914
Mouse monoclonal anti-syntenin-1 (S-31), 0.1 µg/mL	Santa Cruz	sc100336; RRID: AB_2183156

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody, HRP, 1:10,000 dilution	Thermo Fisher Scientific	G-21040
Mouse monoclonal anti-RhoV (F-2), 0.1 µg/mL	Santa Cruz	sc-515072
Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 647, 1:200–1:500 dilution.	Thermo Fisher Scientific	A32787

### Biological samples

Human cancer and adjacent normal tissues and blood	Memorial Sloan Kettering Cancer Center	N/A
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### Chemicals, peptides, and recombinant proteins

PBS	Gibco	10010-023
Uranyl acetate	Electron Microscopy Sciences, Hatfield, PA	22400
RIPA buffer	Sigma-Aldrich	20-188
Protease/phosphatase inhibitors	Thermo Fisher Scientific	78440
Laemmli SDS buffer	Thermo Fisher Scientific	J60015-AC
β-Mercaptoethanol	Sigma-Aldrich	M6250
Tris-glycine SDS-running buffer (10X)	Boston BioProducts	BP-150
Rainbow protein marker	Sigma-Aldrich	GERPN800E
Transfer buffer (10X)	Boston BioProducts	BP-190
Bovine serum albumin (BSA)	Sigma-Aldrich	12659
Tween 20	Sigma-Aldrich	P9416
Tris-buffered saline (TBS) (10X)	Boston BioProducts	BM-300
ECL	Sigma-Aldrich	GERPN2209
Methanol	VWR	BDH1135
Sodium bicarbonate	Sigma-Aldrich	S5761
UltraPure distilled water	Life Technologies	10977
HCl	Sigma-Aldrich	H1758
Triton X-100	Sigma-Aldrich	T8787
Trizma base	Sigma-Aldrich	T1503
NaCl	Millipore	567440
TSA blocking reagent	PerkinElmer	FP1012 or FP1020
Endopeptidase LysC	Wako Chemicals	129-02541
Protein LoBind tubes	Eppendorf	022431081
Sequencing grade modified trypsin	Promega	V5111
Ammonium bicarbonate (ABC)	MilliporeSigma	09830-500G
Urea	MilliporeSigma	U1250-1KG
Dithiothreitol (DTT)	EMD Millipore	1114740025
Iodoacetamide	MilliporeSigma	I6125
Acetonitrile, 100%	MilliporeSigma	34851
Trifluoroacetic acid, 0.1%	MilliporeSigma	76-05-1
Water (LCMS grade)	Fisher Chemical	W64

### Critical commercial assays

Bicinchoninic acid (BCA) assay	Thermo Scientific	23225
Solution A	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
Solution B	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
Solution C	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
Blocking solution	NanoView Biosciences	N/A (in kit #EV-TETRA-C)
NanoSight 100 nm transfer standards	Malvern Panalytical	NTA4088
ExoView chip	NanoView Biosciences	N/A (in kit #EV-TETRA-C)

### Deposited data

Proteomics data of extracellular vesicles and particles	PRIDE (a database of Proteomics Identifications)	PXD018301
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### Experimental models: Cell lines

Human: MDA-MB-231	ATCC	HTB-26
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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human: PANC-1	ATCC	CRL-1469
Human: AsPC-1	ATCC	CRL-1682
Human: A549	ATCC	CRM-CCL-185
Human: HCT116	ATCC	CCL-247
Mouse: B16-F10	ATCC	CRL-6475
<b>Experimental models: Organisms/strains</b>		
Mouse: wild-type BALB/c, female 6–8 weeks old	The Jackson Laboratory	Cat#000651
Mouse: wild-type C57BL/6, female 6–8 weeks old	The Jackson Laboratory	Cat#000664
<b>Software and algorithms</b>		
NanoSight NTA 3.4	Malvern Panalytical	<a href="https://www.malvernpanalytical.com/jp/support/product-support/nanosight-range">https://www.malvernpanalytical.com/jp/support/product-support/nanosight-range</a>
NanoViewer	NanoView Biosciences	<a href="https://www.nanoviewbio.com/exoview-software">https://www.nanoviewbio.com/exoview-software</a>
N Scan	NanoView Biosciences	<a href="https://www.nanoviewbio.com/exoview-software">https://www.nanoviewbio.com/exoview-software</a>
Proteome Discoverer	Thermo Fisher Scientific	<a href="https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/teome-discoverer-software.html">https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/teome-discoverer-software.html</a>
Mascot	Matrix Science	<a href="https://www.matrixscience.com/">https://www.matrixscience.com/</a>
Skyline	MacLean, Bioinformatics 2010	<a href="https://skyline.ms/project/home/begin.view?">https://skyline.ms/project/home/begin.view?</a>
Xcalibur	Thermo Fisher Scientific	<a href="https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/lc-ms-data-acquisition-software/xcalibur-data-acquisition-interpretation-software.html">https://www.thermofisher.com/us/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/lc-ms-data-acquisition-software/xcalibur-data-acquisition-interpretation-software.html</a>
R	The R Foundation	<a href="https://www.r-project.org">https://www.r-project.org</a>
GENE-E	Broad Institute	<a href="https://software.broadinstitute.org/GENE-E/">https://software.broadinstitute.org/GENE-E/</a>
GSEA, MSigDB	UC San Diego and Broad Institute	<a href="http://www.broadinstitute.org/gsea/msigdb/index.jsp">http://www.broadinstitute.org/gsea/msigdb/index.jsp</a>
<b>Other</b>		
Formvar-carbon coated 400 mesh copper grids	Electron Microscopy Sciences, Hatfield, PA	FCF400-Cu
4%–20% Tris-glycine gels	Life Technologies	XP04202BOX
PVDF membrane	Thermo Fisher Scientific	88520
Autoradiography film	Denville Scientific	E3018
Filter paper	Thermo Fisher Scientific	84783
MilliporeSigma Amicon Ultra-4 centrifugal filter units	Fisher Scientific	UFC801024
Milllex PVDF syringe filters	Millipore	SLGV033N
Eco-Heat hot plate with stirrer	Southern Labware	UNEHPLATE-1L
96-well flat clear bottom black polystyrene TC-treated microplates	Corning	3904
Dumont No. N5 tweezers	Electron Microscopy Sciences	0103-N5-PO
Whatman type 1 filter paper	VWR	N/A
Microplate shaker NS-P	AS ONE	1-5173-01
Straight strong point tweezers with plastic tips	Electron Microscopy Sciences	78325-26D, or alternatives which have plastic tips, to avoid damaging the chips
24-well cell culture plate	Corning Incorporated	3526; Alternatively, 6 or 10-cm dishes which have space to allow the addition of water in between each well
6 cm dish	Corning Incorporated	430166
16 gauge, Kel-F hub needle, 2 in, point style 3, 6/PK	Hamilton Company	90516
1702 plunger assembly LT, N, RN, C	Hamilton Company	1122-01

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
5 mL combitip	Eppendorf	30089456
C <sub>18</sub> extraction disk (3M)	Fisher Scientific	14-386-2
Eppendorf microcentrifuge tube 2 mL	Fisher Scientific	10430423
HPLC vial and cap	Thermo Scientific	03452358, 6ARC11NRT1F
Grating replica	Electron Microscopy Sciences	80050
Autoradiography cassette	Cytiva	RPN11650
Eppendorf Thermomixer R Mixer	Marshall Scientific	5355
ExoView	NanoView Biosciences	ExoView R100
SpeedVac vacuum concentrator	Savant	SVC-100H
Microcentrifuge	Sonation	STC-V2
Transmission electron microscope	JEOL	JEM 1400

## MATERIALS AND EQUIPMENT

### TEM

The TEM model used to image the grids is JEOL JEM 1400. Images are captured by a Veleta 2 K × 2 K CCD camera (EMSIS, Munich, Germany).

### LC-MS/MS

The authors used either a Q-Exactive, Q-Exactive HF (Xcalibur v. on 4.4.16.14), or Fusion Lumos mass spectrometer (Xcalibur v. 4.6.67.17) from Thermo Scientific connected to either a Dionex 3000 or Easy-nLC 1200 HPLC.

**Alternatives:** Any nanoflow HPLC operating in reversed-phase chromatography with a C18 column and high-resolution mass spectrometer capable of performing MS/MS may be used.

### Single EVP immuno-detection

The instrument used in this protocol is ExoView R100 (NanoView bioscience).

**Alternatives:** ExoView may be equipped with up to 4 lasers (488 nm, 555 nm, 640 nm, and 750 nm). In addition to Alexa Fluor fluorochromes (Alexa Fluor 488, 555, 647), CF fluorochromes (CF 488, 555, 647), antibodies conjugated to FITC, PE, APC, emGFP, ZsGreen, Cy3, mCherry, Cy5, and near-infrared dyes can be used for ExoView detection.

### Western blot

#### Lysis buffer

Reagent	Final concentration	Amount
RIPA buffer	N/A	990 μL
Protease/Phosphatase inhibitors	N/A	10 μL
<b>Total</b>	<b>N/A</b>	<b>1 mL</b>

**Note:** Lysis buffer should be kept cold on ice or at 4°C and should be prepared and used immediately (within minutes).

#### 4X Loading buffer

Reagent	Final concentration	Amount
Laemmli SDS buffer	N/A	920 μL
β-mercaptoethanol	8%	80 μL
<b>Total</b>	<b>N/A</b>	<b>1 mL</b>

**Note:** Loading buffer should be prepared at room temperature (20°C–22°C) and used within minutes, or stored at –20°C for up to 2 months.

△ **CRITICAL:** β-mercaptoethanol is a hazardous substance and should be handled in a ventilated chemical fume hood.

<b>Running buffer</b>		
Reagent	Final concentration	Amount
10X Tris-glycine SDS running buffer	N/A	100 mL
Ultrapure dH <sub>2</sub> O	N/A	900 mL
<b>Total</b>	<b>N/A</b>	<b>1 L</b>

**Note:** Running buffer can be stored at 4°C for up to 2 months, or aliquoted and stored at –20°C for up to a year.

<b>Transfer buffer</b>		
Reagent	Final concentration	Amount
10X Transfer buffer	N/A	100 mL
Methanol	20%	200 mL
Ultrapure dH <sub>2</sub> O	N/A	700 mL
<b>Total</b>	<b>N/A</b>	<b>1 L</b>

**Note:** Transfer buffer should be placed at 4°C for at least 1 h before use. It can be stored at 4°C for up to a week.

△ **CRITICAL:** Methanol should be handled in a ventilated chemical fume hood.

<b>Washing buffer</b>		
Reagent	Final concentration	Amount
TBS (10X)	N/A	100 mL
Tween 20	0.1%	1 mL
Ultrapure dH <sub>2</sub> O	N/A	900 mL
<b>Total</b>	<b>N/A</b>	<b>1 L</b>

**Note:** The washing buffer can be stored at room temperature (20°C–22°C) for up to a week.

<b>Blocking buffer</b>		
Reagent	Final concentration	Amount
Washing buffer	N/A	50 mL
Bovine Serum Albumin (BSA)	N/A	2.5 g
<b>Total</b>	<b>0.05%</b>	<b>50 mL</b>

**Note:** Blocking buffer can be stored at 4°C for up to a month or at –20°C for up to a year.

The equipment necessary for running western blot consists of Mini-PROTEAN Tetra Vertical Electrophoresis Cell for Mini Precast Gels, with Mini Trans-Blot Module and PowerPac Basic Power Supply (Bio-Rad cat. #1658034), or a similar system.

### ExoELISA

△ CRITICAL: All buffers should be prepared under sterile conditions and kept sterile to avoid contamination.

#### ExoELISA coating buffers

##### 1x Coating buffer (pH 9.4)

Reagent	Final concentration	Amount
Sodium bicarbonate	0.2 M	1.68 g
Ultrapure dH <sub>2</sub> O	N/A	Up to 100 mL
<b>Total</b>	<b>0.2 M</b>	<b>100 mL</b>

##### 2x Coating buffer (pH 9.4)

Reagent	Final concentration	Amount
Sodium bicarbonate	0.4 M	3.36 g
Ultrapure dH <sub>2</sub> O	N/A	Up to 100 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

- To prepare 100 mL of 1x buffer, dissolve 1.68 g of sodium bicarbonate in up to 100 mL ultrapure dH<sub>2</sub>O. Adjust the pH to 9.4 HCl/NaOH and bring the volume to 100 mL with ultrapure dH<sub>2</sub>O.
- Filter the buffer through a 0.22 μm filter into a clean sterile bottle or tube.
- ExoELISA coating buffers can be stored at room temperature (20°C–22°C) for up to 12 months.

#### ExoELISA blocking buffer

##### Tris-HCl 1 M (pH 7.5)

Reagent	Final concentration	Amount
Trizma Base	1 M	12.1 g
Ultrapure dH <sub>2</sub> O	N/A	Up to 100 mL
<b>Total</b>	<b>N/A</b>	<b>100 mL</b>

- Dissolve 12.1 g of Tris Base in 100 mL ultrapure dH<sub>2</sub>O.
- Adjust pH to 7.5 with 36.5%–38% HCl.
- Bring volume up to a total of 100 mL with ultrapure dH<sub>2</sub>O.

##### NaCl 5 M

Reagent	Final concentration	Amount
NaCl	5 M	14.61 g
Ultrapure dH <sub>2</sub> O	N/A	Up to 50 mL
<b>Total</b>	<b>N/A</b>	<b>50 mL</b>

##### TNB blocking buffer

Reagent	Final concentration	Amount
Tris-HCl 1 M (pH 7.5)	0.1 M	20 mL
NaCl 5 M	0.15 M	6 mL
TSA Blocking Reagent	0.5%	1 g
Ultrapure dH <sub>2</sub> O	N/A	Up to 200 mL
<b>Total</b>	<b>N/A</b>	<b>200</b>

- Mix Tris-HCl, NaCl and 100 mL of ultrapure dH<sub>2</sub>O and place solution on a magnetic stirrer with heat control and heat up to 55°C.
- Add TSA blocking reagent slowly and in small amounts and let it dissolve under continuous stirring for 30–60 min.
- Dilute to 200 mL with ultrapure dH<sub>2</sub>O and allow the buffer to cool down to room temperature (20°C–22°C), then aliquot and store at –20°C.
- TNB blocking buffer is stable for several months (<4 months) when stored at –20°C. Bring the buffer back to room temperature (20°C–22°C) before use.

△ **CRITICAL:** HCl and NaOH solutions are highly corrosive and irritant and can cause serious eye and skin damage and respiratory irritation. Handle HCl/NaOH in a chemical hood and use protective clothing, goggles, and gloves.

## STEP-BY-STEP METHOD DETAILS

### NTA (Nanoparticle tracking analysis)

⌚ **Timing:** 15 min per sample

NTA records the Brownian motion of EVPs in solution and measures the size and number of EVPs (Figure 3).

**Note:** The size and number of EVPs from different biological samples have been successfully measured using this NTA protocol, including EVPs from cell culture conditioned media, tissue explant conditioned media, and plasma.<sup>1</sup> Samples from ⌚ in Figure 1 (after 12,000 × g spin) can also be used. EVPs can be aliquoted and stored at –80°C prior to use in the NTA assay.

1. Dilute EVP samples in PBS in a 2 mL Eppendorf tube. A minimum of 1 mL of sample solution should be prepared. See Table 1 for optimal dilution factors.
2. Set focus:
  - a. Dilute standard 100 nm beads 1:500 in PBS.

**Note:** Standard beads are used for focusing and to ensure that the particle size is correct. Use NanoSight Size Transfer Standard beads (Malvern, NTA4088). The starting dilution of standard beads is 1:500, but beads might be further diluted if the concentration is too high for a particular Lot#. Store diluted beads at 4°C for up to one month.

