CELL
APPLICATIONS, INC.

General Instructions for Use

Human Induced Pluripotent Stem Cells (HiPSC)

Be sure to wear face protection mask and gloves when retrieving cryovials from the liquid nitrogen storage tank. The dramatic temperature change from the tank to the room could cause any trapped liquid nitrogen in the cryovials to burst and cause injury.

Open all the packages immediately upon arrival and examine each component for shipping damage. Notify Cell Applications, Inc. or your distributor immediately if there is any problem.

I. KIT CONTENTS AND STORAGE

A. CRYOPRESERVED VIALS (iPS11-10, iPS12-10)

Store the cryovials in a liquid nitrogen storage tank immediately upon arrival.

B. HiPSC GROWTH MEDIUM KIT (015XFK-500)

Store the HiPSC Basal Medium (014-500) at 4°C in the dark immediately upon arrival.
Store the growth supplement (015-GS) at -20°C in the dark immediately upon arrival.
Complete HiPSC growth medium should be kept at 4°C and used within 2 weeks of preparation.

C. HiPSC XENO-FREE COATING SOLUTION STOCK (126XF-005)

Store 50 ul of HiPSC Coating Solution Stock of 100X at -20°C immediately after arrival.
Aliquot after first thaw and store the aliquots at -20°C

D. HiPSC DISSOCIATION SOLUTION (076-5)

Store Dissociation Solution (076-5) at -20°C.

*ROCK Inhibitor: a free sample of 200 µl of 100X is included in the kit for initiating HiPSC culture.
Only add ROCK Inhibitor in Growth Medium for 24 hours:
1X: initial culture from cryopreservation
0.5 X: after subculture

II. PREPARATION FOR CULTURING

1. Make sure the Class II Biological Safety Cabinet, with HEPA filtered laminar airflow, is in proper working condition.
2. Clean the Biological Safety Cabinet with 70% alcohol to ensure it is sterile.
3. Turn the Biological Safety Cabinet blower on for 10 min. before cell culture work.
4. Make sure all serological pipettes, pipette tips and reagent solutions are sterile.
5. Follow standard sterile techniques and safety rules:
   a. Do not pipette with mouth.
   b. Always wear gloves and safety glasses when working with human cells even though all the strains have been tested negative for HIV, Hepatitis B and Hepatitis C.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING HiPSC

A. PREPARING CELL CULTURE FLASKS FOR CULTURING HiPSC

1. Take out HiPSC Coating Solution Stock from the freezer. Briefly centrifuge in microfuge to collect HiPSC Coating Solution Stock.
2. Prepare Working Coating Solution by diluting HiPSC Coating Solution Stock 1 to 100 in sterile PBS Working Coating Solution is only stable for 2 weeks at 4°C.
   Aliquot remaining HiPSC Coating Solution Stock for single use and store at -20°C
3. Prepare one T-25 flask* by pipetting 2.5 ml of HiPSC Working Coating Solution to coat a T-25 flask.
4. Incubate coated T-25 flask at 37ºC for one hour for the coating to stabilize.
5. Aspirate HiPSC Working Coating Solution from the T-25 flask and the coated flask is ready for seeding.
   *Consult Appendix for seeding thawed HiPSC product vial in different culture format.

B. PREPARING GROWTH MEDIUM FOR CULTURING HiPSC

1. Thaw HiPSC Growth Supplements 015-GS and add to the basal medium, mixing gently.
   Do not thaw 015-GS in 37°C water bath.
2. Transfer 5 ml of Growth Medium to coated T-25 flask* prepared in Section IIIA Step 5 and warm up to room temperature*.
   Consult Appendix as seeding quide
3. Transfer 9 ml of HiPSC growth medium to a 15 ml conical tube for diluting freshly thawed HiPSC
   **** Do not warm HiPSC growth medium to 37ºC.***

C. THAWING AND PLATING HiPSC

1. Remove the cryopreserved vial of HiPSC from liquid nitrogen storage tank using proper protection for your eyes and hands.
3. Turn the vial cap a quarter turn to release any liquid nitrogen that may be trapped in the threads, then re-tighten the cap and bury the cryovial in dry ice.
4. Thaw the cells quickly by placing the lower half of the vial in a 37°C water bath and watch the vial closely during the thawing process. This usually takes ~90 sec.
5. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.
6. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
7. Remove the vial cap carefully. Do not touch the rim of the cap or the vial.
8. Transfer the cell suspension gently drop wise to the 15 ml conical tube containing 9 ml HiPSC growth medium prepared in Section III B step 3. Mix gently.
9. Centrifuge cells at 200g for 5 minutes. Remove supernatant carefully by aspiration without disturbing the cell pellet.
10. Resuspend HiPSC in 5 ml HiPSC Growth Medium prepared in Section III B Step 2. Add 50 µL of 100X ROCK Inhibitor to achieve a final concentration of 1X.
11. Transfer 5 ml of HiPSC suspension to the coated T-25 flask. Cap the flask and rock gently to evenly distribute the cells.
12. Place the T-25 flask in a 37ºC, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange. For best results, do not disturb the culture for 24 hours after inoculation.
13. Change to fresh HiPSC Growth Medium without ROCK Inhibitor after 24 hours.
14. Change HiPSC Growth Medium every day until the cells reach 90% confluent.
15. Subculture the cells when the HiPSC reach 90% confluent, usually at day 3.

