

Development of a Highly Sensitive and Rapid Lateral Flow Immunochromatographic Assay for the Quantitative Detection of Small Extracellular Vesicles

Makoto Watanabe

w-makoto@shimadzu.co.jp

Shimadzu Corporation

Yuta Miyazawa

Dai Nippon Toryo Co., Ltd

Takashi Yamasaki

Shimadzu Corporation

Yoshikazu So

Dai Nippon Toryo Co., Ltd

Taka-Aki Sato

Shimadzu Corporation

Article

Keywords:

Posted Date: August 21st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10428701/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Small extracellular vesicles (sEVs) circulated in body fluids have emerged as promising biomarkers for non-invasive clinical diagnostics. However, conventional analysis methods, such as Nanoparticle Tracking Analysis (NTA) and Enzyme-Linked Immunosorbent Assay (ELISA), are often labor-intensive, time-consuming, and costly. In this study, we developed a novel, rapid, and cost-effective lateral flow immunochromatographic assay (LFIA) platform specifically optimized for the quantitative detection of sEV surface antigen (CD9). To overcome the analytical limitations of conventional lateral flow strips, we systematically optimized the sample loading protocol and compared multiple detection modalities.

First, we compared a "One-Step" simultaneous incubation method with a sequential "Two-Step" method. The Two-Step method demonstrated significantly higher signal intensity by mitigating steric hindrance at the nano-bio interface, where pre-mixing gold-conjugated antibodies can mask the sEV surface antigens. Second, to achieve high-sensitivity detection, a chemiluminescence system utilizing enzyme-conjugated antibodies (HRP and ALP) was integrated into the LFIA platform. We compared colorimetric detection using spherical gold nanoparticles (AuSP) and gold nanoplates (AuPL) with chemiluminescent detection. The chemiluminescence system achieved an outstanding detection limit down to the 10^5 particles level, which is 40- to 60-fold more sensitive than the colorimetric method, providing sufficient sensitivity for clinical liquid biopsies. Conversely, the gold nanoparticle-based colorimetric assay offered a wider linear dynamic range. This hybrid LFIA platform provides a strategic dual-modality solution for simple, rapid, and versatile sEV quantification in clinical research.

Introduction

Extracellular vesicles (EVs) are nanoscale membrane vesicles secreted from almost all cell types into various biological fluids, including blood, urine, saliva, and cerebrospinal fluid [1]. Among them, small EVs (sEVs), which primarily include exosome and are typically defined by sizes under 200 nm, are now recognized as critical mediators of intercellular communication, playing vital roles in modulating the tumor microenvironment and driving cancer progression [2, 3]. Consequently, sEVs have attracted significant interest both as therapeutic targets and as highly accessible biomarkers that reflect the molecular composition of their parental cells [4, 5], rapidly advancing as non-invasive tools for early cancer diagnosis [6, 7]

However, the clinical utilization of sEVs as biomarkers requires analytical methods capable of isolating and detecting them from small sample volumes while distinguishing them from abundant non-sEV background proteins. Currently, conventional sEV analysis methodologies are broadly classified into two approaches: the analysis of purified samples and direct detection [8]. Physical characterization techniques like Nanoparticle Tracking Analysis (NTA) determine particle numbers and sizing [9], while Western blotting is standard for measuring the expression of tetraspanin surface markers [10]. For direct detection, flow cytometry, ELISA, Exoscreen, and ExoCounter are commonly utilized [11, 12]. Recently, advanced biosensing technologies, including surface plasmon resonance (SPR) [13, 14], surface-

enhanced Raman scattering (SERS) [15], and microfluidic devices [16], have been developed to achieve high-sensitivity sEV profiling. Although these techniques successfully target sEV surface proteins and offer exceptional sensitivity, they are often constrained by complex operational steps, bulky and expensive instrumentation, and a lack of high-throughput capacity, highlighting an urgent need for rapid, wash-free point-of-care (POC) devices [12].

The lateral flow immunochromatographic assay (LFIA) represents a highly attractive alternative, offering a simple, rapid, and cost-effective point-of-care (POC) device for antigen detection [17]. Recent technological integrations, including novel nanomaterials and signal amplification strategies, have drastically expanded the quantitative capabilities and sensitivity of LFIA [18]. Traditionally utilizing a nitrocellulose membrane and water-absorbent pads, conventional LFIA captures target analytes via immobilized antibodies on a test line, generating a visible signal through the concentration of metal nanoparticles [19]. Despite its advantages, conventional LFIA utilizing gold nanoparticles often exhibits a poor detection limit compared to other specialized methods. Therefore, improving the analytical sensitivity of this method is essential for detecting the low abundance of antigens typically found on circulating sEVs.

In this study, we report a modified LFIA system specifically optimized for the highly sensitive detection and quantification of sEV surface membrane antigens. To overcome the physical limitations of conventional strip-based assays, we evaluated a sequential sample loading protocol designed to minimize antibody masking and steric hindrance on the sEV surface membrane [19, 20]. Furthermore, we combined this structural optimization with a highly sensitive chemiluminescence measurement technique to detect lower expression levels of target antigens. This comprehensive optimization offers a novel, practical framework for versatile sEV quantification.