- b. Place the loading tubing in the bead suspension.
- c. Click on LOAD, CAPTURE, START CAMERA, and move the stage to the ideal laser beam position with even distribution of particles.
- d. Focus on the beads by moving to the ideal imaging plane.

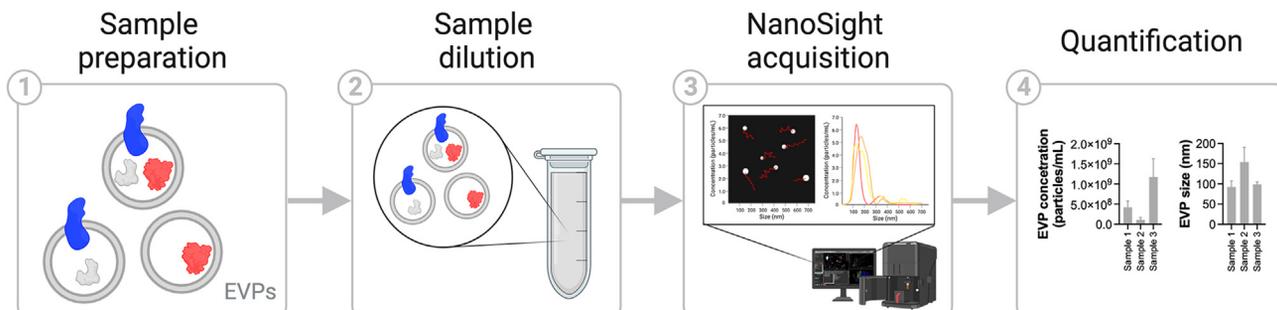


Figure 3. Step-by-step illustration of nanoparticle tracking analysis (NTA)

- e. Adjust the CAMERA LEVEL until the maximum number of EVPs can be seen. The camera level should not be higher than 12 to ensure optimal signal to noise ratio.
  - f. When prompted, transfer the loading tube to the waste and remove the tube containing the beads.
  - g. Click on FLUSH.
3. Run the sample. Each sample should be captured in triplicate for 60 s:
- a. Place the sample in the tube holder and introduce the loading tubing into the sample, ensuring it is fully submerged.
  - b. Click on LOAD, CAPTURE, and START CAMERA. Focus on the particles. SCREEN GAIN: 1 ; CAMERA LEVEL should not be higher than 12.

⚠ **CRITICAL:** The camera level should be kept consistent among samples to be compared.

- c. Go to SOP tab, create an experiment folder and type in the file name. Select the acquisition script.

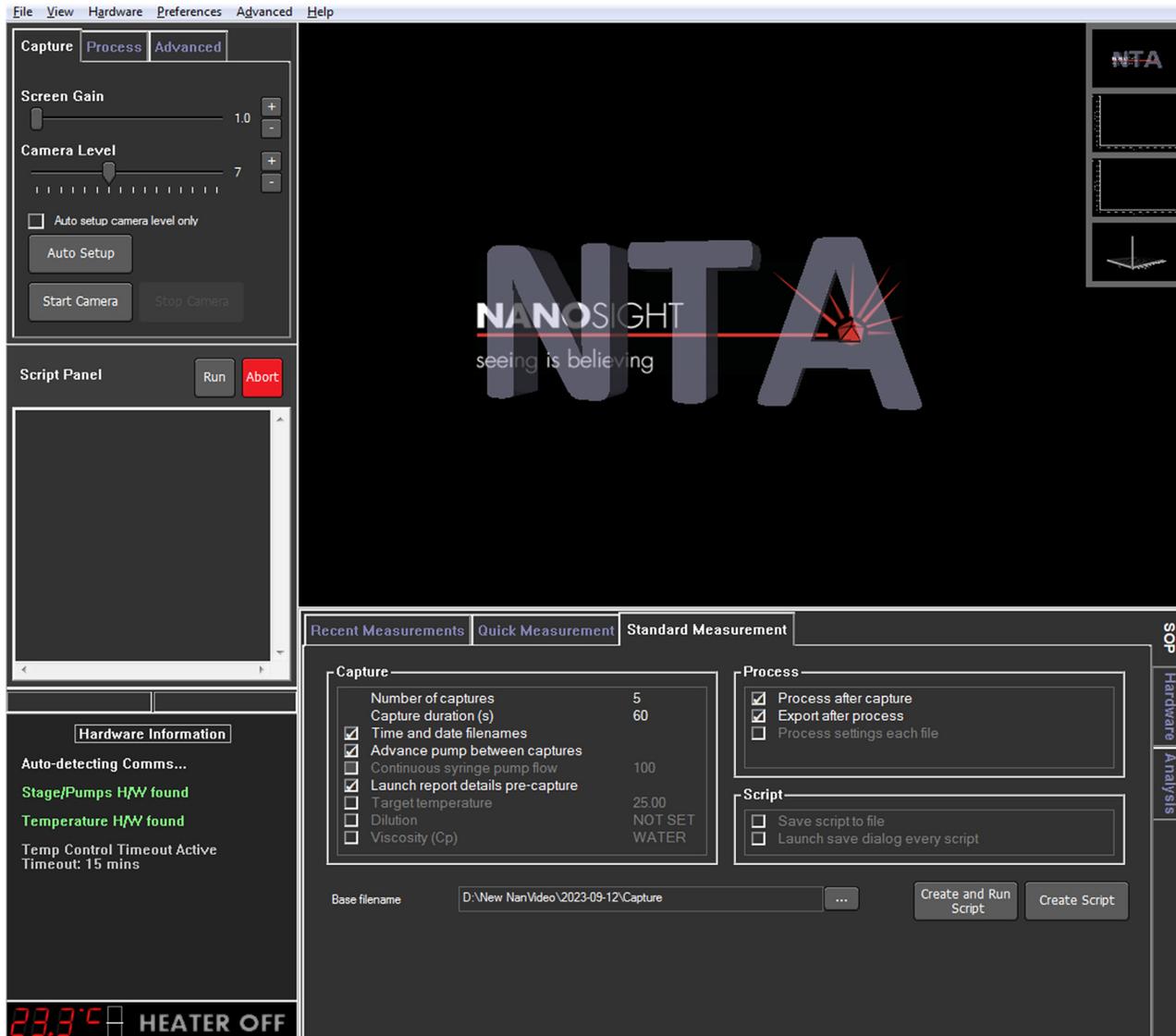


Figure 4. Example of NTA software settings for NS300

**Note:** Example of the script for NS300 (Figure 4).

```
RECORDDILUTION 3000.
SETVISCOSITY WATER.
CAMERASETTINGSMSG.
SYRINGELOAD 1000.
DELAY 20.
SYRINGELOAD 100.
DELAY 10.
REPEATSTART.
CAPTURE 60.
DELAY 1.
REPEAT 2.
SYRINGESTOP.
PROCESSSINGLESETTING.
EXPORTRESULTS.
```

Example of the script for NS500 (fitted with automatic fluidics system).

```
REPEATSTART.
PUMPADV.
DELAY 10.
CAPTURE 60.
REPEAT 2.
LASEROFF.
MESSAGE REMOVE TUBE FROM SAMPLE AND PUT IN WASTE.
PUMPFLUSH.
```

- d. Run script.
4. Analysis:
  - a. Go to ANALYSIS and open the experiment file.
  - b. Process selected files and export results as PDF files.

**Note:** The commands to automatically run the analysis at the end of the acquisition can be added to a script by typing:

```
PROCESSSINGLESETTING.
EXPORTRESULTS.
```

5. Shutdown:
  - a. Click on FLUSH to flush out the remaining sample.
  - b. Disconnect the tube from the PBS bottle and click on EMPTY FLUIDICS.
  - c. Plug in the Milli-Q water bottle to the tubing.
  - d. Click on PRIME FLUIDICS and wait until fluidics is primed.
  - e. Disconnect the tubing from the bottle and click on EMPTY FLUIDICS.
6. Switch off NS500 and the computer after the acquisition is done.

**△ CRITICAL:** To determine the optimal dilution while running your samples, start from the lowest dilution and work towards higher concentrations until the particle number counted by NanoSight is between  $2 \times 10^8$ – $2 \times 10^9$  particles per mL. We recommend preparing serial dilutions to achieve this.

**Note:** Start and shut down the machine by priming it with Milli-Q water, in order to avoid deposition of PBS salt crystals and organic matter from the samples in the tubing and gasket.

**Alternatives:** For NS300, use syringes (1 mL) for all steps. The protocol for acquiring samples on the NS300 is same as for NS500. After running your sample, prime three times with

PBS then two times with Milli-Q water. Machine settings: Set the script to Number of captures: 3; Capture duration (s): 60 sec; syringe speed should be 100  $\mu\text{L/s}$  for running the samples and 1000  $\mu\text{L/s}$  for washing.

**Note:** When using the NS300, syringes should be changed each time a different solution or sample is injected. See [problem 1](#) in [troubleshooting](#) section.

### TEM analysis

TEM is used to characterize EVPs visually and morphologically ([Figure 5](#)).

### Preparation of samples for TEM analyses

⌚ Timing: 10 min

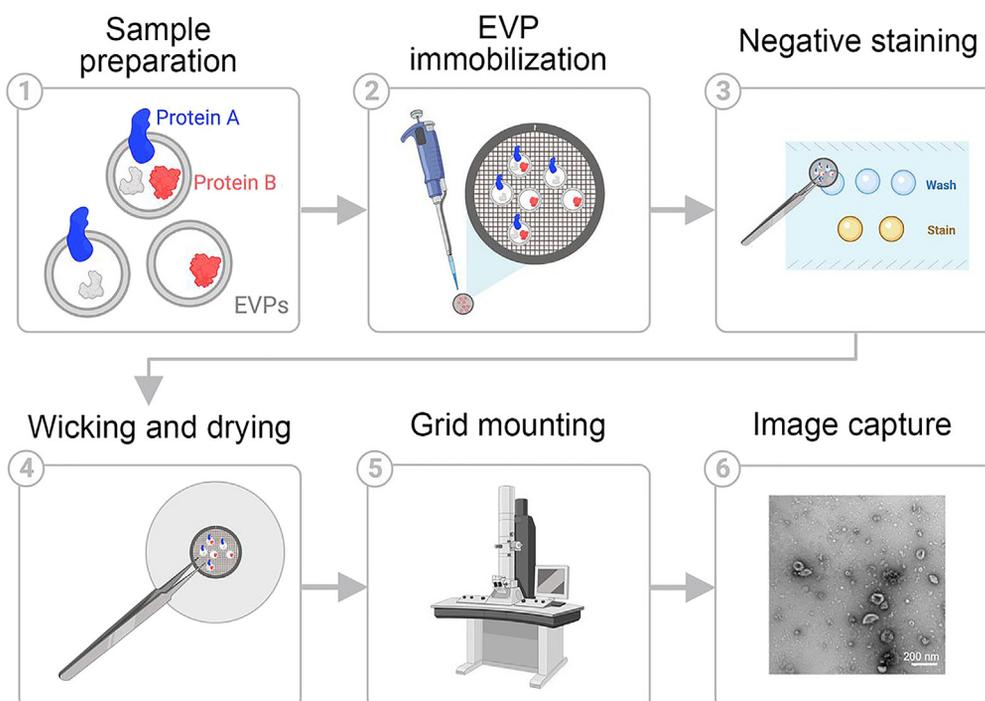
7. Dilute EVP samples (prepared according to [Figure 1](#), step ⌚) in PBS to a final concentration of 0.1  $\mu\text{g}/\mu\text{L}$  of EVP protein (measured using a commercial BCA assay).

**Note:** Freshly isolated samples should be used for best image quality, but samples stored at  $-80^{\circ}\text{C}$  can also be used.

8. Place the required number of coated grids into the chamber of a plasma cleaner (Pelco easiGlow, Ted Pella Inc, CA).
  - a. Run the cycle to neutralize any static charges on the grids.

**Note:** The above step renders the grids hydrophilic and ensures even distribution of the sample across the grid.

9. Place 5  $\mu\text{L}$  of sample on a formvar/carbon-coated 400 mesh copper grid.



**Figure 5.** The main steps required to perform transmission electron microscopy (TEM) on EVPs

- a. Allow to settle for 1 min.
10. Using a wedge of Whatman Grade 1 filter paper:
  - a. Dry the sample by touching the edge of the grid and wicking away the solution.
  - b. Negatively stain with 4 successive drops of 1.5% aqueous solution of uranyl acetate (UrAc).
  - c. Dry between each drop by wicking the solution away.
11. Air-dry grid.

**Note:** When imaging freshly isolated EVPs, a 2% PFA (paraformaldehyde) fixation does not affect image quality. For later imaging, a 2% PFA fixation can be an alternative to  $-80^{\circ}\text{C}$  storage but we have not compared these methods side-by-side.

**△ CRITICAL:** The UrAc is radioactive and must be collected & disposed of accordingly. It is a VERY weak radiation emitter (10'000Bq per gram, [https://ehs.weill.cornell.edu/sites/default/files/uranyl\\_acetate.pdf](https://ehs.weill.cornell.edu/sites/default/files/uranyl_acetate.pdf)), so no special precautions other than wearing gloves are needed (no shielding, or other specific personal protective equipment is required).

**Alternatives:** An alternative protocol can be used in which after the sample has been allowed to settle for 1 minute, the grid is inverted onto a 100  $\mu\text{L}$  droplet of UrAc (on a sheet of parafilm), allowed to sit for 1 minute and then washed by transferring to 3 successive droplets of deionized water, then inverted and air dried. This protocol makes it easier to collect the UrAc for disposal.

**Note:** The grid can be preserved in a grid storage box at room temperature ( $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ ) for 1–2 weeks, although it is preferable to image fresh grids by TEM, if feasible.

## TEM analysis

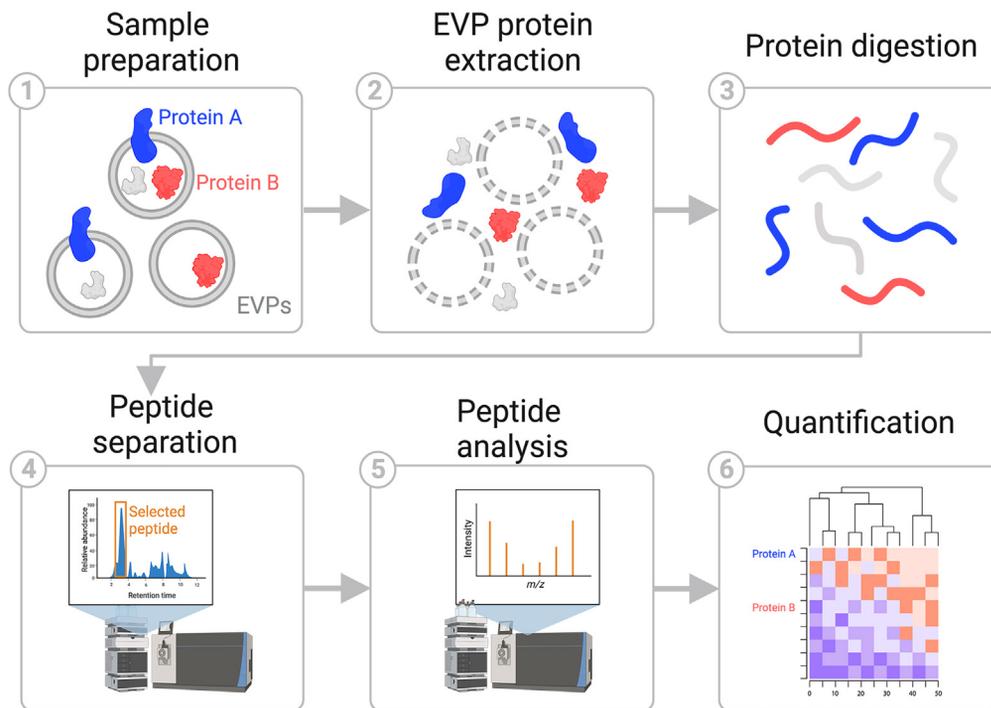
⌚ Timing: 1 h per sample

12. Check TEM alignment according to protocol from JEOL (<https://manualzz.com/doc/4151342/jeol-1400-tem-user-guide>).
13. Mount prepared grid in sample holder.
14. Insert sample holder into the TEM column and wait for the vacuum to equilibrate.
15. Insert camera into TEM column.
16. Turn on the filament.
17. Image the grids with JEOL JEM 1400 (JEOL, USA, Ltd, Peabody, MA) transmission electron microscope operating at 100 kV. Capture images on a Veleta 2 K  $\times$  2 K CCD camera (EMSIS, Munich, Germany).
18. Capture 10–15 images from each of 3 randomly selected areas of each grid at 50,000x and 100,000x lens magnification.
  - a. Calibrate the camera magnification using a grid with a grating replica (such as EMS cat# 80050) with line spacing of 463 nm (2160 lines/mm).

