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Exosome

Research Products Ver.3

FUJIFILM Wako Pure Chemical Corporation

Contents

In recent years, research of extracellular vesicles (EVs) has been advancing at an accelerating pace. While the number of scientific articles on EVs published in 2011 was approximately two hundred, the number increased to more than one thousand in 2016 and involvement of EVs in various physiological functions and pathogenic mechanisms has been suggested. Although EVs are roughly classified into at least two categories: exosomes derived from endosomes and microvesicles derived from the plasma membrane, it is difficult to strictly separate them from each other by differential centrifugation, the technique most frequently used for purification of EVs at present, and the EVs not sedimenting at 10,000×g are called "small EVs" (mainly composed of exosomes) for convenience.[1)] Exosomes are small membrane vesicles (approximately 30-100 nm in diameter) secreted by various cells and present in most body fluids (e.g., blood, urine, and spinal fluid) and cell culture liquids. Exosomes, membrane vesicles surrounded by a lipid bilayer, are generated within intracellular vesicles called "multi-vesicular endosomes" and released into the extracellular space by fusion of multi-vesicular endosomes with the cell membrane. Exosomes contain proteins from secretory cells, including those of endosome origin (e.g., ESCRTs), those involved in intracellular transport (e.g., Rab GTPase), and those of cell membrane origin (e.g., CD63 and CD81), as well as RNAs. Exosomes also contain the cell membrane of secretory cells and lipids from the endosome membrane (cholesterol and sphingomyelin, etc.).[2)] Although exosomes had long been considered to be involved in release of unnecessary cell contents, exosomes are recently attracting attentions of researchers as new mediators of cell-cell communication transporting biomolecules such as lipids, proteins, and RNAs *in vivo*. In addition to clarification of physiological or pathophysiological functions of exosomes, research aiming at clinical application of these functions is rapidly in progress, particularly focusing on diagnostic and therapeutic application as well as development of biomarkers.

Current exosome research covers almost all research areas in biomedical science (immunology, neuroscience, oncology, endocrinology, and cardiovascular research). For example, exosomes derived from immune cells have been shown to contain antigen peptide/MHC complexes and various antigens, which suggests a possibility that exosomes might regulate various immune responses such as activation/inactivation of immune cells in addition to the exchange of antigenic information between immune cells.[3)] In the nervous system, exosomes have been found to be involved not only in regulation of neural circuits[4)] but also in extracellular release of proteins causing various neurodegenerative diseases for subsequent transmission to other cells, a process that might be deeply involved in disease progression.[5)] Exosomes released by cancer cells contain many biomolecules related to angiogenesis and immune evasion, suggesting that they might contribute to construction of microenvironment optimal for cancer cell growth and promotion of cancer progression.[6)] In addition, the expression profile of adhesion molecules on the surface of exosomes from cancer cells has been shown to determine the destination of cancer metastasis.[7)] Recently, exosomes released from adipocytes have been reported to regulate gene expression in the liver.[8)] Furthermore, while many viruses leave cells by utilizing the pathway for exosome production, bacteria and parasites infecting cells are likely to regulate activities of bacteria/parasites infecting other cells via exosomes.[9, 10)]

Most of the above-mentioned functions are mediated by secretory cell-derived biomolecules located within exosomes. In particular, since mRNAs and miRNAs of secretory cell origin were identified in exosomes, potential involvement of exosomes in horizontal transmission of gene expression information between cells has been attracting great research interest.[11)] Since these RNAs are encapsulated within the lipid bilayer membrane of exosomes, they are not susceptible to degradation by RNase and remain intact in blood or other body fluid. Exosomes incorporated into target cells fuse with the endosome membrane to release encapsulated RNAs into the cytosol of target cells. Once released into the cytosol, mRNAs are translated into proteins while miRNAs suppress translation of target genes. Thus, exosomes regulate gene expression within target cells. A single exosome is considered to contain more than several ten-thousands of proteins and more than several thousands of mRNAs and miRNAs. The composition of these biomolecules may vary depending on the type and conditions of a secretory cell which originally harbored the exosome. In addition, the composition of proteins, mRNAs, and miRNAs within an exosome is different from that within the original secretory cell, which suggests the existence of a mechanism selectively loading exosome- specific proteins and mRNAs/miRNAs into exosomes. Such specific composition of exosome RNAs makes them attractive candidates for biomarkers and targets for therapeutic development. While mRNAs within exosomes incorporated into target cells are capable of inducing expression of functional proteins, most miRNA within exosomes are present as precursors of functional miRNA and their physiological significance is under extensive investigation. Thus, since exosomes contain a wide variety of proteins, RNAs, and lipids, construction of an exosome database "ExoCarta" is currently ongoing through classification by cell types. Furthermore, while large scale analysis of exosomes utilizing cutting-edge methodologies in proteomics, transcriptomics, and system biology are separately conducted in laboratories all over the world, EV plugin for FunRich (a stand-alone functional enrichment analysis tool) is distributed aiming at a common and integrated analysis tool. Sharing information among researchers in a wide variety of research fields is essential for promotion of future exosome research.

Development of exosome-based therapeutic and diagnostic methods

In parallel with clarification of exosome functions, efforts to develop therapeutic methods applying exosome functions are being continued in recent years. For example, exosomes released from blood fibrocytes (a population of mesenchymal progenitors) accelerate wound healing by stimulating angiogenesis and inducing migration and proliferation of keratinocytes. Proangiogenic, anti-inflammatory miRNAs as well as a miRNA promoting collagen deposition within these exosomes are reportedly involved in this process.[12)] In addition, exosomes released from dendritic cells in patients with cancer

contain a variety of cancer cell-derived proteins and induce intense activation of cancer cell-specific cytotoxic T lymphocytes. Development of cancer immunotherapy based on this mechanism is currently in the early phase of clinical research.[13] On the other hand, suppression of exosomal functions involved in pathogenesis has also been attempted. For example, apoptosis- inducing TNF-α is accumulated at high concentrations in exosomes released from synovial fibroblasts in patients with rheumatoid arthritis and exacerbates the pathology of rheumatoid arthritis.[14] In addition, since cancer cell derived exosomes contain molecules related to cancer progression and neuron-derived exosomes contain molecules related to neurodegenerative diseases as described above, inhibition or removal of these exosomes may potentially suppress onset of these diseases. Advancement of future research is expected to clarify exosome functions and expand indications of clinically applied exosomes, thereby realizing utilization of exosomes for the treatment of various diseases. Furthermore, delivery of drugs such as siRNAs and anticancer agents to target cells using exosomes has been attempted. Since various cell adhesion molecules are expressed on the surface of exosome membrane and the expression profile of these molecules has been found to determine the target cells for exosome delivery, application of this property to development of a new drug delivery system (DDS) is expected.[15] Exosomes are extremely stable in body fluids, and the exosome lipid bilayer membrane encapsulating proteins and RNAs within vesicles protects them from degradation. Furthermore, exosomes remain relatively intact even in body fluid specimens stored for a long time after collection and are therefore considered as new and promising laboratory biomarkers for diseases. While correlations between exosomes and various diseases have been investigated, cancer cell-derived exosomes released into blood are recently attracting research interest due to difference in constituents from normal cell-derived exosomes and a correlation between constituents of cancer cell-derived exosomes and cancer progression has been extensively investigated as a potential tool for early cancer diagnosis.[16] In addition, exosomes in urine are expected as a new diagnostic marker for renal, prostate, and bladder diseases, while exosomes in cerebrospinal fluid as a new marker for brain tumor and neurodegenerative diseases.

Issues and future perspectives of exosome research

Although many studies on roles of exosomes have already been reported, experiments providing evidence for these reported phenomena use highly concentrated exosomes purified from body fluids and cell culture supernatants. Accordingly, whether these phenomena actually occur *in vivo* remains unclear. The sole approach for clarification of physiological actions of exosomes is to clarify the mechanism of exosome release and physiological phenomena induced by exosome release stimulation/inhibition through modulating the mechanism, which is expected to result in further advancement in exosome research. Another important issue to be addressed in future research and development is *in vivo* kinetics of exosomes (i.e., which exosomes are directed to which target cells).

Conventional methods for exosome purification mainly involved ultracentrifugation and various commercial purification kits using polyethylene glycol (PEG) precipitation technique. However, exosome preparations obtained by these methods contain large amounts of contaminants and careful analysis is required to determine whether experimental results obtained are actually due to actions of exosome constituents per se. Furthermore, ultracentrifugation requiring cumbersome manipulation has several disadvantages including inconsistent recovery interfering quantitative analysis and requirement for an expensive instrument not compatible with high-throughput analysis. Conducting exosome research under these circumstances is difficult and development of technology for easy purification of exosomes at a high purity is urgently needed. We focused on Tim4, an exosome receptor expressed on macrophages, and prepared "Tim4 magnetic beads" by immobilizing the extracellular region of Tim4 on magnetic beads.[17] Since Tim4 binds to phosphatidylserine (PS), a phospholipid on the surface of exosome membrane, in a calcium ion-dependent manner, bound exosomes are released from these beads with an elution buffer containing ethylenediaminetetraacetic acid (EDTA), a chelating agent, to obtain highly purified intact exosomes. In fact, when exosomes released from human leukemia cells were purified by the Tim4-affinity method and compared for purity with exosomes purified by ultracentrifugation and PEG precipitation, the Tim4-affinity method yielded exosome preparations with exosome-specific proteins each exhibiting a band intensity over 10-100 times higher than that obtained by other methods and almost free from non-exosome contaminants, thereby demonstrating reproducible recovery of high-purity exosomes. As a result, many previously unidentified exosome proteins and RNAs could be identified from exosome preparations obtained by this method. Furthermore, application of the strong binding affinity of Tim4 toward exosomes realized high-sensitivity detection and assay of exosomes by enzyme-linked immunosorbent assay (ELISA) and fluorescence-activated cell sorting (FACS). On the other hand, while only crude preparations of microvesicles were conventionally obtained because differential centrifugation was the sole purification technique available, the Tim4-affinity method realized purification of microvesicles at a high purity as well. Details of these techniques are described in this guidebook. We expect that usefulness of these Tim4-affinity-based techniques will be appreciated in the world and greatly contribute to clarification of the original physiological functions of exosomes and microvesicles.

In addition to difficulties in detection and isolation of exosomes, the existence of various classification systems for exosomes and resulting lack of consensus among investigators regarding which method should be used for purification of the extracellular vesicles to be called “exosomes” make interpretation of experimental data and confirmation of reproducibility difficult. To overcome such situation, the International Society for Extracellular Vesicles (ISEV) has recently been established to nurture a global community of EV researchers and “Minimal Information for Studies of EVs” (MISEV) Guidelines has been published as international

standard that investigators who intend to start EV research should consult.[18,19] In addition, as a method for avoiding such confusion, EV-TRACK knowledge database has been constructed to record experimental conditions employed in individual EV-related articles.[20] On the other hand, as EV research has attracted global research interest, a number of large-scale research projects have been launched in various countries. In the United States, National Health Institute (NIH) has initiated a strategic large-scale project "Extracellular RNA Communication" and special interest groups on EV research have been organized at prestigious international conferences such as Gordon Conference and Keystone Symposia since 2016. In Europe, research covering EV has already been conducted as a part of CANCER-ID project supported by "Innovative Medicines Initiative (IMI)," a public-private partnership (PPP) for research and development of medicines. In Japan, EV research has been selected as one of the Research and Development Strategic Objectives in 2017 established by the Ministry of Education, Culture, Sports, Science and Technology Japan and acceleration of future research in this field is expected. In any case, development of solid research methodologies and techniques that constitute the basis of EV research is essentially required for future advancement in this research field, and we expect that the Tim4-affinity method will grow up to be one such technique.

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Introduction

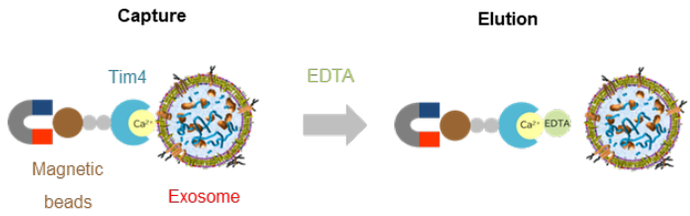
Exosomes are a type of extracellular vesicle (EV) with a diameter of 30-100 nm released from cells. It contains nucleic acids (mRNA, microRNA) and proteins, and there is increasing interest in its role as a tool in intercellular communication and its use as a biomarker in various diseases, including cancer.[1,2] However, exosome experimental technology is still under development and there are many issues.

For example, in exosome purification methods, ultracentrifugation and polymer precipitation (commercially available kits) are known to cause contamination of various substances, which significantly interfere with subsequent experiments. On the other hand, exosomes can be obtained at high purity using antibody affinity and density gradient centrifugation; however, the purified exosomes are not intact, and the physiological function of exosomes cannot be analyzed. Furthermore, western blotting and ELISA are commonly used methods for detecting exosomes, but large amounts of exosomes are required, and marker proteins with low expression levels are difficult to detect. This booklet describes new exosome analysis tools developed by Fujifilm Wako to solve these problems in analytical technologies for exosome research.

Novel Exosome Purification Method Using Phosphatidylserine and Tim4

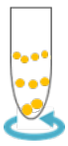
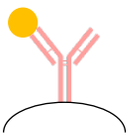
Exosomal membranes contain proteins and lipids derived from secretory cells. Among them, phosphatidylserine (PS) is known to face inside of living cells by the action of the enzyme flippase, whereas it is also exposed to the outside of exosomes.[3] The T-cell immunoglobulin domain and mucin domain-containing protein 4 (Tim4) is a phagocytic receptor of macrophages for apoptotic cells. Tim4 was found to bind to PS via the IgV domain in the extracellular region in a calcium ion-dependent manner.[4]

Based on the above findings, Fujifilm Wako developed a new exosome purification method distinct from existing methods in collaboration with Professor Hanayama of WPI Nano Life Science Institute, Kanazawa University and successfully launched a kit. This method uses magnetic beads coated with Tim4 to capture exosomes in samples such as cell culture supernatant and serum in the presence of calcium ions, and isolates exosomes by adding chelating agents.[5] The PS affinity method employs a simple procedure to achieve a higher purity of intact exosomes than conventional exosome purification methods. It is being established as a new exosome purification method to replace the ultracentrifugation method that has been considered the gold standard.



Isolation and purification of exosomes by the PS affinity methods

Comparison of Exosome Isolation and Purification Methods

	Ultracentrifugation	Density gradient centrifugation	Polymer precipitation	Antibody affinity method	**PS affinity method**
Overview	 Precipitate exosomes by ultracentrifugation at 100,000 x g	 After addition of sucrose, centrifuge and fractionate based on the sedimentation rate	 Precipitate exosomes with polymers such as PEG	 Exosomes are captured with antibodies against antigens on the surface of exosomes	 Exosomes are captured with Tim4, which binds to PS on the surface of exosomes
Purity	++	+++	+	+++	++++
Yield	++	+	++ (Total protein is high)	++	+++ (Total protein is low)
Intactness	Yes (reduce the quality)	Yes	Yes	No	Yes

[Note]
This comparison table is based on our own research. The results may vary depending on the sample, detection target and method, and the performance of each method is not guaranteed.

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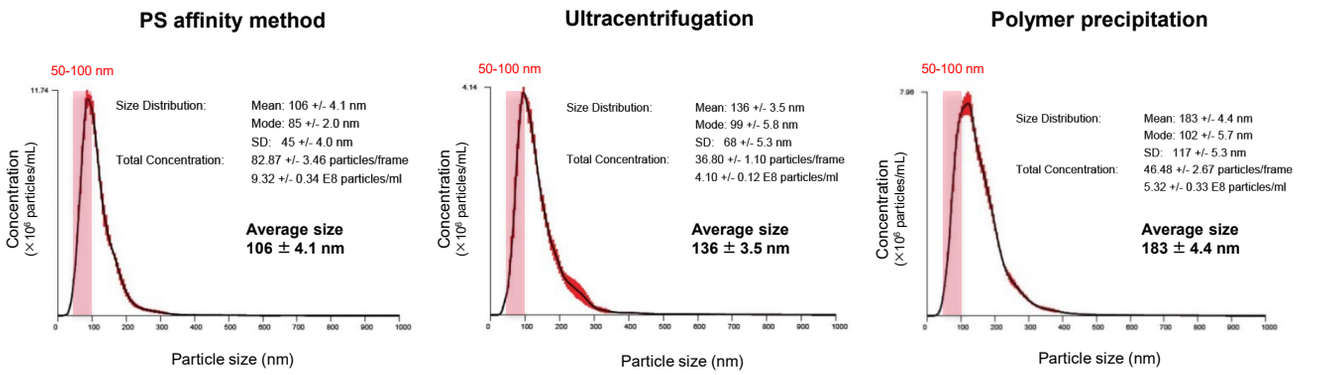
Performance Data for the PS Affinity Method

▼ Particle Analysis by NanoSight

NanoSight is an analytical instrument for the analysis of size and concentration of nanoparticles that functions by visualizing the Brownian motion of nanoparticles in solvent through Nano Tracking Analysis (NTA). Even when the particles in the solvent are mixtures of various materials or polydisperse systems with varying particle sizes, the number of particles having each particle size can be counted using videos of Brownian motion of nanoparticles obtained by the visualization technique.

Exosomes isolated from cell culture supernatants by the PS affinity method, ultracentrifugation, and polymer precipitation method were diluted to appropriate concentrations in ultrapure water, and particle size and concentration were analyzed using NanoSight LM10.



Nakai, W. *et al.*: *Sci. Rep.*, **6**(1), 1(2016).

With the PS affinity method, particles corresponding to the particle size of exosomes (50-100 nm) were more concentrated, indicating higher purity.

▼ Particle Analysis by Electron Microscopy

Electron microscopy is a technique for obtaining magnified images of specimens using an electron beam. As an electromagnetic wave, an electron beam has a very short wavelength, allowing for morphological observation at a much higher magnification than optical microscopes. Electron microscopy is used to observe and analyze all types of samples, from metals and polymers to water-containing materials such as biological tissues, plants, and foods. Below is an example of electron microscopic analysis of exosomes using a transmission electron microscope (TEM).