Results & Discussion

2.1 Effect of Sample Loading Protocol on sEV Detection

We systematically investigated whether the sequential order of sample application onto the immunostrip influences the ultimate analytical sensitivity of sEV detection. Two distinct loading protocols targeting the CD9 surface marker were directly compared under identical conditions: the simultaneous "One-Step" method (pre-mixing the analyte and the Au-labeled probe) and the sequential "Two-Step" method (capturing the analyte on the strip prior to adding the probe) (Fig. 1).

Our experimental results revealed that the sequential Two-Step method yielded a test line signal for CD9 that was significantly stronger ($p < 0.05$, $n = 3$) than that achieved by the One-Step protocol (Fig. 2). Conversely, control evaluations using a blank sample devoid of sEVs yielded no statistically significant difference in background signal between the two application techniques. This clear divergence indicates that the sequential configuration is fundamentally responsible for driving signal enhancement at the test line.

The underlying biophysical mechanism can be explained by steric hindrance and surface masking occurring at the nanoscale bio-interface [19, 20]. In the One-Step protocol, mixing the sample with a high concentration of gold-labeled antibodies causes the highly dense probe molecules to bind and coat almost the entire available surface area of the sEV membrane. This extensive pre-coating induces severe steric hindrance around the remaining uncomplexed tetraspanins. Consequently, when the large sEV-probe complex migrates across the test line, the immobilized capture antibodies on the nitrocellulose membrane face severe physical restriction in accessing their target epitopes, reducing sEV capture efficiency, a phenomenon closely related to the high-dose hook effect often observed in conventional LFIA formats [21, 22]. In contrast, the Two-Step method ensures that intact sEVs freely interact with the flat, immobilized antibody line on the membrane without spatial restrictions. Once the sEVs are successfully immobilized, the subsequently introduced probe antibodies can bind unhindered to the remaining exposed surface antigens, yielding superior capture efficiency and signal intensity. To quantitatively evaluate the efficacy of the sequential loading protocol across different nanomaterial geometries, the signal enhancement factor (defined as the ratio of the Two-Step to One-Step signal intensity) was calculated at a concentration of 1.0×10^9 particles. The Two-Step method demonstrated an 8.3-fold increase in signal intensity for AuSP (183.5 mABS vs. 22.1 mABS) (supplementary data 1), whereas a remarkable 17.8-fold enhancement was observed for AuPL (87.1 mABS vs. 4.9 mABS) (supplementary data 1). This discrepancy suggests that anisotropic nanomaterials like nanoplates are more susceptible to surface masking and subsequent steric hindrance during the pre-mixing phase of the One-Step protocol. Consequently, the mitigation of steric hindrance via the Two-Step protocol yields a more pronounced analytical advantage for gold nanoplates than for conventional spherical nanoparticles [23].

2.2 Integration of Chemiluminescence for High-Sensitivity Detection

Building upon the validation of the sequential Two-Step protocol, we sought to lower the detection limit of the LFIA platform by substituting the conventional gold nanoparticle colorimetric probes with an enzyme-linked chemiluminescence signaling modality. We developed an enzyme-mediated immunochromatographic system using an HRP-conjugated antibody probe.

Our initial evaluations confirmed that a low concentration of sEVs (1.0×10^8 particles), which was completely undetectable by visual inspection under standard colorimetric gold probing, produced a highly discernible, visible band when processed through the chemiluminescence imaging system (Fig. 3). The transition to an enzyme-based signal amplification strategy successfully enabled the visualization of low-abundance targets that fall below the colorimetric detection threshold.

2.3 Comparative Analysis of Modalities and Strategic Selection Guidelines

To fully characterize the versatility of our optimized LFIA platform, we investigated the performance of various antibody labels under the Two-Step protocol. For colorimetric detection, we evaluated anisotropic gold nanoplates (AuPL) alongside standard spherical gold nanoparticles (AuSP). Anisotropic gold nanomaterials often possess superior localized surface plasmon resonance (LSPR) properties, offering higher molar extinction coefficients and enhanced colorimetric sensitivity compared to spherical alternatives [23]. For chemiluminescent detection, an ALP-conjugated antibody system was prepared and evaluated alongside the HRP-conjugated platform.

Our comprehensive evaluations revealed distinct performance trade-offs between the primary signaling modalities (Table 1). Interestingly, changing the specific label within a modality—comparing spherical AuSP against anisotropic AuPL, or HRP against ALP—produced negligible shifts in the absolute analytical detection limits (Table 1). However, the choice between the colorimetric and chemiluminescent modalities resulted in a profound performance divergence.

The chemiluminescence-based system achieved an exceptional detection limit down to the 10^5 particle number range, representing a 40- to 60-fold sensitivity increase over the colorimetric gold nanoparticle system (Table 1). This 10^5 particles order of sensitivity holds great clinical significance. Recent oncology profiles indicate that liquid biopsy targets, such as cancer-specific circulating sEVs expressing HER2 or CEA, often exist within clinical serum ranges of 10^5 to 10^6 particles/mL during early-stage malignancies [24]. Thus, our chemiluminescence LFIA achieves the threshold required for non-invasive clinical screening without requiring massive sample volumes or tedious pre-purification pipelines.

Conversely, evaluating the dynamic range for quantification revealed that the colorimetric gold nanoparticle modality possessed a significantly wider linear quantitative range compared to the chemiluminescence assay.

