The culture of Mammalian cells

All solutions and equipment that contact with the cells must be sterile. Proper sterile technique should be used and worked in a laminar flow hood all along.

1. Protocol for Thawing Frozen Cells

The following protocol describes a general procedure for the thawing of cryopreserved cells. For detailed protocols, always refer to the cell-specific product insert.

1) Take the cryovial containing the frozen cells from liquid nitrogen and immediately place it into a 37°C water bath.

2) Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.

3) Wipe the outside of the vial with 70% ethanol and transfer the vial into a laminar flow hood.

4) Transfer the appropriate amount of pre-warmed complete growth medium and the thawed cells into a centrifuge tube.

5) Centrifuge the cell suspension at approximately 500 × g for 5-10 minutes. The actual centrifugation speed and duration varies depending on the cell type.

6) Discard the supernatant and keep the cell pellet.

7) Gently resuspend the cells with complete growth medium, and transfer into the appropriate culture vessel and into the recommended culture environment.

[Note]:

The actual centrifugation speed and duration varies depending on the cell type.

The appropriate flask size depends on the number of cells frozen in the cryovial.

The culture environment varies based on the cell and medium type.

2. Protocol for Passaging Adherent Cells

1) Discard the spent cell culture medium from the culture vessel.

2) Wash cells with PBS solution without calcium and magnesium (approximately 2 mL per 10 cm2 culture surface area). Gently add wash solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times.

[Note]: The wash step will remove serum, calcium, and magnesium that would inhibit the effect of the dissociation reagent such as trypsin.

3) Remove and discard the wash solution from the culture vessel.

4) Add the pre-warmed trypsin to the side of the flask, the volume of the trypsin could cover the cell layer (approximately 0.5 mL per 10 cm2). Gently shake the vessel to cover the cell layer completely.

5) Incubate the culture vessel at room temperature or 37℃ for approximately 2 minutes. Note that the actual incubation time varies depending on the cell type.

6) Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time and check the dissociation every 30 seconds. You may also pat the vessel to expedite cell detachment.

7) When 90% of the cells detached, tilt the vessel for a minimal length of time to allow the cells to drain. And add the equivalent of 2 volumes (twice the volume of trpsin) of pre-warmed complete growth medium. Disperse the cells by pipetting the medium over the cell layer surface for several times.

8) Transfer the cell suspension to a 15 mL centrifuge tube and then centrifuge at 500 × g for 5 to 10 minutes. Note that the centrifuge speed and time vary depending on the cell type.

9) Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and take a sample for counting the number of cells.

10) Determine the total number of cells and percent viability and add growth medium to the cells to achieve the desired cell concentration.

[Note]: It is recommended to use the Countess Automated Cell Counter to determine the total number of cells and percent viability. Using the same amount of sample with the hemacytometer, the Countess Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells.

11) Dilute cell suspension to the seeding density recommended for the cell line and pipet the appropriate volume into new cell culture vessels, then place the cells to the incubator.

[Note]: If the culture flasks are used, loosen the caps before placing the cells to the incubator to allow proper gas exchange unless vented flasks with gas-permeable caps are used.

3. Protocol for Passaging Suspension Cells

Subculture cells when they are in log-phase growth. The recommended density of different cells before passaging is also different.

1) When the cells are in log-phase growth and ready for passaging, take a sample for counting the number of cells. If cells have settled down before taking the sample, swirl the flask to distribute the cells evenly.

2) Determine the total number of cells and percent viability.

3) Calculate the volume of medium that needed for diluting the cells to seeding density to add to dilute the culture down to the recommended seeding density.

4) Add the appropriate volume of pre-warmed growth medium into the culture flasks, then place the cells to the incubator.

5) Loosen the caps of the culture flasks to allow for proper gas exchange unless vented flasks with gas-permeable caps are used and return the flasks to the incubator.

[Note]: To minimize the accumulation of cell debris and metabolic waste by-products in cultures, gently centrifuge the cell suspension at 500 × g for 5 to 10 minutes, and resuspend the cell pellet in fresh growth medium once every three weeks (or as needed).

