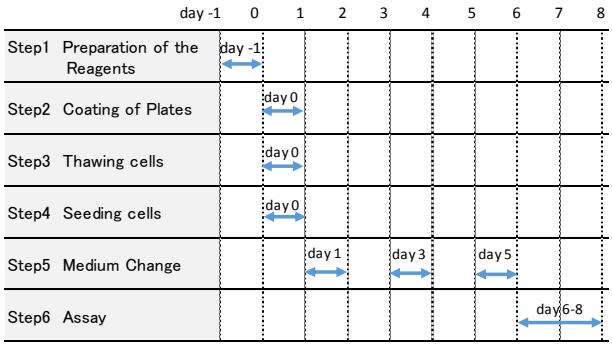
Cell Cultivation Protocol for ReproHepato™

Cat. # RCDH001N
Ver. 1.0

Critical Points for Cell Handling

- To maintain high viability, store cell-vials in liquid nitrogen.
- Before thawing, gently twist the cap a quarter of turn under the laminar flow hood (do not open it completely). This will prevent vial explosion due to liquid nitrogen evaporation & expansion.
- The time interval between thawing and cell transfer into the culture medium must be less than 2 minutes. The total procedures from thawing to seeding must not exceed 30 minutes. Taking too long to complete the total procedure will result in a dramatic loss of cell-viability.
- Pipet repeatedly (but gently) to ensure a single-cell suspension for seeding by using a micropipettor. Make sure that no visible cell aggregates remain.
- The plating density for cell seeding is critical; densities lower than the recommendation, will result in poor recovery and poor performance.

Culture Schedule (Example)



Items not included

Item	Catalog No.	Storage
ReproHepato Culture Medium™	ReproCELL, RCDH101	4°C
ReproHepato Assay Medium™	ReproCELL, RCDH301	4°C
Penicillin Streptomycin	Thermo Fisher (GIBCO), Cat. 15140-122	4°C
Basement Membrane Matrix (Matrigel™)	BD, Cat. 356234	-20°C
Dulbecco's Modified Eagle's Medium - high glucose	SIGMA, Cat. D5796	4°C
CELLSTAR® 24-well Cell Culture Plate (or equivalent)	Greiner, Cat. 662160	RT
Leibovitz's L-15 Culture Medium	Life Technologies, Cat. 21083-027	4°C
Corning PuraMatrix™	Corning, Cat. 354250	4°C

1. Coating 24-well Culture Plates (Matrigel™)

1.1. Thaw Matrigel™ at 4°C overnight.
1.2. Prepare the Matrigel™ as follows:
1.3. In a conical 15 ml tube on ice, add 0.42 mL of 4°C thawed Matrigel™. Add 11.58 mL of chilled DMEM and mix on ice by repeat pipetting the entire solution two times.
1.4. Add the Matrigel™ solution to the wells of the culture plates (250 µL/well for a 24-well plate).
1.5. Add DMEM to the non-cultured wells (250 µL/well for a 24-well plate).
1.6. Incubate the plates at room temperature for 3~4 hours.

Optional: Coating a 24-well Culture Plate with PuraMatrix™ (Corning)

1.1. Decrease the viscosity of PuraMatrix™ stock solution (1% w/v) by vortexing or sonication for a few seconds.
1.2. Dilute the stock solution with distilled water to final concentration of 0.25% w/v.
1.3. Add the solution to the wells of the culture plate (250 µL/well for 24-well plate).
1.4. To promote gel formation, gently overlay ReproHepato Maintenance Medium (250µL/well for 24-well plate).
1.5. Change the Maintenance Medium two times in the next hour to equilibrate the gel with medium (250 µL/well for 24-well plate).

Note. Gelled Corning PuraMatrix™ can be safely stored overnight in an incubator (37℃, 5% CO_2).

2. Preparation of the Reagents

2.1. Thaw all the frozen ReproHepato medium and supplements in a 4°C refrigerator overnight.

Note - If white precipitation is observed in the medium, warm to room temperature or 37ºC. The precipitate is Glutamine (a necessary component), and it will readily re-dissolve. There will be no problem with medium quality or cell culture performance.

2.2. Add 400 µL of the ReproHepato Medium Supplement to the bottle of ReproHepato Culture Medium (Culture Medium).
2.3. Add 300 µL of the Assay Medium Supplement to the bottle of ReproHepato Assay Medium (Assay Medium).
2.4. The complete medium (prepared as described above), should be stored at 4°C and used within 2 weeks. Avoid re-freezing of the complete media.