Specifically, the AuSP system maintained stable, predictable linearity across a broad concentration spectrum spanning over 3 orders of magnitude (from 1.0×10^7 to 1.0×10^{10} particles), proving highly resistant to early signal saturation.

Furthermore, a prominent divergence in high-concentration behavior was observed between the two chemiluminescent enzymes. While the HRP-mediated signal peaked at 1.0×10^8 particles (173.76 a.u.) and rapidly declined at higher concentrations (11.86 a.u. at 1.0×10^9 particles and 2.56 a.u. at 1.0×10^{10} particles), the ALP-mediated system maintained a steady increase in signal intensity up to 1.0×10^{10} particles (129.19 a.u.) (supplementary table 1). The sharp signal attenuation in the HRP system is highly attributable to rapid enzyme-substrate kinetics leading to premature substrate depletion and the suicide inactivation characteristic of peroxidase enzymes under high target concentrations [25, 26]. In contrast, ALP utilizes dioxetane-based substrates to generate a sustained, steady-state chemiluminescence, preventing rapid signal decay and extending the upper limit of quantification [27]. Therefore, although the HRP-based modality offers unparalleled sensitivity for trace antigen detection, the ALP-based or colorimetric systems provide a significantly wider linear dynamic range and higher resistance to signal

saturation, offering distinct advantages depending on the experimental purpose. These observations establish clear guidelines for selecting the optimal assay configuration based on the clinical or experimental objective.

Specifically, the chemiluminescence LFIA modality is ideally suited for applications requiring high-sensitivity screening of trace antigens or early-stage diagnostic detection where sample concentrations are minimal. In contrast, the gold nanoparticle colorimetric modality is highly advantageous for the precise quantification of surface antigens across cohorts known to display broad, highly variable expression ranges among individuals.

Conclusion

In this study, we successfully developed a rapid, simple, and versatile lateral flow immunochromatographic assay platform optimized for the quantitative analysis of sEV surface antigens. By changing from a conventional One-Step mixture profile to a sequential Two-Step loading protocol, we effectively eliminated the limitations imposed by steric hindrance and probe masking on the sEV membrane surface, significantly increasing capture efficiency and signal intensity. Furthermore, by coupling the Two-Step protocol with an advanced chemiluminescence system, we established a highly sensitive analytical platform.

This fully optimized analytical platform offers a dual-modality toolkit for biomarker verification. Investigators can leverage the chemiluminescence configuration to achieve ultra-sensitive detection limits at the 10^5 particle level, meeting the requirements for clinical liquid biopsy applications. Alternatively, users can deploy the gold nanoparticle colorimetric configuration to exploit its broad linear dynamic range for stable quantification across highly variable donor profiles. Given its low operational cost, lack of complex machinery, and high-throughput execution, this optimized LFIA framework represents a significant advancement toward routine, accessible sEV-based diagnostics in clinical and research settings.

Materials & Methods

4.1 Cell Culture and sEV Isolation

The human breast cancer cell line MCF7 was used to obtain cancer-derived sEVs. MCF7 cells were maintained in RPMI 1640 medium (189-02025, FUJIFILM Wako Pure Chemical, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; 10091148, Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin-amphotericin B (PSA; 161-23181, FUJIFILM Wako Pure Chemical, Japan) inside a humidified incubator at 37°C and 5% CO₂. The cells were initially expanded in 100-mm dishes until reaching 90% confluency, and subsequently passaged into ten 150-mm dishes. Upon reaching 80% confluency, the culture supernatant was removed, and the cell monolayers were washed twice with sterile Dulbecco's phosphate-buffered saline without calcium and magnesium (D-PBS(-)). The medium

was then replaced with Advanced RPMI 1640 (12633012, Thermo Fisher Scientific, USA) supplemented with 1% GlutaMAX (35050061, Thermo Fisher Scientific, USA) and 1% PSA. After 72 hours of incubation, 200 mL of the cell culture supernatant was collected for sEV preparation.

sEVs were isolated from the harvested culture supernatant via a series of differential centrifugation steps. First, the supernatant was centrifuged at $10,000 \times g$ at 4°C for 30 minutes, followed by filtration through a 220-nm filter (180E13E, Sartorius AG, Germany). The filtrate was then subjected to ultracentrifugation at $175,000 \times g$ at 4°C for 95 minutes using a SW32Ti rotor (Optima XE-90 Ultracentrifuge, Beckman Coulter, USA). The resulting pellet was resuspended in 4 mL of PBS (166-23555, FUJIFILM Wako Pure Chemical, Japan), washed via a second round of ultracentrifugation at $210,000 \times g$ at 4°C for 45 minutes, and finally resuspended in 200 μL of PBS supplemented with 1% EV-Save (058-09261, FUJIFILM Wako Pure Chemical, Japan) for subsequent analysis.

4.2 Characterization and Validation of Isolated sEVs

The particle concentration and size distribution of the isolated sEVs were measured using a NanoSight LM10 (Malvern Panalytical, UK). The sEV stock solution was diluted 1:1000 with PBS buffer prior to analysis. Three video cycles of 1500 frames were captured under the following tracking parameters: Screen Gain: 1.0, Camera Level: 15, and a Detection Threshold of 5 (Supplementary Fig. 1- (a)).