**Note:** Scale bars on images reflect the magnification at the camera.

## Preparation of EVP samples for LC-MS/MS

Here we describe a simple method using 8 M urea-based lysis of EVPs from EVP-enriched samples diluted in PBS. The protocol is composed of the steps illustrated in [Figure 6](#), use inexpensive chemicals and can be completed in less than 12 h. The advantage of this protocol is that it relies on established proteomics workflows and is done with minimal transfer between vials, thereby minimizing sample loss. Alternatively, sample preparation includes suspension traps (S-trap)<sup>4</sup> or filter-aided sample preparation (FASP),<sup>5</sup> although we have not evaluated these strategies for EVP analysis.



**Figure 6. Major steps in EVP protein cargo characterization by liquid chromatography mass spectrometry (LC-MS/MS)**

### Preparation of EVP samples for LC-MS/MS processing

⌚ Timing: 15 min

⌚ Timing: 2 days (for steps 21–25)

19. Take a volume corresponding to 5  $\mu\text{g}$  of EVP in PBS (samples can be prepared from  $-80^\circ\text{C}$  stored aliquots) and place in a low-protein binding Eppendorf tube.
20. Dry samples without external temperature setting using a vacuum centrifuge.
  - a. At 200 mTorr it will take approximately 1 h to reach dryness.

**Note:** If sample volume is not decreasing, freeze sample vials in liquid nitrogen and repeat vacuum centrifugation.

21. Dissolve, denature, and reduce proteins in EVP sample:
  - a. Add 25  $\mu\text{L}$  denaturing buffer to the dried EVP samples. See [problem 2](#) in [troubleshooting](#) section.
  - b. Incubate at room temperature ( $20^\circ\text{C}$ – $22^\circ\text{C}$ ) for 1 h under vigorous shaking by placing in an Eppendorf thermomixer at room temperature (temperature should not exceed  $30^\circ\text{C}$ ).
22. Alkylate reduced disulfide bonds:
  - a. Prepare a 100 mM iodoacetamide (IAA) solution in a tinted reaction tube by dissolving 18.5 mg IAA in 1 mL 50 mM ABC.
  - b. Add 5  $\mu\text{L}$  IAA solution to each EVP sample.
  - c. Incubate at room temperature ( $20^\circ$ – $22^\circ$ ), in the dark, for 1 h with vigorous shaking. A piece of tinfoil may be placed over the shaker to protect from light.
23. Digest proteins with Endopeptidase Lys-C:
  - a. Lower the urea concentration by adding 30  $\mu\text{L}$  of 50 mM ABC to each sample.

- b. Add 180  $\mu\text{L}$  of 50 mM ABC to the Endopeptidase Lys-C stock to obtain a concentration of 0.1  $\mu\text{g}/\mu\text{L}$ .
  - c. Add 5  $\mu\text{L}$  Lys-C solution to each tube and incubate on a shaker at room temperature (20°C–22°C) overnight.
24. Digest proteins with trypsin:
- a. Lower the concentration of urea further by adding 65  $\mu\text{L}$  of 50 mM ABC to each sample.
  - b. Dissolve the aliquoted lyophilized trypsin in 200  $\mu\text{L}$  of 50 mM ABC.
  - c. Add 5  $\mu\text{L}$  of trypsin solution to each sample and incubate on shaker at room temperature (20°C–22°C) for 6 h.
  - d. Halt digestion by adding 1  $\mu\text{L}$  neat trifluoroacetic acid (TFA).
25. Reverse-phase purification of EVP peptides:
- a. Place the pre-labeled micro-purification tips in a compatible table-top centrifuge (such as Sonation STC-V2, as seen in [Figure 7](#)).

**Alternatives:** If a centrifuge is not compatible with pipette tips, make a hole in the lid of a 2 mL microcentrifuge tube, place the tip in the vial and place the vial in the centrifuge. If the centrifuge does not close, push the tip further into the tube ([Figure 8](#)).

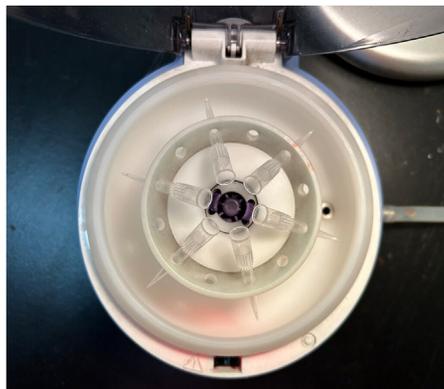
- b. Activate the material by placing the micro-purification tip in the centrifuge and place 70  $\mu\text{L}$  of Activation solvent in the tip.
  - i. Centrifuge until the Activation solvent has passed through but avoid dehydration of the column material, see [problem 2](#) in [troubleshooting](#) section.

**Note:** Keep centrifugation (<3000  $\times g$ ) time short for this step. 10–15 seconds is usually sufficient.

- c. If collecting in a microcentrifuge tube, discard the flow-through. If not, proceed to step d.
- d. Place 70  $\mu\text{L}$  of Equilibration/washing solvent in each tube and centrifuge until the liquid has been pushed through.

**Note:** Spinning time varies depending on centrifuge model, but 30 seconds is usually sufficient.

- e. Discard the flow-through. Repeat step d.
- f. Place a small drop (approx. 10  $\mu\text{L}$ ) of Equilibration/washing solvent in each tube and spin for 1–2 s. *This ensures that the tips do not dry while loading the sample.*
- g. Place the samples in the correct pre-labeled tips.



**Figure 7. Micro-purification tips loaded in tabletop centrifuge capable of holding pipette tips**



**Figure 8.** A D200 pipette tip with a C<sub>18</sub> plug at the bottom, placed in a 2 mL Eppendorf microcentrifuge tube

- i. Centrifuge until all liquid has been pushed through.
- ii. Keep centrifugation times similar to the equilibration step.
- iii. Discard the flow-through.
- h. Wash the tips by placing 70  $\mu$ L of the Equilibration/washing solvent in each tip.
  - i. Continue to centrifuge until the liquid has been pushed through.
  - ii. Keep centrifugation times same as in the equilibration step.
  - iii. Discard the flow-through.
- i. Elute the peptides by connecting the modified combi-tip to the micro-purification tip as seen in [Figure 9A](#) and push 70  $\mu$ L of Elution solvent through the tip, as in [Figure 9B](#).

**Note:** Minimize sample transfer by eluting directly into the HPLC vial, which is loaded into the LC autosampler.

- j. Place samples in vacuum centrifuge at room temperature (20°C–22°C) according to step 20 until completely dry.

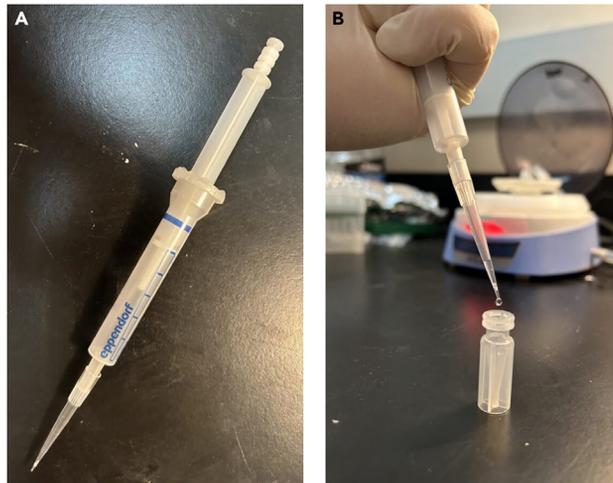
**△ CRITICAL:** Do not allow the C<sub>18</sub> material to dry until after the washing step. Doing so may lower the peptide recovery. See [problem 3](#) in [troubleshooting](#) section.

### LC-MS/MS analysis of EVP peptides

⌚ **Timing:** Time depends on gradient length. Typically, 2–3 h per sample.

In this step the samples are loaded in the autosampler and analyzed by mass spectrometry.

26. Dissolve dry peptides:
  - a. Add 8  $\mu$ L of 0.1% TFA to each HPLC vial containing dried sample and vortex for 5 s. Ensure the liquid is located at the bottom of your tube.
27. Place HPLC tubes in autosampler. 3  $\mu$ L of 8  $\mu$ L are injected per analysis.



**Figure 9. Elution of peptides from micro-purification tip**  
(A) Eppendorf combi-tip connected to micro-purification tip.  
(B) Elution of peptides into HPLC vial.

28. Program mass spectrometer. Use a 90 min\* linear gradient increasing from 1% B/99% A to 35% B/65% A (A: 0.1% formic acid, B: 80% acetonitrile, 0.1% formic acid).

**Note:** \* The duration can be modified based on sample complexity and acquisition speed of the mass spectrometer. See [problem 4](#) in [troubleshooting](#) section.

### Bioinformatic analysis of proteomics data

⌚ **Timing:** Time depends on the number of samples to be analyzed and will typically require 1–2 days.

In this step tandem MS data is queried and analyzed using proteomic databases and statistical software ([Figure 10](#)).

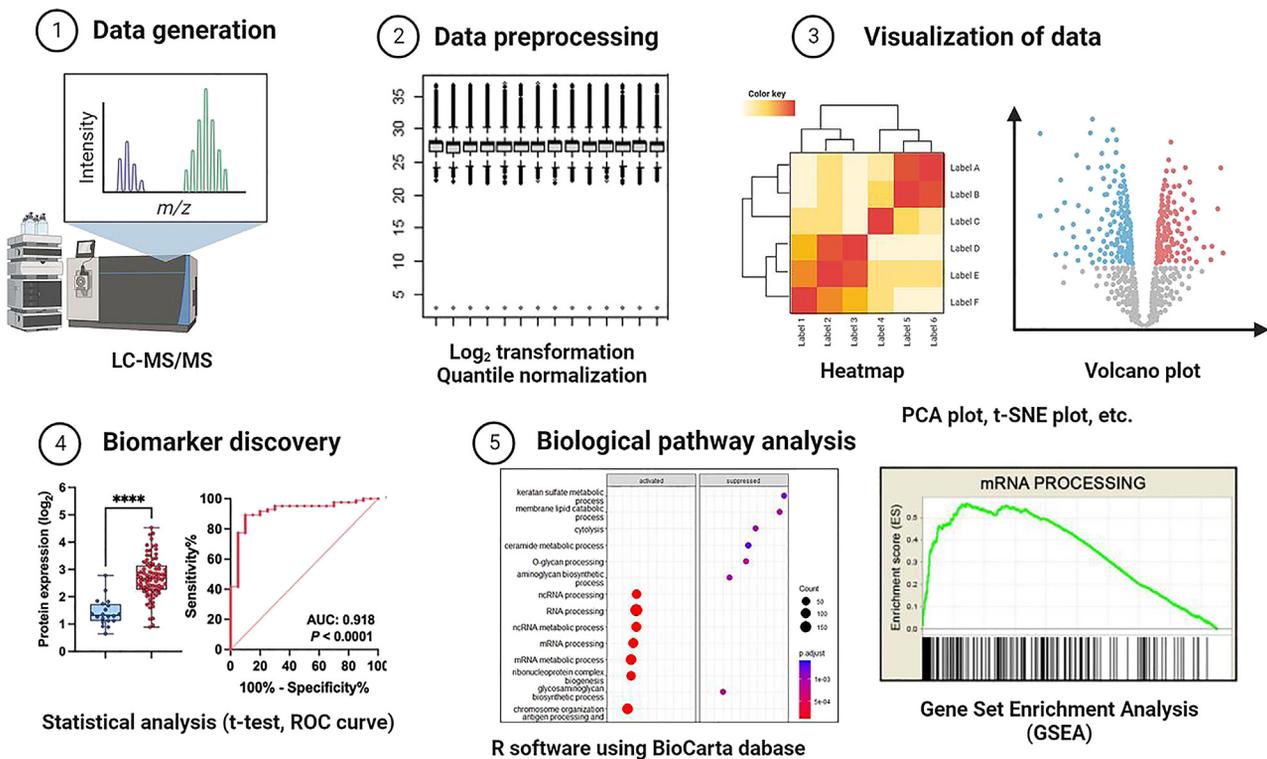
29. Calculate the relative abundance of a given protein.
  - a. Process high resolution/high mass accuracy nano-LC-MS/MS data using Proteome Discoverer 1.4.1.14/Mascot 2.5 software.
    - i. Using this software, calculate the relative abundance of a given protein from the average area of the three most intense peptide signals.

**Note:** This abundance measure has a range of approximately 4 orders of magnitude for this software, resulting in a lower signal range of  $0.8\text{--}1.2 \times 10^6$  that can be integrated for proteins of low abundance.

- ii. Proteins for which area intensities are below the minimum range or were not detected are assigned an area of zero.

**Alternatives:** Other search and quantitation algorithms can also be used for the data analysis,<sup>6</sup> e.g. MaxQuant.<sup>7</sup>

- b. Query human data against the UniProt's Complete HUMAN proteome (<https://www.uniprot.org/proteomes/UP000005640>).



**Figure 10. Step-by-step illustration of bioinformatic analysis of proteomic EVP data**

- c. Query mouse data against UniProt's Complete MOUSE proteome (<https://www.uniprot.org/proteomes/UP000000589>).
  - d. Use the following parameters: Enzyme: Trypsin/P, maximum allowed missed cleavage sites: 2, monoisotopic precursor mass tolerance: 10 ppm, monoisotopic fragment mass tolerance: 0.02 Da, dynamic modifications: Oxidation (M), Acetyl (Protein N-term), static modification: Carbamidomethyl (C).
  - e. Use Percolator to calculate peptide False Discovery Rates (FDR), which are calculated per file. An FDR of 1% is applied to each separate LC-MS/MS file.
  - f. For EVP-enriched samples that have been in contact with Fetal Bovine Serum (FBS, such as samples that originated from cell culture), concatenate a bovine-specific database to the human and mouse databases when querying the data to exclude bovine peptides.
  - g. For plasma and tissue samples, concatenate solely the sequences of porcine trypsin and Endopeptidase Lys-C to the human and mouse databases and use for exclusion.
30. Process proteomic data for further analysis.
- a. For the proteins identified by multiple UniProt IDs, collapse the probe (based on UniProt ID) values at the protein level using the probe with the maximum intensity.
  - b. Following Log<sub>2</sub> transformation, consider quantile normalization if observed changes across samples are due to unwanted technical variability.
  - c. Process the proteomic expression data using the 'Limma' package of the open-source R program (<https://www.r-project.org>).
    - i. Import and normalize proteomic expression data using the 'normalizeBetweenArrays' function (method = quantile).

