ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

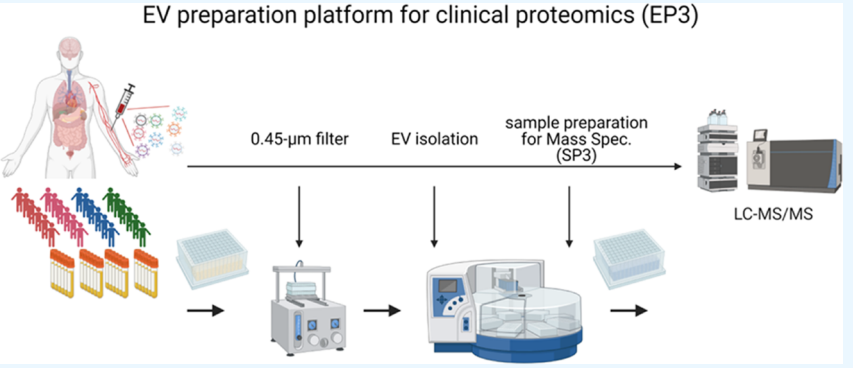
Automated Proteomics Sample Preparation of Phosphatidylserine-Positive Extracellular Vesicles from Human Body Fluids

Satoshi Muraoka, Masayo Hirano, Junko Isoyama, Mimiko Ishida, Takeshi Tomonaga, and Jun Adachi*

Cite This: *ACS Omega* 2022, 7, 41472−41479

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information



ABSTRACT: Extracellular vesicles (EVs) are ubiquitously secreted by almost every cell type and are present in all body fluids. Blood-derived EVs can be used as a promising source for biomarker monitoring in disease. EV proteomics is currently being analyzed in clinical specimens. However, their EV proteomics preparation methods are limited in throughput for human subjects. Here, we introduced a novel automated EV isolation and sample preparation method using a magnetic particle processing robot for automated 96-well processing of magnetic particles for EV proteomics analysis that can be started with a low volume of multiple clinical samples. The automation of EV purification reduced the coefficient of variation of protein quantification from 3.5 to 2.2% compared with manual purification, enabling the quantification of 1120 proteins in 1 h of MS analysis. This automated proteomics EV sample preparation is attractive for processing large cohort samples for biomarker development, validation, and routine testing.

INTRODUCTION

Extracellular vesicles (EVs) are small lipid bilayer-enclosed particles ubiquitously released by almost every cell type and are present in body fluids, including urea, blood, and cerebrospinal fluid (CSF).[1−4] EVs carry nucleic acids, such as microRNA, mRNA, noncoding RNA, lipids, and proteins, which can be transferred to recipient cells for cell-to-cell communication.[5−11] EVs have been extensively investigated for their function and roles in intracellular communication during cancer development and neurodegenerative disease progression.[12−14] Recently, EVs have been used for biomarker discovery, diagnostic development, and therapeutic development for cancer or neuronal disease.

In 2014, International Society for Extracellular Vesicles (ISEV) members published guidelines on the Minimal Information for Studies of Extracellular Vesicles (MISEV), and the guidelines were updated in 2018.[15] To date, the reported methods for separating EVs are differential centrifugation−ultracentrifugation (UC), sucrose gradient UC, size exclusion chromatography (SEC), ultrafiltration, microfluidics, polymer-based precipitation, immunoaffinity capture, affinity capture (AC), and asymmetric-flow field-flow fractionation.[16−26] However, standardization of these protocols for efficient recovery of EVs with high purity from biological samples has not yet been achieved. In addition, an isolation method with high yields of highly pure EVs and automated high throughputs needs to be developed for multisample preparation for biomarker discovery and diagnostic development. The MagCapture exosome isolation method can isolate highly purified EVs without high-abundance proteins over conventional methods, such as UC and polymer-based precipitation. The method uses a magnetic bead immobilizing Tim4, which is bound to calcium-dependent phosphatidylserine (PS) on EV surfaces. The magnetic beads allow automation of the procedure on a robotic liquid handling platform.

Hughes et al. developed single-pot solid-phase-enhanced sample preparation (SP3) for removing extensive handling procedures and unbiased protein retrieval.[27] SP3 provides a

Received: August 16, 2022
Accepted: October 24, 2022
Published: November 3, 2022

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

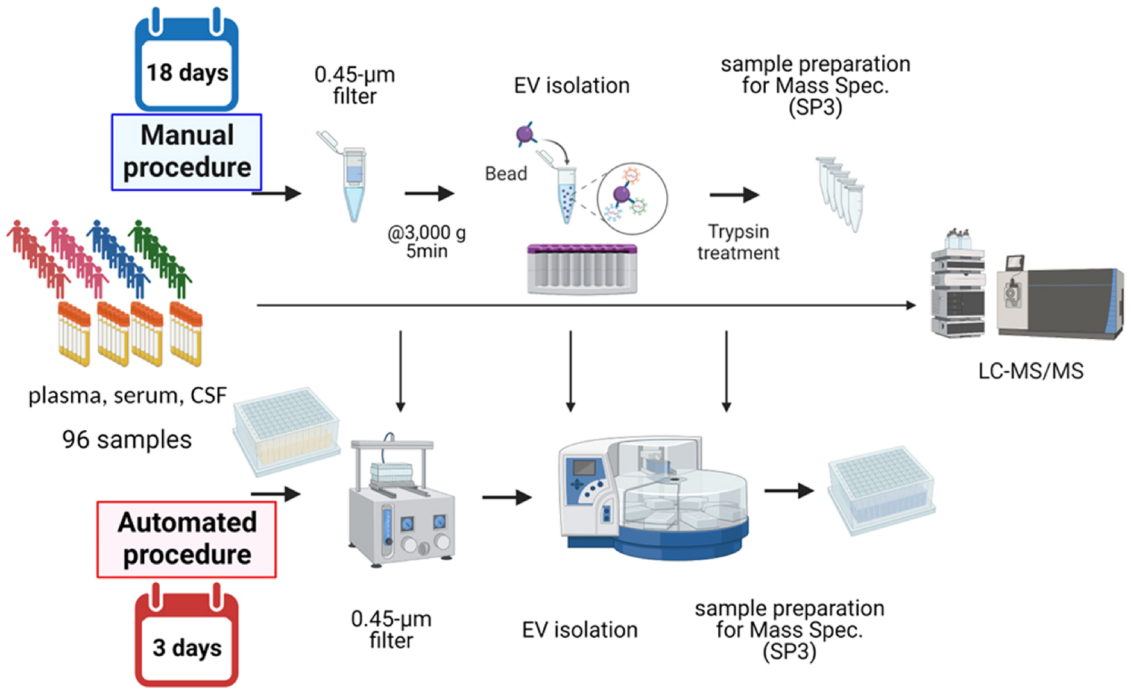


Figure 1. Workflow for automated EV isolation and proteomics sample preparation.

rapid and unbiased means of proteomic sample preparation in terms of efficiency, scalability, speed, throughput, and flexibility. In addition, SP3 uses carboxylate-modified paramagnetic particles and has found broad application in low input proteomics. The SP3 technology was executable in a 96-well format by a magnetic particle processing robot (KingFisher Flex).[28] Leutert et al. established the R2-P1 (rapid-robotic proteomics) and R2-P2 (rapid-robotic phosphoproteomics) automated workflows for protein clean-up and on-bead digestion for proteomic sample preparation approaches.[29]

Here, we developed an automated, high-throughput multi-sample preparation of EVs from biofluid bodies, including serum and plasma, for subsequent mass spectrometry-based proteomics analysis in a 96-well plate-based format. The automated protocol, EV preparation platform for clinical proteomics (EP3), demonstrated the combination systems of the KingFisher Flex platform for 96-well processing of magnetic particles for EV isolation and KingFisher Flex-SP3 technology for proteomics sample preparation. This provides an attractive and high-throughput sample preparation for routine and comprehensive clinical studies.