For Western blot analysis, extracellular vesicles (sEVs) isolated from the culture supernatant of MCF7 cells were analyzed. Purified sEV lysates were prepared using 1.0×10^9 particles as determined by nanoparticle tracking analysis. All samples were mixed with NuPAGE LDS sample buffer (NP0007, Thermo Fisher Scientific, USA) and heated under non-reducing conditions at 95°C for 5 minutes to preserve tetraspanin structures. Proteins were separated by SDS-PAGE using a NuPAGE gel (NP0322, Thermo Fisher Scientific, USA) at a constant voltage of 200 V for 50 minutes, alongside a 3-color High Range Protein Marker (PM2600, Cosmo Bio Co., Ltd., Japan). Separated proteins were transferred onto a PVDF membrane (ISEQ00010, Merck, Germany) using a Trans-Blot Turbo System (1704150, Bio-Rad, USA), at 1.3 A and 25 V for 10 minutes. The membrane was blocked with Tris-buffered saline (1706435, Bio-Rad, USA) containing 0.05% Tween 20 (160-21211, FUJIFILM Wako Pure Chemical, Japan) and 2% skim milk for 60 minutes at room temperature with gentle agitation. The membrane was incubated with a primary antibody against CD9 (SHI-EXO-M01, Cosmo Bio Co., Ltd., Japan) at a concentration of 1 $\mu\text{g}/\text{mL}$ overnight at 4°C . After washing with TBS-T, the membrane was incubated with an HRP-conjugated anti-mouse IgG secondary antibody (NA931, Cytiva, USA) (1:5000 dilution) for 10 minutes at room temperature. After additional washing steps, protein bands were visualized using an enhanced chemiluminescence substrate (ImmunoStar LD, FUJIFILM Wako Pure Chemical, Japan) and detected using an ImageQuant LAS 4000 imaging system (Cytiva, USA) with an exposure time of 10 seconds (Supplementary Fig. 1- (b)).

4.3 Preparation of LFIA Immunostrips

The LFIA immunostrips were fabricated by Hokudo Co.,Ltd (Sapporo, Japan). Briefly, a nitrocellulose membrane (IAB180, ADVANTEC, Tokyo, Japan) and absorbent pads (CFSP223000, Merck, Germany)

were sourced from Advantec Inc.. The test line on the membrane was prepared by dispensing a 2.5 mM phosphate buffer at pH 7.6 (162-27175, FUJIFILM Wako Pure Chemical, Japan) containing 50 µg/mL of anti-CD9 antibody and 5% sucrose (37000-00, Kanto Chemical Co., Inc., Japan) using an automated dispenser (XY3060, BioDot, USA). The membrane was blocked with a 10-fold diluted Blockmaster PA1080 (J-PA1080, MBL, Japan) with gentle agitation at room temperature for 30 minutes, and then dried at 30°C for 30 minutes. Finally, the membrane was assembled onto a backing sheet (Lohmann, Germany) alongside the absorbent pads and precision-cut into individual strips measuring 40 mm in length and 3 mm in width.

4.4 Labeling of Antibodies with Nanoparticles and Enzymes

For colorimetric signaling, anti-CD9 antibodies were conjugated to spherical gold nanoparticles (EM.GC40/7, BBI Solutions, Crumlin, UK) (AuSP, 40 nm) and gold nanoplates (Au-WPPLC1-C, DAI NIPPON TORYO CO., LTD., Japan) (AuPL, 30 nm). One milliliter of each gold nanoparticle dispersion was mixed with 50 µL of 100 mM borate buffer (BB: Boric acid (027-02192, FUJIFILM Wako Pure Chemical, Japan), pH 8.0, prepared in-house) for pH adjustment. Subsequently, 100 µL of anti-CD9 antibody solution (50 µg/mL in 5 mM BB) was added, followed by a 1-hour incubation at room temperature. Stabilizing agents were added sequentially: 50 µL of 5 mM BB containing 0.5% PEG (168-11285, FUJIFILM Wako Pure Chemical, Japan) (30-minute incubation) and 100 µL of 5 mM BB containing 2% BSA (A7030, Merck, Germany) (1-hour incubation). The labeled mixtures were centrifuged, the supernatants were discarded, and the resulting nanosphere/nanoplate pellets were resuspended in 10 mM HEPES buffer (GB60, Dojindo Laboratories, Japan) (pH 7.5) containing 1% BSA, 150 mM NaCl (37144-00, Kanto Chemical Co., Inc., Japan), and 0.05% LIPIDURE-BL802 (NOF, Japan). The concentration of the final dispersions was adjusted to an optical absorbance of 2.0 at their respective maximum absorption wavelengths.

For chemiluminescent signaling, anti-CD9 antibodies were covalently linked to Horseradish Peroxidase (LK33, HRP) or Alkaline Phosphatase (LK12, ALP) using commercial conjugation kits (Dojindo Laboratories, Japan) according to the manufacturer's protocols. The enzyme-antibody conjugates were diluted in a storage buffer (10 mM HEPES, pH 7.5, 1% BSA, 150 mM NaCl, 0.05% LIPIDURE-BL802) to a final antibody concentration of 0.35 µg/mL.

4.5 Lateral Flow Immunoassay Protocols (One-Step vs. Two-Step)

Purified sEV solutions were diluted to the target concentrations using PBS(-) buffer containing 0.05% Tween 20 and 1% BSA.

