Protocol

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Animal Cryopreservation Protocol

For Adherent Cells and Cell Suspensions

Introduction

Cryopreservation is a method for freezing and preserving tissues. Animal cryopreservation is performed to protect or recover extinct or endangered animals, avoid the genetic contamination of lines, have stock in case of natural disasters affecting vivaria, preserve and maintain GMOs and help in the assisted reproduction technology (ART) at the forefront in infertility treatment. Tissues such as semen, embryos, oocytes, somatic cells, nuclear DNA, blood, serum, and tissue can all be cryopreserved.

This protocol for mammalian cells was first reported by Stacey and Masters, 2008, and is a step-by-step guide for both adherent cells* and cell suspensions.

***Adherent cells:** Cells that must be attached to a surface to grow.

Materials

Animal tissues

This protocol can be used for semen, embryos, oocytes, and somatic cells. Remember that although the basic principle is simple, each cell line has its own characteristics, which may affect cryopreservation success. In this sense, regrowth capability and cell viability should be considered when evaluating each animal tissue.

Reagents

- Basic cell culture medium (e.g., DMEM or RPMI-1640)
- Fetal bovine serum (FBS)
- Dimethylsulfoxide (DMSO) (GoldBio Catalog # D-360)
- Trypsin (GoldBio Catalog # T-161)
- Phosphate-buffered saline (PBS) (GoldBio Catalog # P-271)
- Fetal calf serum
- Liquid nitrogen
- Trypan blue

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Equipment

- Horizontal laminar flow hoods
- Controlled freezing device or passive freezing box (Mr. Frosty®)
- CO2 incubator
- Inverted microscope

Trypan blue solution

- Add 0.4% trypan blue to 1X PBS solution.
- Store in a dark bottle. If debris is visible during staining, filter solution with Whatman
- Grade 1 filter paper.

Cryoprotectant medium

- Add 20% vol/vol of fetal calf serum to a basic cell culture (e.g., DMEM or RPMI-1640)
- Add 10% vol/vol of dimethylsulfoxide (DMSO)

Method

Before proceeding with the protocol, ensure you wear and have all the safety materials to manipulate the reagents, such as a lab coat, gloves, thermal gloves to manipulate liquid nitrogen, and safety glasses.

Two methods are detailed below; for cell suspension and for adherent cells:

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For cell suspensions

1. Examine to make sure the cultures are free of contamination.

2. Perform the viability cell counting using trypan blue solution. A **Viability Cell Counting** method is detailed at the end of this protocol.

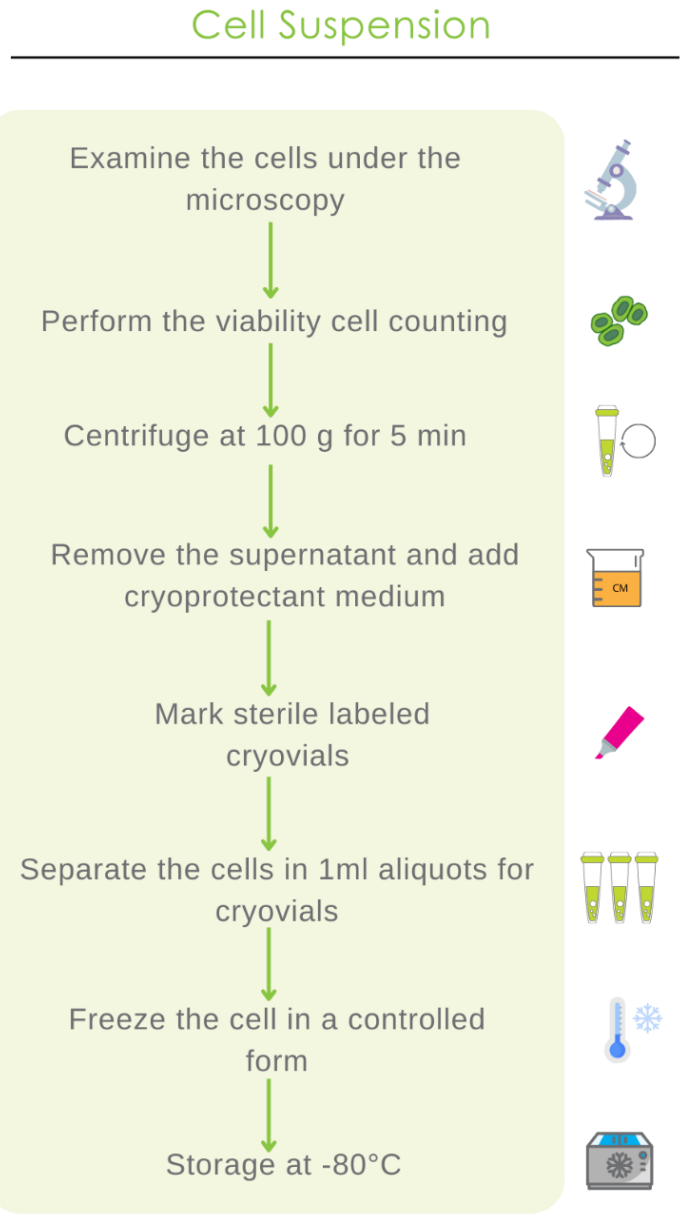
3. Centrifuge the cell suspensions at 100 x g for 5 minutes.

4. Remove the supernatant and resuspend the cell pellet in cryoprotectant medium. The final concentration should be between 2-5 x 10^6.

5. Mark sterile labeled cryovials with name, sample, day, volume.

6. Mix the cell suspension softly and separate the suspension into 1 ml aliquots in sterile labeled cryovials. Seal each vial immediately after it is filled. Do not fill to the top in order to avoid potential contamination.

