# General Guidelines for Culturing Suspension Cells (Nonadherent Cells)

*Certain suspension cells, such as human hematopoietic cells, can be particularly difficult to culture. For these cell lines, please refer to the specific 'Application consideration' provided for each cell line before starting culture.

**Preparation**

- Preheat a 37°C water bath (or a commercially available water-free cell thawing device) in advance.

*Occasionally, there have been incidents where cells were completely damaged due to thawing frozen tubes in an incubator. Without exception, please use a 37°C water bath or cell thawing device for thawing operations.

- Prepare the culture medium in advance and allow it to room temperature. Excessive heating can alter or degrade medium components and cause poor cell growth.

- If an antibiotic Amphotericin B is added to the medium, it can lead to poor cell proliferation. Because some pre-mixed commercially available antibiotic solutions include Amphotericin B, please be cautious when using such antibiotics.

- Use the centrifuge at room temperature without cooling.

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**Thawing Operation**

- Always wear protective equipment (face guard, gloves) during thawing to prepare for unexpected tube bursts.

*In the case of cryotubes, slightly loosen the cap by a 1/4 turn to release the pressure inside, then re-tighten the cap before thawing.

(Please refer to "RIKEN BRC CELL BANK Information Sheet [Thawing procedure].)

- Perform thawing operations one tube at a time (one by one).

- **The thawing time** significantly affects cell viability.

*The basics for cell cryopreservation/resuscitation is 'slow freezing - rapid thawing.'

The thawing time refers to how quickly the cells pass through the critical temperature range above -80°C, where ice re-crystallization can damage the cells. The longer it takes, the cell viability decreases.

**Thawing Time Guidelines**

The cells in our bank are typically stored in 1 mL of cryopreservation medium in either glass ampules or plastic cryotubes. Thawing times are approximately below.

Glass ampules: 30 seconds
Plastic cryotubes: 1-2 minutes

*Thaw until only a small amount of ice remains (about the size of a grain of rice).
If there is excessive ice left in the container, partial ice-recrystallization may occur, damaging the cells. Conversely, continuing to thaw after the ice has completely melted can also cause damage due to the effect of cryoprotectant. The key for successful thawing is to stop thawing just when it seems the contents will fully melt soon, then gently and quickly transfer the cells to the prepared fresh medium.

- Cells are frozen with a cryoprotectant.

*Frozen cells provided by our bank contain DMSO (dimethyl sulfoxide) as a cryoprotectant.

*DMSO has cytotoxic properties.

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After thawing, prolonged exposure or heating of the cryopreservation solution containing DMSO can significantly decrease cell viability and, in some cases, lead to complete cell death.

*DMSO has been reported to induce cellular differentiation.

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For some cell lines, remaining DMSO at the time of thawing may induce differentiation, altering the characteristics of the cell lines.

The most crucial point is **to rapidly, firmly, and gently reduce the concentration of DMSO** while cell metabolism is still suppressed right after thawing.

Minimize the time that cells are exposed to DMSO after thawing, suspend them in the medium, centrifuge, and then promptly replace with fresh medium.
Handle re-suspension with pipetting gently and start culturing immediately after seeding the cells into the culture vessels.

Our general protocol for thawing cells describes suspending the frozen cells in 5 mL of medium, followed by centrifugation at '1000 rpm (approximately 200 xg), for 3 minutes, at room temperature' .
(Please refer to “RIKEN BRC CELL BANK Information Sheet [Thawing procedure].)

The reason for repeating this process twice is to reduce the concentration of DMSO.

However, for some cell lines, such as human hematopoietic cells, of which post-thaw viability is extremely low, it is better not to repeat the centrifugation steps because centrifugation might lead to the physical damage of the cells.
In such cases, resuspend the frozen cells in 10 mL of medium, perform only one centrifugation step, and start culturing promptly.
Please refer to the 'Application consideration' for each cell line for detailed cell information.

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**Seeding to Cell Recovery**

- In general, human hematopoietic cells are susceptible to freeze-thaw damage and often recover slowly.

- **Seeding density** greatly influences cell proliferation immediately after cell thawing.
*In relation to ordinary adherent cells, we usually recommend to seed in either two φ60mm dishes or two T25 $cm^2$ flasks. However, for suspension cells we recommend the use of T25 $cm^2$ flasks.
While the optimal seeding density varies by cell type, the viability of suspension cells immediately after thawing is much lower in general than that of adherent cells. It can be an effective strategy to increase seeding density such as a density covering the visual field full with cells immediately after thawing.

- The day after thawing, the viability may significantly decrease.
*Initially, many dead cells may be observed, including the cells with distorted or shrunken forms that will also degenerate and die.
Most cells recover within a few days, however, the recovery time can vary depending on the damage during thawing. As surviving cells begin to recover and proliferate, the viable cells with distinct, spheroid morphology gradually increase.
Initially observed dead cells gradually lose their structure and disperse into the medium.

- Until cells recover from freeze-thaw damage, **minimal manipulation** often leads to better outcomes.
*"Minimal manipulation" refers to avoiding actions like harvesting, centrifugation, medium exchange, or passaging that can cause physical damage to the cells.
Changing the culturing medium (such as removing or diluting the conditioned medium produced by the cellular metabolism) can also result in poor proliferation.
Until recovery, it is advisable to only add small amounts of fresh medium. Do not perform passage until the cells proliferated sufficiently .

- For cell lines which require cytokines, cytokines are gradually consumed over time. Therefore, add fresh cytokines as needed.
*Typically every 3-4 days or at medium changes. The amounts of cytokines to be added should be calculated based on the total volume of medium in the culture vessels.
(Total medium volume = volume of medium in the culture vessels + volume of fresh medium to be added)

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**Passaging**

**-** The timing for the first passaging is when a vigorous proliferation is observed, and the number of glossy proliferative cells begins to fill the visual field.

- It is preferable to dilute the cells with their conditioned medium rather than completely replace all the medium with fresh medium via centrifugation at passaging. In general, the proportion of dead cells floating in the medium will decrease gradually following passaging.

- The first passage should not reduce the cell density too much, maintaining a reduced dilution ratio (e.g., 1:2).
Once vigorous cell proliferation was observed , the cells can be passaged at the recommended dilution ratio for each cell type.

- Once vigorous cell proliferation was observed, next please be cautious about overgrowth.
Cell overgrowth can lead to a relative deficiency of essential components needed for cell survival and proliferation, ultimately leading to cell death. In relation to rapidly proliferating suspension cells, overgrowth can often result in losing the cells completely.