An Easy-to-Follow
Experimental Protocol

Cell Culture ver. 4.1

Cell Cultivation

01

Cells Aquarium

nacalai tesque
The quality for certainty.

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Protocols with a video icon include an experimental video you can watch.

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Experimental procedures can sometimes be difficult to visualize through text or illustrations. The videos highlight **important points** and **precautions** for each procedure. The **videos are short**, making them ideal for quick checks.

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Basics: Before Starting the Experiment

Cell culture refers to the maintenance and proliferation of cells isolated from living tissues in a culture medium. Cells maintained outside the body are called cultured cells, and the liquid used to support cell growth—designed to mimic extracellular fluid—is called the culture medium. This protocol describes the culture of animal cells (mammalian cells).

What Are Cultured Cells?

Cultured cells are generally classified into primary cells and cell lines. Primary cells are populations of cells directly isolated from living organisms (including tissues and organs). Cell lines, on the other hand, are derived from the passaging of primary cells and consist of populations of cells with consistent characteristics that can be continuously cultured. The term 'cell line' typically refers to immortalized cells; stem cells are usually described separately.
Depending on the culture system, cells that grow attached to the culture vessel are referred to as adherent cells (or attached cells), while cells that grow suspended in the medium are referred to as suspension cells. In general, this reflects their state in vivo: cells that originally formed part of a tissue in the body are typically cultured as adherent cells, whereas cells that circulate in the bloodstream are cultured as suspension cells.

■ Differences Between Primary Cells and Cell Lines

	Primary cell	Cell line
Origin	Cells directly isolated from a living organism and cultured	Cells derived from the passaging of primary cells, capable of being stably cultured.
Features	• They have a finite lifespan and cannot proliferate indefinitely or be maintained in long-term culture • They relatively reflect the characteristics of the tissue of origin • Specialized techniques or media may be required for cell preparation and culture	• They are immortalized cells capable of proliferating almost indefinitely • They often lose the characteristics of their tissue of origin • They can often be cultured in common basal media and are also easy to obtain*
Examples of cell type	Primary hepatocytes, primary neurons, etc.	HeLa cells, CHO cells, HL-60 cells, etc

*Available from the following cell suppliers
ATCC (American Type Culture Collection), ECACC (European Collection of Authenticated Cell Culture)
JCRB Cell Bank and RIKEN BRC,Cell Engineering Division (Japan)

Culture Environment

To perform cell culture, it is necessary to prepare a laboratory suitable for cell culture as well as the appropriate experimental equipment and instruments

■ Laboratory

The laboratory used for cell culture must provide a clean environment to prevent contamination of the cells, and ideally should be separated from ordinary laboratory space. At the same time, in order to minimize or eliminate the risk of exposure of laboratory personnel and the external environment to biohazardous materials, biosafety levels (BSLs) have been established. For detailed information on BSLs, please refer to the World Health Organization (WHO) Laboratory Biosafety Manual, 4th edition.
There is also a similar classification system called the Physical Containment Level (P level). This system is defined under Japanese law, specifically the Cartagena Act, which regulates the use of genetically modified organisms. In this system, experiments are classified based on the combination of the host organism and the source of the introduced genetic material, and each classification specifies the level of physical containment required for safe handling. While P levels are generally comparable to biosafety levels in terms of risk and required facilities, they are based on a different regulatory concept. It is important to note that P levels are legally mandated under Japanese regulations.

Basics: Before Starting the Experiment

■ Essential Equipment for Cell Culture

Name	Details
Clean bench	This is a workbench for performing aseptic operations. When conducting genetic recombination experiments, or depending on the microorganisms or pathogens being handled, it may be necessary to use a biological safety cabinet to prevent cross-contamination from the operator, the environment, the samples themselves, and between samples.
CO_2 incubator	Used to maintain cells at a constant temperature (most commonly 37°C) and CO_2 concentration (5–10%), in conjunction with a CO_2 cylinder. The incubator should be regularly cleaned and sterilized.
Microscope	An inverted phase-contrast microscope is generally used, allowing observation of cell morphology while the cells remain in their culture vessels.
Centrifuge	Used to collect cells during procedures such as passaging or medium exchange.
Water bath	Used to pre-warm culture media and other reagents. It is mainly operated at 37°C, but a model that can also be set to 56°C is recommended for serum inactivation.
Autoclave	Used to sterilize instruments and liquid reagents (limited to those that are heat-resistant).
Refrigerator / Freezer	Used for storing culture media and other reagents.
Ultra-low temperature freezer / Liquid nitrogen storage container	Used for the storage of frozen cells. In general, cells are stored in ultra-low temperature freezers at -80°C, but for longer-term preservation, storage in liquid nitrogen (-196°C) is more suitable.

Aseptic Techniques and Sterilization

In cell culture, contamination refers to the introduction of biological factors other than those intended for culture, which can affect cell growth, function, and experimental outcomes. Microbial contamination is particularly common; therefore, aseptic techniques in a clean bench (or biosafety cabinet) and the use of sterilized reagents and instruments are essential.

≫ For details on contamination, please refer to p. 40.

■ Aseptic Technique

Aseptic work area

To reduce contamination from aerosols such as dust, droplets, and other airborne particles, aseptic techniques should be performed within a clean bench or biosafety cabinet. Below are instructions for the use of clean benches/biosafety cabinets, along with key points for aseptic work areas.

<How to Use a Clean Bench / Biosafety Cabinet>

Before starting operations: Run the circulation fan of the clean bench/biological safety cabinet for at least 5 minutes in advance. Since particles generated during the previous work may serve as a source of contamination, clean the work area as necessary.

During operation: Disinfect all instruments and materials with 70% ethanol (or equivalent) before bringing them into the clean bench/biological safety cabinet.

After operation: Once aseptic procedures are complete, remove any unnecessary items from the clean bench or biosafety cabinet. Disinfect the work surface and the inner side of the front glass with 70% ethanol (or equivalent) to reduce residual sources of contamination. Then, run the circulation fan for at least 5 minutes to remove contaminated air from inside the unit. After stopping the circulation fan, close the front shutter; if desired, switch on the UV lamp to help maintain microbial cleanliness.

<Key Points for Aseptic Work Areas>

- Avoid placing unnecessary items inside the clean bench or biosafety cabinet. These can disturb the airflow and create shaded areas that reduce the effectiveness of the UV lamp, preventing the maintenance of a clean environment.

Basics: Before Starting the Experiment

- If culture medium or reagents are spilled, disinfect immediately with 70% ethanol (or equivalent). Leaving spills unattended can lead to microbial contamination.
- Dispose of used pipettes, tips, and waste liquids immediately after use.
- Be careful of filter clogging in clean benches or biosafety cabinets. Regular inspection is recommended.
- While not considered part of the aseptic work area, water baths used for warming culture media represent a frequent source of contamination. The water bath should always be kept clean, and any media or reagents pre-warmed in it must be disinfected with 70% ethanol (or equivalent) before being brought into the aseptic work area.

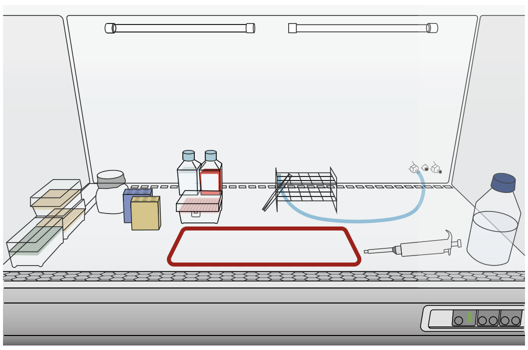
Aseptic technique

Aseptic techniques must be performed carefully yet promptly to prevent microbial contamination. Key points of aseptic operation are outlined below; however, it is more important to practice and become accustomed to these procedures than to learn them only in theory.

When performing aseptic operations for the first time, or if contamination occurs, compare your technique with that of an experienced operator or ask others to observe your handling, in order to verify whether there are any problems with your aseptic technique.

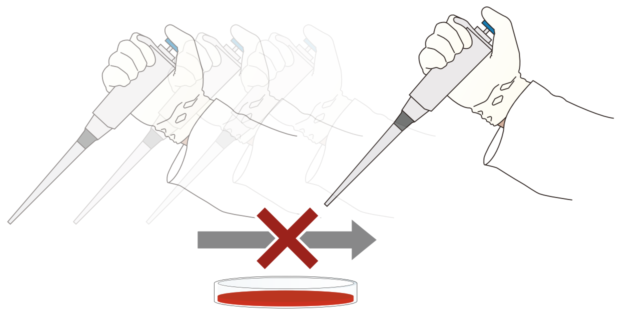
<Key Points of Aseptic Technique>

- Always wash your hands before and after work. Wear personal protective equipment (such as gloves) as necessary. After washing or putting on gloves, take care not to touch your face, hair, or potentially contaminated surfaces such as non-sterile benches.
- During operation within the clean bench or biosafety cabinet, it is essential to maintain laminar airflow. All samples should be placed toward the rear of the work surface (without blocking the rear slit), while bulky items should be arranged to the sides. The central workspace should be kept clear to avoid interference during handling



Ensure that operations are performed within this area.

- To prevent contamination of media and cells by airborne particles or aerosols, open reagent bottles and culture vessel lids only during operation, and avoid passing your hands over the open containers.



Avoid passing your hands unnecessarily over culture dishes containing medium or cells.

- Ensure that the openings of dishes, flasks, tubes, and bottles do not come into contact with your hands, pipettes, or pipette tips.
- To minimize contamination, prepare thoroughly before starting the experiment. Dispense the required amount of medium and other reagents from bottles into centrifuge tubes or similar containers before use.
- Avoid unnecessary actions, such as talking, that may generate particles or disturb airflow either by the operator

performing aseptic work or by those nearby.

- Operations performed under unusual conditions—such as before long holidays, during staff turnover, or under excessive workload—can lead to unexpected errors. Aseptic work should therefore be avoided during such times, or carried out with the utmost caution.

Watch it here!

Basics of Cell Culture

■ Sterilization Methods

Method	Details	Material to be sterilized
Autoclave sterilization	This method sterilizes by direct exposure to high-temperature, high-pressure steam. It is generally performed at 2 atm, 121°C for 15-20 minutes.	• Glassware, heat-resistant plastics, and heat-stable reagents such as salt solutions.
Dry heat sterilization	This method is generally performed at 160-170°C for 120 minutes, or at 180-190°C for 30 minutes.	• Items that are heat-resistant but must not be exposed directly to steam. • Glass pipettes, dishes, CO_2 incubator shelves, trays, etc.
Filtration sterilization	This method sterilizes by filtration through a pre-sterilized membrane filter. Using a 0.2 μm filter, bacteria, fungi, and yeast can be removed; however, some viruses and mycoplasma cannot be eliminated.	• Culture media and other solutions containing heat-labile components.

i Note In addition, sterilization methods such as ethylene oxide gas sterilization and ultraviolet (UV) sterilization are also used. Furthermore, many pre-sterilized reagents for cell culture and pre-sterilized disposable instruments (such as pipettes and flasks) are widely employed.

Endotoxin

Endotoxins are lipopolysaccharides (LPS), structural components of the cell wall of Gram-negative bacteria, and possess a variety of biological activities. When endotoxins contaminate cell cultures, they can induce cytokine production and activate the complement system. Endotoxins are highly heat-resistant; inactivation requires dry heat sterilization at 250°C for at least 30 minutes. Standard autoclave sterilization eliminates bacterial cells, but endotoxin activity may persist. To reduce or avoid the risk of endotoxin contamination, it is essential to pay careful attention to the following potential sources of endotoxins.

■ Major Sources of Endotoxin and Methods of Risk Reduction

Source	Method
Water	Water used for dissolving media, buffers, and cell culture reagents can be a potential source of endotoxin contamination. The risk of contamination can be reduced by purchasing water with a guaranteed endotoxin level or by using a final filter in the ultrapure water system that is capable of removing endotoxins.
Cell culture reagents	Caution is required when using biologically derived materials such as serum and trypsin, or recombinant proteins derived from E. coli. It is recommended to purchase products with guaranteed endotoxin levels.
Laboratory equipment	Use products labeled endotoxin-free (often indicated as pyrogen-free). Alternatively, use equipment sterilized by dry heat at 250°C for at least 30 minutes.
Operator	Human skin, hair, and saliva harbor bacteria, which can serve as sources of endotoxin. During cell culture, proper aseptic technique must be practiced to minimize the contamination of endotoxins.

Basics: Before Starting the Experiment

Culture Medium

Culture medium is a solid or liquid substance that provides the environment necessary for the growth of target organisms such as animal cells, plants, or microorganisms. In this protocol, 'medium' refers specifically to media for animal cell culture. Media supply essential nutrients, growth factors, and hormones required for the maintenance and proliferation of cells, and also establish appropriate conditions for culture such as pH and osmotic pressure. In general, 5–20% animal serum is added as a growth-promoting component. However, serum is expensive, shows significant lot-to-lot variability with poor reproducibility, and carries a high risk of contamination with mycoplasma and other microorganisms. To address these issues, serum-free media and serum substitutes have been developed and are commercially available.

■ Basal Medium

The culture medium to be used should be selected based on the information provided when obtaining or purchasing the cells, as well as relevant literature.
The following are examples of basal media that are commonly used.

Commonly used basal media

Medium	Details
MEM	Eagle developed this medium by investigating the nutritional requirements—such as amino acids, vitamins, sugars, and inorganic salts—of mouse L cells and HeLa cells. Because it contains only the minimum essential amino acids, additional supplements such as MEM essential amino acids or MEM non-essential amino acids are required when culturing cells with higher amino acid demands.
α-MEM	In 1971, Stanners and colleagues modified MEM for the culture of mouse–hamster hybrid cells. α-MEM is based on the formulation of MEM, supplemented with vitamins, non-essential amino acids, deoxyribonucleosides, and ribonucleosides.
DMEM	In 1959, Dulbecco and colleagues modified MEM for the culture of mouse embryonic cells. Compared with the MEM formulation, it contains increased concentrations of amino acids and vitamins. Dulbecco was awarded the Nobel Prize in Physiology or Medicine in 1975. DMEM is widely used for adherent cells.
IMDM	This medium was developed by Iscove and colleagues as a modification of DMEM. Based on the DMEM formulation, it is supplemented with selenium, additional amino acids and vitamins, contains pyruvate and HEPES, and uses potassium nitrate in place of ferric nitrate. It is widely used for the culture of rapidly proliferating cells.
RPMI 1640	This medium was developed at Roswell Park Memorial Institute (RPMI) based on McCoy's 5A. With each modification, it was numbered 1629, 1630, and 1634. RPMI 1640 is characterized by low concentrations of calcium and magnesium, and high levels of phosphate and inositol. It is widely used for the culture of suspension cells.
Ham's F-12	In 1965, Ham modified F-10 medium to create a formulation that allowed CHO cells to form colonies more efficiently. This medium became known as Ham's F-12.
DMEM/Ham's F-12	This medium is a 1:1 mixture of DMEM and Ham's F-12, and is used for the culture of fibroblasts and other cell types.

■ Basal Medium (cont.)

Even media with the same name may vary in formulation, for example in whether certain components are included or excluded, or in their concentrations. The following are key components to consider.

Key components of medium composition

Composition	Details
Glucose	Glucose is an essential nutrient for cell maintenance and proliferation. In DMEM, three formulations are commonly available: high glucose (4500 mg/L), low glucose (1000 mg/L), and glucose-free. Commercial glucose stock solutions are also available and can be used together with glucose-free medium to adjust the glucose concentration as required.
Glutamine	Although L-glutamine is a non-essential amino acid, it serves as an auxiliary energy source during cell division and provides nitrogen for protein and nucleic acid synthesis, making it one of the indispensable components of cell culture. For this reason, it is sometimes referred to as an “essential amino acid” in the context of cell culture. Because L-glutamine is unstable in liquid media, cells that are highly sensitive to glutamine concentration are sometimes cultured in glutamine-free medium supplemented with freshly added L-glutamine. Alternatively, the more stable dipeptide form, L-alanyl-L-glutamine, may be used.
Sodium bicarbonate HEPES	Sodium bicarbonate and HEPES are used to regulate the pH of culture media. In a CO_2 incubator, the combination of atmospheric CO_2 and sodium bicarbonate in the medium maintains the appropriate pH. However, when cells are handled outside the incubator for extended periods, CO_2 levels become insufficient and optimal pH cannot be maintained. In such cases, media containing HEPES can be used to stabilize pH.
Phenol red	Phenol red is used as a pH indicator. It appears yellow under acidic conditions, red at neutral pH, and purple-red under alkaline conditions. When cells proliferate excessively, metabolites acidify the medium; thus, in media containing phenol red, the color can be used as a visual indicator of cell status. Similarly, bacterial contamination also acidifies the medium, causing it to turn yellow. On the other hand, phenol red exhibits estrogen-like activity, so caution is required when culturing hormone-sensitive cells such as MCF-7.
Sodium pyruvate	Sodium pyruvate serves as an alternative carbon source to glucose. It is metabolized through the TCA cycle without passing through glycolysis. In some cases, it is also added as an antioxidant. While certain media formulations (e.g., Leibovitz’s L-15) contain sodium pyruvate by default, it may also be supplemented for specific cell types or experimental systems.