MATERIALS AND METHODS

Sample Selection. Pooled human serum (# 12181201) from 20 healthy subjects was purchased from Cosmo Bio.

Separation of EVs from Human Serum Samples. EVs were separated from pooled human serum using the MagCapture Exosome Isolation Kit PS version 2 (# 290-84103 Fujifilm WAKO Pure Chemical Corporation) with the KingFisher Flex System (Thermo Fisher Scientific). Briefly, 200 μL of serum was added to 300 μL of Tris-buffered saline and centrifuged at 1200 × g for 20 min at 4 °C. The supernatant was filtered through a 0.45-μm filter (# 7820-11001 FastRemover for Protein) using Positive pressure Resolvex M10 96 (TECAN). EVs were isolated from filtered serum using the KingFisher Flex System. The EVs were eluted with 100 μL of elution buffer for proteomics analysis. The program of KingFisher Flex for the EV isolation process is shown in Table S3.

In-Solution Digestion with Singlet-Pot, Solid-Phase-Enhanced Sample Preparation. Separated EV fractions were lysed with lysis buffer (12 mM sodium deoxycholate, 12 mM sodium lauroyl sarcosinate, and 50 mM ammonium bicarbonate), and then the mixed samples were vortexed for 5 min at room temperature followed by spin down and boiling for 10 min at 60 °C. The samples were reduced with 10 mM tris(2-carboxyethyl)phosphine (# 209-19861 WAKO) and alkylated with 20 mM iodoacetamide (# 19302-54 Nacalai Tesque) for 60 min at 37 °C in the dark. Automated SP3 technology was carried out following the program of KingFisher Flex. Then, 100% acetonitrile (ACN) was added to the reduced and alkylated sample tube and vortexed. The samples were bound with SP3 beads for 30 min with medium mixing. The SP3 beads were collected and put into 100% ACN. The mixing beads were placed in 70% ethanol with two repeats, and then trypsin and LysC were added. After 16 h, the digested EV peptide was collected using a Flex system to remove the magnetic beads, desalted via the stop-and-go-extraction tip (StageTip) protocol,[30] dried via vacuum centrifugation, and resuspended in 2% ACN and 1% trifluoroacetic acid (TFA) for liquid chromatography and tandem mass spectrometry (LC−MS/MS) processing. The program of KingFisher Flex for SP3 technology is indicated in Table S4.

Mass Spectrometry. *Nano-LC−MS/MS.* Nano-LC−MS/MS analysis was conducted with an LTQ-Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, USA) equipped with an UltiMate 3000 Nano LC system (Thermo Fisher Scientific, Bremen, Germany) and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Peptides were separated on an analytical column (75 μm × 20 cm, packed in-house with ReproSil-Pur C18-AQ, 1.9 μm resin, Dr. Maisch, Ammerbuch, Germany), and separation was achieved using a 45-min gradient of 5−30% ACN in 0.1% formic acid at

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

Table 1. Time of Each Step for Manual and Automated Procedures

	manual procedure (96 samples/18 days)	automated procedure (96 samples/3 days)
serum preparation step	2 h/16 samples/day (12 h/96 samples)	2 h/96 samples/day
EV isolation step	4 h/16 samples/day (24 h/96 sample)	3 h/96 samples/day
SP3 step (digestion)	2 h + 16 h(digestion)/16 samples/day (12 h + 16 h(digestion)/96 samples)	1 h + 16 h(digestion)/96 samples/day
desalting step	2 h/16 samples/day (12 h/96 samples)	1 h/96 samples/day

a flow rate of 280 nL/min. Data were acquired using data-independent acquisition (DIA). The Orbitrap Fusion Lumos mass spectrometer was used for gas-phase fractionation (GPF)-DIA of a pooled sample for library, and full mass spectra were acquired with the following parameters: a resolution 120,000, an automatic gain control (AGC) target 1×10^6, and an injection time of 250 ms. The five GPF-DIA runs collectively covered 418−782 *m/z* (i.e., 418−494, 490−566, 562−638, 634−710, and 706−782 *m/z*). MS2 spectra were collected with the following parameters: a 2-*m/z* isolation window at 50,000 resolution, an AGC target of 2×10^5 ions, a maximum injection time of 86 ms, and a normalized collision energy of 30. For the individual samples for proteome profiling acquisition, full mass spectra were acquired in the range of 410−780 *m/z* with the following parameters: a resolution of 120,000, an AGC target of 4×10^5, and an injection time of 100 ms. MS2 spectra were collected with the following parameters: a 10-*m/z* isolation window at 30,000 resolution, an AGC target of 2×10^5 ions, a maximum injection time of 54 ms, overlapping window patterns, and normalized collision energy of 30.

MS Data Analysis. MS data (raw file) were processed with Spectronaut software (Ver. 15.4.21 Biognosys, Schlieren, Switzerland).[31] Database searching included all entries from the *Homo sapiens* UniProt database (downloaded in April 2020, taxonomy ID: 9606) and contaminant database.[32] This database was concatenated with one composed of all protein sequences in the reversed order. The search parameters were as follows: up to two missed cleavage sites, 7−52 peptide length, carbamidomethylation of cysteine residues (+57.021 Da) as static modifications, acetylation of N-terminal residues and oxidation of methionine residues as variable modifications, and protein names from FASTA for implicit protein grouping and for quantification strategy. Precursor ions were adjusted to a 1% false discovery rate. Max LFQ was selected for the protein label-free quantitative method. Protein quantitation values were exported for further analysis in Microsoft Excel or Prism 9.

Statistical Analysis. Statistical analysis was conducted using Perseus ver. 1.6.14.0 and GraphPad Prism 9. Bivariate correlation analysis was applied to examine differences between proteins in proteomics data by Pearson's using R studio (ver. 1.4.1103).

RESULTS

Workflow for Automated Isolation and Proteomics Sample Preparation of EVs. To analyze EV proteins from a large number of body fluids, including serum, plasma, and CSF, we developed a method of automated proteomics EV sample preparation with 96 samples in parallel (Figure 1).

Automated EV sample preparation for proteomics analysis was performed by two processes: EV isolation and proteomics sample preparation. For EV isolation, EVs were isolated from plasma and serum using a MagCapture exosome isolation kit in a 96-well format by a magnetic particle processing robot (KingFisher Flex), and for proteomics sample preparation, the EV samples were prepared using the combination of SP3 technology in a 96-well format by a magnetic particle processing robot (KingFisher Flex). The desalting process before LC−MS/MS was performed using C18-SCX StageTips in a 96-well format.