3. Warming the Media

3.1. Using sterile techniques, aliquot 49 mL of L15 Medium to a 50 mL tube A.
3.2. Likewise, aliquot 12 mL of Culture Medium to another tube B.
3.3. Warm tubes A and B in a 37°C water bath for at least 15 minutes.
3.4. Open the lid of tube A in a biological safety cabinet and place the lid on the tube, but do not tighten it, so that the lid can be removed easily.
3.5. Set the micropipette (P-1000) to 1000 µL, and put on a tip; prepare in advance so that it can be used quickly.

Note - Finish all preparations and place the warmed L15 Medium in a biological safety cabinet before beginning to thaw the ReproHepato cells.

4. Thawing the Cells (Thaw only 1 vial at a time)

4.1. Prepare a cryo-reservoir for carrying one vial, and fill it with just enough liquid nitrogen for the vial.
4.2. Take one vial of ReproHepato cells out of the liquid nitrogen and place it in the reservoir. Transport it near to the biological safety cabinet.
4.3. In the safety cabinet, briefly twist the cap a quarter turn to release the internal pressure and then lightly close it again. Return the vial to the liquid nitrogen reservoir again as quickly as possible.

Note - Ensure that this procedure is completed within 30 seconds to prevent temperature increase.

Note - Do not open the cap completely to prevent contamination.

4.4. Transport the reservoir close to the water bath.
4.5. Immerse the vial in the 37°C water bath as quickly as possible, and gently swirl it in the bath for **90 seconds**.

Note - Ensure that this procedure is completed in no more than 90 seconds. The solution should be about half-thawed in 90 seconds. The viability of the cells will decrease if the vial contents are thawed completely at this moment.

4.6. Bring the thawed vial to the safety cabinet as quickly as possible, wipe away water around the vial, and decant the content of the vial into tube A.
4.7. To retrieve remaining cells trapped in the vial, use 1 mL of medium from tube A to again rinse the emptied vial, and transfer the contents back into tube A.

Note - From step 4.5. to step 4.7, be sure to finish within 2 minutes (90 seconds in water bath and 30 seconds in the transfer process).

4.8. Centrifuge tube A at 350 × g for 5 minutes at room temperature.
4.9. While tube A is in the centrifuge, bring tube B containing the Maintenance Medium from the water bath to the safety cabinet.
4.10. After centrifugation is complete, place tube A in the safety cabinet.
4.11. Carefully aspirate all of the supernatant and leave the pellet in tube A. Be careful not to aspirate the pellet itself.
4.12. Transfer 1 mL of Maintenance Medium from tube B to tube A using a P1000 micropipette. Mix the entire content of tube A by pipetting 5 times using a micropipette to obtain a single-cell suspension. When pipetting, withdraw the entire volume and spray it onto the inner wall of the tube to disperse cells. Make sure that no cell aggregates remain.

Note - To make the single-cell suspension, do not use large-size plastic pipettes (e.g. 5 or 10 mL). Use of a P1000 micropipette is required for best results.

4.13. Transfer 10 mL of Maintenance Medium from tube B to tube A using a 10 ml plastic pipet. Mix the entire content of tube A by repeated pipetting 3 times, taking about two seconds each time.

Note - To minimize the total operation time, cell-counting is not recommended at this time.

5. Seeding the Cells in a 24-well plate

5.1. Bring the coated plate (prepared in step 1) to the biological safety cabinet.
5.2. Remove the supernatant solution from one row (6 wells) by using a pipette. Work with one row at a time to avoid drying.
5.3. Immediately add 640 µL of the cell suspension from tube A into each emptied well (take no more than 15

seconds/row). For plates sizes other than 24- wells, please refer to the table below.

5.4. With your micropipette (using the same tip), agitate the cell suspension in tube A by repeated pipetting 4 times to keep the cells from settling or aggregating. Then move on to the second row. The same steps should be repeated for filling the remainder of the rows.

5.5. On the flat surface of the safety cabinet, gently agitate the dish in all horizontal directions several times to uniformly spread the cells. Place the seeded plate in a 37°C, 5% CO_2 incubator to begin the cultivation.