One-Step Method

A pre-mixed solution containing 20 μL of the sEV sample and 30 μL of the labeled antibody conjugate was incubated at room temperature for 30 minutes and then allowed to migrate completely along the immunostrip. Finally, 100 μL of washing buffer (10 mM HEPES, pH 7.5, 1% BSA, 150 mM NaCl, 0.05% LIPIDURE-BL802) was applied to remove excess unbound antibody conjugates from the membrane.

Two-Step Method

First, 20 μL of the sEV sample solution was loaded and fully migrated up the blocked immunostrip to allow target capture. The strip was briefly flushed with 10 μL of washing buffer. Subsequently, 30 μL of the labeled antibody conjugate solution was loaded onto the strip to bind to the immobilized sEVs. A final wash with 100 μL of buffer was applied to clear the membrane background.

Statistical Analysis

All quantitative data are expressed as the mean $\pm$ standard deviation (SD) from three independent technical replicates ($n = 3$). Statistical significance between the One-Step and Two-Step methods was determined using a two-tailed Welch's t-test. A p-value of < 0.05 was considered to indicate statistical significance.

4.6 Signal Detection and Quantitative Image Analysis

Colorimetric signals generated by the test line under the AuSP and AuPL labels were detected and quantified using a specialized immunochromatography (IC) reader (C10066-10, Hamamatsu Photonics, Japan).

For chemiluminescent detection, following the final washing step, the absorbent pad section was removed, and 15 μL of substrate solution (ImmunoStar LD for HRP conjugate or PhsphaGLO Reserve AP Substrate (5430-0053, SeraCare, USA) for ALP conjugate) was evenly applied across the nitrocellulose membrane. Chemiluminescent signals on the test line were captured using an ImageQuant LAS 4000 imager. Quantitative digital image analysis was performed using ImageJ software. Identical regions of interest (ROIs) were defined over the antibody-immobilized test line and an adjacent clear membrane background area to measure mean gray values. Total integrated brightness values were calculated by multiplying the mean intensities by the ROI area. The final analytical detection intensity was calculated by subtracting the integrated background brightness from the integrated test line brightness.

Furthermore, the limit of detection (LOD) for each assay was calculated using the following equation: $3.3 \times (\sigma / S)$, where σ is the standard deviation of the blank responses and S is the slope of the calibration curve, in accordance with the International Conference on Harmonisation (ICH) Q2(R1) guidelines [28].

Statistical Analysis

All quantitative data are expressed as the mean $\pm$ standard deviation (SD) from three independent technical replicates ($n = 3$). Statistical significance between the One-Step and Two-Step methods was

determined using a two-tailed Welch's t-test. A p-value of < 0.05 was considered to indicate statistical significance.

Declarations

Data Availability All data supporting the findings of this study are available within the paper and its Supplementary Information files.

Author Contributions M.W. and Y.M. contributed equally to this work. M.W., Y.M., and T.S. conceptualized the study and designed the experiments. M.W. and T.Y. performed the sEV isolation, characterization, and chemiluminescence assays. Y.M. and Y.S. synthesized the gold nanoplates and performed the colorimetric assays. M.W., Y.M. and T. S. analyzed the data and wrote the main manuscript text. M.W. supervised the project and critically revised the manuscript. All authors reviewed and approved the final manuscript.

Competing Interests M.W., T.Y., and T.S. are employees of Shimadzu Corporation. Y.M. and Y.S. are employees of Dai Nippon Toryo Co., Ltd. The authors declare that the LFIA platform and nanomaterials utilized in this study are related to the commercial research and development activities of their respective employers. The authors declare no other competing interests.

Acknowledgements The authors thank ForDx Inc. for their technical assistance in the fabrication of the LFIA immunostrips.

Funding No specific funding was received for this study.

References

1. Pan, B. T. & Johnstone, R. M. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**, 967–978 (1983).
2. Valadi, H. et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell. Biol.* **9**, 654–659 (2007).
3. Kalluri, R. & LeBleu, V. S. The biology, function, and biomedical applications of exosomes. *Science* **367**, eaau6977 (2020).
4. Kabe, Y. & Handa, H. Exosome analysis for clinical applications. *Clin. Chem.* **64**, 10 (2018).
5. Zheng, Y. et al. The new advance of exosome-based liquid biopsy for cancer diagnosis. *Discover Oncol.* **15**, 12 (2024).
6. Hoshino, A. et al. Extracellular vesicle and particle biomarkers define multiple human cancers. *Cell* **182**, 1044–1061 (2020).
7. Yu, D. et al. Exosomes in cancer diagnostics and therapeutics. *Signal. Transduct. Target. Ther.* **7**, 211 (2022).