PS affinity method

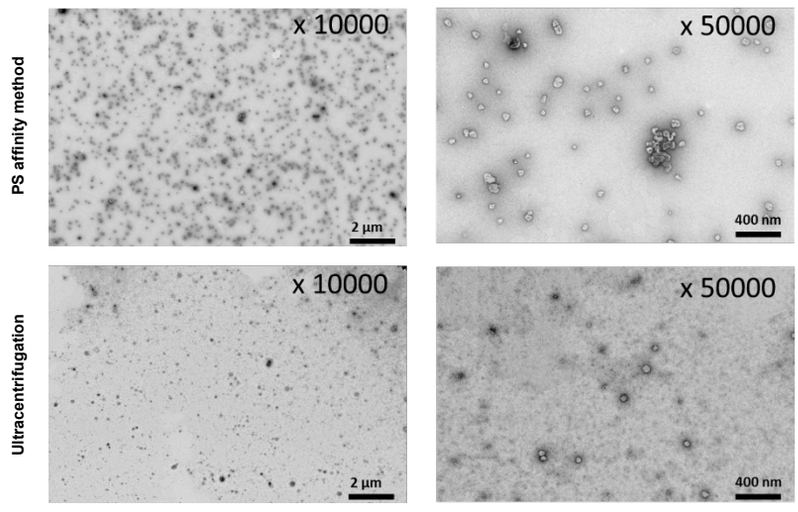
Sample: COLO201 cell culture supernatant 10 mL

Number of particles: 3.69 x 10^{10} particles/mL

Ultracentrifugation

Sample: COLO201 cell culture supernatant 10 mL

Number of particles: 1.68 x 10^{10} particles/mL

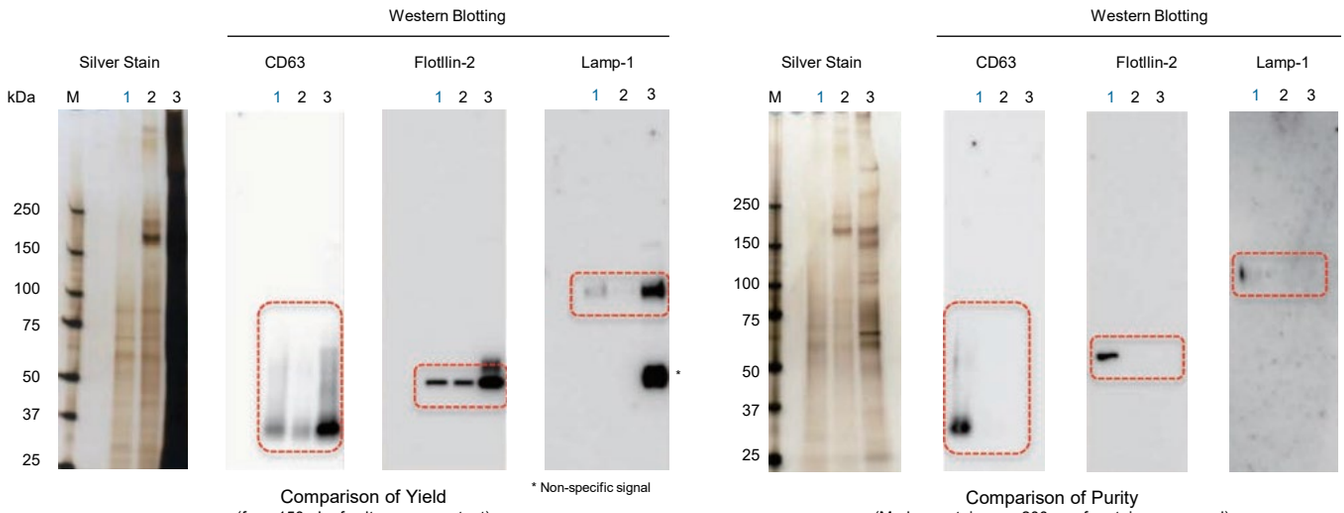


Images were provided by Hanaichi Ultrastructure Research Institute.

A higher number of exosomes was isolated with high purity using the PS affinity method.

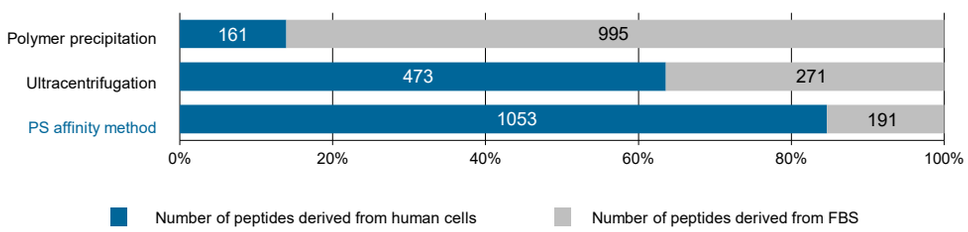
▼ Comparison of Yield and Purity by Western Blotting

Samples prepared from K562 cell culture supernatant (medium supplemented with 10% Exosome-depleted FBS) using ultracentrifugation, polymer precipitation, and the PS affinity method were subjected to electrophoresis followed by detection by silver staining and western blotting (anti-CD63, anti-Flotillin-2, and anti-Lamp-1 antibodies). MS analysis of recovered fractions was also performed to compare the percentage of human peptides derived from K562 cells among all identified peptides.



M: Marker / 1: PS affinity method / 2: Ultracentrifugation / 3: Polymer precipitation (competitor A)

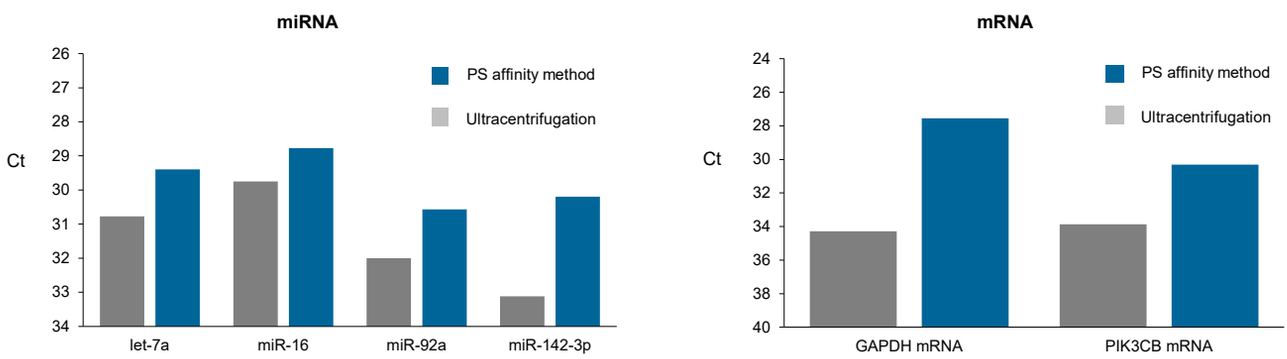
<Supplemental Data> Comparison of human sample-derived peptides identified by MS analysis



Exosomes with high purity were recovered from FBS-containing medium as well using the PS affinity method.

▼ Comparison of Yields of microRNA and mRNA

Exosomes were isolated from healthy human serum using ultracentrifugation and the PS affinity method. RNA was recovered from the isolated exosomes using the microRNA Extractor® SP kit (Product Number: 295-71701), and microRNA (let-7a, miR-16, miR-92a, miR-142-3p) and mRNA levels (GAPDH, PIK3CB) were determined by quantitative PCR, and Ct values were compared.



More microRNA and mRNA were recovered from exosomes isolated using the PS affinity method compared to the ultracentrifugation method.

▼ Proteomics Analysis

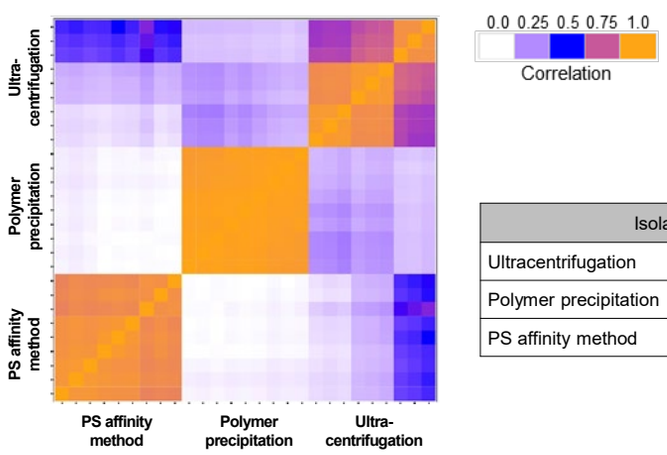
Exosomes were purified from K562 cell culture supernatant (medium supplemented with 10% Exosome-depleted FBS) using ultracentrifugation, polymer precipitation, and the PS affinity method. Purified samples were separated by 10% polyacrylamide electrophoresis, and the entire protein bands were cut out. Subsequently, in-gel digestion was performed, and proteins were identified by LC-MS. The pairwise correlation of identified proteins in exosomes (n = 3) purified by the three methods was also calculated.

(1) Comparison of Top 10 Most Abundant Proteins

Exosome Marker Protein | Exosome-derived Human Protein | FBS-derived protein

	PS affinity method	Ultracentrifugation	Polymer precipitation
1	Heat shock cognate 71 kDa protein	DNA-dependent protein kinase catalytic subunit	Complement C3
2	**Annexin A6**	Transferrin receptor protein 1	Alpha-2-macroglobulin
3	Transferrin receptor protein 1	Serum albumin	Fibronectin
4	V-type proton ATPase catalytic subunit A	ATP-dependent RNA helicase A	Serum albumin
5	**Flotillin-2**	Tubulin beta-5 chain	Thrombospondin-1
6	Programmed cell death 6-interacting protein	Heat shock cognate 71 kDa protein	Complement C4
7	4F2 cell-surface antigen heavy chain	Fatty acid synthase	Alpha-1-antiproteinase
8	**Annexin A1**	4F2 cell-surface antigen heavy chain	Apolipoprotein B-100
9	Kinase D-interacting substrate of 220 kDa	U5 small nuclear ribonucleoprotein helicase	Hemoglobin fetal subunit beta
10	**Annexin A2**	Tubulin beta-4B chain	Tubulin beta-5 chain

(2) Comparison of Pair wise correlation



Isolation methods	Reproducibility
Ultracentrifugation	△
Polymer precipitation	○
PS affinity method	○

Nakai, W. *et al.*: *Sci. Rep.*, **6**(1), 1(2016).

[Column] Total protein amount does not reflect extracellular vesicle amount

To compare EV yields, parameters including particle number, total protein amount, and total lipid amount are generally measured. It should be noted that every method may be affected by contaminants in the original sample, resulting in an overestimation of the EV amount.

We compared EV yields after purification using ultracentrifugation (UC) and PS affinity method (PS), based on total protein amounts measured using BCA (Table 1), particle number counted by NTA (Table 2) and EV marker (CD9, CD63 and CD81) signal level evaluated by ELISA (Figure 1). The results showed that more EVs were recovered in the sample purified using the PS method.

As the total protein amount may include contaminant proteins, comparing of the EV amount based on the total protein amount may yield incorrect results.

Table 1 Comparison of total protein amounts measured using BCA

	PS method	UC method
Protein amount	11.8 μg/mL	30.5 μg/mL

Table 2 Comparison of particle numbers counted using NTA

	PS method	UC method
Number of particles	0.21 × 10^{10} particles/mL	0.09 × 10^{10} particles/mL

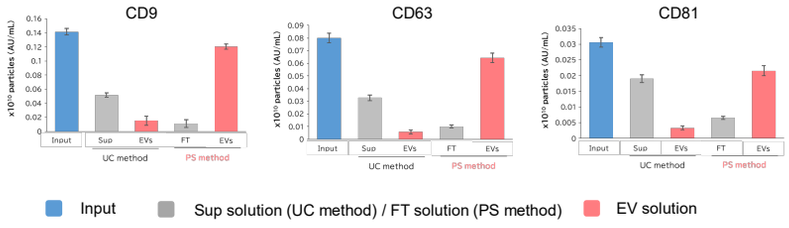


Figure 1 Comparison of EV yields evaluated using tetraspanin ELISA

Advantages of the PS Affinity Method and Frequently Asked Questions (by Prof. Hanayama)

Seven years have passed since Fujifilm Wako commercialized a method for high-performance purification and high-sensitivity quantification using the PS affinity method as a tool for analyzing extracellular vesicles (EVs), including exosomes. During this period, we have received positive feedback from many researchers in Japan and abroad, and we are pleased to have contributed to the advancement of EV research. I would like to take this opportunity to explain the advantages of using the PS affinity method for the analysis of EVs and address some frequently asked questions.

The PS affinity method uses Tim4, one of the receptors for EVs, to capture EVs. Originally evolved as a mechanism to eliminate unwanted molecules in the body, when administered intravenously, EVs are trapped and removed by macrophages in the liver and spleen. Tim4 expressed on macrophages is responsible for this process; it binds to the phospholipid phosphatidylserine (PS) exposed on the EV membrane surface. Thus, the PS affinity method is an analytical technique based on the physiological mechanism of EVs and is distinct from other analytical techniques based on physical characteristics of EVs. In general, analytical methods for EVs using an affinity for molecules on the EV surface offer the advantage of high purity and high specificity. However, EVs are highly heterogeneous, and the expression patterns of tetraspanins (CD9, CD63, CD81, etc.), known as classic EV markers, vary widely depending on the secretory cells present and the sites of secretion. In contrast, the PS affinity method is superior to other affinity analytical methods because it can capture almost all types of EVs equally, regardless of the heterogeneity among EV markers.

A high-performance purification method for EVs known as the MagCapture™ Exosome Isolation Kit PS was developed as the first analytical tool using the affinity method. The unique feature of this tool is that EVs are captured with high efficiency using Tim4-coated beads and then eluted with EDTA, a Ca^{2+} chelating agent, for simple and intact recovery. This method allows for purification of EVs with higher purity compared to ultracentrifugation, and identification of molecules in EVs that could not be identified before is now possible. Other advantages are that high-throughput sample processing is possible, and reproducibility is better compared to ultracentrifugation, which requires expensive equipment and special skills. The PS Capture™ Exosome ELISA Kit was subsequently developed as a highly sensitive quantification method for EVs. This analytical tool is over 100 times more sensitive for the detection of EVs than conventional ELISA with EV capture using anti-tetraspanin antibodies. This is presumably because the binding of Tim4 to EV membranes is stronger and more stable than the binding of antibodies to molecules on EVs.

One advantage of the PS affinity method that is particularly important to emphasize is that the EV purification and quantification methods are based on the same principle. Currently, extensive studies are being conducted around the world to use EVs as a new biomarker of disease. However, EV biomarkers identified using ultracentrifugation frequently fail to be detected by ELISA or other methods. The PS affinity method uses Tim4 to purify and quantify EVs based on the same principle, allowing for seamless identification and detection of EV biomarkers.

Furthermore, a single-particle analysis using flow cytometry is expected to become a mainstream technology for EV analysis in the future. Several companies are currently developing and commercializing high-sensitivity flow cytometers specialized for single-particle analysis of EVs, and they are reaching the level of practical application. With the PS Capture™ Exosome Flow Cytometry Kit, which uses the PS affinity method, bulk (whole) EVs are captured with Tim4 beads, and the sum of signals of EVs can be quantified using conventional flow cytometry. The captured bulk EVs can be easily released from the Tim4 beads by EDTA treatment, and single-particle analysis can be performed with the eluate using a high-sensitivity flow cytometer. In the single-particle analysis of EVs by flow cytometry, washing the EVs after staining with fluorescent antibodies is an important process. This method allows for easy and thorough washing of EVs on Tim4 beads so that specifically stained, intact EVs can be recovered and analyzed in a short time.

Next, I would like to discuss some frequently asked questions about the PS affinity method. The most frequently asked question regards the percentage of EVs that express PS and thus can be captured by the PS affinity method. When the number of EVs in a sample was compared before and after purification by the PS affinity method using the NTA method and ELISA, almost 90% of the EVs were recovered from all cell and body fluid samples. Not only that, EVs purified using the PS affinity method have higher biological activity than EVs purified by ultracentrifugation or other methods, suggesting that more functional EV subpopulations are enriched.

What causes this difference? We set out to examine the difference in EV subpopulations among the various EV purification methods. The compositions of EV subpopulations were analyzed by anion exchange chromatography and compared. As shown in Figure 1, EVs can be fractionated into three subpopulations (non-, low-, and high-anionic) by a salt gradient. Analysis of EV markers and other data indicated that non-anionic EVs contained atypical EVs, low-anionic EVs contained mainly exosomes, and high-anionic EVs contained mainly microvesicles. A higher percentage of low anionic EVs and a lower percentage of high anionic EVs were found in EVs purified by the PS affinity method compared to EVs purified by the ultracentrifugation and size exclusion methods. This is presumably because the binding between Tim4 and high-anionic EVs is very strong in the PS affinity method and cannot be separated by EDTA treatment, resulting in the enrichment of relatively weakly bound low-anionic EVs (mainly exosomes).

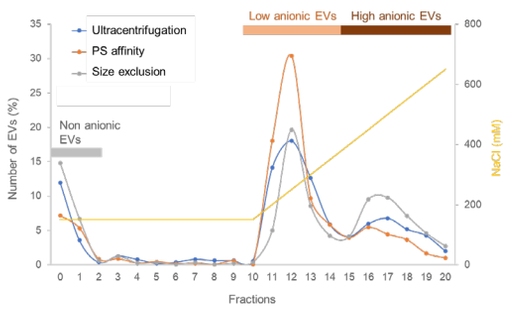


Figure 1 Comparison of EV subpopulations by each isolation method

Another point, researchers seeking EV biomarkers in blood have asked whether the PS affinity method can be used for plasma samples. Since the PS affinity method captures EVs in a Ca^{2+}-dependent manner, we have recommended the use of serum samples rather than plasma samples treated with a Ca^{2+} chelating agent. However, subsequent studies have confirmed that the PS affinity method can be used without problem simply by adding Ca^{2+} to plasma samples, but since the addition of Ca^{2+} promotes blood coagulation, heparin should be added at the same time to avoid coagulation. (Please refer to the respective product manual for specific instructions.)

Thus, the PS affinity method has many advantages and is strongly recommended as a high-performance purification and high-sensitivity quantification method for EVs. On the other hand, quite a few researchers adhere to ultracentrifugation, which has been considered the gold standard method for EV purification. However, at present, no purification method can solve the problem of uneven distribution of EV subpopulations. Currently, there are various analysis methods available for EV research, each of which has advantages and disadvantages. My recommendation is to choose the method that best suits the purpose and application, and the analysis methods that were used must be clearly stated when publishing the results of the research. It is also a good idea to denote EVs analyzed by the PS affinity method as PS^+ EVs, if necessary. What is most important is reproducibility, and if PS^+ EVs show high biological activity or can be used as disease-specific biomarkers with high reproducibility, it is a great research result, and there is no concern.

Recently, the PS affinity method was shown to be a useful analytical tool not only for EVs but also for viruses with envelopes (*Nature*. 2022; 607:345). As seen with ONI's Nanoimager EV Profiler Kit, third-party companies have also begun to use the PS affinity method, and applications are expected to expand further. In particular, the technology to consistently purify large quantities of high-purity EVs must be developed to advance EV-based drug discovery. Further, technologies are being developed to utilize the PS affinity method, which allows for purification of EVs with high biological activity. I hope that the PS affinity method will continue to contribute to the advances of EV research.

Exosome isolation and purification kit with improved exosome yield and purity

MagCapture™ Exosome Isolation Kit PS Ver.2

MagCapture™ Exosome Isolation Kit PS Ver. 2 allows for easy purification of extracellular vesicles such as exosomes with high purity from cell culture supernatants, serum, plasma, and other samples. Purification of intact extracellular vesicles by elution with a chelating agent was achieved by utilizing Tim4 protein, which binds to phosphatidylserine (PS) on the surface of extracellular vesicles in a calcium-dependent manner.