Basics: Before Starting the Experiment

■ Serum and Serum-Free Media

Serum is obtained by allowing blood to clot and then collecting the supernatant. It contains various growth factors, cytoprotective factor, and nutrients that cannot be fully supplemented by medium supplement alone. For this reason, serum is typically added to basal media at 5–20% for cell culture. Commonly used sera include fetal bovine serum (FBS), newborn calf serum (NBCS), and horse serum (HS), with FBS being the most widely used.
Before use, serum may be heat-treated to inactivate complement components that can cause cytotoxicity. This process is referred to as serum inactivation. In addition, since serum quality varies between lots, lot testing is performed as a preliminary experiment to identify the serum best suited for the target cells.
In some cases, serum-free media or serum substitutes are used. Serum-free media are formulated to support cell culture without the need for serum supplementation. Because they exclude the diverse components present in serum, they are particularly useful for studying precise cellular responses and mechanisms of action, as well as for the purification of proteins and bioactive factors. In addition, serum-free media are often employed in contexts such as clinical applications, where preventing contamination by factors derived from non-target species is critical.

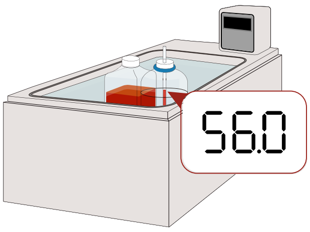
■ Experimental Protocol (Serum Inactivation)

① Thaw the frozen serum.

i Note Thaw the serum slowly at 4°C overnight to one day, or alternatively at room temperature.

② Place the thawed serum in a 56°C water bath and warm until the serum reaches 56°C.

i Note If frozen serum is placed directly into a 56°C water bath, the container may crack. Always thaw the serum completely before warming.



③ Incubate the serum at 56°C for an additional 30 minutes.

④ Aliquot the serum into sterile centrifuge tubes or other sterile containers in appropriate volumes.

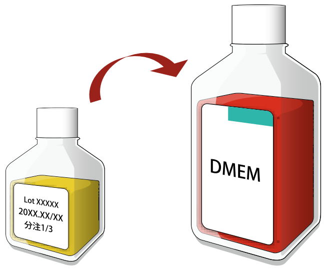
⑤ Store the serum frozen at -20°C.

■ Experimental Protocol (Preparation of Culture Medium)

Prepare culture medium containing 10% FBS.

① Add 55 mL of FBS to 500 mL of basal medium.

≫ For details on basal medium, refer to p. 8 and p. 35 (Appendix: Medium Composition Table).



② For glutamine-free media, add the required amount of L-glutamine solution (Product No. 16948-04) or L-alanyl-L-glutamine solution (Product No. 04260-64) before use. The final concentration of L-glutamine varies depending on the type of medium. Please refer to the table below for the appropriate supplementation.

i Note L-alanyl-L-glutamine is heat-stable and less prone to degradation than L-glutamine, thereby providing a more consistent supply of glutamine required for cell growth. In general, use at the same molar concentration as L-glutamine.

Volume of 200 mM L-Glutamine (or L-Alanyl-L-Glutamine) solution to add for each basal medium

Medium	Final L-Gln concentration	200 mM solution volume (per 500 mL)	Product no. (Our L-Gln-Free Medium)
DMEM	584 mg/L (4 mM)	10 mL	08488, 08489, 08490, 11584, 11585, 16972
Ham's F-12	146 mg/L (1 mM)	2.5 mL	-
DMEM/Ham's F-12	365 mg/L (2.5 mM)	6.25 mL	11583
MEM	292 mg/L (2 mM)	5 mL	-
α-MEM	292 mg/L (2 mM)	5 mL	-
IMDM	584 mg/L (4 mM)	10 mL	-
G-MEM	292 mg/L (2 mM)	5 mL	-
RPMI 1640	300 mg/L (2 mM)	5 mL	05176

③ Add antibiotics/antifungals as needed.

Product name	Product no.	Recommended volume (per 500 mL)
Penicillin-Streptomycin Mixed Solution Penicillin 5,000 u/ml, Streptomycin 5,000 μg/ml	26252-94	10 mL
Penicillin-Streptomycin Mixed Solution Penicillin 10,000 u/ml, Streptomycin 10,000 μg/ml	26253-84	5 mL
Penicillin-Streptomycin Mixed Solution(Stabilized) Penicillin 10,000 u/ml, Streptomycin 10,000 μg/ml	09367-34	5 mL
Antibiotic-Antimycotic Mixed Stock Solution(100x)	02892-54	5 mL
Antibiotic-Antimycotic Mixed Stock Solution(100x)(Stabilized)	09366-44	5 mL
Gentamicin Sulfate Solution(10mg/ml)	16672-04	2.5 mL
Gentamicin Sulfate Solution(50mg/ml)	11980-14	0.5 mL

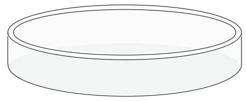
≫ For antibiotics/antifungal agents, refer to p. 40

④ Before use, aliquot as needed and bring to room temperature (or warm to 37°C) to ensure appropriate conditions prior to use.

Culture Vessels

All containers used for cell culture must be sterile. Commonly used culture vessels are introduced below.

Petri Dish (Culture Dish)

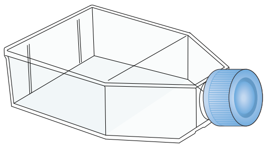


Petri dishes are available in various sizes and are selected according to the culture scale. Glass-bottom dishes, in which part of the base is made of glass, are also available and are suitable for microscopic observation after immunostaining.

Types (diameter)	Culture area (approx.)
35 mm	9 cm^2
60 mm	21 cm^2
100 mm	57 cm^2
150 mm	145 cm^2

Types and culture areas vary by manufacturer.

Culture Flask (T-Flask)

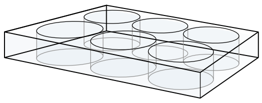


As with Petri dishes, culture flasks are available in various sizes and are selected according to the culture scale. They are often designated as T-25, T-75, etc., where the number indicates the culture area (cm^2). Flasks come with either non-filter caps or filter caps. For non-filter caps, the lid should be loosened during culture to allow gas exchange, whereas filter caps permit use without loosening.

Types	Culture area
T-25	25 cm^2
T-75	75 cm^2
T-175	175 cm^2
T-225	225 cm^2

Types vary by manufacturer.

Culture Multiwell Plate

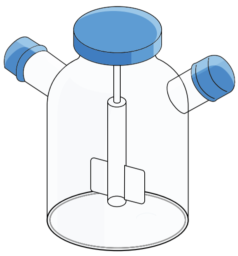


Standard wells have a flat bottom, but round-bottom and V-bottom types are also available. In addition, plates with transparent bottoms and white or black walls are used for detection by fluorescence or luminescence.

Types	Culture area (approx.)
6-well	9.6 cm^2/well
12-well	3.5 cm^2/well
24-well	1.9 cm^2/well
48-well	1.1 cm^2/well

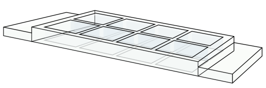
Types and culture areas vary by manufacturer.

Spinner Flask



Used for suspension culture.

Chamber Slide



Used for cell culture on glass slides, typically available in 2- to 8-well formats. Suitable for microscopic observation after immunostaining.

 For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

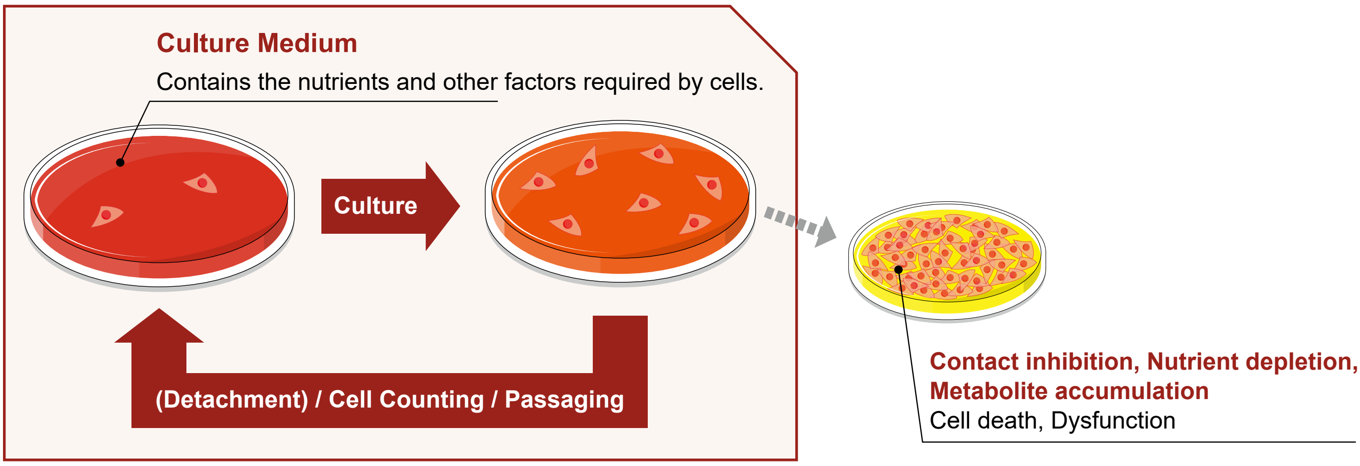
Cell Passaging

To ensure successful experiments using cultured cells, it is essential to maintain and proliferate them properly. This section describes the culture methods primarily for cell lines.

What is Passaging?

During cell culture, when cell density within the culture vessel reaches saturation, cell proliferation may be arrested due to contact inhibition, or cells may die as a result of nutrient depletion and accumulation of metabolites. To avoid this state, cells must be transferred to fresh medium and new culture vessels before reaching such conditions. This process is referred to as passaging (subculture).
This protocol describes passaging methods for cell lines, including both adherent and suspension cells.

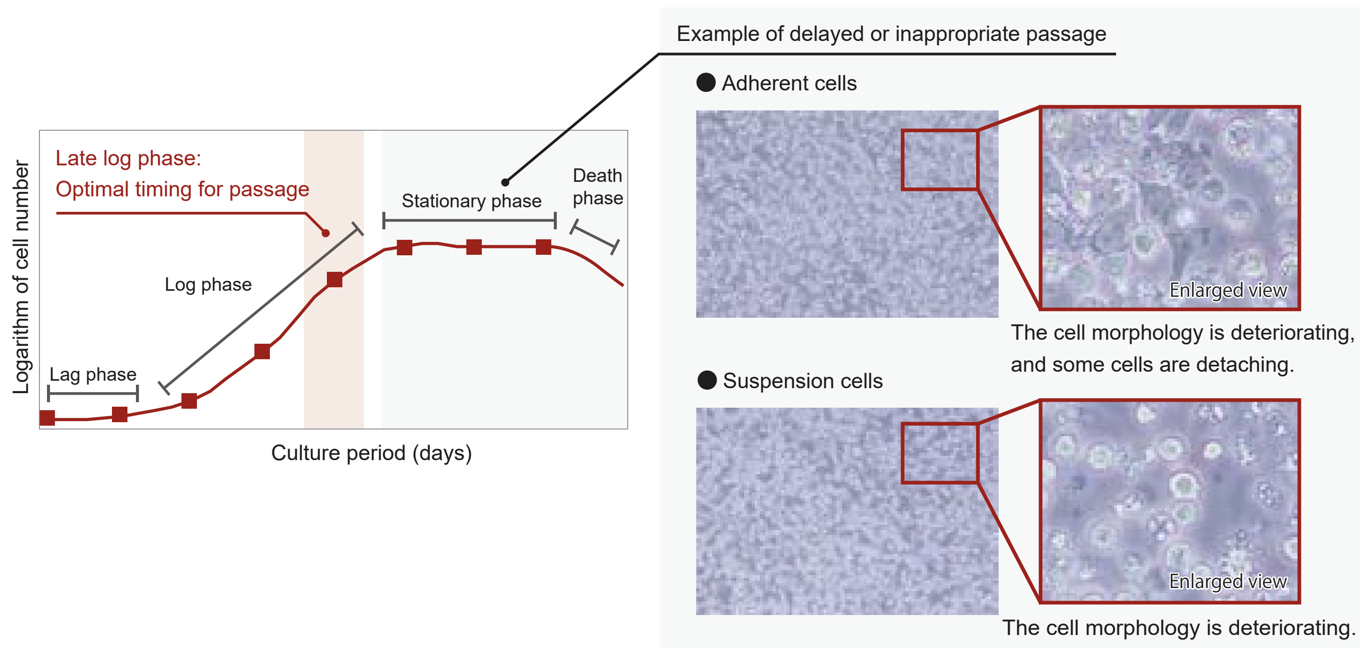
Workflow of cell passaging



Cell Growth Curve and Passaging Timing

In general, when the number of cultured cells is plotted over time, a growth curve is obtained as shown below. The initial phase after seeding is called the lag phase, during which cells adapt to the culture environment and prepare for proliferation. This is followed by the log (exponential) growth phase, where cells proliferate exponentially. Subsequently, due to nutrient depletion in the medium and other factors, cell growth is markedly suppressed or halted, which is referred to as the stationary phase. Finally, the number of cells decreases as dead cells accumulate, known as the death phase. For passaging, it is recommended to subculture cells during the late log growth phase.

Cell growth curve and passaging timing



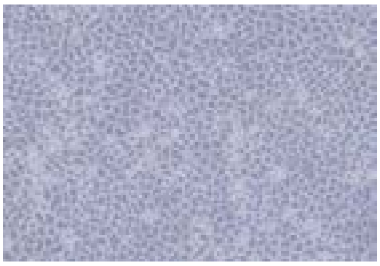
Cell Passaging

Confluent

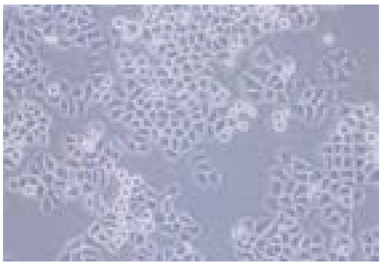
Confluency refers to the state in which cells completely occupy and saturate the available culture surface. The proportion of the culture surface covered by cells is expressed as XX% confluent; for example, when cells occupy 70% of the available culture area, the condition is described as 70% confluent. This terminology is most commonly used in adherent cell culture, and expressions such as “cells are passaged at 70% confluent” are frequently employed.
When cells reach confluency (100% confluent), cell–cell contact induces contact inhibition, leading to growth arrest and a subsequent reduction in proliferative capacity after passaging.

Confluency in adherent cells

Confluency (100% confluent)



70% confluent



Experimental Protocol (Passaging of Adherent Cells)

Adherent cells are attached to the culture vessel and to each other; therefore, detachment and dissociation procedures are required during passaging.

① Confirm the appropriate timing for passage by microscopic observation.

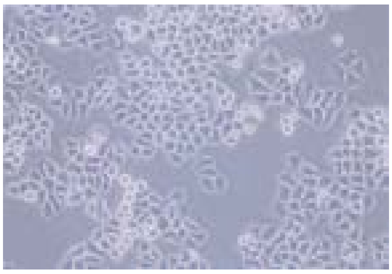
Note The appropriate timing for passage may vary depending on the cell type.

Example) HeLa cells

20-30% confluent

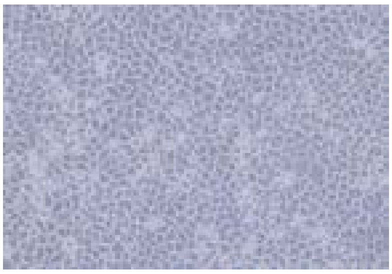
It is too early for passage.
Replace the medium only if required.

70-80% confluent



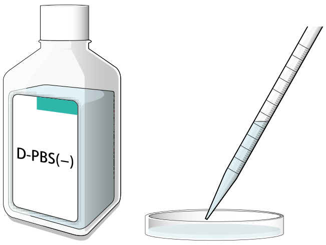
This is the appropriate timing for passage.

100% confluent



The cells have reached confluency
Perform passage before this state is reached.

② Remove the medium from the culture vessel using an aspirator. Gently add D-PBS(-) (Product No.: 14249-95) along the vessel wall to wash the cells, then remove the D-PBS.