7. Freeze the cells using a controlled freezing device or passive freezing box.



8. Transfer the box to a freezer overnight in a location where it will not be disturbed.

9. Storage in liquid nitrogen or freezer below -80°C.

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For adherent cells

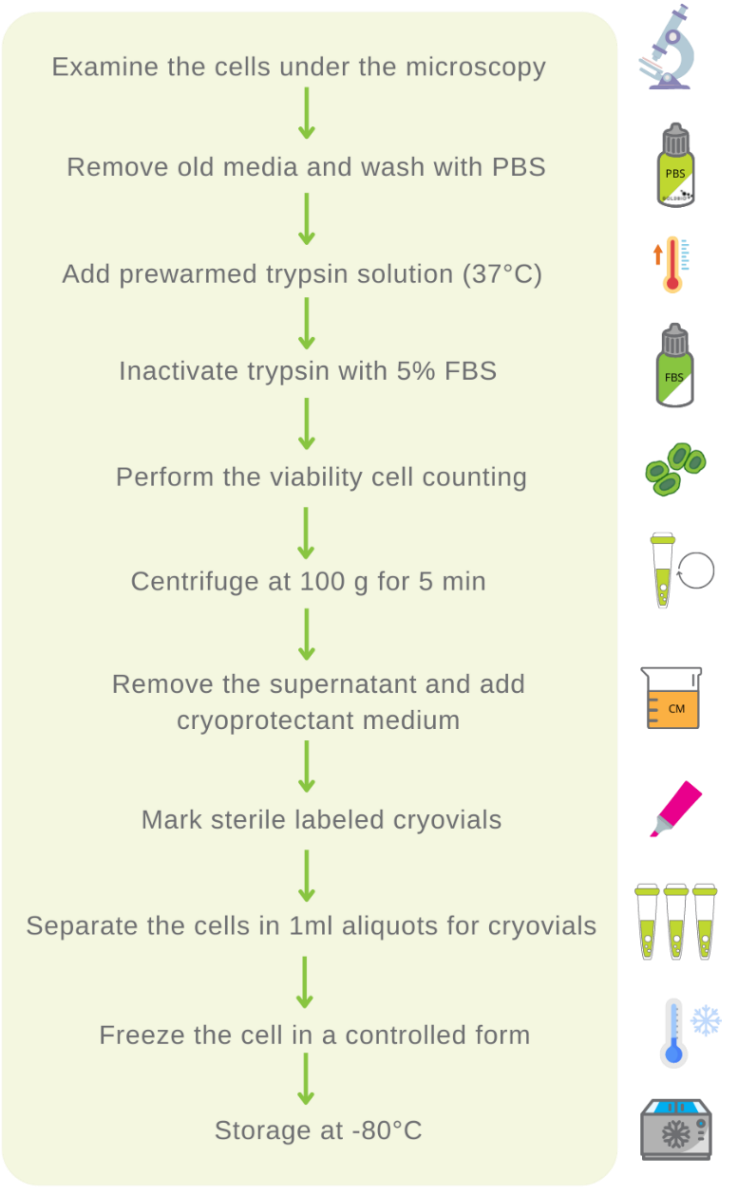
1. This protocol is designed for individual monolayer flasks. First, check the cells under an inverted-phase microscopy. Examine to make sure the cultures are free of contamination.

2. Remove the growth medium from the growing cells and wash twice in PBS.

3. To dislodge the cells, add 0.5 mL of prewarmed trypsin solution (37°C) per 25 cm^2 to cover the cell monolayer, close the flask and incubate at 37°C for 5 minutes. If the cells are not dislodged, continue to incubate for an additional 5 minutes.

4. Collect the cells in 10 ml of your basic cell culture medium containing 5% FBS (or other serum to inactivate trypsin). If there are duplicate flasks, pool cells together.

5. Perform the viability cell counting using trypan blue solution. A **Viability Cell Counting** method is detailed at the end of this protocol.



6. Transfer the cells to a conical tube, mix softly and centrifuge at 100 x g for 5 minutes.

7. Remove the supernatant and resuspend the cell pellet in cryoprotectant medium. The final concentration should be between 2-5 x 10^6.

8. Mark sterile labeled cryovials with name, sample, day, volume.

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9. Mix the cell suspension softly and separate the suspension into 1 ml aliquots in sterile labeled cryovials. Seal each vial immediately after it is filled. Do not fill to the top in order to avoid potential contamination.

10. Freeze the cells using a controlled freezing device or passive freezing box.

11. Transfer the box to a freezer overnight in a location where it will not be disturbed.

12. Storage in liquid nitrogen or freezer below -80°C.

Viability Cell Counting

1. Centrifuge one aliquot of cell suspension (~5-10 ml depending on the cell concentration) at 100 x g for 5 minutes and discard the supernatant.

2. Resuspend the cell pellet in 1 ml PBS or another serum-free complete medium.

3. Mix the trypan blue staining with the cell suspensions in a proportion of 1:1 (e.g., 1 ml:1 ml of each) and incubate for 3 minutes at room temperature.

4. Start the cell counting immediately after incubation, otherwise, the trypan blue can lead to cell death during prolonged incubation.

5. Place a drop of the trypan blue/cell mixture in the hemacytometer.

6. Initiate the cell counting. The unstained cells mean they are viable while blue-stained cells are nonviable.

7. Calculate the percentage of viable cells as follows:

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells per ml of aliquot}}{\text{Total number of cells per ml of aliquot}} \times 100$$

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References

Aljaser, F., 2012. Cryopreservation Methods and Frontiers in the Art of Freezing Life in Animal Models, in: IntechOpen. p. 13.

Masters, J.R., Stacey, G.N., 2007. Changing medium and passaging cell lines. Nat. Protoc. 2, 2276–2284. https://doi.org/10.1038/nprot.2007.319

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