We designed a workflow based on AC for EV isolation and SP3 technology for proteomics sample preparation in a 96-well format by a magnetic particle processing robot (KingFisher Flex system) (Figures 1 and S1). Figure 1 top shows the manual method for EV isolation and proteomics sample preparation, and bottom indicates the automated method using a positive pressure system and magnetic particle processing robot instead of handling. First, to remove large EVs and aggregated proteins, the body fluid samples after low-speed centrifugation were filtered using positive pressure systems for 96-well deep plates. EVs were isolated from the filtrated samples with a MagCapture exosome isolation kit (AC method) using a magnetic particle processing robot for automated 96-well processing of magnetic particles (Figure S2). The AC method uses a magnetic bead immobilizing Tim4, which is bound to calcium-dependent PS on EV surfaces. The characterization of isolated EVs has been shown previously using Nanoparticle tracking analysis, transmission electron microscopy, and Western blotting.[33] Then, the isolated EVs were processed by SP3 technology, which efficiently collects protein samples and performs enzymatic digestion. The desalting process used a C18-SCX StageTip with a 96-sample format. The purified peptides were analyzed with DIA-MS. In addition, Figure 1 shows the experimental time necessary to perform the manual protocol (top) and automated protocol (bottom). In the manual protocol, a total time of 18 days was required to prepare 96 samples. On the other hand, an automated protocol can be performed in only 3 days (Table 1).

Comparison of EV Protein Profiling between Manual and Automated Methods. We performed a DIA label-free quantitative proteomics analysis of EV fractions for proteomic profiling. A total of 1120 proteins were identified with at least two unique peptides and quantified (Figures 2A and S3A, and Tables S1 and S2). Principal component analysis showed a marginal separation of the two groups, which were manual and automated, and high reproducibility between the technical replicates in the automated methods compared to the manual method (Figure 2B). Figure 2C shows the coefficient of variation (CV) in proteins for each individual protein across all samples within manual or automated and in peptides (Figure S3B). Quantitative reproducibility assessed by the distribution of CVs between technical replicates was significantly lower for the automated method (median CV = 2.2%) than for the manual method (median CV = 3.5%). Pearson's correlation coefficient was calculated for the common proteins between the manual and automated methods (Figure 2D). The MS intensity levels were positively correlated between the manual and automated methods ($r = 0.7965$, $p < 0.001$), but several

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

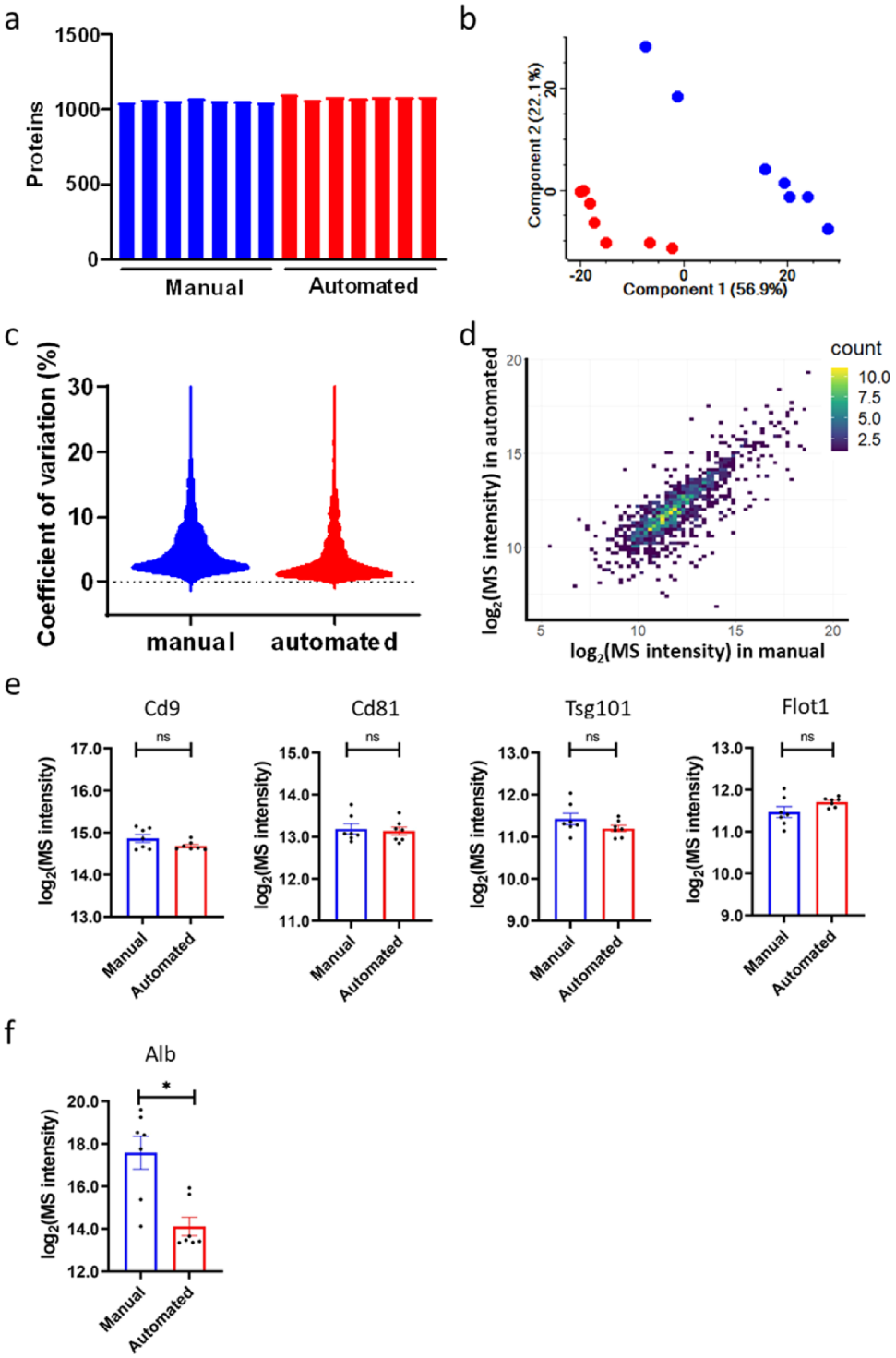


Figure 2. Comparison of protein profiling between manual and automated methods. (a) Number of identified proteins in manual and automated methods ($n = 7$). (b) Principal component analysis for manual and automated methods. (c) Violin plot of the coefficient of variation in quantitative values in manual and automated methods. The y-axis indicates CV (%). (d) Scatter plot of quantitative proteins between manual and automated. (e, f) MS intensity of EV and non-EV marker proteins. (e) EV markers, including Cd9, Cd81, Tsg101, and Flot1. * and ** show $p < 0.01$ and $p < 0.005$. (f) Non-EV marker; Alb.

proteins showed significant differences in quantitative values between these methods. Figure 2E and F show the box plot of the non-EV marker and EV marker proteins. EV marker proteins were not significantly different, but the automated method had a lower CV value than the manual method (Figure 2E). On the other hand, albumin, which is a serum contaminant protein, was reduced approximately eight-fold in the automated methods than in the manual method (Figure 2F). In addition, for albumin (Alb), the manual method has data point dispersion between technical replicates, which might affect the accuracy of the identification and quantification values for other proteins (Figure 2C). The complete workflow

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

takes 3 days to complete for 96 samples for EV isolation and proteomics sample preparation. Thus, the automated method presented here enables an approach to EV marker development using large numbers of clinical samples that would be difficult to perform manually. Therefore, it will be attractive for clinical proteomics laboratories and clinicians of various specialties.