IV. SUBCULTURING HiPSC

Ensure ROCK Inhibitor is available in laboratory.

A. PREPARING SUBCULTURE REAGENTS

1. Take out HiPSC Dissociation Solution from freezer and bring to room temperature.

B. PREPARING CELL CULTURE WARE

1. Decontaminate an aliquot of HiPSC Coating Solution Stock and PBS with 70% alcohol in a sterile hood.
2. Determine the target number of tissue culture wares to be used for expansion, typically 1:8 split is performed.
3. Make enough HiPSC Working Coating Solution by diluting HiPSC Coating Solution Stock 1 to 100 in sterile PBS
4. Coat the tissue culture wares with HiPSC Working Coating Solution.

C. PREPARING PASSAGING MEDIUM (PBS with 0.5X ROCK INHIBITOR)

1. Prepare Passaging Medium by adding 50 µL of 100X ROCK Inhibitor to 10 ml PBS to achieve a final concentration of 0.5X.

D. PREPARING DISSOCIATION SOLUTION

1. Warm Dissociation Solution to 37°C prior to use.

E. PREPARING GROWTH MEDIUM

1. Calculate the amount of Growth Medium needed for expansion and transfer to a 50ml conical tube to warm up to room temperature.
2. Add 100X ROCK Inhibitor to HiPSC Growth Medium prepared in Step 1 to achieve a final concentration of 0.5X.

F. SUBCULTURING HiPSC

1. Aspirate HiPSC Growth Medium.
2. Pipette 2.5 ml warmed HiPSC Dissociation Solution into the T-25 flask. Rock the flask gently to ensure the solution covers all the cells.
3. Re-cap the flask tightly and incubate for 45 seconds at 37°C. Monitor cell detachment during incubation time. If clusters of detaching cells are observed, go to Step 4 immediately.
4. Resuspend cells by gently pipetting to wash down the cells in HiPSC Dissociation Solution 2-3 times If cells do not detach completely, lay flask down and wait 20-30 seconds, then try again. Anh cells which do not detach after a third attempt should be left behind.
5. Dilute cell suspension in T-25 flask by adding 10 ml Passaging Medium prepared in Section IV C Step 1 to the T-25 flask and transfer to a conical tube.
6. Centrifuge cells at 200g for 5 minutes. Remove supernatant carefully by aspiration without disturbing the cell pellet.
7. Resuspend HiPSC in HiPSC Growth Medium with 0.5X Rock Inhibitor prepared in Section IV E Step 2.
8. Aspirate HiPSC Coating Solution from the tissue culture wares.
9. Passage HiPSC in 1:4 ratio (range of 1:4 to 1:6 depending on the cell confluency prior to subculture). Use following table as reference for volume in various tissue culture ware.
10. Place the HiPSC in a 37°C, 5% CO_2 humidified incubator. Loosen the cap to allow gas exchange.
11. Change to fresh HiPSC Growth Medium every 24 hours without ROCK Inhibitor until next subculture.

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APPENDIX

Seeding Guide of Cryopreserved HiPSC Product Vial after Thawing.

TC Vessel	Number of wells per cryovial	Volume per Well		
		Working Coating Solution	HiPSC Growth Medium	HiPSC Dissociation Solution
12-Well Plate	5 wells	0.5 ml	1 ml	0.5 ml
6-Well Plate	3 wells	1 ml	2 ml	1 ml
T-25 Flask	1 flask	2 ml	5 ml	2.5 ml

V. CRYOPRESERVING HiPSC

HiPSC can be cryopreserved when the culture is 80-90% confluent for cell banking.

1. Add 100X ROCK Inhibitor to HiPSC culture to a final concentration of 0.1X for one hour in a 37˚C, 5% CO_2 humidified incubator prior to cryopreservation. Loosen the cap to allow gas exchange.
3. Take out appropriate amount of HiPSC Dissociation Solution and bring to room temperature
4. Aspirate HiPSC Growth Medium .
5. Add warmed Dissociation Solution and rock the flask gently to ensure the solution covers all the cells.
6. Re-cap the flask tightly and incubate for 45 seconds at 37˚C. Monitor cell detachment during incubation time and if clusters of detaching cells are observed, remove Dissociation Solution immediately.
7. Add HiPSC Growth Medium and re-suspend cells by pipetting gently up and down 3-6 times
8. Transfer the cell suspension to a 15 ml conical tube
9. Count the cells.
9. Centrifuge cells at 200g for 5 minutes.
10. Remove supernatant carefully by aspiration without disturbing the cell pellet.
11. Flick the cell pellet at the tip of the conical tube, add HiPSC Freezing Medium and reuspend cell pellet in HiPSC Freezing Medium to achieve the cell density to 1E6-2E6 cells/ml.
12. Aliquote HiPSC suspension to cryovial.
13. Freeze the cells in slow freezing device or by placing the cryovials in a styrofoam box with lid closed in -80˚C freezer for 3 hours or overnight**.
    ** Cells will be compromised by prolonged storage at -80 ˚C.
15. Transfer the frozen cryovials to a liquid nitrogen tank for long-term storage.

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