8. Ueda, K. et al. Proteomic analysis of exosome-like vesicles in human plasma. *Sci. Rep.* **4**, 6232 (2014).
9. Théry, C. et al. 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.* **7**, 1535750 (2018).
10. Andreu, Z. & Yáñez-Mó, M. Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* **5**, 442 (2014).
11. Yoshioka, Y. et al. Ultra-sensitive liquid biopsy of circulating extracellular vesicles using ExoScreen. *Nat. Commun.* **5**, 3591 (2014).
12. Zhang, M. et al. Recent advances in wash-free detection methods of extracellular vesicles. *ACS Sens.* **8**, 3012–3025 (2023).
13. Shao, H. et al. New technologies for analysis of extracellular vesicles. *Chem. Rev.* **118**, 1917–1950 (2018).
14. Im, H. et al. Label-free detection and molecular profiling of exosomes with a nano-plasmonic sensor. *Nat. Biotechnol.* **32**, 490–495 (2014).
15. Kwizera, E. A. et al. Molecular profiling of exosomes using surface-enhanced Raman scattering. *Theranostics* **8**, 2722–2738 (2018).
16. Zhao, Z. et al. ExoSearch: A next-generation multiplexed blood test for blood exosome characterization. *Lab. Chip.* **16**, 489–496 (2016).
17. Oliveira-Rodríguez, M. et al. Development of a rapid lateral flow immunoassay test for detection of exosomes previously enriched from cell culture medium and body fluids. *J. Extracell. Vesicles.* **5**, 31803 (2016).
18. Anfossi, L. et al. Advancements and applications of lateral flow assays (LFAs): a comprehensive review. *Diagnostics* **14**, 520 (2024).
19. Koczula, K. M. & Gallotta, A. Lateral flow assays. *Essays Biochem.* **60**, 111–120 (2016).
20. Parolo, C. et al. Tutorial: design and fabrication of nanoparticle-based lateral-flow immunoassays. *Nat. Protoc.* **15**, 3788–3816 (2020).
21. Bishop, J. D. et al. Sensitivity enhancement in lateral flow assays: a systems perspective. *Lab. Chip.* **19**, 2486–2499 (2019).
22. Rey, S. G. et al. Mitigating the hook effect in lateral flow sandwich immunoassays using real-time reaction kinetics. *Anal. Chem.* **89**, 5095–5100 (2017).
23. Wang, C. et al. Tailoring noble metal nanoparticle designs to enable sensitive lateral flow immunoassay. *Theranostics* **12**, 574–596 (2022).
24. Liu, B. et al. Absolute Quantification of Serum Exosomes in Patients with an SERS-Lateral Flow Strip Biosensor for Noninvasive Clinical Cancer Diagnosis. *Anal. Chem.* **94**, 1234–1242 (2022).
25. Valderrama, B. et al. Suicide inactivation of peroxidases and the challenge of engineering more robust enzymes. *Chem. Biol.* **9**, 555–565 (2002).

26. Kricka, L. J. Clinical applications of chemiluminescence. *Anal. Chim. Acta.* **266**, 239–245 (1993).
27. Hananya, N. & Shabat, D. A glowing trajectory between bio- and chemiluminescence: from luciferin-based probes to triggerable dioxetanes. *Angew Chem. Int. Ed.* **56**, 16454–16463 (2017).
28. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. ICH Harmonised Tripartite Guideline: Validation of Analytical Procedures: Text and Methodology Q2(R1). (2005).

Table 1

Table 1 is available in the Supplementary Files section.