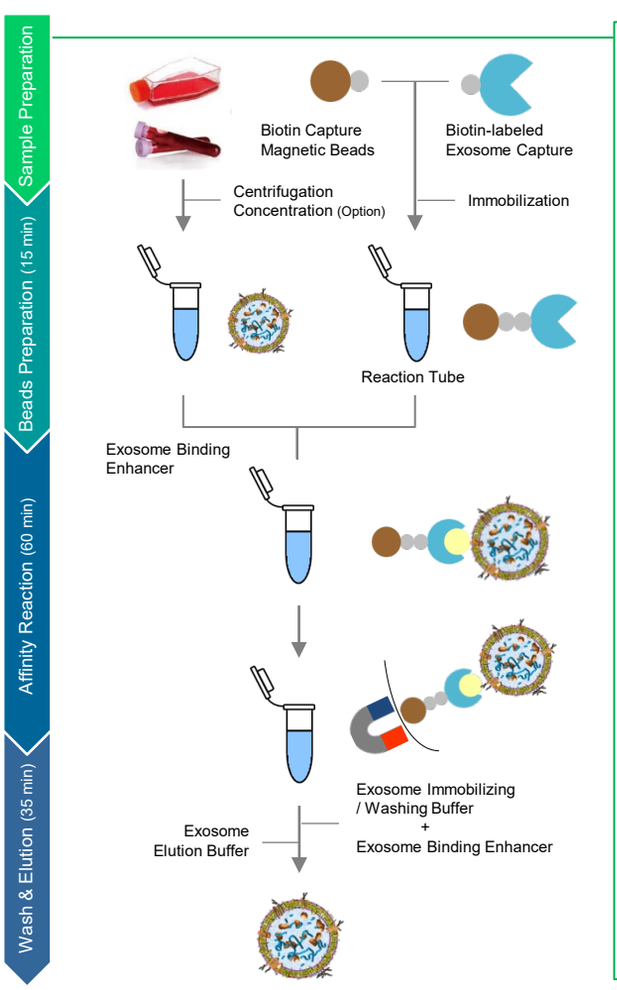
Features

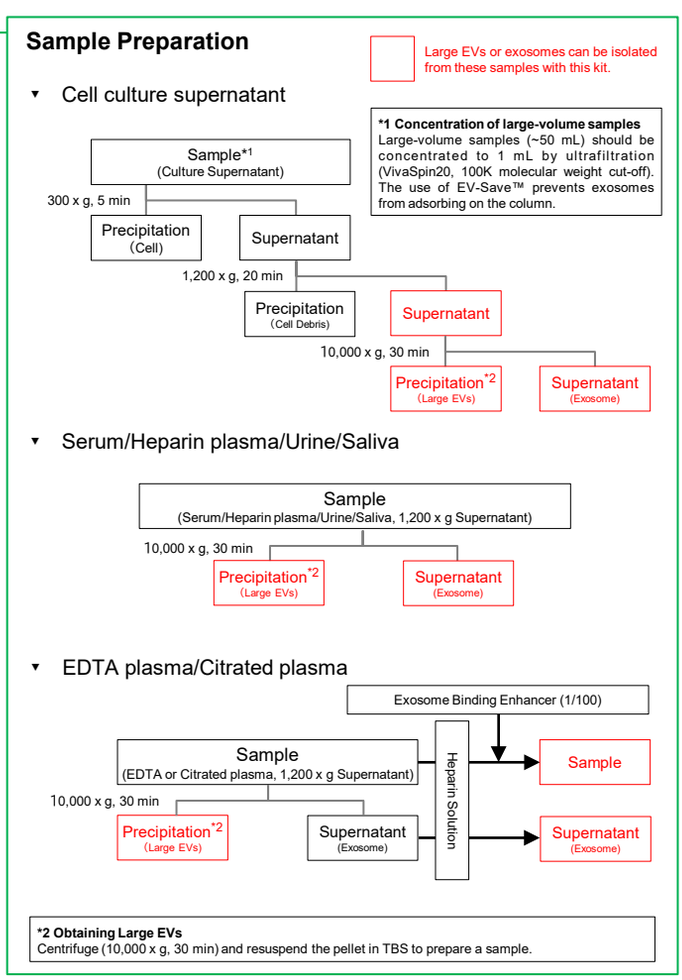
- High purity extracellular vesicles can be obtained by a new affinity method (PS affinity method)
- High purity exosomes can be obtained with a higher yield than by conventional ultracentrifugation
- Intact exosomes can be obtained and used for various applications
- Simple operation with magnetic beads, allowing processing of a large number of samples
- Improved exosome yield and purity compared to the previous kit. Purified exosomes are less cytotoxic and can be added directly to cells

Kit Components

Kit Components	2 tests	10 tests
Biotin Capture Magnetic Beads	120 µL	600 µL
Biotin-labeled Exosome Capture	20 µL	100 µL
Exosome Immobilizing / Washing Buffer (10×)	5 mL	25 mL
Exosome Binding Enhancer (500×)	300 µL	1500 µL
Exosome Elution Buffer (10×)	300 µL	1500 µL
Reaction Tubes	4 tubes	22 tubes

Protocol



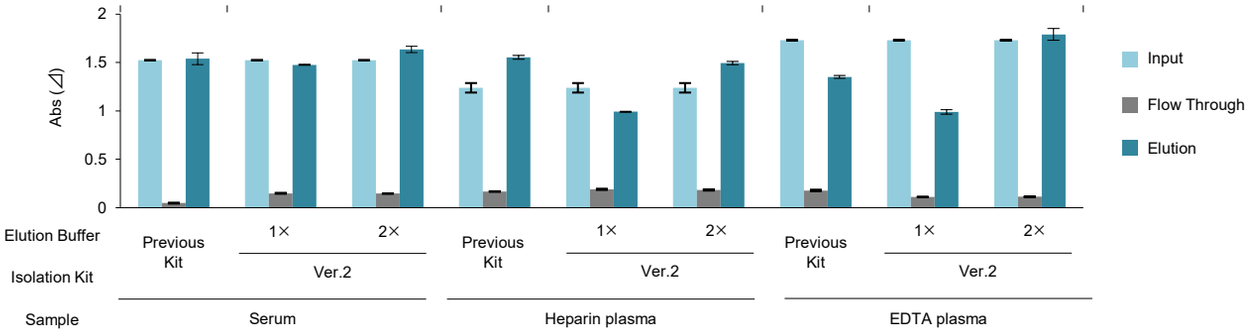


Application Data

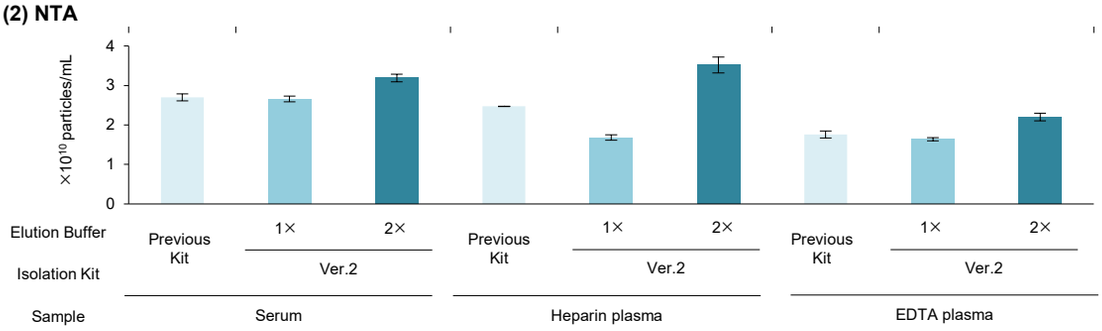
Comparison of the Exosome Yields (Serum and Plasma)

Exosomes were isolated and purified from various human blood samples using MagCapture™ Exosome Isolation Kit PS Ver. 2 (denoted as Ver. 2 below) and MagCapture™ Exosome Isolation Kit PS (denoted as Previous Kit). Two concentrations (1x and 2x) of elution buffer were tested with Ver. 2.

(1) ELISA (PS Capture™ Exosome ELISA Kit)



(2) NTA

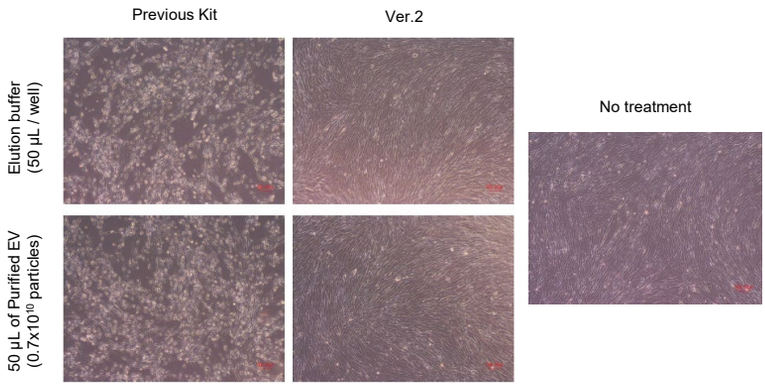


Ver. 2 showed the same or better performance than the previous kit. In particular, the use of a 2x Elution Buffer improved the yield from plasma samples.

Comparison of Cytotoxicity of Purified Exosomes

Exosomes were isolated and purified from COLO201 cell culture supernatants using MagCapture™ Exosome Isolation Kit PS Ver. 2 and the previous version of the kit. Elution Buffer alone or purified exosomes were then added to pre-seeded human normal fibroblasts, and changes in cell morphology were measured after 48 hours.

With the previous kit, cell death occurred 48 hours after the addition of Elution Buffer alone or purified exosomes, but no significant cytotoxicity was observed with Ver. 2.



Product Number	Product Name	Grade	Package Size
294-84101	MagCapture™ Exosome Isolation Kit PS Ver.2	for Genetic Research	2 tests
290-84103			10 tests

Related Product

Product Number	Product Name	Package Size
299-36421	MAGNET STAND	1 pcs

RNA Extraction Kit Optimized for Purified Extracellular Vesicles

microRNA Extractor® Kit for Purified EV

microRNA Extractor® Kit for Purified EV is designed to extract total RNA, including microRNA, from extracellular vesicles (EV), including exosomes, using spin columns. High concentrations of RNA can be recovered with a small amount of elution buffer (20 µL), making it suitable for analyses such as next-generation sequencing (NGS), etc.

Features

- High concentrations of RNA can be recovered with a small amount of elution buffer (20 µL)
- Optimized for RNA extraction from purified exosomes
- Higher yield of RNA compared to conventional products of other companies

Protocol

Lysis

Add Protease Solution and Lysis Solution (+ reducing agent) and incubate at 37˚C for 30 minutes.
Add 1-butanol, enhancer, and 2-propanol in this sequence and spin down briefly.

Binding to the column

Transfer half of the solution into spin column and repeat.
8,000×g, 1 minute, room temperature

Washing 1

Add 500 µL of Washing Buffer I (+ 2-propanol)
8,000×g, 1 minute, room temperature
→ Discard the liquid

Washing 2

Add 700 µL of Washing Buffer II (+ ethanol)
8,000×g, 1 minute, room temperature
→ Discard the liquid
Repeat twice
8,000×g, 3 minutes, room temperature
→ Discard the liquid

Elution

Insert the spin column in a 1.5 mL tube
Add 20 µL of Elution Buffer
Incubate at room temperature for 5 minutes
8,000×g, 1 minute, room temperature

Total RNA solution (20 µL), including microRNA

Application

Sample Exosomal solutions purified from cell culture supernatants, serum, and plasma

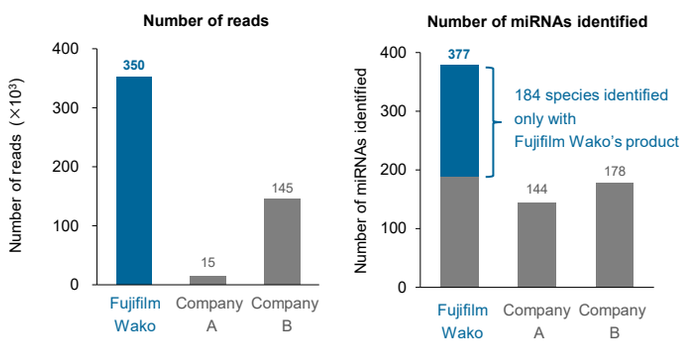
It has not been confirmed whether it can be directly extracted from cell culture supernatant, serum or plasma.

Application RT-qPCR, Microarray, NGS, etc.

Performance Data

Analysis of Exosomal miRNA by NGS

RNA was extracted from the exosomes isolated using MagCapture™ Exosome Isolation Kit PS Ver.2 and analyzed by NGS.

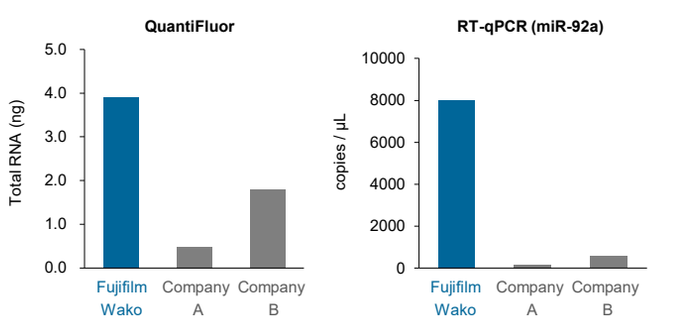


More miRNAs were detected with Fujifilm Wako's product compared to the conventional products of other companies.

Comparison of RNA yield with conventional products

Exosomes were isolated from umbilical cord-derived mesenchymal stem cells using the MagCapture™ Exosome Isolation Kit PS Ver.2, and RNA was extracted using three different kits. RNA was measured by QuantiFluor® RNA System (Promega Corporation) and RT-qPCR.

QuantiFluor® is a trademark or registered trademark of Promega Corporation.



Fujifilm Wako's product showed higher RNA extraction efficiency than the conventional products of other companies.

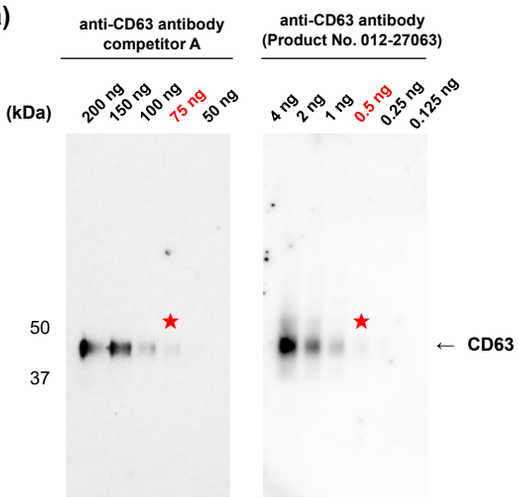
Product Number	Product Name	Grade	Package Size
294-84601	microRNA Extractor® Kit for Purified EV	for Genetic Research	20 tests

Application of the PS Affinity Method to ELISA

Fujifilm Wako developed PS Capture™ Exosome ELISA Kit by applying affinity binding of Tim4 protein with exosomes. This kit can detect exosomes at a sensitivity higher than conventional ELISA methods using immobilized antibodies against exosome surface markers. Exosomes in samples such as culture supernatant and serum are captured by Tim4 protein on a microplate in the presence of calcium ion. The captured exosomes are detected by a primary antibody against an exosome surface marker protein and a labeled secondary antibody. While a mouse anti-CD63 monoclonal antibody is provided with the kit, a user-provided primary antibody against any other exosome surface marker may also be used for exosome detection.

The greatest feature of this kit is that it provides exosome detection with higher sensitivity than that of western blotting analysis and conventional products for exosome ELISA. First, the detection limit for exosomes in western blotting was examined for comparison with this kit (Figures 1a and b). Western blot analysis of exosomes purified from COLO201 cells (of human colon adenocarcinoma origin) with an anti-CD63 monoclonal antibody detected exosomes in an amount as small as 75 ng on the protein basis. Next, the detection limits of this kit for exosomes purified from K562 cells (of human leukemia origin) and COLO201 cells were determined to be 49.9 pg and 10.9 pg, respectively, demonstrating that this kit has a detection sensitivity more than 1,000 times higher than that of western blotting. Considering that the detection limits of conventional products for exosome ELISA range from approximately several ng to several μg (refer to instruction manuals for individual products), the present results demonstrated that this kit utilizing affinity binding of exosomes to Tim4 via PS has a sensitivity more than 100 times higher than those of conventional ELISA methods involving immobilization of an antibody against an exosome surface protein marker.

(a)



(b)

	Detection limit (pg) (Blank＋3.3SD)	Determination limit (pg) (Blank＋10SD)
K562 derived purified exosome	0.05 ng/mL (50 pg)	0.134 ng/mL (134 pg)
COLO201 derived purified exosome	0.011 ng/mL (11 pg)	0.034 ng/mL (34 pg)

Figure 1 Comparison of detection sensitivity between western blotting and ELISA

(a) Detection sensitivity data for western blotting using various anti-CD63 antibodies.

Exosomes purified from COLO201 cell culture supernatant by the PS affinity method were quantified by the BCA method. Exosomes were electrophoresed and detected.
★: Detection limit in western blotting with each antibody

(b) Limit of detection data for Exosome ELISA Kit

K562 and COLO201 derived exosomes were measured using this kit. Standard curves were prepared by measuring the blank (buffer only) and serial dilutions of the standard material. The standard curves were used to calculate the lowest detection sensitivity of purified samples derived from K562 and COLO201. (Each dilution was measured at n=6 and the Blank at n=12.)

Selection Table for Exosome ELISA Kit

Series	PS Capture™ Exosome ELISA Kit		CD9/63/81-Capture Human Exosome ELISA Kit		
Product Number	297-79201	298-80601	296-83701	290-83601	292-83801
Product Name	PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD)	PS Capture™ Exosome ELISA Kit (Streptavidin HRP)	CD9-Capture Human Exosome ELISA Kit (Streptavidin HRP)	CD63-Capture Human Exosome ELISA Kit (Streptavidin HRP)	CD81-Capture Human Exosome ELISA Kit (Streptavidin HRP)
Package Size	96 tests	96 tests	96 tests	96 tests	96 tests
Capture molecule	Tim4 protein	Tim4 protein	Anti-human CD9 antibody (rat)	Anti-human CD63 antibody (mouse)	Anti-human CD81 antibody (mouse)
Detection Antibody (included in the kit)	Anti-human CD63 antibody (mouse)	Anti-human CD63 antibody (mouse)	Anti-human CD9 antibody (rat)	Anti-human CD63 antibody (mouse)	Anti-human CD81 antibody (rat)
Enzyme conjugation method	Secondary antibody	Biotin-Streptavidin	Biotin-Streptavidin	Biotin-Streptavidin	Biotin-Streptavidin
Sample	Purified exosome Culture supernatant*1	Purified exosome Culture supernatant Body Fluid (Serum, Plasma etc.)	Purified exosome Culture supernatant Body Fluid (Serum, Plasma etc.)	Purified exosome Culture supernatant Body Fluid (Serum, Plasma etc.)	Purified exosome Culture supernatant Body Fluid (Serum, Plasma etc.)
Species	Human, Mouse, Rat etc.*2	Human, Mouse, Rat etc.*2	Human	Human	Human
Analyte	PS positive exosome*3	PS positive exosome*3	Human CD9 positive exosome	Human CD63 positive exosome	Human CD81 positive exosome

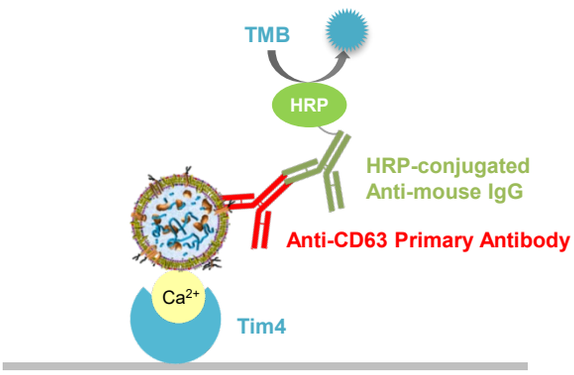
*1 Since the secondary antibody for detection reacts nonspecifically with human, mouse, and rat IgG, serum or plasma samples cannot be used for measurement.
*2 The antibody included in the kit is an anti-human CD63 antibody. Use appropriate antibodies when measuring non-human samples.
*3 If exosome-depleted FBS is not used, PS-positive exosomes derived from FBS are also detected.

