Instruction Manual: Cryopreservation with ReproCryo DMSO Free™

Cat. No. RCHEFM002
Version 1.0

Overview

This protocol describes proper procedures for cryopreservation and thawing of human embryonic stem cells (ES cells) and induced pluripotent stem cells (hiPSCs) with ReproCryo DMSO Free™ medium.

It is strongly recommended that you read and fully understand the entire protocol before beginning your experiments. To maintain sterility, all procedures (except as indicated) should be performed in a sterile biological safety cabinet.

Conditions of Use

This product is for research use only. It should not be used for therapeutic or diagnostic purposes. Sale of this product to a third party, or any other commercial use for this product, is prohibited without prior permission from ReproCELL.

Storage

ReproCryo DMSO Free™ should be stored at -20 °C upon receipt. After thawing, store at 2-8 °C and use within two weeks. Avoid repeated freezing and thawing.

Features of ReproCryo DMSO Free™

- Each lot is culture-tested with human iPS cells as described in Takahashi *et al*., Cell 131:861-72 (2007).
- Control testing of critical criteria, including osmolality, pH, sterility, and mycoplasma has been performed on each lot.
- DMSO Free
- Components are chemically defined and Xeno-free
- For use with slow freezing protocols and instruments

General notes

- ReproCryo DMSO Free™ is a provided in a 2X formulation. Equal volumes should be mixed with your cell suspension in a 15 mL conical tube. Gentle mixing by finger tapping is recommended.
- After mixing the cell suspension with ReproCryo DMSO Free™ medium, incubation for 30 min in a 37°C water bath is required to ensure uptake and equilibration of critical cryopreservation components.
- Vials of cells require slow freezing to -80°C at a rate of -1°C per min using a BICELL, Mr. Frosty or another programmable cell freezer.
- Cryogenic cell vials should be transferred to liquid nitrogen from three to six hours after beginning the freezing process.

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ReproCryo DMSO Free™

Product Description	Cat. No.	Format	Storage
ReproCryo DMSO Free™	RCHEFM002	50 mL	-20 °C

Required Reagents and Equipment

Product Description	Cat. No.	Format	Storage
BICELL, Mr. Frosty or programmable freezer			
ESGRO Complete™ Accutase™	Millipore SF006	100 mL	4 °C
Y27632	-	-	-
PBS (-). Ca^{2+}- and Mg^{2+}-Free	Standard Lab Suppliers	-	-
60 mm Tissue Culture Dish	Standard Lab Suppliers	-	-
Standard cell culture equipment	Standard Lab Suppliers	-	-

ReproCryo DMSO Free™ Protocols

Equipment preparation

1. Equilibrate freezing container or programmable freezer to 4℃.
2. Equilibrate water bath to 37℃.

Cryopreservation with ReproCryo DMSO Free™

Note: Volumes are based on cell growth in a 60 mm tissue culture dish.
Note: Cryopreservation should be performed on human ES/iPS cells that have achieved a typical density that is ready for passage.
Note: ReproCryo DMSO Free™ is a provided in a 2x formulation. Equal volumes should be mixed with your cell suspension in a 15 mL conical tube. Gentle mixing by finger tapping for about 10 sec is recommended.
Note: After mixing the cell suspension with ReproCryo DMSO Free™ medium, incubation for 30 min in a 37°C water bath is required to ensure uptake and equilibration of critical media components.
Note: Vials of cells require slow freezing to -80℃ at a rate of -1℃ per min using a BICELL, Mr. Frosty or another programmable cell freezer.
Note: Cryogenic cell vials should be transferred to liquid nitrogen three or six hours after beginning the freezing process. Maintaining at -80℃ to 3 hours is recommended. Holding at -80℃ beyond six hours is not recommended.

1. Thaw ReproCryo DMSO Free™ at 4℃ before use. If desired, aliquot ReproCryo DMSO Free™ into desired working volumes and stored at -20℃ for future use.
2. Equilibrate freezing container or programmable freezer to 4℃.
3. Equilibrate water bath to 37℃. Aliquot ReproCryo DMSO Free™ into desired volume and warm it.
4. Prepare human ES/iPS cells using feeder or feeder-free culture on 60 mm dishes.
5. Remove the medium from the dish. Wash the cells with 2 mL of calcium and magnesium free phosphate buffered saline (PBS (-)).

6. Remove the PBS (-). Add 1 mL of ESGRO complete ACCUTASE™ to the dish.
7. Incubate for 10 minutes in a 5% CO_2 incubator at 37℃.
8. Add 1 mL of fresh culture medium.
9. Detach all ES/iPS cells and feeder cells from the dish by gentle agitation using a P-1000 pipette; transfer to a 15 mL conical tube.
10. Centrifuge in 300×g for 5 minutes at room temperature. Remove the supernatant as much as possible.
11. Add 0.5 mL of fresh culture medium. Resuspend the cell pellet.
12. Add 0.5 mL of the pre-warmed ReproCryo DMSO Free™ to the cell suspension. Mix gently by tapping 15 mL tube for 10 seconds.
13. Incubate the cell suspension for 30 minutes in a water bath at 37℃.
14. Agitate the cell suspension by pipetting up-and-down twice with a P-1000 pipette every ten minutes to prevent the cells from settling at the bottom of the tube.
15. Transfer the cell suspension to a cryogenic vial after 30 minutes.
16. Place the cryogenic vial into the programmable freezing instrument and start the program to lower the temperature to -80℃ at the rate of -1℃ per minute.
17. Upon achieving -80℃, leave the tube at that temperature until 3 hours has passed from the beginning of the freezing process.
18. Transfer the tube to liquid nitrogen at some time no more than 6 hours after the start of the programmable freezing protocol. Do not wait longer than 6 hours for transfer to liquid nitrogen as this will reduce cell viability.