Note - The thawing process should not exceed 30 minutes maximum. Excessive thawing time reduces the cell viability and attachment efficiency.

Multi-well plates	Culture surface area (cm^2/well)	Medium volume required per well for cell seeding (µL)	Medium volume required per well for medium change (µL)
12 well	3.80	1200	1000
24 well	2.00	640	500
48 well	0.75	240	150
96 well	0.32	100	100

6. Medium Changes

It is necessary to change the culture medium on Days 1, 3, and 5 after seeding.

6.1. Aliquot necessary amount of Maintenance Medium to a 15 mL tube, and start warming the tube in a 37°C water bath at least 15 minutes before placing it under the biological safety cabinet.

6.2. Remove the medium from the wells. Immediately add 500 µL/well (for a 24-well plate) of pre-warmed Maintenance Medium to the emptied wells. Add the medium gently to the side of the wall and not directly to the cells to prevent cells and gels from detaching from the well.

6.3. Return the plate to the 37°C, 5% CO_2 incubator.

Note - Make sure that this medium change is completed in no more than 10 minutes.

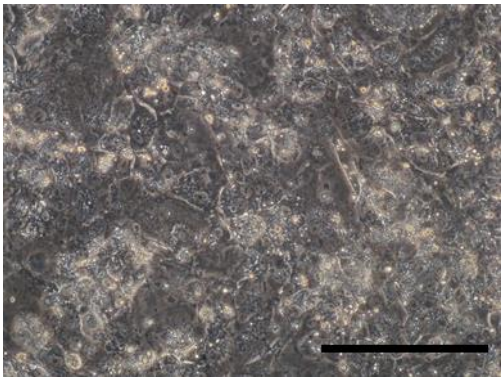
7. Assay

7.1. The ReproHepato cells are ready for use for assay on Day 6. It is recommended to use the complete Assay Medium as prepared as described in Step 2.

7.2. For metabolism testing, start using the cells from Day 6. For induction tests, begin exposure of the cells from Day 6.

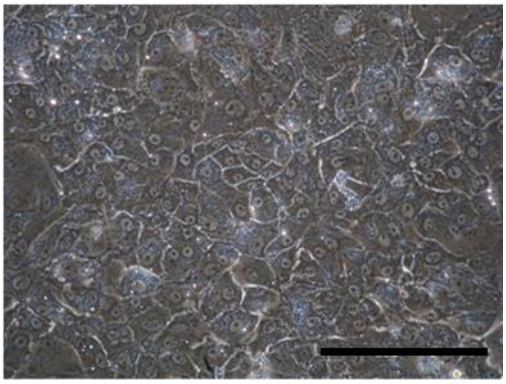
The morphology of the cells after seeding

1. 24 hours after seeding (Day 1); Cells are attached and cover approximately 80% of the surface of the culture plate.



Day 1
Scale bar : 200µm

2. 8 days after seeding (Day 8), the cells have begun to form a cobble stone-like structure.



Day 8
Scale bar : 200µm

FAQ (Frequently Asked Questions)

Q1. Why is thawing time in the water bath so critical?
A1. If shorter than 90 seconds, the content in the cryo-vial may not be thawed, and may not be completely transferred into tube A. If longer than 90 seconds, cell viability and adhesion may be impaired and the cell culture will fail or be unhealthy.

Q2. How do I confirm whether or not I have a single-cell suspension?
A2. By close visual inspection. There should be no particulate matter in the suspension. It requires repeated pipetting at least 5 times with a P1000 micropipette.

Q3. Why is advisable not to count the cells?
A3. The ReproHepato cells within the vial are pre-counted and optimal. By simply adding the recommended volume for each well area, the cell density will be ideal and you can save time for thawing.

Q4. My cells do not appear as those in the attached figure.
A4. Please check all the "Critical Points for Cell Handling" and contact us via e-mail (contact information is provided). Someone will respond by e-mail or phone you.

Q5. Which coating is recommended, Matrigel™ or PuraMatrix™?
A5. It depends on the customers' application. Use of PuraMatrix™ will cause the cells to form spheroid-like 3D structures. This has been shown to increase the basal levels and induction levels of most CYP450 enzymes.

Conditions for Use

This product is for research use only and not for therapeutic or diagnostic purposes. The sale of this product to a third party or its use for commercial purposes is not allowed without prior permission from ReproCELL.

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