Exosome ELISA Kit using the PS Affinity Method

PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD)

PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD) is designed for qualitative analysis of purified exosomes and exosomes in cell culture supernatants and quantitative analysis of exosomes in cell culture supernatants using the PS affinity method.

Assay Principle



Kit Components

Kit Components	96 tests
Exosome Capture 96 Well Plate	1 plate
Plate Seal	4 sheets
Reaction/Washing Buffer (10x)	50 mL×2
Exosome Binding Enhancer (100x)	10 mL
Control Primary Antibody Anti-CD63 (100x)	120 µL
Secondary Antibody HRP-conjugated Anti-mouse IgG (100x)	120 µL
TMB Solution	12 mL
Stop Solution	12 mL

* This kit does not include standard

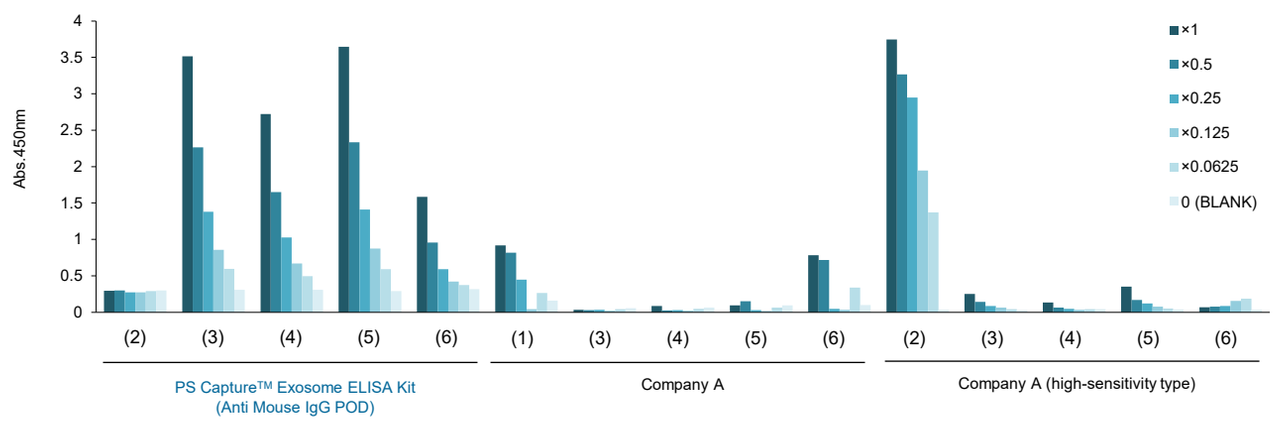
Application Data

Comparison of detection sensitivity using exosomes purified from COLO201 cell culture supernatant and human serum

The following samples were prepared and compared for sensitivity for the detection of the exosome marker CD63 using PS Capture™ Exosome ELISA Kit, an ELISA kit of Company A, and an ELISA kit (high-sensitivity type) of Company A.

<Samples analyzed>
1. Standard included in the ELISA kit of Company A
2. Standard included in the ELISA kit (high-sensitivity type) of Company A
3. Exosomes purified from COLO201 cell culture supernatant using the PS affinity method
4. Exosomes purified from COLO201 cell culture supernatant using the polymer precipitation method
5. Exosomes purified from human serum using the PS affinity method
6. Exosomes purified from human serum using the polymer precipitation method

	(1)	(2)	(3)	(4)	(5)	(6)
×1	1/16	1/1,000	40 ng /mL	160 ng /mL	800 ng /mL	2,000 µg /mL
×0.5	1/32	1/2,000	20 ng /mL	80 ng /mL	400 ng /mL	1,000 µg /mL
×0.25	1/64	1/4,000	10 ng /mL	40 ng /mL	200 ng /mL	500 µg /mL
×0.125	1/128	1/8,000	5 ng /mL	20 ng /mL	100 ng /mL	250 µg /mL
×0.0625	1/256	1/16,000	2.5 ng /mL	10 ng /mL	50 ng /mL	125 µg /mL
Blank	0	0	0	0	0	0



* Note: Data are not listed for values below the limit of detection.

Product Number	Product Name	Grade	Package Size
297-79201	PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD)	for Genetic Research	96 tests

Exosome ELISA Kit using the PS Affinity Method for Direct Measurement of Body Fluid Samples

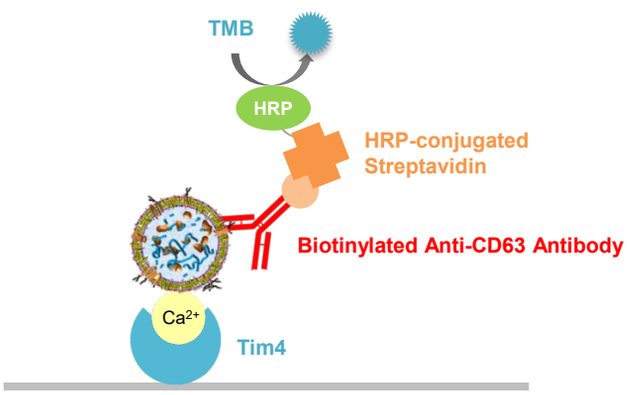
PS Capture™ Exosome ELISA Kit (Streptavidin HRP)

PS Capture™ Exosome ELISA Kit (Streptavidin HRP) is designed for qualitative and quantitative analysis of exosomes in cell culture supernatants and body fluid samples. The conventional PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD) can only use a mouse monoclonal antibody as a detection antibody. This kit can use antibodies and lectins from various animal species for detection as long as they are biotin-labeled. In addition, this kit uses HRP-labeled streptavidin for detection, which shows low nonspecific binding to blood components. As a result, highly sensitive detection of exosomes in blood samples is possible without isolation and purification, which has been difficult with conventional products.

Features

- Highly sensitive qualitative and quantitative analysis
  - 50 to 1,000 times more sensitive than western blotting
  - Exosomes can be detected in the equivalent of 2.5 µL of blood, saving samples
- No need for isolation of exosomes. Cell culture supernatants and body fluid samples can be directly assayed.
- Detection can be done with biotin-labeled antibodies and lectins of various animal species
- Easy operation and high reproducibility
- Wide range of applications
  - Qualitative and quantitative analysis of exosomes in culture supernatants and blood samples
  - Checking residual exosomes in EV-depleted FBS
  - Analysis of glycans in exosomes using lectins

Assay Principle



Kit Components

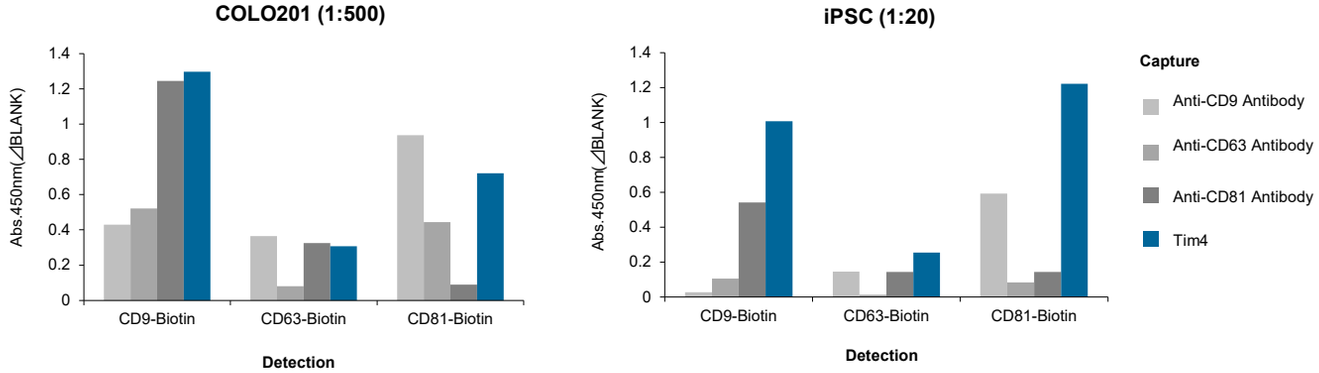
Kit Components	96 tests
Exosome Capture 96 Well Plate	1 plate
Plate Seal	4 sheets
Reaction Buffer	80 mL
Washing Buffer (10x)	100 mL
Exosome Binding Enhancer	10 mL
Control Biotinylated Antibody Anti-CD63 (100x)	120 µL
HRP-conjugated Streptavidin (100x)	240 µL
TMB Solution	12 mL
Stop Solution	12 mL

* This kit does not include standard

Application Data

Exosome Capture Efficiency of the PS Affinity and Antibody Methods

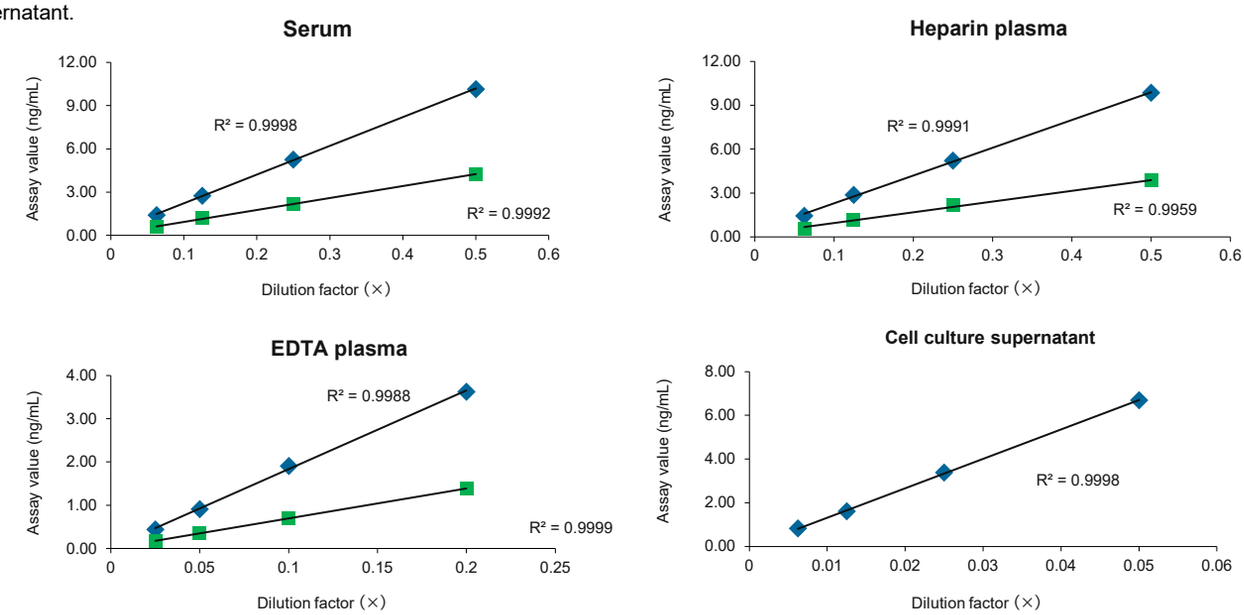
Each cell culture supernatant was pretreated (10,000 x g, 30 min) and placed in microplate wells coated with anti-CD antibodies (CD9, CD63, CD81) or Tim4. Bound exosomes were then detected using biotin-labeled anti-CD antibodies (CD9, CD63, CD81).



The PS affinity method was able to capture exosomes with equal or greater efficiency than the antibody method.

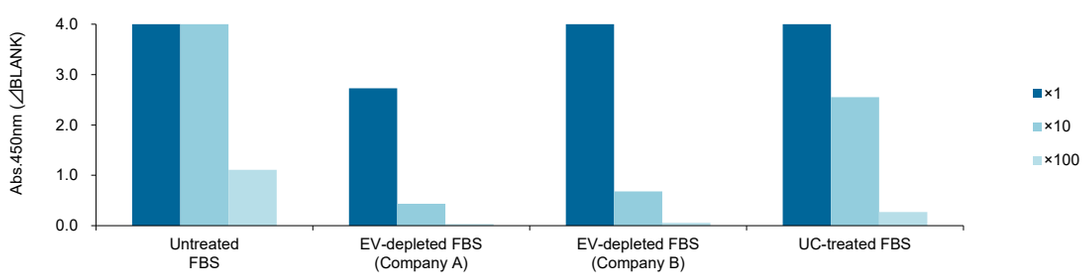
▼ Dilution Linearity for Blood and Cell Culture Supernatant Samples

Based on the standard curve prepared using exosomes purified from COLO201 cell culture supernatant, exosome concentrations were measured (CD63 detection) and the dilution linearity for each sample was evaluated. Below: (1) human serum dilutions, (2) human heparin plasma dilutions, (3) human EDTA plasma dilutions (2 samples each, 4 serial dilutions), and (4) 4 serial dilutions of COLO201 cell culture supernatant.



▼ Checking Residual Exosomes in EV-depleted FBS

Exosomes in untreated FBS, commercially available EV-depleted FBS, and ultracentrifuge-treated FBS (UC-treated FBS, 160,000 x g, 16 hours) were measured with this kit. The detection antibody was an anti-CD9 antibody (Product No. 014-27763) labeled with Biotin Labeling Kit-SH (Product No. 348-90941, Dojindo Laboratories).



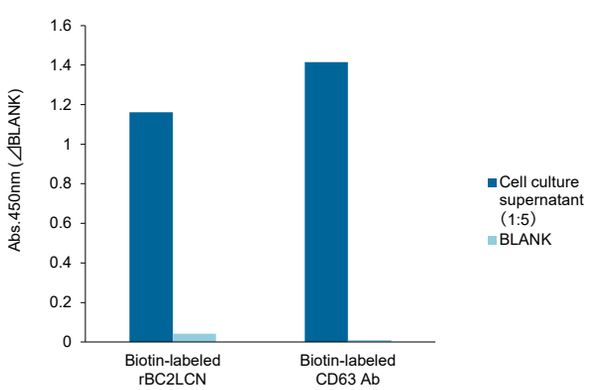
Residual exosomes in FBS were detected with the PS affinity method.

▼ Application for Analysis of Glycans

An iPS cell culture supernatant was diluted 5-fold and pretreated at 10,000 x g for 30 min. Exosomes in this sample were detected using this kit and biotin-labeled anti-CD63 antibody (Product No. 019-27713) and biotin-labeled rBC2LCN lectin.

The biotin-labeled lectin was prepared by labeling rBC2LCN (Product No. 029-18061) using EZ-Link™ Sulfo-NHS-LC-LC-Biotin (Thermo Fisher Scientific).

Glycans on the surface of exosomes were successfully measured.



Product Number	Product Name	Grade	Package Size
298-80601	PS Capture™ Exosome ELISA Kit (Streptavidin HRP)	for Genetic Research	96 tests

Specific Detection and Quantification of Human-derived Exosomes

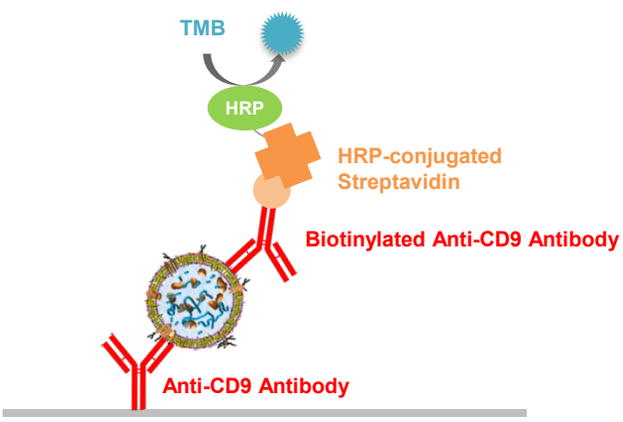
CD9/CD63/CD81-Capture Human Exosome ELISA Kit (Streptavidin HRP)

CD9/CD63/CD81-Capture Exosome ELISA Kit (Streptavidin HRP) is designed for the detection and quantification of human-derived exosomes in cell culture supernatants and body fluid samples. Anti-CD9, CD63, or CD81 antibodies, known as exosome marker proteins, are immobilized to capture exosomes expressing them. These antibodies bind specifically to human CD9, CD63, or CD81, allowing for the detection and quantification of human-derived exosomes.

Features

- Specific detection of human-derived exosomes
- Exosome isolation is not required; exosomes in culture supernatants and blood samples are directly measured
- Quantitative analysis of exosomes is possible when used with exosome standards
- Easy operation and high reproducibility

Assay Principle (CD9-ELISA)



Kit Components (CD9-ELISA)

Kit Components	96 tests
Anti-CD9 Antibody-immobilized 96 Well Plate	1 plate
Plate Seal	4 sheets
Sample Reaction Buffer	50 mL
Antibody Reaction Buffer	50 mL
Washing Buffer (10x)	100 mL
Control Biotinylated Antibody Anti-CD9 (100x)	120 µL
HRP-conjugated Streptavidin (100x)	240 µL
TMB Solution	12 mL
Stop Solution	12 mL

* This kit does not include standard

Application Data

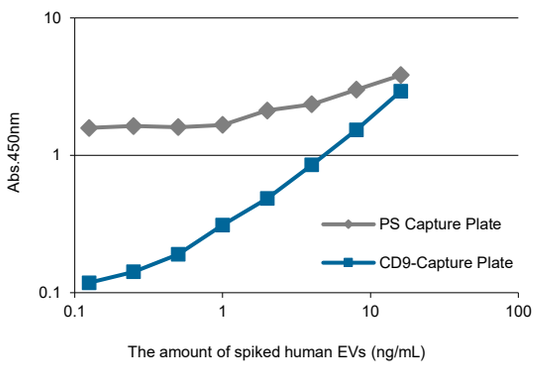
Comparison of Specificity in the Detection of Human-derived Exosomes

D-MEM medium containing 10% FBS (ultracentrifuged) was diluted 2-fold, and various amounts of COLO201 cell-derived, purified exosomes (Product No. 052-09301) were added. The mixtures were reacted on plates in two different ELISA kits, and detected with an anti-CD9 antibody (Clone No. 1K) that cross-reacts with human and bovine CD9.