DISCUSSION

In recent years, EVs separated from body fluids, including serum and plasma, have attracted attention as minimally invasive liquid biopsies for the development of biomarkers in cancer and neurological disease. EVs are capable of transferring disease-related signaling molecules and possess increased capabilities of detecting such disease-associated molecules present at extremely low amounts and from highly complex backgrounds such as patient plasma or serum. It has been reported that candidate EV proteins were identified as several disease markers, but they need more definitive data for use in the clinic. One of the problems is the difficulty of carrying out validation assays on large patient cohorts because there is still no reported methodology for isolating EVs from large sample cohorts. In this work, we have established an automated EV isolation and proteomic sample preparation method that uses magnetic beads for both processes. We successfully isolated EVs from blood using an automated isolation method and developed an automated method for EV proteomic sample preparation (EP3).

Proteome profiling of plasma and serum EVs has been performed following UC, SEC, precipitation, and AC in downstream proteomics analysis.[33−38] However, these methods have not developed automated systems in many sample formats. The automated EV sample preparation method will be attractive for EV-based biomarker discovery and validation with large sample batches. However, if the same EV purity can be isolated, the automated methods are more efficient because they allow for isolation in a short time. In addition, since only a small amount of EVs isolated from plasma or serum can be obtained, the combination of an automated EV isolation method and autoSP3 allows for the processing of low-input samples. Müller and co-workers reported a novel protocol using paramagnetic beads, termed SP3, for rapid, robust, and efficient processing of protein samples for proteomic analysis.[27,39] Recently, Leutert et al. established R2-P2, an automated phosphoproteomic sample preparation method that uses magnetic beads for both protein and peptide clean-up and phosphopeptide enrichment. In addition, they reported that R2-P2 can be performed in a 96-well format for high-throughput and run for 1.5 days from cell lysate to proteomic and phosphoproteomic MS injections.[29] Here, we successfully identified and quantified more than 1000 proteins with a high degree of reproducibility. Moreover, there was no difference in the number of identified EV proteins by the automated methods compared with the manual method, but the CV value of the quantitative method was less than that of the manual methods (Figure 2A). As shown in Figure 2F, albumin in high abundance proteins is reduced by adsorption due to its replacement in the 96-deep well plate, which is thought to stabilize the quantitative values of low abundant proteins. Other contaminant proteins, such as apolipoprotein and calnexin, were identified in the proteomics data, but they were unchanged between the manual and automated methods or higher in the manual methods (Table S1). However, whether contaminant proteins are present as soluble proteins or whether they occur inside or outside EVs has not been analyzed.[40] Our automated process required a shorter time with the same EV purity by manual processing. In addition, EV isolation and proteomic sample preparation methods (EP3) take only 3 days with the automated method, compared to 18 days with the manual methods for 96 serum samples. In addition, to isolate sEVs alone for analysis, it is possible to use a 0.22-μm filter instead of a 0.45-μm filter.

High-throughput workflows for clinical proteomics have been developed for sample preparation, including our EV isolation method, chromatography, mass spectrometry, data acquisition, and data analysis.[28,41−46] Sample preparation based on a 96-well format allowed the preparation of hundreds of samples per day and reduced batch effects. In the MS field, Messner et al. reported that the combination of scanning SWATH and high-flow chromatography created the technological basis for ultrafast and quantitatively precise proteome experiments.[42] In addition, in the LC field, a new LC system from Evosep Biosystems (Denmark) has been developed; the Evosep One LC system elutes peptides from Evotip, which is a special C18 StageTip, at low pressure and flow rates of tens of μL/min and forms a gradient that is stored in an analytical nanoscale column.[46] Bache et al. successfully quantified 5200 proteins in HeLa cell lysates with Evosep One with 60 samples per day.[44] The combination of our automated method with these MS and LC technologies may allow for the processing of several hundred samples per day for EV proteomics on large-scale clinical samples. Thus, it is crucial to develop our automated method (EP3) to stably isolate and prepare EV samples from multiple samples for the discovery and validation of candidate EV biomarker proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05244.

KingFisher Flex configuration for EV isolation and proteomics sample preparation; transmission electron microscopy image of EVs isolated from serum; and identification of peptides in EVs isolated from serum using manual and automated EV (PDF)

Identification and quantification of EV proteins from healthy pooled serum; list of quantified EV peptides in all samples; KingFisher program for EV isolation technology; and KingFisher Flex program for SP3 technology (Excel)

AUTHOR INFORMATION

Corresponding Author

Jun Adachi − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan; Laboratory of Clinical and Analytical Chemistry, Collaborative Research Center for Health and Medicine, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan; Laboratory of Proteomics and Drug Discovery, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto 606-8501, Japan;* orcid.org/0000-0003-1220-3246; Phone: +81-72-641-9862; Email: jun_adachi@nibiohn.go.jp; Fax: +81-72-641-9861

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

Authors

Satoshi Muraoka − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan; Laboratory of Clinical and Analytical Chemistry, Collaborative Research Center for Health and Medicine, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan;* orcid.org/0000-0003-3450-3717

Masayo Hirano − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan*

Junko Isoyama − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan*

Mimiko Ishida − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan*

Takeshi Tomonaga − *Laboratory of Proteomics for Drug Discovery, Center for Drug Design Research, National Institute of Biomedical Innovation, Health and Nutrition, Osaka 567-0085, Japan*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.2c05244

Author Contributions

S.M. and J.A. designed research; S.M., M.H., J.I., and M.I. performed research; S.M. and J.A. analyzed data; S.M. and J.A. wrote the paper; S.M., T.T., and J.A. edited the paper.

Funding

This work is in part funded by AMED under Grant Number JP20cm0106461, JP22fk0210064, and JP22fk0310512.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The author thanks R. Ukekawa and S. Ozaki (Fujifilm Wako Pure Chemical Co.,) for experimental support and S. Kawamoto (Hanaichi UltraStructure Research Institute, Co., Ltd.) for electron microscopic imaging services.

■ REFERENCES

(1) Kowal, J.; Arras, G.; Colombo, M.; Jouve, M.; Morath, J. P.; Primdal-Bengtson, B.; Dingli, F.; Loew, D.; Tkach, M.; Thery, C. Proteomic Comparison Defines Novel Markers to Characterize Heterogeneous Populations of Extracellular Vesicle Subtypes. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, E968−E977.

(2) Muraoka, S.; Jedrychowski, M. P.; Yanamandra, K.; Ikezu, S.; Gygi, S. P.; Ikezu, T. Proteomic Profiling of Extracellular Vesicles Derived from Cerebrospinal Fluid of Alzheimer's Disease Patients: A Pilot Study. *Cell* **2020**, *9*, 1959−2020.

(3) DeLeo, A. M.; Ikezu, T. Extracellular Vesicle Biology in Alzheimer's Disease and Related Tauopathy. *J. Neuroimmune Pharmacol.* **2018**, *13*, 292−308.