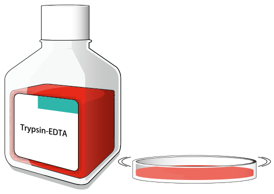
 For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

Cell Passaging

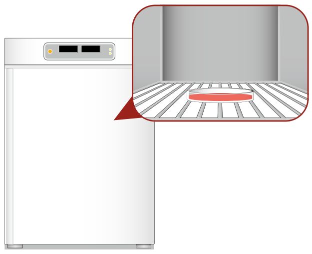
③ Add an appropriate volume of trypsin-EDTA solution (Product No.: 32777-44) and ensure it covers the entire culture surface.

Note Trypsin degrades proteins involved in cell-cell and cell-surface adhesion. EDTA chelates calcium and magnesium ions, which would otherwise inhibit trypsin activity, thereby maintaining its enzymatic function. A variety of products are available, including those with different trypsin/EDTA concentrations and products with gentler dissociation activity than trypsin.

≫ For further details, refer to p. 42 and 43.



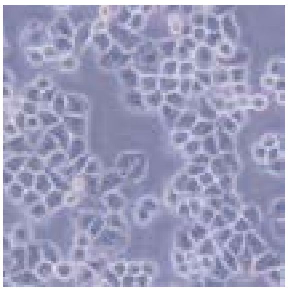
④ Incubate at 37°C in a 5% CO_2 incubator.



⑤ Observe under a microscope and confirm that the cells have rounded up and begun to detach.

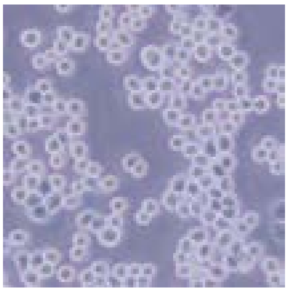
Caution The recommended exposure time to trypsin-EDTA solution is approximately 3–5 minutes. Prolonged exposure may damage the cells.

Before addition



5 minutes after
trypsin-EDTA addition

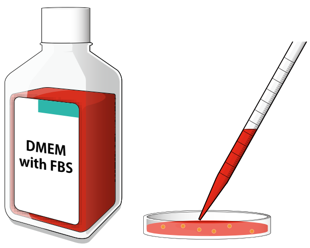
After addition



Cells have become rounded in shape

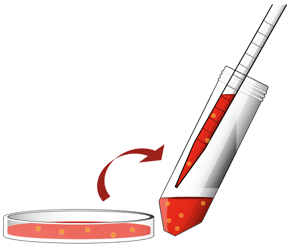
⑥ Gently tap the side of the culture vessel to completely detach the cells. Then, add 5-10 times the volume of serum-containing medium relative to the added trypsin-EDTA solution. Pipette gently to obtain a single-cell suspension.

Note Serum contains protease inhibitors; therefore, the addition of serum-containing medium terminates trypsin activity. When using serum-free medium, use a trypsin inhibitor instead.



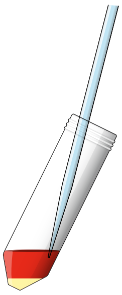
Cell Passaging

⑦ Transfer the suspension to a centrifuge tube.



⑧ Centrifuge at 180 × g for 5 minutes and remove the supernatant.

i Note Appropriate centrifugation conditions may vary depending on the cell type. As a guideline, use 180 × g for 3-10 minutes and adjust as necessary.



⑨ Add an appropriate volume of medium to the cell pellet and gently pipette to disperse the cells.

⑩ Count the cells.

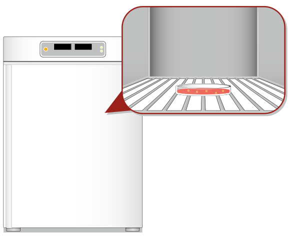
≫ For cell counting using Trypan Blue, refer to p. 21.

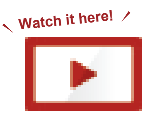
⑪ Dilute the cell suspension with medium to adjust to the appropriate cell density, then seed into a new culture vessel.

i Note Refer to the information provided by the cell supplier for the appropriate cell density at passage.

⑫ Incubate at 37°C in a 5% CO_2 incubator.

Animal cells are generally cultured at 37°C with 5% CO_2; however, conditions may vary depending on the cell type and experimental purpose.





Passaging adherent cells using trypsin

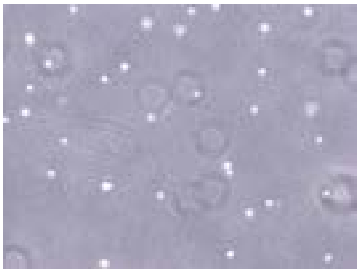
Experimental Protocol (Passaging of Suspension Cells)

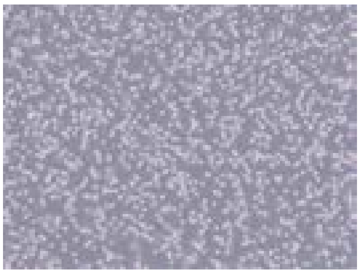
For suspension cells, detachment procedures are not required.

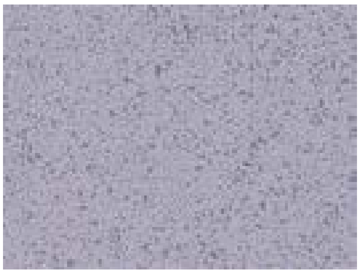
① Confirm the appropriate timing for passage by microscopic observation or cell counting.

Note Refer to the information provided by the cell supplier for the appropriate timing of passage. Depending on the cell type, suspension cells are typically seeded at a density of 2×10^4 to 5×10^5 cells/mL and can be cultured up to approximately 2×10^6 cells/mL.

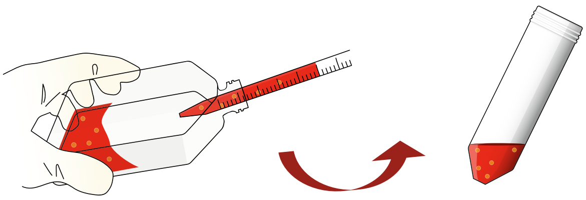
Example) Jurkat cells


It is too early for passage.
Replace the medium only if required.


This is the appropriate timing for passage.

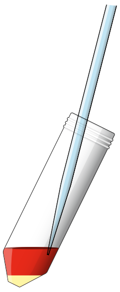

The cells have reached saturation.
Perform passage before this state is reached.

② Gently pipette the culture medium to disperse the cells, then transfer to a centrifuge tube.



③ Centrifuge at 180 × g for 5 minutes and remove the supernatant.

Note Appropriate centrifugation conditions may vary depending on the cell type. As a guideline, use 180 × g for 3-10 minutes and adjust as necessary.



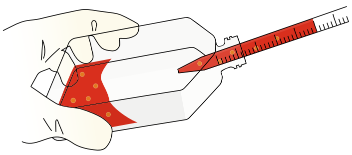
④ Add an appropriate volume of medium to the cell pellet and gently pipette to disperse the cells.

⑤ Count the cells.

≫ Refer to p. 21 for cell counting using trypan blue.

⑥ Dilute the cell suspension with medium, adjust to the appropriate cell density, and seed into a new culture vessel.

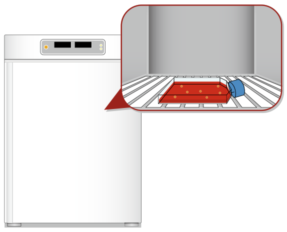
Note Refer to the information provided by the cell bank for the appropriate seeding density. Depending on the cell type, suspension cells are generally seeded at a density of 2×10^4 to 5×10^5 cells/mL.



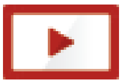
Cell Passaging

① Incubate at 37°C in a 5% CO_2 incubator.

Note Animal cells are generally cultured at 37°C with 5% CO_2, but the optimal conditions may vary depending on the cell type and experimental purpose.



Watch it here!



Passaging Suspension Cells

 For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

Troubleshooting

Slow cell growth

Cause	Solutions
Inappropriate culture medium	Use the culture medium recommended by the cell supplier. Even media with the same name may differ in composition (e.g., glucose-free or varying glucose concentrations). Always check the medium composition table.
Lot-to-lot variation in serum	Cell growth may slow depending on the serum lot. It is recommended to perform lot testing when purchasing serum.
High passage number	Cell growth may slow as the passage number increases. It is recommended to use cells with a low passage number.
Contamination with mycoplasma	If contaminated with mycoplasma, cell growth may be inhibited. Perform mycoplasma testing and removal.

Low cell viability

Cause	Solution
Inappropriate culture medium	Use the culture medium recommended by the cell supplier. Also confirm that the medium has been prepared correctly (e.g., osmolarity, pH).
Improper detachment procedure or detachment solution	If the detachment procedure, composition of the detachment solution, or exposure time is inappropriate, cells may be damaged and viability reduced. Verify the detachment procedure and solution composition.
Cells maintained at high density for an extended period	Prolonged culture at high cell density can damage cells. Perform passaging at the appropriate timing.

Abnormal cell morphology

Cause	Solution
High passage number	With increasing passage number, abnormal cell morphology may appear. Use cells with a lower passage number.
Continued culture after reaching confluency	Once confluency is reached, cells may lose their morphology due to lack of space for growth. Perform passaging at the appropriate timing.

Culture medium turns yellow

Cause	Solution
Excessive cell proliferation	As cells proliferate, metabolites acidify the medium, causing phenol red–containing media to turn yellow. Perform passaging at the appropriate timing.
Contamination	Bacterial contamination also causes the medium to turn yellow. Discard the cells.

Cells do not adhere

Cause	Solution
Residual detachment solution such as trypsin	Residual detachment solution (e.g., trypsin) may remain. After inactivation, remove it thoroughly by centrifugation.
Inappropriate culture vessel	Use culture vessels designed for cell culture.
Improper coating	Some cells require surface coating. Perform appropriate coating according to the information provided by the cell supplier.

Cell Passaging

Cell aggregation

Cause	Solution
Improper passaging procedures or detachment conditions	If passaging or detachment conditions are inappropriate, cells may be damaged and aggregate due to DNA released from dead cells. This can be improved by adding sterile DNase (1 mg/mL aqueous solution). Also review and correct the passaging and detachment conditions.
Prolonged maintenance at high cell density	Cells may aggregate when maintained for extended periods at high density. Perform passaging at the appropriate timing.
Inappropriate centrifugation conditions	Excessive centrifugation force can make it difficult to resuspend cells. Confirm and use suitable centrifugation conditions.

Cells do not detach with trypsin

Cause	Solution
Incomplete removal of serum or trypsin inhibitor	If serum or trypsin inhibitors remain, trypsin activity is weakened. Wash the cells with PBS before trypsinization.
Inappropriate trypsin concentration	Various products with different trypsin and EDTA concentrations are commercially available. Check the product specifications.
Reduced trypsin activity	Trypsin activity decreases with temperature fluctuations and repeated freeze–thaw cycles. Use fresh trypsin.
Insufficient trypsin incubation time	Extending trypsin incubation time can promote cell detachment, but prolonged exposure may damage the cells. Adjust carefully.
Low incubation temperature during trypsinization	Perform trypsinization at 37°C.
Cells maintained in culture after reaching confluency	Cells maintained beyond confluency strengthen cell–cell adhesion, preventing trypsin from accessing the cell–vessel junction. Perform passaging at the appropriate timing.

Cell Counting Using Trypan Blue

During passaging, cryopreservation, or performing analyses using cells, it is crucial to determine the cell number in order to ensure reproducibility and accuracy. This section introduces a cell counting method using Trypan Blue.

About Trypan Blue

Trypan Blue is an azo dye that strongly binds to proteins. Cells with damaged membranes (dead cells) allow Trypan Blue to permeate into the cytoplasm and are unable to expel it, resulting in blue staining. This property enables the distinction between non-vaible and viable cells, allowing both cell number and cell viability to be assessed simultaneously.

Because Trypan Blue enables simple and inexpensive cell counting and viability assessment, it is widely used. However, it has the drawback of being highly cytotoxic: if staining is prolonged, even viable cells may take up the dye and appear blue. As a result, variability in technique can lead to differences in viability assessment.

In addition, since Trypan Blue exclusion assay relies on visual inspection, it is not well suited for counting large numbers of samples. Therefore, drug-induced cytotoxicity is commonly evaluated using indirect methods that measure cell number and condition based on indicators such as metabolic products, enzyme activity, and the release of intracellular components into the extracellular medium.

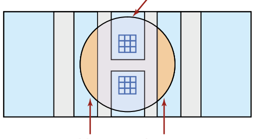
Experimental Protocol

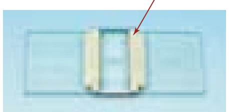
This protocol provides an example using a Bürker-Türk counting chamber.

① Prepare a counting chamber.

Clean the counting chamber and cover glass with ethanol, then wipe them by pressing gently with a KimWipes or equivalent. Place the cover glass on the counting chamber and press it lightly while sliding to create Newton rings, ensuring proper adhesion. Fix with clips if necessary.

Note A counting chamber can be replaced with an automated cell counter. Do not rub the counting chamber or surrounding area of that, as scratches will prevent accurate counting. If Newton rings do not appear, dirt may be present. Wipe again with 70% ethanol or similar disinfectant. Performing the procedure with dirt attached may alter the chamber volume or cause leakage, resulting in inaccurate counts.





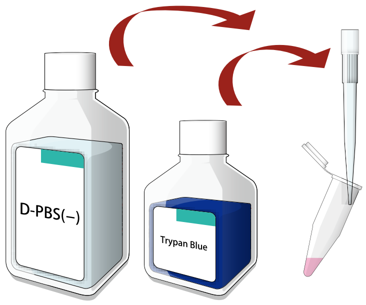
KimWipes is a registered trademark of Kimberly-Clark Worldwide, Inc.

② Transfer an appropriate volume of the cell suspension into a microtube.

Cell Counting Using Trypan Blue

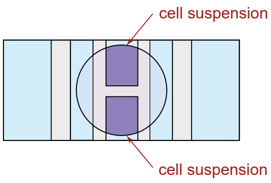
③ If necessary, dilute the collected cell suspension with D-PBS(-). Mix the diluted cell suspension with Trypan Blue Solution (Product No.: 20577-34) at a 1:1 volume ratio by gentle pipetting or similar method.

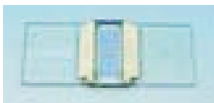
i Note Determine the dilution factor according to the cell density.



④ Inject the cell suspension mixed with Trypan Blue solution into the space between the counting chamber and the cover glass using a micropipette or a similar tool.

! Caution When injecting, avoid overfilling (approximately 10 μL is sufficient) and take care to prevent air bubbles.

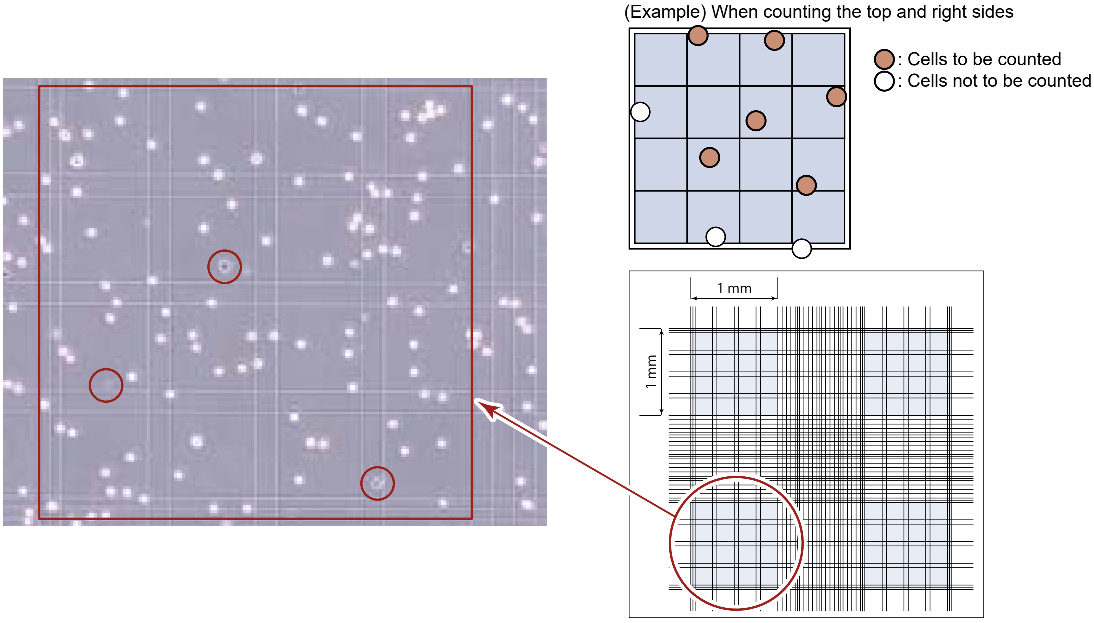




⑤ Observe under a microscope and count the cells in the four corner squares of the counting chamber (each with an area of 1 mm²). Cells that appear bright (unstained) are viable cells, while those stained blue (○) are non-viable (dead) cells. For cells located on the boundary lines of the counting chamber, count only those on one of the two opposing edges. Decide in advance which edges to include (e.g., count the top and right edges).

i Note If the staining time is prolonged, viable cells may also begin to take up the dye. Therefore, complete the counting within approximately 5 minutes after adding trypan blue. For each square, it is appropriate to count around 50–100 cells. If too few cells are counted, errors are likely to occur; if too many are counted, the process becomes time-consuming and reliability decreases. In such cases, reconsider the dilution factor.
When calculating the total cell number or cell viability, count both viable and non-viable cells separately.