■ Kits

PS Capture Plate (PS Affinity Method):
PS Capture™ Exosome ELISA Kit (Streptavidin HRP)

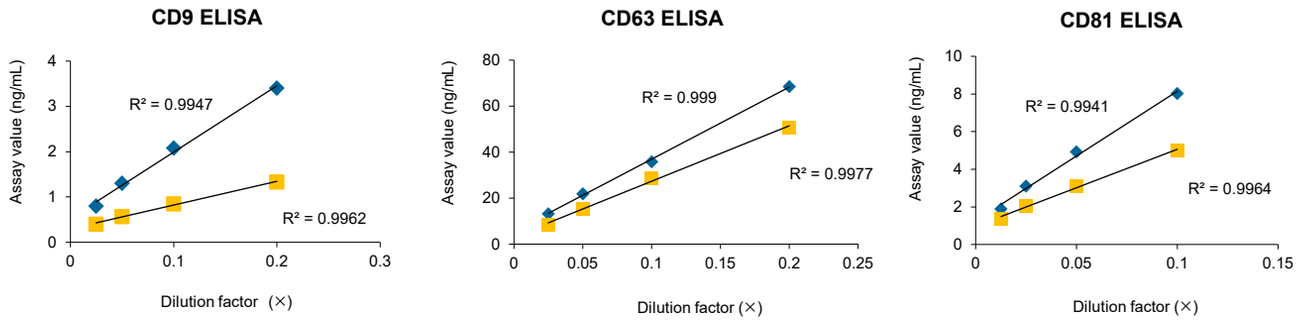
CD9-Capture Plate (This product):
CD9-Capture Human Exosome ELISA Kit (Streptavidin HRP)



PS affinity method also captured bovine-derived exosomes (remaining in ultracentrifuged FBS). CD9-Capture Plate specifically captured human-derived exosomes and detected them with high sensitivity.

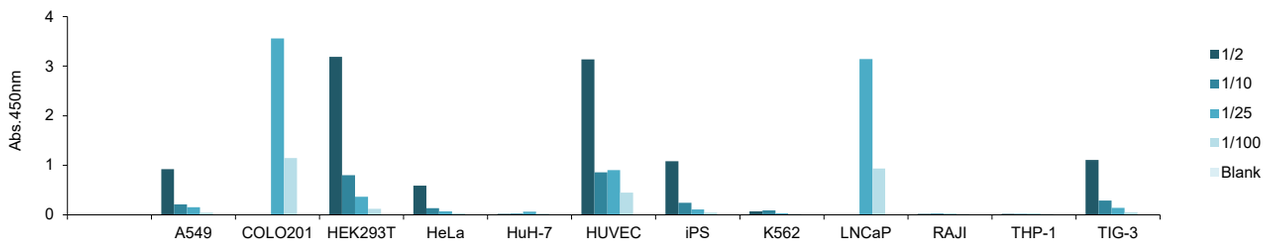
▼ Dilution Linearity of Human Serum Samples

Using the standard curve prepared with exosomes purified from COLO201 cell culture supernatant, exosome concentrations in four serial dilutions of two human serum samples were measured, and the dilution linearity in each sample was evaluated.

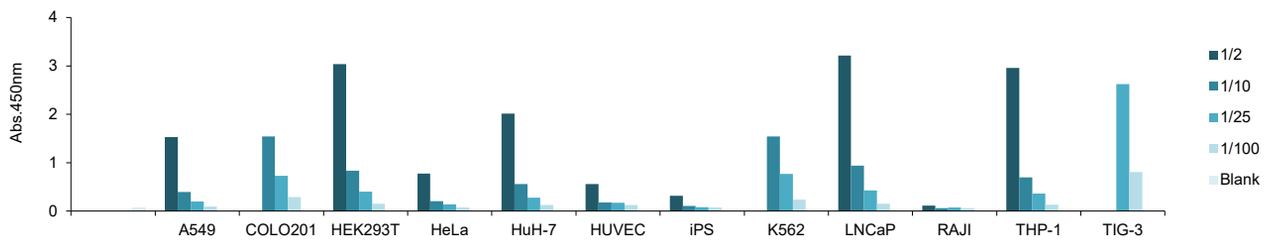


▼ Analysis of Exosome Marker Proteins in Various Cell Types

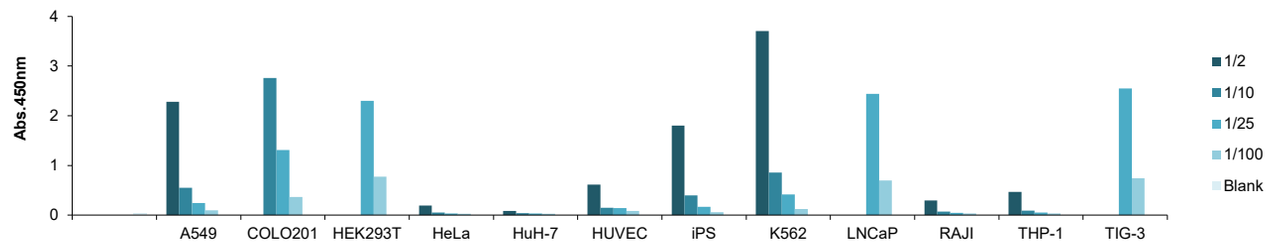
(1) CD9-Capture Human Exosome ELISA Kit (Streptavidin HRP)



(2) CD63-Capture Human Exosome ELISA Kit (Streptavidin HRP)



(3) CD81-Capture Human Exosome ELISA Kit (Streptavidin HRP)



Product Number	Product Name	Grade	Package Size
296-83701	CD9-Capture Human Exosome ELISA Kit (Streptavidin HRP)	for Genetic Research	96 tests
290-83601	CD63-Capture Human Exosome ELISA Kit (Streptavidin HRP)		96 tests
292-83801	CD81-Capture Human Exosome ELISA Kit (Streptavidin HRP)		96 tests

Ideal for Highly Sensitive Qualitative Analysis of Exosomes

PS Capture™ Exosome Flow Cytometry Kit

PS Capture™ Exosome Flow Cytometry Kit is designed for highly sensitive detection of EVs with maker proteins of interest by flow cytometry. It immobilizes exosomes on magnetic beads using the PS affinity method, which utilizes phosphatidylserine-specific binding protein Tim4 and magnetic beads.

* A fluorescent-labeled primary antibody against marker protein, or a primary antibody and a fluorescent-labeled secondary antibody need to be supplied.

Features

- Highly sensitive qualitative analysis by flow cytometry
- Easy operation with magnetic beads
- No purification necessary; samples can be directly measured
- Assay completes in 3 hours from isolation to staining

Assay Principle

1) Isolation of exosomes

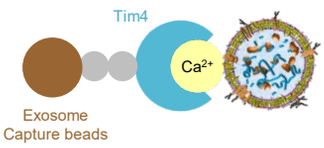
Exosomes are isolated from the sample by Exosome Capture Beads.

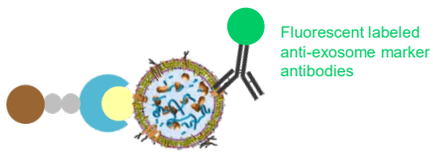
2) Staining of exosomes

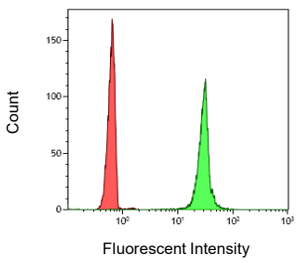
Exosomes are stained with fluorescent-labeled anti-exosome marker antibodies.

3) Flow cytometry analysis

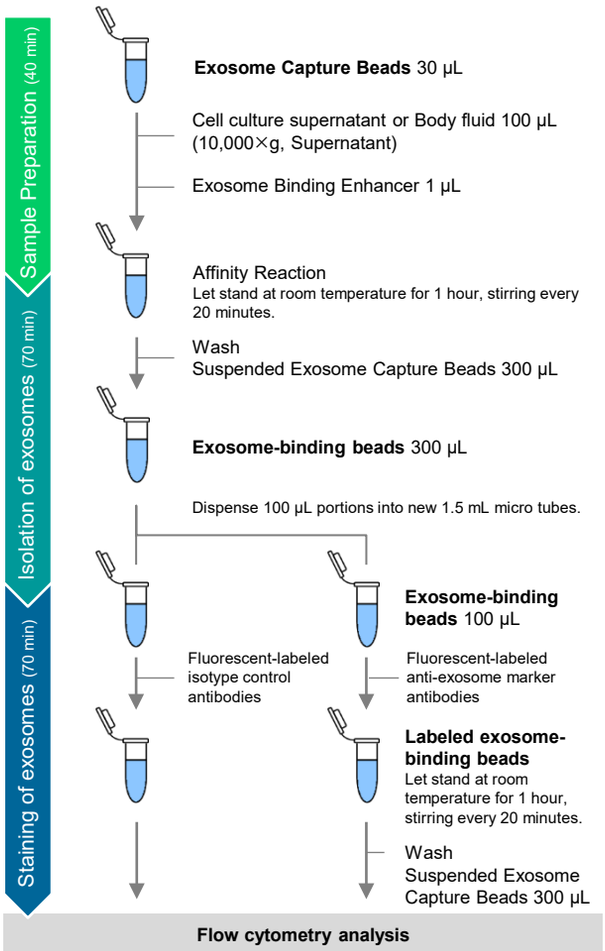
Flow cytometry analysis is performed with exosomes bound to magnetic beads.







Protocol



Sample

- Cell culture supernatant
- Serum
- Heparin plasma
- EDTA plasma

Number of reactions and sample volume

In the exosome isolation process, a standard protocol is set for 1.5 mL microtubes (for two reactions). To increase the scale of reactions, the amount of Exosome Capture Beads and sample volume should be scaled up according to the table below.

Note: The maximum amount is 10 reactions per 1.5 mL microtube.

	Exosome Capture Beads (µL)	Sample Volume (µL)
2 reactions (basic)	30	100
3 reactions	40	133
4 reactions	50	167
5 reactions	60	200
6 reactions	70	233
7 reactions	80	267
8 reactions	90	300
9 reactions	100	333
10 reactions	110	367

▾ Analysis of Surface Antigens of Exosomes in Cell Culture Supernatants

Exosomes in K562 cell culture supernatants were immobilized this kit and with other companies' products (anti-CD9, CD63, and CD81 antibody-coated magnetic beads), and fluorescent-labeled anti-CD9, CD63, and CD81 antibodies were bound for analysis of exosome surface antigen by flow cytometry.

■ Sample

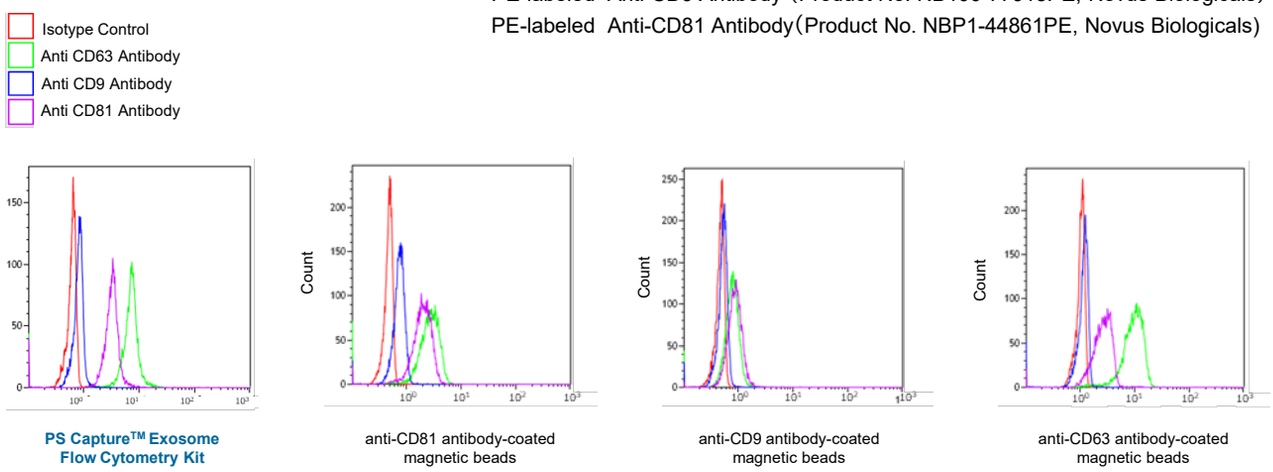
K562 cell culture supernatant 33 µL/assay

■ Detection Antibody

PE-labeled Anti-CD63 Antibody（Product No. 556020, BD Biosciences）

PE-labeled Anti-CD9 Antibody（Product No. NB100-77915PE, Novus Biologicals）

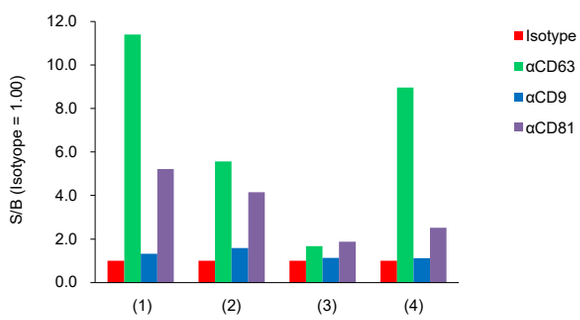
PE-labeled Anti-CD81 Antibody（Product No. NBP1-44861PE, Novus Biologicals）



<Supplemental Data> Comparison of S/B Ratios

(1) PS Capture™ Exosome Flow Cytometry Kit
(2) Competitor: Anti-CD81 antibody-coated magnetic beads
(3) Competitor: Anti-CD9 antibody-coated magnetic beads
(4) Competitor: Anti-CD63 antibody-coated magnetic beads

The PS Capture™ Exosome Flow Cytometry Kit was able to detect exosome surface antigens with higher sensitivity than the competitors' products.



▾ Analysis of Surface Antigens of Exosomes in Serum and Plasma

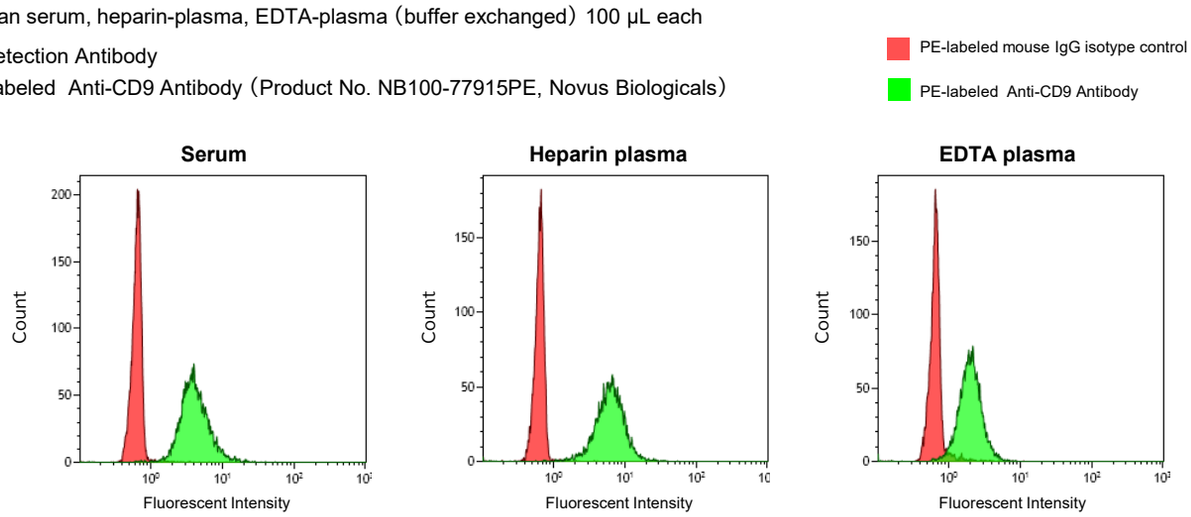
Exosomes in human serum and human plasma (Heparin plasma and EDTA plasma) were immobilized on magnetic beads and then detected by PE-labeled mouse IgG isotype control and PE-labeled anti-human CD9 antibody.

■ Sample

Human serum, heparin-plasma, EDTA-plasma（buffer exchanged）100 µL each

■ Detection Antibody

PE-labeled Anti-CD9 Antibody（Product No. NB100-77915PE, Novus Biologicals）



Product Number	Product Name	Grade	Package Size
297-79701	PS Capture™ Exosome Flow Cytometry Kit	for Genetic Research	300 tests

Highly Sensitive and Specific Exosome Marker Antibodies Produced by DNA Immunization

Exosome Marker Antibodies (CD9/CD63/CD81)

The tetraspanin family members CD9, CD63, and CD81 are exosome marker proteins. Fujifilm Wako offers highly specific monoclonal antibodies established by DNA immunization. These antibodies can be used for western blotting, flow cytometry, ELISA, and immunoprecipitation for exosome analysis.

DNA Immunization

DNA Immunization is a technology in which a gene for a target protein incorporated into an expression vector is introduced into an animal. The target protein is then expressed in the animal, and antibodies are produced against the target protein as an antigen. Because antibodies produced by this method recognize proteins in their native form, the method can be used for production of therapeutic/diagnostic antibodies, antibodies against membrane proteins, neutralizing/functional antibodies, etc.

Features

- High affinity antibodies
- Highly specific
- Recognizes non-reduced samples

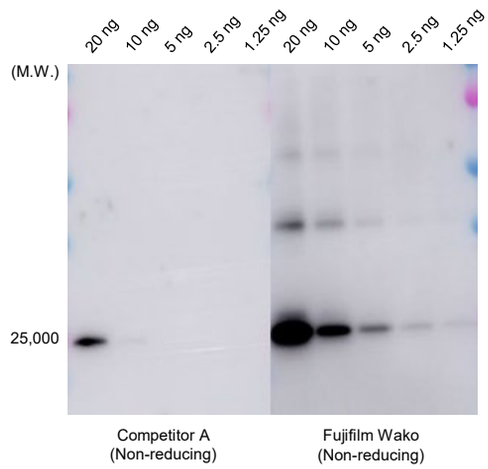
Product Lineup

Antigen		CD9				CD63				CD81		
Clone No.		1K	30B	77B		3-13				17B1	9B	
Host		Mouse	Rat	Rat		Mouse				Mouse	Rat	
Cross-reactivity* (ELISA)	Human	++	++	++		++				++	++	
	Bovine	++	-	-		-				++	±	
	Mouse	-	-	-		-				-	-	
	Rat	+	±	±		-				-	-	
Conjugate		-	Biotin	-	Biotin	-	Biotin	Fluorescein	Red-fluorochrome	-	-	Biotin
Product No.		014-27763	019-27953	013-28171 019-28173	017-28211	012-27063	019-27713	018-27641	011-27751	011-27773	014-28221 010-28223	011-28111

* Cross-reactivity (ELISA): ++: strong reaction, +: weak reaction, ±: slight reaction, -: no reaction

Application Data

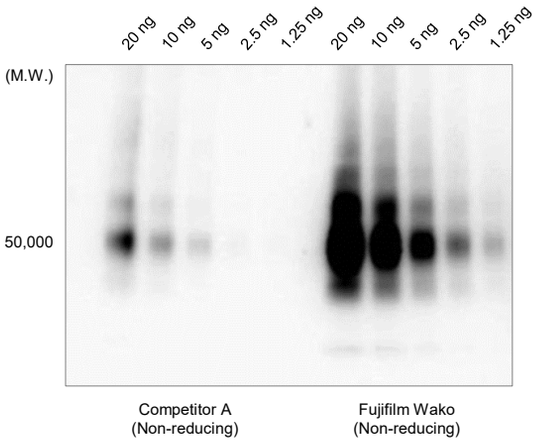
Comparison of Performance of Anti-CD9 Antibody by Western Blotting



Sample: Purified exosomes from cell culture supernatant of COLO201
Isolation: PS Affinity Method
Primary Antibody: Anti CD9, Monoclonal Antibody (1K)
Secondary Antibody: Anti Mouse IgG (H+L), Peroxidase-conjugated
Detection Reagents: ImmunoStar® Zeta (Product No. 291-72401)

Fujifilm Wako's product detected CD9 with higher sensitivity under non-reducing conditions compared to competitor's product A.

