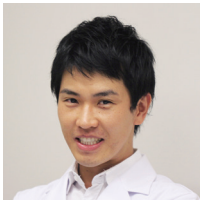
Interview

Applying the Density Gradient Ultracentrifugal Isolation Method in

Exosome Subclass Analysis

Dr. Kiyotaka Shiba

Division Head, Division of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research



Dr. Kazuya Iwai

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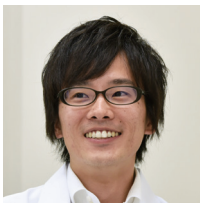
Department of Oral Implantology



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Interview

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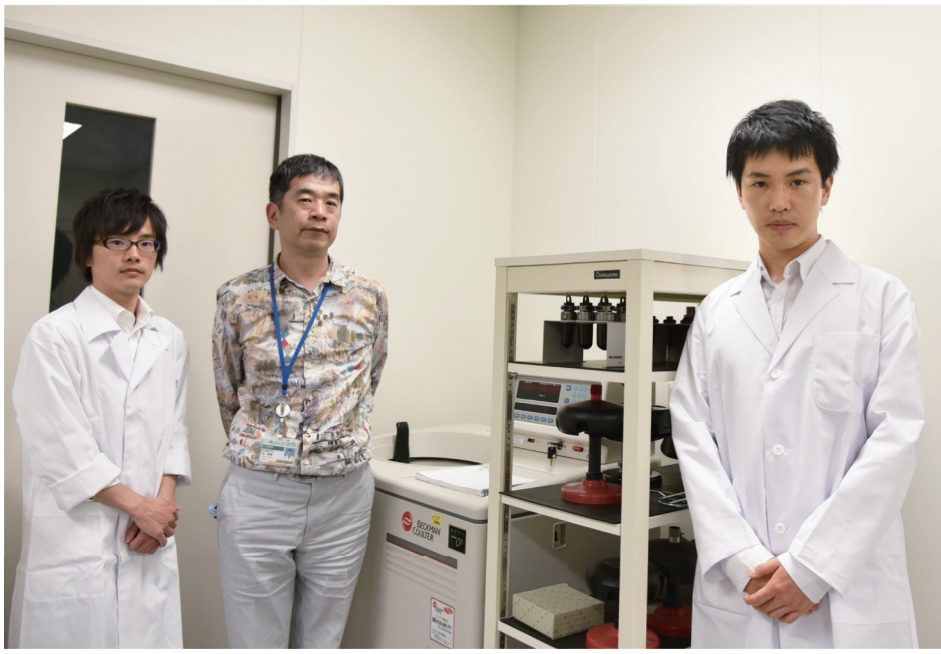
Exosomes are extracellular vesicles that have attracted attention since the late 2000s as communication agents between cells, as well as potential diagnostic/therapeutic tools for various disorders. Much effort has been put into research on their secretion/transport modality, the substance contained within and other characteristics. The reality, however, is that there is no consensus on the fundamental question, "What are exosomes?" In order to define exosomes, research on their isolation method is essential.

Dr. Kiyotaka Shiba conducts research focusing on exosome and leads the Protein Engineering Division at the Cancer Institute of the Japanese Foundation for Cancer Research. Along with research scholars at the institute, Dr. Kazuya Iwai and Dr. Satoshi Yamamoto, from the Department of Oral Implantology at the Tokyo Dental College, Dr. Shiba discusses subclass analysis based on exosome density, as well as purification methods and cautions.



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Dr. Kiyotaka Shiba

Division Head, Division of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research

Profile

1981 • Graduated from the Faculty of Science, Kyoto University

1986 • Completed the second half of the doctoral program at the Graduate School of Science, Kyoto University; obtained a Ph.D. in Science

1986 • Instructor, National Institute of Multimedia Education Development Center

1987 • Assistant (RNA molecular biology), Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo

1989 • Doctoral fellow (Protein engineering), Department of Biology, Massachusetts Institute of Technology

1991 • Researcher, Division of Cell Biology, Cancer Institute, Japanese Foundation for Cancer Research

2001 • Division Head, Division of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research (to the present)

Dr. Kazuya Iwai

Research scholar, Division of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research Department of Oral Implantology, Tokyo Dental College