(4) Fang, Q.; Strand, A.; Law, W.; Faca, V. M.; Fitzgibbon, M. P.; Hamel, N.; Houle, B.; Liu, X.; May, D. H.; Poschmann, G.; Roy, L.; Stühler, K.; Ying, W.; Zhang, J.; Zheng, Z.; Bergeron, J. J. M.; Hanash, S.; He, F.; Leavitt, B. R.; Meyer, H. E.; Qian, X.; McIntosh, M. W. Brain-Specific Proteins Decline in the Cerebrospinal Fluid of Humans with Huntington Disease. *Mol. Cell. Proteomics* **2009**, *8*, 451−466.

(5) Hoshino, A.; Kim, H. S.; Bojmar, L.; Gyan, K. E.; Cioffi, M.; Hernandez, J.; Zambirinis, C. P.; Rodrigues, G.; Molina, H.; Heissel, S.; Mark, M. T.; Steiner, L.; Benito-Martin, A.; Lucotti, S.; Giannatale, A. D.; Offer, K.; Nakajima, M.; Williams, C.; Nogués, L.; Vatter, F. A. P.; Hashimoto, A.; Davies, A. E.; Freitas, D.; Kenific, C. M.; Ararso, Y.; Buehring, W.; Lauritzen, P.; Ogitani, Y.; Sugiura, K.; Takahashi, N.; Alečković, M.; Bailey, K. A.; Jolissant, J. S.; Wang, H.; Harris, A.; Schaeffer, L. M.; García-Santos, G.; Posner, Z.; Balachandran, V. P.; Khakoo, Y.; Raju, G. P.; Scherz, A.; Sagi, I.; Scherz-Shouval, R.; Yarden, Y.; Oren, M.; Malladi, M.; Petriccione, M.; Braganca, K. C. D.; Donzelli, M.; Fischer, C.; Vitolano, S.; Wright, G. P.; Ganshaw, L.; Marrano, M.; Ahmed, A.; DeStefano, J.; Danzer, E.; Roehrl, M. H. A.; Lacayo, N. J.; Vincent, T. C.; Weiser, M. R.; Brady, M. S.; Meyers, P. A.; Wexler, L. H.; Ambati, S. R.; Chou, A. J.; Slotkin, E. K.; Modak, S.; Roberts, S. S.; Basu, E. M.; Diolaiti, D.; Krantz, B. A.; Cardoso, F.; Simpson, A. L.; Berger, M.; Rudin, C. M.; Simeone, D. M.; Jain, M.; Ghajar, C. M.; Batra, S. K.; Stanger, B. Z.; Bui, J.; Brown, K. A.; Rajasekhar, V. K.; Healey, J. H.; de Sousa, M.; Kramer, K.; Sheth, S.; Baisch, J.; Pascual, V.; Heaton, T. E.; Quaglia, M. P. L.; Pisapia, D. J.; Schwartz, R.; Zhang, H.; Liu, Y.; Shukla, A.; Blavier, L.; DeClerck, Y. A.; LaBarge, M.; Bissell, M. J.; Caffrey, T. C.; Grandgenett, P. M.; Hollingsworth, M. A.; Bromberg, J.; Costa-Silva, B.; Peinado, H.; Kang, Y.; Garcia, B. A.; O'Reilly, E. M.; Kelsen, D.; Trippett, T. M.; Jones, D. R.; Matei, I. R.; Jarnagin, W. R.; Lyden, D. Extracellular Vesicle and Particle Biomarkers Define Multiple Human Cancers. *Cell* **2020**, *182*, 1044−1061.e18.

(6) Delpech, J.-C.; Herron, S.; Botros, M. B.; Ikezu, T. Neuroimmune Crosstalk through Extracellular Vesicles in Health and Disease. *Trends Neurosci.* **2019**, *42*, 361−372.

(7) You, Y.; Muraoka, S.; Jedrychowski, M. P.; Hu, J.; McQuade, A. K.; Young-Pearse, T.; Aslebagh, R.; Shaffer, S. A.; Gygi, S. P.; Blurton-Jones, M.; Poon, W. W.; Ikezu, T. Human Neural Cell Type-specific Extracellular Vesicle Proteome Defines Disease-related Molecules Associated with Activated Astrocytes in Alzheimer's Disease Brain. *J. Extracell. Vesicles* **2022**, *11*, No. e12183.

(8) Hosseini, H.; Obradović, M. M. S.; Hoffmann, M.; Harper, K. L.; Sosa, M. S.; Werner-Klein, M.; Nanduri, L. K.; Werno, C.; Ehrl, C.; Maneck, M.; Patwary, N.; Haunschild, G.; Gužvić, M.; Reimelt, C.; Grauvogl, M.; Eichner, N.; Weber, F.; Hartkopf, A. D.; Taran, F.-A.; Brucker, S. Y.; Fehm, T.; Rack, B.; Buchholz, S.; Spang, R.; Meister, G.; Aguirre-Ghiso, J. A.; Klein, C. A. Early Dissemination Seeds Metastasis in Breast Cancer. *Nature* **2016**, *540*, 552−558.

(9) Ikeda, A.; Nagayama, S.; Sumazaki, M.; Konishi, M.; Fujii, R.; Saichi, N.; Muraoka, S.; Saigusa, D.; Shimada, H.; Sakai, Y.; Ueda, K. Colorectal Cancer−Derived CAT1-Positive Extracellular Vesicles Alter Nitric Oxide Metabolism in Endothelial Cells and Promote Angiogenesis. *Mol. Cancer Res.* **2021**, *19*, 834.

(10) Sun, X.; Lin, F.; Sun, W.; Zhu, W.; Fang, D.; Luo, L.; Li, S.; Zhang, W.; Jiang, L. Exosome-Transmitted MiRNA-335-5p Promotes Colorectal Cancer Invasion and Metastasis by Facilitating EMT via Targeting RASA1. *Mol. Ther. Nucleic Acids* **2021**, *24*, 164−174.

(11) Kosaka, N.; Iguchi, H.; Hagiwara, K.; Yoshioka, Y.; Takeshita, F.; Ochiya, T. Neutral Sphingomyelinase 2 (NSMase2)-Dependent Exosomal Transfer of Angiogenic MicroRNAs Regulate Cancer Cell Metastasis*. *J. Biol. Chem.* **2013**, *288*, 10849−10859.

(12) Ruan, Z.; Pathak, D.; Kalavai, S. V.; Yoshii-Kitahara, A.; Muraoka, S.; Bhatt, N.; Takamatsu-Yukawa, K.; Hu, J.; Wang, Y.; Hersh, S.; Ericsson, M.; Gorantla, S.; Gendelman, H. E.; Kayed, R.; Ikezu, S.; Luebke, J. I.; Ikezu, T. Alzheimer's Disease Brain-Derived Extracellular Vesicles Spread Tau Pathology in Interneurons. *Brain* **2021**, *144*, 72.

(13) Hoshino, A.; Costa-Silva, B.; Shen, T.-L.; Rodrigues, G.; Hashimoto, A.; Mark, M. T.; Molina, H.; Kohsaka, S.; Giannatale, A. D.; Ceder, S.; Singh, S.; Williams, C.; Soplop, N.; Uryu, K.; Pharmer, L.; King, T.; Bojmar, L.; Davies, A. E.; Ararso, Y.; Zhang, T.; Zhang, H.; Hernandez, J.; Weiss, J. M.; Dumont-Cole, V. D.; Kramer, K.; Wexler, L. H.; Narendran, A.; Schwartz, G. K.; Healey, J. H.; Sandstrom, P.; Labori, K. J.; Kure, E. H.; Grandgenett, P. M.; Hollingsworth, M. A.; de Sousa, M.; Kaur, S.; Jain, M.; Mallya, K.;

Batra, S. K.; Jarnagin, W. R.; Brady, M. S.; Fodstad, O.; Muller, V.; Pantel, K.; Minn, A. J.; Bissell, M. J.; Garcia, B. A.; Kang, Y.; Rajasekhar, V. K.; Ghajar, C. M.; Matei, I.; Peinado, H.; Bromberg, J.; Lyden, D. Tumour Exosome Integrins Determine Organotropic Metastasis. *Nature* **2015**, *527*, 329−335.