**Note:** It is possible to verify the distribution of protein expression values, a total number of unique proteins or unsupervised clustering to determine potential variability among samples.

If these plots identify outlier samples or substantial deviations from other samples, or when the researchers analyze pooled samples from multiple experiments run at different times, quantile normalization is warranted.

31. Visualize proteomic data.
  - a. Use heatmaps and clustering for data visualization.
    - i. Generate a heatmap using the GENE-E software (<http://www.broadinstitute.org/software/gene-e>).

**Alternatives:** A heatmap can be generated using the open-source R program or other software, such as the Morpheus web-based tool (<https://software.broadinstitute.org/morpheus/>).

- b. Use principal component analysis (PCA) and three-dimensional t-Stochastic Neighbor Embedding (t-SNE) plots for data reduction, simplifying datasets to three dimensions for display purposes.
  - i. Conduct PCA using the 'princomp()' function with default options using the R program.
  - ii. Illustrate PCA results for proteomics data in a three-dimensional plot using the 'rgl' package and 'plot3d()' function.
  - iii. Apply the t-SNE algorithm to generate t-SNE plots using the 'Rtsne' R package.
- c. Conduct consensus clustering to assess whether proteomic expression differs between sample groups using the GENE-E software.

**Note:** Consensus clustering is a resampling-based method for class discovery and one of the unsupervised clustering methods.

**Alternatives:** Python, R, or MATLAB language provides useful packages for computational biology. We recommend generating PCA, t-SNE, hierarchical clustering, or other statistical analyses using these program languages.

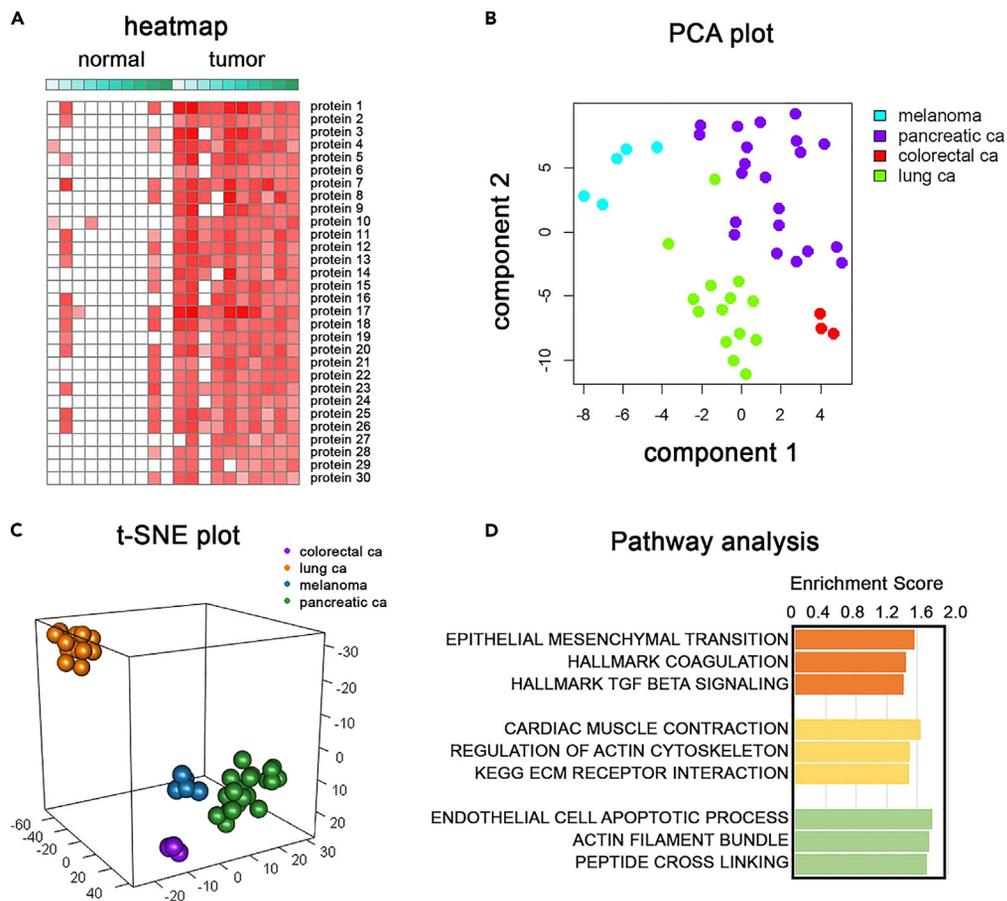
32. Perform biomarker selection and pathway analysis (Figure 11).
  - a. Conduct biomarker selection using the GENE-E software.
    - i. Sort proteins by signal-to-noise statistic,  $(\mu_A - \mu_B)/(\alpha_A + \alpha_B)$  where  $\mu$  and  $\alpha$  represent the mean and standard deviation of proteomic expression, respectively, for each class.<sup>8</sup>
    - ii. Use the signal to noise marker selection tool from GENE-E to identify fraction-specific markers with 1,000 permutations.
    - iii. To identify enriched proteins, apply a fold change cut-off of  $\geq 10$  to select tumor-specific markers (FDR < 0.05).

**Note:** We recommend setting criteria for marker selection. For example, the cutoff to select specific markers is a fold-change  $\geq 10$ , FDR < 0.05, and mean protein expression  $\geq 10^8$  with positivity in  $\geq 80\%$  of the corresponding sample type (i.e., at least eight out of ten samples).

- b. Apply the Random Forest algorithm to identify biomarkers differentiating sample groups (e.g., tumor versus non-tumor samples).

**Note:** Random Forest is a machine learning method that combines the output of an ensemble of regression trees to predict a class variable (e.g., tumor or non-tumor class). This method reduces the risk of over-fitting and makes the method robust to outliers and noise in the input data.

- i. Use Recursive Feature Elimination (RFE) provided by the caret R package for feature selection using default options with ten-fold cross-validation.



**Figure 11. Visualization and pathway analysis of proteomics data**

- (A) An example heatmap is based on proteins found > 50% of pancreatic tumor tissues at expression levels  $\geq 10$ -fold higher in tumor tissues than adjacent tissues (FDR < 0.05). Colored boxes identify sample pairs.
- (B) Principal component analysis (PCA) of proteomic mass spectrometry data of tissue-derived extracellular vesicles and particles (EVP) from melanoma, pancreatic cancer, colorectal cancer, and lung cancer.
- (C) t-SNE plots of proteomic mass spectrometry data of tissue-derived EVPs.
- (D) Gene Set Enrichment Analysis (GSEA) based on the proteomic dataset for pancreatic cancer tissue-derived EVPs.

- ii. Determine the minimal number of top proteins with the best accuracy according to the variable importance measure.
- c. Use Gene Set Enrichment Analysis (GSEA) to identify significant biological functions or pathways related to identified proteins.

**Note:** GSEA ranks all proteins according to their differential expression levels by signal-to-noise statistic,  $(\mu_A - \mu_B)/(\alpha_A + \alpha_B)$  where  $\mu$  and  $\alpha$  represent the mean and standard deviation of proteomic expression, respectively, for each class. GSEA calculates the Kolmogorov-Smirnov statistic to evaluate whether proteins from a pre-determined pathway are significantly overrepresented towards the top or bottom of the ranked gene list.

**Note:** GSEA statistics include enrichment score, normalized enrichment score, false discovery rate, and nominal *P* value (<http://www.gsea-msigdb.org/gsea/doc/GSEAUUserGuideFrame.html>).

**Optional:** Given the lack of coherence in the majority of expression datasets and the limited number of gene sets being evaluated, an FDR threshold of 25% is often adequate. If the researcher utilizes gene set permutation rather than phenotypic permutation, the researcher can use a higher FDR threshold, such as 5%.

- d. Use Gene sets from the Molecular signatures database (MSigDB, <http://www.broadinstitute.org/gsea/msigdb/>) for GSEA (e.g., H: hallmark gene sets; C2:KEGG: canonical pathways from Kyoto Encyclopedia of Genes and Genomes [KEGG] pathway database; C2:Reactome: canonical pathways from the Reactome pathway database; C5: gene sets based on Gene Ontology [GO] term).

**Alternatives:** We recommend comparing between different biological pathway analysis tools, such as DAVID (The Database for Annotation, Visualization and Integrated Discovery; <https://david.ncifcrf.gov/>), a free resource or the commercial Ingenuity Pathway Analysis (QIAGEN IPA; <https://analysis.ingenuity.com/>).

### ExoView analysis

⌚ Timing: 24 h

This protocol provides step-by-step instructions for performing single EVP analysis using the ExoView R100 platform (Unchained Labs). ExoView allows the detection of specific proteins on/within single EVPs using antibodies specific for these proteins (Figure 12). The basic kit includes anti-CD63, -CD9, and -CD81 antibodies but other antibodies of interest can be used to capture EVPs and/or detect antigen presence on/in single EVPs.

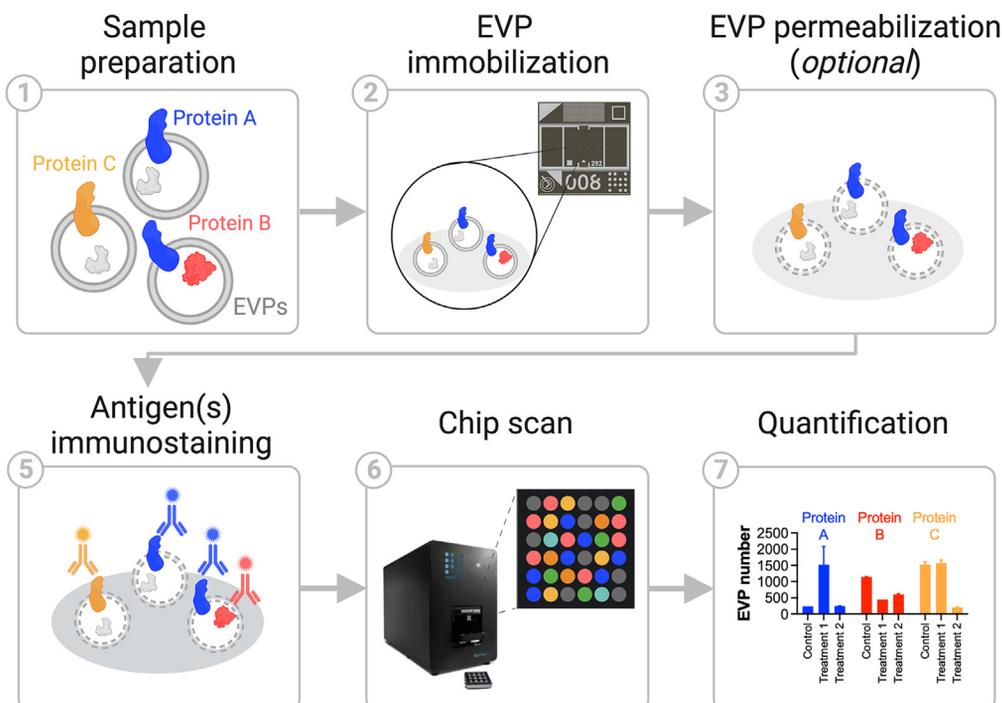


Figure 12. Step-by-step protocol for ExoView analysis

△ **CRITICAL:** For all steps, use tweezers to handle chips. Be careful not to touch the center square of the chip (Figure 13 left, red box) to avoid damaging the antibodies on the surface.

**Note:** The sample should be prepared at different concentrations to find the optimal dilution for each sample type. Samples can be prepared from  $-80^{\circ}\text{C}$  stored aliquots. Table 2 shows examples of recommended sample dilution used by the authors as a reference. Further optimization is needed for other sample types and sources.

### 33. EVP capture:

- To prevent samples from drying, fill the space between the wells (marked in red) of the 24-well plate with  $\text{dH}_2\text{O}$  and use only inner wells (Figure 14).
- Dilute EVP samples in solution A (please see Table 2 for reference).
- Place pre-scanned chips in the inner wells of a 24-well plate. To maintain surface tension of the liquid on the chip at the next steps, leave some space between the corners of the chip and the wall of the well.

△ **CRITICAL:** Chips should not be placed in the outer wells to prevent them from drying (yellow x in Figure 14).

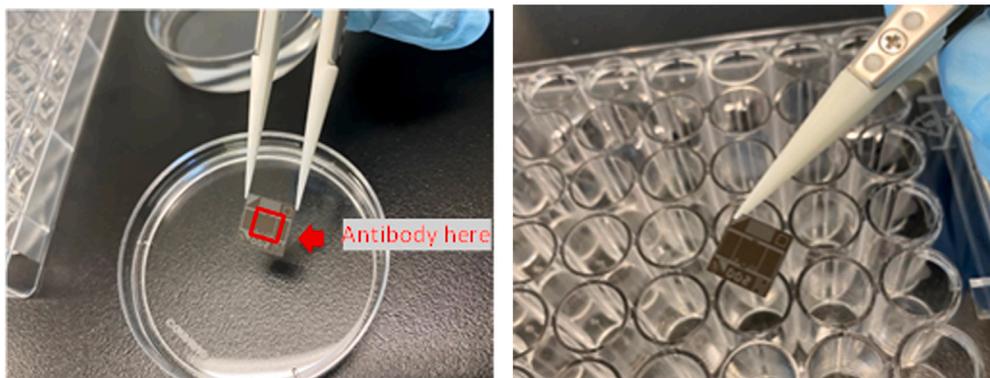
- Carefully load  $35\ \mu\text{L}$  of diluted sample onto the central square of the chip. To prevent damage, avoid touching the chip surface with the pipette tip.

△ **CRITICAL:** Check that the sample is retained on the chip by surface tension (as shown in Figure 15).

- Incubate overnight (16 h) at room temperature ( $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ ). Place a lid and aluminum foil on the plate to protect the samples from light. Avoid contact or movement until the next step.

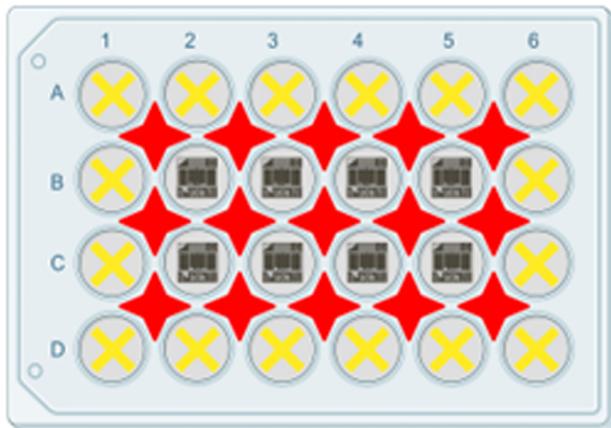
### 34. Immuno-fluorescence staining:

- Add  $1000\ \mu\text{L}$  of solution A to each well containing a chip.
- Place the 24-well plate on an orbital shaker and shake at 500 RPM for 3 min at room temperature ( $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ ).
- Remove  $750\ \mu\text{L}$  of solution A from the well and add  $750\ \mu\text{L}$  of fresh solution A. Be careful not to touch the chip surface with the pipette tip.
- Shake the 24-plate at 500 RPM for 3 min at room temperature ( $20^{\circ}\text{C}$ – $22^{\circ}\text{C}$ ).
- Repeat steps 34c and 34d two more times for a total of 3 washes.



