General Instructions for Use

Human Induced Pluripotent Stem Cells (HiPSC)

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. 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 (018K-500)
   Store the HiPSC Basal Medium (017-500) at 4°C in the dark immediately upon arrival.
   Store the growth supplements GS1 (018-GS1), GS2 (018-GS2) and GS3 (018-GS3) 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 COATING SOLUTION (126-25)
   Store at -20°C immediately after arrival.
   Store at 4°C after thawing.

D. HiPSC DISSOCIATION KIT (091K)
   Store the PBS (060-50) and Dissociation Solution (075-25) at 4°C.

ROCK Inhibitor:
Y27632 can be purchased from RnD Systems, Cat No. 1254
100 X Stock is prepared by as 0.32 mg/ml in nanopure water And sterile filtered through 0.2 filter

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 the standard sterilization technique 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 the HiPSC Coating Solution and HiPSC Growth Medium kit from the refrigerator. Decontaminate the bottle with 70% alcohol in a sterile hood.
   2. Prepare one T-25 flask for culturing HiPSC by pipetting 3 ml of HiPSC Coating Solution to a T-25 flask. See Appendix A for other TC Vessels volumes.
   3. Incubate coated T-25 flask at 37°C for a minimum of 30 minutes for the coating to stabilize.

B. PREPARING GROWTH MEDIUM FOR CULTURING HiPSC
   1. Thaw HiPSC Growth Supplements and add GS1, GS2 and GS3 to the basal medium and mixing gently.
   2. Take out 15 ml of Growth Medium and warm up to room temperature*.
   3. Transfer 9 ml of HiPSC growth medium to a 15 ml conical tube for diluting iPSC

   * Do not warm HiPSC growth medium to 37°C.
C. THAWING AND PLATING HiPSC
1. Remove the cryopreserved vial of HiPSC from the liquid nitrogen storage tank using proper protection for your eyes and hands.
2. 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.
3. 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 about 90 sec.
4. Take the vial out of the water bath when only small amount of ice left in the vial. Do not let cells thaw completely.
5. Decontaminate the vial exterior with 70% alcohol in a sterile Biological Safety Cabinet.
6. Remove the vial cap carefully. Do not touch the rim of the cap or the vial.
7. Transfer the cell suspension drop wise to the 15 ml conical tube containing 9 ml HiPSC growth medium prepared in Section III B step 3. Mix gently.
8. Centrifuge cells at 200g for 5 minutes. Remove supernatant carefully by aspiration without disturbing the cell pellet.
9. Re-suspend 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.
10. Aspirate HiPSC Coating Solution from the T-25 flask prepared in Section III A step 3.
11. Transfer 5 ml of HiPSC suspension to the T-25 flask. Cap the flask and rock gently to evenly distribute the cells.
12. Place the T-25 flask in a 37°C, 5% CO₂ 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
A. PREPARING SUBCULTURE REAGENTS
1. Remove the HiPSC Dissociation Kit from the 4°C and bring to room temperature.

B. PREPARING CELL CULTURE WARE
1. Decontaminate the HiPSC Coating Solution bottle with 70% alcohol in a sterile hood.
2. Determine the target number of tissue culture wares to be used for expansion, typically 1:15 split is performed. Coat the tissue culture wares with HiPSC Coating Solution as in Section III A Step 2 and 3.

C. PREPARING GROWTH MEDIUM with 0.5X ROCK INHIBITOR
1. Calculate the amount of Growth Medium needed for Expansion, add 100X ROCK Inhibitor to the Growth Medium to achieve a final concentration of 0.5X.

D. SUBCULTURING HiPSC
1. Remove the medium from culture flasks by aspiration.
2. Wash the cells with PBS and remove by aspiration.
3. Pipette 2.5 ml of dissociation solution into the T-25 flask. Rock the flask gently to ensure the solution covers all the cells.
4. Re-cap the flask tightly and incubate for 5 min at 37°C. Monitor cell detachment during incubation time and if clusters of detaching cells are observed, remove dissociation solution immediately.
5. Add 6 ml HiPSC Growth Medium with 0.5X ROCK Inhibitor prepared in Section IV C Step 1 to the flask and re-suspend cells by pipetting up and down 3-6 times. If cells do not detach completely, use a cell scraper to gently scrape cells. Break larger cell clusters by pipetting the solution an additional 3-6 times.
6. Count the cells with a hemocytometer or cell counter.
7. Dilute cell to 75,000.cells/ml in HiPSC Growth Medium with 0.5X ROCK Inhibitor prepared in Section IV C Step 1.
8. Aspirate HiPSC Coating Solution from the tissue culture wares
9. Inoculate at 15,000-20,000 cells per cm² for regular seeding use Appendix as reference for seeding.
10. Place the iPSC in a 37°C, 5% CO₂ humidified incubator. Loosen the cap to allow gas exchange. For best results, change to fresh HiPSC Growth Medium after every 24 hours without ROCK Inhibitor until next subculture.

Appendix

<table>
<thead>
<tr>
<th>TC Vessel</th>
<th>Volume per Well</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HiPSC Coating Solution</td>
</tr>
<tr>
<td>T-25 Flask</td>
<td>3 ml</td>
</tr>
<tr>
<td>6 cm Dish</td>
<td>3 ml</td>
</tr>
<tr>
<td>10 cm Dish</td>
<td>6 ml</td>
</tr>
<tr>
<td>15 cm Dish</td>
<td>15 ml</td>
</tr>
<tr>
<td>6-Well Plate</td>
<td>1 ml</td>
</tr>
<tr>
<td>12-Well Plate</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>24-Well Plate</td>
<td>0.25 ml</td>
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</tbody>
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Table: Common tissue culture ware formats with corresponding working volumes.
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.
2. Incubate for one hour in a 37°C, 5% CO₂ humidified incubator. Loosen the cap to allow gas exchange.
3. Take out appropriate amount of PBS and HiPSC Dissociation Solution and bring to room temperature.
4. Aspirate HiPSC Growth Medium and wash cells twice with PBS.
5. Add Dissociation Solution and rock the flask gently to ensure the solution covers all the cells.
6. Re-cap the flask tightly and incubate for 5 min 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 up and down 3-6 times.
8. Transfer the cell suspension to a 15 ml conical tube.
9. Count the cells.
10. Centrifuge cells at 200g for 5 minutes.
11. Remove supernatant carefully by aspiration without disturbing the cell pellet.
12. Flick the cell pellet at the tip of the conical tube, add HiPSC Freezing Medium and re-suspend cell pellet in HiPSC Freezing Medium to achieve the cell density to 1E6-2E6 cells/ml.
13. Aliquot HiPSC suspension to cryovial.
14. 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.