(14) Ma, S.; McGuire, M. H.; Mangala, L. S.; Lee, S.; Stur, E.; Hu, W.; Bayraktar, E.; Villar-Prados, A.; Ivan, C.; Wu, S. Y.; Yokoi, A.; Dasari, S. K.; Jennings, N. B.; Liu, J.; Lopez-Berestein, G.; Ram, P.; Sood, A. K. Gain-of-Function P53 Protein Transferred via Small Extracellular Vesicles Promotes Conversion of Fibroblasts to a Cancer-Associated Phenotype. *Cell Rep.* **2021**, *34*, No. 108726.

(15) Thery, C.; Witwer, K. W.; Aikawa, E.; Alcaraz, M. J.; Anderson, J. D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G. K.; Ayre, D. C.; Bach, J.-M.; Bachurski, D.; Baharvand, H.; Balaj, L.; Baldacchino, S.; Bauer, N. N.; Baxter, A. A.; Bebawy, M.; Beckham, C.; Zavec, A. B.; Benmoussa, A.; Berardi, A. C.; Bergese, P.; Bielska, E.; Blenkiron, C.; Bobis-Wozowicz, S.; Boilard, E.; Boireau, W.; Bongiovanni, A.; Borràs, F. E.; Bosch, S.; Boulanger, C. M.; Breakefield, X.; Breglio, A. M.; Brennan, M. Á.; Brigstock, D. R.; Brisson, A.; Broekman, M. L.; Bromberg, J. F.; Bryl-Górecka, P.; Buch, S.; Buck, A. H.; Burger, D.; Busatto, S.; Buschmann, D.; Bussolati, B.; Buzás, E. I.; Byrd, J. B.; Camussi, G.; Carter, D. R.; Caruso, S.; Chamley, L. W.; Chang, Y.-T.; Chen, C.; Chen, S.; Cheng, L.; Chin, A. R.; Clayton, A.; Clerici, S. P.; Cocks, A.; Cocucci, E.; Coffey, R. J.; Cordeiro-da-Silva, A.; Couch, Y.; Coumans, F. A.; Coyle, B.; Crescitelli, R.; Criado, M. F.; D'Souza-Schorey, C.; Das, S.; Chaudhuri, A. D.; de Candia, P.; Santana, E. F. D.; Wever, O. D.; Portillo, H. A. D.; Demaret, T.; Deville, S.; Devitt, A.; Dhondt, B.; Vizio, D. D.; Dieterich, L. C.; Dolo, V.; Rubio, A. P. D.; Dominici, M.; Dourado, M. R.; Driedonks, T. A.; Duarte, F. V.; Duncan, H. M.; Eichenberger, R. M.; Ekström, K.; Andaloussi, S. E.; Elie-Caille, C.; Erdbrügger, U.; Falcon-Perez, J. M.; Fatima, F.; Fish, J. E.; Flores-Bellver, M.; Försönits, A.; Frelet-Barrand, A.; Fricke, F.; Fuhrmann, G.; Gabrielsson, S.; Gámez-Valero, A.; Gardiner, C.; Gärtner, K.; Gaudin, R.; Gho, Y. S.; Giebel, B.; Gilbert, C.; Gimona, M.; Giusti, I.; Goberdhan, D. C.; Görgens, A.; Gorski, S. M.; Greening, D. W.; Gross, J. C.; Gualerzi, A.; Gupta, G. N.; Gustafson, D.; Handberg, A.; Haraszti, R. A.; Harrison, P.; Hegyesi, H.; Hendrix, A.; Hill, A. F.; Hochberg, F. H.; Hoffmann, K. F.; Holder, B.; Holthofer, H.; Hosseinkhani, B.; Hu, G.; Huang, Y.; Huber, V.; Hunt, S.; Ibrahim, A. G.-E.; Ikezu, T.; Inal, J. M.; Isin, M.; Ivanova, A.; Jackson, H. K.; Jacobsen, S.; Jay, S. M.; Jayachandran, M.; Jenster, G.; Jiang, L.; Johnson, S. M.; Jones, J. C.; Jong, A.; Jovanovic-Talisman, T.; Jung, S.; Kalluri, R.; Kano, S.-I.; Kaur, S.; Kawamura, Y.; Keller, E. T.; Khamari, D.; Khomyakova, E.; Khvorova, A.; Kierulf, P.; Kim, K. P.; Kislinger, T.; Klingeborn, M.; Klinke, D. J.; Kornek, M.; Kosanović, M. M.; Kovács, Á. F.; Krämer-Albers, E.-M.; Krasemann, S.; Krause, M.; Kurochkin, I. V.; Kusuma, G. D.; Kuypers, S.; Laitinen, S.; Langevin, S. M.; Languino, L. R.; Lannigan, J.; Lässer, C.; Laurent, L. C.; Lavieu, G.; Lázaro-Ibáñez, E.; Lay, S. L.; Lee, M.-S.; Lee, Y. X. F.; Lemos, D. S.; Lenassi, M.; Leszczynska, A.; Li, I. T.; Liao, K.; Libregts, S. F.; Ligeti, E.; Lim, R.; Lim, S. K.; Linē, A.; Linnemannstöns, K.; Llorente, A.; Lombard, C. A.; Lorenowicz, M. J.; Lörincz, Á. M.; Lötvall, J.; Lovett, J.; Lowry, M. C.; Loyer, X.; Lu, Q.; Lukomska, B.; Lunavat, T. R.; Maas, S. L.; Malhi, H.; Marcilla, A.; Mariani, J.; Mariscal, J.; Martens-Uzunova, E. S.; Martin-Jaular, L.; Martinez, M. C.; Martins, V. R.; Mathieu, M.; Mathivanan, S.; Maugeri, M.; McGinnis, L. K.; McVey, M. J.; Meckes, D. G.; Meehan, K. L.; Mertens, I.; Minciacchi, V. R.; Möller, A.; Jørgensen, M. M.; Morales-Kastresana, A.; Morhayim, J.; Mullier, F.; Muraca, M.; Musante, L.; Mussack, V.; Muth, D. C.; Myburgh, K. H.; Najrana, T.; Nawaz, M.; Nazarenko, I.; Nejsum, P.; Neri, C.; Neri, T.; Nieuwland, R.; Nimrichter, L.; Nolan, J. P.; Hoen, E. N. N.-T.; Hooten, N. N.; O'Driscoll, L.; O'Grady, T.; O'Loghlen, A.; Ochiya, T.; Olivier, M.; Ortiz, A.; Ortiz, L. A.; Osteikoetxea, X.; Østergaard, O.; Ostrowski, M.; Park, J.; Pegtel, D. M.; Peinado, H.; Perut, F.; Pfaffl, M. W.; Phinney, D. G.; Pieters, B. C.; Pink, R. C.; Pisetsky, D. S.; Von Strandmann, E. P.; Polakovicova, I.; Poon, I. K.; Powell, B. H.; Prada, I.; Pulliam, L.; Quesenberry, P.; Radeghieri, A.; Raffai, R. L.; Raimondo, S.; Rak, J.; Ramirez, M. I.; Raposo, G.; Rayyan, M. S.; Regev-Rudzki, N.; Ricklefs, F. L.; Robbins, P. D.; Roberts, D. D.; Rodrigues, S. C.; Rohde, E.; Rome, S.; Rouschop, K. M.; Rughetti, A.; Russell, A. E.; Saá, P.; Sahoo, S.; Salas-Huenuleo, E.; Sánchez, C.; Saugstad, J. A.; Saul, M. J.; Schiffelers, R. M.; Schneider, R.; Schøyen, T. H.; Scott, A.; Shahaj, E.; Sharma, S.; Shatnyeva, O.; Shekari, F.; Shelke, G. V.; Shetty, A. K.; Shiba, K.; Siljander, P. R.-M.; Silva, A. M.; Skowronek, A.; Snyder, O. L.; Soares, R. P.; Sódar, B. W.; Soekmadji, C.; Sotillo, J.; Stahl, P. D.; Stoorvogel, W.; Stott, S. L.; Strasser, E. F.; Swift, S.; Tahara, H.; Tewari, M.; Timms, K.; Tiwari, S.; Tixeira, R.; Tkach, M.; Toh, W. S.; Tomasini, R.; Torrecilhas, A. C.; Tosar, J. P.; Toxavidis, V.; Urbanelli, L.; Vader, P.; Van Balkom, B. W.; Van Der Grein, S. G.; Deun, J. V.; Van Herwijnen, M. J.; Keuren-Jensen, K. V.; Van Niel, G.; Van Royen, M. E.; Van Wijnen, A. J.; Vasconcelos, M. H.; Vechetti, I. J.; Veit, T. D.; Vella, L. J.; Velot, É.; Verweij, F. J.; Vestad, B.; Viñas, J. L.; Visnovitz, T.; Vukman, K. V.; Wahlgren, J.; Watson, D. C.; Wauben, M. H.; Weaver, A.; Webber, J. P.; Weber, V.; Wehman, A. M.; Weiss, D. J.; Welsh, J. A.; Wendt, S.; Wheelock, A. M.; Wiener, Z.; Witte, L.; Wolfram, J.; Xagorari, A.; Xander, P.; Xu, J.; Yan, X.; Yáñez-Mó, M.; Yin, H.; Yuana, Y.; Zappulli, V.; Zarubova, J.; Žėkas, V.; Zhang, J.-Y.; Zhao, Z.; Zheng, L.; Zheutlin, A. R.; Zickler, A. M.; Zimmermann, P.; Zivkovic, A. M.; Zocco, D.; Zuba-Surma, E. K. Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines. *J. Extracell. Vesicles* **2018**, *7*, No. 1535750.