**Figure 13. Chip should be picked up only by touching their edges**

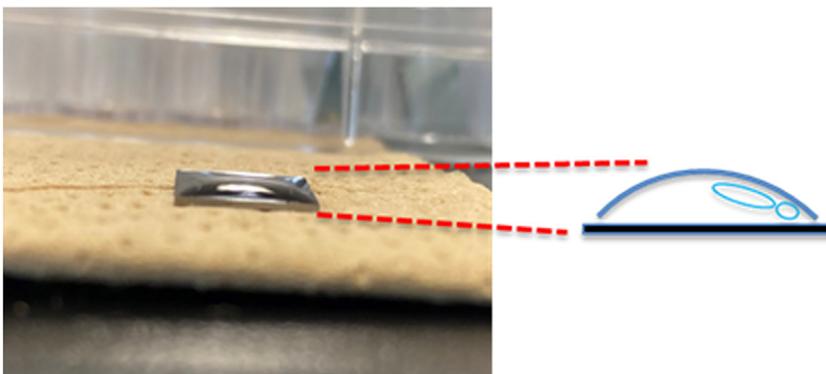
Chips should be handled with extreme care in order not to damage the antibodies on the surface of the center square. Pictures are examples of acceptable methods of handling a chip.



**Figure 14. Example of plate layout for EVP incubation with ExoView chips**

Fill the space shown in red with dH<sub>2</sub>O and avoid using the outer wells.

- f. After the last wash, remove 750  $\mu$ L of solution A.
  - g. Add 250  $\mu$ L of antibody mixture (prepared in the “before you begin” section) to each well. The well already contains 250  $\mu$ L of solution A, so the final volume is 500  $\mu$ L and final antibody concentration is 0.1  $\mu$ g/mL. Pipette solution gently to mix.
  - h. Place a lid on the 24-well plate and wrap in aluminum foil to protect from light, then incubate 1 h at room temperature (20°C–22°C) without shaking.
35. Chip washing:
- a. Add 500  $\mu$ L of solution A to each well and immediately remove 750  $\mu$ L, then add 750  $\mu$ L of fresh solution A.
  - b. Shake the plate at 500 RPM for 3 min at room temperature (20°C–22°C).
  - c. Remove 750  $\mu$ L of solution A and add 750  $\mu$ L of solution B.
  - d. Shake the plate at 500 RPM for 3 min at room temperature (20°C–22°C).
  - e. Repeat 35c and 35d two more times for a total of 3 times.
  - f. Remove 750  $\mu$ L, add 750  $\mu$ L of Milli-Q, and shake the plate at 500 RPM for 3 min at room temperature (20°C–22°C).
  - g. While shaking, fill three 60 cm cell culture dishes with Milli-Q water.
  - h. Wash chips in 60 mm cell culture dishes.
    - i. Transfer one chip to the first dish using tweezers.



**Figure 15. Example of how the sample should be placed on the chip**

Seen from the side, the sample sits on the surface of the chip.

**Note:** Use flat tip tweezers to move chips. In order to pick up a chip, push the chip to the wall and lift up from one side. To transfer the chip, pick it up by holding the sides of the chip and be careful not to touch the chip surface.

- ii. Put the chip in deionized water horizontally and shake gently with tweezers several times (Figure 16).

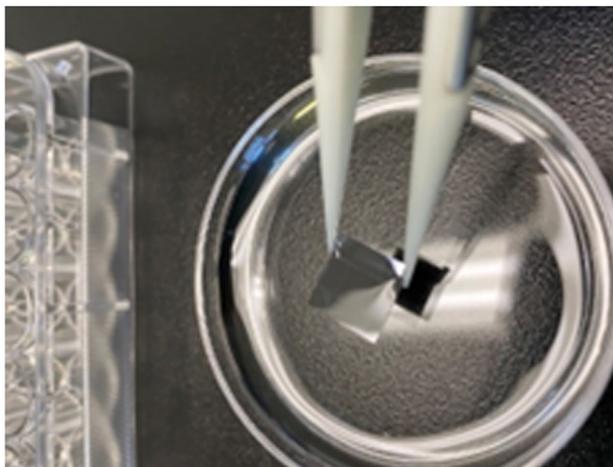
**Note:** Do not allow the chip surface to dry during the washing steps. Ensure that the chip surface is coated with water when moving from one wash to the other (Figure 16).

△ **CRITICAL:** Ensure the chips are always facing up during the washing steps.

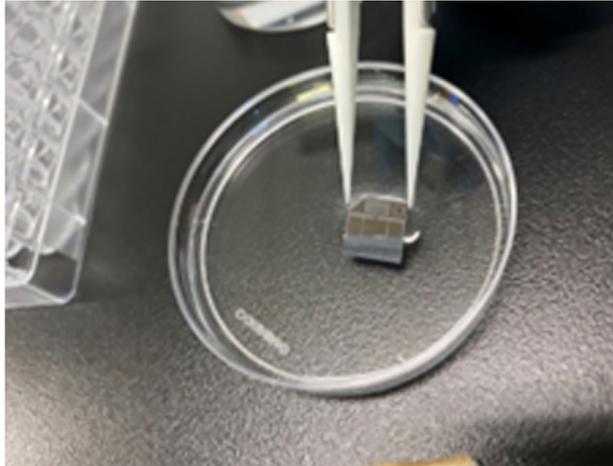
- iii. Repeat 35h.i-ii in the second 6-cm dish.
- iv. Repeat 35h.i-ii in the third 6-cm dish.
- v. Dry the chip by lifting the chip at a 45° angle from the Milli-Q water (Figure 17). Place the chip on paper, facing up, until dry. The chip ID side should be facing up and visible.

△ **CRITICAL:** To avoid scan failure, dry the chips completely without damaging the surface.

36. Chip scan:
  - a. Initiate the ExoScan 2.5.5 acquisition software. Plug the USB supplied with the ExoView kit into the computer. Choose the corresponding chip ID number for the chip to be scanned.
  - b. Place the chip on the stage and start scanning.
37. Acquisition analysis:
  - a. Initiate the ExoViewer 2.5.0 software.
  - b. Set sizing threshold to 50–200 nm diameter.
  - c. Acquire the chip.
  - d. Go over each spot to confirm that they were not damaged during the previous steps and choose the spots to be analyzed by the software. See [problem 5](#) in [troubleshooting](#) section.
  - e. Export the “aggregate” results to an Excel file with particle number, intensity of staining for each of the antibodies, and size of positive EVPs.
  - f. To quantify the number of EVP particles, subtract the baseline (mouse IgG; MIgG) measurement to reduce noise.



**Figure 16.** Moving a chip from one wash to the next



**Figure 17.** Pulling out a chip

### Western blot

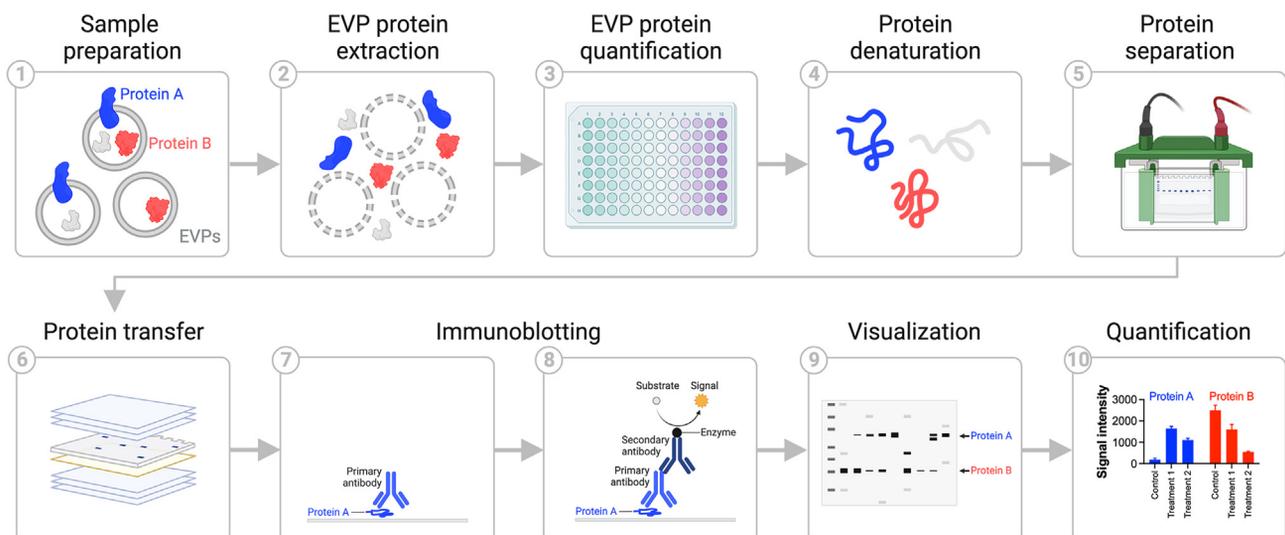
This protocol provides step-by-step instructions for performing western blot analysis of EVP isolated from cell lines, tissue explants, or plasma (Figure 18).

### EVP protein sample preparation

⌚ Timing: 1 h

EVP samples are processed for protein isolation and preparation for western blot analysis.

38. Transfer the necessary amount of EVPs (isolated according to Figure 1, step ②) into new Eppendorf tubes and keep them on ice.
39. Add an equal volume of Lysis buffer to all EVP samples run on the same gel, mix well, place the Eppendorf tube on an orbital shaker and incubate for 30 min at 4°C.



**Figure 18.** Diagram showing the main steps of EVP western blotting

**Note:** Depending on the EVP sample concentration, the amount of lysis buffer to be added may vary. In some cases, lysis buffer addition may not be necessary. As shown in the next steps, either 5  $\mu\text{g}$  or 20  $\mu\text{g}$  of EVP sample will be loaded per well. If the EVP sample concentration is sufficiently high, and thus it is possible to add loading buffer without reaching the maximum loading volume, then adding lysis buffer is recommended. Otherwise directly mix the EVP sample with 4X or 2X sample loading buffer. See [problem 6](#) in [troubleshooting](#) section.

**Note:** Lysis buffer should be kept cold on ice or at 4°C and should be prepared and used immediately (within minutes). Loading buffer should be prepared at room temperature (20°C–22°C) and used within minutes, or stored at –20°C for up to 2 months.

40. Perform BCA assay in a microplate according to manufacturer's instructions for limited sample volumes to measure the protein concentration (BCA assay manual).
41. Transfer 5  $\mu\text{g}$  (for cell line and tissue-derived EVPs) or 20  $\mu\text{g}$  (for plasma-derived EVPs) of EVP protein extracts into new 1.5 mL Eppendorf tubes and keep them on ice.
42. Add loading buffer stock solution (4X or 2X) to the EVP samples to a final concentration of 1X. Place samples in a heat block at 100°C for 5 min.
43. Spin down the samples for 1 min at 12000  $\times g$  at room temperature (20°C–22°C) and load the supernatant into the wells of the commercial polyacrylamide gel.

**Note:** If the protein concentration of the original EVP sample is low, it is possible to skip steps 41–42, and dilute the sample with an equal volume of 4X or 2X Loading buffer. See [problem 7](#) in [troubleshooting](#) section.

### Western blot analysis

⌚ Timing: 24 h

EVP samples are loaded into an electrophoresis gel to separate proteins based on size, proteins are transferred to a PVDF membrane, and specific EVP proteins are detected by immunoblotting.

44. Place a gradient Tris-glycine gel in the electrophoresis tank and add Running buffer up to the top of the gel.
  - a. Rinse the wells with running buffer using a clean loading tip or syringe.

**Note:** Running buffer can be stored at 4°C for up to 2 months, or aliquoted and stored at –20°C for up to a year.

**Note:** The separation range of protein to resolve determines the polyacrylamide concentration to be used. We recommend using a 4%–12% Tris-glycine gel for proteins ranging between 20 and 300 kDa and a 4%–20% Tris-glycine gel for proteins ranging between 5 and 300 kDa.

45. Load wells. For ladder use 5  $\mu\text{L}$  Rainbow protein marker in a separate lane.
46. Run gel for 30 min at 100 V or until bands are stacked, followed by 60 min at 150 V, or until the blue running dye reaches the bottom of the gel.
47. While the gel is running, prepare the PVDF membrane and transfer buffer.

**Note:** Transfer buffer should be placed at 4°C for at least 1 h before use. It can be stored at 4°C for up to a week.

**Note:** When using PVDF membranes, place one membrane in a clean, empty box with a few milliliters of methanol for 10 seconds to activate. Rinse several times with transfer buffer and then rock gently on a shaker at room temperature (20°C–22°C) for at least 2–3 minutes.

48. Soak 2 sponges and 2 rectangles of filter paper in cold transfer buffer.

**Note:** Filter paper needs to have a surface larger than the membrane.

49. Take the gel out of tank, crack open plastic case and cut off the wells and bottom so that the remaining gel is completely flat.

50. Build transfer sandwich from the bottom up in the order depicted in (Figure 19).

51. Insert the sandwich into the gel transfer tank.

**Note:** When building the sandwich, remove all bubbles between the gel and the PVDF membrane by gently rolling a pipette or 15–50 mL tube over the top filter paper.

52. Use fresh transfer buffer to fill in the transfer tank from the top and put on the lid.

a. Run 1 h at 100 V at 4°C in the cold room and/or in a bucket filled with ice.

**Alternatives:** The transfer can be performed overnight (16–18 hours) at 30–40 V at 4°C in the cold room.

53. When the transfer is done, turn off the power source, pull out the sandwich, and unpack it carefully.

a. Place the PVDF membrane, protein side up, in a box containing 5–10 mL blocking buffer (as much as necessary to cover membrane completely).

b. Place on a rocker at room temperature (20°C–22°C) or in the cold room (4°C) for least 1 h.

**Note:** Blocking buffer can be stored at 4°C for up to a month or at –20°C for up to a year.

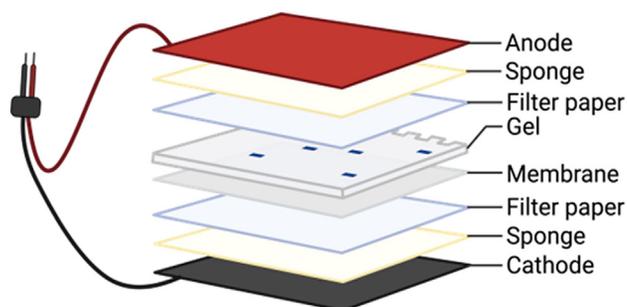
**△ CRITICAL:** Ensure the membrane does not dry out throughout the process, if the incubation box does not have a lid, cover with Saran wrap.

**Note:** Ponceau staining may be done before the blocking step.

54. After 1 h, pour off the blocking buffer and add 5–10 mL primary antibody diluted in blocking buffer to the appropriate concentration.

**Note:** For example, if working with human plasma, the monoclonal mouse anti-human CD9 (clone MM2/57) or monoclonal mouse anti-human syntenin-1 (S-31) can be used at a concentration of 0.1 µg/mL, respectively, as loading controls.

55. Incubate overnight (16–18 h) on a rocking platform in the cold room (4°C).



**Figure 19.** Diagram showing the assembly order of the western blot transfer stack

**Note:** The antibody can normally be re-used (<5 times), especially if containing sodium azide.

56. The following day, pour off the primary antibody, and wash three times, 10 min each, with washing buffer, on a rocking platform, in the cold room (4°C). Discard the washing buffer.
57. Add 5–10 mL HRP-conjugated secondary antibody diluted 1:10000 in washing buffer to the appropriate concentration and incubate at room temperature (20°C–22°C) or in the cold room (4°C), with rocking, for 1–1.5 h.
58. Pour off the secondary antibody, and wash three times, 10 min each, with washing buffer on a rocking platform at room temperature (20°C–22°C) or in the cold room (4°C).
59. During the last wash, prepare the ECL (enhanced chemiluminescence) reagent by mixing equal volumes of each component of HRP substrate (ECL reagent A and B).

