CELL
APPLICATIONS, INC.

IMPORTANT: culturing the 3D organoid system is a detailed, multistep process. Please thoroughly read the below instructions before proceeding with culture.

General Instructions for Use
Induced Human Hepatocytes (i-HH)

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. OVERVIEW

- Store all components of kit appropriately.
- Prepare Recovery Medium.
- Prepare DISCUS materials and set up to print discs.
- Prepare coated cultureware, thaw i-HH and embed in
- DISCUS.
- Allow i-HH to recover for one week.
- Prepare Maturation Medium.
- Remove Recovery Medium and allow i-HH to mature in Maturation Medium for up to one month.
- P450 Metabolic assays using Metabolic Medium may be performed any time after one week of maturation.
- Albumin and urea assays using Maturation Medium may be performed any time after one week of maturation.
- Discs may be dissolved using Dissolution Buffer to retrieve organoids or generate a single cell suspension.

II. STORAGE

A. CRYOPRESERVED i-HH ORGANOID VIAL (i780-05)

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

B. i-HH RECOVERY MEDIUM KIT (i71103K-30)

Store Kit components immediately upon arrival:

Component	Type	Storage
i71102-25	Basal Medium	4°C, dark
i71103-RS1	Recovery Supplement 1	-20°C
i71103-RS2	Recovery Supplement 2	-20°C
i71103-RS3	Recovery Supplement 3	4°C

C. i-HH MATURATION MEDIUM KIT (i71105K-100)

Store Kit components immediately upon arrival:

Component	Type	Storage
i71104-95	Basal Medium	4°C, dark
i71105-MS1*	Maturation Supplement 1	-20°C
i71105-MS2	Maturation Supplement 2	-20°C
i71105-MS3	Maturation Supplement 3	4°C

*contains DMSO; if running P450 metabolic assays use Metabolic Medium Kit (71107K-30)

D. i-HH METABOLIC MEDIUM KIT (i71107K-30)

Store Kit components immediately upon arrival:

Component	Type	Storage
i71106-28	Basal Medium	4°C, dark
i71107-MS1	Metabolic Supplement 1	-20°C
i71107-MS2	Metabolic Supplement 2	-20°C
i71107-MS3	Metabolic Supplement 3	4°C

After complete i-HH media is made (by mixing basal media with supplements), the complete media should be stored at 4°C and used within 4 weeks of preparation.

E. DISCUS™-111 i-HH Kit (DISC1053K)

Store Kit components immediately upon arrival:

Component	Type	Storage
DISC1052	Basal Gel	4°C
DISC1053-P1	Matrix Protein 1	-20°C
DISC1053-P2	Matrix Protein 2	-20°C

F. DISCUS™ DISSOLUTION REAGENT (DISC1050)

Store at 4°C.

G. i-HH NON-STICK COATING SOLUTION (1025-05)

Store at room temperature.

Additionally, prepare 100 mM $CaCl_2$ and 155 mM NaCl before proceeding to culturing cells.

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 cells have been tested negative for HIV, Hepatitis B and Hepatitis C.
   c. Handle all cell culture work in a sterile hood.

III. CULTURING i-HH ORGANOIDS

A. PREPARING LABWARE FOR CULTURING i-HH ORGANOIDS

1. Take out the i-HH Non-Stick Coating Solution and decontaminate the bottle with 70% alcohol in a sterile hood.
2. Prepare one well of a 6-well plate for culturing 5E5 i-HH by pipetting 1 ml of Non-Stick Coating Solution into the well.
3. Swirl Non-Stick Coating Solution briefly around the well, then recover the solution. Used Non-Stick Coating Solution may be kept in a separate tube and re-used up to five times.
4. Rinse well twice with PBS. Leave a small volume of PBS in the well until ready for use.
5. Pipette 2 ml of Non-Stick Coating Solution into a 15 ml conical tube and swirl so that entire inner surface of the 15 ml conical tube has been wetted.
6. Retrieve Non-Stick Coating Solution. Non-Stick Coating Solution may be kept in a separate sterile tube and re-used up to five times. Rinse tube twice with PBS and remove.
7. Coat one p1000 pipette tip by triturating with Non-Stick Coating Solution and rinse with PBS (reserve the tip for use when handling i-HH Organoids).
8. One 100-mm tissue culture plate is required.
9. Chill one large Eppendorf rack at -20°C.
10. Aliquot 10 ml of 100 mM $CaCl_2$ and chill at 4°C.
11. Aliquot 20 ml of sterile 155 mM NaCl.
12. Using a sterile scalpel or pair of sterile scissors, cut off the tip of a p1000 tip to create a wide-bore tip roughly 5 mm in diameter for pipetting the printed discs.