(16) Tian, Y.; Gong, M.; Hu, Y.; Liu, H.; Zhang, W.; Zhang, M.; Hu, X.; Aubert, D.; Zhu, S.; Wu, L.; Yan, X. Quality and Efficiency Assessment of Six Extracellular Vesicle Isolation Methods by Nano-Flow Cytometry. *J. Extracell. Vesicles* **2020**, *9*, No. 1697028.

(17) Nakai, W.; Yoshida, T.; Diez, D.; Miyatake, Y.; Nishibu, T.; Imawaka, N.; Naruse, K.; Sadamura, Y.; Hanayama, R. A Novel Affinity-Based Method for the Isolation of Highly Purified Extracellular Vesicles. *Sci. Rep.* **2016**, *6*, 33935.

(18) Zhang, H.; Lyden, D. Asymmetric-Flow Field-Flow Fractionation Technology for Exomere and Small Extracellular Vesicle Separation and Characterization. *Nat. Protoc.* **2019**, *14*, 1027−1053.

(19) Heinemann, M. L.; Ilmer, M.; Silva, L. P.; Hawke, D. H.; Recio, A.; Vorontsova, M. A.; Alt, E.; Vykoukal, J. Benchtop Isolation and Characterization of Functional Exosomes by Sequential Filtration. *J. Chromatogr. A* **2014**, *1371*, 125−135.

(20) Muraoka, S.; Lin, W.; Chen, M.; Hersh, S. W.; Emili, A.; Xia, W.; Ikezu, T. Assessment of Separation Methods for Extracellular Vesicles from Human and Mouse Brain Tissues and Human Cerebrospinal Fluids. *Methods* **2020**, *177*, 35−49.

(21) Muraoka, S.; DeLeo, A. M.; Sethi, M. K.; Yukawa-Takamatsu, K.; Yang, Z.; Ko, J.; Hogan, J. D.; Ruan, Z.; You, Y.; Wang, Y.; Medalla, M.; Ikezu, S.; Chen, M.; Xia, W.; Gorantla, S.; Gendelman, H. E.; Issadore, D.; Zaia, J.; Ikezu, T. Proteomic and Biological Profiling of Extracellular Vesicles from Alzheimer's Disease Human Brain Tissues. *Alzheimer's Dementia* **2020**, *16*, 896−907.

(22) Kanwar, S. S.; Dunlay, C. J.; Simeone, D. M.; Nagrath, S. Microfluidic Device (ExoChip) for on-Chip Isolation, Quantification and Characterization of Circulating Exosomes. *Lab Chip* **2014**, *14*, 1891−1900.

(23) Bobrie, A.; Colombo, M.; Krumeich, S.; Raposo, G.; Thery, C. Diverse Subpopulations of Vesicles Secreted by Different Intracellular Mechanisms Are Present in Exosome Preparations Obtained by Differential Ultracentrifugation. *J. Extracell. Vesicles* **2012**, *1*, 18397.

(24) Onódi, Z.; Pelyhe, C.; Nagy, C. T.; Brenner, G. B.; Almási, L.; Kittel, Á.; Manček-Keber, M.; Ferdinandy, P.; Buzás, E. I.; Giricz, Z. Isolation of High-Purity Extracellular Vesicles by the Combination of Iodixanol Density Gradient Ultracentrifugation and Bind-Elute Chromatography From Blood Plasma. *Front. Physiol.* **2018**, *9*, 1479.

(25) Wu, M.; Ouyang, Y.; Wang, Z.; Zhang, R.; Huang, P.-H.; Chen, C.; Li, H.; Li, P.; Quinn, D.; Dao, M.; Suresh, S.; Sadovsky, Y.; Huang, T. J. Isolation of Exosomes from Whole Blood by Integrating Acoustics and Microfluidics. *Proc. Natl. Acad. Sci. U. S. A.* **2017**, *114*, 10584−10589.

