



Freezing, cryopreservation, storage and reactivation of cell lines

A- Freezing, Cryopreservation And Storage Of Cell Lines

Cultures for cryopreservation should be healthy, free of contamination and in log phase growth for several days before freezing.

Equipment and Materials

- PBS without Ca^{+2} and Mg^{+2}
- Trypsin-EDTA 1X
- Petri dishes 100 mm
- DMSO
- Falcon tubes 15 ml
- Refrigerated centrifuge
- Haemocytometer
- Cryotubes
- Freezing medium
- Freezer (minus 20-25°C)
- Freezer (minus 70-90°C)
- Dewar 1-2 litres
- Liquid N_2
- Tank for liquid N_2

Freezing medium

to be prepared just before use:

| | | |
|-------------------------------------|------|------|
| A: Foetal bovine serum | 90% | |
| DMSO | | 10% |
| or | | |
| B: Dulbecco's modified Eagle medium | 70 % | |
| Foetal bovine serum | 20 % | |
| DMSO | | 10 % |

Procedure

Suspension cultures

1. Count the number of viable cells which should be in log phase (as described in "Routine Cell counting and assessment of viability"). Centrifuge for 7-10 minutes at 200 g (1000 rpm) to pellet cells. Use a pipette to remove the supernatant without disturbing the pellet.
2. Re-suspend the cells in freezing medium to a concentration of 5×10^6 to 1×10^7 cells/ml.
3. Transfer to sterile cryotubes and close tightly.
4. Write date, cell line code and passage number on cryotubes.
5. Place tubes briefly into a "Cryo 1°C freezing chamber" and place it in a freezer at minus 70-90°C for 2/4 hours. on ice: start the freezing procedure within 5 minutes.
6. The cells should be frozen slowly (at -1°C/min). This is most easily done using a programmable cooler. If such a cooler is not available, transfer the tubes from ice to an insulated pre-cooled box (or wrapped in cotton wool) and place for 2 hours in a freezer at minus 20-25°C; then place in a minus 70-90°C freezer for one or two days. Finally the cryotubes can be transferred to the liquid nitrogen tank for long-term storage.



Adherent cultures

1. Detach cells from 100 mm dish with trypsin-EDTA solution. Subsequently pipette up and down gently and transfer to a 15 ml centrifuge tube.
2. Add complete 5 ml of growth medium to inhibit trypsin.
3. Take sample to determine viability (as described in “Routine Cell counting and assessment of viability”)
4. If sufficiently viable, centrifuge at 1000 rpm for 7-10 minutes; discard the supernatant avoiding disturbing the pellet.
5. Suspend the pellet in freezing medium to a concentration of $5 \times 10^6 - 1 \times 10^7$ cells/ml.
6. Aliquot into sterile cryogenic tubes.
7. Write date, cell line code and passage number on cryotubes.
8. Place tubes on ice and begin Start the freezing procedure using the “Cryo 1°C freezing chamber” within 5 minutes (as described above).

B- REACTIVATION OF FROZEN CELL LINES

Cryopreserved cells are fragile and require gentle handling.

Equipment and materials

- Proliferating medium pre-warmed at 37°C
- Water bath 37-56°C
- Centrifuge
- Laminar flow hood
- CO₂ incubator
- 100 mm Petri dishes
- Pipettes
- Dewar, 1-2 litres
- Proliferating medium
- Haemocytometer

DMEM Proliferating medium

| | |
|--|----------|
| DMEM | 390 ml |
| Foetal bovine serum | 100 ml |
| Penicillin-streptomycin solution 100x | 5 ml |
| Insulin (10 microg/ml final) | 5 ml |
| L-glutamine | 5 ml |
| Human basic fibroblast growth factor | 25 ng/ml |
| Epidermal growth factor | 10 ng/ml |
| Filter and store at +4°C, up to 1 month. | |

Insulin solution

Dissolve 100 mg of insulin in 12 ml of HCl (1N). Add 88 ml of ultrapure water, filter through 0.22 micrometer filters and aliquot (5 ml) into sterile tubes.

Procedure

Cells are kept in liquid nitrogen until they are ready to be thawed, and the thermostated water bath is at 37°C.

Centrifugation method

1. The cryotubes are placed in the water bath at 37°C and the cells allowed to thaw (requires a few minutes).



2. Pipette gently up and down the suspension to homogenize. Transfer to a 15 ml plastic tube with 5 ml of proliferating medium.
3. Centrifuge at 4°C, 200 g (1000 rpm) for 7 minutes.
4. The cells are placed on ice and the supernatant discarded.
5. The pellet is suspended in 1 ml proliferating medium.
6. 25 µl of cell suspension is transferred to an Eppendorf tube for cell counting (not always necessary).
7. The remaining cells are transferred to one or two 100 mm Petri dishes (depending on the viable cell count: there should be at least 3×10^5 cells/ml) containing 8 ml of proliferating medium.
8. The Petri dishes are placed in an incubator at 37°C and cells allowed to adhere to the surface.

Direct plating method

1. The cells are removed from storage and thawed quickly in a 37°C water bath.
2. The cells are then placed directly in complete growth medium (10 ml per 1 ml of frozen cells).
3. The cells are cultured for 12-24 hours. The medium is removed (to remove cryo-preserved) and fresh complete growth medium added, as described in "Primary myoblast culture from fresh human biopsy".