B. PREPARING RECOVERY MEDIUM FOR CULTURING i-HH

1. Thaw i-HH Recovery Supplements 71103-RS1 and 71103-RS2. Add all three Recovery Supplements (including 71103-RS), to the Recovery Basal Medium and mix gently.
2. Store the Complete Medium at 4°C. It should be used within one month following preparation.
3. Transfer 5 ml of Recovery Medium to a 15 ml conical tube and warm up to room temperature.
4. Transfer 9 ml of Recovery Medium to the Non-Stick-coated 15 ml conical tube prepared earlier in Section IIIA Step 6 for diluting freshly thawed i-HH Organoids.

C. PREPARING DISCUS™ GEL

1. Retrieve DISCUS™ Basal Gel (DISC1052) from 4°C and briefly warm in 37°C water bath to return to liquid phase.
2. Retrieve DISCUS™ Matrix Protein 1 (DISC1053-P1) from -20°C and warm to room temperature.
3. Retrieve DISCUS™ Matrix Protein 2 (DISC1053-P2) from -20°C and rapidly warm in 37°C water bath for at least 20 minutes. **Warming at RT will result in congealed reagent that is no longer usable.**
4. Add Matrix Protein 1 (DISC1053-P1) to Basal Gel (DISC1052) and use a p1000 tip to carefully mix up and down 10 times. Do not introduce bubbles, mixture is viscous.
5. Using the same pipette tip, transfer all of Matrix Protein 2 (DISC1053-P2) to the mixture of Basal Gel and Matrix Protein 1 to create the DISCUS™ Gel. Pipette up and down as before; material will become somewhat cloudy, this is normal. Final volume of the complete DISCUS™ Gel is 0.5 ml.

D. PREPARING MATURATION MEDIUM FOR CULTURING i-HH

1. One week after creating DISCUS™ discs containing the i-HH Organoids, thaw i-HH Maturation Supplements 71105-MS1 and 71105-MS2. Add all three Maturation Supplements (including 71105-MS3) to Maturation Basal Medium and mix gently.
2. Store the Complete Medium at 4°C. It should be used within one month following preparation.

E. PREPARING METABOLIC MEDIUM FOR METABOLIC P450 STUDIES OF i-HH

1. One week after incubating the i-HH Organoids in complete Maturation Medium, metabolic assays for P450 studies may be conducted. Maturation Media contains DMSO and is inhibitory to drug metabolism and must be removed during metabolic assays. After the assay is completed, Maturation Medium may be used.
2. Thaw i-HH Metabolic Supplements 71107-MS1 and 71107-MS2. Add all three Metabolic Supplements (including 71107-MS3) to Metabolic Basal Medium and mix gently.

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3. Store the Complete Medium at 4°C. It should be used within one month following preparation.

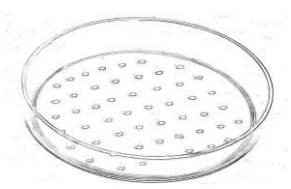
F. THAWING AND ENCAPSULATING i-HH

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.

1. Retrieve the cryopreserved vial of i-HH from the liquid nitrogen storage tank using proper eye and hand protection.
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 a small amount of ice is 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. Using the p1000 pipette tip that was treated with Non-Stick Coating Solution, transfer the cell suspension drop wise to the 15 ml conical tube containing 9 ml i-HH Recovery Medium prepared in Section III A Step 6.
8. Centrifuge cells at 200g for 5 minutes. Remove supernatant carefully by hand without disturbing the cell pellet. Use a p200 tip to remove the last droplets of supernatant.
9. Re-suspend i-HH in 125 ul DISCUS™ Gel prepared in Section III C Step 5 and transfer suspended material to a fresh Eppendorf tube.

The following steps must be completed within 5 minutes.

10. Remove chilled Eppendorf rack from freezer and place 100 mm plate on top of rack inside TC hood.
11. Take lid off plate and using a p20 pipettor, dispense i-HH Organoids in 5 uL drops onto chilled sterile 100 mm tissue culture plate. Prime the pipette tip with suspension by pipetting once or twice to remove initial bubbles. Approximately 80 discs can be printed to one 100mm dish.