(Example) When counting the top and right sides



Counting chamber (Burker-Türk type)

For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

Cell Counting Using Trypan Blue

⑥ Calculate the cell density, cell number, and cell viability as follows.

$$\textbf{Cell density (cells/mL)} = \underbrace{\frac{\text{Total number of cells counted}}{\text{Number of squares counted}}}_{\text{Average number of cells per square}} \times \underbrace{\text{Dilution factor}}_{\text{Dilution ratio of the cell suspension with D-PBS(-) and trypan blue}} \times \underbrace{10^4}_{\text{Conversion from } 0.1\ \text{mm}^3 \text{ to } 1\ \text{mL}}$$

Note (Average number of cells per square)
By counting viable cells, you can determine the viable cell density. For subculturing, use the viable cell density.

Note (Conversion from 0.1 mm³ to 1 mL)
The volume of one square is 1 mm² (area) × 0.1 mm (depth) = 0.1 mm³.

$$\textbf{Cell number (cells)} = \textbf{Cell density (cells/mL)} \times \textbf{Volume of the original cell suspension}$$

$$\textbf{Cell viability (\%)} = \frac{\textbf{Total number of viable cells counted}}{\textbf{Total number of viable and non-viable cells counted}} \times 100$$

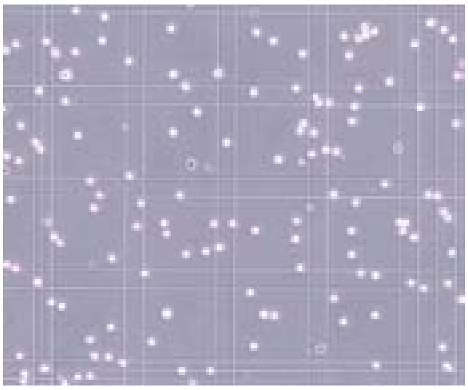
Example

Volume of cell suspension: 5 mL
Dilution factor at counting: 10x
Number of squares counted: 4 squares

Cell suspension	10 µL
D-PBS(-)	40 µL
Trypan blue solution	50 µL

Square	1	2	3	4	Total
Number of viable cells	92	89	96	83	360
Number of total cells	98	95	99	88	380

An actual counted square



$$\textbf{Number of viable cells} = \underbrace{360/4}_{\text{Average number of cells per square}} \times \underbrace{10}_{\text{Dilution factor}} \times \underbrace{10^4}_{\text{Conversion from } 0.1\ \text{mm}^3 \text{ to } 1\ \text{mL}} \times \underbrace{5}_{\text{Volume of the cell suspension}} = \mathbf{4.5 \times 10^7\ cells}$$

$$\textbf{Total cell number} = 380/4 \times 10 \times 10^4 \times 5 = \mathbf{4.75 \times 10^7\ cells}$$

$$\textbf{Cell viability} = 360/380 \times 100 = \mathbf{94.7\%}$$

Watch it here!

Cell counting using trypan blue

Troubleshooting

Many cells are stained with trypan blue

Cause	Solution
Non-viable cells are included	Trypan blue stains non-viable cells. A high proportion of stained cells may indicate that many cells were already dead. In addition, if the cell suspension was diluted with purified water instead of PBS or culture medium, osmotic pressure changes may have caused cell death and staining by trypan blue. Check both the condition of the cells and the type of diluent used.
Too much time elapsed between staining and completion of counting	If the staining time with trypan blue is prolonged, even viable cells may become stained. Complete the counting within approximately 5 minutes after adding trypan blue.

Poor reproducibility

Cause	Solution
The number of cells counted is inappropriate	Counting approximately 50–100 cells per square is appropriate. Reconsider the dilution factor of the cell suspension if necessary.
The time between staining and completion of counting varies	If the staining time with trypan blue is too long, even viable cells may become stained. Complete the counting within approximately 5 minutes after adding trypan blue, and ensure that the counting time is consistent for each measurement.

Pigment-like aggregates are observed

Cause	Solution
Aminoglycoside antibiotics are present	The coexistence of aminoglycoside antibiotics and trypan blue can cause dye precipitation or aggregation. Check whether the culture medium contains aminoglycoside antibiotics such as neomycin sulfate. If present, resuspend and count the cells using a medium that does not contain these antibiotics.

Cannot be measured with a cell counter

Cause	Solution
The trypan blue concentration is inappropriate	Depending on the type of cell counter used, measurement may not be possible if the trypan blue concentration is incorrect. Adjust the concentration to an appropriate level before measurement.

Cell Preservation

In experiments using cultured cells, it is very important to freeze and store cells as a backup to minimize damage in the event of problems such as phenotypic changes caused by long-term passaging or contamination by microorganisms. Frozen cells can be thawed and used again for experiments.

Cell Preservation Methods

There are two main methods for cell preservation: the slow-freezing method and the vitrification method. When cells are frozen in aqueous solutions such as culture medium, ice crystal formation can occur, leading to cellular damage. Therefore, both the slow-freezing and vitrification methods are designed to minimize damage to the cells during the freezing process.

■ Slow-freezing Method and Vitrification Method

	Slow-freezing method	Vitrification method
Characteristics	This is the most commonly used freezing method. A cryoprotectant is added to the freezing medium, and the cooling rate is controlled (-1°C/min) using a programmable freezer or similar equipment. Gradual freezing prevents the formation of large ice crystals and minimizes cellular damage during the freezing process.	This method is used for freezing cells such as stem cells or fertilized ova, which show low survival rates with the slow-freezing method. To avoid ice crystal formation, the cells are frozen in a glass-like state without ice crystallization. A special freezing solution is required, and rapid freezing is performed using liquid nitrogen or similar. The process from suspending the cells in the freezing solution to freezing must be completed within 15 seconds (for DAP213), requiring skilled technique.
Applicable cell types	Cell lines, primary cells, stem cells, etc.	Stem cells (ES cells and iPS cells), fertilized ova.
Examples of freezing solutions	Serum-containing medium with cryoprotectants such as DMSO or glycerol.	Special freezing solution (e.g., DAP213).

Properly preserved cells can generally be stored semi-permanently at -196°C (in liquid nitrogen) or for 1-2 years at -80°C. For storage, use cryopreservation tube (cryovials).

Cell Reservoir One

Our company offers the Cell Reservoir One series of cell preservation solutions, which are free of serum and mammalian-derived components. In the Cell Reservoir One series, sericin, a silk-derived protein, is used in place of serum, providing excellent cryopreservation performance while remaining serum-free.

※ This series is manufactured in collaboration with Seiren Co., Ltd.

Application	For slow-freezing method		For vitrification method
Product name	Cell Reservoir One (with DMSO)	Cell Reservoir One (without DMSO)	Cell Reservoir One, Vitrify
Product no.	07485	07579	11325-62
Features	• High recovery rate (proliferative capacity / functionality) and cell viability • Ideal for preserving serum-free cultured cells • The type without DMSO can also be used for preserving mouse ES cells.		• Suitable for preserving primate ES/iPS cells • Maintains high cell viability even with a 60-second handling time before freezing • Free of DMSO and acetamide, ensuring low cytotoxicity

≫ For details on the types of cells successfully preserved and post-thaw cell viability, refer to p. 28 (Reference: Cell Reservoir One – Cryopreservation Performance Data).

Cell Preservation

Slow Freezing Method Using Cell Reservoir One (With / Without DMSO)

Experimental Protocol (Freezing)

Some primary cells and other types cannot be frozen and thawed. Before freezing, check the datasheet or related literature from the cell provider to confirm whether the cells can be frozen and thawed.

① Confirm under a microscope that the cells are in the logarithmic growth phase. Replace the culture medium if necessary.

i Note If the cells are not in the logarithmic growth phase, continue culturing or passage them as needed.

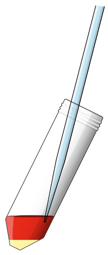
② Collect the cells according to standard procedures and count the cell number.

i Note A viability of 90% or higher is desirable.

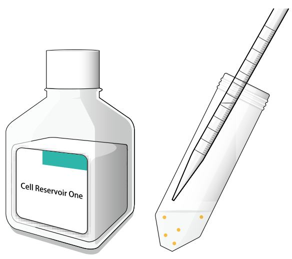
≫ For cell counting using trypan blue, refer to p. 21.

③ Centrifuge at 180 × g for 5 minutes and remove the supernatant.

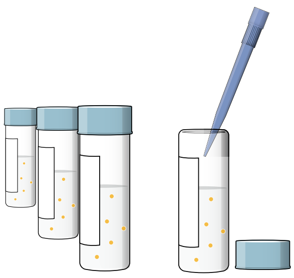
i Note The optimal centrifugation conditions may vary depending on the cell type. Adjust as appropriate, using 180 × g for 3–10 minutes as a general guideline.



④ Add Cell Reservoir One and resuspend the cells to achieve a cell concentration of 5×10^5 to 1×10^7 cells/mL.



⑤ Aliquot the suspension into cryopreservation tube.



⑥ Freeze and store at -80°C. Cells frozen at -80°C can also be stored in liquid nitrogen.

i Note Pre-freezing using a programmable freezer or similar equipment is not required.

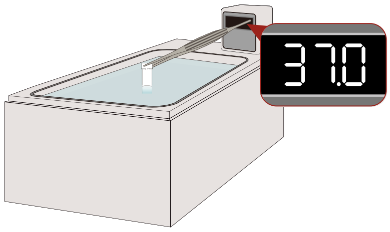
Experimental Protocol (Thawing)

① In advance, aliquot culture medium equivalent to 10 times the volume of the frozen cell suspension into a centrifuge tube..

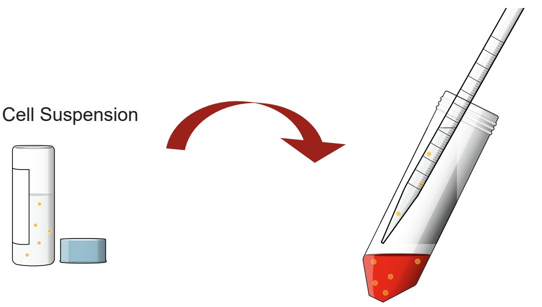
Note This preparation allows the thawed cell suspension to be diluted quickly with the medium.
Example: For HeLa cells (1×10^6 cells / 1 mL / tube), aliquot 10 mL of DMEM containing 10% FBS into a centrifuge tube.

② Quickly thaw the frozen cells in a 37℃ water bath.

Note Do not touch the frozen liquid surface with your hands to prevent gradual melting by body heat.
Thaw for approximately 1–2 minutes at 37°C.
Ideally, remove the tube from the water bath while a small amount of ice remains, so that complete thawing is achieved during step ③ .



③ Immediately transfer the completely thawed cell suspension to the centrifuge tube prepared in step ① .



④ Centrifuge at 180 × g for 5 minutes and remove the supernatant.

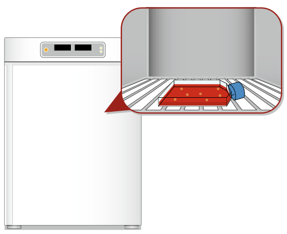
Note The optimal centrifugation conditions may vary depending on the cell type. Adjust as appropriate, using 180 × g for 3–10 minutes as a general guideline.

⑤ Add an appropriate amount of culture medium and gently disperse the cells by pipetting or similar method.

Note Count the cells if necessary.
≫ For cell counting using trypan blue, refer to p. 21.

⑥ After adjusting the cell suspension to the desired cell density, seed the cells into a culture vessel and incubate at 37°C in a 5% CO_2 incubator.

Note Refer to the information provided by the cell supplier for recommended seeding densities.



Watch it here!

Slow-freezing method using Cell Reservoir One

Cell Preservation

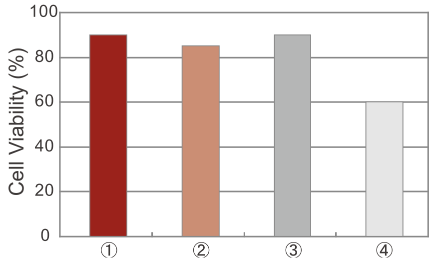
Reference: Cell Reservoir One – Cryopreservation Performance Data

The following section presents the results of cryopreservation tests (cell viability) and cryopreservation experience for cells frozen using Cell Reservoir One.

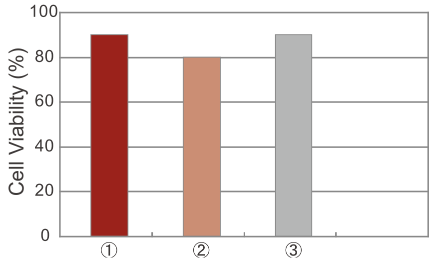
i Note The main component of Cell Reservoir One, sericin, provides a cryoprotective effect comparable to that of serum and also exhibits a mitigating effect on the cytotoxicity of DMSO. However, since DMSO is generally known to affect cell functions, the Cell Reservoir One series includes two product types: with DMSO and without DMSO.

■ Cryopreservation Test (Cell Viability)

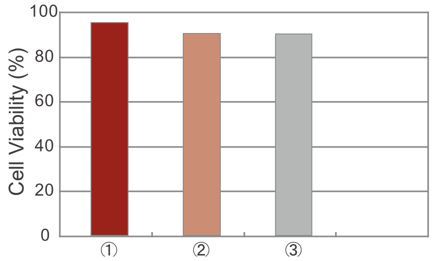
Cell type: Jurkat



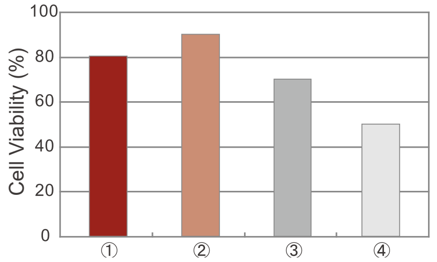
Cell type: HeLa



Cell type: CHO



Cell type: Mouse ES



① Cell Reservoir One (with DMSO) ② Cell Reservoir One (without DMSO) Freezing conditions: -80°C, 1 year
③ Product B (with serum and DMSO) ④ Product D (serum-free / with DMSO)

Species	Cell type	Tissue of origin	Freezing period	Cell Reservoir One viability (%)	
				with DMSO	without DMSO
Human	Jurkat	Blood	1 year	90	85
Human	HL-60	Blood	1 year	90	80
Human	WIL2-NS	Spleen	1 year	90	70
Human	MSC	Bone marrow	1 year	90	70
Human	HeLa	Cervix	1 year	90	80
Human	HepG2	Liver	1 year	85	70
Human	NHDF	Normal skin	1 year	90	75
Mouse	KUSA-A1	Bone marrow	9 months	90	80
Mouse	2E3-O	Hybridoma	1 year	90	80
Mouse	P3U1	Bone marrow	1 year	90	80
Mouse	OP9	Calvaria	10 months	95	85
Mouse	ES	Embryo	1 year	80	90
Rat	PC-12	Adrenal gland	1 year	90	70
Hamster	CHO	Ovary	1 year	95	90
Rabbit	RC4	Cornea	1 year	90	70
Insect	Sf-9	Ovary	1 year	75	70

Storage temperature: -80°C

Examples of cell types preserved using Cell Reservoir One

3T3-L1	Caco-2	HT-1080	MCF-7	NIH/3T3
A-431	COS-7	HuH-7	MDA-MB-231	RAW264.7
A549	HaCaT	HUVEC	MDCK	SH-SY5Y
BHK-21	HCT116	K-562	MEF	SW480
C2C12	HEK293	MC3T3-E1	Neuro-2a	U-87 MG

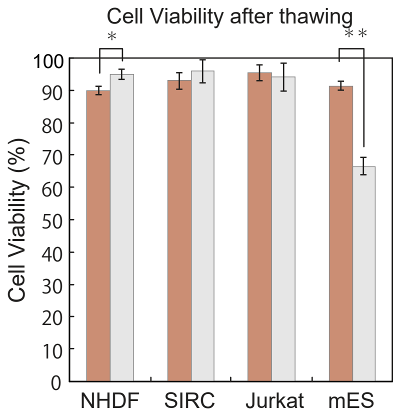
Cell Preservation

Application 1 Application of Cell Reservoir One (without DMSO) to mouse ES cells

Mouse ES cells cryopreserved using Cell Reservoir One (without DMSO) were thawed, and cell viability, proliferative capacity, maintenance of pluripotency, and differentiation into beating cardiomyocytes were evaluated.