Profile

2011 • Graduated from the Tokyo Dental College

2012 • Completed the clinical training in dentistry at the Tokyo Dental College

2012 • Graduate School of Dental Research (Oral Implantation), Tokyo Dental College

2012 • (Research scholar, Division of Protein Engineering) Cancer Institute, Japanese Foundation for Cancer Research

2016 • Completed the doctoral program at the Graduate School of Dental Research, Tokyo Dental College; Ph.D. (Dentistry)

2016 • (Resident, Division of Oral Implantation) Tokyo Dental College Suidobashi Hospital (to the present)

Dr. Satoshi Yamamoto

Research scholar, Division of Protein Engineering, Cancer Institute, Japanese Foundation for Cancer Research Department of Oral Implantology, Tokyo Dental College

Profile

2013 • Graduated from the Tokyo Dental College

2014 • Completed the clinical training in dentistry at the Tokyo Dental College

2014 • Graduate School of Dental Research (Oral Implantation), Tokyo Dental College

2014 • (Research scholar, Division of Protein Engineering) Cancer Institute, Japanese Foundation for Cancer Research (to the present)

Applying the Density Gradient Ultracentrifugal Isolation Method for Exosome Subclass Analysis

On Exosome Research

Exosomes are interesting cells once believed existed relatively independently from each other are actually highly connected via exosomes (Fig. 1). There is a constant traffic of substances between cells via exosomes, and it seems that they communicate with each other. Even distinct living organisms exchange nucleic acids and proteins via exosomes, and I find myself seeing the world differently, as if all living things are connected by exosomes.

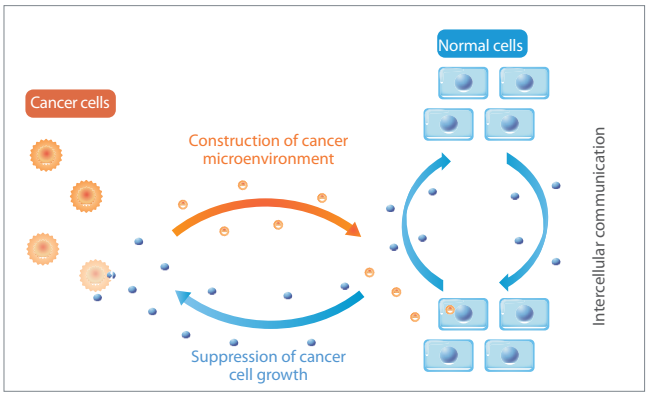


Fig. 1 Intercellular communication via exosomes

There's no doubt that a new era in medicine will be here one day, with exosomes at its center. This includes all areas: prevention, diagnosis and treatment.

With regard to the biological significance of exosomes, there has been a number of excellent research findings reported in the last two to three years that utilized clever genetic approaches. However, for the most part there is not yet an answer to this fundamental cell biology/biochemical question?
- "What are exosomes?" In fact, there is no consensus on the definition of exosomes among exosome researchers, and confusion persists even at a basic level. Having said that, we are certain that "something entangled with the lipid bilayer" that is secreted by cells enables various biological activities of which we were once unaware.

How we purify exosomes is also related to how we define exosomes, and without establishing solid purification and quantitative methods, we will not be able to determine a good definition. Inherently, defining something as diverse as exosomes becomes baffling when people give serious thought to the matter. Considering that difficulties in scientific endeavors that target diverse groups are also present in cancer research, I believe this is an upcoming challenge in the field of biology.

Our team is currently researching exosome purification and quantification methods, and our goal is to establish the basic technology for exosome research. Today we will introduce the exosome purification method we use in our division.

Density-based Exosome Subclass Analysis

One approach to see the "something that is entangled with the lipid bilayer" called the exosome, is to look into fractionation based on density. Exosomes are essentially lipid sacks containing nucleic acid, protein and other materials, and because this lipid sack is light, it's possible to fractionate based on density. For this reason, we use the ultracentrifuge.

