General Cultivation Protocol

The following protocol is recommended for all of CELLnTEC's primary and long-term cell cultures.

Immediately upon Delivery

Check that the vial is still frozen and surrounded by dry ice. The vial may be thawed directly after arrival, or transferred immediately to liquid nitrogen for further storage. Always ensure that the vial remains at -80°C or colder at all times - viability decreases rapidly at warmer temperatures.

General Notes

For optimal performance, cultivate cells at 37°C with 5% CO_2.

To detach cells for passaging, Accutase is recommended. Alternatively, trypsin / EDTA can be used.

Accutase (Cat# CnT-Accutase-100) is a mixture of proteolytic and collagenolytic enzymes isolated from crustaceans. It is free of mammalian components. Accutase is the recommended detachment enzyme due to its gentle action that leaves most surface proteins intact, and the fact that it does not require a separate reagent to stop the reaction (simply dilute with medium immediately after detachment). Accutase is heat senstive, and must be stored at 4°C. Pre-warm only the amount required to 20°C just prior to use. Exposure to higher temperatures or repeated warming / cooling cycles may lead to reduced activity.

Trypsin / EDTA is a solution of proteolytic enzymes containing Trypsin, Chymotrypsin and Elastase. Trypsin is non-defined and of porcine origin. It may vary from lot-to-lot, and must be inactivated with a separate reagent immediately after cell detachment (preferably with an animal-component free inhibitor, alternatively with FCS). Trypsin is much more aggressive than Accutase, resulting in only a very small time window between succssful detachment and irreversible cell damage. If using Trypsin, extra special care must be taken to minimise the exposure time, and ensure rapid inactivation.

Preparation

To identify the correct medium for your cell type, please see corresponding cell data sheet. In the protocols below, the medium is referred to generically as CnT medium.

Before starting to work with the cells, ensure that the amount of medium, buffers and enzymes required have been pre-warmed at least to room temperature.

Always use sterile instruments, aseptic technique, and work in a laminar flow to maintain sterility.

Thawing Cells

1. Add the necessary amount of CnT medium to the desired culture vessels, and equilibrate them for at least 30 min in the 5% CO_2 incubator, to pre-warm the medium and to adjust to physiological pH.
2. Thaw vial with gentle swirling in water bath at 37°C until just melted. Vials must be thawed rapidly, whilst ensuring that the temperature of the cells inside does not rise above 4°C whilst still in the freezing medium (remove vial from water bath whilst some ice crystals remain). For more explanation about the features of an optimal thawing procedure, please review the knowledgebase article in the resources section of www.cellntec.com.
3. Resuspend the cells in the cryo vial by pipetting up and down two to three times and transfer the whole content (1 mL) to the equilibrated cell culture vessels. The DMSO content in the freezing

medium is 10%, after seeding in medium this must be diluted to less than 1% (10x dilution).

4. The volume seeded per vessel should deliver the recommended seeding density listed in the data sheet.

IMPORTANT: CnT medium needs to be changed 6 to 24 h after seeding to remove the residual DMSO from the freezing medium.

Cultivation

During routine cultivation, medium should be changed every 2nd to 3rd day.

Aspirate the old CnT medium and replace it with the same volume of new CnT medium.

For routine cell cultivation, CELLnTEC recommends to work without antibiotics / antimycotics. For isolation, however, we recommend the use of antibiotics / antimycotics up to passage 2.

Passaging

For required CnT medium please see data sheet.

1. Cells should be passaged when they reach 70 to 90% confluency. Do not allow the cells to become 100% confluent, as this will trigger differentiation, and poor growth in the subsequent passage.
2. Aspirate the CnT medium from the culture vessel.
3. Wash the cells by adding 100 µL / cm^2 of PBS (without calcium and magnesium), rotate the culture vessel carefully to rinse the cells, then aspirate the PBS.
4. Add 50 µL / cm^2 of detachment enzyme to the cells. Accutase is recommended (warm to room temp).
5. Incubate at 37°C until cells are rounded and start to detach. Check for cell detachment under the microscope.
6. Tap the vessel to dislodge any remaining attached cells cells (slide vessel sideways into a solid object).
7. When using Accutase: add 2.5 volumes of CnT-PCT medium to the detached cells to deactivate the enzyme. When using Trypsin / EDTA: add the same volume of Trypsin inhibitor.
8. Transfer the cell suspension to a centrifugation tube. Rinse the bottom of the culture vessel 2 to 3 times with the suspension to remove all cells and to separate them into a single cell suspension.
9. Allow to stand for 3 minutes in the centrifuge tube for complete settlement.
10. Centrifuge the cells 5 min, 200 x g at room temperature.
11. Aspirate supernatant and resuspend the pellet in 1 to 2 mL CnT medium.
12. Count the viable cells and seed according to the seeding density listed in the data sheet. Most epithelial cells growing in CnT-PR medium are seeded at 4'000 cells cm^2 after passaging. Fibroblasts growing in CnT-PR-F show the highest growth after seeding at 1'000 cells cm^2.

Freezing

Prior to the freezing process, label and pre-cool the desired number of cryo-vials to 4°C.

1. Only subconfluent cells that are growing well should be frozen.

2. Detach the cells using the passaging protocol above.
3. Centrifuge the cells 5 min, 200 x g at room temperature.
4. Resuspend the cell pellet in 1 mL CnT medium.
5. Place the cells on ice, then take a sample to perform a cell count.
6. Adjust the cell concentration to double the final concentration desired with cold (4°C) CnT medium (e.g. if the desired cell concentration is 1 x10^6 cells / mL then adjust the cell concentration to 2 x 10^6 cells / mL).
7. Add drop-wise the same volume of cold (4°C) 2 x freezing medium (CnT-CRYO-50), while gently swirling the tube. Once all the freezing medium is added, allow to stand for 5 minutes to allow osmotic equilibration in the cytoplasm of the cells. Slow addition and a 5 minute equilibration are important to reach the correct osmotic balance without a shock. For more background on the features of an ideal freezing protocol, please see the knowledgebase article in the resources section of www.cellntec.com.
8. Transfer 1 mL of cell suspension into each of the labeled and pre-cooled cryo tubes.
9. Freeze vials at a cooling rate of 1°C/min either in a controlled-rate freezer or using pre-cooled freezing containers transferred to -80°C (such as "Mr. Frosty" from Nalgene).
10. Allow vials to freeze overnight, then transfer them to liquid nitrogen for long-term storage.

CELLnTEC scientists work with primary cells on a daily basis. They are available for trouble shooting should problems arise. To contact them directly, please email scientist@cellntec.com