**Note:** The washing buffer can be stored at room temperature (20°C–22°C) for up to a week.

**Note:** The total volume of HRP substrate needed depends on the size of the membrane and the number of membranes. The minimum volume is 1 mL A and 1 mL B for one membrane (equivalent for mini gels, i.e., XP04202BOX, Life Technologies).

60. Pour off the washing buffer and add the ECL mix to the membrane.
  - a. Incubate for 1 min (this step does not have to be in the dark), then remove the ECL mix and transfer the membrane to an autoradiography cassette, or alternatively a ChemiDoc station.
61. Develop the autoradiography film in the dark room, determining the optimal exposure time empirically (5 s - 5 min) in the dark room.
62. In order to quantify the individual bands corresponding to proteins of interest, the autoradiography films are acquired using a transparency scanner (scanner with light source on both sides of the bed) with high resolution (600 dpi) in TIFF file format.
  - a. Subsequently, using the software ImageJ, each band is framed as single region of interest and quantified.
  - b. After removing background values, the final relative quantification values are the ratio of net band to net loading control.

### ExoELISA

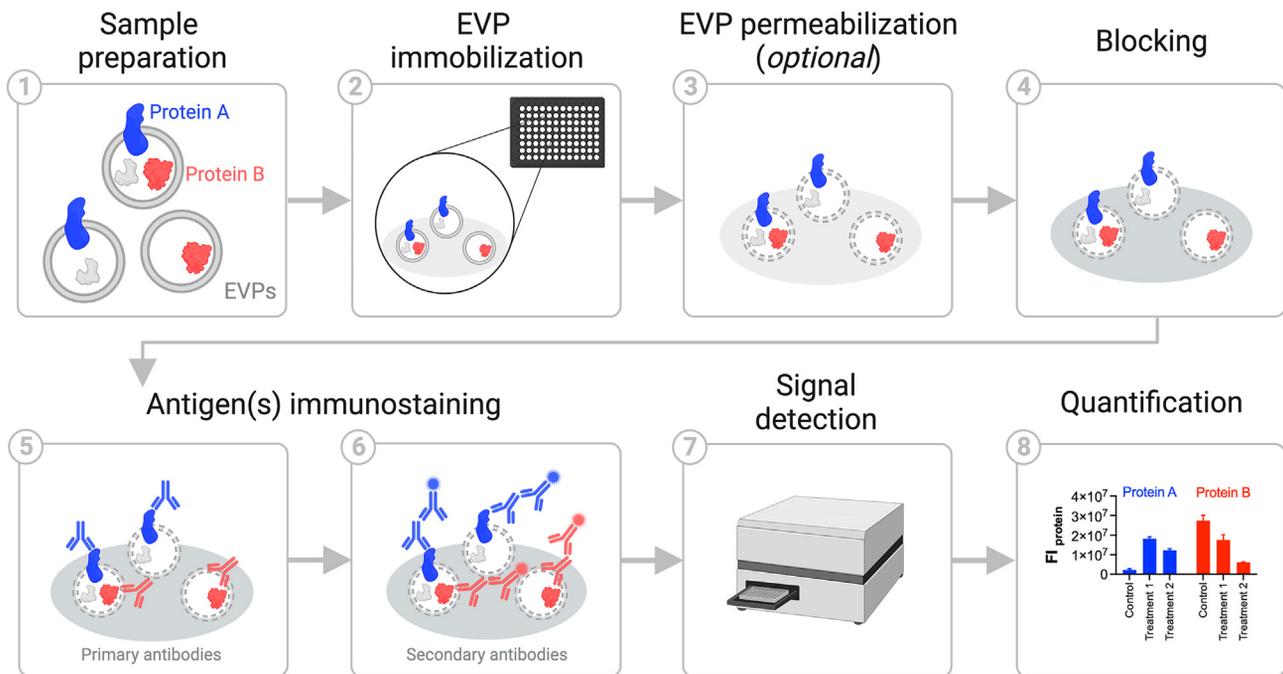
⌚ Timing: 30–48 h

This protocol describes the steps required to perform an “in-house” indirect ELISA (enzyme-linked immunosorbent assay) on EVP proteins (ExoELISA). This method can be used to detect proteins present on the surface or packaged inside EVPs isolated from different sources (including cell culture supernatants, tissue explants, and plasma) when a commercial ELISA kit is not available. [Figure 20](#) depicts the major steps of the protocol: sample preparation (1); EVP immobilization (2), EVP permeabilization (3) and blocking (4); antigen(s) immunostaining (5–6); signal detection (7); quantification (8).

63. Isolate EVPs via ultracentrifugation and measure total protein content by BCA assay as per manufacturer’s instructions (BCA assay manual).

**Note:** EVPs from different biological samples have been successfully measured using this ExoELISA protocol,<sup>1</sup> including EVPs from cell culture conditioned media, tissue explant conditioned media and plasma. Samples from Step ① in [Figure 1](#) (after 12,000 xg spin) can also be used. After isolation, EVPs can be aliquoted and stored at –80°C prior to use for the ExoELISA assay.

64. Sample preparation: dilute EVPs at a concentration of 10–30 µg/mL in coating buffer.



**Figure 20.** Diagram depicting the step-by-step ExoELISA protocol

- For EVP samples with a concentration higher than  $50 \mu\text{g/mL}$ , dilute EVPs in 1x coating buffer to obtain a  $10\text{--}30 \mu\text{g/mL}$  suspension.
- For EVP samples with a concentration lower than  $50 \mu\text{g/mL}$ , including unfractionated plasma and culture supernatant, dilute EVPs at a 1:1 ratio with 2x coating buffer to obtain a  $10\text{--}30 \mu\text{g/mL}$  suspension.

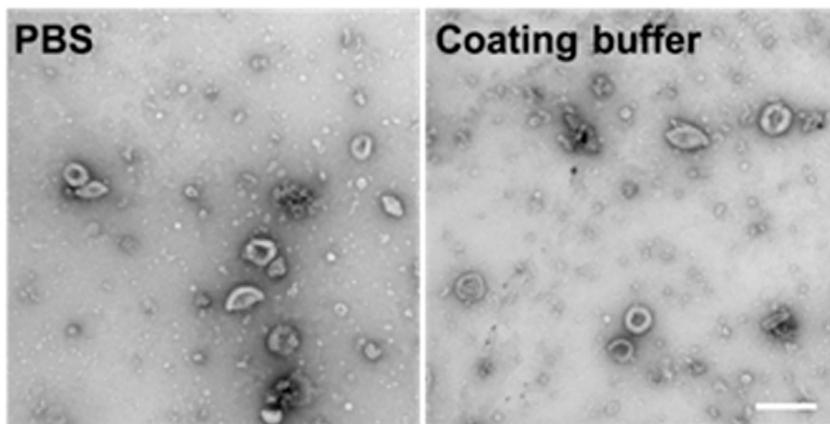
**Note:** ExoELISA coating buffers can be stored at room temperature ( $20^\circ\text{C}\text{--}22^\circ\text{C}$ ) for up to 12 months.

**Note:** The incubation of EVPs with coating buffer does not alter EVP structure, as shown by TEM analysis (Figure 21).

**△ CRITICAL:** The EVP concentration required for ExoELISA measurement can vary according to the sample type, concentration of the target protein per vesicle, presence of contaminants, and EVP dispersion in the EVP suspension. The ideal EVP concentration for immobilization needs to be empirically determined by preparing serial dilutions of the EVP suspension and analyzing the final signal to noise ratio.

- Plate coating (EVP capture):
  - Pipet  $50 \mu\text{L}$  of the EVP suspension (sample) and coating buffer (blank) into the wells of a 96-well plate, ensuring the solution covers the bottom of the well. Each sample should be assayed in triplicate (two wells for protein-specific antibody(ies) [Ab] and at least one well for isotype control(s) [Is. ct]). Use the template in Table 3 to design the sample arrangements in the microwells.

**Note:** Always use assay microplates with flat bottom wells. In addition to plate coating with proteins, polystyrene cell-culture treated plates with slight hydrophilic properties can also be employed for the immobilization of EVPs. The choice of the assay microplate color



**Figure 21. TEM images of EVPs incubated overnight with PBS versus 1x coating buffer**  
Scale bar: 200 nm.

depends on the type of secondary antibody and the nature of the detected signal. Black plates with clear bottom should be used to measure fluorescence signals, white or clear plates should be used to measure colorimetric signals, and white plates should be used to measure chemiluminescent signals.

- b. Incubate the plate overnight (12–20 h) at 4°C, in the dark without shaking.
- c. Remove unbound EVPs by washing 3 times in PBS.
  - i. Carefully aspirate all supernatant from each well.
  - ii. Slowly pipette 100  $\mu$ L of sterile PBS per well, using a multichannel pipettor.
  - iii. Incubate 5 min at room temperature (20°C–22°C).
  - iv. Carefully aspirate the PBS.
  - v. Repeat steps 65c.i–iii two times for a total of 3 washes.

**Note:** We recommend testing the efficiency of EVP plate coating before performing the entire ExoELISA assay. To do this, label the EVP membrane with a lipophilic dye (PKH26, CellVue etc. according to manufacturer's instructions) prior to immobilization, and acquire fluorescent signals (using a plate reader or microscope) after the first wash. Examples of fluorescently-labeled EVPs bound to the multiwell plate are shown in Figure 22.

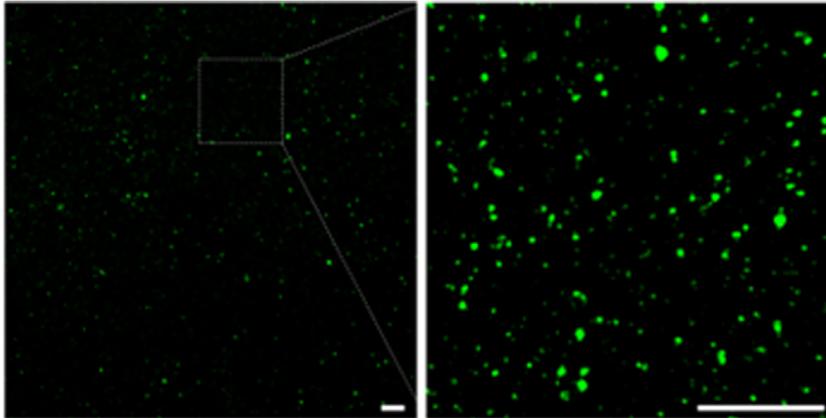
**△ CRITICAL:** All aspiration and pipetting steps for this step and following steps should be performed with extreme care, ensuring not to scratch the surface of the well. We suggest equilibrating the pipet tip with reagent before loading and to pipet buffers slowly onto the wall of the well, allowing liquid to flow to the bottom of the well.

**Alternatives:** This protocol describes how to measure EVP proteins qualitatively, allowing the comparison of their relative levels (expressed as FI, a.u., or RLU) between two or more

**Table 3. Example of ExoELISA plate layout, related to Step 65**

	1	2	3	4	5
A	Blank	Sample 1 <sub>Is. ct</sub>	Sample 1 <sub>Ab</sub> (1)	Sample 1 <sub>Ab</sub> (2)	Sample 1 <sub>Ab</sub> (3) (recommended)
B	Blank	Sample 2 <sub>Is. ct</sub>	Sample 2 <sub>Ab</sub> (1)	Sample 2 <sub>Ab</sub> (2)	Sample 2 <sub>Ab</sub> (3) (recommended)
C	Blank	Sample 3 <sub>Is. ct</sub>	Sample 3 <sub>Ab</sub> (1)	Sample 3 <sub>Ab</sub> (2)	Sample 3 <sub>Ab</sub> (3) (recommended)
D	Blank	Sample 4 <sub>Is. ct</sub>	Sample 4 <sub>Ab</sub> (1)	Sample 4 <sub>Ab</sub> (2)	Sample 4 <sub>Ab</sub> (3) (recommended)
E	...	...	...	...	...

Note that each EVP sample needs to be run in a minimum of two replicates to allow the incubation with protein-specific antibodies (Ab) and their isotype controls (Is. ct). More than one protein can be assayed in the same Sample<sub>Ab</sub> well.



**Figure 22. Epifluorescence image and magnification of fluorescently-labeled EVPs (PKH67, green) immobilized on the surface of a microwell and washed with PBS**

EVPs are passively adsorbed onto microwells via an alkaline bicarbonate coating buffer, allowing the formation of hydrophobic interactions between the EVP proteins and plastic. Scale bar: 50  $\mu\text{m}$ .

experimental groups. For a quantitative ELISA, a set of protein standards should also be prepared and immobilized following steps 64–65. The range of standard concentrations may vary according to the level of protein expression in the EVP sample. A range between 10 and 10000  $\text{pg/mL}$  will cover most EVP protein concentrations, but a narrower range can be empirically determined. At least 7 serial dilutions of the protein standard should be prepared. For quantitative ExoELISA where standards are also immobilized, ELISA microplates with stronger hydrophilic properties could be utilized (Corning 96-well Flat Clear Bottom Black Polystyrene TC-treated Microplates, with Lid, Sterile, Corning #3904).

66. (Optional) EVP permeabilization, to expose internal proteins:
  - a. Aspirate PBS from each well.
  - b. Pipet 100  $\mu\text{L}$  of 0.2% Triton-X in TBS into each well.
  - c. Incubate 5 min at room temperature (20°C–22°C).
  - d. Wash two times with PBS (2  $\times$  5 min incubation at room temperature (20°C–22°C)).

**Note:** The permeabilization step is only necessary if internal EVP proteins are to be detected. Surface proteins can be detected without the permeabilization step.

67. Blocking, to avoid non-specific antibody binding:
  - a. Aspirate PBS from each well.
  - b. Pipet 100  $\mu\text{L}$  of TNB blocking buffer in each well.
  - c. Incubate 1 h at room temperature (20°C–22°C) in the dark.

**Note:** TNB blocking buffer is stable for several months (<4 months) when stored at  $-20^\circ\text{C}$ . Bring the buffer back to room temperature (20°C–22°C) before use.

68. Antigen(s) immunostaining:
  - a. Aspirate TNB buffer from each well.
  - b. Without washing the plate, pipet 30  $\mu\text{L}$  of the primary antibody/isotype control solution into each well. The primary antibody should be diluted 1:50–1:200 in TNB buffer.

**Note:** Each EVP sample should be labeled with the primary antibody/ies in duplicate and their isotype control(s) as a single replicate. The isotype control serves as internal control for non-specific background signal.

c. Incubate the plate overnight (12–20 h) at 4°C in the dark.

**Note:** More than one antigen/antibody can be assayed at the same time providing that the detecting primary antibodies are raised in different hosts and that the detection equipment has 2 channels. Due to the small size of EVPs, we caution against using more than 2 different primary antibodies in the same assay to avoid steric hindrance between antibodies.

d. Wash 3 times with PBS (3 × 5 min incubation at room temperature (20°C–22°C)).

e. Pipet 30 μL of the secondary antibody solution in each well. The secondary antibody should be diluted 1:200–1:500 in TNB buffer.

f. Incubate the plate for 2 h at room temperature (20°C–22°C) in the dark.

g. Aspirate the secondary antibody solution.

h. Wash 3 times with PBS (3 × 5 min incubation at room temperature (20°C–22°C)).

**Note:** Secondary antibodies coupled with different fluorophores and with different species reactivity can be used to recognize multiple primary antibodies in the same assay.

69. Signal detection:

a. Aspirate the PBS from each well.

b. Pipet 200 μL of PBS in each well.

c. Acquire the plate using a plate reader equipped with fluorescence module or a fluorescence imaging system (e.g., Odyssey, Licor). The plate lid should be removed prior to reading. Acquisition parameters should be set as follows: bottom reading; integration time = 1 s; PMT sensitivity = Auto (unless one is comparing different plates); no shaking.