There are other approaches in the market that collect exosomes using kits and reagents. Many of these kits do not disclose the isolation principle or the name of the reagent. I am apprehensive using these kits for exosome research. Because we are conducting research focused on subclassifying exosomes, we achieve purification using ultracentrifugation rather than kits. With ultracentrifugation, it is possible to fractionate exosomes both theoretically and comprehensively without missing any data.[1, 2)]

To sediment exosomes without density fractionation is also occassionally used. However, what is referred to as exosome "crude fractions" sedimented from ultracentrifugation contain various matter, and interpreting them requires careful attention. If the method allows, it's best to further fractionate the exosome "crude fractions" using density gradient ultracentrifugation isolation. As previously stated, exosomes are surrounded by vesicles, therefore they have a low density compared to protein complexes. For this reason, running ultracentrifugation after forming a density gradient over the exosome "crude fractions" would make small exosomes with low density float up, remaining in the layer that corresponds with their density (Fig. 2).

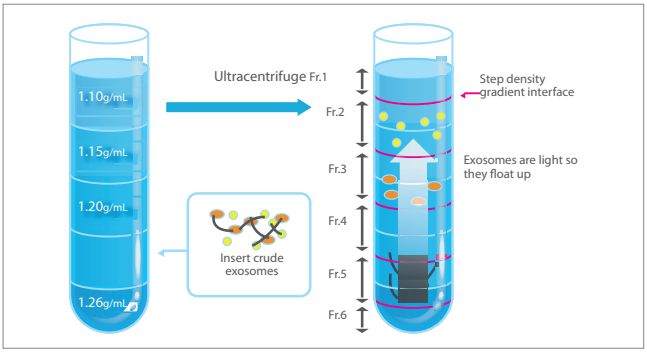


Fig. 2 Overview of equilibrium density gradient ultracentrifugation

We do not consider everything that floats up to be exosomes; instead, we treat them as candidates. We conduct research to define exosome subclasses and non-exosomes among the candidates.

It has been confirmed that there are two classifications for exosomes based on density. There is speculation that the fraction with the lowest density includes a large lipid bilayer sack and an empty sack with almost no protein inside. A larger vesicle is also believed to exist, but it is eliminated in crude centrifugation. To observe large vesicles, it may be better to separate the reagent based on density before running centrifugation.

In addition, it is possible to divide exosome subclasses by their behavior in density gradient centrifugation. For example, with a saliva-derived exosome, the CD9-positive exosome behaves differently from the CD63-positive exosome. It is suggested that these markers determine the exosome subclasses[3].

Subclassification of exosomes dramatically increases the amount of exosome data for diagnosis. Exosomes carry the parental cell data they secrete in "packages," and ideally these data are extracted at the single-particle level. However, this can be a challenging task. For this reason, even extracting data divided into subclasses would significantly increase the volume of data compared to the current bulk analysis that uses the average (Fig. 3).

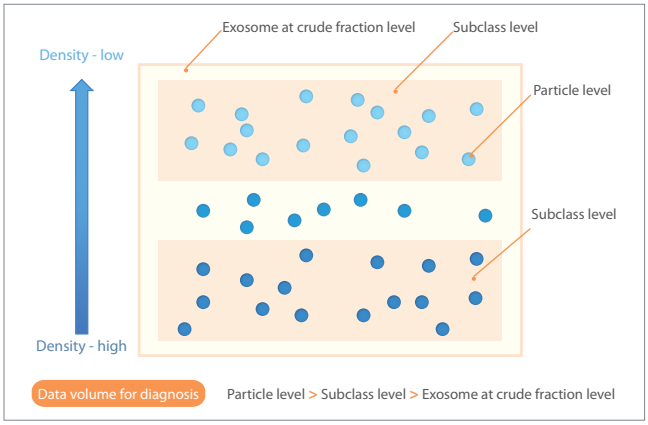


Fig. 3 Data Volume for Diagnosis by Subclass

With density-based subclass analysis of exosomes in saliva, we aspire to put into practice non-invasive diagnostic methods for not only oral diseases, but also systemic diseases such as diabetes and osteoporosis. In the next section, we will describe the purification of exosomes in saliva, which is considered a challenging sample from which to collect exosomes due to the high viscosity.