12. **After printing the last disc**, wait 3-5 minutes for discs to chill. Roughly 20-25 discs should be created.
13. Retrieve 100 mM $CaCl_2$ solution from refrigerator.
14. Slowly cover printed discs with 10 mL chilled 100 mM CaCl2 solution. Carefully dispense along edge of plate to avoid distorting the discs.
15. Remove the $CaCl_2$ solution.
16. Rinse discs twice with 7-10 mL 155 mM NaCl before suspending in 5 mL Recovery Medium. Use the second NaCl wash to gently detach discs from plate surface. Take care to not suck up newly formed discs during rinses.
17. Using the wide-bore p1000 tip, transfer discs to Non-Stick-coated 6-well plate prepared in Section III A, or other sized well as desired.

TC Well Plate	Non-Stick Coating Solution	Disc Number	Medium Volume
6	1 mL	20	3 mL
12	0.5 mL	10	1 mL
24	0.25 mL	5	0.5 mL

18. Place the 6-well plate in a 37ºC, 5% CO_2 humidified incubator.
19. Excess DISCUS gel may be stored at 4°C for up to three weeks.
20. Change i-HH Recovery Medium every other day for one week, using 3 mL per well of a 6-well plate.
21. After one week, change medium to i-HH Maturation Medium to mature hepatoblasts into hepatocytes.
22. Some i-HH Organoids may escape from discs and adhere to the plate, usually within one week. Adhesion can be prevented by carefully transferring discs to a fresh well treated with Non-Stick Coating Solution using a wide-bore p1000 pipette tip, leaving as much media containing escaped organoids behind as possible.
23. Escaped organoids may be recaptured by transferring remaining media to a fresh 15 mL conical tube that was treated with Non-Stick Coating Solution, and briefly spinning down organoids at 200 x g for 1 minute. Re-warm DISCUS™ gel and briefly spin down to remove any precipitates. Follow the method for creating discs as described above. Creating an additional 5-6 discs (25-30 ul of DISCUS™ gel) is usually sufficient.

F. HARVESTING i-HH ORGANOIDS

1. Please note that i-HH Organoids embedded in the DISCUS™ system are suitable for multiple assays *in situ*, including measurement of albumin (Thermofisher Albumin ELISA, Cat# EHALB) and urea (Fisher Scientific Quantichrome Urea Assay, Cat# DIUR-100) production and P450 metabolism (Promega P450 CYP3A4 Luciferin-IPA, Cat# V9001). Four to five discs in 250 uL media are sufficient to assay for activity.
2. i-HH Organoids may be retrieved from the DISCUS™ system quickly and easily.

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3. Bring the DISCUS™ Dissolution Buffer to room temperature.
4. If a single cell suspension is desired, Accutase is a suitable digestion enzyme. Please note that if a single cell suspension is generated, the functional abilities of the organoid system will be abrogated.
5. As detailed in Section III A, prepare a Non-Stick coated 15 ml conical tube, two 50 mL conical tubes and a p1000 tip treated with Non-Stick Coating Solution.
6. Place a 37-micron mesh filter (StemCell Cat# 27250) on one 50 mL conical tube and pre-wet filter with 1 mL Non-Stick solution. Rinse mesh twice with PBS. Move mesh filter to the uncoated 50 mL conical tube.
7. Using a wide-bore p1000 pipette tip, transfer discs to the 15 mL conical tube treated with Non-Stick Coating Solution, and centrifuge for 1 minute.
8. Remove the medium manually and add 1 ml of Dissolution Buffer.
9. After approximately two minutes, pipette up and down with a p1000 tip (that was treated with Non-Stick Coating Solution) 5-10 times to release the organoids. Discs should dissolve within 5 minutes.
10. Using a coated pipette, ransfer the organoids to the prepared 37-micron mesh filter and rinse the mesh twice with cold F12.
11. Move the mesh filter to the second 50 mL coated conical tube and flip the mesh filter over. Rinse organoids off mesh filter using two 2.5 mL cold F12 rinses and transfer the organoids back to the original Non-Stick coated 15 mL conical tube. Spin down organoids for 5 minutes at 200g.
12. Remove supernatant and resuspend organoids in 500 uL Accutase. Incubate organoids at 37°C for 30 minutes with occasional tapping to ensure even exposure to Accutase.
13. Quickly dissociate organoids with at least 40 strokes (<30 seconds), with a p1000 tip (treated with Non-Stick Coating Solution) against the bottom of tube until a single cell suspension is achieved.
14. Wait approximately 5 minutes to allow cells to resume a spherical shape.
15. Count hepatocytes with trypan blue. Remnant matrix debris and small cells will be visible; hepatocytes can be distinguished, as they are bright, larger cells by comparison. The yield is approximately 4,000 hepatocytes per dissolved disc. Cell count can be used for normalization and data analysis.

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