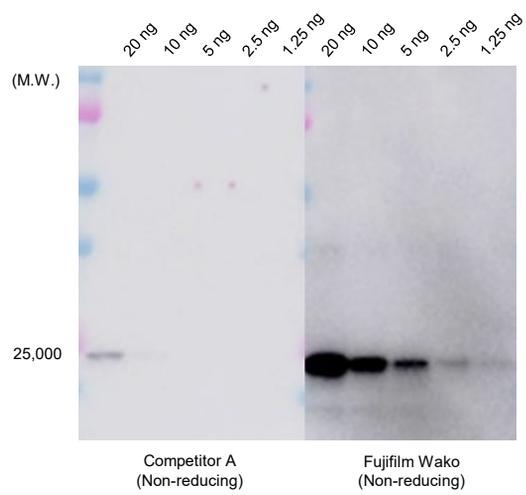
▼ **Comparison of Performance of Anti-CD63 Antibody by Western Blotting**



Sample: Purified exosomes from cell culture supernatant of COLO201
Isolation: PS Affinity Method
Primary Antibody: Anti CD63, Monoclonal Antibody (3-13)
Secondary Antibody: Anti-Mouse IgG (H+L), Peroxidase-conjugated
Detection Reagents：ImmunoStar® Zeta（Product No. 291-72401）

Fujifilm Wako's product detected CD63 with higher sensitivity under non-reducing conditions compared to competitor's product A.

▼ **Comparison of Performance of Anti-CD81 Antibody by Western Blotting**



Sample: Purified exosomes from cell culture supernatant of COLO201
Isolation: PS Affinity Method
Primary Antibody: Anti CD81, Monoclonal Antibody (17B1)
Secondary Antibody: Anti-Mouse IgG (H+L), Peroxidase-conjugated
Detection Reagents：ImmunoStar® Zeta（Product No. 291-72401）

Fujifilm Wako's product detected CD81 with higher sensitivity under non-reducing conditions compared to competitor's product A.

Product Number	Product Name	Grade	Package Size
014-27763	Anti CD9, Monoclonal Antibody (1K)	for Immunochemistry	100 µL
019-27953	Anti CD9, Rat Monoclonal Antibody (30B), Biotin Conjugated		100 µL
013-28171	Anti CD9, Rat Monoclonal Antibody (77B)		20 µL
019-28173			100 µL
017-28211	Anti CD9, Rat Monoclonal Antibody (77B), Biotin-conjugated		50 µL
012-27063	Anti CD63, Monoclonal Antibody (3-13)		100 µL
019-27713	Anti CD63, Monoclonal Antibody (3-13), Biotin Conjugated		100 µL
018-27641	Anti CD63, Monoclonal Antibody (3-13), Fluorescein Conjugated		25 tests
011-27751	Anti CD63, Monoclonal Antibody (3-13), Red Fluorochrome(635) Conjugated		25 tests
011-27773	Anti CD81, Monoclonal Antibody (17B1)		100 µL
014-28221	Anti CD81, Rat Monoclonal Antibody (9B)		20 µL
010-28223			100 µL
011-28111	Anti CD81, Rat Monoclonal Antibody (9B), Biotin-conjugated		50 µL

Blocking reagent with exosome anti-adsorption and cryoprotection effects

EV-Save™ Extracellular Vesicle Blocking Reagent

EV-Save™ Extracellular Vesicle Blocking Reagent is a polymeric reagent that prevents exosomes from adsorbing to tubes, pipette tips, and other labware. It prevents loss of exosomes due to adsorption during processing and storage, thereby increasing the yield. Addition of this product is highly recommended prior to ultrafiltration, purification, and storage.

Features

- Prevents adsorption of exosomes in culture supernatants and purified exosomes onto laboratory equipment
- Protects exosomes from freeze-thaw damage
- Easy to operate, just add to samples
- No interference with the analysis of exosomes in the following analyses:

  - Nanoparticle Tracking Analysis (NTA) / Western blotting/ ELISA / Microarray / Cell culture

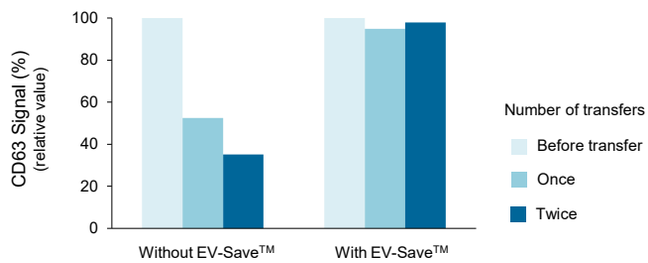
  <Caution>
  When this product is used for serum, plasma, or samples with many contaminants, the anti-adsorption effect may be diminished.
  This product contains polymers. Do not use this product if the presence of polymers may affect experimental results in subsequent processes.

Application Data

Anti-adsorption Effect

EV-Save™ was added to COLO201 cell-derived exosomes purified by the PS affinity method and allowed to stand for 3 minutes. The sample was then transferred to another tube, and the transfer was repeated. The amounts of exosomes (CD63 signal) in the sample tubes were measured by PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD).

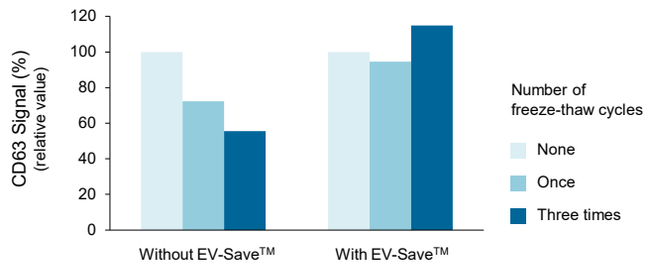
CD63 signal decreased with transfer. The addition of EV-Save™ almost completely prevented the decrease in signal caused by the transfer.



Cryoprotection Effect

EV-Save™ was added to COLO201 cell-derived exosomes purified by the PS affinity method and freeze-thawed. The amounts of exosomes (CD63 signal) in the sample tubes were measured by PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD).

The CD63 signal decreased with freeze-thawing. The addition of EV-Save™ prevented the decrease in signal caused by freeze-thawing.

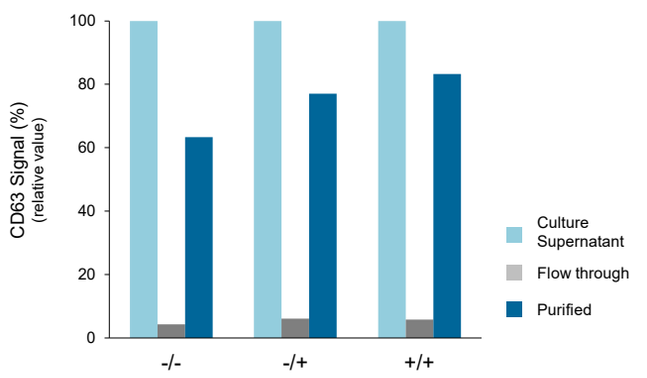


Combination with the PS Affinity Method

A study was conducted to determine whether the addition of EV-Save™ could prevent loss of exosomes during the purification from TIG3 cell culture supernatants using the PS affinity method.

	Addition of EV-Save™ to culture supernatant	Addition of EV-Save™ to elution buffer
−/−	×	×
−/+	×	○
+/+	○	○

When EV-Save™ was added to the cell culture supernatant and the elution buffer of the PS affinity method (+/+), exosome yield was increased by about 20% compared to without the addition of EV-Save™ (-/-).



Product Number	Product Name	Grade	Package Size
058-09261	EV-Save™ Extracellular Vesicle Blocking Reagent	for Genetic Research	1 mL

For administration of exosomes to experimental animals

EV-Save™ Extracellular Vesicle Blocking Reagent for *in vivo*

EV-Save™ Extracellular Vesicle Blocking Reagent for *in vivo* applications contains only components that have been used as pharmaceutical additives. This exosome anti-adsorption and cryoprotectant can be used for administration of exosomes to laboratory animals.

Features

- Contains only ingredients that have been used as pharmaceutical additives
- Prevents adsorption of exosomes in culture supernatants and purified exosomes onto laboratory equipment
- Protects exosomes from freeze-thaw damage
- Easy to operate, just add to samples
- No interference with the analysis of exosomes in the following analyses:

  - Nanoparticle Tracking Analysis (NTA) / ELISA / Fluorescent staining / Addition of exosomes to cells

  <Caution>
  When this product is used for serum, plasma, or samples with many contaminants, the anti-adsorption effect may be diminished.
  This reagent cannot be used for ultrafiltration. If ultrafiltration is required, use EV-Save™ Extracellular Vesicle Blocking Reagent (p. 23).

Comparison of EV-Save™ for *in vivo* and EV-Save™

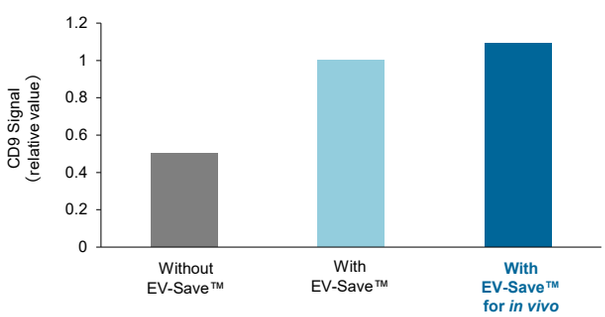
	EV-Save™ for *in vivo* **(Product No. 050-09461)**	EV-Save™ (Product No. 058-09261)
Excretion from the body	**The components are within the range of molecular weights that can be excreted.**	No data
Components	**All ingredients are approved for pharmaceutical use.**	Some ingredients are not approved for pharmaceutical use.
Use in animal experiments	**Applicable**	No data
Use of ultrafiltration	**Not applicable**	Applicable

Application Data

Anti-adsorption Effect

EV-Save™ and EV-Save™ for *in vivo* applications were added to COLO201 cell-derived exosomes purified by the PS affinity method and stored at 4˚C for 16 hours. The amounts of exosomes (CD9 signal) in the sample tubes were measured by PS Capture™ Exosome ELISA Kit (Streptavidin HRP).

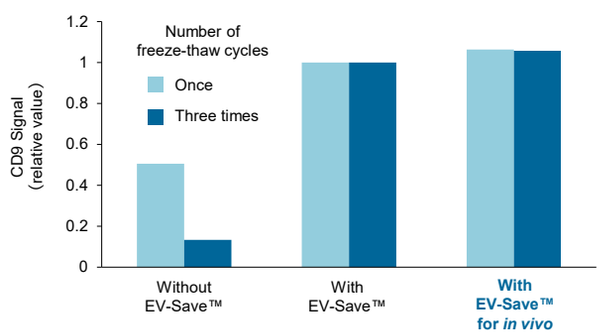
CD9 signal decreased without the addition of EV-Save™. The addition of EV-Save™ or EV-Save™ for *in vivo* applications prevented the decrease in signal.



Cryoprotection Effect

EV-Save™ or EV-Save™ for *in vivo* applications was added to COLO201 cell-derived exosomes purified by the PS affinity method and freeze-thawed once or three times. The amounts of exosomes (CD9 signal) in the sample tubes were measured by PS Capture™ Exosome ELISA Kit (Streptavidin HRP).

In the absence of EV-Save™, freeze-thawing reduced the CD9 signal. This signal reduction was prevented by the addition of EV-Save™ or EV-Save™ for *in vivo* applications.



Product Number	Product Name	Grade	Package Size
050-09461	EV-Save™ Extracellular Vesicle Blocking Reagent for *in vivo*	for Genetic Research	1 mL

Positive control exosomes

Exosomes, from COLO201 cells, purified

High-purity exosomes are derived from COLO201 cells isolated by the PS affinity method.

Features

- High purity
- High stability
- Non-lyophilized
- CD9 / CD63 / CD81 positive exosomes

Specification

Properties: Solution

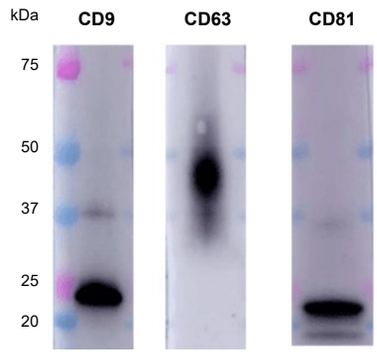
Ingredients: Exosomes, TBS, EDTA, polymer (stabilizer)

Exosome concentration: 10 µg/mL*

* The value is protein concentration corrected by CD63 signal value

Application Data

Detection of Exosome Surface Marker Proteins [CD9 / CD63 / CD81]



Non-reducing SDS-PAGE was performed for 10 ng/Lane of this product, and the exosome marker proteins, CD9 / CD63 / CD81, were detected with antibodies.

■ Primary Antibody

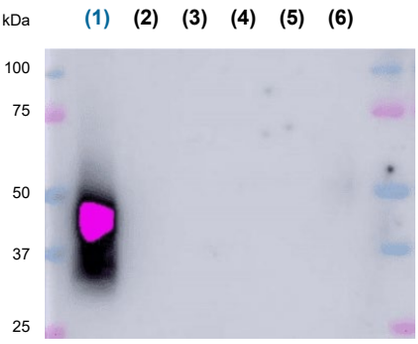
Anti CD9, Monoclonal Antibody (1K) (Product No. 014-27763)
Anti CD63, Monoclonal Antibody (3-13) (Product No. 012-27063)
Anti CD81, Monoclonal Antibody (17B1) (Product No. 011-27773)

■ Secondary Antibody

HRP conjugated anti-mouse IgG

This product shows higher expression of the exosome surface markers.

Detection of Surface Marker Proteins of Exosomes - Comparison with Other Companies' Products



This product and five exosomes from other companies were compared to see whether CD63 signal could be detected. All were applied at a concentration of 10 ng/Lane based on the concentrations determined by each manufacturer.

■ Lane No.

(1) This product
(2) Competitor 1: Exosomes from A549 cells
(3) Competitor 2: Exosomes from HEK293 cells
(4) Competitor 3: Exosomes from A549 cells
(5) Competitor 4: Exosomes from COLO1 cells
(6) Competitor 5: Exosomes from HCT116 cells

■ Primary Antibody

Anti CD63, Monoclonal Antibody (3-13) (Product No. 012-27063)

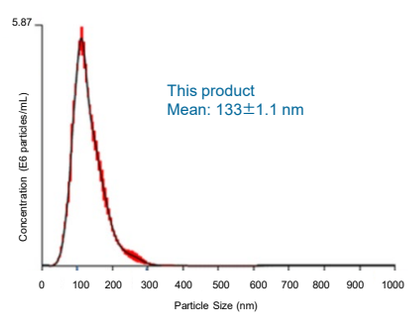
■ Secondary Antibody

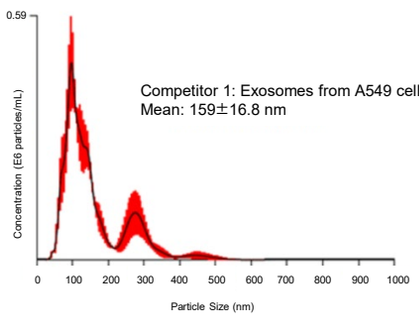
HRP conjugated anti-mouse IgG

CD63 signal was detected only with this product.

Analysis of Exosome Particle Size

Particle size for this product and a competitor's product was analyzed by NanoSight.





This product was uniform in particle size.

Product Number	Product Name	Grade	Package Size
052-09301	Exosomes, from COLO201 cells, purified	for Genetic Research	50 µL

Growth medium for mesenchymal stem cells

MSCulture™ High Growth Medium/MSCulture™ High Growth Supplement

MSCulture™ High Growth Medium is a medium for the efficient expansion of high-quality mesenchymal stem cells (MSCs). It can be used for MSCs derived from various tissues (bone marrow, Adipose tissue, umbilical cord matrix). This product offers higher proliferative potential than conventional MEMα or growth media for MSCs.
After expanding the cells with this product, culture the cells in EV-Up™ MSC EV Production Medium (p. 27) with supplements for efficient production of large quantities of exosomes.

Features

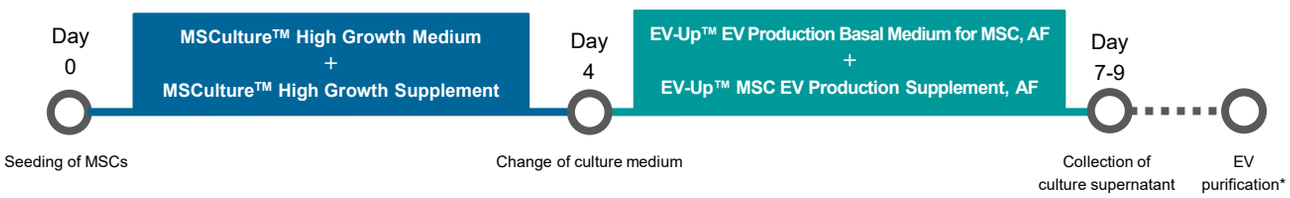
- High proliferative potential
- MSCs can be cultured in high quality without flattening
- Efficient exosome production when used in combination with EV-Up™.
- Animal-free (Animal-free as culture medium before adding serum)

Applicable cells

- Bone marrow-derived MSC
- Adipose tissue-derived MSC
- Umbilical cord matrix-derived MSC

MSCulture™ High Growth Medium can be used for a variety of MSCs, regardless of the species of origin.

Procedure



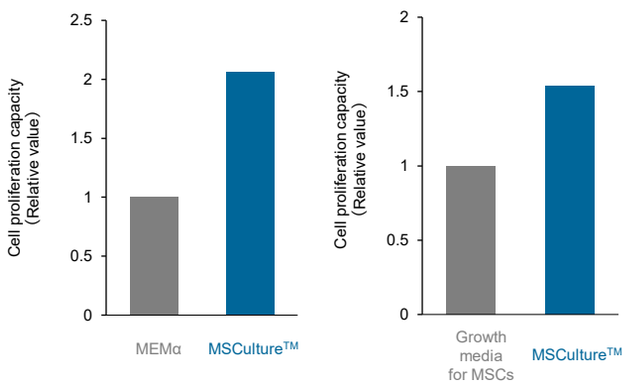
* For purification of recovered exosomes, the PS affinity method using MagCapture™ Exosome Isolation Kit PS Ver. 2 (pp. 10-11) is recommended.