Purification Method and Cautions in using Equilibrium Density Gradient Ultracentrifugal Isolation Method

I. Equilibrium Density Gradient Ultracentrifugal Isolation Method

We mainly conduct fractionation based on density that uses the equilibrium density gradient ultracentrifugal isolation method. It is important to confirm equilibrium, and we are conducting simultaneous experiments in which one places the sample at the bottom, making it float up, and the other places the sample at the top to make it settle down. We judge that a sample has reached equilibrium if the markers that indicate the existence of exosomes (CD63, CD81) are in the fraction with the same density (Fig. 4). Density gradient often uses sucrose as the material, but we use OptiPrep (compound name iodixanol) (Fig. 5) because there have been many cases that reached equilibrium based on the above mentioned criteria when OptiPrep was used. Although sucrose often does not float up sufficiently and stalls midway when running the equilibrium density gradient ultracentrifugal isolation from the bottom, OptiPrep floats up to the appropriate spot and the location corresponds with the location of the sample placed at the top.

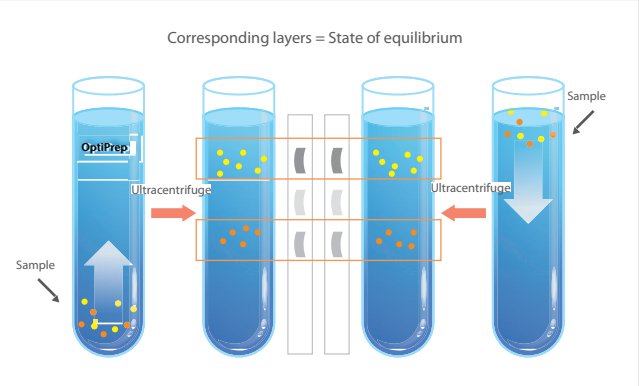


Fig. 4 Confirmation of equilibrium

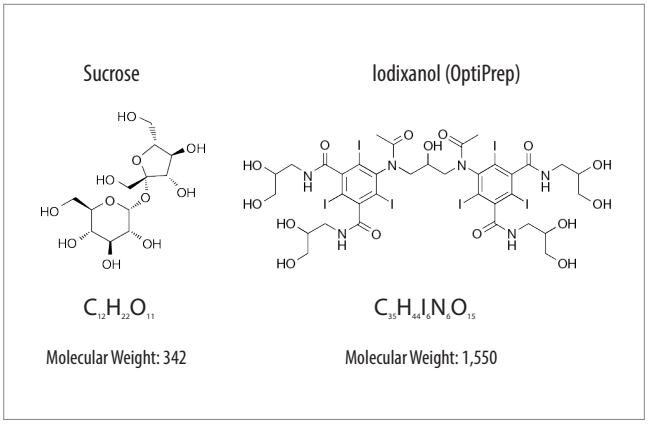


Fig. 5 Density Gradation Media

II. Purification using Swing Rotor

Fig. 6 indicates the protocol for the equilibrium density gradient ultracentrifugal isolation from saliva that we performed.[3] In obtaining exosomes from saliva, it was not possible to directly adopt the protocol for cultured cells. If used without adjustments, the samples would not separate successfully in the density gradient stage. They would also stall midway even if we try to make them float up. We have also confirmed that the fraction that gets stuck shows a high degree of viscosity. For this reason, we treat saliva with ultrasound. Filtration also works, but considering the loss of samples, we have decided to use ultrasound.