**Alternatives:** As alternatives to fluorophore-conjugated secondary antibodies, other types of secondary antibodies can be used, such as enzyme-linked antibodies (e.g., HRP or AP). In this case, the detection of antibody density can be assessed upon incubation with colorimetric substrates (e.g., TMB for HRP) or chemiluminescent substrates (e.g., luminol for HRP), followed by absorbance (a.u.) or luminescence (RFU) reading, respectively, using a plate reader equipped with absorbance and luminescence modules. For absorbance and luminescence reading, the plate can be gently shaken before acquisition and top reading should be selected.

70. Quantification: for each EVP sample replicate, calculate the protein density based on their fluorescence intensity (FI) measurement using the formula:

$$FI_{\text{protein}} = FI_{\text{Ab}} - FI_{\text{Is. ct}} - FI_{\text{Blank}}$$

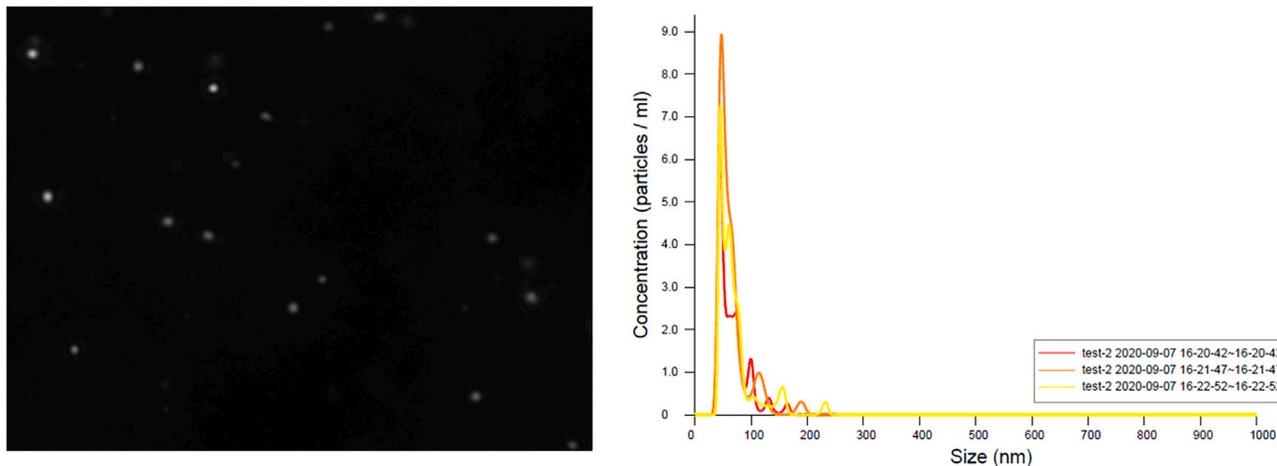
**Note:** If  $FI_{\text{Ab}} < FI_{\text{Is. ct}}$ , then the protein is not present in the samples and the  $FI_{\text{protein}}$  corresponds to 0. A similar calculation applies for signals measured as a.u. or RFU.

**Note:** If protein standards are used, the concentration of the assayed protein can be calculated by plotting the mean FI of standards (y, blank subtracted) and the standard concentration (x), generating the standard curve through a multiparametric curve-fitting model (e.g. four-parameter logistic fit), and then extrapolating sample concentration from their  $FI_{\text{protein}}$  intensity (calculated as indicated above).

## EXPECTED OUTCOMES

### NTA (NanoSight)

NanoSight analysis is used to measure particle size and concentration. Each analysis will generate a datasheet and a movie showing detected particles, their number and size distribution (see [Figure 23](#), [Methods video S1](#), [S2](#), [S3](#), [S4](#), and [S5](#); [Table 4](#) for representative data).



**Figure 23. Example of a caption of EVPs flowing in the NanoSight (left) and an example of the graph of EVP size and count printed in the datasheet (right)**

Methods video [S1](#), [S2](#), [S3](#), [S4](#), and [S5](#). Example videos of NTA, related to step 3.

### TEM

TEM is used to visualize and measure EVP size and morphology. Currently, TEM is the gold standard for confirming EVP isolation (see examples in [Figure 24](#)).

### LC-MS/MS

Expect identification of several hundreds of proteins ([Figure 25](#)). However, depending on your chromatography, mass spectrometer performance and sample composition, one could identify more or fewer proteins.

### Quantification and bioinformatic analysis

The authors used the MASCOT search engine, through Proteome Discoverer 1.4, but other search tools, such as MaxQuant, may also be used. Spectra should be queried against the appropriate organism (may be downloaded in FASTA format from [uniprot.org](http://uniprot.org)) using a sufficiently stringent false discovery rate (the standard is 1%). Include proper contaminant database in FASTA format. If the sample originated from cell culture and has been in contact with media such as FBS, make sure to include common bovine contaminant.<sup>9</sup> If the sample originated from tissue or blood, include only the added proteolytic enzymes as contaminants.

Based on several hundred proteins identified by LC-MS/MS, in any given sample, we could define which proteins are highly expressed in a subset of samples and obtain biological insights based on protein expression profiles.

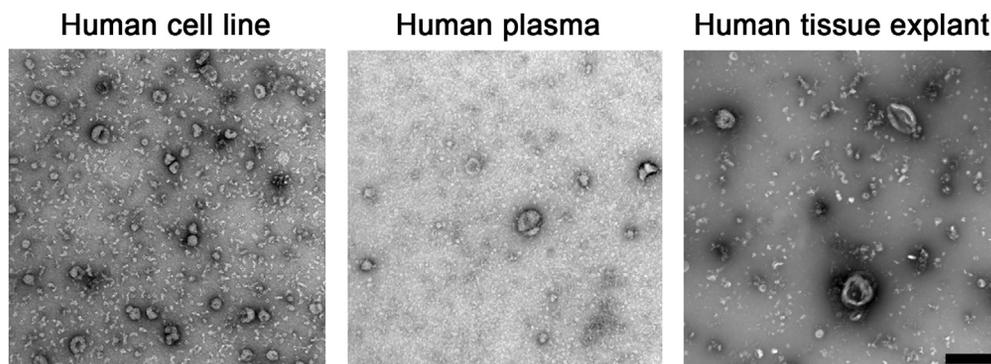
### ExoView analysis

ExoView analysis allows the detection and quantification of proteins of interest on/in single EVPs. EVPs are first captured by either CD63, CD9, or CD81 antibodies printed onto chips, therefore single EVPs containing one or more of these proteins are ultimately detected. The binding of antibodies to

**Table 4. Examples of typical NTA results, related to Expected Outcomes – NTA**

EVP* source	Input	Dilution	Concentration (particles/mL, diluted sample)	Size (nm)
Bodily fluid (plasma)	2–5 $\mu$ L	1:400	$1.58 \times 10^{11} \pm 1.11 \times 10^{10}$	$89.5 \pm 38.5$ nm
Cell line (B16F10)	10 $\mu$ L	1 $\mu$ g/mL	$4.18 \times 10^8 \pm 3.48 \times 10^6$	$116.3 \pm 3$ nm
Tissue explant (lungs, dissected)	50 $\mu$ L	1 $\mu$ g/mL	$7.91 \times 10^8 \pm 3.85 \times 10^7$	$108.2 \pm 0.6$ nm

\*EVP, extracellular vesicle and particle

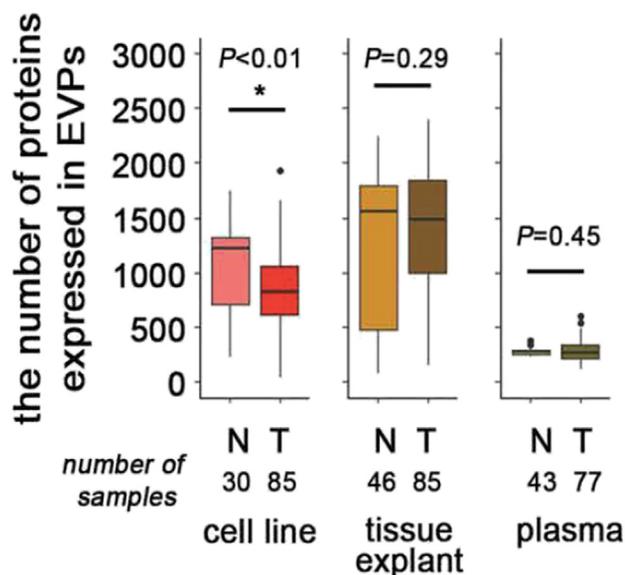


**Figure 24. Examples of human EVP samples analyzed by TEM**

Human cell line (Panc-1), human plasma (colon cancer) and human tumor explant (melanoma) EVP TEM images are shown. Scale bar: 200 nm. Adapted from Hoshino et al., 2020.

antigens representing biomarkers of interest follows a workflow similar to sandwich ELISA, where an additional antibody conjugated to a different fluorochrome is used to detect the presence of a protein of interest on/within the EVPs. The commercial basic kit includes anti-human CD9, CD63, and CD81 fluorescently conjugated antibodies (each antibody is directly conjugated to a different fluorochrome). The relative or absolute numbers of EVPs positive for biomarkers of interest can be quantified for proteins on the surface and within EVPs (Table 5; Figure 26). This approach allows us to determine heterogeneity in terms of EVP protein packaging in any given sample as well as to quantify the level of EVP proteins of interest on/in EVPs of various sizes. By including or not a permeabilization step, between the immobilization and antibody staining, this method also allows us to determine if a given protein of interest is present on the surface or packaged inside EVPs.

**Note:** In Figure 26 the particles were captured by anti-CD9 antibody-coated chips, but the number of particles detected using the anti-CD63 antibody is higher. This phenomenon is sometimes observed in Exoview analysis. The reason for this observation is that EVPs fit in the grooves of the chips and, consequently, CD9 proteins on the EVP surface were masked. Therefore, the EVP number detected by anti-CD9 antibody seems lower than expected.



**Figure 25. The number of proteins detected from different sources with the described LC-MS/MS method** (Modified from Hoshino et al., 2020).

**Table 5. Example of the measurement of breast cancer cell line-derived EVPs using the basic tetraspanin kit, related to Expected Outcomes – ExoView**

Normalized Particles-AVERAGE									
Raw #		ChA(Green)	ChB (blue)	ChC (red)	Baseline removed		ChA (green)	ChB (blue)	ChC (red)
200162001	CD81	1151.4	827.5	301.8	200162001	CD81	1148.7	823.1	296.3
	CD63	50.5	165.4	608.1		CD63	47.8	161	602.6
	CD9	1009.4	2412	947.5		CD9	1006.7	2407.6	942
	MlgG	2.7	4.4	5.5					

The raw particle numbers (left), and the numbers of particles following with baseline (MlgG) removal (right) are depicted.

### Western blot

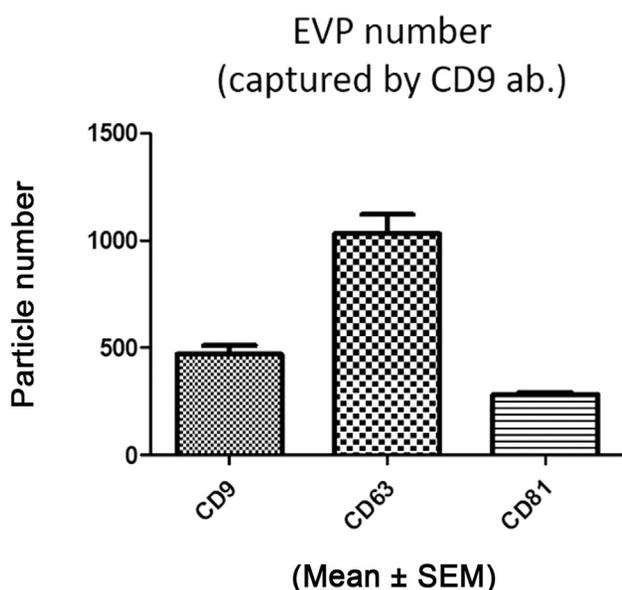
Western blot is used for molecular characterization of EVPs from different sources such as cell lines, tissue explants, and plasma, and for validation of EVP biomarkers of interest identified through unbiased MS analyses of EVPs. In particular, standard (CD9, CD81, TSG101, Syntenin-1) and new (Moesin, Stomatin, Galectin-3-binding protein, b2-microglobulin) EVP markers can be detected and quantified in a specific sample<sup>1</sup> (Figure 27).

### ExoELISA

The ExoELISA protocol allows the detection and quantification of the levels of internal or surface EVP proteins, expressed as fluorescence (FI), absorbance (a.u.) or luminescence (RFU) signal (qualitative ExoELISA) or as protein concentration (quantitative ExoELISA). If fluorescently labeled antibodies are used, more than one protein can be assayed within the same EVP sample. The relative or absolute protein levels can be compared between different EVP samples (Figure 28).

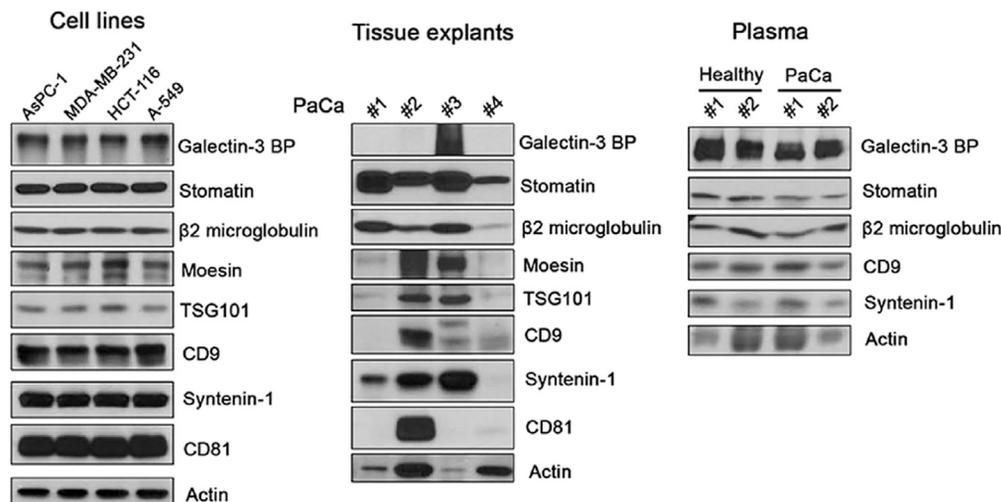
### LIMITATIONS

Western blot, ELISA, and ExoView analyses all require the use of specific antibodies to detect and quantify EVP proteins of interest identified by mass spectrometry. However, occasionally, antibodies specific for a protein of interest are not available or they have not been tested or validated for use in these specific assays or on EVP proteins. Therefore, before conducting the actual experiment, it is crucial to verify that the antibodies work for your sample (see Table 6 for recommendations).



**Figure 26. Typical results of an ExoView analysis of EVPs isolated from human plasma by sequential ultracentrifugation**

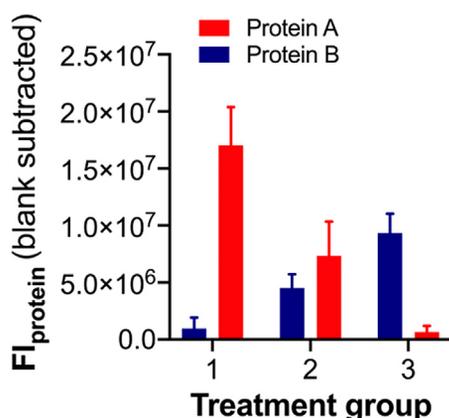
EVPs were captured by anti-human CD9 antibody chips provided in the basic tetraspanin kit.