■ Cell Viability after thawing

Even in mouse ES cells, which are prone to reduced viability after thawing, high cell viability was observed.



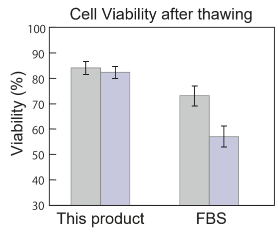
Cell Reservoir One (without DMSO)
FBS / 10% DMSO

∗ $p<0.05$, ∗∗ $p<0.01$

NHDF: Normal human dermal fibroblasts
SIRC : Rabbit corneal cells
Jurkat: Human leukemia cells
mES : Mouse ES cells

Freezing conditions: -80°C, 1 week

i Note During the cell freezing process, it is important to proceed from cell suspension to freezing as quickly as possible. However, Cell Reservoir One minimizes cell damage from long handling before freezing.

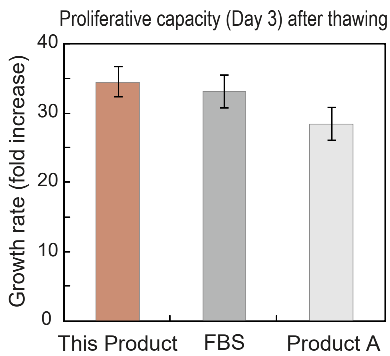


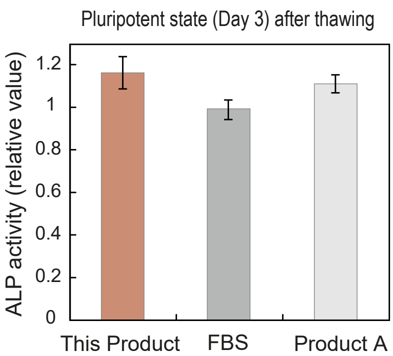
Frozen immediately after suspension
Frozen 30 minutes after suspension

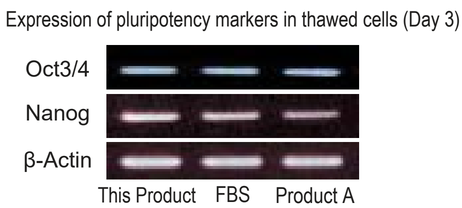
This product: Cell Reservoir One (without DMSO)
FBS: FBS / 10% DMSO
Freezing conditions: -80°C, 3 weeks
Cells: Mouse ES cells

■ Effects on Proliferative Capacity and Pluripotency

After thawing, the cells also maintained their proliferative capacity and undifferentiated state.



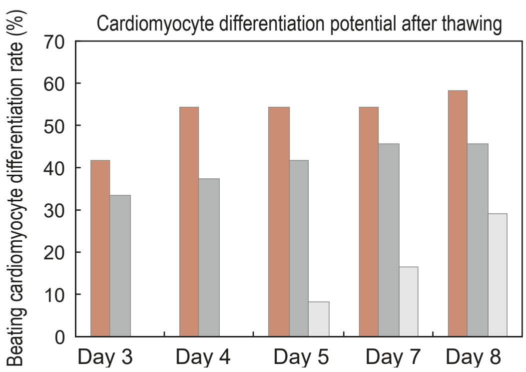




This product : Cell Reservoir One (without DMSO)
FBS : FBS / 10% DMSO
Product A : Serum-free / with DMSO
Freezing conditions : -80°C, 1 week
Cells : Mouse ES cells

■ Effect on differentiation into beating cardiomyocytes

The thawed cells retained their ability to differentiate into cardiomyocytes.



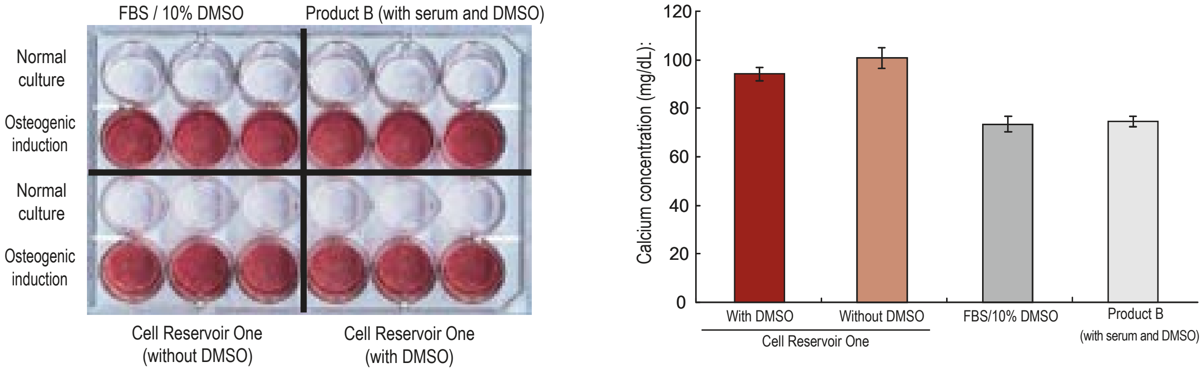
Cell Reservoir One (without DMSO)
FBS / 10% DMSO
Product A (serum-free / with DMSO)

Freezing conditions: -80°C, 1 week
Cells: Mouse ES cells

Cell Preservation

Application 2 Application of Cell Reservoir One to immortalized human mesenchymal stem cells (hMSC)

Immortalized human mesenchymal stem cells (hMSC) cryopreserved using Cell Reservoir One were thawed, and their osteogenic differentiation potential was evaluated. The results showed that the cells maintained their ability to differentiate into bone.



Freezing conditions: -80°C, 1 month

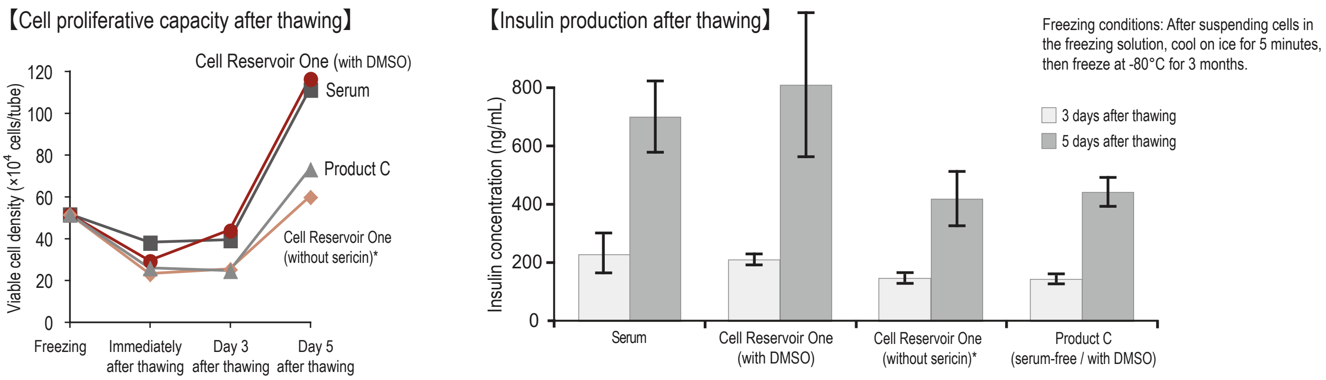
Evaluation method: Cells were stained with Alizarin Red after 4 weeks of osteogenic induction (left), and deposited calcium was quantified after differentiation (right).

Application 3 Application of Cell Reservoir One (with DMSO) to the rat insulinoma–derived cell line (RIN-5F) and the human hepatoma–derived cell line (HepG2)

RIN-5F and HepG2 cells cryopreserved using Cell Reservoir One (with DMSO) were thawed, and the effects on proliferative capacity and protein production ability were evaluated.

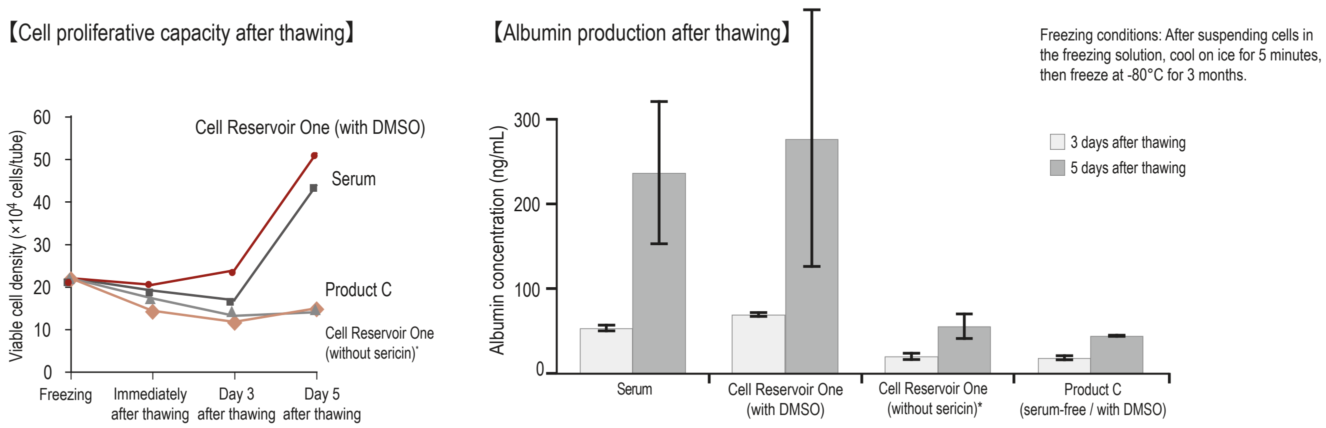
Cells cryopreserved with Cell Reservoir One maintained not only their proliferative capacity but also their insulin and albumin production abilities, both of which were largely attributed to sericin, the main component of Cell Reservoir One.

■ Evaluation using an islet transplantation model with RIN-5F



* Cell Reservoir One (with DMSO) without sericin

■ Evaluation using an artificial liver model with HepG2 cells



Data courtesy of the University of Fukui.

Cell Preservation

Vitrification Method Using Cell Reservoir One, Vitrify

Experimental Protocol (Freezing)

In vitrification, rapid freezing and rapid thawing are essential to maintain high cell viability. To ensure that procedures can be performed promptly, please complete all necessary preparations before beginning the operation.

① Confirm under a microscope that the cells are in the logarithmic growth phase. Replace the culture medium if necessary.

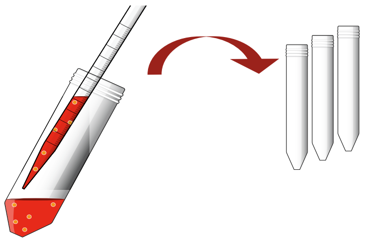
i Note If the cells are not in the logarithmic growth phase, continue culturing or passage them as needed.

② Prepare liquid nitrogen and tweezers near the clean bench.

③ Collect the cells according to standard procedures, and count the cells if necessary.

≫ For cell counting using trypan blue, refer to p. 21.

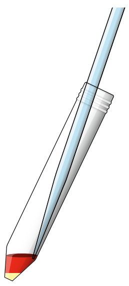
④ Aliquot an appropriate volume of the cell suspension into centrifuge tubes.



⑤ Centrifuge at 180 × g for 5 minutes and remove as much of the supernatant as possible.

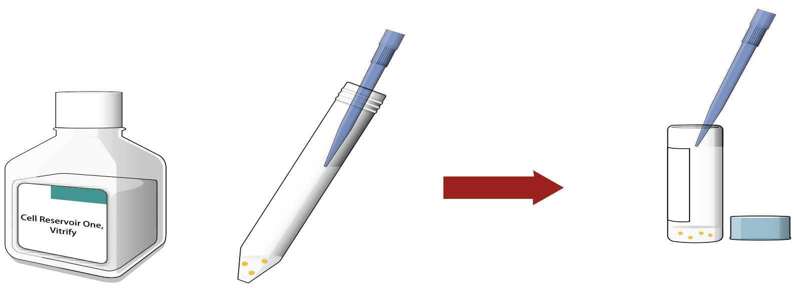
! Caution Steps ⑥ - ⑦ must be completed within 60 seconds; therefore, handle one tube at a time.

i Note Optimal centrifugation conditions may vary depending on the cell type. Use conditions appropriate for your cells.
Residual supernatant may dilute the cell preservation solution and reduce cell viability.



⑥ Add 200 µL of Cell Reservoir One, Vitrify, gently pipette 4–5 times, then transfer the suspension to a cryopreservation tube and securely close the cap.

! Caution Steps ⑥ - ⑦ must be completed within 60 seconds.



⑦ Hold the tube with tweesers and lower it into liquid nitrogen, ensuring that the opening of the tube does not contact the liquid surface. After holding it in this position for 10 seconds, fully immerse the tube in liquid nitrogen to complete freezing.

! Caution Steps ⑥ - ⑦ must be completed within 60 seconds.

⑧ While keeping the tube submerged in liquid nitrogen, quickly transfer it to the liquid nitrogen storage container for long-term storage.

Cell Preservation

Experimental Protocol (Thawing)

In vitrification, rapid freezing and rapid thawing are essential to maintain high cell viability. To ensure that procedures can be performed promptly, please complete all necessary preparations before beginning the operation.

① Warm approximately 20 mL of culture medium to 37°C. For stem cells (such as ES or iPS cells), add Y-27632 (Product No.: 18190) to a final concentration of 10 μM.

② Aliquot 10 mL of the prepared medium into a centrifuge tube.

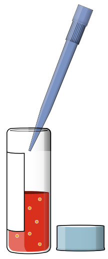
③ Remove the cryopreservation tube containing frozen cells from the liquid nitrogen container, immerse it in aliquoted liquid nitrogen, and carry it near the clean bench.

! Caution Perform steps ③ – ⑥ as quickly as possible.

④ Take the cryopreservation tube out of the liquid nitrogen and place it inside the clean bench. Add 800 μL of the pre-warmed (37°C) medium from step ① , pipetting against the bottom of the tube to thaw rapidly.

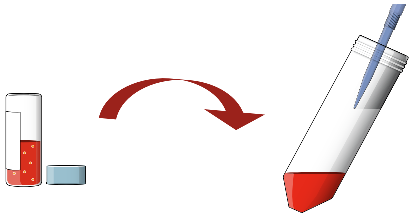
! Caution Perform steps ③ – ⑥ as quickly as possible.

i Note The greater the volume of added medium, the faster the thawing. Adjust the volume added according to the size of the tube used to allow the addition of as much culture medium as possible.



⑤ Transfer the thawed cell suspension to the centrifuge tube prepared in step ② .

! Caution Perform steps ③ – ⑥ as quickly as possible.



⑥ Rinse the cryopreservation tube that contained the cells with the culture medium prepared in step ① , and transfer the rinse to the centrifuge tube from step ⑤ .

! Caution Perform steps ③ – ⑥ as quickly as possible.

⑦ Centrifuge the tube from step ⑤ at 180 × g for 5 minutes and remove as much of the supernatant as possible.

i Note Optimal centrifugation conditions vary depending on the cell type. Adjust as needed, using 180 × g for 3–10 minutes as a guideline.

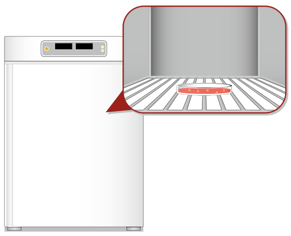
⑧ Add an appropriate volume of the medium prepared in step ① and gently disperse the cells by pipetting.

i Note Count the cells if necessary.

≫ For cell counting using trypan blue, refer to p. 21.

⑨ After adjusting the cell suspension to the desired cell density, seed the cells into a culture vessel and incubate at 37°C in a 5 % CO_2 incubator.

i Note Refer to the information provided by the cell supplier for recommended seeding densities.



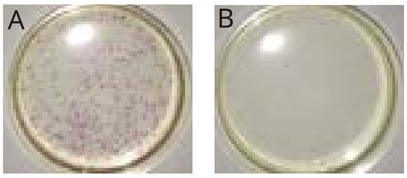
⑩ If Y-27632 was added to the medium, replace it the next day with medium that does not contain Y-27632.