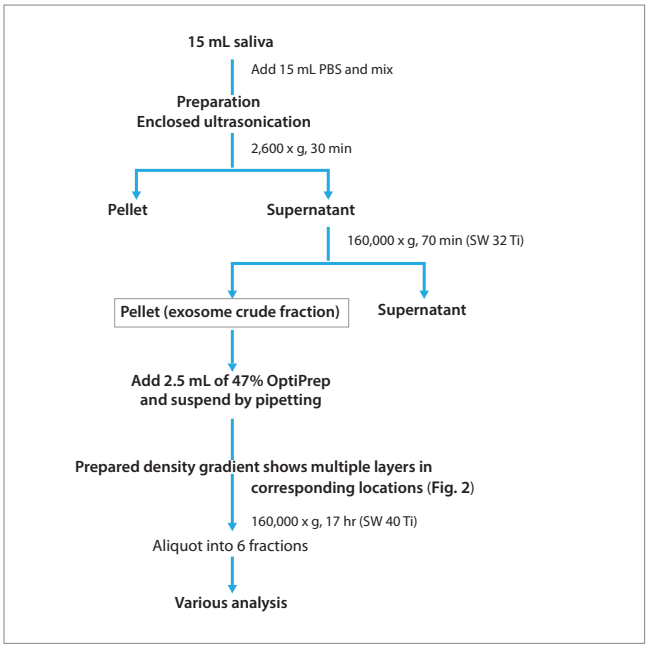


Fig. 6 Equilibrium Density Gradient Centrifugation Protocol using Swing Rotor

After a crude centrifuge of saliva that was treated with ultrasound at 2,600 x g, the supernatant is ultracentrifuged under 160,000 x g for 70 minutes at 4°C on an Optima L-90K ultracentrifuge (Beckman Coulter) with swing rotor SW 32 Ti (38.5 mL Ultra-Clear tubes, #344058). The obtained pellet presents the exosome crude fraction (crude purified fraction, exosome + contaminants). We then suspend this pellet in buffer (47% iodixanol, 0.02 M HEPES/NaOH, pH 7.2) and placed it in the step density gradient buffer (47, 37, 28, 18% iodixanol, 0.02 M HEPES/ NaOH, pH 7.2). In this process we use 14 mL ultra-clear tubes (#344060, Beckman Coulter) and ultracentrifuge at 160,000 x g for 17 hours at 4°C using swing rotor SW 40 Ti. Density gradient may also be prepared using a continuous density gradient preparation device. When there are many samples, the experiment becomes easier when they are prepared in steps. Following centrifugation aliquot fractions from the top every 2.5 mL and confirm using nanoparticle tracking analysis (NTA), western blot and atomic force microscope (AFM).

The fractions obtained may be used in the next experiment as they are, or after running them on an ultracentrifuge, they may be re-suspended in PBS Details are presented later, but because most exosomes are lost when they go through washing, the sample moves straight to the proceeding analysis when miRNA etc. are analyzed. In analyzing NTA etc. as is, it is necessary to correct the viscosity, and because the process requires care, the samples are replaced with PBS before the analysis.

III. Purification using Angle Rotor

The biggest weakness of ultracentrifugation is the throughput. In order to improve the outcome, we have tried to run a density gradient centrifuge with an angle rotor (fixed-angle rotor). Regular swing rotors are able to centrifuge 4 or 6 tubes, and angle rotors are capable of centrifuging more. The tabletop Optima MAX-XP ultracentrifuge and TLA-110 angle rotor enable reductions in sample volume and time. Its protocol is represented in Fig. 7. The procedure is as previously outlined, but we were able to considerably reduce the centrifugation time, and the capacity increased from 6 to 8. We had been limited to one purification a day with the swing rotor but we could run two purifications a day with this. The level of purification is similar to that achieved with the swing rotor, and purification can be performed with no issues.

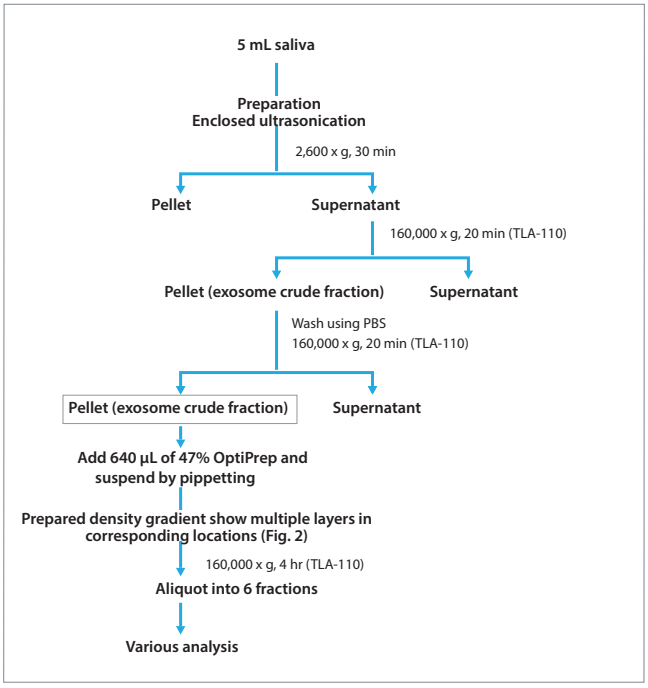


Fig. 7 Equilibrium Density Gradient Centrifugation Protocol using Angle Rotor

IV. Cautions

As a note of caution, there have been some reports that the results differ depending on slight differences in ultracentrifuge conditions. Accordingly, ISEV (International Society for Extracellular Vesicles) and others recommend stating not only the x g and the centrifugation time but also the type of rotor and the tubes used.