Performance Data

Comparison of Cell Proliferation

Bone marrow-derived MSCs were cultured in this product, another company's MEMα, and another company's growth media for MSCs, and cell proliferation capacity was compared.

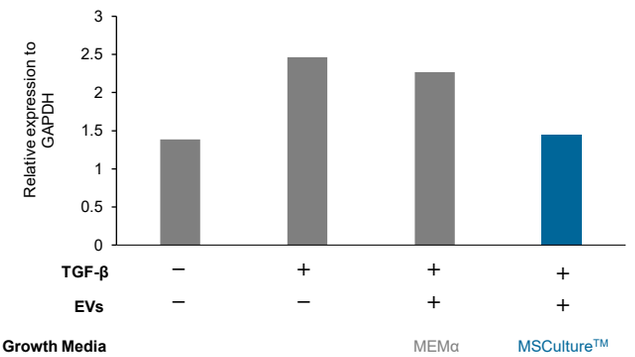
Cells showed a higher cell proliferation capacity when cultured in this product compared to the other company's MEMα and growth media for MSCs.



Comparison of Antifibrotic Activity

Bone marrow-derived MSCs were cultured in this product and another company's MEMα, and the media were replaced with EV-Up™ MSC EV Production Medium for exosome production.
Exosomes were purified from the culture supernatant by the PS affinity method. Purified exosomes ($5x10^7$ particles/mL) were added to human fetal lung-derived fibroblasts stimulated with TGF-β, and antifibrotic activity was compared by quantification of a fibrosis marker (Collagen V) gene expression.

MSC-derived exosomes cultured in this product suppressed the TGF-β-induced collagen V expression.



Product Number	Product Name	Grade	Package Size
132-19345	MSCulture™ High Growth Basal Medium	for Cell Culture	500 mL
133-19331	MSCulture™ High Growth Supplement		5 mL

Exosome Production Medium for Mesenchymal Stem Cells (MSCs)

EV-Up™ EV Production Basal Medium for MSC, AF
EV-Up™ MSC EV Production Supplement, AF

EV-Up™ MSC EV Production Medium is optimized for exosome production by mesenchymal stem cells (MSCs). It is a serum- and animal-derived component-free medium and can be used as a complete medium by adding supplements to the basic medium.

Features

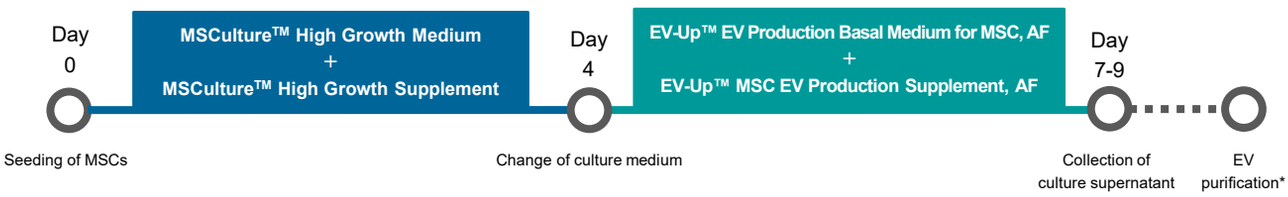
- Higher production of exosomes than serum-containing medium
- Higher biological activity of exosomes
- Maintains high MSC viability
- Serum- and animal-derived component-free

Applicable cells

- Bone marrow-derived MSC
- Adipose tissue-derived MSC
- Umbilical cord matrix-derived MSC

EV-Up™ MSC EV Production Medium can be used for a variety of MSCs, regardless of the species of origin.

Procedure



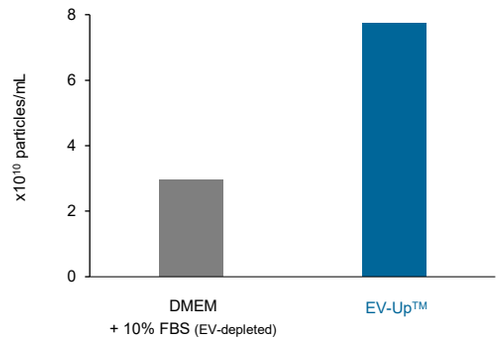
* For purification of recovered exosomes, the PS affinity method using MagCapture™ Exosome Isolation Kit PS Ver. 2 (pp. 10-11) is recommended.

Application Data

Number of Exosome Particles

Exosomes were purified from each culture supernatant using the PS affinity method, and the number of particles was counted by Nano Tracking Analysis using NanoSight.

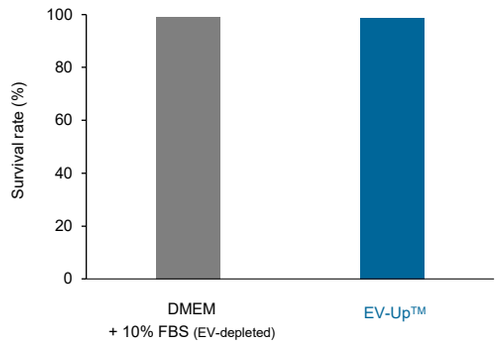
The number of exosome particles released by MSCs cultured in EV-Up™ was approximately 2.6 times that of MSCs cultured in a conventional medium.



Cell Survival Rate

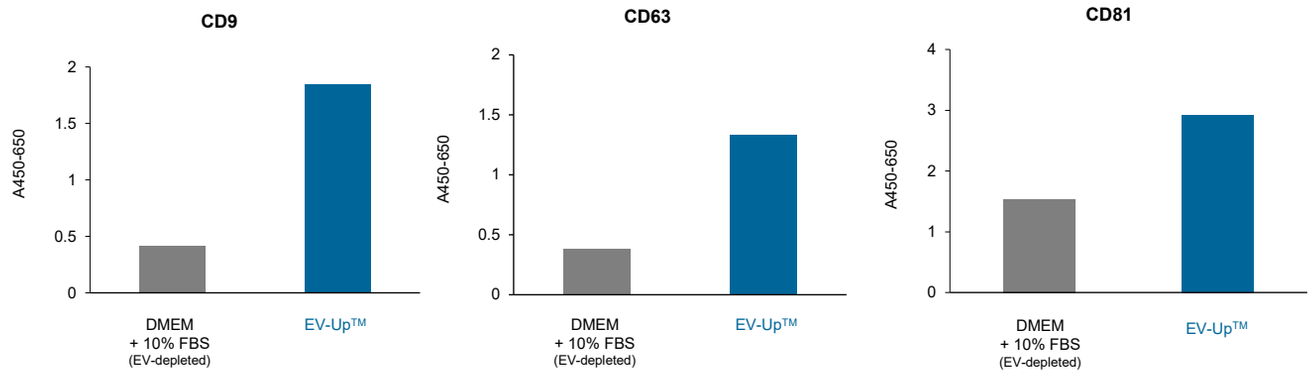
Human bone marrow-derived MSCs were expanded in a serum-containing medium and in EV-Up™. The cells were then cultured in each medium for 5 days for EV production, and the survival rate was measured.

MSCs cultured in EV-Up™ had a high survival rate, similar to cells cultured in a conventional medium.



▾ Expression of Exosome Marker Proteins

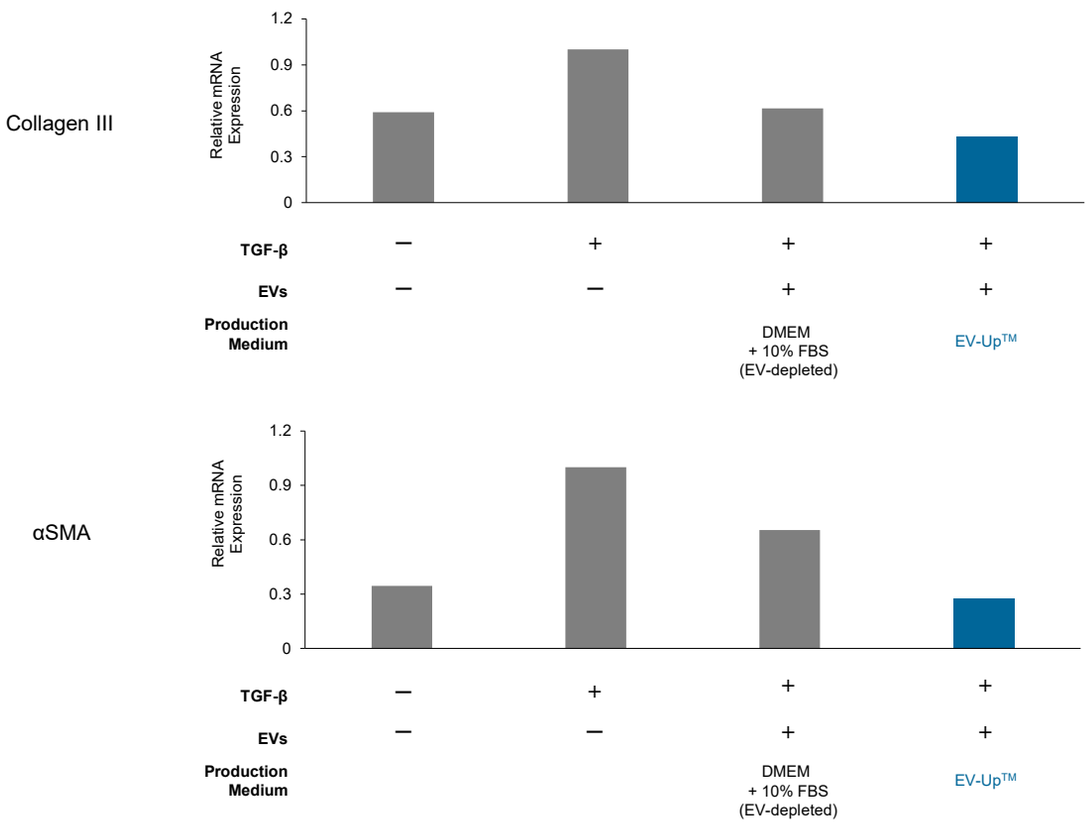
Exosomes were purified from the culture supernatant by the PS affinity method, and the expression of marker proteins CD9, CD63, and CD81 was examined by ELISA.



More exosomal marker proteins were present in the EV-Up™ culture supernatant compared to a conventional medium.

▾ Antifibrotic Activity

Exosomes (5 x 10^8 particles/mL) purified from each culture supernatant by the PS affinity method were added to TGF-β-stimulated human fetal lung-derived fibroblasts (TIG3 cells). Subsequently, mRNAs for fibrosis markers (Collagen III, αSMA) were quantified by RT-PCR, and antifibrotic activity was compared.



Exosomes produced from MSCs cultured in EV-Up™ had higher antifibrotic activity than the exosomes produced from cells cultured in a conventional medium.

Product Number	Product Number	Grade	Package Size
053-09451	EV-Up™ EV Production Basal Medium for MSC, AF	for Cell Culture	95 mL
298-84001	EV-Up™ MSC EV Production Supplement, AF		for 100 mL

Horizontal Co-Culture Plate

UniWells™ Horizontal Co-Culture Plate

UniWells™ Horizontal Co-Culture Plate is a new co-culture plate consisting of two wells connected horizontally. In the case of conventional vertical co-culture vessels,, the same culture medium is shared between the top and bottom compartments, resulting in disadvantages that culture conditions for the two compartments are different, and that simultaneous observation of cells in both chambers is not possible. This product has horizontally connected wells, allowing cells to be cultured under the same conditions and be observed simultaneously.

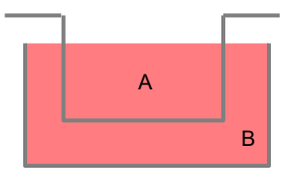
Features

- Simultaneous observation with a time-lapse microscope
  Because there is no need to change the focus, cells in both compartments can be observed simultaneously.
  The plate can be placed under various microscopes, using an adapter for the size of the glass slide used (supplied with the main unit).
- Cells can be cultured under identical conditions.
  Cells can be cultured in the same amount of medium and with the same bottom surface material.
- Any filter can be used.
  Commercially available filters can be inserted to prevent cells from being mixed.
- Cells can be cultured independently.
  Separately cultured cells can be connected in any combination.

Advantages of the Horizontal Plates

The Same Amount of Medium can be Used in Each Well for the Experiment.

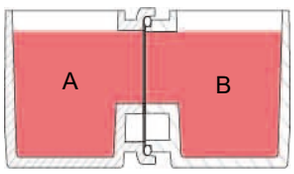
Conventional product



A:B = 1:3

Conventional vertical co-culture vessels require a larger volume of medium in the lower chamber, which dilutes the factors secreted from the cells in the upper chamber.

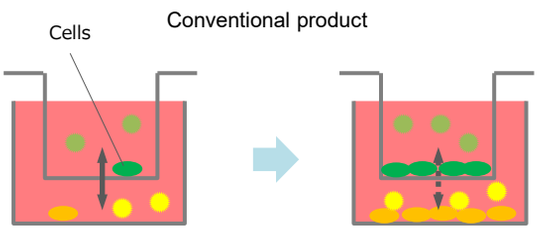
UniWells™



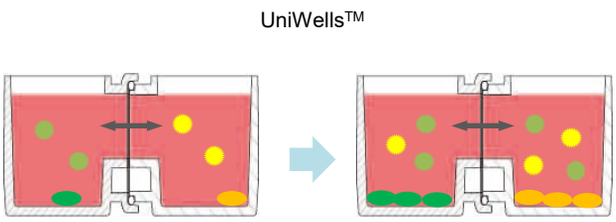
A:B = 1:1

With UniWells™, cells can be cultured with the same amount of medium in both wells.

The Filter is not Clogged by the Cells.



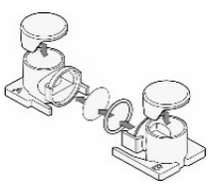
In conventional vertical co-culture vessels, the filter can be clogged by the cells, which blocks the passage of the factors secreted from the cells.



With UniWells™, the filter is not clogged by the cells even when they are confluent.

Usage

Two Connected Wells

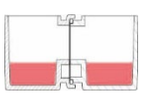


Connect by fitting the filter*, O-ring, and cover. *The filter needs to be purchased separately.

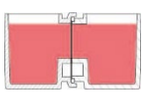
The wells can be connected in two ways.

(1) Aspirate the culture medium from wells that have been cultured separately, and connect.

(2) Connect the two wells first and increase the volume of the culture medium to create a co-culture condition.

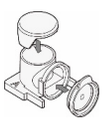


Non-co-culture condition



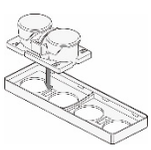
Co-culture condition

Single Well



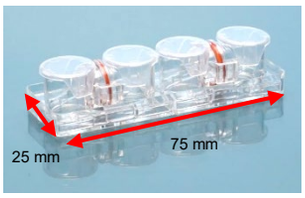
Place a common lid and cover before use.

For Microscopy

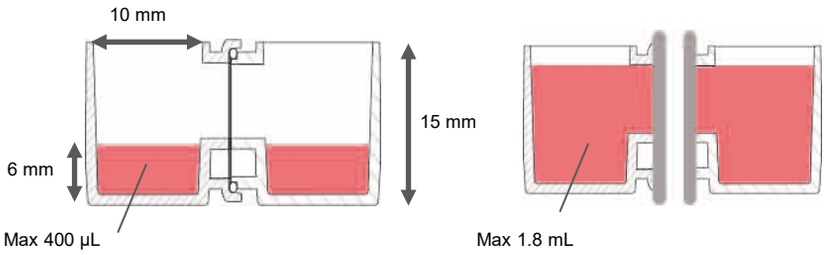


Set the plate on the adapter for the size of the glass slide used, and cells can be easily observed under a microscope.

Size



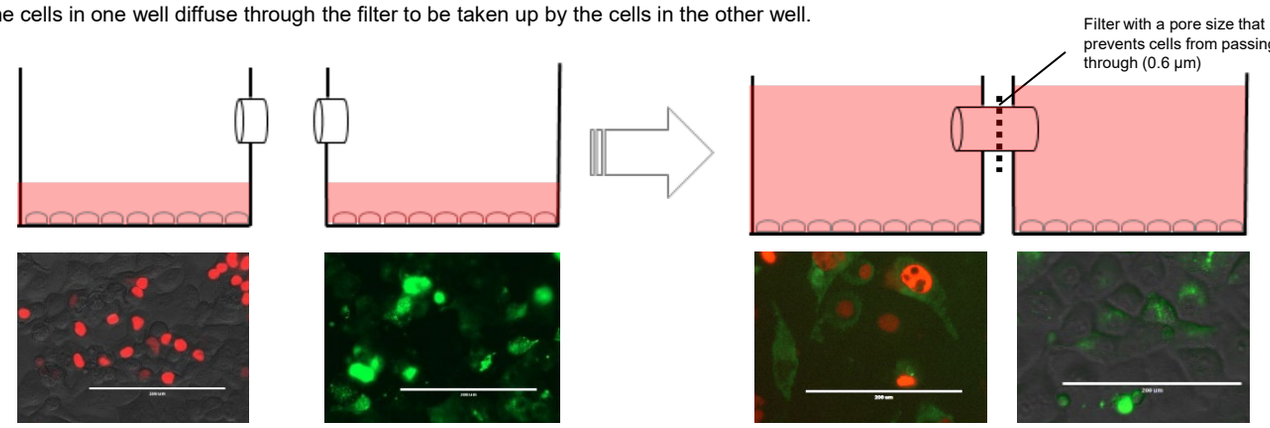
The adapter supplied with the main unit of UniWells™ is the same size as a standard glass slide. The adapter can hold another pair of connected wells, as shown in the photo on the left.



Application Data

Uptake of Exosomes

The following experiments were performed using UniWells™ Horizontal Co-Culture Plate to determine whether exosomes released from the cells in one well diffuse through the filter to be taken up by the cells in the other well.



Cell A
Nuclei are stained in red

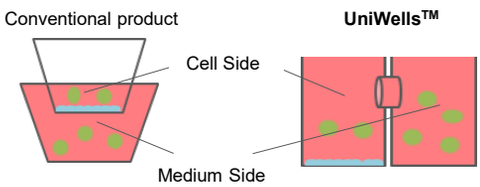
Cell B
Expressing CD63-GFP fusion protein

Exosomes released by Cell B were taken up by Cell A without mixing the cells.

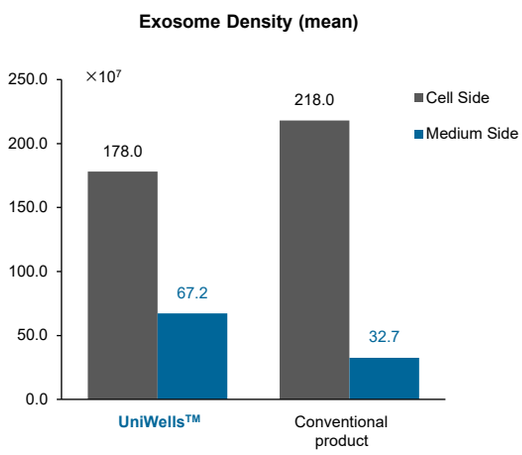
By using UniWells™, only exosomes were allowed to pass through, without mixing the cells.
This allows for easy examination of the uptake of exosomes by different cells.