**Figure 27. Examples of immunoblotting with EVP-specific antibodies on human samples from different sources** (Modified from Hoshino et al., 2020).

For TEM, EVPs are diluted based on the EVP protein concentration. We have listed the recommended concentration in the above protocol; however, due to different amount of proteins contained in different EVP samples, it is important to find the most appropriate concentration of EVPs (by serial dilutions) for clear visualization in a TEM experiment.

For NTA, samples after  $12,000 \times g$  and  $100,000 \times g$  ultracentrifugation can be used. However, note that not all EVPs will be pelleted after  $100,000 \times g$ . Therefore, be aware that there may be some discrepancy between particle number and distribution between samples after  $12,000 \times g$  and  $100,000 \times g$ . Also, while analyzing the samples, although the  $12,000 \times g$  step is designed to remove microvesicles, and thus should not contain large particles, sometimes large particles will be detected due to contamination or EVP aggregation. When a large particle is detected, it will generate a saturating signal and obscure particles in the vicinity, generating errors. In this case, sample detection and analysis will not be accurate. Review the video every time to remove recordings containing these aggregates to ensure accurate sample analysis. If using the LM10 instrument, the acquisition protocol might differ.<sup>10</sup> The NS500 instrument can also detect fluorescently labeled particles.<sup>11</sup>



**Figure 28. Example of the results of an ExoELISA measurement of protein expression on EVP samples from different experimental groups**

**Table 6. Antibodies used for respective immunoassay in this protocol, see Limitations section**

Antibody	Application	Concentration
Anti-CD9 (MM2/57)	Western Blot	0.1 µg/mL
Anti-syntenin-1 (S-31)	Western Blot	0.1 µg/mL
Anti-S100A9 (EPR3555)	ExoView	0.1 µg/mL
Anti-CETP	ExoView	0.1 µg/mL
Anti-B2M (BBM.1)	ExoView	0.1 µg/mL
Anti-RhoV (F-2)	ExoELISA	4 µg/mL

See further information about vendor and catalog number in [key resource table](#)

For LC-MS/MS, as this is a bottom-up proteomics experiment, it is not possible to distinguish between full-length and truncated proteins. Furthermore, the sensitivity of the mass spectrometer limits the depth of analysis. Additionally, this workflow is not designed to study post-translational modifications (PTMs) such as phosphorylation or glycosylation. If PTMs are of primary interest, enrichment-based workflows are recommended.

For western blot analyses, normalization of EVP protein expression is important to allow direct comparison between samples. In particular, patient plasma-derived EVPs show intrinsic inter- and intra-subject variability. EVP-specific proteins such as ALIX or tetraspanins can be used for normalization, however comparative studies seeking optimal normalization strategies of EVP in plasma are lacking. In some cases, a general protein stain such as Ponceau staining can be used to verify equal sample loading and normalize EVP protein amount between samples.

The ExoELISA protocol allows the detection and quantification of a wide range of proteins in EVP samples from different sources, using small sample amounts and providing flexibility in choice of antibodies. Additionally, it offers a good correlation with other techniques of protein quantification. Nevertheless, a pre-requisite for quantification is that the assayed antigen has sufficient density in the EVP samples. This limitation is mainly due to the detection sensitivity of the instrument used for signal measurement (plate reader), which might be lower than other protein measurement techniques, such as mass spectrometry. According to our correlation analysis, this version of the ExoELISA protocol allows quantification of proteins with a mass spectrum area value equal or higher than  $2 \times 10^7$ . To increase the detection power of the ExoELISA protocol, the concentration of EVP suspension for immobilization can be increased above 30 µg/mL. Alternatively, a signal amplification method (e.g., Tyramide Signal Amplification technology, PerkinElmer) could be used to enhance sensitivity.

The ExoELISA protocol is based on the passive adsorption of EVP proteins to the microwell plastics. Thus, the density of EVP surface proteins might affect their immobilization efficiency. For example, membrane-encapsulated extracellular vesicles, which are enriched in membrane-associated proteins, might have better adsorption efficiency than the smaller non-membranous particles. Thus, the ability of ExoELISA to detect non-membranous particle proteins might be reduced. The adsorption of protein-poor particles can be improved via lipid-targeted or capture antibody-based immobilization methods.

In this ExoView protocol, proteins on the EVP are measured. However, many EVP proteins are packaged inside of the EVPs. In that case, refer to Tetraspanin Cargo kit for permeabilizing the EVP membrane prior to fluorescent antibody incubation to detect proteins packaged inside of EVPs. If the localization of the EVP protein is unknown, trying both strategies is recommended. However, should no signal be detected, it would be difficult to determine if the protein is not present or if the antibody is not working. Further experiments are required to expand the range of antibodies that can be used in conjunction with the ExoView platform (see [Table 6](#)).

In summary, though our EVP characterization methods are comprehensive, there are additional/other options. One is to analyze EVPs by nanoparticle flow cytometry (such as the A60 Micro, Apogee system<sup>12</sup>).

These approaches are similar to the ExoView platform in terms of immunostaining and single-particle analysis, and multiple methods to analyze EVPs by flow cytometry are currently under development.<sup>13–19</sup> A recent advancement in the field of single particle analysis is nanoimaging by super-resolution dSTORM microscopy (<https://oni.bio/nanoimager/>). This method allows resolution of single EVPs below 100 nm and analysis of molecule co-expression and localization on a high-throughput scale, thereby furthering our ability to characterize EVPs and understand their functions.

## TROUBLESHOOTING

### Problem 1

In NTA, the liquid is drifting or the background noise is too high (see steps 2–3).

#### Potential solution

- If the liquid is drifting or air bubbles are observed, it means air is present inside the machine. Take out the module and wipe it gently.
- If the background noise is high, set LEVEL to a lower one. A lower LEVEL results in a higher threshold of EVP detection, so be careful not to set LEVEL too low because EVPs will be below the limit of detection.

### Problem 2

In LC-MS/MS, liquid does not dry in vacuum centrifuge (see step 20).

#### Potential solution

- Freeze-trap may not be sufficiently cold, causing insufficient vacuum. If using a dewar, refill with liquid nitrogen.
- Samples do not dry, even though vacuum is sufficient. Flash-freeze samples by dipping the vials shortly in liquid nitrogen (do not allow liquid nitrogen to get into the vial/plate wells) and start vacuum centrifugation while samples are still frozen.

### Problem 3

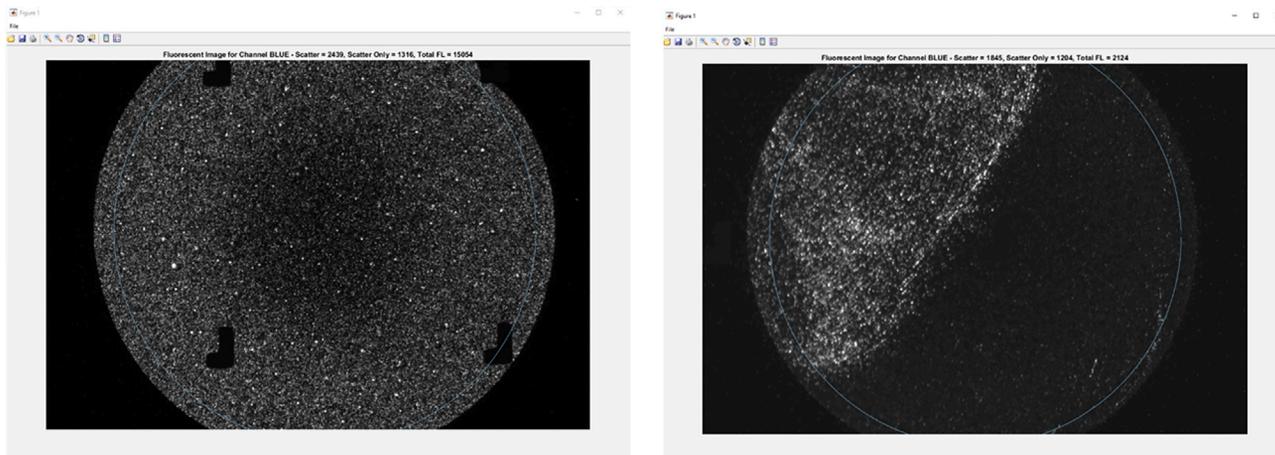
In LC-MS/MS, the number of identified proteins is low.

#### Potential solution

- Digestion is incomplete. Verify that the number of peptides containing no missed cleavages constitute at least 60% of all identified peptides. Verify that the pH of the denaturation buffer is approximately 8.5. If pH is off, remake buffers (Preparing denaturation buffer, steps 7–9).
- Wrong proteome was used in database searching. Download proteome in FASTA format from reliable source. A plethora of databases can be found via [uniprot.org](http://uniprot.org).
- Sensitivity or mass accuracy of mass spectrometer has declined. Include standards at the beginning and end of the sample queue. A suitable standard may include a tryptic digest of either a *Saccharomyces cerevisiae* cell lysate or purified bovine serum albumin. Search standards with appropriate databases and monitor drift of instrument performance. Include weekly calibration of mass spectrometer and maintenance of HPLC.
- Amount of starting material may be lower than anticipated. Inspect whether the autolysis peptides from Lys-C and trypsin are present in high amounts. If so, the starting amounts were most likely lower than anticipated.

### Problem 4

In LC-MS/MS, chromatogram appears empty.



**Figure 29. Example of a fluorescent picture of the intact spot (left) and a fluorescent picture of the failed spot (right)**  
If a picture similar to the right one can be seen, the spot has been damaged.

#### Potential solution

- Peptides may have been lost during solid-phase extraction. When performing the extraction be careful not to allow  $C_{18}$  material to dry, until after the second washing step (before you begin, step 10).
- Autosampler may have drawn liquid from the wrong vial/well. Verify position.
- There may be an issue with spray potential. Verify that the electrode is properly connected and verify spray by turning on potential and monitoring background ions. If spray appears unstable, clean sprayer with methanol or exchange.
- There may still be acetonitrile in sample when it is loaded in the autosampler, causing the peptide to elute immediately. Verify that samples are completely dry before dissolving in loading solvent (Preparing EVP samples for LC-MS/MS, step 20).

#### Problem 5

For ExoView analyses, failed fluorescence spots are after the scan.

#### Potential solution

Start by checking the fluorescent picture of the chip acquired via the ExoViewer software.

- If the fluorescent particles are uniformly spread on the surface of the spot (Figure 29, left panel), then the spot can be used for analysis.
- If particles are not equally distributed or the spot has some parts devoid of particles, as shown in Figure 29 (right panel), do not use the spot.

Most likely during the chip handling, antibodies or the samples were scraped off from the center spot.

#### Problem 6

For western blot, low concentration of the EVP sample, in particular for plasma samples, represents a major problem due to the maximum loading volume in the gel well (see step 39).

#### Potential solution

It is possible to concentrate the EVP sample using specific columns (such as MilliporeSigma Amicon Ultra-4 Centrifugal Filter Units) and use electrophoresis gels that allow larger loading volumes.

Moreover, the use of WedgeWell gels (Thermo Fisher Scientific) allows loading of twice the volume of a normal well.

### Problem 7

In western blotting (and LC-MS/MS), plasma EVP samples can contain higher amounts of non-EVP, free proteins that result in a reduction of the signal for specific EVP proteins (see steps 41–42).

### Potential solution

Include additional purification steps (such as density gradient, size exclusion or affinity binding) during EVP isolation from plasma to reduce free protein contamination and load a larger amount of EVP protein sample on the gel (minimum 20 µg) in order to be able to detect specific EVP proteins.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Lyden ([dcl2001@med.cornell.edu](mailto:dcl2001@med.cornell.edu)).

### Materials availability

This study did not generate any new reagents.

### Data and code availability

All LC-MS raw data acquired for this project has been deposited in the PRIDE repository (identifier [PXD018301](https://doi.org/10.1016/j.xpro.2023.102754)). This study did not generate any unique code.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2023.102754>.

## ACKNOWLEDGMENTS

We thank the Electron Microscopy and Histology services of the Weill Cornell Medicine Microscopy and Image Analysis Core for TEM with NIH Shared Instrumentation Grant (S10RR027699). The Rockefeller University Proteomics Resource Center acknowledges funding from the Leona M. and Harry B. Helmsley Charitable Trust and Sohn Conference Foundation for mass spectrometer instrumentation. We also thank the Medical Illustration & Design, part of the Medical Research Support Services of Yonsei University College of Medicine, Republic of Korea, for generating illustrations for this work. Schematic illustrations were created with [BioRender.com](https://www.biorender.com). The authors gratefully acknowledge support from the National Cancer Institute CA224175 (D.L.), CA210240 (D.L.), CA232093 (D.L.), CA163117 and CA207983 (D.L.), CA163120 (D.L.), CA169416 (D.L.), CA169538 (D.L.), CA218513 (D.L. and H.Z.), and AI144301 (D.L.); the United States Department of Defense W81XWH-13-1-0425 (D.L.), W81XWH-13-1-0427, W81XWH-13-1-0249 (D.L.), and W81XWH-14-1-0199 (D.L.); National Institutes of Health/WCM CTSC (NIH/NCATS) (UL1TR00457) (H.Z.); NIH/NCATS (UL1TR002384) (D.L. and H.Z.); The Hartwell Foundation (D.L.); the Thompson Family Foundation (D.L.); the Starr Cancer Consortium I9-A9-056 (D.L. and H.Z.) and I8-A8-123 (D.L.); the Pediatric Oncology Experimental Therapeutics Investigators' Consortium (D.L.); Alex's Lemonade Stand Foundation (D.L.); the Breast Cancer Research Foundation (D.L.); the Feldstein Medical Foundation (D.L.); the Tortolani Foundation (D.L.); the Clinical and Translational Science Center (D.L. and H.Z.); the Mary Kay Ash Charitable Foundation (D.L. and I.R.M.); the Malcolm Hewitt Wiener Foundation and the Manning Foundation (D.L. and A.H.); the Daniel P. and Nancy C. Paduano Family Foundation, the James Paduano Foundation, the Sohn Conference Foundation, the Fifth District AHEPA Cancer Research Foundation, the Daedalus Fund, Selma and Lawrence Ruben Science to Industry Bridge Award, and the Children's Cancer & Blood Foundation (all to D.L.); the Susan G. Komen Postdoctoral Fellowship PDF15331556, JST PRESTO 30021, Grant-in-Aid for Transformative Research Areas (A) 20H05904, and JSPS KAKENHI JP20H03508 and JP21K19374 and JPMJPR18H9 (A.H.);

the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (2022R1A2C4001879 to H.S.K.); a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (HI22C0353 to H.S.K.); the Bio & Medical Technology Development Program of the National Research Foundation (NRF) and funded by the Korean government (MSIT) (2022M3A9F3016364 to H.S.K.); the Swedish Cancer Society project grant (21 1824 Pj 01 H), the Swedish Research Society starting grant (2021-02356), and the Swedish Society for Medical Research (S21-0079) (L.B.); and the DoD PRCRP Horizon Award, United States (W81XWH-19-PRCRP-HA) (S.L.).

## AUTHOR CONTRIBUTIONS

L.B. and K.S. conceived and wrote the manuscript. H.S.K. wrote the bioinformatics section and drew the graphical abstract. S.H. and H.M. wrote the LC-MS/MS section. S.L. wrote the ExoELISA section, prepared the illustrations, and edited the manuscript. M.C. wrote the western blot section. K.S. wrote NTA and ExoView sections. L.C.-G. wrote the TEM section. K.E.J., H.Z., and I.R.M. edited the manuscript. A.H. and D.L. conceived and edited the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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