One group attempting to precipitate exosomes from breast milk is also creating its own protocol[4], indicating a possible need to develop protocols tailored to each body fluid.

Finally, in conducting exosome research, it is essential to be cognizant of the fact that we are handling highly heterogeneous matter. In conducting basic research, it is necessary to use a purification method that does not miss anything. And this makes an ultracentrifuge the prime choice at this point in time.

Handling Purified Exosomes

I. Adhesion

It is not of concern at the crude isolation stage, but as the degree of purity increases, adhesion to the tube or the pipette chip becomes noticeable. I imagine there are many who have experienced exosomes going missing in the final stage after they reached the density gradient centrifuge stage. Most exosomes with a high purity following a density gradient fractionation disappear when they are condensed using an ultracentrifuge. In other words, they stick to the tube and cannot be resuspended. At the stage of crude purification, I believe various components (serum protein, etc.) that are mixed in large amounts with exosomes coat the tube and prevent exosome adhesion.

I presume all researchers who handle highly pure exosomes have an experience with this adhesive characteristic of exosomes. Aside from ultracentrifugation, regular pipetting and simple operations such as dispensing samples into plastic tubes quickly decrease the number of exosomes, which is a nuisance for researchers.

Because of this we conduct tests on tubes and pipette chips during preparation, and use the ones that are least susceptible to adhesion.

Also, we use ultra-clear tubes that are highly transparent to prioritize pellet collection with the centrifugal tubes. In washing the fraction obtained from density gradient centrifugation, the pellets are at the very bottom when using the swing rotor, and with the angle rotor we take into consideration its angle when making the collection. Adhesion to pipettes is unavoidable in the process, and it is necessary to plan the experiments with this loss in mind. To avoid the loss, if a density gradient medium such as OptiPrep does not influence the proceeding steps, we conduct the experiment without washing. In conducting particle measurement on the unwashed sample using NTA, please do not forget to set the level of viscosity to match the concentration of the medium.

II. Storage

Highly pure exosomes are also very sensitive to frozen storage. There is no noticeable impact at the crude isolation stage, but after fractionating with density gradient centrifugation, freezing would result in losing a percentage of the particles, which is also a source of headaches for researchers.

We use the non-frozen "first squeeze" for the most important experiments, and then break the batch into small portions and use the "second squeeze" that is frozen only once. For experiments that are not influenced by the decrease in the number of particles, we use batches that have been frozen more than twice. For freezing, we use liquid nitrogen and store at -80°C.

Final Remarks

I've been using Beckman Coulter ultracentrifuges since I was a student. In fact, it was a time when ultracentrifuge meant Beckman Coulter. In the past, there was an urban legend that if the balance is not set perfectly, the rotor would fly out and chase after researchers, and ultracentrifugation experiments were a source of distress. However, I believe that newer ultracentrifuges are significantly easier to use compared to earlier models. Until the day we reveal the basic properties of exosomes, I believe many people will continue to depend on ultracentrifuges for some time.

Also, the support system is good and when there is something I do not understand, I immediately call Beckman Coulter and they respond quickly and diligently, so I always make use of their call center.

I used to think that ultracentrifuge is synonymous with Beckman Coulter, but now I recognize them for research/diagnostic devices, and in my view it is an organization that is closely connected to exosome diagnosis research and development.

References

1) Johnstone RM, *et al.* J Biol Chem 1987; 262: 9412-20.
2) Pan BT, and Johnstone RM. Cell 1983; 33: 967-78.
3) K Iwai *et al.* Journal of Extracellular Vesicles 2016; 5: 30829.
4) Marijke I *et al.* Journal of Extracellular Vesicles 2014; 3: 24215.