Cell Preservation

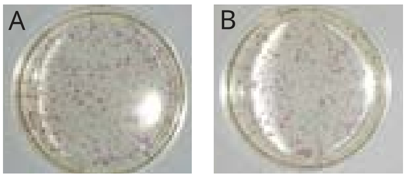
Application 1 Viability of human iPS cell line 201B7

Human iPS cells (line 201B7) were frozen using the vitrification method with Cell Reservoir One, Vitrify and DAP213. After storage in liquid nitrogen for more than two weeks, the cells were thawed and stained for alkaline phosphatase activity on day 4 of culture.
The vitrification method using Cell Reservoir One, Vitrify showed higher cell viability compared with the vitrification method using DAP213, even when the freezing operation time was 60 seconds.

Freezing operation time: 60 seconds



Freezing operation time: 15 seconds



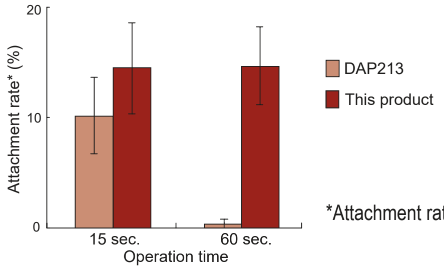
	Preservation Solution Used:	Number of Colonies	
		60 seconds	15 seconds
A	This product	672	563
B	DAP213	37	479

Data courtesy of an external research institution.

Application 2 Analysis of cell attachment rate and pluripotency in human iPS cell line 253G1

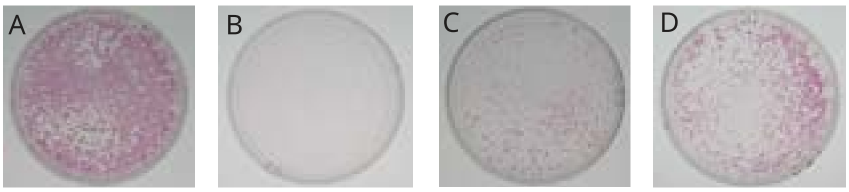
Human iPS cells (line 253G1) were frozen using the vitrification method with Cell Reservoir One, Vitrify and DAP213. After thawing, the cell attachment rate and expression of pluripotency marker genes were evaluated.
The vitrification method using Cell Reservoir One, Vitrify showed a higher attachment rate compared with DAP213, and the results indicated that it was less affected by the duration of the freezing operation. Furthermore, the expression of pluripotency marker genes was confirmed, demonstrating that the undifferentiated state was maintained.

■ Cell attachment rate



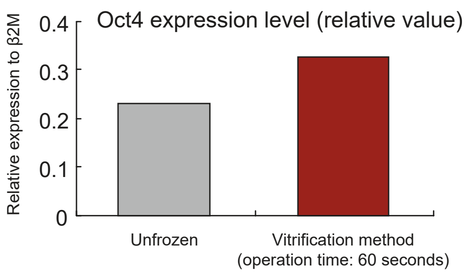
$$\text{*Attachment rate (\%)} = 100 \times \frac{\text{Number of attached colonies after vitrification and thawing}}{\text{Number of attached colonies during normal passaging}}$$

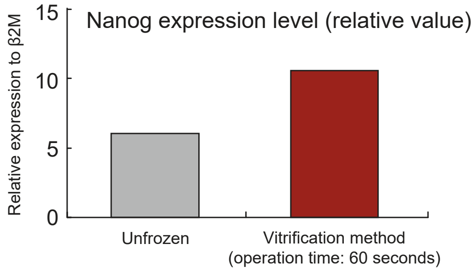
■ Alkaline phosphatase activity



A: Unfrozen
B: FBS / 10% DMSO
(frozen by slow-freezing method)
C: DAP213
(vitrification method, operation time: 15 seconds)
D: This product
(vitrification method, operation time: 15 seconds)

■ Expression of pluripotency marker genes





β2M: β2-microglobulin

Data courtesy of the University of Fukui.

Troubleshooting

No viable or low-viability cells after freeze–thaw

Causes	Solutions
Poor condition of the cells before freezing	Store cells with as few passages as possible that are in the logarithmic growth phase and have a viability of more than 90%.
Inappropriate freezing solution	Use a freezing solution suitable for the cells. Use the freezing solution recommended by the cell supplier or one with a proven track record. Some cells may show toxicity to DMSO, which is used as a cryoprotectant.
Inappropriate cell density during freezing	If the cell density is too low, viability may decrease. It is generally desirable to freeze at a density of 1×10^6 to 1×10^7 cells/mL.
Delay between adding the freezing solution and starting freezing	After suspending the cells in the freezing solution, proceed to freezing as quickly as possible. If this step takes too long, it will cause cell damage. It is recommended to label cryopreservation tubes in advance.
Inappropriate freezing method	Freeze the cells using a method appropriate for the freezing solution used. For conventional freezing solutions such as serum media containing DMSO or glycerin, it is necessary to freeze at a rate of -1°C/min using a programmable freezer to avoid the formation of large ice crystals. When using a freezing solution for vitrification, be sure to perform freezing by the vitrification method. Using the slow freezing method with a vitrification-type solution will cause cell damage.
Inappropriate storage method	If the storage temperature is high, the cells will deteriorate during long-term storage. For storage longer than 1–2 years, it is recommended to store them in liquid nitrogen (-196°C).
Inappropriate thawing method	Thaw the cells using a method appropriate for the freezing solution. Cells thawed in the freezing solution will be damaged over time. If the cell storage location and the thawing location are far apart, transport them while frozen using dry ice or liquid nitrogen, not on ice. Cells frozen by the slow-freezing method should be thawed quickly in a 37°C water bath and diluted as soon as possible with a large volume of pre-prepared medium. Cells frozen by the vitrification method should be rapidly thawed with an appropriate amount of pre-warmed (37°C) medium and immediately diluted with a large volume of medium.
Inappropriate medium used for thawing	Use the culture medium recommended by the cell supplier for thawing. Even if the medium has the same name, its composition (such as glucose content or presence/absence) may differ. Check the medium composition table to confirm.

Culture Medium Composition Table

(mg/L)

<table>
<tr><th colspan="2"></th><th colspan="9">DMEM (High glucose)</th><th colspan="2">DMEM (Low glucose)</th><th>DMEM (No glucose)</th></tr>
<tr><th colspan="2">Product no.</th><th>08457</th><th>08458</th><th>08459</th><th>08488</th><th>08489</th><th>11584</th><th>11585</th><th>16971</th><th>16972</th><th>08456</th><th>08490</th><th>09891</th></tr>
<tr><td rowspan="7">Inorganic salts</td><td>Calcium Chloride・$2H_2O$</td><td colspan="8">265.00</td><td>—</td><td colspan="2">265.00</td><td>265.00</td></tr>
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<tr><td>Magnesium Sulfate</td><td colspan="9">97.67</td><td colspan="2">97.67</td><td>97.67</td></tr>
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<tr><td>Sodium Chloride</td><td>4750.00</td><td colspan="5">6400.00</td><td>4750.00</td><td colspan="2">6400.00</td><td colspan="2">6400.00</td><td>6400.00</td></tr>
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<tr><td>Sodium Hydrogen Carbonate</td><td colspan="7">3700.00</td><td>1500.00</td><td>3700.00</td><td colspan="2">3700.00</td><td>3700.00</td></tr>
<tr><td rowspan="15">Amino acids</td><td>L-Arginine・HCl</td><td colspan="9">84.00</td><td colspan="2">84.00</td><td>84.00</td></tr>
<tr><td>L-Cystine・2HCl</td><td colspan="9">62.60</td><td colspan="2">62.60</td><td>62.60</td></tr>
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<tr><td>L-Lysine・HCl</td><td colspan="9">146.00</td><td colspan="2">146.00</td><td>146.00</td></tr>
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<tr><td>L-Serine</td><td colspan="9">42.00</td><td colspan="2">42.00</td><td>42.00</td></tr>
<tr><td>L-Threonine</td><td colspan="9">95.00</td><td colspan="2">95.00</td><td>95.00</td></tr>
<tr><td>L-Tryptophan</td><td colspan="9">16.00</td><td colspan="2">16.00</td><td>16.00</td></tr>
<tr><td>L-Tyrosine・2Na・$2H_2O$</td><td colspan="9">104.00</td><td colspan="2">104.00</td><td>104.00</td></tr>
<tr><td>L-Valine</td><td colspan="9">94.00</td><td colspan="2">94.00</td><td>94.00</td></tr>
<tr><td rowspan="9">Vitamins</td><td>Choline Chloride</td><td colspan="9">4.00</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>Folic Acid</td><td colspan="9">4.00</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>myo-Inositol</td><td colspan="9">7.20</td><td colspan="2">7.20</td><td>7.20</td></tr>
<tr><td>Nicotinamide</td><td colspan="9">4.00</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>D-Pantothenic Acid Calcium Salt</td><td colspan="8">4.00</td><td>—</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>D-Pantothenic Acid Sodium Salt</td><td colspan="8">—</td><td>4.00</td><td colspan="2">—</td><td>—</td></tr>
<tr><td>Pyridoxine・HCl</td><td colspan="9">4.00</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>Vitamin B_1・HCl</td><td colspan="9">4.00</td><td colspan="2">4.00</td><td>4.00</td></tr>
<tr><td>Vitamin B_2</td><td colspan="9">0.40</td><td colspan="2">0.40</td><td>0.40</td></tr>
<tr><td rowspan="4">Others</td><td>D-Glucose</td><td colspan="9">4500.00</td><td colspan="2">1000.00</td><td>—</td></tr>
<tr><td>HEPES</td><td>5958.00</td><td colspan="5">—</td><td>5958.00</td><td colspan="2">—</td><td colspan="2">—</td><td>—</td></tr>
<tr><td>Phenol Red</td><td colspan="4">14.93</td><td>—</td><td colspan="4">14.93</td><td>14.93</td><td>—</td><td>14.93</td></tr>
<tr><td>Sodium Pyruvate</td><td>—</td><td>110.00</td><td colspan="3">—</td><td>110.00</td><td>—</td><td>110.00</td><td>—</td><td colspan="2">110.00</td><td>—</td></tr>
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Culture Medium Composition Table

(mg/L)

<table>
<tr><th colspan="2"></th><th>Ham's F-12</th><th colspan="7">DMEM/Ham's F-12</th></tr>
<tr><th colspan="2">Product no.</th><th>17458</th><th>05177</th><th>08460</th><th>09893</th><th>11581</th><th>11582</th><th>11583</th></tr>
<tr><td rowspan="12">Inorganic salts</td><td>Calcium Chloride•$2H_2O$</td><td>44.10</td><td colspan="7">154.52</td></tr>
<tr><td>Copper(II) Sulfate•$5H_2O$</td><td>0.0025</td><td colspan="7">0.0013</td></tr>
<tr><td>Iron(III) Nitrate•$9H_2O$</td><td>—</td><td colspan="7">0.05</td></tr>
<tr><td>Iron(II) Sulfate•$7H_2O$</td><td>0.834</td><td colspan="7">0.42</td></tr>
<tr><td>Magnesium Chloride•$6H_2O$</td><td>123.00</td><td colspan="7">61.20</td></tr>
<tr><td>Magnesium Sulfate</td><td>—</td><td colspan="7">48.84</td></tr>
<tr><td>Potassium Chloride</td><td>224.00</td><td colspan="7">311.80</td></tr>
<tr><td>Sodium Chloride</td><td>7599.00</td><td colspan="7">6996.00</td></tr>
<tr><td>Sodium Dihydrogenphosphate</td><td>—</td><td colspan="7">54.30</td></tr>
<tr><td>Sodium Hydrogen Carbonate</td><td>1176.00</td><td colspan="3">1200.00</td><td colspan="2">2483.00</td><td>1200.00</td></tr>
<tr><td>di-Sodium Hydrogenphosphate</td><td>142.04</td><td colspan="7">71.02</td></tr>
<tr><td>Zinc Sulfate•$7H_2O$</td><td>0.863</td><td colspan="7">0.43</td></tr>
<tr><td rowspan="21">Amino acids</td><td>L-Alanine</td><td>9.00</td><td colspan="7">4.45</td></tr>
<tr><td>L-Arginine•HCl</td><td>211.00</td><td colspan="7">147.50</td></tr>
<tr><td>L-Asparagine•H_2O</td><td>15.01</td><td colspan="7">7.50</td></tr>
<tr><td>L-Aspartic Acid</td><td>13.30</td><td colspan="7">6.65</td></tr>
<tr><td>L-Cysteine•HCl•H_2O</td><td>35.00</td><td colspan="7">17.56</td></tr>
<tr><td>L-Cystine•2HCl</td><td>—</td><td colspan="7">31.29</td></tr>
<tr><td>L-Glutamic Acid</td><td>14.70</td><td colspan="7">7.35</td></tr>
<tr><td>L-Glutamine</td><td>146.00</td><td colspan="6">365.00</td><td>—</td></tr>
<tr><td>Glycine</td><td>7.51</td><td colspan="7">18.75</td></tr>
<tr><td>L-Histidine•HCl•H_2O</td><td>20.96</td><td colspan="7">31.48</td></tr>
<tr><td>L-Isoleucine</td><td>3.94</td><td colspan="7">54.47</td></tr>
<tr><td>L-Leucine</td><td>13.10</td><td colspan="7">59.05</td></tr>
<tr><td>L-Lysine•HCl</td><td>36.50</td><td colspan="7">91.25</td></tr>
<tr><td>L-Methionine</td><td>4.48</td><td colspan="7">17.24</td></tr>
<tr><td>L-Phenylalanine</td><td>4.96</td><td colspan="7">35.48</td></tr>
<tr><td>L-Proline</td><td>34.50</td><td colspan="7">17.25</td></tr>
<tr><td>L-Serine</td><td>10.50</td><td colspan="7">26.25</td></tr>
<tr><td>L-Threonine</td><td>11.90</td><td colspan="7">53.45</td></tr>
<tr><td>L-Tryptophan</td><td>2.04</td><td colspan="7">9.02</td></tr>
<tr><td>L-Tyrosine•2Na•$2H_2O$</td><td>7.78</td><td colspan="7">55.79</td></tr>
<tr><td>L-Valine</td><td>11.70</td><td colspan="7">52.85</td></tr>
<tr><td rowspan="10">Vitamins</td><td>D-Biotin</td><td>0.0073</td><td colspan="7">0.0035</td></tr>
<tr><td>Choline Chloride</td><td>13.96</td><td colspan="7">8.98</td></tr>
<tr><td>Folic Acid</td><td>1.32</td><td colspan="7">2.66</td></tr>
<tr><td>myo-Inositol</td><td>18.00</td><td colspan="7">12.60</td></tr>
<tr><td>Nicotinamide</td><td>0.037</td><td colspan="7">2.02</td></tr>
<tr><td>D-Pantothenic Acid Calcium Salt</td><td>0.48</td><td colspan="7">2.24</td></tr>
<tr><td>Pyridoxine•HCl</td><td>0.062</td><td colspan="7">2.03</td></tr>
<tr><td>Vitamin B_1•HCl</td><td>0.34</td><td colspan="7">2.17</td></tr>
<tr><td>Vitamin B_2</td><td>0.038</td><td colspan="7">0.22</td></tr>
<tr><td>Vitamin B_{12}</td><td>1.36</td><td colspan="7">0.68</td></tr>
<tr><td rowspan="9">Others</td><td>D-Glucose</td><td>1802.00</td><td colspan="2">3151.00</td><td>—</td><td colspan="3">3151.00</td></tr>
<tr><td>HEPES</td><td>—</td><td colspan="2">3574.50</td><td colspan="3">—</td><td>3574.50</td></tr>
<tr><td>Hypoxanthine</td><td>4.08</td><td colspan="7">2.10</td></tr>
<tr><td>Linoleic Acid</td><td>0.084</td><td colspan="7">0.042</td></tr>
<tr><td>Lipoic Acid</td><td>0.21</td><td colspan="7">0.11</td></tr>
<tr><td>Phenol Red</td><td>1.30</td><td>—</td><td colspan="3">8.10</td><td>—</td><td>8.10</td></tr>
<tr><td>Putrescine•2HCl</td><td>0.161</td><td colspan="7">0.081</td></tr>
<tr><td>Sodium Pyruvate</td><td>110.00</td><td colspan="7">55.00</td></tr>
<tr><td>Thymidine</td><td>0.73</td><td colspan="7">0.37</td></tr>
</table>

Culture Medium Composition Table

(mg/L)