Comparison of the Efficiency of Exosome Permeability

The same number of cells were seeded in one well of a conventional co-culture vessel or UniWells™. Co-cultures were initiated 24 hours after the seeding. Exosomes in each well of the co-culture vessels were measured by NanoSight 48 hours after the seeding. The total amount of medium is the same in both vessels in this experiment.



More exosomes passed through the filter in UniWells™ than in the conventional product.



Product Number	Product Name	Manufacturer	Package Size
384-14421	UniWells™ Horizontal Co-Culture Plate	Ginreilab Inc.	10 set
381-14431	UniWells™ Filter 0.03 µm		50 sheets
388-14441	UniWells™ Filter 0.6 µm		50 sheets
380-19261	UniWells™ Filter 1.2 µm		50 sheets
388-17001	UniWells™ Adapter 96		1 EA

MagCapture™ Exosome Isolation Kit PS Ver.2

PS Affinity Method

Is it possible to purify exosomes and microvesicles separately?

Exosomes and microvesicles cannot be completely separated, because they are not clearly distinguishable by size. The following simple separation method using this kit is recommended to obtain the major fractions for each:
When purifying small extracellular vesicles (small EVs) such as exosomes, use the supernatant after centrifugation at 10,000 x g as the sample.
To purify large extracellular vesicles (large EVs), including microvesicles, first collect the supernatant after centrifugation at 1,200 x g, and then collect the pellet after centrifugation at 10,000 x g. Suspend the pellet in TBS for use as a sample. If both are purified together, use the supernatant after centrifugation at 1,200 x g as the sample.

Is anything other than exosomes and microvesicles isolated?

Enveloped viruses are also collected. Since phosphatidylserine is also exposed on the surface of enveloped viruses, both are recovered from samples contaminated with enveloped viruses. Due to this property, this kit may be used to isolate enveloped viruses. The purification of viruses using the PS affinity method was reported in the following paper:

<References>
Santiana, M. *et al.*: *Cell Host & Microbe*, **24**, 208(2018).

To isolate a virus, affinity purification with an antibody specific to the virus must be performed after collection with this kit. This is true not only for Fujifilm Wako's kit, but also for purification methods using antibodies against exosome markers such as CD63 that is also present on the envelope.

Do all exosomes have exposed PS?

As described on p. 9, a comparison of the number of EVs in a sample before and after purification by the PS affinity method using the NTA and ELISA methods shows that about 90% of EVs were recovered from all cell or body fluid samples tested.
EVs purified by the PS affinity method showed a higher percentage of low anionic EVs and a lower percentage of high anionic EVs compared to the EVs purified by the ultracentrifugation and size exclusion methods.

What are the advantages of this method compared to other exosome isolation methods?

- **Comparison to Ultracentrifugation**

High purity exosomes can be easily and reproducibly recovered with high efficiency. Exosomes have been recovered from samples that are not suitable for precipitation by ultracentrifugation. The purity of the recovered exosomes is also high; the purity is equivalent to that of the exosomes purified by combined ultracentrifugation and density gradient centrifugation methods.

- **Comparison to Polymer Precipitation**

The yield is higher, and high-purity exosomes can be obtained compared to polymer precipitation.

- **Comparison to Antibody Affinity Methods**

The antibody affinity method uses antibodies against surface antigens of exosomes and requires elution with a denaturing agent or dissociation under acidic conditions for release and recovery of exosomes. With this kit, elution is done with a chelating agent under neutral conditions, allowing for recovery of intact exosomes. Also, contamination of proteins nonspecifically adsorbed on the beads is low, and high purity exosomes can be recovered efficiently.

Sample

What types of samples can be used for purification?

Exosomes have been recovered from cell culture supernatants, serum, plasma (heparin and EDTA), urine, and feces. Users have also reported purification of exosomes from cerebrospinal fluid and saliva.

What animal species are suitable for this kit?

Exosomes have been isolated from samples derived from humans, mice, rats, dogs, and monkeys.

What is the minimum volume of sample needed for purification?

To ensure consistent mixing of the beads and samples, use at least 500 µL of sample when the reaction is performed using a rotator, or 100 µL when the reaction is performed using a tube mixer.
If a smaller volume of sample is used, TBS should be added to bring the volume to the minimum level before reacting with the Exosome Capture-coated beads. It is recommended to add EV-Save™ Extracellular Vesicle Blocking Reagent (p. 23) or EV-Save™ Extracellular Vesicle Blocking Reagent for *in vivo* (p. 24) to the TBS used for filling up the samples.

Is it possible to recover exosomes from large-volume samples?

Although complete recovery is difficult, it is possible after concentration. Up to 50 mL of cell culture supernatants can be used. Concentrate 50 mL of pre-centrifuged cell culture supernatant by ultrafiltration to 1 mL (recommended filter: Sartorius Vivaspin 20, molecular weight cut-off 100 K, Product No: VS2041). Not only serum-free medium, but medium containing 10% FBS can be used. Serum samples cannot be concentrated, and up to 1 mL can be used.

Molecular weight cut-off of 100 K is recommended for concentration by ultrafiltration, but is it possible to use 10 K?

Ultrafiltration filters with cut-off values of 100 K, 300 K, and 1,000 K were tested at Fujifilm Wako, and a 100 K filter is recommended based on the concentration time and the amount of exosomes concentrated. Filters with smaller cut-off values of 10 K and 30 K could be used, but longer concentration times may be required. For media containing albumin, yield of exosomes may be reduced due to concentrated albumin.

Yield of Exosomes

How many exosomes can be isolated from each purification process?

Although it varies greatly depending on the type and volume of the sample, about 30 µg/mL of protein (measured by the BCA method) and 1-2 x 10^{10}/mL of particles (measured by NanoSight LM10) in a purification process were obtained when 5 mL of K562 cell culture supernatant (exosome secretion was stimulated by monensin sodium salt) was concentrated to 1 mL and used for purification. In addition, about 34 µg/mL of protein and 5 x 10^{9}/mL of particles have been recovered from 1 mL of pooled normal human serum in a purification process.

What is the maximum yield of exosomes?

The maximum yield of one purification process is approximately 1-5 x 10^{10} particles.

MagCapture™ Exosome Isolation Kit PS Ver.2

Kit Components and Procedure

What is the composition of the eluent?

It is a PBS-based solution containing 1 mmol/L of chelating agent and salts. If these ingredients interfere with subsequent analysis, replace the buffer with an appropriate buffer by ultrafiltration (Sartorius VivaSpin500, molecular weight cut-off 100 K, Product No.: VS0141) or gel filtration.

What steps should be performed with particular care when using this kit?

In the final part of the washing step after the reaction of the sample with the Exosome Capture-coated beads, the washing solution should be thoroughly removed. Proceed to the elution step after ensuring that the washing solution has been completely removed.
In the elution step, suspend the beads thoroughly after adding the elution solution, making sure the beads are not aggregated.

Is there a step that allows the purification procedure to be carried over to the next day?

The reaction step with the sample and the Exosome Capture-coated beads can be done overnight.

Is it possible to reuse the used Exosome Capture-coated magnetic beads?

It is possible. Used magnetic beads can be reused up to 4 times to recapture the remaining exosomes in the sample. The kit is supplied with enough buffer for up to 50 reactions (in the case of a 10-reaction kit) when the same sample is extracted repeatedly, or when there is no concern about cross-contamination. The reuse is recommended for recovery from samples with volumes greater than 1 mL or from concentrated samples.

Is it possible to store Exosome Capture-coated magnetic beads?

It is possible. When reusing Exosome Capture-coated beads after elution of exosomes, store them in the Washing buffer supplied with the kit or in TBS prepared separately, and under refrigeration. (2 years by in-house test)

Application

What types of analysis are possible with the isolated extracellular vesicles?

Intact extracellular vesicles are obtained and can be used for various analyses.

<Example>

Protein analysis:	SDS-PAGE, Western Blotting, Proteomics analysis, Flow cytometry, ELISA
Genetic analysis:	qPCR, Microarray, NGS
Particle analysis:	Electron microscopy, Nano-tracking analysis
Functional analysis:	*in vitro* or *in vivo* administration

What amount of extracellular vesicles is required for each experiment?

Below are the results of in-house testing.

Experiment	Sample amount (example)
Electron microscopy	2-4 x 10^{10} particles/mL
Microarray	COLO201: 4.6 x 10^{10} particles TIG3: 1.7 x 10^{10} particles iPS: 1.9 x 10^{9} particles
Proteomics analysis	Purified exosome approximately 1 µg
Western Blotting	15 µL amount out of 100 µL of eluate was used.

How many months can isolated extracellular vesicles be stored?

Isolated extracellular vesicles can be stored for 12 months with added EV-Save™ Extracellular Vesicle Blocking Reagent (p. 23).

Is it possible to add the isolated extracellular vesicles directly to the cells in subsequent experiments?

Extracellular vesicles isolated using Ver. 2 have reduced cytotoxicity compared to those using the previous kit, and can be used in *in vitro* and *in vivo* experiments. However, if EDTA in the elution buffer causes a problem, the buffer should be replaced.

Troubleshooting

Purification has been unsuccessful. What can be done?

Please prepare positive controls. Positive controls can be obtained by culturing any cells such as HEK293, preparing the required amount of culture supernatant, and isolating exosomes using the kit. Also, the amount of extracellular vesicles in the medium may be low. Increase scale of the culture.

The total protein content of exosome samples purified with this kit is lower than that of other isolation methods. What is the cause of this?

The total protein content is higher in exosome samples recovered by other methods because of the large number of impurities. In contrast, the total protein content of the exosome samples purified with this kit is lower, but the actual amount of exosomes obtained is not different compared to other isolation methods, because of the high purity of the samples.

The magnetic beads do not stick to the side of the magnetic stand. Are there any possible causes?

It is possible that iron, a component of magnetic beads, has been oxidized by a component in the sample that acts as a chelating agent (e.g., citric acid), resulting in decreased adsorption capacity.

PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD/Streptavidin HRP)

Performance

Which is more sensitive, the PS Capture™ Exosome ELISA Kit or the CD-Capture Exosome ELISA Kit?

PS Capture™ Exosome ELISA Kit is more sensitive. Tim4 captures a wider range of extracellular vesicles more efficiently compared to antibodies against exosome markers (CD9, CD63, CD81).

Sample

What is the minimum amount of samples for detection?

Extracellular vesicles equivalent to 1 ng of proteins can be detected. For reference, the detection limit of extracellular vesicles purified from COLO201 cell culture supernatant was 11 pg. (The detection limit varies depending on the cell line.)

Is it possible to directly measure serum or plasma samples?

It is possible with the PS Capture™ Exosome ELISA Kit (Streptavidin HRP). The PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD) is not suitable for direct measurement of serum and plasma samples, because the secondary antibody for detection provided with the kit reacts non-specifically with human, mouse, and rat IgG.

Is it possible to directly measure cell culture supernatant samples?

Both kits can be used for direct measurement. Since the antibodies supplied with the kit do not react with bovine IgG, cell culture supernatants with a serum-free medium as well as an FBS-supplemented medium can be directly measured.

What cell lines have been successfully used?

The following cell lines have been successfully used in in-house testing:

Cell line	Origin
A549	Human Caucasian lung carcinoma
BxPC-3	Human primary pancreatic adenocarcinoma
COLO201	Human Caucasian colon adenocarcinoma
COS7	African green monkey kidney
FM3A	Mouse C3H mammary carcinoma
HCT116	Human colon carcinoma
HEK293	Human embryo kidney
HEK293T	Human embryo kidney
HeLa	Human cervix carcinoma
HPAF II	Human pancreatic tumor
HuH-7	Human hepatocellular carcinoma
HUVEC	Human umbilical vein endothelial cell
iPSC	induced pluripotent stem cell
K562	Human Caucasian chronic myelogenous leukemia
LNCaP	Human Caucasian prostate carcinoma
P388D1	Mouse leukemia
Panc-1	Human Caucasian pancreas
RAJI	Human Burkitt's lymphoma
SH-SY5Y	Human neuroblastoma
TIG-3	Normal human diploid cells
THP-1	Human monocytic leukemia
U2OS	Human osteosarcoma
BM-MSC	Human Mesenchymal Stem Cells from Bone Marrow
iCell-MSC	Mesenchymal Stem Cells from iPS-01279

How much sample is required for direct measurement of culture supernatant or body fluid samples?

A few µL (1 to 5 µL) of culture supernatant or body fluid sample is sufficient for measurement. This amount is recommended when analyzing the time course of the number of extracellular vesicles in culture medium or when analyzing new cell culture supernatant samples. However, depending on the cell type (such as iPS cells), the number of extracellular vesicles in the medium may be low. When the number of extracellular vesicles in the sample is unknown, a preliminary analysis to determine an appropriate sample volume is recommended.

Kit Component

Why are there no standards included?

This is because the type and amount of surface marker protein of extracellular vesicles secreted by each cell type vary, and standards and assay samples should be derived from the same cell type. Please prepare standards purified from the culture supernatant of cells derived from the same origin as the sample.

Is it necessary to prepare standards? If so, is it necessary to use the PS affinity method?

When performing quantitative measurements, prepare extracellular vesicles as standards. Although extracellular vesicles purified by ultracentrifugation, polymer precipitation, or other methods can be used as standards, it is recommended to use extracellular vesicles purified by the PS affinity method, which uses the same principle as the measurement, as the standard. (For details of the preparation method, please refer to the instruction manual of the kit.)

Can the primary antibodies be changed?

For PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD), select the mouse antibody against the surface marker to be detected, and follow the instruction manual for determining the optimal concentration.
When using the PS Capture™ Exosome ELISA Kit (Streptavidin HRP), select a biotin-labeled antibody for detection of any surface marker, and follow the instruction manual for determining the optimal concentration. If biotin-labeled antibodies are not commercially available, unlabeled antibodies can be biotin-labeled using the Biotin Labeling Kit from Dojindo Laboratories, etc.

Procedure

What is the processing time when using the kit?

The processing time with the PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD) Kit is approximately 5 hours. The processing time with the PS Capture™ Exosome ELISA Kit (Streptavidin HRP) is approximately 6 hours.

Is there a step that allows the procedure to be carried over to the next day?

The step to immobilize each sample on the plate can be done overnight at 4˚C.

Is it possible to reuse the Exosome Capture 96 Well Plate?

Proteins on the plate are denatured by the Stop Solution and cannot be reused.

Troubleshooting

The detection has been unsuccessful. What should be checked?

Please check the following points:

- Whether any of the reagents is expired
- Whether Exosome Binding Enhancer (100 x) was added to Washing Buffer
- If there is no signal with the control antibody supplied with the kit, the expression level of CD63 may be below the detection limit, or there may be other causes.

CD9 / CD63 / CD81-Capture Exosome ELISA Kit (Streptavidin HRP)

Performance

Which is more sensitive, the PS Capture™ Exosome ELISA Kit or the CD-Capture Exosome ELISA Kit?

PS Capture™ Exosome ELISA Kit is more sensitive. Tim4 captures a wider range of extracellular vesicles more efficiently compared to antibodies against exosome markers (CD9, CD63, CD81).

Procedure

Is there a step that allows the procedure to be carried over to the next day?

The step to immobilize each sample on the plate can be done overnight at 4˚C.

PS Capture™ Exosome Flow Cytometry Kit

Sample

What animal species are suitable for this kit?

The kit has been used with samples derived from humans, mice, cows, and monkeys.

What animal species are suitable for this kit?

Approximately 33 µL is required per sample. However, if the amount of extracellular vesicles in the sample is small, pretreat the culture supernatant by centrifugation and concentrate by ultrafiltration to prepare samples (recommended filter: Sartorius Vivaspin 20, molecular weight cut-off 100 K, Product No.: VS2041).

Is there a way to measure purified exosome samples?

Dilute the purified exosomes to an appropriate concentration, and proceed to 2, “Isolation of Extracellular Vesicles," in the instruction manual. To dilute purified exosomes, use the Washing Buffer (10 x) supplied with the kit diluted 10-fold with ultrapure water. Purified exosomes can be detected at concentrations of 125-1,000 ng/mL. Red fluorescent (635)-labeled anti-CD63 monoclonal antibody (clone 3-13) (pp. 21-22) is recommended as the detection antibody.

Kit Components and Procedure

Is it possible to use the MagCapture™ Exosome Isolation Kit PS Ver. 2 for flow cytometry?

It is not possible. Use the PS Capture™ Exosome Flow Cytometry Kit, in which the magnetic beads and the composition of wash buffer are optimized for flow cytometry.

Are antibodies for exosome detection included in this kit?

Fluorescently labeled antibodies for exosome detection are not included in this kit. Please purchase an appropriate fluorescent-labeled antibody such as fluorescein-labeled anti-CD63 antibody or red fluorescent dye-labeled anti-CD63 antibody (pp. 21-22).

Is it possible to use fluorescent-labeled secondary antibodies for detection?

It is possible. After isolating exosomes, follow the protocol below for primary antibody reaction, secondary antibody reaction, and flow cytometry analysis.

1. Mix unlabeled primary antibody and Exosome Capture Beads, and allow to stand at room temperature for 1 hour. Vortex the magnetic beads for about 5 seconds at 20 min, 40 min, and 1 hour.
2. Wash twice with 300 µL of WB (+ Enhancer).
3. Dilute PE-labeled secondary antibody from the Jackson ImmunoResearch Laboratories (Product No.: 115-115-164) 100-fold with WB (+ Enhancer) and add to the magnetic beads in 2.
4. Allow to stand at room temperature for 1 hour. Vortex the magnetic beads for about 5 seconds at 20 min, 40 min, and 1 hour.
5. Wash 3 times with 300 µL of WB (+ Enhancer).
6. Suspend the magnetic beads in 300 µL of WB (+ Enhancer).
7. Analyze by flow cytometry.

Application

Is quantitative analysis possible with this kit?

For quantitative analysis, the number of Exosome Capture factors per bead particle needs to be determined. This product is not guaranteed for it, and quantitative analysis is not possible.

Troubleshooting

Multiple Exosome Capture Beads and exosomes are bound, causing the magnetic beads to aggregate.

Set the gate for singlet bead fraction based on the forward and side-scatter light plots, and detect the fluorescent signal of Exosome Capture Beads within the gate. For a typical sample, the singlet bead fraction represents 50-70% of the total.

The magnetic beads do not stick to the side of the magnetic stand.

The magnetic beads in this kit are optimized for flow cytometry, and the concentration of the magnetic beads is low. It may be difficult to visually confirm whether the magnetic beads are collected. In the washing step, place the tube on the magnetic stand for at least 1 minute, and then slowly pipet so as not to aspirate the magnetic beads.

EV-Save™ Extracellular Vesicle Blocking Reagent

Application

Is it possible to use purified exosome samples for proteome analysis after this reagent has been added?

It is possible after EV-Save™ is removed by the following method:

1. Add 5 times the volume of cold acetone to purified EVs and mix by inversion.
2. Allow to stand at -20℃ for 1 hour.
3. Centrifuge at 1,500 × g for 10 min at 4℃
4. Remove the supernatant
5. Repeat steps 1-4 three times.
6. Open the lid and allow to air dry for 1 hour (RT)
7. Dissolve in 20 µL of 9 M urea, 20 mM HEPES-NaOH, pH 8.2.