About the institution

Japanese Foundation for Cancer Research

Cancer Institute

Location: 3-8-31 Ariake, Koto-ku, Tokyo

The Japan Foundation for Cancer Research was established in 1908 with the mission to "contribute to the welfare of the humanity through eradication of cancer." It is a private non-profit organization. After extensive fundraising efforts, the first cancer institute and hospital in Japan were founded in 1934. Ever since, the Japan Foundation for Cancer Research has been active at the forefront of cancer research and promotion of cancer treatments in Japan.

Exosome-related Products

Automated liquid handling device **Biomek 4000**

Automates various assays from the preparation of precise density gradients to exosome miRNA extraction

An automated liquid handling system that enables automation of various experiments from simple dispensing to complex assays based on accurate handling, flexible expandability using optional devices, and software the allows intuitive operation.It is also possible to automate various nucleic acid extraction, pre-treatment of the next-generation sequencer, qPCR setup etc., including downstream applications.

Specifications

Handling range: 1-1,000 µL single- or 8-channel dispensing
Handling precision: Less than 5% CV when handling 1 µL, less than 2% CV when handling 20 µL

RNA extraction kit from cells **Agencourt RNAdvance Cell v2**

Extraction kit suitable for high-yield, high-purity multiple sample treatment

An RNA extraction kit with high yield/purity based on SPRI technology that uses paramagnetic beads.
Optimized for small quantity cells up to 5×10^4. Suitable for multiple sample handling with a lower cost. The protocol for miRNA extraction enables high-throughput miRNA extraction from exosomes. Needless to say, automation using Biomek instruments is also possible.

Specifications

Agencourt RNAdvance Cell v2 (100 preps)
Agencourt RNAdvance Cell v2 (960 preps)

Floor-model ultracentrifuge **Optima X Series**

An optimum model for extraction of exosomes in culture solution

The large 15" touchscreen allows intuitive operation, and it is also possible to set the sample volume with the eye to run ultracentrifugation. Optimum model for extraction of exosomes in culture solution. Swing rotor SW 41 Ti allows efficient exosome collection. The large-volume swing rotor SW 32 Ti can also be used, enabling centrifugation of a maximum of 6 tubes x 38.5 mL.

Specifications

Optima XPN-100: Maximum rotation speed 100,000 rpm, maximum centrifugal force 802,000 x g
Optima XPN-90: Maximum rotation speed 90,000 rpm, maximum centrifugal force 694,000 x g
Optima XPN-80: Maximum rotation speed 80,000 rpm, maximum centrifugal force 548,300 x g
Optima XE-100: Maximum rotation speed 100,000 rpm, maximum centrifugal force 802,000 x g
Optima XE-90: Maximum rotation speed 90,000 rpm, maximum centrifugal force 694,000 x g

Desktop ultracentrifuge **Optima MAX series**

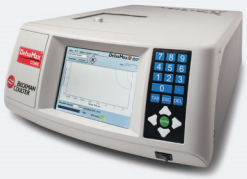
Optimum model for exosome collection from a very small amount of sample, such as blood serum

Touchscreen allows intuitive operation, and it's also possible to set the sample volume with the eye to run ultracentrifugation.
The smallest volume swing rotor in its class, the TLS-55 allows exosome collection from sample volume of 115 µL-2.2 mL

Specifications

Optima MAX-XP: Maximum rotation speed 150,000 rpm, maximum centrifugal force 1,019,000 x g
Optima MAX-TL: Maximum rotation speed 120,000 rpm, maximum centrifugal force 657,000 x g

Dynamic/static light scatter analyzer **DelsaMax CORE**



Achieve real-time particle size analysis with an ultra-small sample

Equipped with two independent detectors and a high-output 100 mW laser, it is possible to perform particle size/molecular weight analysis of samples with a low concentration. Measurement can be taken on just 1 µL of sample, making it suitable for particle size analysis of valuable exosomes, and the collection of sample is possible after the measurement is complete.

Specifications

Measurement range: 0.4 nm - 10 µm (particle size) 300 - 1,000,000 Da (molecular weight)
Sample volume: Quartz cells (1µL, 45 µL), disposable cells (4 µL, 50 µL)

Beckman Coulter, Inc.

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