<table>
<tr><th></th><th></th><th colspan="3">MEM</th><th colspan="2">α-MEM</th><th>IMDM</th><th>G-MEM</th></tr>
<tr><th colspan="2">Product no.</th><th>09848</th><th>21442</th><th>21443</th><th>21444</th><th>21445</th><th>11506</th><th>12965</th></tr>
<tr><td rowspan="10">Inorganic salts</td><td>Calcium Chloride・$2H_2O$</td><td colspan="3">265.00</td><td colspan="2">265.00</td><td>—</td><td>—</td></tr>
<tr><td>Calcium Chloride</td><td colspan="3">—</td><td colspan="2">—</td><td>165.00</td><td>200.00</td></tr>
<tr><td>Iron(III) Nitrate・$9H_2O$</td><td colspan="3">—</td><td colspan="2">—</td><td>—</td><td>0.10</td></tr>
<tr><td>Magnesium Sulfate</td><td colspan="3">97.67</td><td colspan="2">97.67</td><td>97.67</td><td>97.67</td></tr>
<tr><td>Potassium Chloride</td><td colspan="3">400.00</td><td colspan="2">400.00</td><td>330.00</td><td>400.00</td></tr>
<tr><td>Potassium Nitrate</td><td colspan="3">—</td><td colspan="2">—</td><td>0.076</td><td>—</td></tr>
<tr><td>Sodium Chloride</td><td colspan="3">6800.00</td><td colspan="2">6800.00</td><td>4500.00</td><td>6400.00</td></tr>
<tr><td>Sodium Dihydrogenphosphate</td><td colspan="3">122.00</td><td colspan="2">122.00</td><td>109.00</td><td>107.82</td></tr>
<tr><td>Sodium Hydrogen Carbonate</td><td colspan="3">2200.00</td><td colspan="2">2200.00</td><td>3024.00</td><td>2750.00</td></tr>
<tr><td>Sodium Selenite</td><td colspan="3">—</td><td colspan="2">—</td><td>0.0173</td><td>—</td></tr>
<tr><td rowspan="21">Amino acids</td><td>L-Alanine</td><td>8.90</td><td>—</td><td>8.90</td><td colspan="2">25.00</td><td>25.00</td><td>—</td></tr>
<tr><td>L-Arginine・HCl</td><td colspan="3">126.00</td><td colspan="2">126.00</td><td>84.00</td><td>42.00</td></tr>
<tr><td>L-Asparagine・H_2O</td><td>15.00</td><td>—</td><td>15.00</td><td colspan="2">50.00</td><td>28.40</td><td>—</td></tr>
<tr><td>L-Aspartic Acid</td><td>13.30</td><td>—</td><td>13.30</td><td colspan="2">30.00</td><td>30.00</td><td>—</td></tr>
<tr><td>L-Cysteine・HCl・H_2O</td><td colspan="3">—</td><td colspan="2">100.00</td><td>—</td><td>—</td></tr>
<tr><td>L-Cystine・2HCl</td><td colspan="3">31.30</td><td colspan="2">31.30</td><td>91.20</td><td>31.00</td></tr>
<tr><td>L-Glutamic Acid</td><td>14.70</td><td>—</td><td>14.70</td><td colspan="2">75.00</td><td>75.00</td><td>—</td></tr>
<tr><td>L-Glutamine</td><td colspan="3">292.00</td><td colspan="2">292.00</td><td>584.00</td><td>292.00</td></tr>
<tr><td>Glycine</td><td>7.50</td><td>—</td><td>7.50</td><td colspan="2">50.00</td><td>30.00</td><td>—</td></tr>
<tr><td>L-Histidine・HCl・H_2O</td><td colspan="3">42.00</td><td colspan="2">42.00</td><td>42.00</td><td>21.00</td></tr>
<tr><td>L-Isoleucine</td><td colspan="3">52.00</td><td colspan="2">52.00</td><td>105.00</td><td>52.00</td></tr>
<tr><td>L-Leucine</td><td colspan="3">52.00</td><td colspan="2">52.00</td><td>105.00</td><td>52.00</td></tr>
<tr><td>L-Lysine・HCl</td><td colspan="3">72.50</td><td colspan="2">72.50</td><td>146.00</td><td>73.00</td></tr>
<tr><td>L-Methionine</td><td colspan="3">15.00</td><td colspan="2">15.00</td><td>30.00</td><td>15.00</td></tr>
<tr><td>L-Phenylalanine</td><td colspan="3">32.00</td><td colspan="2">32.00</td><td>66.00</td><td>33.00</td></tr>
<tr><td>L-Proline</td><td>11.50</td><td>—</td><td>11.50</td><td colspan="2">40.00</td><td>40.00</td><td>—</td></tr>
<tr><td>L-Serine</td><td>10.50</td><td>—</td><td>10.50</td><td colspan="2">25.00</td><td>42.00</td><td>—</td></tr>
<tr><td>L-Threonine</td><td colspan="3">48.00</td><td colspan="2">48.00</td><td>95.00</td><td>47.60</td></tr>
<tr><td>L-Tryptophan</td><td colspan="3">10.00</td><td colspan="2">10.00</td><td>16.00</td><td>8.00</td></tr>
<tr><td>L-Tyrosine・2Na・$2H_2O$</td><td colspan="3">51.90</td><td colspan="2">51.90</td><td>104.00</td><td>52.00</td></tr>
<tr><td>L-Valine</td><td colspan="3">46.00</td><td colspan="2">46.00</td><td>94.00</td><td>46.80</td></tr>
<tr><td rowspan="11">Vitamins</td><td>L-Ascorbic Acid</td><td colspan="3">—</td><td colspan="2">50.00</td><td>—</td><td>—</td></tr>
<tr><td>D-Biotin</td><td colspan="3">—</td><td colspan="2">0.10</td><td>0.013</td><td>—</td></tr>
<tr><td>Choline Chloride</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>Folic Acid</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>myo-Inositol</td><td colspan="3">2.00</td><td colspan="2">2.00</td><td>7.20</td><td>3.60</td></tr>
<tr><td>Nicotinamide</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>D-Pantothenic Acid Calcium Salt</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>Pyridoxal・HCl</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>Vitamin B_1・HCl</td><td colspan="3">1.00</td><td colspan="2">1.00</td><td>4.00</td><td>2.00</td></tr>
<tr><td>Vitamin B_2</td><td colspan="3">0.10</td><td colspan="2">0.10</td><td>0.40</td><td>0.20</td></tr>
<tr><td>Vitamin B_{12}</td><td colspan="3">—</td><td colspan="2">1.36</td><td>0.013</td><td>—</td></tr>
<tr><td rowspan="13">Others</td><td>Adenosine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>Cytidine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>2'-Deoxyadenosine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>2'-Deoxycytidine・HCl</td><td colspan="3">—</td><td>11.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>2'-Deoxyguanosine・H_2O</td><td colspan="3">—</td><td>10.67</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>D-Glucose</td><td>—</td><td colspan="2">1000.00</td><td colspan="2">1000.00</td><td>4500.00</td><td>4500.00</td></tr>
<tr><td>Guanosine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>Lipoic Acid</td><td colspan="3">—</td><td colspan="2">0.20</td><td>—</td><td>—</td></tr>
<tr><td>Phenol Red</td><td colspan="3">10.00</td><td colspan="2">10.00</td><td>15.00</td><td>15.00</td></tr>
<tr><td>HEPES</td><td colspan="3">—</td><td colspan="2">—</td><td>5958.00</td><td>—</td></tr>
<tr><td>Sodium Pyruvate</td><td colspan="3">—</td><td colspan="2">110.00</td><td>110.00</td><td>—</td></tr>
<tr><td>Thymidine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
<tr><td>Uridine</td><td colspan="3">—</td><td>10.00</td><td>—</td><td>—</td><td>—</td></tr>
</table>

Culture Medium Composition Table

(mg/L)

<table>
<tr><th colspan="2"></th><th colspan="6">RPMI 1640</th></tr>
<tr><th colspan="2">Product no.</th><th>05176</th><th>06261</th><th>09892</th><th>16970</th><th>30263</th><th>30264</th></tr>
<tr><td rowspan="6">Inorganic salts</td><td>Calcium Nitrate・$4H_2O$</td><td colspan="6">100.00</td></tr>
<tr><td>Magnesium Sulfate</td><td colspan="6">48.84</td></tr>
<tr><td>Potassium Chloride</td><td colspan="6">400.00</td></tr>
<tr><td>Sodium Chloride</td><td colspan="4">6000.00</td><td>5650.00</td><td>6000.00</td></tr>
<tr><td>Sodium Hydrogen Carbonate</td><td colspan="3">2000.00</td><td>1500.00</td><td colspan="2">2000.00</td></tr>
<tr><td>di-Sodium Hydrogenphosphate</td><td colspan="6">800.00</td></tr>
<tr><td rowspan="19">Amino acids</td><td>L-Arginine</td><td colspan="6">200.00</td></tr>
<tr><td>L-Asparagine・H_2O</td><td colspan="6">56.82</td></tr>
<tr><td>L-Aspartic Acid</td><td colspan="6">20.00</td></tr>
<tr><td>L-Cystine・2HCl</td><td colspan="6">65.20</td></tr>
<tr><td>L-Glutamic Acid</td><td colspan="6">20.00</td></tr>
<tr><td>L-Glutamine</td><td>—</td><td colspan="5">300.00</td></tr>
<tr><td>Glycine</td><td colspan="6">10.00</td></tr>
<tr><td>L-Histidine</td><td colspan="6">15.00</td></tr>
<tr><td>Hydroxy-L-proline</td><td colspan="6">20.00</td></tr>
<tr><td>L-Isoleucine</td><td colspan="6">50.00</td></tr>
<tr><td>L-Leucine</td><td colspan="6">50.00</td></tr>
<tr><td>L-Lysine・HCl</td><td colspan="6">40.00</td></tr>
<tr><td>L-Methionine</td><td colspan="6">15.00</td></tr>
<tr><td>L-Phenylalanine</td><td colspan="6">15.00</td></tr>
<tr><td>L-Proline</td><td colspan="6">20.00</td></tr>
<tr><td>L-Serine</td><td colspan="6">30.00</td></tr>
<tr><td>L-Threonine</td><td colspan="6">20.00</td></tr>
<tr><td>L-Tryptophan</td><td colspan="6">5.00</td></tr>
<tr><td>L-Tyrosine・2Na・$2H_2O$</td><td colspan="6">28.83</td></tr>
<tr><td></td><td>L-Valine</td><td colspan="6">20.00</td></tr>
<tr><td rowspan="11">Vitamins</td><td>p-Aminobenzoic Acid</td><td colspan="6">1.00</td></tr>
<tr><td>D-Biotin</td><td colspan="6">0.20</td></tr>
<tr><td>Choline Chloride</td><td colspan="6">3.00</td></tr>
<tr><td>Folic Acid</td><td colspan="6">1.00</td></tr>
<tr><td>myo-Inositol</td><td colspan="6">35.00</td></tr>
<tr><td>Nicotinamide</td><td colspan="6">1.00</td></tr>
<tr><td>D-Pantothenic Acid Calcium Salt</td><td colspan="6">0.25</td></tr>
<tr><td>Pyridoxine・HCl</td><td colspan="6">1.00</td></tr>
<tr><td>Vitamin B_1・HCl</td><td colspan="6">1.00</td></tr>
<tr><td>Vitamin B_2</td><td colspan="6">0.20</td></tr>
<tr><td>Vitamin B_{12}</td><td colspan="6">0.005</td></tr>
<tr><td rowspan="5">Others</td><td>D-Glucose</td><td colspan="2">2000.00</td><td>—</td><td>4500.00</td><td colspan="2">2000.00</td></tr>
<tr><td>Glutathione</td><td colspan="6">1.00</td></tr>
<tr><td>HEPES</td><td colspan="3">—</td><td>2383.00</td><td>5958.00</td><td>—</td></tr>
<tr><td>Phenol Red</td><td>5.00</td><td>—</td><td colspan="4">5.00</td></tr>
<tr><td>Sodium Pyruvate</td><td colspan="3">—</td><td>110.00</td><td colspan="2">—</td></tr>
</table>

 For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

Appendix

Balanced Salt Solution Composition Table

(mg/L)

	D-PBS(+)*	D-PBS(-)			PBS		
Product no.	**02492**	**07269**	**11482**	**14249**	**11480**	**11481**	**13397**
References	D-PBS(+) Preparation Reagent(100x)	Powder**	D-PBS(-)(10x)	D-PBS(-)(1x)	PBS(-)(pH 7.2)	PBS(-) (pH 7.2)*** (10x)	PBS(-)(pH 7.4)
Calcium Chloride	10000.00	—			—		
Calcium Chloride・$2H_2O$	—	—			—		
D-Glucose	—	—			—		
Magnesium Chloride・$6H_2O$	10000.00	—			—		
Magnesium Sulfate	—	—			—		
Phenol Red, Sodium	—	—			—		
Potassium Chloride	—	200.00	2000.00	200.00	—		
Potassium Dihydrogenphosphate	—	200.00	2000.00	200.00	210.00	2100.00	144.00
Sodium Chloride	—	8000.00	80000.00	8000.00	9000.00	90000.00	9000.00
Sodium Hydrogen Carbonate	—	—			—		
di-Sodium Hydrogenphosphate	—	1150.00	11500.00	1150.00	—		
di-Sodium Hydrogenphosphate・$7H_2O$	—	—			726.00	7260.00	795.00

* By adding this product to D-PBS(-), D-PBS(+) can be prepared
** D-PBS(-) (1x) can be prepared by dissolving 9.6 g of this product in distilled water and making up the volume to 1 L
*** pH when diluted 10-fold

(mg/L)

	HBSS(+)		HBSS(-)	
Product no.	**09735**	**17459**	**17460**	**17461**
References	HBSS(+) without Phenol Red(1x)	HBSS(+) with Phenol Red(1x)	HBSS(-) with Phenol Red(1x)	HBSS(-) without Phenol Red(1x)
Calcium Chloride	—		—	
Calcium Chloride・$2H_2O$	184.45		—	
D-Glucose	1000.00		1000.00	
Magnesium Chloride・$6H_2O$	—		—	
Magnesium Sulfate	97.67		—	
Phenol Red, Sodium	—	10.20	10.20	—
Potassium Chloride	400.00		400.00	
Potassium Dihydrogenphosphate	60.00		60.00	
Sodium Chloride	8000.00		8000.00	
Sodium Hydrogen Carbonate	350.00		350.00	
di-Sodium Hydrogenphosphate	47.88		47.88	
di-Sodium Hydrogenphosphate・$7H_2O$	—		—	

Appendix

Contamination

In cell culture, contamination refers to the introduction of biological factors other than those intended for culture. Among these, cross-contamination specifically refers to the unintended mixing of different cell types. To prevent contamination, it is essential to obtain cells from a reliable cell bank or supplier and to be thoroughly trained in aseptic techniques.

≫ For details on aseptic techniques and sterilization, refer to p. 5.

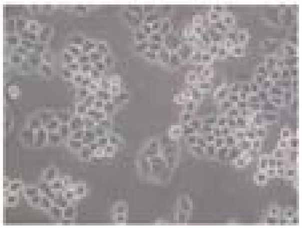
Bacterial / Fungal / Yeast Contamination

■ Confirmation of Contamination

Contamination by bacteria, fungi, or yeast can be confirmed by visual and microscopic observation as follows:

- The medium becomes more acidic or basic than usual (in the case of medium containing phenol red, the color changes to yellow or reddish purple)
- The medium becomes turbid.
- Foreign matter is floating in the medium.
- Under microscopic observation, small dots or cotton-like structures other than the target cells are observed (see figure below)

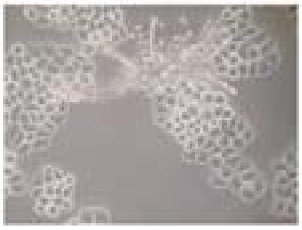
Normal condition



Bacterial contamination

Many small dots present

Fungal contamination



Cotton-like structures present

It is generally recommended to promptly discard culture vessels or containers in which contamination has been confirmed.

■ Prevention of Contamination

Contamination by bacteria, fungi, or yeast can also be prevented by adding antibiotics or antifungal agents to the culture medium. However, these agents may affect the cells or carry the risk of developing drug-resistant microorganisms. Therefore, it is recommended to use antibiotics or antifungal agents only when necessary and for a limited period of time.
The target spectrum and mechanism of action differ depending on the type of antibiotic or antifungal agent; therefore, in some cases, a combination of several antibiotics or antifungal agents may be used.
Commonly used antibiotics and antifungal agents are listed below.

Type	Target organisms	Mechanism of action	Concentration
Amphotericin B	Fungi	Binds to the cell membrane component (ergosterol) to cause membrane damage	0.25 µg/mL
Fungin	Fungi	Inhibits ion exchange across the cell membrane	10 - 50 µg/mL
Gentamicin	Gram-positive bacteria Gram-negative bacteria Mycoplasma	Inhibits protein synthesis	50 µg/mL
Kanamycin Monosulfate	Gram-positive bacteria Gram-negative bacteria Mycoplasma	Inhibits protein synthesis	10 - 100 µg/mL
Penicillin G	Gram-positive bacteria	Inhibits synthesis of the cell wall (peptidoglycan)	50 - 100 units/mL
Streptomycin	Gram-positive bacteria Gram-negative bacteria	Inhibits protein synthesis	100 µg/mL

In addition, certain antibiotics are used for the rapid selection of transfected cells or for obtaining stable expression clones after eliminating transiently transfected cells. These antibiotics are called selection antibiotics.