Figures

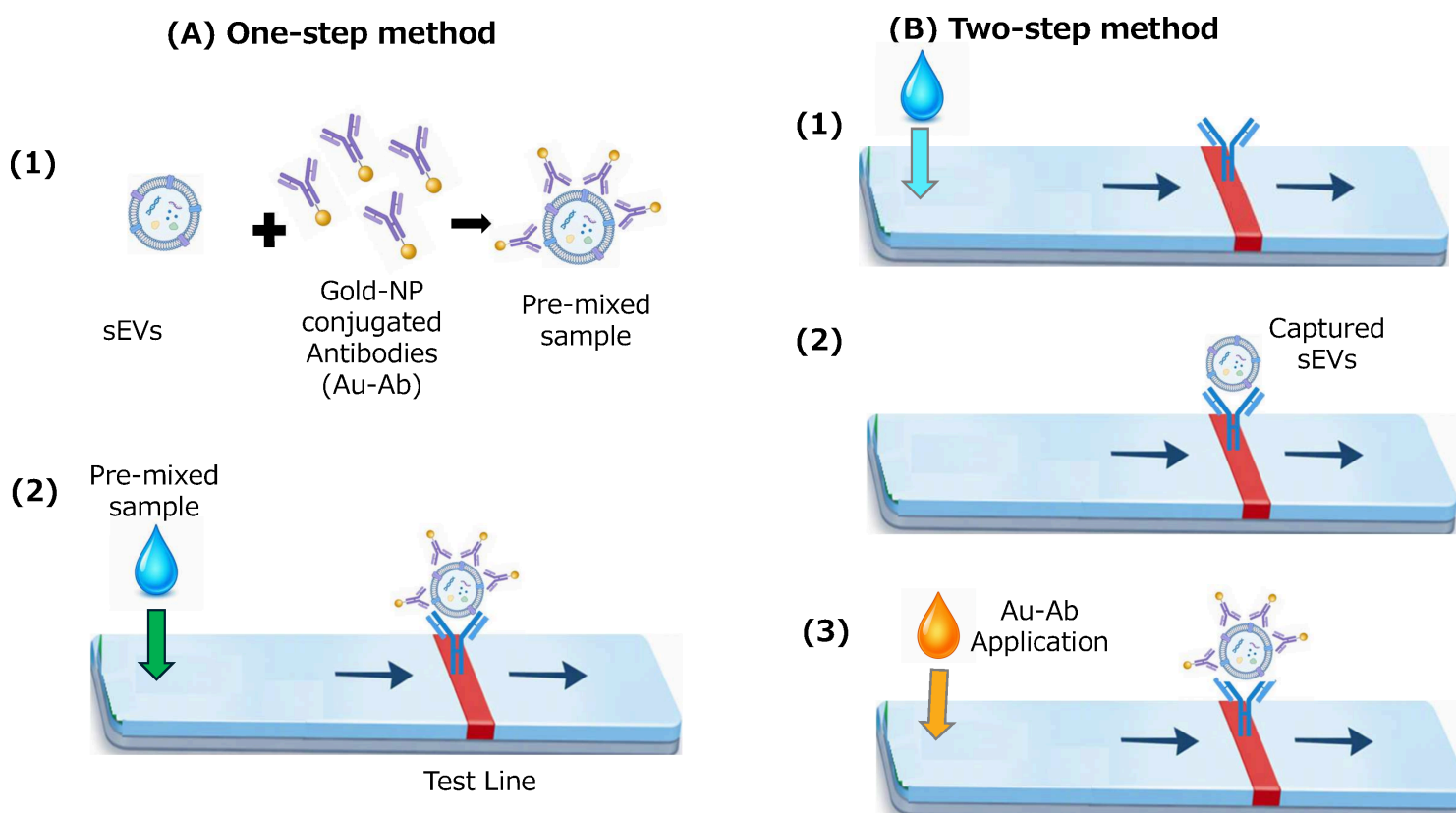


Figure 1

Schematic illustration of the sample loading protocols for the LFIA strip.

(A) One-Step method: The gold-conjugated antibody (Au-Ab) and sEV sample are pre-mixed to allow antigen-antibody complex formation (1). The immunostrip is then immersed in this mixture, and sEVs captured by the immobilized antibody on the test line are detected via colorimetric absorption (2).

(B) Two-Step method: The immunostrip is sequentially immersed in the sEV-containing solution (1) and the Au-Ab solution (2). The captured sEVs are subsequently quantified based on the test line color

density (3).

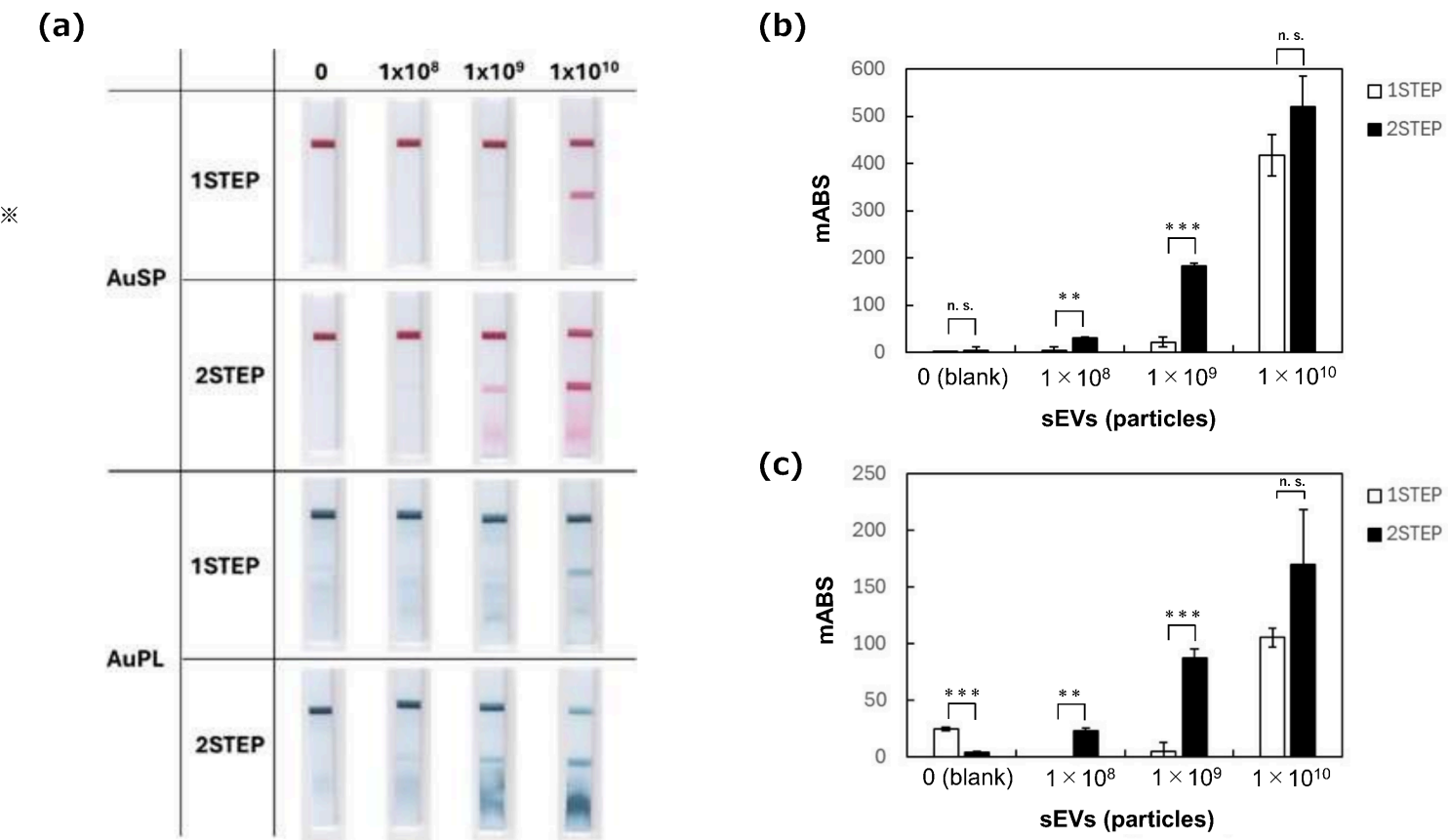


Figure 2

Comparison of LFIA detection sensitivity for sEVs using different sample loading methods.