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479

(26) Cao, F.; Gao, Y.; Chu, Q.; Wu, Q.; Zhao, L.; Lan, T.; Zhao, L. Proteomics Comparison of Exosomes from Serum and Plasma between Ultracentrifugation and Polymer-Based Precipitation Kit Methods. *Electrophoresis* **2019**, *40*, 3092−3098.
(27) Hughes, C. S.; Foehr, S.; Garfield, D. A.; Furlong, E. E.; Steinmetz, L. M.; Krijgsveld, J. Ultrasensitive Proteome Analysis Using Paramagnetic Bead Technology. *Mol. Syst. Biol.* **2014**, *10*, 757.
(28) Müller, T.; Kalxdorf, M.; Longuespée, R.; Kazdal, D. N.; Stenzinger, A.; Krijgsveld, J. Automated Sample Preparation with SP3 for Low-Input Clinical Proteomics. *Mol. Syst. Biol.* **2020**, *16*, No. e9111.
(29) Leutert, M.; Rodríguez-Mias, R. A.; Fukuda, N. K.; Villén, J. R2-P2 Rapid-robotic Phosphoproteomics Enables Multidimensional Cell Signaling Studies. *Mol. Syst. Biol.* **2019**, *15*, No. e9021.
(30) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for Micro-Purification, Enrichment, Pre-Fractionation and Storage of Peptides for Proteomics Using StageTips. *Nat. Protoc.* **2007**, *2*, 1896−1906.
(31) Bruderer, R.; Bernhardt, O. M.; Gandhi, T.; Miladinović, S. M.; Cheng, L.-Y.; Messner, S.; Ehrenberger, T.; Zanotelli, V.; Butscheid, Y.; Escher, C.; Vitek, O.; Rinner, O.; Reiter, L. Extending the Limits of Quantitative Proteome Profiling with Data-Independent Acquisition and Application to Acetaminophen-Treated Three-Dimensional Liver Microtissues*. *Mol. Cell Proteomics* **2015**, *14*, 1400−1410.
(32) Tyanova, S.; Temu, T.; Cox, J. The MaxQuant Computational Platform for Mass Spectrometry-Based Shotgun Proteomics. *Nat. Protoc.* **2016**, *11*, 2301−2319.
(33) Muraoka, S.; Hirano, M.; Isoyama, J.; Nagayama, S.; Tomonaga, T.; Adachi, J. Comprehensive Proteomic Profiling of Plasma and Serum Phosphatidylserine-Positive Extracellular Vesicles Reveals Tissue-Specific Proteins. *Iscience* **2022**, No. 104012.
(34) Ding, M.; Wang, C.; Lu, X.; Zhang, C.; Zhou, Z.; Chen, X.; Zhang, C.-Y.; Zen, K.; Zhang, C. Comparison of Commercial Exosome Isolation Kits for Circulating Exosomal MicroRNA Profiling. *Anal. Bioanal. Chem.* **2018**, *410*, 3805−3814.
(35) Fel, A.; Lewandowska, A. E.; Petrides, P. E.; Wiśniewski, J. R. Comparison of Proteome Composition of Serum Enriched in Extracellular Vesicles Isolated from Polycythemia Vera Patients and Healthy Controls. *Proteomes* **2019**, *7*, 20.
(36) Jeannin, P.; Chaze, T.; Gianetto, Q. G.; Matondo, M.; Gout, O.; Gessain, A.; Afonso, P. V. Proteomic Analysis of Plasma Extracellular Vesicles Reveals Mitochondrial Stress upon HTLV-1 Infection. *Sci. Rep.* **2018**, *8*, 5170−5177.
(37) Pietrowska, M.; Zebrowska, A.; Gawin, M.; Marczak, L.; Sharma, P.; Mondal, S.; Mika, J.; Polańska, J.; Ferrone, S.; Kirkwood, J. M.; Widlak, P.; Whiteside, T. L. Proteomic Profile of Melanoma Cell-Derived Small Extracellular Vesicles in Patients' Plasma: A Potential Correlate of Melanoma Progression. *J. Extracell. Vesicles* **2021**, *10*, No. e12063.
(38) Ebrahimkhani, S.; Vafaee, F.; Hallal, S.; Wei, H.; Lee, M. Y. T.; Young, P. E.; Satgunaseelan, L.; Beadnall, H.; Barnett, M. H.; Shivalingam, B.; Suter, C. M.; Buckland, M. E.; Kaufman, K. L. Deep Sequencing of Circulating Exosomal MicroRNA Allows Non-Invasive Glioblastoma Diagnosis. *NPJ Precis. Oncol.* **2018**, *2*, 28−29.
(39) Hughes, C. S.; Moggridge, S.; Müller, T.; Sorensen, P. H.; Morin, G. B.; Krijgsveld, J. Single-Pot, Solid-Phase-Enhanced Sample Preparation for Proteomics Experiments. *Nat. Protoc.* **2019**, *14*, 68−85.
(40) Ahn, S.-M.; Byun, K.; Cho, K.; Kim, J. Y.; Yoo, J. S.; Kim, D.; Paek, S. H.; Kim, S. U.; Simpson, R. J.; Lee, B. Human Microglial Cells Synthesize Albumin in Brain. *PLoS One* **2008**, *3*, No. e2829.
(41) Gillet, L. C.; Navarro, P.; Tate, S.; Röst, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R. Targeted Data Extraction of the MS/MS Spectra Generated by Data-Independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis*. *Mol. Cell. Proteomics* **2012**, *11*, No. O111.016717.
(42) Messner, C. B.; Demichev, V.; Bloomfield, N.; Yu, J. S. L.; White, M.; Kreidl, M.; Egger, A.-S.; Freiwald, A.; Ivosev, G.; Wasim, F.; Zelezniak, A.; Jürgens, L.; Suttorp, N.; Sander, L. E.; Kurth, F.; Lilley, K. S.; Mülleder, M.; Tate, S.; Ralser, M. Ultra-Fast Proteomics with Scanning SWATH. *Nat. Biotechnol.* **2021**, *39*, 846−854.
(43) Demichev, V.; Messner, C. B.; Vernardis, S. I.; Lilley, K. S.; Ralser, M. DIA-NN: Neural Networks and Interference Correction Enable Deep Proteome Coverage in High Throughput. *Nat. Methods* **2020**, *17*, 41−44.
(44) Bache, N.; Geyer, P. E.; Bekker-Jensen, D. B.; Hoerning, O.; Falkenby, L.; Treit, P. V.; Doll, S.; Paron, I.; Müller, J. B.; Meier, F.; Olsen, J. V.; Vorm, O.; Mann, M. A Novel LC System Embeds Analytes in Pre-Formed Gradients for Rapid, Ultra-Robust Proteomics*. *Mol. Cell. Proteomics* **2018**, *17*, 2284−2296.
(45) Krieger, J. R.; Wybenga-Groot, L. E.; Tong, J.; Bache, N.; Tsao, M. S.; Moran, M. F. Evosep One Enables Robust Deep Proteome Coverage Using Tandem Mass Tags While Significantly Reducing Instrument Time. *J. Proteome Res.* **2019**, *18*, 2346−2353.
(46) Bekker-Jensen, D. B.; Kelstrup, C. D.; Batth, T. S.; Larsen, S. C.; Haldrup, C.; Bramsen, J. B.; Sørensen, K. D.; Høyer, S.; Ørntoft, T. F.; Andersen, C. L.; Nielsen, M. L.; Olsen, J. V. An Optimized Shotgun Strategy for the Rapid Generation of Comprehensive Human Proteomes. *Cell Syst.* **2017**, *4*, 587−599.e4.

https://doi.org/10.1021/acsomega.2c05244
ACS Omega 2022, 7, 41472−41479