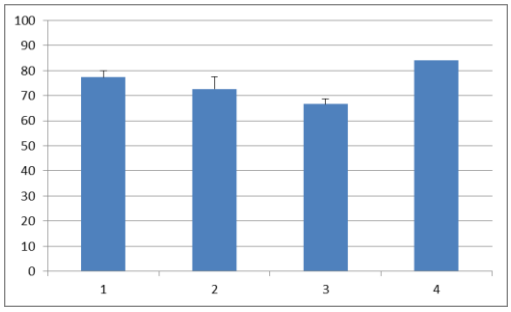
Thawing the cells in ReproCryo DMSO Free™

Note: Volumes are based on culture in a 60 mm tissue culture dish.
Note: After thawing, the slow addition of culture medium (over a period of ~10 sec) is required to avoid severe osmotic shock to the cells.

1. Equilibrate water bath to 37℃. Aliquot culture medium into desired volume and pre-warm it.
2. Prepare a small liquid nitrogen bath. Retrieve the cryogenic vial from the liquid nitrogen storage tank. Move it near to the water bath.
3. Quickly thaw the cryogenic vial by swirling it in the water bath for 2 min 30 sec.
4. Using a P-1000 pipette, resuspend the cell suspension by repeated pipetting (up-and-down) two times gently. Transfer cell suspension to a 15 mL tube.
5. Slowly add 1 mL of pre-warmed culture medium over a period of about 10 seconds, while gently tapping the 15 mL tube to mix.
6. Let stand for about 2 minutes.
7. Again, slowly add 5 mL of pre-warmed culture medium over a period of about 10 seconds, while gently tapping the tube to mix. NOTE: This sequential slow addition of culture medium is required to avoid severe osmotic shock to the cells.
8. Centrifuge in 300×g for 5 minutes at room temperature. Remove the supernatant as much as possible.
9. Add 4 mL of fresh culture medium containing Y-27632 at a 10 µM final concentration. Resuspend the cells pellet by gentle tapping.
10. Add the cell suspension onto a 60 mm dish covered by feeder cells or pre-coated according to the growth requirements of the cell.
11. Swirl the dish to spread the cells evenly and incubate at 37°C in a 5% CO_2 incubator.
12. Change medium after 24 hours. Use of Y-27632 in the growth medium is not required after 24 hours of growth.

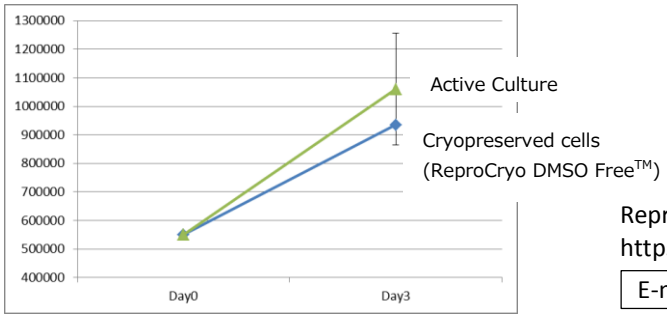
Viability and proliferation after thawing using ReproCryo DMSO Free™

Viability after thawing



Viability of iPSC after thawing has consistently been greater than 60% when tested on four different lots of iPS cells using ReproCryo DMSO Free™ medium. Average viability is close to 80%. (Y axis = % viability; X axis = each lot, n=3)

Proliferation after thawing



Cells (iPSC) that were cryopreserved in ReproCryo DMSO Free™ and recovered (thawed), showed similar proliferation to identical cells that were maintained in active culture for three days incubation. (Y axis = cell count; X axis = culture hours, n=3)

Frequently Asked Questions

Q. **Is it acceptable to keep the cryogenic vial of frozen cells at -80℃ overnight after programmable freezing?**
A. Most of the cells will remain viable and recover after thawing. However viability will be somewhat reduced (10%-20% reduced) if you leave the cells overnight at -80℃. We recommend to transfer the cryogenic vial from -80℃ to a liquid nitrogen storage tank between 3 hours and 6 hours after initiating the programmable freezing.

Q. **Can ES/iPS cells cultivated on feeder or feeder free be cryopreserved with ReproCryo DMSO Free™ Medium?**
A. Yes, both can be effectively cryopreserved.

Q. **Can ReproCryo DMSO Free™ be used in a vitrification method ?**
A. No, it cannot. ReproCryo DMSO Free™ is designed for slow freezing method.

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