4. Cryopreservation of Mammalian Cells

Tips:

a) The following protocol describes a general procedure for the cryopreservation of cultured cells.

b) Prepare freezing medium and store at 2° to 8°C until use. Note that the appropriate freezing medium depends on the cell line.

c) Freeze the cultured cells at a high concentration with a low passage number as possible. Make sure that the percent viability of the cultured cells is more than 90% before freezing. [Note]: The optimal freezing conditions depend on the cell line in use.

d) Store the frozen cells below –70°C; frozen cells begin to deteriorate above –50°C.

e) Always use sterile cryovials for storing frozen cells and the cryovials containing the frozen cells should be stored immersed in liquid nitrogen or in the gas phase above the liquid nitrogen.

f) Please pay attention to personal protective measures.

1) Protocol: Suspension cells

a) Count the number of viable cells to be cryopreserved and calculate required volume of freezing medium according to the viable cell density

[Note]: Cells should be in log phase.

b) Centrifuge the cells at 500 × g for 5-10 min, discard the supernatant and collect the cell pellet.

c) Resuspend the cells with freezing medium at a concentration of 1 x 106 cells/mL.

d) Aliquot the cell suspension into sterile cryovials and place the vials on wet ice or in a 4°C refrigerator, and start the freezing procedure within 5 minutes.

e) Cells are frozen slowly at 1°C/min. This can be achieved with a programmable cooler or by placing vials in an insulated box placed in a –70°C to –90°C freezer for overnight. Then transfer the cryovials to liquid nitrogen for storage.

2) Protocol: Adherent cells

a) Gently detach the cells from the substrate with dissociation reagents (such as trypsin). (It can be refered to the protocol of passaging adherent cells)

b) Resuspend the detached cells with complete growth medium. Count the number of viable cells to be cryopreserved and calculate required volume of freezing medium according to the viable cell density [Note]: Cells should be in log phase.

c) Centrifuge at 500 x g for 5-10 min to pellet cells, discard the supernatant and collect the cell pellet.

d) Resuspend cells with freezing medium at a concentration of 1 x 106 cells/mL.

e) Aliquot the cell suspension into sterile cryovials and place the vials on wet ice or in a 4°C refrigerator, and start the freezing procedure within 5 minutes.

f) Cells are frozen slowly at 1°C/min. This can be achieved with a programmable cooler or by placing vials in an insulated box placed in a –70°C to –90°C freezer for overnight. Then transfer the cryovials to liquid nitrogen for storage.

5. The centrifugation of the cells

To concentrate cells from a suspension culture (or resuspended cells from monolayer culture):

1) Transfer the cell suspension to a sterile centrifuge tube and centrifuge at 500 × g for 10 minutes. [Note]: Certain cell lines are very sensitive to centrifugal force.

2) Discard the supernatant and collect the cell pellet.

3) Gently add appropriate volume of fresh medium to the side of the tube and slowly pipette up and down 2 to 3 times to resuspend the cells.

4) Transfer the cells into the appropriate culture vessels.

6. Count the number of cells with Trypan Blue exclusion

The following protocol describes a procedure to determine the cell viability accurately. Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemacytometer. If the cells take up trypan blue, they are considered non-viable.

1) Clean the chamber and cover slip with alcohol. Dry and fix the coverslip in position.

2) Prepare a 0.4% solution of trypan blue in buffered isotonic salt solution, pH 7.2 to 7.3 (i.e., phosphate-buffered saline).

3) Add 0.1 mL of trypan blue stock solution to 1 mL of cell suspension.

4) Add 10 µL of the cells to the hemacytometer. Do not overfill.

5) Examine immediately under a microscope at low magnification.

6) Count the cells in the large, central gridded square (1 mm2). The gridded square is circled in the graphic below. Multiply by 104 to calculate the number of cells per mL.

[Note]:

1. Prepare duplicate samples and average the count.

2. Count the number of blue staining cells and the number of total cells.

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100