(a) Signal intensities (mABS) obtained using the One-Step and Two-Step methods are compared across various sEV concentrations. Detection was performed using (b) AuSP-labeled antibodies and (c) AuPL-labeled antibodies. Error bars represent standard deviations ($n = 3$). * $p < 0.05$, ** $p < 0.01$ (One-Step vs. Two-Step via two-tailed Welch's t-test). n.s. = not significant.

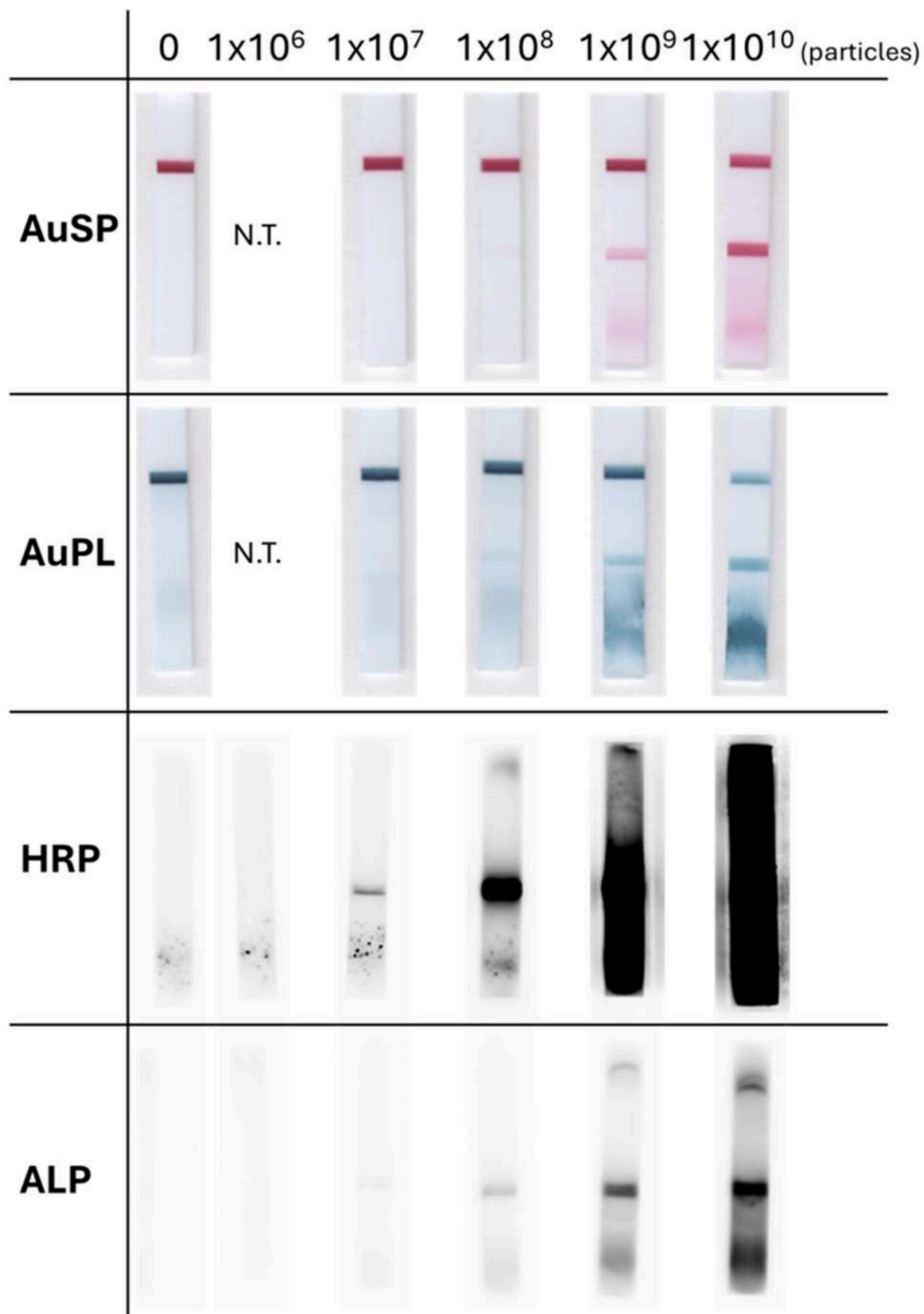


Figure 3

Qualitative comparison of sEV detection using various labeled antibodies.

Representative images of the LFIA strips across different sEV concentrations. Chemiluminescent detection was performed using HRP (exposure time: 30 s) and ALP (exposure time: 120 s). N.T.: not tested.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table.1.pdf](#)
- [SupplementaryInformation.pdf](#)