≫ For selection antibiotics, refer to p. 47.

Contamination

Mycoplasma Contamination

Mycoplasma is a type of bacterium belonging to the class Mollicutes and the order Mycoplasmatales. It lacks a cell wall and is approximately one-tenth the size of common bacteria, making it undetectable under a microscope during routine cell passage observation. In addition, there is no clear visible change in the appearance of the culture medium, and contamination by mycoplasma often goes unnoticed. However, contamination by mycoplasma has a significant impact on experimental results. It can cause abnormal cytokine expression, chromosomal abnormalities, and even cell death (apoptosis). This section introduces methods for detecting mycoplasma contamination.

■ Detection of Mycoplasma Contamination

Methods for confirming mycoplasma contamination include culture method, DNA fluorescence staining method, and nested PCR method. These are listed in the Japanese Pharmacopoeia as methods for mycoplasma detection tests. In addition, commercially available kits using enzyme-based methods or isothermal PCR methods can detect mycoplasma more easily. The characteristics of each method are summarized below.

<table>
<tr><th colspan="2"></th><th>Overview</th><th>Advantages</th><th>Disadvantages</th></tr>
<tr><td colspan="2">Culture method</td><td>Direct culture and counting of mycoplasma-specific “fried egg” colonies.</td><td>• High sensitivity
• Can detect a wide range of cultivable strains
• Listed in JP / USP / EP</td><td>• Requires more than 28 days for evaluation
• Requires a large sample amount</td></tr>
<tr><td colspan="2">DNA staining method using indicator cells</td><td>Co-culture of sample with indicator cells (e.g., Vero cells), followed by nucleic acid staining with Hoechst 33258 or similar dyes for evaluation.</td><td>• Can detect non-cultivable strains
• Requires only a small sample amount
• Low cost
• Listed in JP / USP / EP</td><td>• Less sensitive than the culture method
• Requires about one week for evaluation
• Other nucleic acids are also stained, so interpretation requires experience.</td></tr>
<tr><td rowspan="2">Nested-PCR method</td><td>Endpoint method</td><td>Extraction of nucleic acids from culture supernatant followed by PCR; detection of mycoplasma-derived nucleic acids confirmed by electrophoresis.</td><td>• Rapid (1–2 days)
• Requires only a small sample amount
• Listed in JP / USP / EP
(validation required)</td><td>• Limited to strains with known sequences
• Sensitivity and specificity depend on primers
• Detects dead cells as well</td></tr>
<tr><td>qPCR method</td><td>Extraction of nucleic acids from culture supernatant followed by qPCR; detection of mycoplasma derived nucleic acids.</td><td>• High sensitivity
• Rapid (1–2 days)
• Requires only a small sample amount
• Capable of processing multiple samples
• Listed in JP / USP / EP
(validation required)</td><td>• Limited to strains with known sequences
• Sensitivity and specificity depend on primers
• Detects dead cells as well</td></tr>
<tr><td colspan="2">Enzyme method</td><td>Detection utilizing mycoplasma-specific enzyme reactions.</td><td>• Relatively high sensitivity
• Simple procedure
• Very rapid (within 1 hour)
• Listed in USP
• Detectable regardless of cultivability or sequence knowledge</td><td>• Requires a luminometer
• Possible false positives due to bacterial contamination</td></tr>
<tr><td colspan="2">Isothermal PCR method</td><td>Execution of isothermal PCR using culture supernatant; detection of mycoplasma derived nucleic acids.</td><td>• Relatively high sensitivity
• Simple procedure
• Very rapid (within 1 hour)
• Requires only a small amount of sample</td><td>• Sensitivity and specificity depend on primers
• Detects dead cells as well</td></tr>
</table>

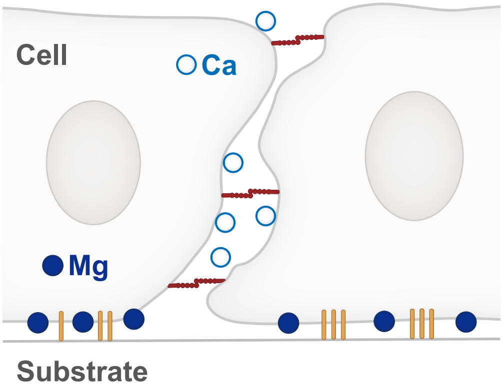
If mycoplasma contamination is confirmed, the infected cells should be discarded or treated to eliminate mycoplasma.
It is also important to verify whether the contamination has spread to other cell cultures.

Appendix

Cell Detachment/Dissociation Reagent: Trypsin-EDTA Solution

Cell–cell or cell–culture vessel adhesion involves proteins. To detach or dissociate cells, it is necessary to degrade the proteins involved in adhesion, and trypsin is used as one example. In some cases, calcium ions and magnesium ions are involved in adhesion. In this case, adding EDTA forms a complex with these metal ions and weakens adhesion. In this way, a trypsin-EDTA solution may be used to enhance the effect of trypsin.

■ Cell adhesion and the role of trypsin / EDTA



Trypsin ➡ Acts on cell-cell junctions

Trypsin ➡ Acts on cell-substrate junctions

EDTA ➡ Forms a complex with metal ions (Ca, Mg)

■ Product Lineup

We sell various formulations of trypsin and EDTA solutions. In general, trypsin is often used at concentrations of 0.05-0.25%, and you can select according to the desired concentration.

Product name	Product no.	Trypsin concentration		EDTA concentration (mmol/L)	Phenol red	Solvent
		(g/L)	(%)			
0.5g/l-Trypsin/0.53mmol/l-EDTA Solution	35553	0.5	0.05	0.53	—	HBSS(-)
0.5g/l-Trypsin/0.53mmol/l-EDTA Solution, with Phenol Red	32778				○	
2.5g/l-Trypsin/1mmol/l-EDTA Solution	35554	2.5	0.25	1.0	—	
2.5g/l-Trypsin/1mmol/l-EDTA Solution, with Phenol Red	32777				○	
2.5g/l-Trypsin Solution	35555			—	—	
5.0g/l-Trypsin/5.3mmol/l-EDTA Solution	35556	5	0.5	5.3	—	
25g/l-Trypsin Solution	18172	25	2.5	—	—	Saline
0.5mmol/l-EDTA/PBS Solution	13567	—	—	0.5	—	PBS(-)
0.2g/l-EDTA Solution	14367	—	—	0.44	—	

 For detailed product information, please search by entering the product number in e-Nacalai Search Version (https://www.e-nacalai.jp/ec2/EC-srchtop.cfm).

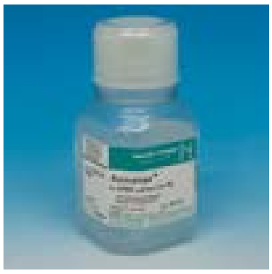
Appendix

Cell Detachment/Dissociation Reagent: Accutase and Accumax

Accutase and Accumax

Accutase and Accumax are gentle cell dissociation solutions composed of enzymes extracted from marine organisms. They are natural enzyme mixtures with proteolytic and collagenolytic activities and do not contain components derived from mammals or bacteria. Accumax has three times higher enzyme concentration than Accutase and is suitable for dissociating cell aggregates.

■ Product Lineup

Product name	Accutase	Accumax
Product no.	12679	17087
Product image		
Features	• Gentle and rapid cell detachment possible • No need for neutralization with serum or inhibitors • Most epitopes are preserved, making it useful for flow cytometry	• Three times higher enzyme concentration compared to Accutase • Suitable for dissociation of cell aggregates and spheroids
Phenol red	○	–
Solvent	D-PBS(-)	
Application record*	Human ES cells, human iPS cells, human mesenchymal stromal cells, bone marrow stem cells, human neural stem cells, neurospheres (Accutase only), macrophages, keratinocytes, vascular smooth muscle cells, vascular endothelial cells, hepatocytes, hepatic progenitor cells, primary chick embryo neuronal cells, fibroblasts, adherent CHO cells, BHK cells, 293 cells, L929 cells, immortalized mouse testicular germ cells, 3T3, Vero, COS, HeLa, NT2, MG63, M24, A375, U251, MRC5 (Accumax only), D54, HT1080, Sf9	

* Some cell types are listed.

≫ For references on use, please refer to the web page.

Accutase **Accumax**

Appendix

Experimental Protocol (Cell Detachment Using Accutase)

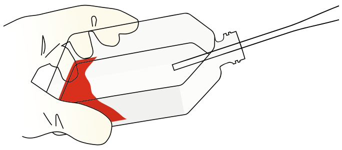
① Thaw Accutase (Product No.: 12679).

! Caution Do not thaw at 37°C. Leaving it at 37°C for more than one hour will result in loss of activity.

i Note After thawing, it can be stored refrigerated for more than two months. After use, be careful not to let the product temperature rise and promptly return it to the refrigerator.

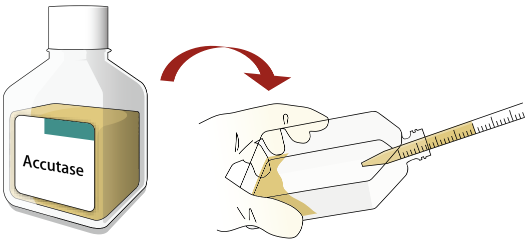
② Remove the medium from the culture vessel using an aspirator or similar equipment.

i Note Unlike trypsin detachment, washing with D-PBS before detachment is not necessary.



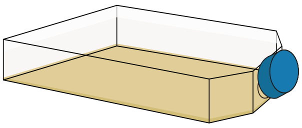
③ Add enough Accutase to cover the cells.

i Note For a T-25 flask, add 2.5–5 mL.
Accutase is a ready-to-use product and can be used without dilution.



④ Incubate at room temperature for 5-10 minutes.

i Note Perform the reaction at room temperature, not at 37°C. The reaction time can be extended up to one hour.



⑤ Observe under a microscope to confirm that the cells have rounded and begun to detach. Then gently tap the side of the culture vessel to completely detach the cells.

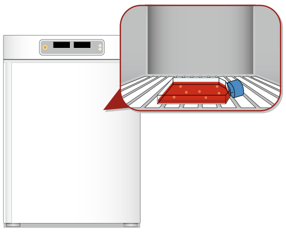
i Note Neutralization with serum or inhibitors is not necessary.

⑥ Collect the cells using standard methods and count the cell number.

≫ Refer to page 21 for cell counting using trypan blue.

⑦ After adjusting the cell suspension to the desired cell density, seed the cells into culture vessels and incubate at 37°C , 5% CO_2.

i Note Refer to the information provided by the cell supplier for the appropriate seeding density.



Appendix

Experimental Protocol (Cell Counting of Aggregates Using Accumax)

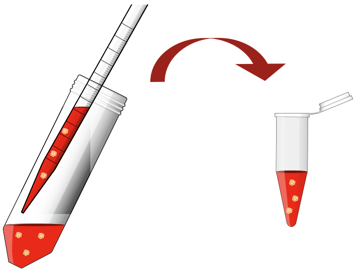
When counting the number of cells in cell aggregates, it is necessary to dissociate the aggregates. Below is a method for dissociating cell aggregates for cell counting.

① Thaw Accumax (Product Number: 17087).

! Caution Do not perform at 37°C. Leaving it at 37°C for more than one hour will result in loss of activity.

i Note After thawing, it can be stored refrigerated for more than two months. After use, be careful of temperature increase and promptly return it to the refrigerator.

② Transfer 0.5-1.0 mL of medium containing cell aggregates into a tube.



③ Mix Accumax with the medium taken in step ② at a 1:1 volume ratio.

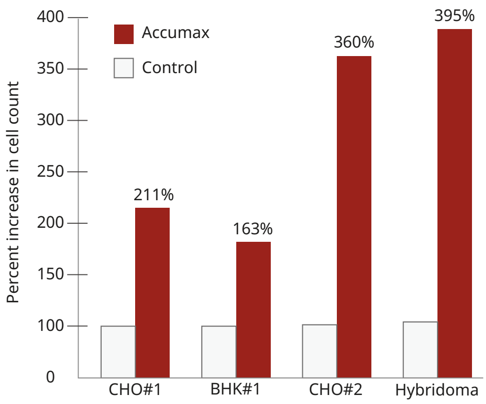
④ Incubate at room temperature for 5-10 minutes to dissociate the cell aggregates.

⑤ Collect the cells using standard methods and count the cell number.

≫ Refer to p. 21 for cell counting using trypan blue.

Application Cell counting of cell aggregates using Accumax

CHO cells, BHK cells, and some hybridoma cells cultured in serum-free or protein-free medium were collected, and the number of cells was counted after incubation (37°C / 5 minutes) with an equal volume of PBS (control) or this product. For more accurate and reproducible cell counting, it is necessary to perform the count with the aggregates fully dissociated.



Experimental Protocol (Dispersion of Spheroids Using Accumax)

① Thaw Accumax (Product Number: 17087).

! Caution Do not perform at 37°C. Leaving it at 37°C for more than one hour will result in loss of activity.

i Note After thawing, it can be stored refrigerated for more than two months. After use, be careful of temperature increase and promptly return it to the refrigerator.

② If spheroids are attached to the culture vessel:

Remove the medium using an aspirator or similar equipment and add enough Accumax to cover the cells. Detachment will take several minutes.

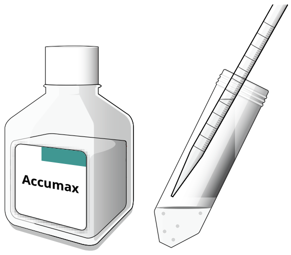
If spheroids are floating:

Transfer the sample to a centrifuge tube, centrifuge, and remove the medium using an aspirator or similar equipment.

③ Add an appropriate amount of Accumax.

Typically, Accumax is added in an amount sufficient to allow the spheroids to float.

i Note For a 50 mL centrifuge tube, add 15-20 mL.



④ At room temperature, gently shake back and forth using a shaker, and observe the structure of the floating spheroids every 5 minutes.

! Caution Do not perform this operation at 37°C.

⑤ When the spheroid structure begins to break apart, fragments will be observed. Gently dissociate the fragments by pipetting or similar methods. Repeat this operation every few minutes to dissociate the spheroids completely.

Selection Antibiotics

Antibiotics used for the rapid selection of transfected cells and for obtaining stable expression clones where transiently transfected cells have been eliminated are called selection antibiotics. By incorporating a resistance gene to the selection antibiotic into the introduced gene in advance, only transfected cells survive in the medium containing the selection antibiotic, allowing for selection. Representative selection antibiotics are introduced below.

Type	Overview	Approximate working concentrations
Blasticidin S	A peptidyl nucleoside antibiotic used for the selection of bacteria and animal cells. Resistance to Blasticidin S is conferred by *B. cereus*-derived Blasticidin S resistance gene (bsr) or *A. terreus*-derived Blasticidin S deaminase gene (bsd).	Bacteria: 25 - 100 μg/mL Animal Cells: 1 - 10 μg/mL
G418 (Geneticin)	An aminoglycoside antibiotic used for the selection of yeast, animal cells, and plant cells. Resistance to G418 is conferred by Neomycin resistance gene (neor).	Animal Cells: 400 - 1000 μg/mL
Hygromycin B	An aminoglycoside antibiotic used for the selection of bacteria, fungi, and animal cells. Resistance to Hygromycin B is conferred by *E. coli*-derived Hygromycin resistance gene (hph or hyg).	Bacteria: 50 - 100 μg/mL Animal Cells: 50 - 200 μg/mL
Phleomycin	A glycopeptide antibiotic used for the selection of bacteria, fungi, animal cells, and plant cells. Useful for selecting filamentous fungi that are less sensitive to Zeocin®. Resistance to Phleomycin is conferred by *S. hindustanus*-derived Bleomycin resistance gene (Sh ble).	Yeast: 10 μg/mL Filamentous Fungi: 25 - 150 μg/mL
Puromycin	An amino nucleoside antibiotic used for the selection of bacteria, animal cells, and insect cells. Resistance to Puromycin is conferred by *S. alboniger*-derived Puromycin N-acetyltransferase gene (pac).	Animal Cells: 1 - 10 μg/mL
Zeocin®	An antibiotic mainly composed of Phleomycin D1, used for the selection of bacteria, yeast, animal cells, and insect cells. Resistance to Zeocin® is conferred by *S. hindustanus*-derived Bleomycin resistance gene (Sh ble).	Bacteria: 25 μg/mL Animal Cells: 50 - 400 μg/mL

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Memo

Memo

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